CHAPTER 3

MATERIALS AND METHODS

Within academic research, the Materials and Methods section is essential for giving readers a thorough understanding of the materials used and the techniques used in an inquiry. It provides comprehensive information on everything from sample collection to experimental design, apparatus, data measurement, and analysis, acting as a kind of blueprint. Gurumani (2019) highlights that the data in this area should be carefully designed to allow other researchers to reproduce the study accurately and precisely. As such, this component is a cornerstone that promotes reproducibility and advances scientific conversation among academics. The aim of this study was to employ Response Surface Methodology in the creation of a Protein-Energy Bar, with the objective of replenishing macronutrients and meeting the dietary needs of individuals engaged in fitness activities. The study was conducted in four stages, beginning with an online market survey on food bars. Subsequently, the Protein-Energy Bar was standardized, and ingredient optimization was conducted utilizing Response Surface Methodology, guided by feedback received. The process involved sensory analysis by a semi-trained panel, alongside physico-chemical, textural, and microbial analysis, followed by a shelf-life study and cost analysis of the final product. Finally, in order to determine the product's appropriateness and appeal in the target market, acceptance trials including athletes, coaches and fitness trainers were carried out.

The study comprised four phases:

Phase 1: Conducting an online market survey on food bars.

Phase II: Standardizing the Protein Energy Bar, optimizing ingredients using Response Surface Methodology and developing the Protein Energy Bar based on the responses.

Phase III: Performing sensory analysis by a semi-trained panel, along with physico-chemical, textural and microbial analysis, as well as conducting a shelf-life study and cost analysis of the final product.

Phase IV: Conducting acceptability trials by athletes, coaches and fitness trainers.

The methodological components of the study have been categorized into the following sections:

3.1 Online Market Survey on Food Bars

3.2 Formulation of Protein-Energy Bars

3.2.1 Concept Generation, Screening and Prototype Development

3.2.2 Procurement of Raw Materials

3.2.2.1 Cereal-Pulses Crispies

3.2.2.2 Whey Protein Concentrate-80 (WPC-80)

3.2.2.3 Other ingredients

3.2.3 Proximate Composition of Raw Materials

3.2.4 Standardization of the Formula

3.2.5 Process Optimization of Protein Energy Bars

3.3 Characterization and Costing

3.3.1 Characterization of Protein Energy Bars

3.3.2 Shelf-Life Analysis

3.4 Acceptability trials involving athletes, coaches and fitness trainers

Study Locale

Raw ingredient extrusion and ingredient optimization using Response Surface Methodology took place at SMC College of Dairy Science, Kamdhenu University, Anand (annexures I. A & I. B). Product formulation, standardization and sensory evaluation were carried out at the Department of Foods and Nutrition, Faculty of Family and Community Sciences, The Maharaja Sayajirao University of Baroda, Vadodara. Nutrient and chemical analysis, as well as shelf-life studies, were performed at the National Institute of Food Technology Entrepreneurship and Management in Thanjavur, Tamil Nadu. Texture Profile analysis was conducted at the College of Food

Processing Technology & Bio-Energy, Anand Agricultural University, Anand (annexure II). Acceptability trials were held with players registered under the Department of Physical Education at The University Pavilion, The Maharaja Sayajirao University of Baroda, Vadodara, as well as fitness trainers from various gymnasiums in urban Vadodara.

Statutory clearance of the study

The research received approval from the Institutional Medical Ethics Committee of the Department of Foods and Nutrition at The Maharaja Sayajirao University of Baroda and was granted Institutional Medical Ethics Committee Number: IECHR/FCSc/PhD/2022/5 (annexures III. A & III. B).

Phase I

3.1 Online Market Survey on Food Bars

An extensive examination was undertaken to investigate the assortment of food bars accessible in the market. This exploration was prompted by the widespread consumption of protein supplements and sports bars among health-conscious individuals, particularly those actively engaged in physical activities. The aim was to gain a comprehensive understanding of the variety of options available to consumers within this category of nutrition bars.

During the exploratory phase of the study, a web survey was conducted to assess the range of food bars available to consumers in India. This survey aimed to achieve the following objectives:

- Identify the diversity of food bars offered by different brands.
- Analyze key features of these food bars, including their protein content, serving size and average cost.

A total of 250 products were listed and categorized into 32 groups according to their respective brands. Additionally, these bars were further organized into 8 distinct groups based on their suggested usage, which included energy bar, protein bar, nuts and seed bar, snack bar, vegan bar, low carb bar, meal replacement bar and gluten-free Bar. Figure 3.1.1 shows the Schematic diagram representing survey of food bars.

