EFFECT OF CHEMICAL MODIFICATION BY POLYPHENOLS ON THE FUNCTIONALITY OF CASEIN

THESIS SUBMITTED TO THE

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

(DEEMED UNIVERSITY)

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY

IN

DAIRY CHEMISTRY

BY

TANUSHREE R

B.Tech. (Dairy Technology)

DAIRY CHEMISTRY DIVISION

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE

(DEEMED UNIVERSITY)

KARNAL-132001(HARYANA), INDIA

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DAIRY CHEMISTRY

Approved by

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CERTIFICATE

This is to certify that the thesis entitled, **"Effect of chemical modification by polyphenols on the functionality of casein"** submitted by **Ms. Tanushree R** towards the partial fulfilment of the award of the degree of **MASTER OF TECHNOLOGY IN DAIRY CHEMISTRY** of the **ICAR-National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated: 23/09/2021

Dr. Richa Singh
MAJOR ADVISOR

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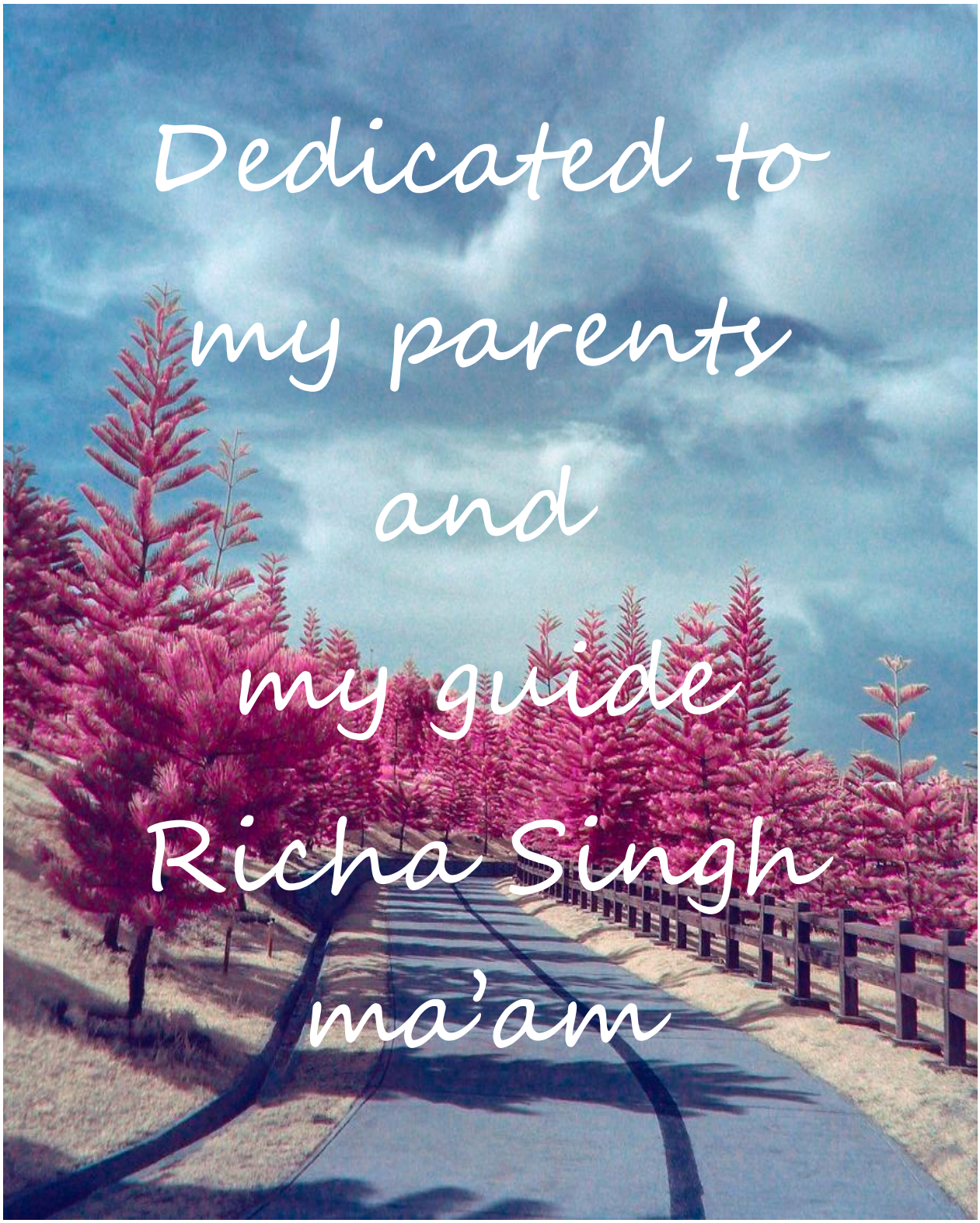
Dedicated to
my parents
and
my guide
Richa Singh
ma'am

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I would say, with all my heart and soul, that I was fortunate enough to study at one of the world's most prestigious institutes in my field, where I laid the groundwork for the rest of my career. This foundation, however, could not be built without the support of the pillars. I'd like to thank those who have helped me and my work get to where we are today.

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Date: 23/09/2021 **(*Tanushree R*)**

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List Of Abbreviations	
Abbreviated Form	**Full Form**
G	Free Energy Changes
H	Enthalpy Changes
S	Entropy Changes
C	Carbon
%	Percentage
LDL	Low Density Lipids
HDL	High Density Lipids
Asc^-	Ascorbate Radicals
°C	Degree Centigrade
EAI	Emulsifying Activity Index
ESI	Emulsion Stability Index
C	Catechin
EC	Epicatechin
EGC	Epigallocatechin
SC	Sodium Caseinate
LG	B-Lactoglobulin
LF	Lactoferrin
LA	A-Lactalbumin
BSA	Bovine Serum Albumin
CD	Circular Dichroism
DLS	Dynamic Light Scattering
FTIR	Fourier Transform Infrared
ITC	Isothermal Titration Calorimetry
NMR	Nuclear Magnetic Resonance
Nm	Nanometre
DSC	Differential Scanning Calorimetry
TGA	Thermo Gravimetric Analysis
SDS- PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
AFM	Atomic Force Microscopy
SEM	Scanning Electron Microscopy
CA	Caffeic Acid
FA	Ferulic Acid

NaCn	Sodium Caseinate
NaCn-CA	Sodium Caseinate - Caffeic Acid
NaCn-FA	Sodium Caseinate-Ferulic Acid
CN	Casein
5-CQA	5-O-Caffeoylquinic Acid
EA	Emulsifying Activity
ES	Emulsion Stability
FC	Foaming Capacity
FS	Foam Stability
AO	Antioxidant Activity
RSI	Radical Scavenging Index
WPI	Whey Protein Isolate
µg	Microgram
µl	Microlitre
et al.	et alii (and others)
g	Gram
i.e.,	Id Est (In Other Words)
M	Meter
mg	Milligram
Min.	Minute
Ml	Millilitre
Mm	Milli Molar
pH	Negative Log Of The Hydrogen Ion Concentration
Rpm	Rotations Per Minute
OH	Hydroxyl Group
A	Alpha
B	Beta
K	Kappa
&	Ampersand
BSA	Bovine Serum Albumin
H_2O_2	Hydrogen Peroxide
EGCG	Epigallocatechin Gallate
Min	Minute
EDC	1-Ethyl-3-(3-Dimethylaminopropyl) Carbodimide Hydrochloride
NHS	*N*-Hydroxy Succinimide
DPPH	2,2-Diphenyl-1-Picrylhydrazyl

ABSTRACT

Proteins and phenolic compounds are two types of food ingredients with distinct functionalities. Proteins are widely used in the food industry as emulsifiers, foaming agents, colloid stabilizers, biodegradable film-forming materials, microencapsulating agents etc. Phenolic–protein conjugates have been demonstrated to possess higher biological activities than proteins alone. Therefore, scope exist that these conjugates can serve as ingredient in formulation of functional foods. In the present study, the functional properties of sodium caseinate (NaCn) were evaluated after its conjugation with caffeic acid (CA) and ferulic acid (FA) using covalent (alkaline, free radical mediated grafting, and chemical coupling methods) and non-covalent (at different pH) approaches. The higher total polyphenol content was observed in non-covalent conjugates of NaCn-FA and NaCn-CA at pH 7; 21.24 ± 0.53 and 25.35 ± 0.92 mg/g of protein. In covalent conjugates, free radical grafting method showed higher total polyphenol content of 28.18 ± 0.49 and 38.69 ± 0.85 mg/g protein for NaCn-FA and NaCn-CA conjugates, respectively. The interactions of NaCn with CA and FA were examined using fluorescence, UV–visible and FTIR spectroscopy. The conjugation between protein and polyphenol was confirmed by decrease in intrinsic fluorescence after addition of polyphenol. The quenching of fluorescence intensity was more in covalent conjugates (>90%) than non-covalent conjugates (~85%). A Red shift of 40nm and 60nm was also observed in NaCn-FA and NaCn-CA conjugates, respectively in maximum wavelength in fluorescence spectra after conjugation. The UV-Vis spectra showed absorption at 270-280 nm and 310-330 nm in non-covalent conjugates and covalent conjugates, with significant absorption in the later approach. In FTIR spectral analysis, no change in the spectra was observed for non-covalent conjugates at all pH values. Whereas, in covalent conjugates shift in amide I and II regions were noticed. Upon conjugation of polyphenol with sodium caseinate, increase in particle size was observed. The increase was more in covalent conjugates. The colloidal stability of the conjugates was improved as there was increase in the negative zeta potential of the conjugates. There was no significant effect of non-covalent (pH 7) conjugation of polyphenol with NaCn, however decrease in solubility was observed in covalent conjugates. Emulsifying activity, foaming capacity, foam stability and radical scavenging activity were found to be higher after conjugation irrespective of type of polyphenol and method of conjugation. Increase in emulsion stability was also observed with non-covalent and alkaline covalent conjugates. Free radical grafting covalent conjugates have shown decrease in emulsion stability. The results have shown the possibility of using CA and FA in improving the functional properties of NaCn. The functionality of the prepared conjugates was found to be affected by type of polyphenol and method of conjugation. Non-covalent conjugates (pH 7) of both CA and FA with NaCn have shown better functional properties than covalent conjugates. Among CA and FA, the conjugation of CA with NaCn has higher functionality. The prepared NaCn-polyphenol conjugates with enhanced functional properties can be used as novel food additives in food products.

सारांश

प्रोटीन और फेनोलिक यौगिक दो प्रकार के खाद्य पदार्थ हैं जिनमें अलग-अलग कार्य होते हैं। खाद्य उद्योग में पायसीकारी, फोमिंग एजेंट, कोलाइड स्टेबलाइजर्स, बायोडिग्रेडेबल फिल्म बनाने वाली सामग्री, माइक्रोएन्कैप्सुलेटिंग एजेंट आदि के रूप में प्रोटीन का व्यापक रूप से उपयोग किया जाता है। फेनोलिक-प्रोटीन संयुग्मों को अकेले प्रोटीन की तुलना में उच्च जैविक गतिविधियों के लिए प्रदर्शित किया गया है।इसलिए, गुंजाइश मौजूद है कि ये संयुग्म कार्यात्मक खाद्य पदार्थों के निर्माण में घटक के रूप में काम कर सकते हैं। वर्तमान अध्ययन में, सोडियम कैसिनेट (NaCn) के कार्यात्मक गुणों का मूल्यांकन कैफिक एसिड (CA) और फेरुलिक एसिड (FA) के साथ सहसंयोजक (क्षारीय, मुक्त कट्टरपंथी मध्यस्थता ग्राफ्टिंग, और रासायनिक युग्मन विधियों) और गैर-सहसंयोजक का उपयोग करके किया गया था। (विभिन्न पीएच पर) दृष्टिकोण। पीएच 7 पर NaCn-FA और NaCn-CA के गैर-सहसंयोजक संयुग्मों में उच्च कुल पॉलीफेनोल सामग्री देखी गई; २१.२४ ± ०.५३ और २५.३५ ± ०.९२ मिलीग्राम/जी प्रोटीन। सहसंयोजक संयुग्मों में, मुक्त मूलक ग्राफ्टिंग विधि ने क्रमशः NaCn-FA और NaCn-CA संयुग्मों के लिए 28.18 ± 0.49 और 38.69 ± 0.85 mg/g प्रोटीन की उच्च कुल पॉलीफेनोल सामग्री दिखाई। प्रतिदीप्ति, यूवी-दृश्यमान और एफटीआईआर स्पेक्ट्रोस्कोपी का उपयोग करके सीए और एफए के साथ NaCn की बातचीत की जांच की गई। प्रोटीन और पॉलीफेनोल के बीच संयुग्मन की पुष्टि पॉलीफेनोल के अतिरिक्त के बाद आंतरिक प्रतिदीप्ति में कमी से हुई थी। प्रतिदीप्ति तीव्रता का शमन गैर-सहसंयोजक संयुग्मों (~ 85%) की तुलना में सहसंयोजक संयुग्मों (> 90%) में अधिक था। संयुग्मन के बाद प्रतिदीप्ति स्पेक्ट्रा में अधिकतम तरंग दैर्ध्य में क्रमशः NaCn-FA और NaCn-CA संयुग्मों में 40nm और 60nm की एक लाल पारी देखी गई। यूवी-विज़ स्पेक्ट्रा ने गैर-सहसंयोजक संयुग्मों और सहसंयोजक संयुग्मों में 270-280 एनएम और 310-330 एनएम पर अवशोषण दिखाया, बाद के दृष्टिकोण में महत्वपूर्ण अवशोषण के साथ। एफटीआईआर वर्णक्रमीय विश्लेषण में, सभी पीएच मानों पर गैर-सहसंयोजक संयुग्मों के लिए स्पेक्ट्रा में कोई परिवर्तन नहीं देखा गया था। जबकि, सहसंयोजी संयुग्मों में एमाइड I और II क्षेत्रों में बदलाव देखा गया। सोडियम कैसिनेट के साथ पॉलीफेनोल के संयुग्मन पर, कण आकार में वृद्धि देखी गई। सहसंयोजक संयुग्मों में वृद्धि अधिक थी। संयुग्मों की नकारात्मक जेट क्षमता में वृद्धि के कारण संयुग्मों की कोलाइडल स्थिरता में सुधार हुआ था। NaCn के साथ पॉलीफेनोल के गैर-सहसंयोजक (पीएच 7) संयुग्मन का कोई महत्वपूर्ण प्रभाव नहीं था, हालांकि सहसंयोजक संयुग्मों में घुलनशीलता में कमी देखी गई थी। पॉलीफेनोल के प्रकार और संयुग्मन की विधि के बावजूद पायसीकारी गतिविधि, फोमिंग क्षमता, फोम स्थिरता और कट्टरपंथी मैला ढोने की गतिविधि संयुग्मन के बाद अधिक पाई गई। गैर-सहसंयोजक और क्षारीय सहसंयोजक संयुग्मों के साथ पायस स्थिरता में वृद्धि भी देखी गई। मुक्त मूलक ग्राफ्टिंग सहसंयोजक संयुग्मों ने पायस स्थिरता में कमी दिखाई है। परिणामों ने NaCn के कार्यात्मक गुणों में सुधार के लिए CA और FA के उपयोग की संभावना को दिखाया है। तैयार संयुग्मों की कार्यक्षमता पॉलीफेनोल के प्रकार और संयुग्मन की विधि से प्रभावित पाई गई। NaCn के साथ CA और FA दोनों के गैर-सहसंयोजक संयुग्म (pH 7) ने सहसंयोजक संयुग्मों की तुलना में बेहतर कार्यात्मक गुण दिखाए हैं। CA और FA के बीच, CA के NaCn के साथ संयुग्मन में उच्च कार्यक्षमता है। तैयार किए गए NaCn-पॉलीफेनोल संयुग्मित कार्यात्मक गुणों के साथ खाद्य उत्पादों में उपन्यास खाद्य योजक के रूप में उपयोग किया जा सकता है |

CHAPTER 1

INTRODUCTION

INTRODUCTION

Milk proteins are divided into two classes which are heterogeneous in nature. Being major proteins, caseins constitute to around 80% of the total protein and they precipitate at pH 4.7. The fraction which remains soluble at pH 4.7 are whey or serum proteins and constitute remaining 20% of the total protein content. Caseins exists as casein micelles in the milk and are classified into different fractions as α_{S1}-, α_{S2}-, β- and k-casein fractions (Huppertz *et al*., 2018). These fractions differ in the composition, number of amino-acids, molecular weight, size but have similar net negative charge. Polyphenols are natural phytochemicals which are synthesized by plants as secondary metabolites. They abundantly present in different fruits, vegetables, tea, coffee, cocoa etc and also in some alcoholic beverages such as beer and wine (Ramos, 2007). Structurally these compounds have an aromatic ring to which one or more hydroxyl groups are being attached and are considered as natural antioxidants. They are also known for other biological functions like antibacterial, anti-inflammatory and anti-cancerous activities because of which they have gained much attention in human diet. In recent years, phenolic compounds have received a lot of attention because of their antioxidant, anti-inflammatory, anti-mutagenic, and anti-clotting properties, which have been linked to a lower risk of cardiovascular disease and cancer development (Ostertag *et al.,* 2010). Fruit juices and extracts have been suggested as potential functional ingredients in the food industry, including the dairy sector (Coisson *et al.,* 2005). However, to manage functionality and acceptability of dairy products added with polyphenol is a difficult task. However, in recent years it has been reported polyphenol can positively influence the functional properties of milk proteins. Therefore, different ways have been used to incorporate polyphenols into foods, such as encapsulation of polyphenols, molecular complexation, etc. When high-quality nutritional proteins are combined with polyphenols, they produce multifunctional ingredients that are more accessible, protected, and bioavailable. Casein, being a member of unstructured protein family, have unfolded structure in their native state. High hydrophobicity of caseins increases their tendency to associate with ligands of other proteins and hydrophobic moieties (Yuksel *et al*., 2010). This is due to low intrinsic hydrophobicity and high net charge of caseins. So, the combination of these characteristics makes caseins a model protein to carry polyphenol and this

"casein-polyphenol conjugate" has recently gained attention as an excellent vehicle for delivering polyphenols into food products. At the same time, conjugation of phenolic chemicals with proteins can also lead to significant structural and physicochemical changes in proteins that may enhance of protein's potential applications.

There can be non-covalent (Hydrogen bonding, electrostatic interactions) or covalent interaction between proteins and polyphenols. Several studies have revealed that the interactions between milk proteins and phenolic compounds can modify protein structure, stability, digestibility and functional properties, antioxidant properties, and phenolic bioavailability (Sęczyk *et al*., 2019). However, the impact on functionality of proteins and polyphenols is also determined by environment parameters such as temperature and pH, as well as the conformation or type of proteins and polyphenols used.

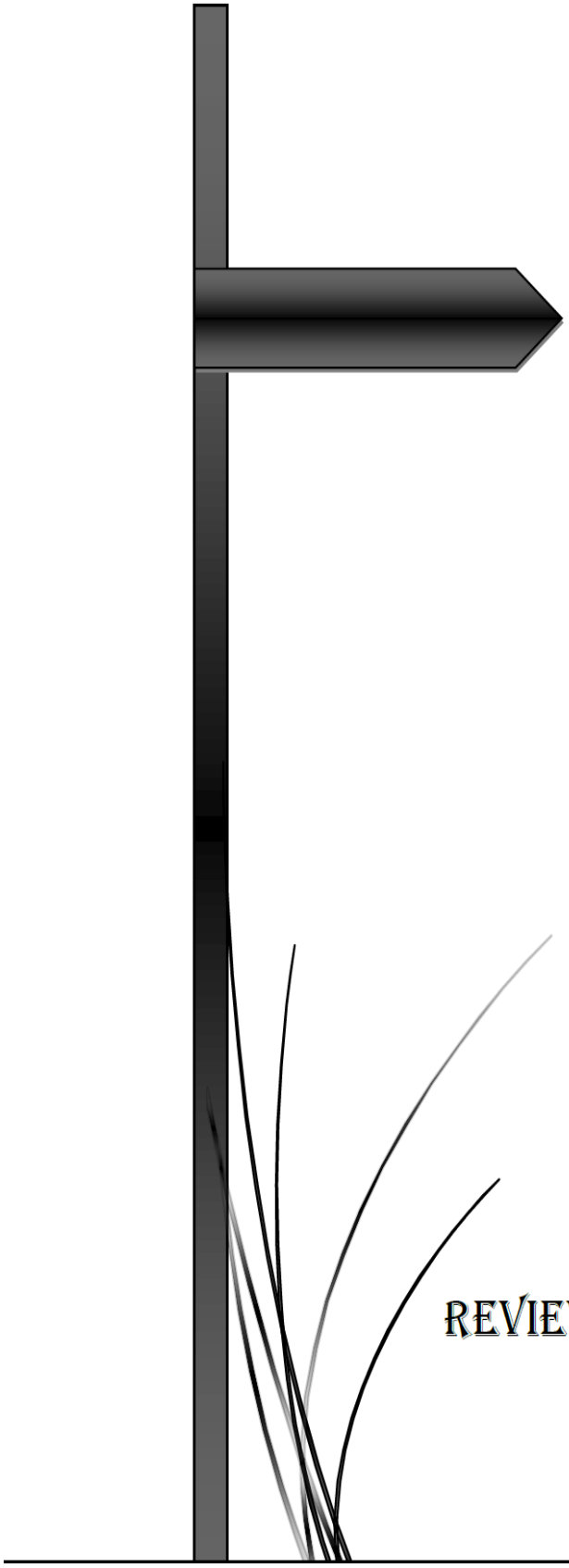
Therefore, casein-polyphenol conjugates with enhanced functional qualities can be used as innovative food additives. Since non-covalent phenolic–protein conjugates are easier to synthesise than covalently coupled phenolic–protein conjugates, the majority of past research has focused on non-covalent interactions. However, covalently linked conjugates, on the other hand, are expected to last longer throughout processing than non-covalent conjugates, due to the fact that non-covalent bonds are weaker than covalent bonds. Hence, the present study aimed to investigate how different procedures for creating casein-polyphenol conjugates alter the functional properties of caseins. Further, these conjugates will be characterized for structural modifications caused by polyphenol. The study was formulated with following two objectives:

Objective 1.

To modify functional properties of casein by its conjugation with polyphenols using covalent and non-covalent approach

Objective 2.

To characterize the prepared casein-polyphenol conjugates



CHAPTER 2

REVIEW OF LITERATURE

2.1 Casein

Milk contains a variety of protein types, with casein accounting for roughly 80% of the total which precipitate at pH 4.6, 30° C, and is organised as micelles with diameters ranging from 50 to 500 nm (Elzoghby *et al*., 2011; Bhat *et al*., 2016). Caseins are heterogeneous in nature and consists of four protein fractions: αS1 -, αS2 -, β -, and k casein (molar ratios of 4:1:4:1), which have different molecular weights (Fox, 2003; Schrieke *et al*., 1985; Singh & Ye, 2009; Dickinson & van Vliet, 2003). The composition, number of amino acids, molecular weight, degree of unfolding, and size of these fractions differ, but they all have a net negative charge. Casein is an unstructured protein that has an unfolded structure in its natural state. Caseins with a high hydrophobicity are more likely to associate with the ligands of other proteins (Yuksel *et al.,* 2010). Two cysteines form intermolecular disulphide bridges in casein, which is a glycoprotein. Caseins have flexible external structures that are described as random, unlike most proteins that show different secondary and tertiary conformations (Sirocic, 2017).

2.2 Polyphenols

Polyphenols, being heterogeneous class of natural antioxidants, are secondary metabolites synthesized by plants basically through Pentose phosphate, Shikimate or Phenyl propanoid pathway (Balasundram *et al*., 2006; Liu *et al*., 2018). Structurally they have an aromatic ring to which one or more hydroxyl groups are being attached. They are present abundantly in many fruits like grapes, strawberry, apple, blue berries, raspberries, olives and vegetables like red onions, spinach and few herbs like cocoa, tea and coffee. Polyphenols are also present in some alcoholic beverages like beer and wine. (Bordenave *et al*., 2014). The term ‘polyphenol’ is in use since 1894 (Webster, 2019). More than 8000 phenolic structures are currently known and out of them nearly 4000 have been identified (Perez-Jimenez *et al*., 2010). In spite of characterizing polyphenols as compounds having phenolic structure features, they are highly diverse and have many sub groups. Classification of polyphenols is done based on chemical structure, source of origin and biological function.

2.2.1 Polyphenol classification

Majorly based on chemical structure, polyphenols are divided into 2 groups; Flavonoids and Non-flavonoids. (Kopustinskiene *et al*., 2020).

Flavonoids: These compounds structurally consist of C6–C3–C6 skeleton. They have 15 carbon atoms along with two aromatic rings which are connected by a 3-Carbon bridge. They are subdivided into flavones, flavanones, flavan-3-ols, flavonols, flavanols, isoflavones, and anthocyanidins (Panche et al., 2016)

Non-flavonoids: Non-flavonoids basically have one aromatic ring in their structure. They include phenolic acids (further divided into benzoic acid derivatives having C6-C1 structure and derivatives of hydroxyl cinnamic acid having C6-C3 structure), stilbenes (have two phenyl moieties that are linked together by a 2-C methylene group), and lignans (consisting of two C6-C3 propyl benzene units which are linked between the β-position in C8 of the propane side chains).

2.2.2 Biological properties of polyphenols: (Lecour and Lamont, 2010)

- They promote the anti-inflammatory effects.
- They are excellent anti-oxidants.
- They possess anti-microbial and anti-carcinogenic properties.
- They are known source of anti-infective agents which are against antibiotic resistant human pathogens.
- They are potential anti-thrombotic agents.
- They are potent inhibitors of low-density lipids (LDL) oxidation and increases high-density lipids (HDL).
- They are also used to treat few diseases like Parkinson’s disease, Alzheimer’s disease and cardiovascular related diseases.
- They fight against cancer and aging.

2.3 Challenges with polyphenol addition to foods

It's not as simple as it sounds to add polyphenols directly to food. Polyphenol addition to food presents a considerable challenge due to their ill-defined structures and absence of commercial standards. Polyphenol addition to foods is limited and faces

several obstacles due to a lack of data on their levels in sources, meals, food-derived products, and dietary supplements. When exposed to light, heat, or different pH levels, polyphenols are prone to chemical destruction. They should therefore be avoided in foods that are processed at high temperatures or exposed to light or oxygen. In addition, certain polyphenols have limited solubility and astringency or harsh flavour, which means they can't be used in a lot of food. If you increase the amount of polyphenols in your diet it may lead to negative sensory features of the food, which could result in a decrease in their consumption. The bitterness and astringency of polyphenol added foods can be modified by adding debittering agents, masking agents or developing debittering processes, which hinders the unacceptable flavours and improves palatability and consumer acceptance. To cover bitterness and astringency, flavourings could be combined with a small amount of natural sugar. Alternatively, according to (Szejtli and Szente, 2005), adding a cyclodextrin causes the bitterness molecules to form an inclusion complex, which prevents them from reacting with the taste buds, resulting in no bitterness being sensed. Use of reducing agents is reported to prevent discoloration caused by polyphenols (Saltveit, 2001). The interaction of polyphenols with amino acid residues can produce a variety of coloured complexes ranging from dark brown to yellow to green. This could be minimised by establishing more thiol group connections with the oxidised polyphenols rather than amino group interactions (Li *et al*., 2016). Adding reducing agents like ascorbic acid or eliminating unbound quinone prevents black or dark brown discoloration (Saltveit, 2001). Excipient substances, such as digestible lipids, might improve polyphenol bioaccessibility by generating mixed micelles in the aqueous phase that can solubilize and transport them (Pandita *et al*., 2014). Molecular complexation (Bourvellec and Renard, 2012) is gaining popularity as a way to enhance the functionality of polyphenol in food matrix by physically (complexation) or chemically (conjugation) linking it to other molecules.

2.4 Casein-polyphenol conjugates as a strategy to deliver polyphenols into food

Molecular modification by conjugation or polymerization is a promising way of synthesising novel materials or changing biomolecules with optimal physical and chemical properties. A certain food component's qualities are maximised, or new

properties are created for specialised needs (Spizzirri *et al*., 2009). Natural food ingredients and conjugated molecules could be combined to improve the nutritional, sensory, and physiochemical properties of foods (Czubinski & Dwiecki, 2017).

There is literature reported regarding interaction between proteins and polyphenols in improving the quality of some foods. Protein-EGCG conjugates, for example, have been shown to have higher anti-oxidant action than unmodified proteins (Gu *et al*., 2017a; Yin *et al*., 2014; You *et al*., 2014). The interaction between proteins and polyphenols could improve the thermal stability and mechanical properties of gelatin gel (Maqsood *et al*., 2013). In addition, it may improve surface-hydrophobicity of the modified proteins and increase the emulsifying properties of native proteins by conjugating proteins with non-polar polyphenols (Ozdal *et al*., 2013). Protein-polyphenol conjugates have been proposed as effective emulsifiers for the prevention of oxidisation in emulsion-driven foods and to locate and act on an oil-water interface (Fan *et al*., 2018b; Feng *et al*., 2018). An oxidation is the main process of protein-polyphenol conjugation, where molecular oxygen oxidises the hydroxyl group of polyphenols to quinone (Prodpran *et al.,* 2012). Casein- polyphenol conjugates can be utilised to improve casein's functional qualities as well as to make functional meals.

