STUDIES ON THE DEVELOPMENT OF L-TRYPTOPHAN-SEROTONIN-MELATONIN RICH NUTRACEUTICAL FOOD AND SUPPLEMENT FROM GAMMA-IRRADIATED GREEN PLANTAINS (*Musa paradisiaca*)

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PROFORMA – 1

"Statement of Originality"

I, **Poulami Sarkar,** registered on **04.09.2019** do hereby declare that this thesis entitled **"Studies on the Development of L-Tryptophan-Serotonin-Melatonin Rich Nutraceutical Food and Supplement from Gamma-Irradiated Green Plantains (*Musa paradisiaca*)"** contains literature survey and original research work done by the undersigned candidate as per Doctoral studies.

All information in this thesis has been obtained and presented in accordance with existing academic rules and ethical conduct. I declare that, as required by these rules and conduct, I have fully cited and referred all materials and results that are not original to this work.

I also declare that I have checked this thesis as per the "Policy on Anti Plagiarism, Jadavpur University, 2019", and the level of similarity as checked by iThenticate software is **6%**.

Signature of Candidate: Poulami Sarkar

Date: 03.04.2024

P. Bhattacharjee 3.4.2024

Certified by Supervisor:

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PROFORMA – 2

CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled **"Studies on the Development of L-Tryptophan-Serotonin-Melatonin Rich Nutraceutical Food and Supplement from Gamma-Irradiated Green Plantains (*Musa paradisiaca*)"** submitted by **Smt. Poulami Sarkar**, who got her name registered on 04.09.2019 for the award of **Ph. D. (Science)** Degree of Jadavpur University, is absolutely based upon her own work under the supervision of **Dr. Paramita Bhattacharjee (Supervisor), Professor, Department of Food Technology and Biochemical Engineering, Jadavpur University, Kolkata, West Bengal** and that neither her thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award before.

P. Bhattacharjee 3.4.24

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Chapter 1

Introduction and Review of Literature

Introduction

Bananas, classified scientifically as *Musa* sp., rank as the world's fourth most vital food crop, trailing only behind staples like rice, wheat, and maize according to sources from Shamla and Nisha (2017) and FAOStat (2023). These fruits belong to the family 'Musaceae' and genus *Musa* (Vilhena *et al*., 2019). Known as the tallest herbaceous, perennial, and monocotyledonous plant (Ajijolakewu *et al*., 2021), bananas feature a distinctive structure with an above-ground pseudostem and an underground rhizome (Lakshmi *et al*., 2015), reaching heights ranging from 3 to 15 meters (Imam and Akter, 2011, Vilhena *et al*., 2019). The banana fruit is characterized by its elongated-cum-curved shape and a flesh that is pulpy and rich in starch. It measures between 8 to 13 cm in length (Mathew and Negi, 2017) and is botanically classified as a berry (Sau *et al*., 2023).

Cultivation and classification

Bananas primarily originate from two wild species, *Musa acuminata* (AA) and *Musa balbisiana* (BB), as documented by Abiodun-Solanke and Falade in 2010. These original species have given rise to the majority of banana cultivars we consume today, either directly or through hybridization processes. One notable hybrid is *Musa paradisiaca*, a genetic cross of *M. acuminata* and *M. balbisiana*, characterized by its polyploid nature predominantly comprising of the AAB (traded plantains), ABB (other cooking plantains), and BBB genomes (Ajijolakewu *et al*., 2021). The ideal conditions for the growth and cultivation areas of *Musa* species have been described elaborately in Table 1.1.

M. paradisiaca, commonly known as the 'cooking banana' or 'plantain,' features a soft, starch-laden, seedless (parthenocarpic) flesh encased in a peel, distinct from the 'dessert banana'. Detailed information on the appearance, consumption, and nutritional factors (Abiodun-

Solanke and Falade, 2010) of plantains have been compiled in Table 1.2. This detailed categorization helps differentiate plantains in terms of their culinary applications and health benefits, emphasising their unique position within the broader banana family. On the day this thesis has been compiled, 1668 research articles have been cited on Scopus on plantains (*Musa paradisiaca*).

Traditional and medicinal usage of plantains

In numerous developing countries worldwide, including Nigeria, plantains serve as a fundamental staple food (Ebun and Santosh, 2011). They enjoy a global appeal, being consumed in various stages of ripeness, ranging from unripe to overripe. This versatility in consumption reflects the plantain's integral role in the dietary habits of diverse cultures, underscoring its significance beyond mere sustenance to being a key component of culinary traditions across the globe.

Beyond its culinary applications as a versatile fruit-cum-vegetable, plantains hold a prominent place in traditional medicine for treating a variety of ailments. According to Ghani (2003) and Khare (2007), they're employed in the management of gastric ulcers, diarrhea, dysentery, and complications associated with ulcerative colitis, diabetes, sprue, uremia, nephritis, gout, hypertension, and cardiac diseases. Ajijolakewu *et al.* (2021) highlights the use of plantains in healing both infectious and non-infectious diseases, as well as in wound care.

Extensive research underscores the antimicrobial properties of plantains against a spectrum of pathogens, including bacteria, fungi, parasites, and viruses, as reported by Ajijolakewu *et al.* (2021). The same author also has reported their anticancer activity, antidiabetic activity, and antiulcer activity. This extensive therapeutic potential positions plantains as a valuable

economic and medicinal resource, promoting their widespread cultivation and research interest globally.

Production and wastage of plantains: Global statistics

According to a report published by FAOStat, 170 million tonnes of banana and plantain (in combination) were produced worldwide in the year 2021, in which India (stood first with 30.5 million tonnes) and China contributed 25% of the global production. Other significant contributors included Uganda, the Philippines, Indonesia, Ecuador, and Brazil (FAOStat, 2023), highlighting the widespread cultivation of these fruits across various climates and regions. In the same year, the global production of plantains was approximately 45.3 million tonnes wherein Uganda contributed around 9.2 million tonnes (FAOStat, 2023). However, distinguishing between banana and plantain production can be challenging, as the top producing countries often do not separate the two in their data. This lack of specificity makes it difficult to obtain precise figures for plantain production alone in countries like India for the year 2021.

In spite of the huge production harvested every year, plantains encounter a high rate of post-harvest losses owing to several multi-faced problems including inaccessibility to production areas, far distances between production areas and customers, inadequate infrastructures for harvesting, and carelessness on the part of harvesters and handlers among others (Abiodun-Solanke and Falade, 2010). In countries like India, one of the top producers, post-harvest wastage is alarmingly high, estimated to be between 25-40% of total production (Kumar and Uma, 2020).

To mitigate this unprecedented post-harvest loss of this important nutritious-cum-highly perishable crop, multiple measures can be adopted such as -extension of shelf-life and

preservation of raw fruits, development of more value-added products and by valorization of the wasted fruits (Kumar and Uma, 2020). Moreover, the peels of plantains, that comprise about 40% of total weight of fresh plantains, are the principal waste materials produced from plantain processing. Currently negligible portions of plantain peels are being used as fertilizer, animal feed, in biorefineries (Martínez-Ruano *et al.,* 2018) and for fiber extraction, while the remaining portion is discarded (Okorie *et al*., 2015). Therefore, there is a pressing need for the comprehensive utilization of both the peel and pulp in the formulation of value-added products. This approach not only aims to reduce waste but also capitalizes on the nutritional and bioactive properties of plantains, which include their intrinsic phytoremediator antioxidant molecules. Achieving this holistic utilization requires a detailed understanding of these components, beyond the basic nutritional value of the fruit. It is opined that this comprehensive approach could significantly enhance the economic and environmental sustainability of the plantain industry.

Phytochemical composition of plantains

On the day this thesis has been compiled, 14 research articles have been cited on Scopus on the phytochemical composition of plantains (*Musa paradisiaca*). Plantain is reported to be a diverse source of phytochemicals (Bennett *et al.,* 2010, Jiménez-Martínez *et al.,* 2017, Oyeyinka and Afolayan, 2019, Pereira and Maraschin, 2015, Shamla and Nisha, 2017, Tsamo *et al.,* 2015) in which not only its pulp, but also its peel serves as the phytochemical and nutrient repository (Behiry *et al.,* 2019, Oyeyinka and Afolayan, 2019, Silva *et al.,* 2014). Among them, anthocyanins (Kitdamrongsont *et al.*, 2008), phenolic acids (Bhaskar *et al.*, 2012, Sheng *et al.*, 2014), flavanones (Ganugapati *et al.*, 2012), and terpenoids (Dutta *et al.*, 1983, Martin *et al.*, 2000, Nazaruk and Borzym-Kluczyk, 2015, Tin *et al.*, 2016) are worth mentioning. It is also rich in antioxidants such as caffeic acid hexoside, ferulic acid hexoside, sinapic acid,

hydroxycinnamic acid derivative, quercetin, myricetin, rutin, kaempferol, isohamnetin-3-0 rutinoside, epicatechin (Tsamo *et al.*, 2015). Another study has reported the presence of natural flavonoid leucocyanidin in unripe plantain as the antiulcerogenic agent (Lewis *et al.*, 1999). The presence of these bioactive molecules confers dietary (nutritional) and therapeutic roles to plantains.

A study highlighted by Lewis and colleagues in 1999 identified leucocyanidin, a natural flavonoid found in unripe plantains, as an antiulcerogenic agent. This discovery points to the presence of bioactive molecules in plantains that offer both nutritional and therapeutic benefits. The identification of such compounds underscores the potential of plantains to contribute to dietary health and to serve as a natural remedy for specific conditions, like ulcers, showcasing the broader significance of this fruit beyond its role as a staple food. This insight into the nutritional and medicinal properties of plantains opens avenues for further research and development in food science and alternative medicine.

Bhattacharjee and Chakraborty's (2019) book chapter unveils that plantains are a reservoir of two crucial yet under-researched biotherapeutic molecules and neurotransmitters serotonin (5-150 µg/g) and melatonin (65.5 ng/100 g) (Bhattacharjee and Chakraborty, 2019) along with their precursor molecule, an essential amino acid L-tryptophan (11 mg in a medium banana-*Musa sp.*). These findings are significant, given the rarity of such compounds in plant sources.

Despite the presence of these vital molecules, which play significant roles in mood regulation, sleep, and various other physiological functions, there has been a noticeable gap in the research exploring plantains as a potential source of these bioactive compounds. This absence of studies, particularly in prominent databases like Scopus and Web of Science, highlights a considerable opportunity for future research. Investigating plantains for their serotonin, melatonin, and L-tryptophan content could open new avenues in both nutritional science and therapeutic

applications, potentially leading to innovative uses of this widely cultivated and consumed fruit.

L-tryptophan, its biological functions, and therapeutic activities

Chemically recognized as an aromatic indolylpropionic acid (Fig. 1.1), tryptophan's discovery dates back to 1901 by scientists Hopkins and Cole, with its chemical structure later elucidated by Ellinger and Flamand. In plants, tryptophan is the end product of the Shikimate pathway (Herrmann and Weaver, 1999) where two carbohydrates, phosphoenolpyruvate and erythrose 4-phosphate are converted into three aromatic amino acids, the other two being phenylalanine and tyrosine. Notably, this pathway is exclusive to plants and microorganisms, absent in animals. This absence underscores the necessity of tryptophan in animal diets, including humans, as they cannot synthesize it internally. Consequently, in human nutrition, tryptophan is deemed an 'essential amino acid,' necessitating its intake through the diet to facilitate the production of vital derivative compounds post-absorption from food (Richard *et al*., 2009). The important biological functions of L-tryptophan in plants and animals have been described in Fig. 1.2.

Serotonin, its biological importance, and therapeutic activities

Serotonin, also known as 5-hydroxytryptamine (5-HT) was first isolated and characterized in 1948 by Maurice Rapport and Irvine Page (Mohammad-Zadeh *et al*., 2008). It is an indoleamine monoamine molecule (Bhattacharjee *et al*., 2016) (Fig. 1.3), a derivative of L-tryptophan and simultaneously acts as a neurotransmitter, a hormone, and a mitogen (Mohammad-Zadeh *et al*., 2008). Many reports have documented notable quantities of serotonin in various plant parts (Bhattacharjee *et al*., 2016). In animals, serotonin is mostly produced in the enterochromaffin cells of the gastrointestinal tract followed by the

serotoninergic neurons of the central nervous system, and the blood platelets. However, unlike tryptophan, serotonin cannot be readily absorbed in the body from consumed foods (Bhattacharjee *et al*., 2016). The extensive involvement of serotonin in various biological functions (Fig. 1.4) further emphasises the complexity and importance of this molecule in maintaining both plant and animal health and well-being.

Melatonin, its biological importance, and therapeutic activities

Melatonin (N-acetyl-5-methoxytryptamine), often considered as the *hormone of darkness*, is an indole hormone (Fig. 1.5) majorly produced in the pineal gland of animal brains and was first discovered in 1958 (Arnao *et al.,* 2023).

In both plants and animals, the presence of melatonin is ubiquitous, and it is derived from L-tryptophan via serotonin (Arnao *et al*., 2023). Melatonin present in plant sources is referred to as 'phytomelatonin' (Blask *et al*., 2004). Notably, melatonin from dietary sources is readily absorbed in the intestinal tract, facilitating its entry into the bloodstream and subsequent physiological effects. This ease of absorption contrasts with serotonin, highlighting melatonin's unique role and potential benefits when consumed through food, including enhanced sleep quality and regulation of biological rhythms. The multiple functions of melatonin are described in Fig. 1.6.

Biosynthesis of serotonin and melatonin from L-tryptophan

Besides these three target biomolecules, each of the intermediate compounds of the conversion pathway have their own functions in plants and animals. The conversion pathway of L-tryptophan to serotonin and melatonin in plants and animals involves a few common along with some different enzymes. The biosynthesis pathway for production of serotonin and melatonin in plants and animals from the precursor L-tryptophan is presented in Fig. 1.7.

Advantages of plant sources over the synthetic supplements of L-tryptophan, serotonin, and melatonin

Given their pivotal roles in maintaining biological homeostasis, tryptophan, and melatonin, particularly in their early discovery phases, could only be sourced from natural origins. This scarcity underlines a notable gap in the market for natural food supplements capable of providing these crucial bioactive molecules in amounts that are both safe and substantial. The available food supplements for L-tryptophan are mostly obtained from synthetic sources, while there is no available commercial food supplement for serotonin. Such supplements, especially those derived from plant sources (phytomelatonin), offer distinct advantages over their chemically synthesized counterparts commonly found in over-the-counter (OTC) medications. Arnao and Hernandez-Ruiz (2018) emphasise these benefits, highlighting the absence of potential contaminants that could arise during the synthetic process; and also, that these natural supplements often come enriched with other beneficial compounds, including antioxidants, vitamins, simple phenols, flavonoids, carotenoids, and tocopherols.

Moreover, the presence of phytomelatonin in natural plant extracts is not just significant for its direct effects but also for its synergistic interactions with co-extracted antioxidants. This synergy enhances the biological efficacy of these substances, making them more effective in their protective roles. Such antioxidants include glutathione, organic acids, flavonoids, phenolic acids, anthocyanins, and lipophilic molecules such as vitamins A and E (Arnao and Hernandez-Ruiz, 2018). The cooperative actions between phytomelatonin and these antioxidants emphasise the holistic health benefits of consuming plant-based supplements, further necessitating their inclusion in dietary regimens for their therapeutic potentials.

Concept of food synergy: Antioxidant synergism in foods

The concept of food synergy, as outlined by Messina *et al.* (2001), emphasises the importance of consuming whole foods to reap the full spectrum of health benefits provided by the phenomenon of natural synergism among antioxidants. This emphatically suggests that the complex interplay of nutrients and bioactive compounds in their natural form can offer more significant health benefits than the sum of their parts. The interaction between antioxidants when isolated from their food matrix can vary, leading to either synergistic or antagonistic effects (Peyrat-Maillard *et al.,* 2003). This variability highlights the complexity of food chemistry and its impact on the efficacy of dietary antioxidants. For instance, Sprung in 2014 highlighted a noteworthy decrease in the therapeutic potential of curcumin, known for its anti-cancer and anti-inflammatory properties, when isolated from turmeric compared to when it is consumed as part of the whole spice. Similarly, research by Gitto *et al.* (2001) demonstrated that lycopene, a potent antioxidant, exhibits diminished bioactivity when extracted from its natural source as opposed to when it is ingested within the food matrix. On the day this thesis has been compiled, 15 research articles have been cited on Scopus on the antioxidant synergy of food.

These findings suggest that the efficacy of natural bioactive compounds is significantly enhanced by their coexistence with other bioactive substances within foods. Supporting this, Chakraborty and Bhattacharjee (2018) and Kadoma and Fujisawa (2011) have indicated that the antioxidant activity of melatonin, for example, is amplified when it interacts synergistically with other antioxidants within a food matrix or biological system.

Therefore, maintaining the integrity of antioxidant synergism in food products is crucial. This approach not only preserves the natural potency of bioactive compounds but also promotes the

consumption of whole foods for optimal health benefits, aligning with the growing body of evidence that favours dietary patterns centred around whole, minimally processed foods.

The investigations of the present study emphasised on maintaining the naturally occurring synergism among the three target bioactive molecules, *i.e.,* L-tryptophan, serotonin, and melatonin, for the holistic utilization and valorization of plantains with the aim to preserve these three '*in house*' beneficial biotherapeutic molecules by means of three 'green' approaches *i.e.,* design of a value-added product from freshly harvested plantains; extension of shelf-life of freshly harvested plantains by a green technology of preservation; and development of a consumable food supplement utilizing an extract of the antioxidant triad from sensorially declined, fully senesced-irradiated plantains.

Plantain preservation through development of value-added food products: Traditional processing to innovative food product design based on antioxidant synergy

On the day this thesis has been compiled, 15 research articles have been cited on Scopus on the preservation of plantains (*Musa paradisiaca*). Substantial literatures exist on the development of plantain-based value-added food products employing various processing methods, such as frying, grilling, boiling, fermentation, and drying (Abiodun-Solanke and Falade, 2010). These food products include wine (Kumar and Uma, 2020); beer (Babayemi *et al*., 2010); ice-cream (Kurniasari *et al*., 2023); pickles (Kumar and Uma, 2020); flours; powders; canned slices; chips; jam and jelly (Abiodun-Solanke and Falade, 2010); mixed flour blends containing plantain, soy-cake, rice-bran, and oat-bran flours (Oluwajuyitan *et al*., 2021); instant foutou (fufu) flour by blending blanched semi-ripe plantain dried flakes, blanched/unblanched cassava dried flakes, and cassava starch (Gnagne *et al*., 2023). Plantain flour is used in production of cookies (Akubor *et al*., 2003); wheat-based biscuits enriched with benniseed and unripe

plantain (Agu and Okoli, 2014); bread (Sarawong *et al*., 2014), and cake (Onwuka and Onwuka, 2005). Few studies have also reported on the utilization of plantain peels in formulation of flour (Agama-Acevedo *et al*., 2016), and functional cookies (Arun *et al*., 2015).

Most reports (*vide supra*) focusing on traditional or fortified plantain-based foods, such as gluten-free flour blends and their derived food products, have primarily concentrated on the processing methods employed, characterization of these food items, sensory acceptability, proximate, physicochemical analyses, and acceptability tests. These studies have seldom delved into the evaluation of antioxidants that survive in these products, consequent to processing. However, a few studies, such as those by Agama-Acevedo *et al*. (2016) and Arun *et al*. (2015), have focused on the antioxidant effectiveness and phenolic contents of plantain flour and its cookies, respectively. Oluwajuyitan *et al*. (2021) have evaluated the glycemic index and hypoglycemic activity of a flour blend incorporating plantain flour. Yet, according to the author's best knowledge, the effects of traditional thermal processing methods on the antioxidants present in plantains, as well as the formulation of plantain-based food products with an emphasis on specific, less studied heat-sensitive antioxidants, have not been thoroughly explored. This research gap spurred the development of an antioxidant-rich, value-added plantain-based food product, notably a sugar-free plantain candy rich in L-tryptophan, serotonin, and melatonin, designed to preserve their natural antioxidant synergy.

The presence of antioxidants in any food product does not ensure their *in vivo* bioavailability post-consumption. Therefore, it is imperative to investigate whether the antioxidants are absorbed *in vivo*. However, not a significant body of research focusses on studies on *in vitro* release kinetics of bioactives from food matrices and their *in vivo* bioavailabilities. Major findings from literature available have been summarized below.

Studies on *in vitro* and *in vivo* bioavailabilities of bioactives from food products

Few studies have investigated the *in vitro* simulated digestion of food and food products and the subsequent changes in the levels of any bioactive molecules. Bermu´dez-Soto *et al*. (2007) reported the stability of phenolic compounds in chokeberry (*Aronia melanocarpa*) after *in vitro* gastric and pancreatic simulated digestion. Peixoto *et al*. (2013) investigated the bioaccessibilities of metallic elements in chocolate drink powder after *in vitro* simulated digestion using spectrometric techniques. Archaina *et al*. (2019) explored *in vitro* oral, gastric, and intestinal simulated digestion of freeze-dried candies from blackcurrant (*Ribes nigrum* L.) and yoghurt and analysed the antioxidant capacities of the treated samples. Similarly, Goulas and Hadjisolomou (2019) studied the changes in targeted phenolic compounds and antioxidant activity of carob fruit (*Ceratonia siliqua* L.) products during *in vitro* oral, gastric, and intestinal simulated digestion. Paz-Yépez *et al*. (2019) reported on the digestibilities of lipids and release of polyphenols after *in vitro* simulated digestion of dark, milk, and white chocolate. Marinelli *et al*. (2021) examined the *in vitro* simulated digestion of watermelon-based candy and antioxidant activity of the treated product. Additionally, two separate studies by Rivero *et al*. (2020 and 2021) investigated the *in vitro* simulated digestion of honey and propolis-containing gummy candies, as well as gelatin candies made with alternative sweeteners and fruit bioactive compounds and evaluated the antioxidant activity of the treated samples.

It is evident that the research highlighted above has predominantly focused on *in vitro* simulated digestion rather than exploring the *in vivo* bioavailabilities of bioactive compounds. This gap in research served as a catalyst for not only examining the *in vitro* release kinetics but also undertaking *in vivo* bioavailability studies (in rat models) for the newly developed plantain-based food product. The objective was to determine if the antioxidants are effectively absorbed by the body, thereby confirming the product's status as genuinely rich in antioxidants.

The research hypothesis of this segment of the study are as follows:

1. The new designer plantain-based value-added food product preserves the natural synergism in the antioxidant triad (L-tryptophan-serotonin-melatonin)

2. The antioxidant triad is truly bioavailable *in vivo*.

In addition to developing value-added food products, extending the shelf-life of raw plantains is crucial for the full utilization of these agricultural produce. The following section of the thesis summarizes literature reports on both traditional and eco-friendly technologies for preserving this agricultural commodity.

Plantain preservation through extension of shelf-life post-harvest: Conventional to green preservation technologies

Traditional methods for preserving plantains, such as drying, boiling, and frying (with the exception of fermentation), involve heat and are therefore detrimental to the heat-sensitive bioactive compounds they contain. However, Dick and Yao (1997) demonstrated that the shelf-life of plantains could be extended to 15 weeks by packaging them in 30 and 100 µm polyethylene bags and storing them at a temperature of 12 °C. It has been widely observed that employing controlled environment storage techniques, like controlled or modified atmosphere storage, significantly benefits the shelf-life extension of plantains (OLorunda, 1996). Some research has also looked into the use of chemical preservatives for plantains, including waxing (Adetuyi and Lola, 2010) and the application of various concentrations of acetic, sorbic, and propionic acid (Nwaiwu and Itumoh, 2017). Although these commonly used conventional food preservation methods, such as fumigation, curing, and chemical treatments, are non-thermal, they can nevertheless lead to a decline in food quality and pose risks to both human health and the environment (Van Kooij *et al*., 1981). In response to the rising global energy crisis and the

need to produce safe, high-quality foods while minimising post-harvest losses, the adoption of low-energy consuming, non-thermal food preservation methods such as radiation (chiefly gamma rays) processing is now increasingly recognized.

Compared to traditional food preservation methods, particularly thermal processing, irradiation stands out as both energy-efficient and equally effective in extending the shelf-life of agricultural commodities and food products (da Silva Aquino, 2012). Besides the shelf-life extension, gamma-irradiation also has a lesser-known application in augmentation of the bioactive molecules in the agro-produces (*vide infra*).

Applications of gamma-irradiation in augmenting synthesis of bioactive molecules in botanicals

On the day this thesis has been compiled, 2 research articles have been cited on Scopus on the effect of gamma-irradiation on bioactive molecules. Our research team previously reported a pioneering application of the non-thermal and cost-effective preservation technology of gamma-irradiation, demonstrating its capability to enhance lutein production by stimulating carotenogenesis in irradiated marigold flowers nearing the end of their senescence cycle (Pal and Bhattacharjee, 2016). In a related study, Gloria and Adão (2013) examined the effects of gamma-irradiation on the alteration of bioactive amines and found that gamma-irradiation at 1.0 kGy significantly increased spermidine levels during storage (likely as a response to stress induced by irradiation) and decreased the rate of serotonin degradation in Prata bananas. Additional research has highlighted the potential for gamma-irradiation to boost the production of bioactive compounds such as phenolics and flavonoids, carotenoids, anthocyanins, antioxidant enzymes including catalase, peroxidase, and superoxide dismutase, as well as organic acids in various agricultural products following treatment (Hussain *et al.,* 2016, Najafabadi *et al*., 2017, Yasmin *et al*., 2019).

The research hypothesis of this segment of the study are as follows:

1. Appreciable extension of shelf-life of fresh harvested plantains by gamma-irradiation

2. Augmentation in contents of the antioxidants, chiefly serotonin and melatonin in gamma-irradiated plantains

If the hypotheses mentioned above are indeed confirmed, the therapeutic bioactive molecules present in agricultural commodities could be utilized as food supplements, even after the commodities have perished. However, senesced commodities often become sensorially unacceptable to consumers. Therefore, the extraction of such therapeutically important molecules from the senesced fruits could reduce the wastage and promote waste valorization. The following section of the thesis cites literature reports on utilization of fully senesced plantains.

Utilization of fully senesced plantains

The literature review reveals very limited applications for over-ripened and senesced plantains, with the majority of these uses found in Africa. In Ghana, three traditional local delicacies-Kaaklo, Ofam, and Tatale incorporate senesced plantains as a key ingredient (Adi *et al*., 2021). In Côte d'Ivoire, West Africa, two other traditional dishes, Dockounou and M'bahou, are popular (Edwige *et al*., 2014, N'Guessan *et al*., 2022). However, to the best of the knowledge of the author, there is no documented evidence on the utilization of senesced (whether irradiated or non-irradiated) plantains as a bioresource for their bioactive compounds, nor on the extraction of bioactive antioxidants from these plantains.

Extraction of bioactives from senesced plantains

Ultrasonication-assisted solvent extraction (UAE) has become a widely adopted and efficient method for the extraction of natural bioactive molecules such as anthocyanins, polyphenols,

phenolics, and carotenoids from a variety of plant materials. This technique provides numerous benefits, including shortened extraction times, lower operational temperatures, reduced energy consumption, and lesser solvent usage (Kumar *et al*., 2021). Furthermore, when the solvent employed in UAE is of Generally Recognized As Safe (GRAS) status, this method offers the added advantage of being considered a green extraction technology. In UAE, ultrasonic energy (with a frequency greater than 20 kHz) causes the extraction medium to undergo a series of compressions and rarefactions (Tiwari, 2015), leading to the formation of thousands of microbubbles within the medium. The process involves continuous changes in pressure and temperature, causing the microbubbles to form, expand, and implode. This phenomenon, known as "acoustic cavitation," facilitates enhanced mass transport by disrupting plant cell walls, which is the fundamental principle behind UAE's efficiency in extracting bioactive compounds (Pringet *et al.,* 2013, Tiwari, 2015).

A study conducted by González-Montelongo *et al*. (2010) focused on optimizing UAE to obtain an extract from the peels of banana (*Musa acuminata* Colla AAA) and characterizing the resultant extract. Despite this advancement, there appears to be no existing literature on the application of UAE for the extraction of L-tryptophan-serotonin-melatonin from senesced plantains ad that too without perturbing the natural antioxidant synergy in the extract.

Use of bioactive-rich UAE extracts in designing food supplements

On the day this thesis has been compiled, 7 research articles have been cited on Scopus on the utilization of UAE extracts in development of food supplements. After the extraction of bioactive compounds, their incorporation into a convenient form for widespread application is essential. Numerous studies have documented the development of antioxidant-rich powders, predominantly through microencapsulation, utilizing spray-drying technology. There exists

literature on the development of microencapsulates from supercritical carbon dioxide extracts of various sources, including the algae *Phormidium valderianum* (Chatterjee *et al.,* 2014), a polyherbal mix of tulsi, bay, and cardamom (Ghosh *et al.,* 2017), small cardamom seeds (Dutta and Bhattacharjee, 2017), and lutein from marigold flowers (Pal and Bhattacharjee, 2018). Among numerous research efforts that have explored the microencapsulation of UAE extracts of natural products, several are particularly noteworthy due to their innovative approaches and significant findings, such as: spray-dried microencapsulates from the UAE extracts of -red pitaya (*Hylocereus polyrhizus*) (Vieira *et al*., 2024), -Sacha inchi shell (Mehta *et al*., 2024), -sugar maple leaves (Yeasmen and Orsat, 2024), -papaya seeds (Hinostroza-Quiñonez *et al*., 2024) and many more.

However, there appears to be a gap in the literature concerning the use of UAE green extracts for the design of food supplements. This gap indicates a potential area for innovative research in the utilization of green extraction methods for the development of bioactive-rich food supplements. The application of heat in the spray-drying process could indeed be detrimental to heat-sensitive molecules such as serotonin and melatonin. Hence, the current work proposes the use of a non-thermal technology, freeze-drying (lyophilization), to obtain a powder form of the antioxidant-rich UAE extract of plantains. This approach aims to preserve the integrity of these sensitive biomolecules. Subsequently, it is envisaged to design a deliverable form of this powder for end use as a food supplement, leveraging the preservation capabilities of freeze-drying to maintain the bioactivity of the extracted compounds for health benefits.

The research hypothesis of this segment of the study are as follows:

1. An antioxidant-rich green extract by UAE with preserved natural antioxidant synergy

2. An antioxidant-rich food supplement from UAE extract of plantains

Based on the above-mentioned research gaps, and the hypotheses framed, the following were the specific objectives of the present study:

- Development of a new nutraceutical antioxidant-rich food product (candy) from freshly harvested green plantains (*Musa paradisiaca*) and its physicochemical characterization, packaging and shelf-life study

- *In vitro* release kinetics and *in vivo* bioavailability studies of the antioxidant triad (L-tryptophan-serotonin-melatonin) from the developed food product

- Optimization of gamma-irradiation processing parameters for extension of shelf-life of '*desi*' variety of green plantains based on their sensory attributes, proximate, physicochemical and phytochemical properties

- Optimization of gamma-irradiation processing parameters to obtain enhanced amount(s) of serotonin and/or melatonin from gamma-irradiated plantains

- Optimization of UAE processing parameters for maximizing the yield of bioactive antioxidants, chiefly melatonin; and to achieve a synergistic blend of antioxidants in the extract using fully ripened, irradiated plantains as the raw material

- Development of a food supplement from the antioxidant-rich plantain extract and its characterization

This thesis has been divided into the following segments:

Development of a nutraceutical antioxidant-rich (specially L-tryptophan-serotonin-melatonin in synergism) food product (candy) from freshly harvested green plantains, its characterization and shelf-life study of the developed candy post-packaging

Utilizing freshly harvested, authenticated '*desi*' variety of green plantains cultivated under controlled conditions, an antioxidant (L-tryptophan, serotonin, and melatonin)-rich sugar-free candy was developed through minimal processing techniques. This approach aimed to preserve heat-sensitive molecules, namely serotonin and melatonin, minimising the use of food additives to maintain the natural (food) synergy of the antioxidant triad and avoid the negative health impacts associated with ultra-processed foods. The resulting plantain candy underwent detailed characterization for its sensory appeal, physicochemical attributes, and phytochemical content, including an analysis of the key antioxidants using high-performance liquid chromatography (HPLC) with a photodiode array (PDA) detector.

Following the development of the antioxidant-rich, sugar-free plantain candy, its shelf-life post-packaging in commercial flexible laminates, predominantly foil wrappers, was evaluated to ensure prolonged storability without compromising the product's physicochemical characteristics and functionalities throughout the storage period. The selected packaging materials were analysed for their mechanical and barrier properties to identify the most effective wrapper, focusing on microbiological safety and sensory appeal of the candy during storage. The best wrapper was ascertained based on the microbiological safety and sensory appeal of the packaged candy during storage. Shelf-life assessment of the packaged candy included assessment of microbial content, sensory attributes, physicochemical characteristics, and the content of key antioxidants (L-tryptophan, serotonin, melatonin) in the candy at regular

time intervals during storage. The methodologies and findings from these comprehensive studies are detailed in **Part 2.1 of Chapter 2** of the thesis.

In-vitro release kinetics and in-vivo bioavailability studies of L-tryptophan-serotonin-melatonin from the designer candy

Part 2.2 of Chapter 2 describes in detail the *in vitro* release kinetics study of the three target biomolecules from the designer plantain candy in simulated gut conditions (in simulated salivary, gastric, intestinal, and rectal buffers). The cumulative percentage of release of each molecule (from the candy) in each buffer was plotted against time, and the data were fitted into various standard kinetic equations. The kinetic model with the highest regression coefficient (R^2) was considered the best model for representing the release of the target biomolecules from the candy. This part of the study also elaborates on the evaluation of the bioavailabilities of the three antioxidant biomolecules in the serum of male Sprague Dawley rat models, post-gavaging of the candy (in suitable form). The levels of the three molecules in the rat blood serum were assessed at different time intervals before and after feeding.

Optimization of gamma-irradiation processing parameters for extension of shelf-life of 'desi' variety of green plantains based on sensory attributes, proximate, physicochemical, and phytochemical properties

The gamma-irradiation experiments were conducted at the National Instruments Limited campus, Jadavpur University, Kolkata, India, using a GC-5000 unit provided by the Board of Radiation and Isotope Technology (BRIT) under the Department of Atomic Energy (DAE), Government of India. This segment of the thesis discusses the application of gamma-irradiation on the '*desi*' variety of green plantains to extend their shelf lives. The process was optimized by carefully selecting processing parameters such as the dose of irradiation, rotation mode

within the irradiation chamber, and the incorporation of a polystyrene cushion in the sample chamber to ensure proper attenuation. The effectiveness of gamma irradiation in extending shelf-life was evaluated by examining the sensory attributes, proximate composition, physicochemical, and phytochemical properties of the irradiated plantains, in comparison to their non-irradiated counterparts. Detailed results of these investigations are provided in **Part 3.1, Chapter 3** of the thesis.

Optimization of gamma-irradiation processing parameters to obtain enhanced amount of serotonin and/or melatonin or both in combination (in antioxidant synergy) from gamma-irradiated plantains

This work reports for the first time on optimization of processing parameters (such as irradiation dose, rotation/static mode of sample chamber, and use of polystyrene cushion in the sample chamber) of gamma-irradiation for augmentation of the said bioactive antioxidants, namely L-tryptophan, serotonin, and melatonin in green plantains. The assessment also concurrently examined the natural antioxidant synergy among the targeted biomolecules in the irradiated plantains. This involved evaluating how the irradiation process influenced the interplay and effectiveness of L-tryptophan, serotonin, and melatonin in acting together to enhance the antioxidant capacity of the plantains. This aspect of the research aimed to understand not only the individual enhancement of these bioactive molecules but also how their combined presence might offer greater health benefits through their synergistic antioxidant effects. The techniques employed and the results of these investigations are thoroughly detailed in **Part 3.2, Chapter 3** of the thesis, offering new insights into the potential of gamma-irradiation for augmenting beneficial compounds in agro produce.

Optimization of UAE processing parameters for maximizing the yield of bioactive antioxidants, chiefly melatonin; and to achieve a synergistic blend of antioxidants in the extract using fully ripened, irradiated plantains as the raw material

UAE was conducted to extract the biotherapeutic molecules L-tryptophan, serotonin, and melatonin from irradiated and fully senesced green plantains using green solvents. This green extraction was facilitated by a probe sonicator, with key parameters including percentage amplitude, extraction time, and the ratio of sample to solvent, each varied at three distinct levels. To optimize these extraction process parameters, a central composite rotatable design (CCRD) was integrated with response surface methodology (RSM). The optimization primarily focused on achieving the highest yield of melatonin in the extract (since the amount of melatonin was highest in completely senesced plantains), leveraging the advanced statistical and experimental design techniques provided by CCRD and RSM to systematically explore and identify the optimal conditions for efficient extraction.

The UAE extract of senesced plantains obtained at the optimized conditions was thereafter subsequently analysed for antioxidant synergy among L-tryptophan, serotonin, and melatonin. This study is pioneering in its approach to optimize UAE parameters specifically to maximize the yield of melatonin while also aiming to obtain an extract that exhibits a synergistic effect among the mentioned antioxidants derived from irradiated and fully senesced plantains. This dual focus on optimization of extraction parameters for maximizing production yield of target molecule(s) and to achieve a synergistic antioxidant activity in the extract, marks a significant advancement in the extraction process of biotherapeutic molecules. The methodologies employed, along with the results of these investigations are thoroughly detailed in **Part 4.1, Chapter 4**, showcasing the novel approach and its outcomes in harnessing the antioxidant properties of green plantains.

This section further delved into the chemical profiling of the UAE extract, obtained under optimized conditions, through several advanced analytical techniques. Energy-dispersive X-ray (EDX) spectroscopy was utilized for the detection of any heavy metals in the extract, ensuring its safety for consumption or use. The presence of the target antioxidants-L-tryptophan, serotonin, and melatonin—was validated through electrospray ionization-time-of-flight mass spectrometry (ESI-TOF-MS), a powerful tool for identifying and quantifying molecules based on their mass-to-charge ratio with high accuracy and resolution. Additionally, the chemical analysis concerning the antioxidant synergy within the extract was conducted, providing insights into how these compounds interact to enhance the overall antioxidant capacity. This comprehensive chemical profiling not only confirms the extract's compositional integrity but also sheds light on its potential health benefits stemming from the antioxidant synergy.

Development of a food supplement from the antioxidant-rich plantain extract and its characterization

The focused aim of this segment of the research was to create a deliverable food supplement from the UAE extract of plantains, leveraging the *in vitro* demonstrated synergistic effects of melatonin with serotonin and L-tryptophan. To achieve this, a freeze-dried powder was prepared using the UAE extract, which preserved the integrity and synergistic antioxidant properties of the bioactive compounds. This powder then served as the basis for developing a food supplement.

The chapter dedicated to this process provides a comprehensive examination of the formulation and physicochemical properties of the freeze-dried powder. Special attention is given to its structure, thermal stability, storage stability, and antioxidant efficacy. Furthermore, it delves into the *in vitro* release kinetics of melatonin, serotonin, and L-tryptophan from the formulated

food supplement, offering insights into how these bioactive molecules could be possibly delivered *in vivo* upon consumption.

This in-depth analysis ensures that the final food supplement is not only effective in delivering the desired health benefits but also stable and safe for consumption over time. The critical details of the formulation process, alongside the physicochemical and release kinetics studies, have been meticulously outlined in **Part 4.2, Chapter 4** of the thesis, providing a clear roadmap for the development of similar health-oriented food supplements in the future.

The overview of the present research study has been shown in Fig. 1.8. The specific research objectives under the aforesaid broad objectives and a brief discussion on novelty (under the section of '**Novelty**') of each investigation have been enumerated in the respective chapters of the thesis. The highlights of the important findings have been summarized in the '**Resume**' section.

Tables:

Table 1.1: Agricultural information of bananas

Species	Preferable growth conditions	Cultivation area
***Musa* sp.**	Tropical humid lowlands (from sea level to an elevation of 2000m. above mean sea level), deep (at least 60 centimeters), rich loamy soil (organic material with high nitrogen content, adequate phosphorus levels, and plenty of potash is good for bananas), having good drainage, adequate fertility, moisture, a pH range of 6.5-7.5 (neither too acidic nor too alkaline) and not compacted. A temperature range of 15 ºC-35 ºC with a relative humidity of 75-85% is recommended (Stover and Simmonds, 1987)	Humid tropical and subtropical regions of Asia, Africa, and America, extend into Europe and Australia, encompassing 135 countries (Ajijolakewu *et al*., 2021)

Table 1.2: Appearance, consumption, and nutritional factors about plantains

Characteristics	Plantain
Scientific name	*Musa paradisiaca*
Physical appearance of fruit	Larger in appearance than banana, have a thicker peel with a firmer, starchy, less-sweet flesh
Consumption	Usually consumed when green and unripe, thus required cooking (boiled, steamed, grilled, or fried)
Composition of fruit pulp (Udo *et al*., 2021)	
Proximate (%)	Moisture- 14.23±0.06 Protein - 8.14±0.53 Ash - 1.34±0.02 Fat - 5.69±0.27 Fiber - 5.03±0.38 Carbohydrate - 65.52±1.09
Amylose content (% of dry weight)	17.51±1.25
Amylopectin content (by difference)	44.29±1.20
Starch (g/100g)	Resistant - 36.01±0.51 Non-resistant - 25.79±0.88 Total - 61.80±1.21
Dietary fiber (g/100g)	8.10±0.20
Pectin content (%)	27.00

Composition of fruit peels (Tsado *et al*., 2021)	
Proximate (%)	Moisture - 4.38±0.03 Ash - 6.17±0.05 Protein - 3.97±0.07 Crude fiber - 8.36±0.04 Fat - 3.01±0.06 Carbohydrate - 74.12±0.56
Mineral content (mg/100g)	Copper - 0.59±0.83 Iron - 7.89±0.79 Manganese - 1.25±0.39 Zinc - 13.30±0.57 Calcium - 14.70±0.25 Magnesium - 45.21±4.36 Sodium - 76.88±0.89 Potassium - 14±2.68 Phosphorus - 28.95±0.94
Amino acid (g/100g)	Leucine - 7.76±0.05 Lysine - 7.90±0.03 Isoleucine - 5.24±0.05 Phenylalanine - 4.79±0.06 Norleucine - 0.02±0.00 Trytophan - 0.58±0.03 Valine - 5.67±0.01 Methionine - 1.60±0.03 Proline - 3.25±0.02 Arginine - 4.99±0.03 Tyrosine - 3.96±0.06 Histidine - 2.11±0.04 Cystine - 0.85±0.02 Alanine - 6.22±0.05 Glutamic acid - 12.72±0.02 Glycine - 3.94±0.01 Threonine - 5.38±0.06 Serine - 4.05±0.04 Aspartic acid - 8.68±0.02 Total - 89.71±5.45

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Figures:

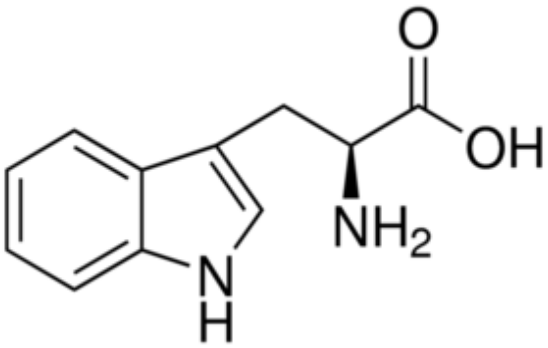

Figure 1.1: Chemical structure of L-tryptophan ($C_{11}H_{12}N_2O_2$)

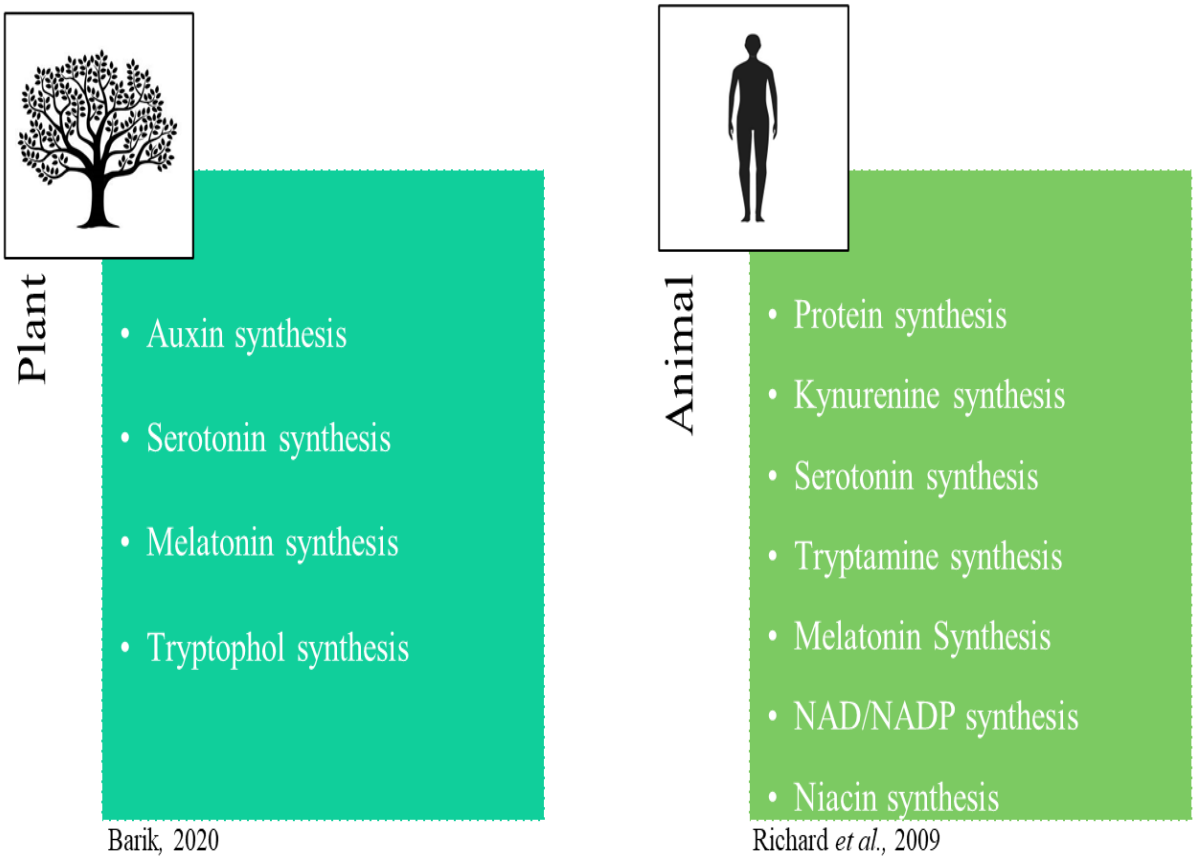

Figure 1.2: Biological functions of L-tryptophan

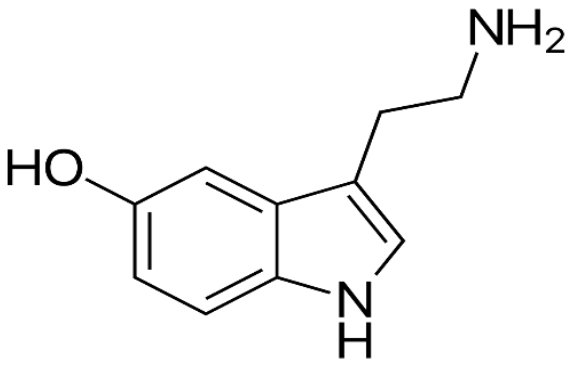

Figure 1.3: Chemical structure of serotonin ($C_{10}H_{12}N_2O$)

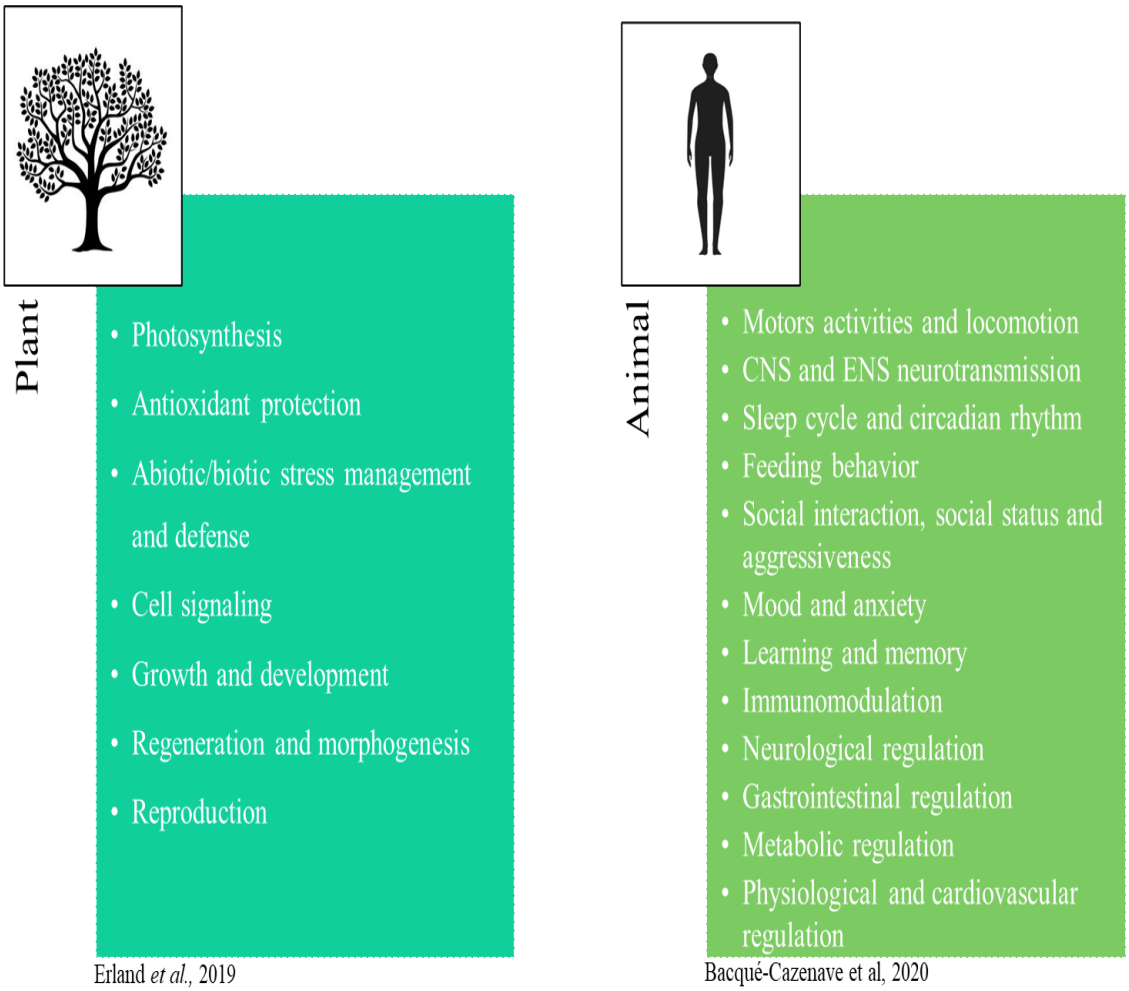

Figure 1.4: Biological functions of serotonin

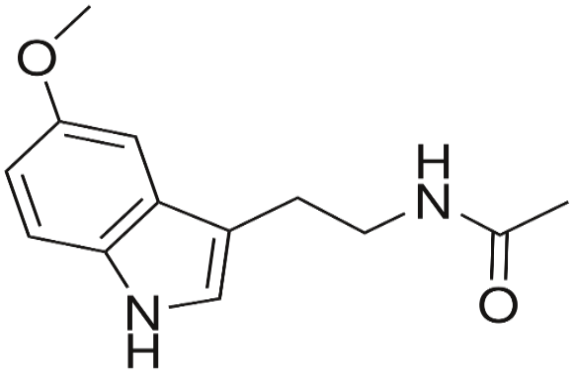

Figure 1.5: Chemical structure of melatonin ($C_{13}H_{16}N_2O_2$)

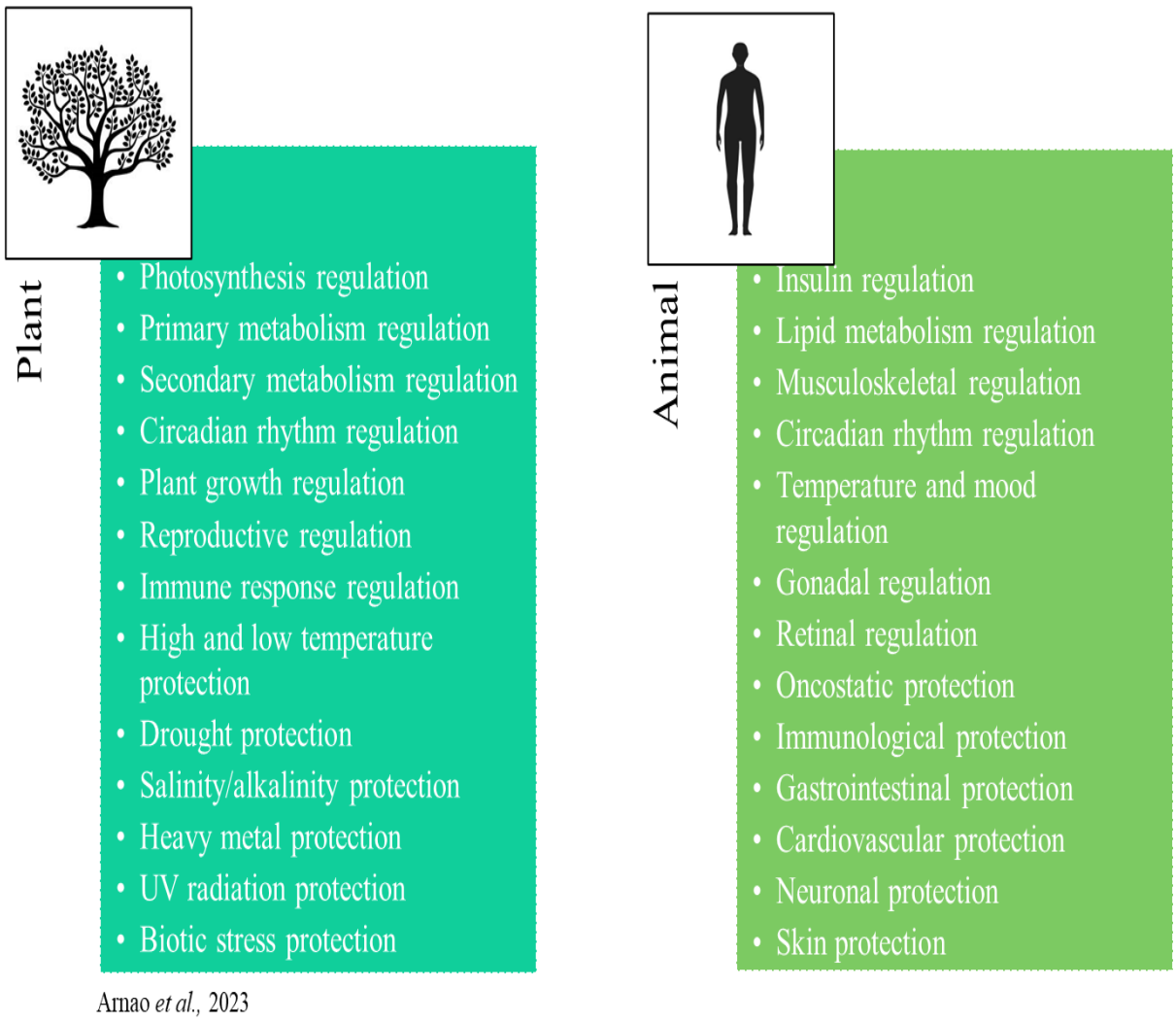

Figure 1.6: Biological functions of melatonin

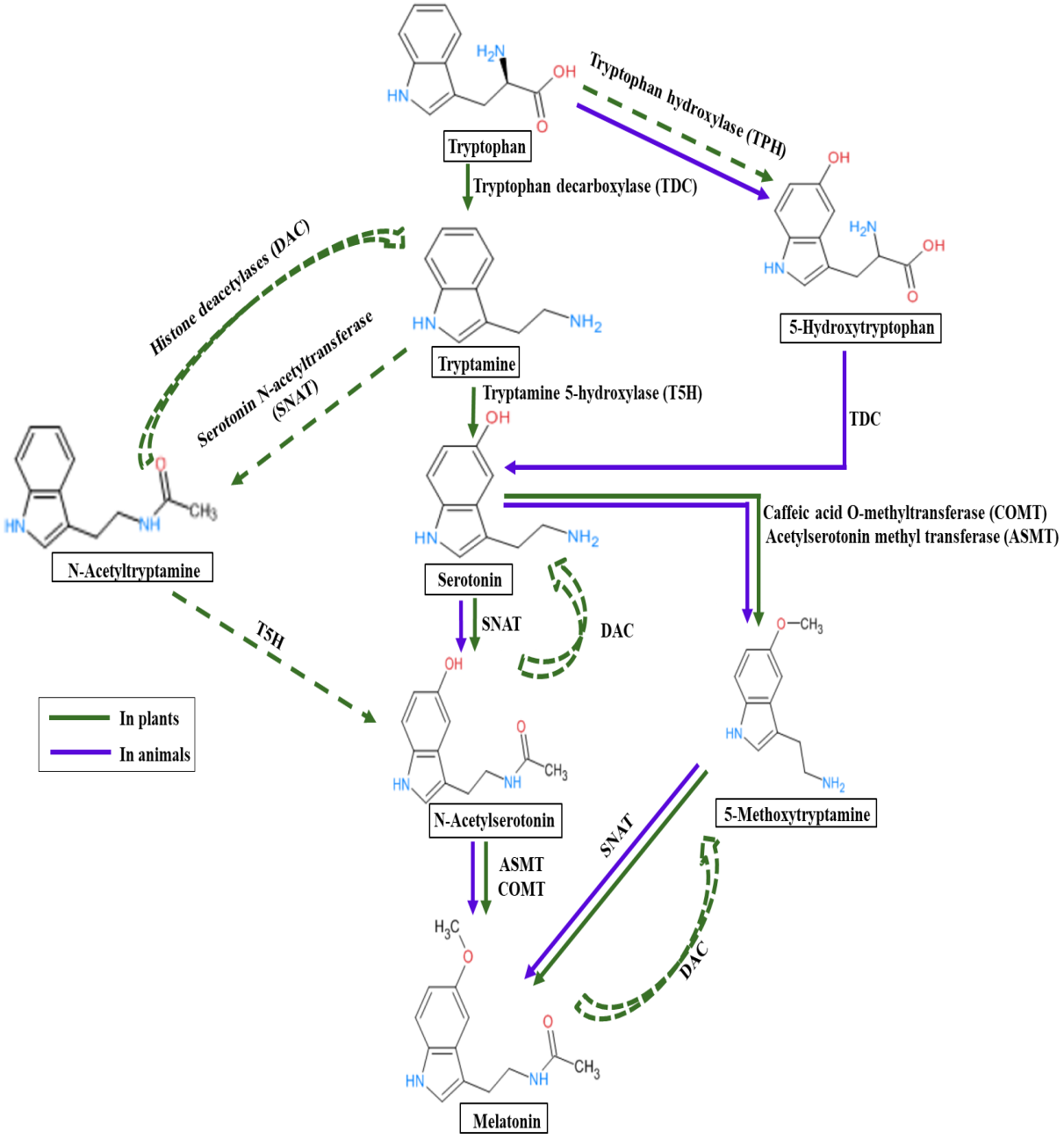

Figure 1.7: Bioconversion of serotonin and melatonin from L-tryptophan in plants: Dashed lines indicate unproven reactions (Arnao *et al*., 2023)

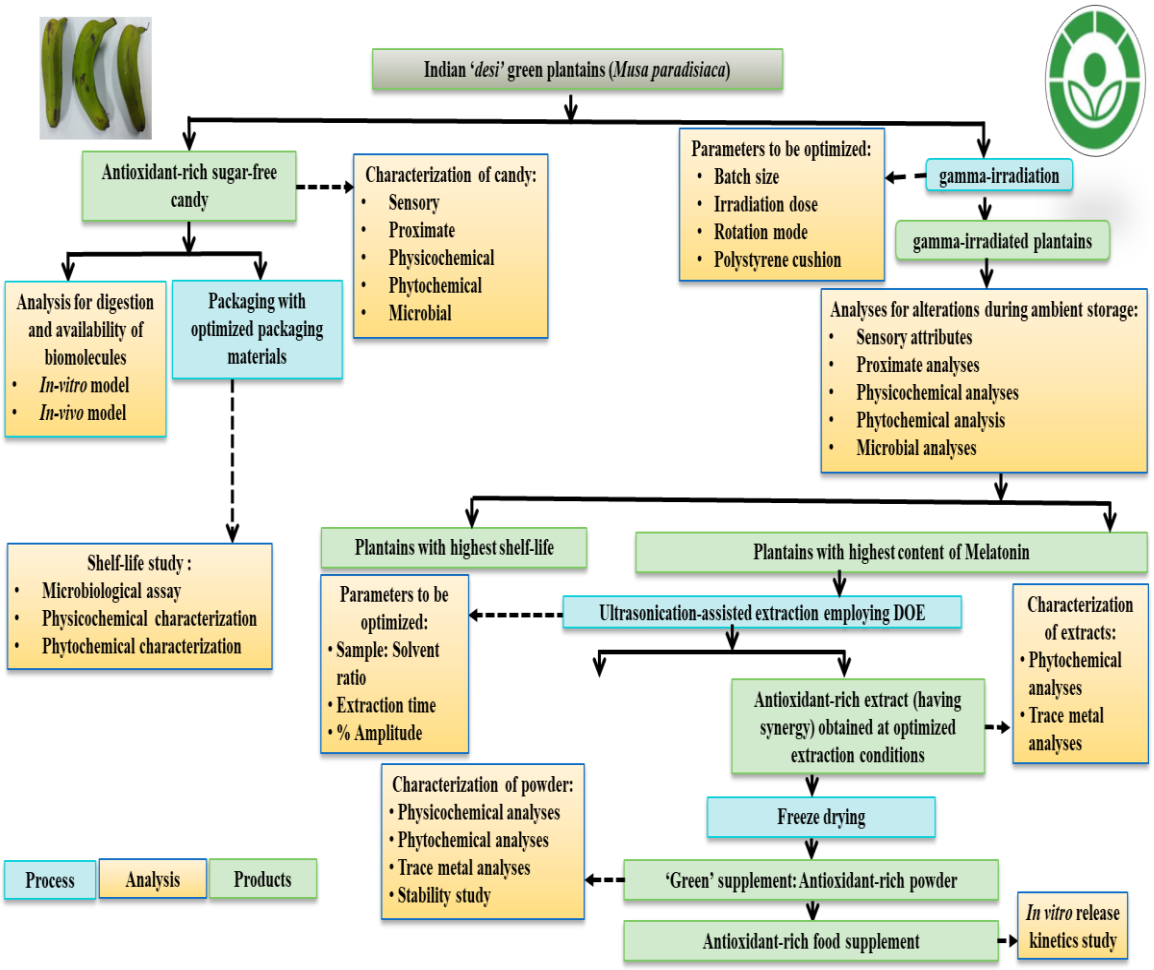

Figure 1.8: Overview of the research work

Chapter 2

Development of an Antioxidant-Rich Sugar-Free Plantain Candy, Assessment of Its Shelf-Life in a Flexible Laminate and Assessment of Its Bioavailability

Introduction

Candies are very popular with all age groups worldwide. In America, 26% of the total population (aged ≥ 2 years) consume an average of 40g of candy (176 kcal) per day (Duyff *et al*., 2015). However, the added sugars and saturated fats present in candies are specifically responsible for several health-debilitating metabolic and cardiovascular disorders (Duyff *et al*., 2015). In addition, the presence of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Alikonis, 1979) in commercially available candies do pose adverse health consequences (Gámez-Meza *et al*., 1999). Even in candies produced from natural fruit or vegetable pulps and juices, the contents of natural antioxidants are compromised consequent to heat degradation that occur during thermal processing. Furthermore, the presence of miscellaneous food additives in them in addition to high fat and sugar, could possibly impede utilization of their health-beneficial antioxidants (if they survive post processing). These do not conform to labelling the fruit/vegetable candies to be antioxidant-rich. We envisage that minimal processing encompassing usage of minimal additives with concomitant low sugar content can render a plantain-based candy to be truly antioxidant-rich and thereby minimize adverse health effects.