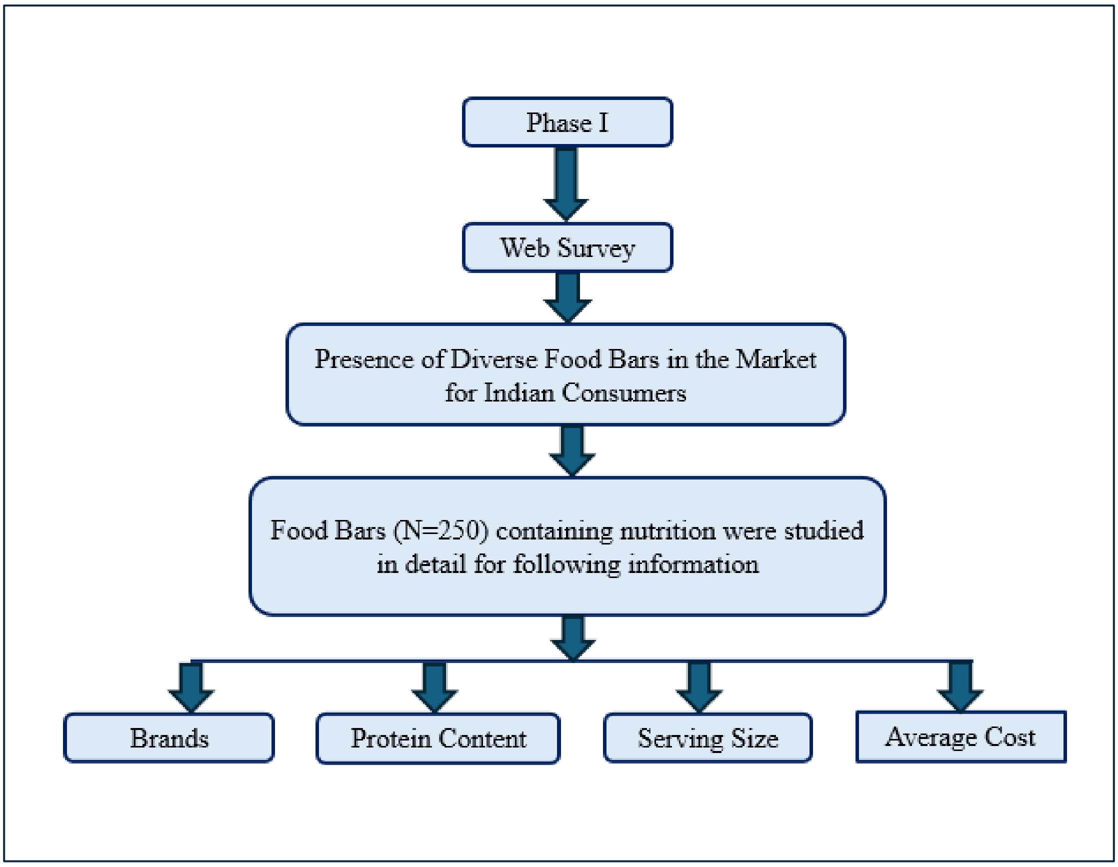

Figure 3.1.1 Schematic Diagram Representing Survey of Food Bars-Phase I

These were then divided into groups according to the concentration of protein in each (low (<10 g per serving), moderate (<10-19 g per serving) and high (>20 g per serving)), average serving size, average amount of protein in each serving and average price of the bars that were part of the survey. Additionally, a list of the common protein sources was identified from the product labels. The protein content range for these sources was referred from a study conducted by Kaur et al. (2022), which examined various proteins and compared them to animal-derived proteins.

Phase II

3.2 Formulation of Protein-Energy Bar (P-E Bar)

Making protein-energy bars involves combining components that provide both protein and energy in a convenient, portable form. This approach employs a formula that has been fine-tuned and optimised using a Central Compostite Rotatable Design, a three-factor Response Surface Methodology, Design-Expert 8.0.3 (Stat-Ease). This process ensures that the bars not only provide the essential nutritional advantages properly, but also taste good. The schematic diagram in figure 3.2.1 outlines the process flow for phase II.

3.2.1 Concept Generation, Screening and Prototype Development

The development of a sports food product formulation involved a comprehensive approach, including gathering insights from experts, reviewing relevant literature, consulting recipe books and exploring online resources. With a focus on addressing the unique dietary needs of physically active individuals, a curated selection of ingredients was chosen to align with the metabolic demands of this demographic, aiming to create a convenient and cost-effective snack option tailored to their requirements. During the evaluation phase, various formulas underwent trials based on the criteria such as serving size, texture, calculated protein content, cost-effectiveness, adherence to hygienic practices and overall appeal. Among the formulations tested, a blend of maize, ragi, gram flour and soy protein isolate stood out, allowing for the creation of a single extrudate using high-temperature short-time cooking, ensuring efficient processing while maintaining nutritional integrity. The feasibility of ingredient usage and preparation conditions was rigorously assessed to ensure optimal product quality throughout the prototype development, which involved rigorous steps from concept generation to screening, aiming to meet the quality standards and fulfil the targeted requirements for convenience, affordability and nutritional value.

3.2.2 Procurement of Raw Materials

Raw materials procurement details, including various materials utilized throughout this research, along with their respective sources, are outlined in this section.

High-quality maize, ragi and Bengal gram were obtained at the Granary Super Store, Amul in Anand, Gujarat. Soy Protein Isolate was obtained from Atuleet Foods Enterprise, which is based in Karamsad, Anand, Gujarat, India. Cereal-pulse crispies were made with flour derived from these sources in a co-rotating twin-screw extruder.