The interactions between phenolic chemicals and proteins can take two forms: non-covalent and covalent. Because they rely on hydrophobic, van der Waals, hydrogen binding, and ionic interactions, non-covalent interactions are weaker than covalent interactions. Non-covalent interactions are also reversible to a large extent. In most cases, however, covalent interactions are irreversible (Czubinski and Dwiecki, 2017). The interactions between proteins and different flavonoids are mostly reported to be dominated by non-covalent interactions such as hydrogen bonding, hydrophobic interactions, and van der Waals attractions. The predominant interaction type between α-lactalbumin and various polyphenols such as genistein, kaempferol, trans-resveratrol, and curcumin has been reported to be H-bond formation (Mohammadi & Moeeni, 2015). (Gallo *et al.,* 2013) demonstrated the creation of covalent bonds between whey proteins and cocoa polyphenols via the free SH groups of protein.

Predominantly, non-covalent interaction forms between polyphenol and protein on mixing together; however, for covalent bond formation different methods like alkaline,

chemical coupling, free radical grafting and enzymatic methods have been reported in literature.

The alkaline approach is a quick and easy way to make phenolic–protein conjugates. The alkaline reaction is typically performed by changing the pH of the reaction solution to 9.0 using NaOH in ambient air. The oxidation of phenolic compounds is the backbone of this approach (Rohn *et al*., 2004). Using the alkaline approach (Wei *et al*.,2015) prepared epigallocatechin-3-gallate (EGCG)–protein conjugates using bovine milk proteins like α-lactalbumin, β-lactoglobulin, lactoferrin and sodium caseinate. With free exposure to air, EGCG is oxidised to its equivalent quinone in an alkaline environment. Then, before the contact with the protein, EGCG dimerizes. Finally, the extremely reactive EGCG dimer quinones can react with nucleophilic side chains of proteins (such as lysine, cysteine, methionine, and tryptophan) (You *et al.,* 2014). According to (Wu *et al*., 2018), EGCG may serve a bridging role in initiating a cross-linking process of β-lactoglobulin after EGCG conjugation, resulting in the development of β-lactoglobulin dimerization. The presence of catechol moieties (ortho-hydroxyl groups) on flavonoids' ring B is required for covalent binding with proteins, whereas catechol moieties on flavonoids' ring A are less reactive and thus unnecessary for grafting (Rawel *et al*., 2003). (Rawel *et al*., 2003) also claimed that quercetin and rutin can bind to the lysine, tryptophan, and cysteine residues in protein. Quercetin was more reactive than rutin when conjugated with protein, demonstrating that the rhamnosylglucoside at the 3-O position has a significant impact on quercetin reactivity.

Another approach for the manufacture of phenolic–protein conjugates is the redox pair induced grafting which involve ascorbic acid and hydrogen peroxide (H_2O_2). Ascorbate radicals (Asc^-) produced by the reaction between ascorbic acid and the redox pair H_2O_2 at pH 6 can attack sensitive residues on the side chains of proteins, resulting in the formation of new macro radical species on the amino-acidic structure, according to (Liu *et al*., 2018; Rai *et al*., 2018). Through covalent linkages, these macro radicals can further conjugate with phenolic compounds (Liu *et al.,* 2018).

When compared to alkaline and free radical mediated grafting methods, the use of enzyme catalysed methods for the synthesis of phenolic–protein conjugates are very limited. Polyphenol oxidases (such as laccase and tyrosinase) are enzymes that

catalyse the conversion of phenolic compounds to electrophilic quinones. Because quinones are freely diffusible, they can undergo non-enzymatic reactions with the nucleophilic amino groups of proteins (Chung *et al.,* 1997). This suggests that the enzyme-catalyzed method's synthetic mechanism is very similar to the alkaline methods. The best reaction conditions are usually determined by the type of enzyme used. At pH 6.0–7.5, the catalysed reaction is frequently carried out.

Phenolic compounds can be easily conjugated with proteins by chemical coupling reagents. Glutaraldehyde, a linear 5-carbon di aldehyde, is more efficient than other aldehydes in generating thermally and chemically stable cross-linkings. Glutaraldehyde can react with nucleophilic side chains of proteins, such as amine, thiol, phenol and imidazole. (Wu *et al.* 2009) successfully synthesize EGCG–collagen conjugate through glutaraldehyde cross-linking. In another study, EGCG–gelatin conjugate was prepared by using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride and *N*-methyl morpholine as the cross-linking reagents in aqueous solution. To conjugate the carboxyl groups of chlorogenic acid with the amino groups of gelatin, (Fu *et al*., 2017) used 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) as coupling reagents. (El-Maksoud *et al*., 2018) reported that EDC/NHS coupling is used to make caffeic acid-β-lactoglobulin conjugates, and the reaction pH has a big impact on conjugation efficiency. Because of the high activity and stability of EDC at pH 6, pH 6 is preferred for binding the maximum caffeic acid units to β-lactoglobulin.

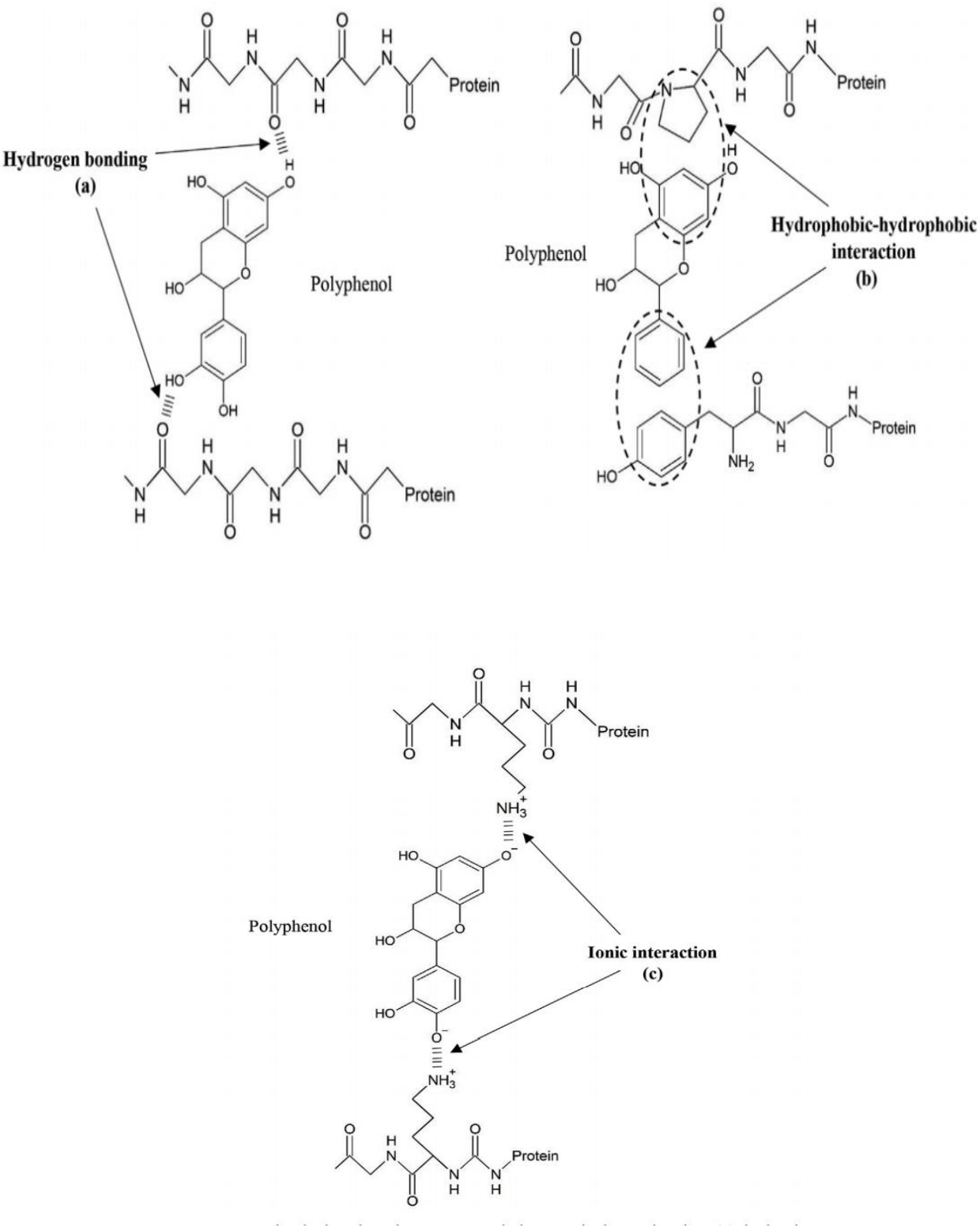


Fig. 2.1 Non-covalent conjugation of proteins and polyphenols and protein cross-linking via a) Hydrogen Bonding, b) Hydrophobic-Hydrophobic Bonding, c) Ionic Interaction.

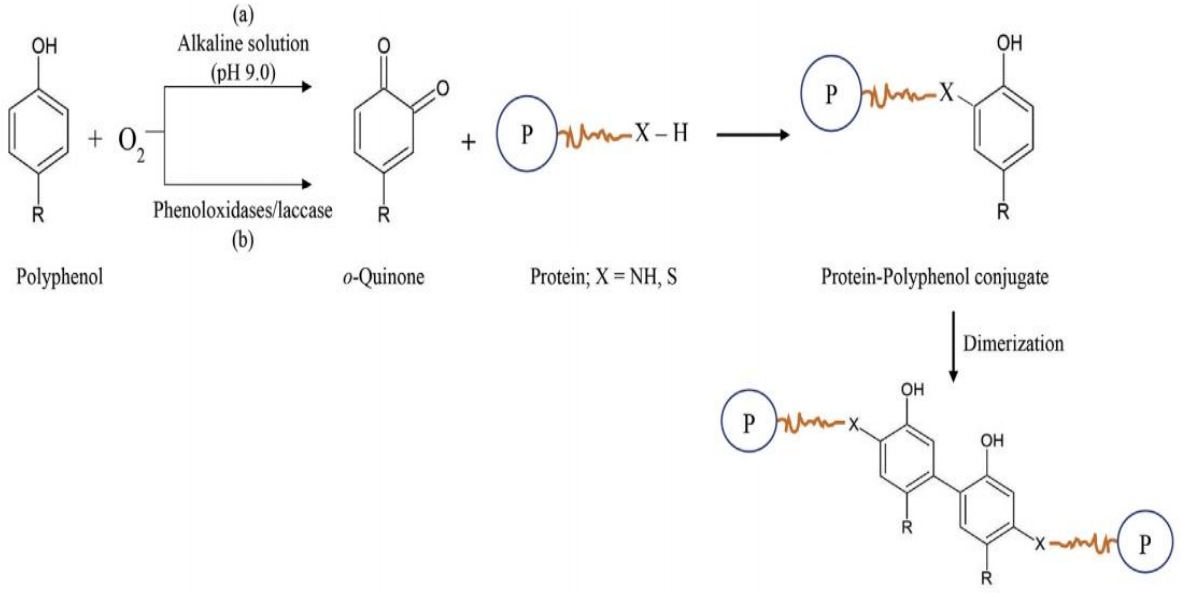


Fig.2. 2 Covalent conjugation of protein and polyphenol and cross linking via a) Alkaline method and b) Enzymatic method.

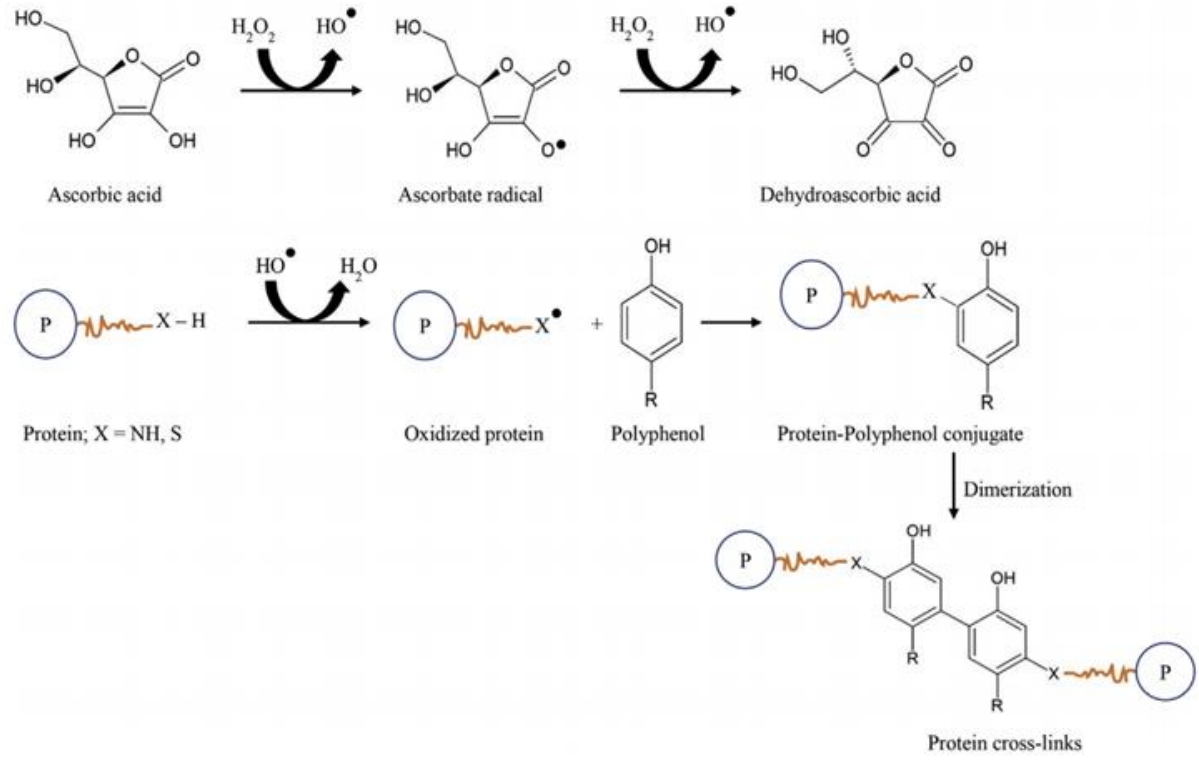


Fig. 2.3 Covalent conjugations of protein and polyphenol through Free Radical Grafting method.

2.5 Effect of the protein-polyphenol conjugate on the functionality of casein

Addition of polyphenols with casein cause significant changes in protein functionality. However, these changes may be negative or positive depending upon the type of polyphenol and the conditions in which interaction has happened between protein and polyphenols. In further sections, the effect of polyphenol addition on functionality of casein is discussed:

2.5.1 Solubility

Depending on the nature of the system, conjugating a polyphenol with a protein can either increase or decrease a protein's water solubility. The electrical characteristics of proteins, specifically the isoelectric point, are affected by covalent attachment of charged polyphenols, causing pH-solubility profiles to fluctuate according to pH (Kroll *et al*., 2003). Proteins' hydrophobicity can be increased by coating them with nonpolar polyphenols, which improves their surface activity while lowering their water solubility (Bandyopadhyay *et al.,* 2012). Proteins can be interconnected by polyphenols and proteins to improve their water solubility (Ozdal et al., 2013). When casein and whey protein isolate interacted noncovalently with chlorogenic acid, their protein solubility increased, according to (Jiang *et al*., 2018). With 240mol/g chlorogenic acid, protein solubility increased by 18.2% and surface hydrophobicity decreased by 16.6% (Jiang *et al*., 2018).

2.5.2 Emulsifying and foaming properties

A number of factors influence the effect of protein-polyphenol conjugation on emulsifying qualities, including pH, temperature, the type of polyphenol and protein involved, their structure, and the conditions under which conjugation occurs, according to the (Quan *et al*., 2019). Casein works well as an emulsifier. Protein conjugation with polyphenols, on the other hand, is being investigated as a viable method for improving protein functionality or changing it in a beneficial way (Quan *et al*., 2019). Lactoferrin–polyphenols (EGCG, chlorogenic acid, and gallic acid) conjugates with greater emulsifying properties than unmodified lactoferrin were created using the free-radical grafting method (Liu *et al*., 2015). When oxidised chlorogenic acid and oxidised tannic acid were conjugated, they significantly increased the emulsifying activity index (EAI) and emulsion stability index (ESI) of porcine plasma protein hydrolysate (Chen *et al*., 2018).

2.5.3 Gelling

Conjugation affects a number of important functional properties, including gelling. According to research, covalently interacted gels are more thermally stable and rigid than non-covalently interacted gels (Zhang *et al*., 2010). According to, protein-polyphenol conjugation produces gels with improved gel properties, such as a lower gelling temperature and a shorter gelling time (Quan *et al*., 2019). Polyphenols increased the rigidity and mechanical strength of gelatin gel significantly (Strauss & Gibson, 2004). Adding tea polyphenols to milk can significantly affect casein processing functionality, such as gelation properties, according to (Haratifar & Corredig, 2014). Polyphenols in meat products are oxidised, resulting in the formation of quinone species. The gelling property is strengthened as a result of the reaction between amino acid groups and quinones, allowing an elastic gel network to form (Cao & Xiong, 2015). Because of the various types of proteins and polyphenols, effective covalent integration of both components is required to create conjugates with improved gelling properties.

Table 2.1. Different polyphenols, proteins, conjugation methods and their overall effect

Protein	**Polyphenols**	**Conjugation Method**	**Effect of conjugation**	**Reference**
α- CN β-CN	Catechin Epicatechin Epigallocatechin Epigallocatechin gallate	Non-covalent method	β-Casein formed stronger complexes with tea polyphenols than α-casein. The order of binding increases as the number of OH group increased with C > EC > EGC > EGCG	Hasni *et al*.,2011
α-l β-lg Lactoferrin	Epigallocatechin-3-gallate	Alkaline method	Covalent protein-EGCG complexes exhibited much stronger antioxidant activity	Wei *et al*., 2015

Sodium caseinate			Stability of emulsions was in the order: SC > LG > LF > LA	
α- CN, β-CN	Resveratrol, Genistein and Curcumin	Non-covalent method	Order of affinity towards protein was: Curcumin> Genistein> Resveratrol Stable complexes were formed with β-CN than α- CN	Bourassa *et al*., 2013
Casein Whey protein Isolate	Chlorogenic acid	Non-covalent	Protein solubility increased by 18.2%. Foaming ability and stability was enhanced.	Jiang *et al*., 2018
α S1-casein β-casein β-lg	Catechin, Epicatechin Epicatechin gallate, EGCG	-	Order of protein affinity towards polyphenol was β-casein> αS1-casein> β-lg	Chanphai *et al*., 2018
Casein Whey protein	Green tea polyphenols	-	Antioxidant acivity of conjuagtes was enhanced Unfolded proteins proteins have stronger affinity to polyphenols than globular proteins	Yildirim-likoglu and Erdem, 2018
α- CN, β-CN BSA α- la β-lg	Epigallocatechin	Non-covalent method	Antioxidant activity BSA, α- lactalbumin, β-lactoglobulin showed weaker antioxidant activity than β-CN and α- CN	Almajano *et al*.,2007

2.6 Controlling functionality of protein-polyphenol conjugates

There are a variety of factors that influence polyphenol-protein interactions like pH, ionic strength, temperature, etc.

Temperature is one of the prime parameters in the polyphenol-protein interactions. It is reported that hydrophobic interactions are increased at a higher temperature. According to (Suryaprakash *et al*., 2000), 10 - 40ºC is favourable for conjugation. At higher temperatures like 40 - 90ºC, the efficiency of conjugation is decreased because polyphenols undergo oxidation at such temperatures. (Prigent *et al*., 2003) determined the effect of temperature on the conjugation of BSA with chlorogenic acid and also, the amount of chlorogenic acid bound to BSA at different temperatures 5ºC, 20ºC, 60ºC. It was reported that higher temperatures decreased the efficiency of conjugation and also the number of molecules of chlorogenic acid binding to BSA was also decreased. (Wang *et al*., 2014) documented that heating approach might also have a negative impact on binding by causing decomposition of polyphenols thereby losing their biological activities and also proteins might undergo polymerization availing fewer binding sites for polyphenols to interact. Hence maintaining proper temperature during conjugation and pre-knowledge on thermal denaturation is necessary to study polyphenols - proteins.

pH is another important parameter which affects the protein-polyphenol interactions. Many authors in the literature studied the effects of pH over different protein-polyphenol conjugates. (Wang *et al*., 2014) reported that conjugation is favourable in a wide pH range of 4 – 10. Specifically lower pH is preferable because proteins undergo dissociation at such pH availing binding sites for polyphenols to interact. (Prigent *et al*., 2003) studied the effect of low pH on the binding of chlorogenic acid with BSA in order to determine the type of interactions involved and reported that higher amount of chlorogenic acid bound to BSA at pH 3 when compared to pH 7 i,e. 10-15 mol of BSA/ mole of BSA and non-covalent interactions were noticed. As the pH is raised towards alkaline side, the polyphenols undergo autooxidation forming quinones or radicals which interact with proteins covalently. It was concluded that there was no significant change on binding affinity at the pH range of 3-7. (Rawel *et al*., 2002) reported similar results where there was no significant change in the binding constant when globular protein BSA was conjugated with chlorogenic acid. (Abd El-

Maksoud *et al*., 2018) performed covalent method i.e. Chemical coupling reaction using EDC/NHS as coupling agents at different pH values (2.5, 6 and 8) to determine the optimal pH conditions and reported that two, eight and three molecules of CA were linked to β-Lg at pH 2.5, pH 6 and pH 8.5, respectively. Thus, it was concluded that pH 6 is optimum for chemical coupling reaction because EDC is having high activity and stability at pH 6 (Hermanson, 2008). (Le Bourvellec & Renard, 2012) documented that at pH 4.9, greater affinity was seen between BSA and tannic acid when compared to pH 7.8. (Wang *et al*., 2013) studied the interactions between α-Lactalbumin and EGCG at pH 6, 7, 8 and reported that EGCG interactions with α-La were more pronounced at pH 8 when compared to pH 6 and 7 confirming that α-La interacted covalently with EGCG at pH 8.

From the above-mentioned studies, it is clear that most of the studies conducted on protein-polyphenol conjugates were mainly focused on whey proteins. However, protein-polyphenol interactions are highly affected by type and surface properties of proteins as protein conformation is critical for conjugation. (Ozdal *et a*l., 2013) documented that protein- polyphenol interactions are influenced by properties like amino acid composition, isoelectric point and also hydrophobicity. Unstructured proteins with open structure showed greater affinity towards polyphenols when compared to closed structured and globular proteins because of the availability of amino acid residues (de Freitas *et al*., 2001). Literature has hypotheses that protein with higher proline residues and open structure bind more phenolic compounds when compared to closed or globular structured proteins. Hence among milk proteins, caseins are being given extra importance recently for their self-association and other properties like high proline content making them unique carriers for polyphenols. Proteins with higher proline content are said to better binding affinity with polyphenols (Bohin *et al*., 2014). Also, prolyl residues and other amino acids like phenylalanine, histidine and arginine are possible sites for polyphenol binding (Yildirim & Erdem, 2018). (Chanphai *et al*., 2018) conducted structural analysis and determined loading efficacies of tea polyphenols catechin, epicatechin, epicatechin gallate and epigallocatechin gallate with milk proteins α-casein, β-casein and β-lactoglobulin and reported the order of binding affinity towards polyphenols i.e, β-casein > α-casein > β-lactoglobulin. Similarly, (Hasni *et al*., 2011) studied the interaction of α-casein and β-caseins with tea polyphenols like catechin, epicatechin, epigallocatechin and

epigallocatechin gallate and reported that β-casein being highly hydrophobic formed stronger complexes with the tea polyphenols when compared to α-caseins because β-casein has more proline residues. (Bohin *et al*., 2012) studied the interactions between animal derived proteins like BSA, β-LG, β-CN, gelatin, ovalbumin, phosvitin and polyphenols like rutin, quercetin, catechin, taxifolin, eriodictyol, luteolin, EGCG. They studied the influence of aminoacid composition and reported that β-CN with higher proline content acted as a potent carrier of EGCG. It also had higher amount of aromatic amino acids and histidine which act as binding sites for polyphenols. It was also reported that proteins with random helical structures appeared to be potent carriers of phenolic compounds when compared to globular proteins. (Bourassa *et al*., 2013) studied the interactions between α- and β-caseins with phenolic compounds resveratrol, genistein, and curcumin and reported that β-CN formed stable complexes when compared to α-CN. However, (Bohin *et al.,* 2013) also reported contrary results that gelatins A and B though they have higher total proline content (sum of proline and hydroxyproline) than β-casein, but displayed a lower affinity to EGCG. This is because the proline repeats reduce the structural stability thereby reducing the binding ability. Thus, it can be concluded that type and conformation of proteins are important for protein-polyphenol interactions.

Therefore, it can be concluded that

- The interaction between polyphenols and proteins is governed by two key factors: hydrophobic interaction and hydrogen bond interaction.
- The extent of these binding forces is determined by the protein and polyphenol molecules' structures, as well as the ratio of them present
- Proteins with a higher proline concentration are more likely to bind polyphenols.

2.7 Polyphenol–protein complex characterization

Understanding the system at a molecular level is critical for probing the interaction between polyphenol and proteins, as well as the nature of complex formation. This necessitates the characterization of the polyphenol–protein system as well as the identification of the chemical modifications that occur in both molecules. This could

help us figure out why polyphenols and proteins have different bioavailability and beneficial activity. Fluorescence, circular dichroism (CD) spectroscopy, dynamic light scattering (DLS), Fourier transform infrared (FTIR) spectroscopy, isothermal titration calorimetry (ITC), nuclear magnetic resonance (NMR), and mass spectroscopy have all been used to characterise the polyphenol–protein complex formation.

2.7.1 Fluorescence spectroscopy

Interactions between phenolic chemicals and proteins can be detected using fluorescence spectroscopy (Choudhury *et al*., 2013). On the one hand, the indole chromophore of aromatic amino acids (e.g., tryptophan) causes protein fluorescence, which is significantly reduced by phenolic substances. On the other hand, some phenolic compounds have intrinsic fluorescence properties that are extremely sensitive to environmental factors such as polarity and hydrogen bonding effects. According to (Liu *et al*., 2017) the fluorescence intensity is determined by the type of phenolic chemical attached, with EGCG–zein being the most common followed by quercetagetin–zein and chlorogenic acid–zein. (Liu *et al*., 2017) recently discovered that fluorescence intensity varies depending on the type of phenolic compound conjugated, with EGCG–zein > quercetagetin–zein > chlorogenic acid–zein being the most common. Furthermore, fluorescence spectroscopy is frequently used to investigate non-covalent interactions and binding affinity between polyphenols and proteins. Trp-19 and Trp-61 are two tryptophan residues in β-lactoglobulin, for example. Trp-19 is in an apolar environment, which accounts for 80% of total fluorescence, whereas Trp-61 is partially exposed to aqueous solvent and contributes only a small amount to Trp fluorescence. α-Casein (a mixture of α-S1 and α-S2 caseins) has two tryptophan residues, Trp-66 and Trp-37 (in α-S1 casein) and Trp-109 and Trp193 (in αS2 casein), whereas β-casein has only one tryptophan residue, Trp-143, which exhibits intrinsic fluorescence (Hasni *et al.,* 2011). (Jiang *et al*., 2018) reported that after interacting with chlorogenic acid, the maximum emission peak for CN (casein) shifted from 348 to 366 nm. The fluorescence intensity was also significantly reduced when chlorogenic acid was added which was due to the fact that protein unfolding and exposing tryptophan residues to more hydrophilic environments in turn resulting in a lower quantum yield.

2.7.2 Differential scanning calorimetry (DSC)

Thermally induced protein transitions are studied using DSC, which is a thermodynamic technique. The thermal behaviour of phenolic–protein conjugates is commonly assessed using DSC. In general, the melting endothermic peak of free phenolic compounds vanished in DSC thermograms of phenolic–protein conjugates. Furthermore, phenolic–protein conjugate DSC profiles frequently occur at higher temperatures than blank proteins, implying that grafting with phenolic chemicals can improve protein thermal stability (Kim, 2012; Ozdal *et al*., 2013; Liu *et al*., 2017). In an ethanol–water solution, (Liu *et al*., 2017) investigated the non-covalent and covalent interactions between zein and polyphenols. The DSC thermogram of zein, according to them, shows peaks that correspond to the protein's thermal denaturation temperature and melting point. The denaturation temperature of the EGCG–zein conjugate, on the other hand, is higher than that of control zein. The EGCG–zein mixture has a lower denaturation temperature than the EGCG–zein conjugate. Thermo gravimetric analysis (TGA), in addition to DSC, is a possible method for determining the thermal properties of phenolic–protein conjugates (Qi *et al.,* 2016).

2.7.3 Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy can be used to investigate the chemical interactions (both non-covalent and covalent) between phenolic substances and proteins. The conjugation of phenolic chemicals complicates the structure of proteins. On the one hand, phenolic compound conjugation can significantly reduce the functional groups in the native side chains of proteins. In contrast, phenolic chemical conjugation can introduce novel spatial and functional groups into protein backbones. FT-IR spectroscopy is a good method for estimating the change in the secondary structures of proteins after conjugation with phenolic chemicals because it analyses the frequency of amide bonds. Various amide bands, such as band I (C=O stretching, 1600–1690 cm-1), band II (N–H bending and C–N stretching, 1480–1575 cm-1), and band III (C– N stretching and N–H bending, 1229–1301 cm-1) are used to investigate the structure of proteins and phenolic–protein conjugates (Kong *et al*., 2007; Liu *et al*.,2016). The amide band I spectral region is the most sensitive to protein secondary structure. The primary peaks in the amide band I region are a-helix (1658–1650 cm-1), b-sheet (1640–1615 cm-1), b-turn (1700–1660 cm-1) and random coil (1650–1640 cm-1) (Zhu *et al*., 2012).

