PROCESS OPTIMIZATION FOR BEE POLLEN INCORPORATED DAIRY PRODUCT

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2025

DECLARATION

I, hereby declare that the presented work in the thesis entitled **“Process Optimization for Bee Pollen Incorporated Dairy Product”** in fulfilment of degree of **Doctor of Philosophy (Ph.D.)** is the outcome of research work carried out by me under the supervision of **Dr. Prasad Rasane**, Professor and Head of Department (Food Technology and Nutrition), School of Agriculture, Lovely Professional University, for the award of degree Ph.D. Food Science and Technology.

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We certify the above statement made by the student is correct to the best of my knowledge and belief**.**

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "Process Optimization for Bee Pollen Incorporated Dairy Product" submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Food Technology and Nutrition, School of Agriculture, is a research work carried out by Jaspreet Kaur, 12009715, is bonafide record of her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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ABBREVIATIONS

%	Percent
/	per
@	at the rate of
μg	micrograms
a_w	water activity
°C	Degree centigrade
AAE	Ascorbic acid equivalents
ABTS	(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACE	Angiotensin-converting enzyme
ADMA	Asymmetric di-methylarginine
AKT–GSK-3β	Protein kinase B-Glycogen synthase kinase-3β
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
AST	Aspartate aminotransferase
ASTM	American Society for Testing and Materials
BPE	Bee pollen extract
BHT	Butylated hydroxytoluene
BUN	Blood urea nitrogen
C3GE	cyanidin-3-glucoside equivalent
cAMP	Cyclic Adenosine Monophosphate
CAT	Catalase
CCD	Central composite design
CE	Catechin equivalent
cfu	Colony forming unit
cm	Centimetre(s)
Cr	Creatinine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Distilled water
EA	Emulsifying activity
EDS	Energy dispersive X-ray spectrometry
EFSA	European Food Safety Authority
ERK–CREB	Extracellular signal-regulated kinase–Cyclic AMP Response Element-Binding Protein
ES	Emulsion stability
et al.	And others
Eth	Ethanol
FC	Foaming capacity
FDA	Food and Drug Administration
Fig.	Figure
FFA	Free fatty acids
FRAP	Ferric reducing antioxidant power
FS	Foam stability
FSSAI	Food safety and standards authority of India
FTIR	Fourier transform infrared spectroscopy
g	grams
GAE	Gallic acid equivalent
GC-MS	Gas chromatography-Mass spectrometry
GI	Gastrointestinal
GSH	Glutathione
HC	Hung curd
HMF	Hydroxymethulfurfural
HPLC	High-performance liquid chromatography
HPP	High pressure processing
ICP-OES	Inductively coupled plasma optical emission spectroscopy
i.e.	That is
Ig	Immunoglobulin
IGF	Insulin-like Growth Factor

KBr	Potassium bromide
Kcal	Kilocalorie
kg	Kilogram
kU	Kilo unit
kV	Kilovolt
l	Liter
LAB	Lactic acid bacteria
LDL	Low-density lipoprotein
Log	Logarithm
MCF-7	Michigan Cancer Foundation-7
MDA	Malondialdehyde
mg	Milligram
min	Minute(s)
MF	Multifloral
MFBP	Multifloral bee pollen
MFBPE	Multifloral bee pollen extract
ml	millilitre
MPa	Megapascal
MRJPs	Major royal jelly proteins
MS	Mustard
MSBP	Mustard bee pollen
MSBPE	Mustard bee pollen extract
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAFLD	Non-alcoholic fatty liver disease
ND	Not Detected
NO	Nitric oxide
OAA	Overall acceptability
OAC	Oil absorption capacity
PBS	Phosphate-Buffered Saline
PCA	Plate count agar
PDA	Potato dextrose agar

PET	Polyethylene terephthalate
pH	Potential of Hydrogen
PFA	Prevention of food adulteration
PMN	Polymorphonuclear
PP	Polypropylene
ppm	Parts per million
FRAP	Ferric reducing antioxidant power
pro-BDNF	pro-brain-derived neurotrophic factor
psi	Pounds per square inch
PTFE	Polytetrafluoroethylene
QE	Quercetin Equivalent
RDA	Recommended dietary allowance
RDI	Reference Daily Intake
ROS	Reactive oxygen species
Rpm	Revolutions per minute
Rs.	Rupees
SEM	Scanning electron microscopy
SOD	Superoxide Dismutase
TA	Tannic acid
TBA	Thiobarbituric acid
TE	Trolox equivalents
TFC	Total flavonoid content
TNF	Tumor necrosis factor
TPC	Total phenolic content
TPZ	Tripyridyltriazine
TSS	Total soluble solids
USD	United states dollar
VRBLA	Violet red bile lactose agar
WAI	Water absorption index

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ABSTRACT

These days, an increased consumer awareness regarding health and wellness has led to a significant rise in the formulation of functional food products. This research was directed to focus on the inclusion of bee pollen, a nutrient-dense functional food ingredient, into a traditional Indian dairy product, Shrikhand. Bee pollen is a rich source of proteins, carbohydrates, lipids, vitamins, minerals and bioactive compounds, which makes it an ideal candidate for enhancing the nutritional potential of dairy products (particularly the phenolic content). However, a few challenges need to be addressed before using it as a food component. These challenges include allergenicity, digestibility and processing limitations. This study was conducted to optimize the process of incorporating bee pollen extract into *Shrikhand*, with the objective of enhancing its total phenolic content and antioxidant activity. Different pre-treatments and process parameters were evaluated to maximize nutritional and sensory attributes. The optimized product was subjected to physicochemical analysis, antioxidant profiling and shelf-life evaluation. The results of the experiment revealed that bee pollen incorporation significantly increased the phenolic content, antioxidant activity and overall acceptability of Shrikhand.

Objective 1 was to screen and analyze two different bee pollen varieties for their potential utilization as functional ingredients. Two varieties, mustard bee pollen (MSBP) and multifloral bee pollen (MFBP), were chosen (depending on the ease of availability) and evaluated for their physicochemical and phytochemical properties. The results indicated that mustard bee pollen (MSBP) has elevated levels of moisture (15.33 ± 0.36%), ash content (4.3 ± 0.014%), total phenolic content (2676 ± 0.58 mg GAE/100 g), and total flavonoids (14.025 ± 0.25 mg QE/g), which contribute to its high antioxidant activity (82.60 ± 0.54%) when compared to multifloral bee pollen (MFBP). Mineral analysis using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) confirmed the presence of essential minerals such as magnesium, calcium, iron, phosphorus, and zinc in the bee pollen samples. Importantly, no traces of toxic heavy metals—including arsenic, mercury, cobalt, or lead—were detected in any of the varieties examined, indicating their safety for potential nutritional applications. Additionally, Fourier Transform Infrared

Spectroscopy (FTIR) facilitated the identification of various stretching, bending, and vibrational characteristics associated with different carbohydrates, protein functional groups, and phenolic compounds.

Objective 2 involved optimizing process parameters for incorporating bee pollen into Shrikhand. Various pre-treatments including- thermal cracking, alkaline pre-treatment and wet heat thermal pre-treatment were applied to enhance digestibility and study their impact on the phenolic content of MSBP and MFBP using HPLC analysis. The conclusions drawn showed that the optimal pre-treatment for MFBP was thermal cracking at 40 °C, yielding the highest concentration of ferulic acid (254.475 mg/L), quercetin (530.719 mg/L), and cinnamic acid (185.962 mg/L). For MSBP, the best results were obtained with thermal cracking at 35 °C, yielding ferulic acid (192.480 mg/L) and cinnamic acid (143.718 mg/L). It was inferred that alkaline and wet heat thermal treatments were less effective as compared to thermal cracking. These findings suggested that controlled pre-treatments effectively enhance the release of phenolic compounds, which enabled the improvement of nutritional quality and functional properties of the bee pollen varieties under this study. After applying these pre-treatments, bee pollen extracts were prepared and evaluated to analyze the phytochemical composition. The optimization was conducted using response surface tool post preliminary trials conducted to determine the range of variables. The extract having highest values of phenolic content and antioxidant activity was incorporated in Shrikhand at concentrations varying from 2.5 ml to 12.5 ml. Numerical optimization of the mustard bee pollen extract-based Shrikhand yielded the optimum product with 10.252 ml mustard bee pollen extract, 77.555 g hung curd and 22.652 g sugar concentration, with 0.914 desirability. While, the optimized formulation with 10.408 ml multifloral bee pollen extract, 75.250 g hung curd and 22.477 g sugar with 0.900 desirability was selected for the development of multifloral bee pollen extract-based Shrikhand. The optimized product showed acceptable results on physicochemical, and sensory basis. The observed values of the different responses for mustard bee pollen extract-based Shrikhand were as following: 36.63% moisture content, 4.36 pH, 1.25% titratable acidity, 0.86% ash content, 67.65 °Brix TSS, 73.01% antioxidant activity, 256.39 mg GAE/100 g total phenolic content, 1133.32 mg QE/100 g total flavonoid content, and 8.15 overall acceptability. The observed values of the different responses for multifloral bee pollen extract-based Shrikhand were as: 33.02% moisture content, 4.30 pH, 1.22% titratable acidity, 0.82% ash content, 65.94 °Brix TSS, 68.95% antioxidant activity, 248.56 mg GAE/100 g total phenolic content, 1096.32 mg QE/100 g total flavonoid content, and 8.05 overall acceptability.

Objective 3 was aimed to determine the shelf-life and sensory attributes (with a 9-point hedonic scale) of the optimized Shrikhand samples. Shrikhand samples were stored at two different temperatures 4 °C and 25 °C in polypropylene (PP) and polyethylene terephthalate (PET) cups for 28 days. The data revealed that PET cups showed the better retention of antioxidant content and total polyphenolic content, and lower degradation of compounds into degradation metabolites such as hydroxymethulfurfural (HMF), thiobarbituric acid (TBA) and free fatty acid (FFA). It was observed that the samples stored in PET cups maintained relatively better scores ($p<0.05$) in terms of flavour, appearance, texture, mouthfeel and overall acceptability in comparison to the PP cups. Microbiological analysis also revealed that the growth of micro-organisms in samples kept at 4 °C were lower than the ones stored at 25 °C. No coliforms were detected in any sample at any stage of the study. The cost analysis showed the total cost for mustard bee pollen extract-based Shrikhand to be ₹25.11 per 100 g and ₹24.29 for multifloral bee pollen extract-based Shrikhand, suggesting that the optimized product was economical while offering valuable nutritional and functional benefits.

This research successfully optimized the incorporation of mustard and multifloral bee pollen into Shrikhand, enhancing its nutritional profile, antioxidant capacity, and sensory properties. Therefore, this study provides valuable insights into developing functional dairy products enriched with bee pollen, which offers potential health benefits and addresses growing consumer demand of functional foods.

Keywords: Bee pollen, Functional food, Mustard bee pollen, Multifloral bee pollen, Shrikhand, Phenolic content, Flavonoid content, Antioxidant activity, Process optimization, Pre-treatment methods, Shelf-life study, Sensory evaluation, Bioactive compounds, Physicochemical analysis

CHAPTER 1

INTRODUCTION

The primary role of diet is not only confined to hunger satisfaction or individual survival but rather also includes disease prevention and promoting the overall well-being of the consumer (Jnawali *et al.*, 2016; Suri *et al.*, 2019). Owing to the increased awareness of the population towards the importance of a healthy lifestyle, the current emphasis is laid on the role of functional foods in overall health promotion (Suri *et al.*, 2019). Thus, the functional food industry is considered to be one of the fastest-growing sectors which aims at the development of biologically active foods, beverages, and supplements by the addition of new components or increasing the amount of existing health-promoting components (Karabagias *et al.*, 2018). Functional foods are destined to have a physiological benefit and alleviate the risk factors responsible for causing chronic health problems besides providing the basic nutrients. Moreover, it should be considered that the functional foods must be similar to traditional/conventional foods and consumed as a part of a daily diet (Karabagias *et al.*, 2018). Along the same line, the current trend is the exploration of bee pollen as a functional ingredient, highlighting its health benefits and nutrient-rich composition (Thakur and Nanda, 2019).

Worker honeybees produce bee pollen by combining flower pollen with salivary secretions and nectar. The complete process of bee pollen collection by worker bees is illustrated in Table 1.1. The chemical and nutritional composition of bee pollen varies significantly and is influenced by several factors, including floral source, seasonal variations, geographical location, soil characteristics, colony type, and beekeeping practices (Karabagias *et al.*, 2018). Bee pollen is often regarded as a nutrient powerhouse because it contains a wide range of macro- and micronutrients *viz*., carbohydrates (40–60%), proteins (20–60%), lipids (1–32%), vitamins, minerals, phenolic compounds, carotenoids and other bioactive compounds (Thakur and Nanda, 2019) (Table 2.1). It exhibits diverse pharmacological properties such as antimicrobial, antioxidant, anti-inflammatory, anticarcinogenic, antimutagenic, hepatoprotective, immunomodulatory as well as local analgesic action (Karabagias *et al.*, 2018; Kaur *et al.*, 2024).

Table 1.1. Process outlining collection of bee pollen (Source: Ismail *et al.*, 2012; De-Melo *et al.*, 2016; Wood *et al.*, 2018; Lau *et al.*, 2019; Thakur and Nanda, 2020)

Process steps	Description
 Foraging by worker honeybees	A colony with 10,000-15,000 worker bees is able to collect about 13-17 kg/year approximately.
 Flower pollen	There are different plant species which belongs to different botanical families from which bees collected pollens by traveling up to 4.0 km radius.
 Honeybee covered with microscopic pollens	The flower pollen is mixed with the nectar and salivary secretions of honeybee.
 Honeybee carrying pollen pellet on the hind leg	The bee-collected pollen is carried on the hindleg of honeybees. A single pollen possess size of about 5–200 µm diameter and showcase a wide variety of colours attributable to the variability of plant species.

 Pollen trap at hive entrance	Upon returning, bees pass through a pollen trap positioned at the entrance of the hive. It consists of a wooden box having slope and two vertical strips having length 32 cm and width 17 cm, with 4 holes/inch.
 Bee-collected pollen	Pollens are collected and specimen identification is done by using light microscopy.

Recent research suggests that bee pollen has potential applications as a functional ingredient in the formulation of diverse products, including fermented and non-fermented beverages, baked goods, confectioneries, and meat-based items (Suri *et al.*, 2019). Although bee pollen is an abundant source of nutrients, however, its consumption is also associated with allergic symptoms and cross-reactions with other allergens. Therefore, it is essential to develop bee pollen-based food products that are affordable, nutritionally balanced, and appealing to consumers. Special attention must be given to ensuring economical, nutritional, and organoleptic attributes as well as compliance with concerned regulatory bodies. Addressing these aspects will help researchers, industrialists, and manufacturers to develop bee pollen-based food products and beverages that are nutritious, safe for consumption, and meet the needs of different age groups. Bee pollen might be regarded as a viable functional food ingredient.

Dairy products like Shrikhand, a popular Indian fermented dessert, provide an excellent medium for incorporating functional ingredients. Shrikhand is widely accepted across age groups due to its appealing taste, texture, and nutritional value. Conventional dairy products lack significant phenolic content and antioxidant activity. Incorporating bee pollen extract into Shrikhand can enhance its nutritional profile, offering consumers a functional food that promotes health and well-being. Shrikhand was chosen as the target dairy product due to its wide consumer acceptance, semi-solid texture suitable for fortification, and its potential to mask the flavor of functional ingredients. Moreover, Shrikhand's composition allows easy incorporation of bioactives without compromising sensory appeal.

The current research aims to optimize a process of incorporating bee pollen into Shrikhand, addressing challenges related to digestibility, sensory attributes, and shelf-life. The research objectives include screening bee pollen varieties, optimizing incorporation parameters, and evaluating the physicochemical properties, antioxidant activity, as well as storage stability of the developed product. The findings of this study will provide guidelines for the food industry to develop bee pollen-based functional dairy products that meet consumer demand for health-promoting foods.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Importance of developing bee pollen-based food products

Bee pollen is widely acknowledged as a functional food ingredient due to its rich composition of bioactive compounds and associated health-promoting properties. Hence, the incorporation of bee pollen for developing different value-added food products will be a step forward to providing more nutrients via foods. It has been reported to be 'the only perfectly complete food' due to the presence of a significant amount of carbohydrates, fats, protein, phytosterols, phenolic compounds, carotenoids, enzymes, bio-elements, vitamins, nucleic acids, and triterpene acids (Nascimento and Luz, 2018).

Table 2.1. Nutritional composition of bee pollen

Components	Amount	Reference
Carbohydrates (g/100 g)	13–55	Bogdanov (2016)
Protein (g/100 g)	10–40	
Crude fibre (g/100 g)	0.3–20	
Fat (g/100 g)	1–13	
Potassium (mg/100 g)	400–2000	Bogdanov (2016)
Calcium (mg/100 g)	20–300	
Zinc (mg/100 g)	3–25	
Iron (mg/100 g)	1.1–17	
Magnesium (mg/100 g)	1.21	Heldt *et al.* (2019)
Copper (mg/100 g)	0.08	
Manganese (mg/100 g)	0.17	
Ascorbic acid (mg/100 g)	0.19–0.21	Komosinska-Vassev *et al.* (2015)
β-carotene (mg/100 g)	1–20	
Tocopherol (vit E) (mg/100 g)	4–32	

Niacin (mg/100 g)	4–14.4	Bogdanov (2016)
Vitamin B9 (mg/100 g)	0.3–1	
Biotin (H) (mg/100 g)	0.05–0.07	
Thiamine (B1)	0.74–0.77	Heldt *et al.* (2019)
Riboflavin (B2)	0.78–0.82	

The presence of these phytonutrients highlights the role of bee pollen in managing various health problems due to its extensive variety of pharmacological activities. It has been confirmed to exhibit antioxidant, antifungal, antimicrobial, anticancer, antidiabetic, antitoxic, antimutagenic, anti-inflammatory, antiradiation, chemo-preventive and hepatoprotective properties (Karabagias *et al.*, 2018; Nascimento and Luz, 2018). The global burden of chronic health problems has increased tremendously as a result of environmental, genetic, behavioural as well as physiological factors (Gogna *et al.*, 2023). The leading causes of death among the adult population are heart diseases, followed by cancer, respiratory tract problems and digestive disorders. Due to this increased burden, the pharmaceutical market has escalated remarkably to cure the aforementioned deadly diseases but at the same time, drug manufacturing companies are becoming extremely diverse, complex, less regulated, and less affordable (George, 2019). Research studies have elucidated a strong interrelation between diet and health status of an individual. Consumers have developed a positive outlook on the consumption of a healthy diet instead of depending upon expensive drugs for disease prevention, which has further increased the demand for functional food products all across the globe. The world functional food market was valued at approximately USD 161.49 billion in 2018 and is projected to reach USD 529.66 billion by 2028, indicating substantial growth in consumer demand for health-promoting food products (Balogh *et al.*, 2020). Based on increased consumer demand and interest in including functional foods as a part of their daily diet, manufacturers and health professionals have emphasized the development or incorporation of functional ingredients in conventional food products. Along the same line, bee pollen is considered to be a valuable front-runner among biologically active ingredients;

therefore, its addition to foods will prove to be beneficial in the management of several medical as well as nutritional problems.

2.2. Health implications of bee pollen

Bee pollen contains essential amino acids that play a crucial role in supporting optimal growth and development, primarily through the regulation of gene expression and cellular signalling pathways. Due to its nutrient-dense chemical composition, bee pollen has gained recognition as a natural dietary supplement with a wide range of therapeutic potentials. Studies have demonstrated its protective effects against various health conditions, including diabetes and obesity (Cheng *et al.*, 2019), non-alcoholic fatty liver disease (Huang *et al.*, 2017), acute myocardial infarction and atherosclerosis (Rzepecka-Stojko *et al.*, 2017), hyperuricemia (Wang *et al.*, 2018), oxidative stress, and certain types of cancer (Khalifa *et al.*, 2021). Additionally, it has shown efficacy in mitigating fluorosis (Khalil and El-Sheikh, 2010), ovarian and testicular dysfunction, intestinal damage (Toman *et al.*, 2015), food-borne mycotoxicity, and prostatitis (Khalifa *et al.*, 2021). Bee pollen also contributes to cognitive improvement (Liao *et al.*, 2019), regulates bone metabolism, and exhibits immunostimulatory properties (Yamaguchi *et al.*, 2006; Khalifa *et al.*, 2021). An overview of its health benefits and underlying mechanisms of action is presented in Figure 2.1.

The bioactive metabolites present in bee pollen have been shown to enhance cognitive function by facilitating the conversion of pro-brain-derived neurotrophic factor (pro-BDNF) to its mature form. This effect is mediated through the activation of key signalling pathways, including the ERK–CREB pathway and the AKT–GSK-3β signalling pathway (Liao *et al.*, 2019). The consumption of bee pollen leads to morphological changes in the small intestine including a relative increase in the epithelial volume, increased depth of Lieberkühn, and longer villi, which in turn increase the surface area for better absorption of nutrients in intestinal mucosa (Toman *et al.*, 2015). It suppresses the activity of enzymes present in the human intestine that break down polysaccharides into simple glucose molecules, thereby preventing a rise in blood sugar levels (Shobana *et al.*, 2009). Research studies have also stated the significant role of bee pollen in the amendment of testicular dysfunction by reducing oxidative stress, enhancing testicular antioxidant defense system, and increasing the levels of spermatids, spermatocytes, spermatogonia, and Sertoli cells (Khalifa *et al.*, 2021). It also contributes to the up-regulation of ovarian secretions (progesterone and estradiol) as well as pro-apoptotic and anti-apoptotic molecules, thus regulating ovarian functions. The phenolic compounds in bee pollen accelerates lipid metabolism, enhances nutrient absorption, and hence promotes weight loss (Cheng *et al.*, 2019). Its consumption (1g/kg body weight) is also linked with ameliorating degenerative changes in hepatocytes, hepatic steatosis, and non-alcoholic fatty liver disease (NAFLD). Moreover, the consumption of high doses of bee pollen aids to decrease the activity of hepatic enzymes, blood urea nitrogen (BUN) as well as creatinine (Cr) levels (Huang *et al.*, 2017).

Bee pollen causes a substantial reduction in the levels of low-density lipoprotein (LDL), total serum cholesterol, asymmetric di-methylarginine (ADMA), angiotensin-II and angiotensin-converting enzyme (ACE) which would otherwise lead to the deposition of atherosclerotic plaques in blood capillaries. Flavonoids and other phenolic compounds have been reported to exert multiple beneficial effects, including the inhibition of platelet aggregation, enhancement of nitric oxide synthesis, suppression of xanthine oxidase activity, promotion of antioxidant defense mechanisms, and exhibition of anti-hyperuricemic properties (Wang *et al.*, 2018). Furthermore, research studies have also testified the utilization of bee pollen in ameliorating the signs of fluorine toxicity by decreasing malondialdehyde (MDA) levels, alkaline phosphatase (ALP) activity, BUN, sodium, potassium, and Cr levels (Khalil and El-Sheikh, 2010). Bee pollen poses anabolic effects on bone tissues (diaphyseal or metaphyseal) by increasing calcium deposition, maintaining calcium homeostasis, and increasing the concentration of ALP which is very essential for bone mineralization (Yamaguchi *et al.*, 2006).

Bee pollen boosts the immune system by increasing lymphocyte proliferation due to its amino acids, vitamins, minerals, phenolics, and flavonoids. It also protects the immune system by increasing total serum protein, globulin, and neutrophil polymorphonuclear leukocyte (PMN)/lymphocyte ratio. Additionally, bee pollen has anti-allergic effects by inhibiting factors such as immunoglobulin E (Ig E) binding with mast cells, tumor necrosis factor (TNF) production, signal transduction pathway, and inflammatory mediator production (Calder, 2006).

The reported health-promoting effects of bee pollen, including antioxidant, anti-inflammatory, and immunomodulatory activities, support its potential use as a functional food ingredient (Khalifa *et al.*, 2021). Nevertheless, most findings are based on in vitro or animal models, with limited application-oriented studies focusing on food incorporation. Translating these bioactive properties into stable and palatable food formulations warrants further investigation.

2.3. Specific considerations for developing bee pollen-based food products

Preparation of bee pollen-based food products is a difficult task for manufacturers with the main challenge being the exine layer which hampers its easy digestibility. Although it contains numerous health compounds, the utilization of bee pollen is limited due to its robust outer covering, exine. The removal of the outer tough coat is necessary to increase its nutritional quality and bioavailability. Various techniques including chemical treatments, high temperature, enzymatic hydrolysis, fermentation, and other physical treatments have been employed so far to destroy the exine layer which allows nutrients to flow freely (Khalifa *et al.*, 2021). Apart from this, another challenge faced by the manufacturers is the safety and acceptability of bee pollen as per the guidelines approved by various regulatory bodies (Zarobkiewicz *et al.*, 2017). Therefore, keeping all the above points in mind, several considerations need to be followed by manufacturers while developing any bee pollen-based food products.

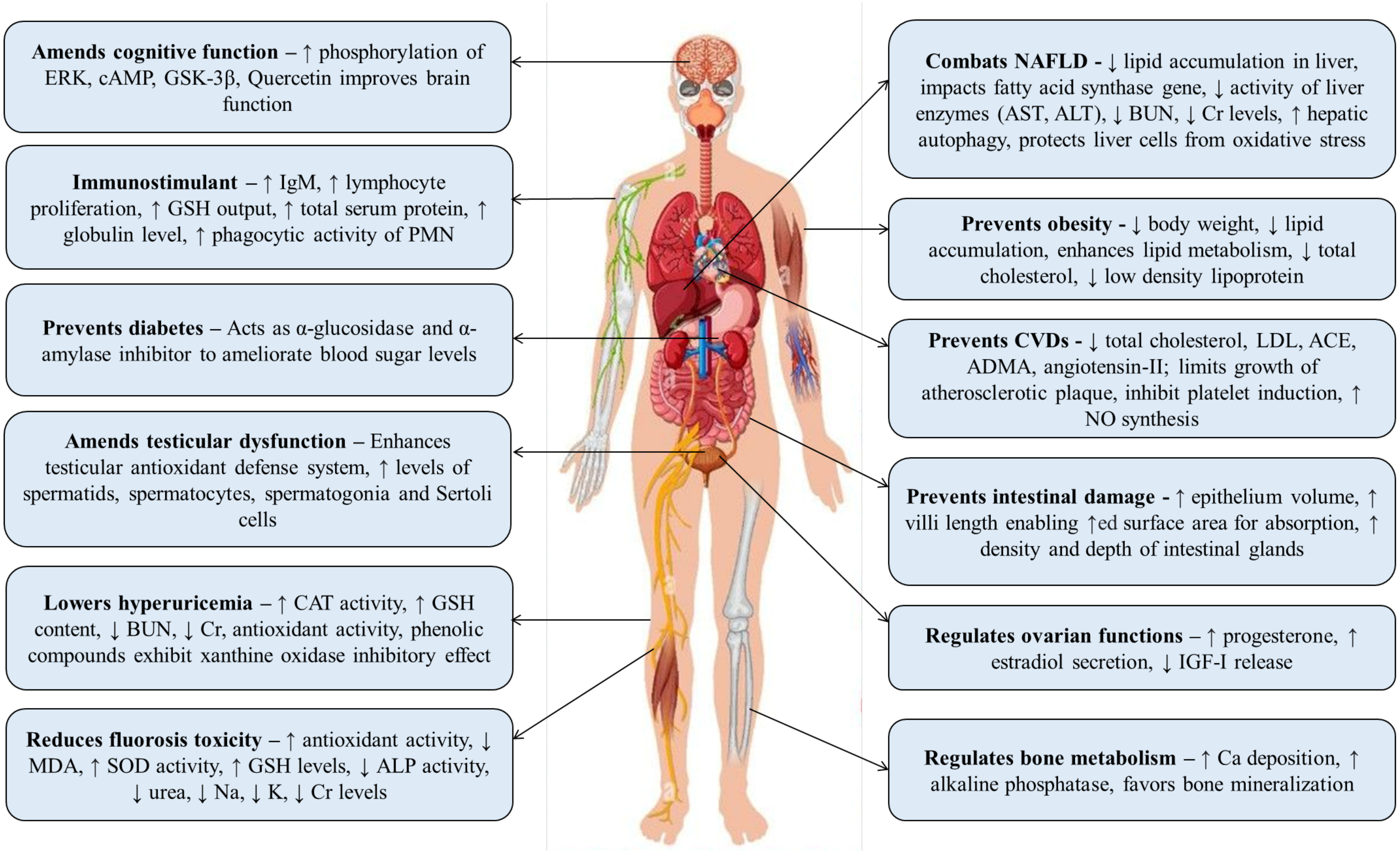


Figure 2.1. Various benefits of bee pollen and their mechanism of action

2.3.1. Digestibility of bee pollen

Pollen cell walls consist of layered concentric structures, with the outmost layer referred to as exine. The pollen coat (exine) is characterized by its stability, flexibility, elasticity, strength, resistance, and firmness, attributed to the presence of sporopollenin. Sporopollenin is composed of aliphatic compounds, phenolics, and hydroxylated fatty acids. It is a strong and inert biopolymer known for its resistance against chemicals, high temperature, microbial action, and enzymatic treatment; which helps to protect the internal compounds of pollen. It consists of two layers, outer sexine and inner nexine (nexine I and nexine II). These biopolymers protect bee pollen from desiccation and unfavourable environmental conditions (Zuluaga *et al.*, 2016). The extraordinary stability and tolerance of exine suggest that bee pollen has to be subjected to processing conditions to make it suitable for human consumption (Wang and Dobritsa, 2018). These processes serve two main purposes: first, to increase the shelf life of the product, and second, to enhance nutritional and functional quality indicators (Benavides-Guevara *et al.*, 2017).

Using chemical treatment to enhance digestibility is considered to be inappropriate while adding bee pollen to food supplements as it involves treatment with monoethanolamine (for 3 hours at 97 ºC), which is a chemical compound. Other chemical methods including acidic pre-treatment and alkaline pre-treatment (3% NaOH) are also employed to enhance the digestibility and also improve the bioavailability of bee pollen nutritional constituents (Benavides-Guevara *et al.*, 2017; Wu *et al.*, 2021). Utilizing acidic solutions for incubation proves to be an effective pre-treatment method for the industrial-scale enzymatic digestion of lignocellulosic materials (Wu *et al.*, 2021). Using concentrated acids might lead to hydrolysis and elimination of additional pollen components, such as polysaccharides, proteins, and lipids found in the intine and pollen coat. This process leaves the sporopollenin-based exine unaffected (Benavides-Guevara *et al.*, 2017). Among the various options, diluted sulfuric acid stands out as the most extensively employed agent for acidic pre-treatment, primarily due to its cost-effectiveness (Wu *et al.*, 2021). Physical methods like high temperature, ultrasound, and supercritical CO_2 have also been used to disrupt the exine layer, however, these methods are not suitable on a small scale due to the associated cost of equipment as well as time (Wu *et al.*, 2021). Another method is the mechanical method which involves the action of shear forces leading to heat generation, which in turn result in the loss of heat-labile nutrients (Dong *et al.*, 2015). Some of the other suitable and affordable methods include fermentation and enzymatic treatment which have been proven to give more remarkable results. The former involves the usage of bacteria (like Lactic acid bacteria, *Apilactobacillus*) for the dissolution of the exine layer; whereas, the latter involves the use of enzymes (protease, cellulase, pectinase, hemicellulase, papain) to breakdown the tough outer coat of pollen grain. Thus, these biotechnological processes may be employed to improve digestibility and aid in the easy diffusion of nutrients under controlled conditions in the gastrointestinal tract (Dong *et al.*, 2015; Filannino *et al.*, 2021; Khalifa *et al.*, 2021). The average degree of carbohydrate and protein digestibility has been reported to be 4% and 53% respectively, which can be further increased by grinding and dissolving the bee pollen in warm water before consumption. It enhances the functionality of pollen by increasing nutrient accessibility to 60–80% (Kostić *et al.*, 2020). The application of such pre-treatments (acidic, alkaline, dry thermal, and wet thermal) helps to break the peptide bonds existing in complex protein structures, thereby increasing the digestibility up to as high as 85–98% (Benavides-Guevara *et al.*, 2017).

In 2016, Zuluaga *et al.* explored how physical and biotechnological processing methods influence the availability of nutrients and bioactive compounds in bee pollen. Their research revealed that subjecting bee pollen to thermal treatment at 121 °C for 10 minutes, coupled with enzymatic hydrolysis, resulted in a comprehensive improvement in terms of digestibility (↑10%), total phenolics (↑14%), and antioxidant capacity (↑13%). Consequently, the modified bee pollen, particularly following thermal and enzymatic treatments, emerges as a potential complete food, due to increased nutritional and bioactive compounds, as compared to its already established nutritional composition. The impact of various pre-treatments on bee pollen's digestibility is explained diagrammatically in Figure 2.2.

2.3.2. Bee pollen allergy

The rising demand for natural products with health-promoting properties drives the increased consumption of bee pollen. Amid concerns about food safety, it is crucial for beekeepers to consistently monitor key stages in beekeeping, implementing good production and processing practices. Ensuring the quality of bee pollen involves prioritizing purity and microbiological safety. Neglecting hygienic standards during early production stages can lead to health risks for consumers. The critical step is pollen collection from traps, where prolonged storage may elevate humidity, fostering microbial growth (Zarobkiewicz *et al.*, 2017; Kostić *et al.*, 2020).

Despite having several beneficial properties as stated in the above-mentioned sections, bee pollen may either be primarily allergic to certain individuals or cross-react with other allergens. People having an intolerance to bee pollen might suffer from health problems such as diarrhoea, abdominal pain, skin allergy, gastroenteritis, nausea, urticaria, malaise, cognitive impairment, rhinitis, dysphagia, myalgia, disorientation etc. (Zarobkiewicz *et al.*, 2017). Moreover, the presence of certain contaminants is another matter of concern that needs to be addressed before incorporating bee pollen into food products. Some of the most common contaminants present in bee-collected pollen include heavy metals, herbicides, ochratoxin A, mycotoxins, pesticides, pyrrolizidine alkaloids, bacteria etc. (Denisow and Denisow-Pietrzyk, 2016). Bee pollen allergies result depending upon where the bee pollen comes from i.e., plant variety (Taketomi *et al.*, 2017).

The possible reason for respiratory symptoms might be anemophilous plants containing water-soluble glycoproteins which evoke IgE antibody-mediated allergic reaction by either of the two mechanisms. The first mechanism involves the expulsion of allergenic particles from the cytoplasm by diffusion (in an isotonic medium) as a result of direct contact between pollen grain and mucosa. In the second mechanism, pollen grain gets hydrated rapidly in a hypotonic medium leading to the expulsion of small-sized inhalable allergic components which reach lower airways and cause asthma. In addition to this, other key factors like pH, time, relative humidity, and temperature may cause the secretion of a remarkable amount of eicosanoid-like constituents which produce allergic reactions by cross-reacting with prostaglandin E2 and leukotriene B4 (Taketomi *et al.*, 2017).

Allergy symptoms may also arise due to cross-reactivity between common epitopes on anemophilous and entomophilous pollens belonging to the same botanical family or reaction to honeybee antigens (derived from bee secretions) present in bee products (here, bee pollen). Some of the major allergen proteins found in different varieties of bee pollen include pollen-specific protein Bnm1, calmodulins, profilin, Sal k2, Sal k3, pollen allergen MetE (cobalamin-independent methionine synthase), and MRJPs (major royal jelly proteins). Moreover, the naturally present pollen enzymes may act as sensitizers that induce allergic symptoms such as rhinitis, sneezing, and asthma in the human respiratory tract (Matuszewska *et al.*, 2022). It has been suggested that the allergic symptoms are found to get resolved after treatment with diphenhydramine, epinephrine, chlorpheniramine, and dexamethasone (Choi *et al.*, 2015). Several case studies on bee pollen-induced anaphylaxis have reported elevated serum-specific IgE levels against a range of pollen sources. Notably high IgE concentrations were observed for *Artemisia vulgaris* (15.5 kU/L), *Lolium perenne* (4.9 kU/L), *Taraxacum officinalis* (14.8 kU/L), *Cupressus arizonica* (2.2 kU/L), *Platanus acerifolia* (3.4 kU/L), *Olea europaea* (6.4 kU/L), *Cynodon dactylon*, *Plantago lanceolata*, *Dactylis glomerata*, *Poa pratensis*, ragweed (*Ambrosia artemisiifolia*, 25.2 kU/L), chrysanthemum (20.6 kU/L), dandelion (11.4 kU/L), sunflower, chamomile, mugwort, mesquite, and willow. These findings underscore the potential allergenic risks associated with the consumption of bee pollen in sensitized individuals. The bee pollen extracts of aforementioned varieties showed positive reactions in the skin-prick test (Martín-Muñoz *et al.*, 2010; Choi *et al.*, 2015). Thus, bee pollen and other bee products must be given very carefully to particularly children and older adults as they may cross-react with other allergens (like pollens and other bee products) (Zarobkiewicz *et al.*, 2017).

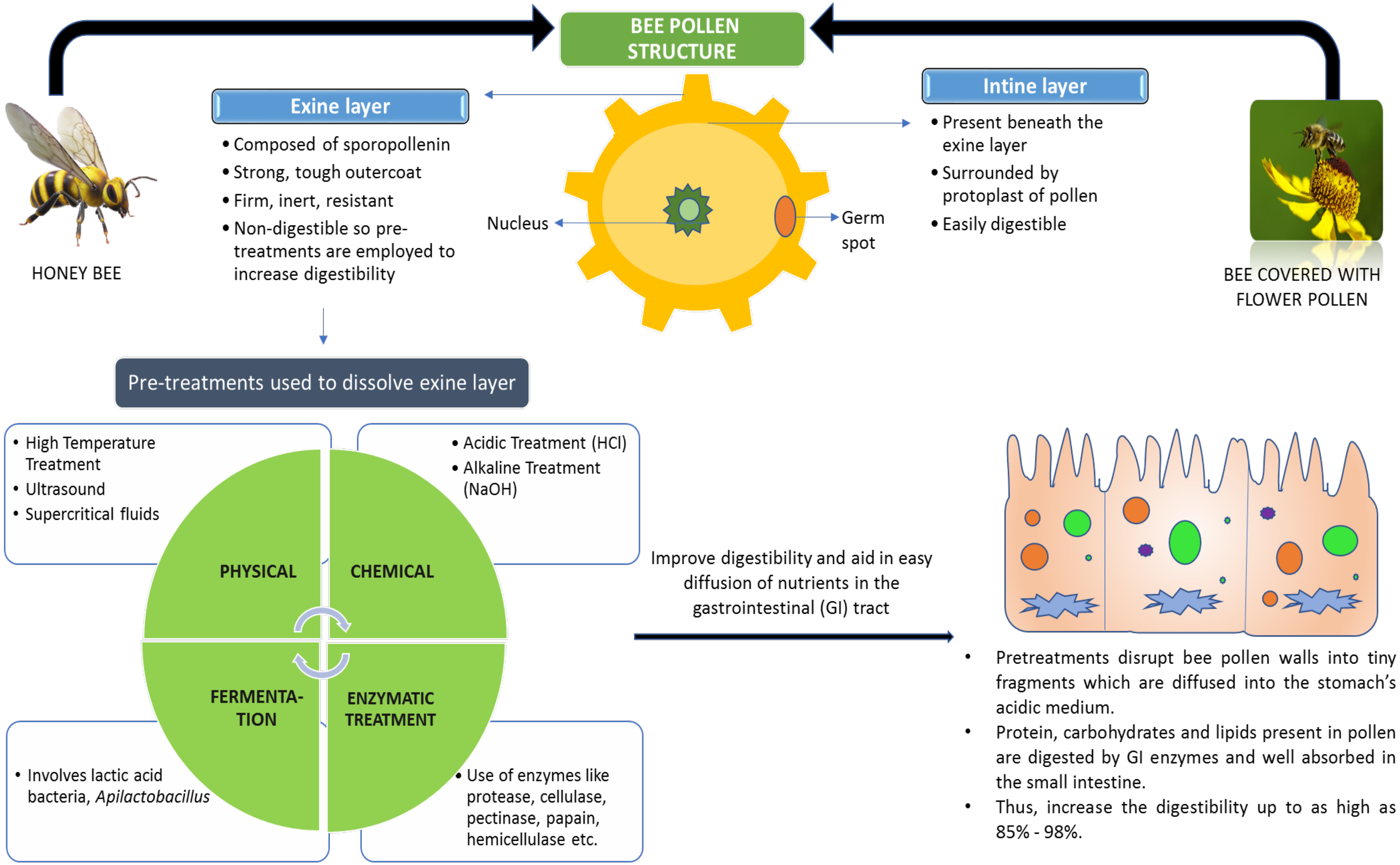


Figure 2.2. Effect of different pre-treatments on the digestibility of bee pollen

2.3.3. Nutritional value and meeting Recommended Dietary Allowance (RDA) requirements

The current trend is the replacement of conventional food ingredients with functional food ingredients having high nutritional significance to appeal the health-conscious consumers (Thakur and Nanda, 2020). Bee pollen, also called the 'life-giving dust'; is an abundant source of nutritional constituents like carbohydrates, protein, lipids, fiber, water-soluble vitamins (B1, B2, B6, C), fat-soluble vitamins (pro-vitamin A, D, E), macro- and micro-minerals (Na, Mg, Ca, P, K, Fe, Se, Zn, Cu, and Mn), phospholipids, phytosterols, 1.6% phenolic compounds (catechins, leukotrienes), flavonoids (quercetin, isorhamnetin, kaempferol), nucleic acids, co-enzymes and other acids (folic acid, nicotinic acid, pantothenic, inositol, archaic, linoleic, γ-linoleic acid, chlorogenic acid) (Khalifa *et al.*, 2021). The main bioactive compounds present in bee pollen are quercetin, quercetin-3-O-rutinoside, kaempferol, isorhamnetin, isorhamnetin-3-O-rutinoside, rhamnetin-3-O-neohesperidoside, quercetin-3-O-neohesperidoside, naringenin etc. (Abd Elhamid and Elbayoumi, 2017).

The Recommended Dietary Allowance (RDA) refers to the average daily intake of a nutrient deemed adequate to fulfil the nutritional needs of nearly 97–98% of healthy individuals within a defined age and population group. Adhering to the RDA values provided by the Indian Council of Medical Research helps to fulfil the daily nutrient requirements of the body and aids in the control or prevention of various non-communicable as well as communicable diseases (hepatitis, influenza, etc.) (Jnawali *et al.*, 2016). In a study conducted by Campos *et al.* (2008), the average nutritional composition of bee pollen was compared with the Reference Daily Intake (RDI) values for essential nutrients. The findings revealed that while carbohydrates and fats were present in relatively lower amounts, bee pollen exhibited a notably higher contribution of protein and crude fiber, which could account for up to 60–70% of the RDI, highlighting its potential as a nutrient-dense supplement. He further reported that the RDI is highly variable and is affected by the composition of bee pollen. A 15 g portion of Spanish pollen has been reported to sufficiently meet the daily requirement for free amino acids (Nagai *et al.*, 2007). In addition to this, consumption of even 50 g of bee pollen is sufficient to meet 50% RDI of most of the essential vitamins as well as minerals except vitamins B5, B6, and calcium. A daily administration of 40 g bee pollen to cardiac patients helps to decrease total cholesterol, serum lipid content, blood viscosity, fibrinogen, and fibrin concentration (Thakur and Nanda, 2020).

2.3.4. Anti-nutritional compounds in bee pollen

Besides the nutritional components, bee pollen also possesses certain anti-nutritional compounds like pyrrolizidine alkaloids, mycotoxins, bacterial toxins, pesticides, metalloids, and other toxic elements. The oral allergic reactions generally subside within 10-30 minutes after consuming bee pollen-based products. But these products are often tolerated by allergic individuals after employing certain cooking methods like boiling and baking. Bee-collected pollen derived from *Echium* and *Senecio* is prone to be hepatotoxic due to the presence of pyrrolizidine alkaloids; whereas, the improper and unhygienic handling of bee pollen increases the possibility of contamination of mycotoxins. Some of the potentially toxic elements present in bee pollen include Al, As, Cd, Cr, Co, Fe, Pb, Hg, Ni, and Sr (Kostić *et al.*, 2020). The acceptable contamination level or maximum tolerance level of these elements in bee pollen is 0.5 mg/kg for Pb and As, 0.1 mg/kg for Cd, and 0.03 mg/kg for Hg. However, there is no established guideline for aluminium intake, a potent neurotoxicant that can be found in notable quantities in pollen samples (Campos *et al.*, 2008). Research by Morgano *et al.* (2010) suggested that the concentration of certain heavy metals and particulate matter were found to be higher in the dry season which is attributed to the air pollution during this period, hence favouring the contamination of bee pollen. Al toxicity in humans has been linked to clinical complications and neurological dysfunctions, including conditions like Alzheimer's disease. The build-up of heavy metals within human body can result in serious health implications, such as carcinogenesis, mental disorders, and various conditions that affect growth, development, metabolism, and the nervous system. These inorganic pollutants vie with vital trace elements for available binding sites within biological systems, resulting in negative impacts on the regulation of homeostasis (Végh *et al.*, 2021). Moreover, the nutritional profile and physicochemical characteristics of bee pollen create a conducive environment for the growth of various microorganisms, including yeasts and mycotoxin-producing molds (Végh *et al.*, 2021). Aflatoxin B1, ochratoxin A, deoxynivalenol, neosolaniol, nivalenol, zearalenone, and fumonisins are among the most commonly occurring carcinogenic mycotoxins present in different pollen samples (Campos *et al.*, 2008). The maximum allowed concentration of aforementioned mycotoxins is 0.00–17.32 µg/kg, 0.00–10.98 µg/kg, 133.30–273.90 µg/kg, 22–30 µg/kg, 1 µg/kg, 115.60–361.30 µg/kg and 6.30–12.60 µg/kg respectively. Aflatoxin contamination of bee pollen is affected by factors like humidity, hygiene practices at the time of pollen harvesting, weather conditions, and processing methods. Therefore, it must be suitably processed as well as properly stored followed by regular quality control before it is used for consumption (Campos *et al.*, 2008; Kostić *et al.*, 2020).

Airborne particulate matter is another pollutant that might contaminate bee pollen and cause a hazardous impact on the human cardiovascular, respiratory as well as gastrointestinal system. Apart from the negative impact on human health, it may also prove to be a major threat to the honeybee's health by bringing about certain cytohistological modifications of the gut epithelium (Papa *et al.*, 2021). Papa *et al.* (2021) attempted to identify the sources and potential risk of inorganic particulate matter in pollen grains obtained from honeybees residing in an industrial area. It was evident from the obtained results that pollen pellets were contaminated by airborne particulate matter such as iron compounds, barite, zinc, antimony oxides, silicon dioxide, lead, calcite, clay minerals, feldspars, quartz, etc. Studies have also suggested the presence of radioactive isotopes (^{137}Cs, ^{40}K) in bee products (bee pollen, honey, propolis) between 1986 and 1990 after the atomic power station accident on April 26, 1986, in Ukraine. Pesticide residue contamination of bee pollen is another area of study that has been widely investigated by several researchers. Furthermore, the most widely occurring active ingredients which serve as the major contaminants of bee pollen are carbendazim (169 µg/kg), thiacloprid (89 µg/kg), tebuconazole (30 µg/kg), acetamiprid (26 µg/kg), chlorpyrifos (16 µg/kg), fluvalinate (11 µg/kg) and thiametoxam (10 µg/kg) (Végh *et al.*, 2021).

Over the past two decades, scientific interest in bee pollen and its associated food safety risks has grown substantially; however, certain aspects remain insufficiently explored and lack comprehensive data. Further studies are required, particularly on alternatives to banned pesticides found in bee pollen. Current literature is insufficient to make definitive conclusions about mycotoxin and mercury contamination. Bee pollen may contain heavy metals and pyrrolizidine alkaloids, posing potential health risks. Hence, it should undergo appropriate processing, proper storage, and routine quality checks before being deemed suitable for consumption (Végh *et al.*, 2021; Papa *et al.*, 2021).

2.3.5. Processing of bee pollen

Water concentration significantly influences the chemical constituents and storage life of a product, with water activity (a_w) closely linked to quality. Higher a_w favours microbial growth, potentially producing mycotoxins and ochratoxins. Fresh pollen pellets contain 20–30% moisture content, which is relatively high and favours the growth of various micro-organisms causing chemical as well as enzymatic reactions, further posing health risks and reduced shelf-life. To ensure safety and retain nutrients, dried pollen should have moisture content between 5% and 9%, with humidity levels ranging from 4% to 8%. Hence, ready-to-consume pollen should maintain a water activity between 0.261 and 0.280 which can be achieved by employing drying techniques like vacuum drying, hot-air drying, freeze-drying, as well as microwave-assisted drying (Thakur and Nanda, 2020; Kostić *et al.*, 2020). Among all, freeze-drying is considered to be the most classic method for drying bee pollen as it helps to retain flavonoids, phenolic compounds, and antioxidant activity when compared to other drying techniques *viz.,* drying at 42 °C (Kostić *et al.*, 2020). On the contrary, subjecting bee pollen to 60 °C results in reduced protein content and vitamin C content up to 43.7% and 31.5% respectively. In addition, significant deterioration has been observed in the physicochemical characteristics, morphological structure, and organoleptic attributes—including color, texture, and odor—of bee pollen (Isik *et al.*, 2019). It has been suggested by Canale *et al.* (2016) that microwave-assisted drying is a suitable procedure when it comes to the preservation of bioactive constituents like rutin, but on the other hand, it also increases hydroxymethylfurfural levels by affecting diastasis activity. Castagna *et al.* (2020) studied the effect of conventional drying on the bioactivity of chestnut pollen (an exceptional source of phenolics) and observed that it resulted in a substantial reduction in the concentration of total phenolic content.

Thakur *et al.* (2021) attempted to optimize the spray drying technique for encapsulating bee pollen and developing functional milk powder. The study concluded that in comparison to fresh pollen, the total phenolic content was found to be decreased in developed powder. This might be attributed to the application of ultrasonication before spray drying leading to the release of phenolic compounds in the sample extract. Another research study aimed to encapsulate pollen pellets in hydrolysed collagen and Arabic gum by using spray drying and lyophilization. The results confirmed that spray-dried pollens encapsulated with collagen and Arabic gum constitute high protein and fiber content; whereas, the lyophilized pollen samples had a more proportional chemical composition of carbohydrates, protein, lipids, and fiber content (Sereia *et al.*, 2018).

Another processing method, i.e., baking might result in decarboxylation and depolymerization of phenolic acids and polyphenols, thereby causing a drastic reduction in the amount of total phenolic constituents. Thus, fortification and enrichment of bakery products with bee pollen could be regarded as a useful option to overcome the losses of thermal treatment (Krystyjan *et al.*, 2015). The impact of enriching biscuits with bee pollen has been explored by researchers. The crude protein and ash content showed a significant increase as the concentration of bee pollen was raised from 0 to 10%. Moreover, bee pollen substitution caused a statistically remarkable rise in total phenolic content as well as antioxidant activity (Végh *et al.*, 2022). Krystyjan *et al.* (2015) proposed that an addition of 10% bee pollen in biscuits helps to increase the polyphenolic content and antioxidant activity remarkably. In terms of physical properties, bee pollen addition gives an intense colour which is caused by a non-enzymatic reaction (Maillard browning) between reducing sugar and amino acids.

2.3.6. Storage of bee pollen

The overall quality of bee pollen gets largely affected by certain factors like cleaning, transportation, packaging, storage conditions, and storage time. de Arruda *et al.* (2013) stated that the functionality and nutritional value of bee pollen declines with time as storage allows the oxidation of phenolic compounds and Maillard reactions to occur. Thus, the concentration of β-carotene, vitamin B2, B6, B12, C, and E decreases. The average loss of nutritional components like β-carotene, vitamin C, and vitamin E has been reported to be 12%, 26%, and 13% respectively in freezer storage conditions. To overcome these challenges, micro- and nano-encapsulation can be employed to prevent the degradation of sensitive phenolic compounds caused by exposure to air, O_2, light, pH, and heat processing techniques (Kostić *et al.*, 2020).

2.3.7. Techno-functional properties of bee pollen

Key techno-functional properties of bee pollen, including hardness, chewiness, adhesiveness, gumminess, springiness, resilience, and cohesiveness, have facilitated its effective integration into a variety of food products. A rise in the concentration of bee pollen leads to a decrease in gumminess, hardness, springiness, and chewiness of meatballs and decreased hardness of biscuits as well as bread. Furthermore, it possesses good emulsifying properties and can therefore be added to natural emulsifiers used for the development of gluten-free bread (Kostić *et al.*, 2015). On the other hand, it does not possess good foaming properties which limits its utilization in ice creams, toppings etc. (Thakur and Nanda, 2019).

Studies have revealed that bee collected-pollen exhibits poor water absorption capacity which is a plus point because functional ingredients having high water absorption capacity might result in brittle and dry food products, particularly during storage. However, bee pollen possesses excellent oil-holding capacity which makes it an ideal ingredient in the preparation of food products for better retention of flavour and good mouthfeel (Kostić *et al.*, 2015; Kostić *et al.*, 2020).

2.3.8. Compliance with FDA and FSSAI guidelines

Compliance with national as well as international standards and regulations is of utmost importance when it comes to the development of any food product. According to FDA (Food and Drug Administration) law, bee pollen can be marked as food because it does not pose any harmful effect on non-allergic individuals. However, FDA thwarts to mention any therapeutic claims regarding its consumption. To date, many promoters have made claims for bee pollen being ‘a giant germ killer’, ‘nature’s most perfect food’, ‘retards aging’, ‘the richest source of protein’, ‘relieves allergy’, ‘improves athletic performance’, but FDA invalidated and disapproved all these claims as there was no scientific evidence in favour of such statements. FDA states that bee pollen and its products must be prepared, packed, and held in a sanitary manner otherwise steps like injunction, seizure or criminal prosecution might be taken against the manufacturer (Sanford, 1995). According to FSSAI (Food Safety and Standards Authority of India) guidelines, the maximum moisture content in bee pollen should be 20% along with a 25,000/gram pollen count (FSSAI, 2018).

2.4. Utilization of bee pollen in various food products

Bee pollen has widely been used as a functional food and feed ingredient. Numerous researchers have attempted to incorporate bee pollen for developing food products including fermented as well as non-fermented foods and beverages *viz.,* Kombucha, mead, wine, yogurt, dietary supplements, white cheese, bee bread, gluten-free bread, biscuits, cookies, juices, sausages, meatballs, etc. (Table 2.2). The deterioration of meat products primarily results from lipid oxidation, leading to a reduction in overall quality and potentially compromising the product's shelf life. To counteract this issue and mitigate damage caused by lipid oxidation, natural antioxidant compounds are commonly incorporated into food formulations (Heldt *et al.*, 2019). The use of bee pollen in meat products is of great significance because its extract shows a strong antioxidative effect which has helped to solve the problems faced by manufacturers of meat products. Although these problems at large, are solved by the use of synthetic additives but consumer awareness has favoured the use of additives of natural origin capable to cease microbial action and uncontrolled oxidation processes (Turhan *et al.*, 2014). Bee pollen serves as a valuable source of antioxidants. The redox properties of these phenolic compounds enable them to function as hydrogen donors, reducing agents, and scavengers of oxygen singlet, directly contributing to antioxidant action (Heldt *et al.*, 2019). Thus, bee pollen has been considered to be a suitable contender to slow down lipid oxidation in meatballs and sausages, that not only extends shelf life but also enhances the diversity of food products offered. This, in turn, holds promise for future industrial advancements and contributes to a deeper understanding of bee pollen as a significant functional food (Turhan *et al.*, 2014; Heldt *et al.*, 2019; Kostić *et al.*, 2020).