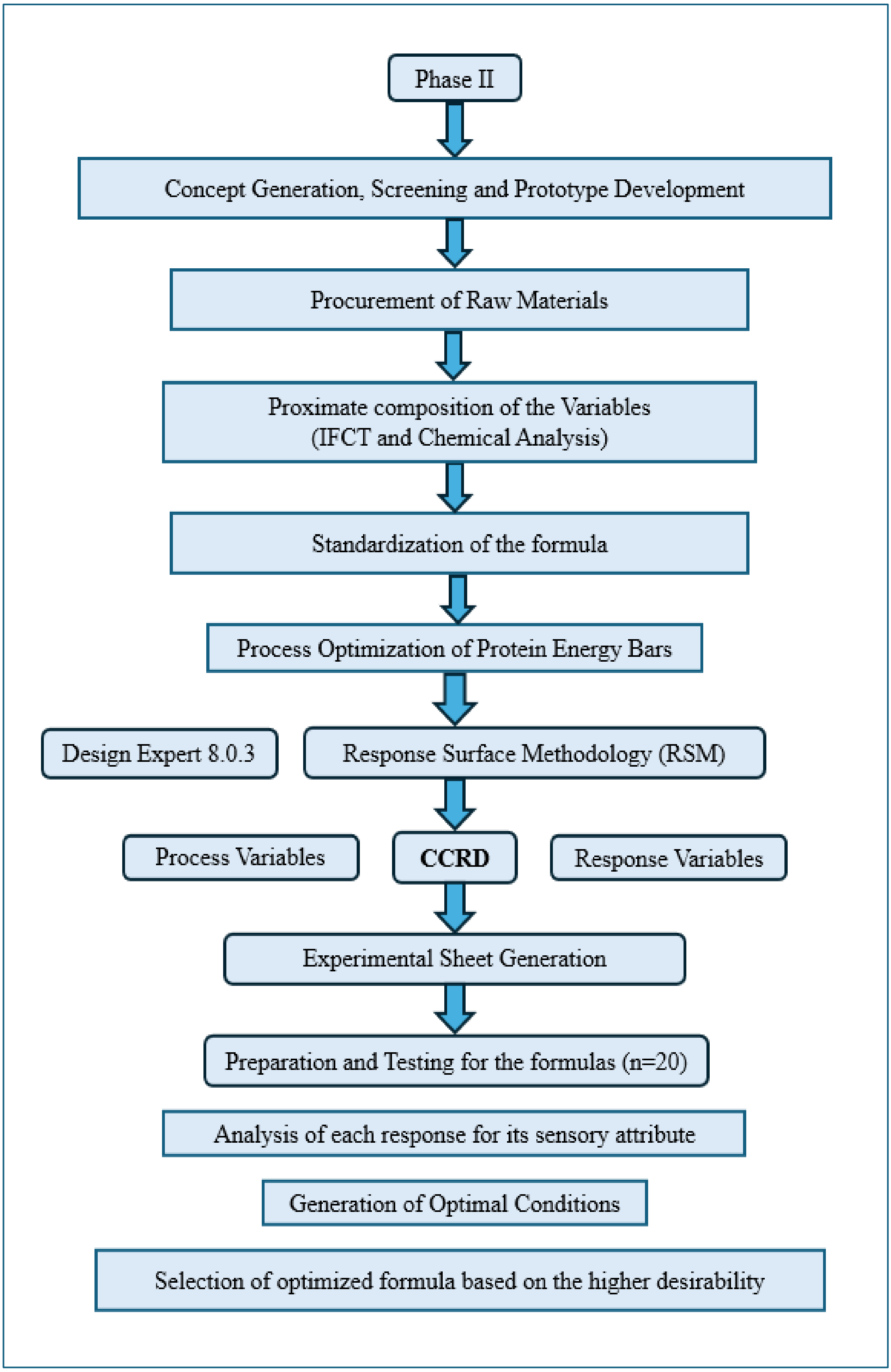

Figure 3.2.1 Phase II Process Flow-Schematic Diagram

3.2.2.1 Cereal-Pulses Crispies

The initial stage of the extrusion process, raw material selection, is crucial as it determines the quality of the final product. Maize (*Zea mays*) flour, ragi (*Eleusine coracana*) flour and gram (*Cicer arietinum*) flour were obtained from the Granary Super Store, Amul in Anand, Gujarat, India. Soy Protein Isolate of NAKPRO brand was acquired from NAKPRO Nutrition, Bengaluru, India, through online purchase (proximate composition in table 4.2.1, chapter 4). Research suggests that incorporating common bean (*Phaseolus vulgaris L*) flour into maize starch-based extruded snacks enhances protein and dietary fiber levels, thereby improving the nutritional profile of the snacks (Anton et al., 2009). The Twin Screw Extruder equipment was used in the laboratory of the SMC College of Dairy Science at Kamdhenu University in Anand (Annexure IV).

Preparation of feed mixture formula:

A combination of cereals and pulses in a ratio of 4:1 was utilized to enhance the protein content and quality, as calculated in the nutritional evaluation of maize flour, ragi (finger millet) flour and gram flour (Berrios, 2006). The proximate principles of soy protein isolate were determined using A.O.A.C. approved methods (George & Latimer, 2019). The cereal and pulses mixture were mixed and then moistened with up to 10 per cent water of the total flour weight. Subsequently, the resulting moistened flour blend underwent a double sieving process to eliminate any coarse or foreign particles that could potentially impede the extrusion process, thereby ensuring smooth operation. The flour blend was then packaged in low-density polyethylene (LDPE) pouches and preconditioned at room temperature for two hours. Figure 3.2.2 outlines the formulation of Crispies.

The product was developed using a co-rotating TSE (BTPL lab type EB-10, built in Kolkata, India) with three zones: feeding zone, heating zone and die zone. The extruder barrel was three feet long and 40 millimetres in diameter. It had a smooth barrel, temperature controls, a variable-speed motor and a cutter.