Some recent studies documented the formulation of candies with substitutes of the commonly used ingredients to circumvent the deleterious effects of candy consumption. Few researchers have successfully incorporated amla powder and green tea extract (Hitlamani *et al*., 2021), sweet orange (Mauliza and Dalimunthe, 2022), spices such as ginger, cinnamon, holy basil along with powdered green tea (Dhawan *et al*., 2023), and a mixture of turmeric, ginger, liquorice, white wormwood, and citrus lemon extracts (Souiy *et al*., 2023) to develop antioxidant-rich hard candies. Several studies have reported the formulation of fruit candies utilizing quince (Mir *et al*., 2015), pumpkin (Muzzaffar *et al*., 2016), amla (Katke *et al*., 2018), and unripe mango (Mahato *et al*., 2020). The juices of sea buckthorn and quince (Lele *et al*., 2018), acetic, alcoholic, and lacto-fermented apple juice products (Bartkiene *et al*., 2021), as

well as fermented algae such as *Spirulina* (Bartkiene *et al*., 2023) have been used in the production of chewy candies. The production of soft candies (gummy jellies) by adding *Psidium guajava* leaf extract (Charoen *et al*., 2015), bovine colostrum, essential oils, and probiotics (Bartkiene *et al*., 2018), and pomegranate juice (Cano-Lamadrid *et al*., 2020) are well-documented. A few researchers have also explored preparation of the same using starch or sugar substitutes such as inulin (Delgado and Bañón, 2018). Furthermore, use of natural antioxidants such as stevia-rosemary extract (Cedeño-Pinos *et al*., 2020) along with fructan fibres (chicory inulin, and fructo-oligosaccharides), green propolis extract with fructans (Cedeño-Pinos *et al*., 2021), and sage extract with inulin-gelatine-fructooligosaccharides (Cedeño-Pinos *et al*., 2023) have also received recognition. However, information on hard and semi-hard candy production in the truest sense is lacking.

Studies on the formulation of hard and semi-hard candies utilizing sugar substitutes are scarce, except for a solitary report by Jeon *et al*. (2021) on the formulation of a nutraceutical hard candy using isomalt, maltitol syrup, and xylitol as sucrose substitutes. Here extracts of *Cudrania tricuspidata* (mandarin melon berry) fruit, lemon, and ginger as sources of natural antioxidants have been utilized. However, to the best of the knowledge of the author, there is no report on the development of natural antioxidant-rich (plantain) sugar-free hard or semi-hard candy emphasising the molecular identities of important biotherapeutic molecules, specifically L-tryptophan, serotonin, and melatonin.

To render a food product truly antioxidant-rich, the bioaccessibility and bioavailability of the biomolecules from the same must be evaluated. Significant reports exist on the bioavailability of melatonin, such as the enhancement of melatonin in chicks when fed a melatonin-rich diet (consisting of corn, milo, beans, and rice) as documented by Hattori *et al*. (1995), and also from walnuts when fed to rats as reported by Reiter *et al*. (2005). Delgado *et al*. (2012) documented increases in serum levels of serotonin and melatonin in both young and aged rats and ring doves after feeding them with a Jerte Valley cherry-based product mix. Continuing the study, Garrido

et al. (2013) found a significant increase in urinary 6-sulfatoxymelatonin levels and actual sleep time in human volunteers after consuming the product twice a day for five days. Bravo *et al*. (2013) reported on the enhancement of both these molecules in 35 human volunteers (aged 55-75 year) when tryptophan-enriched cereals were served as breakfast and dinner for three weeks. However, literature on the bioavailability of the antioxidant triad (L-tryptophan, serotonin, and melatonin) in an animal model from a processed food product developed using a widely consumed farm product (plantain) is lacking. Therefore, a detailed investigation aiming to assess the release of the aforementioned molecules from the food product *in vitro* under simulated gut conditions and also *in vivo* through feeding trials in male Sprague Dawley rats was deemed necessary to render the newly developed candy truly antioxidant rich.

The bioavailability of the above-mentioned biomolecules would depend on the metabolism of the newly designed candy. The liver plays a central role in processing and distributing nutrients throughout the body during food metabolism, while glucose produced after food metabolism is utilized by the brain and gut (Nelson and Cox, 2002). Therefore, in the current study, to evaluate whether the liver, peripheral muscles, brain, and gut are involved centrally during the metabolism of the designer candy, blood glucose and insulin levels were assayed in the animals at specific time intervals after consumption of the designer candy. The data were then fitted into apposite predictive iHOMA2 models (Hill *et al*., 2013).

Therefore, this chapter of the thesis has been divided into the following parts:

- *Development of a nutraceutical antioxidant-rich (specifically L-tryptophan-serotonin-melatonin in synergy) food product (candy) from freshly harvested green plantains, its characterization and shelf-life study post-packaging*

- *Assessment of in vitro release kinetics and in vivo bioavailability of L-tryptophan-serotonin-melatonin from the developed candy*

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Part 2.1

Development of a nutraceutical antioxidant-rich (specially L-tryptophan-serotonin-melatonin in synergism) food product (candy) from freshly harvested green plantains, its characterization and shelf-life study of the developed candy post-packaging

Introduction

The first objective of this part of the present research work was to develop an antioxidant-rich sugar-free candy that could serve as a potential food source of L-tryptophan, serotonin, and melatonin, without perturbing the intrinsic natural antioxidant synergy (Chakraborty and Bhattacharjee, 2018, Paul *et al*., 2019). The chief impediment in designing antioxidant-rich foods lies in the preservation of the natural food synergy (Messina *et al*., 2001), which, if altered, would render the consortium of antioxidants present in the finished food product detrimental rather than beneficial *in vivo* (Chakraborty and Bhattacharjee, 2018, Paul *et al*., 2019). The current investigation therefore focused on the modification of the candy processing parameters by (i) using minimal food additives to aid unimpeded release of antioxidants; (ii) minimizing the losses of heat-sensitive molecules (chiefly serotonin and melatonin); (iii) employing minimal processing not only to preserve the natural antioxidant synergy of the green plantains but also to avert the destructive side effects of consuming ultra-processed food products (Bhattacharya, 2023); and (iv) utilizing substitutes for the classical 'Doctors' (Minifie, 1989) to deliver a nutraceutical yet palatable sugar-free candy.

The second objective of this part of the study was to assess the shelf-life of the newly formulated designer candies after packaging in commercially available flexible laminates (chiefly foil wrappers) to ensure an extended shelf-life without compromising the physicochemical properties of the candies throughout the storage period.

The novelty of this investigation lies in designing a value-added food product using a widely cultivated healthy fruit. This could be a unique confectionary product housing important biotherapeutic antioxidant molecules, besides promoting better utilization of plantains globally.

Materials and methods

Materials

Plantains (*Musa paradisiaca*) of the '*desi*' variety were procured from registered farmers (cultivators) of an organic plantain farm in Narendrapur, South 24 Parganas, West Bengal, India. The plantains were grown at coordinates 22° 43'91" N and 88° 39'68" E, approximately 9 meters above sea level, in the eastern Gangetic plains of India, with a temperature range of 24-35°C and 75-85% relative humidity, in deep, rich loamy, well-drained, moisture-retentive soil with a pH of 6.5-7.5 (Anonymous, 2024). The species of the organically cultivated fruits were authenticated by the West Bengal Food Processing and Horticulture Development Corporation Limited, Kolkata, India. The procured plantains were removed from their respective bunches, and individual plantains were meticulously selected based on visual assessment of colour (an indicator of ripeness: only green, unripe plantains were chosen), surface morphology, and texture (free of blemishes, bruises, and black spots, and turgid), and weight (approximately 175±10 g on average, with a diameter of approximately 120±5 mm). All chosen plantains were at the same stage of maturity.

For candy ingredients, food-grade chemicals such as D-sorbitol, D-mannitol, gum acacia, SiO_2, sorbic acid (all in powder form), and vanilla essence were purchased online from Amazon.in. Specialty chemicals, such as 2,2-diphenylpicrylhydrazyl (DPPH), HPLC-grade acetonitrile, acetic acid, and water; standards such as L-tryptophan, serotonin, melatonin, gallic acid were purchased from M/s Merck, Mumbai, India. Folin Ciocalteu's reagent, culture media for

microbiological analyses, and all other AR grade chemicals were purchased from M/s HiMedia, Mumbai, India. For solid phase extraction (SPE), QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) kits including the SPE tubes and dispersive universal kit, were purchased from M/s Agilent Technologies, Wilmington, USA.

Methods

The experimental design of the present study has been elaborated in Fig. 2.1.1. For the development of a new plantain-based antioxidant-rich sugar-free candy, it was necessary to first assess the antioxidant potential of the raw plantains as well as the content of the desired molecular antioxidants, namely L-tryptophan, serotonin, and melatonin. Three plantain samples were randomly selected from the procured sample lot and analysed for their antioxidant potency; chiefly their total phenolic content (TPC), DPPH radical scavenging activity, and ferric ion reducing antioxidant power (FRAP), as well as their contents of L-tryptophan, serotonin, and melatonin. Detailed methodologies for the same have been described *vide infra*.

Preparation and characterisation of lyophilized plantain powder

The fully matured green plantains were washed thoroughly, then cut into very small cube-shaped pieces along with the skin (except for the stalk and the black tip) and frozen in an ultra-low temperature freezer (Premium C340, M/s New Brunswick Scientific, Enfield, USA) at −80 °C for 24h and finally dried to a final moisture content below 6% using a bench top freeze dryer (FDU-1200, M/s Eyela, Tokyo, Japan) at −45 °C, 14.4 Pa. The freeze-dried plantain pieces were then ground in a mixer-grinder (HL 1618, M/s Philips India Limited, Chennai, India) to obtain a powder with a particle size of <300 µm, and the same was subjected to solvent extraction in accordance with the method described by Dal-Bó and Freire (2022), with few modifications. The lyophilized plantain powder (0.5g) was initially dissolved in dimethyl

sulfoxide (DMSO) in a 1:10 solid/solvent ratio in an incubator shaker (IS 02, M/s Incon Instruments Company, New Delhi, India) at 120 rpm for 2h at 30 °C, following which the mixture was centrifuged (R-8C, M/s Remi, Mumbai, India) at 1,000 × g for 15 min. The supernatant was analysed for its antioxidant activity (in terms of TPC, DPPH radical scavenging activity and FRAP value) in accordance with the procedures described *vide infra.* The lyophilized plantain powder was subsequently used in preparation of the candy (*vide infra*).

Formulation of a plantain-based, antioxidant-rich, sugar-free candy

The ingredients selected for the formulation of the antioxidant-rich candy, their functions, and the safe limits of usage (FSSAI, 2011a) have been presented in Table 2.1.1. Several preliminary trials were conducted to determine the exact amount of each ingredient for the candy formulation. Sugar alcohols such as D-sorbitol and D-mannitol were selected as the base ingredients (Roger, 1973) for the candy formulation, mainly because they provide fewer calories (3 cal/g and 2 cal/g, respectively) (Dwivedi, 1991), have a negligible glycemic index (4 and 2, respectively) (Chéron *et al*., 2019) compared to regular table sugar (4 cal/g; glycemic index: 65); and also serve as plasticizers (Alikonis, 1979). Since D-sorbitol and D-mannitol have a relative sweetness of 0.6 and 0.5-0.6, respectively (Pepper, 1990), compared to sucrose (relative sweetness: 1), it was opined that the sweetness of the formulated candy should not be compromised. Gum acacia as a thickening agent was varied in a concentration range of 3-35%. The preliminary trials established that an amount of 30% (based on the total weight of the candy ingredients) could impart the desired "stand-up'' property to the candy. The amount of lyophilized plantain powder was varied in a concentration range of 55-85% of the total weight of the candy ingredients. It was found that a concentration of 65% conferred desirable sensory properties to the candy. Quantities less or more than 65% imparted improper texture to the

candies, *i.e.*, they were either too hard or poorly formed, *i.e.*, lacking structural integrity (data not shown).

To formulate a plantain-based candy sample (PCS), sorbitol, mannitol, gum acacia, SiO_2, sorbic acid, and water were mixed using a spatula, and the mixture was heated (180-210 ℃) with constant stirring (to prevent lump formation) until it acquired the desired caramel colour. Then the lyophilized plantain powder and vanilla essence were added to the mixture after it had cooled to 60 ℃ to preserve the targeted heat-labile antioxidants. The mixture was then 'seeded' with sorbitol and mannitol powder and allowed to set inside candy moulds for 75 min. In this procedure, the use of food additives was kept to a minimum. No synthetic food colours, organic acids (acidulants), or synthetic antioxidants were used in the formulation of the candies, contrary to those used in the production of commercial candies (Alikonis, 1979). In the formulation of the experimental control candy (without the plantain) sample (CCS), all ingredients and procedures were similar to those used for formulating PCS, except that no plantain powder was used. The weights and dimensions of the resulting CCS and PCS samples were measured using a weighing balance (BSA 224S-CW, M/s Sartorius AG, Göttingen, Germany) and a vernier calliper (532 series, M/s Mitutoyo, Kawasaki, Japan), respectively.

Assessment of the safety parameters of CCS and PCS

The analyses described below were performed to evaluate the safety profile of the newly formulated candies.

Microbiological assessment

The microbial load of the candies for bacteria and fungi (as CFU/g candy) was evaluated in terms of total plate counts of the formulated candies using the pour plate method (Bose *et al*., 2023).

EDX analyses

To ensure the absence of toxic heavy metals such as Pb, Ti, Hg, Ni, As, Si, and Mo, that may be present in the raw material (plantains) or may have been acquired during the production of the candies, EDX analyses of CCS and PCS were performed using INSPECT F50, M/s FEI Company, Hillsboro, USA.

Sensory and physicochemical characterisation of CCS and PCS

The analyses described below were performed to evaluate the eating quality of the newly formulated candy samples primarily in terms of characteristic plantain flavour (assessed by sensory evaluation) and mouthfeel (assessed by both sensory and by textural evaluation).

Sensory evaluation

The sensory evaluation of CCS and PCS was performed using an acceptance test based on an effective method, namely, rating on a 9-point hedonic scale (where 9 denotes 'like extremely' and 1 denotes 'dislike extremely'). The candy samples were served to the participants using the serving protocols used for sensory evaluation of foods and beverages described in E1871-17 ASTM International methodology (ASTM International, 2017). Fifteen men and fifteen women (35-45 years old) were selected from faculty members and research scholars of our university to form a semi-trained sensory panel (Ranganna, 1986). Panellists were selected based on their interest, and their performance was assessed using screening tests conducted with the control sample. Prior to sample evaluation, they were well acquainted with the sensory attributes of the prepared candies and the commercially available hard candies (*Mango bite* and *Poppins*) and asked to evaluate the experimental candies in terms of appearance, texture, colour, taste, flavour, mouthfeel, aftertaste, and overall quality. The sensory evaluation was

conducted in the morning from 10 a.m. to 12 noon in a well-ventilated room with white light. Unsalted crackers, and water (to rinse the palate) as well as expectoration cups (covered) were provided to all participants before each evaluation if they did not wish to swallow the samples (Stone and Sidel, 2004, Bose and Bhattacharjee, 2023). Triplicates of each individual sample were served in each session, and the mean scores (rounded off) were presented graphically in radar plots.

Colour and texture analyses

Instrumental analyses of the colour and texture properties of the newly formulated candy samples were performed to validate the sensory ratings provided by the human judges (as described above) and to remove subjectivity. CIE colour value analysis (L* for lightness, a* for red-green, b* for yellow-blue) of CCS and PCS was conducted using a colour reader (CR-10 Plus, M/s Konica Minolta Inc., Osaka, Japan) (Trisnawati *et al*., 2019). The texture profiles of CCS and PCS were analysed by the two-bite compression test using TA.XT Express Texture Analyser (M/s Stable Micro Systems, Godalming, UK) in accordance with the method described by Rao *et al*. (2020). The entire PCS and CCS were subjected to texture analysis. An aluminium cylinder-shaped probe (P/5) with a 5-mm-diameter was used for the test, and the texture analysis parameters were set as follows: pre-test speed: 1 mm/sec, test speed: 5 mm/sec, post-test speed: 5 mm/sec, target mode: distance, distance: 5 mm, strain: 75%, trigger type: auto (force), trigger force: 5 g, loadcell: 10 kg. The sample was oriented so that the compression was symmetrical to its geometric centre. The TPA graph was generated using Exponent Lite Express version 6.1.4.0 software (M/s Stable Micro Systems Ltd., Godalming, UK). Parameters such as hardness (g), adhesiveness (g.sec), cohesiveness, springiness, gumminess, and chewiness of plantains were determined by the software.

Microstructure analyses of CCS and PCS

The following analyses were carried out for the detailed structural characterisation of the newly formulated candies.

Field emission scanning electron microscopy (FE-SEM)

The surface morphology of the new designer candies was investigated using FE-SEM (INSPECT F50, M/s FEI Company, Hillsboro, Oregon, USA) at an operational voltage of 5 kV. First, the samples were dried under vacuum (6-7 Pa) and then coated with gold using a coating device (Q150R ES, M/s Quorum Technologies Ltd., Ashford, England).

X-Ray diffraction (XRD) analysis

XRD patterns of CCS and PCS were analysed at ambient temperature (23±2 °C) (D8 Advance, M/s Bruker, Massachusetts, USA) using Cu-Kα1 radiation at a wavelength of 0.15406 nm. The measurements were performed at a voltage of 40 kV/40 mA. The XRD data were collected to cover an angular range (2θ) of 30-100 ° at a width of 0.01 ° and a counting time of 0.5 s/step.

Thermal stability assessments of CCS and PCS

It is necessary to study the thermal stability of the candies in order to assess the thermal (and therefore structural) changes that the candies could undergo during storage and transportation, post packaging under ambient conditions (*vide infra*).

Thermogravimetric analysis (TGA)

To predict the thermal stability of CCS and PCS, a TGA was performed using the TGA 4000 (M/s Perkin Elmer, Hopkinton, MA, USA). In this analysis, an empty platinum crucible containing α-alumina powder was used as a reference. The samples, which were individually

placed in a hermetically sealed Al pan, were heated from 51 to 600 °C and scanned at a rate of 10 °C/min under a nitrogen flow of 20 mL/min.

Differential scanning calorimetry (DSC) analysis

For DSC analysis, CCS and PCS were equilibrated at -80 °C for 5 min, then heated to 130 °C at a rate of 5 °C/min and held this temperature for 1 min. The samples were then cooled to −50 °C at a rate of 5 °C/min and held at this temperature for 5 min. The samples were then reheated at a rate of 5 °C/min to 110 °C, using the heat-cool-heat method, which was considered suitable for hard candies (Wang, 2017). For this study, the samples were analysed using a DSC Q2000 (M/s TA Instruments, New Castle, DE, USA).

Analysis of water activities (a_w) of CCS and PCS

The a_w of CCS and PCS were determined according to the AOAC (1978) method to assess their shelf stability.

Proximate analyses of CCS and PCS

Proximate analyses of CCS and PCS were conducted in accordance with the standard AOAC methods, including the estimation of % moisture (dry weight basis-D. W) (AOAC, 1998), % protein (AOAC, 1995), % crude fat (AOAC, 2000), % ash (AACC, 2000), % crude fibre (AOAC, 2005) and total carbohydrates (by difference).

Acidity and solid contents of CCS and PCS

For evaluating acidity and solid contents of the CCS and PCS samples, the same were dissolved in DMSO following the protocol described for the lyophilized plantain powder (*vide supra*). The resulting supernatants were stored at −20 °C prior to analyses. These were subjected to

analyses of total titratable acidity (% TTA) as percent malic acid equivalent (Ranganna, 1986) using standard NaOH (0.1 N), pH (using pH meter, Cyberscan PC510m, M/s Eutech Instruments Pvt. Ltd., Singapore City, Singapore) (Sarkar *et al*., 2021), total insoluble solids (gravimetrically), and total soluble solids as °Brix (Chakraborty and Bhattacharjee, 2018) (using OptiDuo Refractometer, M/s Bellingham + Stanley Ltd., Tunbridge Wells, UK).

Antioxidant properties

Since CCS was chiefly composed of sugar alcohols and contained no additional source of natural antioxidants, the evaluation of antioxidant properties was carried out solely for PCS.

Assessment of antioxidant potency of PCS

The supernatant obtained from the PCS sample (*vide supra*) was also used for the determination of reducing power as µg BHT equivalent/g of D.W. (Ghosh *et al*., 2015); TPC as mg gallic acid equivalent (GAE/100 g D.W.) by the Folin-Ciocalteu method (Aiyegoro and Okoh, 2010); DPPH radical scavenging activity as IC_{50} values (mg/mL) in accordance with Spanos and Wrolstad (1990) and FRAP value as mM $FeSO_4$/100 g D.W. (Benzie and Strain, 1999), using a UV-Vis double Beam Spectrophotometer (Halo DB-20, M/s Dynamica Scientific Ltd., Newport Pagnell, UK).

Determination of antioxidant content (in terms of L-tryptophan, serotonin, melatonin) and antioxidant synergy in PCS

Sample preparation for QuEChERS-SPE extraction of PCS

For the extraction of L-tryptophan, serotonin, and melatonin from PCS, 15g of candy was used. At first, the candy was crushed to powder using a mixer-grinder (HL 1618, M/s Philips India Limited, Chennai, India) and this ground sample was then used for SPE.

QuEChERS-SPE of the ground candy to extract serotonin, and melatonin along with L-tryptophan

QuEChERS-SPE of the ground PCS was carried out according to the method described in AOAC official method 985.22 (2000) and Anastassiades *et al*. (2003). The ground sample was placed in a clean centrifuge tube to which 1% acetic acid in acetonitrile solution and the contents ($MgSO_4$ and NaCl) of an SPE AOAC packet were added. After thorough mixing using a vortex (iSwix VT, M/s Neuation Technologies Pvt. Ltd, Gujarat, India), the tube was centrifuged at 1500 × g for 1 min in a centrifuge (R-8C, M/s Remi, Mumbai, India). One mL of supernatant was withdrawn and added to the dispersive-SPE (dSPE) sample cleanup tube [containing primary secondary amine, C18 sorbent (trifunctionally bonded C18 silica), graphitized carbon black, and $MgSO_4$] and thoroughly mixed. The dSPE sample cleanup tubes were again subjected to centrifugation at 1207 × g for 5 min using a microspin centrifuge (TC-4815D, M/s Eltek, Haryana, India). The supernatant, *i.e.*, the extracted sample was filtered using a micro syringe filter (0.22 µm nylon) and stored in an amber-coloured glass vial at −20 °C for further analyses.

Quantification of L-tryptophan, serotonin, and melatonin by HPLC-PDA analysis

PCS extracts obtained by QuEChERS were subjected to analyses of L-tryptophan, serotonin, and melatonin by HPLC-PDA. For simultaneous quantification of the above-mentioned biomolecules, a new HPLC method has been developed in our laboratory and validated by rigorous chromatography modelling based on mass transfer of analytes (Tamili *et al*., 2023).

The filtered extract (20 µl) was injected into a JASCO C18 reversed phase column (l = 250 mm and i.d = 4.6 mm) HPLC system (LC-Net-2/ADC, PU-4180 HPLC pump, DG- 4000-04 degasser, MD-4015 detector). The pump was operated in SPG mode at a pressure range of 0-5^{e+7} Pa. HPLC-grade methanol and 1% HPLC grade acetic acid in HPLC-grade water were used as mobile phase solvents in gradient mode, each at a flow rate of 1 mL/min. A PDA

detector with a deuterium (D2) lamp set at 280 nm was employed for continuous monitoring of the eluents, adapting the method reported by Huang and Mazza (2011). The peaks of these biomolecules were identified based on the retention time of their corresponding Sigma standards (when 20 µl mixture containing 1mg/mL of each standard was injected and run as per the above programme).

Validation of presence of L-tryptophan-serotonin-melatonin in the designer candy

The presence of a few additional peaks in the HPLC-PDA chromatogram of the QuEChERS-SPE extract of the candy (owing to the presence of antioxidants and constituents other than L-tryptophan-serotonin-melatonin) necessitated cross-validation of the presence of the same in the newly formulated candy. The said extract (previously used for quantification of L-tryptophan-serotonin-melatonin by HPLC-PDA method) was therefore subjected to ESI-TOF-MS analysis employing Xero-G2-Xs-QT, equipped with an ADC-magnetron detector (M/s Waters, Massachusetts, USA) using the programme reported by us (Tamili *et al*., 2023).

Assessment of antioxidant synergism among L-tryptophan, serotonin, and melatonin in PCS

All naturally occurring antioxidants in any food are always present as a synergistic consortium (Messina *et al*., 2001). Once isolated, antioxidants can act either synergistically or antagonistically (Peyrat-Maillard *et al*., 2003). Therefore, it is of utmost importance to achieve antioxidant harmony without perturbing the natural synergy of foods if the benefits of antioxidants *in vivo* are to be experienced. Preservation of the naturally occurring synergism among the three antioxidants mentioned in PCS was one of the prime objectives of this study to ensure that the designer candy is an antioxidant-rich product. To evaluate the 'food synergy' in the developed candy, the synergism among L-tryptophan, serotonin, and melatonin was assessed *in vitro* by determining the synergistic effect (SE) value using the DPPH radical scavenging capacity of pure chemical standards of L-tryptophan, serotonin, and melatonin

separately in varying concentrations similar to those present in PCS and of a mixture comprising of the above antioxidants in the same concentrations (as found by HPLC-PDA analysis of PCS). The experimental scavenging capacity (% ESC), theoretical scavenging capacity (% TSC), and SE value were calculated in accordance with the method described by Liu *et al*., 2008 and Chakraborty and Bhattacharjee (2018). An SE value greater than 1 indicates the preservation of natural synergism amongst these three biomolecules.

Packaging and shelf-life study of packaged candies

For the commercialization of the newly developed candy, it is important to know its storage properties under packaged conditions. For this purpose, two flexible packaging laminates, commonly used for commercial candy packaging, were provided and tested by the Indian Institute of Packaging, Kolkata, India, as used for the study. The flexible 2 and 3-ply packaging laminates in the form of sheets of uniform dimensions consisted of 12 µ non-heat sealable biaxially oriented polypropylene (NHS BOPP)/8 µ white pigmented extrusion-coated polyethylene/15 µ metallized biaxially oriented polypropylene (MET BOPP) (NHS BOPP/MET BOPP); and 12 µ polyethylene terephthalate (PET)/8 µ white pigmented extrusion coated polyethylene/12 µ metalized polyethylene terephthalate (MET PET)/20 µ polyethylene (PE) (PET/MET PET/PE). Their characteristic chemical and mechanical properties [*i.e*., thickness, grammage, water vapour transmission rate (WVTR), oxygen transmission rate (OTR), and tensile strength] were evaluated using standard methods (IS 1060-1, 1966), and are presented in Table 2.1.2.

Selection of the best-packaging laminate (wrapping material) based on the microbiological safety of the packaged candy

To select the best flexible packaging laminate, the microbial load, and sensory attributes of the candy samples (CCS and PCS) packaged in the said laminates were evaluated over a period of

three months. Freshly prepared batches (each batch consisted of 72 CCS samples and 72 PCS samples, where batch size decided based on sample amounts needed for regular physicochemical analysis) of candies were immediately packaged inside foil wrappers of composition NHS BOPP/MET BOPP laminate (36 CCS and 36 PCS) and PET/MET PET/PE laminate (36 CCS and 36 PCS), leaving negligible void space inside the package. It was then sealed with a heat sealer (Delta seal V2, M/s Sevana Traders and Services Pvt. Ltd., Cochin, India). The packaged candies were stored under ambient conditions (27±2 ℃, 80±2 %RH). Three randomly selected samples were subjected to microbial and sensory evaluation on the first day of each week following the methods described above.

Shelf-life assessment of candy packaged in the selected wrapper

The best packaging laminate for the newly designed candy was selected in terms of microbial safety and organoleptic acceptability of the packaged candies. A comprehensive shelf-life study was then conducted with CCS and PCS. Each batch comprised of 40 candies samples (reasons for choice of the said batch size has been explained *vide supra*) wrapped inside the best packaging laminate. Alterations in microbial load, sensory attributes, and physicochemical properties (moisture content, alterations in CIE colour values, TPA); antioxidant efficacy (TPC, DPPH radical scavenging activity, FRAP values); L-tryptophan, serotonin, and melatonin content, and the antioxidant synergy, were assessed throughout the storage period in accordance with the previously described methods (*vide supra*). Each week at regular intervals, three candies were randomly withdrawn for analyses. For candies approaching the end of their shelf lives, microbiological analysis was performed on all days of storage.

Statistical analysis

Development of candies in the best optimized condition, all physicochemical analyses related to the shelf-life study (except the analyses involving high-end instruments such as EDX, FE-

SEM, TGA, DSC, XRD, and ESI-TOF-MS) were performed in triplicate and the results have been reported as mean ± SD of three candy samples, obtained from three sets of independent experiments. Student's t-test to evaluate the individual and interactive effects of two variables, and Duncan's multiple range test to determine significant differences among means were performed using IBM SPSS Statistics Software Version 26 (M/s IBM, New York, USA). A value of $p \leq 0.05$ was considered statistically significant to establish differences in all tests.

Results and Discussion

Antioxidant properties of raw plantains and its lyophilized powder

The plantains procured for this study had a reasonably high TPC value (573.17 mg GAE/100 g D.W.), DPPH radical scavenging activity (IC_{50} value 5.59 mg/mL), FRAP value (3438.12 mM $FeSO_4$/100 g D.W.) and L-tryptophan, serotonin, melatonin contents (102.45 µg/g, 4.56 µg/g and 2.08 µg/g, respectively). The lyophilized plantain powder also exhibited a remarkable TPC value (865.48 mg GAE/100 g D.W.), DPPH radical scavenging activity (IC_{50} value 0.56 mg/mL) and FRAP value (5528.95 mM $FeSO_4$/100 g D.W.), an increase of 50.99%, 89.96% and 60.81%, respectively, over the raw plantains, possibly owing to the concentration effect. A similar increase in antioxidant potency was also reported by Dal-Bó and Freire (2022) in lyophilized avocado pulp powder compared to that of fresh avocado pulp. As a consequence of the enhanced antioxidant activity, homogeneity, as well as low moisture content (<6%) of the lyophilized plantain powder vis-à-vis those of the fresh plantains, the powder was utilized in the development of the designer candy instead of the fresh plantain pulp to achieve an acceptable texture as well as low water activity in the candy (determined from preliminary trials).

Weights and dimensions of CCS and PCS

The weight of CCS from the aforementioned plantains was 20±2 g, whereas that of the PCS was 24±2 g. The dimensions of either type of candy was as follows: length: 4 cm; width: 2 cm, and height: 2 cm. From 100g raw green plantains, 4 pieces of PCS with the above dimensions were obtained (Fig. 2.1.2).

Safety aspects of CCS and PCS

Safety is of paramount importance for all consumable food products. Any microbial or heavy metal contamination beyond safe limits can lead to mild to severe health hazards and can even be fatal to the consumers. The total bacterial and yeast/mould counts (no growth was detected on the first day in either sample) of CCS and PCS were within the safe consumption limits as per the guidelines of FSSAI (2011b), which states that the bacterial and fungal counts in thermally processed (except by pasteurization, which is conducted at a temperature less than 100 °C) fruit and vegetable products should not exceed 1000 and 100 CFU/g of the food product, respectively.

The EDX analyses of CCS and PCS (Fig. 2.1.3a and 2.1.3b, respectively) showed that both candy samples were free of heavy metal contaminants such as Pb, Ti, Hg, Ni, As, Si, and Mo. However, Cu was detected in the PCS sample since plantain is reportedly known to be a source of this micronutrient (Okorie *et al*., 2015).

The findings of the microbiological and EDX analyses assured that the formulated candy was completely safe for human consumption and therefore could be subjected to sensory evaluation.

Sensory and physicochemical characteristics of CCS and PCS

Consumer acceptance is the most important criterion for a new food product. The hedonic scores from the panel responses for CCS and PCS are presented as radar plots (Fig. 2.1.3c and

2.1.3d, respectively). It was evident that PCS was well accepted by the panellists owing to its uniform dark brown colour (L*: 48.5; a*: 5.1; b*: 11.6; c*: 12.7; h: 66.5), rich mouthfeel, pleasant aroma, moderately hard texture, and moderate sweetness. The typical characteristic flavour of green plantains in the processed candy was also moderately appreciated. CCS mimicked the dark caramel-like colour (L*: 51; a*: 3.9; b*: 10.8; c*: 11.5; h: 70.1) (lighter than PCS) and texture of 'hard-boiled candy', tasted extremely sweet, and was sticky in the mouth; while PCS was less hard (more brittle) and not sticky. The caramel-like appearance of the newly developed candies was in consonance with the findings of Ronda *et al*. (2005), who reported that the use of polyols (sorbitol and mannitol) in the cake preparation darkened the crust compared to the cakes prepared with sucrose, primarily owing to the classic Maillard reaction. Additionally, the presence of brown-coloured lyophilized plantain powder (shown in Fig. 2.1.2) also contributed to the formation of darker colour of PCS compared CCS. The findings of the TPA [PCS showed hardness(g): 6803.52±391; adhesiveness (g.sec): −238.80±15.83; springiness: 0.14±0.01; cohesiveness: 0.41±0.02; gumminess: 196.54±32.86; and chewiness: 95.77±2.33] corroborated well with its sensory attributes. CCS exhibited significantly ($p<0.05$) higher hardness (10643.08±893 g), adhesiveness (−3033.96±147 g.sec), springiness (0.97±0.03), cohesiveness (0.18±0.01), gumminess (1899.09±156), and chewiness (1847.51±124) vis-à-vis that of PCS. However, another sugar-free nutraceutical hard candy comprised of isomalt, maltitol syrup, xylitol, and extract of melon berry (Jeon *et al*., 2021) had a harder (in KgF) texture than the candy developed in the present study. Therefore, the newly formulated designer candy was labelled as 'semi-hard'.

The FE-SEM image of CCS (Fig. 2.1.3e) revealed a continuous, uniform, compact, and less porous structure with a smooth surface, which corresponded well with the textural attributes of the sugar-made hard candies (Reinheimer *et al*., 2010, Gu *et al*., 2015). In contrast, PCS (Fig. 2.1.3f) exhibited a grainy, non-homogeneous, and discontinuous (with voids) microstructure,

conferred by the complex composition of plantains (especially the soluble and insoluble fibre content). There is a lack of similar data on sugar-free hard or semi-hard candies to compare these findings.

Fig. 2.1.3g and 2.1.3h present the XRD graphs of CCS and PCS, respectively. Both the graphs revealed few nano-crystalline structures on predominantly amorphous bases. Thus, the candies were inherently amorphous in nature since they were sugar-cum-fat-free candies and free of classical defects such as graininess and crystals. However, they were opaque and owing to the presence of insoluble plantain constituents, little graininess (although the same were not the classical 'blooms') was present in PCS. CCS was thus categorized as 'grained hard candy' (Minifie, 1989), and PCS as a 'grained semi-hard candy', the same being relatively more 'brittle' than CCS. These data obtained could not be validated with literature reports since, to the best of the knowledge of the author, there is no existing XRD data of sugar-free hard candy.

Thermal stability of CCS and PCS

The TGA graphs of both CCS and PCS could be classified under the 'multi-stage decomposition' type (3-stage decomposition), wherein the first decomposition was observed at 125 ℃ for either candy (Fig. 2.1.3i and 2.1.3j), indicating that both candies were thermostable up to very high temperatures.

From the DSC thermograms (Fig. 2.1.3k), for CCS, the onset of glass transition temperature (T_g) was found to be −50 ℃, the T_g midpoint was at −38 ℃, and the T_g endpoint was at −28 ℃ during both the first and second phases of heating. On the other hand, T_g onset, midpoint, and endpoint for PCS (Fig. 2.1.3l) were −35 ℃, −30 ℃, and −25 ℃, respectively, during the first heating phase, and −30 ℃, −25 ℃, and −20 ℃, respectively, during second heating phase. The higher T_g value (mid-point) for CCS affirmed the hard and brittle texture of the candy

during mastication, whereas the lower T_g of PCS was in consonance with the mouthfeel (as assessed by the sensory panel) of the hard-cum-brittle candy, which gradually softened with mastication. The melting peak of PCS was observed to be in the relatively wide range of 94-105 ℃, implying slow melting in the mouth, while that of CCS was in the narrower range of 90-94 ℃ during the first heating phase. During the second heating, CCS exhibited no change in the T_g values; however, there was a slight shift in the T_g and heat flow (w/g) curve for PCS, possibly due to the presence of polysaccharides, dietary fibres, and other complex compounds therein.

The absence of peaks indicated absence of crystallinity of the candies (Wang, 2017), which corroborated well with the findings of the XRD data (*vide supra).* This was owing to the replacement of sucrose as the doctoring agent by sugar alcohols, resulting in the absence of sugar blooms in the new designer candies. These findings of TGA, DSC analysis, and sensory evaluation were similar to those reported by Wang (2017), who had conducted extensive research on sugar-boiled hard candy.

a_w and proximate composition of CCS and PCS

Although CCS were sugar-free, it had an a_w value of 0.43, which was similar to that of conventional hard sugar candies (0.25-0.40). The a_w value of PCS was 0.57, which was similar to that of soft candies (0.46-0.60) (Ergun *et al*., 2010). These results corroborated well with that of the texture analysis data on hardness, based on which PCS was categorised as 'semi-hard'. PCS had a significantly higher ($p<0.05$) a_w value (Table 2.1.3) than CCS, since lyophilized plantain powder (non-packaged) was hygroscopic (Naknaen *et al*., 2016, Lima and Cal-Vidal, 1983). The higher a_w value of PCS would pose a challenge for its shelf-life extension, which was successfully averted by appropriate packaging of the candies (*vide infra*).

The proximate analyses of CCS and PCS (Table 2.1.3) revealed that PCS had significantly higher % moisture ($p < 0.001$), % ash ($p < 0.001$), % crude fibre ($p < 0.001$) and % protein ($p < 0.001$) contents and significantly lower % fat ($p < 0.001$) and total carbohydrate ($p < 0.001$) contents, which rendered PCS nutritionally richer than CCS.

Percentage TTA, pH, and soluble and insoluble solids of CCS and PCS

CCS showed an alkaline pH of 8.1, whereas PCS was acidic (pH = 4.3). A significantly higher ($p < 0.05$) percentage of TTA (0.12 in terms of % malic acid in PCS compared to 0.09 in terms of % malic acid in CCS) and lower pH ($p < 0.05$) was observed for PCS, which could be beneficial for the extension of its shelf-life. These findings were in consonance with the findings documented by Supriyanto *et al*. (2023), who have reported the pH of sucrose-free (with xylitol and glucose syrup) hard candy prepared with Javanese long pepper extract to be 4.3. CCS consisted of mostly of soluble solids (82%), whereas the crude fibre content of the plantains (mostly from its peels) contributed to significantly higher ($p<0.05$) insoluble solids (36.26%) in PCS. The higher insoluble solids as well as crude fibre contents in PCS justified the increased weight of PCS, although it was dimensionally similar to CCS.

Antioxidant properties of PCS

PCS had a considerable amount of reducing power (11.30 µg BHT/g D.W.), TPC value (679.28 mg GAE/100 g D.W.), DPPH radical scavenging activity (IC_{50} value 6.23 mg/mL) and FRAP value (2565.96 mM $FeSO_4$/100 g D.W.), although the processing losses were 18.5%, 11.6%, and 25.4%, respectively, compared to the values obtained for raw plantains. The same were 21.51%, 1012.5% and 53.59%, respectively, compared to the values obtained for the lyophilized plantain powder. The TPC value, DPPH radical scavenging activity, and FRAP value were significantly ($p<0.05$) reduced in PCS compared to the raw and lyophilized

plantains. These findings corroborated well with those reported by Dadwal *et al*. (2023), who found significant decreases ($p<0.05$) in TPC, DPPH, and FRAP values in candies prepared from bamboo shoot compared to the corresponding values of the same for fresh bamboo shoots. These differences could be attributed to the treatment/processing that the ingredients underwent during candy production. Since phenolic content is strongly related to antioxidant activity, the reduction of TPC value during candy preparation also led to a reduction in DPPH radical scavenging activity and FRAP values in the case of PCS (Dadwal *et al*. 2023, Yeo and Shahidi, 2017).

The L-tryptophan, serotonin, and melatonin contents in PCS were 4.54 µg/g, 1.83 µg/g, and 1.23 µg/g, respectively. The ESI-TOF-MS spectra of the QuEChERS extract of the designer candy depicting the presence of L-tryptophan, serotonin, and melatonin are shown in Fig. 2.1.4a, 2.1.4b, and 2.1.4c, respectively. The molecular masses (M) of serotonin and melatonin appeared as their sodium adduct forms $[M + Na]^+$, whereas L-tryptophan appeared as its potassium-cum-hydrogen adduct form $[M + K + H]^+$. Similar ESI spectra after sodium adduct during quantification of serum melatonin have been reported by Yang *et al*. (2002) and De Oliveira *et al*. (2011), who reported potassium adduct forms of fructo-oligosaccharides when the same were analysed in the roots and leaves of *Stevia rebaudiana* (Bert.) Bertoni. Although no report is available for the sodium adduct to serotonin, the adduct of sodium is reportedly a very common phenomenon for many molecules (Erngren *et al*., 2019). Thus, the findings of the ESI-TOF-MS spectra further cross-validated the results obtained by HPLC-PDA analysis of the target biomolecules in the formulated candy.

The contents of L-tryptophan, serotonin, and melatonin present in PCS also exhibited an SE value > 1, indicating that the natural synergism among these three biomolecules is maintained in the processed candies. This finding is in consonance with the findings of Chakraborty and

Bhattacharjee (2018) and Paul *et al*. (2019), who reported on the preservation of antioxidant synergism in processed food products in a nutraceutical beverage formulated using an UAE extract of mustard seeds with lemon and citric acid, and a nutraceutical custard designed using nano-encapsulated supercritical carbon dioxide extract of small cardamom seeds, respectively.

Best packaging laminate for the extension of the shelf-lives of CCS and PCS

During the storage period, the growth of microbes in the candies packaged in the two laminates was restricted for the first three weeks. However, on day 28 of the storage period, NHS BOPP/MET BOPP packaged (both CCS and PCS) candies had considerable bacterial and yeast/mould counts that were beyond the safe limit of consumption as per the guidelines of FSSAI (2011b). The NHS BOPP/MET BOPP packaged candies were therefore discarded on day 28. This study affirmed the suitability of PET/MET PET/PE as a packaging laminate for CCS and PCS.

Based on the properties of the foil wrapper (Table 2.1.2), it was found that the values of the mechanical properties (thickness, grammage, and tensile strength) were significantly higher ($p<0.001$) and the OTR value was significantly lower ($p<0.001$) for the PET/MET PET/PE (Fig. 2.1.5a) laminate, vis-à-vis that of the NHS BOPP/MET BOPP laminate (Fig. 2.1.5b). The high values for thickness, grammage, and tensile strength values of the three-ply PET/MET PET/PE flexible packaging laminate indicated improved mechanical strength, while a low OTR value indicated its good barrier property against oxygen and thus a possible prevention of oxidative degradation of the antioxidants present in the candy. The insignificant difference between the WVTR values suggested similar permeability of the packaging films to atmospheric water vapour. The low OTR and WVTR values of the PET/MET PET/PE laminates aided in the prevention of moisture loss in candies and thus averted the candies from

becoming soft (and therefore sticky). These properties of the wrapper foils proved to be beneficial for the maintenance of the organoleptic wholesomeness of the candy and rendered them safe in terms of microbiological bioburden.

The CCS packaged in PET/MET PET/PE were microbiologically safe for up to 56 days, while the PCS was safe for consumption for up to 63 days (Table 2.1.4). Based on these findings, the storage study period for the candy packaged in the best packaging laminate was ascertained to be 63 days post-packaging in PET/MET PET/PE laminate. A similar packaging laminate (PET/MET PET/PE) is reportedly used for commercial packaging of powdered spice mixes (for export) imparting it a shelf-life of 9-12 months under normal storage condition (Indiramma, 2004).

Shelf-life of 3-ply PET/MET PET/PE packaged CCS and PCS

Alterations in sensory attributes during storage

The response scores of sensory attributes of CCS and PCS during storage are presented as radar plots in Fig. 2.1.5c and 2.1.5d, respectively. On day 56, PCS was disapprovingly dry and brittle to the sensory panel, and CCS was also sensorially unacceptable owing to its increased stickiness and hardness on day 49 of storage. A reduction in the sensory attributes has been reported for *Basella alba* (Malabar spinach) extract-incorporated hard candies when wrapped in a 2-ply laminate of non-stick paper or in an aluminium foil and stored in airtight containers under ambient conditions (Yan *et al*., 2023). The authors attributed the decreases in the sensory scores to deteriorating changes in hardness and adhesiveness, in consonance with the findings of the present study. Henceforth, in this study, the shelf-life of PET/MET PET/PE-packaged PCS was considered to be 56 days, although the same was microbiologically safe up to 63 days.

Alterations in physicochemical properties during storage

For PCS and CCS, significant ($p<0.001$) declinations in percent moisture content during storage (Fig. 2.1.5e) were observed from day 0 until the end of their respective shelf-lives, possibly owing to the migration of moisture through the wrapper(s) into the immediate environment (Ergun *et al*., 2010). Moreover, significant differences ($p<0.001$) were also observed between the percent moisture contents of CCS and PCS on each assessment day. Significant ($p<0.001$) decreases in texture parameters (such as hardness, adhesiveness, springiness, cohesiveness, gumminess, and chewiness) occurred steadily throughout the storage period for both CCS and PCS; although alterations of the same were greater in PCS vis-à-vis CCS (Table 2.1.5). Very little changes ($p < 0.001$) occurred for colour parameters- L*, a*, b*, c*, and h values (Fig. 2.1.5f, 2.1.5g, 2.1.5h, 2.1.5i, 2.1.5j) for both CCS and PCS during storage.

The decline in moisture content during storage of PCS explained the occurrence of dryness, brittleness, and incoherency in the candy structure of the same on the last day of storage. Candies tend to lose moisture to the environment as moisture migrates out of the candy and through the package, making them harder (Ergun *et al*., 2010, Yan *et al*., 2023). In the present study, PCS became brittle instead of being harder, probably owing to the presence of void spaces within, as has been confirmed from its microstructure analysis (Fig. 2.1.3f). Loss of % moisture also led to the loss of adhesiveness (Ergun *et al*., 2010), thereby causing losses in springiness, cohesiveness, gumminess, and chewiness. Changes in colour parameters were possibly due to the occurrence of non-enzymatic browning (Yan *et al*., 2023, Zhou *et al*., 2014), and perhaps accelerated by arabinose, an abundant glycoside present in one of the constituents of candy, *i.e.*, gum acacia (do Nascimento *et al*., 2023). The findings of the present study are in consonance with the findings of Yan *et al*. (2023) described earlier (*vide supra*).

Alterations in antioxidant properties and antioxidant (L-tryptophan, serotonin, and melatonin) synergy during storage

PCS showed appreciable TPC, IC_{50} value of DPPH radical scavenging activity, and FRAP value on day zero, which significantly declined with storage (Fig. 2.1.6a, 2.1.6b, 2.1.6c) until the end of its shelf life (56 days). A similar trend of decline in TPC and antioxidant activities during storage has been reported by Šeremet *et al*. (2020) in candies containing steviol glycosides, sorbitol, inulin, psyllium, citric acid, and white tea extract. At the molecular level, L-tryptophan, serotonin, and melatonin contents decreased significantly ($p<0.001$) from their respective contents (4.54 µg/g, 1.83 µg/g, and 1.23 µg/g, of L-tryptophan, serotonin, and melatonin, respectively) on day 0 until the last day of shelf-life (3.04 µg/g, 0.21 µg/g, and 0.67 µg/g, respectively of L-tryptophan, serotonin, and melatonin) of PCS (Fig. 2.1.6d). The SE value of PCS was found to be greater than unity, signifying that the natural antioxidant synergy was not perturbed during candy processing and also remained unaffected by physicochemical changes in the candy during storage (Table 2.1.6). Thus, the plantain-based candy would be a rich source of the antioxidant triad- L-tryptophan, serotonin, and melatonin even after 56 days of storage inside a commercial foil wrapper. It is anticipated that the candy processing and packaging presented in this work would be a sustainable solution for the delivery of plantain-based antioxidant confectionary.

Conclusion

The newly developed plantain-based candy could be a promising nutraceutical confectionary, rich in three therapeutically important antioxidants, namely L-tryptophan, serotonin, and melatonin, which could be safely stored for 56 days without any physicochemical and considerable antioxidant deterioration when packaged in a 3-ply flexible (PET/MET-PET/PE) laminate wrapper. It is envisaged that the same would be a vehicle for *in vivo* delivery of these

biomolecules since the formulation involves a sugar-free base (of sorbitol and mannitol) and minimal food additives (except for a thickener, a desiccant, and an anti-fungal agent) with appreciable shelf-life. This semi-hard candy could be a novel potential antioxidant-rich food supplement, especially for the geriatric population. This candy was further investigated for the *in vitro* release kinetics of the three biomolecules (L-tryptophan-serotonin-melatonin) using a standard tablet dissolution unit and *in vivo* bioavailabilities of the same molecules in Sprague Dawley rats, and the study has been elaborated in the next part of this chapter.

Novelty

The present study reported for the first time on the development of a semi-hard sugar-free antioxidant rich (especially L-tryptophan-serotonin-melatonin) candy using a widely cultivated healthy fruit, plantain, promoting better utilization of the same globally and rendering this candy less health debilitating. It is envisaged that this newly developed candy would be a food matrix delivering important antioxidants and a substitute of commercial candies consumed globally.

