Formulation and Characterization of Catechins Encapsulated Starch Nanoparticles based Functional Tea Product

Thesis submitted in partial fulfillment of the

requirements for the degree of

DOCTOR OF PHILOSOPHY

by

Kamal Narayan Baruah

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December 2024

Dedicated

To

my beloved brother

Anirban

School of Agro and Rural Technology
Indian Institute of Technology Guwahati
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CERTIFICATE

It is certified that the work contained in this thesis entitled **"Formulation and Characterization of Catechins Encapsulated Starch Nanoparticles based Functional Tea Product"** submitted by **Mr. Kamal Narayan Baruah** for the award of the degree of Doctor of Philosophy has been carried out in the School of Agro and Rural Technology, Indian Institute of Technology Guwahati, and Gifu University Japan, under our supervision. To the best of our knowledge, this work is not submitted elsewhere for the award of any other degree or diploma.

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DISCLAIMER

The experimental, modelling and characterization related data presented in this Ph.D. thesis was carried out by me and is reported after due verification. To the best of my knowledge, the work summarized in this Ph.D. thesis is not submitted elsewhere for the award for any other degree or diploma.

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Ph.D. Thesis Abstract

The aim of this thesis is to improve the physiological impact of catechins using nanotechnology. Catechins, a well known tea polyphenol was encapsulated in starch nanoparticle to enhance its bioaccessibility. The therapeutic benefits of tea catechins are limited due to their low bioavailability, which is a result of their inadequate stability in the gastrointestinal tract and limited capacity to pass through the intestinal barrier.

To retain their functionality in the human gut system, the Ph.D. thesis targets the encapsulation of catechin extracts derived from S3A3 Assam tea cultivar in starch nanoparticle carapace and for their subsequent utility as a key ingredient in the black tea based organoleptic functional tea beverage formulation. Accordingly, the conducted Ph.D. thesis research works involved the following hierarchy: (a) catechins characterization and hot water/enzymatic extraction and characterization of the catechins aqueous extract, (b) preparation and characterization of ultrasound assisted acid hydrolysis based starch nanoparticles system, (c) nanoencapsulation of EGCG and catechin extracts in starch nanoparticles systems, and subsequent evaluation of their micellar cholesterol solubility, and (d) encapsulated catechins extracts based formulation of a novel functional tea beverage with enhanced bio-accessibility. The relevant investigations and important findings have been briefly presented as follows.

Conventional characterization of catechin content in fresh tea leaves refers to the quantification of (-)- Epigallocatechin Gallate (EGCG), (-)- Epicatechin Gallate (ECG), (-)- Epigallocatechin (EGC), (-)- Epicatechin (EC), and (+)- Catechin (C) catechins for their medicinal benefits. The first objective of the Ph.D. thesis targeted a detailed catechin profile in the tea cultivars and their comparative assessment with the best findings in the literature. The investigations affirmed upon

the superiority of the S3A3 tea cultivar in terms of the total catechin concentration and with 329.29 mg/g of dried tea leaves. Further, the enzymatic extraction study was carried out for the selected S3A3 tea cultivar and with the central composite rotatable design (CCRD) based experimental model. Due to their proven enzyme activity, the cell wall degrading enzymes namely cellulase and pectinase were utilised in equal concentrations for enzymatic extraction. Three parameters namely extraction time, temperature, and enzyme concentration were studied as per statistical design approach-based experiments and in the value range of 20 - 40 mins, 60 - 90°C, and 100 - 200 EU/g, respectively. Thereby, the effect of the three responses were evaluated on the epigallocatechin gallate (EGCG), epicatechin Gallate (ECG), epigallocatechin (EGC), epicatechin (EC), catechin (C), and total catechins content in the aqueous extract phase. Accordingly, these entities respectively varied as 73.17 - 144.68, 19.82 - 50.02, 7.37 – 47.84, 0.87 – 24.14, 0.52 - 24.14, 1.3 - 16.86, 128.39 - 260.53 mg/g respectively. Response surface analysis enabled valuable insights into the binary effects of mentioned variables. Numerical integration-based optimization conveyed the best extraction conditions with the adopted technique and thereby affirmed an enhancement in the total catechin and EGCG content by 51.26 and 15.36% with respect to the conventional hot water extraction process.

The second major objective of the Ph.D. thesis involved the synthesis and characterization of potato starch nanoparticles and with the sonication assisted acid hydrolysis method. The experimental studies examined different levels of sulfuric acid molar concentration (ranging from 3.16 to 5 M) and hydrolysis duration (1 to 3 days) and thereby inferred upon the reduction in the preparation time (between 1 to 2 days) in comparison to the conventional hydrolysis process (between 7 to 9 days' time duration). Further, the investigations affirmed improved characteristics of the synthesized starch nanoparticles, a promising feature being desired for their effective utility

as a wall material in the eventually targeted nanoencapsulation of catechins extracts. The relative crystallinity and melting point of the starch nanoparticles increased for the case of enhanced sulfuric acid concentration. X-ray diffraction (XRD) analyses revealed alterations in the starch granule's structure and potential reorganizations during retrogradation. Thereby, the findings conveyed relevant modifications to the crystalline structure of the nanoparticles. The best findings for the starch nanoparticle synthesis have been affirmed for 3 days duration, 4M sulfuric acid and 90 mins of post sonication treatment. Accordingly, the best characterization data refers to the average particle size, PDI, yield, melting point, and relative crystallinity values of 66 nm, 0.362, 29%, 318.99°C, and 64.32%, respectively.

The third major objective involved the micellar cholesterol solubility study of the EGCG and catechin extracts in their raw and nano-encapsulated forms (in the starch nanoparticle system). Inclusion complex method was followed for the nanoencapsulation of EGCG and catechin extracts. The in-vitro micellar solubility study affirmed that the EGCG and catechin extracts were subjected to significantly reduced cholesterol concentration and by 91.63% (2.5mg) and 59.03% (5mg) respectively. Novel insights were achieved in the disappearance of inhibitory action of micellar cholesterol solubility by EGCG/catechin extracts and for the cases of prior to and after nano-encapsulation with starch nanoparticles. The EGCG-SNP complex was further characterized for its morphology, thermal, and crystalline properties. The FESEM results affirmed successful nano-encapsulation of the EGCG-SNP complex and in the particle size range of 20-120 nm diameter. The thermal studies revealed that the EGCG-SNP complex possessed higher thermal stability. The melting point of the EGCG-SNP complex was found to be 282.9°C.

The final Ph.D. thesis objective targeted functional tea beverage formulation that shall possess enhanced bioaccessibility, good organoleptic properties, and eliminates the bitter taste attribute of

the green tea. To do so, firstly, catechin extracts were obtained with the hot water extraction method and with the fresh tea leaves of the S3A3 cultivar. The catechin extracts had a notable level of purity at 56.1%. Thereafter, catechin extracts were encapsulated in the starch nanoparticle carapace and for the targeted enhanced bioaccessibility. The encapsulated catechin extracts were examined for their enhanced bioaccessibility through in-vitro digestion and in terms of the percentage of the antioxidant activity (%AA). The obtained findings affirmed enhancement of AA recovery by 81.94, and 56.01% for CE:SNP (1:1, w/w) and CE:SNP (1:2, w/w) cases respectively, and in comparison to the catechin extracts in their raw form. The encapsulated catechin extracts were also examined for their bio-accessibility status in hot water liquid formulations of the extracts. The findings demonstrated similar enhancement in bioaccessibility for both dry and liquid forms. Finally, a functional tea drink formulation was created, and its sensory qualities were optimized with the 9 point hedonic scale and the fuzzy logic method. Among alternate formulations, the best formulation was achieved at the hot aqueous solution concentration of 1000 µg/mL of CE:SNP (1:1 ratio by weight).

In summary, the Ph.D. thesis recommends an appropriate strategy to enhance bioaccessibility of catechins by two fold as well as make it suitable to add in a sensory acceptable instant tea mix. The findings can inspire design of not just instant tea beverages but other functional beverages and herbal medications.

Novelty Statement

The subjective novelties of the conducted Ph.D. thesis research work have been listed as follows:

1. Optimality of catechins enzymatic extraction in terms of the parametric combinations of extraction time, temperature, and enzyme concentration. Also, for the first time, two cell wall degrading enzymes namely cellulase and pectinase were used in the enzymatic extraction process of catechins from fresh tea leaves. Further, for the first time, the effect of enzymatic extraction on individual catechin types (EGCG, ECG, EGC, EC, and C).were evaluated and through the response surface methodology.
2. The Ph.D. thesis research work further improved the current state-of-the-art in the preparation of starch nanoparticles and in the specified research theme of the sonication assisted acid hydrolysis method. The Ph.D. thesis research work enabled significant reduction in synthesis duration and the achieved nanoparticles had comparatively better attributes in terms of morphology, thermal, and crystalline attributes.
3. For the first time, a simpler process based green encapsulation of EGCG and catechin extracts were achieved with the inclusion complexation method and bioactivity studies were conducted in terms of the in-vitro micellar solubility study to mimic the reduction of gut cholesterol.
4. For the first time, a novel formulation of a novel functional beverage with the encapsulated catechin extracts was achieved with best combinations of in-vitro digestion based bio-accessibility and good organoleptic property being affirmed by 9 point hedonic scale and fuzzy logic sensory methodologies.

The adopted methodology is generic in nature and can be conveniently extended for the nanoencapsulation of herbal and Ayurvedic extracts in functional food products.

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Nomenclature

Abbreviations

AA	Antioxidant activity
ANOVA	Analysis of variance
DSC	Differential Scanning Calorimetry
FESEM	Field emission scanning electron microscopy
HPLC	High pressure liquid chromatography
FTIR	Fourier transform infrared radiation
RSM	Response surface methodology
TPC	Total polyphenolic content
TFC	Total flavonoid content
PDI	Polydispersity index
ISO	International Organization for Standardization

Nomenclature

IC	Inclusion complexation
HWE	Hot water extraction
IG	Ion gelation
ET	Extraction temperature
TCC	Total catechin concentration
RC	Relative crystallinity

EU	Enzymatic unit
AH	Acid hydrolysis
US	Ultrasonic treatment
SNP	Starch nanoparticle
CE	Catechin extracts

Notations

A	Extraction time
B	Temperature
C	Enzyme Concentration
F1	Catechin extracts 250 µg/mL + Black tea 0.5g
F2	Catechin extracts 500 µg/mL+ Black tea 0.5g
F3	Catechin extracts: Starch nanoparticles (1:2), 500 µg/mL+ Black tea 0.5g
F4	Catechin extracts: Starch nanoparticles (1:2), 1000 µg/mL+ Black tea 0.5g
F5	Catechin extracts: Starch nanoparticles (1:1), 500 µg/mL+ Black tea 0.5g
F6	Catechin extracts: Starch nanoparticles (1:1), 1000 µg/mL+ Black tea 0.5g
F7	Control with only black tea 0.5g
R^2	Coefficient of determination

Chapter 1:

Introduction and Literature Review

Chapter 1

Introduction and Literature Review

This chapter discusses the roadmap for the formulation of a catechin based functional tea beverage with enhanced bioavailability and bioactivity. Section 1.1 narrates the importance of tea catechins from the nutritional and medicinal perspective and how efficient, sustainable extraction strategies are needed for the exploration of the true potential of the tea catechins. It also discusses the potential utility of nanotechnology such as the application of starch nanoparticles in improving the bioavailability of the catechins through encapsulation and the influence it would have on a key bioactivity property namely reduction of cholesterol. In section 1.2, the available prior art has been discussed for the catechin profile in Indian tea cultivars, enzymatic extraction avenues for catechin extraction from fresh tea leaves followed by alternate processes being adopted for starch nanoparticle synthesis. It also details upon the contemporary literature in the field of encapsulation techniques for catechins present in tea and its bioactivity studies associated to the reduction of in-vitro cholesterol adsorption and finally formulation of functional tea drinks. In section 1.3, the possible scope for further research on the extraction, encapsulation, and formulation of a catechin based functional tea beverage has been discussed in detail. Thereafter, section 1.4 conveys the broad objectives being set to address the detailed research gaps. Finally, section 1.5 summarizes the details of the thesis content elucidated in various chapters.

1.1 Orientation and Motivation to the Field of Study

In today's world, while some people starve to death, others are bestowed with surplus food but are deprived of basic nutritional requirements. In the modern world, the concept of taste

over nutrition has taken precedence, and eventually, the society largely consumes foods that do not meet the nutritional requirements of human body. Thereby, functional food concepts are to be explored. Functional foods refer to a meal (solid or liquid) providing specific nutritional benefits for human body and therefore regarded as a healthy food. Such foods assure nutritional benefits from the naturally occurring micro or macro nutrients added through fortification and enrichment methods. The macronutrients refer to protein, carbohydrate, and lipids which are consumed in greater proportions . The micronutrients refer to vitamins, minerals, and bioactives which are often consumed in lower proportions. Food bioactives are present in fruits, vegetables or animal foods in smaller constitution and have been proven with medicinal benefits for the human body. Thereby, adopting fortification methods, they can be incorporated as an ingredient in functional foods or drinks. Accordingly, conventional foods can be functionalized such as cookies fortified with vitamin C, rice fortified with iron, salt fortified with iodine etc.

In the northeastern region of India, tea is the most consumed beverage and is also readily available due to wider cultivation. In Assam, over 800 tea estates cultivated tea. Fresh tea leaves contain catechin, a bioactive substance and flavan-3-ol compound. The catechins are responsible for the medicinal benefits of fresh, green, and white tea. In this Ph.D. thesis an endeavor is made to formulate a functional drink using tea catechin extract as an ingredient.

The key features targeted in the Ph.D. thesis aims to overcome the limitation associated to direct consumption of catechin, which is detrimental for derived functionality as more than 95% of the catechin consumed is lost in the complex digestion environment of the human body. Accordingly, the thesis aims to convert encapsulated catechin extracts into a functional drink formulation with enhanced bioavailability and nutritional benefits.

1.2 Targeted Perspectives

Considering the mentioned key features, the targeted perspectives for the Ph.D. thesis have been outlined in the following sections. These methods ensure that the bioactives are enveloped in food grade materials that henceforth serve as a protective environment for the bioactives in the harsh digestive environment. Thus, bioactives can survive during digestion and offer desired nutritional functionality due to minimal loss of their constitution in the human digestive systems.

a. Functional significance of tea catechins
b. Low cost technologies for catechin extraction from tea
c. Low cost synthesis of starch nanoparticles
d. Simpler yet effective encapsulation of tea catechins
e. Bioactivity studies of tea catechins
f. Formulation of catechin rich functional tea beverage

1.2.1 Functional Significance of Tea Catechins

Tea is one of the world's oldest beverages produced from the shoot tips (two leaves and a bud or three leaves and a bud) of the evergreen tea plant. Taxonomically tea is known as *Camellia sinensis* and belongs to the family Theaceae. Commercially tea is found in three cultivars viz. *C. sinensis* L. (Chinese variety), *C. assamica* (Assamese variety), and *C. assamica* ssp. Lasiocalyx (Combod variety). These three cultivars have been interbred several times and accordingly mixed clonal variations ranging from extreme China type to Assam type have been realized and cultivated (Sharma & Venkataramani, 1974; Thwaites et al., 2020).

Tea contains important bioactive substances such as polyphenols, caffeine, and theophylline. These constituents enhance the nutraceutical value of the beverage. Tea polyphenols include

flavonols (quercetin, kaempferol, myricetin), flavan-3-ols (catechins and theaflavins), a smaller percentage of purine alkaloids (caffeine and theobromine), gallic acid derivatives (gallic acid, 5-galloylquinic acid), and hydroxycinnamate quinic esters (caffeoylquinic acids). Among these biochemical substances, catechins and theaflavins are the most important in terms of antioxidant capacity and organoleptic attributes (Higdon & Frei, 2003; Zhang et al., 2018).

Catechins are the most potent antioxidants among all existent polyphenols in the tea. Catechin and its derivatives constitute a majority of the polyphenols present in the tea. The health benefits of catechins are not just limited to their high antioxidant potential. Tea catechins have anti-carcinogenic properties and assist in the reduction of cardiovascular disorders (Higdon & Frei, 2003; Yang & Wang, 2016). As demonstrated in various animal studies, catechins exhibited profound ability to inhibit carcinogenesis of the skin, lung, esophagus, stomach, liver, small intestine, colon, bladder, prostate, and mammary glands demonstrated in various animal studies (Yang et al., 2017; Yang & Wang, 2016). The major catechin types in tea refer to (+)- catechin (C), (-)- epigallocatechin gallate (EGCG), (-)- epigallocatechin (EGC), (-)- epicatechin gallate (ECG), and (-)- epicatechin (EC) (Figure 1.1). A striking limitation of catechins is their low bioavailability. In several in-vivo studies it was analyzed that many alternate catechins do get deteriorated as they travel through the harsh environment of the gastrointestinal tract.

1.2.2 Low Cost Technologies for Catechins Extraction from Tea

Several benefits of the nutraceutical ingredients in tea beverage are subject to the efficient extraction of tea polyphenols during the tea brewing process itself. The quality of tea drink in terms of its nature and bioactive constitution is dependent upon the brewing conditions, temperature, solid/water ratio, and extraction time. Accordingly, considering these variables

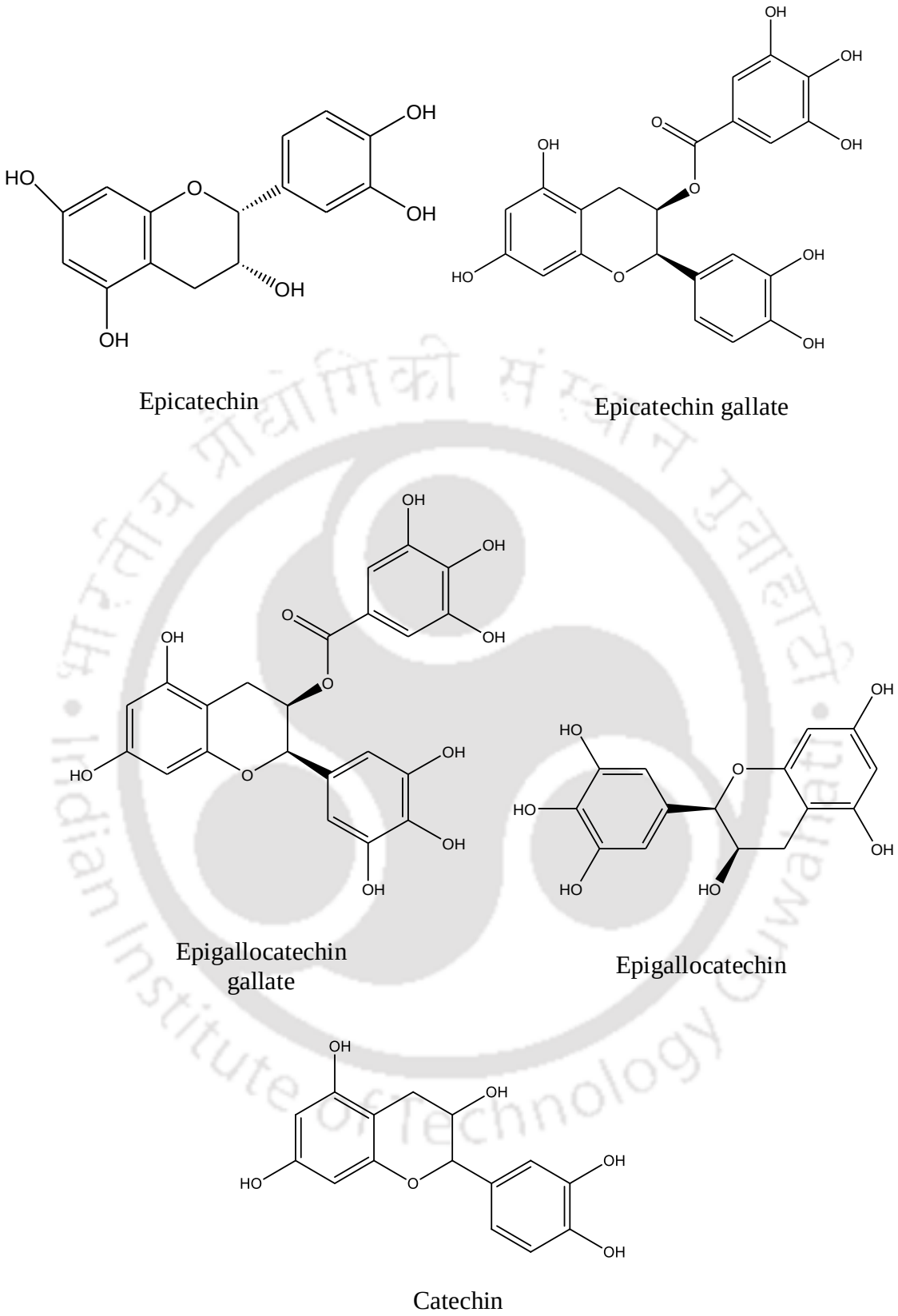


Figure 1.1: Structure of major catechins in tea leaves.

various extraction methods have been developed for the better retention of the bioactives. Thus, both extraction method and the mentioned parameters together have a significant role in the antioxidant potential and organoleptic properties of the tea beverage.

During brewing or extraction, both temperature and time affect the extraction of catechins from tea leaves. Solvents such as methanol, acetonitrile, and water are often used for catechin extraction. However, they are disadvantageous with respect to aqueous extraction, as solvents adopted are often non-green and demand deployment of auxiliary technologies to meet the stringent environmental legislation requirements. Thus, aqueous extraction is the most inexpensive and derived for its p. In the conventional hot water extraction method, tea leaves are placed in a boiling water bath and for extraction time of about 1 – 6 h (Cheng et al., 2023). Often, mechanical agitation is also used during conventional hot water extraction for enhanced extraction yield. Also, higher temperature increases the extraction of the polyphenols. However, catechins are highly degraded at a higher temperature (>100℃) and for longer duration. The catechins undergo structural changes at higher temperature, and do get deteriorated (Liang et al., 2007). Solvent extraction is targeted by applying higher pressure (1000 - 2000 psi) along with higher temperature, (50 - 200℃) for even better and efficient extraction with alternate organic solvents such as ethanol, methanol, ether, and acetonitrile. However, even in such conditions, higher temperature causes degradation of the catechins and such methods are not favorable for the extraction of catechins. Cold brewed tea, on the other hand, provides better organoleptic properties as well as better retention of catechins (Yang, et al., 2014). In summary, extraction at a lower temperature and shorter extraction times has the dual benefits of better taste and preservation of catechin. However, it is not feasible at a large scale due to lower extraction yield. Henceforth, low temperature extraction is modified or combined with several other methods such as higher hydrostatic

pressure, pulsed electric field, ultrasound, and enzymatic treatment (DEMİR et al., 2016; Hong et al., 2013; Koch et al., 2020).

Ultrasound utility in the extraction process profoundly enhances the diffusion rate, and as a result the extraction yield enhances prominently. Alternatively, enzymes are used as an innovative approach for the extraction of catechins. Enzymes such as cellulose, pectinase, amylase, tannase, etc. have been proven for the extraction of polyphenols from plant materials. The cell wall degrading enzymes hydrolyze the cell wall of the tea leaves and accordingly enhance diffusion rates of the catechins. This translates into higher extract yield. Among the mentioned enzymes, tannase enzyme fostered a reduction of gallated enzymes such as EGCG and ECG and their conversion into gallic acid (Ghandahari Yazdi et al., 2019). Considering the importance of EGCG and ECG in many derived health benefits such as reduction of cholesterol adsorption, antidiabetic effect, antimicrobial effect etc., tannase enzyme can be inferred to be not relevant being for the targeted higher yield of EGCG and ECG. On the other hand, pectinase and cellulase enzymes efficacy for the extraction of catechins from fresh tea leaves and associated extraction characteristics of alternate catechin compounds shall be explored.

1.2.3 Starch Nanoparticles Synthesis

Starch is an important ingredient in human food system. Plants utilize starch for energy storage. Plant starch constitutes two biopolymers namely, amylose and amylopectin (Figure 1.2). Among these, amylose has aa linear chain possessing only α, 1-4 glycosidic linkage between two glucose molecules. Further, amylopectin is more common among the two mentioned biopolymers. Usually, 70% of the starch granule constitute amylopectin. Amylopectin possess both α, 1-4 and α, 1-6 glycosidic bonds, and is highly branched (Bertoft, 2017).

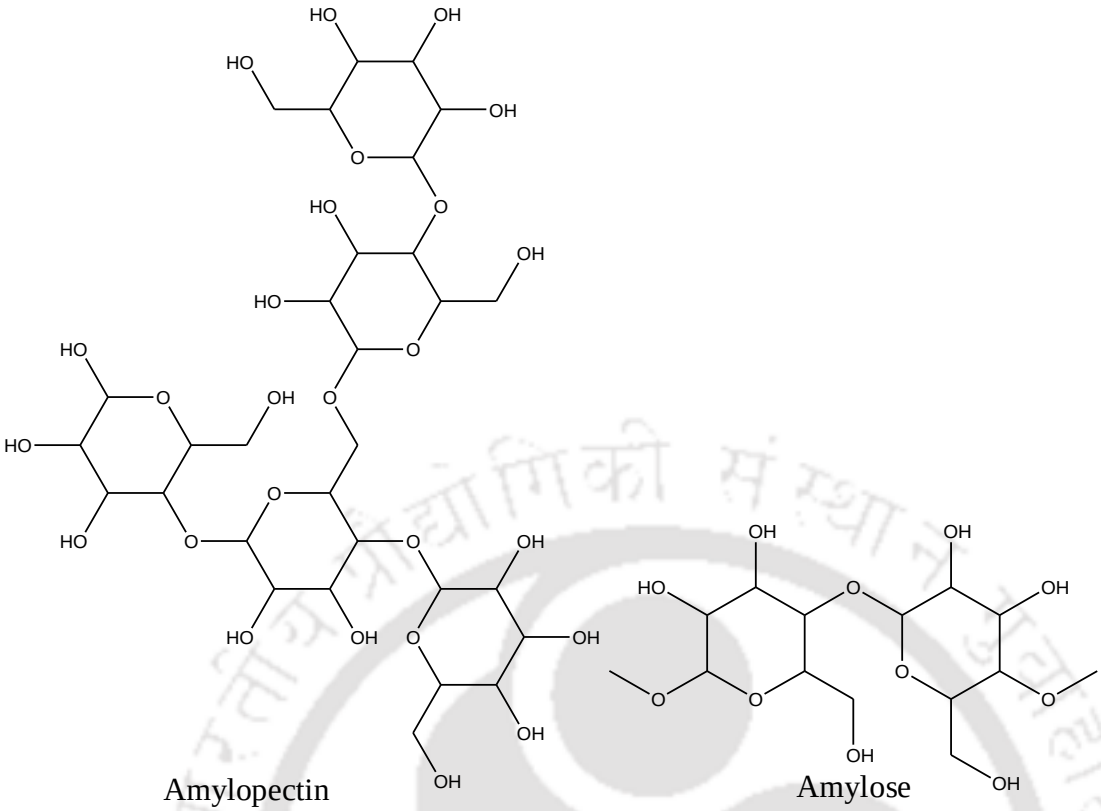


Figure 1.2: Structure of amylose and amylopectin

Compared to other coating materials, starch offers several advantages. Starch is easily available and is cheaper. Starch-based nanoparticles can encapsulate a wide range of substances and henceforth both hydrophilic and hydrophobic substances. The structure of starch has been widely studied and thus has greater customer acceptance. Starch also has many nutritional benefits as it is an integral part of human diet. Starch coated encapsulate has a better controlled release and protection in the harsh environments of gastrointestinal regions. This is due to the capability of the starch molecules to tolerate high temperature or pH environments. On the contrary, other biomolecules such as lipid and protein tend to denature or degrade under similar conditions (Fonseca et al., 2020; Hasanvand et al., 2018; Zhu, 2017).

Starch nanoparticles can be synthesized with both top-down and bottom-up approaches. Among these, the top down approach refers to the preparation of nanoparticles by breaking

down bulk materials into nano sized particles through mechanical or chemical forces. On the other hand, bottom up approach adopt molecular self-assembly strategies for nanoparticle generation with definite shape, size and composition (Abid et al., 2022). Acid hydrolysis is top down method for the preparation of starch nanoparticles. Acid hydrolysis has been successfully used for producing starch nanoparticles. Depending upon the starch source, it takes around 5 to 7 days to realize starch nanoparticles (Duan et al., 2011; Rajisha et al., 2014). The process exhibits two stage kinetics. Also, hydrolysis in the amorphous phase of starch is faster in comparison to the crystalline phase. In order to enhance the formation of starch nanoparticles through acid hydrolysis, several methods are often employed such as enzymatic pre-treatment, ultrasonication, thermal treatment etc. Among these, sonication is a handy tool which can be used in conjunction with acid hydrolysis to reduce the duration required for the preparation of nanoparticles (Kim, Park, et al., 2013).

1.2.4 Encapsulation of Tea Catechins

Catechins are the most constituent polyphenols in the tea and are vital bioactive substances. However, due to the low bioavailability and low permeability of catechins, their utility has not been explored yet to the full potential. This is due to the degradation of catechins during their passage through the gastrointestinal tract. Catechins are highly sensitive to low pH and undergo structural changes during oxidation. During their passage through the human intestinal system after consumption, they get exposed to the high pH environment as well (Cai et al., 2018; Manach et al., 1999; Prakash et al., 2019). Further, the prevalent reactive oxygen species in the intestine system cause high degradation of the catechins (Neilson et al., 2007). In-vitro studies did convey that the bioavailability of tea catechins has been severely affected by gastrointestinal conditions. Net retention of 5.3% was observed for catechins.

Further, the EGC and EGCC exhibited an even more significant decline of 4.6% and 6.1% respectively (Cai et al., 2018).

To resolve this problem, encapsulation has been developed and targeted as an effective solution. Encapsulation facilitates the coating of bioactive substance with a layer of protective material. This material protects the coated substance during the ordeals of storage, processing, and human digestion process itself. Microencapsulation and nanoencapsulation are two well-known approaches being suggested for the encapsulation of polyphenols from tea. The two encapsulation methods differ fundamentally in terms of the size of the deposited or coated particles. The microencapsulation deals with a final particle size ranging in several hundred micrometers (μm). On the other hand, the entrapping nanoparticle substances being entrapped in a protective coating with a radius below 500 nm is targeted in the nanoencapsulation process (Joye et al., 2014). Both methods are very effective to enhance the bioavailability of the tea catechins. However, among these two methods, nanoencapsulation has a comparative edge. Compared to the microencapsulated products, the smaller size of nano-encased particles allows it to get more easily absorbed into the body cells (Galland et al., 2013). Most deployed protective coating materials for encapsulation refer to starch and starch derivatives, proteins, and liposomes. These include inclusion complexation, spray drying, coacervation, antisolvent precipitation, supercritical precipitation, freeze drying, infusion and binding, gel electrophoresis, extrusion, mixing, and adsorption (Puligundla et al., 2017).

1.2.5 Cholesterol Lowering Bioactivity of Tea Catechins

Tea catechins offer many nutritional benefits. These include, antimicrobial, anticancer, antihypertensive, antioxidant properties and anti-atherosclerosis characteristics. Among several such characteristics, a widely studied nutritional property of tea catechins is its ability

to reduce the absorption of cholesterol in the human intestinal system. Being a large molecule, cholesterol is not readily absorbed in the intestinal stage of digestion. Thereby, cholesterol forms a micelle like structure with bile salts, fatty acids, and glycerol (Ogawa et al., 2016). Catechins interact with the micelle structure and result in the coprecipitation of bile salt and cholesterol. This is one of the few medicinal benefits of catechins in which catechin compounds provide their functionality prior to the absorption in the blood plasma. In other words, catechins interact with cholesterol prior to absorption in the intestine and thereby reduce blood cholesterol levels. Accordingly, they reduce the risk of atherosclerosis in the human body (Nagaoka et al., 1999, 2010; Wang et al., 2015).

1.2.6 Tea Catechins Based Functional Beverage

With growing technological advances, the demand for food with health benefits is ever increasing. Functional foods are eatables which upon consumption ensure additional health benefits to human health in lieu of their ingredients. Functional beverages are enriched with ingredients of higher nutritional importance. This is usually achieved through fortification and enrichment. Fortification involves the addition of micronutrients initially absent in the food matrix. On the other hand, enrichment refers to enhancement of already prevalent micronutrient present in the food system but lost in its greater constitution during processing and storage. Ingredients ranging from dietary fiber, polyphenols, proteins, rare minerals, vitamins, etc. are often fortified/enriched in various food products and beverages.

With several important bioactive substances, tea beverages have the potential to provide numerous yet significant health benefits. Catechins are the most important and prevalent nutraceuticals in green tea. More than 90% of the existent polyphenols present in green tea are catechins (Wei et al., 2011). They have higher antioxidant, antimicrobial, anticarcinogenic characteristics (Higdon & Frei, 2003). For these reasons, catechins extracted

from tea can be explored to produce functional tea beverages. Catechins are unstable in aqueous solutions. Catechin stability in bottled tea beverages is always a serious problem. Ascorbic acid is often added to increase the storage life of catechins in bottled tea beverages. Another approach to address this issue is to encapsulate the catechins. Encapsulation will envelope the catechin compounds in a protective carapace and allows it to stay stable in the harsh conditions of the human digestion process. Encapsulation could significantly increase the storage life of catechins significantly. It would also mask the bitter flavor that might arise from catechins. Accordingly, a functional beverage with improved organoleptic properties can be achieved (Viljoen et al., 2017).

1.3 Prior Art

Based on the targeted research perspectives summarized in section 1.1.6, the available prior art can be classified into the following themes:

a. Quantification of the catechin content in tea cultivars.
b. Optimal and efficient extraction of catechins.
c. Preparation and characterization of starch nanoparticles through novel methods
d. Simpler yet effective nano-encapsulation of catechins.
e. Bioactivity studies of cholesterol reduction through catechins.
f. Development of catechins infused functional ready to drink tea beverage.

A brief overview of the available literature in the above-mentioned themes has been presented in the following sub-sections.

1.3.1 Quantification of the Catechin Content in Tea Cultivars.

Tea cultivars are significantly heterogeneous due prolonged mixed breeding amongst the three basic cultivars in any region of their cultivation. These cultivars are well known to be

Assam type, China type, and Combod type. The concentrations of total catechin and individual catechin types vary significantly for alternate types of tea clones and cultivars. Catechin concentration also vary significantly with alterations in location, climate and soil conditions (Wei et al., 2011). A study reported in a relevant prior art has affirmed upon the diversity of catechins in the clonal varieties of north-east India (Sabhapondit et al., 2012). In this study, the authors used ISO 14502:02:2005 method for the estimation of catechins in the fresh tea leaves of the various tea cultivars. Further, the authors delineated upon the catechin profile of ten alternate Assam type clonal varieties namely, TV2, TV12, TV13, TV17, TV21, S3A1, S3A3, Tingamira, TA 17, and T3E3. Amongst these varieties, S3A3 variety had the highest catechin content of 274.0 mg/g of ground tea leaves. Ten China type clonal varieties were also studied by the authors. These refer to TV7, 14/13/3, 14/100/10, 14/100/16, 14/100/6, 317/1, 317/2, 317/3, 317/4, and P126. The China cultivars had comparatively lower catechin content and among the china cultivars, 317/2 had the maximum catechin content of 166.7 mg/g. The nine combod cultivars of tea had intermediate level of catechin concentration of 223.8 mg/g of dry weight. In another study, it was observed that the group of tea cultivars collected from Assam region possessed higher concentration of total catechins of about 22.08% (w/w) of total dry weight. On the other hand, tea cultivars cultivated at Tamilnadu had a comparatively lesser catechin concentration of 12.92% w/w (Gulati et al., 2009).

Catechin content also varies for bud, first leaf, first internode, and second leaf. Previous studies confirmed that the total catechin content for P/11/15 clone of *Camellia sinensis* (L) O. Kuntze is higher for the first internode and first leaf and with a concentration of 20.75% (w/w) and 19.29% (w/w) respectively. On the other hand, catechin content is comparatively lower for the bud (14.35 %, w/w). Epigallocatechin gallate (EGCG) had the highest concentration amongst all catechins. The concentration of EGCG in the bud, first leaf, first

internode, and second leaf were 8.62, 10.25, 11.01, and 9.65 mg/g of dry weight respectively (Samynathan et al., 2016).

Along with the plant location and leaf grade, catechin content is also influenced with the geographical location along with the plant location and leaf grade. In a related prior study, the authors revealed the variation of catechin concentration for tea cultivars from various geographical locations and leaf grade (Zhang et al., 2018). In this study, the authors collected 19 black tea samples from 7 different countries and 28 green tea samples from Sri Lanka and China. Compared to the green tea, catechin content decreased profoundly in black tea (from 193.1 mg/g to 56.07 mg/g). During fermentation process, catechin compounds in the tea leaves converted to other flavonoid compounds and through oxidation and polymerization reactions. However, theaflavin content was higher in black tea. This was due to the conversion of catechins into theaflavins during the oxidation process. The seven types of catechin namely (+)- Catechin, (−)-catechin gallate (CG), (−)-epicatechin gallate (ECG), (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-gallocatechin gallate (GCG), (−)-Epigallocatechin gallate (EGCG) were studied and individual concentration in the tea cultivars were also observed.

Table 1.1 summarizes the available literature with respect to the catechin profile assessed in Indian and north east Indian tea cultivars.

1.3.2 Optimal and Efficient Extraction of Catechins

The method of catechin extraction from tea varies from conventional hot water to enzymatic extraction. Catechins are sensitive to prolonged exposure to high temperature environment. Hence, enzymatic extraction serves as an alternate and pragmatic approach for catechin extraction. Considering these aspects, the prior art has been presented for both conventional (hot water extraction) and enzymatic extraction process and in the following sub-sections.

Table 1.1: Literature data summary in the theme of catechin profile study in various tea cultivars

Sl. No.	Reference	Tea varieties studied	Catechin variants investigated	Highest catechin-best sample
1	Gulati et al., 2009	Combod: UPASI-17, TTL-1, TV-28, UPASI-12, UPASI-18, TV-26 etc. Assam: UPASI-6,UPASI-21, TV-2,TV-3,TV-5, S3A3 , S3A1 etc. China: BS-26, BS-65,BS-1,BS-53,BS-54,BS-14,K-15, HV-39 etc.	ECG, EGCG, EC, EGC	27.03% in TV-26
2	Sabhapondit et al., 2011	Combod: TV9, TV18, TV22, TV23, TV25, TV26, and TV30. Assam: TV2, TV12, TV13, TV17, TV21, S3A1, S3A3,Tingamira, TA 17, and T3E3 China: TV7, 14/13/3, 14/100/10, 14/100/16, 14/100/6, 317/1, 317/2, 317/3, 317/4, and P126.	ECG, EGCG, EC, EGC, +C	27.4 % in S3A3
3	Samynathan et al., 2016	P11-15	ECG, EGCG, EC, EGC, +C	19.51%
4	Zhang et al., 2018	Green tea – Sri Lanka(24), China(4)	ECG, EGCG, EC, EGC, +C, GCG, GC	27.47% in CH grade(Sri-Lanka)

1.3.2.1 Hot Water Extraction of Catechins

The conventional extraction process of polyphenols including catechin compounds from fresh tea leaves and green tea is carried out as hot water extraction. Various other organic solvents such as methanol, acetonitrile, ethyl acetate, chloroform could be also explored for the quality based extraction of polyphenols (Choung et al., 2014). However, in the tea industry for consumer acceptance, hot water extraction method is more commonly used for catechin and polyphenols extraction.

Drying temperature, drying time and solid to water ratio are the most significant independent variables in the hot water extraction process. Several studies considered these three variables and optimized the hot water extraction process performance. In one such study, the authors optimized the hot water extraction conditions for the highest yield of total phenolic and flavonoid content. Accordingly, the best conditions have been reported as 90°C, 40 min, and 1:50 solid to water ratio (S. K. Chandini et al., 2011). In another prior art, the authors have studied the effect of extraction time and temperature on the extraction yield of individual catechin concentrations. It was analyzed that at higher temperatures, gallated catechins such as EGCG and ECG deteriorated significantly in comparison to other catechins. Such losses primarily occurred due to oxidation, polymerization, and epimerization reactions. The optimal yield was achieved at a extraction time, temperature, and tea to water ratio of 30 min, 80°C, and 1:50 respectively (Vuong et al., 2010, 2011). Thus, it can be inferred that the hot water extraction process needs further optimization studies by targeting reduced extraction temperature and time for enhanced catechin recovery.

1.3.2.2 Enzymatic Extraction of Catechins

Enzymatic extraction is a modified form of conventional hot water extraction process. It involves an enzymatic pre-treatment phase prior to the hot water extraction process. The

enzymatic pre-treatment enhances the catechin recovery and reduces the time or temperature required for the hot water extraction of catechins. Various types of enzymes can be used to extract the tea polyphenols. Cell wall degrading enzymes such as cellulase and pectinase were reported in some previous works. Few other researchers also used tannase enzyme and achieved higher polyphenol extraction. Chandini et al., 2011 reported the utility of tannase and pectinase enzymes for the enhanced recovery of polyphenols from black tea. To do so, the extracts were mixed in the enzyme solution (pectinase and tannase) for 2 h incubation period. It was revealed that enzymes namely pectinase and tannase offered enhanced extraction benefits such as extractable solids enhancement by 11.1% and 11.5% respectively. Total polyphenols recovery improved significantly with the tannase enzyme and as 14.3% (w/w). Pectinase didn't assure significant contribution to polyphenol recovery. However, it helps to reduce the briskness index through the simultaneous reduction of caffeine content and improved organoleptic properties of the tea drink. Tea cream reduction was another additional benefit of enzymatic extraction process (65-72% reduction). However, the authors mentioned that the efficiency of tannase reduced significantly for doses greater than 10 U/g.

In another related study, cellulase enzyme was explored for the extraction of polyphenols from old tea leaves (TC et al., 2016). Cellulase enzyme with enzyme activity 700 EU/g and density 1.22 g/mL was used in the conducted study. The results conveyed that the enzyme concentration shall be increased up to optimal point for maximum polyphenol recovery from the old tea leaves.

The maximum polyphenol concentration of 74.45 mg/g was achieved at 2 % (v/w) enzyme concentration. A further increase in the cellulase enzyme concentration had a negative influence on the total polyphenol content. Also, compared to the control case, catechin concentration increased significantly and by 22.53%. The prior art convey the possibility of

using cell wall degrading enzymes as a pre-treatment process in the hot water extraction process to enhance catechin recovery.

Table 1.2 summarizes the prior research findings summary in the field of conventional and enzymatic extraction of catechins from tea.

1.3.3 Preparation and Characterization of Starch Nanoparticles

The selection of proper carapace is very important for the successful encapsulation of a bioactive substance. Various food grade materials such as protein, carbohydrate, and lipid can be used as a wall material for the encapsulation of catechins. Starch is one of the most feasible options due to its easy availability, cost efficiency, and ability to undergo relevant structural modifications. Various top down and bottom up approaches can be adopted to prepare starch nanoparticle which is well known for its ability to encapsulate a variety of materials including hydrophilic and hydrophobic substances. Acid hydrolysis method is conventionally used to prepare starch nanoparticles. Sulfuric acid and hydrochloric acid are commonly deployed acids in the method. The acid hydrolyzes starch particles through a top-down approach for the realization of the starch nanoparticles. In a relevant prior art, the authors utilized 3.16M sulfuric acid at 40°C for 7 days to hydrolyze starch from potato, native maize, and waxy maize. A higher degree of hydrolysis was reported for potato and waxy maize in comparison to the higher amylose containing maize and mungbean. Thus, it was proven that the starch with a higher amylose constitution had a higher resistance to the hydrolysis process. Consequently, nanoparticles obtained from waxy maize were smaller in size (<42 nm), and the high amylose based maize yielded bigger nanoparticles and in the range of 68-72 nm range (Kim et al., 2012). Even though acid hydrolysis has been a well-established method for the preparation of starch nanoparticles, it has certain drawbacks. Firstly, it is not effective due to a longer duration, as it takes around 7-9 days to prepare

Table 1.2: Literature data summary in the field of hot water and enzymatic extraction of catechin

Sl. No.	Reference	Materials	Enzymes used	Independent Variables & range	Dependent Variables & range	Best sample
1	Vuong et al., 2010, 2011	Green tea	None	• Drying temperature • Drying time • Tea/water ratio	• EGCG • ECG • EGC • EC • C	Total catechin: 127.4 mg/g; 80°C, 30 min, 1:50 (Tea/water)
2	Chandni et al., 2011	Black tea (CTC)	Pectinase, Tannase	• Enzyme concentration, • Pectinase: 2-20 U/g • Tannase: 2-100 U/g • Time: 2 h • Temperature: 40°C	• Catechin: 9.9 – 11.29 Control: 10.97 • Polyphenol content: 164.3-186.4 g/kg of black tea Control: 162g/kg • Briskness Index: 15.8-19.9 Control: 13.0 • Turbidity: 30.6-29.4 Control: 32.8 • Tea cream: 0.65-0.53 Control: 1.86	Pectinase: 20 U/g, Tannase: 20 U/g simultaneous, at 40°C for 2 hours. Polyphenol content: 186.4 g/kg of BT Briskness Index:19.1 Catechin: 10.33 g/kg Turbidity:29.4 Tea cream: 0.53
3	TC et al., 2016	Old tea leaves	Cellulase	• Enzyme concentration(%v/w): 0 – 2 • pH: 3.5-7.5 • Time: 0-100 min	• Total polyphenol: 58.75-85.05 mg GAE/g dry matter Control: 58.75 mg GAE/g • Catechins: 11.52-14.32 %dried weight Control: 11.52	Cellulase: 2%(v/w), pH:5.5 at 50°C for 50 Polyphenol content: 85.05 mg GAE/g

nanoparticles of desirable size (<100 nm). Secondly, the produced starch nanoparticles tend to agglomerate, and this results in low storage stability. As such, various works later addressed the resolution of these issues. Sonication has been suggested in conjunction with conventional acid hydrolysis method for the reduction of time. In a relevant study, sonication was used in conjunction with acid hydrolysis and yielded fruitful results in terms of the size and yield of the synthesized nanoparticles. It was observed that the application of bath type sonication at 20 - 40% vibration amplitude resulted in increased yield of starch nanoparticles (from 20 to 70%) and reduced aggregation of nanoparticles and hence higher storage stability. However, the authors did not report any reduction in time required for the preparation of starch nanoparticle (Kim et al., 2013). In another prior art, the authors deployed enzymatic assisted acid hydrolysis for the reduction of time required for the preparation of nanoparticles. Three enzymes namely α amylase, β amylase, and glucoamylase were used and these caused debranching of the starch through the breakdown of the α 1-4 and α 1-6 glycosidic bonds. Thereby, the time needed for the acid hydrolysis was reduced. The authors were able to reduce the time to 2 days in comparison to 7-9 days in the conventional acid hydrolysis. However, they did not report any improvement in the stability of the nanoparticles (Lecorre et al., 2012). In another study, the authors studied the application of probe type sonicator at 220V and 0.7A (3:1 on and off pulse mode) in combination with acid hydrolysis being addressed with the dilute sulfuric acid system (3.16M). The study reported significant reduction in the preparation time (48 h). However, the method was not cost effective and scalable in nature. The prior art revealed that no research addressed studies in the precise theme of molar acid concentration influence on physical and chemical properties of preparation of starch nanoparticles.