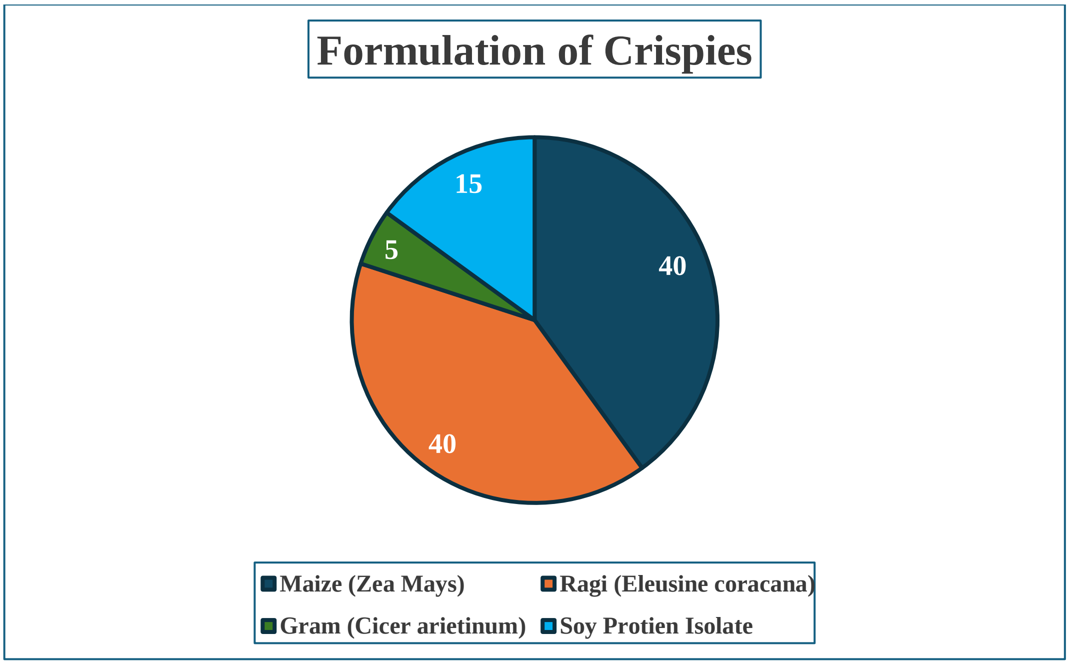

Figure 3.2.2 Formulation of Crispies

The Extrudates (Crispies) were collected and promptly air-dried at 100°C for 30 minutes in a hot air oven to remove any surplus moisture. The process flow diagram for the preparation of the feed material and extrusion for the creation of crispies is depicted in figure 3.2.3, while the Crispies are illustrated in figure 3.2.4. Annexure IV contains detailed operational parameters used during the crispy production process. Proximate composition analysis of extrudate data presented in table 4.2.1 in the subsequent chapter.

3.2.2.2 Whey Protein Concentrate-80 (WPC-80)

Whey Protein Concentrate-80 (WPC-80), with 80 per cent protein content, was obtained from Atuleet Foods Enterprise, located in Karamsad, Anand, Gujarat, India. The composition and additional quality parameters of WPC-80 are detailed in table 4.2.1 in the following chapter.

3.2.2.3 Other ingredients

Amul chocolate chips, butter and chia seeds (*Salvia hispanica*) were obtained from the Granary Super Store, Amul, in Anand, Gujarat. Malt extract was sourced from J.K. Malt Products Pvt. Ltd in Nadiad, Gujarat, India. Liquid glucose and soy lecithin were procured from Deomals, located at Atlantis II in Vadodara, Gujarat. Groundnut (*Arachis* *hypogaea L.*), sugar, ghee, black seeded raisins (*Vitis vinifera*), black sesame seeds (*Sesamum indicum*) and dry dates (*Phoenix dactylifera L.*) were purchased from Apexa Dry Fruit Store in Vadodara, Gujarat. The dates were processed into coarser powder using a food processor. Figures 3.2.5 and 3.2.6 shows the raw ingredients used for the development of Protein-Energy Bars.

3.2.3 Proximate Composition of Raw Ingredients

The independent variables chosen for optimization through RSM encompassed Crispies, WPC-80 and SPI. These materials were subjected to analysis of their proximate composition, following the established protocol outlined in table 3.2.2.

The Indian Food Composition Database (IFCT, Longvah et al., 2017) was utilised to ascertain the approximate composition of the other ingredients, which comprised groundnuts, dates, black raisins and black sesame seeds. The composition of the chia seeds was obtained from the research findings of Agarwal et al., 2023 (annexure V. A). The product label provided the composition of Chocolate chips and product specification of malt (annexure V. B.).

