EFFECT OF CHEMICAL MODIFICATION BY POLYPHENOLS ON FUNCTIONAL PROPERTIES OF WHEY PROTEINS

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

VASAVA HARDIK DILIPKUMAR
B.Tech. (Dairy Technology)

DAIRY CHEMISTRY DIVISION
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL-132001(HARYANA), INDIA

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This is to certify that the thesis entitled, **"Effect of chemical modification by polyphenols on functional properties of whey proteins"** submitted by **Mr. Vasava Hardik Dilipkumar** 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.

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Dedicated to

My Beloved Parents

&

Respected Guide

Dr Richa Ma’am

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Date: 06/09/2021 **(*Hardik Vasava*)**

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List of Abbreviations

Abbreviated form	Full form
WP	Whey Proteins
CA	Caffeic Acid
FA	Ferulic Acid
%	Percentage
°C	Degree centigrade
µg	Microgram
µl	Microlitre
A.R.	Analytical grade
CAGR	Cumulative annual growth rate
et al.	et alii (and others)
g	Gram
i.e.,	id est (in other words)
mg	Milligram
min.	Minute
ml	Millilitre
mM	Milli molar
pH	Negative log of the hydrogen ion concentration
RPM	Rotations per minute
H	Hydrogen
US$	U. S. Dollar
α	Alpha
β	Beta
&	ampersand
BSA	Bovine serum albumin
H_2O_2	hydrogen peroxide
EGCG	Epigallocatechin gallate
Min	Minute
TNF	Tumor Necrosis Factor
pI	isoelectric point
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride
NHS	*N*-hydroxy succinimide
CLA	Chlorogenic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
AO	Antioxidant activity
EA	Emulsifying activity
ES	Emulsion stability
FC	Foaming capacity
FS	Foam stability

ABSTRACT

Proteins are the primary food components, while polyphenols are secondary metabolites, which are abundantly present in plant. During formulation of composite food products, interaction between proteins and polyphenols occur spontaneously and yield “protein-polyphenol conjugate”. These conjugates are known to have an impact on sensorial, functional, and nutraceutical properties of the food products. However, understanding the interaction between proteins and phenolic compounds can help in development of novel conjugates with improved functional properties. In the present study, whey protein was conjugated with plant polyphenols (caffeic and ferulic acid) and the structural and functional characteristics of the conjugates were determined. The conjugation between polyphenol and whey protein concentrate (WPC) was achieved by non-covalent (changing pH) as well as covalent (alkaline, free radical grafting and chemical coupling) methods. The degree of conjugation was higher with caffeic acid than ferulic acid and on the basis of total polyphenol per mg of protein, the binding of both polyphenols was found to be higher in covalent conjugates (free radical grafting>chemical coupling>alkaline method) than non-covalent conjugates (pH 3 and pH 7 >pH 5). Structural analysis of these conjugates was also performed using intrinsic fluorescence, UV spectral analysis and FTIR. The conjugation was confirmed by decreased intrinsic fluorescence intensity (>30%) of protein solution after conjugation with polyphenol. The decrease in intensity was more in covalent conjugates (>80%) than non-covalent conjugates (30-60%) irrespective of polyphenol used. The red shift of 10-30 nm was also observed in maximum wavelength of fluorescence intensity after conjugation. The UV-visible absorbance of the non-covalent conjugate at all three pH was appreciably lower than that of the WPC (control) except at 280-335 nm, where slightly higher absorbance was observed in conjugates than control, providing confirmation of conjugation. Compared to UV-Vis spectra of non-covalent conjugation, UV-Vis spectra of covalent conjugates were more pronounced confirming higher binding of polyphenols in covalent conjugation. In FTIR spectral analysis, no change in the spectra was observed for non-covalent conjugates at all pH values. However, in covalent conjugates of WPC with both polyphenols shifting of band and generation of new peak was observed. There was no significant difference in particle size of WPC after conjugation with polyphenol in both non-covalent and covalent conjugates except at pH 3, where size was significantly higher. In all conjugates, zeta potential decreased in comparison to control WPC. The functional properties of selected conjugates (non-covalent: pH 7; covalent: alkaline and free radical grafting) were also compared with control WPC. The conjugation of both polyphenols has caused decrease in solubility of WPC. However, emulsifying activity, emulsion stability, foaming capacity and radical scavenging activity was found to be higher after conjugation. The foam stability of WPC was not affected significantly after conjugation of polyphenols. As an outcome of study, it has been found that functionality of the WPC-polyphenol conjugate was affected by type of polyphenol and method of conjugation. The functionality was improved maximum in non-covalent conjugates followed by covalent conjugates and WPC-caffeic acid conjugates had better functionality than WPC-ferulic acid conjugates. These prepared whey protein-polyphenol conjugates with enhanced functional properties can be used as novel food additives in food products.

सारांश

प्रोटीन प्राथमिक खाद्य घटक हैं, जबकि पॉलीफेनोल्स द्वितीयक मेटाबोलाइट हैं, जो पौधे में प्रचुर मात्रा में मौजूद होते हैं। मिश्रित खाद्य उत्पादों के निर्माण के दौरान, प्रोटीन और पॉलीफेनोल्स के बीच परस्पर क्रिया अनायास होती है और "प्रोटीन-पॉलीफेनोल संयुग्म" उत्पन्न करती है। इन संयुग्मों का खाद्य उत्पादों के संवेदी, कार्यात्मक और न्यूट्रास्युटिकल गुणों पर प्रभाव के लिए जाना जाता है। हालांकि, प्रोटीन और फेनोलिक यौगिकों के बीच बातचीत को समझने से बेहतर कार्यात्मक गुणों के साथ उपन्यास संयुग्मों के विकास में मदद मिल सकती है। वर्तमान अध्ययन में, मट्ठा प्रोटीन को प्लांट पॉलीफेनोल्स (कैफिक और फेरुलिक एसिड) के साथ संयुग्मित किया गया था और संयुग्मों की संरचनात्मक और कार्यात्मक विशेषताओं को निर्धारित किया गया था। पॉलीफेनोल और मट्ठा प्रोटीन सांद्रता (डब्ल्यूपीसी) के बीच संयुग्मन गैर-सहसंयोजक (बदलते पीएच) के साथ-साथ सहसंयोजक (क्षारीय, मुक्त कट्टरपंथी ग्राफ्टिंग और रासायनिक युग्मन) विधियों द्वारा प्राप्त किया गया था। संयुग्मन की डिग्री फेरुलिक एसिड की तुलना में कैफिक एसिड के साथ अधिक थी और प्रोटीन के प्रति मिलीग्राम कुल पॉलीफेनोल के आधार पर, दोनों पॉलीफेनोल्स का बंधन सहसंयोजक संयुग्मों (फ्री रेडिकल ग्राफ्टिंग> रासायनिक युग्मन> क्षारीय विधि) में गैर की तुलना में अधिक पाया गया। -सहसंयोजक संयुग्म (पीएच 3 और पीएच 7> पीएच 5)। इन संयुग्मों का संरचनात्मक विश्लेषण भी आंतरिक प्रतिदीप्ति, यूवी वर्णक्रमीय विश्लेषण और एफटीआईआर का उपयोग करके किया गया था। पॉलीफेनोल के साथ संयुग्मन के बाद प्रोटीन समाधान की आंतरिक प्रतिदीप्ति तीव्रता (> 30%) में कमी द्वारा संयुग्मन की पुष्टि की गई थी। तीव्रता में कमी सहसंयोजक संयुग्मों (> 80%) में गैर-सहसंयोजक संयुग्मों (30-60%) की तुलना में अधिक थी, भले ही पॉलीफेनोल का उपयोग किया गया हो। संयुग्मन के बाद प्रतिदीप्ति तीव्रता की अधिकतम तरंग दैर्ध्य में 10-30 एनएम की लाल पारी भी देखी गई। तीनों पीएच पर गैर-सहसंयोजक संयुग्म का यूवी-दृश्य अवशोषण 280-335 एनएम को छोड़कर डब्ल्यूपीसी (नियंत्रण) की तुलना में काफी कम था, जहां संयुग्मन की पुष्टि प्रदान करते हुए नियंत्रण से संयुग्मों में थोड़ा अधिक अवशोषण देखा गया था। गैर-सहसंयोजक संयुग्मन के यूवी-विज़ स्पेक्ट्रा की तुलना में, सहसंयोजक संयुग्मों के यूवी-विज़ स्पेक्ट्रा सहसंयोजक संयुग्मन में पॉलीफेनोल्स के उच्च बंधन की पुष्टि करते हुए अधिक स्पष्ट थे। एफटीआईआर वर्णक्रमीय विश्लेषण में, सभी पीएच मानों पर गैर-सहसंयोजक संयुग्मों के लिए स्पेक्ट्रा में कोई परिवर्तन नहीं देखा गया था। हालांकि, डब्ल्यूपीसी के सहसंयोजक संयुग्मों में दोनों पॉलीफेनोल्स बैंड के स्थानांतरण और नए शिखर की पीढ़ी को देखा गया था। पीएच 3 को छोड़कर, जहां आकार काफी अधिक था, गैर-सहसंयोजक और सहसंयोजक संयुग्म दोनों में पॉलीफेनोल के साथ संयुग्मन के बाद डब्ल्यूपीसी के कण आकार में कोई महत्वपूर्ण अंतर नहीं था। सभी संयुग्मों में, डब्ल्यूपीसी को नियंत्रित करने की तुलना में जीटा क्षमता में कमी आई है। चयनित संयुग्मों (गैर-सहसंयोजक: पीएच 7; सहसंयोजक: क्षारीय और मुक्त कट्टरपंथी ग्राफ्टिंग) के कार्यात्मक गुणों की भी नियंत्रण डब्ल्यूपीसी के साथ तुलना की गई थी। दोनों पॉलीफेनोल्स के संयुग्मन से डब्ल्यूपीसी की घुलनशीलता में कमी आई है। हालांकि, पायसीकारी गतिविधि, इमल्शन स्थिरता, झाग क्षमता और कट्टरपंथी मैला ढोने की गतिविधि संयुग्मन के बाद अधिक पाई गई। पॉलीफेनोल्स के संयुग्मन के बाद डब्ल्यूपीसी की फोम स्थिरता महत्वपूर्ण रूप से प्रभावित नहीं हुई थी। अध्ययन के परिणाम के रूप में, यह पाया गया है कि डब्ल्यूपीसी-पॉलीफेनोल संयुग्म की कार्यक्षमता पॉलीफेनोल के प्रकार और संयुग्मन की विधि से प्रभावित थी। गैर-सहसंयोजक संयुग्मों के बाद सहसंयोजक संयुग्मों में कार्यक्षमता में अधिकतम सुधार हुआ था और डब्ल्यूपीसी-कैफिक एसिड संयुग्मों में डब्ल्यूपीसी-फेरुलिक एसिड संयुग्मों की तुलना में बेहतर कार्यक्षमता थी। इन तैयार व्हे प्रोटीन-पॉलीफेनोल संयुग्मित कार्यात्मक गुणों के साथ खाद्य उत्पादों में उपन्यास खाद्य योजक के रूप में उपयोग किया जा सकता है।

INTRODUCTION

Now a Days People become more aware about health and the focus is shifting towards nutritional and healthy diets including functional foods. Functional foods are those foods which provides basic nutrition and energy along with beneficial ingredients that modulate one or more targeted functions in the body (Nicoletti, 2012) As per market survey in 2020, the global whey protein ingredients market was estimated 9.7 billion US$, which is estimated to reach 16.4 billion US$ by 2027 with 7.8% CAGR (Compound Annual Growth Rate). Similarly, the Global functional food market size was valued at 177 billion US$ in 2019, and it is estimated to reach 267 billion US$ by 2027 at 6.7% CAGR. The polyphenol-based product shares 33% of total functional food market.

Whey proteins shares 20% of total milk proteins and also referred as serum proteins or non-casein proteins. The major families of proteins included in this class were originally the β-lactoglobulin, α-lactalbumin, and blood serum albumins, represent 50, 20 and 10 % of whey protein fractions. Other minor proteins are immunoglobulins, lactoferrin, and protease-peptones. Whey proteins exhibits many beneficial functional properties e.g., solubility, emulsifying properties and these finds applications as thickening agents, gelling agents, texture modifiers, and foaming agents in food preparations. Phenolic compounds are secondary metabolites in plants and possess many valuable biological activities, such as antioxidant, antimicrobial, antimutagenic, anticancer etc. Fruits or fruit extracts are good sources of bioactive compounds with antioxidant activity and are thus commonly included into dairy product formulations to improve their health-related properties (Lila *et al*., 2017). However, the incorporation of polyphenol into dairy matrix is a challenging task in terms of functionality and acceptability. However, in recent years it has been reported polyphenol can positively influence the functional properties of whey proteins. Jiang *et al*., (2018) reported improved solubility, foaming capability and foam stability of whey proteins after addition of chlorogenic acid. Different strategies e.g., encapsulation of polyphenols, molecular complexation etc**.** have been used to incorporate polyphenols in foods. Interaction between whey proteins and polyphenols, yielding “whey protein-polyphenol conjugate” has recently gained attention as an excellent vehicle to deliver polyphenols into food products as the combination of high-quality nutritional proteins with health-promoting polyphenols yields multifunctional ingredients which are more

accessible, protected, and more bioavailable. The conjugation of phenolic compounds with proteins can cause significant changes in the structural and physicochemical properties and also widen the applications of proteins. The interaction of whey proteins and polyphenols can be non-covalent (H bonding, electrostatic interactions) or covalent. The functionality of the conjugates is highly dependent on environmental factors like temperature and pH, as well as the conformation or type of proteins and polyphenols used.

The whey protein-polyphenol conjugates with enhanced functional properties can be used as novel food additives in food products. The majority of previous research has concentrated on non-covalent interactions between phenolic molecules and proteins, which is likely due to the fact that non-covalent phenolic–protein conjugates are easier to make than covalently coupled phenolic–protein conjugates. However, it anticipated that covalently linked conjugates will survive more during processing of products in which they are added in comparison to noncovalent conjugates due to the established fact that non-covalent bonds are weaker than covalent bonds. Therefore, the present study is undertaken to understand the modification in functional properties of whey proteins when different methods used for preparation of whey protein-polyphenol conjugates. Further, these conjugates will be characterized for structural modifications caused by polyphenol. The study is formulated with following two objectives:

Objective 1.
To modify functional property of whey protein using covalent and non-covalent conjugation with polyphenol

Objective 2.
To characterize the prepared whey protein-polyphenol conjugates

2.1 Whey protein

Whey proteins include whey protein concentrate (WPC) (30-85% protein by weight), whey protein isolate (WPI) (>90 percent), and whey protein hydrolysate (digestive enzyme-treated) (WPH) (EFSA, 2010). β-lactoglobulin (β-lg), α-lactalbumin (α-la), bovine serum albumin (BSA) are the three primary individual protein components (Haug, Hstmark, & Harstad, 2007). Whey proteins are commonly used in formulated foods due to their excellent nutritional qualities, numerous functional features, wide availability, and cost-effectiveness (Ali, Homann, Khalil, Kruse, & Rawel, 2013). And these whey protein ingredients (WPC, WPI, and WPH) as well as individual components (β-lg, α-la, BSA, and LA) are highly soluble at a wide range of food pH, allowing for more molecular level interaction (Chevalier *et al*., 2019; Jiang *et al*., 2020; Xu *et al*., 2019).

2.2 Phenolic compounds

Polyphenols, heterogeneous class of natural antioxidants, are secondary metabolites synthesized by plants basically through pentose phosphate, shikimate or phenyl propanoid pathways (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).

Currently, more than 8000 phenolic structures are 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. Majorly based on chemical structure, polyphenols are divided into 2 groups: Flavonoids and Non-flavonoids (Serra *et al*., 2020).

1. 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 (Cutrim & Cortez, 2018).

2. Non-flavonoids: non-flavonoids basically have one aromatic ring in their structure. They include phenolic acids (further divided into benzoic acid derivatives having C6C1 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).

2.3 Challenges in addition of polyphenols into food

Polyphenols are sensitive compounds that are susceptible to chemical degradation in presence of light, heat, or varied pH conditions. Therefore, their direct addition into food may cause loss during processing of food and high temperature processing and exposure to light and oxygen should be minimized Another concern is the low solubility and astringency or bitter taste of some polyphenol restricts their incorporation in many foods. Polyphenols also react with other components of foods and forms coloured complex and increasing their content in foods may leads to undesirable sensory characteristics of foods, which may reduce their consumption.

The stability, palatability, bioavailability, and bioactivity of polyphenols can be improved using several strategies. Processing technologies like freeze drying, spray drying, and microwave drying (Wais *et al*., 2016) can be used to increase stability of polyphenols during food processing. Encapsulation of polyphenols (Fang *et al*., 2010) is considered to be the easy way to prevent polyphenol from external damage. Excipient ingredients (Pandita *et al*., 2014) like digestible lipids that can increase the bio accessibility of polyphenols by forming mixed micelles in the aqueous phase that can solubilize and transport them. Molecular complexation (Bourvellec & Renard, 2012) by physically (complexation) or chemically (conjugation) linked to other molecules is gaining popularity to improve the functionality of polyphenol in Food matrix.

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

The promising method of synthesising novel materials or modifying biomolecules with suitable physical and chemical properties is molecular modification by conjugation or polymerization. This method tries to maximize the properties of a specific food component or produce new properties for specific purposes (Spizzirri *et al*., 2009). The nutritional, sensory, and physiochemical qualities of food products could be improved by combining the characteristics of natural food elements and conjugated molecules (Czubinski & Dwiecki, 2017). Whey Protein is an important macronutrient found in cow's milk. Proteins have different structure, conformation, and functioning due to differences in amino acid compositions and sequences. One of the most important features obtained by proteins following conjugation with polyphenols is antioxidant activity. The incorporation of antioxidant molecules into protein structure increases the functionality and bioactive properties of proteins, expanding their uses in the pharmaceutical and biomedical fields (Liu *et al*., 2015).

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). Furthermore, conjugating proteins with nonpolar polyphenols may improve the surface hydrophobicity of the modified proteins, increasing the native protein’s emulsifying characteristics (Ozdal *et al*., 2013). Protein polyphenol conjugates have been proposed as effective antioxidant emulsifiers that can locate and act at the oil-water interface, preventing oxidation in emulsion-based foods (Fan *et al*., 2018; Feng *et al*., 2018). The primary process of protein polyphenol conjugation is thought to be driven by oxidation, in which molecular oxygen oxidises the polyphenol's hydroxyl group to quinone (Prodpran *et al*., 2012). Whey protein polyphenol conjugates can be utilised to improve whey protein's functional qualities as well as to make functional meals.

Non-covalent and covalent contacts are the two forms of interactions between phenolic chemicals and proteins. Non-covalent interactions are weaker than covalent

interactions because they are based on hydrophobic, van der Waals, hydrogen binding, and ionic interactions. Furthermore, non-covalent interactions are largely reversible. Covalent interactions, on the other hand, are usually irreversible (Czubinski & Dwiecki, 2017). The interactions between whey 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. 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 Tao *et al*., (2019) 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. Rawel *et al*., (2003) compared the reactivity of several flavonoids under alkaline conditions and discover that the presence of catechol moieties (ortho-hydroxyl groups) on flavonoid’s 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) also claimed that quercetin and rutin can react with whey protein's lysine, tryptophan, and cysteine residues. When quercetin is conjugated with whey protein, it was more

reactive than rutin, 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). Liu *et al*., (2018) reported that ascorbate radicals (Asc^-) produced by the reaction between ascorbic acid and the redox pair H_2O_2 at pH 6 can attack the sensitive residues on the side chains of proteins, resulting in the formation of new macro radical species on the amino-acidic structure. These macro radicals can further conjugate with phenolic compounds through covalent linkages (Liu & Pu, 2018). However, the exact linkage positions between phenolic compounds and proteins are still unclear up to now (Spizzirri *et al., 2009).*

The application of enzyme catalyzed method for the synthesis of phenolic–protein conjugates are very limited, as compared with alkaline and free radical mediated grafting methods. Polyphenol oxidases (*e.g.,* laccase and tyrosinase) can catalyze phenolic compounds into electrophilic quinones. The quinones are freely diffusible so they can further undergo reaction with the nucleophilic amino groups of protein through non-enzymatic reactions. (Chung *et al*., 2003). The covalent bonds formed between phenolic compounds and proteins are Schiff-bases (C=N) and Michael type adducts (C–NH) (Qi & Wang, 2016). This indicates the synthetic mechanism of enzyme catalyzed method is much similar to that of alkaline method. The optimal reaction conditions usually depend on the type of enzyme selected. The catalyzed reaction is often carried out at pH 6.0–7.5.