Existing studies have explored the addition of bee pollen to products such as bakery items, beverages, and meat analogues. However, there is a limited work on its integration into fermented dairy products, which offer a favorable medium for nutrient delivery. Furthermore, optimization of incorporation levels, sensory acceptability, and storage stability of bee pollen-enriched dairy products has not been comprehensively studied.

2.5.Conclusion

Bee pollen is a natural ingredient having excellent nutritional value based on the geographic origin and floral species. Thus, it can be potentially incorporated into food products and accepted as a part of our daily diet. However, it is very important to acknowledge the other aspects related to pollen contamination, allergenicity, processing, health implications, digestibility as well as storage. The review of the literature has revealed that more emphasis should be laid on extending the existing guidelines and regulations for determining the acceptable upper levels of potentially harmful compounds in bee pollen before human consumption. Intensive research on the unexplored aspects will help in enriching the knowledge of researchers, industrialists, and manufacturers to develop healthy, nutritious, and safe bee pollen-based food products.

Previous research has rarely addressed the optimization of pre-treatment methods, incorporation levels, or sensory-functional balance in bee pollen–based products. Additionally, studies on shelf-life and storage stability are scarce. These gaps highlight the need for systematic research focusing on process optimization and quality evaluation of bee pollen-incorporated Shrikhand, which forms the basis of the present investigation.

Table 2.2. Various food products incorporated with bee pollen

Bee pollen-based product	Objective of the study	Bacterial culture	Storage temp.	Significant findings	Source
Chicken breast meat	To determine the effect of supplementation of bee pollen or propolis along with probiotic on the breast meat.	*Lactobacillus fermentum*	-18 °C	• Breast meat was supplemented with probiotic (3.3 g) and bee pollen or propolis (400 mg/kg meat). • The breast meat of chickens supplemented with bee pollen and probiotics was found to have the lowest sensory attribute scores. • Moreover, no significant difference was found in physicochemical parameters except for the fat content (which was increased in bee pollen supplementation).	Trembecká *et al.* (2017)
Bee pollen supplemented traditional home-made yoghurt	To examine the physical and chemical properties of bee pollen supplemented traditional home-made yoghurt.	*Lactobacillus delbrueckeii* subsp *bulgaricus* and *Streptococcus Thermophilus*	4 °C	• Different concentrations of bee pollen, i.e., 0.5, 1.0, 1.5, 2.0, 2.5 and 3%, were incorporated. • 3% pollen supplementation showed the highest values of minerals such as, zinc, copper, manganese, iron, and sulphur. • Addition of pollen also decreased the pH and titratable acidity.	Özcan *et al.* (2020)

Polyphenol-rich milk powder	To assess the impact of bee pollen concentration on milk powder, bacterial cultures, and its physicochemical and functional properties.	-	4 °C	• The addition of 5%, 10%, and 15% bee pollen to milk powder, under varying temperature and pressure conditions, negatively impacted solubility, while the apparent density remained within the acceptable range for powdered foods. • Addition of bee pollen resulted in an increased concentration of low molecular weight sugars, thereby enhancing the hygroscopic nature of the resulting powder.	Thakur and Nanda (2019)
Yoghurt (cow, goat and sheep)	To evaluate the impact of bee pollen concentration on yogurt made from cow, goat, and sheep milk, as well as its effects on bacterial cultures and physicochemical properties.	*S. thermophilus* and *L. bulgaricus*	4 °C	• Different concentrations of bee pollen, i.e., 0.5, 1.0, 2.5 and 3.0%, were added into yoghurt prepared from cow, goat and sheep's milk. • Significant improvement in the product's phenolic content and antioxidant activity along with the taste, odor, appearance, and cohesion was observed. • Sensorial evaluation identified that bee pollen concentration of 0.5–1% could be ideal and may help in treating several disorders.	Karabagias *et al.* (2018)

Bee pollen fermented Kombucha consortium	To determine the effect of bee pollen produced by fermentation with a Kombucha Consortium on microbial quality and polyphenols.	*Lactobacillus* sp., *Lactococcus* sp., *Gluconoacetobacter*, and *Komagataeibacter*	-	• Bee pollen was added in Kombucha consortium post inoculation after 20 days of fermentation. • Addition bee pollen into the product led to a significant increase in polyphenol, flavonoid, and antioxidant content, which demonstrated an anti-tumoral effect on Caco-2 cells. • The addition of pollen increased lactic acid bacteria (LAB) proportion. Larger microbial diversity of bee pollen fermented Kombucha also helped in the formation of short-chain fatty acids, illustrating postbiotics.	Uțoiu *et al.* (2018)
Yoghurt	To evaluate the impact of varying concentrations of bee pollen on the bacterial cultures and physicochemical properties of yoghurt of Egyptian origin.	*B. angulatum*, *L. gasseri* and *L. rhamnosus*	5 °C	• When 0.6% of royal jelly and 0.8% of bee pollen was added into yoghurt of Egyptian origin, an increase in the nutritional profile including amino acids and fatty acids was noticed.	Atallah (2018)

Yoghurt	To examine the influence of varying bee pollen concentrations on the bacterial cultures and physicochemical characteristics of yoghurt of Bulgarian origin.	*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*	-	• The organoleptic properties of Bulgarian yogurt remained unchanged with the addition of 5% honey and 0.4% bee pollen, despite testing various concentrations of honey (5%, 10%, and 15%) and bee pollen (0.4%, 0.6%, and 0.8%). • Enhancement in vitamin C content of yoghurt was observed after the addition of bee pollen.	Zlatev *et al.* (2018)
White cheese (camel and cow milk)	To check the effect of bee pollen on white cheese of camel and cow milk on bacterial cultures and physicochemical properties.	*L. bulgaricus*, *S. thermophilus*	10 °C	• The addition of bee pollen at concentrations of 0%, 0.5%, 1.0%, 1.5%, and 2% into white cheese prepared from camel and cow milk, followed by storage for 45 days, revealed enhanced antibacterial activity—particularly at the 2% level—against *Salmonella typhimurium* and *Escherichia coli*. • The total phenolic content increased by up to 31-fold, reaching 46.12 mg/g; however, this enhancement was accompanied by adverse effects on sensory attributes, including texture, taste, overall acceptability, and odor.	Abd Elhamid and Elbayoumi (2017)

Yoghurt	To evaluate the bee pollen concentration on yoghurt of Egyptian origin on bacterial cultures and physicochemical properties.	*S. thermophilus*, *L. delbrueckii* spp. *bulgaricus*, *L. rhamnosus, Bifidobacterium angulatum*, and *L. gasseri*	5 °C	• With the addition of 0.6% of royal jelly and 0.8% of bee pollen into yoghurt of Egyptian origin followed by storage of 21 days, an increase in the hardness and chewiness was observed which resulted in better texture and less syneresis, due to refrigerated conditions.	Atallah and Morsy (2017)
Yoghurt	To evaluate the impact of varying bee pollen concentrations on the physicochemical characteristics and bacterial cultures of yoghurt.	*L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* (1:1)	5 °C	• When 0.6% of royal jelly and 0.8% of bee pollen was added into yoghurt of Egyptian origin and stored for a time period of 21 days, decrease in the count of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* was observed. • Increase in total solids, ash content, fat, protein, and titratable acidity was observed. • Maximum acceptability of the product was noticed when stored for up to 7 days.	Atallah (2016)

Fermented milk beverages	To check the effect of bee pollen concentration in fermented milk beverage, on physicochemical properties.	*L. acidophilus*, *Bifidobacterium animalis* subsp. *lactis* and *S. thermophilus* (ABT-1)	4 °C	• With the addition of 2.5, 5.0, 7.5, 10, 20 mg/mL of bee pollen of Turkish origin into fermented milk beverages followed by storage of 21 days, an increase in TSS content, viscosity, and proteolytic activity was observed. • Antimicrobial effect against *B. lactis* was also observed.	Yerlikaya (2014)
Beef burger	To check the effect of beef burger fortified with bee pollen from *Apis mellifera* L. on oxidative stability and physicochemical properties.	-	-12 °C	• The addition of 10 g of bee pollen to beef burgers, followed by 42 days of storage, resulted in an increase in total phenolic content and antioxidant activity, which may help prevent the formation of free radicals. • A greater inhibitory effect on lipid oxidation (by 31.78%) was observed on beef burgers fortified with bee pollen rather than beef burgers incorporated with BHT. • Therefore, adding bee pollen to meat products could be identified as innovative strategy to improve health promoting substances.	Heldt *et al.* (2019)

Bee pollen-based beverage	The objective of the study was to evaluate the impact of high-pressure processing on phenolic compounds, carotenoid content, antioxidant capacity, and microbial load in both bee pollen paste and bee pollen-based beverages.	*Salmonella typhimurium* (CECT 443) and *Zygosaccharomyces rouxii* (CECT 1229)	4°C	• Different concentrations of bee pollen, i.e., 5% and 10% (w/v), were added into pineapple juice sample and a significant increase in the bioactive components, and antioxidant capacity was observed due to High pressure processing (HPP) at 400 MPa for 15 mins. • Due to HPP, extraction of carotenoids and phenolic compounds increased by the process of diffusion. • With 5% bee pollen addition, carotenoid content increased to 80.14 mg β-carotene/kg, whereas with 10% addition, the increase was approximately 86.60 mg β-carotene/kg. • Treatment at 315 MPa for 14.5 minutes in the presence of 8% (w/v) bee pollen resulted in a 5-log reduction of *Salmonella* and *Zygosaccharomyces rouxii*, while simultaneously enhancing the levels of carotenoids, phenolic compounds, and overall antioxidant activity.	Zuluaga *et al.* (2016)

Bee pollen fortified biscuits	The aim was to investigate how the fortification of biscuits with varying concentrations of bee pollen affects their physicochemical and antioxidant properties.	-	21°C	• Bee pollen was incorporated into the biscuit dough at concentrations of 2.5%, 5.0%, 7.5%, and 10%, and the dough was stored for 2 months. • Significant increase in the protein, ash content and soluble and insoluble fraction of dietary fiber of bee pollen fortified biscuit was observed. • Results indicated that 10% addition of pollen was most effective in enhancing the phenolic compounds and improving the antioxidant activity. However, only till 5% of bee pollen addition had the same taste as that of the control.	Krystyjan *et al.* (2015)

CHAPTER 3

RESEARCH GAP

Owing to the rich polyphenol composition, bee pollen is characterized by high biological activity. Despite the fact that milk and milk products contain appreciable amounts of macro- and micronutrients, they are poor sources of phenolic compounds. Therefore, incorporating bee pollen extract into a suitable dairy-based food product such as Shrikhand can help enhance its phenolic content and functional value. Shrikhand, a popular Indian fermented dessert, is widely accepted by children, adults, and elderly people due to its appealing taste, texture, and flavour, making it a suitable contender for delivering valuable nutrients from bee pollen.

Although several studies have examined the compositional and nutritional aspects of bee pollen, there is a lack of systematic research on its incorporation into fermented dairy matrices such as Shrikhand. The levels of bee pollen incorporation required to achieve an optimal balance between nutritional enhancement and sensory acceptability have not been well established. In addition, very few studies have investigated the effect of different pre-treatments on bee pollen for improving its suitability in food applications. Furthermore, there is limited information available on the shelf-life, storage stability, and consumer acceptance of bee pollen-fortified dairy products.

Hence, this research was undertaken to address these gaps by developing and optimizing a process for incorporating bee pollen extracts into Shrikhand, with the objective of achieving desirable physicochemical, phytochemical, and sensory attributes while ensuring product stability during storage.

CHAPTER 4

RESEARCH OBJECTIVES

The following objectives were undertaken:

Objective 1. To screen the bee pollen varieties for incorporation as a functional ingredient in a food product

Objective 2. To optimize the levels of bee pollen extract, hung curd, and sugar concentrations for the development of a functional food product using response surface methodology

Objective 3. To determine the shelf-life of the optimized food product

Expected research outcomes:

1. A comprehensive database of the physico-chemical, phytochemical properties, mineral and textural profile of bee pollen varieties will be generated.
2. The proposed research work will determine a process to formulate a novel food product.
3. The work will focus on the addition of bee pollen extract into food product. Integrating bee pollen is anticipated to remarkably increase the phenolic content of Shrikhand, making it a more nutritionally beneficial product.
4. It will provide guidelines for the food industry to effectively use bee pollen as a functional ingredient and tap into a growing consumer demand for functional foods.

CHAPTER 5

MATERIALS AND METHODOLOGY

5.1. Procurement of sample

The two varieties of bee pollen, *viz.* Multifloral bee pollen (MFBP) and Mustard bee pollen (MSBP), both weighing 1 kg each, were sourced from Honey Bee Web located in Indore, Madhya Pradesh–452010, India (Figure 5.1). Upon collection, the pollen pellets were kept in zip-lock laminates at a refrigerated temperature of 4 ºC until they were required for further analysis. For the analysis, samples were initially ground into a fine powder using a laboratory-scale mechanical grinder, followed by sieving through a 22-mesh screen using a sieve shaker (8" Gilson SS-15). The processed samples were then stored at 4 °C until further use.

(a) Mustard bee pollen **(b) Multifloral bee pollen**

Figure 5.1. Varieties of bee pollen

5.2. Techno-functional properties of bee pollen

5.2.1. Angle of repose

To calculate the angle of repose (θ), pollen pellets were slowly discharged through a bottomless cylinder onto a flat surface, forming a conical heap. The diameter (D) and height (H) of the heap were recorded, and the final value was computed using the formula provided by Thakur and Nanda (2018), as shown in equation 1.

$$\theta = \tan^{-1}(2H/D) \quad (1)$$

5.2.2. Water absorption index

The water absorption index (WAI) was assessed by mixing 3 g of bee pollen powder with 30 ml of distilled water using a vortex mixer (Sharma *et al.,* 2015). The mixture was allowed to rest for 30 minutes in a water bath set at 30 °C, later centrifuged in a refrigerated centrifuge for 10 minutes at 4500 rpm and 4 °C temperature. The final value was obtained by using the formula (equation 2):

$$WAI\ (g/g) = \frac{weight\ of\ hydrated\ residue}{weight\ of\ dry\ bee\ pollen\ sample} \quad (2)$$

5.2.3. Oil absorption capacity

Oil absorption capacity (OAC) was determined using 1 g of the powdered sample and mixed with 10 ml of refined soybean oil (Baljeet *et al.*, 2014). The mixture was further incubated for 30 minutes at temperature 37 °C. In continuation, sample was centrifuged for 10 minutes at 2060 rpm. The amount of oil retained per gram of sample was used to calculate the oil absorption capacity, as outlined in equation 3.

$$OAC\ (g/g) = \frac{weight\ of\ sample + oil}{weight\ of\ dry\ bee\ pollen\ sample} \quad (3)$$

5.2.4. Water-/oil-holding index

The water-/oil-holding index was determined by the ratio of water holding capacity and oil holding capacity.

5.2.5. Wettability

The wettability of the bee pollen varieties was assessed following the procedure described by Thakur and Nanda (2018). Wettability was defined as the time required for 1 g of bee pollen sample to become fully submerged in 400 ml of distilled water maintained at 25 ± 1 °C.

5.2.6. Dispersibility

1 g of bee pollen sample was dispersed in 10 ml of distilled water maintained at 25 ± 1 °C and vigorously stirred to form a homogeneous slurry. The resulting slurry was passed through a 212 µm sieve into an aluminium pan and subsequently dried in a hot air oven at 105 ± 1 °C for 4 hours (Thakur and Nanda, 2018). The dispersibility of the samples was then calculated using the following formula (equation 4):

$$Dispersibility\ (\%) = [(10 + a)\%TS]/[\frac{a(100-b)}{100}] \quad (4)$$

where, a is the weight of sample in gram, b is the moisture content of bee pollen, %TS is the dry matter (%).

5.2.7. Emulsifying activity and emulsion stability

To determine the emulsifying properties, the methods described by Thakur and Nanda (2018) were used. 2% bee pollen solution (w/v) was prepared initially. This solution was mixed with pure sunflower oil in 1:1 ratio (5 ml each) and centrifuged at 3,500 × g for 5 minutes. Emulsifying activity (EA) was determined by measuring the height of the emulsified layer relative to the total contents in the centrifuge tube, as calculated using equation 5. Thereafter, the emulsion so formed was subjected to heat treatment for 30 minutes at 80 °C followed by centrifugation at 3,500 × g for 5 minutes. The formula stated in equation 6 was used to calculate the emulsion stability (ES).

$$EA\ (\%) = \frac{Height\ of\ emulsified\ layer}{Height\ of\ total\ contents\ in\ centrifuge\ tube} \times 100 \quad (5)$$

$$ES\ (\%) = \frac{Height\ of\ emulsified\ layer}{Height\ of\ total\ contents\ in\ centrifuge\ tube} \times 100 \quad (6)$$

5.2.8. Foaming capacity and foam stability

The foaming properties of samples were evaluated following the method described by Singh *et al.* (2022). A 3 g portion of bee pollen was dissolved in 100 ml of distilled water and subjected to high-speed whipping using a mechanical blender for 5 minutes. The resulting mixture was transferred to a 250 ml graduated cylinder, and the foam volume was recorded. Foaming capacity (FC) was then calculated using equation 7. Thereafter, any changes in the foam volume were also recorded after 15 minutes to determine the foam stability (FS) using equation 8.

$$FC\ (\%) = \frac{Volume\ of\ foam\ after\ whipping - Initial\ volume}{Initial\ volume} \times 100 \quad (7)$$

$$FS\ (\%) = \frac{Volume\ of\ foam\ after\ 15\ minutes}{Initial\ volume\ of\ foam} \times 100 \quad (8)$$

5.3. Color analysis of bee pollen varieties

The color of the bee pollen pellets was measured in triplicate using a Hunter LAB colorimeter (Model CM-508d, Minolta, Japan). Color values were recorded in the CIE L*a*b* color space, where the L* axis represents lightness, ranging from 0 (black) to 100 (white). The a* and b* axes denote the chromatic coordinates, extending from green (–a*) to red (+a*) and from blue (–b*) to yellow (+b*), respectively. Chroma (C*) and hue angle (h°) were calculated using equations 9 and 10, as described by Thakur and Nanda (2018) and Singh *et al.* (2022). The R^2 value was found to be greater than 0.98.

$$C^* = (a^{*2} + b^{*2})^{1/2} \quad (9)$$

$$h^{\circ} = tan^{-1}(b^*/\ a^*) \quad (10)$$

5.4. Fourier Transform Infrared Spectroscopy (FTIR) analysis of bee pollen

Qualitative characterization of bee pollen was performed using a Shimadzu 8400S FTIR spectrometer, which was fitted with a KBr beam splitter. A pellet consisting of the powdered bee pollen (separately from mustard and multifloral varieties) was prepared by mixing it with 5 mg of potassium bromide (KBr). The FTIR spectrophotometer operated at a maximum resolution of 0.85 cm^{-1} within a spectral range of 4000–400 cm^{-1}. The resulting spectra were interpreted using the standards established by Stuart (2004) for spectral band analysis.

5.5. Scanning electron microscopy (SEM)

A field emission scanning electron microscope (JSM-6510 LV SEM) integrated with energy dispersive X-ray spectroscopy (EDS) was employed to investigate the morphological features of the bee pollen samples. The samples were mounted on the SEM aluminium stubs followed by coating with thin platinum layer using platinum sputtering. The micrographs of both bee pollen varieties were taken at different magnifications (10X, 1000X, and 2000X) by applying an accelerating voltage of 20.0

kV. In addition to capturing the high-resolution surface morphological images of the samples, the presence of certain elements was also detected (Thakur *et al.*, 2024).

5.6. Texture analysis

Textural properties of the bee pollen samples—including hardness, cohesiveness, chewiness, gumminess, and springiness—were evaluated using a Texture Analyzer (TA-XT2) fitted with a 35 mm cylindrical probe (Figure 5.2), following the method described by Singh *et al.* (2022). To simulate the mastication process, a double compression cycle was performed at a crosshead speed of 1 mm/s in which each sample was compressed to 50% of its original height. The pollen pellet was centrally placed on the testing platform. All measurements were conducted in triplicate at a temperature of 27 ± 1 °C, and the mean values were calculated for reporting.

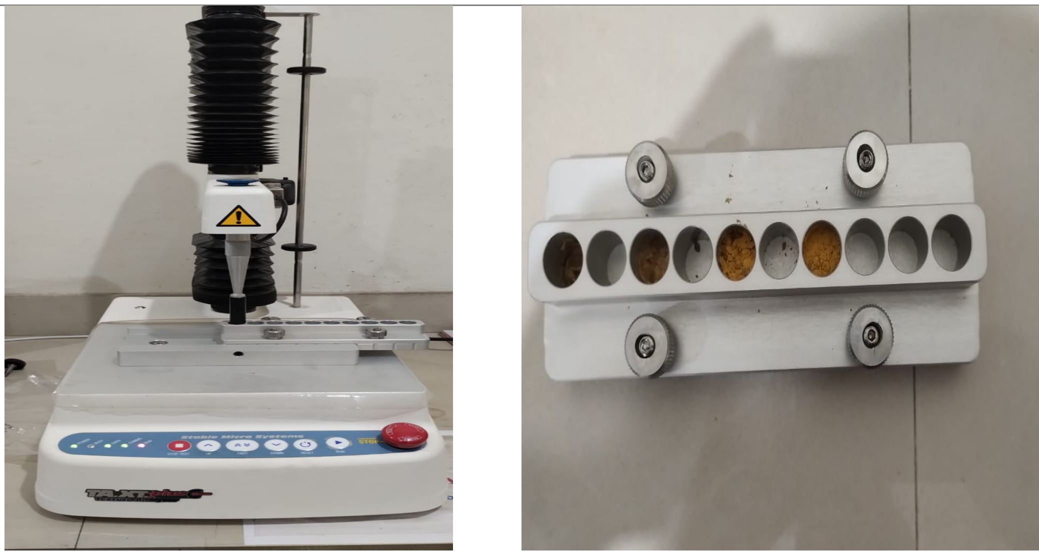


Figure 5.2. Texture Analyzer

5.7. Mineral analysis of bee pollen

The mineral composition of the bee pollen samples was analyzed following the procedure outlined by Singh *et al.* (2022). A total of 100 mg of bee pollen was subjected to ashing for 5-7 hours at 420–450 ºC in a temperature-controlled muffle furnace as part of the sample preparation process. The ash was dissolved in 5 ml of concentrated nitric acid (HNO_3) before the final volume of deionized water was added to bring it to 100 ml. The mixture was filtered to obtain a clear solution, after which 1 ml of the filtrate was diluted with 10 ml of deionized water. Thermo iCAP 7000 Duo

Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (Thermo Fisher Scientific, Bremen, Germany) was used to examine the final sample to assess the mineral content of the bee pollen sample. Plasma generation was carried out using high-purity argon gas sourced from Himalayan Gases, Baddi, India. Microwave-assisted digestion was performed in PTFE vessels using a Multiwave Pro system (Anton Paar, Graz, Austria). All reagent solutions were prepared with deionized water conforming to ASM Type I standards (18.2 mΩ, Aurium mini, Sartorius, Germany). For the digestion process, 69% HNO_3 was employed. Analytical solutions were prepared by diluting certified standard solutions of different elements, all obtained from Alfa Aesar, Specure, MA, USA. 0.5 g sample masses were directly weighed into the PTFE containers, and then 7 ml of HNO_3 was added. The five-step heating protocol for the microwave-assisted digestion process included a 10-minute ramp to 150 °C, a 10-minute hold at 150 °C, a 10-minute ramp to 180 °C, a 10-minute stay at 180 °C, and a 21-minute ramp to a cooling temperature of 55 °C. After cooling to room temperature, the solutions were transferred to polyethylene tubes and diluted to a final volume of 30 ml using ultrapure water conforming to ASTM Type I standards. ICP-OES measurements were conducted in axial view mode, with the plasma sustained by a radio frequency power of 1250 W. After the samples had been digested, the following parameters were set: sample flow rate of 0.50 L/min, plasma gas flow rate of 12 L/min, analysis pump rate of 50 rpm, nebulizer gas flow rate of 0.5 L/min, auxiliary gas flow rate of 0.5 L/min, integration time of 15 sec, stabilization time of 5 sec, and nebulization pressure of 20 psi. Ca (293.366, 396.847 nm), Cu (324.754 nm), Fe (259.953 nm), K (766.490 nm), Mg (279.553 nm), Mn (257.610 nm), Na (588.995, 589.592 nm), and Zn (213.856 nm) were the elements that the suggested technique monitored for analysis.

5.8. Physico-chemical analysis of bee pollen varieties

5.8.1. Moisture content

The pollen samples were analysed to assess a range of physicochemical and phytochemical properties. Moisture content in the bee pollen samples was assessed following the AOAC official method 925.10 (AOAC, 2005). Approximately 5 grams of each sample (in triplicate) was placed in a petri-plate and dried in a hot air oven at

110 ± 2 °C for 4 hours. Later, samples were cooled down to room temperature in a desiccator over silica gel and then weighed. This cycle of cooling, drying, and weighing was repeated until the weight difference between two consecutive measurements was less than 1 mg. The difference between the weight of petri-plate with sample before and after drying provided the loss in moisture content. The final moisture content was calculated using equation 11.

$$Moisture\ content\ \% = \frac{Loss\ in\ moisture}{Initial\ weight\ of\ sample} \times 100 \quad (11)$$

5.8.2. Ash content

The ash content of the two varieties of bee pollen, multifloral and mustard, was measured following the guidelines outlined in method 923.03 by AOAC (2005). Accurately weight 1 g sample was taken in a pre-weighed silica crucible followed by charring and ashing (to obtain white ash residues) in muffle furnace for 6 hours at 450 °C. Weight of ash was determined by taking the difference of weight of crucible with sample before and after ashing. Equation 12 was utilized in calculating total ash content of the sample.

$$Ash\ content\ \% = \frac{Weight\ of\ ash}{Initial\ weight\ of\ sample} \times 100 \quad (12)$$

5.8.3. Fat content

Soxhlet extraction method was used to determine fat content of both varieties of pollen (Singh *et al.*, 2022). 80 ml petroleum ether was taken in a pre-weighed empty beaker. 2 g of powdered sample was added in thimble and placed inside the thimble holder (i.e., beaker). It was then set up in the Soxhlet apparatus after ensuring continuous supply of water and knobs closed. The extraction was carried out for 1 hour, beginning at 80 °C and further increasing the temperature to 180 °C after 30 minutes of starting the process. The beaker containing extracted fat was placed in hot air oven maintained at 100 °C for 30 minutes, which was followed by placing it in desiccator for 15 minutes to allow cooling. Finally, weight of extracted fat was taken. Equation 13 was used to calculate the total fat content.

$$Fat\ content\ (g) = \frac{Weight\ of\ extracted\ fat\ (g)}{Initial\ weight\ of\ sample\ (g)} \quad (13)$$

5.8.4. Total protein content

Kjeldhal method involving digestion, distillation and titration was used for protein estimation (AOAC, 2005). Sample digestion was carried out by mixing 5 g bee pollen sample with 10 ml conc. H_2SO_4 and digestion mixture in a digestion flask. The samples were allowed to digest for 2 hours till bluish green colour was observed. This was followed by cooling the samples and then distilling for 9 minutes by using 4% boric acid and mixed indicator (methyl red and bromocresol green). Finally, the distilled samples were titrated against 0.1 N HCl till end point was reached (pink colour). The total protein content was calculated by applying a conversion factor of 6.25 to the measured nitrogen content.

$$Nitrogen\ \% = \frac{(Sample\ titre - Blank\ titre) \times Normality\ of\ HCl \times 14 \times 100}{Weight\ of\ the\ sample \times 1000} \quad (14)$$

$$Protein\ \% = Nitrogen\ \% \times 6.25 \quad (15)$$

5.8.5. Carbohydrate content

The carbohydrate content was calculated using the following equation:

$$Carbohydrate\ content\ (g) = 100 - (moisture + fat + crude\ protein + ash)$$

5.8.6. Crude fibre

Approximately 2 g of defatted and moisture-free sample was treated with 200 ml of 1.25% sulphuric acid and gently boiled for about 30 minutes. Later, mixture was filtered and resulting residue thoroughly washed with hot distilled water. This step was repeated several times to ensure that residues are free from acid. 200 ml of 1.20% NaOH was added to the residues for treating again for 30 minutes followed by proper washing of residues with hot distilled water to make them free from alkali. Finally, the residue was dried overnight in an oven maintained at 100 °C and weighed next day (AOAC, 2005). The following equation was used to calculate the final values:

$$Crude\ fibre = \frac{Final\ weight\ of\ residue\ after\ drying\ (g)}{Initial\ weight\ of\ sample\ (g)} \times 100 \quad (16)$$

5.8.7. Total phenolic content

Total phenolic content (TPC) was estimated following the method proposed by Sharma *et al.* (2022). The samples were initially extracted using 80% ethanol. Later, extract mixed with 1.0 ml Folin-Ciocalteu reagent and a solution of 7.5% sodium carbonate were added. The mixture was then left undisturbed at ambient temperature for 30 minutes to allow the reaction to proceed. UV-Visible spectrophotometer (Model: Shimadzu, Japan) was utilised to measure absorbance at 675 nm, while employing gallic acid as the calibration standard. A standard curve was plotted to calculate TPC in the bee pollen sample (R^2 = 0.998). Results were expressed as mg GAE/100 g of sample.

5.8.8. Total flavonoid content

Total flavonoid content (TFC) was determined using method described by Ranganna (2006) and Kaur *et al.* (2021). 1 g of each sample was extracted with 80% ethanol. From this extract, 0.25 ml was mixed with 1.25 ml of distilled water and 0.75 ml of 5% sodium nitrate solution, and the mixture was allowed to stand for 5 minutes. Subsequently, 0.15 ml of aluminium chloride was added, and the reaction mixture was left undisturbed for another 5 minutes. After that, 0.5 ml of 1N NaOH and 0.275 ml of distilled water were added. Absorbance was measured at 510 nm using a UV-Visible spectrophotometer. Flavonoid content was quantified using a calibration curve (R^2 = 0.996) and expressed as mg QE/100 g of sample.

5.8.9. Antioxidant activity (DPPH)

The DPPH free radical scavenging activity, used to evaluate antioxidant potential, was measured according to the procedure described by Brand-Williams *et al.* (1995). The sample extract prepared using 80% ethanol was mixed with 1 ml of DPPH solution and 2.9 ml ethanol. The contents were mixed thoroughly to ensure uniform mixing and allowed to stand undisturbed for 60 minutes in the dark. The absorbance of the reaction mixture was measured at 517 nm, using a blank as the reference. The DPPH radical scavenging activity was then calculated as a percentage of inhibition using the following formula:

$$Antioxidant\ activity(\%) = \frac{Absorbance\ of\ blank - Absorbance\ of\ sample}{Absorbance\ of\ blank} \times 100 \qquad (17)$$

5.8.10. Ferric reducing antioxidant power (FRAP)

This assay is based on the principle that antioxidants are responsible for the reduction of ferric ions to ferrous ions which form blue-coloured complexes with tripyridyltriazine (TPTZ) (Singh *et al.*, 2022). The FRAP reagent was prepared by combining acetate buffer (300 mM, pH 3.6), TPTZ dissolved in hydrochloric acid, and ferric chloride solution (20 mM) in a 10:1:1 ratio. 3.995 ml of this freshly prepared reagent was added to 5 µl sample extract and mixed thoroughly to obtain an intense blue colour. The reaction mixture was incubated at 37 °C for 30 minutes, followed by measurement of absorbance at 593 nm. The absorbance of different concentrations of Trolox recorded at 593 nm was plotted to prepare the calibration curve ($R^2 = 0.998$). The final values were expressed as mg TE/g.

5.8.11. Total carotenoid content and chlorophyll content

To 1 g of accurately weighed bee pollen sample, 9 ml of acetone was added in a test tube. The mixture was agitated for 1 minute and then subjected to centrifugation at 3500 rpm for 5 minutes. The clear supernatant was collected in another test tube to measure the absorbance at three different wavelengths: 470 nm, 662 nm and 645 nm. The estimation of total chlorophyll content, total carotenoid content, chlorophyll a and chlorophyll b were done spectrophotometrically by the method suggested by Martínez-Ispizua *et al.* (2022) and β-carotene was used as the standard reagent while 80% acetone (v/v) was utilized as blank for this analysis.

5.8.12. Total anthocyanin content

A series of standard solutions of cyanidin-3-glucoside at different concentrations were prepared to create a calibration curve (Singh *et al.*, 2022). 5 ml of bee pollen sample extract was diluted with 95 ml of 1% HCl in methanol followed by stirring for 30 minutes to ensure thorough mixing. The mixture was centrifuged at 3000 rpm for 10 minutes. The resulting supernatant was carefully collected and diluted to record absorbance values between 0.500 and 1.000 measured at 530 nm. The final value was calculated using the following equation:

$$Anthocyanin\ content\ (mg/g) = \frac{C \times V}{W} \quad (18)$$

where, C: anthocyanin concentration from the calibration curve, V: extract total volume, W: sample weight used for extraction.

5.8.13. Ascorbic acid content (Vitamin C)

Ascorbic acid content was estimated using the AOAC (2005) standard method. The sample, extracted with 4% oxalic acid, was titrated against 2,6-dichloroindophenol dye solution. The endpoint of the titration was identified by the appearance of a stable pink color, indicating the completion of the reaction. The final concentration (mg/100 g) was calculated using equation 19.

$$Ascorbic\ acid\ content = \frac{Titre \times Dye\ factor \times\ Volume\ made\ up}{Aliquot\ of\ sample\ extract \times Weight\ of\ sample} \times 100 \quad (19)$$

5.9. Anti-nutritional factors

5.9.1. Alkaloids

A total of 5 g of finely powdered sample was extracted using 100 ml of 1% hydrochloric acid solution. It was stirred for 1 hour to allow proper dissolution of alkaloids. The mixture was then filtered to obtain liquid extract containing alkaloids. The pH was adjusted between 7 to 8 using NaOH to help in the recovery of alkaloids. Thereafter, chloroform was added to the above neutralized solution and shaken well to ensure uniform mixing. The layers were allowed to separate and the organic layer containing alkaloids was collected for further analysis. The organic solvent i.e., chloroform was evaporated using water bath and finally the concentrated alkaloid extract was recovered. The absorbance was measured using spectrophotometer and alkaloid content was calculated (AOAC, 2005).

5.9.2. Phytic acid

The standard AOAC (2005) protocol was followed to estimate the phytic acid content of the samples. A 5 g portion of the powdered sample was stirred with 25 ml of 0.5 M hydrochloric acid for a minimum of one hour at room temperature to facilitate extraction. Afterward, the mixture was centrifuged for 10 minutes at 3000 rpm, and the supernatant was carefully collected and filtered to obtain a clear extract for analysis. Thereafter, 0.3% ferric chloride solution was added, and mixture was kept for 10–15 minutes allowing the reaction to take place. Finally, the absorbance of coloured mixture was recorded at 520 nm. The final readings were calculated using the calibration curve plotted by preparing standard solutions of sodium phytate at known concentrations ($R^2 = 0.995$). The phytic acid content in bee pollen samples was calculated using the following formula:

$$Phytic\ acid\ content\ (\%) = \frac{C \times V}{W} \times 100 \qquad (20)$$

where, C: phytic acid concentration from calibration curve, V: extract total volume, W: sample weight used for extraction.

5.9.3. Total tannin content

The total tannin content was estimated using the colorimetric method involving Folin-Denis reagent, following the protocol outlined by Eshbah *et al.* (2021). A 5 ml aliquot of the sample extract was mixed with 5 ml of freshly prepared Folin-Denis reagent and 10 ml of 20% sodium carbonate solution. The final volume was adjusted 100 ml using distilled water. The resulting mixture was incubated at ambient temperature for 30 minutes. Subsequently, absorbance was recorded at 760 nm. A standard calibration curve was prepared using different concentrations of tannic acid ($R^2 = 0.998$), and the total tannin content was calculated accordingly using the specified equation.

$$Tannin\ content\ (\%) = \frac{C \times V}{W} \times 100 \qquad (21)$$

where, C: tannin concentration from the calibration curve, V: extract total volume, W: sample weight used for extraction.

5.10. Antimicrobial analysis

The antimicrobial activity of different bee pollen varieties was evaluated using the agar well diffusion method, as described by Najda *et al.* (2021). Petri plates containing Mueller Hinton Agar, enriched with 4% sodium chloride, were inoculated with pathogenic microbial strains at a concentration of 1.5×10^8 CFU/ml. Wells were created in the agar using a sterile borer. Bee pollen extracts were prepared by dissolving 10 mg of sample in 10 ml of 5% DMSO, and 50 µL of this solution was introduced into each well using a micropipette. Streptomycin (1 µg/ml) and 5% DMSO served as positive and negative controls, respectively. Plates inoculated with bacterial strains were incubated at 37 °C for 24 hours, while those containing fungal strains were incubated at 27 °C for 72 hours. Antimicrobial activity was determined by measuring the diameter of the inhibition zones (in mm) surrounding each well.

5.11. Cytotoxicity of bee pollen varieties

The cytotoxicity of bee pollen extract was determined against MCF-7, a human breast cancer cell line (Omar *et al.*, 2016; Kumar *et al.*, 2020). Cells (1×10^6) were seeded in 96-well plates and incubated at 37 °C. Varying concentrations of sample extract (5, 10, 20, 30 and 40 mg/ml) were added to the wells and incubated in an CO_2 incubator for 20 hours. The media was then discarded by inverting and blotting the plate, and the cells were washed with 100 µl PBS. Subsequently, 90 µl of fresh media and 10 µl of MTT (5 mg/ml) were added, and the plate was shaken gently before incubating for 4 hours at 37 °C. Thereafter, 70 µl of the supernatant was taken from each well, and to dissolve the formazan crystals, 10 µl of DMSO was added. The optical density was subsequently measured at 570 nm to assess cell viability after incubating the plate again for 10 minutes at 37 °C.

5.12. To evaluate the effect of different food processing conditions on bee pollen varieties using analytical techniques

5.12.1. Cracking method

The bee pollen samples were subjected to thermal cracking at different temperatures viz. 35 °C, 40 °C and 45 °C for 230 minutes (3.8 hours), 210 minutes (3.5 hours) and 170 minutes (2.8 hours) respectively (Barajas *et al.*, 2012).

5.12.2. Alkaline pre-treatment

To hydrolyse bee pollen, a 3% (w/v) solution of food-grade sodium carbonate was prepared. The prepared solution was mixed with 1 g of pollen sample in a 1:2 ratio and subsequently heated in a water bath at 70 °C for 10 minutes. After the completion of hydrolysis, the mixture was diluted by adding an equal volume of distilled water to it. Finally, the alkali-treated bee pollen samples were sterilized at 121 °C for 15 minutes (Benavides-Guevara *et al.*, 2017).

5.12.3. Wet heat thermal pre-treatment

The method outlined by Benavides-Guevara *et al.* (2017) was used with certain modifications to employ wet heat thermal pre-treatment. Bee pollen sample was mixed with distilled water in 1:2 ratio and placed in a closed container at 130 °C and 2 atm pressure for 2 minutes. It was then neutralized with sodium carbonate and sterilized at 121 °C for 15 minutes.

5.12.4. High performance liquid chromatography (HPLC)

1 g of coarsely powdered bee pollen sample was extracted using 5 ml of HPLC grade methanol by continuous stirring on an orbital shaker for 24 hours at 28 ± 3 °C. The resulting extract was then filtered through a 0.45 µm Millipore membrane. The same steps were repeated once again on the residue for maximum extraction of phenolics. Following two successive extractions, the resulting extract was filtered through a 0.20 µm syringe filter and transferred into amber vials for HPLC analysis. The mobile phase consisted of Solvent A (1% aqueous acetic acid prepared with HPLC-grade water) and Solvent B (acetonitrile). The flow rate was modified to a setting of 0.7 ml/min, sample injection volume 10 µl, 1700 psi pressure for gradient program, and the column was thermostatically maintained at 28 °C. HPLC chromatograms were recorded by detecting the absorption maxima of the analytes using a photodiode array (PDA) UV detector set at multiple wavelengths. Each compound was identifying its characteristics to those of established standards, including cinnamic acid, caffeic acid, ferulic acid, gallic acid, etc. The identification was ascertained using a calibration curve and retention times (Seal, 2016; Kaur *et al.*, 2021). All the measurements were conducted in triplicates.

5.13. Preparation of bee pollen extract

Various extracts of the two bee pollen varieties (mustard bee pollen and multifloral bee pollen) were prepared by altering the concentration of two solvents: distilled water and ethanol (Figure 5.3 and 5.4). The objective was to evaluate how different combinations affect the extraction efficiency and the composition of bioactive compounds present in bee pollen. The solvent concentration was varied from 0% to 100%, which resulted in 6 distinct solvent combinations as outlined in Table 5.1. The specific combinations were designed to cover a range of polarities, enabling the better extraction of a broad range of phytochemicals present in bee pollen samples. The following steps were involved in the preparation of various extracts. First, the mortar was placed in a tray filled with ice-cold water to prepare the sample extract using cold maceration technique. 1 g of the sample was added to the mortar, and it was then treated with a specific ratio of solvents. The mixture was filtered through Whatman filter paper No. 1 to separate the liquid extract from the solid residue. The filtrate was then collected in culture tubes and stored at a refrigerated temperature of 4 °C in stand-alone culture tubes. Also, the weight of residue was determined after drying it in a tray dryer in order to determine the extract concentration.

Table 5.1. Ratio of solvents for the preparation of different sample extracts

Sample code	Distilled water (DW)	Ethanol (Eth)	Ratio
MS1, MF1	20 ml	0 ml	100% D.W., 0% Eth
MS2, MF2	16 ml	4 ml	80% D.W., 20% Eth
MS3, MF3	12 ml	8 ml	60% D.W., 40% Eth
MS4, MF4	8 ml	12 ml	40% D.W., 60% Eth
MS5, MF5	4 ml	16 ml	20% D.W., 80% Eth
MS6, MF6	0 ml	20 ml	0% D.W., 100% Eth

MS- mustard bee pollen, MF- multifloral bee pollen

5.13.1. Phytochemical analysis

The various sample extracts prepared were analysed for determining TFC, TPC, antioxidant activity, FRAP, total carotenoid content, total chlorophyll content, chlorophyll a, and chlorophyll b according to the procedures stated in above methodology section 5.6.7, 5.6.8, 5.6.9, 5.6.10 and 5.6.11, respectively.

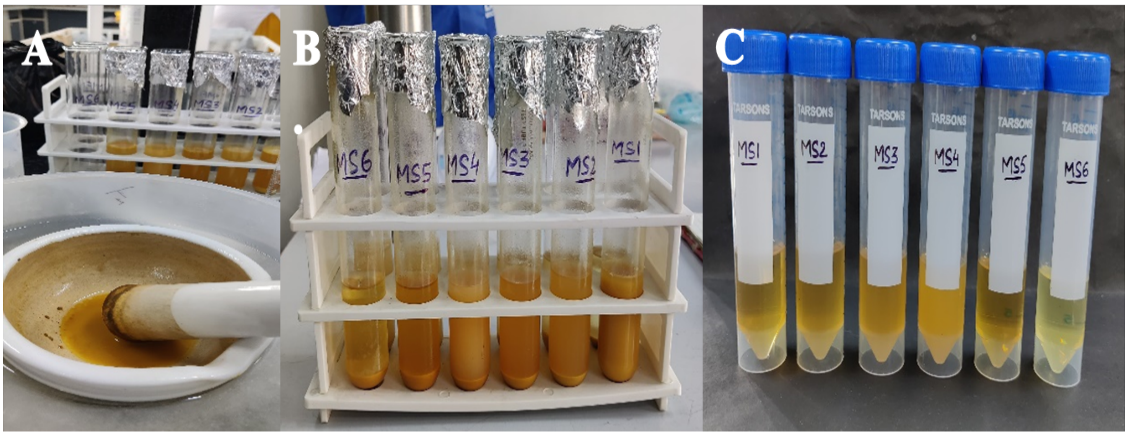


Figure 5.3. Mustard bee pollen extract: (A.) Sample preparation, (B.) Sample extract in test tubes before filtration, (C.) Clear filtrate collected in stand-alone culture tubes

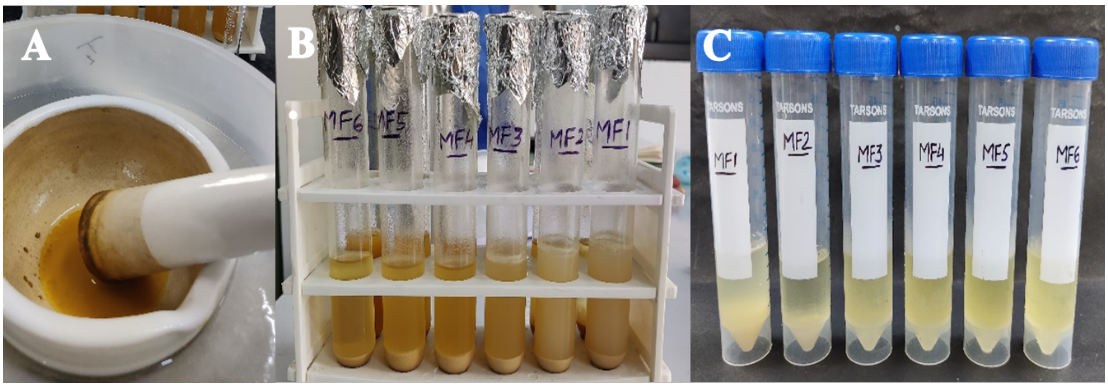


Figure 5.4. Multifloral bee pollen extract: (A.) Sample preparation, (B.) Sample extract in test tubes before filtration, (C.) Clear filtrate collected in stand-alone culture tubes

5.14. Optimization of bee pollen-based dairy product

5.14.1. Preparation of Shrikhand

For the preparation of Shrikhand, one litre buffalo milk (having 6% fat) was heated at 71 ± 5 °C for 10 minutes followed by cooling at 30 ± 2 °C. The milk was inoculated with 1% starter culture to initiate lactic acid fermentation and incubated for 12 hours at 30 ± 2 °C to set curd. The curd obtained was then hung in muslin cloth for 8 hours to drain off the whey. The resulting semi-solid product called hung curd/chakka having a creamy consistency, was removed from muslin cloth to further add sugar and bee pollen extract. The final product was stored at 4 °C in the refrigerator till further utilization for analysis. Pictorial representation of the process is given in Figure 5.5.

5.14.2. Optimization of different variables for the preparation of Shrikhand

Pre-liminary trial was carried out in order to find out the range of process variables for the preparation of Shrikhand. The variable defined for this product were bee pollen extract (A), hung curd/chakka (B) and sugar (C). To determine the acceptable range of bee pollen extract concentration, it was varied as 1, 5, 10, 15, 20 and 25 ml. Different treatments were formulated which were further subjected to sensory analysis using 9-point hedonic scale method. For both types of extracts (mustard and multifloral), at 1 ml level, the change in taste was barely noticeable and was similar to the control, while at 20 and 25 ml concentration, the taste was unpleasant and unacceptable. So, for the preparation of final product, the extract concentration was varied between 2.5 to 12.5 ml for both varieties.

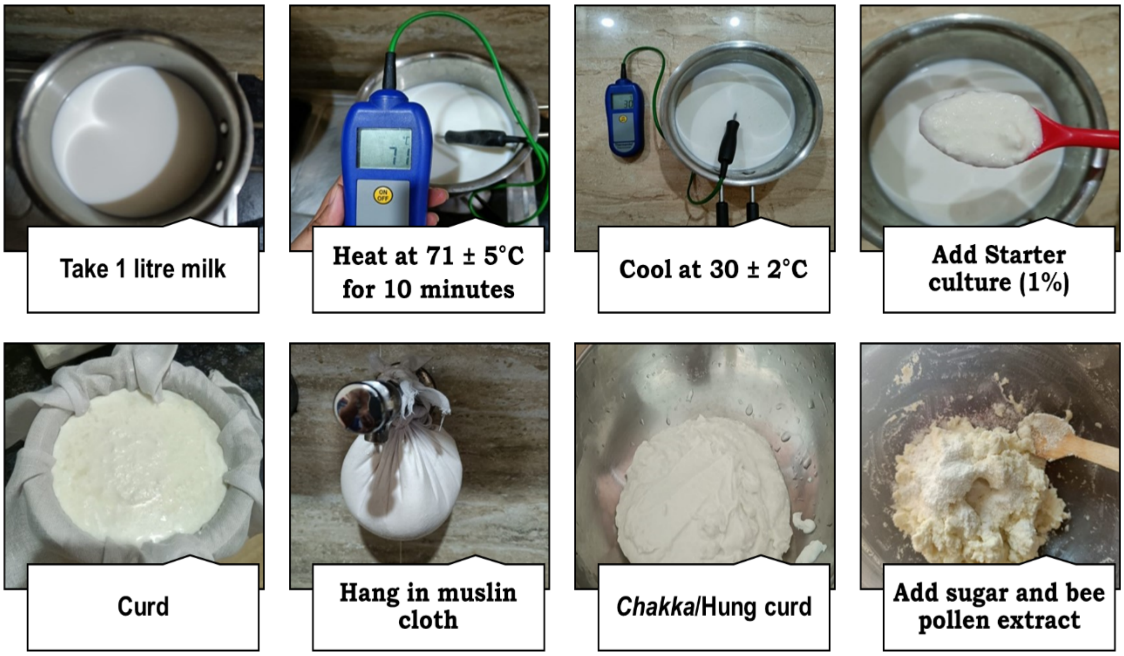


Figure 5.5. Pictorial representation of the process followed for the preparation of Shrikhand

The maximum acceptability of sweetness was obtained with 25 g. Factor A (bee pollen extract concentration), factor B (chakka/hung curd) and factor C (sugar content) were varied from 2.5 to 12.5 ml, 60 to 80 g and 20 to 25 g, respectively. 20 different formulations were predicted by using central composite design type, quadratic design model and response surface methodology in STAT-EASE 360 software. The formulations are presented in Table 5.2, along with the independent variables/factors and their corresponding levels shown in Table 5.3.

A 9-point hedonic scale was employed to evaluate the sensorial characteristics of various Shrikhand samples (Amerine *et al.*, 2013), where 9 corresponds to 'like extremely' and 1 corresponds to 'dislike extremely'. A panel of 50 semi-trained evaluators (25 males and 25 females), comprising faculty members and postgraduate students aged between 22 and 35 years from the Department of Food Technology and Nutrition at Lovely Professional University, assessed the samples at a temperature of 27 ± 2 °C. The same panel was used for sensory evaluation throughout the study, and plain water was provided for rinsing their mouths between sample evaluations. Discussions were not permitted during the evaluation process. The different sensory parameters were colour/appearance, flavour, body/texture, mouthfeel and overall acceptability (OAA) of the product (Table 5.4). All the panelists were briefly explained about the required sensorial characteristics. The appearance of the product was required to be free from any signs of fat, water seepage or mouldiness, and the material should be white to pale yellow. For flavour, the product should have been free from objectionable flavours and odours. The product should have a good body/texture, uniform consistency and free from coarseness. Shrikhand's mouthfeel should have been creamy and smooth, therefore providing a rich texture. Finally, OAA of the product was determined using the average score of all sensory attributes.

5.14.3. Physicochemical and Phytochemical Analysis

The final products (by incorporating bee pollen extract in different concentrations) were subjected to physicochemical and phytochemical analysis. The analysis was conducted according to the procedures already stated in section 5.8. A digital pH meter was used to determine the pH of the samples, with measurements conducted in triplicate. Total soluble solids (TSS) were assessed using a calibrated hand-held refractometer, and the values were reported in °Brix. Acidity of the samples was estimated with titration method using 0.01 N NaOH solution and final values were derived by the following equation:

$$Titratable\ acidity(\%) = \frac{Titre\ value \times N\ NaOH \times\ Volume \times Eq.weight\ (Citric\ acid)}{Sample\ weight \times Aliquot\ taken\ for\ titration \times 100} \times 100$$

Table 5.2. Details of various treatment combinations

Run	Space type	Factor A: BPE	Factor B: Hung curd	Factor C: Sugar
1	Center	7.5	70	22.5
2	Axial	7.5	86.8179	22.5
3	Center	7.5	70	22.5
4	Factorial	12.5	80	25
5	Center	7.5	70	22.5
6	Factorial	2.5	60	25
7	Axial	7.5	70	26.7045
8	Center	7.5	70	22.5
9	Center	7.5	70	22.5
10	Factorial	2.5	60	20
11	Factorial	2.5	80	20
12	Axial	7.5	53.1821	22.5
13	Axial	7.5	70	18.2955
14	Factorial	12.5	60	20
15	Axial	-0.9089	70	22.5
16	Factorial	12.5	80	20
17	Factorial	2.5	80	25
18	Center	7.5	70	22.5
19	Axial	15.909	70	22.5
20	Factorial	12.5	60	25

Table 5.3. Independent variables and their corresponding levels

Independent variable	Symbol	Coded levels				
		-α	-1	0	+1	+α
Bee pollen extract (ml)	A	-0.9089	2.5	6	12.5	15.909
Hung curd/Chakka (g)	B	53.1821	60	6	80	86.8179
Sugar (g)	C	18.2955	20	6	25	26.7045

5.14.4. Sensory evaluation of the different combinations of bee pollen-based Shrikhand

Table 5.4. Sensory analysis of the different combinations of bee pollen-based Shrikhand

Run	Colour/Appearance	Flavour	Body/Texture	Mouthfeel	OAA
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	-	-	-
6	-	-	-	-	-
7	-	-	-	-	-
8	-	-	-	-	-
9	-	-	-	-	-
10	-	-	-	-	-
11	-	-	-	-	-
12	-	-	-	-	-
13	-	-	-	-	-
14	-	-	-	-	-
15	-	-	-	-	-
16	-	-	-	-	-
17	-	-	-	-	-
18	-	-	-	-	-
19	-	-	-	-	-
20	-	-	-	-	-

5.15. Determination of shelf-life of Shrikhand

The optimized food product was subjected to shelf-life estimation. Shrikhand was packed in different packaging materials including polypropylene cups and polyethylene terephthalate cups with lids. The samples were stored for 28 days at two different temperatures *viz.* refrigerated condition (4 °C) and ambient temperature (25 °C) and analysed for storage stability at an interval of 7 days. The following set of analysis were performed.

5.15.1. Microbial assay

5.15.1.1. Total plate count

To begin, the workspace was sterilized using 70% alcohol. Accurately weighed 2.4 g of Plate Count Agar (PCA) was dissolved in 100 ml distilled water and was autoclaved at 121 °C for an hour. The mixture was then cooled to approximately 45 °C, after which PCA was poured into a petriplate and gently combined with the sample. Once solidified, the gel was incubated at 37 °C for 24 hours. After this period, bacterial growth was quantified in terms of colony-forming units (CFU/g) (Bhagwan *et al.*, 2020; Singh *et al.*, 2024).

5.15.1.2. Total yeast count

The workspace was initially sterilized with 70% alcohol. For total yeast count, 4.2 g of Potato Dextrose Agar (PDA) was used. It was dissolved in 100 ml distilled water followed by autoclaving at 121 °C for an hour and later allowed to cool to 45 °C. The petriplate containing PDA and test sample was swirled gently to ensure proper mixing and allow the formation of gel. This step was followed by 24-hour incubation at 37 °C. After 24 hours, the yeasts were quantified as CFU/g (Bhagwan *et al.*, 2020; Singh *et al.*, 2024).