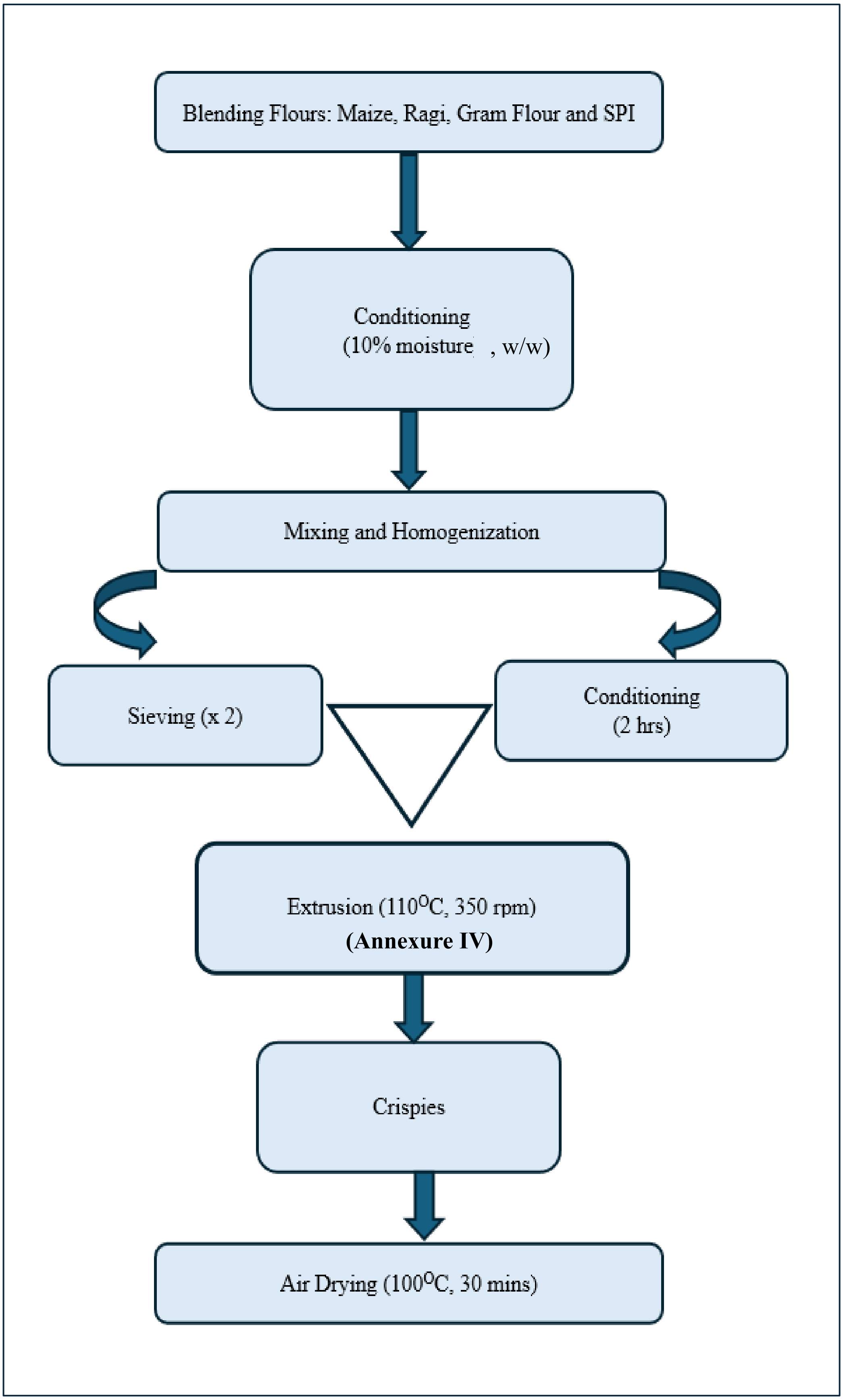

Figure 3.2.3 Procedure for Preparation of Crispies

Figure 3.2.4 Extrusion of Crispies– Twin Screw Extruder

Table 3.2.1 Chemical Quality Analysis of Raw Materials Selected as Independent Variables for the Process of Optimization

Sr. No.	Parameter Analyzed	Method of Analysis	Annexure no.
1.	Moisture	AOAC, 21st Edn, 2019 (George & Latimer, 2019), (Gupta & Sridevi, 2022)	VI
2.	Protein	AOAC, 21st Edn, 2019, (George & Latimer, 2019), (Gupta & Sridevi, 2022)	VII
3.	Ash	AOAC, 21st Edn, 2019, 923.03; Cha 32.1.05; Vol II; Pg: 2, (George & Latimer, 2019), (Gupta & Sridevi, 2022)	VIII
4.	Crude Fat	AOAC, 21st Edn, 2019, (George & Latimer, 2019), (Gupta & Sridevi, 2022)	IX
5.	Crude Fiber	AOAC, 21st Edn, 2019, (George & Latimer, 2019), (Gupta & Sridevi, 2022)	X
6.	Carbohydrate	By difference method. (Menezes et al., 2004)	XI
7.	Energy	Food Labeling – Requirements for FDA Regulated products, by James L. Vetter, E. M. Melran, Ed., AIB International. Manhattan, K.S, 2007 (Vetter & Meloan, (2007), (Gupta & Sridevi, 2022)	XII

3.2.4 Standardization of the Formula

Product standardization and testing represent crucial steps in food product development. A standardized recipe is defined as one that has been repeatedly tested and adopted by a specific food service operation, consistently yielding the same satisfactory results when the exact procedures, equipment and quantity and quality of ingredients are employed (Patil & Pol, 2014). In the standardization process for P-E Bars, the formula developed for the prototype was thoroughly reviewed, replicated in the laboratory and its yield was confirmed. Based on preliminary trials, standardization methods, existing literature and the protein content, Crispies, WPC-80 and SPI were opted as independent variables, the proportions of the remaining ingredients were kept at constant (table 3.2.3). Peanuts were opted to be included by difference in the formulation. Soy lecithin, serving as an emulsifier, was incorporated at a rate of 0.2 per cent. The standardized formula was further subjected to optimization process using RSM.

Table 3.2.2 Proportions of Ingredient Set as Constant

Component	Proportion (%)
Chocolate chips	12.9
Sugar	12.9
Ghee	9.7
Malt	9.7
Butter	4.8
Liquid glucose	4
Date powder	0.8
Sesame seeds	0.8
Raisin (black, seeded)	0.8
Chia seeds	0.3

Figure 3.2.5 Raw Ingredients

3.2.5 Process Optimization of Protein Energy Bars

RSM is widely recognised as an effective process optimisation technique, especially when independent variables like the sources of protein are expected to have separate or combined effects on the intended results (Nadeem et al., 2012). In the present study, the advanced statistical software program named Design Expert-8.0.3 (Stat-Ease, Inc., Minneapolis) enabled process optimisation using RSM was employed to determine the ideal formula for developing P-E Bars. Based on these fundamental concepts, the research was designed to produce a nutrient-dense bar with a good sensory appeal and an economic potential.

Optimization of Levels of Crispies, WPC-80 and SPI for Development of P-E Bars using RSM

RSM using the CCRD (three factor RSM) was applied to optimize the levels of Crispies (6.5-8.1 per cent), WPC-80 (14 to 15 per cent) and SPI (14 to 15 per cent). It uses statistical methods such as regression analysis to fit a mathematical model to the acquired data.

Researchers often require understanding how various variables influence a desired outcome or reaction. To achieve this, they use experimental designs such as the Central CCRD. This design requires adjusting three variables, which are commonly classified as low, moderate and high levels, to determine how they influence the response variable. CCRD is valuable since it predicts and optimises outcomes by systematically altering these variables and observing the results. By performing trials at various positions on a sphere, CCRD allows for an in-depth analysis of the interactions between the components and the response variable. (Yolmeh and Jafari, 2017)

CCRD consists of factorial points, centre points and star points, which aids in curvature estimate. This design calculates the total number of experiments using the formula $(2)^n + 2n$ + central points, where n is the number of variables. For three variables, each important component requires a total of 20 trials (Gupta, 2016).