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-linking's.

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-methylmorpholinium chloride and *N*-methyl morpholine as the cross-linking reagents in aqueous solution. Fu *et al.,* (2017) used 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride (EDC) and *N*-hydroxy succinimide (NHS) as the coupling

reagents to conjugate the carboxyl groups of chlorogenic acid with the amino groups of gelatin.

Table.1. Different Whey protein, polyphenols, conjugation methods and their overall effect

Whey protein	Polyphenol	Method of Conjugation	Results	Reference
Bovine serum albumin (BSA)	Chlorogenic acid	Covalent method	Improved Solubility, emulsion & foaming activity.	Rawel *et al*., 2002
β-lactoglobulin	Epigallocatechin gallate	Non-Covalent method	Increase in β- sheet and α-helix, leading to protein structural stabilization. β-lg structural changes is major factor, affecting on the antioxidant capacity.	Kanakis *et al., 2011*
β-lactoglobulin	Catechin, epigallocatechin gallate	Non-covalent method	Accelerated gelation of β-lactoglobulin and affected gel viscoelasticity. Galloylation of EGCG increase hydrophobicity of the flavonoid.	Bohin *et al.*, 2012
Bovine serum albumin (BSA)	Epigallocatechin gallate	Covalent method	Increased heat stability & emulsifying properties.	Wang *et al*., 2014
Lactoferrin	Gallic acid, Chlorogenic acid, EGCG	Covalent method	Improved thermal stability, significant increases in solubility. 0.23 to 2.10-Fold increase ABTS scavenging activity.	Liu *et al*., 2016

α-lactalbumin	Epigallocatechin-3-gallate	Non-Covalent method	Protein secondary structural changes. Release EGCG during digestion process and improve uptake.	Al-Hanish *et al*., 2016
Whey protein	CLA quinone	Covalent method	Enhanced emulsifying and foaming capacity. Improved antioxidant activity.	Xu *et al*.,2019
Bovine serum albumin	Caffeic acid	Free radical grafting method	Improved Antioxidant activity.	Fan *et al*., 2018
α-lactalbumin	Caffeic acid	Covalent method	Increased thermal stability and More stable to prevent oxidation.	Abd El Maksoud *et al*., 2018

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

Addition of polyphenols with whey proteins cause significant changes 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 whey protein functionality is discussed:

2.5.1 Emulsifying and foaming properties

Through covalent contact, polyphenols have a favourable effect on the physical and chemical emulsion stability of proteins. When covalent CLA quinone was added to whey proteins, it resulted in a more open protein structure, which improved emulsifying and foaming capabilities (Xu *et al*., 2019). Gelatine covalently treated with oxidised tannic acid, caffeic acid, or ferulic acid reduced the protein's hydrophobicity, increasing surface activity in emulsions and reducing the size of

emulsion oil droplet sizes (Aewsiri *et al*., 2013). Liu *et al*., (2016) reported improved creaming stability by covalent adducts of α-lactalbumin with EGCG or CLA.

2.5.2 Gelling

According to Strauss & Gibson (2004), the covalent modification of proteins with polyphenols also increased gel strength due to the oxidised polyphenols' known crosslinking activities. This was discovered in gelatine gels treated with rutin hydrate, caffeic acid, ferulic acid, or phenols from coffee or grape juice. After covalent attachment of oxidised ferulic acid, tannic acid, catechin, or caffeic acid to the fish protein, improved gelling properties (increased gel breaking force and deformation) were seen in gels. Polyphenols that generated multidentate binding were shown to be more effective than smaller phenolic acids (Balange & Benjakul, 2009). The gel deformation and breaking force decreased as the polyphenol/protein ratio increased, as did the water holding capacity. Because of the high degree of modification with polyphenols, there was a large loss of free thiol groups in proteins, resulting in a loss of intrinsic protein crosslinking potential. Furthermore, the phenolic compound's self-aggregation hampered the creation of a homogeneous gel (Tang *et al*., 2017; Wang *et al*., 2018; Zhang *et al*., 2018)

2.5.3 Solubility

Depending on the nature of the system, conjugating a protein with a polyphenol can either improve or reduce the protein's water solubility. According to Kroll *et al*., (2003) covalent attachment of charged polyphenols alters the electrical properties of proteins, especially the isoelectric point, causing pH-solubility profiles to vary. Attaching nonpolar polyphenols to proteins increases their surface hydrophobicity, which may boost their surface activity while decreasing their water solubility (Bandyopadhyay *et al*., 2012). Protein cross-linking may occur as a result of the reaction between polyphenols and proteins, altering their water solubility (Ozdal *et al*., 2013). Lactoferrin conjugation with chlorogenic acid and epigallocatechin-3-gallate (EGCG) significantly increased its solubility (Liu *et al*., 2016). The conjugates had a good solubility from pH 7 to 11 and from pH 2 to 3, but a low solubility at pH 4 and 5.

2.6 Controlling functionality of protein-polyphenol conjugates

Polyphenol–protein interactions are influenced by a number of factors, including polyphenol structure, protein structure, and some solution conditions (pH, ionic strength, temperature, etc.). Poncet-Legrand *et al*. used ITC to investigate the interaction of four flavan-3-ol monomers [catechin (C), epicatechin (EC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG)] and the oligomeric fraction of tannins with a model proline-rich protein poly(L-proline). They discovered that adding non-galloylated monomers (C and EC) to the poly (L-proline) resulted in insignificant heat changes compared to adding galloylated monomers (ECG, EGCG, etc.) to the poly (L-proline). Galloylated monomers have a stronger affinity for proline rich proteins than non-galloylated monomers, according to this experimental finding. Furthermore, the binding parameters show that polyphenols (ECG and EGCG) have a significantly greater binding constant with proline-rich proteins than with any other protein. This also shows that more proline in the protein chain promotes polyphenol binding.

Soares et al. reported binding of polyphenol (C, EC, ECG, EGCG) with protein [bovine serum albumin (BSA) and human salivary a-amylase (HSaA)]. BSA and HSaA are globular proteins with nearly identical sizes and three-dimensional distributions of distinct amino acid residues. The authors indicated that the distinct three-dimensional networks of BSA and HSaA cause unequal binding of the identical polyphenol. Proline residues strewn around the cavity's surface serve as binding sites for the multidentate ligand (polyphenols). Because small molecules like catechin can't create as many bonds in the huge pocket as massive tannic acid, a large cavity of BSA promotes bulky polyphenols (like EGCG) over small ones (like catechin).

The mode of polyphenol-protein interaction is determined not only by the structure of the polyphenol and protein, but also by the ratio of polyphenol to protein. Pascal et al. used DLS, ITC, and CD spectroscopy to investigate the interaction of human salivary proline rich protein with flavan-3-ol monomer (EGCG) in 2008. Agglomeration between protein and polyphenol took three steps at low protein concentrations, depending on the EGCG/protein ratio. When the EGCG/protein ratio is low, the interaction sites in the protein chain saturate due to EGCG binding (first stage, I), then as the EGCG/protein ratio rises, EGCG bridges the soluble particles (saturated protein chains), resulting in metastable colloids (second stage, II), and finally when

the EGCG/protein ratio is very high, haze forms (third stage, III) (third stage, III). EGCG, on the other hand, bridges the protein chains far before the saturation of interaction sites occurs at high protein concentrations, resulting in aggregation formation at much lower EGCG/protein ratios. As a result, the turbidity threshold lowered to a low EGCG/protein ratio at greater protein concentrations compared to low protein concentrations.

Wang *et al*, (2007) studied influence of temperature, salt content, and pH on the binding of EGCG to the BSA surface. They concluded that increasing the temperature leads the BSA chain to unfold, exposing the protein's hydrophobic surface. This allows more EGCG to attach to the protein surface at higher temperatures. EGCG adsorption on protein surface is suppressed by the addition of salt, which reduces the self-agglomeration probability of BSA (pH4.9, around its pI). EGCG adsorption capacity on the BSA surface varied with pH as well, with the highest adsorption capacity at pH 4.9 (around the pI of BSA), followed by pH 3 and pH 7.

Staszewski *et al*. (2011) used DLS and dynamic rheometry to investigate the effect of green tea polyphenols on the colloidal stability and gelation of whey protein concentrate (WPC). They discovered that particles generated at pH 6 (away from the pI of WPC) are smaller and have a higher charge than those created at pH 4.5 (near the pI of WPC), resulting in less polyphenol-protein complex precipitation at pH 6 than at pH 4.5. It has previously been observed that maximum binding of polyphenols by protein occurs at pH levels near the protein's isoelectric point.

Using fluorescence spectroscopy and DLS, Shpigelman *et al*, (2012) investigated the influence of temperature on the interaction of EGCG with b-lactoglobulin (β-lg). They discovered that EGCG binding was greater in the warmed b-lg than in the native protein at room temperature. When compared to room temperature, the binding constant at 70–80C was 3.2 times higher. They hypothesised that as protein molecules are heated, certain inner and non-polar domains are exposed, increasing the likelihood of EGCG binding to the protein molecule at higher temperatures.

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 in a given media.
- Proteins with a higher proline concentration are more likely to bind polyphenols.
- Galloylated polyphenolic compounds (ECG, EGC, EGCG) show a higher affinity for protein binding than non-galloylated polyphenolic compounds (C, EC).
- Polyphenol binding has an impact on the secondary and tertiary structure of protein molecules. The degree to which the protein structure is denaturized is determined by the polyphenol structure and the type of protein molecule studied.

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

Fluorescence spectroscopy can be used to detect interactions between phenolic chemicals and proteins (Choudhury *et al*., 2013). On the one hand, protein fluorescence is mainly caused by the indole chromophore of aromatic amino acids (e.g., tryptophan), which is substantially quenched by phenolic substances. Some phenolic compounds, on the other hand, have intrinsic fluorescence properties that are extremely sensitive to environmental conditions like polarity and hydrogen bonding effects. According to Liu *et al*., (2016) the fluorescence intensity is dependent on the type of phenolic chemical attached, with EGCG–zein > quercetagetin–zein > chlorogenic acid–zein being the most common. Furthermore, fluorescence spectroscopy is frequently employed to analyse non-covalent interactions and binding affinities between polyphenols and proteins. Trp-19 and Trp-61 are two tryptophan residues in b-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 just a little amount of Trp fluorescence. Tryptophan fluorescence may change when polyphenols interact with b-lactoglobulin, depending on the influence of non-covalent interactions on the protein structure.

2.7.2 Differential scanning calorimetry (DSC)

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

2.7.3 Fourier transform infrared (FT-IR) spectroscopy

The chemical interactions (including non-covalent and covalent interactions) between phenolic substances and proteins can be studied using FT-IR spectroscopy. The structure of proteins is complicated by the conjugation of phenolic chemicals. On the one hand, phenolic compound conjugation can dramatically diminish the functional groups in proteins' native side chains. Conjugation of phenolic chemicals, on the other hand, can introduce novel spatial and functional groups into protein backbones. By analysing the frequency of amide bonds, FT-IR spectroscopy is a good approach for estimating the change in the secondary structures of proteins after conjugation with phenolic chemicals. To examine the structure of proteins and phenolic–protein

conjugates, 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 (Kong *et al*.,2007; Liu *et al*.,2016). The amide group I is the most sensitive spectral region for secondary structure of proteins. α-helix (1658–1650 cm-1), β-sheet (1640–1615 cm-1), β -turn (1700–1660 cm-1) and random coil (1650–1640 cm-1) are the primary peaks in the region of amide band I (Zhu *et al*., 2012). Lactoferrin is protected against heat aggregation by polyphenol conjugation at neutral pH. In comparison to lactoferrin alone, the FT-IR spectra of EGCG–lactoferrin conjugate shows less modification after heat treatment (particularly b-sheet) (Liu *et al*, 2017). When tea polyphenols interact non-covalently with b-lactoglobulin, FT-IR spectroscopy shows a significant increase in the b-sheet and a slight rise in the a-helix (Kanakis *et al*., 2017).

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

The molecular weights of proteins and their phenolic conjugated analogues are routinely determined using electrophoresis techniques, particularly SDS-PAGE (Zhu *et al*., 2012). When SDS is allowed to completely react with proteins, the resultant SDS–protein complexes have identical charge densities, which inhibits non-covalent interactions between phenolic chemicals and proteins. As a result, the mobility of the sample on SDS-PAGE is determined by the sample size. SDS-PAGE can discriminate between non-covalent and covalent interactions between phenolic chemicals and proteins.

Gu *et al*. discovered in 2017 that 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. Because the non-covalent connections between catechin and egg white proteins are disrupted by adding SDS, the SDS-PAGE profile of catechin-egg white protein mixtures is identical to that of egg white proteins. This demonstrates that when proteins are conjugated with phenolic chemicals, their molecular weights rise. The covalently bonded phenolic moieties are responsible for the rise in the molecular weights of phenolic–protein conjugates.

2.7.5 Microscopic methods

Another method that provides insight into complicated anatomy, aggregation form, and the influence of polyphenol conjugation on proteins is atomic force microscopy

(AFM). It's a good way to track protein aggregation, which can be induced or halted by a variety of tiny chemicals, including polyphenols. The method provides information on the conjugates' shape and size. However, because AFM imaging is performed on dehydrated samples, the results cannot be compared to hydrodynamic light diffusion measurements in dispersions (Wobst *et al*., 2015). The microstructure of the conjugated samples can also be characterised using a scan electron microscopy (SEM).

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 is based on calculating the heat generated during the chemical combination (Al-Hanish *et al.,* 2016). In titration mode, the ligand, polyphenol, has been titrated into a macromolecule (Protein), and numerous injections have been made at various times. A cell containing simply a buffer determines the heat emitted or absorbed into the sample cell where the complexation reaction occurs at any injection. The thermodynamic characteristics of interactions (free energy changes (G), enthalpy changes (H), and entropy changes (S)) are critical to understand the binding mode. Interactions may entail hydrophobic forces, static interactions, interactions with van der Waals, and hydrogen bonds. The fact that variations in free energy have a negative sign suggests that the contact mechanism is random. The connection between positive enthalpy and entropy changes is aided by hydrophobic forces (Bose, 2016). Exothermic enthalpy has been found to improve with the production of α -lactalbumin and epigalocatechin-3- gallate (EGCG) complexes (20 mM phosphate buffer, pH 7.2). (Al-Hanish *et al*, 2016). It was also discovered that EGCG binding to lipase is a phenomenon. During the ITC analyses, the thermal stability of the BSA following contact with ferulic acid (pH 7.4) was improved. ITC has been used in conjunction with other approaches to investigate the interactions of tiny compounds, such as Tea Catechin as a catalase inhibitor (Pal *et al*., 2014).

MATERIALS AND METHODS

This chapter deals with the materials, experimental methodologies, procedures, and techniques employed during the present investigation.

3.1 Materials

Regarding various ingredients used during the study are delineated here under:

3.1.1 Whey Protein Concentrate (WPC 70%)

Whey Protein Concentrate was procured from the Modern Dairy Pvt. Ltd. Karnal, Haryana.

3.1.2 Polyphenols

Caffeic Acid and Ferulic Acid standard compounds were taken.

3.1.2.1 Caffeic Acid

Caffeic Acid was procured from Sigma Aldrich.

3.1.2.2 Ferulic Acid

Ferulic Acid was procured from Sigma Aldrich.

3.1.3 Chemicals

All the chemicals used during investigation were of AR grade and obtained from standard supplier.

3.2 Analysis of Whey Protein Concentrate

WPC was analysed for total protein content.

3.2.1 Estimation of protein by Kjeldahl method

Total nitrogen was estimated using Kjeldahl method and the protein content was determined by multiplying the estimated total nitrogen with the factor of
6.38.

3.2.1.1 Reagents: -

Concentrated sulphuric acid, potassium sulphate, copper sulphate solution (5%), mixed indicator (methyl red- saturated alcoholic methyl red dye, methylene blue-

0.2% solution in ethanol), saturated boric acid (4%) and sodium hydroxide (40%), hydrochloric acid (0.1 N).

3.2.1.2 Procedure: -

A. Test portion and pre-treatment: -

To the clean and dry digestion tubes, 12 g of potassium sulphate and 1 ml of copper sulphate solution was added. About 0.15 g ± 0.05 g of the sample was accurately weighed and was added into digestion tube. Then, 20 ml concentrated sulphuric acid was poured along the sides of the digestion flask to wash down any copper sulphate solution, potassium sulphate or test portion left on the neck of the flask and contents were mixed gently and tube was left at room temperature for 10 min.

B. Digestion: -

1. Digestion tubes were fixed in the digestion block and the main switch of instrument was turned on. Digestion block was set at a low initial temperature to control foaming (at approximately between 180°C and 230°C).
2. Test portion was digested for 30 min or until white fumes develop. Then temperature of digestion block increased to between 410°C and 430°C. Test portion was digested until clear and free of undigested remnant was obtained.
3. The digestion process was completed in 2-2.5 hours.
4. The digestion tube was removed from the block with the exhaust manifold in place and allowed to cool to room temperature for approximately 30 min.

C. Distillation: -

1. Turned on the condenser water for the distillation apparatus. Digestion tube containing digested test portion was attached to distillation unit and automatic programme was started in distillation unit.
2. Conical flask placed under the outlet of condenser where 40 ml of boric acid solution was delivered automatically as set in method.
3. In distillation unit, method was set to dispense 70 ml of 40% sodium hydroxide solution in the digestion tube.

4. Liberated ammonia was collected in the conical flask containing excess boric acid solution with indicator.

5. Conical flask was removed for further titration.

D. Titration: -

1. 0.1 N Hydrochloric acid was filled in burette of 25 ml with the help of funnel.

2. Then the content of the conical flask was titrated against 0.1 N Hydrochloric acid till the appearance of pink colour.

3. The amount of 0.1N HCL used for titration was measured.

3.2.1.3 Blank test: -

Blank test was carried out by following the same procedure as described above, taking 5 ml of water and about 0.85 g of sucrose instead of test portion.

Calculations: -

Nitrogen content was calculated by following equation:

$$1.4007 * (Vb - Vs) * N/\mathrm{W_n}$$

here,

W_n = nitrogen content of the sample, expressed as percentage by mass,

V_s = millilitre of standard hydrochloric acid used for WPC sample

V_b = millilitre of standard hydrochloric acid used for blank sample

Calculate the protein content W_p using the following equation:

$$Wp = Wn * 6.38$$

3.3 Preparation of whey protein polyphenol conjugates by different methods.

3.3.1 Non-Covalent Method (Ferraro *et al*., 2015)

3.3.1.1 Apparatus: -

Balance, capable of weighing to the nearest 1 mg; Beaker, 50ml capacity; Conical Flask, 50ml capacity; Dialysis tubing membrane; Amber coloured Centrifuge tubes,15ml capacity;

3.3.1.2 Reagents: -

Potassium Phosphate; Potassium dihydrogen Phosphate,

3.3.1.3 Procedure: -

To Prepare Non-Covalent conjugates, WPC powder (1.42g in 100ml) was dispersed in phosphate buffer (50 mM, pH3, pH5 & pH7) to make 1% solution. The stock solution of WPC was stored 24hr at 4°C for Hydration, to ensure complete dispersion and dissolution, while sodium azide (0.02, w/w) was added to prevent microbial growth. Then 0.2% solution of caffeic and ferulic acid individually was mixed with the protein dispersion and then the pH3, pH5 & pH7 was readjusted using 0.1N NaOH &0.1N HCL. Incubation at 4 °C for 24 hr was done to allow interaction between Protein and
Polyphenol. Free unreacted polyphenol in reaction solution was removed by dialysis (5Kda) against Distilled water

3.3.2 Covalent methods

3.3.2.1 Apparatus: -

Balance, capable of weighing to the nearest 1 mg; Beaker, 50ml capacity; Conical Flask, 50ml capacity; Dialysis tubing membrane; Ambered coloured Centrifuge tubes,15ml capacity;

3.3.2.2 Reagents: -

Potassium Phosphate; Potassium dihydrogen Phosphate; Ethylcarbodimide; Hydroxy succinimide; ascorbic acid; hydrogen peroxide

I. Alkaline Method (Abd El- Maksoud *et al*., 2018)

WPC powder (1.42g in 100ml) was dispersed in phosphate buffer (50 mM& pH9) to make 1% solution. The stock solution of WPC was stored 24hr at 4°C for Hydration, to ensure complete dispersion and dissolution, while sodium azide (0.02, w/w) was added to prevent microbial growth. Then 0.2% solution of caffeic and ferulic acid individually was mixed with the protein dispersion and then the pH9 was readjusted using 0.1N NaOH. Incubation was done at room temperature for 24hr. Free unreacted polyphenol in reaction solution was removed by dialysis (5Kda) against Distilled water.