Polyphenol conjugation protects lactoferrin from heat aggregation at neutral pH. FT-IR spectra of EGCG–lactoferrin conjugate show less modification after heat treatment (particularly β-sheet) than lactoferrin alone (Liu *et al*., 2017). When tea polyphenols interact with β-lactoglobulin non-covalently, FT-IR spectroscopy reveals a significant increase in the β-sheet and a slight increase in the α-helix (Kanakis *et al.,* 2017).

2.7.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis techniques, particularly SDS-PAGE, are commonly used to determine the molecular weights of proteins and their phenolic conjugated analogues (Zhu *et al*., 2012). When SDS is allowed to completely react with proteins, the resulting SDS–protein complexes have identical charge densities, which prevents phenolic chemicals from interacting with proteins non-covalently. As a result, the sample size determines the sample's mobility on SDS-PAGE. Non-covalent and covalent interactions between phenolic chemicals and proteins can be distinguished using SDS-PAGE.

The SDS-PAGE profile of catechin-egg white protein conjugates differs significantly from that of egg white proteins, with some bands diffusing and rising upwards, according to (Gu *et al.,* 2017). The SDS-PAGE profile of catechin-egg white protein mixtures is identical to that of egg white proteins because the non-covalent connections between catechin and egg white proteins are disrupted by adding SDS. This shows that when phenolic chemicals are conjugated to proteins, their molecular weights increase. The rise in the molecular weights of phenolic–protein conjugates is due to covalently bonded phenolic moieties.

2.7.5 Microscopic methods

Atomic force microscopy is another technique that can reveal complex anatomy, aggregation form, and the impact of polyphenol conjugation on proteins (AFM). It's a useful tool for monitoring protein aggregation, which can be triggered or stopped by a variety of small chemicals, including polyphenols. The method provides information on the shape and size of the conjugates. The results cannot be compared to hydrodynamic light diffusion measurements in dispersions because AFM imaging is performed on dehydrated samples. Scanning electron microscopy (SEM) can also be used to examine the microstructure of the conjugated samples.

2.7.6 Thermodynamic methods

When a phenolic chemical is added to a protein solution, it might cause a thermodynamic effect called enthalpy shift (Ojha *et al*., 2012). Isothermal calorimetric titration can be used to investigate the thermodynamic properties of the connection between phenolic chemicals and proteins.

2.7.6.1 Isothermal Titration Calorimetry (ITC)

The method works by calculating the amount of heat produced during the chemical reaction (Al-Hanish *et al*., 2016). The ligand, polyphenol, was titrated into a macromolecule (Protein) in titration mode, and numerous injections were made at various times. The heat emitted or absorbed into the sample cell where the complexation reaction occurs at any injection is determined by a cell containing only a buffer. Understanding the binding mode requires an understanding of the thermodynamic characteristics of interactions (free energy changes (G), enthalpy changes (H), and entropy changes (S). Hydrophobic forces, static interactions, van der Waals interactions, and hydrogen bonds are all examples of interactions. Because variations in free energy have a negative sign, the contact mechanism appears to be random. Hydrophobic forces aid the connection between positive enthalpy and entropy changes (Bose, 2016). The production of α-lactalbumin and epigalocatechin-3-gallate (EGCG) complexes has been found to improve exothermic enthalpy (20 mM phosphate buffer, pH 7.2) (Al-Hanish *et al.*, 2016). The phenomenon of EGCG binding to lipase was also discovered. The thermal stability of the BSA after contact with ferulic acid (pH 7.4) was improved during the ITC analyses. Tea Catechin as a catalase inhibitor was studied using ITC in combination with other approaches (Pal *et al*., 2014).

CHAPTER 3

MATERIALS AND METHODS

3. Materials and methods

For the present research on “Effect of chemical modification by polyphenols on the functionality of casein”, the materials and methodologies employed are dealt in this chapter.

3.1. Sample preparation

The conjugates of casein and polyphenols like Caffeic acid and Ferulic acid were prepared by two categories as mentioned below:

a. Non- covalent method
b. Covalent method
    - Free Radical Grafting method
    - Chemical coupling method
    - Alkaline method

3.1.1. Preparation of conjugates by non-covalent method

3.1.1.1 Apparatus

Balance, capable of weighing to the nearest 0.1mg; centrifuge tube,15 ml capacity; micropipette, of capacity 1 ml and 10 ml; beakers, of capacity 25ml; magnetic stirrer; dialysis membrane (cellulose), of capacity 5kDa; UV-Visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan)

3.1.1.2 Materials

Sodium Caseinate was obtained from SRL Pvt. Ltd, Caffeic acid and Ferulic acid was purchased from Sigma Aldrich. Sodium azide was purchased from HI Media. Ethanol (C2H5OH), 99.9 %. Salts like Potassium Dihydrogen Ortho Phosphate (KH_2PO_4) and Dipotassium Hydrogen Phosphate (K_2HPO_4) were purchased from Merck life science Pvt, Ltd. India. Ethanol was purchased Thermo Fisher Scientific Pvt, Ltd.

3.1.1.3 Sample preparation

Sodium caseinate of (10mg/mL) was dissolved in 50mM phosphate buffer of pH 6, 7, and 8 to obtain a protein solution of 1%. To this 0.01% of sodium azide was added and the solution was stored at 4ºC / 24hours for hydration. Caffeic acid and Ferulic acid of 2mg/mL was dissolved in 50mM phosphate buffer of pH 6, 7 and 8 to get 0.2% solution.

3.1.1.4 Procedure

7mL of casein 1% casein solution of each pH 6, 7, and 8 was taken in a 15ml centrifuge tubes. To this 3 mL of 0.2% caffeic acid of corresponding pH was added. Similarly, 7mL of casein 1% casein solution of each pH 6, 7, and 8 was taken in a 15ml centrifuge tubes. To this 3 mL of 0.2% caffeic acid of corresponding pH was added. Immediately the tubes were closed and are mixed gently to get NaCn-CA and NaCn-FA conjugate solution. These tubes were incubated at 4ºC for 24hrs. Followed, dialysis of these solutions was done against distilled water using 5k DA membranes for 24hrs to remove unbound polyphenols. To ensure the complete removal of free polyphenols, total polyphenol content of outside solution was determined by recording the absorbance using UV-Visible spectrophotometer. Later, the solutions were drawn in centrifuge tubes and were stored at 4ºC for further analysis.

3.1.2 Preparation of conjugates by Covalent method

3.1.2.1 Free radical grafting method

3.1.2.1.1 Apparatus

Balance, capable of weighing to the nearest 0.1mg; centrifuge tube,15 ml capacity; micropipette, of capacity 1 ml and 10 ml; beakers, of capacity 25ml and 50ml; magnetic stirrer; dialysis membrane(cellulose), of capacity 5kDa; UV-Visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan)

3.1.2.1.2 Materials

Sodium Caseinate was obtained from SRL Pvt. Ltd, Caffeic acid, Ferulic acid and Ascorbic acid was purchased from Sigma Aldrich. Sodium azide was purchased from HI Media, Hydrogen peroxide (30%) was purchased from Merck life science Pvt, Ltd. India

3.1.2.1.3 Procedure

50mg of ascorbic acid and 200µL of 5mol/L hydrogen peroxide were added to 20mL of casein solution of pH 7. The mixture was then stirred on a magnetic stirrer for 2 h at 4ºC. Thereafter, 7mL of above mixture was taken in two separate 50mL beakers. Later 3 mL of 0.2% caffeic acid (pH 7) was added to one beaker and to another 0.2% ferulic acid was added. Thereafter, these solutions were incubated by continuous magnetic stirring at 4º C for 24hrs. Followed, dialysis of these solutions was done against distilled water using 5k DA membranes for 24hrs to remove unbound polyphenols. To ensure the complete removal of free polyphenols, total polyphenol content of outside solution was determined by recording the absorbance using UV-Visible spectrophotometer. Later, the solutions were drawn in centrifuge tubes and were stored at 4ºC for further analysis.

3.1.2.2 Chemical Coupling method

3.1.2.2.1 Apparatus

Balance, capable of weighing to the nearest 0.1mg; centrifuge tube,15 ml capacity; micropipette, of capacity 1 ml and 10 ml; beakers, of capacity 25ml; magnetic stirrer; dialysis membrane(cellulose), of capacity 5kDa; UV-Visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan)

3.1.2.2.2 Materials

Sodium Caseinate was obtained from SRL Pvt. Ltd, Caffeic acid and Ferulic acid was purchased from Sigma Aldrich. Sodium azide was purchased from HI Media, EDC [N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride] and NHS (N-Hydroxysuccinimide) were purchased from Sigma Aldrich.

3.1.2.2.3 Procedure

20.916mg of EDC and 12.556mg of NHS was added to 10mL of casein solution (10mg/mL) of pH 7. The solution was mixed thoroughly. 7 ml of the above solution was drawn into 15ml centrifuge tube. To this 3mL of 0.2% Caffeic acid (pH 7) was added was gently mixed to get NaCn-CA conjugate solution. Similarly, Ferulic acid was added to get NaCn-FA conjugate solution. Later, these solutions were incubated at 4º for 24hrs. Followed, dialysis of these conjugate solutions was done against distilled

water using 5k DA membranes to remove unbound polyphenols. To ensure the complete removal of free polyphenols, total polyphenol content of outside solution was determined by recording the absorbance using UV-Visible spectrophotometer. Later, the solutions were drawn in centrifuge tubes and were stored at 4ºC for further analysis.

3.1.2.3. Alkaline method

3.1.2.3.1 Apparatus

Balance, capable of weighing to the nearest 0.1mg; centrifuge tube,15 ml capacity; micropipette, of capacity 1 ml and 10 ml; beakers, of capacity 25ml; magnetic stirrer; dialysis membrane(cellulose), of capacity 5kDa; UV-Visible Spectrophotometer (Shimadzu Corporation, Kyoto, Japan)

3.1.2.3.2 Materials

Sodium Caseinate was obtained from SRL Pvt. Ltd, Caffeic acid and Ferulic acid was purchased from Sigma Aldrich. Sodium azide was purchased from HI Media, Petri plates; Media shaker.

3.1.2.3.3 Procedure

7ml of casein solution(10mg/mL) of pH 9 was drawn into a Petri plate. To this 3mL of 0.2% Caffeic acid (pH 9) was added to get NaCN-CA solution. Similarly, 0.2% Ferulic acid was added to prepare NaCN-FA conjugate solution. These Petri plates were kept open at room temperature under continuous shaking on media shaker for 6 hrs for oxygen to incorporate. Later these solutions were transferred to 15ml centrifuge tubes and were incubated at 4º C for 24hrs. Followed, dialysis of these conjugate solutions was done against distilled water using 5k DA membranes to remove unbound polyphenols. To ensure the complete removal of free polyphenols, total polyphenol content of outside solution was determined by recording the absorbance using UV-Visible spectrophotometer. Later, the solutions were drawn in centrifuge tubes and were stored at 4ºC for further analysis.

3.2 Determination of total polyphenol content

Total polyphenol content of the above prepared NaCn-CA and NACn-FA conjugates were determined by Folin Ciocalteu method as described by (Wang *et al*., 2014).

3.2.1 Apparatus

Test tubes,10 ml capacity; volumetric flasks ,10 ml capacity; micropipette, of capacity 1 ml and 10 ml; beakers, of capacity 25ml and 50ml; Measuring cylinder, of capacity 25ml; Vortex mixer; UV-Visible Spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

3.2.2 Reagents

a) 7.5% Sodium carbonate: 7.5g of Na_2CO_3 was dissolved in100mL of distilled water

b) 0.2 N Folin's reagent: Commercially available 2N Folin's reagent (Sigma Aldrich) was diluted 10 times to obtain 0.2N working reagent

c) Standard solution: Caffeic acid and Ferulic acid of concentration 0.1 mg/ml was used as standard.

3.2.3 Procedure

3.2.3.1 Standard curve

1. Take 10 volumetric flasks and mark different concentrations of 10µg, 20µg, 30µg, 50µg and 100µg. To get 10µg add 1mL of standard caffeic acid solution and makeup to 10ml using distilled water. For 20µg, add 2mL and make up to 10mL, similarly up to 100µg standard curve was prepared. Similarly, for ferulic acid standard curve is drawn using Ferulic acid as standard solution.
2. 500µl of each standard solution from each flask was taken in separate test tubes.
3. 2.5ml of 0.2N Folin-Ciocalteu reagent was added.
4. 2ml of 7.5% Sodium carbonate solution was added and mixed the content using vortex mixer.
5. Solution was incubated at 25°C for 20-30 minutes for reaction to complete.
6. Absorbance was measured at 760 nm against blank and a graph was plotted between absorbance vs caffeic/ferulic acid concentration.

3.2.3.2 Sample preparation

For estimation of total polyphenol content in prepared conjugate solutions, initially, the NaCn-CA and NaCn-FA conjugate solutions were diluted to 1:10 ratio with corresponding pH of 50mM phosphate buffer in which the conjugates were prepared. At room temperature, 0.5 ml of the properly diluted sample was combined with 2.5 ml of freshly made Folin–Ciocalteu reagent. Later, 2 mL of sodium carbonate was added, vortexed, and allowed to stand for 30min at room temperature in the dark. A Shimadzu UV-1800 UV–vis spectrophotometer was used to detect the absorbance at 760 nm. The total polyphenol content was calculated using the standard curve in the Fig 3.1 and 3.2 for NaCn-CA and NaCn-FA conjugates, respectively.

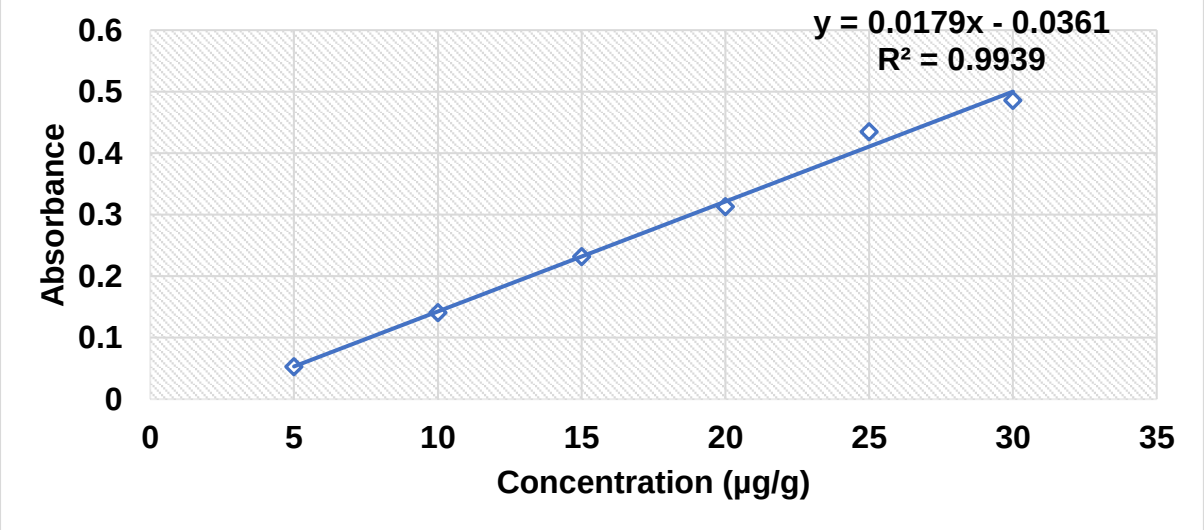


Fig. 3.1 Standard curve for Caffeic acid for total polyphenol content estimation of NaCn-CA conjugates

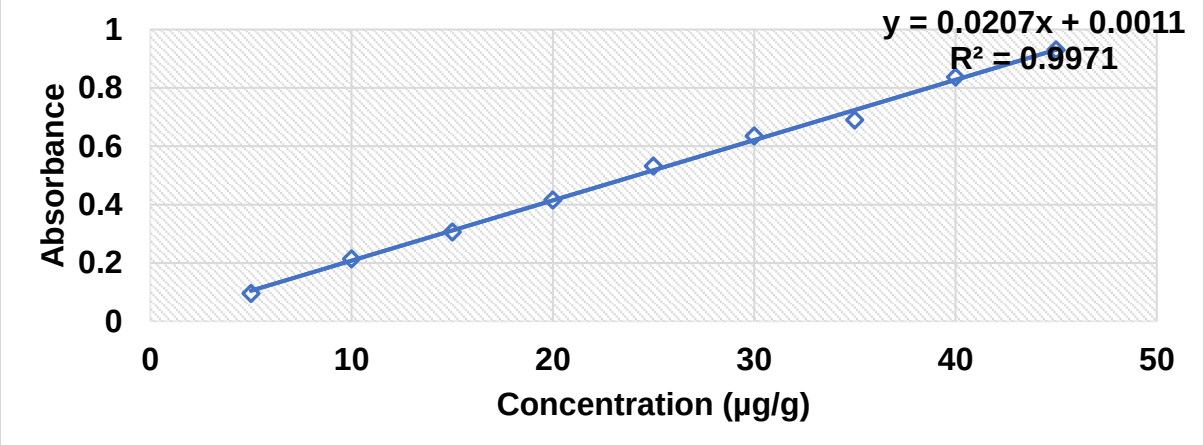


Fig. 3.2 Standard curve for Ferulic acid for total polyphenol content estimation of NaCn-FA conjugates

3.3. Characterization of casein-polyphenol (caffeic acid/ferulic acid) conjugates

The conjugation between polyphenol (CA and FA) and whey protein was confirmed by using fluorescence spectroscopy, UV-VIS spectroscopy and Fourier transform infrared spectroscopy (FTIR). The prepared conjugates were further characterized for particle size and zeta potential.

3.3.1 Intrinsic fluorescence spectroscopy

3.3.1.1. Materials

Cuvettes; Spectro-fluorimeter

3.3.1.2. Procedure

The fluorescence spectra of the samples were recorded using spectrofluorometer according to the procedure of (Arroyo-Maya *et al*., 2016) with slight modifications. The concentration of the sample was maintained at 10mg/ml prepared in 50mM phosphate buffer of respective pH. The samples were drawn in a specially designed cuvette of wavelength 1 cm and carefully placed in the sample cell. The sample solutions were excited at 280 nm and emission spectra were recorded from 300 to 500 nm with a scanning speed of 12000nm/min.

3.3.2. UV-Visible spectroscopy

3.3.2.1 Materials

Quartz cuvettes, Shimadzu UV-1800 UV–vis spectrophotometer.

3.3.2.2 Procedure

The UV Vis absorption of the prepared NaCn-polyphenol conjugates were determined using UV-1800 UV–vis spectrophotometer as the method described by (Liu *et al*., 2017) with slight modifications. The conjugate solutions prepared initially were diluted to 1:10 using 50mM phosphate buffer of respective pH at room temperature. Later the samples are drawn into cuvettes and cuvettes are placed in the sample testing cell. The samples were scanned between 200nm to 600nm against the buffer of corresponding pH which was used as blank.

3.3.3 FTIR spectra

3.3.3.1 Materials

Micropipette of capacity 250µl; FTIR (IR Affinity-01, Shimadzu, Japan)

3.3.3.2 Procedure

FTIR spectrum was determined using the method described by (Jiang *et al*., 2018) with modifications. Here the control protein solutions of concentration (10mg/ml) and the conjugate solutions were directly scanned in the range 4000 cm -1 to 400 cm -1 at a data acquisition rate of 2 cm−1 per point. Each sample was scanned 32 times.

3.3.4 Determination of size of the conjugates

3.3.4.1 Materials

Micropipette, of capacity 1ml; Cuvettes; Zetasizer (Malvern Instruments).

3.3.4.2 Procedure

The particle size of the prepared NaCn-polyphenol conjugates was determined using a Zetasizer (Malvern Instruments). The prepared conjugate solution was diluted to 1:10 using 50mM phosphate buffer of respective pH before analysing the particle size. Later the samples are drawn into cuvettes and cuvettes are placed in the sample testing cell. Each sample was run for 25 scans and the experiment results were recorded.

3.3.5 Determination of zeta potential of the conjugates

3.3.5.1 Materials

Micropipette, of capacity 1ml; Cuvettes; Zetasizer (Malvern Instruments).

3.3.5.2 Procedure

The zeta potential of the prepared NaCn-polyphenol conjugates was determined using a Zetasizer (Malvern Instruments). The prepared conjugate solution was diluted to 1:10 using 50mM phosphate buffer of respective pH before analysing the zeta

potential. Later the samples are drawn into specially designed cuvettes for zeta potential analysis and cuvettes are placed in the sample testing cell. Each sample was run for 25 scans and the experiment results were recorded.

3.4 Determination of functional properties of the conjugates

3.4.1 Determination of antioxidant activity of the conjugates

Antioxidant activity based on 2, 2 diphenyl-1-picryl hydrazyl (DPPH) radical scavenging for peptides was analyzed as per the method given by (Wu *et al.,* 2003).

3.4.1.1 Apparatus

Balance, capable of weighing to the nearest 0.1mg; centrifuge tube,15 ml capacity; micropipette, of capacity 1 ml and 10 ml; beakers, of capacity 25ml; vortex mixer; UV-Visible Spectrophotometer (Shimadzu Corporation, Kyoto, Japan)

3.4.1.2 Reagents

a) DPPH (2, 2 Diphenyl-1-picryl-1-hydrazyl) Stock solution (2mM)

78.864 mg of DPPH was dissolved in 50 mL 70% methanol by stirring over magnetic stirrer overnight at 4ºC. The final volume was adjusted with 70% methanol to 100 mL using volumetric flask and the stock solution was stored at 4 ºC in an amber coloured reagent bottle.

b) Preparation of DPPH working solution (0.2mM)

One millilitre of stock solution was diluted with 70% methanol to 10 mL in a volumetric flask to get 0.2mM. Working solution was prepared freshly prior to analysis and kept in amber glass bottle.

3.4.1.3. Procedure

1mL of Casein-Polyphenol Conjugate Solution was taken in a Test-tube. 2ml of 70% methanol was added & mixed thoroughly. Then 1mL of 0.2mM DPPH dissolved in methanol was added to the mixture & mixed thoroughly for 10 s, then content was incubated in the dark for 30min at room temperature. Absorbance was recorded at 517nm using Spectrophotometer (UV 2700, Shimadzu, Japan).

For control, DPPH solution was used, whereas for blank 70% methanol was taken. The absorbance was recorded at 517 nm against the blank using the UV-Vis spectrophotometer. Then the % radical activity was calculated using below mentioned formula.

3.3.1.4 Calculation

The DPPH radical scavenging activity was calculated using the following equation:

Percent inhibition or DPPH radical scavenging activity was calculated using the formula:

$$\% \text{ DPPH radical scavenging activity} = \frac{(A\,c - A\,s)}{A\,c} \times 100$$

Here,

A c = denotes the absorbance of control (0.2mM DPPH solution)

A s = denotes the absorbance of sample

3.4.2 Determination of the solubility of the conjugates

The solubility of the conjugates and the control protein was determined according to the method as described by (Jiang *et al*.,2018) with slight modifications.

3.4.2.1 Materials

Centrifuge tube, of capacity 15ml; centrifuge bottle, of capacity 50ml; High Speed Refrigerated centrifuge (KUBOTA TA-6500, Tokyo, Japan)

3.4.2.2 Procedure

The conjugate solutions and the control protein solution were taken in centrifuge tubes and were subjected to centrifugation at 10,000g for 20 min. Later, the protein concentration in the supernatant and in the original samples were measured using the Kjeldahl method.

3.4.2.2.1 Kjeldahl method for estimation of protein content

3.4.2.2.1.1 Reagents

a) Sulphuric acid (H_2SO_4)

b) Digestion mixture: Mix 3.5g of potassium sulphate (K_2SO_4) anhydrous, with 0.105g of anhydrous copper sulphate ($CuSO_4$)

c) NaOH solution (40% w/v): Dissolve 400g sodium hydroxide (NaOH) in 1000mL distilled water.

d) Boric acid (4% w/v): Dissolve 40g of Boric acid in 1000mL distilled water.

g) Standard 0.1N HCL

h) Indicator Mix 100 ml of 0.1 % methyl red (in 95% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol).

3.4.2.2.1.2 Procedure

The solubility of NaCn before and after the incubation with polyphenol was determined according to (Chen *et al*., 2019). Control protein solution and Protein-Polyphenol conjugate solution was centrifuged at 10,000g at 20^{o}C for 30 min. Protein content of supernatant and initial dispersion was analyzed using Kjeldahl method (ISO 8968-1:2014).

3.4.2.2.1.3 Calculation

Protein nitrogen = $\frac{((b - a) \times 0.1 \times 1.4007)}{(Ws)} \times 100$

Here,

Ws = weight (g) or volume (ml) of sample

a = volume (ml) of 0.1N HCl used in blank titration

b = volume (ml) of 0.1N HCl used in sample titration

0.1= normality of HCl

Protein %= Protein Nitrogen x 6.38

3.4.2.3 Calculation

Solubility was defined as the percentage of the protein concentration of the supernatant over that of total protein (before centrifugation) as followed.

Solubility (%) = $\frac{(\text{Protein concentration in the supernatant})}{(\text{Total protein before centrifugation})} \times 100$

3.4.3 Determination of emulsifying ability and emulsifying stability of NaCn-polyphenol conjugates

The emulsifying ability and emulsifying stability of the conjugates were determined according to the method as described by (Dalev and Simeonova, 1995) with slight modifications.

3.4.3.1 Materials

Measuring cylinder, of capacity 100ml; beaker, of capacity 100ml; centrifuge tube, pf capacity 15ml; Soya bean oil, Magnetic stirrer, Probe sonicator, Centrifuge (Eltek, Kapali Trading Corporation).

3.4.3.2 Procedure

Emulsifying activity and emulsion stability of NaCn-Polyphenol conjugates were determined according to method of (Dalev and Simeonova, 1995) with some modifications. For emulsifying activity (EA) emulsion was prepared by taking 50ml of sodium caseinate -polyphenol conjugate solution and 25ml of Soyabean oil in a beaker and mixture was allowed to rest for 20-30 min on magnetic stirring. Further, emulsions were prepared using probe sonicator for 30min with a pulse rate of 5 sec and an amplitude of 50% at 20ºC. Once the emulsions were prepared, 10mL of the emulsion was taken in graduated tube and centrifuged at 2600g/10min. Later, the whole volume of the tube and the volume of the emulsion phase was measured. The emulsion capacity was determined by the equation 3.3.3.3. In order to determine the Emulsion Stability, the emulsion is heated to 80º C for 30min, then cooled immediately by placing it in ice bath for 5- 10min. Emulsion was transferred in a graduated tube and centrifuge it at 1300g/10min. Later, the whole volume of the tube and the volume of the emulsion phase was measured. The emulsion stability was determined by the equation 3.3.3.3.

3.4.3.3 Calculation

Emulsion Capacity (%) = $\frac{\mathrm{EPV}}{\mathrm{WV}} \mathrm{X}\ 100$

Here,

EPV= denotes the volume of the emulsion phase

WV= denotes the whole volume of the tube

Emulsion Stability (%) = $\frac{\mathrm{EPVh}}{\mathrm{WVh}} \mathrm{X}\ 100$

Here,

EPVh = denotes the volume of the emulsion phase after heating

WVh = denotes the whole volume of the tube after heating

3.4.4 Determination of foaming activity and stability of NaCn-polyphenol conjugates

Foaming capacity and stability of the conjugates was determined as the method described by (Jiang *et al*., 2018) with slight modifications.

3.3.4.1 Apparatus

Measuring cylinder, of capacity 100ml and 1000ml; beaker, of capacity 250ml; Ultra Turrax-T25 (IKA) homogenizer.

3.3.4.2 Procedure

Initially 60 mL of each conjugate solution was transferred into a beaker and was blended using Ultra-Turrax homogenizer for 120 seconds at a high speed of 17,500 r/min. Foam was prepared for control protein solution in a same way. Later, Foaming Capacity (FC) was calculated according to the equation 3.3.4.3. In order to determine the Foam Stability (%), the foam after being prepared, are allowed to stand undisturbed for 15min. Later, the Foaming stability was determined using the following equation 3.3.4.3.

3.3.4.3 Calculation

Foam Capacity (%) = $\frac{\mathrm{V_o - V)}}{\mathrm{V}} \mathrm{X}\ 100$

Here,

V_o= denotes the volume generated after being subjected to blending

V= denotes the initial taken volume i.e. 60mL

Foam Stability (%) = $\frac{\mathrm{V15}}{\mathrm{V0}} \mathrm{X}\ 100$

Here,

V_o = denotes the volume generated immediately after blending

V_{15}= denotes the remaining foam volume after the foams were left undisturbed for 15 min.

3.5 Statistical Analysis

All statistical analysis was performed using MS-EXCEL-2019. Results are presented as mean ± standard deviation (SD) of three replicates, and significance was tested by employing analysis of variance (ANOVA).

CHAPTER 4

RESULTS AND DISCUSSION

The results obtained from the different experiments performed according to the objectives mentioned in chapter 1 and the following methods are described in the chapter 3 are presented in this chapter.

4.1 Total phenolic content of casein-polyphenol (caffeic acid/ferulic acid) conjugates

The total phenolic content of casein-caffeic acid and casein-ferulic acid conjugates prepared by non-covalent and covalent methods was determined using the Folin–Ciocalteu's method.

