PREPARATION OF WHEY PROTEIN BASED CONJUGATES AND EVALUATION OF THEIR EFFICACY IN W/O/W DOUBLE EMULSION

THESIS SUBMITTED TO THE

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

(DEEMED UNIVERSITY)

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY

IN

DAIRY TECHNOLOGY

BY

VAHORA AMIR MAHMADSHAFI

B. Tech (Dairy Technology)

DAIRY TECHNOLOGY DIVISION

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE

(DEEMED UNIVERSITY)

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Approved by:

(25/7/15)
EXTERNAL EXAMINER

25/7/15
(Dr. Latha Sabikhi)
MAJOR ADVISOR AND CHAIRMAN
(GUIDE)

Members of Advisory Committee

1) **Dr. A. K. Singh**
   Principal Scientist, DT Division — 25/7/2015
2) **Mr. Sathish Kumar M. H.**
   Scientist, DT Division — 25/7/15
3) **Dr. Rajan Sharma**
   Principal Scientist, DT Division — 25/7/15
4) **Er. P. S. Minz (JD Nominee)**
   Scientist, DE Division — 25/7/15

DAIRY TECHNOLOGY DIVISION
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
(Indian Council of Agricultural Research)
KARNAL -132001 (HARYANA), INDIA

Dr. Latha Sabikhi
Principal Scientist and Head

CERTIFICATE

This is to certify that the thesis entitled **"PREPARATION OF WHEY PROTEIN BASED CONJUGATES AND EVALUATION OF THEIR EFFICACY IN W/O/W DOUBLE EMULSION"** submitted by **Mr. AMIR MAHMADSHAFI VAHORA** towards the partial fulfilment of the award of the degree of **Master of Technology in Dairy Technology** 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 no part of the thesis has been submitted for any other diploma or degree.

27/7/15

Dated: July 27, 2015

(Dr. Latha Sabikhi)
MAJOR ADVISOR AND CHAIRMAN
(GUIDE)

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ABSTRACT

Over the past few decades, human lifestyles have changed drastically due to industrialization and changing work cultures, which have led to increased lifestyle related and other degenerative diseases. With the increasing health awareness, shift towards preventive health care and increased regulatory clarity in food safety and standards, people have moved to better alternatives such as nutraceutical over pharmaceuticals. *Brahmi* has been used in the Ayurvedic system of medicine for centuries and is classified as a *medhyarasayana*, a drug used to improve memory and intellect (*medhya*). The present study was envisaged to develop w/o/w double emulsion as an effective delivery system for *brahmi* extract using whey protein based conjugates as hydrophilic emulsifiers. Different polysaccharides such as maltodextrin, high methoxyl pectin and gum arabic were conjugated with whey proteins at protein: polysaccharide weight ratio of 1:2, 1:1 and 2:1. Whey protein concentrate-high methoxyl pectin (WPC-HMP) conjugate at the weight ratio of 1:2 was found to have better emulsifying properties (84.44% emulsifying activity and 78.33% emulsion stability) compared to other whey protein based conjugates and whey protein concentrate alone. WPC-HMP (1:2) conjugate also had better solubility around the iso-electric pH of whey proteins. SDS-PAGE of all the whey protein based conjugates confirmed the covalent linking of whey proteins and polysaccharides. Hence, based on the results WPC-HMP (1:2) conjugate was selected for use as hydrophilic emulsifier and evaluate its efficacy in optimization of w/o/w double emulsion. Further, optimization using RSM showed that optimized formulation containing 4% PGPR, 0.4% salt, 2% conjugate and 22000 rpm speed had higher zeta potential (-32.7 mV) and 100% sedimentation stability at 7°C and 37°C for one month. The study resulted in an optimized double emulsion as a potential delivery system for *brahmi* extract which can be further applied in dairy and food products.

व्हे प्रोटीन आधारित कांजुगेट कि तैयारी और उसका जल/वसा/जल द्विस्तरीय पायस में प्रभाव का मूल्यांकन