II. Chemical Coupling Method (**Abd El- Maksoud *et al*., 2018)**

WPC powder (1.42g in 100ml) was dispersed in phosphate buffer (50 mM& pH9) to make 1% solution. The stock solution of WPC was stored 24hr at 4°C for Hydration, to ensure complete dispersion and dissolution, while sodium azide (0.02, w/w) was added to prevent microbial growth. Then coupling reagent (Ethylcarbodimide 20mg/10ml & Hydroxy succinimide12mg/ml) was added. After That 0.2% solution of caffeic and ferulic acid individually was mixed with the protein dispersion and then the pH9 was readjusted using 0.1N NaOH. Free unreacted polyphenol in reaction solution was removed by dialysis (5Kda) against Distilled water.

III. Free Radical Grafting Method (Fan *et al*., 2018)

WPC powder (1.42g in 100ml) was dispersed in phosphate buffer (50 mM& pH7) to make 1% solution. The stock solution of WPC was stored 24hr at 4°C for Hydration, to ensure complete dispersion and dissolution, while sodium azide (0.02, w/w) was added to prevent microbial growth. Then 0.25g ascorbic acid and 1mL of 5M hydrogen peroxide per 100ml Protein solution was added. The mixture was Incubated at Room Temperature under continuous magnetic stirring for 2hr. After That 0.2% solution of caffeic and ferulic acid individually was mixed with the protein dispersion and then the pH7 was readjusted using 0.1N NaOH. Free unreacted polyphenol in reaction solution was removed by dialysis (5Kda) against Distilled water.

3.4 Determination of Total Phenolic content (Hu *et al*., 2016)

3.4.1 Apparatus: -

Test tubes**,**10ml Capacity; Test tube Stand; Volumetric Flask, 10ml Capacity; Vortex mixture; Incubator, maintained at 25°C, Quartz Cuvettes; Spectrophotometer;

3.4.2 Reagents: -

Folin-Ciocalteu reagent (2N was diluted to 0.2N), Sodium carbonate solution (7.5%), Gallic Acid.

3.4.3 Procedure: -

I. 500µl of the Sample was taken in a test tube.

II. 2.5ml of 0.2N Folin-Ciocalteu reagent was added.

III. 2ml of 7.5% Sodium carbonate solution was added and mixed the content using vortex mixer.

IV. Then the solution was incubated at 25°C for 20-30 minutes for reaction to complete.

V. Then Absorbance was measured at 765nm against blank.

VI. The obtained absorbance of the sample was used to determine total polyphenol content in sample using standard curve.

3.4.4 Preparation of Gallic Acid Standard Curve: -

3.4.4.1 Procedure: -

Preparation of standard gallic acid solution stock solution (10mg/ml): 1g of gallic acid was dissolved into 10ml distilled water.

1. 10 volumetric flasks of 10ml capacity were taken.
2. For 10mg/ml concentration 1ml of standard stock solution, for 20mg/ml concentration 2ml of standard stock solution were taken into different 10ml flasks subsequently for 30mg/ml, 40mg/ml, 50mg/ml, 60mg/ml, 70mg/ml, 80mg/ml, 90mg/ml and 100mg/ml the 3ml, 4ml, 5ml, 6ml, 7ml, 8ml, 9ml and 10ml standard stock solution was added and the volume of flask is made up to 10ml using distilled water.
3. 500µl of the solution from each flask was taken in separate test tubes.
4. 2.5ml of 0.2N Folin-Ciocalteu reagent was added.
5. 2ml of 7.5% Sodium carbonate solution was added and mixed the content using
6. vortex mixer.
7. Then the solution was incubated at 25°C for 20-30 minutes for reaction to complete.
8. Then Absorbance was measured at 765nm against blank.
9. Then absorbance vs mg gallic acid Concentration graph was plotted.

3.5 CHARACTERIZATION OF WHEY PROTEIN POLYPHENOL CONJUGATES

3.5.1 Structural characterization

3.5.1.1 Spectrofluorometric analysis (Arroyo-Maya *et al*., 2016)

The fluorescence spectroscopy of WPC-Caffeic Acid Complexes and WPC-Ferulic Acid Complexes for Covalent as well as Non-Covalent Conjugation were recorded using a Spectrofluorometer. The excitation wavelength was 280 nm, and the emission spectra were scanned in the range of 300–500 nm with a scanning speed of 12,000 nm/min. Analysis of second derivative spectra and Peak fitting analysis was done using OriginPro software.

3.5.1.2 UV-visible spectroscopy (Liu *et al*., 2017)

UV-visible absorbance measurements were carried out using a spectrophotometer (UV 2700, Shimadzu, Japan) with a 1 cm optical path length. The spectra of WPC, WPC-polyphenol conjugates solution was determined after an appropriate dilution (1:50).

The wavelength scanning range was 200 to 600 nm.

3.5.1.3 FTIR spectroscopic measurements (Kanakis *et al*., 2011)

Spectra of samples were measured using FTIR spectrometer (IR Affinity-01, Shimadzu, Japan) with inbuilt IR-Solution software at 4.0 cm-1 resolution. Spectra were measured in terms of absorption. Each spectrum was averaged over 30 scans with a 4.0 cm-1 resolution. Spectra in the wavenumber range from 400 to 4000 cm^{-1}.

3.5.2 Determination of particle size distribution

The mean particle diameter, particle size distribution, Z- average, zeta-potential and were measured by using Malvern Nano ZS90 Zetasizer (Malvern Instruments). The experiments were carried out on the 1:10 diluted freshly prepared Samples. It measures particle diameter and particle distribution width, realizing this wide dynamic range by means of photon correlation spectroscopy. Dispersed particles suspended in solution moving due to Brownian motion are irradiated with laser light, and light scattered from the particles is detected using a photomultiplier tube as detector and semiconductor laser excitation solid laser (532 nm) as the light source. Disposable four-side plain cuvettes were used under an operating temperature of 25°C and

humidity 85%. Particle size measurements were carried out in triplicate for each Samples and represented as mean ± standard deviation (SD) of five replicates.

3.5.3 Determination of zeta potential

The electrical charge on the oil droplets in the emulsions was determined using Malvern Nano ZS90 Zetasizer (Malvern Instruments) in the form of zeta potential and conductivity of the prepared Conjugates. The experiments were carried out on the 1:10 diluted freshly prepared Conjugates. It is based on the principle of Laser Doppler electrophoresis. In this method, sample particles suspended in a solvent are irradiated with laser light and an electric field is applied. When the frequency shift at angle θ is measured once the electric field is applied, the following relationship between particle motion velocity (V) and mobility (U=V/E) is formed. The analyser uses a heterodyne optical system to observe particle motion velocity and calculate electrical mobility from the resulting frequency intensity distribution.

The zeta potential is determined by measuring the velocity of the droplet when in an applied electric field. The measurement was carried out under holder at 25°C and electric voltage 3.9 V. A parallel plate electrode (0.45 cm-2 square platinum plates with a 0.4 cm gap) was inserted and the cuvette was placed in a temperature-controlled holder at 25°C. Measurements were carried out for each sample and results were reported in mV. The Final results were represented as mean ± standard deviation (SD) of five replicates

3.6 ANALYSIS FOR FUNCTIONALITY OF WHEY PROTEIN-POLYPHENOL CONJUGATES

3.6.1 Antioxidant activity

3.6.1.1 Apparatus: -

Test tubes,10ml Capacity; Test tube Stand; Volumetric Flask, 100ml Capacity; Vortex mixture; Quartz Cuvettes; Spectrophotometer;

3.6.1.2 Reagents: -

Methanol,70% solution; DPPH (2,2-diphenyl-1-picrylhydrazyl) solution, 0.2mM;

3.6.1.3 Procedure: -

The antioxidant activities were measured in terms of DPPH (2,2-diphenyl-1picrylhydrazyl) Radical scavenging Activity (RSA) according to Abd El-Maksoud et al.,2018.1mL of WPC-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 ethanol 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). % Radical Scavenging activity was calculated using formula: -

$$\%\ Radical\ Scavenging\ Activity = A0 - A1 * 100 / A0$$

Here, A0= the absorbance of the control reaction (containing all reagents except the test compounds)

A1= absorption of test extract solution (t=30 mints)

3.6.2 Solubility (Chen *et al*., 2019)

3.6.2.1 Apparatus: -

Centrifuge tubes,20ml Capacity; Centrifuge;

3.6.2.2 Procedure: -

The solubility of WPC 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 analysed using Kjeldahl method (ISO 8968-1:2014). Solubility was calculated using formula: -

$$\%\ Solubility = \text{Protein (\%) in Supernatant} * 100\ / Protein\ (\%)\ in\ initial\ Dispersion$$

3.6.3 Emulsifying activity and emulsion stability

3.6.3.1 Apparatus: -

Beaker,50ml capacity; Magnetic stirrer; Ultrasonicator; Centrifuge;

3.6.3.2 Reagents: -

Soybean Oil;

3.6.3.3 Procedure: -

Emulsifying activity and emulsion stability of WP-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 Whey Protein -Polyphenol Conjugate Solution and 25ml of Soybean oil in a beaker. Allowed the mixture for 20-30 min for magnetic stirring then after Ultrasonication (Sonics VCX-750 watt) was done for 15min (27°C & 50% amplitude) in Ice bath.10ml of Prepared Emulsion was taken in graduated tubes and centrifuged at 2600g for 10min and volume of the emulsion phase (EPV) were measured. Emulsifying Activity was calculated according to formula: -

$$Emulsion\ activity = \text{Emulsion Phase Volume} / Total\ Volume$$

For emulsion stability (ES) emulsions were prepared in the same way as in the emulsion activity test and kept at 80°C for 30 min, then cooled in an ice bath for 15min and finally centrifuged at 1300 x g for 10 min and volume of the emulsion phase (EPV) were measured. Emulsion stability was calculated according to formula:

$$Emulsion\ Stability = \text{Volume of Emulsified Layer after Heating / Volume of Emulsified Layer before Heating}$$

3.6.4 Foaming capacity (Jiang *et al*., 2018)

3.6.4.1 Apparatus: -

Beaker,250ml capacity; Measuring Cylinder; 250ml capacity; IKA T-25 ULTRA-TURRAX Digital High-Speed Homogenizer

3.6.4.2 Procedure: -

Foams were prepared by blending 60mL of WPC Conjugate and Control WPC solutions for 120s at a high speed (12,500 r/min) using IKA T-25 ULTRA-TURRAX Digital High-Speed Homogenizer (Jiang et al., 2018). Foaming capacity (FC) and foam stability (FS) were calculated according to the following equations:

$$Foaming\ Capacit(FC) = (V0 - V) * 100 / V$$

$$Foaming\ Stabilit(FS) = V30 * 100 / V$$

Here,
V= the applied volume of protein solution (60mL)
V0 = the generated foam volume immediately after blending,
V30 = represents the remaining foam volume after the foams were set for 30min.

3.7 STATISTICAL ANALYSIS

All statistical analysis was performed using MS-EXCEL-2016 package. Results are presented as mean ± standard deviation (SD) of five replicates, and significance was tested by employing analysis of variance (ANOVA).

RESULTS AND DISCUSSIONS

In the present study, whey protein was conjugated with plant polyphenols (caffeic and ferulic acid) and the structural and functional characteristics of the conjugates were determined. The conjugation between polyphenol and whey protein concentrate (WPC) was achieved by non-covalent (pH 3, 5 and 7) as well as covalent (alkaline, free radical grafting and chemical coupling) methods.

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

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

In the present study, the whey protein-polyphenol conjugates were prepared using both non-covalent and covalent approach. In non-covalent approach, the protein (1%) solution of pH 3, 5 and 7 was mixed with 0.2% solution of caffeic and ferulic acid individually and incubated at 4ºC 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 3.3.2. The unreacted caffeic and ferulic acid was removed by dialysis using 5KDa membrane.

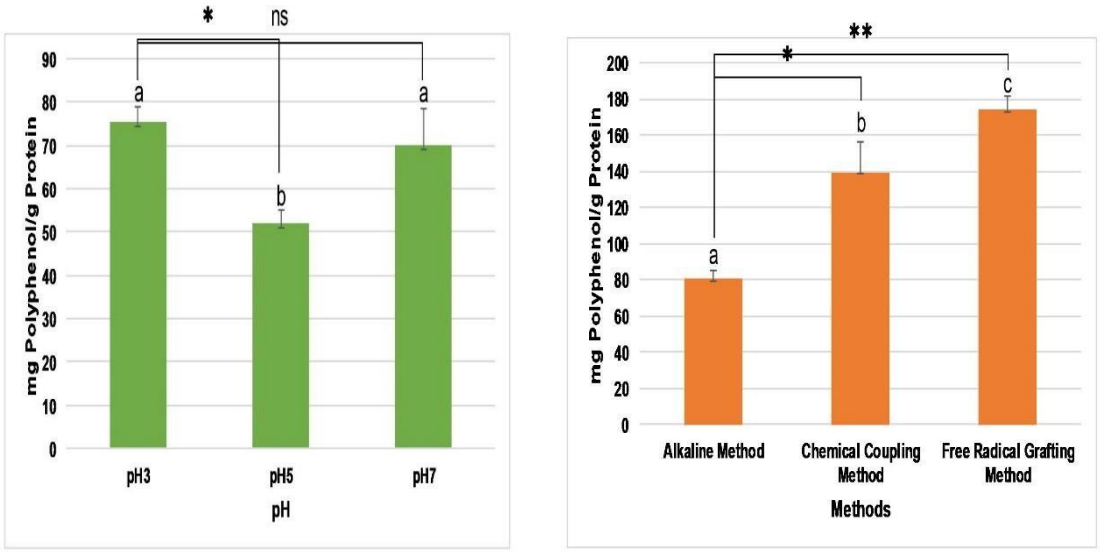
The polyphenol content was measured in conjugates using folin's method. The protein (1%) solution of without polyphenol was used as control. The polyphenol in whey protein-caffeic acid (WP-CA) and whey protein-ferulic acid (WP-FA) conjugates was calculated as mg polyphenol per g of protein in and presented in Figure 4.1 and 4.2, respectively.

As shown in Fig. 4.1.1 (A) The total phenolic contents of the WP-CA Non-covalent conjugates produced at pH 3.0 and 7.0 were higher than those at pH 5.0. This result indicated that binding of caffeic acid with whey protein was higher at pH 3 and 7 in comparison to pH 5. At pH 3, higher binding may be attributed to dissociation of protein that has provided more binding sites to polyphenol (Sastry & Rao, 1990). Similar Results reported by de Morais et al., 2020 that FCRRS (Folin Ciocalteu reagent reducing substances) values of WPI:CA-3.5 pH complexes were higher than those of WPI:CA-7.0 pH. Ozdal *et al*., (2013) said that Protein dissociation occurs at

low pH (pH < 7.0), exposing binding sites for interaction with polyphenols via electrostatic interactions led to stronger binding. For covalent conjugates, as shown in Fig. 4.1.1 (B) highest interaction of caffeic acid with whey protein were observed in free radical grafting method compared to conjugates prepared by alkaline and chemical coupling methods. The phenolic content of all covalent conjugates was higher than that of non-Covalent conjugates, which indicated that more binding of the caffeic acid with whey protein in covalent methods. Because Non-covalent protein-polyphenol interactions are usually reversible and are weaker than covalent interactions (Czubinski & Dwiecki, 2017; Jakobek, 2015).

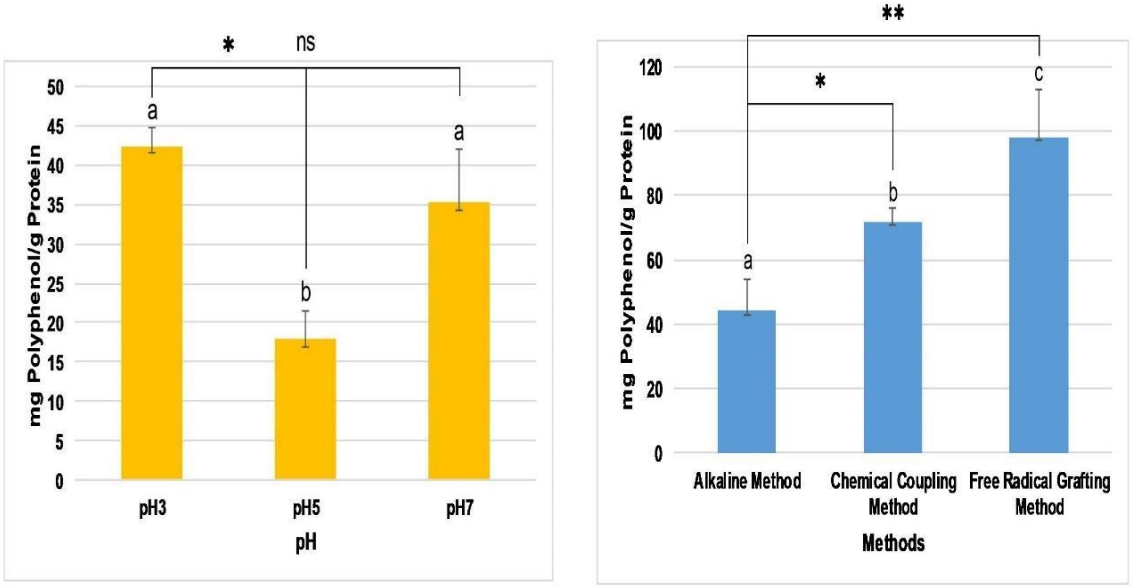
As shown in Fig. 4.1.2, the total phenolic contents of the WP-FA for non-covalent as well as covalent conjugates was lower than that of WP-CA conjugates. From, the results, it can be described that WP interacted with FA but the interaction was lower in comparison to CA. This may be ascribed to one extra -OH group present in the caffeic acid structure (Horbury *et al.*,2016)

Among non-covalent conjugates of WP-FA, the results were similar to WP-CA conjugates and higher binding was observed at pH 3 and 7 in comparison to pH 5. The binding of FA was found to be higher in conjugates prepared by covalent methods then non-covalent methods. Among covalent conjugates of WP-FA, maximum binding was observed in free radical grafting method than alkaline and chemical coupling methods.



(A) (B)

Fig. 4.1.1 Polyphenol content (mg/g protein) in WP-CA conjugates prepared by A) non-covalent method (at different pH 3.0, 5.0 and 7.0) B) covalent method (alkaline, chemical coupling, free radical grafting method)



(A) (B)

Fig. 4.1.2 Polyphenol content (mg/g protein) in WP-FA conjugates prepared by A) non-covalent method (at different pH 3.0, 5.0 and 7.0) B) covalent method (alkaline, chemical coupling, free radical grafting method)

4.2 Characterization of whey protein-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.

4.2.1 Changes in Fluorescence Spectra

The fluorescence spectroscopy of WP-CA and WP-FA conjugates prepared by covalent as well as non-covalent method were recorded using a spectrofluorometer. The excitation wavelength was 280 nm, and the emission spectra were scanned in the range of 300–500 nm with a scanning speed of 12,000 nm/min (Jiang *et al*., 2018). Intrinsic fluorescence characteristics of proteins are sensitive to the polar microenvironment and have been widely exploited to identify changes in protein tertiary structure. At a specific excitation wavelength, aromatic amino acids (tryptophan, tyrosine, and phenylalanine) which have a benzene ring structure with conjugated double bonds, can create intrinsic fluorescence (Jiang *et al*., 2018). The interaction of polyphenol with proteins interferes in the intrinsic fluorescence of protein, therefore, can be used as an effective tool for confirming the conjugation between proteins and polyphenols.