In the present study, the casein-polyphenol conjugates were prepared using both non-covalent and covalent approach. In non-covalent approach, the sodium caseinate (1%) solution of pH 6, 7 and 8 was mixed with 0.2% solution of caffeic and ferulic acid individually and incubated at 4^{o}C for 24 h. In covalent approach the protein (1%) solution of pH 7 was mixed with 0.2% solution of caffeic and ferulic acid individually and further processing was done as per the method described for alkaline method, free radical grafting and chemical coupling methods in section 3. The unreacted caffeic and ferulic acid was removed by dialysis using 5KDa membrane. The polyphenol content in conjugates was measured using Folin's method. The protein (1%) solution of without polyphenol was used as control. The polyphenol in sodium caseinate-caffeic acid (NaCn-CA) and sodium caseinate-ferulic acid (NaCn-FA) conjugates was calculated as mg polyphenol per g of protein and results are presented in Table 4.1.1 and Fig. 4.1.1 and 4.1.2, respectively. Among non-covalent conjugates of NaCn-CA, the total polyphenol content was found to be 21.02 ± 0.93, 25.36 ± 0.46 and 19.25 ± 0.78 mg/g protein at pH 6, 7 and 8, respectively. Among covalent conjugates of NaCn-CA, the total polyphenol content was 35.75 ± 0.14, 38.69 ± 0.68 and 26.88 ± 0.24 mg/g protein in chemical coupling method, free radical grafting and alkaline method, respectively. Among non-covalent conjugates of NaCn-FA, the total polyphenol content was 18.83 ±0.59, 21.35 ± 0.52 and 16.66 ± 0.72 mg/g protein at pH 6, 7 and 8 whereas the total polyphenol content of covalent conjugates of NaCn-FA was 23.2 ± 0.75, 28.18 ± 0.49 and 20.28 ± 0.32 mg/ml in chemical coupling method, free radical grafting and alkaline method, respectively. In non-covalent conjugates, the polyphenol

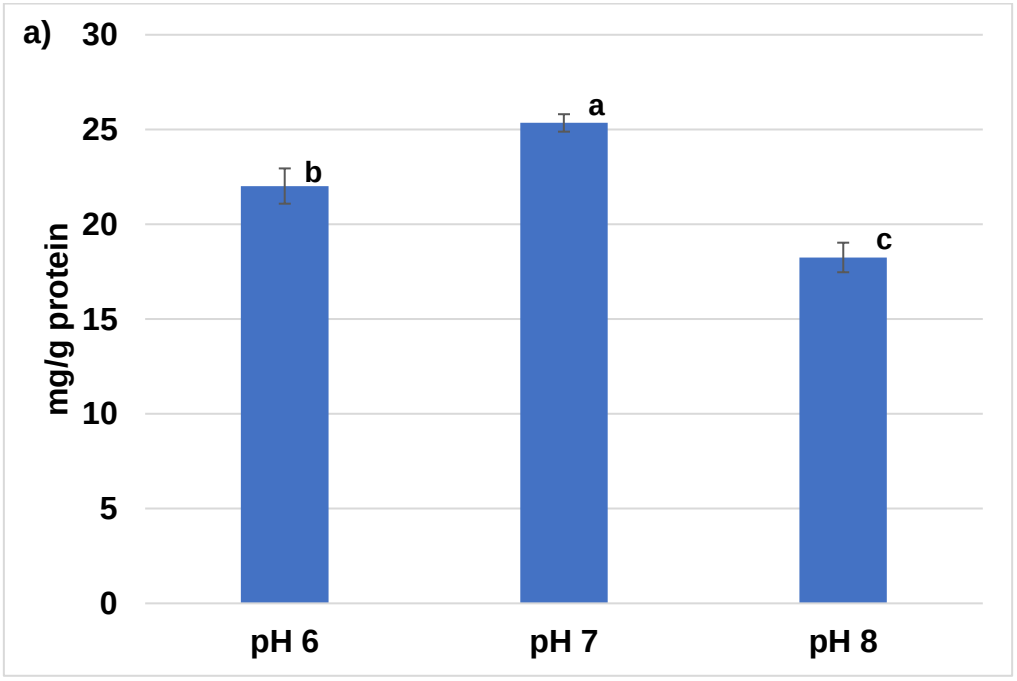
content was higher at pH 7 followed by pH 6 and pH 8 for both CA and FA. The difference in binding of polyphenol with respect to pH was because the ionisation status of specific protein residues and intra-protein interactions are modified by pH variations (Abd El-Maksoud *et al*., 2018) and more binding at pH 6 and 7 may be attributed to dissociation of proteins at pH ≤ 7 leads to the exposure of more binding sites for polyphenol association (Ozdal *et al*., 2013). (Sastry & Rao, 1990) studied the binding of chlorogenic acid by the polyphenol-free 11s protein of sunflower seed as a function of pH and reported that as the pH was lowered, availability of binding sites is more. In contrast, the total phenolic content of α-La–EGCG non-covalent complexes was measured by (Wang *et al*., 2014) at three pH levels (6.0, 7.0, and 8.0), with the total polyphenol content being higher at pH 8. They also discovered that at pH 8.0, α-La interacted covalently with EGCG, increasing the total phenolic content of α-La. (Prigent *et al*., 2003) found that the amount of 5-CQA (5-O-Caffeoylquinic Acid) bound per BSA molecule was slightly higher at pH 3.0 than at pH 7.0. Among covalent conjugates, maximum binding of polyphenol occurred in conjugates prepared by free radical grafting method followed by chemical coupling and alkaline method, respectively for both NaCn-CA and NaCn-FA conjugates. The total polyphenol content of NaCn-CA was more than NaCn-FA conjugates and the difference was due to structure of polyphenol. CA and FA both are phenolic acids having a very less difference in molecular weight but differ in structure. CA has extra OH group at position 3 which is replaced by methoxy group in FA. Between covalent and non-covalent conjugates of NaCn-CA and NaCn-FA, the binding of polyphenol was more with covalent conjugates than the non-covalent conjugates. This may be attributed to the binding of phenolic compounds to to certain amino acids covalently only (Wei *et al*.,2016) and non-covalent interactions between proteins and polyphenols are usually reversible and less strong than covalent ones (Czubinski & Dwiecki, 2017; Jakobek, 2015).

Table.4.1.1. Total polyphenol content of NaCn-CA and NaCn-FA conjugates

	Sample	NaCn-CA mg/g	NaCn-FA mg/g
Non-covalent	pH 6	21.01 ± 0.93[a]	18.83 ± 0.59[b]
	pH 7	25.36 ± 0.46[a]	21.35 ± 0.52[b]
	pH8	19.25 ± 0.78[a]	16.67 ± 0.72[b]
Covalent	Chemical coupling	35.75 ± 0.14[a]	23.27 ±0.75[b]
	Free radical	38.69 ± 0.68[a]	28.18 ± 0.49[b]
	Alkaline	26.89 ± 0.24[a]	20.28 ± 0.32[b]

Mean ± S.D, n=3,

means with different superscripts (a,b,) in each row differ significantly ($p<0.05$) from each other



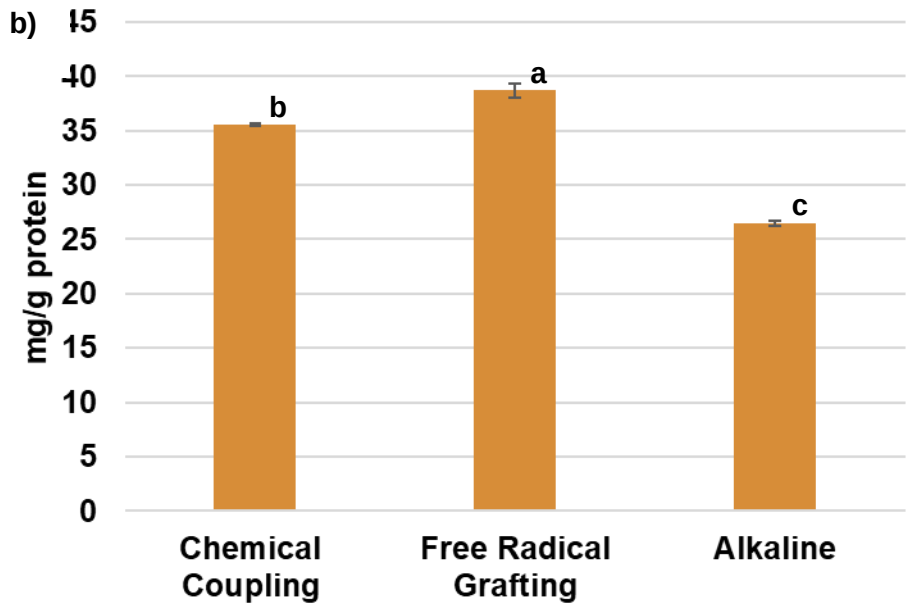
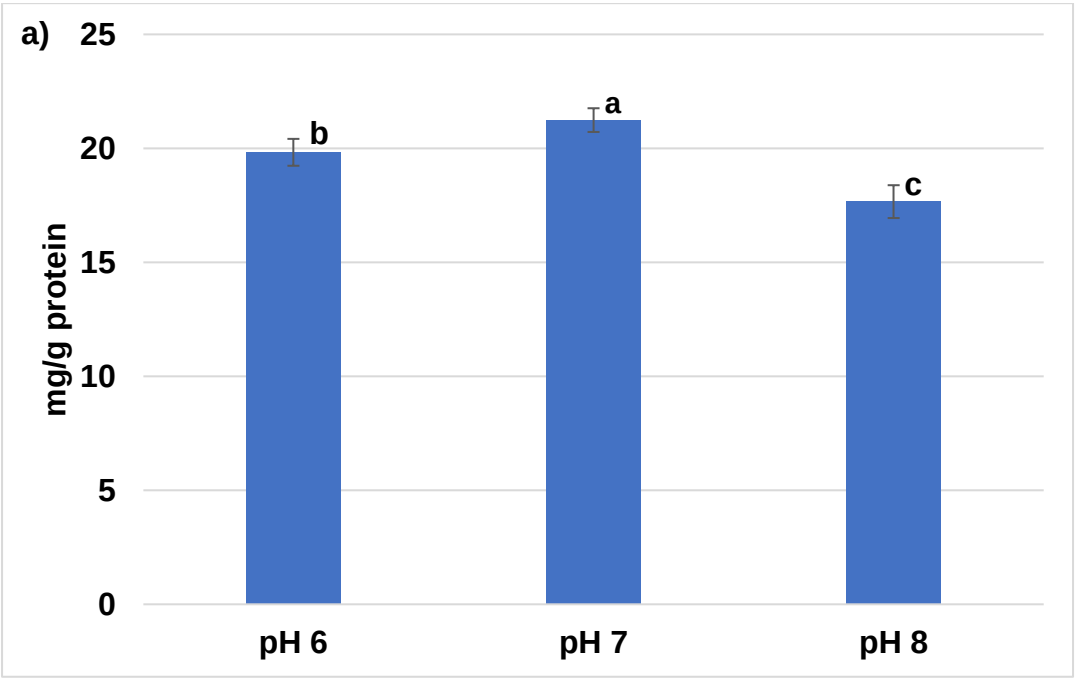


Fig. 4.1.1 Polyphenol content (mg/g protein) in NaCn-CA conjugates prepared by a) non-covalent method (at different pH 6, 7 and 8) b) covalent method (Chemical coupling, Alkaline, Free radical grafting method)



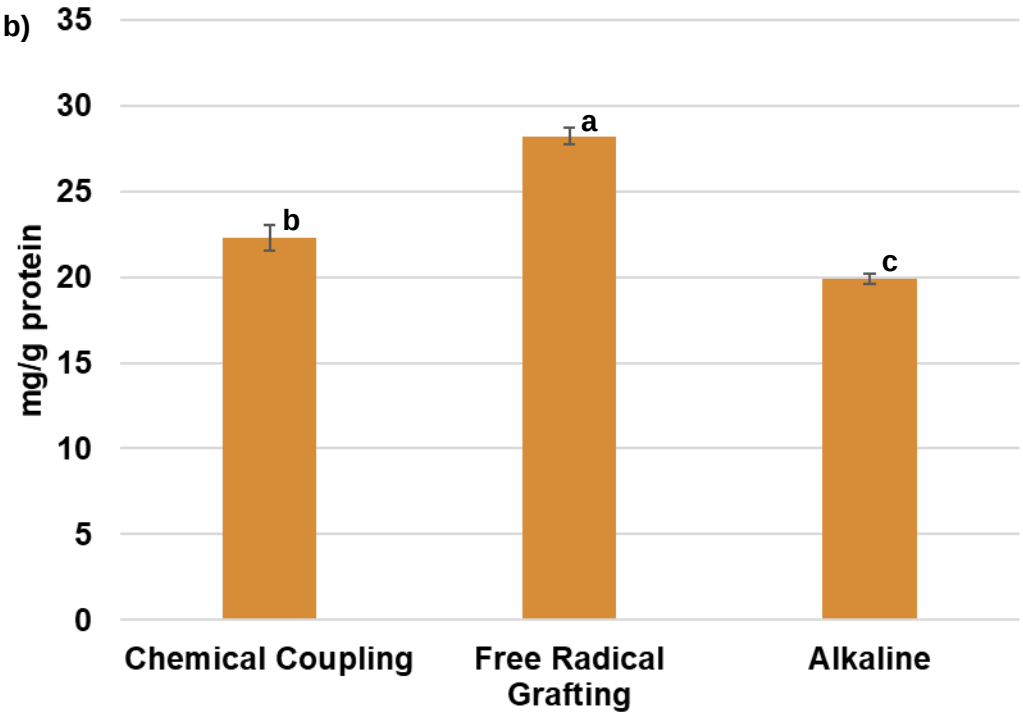


Fig. 4.1.2 Polyphenol content (mg/g protein) in NaCn-FA conjugates prepared by a) non-covalent method (at different pH 6, 7 and 8) b) covalent method (Chemical coupling, Alkaline, Free radical grafting method)

4.2 Characterization of casein-polyphenol (caffeic acid/ferulic acid) conjugates

The conjugation between polyphenol (CA and FA) and sodium caseinate was confirmed by using fluorescence spectroscopy, UV-VIS spectroscopy and Fourier transform infrared spectroscopy (FTIR). The prepared conjugates were further characterized for particle size and zeta potential.

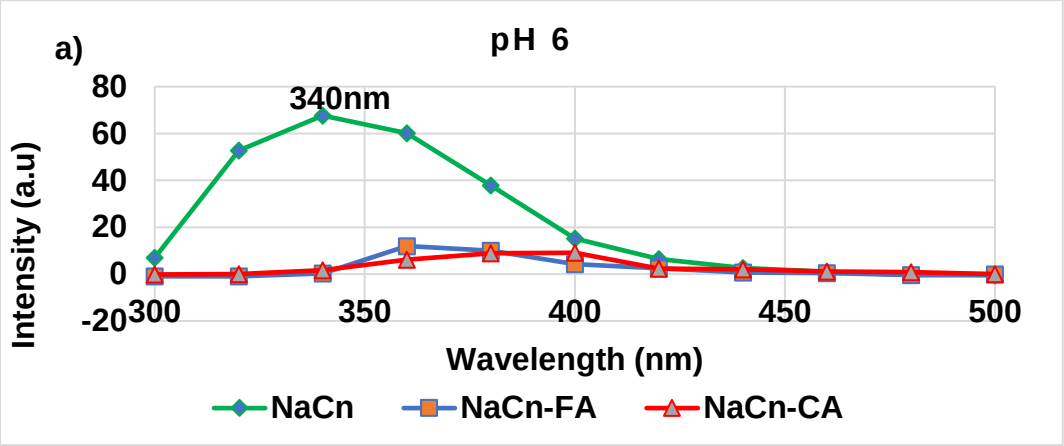
4.2.1 Changes in Fluorescence Spectra

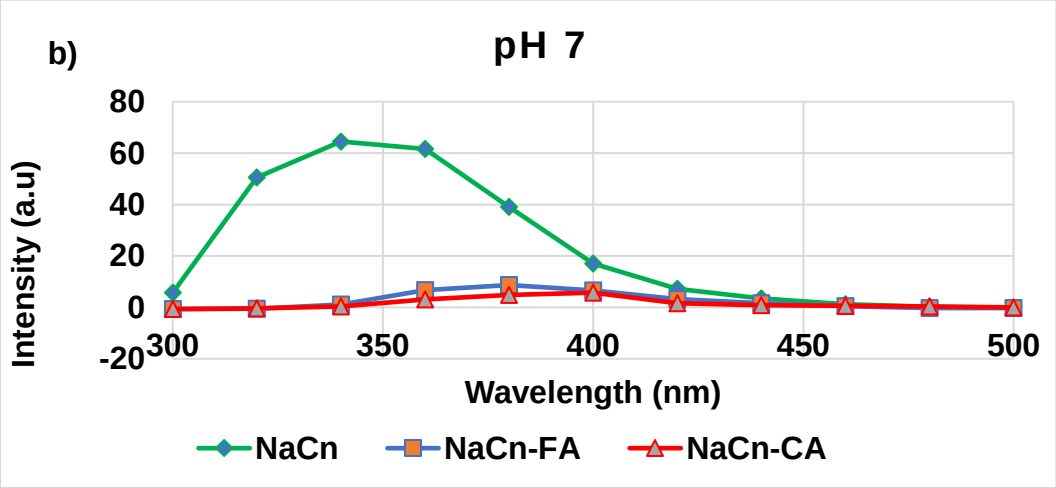
The indole chromophore of aromatic amino acids like tryptophan, tyrosine, and phenylalanine causes protein intrinsic fluorescence (Liu *et al.,* 2019). α-Casein (a mixture of α_{S1} and α_{S2} caseins) has two tryptophan residues, Trp-66 and Trp-37 (in α_{S1} casein) and Trp-109 and Trp193 (in α_{S2} casein), whereas β-casein has only one tryptophan residue, Trp143, which exhibits intrinsic fluorescence (Hasni *et al*.,2011). On conjugation of phenolic compounds to NaCn in NaCn-CA and NaCn-FA, the decrease in intrinsic fluorescence was observed in both non-covalent and covalent conjugates. The drop in intrinsic fluorescence intensity could have been induced by

the attachment of phenolic moieties to protein, which caused a quenching effect and so reduced the emission intensity (Jia *et al*., 2016).

Emission spectra from 300 to 500 nm of control, NaCn-CA and NaCn-FA conjugates prepared by non-covalent approach (pH 6, 7 and 8) are shown in Fig 4.2.1.1. Control NaCn has shown maximum intensity at wavelength (λ) 340nm. On conjugation of polyphenol (both CA and FA), decreased intrinsic fluorescence intensity was observed at all pH values. On conjugation with CA and FA, with decrease in fluorescence intensity, shift in maximum wavelength was also observed. NaCn-CA and NaCn-FA have shown red shift of 60 nm and 40nm at all the pH, respectively. The red shift suggested an increase in polarity of the microenvironment around the tryptophan residues making it more hydrophilic after binding of polyphenols with NaCn. CA being more hydrophilic due to presence of extra OH group than FA has shown higher red shift. Similar results were reported by (Hasni *et al*., 2011) where conjugates of EGCG with casein (α and β) showed upper shift than those with Catechin (C), Epicatechin (EC) and Epigallocatechin (EGC) because EGCG is bulkier than the other polyphenols.(Hasni *et al*., 2011) reported that the emission band of the free protein at 350 nm (α-casein) and 348 nm (β-casein) shifted towards a lower wavelength in the catechin and epicatechin complexes of α- and β-caseins, at 346 nm (C–casein) and 348 nm (EC) for a-casein, and at 334 nm (C–casein) and 343 nm (EC–casein) .The emission band of free caseins, on the other hand, shifted towards a higher wavelength for α-casein at 351 nm (EGC–casein) and 365 nm (EGCG–casein), and for β-casein complexes at 349 nm (EGC–casein) and 367 nm (EGCG–casein). (Jiang *et al.,* 2018) reported that after interacting with chlorogenic acid, the maximum emission peak for CN (casein) shifted from 348 to 366 nm. The fluorescence intensity was also significantly reduced when chlorogenic acid was added which was due to the fact that protein unfolding and exposing tryptophan residues to more hydrophilic environments in turn resulting in a lower quantum yield. Quenching of intrinsic fluorescence of NaCn was more significant in covalent conjugates as shown in Fig. 4.2.1.2 for both NaCn-CA and NaCn-FA. The quenching of fluorescence intensity was >90% in covalent conjugates and ~ 85% in non-covalent conjugates. This can be hypothesized that in non-covalent conjugates, polyphenols bind largely to fluorophores on the surface of caseins due to intramolecular interactions, such as hydrogen bonds that tighten the protein structure and bury tryptophan in a more hydrophobic environment (Hasni *et*

al., 2011). However, the hidden tryptophan is likely to be revealed in covalent conjugates, resulting in higher quenching of fluorescence. The NaCn-CA conjugates have shown higher quenching than NaCn-FA conjugates irrespective of the method used and this may be attributed to higher binding of CA than FA which was also confirmed by higher total phenol content in NaCn-CA.





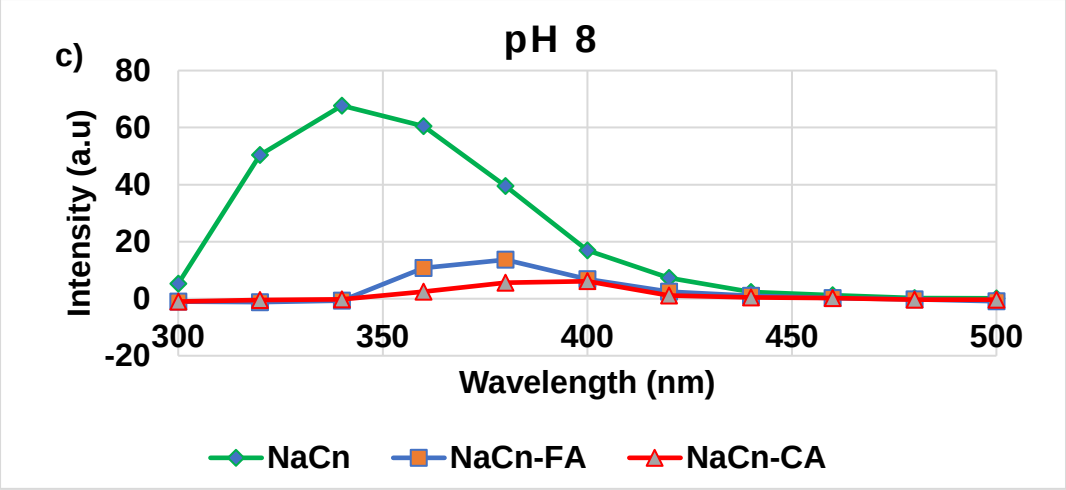
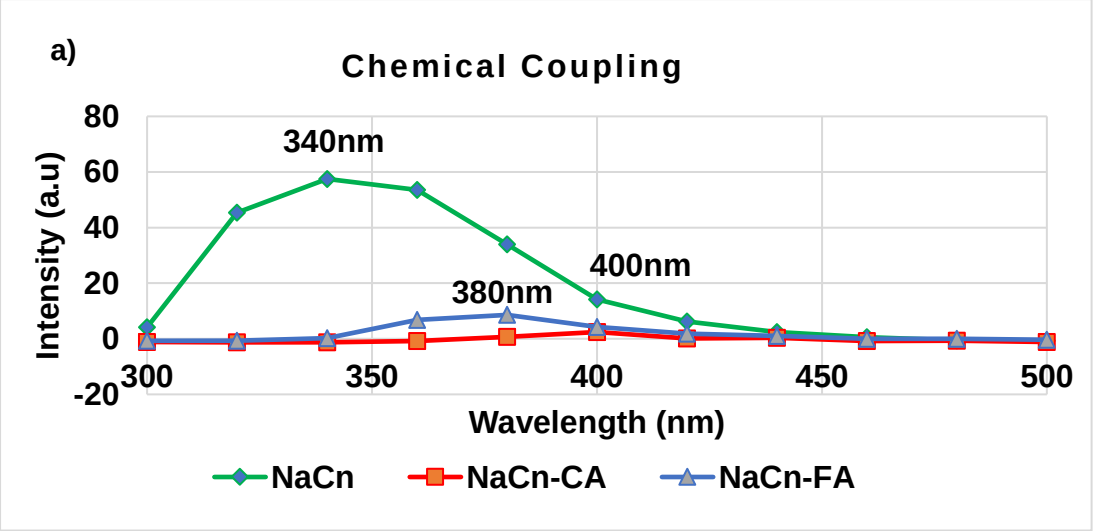
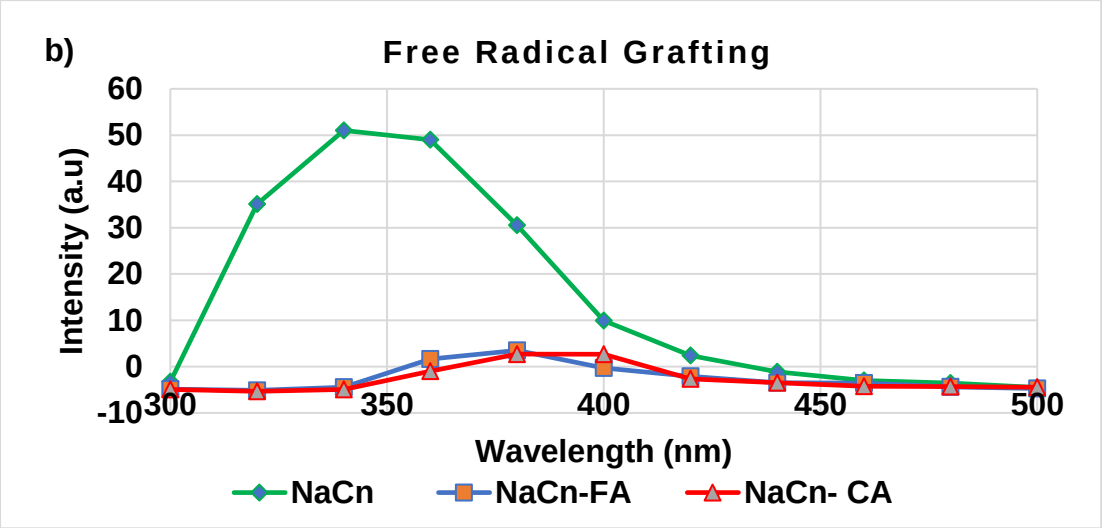


Fig.4.2.1.1. Intrinsic fluorescence of control NaCn and non-covalent conjugates of NaCn-FA and NaCn-CA at a) pH 6, b) pH 7 and c) pH 8





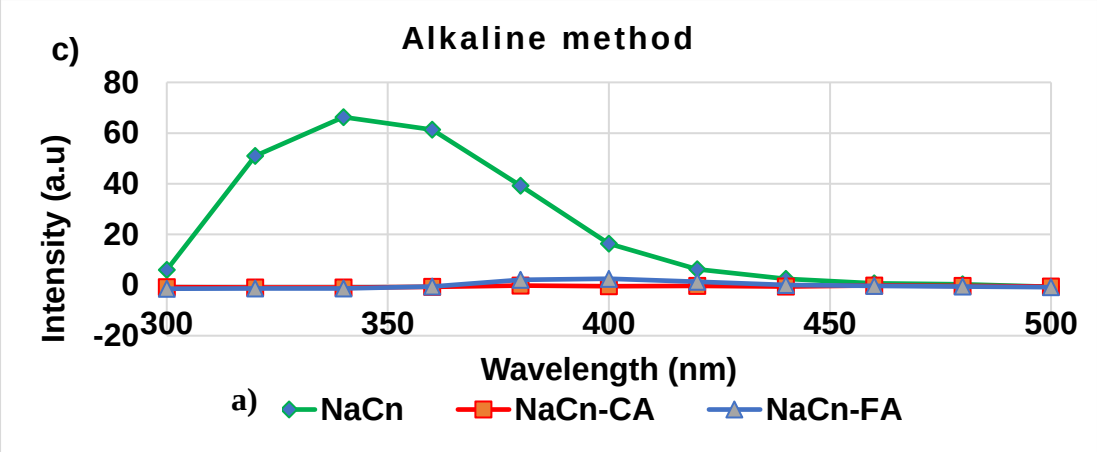


Fig.4.2.1.2. Intrinsic fluorescence of control NaCn, covalent conjugates of NaCn-CA and NaCn-FA at a) Chemical Coupling b) Free Radical Grafting and c) Alkaline method

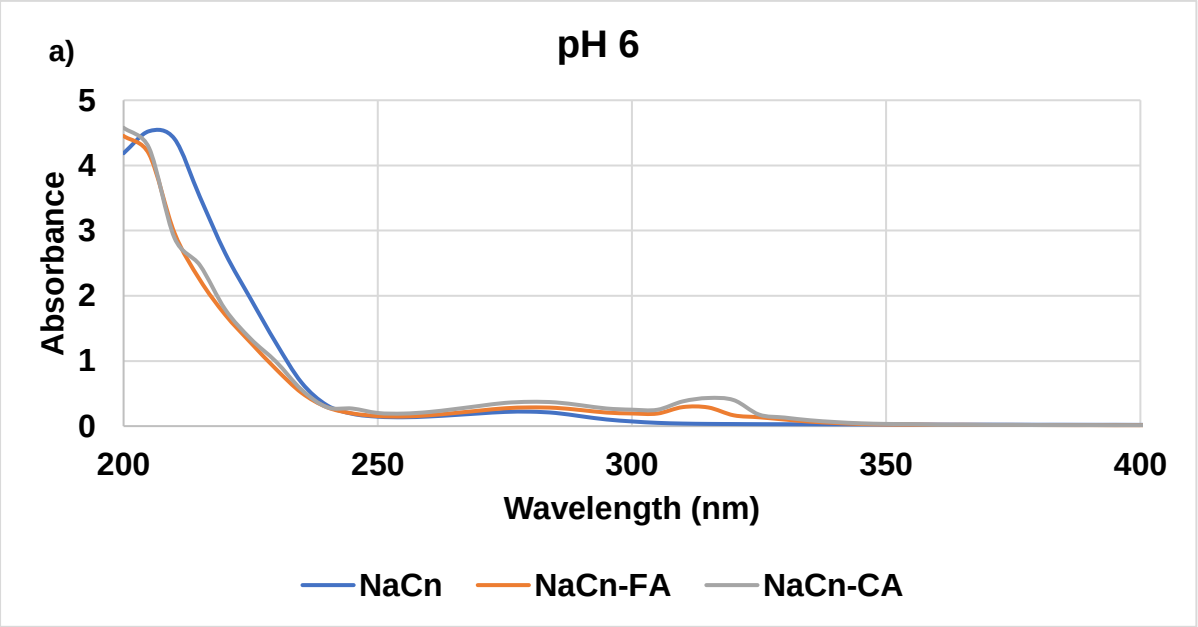
4.2.2 Change in UV-visible spectra

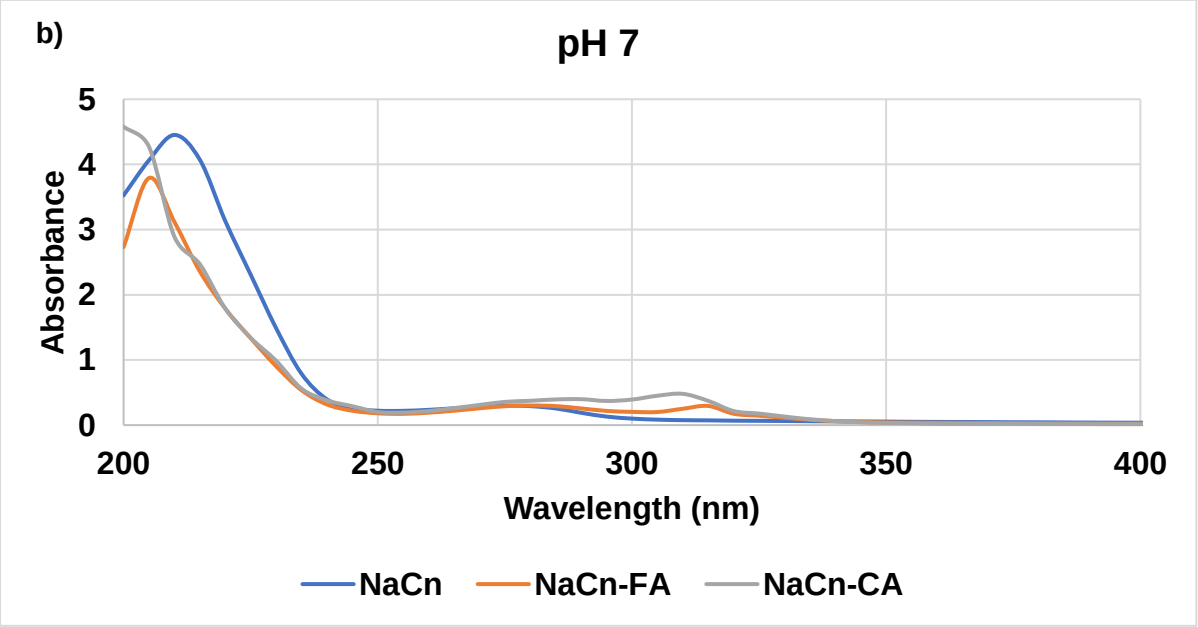
The phenolic–protein conjugates can be confirmed using UV spectroscopy. Phenolic molecules differ substantially from proteins in terms of UV spectroscopy. UV absorption maxima for proteins are 214 nm for peptide bond absorption and 280 nm for aromatic amino acids tryptophan, tyrosine, and phenylalanine absorption (Liu *et al*., 2019). CA have UV absorption maxima of 295 and 327 nm, respectively, whereas FA have UV absorption maxima of 287 and 312 nm.