The available literature data in the field of the starch nanoparticle synthesis with acid hydrolysis and sonication methods has been summarized in Table 1.3.

Table 1.3: Literature data summary in the theme of starch nanoparticle preparation

Sl. No.	Reference	Material	Method	Degree of Hydrolysis(DOH)	Size (nm)	Best sample
1	Kim et al., 2012	Waxy maize, Normal maize, High AM maize, Potato, Mung bean starches	Acid hydrolysis Sulfuric acid, 3.16 M. 7 days, 40°C	Waxy maize: 90.9 Potato: 89.8 AM maize: 61.4 Normal maize: 85.3 Mung bean maize: 73.9	40-70	Potato DOH: 89.8 Size: 20-50 nm
2	Kim et al., 2013	Waxy maize	Acid hydrolysis Ultrasonication	Waxy maize: 95%	70	40% Amplitude, 60 min sonication/day 7 days Size: <100 nm
3	LeCorre et al., 2012	Waxy maize starch	Enzyme + Acid hydrolysis Sulfuric acid, 3.16 M. 5 days, 40°C	84.2% to 41.8%	50-100	Gluco-amylase

1.3.4 Nano-encapsulation of Catechins in Starch Carapace

Inclusion complexation is a bottom-up approach being followed for the encapsulation of bioactives in wall materials such as β cyclodextrin, starch, and amylose. In a relevant prior art, the authors used β cyclodextrin to encapsulate catechin with the molecular inclusion method. To do so, catechin and β cyclodextrin were mixed in 1:1 ratio and thereby an average particle size of 518 nm was achieved in the prepared powder. Also, the study reported enhanced thermal properties in the encapsulated system. An increase in the melting temperature at 250.4℃ were reported along with relevant changes in the crystalline pattern. The study was the first to provide insights into the catechins and β cyclodextrin encapsulation. It furthered opportunities for application of inclusion complex interactions with starch systems for further research in the field of encapsulation (Krishnaswamy et al., 2012).

In another study, the authors conducted a detailed analysis on the preparation and application of the nano encapsulated inclusion complex of β cyclodextrin and catechin in a variety of food systems such as cheese, yogurt, and milk. Catechin and β cyclodextrin were mixed in 1:1 ratio and in molar concentration. Thereafter, the system was dried in a vacuum dryer. Subsequently, the dried encapsulates were reported with an yield and encapsulation efficiency of 84 and 94% respectively. Morphology studies revealed that the original structure of catechin and β cyclodextrin was completely lost and was replaced by irregular aggregates in the size range of 5-100 µm. The authors also reported enhancement of thermal properties with increased melting temperatures at 202°C. The encapsulated catechin - β cyclodextrin complex sustained 94% retention of antioxidant capacity after an exposure to the high temperature environment at 100℃ for 60 min (Ho et al., 2017). The authors in their extended work applied encapsulated catechins in various food systems. The findings

conveyed higher stability of the encapsulated catechins in solid food system i.e. cheese in comparison to solid-liquid and liquid food systems (Ho et al., 2019).

Researchers also explored the possibility of V type carbohydrates such as amylose as a wall material for the encapsulation of catechins with inclusion complexation method. In a prior art, the authors used potato amylose to encapsulate catechin hydrate and studied the in-vitro digestion profile after encapsulation. Prior to the encapsulation, catechin hydrate was lipophilized into hexadecyl catechin. Accordingly, it improved the thermal properties and photostability after encapsulation. The helical V shaped amylose structure was able to successfully entrap the hexadecyl catechin hydrates with an encapsulation efficiency of 86.5% (Wang et al., 2021). Thus, these findings paved the pathway for a possible application of starch nanoparticles as a wall material for encapsulation of catechins.

A brief summary of the findings on the nano-encapsulation of catechins has been summarized in Table 1.4.

1.3.5 Bioactivity of Tea Catechins

Multiple studies have been conducted to study the bioactivity of various nutrients in the reduction of cholesterol absorption in human body. This was based on both via in-vitro and in-vivo methods. In one study, researchers studied the ability of soy protein hydrolysate (SPH) to reduce the micellar solubility of cholesterol along with binding activity of the SPH with taurocholic acid. The study considered both in-vitro and in-vivo phases and obtained significant findings. These confirmed that the SPH was able to reduce the absorption of cholesterol. Cholestyramine was used as a positive standard to compare the effect of SPH, casein, and soy protein on the micellar solubility of cholesterol. In-vitro findings demonstrated significant reduction in cholesterol uptake in caco 2 cells by SPH (5.7

Table 1.4: Literature data summary in the theme of catechins nano-encapsulation in starch carapace

Sl. No.	Reference	Bioactive molecule	Wall material	Method	Encapsulation efficiency (%)	Size(nm)	Melting point, °C	Best sample
1	Krishnaswamy et al., 2012	Catechin	β-cyclodextrin	Inclusion complexation	-	518.78	242.69	Catechin:β-cyclodextrin 1:1
2	Ho et al., 2017	Catechin	• β-cyclodextrin (β-CD) • Hydroxypropyl β-cyclodextrin(HPβ-CD) • Methyl β-cyclodextrin(Mβ-CD)	Inclusion complexation	• 86 - β-CD • 89 - HPβ-CD • 94 - Mβ-CD	5-100 µm	• 202 - β-CD • 196- HPβ-CD • 197- Mβ-CD	Cat – Mβ-CD
3	Wang et al., 2021	Catechin; Hexadecyl catechin	Amylose from potato	Inclusion complexation	86.5% - amylose HC IC*	-	-	Hexadecyl catechin amylose complex

* Amylose hexadecyl catechin inclusion complex

pmol/well) in comparison to casein and soy protein (9.7 pmol/well and 8.12 pmol/well). The in-vivo findings with rat animal models also conveyed similar trends (Nagaoka et al., 1999). To understand the molecular mechanisms that prompt upon the effective reduction of cholesterol absorption in human body by the tea polyphenols, a relevant prior art studied the relationship between taurocholic acid and gallated catechins and further consequence on micellar solubility. The NMR findings suggested strong correlation between EGCG and oolongtheanins with the bile acid and such a correlation may lead to reduced micellar solubility. Thus, the findings revealed the pathway for the interaction between tea polyphenols and micelle, subsequent precipitation of cholesterol and phosphatidyl choline. It was suggested that EGCG and oolongtheanins gallate could effectively create a hydrophobic zone for the capture of taurocholic acid and such an interaction finally led to the precipitation of cholesterol (Ogawa et al., 2016). Table 1.5 summarizes the available literature in the field of the bioactivity study of tea catechins. The table delineates upon the quantitative and qualitative influences of tea catechins and soy protein compounds on the micellar solubility of cholesterol.

1.3.6 Functional Ready to Drink Tea Beverage Formulations

Till date, several attempts targeted an enhancement in the nutritional properties of the traditional tea beverage and thereby, an antioxidant rich formulation was saught. In one such attempt, the researchers developed a functional tea beverage by infusing *Averrhoa bilimbi* L(AB) extract with black tea extract (Anggraini et al., 2016). The authors targeted grade III black tea, as it constitutes lesser antioxidants with respect to good grade black tea. Thus, the authors designed the formulation with fixed concentrations of tea extracts (340 mL), sugar (180 g), sodium benzoate (50 mg), and varied the concentration of *Averrhoa bilimbi* L extracts as 2, 4, 6, 8, and, 10%. Thereby, a significant rise in the antioxidant activity of the

beverage with increasing AB extract was recorded and highest antioxidant activity was found in the AB tea with 10% AB extracts (22.82% at 0.125mg/mL). However, sensory evaluation findings confirmed that the best formulation was the beverage prepared with 8% AB extracts.

The nutritional constitution of black tea is entirely different from green tea. This is due to the fact that during fermentation, catechins in green tea undergo structural changes. Thereby, the black tea gets enriched with theaflavins and thearubigins. The nutritional benefits of these compounds differ significantly as well despite them being potent antioxidants. To enhance the quality of black tea with the benefits of green tea, in a relevant prior art, another research group formulated a functional tea beverage with black tea extract, and orange peel extracts (Rasouli Ghahroudi et al., 2017). The authors adopted complex coacervation method to microencapsulate the green tea extracts and orange-peel extracts. Subsequently, the encapsulated extracts were used to formulate a functional drink with enhanced antioxidant capacity, and bioavailability. The sensory analysis further revealed that the encapsulation process was able to mask the bitterness of green tea and orange peel extracts and enhanced the overall organoleptic properties of the formulation. The formulations also exhibited enhanced antioxidant activity and flavonoid constitution as 84%, and 15.5 mg QE g^{-1} dry weight respectively.

The organoleptic properties of a drink or food product is very significant in terms of consumer acceptance. Henceforth, it needs equal significance with respect to nutritional benefits. In another relevant study, researchers formulated rooibos iced tea enriched with encapsulated aspalathin from green rooibos extract and conducted a sensory analysis to optimize the organoleptic properties of the formulation. However, the authors also addressed the relevance of nutritional benefits. The control sample being prepared with green rooibos extract and plain aspalathin performed poorly in the sensory analysis in comparison to the nano encapsulated aspalithin (in nano micelles) enriched rooibos tea. The significant

Table 1.5: Literature data summary in the field of tea catechins effect on micellar solubility of cholesterol

Sl. No.	Reference	Mechanism studied	Controls	Sample	Micellar solubility of cholesterol	Taurocholic acid binding activity	Best sample
1	Nagaoka et al., 1999	Micellar solubility of cholesterol by soy protein	Casein, Cholestyramine	Soy protein (SP) Soy protein peptic hydrolysate (SPH), Soy protein peptic hydrolysate with bound phospholipid (SPHP)	Casein: 0.45 mmol/L Cholestyramine: 0.02 mmol/L SP: 0.39 mmol/L SPH: 0.19 mmol/L SPHP: 0.20 mmol/L	Casein: 6.17 mmol/L SP: 5.52 mmol/L SPH: 5.88 mmol/L SPHP: 5.65 mmol/L	SPH Cholesterol: 0.19 mmol/L Taurocholic acid: 5.88 mmol/L
2	Ogawa et al., 2016	Micellar solubility of cholesterol by tea polyphenols	-	EGCG EGC Oolongtheanin-3-O-gallate Desgalloyl oolongtheanin	Degree of change as per NMR results- Oolongtheanin-3-O-gallate>EGCG>Desgallolyl oolongtheanins>EGC	Regiospecific interaction between EGCG (optimum at 10mM)& Oolongtheanin-3-O-gallate (optimum at 13.5 mM) and Taurocholic acid	EGCG (10mM) and Oolongtheanin-3-O-gallate (13.5 mM)

difference in their organoleptic properties were due to the encapsulation of the aspalithin. Table 1.6 summarizes the available prior art data in the field of the catechins based functional tea drink formulation in which the catechins have been extracted from fresh tea leaves.

1.4 Possible Scope for Further Research

1.4.1 Study of the Catechin Content in North-East Indian Tea Cultivars

Till date, very few researchers studied the complete catechin profile in the tea cultivars of North-east India. Assam tea is superior to China and Combod tea varieties in terms of catechin and polyphenol constitution. The conducted studies did confirm that the total polyphenol content was the highest in S3A3 cultivar which is an Assam type tea variety (Sabhapondit et al., 2012). Also, the polyphenol content was higher in the second flush in comparison to first flush, autumn flush and rain flush (Deka et al., 2021). Further, research was also addressed with respect to the catechin profile of north east Indian tea cultivars and for EGCG, ECG, EGC, EC, and C. The results affirmed that gallated catechins dominated the catechin fractions in the tea cultivars (Sabhapondit et al., 2012). However, concentrations of various catechins in tea leaves are subject to alteration with variation in soil conditions, environmental factors, cultivar type, and location. Also, very few studies were conducted to evaluate the catechin profile of the northeast Indian tea cultivars. Thus, based on the limitations of the available prior studies, the evaluation of catechin profile of the selected tea cultivars shall be targeted in this Ph.D. thesis and as a part of the thesis first objective.

1.4.2 Extraction of Catechins by Enzymatic Method

Cellulase, pectinase, and tannase were the most studied enzymes in the enzymatic extraction of catechins from tea. Multiple studies elucidated upon the catechins extraction from various

Table 1.6: Literature data summary in the field of functional ready to drink tea beverage

Sl. No.	Reference	Materials	Functional component	Formulation	Method	Total polyphenols & Total flavonoids	Catechin content	Physico-chemical properties	Best formulation
1	Anggraini et al., 2016	Black tea	*Averrhoa bilimbi* L Extract	Tea extract(mL): 340 Sugar(mg): 180 Na-Benzoate(mg): 50 Water(mL): 640 AB extract(mL): 20,40,60,80,100	Mixing	TP: 512 - 572 mg/L of tea	A (2%)- 284.527 B (4%)- 280.747 C (6%)- 252.130 D (8%)- 259.654 E (10%)- 225.446	Sugar content(°Brix) :A:11.17,B:11.37,C:12.1,D: 12.27,E:12.76	D Formulation: Sensory:225% Sugar content: 12.2,TP:51.42, Antioxidant activity: 91.44
2	Ghahroudi et al., 2017	Green tea	Orange peel	Total concentration of wall materials (% w/w): 2.5-5 Core/wall materials ratio (% w/w): 50,75,100	Micro-encapsulation by complex coacervation	TP: 393 mg of GAE g^{-1} of dry tea weight TF: 10.8(OP),11.7(GT),15.5(M)	-	Water activity: 0.27-0.35 Solubility: 77–83% Encapsulation efficiency:67.1 to 16.1%	D formulation Wall material concentration: 5%(w/w) Core/wall ratio(%w/w): 50
3	Viljoen et al., 2017	Rooibos iced tea	Aspalathin	Fermented Rooibos(g/L): 0.625,1.25 Aspalathin-enriched green rooibos(g/L): 0.625 Aspalathin enriched green rooibos extract solubilised in nanomicelles(g/L): 1.1,2.2 Lemon Flavor (mL/L): 3.25	Nano-micelles	338.05-508.76 mg gallic acid equivalents/L) TF(mg/mL): 32.4-76.8	-	Sensory analysis: Plant like, hay like, rooibo woody, Astringent, Lemon	F/lemon and N+F/lemon Flavonoid in F/lemon(mg/L): 32.4 N+F/lemon(mg/L): 54.6

sources of tea leaves such as old tea, black tea leaves, and green tea leaves and with cell wall degrading enzymes such as cellulase and pectinase (S. K. Chandini et al., 2011; Ghandahari Yazdi et al., 2019; Shao et al., 2020; TC et al., 2016). The cellulase enzyme was used to extract catechins from old tea leaves and thereby resulted in 26.7% TPC enhancement in comparison to the untreated samples (TC et al., 2016). Cell wall degrading enzymes such as cellulase and pectinase function through the disassociation of the leaves' cellular structure and thereby accelerate the extraction process. Thus, the enzymes have been deployed in low temperature applications for temperature sensitive bioactives extraction. Also, another investigation inferred that the enzymes do not cause structural changes in the extracted catechins (S. K. Chandini et al., 2011).

Few researchers conveyed that the tannase enzyme resulted in higher yield of both total polyphenol content as well as antioxidant activity (Baik et al., 2015; Shantha Kumar Chandini et al., 2011; Ghandahari Yazdi et al., 2019). The tannase enzyme was used in multiple studies for the extraction of phenolic compounds from tea. It had been observed that the tannase enzyme resulted in the substantial simultaneous enhancement in the total phenolic content, extract yield, and antioxidant activity. It also resulted in the transformation of the gallated catechins i.e. EGCG and ECG into gallic acid (Lu & Chen, 2008; Shao et al., 2020). In an appropriate prior art, upon tannase enzymatic treatment, the concentration of EGCG reduced from 3665.1 to 60 µg/mL with a simultaneous increase in the gallic acid concentration from 314.5 to 4076 µg/mL (Baik et al., 2015). These results confirm that while tannase enzyme positively affected the total phenolic content and antioxidant activity, it reduced the concentrations of gallated catechins, a desired set of catechins for functional nutritional benefits.

The previously mentioned prior art targeted the influence of enzymatic treatment on the overall polyphenol content, flavonoid content or total catechin concentration. Also, the

combinatorial influences of multiple enzymes have not been explored. Further, in a wider context, no literature exists to delineate upon the effect of enzyme concentration, time, and temperature on the individual concentrations of the five catechin variants namely EGCG, ECG, EGC, EC and C. Moreover, parametric optimization of the enzymatic treatment-based extraction was not targeted. Such detailed investigations and useful insights can provide generic guidelines-based selection of optimal process-product parametric and characteristics. Considering the mentioned lacunae in the mentioned literature, the optimization of the enzymatic extraction process with respect to extraction temperature, time, and enzyme concentration was not studied in the context of catechin profile and shall be considered as the second part of the first objective of this Ph.D. thesis.

1.4.3 Acid Hydrolysis based Starch Nanoparticle Synthesis

A reported study in starch nanoparticle preparation adopted conventional acid hydrolysis method and this performed poorly in terms of duration (7-9 days) for the preparation of starch nanoparticles (<100 nm size) (Kim et al., 2012). The study reported comparison of acid hydrolysis for various starch sources namely waxy maize, potato, mungbean, and high amylose maize. High amylose concentration in starch sources negatively affected the degree of hydrolysis and nanoparticles prepared from waxy maize, normal maize, and potato starch sources were smaller in size (< 44 nm). However, the synthesized nanoparticles formed aggregates after being prepared with acid hydrolysis. To counter this issue, in a related prior art, the authors adopted sonication assisted acid hydrolysis to prepare starch nanoparticles (Kim et al., 2013). The nanoparticle aggregation issue was thereby resolved but the preparation time did not reduce (2 – 6 days). The mentioned prior art in the field of ultrasound assisted post-treatment process that followed acid hydrolysis for starch nanoparticle preparation had the following limitations. Firstly, the acid concentration was

fixed (3.16M) and was not investigated to infer upon its sensitive influence on the fabricated starch nanoparticle properties. Secondly, the post treatment process reported in the literature took a significant fabrication time of 7 - 9 days and thus requires critical attention. Thirdly, intermittent sonication can be eliminated to ensure upon a simpler yet effective process. Fourthly, while bath type sonication was used in previous studies, which is promising from scale up and cost effectiveness, its comparative performance in terms of continuous and pulse modes of operation was not addressed. Fifthly, the influence of higher acid concentration in conjunction with sonication post treatment has not been addressed in the relevant prior art. In this regard, previous studies affirmed that sonication treatment after acid hydrolysis facilitated a reduction in the agglomeration of starch nanoparticles and thereby ensured lower range of the particle size (Kim et al., 2012). The influence of acid concentration during sonication post treatment has not been addressed in the literature. In this regard, it can be hypothesized that higher acid concentrations during sonication pre-treatment can be counterproductive to the quality of starch nanoparticle fabrication. Finally, previous studies did not report characterization data in terms of polydispersity index, melting point, delta C_p. These are very important parameters to judge upon the quality of starch nanoparticle fabrication. Considering the above-mentioned research gaps, the preparation of starch nanoparticles with acid hydrolysis and post sonication treatment shall be addressed and the effect of sulfuric acid molar concentration on the nanoparticle synthesis time, and quality of starch nanoparticles shall be addressed as the second objective of the Ph.D. thesis.

1.4.4 Catechin Encapsulation in Starch Nanoparticle Carapace

Substantial research was conducted till date in the field of tea polyphenols encapsulation. Various methods have been explored for the encapsulation of tea polyphenols. These refer to nanoliposomes, pickering emulsions, antisolvent precipitation method etc. (M. Ahmad et al.,

2019; S. Ahmad et al., 2016; Rashidinejad et al., 2014; Shao et al., 2020). Encapsulation is well known to be an expensive process as it involves the utilization of expensive chemicals and drying systems. Very few literatures addressed cost effective chemical free/minimal chemical-based processes for the encapsulation of catechin extracts. In a relevant prior art, the authors prepared catechin encapsulate with β-cyclodextrin as the wall material and with inclusion complexation method (Ho et al., 2019). Till date, no literature reported encapsulation of catechin extracts from fresh tea leaves and with inclusion complexation method. Also, the application of starch as a wall material to encapsulate catechin extracts and EGCG with the inclusion method was not explored. Thirdly, the characterization including thermal, physical, crystalline properties, and scalability of the inclusion complexation method for encapsulation of catechin extracts, EGCG shall be considered in such research endeavors. Furthermore, encapsulated catechins could be potentially used as an ingredient for the formulation of a functional tea drink. Thus, the lacunae clearly state that nano-encapsulation of catechin extracts in starch nanoparticle as a wall material with the inclusion complexation method was not studied till date and this shall be set as the 3rd objective of the thesis.

1.4.5 Effect of Tea Catechins on Micellar Solubility of Cholesterol

The micellar solubility study provides insights into the ability of the catechin compounds for cholesterol reduction during its absorption in the intestinal digestion stage. In the cited literature, the authors reported cholesterol micellar solubility effect of soy protein hydrolysate, cholestyramine, EGCG, EGC, Oolongtheanins-3-O-gallate (Ogawa et al., 2016). However, no literature addressed the effect of encapsulated EGCG and catechin extracts derived from tea leaves on the micellar solubility of cholesterol. The effect of encapsulation of catechin extracts, which being a combination of five types of catechin namely EGCG, ECG, EGC, EC, and C on the micellar solubility of cholesterol needs to be studied in detail.

Moreover, the bile acid binding activity of encapsulated EGCG and catechin extracts needs further investigations. Considering these lacunae, it can be concluded that the effect of prepared starch nanoparticle encapsulated catechin extracts on the micellar solubility of cholesterol shall be addressed and set as the fourth objective of the Ph.D. thesis.

1.4.6 Functional Tea Beverage Formulation with Encapsulated Catechins

Several benefits of tea are often not explored due to the conventional extraction processes and also due to adopted procedures for beverage formulation. In a previous study, the authors made an attempt to improve black tea nutritional profile through the infusion of *Averrhoa bilimbi* L extracts which are high in antioxidant constituents (Anggraini et al., 2016). In another study, green tea and orange-peel extract has been encapsulated and added to black tea to prepare a functional beverage with better combinations of organoleptic qualities and nutritional qualities (Rasouli Ghahroudi et al., 2017). Further, nano-encapsulated products have an edge with respect to microencapsulated products as nanoencapsulation provides higher bioavailability indices. The smaller size of the nanoparticles allows it to get easily absorbed by the cell matrix. Till date, no study targeted the formulation of functional beverage with nano-encapsulated tea catechins being extracted from fresh tea leaves. Secondly, sensory optimization of the formulation with enhanced bioavailibity due to fortification by encapsulated catechin extract needs to be studied in detail. Thirdly, in-vitro digestion study of the optimally formulated beverage with encapsulated catechin extracts as an ingredient was also not studied in the available prior art. As such, the lacunae in this theme direct towards the formulation research for a sensory study based optimally prepared functional tea drink with encapsulated catechin extracts. Further research in this area will thus be quintessential for the functional beverage research. In a nutshell, the formulation of a

functional tea drink beverage with encapsulated catechin extracts and its bioavailability characteristics shall be addressed as the fifth objective of this Ph.D. thesis.

1.5 Objectives

Considering the final objective of the Ph.D. thesis as the optimal formulation of a functional tea beverage, this Ph.D. thesis delineates upon the fulfillment of the following objectives.

1. Evaluation and identification of the north-east Indian tea cultivar with the best catechin profile and optimization of enzymatic extraction of catechins from Assam tea leaves.
    - Catechin profile evaluation of S3A3 and TV18 Assam tea cultivars with HPLC method.
    - Response surface analysis-based optimization of extraction time, temperature, and enzyme concentration for the best achievable catechin profile (EGCG, ECG, EGC, EC, C) in the extract prepared with S3A3 tea leaves.
2. Preparation and characterization of potato starch nanoparticles prepared using acid hydrolysis followed with sonication post treatment process. To do so, the following shall be targeted.
    - Influence of sonication post treatment and acid concentration on the fabricated starch nanoparticles characteristics namely yield, particle size, polydispersity index, morphology, thermal and crystalline properties.
    - Characterization of the optimally synthesized starch nanoparticles in terms of, particle size, polydispersity index, morphology, thermal and crystalline properties.
3. Inclusion complexation-based encapsulation of starch nanoparticles with catechin extracts from tea leaves and subsequent characterization of the product.

4. In-vitro bioactivity studies of catechin extract prior to and after encapsulation. To do so, following shall be targeted.
    - The effect of EGCG, catechin extracts prior to and after encapsulation with starch nanoparticle on the micellar solubility of cholesterol.
    - The effect of EGCG and catechin extracts on the bile acid binding activity.
5. Sensory optimization based optimal formulation of a functional tea drink being prepared with encapsulated catechin extracts as an ingredient. To do so, following shall be targeted.
    - 9-point hedonic scale based sensory optimization.
    - In-vitro digestion study of the encapsulated catechin extracts and optimally formulated functional tea drink.

1.6 Organization of the Thesis

The Ph.D. thesis targeted the preparation and formulation of a functional tea beverage with improved bioaccessibility of cholesterol-reducing components using green nano-encapsulation techniques. This goal is based on identified research gaps, the scope of further research, and established objectives. To achieve these objectives, the Ph.D. thesis has been organized into 7 chapters.

Chapter 1 addresses the medicinal and nutritional benefits of tea catechins followed by the importance to solve the problem of tea catechin's poor bioavailability. Thereby, the concept of a functional tea beverage formulation using nano-encapsulated bioactives has been discussed in the targeted perspectives section. Relevant prior arts were further discussed in detail with respect to the identification and extraction of catechins from Assam tea, preparation of novel starch nanoparticles suitable as a carrier vehicle or wall material,

catechin's nano-encapsulation, and finally the functional tea beverage formulation. Subsequently, the objectives of the Ph.D. thesis were finalized with the end goal of a nanotechnology enabled functional tea beverage.

Chapter 2 explores the specific materials and procedures that were used to achieve the objectives of the thesis. The necessary methods to accomplish each objective were meticulously outlined in various sections of the chapter. Firstly, these techniques pertain to the methods used to determine the catechin profile of the S3A3 Assam tea cultivar and extract it via enzyme assisted hot water extraction. Subsequently, a methodology utilizing sonication-assisted acid hydrolysis has been outlined for the preparation of a wall material that is suitable for encapsulating catechins. These were followed by methods used to encapsulate the tea catechins and create the functional tea beverage, which was then optimized for the organoleptic qualities.

Chapter 3 provides a concise overview of the main research breakthroughs pertaining to the analysis of catechin composition in the two Assam tea varieties, S3A3 and TV18. Subsequently, a thorough analysis was conducted to examine the outcomes achieved in the process of optimizing the enzymatic aided extraction of tea catechins. The results from hot water extractions were compared to enzymatic extractions, and an in-depth study was undertaken to examine the impact of enzymatic pre-treatment on the extraction of specific types of catechins.

Chapter 4 examines the research achievements on the creation of novel potato starch nanoparticles using sonication assisted acid hydrolysis method. Thereby, the text outlines a comprehensive discussion on the characterization of the synthesized starch nanoparticles, including their average particle size, PDI, shape, thermal characteristics, and crystalline properties.

Chapter 5 provides a concise overview of the research findings related to the nano-encapsulation of EGCG and catechin extracts, as well as their subsequent characterization. The characterization studies involve a thorough evaluation of the size, morphology, thermal, and crystalline properties of the encapsulated EGCG/catechin extracts. Subsequently, a thorough discussion was conducted on the significant findings gained from the study on the micellar solubility of cholesterol for EGCG/catechin extracts before and after encapsulation.

Chapter 6 elaborates the results obtained for the preparation of the functional tea beverage using the encapsulated catechin extracts and its subsequent characterization. These were followed by a detailed discussion on the in vitro digestion results obtained for the dry and liquid formulations. Subsequently, sensory optimization studies were discussed for the functional tea drink prepared with black tea and encapsulated catechin extracts using 9 point hedonic scale and fuzzy logic based method.

Chapter 7 outlines the major conclusions for the obtained research findings of the Ph.D. thesis. Thereby, the chapter enlists the possible research pathways in the field of the enzymatic extraction of tea bioactives and nanotechnology based functional food product development.

Chapter 2:

Materials and Methods

Chapter 2

Materials and Methods

This chapter summarizes the materials and methods adopted for the fulfillment of the Ph.D. thesis objectives. The chapter is divided into the following sections – (a) materials required for the thesis work (b) procedures adopted for the evaluation of catechin profile of north east Indian tea cultivars – S3A3 and TV18 (c) methodology adopted for the response surface methodology based optimization of the enzymatic extraction of catechins (d) methodology followed for the preparation of starch nanoparticles using ultrasonic assisted acid hydrolysis (e) inclusion complexation based nano encapsulation of EGCG and catechin extracts followed by its characterization (f) procedures adopted to study the sensitive influence of EGCG and catechin extracts (prior to and after encapsulation) on the in-vitro micellar solubility of cholesterol adsorption (g) In-vitro digestion study and sensory analysis based optimization for an instant mix formulation of black tea fortified with nano-encapsulated catechin extracts.

2.1 Materials

Fresh tea leaf samples (three leaves and a bud) were procured from Mazbat Tea Estate (26°79′N, 92°28′E, 118 MSL), a finest region in Assam, India that hosts good quality CTC and green tea cultivars. For the conducted investigations, an Assam type (S3A3) and a Combod type (TV18) tea cultivar were selected. The primary catechin standards namely (-) - Epigallocatechin gallate (EGCG), (-) - Epigallocatechin (EGC), (-) - Epicatechin gallate (ECG), (-) - Epicatechin (EC) and (+) - Catechin (C) were purchased from Sigma Aldrich. Further, acetonitrile (HPLC grade), acetic acid (HPLC grade), EDTA and ascorbic acid were also supplied by Sigma Aldrich, India. Cellulase enzyme from Aspergillus niger (Enzyme

Commission number: 3.2.1.4) and pectinase enzyme from Aspergillus niger (Enzyme commission number: 3.2.1.15) were purchased from Sigma Aldrich, India. The cellulase and pectinase enzymes had an activity of 300 and 300 EU/g/g. Potato starch powder, H_2SO_4 (99% purity), and NaOH were purchased from Merck chemicals, India. These were the chemicals purchased in India for the objectives completed in IIT Guwahati, Assam, India.

The following chemicals were purchased in Japan for the objectives completed at Gifu University, Japan. Taurocholic acid (SIGMA T-4009), Oleic acid (SIGMA O-1383), L-α-phosphatidyl choline (SIGMA P-2772), Mono olein (SIGMA M-7765), Cholestyramine (SIGMA C4650), Potato starch (SIGMA S2004), Dimethyl sulfoxide (SIGMA D2650) and EGCG (SIGMA E4143) were purchased from Sigma Aldrich, Japan. NaOH (Wako 198-13765), Chloroform (Wako 033-02617), NaH2PO4.2H2O (Wako 192-02815), Ethanol (Wako 057-00451) and Toluene (Wako, 204-01861) were purchased from Wako, Japan. Sulfuric acid (325-19) and Polyethylene glycol mono-p-isooctylphenyl ether (28228-35) were purchased from Nacalai tesque, Japan. DPO(DOJINDO 344-01112), POPOP(DOJINDO 342-02252) were purchased from DOJINDO, Japan. Insta-Gel Plus (Perkin Elmer Product No.6013399), [14C]-cholesterol (Perkin Elmer NEC-018) were purchased from Perkin Elmer Life Sciences Inc., Japan [14C] Taurocholic acid was purchased from Amersham Life Science, Japan and Cholesterol (Katayama 9859) was purchased from Katayama Chemical, Japan.

2.2 Methodology for the Catechin Profile Study of S3A3 and TV18 Tea Cultivars and Response Surface Optimization of the Enzymatic Extraction of Catechins

2.2.1 Drying of Tea Leaves

The tea leaves of the S3A3 tea cultivar were dried at 60°C for 3.5 h in an assembled tray dryer (International Commercial Traders, Kolkata, India, intermittent airflow at 4.5 m/s and gap of

20 s). The heat treatment conditions have been justified with the reason that the catechins present in tea leaves are relatively stable at higher temperatures and are affected at temperatures beyond 100°C (Su et al. 2003). In summary, the tea leaves dried at 60°C under intermittent airflow conditions, ascertained upon an insignificant loss of catechins.

2.2.2 Methanolic/Hot Water Extraction and Isolation of Catechin Extracts

For the methanolic extraction, firstly, 0.2 g of dried tea leaves of each cultivar (S3A3 and TV18) was taken in a test tube. To this, 5 mL of 70% methanol was added, and the mixture was kept for 30 min to equilibrate. Subsequently, the grounded tea leaves were extracted at 70°C for 10 mins. In due course of such extraction, the solution in the test tube was mixed with a vortex mixer (Tarsons Spinix vortex shaker, Cat. 3020) for an intermittent duration of 5 mins. Thereby, the samples were centrifuged for 12 mins at 3500 rpm (Sigma 2-16P). Following this, the supernatant was separated, and the remaining solid particles were again subjected to extraction for 10 mins at 70°C and with intermittent mixing after every 5 min interval. After final centrifugation at 3500 rpm for 12 mins, the obtained supernatant was mixed with the prior obtained extract solution. Finally, the total extracted solution was subjected to HPLC analysis for the determination of catechin profiles.

Catechins were extracted from S3A3 Assam fresh tea leaves with a modified hot water extraction method (Chandini et al. 2011). To do so, 10 g of dried tea leaves were solubilized in 500 mL of water and were subjected to extraction in a water bath (Equitron S7514) at 90°C for 40 minutes. The extract solution was then filtered with whatman filter paper. Thereafter, the extract solution was concentrated in a rotary evaporator dryer system (Make: Buchi Rotavapor Model: R 300) at 60°C. The concentrated solution was eventually dried in an oven dryer for 12 h at 60°C. The dried catechin extracts were stored at -19°C for a maximum duration of 2 weeks to facilitate further characterization.

2.2.3 Enzymatic Extraction of Catechins

The enzymatic extraction of tea leaves can be accomplished in two major sequential steps. The first step involved the enzymatic incubation stage. In this stage, cellulase and pectinase enzymes act upon the grounded dry tea leaves being dispersed in a distilled water system for 2 h duration at the optimal temperature (35°C). During the pretreatment stage, while the incubation period and temperature remained constant, the enzymatic concentration of the two enzymes was varied as per the central composite rotatable design (CCRD) model. After incubation, the enzymes were inactivated by treatment with the boiling water. Thereafter, the second step involved hot water extraction of the enzyme-treated tea leaves solution system. The details of the experimental design including range of independent variables extraction time, temperature, and enzymatic concentration are discussed later in section 2.2.5 of the chapter.

To do so, firstly, 0.2 g of dried tea leaves were added to 10 mL of distilled water, followed by the addition of cellulase and pectinase enzymes at concentrations as per the experimental design. Thereby, the tea leaves solutions were incubated (Make: Das & Co. model: NM) at a temperature of 35°C for 2 h. Post incubation, the enzymes were inactivated at 100°C for 1.5 mins (Chandini et al. 2011). During the incubation process, the samples were mixed with a vortex stirrer at 30 min intervals. Subsequently, the tea leaves were extracted in a pre-heated water bath and as per the experimental design. Thereby, the tea extract solutions were cooled to room temperature and were centrifuged (Make: Sigma Model: 2-16P) at 3500 rpm for 10 mins. Finally, the supernatant was separated and filtered (Syringe filter 0.45 µm).

2.2.4 HPLC Analysis for Catechin Profile Study

The HPLC analysis was carried out using Shimadzu High-performance Liquid Chromatography system equipped with Luna 5 µm Phenyl-Hexyl column (250 × 4.6 mm).

Thereby, as per the ISO method, the samples were detected with a UV detector system at 278 nm (Sabhapondit et al. 2012). A gradient elution method was followed for the catechin profile analysis.

The HPLC analysis involved the following steps. Using a stabilizing solution, the extracted solution was first subjected to four times dilution. After dilution, the extract solution was filtered with a 0.45 µm syringe filter. Subsequently, a manual injector was used to inject 20 µL of the solution into the HPLC system. Both mobile phases A and B being used in the HPLC analysis were prepared by following the procedure delineated in the ISO method. For the first 10 minutes duration, 100 % mobile phase A was eluted. Thereafter, for the next 15 min, mobile phase B elution was gradually increased to 32% (68% of mobile phase A). Subsequently, for the next 10 mins, the elution ratio was kept constant. Thereafter, mobile phase A elution was once again increased to 100 % (0% mobile phase B). In the entire analysis procedure, the flow rate of the eluent system was maintained constant at a constant value of 1 mL/min.

2.2.5 Experimental Design for Enzymatic Extraction

The experiments were conducted as per the Central Composite Rotatable Design (CCRD) tool in the design expert software environment (version 13.0). Extraction time, enzyme concentration, and temperature have been chosen as three independent variables in the conducted study. Thereby, responses were experimentally determined for total catechin content (TCC), EGCG, ECG, EGC, EC, and C content. The independent variable range was set as per the previous work data (Baruah, Singha, and Uppaluri 2023; Hu, Zhou, and Chen 2009). The extraction time, temperature, and enzyme concentration were altered from 13.18 - 46.82 min, 49.77 - 100.23°C, and 65.91 – 234.09 EU/g, respectively. A numerical optimization tool was used to optimize the three independent variables for the assessment of best extraction yield (Teng et al. 2021). The tea extract to water ratio was chosen as a fixed value (1:50) and as per

the reported optimized conditions (Chandini et al. 2011). The extraction time and enzymatic concentration range were chosen as per the previously conducted studies (Baruah, Singha, and Uppaluri 2023; Liang et al. 2007). For the experimental design, the number of experiments (N) was obtained as per the following equation.

$$N = 2^k + K + N_o \quad (2.1)$$

where K = 3 (number of independent variables) and N_o = 6 (number of center points).

To ascertain upon the specificity and range of independent variables, the – alpha and + alpha values have been set as in Table 1. The percentage desirability value for all responses was calculated with the following expression:

$$D = (d_1 \times d_2 \times d_3 \times \dots \dots d_n)^{\frac{1}{n}} \quad (2.2)$$

where d_1, d_2…. d_n denote the desirability values of individual responses, and D is the overall desirability.

Table 2.1: Experimental model for enzymatic extraction

Name	Units	Low	High	-alpha	+alpha
Extraction Time	Min	20	40	13.18	46.82
Enzyme concentration	EU*/g	100	200	65.91	234.09
Temperature	°C	60	90	49.77	100.23

2.2.6 Statistical Analysis

To judge upon the linear and other interaction effects of analysis of variance (ANOVA) and multiple regression, statistical analysis was conducted for the variables with the evaluated parameters and for a significance level of $p < 0.05$. The coefficient of determination, R^2, was assessed to ensure upon model adequacy. Design Expert Statistical Software package 13.0 version (Stat Ease Inc., Minneapolis, MN, USA) was used to perform the statistical analysis.

All experiments were performed in triplicates, and the findings have been represented as mean ± standard deviation.

2.3 Preparation of Starch Nanoparticles

2.3.1 Ultrasonic Assisted Acid Hydrolysis of Starch

Starch nanoparticles were prepared using acid hydrolysis-sonication post-treatment method being reported in the literature (Kim et al. 2012, 2013) and with few modifications such as variations in acid concentration, reaction time, and sonication post treatment process. The current work targeted the effect of higher concentration based acid hydrolysis on the assessed parameters of the fabricated starch nanoparticles. Previous studies focused upon the preparation of starch nanoparticles at lower H_2SO_4 concentrations (Kim et al. 2012; Rajisha et al. 2014; Shabana et al. 2019). In only one article (Saeng-on and Aht-Ong 2017) starch nanoparticles were prepared from banana and tapioca starch with varied concentrations of H_2SO_4 and HCl. The method developed in this work explored the possibility of acid hydrolysis using higher concentrations of H_2SO_4 in the process being supplemented with the ultrasonic post treatment process. The motivation for such studies is in terms of lower duration for starch nanoparticles along with better characteristics such as particle size range, polydispersity index, yield, crystalline and thermal properties

Preliminary studies undertaken in the thesis work confirmed that the pulse mode sonication has better characteristics in terms of temperature control (below 40 °C) of the system. Thereby, it was easy to conduct the experiments. However, the average particle size did not vary significantly, and the polydispersity index enhanced marginally for the case. The starch hydrolysis experiments were conducted on a trial-and-error basis (based on few preliminary investigations) and with variant combinations of sulfuric acid concentration (3.16 – 5 M) and duration (24 – 72 h). For the case, a fixed choice of system temperature 25°C and starch

concentration (15% w/v) were considered. The post-treatment of the solution system was conducted with a bath type sonicator (S 30 H, Elmasonic) for 1.5 h at 40°C, 280W and 37 kHz frequency. Ultrasonic bath was used in the present work due to its scalability and low cost. The bath type ultrasonicator did not consume much power (1-5 W/cm^2) in comparison to probe type sonicator (Harun et al. 2022). Hence, after few trial-and-error experiments, a duration of 1.5 h was selected for the post treatments. The pulse mode of sonication had a frequency of 1.6 pulses/s and also ensured equal intermittent periods of zero intensity. Subsequently, the solution pH was neutralized with 2M NaOH solution. Thereafter, the solution was centrifuged at 12000 rpm for 12 mins and for 3 cycles. After each cycle, the solution supernatant was discarded, and distilled water was added in equal volume to facilitate complete removal of sulfates. Thereafter, the mixture was dried in a vacuum dryer at 25 psi and 50°C for 12 h to achieve dried starch nanoparticles. The dried starch nanoparticle samples were stored in a deep freezer at -20°C for further characterization and analysis. Also, the chosen conditions and drying systems were based on few preliminary trials using alternate drying methods such as oven drying, tray drying, and vacuum drying. Further, freeze drying was not considered due to its poor economic effectiveness and scalability.

2.3.2 Characterization of the Starch Nanoparticles

The starch nanoparticle characterization was conducted in terms of its morphology, average particle size, polydispersity index (PDI), crystallinity and thermal properties.

2.3.2.1 Morphology

The morphology of the starch nanoparticles was studied with the Gemini 300 FESEM instrument (make – Zeiss, model - Gemini) (Rajisha et al. 2014). The starch nanoparticles were first drop casted with a piece of glass and aluminium foil. To do so, 0.1 g of the sample was mixed with 40 mL of water and the mixture was subjected to sonication for 10 minutes.

Subsequently, the mixture was subjected to casting on a glass piece covered with an aluminium foil. Eventually, the drop casted sample was attached to the stub with the double sided carbon tape. Finally, the samples were gold coated under vacuum to achieve the pre-treated sample for analysis with Gemini FESEM instrument.

2.3.2.2 Average Particle Size, PDI, Yield Study

The average particle size and Polydispersity index (PDI) of the starch nanoparticles were evaluated with the Delsa Nano C particle analyzer (dynamic light scattering method) (Ge et al. 2017). To do so, 0.1 g of sample was mixed with 20 mL water and sonicated for 10 mins and was thereafter transferred into a cuvette for analysis at 25°C and 170° scattering angle. For these experiments, the deployed diluent water possessed a refractive index and viscosity of 1.3328 and 0.8818 cP respectively. The yield of the starch nanoparticles was evaluated as a ratio of the weight of the total amount of nanoparticle produced after vacuum drying to the total weight of the initial raw material provided.

$$\text{Yield, } Y = \frac{Weight\ of\ starch\ nanoparticle\ produced, W_f}{Weight\ of\ inital\ starch\ added\ W_i} \quad (2.3)$$

2.3.2.3 XRD and DSC Analysis

The starch nanoparticles were analyzed with X ray diffractometer (make: Rigaku, model: Micromax-007HF). The obtained diffractograms were analyzed with Origin pro software (version 9.0) for the evaluation of the crystallinity of the sample. The XRD measurements were conducted at 40 kV and 100 mA conditions of the instrument and in the 2Θ range of 3 - 40°C. The thermal properties of the starch nanoparticles were analysed with a differential scanning calorimeter (make: NETZSCH model: DSC 3500 Sirious), differential scanning calorimeter. To do so, 7 mg of sample was placed in a 40 µL concave aluminium crucible with a lid. A hole

was pierced in the lid and following that, the system was placed inside the DSC pan for the thermal analysis in the temperature range and heating rate of 30 - 350°C and 10°C/min respectively. In the DSC experiment, the environment was maintained at a flow rate of 60 mL/min of N_2 (protective gas).

2.4 Inclusion Complexation based Nanoencapsulation of EGCG and Catechin Extracts and its Characterization

2.4.1 Preparation of EGCG/CE-SNP Complex

To prepare the SNP-EGCG and SNP-Catechin extract, a marginally modified inclusion complexation method was followed with the starch nanoparticles as the wall material (Mukherjee, Narayan, and Ramagopal 2023; Q. Wang et al. 2022; Y. Wang et al. 2021). In the current work, EGCG and catechin extracts were encapsulated by using DMSO solvent based inclusion complex mechanism. For this purpose, firstly, 25 mg SNP was mixed in 1 mL 95% DMSO solution and was subjected to stirring for 60 min at 500 rpm. After an hour, the nanoparticles were properly mixed with the solvent and appropriate amounts of EGCG/Catechin extracts were added (1 mg and 2.5 mg). Thereafter, the mixture was covered with aluminum foil to ensure a dark environment during the experiment. The stirring was continued for another 60 min for further mixing of the components. Thereafter, 2.5 mL deionized water is added to the mixture and the stirring was continued for another 4 h in the same dark environment. Consequently, stirring was stopped and the mixture was allowed to settle for 12h in a similar environment. The solution mix was then centrifuged at 3000 rpm and the supernatant was replaced with 50% ethanol. Centrifugation was repeated for two more times to facilitate the complete removal of DMSO solvents from the solution mix. Thereafter, the precipitate was dried at 50°C for 24h in hot air oven and the dried SNP-EGCG/CE powders were stored in deep freezer at -79°C and were used for further analysis.

2.4.2 Characterization

EGCG, SNP, and EGCG/CE-SNP complex samples were subjected to FTIR, morphology, thermal properties, and crystallinity analyses. The methods adopted for morphology, thermal properties (DSC), and crystallinity (XRD) have been explained in section 2.3.2 of this chapter.

2.4.2.1 FTIR analysis

The infrared spectra of the EGCG, starch nanoparticles, and EGCG-SNP encapsulates were measured with the FTIR spectrophotometer instrument (Shimadzu, Japan). The samples were scanned directly in the FTIR spectrophotometer (wavelength range of 4000 to 400 cm^{-1}) and the spectra of all samples were thereafter analysed with the Origin 2022b software.