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Tables:

Table 2.1.1: Details of ingredients used in candy formulation

Ingredients	Function	Permitted amount	Used amount	Reference
Sorbitol	Low calorie sweetening agent, forms the body of candy	GMP (FSSAI)	Sorbitol: D-mannitol= 3:1 (% by mass)	Roger, 1973
D-Mannitol	Low calorie sweetening agent, forms the base of candy	GMP (FSSAI)		
Gum acacia	Thickening agent, stabilizer, emulsifier	-	30%	FSSAI, 2011a
SiO_2	Desiccant, anticaking agent	-	0.5%	
Sorbic acid	Antifungal agent	1000 ppm (FSSAI)	0.1%	
Lyophilized green plantain powder	Principle fortifying material rich in antioxidants	-	65%	-
Potable water	To solubilize the ingredients	-	50%	-
Vanilla essence	Flavouring agent	-	100 µl	-

FSSAI: Food Safety and Standards Authority of India

Table 2.1.2: Mechanical and chemical properties of different packaging laminates*

Test	PET/MET PET/PE	BOPP/MET BOPP
Structure	12 Micron polyethylene terephthalate (PET)/ 8W. Ext. Coting/12 Micron metalized polyester (MET PET)/20 Micron N polyethylene (PE)	12 Micron NHS Biaxially oriented polypropylene (BOPP)/8W. Ext. Coating/15 Micron metallized bi-orientated polypropylene (MET BOPP)
Thickness (micron)	52 ± 0.54^a	40 ± 0.97^b
Grammage (g/m^2)	59 ± 0.42^a	35 ± 0.75^b
Water vapour transmission rate (g/m^2/day at 38± 1 °C and 90±2 s% RH)	0.127 ± 0.05^a	0.124 ± 0.08^a
Oxygen transmission rate (cc/ (m^2/24h/atm) at 25 °C)	01.08 ± 0.05^a	56.67 ± 1.56^b
Tensile strength (kg/cm^2)	Machine direction: 1028.00 ± 2.56^a	Machine direction: 725 ± 2.58^b
	Transverse direction: 875 ± 1.86^a	Transverse direction: 645 ± 1.46^b

*Data are mean ±SD of three samples
Different letters in a row indicate significant differences at $p<0.05$ level

Table 2.1.3: Proximate composition and water activity of control candy sample and plantain-candy sample

Analysis parameters	Composition (% on fresh weight basis) *	
	Control candy sample	Plantain-candy sample
Moisture	3.65 ± 0.84^{a}	14.74 ± 1.53^{b}
Fat	0.66 ± 0.03^{a}	0.16 ± 0.06^{b}
Protein	0.29 ± 0.02^{a}	2.6 ± 0.35^{b}
Crude Fibre	-	2.16 ± 0.73^{a}
Ash	0.90 ± 0.02^{a}	4.17 ± 0.88^{b}
Carbohydrate (by difference)	94.51 ± 1.43^{a}	76.17 ± 2.85^{b}
Water activity	0.43 ± 0.01^{a}	0.57 ± 0.02^{b}

*Data are mean ±SD of three samples of each set
Different letters in a row indicate significant differences at $p<0.05$ level

Table 2.1.4: Microbial counts in PET/MET PET/PE packaged candies during storage

Days	Control candy sample		Plantain candy sample	
	Bacteria Count* (CFU/g)	Yeast/Mold Count* (CFU/g)	Bacteria Count* (CFU/g)	Yeast/Mold Count* (CFU/g)
0	0^{a}	0^{k}	0^{a}	0^{k}
7	0^{a}	0^{k}	0^{a}	0^{k}
14	0^{a}	0^{k}	0^{a}	0^{k}
21	0^{a}	0^{k}	0^{a}	0^{k}
28	187.5^{b}	0^{k}	0^{a}	0^{k}
35	250^{c}	0^{k}	187.5^{b}	0^{k}
42	500^{d}	0^{k}	437.5^{g}	0^{k}
49	562.5^{e}	0^{k}	812.5^{h}	0^{k}
56	1750^{f}	937.5^{l}	687.5^{i}	0^{k}
63	-	-	1062.5^{j}	312.5^{m}

*Data are mean ±SD of three samples of each set
Different letters indicate significant differences at $p<0.05$ level
PET – Polyethylene terephthalate; MET PET – Metalized polyester; PE – Polyethylene

Table 2.1.5: Texture profile of control candy sample and plantain candy sample during storage

Days	Hardness (g)	Adhesiveness (g.sec)	Springiness	Cohesiveness	Gumminess	Chewiness
Control candy sample						
0	10643.08 ± 893^{a}	-3033.96 ± 147^{a}	0.97 ± 0.03^{a}	0.18 ± 0.01^{a}	1899.09 ± 156^{a}	1847.51 ± 124^{a}
7	10653.25 ± 683^{b}	-3075.25 ± 285^{b}	0.99 ± 0.03^{b}	0.19 ± 0.02^{b}	1893.48 ± 135^{a}	1839.93 ± 131^{b}
14	10514.48 ± 844^{c}	-3012.23 ± 216^{c}	0.97 ± 0.05^{c}	0.18 ± 0.01^{c}	1886.75 ± 128^{b}	1824.03 ± 103^{c}
21	10541.88 ± 580^{c}	-2986.85 ±194^{d}	0.95 ± 0.02^{d}	0.17 ± 0.02^{c}	1872.56 ± 117^{b}	1798.28 ± 116^{d}
28	10442.26 ± 719^{e}	-2964.47 ± 201^{e}	0.92 ± 0.01^{e}	0.17 ± 0.02^{d}	1856.37 ± 123^{c}	1782.47 ± 119^{e}
35	10475.84 ± 904^{f}	-2993.26 ± 178^{f}	0.93 ± 0.06^{d}	0.17 ± 0.02^{df}	1839.25 ± 169^{d}	1777.24 ± 136^{f}
42	10344.31 ± 729^{g}	-2955.19 ±255^{g}	0.91 ± 0.06^{f}	0.16 ± 0.01^{e}	1823.07 ±105^{d}	1763.07 ± 148^{g}
49	10243.24 ± 812^{h}	-2913.43 ± 242^{h}	0.90 ± 0.08^{g}	0.19 ± 0.02^{e}	1815.65 ± 122^{e}	1758.25 ± 152^{h}
56	10181.10 ± 914^{i}	-2879.37 ± 268^{d}	0.90 ± 0.03^{g}	0.16 ± 0.02^{f}	1804.36 ± 108^{d}	1752.72 ± 150^{h}
Plantain candy sample						
0	6803.52 ± 391^{j}	-238.80 ± 15.83^{i}	0.14 ± 0.01^{h}	0.41 ± 0.02^{g}	196.54 ± 32.86^{e}	95.77 ± 2.33^{i}
7	6888.84 ± 268^{k}	-242.27 ± 17.15^{j}	0.18 ± 0.02^{i}	0.07 ± 0.03^{h}	486.12 ± 25.92^{e}	85.22 ± 4.21^{j}
14	6743.49 ± 342^{l}	-175.63 ± 10.52^{k}	0.16 ± 0.02^{j}	0.06 ± 0.01^{i}	389.75 ± 20.86^{f}	64.29 ± 1.53^{k}
21	6535.92 ± 184^{m}	-115.09 ± 9.92^{l}	0.11 ± 0.01^{k}	0.06 ± 0.01^{i}	415.98 ± 21.05^{f}	46.22 ± 2.65^{l}
28	6315.33 ± 165^{n}	-125.94 ± 11.05^{m}	0.15 ± 0.01^{l}	0.04 ± 0.01^{j}	228.55 ± 18.84^{g}	33.30 ± 2.14^{m}
35	6440.39 ± 228^{o}	-130.14 ± 4.86^{n}	0.13 ± 0.01^{k}	0.05 ± 0.01^{jk}	359.95 ± 2.10^{h}	45.33 ± 3.16^{n}
42	6325.23 ± 271^{p}	-103.04 ± 7.25^{o}	0.09 ± 0.02^{m}	0.07 ± 0.01^{i}	234.17 ± 16.85^{h}	21.39 ± 1.32^{o}
49	6057.72 ± 193^{q}	-120.09 ± 9.92^{p}	0.11 ± 0.01^{n}	0.05 ± 0.01^{i}	314.86 ± 28.44^{g}	37.32 ± 2.96^{p}
56	4683.31 ± 158^{r}	-223.50 ± 5.27^{q}	0.12 ± 0.01^{n}	0.07 ± 0.01^{k}	339.75 ± 22.85^{h}	40.27 ± 3.13^{p}

*Data are mean ±SD of three samples of each set
Different letters in a column indicate significant differences at $p<0.05$ level

Table 2.1.6: The *in vitro* synergistic effect of L-tryptophan-serotonin-melatonin in plantain candy

Sample Name	Serotonin (µg/mL)	L-tryptophan (µg/mL)	Melatonin (µg/mL)	Synergistic effect
Serotonin1	1.87	0.00	0.00	-
Serotonin2	0.35	0.00	0.00	-
L-tryptophan1	0.00	4.54	0.00	-
L-tryptophan2	0.00	3.04	0.00	-
Melatonin1	0.00	0.00	1.23	-
Melatonin2	0.00	0.00	0.67	-
Plantain candy sample on day 0	1.87	4.54	1.23	1.03 ± 0.05^{a}
Plantain candy sample on day 56	0.35	3.04	0.67	1.01 ± 0.07^{b}

*Data are mean ±SD of three samples of each set.
Different letters in a column indicate significant differences at $p<0.05$ level

Figures:

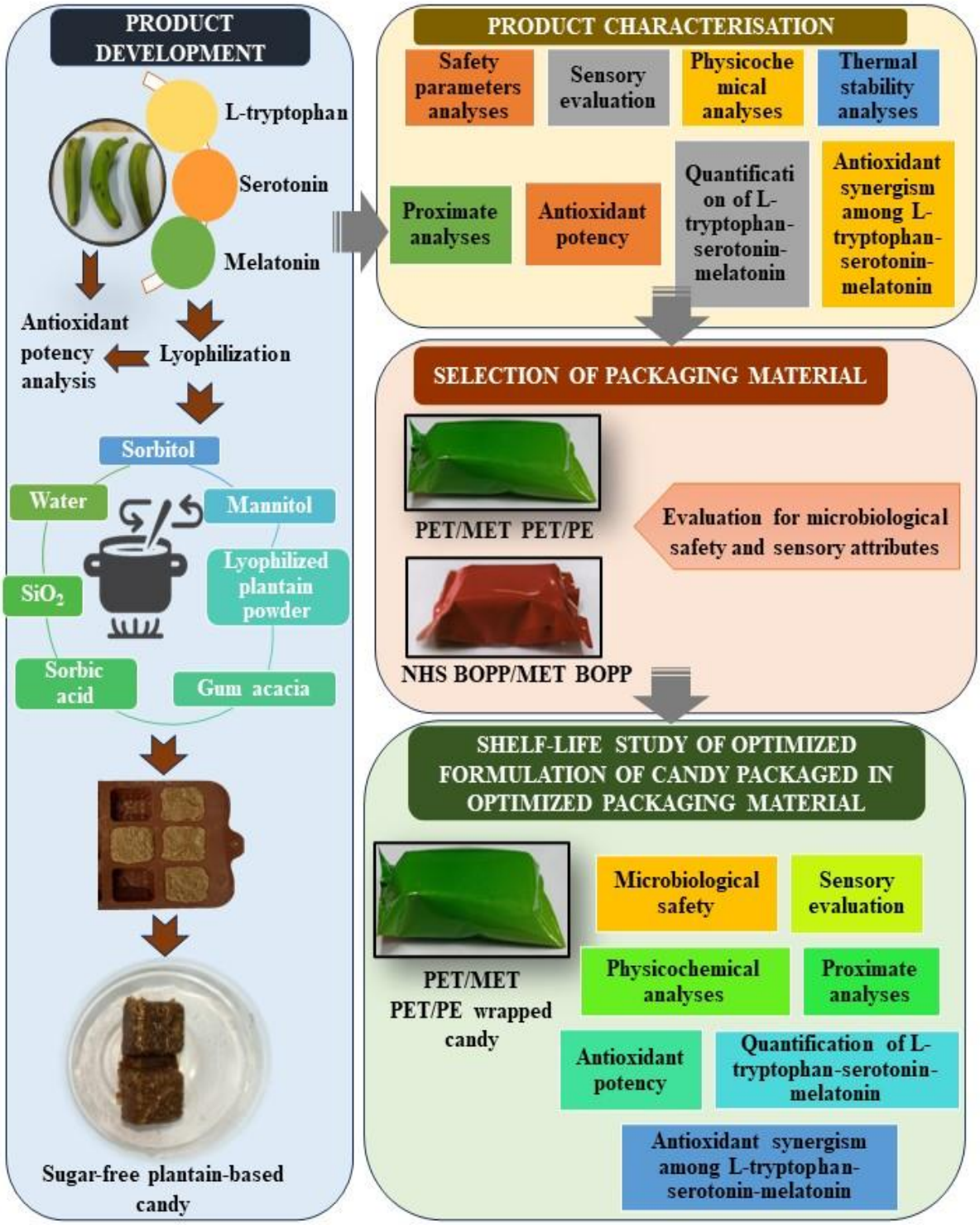

Figure 2.1.1: Experimental design of the present study

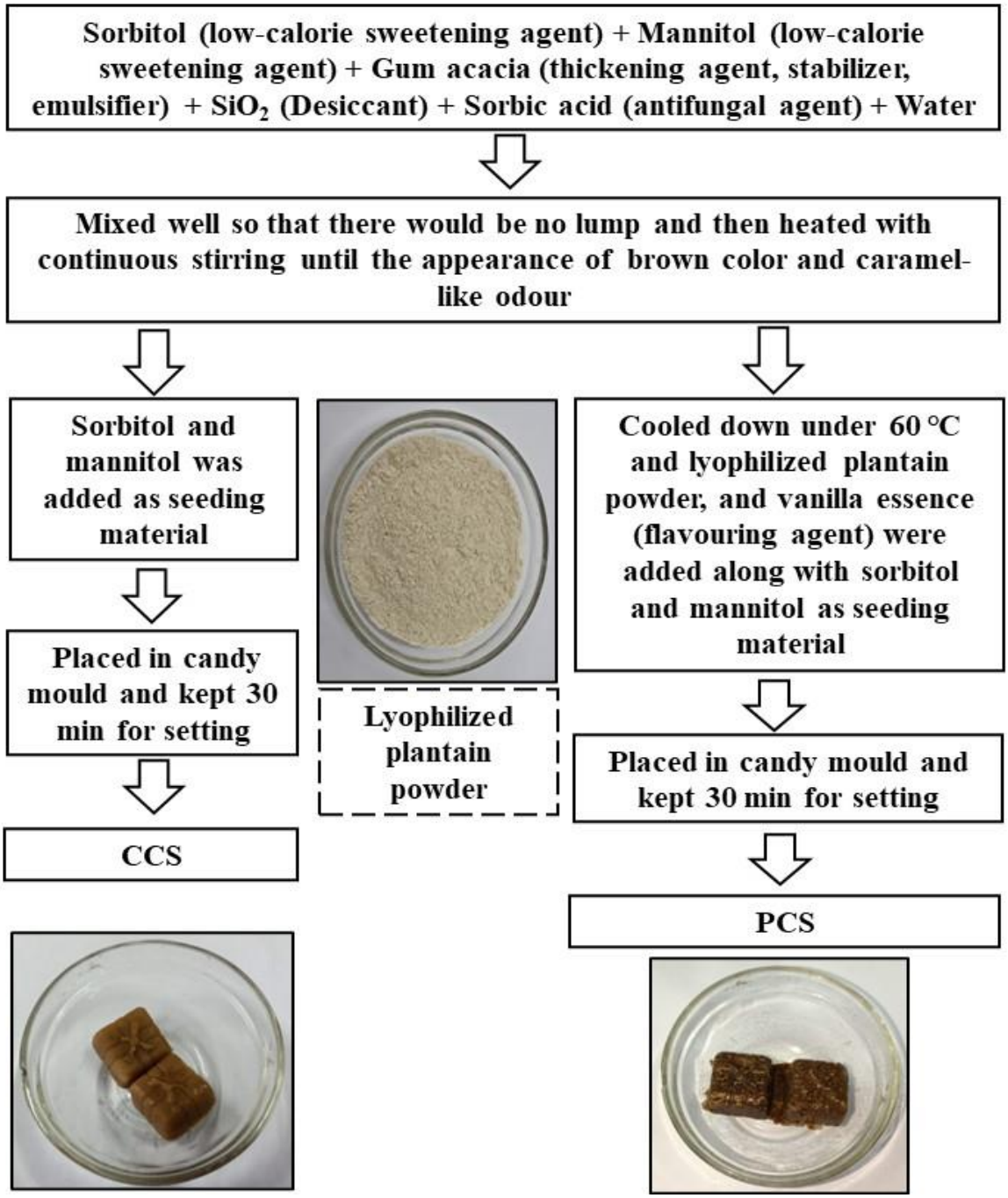

Figure 2.1.2: Methods of preparation of control candy sample and plantain-candy sample and the relevant pictures of the final products

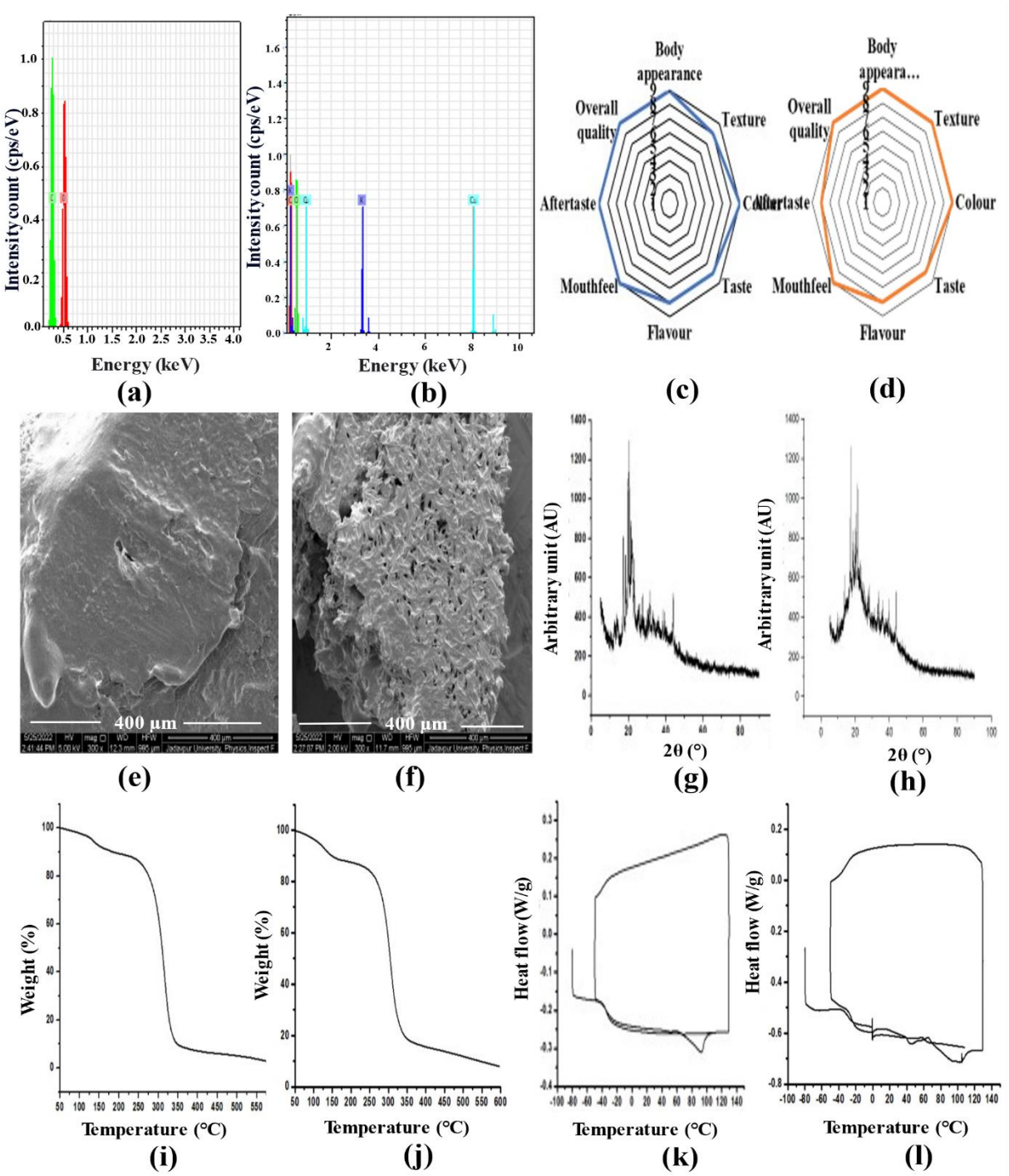

Figure 2.1.3: Physicochemical properties of control candy sample and plantain-candy sample after formulation- energy dispersive X-ray spectra of a) control candy sample and b) plantain candy sample; radar plots of hedonic scores obtained by sensory analyses of c) control candy sample and d) plantain candy sample where each value represents mean ± SD of three sets of experimental data; field emission scanning electron microscopy images of e) control candy sample and f) plantain candy sample; X-Ray diffraction spectra of g) control candy sample and h) plantain candy sample; thermogravimetric graphs of i) control candy sample and j) plantain candy sample; differential scanning calorimetry thermographs of k) control candy sample and l) plantain candy sample.

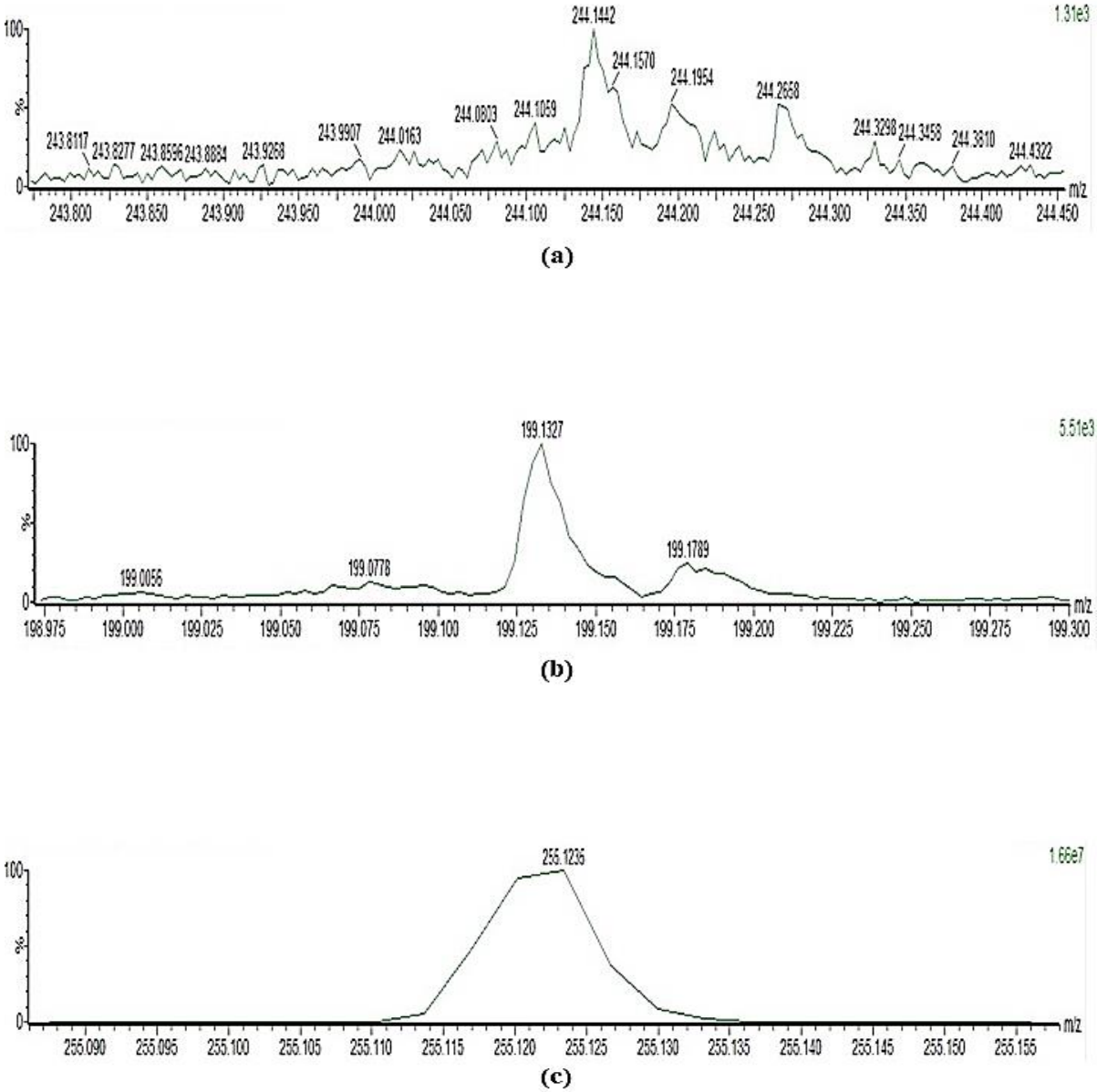

Figure 2.1.4: ESI-TOF-MS spectra of QuEChERS extract of sugar-free plantain-based candy indicating the peaks for a) L-tryptophan $[M + K + H]^+$, b) serotonin $[M + Na]^+$, and c) melatonin $[M + Na]^+$

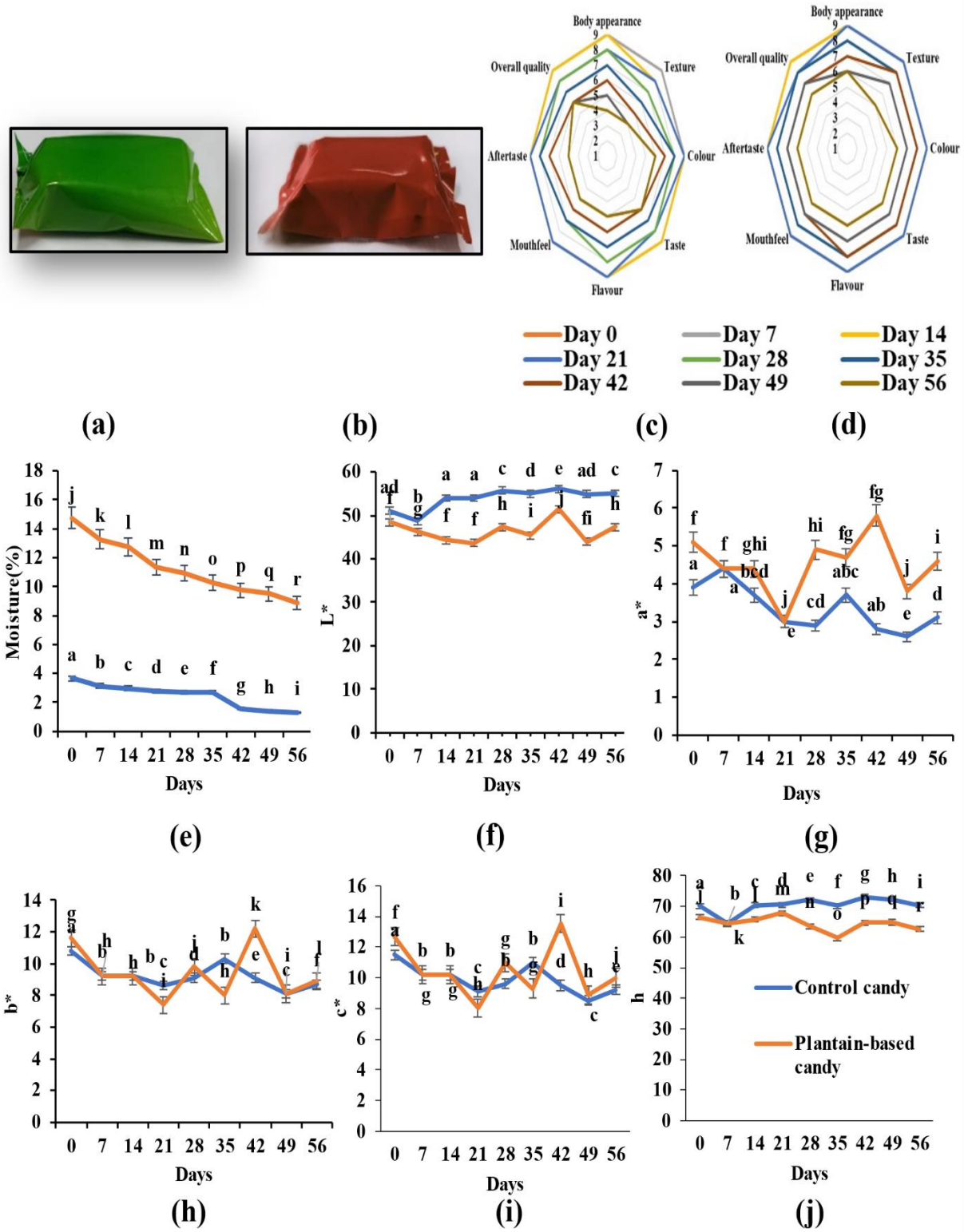

Figure 2.1.5: a) Polyethylene terephthalate/metalized polyester/polyethylene packaged candy; b) biaxially oriented polypropylene/metallized bi-orientated polypropylene packaged candy; radar plot of hedonic scores obtained by sensory analyses of c) control candy sample and d) plantain candy sample during storage at 27±2 °C, 80±2% RH; e) alterations in % moisture content (on wet weight basis) during storage of control candy sample and plantain candy sample; alterations in colour parameters f) L* value, g) a* value, h) b* value, i) c* value, and j) h value of control candy sample and plantain candy sample during storage.

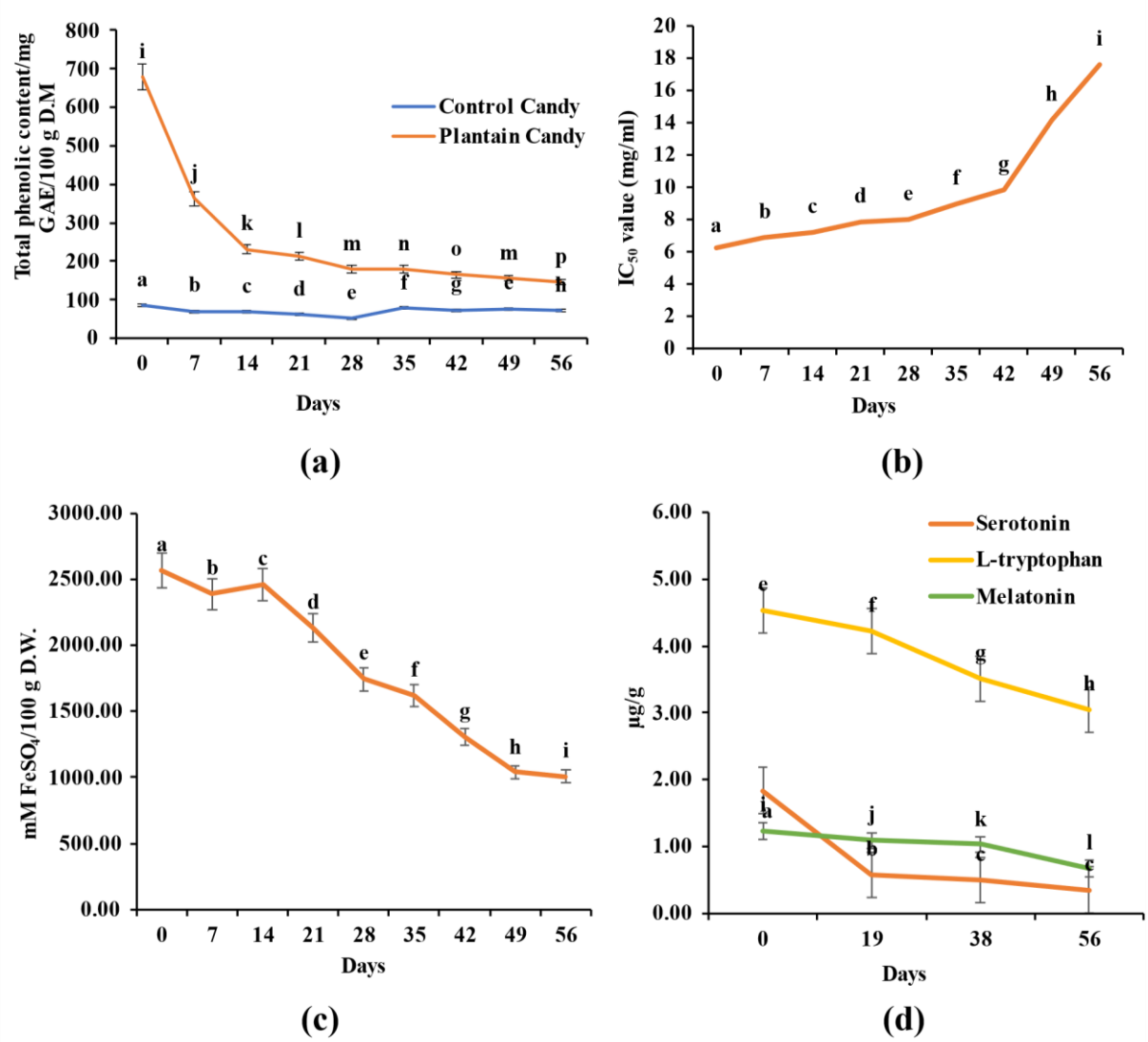

Figure 2.1.6: Phytochemical analysis of plantain candy sample during storage- a) total phenolic content (mg GAE/100 g D.W.); b) IC_{50} value by DPPH (mg/mL); c) FRAP value (mM $FeSO_4$/100 g D.W.); d) L-tryptophan, serotonin, and melatonin (µg/g D.W.) in plantain candy sample during storage. %RSD of high-performance of liquid chromatography analyses results: ≤3%. The limits of quantification for L-tryptophan, serotonin, and melatonin were 1.88, 0.89, and 0.51 µg/L, respectively. Each value is mean ± SD of three sets of samples. Different alphabets denote that mean values belong to different subsets at $p < 0.05$.

Part 2.2

In vitro release kinetics and *in vivo* bioavailability studies of L-tryptophan-serotonin-melatonin from the designer candy

Introduction

The previous part of this chapter reported on successful formulation of a L-tryptophan-serotonin-melatonin rich plantain-based sugar-free candy and its detailed characterization. Therefore, the specific objectives of the this part of the study were to investigate – the release kinetics of L-tryptophan, serotonin, and melatonin from the new designer plantain-based candy by *in vitro* dissolution assays; - *in vivo* releases of the biomolecules in the blood serum of rodent (rats) models post-consumption of the candy; and finally to ascertain the role(s) of the liver and/or periphery as the functional centralities of metabolism, and of the primary site (brain and/or gut) of glucose uptake during metabolism by non-invasive predictive modelling using iHOMA2. These aspects of molecular nutrition for the said biomolecules from a plantain-based product are being investigated for the first time.

Materials and methods

Materials

The plantains, raw materials and chemicals used in this study were the same as described in Part 2.1 of Chapter 2, unless specified. The ELISA kit for melatonin (with an assay sensitivity of 2 pg/mL) was procured from M/s SunLong Biotech Co. Ltd., Zhejiang, China, and ELISA kits for serotonin (with an assay sensitivity of 230 pg/mL) and L-tryptophan (with an assay sensitivity of 4.18 pg/mL) were purchased from M/s Bioassay Technology Laboratory, Zhejiang, China. The ELISA kit for insulin (with an assay sensitivity of 3.75 pg/mL) was

purchased from M/s Elabscience, Texas, USA. The blood glucose measuring device from M/s AccuSure Simple glucometer was used in the present study. Walnuts (kernel chile) were procured from Spencer's Retail Limited, Kolkata, India.

Methods

Studies on the release kinetics of L-tryptophan, serotonin, and melatonin from the designer candy

To ascertain the bioavailabilities of the three molecules *in vivo*, it is imperative to first determine whether they are released *in vitro* under simulated gut buffers. Based on preliminary trials (conducted with a magnetic stirrer), dissolution of the designer candy in simulated oral, gastric, intestinal, and rectal buffers was conducted (details *vide infra*). Except for gastric buffer, all buffers were kept at neutral pH (7), which would be conducive to the stability of serotonin and L-tryptophan, which are reportedly known to be more stable at neutral pH than at acidic pH (Huang and Kissinger, 1996, Lazzari *et al*., 2019). The stability of melatonin does not depend on the pH of the buffer (Daya *et al*., 2001), and thus its release can be well investigated in gut-simulated buffers of varying pH (Daya *et al*., 2001).

Investigations on phenolics and antioxidant activity of bamboo leaf soup by *in vitro* digestion revealed no significant differences in antioxidant activities when the soup was subjected to digestion with and without enzymes (*i.e*., treated with buffers of different pH), attesting to the fact that the release of antioxidants from the food product is more dependent on the pH of the dissolution buffer than on enzymatic digestion of the same (Ma *et al*., 2020). Moreover, the addition of enzymes was avoided since the same would incur complex downstream purification of buffer aliquots (*vide infra*), which would render HPLC-PDA analyses of L-tryptophan, serotonin, and melatonin erroneous (Tamili *et al*., 2023). It is hereby conjectured that if the

target biomolecules are 'released' unaided by enzymes, it will certainly be so in the presence of digestive enzymes *in vivo*.

Therefore, in the current investigation, *in vitro* dissolution studies have been performed to assess the release of the target molecules in four different buffer systems, *viz.*, oral, gastric, small intestinal (duodenum), and rectal buffers. Since it is reportedly known that maximum absorption of melatonin occurs in the rectum of male Sprague Dawley rats (Tran *et al*., 2009) and pig-barrow (Bubenik *et al*., 1996), the other regions of both small and large intestines have not been considered in the present study. Moreover, corroboration of these findings with actual gut absorption of the said biomolecules would necessitate not only animal sacrifice but also detailed histological examination of tissues of each section of the intestines (Tran *et al*., 2009), which has not been conducted here in sync with current global legislation based on the "3R" principle of "Refining, Reducing and Replacing" emphasising on the reduction of animal studies (Aerts *et al*., 2022). Additionally, the current experimental scheme comprises four separate enzyme-unaided digestion phases instead of one continuous complete digestion since the latter inadvertently led to analytical errors consequent to the dilution effect of the said biomolecules (present in micrograms in the original food product) in the respective buffers.

In the preliminary trials, the release kinetics study was performed using simulated gut buffers (gently agitated using a magnetic stirrer) without the use of enzymes. Simulated phosphate saline buffers (PBS) such as simulated salivary buffer (SSB), simulated gastric buffer (SGB), and simulated intestinal buffer (SIB) with multiple electrolytes rendering strong buffering capacities were prepared as per the composition described by Peixoto *et al*. (2013) with few modifications, and their corresponding pH values were set to 7, 1, and 7.4 to simulate the digestive environments inside the mouth, stomach, and intestine, respectively. Phosphate

buffer with minimal buffering capacity and a pH of 7.4 (Purohit *et al*., 2018) was used as simulated rectal buffer (SRB).

To simulate the mastication-cum-mouth-melting attribute of the candy, the same was comminuted into small pieces using a mortar and pestle prior to the dissolution study. The pulverised plantain candy (5g) thus obtained was placed in 250 mL beakers containing 100 mL of SSB and subjected to constant stirring using a heat-controlled magnetic stirrer (IKA RCT Basic, M/s IKA-Werke, Staufen, Germany) at 75 rpm at 37 °C (Kong and Singh, 2008) for 10 min. Aliquots (5 mL of buffer) were withdrawn from the beaker at regular time intervals (and each time an equal volume of fresh buffer was replaced) of 2, 5, 7, and 10 min (the end time was decided based on complete dissolution of the candy in the mouth as assessed by the sensory panel), and cold centrifugation (C-24BL, M/s Remi, Mumbai, India) was performed at 12300×g at 4 °C for 10 min. The supernatant was collected, filtered through a 0.22 µ syringe filter, and stored at -20 °C prior to HPLC-PDA analysis. Following this, the release of the target biomolecules was ascertained in a similar manner in SGB, SIB, and SRB, except that the aliquots were withdrawn at time intervals of 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120 min. Analyses of L-tryptophan, serotonin, and melatonin contents in the buffers revealed that very low amounts of the said molecules were released (Fig. 2.2.1) beyond 60 min, 70 min, and 90 min from the candy in SGB, SIB, and SRB, respectively.

Although the release of the molecules from the food matrix was confirmed in this study, a scale-up study employing a standard tablet dissolution unit equipped with a paddle agitator (Inspire 8 Basic, M/s Electrolab, Mumbai, India) and a buffer volume of 500 mL was conducted. This part of the work was conducted at TAAB Biostudy, Kolkata, West Bengal, India. In this method, three different batch sizes of pre-treated candy (*vide supra*) were subjected to dissolution inside the standard 80-vessel dissolution unit housing 500 mL of

buffer(s) per vessel under continuous stirring conditions at 37 °C (maintained by a thermostatically controlled water bath). In a typical paddle method, 500-1000 mL of buffer volume is recommended (Siewert, 1996); in the current study, 500 mL was taken to minimize analytical errors that might arise by high dilution of the target analytes.

The paddle speed was set to the maximum limit of 75 rpm for SSB, SGB, and SIB and 60 rpm for SRB since the batch sizes were larger than those in typical pharmaceutical formulations and also because the feed contained relatively dense particles that could otherwise turn lumpy (Klancke, 2003). Then aliquots (5 mL buffer) were withdrawn at regular time intervals (same as above), centrifuged (*vide supra*), and stored at -20 °C. This procedure was followed during the dissolution of the candy in the SSB, where aliquots were withdrawn at 2, 5, 7, and 10 min; and in the SGB, SIB, and SRB, except that the aliquots were withdrawn until 60 min, 70 min, and 90 min, respectively, at 10 min intervals (the end time chosen based on the findings discussed *vide supra*). However, analyses of serotonin, melatonin, and L-tryptophan contents in the buffers revealed that their amounts in SGB beyond 60 min, in SIB beyond 70 min, and in SRB beyond 90 min did not follow a specific trend, possibly because of analytical uncertainties that arose consequent to the presence of co-extractants and/or interfering pigments (confirmed by the presence of extra peaks in the HPLC-PDA chromatogram) and chemical absorption or physical adsorption of the analytes with the food matrix.

Three different serving sizes of the candy were initially chosen to ascertain the serving size that contributed to the maximum release of the said biomolecules. The batch sizes chosen were 24 g, 48 g, and 72 g (considering a maximum consumption limit of three candies, corresponding to 72 g at a time by an adult man of 60 kg body weight). It was evident that the lowest serving size allowed maximum release of the discussed biomolecules (Fig. 2.2.2, 2.2.3, and 2.2.4), and thus, 24 g was selected as the batch size for evaluating their release kinetic models.

The aliquots withdrawn were filtered through a double syringe filtration system (0.45 and 0.22 µm) and were then subjected to HPLC-PDA analysis to quantify the amounts of L-tryptophan, serotonin, and melatonin individually. The cumulative percentage of release of each molecule (from the candy) in each buffer was plotted against time, and the data were fitted into various standard kinetic equations, such as zero order ($Qt = K_0 \cdot t$), first order ($\ln Q = K_1 \cdot t$), sub-types of first order subtypes such as the Higuchi model ($Qt = K_H \cdot t_{1/2}$), Hixson-Crowell's cube root model ($Q_{1/3} = K_{HC} \cdot t$), and Korsmeyer-Peppas ($Qt = K_k \cdot t_n$) equations, where Qt is the amount of the biomolecule released at time t and Q is the amount of the same present in the candy, K is the constant for each model, and n is the diffusion or release exponent of each biomolecule. The kinetic model with the highest regression coefficient (R^2) was considered the best model for representing the release of the target biomolecules from the candy.

Assessment of *in vivo* bioavailabilities of L-tryptophan, serotonin, and melatonin from the designer candy

In vitro release kinetics of the said biomolecules in gut simulated buffers (with no digestive enzymes) do not truly mimic *in vivo* gut-digestion involving a myriad of enzymes. This necessitated an assessment of the release of the above-discussed biomolecules from the designer plantain candy post-absorption in actual gut conditions, and thus feeding trials involving male Sprague-Dawley rats and subsequent periodic assessments of the serum levels of the three target biomolecules were conducted.

This part of the work was planned after consultation with TAAB Biostudy, Kolkata, and conducted under the supervision of professional veterinary personnel in the facility of NSHM Knowledge Campus, Kolkata, West Bengal, India, in March-April 2023. The researchers adhered to the EU Directive 2010/63/EU and Institutional Animal Ethical Committee (IAEC) guidelines (registration no. NCPT/IAEC-012/22023) for all animal experiments, care, and use

of experimental animals, ensuring the well-being of the animals and their personal hygiene. Two-month-old male Sprague-Dawley rats (n=30) with an average body weight of 175-200 g were procured from M/s Saha Enterprise, Kolkata, India. The detailed study design (including animal grouping and time of blood withdrawal) has been presented in Fig. 2.2.5. The frequency of blood collection (Table 2.2.1) and volume of blood collected were set according to the IACUC guidelines for blood collection from rats, whereby a maximum of 10% of the total blood volume was withdrawn from each rat in two weeks (IACUC, 2022).

For the first week, the animals [two rats were kept in a polyacrylic cage (435x290x160 mm)] were acclimatized under standard environmental conditions (17-23 °C, 60±5% RH) with 12 h: 12 h dark-light (180 to 200 lux at daytime) cycles during which they had free access to a standard rat diet (a mixture of wheat flour, Bengal gram flour, milk powder, salt, and distilled water, on a weight basis) and fresh water.

Post-acclimatization, each rat was randomly selected (since all rats were healthy, active, and had similar body weights) and assigned to one of the five groups [control group, positive control group, test group, control group for iHOMA2 (CH), and test group for iHOMA2 (TH)]. Each group was comprised of six male rats. The control, positive control, and test groups were used for experiments related to L-tryptophan, serotonin, and melatonin levels in the serum, whereas the CH and TH groups were used solely for experiments related to iHOMA2 studies (*vide infra*).

At the end of 7 days of acclimatization with the standard rat diet, the endogenous levels of the three biomolecules in the rat serum were ascertained using apposite standard ELISA kits (in conjunction with an ELISA microplate reader of Merilyzer Eiaquant, M/s Meril, Vapi, India). Blood was withdrawn from the tail vein of one rat (selected randomly) from each group, and the serum was separated by cold centrifugation (at 4 °C at 1,000 x g for 20 min) and stored at

-20 ℃ for further analysis. Thereafter, all the animals in the five groups were provided with L-tryptophan, serotonin, and melatonin-lean raw noodles (prepared in our laboratory using tapioca flour, hydrocolloids, salt, oil, and water) and water *ad libitum* for three consecutive days instead of the standard rat diet (owing to the presence of L-tryptophan and melatonin in the same), following which the said biomolecules were analysed again in the blood serum at 9 a.m. and 3 a.m. in the blood serum. The animals were then kept for fasting for 24 h, followed by immediate withdrawal of blood for analyses of L-tryptophan, serotonin, and melatonin levels.

Subsequently, the rats of the control and CH groups were fed with L-tryptophan, serotonin, and melatonin-lean noodles; those of the positive control group with melatonin-rich nuts, *viz*. walnuts (Reiter *et al*., 2005); and those of the test and TH groups with the newly designed plantain-based candy (pre-treated for swallowable consistency, *vide supra*) by gavaging (at 9 a.m.), which is reportedly (Turner *et al*., 2011) a reliable method for administering substances into the GI tract. Two additional positive control groups for feeding trials with serotonin-rich or L-tryptophan-rich food or food products were not included in the absence of commercially available apposite food products (although several food or food products are reportedly known to be L-tryptophan and/or serotonin-rich).

Blood was withdrawn from the control, positive control, and test groups after 30 min, 1h, and 2h post-feeding, and then at a regular time interval of 6h up to 24h of feeding, for analysis of L-tryptophan, serotonin, and melatonin levels. After 6h of feeding, all rats were provided with L-tryptophan, serotonin, and melatonin-lean raw noodles and water *ad libitum* for the next 18h. The said time was fixed based on the studies of Padmanabhan *et al*. (2013) and Munakata *et al*. (1995), who reported that a 6h time span was the average GI transit time in 129SvEv mice and rats, respectively. Details of the gavaging procedure and subsequent blood sample withdrawal have been elaborated in Table 2.2.1.

Concomitantly, the behavioural (salivation, lethargy, sleep, convolutions) and physical (locomotion, tremor, diarrhoea, and mortality) wellness parameters of the rats were recorded systematically at 30 min, 60 min, 2h, 6h, 12h, and 24h after candy feeding, and otherwise once regularly throughout the entire experimental period following the procedures elaborated by Paul *et al*. (2021). This entire experimental scheme of feeding and assessments of the bioavailabilities of L-tryptophan, serotonin, and melatonin was conducted twice with fresh groups of animals in each scheme following similar procedures and guidelines, as stated above.

Food safety assessment of the designer candy in terms of L-tryptophan, serotonin, and melatonin intake

The 'no observed adverse effect level' (NOAEL) values of L-tryptophan and melatonin are reportedly known to be 779 mg/day/kg body weight and 5 mg/day/kg body weight, respectively (Bodin *et al*., 2021, Shibui *et al*., 2018). Their acceptable daily intake (ADI) values have been calculated using equation 1 (Chemsafety Pro, 2018):

$$\text{ADI} = \text{NOAEL value (mg/day/kg body weight)} / \text{safety factor} \quad (1)$$

where the safety factor is 100 for extrapolation from animal to human. The ADI values thus obtained were compared with concentrations of the target biomolecules present in the serving size of designer candy (24g) to assess their safe levels of intake by an adult human being (of 60kg body weight). A similar endeavour could not be conducted for serotonin in the absence of its NOAEL value.

Assessment of effects of consumption of plantain candy on β-cell function, insulin sensitivity, and glucose uptake using iHOMA2 model

In the present study, the *in vitro* non-invasive predictive model of iHOMA2 has been used to evaluate the effects of consumption of the designer candy on insulin sensitivity and glucose uptake in the rats without sacrificing the animals. Since the liver plays a central processing role

in metabolism, including a few other extrahepatic or peripheral tissues and organs (Nelson and Cox, 2002), it is necessary to adjudge whether the metabolism of the designer candy involved the functional centralities of the liver. Therefore, the breakdown and release of glucose from the plantain candy matrix and its consequent impact on glucose uptake (primarily liver and peripheral) and/or modulations in insulin sensitivity need to be assessed for this new designer candy. Additionally, since circulating melatonin is rapidly metabolised in the liver (Barret, 2010), assessment of the involvement of the liver with or without its peripheral tissues and organs is of utmost importance and was therefore conducted using iHOMA2.

Post-feeding of the L-tryptophan, serotonin, and melatonin-lean raw noodles and the designer candy to the animals of the CH and TH groups, respectively, the animals were allowed to metabolise the food completely for 6h (*vide supra*). The glucose and insulin values were assayed in the CH and TH groups just prior to feeding and then after 2h, 4h, and 6h post-feeding of the appropriate food product. In this study, %S (insulin sensitivity) and %β (β-cell function) were calculated from experimental insulin (using standard ELISA kits) and glucose (by glucose strips) values using iHOMA2 software in predictive mode (Mathews, 1985, Levy, 1998, Hill *et al*., 2013). Six predictive models based on the possible sites of metabolism were considered: 1. Alterations in insulin sensitivity equally partitioned at the liver and periphery; 2. Alterations in insulin sensitivity only at the liver; 3. Alterations in insulin sensitivity only at the periphery; 4. Alterations in glucose uptake equally partitioned at the brain and gut; 5. Alterations in glucose uptake only at the brain; and 6. Alterations in glucose uptake only in the gut, were hypothesised for increased insulin sensitivity and changes in glucose uptake (Paul *et al*., 2021). The predicted values of glucose and insulin obtained by predictive mode of iHOMA2 software were compared with their experimental values obtained by glucose strips and by ELISA kit assay, respectively, using Bland-Altman plots to assess the agreement between the two methods of glucose and insulin estimation. The predicted values of glucose and insulin were

also analysed by the F-test statistic to obtain the best fit model, as described by Hill *et al*. (2013).

Statistical analysis

In vitro analyses were conducted in triplicate, and the results have been reported as mean ± SD of three sets of independent experiments. *In vivo* experiments were conducted in duplicate. Each time blood was withdrawn from two different animals in each independent experiment and the results have been expressed as mean ± SD of four independent data obtained from the blood serum of rats in the same group. Duncan's multiple range test was conducted to determine significant differences among the means of the concentrations of the said biomolecules present in the rat serum. A value of $p \leq 0.05$ was considered significant to establish the differences in all tests including Student's t-tests. The F-value was ascertained to predict the best-fit model obtained from iHOMA2. All statistical tests were performed by IBM SPSS Statistics Software Version 26 (M/s IBM, New York, USA).

Results and Discussion

***In vitro* release kinetics models of L-tryptophan, serotonin, and melatonin from the candy in a standard dissolution apparatus**

Fig. 2.2.6a presents the percentage releases of the three target biomolecules from the designer candy in SSB. The amounts of L-tryptophan, serotonin, and melatonin released in 500 mL of SSB immediately after 2 min of dissolution were 9.40 µg, 8.25 µg, and 2.94 µg, respectively, whereas after 10 min, the amounts of the same were 58.74 µg, 14.62 µg, and 13.34 µg, respectively, which corresponded to 53.95%, 33.22%, and 45.12%, respectively, of their original content(s) present in the candy.

This clearly reflects moderate releases of the desirable molecules from the matrix into the SSB. Vakkuri (1985) and Vakkuri *et al*. (1985) have reported a release of 4.835×10^4 pg/mL of

melatonin in the human saliva after 60 min of consumption of 100 mg of synthetic melatonin (in a single gelatine capsule). Thus, it could be conjectured that melatonin would be inadvertently released from the designer candy in human saliva within 10 min of its residence time in the oral cavity. There is no existing report on similar lines for L-tryptophan and serotonin to substantiate the present findings.

Fig. 2.2.6b presents the percentage release of the said biomolecules in SGB. The amounts of L-tryptophan, serotonin, and melatonin released in 500 mL SGB were 51.37 µg, 3.81 µg, and 5.23 µg after 10 min of dissolution; and 105.81 µg, 27.54 µg, and 28.10 µg, respectively, after 60 min, corroborating to appreciable releases of L-tryptophan (97.17%), serotonin (62.59%), and melatonin (95.05%).

Fig. 2.2.6c represents the percentage release of the target biomolecules in SIB. The amounts of L-tryptophan, serotonin, and melatonin released in 500 mL SIB were 11.38, 16.43, and 6.9 µg after 10 min of dissolution, and after 70 min, they were 98.18, 43.88, and 26.83 µg, respectively, attesting to appreciable releases of L-tryptophan (90.16%), serotonin (99.73%), and melatonin (90.78%). Fig. 2.2.6d represents the percentage release of the target biomolecules in SRB. The amounts of L-tryptophan, serotonin, and melatonin released in 500 mL SRB were 42.7, 17.53, and 2.93 µg, respectively, after 10 min of dissolution and after 90 min were 96.45, 32.25, and 28.28 µg, respectively, corresponding to 88.57% release of L-tryptophan, 71.52% of serotonin, and 95.66% of melatonin. For model fitting, the end time (60 min for SGB, 70 min for SIB, and 90 min for SRB) coincided with the time beyond which the amounts of the biomolecules were found to significantly decrease (possibly owing to instabilities of the molecules on prolonged dissolution in the buffers).

Post-fitting of the cumulative percentage release kinetics data into different kinetics models *(vide supra),* it was found that the release of L-tryptophan and melatonin from the plantain

candy in the oral phase (SSB) followed two different models of first-order kinetics, *i.e.*, L-tryptophan followed the Korsmeyer-Peppas model (R^2 = 0.98) and melatonin followed the Hixon-Crowell model (R^2 = 0.99), whereas zero-order kinetics (R^2 = 0.99) was evident during release of serotonin. However, in the gastric phase (SGB), all three molecules followed Korsmeyer-Peppas model kinetics (R^2 for L-tryptophan, serotonin, and melatonin were 0.98, 0.99, and 0.99, respectively).

In the intestinal phase (SIB), Korsmeyer-Peppas model kinetics were obeyed by L-tryptophan (R^2 = 0.95) and serotonin (R^2 = 0.99), whereas melatonin followed the Higuchi model kinetics (R^2 = 96). In the rectal phase (SRB), release of L-tryptophan from the plantain candy followed the Higuchi model (R^2 = 0.96); serotonin followed the Korsmeyer-Peppas model (R^2 = 0.99); and melatonin followed Hixon Crowell (R^2 = 0.99) model of release kinetics.

There is no existing literature on the release of the said biomolecules from sugar alcohol-hydrocolloid-fruit matrices to substantiate these findings. However, the release of natural and pharmaceutical active ingredients followed similar models, such as the release of anthocyanins from a chewing gum prepared with encapsulated black jamun (*Syzygium cumini*) pulp extract following the Korsmeyer-Peppas model in phosphate saline buffer at pH 6.8 (Sharma *et al*., 2024); the release of metoprolol tartrate from a tablet containing 40% xanthan gum following the Hixon-Crowell model in phosphate buffer of pH 6.8 (Varshosaz *et al*., 2006); and the release of theophylline from a quince seed hydrogel formulation following zero order kinetics at pH 6.8 and 7.4 (Ashraf *et al*., 2018). Adiba *et al*. (2011) studied the release of phycocyanin from a food tablet composed of date and spirulina powder in various solvent systems of different pH including acidic (0.1 N HCl) and found that the diffusion of phycocyanin followed the Korsmeyer's Peppas model. Banala *et al*. (2018) reported Higuchi release kinetics model for the drug duloxetine hydrochloride in buccal mucosa at neutral pH, and the same model was

followed by L-tryptophan when released from a hydrogel into a neutral phosphate buffer solution (Larrañeta *et al*., 2014).

According to previous reports by Huang and Kissinger (1996), serotonin is more stable at neutral pH, *i.e.*, pH 7, and less stable at acidic pH, whereas the stability of melatonin is not much affected by an alteration in pH (Daya *et al*., 2001). Accordingly, serotonin followed zero order release kinetics in SSB (pH 7), indicating its continuous release from the candy matrix irrespective of its source concentration, and in SIB (pH 7.4) and SRB (pH 7), it followed the Korsmeyer-Peppas model, indicating diffusion-dependant release. The release of melatonin followed the Hixon Crowell model of sustained release kinetics in both SSB and SRB (pH 7), indicating that the release was majorly dependent on the alterations in the size and surface area of the comminuted candy pieces during dissolution. However, in SIB, melatonin followed the Higuchi model, indicating the release of biomolecules as a square root of a time-dependent process based on Fickian diffusion. A similar model was followed by L-tryptophan in SRB (pH 7). L-tryptophan is known to demonstrate better stability in neutral pH (Lazzari *et al*., 2019); however, it followed the Korsmeyer-Peppas model of release kinetics, indicating a diffusion-dependant release in SSB and SIB. On the contrary, in the acidic pH of SGB (pH 1), all three molecules obeyed a single model of release kinetics, *viz*., the Korsmeyer Peppas model, indicating anomalous non-linear trends.

The exact explanation of the release kinetics models thus obtained is intriguing, possibly owing to a complex interplay of several factors, such as varying macro- and micro-structures of the food matrix (varying particle sizes and surface areas exposed to the dissolution buffers with time), alterations in viscosities and consistencies of the dissolution buffers with time of dissolution, the abundance of the said biomolecules present in the processed food matrix, interactions among the food components and with additives, and the relative bulkiness of the food constituents with respect to the buffer (and hence their diffusional resistances).

In vivo bioavailabilities of L-tryptophan, serotonin, and melatonin from the designer candy in male Sprague-Dawley rats

Immediately following acclimatization, the mean blood melatonin level in three randomly chosen animals at 3 a.m. was found to be 44.38±3.26 pg/mL. Serum levels of tryptophan and serotonin at the same clock time were found to be 275.3±23.98 pg/mL and 8930±765 pg/mL, respectively. Fig. 2.2.7a-c present the alterations in melatonin, serotonin, and L-tryptophan levels, respectively, in the blood serum levels of the rats during the entire experimental period of 6 days (post-acclimatization). After 3 days of consumption of the L-tryptophan, serotonin-melatonin-lean diet, the serum levels of the above-mentioned molecules decreased significantly ($p \leq 0.05$) on day 10, although the natural trends of increase and decrease of serum serotonin and melatonin during different times of the day (when monitored at 9 a.m. in the morning and 3 a.m. at midnight) remained unperturbed (Grivas and Savvidou, 2007, Mateos *et al*., 2009). Also, the wellness parameters did not reflect any abnormalities in physiological, neurological, and behavioural patterns implying that the rats remained healthy and active throughout the study period, which was possibly owing to an interplay of the presence of dopamine ($[M + K + H]^+$) in the plantain candy as has been identified in the ESI-TOF-MS spectra (Fig. 2.2.8 in previous chapter); the presence of serotonin above its endogenous level (*vide infra)*; and the presence of melatonin *(vide infra*) at levels that did not perturb the sleep-wake cycle.

The alterations in serum melatonin levels during the entire experimental period (6 days post-acclimatization) are presented in Fig. 2.2.7a. The serum melatonin level of the positive control group (walnut-fed rats) followed a natural trend of high and low (*vide supra*) throughout the tenure of the experimental period, except on the day of feeding. Post-feeding of walnuts, the serum melatonin level was observed to be significantly ($p<0.05$) increased after 30 min vis-à-vis that of the control (*i.e.*, the endogenous level) and test groups; however, the level was significantly ($p<0.05$) low after 60 min compared to that of the test group, and thereafter, the

levels followed the trends of increase and decrease similar to those of the control group of rats. These findings are in consonance with the findings of Hattori *et al.* (1995), where blood melatonin levels of chicks were measurably enhanced after consumption of melatonin-containing food products such as corn, milo, beans, and rice, and with the study of Reiter *et al.* (2005), who found that the serum melatonin levels in Sprague-Dawley rats appreciably increased after feeding walnuts. However, the melatonin levels in the control group of rats followed the regular trend of up and down cycles for the entire experimental tenure, with no exemption on the day of treatment. Post-gavaging of the test group (candy-fed rats), a significant ($p<0.05$) increase in serum melatonin level was observed both after 30 min and 60 min compared to that of the control group (endogenous level). After 1 h of feeding, the trend of increase and decrease in serum melatonin level of the test group was similar to that of the control and positive control groups; however, the levels were relatively higher ($p<0.05$) vis-à-vis those observed in the latter groups. Thereafter, the serum melatonin level significantly decreased after 120 min ($p<0.05$) and at 3 p.m. ($p<0.05$). Subsequently, from 9 p.m., the serum melatonin levels in the test group of rats increased significantly ($p<0.05$) and reached their peak ($p<0.05$) at 3 a.m. (18 h post-consumption). The trend of increase in serum melatonin post-feeding of the candy was possibly owing to the fact that the biomolecule being amphiphilic (Ritwiset *et al*., 2021) was readily absorbed by the body when taken orally.

Increases in blood serum levels of melatonin in human volunteers (18-25 years old male) were observed post-consumption of two whole peeled bananas (stage of ripening and species of the fruit and amount consumed have not been reported by the investigators); orange juice and pineapple juice (amounts consumed not known) extracted from one kg of the respective fruits, when each food product was consumed individually with intermittent wash-out periods (Sae-teaw *et al*., 2013). Interestingly, the plantain-based new designer candy could release the same within half the reported time in Sprague Dawley rats. This difference could be attributed to the fact that processing may have rendered the food matrix(s) more bioamenable for the release of

melatonin, notwithstanding the fact that animal to human extrapolation involves uncertainties owing to several biochemical variabilities. The decline in the melatonin concentration after 60 min of feeding is in sync with the well-known fact that melatonin is a short-lived molecule with an average life of about 20-40 min in the blood stream (Maldonado *et al*., 2009). The increase in the melatonin level in the rats, however, did not induce lethargy, sleep, or impaired locomotion activities in the animals. This certainly attests to the nutraceutical efficacy of the designer candy. Moreover, the routine peak of melatonin occurred at 3 a.m. (at its acrophase) the following day, signifying perfect hormonal homeostasis in the animals (Cutolo *et al*., 2005).

Fig. 2.2.7b depicts the alterations in the serum levels of serotonin in the control, positive control, and test groups of rats during the post-acclimatization experimental period. No effect of feeding was observed in the control and walnut-fed positive control groups of rats; rather, the changes in serum serotonin levels in these two groups followed the natural physiological cycle (Mateos *et al*., 2009). It was observed that after feeding, the test group (candy-fed rats) showed a significant ($p<0.05$) increase in serum serotonin level both after 30 min and 60 min of candy consumption. The level was observed to decrease significantly ($p<0.05$) thereafter, *i.e.*, after 120 min of post-consumption and thereafter at 3 p.m. and 9 p.m. This finding is very promising since serotonin does not have a synthetic drug counterpart, and its deficiency is known to cause neural complications such as attention deficit hyperactivity disorder (ADHD) (Duff, 2014). Processed food products are unlikely to deliver serotonin at desirable levels primarily because they are impaired by the presence of several chemical additives, including preservatives, and possibly also owing to the absence of serotonin-metabolism elicitor(s) either in the consumer or in the food product. The tapioca-based L-tryptophan, serotonin, and melatonin-lean diet fed to the rats (prior to gavaging the designer candy) is known to house serotonin elicitors such as zinc and magnesium (Oboh and Elusiyan, 2007), which could have aided in the bioavailability of serotonin from the designer candy (Duff, 2014, Bhattacharjee *et*

al., 2016). Therefore, this minimally processed designer candy (with minimal food additives) could not only be a nutraceutical product for melatonin delivery but for serotonin as well.

A nutraceutical powder mix of Jerty Valley cherries was found to augment blood serotonin level (230×10^3~ to ~280×10^3 pg/mL) at 7 p.m. and melatonin level (~200 to ~ 280 pg/mL) in its acrophase in young (6-7 months) male Wistar rats when fed continuously for ten days along with their standard diet (Delgado *et al*., 2012); and also increased the urinary 6-sulfatoxymelatonin levels in human volunteers post consumption of the same twice a day for five days (Garrido *et al*., 2013). The designer candy, however, augmented the levels of the said hormones within 30 min of consumption.

From Fig. 2.2.7c, it can be observed that the control group showed no elevation in serum levels of L-tryptophan throughout the entire tenure of the experiment. However, the same increased sharply in the apposite groups of animals immediately after feeding walnuts and the designer candy. The serum levels of L-tryptophan were observed to increase significantly ($p<0.05$) after 30 min and 60 min of consumption of the food products in the positive control (walnut-fed rats) and in the test (candy-fed rats) groups, respectively. However, the test group showed a higher increase in blood L-tryptophan level vis-à-vis that of the positive control group. Among the three target biomolecules, the absorption of L-tryptophan was highest (in the test group), which is attributed to the fact that the food product was richest in L-tryptophan. The level of the same then reduced significantly ($p<0.05$) at 9 p.m. after which the levels were similar to those of the control group. These findings attest to an increase in serum tryptophan level post-feeding of the designer candy, as has been known to occur after oral administration of synthetic L-tryptophan to a mutant strain of Sprague-Dawley rats (Emori *et al*., 1983). There is no literature to directly corroborate this finding with tryptophan-rich foods. Researchers such as Bravo *et al*. (2013) have monitored levels of serotonin-melatonin derivatives (and not of

tryptophan) in the urine of human volunteers after being served with tryptophan-enriched cereals; and similarly, Garrido *et al*. (2013) too (*vide supra*), to cite a few.

Since the standard rat diet was devoid of L-tryptophan, serotonin, and melatonin content, it can be concluded that the alterations in the blood levels of these three target biomolecules were the effect of consuming walnuts (for the positive control group) and designer candy (by the test group).

Calculated safe levels of L-tryptophan and serotonin post-consumption of the designer candy

The ADI values for L-tryptophan and melatonin were found to be 0.779 mg/day/kg of body weight and 0.05 mg/day/kg of body weight, respectively, which corroborates to 46.74 mg of L-tryptophan and 3 mg of melatonin for an adult human (60 kg body weight). The ADI values for L-tryptophan and melatonin in adult humans are much higher than their respective amounts present in the serving size of candy, and thus consumption of the designer candy will not invite amino-acid antagonism (Damodaran, 2008) and would also safely leave provision for the individual to consume L-tryptophan from other food sources.

Interrelationships among intake of tryptophan-rich foods, exposure to light, and melatonin level

Previous studies have reported that the amount of melatonin secretion at night is largely dependent on the amount of tryptophan intake in the morning (first meal) and exposure to bright light (Fukushige *et al*., 2014, Fukuda and Morita, 2016). In the present study, on the consecutive day of feeding, the levels of serum melatonin at 3 a.m. (at its acrophase) were higher in both the test and positive control groups of rats vis-à-vis that (endogenous level) of the control group of rats, indicating the combination effect of consumption of a L-tryptophan-rich diet (leading to production of melatonin via serotonin) and the time of feeding, *viz*., in the morning, thereby justifying that there exists a strong correlation between the time of

consumption of the L-tryptophan, serotonin, and melatonin-rich diet and the exposure to bright light on the enhancement of melatonin in the rat blood serum.

Effects of candy consumption on serum insulin level and glucose uptake

Alterations in serum insulin and blood glucose levels of the rats along with their respective %S and %β values at 0h (before feeding), 2h, 4h, and 6h post-feeding of the plantain candy are presented in Table 2.2.2. The serum insulin values were lower for rats fed with a L-tryptophan, serotonin, and melatonin-lean diet (control), whereas the values increased in the candy-fed rats (test group). The Bland-Altman plots showed good fit between the experimental and predictive values of glucose and insulin in rats indicating an agreement between the methods. Table 2.2.3 elaborates on the predictive results from iHOMA2 and the F-values of model fitting. From the obtained F-values, it can be concluded that the models based on the second hypothesis (insulin sensitivity increased in the liver) and the sixth hypothesis (glucose uptake increased in the brain) fitted well with the experimental data of glucose and insulin levels in the candy-fed rats (Table 2.2.3). Consumption of the designer candy thus involved the central organ of metabolism, the liver (Nelson and Cox, 2002). Therefore, in the present study, the findings of the non-invasive predictive model of iHOMA2 strongly indicated that post-consumption, the newly developed candy enhances either insulin sensitivity in the liver or glucose uptake in the brain or both, in consonance with the changes that are known to occur post-oral administration of synthetic melatonin to male Wister rats (Zanuto *et al*., 2013). The second and sixth predictive hypothesis models of iHOMA2 confirmed involvement of the liver and the brain in the metabolism of the designer candy, and thus the post-consumption increases in serum L-tryptophan, serotonin, and melatonin levels are not invalid metabolic panaceas (Bisson *et al*., 2016).