Emission spectra from 300 to 500 nm of control, WP-CA and WP-FA Conjugates prepared by non-covalent approach (pH 3.0, 5.0, 7.0) are shown in Fig 4.2.1.1
Control WP have shown maximum intensity at wavelength (λ) 360 nm, which was close to the reported maximum wavelength (λ) 350 nm (Arroyo-Maya *et al*., 2018). On conjugation of polyphenol (both CA and FA), decreased intrinsic fluorescence intensity was observed at all pH values. Protein fluorescent emission within the range of 340-350 nm can be attributed to a decrease in this range of fluorescence emissions due to the presence of aromatic amino acids and ligand interactions (Rawel et al., 2002; Liu *et al*., 2010; Czubinski *et al*., 2014).

The intrinsic fluorescence intensity of WP decreased with the addition of CA and FA, which suggested that conjugation with CA and FA resulted in the unfolding protein structure of WP and its interaction with polyphenol that has blocked the fluorophore amino acids (Xu *et al*., 2019). Fluorescence intensity of WP-FA conjugates was

higher throughout wavelength 300-500nm compared to that of the WP-CA, which may be due to the less interaction between FA with WP compared to CA. The results were supported by total phenolic content in WP-CA and WP-FA conjugates, which was higher in WP-CA conjugates. On conjugation of CA and FA with WP, the maximum wavelength was shifted to higher wavelength, which is known as red shift. The red shift indicates that tyrosine and tryptophan residues transferred to a more hydrophilic environment means unfolding of protein and distortion of the tertiary and quaternary structure (Royer, 2006). A red shift of 20 nm was observed for WP-CA and WP-FA conjugates at all pH values.

As Shown in Fig 4.2.1.2, the fluorescence intensity of covalent conjugates was lower as compared to non-covalent conjugates. This was in accordance to the total phenolic content in WP-CA and WP-FA conjugates which was higher in covalent conjugates than non-covalent conjugates. Similar to non-covalent conjugates, in covalent conjugates also the shift in maximum wavelength was also observed. In conjugates prepared by chemical coupling method, a red shift was of 30 nm and 10 nm was observed for WP-CA and WP-FA conjugates, respectively. In conjugates prepared by alkaline method, a red shift of 10 nm observed for both WP-CA and WP-FA conjugates, whereas no red shift was observed for conjugates prepared by free radical grafting method. Also, fluorescence intensity of WP-CA conjugates was lower as compared to WP-FA conjugates. Kristo, Hazizaj, & Corredig, (2012) also reported decrease in fluorescence intensity indicates that the interaction of chlorogenic acid and proteins caused fluorescence quenching of tryptophan. A small decrease in the FI% (fluorescence intensity) of the noncovalent complex of β-lg and CA (caffeic acid) (β-Lg/CA; 225% FI) compared to native β-lg (250% FI) (Abd El-Maksouda et al., 2018). Arroyo-Maya *et al*., (2016) found that the protein fluorescence intensity, which is dominated by the emission of the two tryptophan residues present in its structure, decreases appreciably with increasing pelargonidin concentration whereas there was only a slight wavelength shift (2 nm) of the spectral maximum.

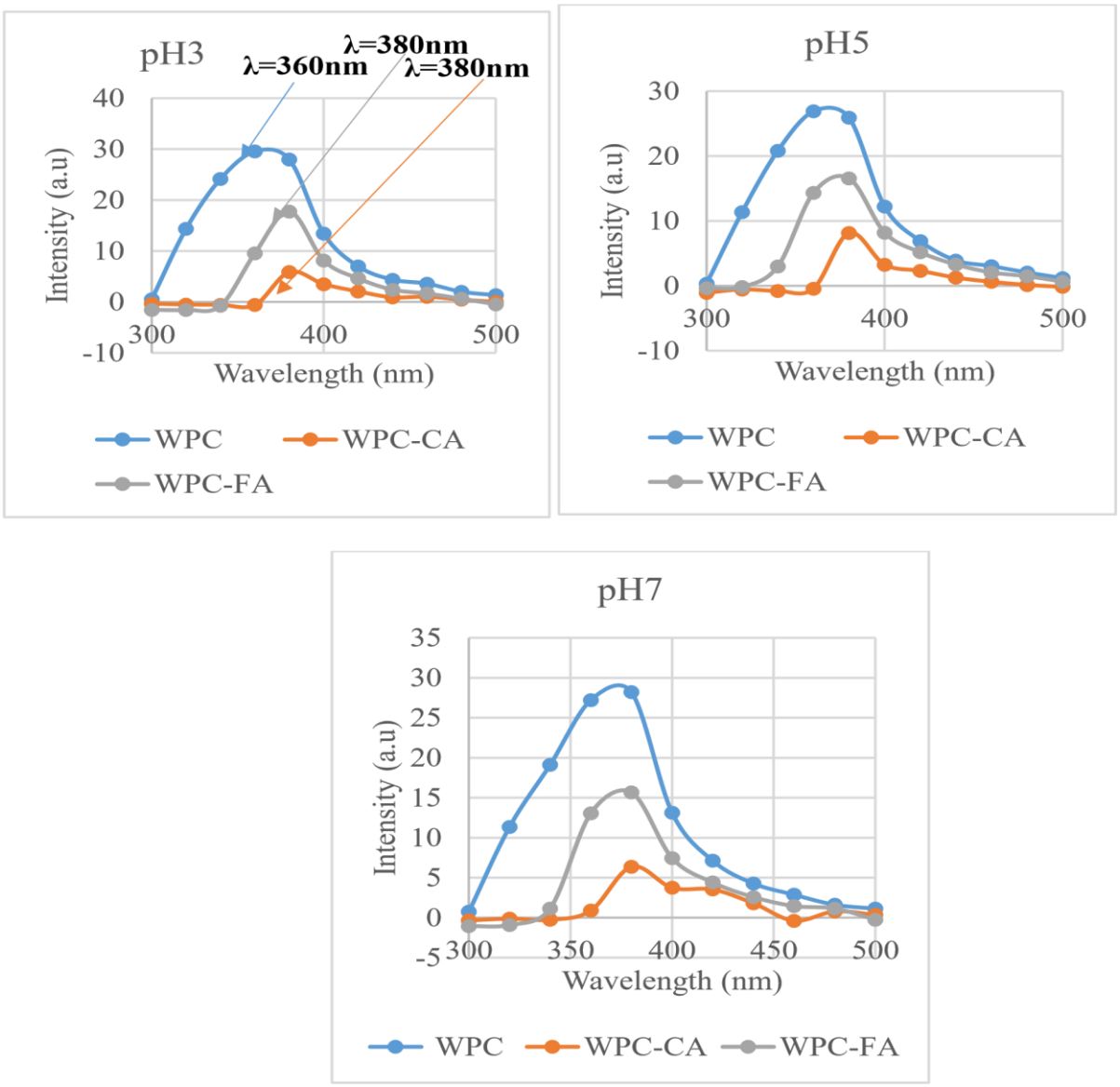


Fig. 4.2.1.1 Fluorescence Spectra of WP-Caffeic Acid & WP-Ferulic Acid Non-Covalent Conjugates and Control WP at pH 3.0,5.0.,7.0.

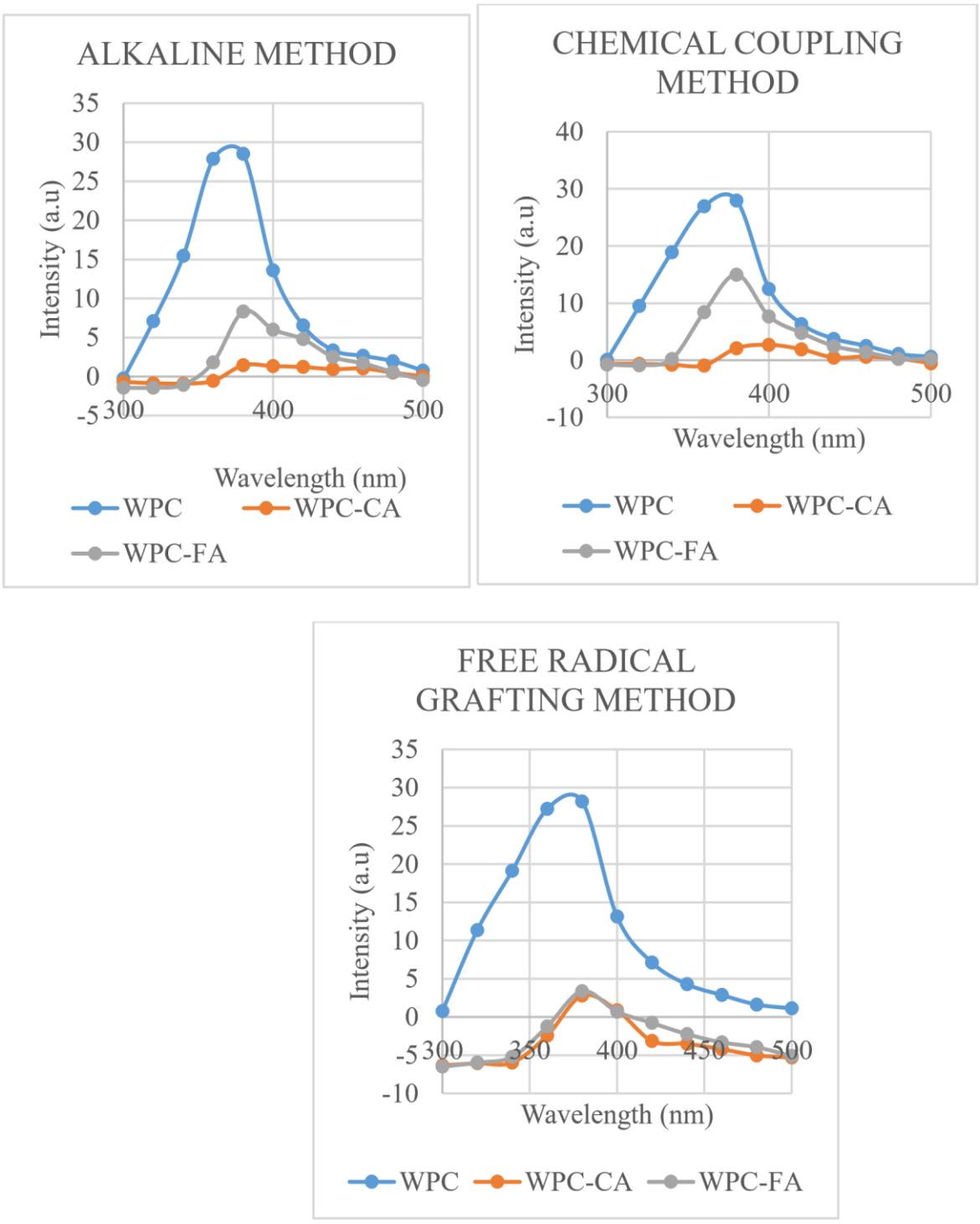


Fig. 4.2.1.2 Fluorescence Spectra of WP-Caffeic Acid & WP-Ferulic Acid Covalent Complexes and Control (Alkaline Method, Chemical Coupling Method, Free radical Grafting Method).

4.2.2 Change in UV-visible spectra

UV-visible absorbance measurements were carried out using a Spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) with a 1 cm optical path length). The wavelength scanning range was 200 to 600 nm. UV–visible spectroscopy was used to provide

some insights into the impact of polyphenol type and the nature of the interactions on molecular structure.

As shown in the Fig. 4.2.2.1 the absorbance of the non-covalent conjugate (WPCA and WP-FA) at all pH values was appreciably lower than that of the control WP.
Also, the conjugates (WP-CA and WP-FA) have shown higher absorbance at 280-335 wavelength that may be due to strong absorptions of CA and FA in the wavelength of 315-400 nm (Horbury *et al*., 2016).

The Fig 4.2.2.2 represent the UV-Vis spectra of covalent conjugates of WP-CA and WP-FA. With comparison to control WP, the changes in UV-Visible spectra of covalent conjugates were more pronounced as compared to non-covalent conjugates. The higher changes have provided information, that structure of WP was altered and binding with polyphenol was more in covalent conjugates. For all wavelengths, in all the method of covalent conjugation, appreciably higher absorbance was observed than control WP and intense peaks were observed at 280 and 325 nm.

The UV–Visible spectra of WPI-CQA (Chlorogenic acid), WPI-RA (Rosmarinic acid), and WPI-Q (quercetin dihydrate) conjugates, formed at alkaline compared to the control of WPI (280 nm), shows maximum absorption around 325, 330 and 350 nm for modified WPI respectively (Ali, 2019). After varied catechin doses were added, the absorbance of β-lg was significantly increased, with a red shift to the maximum wavelength. The increased absorption and modification in the maximum wavelength indicate that complex formation is also modified between β-lg and catechin and the microenvironment polarity (Al-Shabib *et al*., 2020). For the zein-EGCG system, the conjugate had an appreciably higher absorbance than the physical mixture, which suggested that the formation of a covalent complex altered the structure of zein (Liu *et al*., 2016)

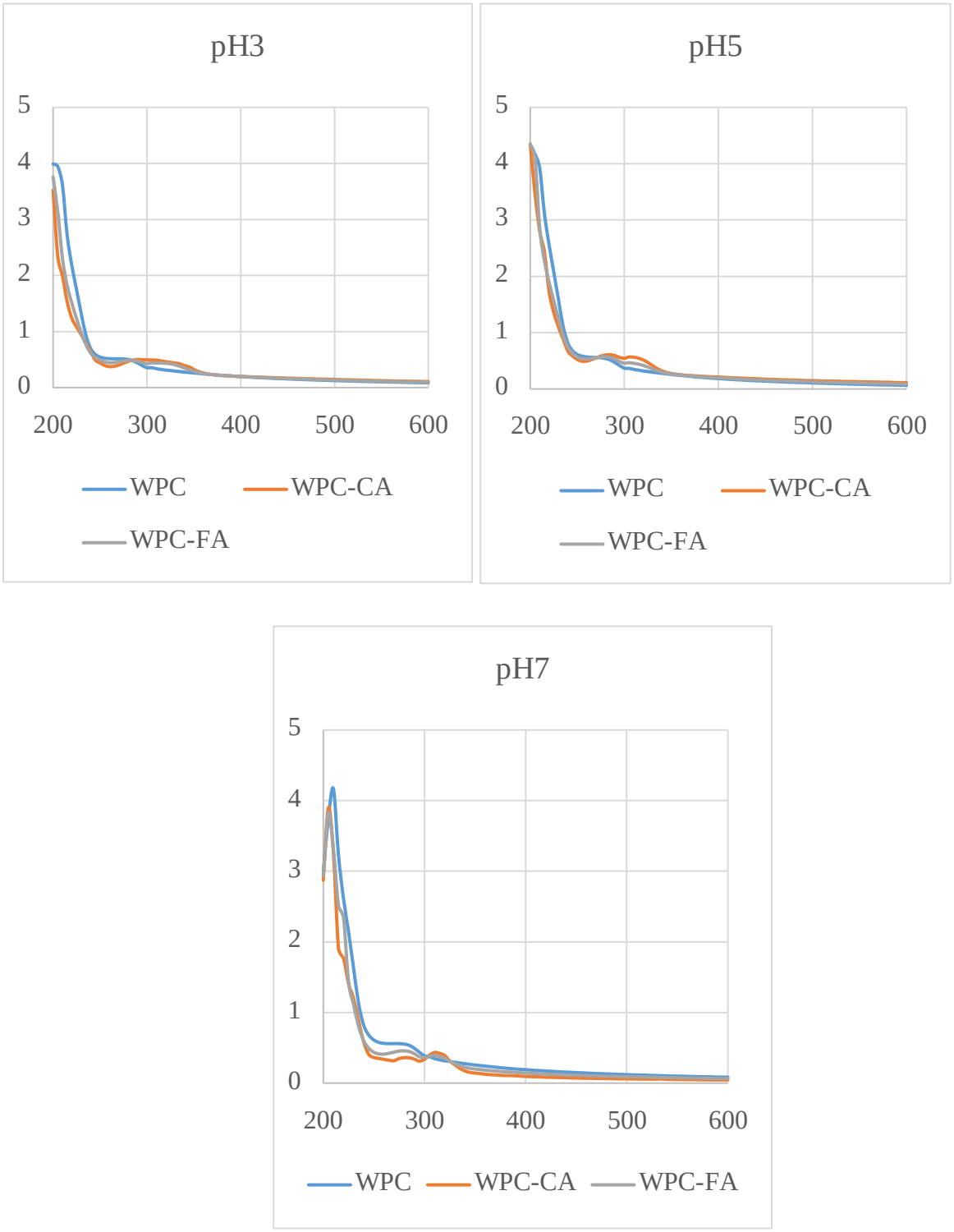


Fig. 4.2.2.1 UV-Visible Spectra of WP-Caffeic Acid & WP-Ferulic Acid Non-Covalent Conjugates and Control WP at pH 3.0,5.0.,7.0.

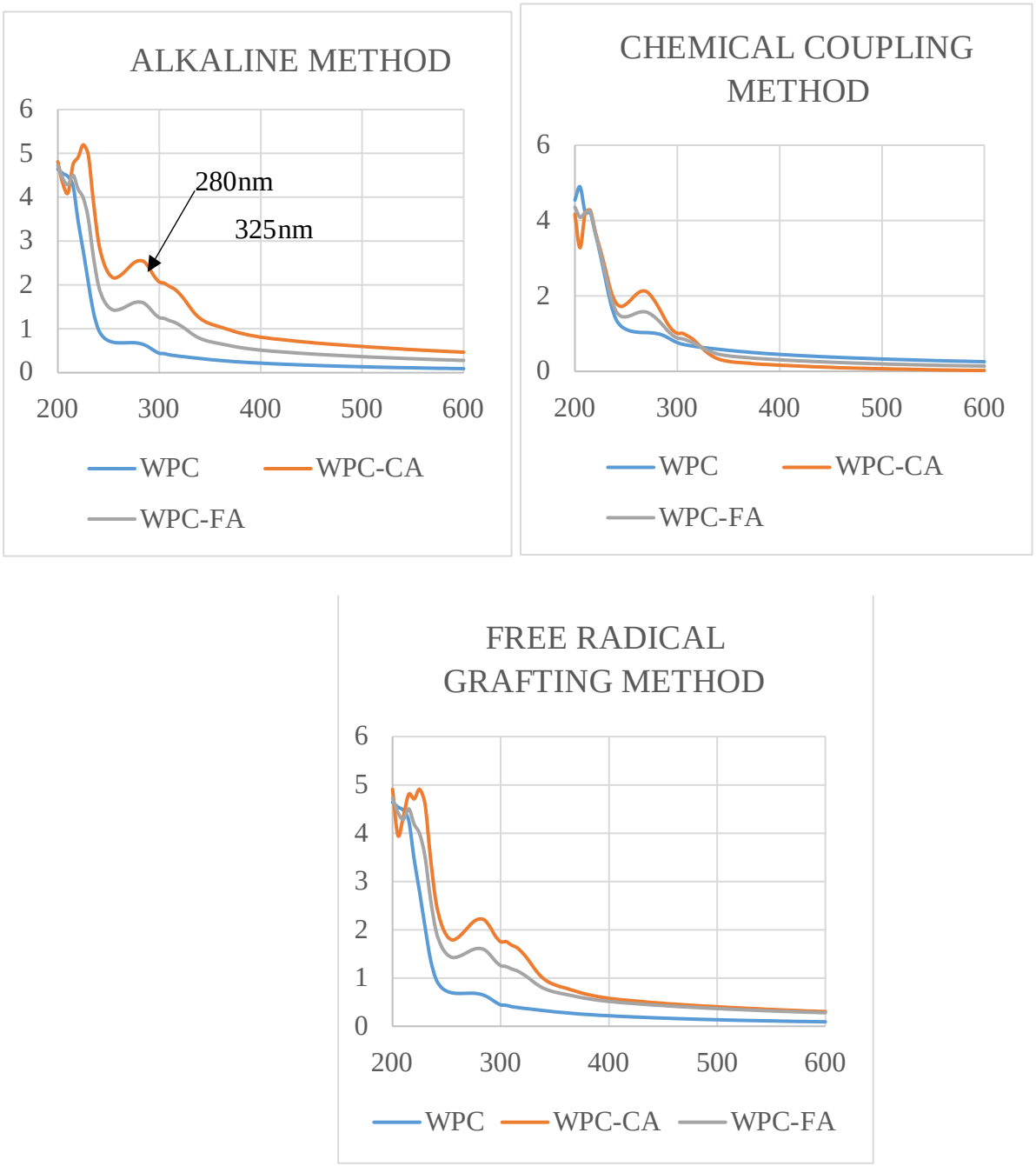


Fig. 4.2.2.2 UV-Visible Spectra of WP-Caffeic Acid & WP-Ferulic Acid Covalent Complexes and Control (Alkaline Method, Chemical Coupling Method, Free radical Grafting Method).