2.5 The Impact of EGCG and Catechin Extracts (Before and After Encapsulation) on Micellar Solubility of Cholesterol Adsorption, an In-Vitro Study

2.5.1 Taurocholic Acid Binding Activity

The binding activity of the EGCG and catechin extracts with taurocholic acid was determined as per the literature reported procedure (Nagaoka et al. 2010; J. Wang et al. 2015). To do so, first, sodium taurocholic acid (0.1 mol/L) was mixed with tris-HCl buffer (0.1 mol/L) at pH 7.4 followed by the addition of 1.85 kBq of tauro [carbonyl-^{14}C]cholic acid. The samples including Cholestyramine as positive control, EGCG, catechin extracts were added to the mixture and incubated for 2 h at 37°C. After incubation of the samples, they were centrifuged at 15000 rpm for 15 min and the supernatant was used to measure the radioactivity of the system with the liquid scintillation counter (Make: Perkin Elmer model: Tri-carb 2900TR).

2.5.2 Assay for Micellar Solubility of Cholesterol

The micellar solubility of cholesterol with EGCG, catechin extracts (CE), starch nanoparticles (SNP), and EGCG/CE-SNP complex was measured with the literature reported radioactivity method (Nagaoka et al. 1999). To ensure that the final concentrations are as indicated in the parentheses, a micellar solution was prepared by mixing cholesterol (0.1 mM), sodium taurocholate (6 mM), oleic acid (1 mM), phosphatidylcholine (0.06 mM), mono olein (0.5 mM), and [^{14}C] – cholesterol (2µM), followed by nitrogen drying. The dried mix was dissolved in taurocholic acid phosphate buffer solution and was further sonicated for 180 s to ensure upon better mixing of the solution system. The solution was then incubated in a water bath for 24 h at 37°C. Post incubation, samples including EGCG, catechin extract, SNP, EGCG/CE-SNP complex, cholestyramine (as positive control) were added to the micellar solution followed by sonication for better dispersion. The micellar solution was then incubated for 1 h at 37°C followed by centrifugation at 100000 × g for 1 h and at 37°C. The supernatant was collected, and its radioactivity was counted using a liquid scintillation counter for the determination of [^{14}C] – cholesterol.

2.6 In-Vitro Digestion Study and Sensory Based Optimization for an Instant Mix Formulation of Long Leaf Black Tea Fortified with Nano-Encapsulated Catechin Extracts

2.6.1 Nanoencapsulation of Catechin Extracts in Starch Nanoparticles

A modified inclusion complex method was followed to encapsulate the catechin extract (Ho et al. 2019; Zhu 2017). The literature reported methods adopted a 1:1 ratio (on a molecular weight basis) for the wall material and bioactive compound at 0.006 mol/L. Nevertheless, considering the fact that the catechin extract consists of a combination of various catechin compounds, including EGCG, ECG, EGC, EC, C, and other polyphenols, a fixed weight basis was

established in this study for the catechin extracts. Thus, a precise molecular weight basis was not considered and the molecular weight of the catechin being reported in previous studies was presumed (Mukherjee, Narayan, and Ramagopal 2023). Accordingly, catechin extracts and starch nanoparticles were mixed at the ratios of 1:1 and 1:2 and encapsulated with the inclusion complexation method. To do so, firstly, the starch nanoparticles were dissolved in distilled water at 10 mg/mL and stirred in a dark environment for 60 min duration. Catechin extracts at desired ratio (1:1 or 1:2) were added after 60 mins and the stirring was continued for another 60 mins. Subsequently, equal volume of distilled water in comparison to the initial ratio were added and the stirring was continued for 4 h. Consequently, the stirring was terminated, and the solution was kept in the dark environment for 12 h. Eventually, the solution was concentrated with a rotary evaporator at 60°C and was subsequently dried in an oven dryer at 60°C.

2.6.2 Characterization

The catechin extracts encapsulated in starch nanoparticles were characterized for its morphology, FTIR, thermal and crystalline properties. The methodology being adopted to evaluate the mentioned characterizations have been explained in section 2.3.2 and 2.4.2 of this chapter.

2.6.3 In-Vitro Digestion Study

The in-vitro digestion studies for the catechin extracts, encapsulated catechin extracts were conducted by following a previously reported method and with a few modifications (Sollano-mendieta et al. 2021). With such modifications, an in-vitro approach that simulates gastrointestinal digestion was adopted to assess the bioaccessibility of the catechin extracts and

catechin extracts - SNP encapsulates. Adopting digestive enzymes, pH adjustments, digestion times, and salt concentrations, stimulated environments have been achieved for gastric and intestinal phases of digestion. A brief account of these phases are as follows.

Sample preparation: Both dry and liquid forms of the encapsulated catechin extractions were subjected to in vitro studies. In the dry form trials, raw catechin extracts weighing 0.025 and 0.017 g were utilized for the in vitro digestion investigation. In the in vitro digestion investigation, 0.05 g of CE:SNP (1:1 w/w) and CE:SNP (1:2 w/w) were measured. The liquid form investigation involved dissolving raw catechin extracts, CE:SNP (1:1 w/w), and CE:SNP (1:2 w/w) in boiling water at a concentration of 500 µg/mL for a duration of one minute. Subsequently, the sample was subjected to an in vitro digestion investigation.

Gastric phase: In the gastric phase, the solution was mixed with 30 mL solution of 140Mm NaCl and 5Mm KCl. Subsequently, 0.5 mL of 11000 U/mL Himedia pepsin 1: 1000, EC 232-629-3 solution prepared in SGF electrolyte stock solution was added to the mixture sample. After thorough mixing, the pH was lowered to 5 using 6M HCl. In a water bath, the final mixture containing vessel was shaken at 100 rpm for two hours at a temperature of 37 °C.

Intestinal phase: After the gastric phase, the solution was well mixed with 2.5 mL of pancreatin (Pancreatin from Porcine Pancreas, P7545-25G, Sigma Aldrich) solution prepared in the SIF electrolyte stock solution. To this system, 40 µL of 0.3M CaCl2 was added and the pH of the mixture was adjusted to 7 through the addition of 0.5 cc of 1M NaOH. Finally, shaking procedure similar to that mentioned for the gastric phase was conducted.

After completing enzymatic process, the sample solution vessel was submerged in a cold bath for 10 minutes. Eventually, the sample solution was then centrifuged for 40 minutes at 5000 rpm. The resultant supernatant was then tested for total phenolic content and radical antioxidant activity (DPPH scavenging method).

The bioaccessibility index (BI) was used to assess upon the impact of in-vitro gastrointestinal digestion of the content of RSA and was determined with the following equation (Martínez-Las Heras et al. 2017):

$$Bioaccesibility\ index = \frac{A}{B} \times 100 \quad (2.4)$$

where A and B are the values s of the radical antioxidant activities (DPPH) of samples prior to and after digestion (in-vitro) respectively.

2.6.4 Sensory Evaluation

A panel of 28 semi-expert evaluators (12 women and 16 men) with appropriate sensory sensitivity and ages ranging from 15 to 70 were engaged for the sensory evaluation. The subjects provided the quality descriptors and weightage coefficients for a 9-point hedonic and fuzzy logic evaluation scale. The methodology were adopted as per the literature reported approach (Akasapu and Uppaluri 2023).

Chapter 3:

Evaluation of Catechin Profile in S3A3 and TV18 Tea Cultivar and Optimality of Catechins Enzymatic Extraction

Chapter 3

Evaluation of Catechin Profile in S3A3 and TV18 Tea Cultivar and Optimality of Catechins Enzymatic Extraction

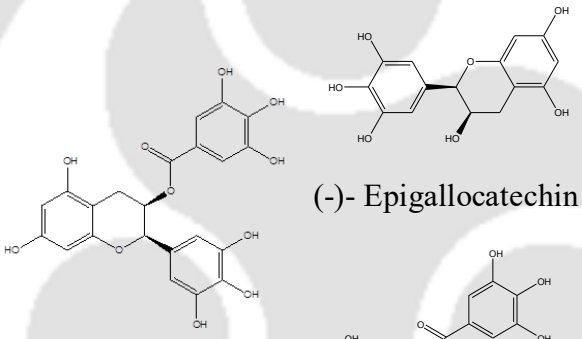
In this chapter, the catechin profile of two Assam tea cultivars has been presented. Following this, optimality of enzymatic extraction of catechins from the fresh S3A3 tea leaves has been addressed. A concise introduction has been presented in section 3.1 of the chapter. Thereafter, the results obtained for the catechin profile study of the two mentioned Assam tea cultivars has been summarized in section 3.2. Section 3.3 entails the results and discussion for the optimality of enzymatic extraction of catechins. Accordingly, section 3.3.1 and 3.3.2 delineate upon the experimental results obtained during the enzymatic extraction and statistical analysis using ANOVA methodology respectively. Section 3.3.3 encompasses the response surface analysis for individual catechin types with respect to the three independent variables namely extraction time, temperature, and enzyme concentration. Section 3.3.4 presents a comparative assessment of the findings associated to the enzymatic and hot water extraction processes. Thereafter, in section 3.3.5, a brief literature comparison has been addressed to clearly mention upon the novelty of the current investigation. Finally, the significant findings of the conducted investigations has been summarized in section 3.4 of the chapter.

Overview

S3A3 and TV18 are two of the most commonly cultivated tea cultivars among the north eastern tea estates. They belong to the category of Assam tea cultivars. This chapter elaborates upon the detailed catechin profile in the fresh tea leaves of the two cultivars. Consequently, the chapter entails upon the optimization of the enzymatic extraction of catechins from S3A3 tea

leaves. In such efforts, enzymatic concentration, extraction time, and temperature have been considered as the independent variables. Furthermore, a comparative study has been addressed for both conventional hot water and enzymatic extraction. The experimental conditions were designed using the central composite rotatable design (CCRD, an efficient tool for the reduction in the number of the experiments and as for enhanced robustness in parametric optimization procedures. Two enzymes namely cellulase and pectinase were used in the enzymatic extraction process. These two enzymes are important cell wall degrading enzymes and do enhance the phenolic recovery through the increased extraction rate of catechins such (-)- Epigallocatechin gallate, (-)- Epicatechin gallate, (-)- Epigallocatechin, (-)- Epicatechin, and (+)- Catechin.

Fresh tea leaves of S3A3 /TV18



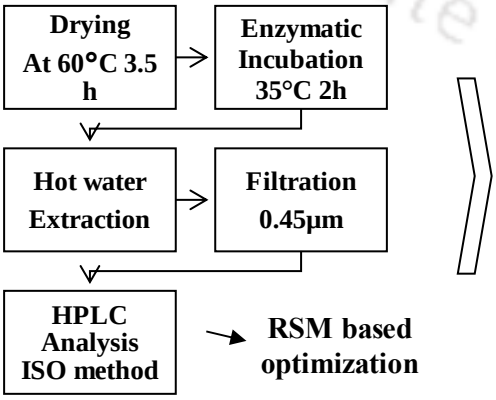
(-)- Epigallocatechin

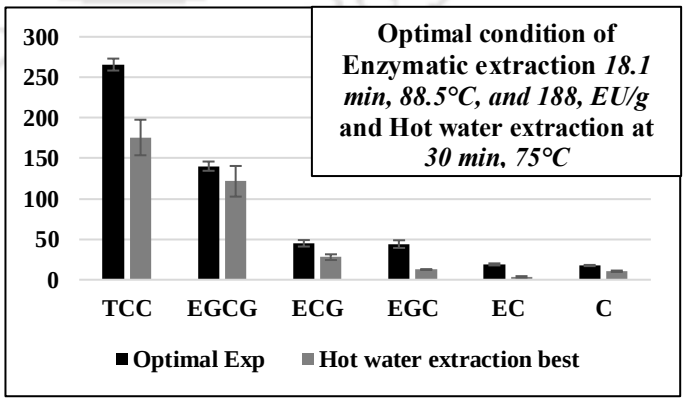
(-)- Epicatechin

(-)- Epigallocatechin 3 Gallate

(-)- Epicatechin Gallate

(+)- Catechin





3.1 Introduction

Among various phenolic compounds that exist in the fresh tea leaves, major constituents include (-) - Epigallocatechin gallate (EGCG), (-) – Epigallocatechin (EGC), (-) - Epicatechin gallate (ECG), (-) - Epicatechin (EC) and (+) - Catechin (C) (Sabhapondit et al. 2012). The concentrations of the mentioned catechins do vary significantly and as per the location, climate, soil conditions, and type of tea cultivar. In this chapter, the catechin profile evaluated for the two Assam tea cultivars S3A3 and TV18 using ISO 14502:2:2005 (HPLC based method) has been presented. Thereafter, the chapter discusses the results obtained for the optimality of enzymatic extraction of catechins from the S3A3 fresh tea leaves. Such investigations were directed towards the effect of enzymatic concentration, time, and temperature on the total catechin concentration as well as on the individual catechin yields with enzymatic and alternate conventional yet inexpensive hot water extraction (HWE) processes. Further, the conducted investigations aim towards the identification of the optimal conditions of extraction in terms of enzyme concentration, time, and temperature. This has been targeted with numerical optimization tool and CCRD-based response surface methodology (Table 3.1). The CCRD model is an excellent choice for the optimization of process parameters. It reduces the number of experiments and, in lieu of its rotatable properties, it helps in the optimization of bioprocesses such as the extraction of bioactive (Rasheed et al. 2023). As mentioned in the relevant prior art, the method of drying and inactivation of enzymes present in the tea leaves affect the polyphenol and catechin concentration of tea leaves. Studies affirmed that the oven drying or tray drying at 60°C resulted in the minimal loss of total polyphenol concentrations. Also, freeze drying was found to be superior for better retention of the phenolic profile (Roslan et al. 2020). However, due to its expensive and unscalable nature, the tray drying method has been opted in the current work for the drying of the fresh tea leaves prior to the extraction process. The grounded tea leaves were extracted with a hybrid extraction method that included

Table 3.1: CCRD based ranges of independent variables for enzymatic extraction of catechins

Name	Units	Low	High	-alpha	+alpha
Extraction Time	Min	20	40	13.18	46.82
Enzyme concentration	EU*/g	100	200	65.91	234.09
Temperature	°C	60	90	49.77	100.23

*An enzymatic unit is defined as enzyme quantity that could catalyze the conversion of 1 µmol of substrate per minute.

an enzymatic pre-treatment followed by hot water extraction under conditions specified by the CCRD model.

3.2 Catechin Profile Analysis for S3A3 and TV18 Assam Tea cultivars

3.2.1 Catechins Constitution in TV18 and S3A3 Tea Cultivars

As per the ISO method, standards curves were prepared for the five catechin constituents of the tea leaves (Appendix A). For these curves, Table 3.2 summarizes intercept, slope, and R^2. Affirming good fitness, the R^2 values for the standard curves have been obtained as 0.99, 0.99, 0.96, 0.98, 0.99 for (-)- epigallocatechin gallate (EGCG), (-)- epicatechin gallate (ECG), (-)- epigallocatechin (EGC), (-) epicatechin (EC), and (+)- Catechin (C) cases respectively.

Table 3.2: Intercept, Slope, and R^2 values for the plotted calibration curves of catechin standards (HPLC grade) EGCG, ECG, EGC, EC, and C

Standard	Intercept	Slope	R^2
EGCG	984428	29225	0.99
ECG	431191	42180	0.99
EGC	182396	7954	0.96
EC	313047	17862	0.98
C	3894	15616	0.99

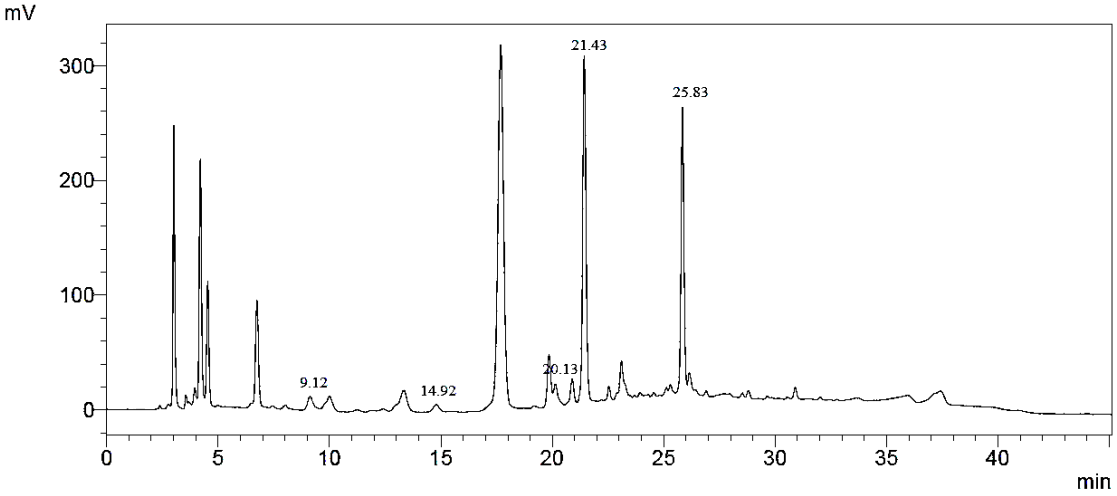


Figure 3.1: HPLC chromatogram of S3A3 tea cultivar

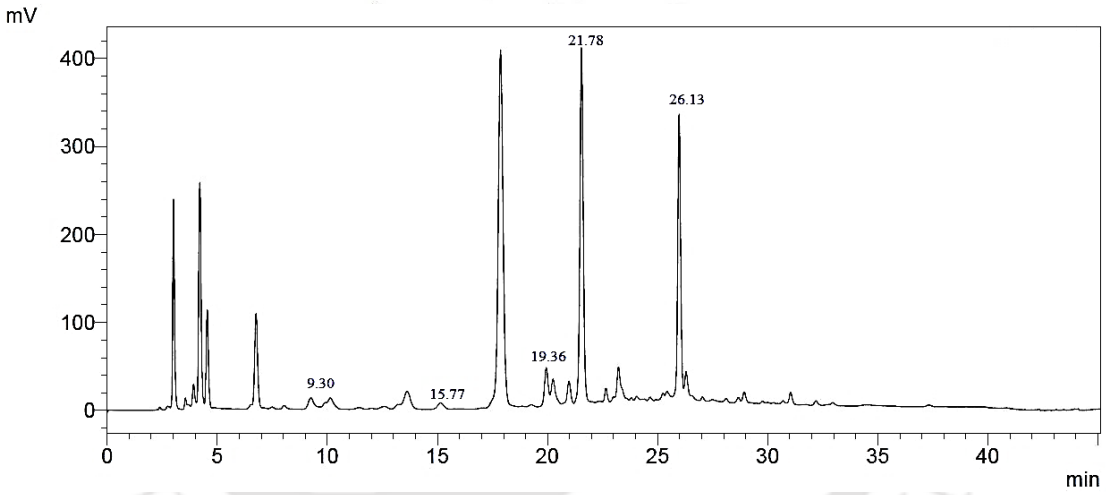


Figure 3.2: HPLC chromatogram of TV18 cultivar

For the S3A3 tea cultivars, the HPLC chromatogram has been illustrated in Figure 3.1. Thereby, the peaks for EGC, C, EC, EGCG, ECG have been analyzed to be at elution times of 9.12, 14.92, 20.13, 21.43 and 25.83 min, respectively. Thus, the S3A3 tea cultivar has been affirmed to constitute a major concentration of the galloyl catechins. Similarly, the HPLC chromatogram of the TV18 tea cultivars has also been analyzed to indicate the retention times of EGC, C, EC, EGCG, ECG as 9.30, 15.77, 19.36, 21.78 and 26.13 min, respectively (Figure 3.2).

Figure 3.3 elucidates upon the catechin profile of both cultivars in conjunction with the data being presented in the best available prior art. For the catechin profile analysis, three leaves and a bud were considered for each cultivar plucked in the spring season flush. The results of the unpaired t test conveyed significant differences between the two cultivars TV18 and S3A3 in terms of C, EC, and EGC concentration (p values of 0.0011, 0.0088, and 0.0034 respectively). As per the analysis, total catechin concentration, EGCG and ECG concentrations among the two cultivars were not significantly different (p values of 0.4768, 0.1042, and 0.0760 respectively). In summary, the findings inferred that the tea cultivars S3A3 and TV18 being commonly cultivated in northeast India possess a better catechin profile in comparison with any other cultivars being studied till date. In this regard, it shall be once again noted that (Wei et al. 2011) the location, climate and overall environment do influence the catechin constituent profile in the fresh tea leaves (Zhang et al. 2018). Similar seasonal variance inference has been affirmed for the tea catechin profile of the cultivars being investigated in different countries. Previous studies have affirmed that three leaves and a bud possess higher total catechin concentrations and phenolic profile in comparison to plucking with two leaves and a bud and one leaf and a bud (Wei et al. 2011). Also, it has been noted that the tea leaves plucked in the first flush during the spring season possess higher phenolic content and better catechin profile in comparison to later flushes of the year (Jayasinghe and Kumar 2021). These could be among few reasons to corroborate with the high concentration of various catechins in the present work. Also, in India and many other major tea producing countries such as China, Bangladesh, the climate alterations play a major role in the polyphenolic nutrition profile of the tea leaves (Bora, Ragaee, and Marcone 2019; Kfoury et al. 2019). Climate studies reported that the average highest and lowest temperature at Mazbat town, Assam are 29.3°C and 16.3°C respectively in the March month for a span of 30 years from 1991-2020 (data collected from Indian Meteorological Department). In this regard, it shall be noted that the profile of tea leaves do

convey upon a direct dependence on temperature. Higher temperature in the spring season were correlated with higher concentrations of gallated catechins (EGCG & ECG). However, it had a negative influence on the EGC concentration. Similar trends can be observed for the findings summarized in Table 3.2. Thereby, while the concentration of EGCG and ECG had increased, the EGC concentration reduced in comparison to the relevant prior art data (Sabhapondit et al. 2012). Prior art also considered rainfall as one of the significant factors to significantly contribute to the alteration in the phenolic profile of the tea cultivars. Alterations in rainfall during spring and monsoon seasons contributed to a reduced total catechin concentration as well as tea yield (Bora, Ragaee, and Marcone 2019). The average mean rainfall in the Mazbat town have been reported to be 51.7 mm (average of 4.4 days of rainfall/month) for a span of 30 years ranging from 1991-2020 (data collected from Indian Meteorological Department). The stable rainfall and optimal temperatures have been major environmental factors contributing to the rich phenolic and catechin profile of the Assam Tea cultivars. Thereby, pertinent variations in the catechin profile characteristics of tea cultivars of North-east India can be understood.

Among five alternate catechins being investigated, the EGCG has been analysed to have the highest constitution in both cultivars. The HPLC analysis confirmed that comparatively, TV18 tea cultivar possessed higher EGCG (197.77 mg/g) in comparison with the S3A3 (161.45 mg/g). Compared to the most relevant prior art (Sabhapondit et al. 2012), S3A3 and TV18 possessed 9.82 and 20.89% higher catechin constitutions. Further, it shall be noted that till date, no other prior art reported a higher EGCG value than that being prevalent in the TV18 cultivar (163.6 mg/g of dry matter EGCG concentration in TV 18 as per (Sabhapondit et al. 2012).

Among the galloyl catechins being extracted from tea, after EGCG, the ECG is the most extensively investigated. This is due to its multi-nutritional and medicinal properties. Among both cultivars investigated in this work, the S3A3 possessed a better ECG concentration than the TV18. Compared to the best reported literature value (43.6 mg/g), the S3A3 possessed

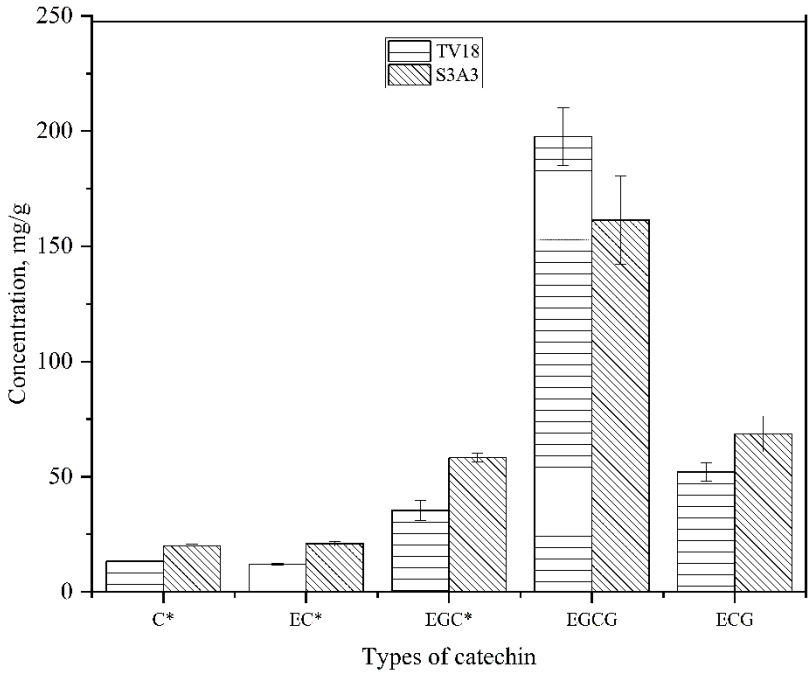


Figure 3.3: Catechin profile in S3A3 and TV18 cultivar; asterisks indicate statistically significant differences (unpaired t test)

about 57.4% higher ECG concentration (68.63 mg/g). Comparatively, the TV18 possessed a lower ECG concentration (52.14 mg/g or 14.24% lower) in conjunction with the literature reported value of 60.8 mg/g (Sabhapondit et al. 2012).

EGC is one of the dominant catechins in the tea cultivars of NE India. Both S3A3 and TV18 exhibited excellent EGC constitutions. Compared to the literature reported 63.1 mg/g (Sabhapondit et al. 2012), the S3A3 exhibited 58.32 mg/g of EGC concentration and hence a 7.57% reduction in the EGC content. For the TV18 cultivar, the EGC constitution has been 35.35 mg/g which corresponds to 14.03% from the literature reported value (Sabhapondit et al. 2012). The literature also indicated that the S3A1 tea cultivar being commonly cultivated in northeast India also had a high EGC constitution of 62.3 mg/g.

In both S3A3 and TV18, both epicatechin and catechin constitution was found in significant proportions. The reported literature data for the mentioned Assam and Combod type tea

indicate a lower constitution of both EC and C. While S3A3 constituted a better EC profile of 20.89 mg/g, the TV18 has been analysed to consist of 11.98 mg/g of EC. Comparatively, the literature reported data refers to 7.7 mg/g of EC for S3A3 and 5.7 mg/g of EC for the TV18 cultivar. Among alternate tea cultivars reported in the prior art, TV23 possessed the highest constitution of EC (19.6 mg/g).

In comparison with EGCG, ECG and EGC, (+-) catechin was found in lower concentration in the tea leaves. Its constitution was analysed to be 20.1 and 13.3 mg/g in S3A3 and TV18 respectively. These values were significantly higher than those being reported for the same cultivars. The literature reported data for the highest catechin concentration corresponds to Fudingdabaicha China tea cultivar being cultivated in the Wuyi province of China (22.7 mg/g). In this regard, it shall be noted that very few studies targeted complete catechin profile of tea cultivars of NE India and most of them deployed green tea for the analysis but not fresh tea leaves.

The literature reported trends for the tea cultivars of NE India affirmed lower epicatechin and catechin constitution as shown in Table 3.3 (Sabhapondit et al. 2012). However, the results inferred that both TV18 and S3A3 exhibit better constitutions of epicatechin and catechin which are well known to be highly sensitive to high temperature and high pH environments. Also, this work considered extraction from dried tea leaves at 60°C for 3.5 h based on several trials and subsequent analysis-based optimization for catechin profiles. The relevant prior art also justified the approach with the observation that the catechins don't change their structure below 70°C (Gadkari and Balaraman 2015).

Table 3.3: Literature comparison table for catechin profile of S3A3 and TV18 cultivar

Cultivar name	EGCG, mg/g	ECG, mg/g	EGC, mg/g	EC, mg/g	C, mg/g	Total catechin, mg/g	Reference
S3A3	147.0	43.6	63.1	12.5	7.7	274.0	Sabhapondit et al., 2011
S3A3	161.45 ± 19.17, 9.82% ↑	68.63 ± 7.79, 57.4% ↑	58.32 ± 1.81, 7.57% ↓	20.898 ± 1.12, 67.1% ↑	20.1 ± 0.69, 161% ↑	329.39 ± 24.64, 20.21% ↑	This work
TV18	163.6	60.8	31.0	5.7	4.3	265.4	Sabhapondit et al., 2011
TV18	197.77 ± 12.49, 20.89% ↑	52.14 ± 4.05, 14.24% ↓	35.35 ± 4.45, 14.03% ↑	11.98 ± 0.4, 110% ↑	13.3 ± 0.26, 216.2% ↑	310.54 ± 21.80, 17.16% ↑	This work
S3A1	142.5	46.2	62.3	14.6	3.5	269.2	Sabhapondit et al., 2011
TV23	120.1	41.7	39.1	19.8	4.9	220.6	Sabhapondit et al., 2011
Fudingdabaicha	50.8	21.6	17.1	7.1	22.7	119.2	Wei et al., 2011

3.3 Optimization of the Enzymatic Extraction of Catechins from Fresh S3A3 Tea Leaves

3.3.1 Experimental Data Analysis for Enzymatic and Hot Water Extraction

This investigation provides very useful insights into the influence of enzymatic extraction conditions on the total catechin and individual catechin concentration profiles in the tea leaves extract. The extraction time, enzyme concentration, and extraction temperature had a significant influence on the yield of total catechin, EGCG, ECG, EGC, EC, and C. For an alteration in extraction temperature, extraction time, enzyme concentration from 49.80 - 100.20°C, 13.2 - 46.8 mins, and 65.9 - 234 EU/g, the total catechin concentration, EGCG, ECG, EGC, EC and C altered as, 128.39 - 260.53, 73.17 - 144.68, 19.82 - 50.02, 7.37 - 47.84, 0.87 - 24.14, and 1.30 - 16.86 mg/g respectively.

It was observed that enzymatic extraction achieved a significant increase in the individual catechin profile and total catechin concentrations in comparison to conventional hot water extraction, as shown in Table 3.4. While the EGCG, ECG, EGC, EC, and C contributed about

121.57, 27.87, 12.55, 3.21, and 10.46 mg/g during hot water extraction at 30 min - 75°C, they altered to 134.77, 42.55, 47.84, 18.51, and 16.86 during the enzymatic extraction at 20 min - 90°C – 200 EU/g (Table 3.4). Also, higher TCC was achieved for the later case. In hot water extraction, the best result was obtained at 30 min - 75°C condition, which yielded a total catechin content in the extract of 175.66 mg/g. At this condition, percentage yields of major catechin fractions were in the order of EGCG> EC>EC>C>EGC. Among the CCRD design based on 24 alternate experimental datasets (with 4 control), the best case corresponds to 20 min - 90°C - 200 EU/g that ensured the highest total catechin content in the extract as 260.53 mg/g. At this condition, percentage yields of major catechin fractions in the extract were in the order of EGCG>EGC>ECG>EC>C, and the gallated catechins (EGCG & ECG) constituted about 68.02% of the total catechin concentrations. In comparison to hot water extraction at 30 min 75°C, extraction at 20 min, 90°C, and 200 EU/g conditions improved TCC, EGCG, ECG, EGC, EC, and C concentration by 48.31, 10.86, 52.67, 281.19, 476.63, 61.18 % respectively. The tabulated data (Table 3.4) conveyed that the exposure to high temperatures (≥ 90°C) and for longer durations (≥ 40min) resulted in the significant loss of catechins in the extract. Prior to this study, only hot water extraction was targeted for the evaluation of the influence of time and temperature on catechin yield. Similar trends were reported for hot water extraction based on total catechin yield, and the best condition reported were 80°C and 30 mins for extraction temperature and time, respectively (Vuong et al. 2011).

The cell wall degrading enzymes cellulase and pectinase acted upon the tea leaves during the incubation stage of extraction (H. Liang et al. 2007; TC et al. 2016). Hence, a significant increase in the yield of individual catechins and total catechins was observed with increasing concentrations of the enzymes. For the 20 min - 90°C condition and for an increase in the enzymatic concentration from 100 - 200 EU/g, the total catechin content, EGCG, EGC, EC, and C increased by 6.81, 9.18, 58.2, 5.74, 24.51 % respectively. On the contrary, for the 40

min and 60°C cases, the corresponding enhancement in total catechin content, EGCG, and ECG were 7.3, 17.16, and 25.59 %, respectively. For all other combinations involving either a combination of short duration and low temperature or long duration and higher temperature, the effects of enzymatic pretreatment were not significant. The literature reported a trend for hot water extraction indicated poor performance for the higher temperature and longer duration conditions. Henceforth, the observations in this work are novel for the specific condition of higher temperature and longer duration (Vuong et al. 2010, 2011).

3.3.2 ANOVA Studies & Statistical Analysis of Models

The analysis of variance (ANOVA) method was used to evaluate the validity of the statistical parameters for the total catechin, EGCG, ECG, EGC, EC, and C content in the extract. These have been summarized in Table 3.4. A brief account of the statistical analysis-based parameters has been presented in the following sub-sections. Thereby, confidence in the modeling efforts has been affirmed.

3.3.2.1 Statistical Analysis for Total Catechin Content

A quadratic model was suggested as per the ANOVA results for the total catechin content concentration being achieved with the CCRD. The coefficient of correlation (R^2) value of the best fit quadratic model was found to be 0.957 with a significant p-value ($p<0.0001$) and insignificant lack of fit. The F value and adequate precision of the model were 25.06 and 18.04, respectively, and thereby affirmed the significance and adequacy of the model. The F and p values of the variables, namely extraction time (A), temperature (B), and enzyme concentration (C), and their interactions have also been presented in Table 3.4. The linear influence of temperature and enzyme concentration on the total catechin concentration indicated moderate influence in comparison with BC and AC terms. Incidentally, the p-value for the AB term is

Table 3.4: Two way ANOVA (quadratic model) table describing the F value, p value (significance) for the independent variables, extraction time (A), temperature (B), enzyme concentration (C), and their quadratic interactive terms along with adequate precision, lack of fit and standard deviation values for the enzymatic extraction of catechins in terms of TCC, EGCG, ECG, EGC, EC, and C

Components	TCC		EGCG		ECG		EGC		EC		C	
	F value	**p value**	**F value**	**p value**	**F value**	**p value**	**F value**	**p value**	**F value**	**p value**	**F value**	**p value**
Model	25.060	< 0.0001	18.120	< 0.0001	22.330	< 0.0001	6.100	0.005	16.380	< 0.0001	18.810	< 0.0001
A	4.040	0.072	17.230	0.002	0.329	0.579	0.759	0.404	25.530	0.001	4.660	0.056
B	15.160	0.003	0.523	0.486	1.050	0.330	20.730	0.001	4.970	0.050	0.107	0.750
C	6.410	0.030	7.180	0.023	0.332	0.577	0.514	0.490	0.157	0.701	0.040	0.845
AB	175.140	< 0.0001	39.170	< 0.0001	109.560	< 0.0001	15.750	0.003	70.130	< 0.0001	150.500	< 0.0001
AC	0.013	0.913	0.116	0.741	4.600	0.058	0.005	0.944	8.510	0.015	0.274	0.612
BC	0.041	0.845	0.021	0.887	4.310	0.065	0.047	0.833	6.930	0.025	2.320	0.159
A^2	0.105	0.753	43.790	< 0.0001	78.290	< 0.0001	14.750	0.003	24.430	0.001	5.500	0.041
B^2	24.080	0.001	64.520	< 0.0001	0.136	0.720	3.260	0.101	8.300	0.016	6.950	0.025
C^2	0.122	0.735	2.760	0.128	2.900	0.120	1.050	0.329	0.070	0.796	0.320	0.584
R^2	0.958		0.942		0.953		0.846		0.937		0.944	
Adequate precision	18.040		11.639		13.327		8.074		15.176		15.597	
Lack of fit	0.132		0.352		0.053		0.115		0.055		0.133	
Standard deviation	10.540		8.000		6.360		3.310		7.550		1.360	

less than 0.001. Thus, among all terms, B and C linear, B^2 quadratic, and AB bi-linear terms had a significant coefficient (negative) and thereby affirmed significant influence in comparison with BC and AC terms. Incidentally, the p-value for the AB term is less than 0.001. Thus, among all terms, B and C linear, B^2 quadratic, and AB bi-linear terms had a profound influence on the total catechin content. Accordingly, the best quadratic model to represent total catechin content as a function of the three independent variables was obtained as follows (coded equation):

$$TCC = 195.56 - 5.73 \times A + 11.11 \times B + 7.23 \times C - 49.34 \times A \times B + 0.4178 \times A \times C + 0.7502 \times B \times C + 0.90 \times A^2 - 13.63 \times B^2 - 0.9684 \times C^2 \quad (3.1)$$

where A, B, and C refer to time (min), temperature (°C), and enzyme concentration (EU/g).

3.3.2.2 Statistical Analysis for Epigallocatechin Gallate

A quadratic model was suggested as per the ANOVA results for the EGCG concentration being achieved with the CCRD. The coefficient of correlation (R^2) value of the best fit quadratic model was found to be 0.942 with a significant p-value ($p<0.0001$) and insignificant lack of fit. The F value and adequate precision of the model were 18.12 and 11.639, respectively, and thereby affirmed the significance and adequacy of the model. The F and p values of the variables, namely extraction time (A), temperature (B), and enzyme concentration (C), and their interactions have also been presented in Table 3.4. The linear influence of extraction time and enzyme concentration on the EGCG concentration indicated moderate competence and acceptable p values (< 0.005 for A and < 0.05 for C). Among bi-linear terms, the AB term had a significant coefficient (negative) and thereby affirmed significant influence in comparison with BC and AC terms. Incidentally, the p-value for the AB term is less than 0.0001. Among the quadratic terms, the A^2 and B^2 terms had a significant coefficient (negative) and thereby affirmed significant influence in comparison with the C^2 term. The p values for the A^2 and B^2

terms are less than 0.0001. Thus, among all terms, A and C linear, A^2 and B^2 quadratic, and AB bi-linear terms had a profound influence on the EGCG concentration. Accordingly, the best quadratic model to represent EGCG concentration as a function of the three independent variables was obtained as follows (coded equation):

$$EGCG = 135.72 - 8.99 \times A + 1.57 \times B + 5.8 \times C - 17.7 \times A \times B + 0.96 \times A \times C - 0.41 \times B \times C - 13.95 \times A^2 - 16.93 \times B^2 - 3.5 \times C^2 \quad (3.2)$$

Where A, B, and C refer to time (min), temperature (°C), and enzyme concentration (EU/g).

3.3.2.3 Statistical Analysis for Epicatechin Gallate

A quadratic model was suggested as per the ANOVA results for the ECG concentration being achieved with the CCRD. The coefficient of correlation (R^2) value of the best fit quadratic model was found to be 0.953 with a significant p-value ($p<0.0001$) and insignificant lack of fit. The F value and adequate precision of the model were 22.33 and 13.327, respectively, and thereby affirmed the significance and adequacy of the model. The F and p values of the variables, namely extraction time (A), temperature (B), and enzyme concentration (C), and their interactions have also been presented in Table 3.4. None of the linear terms had a significant influence on the ECG concentration ($p>0.05$). Among bi-linear terms, the AB term had a significant coefficient (negative) and thereby affirmed significant influence in comparison with BC and AC terms. Incidentally, the p-value for the AB term is less than 0.0001. Among the quadratic terms, the A^2 term had a significant coefficient (positive) and thereby affirmed significant influence in comparison with B^2 and C^2 terms. The p-value for the A^2 term is less than 0.0001. Thus, among all terms, A^2 quadratic and AB bi-linear terms had a profound influence on the ECG concentration. Accordingly, the best quadratic model to represent ECG concentration as a function of the three independent variables was obtained as follows (coded equation):

$$ECG = 24.49 - 0.4514 \times a + 0.8058 \times B + 0.4534 \times C - 10.76 \times A \times B - 2.20 \times A \times C - 2.13 \times B \times C + 6.77 \times A^2 - 0.2824 \times B^2 + 1.30 \times C^2 \quad (3.3)$$

where A, B, and C refer to time (min), temperature (°C), and enzyme concentration (EU/g).

3.3.2.4 Statistical Analysis for Epigallocatechin

A quadratic model was suggested as per the ANOVA results for the EGC concentration being achieved with the CCRD. The coefficient of correlation (R^2) value of the best fit quadratic model was found to be 0.846 with a significant p-value ($p<0.0001$) and insignificant lack of fit. The F value and adequate precision of the model were 6.1 and 8.074, respectively, and thereby affirmed the significance and adequacy of the model. The F and p values of the variables, namely extraction time (A), temperature (B), and enzyme concentration (C), and their interactions have also been presented in Table 3.4. The linear influence of temperature on the EGC concentration indicated moderate competence and acceptable p values (< 0.005). Among bi-linear terms, the AB term had a significant coefficient (negative) and thereby affirmed significant influence in comparison with BC and AC terms. Incidentally, the p-value for the AB term is less than 0.0001. Among the quadratic terms, the A^2 term had a significant coefficient (positive) and thereby affirmed significant influence in comparison with B^2 and C^2 terms. The p-value for the A^2 term is less than 0.005. Thus, among all terms, B linear, A^2 quadratic, and AB bi-linear terms had a profound influence on the EGC concentration. Accordingly, the best quadratic model to represent EGC concentration as a function of the three independent variables was obtained as follows (coded equation):

$$EGC = 18.33 + 1.39 \times A + 7.25 \times B + 1.14 \times C - 8.25 \times A \times B - 0.15 \times A \times C + 0.45 \times B \times C + 5.95 \times A^2 + 2.80 \times B^2 + 1.59 \times C^2 \quad (3.4)$$

Where A, B, and C refer to time (min), temperature (°C), and enzyme concentration (EU/g).

3.3.2.5 Statistical Analysis for Epicatechin

A quadratic model was suggested as per the ANOVA results for the EC concentration being achieved with the CCRD. The coefficient of correlation (R^2) value of the best fit quadratic model was found to be 0.936 with a significant p-value ($p<0.0001$) and insignificant lack of fit. The F value and adequate precision of the model were 16.38 and 15.176, respectively, and thereby affirmed the significance and adequacy of the model. The F and p values of the variables, namely extraction time (A), temperature (B), and enzyme concentration (C), and their interactions have also been presented in Table 3.4. The linear influence of extraction time and temperature on the EC concentration indicated acceptable p values (≤ 0.0005 for A and < 0.05 for B) and high and moderate competence, respectively. Among bi-linear terms, the AB term had a significant coefficient (negative) and p-value less than 0.0001. The bi-linear influence of the AC and BC terms on EC concentration indicated moderate competence and acceptable p values (< 0.05 for AC and < 0.05 for BC). Among the quadratic terms, the A^2 term had a significant coefficient (negative) and p-value less than 0.001. The term B^2 had moderate competence and acceptable p values (< 0.05). Thus, among all terms, A and B linear, A^2 and B^2 quadratic, and AB, AC, and BC bi-linear terms had a profound influence on the EC concentration. Accordingly, the best quadratic model to represent EC concentration as a function of the three independent variables was obtained as follows (coded equation):

$$EC = 5.55 + 3.11 \times A + 1.37 \times B - 0.2435 \times C - 6.73 \times A \times B - 2.35 \times A \times C + 2.12 \times B \times C + 2.96 \times A^2 + 1.73 \times B^2 - 0.1589 \times C^2 \quad (3.5)$$

where A, B, and C refer to time (min), temperature (°C), and enzyme concentration (EU/g).

3.3.2.6 Statistical Analysis for Catechin

A quadratic model was suggested as per the ANOVA results for the catechin concentration being achieved with the CCRD. The coefficient of correlation (R^2) value of the best fit quadratic model was found to be 0.944 with a significant p-value ($p<0.0001$) and insignificant lack of fit. The F value and adequate precision of the model were 18.81 and 15.597, respectively, and thereby affirmed the significance and adequacy of the model. The F and p values of the variables, namely extraction time (A), temperature (B), and enzyme concentration (C), and their interactions have also been presented in Table 3.4 None of the linear terms had a significant influence on catechin concentration ($p > 0.05$). Among bi-linear terms, the AB term had a significant coefficient (negative) and thereby affirmed significant influence in comparison with BC and AC terms. Incidentally, the p-value for the AB term is less than 0.0001. Among the quadratic terms, the A^2 and B^2 terms had a significant coefficient (negative) and thereby affirmed significant influence in comparison with the C^2 term. The p values for the A^2 and B^2 terms are less than 0.05. Thus, among all terms, A^2 and B^2 quadratic and AB bi-linear terms had a profound influence on the catechin concentration. Accordingly, the best quadratic model to represent catechin concentration as a function of the three independent variables was obtained as follows (coded equation):

$$\text{Catechin} = 11.46 - 0.7931 \times \text{A} + 0.1203 \times \text{B} + 0.0736 \times \text{C} - 5.89 \times \text{A} \times \text{B} - 0.2512 \times \text{A} \times \text{C} + 0.7313 \times \text{B} \times \text{C} - 0.8388 \times A^2 - 0.9431 \times B^2 - 0.2024 \times C^2 \quad (3.6)$$

where A, B, and C refer to time (min), temperature (°C), and enzyme concentration (EU/g).

3.3.3 Response Surface Analysis for Enzymatic Extraction

Response surface plots were captured using design expert software (version 13.0) that describes the relationship between the independent variables viz. extraction time (T), temperature (t), enzyme concentration (EU/g), and the responses, namely total catechin content, EGCG, ECG,

EGC, EC and C catechins in the extract. A brief account of the pertinent responses has been presented in the following sub-section:

3.3.3.1 Total Catechin Content

The total catechin content surface plot is shown in Figure 3.4 A – C with two of the three degrees of freedom extraction time, temperature, and enzyme concentration varying. The total catechin content varied from 128.12 – 138.87 mg/g, respectively, for a variation in extraction time and temperature (at 150 EU/g) from 20 min - 60°C and 40 min – 90°C, with a peak concentration of 249.01 mg/g at 20 min – 90°C. Similarly, for a variation in temperature and enzyme concentration from 60°C – 100 EU/g and 90°C – 200 EU/g (at 20 min), TCC altered as 121.09 – 255.59 mg/g respectively. Also, for a variation in extraction time and enzyme concentration from 20 min – 100 EU/g and 40 min – 200 EU/g (at 90°C), the total catechin content altered as 221.25 – 129.5 mg/g with a peak concentration of 255.59 mg/g at 20 min – 200 EU/g respectively.