The formulated plantain candy, being a completely sugar-free confectionary, did not cause an excess increase in the blood sugar level or serum insulin level post-consumption (Table 2.2.2).

All the ingredients utilized for the development of the plantain candy reportedly possessed low to very low glycemic indices, such as 40 for unripe plantains (Barine and Yorte, 2016), 9 for sorbitol, 0 for mannitol (Msomi *et al*., 2021), and 0 for gum acacia (Salama *et al*., 2021). From these values, it could be supposed that the glycemic index value of the formulated plantain candy would also be low.

A processed food product as a vehicle of L-tryptophan, serotonin, and melatonin: promises and concerns

The new designer plantain candy could be a promising food vehicle for serotonin and/or melatonin deficient populations. A confectionary as a food matrix for molecular nutrition (Norheim *et al*., 2012) of L-tryptophan, serotonin, and melatonin is the first of its kind. The semi-hard texture of the candy would render it suitable for consumers of all ages.

It is reportedly known that under conditions of enhanced insulin in the blood, tryptophan is largely transported to the brain, where it stimulates the synthesis of serotonin (Wurtman and Wurtman, 1995). This raises concern for the designer candy, which, during its metabolism, has inadvertently enhanced blood insulin levels and could possibly augment serotonin production, which therefore may lead to perturbation in the hormonal homeostasis if the candy is consumed on a long-term basis. Moreover, a greater amount of melatonin resides in the GI tract than in the pineal gland, especially under high serum tryptophan levels (Bhattacharjee *et al*., 2016), necessitating the execution of continuous animal feeding trials to assess the adverse effects of prolonged consumption of the designer candy prior to conducting human trials.

Conclusion

The findings of the present study strongly established the newly designed sugar-free plantain-based candy as a truly antioxidant-rich food product since it exhibited appreciable antioxidant activity with considerable release of the three antioxidants, namely, L-tryptophan, serotonin, and melatonin, under *in vitro* simulated gut conditions. *In vivo* feeding studies in male Sprague-

Dawley rats confirmed the true bioavailabilities of the said biomolecules. The non-invasive predictive model of iHOMA2 confirmed the involvement of the functional centralities of metabolism in candy digestion.

The designer candy is indeed a true functional food, providing molecular nutrition of L-tryptophan, serotonin, and melatonin in animals and can hold promise of delivery of the same to humans. It could be particularly beneficial for serotonin-melatonin-compromised people. Moreover, this confectionary did not increase the blood glucose beyond the normal physiological level and maintained perfect hormonal homeostasis, and hence could safely be consumed by consumers at large. However, more detailed investigations on glycemic index post-consumption, metabolic changes and physiological effects in animals and human are warranted to render the newly formulated sugar-free candy as a "brain food" for consumers *en masse*. Nevertheless, the detailed methodology described in this work can be safely extrapolated to assessment of bioavailabilities of the said or other biomolecules from other processed food products.

Novelty

The uniqueness of the present study lies in firmly establishing the new designer sugar-free plantain-based candy as truly antioxidant-rich, capable of successfully enhancing blood levels of L-tryptophan, serotonin, and melatonin post-consumption. More specifically, this work represents a venture into 'molecular nutrition', since the bioavailabilities of specific biomolecules have been targeted and accomplished through a designer food product for the first time. Furthermore, this endeavour can be counted as yet another unique plantain-based food product that can increase the overall utilization of this nutritious agro-commodity.

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Tables:

Table 2.2.1: Details of gavaging procedure and time of blood withdrawal from male Sprague Dawley rats

Day	Diet	Action	Blood collection time	Rat number 1#	Rat number 2#
1-7	Standard rat diet	Acclimatization		All rats	All rats
7		Blood withdrawn	3:00 am	R1	R6
8-10	Tapioca-based noodle diet flour	Acclimatization with L-tryptophan, serotonin, and melatonin-lean diet		All rats	All rats
8		Blood withdrawn	9:00 am	R2	R5
10			3:00 am	R3	R4
11	-	Fasting		All rats	All rats
12	Gavaging with designer candy	Feeding	9:00 am	All rats	All rats
	-	Blood withdrawn	9:30 am	R4	R3
			10:00 am	R5	R2
			11:00 am	R6	R1
	Tapioca-based noodle diet flour		3:00 pm	R1	R5
			9:00 pm	R2	R6
13			3:00 am	R3	R1
			10 am	R4	R5

#R1-R6 denotes six rats from each (control, positive control, and test) group.

Table 2.2.2: Blood glucose level, serum insulin level, insulin sensitivity and β-cell function of rats

Group	Blood glucose* (mmol/L)	Serum insulin* (pmol/L)	%S	%β
0h	5.1 ± 0.49	102.16 ± 8.35	53.8	144.6
2h	7.2 ± 0.63	108.11 ± 9.22	46.8	77.4
4h	6.3 ± 0.51	99.74 ± 8.45	52.2	94.3
6h	5.3 ± 0.49	90.34 ± 7.87	59.9	123.2

*Data are mean ±SD of six rats
%S - insulin sensitivity; %B - β-cell function

Table 2.2.3: Results of iHOMA2

Hypotheses	Predicted glucose (mmol/L)[#]	Experimental glucose (mmol/L)[##]	*F value*	Predicted insulin (pmol/L)[#]	Experimental insulin (pmol/L)[##]	*F value*
After feeding plantain-based candy						
Increase in insulin sensitivity is equally partitioned between liver and periphery	5.37	6.27	78408.34	66.73	99.40	59228.89
Increase in insulin sensitivity is only sited at the liver	5.83	6.27	880.65	83.2	99.40	59371.14
Increase in insulin sensitivity is only sited at the periphery	5.67	6.27	78408.34	76.13	99.40	246937.4
Glucose uptake is equally partitioned between brain and gut	3.23	6.27	14.43	14.57	99.40	40.07
Glucose uptake is only sited at the gut	5.47	6.27	94.66	68.5	99.40	13.67
Glucose uptake is only sited at the brain	4.07	6.27	15.48	29.07	99.40	2.12

#Predicted values of glucose (mmol/L) and insulin (pmol/L): Values of glucose and insulin as obtained from iHOMA2 in predictive mode

##Experimental value of glucose (mmol/L) and insulin (pmol/L): Experimental values of glucose was measured by glucose strips and values of insulin by using standard ELISA kits

Figures:

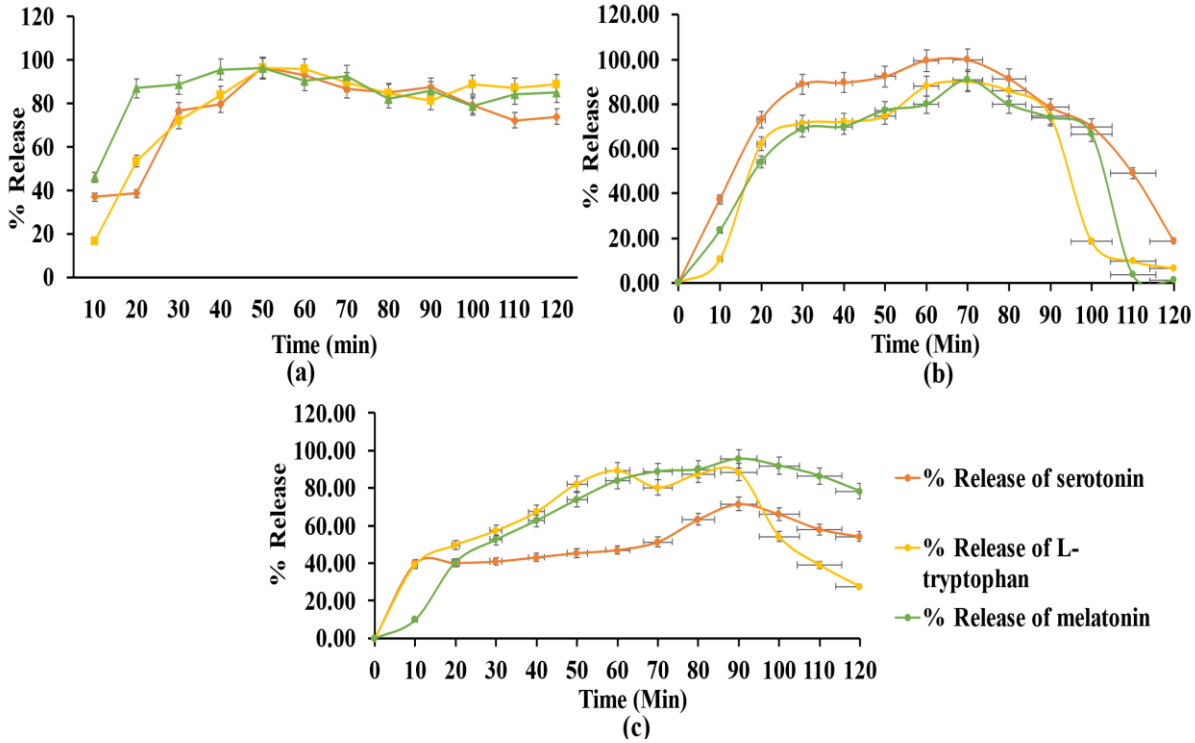

Figure 2.2.1: Release profile of L-tryptophan-serotonin-melatonin from plantain-based candy during preliminary dissolution study for 120 min in a) simulated gastric buffer; b) simulated intestinal buffer; and c) simulated rectal buffer

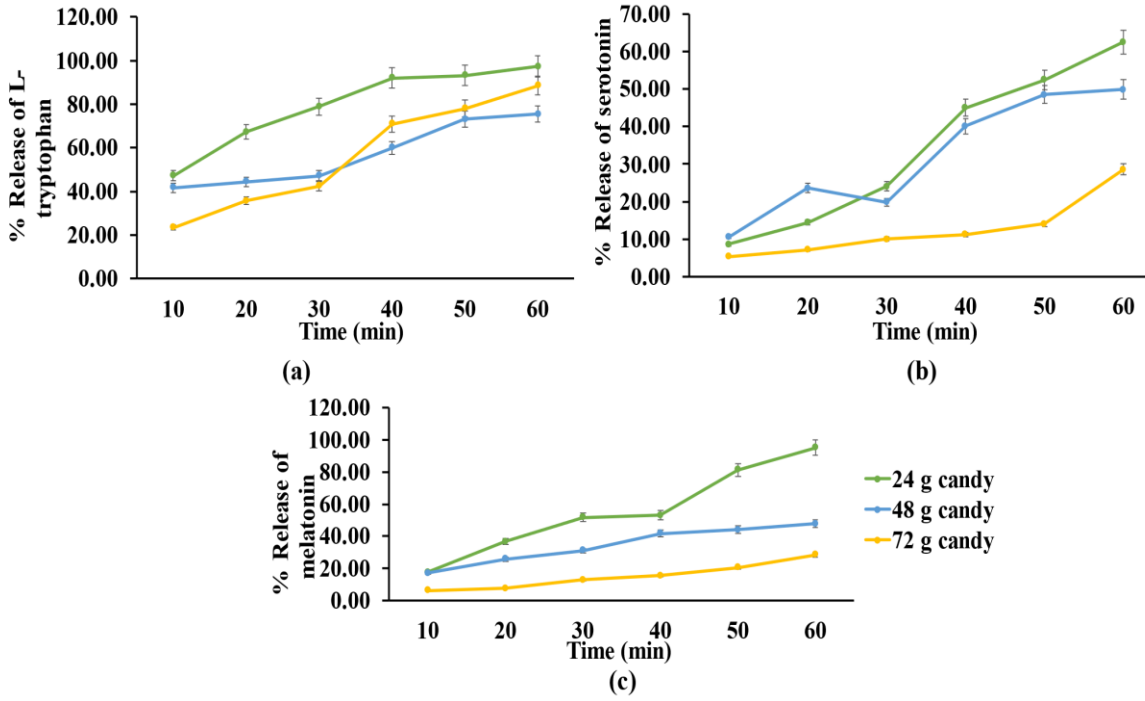

Figure 2.2.2: Release profile of a) L-tryptophan, b) serotonin, and c) melatonin from different serving size of plantain-based candy during preliminary dissolution study in simulated gastric buffer

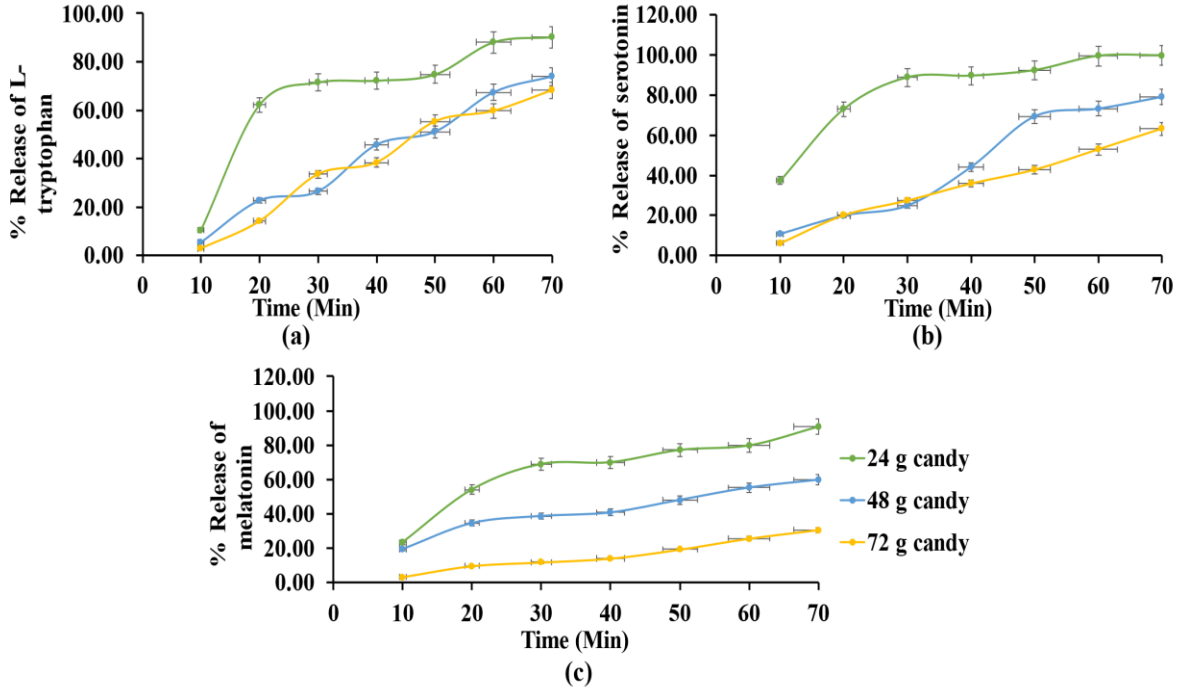

Figure 2.2.3: Release profile of a) L-tryptophan, b) serotonin, and c) melatonin from different serving size of plantain-based candy during preliminary dissolution study in simulated intestinal buffer

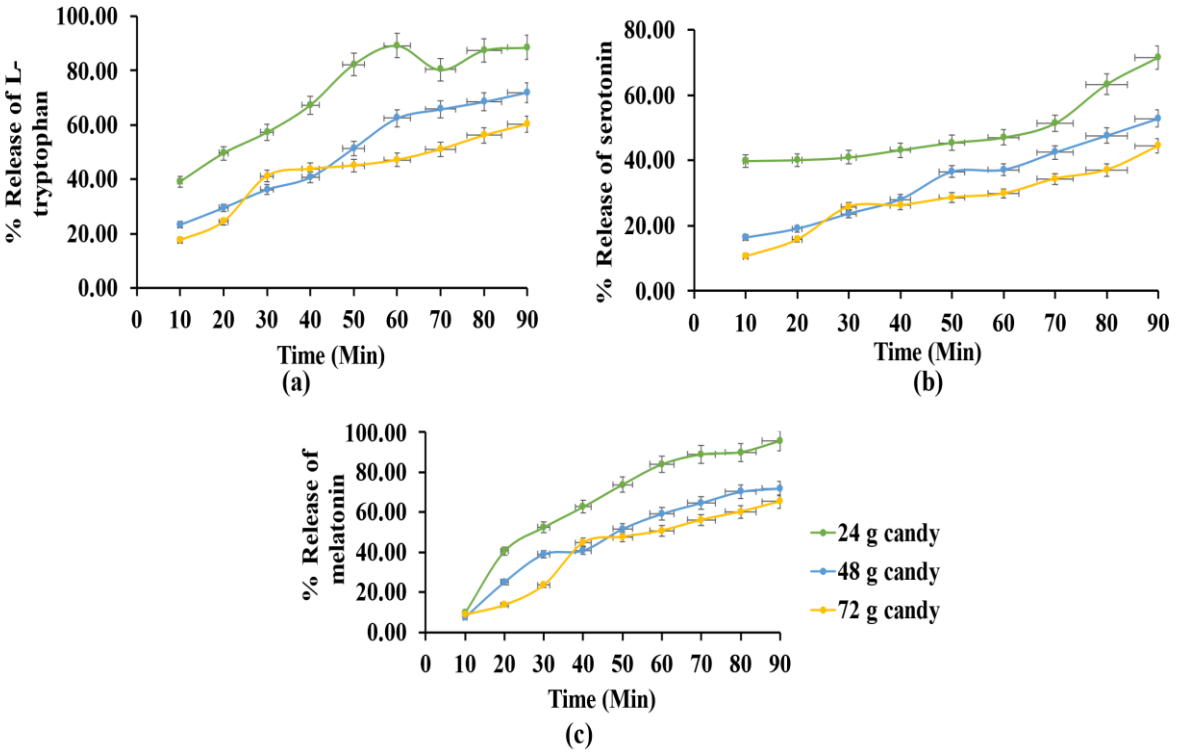

Figure 2.2.4: Release profile of a) L-tryptophan, b) serotonin, and c) melatonin from different serving size of plantain-based candy during preliminary dissolution study in simulated rectal buffer

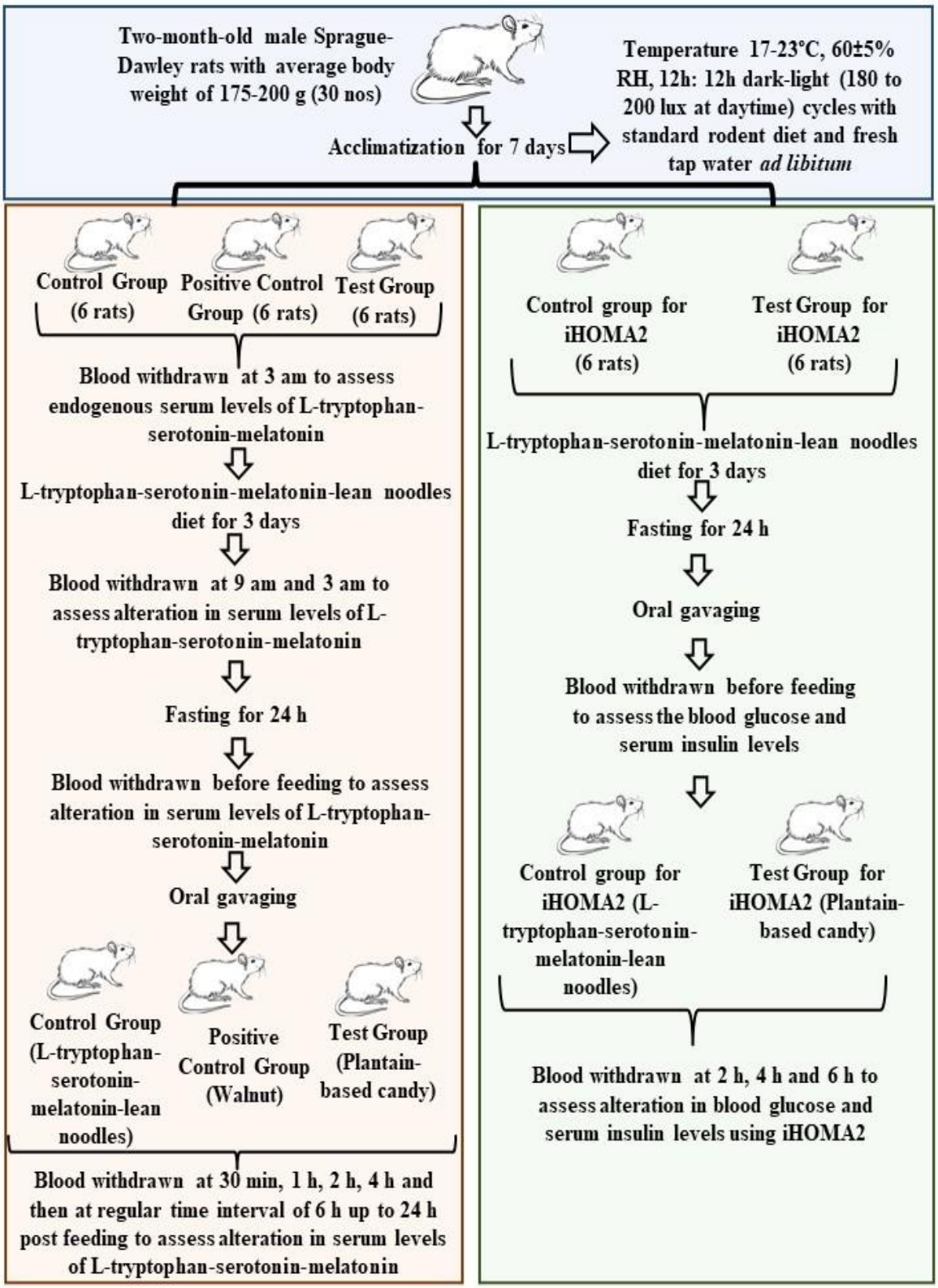

Figure 2.2.5: Experimental design for i*n vivo* study of bioavailabilities of L-tryptophan, serotonin, and melatonin in male Sprague-Dawley rats

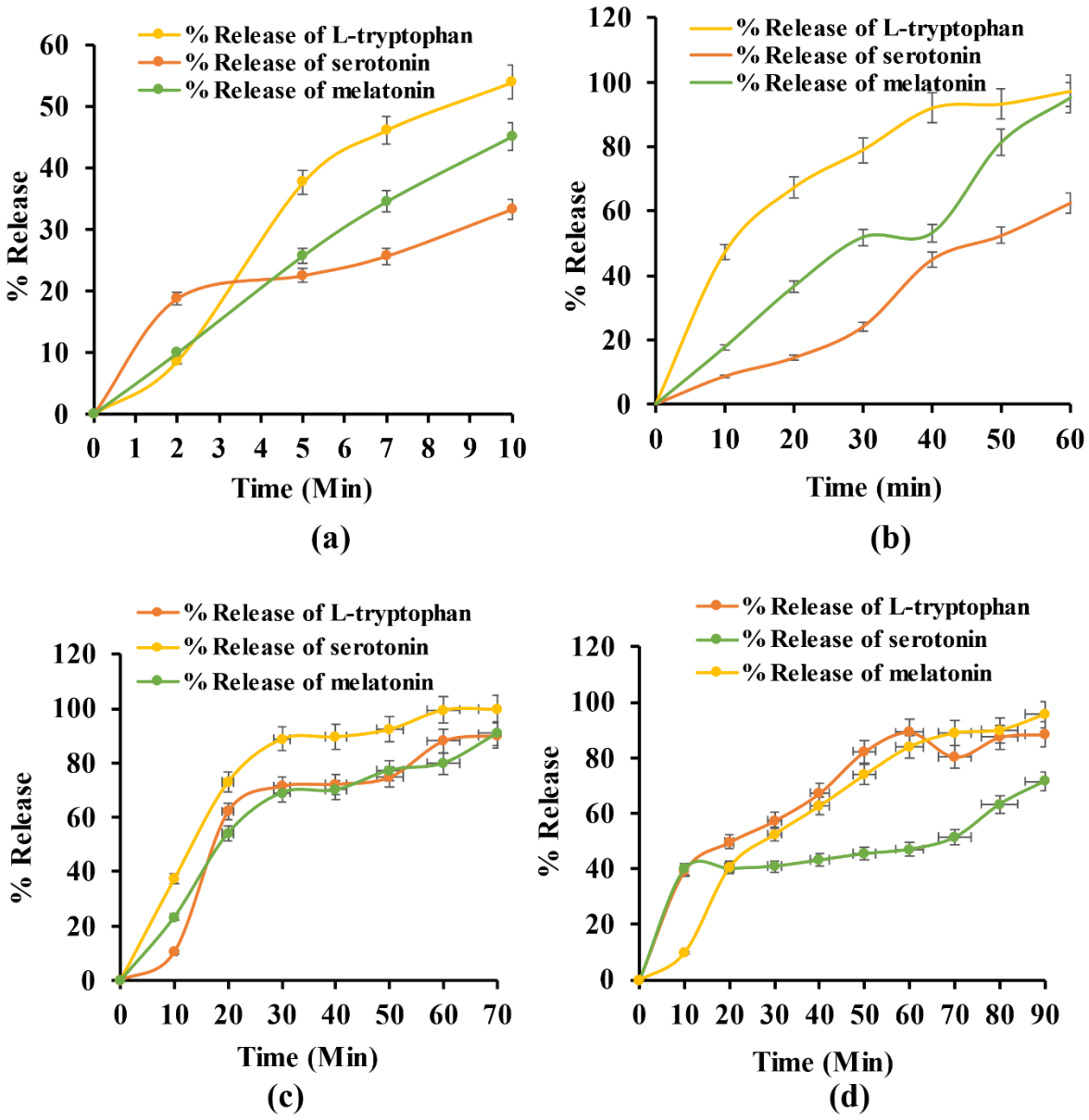

Figure 2.2.6: Release profiles of L-tryptophan, serotonin, and melatonin from plantain-based candy during dissolution a) in simulated salivary buffer, b) in simulated gastric buffer, c) in simulated intestinal buffer, and d) in simulated rectal buffer

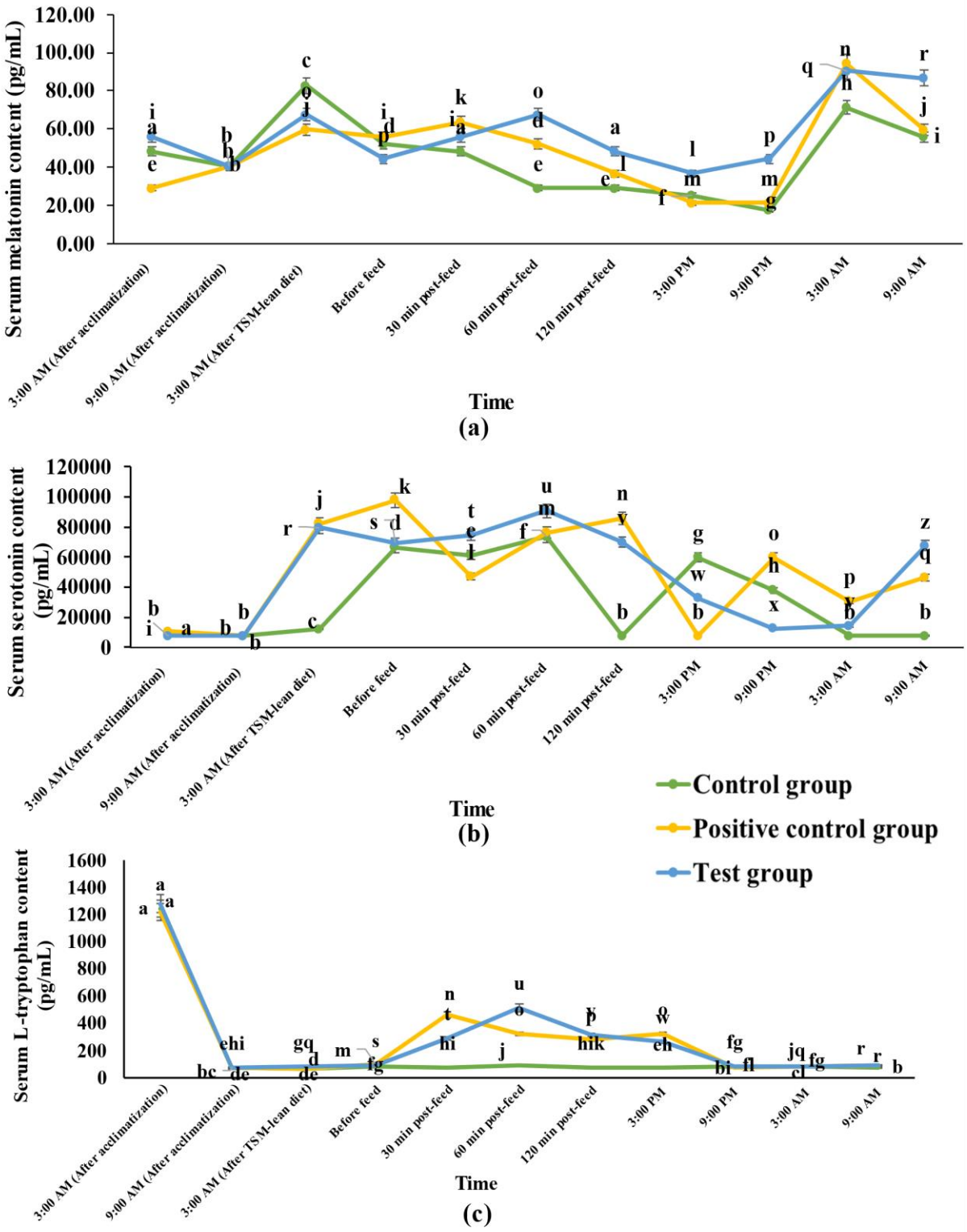

Figure 2.2.7: Alterations in serum levels of a) melatonin, b) serotonin and c) L-tryptophan in Sprague-Dawley rats with time- before feeding L-tryptophan-serotonin-melatonin-lean noodles, after feeding L-tryptophan-serotonin-melatonin-lean noodles, after fasting and after gavaging the plantain-based candy

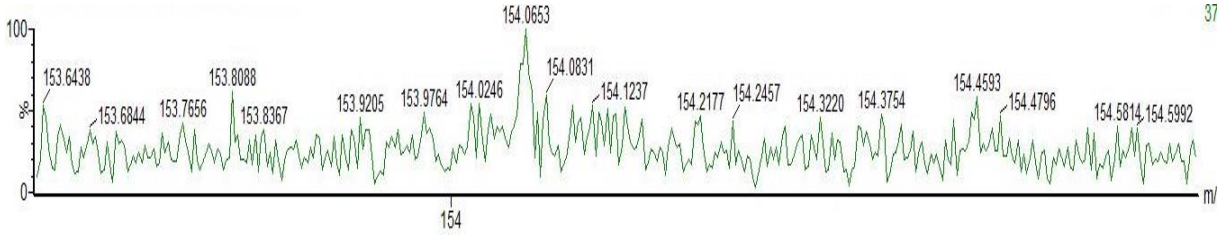

Figure 2.2.8: ESI-TOF-MS spectra of QuEChERS extract of sugar-free plantain-based candy indicating the peaks for dopamine $[M + K + H]^+$

Chapter 3

Gamma-Irradiation Processing of 'Desi' Variety of Green Plantains

Introduction

Utilization of the raw green plantains in developing a new confectionary such as candy containing three bioactive molecules namely, L-tryptophan, serotonin, and melatonin, and the bioavailability of the same after consumption have been elaborately discussed in the preceding chapter (Chapter 2). Apart from producing value-added products (such as candy) from freshly harvested plantains, extending their shelf-life could lead to delayed senescence and thus can reduce their wastage. Literature reports suggest that application of the unconventional non-thermal eco-friendly–cum-low-cost preservation technology of gamma-irradiation can enhance the overall shelf-life of agro produces and also that of plantains. Aina *et al*. (1999) reported that an optimized dose range of 0.15-0.30 kGy could delay the ripening of three plantain (*Musa paradisiaca*) cultivars namely, Agbagba, Obino L'ewai and Cardaba for 10-12 days. Another study by Zaman *et al*. (2007) reported that the shelf-life of banana (*Musa sapientum*) could be increased by 20 days when they were subjected to gamma-irradiation at doses of 0.3, 0.4, and 0.5 kGy and were successively sealed tightly inside cellophane bags. Sunyoto *et al*. (2019) stated a close relation between the shelf-life (for 6 days) of bananas (*Musa acuminata* colla) at various maturity levels to their physicochemical characteristics when subjected to gamma-irradiation at 0.3 kGy. Similarly, Lwin and Maw (2019) studied the effects of gamma-irradiation on banana (*Musa sinensis* Sw.) and reported an extended shelf-life of 6 to 8 days without any nutritional change for the doses of 0.25 and 0.35 kGy, respectively. Sopi and Adenan (2021) have worked on bananas (*Musa acuminata* Colla) and reported that 0.4 kGy was the most effective dose of gamma-irradiation to delay the ripening compared to 0.8 and 1.2 kGy doses. Therefore, it could be seen that the optimized irradiation dose and the shelf-life period vary based on the geographic area of cultivation, the agro-climatic conditions, the variety of the fruit grown, and the storage conditions. To the best of the knowledge of the

author, there is no published literatusre on the optimized dose of gamma-irradiation for shelf-life extension of green plantains (*Musa paradisiaca)* cultivated in India, especially those in West Bengal, and stored without any packaging under ambient conditions.

Effects of gamma-irradiation on the contents of bioactive compounds such as lutein, phenolic and flavonoid compounds, carotenoids, anthocyanins, antioxidant enzymes such as catalase, peroxidase, and superoxide dismutase, as well as on organic acids in agricultural produces present in various agro-commodities have been elaborately discussed in the previous chapter (Chapter 1). Therefore, it could be hypothesized that the biosynthesis of important therapeutic molecules presents in plantains, namely serotonin, and melatonin, could also be augmented consequent to gamma-irradiation. The gamma-irradiation induced enhancement in the production of reactive oxygen species would probably lead to the increased production of these plant antioxidant molecules as a part of the natural defence mechanism of the plantain. There is no literature report till date on the augmentation of serotonin and melatonin in gamma-irradiated plantains.

The plantains with enhanced amount of these phytoremediator molecules could be a potential food source of these antioxidants and thereby either serve as a direct nutraceutical food (when the antioxidants are in synergistic combination) or as a bioresource for production of value-added antioxidant-rich products (when the mentioned antioxidants are not in synergy). Furthermore, the senesced green plantains (at the end of their shelf-lives post irradiation), which are sensorially compromised could still be valued as a reserve of these phytoremediators and can be harnessed as a source of food antioxidants. This would thus provide a possible means of valorization of green plantains, post senescence.

Therefore, this chapter of the thesis has been divided into following parts:

- *Optimization of gamma-irradiation processing parameters for extension of shelf-life of 'desi' variety of green plantains based on sensory attributes, proximate, physicochemical, and phytochemical properties*

- *Optimization of gamma-irradiation processing parameters to obtain enhanced amount of serotonin and/or melatonin or both in combination (in antioxidant synergy) in gamma-irradiated plantains*

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Part 3.1

Optimization of gamma-irradiation processing parameters for extension of shelf-life of '*desi*' variety of green plantains based on sensory attributes, proximate, physicochemical, and phytochemical properties

Introduction

The objective of this part of the study was to optimize the dose of gamma-irradiation for extension of shelf-life of '*desi*' variety of green plantains (*Musa paradisiaca)* by evaluating sensory, proximate, physicochemical, and phytochemical (including antioxidant) properties of samples at regular time intervals when stored at ambient storage condition post irradiation.

Materials and methods

Materials

The details of authentication, procurement, selection of plantains, raw materials and chemicals used in this study for microbial, physicochemical, biochemical, and antioxidant analyses have been described in part 2.1 of chapter 2. Thiobarbituric acid (TBA), trichloroacetic acid (TCA), ethylene diamine tetraacetic acid (EDTA), ascorbic acid, and 30% hydrogen peroxide (H_2O_2) were procured from M/s Merck, Mumbai, India.

For irradiation, the selected individual plantains were swiftly transported into the gamma-irradiation laboratory (*vide infra*) in the university campus in cane baskets after harvesting.

Methods

Irradiation with ^{60}Co as the radioactive source was conducted in gamma chamber GC-5000 unit [manufactured by Board of Radiation and Isotope Technology (BRIT), Mumbai, India] in the Food Irradiation Laboratory of National Instruments Ltd. (NIL) campus, Jadavpur

University, Kolkata, India [having dose rate at maximum capacity of ~9kGy/h and dose rate uniformity of +25% radially and –25% axially (Anonymous, 2024)]. The surface radiation from GC-5000 was measured using a Minirad survey meter (M/s Pulsecho, Mumbai, India) (Ghosh *et al*., 2017) and was within its safe operational limits *i.e.*, <2 mrad throughout the study period. The calibration including attenuation and dose rate certification were regulated and supervised by BRIT employing standard Fricke's dosimeter and the uncertainty in the measured dose was recorded at ±5%.

In a preliminary study, the green plantains were gamma-irradiated in the dose range of 0.02 kGy-1 kGy (*i.e.*, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 kGy) which revealed that very low doses (below 0.1 kGy) of gamma-irradiation were effective for the enhancement of shelf-life of the plantains by approximately 5 days; whereas, comparatively higher dose range (above 0.1 kGy) showed compromised shelf-life consequent to severe tissue damage, softening, and skin discolouration. These findings corroborated well with those reported by Aina *et al*. (1999) who also found that the plantains irradiated at relatively higher dose (above 0.5 kGy) had lower shelf-life. Therefore, irradiation was conducted at a 'very low dose range' of 0.04-0.08 kGy for extension of shelf-life of green plantains.

To meet the objective, the experiments were conducted thrice as per the design of experiments (DoE) presented as a block diagram in Fig. 3.1.1. Based on the preliminary study, the current study was designed for 25 days. The bulk sample set comprised of a set of 75 plantains for each of the three doses of irradiation, *i.e.*, 225 plantains along with 75 non-irradiated (control) plantains. The bulk sample sets were temporarily stored at 23±2 °C, 70±2 % RH in an environmental chamber prior to gamma-irradiation. The bulk sample set (apart from non-irradiated plantains) were then subjected to gamma-irradiation processing on day 0. Therefore, for each dose of gamma-irradiation, 25 batches of green plantains were treated to meet each

objective. Each day, three plantains were randomly withdrawn from the processed sample sets which constituted the analytical sample set.

For each batch, the stalks of three (randomly selected from bulk) green plantains (average weight of 525±20 g) were sized and gamma-irradiated after placing in an amber-coloured beaker with polystyrene cushions on the inside walls. The beaker was positioned at the geometric centre of the sample chamber of the GC-5000 unit and covered using the removable lid of the chamber. Irradiation was conducted at 33±3 °C keeping the chamber under rotation (60 rpm) at a rate of 3.062 kGy/h. Rotation aided proper attenuation and thus ensured uniform distribution of gamma-rays in the sample chamber together with polystyrene cushioning which restricted movement of plantains inside the beaker by minimizing the free space (IAEA, 2002). Post-irradiation, the processed sample sets (irradiated and non-irradiated plantains) were stored at 23±2°C, 70±2 % RH in an environmental chamber for a total period of 25 days.

From day 0 onwards, three plantains were randomly withdrawn from the processed samples sets for shelf-life assessment (by evaluation of sensory, proximate, physicochemical, and phytochemical properties). The studies were conducted simultaneously for a three-month duration (*viz*. the above DoE was repeated thrice).

Experimental plan for selection of best dose of gamma-irradiation for enhancement of shelf-life of green plantains

Whole green plantains were subjected to sensory evaluation and also for assessment of colour, texture, true density, and pH. All assays including sensory, proximate, physicochemical, and phytochemical analyses were carried out at an interval of 3 days for each analytical sample set and on the last day of their sensorial rejection. 10 g plantain from each set of analytical sample

set *i.e.*, a total of 30 g (10 x 3) plantains were diced, reshuffled and mixed and then required amounts were subjected to determination of moisture content, fat content, ash content, and percent membrane stability index (%MSI). The remaining plantains were homogenously ground in a mortar-pestle (along with their skin) and a pulpy mass was obtained. An extract prepared from this pulp (*vide infra*) was used for determination of protein content, reducing sugar, titratable acidity, and antioxidant potency (assessed by TPC, IC_{50} value and FRAP value). Using the remaining homogeneous pulpy mass, total pectin content as calcium pectate, vitamin C content, and enzyme activities were evaluated. For each analytical sample set, the relatively stable parameters (*i.e.*, crude fat, ash, crude fiber, total pectin content as calcium pectate, and ascorbic acid contents) which did not show significant changes in a 3-day interval (assessed from the preliminary trial) were analysed at an interval of 5 days and also on the last day when they had to be sensorially rejected.

Sensory evaluation of gamma-irradiated green plantains

Sensory evaluation of each analytical set of plantains was performed by rating on a 9-point hedonic scale (9-like extremely and 1-dislike extremely) according to the method elaborated in the preceding part (part 2.1 of chapter 2). The panelists were not formally trained by any company or institute. However, they were all very familiar with the quality parameters of green plantains (viz. colour, odour, texture, browning, shrinkage, and overall appearance) since the same are a part of their diet.

Proximate analyses of gamma-irradiated green plantains

Proximate analyses of the non-irradiated green plantains were conducted on day 0 in accordance with previously described methods (part 2.1 of chapter 2) which included

estimation of %moisture on dry weight basis (D.W), protein (g/ 100 g D.W.), %crude fat, %ash, %crude fiber, total carbohydrates (by difference) and reducing sugar content (g/ 100 g D.W.), in accordance with the method described by Pal and Bhattacharjee (2018). During the storage study, sample sets were similarly analysed for their proximate constituents following AOAC methods except for moisture content which was analysed using IR moisture analyser (MB 200C, M/s Citizen-Aczet, Mumbai, India) according to the method of Latorre *et al*. (2010) and was reported on a %wet weight basis.

Physicochemical analyses of gamma-irradiated green plantains

To analyse the changes in texture profiles of plantains with storage, TPA by two-bite compression test of plantains were conducted employing a TA.XT Express texture analyser (M/s Stable Micro Systems, Godalming, UK) in accordance with the previously elaborated method described in the part 2.1 of chapter 2. Whole plantains were subjected to texture analysis and the TPA graph was executed by software of Exponent Lite Express.

CIE colour values of plantains were determined with a portable CR-10 Plus Colour reader (M/s Konica Minolta Inc., Osaka, Japan) as described in part 2.1 of chapter 2. Other physicochemical properties assayed were: pulp to peel ratio (Abdullah *et al*., 2017), true density (g/cm^3) (Wasala *et al*., 2012), %MSI by electrical PC 510 conductivity meter (M/s Eutech Instruments Pvt Ltd, Singaore City, Singapore) (Pal and Bhattacharjee, 2018), % TTA as % malic acid equivalent, pH (using pH meter, Cyberscan PC 510 m, M/s Eutech Instruments Pvt. Ltd., Singapore City, Singapore) (previously described in part 2.2 of chapter 2), and total pectin content as % calcium pectate (Sadasivam and Manickam, 2005).

Phytochemical analyses of gamma-irradiated green plantains

Preparation of extract

To obtain an extract of green plantains for phytochemical analyses, three plantains from each analytical set and from the control set were randomly withdrawn each day. The extract was used for estimation of TPC and for antioxidant assays in accordance with the procedures described by Ranganna (2000) and Shian *et al.* (2012). Whole plantains (comprising of pulp and peel) were cut into small pieces and crushed in a mortar-pestle into a pulpy mass. The pulpy mass (20 g) was then homogenized in 100 mL of 70% ethanol: water (v/v) using a homogenizer (T50 digital Ultra-turrax®, M/s Ika, Staufen, Germany) for 1 min at 24,000 rpm followed by 10 min of centrifugation at 4750 × g using a centrifuge (R-8C, M/s Remi, Mumbai, India). The supernatant was collected and stored at -20 °C for the following analyses.

Determination of TPC

TPC of the plantains was estimated spectrophotometrically by UV-Vis double beam spectrophotometer (Halo DB-20, M/s Dynamica Scientific Ltd., Newport Pagnell, UK) by Folin-Ciocalteu method as described previously in part 2.1 of chapter 2 and was expressed as mg GAE/100 g D.W.

Determination of antioxidant activities

The antioxidant activities of the control and gamma-irradiated green plantains were carried out by assaying IC_{50} values (mg/mL) of DPPH radical scavenging activities of the extracts (*vide supra*) and by FRAP values expressed as mM $FeSO_4$/100 g D.W. in accordance with the methods elaborated previously in part 2.1 of chapter 2.

Determination of total ascorbic acid content

The total ascorbic acid contents of the plantains were estimated spectrophotometrically (Halo DB-20, M/s Dynamica Scientific Ltd., Newport Pagnell, UK) by the method elaborated by Chakraborty and Bhattacharjee (2018) and Kapur *et al*. (2012) and were expressed as mg/100 g D.W.

Determination of enzyme activities of gamma-irradiated green plantains

Ascorbic peroxidase (APX) activities of the plantains were evaluated according to the method followed by Pal and Bhattacharjee (2016) and were expressed as micromole ascorbate oxidized/mg protein/min. Polyphenol oxidase (PPO) activity was determined according to the method described by Galeazzi *et al.* (1981) and was expressed as IU/100 gm D.W.

Microbial assessment of the irradiated plantains

On the same day of irradiation, the bacterial loads (expressed as CFU/g of plantain) in the irradiated (at lowest dose *i.e.*, 0.04 kGy) and control sets of plantains were evaluated in terms of total plate counts using the pour plate method as described in part 2.1 in chapter 2.

Statistical analysis

All experiments (including processes and analyses) in this part of the study were performed in triplicate and all data reported are mean ±SD of data obtained from the three independent consortium of experiments as explained in Fig. 3.1.1. Two-way ANOVA was performed by Statistica 8.0 software (M/s Statsoft, Oklahoma, USA) to analyse the individual and interactive effects of two variables *i.e.*, shelf-life period and dose of gamma-irradiation on each assay parameter. Duncan's multiple range test was employed to determine significant differences

among means by employing IBM SPSS Statistics software version 26 (M/s IBM, New York, USA). A value of $p \leq 0.05$ was considered significant to establish differences in all tests.

Results and Discussion

Estimation of best dose of gamma-irradiation to enhance shelf-life of green plantains by assessing alterations in their quality parameters during storage

With progression of storage (total storage period of 25 days) the control set and 0.04, 0.06, and 0.08 kGy irradiated green plantains had to be discarded after 17, 22, 22, and 21 days, respectively, based on the sensory evaluation. The said samples were ineligible for further analyses owing to the development of unacceptable black colour, foul odour, and shrunken-over-softened texture. These characteristic changes were clear indications of completion of their respective shelf-life. Proximate, physicochemical, and phytochemical analyses were carried out for the control set and 0.04, 0.06, and 0.08 kGy irradiated green plantains for 17, 22, 22, and 21 days, respectively (henceforth this time period has been nomenclatured as 'assessment period') to finally ascertain the day until which the plantains could be consumed safely for nutraceutical benefits (henceforth this time period from day 0 until the day the samples were suitable for consumption has been denoted as 'shelf-life'). The visual changes of green plantains with progression of senescence during storage have been presented in Fig. 3.1.2.

Alterations in sensory attributes

The hedonic scores of sensory attributes of gamma-irradiated (0.04, 0.06, and 0.08 kGy) and control sets of green plantains on days 0, 3, 6, 9, 12, 15, 17, 18, 21, and 22 have been presented in radar plots (Fig. 3.1.3). Development of yellow colour which is an indicator of ripening was observed from day 3 onwards in control set of samples; while 0.04 and 0.06 kGy-irradiated

plantains showed significant delay in development of yellow colour which commenced on day 9. From day 5 onwards, the control set of plantains exhibited enhanced enzymatic browning *i.e.*, development of unacceptable brown colour in corroboration with increased PPO activity (discussed *vide infra*). 0.04 and 0.06 kGy-irradiated plantains showed delayed ripening in tandem with delayed onset of browning (from day 12 onwards). All analytical sample sets of plantains were subjected to sensory evaluation until they scored ≤1 (*i.e.*, dislike extremely) on the 9-point hedonic scale. The control and 0.08 kGy-irradiated plantains were sensorially rejected on days 17 and day 21; while the 0.04 and 0.06 kGy-irradiated plantains were rejected on day 22 owing to development of black colour, foul odour, and dry texture. It was found that the overall sensory scores of the panelists were highest for 0.04 kGy-irradiated plantains throughout the assessment period.

Alterations in proximate constituents

The trends in changes in proximate constituents [%moisture on wet weight basis, protein (g/100 g D.W.), %crude fat, %ash, %crude fiber, carbohydrate (by difference) and reducing sugar contents (g/100 g D.W.)] during storage have been graphically represented in Fig. 3.1.4. The proximate constituents of non-irradiated plantains on day 0 on %D.W. basis were as follows: 79.313±1.82 % moisture, 3.06±0.97 % protein, 0.65±0.09 % crude fat, 6.501±0.78 % ash and 6.52±2.24 % total carbohydrates (by difference). No significant changes in moisture contents were observed among the control and irradiated plantain sets on the same day of study, which signified that there were no differences in moisture contents among the irradiated and the non-irradiated plantains on the same day of storage. These findings support those of Maxwell *et al*. (2017), who studied effects of different doses of gamma-irradiation (0.05, 0.1, 0.3, and 0.8 kGy) on vitamin C, acidity, and moisture contents of four different varieties of mangoes (flat mangoes, *Binta* sugar mangoes, *Barki Akus* mangoes, and *Cameron* mangoes) and reported no

difference in moisture contents among the control and irradiated sample sets. A significant declination (p<0.05) in moisture contents were observed in both gamma-irradiated and non-irradiated plantains with progression of senescence (Fig. 3.1.4a) in consonance with the sensory panelists' scores on shrinkage (discussed *vide supra*).

There were no differences in protein contents among the control and irradiated sample sets immediately after gamma-irradiation (on day 0). However, protein contents of both the control and irradiated sample sets had decreased significantly (p<0.05) with advancement of senescence. The depletion of protein content in the control set could be attributed to oxidative degradation of cellular protein, which is reportedly a common phenomenon of senescence (Jajic *et al*., 2015); whereas, in irradiated plantains, structural changes such as cross-linking and aggregation of proteins (not investigated in the present study) consequent to gamma-irradiation (Ciesta *et al*., 2000) perhaps slowed down protein degradation. Among all sample sets, 0.04 kGy-irradiated plantains had the highest protein contents followed by those of 0.06 and 0.08 kGy-irradiated sets, *vis-à-vis* the control set (Fig. 3.1.4b). The trend of declination in protein contents of irradiated plantains was in consonance with the finding of Pal and Bhattacharjee (2020) who studied effects of gamma-irradiation (1.0, 3.0, 5.0, 8.0 and 10.0 kGy) on shelf-life of yellow corn (*Zea mays*) kernels. The findings on protein contents corroborated well with the data obtained by MSI analyses (*vide infra*).

No significant changes (p<0.05) in %crude fat (Fig. 3.1.4c), %ash (Fig. 3.1.4d) and %crude fiber (Fig. 3.1.4e) was observed among the gamma-irradiated and control sets of plantains which indicated that gamma-irradiation did not have significant effects on these parameters. However, these parameters showed declination trends (p<0.05) in both control and gamma-irradiated plantains with progression of storage. Insignificant changes in the said parameters in

the control and irradiated sets of plantains were in compliance with the findings of Zaman *et al*. (2007) and Ihsanullah *et al*. (2005). Zaman *et al*. (2007) had reported insignificant changes in %crude fat during investigation of extension of shelf-life of bananas (*Musa sapientum)* employing low dose gamma-irradiation (0.3 kGy, 0.4 kGy, and 0.5 kGy) and post storage at room conditions (25±2 °C, 80±5 %RH). Ihsanullah *et al*. (2005) investigated effects of gamma-irradiation (0.2, 0.5, 1, 2, and 3 kGy) on nutrients of Pakistani dates (*Pheonix dactylera* L.) when stored in white polythene for five months and reported insignificant changes in %crude fiber contents. Similarly, negligible changes in %ash contents in irradiated plantains during storage agreed well with the findings of Al-Bachir (2004) who studied effects of gamma-irradiation (0.5, 1.0, 1.5, and 2.0 kGy) on proximate constituents of walnuts (*Juglans regia L*.).

Percentage total carbohydrates increased ($p<0.05$) with the progression of senescence (Fig. 3.1.4f) owing to concentration effect brought about by loss of moisture in the samples with storage. The amounts of reducing sugar in the control set increased significantly ($p<0.05$) from day 9 (mid-senescence) onwards and remained higher than the remaining sets (Fig. 3.1.4g) indicating enhanced ripening in non-irradiated samples; whereas the rise in reducing sugar levels in gamma-irradiated plantains commenced after day 15 (late senescence) owing to delayed onset of ripening in the same. This trend of enhancement in reducing sugar content is the result of enzymatic conversation of higher polysaccharides (such as starch present in plantains) into simple sugars with progression of storage (Hussain *et al*., 2008), which complied well with the findings of Pal and Bhattacharjee (2020) on reducing sugar contents of irradiated corn. However, 0.04 kGy-irradiated plantains exhibited lowest content of reducing sugar indicating delayed ripening of the same which corroborated well with the changes we obtained for pulp to peel ratios (*vide infra*).

Alterations in physicochemical properties of gamma-irradiated plantains

Alterations in texture profiles

Hardness, cohesiveness, gumminess, and chewiness of plantains for both gamma-irradiated and control sets had decreased significantly ($p<0.05$); whereas adhesiveness and springiness of the said samples had increased significantly ($p<0.05$) throughout the assessment period. These results were in good agreement with those reported by Watharkar *et al*. (2021). The reduction in hardness is attributed to the chemical changes such as hydrolysis of starch into sugar, conversion of insoluble protopectin to soluble pectin during fruit ripening and the losses in the crude fiber content due to structural changes (Mustaffa *et al*., 1998, Siriamornpun and Kaewseejan, 2017). Considering the entire processed sample set, plantains gamma-irradiated at 0.04 kGy showed lower adhesiveness, springiness and higher hardness, chewiness, gumminess, and cohesiveness vis-à-vis the other sets during storage (Fig. 3.1.5). This finding indicated best retention of characteristic texture of the gamma-irradiated plantains at the aforesaid dose, which were in consonance with the responses of the sensory panel on textural attribute (discussed *vide supra*).

Alterations in colour profiles

With progression of ripening (senescence), green colour of plantains turned yellow, substantiated by their higher ($p<0.05$) b* values. Additionally, a* values of the gamma-irradiated as well as control sets of plantains had increased significantly ($p<0.05$) during senescence. These results correlated well with the development of browning (Fig. 3.1.6) in the above samples as have also been reported by Kajuna *et el*. (1998). Plantains irradiated at 0.04 kGy remained green (negative a*) up to day 15 (late senescence) and possessed relatively lower b* values (yellowness) and lower L* values (lightness) throughout the storage period which

correlated well with the sensory evaluation scores on colour. Delayed onset of ripening and thus better retention of characteristic green colour was observed for 0.04 kGy-irradiated plantains compared to both control and other irradiated sample sets. These results further validated the results obtained by sensory analyses and that of PPO activity (discussed *vide supra* and *vide infra*, respectively).

Alterations in pulp to peel ratios

The pulp to peel ratio increased with advancement of senescence due to increase of pulp weight and decrease of peel weight. At the time of post-harvest ripening, sugar content increases in the pulp which increases osmotic pressure and movement of moisture from peel to pulp thereby increasing pulp weight and concomitantly, transpiration results in moisture loss in peels (Dadzie and Orchard, 1997). Breaking down of the cell wall and formation of air gap in the middle lamella could be attributed to the thinning of peel and its subsequent weight loss during the ripening period (Adi *et al*., 2019). It was found that the rate of increase ($p<0.05$) of pulp to peel ratio in the control sets was faster than their irradiated counterparts (Fig. 3.1.7a) since the non-irradiated samples ripened faster than the irradiated ones. Increase in pulp to peel ratio and ripening of fruits are strongly correlated. It is also evident from the findings of colour, texture and %MSI analyses that ripening in control sets were faster than their irradiated counterparts. The plantains irradiated at 0.04 kGy exhibited the maximum delay in rise of pulp to peel ratios in comparison with those of the control and irradiated sample sets. This finding was in consonance with that reported by Abdullah *et al*. (2017) who investigated the ripening behavior of gamma-irradiated (0.5, 0.75, and 1.0 kGy) green bananas (*Musa sapientum* cultivar 'Dwarf Cavendish') when stored at room temperature and found delayed increase in pulp to peel ratios vis-à-vis the non-irradiated ones.

Alterations in true density

The true density values of gamma-irradiated and control sets of plantains increased significantly ($p<0.05$) during senescence (Fig. 3.1.7b) in the range of 1.29 to 1.88 g/cm^3. The control set showed maximum increase ($p<0.05$); whereas 0.04 kGy-irradiated plantains exhibited minimum increase ($p<0.05$). True density calculated as mass per unit volume is inversely proportional to moisture content (Boukouvalas *et al*., 2006). Thus, in non-irradiated samples, true density showed a faster increase with decrease in moisture content in contrast to the remaining sample sets. On the other hand, 0.04 kGy-irradiated samples showed minimum increase in true densities since the same also showed minimum decrease in moisture contents compared to the remaining samples sets.

Alterations in MSI

Membrane stability had decreased significantly ($p<0.05$) from day 0 in both gamma-irradiated and control sets of plantains (Fig. 3.1.7c) since it is reportedly known that with progression of senescence, cell membrane weakens resulting in loss of the ability of selective leakage of intracellular ions (Chakrabarty *et al*., 2009). However, among all treated plantains, the highest %MSI was observed in plantains gamma-irradiated at 0.04 kGy attesting it to be the best dose of gamma-irradiation in maintaining characteristic membrane integrity during storage. Deterioration of proteins in botanicals (*vide supra*) could be attributed to enhanced rate of membrane permeability and subsequent loss of ions (Shahri and Tahir, 2011), which corroborated well with the present findings of %MSI values. Ghosh *et al*. (2017) in their study on shelf-life extension of tuberose flowers using a combination of gamma-irradiation and generally regarded as safe (GRAS) preservatives, also reported that treated flowers exhibited higher membrane stabilities compared to their non-irradiated counterparts.

Alterations in TTA contents and pH

Percentage TTA of plantains in terms of %malic acid had increased in a significant ($p<0.05$) manner (Fig. 3.1.7d); whereas significant ($p<0.05$) decreases in pH were observed from day 9 (mid-senescence) onwards with the advancement of ripening in both gamma-irradiated and control sets of plantains. pH values of gamma-irradiated and control sets of plantains were in the range of 6.82-4.09 (Fig. 3.1.7e) during the storage period. This increase in acidity is attributed to starch degeneration via Krebs cycle and concomitant production of organic acids such as malic acid and citric acid in the pulp during ripening (Lustre *et al*., 1976, Seymour, 1993). However, there are no significant differences ($p<0.05$) in %TTA and pH values with storage among the control and irradiated sets of plantains on the same day. This finding was in consonance with those of Maraei and Elsawy (2017) who studied chemical quality and nutritional composition of gamma-irradiated (0, 0.3, 0.6, and 0.9 kGy) strawberries (*Fragaria* x *ananassa* cv. Festival) during storage at 10 ºC.

Alterations in total pectin content as calcium pectate

In the current study, total pectin contents (in terms of calcium pectate) had decreased significantly ($p<0.05$) in both gamma-irradiated and control sets of plantains (Fig. 3.1.7f) from early to late senescence. This could be attributed to the transformation of insoluble pectin into soluble pectin and a concomitant reduction in the amount of calcium pectate in the latter days of senescence which agreed well with the findings of Inari *et al*. (2002) who studied pectin contents of cherry tomatoes. Similar trends in reduction of chelator soluble and alkali-soluble pectin contents in gamma-irradiated (0.25, 0.50, 0.75, 1.0, and 1.5 kGy) papayas during ripening have been reported by Zhao *et al*. (1996). Plantains gamma-irradiated at 0.04 kGy had higher pectin contents throughout the senescence period indicating delayed ripening in the

same vis-à-vis the remaining sample sets. These results corroborated well with the sensory (discussed *vide supra*) and texture profile analyses data (*vide supra*).

Phytochemical properties of gamma-irradiated green plantains

Alterations in TPC

TPC of the irradiated plantain sets showed a significant (p<0.05) increase during senescence (up to day 12), followed by a gradual decrease (Fig. 3.1.8a). Plantains irradiated at 0.04 kGy dose exhibited higher TPC values throughout the storage period vis-à-vis the control and other irradiated sets and the highest value was recorded on day 12 (mid-senescence) of storage. Wi *et al*. (2007) in their study on the effect of gamma-irradiation on the morphological changes and biochemical responses of plants, concluded that the alterations in the cell structure and metabolism could have resulted in accumulation of phenolic compounds in gamma-irradiated post-harvest produce during the initial days of storage, while oxidative degradation of TPC led to its decrease during later days of storage.