4.2.3 Change in Fourier transform infrared spectroscopy (FTIR) spectra

FT-IR spectroscopy is a useful method to estimate the change in the secondary structures of proteins after conjugation with phenolic compounds by inspecting the frequencies of amide bonds. The amide band I (1600-1700 cm-1) and amide 2 (1500-1600 cm-1) is the most sensitive spectral region for the secondary structure of proteins (Liu *et al*., 2015).

The FTIR spectra (1500-1700 cm-1 wavenumber) for non-covalent conjugates and covalent conjugates of WP-CA and WP-FA is presented in Fig. 4.2.3.1 and 4.2.3.2, respectively. The spectral changes were more visible in covalent conjugates of WP-CA and WP-FA than non-covalent conjugates. As shown in Fig. 4.2.3.2, two strong bands at 1648 and 1538 cm^{-1} at the frequencies of amide I and amide II, were observed for WP-CA and WP-FA covalent conjugates compared to control WP. 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 4.2.3.6 for both non-covalent and covalent conjugates of WP-CA and WP-FA. As shown in the Fig 4.2.3.3 for control WP, peaks were observed near at 1614, 1631, 1641, 1662 and 1687. For all non-covalent conjugates of WP-CA, similar peaks were observed except for pH 3 (Fig 4.2.3.3) where peak at 1662 was shifted to 1660 cm^{-1} and peak at 1687 was shifted to 1689 at all pH values. For non-covalent conjugates of WP-FA, no appreciable difference was observed in comparison to control except at pH 3 (Fig 4.2.3.4) where peak at 1662 was shifted to 1660 cm^{-1}. On analyzing peaks, it was observed that shifting of peaks was more in covalent conjugates of both CA and FA. As shown in the Fig 4.2.3.5 covalent conjugate of WP-CA prepared by Chemical coupling method have shown one extra peak near 1654 cm^{-1}, and shifting of peak from 1687 to 1689 compared to control WP was observed. For the conjugates prepared by alkaline method the shifting of peak from 1662 to 1660 and 1687 to 1689 was observed. For free radical grafting method shifting of 1662 to 1660 and 1687 to 1685 was observed. For WP-FA conjugates (as shown in Fig. 4.2.3.6) prepared by chemical coupling method have shown one extra peak near 1654 cm^{-1}. The conjugates prepared by alkaline method has shown the shifting of peak from 1662 to 1660 and 1687 to 1685. In conjugates prepared by free radical grafting method, shifting of peak from 1687 to 1689 and one extra peak near 1656 was observed.

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 WP-CA and WP-FA is presented in Fig 4.2.3.7 and 4.2.3.8, respectively.

The second derivative spectra of control WP and covalent conjugates of WP-CA and WP-FA by different methods shown in fig 4.3.2.8 which clearly revealed several bands were present at 1670, 1654, 1635, and 1624 cm^{-1} in the amide I region. The band at around 1670 cm^{-1} was due to β-turn structures, and 1654 cm^{-1} was associated with α-helical structure. The band at 1635 cm^{-1} was assigned to β-sheet structure, and the band at 1623 cm-1 was attributed to intermolecular βsheet structure. The absorbance changes around these wavenumbers confirmed the changes in secondary structure of protein brought about by the covalent conjugation. (Jia *et al*., 2016)

Non-covalently conjugated WPI samples with different CA levels showed a similar FTIR spectrum pattern to their protein control, indicating that there were no new covalent bonds generated (Jiang *et al*., 2018). The EGCG modified WPI spectrum, due to the O-H stretching vibration from the phenolic groups, produced an additional 3399.0 cm^{-1} absorption range. The new band confirmed that polyphenol bind via the covalent link to the protein components in WPI. The frequencies of amide I and amide II for modified WPI shifted to 1651.1 and 1538.2 cm^{-1}, respectively. This indicated that the secondary structure of WPI was changed after modification (Jia *et al*., 2016). In another study with the addition of EGCG, a shift to a larger wave number was observed for the shoulder around 1692 cm-1, and the intensity of the band at 1655 cm^{-1} increased. The results indicated that the reaction between EGCG and α-la changed the secondary structure of α-la (Wang et al.,2014).

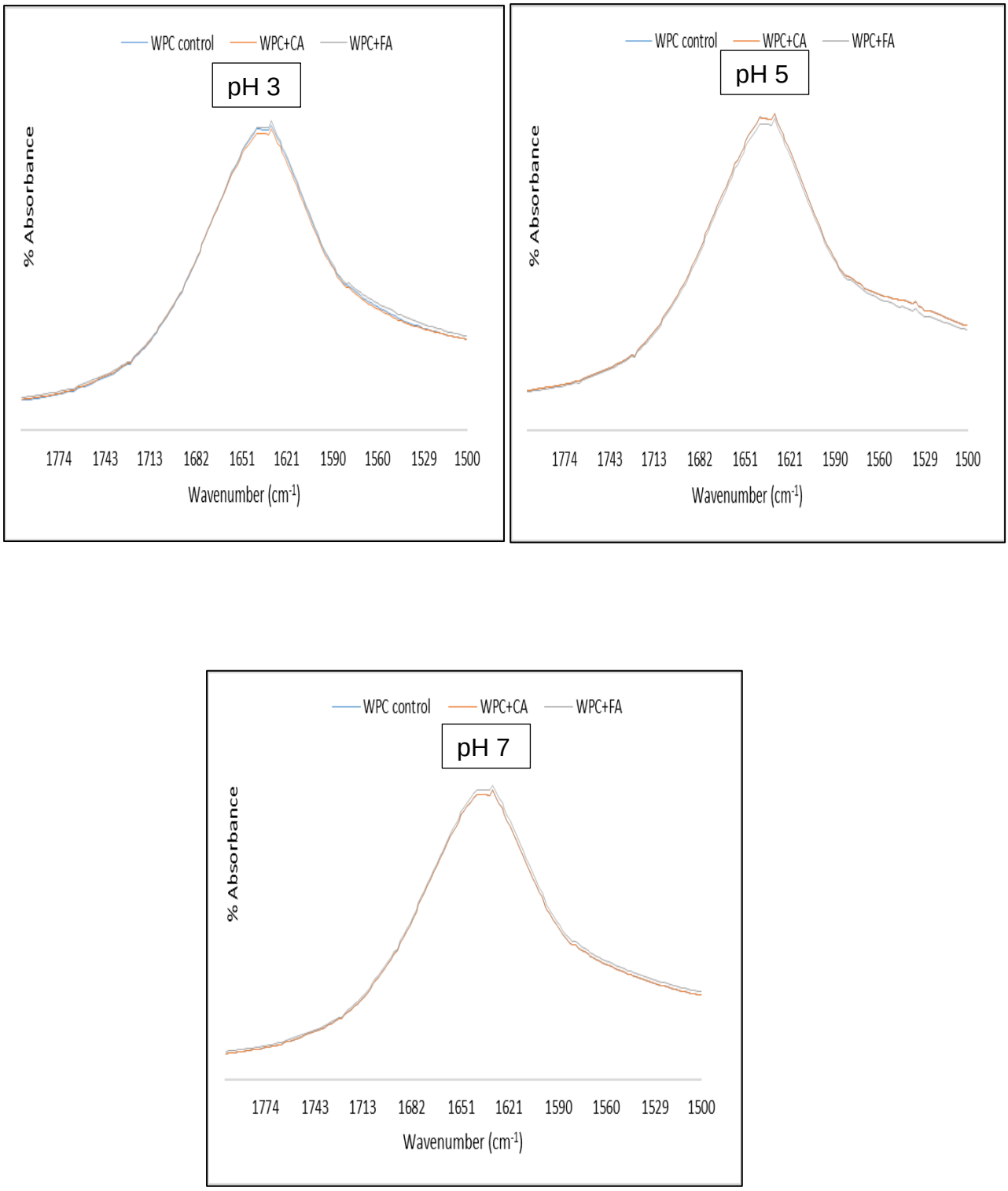
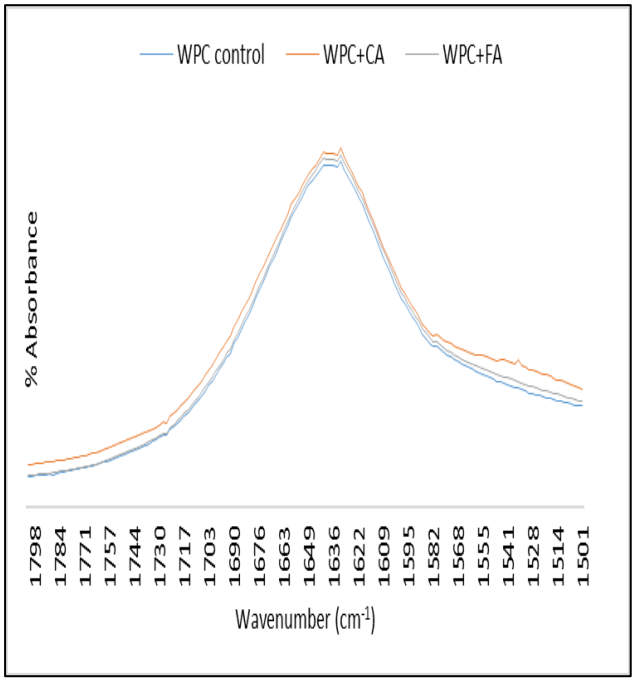
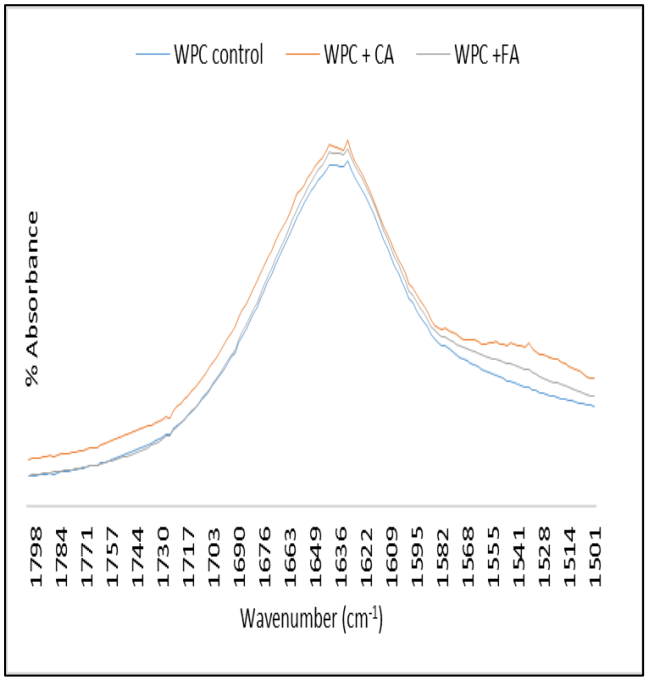


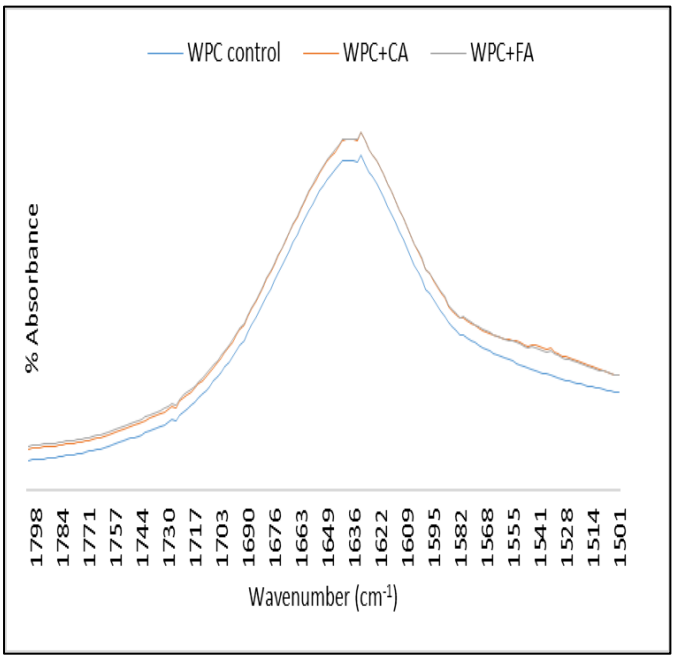
Fig. 4.2.3.1 FTIR Spectra of WP-Caffeic Acid & WP-Ferulic Acid Non-covalent Conjugates and Control WP at pH 3.0,5.0.,7.0.



Alkaline method

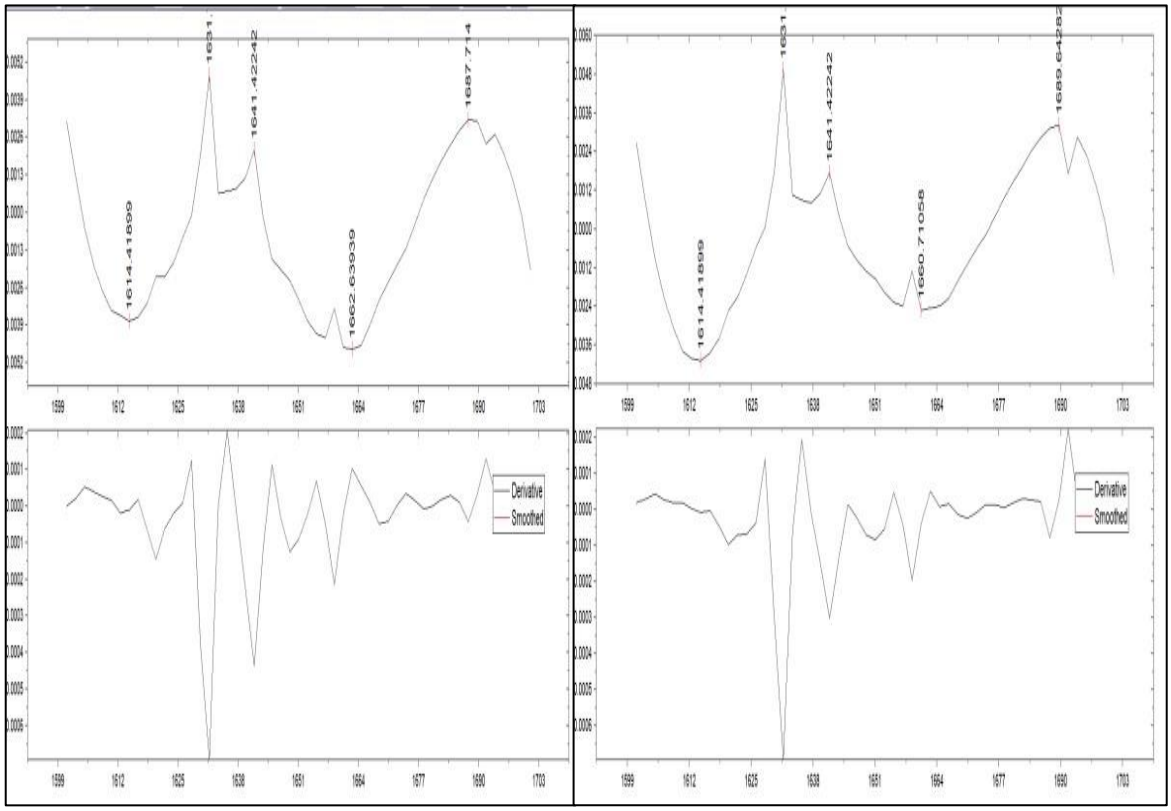


Chemical Coupling method



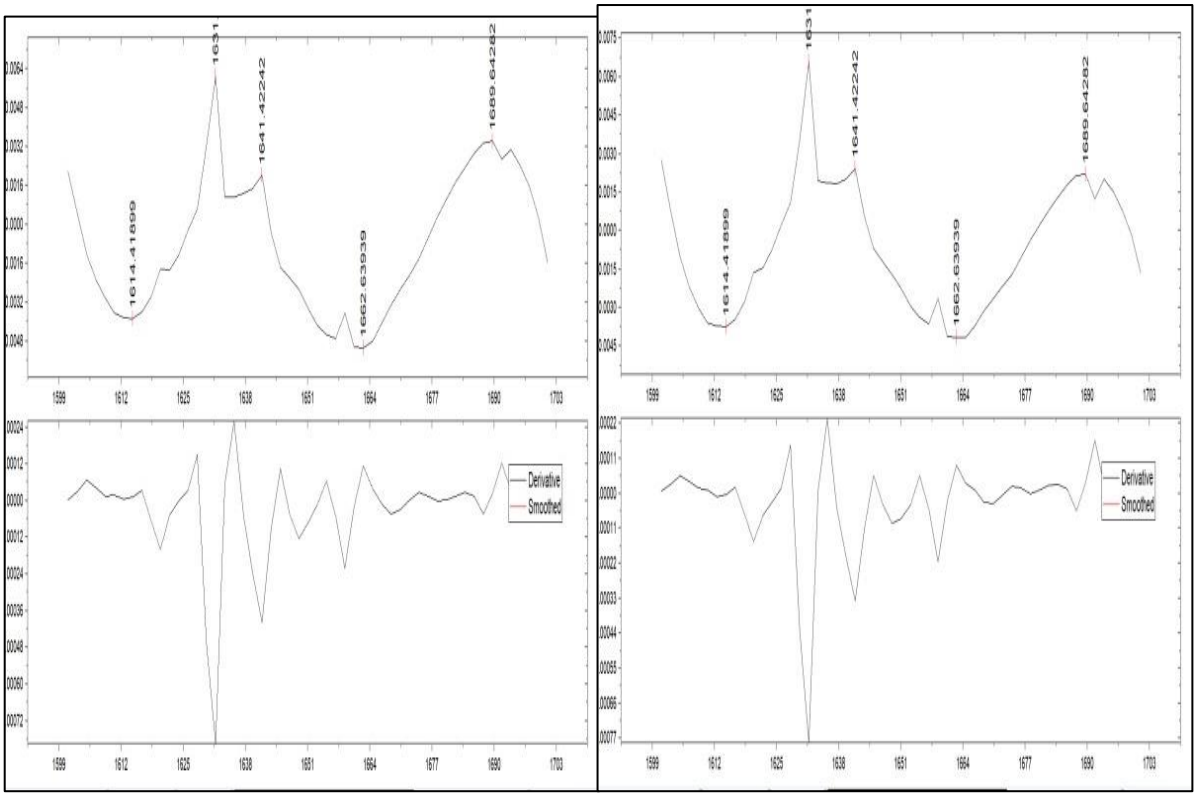
Free Radical grafting

Fig. 4.2.3.2 FTIR Spectra of WP-Caffeic Acid & WP-Ferulic Covalent Complexes and Control (Alkaline Method, Chemical Coupling Method, Free radical Grafting Method).