Temperature and time displayed strong non-linear and linear beneficial effects on the total catechin concentration, respectively, as shown in Figure 3.4 A-C. The total catechin concentration showed a moderately positive linear influence by the extraction time. Additionally, it was noted that higher temperatures and longer extraction durations confirmed a negative correlation between the total catechin concentration in the extract and both factors. Due to the quick cell wall softening and easy release of phenolic compounds into the extraction medium, high extraction temperatures typically result in large yields of polyphenol content. As a result, the total catechin content considerably increased at shorter extraction times for a higher temperature, which is consistent with the trend shown in the pertinent study (Astill et al. 2001; Baruah, Singha, and Uppaluri 2023; Lu and Chen 2008). The gallate catechins have, however, clearly deteriorated as a result of the extended extraction time and higher temperature. This is

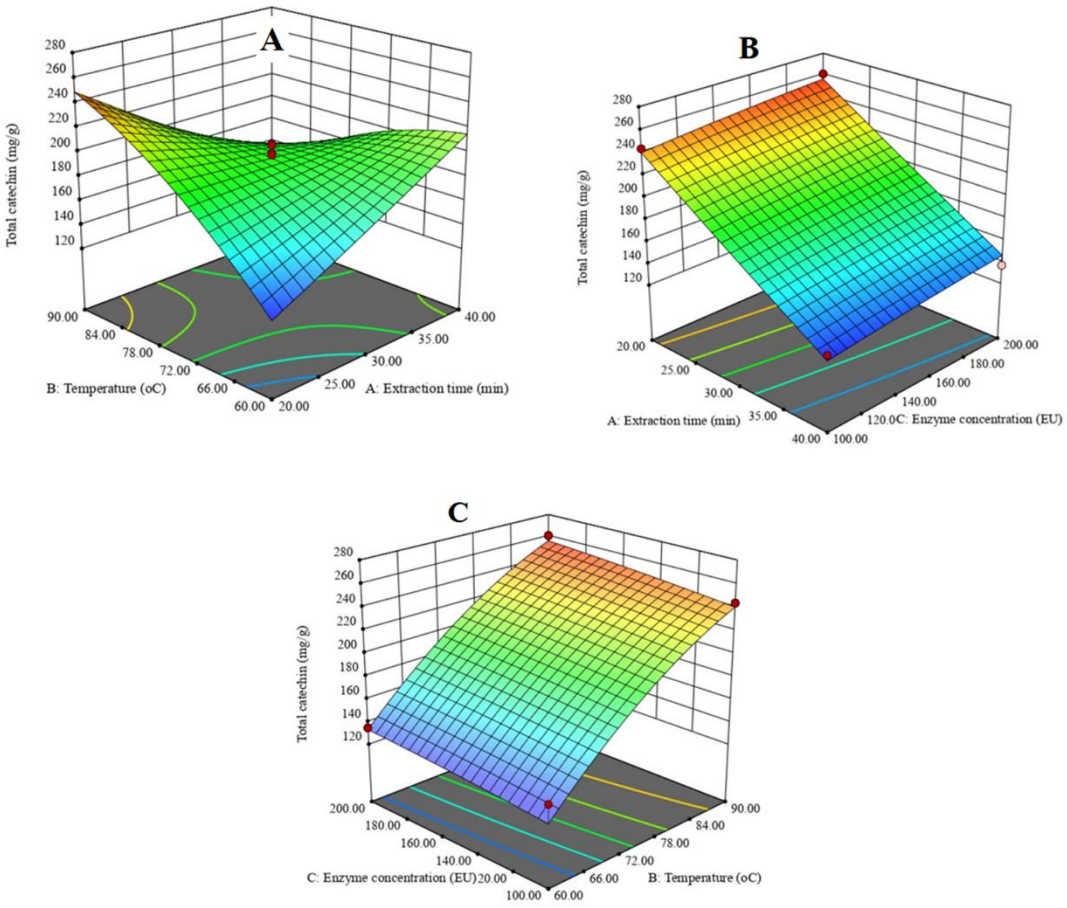


Figure 3.4: A - C Response surface plots for interaction of different parameters w.r.t total catechin content A – Temperature vs Time at EC = 150 EU, B – Extraction time vs Enzyme concentration at t = 90°C, C – Enzyme concentration vs Temperature at T = 20 min

because under these circumstances, the gallate catechins, EGCG and ECG, go through an epimerization process and change into gallocatechin gallate and catechin gallate, respectively. As a result, the extract's overall catechin content decreases (Tian et al. 2021). Catechins are oxidized to tea pigments such as theaflavins, thearubigins, theasinensins, theacitrins, and oolongtheanins, in addition to isomerization and conversion at elevated temperatures. This reaction of conversion of tea catechins into tea pigments is facilitated by polyphenol oxidase and peroxidase enzymes.

In the current work, tea leaves were dried at 60°C for 3.5 h before extraction. During the enzymatic extraction process, post-incubation (2h at 35°C), the enzymes were inactivated by treating them with boiling water. With these processes, PPO and POD activity is reduced

drastically, as stated in previous studies (Andreaus, Azevedo, and Cavaco-Paulo 1999; Khan and Barate 2016).

3.3.3.2 Epigallocatechin Gallate Content

Epigallocatechin gallate is one of the most abundant catechins found in the tea leaves. It constitutes nearly 50 % of the total catechin concentration and is a very powerful antioxidant (Nagle, Ferreira, and Zhou 2006; Su et al. 2003). The EGCG concentration surface plots for the variation of two of the independent variables while keeping the third variable fixed are shown in Figure 3.5 A – C. For a variation in extraction time and temperature (at 150 EU/g) from 20 min - 60°C and 40 min – 90°C, the EGCG concentration varied from 94.56 – 111.99 mg/g respectively. As observed in the response surface plot, EGCG concentration showed a peak concentration of 143.06 mg/g at 30 min and 75°C. Similarly, for a variation in temperature and enzyme concentration from 60°C – 100 EU/g and 90°C – 200 EU/g (at 30 min), the EGCG concentration altered as 85.80 – 134.02 mg/g, respectively. Lastly, upon variation of extraction time and enzymatic concentration from 20 min – 100 EU/g to 40 min – 200 EU/g (at 75°C), EGCG concentration altered as 123.43 – 82.56 mg/g with a peak concentration of 134.02 mg/g at 20 min – 200 EU/g respectively.

As shown in Figure 3.5 A - C, temperature and time had a strongly positive non-linear influence, while enzymatic concentration incurred a moderately positive non-linear influence on the EGCG concentration. EGCG concentration reduced significantly with a simultaneous increase in extraction time and temperature. Previous studies had also reported similar results and explained that extraction at higher temperatures increased cell wall permeability and enhanced the solubility and diffusion coefficients of tea catechins (Astill et al. 2001; Shao et al. 2020). This process is further expedited with enzymatic pretreatment and subsequently results in the higher release of catechins into the extracting solution (Gligor et al. 2019). Previous studies reported that prolonged exposure at higher temperatures (≥80°C) could cause

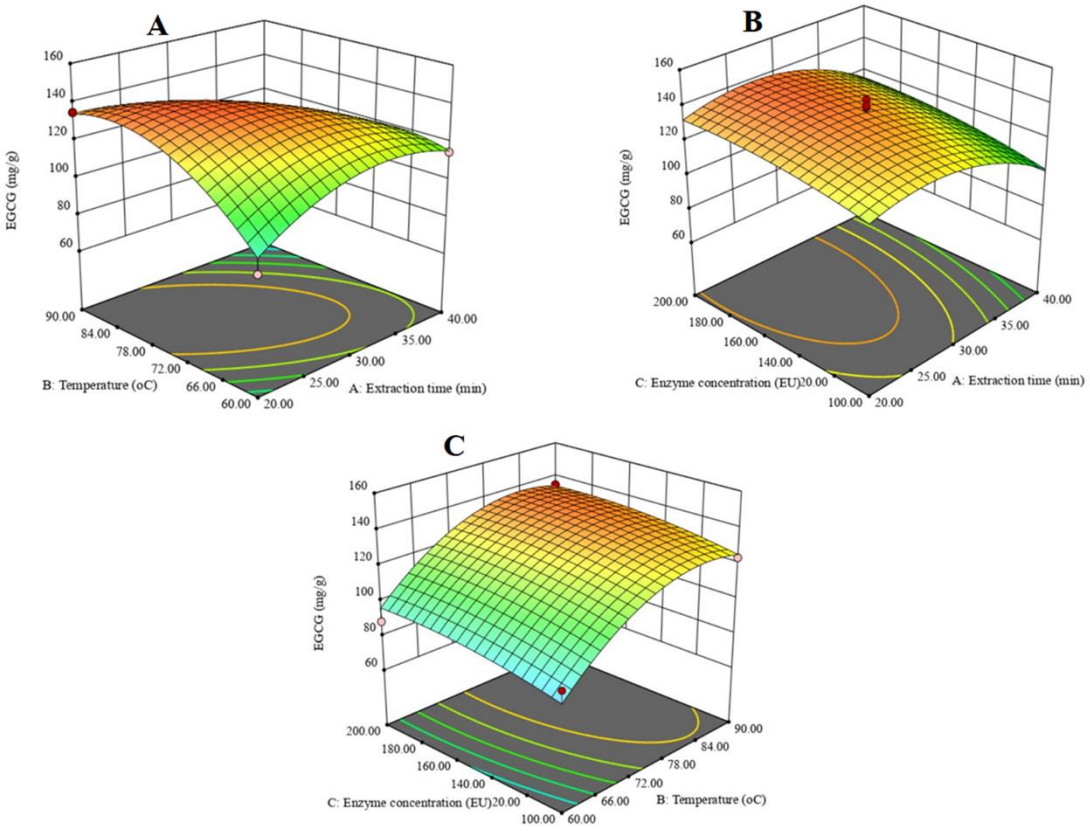


Figure 3.5: A - C Response surface plots for interaction of different parameters w.r.t EGCG content A – Temperature vs Time at EC = 150 EU, B – Extraction time vs Enzyme concentration at t = 75°C, C – Enzyme concentration vs Temperature at T = 30 min

a stereochemical conversion of EGCG into gallocatechin gallates through epimerization and loss in extraction yield (Astill et al. 2001; Tian et al. 2021).

As shown in Figure 3.5 A, the optimal EGCG concentrations were recorded in the areas with lower extraction time and higher temperature (EC = 150EU/g). Figure 3.5 B showed the optimal EGCG concentrations at regions with higher enzyme concentration and lower extraction time (t = 75°C). Also, upon alteration of enzymatic concentration and temperature

at 30 min, the optimal EGCG concentrations were recorded at higher enzymatic concentrations and mid temperatures.

3.3.3.3 Epicatechin Gallate Content

Figure 3.6 A - C illustrates the ECG concentration surface plots. Epicatechin gallate is one of the most common catechin compounds present in the tea leaves of Assam Tea cultivars and the second highest after EGCG (Sabhapondit et al. 2012). The ECG concentration varied from 19.87 – 20.58 mg/g for an alteration in extraction time and temperature from 20 min - 60°C and 40 min – 90°C at a fixed enzyme concentration of 150 EU/g respectively. Response surface plots revealed a peak ECG concentration of 43.07 mg/g at 20 min – 90°C and 150 EU/g. A maximum to minimum variation in concentration from 20.79 to 40.41 mg/g was observed upon variation of temperature and enzyme concentration between 60°C – 100 EU/g and 90°C – 200 EU/g (at 40 min). While, for changes in extraction time and enzyme concentrations at fixed temperatures (90°C), from 20 min – 100 EU/g to 40 min – 200 EU/g, the responses varied as 48.18 – 22.41 mg/g respectively.

Temperature and time had a strongly positive linear and non-linear influence on the ECG concentration, while enzymatic concentration had a moderately positive linear influence, as observed in the response surface plots (Figure 3.6 A - C). Similar to EGCG, ECG being a gallated catechin, undergoes stereochemical changes when exposed to high temperatures for longer periods. The major reactions responsible for such stereochemical changes are epimerization, oxidation, and isomerization (H. Liang et al. 2007). The optimal ECG concentration was recorded at either lower temperature and higher extraction time or higher temperature and lower extraction time conditions. The highest ECG concentration (50.02 mg/g) was recorded at 20 min, 90°C, and 100 EU/g conditions, and the second best yield (48.48 mg/g) was obtained under contrasting extraction conditions at 40 min, 60°C and 200 EU/g. Previous

studies conducted on hot water extraction from green tea leaves also reported similar trends in the effect of temperature and time on the ECG concentration (Vuong et al. 2011).

3.3.3.4 Epigallocatechin Content

EGC response surface plots are shown in Figure 3.7 A – C, and it displays the extraction behaviour of the catechins present in tea leaves with changes to the extraction time, temperature, and enzyme concentration. Upon variation of extraction time and temperature (20 min - 60°C to 40 min – 90°C), at fixed enzyme concentrations (150 EU/g), EGC yield altered from 10.19 – 27.46 mg/g while registering a peak concentration of 41.19 mg/g at 20 min and 90°C. The responses altered from 10.94 – 44.52 mg/g when temperature and enzyme concentration were varied from a low to a high combination of 60°C – 100 EU/g and 90°C – 200 EU/g (at 20 min). Also, EGC concentrations varied from 41.04 – 30.49 mg/g respectively for changes in the extraction time and enzyme concentration from 20 min – 100 EU/g and 40 min – 200 EU/g (at 90°C).

Temperature and time had a highly positive non-linear while enzymatic concentration had a moderately positive non-linear influence on the EGC concentration. The optimal EGC concentrations were obtained at higher temperatures and lower extraction times. Long exposure to high temperatures resulted in a significant loss of EGC concentration.

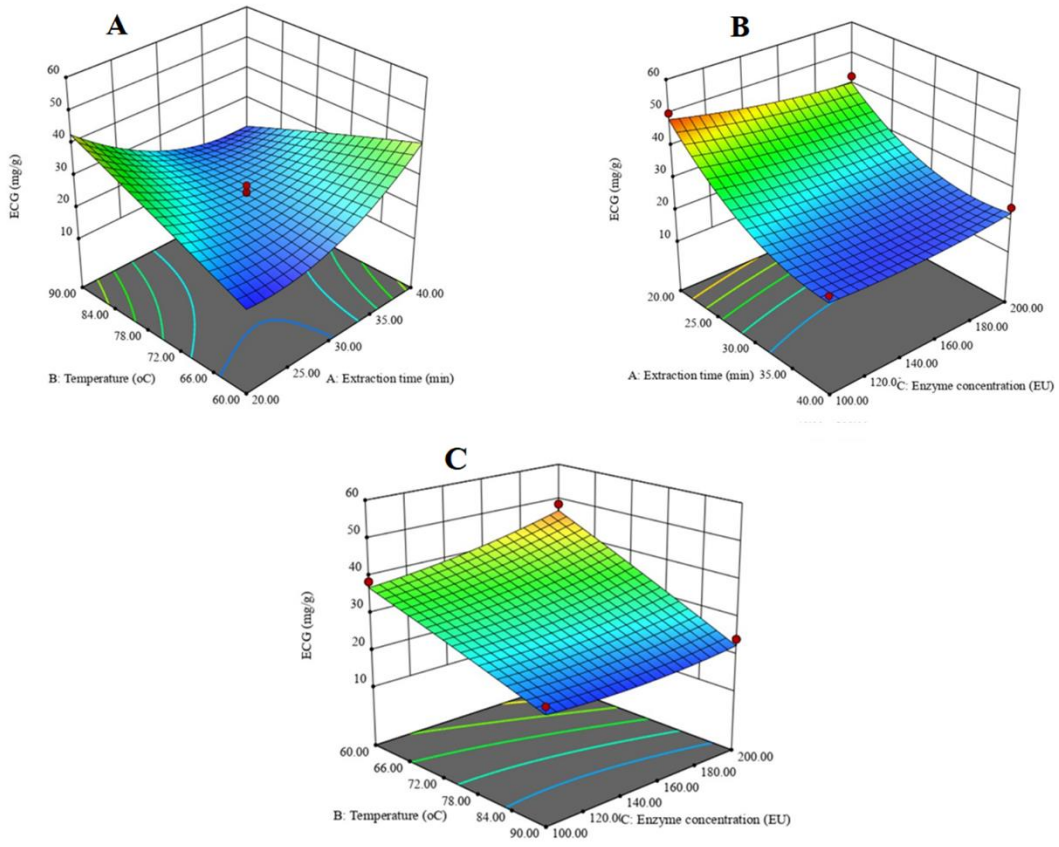


Figure 3.6: A – C Response surface plots for interaction of different parameters w.r.t ECG content A – Temperature vs Time at EC = 150 EU, B – Extraction time vs Enzyme concentration at t = 90°C, C – Enzyme concentration vs Temperature at T = 40 min

Previous studies on hot water extraction and catechin profile study using green tea leaves had also reported similar agreements on the effect of temperature and time on the EGC concentration (Vuong et al. 2011). At higher extraction temperatures, gallate catechins are epimerized, and interconversion leads to a higher yield of EGC concentration (Astill et al. 2001; Shao et al. 2020). The highest EGC concentration (47.84 mg/g) was obtained at 20 min - 90°C - 200 EU/g condition.

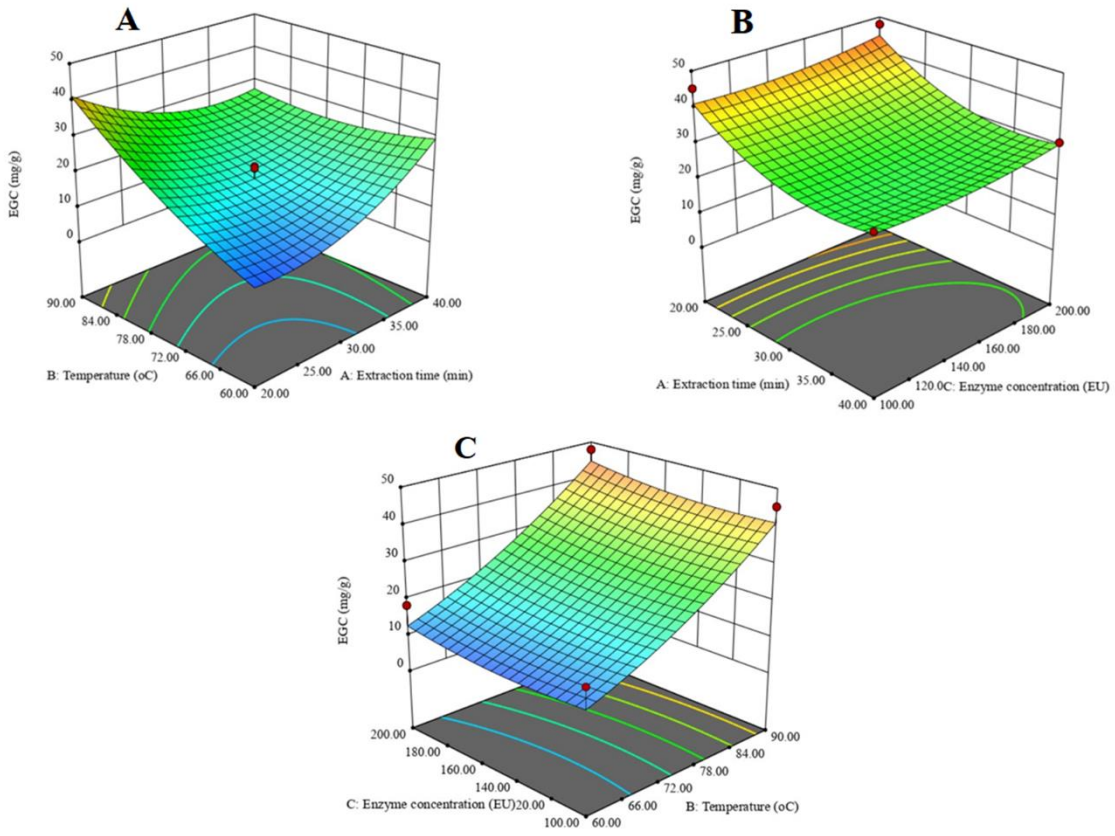


Figure 3.7: A – C Response surface plots for interaction of different parameters w.r.t EGC content A – Temperature vs Time at EC = 150 EU, B – Extraction time vs Enzyme concentration at t = 90°C, C – Enzyme concentration vs Temperature at T = 20 min

3.3.3.5 Epicatechin Content

The response surface plots for epicatechin responses with changes in extraction time, temperature, and enzyme concentration were shown in Figure 3.8 A – C. EC concentration ranged from -0.97 – 7.98 mg/g for a change in extraction time and temperature from 20 min - 60°C and 40 min – 90°C at 150 EU/g. Peak EC concentration was observed as 18.71 mg/g at 40 min, 60°C, and 150 EU/g. For a similar variation in temperature and enzyme concentration (60°C – 100 EU/g and 90°C – 200 EU/g) at fixed extraction time (40 min), EC concentration

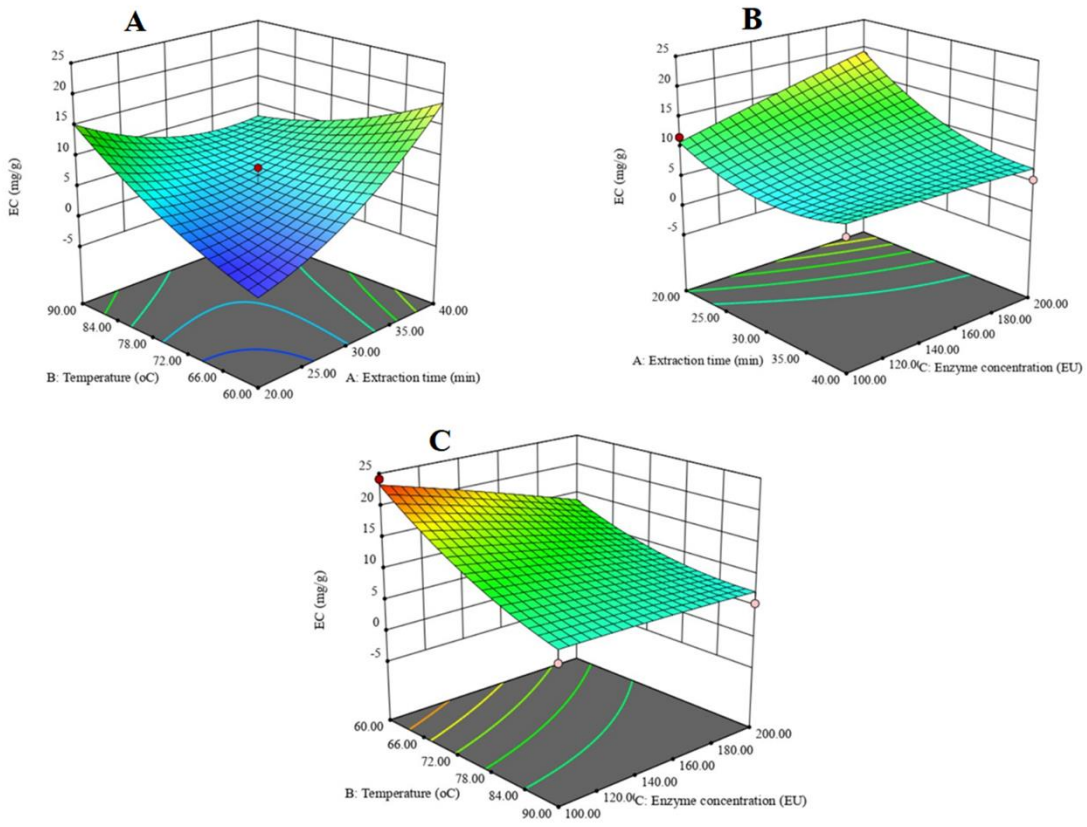


Figure 3.8: A - C Response surface plots for interaction of different parameters w.r.t EC content A – Temperature vs Time at EC = 150 EU, B – Extraction time vs Enzyme concentration at t = 90°C, C – Enzyme concentration vs Temperature at T = 20 min

changed from -1.12 to 19.29 mg/g, respectively. While with alteration in extraction time and enzyme concentration as discussed in the previous sections, at a fixed temperature (90°C), EC concentration varied from 10.85 to 7.35 mg/g respectively and registered a peak concentration of 19.29 mg/g at 20 min and 200 EU/g. Enzyme concentration had a moderately positive linear influence on EC concentration, while temperature and time incurred a highly positive non-linear influence. Optimal concentrations of EC were recorded at higher temperatures and lower extraction time conditions (EC=150EU/g). With the alteration of time and enzyme concentration at 90°C, optimal conditions of EC extraction were recorded at lower time and higher enzyme concentration (Figure 3.8 B). While, with the alteration of temperature and

enzyme concentration at 40 min, optimal concentrations of EC were recorded at lower temperatures and lower enzymatic concentration (Figure 3.8 C). Previous studies on hot water extraction reported similar trends with time and temperature combinations (Vuong et al. 2010). At higher temperatures, the results indicated a moderate increase in EC concentration upon an increase in enzyme concentration. The highest EC concentration was obtained at 40 min - 60°C - 100EU/g condition.

3.3.3.6 Catechin Content

Changes in catechin content with lowest to highest combinations of extraction time, temperature, and enzyme concentration are illustrated in the response surface plots in Figure 3.9 A - C. Catechin content altered from 4.46 - 3.11 mg/g with alteration in extraction time and temperature from 20 min - 60°C and 40 min – 90°C at 150 EU/g respectively with a peak value of 16.48 mg/g at 20 min – 90°C. Upon similar variation of temperature and enzyme concentration from 60°C – 100 EU/g and 90°C – 200 EU/g at 40 min extraction time, the catechin content ranged from 9.79 to 17.33 mg/g respectively. Also, upon alteration of extraction time and enzyme concentration from 20 min – 100 EU/g and 40 min – 200 EU/g at a fixed temperature of 90°C, the EC concentrations altered as 15.22 – 3.46 mg/g, respectively, with a peak value of 17.33 mg/g at 20 min and 200 EU/g.

Temperature and time had a strong positive non-linear influence on the catechin concentration while enzyme concentration had a moderately linear influence on it. The trans-configured catechin is a major isomer form present in tea leaves. Being non-epistructured, catechins were more stable at higher temperatures (Astill et al. 2001; Nagle, Ferreira, and Zhou 2006). As shown in Figure 3.9 A, with changes in time and temperature at 150 EU/g, optimal catechin concentrations were achieved at either higher temperature and low extraction time or lower temperature and higher extraction time conditions. Although relatively stable, extended

exposure to high temperatures (≥ 90°C) resulted in a significant loss of C concentration. Optimal catechin concentrations were observed at low extraction time and high enzymatic concentrations when time and enzymatic concentrations were varied at fixed temperatures (90°C), as shown in Figure 3.9 B. Figure 3.9 C showed that the catechin concentrations were higher at longer extraction time and lower enzymatic concentrations when temperature and enzyme concentration were altered at 40 min extraction time. The response surfaces revealed that an increase in enzymatic concentration didn't have a significant impact on the C extraction. Previous studies on hot water extraction of catechins have also reported similar trends in the extraction behaviour of catechin by time and temperature (Li et al. 2012).

3.3.4 Extraction Efficacy of the Enzymatic Method in Comparison to the Hot Water Extraction Process

The extraction experiments were conducted for a total of 24 data sets (4 control) and for various combinations of time, temperature, and enzymatic concentration being set with the CCRD model and control conditions, as shown in table 3.5. The control experiments were conducted with both water and methanol as the extraction solvent for comparison purposes. However, for the current research work, since all the experiments in enzymatic extraction were carried out using water as the extraction solvent, methanolic extraction control data is not used for comparison with the enzymatic extraction process.

3.3.4.1 Comparison of Hot Water and Methanolic Extraction (Control)

Table 3.6 shows the extraction yields for EGCG, ECG, EGC, EC, C, and TCC under methanolic extraction of catechins from fresh tea at 30 min - 75°C, 30 min - 100.2°C, 40 min-90°C, and 20 min - 60°C conditions. In comparison to hot water extraction, TCC increased in 20 min - 60°C, 40 min - 90°C, and 30 min - 75°C by 65.9, 28.84, and 14.71%, respectively.

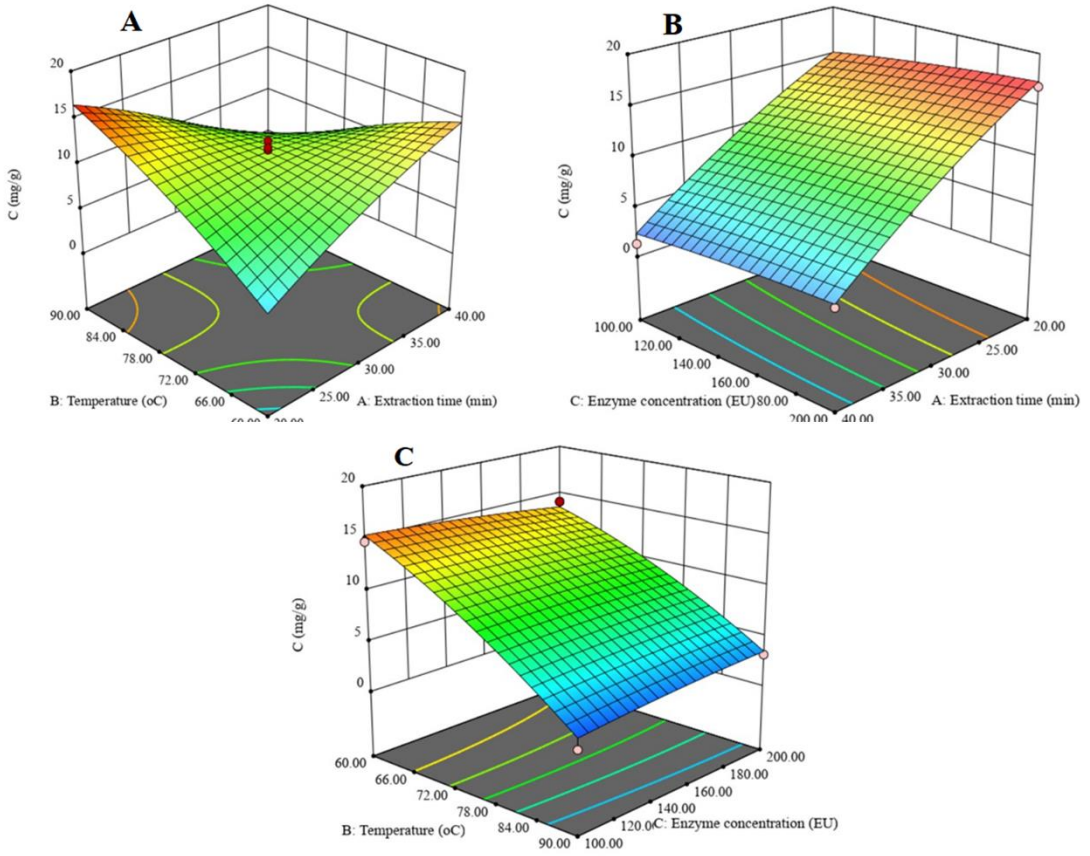


Figure 3.9: A - C Response surface plots for interaction of different parameters w.r.t C content A – Temperature vs Time at EC = 150 EU, B – Extraction time vs Enzyme concentration at t = 90°C, C – Enzyme concentration vs Temperature at T = 20 min

These results can be explained due to the superiority of methanol as an extraction solvent (Koch et al. 2020; Roedig-Penman and Gordon 1997). However, for 30 min – 100.2°C condition, TCC reduced significantly in comparison to HWE. This can be explained due to the loss of catechins at higher temperatures with better extraction rates in the methanolic extraction process (Vuong et al. 2011). Previous studies on S3A3 and TV18 tea cultivars using methanolic extraction reported the best extraction conditions at 20 min and 70°C as per ISO 14502:2:2005 (Baruah, Singha, and Uppaluri 2023).

Table 3.5: Extraction yields for EGCG, ECG, EGC, EC, C, and TCC under methanolic extraction of catechins from fresh tea leaves

Time min	Temperature °C	EGCG	ECG	EGC	EC	C	TCC
30	75	85.43 ± 0.88	44.47 ± 0.06	44.65 ± 7.10	10.23 ± 2.80	16.73 ± 1.44	201.50 ± 7.63
30	100.2	8.42 ± 3.51	32.46 ± 1.68	24.87 ± 3.22	1.05 ± 0.19	2.34 ± 0.11	69.15 ± 8.50
40	90	59.65 ± 18.21	37.59 ± 0.70	28.78 ± 3.16	2.65 ± 0.80	1.46 ± 0.35	130.13 ±23.22
20	60	80.58 ± 0.68	36.24 ± 1.92	34.27 ± 8.98	9.10 ± 1.92	15.88 ± 1.00	176.07 ±14.51

3.3.4.2 Total Catechin Content

For the total catechin content profile being obtained with the HPLC, Figure A1 presents the pertinent trends. Compared to the control experiments at 20 min - 60°C, 30 min - 75°C, 30 min - 100.2°C, and 40 min – 90°C conditions, the data obtained for the enzymatic pretreatment cases involved a significant increase in total catechin concentration. The maximum value of total catechin content was observed for the combination of 20 min - 90°C – 200 EU/g (260.52 mg/g of dried tea extract). For the 20 min - 60°C -100 EU/g combination case, the total catechin content increased by 29.02% upon enzymatic pretreatment (100EU/g) in conjunction with the hot water extraction. However, the values did not differ significantly for the case that referred to the higher enzyme dosage of 200 EU/g. For the combination of 30 min - 100.2°C - 150 EU/g, the total catechin content increased by 14.62 % with respect to those obtained with the hot water extraction method. For the 30 min - 75°C combination, it was observed that the enzymatic treatment with 150 and 234 EU/g improved TCC by 11.47, and 18.41%, respectively, in comparison to hot water extraction. These findings are similar to those reported in the earlier studies for the total polyphenol and flavonoid yield being reported for the green tea extract (Baruah, Singha, and Uppaluri 2023; Y. R. Liang et al. 2008).

Table 3.6: Extraction yields of EGCG, ECG, EC, EGC, and C at CCRD based combinations of time, temperature, and enzyme concentration and control conditions

Run	Extraction time, min	Temperature, °C	Enzyme concentration, EU/g	EGCG, mg/g	ECG, mg/g	EC, mg/g	EGC, mg/g	C, mg/g	TCC, mg/g
1	30	75	150	138.93 ± 10.74	27.29 ± 3.24	5.29 ± 1.96	11.03 ± 0.32	9.90 ± 1.06	192.45 ± 8.72
2	20	90	100	123.43 ± 3.75	50.02 ± 2.89	11.70 ± 3.29	45.24 ± 2.61	13.54 ± 3.61	243.93 ± 2.35
3	30	75	100	138.69 ± 9.61	24.15 ± 1.31	4.70 ± 0.85	21.36 ± 3.46	10.70 ± 0.25	199.60 ± 8.06
4	30	49.77	150	86.94 ± 8.94	19.82 ± 2.43	5.66 ± 0.83	7.37 ± 1.94	8.60 ± 0.09	128.39 ± 14.26
5	30	75	65.91	110.43 ± 2.65	24.82 ± 0.62	4.54 ± 1.78	16.01 ± 2.73	12.71 ± 1.74	168.51 ± 6.03
6	20	60	200	88.37 ± 15.68	23.33 ± 2.68	0.95 ± 0.13	18.33 ± 0.43	3.80 ± 0.00	134.78 ± 18.93
7	20	90	200	134.77 ± 8.74	42.55 ± 6.78	18.51 ± 1.12	47.84 ± 7.71	16.86 ± 0.83	260.53 ± 9.94
8	46.82	75	150	84.62 ± 2.01	40.41 ± 4.71	21.44 ± 3.89	37.01 ± 0.54	8.09 ± 0.57	191.57 ± 7.53
9	40	60	200	114.96 ± 8.15	48.48 ± 3.66	13.10 ± 2.77	33.59 ± 6.50	14.11 ± 0.65	224.25 ± 21.74
10	13.18	75	150	111.39 ± 1.91	41.62 ± 0.48	6.28 ± 0.04	24.56 ± 1.03	11.67 ± 0.31	195.52 ± 1.63
11	40	90	100	75.36 ± 7.15	23.33 ± 0.49	6.3 ± 0.61	28.09 ± 3.08	1.30 ± 0.44	134.37 ± 8.70
12	30	75	150	143.06 ± 2.79	25.22 ± 0.27	5.33 ± 0.12	21.19 ± 4.46	11.66 ± 0.77	206.47 ± 7.62
13	20	60	100	92.75 ± 7.59	22.79 ± 0.28	0.87 ± 1.17	16.64 ± 1.74	3.88 ± 0.64	136.93 ± 11.43
14	30.00	100.23	150.00	92.20 ± 0.11	21.29 ± 1.15	16.07 ± 2.40	36.37 ± 1.70	10.57 ± 1.50	176.50 ± 2.06
15	30.00	75.00	234.09	144.68 ± 1.14	26.26 ± 0.16	5.53 ± 0.12	20.89 ± 0.18	10.65 ± 0.20	208.01 ± 1.10
16	30.00	75.00	150.00	129.15 ± 2.23	22.10 ± 0.41	8.21 ± 2.66	21.84 ± 1.73	12.65 ± 0.14	193.95 ± 7.19
17	30.00	75.00	150.00	124.24 ± 19.24	24.94 ± 0.18	5.17 ± 0.88	18.20 ± 2.45	11.67 ± 0.07	184.21 ± 20.55
18	30.00	75.00	150.00	139.66 ± 1.41	24.17 ± 0.22	4.64 ± 0.64	17.85 ± 11.78	11.92 ± 1.54	198.24 ± 13.87
19	40.00	90.00	200.00	73.17 ± 0.71	24.15 ± 0.55	5.46 ± 0.24	30.98 ± 4.58	3.14 ± 0.18	136.90 ± 3.26
20	40.00	60.00	100.00	98.12 ± 15.92	38.60 ± 7.68	24.14 ± 1.99	33.39 ± 2.71	14.72 ± 1.04	208.97 ± 19.95
				Control extraction (HWE)					
21	30	75	-	121.57 ± 18.93	27.87 ± 3.51	3.21 ± 0.14	12.55 ± 1.22	10.46 ± 0.87	175.66 ± 21.95
22	40	90	-	46.96 ± 1.71	18.51 ± 0.46	9.37 ± 4.51	12.21 ± 2.30	13.94 ± 1.28	101.00 ± 2.24
23	20	60	-	62.20 ± 0.02	24.34 ± 0.04	0.52 ± 0.43	17.43 ± 2.03	1.63 ± 0.01	106.13 ± 1.62
24	30	100.23	-	87.87 ± 4.37	19.43 ± 1.12	10.75 ± 0.36	25.76 ± 0.56	10.17 ± 0.01	153.99 ± 5.68

3.3.4.3 Epigallocatechin Gallate

The effects of enzymatic pretreatment had been most prominent in EGCG extraction, and the comparison with hot extraction reaffirms the obtained trends. As shown in Figure A2, in comparison to hot water extraction at 20 min - 60°C and 40 min - 90°C conditions, the use of enzymatic pretreatment (100 EU/g) was able to significantly enhance EGCG concentration by 49.11% and 60.47% respectively. Also, at 30 min - 100.2°C and 30 min - 75°C control conditions, the use of enzymatic pretreatment (150 EU/g) improved EGCG concentration by 4.69% and 11.55%, respectively. An increase in enzymatic concentration (100 - 200 EU/g) at higher temperatures and a longer time resulted in a slight loss of EGCG concentration (3 % at 40 min – 90°C). Cellulase and pectinase enzymes cause cell wall degradation, which allows rapid release of catechin compounds during the extraction and, as a result, becomes more susceptible to loss under prolonged exposure at higher temperatures (≥ 90°C). The maximum concentration of EGCG was obtained at 30 min - 75°C – 234 EU/g (144.68 mg/g).

3.3.4.4 Epicatechin Gallate

As shown in Figure A3, the results obtained indicated that enzymatic pretreatment did not have significant bearings in ECG extraction. For instance, when compared to 30 min -75°C, 20 min - 60°C control case, enzymatic pretreatment did not result in any significant increment in the extraction process with an increase in enzymatic concentration from 65.9 - 234 EU/g. At 40 min – 90°C and 30 min - 100.23°C conditions, enzymatic treatment improved ECG concentration by 30.47 and 9.57 %, respectively.

3.3.4.5 Epigallocatechin

The comparison of EGC extraction under enzymatic and hot water extraction methods is shown in Figure A4. In comparison to hot water extraction, at 30 min - 75°C, EGC yield increased by 66.45% at enzymatic pretreatment of 234EU/g. While in the case of 30 - 100.2 control experiments, enzymatic pretreatment resulted in an EGC concentration increment of 41.19% upon enzymatic pretreatment (150EU/g). Also, in comparison to 40 min - 90°C control cases, EGC concentration increased by 130.05 and 153.72% under enzymatic pretreatment at 100 and 200 EU/g, respectively. Previous studies reported the interconversion of gallate catechins into EGC as a result of stereochemical reactions such as epimerization under prolonged exposure at high temperatures (Hong et al. 2013). Enzymatic pretreatment causes a rapid release of catechin compounds through cell wall degradation and increases the rate of interconversion of gallated catechins into EGC through epimerization reactions. At low-temperature, short-duration combinations, interconversion of gallate catechins into EGC did not occur. As such, in the current study, at 20 min - 60°C control case, there was no significant change observed in EGC yield upon enzymatic pretreatment.

3.3.4.6 Epicatechin

Figure A5 illustrates the comparison of EC extraction under enzymatic and hot water extraction methods. Temperature and extraction time played a major role in EC extraction along with enzymatic pretreatment. The comparison of enzymatic extraction data sets with hot water extraction methods reaffirms the effect of enzymatic pretreatment on EC yield. EC concentration increased by 73.10% under enzymatic treatment at 150 EU/g in comparison to hot water extraction at 30 min and 75°C. Further increase in enzyme concentration to 234 EU/g didn't have a significant impact on EC concentration. In comparison to control experiments at 30 min - 100.2°C and 20 min – 60°C, enzymatic pretreatment at 150 EU/g and 100 EU/g

improved EC concentration by 49.48, and 82.69%, respectively. On the contrary, in comparison to the 40 min – 90°C control case, enzymatic pretreatment (100 & 200EU/g) resulted in a decrease in EC concentration. Prolonged exposure to high temperatures causes EC degradation through various stereochemical changes such as epimerization, isomerization, etc. These processes were expedited by enzymatic pretreatment and resulted in a higher loss of EC (Astill et al. 2001; Nagle, Ferreira, and Zhou 2006).

3.3.4.7 Catechin

The comparison of catechin extraction under enzymatic and hot water extraction methods is plotted in Figure A6. The results indicated that enzymatic pretreatment did not have a significant impact on C concentration. In comparison with 20 min – 60°C control case, upon enzymatic pretreatment at 100 EU/g C, concentration increased by 138.03%. Further increase in enzyme concentration at 200 EU/g did not improve EC concentration significantly. In all the other control experiments, enzymatic pretreatment did not have a significant impact on C concentration. Post enzymatic treatment, catechins become more susceptible to degradation under prolonged exposure at higher temperatures. Previous studies reported that catechin degradation at high temperatures occurs primarily due to epimerization, polymerization, and oxidation reactions (Astill et al. 2001; Nagle, Ferreira, and Zhou 2006).

3.3.4.8 Effect of Enzymatic Pretreatment on Different Catechins

Enzymatic pretreatment had a significant impact on the extraction efficiency of EGCG alone and did not bear much significance for ECG, EGC, EC, and C. However, control extraction experiments revealed that enzymatic pretreatment had a moderate effect on the extraction efficiency of EGC. As discussed in the previous sections, an increase in EGC concentrations is also associated with the interconversion of gallate catechins at higher temperatures which is

further ameliorated by the enzymatic pretreatment in the extraction process. Enzymatic pretreatment did not have a significant impact on the extraction efficacy of ECG, EC, and C. These results suggested that enzymatic pretreatment had no major distinction between gallated catechins and non-gallated catechins. While the distribution of the catechin compounds in the subcellular structure of tea leaves may have caused differential effects on the extraction efficiency of the catechins. Previous studies have stated that EGCG and EGC, which are higher in concentrations in tea leaves (Vuong et al. 2011), were located in the large vacuoles inside the mesophyll cells (Khan and Barate 2016; Roedig-Penman and Gordon 1997). Catechin compounds with low concentrations are possibly located in smaller vacuoles (Suzuki et al. 2003). Hence, cell wall degrading enzymes may have comparatively easier access to EGCG and EGC present inside the larger vacuoles in the mesophyll cells.

3.3.5 Optimization of the Extraction Conditions for Total Catechin Content

Response surface optimization was carried out using design expert software for the chosen three degrees of freedom, namely time(T), temperature(t), and enzyme concentration (EC). Table 3.7 summarizes the relevant criteria and numerical optimization outcomes for the enzymatic extraction process. During the study, three parameters, namely extraction time, temperature, and enzyme concentration, were altered as 13.18 - 46.82 min, 49.77 - 100.2°C, and 65.91 - 234 EU/g, respectively. The total catechin content had been assigned an importance level of 4 as the model was set to get the best extraction profile for all catechins. All other parameters, including dependent and independent variables, were set to an important level of 3. For the given inputs and optimization criteria, the model predicted best conditions were 18.1 min, 88.5°C, and 188.0 EU/g of enzyme concentration (with a desirability of 0.926). Corresponding predicted optimal catechins content are 260.52, 134.55, 44.30, 44.96, 19.18, and 17.53 mg/g for TCC, EGCG, ECG, EGC, EC, and C, respectively.

Table 3.7: Criteria and outputs of numerical optimization of the responses for enzymatic extraction of catechins analysed at ($p < 0.05$)

	Parameters	Goal	Importance	Predicted value, mg/g
Independent Variables	A. Extraction time, min	In range	3	18.08
	B. Temperature, ℃	In range	3	88.47
	C. Enzyme concentration, EU	In range	3	188.08
	Desirability			0.926
Dependent variables	Y1: Total catechin content, mg/g	Maximize	4	260.52
	Y2: EGCG, mg/g	Maximize	3	134.55
	Y3: ECG, mg/g	Maximize	3	44.30
	Y4: EGC, mg/g	Maximize	3	44.96
	Y5: EC, mg/g	Maximize	3	19.18
	Y6: C, mg/g	Maximize	3	17.53

Table 3.8 presents a comparative summary of extracted catechin yields under the experimental best conditions, best conditions as per the numerical optimization, and hot water extraction. Among the 20 experiments conducted as per the CCRD model, the best experimental data-based extraction conditions were 20 min, 90°C, and 200 EU/g. Under these circumstances, the total catechin concentration was found to be 260.53 mg/g. The predicted yield of TCC at the optimal conditions was similar (260.53 mg/g) but with marginally lower values of extraction time (18.1 min), temperature (88.5°C), and enzyme concentration (188 EU/g). Upon experimental validation at the optimal condition, the catechin content was obtained as 265.7, 140.25, 44.98, 43.98, 18.94, and 17.55 mg/g for TCC, EGCG, ECG, EGC, EC, and C, respectively. In comparison to these results, the best results with hot water extraction were obtained for the condition of 30 min and 75°C. These are in agreement with the data reported in the previous work on hot water extraction (Vuong et al. 2011). Catechin yields under these conditions were 175.66, 121.57, 27.87, 12.55, 3.21, and 10.46 mg/g for TCC, EGCG, ECG,

EGC, EC, and C, respectively. Extraction time and temperature played a major role in the yield of various catechins in the undertaken research work and did corroborate with the trends reported in the literature (Baruah, Singha, and Uppaluri 2023; Shao et al. 2020). With enzymatic extraction, the present work conveyed a significant increase in the yield of TCC and EGCG, which is comparable to previous works that conveyed that a significant increase in the TPC and TFC yield was apparent due to the usage of cell wall degrading enzymes such as cellulase and pectinase (Chandini, Jaganmohan Rao, and Subramanian 2011; Hu et al. 2018; TC et al. 2016).