5.15.1.3. Total coliform count

The workspace was disinfected using 70% alcohol. To determine total coliform count, 6.2 g of Violet Red Bile Lactose Agar (VRBLA) was dissolved in 150 ml distilled water by shaking while boiling to ensure proper dissolution. Thereafter, it was cooled down to 45 °C. VRBLA and sample were poured into a petriplate after proper mixing to form gel. This step was followed by 24-hour incubation at 42 °C. The results were expressed as CFU/g (Bhagwan *et al.*, 2020; Singh *et al.*, 2024).

5.15.2. Physicochemical and phytochemical analysis

The analysis was performed as per the standard protocols outlined in detail in section 5.8.

5.15.3. Chemical analysis

5.15.3.1. Hydroxymethylfurfural content (HMF)

The concentration of hydroxymethylfurfural (HMF) was measured using a 3 g sample of bee pollen-enriched Shrikhand, which was mixed with 7 ml of distilled water and 5 ml of 0.3 mol/L oxalic acid in a test tube (Rasane *et al.*, 2019; and Singh *et al.*, 2024). The mixture was heated in a boiling water bath for 1 hour. After allowing the solution to cool to room temperature, 5 ml of a trichloroacetic acid solution—prepared by dissolving 40 g of trichloroacetic acid in 100 ml of distilled water—was added. The resulting precipitate was removed by filtration using Whatman No. 42 filter paper. Then, 0.5 ml of the filtrate was transferred to a separate test tube and combined with 3.5 ml of distilled water and 1 ml of 0.05 mol/L thiobarbituric acid solution. This reaction mixture was incubated in a water bath at 40 °C for 50 minutes. After cooling, the absorbance was measured at 443 nm using a spectrophotometer.

5.15.3.2. Thiobarbituric acid test

In 50 ml of 20% trichloroacetic acid solution, accurately weighed 2 g of the sample was blended and left undisturbed for around 10 minutes. The mixture was filtered using Whatman No. 1 filter paper, and 5 ml of the resulting filtrate was mixed with 5 ml of 0.01 M 2-thiobarbituric acid solution. The test tubes were then placed in a water bath at 100 °C for 30 minutes to facilitate color development. After cooling to room temperature, the absorbance was measured at 532 nm (Rasane *et al.*, 2019).

5.15.3.3. Free fatty acid content

Free fatty acid content was estimated using 2 g sample, which was dissolved in 50 ml of ethanol, and a few drops of phenolphthalein indicator were added (Singh *et al.*, 2024). The mixture was then titrated with 0.10 N potassium hydroxide solution until a persistent pink endpoint, lasting for at least 15 seconds, was observed. The final value was calculated using the corresponding equation 22.

$$Acid\ value\ (mg\ KOH/g) = \frac{Titre\ value \times N\ of\ KOH \times 56.1}{Weight\ of\ sample\ (g)} \quad (22)$$

5.15.4. Effect of storage time on sensory characteristics

To evaluate the effect of storage conditions—specifically temperature and duration—on the sensory attributes of Shrikhand, organoleptic analysis was performed in accordance with the procedure described in Section 5.15.3.

5.16. Cost analysis

The cost of final product was estimated taking into account the cost of individual raw materials as well as other charges like overhead charges and taxation.

5.17. Statistical analysis

Each experiment was performed in triplicate ($n = 3$), and the resulting data were analysed using SPSS Version 22 along with Design Expert software. The outcomes are expressed as mean values accompanied by standard deviation. Statistical significance among different groups was assessed using one-way ANOVA, with significant differences at $p < 0.05$.

CHAPTER-6

RESULTS AND DISCUSSION

6.1. Techno-functional properties

6.1.1. Angle of repose

The results about the techno-functional properties *viz.,* angle of repose, WAI, and OAC are significant to understand the methods desirable for processing, handling, transportation as well as storage of bee pollen. It is evident from the results that the MSBP variety had a lower angle of repose (19.045°) in comparison to MFBP (33.04°) (Table 6.1). This suggests that MSBP tends to form flatter heaps or spread out wider over any flat surface which demonstrates its better transfer characteristics than MFBP. This is because belt-conveying systems favor materials that are more easily spread (Thakur and Nanda, 2018). Bart-Plange and Baryeh (2003) emphasized the benefits of low angle of repose in belt conveying, particularly with cocoa beans. The internal friction angle, pollen pellet size, and shape, density, moisture content, interface friction angle, stratification, base roughness, material segregation, the pull-out velocity of the hollow cylinder (used for measuring angle of repose), material mass, pouring height, and morphology of bee pollen are among the variables that can affect the angle of repose (Al-Hashemi and Al-Amoudi, 2018).

6.1.2. Water absorption index

WAI of MSBP and MFBP was found to be 1.43 ± 0.014 g/g and 1.31 ± 0.014 g/g respectively which suggests that the former has a higher affinity towards water as compared to the latter (Table 6.1). This property is crucial for determining the utilization of bee pollen in the food industry in terms of the moistness of the product (Sharma *et al.*, 2015). The solubility of bee pollen largely depends on the balance between its soluble and insoluble protein and carbohydrate fractions, as well as their interactions with other constituents within the matrix. Substances like honey or nectar, soluble polysaccharides, low molecular weight protein structures, vitamins, etc. are the soluble components. On the other hand, the non-soluble fractions comprise high molecular weight proteins, complex polysaccharides (lignin, cellulose, and sporopollenin), lipids, and other compounds (Kostić *et al.*, 2015; Laaroussi *et al.*, 2023).

6.1.3. Oil absorption capacity

The OAC of both MSBP and MFBP were similar, with values of 2.26 ± 0.03 g/g and 2.02 ± 0.025 g/g, respectively (Table 6.1). In a study conducted by Thakur and Nanda (2018), the OAC values ranged from 1.31 g/g to 2.13 g/g, which are comparable to the results of the present investigation. This property of bee pollen is largely influenced by intrinsic factors such as the nutritional composition, protein conformation, and hydrophobicity (Laaroussi *et al.*, 2023). Additionally, the OAC of pollen is attributed to sporopollenin, which constitutes over 20% of the pollen's dry weight. Sporopollenin (the major component of bee pollen exine) is a complex polymer with varying degrees of polymerization and cross-linking, effectively trapping oil within its matrix. Thus, results confirm that bee pollen has the potential to enhance mouthfeel and retain flavors, making it a promising functional ingredient in various food preparations (Kostić *et al.*, 2015; Laaroussi *et al.*, 2023).

6.1.4. Water-/oil-holding index

The water/oil holding index for both the varieties was calculated as the ratio of water holding index to oil holding capacity. The results were comparable, with values of 0.63 ± 0.02 for MSBP and 0.65 ± 0.015 for MFBP (Table 6.1).

6.1.5. Solubility, Dispersibility and Wettability

The solubility and dispersibility data indicated that MSBP had higher values, with 85.20 ± 0.39% solubility and 43.80 ± 0.26% dispersibility, compared to MFBP, which had 84.33 ± 0.65% solubility and 38.59 ± 0.29% dispersibility (Table 6.1). In contrast, the wettability of MFBP (689.31 ± 10.39 sec) was higher than MSBP (346.88 ± 11.33 sec). All the values were found to be comparable with the ones reported by Thakur and Nanda (2018). These differences might be attributed to various factors including the diverse chemical composition of varieties under study, its texture, structure, surface area as well as the type of cultivar.

6.1.6. Emulsifying properties

Emulsions are of great value as they are responsible for providing a preferable mouthfeel and structure to food products like desserts, beverages, mayonnaise, coffee creamers, ice-creams, etc. Specific conditions need to be maintained while preparing such products otherwise emulsion instability can badly affect the quality of product over time (Laaroussi *et al.*, 2023). Thus, in this study, the emulsifying properties of bee pollen varieties were assessed as they are very important in understanding the functionality of different food constituents. For both parameters *viz.* emulsifying activity and emulsion stability, the values for MSBP were 40.38 ± 0.16% and 19.67 ± 0.33%, respectively. In comparison, MFBP showed slightly higher values of 41.35 ± 0.13% for emulsifying activity and 21.28 ± 0.32% for emulsion stability (Table 6.1). These values were within the range reported by Thakur and Nanda (2018).

6.1.7. Foaming properties

Due to the complex microstructure of foam bubbles and their surrounding environment, maintaining foam in a stable and defined state presents a significant challenge. The preparation of food products like whipped toppings on desserts, marshmallow, mousse and ice-creams require air incorporation to provide a desirable texture and structure to these foods (Kostić *et al.*, 2015). Therefore, evaluating the foaming properties plays a crucial role in the development of food products. The foam capacity and foam stability were higher in MSBP (8.11 ± 0.10% and 17.08 ± 0.35%, respectively) compared to MFBP, which showed values of 7.86 ± 0.15% and 16.98 ± 0.29%, respectively (Table 6.1). Similar findings have been reported by Thakur and Nanda (2018) in their study of techno-functional properties of bee pollen from different botanical sources. Another study by Liang *et al.* (2013) concluded that bee pollen didn’t showcase foaming properties and didn’t form foams. It was suggested in their study that bee pollen could find its potential application as a foam depressant in foods where these properties are undesirable. The variability in the values might be attributed to differences in the protein, carbohydrate and lipid content of both varieties. Proteins tend to contribute to foaming properties by lowering surface tension at the water-air interface and creating a stable, cohesive film around air bubbles. In contrast, lipids tend to decrease foam stability. Their higher surface activity compared to proteins causes them to displace proteins at the water-air interface, which weakens the film by reducing its thickness and disrupting its cohesiveness. Lipoproteins and phospholipids, in particular, are known to negatively impact foam formation and stability (Kostić *et al.*, 2015; Thakur and Nanda, 2018).

Table 6.1. Physicochemical analysis and antioxidant profile of bee pollen varieties

Parameters	Mustard bee pollen	Multifloral bee pollen
Angle of Repose (Θ)	19.045 ± 0.36^{b}	33.04 ± 0.58^{a}
Water absorption index (WAI) (g/g)	1.43 ± 0.014^{a}	1.31 ± 0.014^{b}
Oil absorption capacity (OAC) (g/g)	2.26 ± 0.03^{a}	2.02 ± 0.025^{b}
Water/oil holding index	0.63 ± 0.02^{b}	0.65 ± 0.01^{a}
Wettability (sec)	346.88 ± 11.33^{b}	689.31 ± 10.39^{a}
Solubility (%)	85.20 ± 0.39^{a}	84.33 ± 0.65^{b}
Dispersibility (%)	43.80 ± 0.26^{a}	38.59 ± 0.29^{b}
Emulsifying activity (%)	40.38 ± 0.16^{b}	41.35 ± 0.13^{a}
Emulsion stability (%)	19.67 ± 0.33^{b}	21.28 ± 0.32^{a}
Foam capacity (%)	8.11 ± 0.10^{a}	7.86 ± 0.15^{b}
Foam stability (%)	17.08 ± 0.35^{a}	16.98 ± 0.29^{b}
Moisture content (%)	15.33 ± 0.36^{a}	7.6 ± 0.58^{b}
Ash content (%)	4.3 ± 0.014^{a}	3.3 ± 0.014^{b}
Total fat content (%)	1.5 ± 0.33^{b}	2.0 ± 0.28^{a}
Total protein content (g/100 g)	21.88 ± 0.83^{a}	20.65 ± 0.95^{b}
Carbohydrate content (g/100 g)	43.78 ± 0.62^{a}	41.98 ± 0.88^{b}
Crude fibre (g/100 g)	3.53 ± 0.52^{a}	3.042 ± 0.33^{b}
TPC (mg GAE/100 g)	2676 ± 0.58^{a}	2466 ± 0.34^{b}

TFC (mg QE/g)	14.025 ± 0.25^{a}	8.38 ± 0.41^{b}
Antioxidant activity (DPPH) (%)	82.6 ± 0.54^{a}	64.81 ± 0.25^{b}
Total carotenoids (µg/g)	30.0 ± 0.33^{b}	38.0 ± 0.58^{a}
Chlorophyll a (mg/100 g)	0.015 ± 0.01^{a}	0.004 ± 0.012^{b}
Chlorophyll b (mg/100 g)	0.014 ± 0.01^{a}	0.006 ± 0.01^{b}
Anthocyanin content (mg/100 g)	2.44 ± 0.10^{b}	5.9 ± 0.05^{a}
Ascorbic acid content (µg/g)	76.6 ± 0.32^{a}	58.4 ± 0.43^{b}

The data are presented as mean ± standard deviation, calculated from three independent experiments (n=3).
Values within the same row that are marked with distinct superscripts (a, b) indicate a statistically significant difference ($p<0.05$).

6.2. Colour analysis

The colour of bee pollen is determined by the pigments (carotenoids, chlorophyll, anthocyanins) present in the pollen grains which itself is dependent upon the botanical species and degree of maturity. If the pollen grains have the same colour, the bee pollen is considered monochromatic. On the other hand, if the pollen grains have different colours, the bee pollen is referred to as polychromatic (Bleha *et al.*, 2021). The colour of bee pollen is of great significance since it has a substantial impact on the product's quality and sensory appeal. Table 6.2 presents the colour properties of MSBP and MFBP, including L*, a*, b*, C* and h°. It is evident from the results that MSBP exhibited higher values for all the colour parameters (L*= 58.00 ± 0.47, a*= 7.00 ± 0.28, b*= 24.93 ± 0.73, C*= 25.89 ± 0.76 and h°= 74.31 ± 0.33) compared to MFBP (L*= 53.75 ± 0.69, a*= 4.74 ± 0.09, b*= 13.05 ± 0.57, C*= 13.89 ± 0.42 and h°= 70.02 ± 0.86). The values suggest that the former had a brighter appearance and was more pronounced in terms of red and yellow hues compared to the latter. It can be inferred from the results that mustard bee pollen possesses higher color parameter values, attributable to the fact that it is derived primarily from mustard plants which impart specific pigments. In contrast, the presence of various pigments in multifloral bee pollen likely accounts for its less uniform and more subdued colour. Thus, these variations can be associated to the distinct genetic make-up of the bee pollen varieties studied (Barth, 2004; Campos *et al.*, 2008; Bleha *et al.*, 2021).

Table 6.2. Colour analysis of bee pollen varieties

Sample code	L* (lightness)	a* (green-redness)	b* (blue-yellowness)	C* (chroma value)	h° (hue angle)
MSBP	58.00 ± 0.47[a]	7.00 ± 0.28[a]	24.93 ± 0.73[a]	25.89 ± 0.76[a]	74.31 ± 0.33[a]
MFBP	53.75 ± 0.69[b]	4.74 ± 0.09[b]	13.05 ± 0.57[b]	13.89 ±0.42[b]	70.02 ± 0.86[b]

MSBP – mustard bee pollen, MFBP – multifloral bee pollen,
The data are presented as mean ± standard deviation, calculated from three independent experiments (n=3).
Values within the same column that are marked with distinct superscripts (a, b) indicate a statistically significant difference ($p<0.05$).

6.3. FTIR analysis

One of the most significant and useful analytical methods for examining the conformational and physicochemical characteristics of any food sample is FTIR analysis. The FTIR spectra of MSBP and MFBP, shown in Figures 6.1(a) and 6.1(b) respectively, confirm the presence of significant functional groups in both types of bee pollen. The detailed interpretation of the FTIR analysis for MSBP and MFBP is presented in Tables 6.3 and 6.4, respectively. According to the analysis of the peaks and their intensities, the peak at 3272.60 cm^{-1} depicts the stretching vibrations of N–H bonds in protein structures and O–H bonds in carbohydrates, particularly glucose, fructose, and sucrose (Thakur and Nanda, 2020b; Laaroussi *et al.*, 2023). The peak at 2922.23 cm^{-1}, which can be attributed to a variety of bee pollen constituents including lipids, carbohydrates, fatty acids, cellulose, and other long chain structures, is due to the stretching vibrations of C–H bonds. Because it shows the stretching of the C–H bonds in functional groups like aldehydes, as well as in lipids and carbohydrates, the absorption peak at 2851.41 cm^{-1} is important (Anjos *et al.*, 2017; Laaroussi *et al.*, 2023). The stretching vibrations of phosphorus acid and ester P–H bonds, phosphorus acid, and ester O–H bonds, and a combination of C–H stretching vibrations were observed at a wavelength of 2359.40 cm^{-1}. The stretching vibrations of aliphatic aldehyde, lipids, ester, and C=O bonds of flavonoids are attributed to the peak at 1733.21 cm^{-1} (Anjos *et al.*, 2017; Thakur and Nanda, 2020b; Laaroussi *et al.*, 2023). Furthermore, the absorption peak at 1636.30 cm^{-1} is attributed to amide I vibrations, indicating the vibrations of C=O and C–N groups in the protein fraction of bee pollen (Carbonaro and Nucara, 2010). The peak at 1543.11 cm^{-1} and 1420.11 cm^{-1} corresponds to the stretching vibrations of C=C bonds, as well as the asymmetric stretching vibrations of aliphatic nitro compounds' NO_2 groups, the stretching vibrations of azo compounds' N=N bonds, and the first overtones of N-H and O-H stretching vibrations, along with a combination of C–H stretching vibrations. In addition to the deformation vibrations of N–H and C–H bonds in proteins, the significant peak at 1028.74 cm^{-1} also shows the stretching vibrations of aliphatic C–N bonds. The skeletal C–C stretching vibrations of the carbon skeleton in monosaccharides, notably glucose and fructose, are responsible for the vibrations detected between 920 cm^{-1} and 865 cm^{-1} (Laaroussi *et al.*, 2023).

Table 6.3. Interpretation of FTIR spectra of mustard bee pollen

Peak number	Wave number	Functional group
1	775.28	Third overtone N–H stretching, Phenylalanine- Benzene ring vibrations, Aliphatic symmetric P–O–C stretching, P=S stretching, =C–H out-of-plane bending, NH_2 wagging and twisting
2	816.28	P=S stretching, P–F stretching, NH_2 wagging and twisting, third overtone N–H stretching
3	864.74	=C–H out-of-plane bending, Nitrate N–O stretching, third overtone C–H stretching
4	1028.74	Aliphatic asymmetric P–O–C stretching, Phosphorus ester P–OH stretching, P–F stretching, Si–O–Si asymmetric stretching, Aliphatic C–N stretching
5	1237.47	Aromatic P–O stretching, Aromatic C–O stretching, second overtone C–H stretching
6	1420.11	Azo compound N=N stretching, first overtone N–H stretching, first overtone O–H stretching, Combination C–H stretching
7	1543.11	C=C stretching, Aliphatic nitro compound NO_2 asymmetric stretching
8	1636.30	Aliphatic hydrocarbons C=C stretching, Nitrate NO_2 asymmetric stretching, Oxime C=N–OH stretching, Pectin
9	1733.21	Aliphatic aldehyde C=O stretching, Aliphatic ester C=O stretching, first overtone C–H stretching
10	2359.40	Phosphorus acid and ester P–H stretching, Phosphorus acid and ester O–H stretching, Combination C–H stretching
11	2922.23	Carboxylic acid O–H stretching, Carbohydrates C–H stretching, Fats C–H stretching, CH_2; CH_3 stretching
12	3272.60	Carboxylic acid O–H stretching, Water O–H stretching

Table 6.4. Interpretation of FTIR spectra of multifloral bee pollen

Peak number	Wave number	Functional group
1	775.28	Third overtone N–H stretching, Phenylalanine- Benzene ring vibrations, Aliphatic symmetric P–O–C stretching, P=S stretching, =C–H out-of-plane bending, NH_2 wagging and twisting
2	820.01	P=S stretching, P–F stretching, NH_2 wagging and twisting, third overtone N–H stretching
3	920.65	Phosphine P–H wagging, Aromatic P–O stretching
4	1028.74	Aliphatic asymmetric P–O–C stretching, Phosphorus ester P–OH stretching, P–F stretching, Si–O–Si asymmetric stretching, Aliphatic C–N stretching
5	1256.11	Aromatic P–O stretching, Aromatic C–O stretching, second overtone C–H stretching
6	1420.11	Azo compound N=N stretching, first overtone N–H stretching, first overtone O–H stretching, C–H stretching
7	1509.57	C=C stretching, Aliphatic nitro compound NO_2 asymmetric stretching
8	1636.30	Aliphatic hydrocarbons C=C stretching, Nitrate NO_2 asymmetric stretching, Oxime C=N–OH stretching, Pectin
9	2102.21	Si–H stretching, Aromatic isonitrile –N≡C stretching
10	2359.40	Phosphorus acid and ester P–H stretching, Phosphorus acid and ester O–H stretching, Combination C–H stretching
11	2851.41	Aldehyde C–H stretching, Fats C–H stretching, Carbohydrates C–H stretching
12	2922.23	Aldehyde C–H stretching, Methylene asymmetric C–H stretching
13	3272.60	O–H stretching of carboxylic acids, Water O–H stretching, Si–OH stretching
14	3738.51	N–H stretching, N–H bending

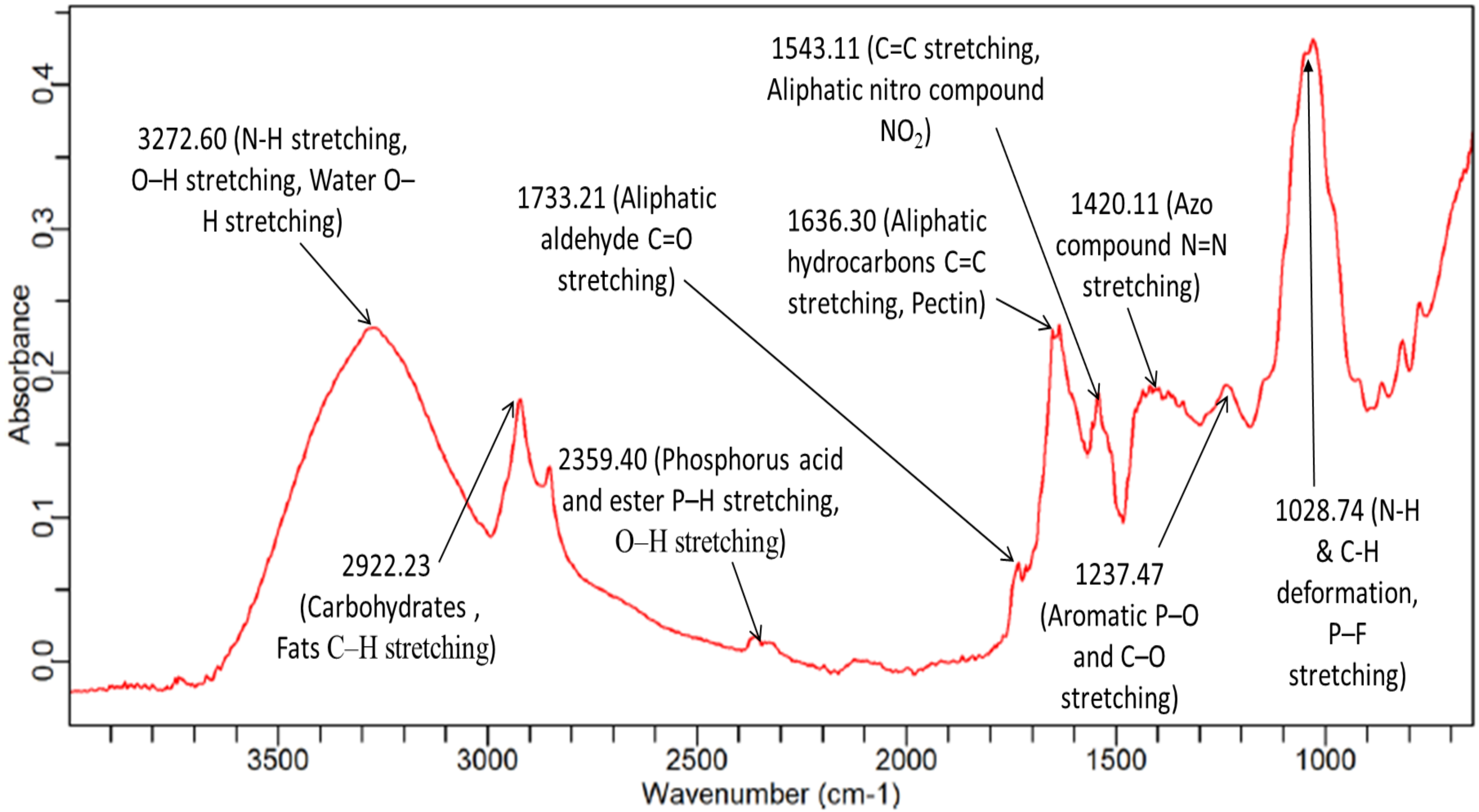


Figure 6.1(a). FTIR spectra of mustard bee pollen

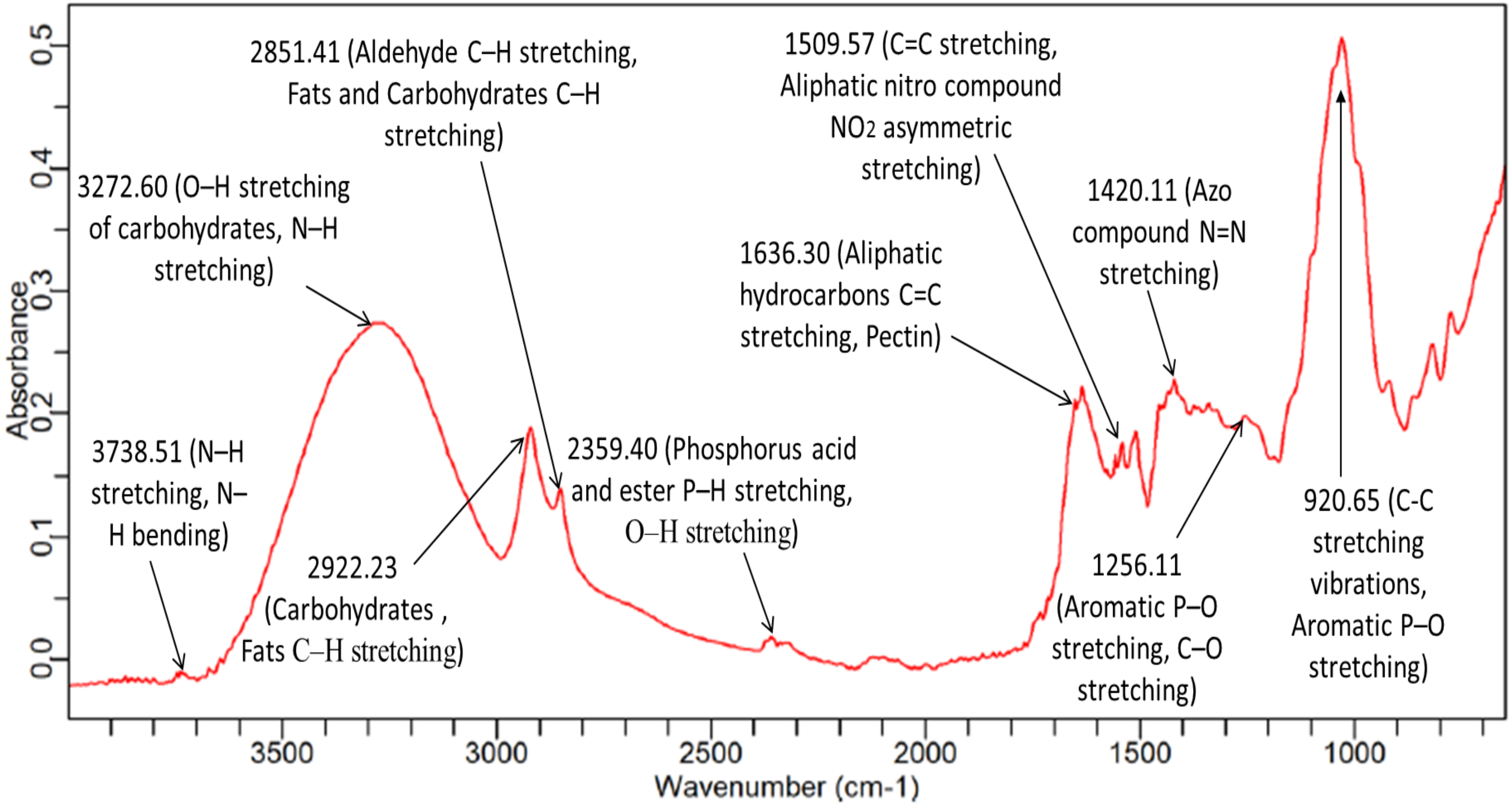


Figure 6.1(b). FTIR spectra of multifloral bee pollen

6.4. Scanning electron microscopy (SEM)

In this study, SEM helped to study the surface morphology and texture of the samples, which can reveal insights into their quality and potential applications. The SEM micrographs of mustard and multifloral bee pollen are illustrated in Figure 6.2 and 6.3 respectively. For mustard bee pollen, the morphological examinations revealed the pollen grain's structural integrity having a nearly ovoid shape and depressions on its surface. The cell wall was observed to have concentric layers, with the outermost referred to as exine. The exine sculpturing of this type is called as 'Faveolate', in which small, polygonal pits/depressions are present on the surface. This pattern is uniform and regular, similar to the cells of a honeycomb. On the other hand, the morphological examination of multifloral bee pollen revealed the pollen grain's structural integrity having a nearly prolate spheroid shape. This bee pollen variety displays a relatively smooth surface with sparse granules and vertical striations. This suggests that there were significant differences in the surface texture among both varieties, which could further impact their functionality and applications in food product formulations. A similar kind of scanning electron microscopic study was conducted by Saklani and Mattu (2020), in which they investigated the surface morphology and exine sculptures of eight different bee pollen varieties in order to create a reference pollen data valuable in palynology and melissopalynology. The conclusions of our observations were also drawn from the explanations and results reported by Saklani and Mattu (2020). The morphological features of MSBP were similar to the ones observed by Spulber *et al.* (2020).

Additionally, the SEM-EDS images represented the elemental composition of the pollen samples. It provides the data of atomic % and weight % of each element in the sample. The images reveal that the elements detected in mustard bee pollen include K, Ca, Cu, Mg, Na, and Zn, with atomic percentages of 29.25%, 28.48%, 15.25%, 9.74%, 9.53%, and 7.76%, respectively. The corresponding weight percentages were 27.12% ± 5.59, 27.07% ± 5.79, 22.98% ± 8.18, 5.61% ± 5.65, 5.20% ± 7.82, and 12.02% ± 10.25 (Figure 6.4). Similarly, in multifloral variety, elements including Fe, Ca, K, and Zn were present in significant amounts along with the presence of Cu, Mg and Na in traces. The atomic % of these elements were: Fe dominates with 52.52%, followed by Ca (20.28%), K (16.56%), Zn (5.92%), Mg (3.35%), and Cu (1.36%). The weight percentages of the elements detected were: Fe (59.27% ± 5.64), Ca (16.43% ± 2.30), K (13.09% ± 2.01), Zn (7.82% ± 5.68), Cu (1.75% ± 4.99), and Mg (1.65% ± 3.27) (Figure 6.5).

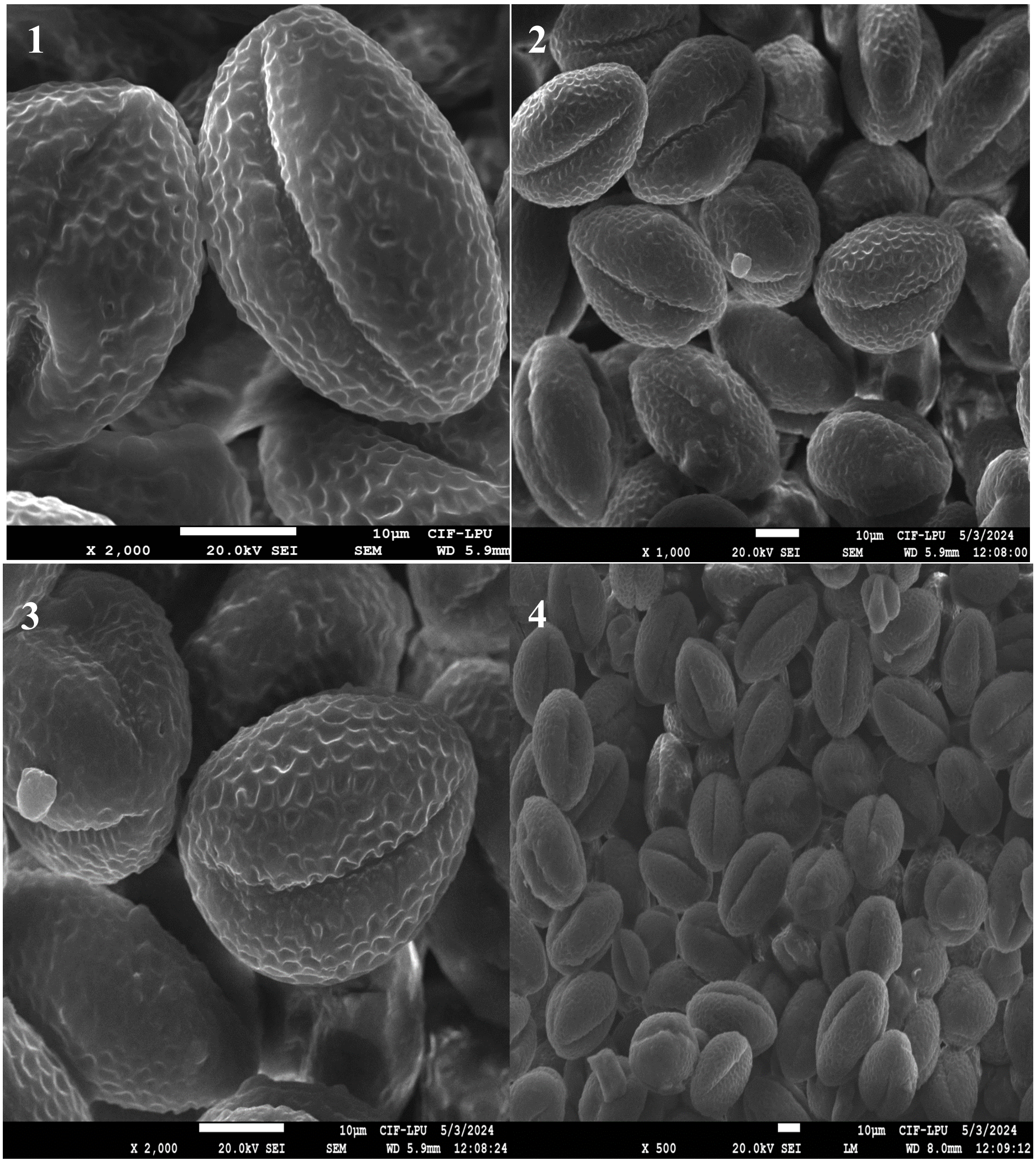


Figure 6.2. Scanning electron micrographs of mustard bee pollen variety: (1) magnification 2000X, scale bar 10 µm, 20.0 kV, WD 5.9 mm; (2) magnification 1000X, scale bar 10 µm, 20.0 kV, WD 5.9 mm; (3) magnification 2000X, scale bar 10 µm, 20.0 kV, WD 5.9 mm; (4) magnification 500X, scale bar 10 µm, 20.0 kV, WD 8.0 mm

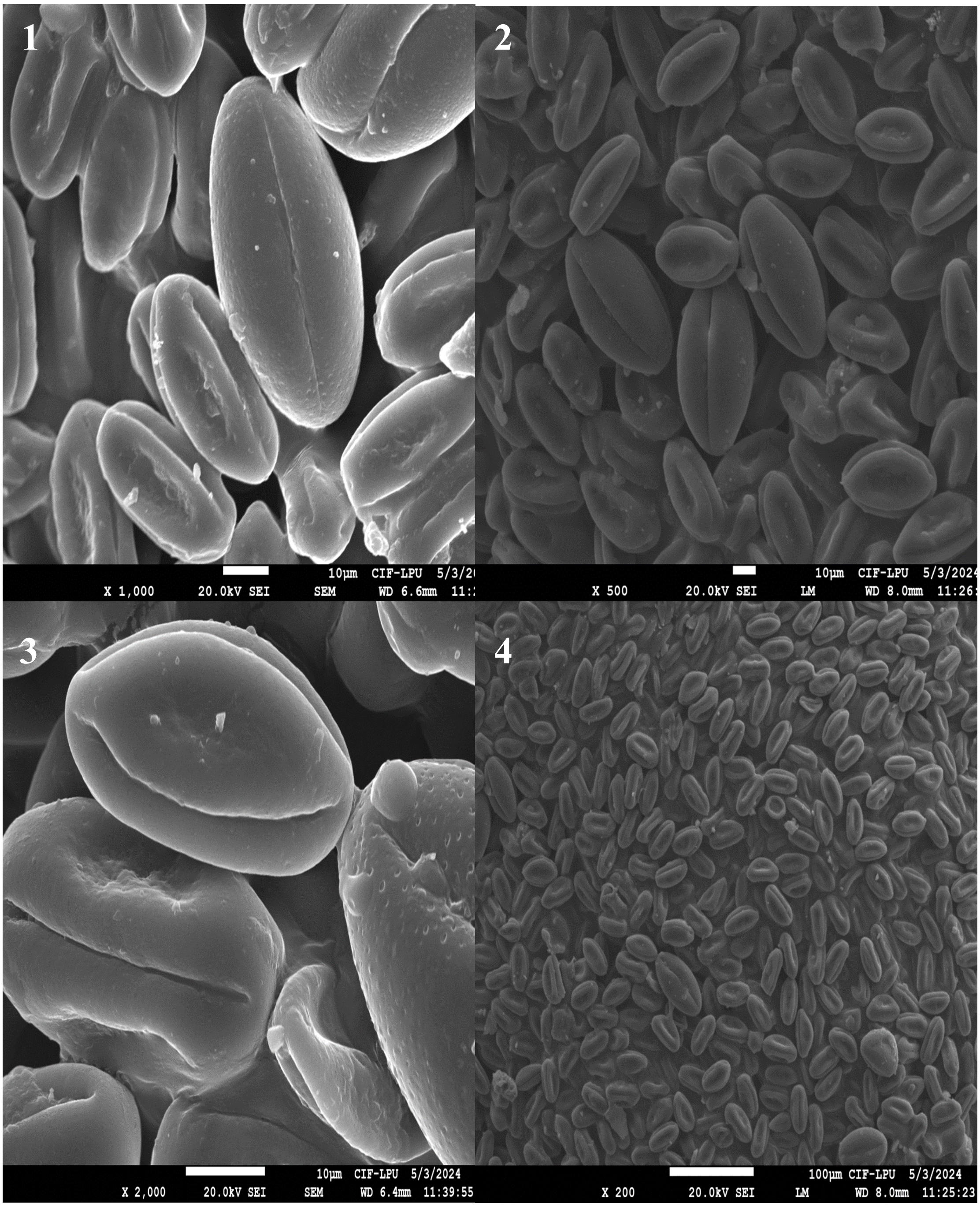


Figure 6.3. Scanning electron micrographs of multifloral bee pollen variety: (1) magnification 1000X, scale bar 10 µm, 20.0 kV, WD 6.6 mm; (2) magnification 500X, scale bar 10 µm, 20.0 kV, WD 8.0 mm; (3) magnification 2000X, scale bar 10 µm, 20.0 kV, WD 6.4 mm; (4) magnification 200X, scale bar 10 µm, 20.0 kV, WD 8.0 mm

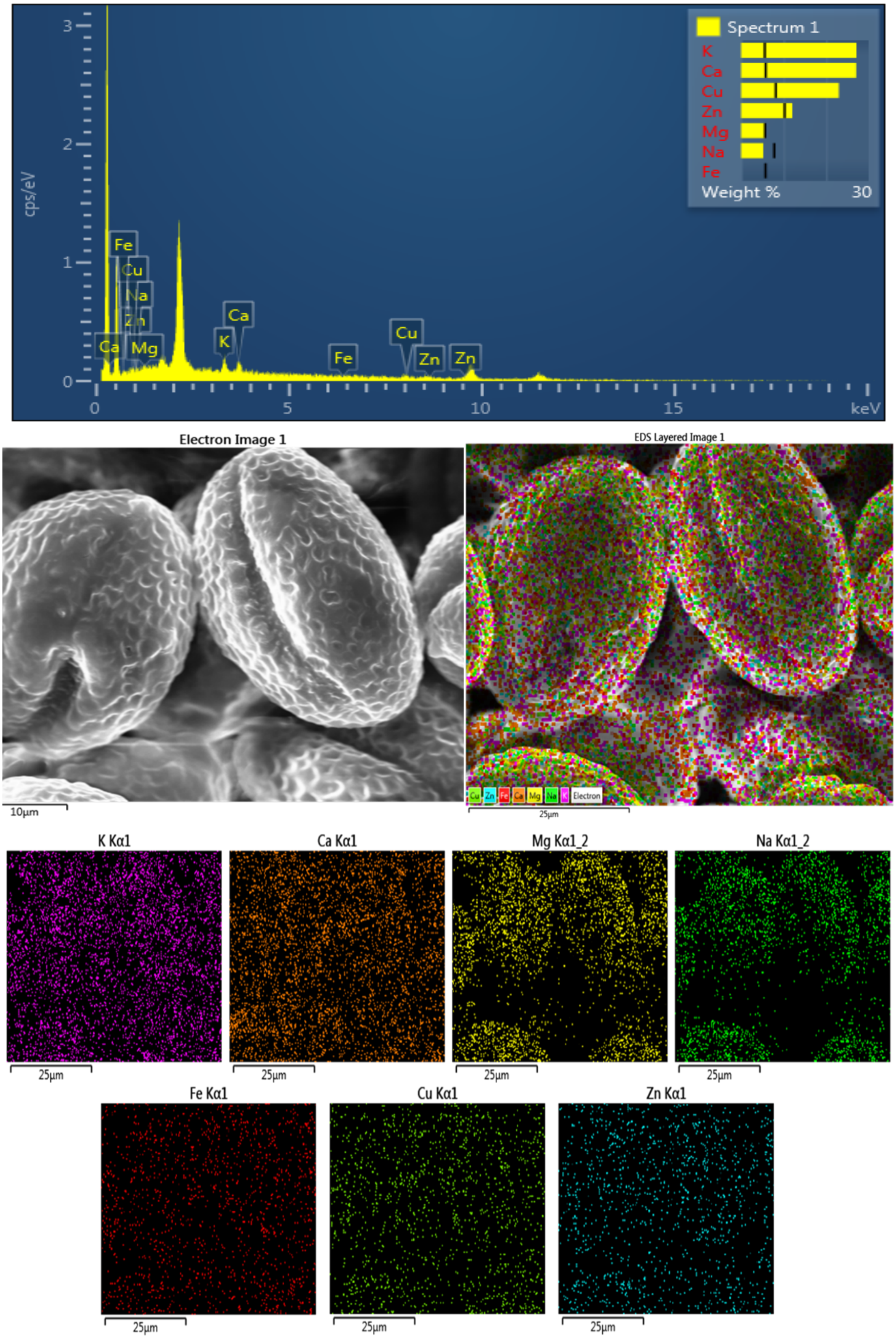


Figure 6.4. Elemental mapping of mustard bee pollen using energy-dispersive X-ray spectroscopy (EDS)

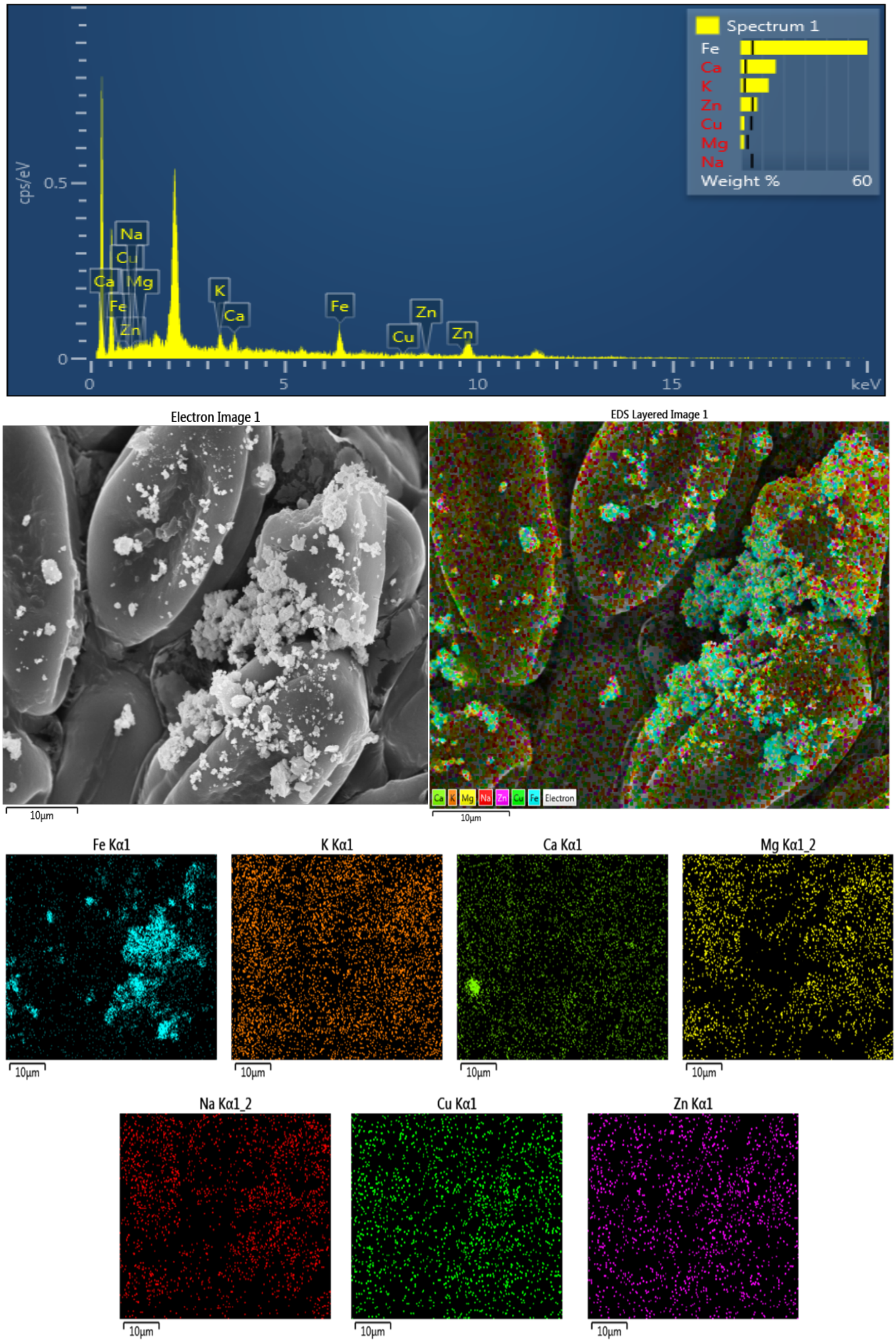


Figure 6.5. Elemental mapping of multifloral bee pollen using energy-dispersive X-ray spectroscopy (EDS)

6.5. Texture analysis

The texture analysis of bee pollen samples allows us to better understand their textural properties, which are crucial for determining their potential applications in food product development. Bee pollen can be consumed in various forms, including as whole pellets, in powdered form, or as an extract prepared using food-grade solvents such as ethanol or distilled water. According to the results obtained (Table 6.5), hardness of MSBP and MFBP was found to be 15.81 ± 1.69 N and 23.88 ± 2.34 N respectively. This difference might be attributed to the moisture content of the samples. Moisture content has been reported to be negatively correlated with hardness; therefore, mustard bee pollen, having high moisture was less hard in comparison to the multifloral variety. Water, acting as a plasticizer in the amorphous regions, facilitates hydrogen bond breakage, which further leads to the formation of new hydrogen bonds between water molecules and the polysaccharide chains. Similar trend of hardness was observed by Singh *et al.* (2013) as well as Thakur and Nanda (2018). The cohesiveness of MFBP was higher (1.32 ± 0.07) than MSBP (0.18 ± 0.05) due to the varying structural integrity of pollen samples. The conclusion drawn from this observation is that the former variety is capable of withstanding the stresses associated with processing, packaging, and logistics, allowing it to be delivered to consumers in their natural state without damage (Thakur and Nanda, 2018). The other values corresponding to the different textural attributes of MSBP are as follows: compressibility 3474.67 ± 2.47%, relaxation 33.83 ± 1.28%, stiffness 11.08 ± 2.73 MPa, and elastic recovery 1.49 ± 1.76%. In contrast, the values for MFBP were found to be compressibility 2385.90 ± 2.69%, relaxation 23.42 ± 1.09%, stiffness 18.18 ± 1.57 MPa, and elastic recovery 0.46 ± 1.42%. The distinct chemical composition of the samples, surface characteristics and cultivar are the major factors known to largely affect all the aforementioned textural attributes (Thakur and Nanda, 2018).

6.6. Mineral analysis

Table 6.6 illustrates the results of mineral analysis of bee pollen, which indicates that both samples lacked toxic minerals such as arsenic, mercury, nickel, and lead. This suggests that they are suitable for inclusion in the daily diet.

Table 6.5. Texture analysis of bee pollen samples

Sample code	Textural attributes					
	Compressibility (%)	Relaxation (%)	Stiffness (MPa)	Elastic recovery (%)	Hardness (N)	Cohesiveness
MSBP	3474.67 ± 2.47[a]	33.83 ± 1.28[a]	11.08 ± 2.73[b]	1.49 ± 1.76[a]	15.81 ± 1.69[b]	0.18 ± 0.05[b]
MFBP	2385.90 ± 2.69[b]	23.42 ± 1.09[b]	18.18 ± 1.57[a]	0.46 ± 1.42[b]	23.88 ± 2.34[a]	1.32 ± 0.07[a]

MSBP – mustard bee pollen, MFBP – multifloral bee pollen
The data are presented as mean ± standard deviation, calculated from three independent experiments (n=3).
Values within the same column that are marked with distinct superscripts (a, b) indicate a statistically significant difference ($p<0.05$).

The calcium content of MSBP and MFBP was almost identical i.e., 816.3 ± 0.86 mg/100 g and 882.2 ± 1.15 mg/100 g respectively. The results also reveal that MSBP had higher phosphorus (1973.5 ± 0.66 mg/100 g), magnesium (386.3 ± 1.04 mg/100 g), iron (15.52 ± 0.56 mg/100 g), and zinc (24.4 ± 0.21 mg/100 g) content than MFBP. Whereas the sodium content was reported to be 927.4 ± 1.22 mg/100 g in MFBP while it was found to be zero in MSBP. Research studies suggest that soil, climate, geographic origin, harvest period, and plant species are the key determinants of the mineral composition of bee pollen; which may explain the difference observed between the studied samples (Valverde *et al.*, 2023; Laaroussi *et al.*, 2023). Minerals are essential components of our daily diet, crucial for maintaining proper physiological and metabolic functions. Iron is vital for synthesizing hemoglobin, zinc protects against superoxide radicals, potassium helps manage blood pressure, magnesium maintains blood glucose levels, and sodium regulates the acid-base balance in the human body (Bakour *et al.*, 2022; Laaroussi *et al.*, 2023). Due to adulteration with pollen from unreported origins, the mineral profile can be utilized as a biomarker of the botanical and geographic origins of bee pollen. Thus, it is useful in terms of preventing fraud in the beekeeping business (Valverde *et al.*, 2023). It is further important for assessing the quality and safety of consumption of bee pollen (Wang *et al.*, 2022).

Table 6.6. Mineral analysis of bee pollen varieties

Sample code	Ag (mg/100 g)	As (mg/100 g)	Ca (mg/100 g)	Fe (mg/100 g)	Hg (mg/100 g)	Mg (mg/100 g)	Ni (mg/100 g)	P (mg/100 g)	Pb (mg/100 g)	Zn (mg/100 g)	Co (mg/100 g)	Cu (mg/100 g)	Mn (mg/100 g)	Na (mg/100 g)
MSBP	0	0	816.3 ± 0.86^b	15.52 ± 0.56^a	0	386.3 ± 1.04^a	0	1973.5 ± 0.66^a	0	24.4 ± 0.21^a	0	0	0	0
MFBP	0	0	882.2 ± 1.15^a	8.4 ± 0.19^b	0	204.7 ± 1.06^b	0	713.4 ± 0.63^b	0	24.1 ± 0.54^b	0	0	0	927.4 ± 1.22

MSBP – mustard bee pollen, MFBP – multifloral bee pollen
The data are presented as mean ± standard deviation, calculated from three independent experiments (n=3).
Values within the same column that are marked with distinct superscripts (a, b) indicate a statistically significant difference ($p<0.05$).

6.7. Physicochemical and phytochemical analysis

Bee pollen primarily consists of carbohydrates and water, along with minor compounds including proteins, minerals, organic acids, enzymes, and secondary metabolites. Despite their relatively low concentrations, bioactive compounds such as flavonoids, carotenoids, ascorbic acid, and phenolic compounds significantly contribute to the health-promoting properties of bee pollen. The physicochemical properties of the different bee pollen varieties analysed in this study are summarized in Table 6.1. The moisture content for MSBP was measured at 15.33 ± 0.36%, while MFBP had a moisture content of 7.6 ± 0.58%. In terms of ash content, MSBP recorded 4.3 ± 0.014%, compared to 3.3 ± 0.014% for MFBP. The difference in the values might be attributed to the different compositions of both varieties. The results were consistent with the values reported by Thakur and Nanda (2018) and Dulger Altiner *et al.* (2020). Ash content indicates the inorganic constituents in these bee pollen which are significantly impacted by the soil type and botanical origin; thus, it is a crucial quality criterion (Bakour *et al.*, 2022). The total fat content data indicated that MSBP and MFBP had values of 1.5 ± 0.33% and 2.0 ± 0.28%, respectively. These fat content measurements align with the findings of Barajas *et al.* (2012), which reported a range of 1.14 ± 1.1% to 5.1 ± 1.8%.

The recorded value for protein content of MSBP and MFBP was 21.88 ± 0.83 g/100 g and 20.65 ± 0.95 g/100 g, respectively. These values align with the ranges reported by various studies: 14.81–37.25 g/100 g as reported by Kostić *et al.* (2015), 10–40 g/100 g as reported by Bogdanov (2016), 21.09 ± 0.16 g/100 g recorded by Darwish *et al.* (2023), and 16.63 ± 3.66 to 31.17 ± 3.81 g/100 g as reported by Laaroussi *et al.* (2023). The data pertaining to carbohydrate content showed the values of MSBP and MFBP as 43.78 ± 0.62 g/100 g and 41.98 ± 0.88 g/100 g respectively. The values were similar to the range (18.52 ± 0.19 g/100 g to 46.44 ± 0.55 g/100 g) reported by Laaroussi *et al.* (2023). The date regarding crude fibre showed that MSBP (3.53 ± 0.52 g/100 g) had slightly higher values compared to MFBP (3.042 ± 0.33 g/100 g). These findings align with those reported by Thakur and Nanda (2018).

The total phenolic content was found to be 2676 ± 0.58 mg GAE/100 g for MSBP and 2466 ± 0.34 mg GAE/100 g for MFBP. Previous studies have reported that the phenolic content of bee pollen can reach up to 4300 mg GAE/100 g (Habryka *et al.*, 2021). However, the type and region of the plant sources from which bee pollen is derived have a considerable impact on its phenolic content. Literature reports highlight considerable variation in TPC of bee pollen, which is largely influenced by botanical origin. For instance, phenolic content has been reported to range from 420 mg GAE/100 g in pollen collected from magnolia flowers to 2960 mg GAE/100 g in pollen derived from white nettle (*Lamium*) blossoms (Habryka *et al.*, 2021). Additionally, a study by LeBlanc *et al.* (2009) reported a phenolic content of 3485 mg GAE/100 g in bee pollen obtained from mimosa flowers. Beyond their ability to neutralize organic radicals and scavenge reactive oxygen species, polyphenols also have the capacity to chelate metal ions that promote oxidation reactions, as noted by Soares de Arruda *et al.* (2021). Numerous studies have established a correlation between increased consumption of phenolic antioxidants and a reduced risk of vascular diseases and certain types of cancer (Mărgăoan *et al.*, 2010).