सारांश

पिछले कुछ दशकों में, मानव की जीवन शैली में औद्योगीकरण और बदलते कार्य संस्कृतियों की वजह से काफी बदलाव हुए है जिससे जीवन शैली सम्बंधित और अन्य अपक्षयी रोगों में काफी वृद्धि हो गई है। बढ़ रही स्वास्थ्य के प्रति जागरूकता ने स्वास्थ्य देखभाल की ओर लोगों का ध्यानाकर्षण किया है, और खाद्य सुरक्षा एवं मानकों में स्पष्टता की वजह से, लोग दवाइयों से अधिक न्यूट्रास्युटिकल को बेहतर विकल्प के रूप में चुन रहे हैं। ब्राह्मी सदियों से आयुर्वेदिक प्रणाली मे मेध्यरसयाना औषधि की तरह इस्तेमाल किया गया है, एवम इसका उपयोग स्मृति और बुद्धि (मेध्य) में सुधार करने के लिए किया जाता है। वर्तमान अध्ययन जल/वसा/जल द्विस्तरीय पायस को ब्राह्मी के लिए एक प्रभावी वितरण प्रणाली हाइड्रोफिलिक पायसीकारी के रूप में व्हे प्रोटीन आधारित सगुणित के प्रयोग से विकसित करने की परिकल्पना की गई थी। विभिन्न पॉलीसैकराइड के रूप में माल्टोडेक्सट्रिन, उच्च मिथोक्षिल पेक्टिन और अरबी गोंद को व्हे प्रोटीन के साथ प्रोटीन-पॉलीसैक्राइड को विभिन्न अनुपात 1:2, 1:1 और 2:1 में संयुग्मित किया था। व्हे प्रोटीन कंसन्ट्रेट-उच्च मिथोक्षिल पेक्टिन (डब्ल्यूपीसी-एच ऍम पी) के 1:2 अनुपात द्वारा विकसित सगुणक का पायसीकारी गुण (84.44% पायसीकारी गतिविधि और 78.33% पायस स्थिरता) अन्य व्हे प्रोटीन आधारित कोंजुगेट और अकेले व्हे प्रोटीन कंसन्ट्रेट की तुलना में बेहतर पाया गया था। डब्ल्यूपीसी- एच ऍम पी (1: 2) कोंजुगेट की घुलनशीलता भी व्हे प्रोटीन के आइसो-इलेक्ट्रिक पीएच के आसपास बेहतर थी। एसडीएस-पेज ने सभी व्हे प्रोटीन आधारित कोंजुगेट में व्हे प्रोटीन और पॉलीसैक्राइड के सहसंयोजक बनने की पुष्टि की। इसलिए, डब्ल्यूपीसी-एच ऍम पी (1:2) को हाइड्रोफिलिक पायसीकारकों के रूप में उपयोग के लिए चुना गया और जल/वसा/जल द्विस्तरीय पायस के अनुकूलन में उनकी प्रभावकारिता का मूल्यांकन किया गया था। इसके अलावा, प्रयोगों द्वारा प्राप्त सांख्यिकीय परिणामो के अध्ययन के आधार पर 4% पी जी पी आर (पायसीकारक), 0.4% नमक, 2% कोंजुगेट और 22000 आरपीएम की गति से उच्च जीटा विभव (-32.7 एम वी) और 100% अवसादन स्थिरता दोनों तापमानों (7 डिग्री सेल्सियस और 37 डिग्री सेल्सियस) पर एक महीने के लिए था। इस अध्ययन से ब्राह्मी के लिए एक वितरण प्रणाली के रूप में एक अनुकूलित द्विस्तरीय पायस प्राप्त हुआ जिसका डेयरी और खाद्य उत्पादों में इस्तेमाल किया जा सकता है।

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INTRODUCTION

1. INTRODUCTION

Recently, the functional food and nutraceutical market has increased due to the increased risk of lifestyle and age-related diseases. The new lifestyle and aging have considerable impact on health, which led to interest in herbal alternatives with increasing health awareness across the world. A wide variety of active phytochemicals have been identified in different herbs that include flavonoids, terpenoids, lignans, sulfides, polyphenolics, carotenoids, coumarins, saponins, plant sterols, curcumins and phthalides (Craig, 1999). Herbal nutraceuticals are known not only for their health benefits to reduce the risk of cancer, heart diseases and other related ailments, but also to prevent or treat hypertension, high cholesterol, excessive weight, osteoporosis, diabetes, arthritis, macular degeneration, cataracts, menopausal symptoms, insomnia, diminished memory and concentration, digestive upsets and constipation. Bitter taste and obnoxious flavour of most of the herbs restrict their direct consumption and application in food products. Therefore, a delivery system is required for incorporation of such herbs in foods. Encapsulation of herbs with water-in-oil-in-water (W/O/W) double emulsions offer several strategic advantages for use in food applications: (i) incorporation of oil- and water-soluble sensitive ingredients separately in a single system (Muschiolik, 2007); (ii) controlled release of the encapsulated ingredients during eating and digestion (Dickinson, 2011); (iii) protection of sensitive compounds from detrimental environmental factors that may degrade them through the design of protective membranes (McClements *et al*., 2007). The stability of W/O/W double emulsion still remains a challenge using food-grade emulsifiers and stabilizers instead of synthetic surfactants and polymers used in other industries. Polymeric hydrophilic emulsifiers, mainly proteins and polysaccharides, have been known to provide better encapsulation and stability to emulsions. But proteins are not effective emulsifiers near their iso-electric pH which limits their application in acidic foods. Hence, protein-polysaccharide conjugates formed through maillard type reactions are suitable as they are very stable to changes in pH, ionic strength and temperature (Dickinson and Euston, 1991; Schmitt *et al*., 1998). Such conjugates have high molecular weights and when used as emulsifiers, show combined properties of both proteins and polysaccharides. The protein-polysaccharide conjugates provide better emulsification and solubility even near the

iso-electric pH of the protein. They produce stable emulsions preventing creaming, flocculation and coalescence by steric stabilization (Dickinson 1993).