The software recommended 20 runs (trials) for the preparation of P-E Bars, as outlined in the design matrix of the three-factor CCRD provided in table 3.2.3. P-E Bars were prepared as per the procedure outlined in figure 3.2.6. Sensory attributes hold considerable importance in determining the acceptability of Protein Energy Bars during their development. A panel consisting of 37 semi-trained members from the Department of Foods and Nutrition, The Maharaja Sayajirao University of Baroda conducted a sensory evaluation of the P-E Bars, based on their run order. Consent was obtained to enrol the panellist (annexure XIII and XIV). This evaluation employed both a Composite Score Card (CSC) (annexure XV) and a 9-Point Hedonic Scale (9-PHS) (annexure XVI). Mean responses from the panellists were collected and thereupon entered into software to derive regression equations for the attributes. This data was utilized for further optimization processes. The software gave a regression equation by optimizing varying levels of the independent variables and describing their effect on the plotted three-dimensional (3D) graphs. The results were further validated by conducting a sensory analysis and using the mean of the seven trials.

Table 3.2.3 Experimental design matrix (CCRD) for levels of factors: Crispies, WPC-80 and SPI

Run No.	A: Crispies (%)	B: WPC-80 (%)	C: SPI (%)
1	7.3	14.5	14.5
2	7.3	15.3	14.5
3	7.3	14.5	14.5
4	7.3	13.7	14.5
5	6.5	15.3	13.7
6	7.3	14.5	15.3
7	7.3	14.5	13.7
8	8.1	13.7	13.7
9	7.3	14.5	14.5
10	6.5	13.7	13.7
11	7.3	14.5	14.5
12	8.1	13.7	15.3
13	7.3	14.5	14.5
14	6.5	14.5	14.5
15	8.1	15.3	13.7
16	6.5	13.7	15.3
17	8.1	14.5	14.5
18	8.1	15.3	15.3
19	6.5	15.3	15.3
20	7.3	14.5	14.5

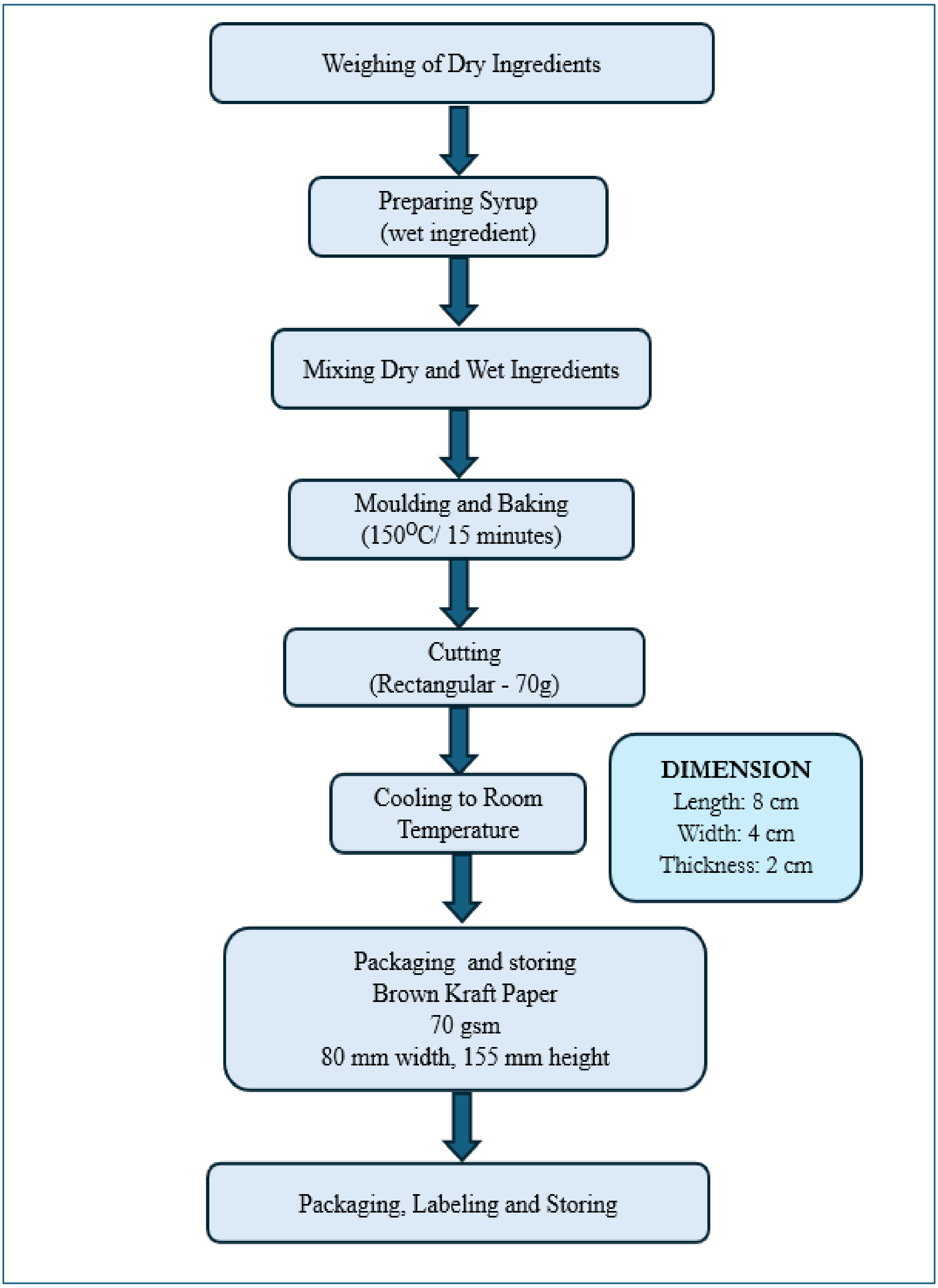

Figure 3.2.6 Tentative Flow Diagram of the Process of Development of Protein Energy Bars