A

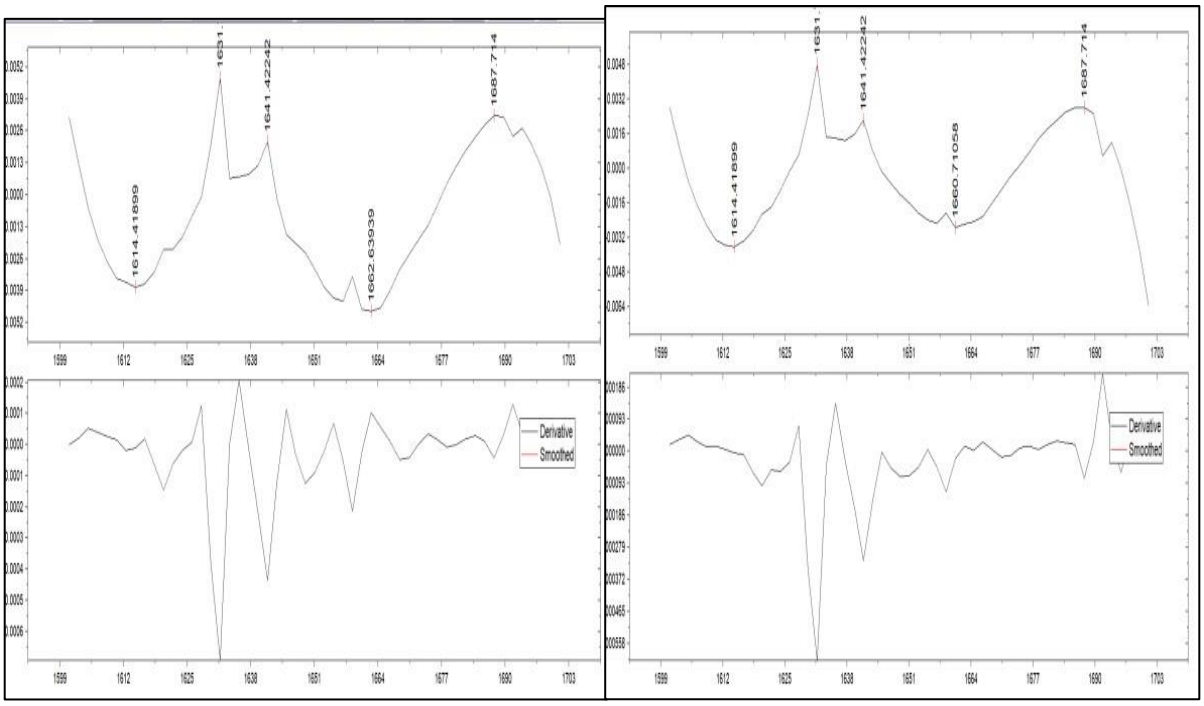
B



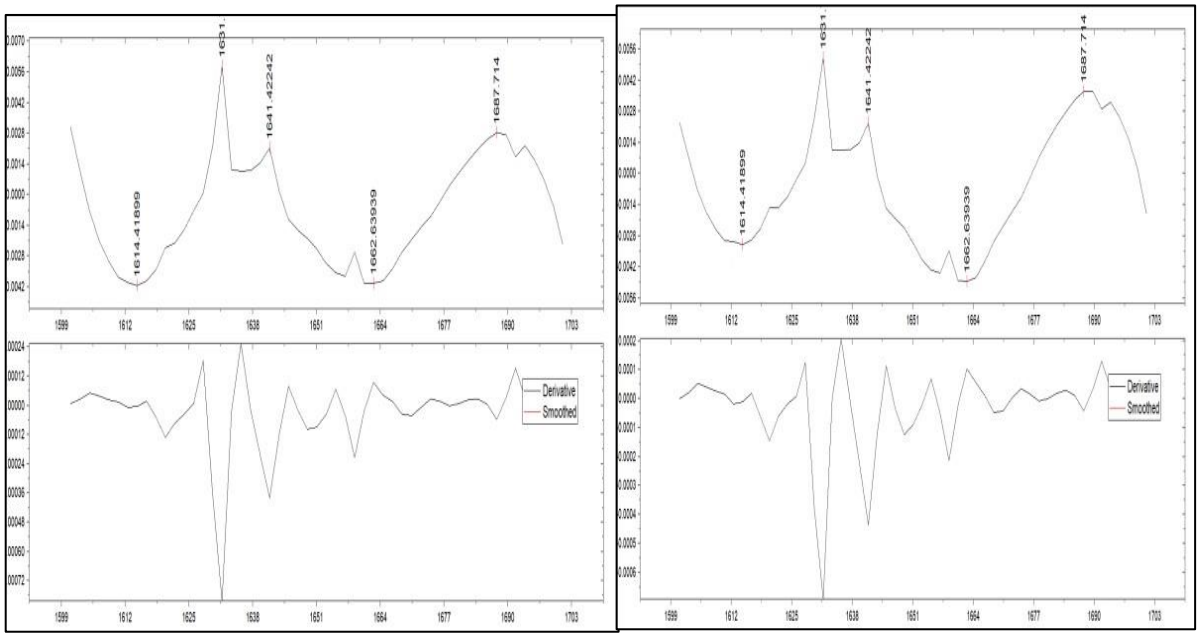
C

D

Fig. 4.2.3.3 Peak fitting analysis in FTIR spectra of (A: Control WP, B: WP-CA pH 3, C: WP-CA pH 5, D: WP-CA pH 7)

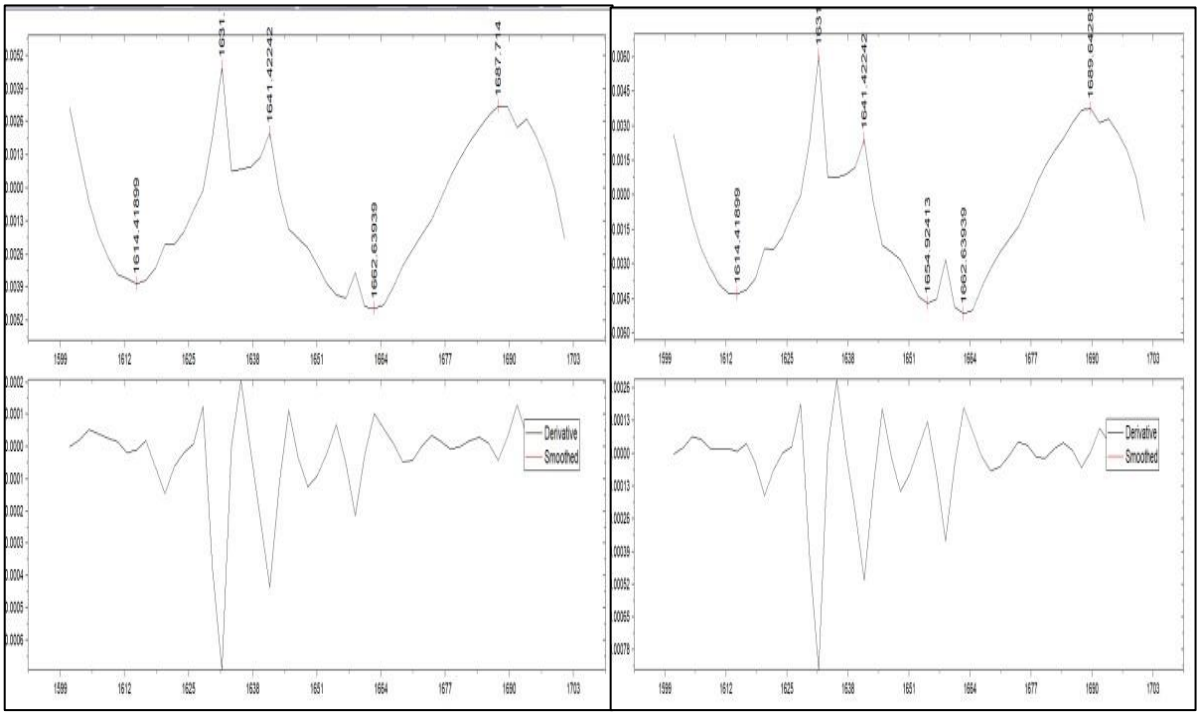


A B

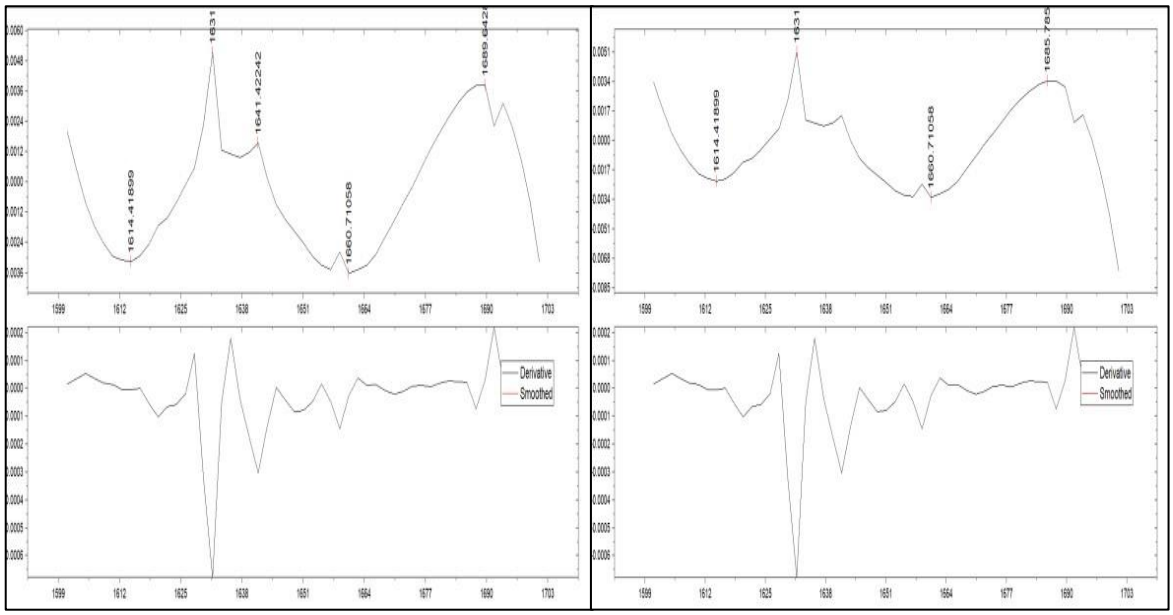


C D

Fig. 4.2.3.4 Peak fitting analysis in FTIR spectra of (A: Control WP, B: WP-FA pH 3, C: WP-FA pH 5, D: WP-FA pH 7)

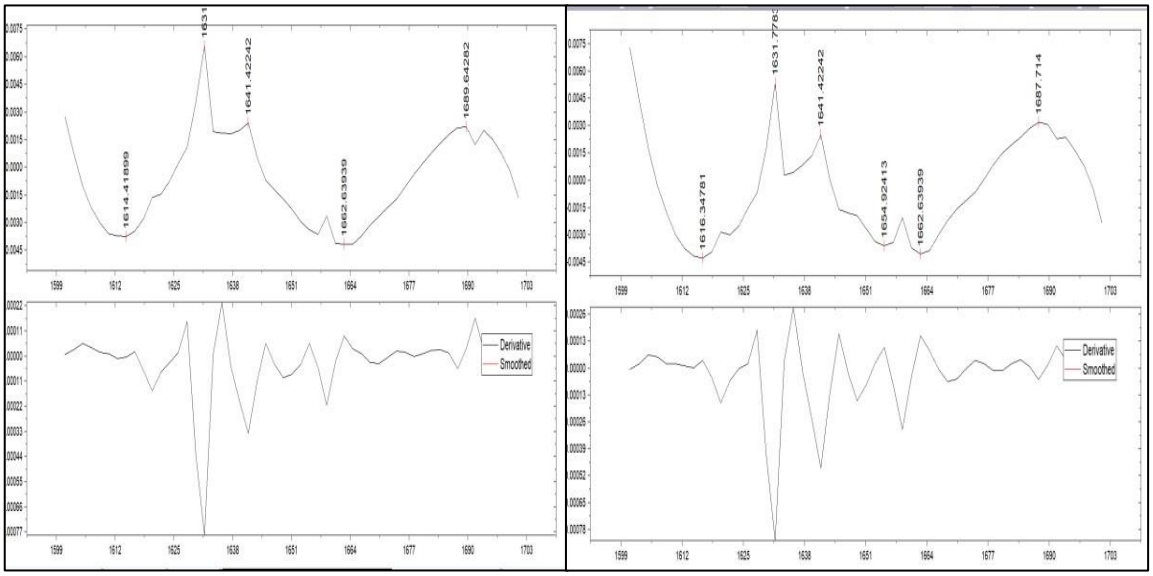


A B

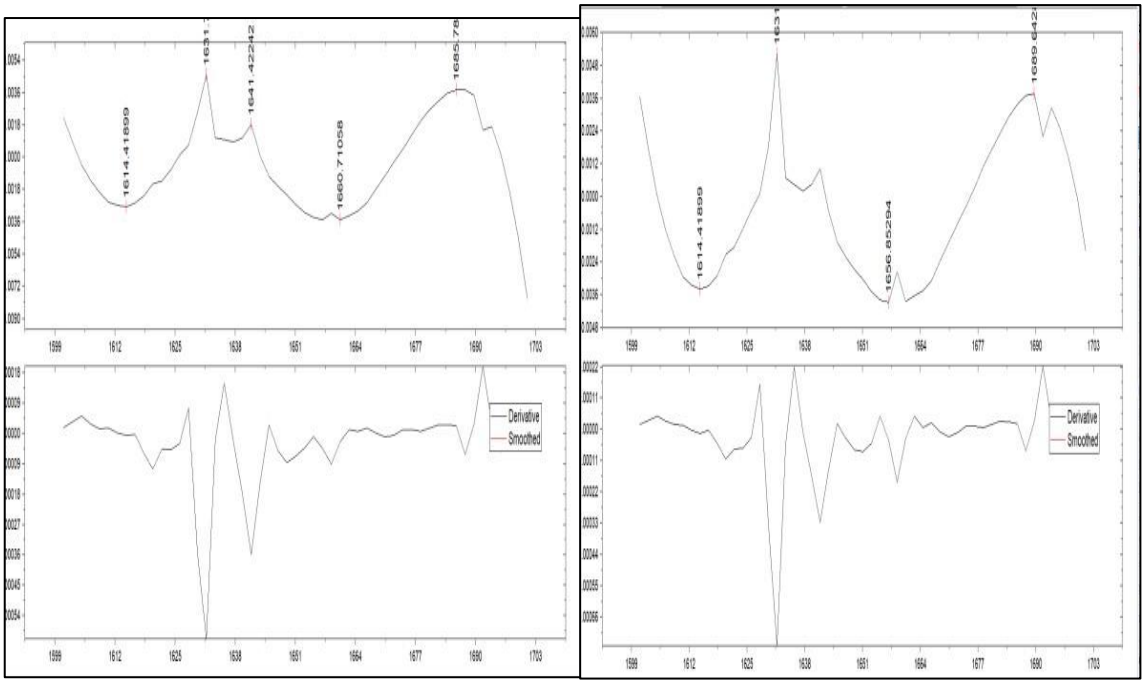


C D

Fig. 4.2.3.5 Peak fitting analysis in FTIR spectra of (A: Control WP, B: WP-CA by chemical coupling method, C: WP-CA by alkaline method, D: WP-CA by free radical grafting method)



A B



C D

Fig. 4.2.3.6 Peak fitting analysis in FTIR spectra of (A: Control WP, B: WP-FA by chemical coupling method, C: WP-FA by alkaline method, D: WP-FA by free radical grafting method)

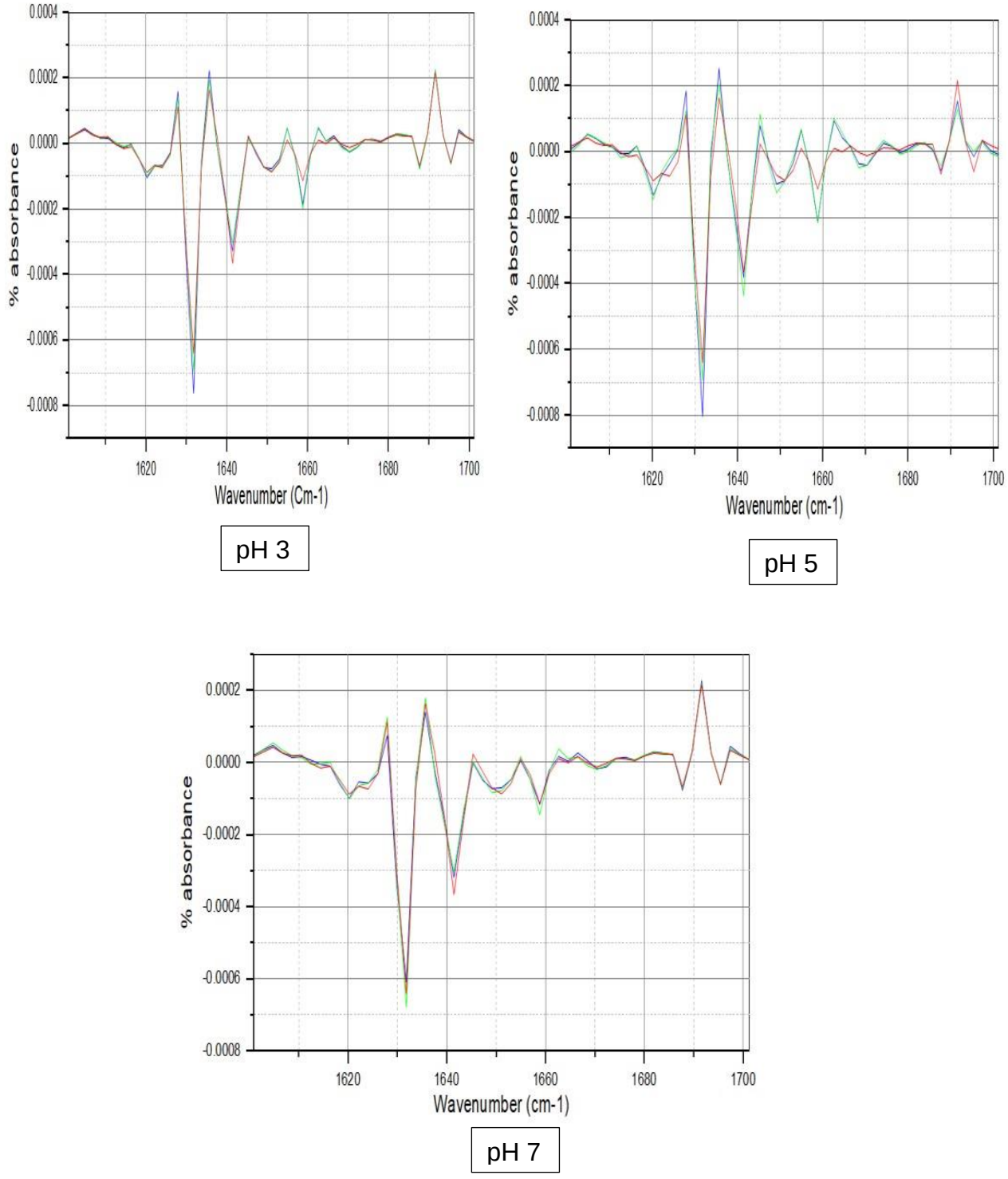


Fig. 4.2.3.7 Second derivative FTIR Spectra of WP-Caffeic Acid & WP-Ferulic Acid Non-Covalent Conjugates and Control WP at pH 3.0, 5.0, 7.0.