The data obtained revealed that the TFC was 14.025 ± 0.25 mg QE/g for MSBP and 8.38 ± 0.41 mg QE/g for MFBP. In addition to this, the antioxidant activity (DPPH) of MSBP and MFBP was observed to be 82.6 ± 0.54% and 64.81 ± 0.25% respectively. The rich content of polyphenols and flavonoids (chlorogenic acid, caffeic acid, catechin, chrysin, isorhamnetin, kaempferol, naringenin, and quercetin) contributes to the enhanced antioxidant activity observed in different bee pollen varieties (Leja *et al.*, 2007; Harif Fadzilah *et al.*, 2017; Dulger Altiner *et al.*, 2020). These antioxidants protect cell organelles, cytoplasm, and extracellular fluids from damage caused by free radicals (Soares de Arruda *et al.*, 2021).

The literature states that the total carotenoid content in fresh bee pollen ranges from 27 to 35 µg/g and 25.34 to 368.5 µg/g in processed samples (Melo and Almeida-Muradian, 2010). In the present study, it was observed that the values obtained for total carotenoid content of MSBP (30 ± 0.33 µg/g) and MFBP (38 ± 0.58 µg/g) were also found to be in the same range. The amount of chlorophyll a and b was reported to be 0.015 ± 0.01 mg/100 g and 0.014 ± 0.01 mg/100 g for MSBP, while 0.004 ± 0.012 mg/100 g and 0.006 ± 0.01 mg/100 g MFBP. The colour of pollen pellets is influenced by the presence of pigments like chlorophyll, flavonoids, carotenoids and anthocyanins. Chlorophyll imparts green colour and flavonoids are responsible for colours such as red, pink, purple, and blue (Mărgăoan *et al.*, 2010; Thakur and Nanda, 2018). Carotenoids constitute a diverse class of lipid-soluble compounds derived from isoprenoids, responsible for imparting a broad spectrum of colors ranging from pale yellow to deep red, including various shades of orange and yellow-orange. In addition to their pigmentation properties, carotenoids have been associated with a reduced risk of certain types of cancer (Mărgăoan *et al.*, 2010).

Owing to the fact that anthocyanins possess biological properties like antioxidant and anti-inflammatory, these pigments are of great importance. It is primarily found in flowers, fruits, and vegetables and is responsible for imparting blue, red, and purple colours. Regarding anthocyanin content, MFBP had a higher value of 5.9 ± 0.05

mg/100 g compared to MSBP, which had 2.44 ± 0.10 mg/100 g. Di Paola-Naranjo *et al.* (2004) attempted to quantify anthocyanins in dark blue pollen from *Echium plantagineum* and found that the levels ranged from 45 to 80 mg/100 g. The study confirmed that the selected variety was an abundantly rich source of phytochemicals. A study by Chelucci *et al.* (2023) reported the anthocyanin content of Tuscan bee pollen to be 58.16 ± 1.45 mg C3GE/L. Among the various floral sources analysed, *Rubus* bee pollen exhibited a concentration of 53.44 ± 2.36 mg C3GE/L, *Cistus* pollen contained 57.19 ± 5.84 mg C3GE/L, and the highest value was observed in *Castanea* bee pollen at 77.37 ± 2.55 mg C3GE/L. The values obtained in our study were significantly lower, which may be attributed to differences in the geographical locations where the bee pollen was collected which ultimately affects the chemical composition of pollen samples.

Vitamin C, or ascorbic acid, is a crucial water-soluble vitamin. It gets oxidized to dehydroascorbic acid on exposure to oxygen, light, high pH and high temperature (Zhu *et al.*, 2020). In our study, the results for ascorbic acid content indicated that MSBP possessed higher values, measuring 76.6 ± 0.32 µg/g, compared to MFBP, which had 58.4 ± 0.43 µg/g. Similar values were found for rape and lotus bee pollen, with ranges oscillating between 17.54 to 94.01 µg/g and 66.01 to 111.66 µg/g, respectively (Zhu *et al.*, 2020). The literature also states that fresh bee pollen, when stored in a freezer for an extended period, loses its vitamin C content significantly, whereas dried bee pollen has a higher ascorbic acid content. Dehydration helps preserve the levels of this vitamin (Melo *et al.*, 2010).

6.8. Antinutritional factors in bee pollen varieties

6.8.1. Alkaloids

Although bee pollen contains numerous health-promoting compounds, like many natural products, it may also contain anti-nutritional components that could be detrimental to human health. One of these compounds is pyrrolizidine alkaloids, which are secondary metabolites of natural origin produced by plants to protect against herbivores (Inacio *et al.*, 2020). It has been reported in research studies that the concentration of pyrrolizidine alkaloids in bee pollen is much higher than its amount present in honey (Mulder *et al.*, 2015). The European Food Safety Authority

(EFSA) has established a maximum recommended intake for pyrrolizidine alkaloids at 0.007 µg/kg of body weight per day to mitigate potential carcinogenic risks. Based on this guideline, the tolerable daily intake for an average adult male weighing 60 kg should not exceed 0.42 µg (Inácio *et al.*, 2020). Dübecke *et al.* (2011) states that the safe limit for consuming pyrrolizidine alkaloids is 1 µg/day if consumed for six weeks, and 0.1 µg/day if consumed for longer than six weeks. In our study, the results indicated that the alkaloid content in MSBP and MFBP was found to be 0.33 ± 0.002 mg/100 g and 0.16 ± 0.004 mg/100 g respectively (Table 6.7). The results of the present study align with those reported by Inácio *et al.* (2020), supporting the conclusion that the examined bee pollen varieties are safe for human consumption.

6.8.2. Phytic acid

Due to the role of phytic acid in forming insoluble complexes with other food nutrients (like iron) and reducing their bioavailability, it is regarded as an anti-nutritional factor. But literature also suggests that it has proven effects against cardiovascular diseases, diabetes, kidney stone and cancer (Bloot *et al.*, 2023). Jackson and Linskens (1982) reported that pollen from plants having style length >5 mm contains significant amounts of phytic acid, whereas pollen from plants with very short style lengths possesses little or no phytic acid in them. Regarding phytic acid content, the values obtained were 0.347 ± 0.001 mg/100 g and 0.245 ± 0.003 mg/100 g for MSBP and MFBP, respectively (Table 6.7). The minor variability could be due to differences in the plant sources from which the pollen is collected. The specific values of phytic acid concentration in bee pollen samples have not been reported, but studies suggest that the amount is typically lower compared to other plant sources, such as cereals and legumes.

6.8.3. Tannins

Besides having anti-nutritional effects, such as reduced digestibility, tannins are a class of water-soluble phenolic constituents that exhibit antioxidant, anti-inflammatory, anti-helminthic, anti-allergic, anti-cancer and antimicrobial properties. It also possesses wound healing properties (Sharma *et al.*, 2021; Ogidi *et al.*, 2019). The data pertaining to the total tannin content in mustard and multifloral bee pollen is not available. The results in current study showed that MSBP contained 0.605 ± 0.068 mg TA/100 g and MFBP contained 0.370 ± 0.002 mg TA/100 g (Table 6.7). The variation in values was linked to the plant source from which the pollen was obtained. A study conducted by Ogidi *et al.* (2019) reported that the tannin content in brown mustard seeds is 7.75 ± 0.06%. In another study, Pascoal *et al.* (2014) reported the tannin concentration in clover pollen and maize pollen with values corresponding to 752.21 mg/100 g and 447.10 mg/100 g, respectively.

Table 6.7. Antinutritional factors present in bee pollen varieties

Sample	Alkaloids (mg/100 g)	Phytic acid (mg/100 g)	Tannins (mg TA/100 g)
MSBP	0.33 ± 0.002^{a}	0.347 ± 0.001^{a}	0.605 ± 0.068^{a}
MFBP	0.16 ± 0.004^{b}	0.245 ± 0.003^{b}	0.370 ± 0.002^{b}

MSBP – mustard bee pollen, MFBP – multifloral bee pollen
The data are presented as mean ± standard deviation, calculated from three independent experiments (n=3).
Values within the same column that are marked with distinct superscripts (a, b) indicate a statistically significant difference (p<0.05).

6.9. Antimicrobial activity

Exploring the antimicrobial properties of natural food products is essential due to the increasing emergence of antimicrobial resistance, which is a global concern. The antimicrobial activity of bee pollen is influenced by several factors, including its botanical origin, chemical composition, extraction procedure, and the type of solvent employed (Didaras *et al.*, 2020). In the present study, bee pollen powder was assessed for its antibacterial activity against selected pathogenic microorganisms, comprising both gram-positive (*Staphylococcus aureus*, *Bacillus cereus*) and gram-negative (*Escherichia coli*, *Salmonella typhi*) bacterial strains. The diameter of the inhibition zone (in mm) surrounding each well on the agar plate was recorded, as illustrated in Figure 6.6. The positive control (streptomycin, a broad-spectrum antibiotic) had a considerably high zone of inhibition corresponding to 22.8 mm, 22.0 mm, 20.0 mm, 18.5 mm for *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhi and Escherichia coli*, respectively. For MSBP, it was observed that *Staphylococcus aureus* had the highest zone of inhibition (18.00 ± 0.14 mm) followed by *Bacillus cereus*

(15.3 ± 0.14 mm), *Salmonella typhi* (17.65 ± 0.07 mm) and *Escherichia coli* (15.00 ± 0.14 mm) (Figure 6.7). For MFBP, the results of zone of inhibition were observed as: 17.7 ± 0.21 mm against *Staphylococcus aureus*, 14.6 ± 0.14 mm against *Bacillus cereus*, 17.0 ± 0.14 mm against *Salmonella typhi* and 14.00 ± 0.21 mm against *Escherichia coli* (Figure 6.8). Thus, it was inferred from the results that gram positive bacterial strains were more susceptible than the gram-negative ones. The results were in accordance with the ones reported by Didaras *et al.* (2020). However, there are certain exceptions. For instance, Kačániová *et al.* (2012) observed that, depending on the variability in extract concentration, *E. coli* was most susceptible when tested against a 70% ethanolic extract of bee pollen, but became more resistant when the extract concentration was increased to 96%. In another study carried out by Erkmen and Ozcan (2008), the antimicrobial effect of Turkish pollen was found to be negligible. The major compounds responsible for the antimicrobial activity of bee pollen include flavonoids such as quercetin glycosides, kaempferol glycosides, luteolin, myricetin, apigenin, galangin, and others. These compounds showcase antimicrobial effects through various mechanisms such as bacterial cell membrane mutilation, preventing the formation of biofilms, inhibiting *E. coli* DnaB helicase, aggregation of bacterial cells, and destabilization of cell wall constituents. In addition to these flavonoids, other phenolic compounds (ferulic acid, p-coumaric acid, gallic acid, and esters of caffeic acid) also contribute to the antimicrobial effects of bee pollen. Their mechanism of action involves disruption of bacterial cell wall, changes in hydrophobicity, binding to genomic DNA of bacteria or inducing oxidative stress (Didaras *et al.*, 2020).

Similarly, the antifungal activity of bee pollen varieties was evaluated against *Candida albicans*, which is reported to be one of the most resistant fungal strains. Literature also confirms that fungal strains are comparatively more resistant to the effect of bee pollen extract than the bacterial strains (Kaškonienė *et al.*, 2020). In our study, the diameter of inhibition zone formed against *Candida albicans* was measured to be 5.2 ± 0.28 mm for MSBP and 4.7 ± 0.21 mm for MFBP, with a positive control value of 10.5 mm (Figures 6.7 and 6.8). The antifungal activity is attributed to the high phenolic content and high antioxidant potential of bee pollen varieties (Didaras *et al.*, 2020). A number of studies have presented evidence that bee pollen exhibits strong antifungal properties. Kacániová *et al.* (2012) reported that the inhibition zone of the positive control against *Candida albicans* measured 12.33 ± 0.58 mm, whereas the inhibition zone for the bee pollen extract—primarily composed of monofloral pollen from sunflower, poppy, and rape—was recorded at 2.17 ± 0.29 mm. The values obtained in our study were comparable to these results, with slight variations likely due to differences in the botanical source and chemical composition of the bee pollen. Ilie *et al.* (2022) also reported similar results stating that gram +ve bacterial strains were more sensitive in comparison to gram -ve pathogenic strains. The higher resistance of gram-negative strains is likely due to their complex cell wall made up of peptidoglycans and lipoproteins, which acts as a barrier and limits the permeability of external constituents.

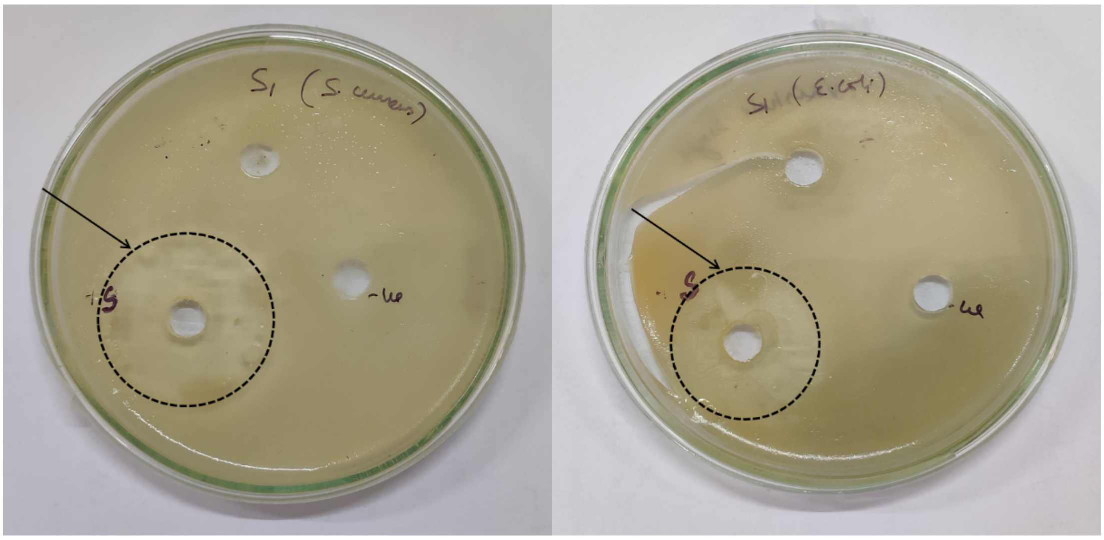


Figure 6.6. Inhibition zone against *Staphylococcus aureus* and *Escherichia coli*

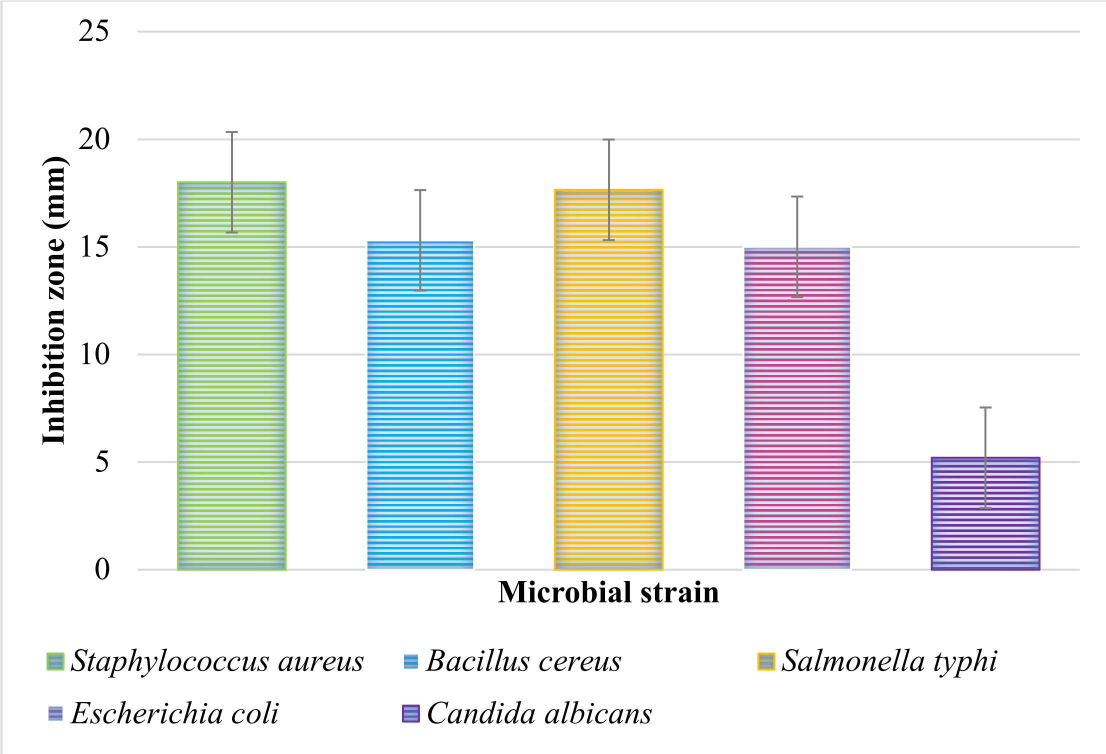


Figure 6.7. Antimicrobial activity of mustard bee pollen

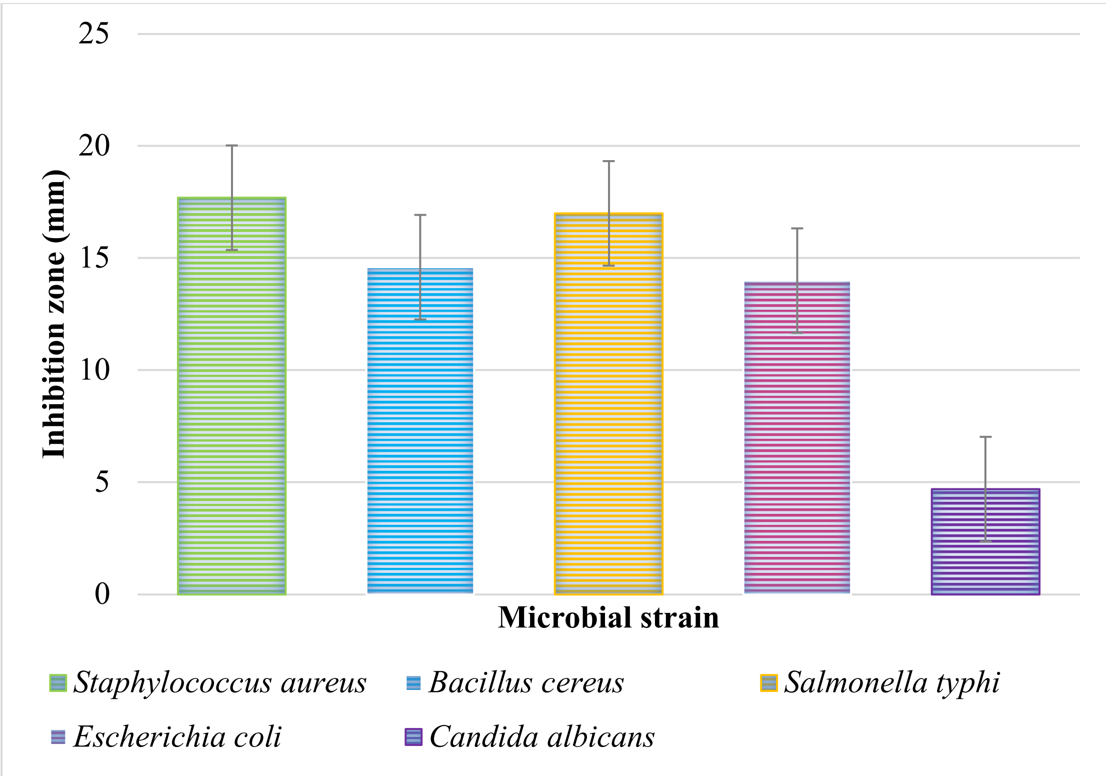


Figure 6.8. Antimicrobial activity of multifloral bee pollen

6.10. Cytotoxicity of bee pollen varieties

MTT assay is based on the principle of conversion of MTT to formazan in the presence of enzyme mitochondrial dehydrogenase. This causes a colour change from yellow to purple. The %age of living cells are compared to control and further expressed as cell viability (Ilie *et al.*, 2022). The *in-vitro* results of anti-cancer effect of bee pollen extracts revealed that both varieties inhibited the growth of MCF-7 cancer cell line in a dose-dependent manner (mg/ml) (Figure 6.9). MCF-7 is a widely used human breast cancer cell line in research studies. It was observed that at low concentration, i.e., 5 mg/ml, the cell proliferation was close to 100%, indicating minimal impact on the viability of MCF-7 cells. With further increase in sample extract concentration, the cell proliferation decreased to 69% for MSBP and 79% for MFBP. At concentration around 20 mg/ml, cell proliferation dropped to 50% and 61% for MSBP and MFBP, respectively. From 20 to 40 mg/ml, it decreased further, reaching 26% and 37% for MSBP and MFBP, respectively at higher concentration (40 mg/ml). Thus, it was concluded that at higher concentration, bee pollen extract exhibited a strong inhibitory effect on MCF-7 cell growth and warrants its anti-cancer properties. The IC50 values of MSBP and MFBP were inferred from the provided data points based on the concentration at which the % cell proliferation was halved. The cytotoxic effect of bee pollen is associated with the high phenolic content, bioactive compounds and antioxidant activity of the varieties under study. The phenolics and flavonoids found in bee pollen are crucial in triggering apoptosis in cancer cells, thereby inhibiting their growth and proliferation (Nguyen *et al.*, 2022).

The findings of this study are consistent with those reported by Omar *et al.* (2016), who demonstrated that bee pollen extract exhibited anti-proliferative activity against both cancerous (MCF-7) and normal (L929) cell lines, with IC_{50} values of 15 mg/ml and 26 mg/ml, respectively. Additionally, the extract enhanced the cytotoxic effect of cisplatin on MCF-7 cells across multiple concentrations, indicating a synergistic interaction. Similarly, Amalia *et al.* (2020) investigated the pharmacological effects of a water-soluble bee pollen extract derived from *Trigona* spp. on the MCF-7 human breast cancer cell line, further supporting the potential therapeutic applications of bee pollen.

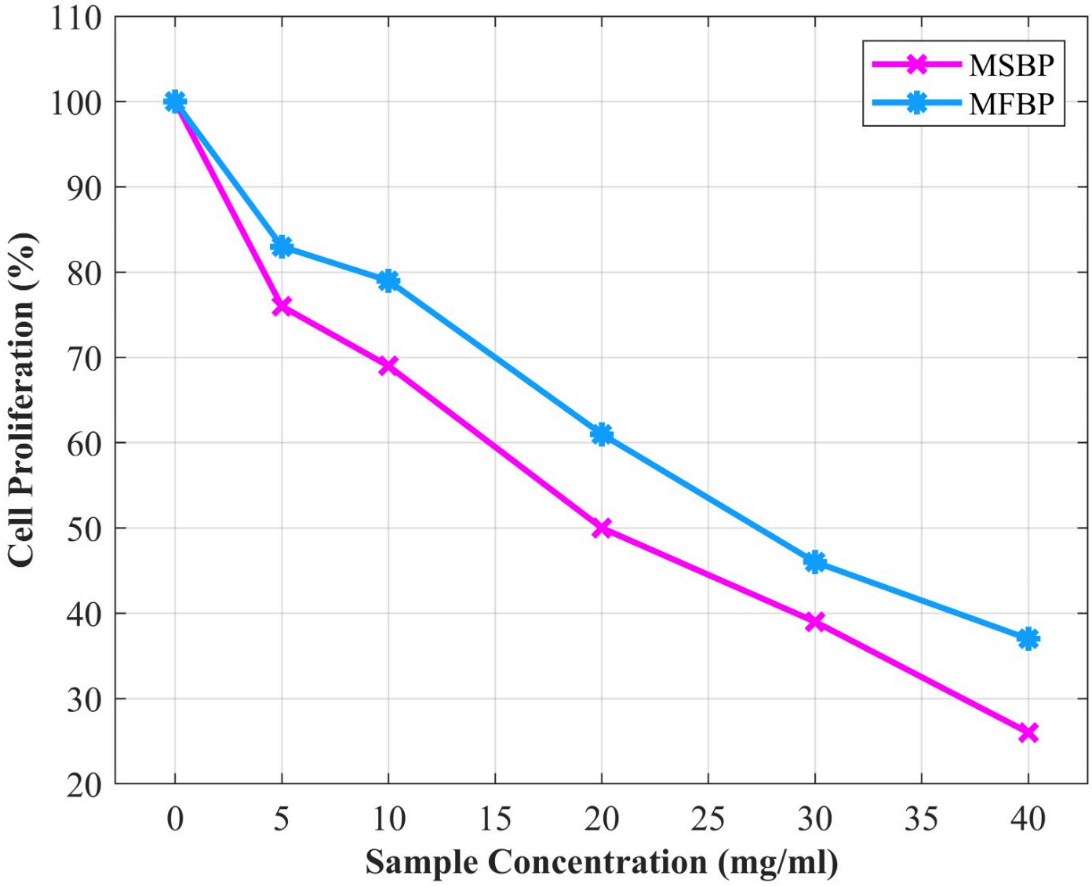


Figure 6.9. Antiproliferative activity of MSBP and MFBP against MCF-7

The bee pollen extract exhibited strong antioxidant activity, with an IC_{50} value of 18.6 mg/ml, and demonstrated notable cytotoxic effects, particularly after 24 hours of exposure. Nguyen *et al.* (2022) further confirmed the anti-proliferative potential of bee pollen, reporting its effectiveness against three distinct breast cancer cell lines: MCF-7, BT-20, and Hs 578T. Their findings underscore the potential of bee pollen as a natural bioactive compound with significant anticancer properties, capable of inhibiting the growth of diverse breast cancer cell types.

6.11. Results of effect of different pre-treatments on bee pollen varieties using HPLC

The structure of bee pollen, particularly its outermost layer (exine) or cell wall, consists of stratified concentric layers. It is primarily made up of sporopollenin, which contains phenolics, aliphatic compounds, and fatty acids. This exceptionally strong biopolymer resists high temperatures, chemical degradation, enzymatic action, and microbial effects. Due to its extraordinary stability and resistance, various pre-treatments or processing methods must be applied to bee pollen to enhance its nutritional quality, functional properties, and digestibility. These treatments ultimately extend the product's shelf life and make it more suitable for human consumption (Benavides-Guevara *et al.*, 2017; Kostić *et al.*, 2020; Kaur *et al.*, 2024).

In this study, the bee pollen varieties were subjected to three different pre-treatments: thermal cracking at three different temperatures, alkaline pre-treatment, and wet heat thermal pre-treatment, with the aim of improving the digestibility and functionality of bee pollen. HPLC was then used to analyse the effect of these processing conditions or pre-treatments on the phenolic content. Alkaline pre-treatment, a cost-effective and viable method, enhances the solubilization of complex carbohydrates (lignocellulosic materials) and reduces crystallinity (Modenbach and Nokes, 2014). High temperature is a low-cost and relatively simple method which helps to disrupt the tough outer coat (exine) on a laboratory scale. It also causes the unfolding of polypeptides and can expose active sites for further penetration by digestive enzymes. Heating can impact the structural and mechanical properties of bee pollen, and exposure to excessively high temperatures may cause the breakdown of certain heat-sensitive compounds (Aylanc *et al.*, 2021; Zhang *et al.*, 2023). According to published literature, all the pre-treatments selected in the current study have been reported to increase digestibility to as high as 85–98%. The process involves the breakdown of complex protein structures into simpler components by cleaving peptide bonds, which enhances the accessibility of nutrients and facilitates the diffusion of nutritional elements in the gastrointestinal tract (Benavides-Guevara *et al.*, 2017; Kaur *et al.*, 2024).

6.11.1. Cracking method

HPLC chromatograms obtained after subjecting both varieties of bee pollen to thermal cracking pre-treatments revealed that, in multifloral sample subjected to 35 °C temperature, the concentration of phenolics varied as- ferulic acid in highest amount (252.241 mg/l) followed by cinnamic acid (185.962 mg/l), quercetin (135.757 mg/l) and caffeic acid (23.282 mg/l) (Figure 6.10). Ferulic acid is beneficial for humans as it possesses pharmacological properties such as, anti-inflammatory, anticancer, anti-oxidative, anti-fibrotic, and anti-allergic. It is essential for providing rigidity and structure to the plant cells being closely linked to the plant polysaccharides (Zhai *et al.*, 2023). Quercetin and cinnamic acid have antioxidant and anti-inflammatory effects and help prevent heart disease and cancer. Caffeic acid has in addition of these, anti-viral properties through phytochemical action (Pyo *et al.*, 2024). On increasing the temperature to 40 °C, the concentration of caffeic acid, ferulic acid and quercetin were found to be increased to values corresponding to 41.883 mg/l, 254.475 mg/l and 530.719 mg/l, respectively (Figure 6.10). Further increasing the temperature to 45 °C, the concentration of all the phenolics except cinnamic acid was decreased. The decrease in phenolic content when the temperature increased to 45 °C may be due to the fact that the initial rise in temperature helps release phenolic compounds bound to lignocellulosic material by degrading the polysaccharides present in the cell wall, thereby increasing the phenolic yield. However, a further increase in temperature likely led to the degradation of phenolic compounds (Antony and Farid, 2022). This suggests that, among all the temperatures tested, 40 °C was the most appropriate for treating the multifloral bee pollen variety. In the mustard bee pollen variety, three major phenolic compounds were quantified, ferulic acid (192.480 mg/l), quercetin (51.916 mg/l) and cinnamic acid (143.718 mg/l) at 35 °C. Quercetin was found to be less concentrated in mustard variety in comparison to the multifloral variety. It was noted that when the temperature was raised to 40 °C, only ferulic acid (303.483 mg/l) and cinnamic acid (15.876 mg/l) were present. At 45 °C, the concentration of ferulic acid was found to be higher (702.823 mg/l) but there was no other major phenol detected (Figure 6.10). This suggested that among all temperatures employed, 35 °C was the most appropriate temperature for treating mustard bee pollen variety. The increase in ferulic acid concentration with an increase in temperature might be attributed to the aforementioned reason i.e., release of phenolic compounds from the cell wall structure (Maghsoudlou *et al.*, 2019; Antony and Farid, 2022). The reason quercetin was not quantified at high temperatures may be due to its degradation into other compounds upon exposure to high temperature, oxygen, or light. This is also one of the main disadvantages of incorporating quercetin for the development of functional foods (Aceituno-Medina *et al.*, 2015).

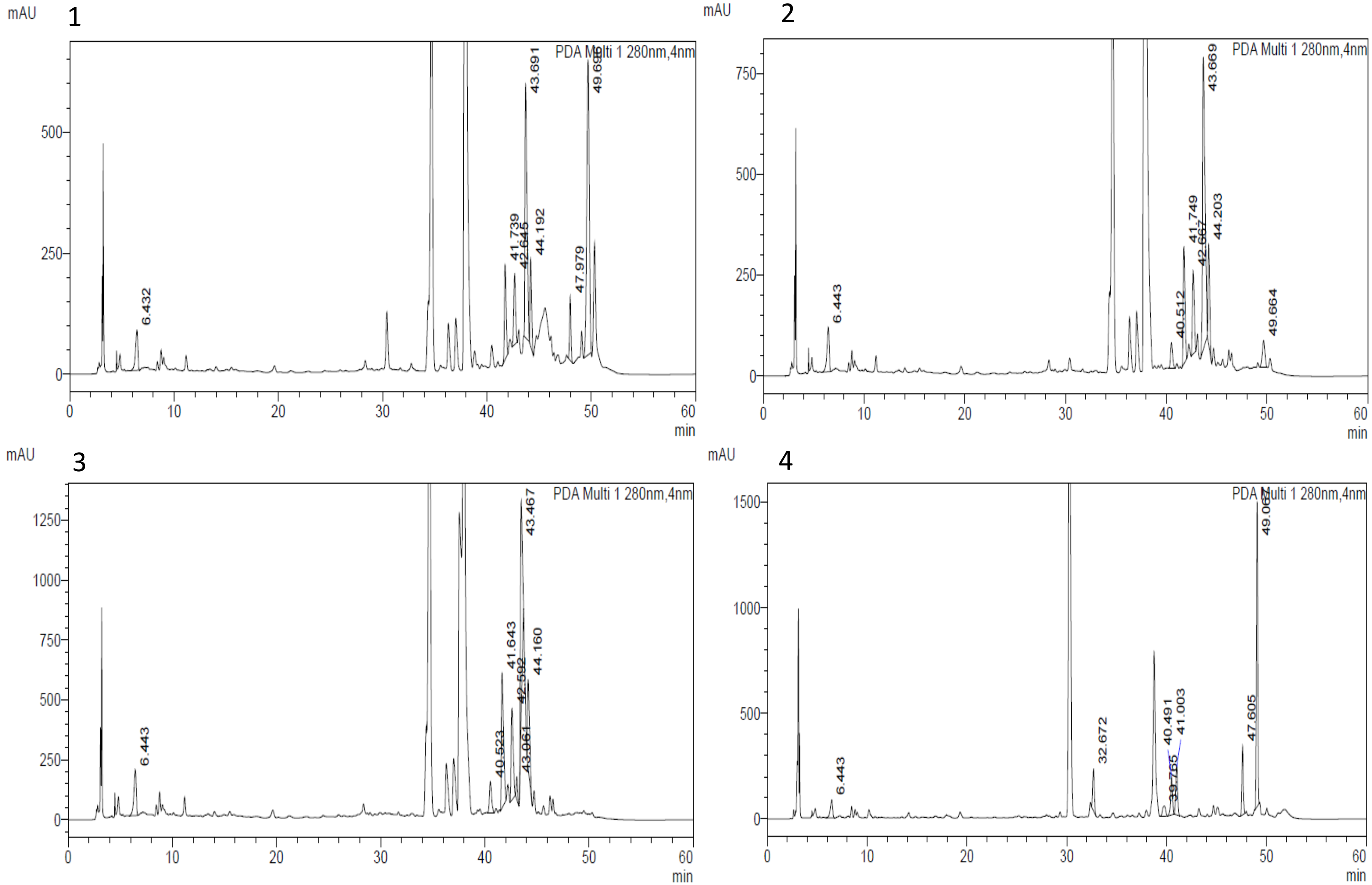
6.11.2. Alkaline pre-treatment

Upon subjecting mustard bee pollen to alkaline pre-treatment, it was observed that two phenolic compounds, gallic acid and ferulic acid, were present in amounts of 20.6 mg/L and 95.7 mg/L, respectively. In the multifloral variety treated with alkali, gallic acid (7.430 mg/L), ferulic acid (23.681 mg/L), and quercetin (152.104 mg/L) were quantified (Figure 6.10). A research study by Benavides-Guevara *et al.* (2017) reported that alkaline pre-treatment led to an increase in phenolic compounds due to a mechanism in which alkali treatment causes cell wall degradation, fracturing the polymer lignin and releasing phenolics bound to the complex polymer of exine, which is composed of fatty acids and lignins of phenolic compounds. They also noted a significant increase in the release of phenolic compounds and digestibility following the application of various pre-treatments, such as thermal, chemical, and enzymatic methods. These effects were attributable to the structural modifications of the exine layer.

6.11.3. Wet heat thermal pre-treatment

Wet thermal pre-treatment of mustard and multifloral variety, helped to quantify ferulic acid (199.072 mg/l) and cinnamic acid (15.747 mg/l) (Figure 6.10). Studies suggest that the functionality and accessibility of bee pollen can increase to as much as 60–80%, achieved by grinding and dissolving the pollen in warm water (Benavides-Guevara *et al.*, 2017; Kaur *et al.*, 2024).

When comparing the phenolic concentrations obtained after the various pre-treatments discussed so far, it was concluded that for multifloral bee pollen, the most suitable method was heating treatment (cracking method) at 40°C, while for mustard bee pollen, the most suitable method was heating treatment at 35°C. All subsequent experiments were conducted after subjecting the pollen varieties to these pre-treatments.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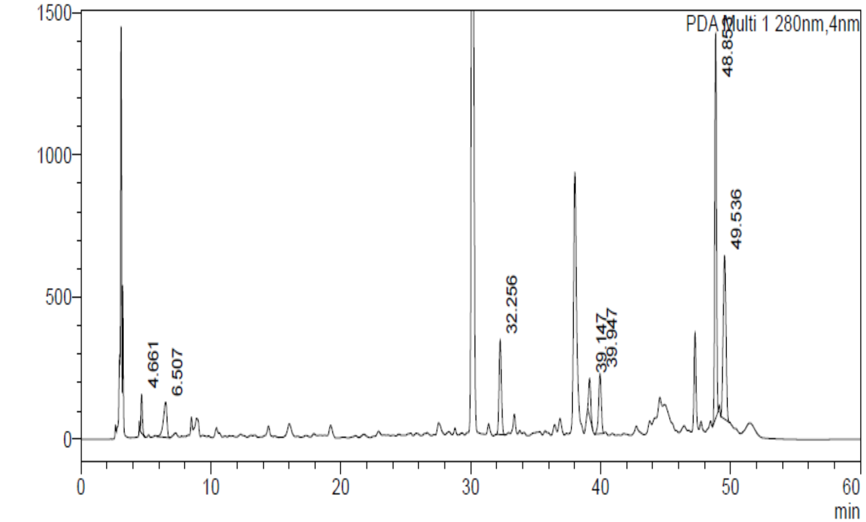
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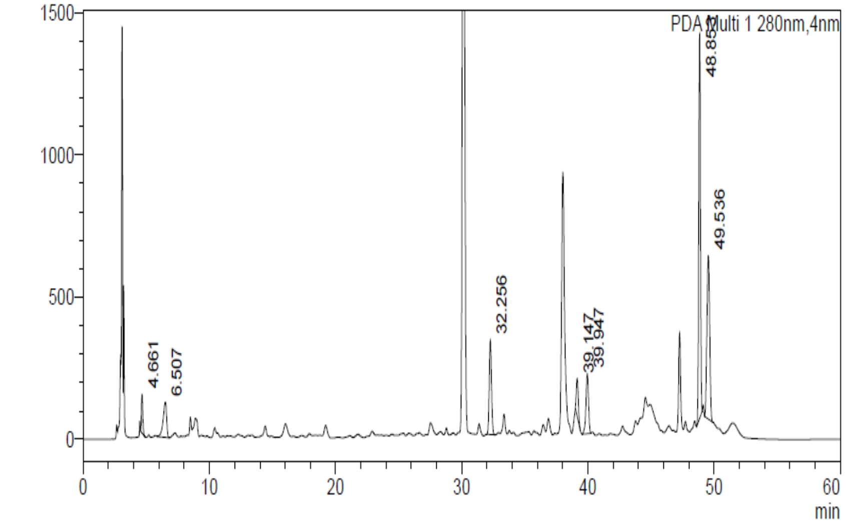
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min

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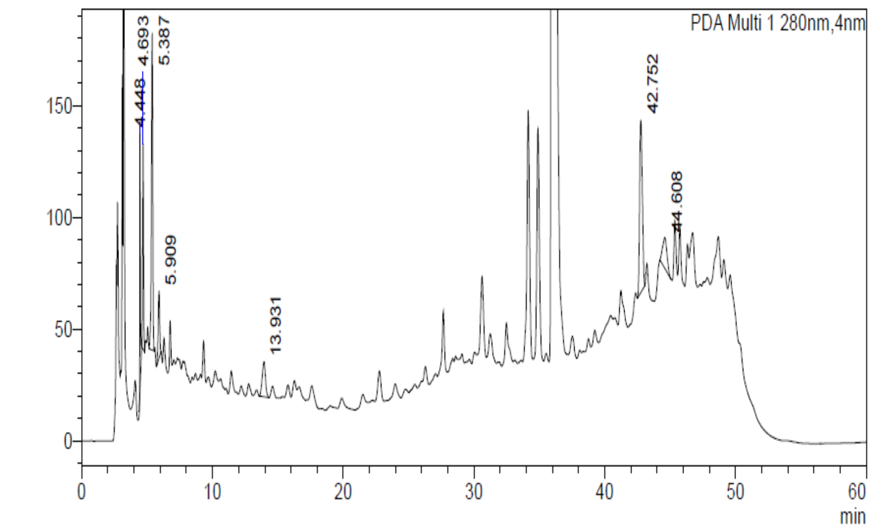
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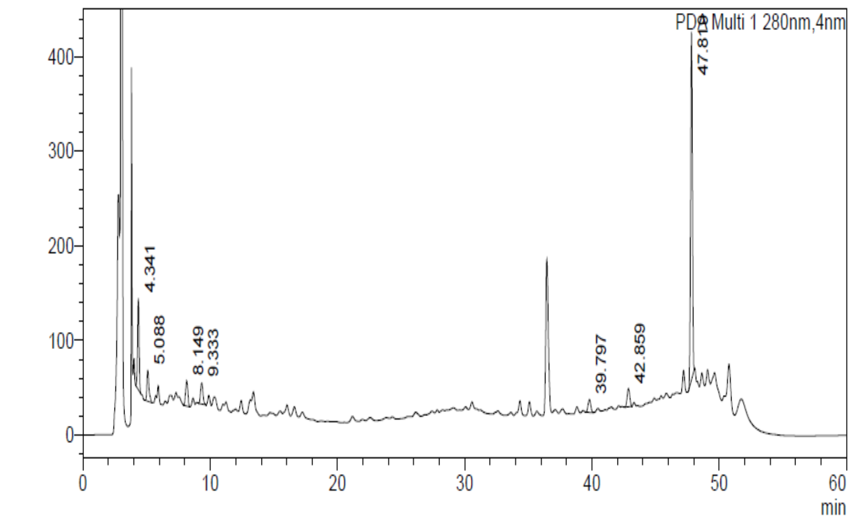
8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47.813
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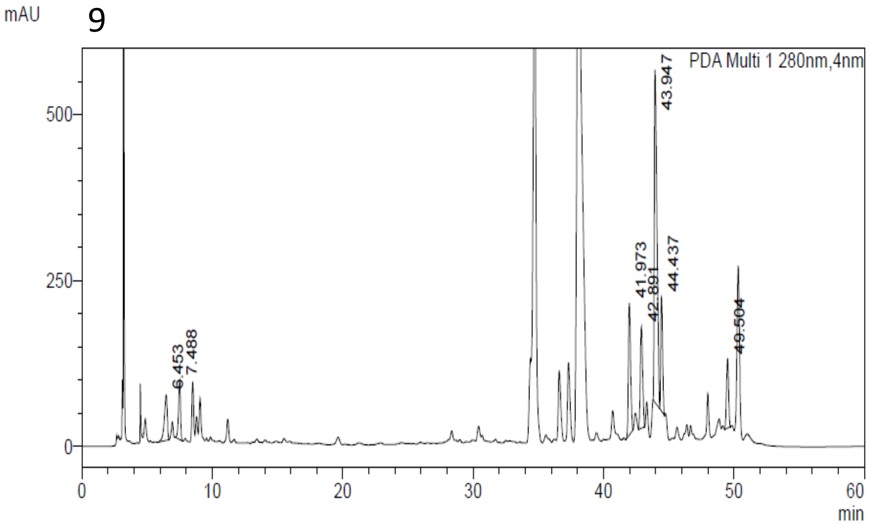


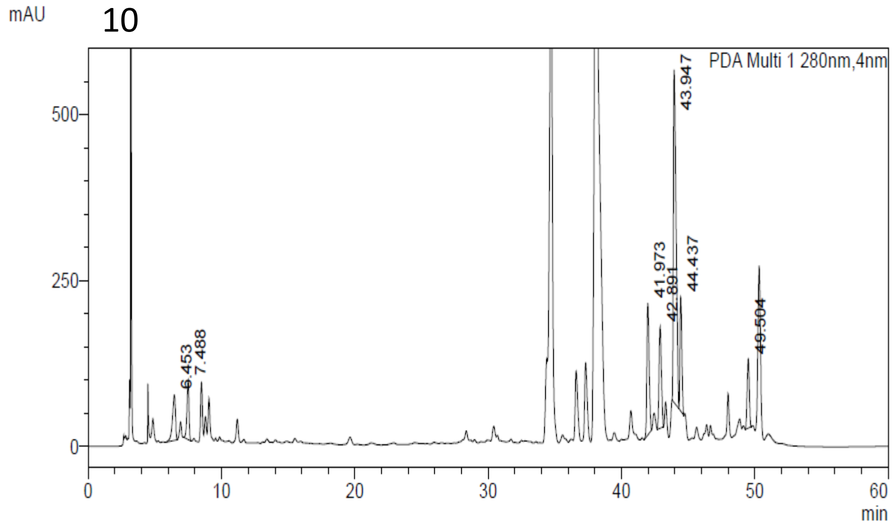


Figure 6.10. HPLC chromatograms of (1.) cracking MS bee pollen at 35 °C, (2.) cracking MS bee pollen at 40 °C, (3.) cracking MS bee pollen at 45 °C, (4.) cracking MF bee pollen at 35 °C, (5.) cracking MF bee pollen at 40 °C, (6.) cracking MS bee pollen at 45 °C, (7.) alkaline pre-treatment of MS bee pollen; (8.) alkaline pre-treatment of MF bee pollen; (9.) wet thermal pre-treatment of MS bee pollen, and (10.) wet thermal pre-treatment of MF bee pollen

6.12. Phytochemical analysis of the bee pollen extracts prepared by varying solvent concentration

Different extracts of bee pollen varieties were prepared by varying the amount of two solvents i.e., distilled water and ethanol. The concentration of both the solvents was varied from 0 to 100%. The samples prepared were analysed for the determination of total carotenoid content, total chlorophyll content, chlorophyll a, chlorophyll b, TPC, TFC, antioxidant activity, and FRAP. Water, being non-toxic, cost-effective, and easy to handle, is regarded as the most preferred solvent for the extraction of bioactive constituents. However, it is more efficient at extracting polar compounds, whereas organic or binary solvents have better extraction efficiency for less polar and non-polar molecules (Plaskova and Mlcek, 2023).

6.12.1. Total carotenoid content

The results obtained from the analysis of MSBP revealed that the total carotenoid content increased steadily from MS1-E (28.49 ± 0.43 μg/g) to MS6-E (43.73 ± 0.24 μg/g) which suggest that ethanol was found to be more effective at extracting carotenoids from bee pollen as compared to distilled water (Table 6.8 and Figure 6.11). Statistically significant differences ($p<0.05$) were observed in the values across all samples. Carotenoids are non-polar or lipophilic due to the presence of long chain of polyunsaturated hydrocarbon in its structure. Thus, this might be the reason of higher solubility of carotenoids in organic solvents (petroleum ether and hexane) as well as semi-polar solvents like ethanol which led to its increased concentration (Miękus *et al.*, 2019). Salazar-González *et al.* (2022) conducted a study on Andean bee pollen to characterize its carotenoid profile and α-tocopherol content. In another study by Melo *et al.* (2023), the total carotenoid content was observed to range from 0.81 to 22.82 μg/g. Our values were comparatively higher, likely due to varietal differences and the chemical composition of the bee pollen varieties. Owayss *et al.* (2004) reported values of total carotenoid content in the range of 50–150 μg/g.

6.12.2. Total chlorophyll content

The total chlorophyll content and its two components (chlorophyll a and chlorophyll b) also increased with an increase in ethanol content, from MS1-E to MS6-E (Figure

6.11). The values for total chlorophyll content, chlorophyll and b ranged from 1.26 ± 0.01 to 1.46 ± 0.02 µg/g, 0.48 ± 0.005 to 0.57 ± 0.006 µg/g, 0.84 ± 0.005 to 0.97 ± 0.005 µg/g, respectively (Table 6.8). The highest and lowest chlorophyll a values were significantly different ($p<0.05$), whereas the intermediate values did not exhibit statistically significant differences ($p>0.05$). For total chlorophyll content and chlorophyll b, statistically significant differences ($p<0.05$) were observed across all samples. The increased trend in total chlorophyll content can be explained by the fact that chlorophylls, being lipophilic pigments, are better extracted with ethanol, which is less polar than water (Kim *et al.*, 2020). Owayss *et al.* (2004) reported that the acetone extract of citrus, clover, and cotton bee pollen contained chlorophyll a and b, with values of 4.63 and 6.99 µg/g for citrus, 1.08 and 1.62 µg/g for clover, and 22.68 and 47.01 µg/g for cotton bee pollen, respectively.

6.12.3. Total flavonoid content

The data pertaining to TFC showed a similar trend, increasing from 8.69 ± 0.28 mg QE/g in MS1-E to 17.12 ± 0.29 mg QE/g in MS6-E (Table 6.8 and Figure 6.11). Statistically significant differences ($p<0.05$) were noted among the values across all samples. Like carotenoids, flavonoid compounds (including isoflavones, flavanols, etc.) are better extracted with pure alcohols and other organic solvents like chloroform, ethyl acetate, dichloromethane, and diethyl ether (Plaskova and Mlcek, 2023). Prelipcean (2011) investigated the flavonoid content in a polyfloral bee pollen load extracted with methanol and found that fresh bee pollen had values equivalent to 20.44 mg QE/g pollen. In another study by Spulber *et al.* (2020), it was recorded that the flavonoid content of different bee pollen samples extracted with ethanol was around 13.65 and 15.11 mg QE/g pollen. Similarly, in Transylvania, the same extraction solvent was utilized, revealing that *Malus domestica* pollen had the highest concentration of total flavonoids. Another study by Kim *et al.* (2015) confirmed that ethanol was more effective in the extraction of flavonoids from *Acorn* and *Actinidia* bee pollen, in comparison to distilled water. The flavonoid content of *Acorn* pollen extracted with ethanol was 8.99 mg CE/ml, while with water, it was 0.36 mg CE/ml. For *Actinidia*, the flavonoid content was 4.70 mg CE/ml with ethanol and 0.62 mg CE/ml with water.

6.12.4. Total phenolic content

The total phenolic content also increased with an increase in ethanol concentration (Figure 6.11), starting from 1841.40 ± 0.71 mg GAE/100 g for MS1-E and rising to 2747.33 ± 0.97 mg GAE/100 g for MS6-E (Table 6.8). Statistically significant differences ($p<0.05$) were noted among the values across all samples. The increased trend might be attributed to the fact that phenolic compounds tend to be more soluble in ethanol, allowing better extraction efficiency as ethanol concentration rises (de Melo *et al.*, 2023). Kim *et al.* (2015) quantified the total polyphenol content of *Acorn* and *Actinidia* bee pollen extracted with water and ethanol. It was reported that values were higher for ethanolic extract as compared to aqueous extract. According to Spulber *et al.* (2020), pollen from *Brassica* species exhibited the highest TPC, measuring 26.8 ± 0.9 mg GAE/g of pollen, while the lowest value was found in *Zea mays* pollen samples, with values of 11.0 ± 0.4 mg GAE/g. In a study published by Melo *et al.* (2023), it was reported that TPC values were low due to the low extraction efficiency when using water as a solvent. Carpes *et al.* (2009) reported higher TPC values for ethanolic extracts of dehydrated pollen, with an average of 30.77 mg GAE/g. Similarly, in another study by de Melo *et al.* (2016), the values ranged between 9.2 and 19.7 mg GAE/g. The values obtained in our study were comparable to these stated results, with slight variations attributed to factors such as climatic conditions, pollen variety, and composition.

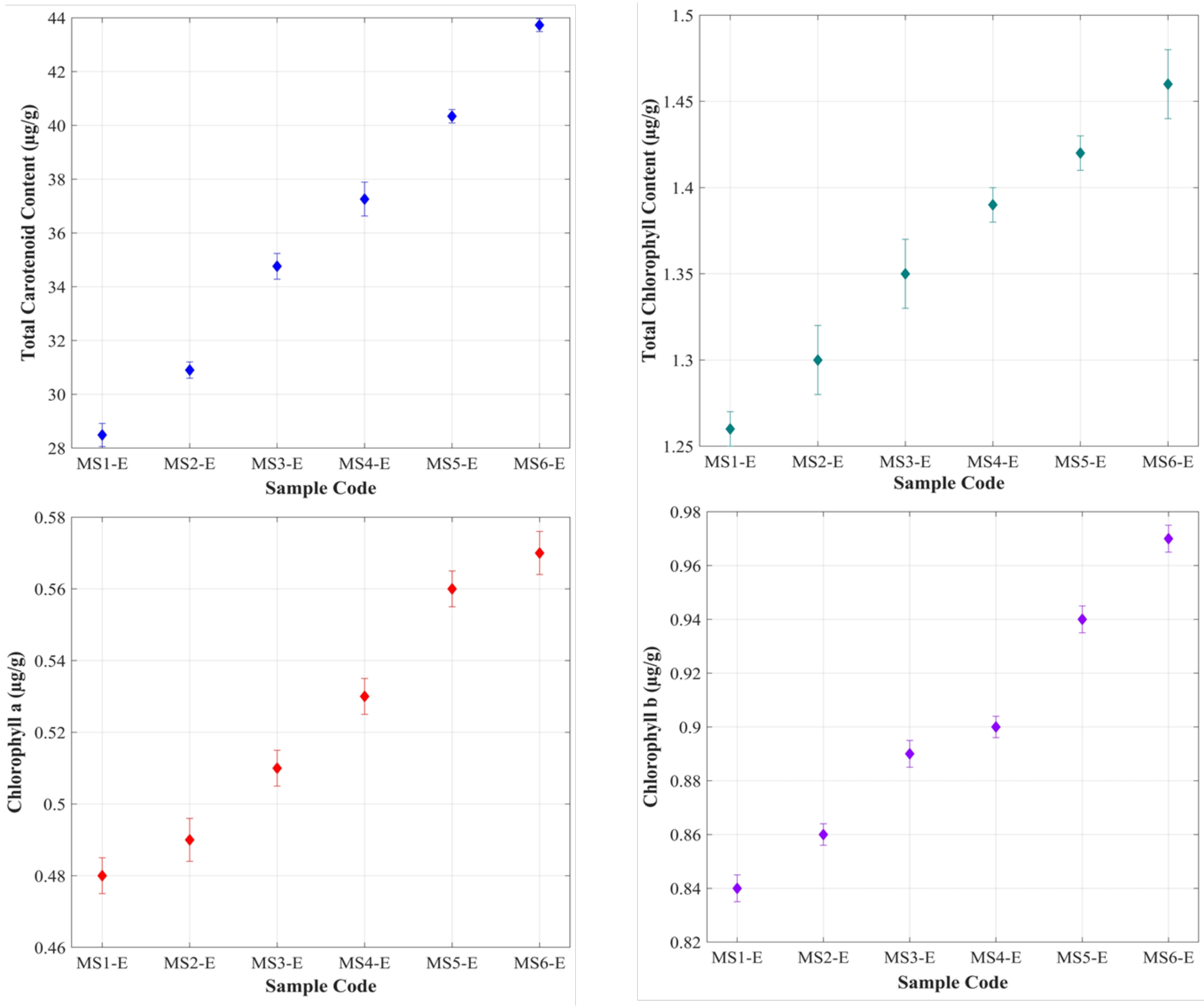
6.12.5. Antioxidant activity

The values obtained for the DPPH and FRAP also followed a similar trend with the former ranged from 69.10 ± 0.60 to 91.71 ± 0.58%, while the latter ranging from 33.39 ± 0.53 to 64.94 ± 0.57 mg TE/100 g (Table 6.8 and Figure 6.11). Statistically significant differences ($p<0.05$) were noted among the values across all samples. The higher values of these parameters can be attributed to the more effective extraction of antioxidant compounds *viz.* flavonoids and phenolic compounds. It is primarily influenced by the botanical origin of the bee pollen (Spulber *et al.*, 2020). As the concentration of ethanol increased, it changed the overall polarity of the solvent mixture. Some antioxidants might be more soluble or more easily extracted in a less polar environment, which could enhance their extraction efficiency. According to the results reported by Spulber *et al.* (2020), *Brassica* sp. pollen extracts showed superior antioxidant activity compared to other extracts, as measured by DPPH and ABTS methods.