Mixing of the Ingredients

Sesame seeds, raisins and chia seeds were individually roasted. Preheating the oven to 350°F (175°C), sesame seeds and chia seeds were spread evenly on separate baking sheets lined with parchment paper or silicone baking mats. Roasting for approximately 8-10 minutes, with occasional stirring, resulted in a golden brown and fragrant outcome. For black raisins, the oven was preheated to 275°F (135°C), with the raisins spread evenly on a prepared baking sheet. Roasting for about 15-20 minutes and stirring periodically, produced plump and caramelized raisins. Sugar underwent conversion into caramelized syrup at 150°C for 20 minutes, contributing as a moist ingredient.

Dry components such as crispies, WPC-80, SPI, peanuts, date powder, sesame seeds, raisins and chia seeds were weighed according to predetermined rates derived from RSM. Additionally, caramelized sugar syrup, melted chocolate chips, ghee, malt, butter, liquid glucose and soy lecithin were weighed according to their respective addition rates, forming the wet ingredient. The warm syrup was then thoroughly combined with the pre-weighed dry ingredients, ensuring a homogenous mixture.

Moulding and Baking

Following baking, the contents were allowed to cool down to room temperature and then cut into 70g rectangular pieces. The dimensions of the P-E Bars were 8 cm in length, 4 cm in width and 2 cm in thickness.

Packaging and Storage

The Protein-Energy Bars were then packaged and sealed in laminated brown kraft paper, which had a weight of 70 gsm and dimensions of 80 mm in width and 155 mm in height.

Equipment / Instruments Used

Details regarding the equipment and instruments utilized in the study for the development of Protein-Energy Bars are provided in table 3.2.4. In phase III of the study, the P-E Bars underwent analytical study, involving chemical, physical, organoleptic, microbial, shelf life and cost analysis.

Hypotheses- Phase II

Null Hypothesis (H_0): No significant relationship exists between the ingredients of the Protein Energy Bar and its sensory attributes, indicating that adjusting ingredient proportions does not significantly alter sensory characteristics.

Alternative Hypothesis (H_1): There is a significant relationship between the ingredients of the Protein Energy Bar and its sensory attributes, suggesting that adjusting ingredient proportions leads to noticeable improvements in sensory characteristics.

Figure 3.2.7 Production of Protein-Energy Bars

Table 3.2.4 Equipment/Instruments for P-E Bar Development Process

Sr. No	Equipment/ Instruments and Software	Details
1.	Analytical Balance	Sartorius, England
2.	Co- rotating twin screw extruder	BTPL lab model, Basic Technology Pvt. Ltd. Kolkata, India
3.	Hot Air Oven	Model No. IK-III, Make: IKON, India
4.	Food Processor	Bajaj Nx 01 300 W Mixer Grinder
5.	Oven	Morphy Richards 60-Litre 60 RCSS 60L Luxe Series OTG Oven Toaster Grill (OTG)

Phase III

3.3 Characterization and Costing

The third phase of this study aims to comprehensively evaluate the P-E Bars through a multi-faceted approach encompassing the chemical, physical, textural, organoleptical, microbial, shelf-life and cost analysis. This phase serves as a critical step in assessing the overall quality, safety and economic feasibility of the formulated bars.

3.3.1 Characterization of Protein Energy Bars

A semi-trained panel conducted a sensory evaluation of the Protein Energy Bars using a CSC and a 9-PHS. Proximate analysis was performed to determine the levels of moisture, protein, ash, crude fat and crude fiber and nitrogen following the methods outlined in AOAC, 21st Edition, 2019. Carbohydrate content was determined using the difference method, while energy values were calculated based on FDA-regulated product Labeling requirements (annexures VI to XII and XVII). Physicochemical properties such as peroxide value, free fatty acid content (expressed as oleic acid), acid value and water activity (annexures XVIII to XXI) were assessed according to AOAC guidelines and a study by Pardo et al. (2004). Texture analysis was conducted using a

Texture Analyser from Stable Micro Systems, located in Surrey, UK (annexure XXII). Microbial analysis involved testing for yeast and mold according to IS 5403:1999 reaffirmed in 2018 (annexure XXIII), total coliforms following IS 5401 (Part 1) (annexure XXIV) and total bacterial counts (TBA) using IS 5402 (Part 1) (annexure XXV).

3.3.2 Shelf-Life Analysis

The shelf life of a food product is defined as the duration during which it maintains an acceptable level of eating quality, as assessed through sensory (panel of 18 semi trained members), physical, functional, chemical and microbial evaluation. This period is influenced by several key factors, including the product's formulation, processing methods, packaging, storage and distribution logistics management (Gilbert and Prusa, 2021). To accurately assess the shelf life, the food product can be exposed to controlled environments where one or more extrinsic factors, such as temperature or humidity, are maintained at higher-than-normal levels. This allows for the quantification of deterioration rates and the calculation of acceleration magnitude, which in turn helps in estimating the "true" shelf life of the product under normal conditions (Robertson, 2005). However, to reduce the time needed to estimate a shelf life, which would otherwise take an unreasonably lengthy period to ascertain (at least in terms of new product development), accelerated shelf-life testing, or ASLT, is utilised (Gilbert and Prusa, 2021). The data were presented as mean ± SD and analyzed using Student's t-test in MS Excel. For one-way ANOVA, significance of mean differences during storage period was assessed by Duncan's post-hoc test, with $p \leq 0.05$ considered as the significant level of difference, also performed using SPSS 20.