Table 3.8: Comparison of catechin yields extracted under the best Experimental enzymatic extraction (EE), Optimized, and Hot water extraction conditions

Catechin type	Experimental EE, mg/g	Optimal EE, mg/g	Hot water extraction, mg/g
TCC	260.53	265.7	175.66
EGCG	134.77	140.25	121.57
ECG	42.55	44.98	27.87
EGC	47.84	43.98	12.55
EC	18.51	18.94	3.21
C	16.86	17.55	10.46

3.3.6 Literature Comparison

Table 3.9 summarizes the literature comparison of the available studies with the findings of the current research work under optimal conditions. Previous works only reported catechin profile studies using hot water extraction. TCC under enzymatic extraction improved significantly by 29.02, 18.41, 35.54, and 14.62% at 20 min – 60°C – 200 EU/g, 30 min -75°C – 234 EU/g, 40 min – 90°C – 200 EU/g, and 30 min – 100.2°C – 150 EU/g respectively in comparison to hot water extraction at 20 min – 60°C, 30 min - 75°C, 40 min – 90°C, and 30 min – 100.2°C respectively. Among the catechin compounds, EGCG concentration improved significantly

Table 3.9: Literature comparison table for the best conditions of hot water and enzymatic extraction with previously reported works

Extraction Condition (Min - °C - EU)	EGCG, mg/g	ECG, mg/g	EC, mg/g	EGC, mg/g	C, mg/g	Total catechin, mg/g	Optimized Conditions	Reference
30-75 - control	121.57 ±18.93	27.87 ± 3.51	3.21 ± 0.14	12.55 ± 1.22	10.46 ± 0.87	175.66 ±21.95	18.1 mins 88.5°C 188 EU	Current work
40-90-control	46.96 ± 1.71	18.51 ± 0.46	9.37 ± 4.51	12.21 ± 2.30	13.94 ± 1.28	101.00 ± 2.24		
30-75-234	144.68 ± 1.14	25.26 ± 0.16	5.53 ± 0.12	20.89 ± 0.18	10.65 ± 0.20	207.01 ± 1.10		
40-60-200	114.96 ± 8.15	48.48 ± 3.66	13.10 ± 2.77	33.59 ± 6.50	14.11 ± 0.65	224.25 ±21.74		
20-90-200	134.77 ± 8.74	42.55 ± 6.78	18.51 ± 1.12	47.84 ± 7.71	16.86 ± 0.83	260.53 ± 9.94		
Literature study								
40-90 (Hot water extraction)	1.58	4.21	1.47	3.69	-	10.95	Chandini et al., 2011	
Hot water extraction	55.7	12.7	9.7	26.2	6.5	111.1	30 min - 80°C	Vuong et al, 2011

with enzymatic pretreatment (234 & 100 EU/g) in the order of 19.0 and 60.47% in comparison to control cases at 30 min – 75°C and 40 min – 90°C respectively. The detailed explanation behind the mechanism is cited in section 3.5. In comparison to previous works conducted (Vuong et al. 2011), TCC improved by 134.50 and 139.15% for the best experimental data and optimal condition as per numerical integration, respectively. The possible reasons for such an increase in TCC might be due to the use of tea leaves, enzymatic pretreatment, and higher solid-to-water ratio (260.53 and 265.7 mg/g in this case for the experimental best and optimal condition in comparison to 111.1 mg/g in the literature), etc. in conjunction with the literature values.

3.4 Thematic Summary

The current investigation considered equal concentrations of the cell wall degrading enzymes cellulase and pectinase in the enzymatic extraction process. Thus, the carried-out investigation proved the efficacy of the enzymes in improving catechin recovery when combined with conventional hot water extraction. However, future researches may be carried out to understand both the individual effect of cell wall degrading enzymes on the recovery of phenolic bioactives such as catechins in a dose dependent manner and their effect on the different catechin types when used individually as well as combined.

Chapter 4:

Preparation of Potato Starch Nanoparticles using Acid Hydrolysis and Ultrasonic Post-treatment

Chapter 4

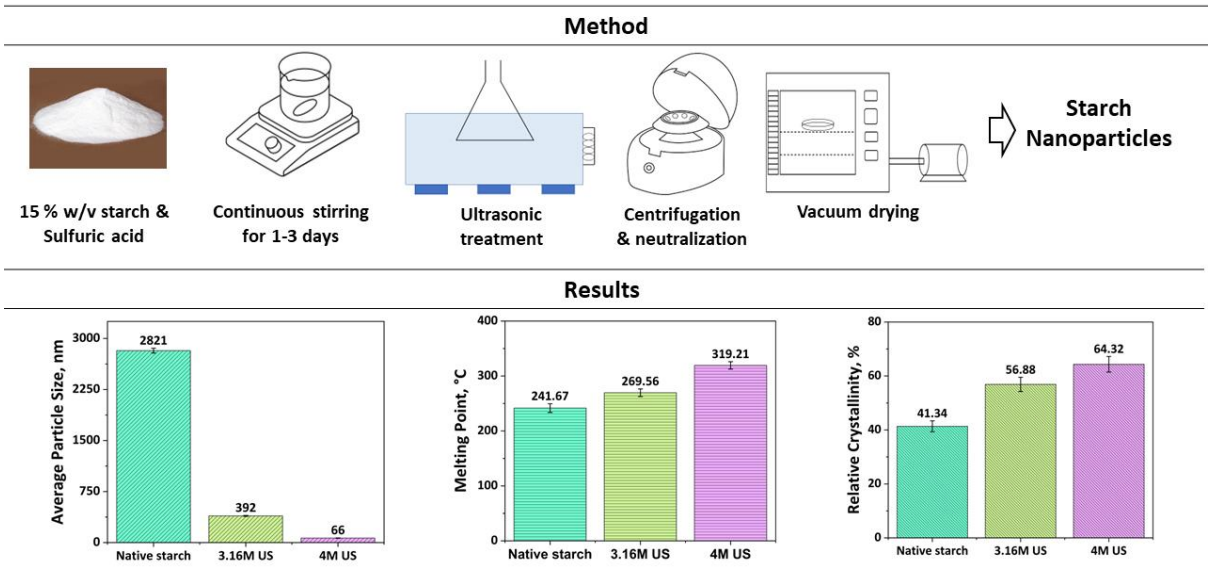
Preparation of Potato Starch Nanoparticles using Acid Hydrolysis and Ultrasonic Post-treatment

In this chapter potato starch nanoparticles prepared using acid hydrolysis and ultrasonication post treatment has been characterized for its physical and chemical properties. They include size, polydispersity index (PDI), morphology, thermal, and crystalline properties. Section 4.1 presents a brief introduction on the preparation of starch nanoparticles using acid hydrolysis and ultrasonication post treatment. Thereafter, section 4.2 contains two sub sections 4.2.1 and 4.2.2 which entails a detailed analysis on the effect of duration, molar concentration, and ultrasonication on the quality of the prepared starch nanoparticles in terms of its size, PDI, and yield. Section 4.3 presents the morphological changes occurred during the preparation of the starch nanoparticles. Section 4.4 and 4.5 addresses a detailed analysis of the crystalline and thermal properties of the prepared starch nanoparticles respectively. Finally, the significant findings of the conducted investigations are summarized in the section 4.6.

Overview

Starch nanoparticles are one of the most commonly synthesized material for its wide array of applications in the food processing as a carrier vehicle for functional bioactives. Starch nanoparticles are preferred for the encapsulation of a diverse group of compounds in lieu of its modifiable nature, cheaper costs, and easy availability. The size, polydispersity index (PDI), thermal and crystalline properties were one of the key targets in the recent studies. Also, compared to conventional starch which has a high glycemic index, starch nanoparticles can be modified to have higher resistant starch content through methods such as acid hydrolysis.

In the current chapter, potato starch nanoparticles were prepared using a combination of acid hydrolysis and ultrasonication post-treatment. The prepared starch nanoparticles were characterized for its size, PDI, morphology, thermal, and crystalline properties.



4.1 Introduction

Starch biomaterial is a promising component in food products due to its non-toxic, cheap, appropriate physical, and chemical properties and safe consumption characteristics. (Zhu 2017). Functional foods based on starch to a large extent explore its constitution in terms of amylopectin and amylose. Further, starch offers additional flexibility to undergo structural modifications through simpler methods such as acetylation, crosslinking, acid hydrolysis, retrogradation, and enzymatic treatment (Acevedo-Guevara et al. 2018; Morán et al. 2021). Thereby, appropriate modifications to the physical and chemical characteristics of modified starch-based substances can be efficiently targeted towards customized food product applications. Among various botanical sources being deployed for starch nanoparticles preparation, potato starch is beneficial from the perspectives of its lower cost, ease of modification, and nutritional characteristics (Jiang et al. 2016; KIM et al. 1995; Sani et al. 2021). Promising applications have been reported till date for potato starch nanoparticles and

these refer to encapsulation of vitamin D3 using sonication assisted nanoprecipitation method and encapsulation of bioactive substances constituting functional groups such as secosteroids, polyphenols (Fonseca et al. 2020; Hasanvand, Fathi, and Bassiri 2018; Sani et al. 2021).

With functional applications such as nanocarriers, emulsion stabilizers and film characteristics enhancers, starch nanoparticles received significant emphasis in food product applications. It is well known that the characteristics of starch nanoparticles are strongly dependent upon the fabrication process being adopted. Compared to the conventional acid hydrolysis method, the modern methods such as enzyme debranching, sonication, thermo-sonication, milling, nanoprecipitation provide enhanced process features. Needless to convey, conventional starch hydrolysis process takes about 7 – 9 days to achieve 40-70 nm sized starch nanoparticles through the deployment of 3.16M sulfuric acid solution at 40°C (Campelo, Sant'Ana, and Pedrosa Silva Clerici 2020). During acid hydrolysis process, the hydroxonium ion (H^3O^+) breaks down the α 1-4 and α 1-6 glycosidic linkages that forms the starch polymer. These results in the generation of numerous short linear chains of starch molecule and subsequently causes the formation of nanoparticles.

The current work aims to shorten the preparation time and also to enhance the quality of the starch nanoparticles in terms of its various physical (size, PDI, Crystallinity, thermal degradation point) and chemical properties (Delta C_p). In order to do so, the present work investigates the effect of high H_2SO_4 acid concentration (3-5M) in combination with ultrasonication post-treatment (90mins) towards the preparation of starch nanoparticles.

4.2 Effect of Sulfuric Acid Concentration, Hydrolysis Duration, and Sonication on Size, PDI, and Yield

Potato starch nanoparticles were prepared using ultrasound assisted acid hydrolysis method. The effect of change in molar concentration of sulfuric acid, duration, and ultrasonication on

size, polydispersity index, and yield had been studied in the reported work.

4.2.1 Effect of Change in Duration and Sulfuric Acid Concentration

In the present work, various molar concentrations of sulfuric acid have been used for the acid hydrolysis of starch, viz. 3.16M, 4M, 5M, and control experiments without ultrasonication post-treatment for 3.16M and 4M concentrations (Table 1). Preliminary experiments were done, and it was found that above this range, increasing molar concentration further produced nanoparticles with a very insignificant yield (less than 0.2%). And below 3.16M concentration, it took a very long duration for the preparation of nanoparticles. The sulfuric acid concentration had a positive effect on the average particle size and PDI of the starch nanoparticles while negatively affected the yield of the nanoparticles. The starch nanoparticles have been observed to be substantially influenced with the hydrolysis duration as well (Figure 4.1 A - C). It can be observed that for an alteration in hydrolysis duration from 24 to 72 h, the average particle size reduced from 1378 - 370, 180 - 66, 102 - 13, 1550 - 392, 323 - 146 nm for 3.16M, 4M, 5M, 3.16M control, and 4M control cases respectively (Table 1). The reported findings in the prior art are in agreement with the results obtained for the control case i.e., 3.16M sulfuric acid without sonication (Copeland et al. 2009; Kim et al. 2012). It has been also observed that the particle size reduction has been predominant for the hydrolysis duration of 24 - 48 h but not for 48 - 72 h. The best dataset in terms of average particle size corresponds to 5M AH+US and 4M AH+US cases (Table 1).

The polydispersity index of the nanoparticles also varied in accordance with changes in the duration and molar concentration of the hydrolysis process. For an increase in the hydrolysis duration from 24 - 72 h, the polydispersity index varied from 0.496 - 0.334, 0.414 - 0.362, 0.527 - 0.327, 0.383 - 0.354, 0.611 - 0.747 for 3.16M, 4M, 5M, 4M control and 3.16M control respectively (Table 1). The PDI of starch nanoparticles mostly varied linearly and reduced

along with average particle size with increasing duration. However, in some cases, the PDI values increased from time duration alteration from 2 - 3 days. The high PDI values meant wider distribution of sizes of the starch nanoparticles. It can be concluded that PDI depends on multiple factors, including duration, molar concentration of sulfuric acid, and sonication, and hence the definitive linear trend doesn't exist for the PDI.

The yield of the starch nanoparticles reduced linearly with increasing duration for all experimental conditions. It had been observed that for a variation of duration from 1 - 3 days, the yield of the starch particles altered from 82 - 65, 59 - 29, 9 - 4, 55 - 30, 83 - 66% for the 3.16M, 4M, 5M, 4M control, 3.16M control conditions of acid hydrolysis respectively (Table 1). The yield of the starch nanoparticles was inversely proportional to the hydrolysis duration. The same trend was apparent in the relevant prior art (Kim et al. 2012; Kim, Han, et al. 2013). In due course of starch nanoparticles preparation, yield along with average particle size and PDI have been considered as very important parameters. Further, the 5M sulfuric acid case reported yield less than 10% despite affirming better average particle size. Hence, it was considered to be non-optimal for the preparation of starch nanoparticles.

The optimum results with respect to the size of the starch nanoparticles were obtained in the 5M control hydrolysis for 3 days duration, where the hydrolysis resulted in the production of nanoparticles of 13 nm size. In terms of polydispersity index, the best results were obtained in 5M control hydrolysis for 2 days with a polydispersity index of 0.211. In terms of yield, however, in 5M control hydrolysis, the yield was very poor. As the yield changes negatively with an increase in molar concentration, the best yield was observed in 3.16M control hydrolysis for a duration of 1 day with 83% yield.

4.2.2 Effect of Ultrasonication

The average size of particles obtained varied vastly with ultrasonication, change in molar

Table 4.1: Literature comparison for starch nanoparticle prepared using ultrasound-assisted acid hydrolysis.

S. No.	Optimum Fabrication Conditions	Days	Average Particle size, nm	PDI	Yield, %	Crystallinity Index, %	Crystalline pattern	Thermal degradation point, °C	Onset temperature, °C	Peak temperature, °C	End temperature °C	Delta C_p, J/g/k	Reference
1	Native starch	-	2821±35.35	0.257±0.01	-	41.34±2.02	B type	245.71±7.82	39.1±1.55	99.2±4.58	169.71± 3.81	0.872 ±0.03	This work
2	3.16M AH control	1	1550±24.04	0.611±0.02	83±4.24	-		-					
		2	564±15.55	0.419±0.01	79±1.41								
		3	392±7.07	0.747±0.01	66±3.54								
3	3.16M US+AH	1	1378±18.38	0.496±0.02	82±1.41	56.88±2.64	B type	259.11±2.26	39.7±1.52	82.2±2.8	136.21± 2.70	0.535±0.02	
		2	489±14.14	0.451±0.02	75±1.41								
		3	370±7.07	0.334±0.01	65±2.69								
4	4M AH control	1	323±7.07	0.414±0.01	55±1.41	-		-					
		2	199.1±8.48	0.381±0.01	47±2.82								
		3	146±4.24	0.354±0.02	30±1.84								
5	4M US+AH	1	180±2.83	0.414±0.01	59±1.41	64.32±2.90	Undefined	318.99±6.79	40.1±1.87	91.2±3.15	185.71± 4.38	0.144±0.01	
		2	111±12.73	0.381±0.01	51±2.12								
		3	66±2.83	0.362±0.01	29±1.55								
6	5M US+AH	1	102±5.66	0.527±0.01	09±1.41	-		-	-			-	
		2	26.1±4.24	0.211±0.01	4.3±0.28								
		3	13±1.41	0.327±0.01	04±1.27								
7	3.16M AH	7	43.2	-	-	-		-	59.1 NS			-	Kim et al., 2011
8	Enzymolysis + AH 3.16M 45 h	-	145	-	-	37.2, WMS* 46.75, AH		-	-			-	LeCorre et al.,2012

*Waxy maize starch, Results are found significant (p value < 0.05)

concentration of sulfuric acid, and duration. The polydispersity index (PDI) and yield also varied and were important parameters for nanoparticle characterization. Ultrasonication of the acid hydrolyzed starch solution resulted in better average particle size of nanoparticles obtained. As shown in Table 1, in comparison to the control experiment at 3.16M H_2SO_4 condition, the average particle size improved significantly and altered as 1550 to 1378, 564 to 489, 392 to 370 nm for 1, 2 and 3 days respectively upon hydrolysis supplemented with sonication treatment. At 4M H_2SO_4 condition, the average particle size improved in comparison to control experiments and altered as 323 to 180, 199.1 to 111, 146 to 66 nm for 1, 2 and 3 days respectively upon hydrolysis supplemented with sonication treatment. During sonication treatment, the wave passage affirmed by the transducer generated cavities. These cavities in due course of their formation, growth, and collapse do affect the molecules present in the solution and thereby resulted in the reduction of particle sizes and narrow range nanoparticle formation (Kim, Park, et al. 2013; Li et al. 2020). Sonication treatment post hydrolysis effectively reduces the agglomeration of the starch nanoparticles through the rupturing of the hydrogen bonds, and linkages between molecules that tend to further enhance agglomeration. Previous studies conducted with sonication treatment at 20 kHz affirmed similar results(Gonçalves et al. 2014; Jambrak et al. 2010). Ultrasonication also positively affected the polydispersity index. Ultrasonication helped in reducing the polydispersity index for both 3.16M H_2SO_4 for day 1, day 2, and day 3 studies. For 4M H_2SO_4 on day 1 and day 2,
the PDI was reduced in comparison to control (Without US). For the 4M H_2SO_4 day 3, the average particle size reduced significantly from 146 nm in control to 66 nm in pulse mode ultrasonic assisted acid hydrolysis. This explains the minimal change of PDI under the 4M day 3 acid hydrolysis. This meant that the ultrasonication treatment was able to break down the starch solution nanoparticles and thus helped in increment of uniformity of particle size. The effect of ultrasonication was not significant in the yield of the particles for both 3.16M and 4M

H_2SO_4 acid hydrolysis conditions.

Acid hydrolysis of potato starch under mild acid treatment occurs due to the erosion of the amorphous and crystalline regions of the starch granule. The amorphous region of the starch granule consists of amylose molecules of a linear structure with α 1 - 4 glycosidic bonds, and the crystalline regions of the starch are formed by amylopectin molecules with both α 1 - 4 & α 1 - 6 glycosidic bonds and have a branched structure (Bertoft 2017). It is hypothesized that amorphous regions having a simpler linear structure is more susceptible to acid hydrolysis compared to the branched amylopectin regions of the starch granule[12]. During acid hydrolysis, at mildly acidic conditions (3.16M H_2SO_4), for the first 3 days, the hydrolysis of the starch granule occurs dominantly in the amorphous regions and is termed fast hydrolysis. During this process, numerous linear chains of starch molecule are generated that causes the formation of starch nanoparticles. Later, between 3 - 9 days of acid hydrolysis, erosion of the crystalline regions of the starch granule occurs, which is termed as slow hydrolysis and is not desirable in terms of the quality of the fabricated nanoparticles (Harun et al. 2022). In acid hydrolysis with higher acidic concentration, it can be hypothesized that the erosion of the amorphous regions occurs at a faster rate, and as a result, starch nanoparticles are formed after a duration of 24 - 48 h in comparison to 5 - 7 days in mildly acidic conditions. No literature reported trends exist for the mentioned trends in this work for acid hydrolysis at higher concentrations in combinations with post ultrasonication, and hence these observations can serve as useful guidelines in future research.

4.3 Morphological Analysis of Starch Nanoparticles

FESEM images were taken for the best results obtained in starch nanoparticle preparation and shown in Figure 4.2 A - E. The particle sizes of the starch nanoparticles were analysed using ImageJ software (1.53t). Acid hydrolysis breaks down the amorphous regions of the starch

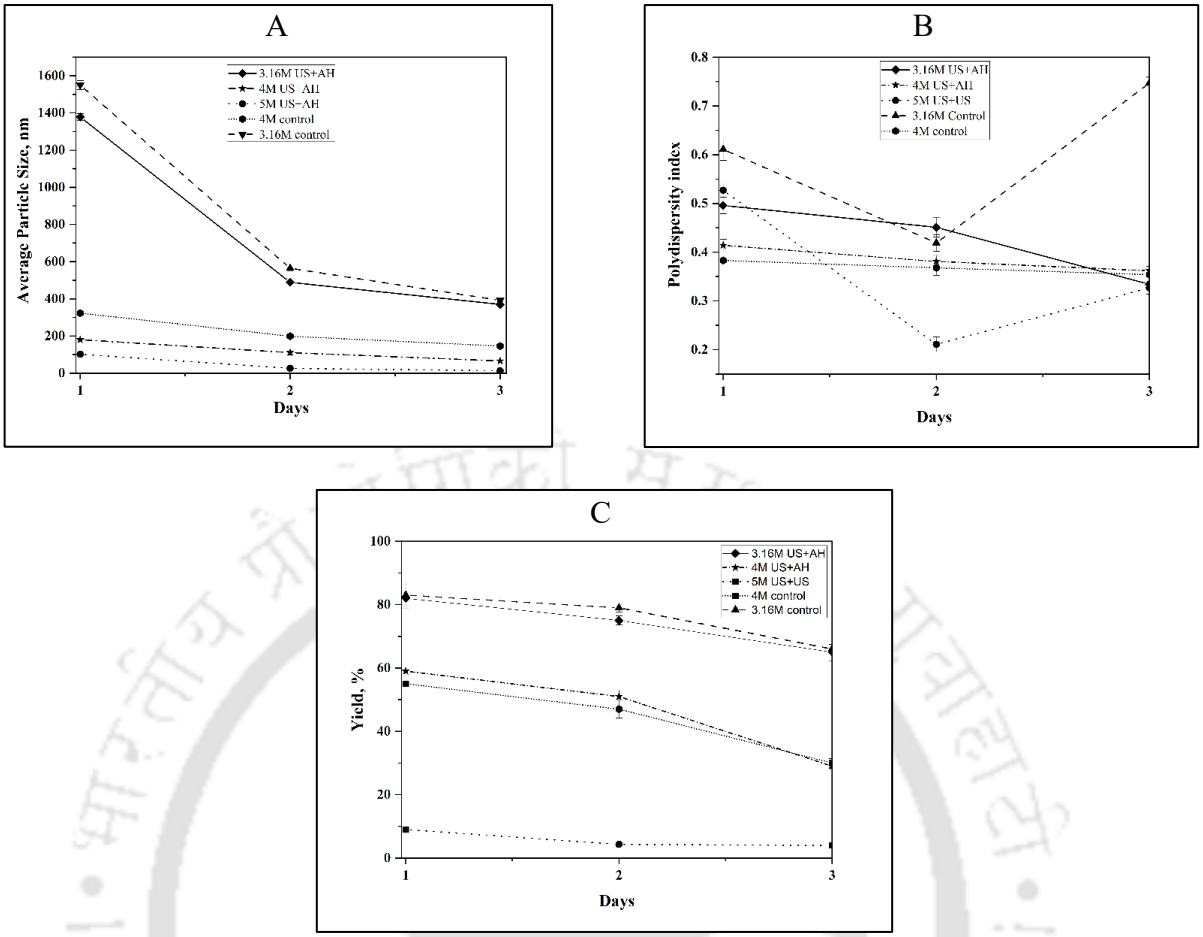


Figure 4.1: A- Size vs no. of days, B- Polydispersity index vs no. of days and C- Yield vs no. of days for different conditions in acid hydrolysis

granule resulting in the formation of nano structured starch particles. The smooth edges of the starch granule broke down after acid hydrolysis with 4M H_2SO_4 (1 day) and post ultrasonication treatment resulting in the formation of short chained starch particles around the granules, as can be observed from Figure 4.2 A - B. SEM images from starch particles formed with ultrasound assisted 3.16M acid hydrolysis show particles with varying sizes ranging from 100 nm to 1100 nm (Figure 4.2 C). This is also in line with the average particle sizes obtained by DLS method (370 nm). Previous literature using 3.16M H_2SO_4 for 3 days had also obtained similar results (Kim et al. 2012; Kim, Park, et al. 2013). It was observed that the particle size obtained by Dynamic light scattering method was in line with the results obtained in FESEM study. The nanocrystal sizes varied from 40 - 100 nm for the particles formed during ultrasound

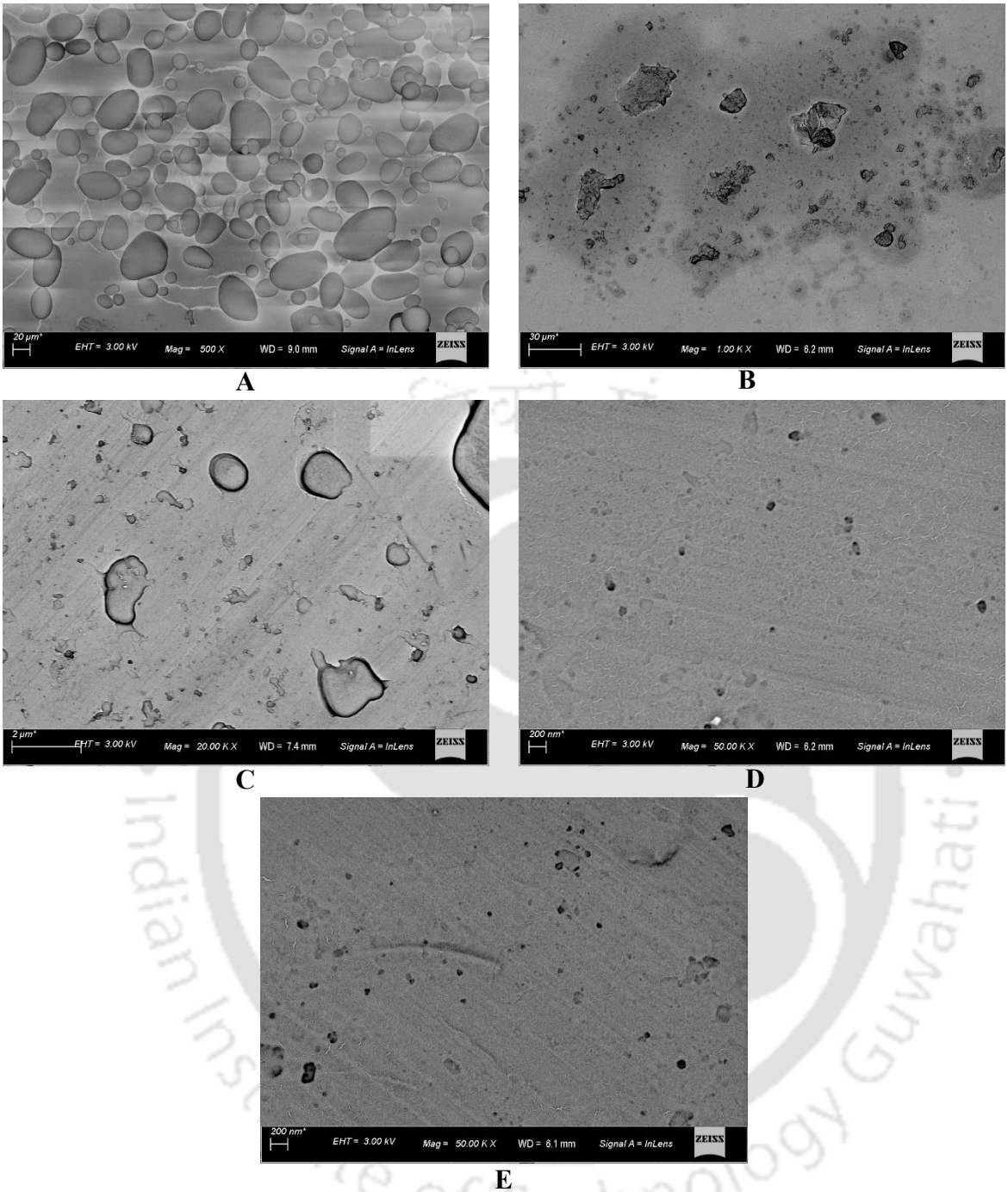


Figure 4.2: FESEM images of A - Native starch; B – Starch prepared in 4M 1day AH+US, C – 3.16M 3 days AH+US, D – 4M 3 days AH control (without US), E – 4M 3 days AH+US

assisted acid hydrolysis with 4M sulfuric acid, which is in line with the results of average particle size obtained using DLS method (66 nm). The control sample without ultrasonication and acid hydrolysis for 3 days with 4M H_2SO_4 had particle sizes ranging from 90 nm to 170 nm which was also in line with the results obtained using DLS method (146 nm).

4.4 Crystalline Properties of Starch Nanoparticles

Crystalline properties of native starch, starch nanoparticles prepared with 3.16M, 4M sulfuric acid, and ultrasonication post-treatment were studied and shown in Figure 4.3 A - C. For native starch, diffraction peaks were observed in 2θ values of 5.68°, 14.92°, 16.98°, 19.44°, 22.00, and 23.98° Bragg angles. The results confirmed that the native potato starch had a B type semi crystalline structure (Kim, Park, et al. 2013; Manek et al. 2012). The relative crystallinity upon derivation using origin software was found to be 41.34 %.

Starch nanoparticles prepared using ultrasound assisted acid hydrolysis with 3.16M H_2SO_4 for 3 days were studied in X ray diffractometer. The diffractogram of the SNP revealed a sharp increase in many peaks as compared to the native starch. Peaks were observed in Braggs angles of 5.74°, 14.92°, 17.12°, 19.46°, 24.06°, 26.12°, 27.96°, 28.88°, 32.10°, 33.88°, and 38.66° as shown in Figure 4.3. New peaks were observed along with increase in the peaks that were observed in native starch. The generation of new peaks and a similar increase in relative crystallinity were observed in previous studies in case of acid hydrolysis using 3.5M H_2SO_4 (Saeng-on and Aht-Ong 2017). Relative crystallinity upon calculation is found to be 56.88.

The starch nanoparticles prepared using ultrasound assisted acid hydrolysis with 4M H_2SO_4 solution were studied using the XRD method for its crystalline structure and relative crystallinity. Diffractions peaks are observed in 2θ values of 16.96°, 19.04°, 23.18°, 28.04°, 29.02°, 32.14°, 33.86°, and 38.62° Bragg angles as shown in Figure 4.3. The XRD pattern of starch nanoparticles prepared at 4M conditions had similar pattern of peaks with that of 3.16M

conditions except for the missing peak near 5° 2θ and compared to 3.16M conditions the peaks generated at 4M conditions were sharper with high intensity. The absence of the peak near 5° 2θ for 4M sulfuric acid hydrolysis showed that the crystalline structure of the starch changed after getting exposed to high acid concentrations for 3 days. The A type crystalline structure found in maize, corn, etc., does not contain the peak at 5° 2θ (Manek et al. 2012). The pattern of 4M sulfuric acid hydrolysed starch showed a hybrid crystalline structure which had features of both A and B type crystalline structure. A type crystalline structures are more compact and possess higher relative crystallinity than B type structures. The hydrolysis process gets expedited at strong acid concentrations and it can be hypothesized that the alterations of the crystalline structure were because of fast hydrolysis in the amorphous regions of starch granule. During fast hydrolysis, the hydroxonium ion attacks the amorphous regions containing α 1 - 4 glycosidic bonds and results in the rapid formation of starch nanoparticles (Harun et al. 2022; Kim et al. 2012). Relative crystallinity was found to be 64.32% for 4M H_2SO_4 acid hydrolysis, which shows a significant increase than native starch and starch nanoparticles from 3.16M acid hydrolysis. Previous studies on banana starch and tapioca starch also showed an increase in the relative crystallinity (60.06%) when exposed high concentrations of H_2SO_4 (Saeng-on and Aht-Ong 2017). Starch nanocrystals prepared from waxy maize starch after 6 and 10 days of acid hydrolysis at 3.16M also exhibited high relative crystallinity values of 63 and 79% respectively (Duan et al. 2011). However, in the present work high relative crystallinity was obtained at a relatively less duration of 3 days with strong acid hydrolysis in combination with ultrasonication post-treatment.

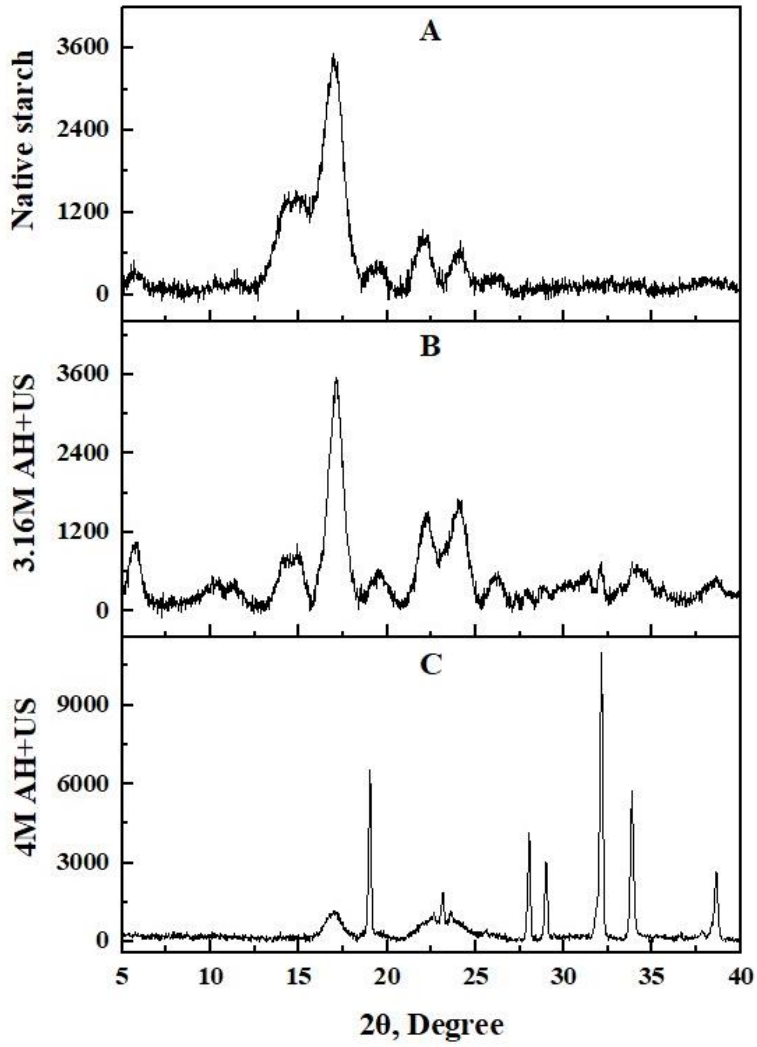


Figure 4.3: A - C XRD diffractogram of A - Native starch, B - starch nanoparticle prepared using ultrasonication (US) assisted acid hydrolysis (AH) at 3.16M H_2SO_4 for 3 days, and C - starch nanoparticle prepared using ultrasonication assisted acid hydrolysis at 4M H_2SO_4 for 3 days

4.5 Thermal Properties of Starch Nanoparticles

The thermal properties of native starch were compared with the starch nanoparticles prepared using ultrasound assisted acid hydrolysis process as can be seen in Table 1. Figure 4.4 A - C depicts a comparison of DSC curves for native starch and starch nanoparticles prepared with

3.16M and 4M conditions and with sonication post-treatment. The broader endotherms of DSC curves indicated that the gelatinization of the crystalline regions was affected by the recrystallization of the unbranched starch chains and subsequent formation of short linear glucans. This also inferred that the amorphous regions of the starch structure being reduced due to acid hydrolysis supplemented with sonication (KIM et al. 1995). An initial endothermic peak existed between 35 - 40°C. This was due to the initial water loss from the starch powders. Such a loss was due to the non-airtight condition of the pan being deployed in the manually sampled DSC instrument system (Ho et al. 2019). The gelatinization properties of the starch particles also get changed with acid hydrolysis and size reduction. The changes occurred in the gelatinization properties depend upon the mechanism of hydrolysis, viz., fast, and slow hydrolysis (Li et al. 2020). The onset temperature remains quite similar in both native starch and starch nanoparticles, while the peaks were found in higher starch nanoparticles in the order of SNP4M>SNP3.16M>NS. The end temperatures were found higher in SNP4M in comparison to SNP3.16M and native starch. It further showed that starch nanoparticles prepared at 4M sulfuric acid and post ultrasonication had improved thermal characteristics (Kim et al. 2012; Saeng-on and Aht-Ong 2017). An exothermic peak is observed at 240.21°C in native starch which may have occurred due to thermal decomposition and subsequent burning of glucose molecules present in native starch. As starch nanoparticles prepared have higher crystallinity and better thermal resistance (Saeng-on and Aht-Ong 2017), the exothermic peak was not observed in 3.16M, and 4M hydrolysed prepared starch nanoparticles. An endothermic peak had initiated at 245.71°C in native starch confirmed the beginning of thermal degradation through thermal decomposition. As shown in Figure 4 B - C endothermic peaks initiated at 259.11°C and 318.99°C for starch nanoparticles prepared using 3.16M and 4M with ultrasonication conditions respectively. These corroborates with the initiation thermal degradation of the starch nanoparticles. Previous studies have also confirmed similar initiation

of melting for starch gelatinization using sodium silicate (Rashid et al. 2012). This increase in its thermal properties and thermal degradation point may be due to the change in the structure of the starch during the acid hydrolysis process, which is quite evident from the XRD analysis. Similar trends were observed for maize starch at 270°C (Li et al. 2020). The crystalline structure of starch had been hypothesised to change from a more open B type structure to a hybrid type crystalline structure which was more compact and had a different diffractogram than A, B, or C type crystalline structures. With the increase in relative crystallinity, the thermal degradation point also increased, which was clear from the results obtained. Previous works also reported that an increase in crystallinity resulted in an increase in the thermal degradation point (Jiang et al. 2016). With acid hydrolysis for 3 days XRD results already conveyed the increase in crystallinity. Previous studies on proso millet starch reported a decrease in enthalpy with an increase in crystallinity of starch nanoparticles in comparison to native starch prepared using enzymatic hydrolysis, which has a similar working mechanism with acid hydrolysis (Sun et al. 2014). The endothermic enthalpy is directly proportional to delta Cp and its measurement is important in the estimation of different other thermodynamic properties such as entropy, energy, free energy, and Gibbs free energy[29]. The decrease in Cp in the present work could be associated with a decrease in ordering and stabilized double helical structures in the prepared starch nanoparticles through different molecular forces, such as hydrogen bonds (Mutungi et al. 2009; Shi et al. 2012).

4.6 Thematic Summary

The novel method for the generation of potato starch nanoparticles using ultrasound as a post-treatment method produced repeatable and fruitful results. Ultrasonication proved to be very effective in reducing the average particle size of the particles as well as the polydispersity index

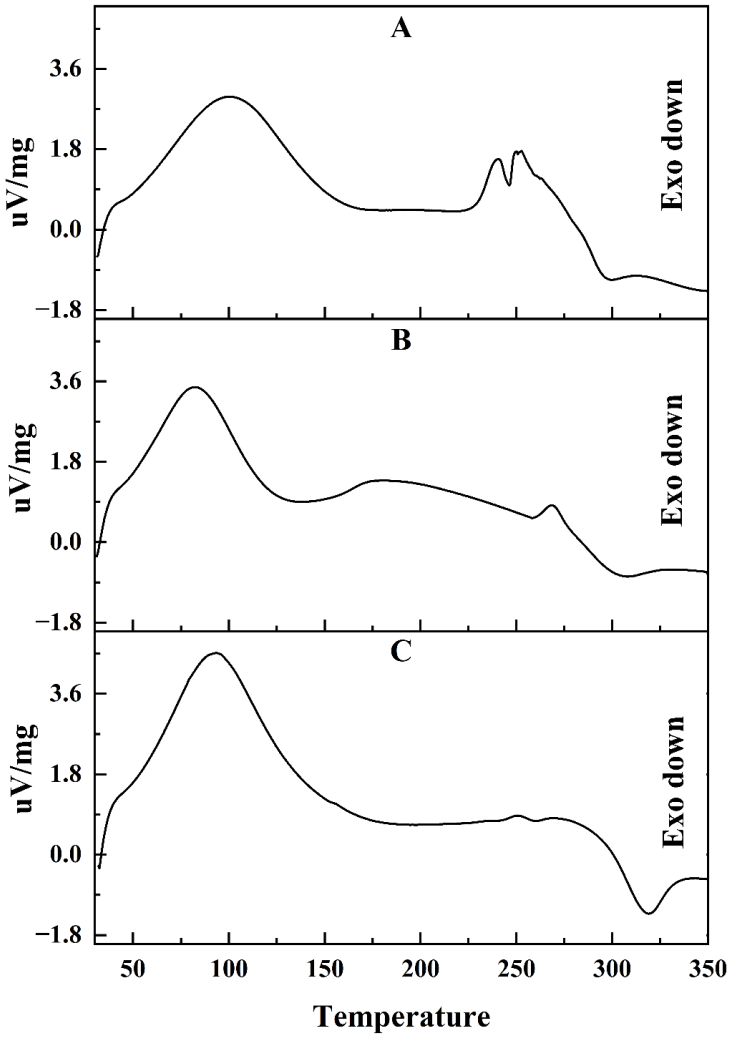


Figure 4.4: A - C DSC thermograms of A - Native starch, B - starch nanoparticles prepared with 3.16M sulfuric acid with ultrasonication for 3 days and C - 4M sulfuric acid with ultrasonication for 3 days

but didn't have much effect on the yield. The yield was mainly affected by the hydrolysis process itself more than any other factor. Nanoparticles were prepared with good polydispersity index, and the properties of the starch itself was modified in a way that will suit its application in many areas, such as encapsulation of bioactive substances. The starch nanoparticles had higher relative crystallinity due to the modification of the crystalline structure from B type in native starch to hybrid type in dried starch nanoparticles. Due to increase in the relative

crystallinity thermal properties such as thermal degradation point were also enhanced. There was a definite increase in the thermal degradation point in the starch nanoparticles prepared using acid hydrolysis, and it increased with increase in the molar concentration of sulfuric acid. Higher thermal resistant nanoparticles will be suitable for targeted delivery of functional bioactive substances and thus opens its door for the application as a carapace material for encapsulation.

Chapter 5:

Nano-encapsulation of EGCG and Catechin Extracts using Starch Nanoparticles and its Effect on Micellar Cholesterol Solubility In-Vitro

Chapter 5

Nano-encapsulation of EGCG and Catechin Extracts using Starch Nanoparticles and its Effect on Micellar Cholesterol Solubility In-Vitro

This chapter discusses the process of encapsulating EGCG and catechin extracts in starch nanoparticles by the use of the inclusion complex approach. Subsequently, the effectiveness of EGCG and catechin extracts towards micellar cholesterol inhibition has been examined using a radioactive isotope technique, both before and after encapsulation. Section 5.1 provides a concise introduction of the chapter. Section 5.2 provides a detailed explanation of the morphological, FTIR, thermal, and crystalline properties of the EGCG, SNP, and EGCG-SNP complex. Subsequently, section 5.3 and 5.4 go into a comprehensive analysis of the taurocholic acid binding activity and micellar solubility experiments on the precipitation of cholesterol. This analysis specifically focuses on the EGCG, CE, and EGCG/CE-SNP complex. Section 5.5 provides a concise summary of the chapter.

Overview

The present work investigates the cholesterol micellar inhibition effect of (-)- Epigallocatechin Gallate (EGCG) and catechin extracts derived from S3A3 Assam tea leaves before and after nano-encapsulation with starch nanoparticle carapace. Potato starch nanoparticles were used as wall material for the encapsulation of EGCG and catechin extracts. The starch nanoparticles

were prepared using acid hydrolysis and post ultrasonication treatment. The EGCG/CE-SNP encapsulation was achieved through inclusion complexation method. The EGCG and catechin extracts were encapsulated and preserved as dry powder for subsequent analysis and investigation of their micellar inhibition properties. The efficacy of EGCG and catechin extracts towards precipitation of cholesterol in-vitro were studied using radioactive isotope method. Further, the EGCG-SNP complex was characterized for the morphology, thermal, and crystalline properties.

5.1 Introduction

Cardiovascular disorders and excessive blood pressure have been significant contributors to illness worldwide (Fuchs and Whelton 2020; Nabel 2003). Elevated levels of low-density lipoprotein cholesterol (LDL-C) significantly contribute to the progression of atherosclerosis, hence increasing the risk of cardiovascular disease and stroke. Tea is a widely used functional beverage that offers several nutritional advantages, such as lowering the content of LDL cholesterol in our body. Tea leaves include catechins, which are flavan-3-ol molecules that have properties that can lower blood pressure and reduce high levels of lipids in the blood. (Matsui 2022).

The primary catechins found in tea are Epigallocatechin gallate (EGCG), Epicatechin gallate (ECG), Epigallocatechin (EGC), Epicatechin (EC), and Catechin (C) (Lee et al. 2014; Sabhapondit et al. 2012). Catechin substances, specifically gallated catechins like EGCG and ECG, have garnered significant interest among researchers due to their capacity to diminish cholesterol absorption inside the body (Dey et al. 2022; Nagle, Ferreira, and Zhou 2006). Prior research has demonstrated that EGCG decreases blood cholesterol levels through a process known as micellar precipitation (Ogawa et al. 2016).