A similar trend of increased values (Figure 6.12) of all these parameters was also observed in the multifloral bee pollen variety, where the values of total carotenoid content, total chlorophyll, chlorophyll a, chlorophyll b, TFC, TPC, antioxidant activity, and FRAP increased (MF1-E to MF6-E) from 38.46 ± 0.32 to 47.63 ± 0.14 µg/g, 1.35 ± 0.01 to 1.52 ± 0.01 µg/g, 0.45 ± 0.005 to 0.54 ± 0.005 µg/g, 0.79 ± 0.005 to 0.92 ± 0.005 µg/g, 6.12 ± 0.23 to 12.79 ± 0.18 mg QE/g, 1501.99 ± 0.95 to 2541.45 ± 0.84 mg GAE/100 g, 59.28 ± 0.74 to 87.45 ± 0.84% and, 23.44 ± 0.63 to 56.88 ± 0.83 mg TE/100 g, respectively (Table 6.9). Statistically significant differences ($p<0.05$) were noted for all parameters among all samples, with the exception of chlorophyll a content, which showed no significance ($p>0.05$).

6.13. Optimization of bee pollen-based dairy product

In pre-liminary trial, for both types of extracts (mustard and multifloral), at 1 ml concentration, the change in taste was barely noticeable and was similar to the control, while at 20 and 25 ml concentration, the taste was unpleasant and unacceptable. So, for the preparation of final product, the extract concentration was varied between 2.5 to 12.5 ml for both varieties. The maximum acceptability of sweetness was obtained with 25 g. Factor A (bee pollen extract concentration), factor B (chakka/hung curd) and factor C (sugar content) were varied from 2.5 to 12.5 ml, 60 to 80 g and 20 to 25 g, respectively. 20 different formulations were predicted by using central composite design (CCD) type, quadratic design model and response surface methodology in STAT-EASE 360 software. The sensory scores of bee pollen extract-based Shrikhand along with the physicochemical and phytochemical properties were analysed in response to the variables.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30
32
34
36
38
40
42
44
Total Chlorophyll Content (µg/g)
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1.35
1.4
1.45
1.5
Chlorophyll a (µg/g)
0.46
0.48
0.5
0.52
0.54
0.56
0.58
Chlorophyll b (µg/g)
0.82
0.84
0.86
0.88
0.9
0.92
0.94
0.96
0.98
MS1-E
MS2-E
MS3-E
MS4-E
MS5-E
MS6-E
Sample Code

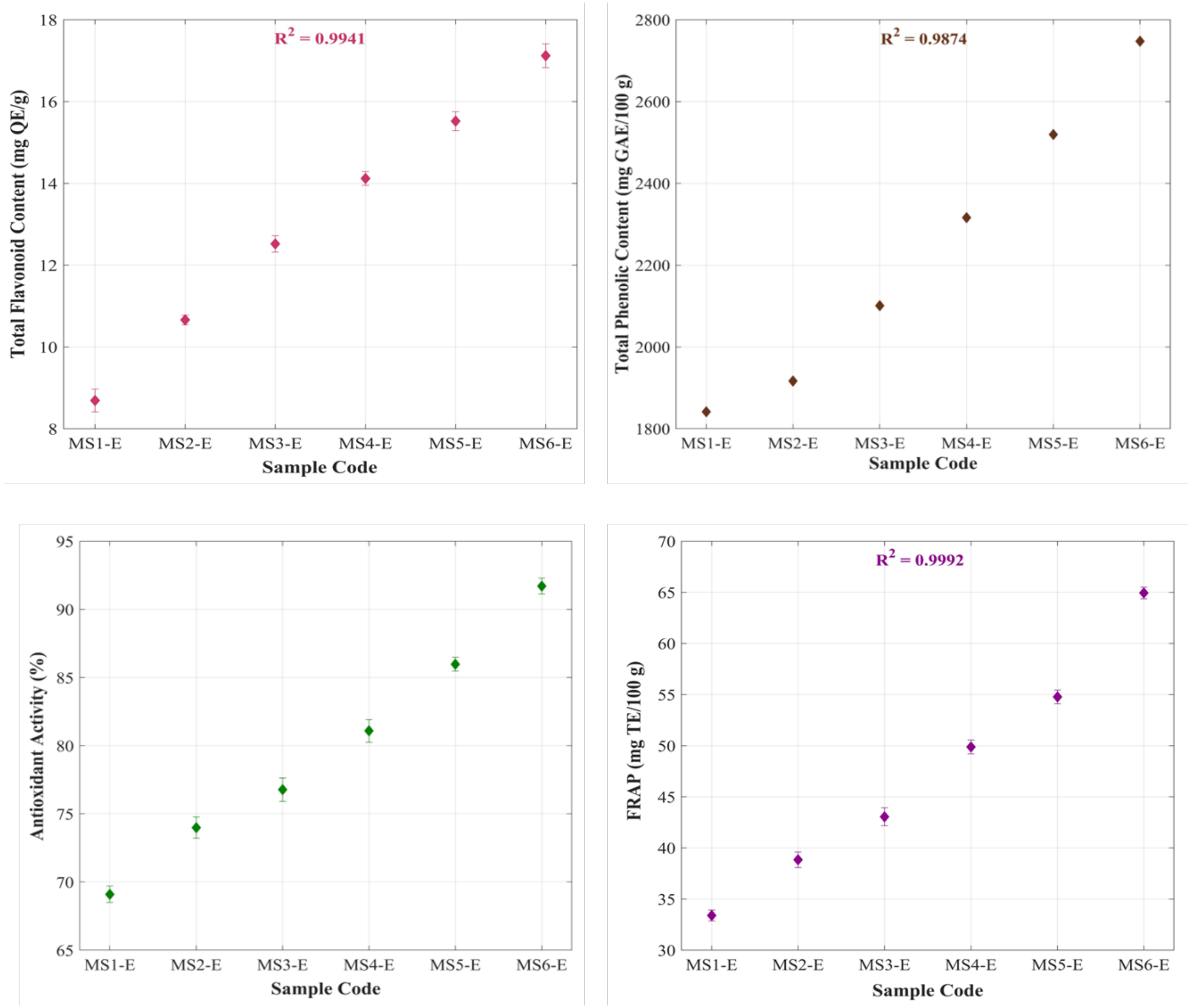


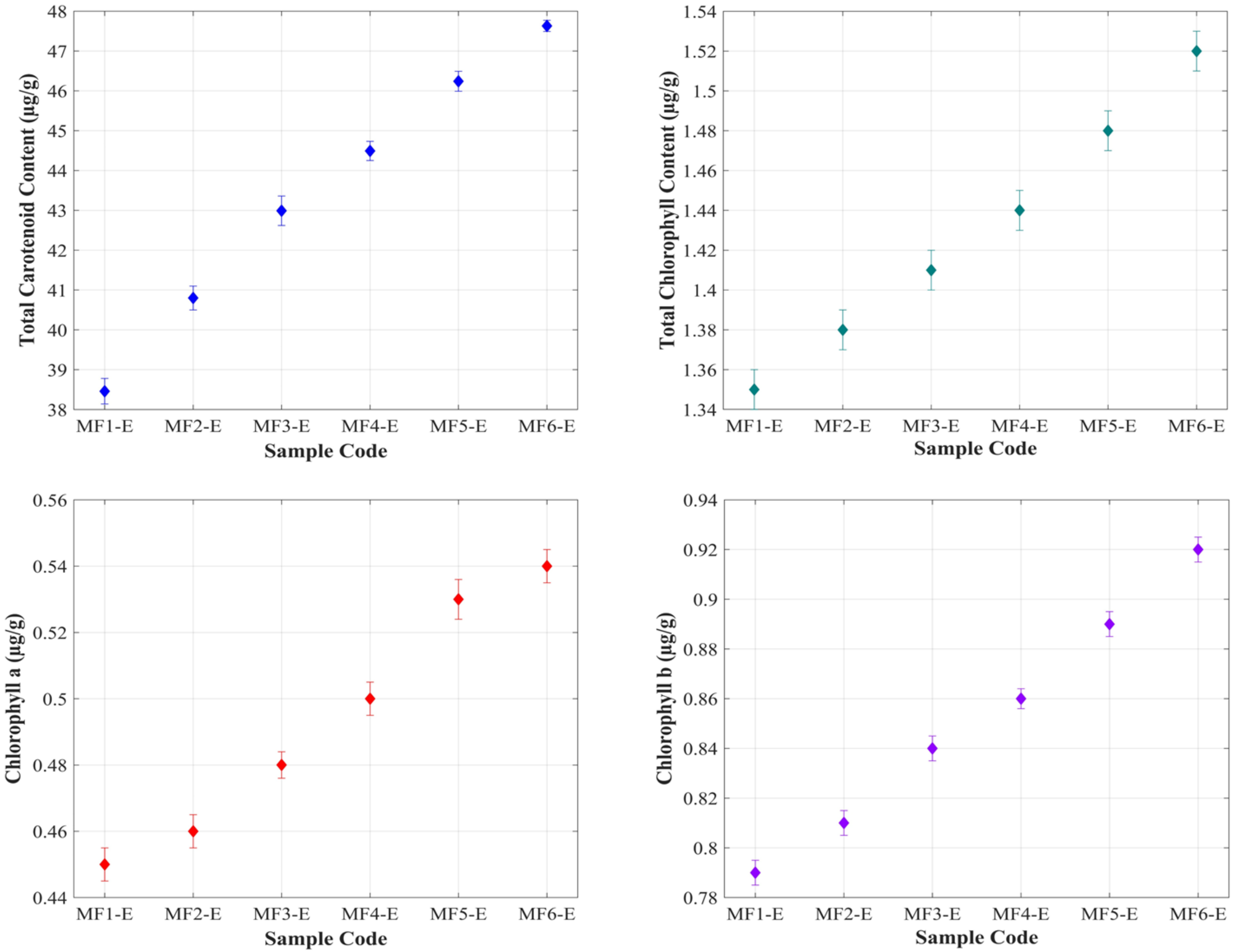
Figure 6.11. Phytochemical analysis of mustard bee pollen extract prepared by varying solvent concentration

Table 6.8. Phytochemical analysis of mustard bee pollen extract prepared by varying solvent concentration

Sample code	Carotenoid content (µg/g)	Total chlorophyll (µg/g)	Chlorophyll a (µg/g)	Chlorophyll b (µg/g)	TFC (mg QE/g)	TPC (mg GAE/100 g)	Antioxidant activity (%)	FRAP (mg TE/100 g)
MS1-E	28.49 ± 0.43^{f}	1.26 ± 0.01^{f}	0.48 ± 0.005^{d}	0.84 ± 0.005^{f}	8.69 ± 0.28^{f}	1841.40 ± 0.71^{f}	69.10 ± 0.60^{f}	33.39 ± 0.53^{f}
MS2-E	30.90 ± 0.30^{e}	1.30 ± 0.02^{e}	0.49 ± 0.006^{d}	0.86 ± 0.004^{e}	10.66 ± 0.12^{e}	1916.67 ± 0.93^{e}	73.99 ± 0.78^{e}	38.84 ± 0.76^{e}
MS3-E	34.76 ± 0.48^{d}	1.35 ± 0.02^{d}	0.51 ± 0.005^{c}	0.89 ± 0.005^{d}	12.52 ± 0.20^{d}	2100.99 ± 0.58^{d}	76.78 ± 0.86^{d}	43.05 ± 0.88^{d}
MS4-E	37.26 ± 0.63^{c}	1.39 ± 0.01^{c}	0.53 ± 0.005^{b}	0.90 ± 0.004^{c}	14.12 ± 0.17^{c}	2316.09 ± 0.85^{c}	81.09 ± 0.83^{c}	49.88 ± 0.68^{c}
MS5-E	40.34 ± 0.25^{b}	1.42 ± 0.01^{b}	0.56 ± 0.005^{a}	0.94 ± 0.005^{b}	15.52 ± 0.23^{b}	2519.26 ± 0.74^{b}	85.98 ± 0.51^{b}	54.77 ± 0.67^{b}
MS6-E	43.73 ± 0.24^{a}	1.46 ± 0.02^{a}	0.57 ± 0.006^{a}	0.97 ± 0.005^{a}	17.12 ± 0.29^{a}	2747.33 ± 0.97^{a}	91.71 ± 0.58^{a}	64.94 ± 0.57^{a}

Table 6.9. Phytochemical analysis of multifloral bee pollen extract prepared by varying solvent concentration

Sample code	Carotenoid content (µg/g)	Total chlorophyll (µg/g)	Chlorophyll a (µg/g)	Chlorophyll b (µg/g)	TFC (mg QE/g)	TPC (mg GAE/100 g)	Antioxidant activity (%)	FRAP (mg TE/100 g)
MF1-E	38.46 ± 0.32^{f}	1.35 ± 0.01^{f}	0.45 ± 0.005^{d}	0.79 ± 0.005^{f}	6.12 ± 0.23^{f}	1501.99 ± 0.95^{f}	59.28 ± 0.74^{f}	23.44 ± 0.63^{f}
MF2-E	40.80 ± 0.30^{e}	1.38 ± 0.01^{e}	0.46 ± 0.005^{d}	0.81 ± 0.005^{e}	7.11 ± 0.12^{e}	1652.06 ± 0.88^{e}	64.35 ± 0.92^{e}	29.42 ± 0.71^{e}
MF3-E	42.99 ± 0.37^{d}	1.41 ± 0.01^{d}	0.48 ± 0.004^{c}	0.84 ± 0.005^{d}	8.54 ± 0.19^{d}	1850.88 ± 0.91^{d}	70.39 ± 0.70^{d}	35.67 ± 0.58^{d}
MF4-E	44.49 ± 0.24^{c}	1.44 ± 0.01^{c}	0.50 ± 0.005^{b}	0.86 ± 0.004^{c}	9.72 ± 0.26^{c}	2101.97 ± 0.80^{c}	77.38 ± 0.89^{c}	42.85 ± 0.61^{c}
MF5-E	46.24 ± 0.25^{b}	1.48 ± 0.01^{b}	0.53 ± 0.006^{a}	0.89 ± 0.005^{b}	11.12 ± 0.38^{b}	2339.12 ± 0.94^{b}	83.57 ± 0.50^{b}	49.96 ± 0.56^{b}
MF6-E	47.63 ± 0.14^{a}	1.52 ± 0.01^{a}	0.54 ± 0.005^{a}	0.92 ± 0.005^{a}	12.79 ± 0.18^{a}	2541.45 ± 0.84^{a}	87.45 ± 0.84^{a}	56.88 ± 0.83^{a}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47
46
45
44
43
42
41
40
39
38
MF1-E
MF2-E
MF3-E
MF4-E
MF5-E
MF6-E
Sample Code
Total Chlorophyll Content (µg/g)
1.54
1.52
1.5
1.48
1.46
1.44
1.42
1.4
1.38
1.36
1.34
MF1-E
MF2-E
MF3-E
MF4-E
MF5-E
MF6-E
Sample Code
Chlorophyll a (µg/g)
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0.54
0.52
0.5
0.48
0.46
0.44
MF1-E
MF2-E
MF3-E
MF4-E
MF5-E
MF6-E
Sample Code
Chlorophyll b (µg/g)
0.94
0.92
0.9
0.88
0.86
0.84
0.82
0.8
0.78
MF1-E
MF2-E
MF3-E
MF4-E
MF5-E
MF6-E
Sample Code

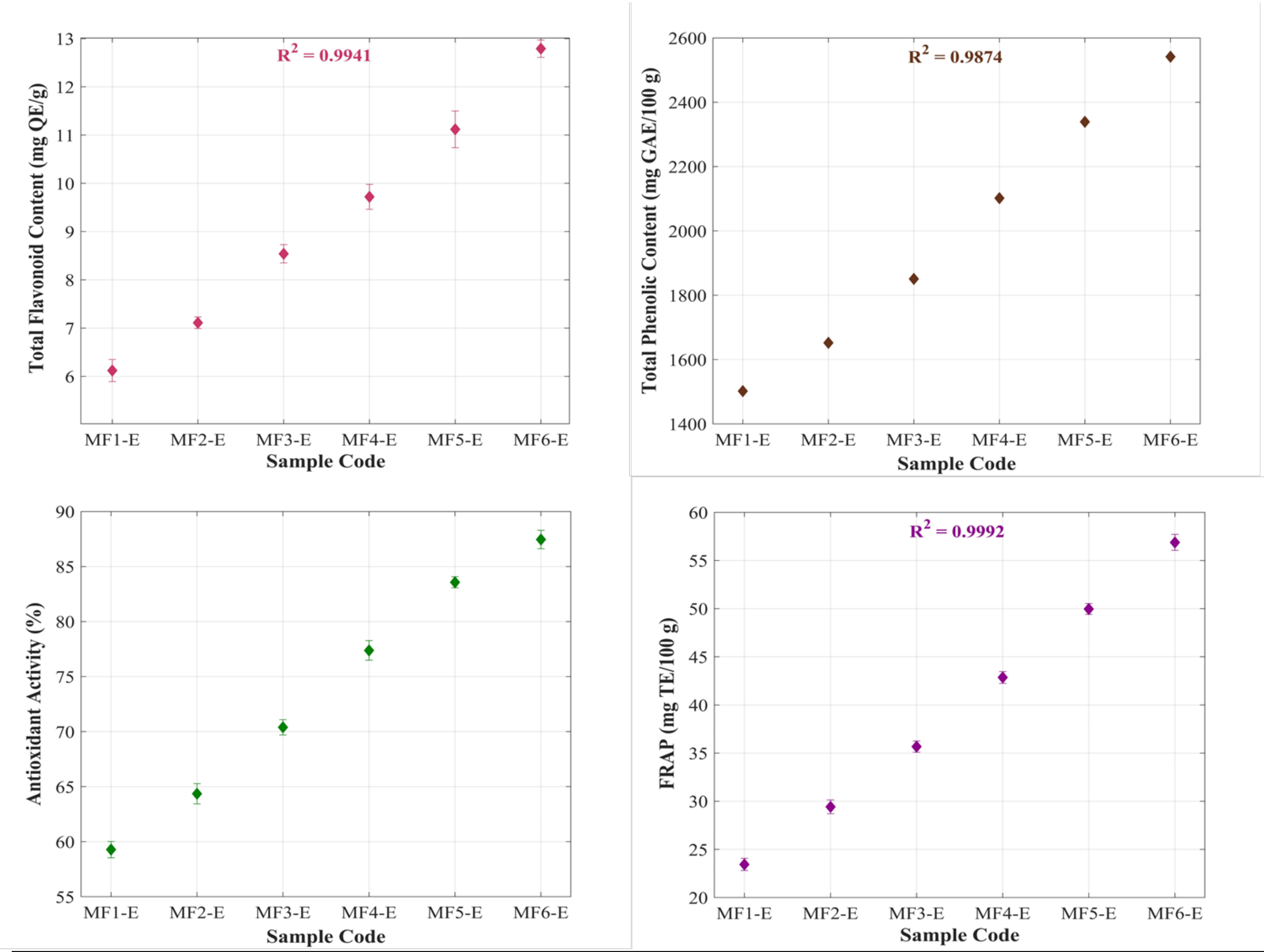


Figure 6.12. Phytochemical analysis of multifloral bee pollen extract prepared by varying solvent concentration

6.14. Sensory analysis of Shrikhand

To determine the acceptable range of bee pollen extract concentration, varying levels of 1, 5, 10, 15, 20, and 25 ml were tested. For both mustard and multifloral bee pollen extracts, the 1 ml concentration caused minimal noticeable change in taste and was similar to the control. However, concentrations of 20 and 25 ml resulted in unpleasant and unacceptable flavours. Based on these findings, the extract concentration for the preparation of Shrikhand was adjusted to a range of 2.5 to 12.5 ml for both types of extracts. Table 6.10a, Figure 6.13a and Table 6.10b, Figure 6.13b presents the sensory evaluation results from the preliminary trials of shrikhand incorporated with mustard and multifloral bee pollen extract, respectively.

Table 6.10a. Preliminary trial of mustard bee pollen extract based-Shrikhand

Sample code	Appearance	Flavour	Texture	Mouthfeel	OAA
Control	7.33 ± 0.06^{c}	7.15 ± 0.05^{c}	7.38 ± 0.08^{d}	7.18 ± 0.03^{b}	7.83 ± 0.06^{ab}
1 ml	7.58 ± 0.03^{bc}	7.43 ± 0.06^{bc}	7.65 ± 0.05^{c}	7.58 ± 0.03^{ab}	7.57 ± 0.06^{b}
5 ml	8.58 ± 0.03^{a}	8.60 ± 0.10^{a}	8.48 ± 0.03^{a}	7.93 ± 0.06^{a}	8.35 ± 0.05^{a}
10 ml	7.83 ± 0.06^{b}	7.62 ± 0.03^{b}	7.83 ± 0.06^{b}	7.85 ± 0.05^{ab}	7.73 ± 0.06^{ab}
15 ml	6.63 ± 0.23^{d}	6.58 ± 0.08^{d}	7.00 ± 0.00^{e}	6.33 ± 0.29^{c}	6.50 ± 0.00^{c}
20 ml	6.47 ± 0.06^{d}	5.17 ± 0.29^{e}	6.13 ± 0.12^{f}	5.57 ± 0.12^{d}	5.83 ± 0.29^{c}
25 ml	6.00 ± 0.00^{e}	4.33 ± 0.29^{f}	6.00 ± 0.00^{f}	4.17 ± 0.29^{e}	4.67 ± 0.58^{d}

Values within the same column marked with different superscripts (a, b) indicate a statistically significant difference ($p<0.05$).

Table 6.10b. Preliminary trial of multifloral bee pollen extract based-Shrikhand

Sample code	Appearance	Flavour	Texture	Mouthfeel	OAA
Control	7.33 ± 0.06^{c}	7.15 ± 0.05^{bc}	7.38 ± 0.08^{b}	7.18 ± 0.03^{cd}	7.83 ± 0.05^{b}
1 ml	7.57 ± 0.06^{bc}	7.37 ± 0.06^{b}	7.65 ± 0.05^{b}	7.35 ± 0.05^{bc}	7.85 ± 0.05^{b}
5 ml	8.68 ± 0.08^{a}	8.65 ± 0.05^{a}	8.52 ± 0.03^{a}	8.17 ± 0.29^{a}	8.45 ± 0.14^{a}
10 ml	7.88 ± 0.03^{b}	7.58 ± 0.03^{b}	7.78 ± 0.03^{b}	7.88 ± 0.03^{ab}	7.58 ± 0.29^{b}
15 ml	6.67 ± 0.29^{d}	6.67 ± 0.29^{c}	6.83 ± 0.29^{c}	6.67 ± 0.29^{d}	6.67 ± 0.29^{c}
20 ml	6.48 ± 0.03^{de}	5.33 ± 0.29^{d}	6.17 ± 0.29^{d}	5.33 ± 0.29^{e}	5.83 ± 0.29^{d}
25 ml	6.17 ± 0.29^{e}	4.33 ± 0.29^{e}	6.00 ± 0.00^{d}	4.33 ± 0.29^{f}	4.67 ± 0.29^{e}

Values within the same column marked with different superscripts (a, b) indicate a statistically significant difference ($p<0.05$).

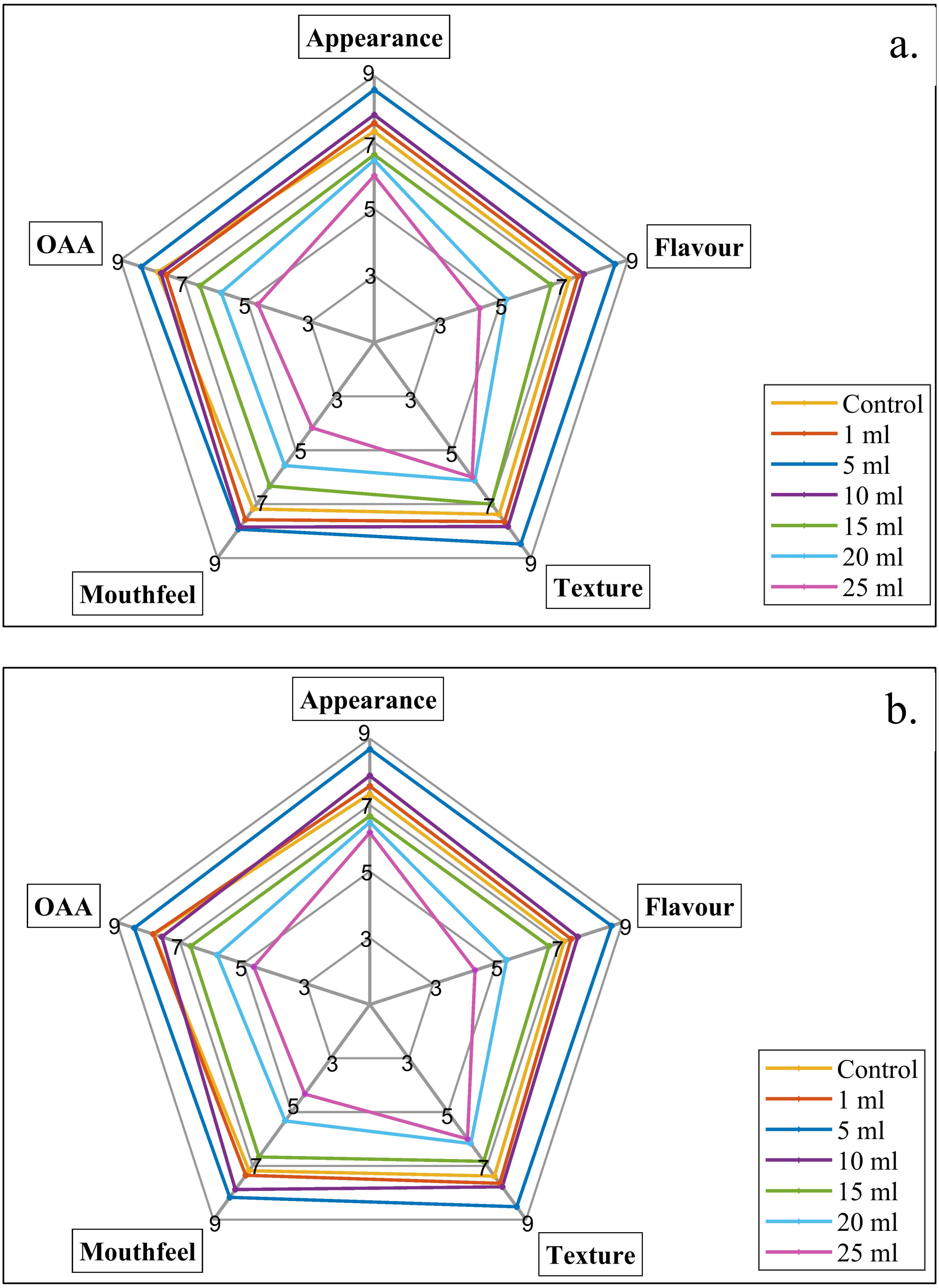


Figure 6.13. Radar Chart depicting preliminary trial results of shrikhand with (a.) mustard and (b.) multifloral bee pollen extracts

6.14.1. Sensory analysis of mustard bee pollen extract-based Shrikhand using 9-point hedonic scale

Sensory evaluation was conducted on 20 different sample runs, assessing attributes such as flavor, appearance, body/texture, mouthfeel, and overall acceptability. A 9-point hedonic scale was used for rating, with scores ranging from 1 (dislike extremely) to 9 (like extremely). Figure 6.15(a) summarizes the sensory evaluation of Shrikhand made with varying concentrations of mustard bee pollen extract along with the standard deviations and, superscripts indicate statistically significant differences between the samples. All attributes showed similar trends with scores ranging mostly between 6 and 8, indicating a generally favourable acceptance of the Shrikhand samples across the sensory parameters. However, runs 1, 3, 5, 8, 9 and 18 achieved the highest overall acceptability scores of 8.41 ± 0.21, 8.47 ± 0.03, 8.51 ± 0.03, 8.45 ± 0.03, 8.53 ± 0.04 and 8.46 ± 0.03 respectively, indicating that the combination of 7.5 ml mustard bee pollen extract, 70 g hung curd and 22.5 g sugar created the most pleasing balance for the sensory panel. This implies that moderately high pollen extract concentration as well as balanced sugar and curd content contributed to superior flavour, mouthfeel and overall texture of the final product.

Run 19 with the highest concentration of 15.909 ml bee pollen extract, 60 g hung curd and 22.5 g sugar received the lowest overall acceptability score (7.00 ± 0.03). The extract concentration significantly affected the flavour (6.95 ± 0.03) and mouthfeel (6.56 ± 0.03), as seen in the low scores. Bee pollen extract when added in higher concentration might have led to unpleasant taste notes, making this sample less appealing to the panelists. Run 12 also received a low overall acceptability score (7.26 ± 0.03) as the amount of hung curd was less as compared to bee pollen extract concentration. Thus, bee pollen extract concentration had the most noticeable impact on flavour and overall acceptability ($p<0.05$). As observed in the highest and lowest scoring samples, 7.5 ml bee pollen extract concentration was associated with high sensory scores, while higher concentrations (12.5 ml and above) negatively impacted the flavour. Furthermore, hung curd percentage was found to influence the texture and mouthfeel in a non-significant manner. As observed in the results, samples with high hung curd concentration (70 g) achieved better scores for texture and mouthfeel, likely due to the creamier and smoother consistency. The samples having lower hung curd levels (60 g and low in Runs 12 and 19) resulted in lower scores for these attributes which might be attributed to the thinner or less desirable texture. But the effect of hung curd on texture was found to be less significant (p>0.05). Although sugar concentration was relatively constant across samples (20–25 g), its slight variation between samples didn't appear to have a major influence on the overall acceptability.

Overall, the mean scores for all sensory attributes were relatively high for each response, with low standard deviations, which indicated consistent sensory quality and good overall acceptability of the samples.

Figure 6.14(a). Mustard bee pollen extract-based Shrikhand

6.14.2. Sensory analysis of multifloral bee pollen extract-based Shrikhand using 9-point hedonic scale

Figure 6.15(b) summarizes the sensory evaluation of Shrikhand made with varying concentrations of multifloral bee pollen extract along with superscripts indicating statistically significant differences between the samples. It was observed that the samples showed results comparable to the Shrikhand samples incorporated with mustard bee pollen extract. The samples with highest overall acceptability scores were Runs 1 (8.46 ± 0.04), 3 (8.38 ± 0.06), 5 (8.42 ± 0.07), 8 (8.37 ± 0.07), 9 (8.44 ± 0.06) and 18 (8.37 ± 0.09), indicating that the combination of 7.5 ml multifloral bee pollen extract, 86.8179 g hung curd, and 22.5 g sugar created a highly appealing product. It

was evident from the results that 7.5 ml bee pollen extract concentration appears to be ideal for maximizing flavour without negatively affecting the texture and mouthfeel.
With a lower overall acceptability score, runs 14 (7.14 ± 0.08) and 19 (7.05 ± 0.09) showed that a high concentration of 12.5 ml and 15.909 ml multifloral bee pollen extract might negatively influence the sensory attributes, especially flavour. Additionally, run 12 had a lower flavour score (6.96 ± 0.06) and was one of the least acceptable overall (7.08 ± 0.07). The lower hung curd content (53.18 g) might have affected the texture and mouthfeel as well, making the samples less creamy as compared to the most acceptable samples. Thus, it was inferred from the results that the variable, multifloral bee pollen extract concentration, had the greatest influence on flavour and overall acceptability. Moderate concentrations produced the most desirable sensory attributes, whereas, excessive bee pollen extract concentration (15.909 ml in Run 19) led to diminished sensory appeal. On the other hand, samples with higher curd content, resulted in smoother textures and better mouthfeel, making the product more desirable. Lower hung curd levels (53.18 g in Run 12, 60 g in Runs 14 and 20) likely produced a less smooth texture, resulting in lower scores for these attributes.
Overall, the average scores of Shrikhand incorporated with multifloral bee pollen extract suggested that all the samples were well-received, with a particularly high score for overall acceptability.

Figure 6.14(b). Multifloral bee pollen extract-based Shrikhand

6.15. Effect of variables on the physicochemical and phytochemical parameters of Shrikhand incorporated with mustard and multifloral bee pollen extract

The effects of different factors on the physicochemical and phytochemical characteristics of Shrikhand fortified with mustard bee pollen extract and multifloral bee pollen extract are presented in Tables 6.11 and 6.12, respectively. Due to the effects of different variables (mustard bee pollen extract concentration, hung curd, and sugar), the moisture content across 20 runs ranged from 34.66% to 39.955%. The pH ranged from 4.065 to 4.675, titratable acidity from 1.185 to 1.29%, ash content from 0.755% to 0.915%, TSS from 66 to 69.11 °Brix, antioxidant activity from 56.085% to 76.48%, TPC from 1.55 to 439.55 mg GAE/100 g, TFC from 0.5 to 1321.7 mg QE/100 g and, overall acceptability from 7 to 8.53. Similarly, the moisture content values for 20 runs of Shrikhand incorporated with multifloral bee pollen extract ranged from 30.73% to 35.905%. The pH ranged from 4.033 to 4.645, while the titratable acidity varied from 1.162% to 1.265%. The ash content was between 0.725% and 0.885%, and the TSS ranged from 63.75 to 66.52 °Brix. Antioxidant activity varied from 51.3% to 72.4%, TPC ranged from 1.55 to 419.64 mg GAE/100 g, TFC from 0.5 to 1162 mg QE/100 g, and overall acceptability was between 7.05 and 8.46. The comparison between the actual experimental values and predicted values of MSBPE and MFBPE-based Shrikhand is presented in Figures 6.23 and 6.24, respectively. The effect of different variables on the aforementioned responses was studied at linear (A, B, C), interactive (AB, BC, AC), and quadratic levels (A^2, B^2, C^2).

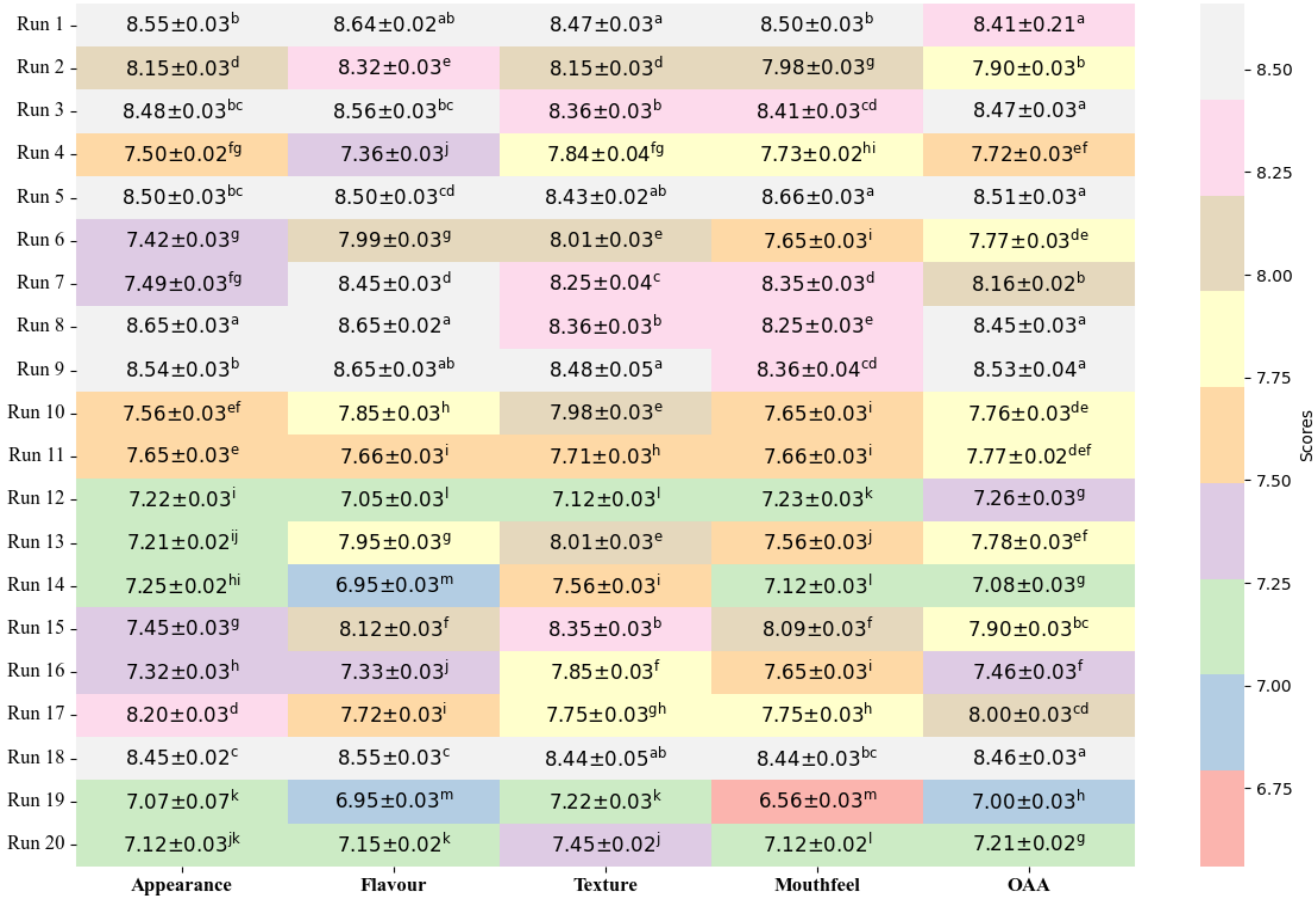


Figure 6.15(a). Sensory evaluation of mustard bee pollen extract-based Shrikhand using 9-point hedonic scale

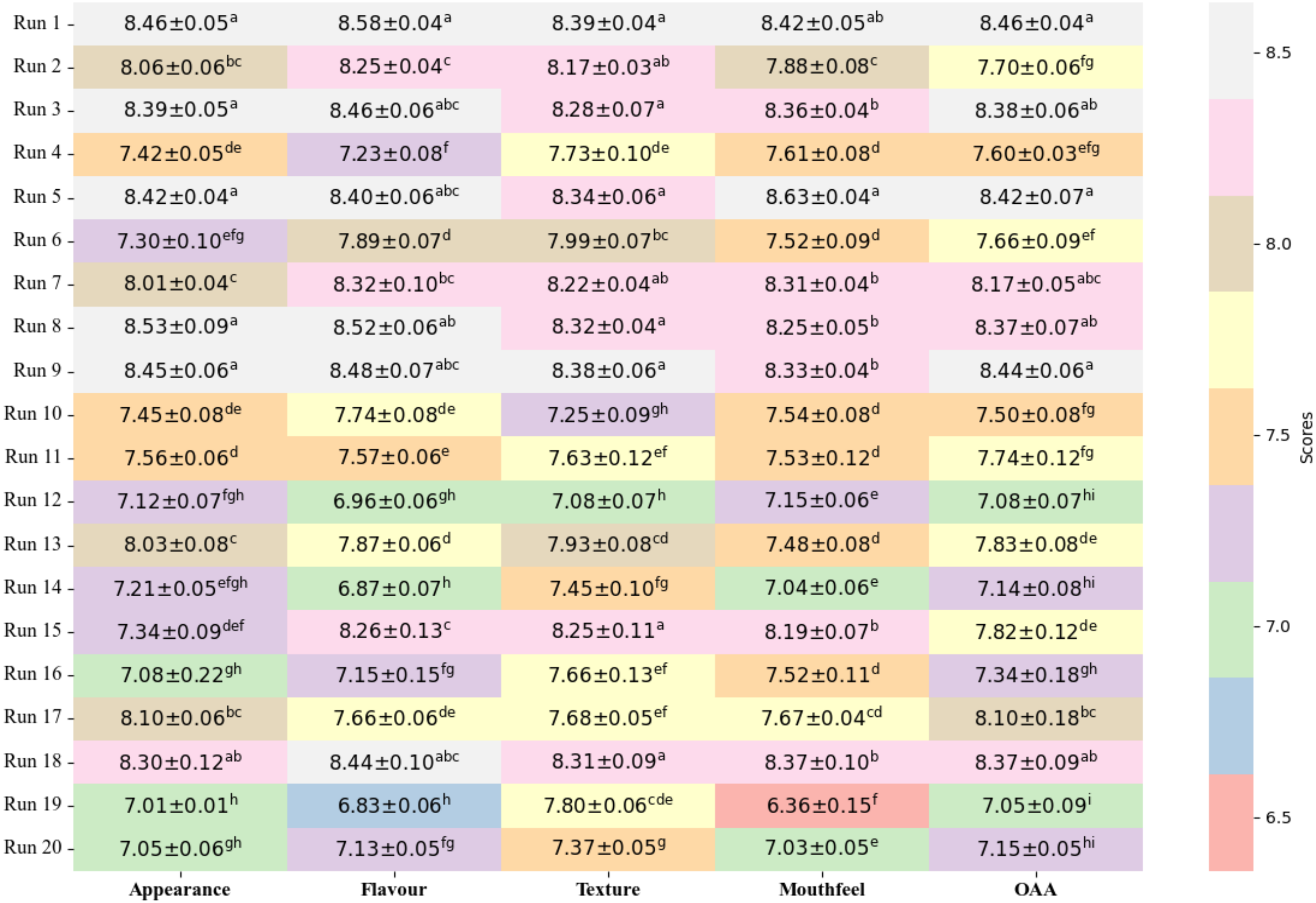


Figure 6.15(b). Sensory evaluation of multifloral bee pollen extract-based Shrikhand using 9-point hedonic scale

Table 6.11. Physicochemical and phytochemical analysis of Shrikhand incorporated with mustard bee pollen extract

Run	Factor 1	Factor 2	Factor 3	Moisture (%)	pH	Titratable acidity (%)	Ash content (%)	TSS (°Brix)	Antioxidant activity (%)	TPC (mg GAE/100 g)	TFC (mg QE/100 g)	OAA
1	7.5	70	22.5	37.5	4.51	1.225	0.85	67.655	70.645	190.45	1082.35	8.41
2	7.5	86.817	22.5	34.66	4.065	1.29	0.83	67.945	72.325	196.86	948.18	7.90
3	7.5	70	22.5	37.445	4.515	1.235	0.845	67.645	70.65	190.5	1082.4	8.47
4	12.5	80	25	37.465	4.15	1.275	0.875	68.72	76.125	335.95	1227.75	7.72
5	7.5	70	22.5	37.515	4.505	1.235	0.845	67.64	70.63	190.45	1082.35	8.51
6	2.5	60	25	39.795	4.65	1.195	0.785	67.63	61.96	72.4	443.26	7.77
7	7.5	70	26.704	38.715	4.525	1.215	0.855	69.11	70.665	192.5	942.24	8.16
8	7.5	70	22.5	37.54	4.505	1.23	0.845	67.66	70.55	190.45	1082.4	8.45
9	7.5	70	22.5	37.525	4.51	1.225	0.855	67.625	70.56	190.45	1082.4	8.53
10	2.5	60	20	38.975	4.66	1.2	0.785	66.915	62.01	72.19	442.69	7.76
11	2.5	80	20	34.81	4.4	1.265	0.795	67.49	64.135	73.76	440.66	7.77
12	7.5	53.182	22.5	39.955	4.675	1.185	0.81	67.515	68.89	192.25	947.99	7.26
13	7.5	70	18.295	36.945	4.515	1.235	0.835	66.0	70.605	191.6	943.34	7.78
14	12.5	60	20	38.55	4.61	1.215	0.865	66.945	74.2	330.55	1234.62	7.08
15	-0.908	70	22.5	37.455	4.61	1.22	0.755	67.455	56.085	1.55	0.5	7.90
16	12.5	80	20	36.11	4.135	1.285	0.865	67.56	76.035	335.05	1229.65	7.46
17	2.5	80	25	35.78	4.405	1.255	0.785	68.12	64.09	73.4	448.5	8.00
18	7.5	70	22.5	37.505	4.51	1.235	0.845	66.64	70.595	190.45	1082.4	8.46
19	15.909	70	22.5	38.41	4.355	1.255	0.915	67.875	76.48	439.55	1321.7	7.00
20	12.5	60	25	39.655	4.62	1.195	0.885	68.61	74.23	331.95	1225.5	7.21

Table 6.12. Physicochemical and phytochemical analysis of Shrikhand incorporated with multifloral bee pollen extract

Run	Factor 1	Factor 2	Factor 3	Moisture (%)	pH	Titratable acidity (%)	Ash content (%)	TSS (°Brix)	Antioxidant activity (%)	TPC (mg GAE/100 g)	TFC (mg QE/100 g)	OAA
1	7.5	70	22.5	33.45	4.505	1.205	0.82	65.455	66.015	178.05	981.35	8.46
2	7.5	86.817	22.5	30.73	4.033	1.265	0.81	65.745	67.9	174.1	806.25	7.70
3	7.5	70	22.5	33.395	4.495	1.215	0.815	65.445	66.02	178.1	981.3	8.38
4	12.5	80	25	33.46	4.1585	1.255	0.855	66.52	72.4	309.3	1069.91	7.60
5	7.5	70	22.5	33.465	4.485	1.215	0.815	65.44	66	178.05	981.3	8.42
6	2.5	60	25	35.745	4.618	1.175	0.755	65.425	56.5	69.71	368.44	7.66
7	7.5	70	26.704	34.665	4.505	1.195	0.825	66.51	66.035	179.5	831.48	8.17
8	7.5	70	22.5	33.49	4.485	1.21	0.815	65.46	65.92	178.05	981.35	8.37
9	7.5	70	22.5	33.475	4.49	1.205	0.825	65.425	65.93	178.05	981.3	8.44
10	2.5	60	20	34.925	4.645	1.185	0.755	64.715	57.7	63.94	375.43	7.50
11	2.5	80	20	30.81	4.38	1.245	0.765	65.29	59.505	63.12	388.35	7.74
12	7.5	53.182	22.5	35.905	4.635	1.162	0.78	65.315	64.05	180.17	795.61	7.08
13	7.5	70	18.295	32.895	4.495	1.21	0.805	63.75	65.975	179	835.59	7.83
14	12.5	60	20	34.565	4.585	1.195	0.835	64.745	69.57	316.43	1068.72	7.14
15	-0.908	70	22.5	33.405	4.6	1.185	0.725	65.255	51.3	1.55	0.5	7.82
16	12.5	80	20	32.15	4.102	1.265	0.84	65.36	71.1	314.5	1067.69	7.34
17	2.5	80	25	31.91	4.385	1.235	0.76	65.92	59.46	64.45	382.04	8.10
18	7.5	70	22.5	33.455	4.49	1.215	0.815	64.445	65.965	178.05	981.35	8.37
19	15.909	70	22.5	34.36	4.368	1.235	0.885	65.675	72.1	419.64	1162	7.05
20	12.5	60	25	35.605	4.6	1.175	0.855	66.41	69.595	315.65	1070.25	7.15

Table 6.13. Coefficient of regression of physicochemical and phytochemical parameters of MSBPE-based Shrikhand

Factor	Moisture	pH	Titratable acidity	Ash content	TSS	Antioxidant activity	TPC	TFC	OAA
Intercept	37.51	4.51	1.23	0.8476	67.47	70.6	190.46	1082.38	4.67
A	0.2948*	-0.0753*	0.0083*	0.0446*	0.1747	6.06*	130.22*	392.8*	0.8186*
B	-1.59*	-0.1813*	0.0331*	0.0025*	0.184	1.01*	1.38	0.0593	0.3564*
C	0.5292*	0.0027*	-0.0058*	0.0039	0.6883*	0.0092	0.2683	-0.3266	0.1179*
AB	0.4437*	-0.055*	0.0031*	-0.0025	-0.0425	-0.0656*	0.7413	-0.7412	0.0528*
AC	0.0838	0.0038*	-0.0019	0.005	0.185	0.0269	0.3062	-2.43	0.0028
BC	0.05	0.0025*	0.0006	-0.0025	-0.0738	0.0081	-0.1337	1.81	0.0153*
A^2	0.1388	-0.0096*	0.0028	-0.0049*	0.0849	-1.53*	10.65*	-148.95*	1.93*
B^2	-0.0822	-0.0493*	0.0028	-0.0102*	0.1079	0.003	1.46	-47.49	1.47*
C^2	0.1025	0.0037*	-0.0016	-0.0014	0.046	0.0127	0.5728	-49.36	0.4769*
R^2	0.9995	0.9999	0.9589	0.9883	0.7799	1.000	1.000	1.000	0.9939

*Represents significant difference ($p<0.05$), MSBPE – mustard bee pollen extract

The coefficient of regression of different response parameters of mustard and multifloral bee pollen extract-based Shrikhand is presented in Table 6.13 and 6.14, respectively. The sign and magnitude of the coefficients indicate the expected change in the response variable resulting from a one-unit change in the corresponding factor, while keeping all other factors constant. The VIFs for all the factors were found to be equal to 1.00, which, according to a rough rule, are considered tolerable if they are less than 10. This indicates that the values are acceptable. The suitability of the model was assessed based on statistical parameters including F-values, standard deviation, means, coefficient of correlation, R^2, adjusted R^2, and the lack-of-fit test. Based on these evaluations, quadratic response surface models were selected for further statistical analysis.

Table 6.14. Coefficient of regression of physicochemical and phytochemical parameters of MFBPE-based Shrikhand

Factor	Moisture	pH	Titratable acidity	Ash content	TSS	Antioxidant activity	TPC	TFC	OAA
Intercept	33.46	4.49	1.21	0.8176	65.27	65.97	178.06	981.32	8.41
A	0.2926*	-0.0712*	0.0098*	0.0453*	0.1751	6.19*	124.32*	345.3*	0.2244*
B	-1.55*	-0.1783*	0.0325*	0.0052*	0.1844	1.14*	-1.8	3.15	0.1737*
C	0.5306*	0.0049*	-0.0055*	0.0047	0.6449*	0.0132	0.1436	-1.21	0.0997*
AB	0.4237*	-0.0533*	0.0037*	-0.0012	-0.0431	-0.0538*	-0.275	-3.49	-0.0038*
AC	0.0537	0.0117*	-0.0012	0.005	0.1856	0.3213	-1.64	2.13	-0.0312
BC	0.0687	0.0092*	0.0013	-0.0012	-0.0731	0.3037	-1.11	0.1713	0.0562*
A^2	0.1456	-0.0031*	0.0013	-0.0048*	0.1013	-1.51*	11.5*	-141.45*	-0.3501*
B^2	-0.0541	-0.0562*	0.0026	-0.0083*	0.1243	0.001	-0.3305	-63.78	-0.366*
C^2	0.1094	0.0025*	-0.0013	-0.0013	-0.0171	0.0116	0.4173	-52.25	-0.1504*
R^2	0.9997	0.9993	0.9469	0.9902	0.7672	1.000	1.000	1.000	0.9974

*Represents significant difference (p<0.05), MFBPE – multifloral bee pollen extract

A statistical regression analysis was subsequently performed on the experiments, and the relationship of the responses with the independent variables was estimated using equations 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 and 40.

$$\textit{Moisture content} = 56.959 - 0.796A - 0.155B - 0.716C + 0.008AB + 0.006AC + 0.002BC + 0.005A^2 - 0.001B^2 + 0.016C^2 \quad (23)$$

$$\textit{pH} = 3.358 + 0.061A + 0.056B - 0.035C - 0.001AB + 0.0003AC + 0.0001 * BC - 0.0003A^2 - 0.0005B^2 + 0.0005C^2 \quad (24)$$

$$\textit{Titratable acidity} = 1.099 - 0.001A - 0.002B + 0.008C + 6.25AB - 0.0001AC + 2.50BC + 0.0001A^2 + 2.808B^2 - 0.0002C^2 \quad (25)$$

Ash content = -0.015 + 0.006A + 0.017B + 0.015C - 5.00AB + 0.0004AC - 0.0001BC - 0.0002A^2 - 0.0001B^2 - 0.0002C^2 (26)

TSS = 59.892 + 0.035A + 0.018B + 0.275C (27)

Antioxidant activity = 52.309 + 2.170A + 0.099B - 0.126C - 0.0013AB + 0.0021AC + 0.0003BC - 0.061A^2 + 2.966B^2 + 0.002C^2 (28)

TPC = 128.382 + 18.065A - 1.895B - 3.826C + 0.0148AB + 0.0245AC - 0.005BC + 0.426A^2 + 0.014B^2 + 0.0916C^2 (29)

TFC = 173.339A + 64.968B + 351.636C - 0.0148AB - 0.1943AC + 0.072BC - 5.958A^2 - 0.474B^2 - 7.897C^2 (30)

Overall acceptability = -20.494 + 0.023A + 0.412B + 1.213C+ 0.002AB + 0.0015AC + 0.002BC - 0.015A^2 - 0.0032B^2 - 0.029C^2 (31)

where A, B and C represents mustard bee pollen extract concentration, hung curd and sugar concentration, respectively. Similarly, the equations for statistical regression analysis of Shrikhand incorporated with multifloral bee pollen extract are as following:

Moisture content = 55.155 - 0.718A - 0.205B - 0.800C + 0.008AB + 0.004AC + 0.003BC + 0.006A^2 - 0.0005B^2 + 0.017C^2 (32)

pH = 3.425 + 0.041A + 0.060B - 0.049C - 0.001AB + 0.001AC + 0.0004BC - 0.0001A^2 - 0.0006B^2 + 0.0004C^2 (33)

Titratable acidity = 1.142 - 0.002A - 0.002B + 0.004C + 7.50AB - 9.999AC + 5.00BC + 5.333A^2 + 2.570B^2 - 0.0002C^2 (34)

Ash content = 0.127 + 0.005A + 0.013B + 0.011C - 2.50AB + 0.0004AC - 5.00BC - 0.0002A^2 - 8.322B^2 - 0.0002C^2 (35)

TSS = 60.290 - 0.299A - 0.083B + 0.475C - 0.0008AB + 0.015AC -0.003BC + 0.004A^2 + 0.001B^2 - 0.0027C^2 (36)

$$Antioxidant\ activity = 69.088 + 1.640A - 0.153B - 1.121C - 0.0011AB + 0.0257AC + 0.0121BC - 0.060A^2 + 9.673B^2 + 0.002C^2 \quad (37)$$

$$TPC = -48.372 + 21.292A + 1.321B + 1.135C - 0.005AB - 0.131AC - 0.044BC + 0.459A^2 - 0.003B^2 + 0.066C^2 \quad (38)$$

$$TFC = -7220.499 + 154.972A + 89.972B + 373.960C - 0.069AB + 0.170AC + 0.006BC - 5.657A^2 - 0.637B^2 - 8.360C^2 \quad (39)$$

$$\text{Overall acceptability} = -21.188 + 0.226A + 0.479B + 0.983C - 7.50AB - 0.002AC + 0.002BC - 0.014A^2 - 0.004B^2 - 0.024C^2 \quad (40)$$

where A, B and C represents multifloral bee pollen extract concentration, hung curd and sugar concentration, respectively.