Alterations in antioxidant activities

The IC_{50} values of irradiated plantains showed significant (p<0.05) decreases (*i.e.*, increased DPPH radical scavenging activities) from day 1(early senescence) up to day 12 (mid-senescence) followed by a gradual increase (decreased radical scavenging activity); while the control set showed decrease (p<0.05) on day 12 followed by a sharp increase in the same until day 17 (late senescence) which was the last day of the assessment period for the control set (Fig. 3.1.8b). On the other hand, FRAP values for both irradiated and control sets significantly (p<0.05) increased up to day 12 (mid-senescence) (Fig. 3.1.8c), after which the values started to decline continuously until the end of the assessment period. Plantains irradiated at 0.08 kGy exhibited the highest antioxidant activities in terms of DPPH radical scavenging activity on

day 12, although the phenolic contents and FRAP values were the highest in 0.04 kGy-irradiated plantains on day 12. Antioxidants present in plantains other than phenolics, such as vitamin C, carotenoids, and xanthophylls contribute to DPPH radical scavenging activity (Kondo *et al*., 2005, Ali *et al*., 2019). The increases in DPPH radical scavenging activities of the irradiated plantains (compared to the control ones) possibly could be attributed to enhanced activities of enzymes such as ammonialyase and peroxidase or due to irradiation-induced enhancement in tissue extractability caused by dissolution and depolymerization of cell wall polysaccharides (Alothman *et al*., 2009). While DPPH radical scavenging activity is primarily due to antioxidants other than phenolics, FRAP values are chiefly due to the activities of phenolic compounds. In agreement with literature reports, the results of TPC (*vide supra*) and FRAP values were found to be strongly correlated. Similar findings on higher DPPH radical scavenging activities and FRAP values have been reported for 3 kGy-irradiated tomato pomace compared to its non-irradiated counterpart (Khalaf *et al*., 2014).

Alterations in total ascorbic acid contents

The amounts of ascorbic acid contents of the control and gamma-irradiated sets of plantains during storage (assessment period for sample acceptability) are presented in Fig. 3.1.8d. Ascorbic acid contents in the plantains reduced significantly ($p<0.05$) with progression of senescence. The amounts of ascorbic acid in the control set were found to be higher than those of the irradiated sets up to day 9 (mid-senescence), followed by a steady decrease of the same until the end of the assessment period. During irradiation, the ionizing radiation reacts with water molecules present in foods and releases electrons which leads to production of highly reactive free radicals. These free radicals then react with antioxidants such as vitamin C, change their structures, and reduce their activities (da Silva Aquino, 2012). Since this degradation continues beyond the time of irradiation, loss of vitamin C in irradiated foods was found to

occur during storage but this effect was less pronounced in non-irradiated foods (Diehl, 1967). This finding is supported by de Figueiredo *et al*. (2014) who studied the effects of gamma-irradiation (0.8 kGy) on carotenoids and vitamin C contents of papayas (*Carica papaya* L.) when stored at 24±2° C and found that irradiation reduces the vitamin C contents in these fruits more than their non-irradiated counterparts.

Alterations in APX and PPO activities of gamma-irradiated green plantains

Alterations in APX activities

APX activities significantly ($p< 0.05$) increased in gamma-irradiated sets up to day 9 (Fig. 3.1.8e) and then decreased, whereas in the control set, activities steadily decreased from day 1. The APX activity was higher in 0.04 kGy-irradiated plantains throughout the storage period. Identical findings have also been reported by Pal and Bhattacharjee (2016) who studied the effect of gamma-irradiation (0.02-2.5 kGy) on sensory and physicochemical properties of marigold (*Tagetes erecta* l.) cut flowers and reported that APX activities in gamma-irradiated flowers increased during storage up to day 8, after which the activities declined. This phenomenon of increase in APX enzyme activity during the initial days of storage could be owing to the prevention of oxidation in cells (Pal and Bhattacharjee, 2016). During later days of storage, the reduction in the activity of APX is possibly due to the increase in concentration of H_2O_2 (Wi *et al*., 2007).

Alterations in PPO Activities

Occurrence of browning in the plantains was chiefly due to oxidation of phenolic compounds to melanin catalyzed by PPO (Thomas and Nair, 1971). The PPO activities of both gamma-irradiated and control sets of plantains were found to increase significantly ($p<0.05$) with the advancement of ripening (Fig. 3.1.8f) up to day 18 (late senescence) followed by a steady

decrease until the end of their respective shelf-lives. PPO activity was observed to be the highest in the control set followed by those in 0.08, 0.06, and 0.04 kGy-irradiated plantains. Higher PPO activity indicates higher rate of browning and ripening in the plantains which further validates the findings on colour and texture profiles (discussed *vide supra*). These results are in consonance with the findings of Lu *et al*. (2005), who investigated the effects of gamma-irradiation (0.5, 1.0, and 1.5 kGy) on PPO activity of fresh-cut celery when stored at 4 °C.

In the present study, the assessment period of the control set and 0.04, 0.06, and 0.08 kGy-irradiated green plantains were 17, 22, 22, and 21 days, respectively based on sensory properties. However, physicochemical and biochemical assays including the evaluation of antioxidant properties revealed that 0.04 kGy-irradiated plantains were acceptable for direct consumption up to 20 days, whereas, the control, 0.06 and 0.08 kGy-irradiated plantains were acceptable for 12, 18 and 17 days, respectively. Thus, it could be concluded that 0.04 kGy was the most effective dose of gamma-irradiation for enhancing the shelf-life of plantains up to 20 days, viz. a lead of 8 days with respect to the control set of plantains (12 days). Although published literature states 0.15-0.30 kGy to be the optimum dose range for extension of shelf-life of *Musa paradisiaca* (Aina *et al*., 1999, Viveka *et al*., 2014), the present studies indicate a very low dose of 0.04 kGy to be the best dose for the local '*desi*' variety of *Musa paradisiaca* of West Bengal.

Alterations in microbial loads

Fig. 3.1.9a and 3.1.9b present the total bacterial counts of the control and 0.04-kGy irradiated sets of plantains, respectively. The total bacterial count in the control set of plantains was 5365.69 CFU/g of plantains; whereas, in 0.04 kGy-irradiated plantains, it was 676.75 CFU/g of plantains, *i.e.*, a reduction of 87.39% in bacterial count was observed post-irradiation. This

finding corroborated well with the report of Mohamed and Mahmoud (2010), wherein ~ 99.2% decrease in total bacterial count was observed in gamma-irradiated (0.25 kGy) bananas compared to the control set of bananas. Besides directly affecting the microbes by inducing mutation and structural alterations, gamma-irradiation can also indirectly affect the micro-organisms by ionizing the water molecules present, transforming them into 'activated water', and resulting in concomitant formation of highly reactive hydrogen and hydroxyl radicals by free radical mechanism, which can be lethal for the microbes (Sarkar *et al*., 2023).

Conclusion

The findings of the present study render gamma-irradiation an effective non-thermal technology for freshly harvested plantains, extending their shelf-life and preserving their physicochemical and biochemical properties without the use of any packaging or controlled environment of storage. Thus, low-dose gamma-irradiation could serve as an alternative to high-energy-consuming cold storage technology. Furthermore, the effects of relatively high doses of gamma irradiation on the less explored '*in-house*' antioxidant contents (L-tryptophan-serotonin-melatonin) of plantains were further investigated and elaborated in the next part of this chapter.

Novelty

The present study reported for the first time on 8 days of extended shelf-life of 'desi' variety of plantains cultivated in West Bengal by employing the non-thermal preservation technology of gamma-irradiation. This study also extensively documented for the first time on the associated alterations in sensory, proximate, physicochemical, and phytochemical (antioxidant) characteristics of both irradiated and non-irradiated 'desi' variety of non-packaged plantains with the progression of senescence during storage under ambient conditions.

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Figures:

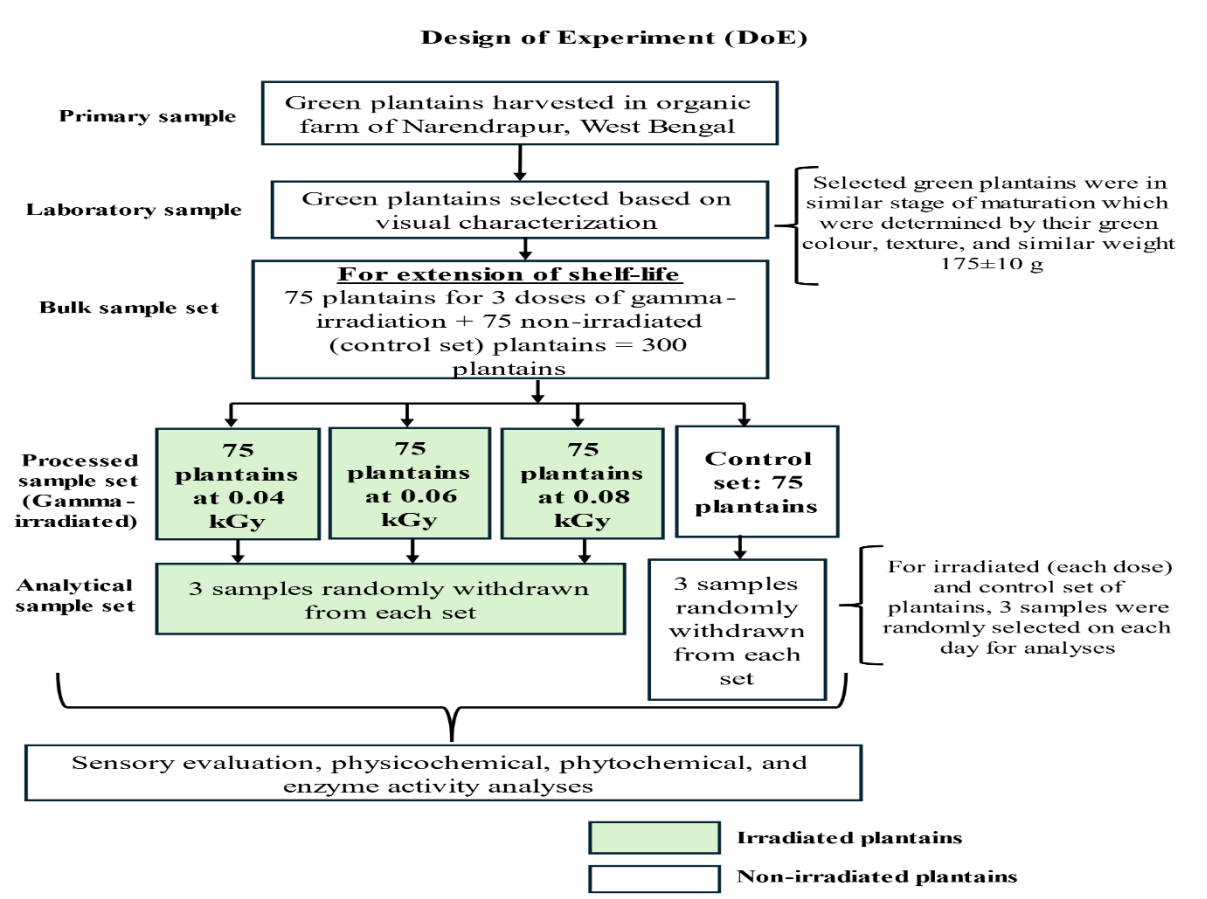


Figure 3.1.1: Experimental design for gamma-irradiation of green plantains. Each consortium of experiments (apposite to its objective) was conducted thrice as per the DoE and results are reported as mean ±SD of data obtained by the three independent runs of each experiment consortium.

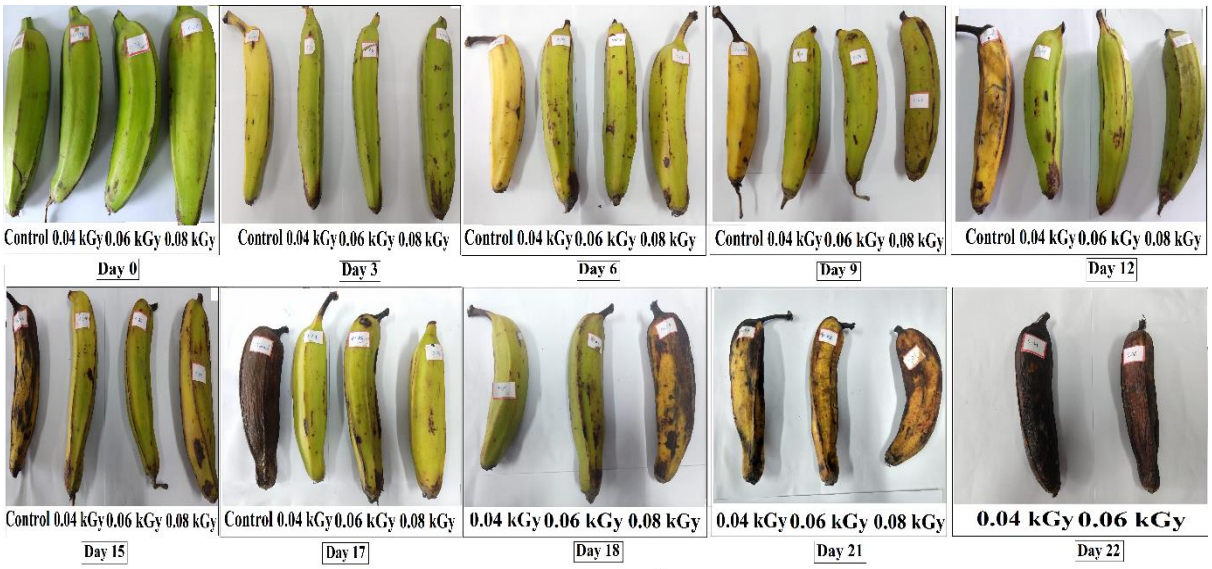


Figure 3.1.2: Physical changes in gamma-irradiated (0.04-0.08 kGy) plantains and control samples during 22 days of storage when stored at 23±2 °C, 70±2 %RH in an environmental chamber.

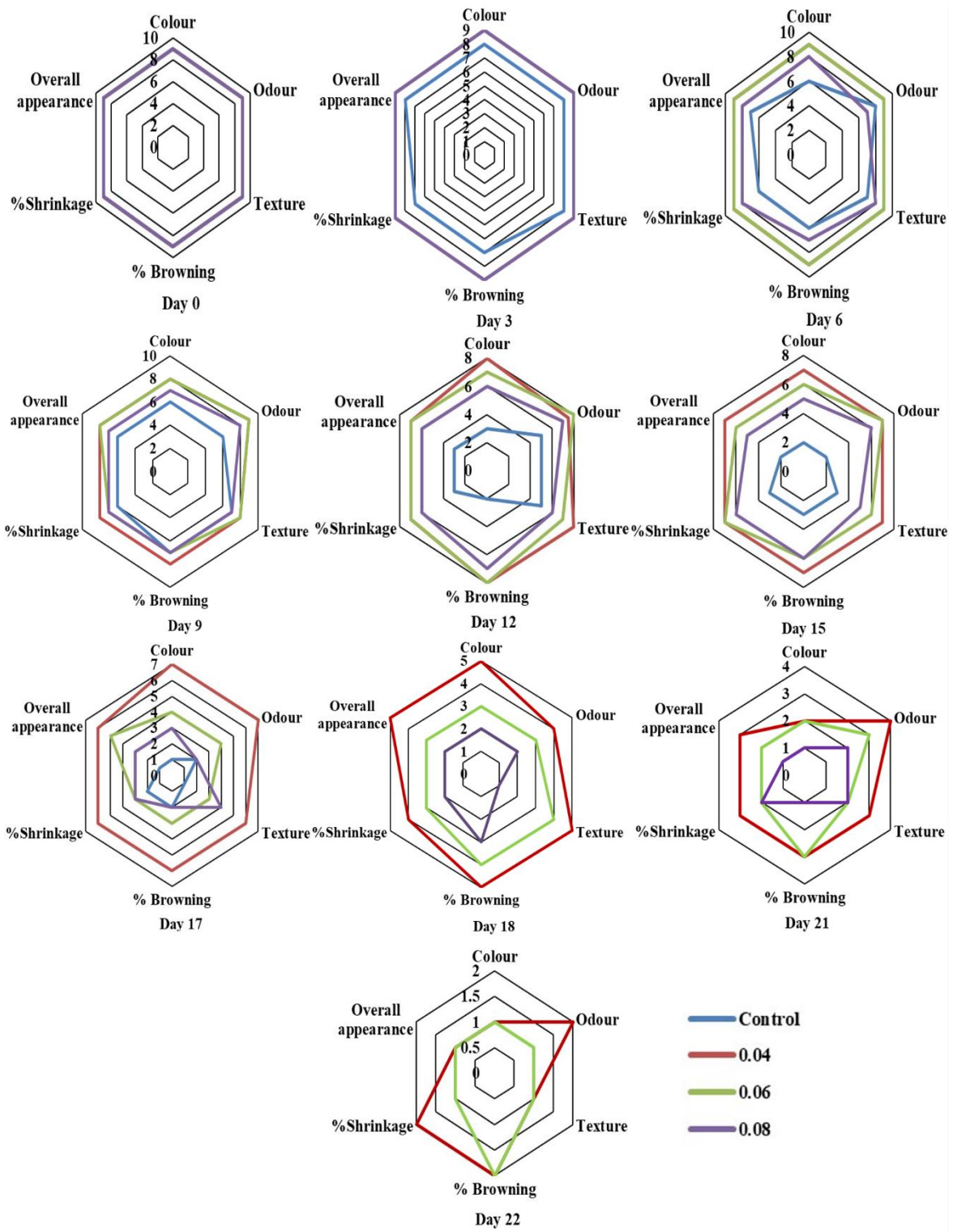


Figure 3.1.3: Radar plots of hedonic scores obtained by sensory analyses of control and gamma-irradiated (0.04, 0.06 and 0.08 kGy) green plantains for 22 days when stored at 23±2 °C, 70±2 %RH in an environmental chamber. Since both control and irradiated samples scored same on day zero and day three, there is only single line in radar plot on that day (except the parameter 'colour' on day three). The green line on day 6 represents both 0.04 and 0.06 kGy since their scores were identical. Each value represents mean ±SD of three sets of experimental data.

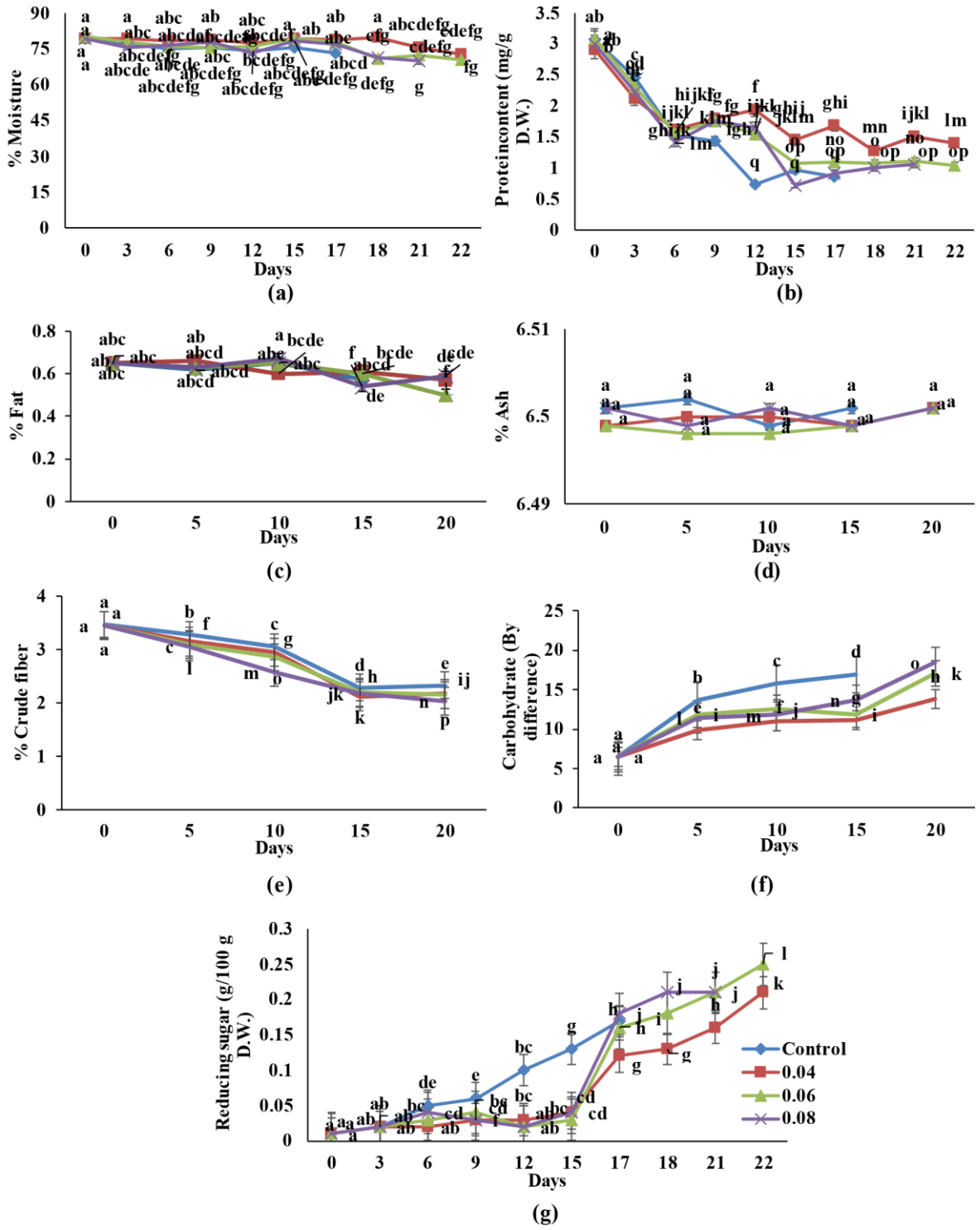


Figure 3.1.4: Analyses of proximate constituents of gamma-irradiated plantains and control samples during 22 days of storage- a) %moisture content (on wet weight basis); b) protein content (g/100 g D.W.); c) %crude fat content; d) %ash content; e) %crude fiber; f) total carbohydrates by difference and g) reducing sugar content (g/100 g D.W.). Each value represents mean ±SD of three sets of experimental data. Different alphabets specify that mean values belong to different subsets at $p<0.05$.

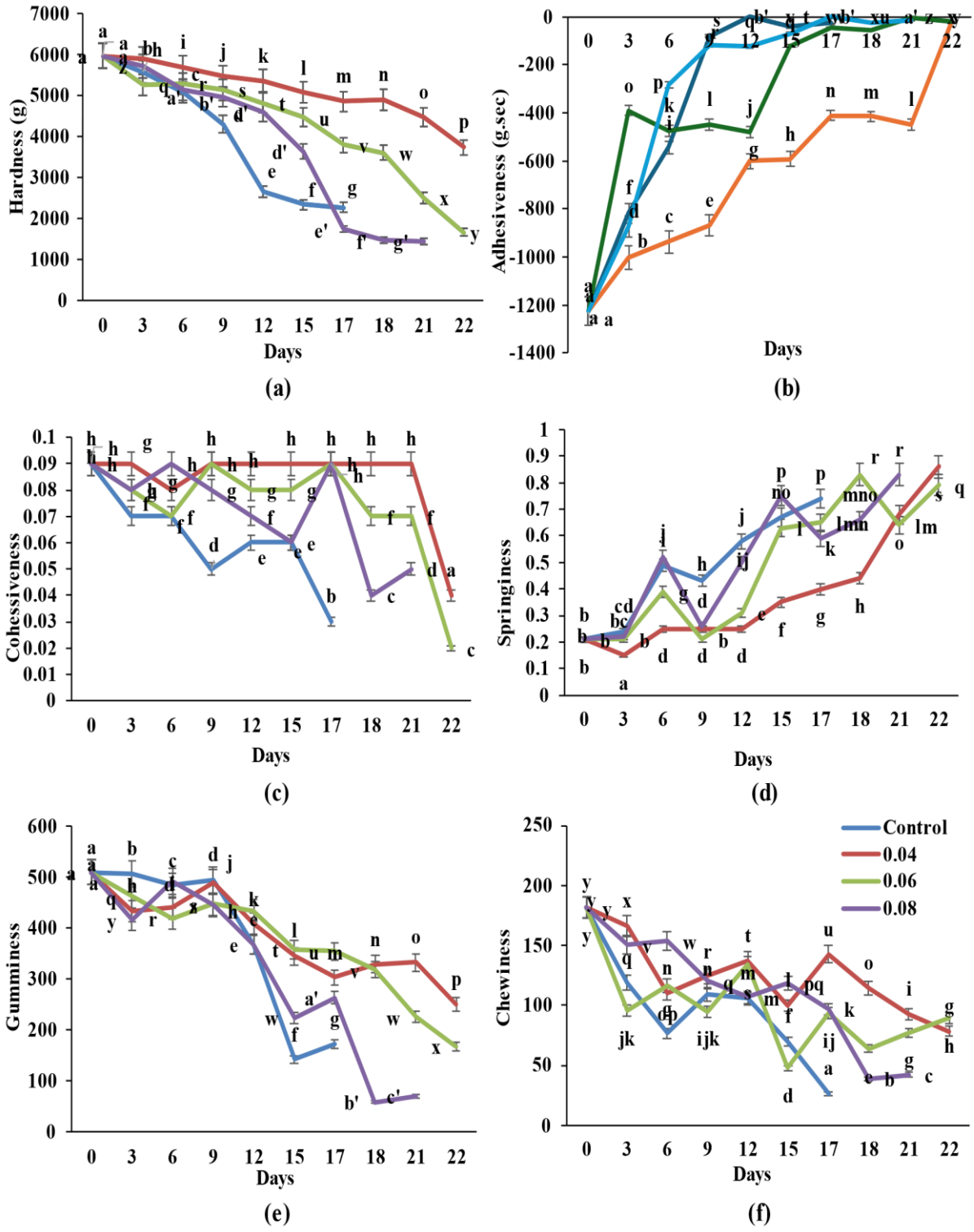


Figure 3.1.5: Analyses of texture of gamma-irradiated plantains and control samples during 22 days of storage- a) hardness (g); b) adhesiveness (g.sec); c) cohesiveness; d) springiness; e) gumminess; f) chewiness. Each value represents mean ±SD of three sets of experimental data. Different alphabets denote that mean values belong to different subsets at $p<0.05$.

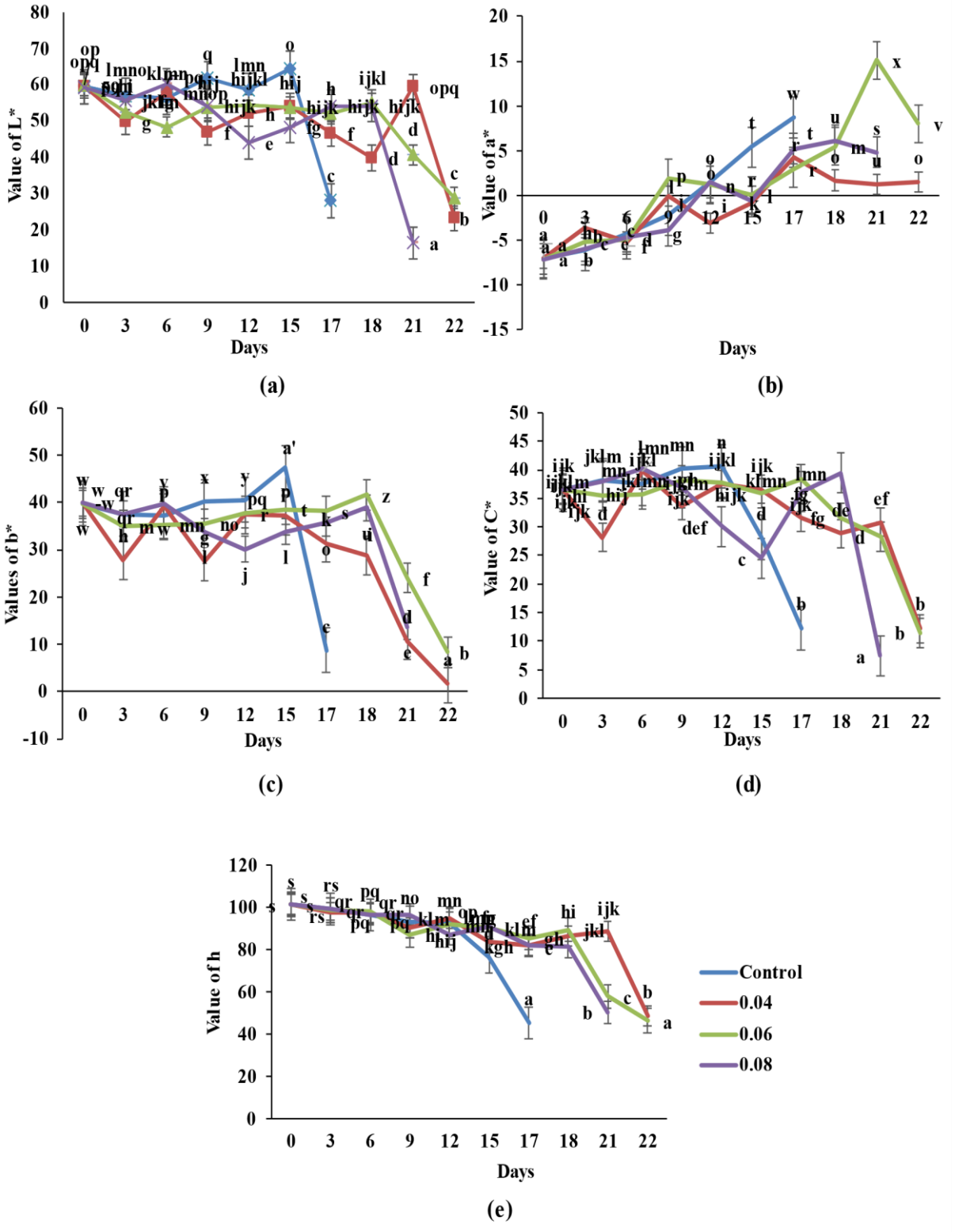


Figure 3.1.6: Colour parameters a) L* value, b) a* value, c) b*, d) c*, and e) h values of gamma-irradiated plantains and control samples. Each value represents mean ±SD of the data obtained from three independent sets of experimental data during a storage period of 22 days. Different alphabets denote that mean values belong to different subsets at $p<0.05$.

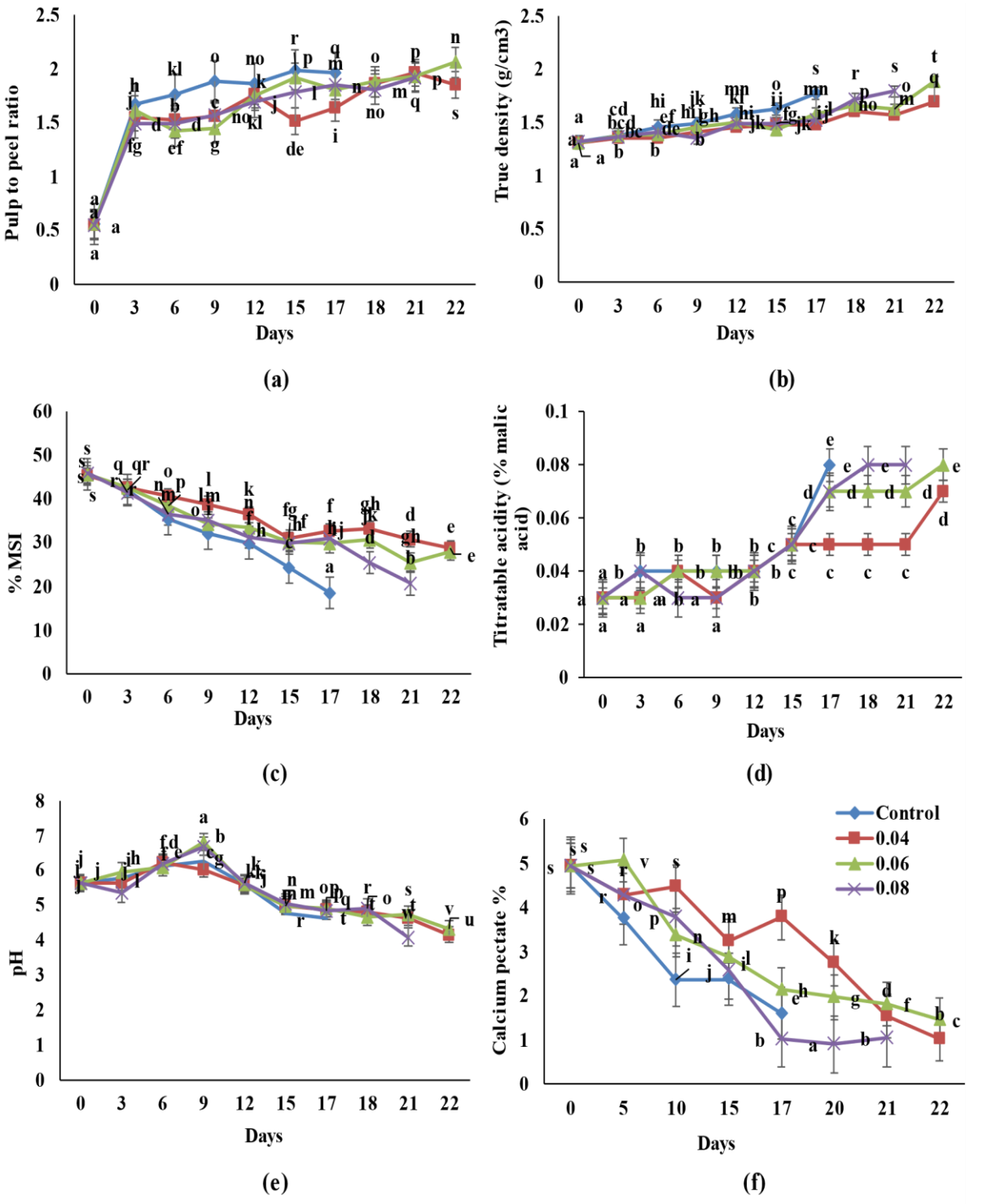


Figure 3.1.7: Physicochemical analyses of gamma-irradiated and control plantains during 22 days of storage- a) pulp to peel ratio; b) true density (g/cm^3); c) % membrane stability index (MSI); d) titratable acidity (%malic acid); e) pH and f) % calcium pectate. Each value stands for mean ±SD of three sets of experimental data. Different alphabets denote that mean values belong to different subsets at $p<0.05$.

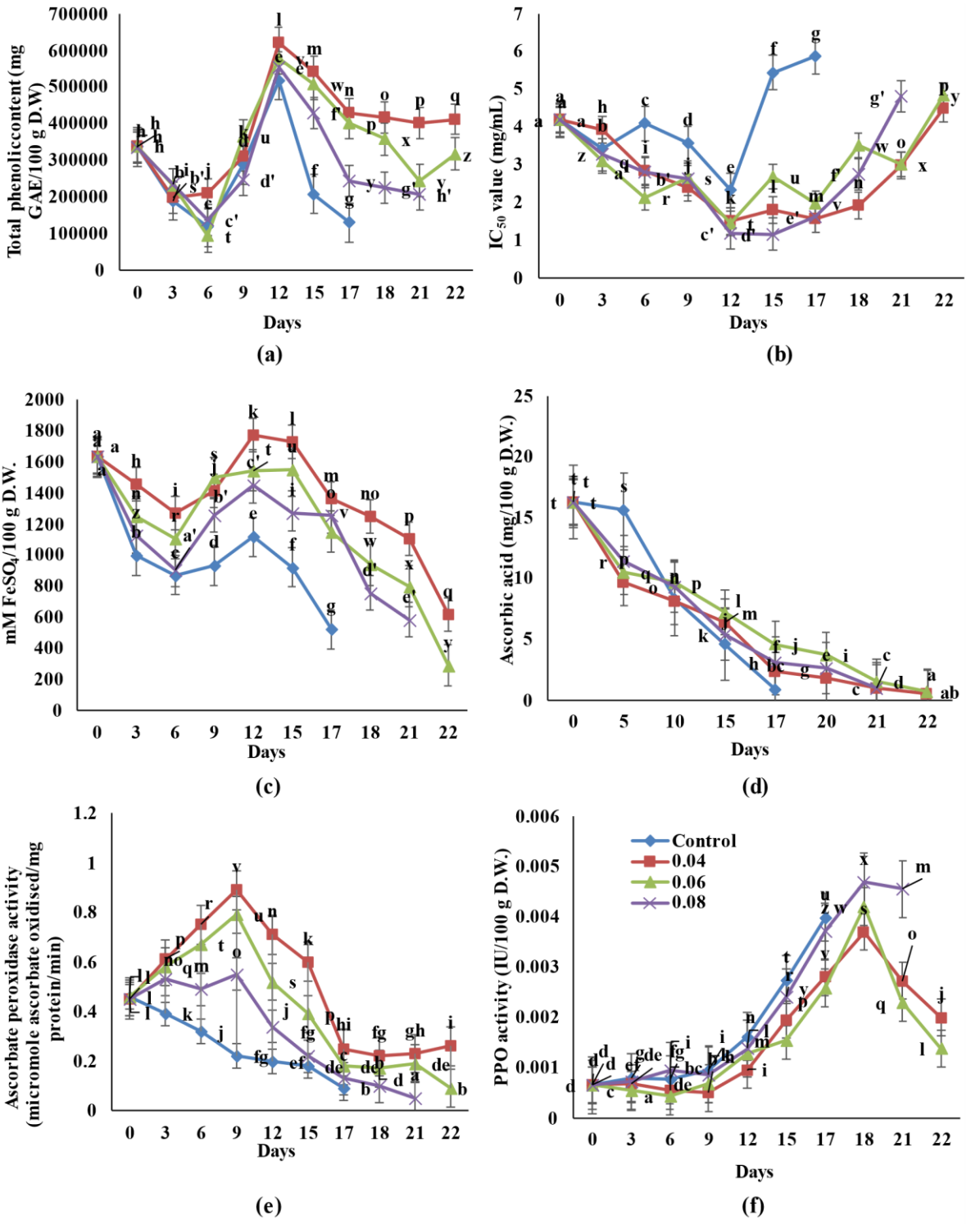
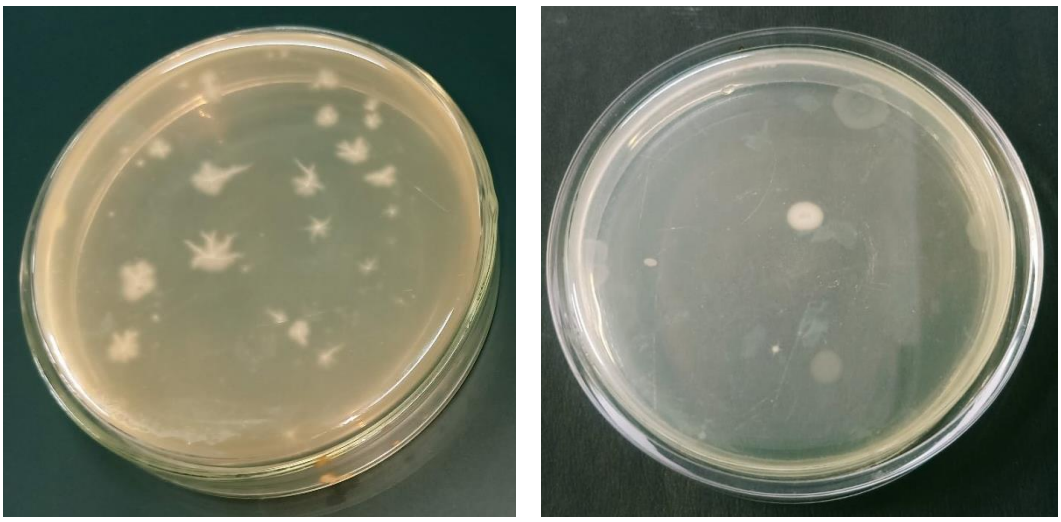


Figure 3.1.8: Phytochemical and enzyme activity analyses of gamma-irradiated plantains and control sets during 22 days of storage- a) total phenolic content (mg GAE(gallic acid equivalent)/100 g D.W.); b) IC_{50} value by DPPH (mg/mL); c) ferric reducing antioxidant power (FRAP) value (mM $FeSO_4$/100 g D.W.); d) ascorbic acid content (mg/100 g D.W.); e) ascorbic peroxidase activity (micromole ascorbate oxidized/mg protein/min) and f) polyphenol peroxidase (PPO) activity assay (IU/100 g D.W.). Each value stands for mean ±SD of three sets of experimental data. Different alphabets denote that mean values belong to different subsets at $p<0.05$.



(a) **(b)**

Figure 3.1.9: Microbial assessment of a) control and b) gamma-irradiated (0.04 kGy) plantains

Part 3.2

Optimization of gamma-irradiation processing parameters to obtain enhanced amount of serotonin and/or melatonin or both in combination (in antioxidant synergy) from gamma-irradiated plantains

Introduction

Previous part of this chapter reported that the shelf-life of '*desi'* variety of plantains (grown in West Bengal) can be successfully extended by 8 days (*i.e.*, a lead of 8 days of shelf life with respect to non-irradiated counterparts) by employing gamma-irradiation. Therefore, the specific objective of this part of the study was to obtain the best dose of gamma-irradiation which would enhance the contents of serotonin and melatonin in plantains to the maximum extent possible. This is the first study on the application of gamma-irradiation technology for the augmentation of contents of antioxidants in green plantains.

Materials and methods

Materials

The details of authentication, procurement, selection of plantains, raw materials and chemicals used in this study for QuEChERS-SPE and HPLC-PDA analyses have been described in part 2.1 of chapter 2. Liquid nitrogen was procured from liquid nitrogen plant, National Instruments Limited (NIL) campus, Jadavpur University, Kolkata, India.

Methods

Gamma-irradiation of the freshly harvested plantains was carried out in accordance with the method described in the preceding part of this chapter (part 3.1 of chapter 3). It had been

reportedly established that relatively higher doses of gamma-irradiation can enhance biosynthesis of natural antioxidants in botanicals, *viz.* production of phytoremediators as defence molecules against radiation-induced stresses is triggered at high radiation doses (Bhattacharjee and Chakraborty, 2019). Therefore, a 'low dose range' of 0.6-1 kGy, which was relatively higher compared to that used in the previous part of study was chosen for enhancement of serotonin-melatonin contents in it. The DoE for the present study was the same as described in the preceding part (part 3.1, chapter 3) and presented as a block diagram in Fig. 3.2.1. From day 0 onwards, three plantains were randomly withdrawn from the processed samples set for quantification of the content of biomolecules, namely L-tryptophan, serotonin, and melatonin.

Experimental plan for determination of the best dose of gamma-irradiation of green plantains for enhancement of contents of serotonin and melatonin individually

Contents of serotonin, melatonin, and their precursor molecule L-tryptophan in gamma-irradiated (doses: 0.6 - 1 kGy) and control sets of green plantains were quantified at an interval of 2 days for the entire storage period (25 days). Since the amounts of these bioactive antioxidant molecules changed more rapidly *vis-à-vis* their shelf-life parameters, it was necessary to conduct analyses of the said bioactives at a comparatively smaller time interval (*viz*. 2 days) for reliable elucidation of the trend (enhancement) of production of these biomolecules post irradiation.

QuEChERS-SPE extraction followed by HPLC-PDA analysis of irradiated plantains

For extraction of serotonin and melatonin from green plantains, whole fruits were used along with their peel, except the stalk and the black tip (remains of perianth, stigma and style). At first, the fruits were diced, placed inside a polystyrene box (30 cm * 30 cm * 25 cm) and adequate liquid nitrogen (to freeze the samples) was poured using a canister followed by

immediate grinding to powder in a mixer-grinder (HL 1618, M/s Philips India Limited, Chennai, India) in accordance with the method described by Okazaki and Ezura (2009), with few modifications. This cryo-ground sample was used for QuEChERS-SPE and then the extract was subjected to HPLC-PDA analyses for quantification of L-tryptophan-serotonin-melatonin. The detailed procedure for the extraction was demonstrated in part 2.1, chapter 2.

Determination of SE value

To evaluate the direct impact of gamma-irradiation on synergism among the three antioxidant molecules, namely serotonin, melatonin, and L-tryptophan in plantains, *in vitro* SE value was ascertained using pure chemical standards separately in varying concentrations similar to those present in the apposite extracts of plantain samples, *viz.* control sample on day 0, control sample on last day of storage, sample possessing highest amount of serotonin, and sample possessing highest amount of melatonin, and of mixtures comprising of the above antioxidants in the same concentrations (as analysed by HPLC-PDA). The detailed procedure for the analysis was demonstrated in part 2.1, chapter 2.

Statistical analysis

All experiments (including processes and analyses) in this part of the study were performed in triplicate and all data reported are mean ±SD of data obtained from the three independent sets of experiments as explained in Fig. 3.2.1. Two-way ANOVA was performed by IBM SPSS Statistics Software Version 26 (M/s IBM, New York, USA) to analyse the individual and interactive effects of two variables. A value of $p \leq 0.05$ was considered significant to establish differences in all tests.

Results and Discussion

Estimation of the best doses of gamma-irradiation to be applied on green plantains for enhancement of serotonin-melatonin contents individually

From the preliminary studies, it was observed that gamma-irradiation doses above 0.5 kGy enhanced the contents of the above-mentioned antioxidant molecules. Thus, the study was designed for 0.6, 0.8 and 1 kGy-irradiation doses for 25 days. The analyses for contents of L-tryptophan (as precursor)-serotonin-melatonin for the control set and the above-mentioned irradiated sample sets were conducted for 17, 18, 17, and 15 days (only), respectively, since the samples thereafter were completely spoiled and could not be analysed.

L-tryptophan, serotonin, and melatonin contents in gamma-irradiated green plantains

Fig. 3.2.2 depicts the visual changes in control and gamma-irradiated (0.6- 1 kGy) sets of plantains. Fig. 3.2.3a and 3.2.3b represent the chromatograms of a mixture of pure standards of L-tryptophan, serotonin, and melatonin; and the QuEChERS-SPE extract of 0.6 kGy-irradiated green plantains on day 12, respectively.

In the preliminary study, it was observed that, plantains gamma-irradiated under rotation mode with polystyrene cushions had relatively higher contents of serotonin and melatonin than those which were irradiated without polystyrene cushions and in static (no-rotation) mode. This was possibly owing to better attenuation (ensured from manufacturer's end and enhanced by using polystyrene cushions) and greater uniformity of dose distribution (under rotation mode) achieved during gamma-radiation of green plantains resulting in uniform irradiation of the samples (Anonymous, 2024) and thus enhancement in antioxidant production.

The highest content of L-tryptophan (121.87 µg/g D.W) was observed in plantains on day 0 which decreased with progression of senescence. The highest content of serotonin was observed in 1 kGy-irradiated plantains (135.03 µg/g D.W, *i.e.*, 44.10% enhanced content vis-

à-vis that in non-irradiated plantains) on day 10 followed by that in 0.8 kGy-irradiated plantains (128.86 µg/g D.W) on the same day. Melatonin content was found to be the highest in 0.6 kGy-irradiated plantains (7.20 µg/g D.W, *i.e.*, 146.57% enhanced content vis-à-vis that in non-irradiated plantains) on day 18 followed by that in 0.8 kGy-irradiated plantains (6.53 µg/g D.W) on day 17. The changes in serotonin, L-tryptophan, and melatonin contents with the progression of storage have been depicted in Fig. 3.2.3c, 3.2.3d and 3.2.3e, respectively.

Plantains possessed the highest content of L-tryptophan (121.87 µg/g D.W) in their initial stage of senescence, which decreased gradually (4.38 µg/g D.W) with storage (Fig. 3.2.3d). Serotonin contents in plantains increased and attained maximum value on day 10 (mid-senescence) which decreased steadily thereafter (Fig. 3.2.3c). However, melatonin content steadily increased with senescence (Fig. 3.2.3e). These findings of the present study corroborated well with those of Foy and Parratt (1960) and Adao and Gloria (2005) who showed that the amounts of serotonin in plantains increased during ripening followed by decrease during over-ripening. The increase of melatonin during ripening also supports the findings of Van Tassel *et al*. (2001) who found enhancement of amount of melatonin during ripening and over-ripening phases in two cultivars of tomato. Besides, bioconversion of L-tryptophan to melatonin via serotonin (Nawaz *et al*., 2016) enhances contents of melatonin in plants in the last phase of senescence with concomitant reduction in both L-tryptophan and serotonin contents. Although literature exists on enhancement of contents of melatonin production with ripening, there is no report on its enhancement consequent to gamma-irradiation. This study reports for the first time on the effectiveness of gamma-irradiation treatment in enhancing the amounts of the phytoremediator antioxidant molecules, namely serotonin and melatonin in green plantains.

The findings of the current investigation were also in harmony with this bioconversion pathway of melatonin production in plants. Besides it is known that phytohormone melatonin functions as an effective radioprotector (El-Desouky *et al*., 2014) and thus the increased production of

serotonin and melatonin in irradiated plantains for them to act as remediators (or scavengers) is a part of the protective mechanism against irradiation-induced overproduction of reactive oxygen species.

Antioxidant synergy in gamma-irradiated plantain extract by QuEChERS-SPE

In the present study, the SE values revealed that samples having the highest amount of serotonin (1-kGy gamma-irradiated green plantains on day 10) as well the non-irradiated control set of plantains (on day 0) possessed a synergistic consortium of the three antioxidants (serotonin, melatonin, and L-tryptophan) (Table 3.2.1). These results indicated that even after treatment with gamma-radiation, the natural food (antioxidant) synergy in the plantains was unperturbed. In contrast, the non-irradiated set of plantains on their last day of analysis (late senescence) as well as the samples having highest amount of melatonin [0.6-kGy gamma-irradiated green plantains on day 18 (late senescence] did not exhibit synergism among the said antioxidants.

Irradiation doses of 1 kGy and 0.6 kGy were the best doses that triggered the antioxidant(s) production pathways and samples irradiated with the said doses thus exhibited enhancement in contents of serotonin [1.44 folds (44.10%)] and melatonin [2.45 folds (146.58%)] vis-à-vis their non-irradiated counterparts. Furthermore, 1 kGy-irradiated plantains on day 10 (possessing highest serotonin) continued to exhibit natural synergism (viz. food synergy) among the three antioxidants (L-tryptophan, serotonin, and melatonin) as was present in the non-irradiated control set on day zero. Plantains irradiated at 0.6 kGy on day 18 (late senescence), which contained the highest amount of melatonin, although was unfit for direct human consumption, could be harvested as a potential source of phytomelatonin. Thus, this investigation also encompasses waste-valorization through utilization of completely senesced plantains as potential sources of biotherapeutic molecules which have promising uses as nutraceutical food-cum-therapeutic supplements.

Conclusion

Low doses of gamma-irradiation *viz.* 0.6 and 1 kGy contributed to enhancement of its contents of phyto-antioxidants, chiefly melatonin, and serotonin, respectively, additionally preserving antioxidant synergy in the latter. The findings of the present study strongly indicate that low dose of gamma-irradiation could augment production of the phytoremediator molecules, namely serotonin [1.44 folds (44.10%)] and melatonin [2.45 folds (146.58%)] in green plantains conferring irradiated plantain to be a potential source of food antioxidants. However, the plantains in late senescence which otherwise were sensorially unacceptable, were further utilized as potential sources of these important biotherapeutic molecules and has been elaborated in the following chapter (Chapter 4). This way, even the wasted plantains could be upcycled by utilizing them as sources of nutraceuticals.

Novelty

This work reports for the first time that gamma-irradiation can enhance the contents of phytoremediator molecules such as serotonin and melatonin in the produce thereby conferring the irradiated agro-produce to be a potential source of food antioxidants. This investigation also encompasses waste-valorization through utilization of completely senesced plantains (which otherwise would have been wasted) as potential sources of these biotherapeutic molecules which have promising uses as nutraceutical food-cum-therapeutic supplements. The findings of this study suggest novel uses of gamma-irradiation beneficial for both farmers and consumers alike and also propose a new benefit of gamma-irradiation to commercial food irradiators who can commercialize irradiated plantains for use as enhanced sources of serotonin and melatonin for food and pharmaceutical industries.

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Tables:

Table 3.2.1: The *in vitro* synergistic effect (SE) of antioxidants

Sample Name	Serotonin (µg/mL)	L-tryptophan (µg/mL)	Melatonin (µg/mL)	SE[f]
Serotonin1	5.52	0.00	0.00	-
Serotonin2	1.10	0.00	0.00	-
Serotonin3	135.03	0.00	0.00	-
Serotonin4	3.68	0.00	0.00	-
L-trytophan1	0.00	121.87	0.00	-
L-trytophan2	0.00	4.38	0.00	-
L-trytophan3	0.00	16.99	0.00	-
L-trytophan4	0.00	14.76	0.00	-
Melatonin1	0.00	0.00	0.52	-
Melatonin2	0.00	0.00	1.09	-
Melatonin3	0.00	0.00	3.38	-
Melatonin4	0.00	0.00	7.20	-
Sample 1 (Control set on day 0)	5.52	121.87	0.52	1.755 ± 0.004^{a}
Sample 2 (Control set on day 17)	1.10	4.38	1.09	0.100 ± 0.010^{b}
Sample 3 (1 kGy-irradiated sample on day 10)	135.03	16.99	3.38	1.096 ± 0.004^{c}
Sample 4 (0.6 kGy-irradiated sample on day 18)	3.68	14.76	7.20	-1.322 ± 0.003^{d}

a,b,c,d Different letters in a column indicate significant differences at $p<0.05$ level
[f]SE values are the mean ± SD values of three independent samples

Figures:

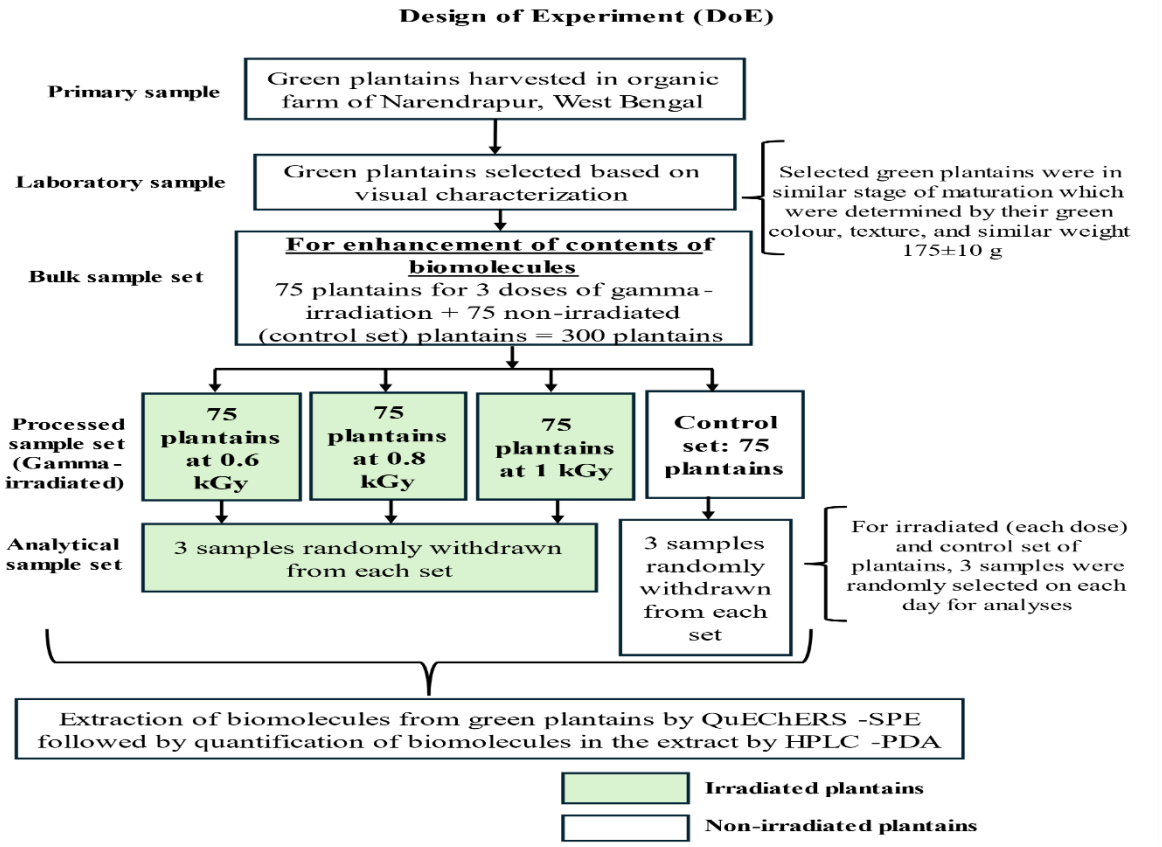

Figure 3.2.1: Experimental design for gamma-irradiation of green plantains. Each consortium of experiments (apposite to its objective) was conducted thrice as per the DoE and results are reported as mean ±SD of data obtained by the three independent runs of each experiment consortium. #QuEChERS-SPE- Quick, easy, cheap, effective, rugged, and safe- solid phase extraction. HPLC-PDA- High performance liquid chromatography-photo diode array

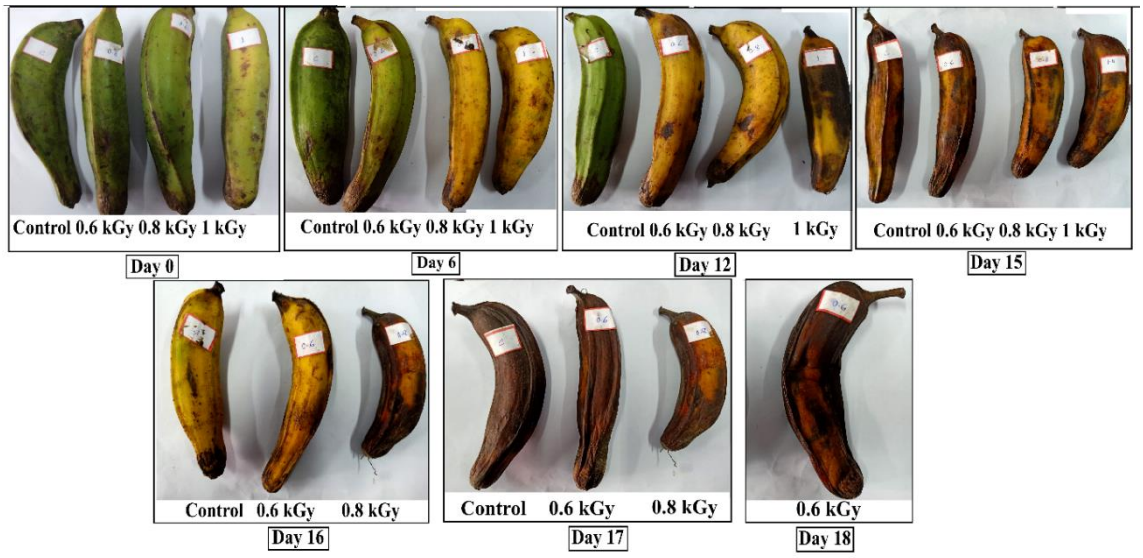

Figure 3.2.2: Physical changes in gamma-irradiated (0.6-1 kGy) plantains and control samples during 18 days of storage when stored at 23±2 °C, 70±2 % RH in an environmental chamber.

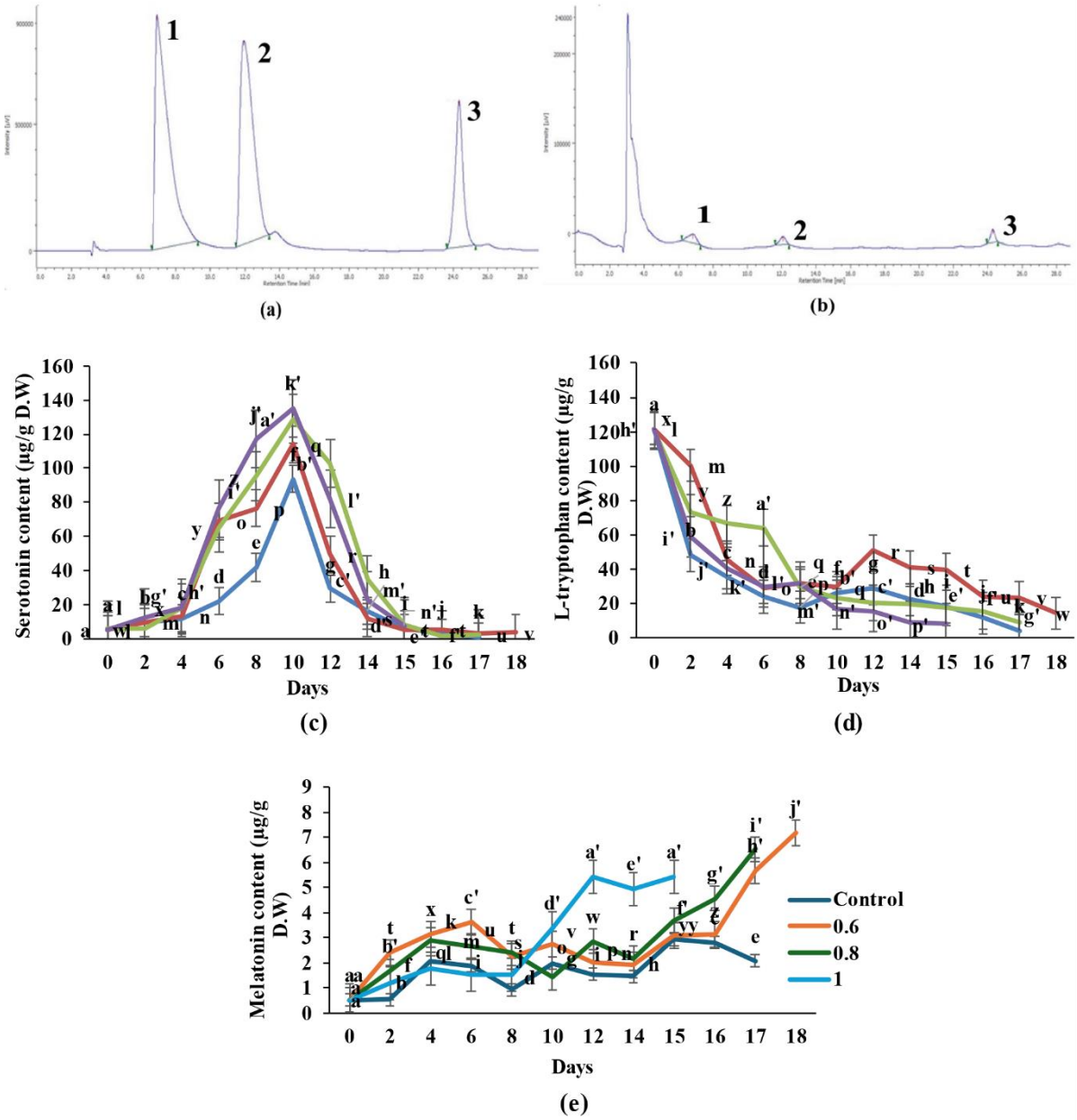

Figure 3.2.3: HPLC chromatogram of a mixture of - a) standard serotonin (1), L- tryptophan (2) and melatonin (3); b) HPLC chromatogram of QuEChERS-SPE extract of 0.6 kGy-irradiated green plantains on day 12 containing serotonin (1), L- tryptophan (2) and melatonin (3); c) serotonin (µg/g D.W); d) L-tryptophan (µg/g D.W) and e) melatonin (µg/g D.W) in gamma-irradiated (0.6, 0.8, and 1 kGy) plantains and control sets during 18 days of storage. The limits of quantification (LOQ) for L-tryptophan, serotonin and melatonin were 1.88 µg/L, 0.89 µg/L, and 0.51 µg/L, respectively. Each value stands for mean ±SD of three sets of experimental data. Different alphabets denote that mean values belong to different subsets at $p<0.05$.