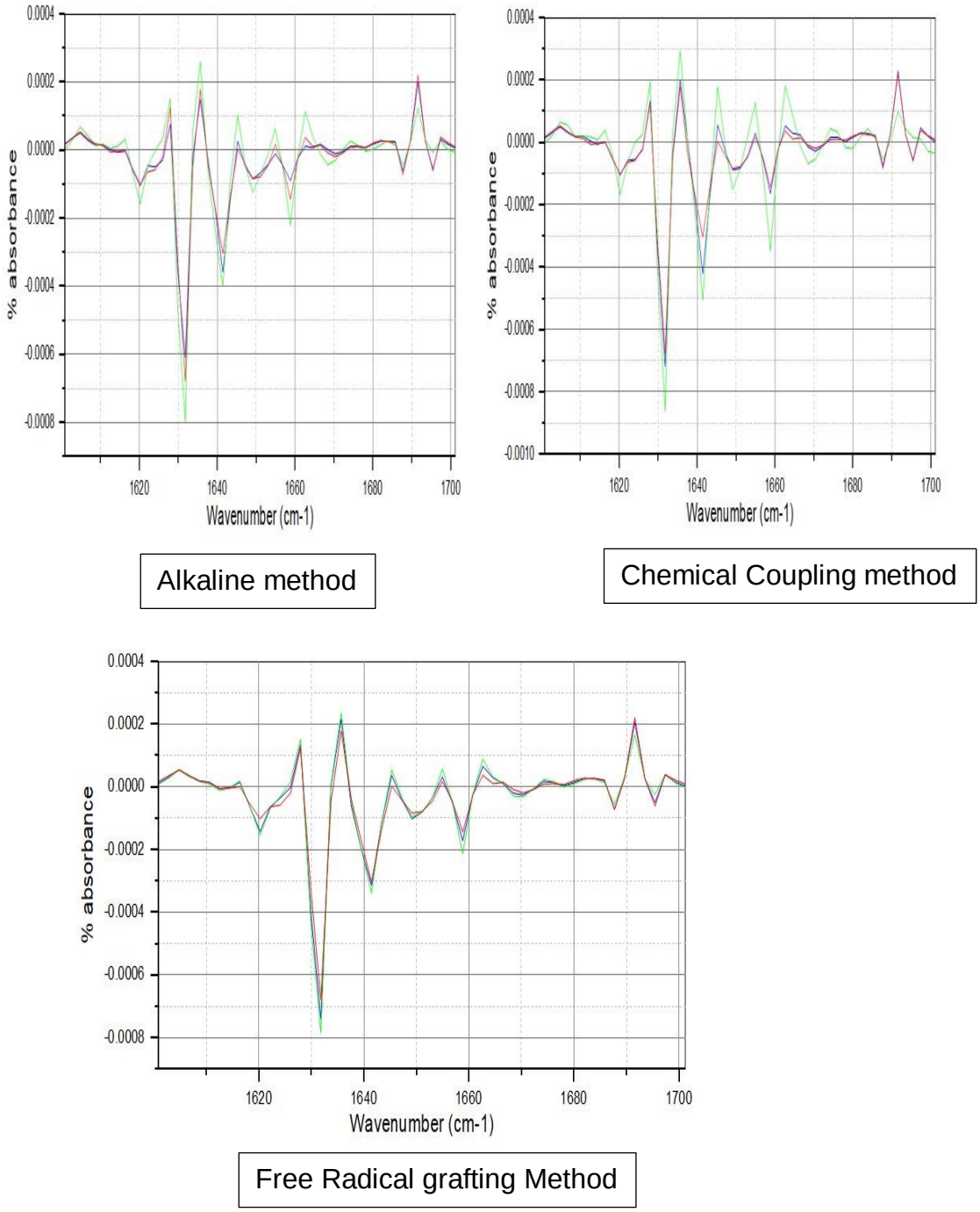


Fig. 4.2.3.8 Second derivative FTIR Spectra of WP-Caffeic Acid Covalent Complexes and Control (Alkaline Method, Chemical Coupling Method, Free radical Grafting Method).

4.2.4 Changes in Particle Size

The particle size of prepared conjugates of WP-CA and WP-FA was measured using Zetasizer and results were expressed as the volume-surface average Particle size (Z). The particle size of WP-CA and WP-FA non-covalent and covalent conjugates are shown in Fig. 4.2.4.1 and 4.2.4.2, respectively. For non-covalent conjugates of both WP-CA and WP-FA, significant increase in particle size was observed at pH 3.0. This may be due to the greater electrostatic attraction between CA, FA and WP at lower pH, which makes the conjugates bigger, unstable, and close to flocculation (Ferraro *et al,*2015). At pH 5 and 7 there was no significant differences between particle Size of WP and its conjugates with CA and FA.

As shown in the fig. 4.2.4.2 there was no significant difference in average particle size of covalent conjugates prepared by alkaline and chemical coupling method in comparison to control WP, however, average particle size of covalent conjugates of WP-CA and WP-FA prepared by free radical grafting method was decreased.
Similar decrease in particle size of β-Lactoglobulin–chlorogenic acid conjugate by free radical grafting method was also reported by Fan *et al*., 2018.

For the WPI–RosA (Rosmarinic Acid) conjugate prepared by alkaline method, no significant change in the particle size was found (Ali *et al*., 2018). The size of AITC (allyl isothiocyanate) - covalently modified WPI was not significantly different from that of native WPI (Keppler *et al*.,2017).

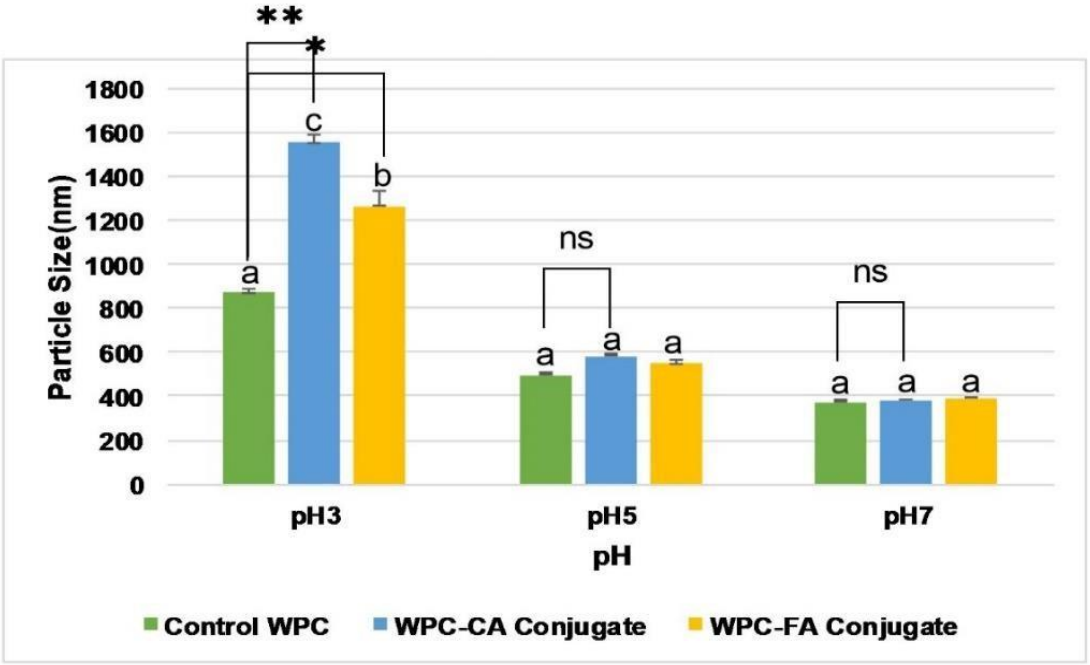


Fig. 4.2.4.1 Particle size of control WP, WP-CA and WP-FA Non-covalent Conjugates

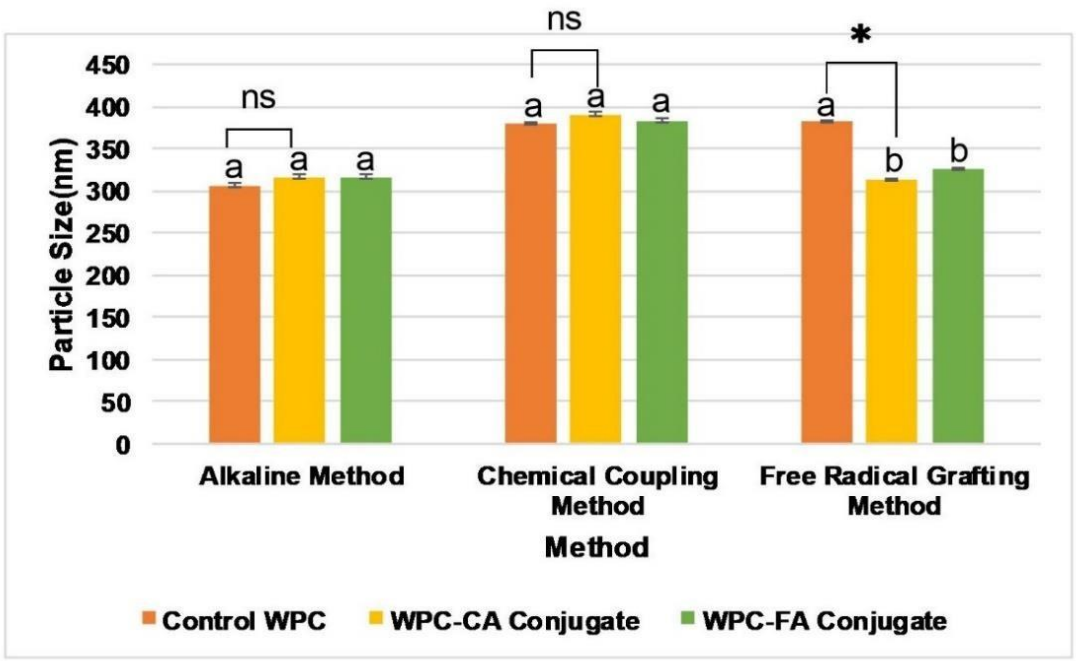


Fig. 4.2.4.2 Particle size of control WP, WP-CA and WP-FA Covalent Conjugates

4.2.5 Changes in Zeta Potential

The Zeta Potential of WP-CA and WP-FA non-covalent and covalent conjugates are shown in Table 4.2.5.1 and 4.2.5.2, respectively. At pH 7, the zeta potential was -9.83 for control WP and on decreasing pH to pH 5 zeta potential decreased and on further decrease in pH to 3, zeta potential became positive (+6.73) which was also similar to earlier findings that from pH 1 to 4, the zeta potential was positive and took a negative value at pH 5 (-5.9 ± 0.3 mV) and above, also zeta potential taken a zero value between pH 4 and 5 corresponding to the isoelectric point of the protein which was around pH 4.6 (Gbassi *et al*., 2012). On conjugation with polyphenol both CA and FA, zeta potential was more negative as compared to control WP. The shift to a more negative value in conjugates can be explained by polyphenol binding to free amine groups, which removes part of the protein's positive charges from the overall charge (Keppler *et al*.,2017). For conjugates prepared by covalent methods, insignificant changes in the zeta potential were observed for WP-CA and WP-FA conjugate prepared by alkaline method; however, for chemical coupling method and free radical grafting method the zeta potential shifted towards negative side compared to control WP for both the WP-CA and WP-FA conjugates.

Insignificant changes in the zeta potential were observed for WPI–RosA (Rosmarinic Acid) conjugate at alkaline conditions (~ −9mV) compared to unmodified WPI (Whey Protein Isolate) (~ −10 mV) (Ali *et al*., 2018). Zeta potential [mV] of 10 gL^{-1} freeze-dried native whey protein isolate (WPI) decreased, after covalent modification with AITC (allyl isothiocyanate) (Keppler *et al*.,2017).

Table 4.2.5.1 Zeta Potential of Non-Covalent Conjugation of WP, Caffeic Acid and Ferulic Acid

Sample	**pH 3**	**pH 5**	**pH 7**
Control WPC	6.72 ±0.40	-7.69 ±0.13	-9.83 ±0.08
WPC-CA Conjugate	5.05 ±0.15	-8.32 ±0.04	-9.94 ±0.02
WPC-FA Conjugate	6.38 ±0.46	-10.48 ±0.08	-11.04 ±0.56

Table 4.2.5.2 Zeta Potential of Covalent Conjugation of WP, Caffeic Acid and Ferulic Acid

Sample	Alkaline Method	Chemical Coupling Method	Free Radical Grafting Method
Control WPC	-12.36 ±0.77	-10.3 ±0.10	-10.32 ±0.08
WPC-CA Conjugate	-12.16 ±0.33	-11.32 ±0.22	-11.28 ±0.13
WPC-FA Conjugate	-12.00±0.33	-11.29 ±1.66	-10.53 ±0.05

4.3 Changes in Functional Properties

On the basis of size and zeta potential, it has been found that non-covalent conjugates at pH 5 and 7 were more stable than pH 3 for both WP-CA and WPFA. The non-covalent conjugates of WP-CA and WP-FA of pH 7 has significantly higher retention of polyphenol than pH 5. Therefore, non-covalent conjugate prepared at pH 7 of both WP-CA and WP-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.

WP-CA and WP-FA conjugates selected for functional property analysis were noncovalent 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

The antioxidant activities were measured in terms of DPPH Radical scavenging activity (RSA).

The antioxidant activity (AO) of control WP, WP-CA and WP-FA is presented in Fig. 4.3.1. The AO of control WP was 38% RSA. On conjugation with CA and FA, the AO increased significantly. There was no significant difference in AO of WPCA and WP-FA conjugates in all the method of preparation.

On conjugation with CA, the AO was found to be significantly increased up to 59.44, 68.39, 67.90 % RSA in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively.

Similarly, on conjugation with FA, the AO was found to be significantly increased up to 58.64, 62.96, 66.17 % RSA in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively.

The addition of hydroxyl groups of phenolics to protein structure was responsible for improving the antioxidant activities of protein-phenolics conjugates. Also, the WP-CA conjugates showed higher % radical scavenging activity than WP-FA conjugates. That may be due to higher affinity of CA to bind with WP than FA. which was also confirmed from total phenolic content described in section 4.1.

BSA-CA conjugate's DPPH scavenging activity was noticeably greater than BSA (bovine serum albumin), at 0.4 mg / mL DPPH scavenging activity Values Were 89.7% and 91.9% for BSA-CA conjugate, suggesting a significant improvement of BSA's chemical antioxidant activities following conjugation with CA (caffeic acid) (Fan *et al*.,2018).The covalent incorporation of EGCG (epigallocatechin gallate) into α-La at alkaline pH has contributed to the growth of α-La antioxidant activity (Wang *et al*.,2014). The ability of β-Lg-CA conjugates to inhibit oxidation (%DPPH Scavenging index > 76.2) was higher compared with the non-covalent complex (% DPPH Scavenging index of β-Lg/CA = 71.3) (Abd El-Maksoud *et al*.,2017).

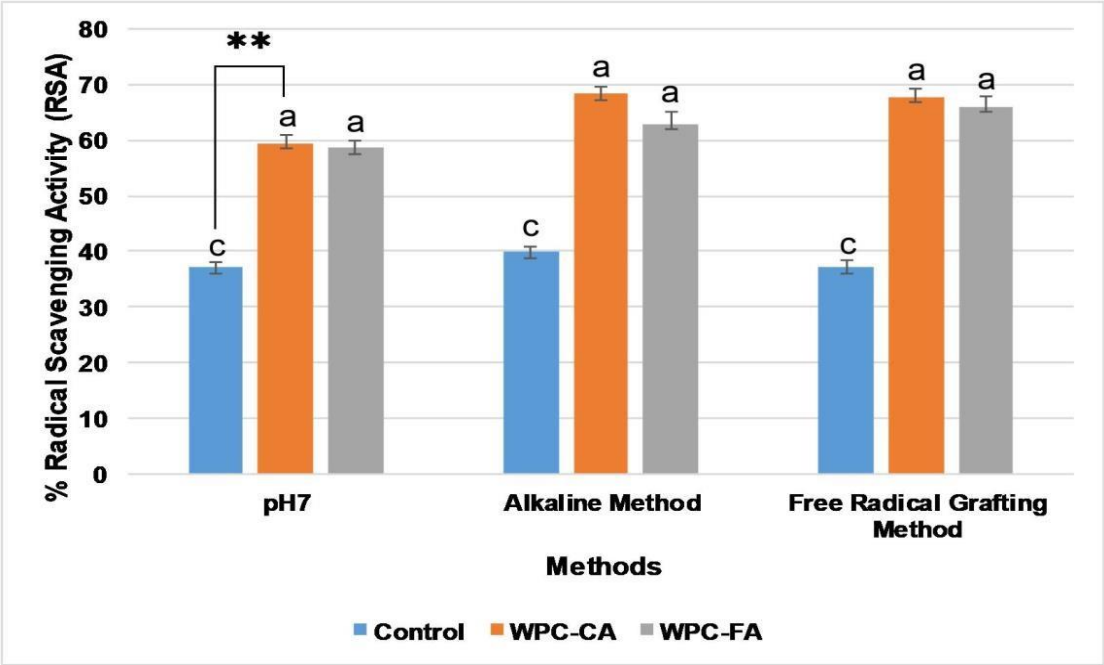


Fig. 4.3.1.1 Antioxidant activity (AO) of control WP, WP-CA and WP-FA

4.3.2 Solubility

The solubility of control WP, WP-CA and WP-FA is presented in Fig. 4.3.2. The solubility of control WP was 85-92%. On conjugation with CA and FA, the solubility decreased. There was no significant difference in solubility of WP-CA and WP-FA conjugates irrespective of the method of preparation.

On conjugation with CA, the solubility was not significantly decreased and found to be 86.77, 84.85, 79.67% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively.

Similarly, on conjugation with FA, the solubility was Not significantly decreased and found to be 83.25, 83.1, 73.7 % in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively.

The decrease in solubility after conjugation with CA and FA may be due to the attachment of non-polar polyphenols that can improve the surface hydrophobicity of proteins, thereby reducing their water solubility (Jiang *et al*., 2018)

Solubility profiles of the CQA modified β-lactoglobulins and alkaline-modulated βlactoglobulin sample showed a decrease in solubility of 20% (Ali *et al*., 2013). When proteins were modified by CQA (chlorogenic acid), oxidised at alkaline pH 9

(quinones formation), resulted in a shift of the isoelectric pH to lower values, causing the solubility of α-lactalbumin at pH = pI to be strongly reduced, whereas the solubility of BSA was only slightly affected (Prigent et al., 2007).

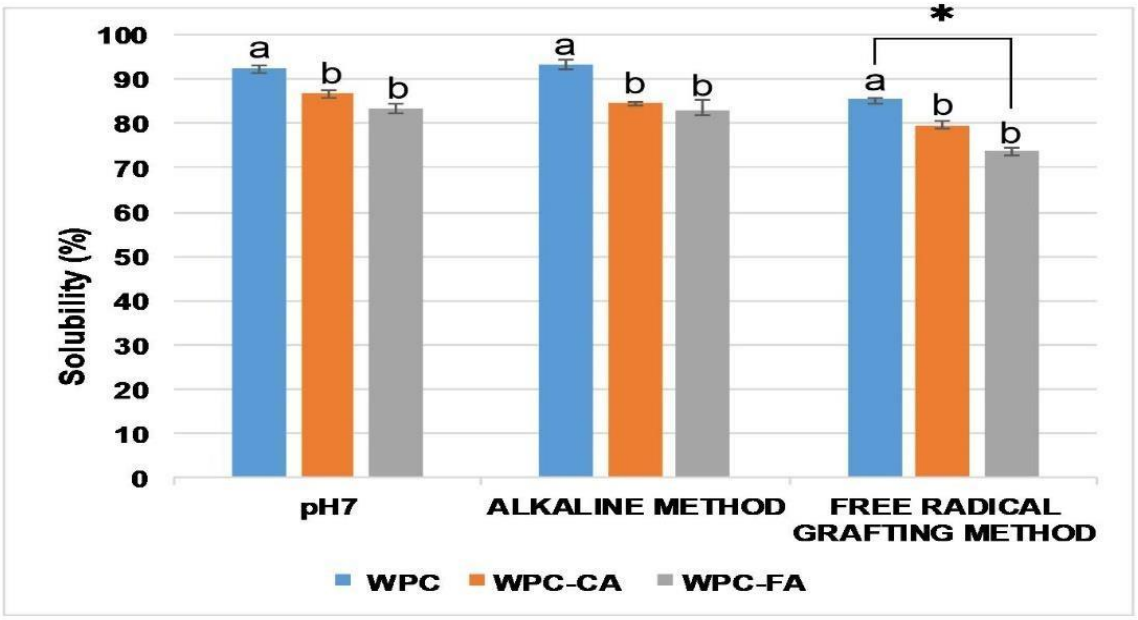


Fig. 4.3.2.1 Solubility (%) of control WP, WP-CA and WP-FA

4.3.3 Emulsifying Activity and Emulsion Stability

The emulsifying activity (EA) and emulsion stability (ES) of control WP, WP-CA and WP-FA is presented in Fig. 4.3.3. The EA and ES of control WP was 85.99 and 84.33%, respectively. On conjugation with CA and FA, both EA and ES increased. There was no significant difference in EA and ES of WP-CA and WP-FA conjugates irrespective of the method of preparation.