Moisture content, pH, titratable acidity, ash content and TSS were selected as the physicochemical parameters. The moisture content was found to be significantly dependent on all primary factors ($p<0.05$), particularly hung curd ($p<0.0001$), which had a negative coefficient (-1.59* and -1.55* for MSBPE and MFBPE-based samples respectively), indicating that moisture content decreased with an increase in hung curd concentration. On the contrary, bee pollen extract concentration and sugar positively influenced the moisture content (Figures 6.16a and b). The interaction term AB also showed significance ($p<0.05$), hence impacting moisture content. The hung curd concentration likely affected the moisture content because much of the excess water is already expelled from it during straining, resulting in a solid mass, having low moisture content. Consequently, the overall moisture content of Shrikhand was lowered with an increase in the concentration of hung curd (Arab *et al.*, 2023). The positive effect of bee pollen extract concentration might be attributed to the hygroscopic nature of bee pollen owing to the presence of proteins, polysaccharides and natural sugars in it (Kieliszek *et al.*, 2023). Similarly, sugar also possess the characteristic feature of attracting and holding water in the food products, especially ice-creams and frozen desserts (Goldfein and Slavin, 2015). Thus, sugar concentration was observed to positively influence the moisture content of Shrikhand.

The pH values of mustard and multifloral bee pollen extract-based Shrikhand were significantly affected by all the parameters at linear ($p<0.05$), interactive ($p<0.05$) and quadratic level ($p<0.05$). The coefficient values indicate that all factors, especially the bee pollen extract concentration and the amount of hung curd, showed both individual and combined effects on pH changes in the product (Figures 6.16a and b). The pH values obtained in our analysis align with those reported by Zlatev *et al.* (2018), who found that the pH of yogurt incorporated with bee pollen ranged from 4.1 to 4.5. Melo *et al.* (2023) also reported in their findings that the average pH values of bee pollen samples were between 4.55 and 5.09. The differences are attributable to the inherent acidity of different bee pollen varieties.

Titratable acidity was positively affected by bee pollen extract concentration and hung curd at linear level with coefficient values corresponding to 0.0083* and 0.0331* ($p<0.05$) for mustard, and 0.0098* and 0.0325* ($p<0.05$) for multifloral bee pollen extract-based samples respectively. The impact of sugar was negatively correlated but had a minimal effect (-0.0058* for mustard and -0.0055* for multifloral). pH and titratable acidity are interrelated to each other, thus the Shrikhand samples having low pH had high values of titratable acidity. Matela *et al.* (2019), in their research study, estimated the values of titratable acidity of yoghurt samples in terms of percentage of lactic acid present in it and found that the values were in the range of 0.69 to 1.81%. The inherent acidity of bee pollen sample might have impacted the values in our analysis (Melo *et al.*, 2023).

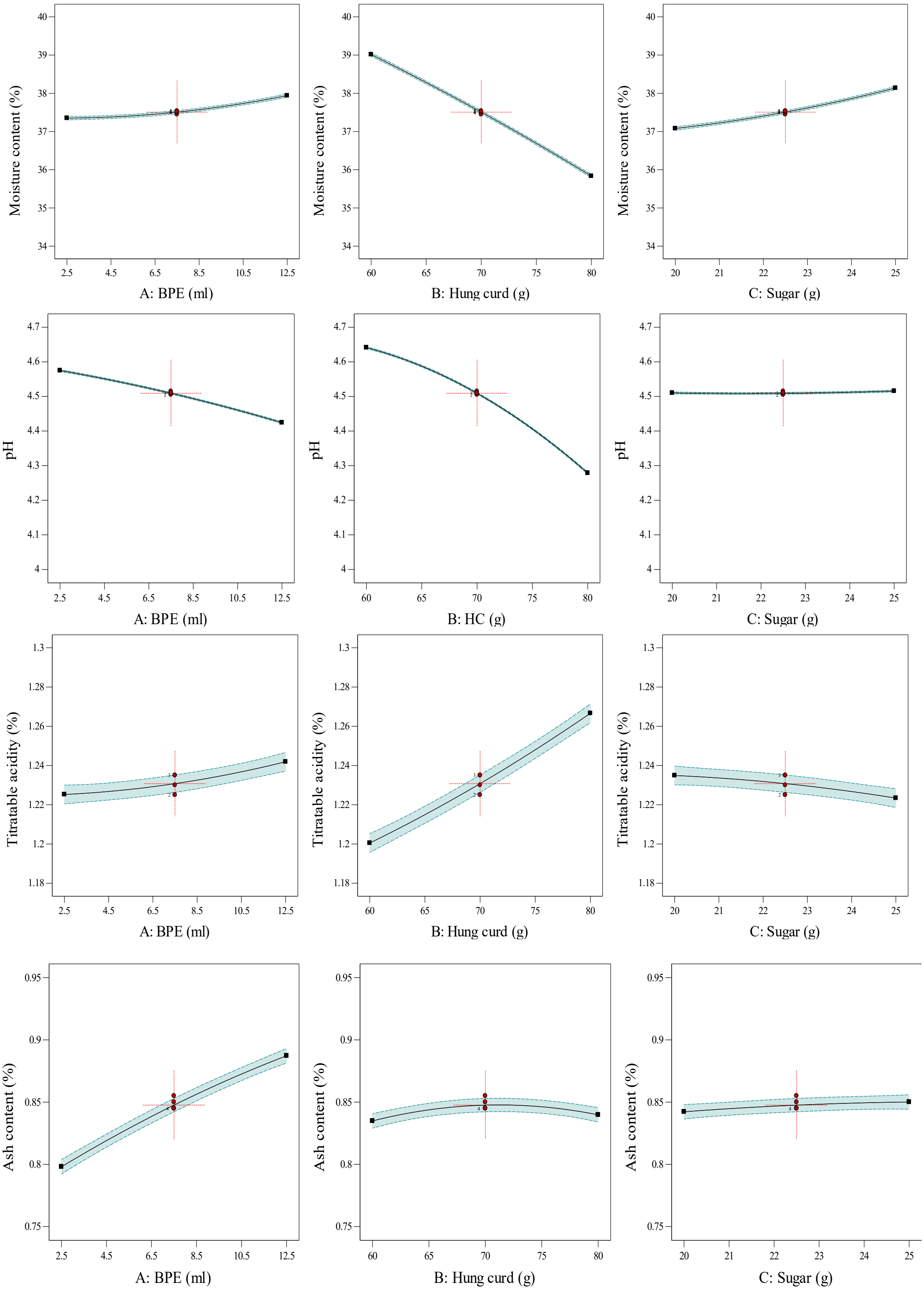
Bee pollen extract concentration and hung curd were observed to significantly impact the ash content (0.0446* and 0.0025* for mustard, 0.0453* and 0.0052* for multifloral, respectively) at linear ($p<0.05$) and quadratic level ($p<0.05$), suggesting that an increase in the concentration of mustard and multifloral bee pollen extract tend to increase the mineral content of Shrikhand, as ash content represents the mineral residue. Hung curd, being a source of important macro and micronutrients (Savarino *et al.*, 2021), may have contributed to the increase in ash content with higher concentrations. The amount of sugar didn't pose any significant effect on this parameter at any level (linear, interactive or quadratic) as the p-values were greater than 0.05. The ash content of mustard bee pollen used for the preparation of Shrikhand was 4.3% which reflects the presence of important minerals (Kaur *et al.*, 2024). The values obtained in our study were in accordance with the ones reported by Ogunlakin *et al.* (2022), who observed that the ash content of yoghurt samples ranged between 0.60 to 0.89%.

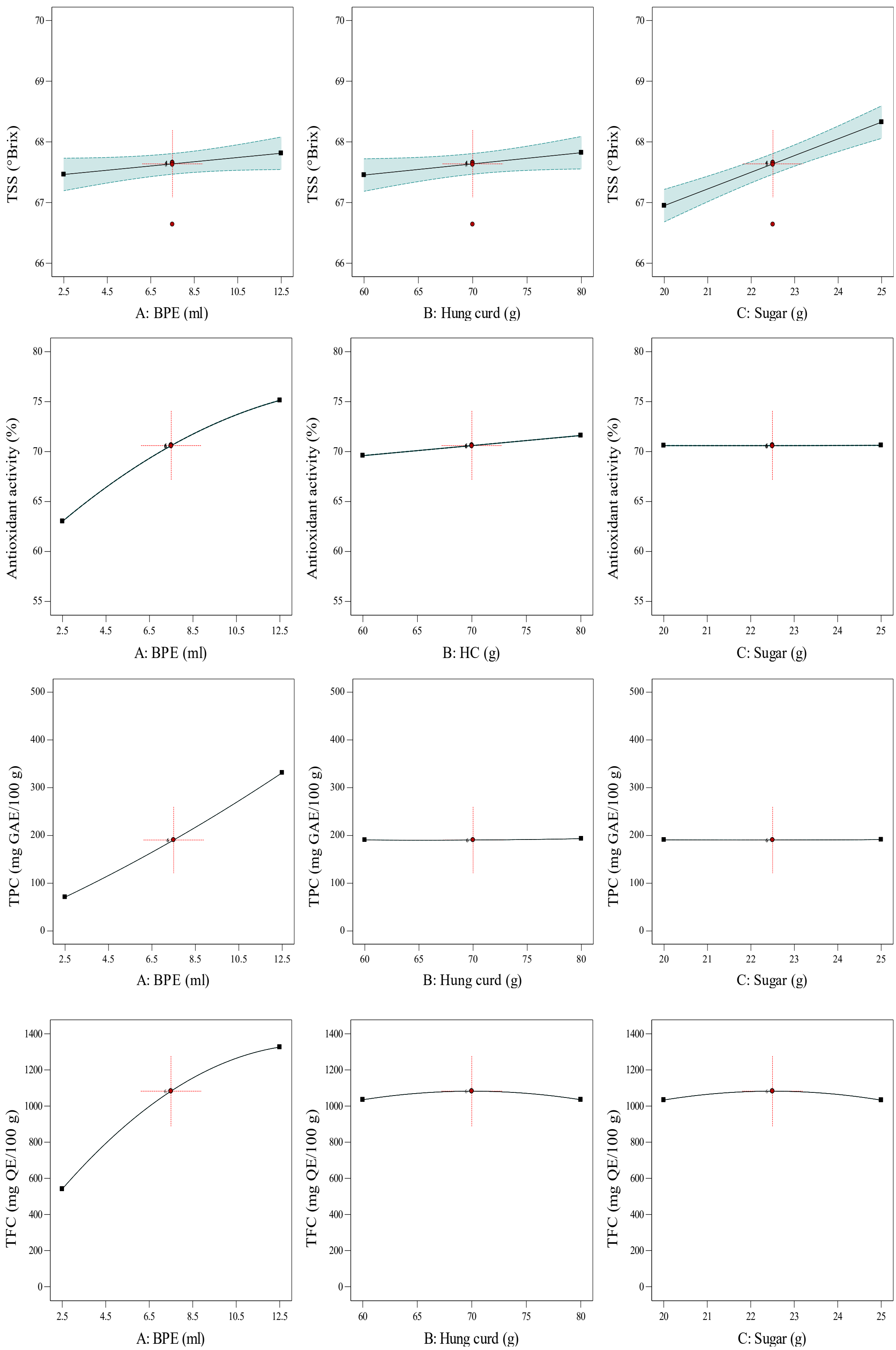
For Total Soluble Solids (TSS), sugar was found to have the most substantial positive impact (0.6883* for mustard and 0.6449* for multifloral bee pollen extract-based samples) at linear level ($p<0.05$), which aligns with the fact that high sugar levels increase the value of TSS. The other two factors *viz.* bee pollen extract and hung curd were observed to have minor effects ($p>0.05$). The interaction and quadratic terms showed limited influence ($p>0.05$), suggesting that TSS values were primarily governed by sugar concentration and a slight amount of amino acids, proteins and other organic as well as inorganic substances (Lo and Sekeh, 2024). Kusumiyati *et al.* (2020) also observed a notable increase in the TSS values of slice jam with higher sugar concentrations.

TPC, TFC, and antioxidant activity were chosen as key phytochemical parameters for evaluation. The antioxidant activity was observed to be significantly enhanced by bee pollen extract concentration ($p<0.05$), with a positive coefficient (6.06* for mustard and 6.19* for multifloral) at linear, interactive (AB) and quadratic levels, indicating that bee pollen extract contributed to the desirable antioxidant properties of the product. The effect of hung curd was also significant ($p<0.05$) at linear level, but minor, with a positive coefficient (1.01* mustard and 1.14* for multifloral). The antioxidant activity of Shrikhand increased with higher concentrations of bee pollen extract, attributable to the presence of antioxidant-rich compounds in bee pollen, such as vitamin A, vitamin E, polyphenols, and quercetin. This antioxidant property helps in the prevention of free radical production and damage caused by them (Habryka *et al.*, 2021; Kaur *et al.*, 2024). It was reported in a study conducted by Habryka *et al.* (2021) the addition of bee pollen in honey significantly affected its antioxidant activity in a positive manner, causing an increase in the value from 9.90 to 12.41 mmol AAE/100 g.

TPC and flavonoid content were remarkably influenced by the concentration of mustard bee pollen extract ($p<0.05$) at linear level, with a significantly high positive coefficient value (130.22* and 392.8* respectively), which confirmed its role in enhancing phenolic and flavonoid content of the product. Similarly, for samples incorporated with multifloral bee pollen extract, the coefficient values of TPC and TFC for factor A were 124.32* and 345.3*, respectively. The other factors didn't contribute to the phenolic content ($p>0.05$), and the interactions were also non-significant. This suggested that phenolics in Shrikhand were chiefly driven by the inclusion of mustard and multifloral bee pollen extract. Sugar posed a minor negative effect ($p<0.05$) on the TFC (-0.3266* for mustard and -1.21* for multifloral). The interaction (AB, BC, AC) and quadratic terms (A^2, B^2, C^2) also contributed to slight variations in flavonoid content ($p<0.05$). Bee pollen is an abundant source of polyphenols which are crucial for the reduction and prevention of inflammation by inhibiting the causative enzymes, reduces total cholesterol content by exhibiting anti-atherosclerotic effect, and controls the damage caused by pathogenic microbes by showcasing antibiotic effect (Habryka *et al.*, 2021; Kaur *et al.*, 2024). Habryka *et al.* (2021) studied the impact of adding bee pollen to honey and observed that the values of TPC were significantly increased from 30.75 to 178.26 mg GAE/100 g. Similarly, the augmentation in the values of TFC was also remarkable, which increased from 2.77 to 16.39 mg QE/100 g.

The overall acceptability, representing the palatability of MSBPE-based and MFBPE-based Shrikhand, was influenced by all three independent variables ($p<0.05$). A positive coefficient for bee pollen extract concentration (0.8186* for mustard, 0.2244* for multifloral) suggested that higher concentrations of bee pollen extract, up to 12.5 ml, enhanced overall acceptability, potentially due to its nutritional benefits and flavour contributions. The negative coefficient for hung curd indicated that increasing its concentration likely led to textural and flavour changes that were less preferred by the panelists, thereby decreasing the product's overall acceptability. Conversely, sugar had a positive impact on overall acceptability, suggesting that it improved sweetness and flavour, making the product more appealing. The interaction between bee pollen extract concentration and hung curd showed a slight negative effect, indicating that while both factors were beneficial individually, their combination might not be as favourable. At the quadratic level, the negative coefficient for hung curd (B^2) suggested that very high levels of hung curd could detract from overall acceptability, possibly due to over-concentration.


Moisture content (%)
A: BPE (ml)
B: Hung curd (g)
C: Sugar (g)
pH
A: BPE (ml)
B: HC (g)
C: Sugar (g)
Titratable acidity (%)
A: BPE (ml)
B: Hung curd (g)
C: Sugar (g)
Ash content (%)
A: BPE (ml)
B: Hung curd (g)
C: Sugar (g)


TSS (°Brix)
A: BPE (ml)
TSS (°Brix)
B: Hung curd (g)
TSS (°Brix)
C: Sugar (g)
Antioxidant activity (%)
A: BPE (ml)
Antioxidant activity (%)
B: HC (g)
Antioxidant activity (%)
C: Sugar (g)
TPC (mg GAE/100 g)
A: BPE (ml)
TPC (mg GAE/100 g)
B: Hung curd (g)
TPC (mg GAE/100 g)
C: Sugar (g)
TFC (mg QE/100 g)
A: BPE (ml)
TFC (mg QE/100 g)
B: Hung curd (g)
TFC (mg QE/100 g)
C: Sugar (g)

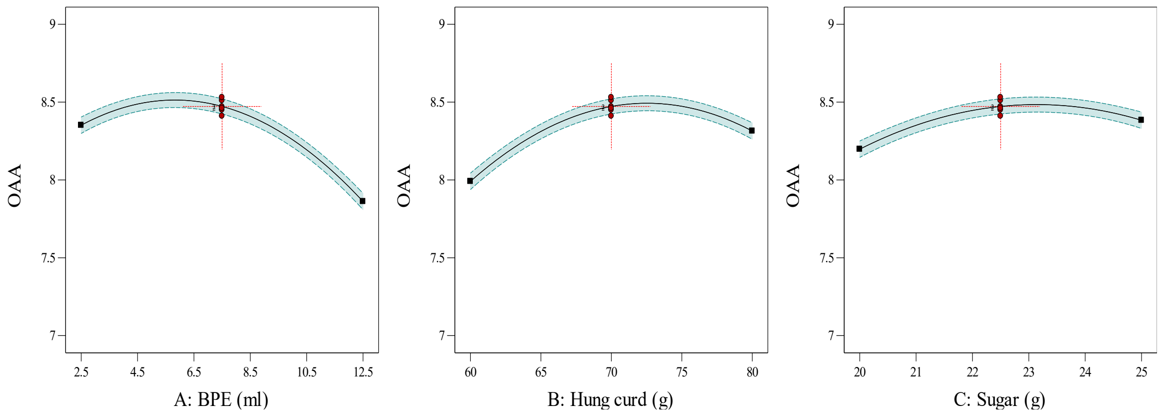
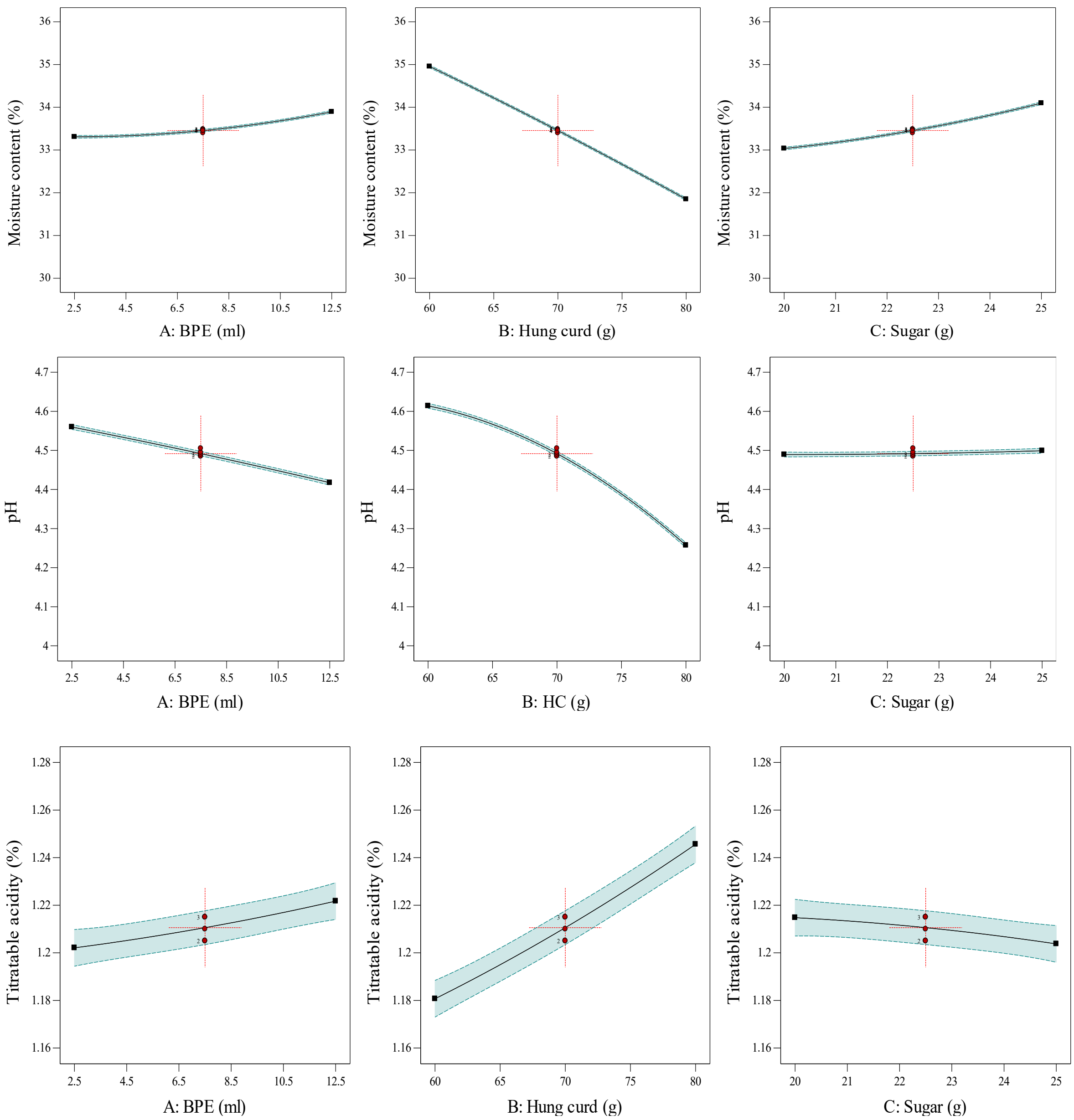
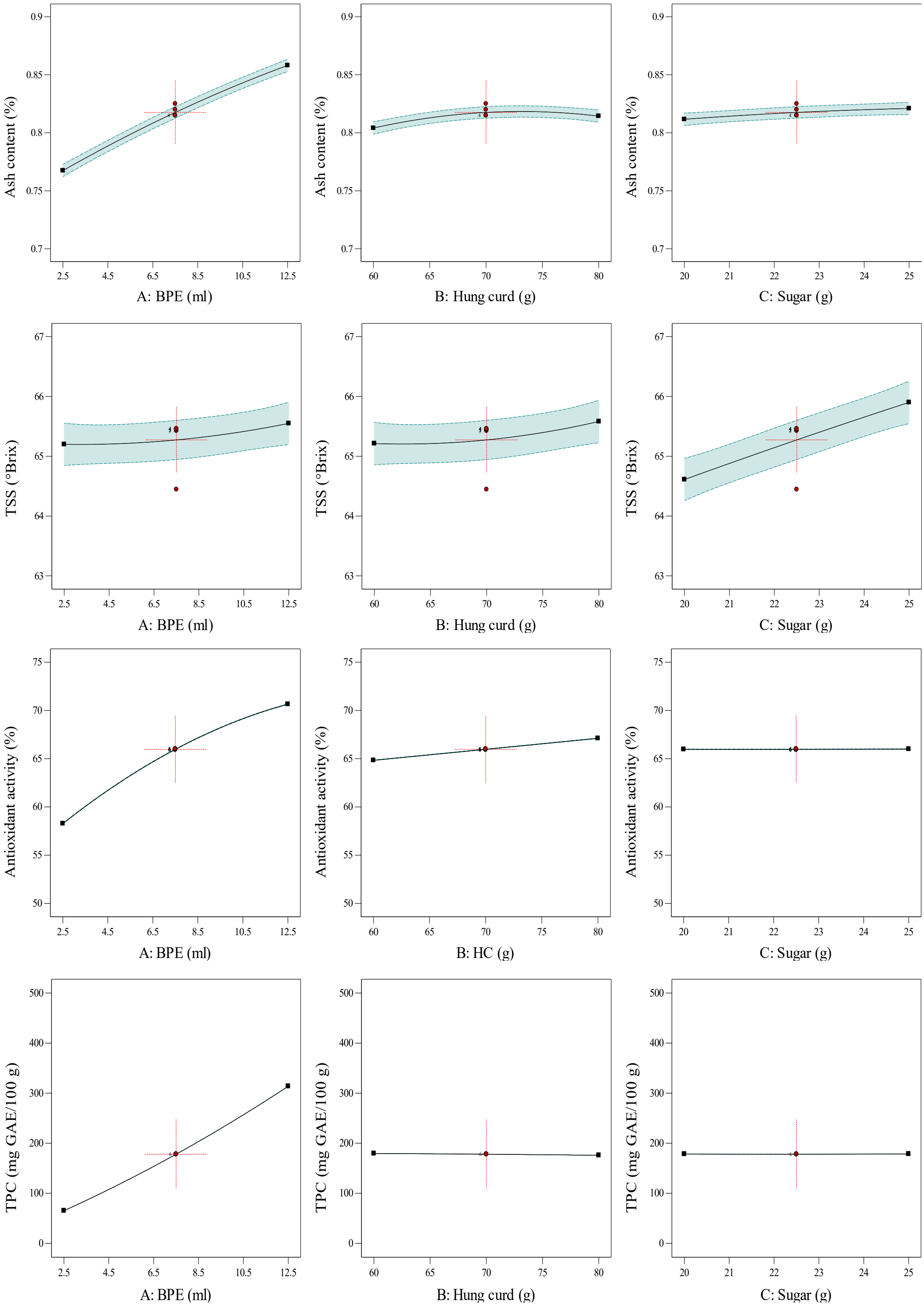


Figure 6.16(a). Linear correlation of process variables with various parameters




Ash content (%)
A: BPE (ml)
B: Hung curd (g)
C: Sugar (g)
TSS (°Brix)
Antioxidant activity (%)
B: HC (g)
TPC (mg GAE/100 g)

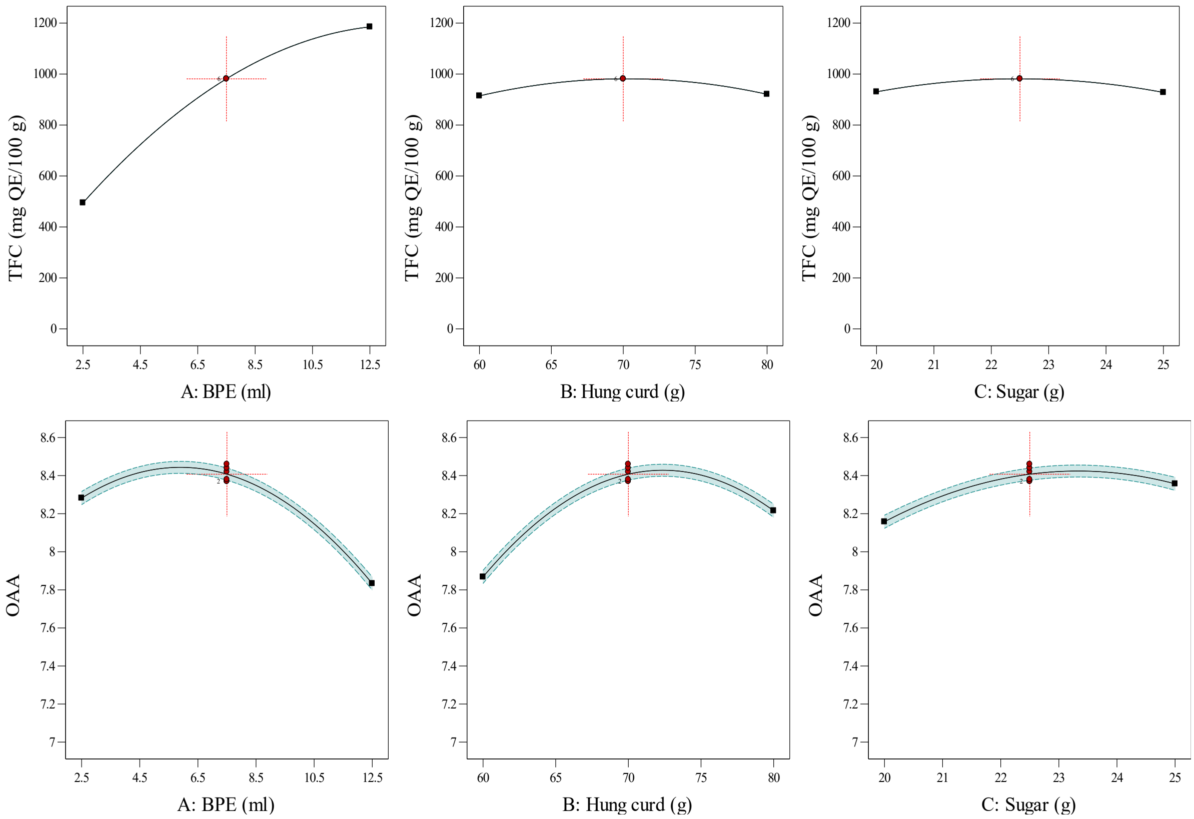


Figure 6.16(b). Linear correlation of process variables with physicochemical and phytochemical responses of MFBPE-based Shrikhand

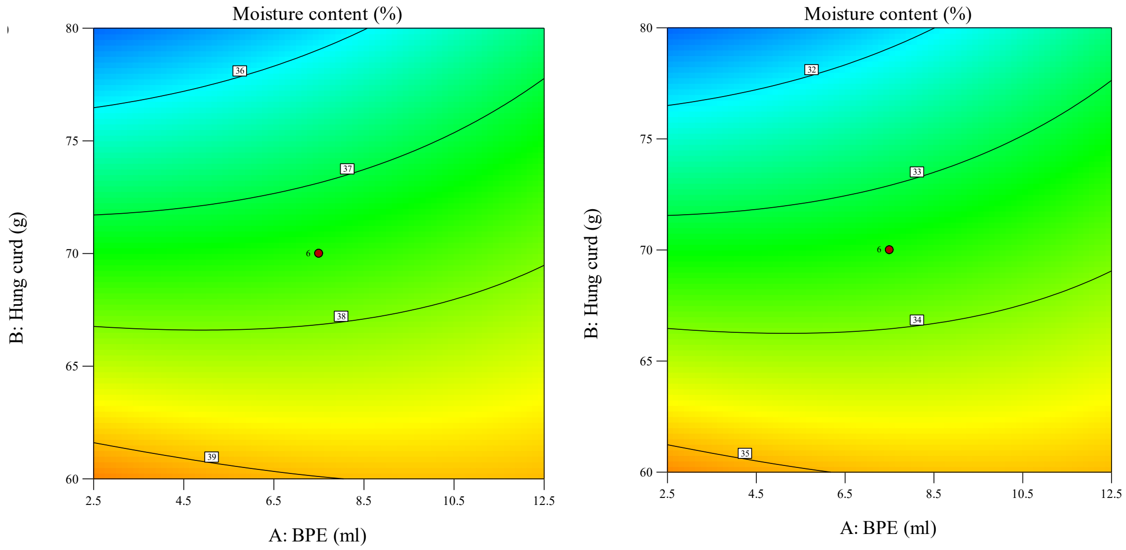


Figure 6.17. Interactive effect of bee pollen extract and hung curd on moisture content of (a) MSBPE-based Shrikhand, and (b) MFBPE-based Shrikhand

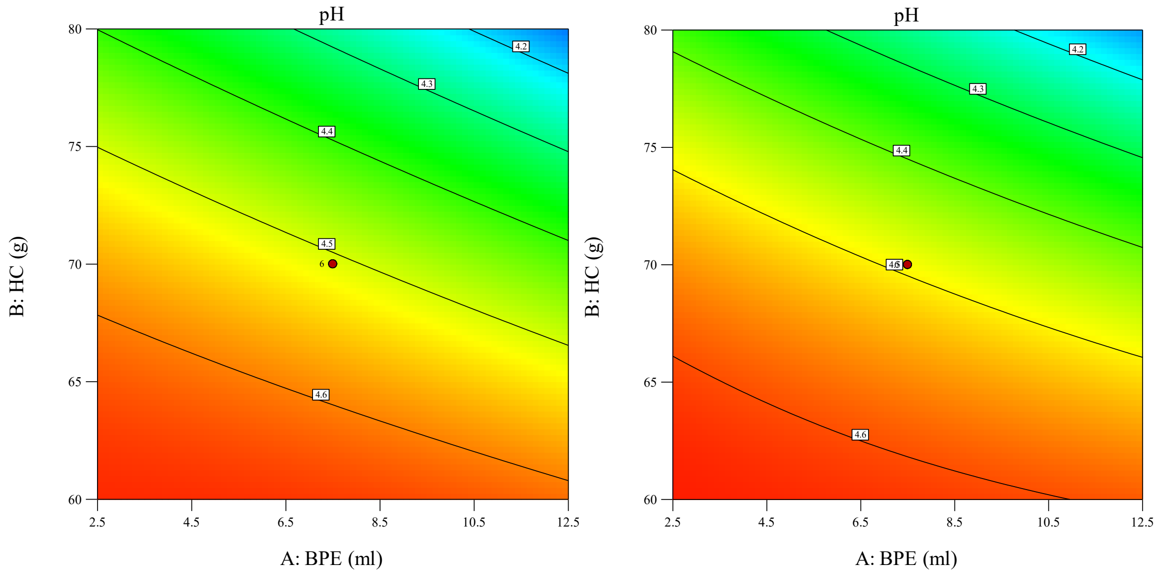


Figure 6.18. Interactive effect of bee pollen extract and hung curd on pH of (a) MSBPE-based Shrikhand, and (b) MFBPE-based Shrikhand

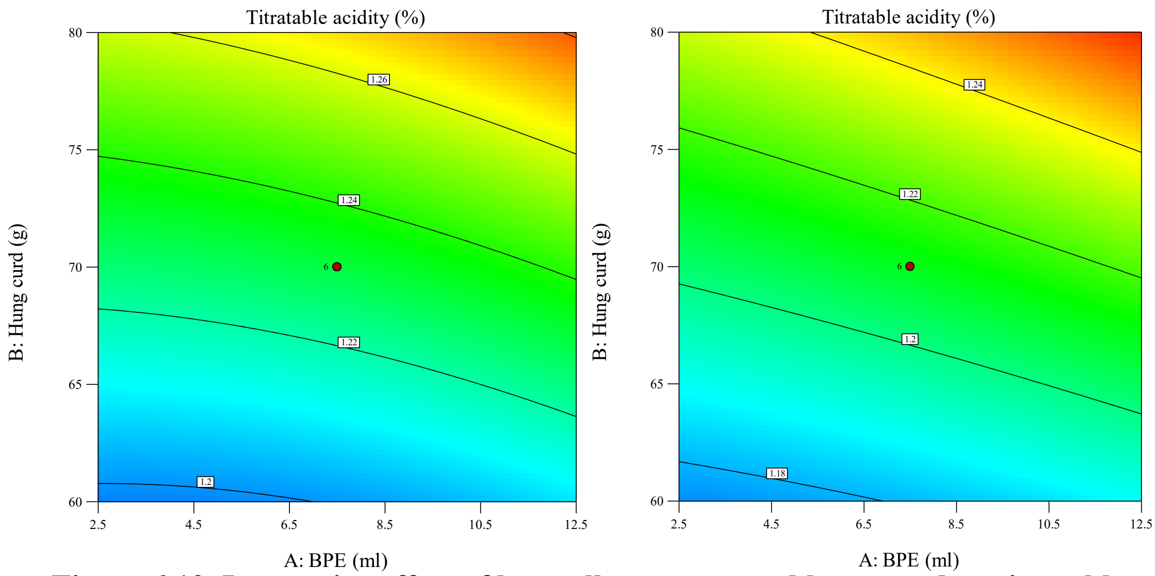


Figure 6.19. Interactive effect of bee pollen extract and hung curd on titratable acidity of (a) MSBPE-based Shrikhand, and (b) MFBPE-based Shrikhand

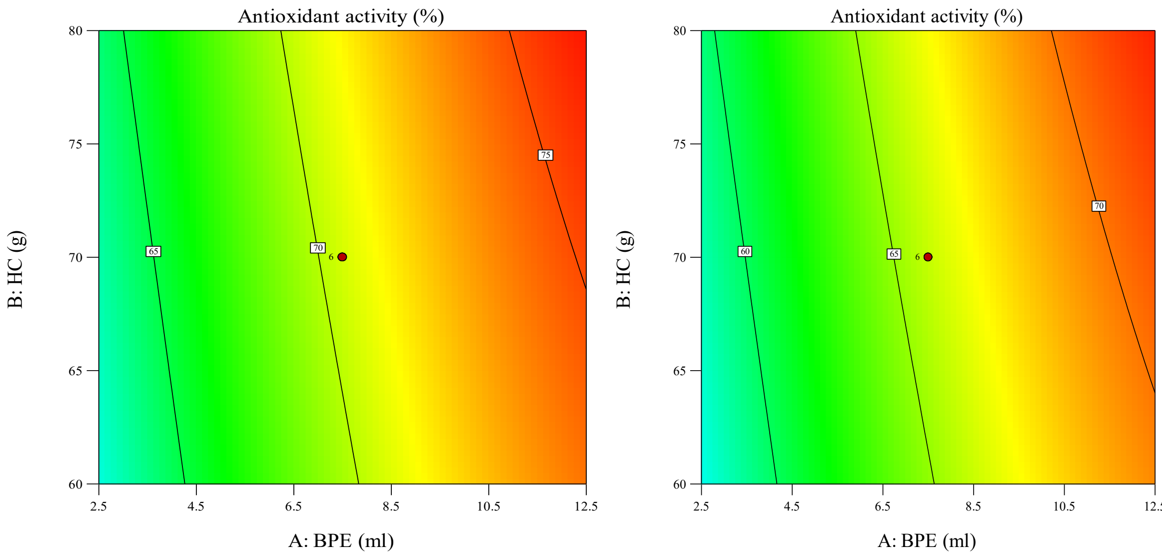


Figure 6.20. Interactive effect of bee pollen extract and hung curd on antioxidant activity of (a) MSBPE-based Shrikhand, and (b) MFBPE-based Shrikhand

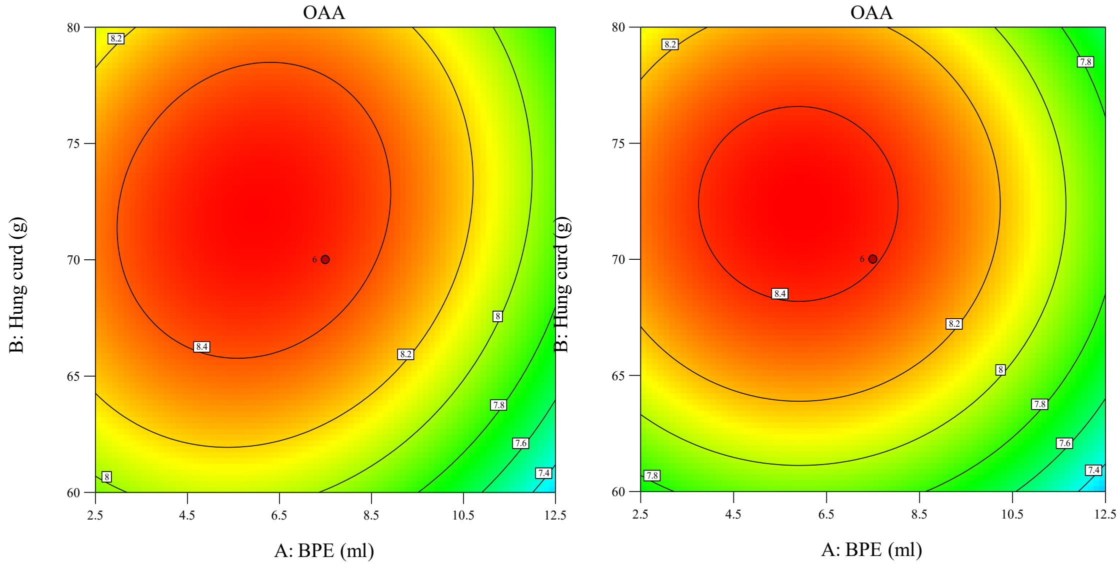


Figure 6.21. Interactive effect of bee pollen extract and hung curd on overall acceptability of (a) MSBPE-based Shrikhand, and (b) MFBPE-based Shrikhand

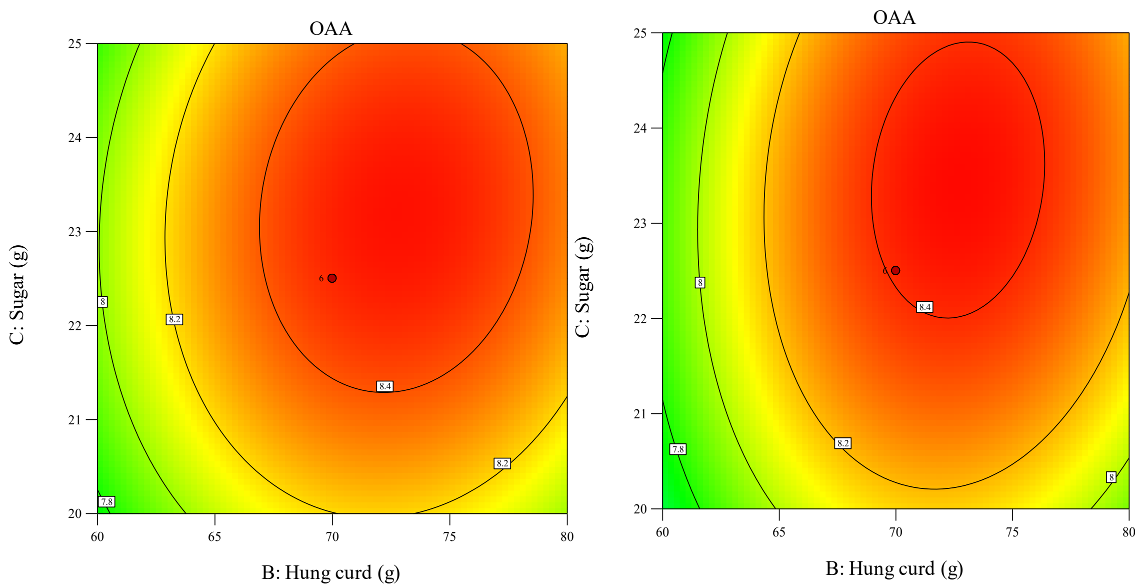


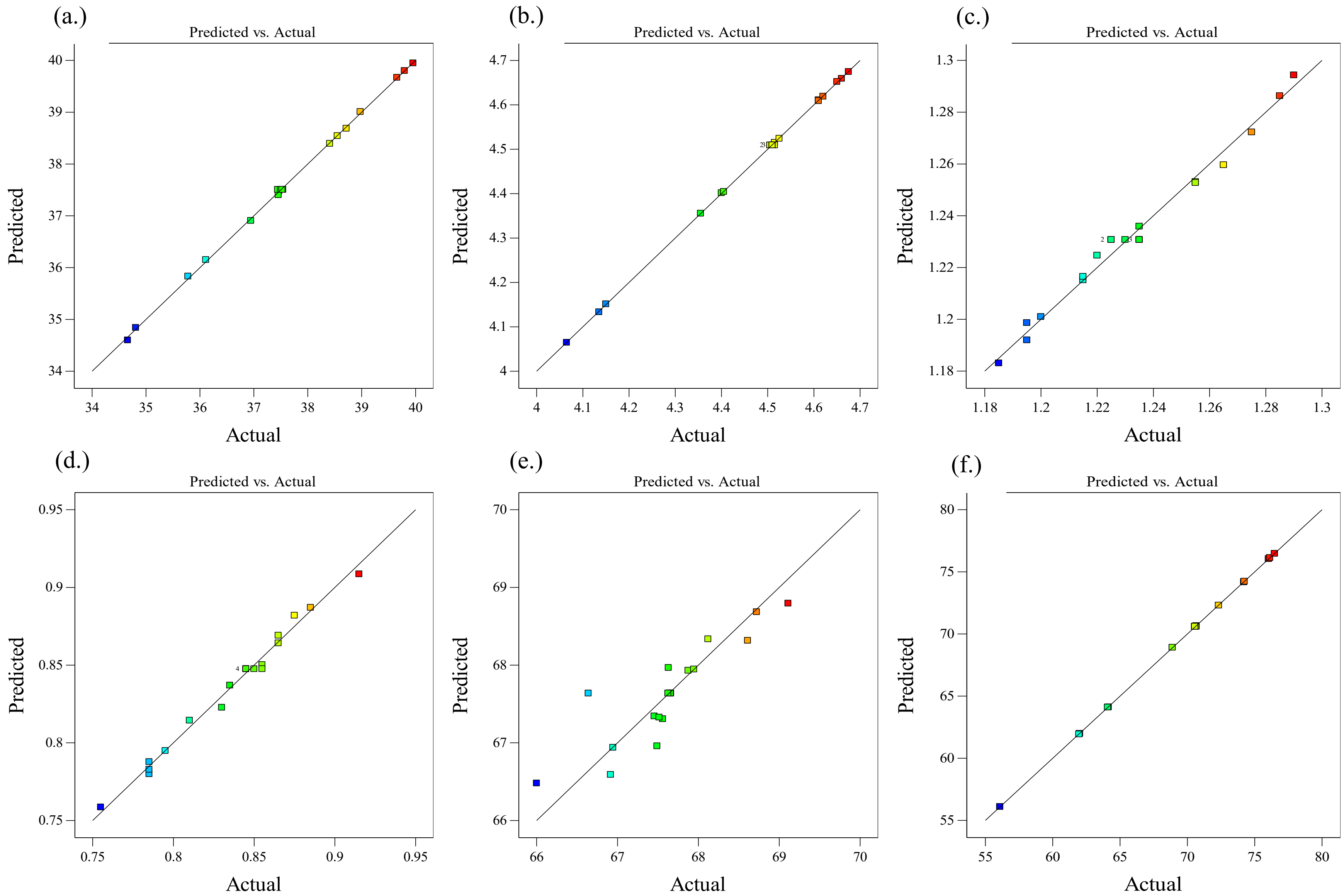
Figure 6.22. Interactive effect of hung curd and sugar on overall acceptability of (a) MSBPE-based Shrikhand, and (b) MFBPE-based Shrikhand

6.16. Optimum levels of variables for MSBPE-based Shrikhand

To formulate *Shrikhand* enriched with mustard bee pollen extract, numerical optimization of the process parameters was conducted to obtain the most desirable product outcome. Specific goals were defined for each factor and response variable based on their respective characteristics, with differential weighting applied to reflect their relative importance in determining overall desirability. All independent variables were kept within specified ranges to assess their impact on the overall product quality. For the responses, the target was to minimize moisture content to enhance shelf life and texture. While, pH, titratable acidity, ash content, and TSS were maintained within their designated ranges. The aim was to maximize antioxidant activity, TFC, and TPC to boost health-promoting properties, as well as overall acceptability to improve the product's palatability. The software generated optimum conditions i.e., 10.252 ml mustard bee pollen extract, 77.555 g hung curd and 22.625 g sugar concentration with 0.914 desirability for the development of mustard bee pollen extract-based Shrikhand (Figure 6.25). The predicted values of the different responses were as following: 36.679% moisture content, 4.277 pH, 1.264% titratable acidity, 0.866% ash content, 67.907 °Brix TSS, 74.212% antioxidant activity, 267.548 mg GAE/100 g TPC, 1225.942 mg QE/100 g TFC, and 8.207 overall acceptability. A comparison between the observed and predicted values is presented in Table 6.15, which shows that there were no significant differences between them.

6.17. Optimum levels of variables for MFBPE-based Shrikhand

As presented in Figure 6.26, the optimal levels of factors/variables to develop the most desirable optimized product includes 10.408 ml multifloral bee pollen extract, 75.350 g hung curd and 22.477 g sugar. A desirability value of 0.900 was achieved for the specified formulation. The predicted values of responses were: 32.955% moisture content, 4.321 pH, 1.236% titratable acidity, 0.842% ash content, 65.523 °Brix TSS, 69.652% antioxidant activity, 253.124 mg GAE/100 g TPC, 1116.647 mg QE/100 g TFC, and 8.145 overall acceptability. As presented in Table 6.16, no significant differences were observed between the experimental and predicted values, indicating good agreement between the model and actual results.