Chapter 4

Ultrasonication-Assisted Solvent Extraction from Gamma-Irradiated-Cum-Senesced Plantains and Development of a Food Supplement

Introduction

Extension of shelf-life of freshly harvested plantains by 8 days and enhancement of serotonin [1.44 folds (44.10%)] and melatonin [2.45 folds (146.58%)] contents in the irradiated plantains in their mid-senescence and late-senescence, respectively, by employing gamma-irradiation have been elaborately discussed in the preceding chapter (Chapter 3). From the findings, it was evident that the highest content of serotonin was present in the irradiated (at 1 kGy) plantains in their mid-senescence (on day 10 of storage period) when they were suitable for consumption. However, the highest melatonin content was present in irradiated (at 0.6 kGy) and completely senesced (on day 18) plantains, when they were sensorially unacceptable and unfit for direct consumption. Therefore, for utilization of these senesced-yet-valuable-irradiated plantains, the extraction technology of UAE using green solvents could be employed to extract these highly important bioactive antioxidant molecules (chiefly melatonin) and deliver them in a suitable form to the consumers as a source of phytomelatonin along with serotonin and L-tryptophan.

A previous study reported the extraction of L-tryptophan and its derivatives including tryptamine, oxitriptan or 5-hydroxytryptophan, serotonin, and indole-3-acetic acid from rice grains using UAE (Setyaningsih *et al*., 2017). Melatonin has been extracted from grape skins (Iriti *et al.*, 2006), strawberries (Stürtz *et al*., 2011), red rice grains (Setyaningsih *et al*., 2016), and yellow and black mustard seeds (Chakraborty and Bhattacharjee, 2020) using UAE. To the best of the knowledge of the author, there is no literature report till date stating the simultaneous extraction of L-tryptophan-serotonin-melatonin using UAE from any source, including that from plantain pulp and peel.

For the formulation of a deliverable food supplement using the antioxidant-rich UAE extract, it needs to be transformed into a dried form, say a powder. For this purpose, a popular and widely used drying method of freeze-drying or lyophilization has been chosen for this study.

The non-thermal characteristics of lyophilization make it suitable for drying natural extracts containing thermosensitive bioactive molecules such as natural pigments and antioxidants (Shofinita *et al*., 2020). According to the literature reports, this technology has been used for formulation of food colorant powder from dragon fruit skin extract (Shofinita *et al*., 2020), powder from *Hibiscus sabdariffa* (Roselle) extract (Cid-Ortega and Guerrero-Beltran, 2022), encapsulate of *Indigofera tinctoria* L. extract (Shadordizadeh *et al*., 2022), microencapsulate of firethorn (*Pyracantha coccinea* Roemer var. Lalandi) extract (Dincera and Temiz, 2023) and encapsulate of *Amaranthus quitensis* L. (Quelal *et al*., 2023). To the best of the knowledge of the author, there is no available literature on the formulation of a food supplement containing the antioxidant triad L-tryptophan-serotonin-melatonin, and none on the extract of this consortium from green plantains.

Therefore, this chapter of the thesis has been divided into the following parts:

- *Optimization of processing parameters of UAE to obtain an extract having synergy among L-tryptophan-serotonin-melatonin from gamma-irradiated plantains*
- *Development of a food supplement in dried form from the antioxidant-rich plantain extract and its characterization*

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Part 4.1

Optimization of UAE processing parameters for maximizing the yield of bioactive antioxidants, chiefly melatonin, and to achieve a synergistic blend of antioxidants in the extract using fully ripened, irradiated plantains as the raw material

Introduction

The objective of this part of the study was to optimize the processing parameters of UAE by employing CCRD and RSM to obtain an extract containing L-tryptophan-serotonin-melatonin from the gamma-irradiation-cum completely senesced plantains and to evaluate the antioxidant synergy among the three antioxidants.

Materials and methods

Materials

The details of authentication, procurement, selection of plantains, raw materials and chemicals used in this study for UAE extraction, QuEChERS-SPE extraction, HPLC-PDA analyses, and antioxidant assessment have been described in part 2.1 of chapter 2. The details of irradiation were described earlier in part 3.1 of chapter 3.

Methods

Gamma irradiation of raw green plantains to enhance melatonin production

Based on the findings from the preceding chapter (part 3.2 of chapter 3), irradiated (at 0.6 kGy) and completely senesced (on day 20) plantains were used as raw material for this study.

Gamma-irradiation of the freshly harvested plantains was carried out in accordance with the method described in the preceding part of this chapter (part 3.2 of chapter 3).

Preliminary trial runs for UAE extraction

The 20-day stored irradiated-cum-completely senesced plantains having the highest melatonin content was subjected to UAE. The extractions were performed using a titanium probe sonicator (Labsonic M, M/s Sartorius, Melsungen, Germany) having a length of 80 mm and a tip diameter of 3 mm. The said equipment was compatible with batch size of 5 to 200 g of plantains and can be operated at a maximum power of 100 W at a maximum frequency of 30 kHz. To ensure safe end usage of the UAE extract for direct consumption (or as a deliverable to food industries), a GRAS status solvent such as ethanol was used for extraction. Few processing parameters of UAE such as batch size of sample (1 to 10 g), solvent composition (with varying ratio of ethanol: water:: 1:0, 0:1, 1:1, 0.5:1, 1:0.5), and working mode of ultrasonication (either continuous or discontinuous with pulse duration settings) were optimized through several preliminary trials. A minimum batch size of 2g was deemed necessary for reliable quantification of each of the three biomolecules of the antioxidant consortium since the same ensured their presence in amounts greater than their limits of detection (LOD) and limits of quantification (LOQ) of the HPLC-PDA analysis method used in the study (Tamili *et al*., 2023).

A sample size of 4g was found to be most ideal for the said extraction process, considering sample: extracting solvent ratio and subsequent quantitative recovery of melatonin in the extract. Among all solvent combinations experimented with, pure ethanol yielded maximum amount of melatonin in the extract. No significant difference in melatonin yields was obtained when the probe sonication was operated in continuous or pulsed mode and therefore all subsequent experimental runs were conducted in continuous mode for ease of operation.

Validation of the presence of the antioxidant triad in the UAE extracts

A few additional peaks in HPLC-PDA chromatograms of the UAE extracts perhaps of co-extracted molecules (other than the three target biomolecules), necessitated validation of the presence of melatonin, serotonin, and L-tryptophan in the extracts. To accomplish this, the UAE extract having the highest content of melatonin was dissolved in methanol, filtered through a double syringe filtration system (0.45 and 0.22 μm) and subjected to ESI-TOF-MS analysis using Xero-G2-Xs-QT, equipped with an ADC-magnetron detector (M/s Waters, Massachusetts, USA), following the programme previously reported by us (Tamili *et al*., 2023).

Optimization of UAE extraction parameters for obtaining the highest yield of phytomelatonin from 20 day stored (post irradiation) plantains using CCRD and RSM

Based on the above findings of the preliminary runs, three process variables were chosen and varied at three levels (3^3) for CCRD. These variables included the ratio of weight of the sample to the volume of extracting solvent (1:3, 1:5, 1:7 w/v), time of extraction (5, 10, and 15 min) and %amplitude of ultrasonic waves (85, 90, and 95) and fitted into a CCRD. The DoE matrix comprised of 17 runs (3 blocks), wherein factorial points were encoded as ±1 (total number=8), star or axial points were encoded as ±α (total number=6), and central points were encoded as 0 (total number=3). The factors and their levels are presented in Table 4.1.1. Triplicate runs for central points were conducted to identify experimental error(s), if any. Optimization was carried out independently with each response taken individually.

For extraction, 4g (*vide supra*) of cryo-ground plantains was mixed thoroughly with varying volumes of extracting solvent (ethanol) (Table 4.1.2) on an incubator shaker (IS 02, Incon Instruments, Delhi, India) at 500 rpm for 5 min in 15 mL amber-coloured glass vials. These samples were then subjected to extraction of melatonin using the probe sonicator under conditions described in Table 4.1.2. Throughout the ultrasonication process, it was assured that

the probe did not touch the sample vial surface, maintaining a distance of 1 cm between the probe-tip and the base (as well as the walls) of the extraction vial, to ensure appropriate impact of sound waves on the samples (Tiwari, 2015). A temperature range of 1-4 ℃ was maintained using an ice bath during extraction to prevent heat production and thus loss of heat sensitive melatonin. The UAE extracts thus obtained were then centrifuged in a cold centrifuge (C-24BL, M/s Remi, Mumbai, India) at 10,000 x g for 15 min at 4 ℃. The supernatant was concentrated in a rotary vacuum evaporator (PBU-6D, M/s Superfit Continental Private Ltd., Mumbai, India) at 50 mbar, 50±2 ℃ (water bath temperature) for 20-30 min; and further concentrated so as to completely remove the solvent residue by purging nitrogen. The extracts thus obtained were further purified prior to analysis of melatonin by HPLC-PDA.

To eliminate co-extracted natural colorants and interfering biomolecules (except melatonin, serotonin, and L-tryptophan) present in the UAE extract, which may impede the quantification of the three target biomolecules by HPLC-PDA analysis, SPE was adopted for extract purification. A reversed-phase polypropylene straight barrel SPE cartridge with bed mass 200 mg, bed mesh size 45 µm, and column capacity 6 mL was used for purification of the extracts (2g loaded) in accordance with the procedure developed by Chakraborty and Bhattacharjee (2020). Melatonin was eluted using 80:20::methanol: water; the eluate was filtered through a 0.22 µm syringe microfilter, and stored in a -20 ℃ freezer for further analyses. The phytomelatonin contents of the UAE extracts were quantified by HPLC-PDA analysis of the eluates following the method described by Tamili *et al*. (2023).

The software ‘Statistica’ was used to generate the 3-d quadratic (spline) response curves from the yields of melatonin obtained by HPLC-PDA analysis of the extracts. The response surfaces are illustrated as a function of two factors keeping the third factor unchanged. Second order multiple regression equations were used to model the response surfaces and further

characterized using canonical forms of the regression equations to determine the optimum extraction conditions needed for the highest recovery of phytomelatonin from green plantains.

Evaluation of antioxidant synergy among melatonin, serotonin, and L-tryptophan in the UAE extracts

Although the UAE extraction was aimed in maximising yield of melatonin in the extracts, co-extraction of its precursor molecules *viz*. serotonin and L-tryptophan inadvertently occurred in all extractions. This necessitated assessment of synergism in the antioxidant consortium triad by evaluating the SE value of the extract which must be greater than unity to ensure antioxidants to act in synergism. Therefore, each UAE extract was analysed for its SE value following the previously described method (Sarkar *et al*., 2021, Liu *et al*., 2008).

Re-optimization of UAE extraction parameters to obtain highest SE value by RSM

It is known that concentration of individual antioxidants has a direct effect on SE value and also that lowest concentration of any antioxidant in the consortium triad could produce the greatest synergistic effect (Liu *et al*., 2008). Absence of synergism among the co-extracted antioxidants could render usage of the UAE extract health- debilitating. This necessitated implementation of RSM for re-optimization of the UAE parameters, considering SE value as the response. The UAE exacts thus obtained above were all assessed for their SE values (*vide supra*) and it was found that the UAE extract having the highest recoverable yield of melatonin lacked synergy with its co-extracted precursors present in the antioxidant triad. Thus, for safe usage of the UAE extracts, it was mandatory to reevaluate them in terms of SE values and repeat the RSM exercise to deliver extraction conditions optimum for obtaining an UAE extract which may not be rich in either of the antioxidant molecule (from the triad) but would have a SE value greater than unity (higher the value, greater is the synergism). The extract thus

obtained by this endeavour was successively characterized and utilized for product development. This extract has been henceforth designated as U_{Best}.

Assessment of safety and antioxidant activity of U_{Best}

There was a chance of occurrence of heavy metal contaminant especially titanium, due to the use of a titanium probe during ultrasonication. Thus, to identify the presence of heavy toxic metals such as Ti, Pb, Hg, Ni, As, Si, and Mo in U_{Best}, EDX analysis was performed using INSPECT F50, M/s FEI Company, Hillsboro, USA.

The antioxidant activity of U_{Best} was ascertained in terms of its TPC, DPPH radical scavenging activity, FRAP value and reducing power according to the methods described in part 2.1 in chapter 2.

Estimation of ultrasonic intensity (UI) and acoustic energy density (AED) utilized to obtain U_{Best}

To ensure whether the UAE technology is a 'green', energy saving one, it was important to assess energy utilized during ultrasonication process. It is assumed that the UAE system has no heat losses and hence, a rough estimate of energy introduced into the extraction system can be expressed as UI (in W/cm^2) or AED (in W/cm^3 or W/mL) (Tiwari, 2015). The intensity of ultrasonication and acoustic energy density were determined using standard equations (Tiwari, 2015). At first, acoustic power (P) consumption was estimated by calorimetric method (Eq. 1), this was followed by evaluation of applied UI (Eq. 2) and AED (Eq. 3) using the above estimated power consumption (Tiwari, 2015).

$$P = m.Cp.[\frac{dT}{dt}]_{t=0} \quad (1)$$

$$UI = P \quad (2)$$

$$AED = P/V \quad (3)$$

where, Cp denotes heat capacity of the solvent when the pressure is constant (J kg^{-1} K^{-1}); m defines the mass of sample (kg); T is the temperature (°C) of the extraction medium; t denotes the extraction time (s), dT/dt defines temperature change per second (°C/s); A is the area of the tip of the probe (cm^2) and V is the volume of the sample present in the extraction vial (mL).

Statistical analyses

All experiments including processes and analyses (except the analyses involving high-end instruments such as EDX and ESI-TOF-MS) were conducted in triplicate, and the results are reported as the mean ±SD of three values obtained from three independent experiments. Statistical analysis, including the determination of significant differences between means was performed using one-way ANOVA, Duncan's multiple-range test, and RSM with regression modelling. IBM SPSS Statistics Software Version 26 (M/s IBM, New York, USA) was used for ANOVA and Duncan's test, while STATISTICA 8.0 software (M/s Statsoft, Oklahoma, USA) was utilized for RSM and regression modelling. A p-value of <0.05 was considered significant.

Results and Discussion

The HPLC-PDA chromatogram of the QuEChERS-SPE extract of plantains irradiated at 0.6 kGy (after 20 days of storage, *i.e.*, at the end of senescence) revealed the presence of 7.22 µg/g of melatonin, 5.91 µg/g of serotonin, and 12.68 µg/g of L-tryptophan therein. The antioxidant synergy however was destroyed in the same since SE value of the antioxidant triad was found to be less than unity. These findings are in sync with the findings of the previous publication (Sarkar *et al*., 2021).

Generation of response surface curves

Fig. 4.1.1a, 4.1.1b, and 4.1.1c depict the spectra of melatonin, serotonin, and L-tryptophan, respectively, obtained by ESI-TOF-MS analysis of the methanolic UAE extract of plantains.

The molecular mass (M) of melatonin and L-tryptophan appeared as their sodium-adduct [M + Na] + form; whereas serotonin appeared as its proton adduct form [M + H] +. Sodium adduct of melatonin has been previously reported by Yang *et al*. (2002) during quantification of melatonin in blood serum. Although no report is available for sodium adduct of L-tryptophan, presence of sodium adducts is reportedly a very common phenomenon for many molecules (Kruve and Kaupmees, 2017). Similar ESI spectra for proton adduct of serotonin has been reported by Ramakrishna *et al*. (2012).

The whole experimental programme of CCRD model and the results (L-tryptophan, serotonin, and melatonin content of the extracts under different conditions of UAE along with their respective SE value) are presented in Table 4.1.2. As is evident from the data, the content of melatonin extracted increased with increasing %amplitude and time. Among all the experimental runs, the highest melatonin yield (6.65 µg/g) along with its co-extracted serotonin (4.05 µg/g) and L-tryptophan (3.22 µg/g) was achieved under conditions having sample weight to extracting solvent volume ratio of 1:5, an extraction time of 10 min, and an ultrasonication amplitude of 90% (Table 4.1.2). Based on the yield of melatonin thus obtained in each of the 15 runs of CCRD, regression modelling was employed.

Regression modelling

Each parameter of UAE, namely the sample weight to extracting solvent volume (X_1), time required for extraction (X_2) and % amplitude of ultrasonic waves (X_3), exhibited appreciable effects on the extraction yield of melatonin and have been presented as response surfaces in Fig. 4.1.2. The sample weight to extracting solvent volume (in mL), time required for extraction (in min) and %amplitude of ultrasonic waves (in %) were fixed at their median values of 5 mL, 10 min and 90% respectively, in the corresponding stereoscopic figures. Regression modelling

was employed to characterize the response surfaces, and the second-order polynomial equation that best fit the experimental variables is provided in Eq. 4.

$$Y = B_0 + \Sigma B_i X_i + \Sigma B_{ii} X_i^2 + \Sigma B_{ij} X_i X_j \quad (4)$$

Here, Y denotes the experimental response *i.e.*, yield of melatonin from senesced (irradiated) plantains. B_0, B_i, B_{ii}, and B_{ij} present the constants and regression coefficients of the model while X_i and X_j are two independent variables in coded forms. The expanded models, including linear, quadratic, and cross-product terms, are as below (with intercept):

$$Y_{Melatonin} = 6.11610 + 0.48148\ X_1 - 1.14527\ X_1^2 + 1.17354\ X_2 - 0.77431\ X_2^2 + 1.79674\ X_3 - 0.75295\ X_3^2 + 0.32500\ X_1X_2 - 0.76717\ X_1X_3 + 0.41112\ X_2X_3 \quad (5)$$

The effects of the UAE processing parameters and their interactions on the final yield of melatonin were studied. Eq. 5 shows the effects of X_1, X_2 and X_3 on the response Y. Additionally, an evaluation of the significance level of the investigated independent factors and their interactions were carried out. Table 4.1.3 presents the two-way ANOVA results for the model.

It was evident that the yield of melatonin was significantly ($p<0.05$) dependant on all the aforementioned UAE processing parameters (X_1, X_2, X_3) including their second order terms (X_1^2, X_2^2, X_3^2). The two-level interaction factors of involving solvent amount and time with % amplitude of ultrasonic waves *i.e.,* X_1X_3 and X_2X_3, exhibited significant ($p<0.05$) effects on the final yield of melatonin. However, the interaction between solvent amount and extraction time (X_1X_2) showed no significant ($p<0.05$) effect on the yield of melatonin. The ANOVA table (Table 4.1.3) presents high F values (Fisher's variance ratio) ranging from 15.28 to 212.36 for all the first order (X_1, X_2, X_3) terms as well as second order terms (X_1^2, X_2^2 and X_3^2), along with two-level interactions of X_1X_3 and X_2X_3 for all the three parameters of UAE. This

validates their significance and importance on the final yield. The highest F value (Table 4.1.3) indicates that the %amplitude of ultrasonic waves (F=212.36) was the most important factor, followed by extraction time (F=90.59) that influenced melatonin yield.

To evaluate the competence of the obtained regression model and violations of the basic assumptions of the same, 'residual analyses' was carried out with the experimental data following to the method described by Montgomery (2001a). The plots of residual vs. fitted values exhibited a "structure less" pattern, showing no obvious trends (Fig. 4.1.3a). This randomness in the plot further validated the competency of the model. The graph plots of predicted yields (µg/g of plantain D.W.) and observed yields (µg/g of plantain D.W.) showed very close fits (Fig. 4.1.3b). Hence, multiple regression relationships (R^2=0.98) among the independent and responding variables were statistically significant. The complete quadratic models also exhibited a very good fit denoting a statistically significant multiple regression relationship between the independent variables and the responding variables. From the test statistics for the regression models (F-test, t-test, and ANOVA), it is seen that % amplitude of ultrasonic wave had the most significant effect on the yield of melatonin than any other combination of extraction parameters.

Characteristics of response surfaces

For the characteristic evaluation of the response surfaces, the nature of the obtained stationary points needs to be analysed to determine whether it is a point of maxima, minima, or saddle points. To achieve this, the regression equations were converted into their canonical forms, and the eigenvalues were calculated according to the method reported by Montgomery (2001a). The eigenvalues obtained for the yields of melatonin (-0.010406, -0.016760 and -0.146537) in the UAE extract of plantain were found to be negative. This finding indicate that the X_s represent a point of maxima.

Optimal processing conditions for obtaining UAE extract with the highest phytomelatonin yield

For the optimization of the UAE process conditions, the second order regression equation was converted into its matrix form according to Montgomery (2001b) and obtained stationary points were as follows: X_{1S} = 4.68 mL, X_{2S} = 15.72 min; X_{3S} = 97.93%. The predicted yield of melatonin at these conditions was 7.15 µg/g of plantain D.W., which aligned well with the melatonin content of the extract thus obtained under similar experimental conditions (7.03 µg/g of plantain D.W.). This optimized UAE condition successfully recovered 97.36% of melatonin from its actual content present in irradiated plantains (analysed by QuEChERS-SPE followed by HPLC-PDA) on the same day.

This finding is similar to the results described by Oroian *et al*. (2020) who reported that the ultrasound amplitude had significant ($p<0.05$) influence on the TPC, total flavonoid content and balsam content extracted from propolis (bee glue). Ultrasonic waves lead to the formation of small bubbles subjected to fast adiabatic compression and expansion, triggering fast localized enhancement of temperature and pressure, which leads to breakage in the plantain cell wall. This cavitation-like process occurs as a result of compression and rarefaction cycle of ultrasonic waves which in turn depends on the amplitude of the ultrasonic waves. Thus, with increasing ultrasonic amplitude, the number of cavities also enhanced, which subsequently resulted in achieving a maximum extraction yield.

Despite having the highest yield of phytomelatonin, the synergy among L-tryptophan, serotonin, and melatonin was not preserved (SE= −0.62) in the extract thus obtained under the above-discussed optimized conditions, assessed by its DPPH radical scavenging activity. Due to the presence of a phenolic hydroxyl group, serotonin is having higher radical scavenging activity and reducing activity compared to melatonin containing aromatic indole ring (Gülçin,

2008) which possible attributed to the above loss in synergy. Therefore, it is necessary to re-optimize the UAE processing conditions based on the SE values of the UAE extracts under different conditions of the CCRD DoE (Table 4.1.2).

Re-optimized processing conditions for obtaining UAE extract with synergistic consortium among L-tryptophan-serotonin-melatonin

RSM was re-performed based on the SE values. Y in Eq. 6 denotes the SE values of the extracts and other parameters were similar to those described *vide supra*. The expanded models, including linear, quadratic, and cross-product terms are as below (with intercept):

$$Y_{SE} = -0.154872 - 0.287042\ X_1 + 0.350518\ X_1^2 + 0.460206\ X_2 + 0.390560\ X_2^2 - 0.972152\ X_3 + 0.586989\ X_3^2 - 0.270000\ X_1X_2 + 0.515000\ X_1X_3 - 0.625000\ X_2X_3 \quad (6)$$

The effects of the UAE processing parameters and their interactions on the SE value of the extract were studied. The Eq. 6 revealed the effects of X_1, X_2 and X_3 on the response Y representing SE value of the extract and have been presented as response surfaces in Fig. 4.1.4. Additionally, an evaluation of the significance level of the investigated independent factors and their interactions were carried out. Table 4.1.4 presents the two-way ANOVA results for the models of SE values.

It was evident that the preservation of SE value (SE>1) was significantly ($p<0.05$) dependant only on the % amplitude (X_3) of ultrasonication. The ANOVA table (Table 4.1.4) presents F value of 12.48 for this parameter. The plots of residual vs. fitted SE values exhibited a "structure less" pattern, showing no obvious trends (Fig. 4.1.5a). The graph plots of predicted and observed SE values showed very close fits (Fig. 4.1.5b). Hence, it could be concluded that the multiple regression relationship (R^2=0.79) among the independent and responding variables

was statistically significant. From the test statistics for the regression models (F-test, t-test, and ANOVA) as discussed above, it is seen that % amplitude of ultrasonic wave had the most significant effect on SE value than any other combination of extraction parameters. The eigenvalues obtained for the SE values of the UAE extracts are 0.050043, 0.010286 and 0.003036 indicating that the X_s represent a point of minima.

The obtained stationary points were as follows: X_{1S} = 4.41 mL, X_{2S} = 10.65 min; X_{3S} = 95.13%. The predicted SE value of the extract under these extraction conditions was -0.37, which is similar to that of the obtained value (-0.32) under similar conditions. Therefore, any conclusive extrapolation of UAE processing parameters for obtaining the highest amount of synergy cannot be drawn despite obtaining X_s as a point of minima.

Safety of UAE extract of plantain for consumption and its antioxidant property

The results of the EDX analysis confirmed that the UAE extract exhibited no traces of toxic heavy metal contaminants (Fig. 4.1.6). This finding establishes the UAE extract from senesced (irradiated) plantain to be completely safe for human consumption.

The reducing power (4.63±0.04 mg BHT/g of plantain in D.W.) and FRAP values (97.58±0.65 mmol $FeSO_4$ equivalent/g of plantain in D.W.) confirmed that the extract indeed had strong antioxidant potency. However, the values for TPC (1.25±0.05 mg of GAE/g of plantain in D.W.) and the DPPH radical scavenging activity in terms of IC_{50} value (30.49 ± 0.04 mg/mL) were not as appreciable. The difference between DPPH radical scavenging activity and FRAP value can be attributed to the explanation provided by Ali *et al*. (2019). They described that the non-phenolic antioxidants such as xanthophylls, carotenoids, and vitamin C are primarily responsible for DPPH radical scavenging activity. On the other hand, phenolic compounds, which can donate a single electron to the Fe^{3+}-TPTZ complex to reduce it into the blue Fe^{2+}-

TPTZ complex (Gu *et al*., 2019), contribute to the ferric reducing power activity. Bhebhe *et al*. (2016) also reported that TPC and antioxidant activity are not always directly proportional, who found that despite having a low quantity of phenolic compounds, a 50% ethanolic extract of *Syzygium jambolanum* exhibited better free radical scavenging activity. This phenomenon of having high antioxidant activity with low phenolic content has also erstwhile reported from our laboratory for UAE extracts of yellow and black mustard seeds (Chakraborty and Bhattacharjee, 2020).

Industrial feasibility of the optimized UAE conditions

From the above-discussed equations, the UI required under the optimized conditions of UAE extraction was 12.39 W/cm^2. The energy requirement was similar to that reported by Salehan *et al*. (2016) who extracted gallic acid from *Labisia pumila* using UAE with a power intensity of 8.66 W/cm^2. Similar energy level was also reported by Li *et al*. (2004), who extracted oil from soyabeans using UAE with UI levels ranging from 16.4 to 47.6 W/cm^2. According to Tiwari (2015), ultrasound can be either of low-intensity sonication, *i.e.*, <1 W/cm^2, primarily employed for non-destructive analytical techniques for quality assurance and process control, or the high-intensity sonication (10-1000 W/cm^2) used for extraction and processing applications. The extraction of melatonin, serotonin, and L-tryptophan by ultrasonication require high-intensity acoustic energy, leading to the production of high cavitation energy in the extraction medium (Tiwari, 2015). This high cavitation energy possibly improved solvent penetration across cells by rupturing the cells and cell membranes of plantains. The AED calculated at the optimized UAE condition was 1.47 W/cm^3. Therefore, the present study demonstrates that UAE of melatonin, serotonin, and L-tryptophan from irradiated-senesced green plantains consumes appreciably low energy.

Conclusion

The present study delivers the optimized parameters of UAE extraction for obtaining two extracts: one with the highest yield of phytomelatonin and another having a synergistic consortium of antioxidants- phytomelatonin, serotonin, and L-tryptophan, from gamma-irradiated (0.6 kGy)-cum-completely senesced plantains, documenting the first report of its kind. The development of a deliverable food supplement from the extract U_{Best}, its characterization and *in vitro* release kinetics of the antioxidant triad from the same have been further investigated and elaborated in the next part of this chapter.

Novelty

The utilization of gamma-irradiated (0.6 kGy)-cum-completely senesced plantains by extraction of phytomelatonin-serotonin-L-tryptophan as a consortium by employing UAE using green solvent) is being reported for the first time. The present endeavour also delivered for the first time the optimized conditions of UAE for recovery of two extracts- one rich in melatonin and the other possessing the highest SE value among the three health-beneficial antioxidants.

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Tables:

Table 4.1.1: Three level CCRD design utilized in the present study with codes and levels of investigated independent variables

Independent variables	Codes	Levels[a]				
		−α (-1.67)	**-1**	**0**	**+1**	**+α (+1.67)**
Extraction time (min)	X_1	1.63	5	10	15	18.36
%Amplitude	X_2	81.63	85	90	95	98.36
Volume of extracting solvent (mL)*	X_3	1.65	3	5	7	8.35

[a]The CCRD model was consisted of 17 runs (number of blocks 3) including eight factorial points (enciphered as ±1), six star or axial points (enciphered as ±α) and three centre points (enciphered as 0). *Sample to solvent ratios of 1:3, 1:5, 1:7 w/v corresponds to 4g of ground plantain extracted with 12 mL, 20 mL, and 28 mL of extracting solvent, respectively.

Table 4.1.2: Yields of melatonin, serotonin, and L-tryptophan from plantains and their respective SE value

No of Runs	Sample: Solvent (w/v)	Time (min)	%Amplitude	Melatonin (µg/g D.W. of plantain)*	Serotonin (µg/g D.W. of plantain)*	L-tryptophan (µg/g D.W. of plantain)*	SE Value
Factorial Points							
1	1:3	5	85	3.02 ± 0.05^{a}	1.64 ± 0.01^{p}	$1.29 \pm 0.02^{d'}$	0.31
2	1:7	5	95	4.79 ± 0.02^{b}	2.65 ± 0.04^{q}	$2.06 \pm 0.09^{e'}$	0.16
3	1:7	15	85	4.96 ± 0.06^{c}	2.42 ± 0.04^{r}	$2.14 \pm 0.02^{f'}$	0.76
4	1:3	15	95	6.65 ± 0.05^{d}	4.05 ± 0.01^{s}	$3.12 \pm 0.05^{g'}$	0.32
5	1:7	5	85	3.80 ± 0.02^{e}	1.94 ± 0.02^{t}	$1.78 \pm 0.03^{h'}$	-0.12
6	1:3	5	95	5.32 ± 0.08^{f}	2.31 ± 0.06^{u}	$2.57 \pm 0.07^{i'}$	0.15
7	1:3	15	85	3.31 ± 0.09^{g}	2.45 ± 0.05^{v}	$1.84 \pm 0.03^{j'}$	2.32
8	1:7	15	95	6.55 ± 0.01^{h}	3.93 ± 0.03^{w}	$2.76 \pm 0.02^{k'}$	0.38
Axial Points							
9	1:5	1.63	90	3.97 ± 0.04^{i}	1.82 ± 0.05^{x}	$1.68 \pm 0.01^{l'}$	0.4
10	1:5	18.37	90	6.03 ± 0.05^{j}	3.56 ± 0.07^{y}	$2.76 \pm 0.02^{k'}$	0.31
11	1:5	10	81.63	3.83 ± 0.02^{k}	2.18 ± 0.06^{z}	$1.15 \pm 0.03^{m'}$	1.93
12	1:5	10	98.37	6.23 ± 0.06^{l}	$3.97 \pm 0.04^{a'}$	$3.22 \pm 0.06^{n'}$	-0.67
13	1:1.65	10	90	4.04 ± 0.06^{m}	2.29 ± 0.08^{u}	$2.31 \pm 0.02^{o'}$	0.31
14	1:8.35	10	90	4.92 ± 0.03^{n}	3.20 ± 0.07^{b}	$2.36 \pm 0.05^{p'}$	0.29
Centre Points							
15	1:5	10	90	6.11 ± 0.02^{o}	$3.89 \pm 0.03^{c'}$	$2.87 \pm 0.01^{q'}$	-0.15
16	1:5	10	90	6.12 ± 0.01^{o}	$3.90 \pm 0.02^{c'}$	$2.88 \pm 0.02^{q'}$	-0.15
17	1:5	10	90	6.13 ± 0.03^{o}	$3.91 \pm 0.02^{wc'}$	$2.89 \pm 0.02^{q'}$	-0.15

Different letters in a column indicate significant differences at $p<0.05$ level.
*Yields of melatonin, serotonin and L-tryptophan from green plantains were mean ±SD of independent extractions in triplicate

Table 4.1.3: ANOVA table of response surface methodology of melatonin extraction from plantain

Effect of parameters*	Yield of melatonin from plantain (µg/g of plantain in D.W.)				
	SS	Degree of freedom	MS	F	p
X_1	0.78886	1	0.78886	15.2624	0.005848
X_1^2	3.66023	1	3.66023	70.8153	0.000066
X_2	4.68250	1	4.68250	90.5936	0.000030
X_2^2	1.66750	1	1.66750	32.2615	0.000751
X_3	10.97617	1	10.97617	212.3587	0.000002
X_3^2	1.57677	1	1.57677	30.5062	0.000885
X_1X_2	0.21126	1	0.21126	4.0872	0.082924
X_1X_3	1.17711	1	1.17711	22.7738	0.002031
X_2X_3	0.33803	1	0.33803	6.5400	0.037697
Error	0.36181	7	0.05169		
Total SS	23.11438	16			

*X_1 = Solvent amount (mL); X_2= Time (min); X_3= % Amplitude of ultrasonication

Table 4.1.4: ANOVA table of response surface methodology of synergistic efficacy values of UAE extracts of plantain

Effect of parameters*	SE value of extracts				
	SS	Degree of freedom	MS	F	p
X_1	0.280368	1	0.280368	1.08899	0.331389
X_1^2	0.342858	1	0.342858	1.33171	0.286375
X_2	0.720085	1	0.720085	2.79690	0.138366
X_2^2	0.424237	1	0.424237	1.64779	0.240116
X_3	3.213269	1	3.213269	12.48076	0.009562
X_3^2	0.958279	1	0.958279	3.72208	0.095024
X_1X_2	0.145800	1	0.145800	0.56631	0.476270
X_1X_3	0.530450	1	0.530450	2.06034	0.194313
X_2X_3	0.781250	1	0.781250	3.03448	0.125048
Error	1.802205	7	0.257458		
Total SS	8.633588	16			

*X_1 = Solvent amount (mL); X_2= Time (min); X_3= % Amplitude of ultrasonication

Figures:

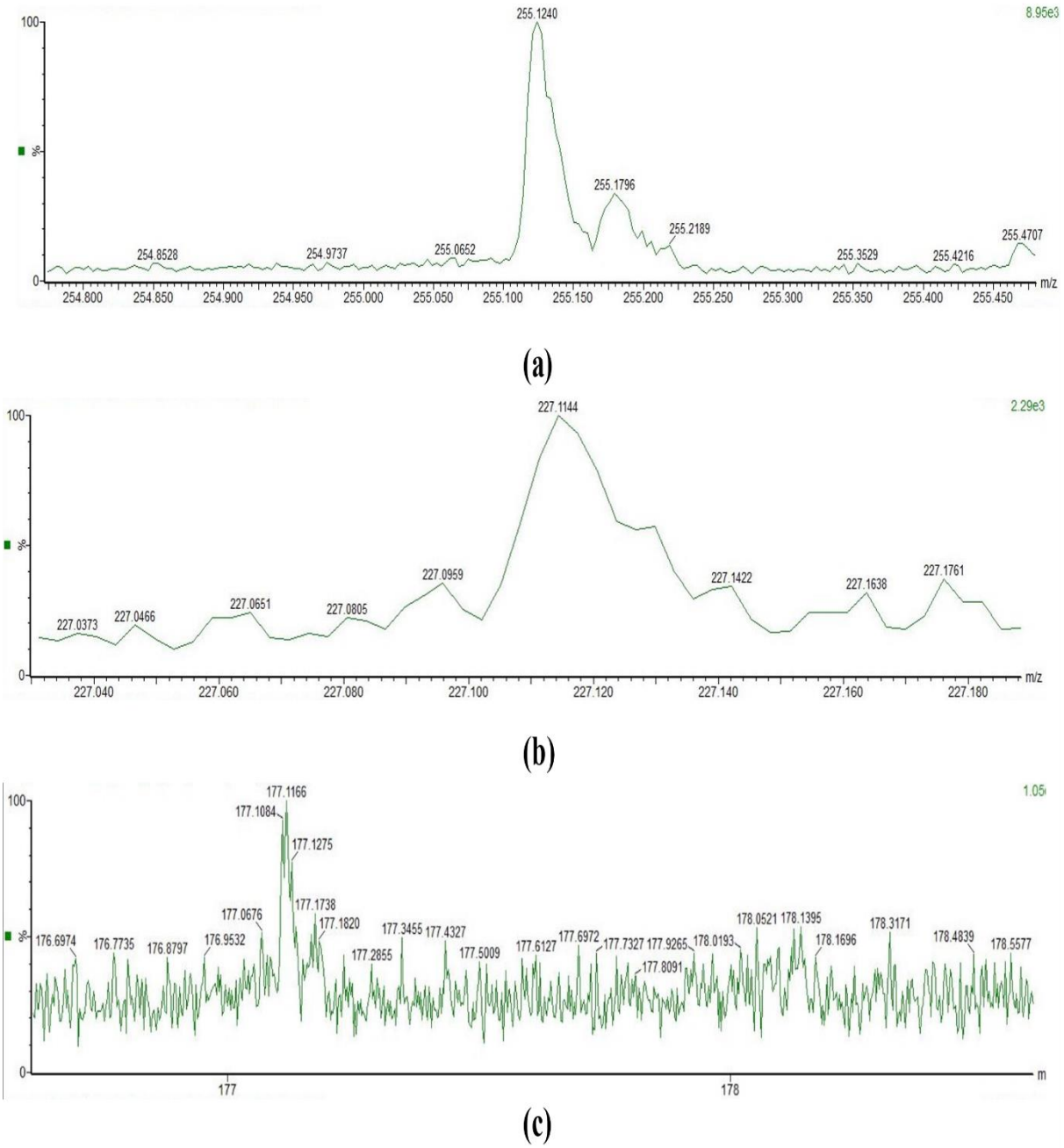

Figure 4.1.1: Electrospray ionization-time-of-flight-mass spectrum of ultrasonication-assisted solvent extract from irradiated-cum-completely senesced plantains under the optimized extraction condition indicating the peaks for a) melatonin [M + Na] +, b) serotonin [M + H] +, and c) L-tryptophan [M + Na] +

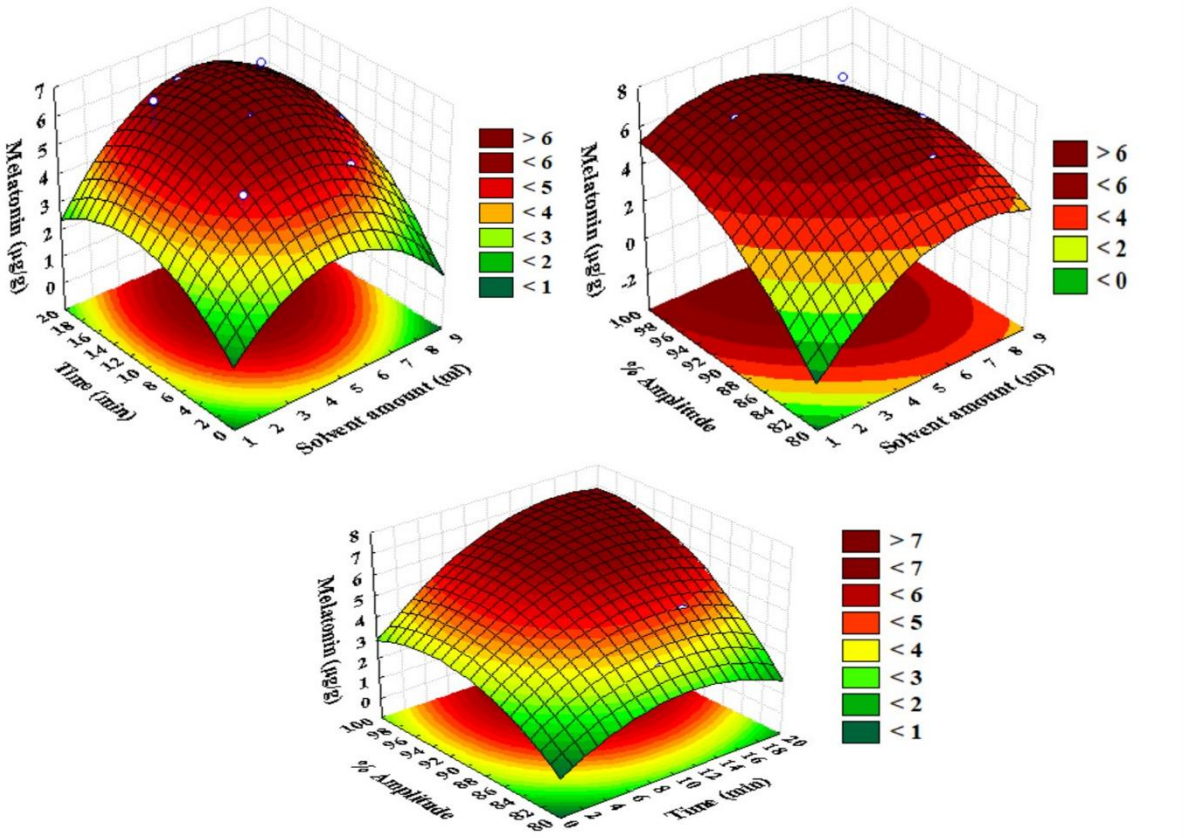

Figure 4.1.2: Response surfaces indicating yields of melatonin from irradiated-cum-completely senesced plantains as a function of ratio of sample weight to solvent volume (ratios of 1:3, 1:5, 1:7 w/v), % amplitude of ultrasonication (85, 90, and 95) and extraction time (5, 10, and 15 min)

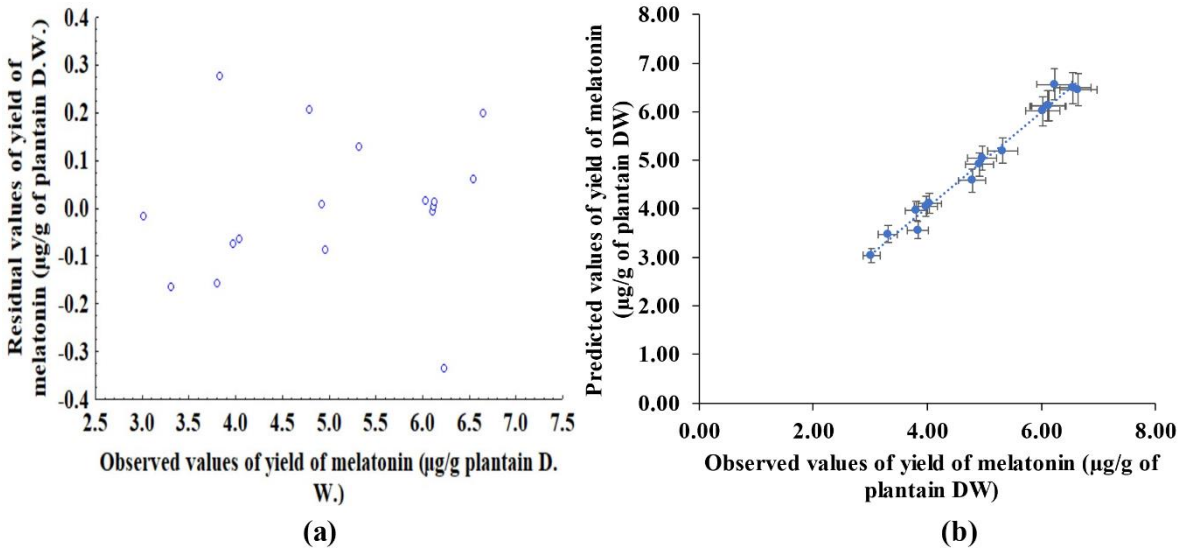

Figure 4.1.3: Plots for a) the residual and observed values of the yield of melatonin and b) the predicted and observed yields of melatonin from the irradiated-cum-completely senesced plantains

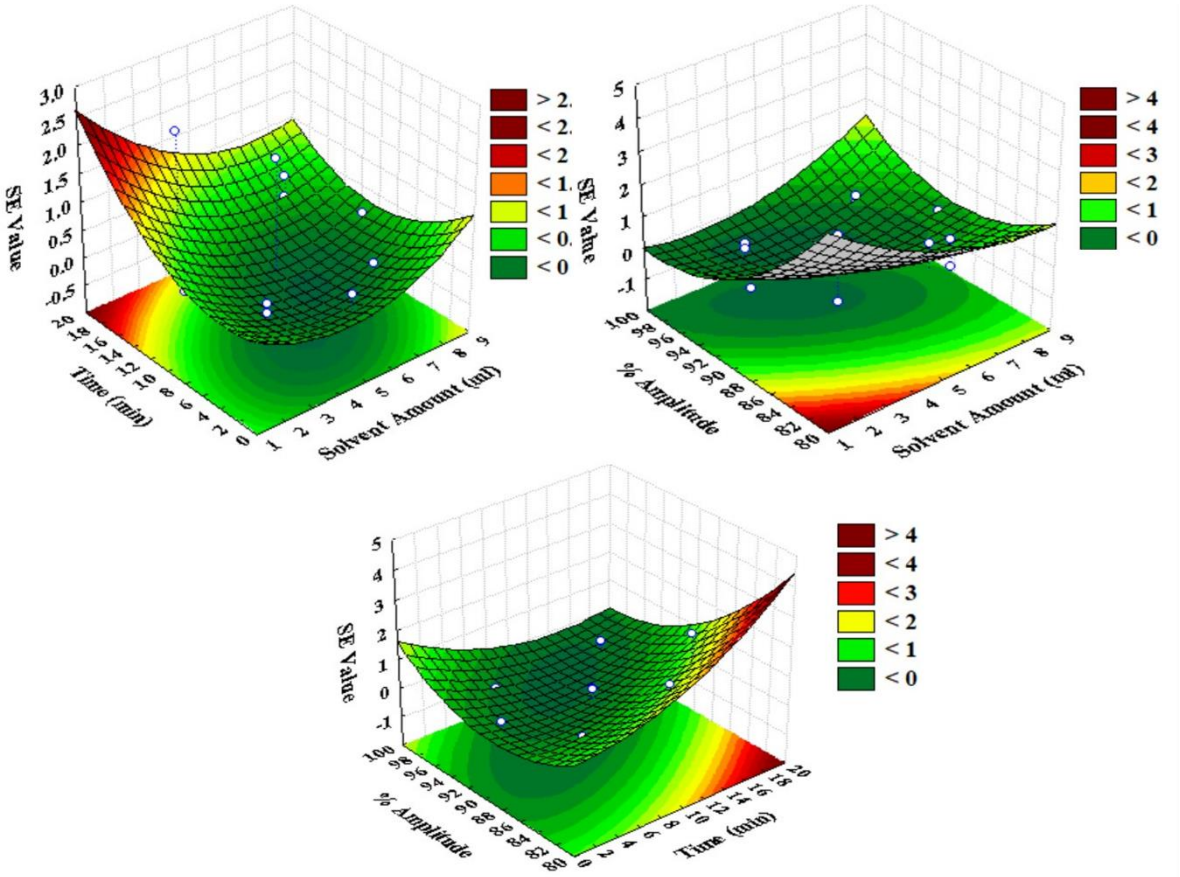

Figure 4.1.4: Response surfaces indicating the effects of ratio of sample weight to solvent volume (ratios of 1:3, 1:5, 1:7 w/v), % amplitude of ultrasonication (85, 90, and 95) and extraction time (5, 10, and 15 min) on the SE value of the UAE extracts of the irradiated-cum-completely senesced plantains

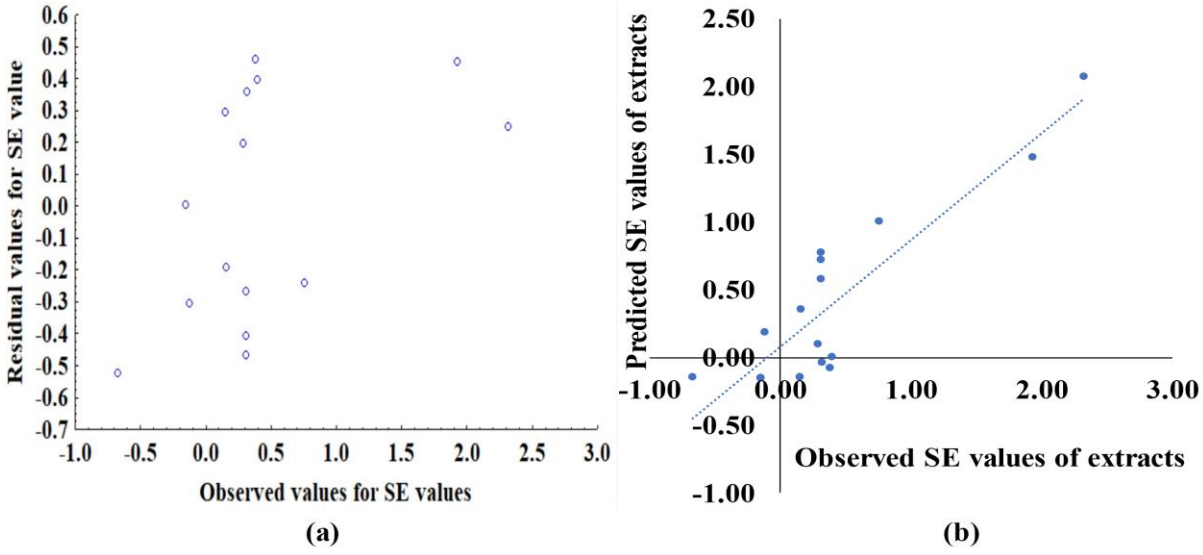

Figure 4.1.5: Plots for a) the residual and observed values for the SE values and b) the predicted and observed SE values from the irradiated-cum-completely senesced plantains

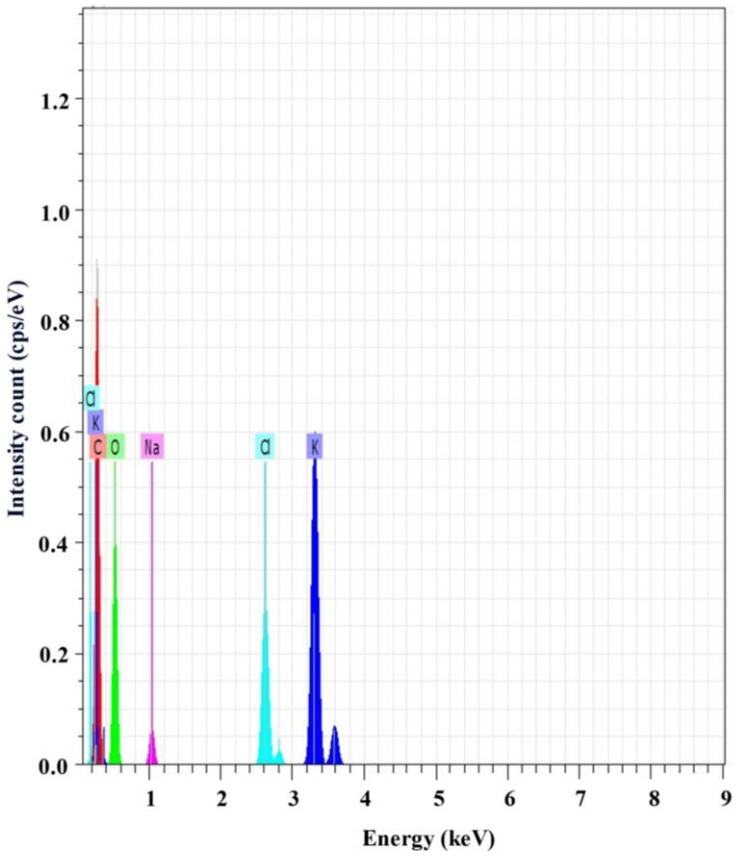

Figure 4.1.6: Energy dispersive X-ray spectra of ultrasonication-assisted solvent extract of the irradiated-cum-completely senesced plantains under the optimized extraction condition

Part 4.2

Development of a food supplement from the antioxidant-rich plantain extract and its characterization

Introduction

The first objective of this part of the study was to formulate a powder from the previously obtained antioxidant-rich UAE extract U_{Best} (having synergy among L-tryptophan-serotonin-melatonin) and perform its characterization. The second objective was to develop a deliverable form of the powder, *i.e.*, a food supplement and evaluate its physicochemical properties, including *in vitro* release kinetics of the three target antioxidant molecules from it.

Materials and methods

Materials

The UAE extract (U_{Best}) obtained in the preceding part (part 4.1 chapter 4) was used as the raw material for this part of the study. The chemicals used for the development and characterization of the powder, and that for the *in vitro* release kinetics study are similar to those described in part 2.1 of chapter 2.

Methods

Development of powder using U_{Best}

To deliver U_{Best} in a convenient dried form as a powder, it was subjected to freeze-drying or lyophilization. Spray drying technology was avoided in this study to eliminate heat exposure during processing (as discussed in the introduction chapter 1). However, the addition of an excipient, especially a bulking agent, was necessary before freeze-drying to prevent product

"blowout" (Sadikoglu *et al*., 2006, Nongkhlaw *et al*., 2020), since the concentration of the active biomolecules were very low in U_{Best} (part 4.1 in chapter 4). Minimal usage of excipients was targeted to aid in easy dissolution.

Maltodextrin, one of the most popular bulking agents in food industries was not used in this study for its high glycemic index. As a substitute, D-mannitol was chosen for its low calorific value (2 cal/g) (Dwivedi, 1991), negligible glycemic index (2) (Chéron *et al*., 2019) and low hygroscopicity. It was used at 10% concentration of the extract to achieve both better tabletability and considerable release of the biomolecules (Kaialy *et al*., 2016).

After purification of U_{Best} by SPE (discussed in part 4.1 in chapter 4), the collected extract was subjected to rotary vacuum evaporator (PBU-6D, M/s Superfit Continental Private Ltd., Mumbai, India) at 50 mbar, 50±2 ℃ (water bath temperature) for 10-15 min to eliminate the ethanol present in it. Thereafter, 10% D-mannitol was mixed with the extract, frozen in an ultra-low temperature freezer (Premium C340, M/s New Brunswick Scientific, Connecticut, USA) at −80 °C for 24 h and lyophilized using a bench top freeze dryer (FDU-1200, M/s Eyela, Tokyo, Japan) at −45 °C, 14.4 Pa, to a final moisture content below 6%. The final freeze-dried powder was subsequently packaged in an aluminium foil and thereafter placed inside Ziploc pouches, flushed with nitrogen, and stored at 23±2 °C in a desiccator and kept in the dark until further analyses.

Characterisation of the powder in terms of its physicochemical properties

The percent yield of the lyophilized powder from U_{Best} was determined gravimetrically. For characterisation of the powder, indicators such as Carr index (CI), Hausner ratio (HR), bulk density (BD), and tapped density (TD) were determined according to the methods described by Barman *et al*. (2014).

The remaining physicochemical characterisations, such as moisture content; CIE colour value; EDX analysis for evaluating the presence of important '*in-house*' micronutrients of plantains (such as Na, K, Fe, Mg, Cl, Zn, Cu) in the powder; FE-SEM analysis for elucidation of surface morphology, shape, size, and crystallinity; and XRD analysis to evaluate whether the powder was crystalline or amorphous were performed according to the methods described previously in part 2.1 in chapter 2.

Assessment of thermal stability is necessary to determine the decomposition or changes in the powder with changes in temperature during storage. For this, TGA was performed according to previously described method in part 2.1 in chapter 2. To determine the amount of energy the powder absorbs or releases during heating or cooling, DSC analysis was performed. After equilibrating at a temperature of -29 ℃, the powder was heated to 258 ℃ at a rate of 5 ℃/min and held at that temperature for 1 min, followed by cooling at a rate of 5 ℃/min to room temperature (27 ℃) employing a DSC Q2000 (M/s TA Instruments, New Castle, USA). The temperatures for heating and cooling were determined based on the TGA thermogram.

Evaluation of antioxidant activity, contents of melatonin, serotonin, and L-tryptophan, and their antioxidant synergism in the powder

The evaluation of antioxidant activity in terms of TPC, DPPH radical scavenging activity, and FRAP value were conducted in accordance with the previously described methods (part 2.1 in chapter 2).

For HPLC-PDA analysis, 1g of freeze-dried powder was dissolved in 5 mL of distilled water, agitated using a vortex (iSwix VT, M/s Neuation Technologies Pvt. Ltd, Gujarat, India) at 500 rpm for 5 min and the resulting mixture was then filtered through a double syringe filtration system (0.45 and 0.22 μm). Thereafter, the mixture was subjected to HPLC-PDA analysis to

quantify the target biomolecules *i.e.,* melatonin, serotonin, and L-tryptophan and subsequently, the antioxidant synergy among them was also evaluated in accordance with the methods described in part 2.1 in chapter 2.

Development of a food supplement from the powder

The powder was subjected to compression using an automatic tablet press machine (Rimek Mini Press I, M/s Karnavati Engineering Pvt. Ltd., Gujarat, India) fitted with 7 mm diameter flat–faced punches to produce round tablets with a target weight of (250±0.5) mg. Before compression, the compaction surfaces were lubricated with 1% (w/v) magnesium stearate in acetone to reduce the friction between the die and the product, and to prevent the produced tablets from sticking to the die walls and punch faces. The developed food supplement in the form of tablets were subsequently packaged in aluminium foils, placed inside Ziploc pouches, flushed with nitrogen, and stored at 23±2 °C in a desiccator in the dark, until further analyses.

Physiochemical properties of the food supplement

The weight and dimensions of the developed food supplement (in form of tablet) was measured using weighing balance (M/s Sartorius AG, Göttingen, Germany) and vernier callipers (BSA 224S-CW, M/s Mitutoyo, Kawasaki, Japan), respectively. Thereafter, the food supplement was subjected to TPA analysis using a P/36 probe in accordance with the method described in part 2.1 in chapter 2. The antioxidant properties of the same in terms of L-tryptophan, serotonin, and melatonin contents, were evaluated by HPLC-PDA analyses according to the method elaborately discussed in part 2.1 in chapter 2.

Assessment of the stability of the food supplement

The storage stability of the food supplement was determined by $T_{1/2}$ analysis (with respect to the melatonin, serotonin, and L-tryptophan content) at an ambient storage temperature of 25±2

°C (Pal and Bhattacharjee, 2019) and when stored at 4 °C in the dark (Pal and Bhattacharjee, 2019). The accelerated stability study for the food supplement was performed at 60±2 °C, 75% RH for 6 h in accordance with the methods reported by Pal and Bhattacharjee (2019) and Aman *et al*. (2005). The storage temperature of 60±2 °C was selected owing to the heat-sensitivity of melatonin and serotonin (Pranil *et al*., 2020). Contents of the antioxidant-triad were determined by HPLC-PDA analysis at intervals 10 days for samples stored at 25 °C and 4 ºC and at interval of 2 h for accelerated storage study. The ratio of the biomolecules on day 0 (TA_0) and on day (t) (TA_t) were estimated and the natural logarithm of TA_0 /TA_t were plotted against storage time (t). The slope of the line (k) was used to obtain the half-life ($T_{1/2}$ = ln 2/k) of the respective biomolecules in the samples.

Studies on the *in vitro* release kinetics of L-tryptophan, serotonin, and melatonin from the developed food supplement

The *in vitro* study on release kinetics was performed using a magnetic stirrer. For this study, 1 food supplement weighing 250 mg was added to 50 mL of the respective buffers (SGB, SIB, and SRB) and was subjected to continuous stirring under controlled heat for 2 h. Since the food supplement is in the form of a tablet that must be swallowed, the dissolution study with SSB was not performed. The buffers were withdrawn at regular time intervals and subjected to HPLC-PDA analysis to determine the content of the antioxidant-triad. The methodology has been elaborately described in part 2.2 in chapter 2.