As shown in the Fig. 4.2.2.1 the absorbance of the non-covalent conjugate (NaCn-CA and NaCn-FA) at all pH values was appreciably lower near 214 nm and slightly higher at 270-280 nm and 305–330 nm than that of the control NaCn. The decreased absorbance near 214 nm was due to conjugation of phenolic compounds with proteins and slightly increased absorbance at 280-335 wavelength that may be due to strong absorptions of CA and FA in the wavelength of 315-400 nm (Michael *et al*., 2016).

The Fig 4.2.2.2 represent the UV-Vis spectra of covalent conjugates of NaCn-CA and NaCn-FA. With comparison to control NaCn, the changes in UV-Visible spectra of covalent conjugates were more pronounced as compared to non-covalent conjugates. In covalent conjugates, higher absorbance at 215 – 400 nm was due to more exposure of hidden tryptophan residues in covalent method of preparation of conjugates. Significant absorption was seen in all covalent conjugates in the 280-290 nm and 310-

330nm. This was due to the formation of covalent linkages between heteroatom in the side chains of proteins and aromatic ring of phenolic compounds (Lui *et al*., 2019)





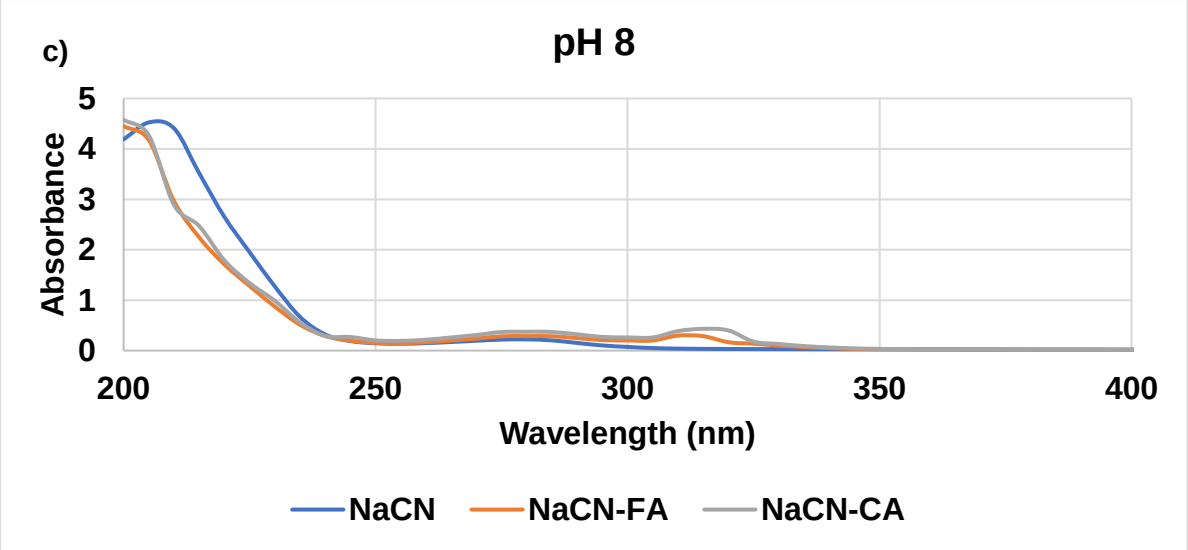
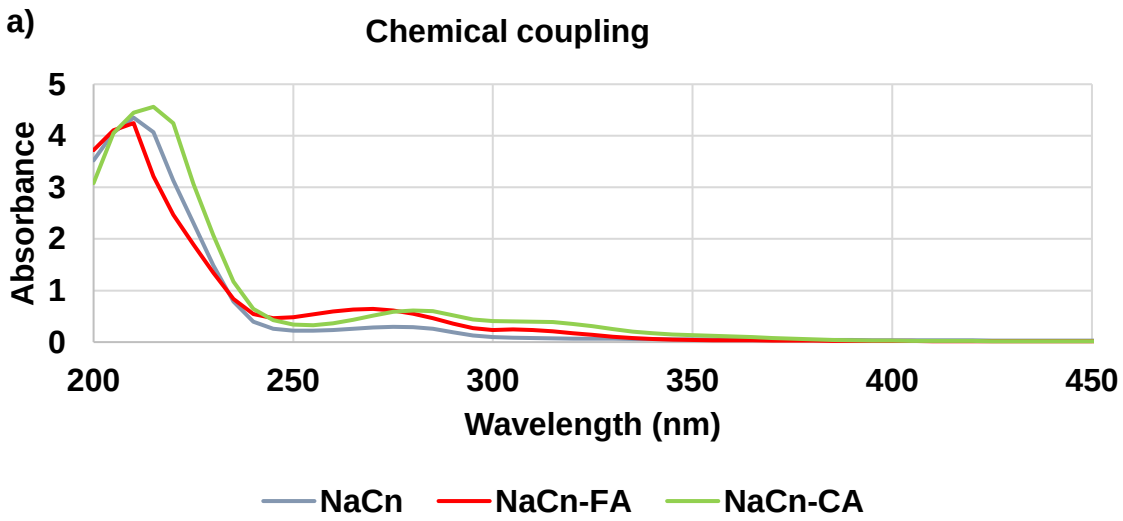
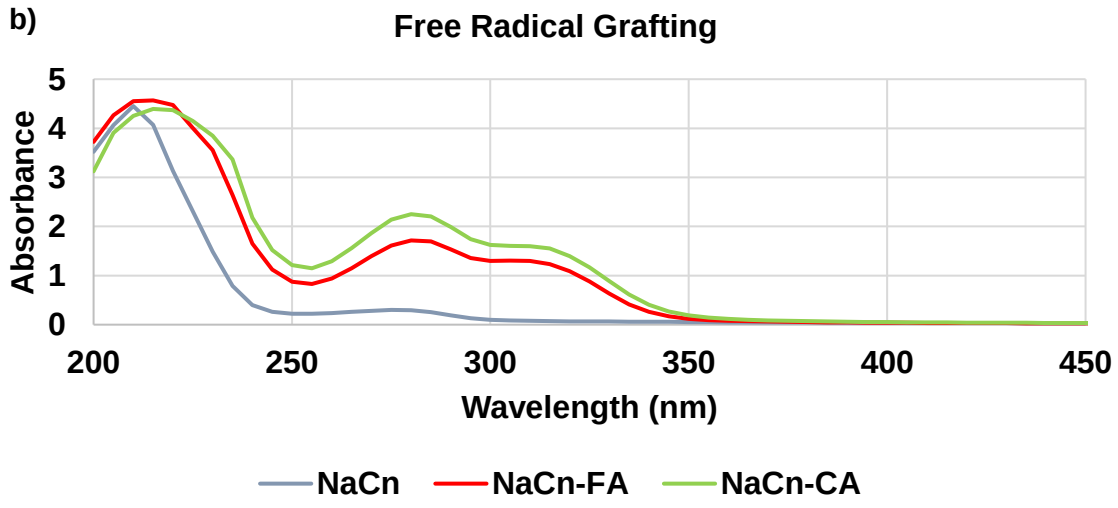


Fig. 4.2.2.1. UV-Visible spectroscopy of control NaCn and non-covalent conjugates of NaCn-FA, NaCn-CA at a) pH 6, b) pH 7 and c) pH 8





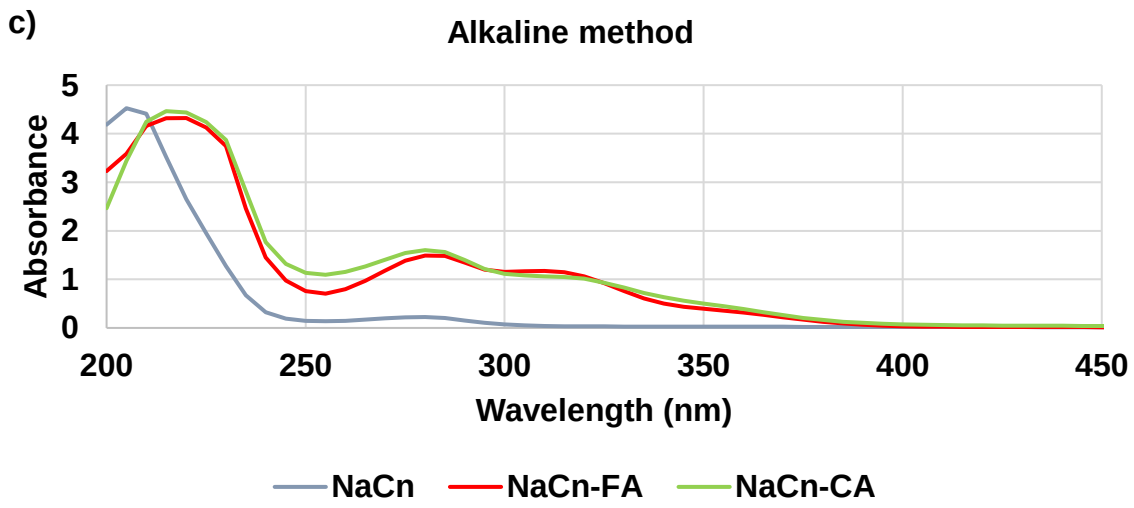


Fig. 4.2.2.2. UV-Visible spectroscopy of control NaCn and Covalent conjugates of NaCn-FA, NaCn-CA at a) Chemical Coupling b) Free Radical Grafting and c) Alkaline method

4.2.3 Change in Fourier transform infrared spectroscopy (FTIR) spectra

FT-IR spectroscopy is a good approach for measuring the change in the secondary structures of proteins following conjugation with phenolic chemicals by analysing the frequency of amide bonds. FT-IR spectroscopy can also be used to investigate the molecular interactions between phenolic chemicals and proteins (Liu *et al*., 2019). The amide I band (1600–1700 cm-1) represents the vibration of the peptide bond's C=O stretching, whereas the amide II band (1500–1600 cm-1) is principally responsible for the vibrations of N-H bending and C-N stretching. Both the amide I and amide II bands are composed of overlapping bands with different frequencies that correspond to secondary structure elements such as α-helices, β-sheets, turns, and disordered structures. As a result, the amide I and amide II bands' frequencies are particularly sensitive to the secondary structure of the polypeptide chain.

The FTIR spectra (1500-1800 cm-1 wavenumber) for non-covalent conjugates and covalent conjugates of NaCn-CA and NaCn-FA is presented in Fig. 4.2.3.1 and 4.2.3.2, respectively. The spectral changes were more visible in covalent conjugates than non-covalent conjugates. Along with decrease in the intensity for amide I and amide II region in the conjugates spectral shift from 1638 cm-1 to 1635 cm-1 and from 1535 cm-1 to 1527 cm-1 in amide I and amide II was observed, respectively.

Further, peak fitting analysis was performed in amide I region (1600-1700 cm-1) using Originpro software and results were presented in Fig 4.2.3.3 to 4.2.3.8 for both non-covalent and covalent conjugates of NaCn-CA and NaCn-FA.

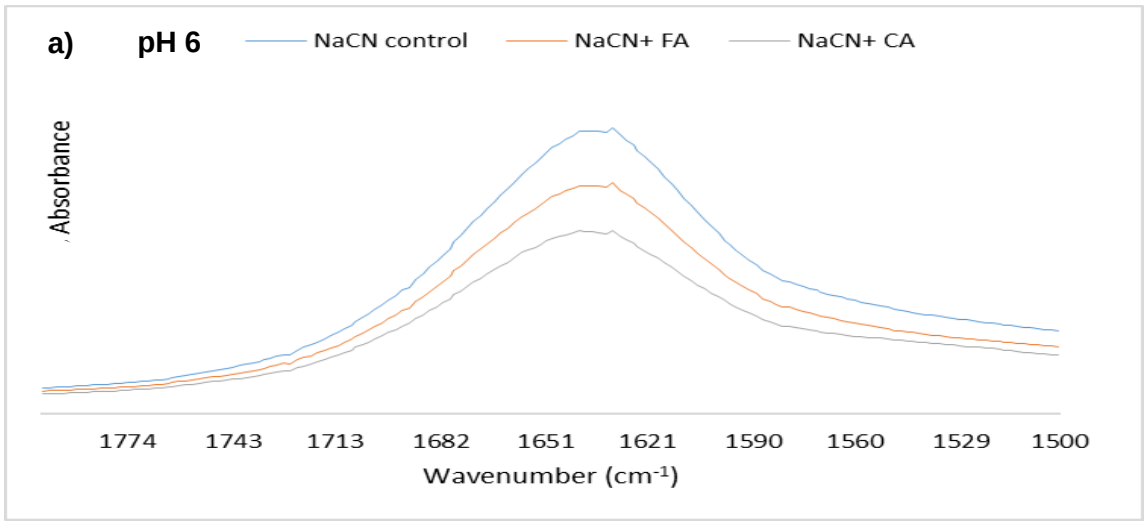
As shown in the Fig 4.2.3.5 for control NaCn, peaks were observed near at 1614, 1631, 1641, 1664 and 1689. For all non-covalent conjugates of NaCn-CA, similar peaks were observed except for pH 6 and 8 (Fig 4.2.3.5) where peak at 1664 was shifted to 1660 cm-1. For non-covalent conjugates of NaCn-FA (Fig. 4.2.3.6), similar peaks were observed except peak at 1664 was shifted to 1662 at all pH values and peak at 1689 shifted to 1687 at pH 7 and 8. On analysing peaks, it was observed that shifting of peaks was more in covalent conjugates of both CA and FA. As shown in the Fig 4.2.3.7 covalent conjugate of NaCn-CA prepared by chemical coupling method have shown one extra peak near 1654 cm-1, and shifting of peak from 1689 to 1687 compared to control NaCn was observed. For the conjugates prepared by alkaline method the shifting of peak from 1664 to 1656 and 1689 to 1687 was observed. For free radical grafting method shifting of 1664 to 1660 and 1689 to 1687 was observed. For NaCn-FA conjugates (as shown in Fig. 4.2.3.6) prepared by chemical coupling method have shown shifting of peak from 1689 to 1687 and 1664 to 1660. In the conjugates prepared by alkaline method, the peak at 1641 and 1689 has disappeared and peak from 1664 was shifted to 1656. In conjugates prepared by free radical grafting method, shifting of peak from 1664 to 1662 was observed and peak near 1641 was disappeared.

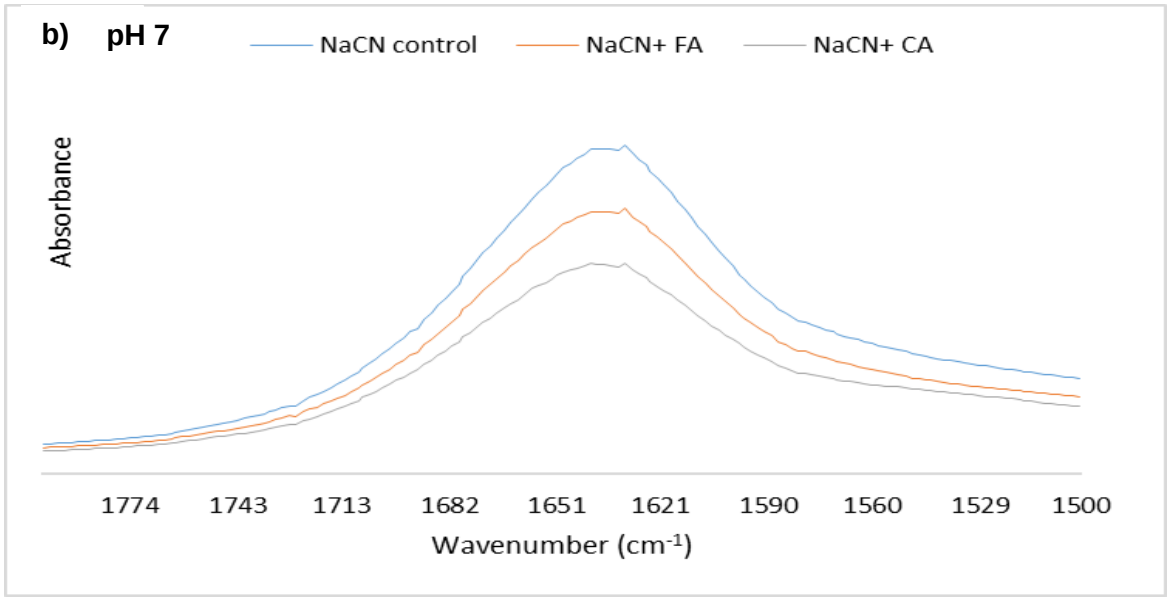
The individual underlying secondary structure components cannot be readily observed in the amide I band as shown in the Fig 4.2.3.1 and 4.2.3.2. This is due to the fact that the width of the various component bands is greater than the gap between the peaks of the individual component bands. To find the hidden peaks, the second derivative of the spectrum was constructed as a band narrowing/peak sharpening method. The second derivative spectra of non-covalent and covalent conjugates of NaCn-CA and NaCn-FA is presented in Fig 4.2.3.7 and 4.2.3.8, respectively.

(Jiang *et al*., 2018) studied the effects of the interaction of casein (CS) with chlorogenic acid and reported similar results. They reported that the amide I and amide II of casein shifted from 1638.4 cm-1 to 1635.1 cm-1 and from 1534.7 cm-1 to 1516.9 cm-1 with the addition of chlorogenic acid (CA), respectively. The movement of the

amide I and amide II indicate that the β-sheet content is increasing while the α-helix content is decreasing. Polyphenols cause a decrease in α-helix content, which indicates that protein structure is loosening.

(Hasni *et al*., 2011) studied the interaction between α and β-caseins with tea polyphenols like catechin (C), epicatechin (EC), epigallocatechin (EGC) and epigallocatechin gallate (EGCG) and reported that decrease intensity at amide band I suggests a major reduction of protein α-helical structure which basically happens at high polyphenol concentrations. They also reported that the conformational changes observed in ECG and EGCG complexes were more pronounced than in C and EC complexes which indicates that larger and bulkier polyphenols have a greater impact on the secondary structure of casein.





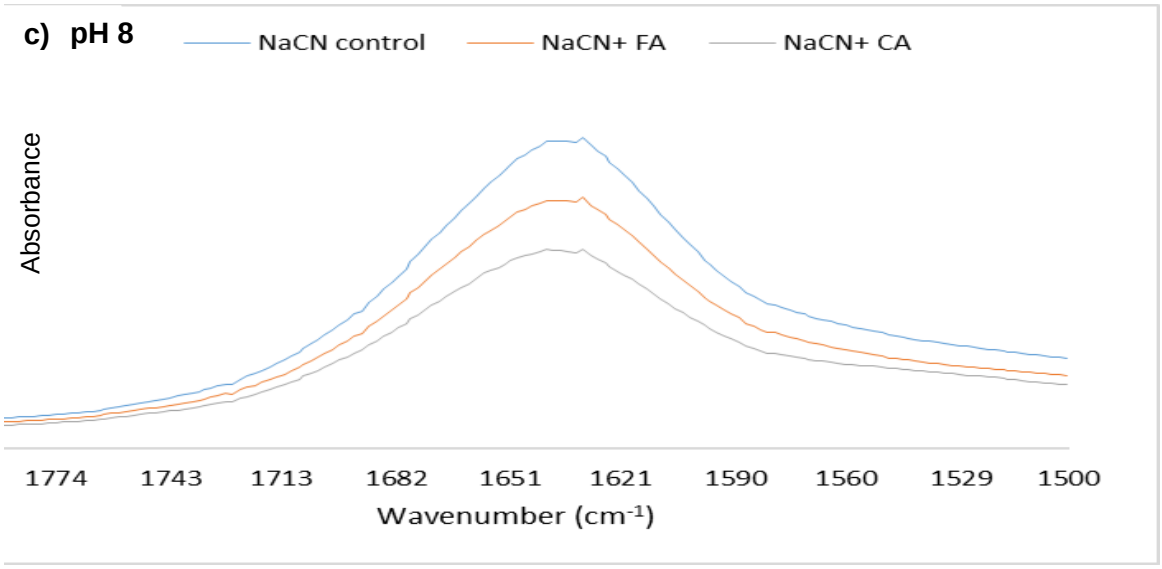
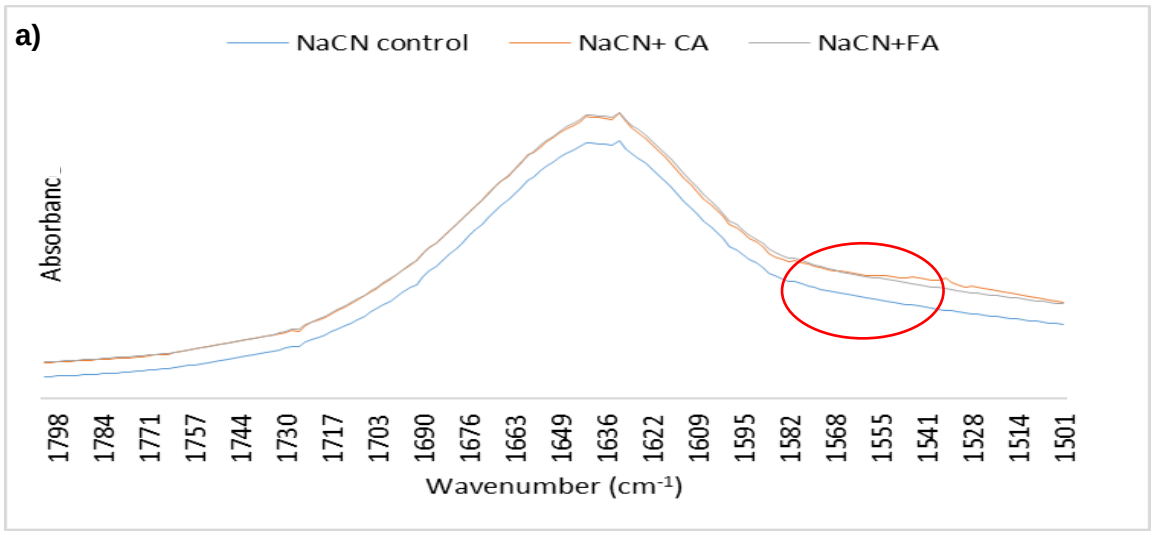
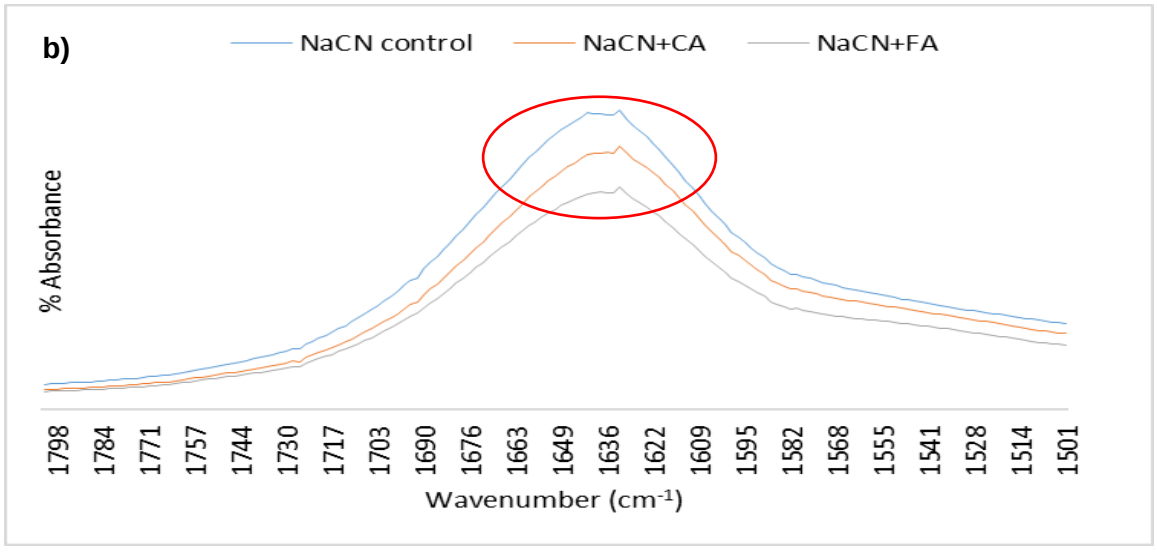


Fig. 4.2.3.1. FTIR spectra of control NaCn and non-covalent conjugates of NaCn-CA and NaCn-FA at a) pH 6, b) pH 7 and c) pH 8