(a.)
Predicted vs. Actual
Predicted
Actual
(b.)
Predicted vs. Actual
Predicted
Actual
(c.)
Predicted vs. Actual
Predicted
Actual
(d.)
Predicted vs. Actual
Predicted
Actual
(e.)
Predicted vs. Actual
Predicted
Actual
(f.)
Predicted vs. Actual
Predicted
Actual

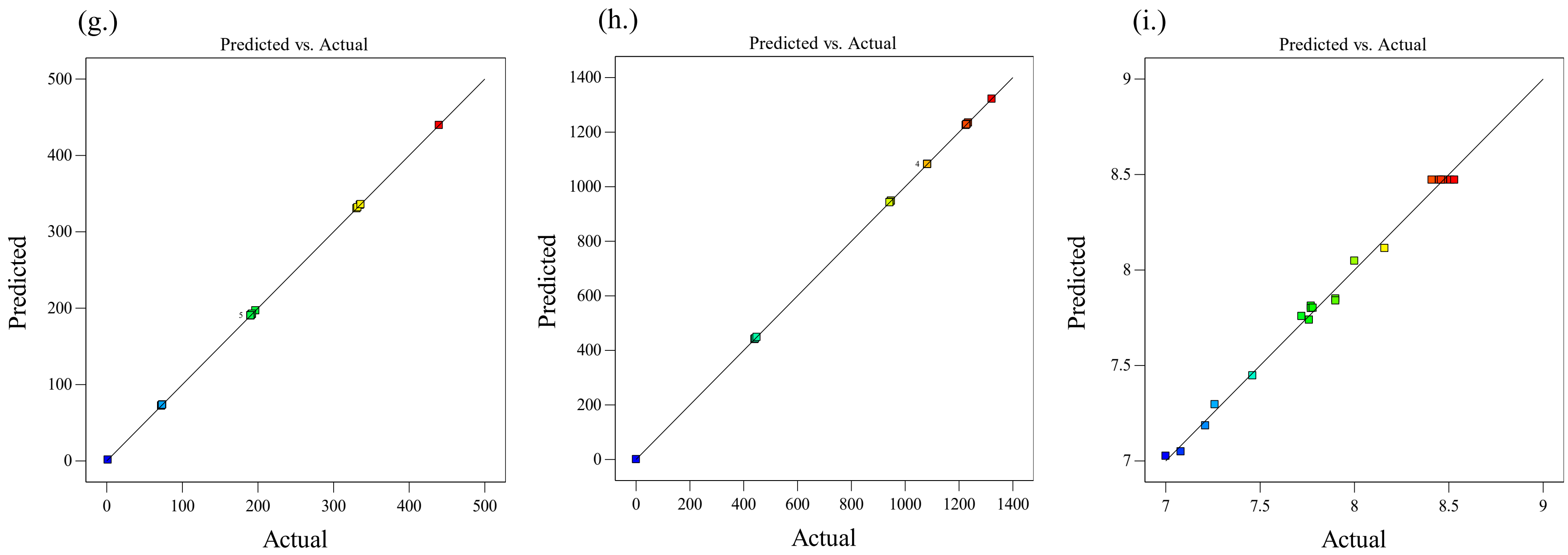
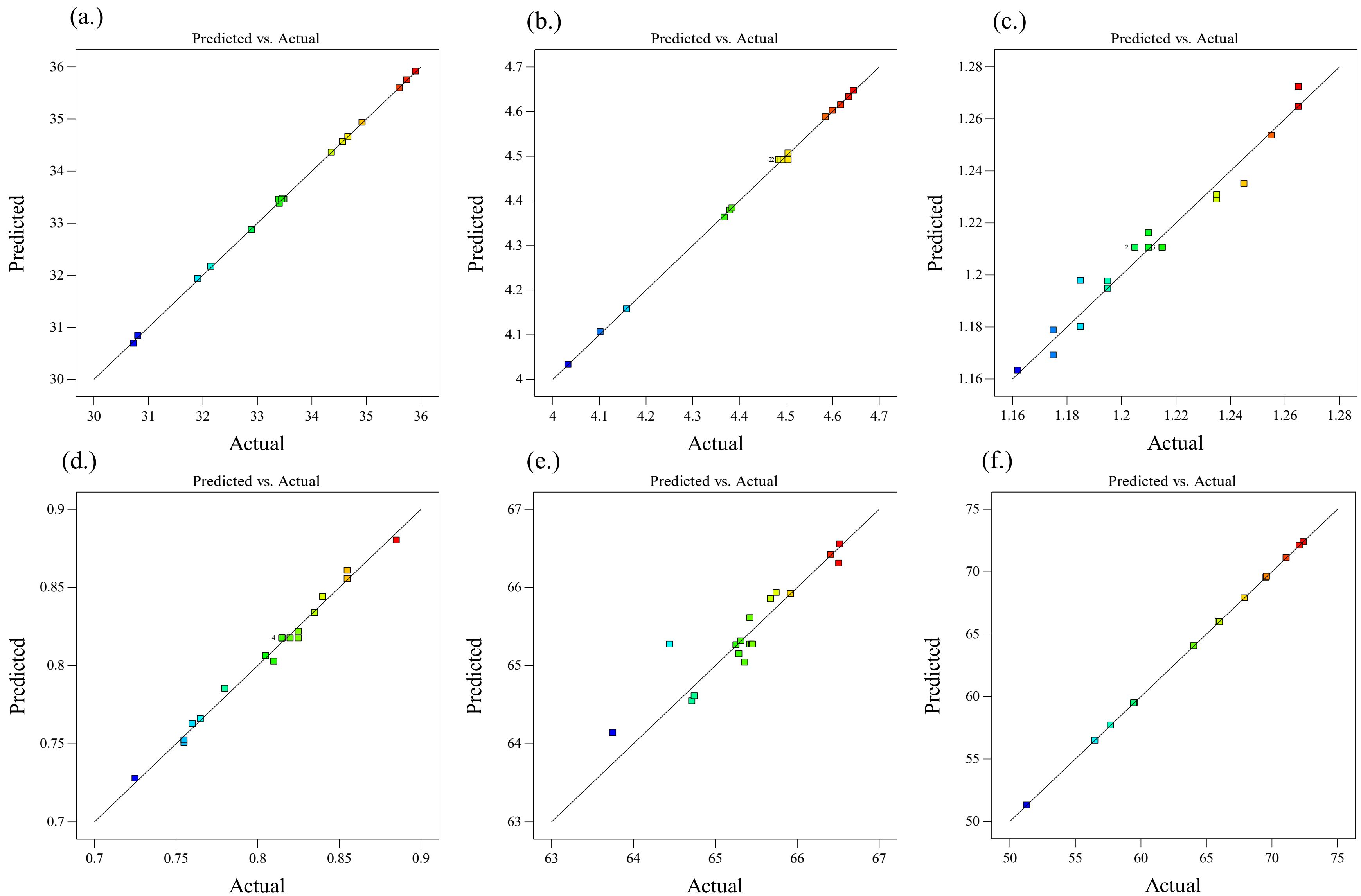


Figure 6.23. Comparison between actual experimental and predicted data of (a) moisture content, (b) pH, (c) titratable acidity, (d) ash content, (e) TSS, (f) antioxidant activity, (g) TPC, (h) TFC, and (i) overall acceptability of MSBPE-based Shrikhand


(a.)
Predicted vs. Actual
Predicted
36
35
34
33
32
31
30
30 31 32 33 34 35 36
Actual
(b.)
Predicted vs. Actual
Predicted
4.7
4.6
4.5
4.4
4.3
4.2
4.1
4
4 4.1 4.2 4.3 4.4 4.5 4.6 4.7
Actual
(c.)
Predicted vs. Actual
Predicted
1.28
1.26
1.24
1.22
1.2
1.18
1.16
1.16 1.18 1.2 1.22 1.24 1.26 1.28
Actual
(d.)
Predicted vs. Actual
Predicted
0.9
0.85
0.8
0.75
0.7
0.7 0.75 0.8 0.85 0.9
Actual
(e.)
Predicted vs. Actual
Predicted
67
66
65
64
63
63 64 65 66 67
Actual
(f.)
Predicted vs. Actual
Predicted
75
70
65
60
55
50
50 55 60 65 70 75
Actual

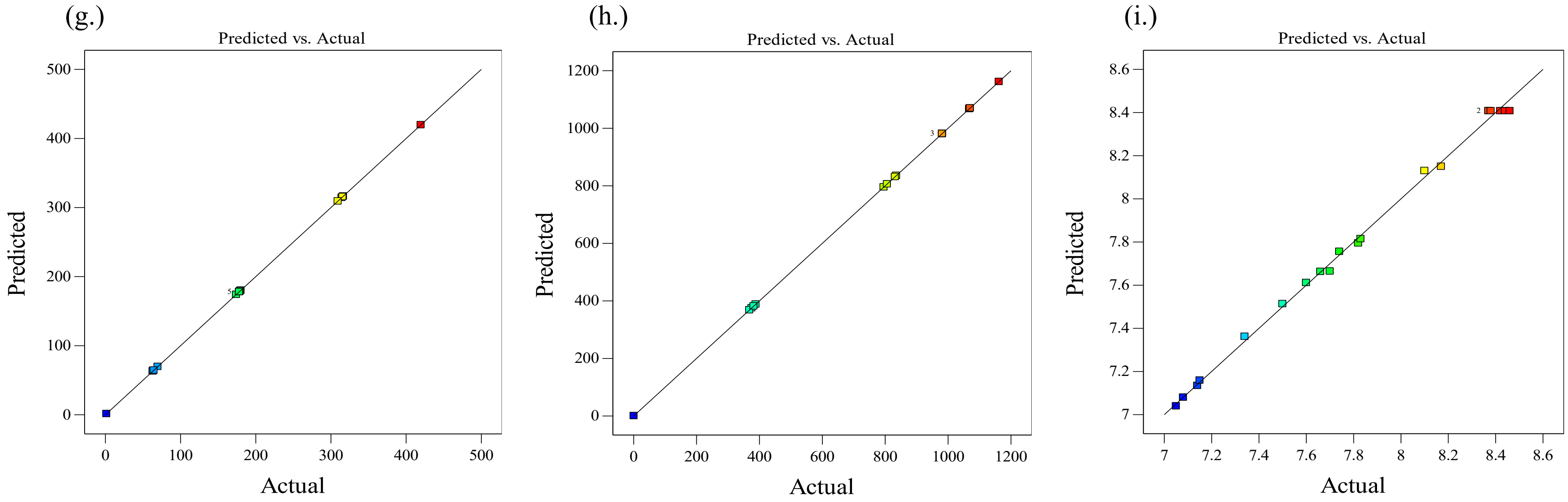


Figure 6.24. Comparison between actual experimental and predicted data of (a) moisture content, (b) pH, (c) titratable acidity, (d) ash content, (e) TSS, (f) antioxidant activity, (g) TPC, (h) TFC, and (i) overall acceptability of MFBPE-based Shrikhand

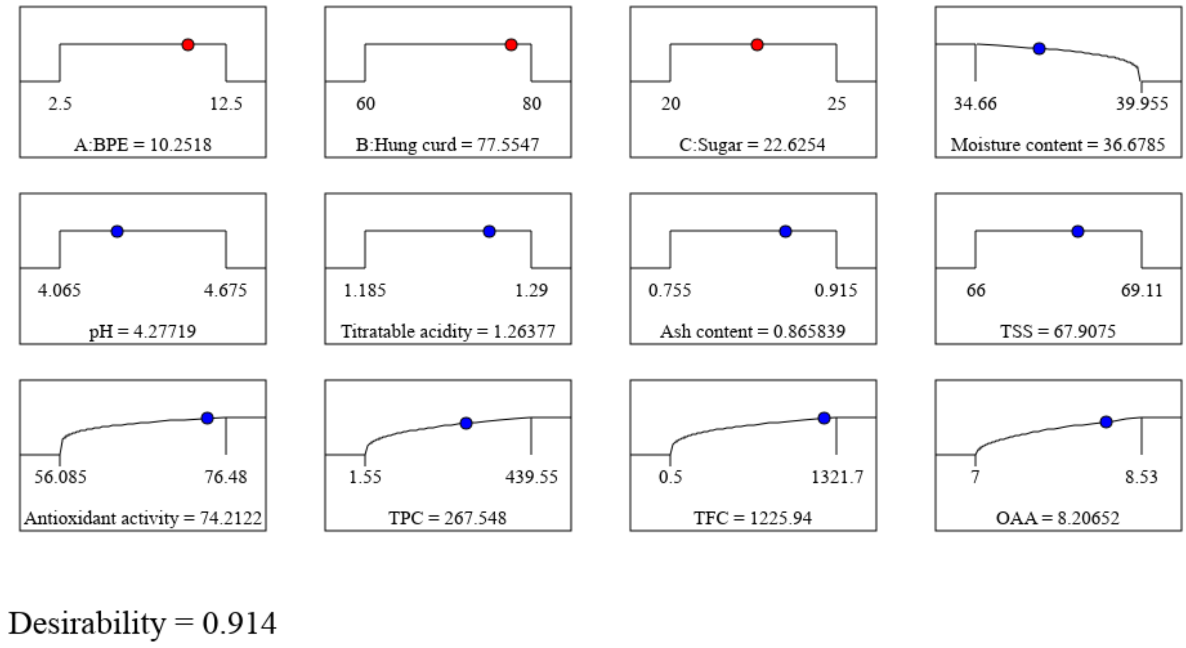


Figure 6.25. Optimum variable levels for MSBPE-based Shrikhand

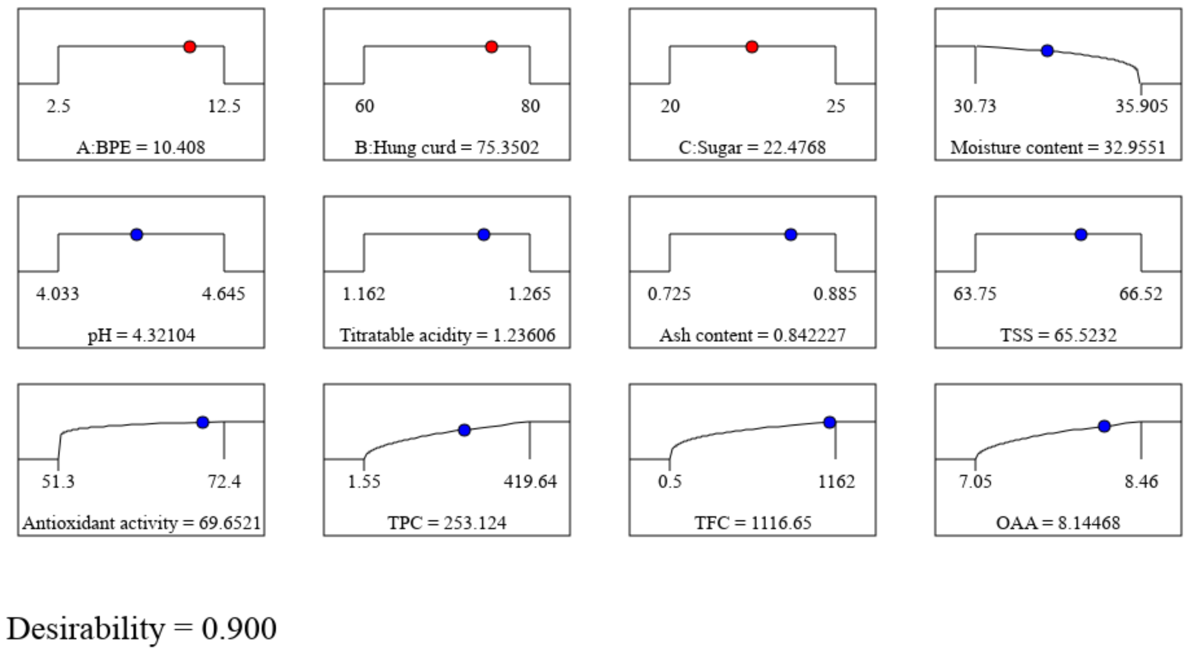


Figure 6.26. Optimum variable levels for MFBPE-based Shrikhand

Table 6.15. Predicted and observed optimum responses of Shrikhand incorporated with mustard bee pollen extract

Responses	Predicted values	Observed values
Moisture content (%)	36.679	36.63
pH	4.277	4.36
Titratable acidity (%)	1.264	1.25
Ash content (%)	0.866	0.86
TSS (°Brix)	67.907	67.65
Antioxidant activity (%)	74.212	73.01
TPC (mg GAE/100 g)	267.548	256.39
TFC (mg QE/100 g)	1225.942	1133.32
Overall acceptability	8.207	8.15

Table 6.16. Predicted and observed optimum responses of Shrikhand incorporated with multifloral bee pollen extract

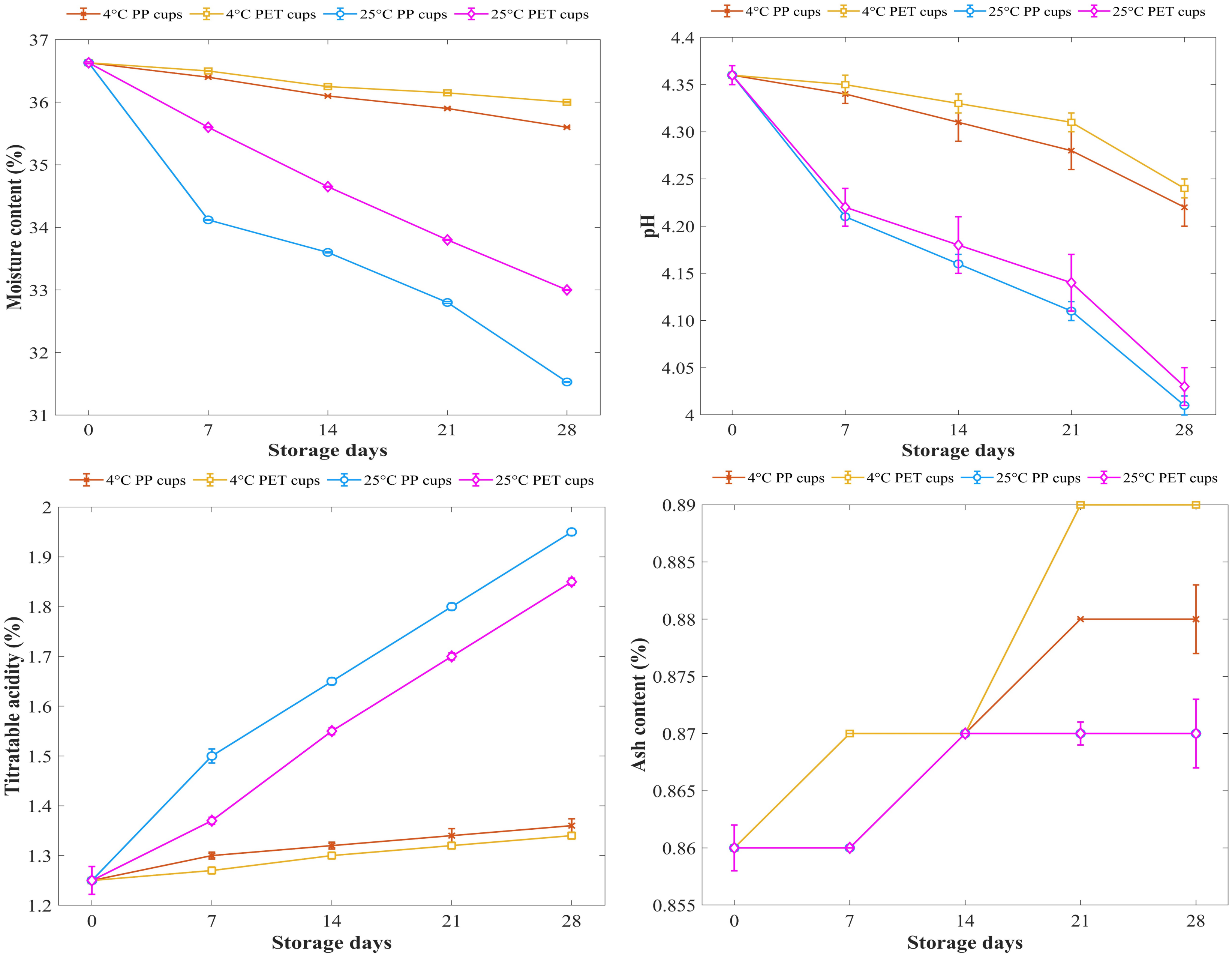
Responses	Predicted values	Observed values
Moisture content (%)	32.955	33.02
pH	4.321	4.30
Titratable acidity (%)	1.236	1.22
Ash content (%)	0.842	0.82
TSS (°Brix)	65.523	65.94
Antioxidant activity (%)	69.652	68.95
TPC (mg GAE/100 g)	253.124	248.56
TFC (mg QE/100 g)	1116.647	1096.32
Overall acceptability	8.145	8.05

6.18. Effect of storage on the physicochemical and phytochemical parameters of MSBPE and MFBPE-based Shrikhand

The shelf-life study of mustard bee pollen extract-based Shrikhand and multifloral bee pollen extract-based Shrikhand was conducted over a period of 28 days at 4 °C and 25 °C in two different packaging materials: polypropylene (PP) cups and polyethylene terephthalate (PET) cups. Table 6.17, Figures 6.27 and 6.28 presents the effect of storage conditions on the physicochemical (moisture content, pH, titratable acidity, ash content, TSS) and phytochemical parameters of the developed food product as recorded on 0^{th}, 7^{th}, 14^{th}, 21^{st} and 28^{th} day.

Moisture content is a key parameter influencing the storage stability and shelf life of a food product. It was observed that the moisture content of MSBPE-based Shrikhand varied from 36.63 ± 0.014% to 35.60 ± 0.007% in PP cups at 4 °C, 36.63 ± 0.014% to 36.00 ± 0.007% in PET cups at 4 °C, which showed that this parameter remained relatively stable at low temperature. Whereas, at 25 °C, a more pronounced reduction in moisture content ($p<0.05$) was noted, particularly in PP cups, where the values dropped from 36.63 ± 0.014% to 31.53 ± 0.007%, suggesting that higher temperature led to greater water loss. Similar changes in moisture content were observed in MFBPE-based Shrikhand, where the values dropped from 33.02 ± 0.014% to 29.98 ± 0.007% at 25 °C in PP cups. Singh and Rashmi (2024) also observed a similar trend of reduction in moisture content values (39.74 to 38.68%) in soy-fortified shrikhand with increased storage period. In another study, Venkatesh *et al.* (2018) stated a significant decrease in moisture content (41.53 to 37.69%) in microwave-treated enriched Shrikhand stored at 30 °C.

The pH of samples was the highest when recorded on 0^{th} day in both MSBPE and MFBPE-based Shrikhand. The values reduced remarkably ($p<0.05$) with a rise in the storage period (28^{th} day). It was also observed that the change in pH values was gradual at low temperature, whereas, the values dropped more substantially at 25 °C in PP cups, reaching 4.01 ± 0.01 in both MSBPE and MFBPE-based Shrikhand. The changes in pH might be attributed to the increase in microbial activity with extended period of storage. Yadav *et al.* (2021) experienced a comparable trend of decline in pH values of market-based and laboratory prepared Shrikhand samples. The decline in values was possibly due to the microbial activity and fermentation in hung curd during storage (Kumar *et al.*, 2019; Yadav *et al.*, 2021; Ajmi *et al.*, 2022; Singh and Rashmi, 2024).


4°C PP cups
4°C PET cups
25°C PP cups
25°C PET cups
Moisture content (%)
Storage days
pH
Storage days
Titratable acidity (%)
Storage days
Ash content (%)
Storage days

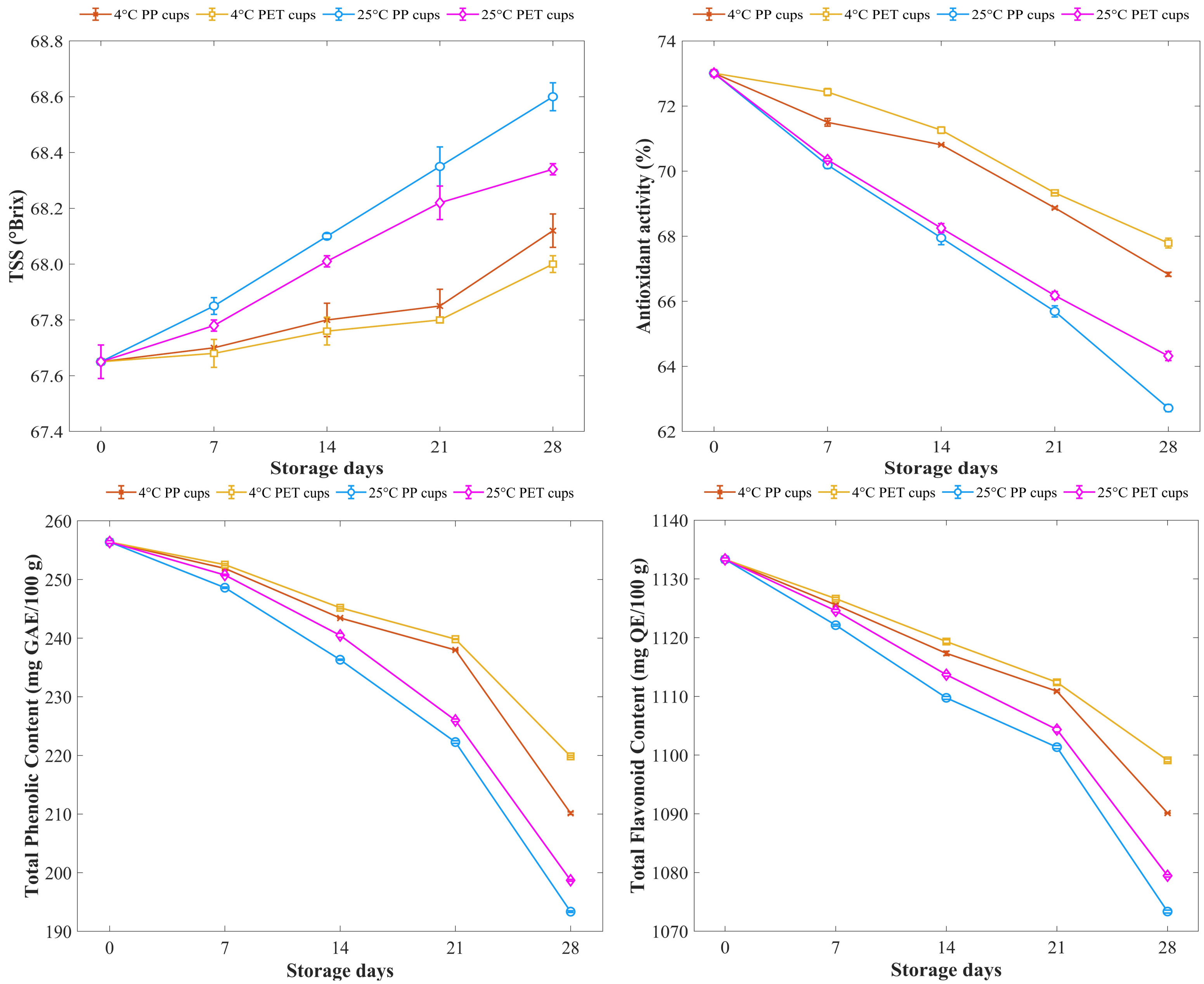
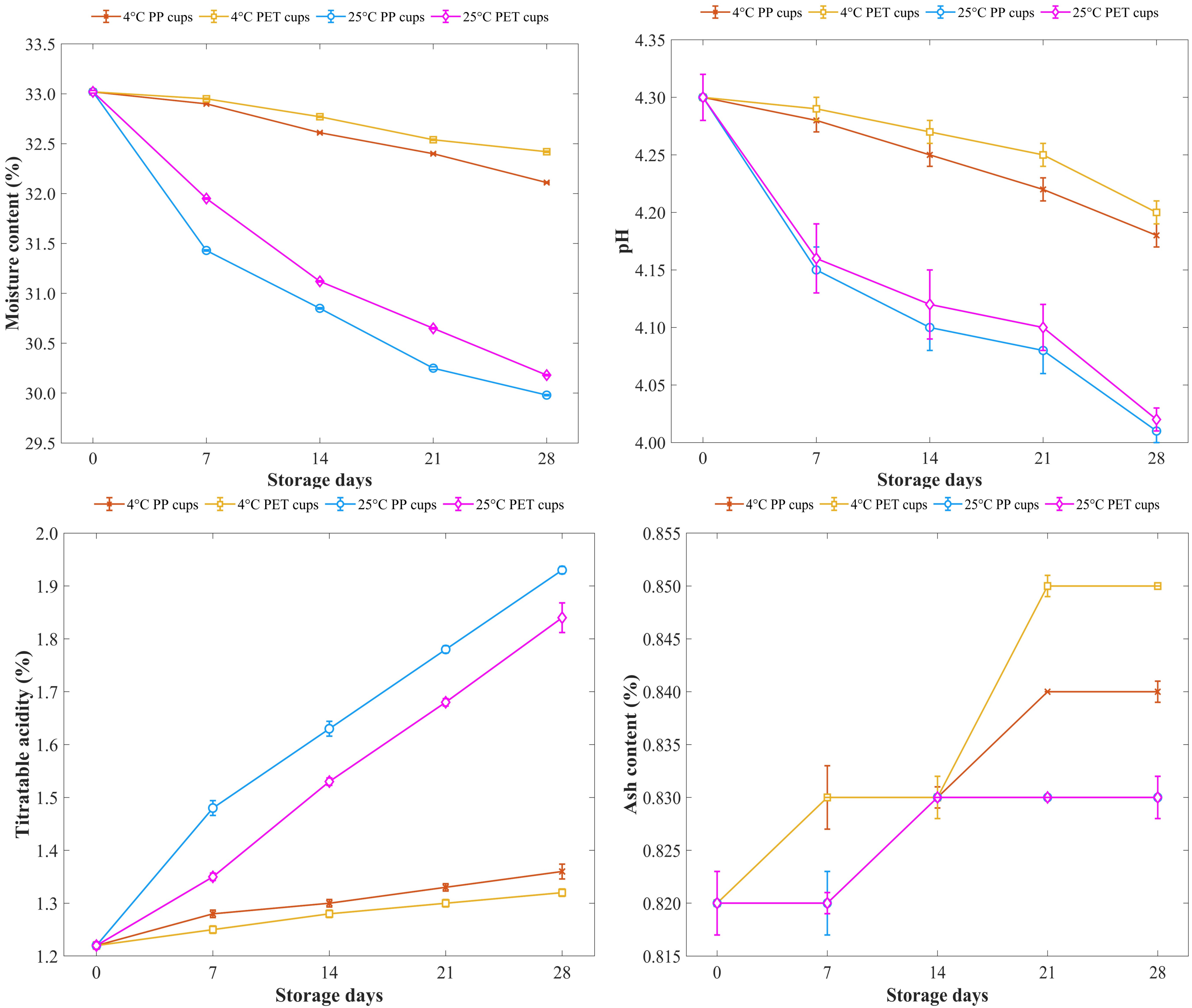


Figure 6.27. Effect of storage on various parameters of MSBPE-based Shrikhand


4°C PP cups
4°C PET cups
25°C PP cups
25°C PET cups
Moisture content (%)
Storage days
pH
Titratable acidity (%)
Ash content (%)
0
7
14
21
28

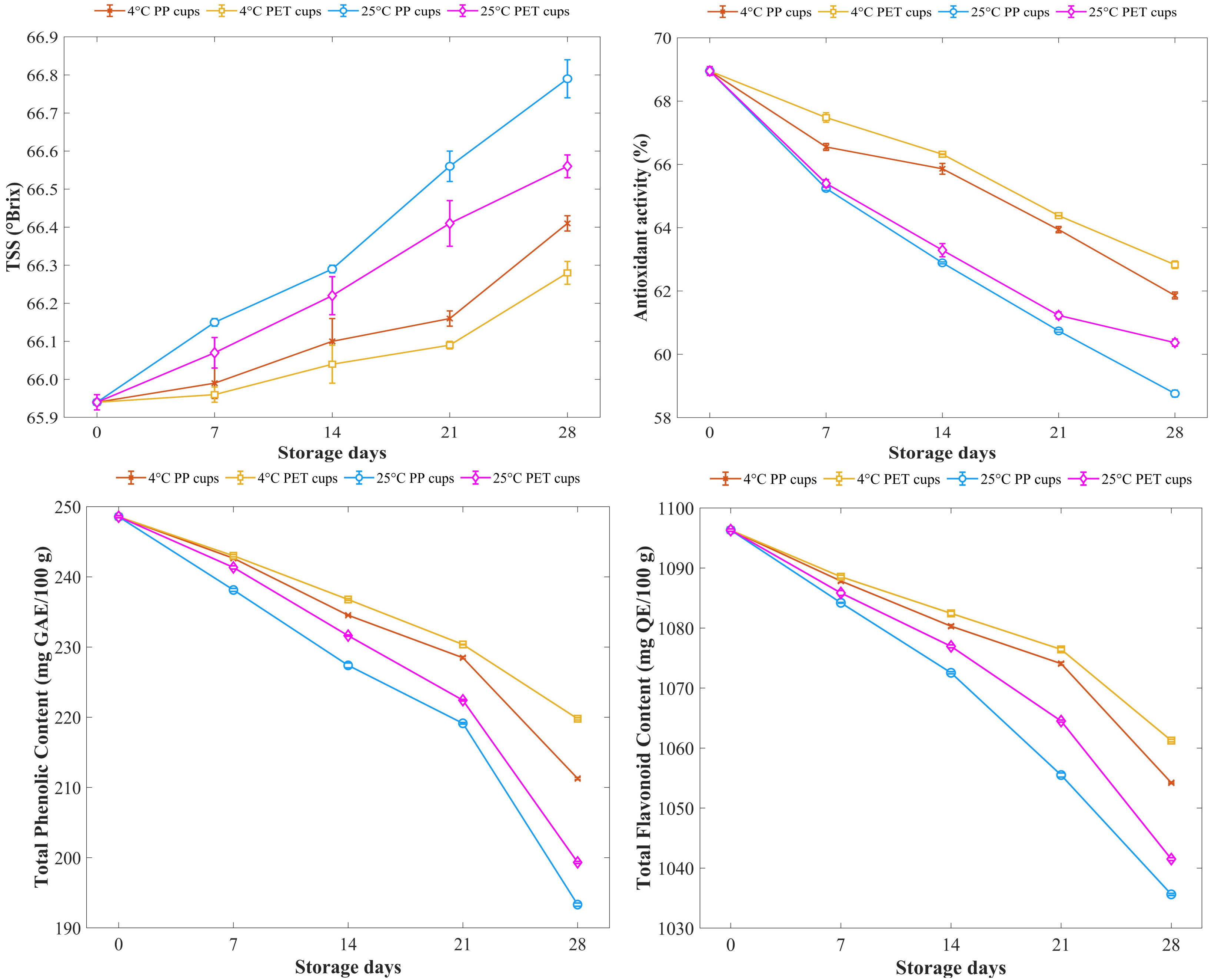


Figure 6.28. Effect of storage on various parameters of MFBPE-based Shrikhand

Table 6.17. Effect of storage on physicochemical and phytochemical parameters of MSBPE-based Shrikhand

Storage days	Temp of storage	Packaging material	Moisture (%)	pH	Titratable acidity (%)	Ash content (%)	TSS (°Brix)	Antioxidant activity (%)	TPC (mg GAE/100 g)	TFC (mg QE/100 g)
0th day	4 °C	PP	36.63 ± 0.014[a]	4.36 ± 0.01[a]	1.25 ± 0.028[k]	0.86 ± 0.002[d]	67.65 ± 0.06[m]	73.01 ± 0.11[a]	256.39 ± 0.23[a]	1133.32 ± 0.25[a]
		PET	36.63 ± 0.014[a]	4.36 ± 0.01[a]	1.25 ± 0.028[k]	0.86 ± 0.002[d]	67.65 ± 0.06[m]	73.01 ± 0.11[a]	256.39 ± 0.23[a]	1133.32 ± 0.25[a]
	25 °C	PP	36.63 ± 0.014[a]	4.36 ± 0.01[a]	1.25 ± 0.028[k]	0.86 ± 0.002[d]	67.65 ± 0.06[m]	73.01 ± 0.11[a]	256.39 ± 0.23[a]	1133.32 ± 0.25[a]
		PET	36.63 ± 0.014[a]	4.36 ± 0.01[a]	1.25 ± 0.028[k]	0.86 ± 0.002[d]	67.65 ± 0.06[m]	73.01 ± 0.11[a]	256.39 ± 0.23[a]	1133.32 ± 0.25[a]
7th day	4 °C	PP	36.40 ± 0.007[c]	4.34 ± 0.01[c]	1.30 ± 0.007[ij]	0.87 ± 0.000[c]	67.70 ± 0.03[k]	71.50 ± 0.12[c]	251.90 ± 0.17[c]	1125.58 ± 0.17[c]
		PET	36.50 ± 0.007[b]	4.35 ± 0.01[b]	1.27 ± 0.007[j]	0.87 ± 0.000[c]	67.68 ± 0.05[l]	72.43 ± 0.11[b]	252.53 ± 0.16[b]	1126.65 ± 0.21[b]
	25 °C	PP	34.12 ± 0.007k	4.21 ± 0.01[h]	1.50 ± 0.014[g]	0.86 ± 0.000[d]	67.85 ± 0.03[g]	70.19 ± 0.11[g]	248.60 ± 0.11[e]	1122.12 ± 0.15[e]
		PET	35.60 ± 0.007i	4.22 ± 0.02[h]	1.37 ± 0.007[h]	0.86 ± 0.000[d]	67.78 ± 0.02[i]	70.35 ± 0.04[f]	250.77 ± 0.12[d]	1124.58 ± 0.25[d]
14th day	4 °C	PP	36.10 ± 0.007f	4.31 ± 0.02[e]	1.32 ± 0.007[i]	0.87 ± 0.000[c]	67.80 ± 0.16[h]	70.81 ± 0.01[e]	243.43 ± 0.04[g]	1117.33 ± 0.35[g]
		PET	36.25 ± 0.007[d]	4.33 ± 0.01[d]	1.30 ± 0.007[ij]	0.87 ± 0.000[c]	67.76 ± 0.07[j]	71.26 ± 0.10[d]	245.17 ± 0.17[f]	1119.34 ± 0.31[f]

	25 °C	PP	33.60 ± 0.007^{m}	4.16 ± 0.01^{j}	1.65 ± 0.007^{e}	0.87 ± 0.000^{c}	68.10 ± 0.01^{e}	67.95 ± 0.21^{k}	236.32 ± 0.10^{k}	1109.75 ± 0.25^{k}
		PET	34.65 ± 0.007^{j}	4.18 ± 0.03^{i}	1.55 ± 0.007^{f}	0.87 ± 0.000^{c}	68.01 ± 0.02^{f}	68.25 ± 0.14^{j}	240.48 ± 0.35^{h}	1113.69 ± 0.25^{h}
21st day	4 °C	PP	35.90 ± 0.007^{h}	4.28 ± 0.02^{f}	1.34 ± 0.014^{hi}	0.88 ± 0.000^{b}	67.85 ± 0.07^{g}	68.87 ± 0.03^{i}	237.98 ± 0.12^{j}	1110.88 ± 0.18^{j}
		PET	36.15 ± 0.007^{e}	4.31 ± 0.01^{e}	1.32 ± 0.007^{ij}	0.89 ± 0.000^{a}	67.80 ± 0.01^{h}	69.33 ± 0.01^{h}	239.83 ± 0.07^{i}	1112.40 ± 0.35^{i}
	25 °C	PP	32.80 ± 0.014^{o}	4.11 ± 0.01^{l}	1.80 ± 0.007^{c}	0.87 ± 0.001^{c}	68.35 ± 0.23^{b}	65.69 ± 0.17^{o}	222.28 ± 0.23^{m}	1101.35 ± 0.26^{m}
		PET	33.80 ± 0.007^{l}	4.14 ± 0.03^{k}	1.70 ± 0.007^{d}	0.87 ± 0.001^{c}	68.22 ± 0.17^{c}	66.18 ± 0.12^{n}	225.98 ± 0.23^{l}	1104.35 ± 0.42^{l}
28th day	4 °C	PP	35.60 ± 0.007^{i}	4.22 ± 0.02^{h}	1.36 ± 0.014^{h}	0.88 ± 0.003^{b}	68.12 ± 0.06^{d}	66.83 ± 0.06^{m}	200.15 ± 0.21^{o}	1080.12 ± 0.06^{o}
		PET	36.00 ± 0.007^{g}	4.24 ± 0.01^{g}	1.34 ± 0.007^{hi}	0.89 ± 0.000^{a}	68.00 ± 0.03^{f}	67.79 ± 0.15^{l}	204.85 ± 0.17^{n}	1084.10 ± 0.24^{n}
	25 °C	PP	31.53 ± 0.007^{p}	4.01 ± 0.01^{n}	1.95 ± 0.007^{a}	0.87 ± 0.003^{c}	68.60 ± 0.05^{a}	62.72 ± 0.11^{q}	193.36 ± 0.14^{q}	1073.36 ± 0.25^{q}
		PET	33.00 ± 0.007^{n}	4.03 ± 0.02^{m}	1.85 ± 0.007^{b}	0.87 ± 0.003^{c}	68.34 ± 0.02^{b}	64.32 ± 0.14^{p}	198.71 ± 0.15^{p}	1079.46 ± 0.21^{p}

Table 6.18. Effect of storage on physicochemical and phytochemical parameters of MFBPE-based Shrikhand

Storage days	Temp of storage	Packaging material	Moisture (%)	pH	Titratable acidity (%)	Ash content (%)	TSS (°Brix)	Antioxidant activity (%)	TPC (mg GAE/100 g)	TFC (mg QE/100 g)
0th day	4 °C	PP	33.02 ± 0.014^{a}	4.30 ± 0.02^{a}	1.22 ± 0.007^{k}	0.82 ± 0.003^{d}	65.94 ± 0.02^{m}	68.95 ± 0.14^{a}	248.56 ± 0.15^{a}	1096.32 ± 0.21^{a}
		PET	33.02 ± 0.014^{a}	4.30 ± 0.02^{a}	1.22 ± 0.007^{k}	0.82 ± 0.003^{d}	65.94 ± 0.02^{m}	68.95 ± 0.14^{a}	248.56 ± 0.15^{a}	1096.32 ± 0.21^{a}
	25 °C	PP	33.02 ± 0.014^{a}	4.30 ± 0.02^{a}	1.22 ± 0.007^{k}	0.82 ± 0.003^{d}	65.94 ± 0.02^{m}	68.95 ± 0.14^{a}	248.56 ± 0.15^{a}	1096.32 ± 0.21^{a}
		PET	33.02 ± 0.014^{a}	4.30 ± 0.02^{a}	1.22 ± 0.007^{k}	0.82 ± 0.003^{d}	65.94 ± 0.02^{m}	68.95 ± 0.14^{a}	248.56 ± 0.15^{a}	1096.32 ± 0.21^{a}
7th day	4 °C	PP	32.90 ± 0.007^{c}	4.28 ± 0.01^{c}	1.28 ± 0.007^{ij}	0.83 ± 0.003^{c}	65.99 ± 0.05^{k}	66.55 ± 0.11^{c}	242.65 ± 0.14^{c}	1087.85 ± 0.25^{c}
		PET	32.95 ± 0.007^{b}	4.29 ± 0.01^{b}	1.25 ± 0.007^{j}	0.83 ± 0.000^{c}	65.96 ± 0.03^{l}	67.48 ± 0.15^{b}	243.01 ± 0.17^{b}	1088.56 ± 0.24^{b}
	25 °C	PP	31.43 ± 0.007^{k}	4.15 ± 0.02^{h}	1.48 ± 0.014^{g}	0.82 ± 0.003^{d}	66.15 ± 0.01^{g}	65.24 ± 0.06^{g}	238.13 ± 0.21^{e}	1084.21 ± 0.06^{e}
		PET	31.95 ± 0.007^{i}	4.16 ± 0.03^{h}	1.35 ± 0.007^{h}	0.82 ± 0.001^{d}	66.07 ± 0.06^{i}	65.40 ± 0.12^{f}	241.34 ± 0.23^{d}	1085.85 ± 0.42^{d}
14th day	4 °C	PP	32.61 ± 0.007^{f}	4.25 ± 0.01^{e}	1.30 ± 0.007^{i}	0.83 ± 0.001^{c}	66.10 ± 0.17^{h}	65.86 ± 0.17^{e}	234.53 ± 0.07^{g}	1080.30 ± 0.26^{g}

		PET	32.77 ± 0.007^{d}	4.27 ± 0.01^{d}	1.28 ± 0.007^{ij}	0.83 ± 0.002^{c}	66.04 ± 0.22^{j}	66.32 ± 0.01^{d}	236.77 ± 0.12^{f}	1082.45 ± 0.35^{f}
	25 °C	PP	31.02 ± 0.007^{m}	4.10 ± 0.02^{j}	1.63 ± 0.014^{e}	0.83 ± 0.000^{c}	66.29 ± 0.01^{e}	62.89 ± 0.03^{k}	227.39 ± 0.35^{k}	1072.55 ± 0.18^{k}
		PET	31.12 ± 0.007^{j}	4.12 ± 0.03^{i}	1.53 ± 0.007^{f}	0.83 ± 0.000^{c}	66.22 ± 0.07^{f}	63.29 ± 0.21^{j}	231.63 ± 0.10^{h}	1076.96 ± 0.25^{h}
21st day	4 °C	PP	32.4 ± 0.007^{h}	4.22 ± 0.01^{f}	1.33 ± 0.007^{hi}	0.84 ± 0.000^{b}	66.16 ± 0.02^{g}	63.94 ± 0.10^{i}	228.48 ± 0.17^{j}	1074.08 ± 0.25^{j}
		PET	32.54 ± 0.007^{e}	4.25 ± 0.01^{e}	1.30 ± 0.007^{ij}	0.85 ± 0.001^{a}	66.09 ± 0.01^{h}	64.38 ± 0.01^{h}	230.36 ± 0.04^{i}	1076.45 ± 0.31^{i}
	25 °C	PP	30.25 ± 0.014^{o}	4.08 ± 0.02^{l}	1.78 ± 0.007^{c}	0.83 ± 0.000^{c}	66.56 ± 0.07^{b}	60.74 ± 0.04^{o}	219.14 ± 0.12^{m}	1063.51 ± 0.35^{m}
		PET	30.65 ± 0.007^{l}	4.10 ± 0.02^{k}	1.68 ± 0.007^{d}	0.83 ± 0.000^{c}	66.41 ± 0.16^{c}	61.23 ± 0.11^{n}	222.43 ± 0.11^{l}	1066.50 ± 0.25^{l}
28th day	4 °C	PP	32.11 ± 0.007^{i}	4.18 ± 0.01^{h}	1.36 ± 0.014^{h}	0.84 ± 0.001^{b}	66.41 ± 0.02^{d}	61.86 ± 0.11^{m}	201.27 ± 0.16^{o}	1042.22 ± 0.15^{o}
		PET	32.42 ± 0.007^{g}	4.20 ± 0.01^{g}	1.32 ± 0.007^{hi}	0.85 ± 0.000^{a}	66.28 ± 0.03^{f}	62.83 ± 0.12^{l}	205.78 ± 0.17^{n}	1046.25 ± 0.21^{n}
	25 °C	PP	29.98 ± 0.007^{p}	4.01 ± 0.01^{n}	1.93 ± 0.007^{a}	0.83 ± 0.002^{c}	66.79 ± 0.05^{a}	58.76 ± 0.11^{q}	193.32 ± 0.23^{q}	1035.63 ± 0.17^{q}
		PET	30.18 ± 0.007^{n}	4.02 ± 0.01^{m}	1.84 ± 0.028^{b}	0.83 ± 0.002^{c}	66.56 ± 0.03^{b}	60.37 ± 0.11^{p}	199.33 ± 0.15^{p}	1041.50 ± 0.25^{p}

Table 6.19. Effect of storage on sensory attributes of MSBPE-based Shrikhand

Storage days	Temperature of storage	Packaging material	Appearance	Flavour	Texture	Mouthfeel	Overall acceptability
0th day	4 °C	PP	8.32 ± 0.03ᵃ	8.20 ± 0.00ᵃ	8.30 ± 0.02ᵃ	8.20 ± 0.01ᵃ	8.15 ± 0.00ᵃ
		PET	8.32 ± 0.03ᵃ	8.20 ± 0.00ᵃ	8.30 ± 0.02ᵃ	8.20 ± 0.01ᵃ	8.15 ± 0.00ᵃ
7th day	4 °C	PP	8.20 ± 0.01ᵇ	7.90 ± 0.01ᶜ	7.81 ± 0.01ᶜ	7.80 ± 0.01ᶜ	7.81 ± 0.01ᶜ
		PET	8.30 ± 0.00ᵃ	8.11 ± 0.01ᵇ	8.09 ± 0.01ᵇ	8.11 ± 0.01ᵇ	8.05 ± 0.01ᵇ
14th day	4 °C	PP	7.80 ± 0.01ᵈ	7.51 ± 0.01ᵈ	7.51 ± 0.01ᵉ	7.60 ± 0.01ᵉ	7.50 ± 0.01ᵉ
		PET	8.00 ± 0.00ᶜ	7.91 ± 0.01ᶜ	7.71 ± 0.01ᵈ	7.71 ± 0.02ᵈ	7.70 ± 0.01ᵈ
21st day	4 °C	PP	7.50 ± 0.01ᵉ	6.60 ± 0.01ᶠ	6.50 ± 0.01ᵍ	6.49 ± 0.01ᵍ	6.61 ± 0.01ᵍ
		PET	7.81 ± 0.01ᵈ	7.11 ± 0.01ᵉ	7.30 ± 0.02ᶠ	7.11 ± 0.01ᶠ	7.10 ± 0.01ᶠ
28th day	4 °C	PP	NA	NA	NA	NA	NA
		PET	7.45 ± 0.01ᶠ	6.80 ± 0.01ᵍ	6.80 ± 0.01ʰ	6.85 ± 0.01ʰ	6.85 ± 0.01ʰ

MSBPE – mustard bee pollen extract

Table 6.20. Effect of storage on sensory attributes of MFBPE-based Shrikhand

Storage days	Temperature of storage	Packaging material	Appearance	Flavour	Texture	Mouthfeel	Overall acceptability
0th day	4 °C	PP	8.11 ± 0.01[a]	8.05 ± 0.07[a]	8.11 ± 0.01[a]	8.03 ± 0.04[a]	8.05 ± 0.00[a]
		PET	8.11 ± 0.01[a]	8.05 ± 0.07[a]	8.11 ± 0.01[a]	8.03 ± 0.04[a]	8.05 ± 0.00[a]
7th day	4 °C	PP	7.81 ± 0.01[c]	7.51 ± 0.01[c]	7.81 ± 0.01[c]	7.51 ± 0.01[c]	7.50 ± 0.01[c]
		PET	8.00 ± 0.00[b]	7.82 ± 0.02[b]	8.03 ± 0.04[b]	7.81 ± 0.01[b]	7.81 ± 0.01[b]
14th day	4 °C	PP	7.61 ± 0.01[d]	7.11 ± 0.01[d]	7.19 ± 0.01[e]	7.11 ± 0.01[e]	7.11 ± 0.01[e]
		PET	7.81 ± 0.01[c]	7.53 ± 0.04[c]	7.71 ± 0.01[d]	7.71 ± 0.01[c]	7.67 ± 0.02[c]
21st day	4 °C	PP	7.00 ± 0.00[f]	6.03 ± 0.04[f]	6.51 ± 0.01[g]	6.03 ± 0.04[g]	6.47 ± 0.02[g]
		PET	7.50 ± 0.00[e]	6.51 ± 0.01[e]	7.03 ± 0.04[f]	6.51 ± 0.01[f]	6.91 ± 0.01[f]
28th day	4 °C	PP	NA	NA	NA	NA	NA
		PET	7.45 ± 0.00[g]	6.43 ± 0.04[g]	6.90 ± 0.01[h]	6.45 ± 0.04[h]	6.70 ± 0.02[h]

MFBPE – multifloral bee pollen extract

The data pertaining to titratable acidity of MSBPE-based Shrikhand suggested that at 4 °C, the values showed a gradual increase over time, reaching 1.36 ± 0.014% and 1.34 ± 0.007% on 28th day in PP and PET cups, respectively. The same trend was observed in MFBPE-based Shrikhand as well. The increase in this parameter was more pronounced at 25 °C, particularly in PP cups. It was observed that the rise in titratable acidity values was consistent with the recorded pH decrease at higher temperatures. The rise in values could be linked to microbial activity leading to spoilage of milk-based food products like Shrikhand. Additionally, the conversion of lactose to lactic acid may account for the heightened acidity observed in the samples (Kumar *et al.*, 2019; Yadav *et al.*, 2021). Similar findings have been described by Singh and Rashmi (2024), Ajmi *et al.* (2022) and Kumar *et al.* (2011) in Shrikhand, as well as Tarun (2014) in barfi. The Arrhenius plot for titratable acidity in the MSBPE- and MFBPE-based shrikhand stored in PP and PET cups is displayed in Figures 6.29 and 6.30.

The values of ash content increased slightly with an increase in storage period but the differences in those values were highly non-significant (p>0.05). The values varied between 0.86 to 0.89% in MSBPE and 0.82-0.85% in MFBPE-based Shrikhand. Thus, minimal variations were observed regardless of packaging material and storage temperature. The results of our study align with the findings of Kumar *et al.* (2019) as well as Singh and Rashmi (2024), who also reported that no significant changes were observed in ash content values over prolonged storage periods.

TSS values were observed to remain stable in MSBPE-based Shrikhand at 4 °C with minor variations from 67.65 ± 0.06 to 68.12 ± 0.06 °Brix in PP cups and 67.65 ± 0.06 to 68.00 ± 0.03 °Brix in PET cups from 0th to 28th day. Similar trend was observed for the multifloral variety as well. Whereas, at 25 °C, the increase in TSS values was slightly significant by 28th day, which indicated a possible concentration due to moisture loss resulting at higher temperature. The results of our analysis were found to be comparable with the results of Kumar *et al.* (2011), Kumar *et al.* (2019), Ajmi *et al.* (2022) and, Singh and Rashmi (2024).

At 4 °C, the antioxidant activity of MSBPE-based Shrikhand showed a decline from 73.01 ± 0.11 to 66.83 ± 0.06% in PP cups and 67.79 ± 0.15% in PET cups from 0th to 28th day. Similarly, in MFBPE-based Shrikhand the values dropped from 68.95 ±

0.14% to 61.86 ± 0.11% in PP cups and 62.83 ± 0.12% in PET cups. At 25 °C, a more substantial decline ($p<0.05$) in antioxidant activity was noted, especially in PP cups, where it decreased to 62.72 ± 0.11% and 58.76 ± 0.11% in mustard and multifloral incorporated product, respectively. This trend suggested possible degradation of antioxidant compounds at higher temperatures. Our observations were in contrast with the ones stated by Kumar *et al.* (2019), who observed that the antioxidant activity of shrikhand increased from 0^{th} to 42^{nd} day in an insignificant manner. But the findings of our study were comparable to the results of Pugazhenthi *et al.* (2020) as well as Sarkar and Ghosh (2020), who observed a decline in the antioxidant activity with prolonged storage period. This loss was attributable to the degradation of unstable compounds as a result of chemical reactions with due course of time (Pugazhenthi *et al.*, 2020).

The total phenolic content of MSBPE and MFBPE-based Shrikhand was initially recorded as 256.39 ± 0.23 and 248.56 ± 0.15 mg GAE/100 g. It was observed that the values gradually declined to 200.15 ± 0.21 in mustard-based and 201.27 ± 0.16 in multifloral-based Shrikhand stored in PP cups at 4 °C by the end of 28 days. A more marked decrease ($p<0.05$) was observed at 25 °C, indicating that higher temperature led to the degradation of phenolic compounds. Results obtained were in accordance with the findings of Pugazhenthi *et al.* (2020). This decrease was probably caused by various chemical and enzymatic reactions that led to the breakdown of unstable compounds (Pugazhenthi *et al.*, 2020).

The initial value of TFC of MSBPE and MFBPE-based Shrikhand was 1133.32 ± 0.25 mg QE/100 g and 1096.32 ± 0.21 mg QE/100 g, respectively. A trend similar to the antioxidant activity and TPC was also observed for TFC, where a substantial decrease in the values ($p<0.05$) was noted by the 28^{th} day at 25 °C, which suggested that flavonoids are better preserved at lower temperatures (4 °C). The flavonoid content decreased in parallel with the loss of antioxidant activity and the degradation of phenolic compounds.

Given these findings, it was inferred that most parameters showed significant variations at higher temperature of 25 °C over the 28-day storage period. Moreover, results also indicated that PET cups preserved the moisture content, antioxidant activity, TFC, and TPC in a better way by providing protection against environmental factors as compared to PP cups. The better performance of PET cups compared to PP cups may be attributed to their lower oxygen and moisture permeability, which reduces oxidative degradation and microbial growth during storage. PET's higher barrier properties also help preserve antioxidant compounds and maintain product quality over time (Farrell *et al.*, 2024).

6.19. Effect of storage on sensory attributes of MSBPE-based Shrikhand

Table 6.18 illustrates the impact of storage on the sensory characteristics of Shrikhand incorporated with mustard bee pollen extract. The product was stored in two different packaging materials *viz.* PP cups and PET cups over a 28-day storage period. Sensory analysis was conducted using a 9-point hedonic scale and each sample was coded in order to conceal its identity.

The sensory evaluation of Shrikhand stored at room temperature was done on 0^{th} and 7^{th} day because the samples deteriorated at a faster rate and samples started tasting sour as the acidity was increased to 1.50, thereby making them unacceptable. Initially, the sensory scores were observed to be high with an overall acceptability of 8.15. This suggested that the freshly prepared Shrikhand sample had a pleasant flavour, desired texture and good mouthfeel. The sensory scores were observed to decline ($p<0.05$) slightly by the 7^{th} day with an overall acceptability score of 7.81 ± 0.01 in PP cups and 8.05 ± 0.01 in PET cups. A further decline in the sensory attributes was observed on 14^{th} day, with samples stored in PP cups scoring noticeably less than PET cups among all parameters ($p<0.05$). On 21^{st} day, a marked decrease ($p<0.05$) in the sensory attributes was observed with scores reaching 6.61 ± 0.01 in PP cups and 7.10 ± 0.01 in PET cups. By the 28^{th} day, the samples stored in PP cups were found to be unacceptable due to changes in taste, whereas, the samples stored in PET cups were still acceptable with an overall acceptability score of 6.85 ± 0.01. Although there was a substantial decrease in all sensory attributes of PET stored samples ($p<0.05$), but it still maintained an acceptable flavour and texture, indicating that PET cups might prove to be a better packaging material to preserve the product's quality for a prolonged storage period.

Table 6.21. MSBPE-based Shrikhand samples stored at 4 °C and 25 °C (28 days)

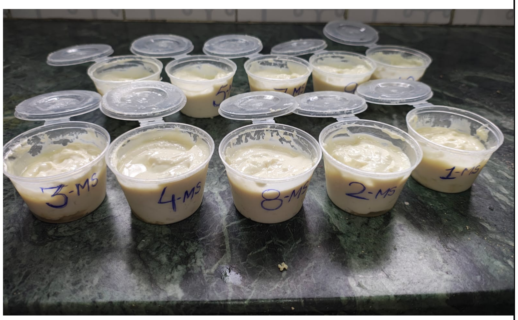
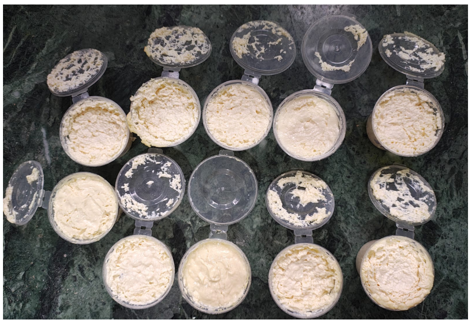
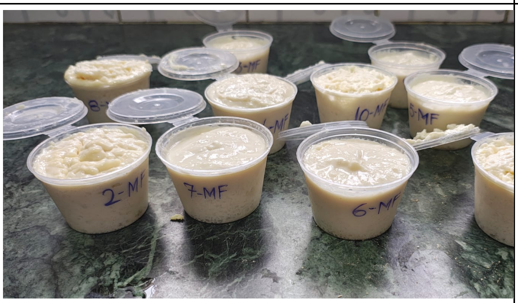
Storage days	4 °C	25 °C
Day 1		
Day 7		
Day 14		
Day 21		
Day 28		

Table 6.22. Photographic representation of Shrikhand samples containing multifloral bee pollen extract stored at 4 °C and 25 °C over a 28-day period (Day 1 to Day 28)

Storage days	4 °C	25 °C
Day 1		
Day 7		
Day 14		
Day 21		
Day 28		

6.20. Effect of storage on sensory attributes of MFBPE-based Shrikhand

For the samples stored at 4°C, similar sensory quality (8.05 ± 0.00) was observed across all the parameters, suggesting high acceptability of the product. A slight decline in the sensory scores was observed for samples stored in both packaging materials on 7th day. However, the scores of samples stored in PET cups were slightly higher ($p<0.05$) in terms of appearance, flavour, texture, mouthfeel and overall acceptability compared to PP cups. A more remarkable reduction in sensory attributes, particularly for flavour and mouthfeel, was noted on the 14th day. There was a substantial decline ($p<0.05$) in the sensory quality of Shrikhand stored in both packaging materials by the end of 21st day. Substantial reduction of flavour (6.03 ± 0.04) and mouthfeel (6.03 ± 0.04) scores was observed in samples stored in PP cups. Whereas, samples stored in PET cups maintained relatively better scores with overall acceptability of 6.91 ± 0.01. The sensory analysis was not performed on 28th day because samples stored in PP cups tasted sour and became unacceptable, while the ones stored in PET cups had acceptable scores (6.70 ± 0.02). Given the findings, it was inferred that PET cups were found to be more suitable for the storage of Shrikhand.

6.21. Microbiological changes in MSBPE- and MFBPE-based Shrikhand during storage

The microbial changes in MSBPE- and MFBPE-based shrikhand were observed on a regular basis throughout storage (28 days), and it was observed to increase dramatically with time. Table 6.23 presents the microbial analysis results, including total plate count, yeast and mold count, and coliform count. It was inferred from the microbial analysis of samples that the growth of micro-organisms was lower at 4 °C and higher at 25 °C, with variations between different packaging materials.

6.21.1. Total Plate Count

The total plate count indicates the total bacterial growth in the samples over time. With extended storage and higher temperatures, the values were observed to increase, particularly at 25 °C. The total plate count of MSBPE- and MFBPE-based shrikhand stored at 25 °C exhibited higher values in comparison to those stored at 4 °C. The highest count was observed on 28th day with values equal to 3.56 ± 0.72 and 3.63 ± 0.24 (PP cups), and 3.48 ± 0.28 and 3.55 ± 0.35 (PET cups) in MSBPE- and MFBPE-based shrikhand respectively. The results obtained in our study aligned with the findings of Srinivas *et al.* (2018), who stated that the total plate count values increased in both the control and value-added shrikhand by 18% on 7th, 25% on 15th and 49% on 21st day. They also stated that the storage stability of value-added shrikhand (in terms of microbial activity) is longer than 15 days. Similar results were also reported by Ajmi *et al.* (2022), who observed that the values increased from 3.24 to 4.32 log10 cfu/g in calcium and vitamin D fortified shrikhand over 28 days of storage.