S3A3 is a widely utilized tea variety in the northeastern region of India, known for its high co-

ncentration of gallated catechins. These compounds make up approximately 70% of the overall catechin content (Sabhapondit et al. 2012). The comprehensive composition of catechins in the S3A3 tea cultivar has been elucidated in section 3.2 of chapter 3 inside this thesis. The utilization of tea catechins, including EGCG, was restricted due to their inadequate stability during the process of digestion, resulting in significant loss prior to absorption in the small intestine (Chen et al. 2001; Su et al. 2003). Encapsulation is a nascent technology that employs proteins, lipids, or carbohydrates as a safeguarding layer to protect bioactive molecules from damage during storage and digestion in the intestines. (Hasanvand, Fathi, and Bassiri 2018; Rashidinejad et al. 2014). Food grade starches are one of the versatile compounds used for the encapsulation of the catechins using various methods such as spray drying, freeze drying, inclusion complexation, antisolvent precipitation (Ho et al. 2019; Krishnaswamy, Orsat, and Thangavel 2012; Rashidinejad et al. 2014; Wang et al. 2021) etc. The authors have synthesized potato starch nanoparticles in chapter 4 of the thesis, which exhibit improved thermal and crystalline characteristics. The potato starch nanoparticles, once manufactured, will serve as a wall material for encapsulating EGCG and catechin extracts.

Prior studies have examined the impact of EGCG on the micellar solubility of cholesterol. However, no research has investigated the influence of encapsulated EGCG/catechin extracts on the binding activity of taurocholate and the micellar solubility of cholesterol and its subsequent precipitation. Furthermore, no earlier reports have documented the impact of catechin extracts obtained from S3A3 tea leaves on taurocholate binding activity and cholesterol micellar solubility, both before and after encapsulation with starch nanoparticles, as well as their characterization. The inclusion complexation method is considered a green and acceptable approach for encapsulating catechin extracts/EGCG due to its minimum use of chemicals. (Ho et al. 2017, 2019). Inclusion complexation method uses the self-assembly mechanism to prepare a complex of the host molecule and the bioactive substance. It is a simple

and green method suitable for the encapsulation of green tea extracts and EGCG as stated by previous studies (Ho et al. 2019; Krishnaswamy, Orsat, and Thangavel 2012; Song et al. 2009). As such, in this work, starch nanoparticles were used to encapsulate the tea catechin extracts and EGCG with inclusion complexation method. Furthermore, to evaluate the cholesterol lowering effects of EGCG, S3A3 catechin extracts before and after encapsulation we conducted micellar cholesterol solubility tests and bile acid binding assays using radioactive isotope method (Ogawa et al. 2016).

5.2 Characterization of EGCG, SNP, and SNP-EGCG/CE Complex

The EGCG and catechin extracts were analysed after encapsulation to determine their physical and chemical properties, such as morphology, FTIR, thermal, and crystalline properties. All of these concepts will be further explained in the subsequent sections.

5.2.1 Morphology of EGCG, SNP, and EGCG-SNP Complex

The morphology of EGCG, starch nanoparticles, and EGCG-SNP complex were studied through the FESEM images as shown in Figure 5.1 A-C. The particle size of the samples were analyzed using ImageJ software (1.53t). EGCG particles were studied at 1500X zoom, 4.3 mm working distance, and 5 kV electron high tension. The EGCG particle sizes ranged from 12.5 – 19.5 µm and the shape varied from round to irregular triangular in various particles. The starch nanoparticle images were taken at 10000X zoom, 4.5 mm working distance, and 5 kV EHT. The starch nanoparticles majorly constituted rod-shaped structures with diameter ranging from 90-150 nm as observed in Figure 5.1B. The FESEM images taken for EGCG-SNP complex showed the irregular shapes of the nano-structures. It was observed that the particle diameter ranged from 20-120 nm. The rod-shaped starch nanoparticles lost their shape during the inclusion complexation process and acquired irregular shapes with smaller particle size.

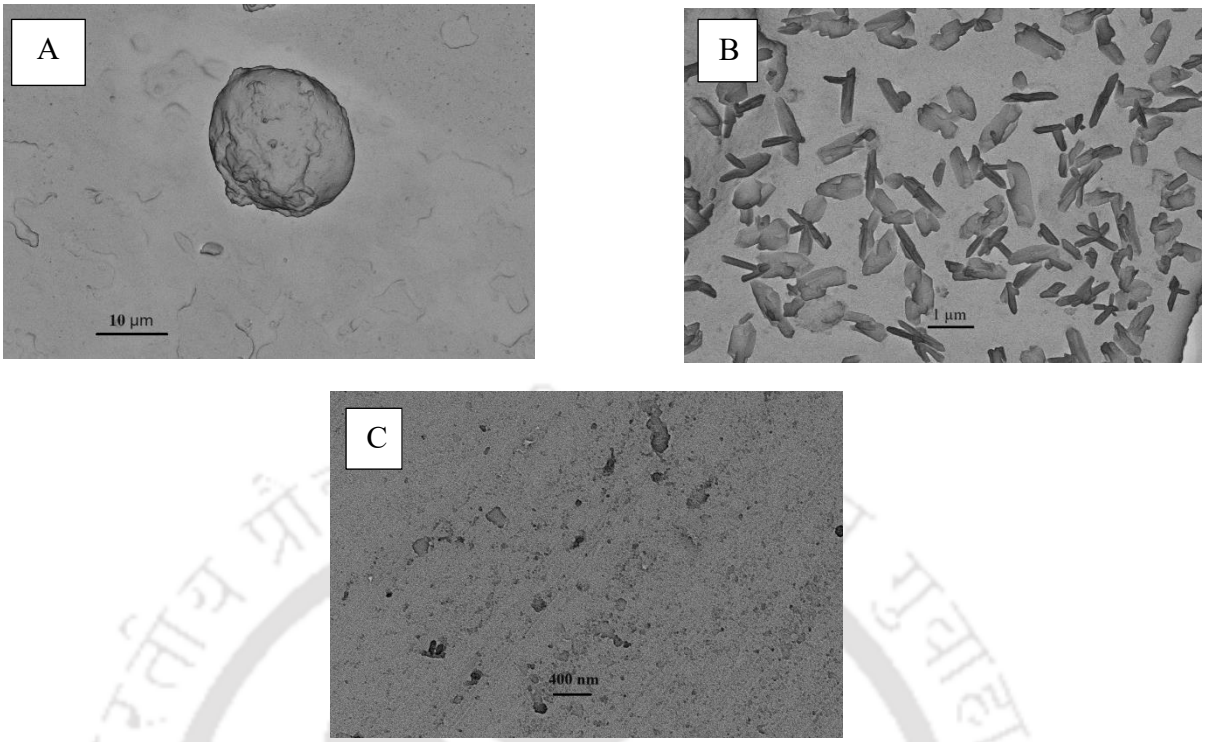


Figure 5.1: FESEM images of A. EGCG, B. Starch nanoparticles (SNP), C. EGCG-SNP

The reduction in particle size is favourable and could be attributed to the formation of EGCG-SNP complex through the aggregation of the EGCG molecules on the host starch nanoparticles (Krishnaswamy, Orsat, and Thangavel 2012).

5.2.2 FTIR Study of EGCG, SNP, and EGCG-SNP Complex

The FTIR spectra of EGCG, SNP, and EGCG-SNP are displayed in Figure 5.2. The FTIR plots provide evidence of the existence of an inclusion complex formed between EGCG and SNP. Figure 5.2 demonstrates that the wide peaks observed at 3250-3625 cm^{-1} are indicative of the existence of water. The peaks in EGCG and EGCG-SNP are wider than those in SNP, indicating towards its high moisture concentration. The EGCG plot displayed peaks at 1452 and 1339 cm^{-1}, indicating the presence of hydroxyl molecules in EGCG. Furthermore, the presence of C-O stretching, a strong indicator of EGCG, was confirmed by the observation of numerous peaks at 1143 and 1091 cm^{-1}. The existence of EGCG and the development of the

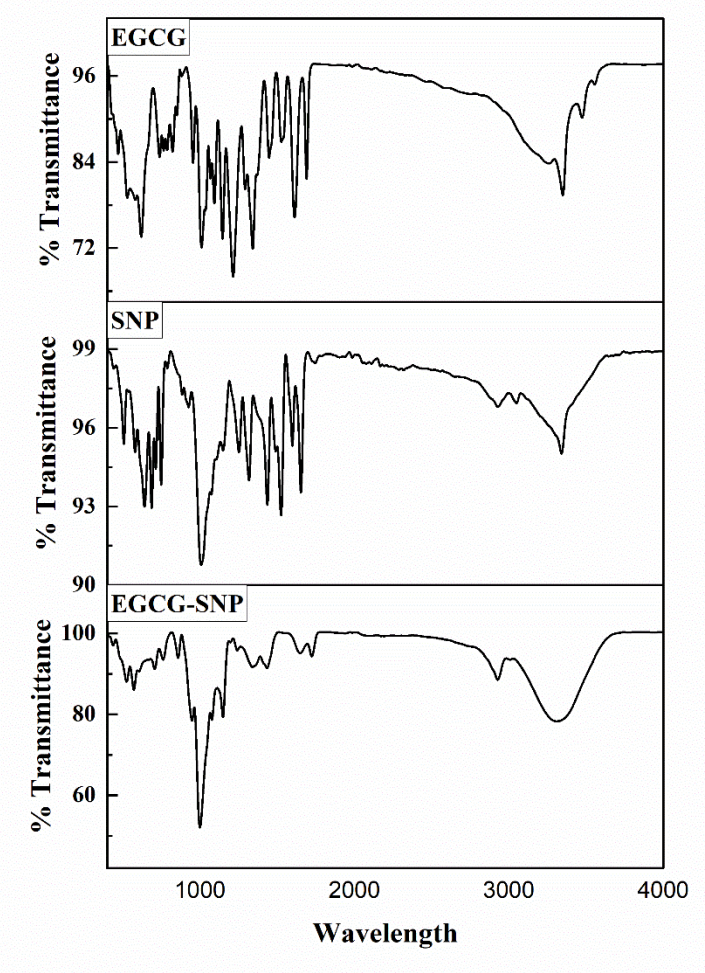


Figure 5.2: FTIR plots for EGCG, Starch nanoparticle, EGCG-SNP

EGCG-SNP complex were confirmed by the observation of similar peaks at wavelengths of 1146 and 1080 cm^{-1} on the EGCG-SNP plot. Furthermore, a distinct peak at 2928 cm^{-1} was detected for EGCG-SNP, indicating the presence of strong O-H stretching. The starch nanoparticles exhibited typical peaks at 1658 and 1602 cm^{-1}, confirming the presence of C=C stretching. The existence of hydroxyl compounds is indicated by peaks seen at 2922 cm^{-1}, while C-H stretching is indicated by peaks at 751 cm^{-1}. Peaks with similar characteristics were also seen on the EGCG-SNP graph, specifically at 2927, 1646, and 768 cm^{-1}. These peaks confirm the existence of SNP and its subsequent formation of a complex with EGCG.

5.2.3 Thermal Properties of EGCG, SNP, and EGCG-SNP Complex

The thermal properties of EGCG, SNP and EGCG-SNP complex were studied and were illustrated in Fig. 5.3. The thermograms reveal the interactions of the starch nanoparticles with the EGCG compound. As observed in the plot, the initial peak at 33.5°C, 35.3°C, 37.9°C for EGCG, SNP, and EGCG-SNP is due to the loss of water (Krishnaswamy et al., 2012). For the EGCG case, the onset temperature for EGCG glass transition was apparent at 89°C. The endothermic peak being observed at 116.6°C and at -0.99 mW/mg confirms upon the glass transition temperature (Table 5.1). Another endothermic peak at 225.7°C signified the thermal degradation point and initiation of thermal decomposition for the EGCG. The broad endothermic peak from 230-270°C signifies the melting decomposition of EGCG. For the SNP, after the initial loss of water, an endothermic peak was observed at 164.5°C. This is indicative towards the glass transition temperature. Consequently, another endothermic peak was observed at 267°C. This corroborates to its thermal degradation point and initiation of thermal decomposition (Narayan Baruah et al., 2023). The EGCG-SNP complex exhibited improved thermal characteristics in comparison to EGCG and SNP and after the inclusion complex formation. As observed in Fig. 2, EGCG-SNP complex showed a broad endothermic peak at 99.7°C that confirms a broad glass transition phase. Another endothermic peak at 282.9°C affirms the thermal degradation point of the EGCG-SNP complex and the initiation of thermal decomposition of the complex. The results from the thermograph indicated novel thermal properties for the EGCG-SNP inclusion complex and in terms of the enhanced thermal degradation point and thermal stability at higher temperatures.

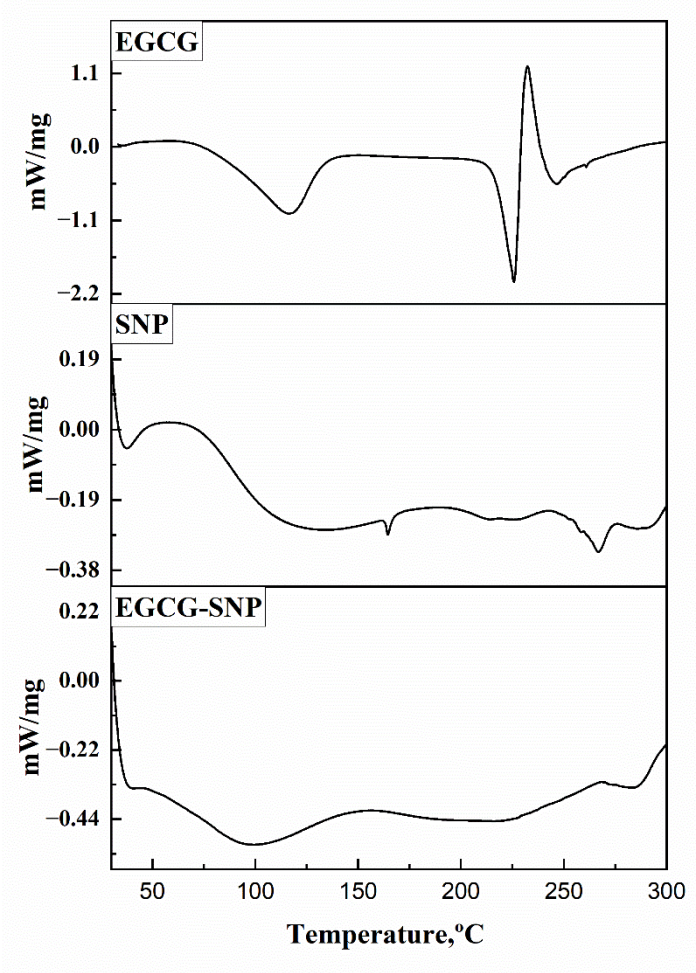


Figure 5.3: Differential scanning calorimetry thermograms for EGCG, SNP, EGCG-SNP complex

5.2.4 Crystallinity Study of EGCG, SNP, and EGCG-SNP Complex

X-ray diffractograms were plotted for EGCG, SNP, and EGCG-SNP as shown in Figure 5.4. The crystallography plots for EGCG exhibited strong peaks at 2θ values of 19.09, 28.11, 29.02, 32.22, and 34° which are characteristic of the crystalline nature of EGCG as cited in previous studies (He et al. 2020). The starch nanoparticles prepared using acid hydrolysis showed crystalline nature, and had sharp peaks at 2θ values of 19.06, 28.06, 29.02, 32.14, 33.88, 38.64° which is characteristic of its transformed crystalline nature. The 4th chapter of the thesis extensively examines the process of manufacturing starch nanoparticles and their associated

properties. The research demonstrated that the acid hydrolysis process led to the reorganization of the non-crystalline areas of the starch, resulting in a higher degree of crystallinity compared to the original starch, which possessed partially crystalline characteristics. The crystalline nature of the starch nanoparticles is one of key factors for the selection of starch nanoparticles as a novel carapace for the encapsulation of catechins from tea leaves. Starch nanoparticles also have enhanced thermal stability due to its enhanced crystallinity. The EGCG-SNP plots for the XRD analysis exhibits characteristic peaks for both EGCG and SNP with 2θ values of 19.54, 20.7, 21.5, 28.14, 28.9, 29.5, 35.49°. These reaffirms the complex formation between EGCG and SNP. Also new peaks were observed at 2θ values of 12.06, 15.58, 17° which may have been caused due to the molecular rearrangement of EGCG and SNP during the inclusion complexation process (Krishnaswamy, Orsat, and Thangavel 2012).

5.3 Taurocholic Acid Binding Activity

Cholestyramine was used as a positive control to assess its ability to bind to taurocholic acid. The binding activity of taurocholic acid is directly associated with the formation of cholesterol precipitation. Catechin extract and EGCG were tested for their taurocholate binding activity at 5mg/mL concentration (Figure 5). Cholestyramine which is proven to affect the taurocholate binding activity showed a binding ability of 85.58% which is in line with the findings of the previous study (Nagaoka et al. 2010). The results indicated that precipitation of cholesterol during its intestinal digestion is not caused by binding of taurocholic acid with EGCG or Catechin extracts.

5.4 In-Vitro Micellar Cholesterol Solubility Study

The effect of EGCG, catechin extract, starch nanoparticle, CE-SNP complex, and EGCG-SNP complex on the micellar solubility of cholesterol were studied as shown in Figure 5.7 A-C

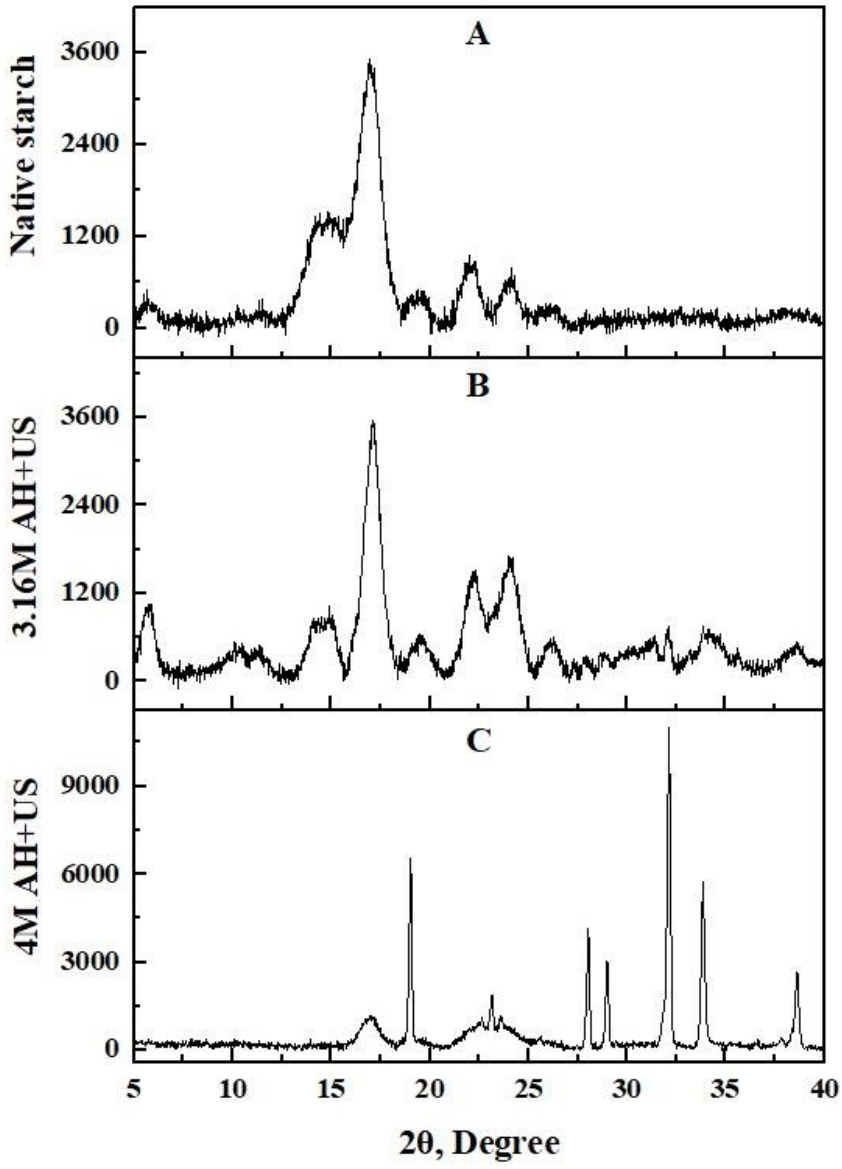


Figure 5.4 XRD plots for EGCG, Starch nanoparticle, EGCG-SNP

(Table 1). The effect of cholestyramine as a positive control on the micellar cholesterol solubility were also studied (Figure 5.6). Previous studies confirmed that cholestyramine has a proven effect on the micellar solubility of cholesterol (Nagaoka et al. 1999, 2010). The experimental findings in the current work indicated cholestyramine's (5 mg/mL) strong effect on the inhibitory action of micellar cholesterol solubility at 15.5%. Also, cholestyramine has a simple structure and had been extensively studied for various health benefits including its effect

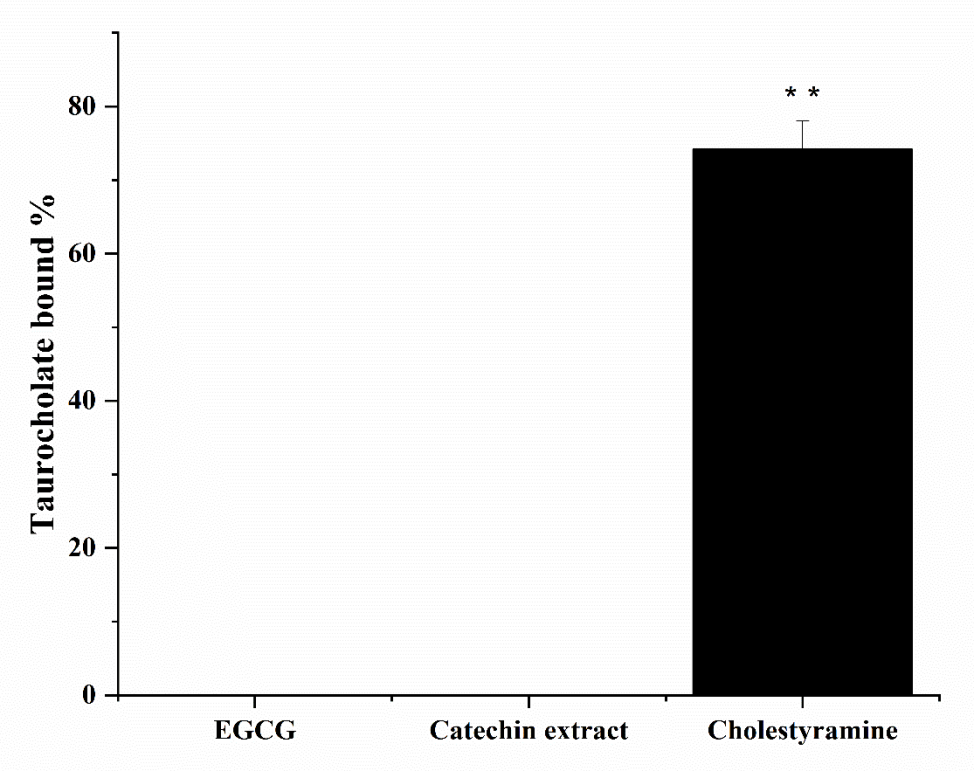


Figure 5.5: Taurocholic acid binding ability of EGCG, Catechin extract and Cholestyramine

on the cholesterol intake inhibition through micellar precipitation. These factors were pivotal for its selection as a positive control. Catechin extracts, and EGCG at 1-2.5 mg/mL concentrations were evaluated for their effect on micellar cholesterol solubility. It was observed that catechin extracts and EGCG had a dose dependent effect on the micellar cholesterol solubility. Catechin extracts exhibited 64.37, 40.96% at 5, 2.5 mg/mL concentrations. While EGCG exhibited 8.87, 31.15% at 2.5, 1 mg/mL concentrations. The S3A3 leaves catechin extract contained a mix of various catechins such as EGCG, ECG, EGC, EC and C along with other cellular substances. As such the effect of EGCG was found stronger in comparison to catechin extracts. Starch nanoparticles did not have any effect on the reduction of cholesterol via micellar cholesterol solubility which was evident from the results. After the inclusion complex encapsulation of EGCG and catechin extracts by using SNP as wall material, their effect on the micellar cholesterol solubility had been studied as shown in Figure 5.7. The results indicated that post encapsulation the encapsulate complex containing the EGCG and

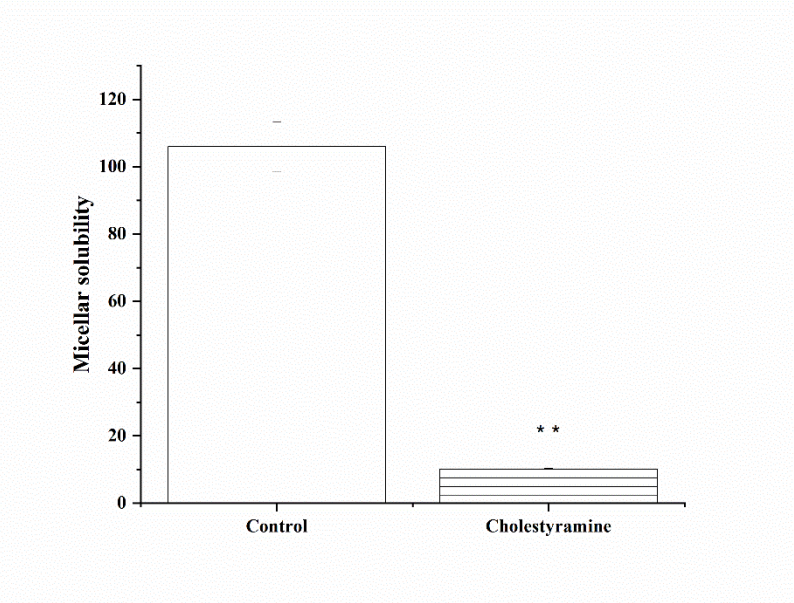


Figure 5.6: Micellar solubility study of control and cholestyramine

catechin extracts lost its ability to reduce the cholesterol absorption. These reaffirms the successful encapsulation of EGCG and catechin extracts. Also, encapsulated EGCG and catechin extracts have the potential to exhibit better cholesterol reduction as during the digestion process most of the catechin compounds are lost due to harsh environments in the digestion process (Ahmad et al. 2019). In the current work, EGCG and catechin extracts were encapsulated using 1:10 and 1:25 ratio of EGCG/CE:SNP. It was observed that at both the ratio's the effect of the encapsulation on the micellar cholesterol solubility did not have any significant change. Finally, we expect that nanoencapsulation will help protect catechins from the gastric environment and retain their cholesterol-lowering properties during the digestive process.

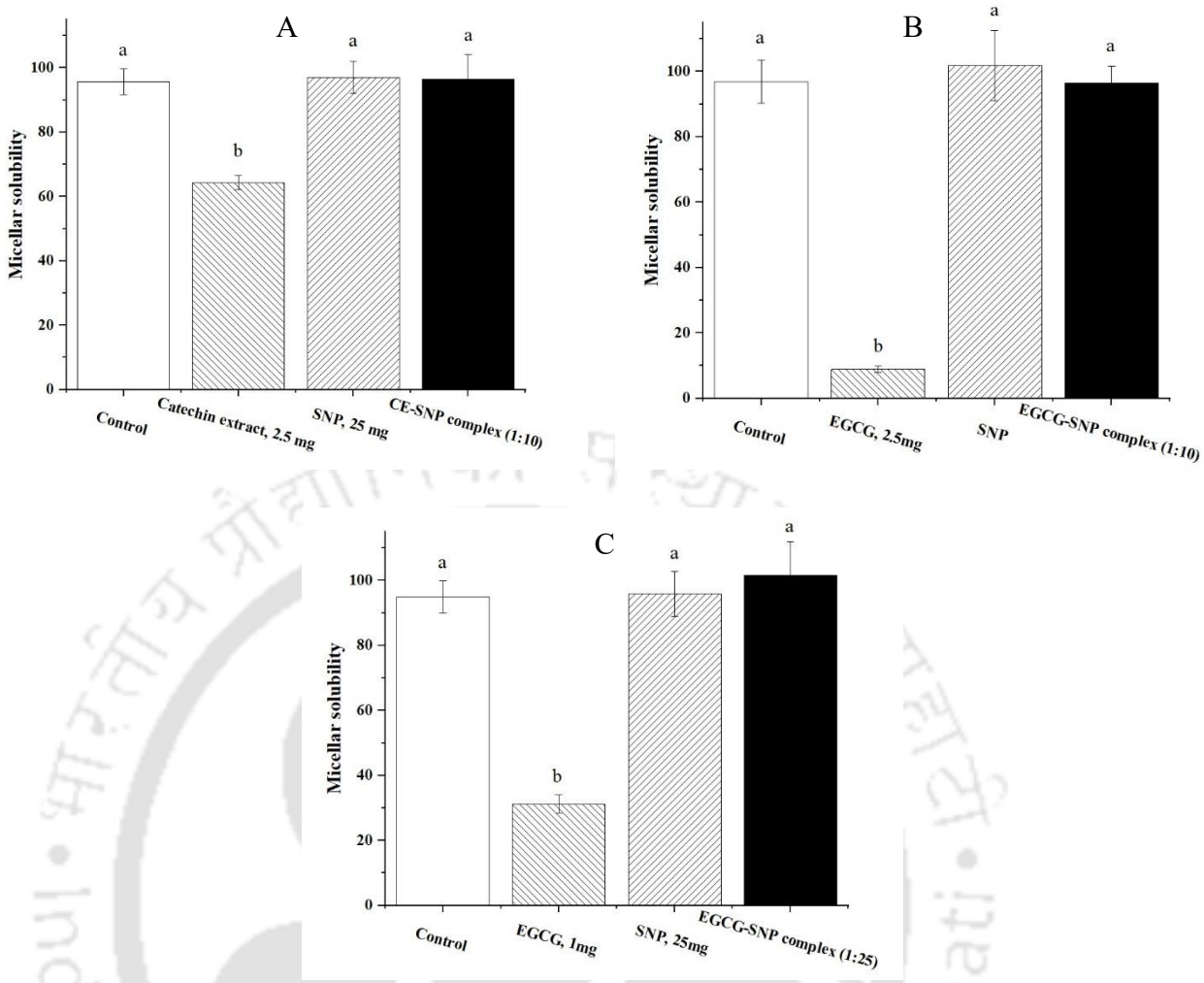


Figure 5.7: Micellar solubility study of A. Control, Catechin extract (CE), Starch nanoparticle (SNP) and CE-SNP complex at 1:10 ratio; B. Control, EGCG, Starch nanoparticle (SNP) and EGCG-SNP complex at 1:10 ratio; C. Control, EGCG, SNP and EGCG-SNP complex at 1:25 ratio

5.5 Thematic Summary

The current work provided insightful information on the effect of EGCG and catechin extracts derived from Assam tea leaves on the micellar solubility of cholesterol. It was interesting to observe the effect of encapsulation on the in-vitro micellar solubility as well. The results indicated that EGCG had a strong effect on the micellar cholesterol solubility but did not affect

Table 5.1: Literature comparison table for EGCG/CE encapsulation , characterization , taurocholate binding, and micellar solubility study

S. No.	Samples	Concentration, mg/mL	Size	Glass transition		Point of thermal degradation, °C	C_p J/(g*K)	Taurocholate Binding activity, %	Micellar Solubility study, %	Reference
				Onset, °C	Peak, °C					
1	EGCG	2.5	12.5-19.5µm	89 ± 3.6	116.6 ± 3.21	225.7 ± 5.7	1.50 ± 0.179	0	8.87 ± 0.91	This work
2	Catechin extract	2.5	-	-	-	-	-	0	64.37 ± 2.23	
3	SNP	25	90-150 nm	90.1 ± 3.01	164.5 ± 2.72	267 ± 5.39	0.753 ± 0.042	85.58 ± 0.94	97.09 ± 5	
4	EGCG-SNP(1:10)	2.5:25	20-120 nm	66.17 ± 3.92	99.7 ± 3.21	282.9 ± 2.8	0.833 ± 0.02	-	96.51 ± 5.02	
5	CE-SNP(1:10)	2.5:25	-	-	-	-	-	-	96.49 ± 7.55	
7	Cholestyramine	25	-	-	-	-	-	-	2 ± 0.1	Nagaoka et al., 1999

the taurocholic acid binding. EGCG effectively reduced the cholesterol in the micelle to 8.87% at only 2.5 mg/mL. The radioisotope-based inhibitory action of micellar cholesterol solubility in-vitro study also reaffirmed the EGCG-SNP complex formation as it nullified the inhibitory properties of EGCG post encapsulation. The FTIR and XRD results also indicated the formation of the complex between the guest EGCG molecule and the host starch nanoparticle. EGCG and starch nanoparticle had a relatively crystalline structure and the resulted EGCG-SNP complex also retained its crystalline attributes. The thermal properties evaluated for EGCG-SNP complex indicated novel properties with enhanced thermal stability. The thermal degradation point of EGCG-SNP complex was defined at 282.9°C in comparison to 225.7, 275.9°C for EGCG and SNP respectively. The current work envisages a path for future research concerning the effect of encapsulated catechin extracts or EGCG on the inhibitory action of micellar cholesterol solubility. Potential studies may also be carried out on the in-vivo effect of inhibition of cholesterol absorption through the application of encapsulated EGCG/catechin extracts in various food matrices.

Chapter 6:

Formulation of an Instant Functional Black Tea Drink Formulation Fortified with Nano-Encapsulated Catechin Extracts and its Associated Characterization

Chapter 6

Formulation of an Instant Functional Black Tea Drink Formulation Fortified with Nano-Encapsulated Catechin Extracts and its Associated Characterization

The chapter explores the development of a functional tea drink with the encapsulated catechin extracts. Section 6.1 presents a concise overview of the creation and utility of functional tea beverages. Section 6.2 provides an in-depth analysis of the catechin extract, starch nanoparticles, and catechin extract-starch nanoparticles (CE-SNP) complex. The addressed characterization in this section refer to morphological, thermal, and crystalline analyses. Section 6.3 presents the in-vitro digestion outcomes of dry encapsulated catechin extracts and their solubilized liquid formulations. Section 6.4 outlines the sensory evaluation findings for the formulated functional tea drink.

Overview

The chapter explores the utility of starch nanoparticles as a wall material for the encapsulation of catechin extracts. The inclusion complex approach, a green and sustainable option for the encapsulation functional products has been investigated for the preparation of encapsulated catechin extracts. The encapsulated catechin extracts were analysed for their morphology, size, thermal characteristics, and crystalline qualities. An in-vitro digestion study was conducted to evaluate the sensitive influence of encapsulation on the retention of nutrients in catechin

extracts, and for the comparative assessment of dry and solubilized forms of the product under simulated digestion conditions. The encapsulated catechin extracts were utilized to strengthen and effectively create a functional tea drink formulation. For this purpose, long black tea leaves were used as the foundation component. These were subsequently fortified with encapsulated catechin extracts that possessed higher constitution of the catechins. Thereafter, adopting sensory evaluation techniques, the prepared formulations were optimized to determine the ideal constitution of the product palette.

6.1 Introduction

On a worldwide basis, tea is recognized as one of the oldest and most widely consumed beverages. Continued and ever increasing awareness of the diverse health advantages of the tea drink fostered substantial research on the greater exploration of its functional properties. Well known tea beverages refer to unfermented green tea, partially fermented oolong tea, and fully fermented black tea (Jayasinghe & Kumar, 2021). Each of these tea products have unique nutritional palette and advantages in terms of the constituent alternate phenolic components. The catechin compounds are the major phenolic chemicals in the fresh tea leaves. According to the best findings reported in the Chapter 3 of the Ph.D. thesis, the S3A3 Assam tea cultivar is one among the best for the higher constitution of alternate catechin compounds.

In due course of their utility as a functional ingredient in drink formulations, the bioavailability of the catechins is a primary challenge. Tea catechins are vulnerable to degradation and lose their desired functional characteristics during the rigorous digestive processes in the human gut system (Shim et al., 2012). To address this issue, edible wall materials can be explored for the inexpensive encapsulation of tea phenolics. In this regard, numerous studies conducted till date inferred significant improvements in the bioavailability of green tea extracts, EGCG, and catechin components. This is accomplished through the utilization of various wall materials

such as maltodextrins, cyclodextrins, chitosan nano systems, and liposomes (He et al., 2020; Mukherjee et al., 2023; Rashidinejad et al., 2014). Among various encapsulation techniques, micro and nano encapsulation techniques have been studied for tea catechins, green tea, and various tea phenolic components such as EGCG and ECG (Safer et al., 2019). Among these, the nano encapsulation is favored due to its ability to offer a larger surface area for greater absorption of desired constituents during the gut digestion process (Leclercq et al., 2009; Safer et al., 2019).

In the field of either encapsulation based functional tea product development or catechins encapsulation in other food matrices, very few literatures exist. A brief account of these are as follows. A research group created a rooibos iced tea (a herbal tea) through the incorporation of nano-encapsulated rooibos extract as a fortifying constituent (Viljoen et al., 2017). Further, the incorporation of encapsulated catechin extracts in various food matrices such as yoghurt, cheese and orange peels has been as well addressed (I. Ahmad et al., 2022; Ho et al., 2019; Rasouli Ghahroudi et al., 2017). Despite such experimental advances and confidence ascertaining strategies, the functional tea encapsulation in food matrices is highly restricted due to the non-availability of chemically safe and consumer-friendly encapsulation matrix or technology. Further, in the specific research theme of encapsulated catechin extracts or tea phenolics into functional tea beverages, no literature exists to the best of the knowledge of the author. In this regard, it can be noted that for such application, it has been already inferred that the starch nanoparticles serve as a viable medium for catechins encapsulation (M. Ahmad et al., 2019). Accordingly, the best findings of the Chapter 4 of the Ph.D. thesis delineate upon the cost effective production of potato starch nanoparticles for their intended utility as a carrier for the encapsulation of tea catechins.

The targeted research of catechins encapsulated starch nano-particle systems for functional tea product development is justified with the following lacunae. Firstly, limited studies exist for

catechins encapsulation in starch nanoparticles (M. Ahmad et al., 2019). However, such an encapsulation was based on catechin chemicals available in the market but not catechins extracted from green tea leaves such as the Assam tea cultivars. Also, anti-solvent precipitation method was used and comparatively inclusion complexation, a green method was not targeted. Secondly, the conducted studies till date did not delineate upon the detailed characterization of the prepared encapsulated materials in terms of morphology, size, thermal, and crystalline properties. Thirdly, the green encapsulation technology offers an additional benefit in terms of its consumer acceptance for utility in various food matrices. Fourthly, very few studies addressed encapsulated catechin system in the black tea food matrix system. This system has a specific advantage in terms of its lower catechin constitution and higher constitution of other desired compounds such as theaflavins and thearubigins. Therefore, the black tea food matrix is ideal to offer best combinations of both encapsulated catechins and theaflavins and thearubigins. Fifthly, no prior research optimized functional tea beverage has been prepared with the encapsulated catechin extracts and with the sensory evaluation methodology. Finally, and most importantly, the in-vitro digestion assessment of both dry and solubilized catechins encapsulated functional tea product has been scarcely addressed in the literature (Green et al., 2007).

Considering the above mentioned lacunae, the Ph.D. thesis targets the creation of an efficient tea mix with the black tea leaves as the primary ingredient to host catechin extracts functionally encapsulated in starch nano particle systems. The Hedonic scale and fuzzy logic approach based sensory evaluation methodologies were considered for the determination of the formulation optimality in terms of the loading of the functionalized nanoparticle system. Since green and chemical free inclusion complexation technique was adopted for the encapsulation of catechin extracts in starch nano-particles being as well prepared with a green and non-chemical method, the developed product with the other black tea ingredient can be considered

to be green for the case in which organic black tea can be resourced. Finally, the sensory evaluation based optimized formulation was subjected to in-vitro digestion study for the validation of its bio-availability efficacy in both liquid and solid forms as a functional tea beverage.

Prior to the conduct of the sensory evaluation study, the catechins encapsulated starch nanoparticle systems (termed CE:SNP complex) were subjected to characterization studies. For comparison purposes, both catechin extracts (CE) and starch nanoparticle systems (SNP) were also considered. A summary of the associated findings have been delineated in the following section.

6.2 Characterization of the Catechin Extracts, Starch Nanoparticles and CE:SNP Encapsulate

The CE:SNP complexes at 1:1 and 1:2 weight ratio were considered for the characterization studies. The ratios were based on the previous findings where catechin extracts were encapsulated using maltodextrins using inclusion complex method (Mukherjee et al., 2023). For all three cases namely CE, SNP and CE:SNP complexes, morphological, thermal, and crystalline properties were assessed. The morphological findings have been summarized in the following sub-section.

6.2.1 Morphological Studies

Figure 6.1 displays the morphology of catechin extracts, starch nanoparticles, and catechin extracts functionalized starch nanoparticles systems. The images were captured with a SIGMA 300 FESEM device and were thereafter analysed with the Image J software. The CE displayed a circular shape but with particle sizes that altered from 12 to 50 µm. Accordingly, the average

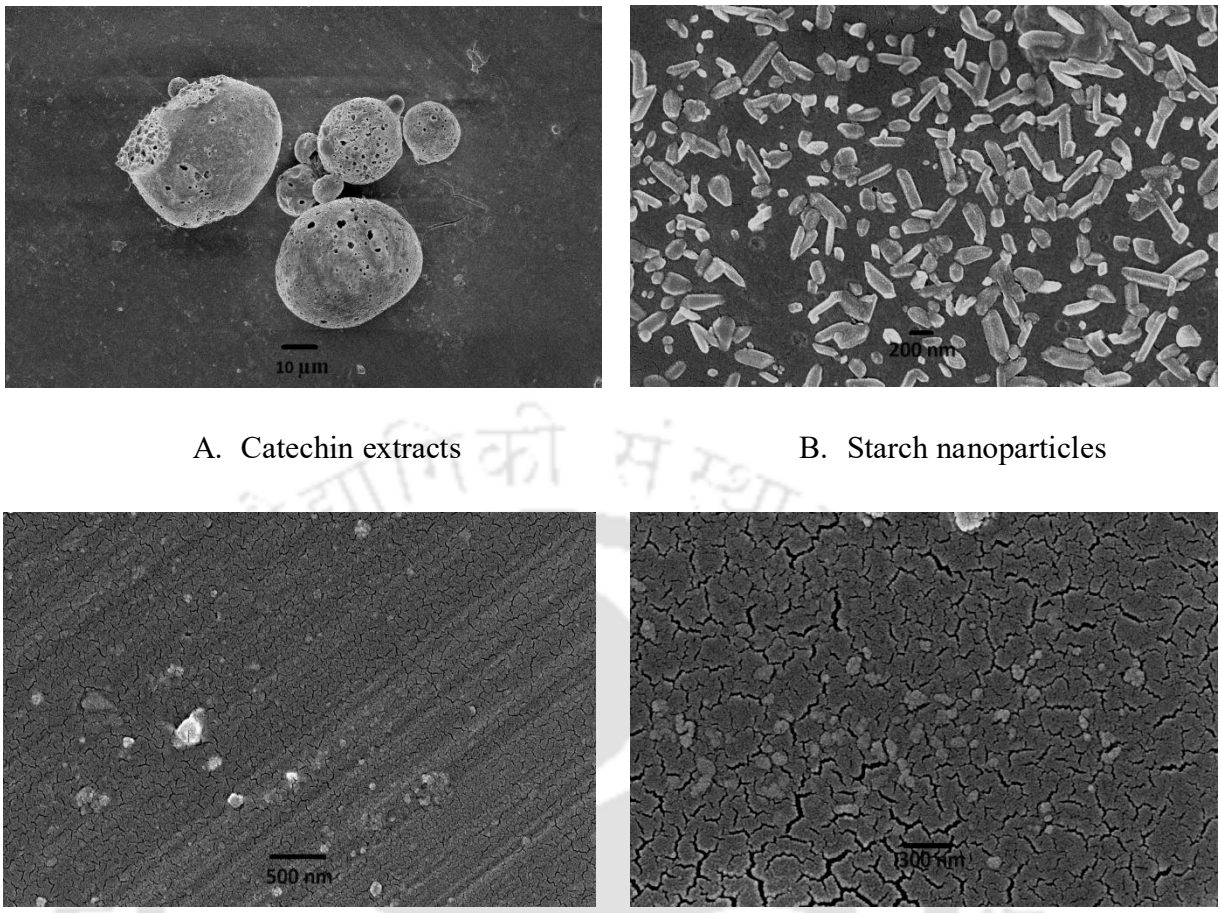
particle size of the CE was 30 µm. These findings are in agreement with the reported dimensions of the CE in a recent study (Mukherjee et al., 2023).

The SNP were prepared as per the procedures summarized in section 2.3 of the Ph.D. thesis. The FESEM images affirmed cylindrical shape of the nanoparticles and with particle length sizes ranging from 39 to 146 nm and particle length sizes ranging from 82 to 495 nm. Accordingly, the average size of the SNP were assessed to be 93.6 nm in length and 295.8 nm in breadth.

The CE:SNP complex with 1:1 weight ratio constitution possessed a particle size range of 43 – 210 nm and an average particle size of 79.4 nm. On the other hand, the CE:SNP complex with 1:2 weight ratio constitution possessed a particle size range of 22 - 102 nm and an average particle size of 61.8 nm. Further, the images affirmed effective encapsulation of the SNP with the CE. Also, for the sub-case of 1:2 weight ratio of the CE:SNP complex, the particle size reduced due to enhanced concentration of the SNP. The FESEM pictures clearly depicted that the SNP no longer had their distinctive rod-shaped structure after CE encapsulation with the inclusion complex approach. This serves as a direct proof for the effective CE encapsulation in the nanoparticle system.

6.2.2 Thermal Properties of the Catechin Extracts, Starch Nanoparticles, and CE:SNP Encapsulate

The microcapsules being prepared in the Ph.D. thesis research work were achieved with the boiled water. Henceforth, temperatures higher than 100 °C were not encountered. However, catechin SNP encapsulates can also be incorporated in baked products such as cookies, chips etc. In such baked products, the encapsulated catechin extracts would be exposed to higher temperatures. This justifies the need for the conducted thermal degradation study at higher temperatures.