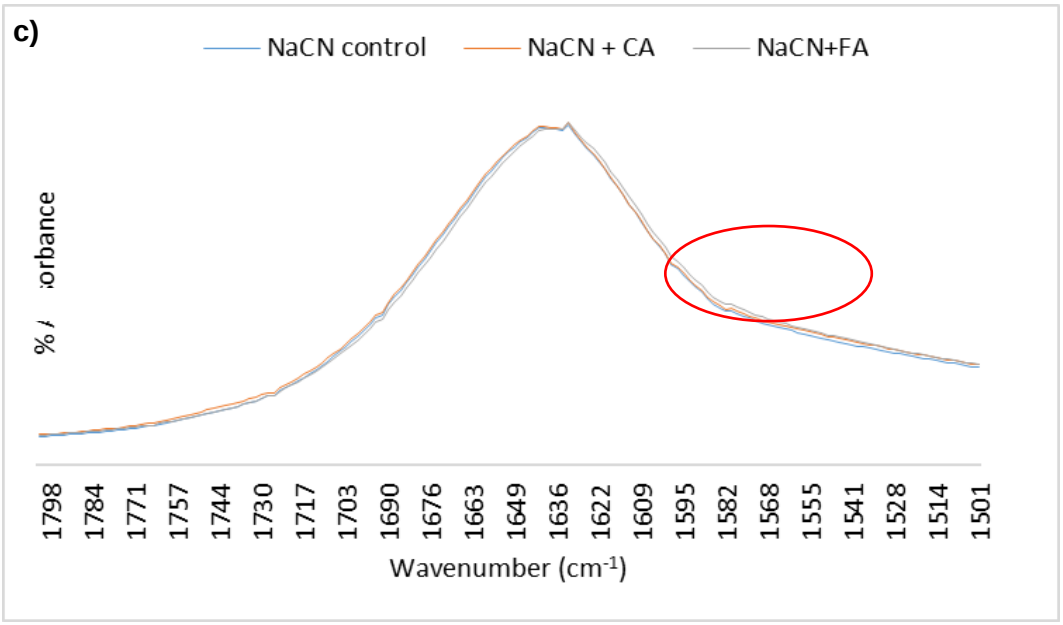
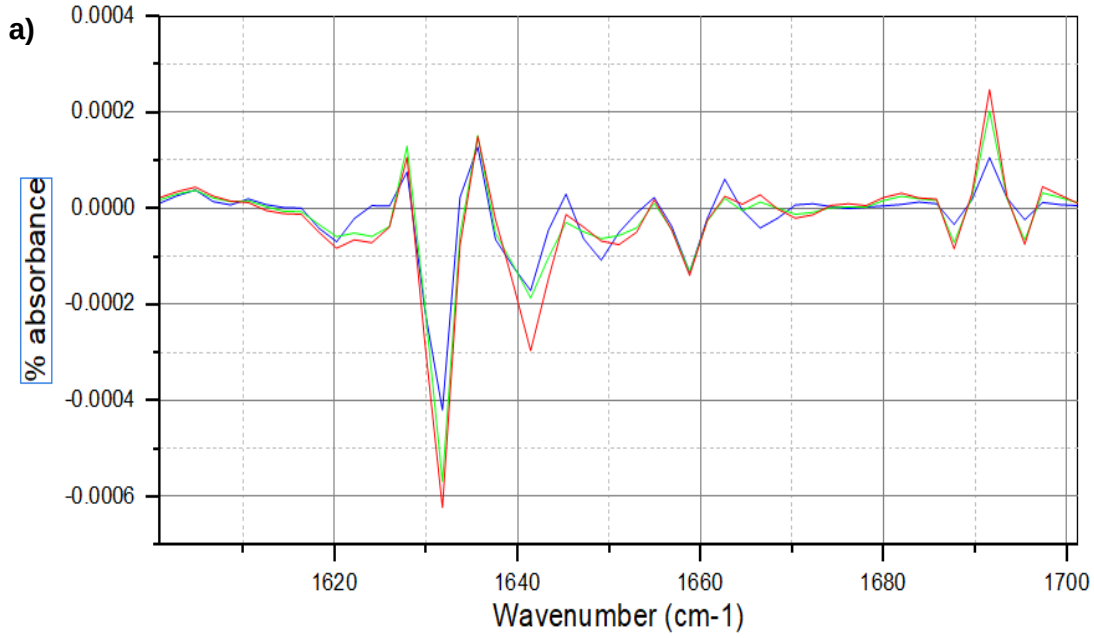


Fig. 4.2.3.2. FTIR spectra of control NaCn and covalent conjugates of NaCn-CA and NaCn-FA under a) Chemical Coupling b) Free Radical Grafting and c) Alkaline method



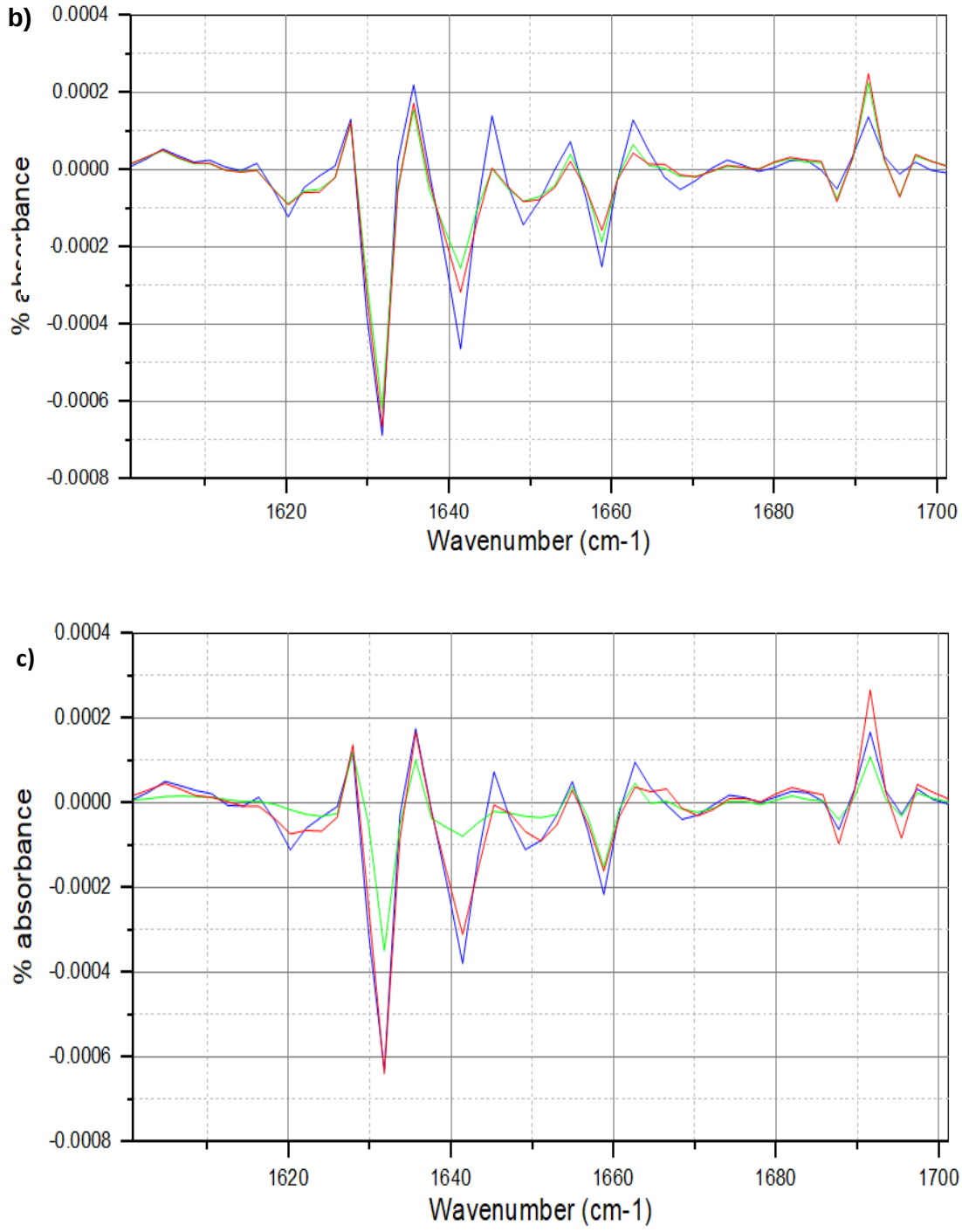


Fig. 4.2.3.3. Second derivative spectral analysis of FTIR spectra of non-covalent NaCn-CA and NaCn-FA conjugates at a) pH 6, b) pH 7 and c) pH 8

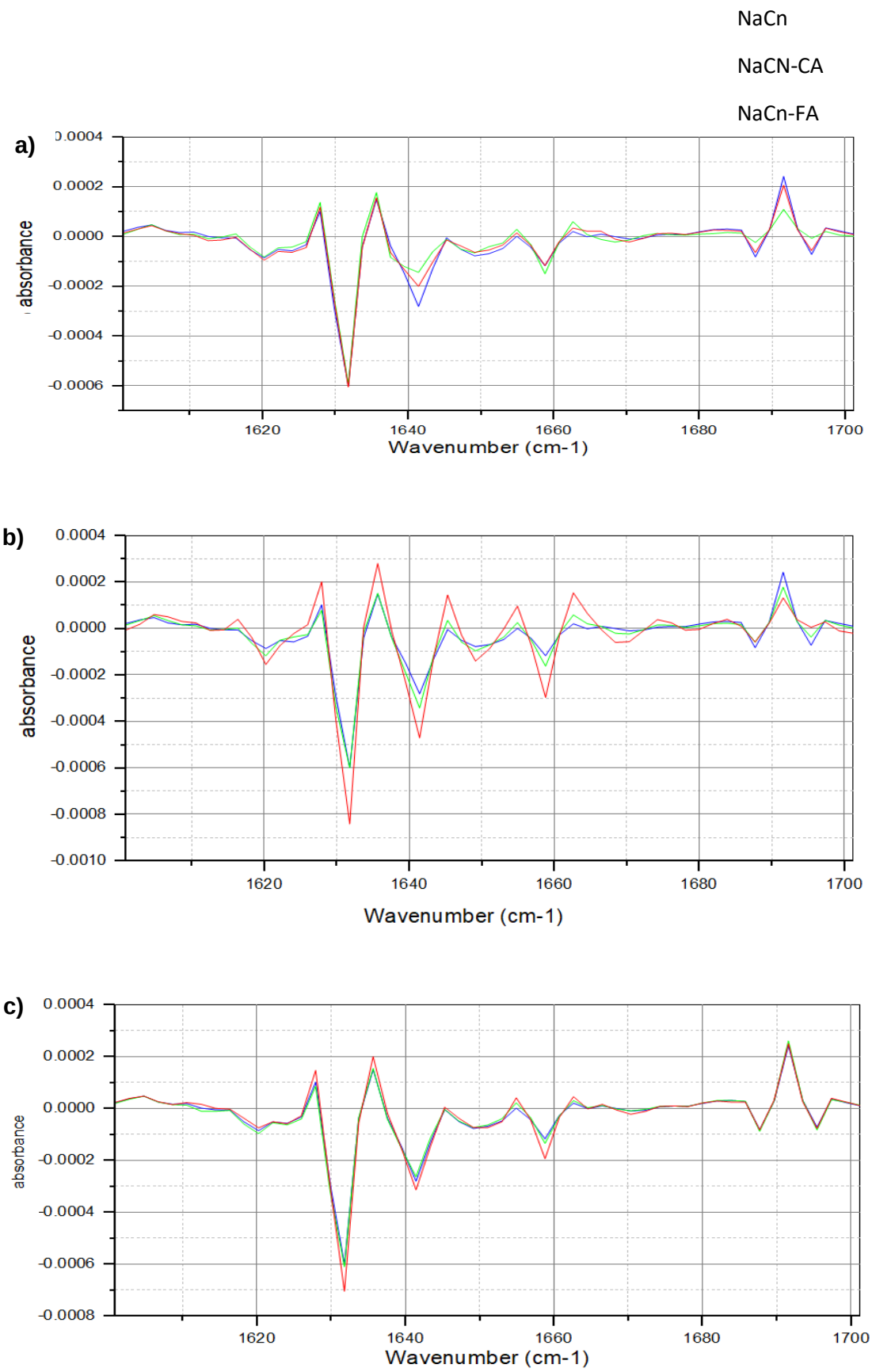
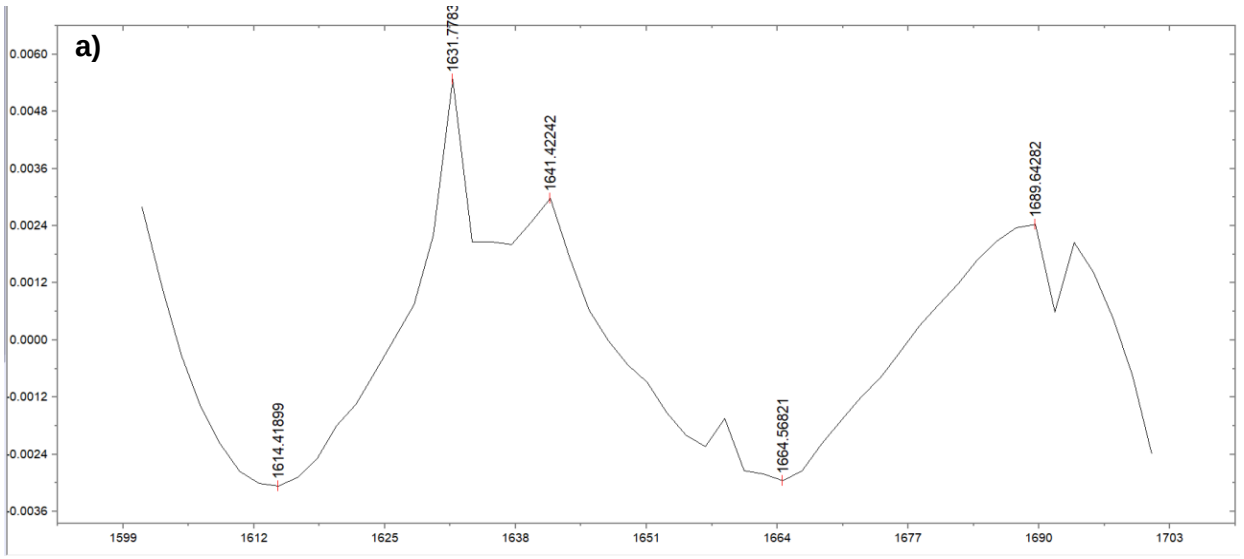
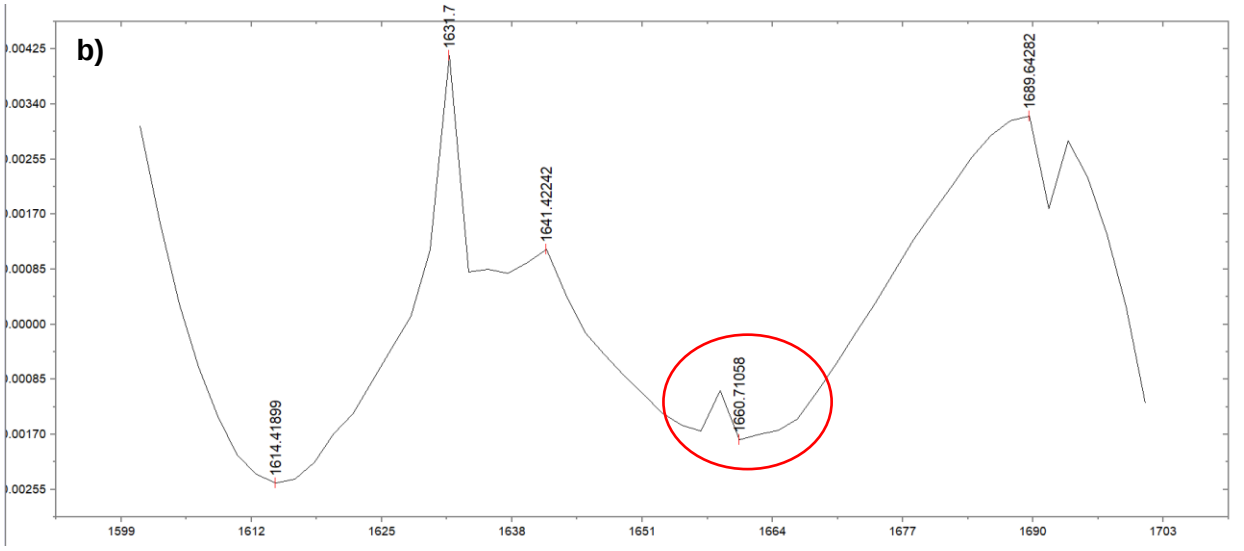
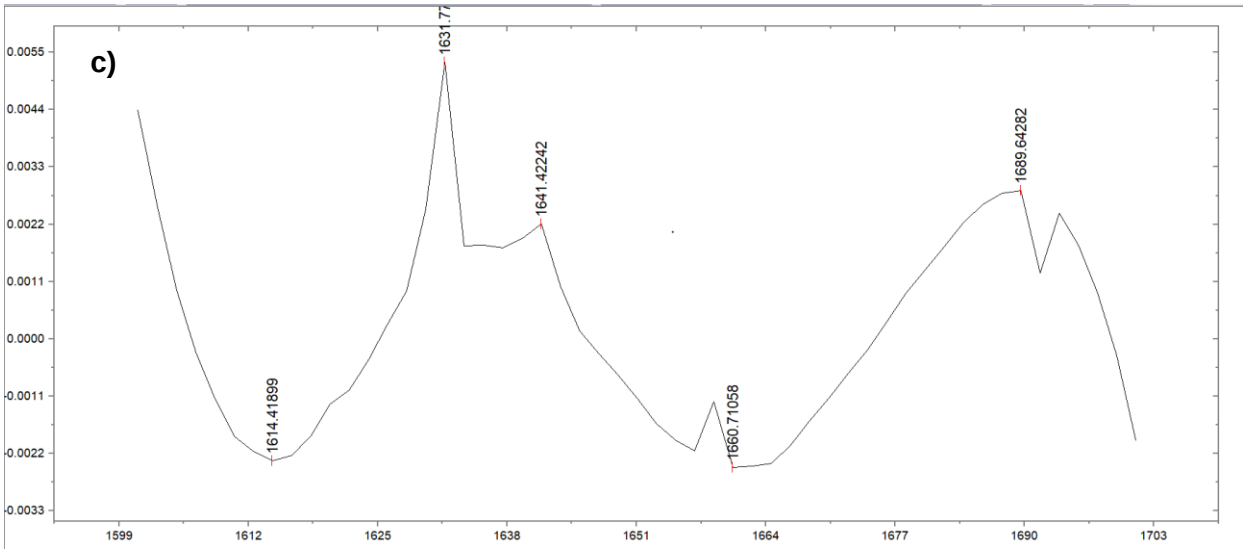


Fig. 4.3.3.4. Second derivative spectral analysis of FTIR spectra of covalent NaCn-CA and NaCn-FA conjugates prepared by a) Chemical Coupling, b) Free Radical Grafting and c) Alkaline method

Peak fitting analysis in FTIR spectra of conjugates







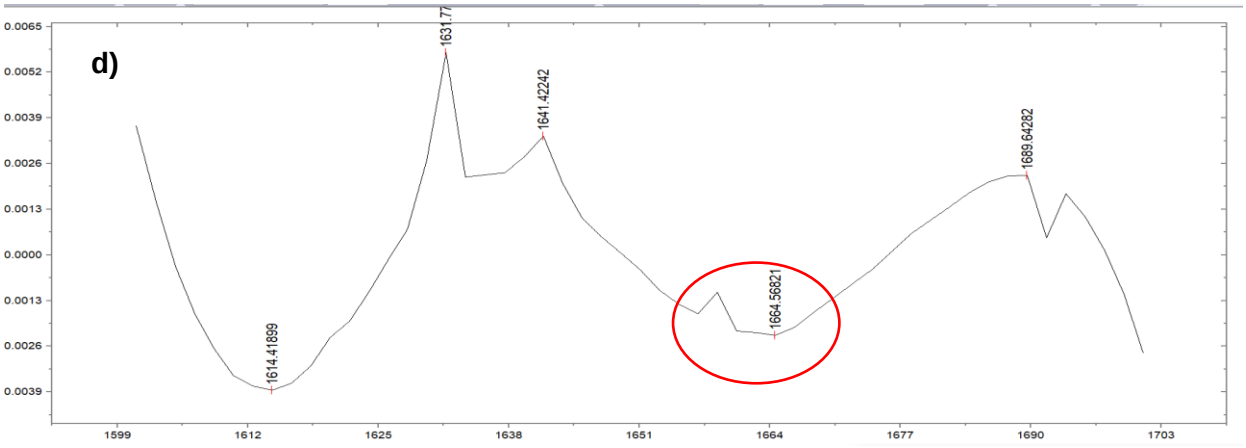
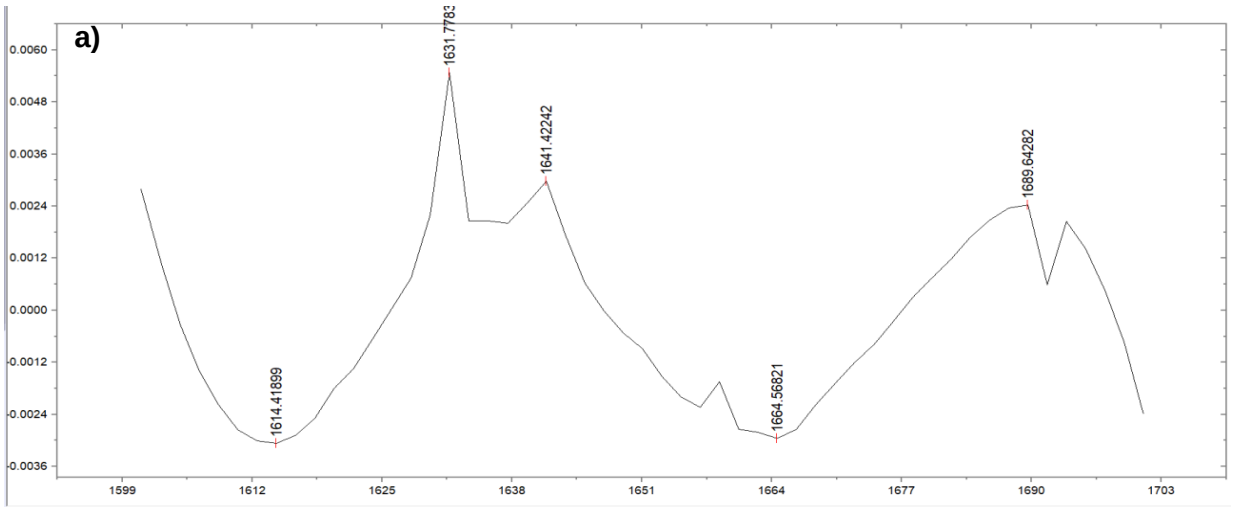
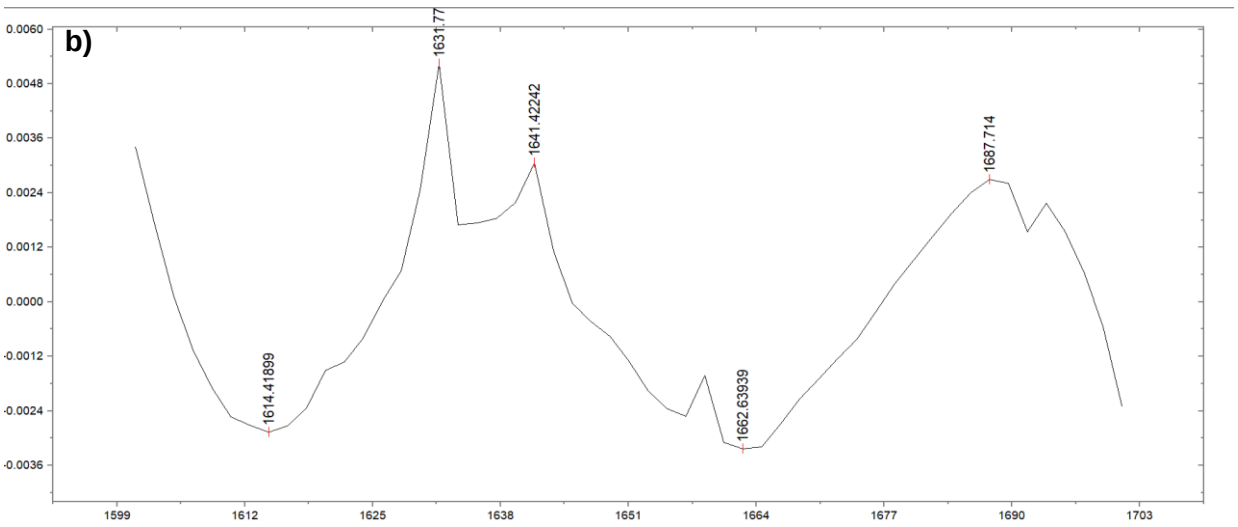


Fig 4.3.3.5 Peak fitting analysis in FTIR spectra of control NaCn (a) and non-covalent conjugates of NaCn-CA at b) pH 6, c) pH 7 and d) pH 8





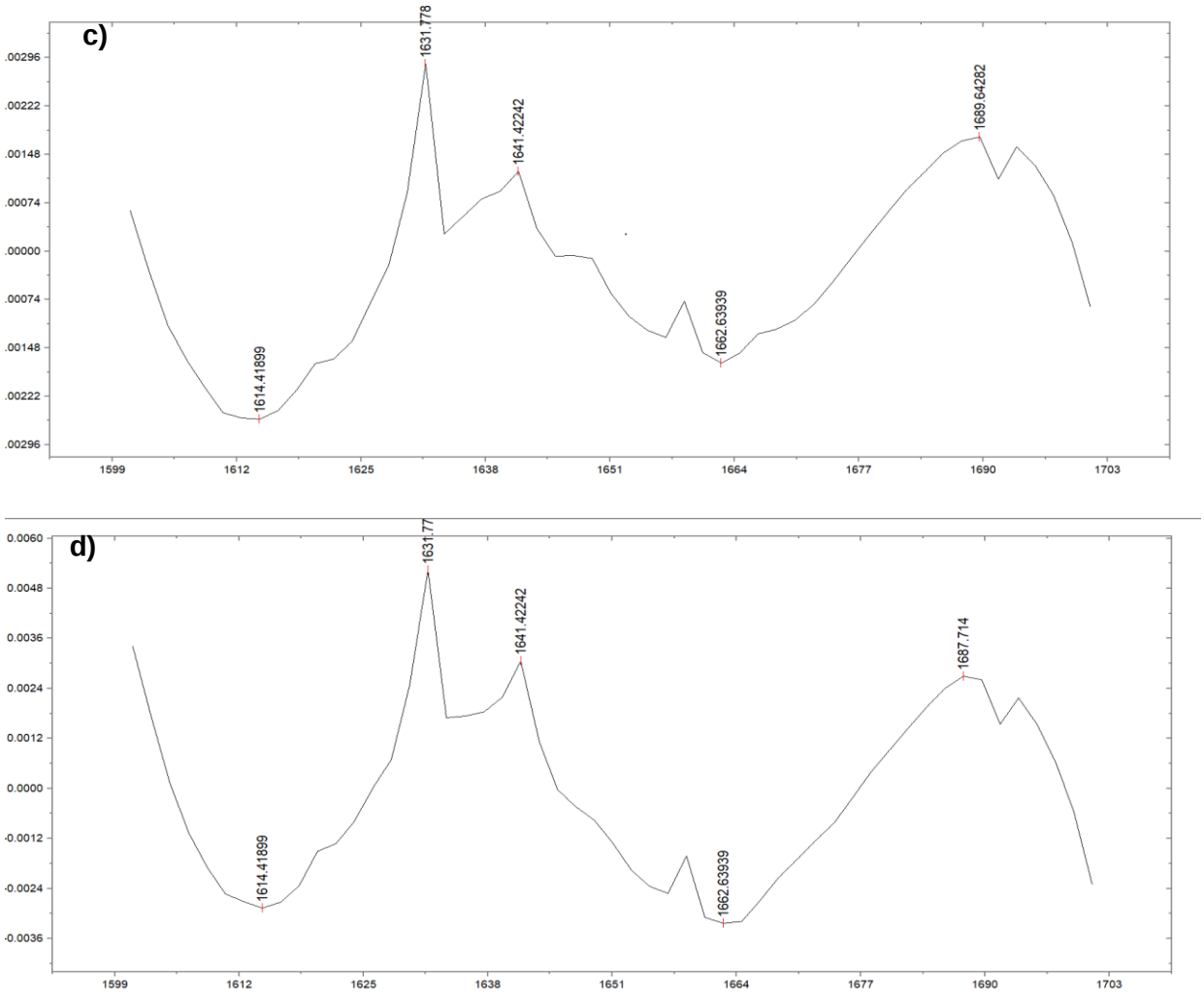
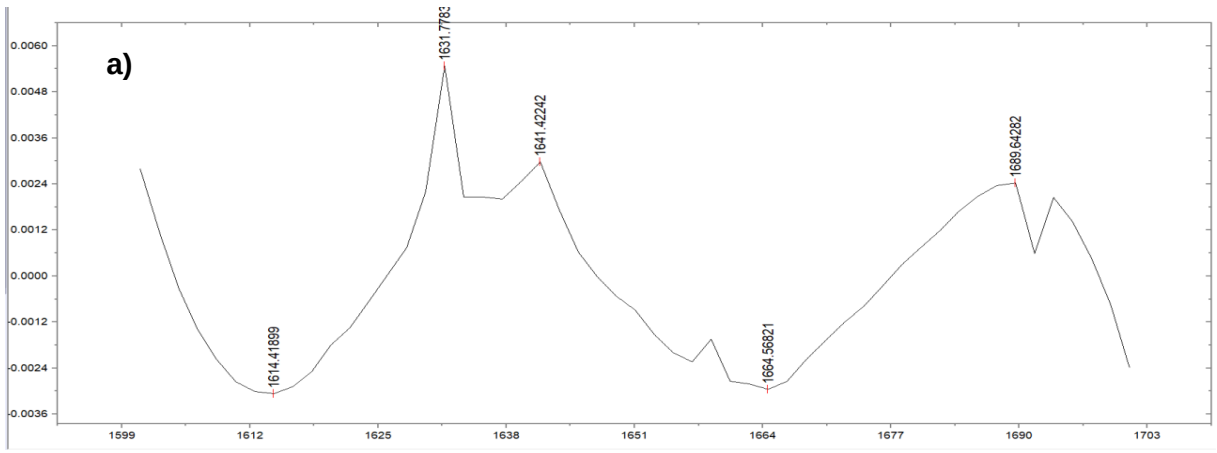