Statistical analysis

All physicochemical analyses of powder and food supplement (except the analyses involving high-end instruments such as EDX, FE-SEM, TGA, DSC, and XRD) were conducted in triplicate, and the results are reported as the mean ±SD of three values obtained from three

independent sets of experiments. IBM SPSS Statistics Software Version 26 (M/s IBM, New York, USA) was used. Duncan's test and one-way ANOVA. A *p*-value of <0.05 was considered significant.

Results and Discussion

Antioxidant-rich lyophilized powder from the optimized UAE extract of plantains

The lyophilized powder obtained from U_{Best} (Fig. 4.2.1a) was white in colour, odourless, fluffy, and moderately sweet in taste. The detailed characteristics were discussed below.

Physicochemical characteristics of the powder

The yield of the lyophilized powder of U_{Best} was 17.53±1.23% (D.W.). The EDX spectrum of the powder confirmed the presence of micronutrients such as Na, K, Mg, Cl, Fe, Cu, and Zn in it (Fig. 4.2.1b). The moisture content of the powder was 3.64±0.25% D.W., indicating the effectiveness of freeze-drying. The CIE colour values of the powder were L*: 91.8; a*: -0.2, b*: 11.2, c*: 11.2, and h: 91.2 which were in consonance with the report of calibration with a white plate (L*: 94.90, a*: –0.39, b*: 3.88) by Choi *et al*. (2020). The results further validated the white colour of the powder with very light tint of yellowness, which was otherwise not detectable with bare eyes (Fig. 4.2.1a). The BD of the lyophilized powder was 0.02±0.01 g/mL, while its TD was 0.07 ±0.02 g/mL. From these values of BD and TD, CI and HR were calculated, which were 71.43±6.05 and 3.5±0.25, respectively, classifying as extremely poor flowability (Sonawane *et al*., 2021) of the powder. These findings were in consonance with the study by Kaialy *et al*. (2016) who reported similar extremely poor flowability of lyophilized powder of D-mannitol. FE-SEM images of the powder (Fig. 4.2.1c) revealed a porous, needle shaped crystalline morphology, which was also in agreement with the FE-SEM images of recrystallised mannitol reported by Kaialy *et al*. (2016) and Cares-Pacheco *et al*. (2014).

Additionally, the XRD graph also confirmed prominent crystalline structure (Fig. 4.2.1d) with 73.5% crystallinity of the powder which is in consonance with the XRD graphs of various physical forms of D-mannitol reported by Cares-Pacheco *et al*. (2014) and Thakral *et al*. (2023). Despite having extremely poor flowability, this kind of fluffy, porous powder is known as an apposite material for food supplement (in the form of a tablet) formation due to the increase in the powder bed porosity, allowing particle–particle interactions to a great extent during the subsequent stage of compression, resulting in higher tabletability (Kaialy *et al*., 2016).

Fig. 4.2.1e presents the TGA thermograph of the powder, revealing its stability below 250 °C, and indicating a broad temperature range of stability. Degradation of the powder initiates at approximately 250 °C, with rapid weight loss beyond 300 °C. This contrasts with findings reported by Kumaresan *et al*. (2011) and Mojiri *et al*. (2018) who identified decomposition initiation temperatures for pure D-mannitol at 300 °C and 207 °C, respectively. Fig. 4.2.1f displays the DSC thermograph, indicating a melting point onset at 160 °C and solidification onset at 120 °C. These results corroborated well with the study by Mojiri *et al*. (2018), where the temperature(s) for melting and solidification were reported as 168 °C and 110-120 °C, respectively for pure D-mannitol. Thus, the powder developed from UAE extract of plantains is confirmed to be a heat-stable formulation.

Antioxidant properties of the freeze-dried powder

The TPC value of the powder (63.66±9.05 mg of GAE/g of powder in D.W.), IC_{50} value for DPPH radical scavenging activity (17.12±2.04 mg/mL) and FRAP assay result (267.58±43.65 mmol $FeSO_4$/g of powder in D.W.) confirmed its significantly ($p<0.05$) higher antioxidant activity compared to the UAE extract. This increase in antioxidant potency aligns with the findings of Dal-Bó and Freire (2022) for lyophilized avocado pulp powder compared to fresh

avocado pulp. The melatonin, serotonin, and L-tryptophan contents were found to be 43.07 µg/g, 40.59 µg/g, and 32.98 µg/g of powder D.W., respectively, significantly ($p<0.05$) higher than those in the UAE extract thus obtained under optimized conditions. This augmentation in concentration of the bioactive antioxidant molecules in the lyophilized powder, compared to the extract, is consistent with the results reported by Pal and Bhattacharjee (2018), where lutein content in a lyophilized food supplement from supercritical carbon dioxide extract of yellow corn exceeded that in the extract itself. The presence of melatonin, serotonin, and L-tryptophan in the lyophilized powder also exhibited antioxidant synergism (SE>1) among themselves, affirming that the formulated powder to be a truly antioxidant-rich product.

The food supplement: Physicochemical properties

The weight of the developed food supplement (Fig. 4.2.2a) was 250±4 mg and the dimensions were as follows: diameter: 7.05±0.05 mm and thickness: 3.05±0.06 mm. The TPA analysis of the food supplement assessed its hardness(g): 2763.175±158.35, cohesiveness: 0.934±0.06, springiness: 0.214±0.01, gumminess: 2580.097±190.34, chewiness: 552.878±49.11, and resilience: 1.479±0.08. The contents of melatonin, L-tryptophan, and serotonin present in the 250 mg of the tablet-formed supplement as ascertained by HPLC-PDA analysis were 10.80 µg, 8.26 µg, and 10.18 µg, respectively.

Stability of the food supplement during storage

The HPLC analysis revealed that the half-life ($T_{1/2}$) values of the food supplement with respect to melatonin were 236 days at 4 °C and 41 days at ambient conditions (23±2 °C, 80% R.H.) in dark; with respect to serotonin, 311 days at 4 °C and 203 days at ambient conditions in dark and with respect to L-tryptophan, 460 days at 4 °C and 340 days at ambient conditions in the dark. At a higher temperature of 60 °C, the $T_{1/2}$ values for the food supplement with respect to

melatonin, serotonin, and L-tryptophan were 2.05h, 5.69h, and 9.2h, respectively. These findings confirm better shelf-stability of the food supplement when stored at 4 °C in the dark compared to the ambient conditions (23±2 °C, 80% R.H.). These results were in perfect consonance with the findings of Cavallo and Hassan (1995) who reported that the aqueous solution of melatonin was stable up to 6 months when stored at 4 °C or at -70 °C in sterile, pyrogen-free vials. In contrast, Pranil *et al*. (2020), indicated a $T_{1/2}$ value of approximately 14 days for melatonin in fruit juices at room temperature, although the accelerated temperature stability exceeded 12 hours in their study at 60 °C.

***In vitro* release kinetics study of L-tryptophan, serotonin, and melatonin from the food supplement**

In the preliminary trials, the buffer aliquots from SGB, SIB, and SRB were withdrawn at regular time intervals of 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120 min. Analyses of L-tryptophan, serotonin, and melatonin contents in the buffers revealed that very low amounts of the said molecules were released (Fig. 4.2.2b- 4.2.2d) beyond 50 min, 40 min, and 60 min from the food supplement in SGB, SIB, and SRB, respectively. Therefore, in the final experiment, the aliquots were withdrawn until 50 min, 40 min, and 60 min from SGB, SIB, and SRB, at an interval of 10 min.

Fig. 4.2.3a presents the percentage releases of the three target biomolecules from the developed food supplement in SGB. The amounts of L-tryptophan, serotonin, and melatonin released in 50 mL of SGB immediately after 10 min of dissolution were 1.21 µg, 1.28 µg, and 5.87 µg, respectively; whereas, after 50 min, the amounts of the same were 8.08 µg, 2.48 µg, and 9.05 µg, respectively, which corresponded to 97.78%, 24.33%, and 83.78%, respectively, of their original content(s) present in the food supplement.

Fig. 4.2.3b represents the percentage release of the target biomolecules in SIB. The amounts of L-tryptophan, serotonin, and melatonin released in 50 mL SIB were 1.94, 1.49, and 0.66 µg after 10 min of dissolution; and after 40 min, they were 4.97, 4.44, and 7.39 µg, respectively, attesting to appreciable releases of L-tryptophan (60.21%), and serotonin (43.63%), and melatonin (68.45%). Fig. 4.2.3c represents the percentage release of the target biomolecules in SRB. The amounts of L-tryptophan, serotonin, and melatonin released in 50 mL SRB were 4.60, 3.09, and 3.30 µg, respectively, after 10 min of dissolution; and after 60 min were 7.47, 9.68, and 10.55 µg, respectively, corresponding to 90.41% releases of L-tryptophan, 95.10% of serotonin, and 97.71% of melatonin. For model fitting, the end time when the release of the molecule(s) reached its (their) peak(s) (50 min for SGB, 40 min for SIB, and 60 min for SRB) coincided with the time beyond which the amounts of the biomolecules were found to significantly decrease, possibly owing to instabilities of the molecules on prolonged dissolution in the buffers.

Post-fitting of the cumulative percentage release kinetics data into different kinetics models (described in detail in part 2.2 in chapter 2), it was found that the antioxidant triad, *i.e.*, L-tryptophan, serotonin, and melatonin in SGB (R^2 = 0.99, 0.99, 0.99, respectively) and SIB (R^2 = 0.99, 0.96, 0.96, respectively) followed Korsmeyer-Peppas model of release kinetics indicating a combination of diffusion as well as erosion mechanism.

In SRB, serotonin (R^2 = 0.99) followed Korsmeyer-Peppas model; whereas, the rest of the molecules, *i.e.*, L-tryptophan (R^2 = 0.91) and melatonin (R^2 = 0.99) followed Higuchi model of release kinetics indicating the release of biomolecules as a square root of a time-dependent process based on Fickian diffusion.

These findings corroborated well with that of Fathi *et al*. (2022) who reported the release of punicalagin (obtained from pomegranate peel) from biopolymer based microparticles to the

release medium (deionized water) followed Korsmeyer Peppas model of release kinetics. Another study by Singh *et al.* (2018) documented Higuchi model of release kinetics at pH 7 of iron from ferrous sulphate when microencapsulated with two reverse-enteric coating materials, namely chitosan and Eudragit EPO. This outcome is in sync with the findings of the present study where the release of L-tryptophan and melatonin in SRB also adhered to the Higuchi model.

Conclusion

The findings of the present study reported on the development of an antioxidant-rich food supplement in the form of a tablet from a lyophilized powder obtained from the UAE extract of an antioxidant-triad - L-tryptophan, serotonin, and melatonin. Furthermore, this study also confirmed the *in vitro* release mechanism of the three above-mentioned molecules in simulated gastric, intestinal, and rectal buffers, post-consumption. Therefore, these findings strongly established the newly designed food supplement as truly antioxidant-rich which could be particularly beneficial for serotonin-melatonin-compromised people.

Novelty

This work reports for the first time on the formulation of a food supplement in the form of a tablet containing lyophilized powder of an UAE extract of L-tryptophan, serotonin, and melatonin from irradiated-cum-completely senesced plantains. This study has delivered a unique food supplement obtained from otherwise wasted plantains.

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Figures:

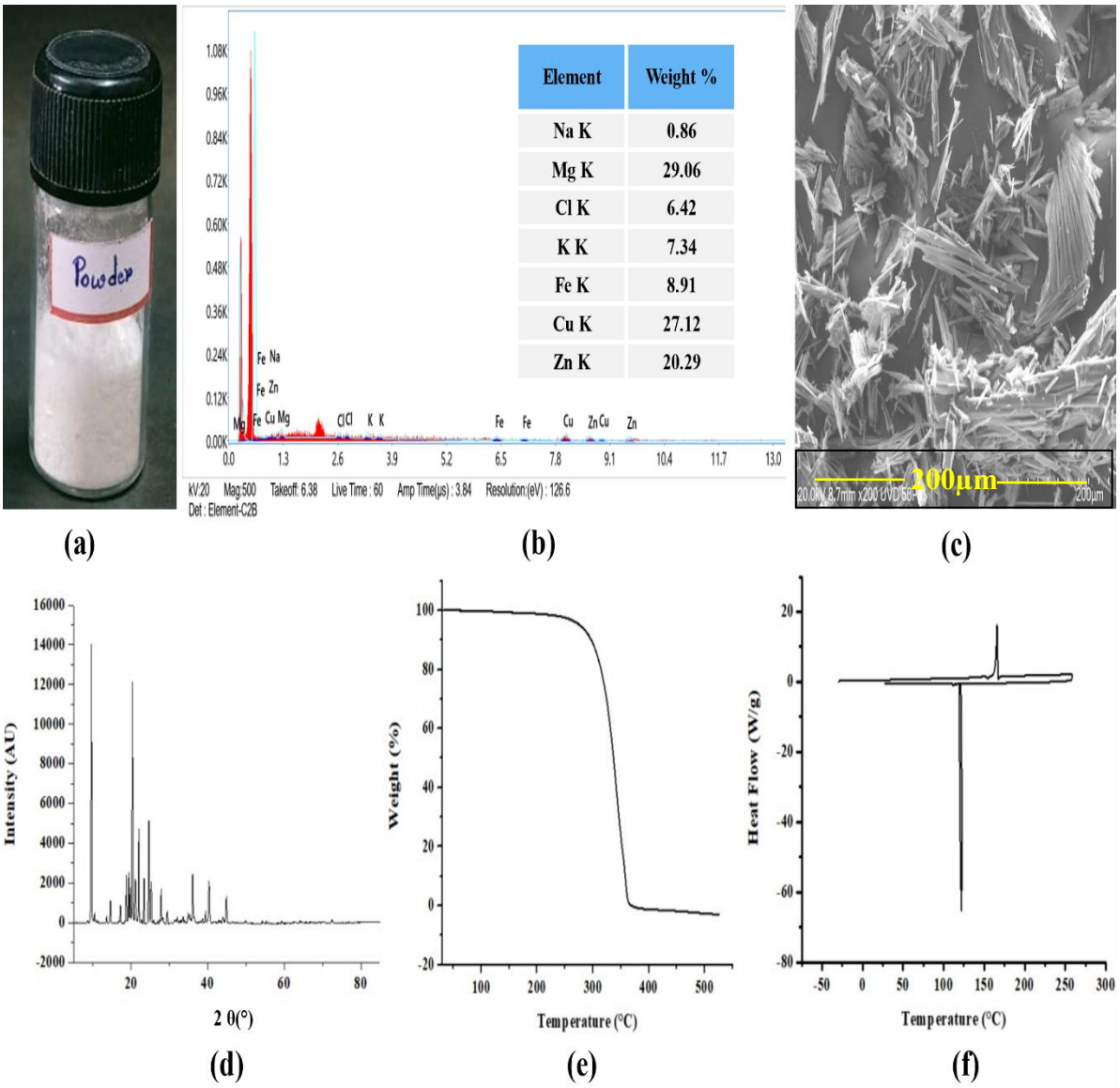


Figure 4.2.1: Physicochemical characteristics of the lyophilized powder- a) image of the powder, b) energy dispersive X-ray spectra, c) field emission scanning electron microscopy image, d) X-ray diffraction spectrum, e) thermogravimetric graph, f) differential scanning calorimetry thermograph of the powder

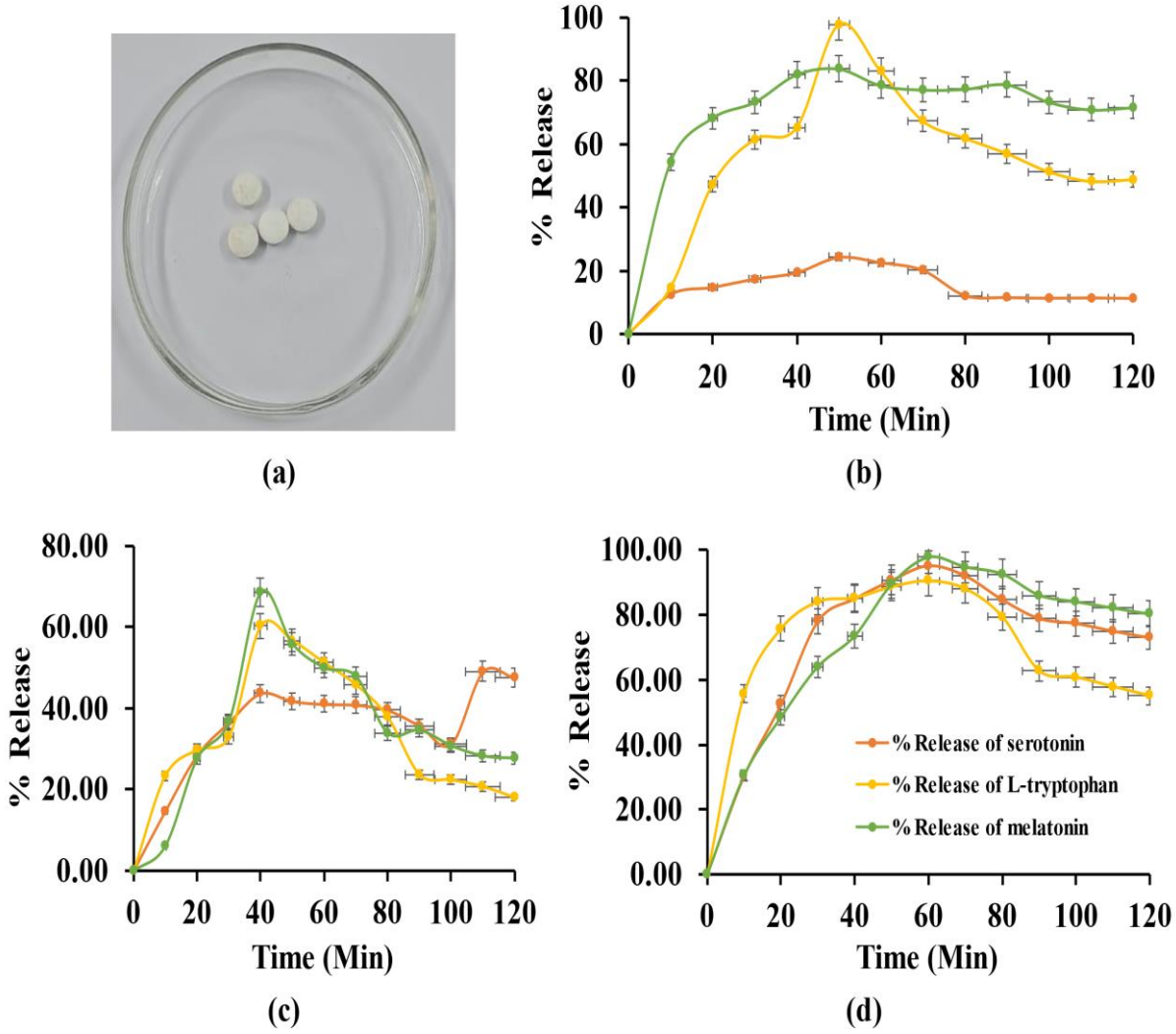


Figure 4.2.2: a) Image of the food supplement; release profiles of L-tryptophan, serotonin, and melatonin from the food supplement during dissolution for 120 min b) in simulated gastric buffer; c) in simulated intestinal buffer, and d) in simulated rectal buffer

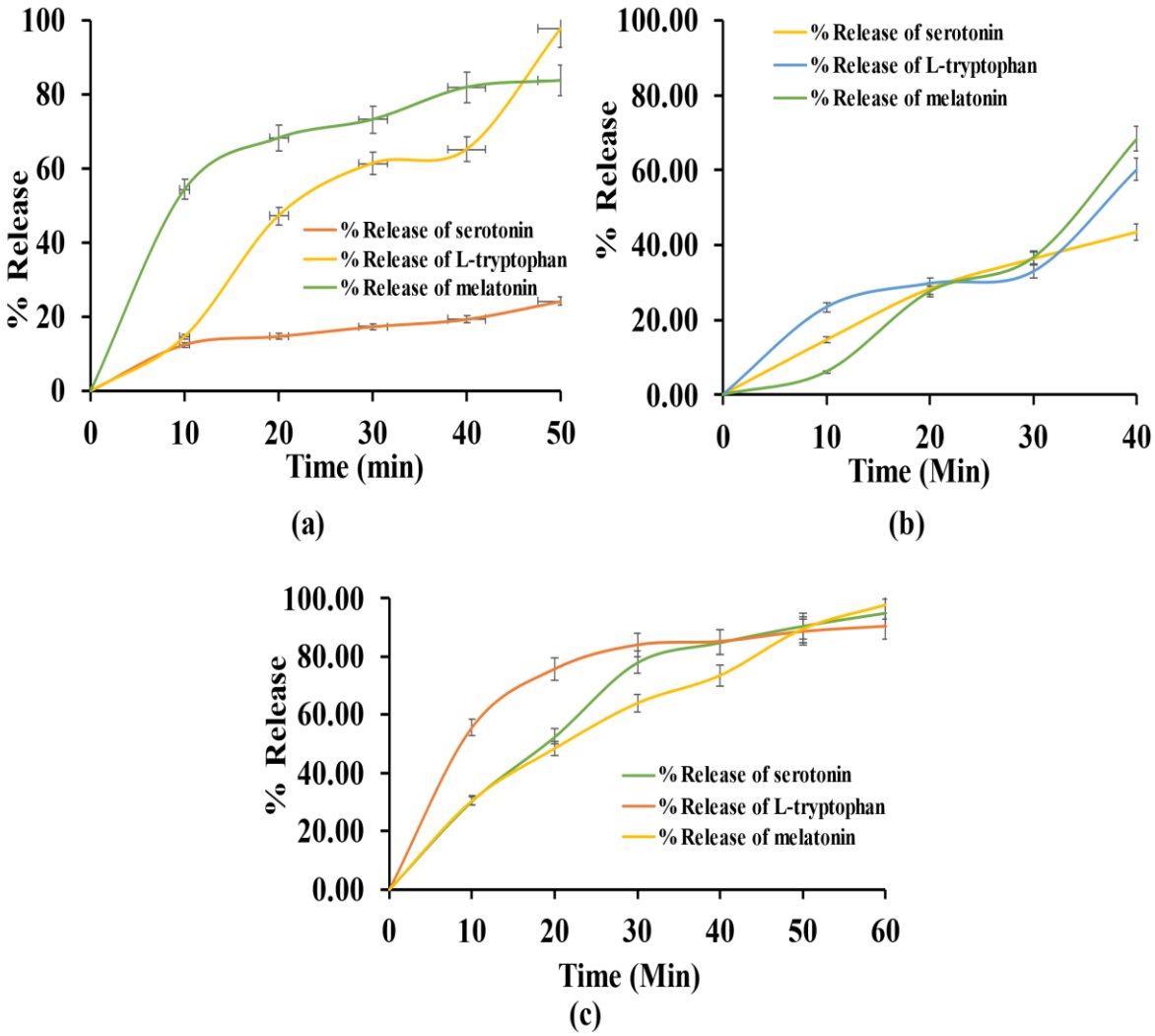
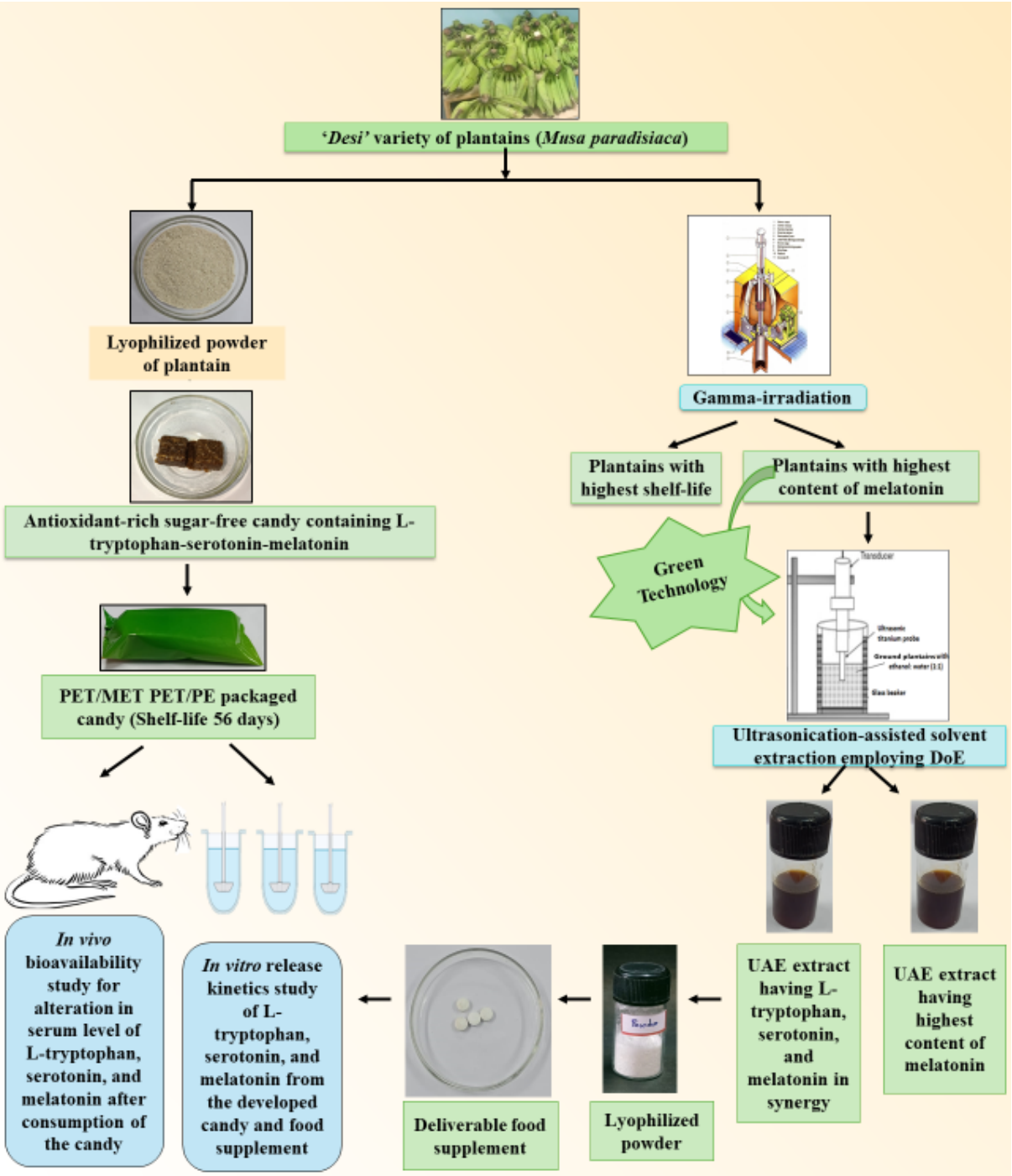


Figure 4.2.3: Release profiles of L-tryptophan, serotonin, and melatonin from the food supplement during dissolution a) in simulated gastric buffer; b) in simulated intestinal buffer, and c) in simulated rectal buffer

Overall Conclusion



This work is of significance since it emphasised on maintaining the naturally occurring synergism among the three target bioactive molecules, *i.e.,* L-tryptophan, serotonin, and melatonin, for the holistic utilization and valorization of plantains with the aim to preserve these three '*in house*' beneficial biotherapeutic molecules by means of three 'green' approaches *i.e.,* design of a value-added product from freshly harvested plantains; extension of shelf-life

of freshly harvested plantains by a green technology of preservation; and development of a consumable food supplement utilizing an extract of the antioxidant triad from sensorially declined, fully senesced-irradiated plantains. The present work delivers five novel food products and supplements along with seven novel process for food processors and quality control personnel.

The five novel food products and supplements delivered from this study include: 1. A semi-hard sugar-free antioxidant rich (especially L-tryptophan-serotonin-melatonin) plantain-based candy, 2. Gamma-irradiated plantains rich in serotonin and melatonin, 3. An UAE extract rich in phytomelatonin, 4. An UAE extract with a synergistic mix of antioxidants, namely L-tryptophan, serotonin, and melatonin, 5. A food supplement rich in L-tryptophan-serotonin-melatonin.

The seven novel processes that the current investigation documented for the first time are: 1. The SOP for development of the semi-hard sugar-free antioxidant rich (especially L-tryptophan-serotonin-melatonin) plantain-based candy, 2. *In vitro* release kinetics study in simulated buffers to evaluate the release and *in vivo* study involving rat model to evaluate the bioavailabilities of L-tryptophan, serotonin, and melatonin, 3. The optimized processing parameters of gamma-irradiation for extension of shelf-life of plantains, 4. The optimized processing parameters of gamma-irradiation for augmentation of the contents of serotonin and melatonin in plantains; 5. The optimized processing parameters for UAE extraction to obtain an extract rich in phytomelatonin from fully ripened, irradiated plantains, 6. The optimized processing parameters for UAE extraction to obtain an extract with a synergistic blend of phytomelatonin-serotonin-L-tryptophan from completely senesced-cum-irradiated plantains, 7. SOP for developing lyophilized powder and a food supplement from the UAE extract of plantains.

Resume

The present investigation focuses on maintaining the naturally occurring synergism among the three target bioactive molecules, *i.e.,* L-tryptophan, serotonin, and melatonin, for the holistic utilization and valorization of '*desi'* variety of plantains (*Musa paradisiaca* L.) with the aim to preserve these three '*in house*' beneficial biotherapeutic molecules by means of three 'green' approaches *i.e.,* design of a value-added product from freshly harvested plantains; extension of shelf-life of freshly harvested plantains by a green technology of preservation; and development of a consumable food supplement utilizing an extract of the antioxidant triad from sensorially declined, fully senesced-irradiated plantains. The SOP for the development of the antioxidant (L-tryptophan, serotonin, and melatonin)-rich sugar-free plantain-based candy was optimized based on the sensory attributes and thereafter, its detailed physicochemical characteristics, followed by the *in vitro* release kinetics (using simulated buffers) and *in vivo* release (using Sprague-Dawley rat model) of the antioxidant-triad were also investigated. Very low and low doses of gamma-irradiation were employed on plantains and their shelf-life was evaluated based on sensory and physiochemical characteristics. Additionally, the content of antioxidant-triad was evaluated during the enstire storage period. The completely senesced-cum-irradiated plantains thereafter was subjected to UAE extraction to obtain an extract with this antioxidant-triad. To develop a consumable food supplement, the extract thus obtained was then subjected to lyophilization followed by its thorough physicochemical characterization and *in vitro* release kinetics study of the three antioxidants.

The present research work comprises of the following:

Development of a nutraceutical antioxidant-rich (specially L-tryptophan-serotonin-melatonin in synergism) food product (candy) from freshly harvested green plantains, its characterization and shelf-life study of the developed candy post-packaging

- Development of a nutraceutical sugar-free plantain-based candy was performed using the lyophilized powder of freshly harvested plantains employing minimal processing techniques.

- The findings of microbial assessment and EDX analysis render the candy safe for human consumption.
- The newly designed candy was sensorially well-accepted owing to its uniform dark brown colour, rich mouthfeel, pleasant aroma, moderately hard texture, and moderate sweetness.
- The FE-SEM image of the candy revealed a grainy, non-homogeneous, and discontinuous (with voids) microstructure, conferred by the complex composition of plantains.
- The XRD graph of the candy showed few nano-crystalline structures on predominantly amorphous bases.
- The DSC thermogram revealed that the melting of the candy occurred over a wide temperature regime of 94-105 ℃ indicating its slow melting property in the mouth.
- The candy showed appreciable antioxidant activity in terms of TPC, DPPH scavenging activity, and FRAP values.
- HPLC-PDA analysis of the QuEChERS-SPE extract of the candy indicated presence of L-tryptophan, serotonin, and melatonin (4.54 µg/g, 1.83 µg/g, and 1.23 µg/g, respectively). The identities of these antioxidants were further confirmed by ESI-TOF-MS analysis.
- SE value of the QuEChERS-SPE extract of the candy revealed that the antioxidants were present as a synergistic (SE >1) consortium in the developed candy.
- The plantain-candy could be safely stored for 56 days without any physicochemical and considerable antioxidant deterioration when packaged in a 3-ply flexible (PET/MET-PET/PE) laminate wrapper.

- The antioxidant synergy was unperturbed on the first day as well as on the end day of the shelf-life of the candy.

In-vitro release kinetics and in-vivo bioavailability studies of L-tryptophan-serotonin-melatonin from the designer candy

- This designed candy showed considerable release of L-tryptophan, serotonin, and melatonin in simulate salivary (53.95%, 33.22%, and 45.12%, respectively, after 10 min), simulated gastric (97.17%, 62.59%, and 95.05%, respectively, after 60 min), intestinal (90.16%, 99.73%, and 90.78%, respectively, after 60 min), and rectal (88.57%, 71.52%, and 95.66%, respectively, after 90 min) buffers mimicking the digestive process *in vitro.*
- In *in vivo* study involving feeding of Sprague Dawley rats, a considerable enhancement in levels of L-tryptophan, serotonin, and melatonin in the rat blood serum was observed after 30 min of consuming the designer candy; and their levels started to decline 60 min onwards.
- Additionally, the effect of the candy consumption on insulin sensitivity as well as glucose uptake were also explored using the non-invasive model of iHOMA2, which predicted an increase in insulin sensitivity in the liver and glucose uptake in the brain post candy consumption.

Optimization of gamma-irradiation processing parameters for extension of shelf-life of 'desi' variety of green plantains based on sensory attributes, proximate, physicochemical, and phytochemical properties

- By employing the non-thermal green technology of gamma-irradiation (0.04 kGy), the shelf-life of freshly harvested plantains can be extended by 8 days (when stored at

23±2 °C, 70±2 % RH), determined by extensive analyses of sensory, physicochemical, and biochemical characteristics. This was achieved without the use of packaging or controlled storage conditions.

Optimization of gamma-irradiation processing parameters to obtain enhanced amount of serotonin and/or melatonin or both in combination (in antioxidant synergy) from gamma-irradiated plantains

- By employing gamma-irradiation at 1 kGy and 0.6 kGy doses, the contents of the bioactive antioxidants *viz.* serotonin and melatonin could be enhanced by 1.44 fold (44.10%) and 2.45 fold (146.58%), respectively, in the irradiated plantains when stored at 23±2 °C, 70±2 % RH.

Optimization of UAE processing parameters for maximizing the yield of bioactive antioxidants, chiefly melatonin, and to achieve a synergistic blend of antioxidants in the extract using fully ripened, irradiated plantains as the raw material

- The extraction technology of UAE has been successfully employed to extract a synergistic consortium of bioactive antioxidants (L-tryptophan, serotonin, and melatonin) from fully ripened, irradiated plantains.
- This endeavour involved judicious choice of the extraction process parameters (and their ranges of operations) and their optimization using statistical tools namely, CCRD-RSM.
- This study delivered for the first time the optimized parameters of UAE extraction for obtaining two extracts: one with the highest yield of phytomelatonin (7.15 µg/g) and another having a synergistic blend of L-tryptophan, serotonin, and phytomelatonin

(1.84 µg/g, 2.45 µg/g, and 3.31 µg/g, respectively), from gamma-irradiated (0.6 kGy), fully senesced plantains.

- HPLC-PDA analysis of the UAE extract indicated presence of L-tryptophan, serotonin, and melatonin. The identities of these antioxidants were further confirmed by ESI-TOF-MS analysis.
- SE value of the UAE extract revealed that a synergistic (SE >1) consortium of antioxidants was present in the extract.
- EDX analysis confirmed the absence of any heavy metal in the extract rendering it safe for human consumption.

Development of a food supplement from the antioxidant-rich plantain extract and its characterization

- The UAE extract having synergism among L-tryptophan, serotonin, and melatonin was utilized to develop a consumable food supplement (containing 32.98 µg/g, 40.59 µg/g, and 43.07 µg/g of L-tryptophan, serotonin, and melatonin, respectively).
- By employing the non-thermal technology of lyophilization, a powder was developed from the UAE extract and the powder was subsequently used for development of a food supplement.
- The lyophilized powder thus obtained was white in colour, odourless, fluffy, moderately sweet in taste, and had poor flowability.
- The EDX spectrum of the powder confirmed the presence of micronutrients such as Na, K, Mg, Cl, Fe, Cu, and Zn in it.

- The XRD graph of the powder confirmed prominent crystalline structure with 73.5% crystallinity.
- The DSC thermograph of the powder indicated a melting point onset at 160 °C and solidification onset at 120 °C.
- The powder showed appreciable antioxidant activity in terms of TPC, DPPH scavenging activity, FRAP values and also exhibited antioxidant synergism (SE>1) among L-tryptophan, serotonin, and melatonin
- This food supplement showed considerable release of L-tryptophan, serotonin, and melatonin in gut simulated gastric (97.78%, 24.33%, and 83.78%, respectively, after 50 min), intestinal (60.21%, 43.63%, 68.45%, respectively, after 40 min), and rectal (90.41%, 95.10%, and 97.71%, respectively, after 60 min) buffers *in vitro.*

Suggestions for Future Work

- The antioxidant-rich, sugar-free candy derived from plantains is now prepared for human nutrition studies. This will allow for the clinical testing of increases in serum antioxidant levels and the evaluation of its glycemic index.
- The standardized operating procedures (SOPs) developed for the candy production and the ultrasonic-assisted extraction (UAE) of a synergistic antioxidant mixture are ready to be shared with industry partners for manufacturing, which will require scaling up these processes from lab to industrial scale.
- Transcriptomic analysis could be conducted on plantains that have been exposed to gamma irradiation at very low (0.04 kGy) and low (0.6 and 1 kGy) doses. This analysis would aim to uncover the genetic changes that enhance the levels of L-tryptophan, serotonin, and melatonin.

Appendices

List of Abbreviations

%S :	Insulin sensitivity in percentage
%β :	β-cell function in percentage
ADHD :	Aattention deficit hyperactivity disorder
ADI :	Acceptable daily intake
AED :	Acoustic energy density
ANOVA :	One-way analysis of variance
AOAC :	Association of Analytical Communities
APX :	Ascorbic peroxidase
AR :	Analytical reagents
a_w :	Water activity
BD :	Bulk density
BHA :	Butylated hydroxyanisole
BHT :	Butylated hydroxytoluene
BRIT :	Board of Radiation and Isotope Technology
CCRD :	Central composite rotatable design
CCS :	Control candy sample
CFU :	Colony-forming units
CH :	Control group for iHOMA2
CI :	Carr index
CIE :	Commission Internationale de l'Éclairage
DAE :	Department of Atomic Energy
DMSO :	Dimethyl sulfoxide
DoE :	Design of Experiment
DPPH :	2,2-diphenylpicrylhydrazyl
DSC :	Differential scanning calorimetry

dSPE :	dispersive-Solid phase extraction
D.W. :	Dry weight
EDTA :	Ethylene diamine tetraacetic acid
EDX :	Energy dispersive X-ray
ELISA :	Enzyme-linked immunoassay
ESC :	Experimental scavenging capacity
ESI-TOF-MS :	Electrospray ionization-time-of-flight-mass spectrometer
FE-SEM :	Field emission scanning electron microscopy
FRAP :	Ferric reducing antioxidant power
FSSAI :	Food Safety and Standards Authority of India
GAE :	Gallic acid equivalent
GI :	Gastrointestinal
GRAS :	Generally regarded as safe
HR :	Hausner ratio
IAEC :	Institutional Animal Ethical Committee
IC_{50} :	Concentration that required for 50% inhibition in vitro
iHOMA :	Homeostatic model assessment
HPLC :	High performance liquid chromatography
IACUC :	Institutional Animal Care & Use Committee
IAEC :	Institutional Animal Ethical Committee
LOD :	Limits of detection
LOQ :	Limits of quantification
M :	Molecular mass
MET BOPP :	Metallized biaxially orientated polypropylene
MET PET :	Metalized polyethylene terephthalate
NHS BOPP :	Non-heat sealable biaxially oriented polypropylene
NIL :	National Instruments Limited

NOAEL :	No observed adverse effect level
OTC :	Over-the-counter
OTR :	Oxygen transmission rate
PBS :	Phosphate saline buffer
PCS :	Plantain-based candy sample
PDA :	Photodiode array
PE :	Polyethylene
PET :	Polyethylene terephthalate
PPO :	Polyphenol oxidase
QuEChERS :	Quick, Easy, Cheap, Effective, Rugged and Safe
RH :	Relative humidity
RSM :	Response surface methodology
SD :	Standard deviation
SE :	Synergistic effect
SGB :	Simulated gastric buffer
SIB :	Simulated intestinal buffer
SOP :	Standard operating procedure
SPE :	Solid phase extraction
SRB :	Simulated rectal buffer
SSB :	Simulated salivary buffer
TBA :	Thiobarbituric acid
TCA :	Trichloroacetic acid
TD :	Tapped density
T_g :	Glass transition temperature
TGA :	Thermogravimetric analysis
TH :	Test group for iHOMA2
TPA :	Texture profile analysis

TPC :	Total phenolic content
TPTZ :	2,4,6-Tripyridyl-S-triazine
TSC :	Theoretical scavenging capacity
TSM :	L-tryptophan-serotonin-melatonin
TTA :	Total titratable acidity
UAE :	Ultrasonication assisted solvent extraction
UI :	Ultrasonic intensity
WVTR :	Water vapor transmission rate
XRD :	X-Ray diffraction

Glossary

Ad Libitum :	Free choice.
ELISA :	Enzyme-linked immunosorbent assay. It is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies, and hormones.
iHOMA2 :	iHOMA2 is a Homeostatic Model of Assessment which can be used to evaluate therapeutic agents and predict effects on fasting glucose and insulin and on beta cell function and insulin sensitivity.
Vide infra :	See below/further on
Vide supra :	See above/earlier

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ORIGINAL ARTICLE

Low dose gamma-irradiation enhances shelf-life and contents of serotonin and melatonin in green plantains (*Musa paradisiaca*): A study involving antioxidant synergy

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Abstract

Green plantains (*Musa paradisiaca*) were gamma-irradiated in the dose range of 0.04–1 kGy and stored under ambient condition (23 ± 2°C, 70 ± 2% RH) in an environmental chamber. The objectives of the present study were to extend the shelf-life of plantains and to enhance its contents of bioactive antioxidant molecules (namely serotonin and melatonin) by gamma-irradiation. Several physicochemical and phytochemical assays conducted with the irradiated samples (and non-irradiated control set) established 0.04 kGy-irradiated plantains with a lead of 8 days in shelf-life to be the "best shelf-stable". The plantains when irradiated at 0.6 and 1 kGy had significantly enhanced contents of melatonin (2.45 folds [146.58%]) and serotonin (1.44 folds [44.10%]), respectively. Moreover, in the plantains having highest serotonin (1 kGy-irradiated on day 10), synergism among the said antioxidants was also preserved. This is the first report on utilization of gamma-irradiation for enhanced production of phyto-antioxidants in green plantains.

Novelty impact statement: Gamma-irradiation is a well-known technology of food preservation; however, very few studies have reported on its effectiveness in enhancement of contents of phytoremediator therapeutic molecules present in agricultural produces. This study aims to employ gamma-irradiation not only for extension of shelf-life of an agro produce but also aims in exploring its usage for enhancement of contents of bioactive antioxidant molecules of immense therapeutic importance (serotonin and melatonin) in the produce thereby conferring the irradiated agro-produce to be a potential source of food antioxidants. This study also provides a possible means of valorization of green plantains, post senescence through utilization of completely senesced plantains as potential sources of these biotherapeutic molecules which have promising uses as nutraceutical food-cum-therapeutic supplements. Thus, induction of overproduction of phyto-antioxidants leading to utilization of plantain waste is the novelty of this work.

Abbreviations: APX, ascorbic peroxidase; D.W, dry weight; DPPH, 1-diphenyl-2-picrylhydrazyl; DPPH-RSA, DPPH radical scavenging activities; dSPE, dispersive-Solid phase extraction; EDTA, ethylene diamine tetraacetic acid; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalent; GRAS, generally regarded as safe; HPLC, high performance liquid chromatography; NIL, national Instruments Limited; PPO, polyphenol oxidase; QuEChERS, quick, easy, cheap, effective, rugged, and safe; SE, synergism effect; SPE, solid phase extraction; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TPA, texture profile analysis; TPC, total phenolic content; TTA, total titratable acidity; γ-irradiation, gamma-irradiation.

1 | INTRODUCTION

The local or *desi* variety of Indian green plantains (*Musa paradisiaca L.)* of the family *Musaceae* is extensively cultivated in India including West Bengal. For being low-priced and rich in phytochemicals and carbohydrates, it has been explored since ages for its numerous health benefits and has been extensively utilized in preparation of medicines, beverages, flavorings, foods, fermentable sugars, and in cooked preparations (Nelson et al., 2006; Phillip et al., 2009). However, plantains have a limited shelf-life of 3–5 days (Baez-Sañudo et al., 2009). Not only India, other Asian, Latin American and African countries such as China, Philippines, Ecuador, Brazil, Costa Rica, Colombia, Guatemala also produce large quantities of bananas and plantains. In poorly developed, food-deficit countries, plantains are also used as a staple food (Anonymous, 2021a). According to a report by Kumar and Uma (2020), in India alone around 25%–40% of the total produce of bananas (including plantains) are wasted due to mishandling. Traditional way of preserving plantains includes drying, especially sun-drying and oven or fire-drying or fermentation of the dried plantains. Plantains are also preserved in the form of flour, chips, sweet figs, and beer in areas of Uganda, Western Samoa, and Rwanda where utilization of green bananas is particularly high (Anonymous, 2021b). Thus, there is a compelling need to preserve this important natural resource and minimize its post-harvest wastage which impedes fullest utility of this crop and also encumbers utilization of its nutraceutical contents. Literature reports suggest that application of the unconventional eco-friendly–cum-low-cost preservation technology of gamma (ɣ)-irradiation can enhance the overall shelf-life of agro produces including that of plantains. However, the optimized irradiation dose and the shelf-life period vary based on the geographic area of cultivation, the agro-climatic conditions and the variety of the fruit grown (Aina et al., 1999; Sunyoto et al., 2019; Zaman et al., 2007). To the best of our knowledge, there is no published literature on the optimized dose of ɣ-irradiation for shelf-life extension of green plantains (*Musa paradisiaca)* cultivated in India, especially those in West Bengal.

In plants, the amino acid L-tryptophan synthesizes serotonin through a cascade of biological reactions and is finally converted into melatonin (Nawaz et al., 2016). L-tryptophan, serotonin, and melatonin are bioactive compounds and serve numerous benefits to plants as well as to human (D'Souza & Craig, 2006, Luck, 1998; Bhattacharjee et al., 2016). Green plantain is also a rich source of serotonin (5–150 μg/g) and melatonin (65.5 ng/100 g) (Bhattacharjee & Chakraborty, 2019) besides phenolic and flavonoid compounds (Bhattacharjee et al., 2016, Bravo et al., 2015). Previous research conducted by our research team had reported for the first time a novel application of this non-thermal-cum-low-cost preservation technology of ɣ-irradiation in enhancing production of lutein by stimulating carotenogenesis in irradiated marigold flowers nearing end of senescence (Pal & Bhattacharjee, 2016). A few more studies have also reported on enhanced production of bioactive compounds such as phenolic and flavonoid compounds, carotenoids, anthocyanins, as well as organic acids in agricultural produces post ɣ-irradiation treatment (Hussain et al., 2016; Najafabadi et al., 2017).

The above findings led us to logically hypothesize that ɣ-irradiation could also augment biosynthesis of these antioxidants viz. serotonin and melatonin in plantains. The plantains with enhanced amount of these phytoremediator molecules could be a potential food source of these antioxidants and thereby either serve as a direct nutraceutical food (when the antioxidants are in synergistic combination) or can be utilized for production of value-added antioxidant-rich products (when the mentioned antioxidants are not in synergy). Irradiated plantains could thus be healthy alternatives to the synthetic counterparts of serotonin and melatonin widely used as therapeutics. Furthermore, the senesced green plantains (at the end of their shelf-lives post irradiation), which are sensorically compromised could still be valued as a source of these phytoremediators and can be harnessed as a source of food antioxidants. This would provide a possible means of valorization of green plantains, post senescence.

Therefore, the first objective of the present study was to optimize the dose of ɣ-irradiation for extension of shelf-life of Indian *desi* variety of green plantains (*Musa paradisiaca)* by evaluating sensory, proximate, physicochemical, and phytochemical (including antioxidant) properties of samples at regular time intervals when stored at ambient storage (23 ± 2°C, 70 ± 2% RH) condition post irradiation. The second objective of the study was to obtain the best dose of ɣ-irradiation which would enhance the contents of serotonin and melatonin in plantains to the maximum extent possible under said experimental condition. This is the first study on application of ɣ-irradiation technology for augmentation of contents of antioxidants, chiefly serotonin and melatonin in green plantains.

2 | MATERIALS AND METHODS

2.1 | Materials

For ɣ-irradiation, green plantains (*Musa paradisiaca*) of *desi* variety were procured from registered farmers (cultivators) of an organic plantain farm of Narendrapur, South 24 Parganas, West Bengal, India. The plantains were grown at 22° 4,391′ N and 88° 3,968′ E, at about 9 m height from sea level in the eastern Gangetic plains of India at a temperature of 24–35°C and 75%–85% RH in deep, rich loamy, well-drained, moisture-retentive soil of pH 6.5–7.5 (Anonymous, 2021c). The species of the organically cultivated fruits were authenticated by West Bengal Food Processing and Horticulture Development Corporation Limited, Kolkata, India. Plantains were harvested thrice from March to May 2019 for triplicate sets of experiments (described later). The procured plantains were removed from their respective bunches and individual plantains were meticulously selected based on visual assessment of color (indicator of stage of ripening: only green unripe were chosen), surface morphology and texture (free of blemishes, bruises, and black spots and were turgid), and weight

(~175 ± 10 g on an average having ~120 ± 5 mm diameter). The chosen plantains were all in the same stage of maturity. The selected individual plantains were swiftly transported to the ɣ-irradiation laboratory in our university campus in cane baskets.

L-tryptophan, serotonin, melatonin, 1, 1- diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), and other specialty chemicals such as acetonitrile (99.9% pure, HPLC grade), acetic acid of HPLC grade (99.8% pure), Folin Ciocalteu's reagent, trichloroacetic acid (TCA), ethylene diamine tetraacetic acid (EDTA), ascorbic acid, 30% hydrogen peroxide (H_2O_2) were procured from M/s Sigma-Aldrich, Munich, Germany. For solid phase extraction, Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) kit including the solid phase extraction (SPE) tubes and dispersive universal kit were purchased from M/s Agilent Technologies, Wilmington, USA. Liquid nitrogen was procured from liquid nitrogen plant, National Instruments Limited (NIL) campus, Jadavpur University, Kolkata, West Bengal. All AR grade chemicals were used in this work unless specified.

2.2 | Methods

Irradiation with ^{60}Co as the radioactive source was conducted in gamma chamber GC-5000 unit (manufactured by Board of Radiation and Isotope Technology [BRIT], India) in the Food Irradiation Laboratory of National Instruments Ltd. (NIL) campus, Jadavpur University, Kolkata, India (having dose rate at maximum capacity of ~9kGy/h and dose rate uniformity of +25% radially and −25% axially [Anonymous, 2021d]). The surface radiation from GC-5000 was measured using a Pulsecho, India made Minirad survey meter (Ghosh et al., 2017) and was within its safe operational limits, that is, <2 m rad throughout the study period. The calibration including attenuation and dose rate certification were regulated and supervised by BRIT employing standard Fricke's dosimeter and the uncertainty in the measured dose was recorded at ±5%.

In a preliminary study, green plantains were ɣ-irradiated in the dose range of 0.02 kGy–1 kGy (i.e., 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 kGy) which revealed that very low doses (below 0.1 kGy) of ɣ-irradiation were effective for enhancement of shelf-life of the plantains by approximately 5 days; whereas, comparatively higher dose range (above 0.1 kGy) showed compromised shelf-life consequent to severe tissue damage, softening, and skin discoloration. These findings corroborated well with those reported by Aina et al. (1999) who also found that plantains irradiated at relatively higher dose (above 0.5 kGy) had lower shelf-life. It had also been reportedly established that relatively higher doses of ɣ-irradiation can enhance biosynthesis of natural antioxidants in botanicals, viz. production of phytoremediators as defense molecules against radiation-induced stresses is triggered at high radiation doses (Bhattacharjee & Chakraborty, 2019). Therefore, irradiation was conducted at a "very low dose range" of 0.04–0.08 kGy for extension of shelf-life of green plantains and also at a "low dose range" of 0.6–1 kGy for enhancement of serotonin-melatonin contents.

To meet the duo objectives, each consortium of experiments (apposite to its objective) was conducted thrice as per the design of experiments (DOE) presented as a block diagram in Figure 1. Based on the preliminary study, the current studies were designed for 25 days. The bulk sample set comprised of a set of 75 plantains for each of the three doses of irradiation (to meet each objective) that is, 225 plantains along with 75 non-irradiated (control) plantains for each objective. The bulk sample sets were temporarily stored at 23 ± 2°C, 70 ± 2% RH in an environmental chamber prior to ɣ-irradiation. The bulk sample set (apart from non-irradiated plantains) were then subjected to ɣ-irradiation processing on day 0. Therefore, for each dose of ɣ-irradiation, 25 batches of green plantains were treated to meet each objective. Each day, three plantains were randomly withdrawn from the processed sample sets which constituted the analytical sample set.

For each batch, stalks of three (randomly selected from bulk) green plantains (average weight of 525 ± 20 g) were sized and ɣ-irradiated after placing in an amber-colored beaker with polystyrene cushions on the inside walls. The beaker was positioned at the geometric center of the sample chamber of the GC-5000 unit and covered using the removable lid of the chamber. Irradiation was conducted at 33 ± 3°C keeping the chamber under rotation (60 rpm) at a rate of 3.062 kGy/h. Rotation aided proper attenuation and thus ensured uniform distribution of ɣ-rays in the sample chamber together with polystyrene cushioning which restricted movement of plantains inside the beaker by minimizing the free space (IAEA, 2002). Post-irradiation, the processed sample sets (irradiated and non-irradiated plantains) were stored at 23 ± 2°C, 70 ± 2% RH in an environmental chamber for a total period of 25 days.

From day 0 onwards, three plantains were randomly withdrawn from the processed sample sets for shelf-life assessment (by evaluation of sensory, proximate, physicochemical, and phytochemical properties) as well as for quantification of the content of biomolecules, namely L-tryptophan, serotonin, and melatonin. The studies were conducted simultaneously for a 3-month duration (viz. the above DOE was repeated thrice). A two-day interval was maintained between irradiation treatments at "very low dose" and "low dose" to facilitate sample handling and analyses.

2.3 | Experimental plan for selection of best dose of ɣ-irradiation for enhancement of shelf-life of green plantains

Whole green plantains were subjected to sensory evaluation and also for assessment of color, texture, true density, and pH. All assays including sensory, proximate, physicochemical, and phytochemical analyses were carried out at an interval of 3 days for each analytical sample set and on the last day of their sensorical rejection. 10 g plantain from each set of analytical sample set, that is, a total of 30 g (10 × 3) plantains were diced, reshuffled, and mixed and then required amounts were subjected to determination of moisture, fat, ash contents, and percent membrane stability index (% MSI). The

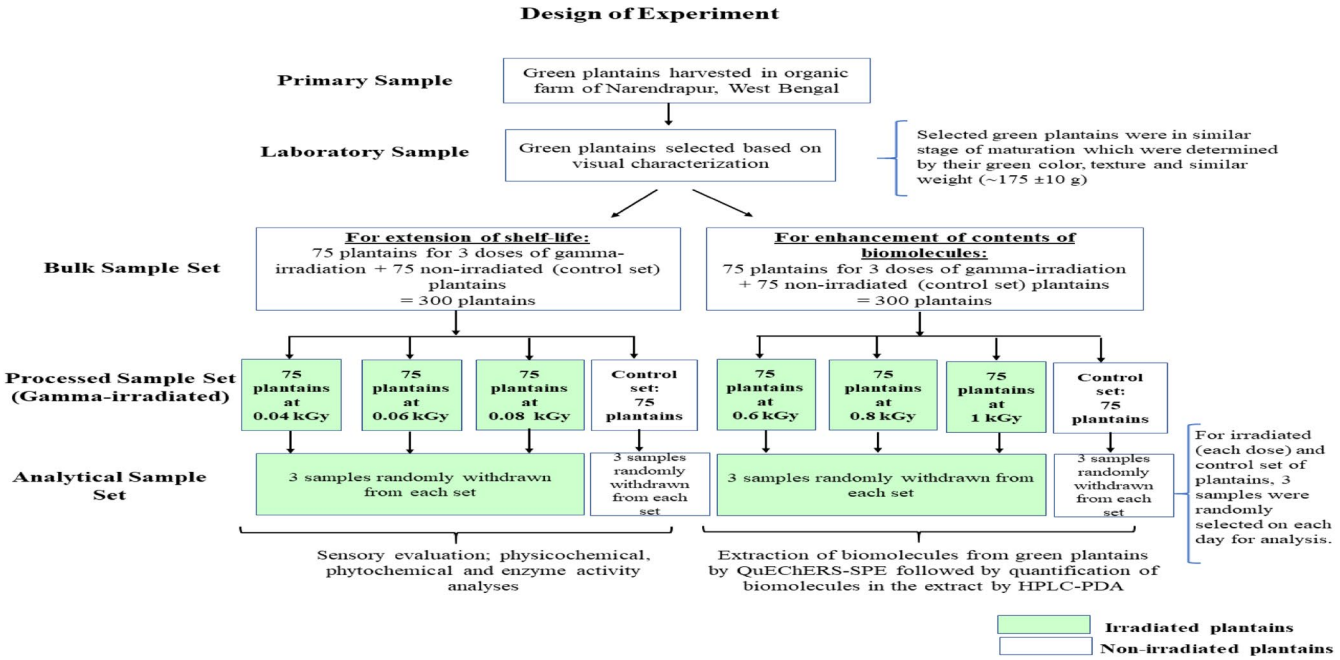

FIGURE 1 Experimental design for ɣ-irradiation of green plantains. Each consortium of experiments (apposite to its objective) was conducted thrice as per the design of experiments and results reported are mean ± *SD* of data obtained by the three independent runs of each experiment consortium

remaining plantains were homogenously ground in a mortar-pestle (along with their skin) and a pulpy mass was obtained. An extract prepared from this pulp (described later) was used for determination of protein content, reducing sugar, titratable acidity, and antioxidant potency (assessed by IC_{50} value, ferric reducing antioxidant power, and total phenolic content [TPC]). Using the remaining homogeneous pulpy mass, total pectin content as calcium pectate, vitamin C content, and enzyme activities were evaluated. For each analytical sample set, the relatively stable parameters (i.e., crude fat, ash and crude fiber contents; total pectin content as calcium pectate; and ascorbic acid content) which did not show significant changes in a 3-day interval (assessed from the preliminary trial) were analyzed at an interval of 5 days and also on the last day when they had to be sensorically rejected.

2.3.1 | Sensory evaluation of ɣ-irradiated green plantains

Sensory evaluation of each analytical set of plantains was performed by rating on a 9-point hedonic scale (9-like extremely and 1-dislike extremely) according to the method reported by Stone and Sidel (2004), Pal and Bhattacharjee (2018) and Ghosh et al. (2017). The sensory panel consisted of semi-trained university faculties and research scholars (15 men and 15 women) in the age group of 20–45. The panelists were not formally trained by any company or institute. However, they were all very well familiar with the quality parameters of green plantains (viz. color, odor, texture, browning, shrinkage, and overall appearance) since the same are a part of their diet.

2.3.2 | Proximate analyses of ɣ-irradiated green plantains

Proximate analyses of the non-irradiated green plantains were conducted on day 0 in accordance with AOAC methods which included estimation of % moisture on dry weight basis (D.W) (Association of Official Analytical Chemists [AOAC], 1998), protein (g/ 100 g D.W.) (Association of Official Analytical Chemists [AOAC], 1995), % crude fat (Association of Official Analytical Chemists [AOAC] method 920.39, 2000a), % ash (AACC, 2000), % crude fiber (Association of Official Analytical Chemists [AOAC] method 978.10, 2005; Nankar et al., 2016), % total carbohydrates (by difference), and reducing sugar content (g/ 100 g D.W.), in accordance with the methods described by Pal and Bhattacharjee (2018). During the storage study, sample sets were similarly analyzed for their proximate constituents following AOAC methods except for moisture content which was analyzed using IR moisture analyzer (Citizen MB 200 C) according to the method of Latorre et al. (2010) and reported on a % wet weight basis.

2.3.3 | Physicochemical analyses of ɣ-irradiated green plantains

To analyze the changes in texture profiles of plantains with storage, texture profile analysis (TPA) by two-bite compression test of plantains were conducted employing a TA.XT Express Texture Analyzer (Stable Micro Systems, Godalming, UK) in accordance with the method described by Rao et al. (2020). Whole plantains were subjected to texture analysis. An aluminum cylinder-shaped probe (P/5)

with 5-mm-diameter was used for the test and the texture analysis parameters were set to: pre-test speed: 1 mm/s, test speed: 5 mm/s, post-test speed: 5 mm/s target mode: distance, distance: 5 mm, strain: 75%, trigger type: auto (force), trigger force: 5 g, loadcell: 10 kg. The sample was aligned such that compression was effected symmetrically w.r.t it's geometric center. The TPA graph was executed by software of Exponent Lite Express (version 6.1.4.0). Parameters such as hardness (g), adhesiveness (g.sec), cohesiveness, springiness, gumminess, and chewiness of plantains were ascertained by the software.