On conjugation with CA, the EA and ES was increased significantly and found to be 97.03 and 97.13, 91.21 and 89.53, 97.15 and 92.33% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively.

On conjugation with FA, the EA and ES was increased significantly and found to be 95.33 and 96.66, 85.66 and 86.33., 95.66 and 91.33% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively.

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 WP (Afizah & Rizvi, 2014). The conjugation of polyphenol has caused the unfolding of WP structure, therefore exposed more aromatic residues and improved the emulsifying activity and emulsion stability of WP.

At all concentrations assayed, the increases of d32 values of modified WPI emulsions were lower than those of unmodified WPI emulsions, indicating EGCG modification improved the emulsion stability of WPI (Jia et al., 2016). emulsifying property of α-La was improved owing to the covalent interaction between a-La and EGCG at pH 8.0 (Wang et al., 2014)

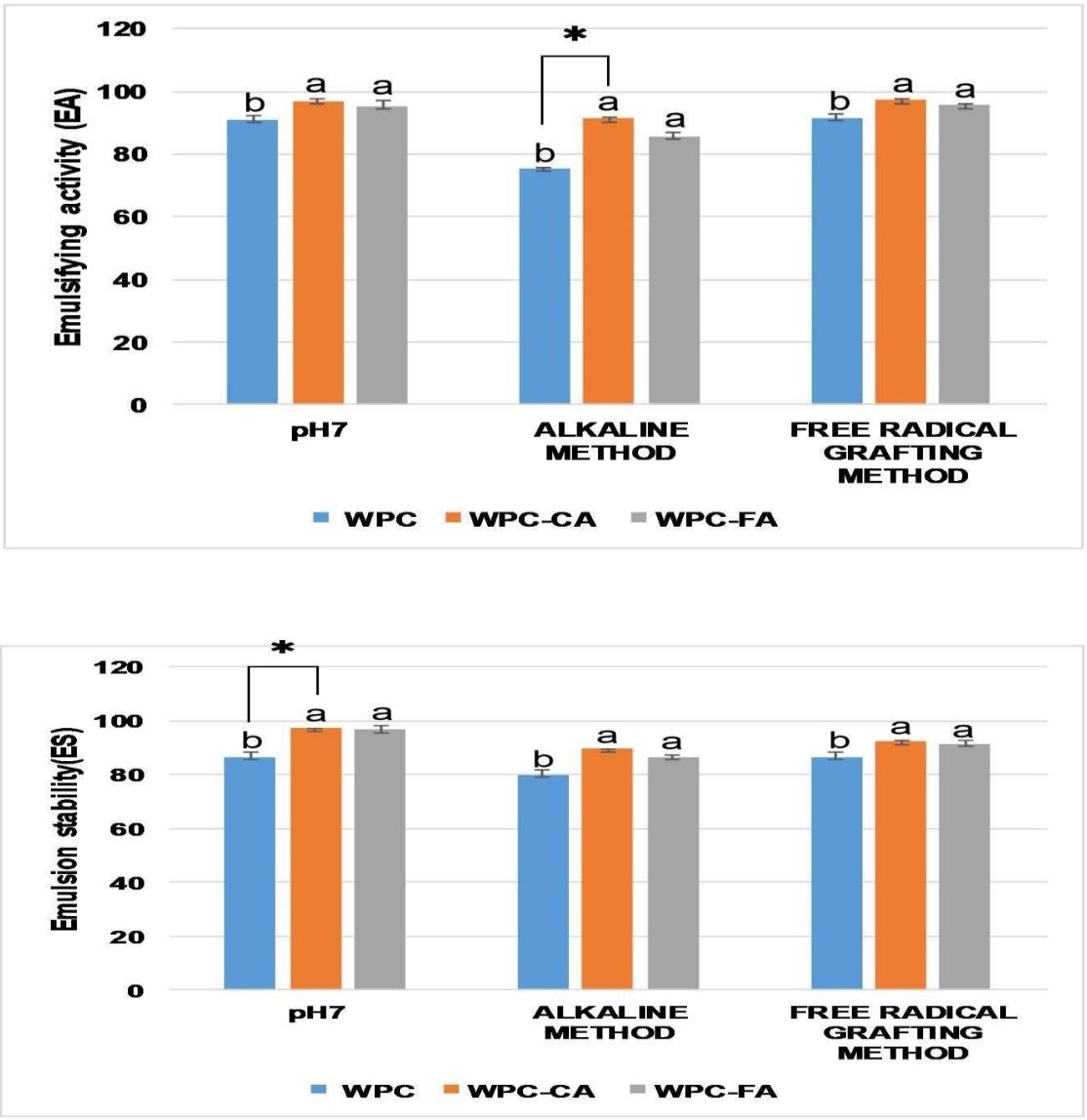


Fig. 4.3.3.1 Emulsifying activity (EA) and emulsion stability (ES) of control WP, WP-CA and WP-FA

4.3.4 Foaming Capacity and Foam Stability

The foaming capacity (FC) and foam stability (FS) of control WP, WP-CA and WPFA is presented in Fig. 4.3.4. The FC and FS of control WP was 66.93 and 60.33 %, respectively. On conjugation with CA and FA, FC increased but there was no significant difference in FS. There was no significant difference in EA and ES of WP-CA and WP-FA conjugates irrespective of the method of preparation.

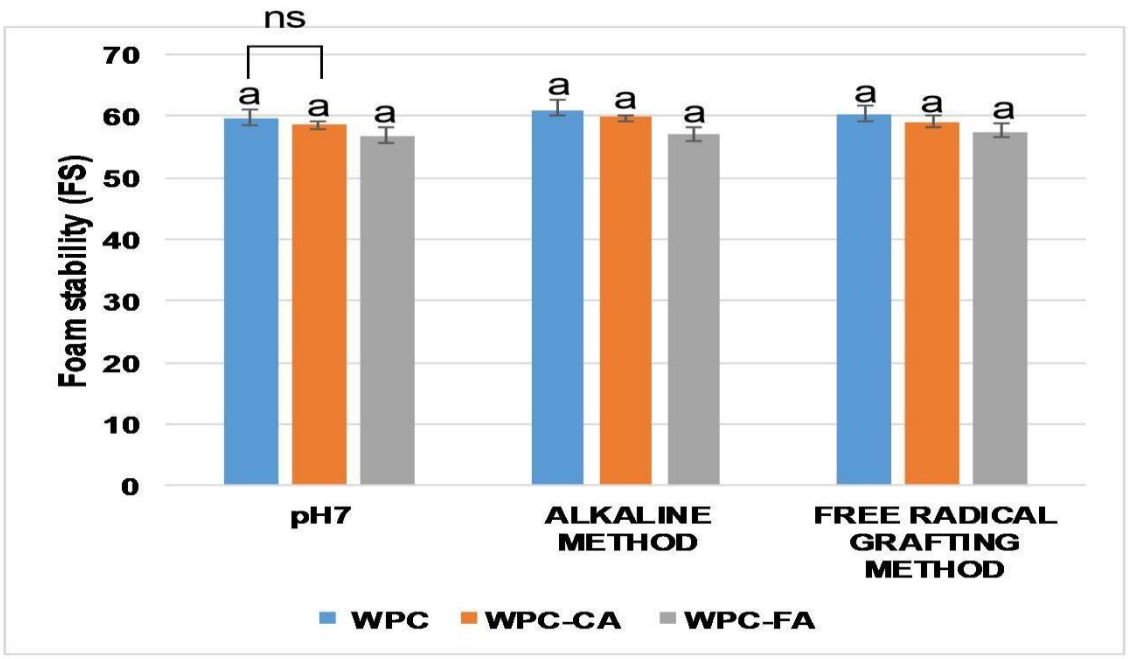
On conjugation with CA, the FC and FS was found to be 82.38 and 58.86, 76.37 and 60.08, 76.7 and 59.15% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. The significant increase in foaming capacity (FC) and non-significant decrease was observed for foam stability (FS).

On conjugation with FA, the FC and FS was found to be 79 and 56.82, 74.33 and 57.14, 75.33 and 57.5% In conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. The results were similar to CA that significant increase in foaming capacity (FC) and non-significant decrease was observed for foam stability (FS).

Foaming capacity exhibited a clear trend of enhancement and Foam stability little bit decreased for all the Covalent as well As Non-covalent conjugates of WPCFerulic acid. Some studies have also shown that polyphenols could improve protein foaming properties through the promotion of protein cross-linking.

The increase in FC after conjugation with CA and FA was due to promotion of protein crosslinking after conjugation with polyphenols (Kuan, Bhat, & Karim, 2011). A similar result was reported for WPI treated with EGCG (Jia *et al*., 2016).

For Whey protein isolate, foaming capacity as well as foam stability exhibited a clear trend of enhancement with CA (Chlorogenic acid) levels (Jiang *et al*., 2018). The WPI was significantly modified by the cross-linking reaction induced by EGCG and results revealed that the foaming properties of WPI were greatly improved by modification (Jia *et al*., 2016).



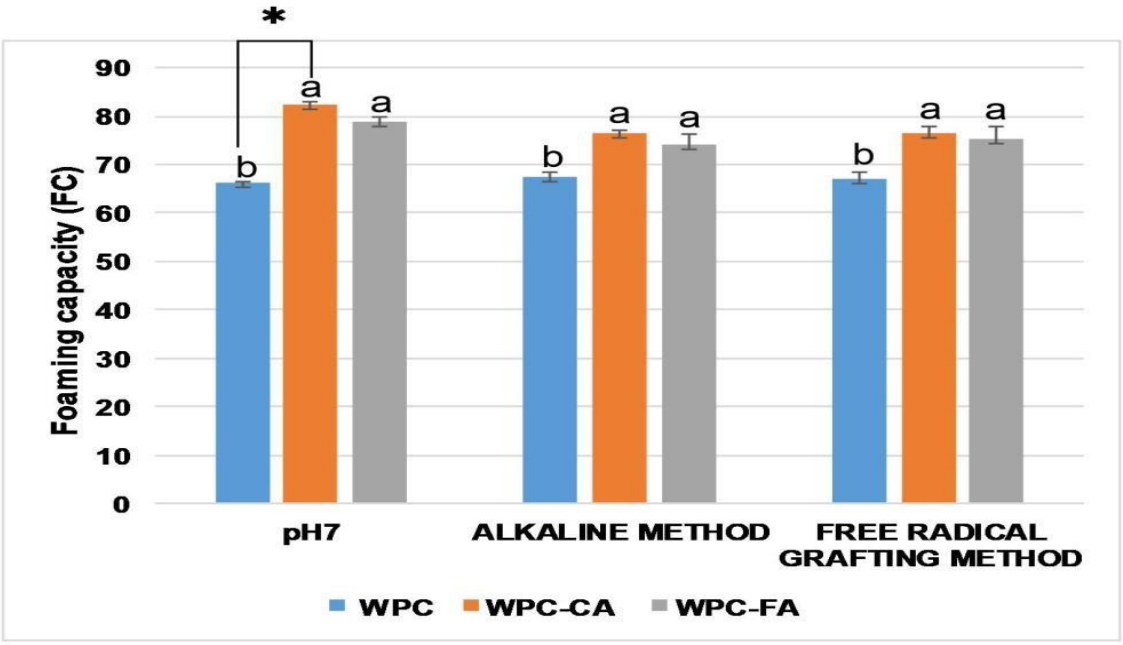


Fig. 4.3.4 Foaming capacity (FC) and foam stability (FS) of control WP, WP-CA and WP-FA

The Phenolic content results confirmed that WPC interacted with Caffeic Acid as well as ferulic acid at both non-covalent and covalent conditions. The total phenolic contents of the WP-CA Non-covalent conjugates produced at pH 3.0 and 7.0 were higher than those at pH 5.0. This result indicated that binding of caffeic acid with whey protein was higher at pH 3 and 7 in comparison to pH 5. For covalent conjugates, highest interaction of caffeic acid with whey protein were observed in free radical grafting method compared to conjugates prepared by alkaline and chemical coupling methods. The phenolic content of all covalent conjugates was higher than that of non-Covalent conjugates, which indicated that more binding of the caffeic acid with whey protein in covalent methods. The total phenolic contents of the WP-FA for non-covalent as well as covalent conjugates was lower than that of WP-CA conjugates. Among non-covalent conjugates of WP-FA, the results were similar to WP-CA conjugates and higher binding was observed at pH 3 and 7 in comparison to pH 5. The binding of FA was found to be higher in conjugates prepared by covalent methods then non-covalent methods. Among covalent conjugates of WP-FA, maximum binding was observed in free radical grafting method than alkaline and chemical coupling methods.

In Spectrofluorometric analysis Control WP has shown maximum intensity at wavelength (λ) 360 nm. On conjugation of polyphenol (both CA and FA) in noncovalent conjugation, decreased intrinsic fluorescence intensity was observed at all pH values. Fluorescence intensity of WP-FA conjugates was higher throughout wavelength 300-500nm compared to that of the WP-CA, which may be due to the less interaction between FA with WP compared to CA. On conjugation of CA and FA with WP, the maximum wavelength was shifted to higher wavelength, which is known as red shift. A red shift of 20 nm was observed for WP-CA and WP-FA conjugates at all pH values. The fluorescence intensity of covalent conjugates was lower as compared to non-covalent conjugates. Similar to non-covalent conjugates, in covalent conjugates also the shift in maximum wavelength was also observed. In conjugates prepared by chemical coupling method, a red shift was of 30 nm and 10 nm was observed for WP-CA and WP-FA conjugates, respectively. In conjugates prepared by alkaline method, a red shift of 10 nm observed for both WP-CA and WP-FA conjugates, whereas no red shift was observed for conjugates prepared by free

radical grafting method. Also, fluorescence intensity of WP-CA conjugates was lower as compared to WP-FA conjugates.

With comparison to control WP, the changes in UV-Visible spectra of covalent conjugates were more pronounced as compared to non-covalent conjugates. The higher changes have provided information, that structure of WP was altered and binding with polyphenol was more in covalent conjugates. For all wavelengths, in all the method of covalent conjugation, appreciably higher absorbance was observed than control WP and intense peaks were observed at 280 and 325 nm.

The FTIR (1500-1700 cm^{-1} wavenumber) spectral changes were more visible in covalent conjugates of WP-CA and WP-FA than non-covalent conjugates. Two strong bands at 1648 and 1538 cm^{-1} at the frequencies of amide I and amide II, were observed for WP-CA and WP-FA covalent conjugates compared to control WP. Further, peak fitting analysis was performed in amide I region (1600-1700 cm1) using OriginPro software and in control WP, peaks were observed near at 1614, 1631, 1641, 1662 and 1687. For all non-covalent conjugates of WP-CA, similar peaks were observed except for pH 3, where peak at 1662 was shifted to 1660 cm1 and peak at 1687 was shifted to 1689 at all pH values. For non-covalent conjugates of WP-FA, no appreciable difference was observed in comparison to control except at pH 3 where peak at 1662 was shifted to 1660 cm^{-1}. On analyzing peaks, it was observed that shifting of peaks was more in covalent conjugates of both CA and FA. Covalent conjugate of WP-CA prepared by chemical coupling method have shown one extra peak near 1654 cm^{-1}, and shifting of peak from 1687 to 1689 compared to control WP was observed. For the conjugates prepared by alkaline method the shifting of peak from 1662 to 1660 and 1687 to 1689 was observed. For free radical grafting method shifting of 1662 to 1660 and 1687 to 1685 was observed. For WP-FA conjugates prepared by chemical coupling method have shown one extra peak near 1654 cm^{-1}. The conjugates prepared by alkaline method has shown the shifting of peak from 1662 to 1660 and 1687 to 1685. In conjugates prepared by free radical grafting method, shifting of peak from 1687 to 1689 and one extra peak near 1656 was observed. The second derivative spectra of control WP and covalent conjugates of WP-CA and WP-FA by different methods clearly revealed several bands were present at 1670, 1654, 1635, and 1624 cm^{-1} in the amide I region. The band at around 1670 cm-1 was due to β-turn structures, and 1654 cm^{-1} was associated with

α-helical structure. The band at 1635 cm-1 was assigned to β-sheet structure, and the band at 1623 cm-1 was attributed to intermolecular β-sheet structure.

For non-covalent conjugates of both WP-CA and WP-FA, significant increase in particle size was observed at pH 3.0. At pH 5 and 7 there was no significant differences between particle Size of WP and its conjugates with CA and FA. There was no significant difference in average particle size of covalent conjugates prepared by alkaline and chemical coupling method in comparison to control WP, however, average particle size of covalent conjugates of WP-CA and WP-FA prepared by free radical grafting method was decreased.

At pH 7, the zeta potential was -9.83 for control WP and on decreasing pH to pH 5 zeta potential decreased and on further decrease in pH to 3, zeta potential became positive (+6.73). On conjugation with polyphenol both CA and FA, zeta potential was more negative as compared to control WP. For conjugates prepared by covalent methods, insignificant changes in the zeta potential were observed for WP-CA and WP-FA conjugate prepared by alkaline method; however, for chemical coupling method and free radical grafting method the zeta potential shifted towards negative side compared to control WP for both the WP-CA and WP-FA conjugates.

The non-covalent conjugates at pH 7 and covalent conjugates prepared by alkaline and free radical grafting method were selected for characterization of functional properties.

The AO of control WP was 38% RSA. On conjugation with CA and FA, the AO increased significantly. There was no significant difference in AO of WP-CA and WP-FA conjugates in all the method of preparation. On conjugation with CA, the AO was found to be significantly increased up to 59.44, 68.39, 67.90 % RSA in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. Similarly, on conjugation with FA, the AO was found to be significantly increased up to 58.64, 62.96, 66.17 % RSA in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively.

The solubility of control WP was 85-92%. On conjugation with CA and FA, the solubility decreased. There was no significant difference in solubility of WP-CA and WP-FA conjugates irrespective of the method of preparation. On conjugation with CA, the solubility was not significantly decreased and found to be 86.77, 84.85, 79.67% in conjugates prepared by non-covalent (pH 7), alkaline and free radical

grafting method, respectively. Similarly, on conjugation with FA, the solubility was Not significantly decreased and found to be 83.25, 83.1, 73.7 % in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively.

The EA and ES of control WP was 85.99 and 84.33%, respectively. On conjugation with CA and FA, both EA and ES increased. There was no significant difference in EA and ES of WP-CA and WP-FA conjugates irrespective of the method of preparation. On conjugation with CA, the EA and ES was increased significantly and found to be 97.03 and 97.13, 91.21 and 89.53, 97.15 and 92.33% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. On conjugation with FA, the EA and ES was increased significantly and found to be 95.33 and 96.66, 85.66 and 86.33., 95.66 and 91.33% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively.

The FC and FS of control WP was 66.93 and 60.33 %, respectively. On conjugation with CA and FA, FC increased but there was no significant difference in FS. There was no significant difference in EA and ES of WP-CA and WP-FA conjugates irrespective of the method of preparation. On conjugation with CA, the FC and FS was found to be 82.38 and 58.86, 76.37 and 60.08, 76.7 and 59.15% in conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. The significant increase in foaming capacity (FC) and nonsignificant decrease was observed for foam stability (FS). On conjugation with FA, the FC and FS was found to be 79 and 56.82, 74.33 and 57.14, 75.33 and 57.5% In conjugates prepared by non-covalent (pH 7), alkaline and free radical grafting method, respectively. The results were similar to CA that significant increase in foaming capacity (FC) and non-significant decrease was observed for foam stability (FS).

In this study, results indicated the possibility of using Caffeic acid and Ferulic acid in improving the functional properties of WPC.The functionality of the prepared conjugates was found to be affected by type of polyphenol and method of conjugation. The functionality was improved maximum in non-covalent conjugates followed by covalent conjugates. The WPC-caffeic acid conjugates had better functionality than Ferulic acid conjugates. The whey protein-polyphenol conjugates with enhanced functional properties can be used as novel food additives in food products.

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