A. Catechin extracts B. Starch nanoparticles

C. CE:SNP 1:1 D. CE:SNP 1:2

Figure 6.1: FESEM images of (A) Catechin extracts (B) Starch nanoparticles (C) CE:SNP (1:1 w/w) and (D) CE:SNP system (1:2 w/w)

Figure 6.2 displays the thermograms of CE, SNP, CE:SNP (1:1), and CE:SNP (1:2) systems. In the respective thermogram, the CE from S3A3 tea cultivars affirmed a peak at 37.7°C. This is due to the water loss from the system. The gelatinization of catechin extracts started at 69.5°C and ended at 133.1°C. The CE had a thermal degradation point of 178.4°C. The thermal degradation of catechin extracts started at the glass transition temperature (133.1°C, as stated by Mukherjee et al. (2023). The findings in section 4.5 of the Ph.D. thesis affirmed enhanced thermal properties of the SNP. The SNP conveyed the initiation of glass transition at 92.9°C. Also, the peak at 164.5°C affirmed the conclusion of the glass transition phase. The thermal degradation

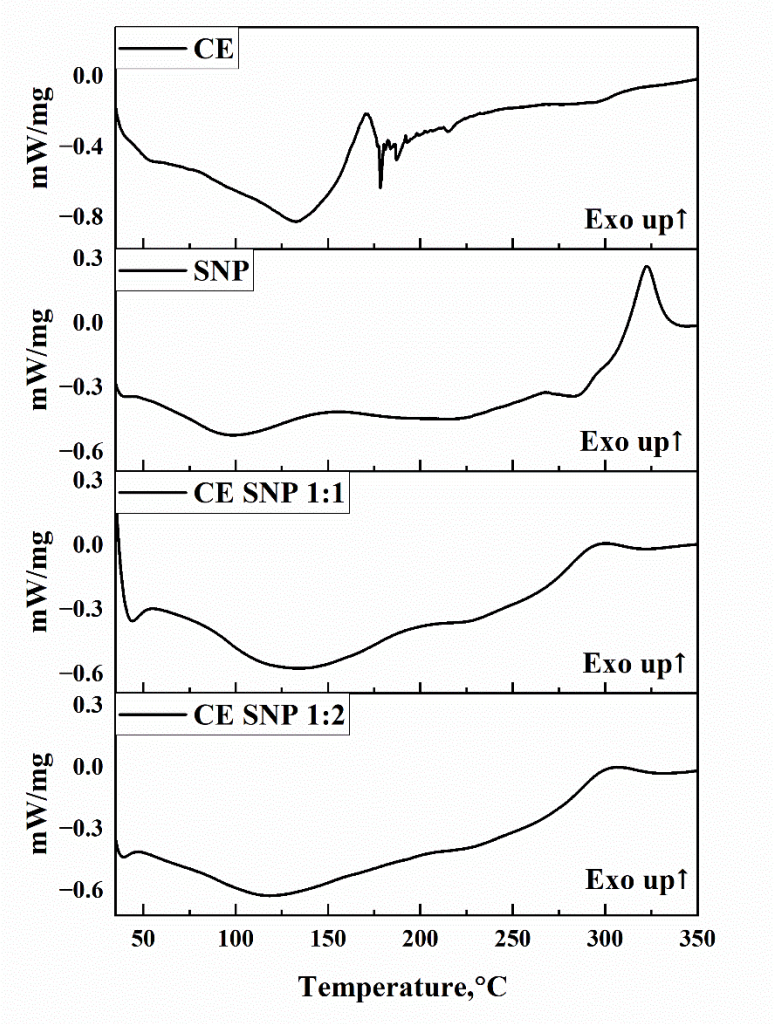


Figure 6.2: DSC thermograms for CE, SNP, CE:SNP (1:1), and CE:SNP (1:2)

point was determined to be 275.9 °C and from the onset of an endothermic peak in the thermogram. After encapsulation, important changes transpired in the CE:SNP encapsulate. The glass transition conditions in CE:SNP (1:1) did not vary significantly in comparison to the CE case. The initial temperature and final temperatures were noted at 61.3 and 133.2°C respectively. An additional endothermic peak at 228.4°C validated it to be the beginning point of thermal degradation of CE:SNP complex at 1:1 weight ratio constitution. Thus, in comparison with the CE, the thermal degradation point significantly enhanced for the case and thereby affirmed enhanced thermal stability. For the sub-case of CE:SNP with 1:2 weight ratio, the beginning temperature was at 47.2 °C and the end temperature was at 118.7 °C. The glass

transition temperature for the case did not exhibit significant alteration. Another endothermic peak at 230.2°C affirmed it to be the beginning of the thermal degradation for the sub-case. The thermal degradation points were similar for both sub-cases of the CE:SNP complexes. This is possibly due to inadequate enrichment in the encapsulation for the second sub-case. In other words, CE:SNP complex constitution of 1:1 weight ratio is optimal from the perspective of the thermal properties.

6.2.3 Crystalline Properties of the Catechin extracts, Starch nanoparticles and CE:SNP Encapsulate

Figure 6.3 depicts X-ray diffractograms of CE, SNP, CE:SNP (1:1 weight ratio), and CE:SNP (1:2 weight ratio) systems. The CE demonstrated peaks at 2θ values of 14.3, 20.8, 22.68, 28.84, 32.18, and 37.02°. The greatest intensity of the peak as 3468.95 was detected at a 2θ value of 14.3°. The CE diffractogram confirmed that the CE had a semi-crystalline structure. Similar inference has been cited in the relevant prior art that conveyed a comparable crystalline structure of the CE comprising of several kinds of catechins (Mukherjee et al., 2023).

For the SNP case, the crystalline peaks were detected at 2θ values of 19.06, 22.64, 23.16, 23.62, 25.58, 28.06, 29.02, 31.86, 32.14, 33.88, 37.8, and 38.64°. The SNP obtained from acid hydrolysis and ultrasonic post treatment affirmed very good crystalline structure. This is due to the existence of intense peaks. For this, the reasoning provided in section 4.4 of the Ph.D. thesis that conveyed that the recrystallization in the SNP has been mostly due to the reorganization of the amorphous areas of potato starch granules during the drying process that followed the acid hydrolysis. The maximum intensity peak of 58688 was detected at 32.14°.

For the CE:SNP with 1:1 weight ratio sub-case, the crystalline peaks were detected at 2θ values of 14.24, 22.32, 25.12, 28.8, 31.34, 32.2, and 34.64°. The obtained high intensity peaks affirmed a typical crystalline structure. Also, it is apparent that the crystalline structure of starch

nanoparticles influenced the crystalline structure of the CE-SNP encapsulate. The intensity reduced significantly in comparison to the SNP case. The highest peak intensity value of 23230 was obtained for the case at a 2θ value of 22.32°. Also, the crystalline pattern affirming distinct peaks at 2θ values of 14.24, 25.12, 28.8, and 32.2° demonstrated greater intensity in comparison with the CE. Due to this effect, it may be the case that a new compound may have been generated between the CE and SNP. For the sub-case of the CE:SNP at 1:2 weight ratio, the crystalline peaks exist at 2θ values of 14.36, 17.06, 22.54, 25.38, 31.7, and 32.22°. These

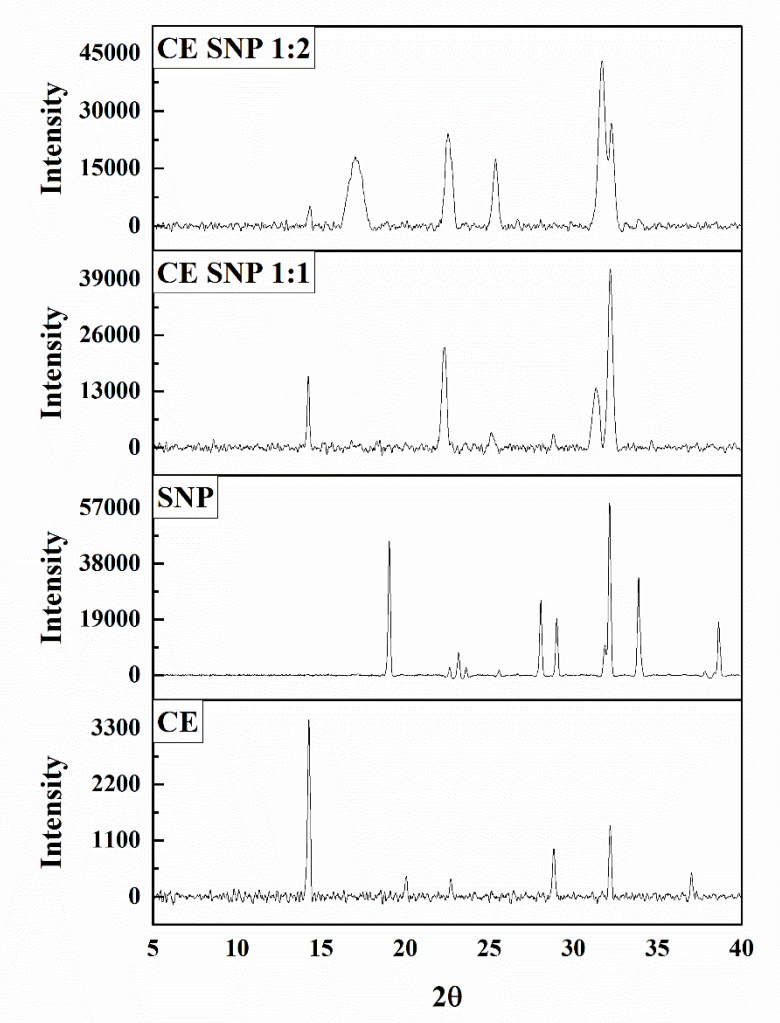


Figure 6.3: X ray diffraction patterns for CE, SNP, CE:SNP (1:1), and CE: SNP (1:2)

closely resemble the corresponding peaks obtained for the CE:SNP complex with 1:1 weight ratio. The highest intensity of 42956.17 has been significantly higher at a 2θ value of 31.7°.

The CE-SNP complex displayed characteristic peaks at 2θ values of 14.36, 25.12, and 32.22° and thereby affirmed the presence of catechin extracts and in the form of encapsulated entity.

6.3 In-Vitro Digestion Studies for CE and CE:SNP

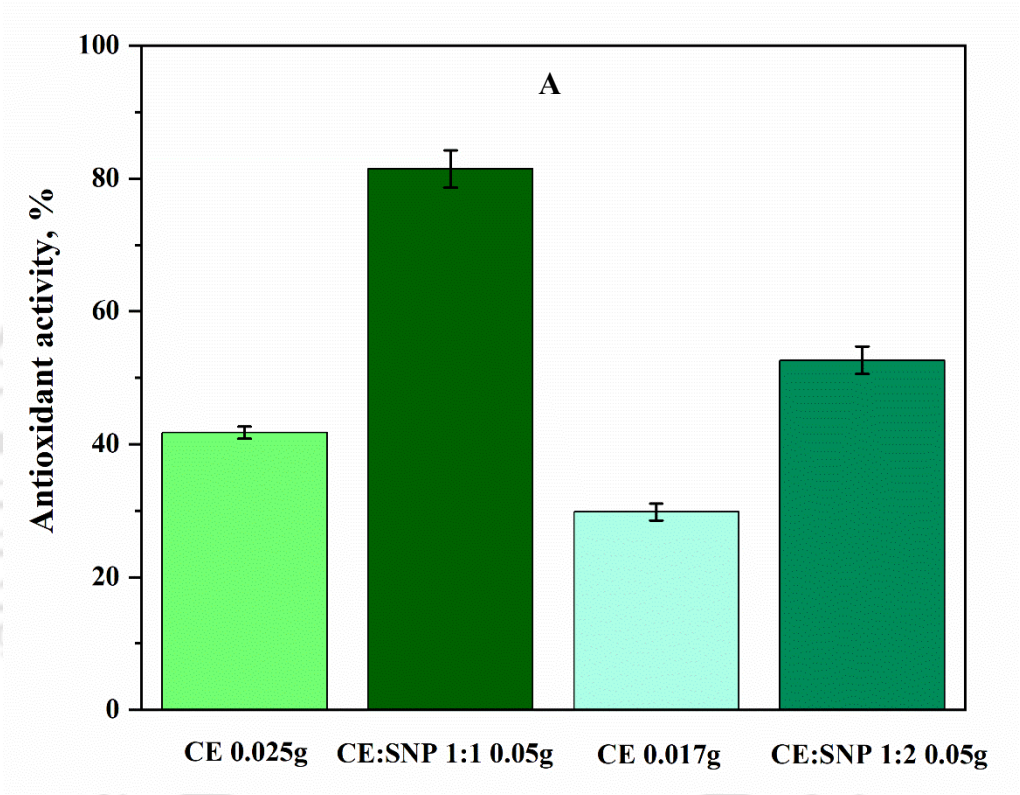
Figure 6.4 A and B respectively depict the findings obtained from the in-vitro digestion studies of CE and CE:SNP in dry powder (Figure 6.4 A) and liquid forms (Figure 6.4B). The liquid form of the product was obtained through the dissolution of the CE:SNP dry mix (with appropriate amount mentioned in the figure) in 100 mL of boiling water. Thus, two liquid samples were generated and for CE:SNP weight ratios of 1:1 and 1:2. Thereby, the liquid form was prepared to ascertain a solids concentration of 500 µg/mL.

The figures depict very interesting and promising outcomes. Outstanding antioxidant potential characteristics have been demonstrated by the CE:SNP complex with 1:1 weight ratio. For the dry sample's cases of catechin extracts (0.025 g and 0.017g), CE:SNP (1:1 weight ratio and 0.05 g), and CE:SNP (1:2 weight ratio and 0.05 g), the antioxidant activity after in-vitro digestion have been obtained as 41.75, 29.82, 81.46, 52.63% respectively. In other words, it is apparent that the dry form of the CE at the lower loading had lower AA in comparison to that obtained at the higher loading. This is due to the inadequate loading having detrimental influence of the AA retention. Comparatively, the CE:SNP complex with 1:1 and 1:2 weight ratio constitutions demonstrated the AA of 81.46 and 52.63% respectively after in-vitro digestion. Compared to the non-encapsulated CE case, the CE:SNP case (1:1 weight ratio sub-case) ascertained 95.11% enhancement in the AA retention. Comparatively, the enhancement has been 76.5% for the CE:SNP sub-case with 1:2 weight ratio constitution. The retention of about 52.63% AA after in-vitro digestion for the former case is promising. However, this shall be ensured after contacting with the boiling water. Also, for the later case, the AA retention has been poor due to lower loading of the CE in the complex.

Figure 6.4B depicts the %AA recovery of various liquid samples of CE and CE:SNP complexes. For the CE, CE:SNP (1:1 weight ratio) and CE:SNP (1:2 weight ratio) cases, the AA after in-vitro digestion were 43.2, 78.6, and 67.4% respectively. The encapsulated catechin extracts ascertained increased antioxidant activity in comparison to the liquid CE sample (81.94 and 56.01% for CE: SNP complex with 1:1 and 1:2 weight ratio constitutions respectively). Once again, highest AA retention has been apparent for the CE:SNP complex with 1:1 weight ratio constitution. The in-vitro AA results for the encapsulated catechin extracts in the dissolved state at 500 µg/mL exhibits similar antioxidant activity post digestion in comparison to the dry form for the raw catechin and CE:SNP (1:1 w/w) case. The results indicated that catechin extracts in its raw and encapsulated form had been stable in its dissolved state and while going through the simulated digestion condition. However, in CE:SNP (1:2 w/w) case, the antioxidant activity in the liquid form was found to be higher than that of the dry form. CE:SNP (1:2 w/w) in the liquid state ascertained an enhanced AA of 28.06% in comparison to the dry form. For the case of CE:SNP with higher starch nanoparticles ratio (1:2), the stability of the complex improved even further and hence an increase in the bioactive retention could be observed in terms of enhanced antioxidant activity. Previous works on in-vitro study of catechin mix in dissolved state reported the enhanced recovery of bioactives due the presence of additives such as ascorbic acid, soy, and polysaccharides (Green et al., 2007; Oh et al., 2021).

The retention of the AA for liquid form for the case demonstrates the prominence of the wall material to retain the AA even after exposure to the harsh thermal environment. In other words, with very good retention of the AA, the product is expected to provide best nutritional functional efficacy in the human gut system. Thus, the findings affirmed that the catechin extracts after encapsulation facilitated the better preservation of the catechin components that ascertain higher AA values even after being subjected to simulated digestion condition. Earlier investigations affirmed comparable data with respect to the catechins release in simulated

digestion environment (S. Ahmad et al., 2016; Shim et al., 2012). The encapsulated CE affirmed reduced AA of the liquid samples in comparison with the dried form. This is possibly due to the higher dilution of the encapsulated catechin extracts in 100 mL of the boiling water. The boiling water sample may have diminished the effectiveness of the encapsulated catechin extracts. The results demonstrated novel enhancements in a liquid functional tea drink formulation through the utilization encapsulated catechin extracts.



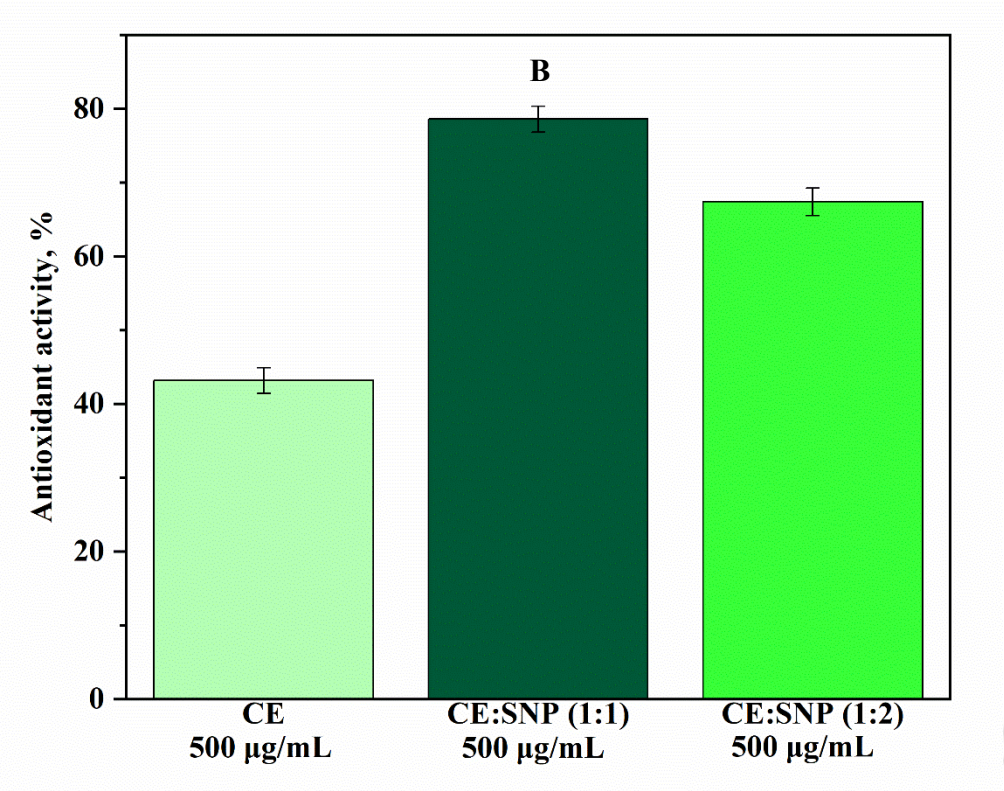


Figure 6.4: In-vitro digestion findings of (A) catechin extracts (0.025 g and 0.017g), CE:SNP (1:1) 0.05 g, and CE:SNP (1:2) 0.05 g in dry form (B) Liquid samples - Catechin extracts: 500 µg/mL, CE:SNP (1:1): 500 µg/mL, and CE:SNP (1:2): 500 µg/mL

6.4 9-Point Hedonic Scale and Fuzzy Logic Based Sensory Analysis

For the sensory evaluation studies, seven samples namely F1-F7 were considered. These referred to F1 - CE, 250 µg/mL; F2 - CE, 500 µg/mL; F3 - CE:SNP (1:2), 500 µg/mL; F4 - CE: SNP (1:2), 1000 µg/mL; F5 - CE:SNP (1:1), 500 µg/mL, F6 - CE:SNP (1:1), 1000 µg/mL and F7 – control sample prepared with only the commercial black tea leaves. Table 6.2 summarizes the findings obtained from the hedonic scale based sensory studies for the mentioned F1-F7 samples. Among all cases, the F6 formulation prepared with the encapsulated catechin extracts affirmed highest overall acceptability value of 7.2. This is followed by the F3 and F4 formulation with a score of 7 and 6.8 respectively. The F6 formulation exhibited superior sensory attributes in terms of flavour, colour, scent, appearance, taste, and aftertaste. These

were 7, 7.2, 7.4, 7, 6.6, 6.8, and 7.2 respectively in the hedonic rating scale. The control sample with the commercial tea leaves received the highest overall acceptability sensory rating of 7.6. The findings of the fuzzy logic study demonstrated the superiority of the F6 sample (Table 6.1). The F6 sample utilized catechin extracts and starch nanoparticles at a 1:1 weight ratio along with commercial black tea leaves (concentration of 1000 µg/mL). The F6 formulation achieved a satisfactory rating of 0.697, which was the highest fuzzy logic rating among all the formulations. All the formulations demonstrated satisfactory rating, which conveys the highest priority. The formulations F1, F2, F3, F4, F5, and F7 were assigned fuzzy logic ratings of 0.611, 0.563, 0.579, 0.645, 0.591, and 0.624 respectively. The results demonstrated that the formulations containing non-encapsulated catechin extracts exhibited inferior performance in comparison to the formulations containing encapsulated catechin extracts. The bitter taste of the pure catechin extracts is masked through nano encapsulation, for this reason, enhanced sensory qualities were apparent in F6 and F4. The inclusion of starch nanoparticles did not affect the flavour of the formulations. This is confirmed by sensory analysis with both hedonic scale and fuzzy logic methods. Unlike native starch, the starch nano-

Table 6.1: Fuzzy logic sensory data for catechin extract, and encapsulated catechin extract based functional tea drink formulations

Samples	Score					
	Not Satisfactory	Fair	Satisfactory	Good	Very Good	Excellent
F1	0.080	0.439	0.611	0.461	0.272	0.080
F2	0.064	0.393	0.563	0.476	0.323	0.117
F3	0.062	0.387	0.579	0.523	0.376	0.144
F4	0.094	0.553	0.645	0.614	0.211	0.039
F5	0.073	0.444	0.591	0.469	0.297	0.099
F6	0.091	0.550	0.697	0.523	0.306	0.090
F7	0.088	0.437	0.624	0.483	0.259	0.069

*F1: CE, 250 µg/mL, F2: CE: 500 µg/mL, F3: CE:SNP (1:2), 500 µg/mL, F4: CE:SNP (1:2), 1000 µg/mL, F5: CE:SNP (1:1), 500 µg/mL, F6: CE:SNP (1:1), 1000 µg/mL, F7: Control

particles have high solubility in water. When combined with catechin extracts, they do not introduce any undesirable flavour in the functional tea drink compositions.

6.5 Literature Comparison

Table 6.2 provides a concise summary of the comparison between the existing literature and the best experimental findings acquired in the present study. This study presents new findings on the development of a functional tea beverage by incorporating encapsulated catechin extracts as a functional ingredient and fortificants. The thermal characteristics of catechin extracts and starch nanoparticles were consistent with recent studies (Mukherjee et al., 2023). A substantial increase in the thermal degradation point was noticed in contrast to the values published in the literature. The thermal degradation points were measured at 228.4 and 230.2°C, and thereby affirmed a substantial increase with respect to the pure catechin extracts. The in-vitro bioaccessibility index, measured in terms of the percentage of antioxidant activity, affirmed a considerable increase to 95.11% for CE:SNP produced with 0.05 g of the dry form.

Table 6.2: Nine point Hedonic scale sensory data of catechin extract, and encapsulated catechin extract based functional tea drink formulations

Samples	F1	F2	F3	F4	F5	F6	F7
Flavour	6.2 ± 0.83	6.4 ± 1.34	6.8 ± 1.30	6.6 ± 1.34	6.2 ± 1.30	7 ± 1	7.6 ± 0.54
Colour	7 ± 0.71	7 ± 1	6.8 ± 1.30	6.4 ± 1.14	7 ± 1.22	7.2 ± 0.83	7.4 ± 0.89
Aroma	6.3 ± 1.39	6.1 ± 1.14	6.4 ± 1.51	6.3 ± 1.56	6.8 ± 1.30	7.4 ± 0.89	7.2 ± 0.83
Appearance	6.6 ± 1.14	6.6 ± 1.67	7 ± 1.22	6.8 ± 1.30	6.6 ± 1.51	7 ± 1	7.6 ± 0.54
Taste	6 ± 1	6.3 ± 0.97	6.6 ± 1.14	6.4 ± 1.14	6.2 ± 1.30	6.6 ± 1.14	7.4 ± 0.89
Aftertaste	6.4 ± 1.34	6.2 ± 1.30	6.6 ± 1.51	6.4 ± 1.51	6.2 ± 1.30	6.8 ± 1.30	7 ± 1
Overall acceptability	6.5 ± 1.22	6.4 ± 1.08	7 ± 1.41	6.8 ± 1.09	6.7 ± 1.20	7.2 ± 1.09	7.6 ± 0.54

*F1: CE, 250 µg/mL, F2: CE: 500 µg/mL, F3: CE:SNP (1:2), 500 µg/mL, F4: CE:SNP (1:2), 1000 µg/mL, F5: CE:SNP (1:1), 500 µg/mL, F6: CE:SNP (1:1), 1000 µg/mL.

Table 6.3: A summary of characterization and in-vitro digestion literature and best findings data summary for catechins extracts, starch nanoparticles, and CE:SNP encapsulate

S. No.	Samples	Size, nm	Glass transition		Thermal degradation point, °C	C_p J/(g*K)	In-vitro digestion, % AA		Reference
			Onset, °C	End, °C			Dry form	Solubilized form, 500 µg/mL	
1	CE, 0.025 g	3000	69.5	133.1	178.4	2.49	41.75 ± 0.9	58 ± 2.78	This work
2	SNP	93.59	92.9	164.5	275.9	0.753	-	-	
3	CE:SNP (1:1), 0.05 g	79.39	65.1	133.2	228.4	2.37	81.46 ± 2.79	78.6 ± 1.75	
4	CE:SNP (1:2), 0.05 g	61.83	47.6	118.7	230.2	2.969	52.63 ± 2.05	67.4 ± 1.86	
5	Catechin – Chest nut starch	322.7	-	-	161	-	4.8 mg catechin/100mg	-	Ahmad et al., 2019

Table 6.4: Literature and best findings data summary for the functional tea drink formulation

Formulations	Composition	Sensory properties		In-vitro digestion		References
		Hedonic	Fuzzy	Dry (0.05 g)	Liquid (500 µg/mL)	
F1	Water:100 mL CE:SNP (1:1 w/w): 100 mg	7.2	0.697 (Satisfactory)	81.46 (%AA)	78.6 (%AA)	This work
F4	Water:100 mL CE:SNP (1:2 w/w): 100 mg	6.8	0.645 (Satisfactory)	52.63 (%AA)	67.4 (%AA)	
Green Tea Extract	1 g/115 mL Water, 318.15 mg catechins, at 20°C	-	-	-	22.05% (mg of catechins)	(Oh et al., 2021)
Green Tea Extract	50 mg/100 mL, at 100°C	-	-	-	19.6% (mg of catechins)	(Green et al., 2007)

Table 6.4 presents a comparison between the most effective formulations of the study and the formulations that are described in the literature. Previous studies have not examined the in-vitro digestion or bioavailability of the combination of catechin extract encapsulation and functional tea drink formulation in both powdered and liquid states. Furthermore, earlier investigations did not provide any sensory evaluation data for formulations including catechin extract. The present study incorporates both sensory-based optimization with the hedonic scale and fuzzy logic methods. Additionally, this work integrates an in-vitro digestion analysis that conveys the advantage of nano encapsulation of catechin extracts in terms of their bioaccessibility.

6.5 Thematic Summary

The chapter successfully demonstrated the utilization of sustainable technologies and consumer approval methodologies for the creation of a functional tea drink product. Notable findings of

the chapter are as follows. Firstly, the chapter demonstrated the possibility to prepare a functional tea drink without utilizing chemical ingredients and thereby ascertained the product to be a environmentally friendly option. Secondly, the conducted characterization ascertained unique thermal and crystalline properties for the encapsulated catechin extracts prepared with both 1:1 and 1:2 weight ratios of catechin extracts and starch nanoparticles. Thereby, with improved thermal stability, the functionalized materials shall aid in the preservation of nutrients in the simulated digestion environment. Thirdly, in-vitro studies ascertained very good retention of antioxidant activity in the encapsulated starch nanoparticles systems in comparison to the catechin extracts. Further, the retention of the AA in the liquid forms after in-vitro studies in the encapsulated catechin extracts infers upon the nutrient preservation in even simulated stomach, and intestinal digestion phases. Fourthly, the sensory evaluation findings affirmed that the addition of encapsulated catechin extracts to traditional black tea did not significantly alter the sensory characteristics of the formulated functional tea drink product. Fifthly, the prepared functional tea product has balanced nutritional attributes due to the richer constitution of catechins, theaflavins and thearubigins. This is due to the fact that the lost catechins due to the fermentation step of the black tea were supplemented with the encapsulated catechins in the starch nanoparticle systems. Thus, the nutritional balance in the product is even attractive from the perspective of the consumer wish list. In summary, the prepared functional tea drink product is effective for its desired application due to nutritional, morphological, thermal, and sensory properties. Similar research studies can be addressed for encapsulated catechin extracts in different food products such as baked goods and soups. Such studies can enhance their functionality in terms of the preservation of nutritional properties in even human gut environment.

Chapter 7:

Conclusions and Future Work

Chapter 7

Overall Conclusions and Future Work

In section 7.1, the chapter summarizes the significant conclusions being deduced from the work conducted in the Ph.D. thesis. Subsequently, section 7.2 entails upon the possible scope for future work in the chosen research themes.

7.1 Conclusions

The Ph.D. thesis fulfilled the ultimate objective of a functional tea beverage formulation specification with the black tea and encapsulated catechin extracts. The conducted research work carried has been able to address the pertinent gaps in the field of nano-encapsulation based functional tea beverage formulation. Important conclusive findings of the conducted research work have been presented in the following sub-sections.

7.1.1 Evaluation of Catechin Profile in S3A3 and TV18 Tea Cultivar and Optimality of Catechins Enzymatic Extraction

The investigations carried out in this research theme delineate upon the catechin rich profile of Assam tea cultivars and subsequent parametric optimization of the enzymatic extraction process. The results convey novel findings with respect to the enzymatic extraction, its sensitive influence upon individual catechin types, and the optimization of the extraction conditions in terms of the time, temperature, and enzyme concentration. The following conclusions can be enlisted from the detailed assessments:

- The TV18 cultivar possessed higher EGCG content as 197.77 mg/g and S3A3 tea cultivar constituted higher ECG, EGC, EC, and C content and as 68.63, 58.32, 20.90, and 20.1 mg/g

respectively.

- The S3A3 and TV18 tea cultivars respectively possessed 70% and 80% catechins as (-)-EGCG and (-)- ECG. These are well known catechins for their therapeutic applications.
- The response surface methodology based optimization of the enzymatic extraction operating conditions have been obtained as 18.1 min, 88.5°C, and 188 EU/g and for 0.926 desirability. At this optimized condition, the extraction yields of TCC, EGCG, EGC, ECG, EC, and C have been achieved as 265.7, 140.25, 44.98, 43.98, 18.94, and 17.55 mg/g, respectively.
- For the hot water extraction process, the optimal process conditions of 30 min and 75°C yielded TCC, EGCG, EGC, ECG, EC, and C content in the aqueous extract as 175.66, 121.57, 27.87, 3.21, 12.55, and 10.46 respectively. The yield obtained at optimal point were 51.25% higher in comparison to the best yield obtained from trial and error methods.
- The enzyme concentration had a significant influence on the TCC and EGCG concentration ($p<0.05$). However, it did not have any significant influence upon the other types of catechins. Henceforth, the study affirmed the efficacy of cellulase and pectinase enzymes in enhancing the extraction efficiency of individual catechins that exist in tea leaves.
- The linear and quadratic influence of time and temperature respectively has been significant on the extraction of both total catechin and individual catechins.

7.1.2 Preparation of Potato Starch Nanoparticles using Acid Hydrolysis and Ultrasonic Post-Treatment

Following are the notable conclusions of the research work conducted in the field of the potato starch nanoparticles preparation with sonication assisted acid hydrolysis method.

- The starch nanoparticles were synthesized in reduced duration of 3 days and with increased concentration of sulfuric acid. The results conveyed strong influence of duration on the average particle size. Accordingly, the parameter altered from 1378 - 370, 180 - 66, 102 -

13, 1550 - 392, 323 - 146 nm for 3.16M, 4M, 5M, 3.16M control, and 4M control cases respectively.

- An increase in the the concentration of sulfuric acid resulted in reduced average particle size, polydispersity index and the yield of the starch nanoparticles. The yield has been the lowest for 5M sulfuric acid based hydrolysis (4%).
- Best findings have been obtained for 3 days of 4M sulfuric acid based hydrolysis and 90 mins duration of the post sonication treatment. At this condition, average particle size, PDI, yield, thermal degradation point, and relative crystallinity have been assessed as 66 nm, 0.362, 29%, 318.99°C, and 64.32%, respectively.
- The FESEM results also confirmed the findings obtained through the DLS method. The particle size range of starch altered from 100-1100 nm (for 3.16M sulfuric acid based hydrolysis) to 40-100 nm (4M sulfuric acid based hydrolysis at 3 days and 90 mins of sonication).
- The thermal stability of the starch nanoparticles increased with enhanced concentrations of sulfuric acid. The DSC results corroborate with the thermal degradation point enhancement from 245.71 (native starch) to 318.99°C (for SNP prepared with 3 days of 4M sulfuric acid based hydrolysis).

7.1.3 Nano-Encapsulation of EGCG and Catechin Extracts using Starch Nanoparticles and its Effect on In-Vitro Micellar Cholesterol Solubility

Important findings in the research theme of EGCG and catechin extracts' nano encapsulation and subsequent in-vitro micellar solubility have been presented as follows.

- The encapsulation of EGCG with starch nanoparticles resulted in 20-120 nm sized nano structures. The starch nanoparticles had distinct rod shaped sizes, and they altered in the range of 90-150 nm. Upon encapsulation with the IC method, the starch nanoparticles lost

their shape and constituted irregular shaped structures.

- The successful encapsulation of EGCG with starch nanoparticles depend upon the formation of H bonds that further facilitate the complex formation between EGCG and SNP (confirmed through the FTIR analysis). For the starch nanoparticles, peaks observed at 3637, 2922, 1658, 1521 cm^{-1} can be associated with the stretching vibration of hydroxyl groups, C–H, C=O, C–N. However, in the EGCG loaded starch nanoparticles the observed peaks shifted marginally to a higher wave number (peaks were observed at 3675, 2927, 1730, 1646 cm^{-1}). These peaks correspond to the formation of new hydrogen bonds between the EGCG and starch nanoparticles.
- The DSC studies revealed enhanced thermal stability of the encapsulated EGCG. This is evident in the enhanced thermal degradation point from 225.7°C to 282.9°C for EGCG and EGCG-SNP respectively. Such an effect is primarily due to the formation of new H bonds between EGCG and SNP and subsequent complex formation.
- The XRD findings affirmed enhanced crystalline properties of EGCG after encapsulation. The EGCG-SNP exhibited characteristic peaks for both EGCG and SNP and at 2θ values of 19.54, 20.7, 21.5, 28.14, 28.9, 29.5, 35.49°. Such findings infer upon the complex formation between EGCG and SNP.
- The catechin extracts and EGCG had a strong dose dependent effect on the micellar cholesterol solubility (MCS). The catechin extracts exhibited 64.37 and 40.96% MCS at 5, 2.5 mg/mL concentrations, respectively. However, EGCG exhibited 8.87 and 31.15% MCS at 2.5 and 1 mg/mL concentrations respectively.
- The starch nanoparticles did not have any effect on the reduction of cholesterol via micellar cholesterol solubility. Similar results were apparent for the encapsulated CE and EGCG cases. Also, post encapsulation, the encapsulate complex containing the EGCG and catechin extracts lost its ability to reduce the cholesterol absorption.

7.1.4 Formulation of Instant Functional Black Tea Formulation with Nano-Encapsulated Catechin Extracts and its Associated Characterization

Notable findings in the research theme refer to the following enlisted conclusions.

- The characterization of the encapsulated catechin extracts confirm upon the formation of nanostructures between CE and SNP and through the self-assembly mechanism. The particle size altered from 43-210 nm and 22-102 for CE:SNP (1:1, w/w) and CE:SNP (1:2, w/w) respectively (FESEM findings). The thermal properties were also significantly enhanced in terms of the enhanced thermal degradation point from 178.4 to 228.4°C for CE and CE:SNP (1:1, w/w) respectively.
- In comparison to the raw catechin extracts, the bioactives retention (%AA) enhanced by 95.11% for the CE:SNP (1:1, w/w) case. This affirmed the efficiency of the nano-encapsulation system in terms of the enhanced bioaccessibility for the protection of catechin extracts in the harsh digestion environments.
- In comparison to the dry form of encapsulated catechin extracts, the liquid form did not alter the bioactivity retention in terms of %AA for the raw catechin extracts and CE:SNP (1:1 w/w). Thereby, good stability of the encapsulated catechin extracts in hot water system can be ascertained.
- For the case of CE:SNP (1:2, w/w), the in-vitro digestion results for liquid form conveyed an enhanced recovery of bioactives (%AA) in comparison to the dry form. A 28.06% enhancement has been apparent for the CE:SNP and liquid system case. The enhanced concentration of starch nanoparticles may have affected the bioactive recovery. For the case of CE:SNP with higher concentration of the starch nanoparticles (1:2, w/w), the stability of the complex improved even further due to increased starch nanoparticle concentration. Henceforth, the bioactives retention increased further to infer upon the enhanced antioxidant

activity.

- Among all cases subjected to sensory analysis, the F6 formulation being prepared with 0.5 g of black tea and 100 mg of encapsulated catechin extracts at 1:1, w/w affirmed highest overall acceptability value of 7.2. The F6 formulation exhibited superior sensory attributes in terms of flavour, colour, scent, appearance, taste, and aftertaste sensory parameters. These were 7, 7.2, 7.4, 7, 6.6, 6.8, and 7.2 respectively in the hedonic rating scale.
- Among all tested formulations, F6 formulation achieved a satisfactory and the highest fuzzy score of 0.697. All the formulations demonstrated satisfactory rating, and henceforth demonstrate highest priority in the fuzzy sensory scale.

7.2 Future work

The following list elucidates upon the possible scope for further research in the addressed research themes of the Ph.D. thesis:

- The concentration of catechins in the North-east India tea leaves vary vastly with respect to climate, flush type, soil conditions, and types of tea cultivar. A detailed analysis on the effect of these parameters on the quality of tea leaves in terms of its polyphenolic constitution can be duly addressed.
- The Ph.D. thesis considered equal concentrations of cellulase and pectinase in the enzymatic pre-treatment process. Dose dependent influence of the individual enzymes such as cellulase and pectinase on the catechin profile of the tea leaves during the enzymatic hot water extraction can be targeted in future investigations.
- Also, the current Ph.D. thesis considered 5 types of catechins that exist majorly in the tea leaves. Future work may target and assess minor types of catechins that exist in the tea leaves.
- Further studies on the exact mechanism of the release of different types of catechins from

the tea leaves matrix could be addressed. Detailed molecular studies shall be addressed to understand the pertinent mechanism associated to the altered extraction rates for catechin in an enzyme based extraction process.

- The demonstrated novel synthesis process for the preparation of starch nanoparticles may be applied to different starch sources such as maltodextrin, maize, corn etc. These may result in a variegated category of starch nanoparticles in terms of their properties for applications in the food industry.
- The acid hydrolysis process enhances the concentration of resistant starch and through the recrystallization and rearrangement of amylose and amylopectin branches. Further studies may be directed towards the effect of increased concentrations of sulfuric acid on the concentration of resistant starch.
- The enhancement in bioavailability directly increases the concentration of bioactive compounds in the intestinal absorption stage. Further studies can be directed to understand the effect of enhanced bioaccessibility of catechin extracts on in-vitro micellar solubility of cholesterol. Thereby, the degree of influence of bioavailability can be sought.
- Detailed molecular studies can be addressed to understand the complex formation between catechin extracts containing a mixture of EGCG, ECG, EGC, EC and C with starch nanoparticles and how it effects the bioaccessibility.
- In-vivo bioavailability studies can be carried out to understand the effect of encapsulation of catechin extracts in animal body. Thereby, its effect on the reduction of cholesterol concentration in blood can be assessed.
- Adopt Nano-encapsulation method based on inclusion complexation process and conduct studies on ayurvedic extracts of Arjuna, Ashwagandha for their enhanced therapeutic applications with starch/maltodextrin/sunflower lecithin as the wall material.

- Extend applications of catechins nano encapsulated starch system in baked products such as bread, chips, cookies, and pan cakes. Thereby, address characterization studies to infer upon the efficacy of functional food product diversification and customization.

In summary, the Ph.D. thesis work paves the way for the formulation of nanotechnology based functional beverage. In order to achieve this objective, starch nanoparticles were created through the process of sonication-assisted acid hydrolysis. This resulted in improved thermal and crystalline characteristics. The starch nanoparticles that were created were used as the wall material to encapsulate catechin extracts. This expands the potential for future research on the production of starch nanoparticles that effectively transport various bioactive constituents. Customizing the thermal and physical parameters of a carrier vehicle for certain bioactive compounds can be potentially met. In addition, the combination of acid hydrolysis process and sonication can be utilized to produce starch nanoparticles that have a higher concentration of resistant starch.

There are numerous potential research opportunities in the realm of enzymatic extraction of catechins. Further investigation into enzymatic extraction can target dose-dependent tests and with various cell wall disintegrating enzymes. Also, enzymatic extraction has a significant role for the extraction of bioactives from aged tea leaves. This area can also be explored.

The Ph.D. thesis investigated the sensitive influence of encapsulation on enhanced bioaccessibility of nutrients and with the in-vitro digestion study. Future research can be undertaken using in-vivo animal models for the assessed interaction of encapsulated catechin extracts inside the pathways of the animal body. In summary, the Ph.D. thesis proposes a new approach to create a functional tea beverage formulation with the principle of nano-encapsulation. Such a concept can be widely utilized to enclose a variety of other biologically active substances and for diversified functional food applications. Accordingly, thorough

investigations will enhance the development of nanotechnology based functional food products for wider consumer acceptance.

Publications

List of Publications

A) Publications

1) **Kamal Narayan Baruah**, Siddhartha Singha, Ramagopal V.S. Uppaluri (2023). Preparation of Potato Starch Nanoparticles Using Acid Hydrolysis and Ultrasonic Post-treatment. *ACS Food Science and Technology*, 3(4), 626-634. DOI: https://doi.org/10.1021/acsfoodscitech.2c00369
2) **Kamal Narayan Baruah**, Siddhartha Singha, Ramagopal V.S. Uppaluri (2023). Determination of Catechin Contents in S3A3 and TV18 Tea Cultivar Using HPLC Method. *Agricultural Engineering*, 55, 11-18. DOI: https://doi.org/10.15544/ageng.2023.55.4.
3) **Kamal Narayan Baruah**, Siddhartha Singha, Paushali Mukherjee, Ramagopal V.S. Uppaluri (2023). Optimization of the enzymatic extraction of catechins from Assam tea leaves. *Biomass conversion and biorefinery*, https://doi.org/10.1007/s13399-023-04684-x
4) **Kamal Narayan Baruah**, Satoshi Nagaoka, Arata Banno, Siddhartha Singha, Ramagopal V.S. Uppaluri (2024). Nano-encapsulation of EGCG using starch nanoparticles: characterization and insights on in vitro micellar cholesterol solubility. *Journal of Food Science*. DOI: https://doi.org/10.1111/1750-3841.17260
5) Paushali Mukherjee, **Kamal Narayan Baruah**, Ramagopal V.S. Uppaluri (2023). Encapsulation and Characterization of Commercial Green Tea Extracts Using Green Methods: A Comparative Study of Inclusion Complexation and Ion Gelation. *Journal of Food Measurement and Characterization*. DOI: https://doi.org/10.1007/s11694-023-02250-7

B) Articles to be submitted

1) **Kamal Narayan Baruah,** Satoshi Nagaoka, Siddhartha Singha, Emiko Yanase, Ramagopal V.S. Uppaluri (2024). Formulation of Instant Functional Black Tea Drink Fortified with Nano-Encapsulated Catechin Extracts and its Associated Characterization.

C) Indian Patent to be submitted

1) **Kamal Narayan Baruah,** Siddhartha Singha, Ramagopal V.S. Uppaluri (2024). Formulation of Instant Functional Black Tea Drink Fortified with Nano-Encapsulated Catechin Extracts and its Associated Characterization.

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Appendices

Appendix A: HPLC Standard Curves for EGCG, ECG, EGC, EC, And C

HPLC chromatogram of mixed standards at 278 nm as per the ISO 14502:2:2005 method.