CIE color (L^* for lightness axis; a^* for red-green axis; b^* for yellow-blue axis) values of plantains were determined with a portable CR-10 Plus Color reader (M/s Konica Minolta Inc., Osaka, Japan) (Trisnawati et al., 2019). Other physicochemical properties assayed were: pulp to peel ratio (Abdullah et al., 2017), true density (g/cm^3) (Wasala et al., 2012), % MSI by electrical PC 510 conductivity meter, M/s Eutech Instruments, Malaysia (Pal & Bhattacharjee, 2018), % total titratable acidity (TTA) as % malic acid equivalent (Ranganna, 2000), pH (using pH meter, Cyberscan PC 510 m, M/s Eutech Instruments Pvt. Ltd., Singapore) (Chakraborty & Bhattacharjee, 2018), and total pectin content as % calcium pectate (Sadasivam & Manickam, 2005).

2.3.4 | Phytochemical analyses of ɣ-irradiated green plantains

Preparation of extract

To obtain an extract of green plantains for phytochemical analyses, three plantains from each analytical set and from the control set were randomly withdrawn each day. The extract was used for estimation of TPC and for antioxidant assays in accordance with the procedures described by Ranganna (2000) and Shian et al. (2012). Whole plantains (comprising of pulp and peel) were cut into small pieces and crushed in a mortar-pestle into a pulpy mass. The pulpy mass (20 g) was then homogenized in 100 ml of 70% ethanol: water (vol/vol) using a homogenizer (T 50 digital Ultra-turrax, M/s Ika, Staufen, Germany) for 1 min at 24,000 rpm followed by 10 min of centrifugation at 4,750×*g* using a centrifuge (R-8C, M/s Remi, Mumbai, India). The supernatant was collected and stored at −20°C before analyses.

Determination of TPC

TPC of the plantains was estimated spectrophotometrically (Halo DB-20, UV-Vis Double Beam Spectrophotometer, Dynamica Scientific Ltd., Newport Pagnell, UK) by Folin-Ciocalteu method as elaborated by Aiyegoro and Okoh (2010) and was expressed as mg gallic acid equivalent (GAE)/100 g D.W.

Determination of antioxidant activities

The antioxidant activities of the control and ɣ-irradiated green plantains were carried out by assaying IC_{50} values (mg/ml) of DPPH radical scavenging activities (DPPH-RSA) of the extracts (described previously) and by ferric reducing antioxidant power (FRAP) values expressed as mM $FeSO_4$/100 g D.W. in accordance with the methods described by Spanos and Wrolstad (1990), and Benzie and Strain (1999), respectively.

Determination of total ascorbic acid content

The total ascorbic acid contents of the plantains were estimated spectrophotometrically by the method elaborated by Chakraborty and Bhattacharjee (2018) and Kapur et al. (2012) and were expressed as mg/100 g D.W.

Determination of enzyme activities of ɣ-irradiated green plantains

Ascorbic peroxidase (APX) activities of the plantains were evaluated according to the method followed by Pal and Bhattacharjee (2016) and were expressed as micromole ascorbate oxidized/mg protein/min. Polyphenol oxidase (PPO) activity was determined according to the method described by Galeazzi and Sgarbieri (1981) and was expressed as IU/100 g D.W.

2.4 | Experimental plan for determination of best dose of ɣ-irradiation of green plantains for enhancement of contents of serotonin and melatonin individually

Contents of serotonin, melatonin, and their precursor molecule L-tryptophan in ɣ-irradiated (dose range: 0.6–1 kGy) and control sets of green plantains were quantified at an interval of 2 days for the entire storage period (25 days). Since the amounts of these bioactive molecules changed more rapidly vis-à-vis their shelf-life parameters, it was necessary to conduct analyses of the said bioactives at a comparatively smaller time interval (viz. 2 days) for reliable elucidation of the trend (enhancement if any) of production of these biomolecules post irradiation.

2.4.1 | Sample preparation for QuEChERS-SPE extraction of green plantains

For extraction of serotonin and melatonin from green plantains, whole fruits were used along with their peel, except the stalk and the black tip (remains of perianth, stigma and style). The fruits were diced, placed inside a polystyrene box (30 × 30 × 25 cm) and adequate liquid nitrogen (to freeze the samples) was poured over them using a canister followed by immediate grinding of the sample to powder in a mixer-grinder (Philips Mixer Grinder, HL 1618, Philips India Limited, Chennai, India) in accordance with the method described by Okazaki and Ezura (2009), with few modifications. This cryo-ground sample was used for SPE.

2.4.2 | QuEChERS-SPE of the cryo-ground green plantain to extract serotonin, and melatonin

QuEChERS-SPE of the cryo-ground plantain sample was carried out according to the method described in Association of Official Analytical Chemists (AOAC) method 985.22 (2000b) and Anastassiades et al. (2003). Cryo-ground sample was taken in a clean

centrifuge tube where 1% acetic acid in acetonitrile solution and a SPE AOAC packet (containing $MgSO_4$ and NaCl) were added. The mixture was thoroughly vortexed. The tube was then centrifuged at 1,500 × g for 1 min using a R-8C centrifuge. One ml filtrate was withdrawn and added to the dispersive-SPE (dSPE) sample cleanup tube (containing primary secondary amine, C18 sorbent [trifunctionally-bonded C18 silica], graphitized carbon black and $MgSO_4$) and thoroughly mixed. The dSPE sample cleanup tubes were then subjected to centrifugation at 1,207×*g* for 5 min using a microspin TC- 4815D centrifuge (Eltek, Haryana, India). The supernatant that is, the extracted sample was filtered using a micro syringe filter (0.2 μm nylon) and kept in amber-colored glass vials at −20°C for further analyses.

2.4.3 | Quantification of serotonin and melatonin by high performance liquid chromatography (HPLC)

Plantain extracts obtained by QuEChERS were subjected to analyses of L-tryptophan, serotonin, and melatonin by HPLC-PDA. For simultaneous quantification of the above mentioned biomolecules, a new HPLC method has been developed in our laboratory and validated by rigorous chromatography modeling based on mass transfer of analytes (unpublished data).

The filtered extract (20 μl) was injected into JASCO C18 reversed phase column (l = 250 mm and i.d = 4.6 mm) HPLC (LC-Net-2/ADC, PU-4180 HPLC pump, DG- 4000–04 degasser, MD-4015 detector) system. The pump was operated in SPG (Shirasu porous glass) mode at a pressure range of 0–5^{e+7} Pa. HPLC grade methanol and 1% HPLC grade acetic acid in distilled water were used as mobile phase solvents in gradient mode, each at a flow rate of 1 ml/min. PDA detector having a D2 lamp at 280 nm was employed to monitor the eluents in a continuous manner, adapting the method reported by Huang and Mazza (2011). Peaks of these biomolecules were identified based on retention time of Sigma standards of L-tryptophan, serotonin, and melatonin (when 20 μl mixture containing 1 mg ml^{-1} of each standard was injected and run as per the above program).

2.4.4 | Determination of synergism effect (SE) value

Synergism among antioxidants is naturally present in all foods (Messina et al., 2001). When isolated, antioxidants may either act in synergism or in antagonism (Peyrat-Maillard et al., 2003). To evaluate the direct impact of ɣ-irradiation on synergism among the three antioxidant molecules, namely serotonin, melatonin, and L-tryptophan in plantains, in vitro synergistic effect was ascertained using pure chemical standards of these in concentrations present in the apposite extracts of plantain samples, viz. control sample on day 0, control sample on last day of storage, sample possessing highest amount of serotonin, and sample possessing highest amount of melatonin, in accordance with the method reported by Chakraborty and Bhattacharjee (2018).

2.5 | Statistical analysis

All data reported are mean ± *SD* of data obtained from the three independent consortium of experiments as explained in Figure 1. Two-way ANOVA was performed by STATISTICA 8.0 software (Statsoft, Oklahoma, USA) to analyze the individual and interactive effects of two variables that is, shelf-life period and dose of ɣ-irradiation on each assay parameter. Duncan's multiple range test was employed to determine significant differences among means by employing IBM SPSS Statistics Software Version 26 (IBM, USA). A value of $p \leq .05$ was considered significant to establish differences in all tests.

3 | RESULTS AND DISCUSSION

3.1 | Estimation of best dose of ɣ-irradiation to enhance shelf-life of green plantains by assessing alterations in their quality parameters during storage

With progression of storage (total storage period of 25 days) the control set and 0.04, 0.06, and 0.08 kGy irradiated green plantains had to be discarded after 17, 22, 22, and 21 days, respectively, based on sensory evaluation. The said samples were ineligible for further analyses owing to development of unacceptable black color, foul odor, and shrunken-over-softened texture. These characteristic changes were clear indications of completion of their respective shelf-life. Proximate, physicochemical, and phytochemical analyses were carried out for the control set and 0.04, 0.06, and 0.08 kGy irradiated green plantains for 17, 22, 22, and 21 days, respectively (henceforth this time period has been nomenclatured as "assessment period") to finally ascertain the day until which the plantains could be consumed safely for nutraceutical benefits (henceforth this time period from day 0 until the day the samples were suitable for consumption has been denoted as "shelf-life"). The visual changes of green plantains with progression of senescence during storage have been presented in Figure 2.

3.1.1 | Alterations in sensory attributes

The hedonic scores of sensory attributes of ɣ-irradiated (0.04, 0.06 and 0.08 kGy) and control sets of green plantains on days 0, 3, 6, 9, 12, 15, 17, 18, 21, and 22 have been presented in radar plots (Figure 3). Development of yellow color which is an indicator of ripening was observed from day 3 onwards in control set of samples; while 0.04 and 0.06 kGy-irradiated plantains showed significant delay in development of yellow color which commenced on day 9. From day 5 onwards, the control set of plantains exhibited enhanced enzymatic browning, that is, development of unacceptable brown color in corroboration with increased PPO activity (discussed in Section 3.1.4). 0.04 and 0.06 kGy-irradiated plantains showed delayed ripening in tandem with delayed onset of browning (from day 12 onwards). All analytical sample sets of plantains were subjected to sensory evaluation until they scored ≤1 (i.e., dislike extremely) on the 9-point hedonic

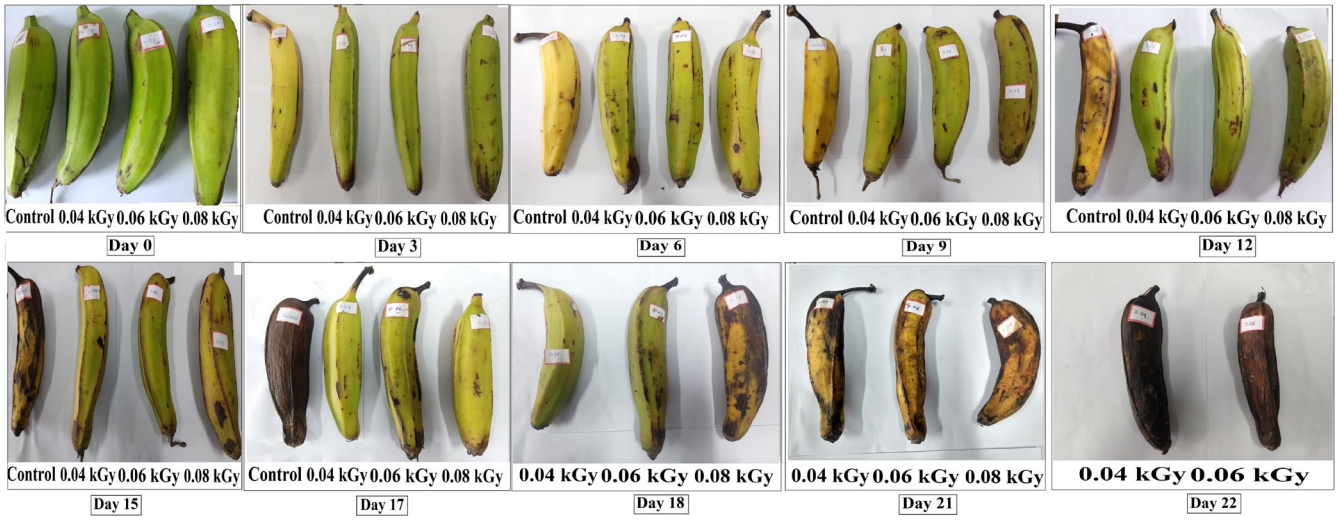

FIGURE 2 Physical changes in ɣ-irradiated (0.04–0.08 kGy) plantains and control samples during 22 days of storage when stored at 23 ± 2°C, 70 ± 2% RH in an environmental chamber

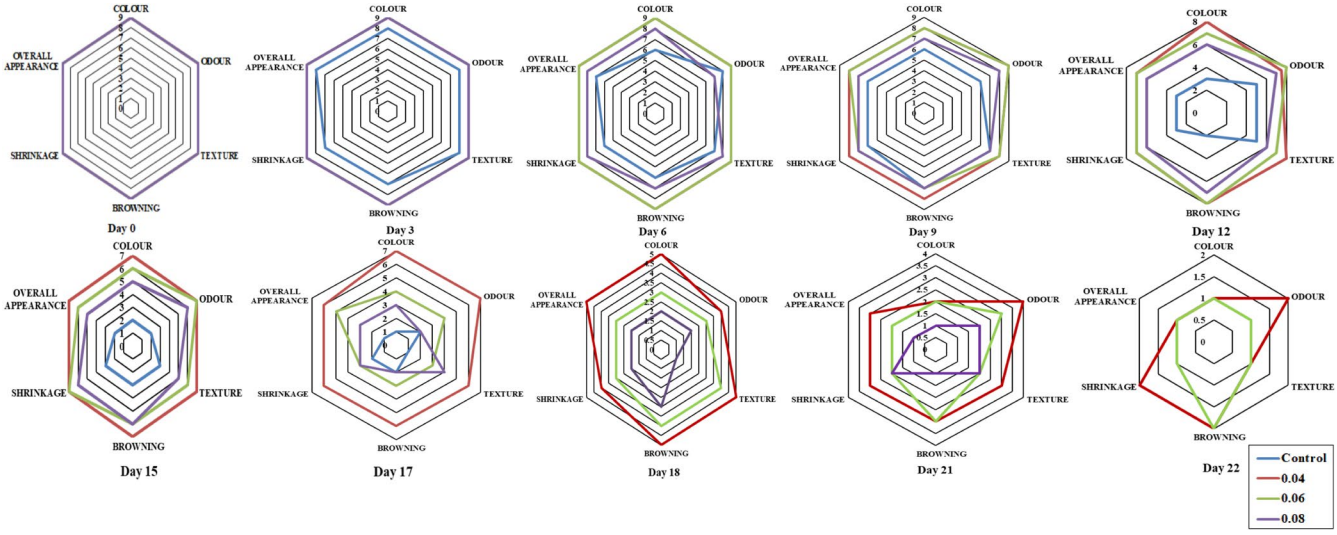

FIGURE 3 Radar plots of hedonic scores obtained by sensory analyses of control and ɣ-irradiated (0.04, 0.06, and 0.08 kGy) green plantains for 22 days when stored at 23 ± 2°C, 70 ± 2% RH in an environmental chamber. Since both control and irradiated samples scored same on day zero and day three, there is only single line in radar plot on that day (except the parameter "color" on day three). The green line on day 6 represents both 0.04 and 0.06 kGy since their scores were identical. Each value represents mean ± *SD* of three sets of experimental data

scale. The control and 0.08 kGy-irradiated plantains were sensorically rejected on days 17 and day 21; while the 0.04 and 0.06 kGy-irradiated plantains were rejected on day 22 owing to development of black color, foul odor, and dry texture. It was found that the overall sensory scores of the panelists were highest for 0.04 kGy-irradiated plantains throughout the assessment period.

3.1.2 | Alterations in proximate constituents

The trends in changes in proximate constituents (% moisture on wet weight basis, protein [g/100 g D.W.], % crude fat, % ash, % crude fiber, carbohydrate [by difference] and reducing sugar contents [g/100 g D.W.]) during storage have been graphically represented in Figure 4. The proximate constituents of non-irradiated plantains on day 0 on % D.W. basis were as follows: 79.313% ± 1.82% moisture, 3.06% ± 0.97% protein, 0.65% ± 0.09% crude fat, 6.501% ± 0.78% ash, and 6.52% ± 2.24% total carbohydrates (by difference). No significant changes in moisture contents were observed among the control and irradiated plantain sets on the same day of study, which signified that there were no differences in moisture contents among the irradiated and the non-irradiated plantains on the same day of storage. These findings support those of Maxwell et al. (2017), who studied effects of different doses of ɣ-irradiation (0.05, 0.1, 0.3, and

0.8 kGy) on vitamin C, acidity, and moisture contents of four different varieties of mangoes (flat mangoes, *Binta* sugar mangoes, *Barki Akus* mangoes, and *Cameron* mangoes) and reported no difference in moisture contents among the control and irradiated sample sets. A significant declination ($p < .05$) in moisture contents were observed in both ɣ-irradiated and non-irradiated plantains with progression of senescence (Figure 4a) in consonance with our sensory panelists' scores on shrinkage (discussed in Section 3.1.1).

There were no differences in protein contents among the control and irradiated sample sets immediately after ɣ-irradiation (on day 0). However, protein contents of both the control and irradiated sample sets had decreased significantly ($p < .05$) with advancement of senescence. The depletion of protein content in the control set could be attributed to oxidative degradation of cellular protein, which is reportedly a common phenomenon of senescence (Jajic et al., 2015); whereas, in the irradiated plantains, structural changes such as cross-linking and aggregation of proteins (not investigated in the present study) consequent to ɣ-irradiation (Ciesta et al., 2000) perhaps slowed down protein degradation. Among all sample sets, 0.04 kGy-irradiated plantains had the highest protein contents followed by those of 0.06 and 0.08 kGy-irradiated sets, vis-à-vis the control set (Figure 4b). The trend of declination in protein contents of irradiated plantains was in consonance with the findings of Pal and Bhattacharjee (2020) who studied effects of ɣ-irradiation (1.0, 3.0, 5.0, 8.0, and 10.0 kGy) on shelf-life of yellow corn (*Zea mays*) kernels. The obtained protein contents corroborated well with the data obtained by MSI analyses (discussed in Section 3.1.3).

No significant changes ($p < .05$) in % crude fat (Figure 4c), % ash (Figure 4d), and % crude fiber (Figure 4e) were observed among the ɣ-irradiated and control sets of plantains which indicated that ɣ-irradiation did not have significant effects on these parameters. However, these parameters showed declination trends ($p < .05$) in both control and ɣ-irradiated plantains with progression of storage. Insignificant changes in the said parameters in the control and irradiated sets of plantains were in compliance with the findings of Zaman et al. (2007) and Ihsanullah et al. (2005). Zaman et al. (2007) had reported insignificant changes in % crude fat during investigation of extension of shelf-life of bananas (*Musa sapientum)* employing low dose ɣ-irradiation (0.3, 0.4, and 0.5 kGy) and post storage at room conditions (25 ± 2°C, 80% ± 5% RH). Ihsanullah et al. (2005) investigated effects of ɣ-irradiation (0.2, 0.5, 1, 2, and 3 kGy) on nutrients of Pakistani dates (*Pheonix dactylera* L.) when stored in white polythene for five months and reported insignificant changes in % crude fiber contents. Similarly, negligible changes in % ash contents in irradiated plantains during storage agreed well with the findings of Al-Bachir (2004) who studied the effects of ɣ-irradiation (0.5, 1.0, 1.5, and 2.0 kGy) on proximate constituents of walnuts (*Juglans regia L.*).

Percentage total carbohydrates increased ($p < .05$) with the progression of senescence (Figure 4f) owing to concentration effect brought about by loss of moisture in the samples with storage. The amounts of reducing sugar in the control set increased significantly ($p < .05$) from day 9 (mid-senescence) onwards and remained higher than the remaining sets (Figure 4g) indicating enhanced ripening in non-irradiated samples; whereas, the rise in reducing sugar levels in ɣ-irradiated plantains commenced after day 15 (late senescence) owing to delayed onset of ripening in the same. This trend of enhancement in reducing sugar content is the result of enzymatic conversation of higher polysaccharides (such as starch present in plantains) into simple sugars with progression of storage (Hussain et al., 2008), which complied well with the findings of Pal and Bhattacharjee (2020) on reducing sugar contents of irradiated corn. However, 0.04 kGy-irradiated plantains exhibited lowest content of reducing sugar indicating delayed ripening of the same which

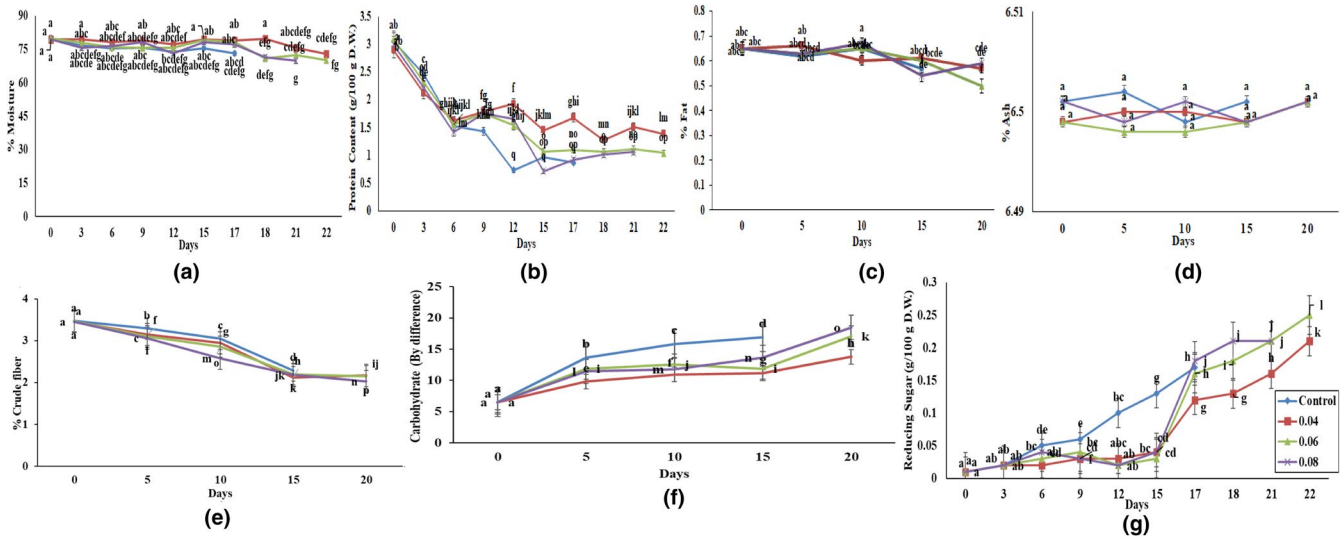

FIGURE 4 Analyses of proximate constituents of ɣ-irradiated plantains and control samples during 22 days of storage- (a) % Moisture content (on wet weight basis); (b) protein content (g/100 g D.W.); (c) % crude fat content; (d) % ash content; (e) % crude fiber; (f) total carbohydrates by difference and (g) reducing sugar content (g/100 g D.W.). Each value represents mean ± *SD* of three sets of experimental data. Different alphabets specify that mean values belong to different subsets at $p < .05$

corroborated well with the changes obtained for pulp to peel ratios (discussed in Section 3.1.3).

3.1.3 | Alterations in physicochemical properties of ɣ-irradiated plantains

Alterations in texture profiles

Hardness, cohesiveness, gumminess, and chewiness of plantains for both ɣ-irradiated and control sets had decreased significantly ($p < .05$); whereas adhesiveness and springiness of the said samples had increased significantly ($p < .05$) throughout the assessment period. These results were in good agreement with those reported by Watharkar et al. (2021). The reduction in hardness is attributed to the chemical changes such as hydrolysis of starch into sugar, conversion of insoluble protopectin to soluble pectin during fruit ripening and the losses in the crude fiber content due to structural changes (Mustaffa et al., 1998; Siriamornpun & Kaewseejan, 2017). Considering the entire processed sample set, plantains ɣ-irradiated at 0.04 kGy showed lower adhesiveness, springiness and higher hardness, chewiness, gumminess and cohesiveness vis-à-vis the other sets during storage (Figure 5). This finding indicated best retention of characteristic texture of the ɣ-irradiated plantains at the aforesaid dose, which were in consonance with the responses of the sensory panel on texture parameters (discussed in Section 3.1.1).

Alterations in color profiles

With progression of ripening (senescence), green color of plantains turned yellow, substantiated by their higher ($p < .05$) b^* values. Additionally, a^* values of the ɣ-irradiated as well as control sets of plantains had increased significantly ($p < .05$) during senescence. These results correlated well with the development of browning (Figure 6) in the above samples as have also been reported by Kajuna et al. (1998). Plantains irradiated at 0.04 kGy remained green (negative a^*) up to day 15 (late senescence) and possessed relatively lower b^* values (yellowness) and lower L^* values (lightness) throughout the storage period which correlated well with our sensory evaluation scores on color. Delayed onset of ripening and thus better retention of characteristic green color was observed for 0.04 kGy-irradiated plantains compared to both control and other irradiated sample sets. These results further validated those obtained by sensory analyses and PPO activities (discussed in Section 3.1.1 and 3.1.4, respectively).

Alterations in pulp to peel ratios. The pulp to peel ratio increased with advancement of senescence due to increase of pulp weight and decrease of peel weight. At the time of post-harvest ripening, sugar content increases in the pulp which increases osmotic pressure and movement of moisture from peel to pulp thereby increasing pulp weight and concomitantly, transpiration results in moisture loss in peels (Dadzie & Orchard, 1997). Breaking down of the cell wall and formation of air gap in the middle lamella could be attributed to the thinning of peel and its subsequent weight loss during the ripening period (Adi et al., 2019). It was found that the rate of increase ($p < .05$) of pulp to peel ratio in the control sets was faster than their irradiated counterparts (Figure 7a) since the non-irradiated samples ripened faster than the irradiated ones. Increase in pulp to peel ratio and ripening of fruits are strongly correlated. It is also evident from the findings of color, texture and % MSI analyses that ripening in control sets were faster than their irradiated counterparts. The plantains irradiated at 0.04 kGy exhibited the maximum delay in rise of pulp to peel ratios in comparison with those of the control and irradiated sample sets. This finding was in consonance with that reported by Abdullah et al. (2017) who investigated the ripening behavior of ɣ-irradiated (0.5, 0.75 and 1.0 kGy) green bananas (*Musa sapientum* cultivar "Dwarf Cavendish") when stored at room temperature and found delayed increase in pulp to peel ratios vis-à-vis the non-irradiated ones.

Alterations in true density

The true density values of ɣ-irradiated and control sets of plantains increased significantly ($p < .05$) during senescence (Figure 7b) in the range of 1.29 to 1.88 g/cm^3. The control set showed maximum increase ($p < .05$); whereas 0.04 kGy-irradiated plantains exhibited minimum increase ($p < .05$). True density calculated as mass per unit volume is inversely proportional to moisture content (Boukouvalas et al., 2006). Thus in non-irradiated samples, true density showed a faster increase with decrease in moisture content in contrast with the remaining sample sets. On the other hand, 0.04 kGy-irradiated samples showed minimum increase in true densities since the same also showed minimum decrease in moisture contents compared to the remaining samples sets.

Alterations in MSI

Membrane stability had decreased significantly ($p < .05$) from day 0 in both ɣ-irradiated and control sets of plantains (Figure 7c) since it is reportedly known that with progression of senescence, cell membrane weakens resulting in loss of the ability of selective leakage of intracellular ions (Chakrabarty et al., 2009). However, among all treated plantains, the highest % MSI was observed in plantains ɣ-irradiated at 0.04 kGy attesting it to be the best dose of ɣ-irradiation in maintaining characteristic membrane integrity during storage. Deterioration of proteins in botanicals (discussed in Section 3.1.2) could be attributed to enhanced rate of membrane permeability and subsequent loss of ions (Shahri & Tahir, 2011), which corroborated well with the present findings of % MSI values. Ghosh et al. (2017) in their study on shelf-life extension of tuberose flowers using a combination of ɣ-irradiation and generally regarded as safe (GRAS) preservatives, also reported that treated flowers exhibited higher membrane stabilities compared to their non-irradiated counterparts.

Alterations in TTA contents and pH

Percentage TTA of plantains in terms of % malic acid had increased in a significant ($p < .05$) manner (Figure 7d); whereas

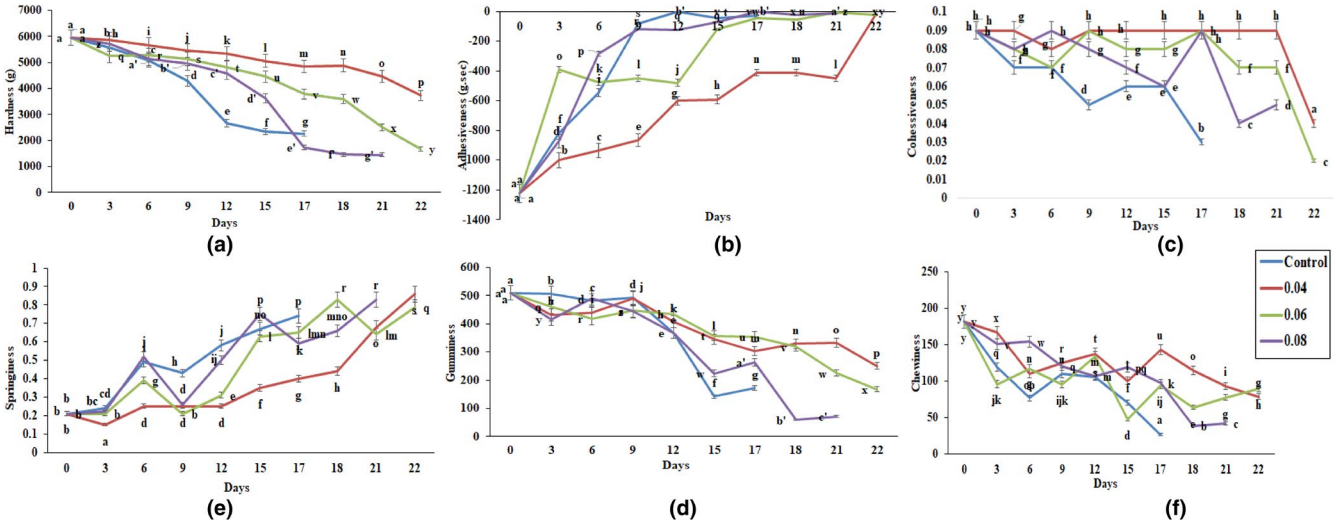

FIGURE 5 Analyses of texture of ɣ-irradiated plantains and control samples during 22 days of storage- (a) Hardness (g); (b) adhesiveness (g.sec); (c) cohesiveness; (d) springiness; (e) gumminess; (f) chewiness. Each value represents mean ± *SD* of three sets of experimental data. Different alphabets denote that mean values belong to different subsets at $p < .05$

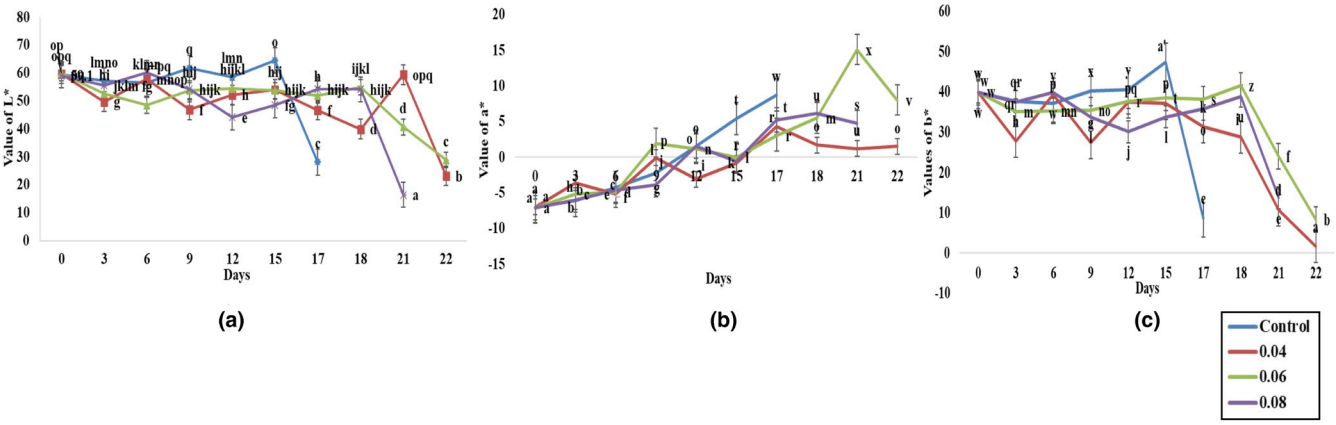

FIGURE 6 Color parameters (a) *L** value, (b) *a** value and (c) *b** values of ɣ-irradiated plantains and control samples are mean ± *SD* of the data obtained from three independent sets of experimental data during a storage period of 22 days. Different alphabets denote that mean values belong to different subsets at $p < .05$

significant ($p < .05$) decreases in pH were observed from day 9 (mid-senescence) onwards with the advancement of ripening in both ɣ-irradiated and control sets of plantains. pH values of ɣ-irradiated and control sets of plantains were in the range of 6.82–4.09 (Figure 7e) during the storage period. This increase in acidity is attributed to starch degeneration via Krebs cycle and concomitant production of organic acids such as malic acid and citric acid in the pulp during ripening (Lustre et al., 1976; Seymour, 1993). However, there are no significant differences ($p < .05$) in % TTA and pH values with storage among the control and irradiated sets of plantains on the same day. This finding was in consonance with those of Maraei and Elsawy (2017) who studied chemical quality and nutritional composition of ɣ-irradiated (0, 0.3, 0.6, and 0.9 kGy) strawberries (Fragaria × ananassa cv. Festival) during storage at 10°C.

Alterations in total pectin content as calcium pectate

In the current study, total pectin contents (in terms of calcium pectate) had decreased significantly ($p < .05$) in both ɣ-irradiated and control sets of plantains (Figure 7f) from early to late senescence. This could be attributed to the transformation of insoluble pectin into soluble pectin and a concomitant reduction in the amount of calcium pectate in the latter days of senescence which agreed well with the findings of Inari et al. (2002) who studied pectin contents of cherry tomatoes. Similar trends in reduction of chelator soluble and alkali-soluble pectin contents in ɣ-irradiated (0.25, 0.50, 0.75, 1.0, and 1.5 kGy) papayas during ripening have been reported by Zhao et al. (1996). Plantains ɣ-irradiated at 0.04 kGy had higher pectin contents throughout the senescence period indicating delayed ripening in the same vis-à-vis the remaining sample sets. These results corroborated well with our sensory (discussed in Section 3.1.1) and texture profile analyses data (discussed in Section 3.1.3).

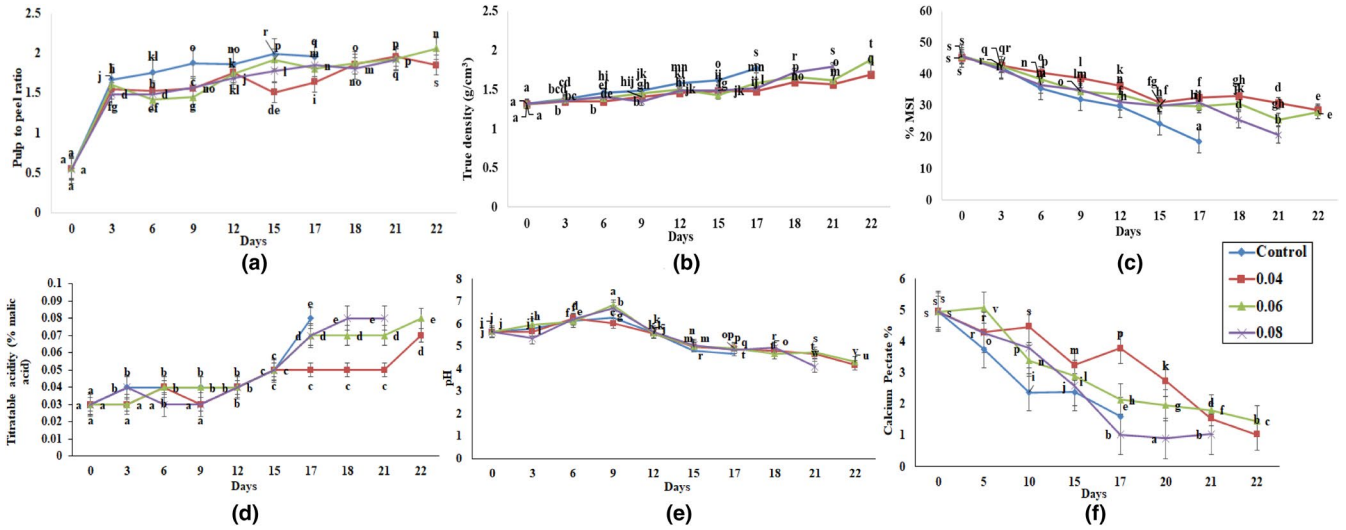

FIGURE 7 Physicochemical analyses of ɣ-irradiated and control plantains during 22 days of storage- (a) Pulp to peel ratio; (b) true density (g/cm^3); (c) % membrane stability index; (d) titratable acidity (% malic acid); (e) pH, and (f) % calcium pectate. Each value stands for mean ± *SD* of three sets of experimental data. Different alphabets denote that mean values belong to different subsets at $p < .05$

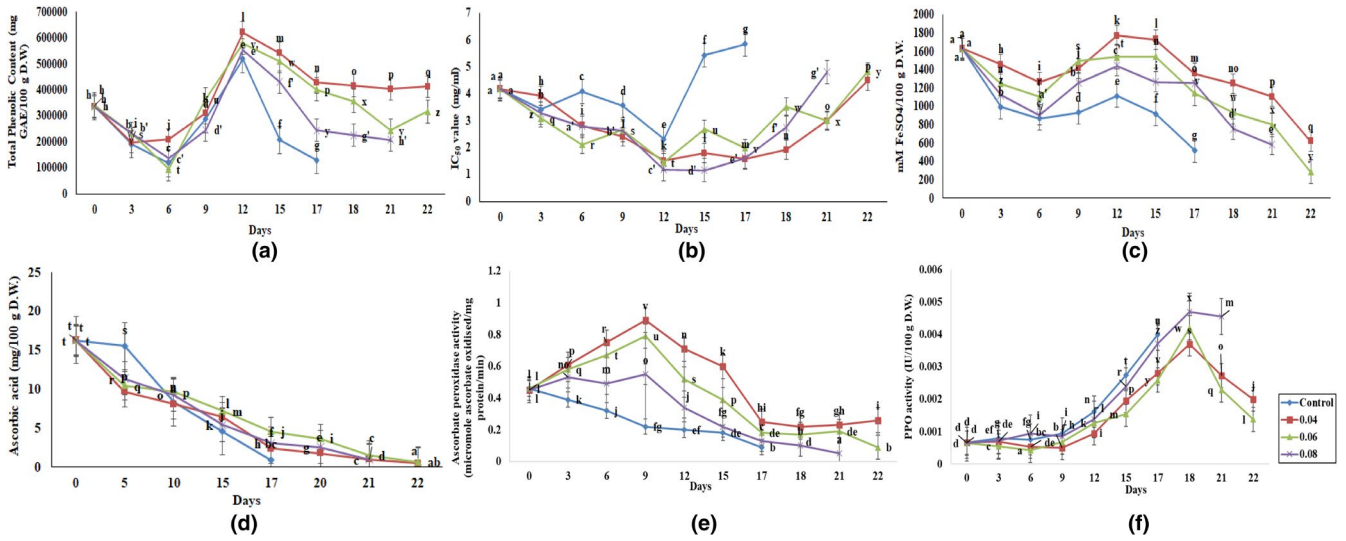

FIGURE 8 Phytochemical and enzyme activity analyses of ɣ-irradiated plantains and control sets during 22 days of storage- (a) Total phenolic content (mg GAE/100 g D.W.); (b) IC_{50} value by 1-diphenyl-2-picrylhydrazyl (mg/ml); (c) ferric reducing antioxidant power value (mM $FeSO_4$/100 g D.W.); (d) ascorbic acid content (mg/100 g D.W.); (e) ascorbic peroxidase activity (micromole ascorbate oxidized/mg protein/min), and (f) polyphenol oxidase activity assay (IU/100 g D.W.). Each value stands for mean ± *SD* of three sets of experimental data. Different alphabets denote that mean values belong to different subsets at $p < .05$

3.1.4 | Phytochemical properties of ɣ-irradiated green plantains

Alterations in TPC

TPC of the irradiated plantain sets showed a significant ($p < .05$) increase during senescence (up to day 12), followed by a gradual decrease (Figure 8a). Plantains irradiated at 0.04 kGy dose exhibited higher TPC values throughout the storage period vis-à-vis the control and other irradiated sets and the highest value was recorded on day 12 (mid-senescence) of storage. Wi et al. (2007) in their study on the effect of ɣ-irradiation on the morphological changes and biochemical responses of plants, concluded that the alterations in the cell structure and metabolism could have resulted in accumulation of phenolic compounds in ɣ-irradiated post-harvest produce during the initial days of storage, while oxidative degradation of TPC led to its decrease during later days of storage.

Alterations in antioxidant activities

The IC_{50} values of irradiated plantains showed significant ($p < .05$) decreases (i.e., increased DPPH-RSA) from day 1(early senescence) up to day 12 (mid-senescence) followed by a gradual increase (decreased radical scavenging activity); while the

control set showed decrease ($p < .05$) on day 12 followed by a sharp increase in the same until day 17 (late senescence), which was the last day of the assessment period for the control set (Figure 8b). On the other hand, FRAP values for both irradiated and control sets significantly ($p < .05$) increased up to day 12 (mid-senescence) (Figure 8c), after which the values started to decline continuously until the end of the assessment period. Plantains irradiated at 0.08 kGy exhibited highest antioxidant activities in terms of DPPH radical scavenging activity on day 12, although the phenolic contents and FRAP values were the highest in 0.04 kGy-irradiated plantains on day 12. Antioxidants present in plantains other than phenolics, such as vitamin C, carotenoids, and xanthophylls contribute to DPPH radical scavenging activity (Ali et al., 2019; Kondo et al., 2005). The increases in DPPH radical scavenging activities of the irradiated plantains (compared to the control ones) possibly could be attributed to enhanced activities of enzymes such as ammonialyase and peroxidase or due to irradiation-induced enhancement in tissue extractability caused by dissolution and depolymerization of cell wall polysaccharides (Alothman et al., 2009). While DPPH radical scavenging activity is primarily due to antioxidants other than phenolics, FRAP values are chiefly due to the activities of phenolic compounds (Ali et al., 2019). In agreement with literature reports, the results of TPC (discussed in Section 3.1.4) and FRAP values were found to be strongly correlated. Similar findings on higher DPPH radical scavenging activities and FRAP values have been reported for 3 kGy-irradiated tomato pomace compared to its non-irradiated counterpart (Khalaf et al., 2014).

Alterations in total ascorbic acid contents

The amounts of ascorbic acid contents of the control and ɣ-irradiated sets of plantains during storage (assessment period for sample acceptability) are presented in Figure 8d. Ascorbic acid contents in the plantains reduced significantly ($p < .05$) with progression of senescence. The amounts of ascorbic acid in the control set were found to be higher than those of the irradiated sets up to day 9 (mid-senescence), followed by a steady decrease of the same until the end of the assessment period. During irradiation, the ionizing radiation reacts with water molecules present in foods and releases electrons which leads to production of highly reactive free radicals. These free radicals then react with the antioxidants (such as vitamin C), change their structures, and reduce their activities (da Silva Aquino, 2012). Since this degradation continues beyond the time of irradiation, loss of vitamin C in irradiated foods was found to occur during storage but this effect was less pronounced in non-irradiated foods (Diehl, 1967). This finding is supported by de Figueiredo et al. (2014) who studied the effects of ɣ-irradiation (0.8 kGy) on carotenoids and vitamin C contents of papayas (*Carica papaya* L.) when stored at 24 ± 2°C and found that irradiation reduces the vitamin C contents in these fruits more than their non-irradiated counterparts.

Alterations in APX and PPO activities of ɣ-irradiated green plantains

Alterations in APX activities. APX activities significantly ($p < .05$) increased in ɣ-irradiated sets up to day 9 (Figure 8e) and then decreased; whereas in the control set, activities steadily decreased from day 1. The APX activity was higher in 0.04 kGy-irradiated plantains throughout the storage period. Identical findings have also been reported from our laboratory by Pal and Bhattacharjee (2016) who studied the effect of ɣ-irradiation (0.02–2.5 kGy) on sensory and physicochemical properties of marigold (*Tagetes erecta* l.) cut flowers and reported that APX activities in ɣ-irradiated flowers increased during storage up to day 8, after which the activities declined. This phenomenon of increase in APX enzyme activity during the initial days of storage could be owing to the prevention of oxidation in cells (Pal & Bhattacharjee, 2016). During later days of storage, the reduction in the activity of APX is possibly due to the increase in concentration of H_2O_2 (Wi et al., 2007).

Alterations in PPO Activities. Occurrence of browning in the plantains was chiefly due to oxidation of phenolic compounds to melanin catalyzed by PPO (Thomas & Nair, 1971). The PPO activities of both ɣ-irradiated and control sets of plantains were found to increase significantly ($p < .05$) with the advancement of ripening (Figure 8f) up to day 18 (late senescence) followed by a steady decrease until the end of their respective shelf-lives. PPO activity was observed to be the highest in the control set followed by those in 0.08, 0.06, and 0.04 kGy-irradiated plantains. Higher PPO activity indicates higher rate of browning and ripening in the plantains which further validates our findings on color and texture profiles (discussed in Section 3.1.3, respectively). These results are in consonance with the findings of Lu et al. (2005), who investigated the effects of ɣ-irradiation (0.5, 1.0, and 1.5 kGy) on PPO activity of fresh-cut celery when stored at 4°C.

In summary, the assessment period of the control set and 0.04, 0.06, and 0.08 kGy-irradiated green plantains were 17, 22, 22, and 21 days, respectively, based on sensory properties. However, physicochemical and biochemical assays including evaluation of antioxidant properties revealed that 0.04 kGy-irradiated plantains were acceptable for direct consumption up to 20 days; whereas, the control, 0.06 and 0.08 kGy-irradiated plantains were acceptable for 12, 18, and 17 days, respectively. Thus it could be concluded that 0.04 kGy was the most effective dose of ɣ-irradiation for enhancing the shelf-life of plantains up to 20 days, viz. a lead of 8 days with respect to the control set of plantains (12 days). Although published literature states 0.15–0.30 kGy to be the optimum dose range for extension of shelf-life of *Musa paradisiaca* (Aina et al., 1999 and Viveka et al., 2014), our studies indicate a very low dose of 0.04 kGy to be the best dose for the local *desi* variety of *Musa paradisiaca* of West Bengal. Thus, low dose ɣ-irradiation could be effective for preservation of agro produce, alternative to high-energy consuming cold storage technology.

3.2 | Estimation of best doses of ɣ-irradiation to be applied on green plantains for enhancement of serotonin-melatonin contents individually

From the preliminary studies, it was observed that ɣ-irradiation doses above 0.5 kGy enhanced the contents of the above-mentioned antioxidant molecules. Thus, the study was designed for 0.6, 0.8, and 1 kGy-irradiation doses for 25 days. The analyses for contents of L-tryptophan (as precursor)-serotonin-melatonin for the control set and the above-mentioned irradiated sample sets were conducted for 17, 18, 17, and 15 days (only), respectively, since the samples thereafter were completely spoiled and could not be analyzed.

3.2.1 | L-tryptophan, serotonin, and melatonin contents in ɣ-irradiated green plantains

Figure 9 depicts the visual changes in control and ɣ-irradiated (0.6–1 kGy) sets of plantains. Figure 10a,b represent the chromatograms of a mixture of pure standards of L-tryptophan, serotonin, and melatonin; and the QuEChERS-SPE extract of 0.6 kGy-irradiated green plantains on day 12, respectively.

In the preliminary study, it was observed that, plantains ɣ-irradiated under rotation mode with polystyrene cushions had relatively higher contents of serotonin and melatonin than those which were irradiated without polystyrene cushions and in static (no-rotation) mode. This was possibly owing to better attenuation (ensured from manufacturer's end and enhanced by using polystyrene cushions) and greater uniformity of dose distribution (under rotation mode) achieved during ɣ-radiation of green plantains resulting in uniform irradiation of the samples (Anonymous, 2021e) and thus enhancement in antioxidant production.

The highest content of L-tryptophan (121.87 μg/g D.W) was observed in plantains on day 0 which decreased with progression of senescence. The highest content of serotonin was observed in 1 kGy-irradiated plantains (135.03 μg/g D.W, i.e., 44.10% enhanced content vis-à-vis that in non-irradiated plantains) on day 10 followed by that in 0.8 kGy-irradiated plantains (128.86 μg/g D.W) on the same day. Melatonin content was found to be the highest in 0.6 kGy-irradiated plantains (7.20 μg/g D.W, i.e., 146.57% enhanced content vis-à-vis that in non-irradiated plantains) on day 18 followed by that in 0.8 kGy-irradiated plantains (6.53 μg/g D.W) on day 17. The changes in serotonin, L-tryptophan, and melatonin contents with the progression of storage have been depicted in Figure 10c–e, respectively.

Plantains possessed the highest content of L-tryptophan (121.87 μg/g D.W) in their initial stage of senescence, which decreased gradually (4.38 μg/g D.W) with storage (Figure 10d). Serotonin contents in plantains increased and attained maximum value on day 10 (mid- senescence) which decreased steadily thereafter (Figure 10c). However, melatonin contents steadily increased with senescence (Figure 10e). These findings of the present study corroborated well with those of Foy and Parratt (1960) and Adao and Gloria (2005) who showed that the amounts of serotonin in plantains increased during ripening followed by decrease during over-ripening. The increase of melatonin during ripening also supports the findings of Van Tassel et al. (2001) who found enhancement of amount of melatonin during ripening and over-ripening phases in two cultivars of tomato. Besides, bioconversion of L-tryptophan to melatonin via serotonin (Nawaz et al., 2016) enhances contents of melatonin in plants in the last phase of senescence with concomitant reduction

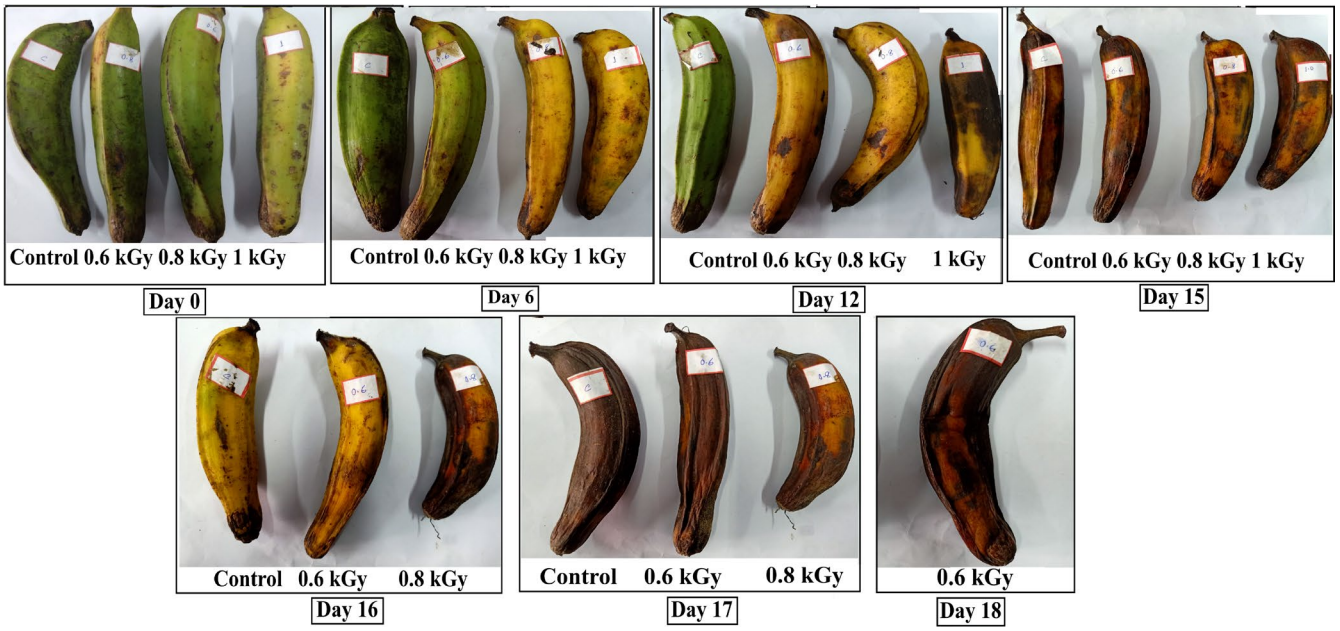

FIGURE 9 Physical changes in ɣ-irradiated (0.6–1 kGy) plantains and control samples during 18 days of storage when stored at 23 ± 2°C, 70 ± 2% RH in an environmental chamber

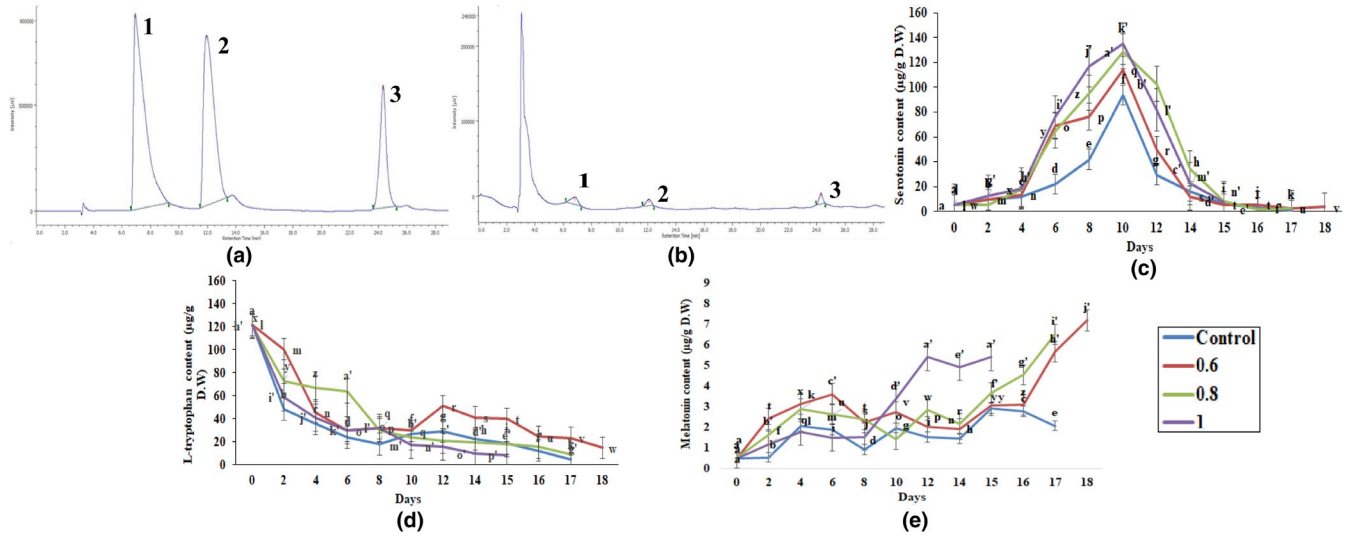

FIGURE 10 High performance liquid chromatography (HPLC) chromatogram of a mixture of—(a) Standard serotonin (1), L- tryptophan (2) and melatonin (3); (b) HPLC chromatogram of QuEChERS-SPE extract of 0.6 kGy-irradiated green plantains on day 12 containing serotonin (1), L- tryptophan (2) and melatonin (3); (c) Serotonin (µg/g D.W); (d) L-tryptophan (µg/g D.W); and (e) Melatonin (µg/g D.W) in ɣ-irradiated (0.6, 0.8, and 1 kGy) plantains and control sets during 18 days of storage; %RSD of HPLC analyses results: ≤3%. The limits of quantification for L-tryptophan, serotonin, and melatonin were 1.88, 0.89, and 0.51 µg/L, respectively. Each value stands for mean ± *SD* of three sets of experimental data. Different alphabets denote that mean values belong to different subsets at $p < .05$

TABLE 1 The in vitro synergistic effect (SE) of antioxidants

Sample name	Serotonin (µg/ml)	L-tryptophan (µg/ml)	Melatonin (µg/ml)	SE[f]
Serotonin1	5.52	0.00	0.00	–
Serotonin2	1.10	0.00	0.00	–
Serotonin3	135.03	0.00	0.00	–
Serotonin4	3.68	0.00	0.00	–
L-trytophan1	0.00	121.87	0.00	–
L-trytophan2	0.00	4.38	0.00	–
L-trytophan3	0.00	16.99	0.00	–
L-trytophan4	0.00	14.76	0.00	–
Melatonin1	0.00	0.00	0.52	–
Melatonin2	0.00	0.00	1.09	–
Melatonin3	0.00	0.00	3.38	–
Melatonin4	0.00	0.00	7.20	–
Sample 1 (Control set on day 0)	5.52	121.87	0.52	1.755 ± 0.004^{a}
Sample 2 (Control set on day 17)	1.10	4.38	1.09	0.100 ± 0.010^{b}
Sample 3 (1 kGy-irradiated sample on day 10)	135.03	16.99	3.38	1.096 ± 0.004^{c}
Sample 4 (0.6 kGy-irradiated sample on day 18)	3.68	14.76	7.20	-1.322 ± 0.003^{d}

Note: [a–d]Different letters in a column indicate significant differences at $p < .05$ level. [f]SE values are the mean ± *SD* values of three independent samples.

in both L-tryptophan and serotonin contents. Although literature exists on enhancement of contents of melatonin production with ripening, there is no report on its enhancement consequent to ɣ-irradiation. This study reports for the first time on the effectiveness

of ɣ-irradiation treatment in enhancing the amounts of the phytoremediator antioxidant molecules, namely serotonin and melatonin in green plantains.

Thus the findings of the current investigation are in harmony with the bioconversion pathway of melatonin production in plants. Besides it is known that phytohormone melatonin functions as an effective radioprotector (El-Desouky et al., 2014) and thus the increased production of serotonin and melatonin in irradiated plantains for them to act as remediators (or scavengers) is a part of the protective mechanism against irradiation-induced overproduction of reactive oxygen species.

3.2.2 | Antioxidant synergy in ɣ-irradiated plantain extract obtained by QuEChERS- SPE

In the present study, the SE values revealed that samples having the highest amount of serotonin (1-kGy ɣ-irradiated green plantains on day 10) as well the non-irradiated control set of plantains (on day 0) possessed a synergistic consortium of the three antioxidants (serotonin, melatonin, and L-tryptophan) (Table 1). These results indicated that even after treatment with ɣ-radiation, the natural food (antioxidant) synergy in the plantains was unperturbed. In contrast, the non-irradiated set of plantains on their last day of analysis (late senescence) as well as the samples having highest amount of melatonin (0.6-kGy ɣ-irradiated green plantains on day 18 [late senescence]) did not exhibit synergism among the said antioxidants.

Irradiation doses of 1 kGy and 0.6 kGy were the best doses that triggered the antioxidant(s) production pathways and samples irradiated with the said doses thus exhibited enhancement in contents of serotonin (1.44 folds [44.10%]) and melatonin (2.45 folds [146.58%]) vis-à-vis their non-irradiated counterparts. Furthermore, 1 kGy-irradiated plantains on day 10 (possessing highest serotonin) continued to exhibit natural synergism (viz. food synergy) among the three antioxidants (L-tryptophan, serotonin, and melatonin) as was present in the non-irradiated control set on day zero. Plantains irradiated at 0.6 kGy on day 18 (late senescence), which contained highest amount of melatonin, although was unfit for direct human consumption, could be harvested as a potential source of phytomelatonin. Thus, this investigation also encompasses waste-valorization through utilization of completely senesced plantains as potential sources of biotherapeutic molecules which have promising uses as nutraceutical food-cum-therapeutic supplements.

4 | CONCLUSION

Very low dose of ɣ-irradiation viz. 0.04 kGy was ideal for shelf-life extension of the *desi* variety of green plantains; whereas, low doses of 0.6 and 1 kGy contributed to enhancement of its contents of phyto-antioxidants, chiefly melatonin, and serotonin, respectively, additionally preserving antioxidant synergy in 1 kGy-irradiated samples. Our findings strongly indicate that low dose ɣ-irradiation could augment production of the phytoremediator molecules, namely serotonin and melatonin in green plantains conferring irradiated plantain to be a potential source of food antioxidants. Plantains in late senescence which otherwise are sensorically unacceptable could be utilized as potential source of these important biotherapeutic molecules. This way, the wasted plantains could be upcycled by utilizing them as nutraceutical products.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

AUTHOR CONTRIBUTIONS

Poulami Sarkar: Data curation; Formal analysis; Investigation; Methodology; Validation; Writing-original draft. **Dipshikha Tamili:** Data curation; Formal analysis; Investigation; Methodology; Validation; Writing-original draft. **Paramita Bhattacharjee:** Conceptualization; Supervision; Validation; Writing-review & editing.

DATA AVAILABILITY STATEMENT

All data are provided in the manuscript in tables and figures.

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Dear Prof. Bhattacharjee

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Thank you for submitting your paper for publication in our journal. I am pleased to say that it has been reviewed and accepted for publication as an **original scientific paper** in Vol. 62 Issue 2/2024 of *Food Technology and Biotechnology*.

Thank you once again for your contribution.

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