To hold 70 g of Protein Energy Bars (recommended serving size), brown Kraft paper with a 12 mm aluminium surface barrier coating was 80 mm wide and 155 mm height. They were stored at 35 ± 2°C and 70 per cent relative humidity (RH) for 70 days. In an accelerated shelf-life research, materials were analysed for chemical composition, texture, sensory characteristics and microbiological quality every 7 days.

3.3.3 Cost Analysis

The cost analysis method utilized in this study involved a comprehensive breakdown of expenses associated with producing one batch of P-E Bars. Initially, raw material costs were calculated based on the quantities and rates of each ingredient used per batch. Subsequently, utility costs were determined, incorporating expenses for electricity, heat energy, packaging and labeling. Overhead costs were then added, comprising 10 per cent of the combined raw material and utility costs. The total cost for one batch of P-E Bars was obtained by summing the raw material, utility (Electricity cost, annexure XXVI) and overhead costs. Finally, the cost per individual P-E Bar (weighing 70g) was calculated by dividing the total batch cost by the number of bars produced in one batch.

Labelling plays a vital role in providing consumers, including athletes, fitness enthusiasts and individuals seeking convenient and nutritious snacks, with essential information about P-E Bar. It includes details regarding the product's ingredients, nutritional content, price, expiry date and suitability for vegetarians. The Nutrition information along with the shelf stability were mentioned in the food label curated for the P-E Bars.

Table 3.3.1 Parameters ant Tools used in Characterization and Costing of P-E Bars

Parameters	Tools
Sensory Evaluation (Semi Trained Panel)	Composite Score Card
	9 – Point Hedonic Scale
Proximate Analysis	Protein, Crude Fat, Crude Fiber, Moisture and Ash: AOAC, 21st Edn, 2019
	Carbohydrate: By difference method
	Energy: Food Labeling Requirements for FDA Regulated products
Physico Chemical Properties	Peroxide value, Free Fatty Acid (as oleic acid), Acid value: AOAC, 21st Edn, 2019
	Water activity: Pardo, E., et., al., (2004)
Texture Analysis:	Texture Analyser - Stable Micro Systems, Surrey, UK
Microbial Analysis:	Yeast and Mould: IS 5403:1999 reaffirmed 2018
	Total Coliforms: IS 5401 (Part 1)
	Total Bacteria Found: IS 5402 (Part 1)
Shelf-life Study:	In the ASLT, samples were analyzed for sensory attributes, physico-chemical characteristics, texture and microbial quality every seventh day for 70 days.
Cost Analysis	Calculation of raw material, utility, overhead, packaging and Labeling cost per batch of 10 kg material, then dividing it with the per unit weigh of 70 g.

Hypotheses- Phase III

1 **Null Hypothesis (H_0):** There will be no significant effect of varying levels of milk and pulse protein on the quality of the protein bars.

1 **Alternative Hypothesis (H_1):** Varying levels of milk and pulse protein will significantly affect the quality of the protein bars.

2 **Null Hypothesis (H_0):** The developed protein-energy bar will not possess commercial value.

2 **Alternative Hypothesis (H_1)**: The developed protein-energy bar will possess commercial value.

3 **Null Hypothesis (H_0):** The Protein-Energy Bars will receive low acceptability scores on a Composite Score Card and a 9-Point Hedonic Scale as assessed by the semi-trained panel.

3 **Alternative Hypothesis (H_1):** The Protein-Energy Bars will receive high acceptability scores on a Composite Score Card and a 9-Point Hedonic Scale as assessed by the semi-trained panel.

4 **Null Hypothesis (H_0):** The texture of the P-E Bars will not show acceptable scores in terms of hardness and fracturability on the instrumental analysis.

4 **Alternative Hypothesis (H_1):** The texture of the P-E Bars will show acceptable scores in terms of hardness and fracturability on the instrumental analysis.

5 **Null Hypothesis (H_0):** The shelf life of the standardized product will be outside the acceptable range.

5 **Alternative Hypothesis (H_1):** The shelf life of the standardized product will be within the acceptable range.

Phase IV

3.4 Acceptability Trials Involving Athletes, Coaches and Fitness Trainers

Acceptability trials are a crucial phase in the development and refinement of products, services, or interventions aimed at enhancing athletic performance, fitness, or overall health (Géci, et al. 2020). Hence, in this phase, the crucial aspect of acceptability trials conducted to assess the reception and preferences of physically active individuals towards the study bar was addressed. The study was carried out using a semi-structured questionnaire (annexure XXVII) that included a wide range of questions, covering demographics, snack preferences, propensity towards healthy bars and satisfaction with study bars. In addition, the study examined the participants' exercise habits, their perception of food bars and their sensitivity to cost. The protein energy bar was assessed using a 9-point hedonic scale (annexure XXVIII), which provided information on its palatability and overall appeal. A group of 120 persons who engage in regular physical activity participated in this study phase and were willing to participate along with the consent (annexures XXIX and XXX), which aimed to provide a thorough knowledge of the bar's ability to suit the needs of health-conscious customers within the active community.

The target population comprised of athletes from the Department of Physical Education, University Pavilion, The Maharaja Sayajirao University of Baroda and The Fitness trainers belonging to various gymnasiums of Vadodara City. By interacting with end-users that the final product aligns with their requirements, preferences and expectations.

Hypotheses- Phase IV

Null Hypothesis (H_0): The Protein-Energy Bars will not meet acceptability standards among athletes, coaches and fitness trainers regarding sensory attributes and cost-effectiveness.

Alternative Hypothesis (H_1): The Protein-Energy Bars will meet acceptability standards among athletes, coaches and fitness trainers regarding sensory attributes and cost-effectiveness.