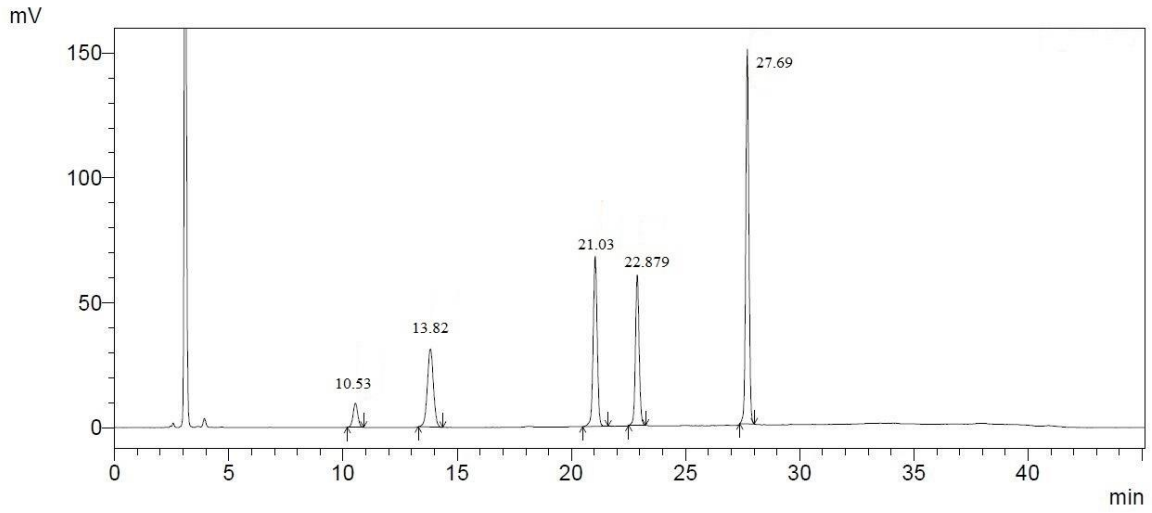


Figure A1: Standard curve at 50 ppm concentration of mixed standards

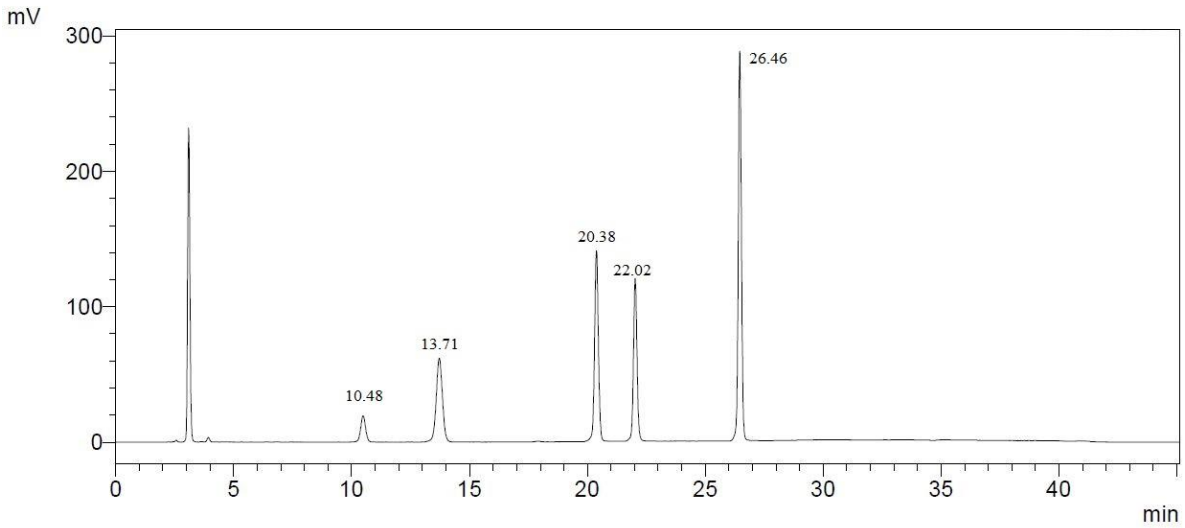


Figure A2: Standard curves at 100 ppm of mixed standards

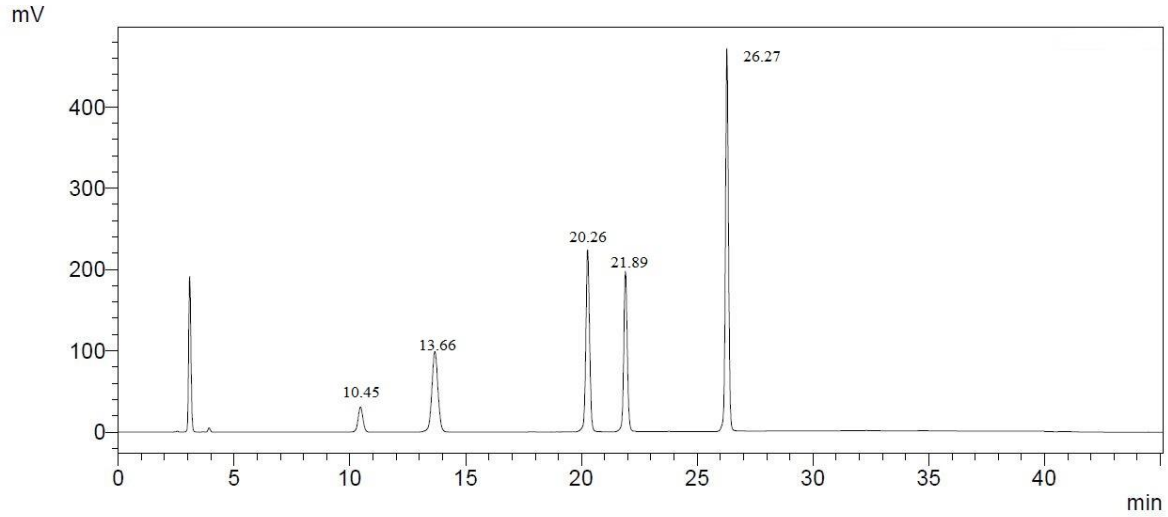


Figure A3: Standard curves at 150 ppm of mixed standards

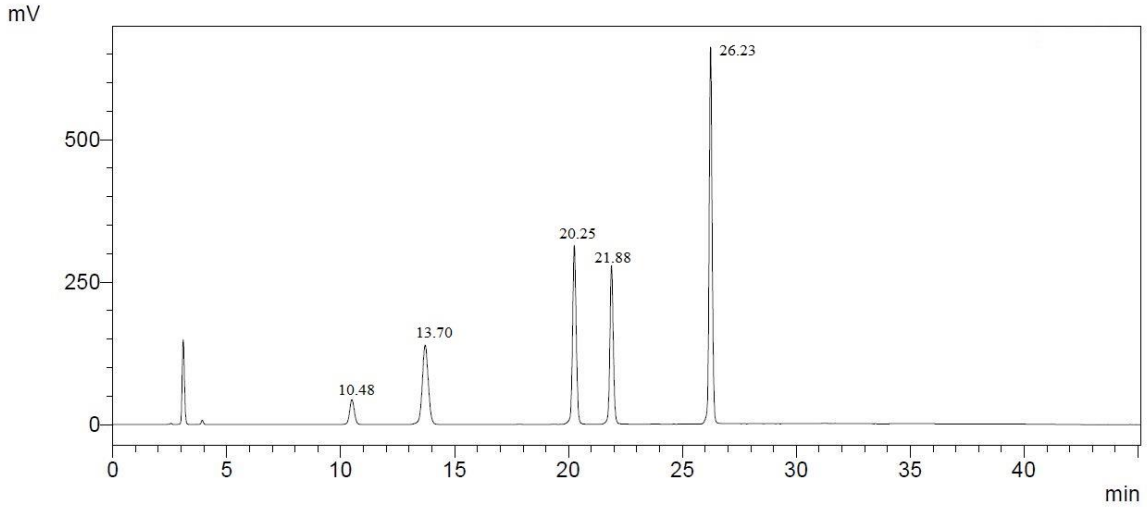


Figure A4: Standard curves at 200 ppm of mixed standards

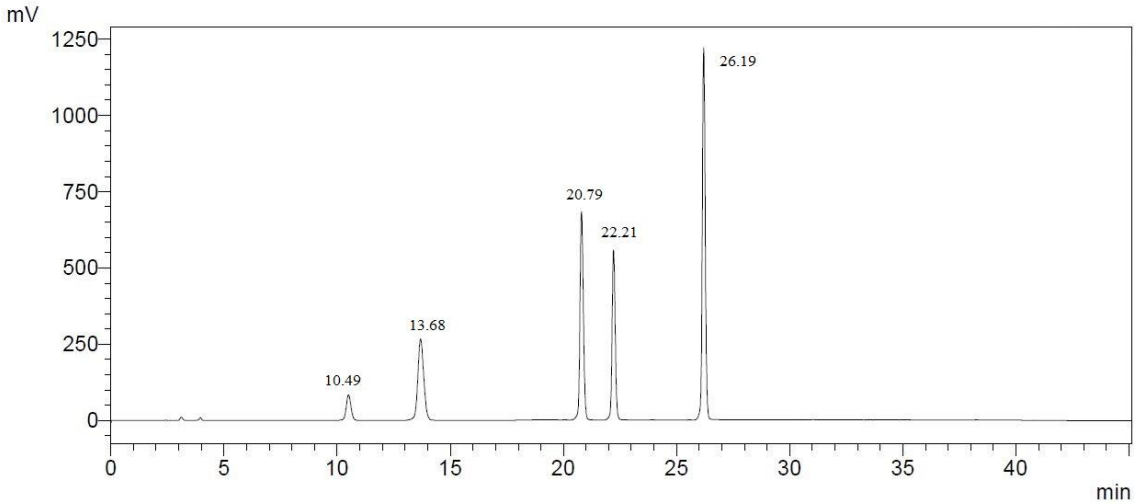


Figure A5: Standard curves at 300 ppm of mixed standards

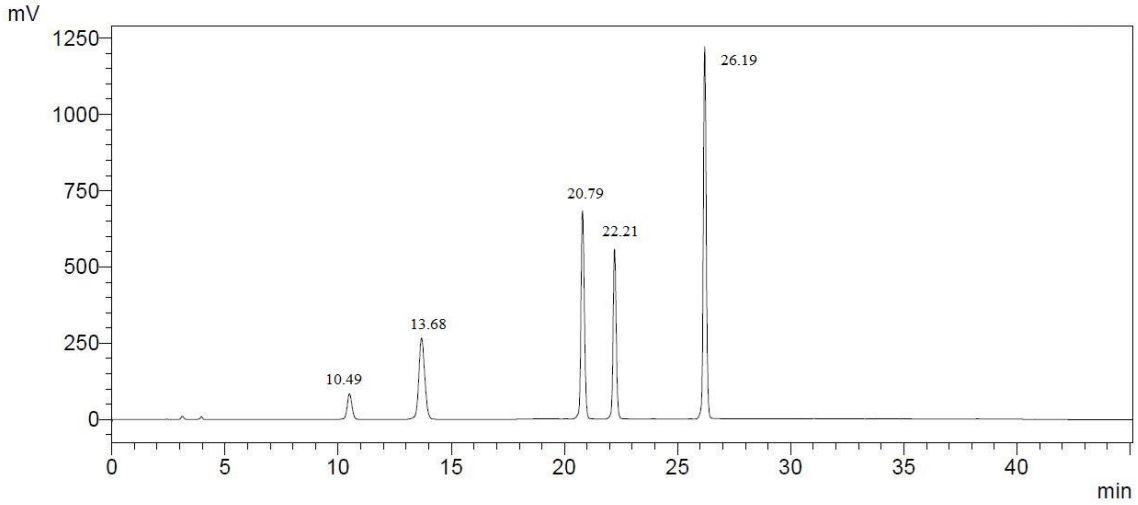


Figure A6: Standard curves at 500 ppm of mixed standards

Appendix B: Catechins Profile under Enzymatic assisted Extraction at Different Combinations of Time, Temperature and Enzyme Concentration

In this section, the results obtained for enzymatic and hot water extraction at different conditions have been presented in the following column graphs individually for each catechin types.

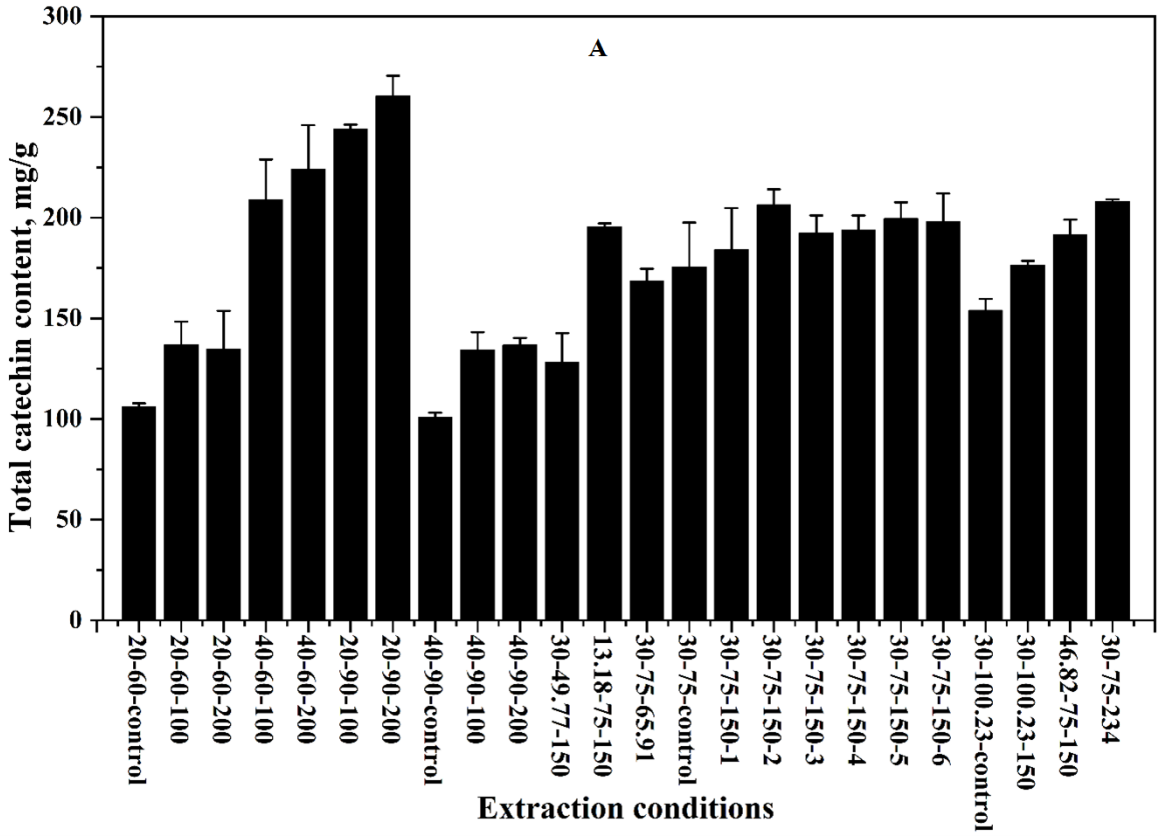


Figure B1: Total catechin content under enzymatic and hot water extraction

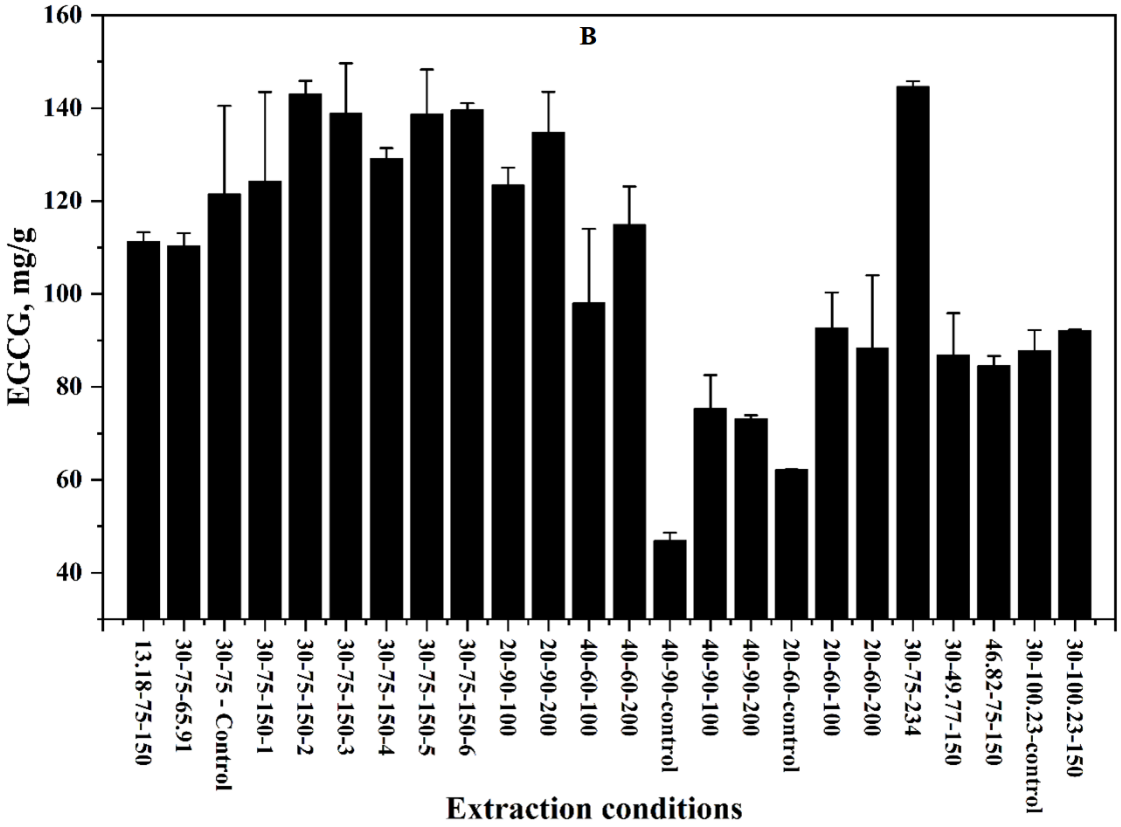


Figure B2: EGCG content under enzymatic and hot water extraction

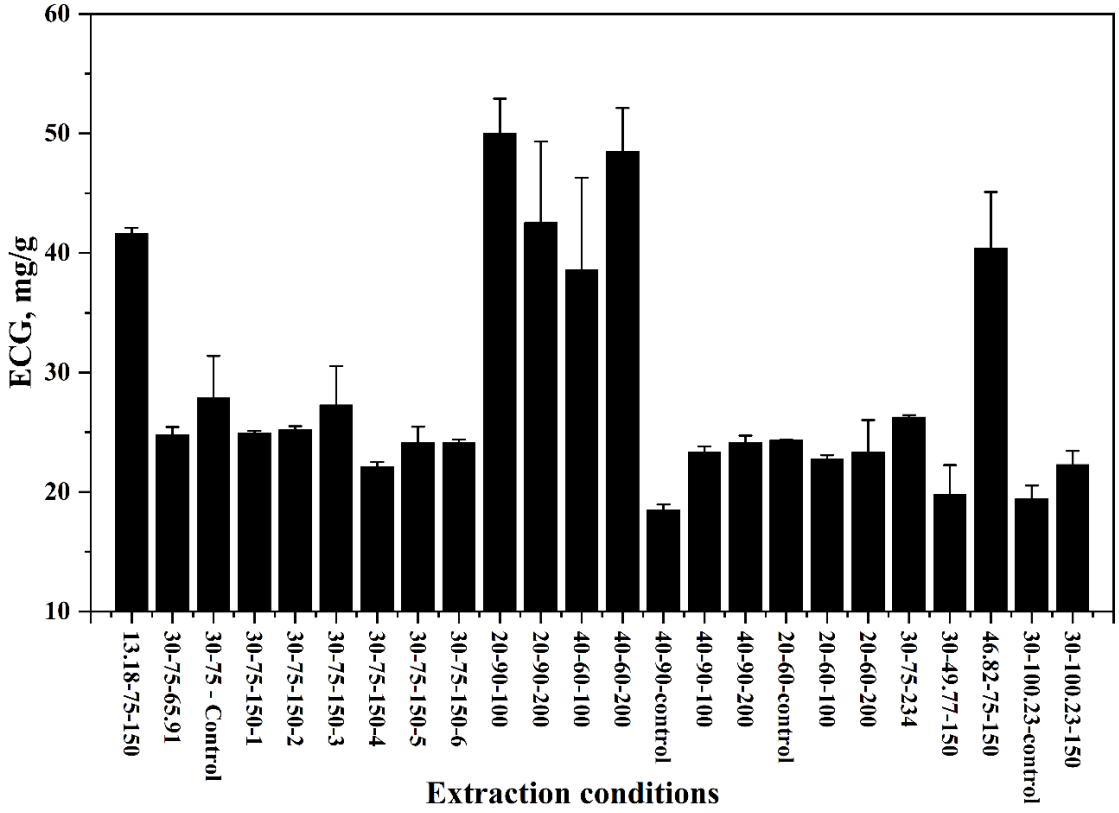


Figure B3: ECG content under enzymatic and hot water extraction

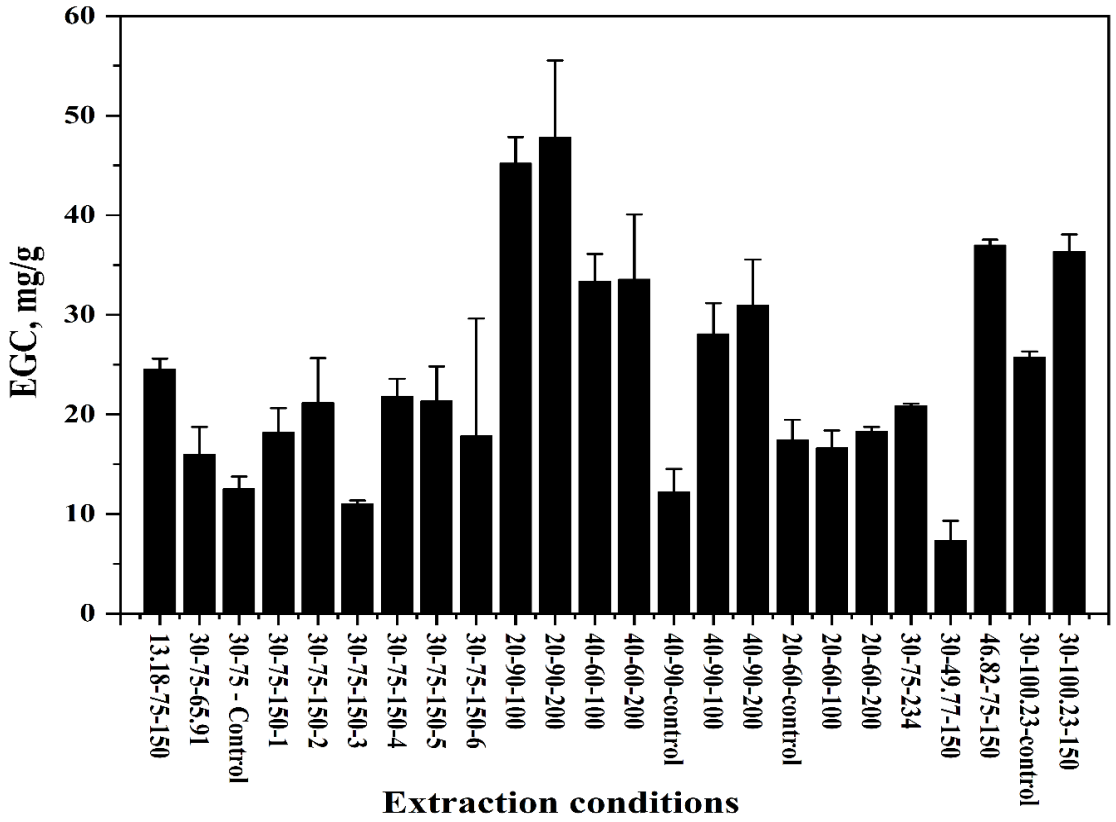


Figure B4: EGC content under enzymatic and hot water extraction

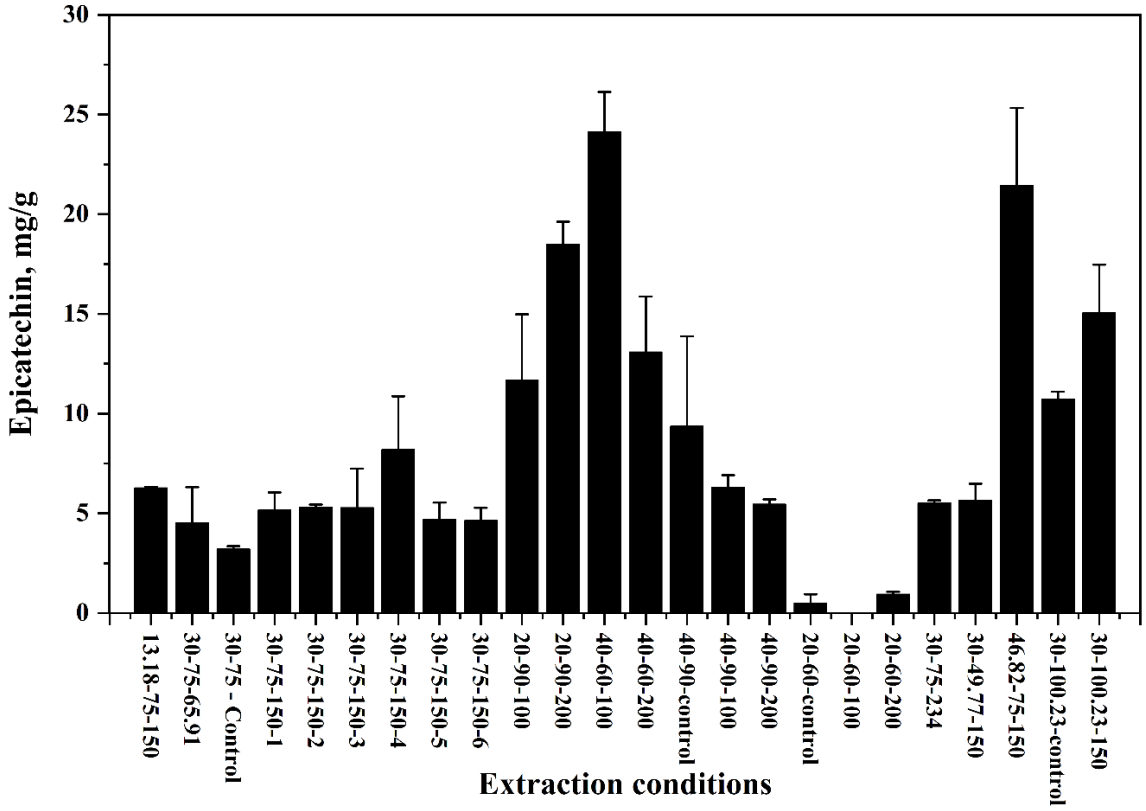


Figure B5: Epicatechin content under enzymatic and hot water extraction

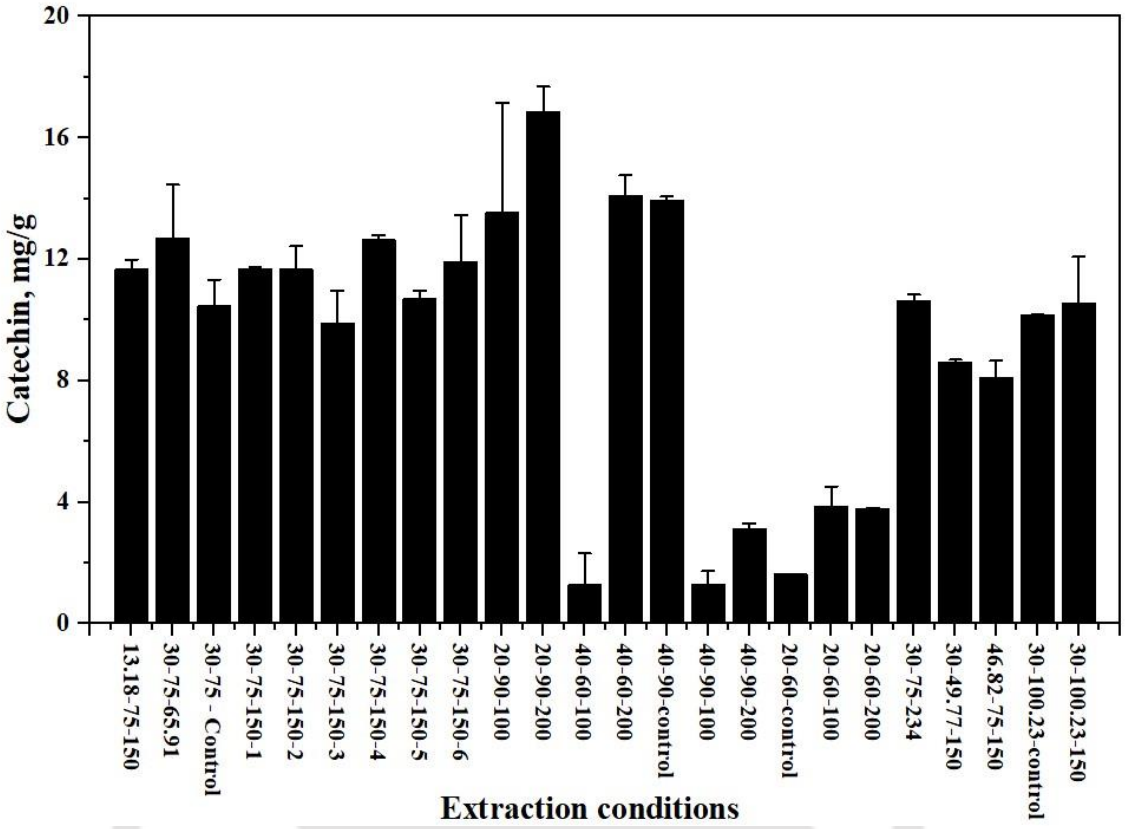
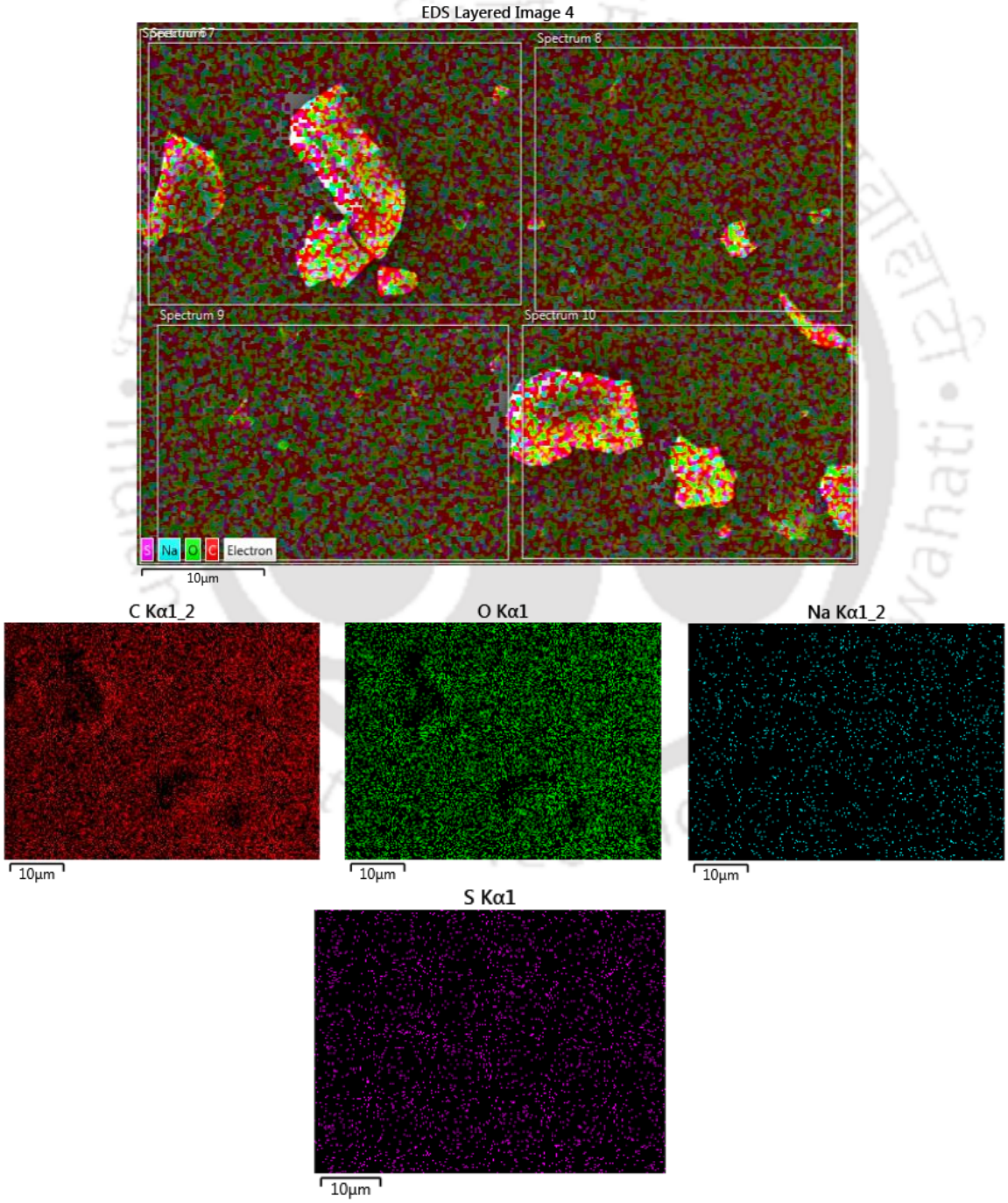


Figure B6: Catechin content under enzymatic and hot water extraction

Appendix C: FESEM EDS Analysis for the Encapsulated Catechin Extracts

In this section, the FESEM EDS analysis results for catechin extracts, starch nanoparticles, and encapsulated catechin extracts in 1:1, w/w and 1:2, w/w are presented.



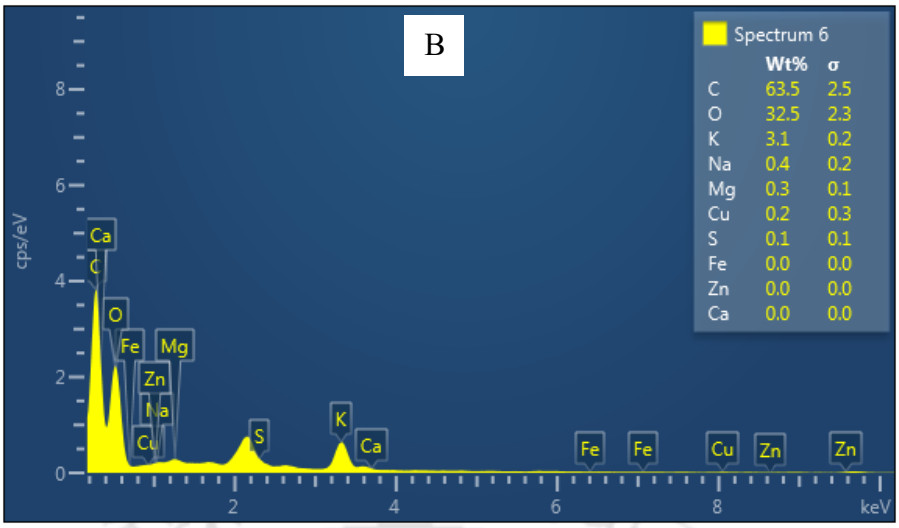
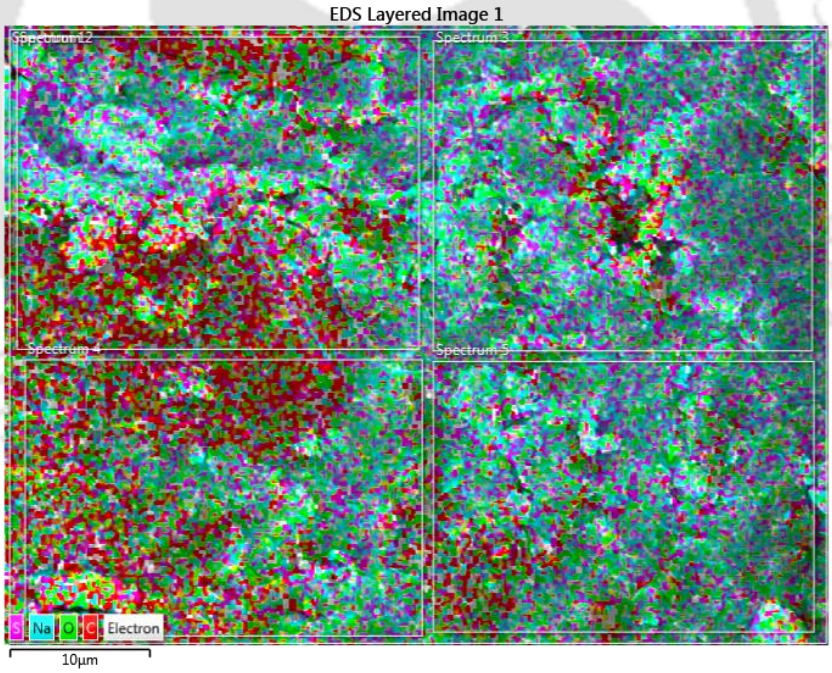


Figure C1: FESEM EDS report for catechin extracts, A) Map B) Weight % for C, O, K, Na, Mg, Cu, S, Fe, Zn, and Ca



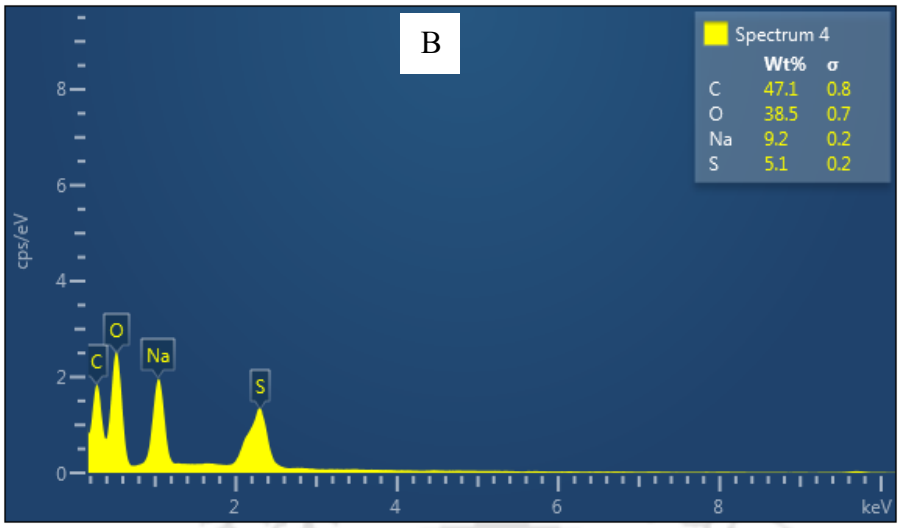
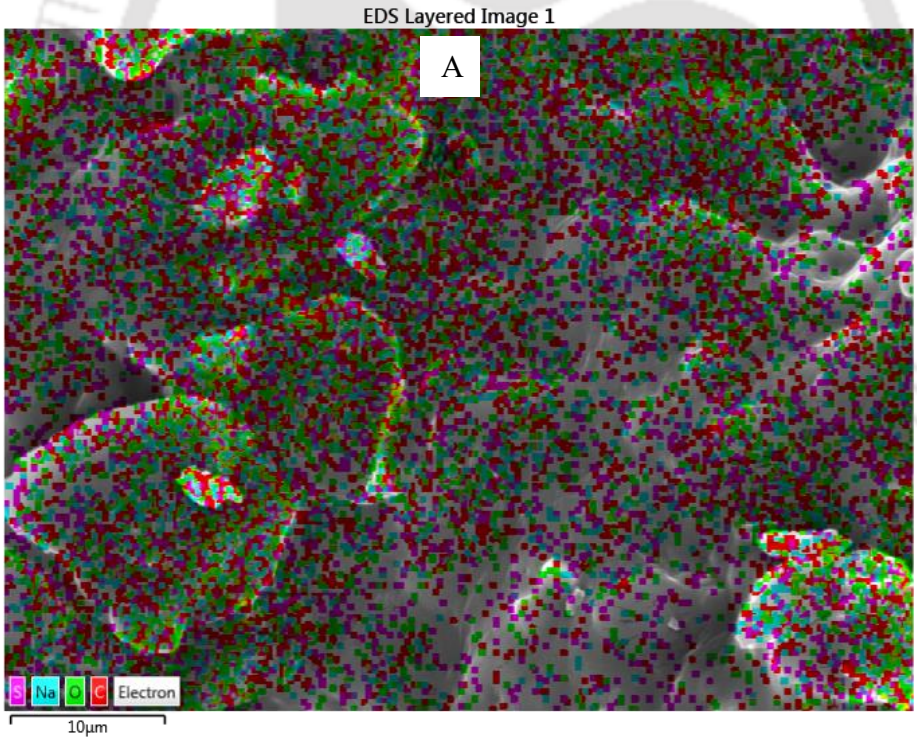


Figure C2: FESEM EDS report for starch nanoparticles A) Map B) Weight % for C, O, K, Na, Mg, Cu, S, Fe, Zn, and Ca



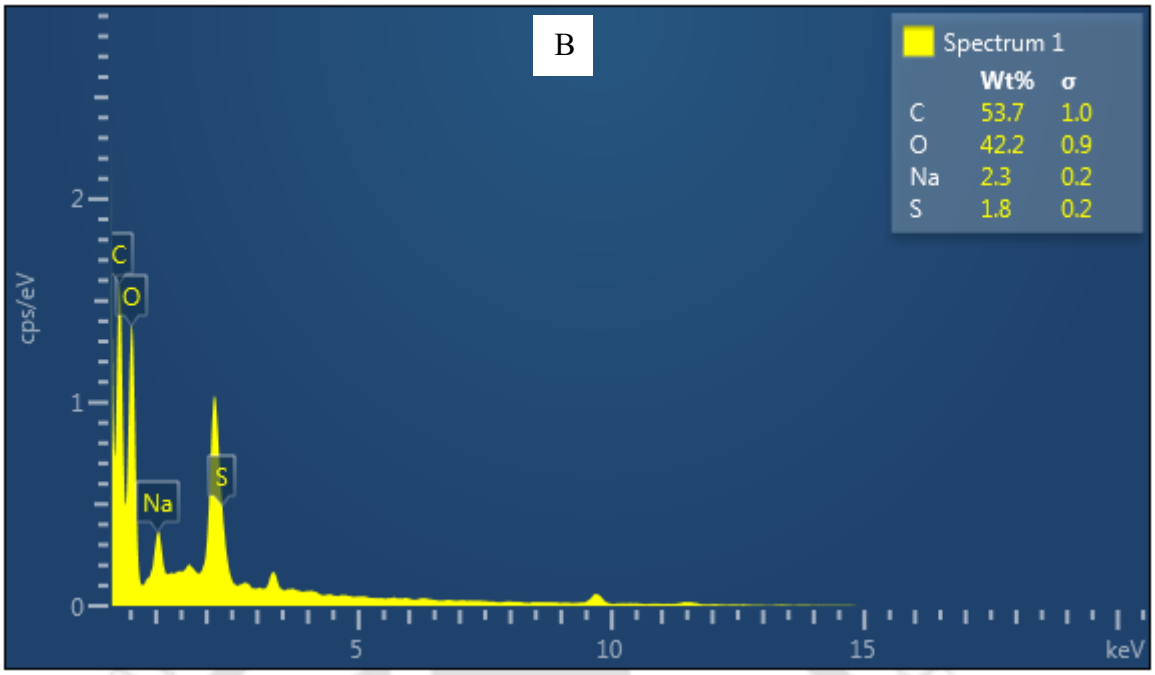
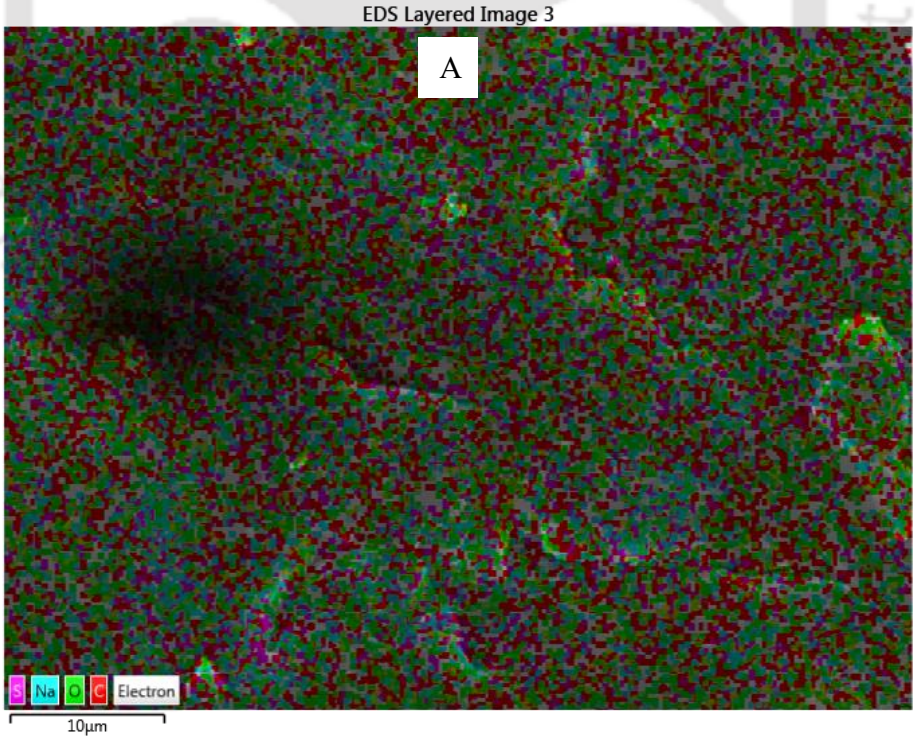


Figure C3: FESEM EDS report for CE:SNP, 1:1 w/w A) Map B) Weight % for C, O, K, Na, Mg, Cu, S, Fe, Zn, and Ca



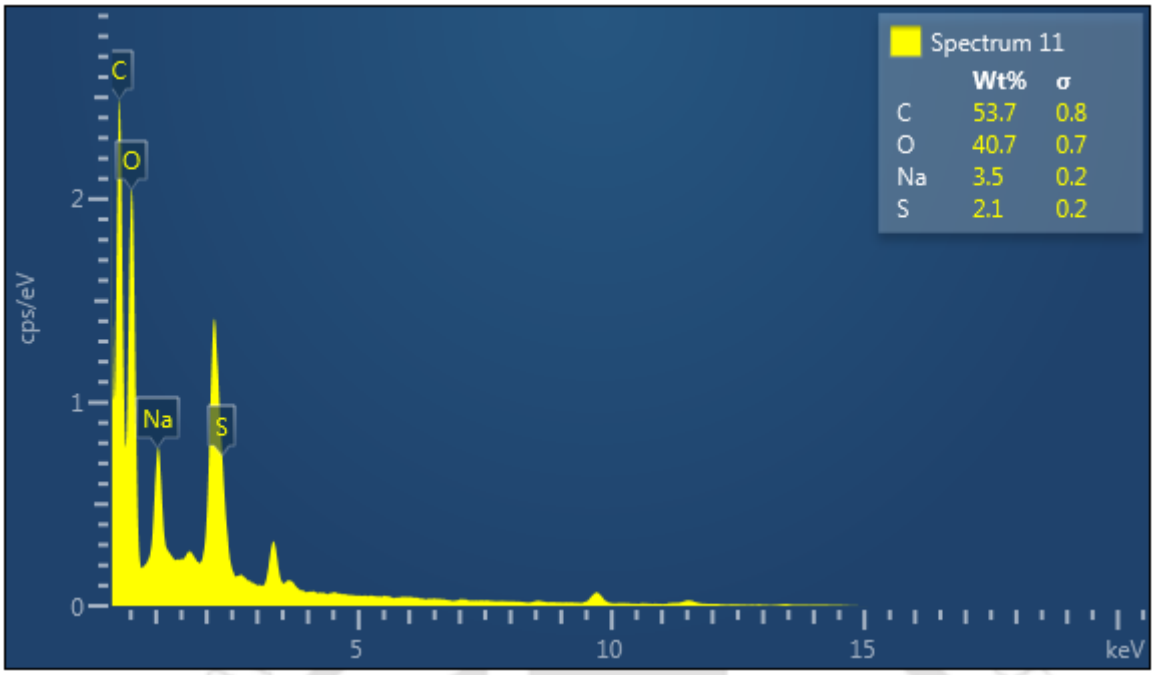


Figure C4: FESEM EDS report for CE:SNP, 1:2 w/w A) Map B) Weight % for C, O, K, Na, Mg, Cu, S, Fe, Zn, and Ca