6.21.2. Yeast and mold Count

The yeast and mold count helps to assess the growth of yeast and mold throughout storage. It was observed that the values increased over time and the growth was more noticeable at 25 °C than at 4 °C. Additionally, the count was higher in samples stored in PP cups than in PET cups, with the highest count recorded on the 28th day at 25 °C (5.25 ± 0.03 and 5.32 ± 0.12 in MSBPE- and MFBPE-based shrikhand, respectively). Our findings were in agreement with the results of Srinivas *et al.* (2018). They observed that the yeast and molds were absent initially but growth was detected on 7th day which gradually rose as the storage period was extended, reaching 9.25×10^1/gram by the end of 21st day. The increase in the values was significant and rate of growth was even faster at high temperature. Based on the prescribed limits proposed by Prevention of Food Adulteration Act (2009), the values of yeast and mould count for shrikhand shouldn't exceed 50,000/g. The growth is favoured by factors including enough moisture and increased acidity of samples during storage. In another research study by Kumar *et al.* (2019), it was revealed that there was a substantial increase ($p<0.05$) in yeast and mold count in shrikhand samples over the storage period of 42 days at 5–7 °C, with values varying between 3.50 to 9.00. Comparable results were also stated by Ajmi *et al.* (2022), who observed that the values increased from 0.95 to 1.67 log10 cfu/g in calcium and vitamin D fortified shrikhand over 28 days of storage.

6.21.3. Coliform count

The data demonstrated that coliforms were not detected in the samples across different storage days (0^{th}, 7^{th}, 14^{th}, 21^{st}, and 28^{th} day). This indicated that the samples were free from coliform bacteria contamination in both MSBPE- and MFBPE-based shrikhand under all the conditions tested. Our results align with the observation of Srinivas *et al.* (2018) as they didn't detect the presence of coliform bacteria in their samples up to two weeks of refrigeration. Thereafter, coliform was detected within the permitted limits. Kumar *et al.* (2019) reported comparable findings. Owing to the high acidity and low pH of fermented dairy products, they aren't suitable for the growth of coliform bacteria. Thus, the absence of this micro-organism confirmed that no source of faecal contamination was present in the shrikhand samples (Kumar *et al.*, 2019; Yadav *et al.*, 2024).

6.22. Predictive modelling by chemical kinetics

Quality parameters significantly impact shelf-life prediction modeling, largely due to the effects of storage time and temperature. The shelf-life of the product was predicted by developing verifiable mathematical equations combined with kinetic functions. Subsequently, these equations were incorporated into the Arrhenius relationship, which connected various storage temperatures (4 °C and 25 °C) to the rate constants (ln k) for different variables. The impact of storage on the content of free fatty acids (FFA), thiobarbituric acid (TBA), and hydroxymethylfurfural (HMF) in Shrikhand with mustard and multifloral bee pollen extract is illustrated in Table 6.24. Data were gathered during a 28-day storage period at both 4 °C and 25 °C, utilizing two distinct packaging materials: PP cups and PET cups.

6.22.1. Changes in free fatty acid (FFA) value of MSBPE- and MFBPE-based Shrikhand

Analysing the shelf-life of the product heavily relies on the determination of FFA and TBA levels. These levels provide insights into the extent of changes resulting due to lipolysis in shrikhand as fermented dairy products containing fat generally undergo lipolytic changes, which might lead to spoilage of the product (Yadav *et al.*, 2021).

In MSBPE-based shrikhand, the FFA values were observed to increase steadily throughout the storage period from 1.25 on 0th day to 1.36 (4 °C in PP cups), 1.29 (4 °C in PET cups), 1.56 (25 °C in PP cups), and 1.49 (25 °C in PET cups) on the 28th day. Similarly, in MFBPE-based shrikhand, the FFA values were observed to increase steadily throughout the storage period from 1.75 on 0th day to 1.87 (4 °C in PP cups), 1.81 (4 °C in PET cups), 2.15 (25 °C in PP cups), and 2.02 (25 °C in PET cups) on the 28th day (Table 6.24). A similar trend of increase in FFA content was observed by Yadav *et al.* (2021) examined how storage conditions influenced the FFA content in both market and laboratory-prepared shrikhand samples kept at a temperature of 5 °C. It was noticed that the values increased from 1.82 to 2.42 meq/g over a period of 20 days. The factors known to be responsible for this increase in values include high moisture content and release of free fat during product formulation (Yadav *et al.*, 2021). The findings of this study were consistent with those reported by Ajmi *et al.* (2022). Figures 6.30 and 6.31 illustrate the Arrhenius plots corresponding to the FFA values.

Table 6.23. Microbiological changes in MSBPE- and MFBPE-based Shrikhand during storage

Storage days	Temperature of storage	Packaging material	Total plate count (log 10 cfu/g)		Yeast and mold count (log 10 cfu/g)		Coliform count (cfu/g)	
			MSBPE-based shrikhand	MFBPE-based shrikhand	MSBPE-based shrikhand	MFBPE-based shrikhand	MSBPE-based shrikhand	MFBPE-based shrikhand
0th day	4 °C	PP	1.21 ± 0.01[o]	1.25 ± 0.01[o]	0.40 ± 0.00[o]	0.50 ± 0.02[o]	Nil	Nil
		PET	1.21 ± 0.01[o]	1.25 ± 0.01[o]	0.40 ± 0.00[o]	0.50 ± 0.02[o]	Nil	Nil
	25 °C	PP	1.21 ± 0.01[o]	1.25 ± 0.01[o]	0.40 ± 0.00[o]	0.50 ± 0.02[o]	Nil	Nil
		PET	1.21 ± 0.01[o]	1.25 ± 0.01[o]	0.40 ± 0.00[o]	0.50 ± 0.02[o]	Nil	Nil
7th day	4 °C	PP	1.38 ± 0.00[m]	1.45 ± 0.07[m]	1.15 ± 0.02[m]	1.22 ± 0.03[m]	Nil	Nil
		PET	1.28 ± 0.03[n]	1.35 ± 0.04[n]	1.05 ± 0.01[n]	1.12 ± 0.12[n]	Nil	Nil
	25 °C	PP	1.48 ± 0.00[k]	1.55 ± 0.25[k]	2.25 ± 0.01[k]	2.32 ± 0.17[k]	Nil	Nil
		PET	1.43 ± 0.01[l]	1.50 ± 0.12[l]	2.10 ± 0.01[l]	2.17 ± 0.11[l]	Nil	Nil
14th day	4 °C	PP	1.56 ± 0.04[j]	1.63 ± 0.22[j]	2.25 ± 0.02[k]	2.31 ± 0.32[k]	Nil	Nil
		PET	1.45 ± 0.08[l]	1.52 ± 0.37[l]	2.10 ± 0.02[l]	2.15 ± 0.21[l]	Nil	Nil
	25 °C	PP	2.78 ± 0.12[e]	2.85 ± 0.11[e]	3.56 ± 0.01[g]	3.63 ± 0.07[g]	Nil	Nil
		PET	2.63 ± 0.09[f]	2.70 ± 0.07[f]	3.12 ± 0.04[j]	3.19 ± 0.07[j]	Nil	Nil
21st day	4 °C	PP	1.78 ± 0.14[h]	1.85 ± 0.02[h]	3.50 ± 0.02[h]	3.57 ± 0.01[h]	Nil	Nil
		PET	1.57 ± 0.05[j]	1.64 ± 0.03[j]	3.26 ± 0.03[i]	3.33 ± 0.01[i]	Nil	Nil
	25 °C	PP	3.08 ± 0.12[c]	3.15 ± 0.03[c]	4.09 ± 0.08[e]	4.16 ± 0.12[e]	Nil	Nil
		PET	3.01 ± 0.10[d]	3.05 ± 0.25[d]	3.85 ± 0.06[f]	3.92 ± 0.12[f]	Nil	Nil
28th day	4 °C	PP	2.03 ± 0.14[g]	2.10 ± 0.24[g]	4.66 ± 0.11[c]	4.72 ± 0.11[c]	Nil	Nil
		PET	1.73 ± 0.30[i]	1.80 ± 0.32[i]	4.50 ± 0.08[d]	4.57 ± 0.09[d]	Nil	Nil
	25 °C	PP	3.56 ± 0.72[a]	3.63 ± 0.24[a]	5.25 ± 0.03[a]	5.32 ± 0.12[a]	Nil	Nil
		PET	3.48 ± 0.28[b]	3.55 ± 0.35[b]	4.92 ± 0.02[b]	4.99 ± 0.11[b]	Nil	Nil

Table 6.24. Effect of storage on TBA, FFA and HMF levels of MSBPE- and MFBPE-based Shrikhand

Storage days	Temperature of storage	Packaging material	TBA (mg/kg)		FFA (μeq/g)		HMF (μg/g)	
			MSBPE-based shrikhand	MFBPE-based shrikhand	MSBPE-based shrikhand	MFBPE-based shrikhand	MSBPE-based shrikhand	MFBPE-based shrikhand
0th day	4 °C	PP	0.232	0.268	1.25	1.75	2.10	1.90
		PET	0.232	0.268	1.25	1.75	2.10	1.90
	25 °C	PP	0.232	0.268	1.25	1.75	2.10	1.90
		PET	0.232	0.268	1.25	1.75	2.10	1.90
7th day	4 °C	PP	0.326	0.357	1.28	1.78	2.95	2.80
		PET	0.301	0.335	1.26	1.76	2.80	2.60
	25 °C	PP	0.448	0.479	1.32	1.85	4.23	4.10
		PET	0.413	0.445	1.30	1.80	3.90	3.80
14th day	4 °C	PP	0.418	0.442	1.30	1.80	3.81	3.60
		PET	0.365	0.382	1.27	1.77	3.50	3.30
	25 °C	PP	0.678	0.696	1.40	1.95	6.80	6.70
		PET	0.496	0.525	1.36	1.88	6.20	6.20
21st day	4 °C	PP	0.462	0.497	1.33	1.83	4.75	4.80
		PET	0.426	0.453	1.28	1.79	4.10	4.40
	25 °C	PP	0.703	0.726	1.48	2.05	9.54	9.40
		PET	0.596	0.623	1.42	1.95	8.50	8.60
28th day	4 °C	PP	0.547	0.577	1.36	1.87	5.82	5.90
		PET	0.505	0.539	1.29	1.81	5.10	5.30
	25 °C	PP	0.805	0.834	1.56	2.15	12.21	12.40
		PET	0.785	0.814	1.49	2.02	11.00	11.50

6.22.2. Changes in thiobarbituric acid (TBA) value of MSBPE- and MFBPE-based shrikhand

In the same line, results revealed that TBA values (measured at 532 nm) increased over time for both types of shrikhand, with a more significant rise at 25 °C in comparison to the samples stored at 4 °C (Table 6.24). After 28 days of storage, the TBA value for MSBPE-based shrikhand stored at 25 °C increased from 0.232 to 0.805 mg/kg in PP cups and 0.232 to 0.785 mg/kg in PET cups. While at 4 °C, the values increased from 0.232 to 0.547 mg/kg in PP cups and 0.505 to mg/kg in PET cups. Similarly, for MFBPE-based shrikhand, there was a significant increase in TBA values on 28th day at 25 °C in PP cups, increasing from 0.268 to 0.834 mg/kg and 0.268 to 0.814 mg/kg in PET cups. Results obtained were aligned with the observations of Ajmi *et al.* (2022), who reported that TBARS value increased significantly from 0.42 to 0.90 in control and 0.32 to 0.71 in optimized shrikhand stored at 7 °C. TBA reactive substances are the indicators of the production of secondary oxidation products. Processing and storage can induce various physical and chemical changes, such as the auto-oxidation of milk lipids and the formation of trans-fatty acids. These alterations may contribute to the generation of low molecular weight chemical compounds. Additionally, they can lead to a deterioration in sensory quality over an extended period (Semma, 2002). The Arrhenius plot for TBA values is shown in Figures 6.30 and 6.31.

6.22.3. Changes in hydroxymethylfurfural (HMF) content in MSBPE- and MFBPE-based Shrikhand

Hydroxymethylfurfural (HMF) is a key parameter that significantly impacts the quality of dairy products such as kheer, ice cream, shrikhand, and basundi. The formation occurs due to the Maillard reaction, which takes place when amino acids interact with reducing sugars under heated conditions. In addition to temperature, other factors influencing the rate of HMF formation include storage time, ingredients used and the fermentation process (Terzioğlu *et al.*, 2022). The HMF content of MSBPE- and MFBPE-based shrikhand is presented in Table 6.24. It was observed that the HMF levels in MSBPE-based shrikhand increased over the storage period, rising from 2.10 on day 0 to 5.82 (4 °C, PP cups), 5.10 (4 °C, PET cups), 12.21 (25

°C, PP cups), and 11.00 (25 °C, PET cups) by day 28. Similarly, HMF levels in MFBPE-based shrikhand also increased over time, from 1.90 on day 0 to 5.90 (4 °C, PP cups), 5.30 (4 °C, PET cups), 12.40 (25 °C, PP cups), and 11.50 (25 °C, PET cups) on day 28. Research on HMF content in shrikhand or yoghurt samples is relatively scarce in the literature. However, Cui *et al.* (2021) reported HMF levels in yogurt samples reaching up to 3.43 mg/kg. As established by the European Food Safety Authority, the maximum permissible limit for HMF in milk and milk-based products is set at 15 mg/kg due to its proven toxicity. In line with this standard, the HMF content of all experimental shrikhand samples in our study was found to be below this threshold (Terzioğlu *et al.*, 2022). The Arrhenius plot for HMF values is shown in Figures 6.30 and 6.31.

Given the findings, it was inferred that PET cups tend to maintain lower TBA, FFA and HMF values as compared to PP cups at low temperature (4 °C), thereby providing better protection from fat hydrolysis and oxidation of lipids. It was also evident from the results that shrikhand incorporated with multifloral bee pollen extract exhibited higher TBA as well as FFA values.

The TBA, FFA, HMF content, and titratable acidity were observed to increase with storage time, and the values for MSBPE-based shrikhand changes were observed to adhere to zero-order reaction kinetics (n=0). Furthermore, the Arrhenius plot (ln k vs. 1/T) for all these parameters exhibited a straight-line relationship (Figures 6.30 and 6.31).

The fitting curve and R^2 values provided in Figure 6.29 and Table 6.25 respectively, illustrates the reaction kinetics of TBA, FFA and HMF values in MSBPE- and MFBPE-based Shrikhand stored at 4 °C and 25 °C in PP and PET cups. In order to determine the best fit for each parameter, the regression analysis was conducted for zero-order, first-order, and second-order reaction models. The fitting curve (Figure 6.29) specifically represents TBA levels in MSBPE-based Shrikhand stored in PP cups at 4 °C. The R^2 values indicate the goodness of fit for each reaction order, with the zero-order reaction model showing the highest R^2 corresponding to 0.9873, followed by the first-order (0.9533) and second-order (0.8928) models. The higher R^2 values for the zero-order model suggests that the degradation of TBA levels follows zero-order reaction kinetics under these conditions. Similarly, regression analyses were performed for other parameters and storage conditions (as presented in Figure 6.29), to identify the most appropriate kinetic model for each case.

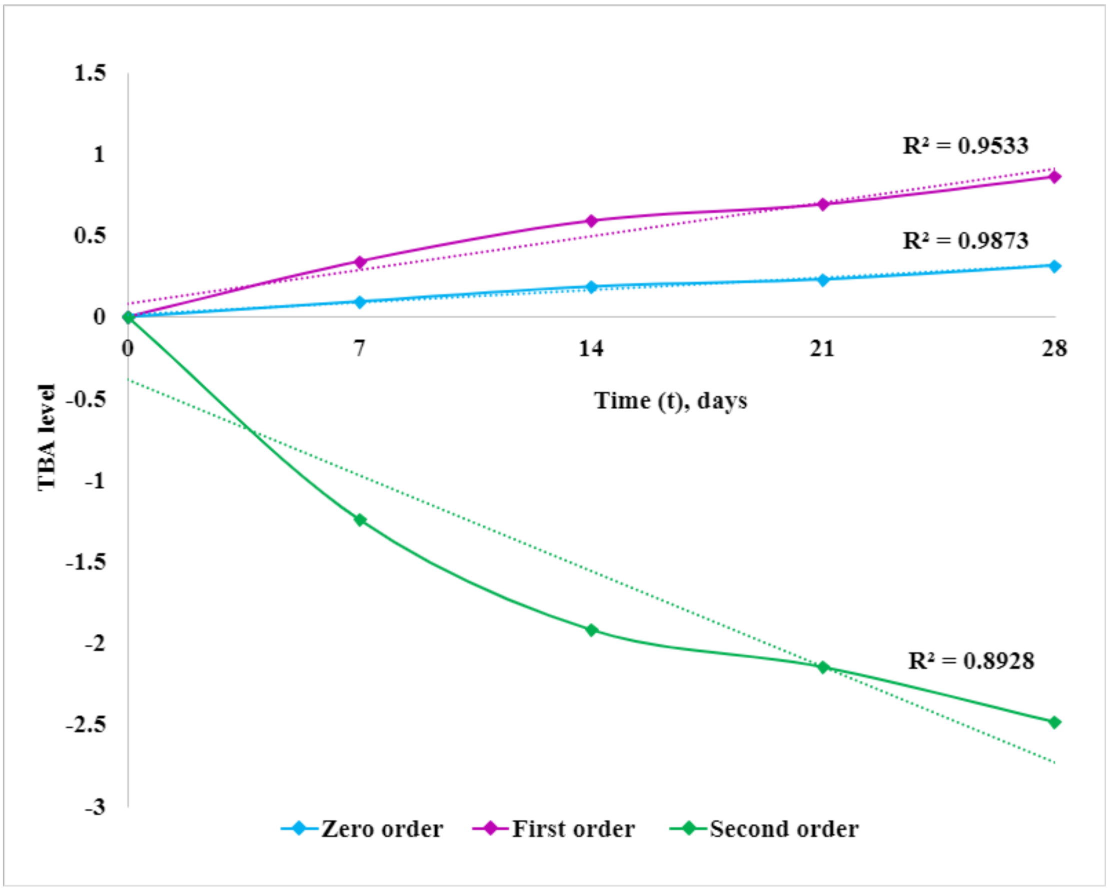
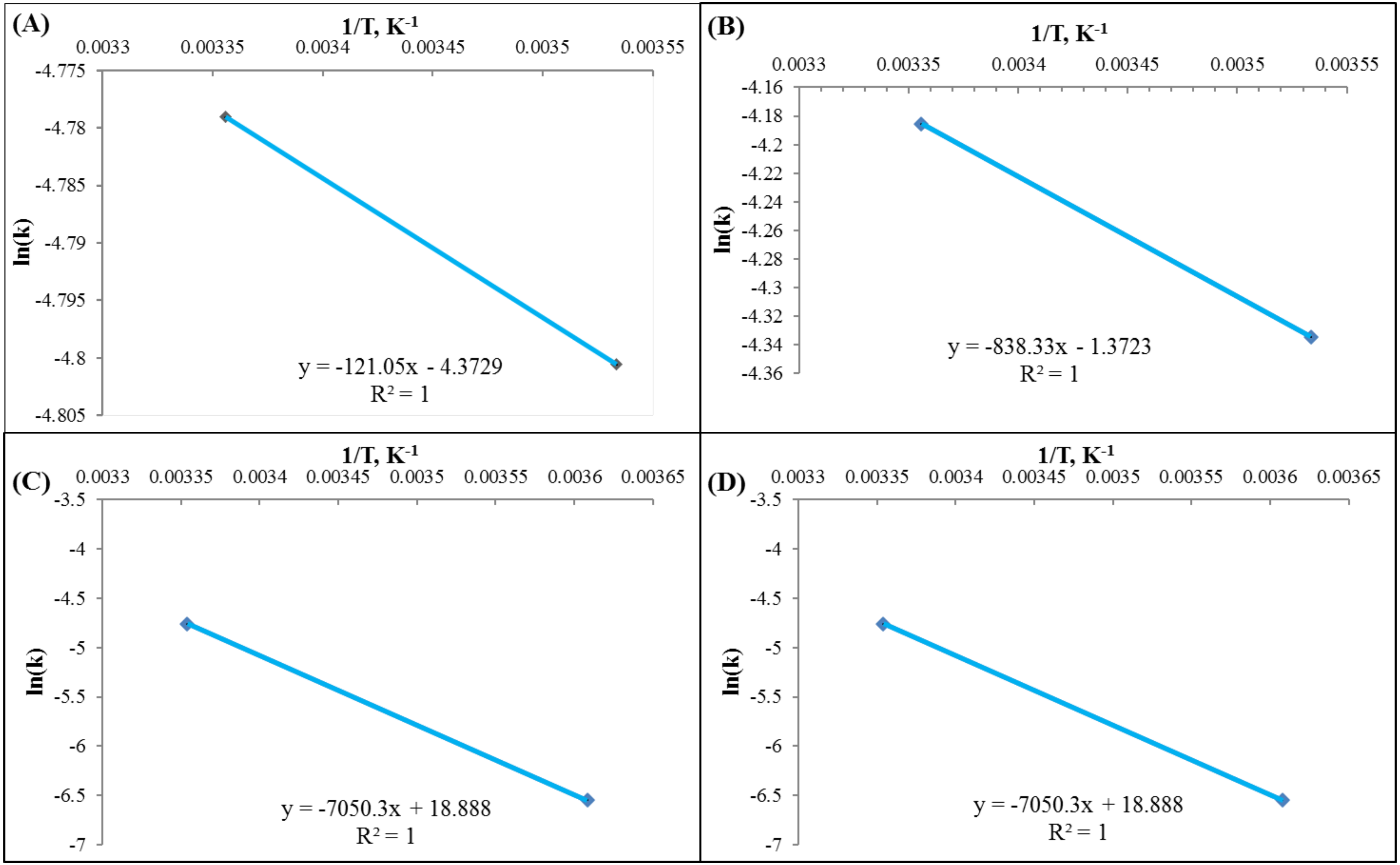


Figure 6.29. Fitting curves for zero-order, first-order, and second-order reaction kinetics of TBA levels

Table 6.25. R^2 values for zero-order, first-order, and second-order reaction kinetics of quality parameters (TBA, FFA, and HMF) in MSBPE-based and MFBPE-based Shrikhand stored in PP cups and PET cups at 4°C and 25°C

Parameter	Packaging material	Temp. (°C)	R^2 values		
			Zero-order	First-order	Second-order
MSBPE-based Shrikhand					
TBA	PP cups	4	0.9873	0.9533	0.8928
		25	0.9187	0.8422	0.7443
	PET cups	4	0.9983	0.9888	0.9518
		25	0.9788	0.9394	0.8343
FFA	PP cups	4	0.9967	0.9965	0.9959
		25	0.9997	0.9993	0.9980
	PET cups	4	1.0000	1.0000	0.9999
		25	0.9998	0.9989	0.9967
HMF	PP cups	4	0.9976	0.9886	0.9356
		25	0.9979	0.9594	0.8222
	PET cups	4	0.9927	0.9899	0.9496
		25	0.9969	0.9681	0.8477
MFBPE-based Shrikhand					
TBA	PP cups	4	0.9941	0.9686	0.9200
		25	0.9289	0.8583	0.7674
	PET cups	4	0.9946	0.9901	0.9744
		25	0.9771	0.9477	0.8584
FFA	PP cups	4	0.9904	0.9888	0.9871
		25	1.0000	0.9991	0.9963
	PET cups	4	0.9727	0.9713	0.9698
		25	0.9969	0.9969	0.9960
HMF	PP cups	4	0.9940	0.9885	0.9285
		25	0.9970	0.9558	0.8036
	PET cups	4	0.9938	0.9914	0.9537
		25	0.9948	0.9655	0.8281


(A)
1/T, K-1
0.0033
0.00335
0.0034
0.00345
0.0035
0.00355
-4.775
-4.78
-4.785
-4.79
-4.795
-4.8
-4.805
ln(k)
y = -121.05x - 4.3729
R² = 1
(B)
1/T, K-1
0.0033
0.00335
0.0034
0.00345
0.0035
0.00355
-4.16
-4.18
-4.2
-4.22
-4.24
-4.26
-4.28
-4.3
-4.32
-4.34
-4.36
ln(k)
y = -838.33x - 1.3723
R² = 1
(C)
1/T, K-1
0.0033
0.00335
0.0034
0.00345
0.0035
0.00355
0.0036
0.00365
-3.5
-4
-4.5
-5
-5.5
-6
-6.5
-7
ln(k)
y = -7050.3x + 18.888
R² = 1
(D)
1/T, K-1
0.0033
0.00335
0.0034
0.00345
0.0035
0.00355
0.0036
0.00365
-3.5
-4
-4.5
-5
-5.5
-6
-6.5
-7
ln(k)
y = -7050.3x + 18.888
R² = 1

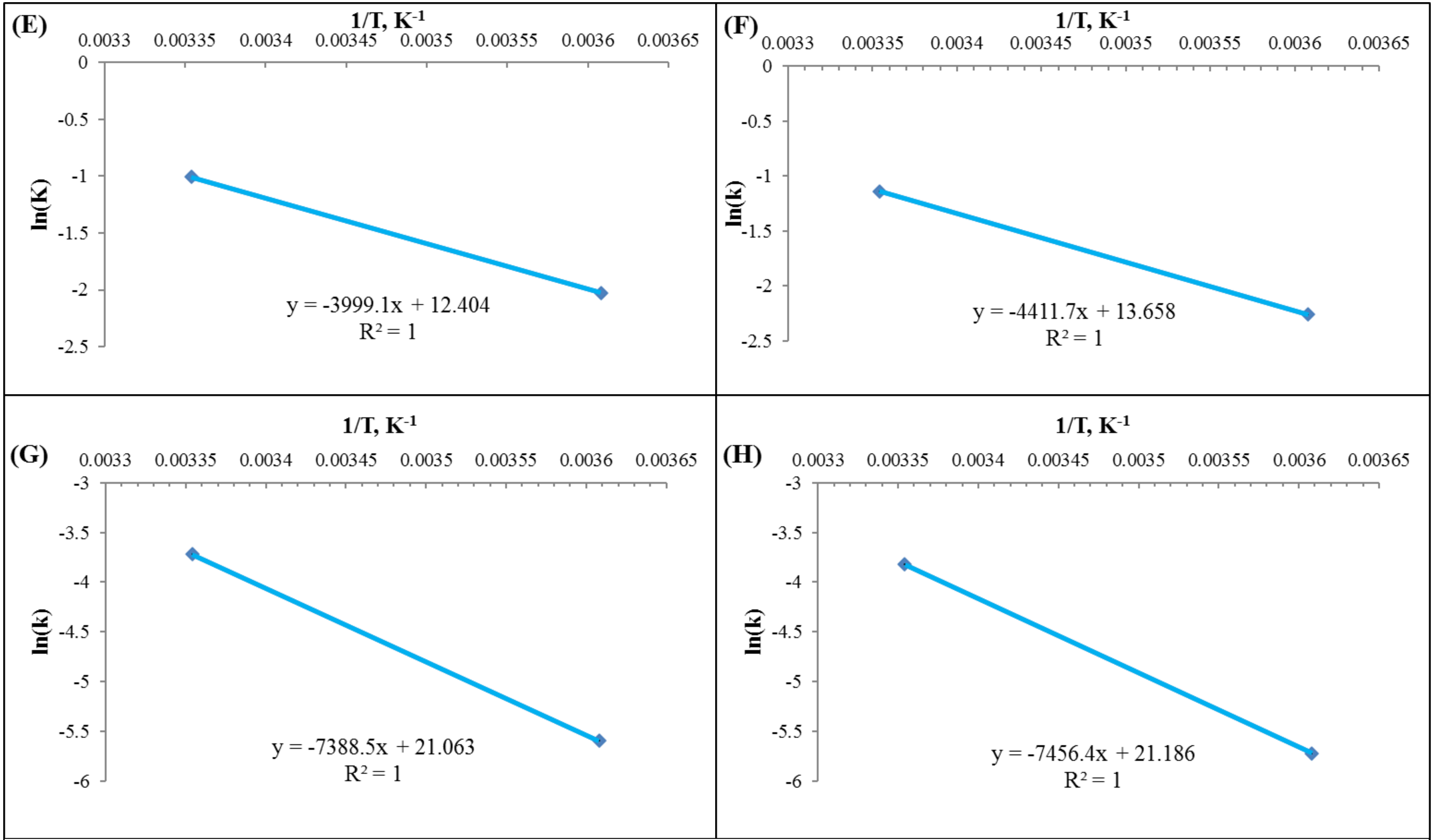
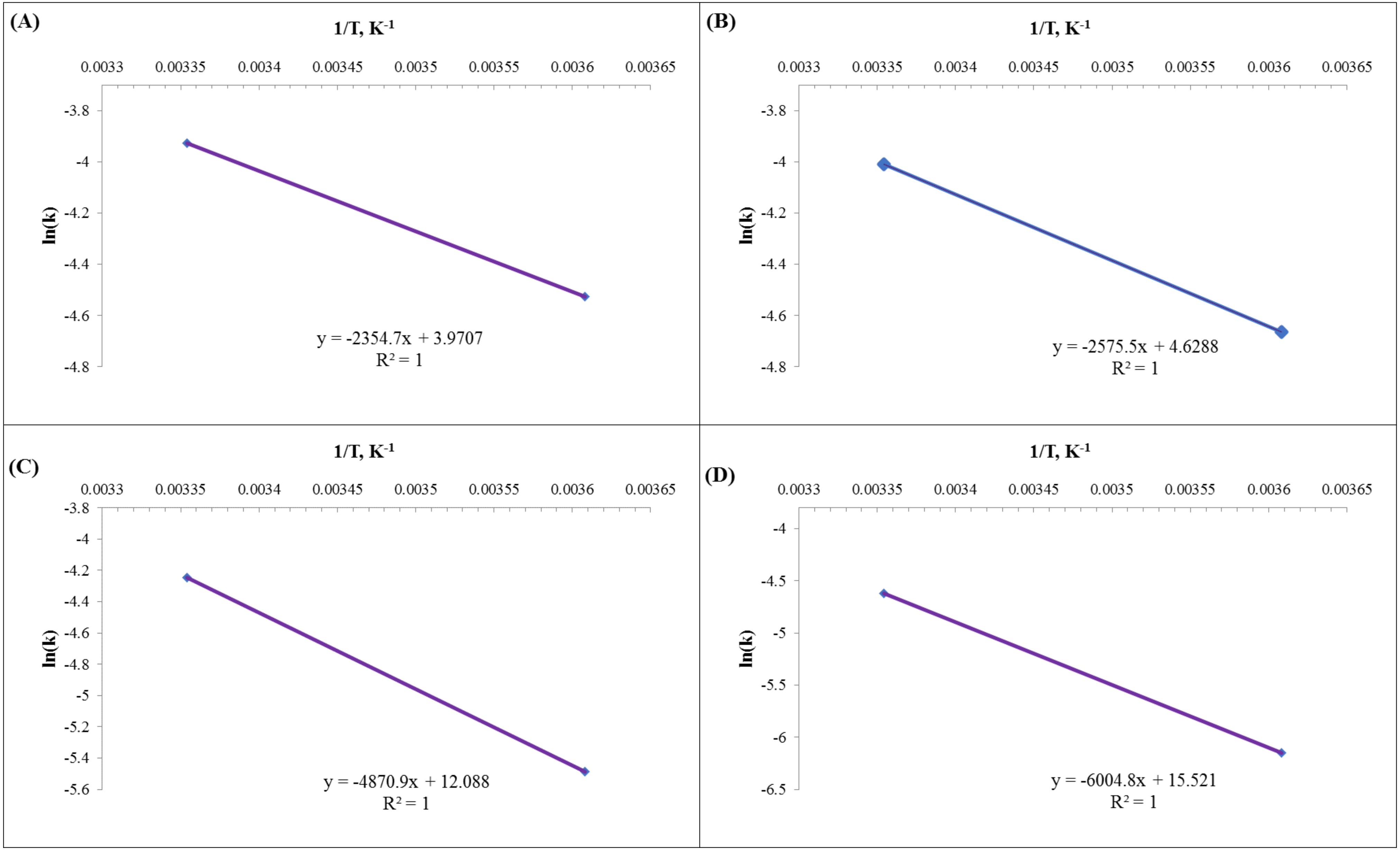


Figure 6.30. Arrhenius plots for parameters of MSBPE-based shrikhand (A) TBA value of samples stored in PP cups; (B) TBA value of samples stored in PET cups; (C) FFA value of samples stored in PP cups; (D) FFA value of samples stored in PET cups; (E) HMF content of samples stored in PP cups; (F) HMF content of samples stored in PET cups; (G) Titratable acidity of samples stored in PP cups; and (H) Titratable acidity of samples stored in PET cups


(A)
1/T, K-1
0.0033 0.00335 0.0034 0.00345 0.0035 0.00355 0.0036 0.00365
-3.8 -4 -4.2 -4.4 -4.6 -4.8
ln(k)
y = -2354.7x + 3.9707
$R^2 = 1$
(B)
1/T, K-1
0.0033 0.00335 0.0034 0.00345 0.0035 0.00355 0.0036 0.00365
-3.8 -4 -4.2 -4.4 -4.6 -4.8
ln(k)
y = -2575.5x + 4.6288
$R^2 = 1$
(C)
1/T, K-1
0.0033 0.00335 0.0034 0.00345 0.0035 0.00355 0.0036 0.00365
-3.8 -4 -4.2 -4.4 -4.6 -4.8 -5 -5.2 -5.4 -5.6
ln(k)
y = -4870.9x + 12.088
$R^2 = 1$
(D)
1/T, K-1
0.0033 0.00335 0.0034 0.00345 0.0035 0.00355 0.0036 0.00365
-4 -4.5 -5 -5.5 -6 -6.5
ln(k)
y = -6004.8x + 15.521
$R^2 = 1$

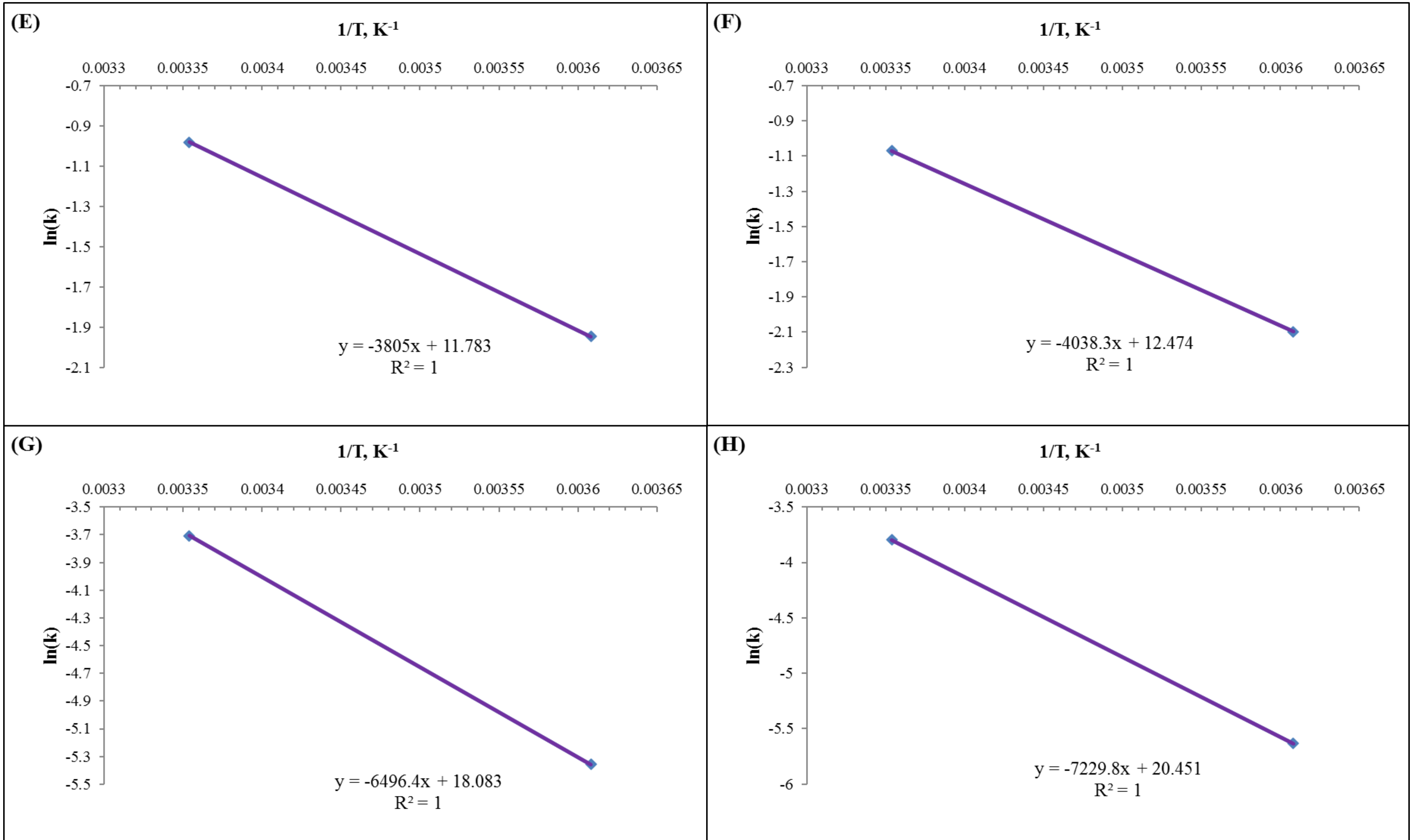


Figure 6.31. Arrhenius plots for parameters of MFBPE-based shrikhand (A) TBA value of samples stored in PP cups; (B) TBA value of samples stored in PET cups; (C) FFA value of samples stored in PP cups; (D) FFA value of samples stored in PET cups; (E) HMF content of samples stored in PP cups; (F) HMF content of samples stored in PET cups; (G) Titratable acidity of samples stored in PP cups; and (H) Titratable acidity of samples stored in PET cups

Table 6.26. Kinetic parameters related to changes in TBA, FFA and HMF formation during storage

	Variables						
	Parameter	**Temp. (K)**	**Rate constant, k**	**Apparent activation energy, E_a (kJ/mol)**	**ΔH (kJ/mol)**	**ΔS (J/mol K)**	**ΔG (kJ/mol)**
MSBPE-based Shrikhand	TBA (PP)	277.15	0.01094	1.01	-1.30	-286.52	79.41
		298.15	0.02001	1.01	-1.47	-282.36	84.18
	TBA (PET)	277.15	0.00959	6.97	4.67	-266.10	73.75
		298.15	0.01841	6.97	4.49	-263.05	78.43
	FFA (PP)	277.15	0.00386	34.71	32.40	-173.59	48.14
		298.15	0.01114	34.71	32.23	-174.20	51.97
	FFA (PET)	277.15	0.00143	58.62	56.31	-95.58	26.55
		298.15	0.00857	58.62	56.14	-96.18	28.73
	HMF (PP)	277.15	0.13200	33.25	30.94	-149.48	41.46
		298.15	0.36471	33.25	30.77	-150.09	44.78
	HMF (PET)	277.15	0.10429	36.68	34.37	-139.06	38.57
		298.15	0.32000	36.68	34.20	-139.67	41.68
MFBPE-based Shrikhand	TBA (PP)	277.15	0.01083	19.58	17.27	-219.60	60.88
		298.15	0.01970	19.58	17.10	-220.20	65.67
	TBA (PET)	277.15	0.00943	21.41	19.11	-214.12	59.36
		298.15	0.01814	21.41	18.93	-214.73	64.04
	FFA (PP)	277.15	0.00414	40.50	38.19	-152.10	42.19
		298.15	0.01429	40.50	38.02	-152.71	45.57
	FFA (PET)	277.15	0.00214	49.92	47.62	-123.57	34.29
		298.15	0.00986	49.92	47.45	-124.18	37.07
	HMF (PP)	277.15	0.14286	31.63	29.33	-154.64	42.89
		298.15	0.37571	31.63	29.16	-155.25	46.32
	HMF (PET)	277.15	0.12286	33.57	31.27	-148.90	41.30
		298.15	0.34286	33.57	31.10	-149.51	44.61

MSBPE – mustard bee pollen extract, MFBPE – multifloral bee pollen extract

Table 6.26 presents the kinetic parameters related to the formation of TBA, FFA and HMF in MSBPE- and MFBPE-based Shrikhand. The parameters were best represented by a zero-order reaction (n=0). A straight line was obtained by the Arrhenius plots (ln k versus 1/T) (Figures 6.29 and 6.30). Rate constant (k) indicates the rate of change for each parameter, apparent activation energy refers to the energy necessary for the reactions to take place, enthalpy change (ΔH) is the energy change during the reaction; entropy change (ΔS) indicates disorder in the system, and Gibbs free energy helps determine the spontaneity of the reaction (ΔG). For MSBPE-based Shrikhand, the rate of TBA formation is higher at 25 °C than at 4 °C, indicating an increase in lipid oxidation at higher temperatures. The activation energy for PET (6.97 kJ/mol) is significantly higher for PP cups (1.01 kJ/mol), implying that lipid oxidation in PET packaging is less likely to occur spontaneously. Similar trends were observed for MFBPE-based Shrikhand. PET packaging shows slightly higher activation energies (21.41 kJ/mol) compared to PP (19.58 kJ/mol), confirming PET's effectiveness in reducing oxidation rates. In MSBPE-based Shrikhand, the activation energy for FFA formation is highest in PET packaging (58.62 kJ/mol), indicating that PET packaging offers better resistance to lipid hydrolysis compared to PP (34.71 kJ/mol). Similar results were observed in MFBPE-based Shrikhand.

For MSBPE-based Shrikhand, PET packaging shows higher activation energy (36.68 kJ/mol) compared to PP (33.25 kJ/mol), suggesting PET reduces the rate of the Maillard reaction. Similar trends are observed with slightly lower activation energies (33.57 kJ/mol for PET and 31.63 kJ/mol for PP). Ruhil *et al.* (2011), Rasane *et al.* (2019) and Singh *et al.* (2024) observed similar results in their studies on basundi mix, nutricereal-based fermented baby food pre mix and corn silk-based instant mix, respectively.

Values of ΔH that are positive suggest that the reactions are endothermic in nature, implying that they require heat to proceed. Less negative values of ΔS for PET packaging suggests that it offers a more stable storage environment. Positive values of ΔG suggest that the formation of these compounds is accelerated at higher temperatures, despite the reactions being non-spontaneous.

6.23. Cost analysis of MSBPE and MFBPE-based shrikhand

Tables 6.27 presents the cost of shrikhand incorporated with mustard bee pollen extract and multifloral bee pollen extract, respectively. Similar ingredients were used in both the products but the total cost of MSBPE-based shrikhand is slightly higher at ₹25.11 per 100 g compared to ₹24.29 for MFBPE-based shrikhand. The difference is primarily due to the higher price of mustard bee pollen and slightly larger quantities of some ingredients used in the MSBPE variant. For comparison, the cost of Amul Shrikhand available in the market is ₹123 for 500 g, which translates to ₹246 per kg.

This indicates that the cost of the Shrikhand developed in this study is almost comparable to that of commercially available products.

The developed Shrikhand formulations were found to be economically viable while also offering enhanced nutritional value due to the incorporation of bee pollen. This makes them attractive for consumers seeking functional foods that are both health-promoting and affordable. Considering their comparable cost to market products and their additional nutritional benefits, these Shrikhand formulations have strong potential for commercial-scale production. They can particularly appeal to health-conscious consumers who are interested in natural, nutrient-enriched products without incurring a significant price premium. Overall, these findings suggest that the incorporation of bee pollen not only enhances the nutritional profile of Shrikhand but also maintains its economic feasibility, making it a promising option for wider adoption in the functional food market.

Table 6.27. Cost analysis of MSBPE- and MFBPE-based Shrikhand

Ingredients	Amount of ingredient used	Total cost per kg/l (₹)	Cost as per amount used (₹)
Mustard bee pollen	1.0 g	1500 per kg	1.5
Sugar	22.6254 g	40 per kg	0.91
Hung curd	77.5547 g	175 per kg	13.57
Solvent (ethanol)	10.2518 ml	500 per litre	5.13
Overhead charges (Electricity/cooking gas)	-	-	4.0
Total cost of MSBPE-based shrikhand			**₹ 25.11**
Multifloral bee pollen	1.0 g	1000 per kg	1.0
Sugar	22.4768 g	40 per kg	0.90
Hung curd	75.3502 g	175 per kg	13.19
Solvent (ethanol)	10.408 ml	500 per litre	5.20
Overhead charges (Electricity/cooking gas)	-	-	4.0
Total cost of MFBPE-based shrikhand			**₹ 24.29**

Conclusion

This research study successfully demonstrated the potential of incorporating bee pollen extract into Shrikhand for the development of nutritionally enhanced functional dairy product. The comprehensive screening of mustard bee pollen and multifloral bee pollen revealed their rich profiles of protein content, phenolic compounds, flavonoids and antioxidant activity. The findings indicated that MSBP exhibited enhanced properties, including elevated moisture content, ash content, TPC, and TFC, all of which contributed to its superior antioxidant activity compared to MFBP. The analysis of minerals verified the existence of key minerals such as magnesium, iron, calcium, phosphorus, and zinc. The analysis also confirmed the absence of toxic elements such as arsenic, mercury, cobalt and lead. FTIR analysis revealed the presence of distinct functional groups corresponding to proteins , carbohydrates, lipids, and phenolic compounds.

Pre-treatment processes such as thermal cracking, alkaline pre-treatment and wet thermal pre-treatment effectively improved the phenolic profile and improved digestibility. Among all, thermal cracking at 40 °C yielded the highest concentrations of ferulic acid, quercetin and cinnamic acid for mustard bee pollen, and thermal cracking at 35 °C resulted in highest concentration of ferulic acid and cinnamic acid. The results obtained from the analysis of MSBP and MFBP extract revealed that the total carotenoid content, total chlorophyll content, chlorophyll a, chlorophyll b, TFC, TPC, antioxidant activity and FRAP values increased steadily suggesting that ethanol was more effective at extracting bioactive compounds as compared to distilled water. All the parameters examined exhibited statistically significant differences ($p<0.05$) across every sample. Numerical optimization using response surface methodology determined the best formulations for Shrikhand. It constituted of bee pollen extract along with hung curd and sugar content. The optimized mustard bee pollen Shrikhand formulation included 10.252 ml bee pollen extract, 77.555 g hung curd, and 22.652 g sugar, with a desirability of 0.914. For multifloral bee pollen Shrikhand, the optimized formulation consisted of 10.408 ml bee pollen extract, 75.250 g hung curd, and 22.477 g sugar, with a desirability of 0.900.

The shelf-life study was conducted on the optimized products at two distinct temperatures (4 °C and 25 °C), and suitable packaging materials (PP cups and PET cups). PET cups showed comparatively better results when stored at 4 °C. PET containers better preserved the antioxidant content and TPC, while minimizing degradation into metabolites like HMF, FFA, and TBA. The chemical reaction kinetics was conducted to predict the shelf-life of the product in terms of HMF, FFA and TBA content. Sensory evaluation scores indicated that PET-stored samples maintained higher acceptability for appearance, flavour, texture, mouthfeel, and overall acceptability ($p<0.05$). Microbiological analysis confirmed the safety of the product, with no coliforms detected throughout the study period. The cost analysis of both the products suggests a cost of production ₹25.11 per 100 g for the mustard bee pollen extract-based Shrikhand, and ₹24.29 per 100 g for multifloral bee pollen extract-based Shrikhand.

In conclusion, this research successfully optimized the incorporation of bee pollen into Shrikhand, enhancing its antioxidant activity, phenolic content and sensory properties, while maintaining storage stability and health-promoting foods, paving the way for innovation in the functional food industry. Future research could explore the incorporation of bee pollen extract into other dairy matrices such as yogurt, lassi, or ice cream to enhance their functional potential. Such investigations could help in developing a broader range of functional dairy products that cater to diverse consumer preferences while delivering added health benefits. Further scaling-up trials and consumer studies can help in commercial adoption. It will be crucial to evaluate process feasibility, cost-effectiveness, and product stability under real-world production conditions. These efforts would contribute toward sustainable development of functional dairy products.

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PRIMARY SOURCES

1	Jaspreet Kaur, Jyoti Singh, Sawinder Kaur, Vikas Nanda, Prasad Rasane. "Evaluation and comparative analysis of the physicochemical and phytochemical characteristics of mustard and multi-floral bee pollen", South African Journal of Botany, 2024 Publication	7%
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LIST OF PUBLICATIONS

Sr. No.	Title of paper with author names	Name of journal	Published Date	Volume & Issue Number	ISSN/ISBN Number	Journal indexing (Scopus/UGC/ Web of Science)
1	Evaluation and comparative analysis of the physicochemical and phytochemical characteristics of mustard and multi floral bee pollen Jaspreet Kaur, Jyoti Singh, Sawinder Kaur, Vikas Nanda, Prasad Rasane	South African Journal of Botany	21/09/2024	Volume 174	0254-6299	Scopus
2	Exploring the Health Benefits of Bee Pollen and Its Viability as a Functional Food Ingredient Jaspreet Kaur, Prasad Rasane, Vikas Kumar, Vikas Nanda, Vishesh Bhadariya, Sawinder Kaur, Jyoti Singh	Reviews in Agricultural Science	15/05/2024	Volume 12	2187-090X	Scopus

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Evaluation and comparative analysis of the physicochemical and phytochemical characteristics of mustard and multi-floral bee pollen

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ABSTRACT

In today's health-conscious era, consumers are inclined towards value-added products that offer the option to substitute conventional food products with high-nutritional value components, thereby enhancing the quality of existing processed products. Bee Pollen is a suitable contender, which is a biologically active food ingredient comprising macronutrients, micronutrients, and phenolic compounds. It has been utilized in traditional medicine for its therapeutic properties including hepatoprotective and wound healing. This study aims to unveil the physicochemical, and phytochemical characteristics of two different varieties of bee pollen (*viz.* mustard and multifloral) in addition to the color, mineral, and Fourier Transform Infrared Spectroscopy (FTIR) analysis. The results indicate that mustard bee pollen (MSBP) possesses high moisture (15.33 ± 0.36 %), ash (4.3 ± 0.014 %), total phenol (2676 ± 0.58 mg GAE/100 g) and total flavonoid content (14.025 ± 0.25 mg QE/g) which is responsible for its high antioxidant activity (82.6 ± 0.54 %) as compared to multifloral bee pollen (MFBP). It was further confirmed from mineral analysis performed by using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) quantification procedure that health-promoting minerals like Ca, Mg, Fe, P, and Zn were present; whereas, the toxic minerals including As, Hg, Co, and Pb were absent in the pollen varieties under study. Moreover, FTIR allowed for the identification of stretching, bending, and vibrations associated with the numerous carbohydrates, protein functional groups as well as phenolic compounds. Therefore, the production of bee pollen can be scaled up to an industrial level due to its functional properties for utilization in food formulations, the pharmaceutical industry, and the nutraceutical sector.

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REVIEWS **OPEN ACCESS**

Exploring the Health Benefits of Bee Pollen and Its Viability as a Functional Food Ingredient

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ABSTRACT

Bee pollen, also known as 'life-giving dust', is a treasure trove of nutrients and bioactive compounds. It is regarded as a valuable functional food ingredient owing to its various health-promoting effects. Thus, it can be incorporated into different food products for the development of functional foods. The nutritional and bioactive constituents of bee pollen contribute to its extensive health benefits, including its role against cancer, diabetes, liver disease, cardiovascular disorders, etc. Despite having numerous positive health implications, its utilization as a functional ingredient in food products needs to be critically evaluated in terms of clinical effects and safety profile. The exine layer of bee pollen limits its utilization and contributes to the low bioavailability of key nutrients. Processing techniques (chemical, physical, enzymatic) break down the robust outer coat, improves digestibility, and allow the diffusion of nutrients in the GI tract. In addition, 50 g of bee pollen is sufficient to fulfill 50% reference daily intake (RDI) of most vitamins and minerals. Overall, the use of bee pollen is safe and its use seems promising for coping with various nutritional inadequacies. This review focuses on the important aspects and specific considerations which are required to be taken into account before the development of bee pollen-based food products. Particular attention must be paid to nutritional adequacy, sensory attributes, health effects, allergenicity, digestibility, and compliance with regulatory bodies.

LIST OF CONFERENCES

Sr. No.	Name of the conference	Organized by	Title of oral/poster presentation	Date of conference
1	Global Indian Young Scientists Research and Innovation Conference	Global Indian Scientists & Technocrats Forum (GIST), New Delhi, India	Assessment of physicochemical properties and antioxidant profile of bee pollen from India	31/05/2023
2	International Conference On Emerging Techniques in Food Processing-II (ETFP-2022)	Ghani Khan Choudhury Institute of Engineering and Technology Malda, India	An overview of the nutritional composition, types, health benefits and utilization of bee pollen in value-added food products	25/03/2022
3	INTERNATIONAL DİCLE SCIENTIFIC STUDIES AND INNOVATION CONGRESS, ISARC (2022)	INTERNATIONAL SCIENCE AND ART RESEARCH CENTER, TURKEY	Physicochemical properties and antioxidant profile of bee pollen from India	28/05/2022

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Certificate of Participation

Prof./Dr./Mr./Mrs./Ms. ***Jaspreet Kaur*** *from* ***Lovely Professional University***

presented a ***ORAL/POSTER*** *entitled* ***Assessment of physico-chemical properties and antioxidant profile of bee pollen from India*** *during the conference.*

Dr. Yelloji- Rao Mirajkar
International Coordinator
GIST Forum, USA

Dr. Rajnish Chaturvedi
Convenor, GI-YSRI 2023

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Prof./Dr./Mr./Mrs./Ms JASPREET KAUR from LOVELY PROFESSIONAL UNIVERSITY presented a **POSTER** entitled ASSESSMENT OF PHYSICO-CHEMICAL PROPERTIES AND ANTIOXIDANT PROFILE OF BEE POLLEN during the conference.

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Dr. Yelloji- Rao Mirajkar
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Department of Food Processing Technology, Ghani Khan Choudhury Institute of Engineering and Technology

(A Centrally Funded Technical Institute, Ministry of Education, Government of India)

Narayanpur, Malda, West Bengal: 732141

Certificate for Presentation

Certificate No: *GKCIET/FPT/ETFP-II/219*

This is to certify that ***Jaspreet Kaur*** *of* ***Lovely Professional University, Phagwara, Punjab (India)*** *has given a* ***Oral presentation*** *in the International Conference on* ***"Emerging Technologies in Food Processing-II (ETFP-2022)"*** *organized by the* ***Department of Food Processing Technology****, Ghani Khan Choudhury Institute of Engineering and Technology, Malda, West Bengal during 25th -26th March, 2022.*

Title: An overview of the nutritional composition, types, health benefits and utilization of bee pollen in value-added food products

Authors: Jaspreet Kaur

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'' Assessment of physico-chemical properties and antioxidant profile of bee pollen from India ''

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APPLICATION TYPE	ORDINARY APPLICATION
DATE OF FILING	17/12/2022
APPLICANT NAME	**LOVELY PROFESSIONAL UNIVERSITY**
TITLE OF INVENTION	AN APPARATUS AND METHOD FOR PRODUCTION OF BEE POLLEN POWDER
FIELD OF INVENTION	FOOD
E-MAIL (As Per Record)	director@bansalip.com
ADDITIONAL-EMAIL (As Per Record)	dip@lpu.co.in
E-MAIL (UPDATED Online)	
PRIORITY DATE	
REQUEST FOR EXAMINATION DATE	--
PUBLICATION DATE (U/S 11A)	30/12/2022

Application Status	
APPLICATION STATUS	**Awaiting Request for Examination**

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1	Form-XIV	39805/2024-CO/L	172986	Process Optimization for Bee Pollen Incorporated Dairy Product.	500
Amount in Words			Rupees Five Hundreds		500

PAYMENT MODE	Transaction Id	CIN
Online	C-0000191166	1812240050320

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