Fig 4.3.3.6. Peak fitting analysis in FTIR spectra of control NaCn (a) and non-covalent conjugates of NaCn-FA at (b) pH 6, (c) pH 7 and (d) pH 8



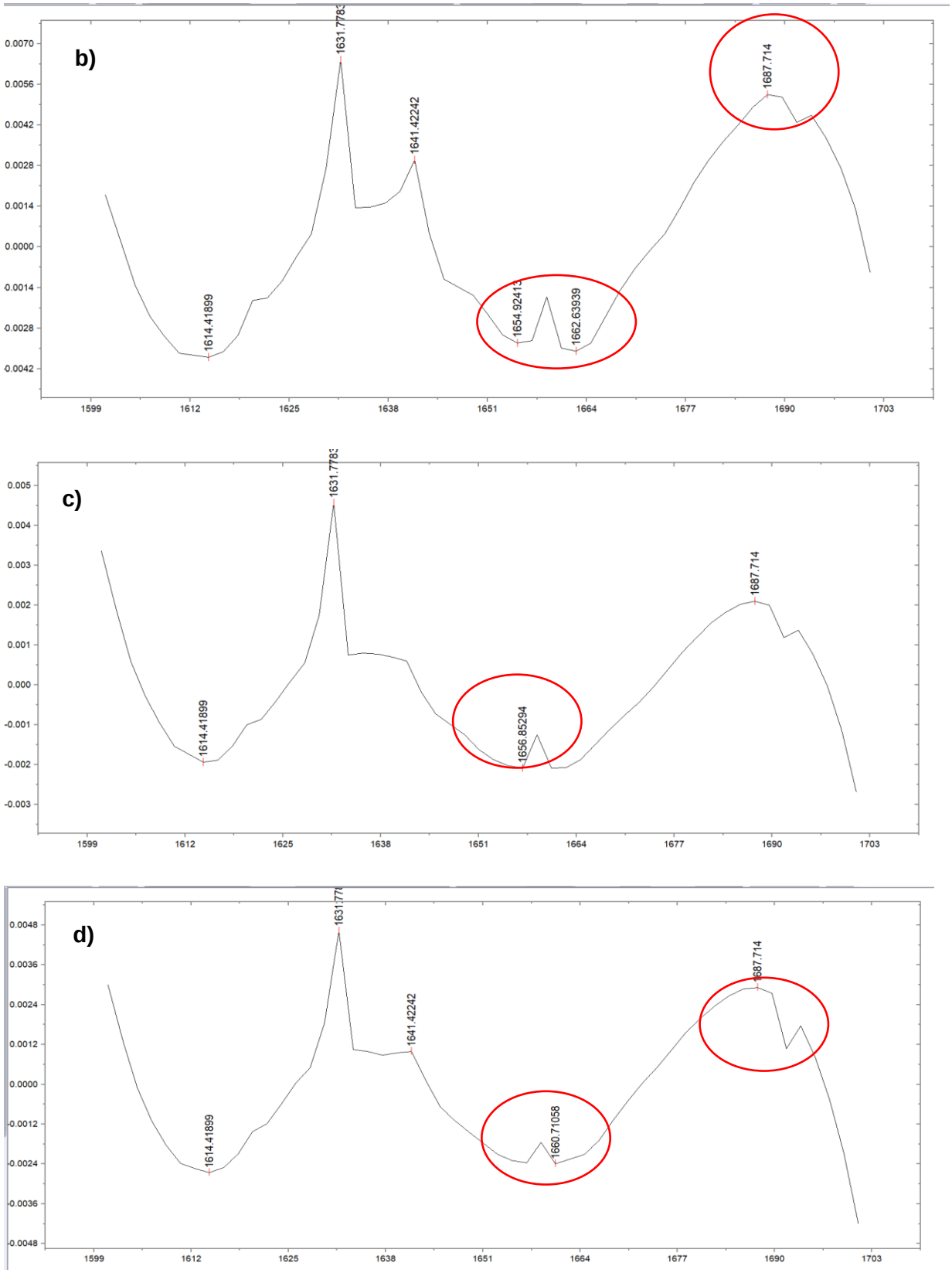
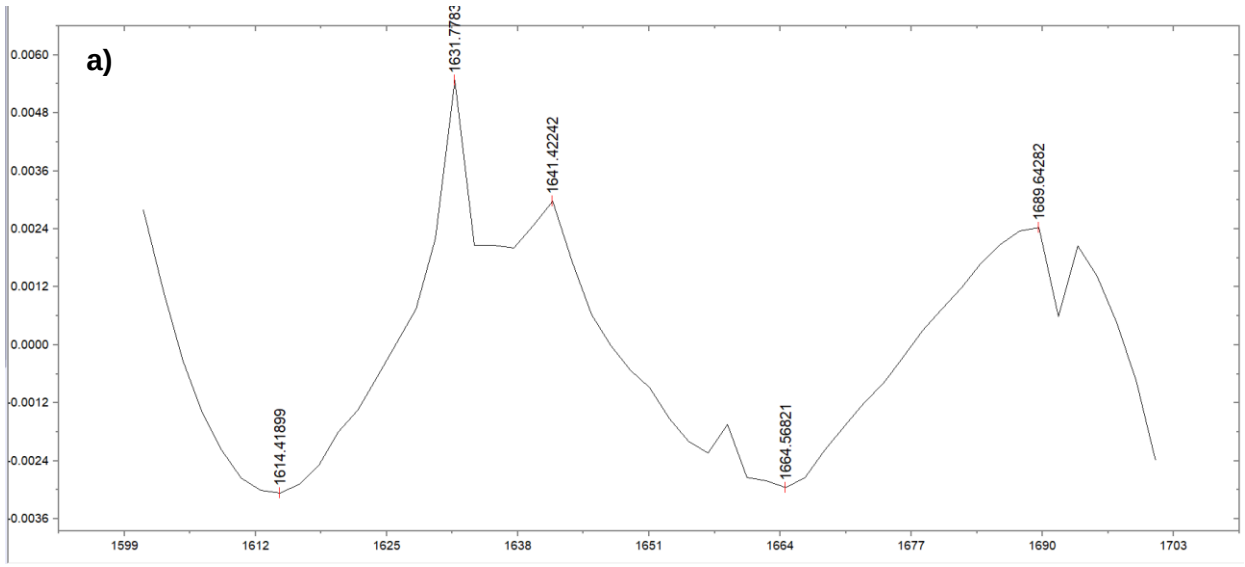


Fig 4.3.3.7 Peak fitting analysis in FTIR spectra of control NaCn (a) and covalent conjugates of NaCn-CA prepared by (b) Chemical Coupling, (c) Free Radical Grafting and (d) Alkaline method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0.0012
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-0.0036
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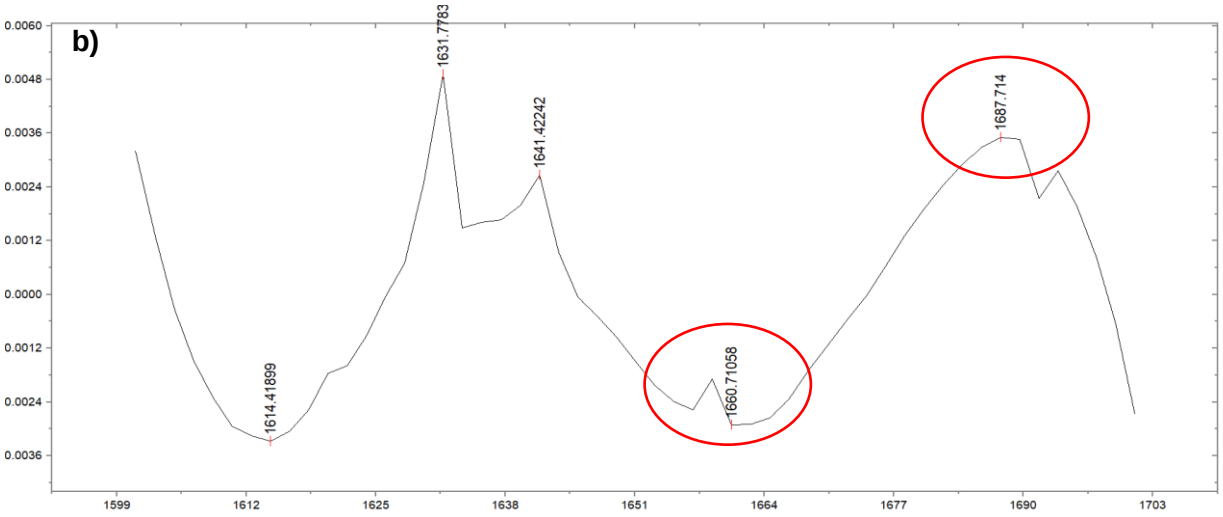
1703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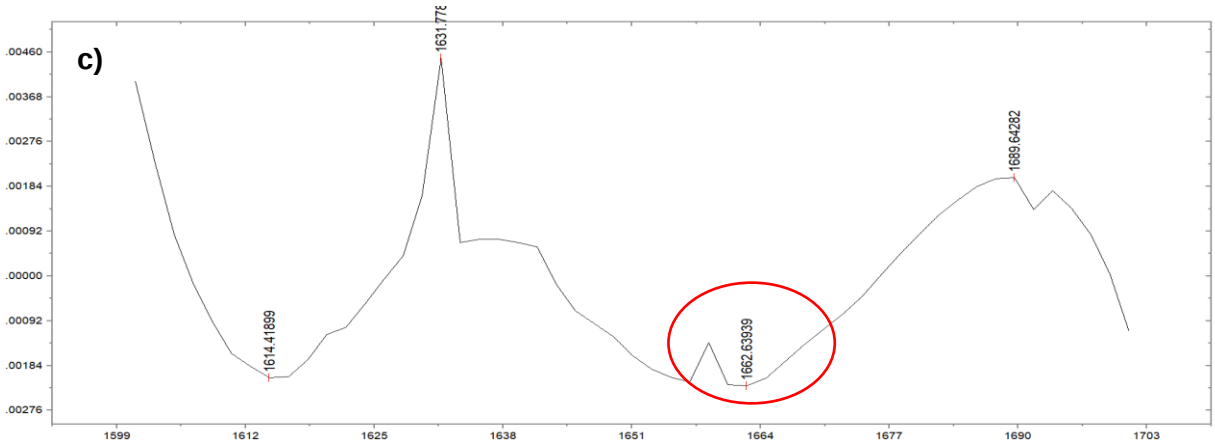
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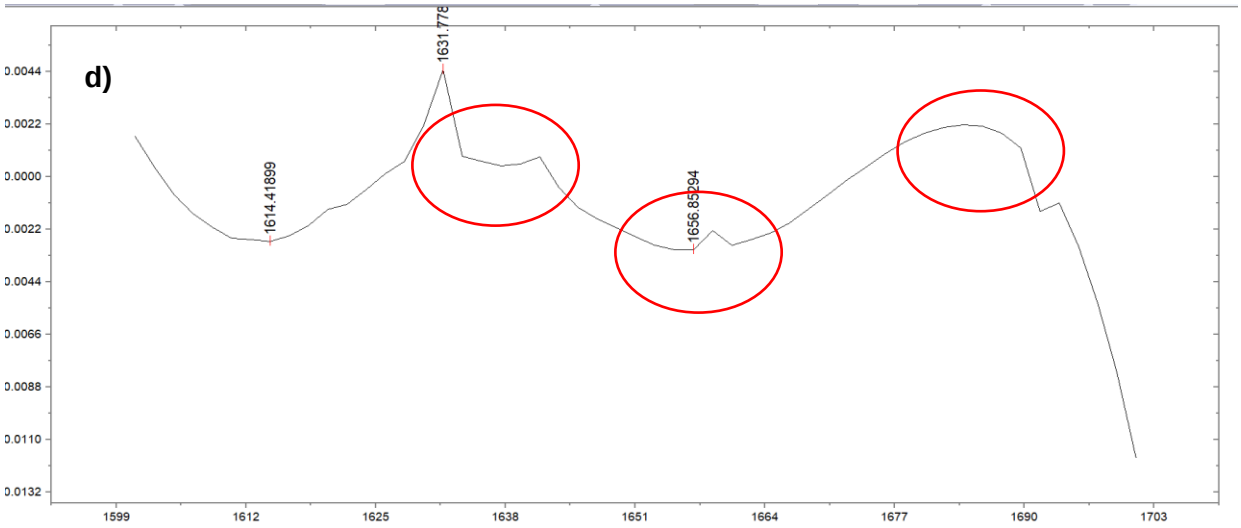

Fig. 4.3.3.8 Peak fitting analysis in FTIR spectra of control NaCn (a) and covalent conjugates of NaCn-FA prepared by (b) Chemical Coupling, (d) Free Radical Grafting and (c) Alkaline method

4.2.4 Particle Size

The particle size of prepared conjugates of NaCn-CA and NaCn-FA was measured using Zetasizer and results were expressed as the volume-surface average Particle size (Z). The particle size of NaCn-CA and NaCn-FA non-covalent and covalent conjugates are shown in Fig. 4.2.4.1 and 4.2.4.2, respectively. As shown in the Table 4.2.4.1 the particle size of the control protein NaCn was 189 ± 1.64, 197.8±0.94 and 208.2±1.92 nm at pH 6, 7 and 8 respectively. The decrease in particle size was observed in non-covalent conjugation at all pH values and covalent conjugation by alkaline method for both NaCn-CA and NaCn-FA. In case of covalent conjugates of NaCn-CA and NaCn-FA, the particle size was increased in conjugates prepared by chemical coupling and free radical grafting method that may be due to involvement of the crosslinking agents in both cases.

Table no. 4.2.4.1. Particle size of control NaCn. NaCn-CA and NaCn-FA conjugates

Method	Sample	NaCn (nm)	NaCn-CA (nm)	NaCn-FA (nm)
Non-covalent	pH 6	189 ± 1.64^{a}	152.7±2.37^{b}	154.8±2.3^{b}
	pH 7	197.8±0.94^{a}	114.2±0.69^{c}	150.7±1.25^{b}
	pH 8	208.2±1.92^{a}	187.4±1.09^{b}	219.6±1.52^{a}
Covalent	Chemical coupling	206.2±6.69^{a}	138.8±6.82^{c}	165.58 ±4.89^{b}
	Free radical grafting	206.2±6.69^{c}	439.54±4.83^{b}	552.1±4.29^{a}
	Alkaline	211.9±3.323^{c}	229.34±2.4^{b}	267.44±3.37^{a}

Mean ± S.D, n=3,

means with different superscripts (a,b,c) in each row differ significantly ($p<0.05$) from each other

4.2.5 Zeta Potential

The colloidal stability of the conjugates is shown by the zeta potential. Zeta potential, also known as electro-potential in colloidal systems, reveals the difference between the dispersion medium and the stationary layer of fluid associated to the dispersed particle. The Zeta potential of NaCn-CA and NaCn-FA non-covalent and covalent conjugates are shown in Table 4.2.5.1. All NaCn-CA and NaCn-FA under covalent and non-covalent conjugates exhibited more negative zeta potential than control NaCn. This might be due conjugation of polyphenols with NaCn led to unfolding of protein, thus more charged regions were exposed resulting in more negative zeta-potential. (Wang *et al*., 2014) studied the zeta potential of α-La–EGCG complexes and reported that the net charges of the emulsion stabilised by the control α-Lactalbumin at pH 6.0 and 8.0, 60ºC, were -18.20± 0.32 and -19.80± 0.47 mV, respectively,

whereas the net charges of emulsions stabilised by the α-La–EGCG complexes at 60º C, pH 6.0, 8.0, were -20.80± 0.50, -21.30± 1.20 mV, respectively. Stronger negative zeta potential could enhance colloidal stability of protein dispersions by enhancing electrostatic repulsion between colloidal particles.

Table No. 4.2.5.1 Zeta potential of control NaCn, NaCn-CA and NaCn-FA conjugates

Method	Sample	NaCn (mV)	NaCn-CA (mV)	NaCn-FA (mV)
Non-covalent	**pH 6**	-9.56 ±0.11^{b}	-12±0.46^{a}	-13.8±0.44^{a}
	pH 7	-10±0.107^{b}	-11.0±0.39^{b}	-13.5±0.365^{a}
	pH8	-11.56±0.32^{b}	-12.72±0.28^{b}	-13.64±0.76^{a}
Covalent	**Chemical coupling**	-10.1±0.42^{b}	-11.4±0.209^{b}	-12±0.38^{a}
	Free radical grafting	-10.2±0.42^{b}	-13.4±0.15^{a}	-13.3±0.256^{a}
	Alkaline	-11.1±0.48^{a}	-12.7±0.108^{a}	-12.39±0.318^{a}

Mean ± S.D, n=3,

means with different superscripts (a,b,c) in each row differ significantly ($p<0.05$) from each other

4.3 Functional Properties

On the basis of size and zeta potential, it has been found that non-covalent conjugates at pH 6 and 7 were more stable than pH 8 for both NaCn-CA and NaCn-FA. The non-covalent conjugates of NaCn-CA and NaCn-FA of pH 7 has significantly higher retention of polyphenol than pH 6. Therefore, non-covalent conjugate prepared at pH 7 of both NaCn-CA and NaCn-FA was selected for characterization of functional properties. Among, covalent conjugates, all have higher retention of polyphenol but in the chemical coupling method the non-food grade chemicals are used (EDC and NHS), therefore, it was not considered for analysis of functional properties.

NaCn-CA and NaCn-FA conjugates selected for functional property analysis were non-covalent conjugate of pH 7 and covalent conjugates prepared by alkaline and free radical grafting method. The functional properties like antioxidant activity, solubility, emulsifying activity (EA), emulsion stability (ES), foaming capacity (FC) and foam stability (FS) were studied.

4.3.1 Antioxidant activity

One of the most important biological features of the protein-polyphenol conjugate is its antioxidant capacity. Polyphenols can scavenge free radicals, preventing oxidation of dietary biomolecules. The antioxidant capability of the NaCn-polyphenol complexes was calculated using the DPPH radical scavenging ability.

The antioxidant activity (AO) of control NaCn, NaCn-CA and NaCn-FA is presented in Fig. 4.3.1.1**.** The AO of control NaCn was 9.57±0.32% and 10.96±0.47% at pH 7 and pH 9 respectively. On conjugation with CA and FA, the AO increased significantly. There was no significant difference in AO of NaCn-CA and NaCn-FA conjugates in all the method of preparation.

On conjugation with CA, the AO was found to be 38.92±0.95%, 34.41±1.12% and 43.11±1.07 % in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. The AO was significantly high in free radical method whereas in non-covalent (pH 7) and alkaline method showed non-significantly higher AO.

Similarly, on conjugation with FA, the AO was found to be 37.33±1.25%, 31.83±1.38% and 41.26±0.89% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. Similar trend was seen in NaCn-FA conjugates where the AO was significantly high in free radical method whereas in non-covalent (pH 7) and alkaline method showed non-significantly higher AO.

The covalent conjugates of NaCn-CA and NaCn-FA prepared by free radical method possessed maximum radical scavenging index (RSI) than conjugates prepared by non-covalent (pH 7) and alkaline methods. These results were in line with the total polyphenol content. There was more binding of polyphenols when conjugates were prepared by covalent approach using free radical method.

(Almajano *et al*., 2007) investigated the interactions of EGCG with α-casein, and β-casein and discovered that the antioxidant activity of the protein components was in the following order: α -casein > β-casein before storage. It has been proposed that when polyphenols are bound to proteins, free hydroxyl groups connected to the benzene ring can exert antioxidant functions (Almajano *et al*., 2007), and that polyphenol-protein systems can clear free radicals due to the formation of more stable reaction products, thereby terminating the free-radical chain reaction (Jiang *et al*., 2018).

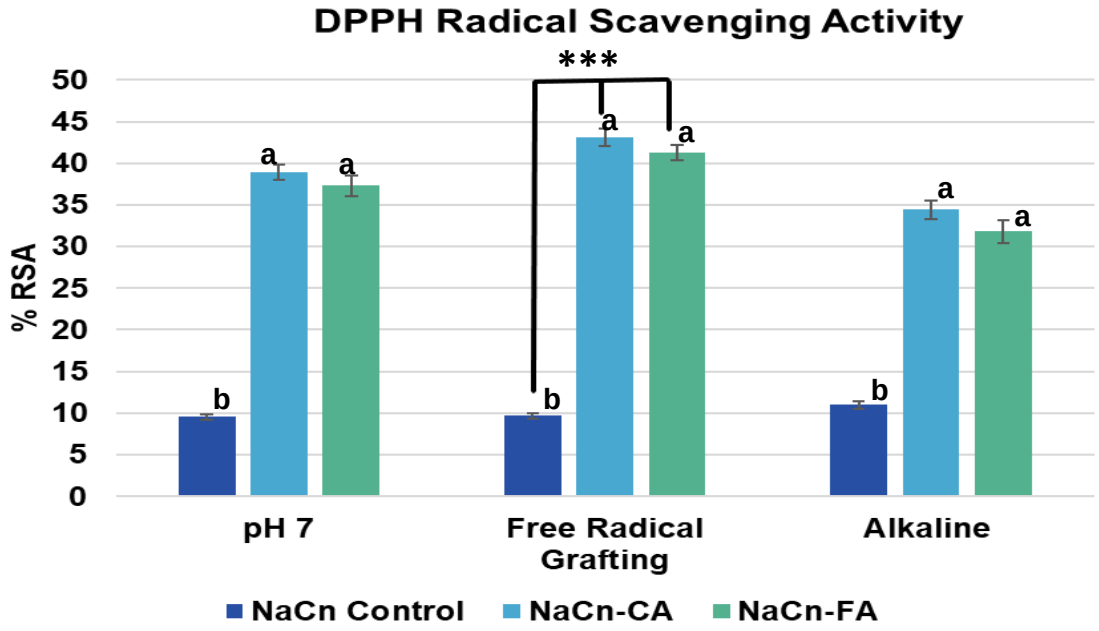

Fig.No.4.3.1.1. DPPH Radical Scavenging Activity of control NaCn, covalent and non-covalent conjugates of NaCn-CA and NaCn-FA

4.3.2 Solubility

The solubility of control NaCn, NaCn-CA and NaCn-FA is presented in the Fig. 4.3.2.1. The solubility of control NaCn was 93.06 ± 0.28 and 90.132 ± 0.7 % at pH 7 and pH 9, respectively. On conjugation with CA and FA, the solubility decreased significantly in covalent conjugates and no significant difference was observed in non-covalent conjugates. There was significant difference in solubility of NaCn-CA and NaCn-FA conjugates in covalent conjugation method of preparation and decrease was less in FA conjugation than CA conjugation with NaCn.

On conjugation with CA, the solubility was found to be 96.98±0.38%, 87.42±0.92% and 84.65±0.63% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. Non-covalent (pH 7) conjugates showed insignificantly higher solubility whereas covalent conjugates under alkaline method and free radical method showed significantly lower solubility.

Similarly, on conjugation with FA, the solubility was found to be 94.21±0.39%, 85.91±0.13% and 81.88±1.1% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively.

Solubility of non-covalent conjugates of NaCn-CA and NaCn-FA was insignificantly higher by 3.39±0.4% and 1.34±0.27%, respectively. This increase may be due to increase in hydrophilicity of caseins by incorporation of OH groups through polyphenols. Similar results were reported by (Jiang *et al*., 2018) where casein (CN) solubility was increased by 18.2% when the polyphenol chlorogenic acid was non-covalently interacted with casein. The reduced solubility in conjugates prepared by alkaline method may be associated with the oxidation of polyphenols acid to form quinones. Similar results were reported by (Prigent *et al*., 2007), where the solubility of lysozyme was decreased in the presence of chlorogenic acid at pH 8.0, which was linked to the oxidation of chlorogenic acid to form quinones.

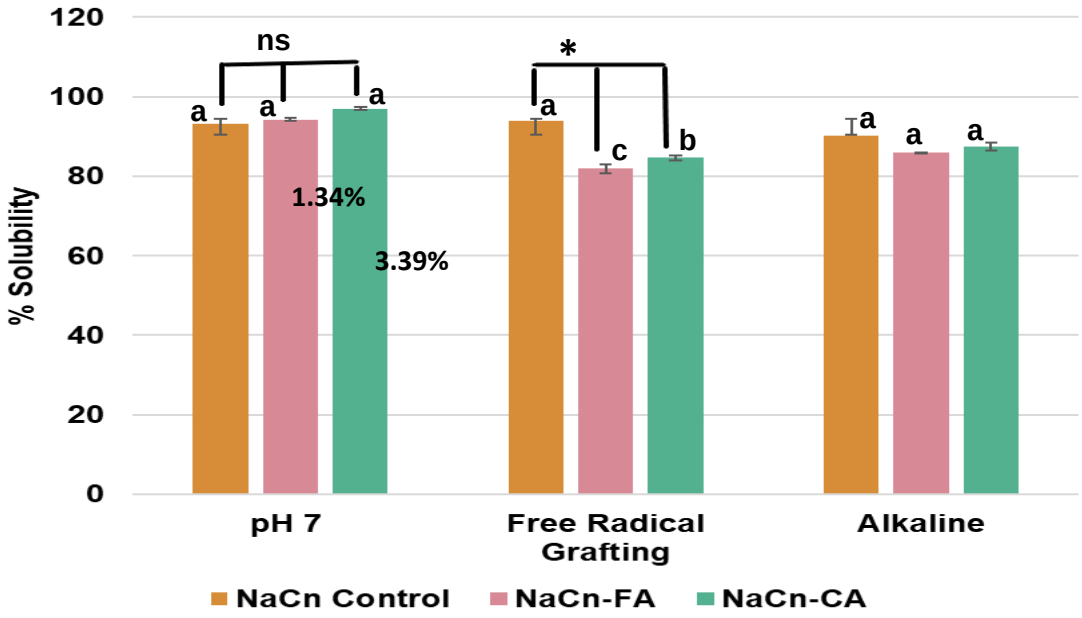

Fig No.4.3.2.1. Solubility of control NaCn, covalent and non-covalent conjugates of NaCn-CA and NaCn-FA

4.3.3 Emulsifying Activity and Emulsion Stability

The emulsifying activity (EA) and emulsion stability (ES) of control NaCn, NaCn-CA and NaCn-FA is presented in Fig. 4.3.3.1. and Fig. 4.3.3.2, respectively. The EA of control NaCn was 55.5± 0.83% and 65± 0.71% at pH 7 and pH 9, respectively whereas ES of control NaCn was 89.8± 0.836 % and 90.9± 1.24% at pH 7 and pH 9, respectively. On conjugation with CA and FA, both EA and ES increased, except in conjugates prepared by free radical grafting where decrease in ES was observed. There was significant difference in EA of NaCn-CA and NaCn-FA conjugates irrespective of the method of preparation.

On conjugation with CA, the EA and ES was found to be 84.54± 0.89% and 98.92± 1.01%, 93.26± 0.7 % and 97.45± 0.59 %, 89.21± 1.01 % and 84.54± 0.96 % in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. EA was significantly higher in NaCn-CA conjugates under free radical and alkaline method and non-significantly lower in non-covalent conjugates.

On conjugation with FA, the EA and ES was found to be 81.1± 1.02 % and 96± 1.17 %, 89.1± 0.89 % and 95.7± 0.67 %, 86.2± 1.03 % and 81.1± 1.02 % in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. Similar to NaCn-CA conjugates, in NaCn-FA conjugates, EA was significantly higher in NaCn-FA conjugates under free radical and alkaline method and non-significantly lower in non-covalent conjugates.

The increase in EA and ES after conjugation with CA and FA was due to increase in exposure of aromatic amino acid residues which have increased the affinity of the proteins toward the oil/water interface and improved the emulsifying activity of NaCn. (Afizah & Rizvi, 2014). The conjugation of polyphenol has caused the addition of OH groups in structure of protein thus improving the emulsifying activity of NaCn.

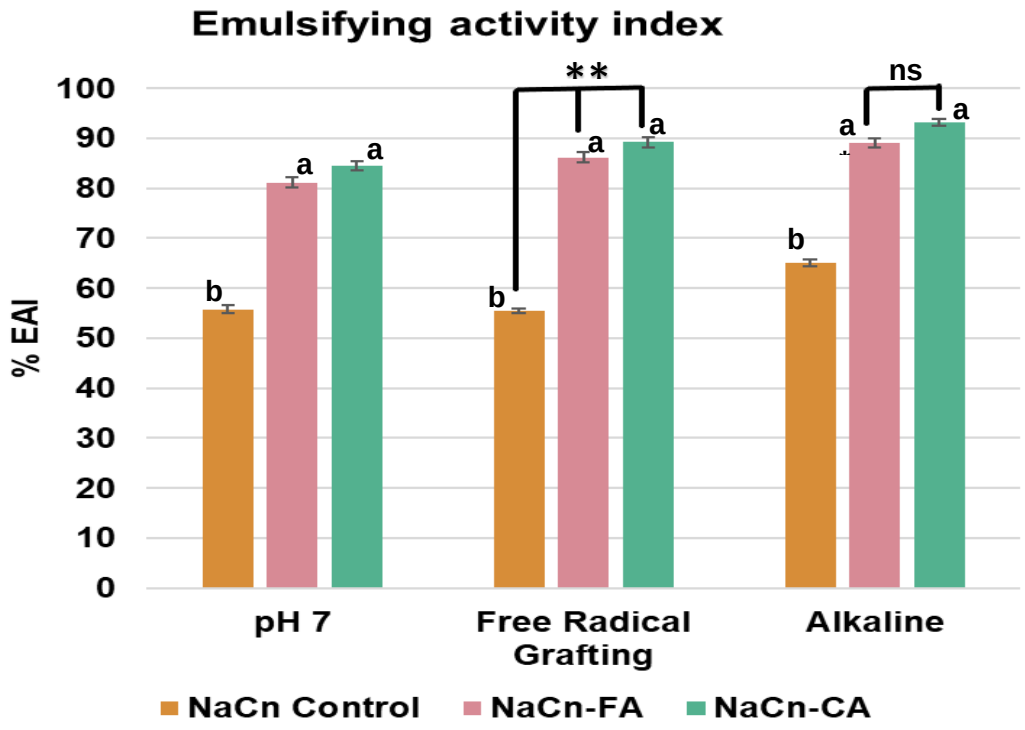

Fig.No.4.3.3.1. Emulsifying activity index of control NaCn, covalent and non-covalent conjugates of NaCn-CA and NaCn-FA

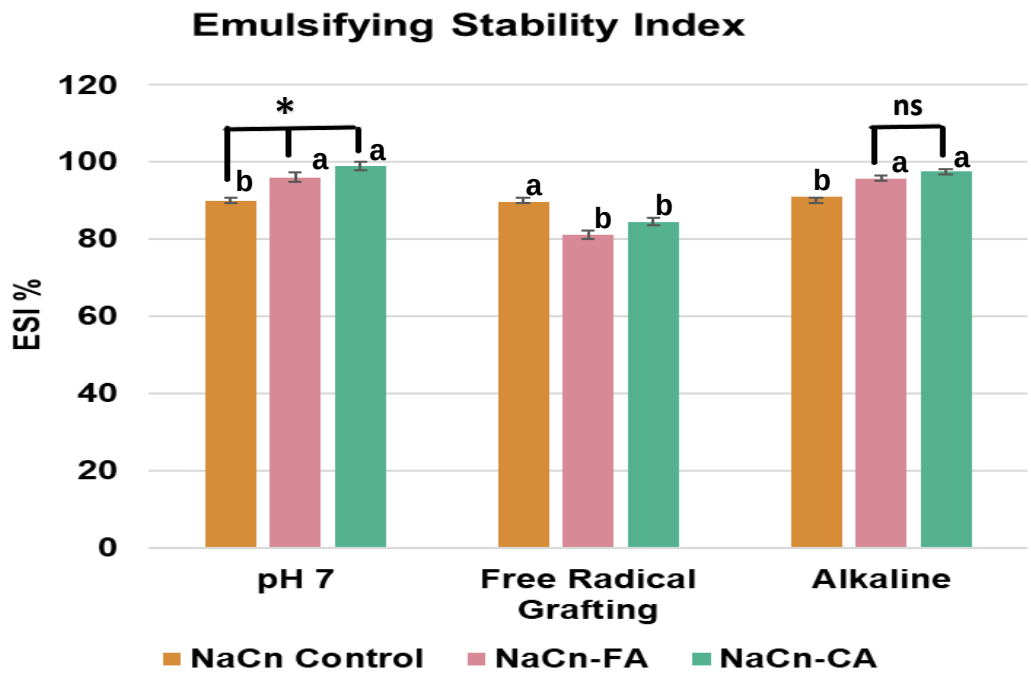

Fig.No.4.3.3.2. Emulsifying stability index of control NaCn, covalent and non-covalent conjugates of NaCn-CA and NaCn-FA

4.3.4 Foaming Capacity and Foam Stability

The foaming capacity (FC) and foam stability (FS) of control NaCn, NaCn-CA and NaCn-FA is presented in Fig. 4.3.4.1 and 4.3.4.2, respectively. The FC and FS of control NaCn was 123.15 ± 2.09% and 92.35± 1.4% at pH 7 and 127.64±1.06 and 91.9785± 1.27 % at pH 9, respectively. On conjugation with CA and FA, both FC and FS increased. There was no significant difference in FC and FS of NaCn-CA and NaCn-FA conjugates irrespective of the method of preparation.

On conjugation with CA, the FC and FS was found to be 149.54± 1.96% and 95.98± 1.07 %, 142.36 ± 2.05 % and 93.53± 0.94%, and 136.34± 1.56 and 93.941± 1.25% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. FC was higher in the non-covalent (pH 7) conjugates of NaCn-CA and was non-significantly lower in other covalent conjugates.

On conjugation with FA, the FC and FS was found to be 142.53± 0.93% and 94.17±0.58%, 137.33 ± 1.08% and 92.59± 1.2 %, 134.67± 2.41% and 93.185 ±1.72% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. FC was higher in the non-covalent (pH 7) conjugates of NaCn-CA and NaCn-FA and was non-significantly lower in other covalent conjugates. Meanwhile, all conjugates of NaCn-CA and NaCn-FA have non-significantly higher FS than the control NaCn.

The increase in FC after conjugation with CA and FA was due to promotion of protein crosslinking after conjugation with polyphenols (Kuan *et al.,* 2011). When compared to the other conjugates, covalent conjugates of NaCn-CA and NaCn-FA under free radical method showed lower foaming capacity as the particle size was more in those conjugates. Foaming stability is the stability of the foam after a predetermined time.

Similar to FC, all conjugates of NaCn-CA and NaCn-FA have non-significantly higher FS than the control NaCn. This may be most likely due to an increase in negative zeta charge. Particles with a larger negative zeta potential are more stable due to the stronger repulsion between them. The bubbles were unable to approach each other due to the repulsion forces. As a result, the foaming stability of all conjugates was improved (Wang, 2013).

(Jiang *et al.,* 2018) studied the effects of the interaction of whey protein isolate (WPI) and casein (CN) with chlorogenic acid. They reported that CN showed higher foaming capacity and foaming stability when compared to WPI maybe due to higher water solubility of the protein-polyphenol mixture, allowing for a more efficient transfer of protein molecules to the air-water interface thereby improving the foaming properties. Some researchers have found that polyphenols have the ability to improve protein foaming properties by promoting protein cross-linking (Kuan *et al*., 2011. (Jia *et al*., 2018) studied foaming activities of WPI covalently modified by polyphenols under alkaline method. They reported that cross-linking treatment is used to manipulate the foaming behaviour of proteins. By suppressing coalescence and disproportionation, the cross-linking treatment increases interfacial elasticity and foam formation. (Jia *et al*., 2016; Moracles *et al*., 2015) also discovered that the protein's molecular size is another important factor that influences foaming properties. The larger the particle size, the slower the protein diffuses to the interface, reducing foaming ability.

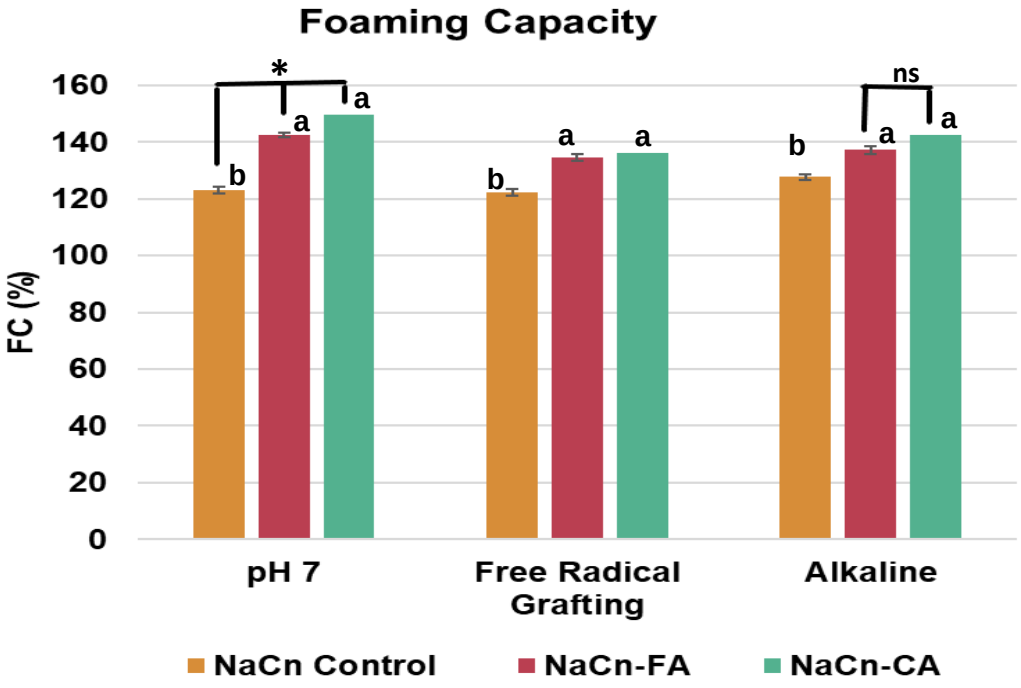

Fig.No.4.3.4.1. Foaming capacity of control NaCn, covalent and non-covalent conjugates of NaCn-CA and NaCn-FA

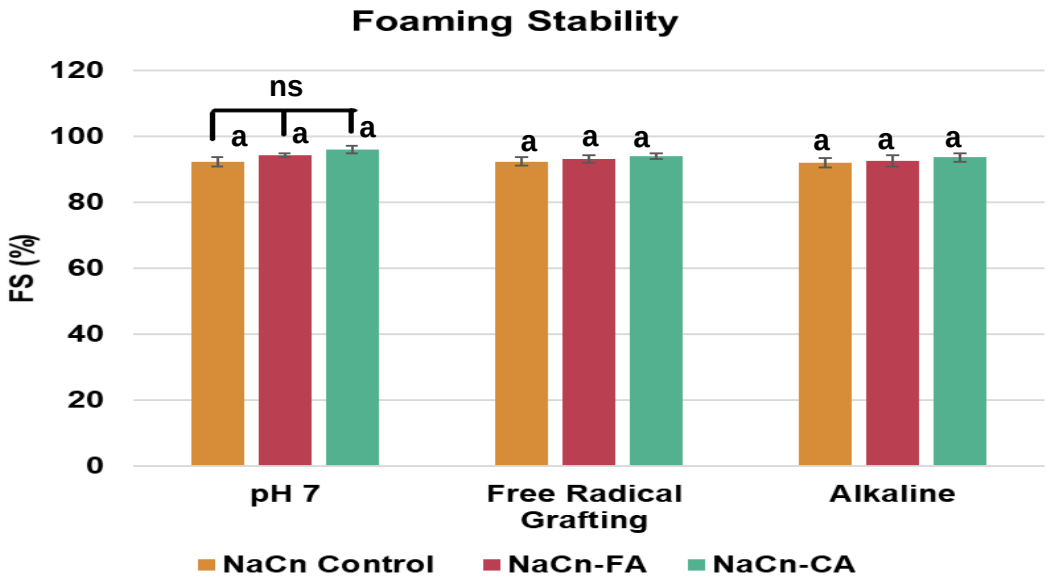

Fig.No.4.3.4.2. Foaming stability of control NaCn, covalent and non-covalent conjugates of NaCn-CA and NaCn-FA

CHAPTER 5

SUMMARY AND CONCLUSION

The Phenolic content results confirmed that casein interacted with Caffeic Acid as well as ferulic acid at both non-covalent and covalent conditions. Among non-covalent conjugates of NaCn-CA, the total polyphenol content was found to be 21.02 ± 0.93, 25.36 ± 0.46 and 19.25 ± 0.78 mg/g protein at pH 6, 7 and 8, respectively. Among covalent conjugates of NaCn-CA, the total polyphenol content was 35.75 ± 0.14, 38.69 ± 0.68 and 26.88 ± 0.24 mg/g protein in chemical coupling method, free radical grafting and alkaline method, respectively. Among non-covalent conjugates of NaCn-FA, the total polyphenol content was 18.83 ±0.59, 21.35 ± 0.52 and 16.66 ± 0.72 mg/g protein at pH 6, 7 and 8 whereas the total polyphenol content of covalent conjugates of NaCn-FA was 23.2 ± 0.75, 28.18 ± 0.49 and 20.28 ± 0.32 in chemical coupling method, free radical grafting and alkaline method, respectively. Among caffeic and ferulic acid, the conjugation of sodium caseinate was higher with caffeic acid that may be due to presence of extra OH group in Caffeic acid. Among non-covalent conjugates, at pH 7 both caffeic and ferulic acids have shown higher interaction. Between covalent and non-covalent conjugates, binding of polyphenol was more in covalent conjugates than non-covalent.

The conjugation between, polyphenol and sodium caseinate was confirmed by decrease in intrinsic fluorescence intensity of control protein NaCn after conjugation with polyphenols Caffeic acid and Ferulic acid. Control NaCn has shown maximum intensity at wavelength (λ) 340nm. On conjugation with CA and FA, with decrease in fluorescence intensity, shift in maximum wavelength was also observed. NaCn-CA and NaCn-FA have shown red shift of 60 nm and 40nm at all the pH, respectively. CA being more hydrophilic due to presence of extra OH group than FA has shown higher red shift. Quenching of intrinsic fluorescence of NaCn was more significant in covalent conjugates for both NaCn-CA and NaCn-FA. The quenching of fluorescence intensity was >90% in covalent conjugates and ~ 85% in non-covalent conjugates.

Conjugation was also confirmed inspecting by the changes in UV-Visible spectra. The absorbance of the non-covalent conjugate (NaCn-CA and NaCn-FA) at all pH values was appreciably lower near 214 nm and slightly higher at 270-280 nm and 305–330 nm than that of the control NaCn. Significant absorption was seen in all covalent

conjugates in the 280-290 nm and 310-330nm. With comparison to control NaCn, the changes in UV-Visible spectra of covalent conjugates were more pronounced as compared to non-covalent conjugates.

In FTIR spectral analysis, the spectral changes were more visible in covalent conjugates than non-covalent conjugates. Along with decrease in the intensity for amide I and amide II region in the conjugates, spectral shift from 1638 cm-1 to 1635 cm-1 and from 1535 cm-1 to 1527 cm-1 in amide I and amide II was observed, respectively. No major changes in the spectra were observed for non-covalent conjugates of NaCn-CA and NaCn-FA at all pH values.

Further, peak fitting analysis was performed in amide I region (1600-1700 cm-1) and peaks were observed near at 1614, 1631, 1641, 1664 and 1689 cm-1 for control NaCn. For all non-covalent conjugates of NaCn-CA, similar peaks were observed except for pH 6 and 8 whereas peak at 1664 was shifted to 1660 cm-1. For non-covalent conjugates of NaCn-FA, similar peaks were observed except peak at 1664 was shifted to 1662 at all pH values and peak at 1689 shifted to 1687 at pH 7 and 8.

On analysing peaks, it was observed that shifting of peaks was more in covalent conjugates of both CA and FA. Covalent conjugates of NaCn-CA prepared by chemical coupling method have shown one extra peak near 1654 cm-1, and shifting of peak from 1689 to 1687 compared to control NaCn was observed. For the conjugates prepared by alkaline method the shifting of peak from 1664 to 1656 and 1689 to 1687 was observed. For free radical grafting method shifting of 1664 to 1660 and 1689 to 1687 was observed. Meanwhile, NaCn-FA conjugates prepared by chemical coupling method have shown shifting of peak from 1689 to 1687 and 1664 to 1660. In the conjugates prepared by alkaline method, the peak at 1641 and 1689 has disappeared and peak from 1664 was shifted to 1656. In conjugates prepared by free radical grafting method, shifting of peak from 1664 to 1662 was observed and peak near 1641 was disappeared.

The particle size of NaCn-CA and NaCn-FA non-covalent and covalent conjugates were analysed. The particle size of the control protein NaCn was 189 ± 1.64, 197.8±0.94 and 208.2±1.92 nm at pH 6, 7 and 8 respectively. Non-covalent conjugates of NaCn-CA showed particle size of 152.7±2.37, 114.2±0.69 and 187.4±1.09 nm at pH 6, 7 and 8, respectively whereas non-covalent conjugates of NaCn-FA showed

particle size of NaCn-FA 154.8±2.3, 150.7±1.25 and 219.6±1.52 nm at pH 6, 7 and 8, respectively. Covalent conjugates of NaCn-CA showed particle size of 138.8±6.82, 439.54±4.83 and 229.34±2.4 nm in chemical coupling, free radical and alkaline method, respectively whereas covalent conjugates of NaCn-FA gave particle size of 165.58 ±4.89, 552.1±4.29 and 267.44±3.37 nm in chemical coupling, free radical and alkaline method, respectively. In case of covalent conjugates of NaCn-CA and NaCn-FA, the particle size was increased in conjugates prepared by chemical coupling and free radical grafting method.

The colloidal stability of the conjugates is shown by the zeta potential. Zeta potential of control NaCn was found to be -9.56 ±0.11, -10±0.107 and -11.56±0.32 mV at pH 6, 7 and 8, respectively. Zeta potential of non-covalent conjugates of NaCn-CA was found to be -12±0.46, -11.0±0.39 and -12.72±0.28 mV at pH 6, 7 and 8, respectively. Meanwhile, non-covalent conjugates of NaCn-FA showed the zeta potential of -13.8±0.44, -13.5±0.365 and -13.64±0.76mV in chemical coupling, free radical and alkaline method, respectively whereas covalent conjugates of NaCn-FA gave zeta potential of -12±0.38, -13.3±0.26 and -12.39±0.32 mV in chemical coupling, free radical and alkaline method, respectively.

The antioxidant capability of the NaCn-polyphenol complexes was calculated using the DPPH radical scavenging ability. On conjugation with CA, the AO was found to be 38.92±0.95%, 34.41±1.12% and 43.11±1.07 % in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. The AO was significantly high in free radical method whereas in non-covalent (pH 7) and alkaline method showed non-significantly higher AO. Similarly, on conjugation with FA, the AO was found to be 37.33±1.25%, 31.83±1.38% and 41.26±0.89% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. Similar trend was seen in NaCn-FA conjugates where the AO was significantly high in free radical method whereas in non-covalent (pH 7) and alkaline method showed non-significantly higher AO. There was no significant difference in AO of NaCn-CA and NaCn-FA conjugates in all the method of preparation. The covalent conjugates of NaCn-CA and NaCn-FA prepared by free radical method possessed maximum radical scavenging index (RSI) than conjugates prepared by non-covalent (pH 7) and alkaline methods.

The solubility of control NaCn was 93.06 ± 0.28 and 90.132 ± 0.7 % at pH 7 and pH 9, respectively. On conjugation with CA, the solubility was found to be 96.98±0.38%, 87.42±0.92% and 84.65±0.63% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. Non-covalent (pH 7) conjugates showed insignificantly higher solubility whereas covalent conjugates under alkaline method and free radical method showed significantly lower solubility. Similarly, on conjugation with FA, the solubility was found to be 94.21±0.39%, 85.91±0.13% and 81.88±1.1% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. Solubility of non-covalent conjugates of NaCn-CA and NaCn-FA was insignificantly higher by 3.39±0.4% and 1.34±0.27%, respectively. On conjugation with CA and FA, the solubility decreased significantly in covalent conjugates and no significant difference was observed in non-covalent conjugates. There was significant difference in solubility of NaCn-CA and NaCn-FA conjugates in covalent conjugation method of preparation and decrease was less in FA conjugation than CA conjugation with NaCn.

The Emulsifying activity (EA) of control NaCn was 55.5± 0.83% and 65± 0.71% at pH 7 and pH 9, respectively whereas Emulsifying capacity (ES) of control NaCn was 89.8± 0.836 % and 90.9± 1.24% at pH 7 and pH 9, respectively. On conjugation with CA, the EA and ES was found to be 84.54± 0.89% and 98.92± 1.01%, 93.26± 0.7 % and 97.45± 0.59 %, 89.21± 1.01 % and 84.54± 0.96 % in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. EA was significantly higher in NaCn-CA conjugates under free radical and alkaline method and non-significantly lower in non-covalent conjugates. On conjugation with FA, the EA and ES was found to be 81.1± 1.02 % and 96± 1.17 %, 89.1± 0.89 % and 95.7± 0.67 %, 86.2± 1.03 % and 81.1± 1.02 % in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. Similar to NaCn-CA conjugates, in NaCn-FA conjugates, EA was significantly higher in NaCn-FA conjugates under free radical and alkaline method and non-significantly lower in non-covalent conjugates. On conjugation with CA and FA, both EA and ES increased, except in conjugates prepared by free radical grafting where decrease in ES was observed. There was significant difference in EA of NaCn-CA and NaCn-FA conjugates irrespective of the method of preparation.

On conjugation with CA, the FC and FS was found to be 149.54± 1.96% and 95.98± 1.07 %, 142.36 ± 2.05 % and 93.53± 0.94%, and 136.34± 1.56 and 93.941± 1.25% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. FC was higher in the non-covalent (pH 7) conjugates of NaCn-CA and was non-significantly lower in other covalent conjugates.

On conjugation with FA, the FC and FS was found to be 142.53± 0.93% and 94.17±0.58%, 137.33 ± 1.08% and 92.59± 1.2 %, 134.67± 2.41% and 93.185 ±1.72% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. FC was higher in the non-covalent (pH 7) conjugates of NaCn-CA and NaCn-FA and was non-significantly lower in other covalent conjugates. Meanwhile, all conjugates of NaCn-CA and NaCn-FA have non-significantly higher FS than the control NaCn.

The results have shown the possibility of using caffeic acid and ferulic acid in improving the functional properties of sodium caseinate. Thereby it was noticed that functionality of the prepared conjugates was found to be affected by conditions like type of polyphenol and method of conjugation. Among Non-covalent conjugates at pH 7 of both CA and FA have shown better functional properties than covalent conjugates. Among CA and FA, the conjugation of CA with sodium caseinate has higher functionality. In covalent method, alkaline method is observed to give better solubility and emulsifying properties but free radical method gave higher foaming capacity and antioxidant activity. So, this concludes that the prepared NaCn-polyphenol conjugates with enhanced functional properties can be used as novel food additives in food products.

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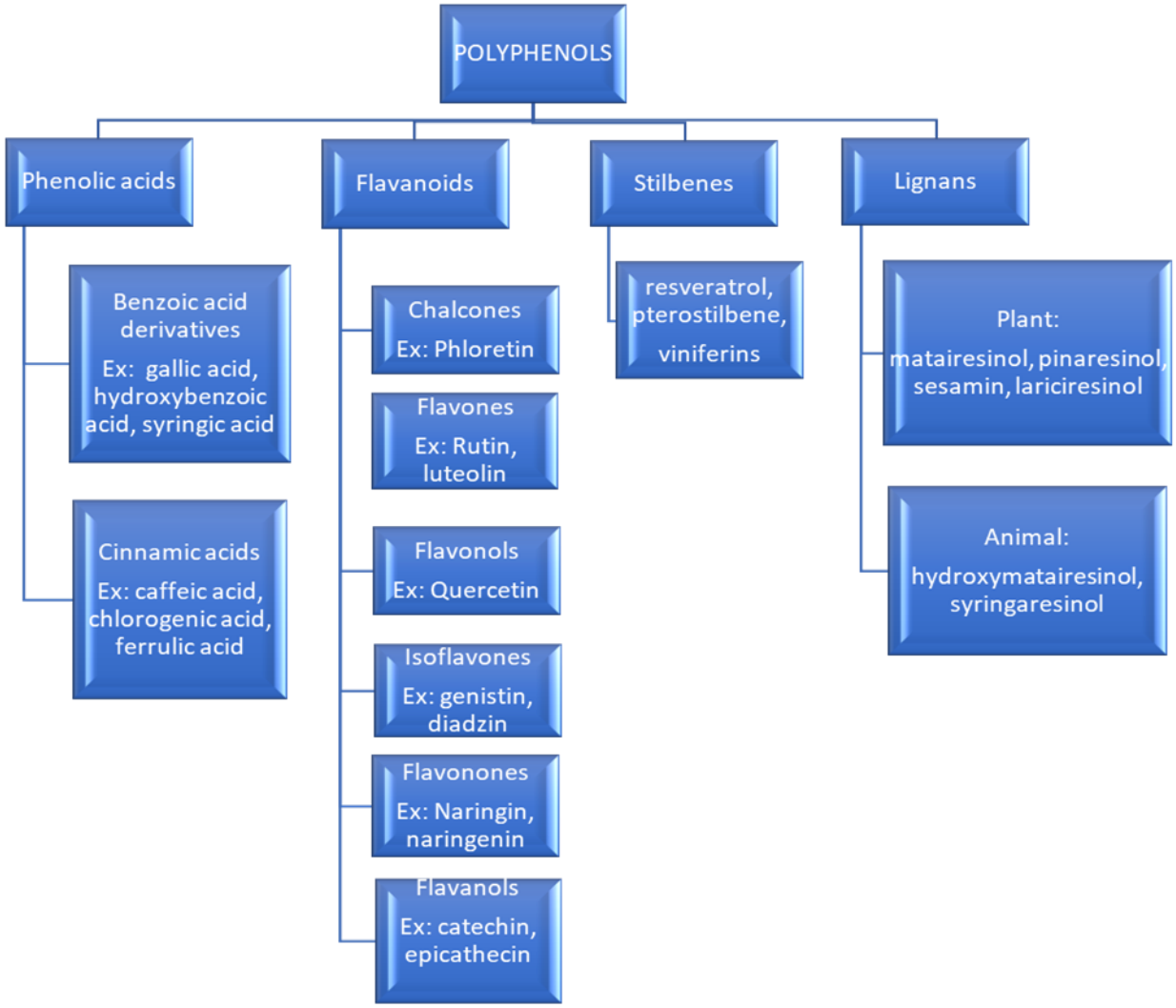
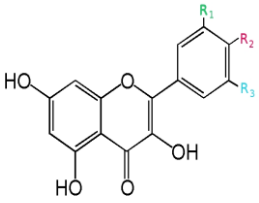
Outline of polyphenol classification

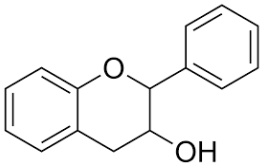


Flavonoids:

They are subdivided into flavanols, anthocyanidins, anthocyanins, isoflavones, flavones, flavonols, flavanones, and flavanonols.



Flavonols



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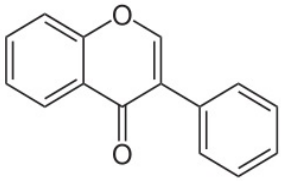
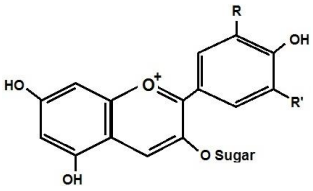
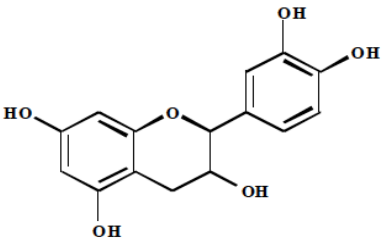


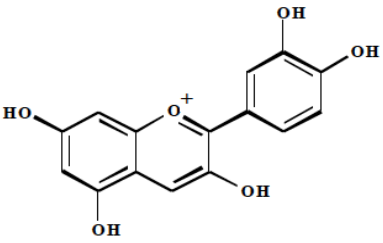
Isoflavones



Anthocyanin

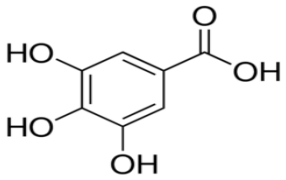


Flavan-3-ols

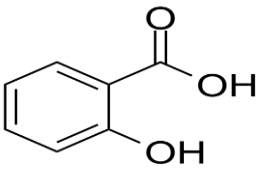


Anthocyanidin

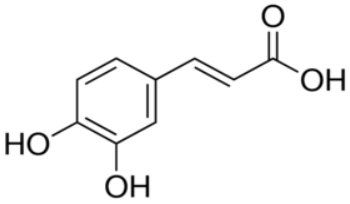
Phenolic acids:



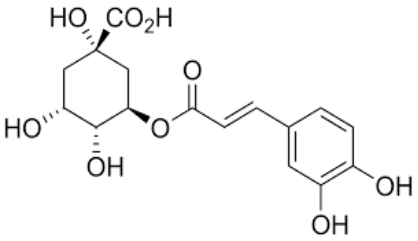
Gallic



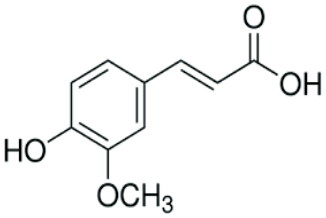
Hydroxy benzoic



Caffeic acid

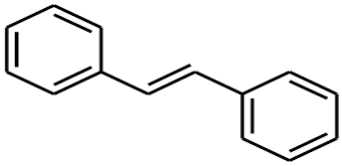


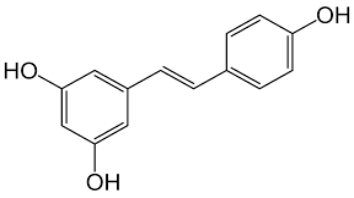
Chlorogenic acid



Ferulic acid

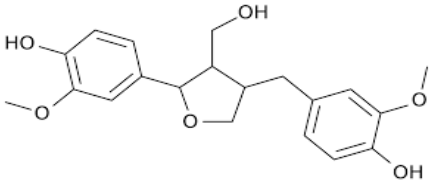
Stilbenes:



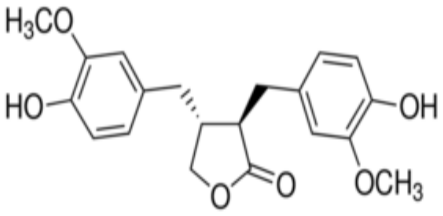


Resveretrol

Lignan:



Lariciresinol



Metaraminol