Considering the present need, development of stable W/O/W double emulsion for the delivery of *brahmi* extract using whey protein-polysaccharide conjugates was undertaken for its potential application in food. The present study 'Preparation of whey protein based conjugates and evaluation of their efficacy in W/O/W double emulsion' was planned with the following objectives:

1. To optimize the formation of whey protein based conjugates and characterize their emulsifying properties
2. To evaluate the efficacy of whey protein based conjugates in W/O/W double emulsion containing *brahmi* extract

CHAPTER-2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Overview

Over the past few decades, human lifestyles have changed drastically due to industrialization and changing work cultures, which have led to increased lifestyle related and other degenerative diseases. With the increasing health awareness, shift towards preventive health care and increased regulatory clarity in food safety and standards, people have moved to better alternatives such as nutraceutical over pharmaceuticals. Thus, Indian nutraceutical market has experienced tremendous growth over past few years. According to a report by business research and consulting firm Frost and Sullivan, the Indian nutraceuticals market was $ 1,480 million in 2011 and is expected to grow to $ 2,731 million in 2016. The industry still in a budding stage holds ample opportunity to grow in future. There is an excellent market potential for the nutraceuticals in India with a strong need of developing customized products, affordable pricing and distribution strategy.

2.2 Nutraceuticals

The term 'nutraceutical' was coined in 1979 by Stephen DeFelice, Founder and Chairman of the Foundation for Innovation in Medicine. It is defined as 'a food or part of a food that provides medical or health benefits, including the prevention and treatment of disease'. Nutraceuticals may range from isolated nutrients, dietary supplements and specific diets to genetically engineered designer foods, herbal products, and processed products such as cereals, soups and beverages (DeFelice, 1995). Nutraceuticals are found in a variety of products emerging from the food industry, herbal and dietary supplement market, pharmaceutical industry, and the newly emerged pharmaceutical/ agribusiness/ nutrition conglomerates (Dureja *et al.*, 2003). Nutraceutical products are known not only for their health benefits to reduce the risk of cancer, heart diseases and other related ailments, but also to prevent or treat hypertension, high cholesterol, excessive weight, osteoporosis, diabetes, arthritis, macular degeneration, cataracts, menopausal symptoms, insomnia, diminished memory and concentration, digestive upsets and constipation. Nutraceuticals have also gained considerable trust in treating headaches and migraines resulting from stress (Prabu *et al.*, 2012). Nutraceuticals are marketed in concentrated forms either as a single substance or

as combination preparations (Stephen, 1998). They are categorized based on their chemical constituents into nutrients, herbals and dietary supplements (Hathcock, 2001).

2.3 Herbal nutraceuticals

Herbs have been used as food and as medicine for centuries. Herbals have been an integral part of the society since the beginning of human civilization, valued for both their culinary and medicinal properties. They have played a significant role in maintaining human health, improving the quality of human life and serving us with valuable components of seasonings, beverages, cosmetics, dyes, and medicines. A wide variety of active phytochemicals have been identified in different herbs that include flavonoids, terpenoids, lignans, sulfides, polyphenolics, carotenoids, coumarins, saponins, plant sterols, curcumins and phthalides (Craig, 1999).

India has often been referred to as the Botanical Garden of the world having enormous wealth of medicinal plants. It was estimated that the primary health care of about 80% of the world's population still depended on herbal medicines (WHO, 2002). Increasing awareness about health and side effects of modern medicines has created a major shift of consumers toward herbal and natural products in the international market. According to the World Health Organization (WHO), the global market for herbal products is continuously expanding and it is expected to reach US$ 5 trillion mark by the year 2050. Despite being a major player, India's share in the global market of medicinal plants and products is mere 2.5%. India's export of medicinal herbs and their products was US$ 232.14 million in the year 2012-13, as informed by the Minister of State for Commerce and Industry, Dr. E.M. Sudarsana Natchiappan in Lok Sabha on December 9, 2013. The market for Indian herbal industry is estimated to be Rs. 15,000 crore in 2015 with an expected compounded annual growth rate of about 20% (SME Times, 2010).

2.4 Bacopa monnieri Linn. or *Brahmi*

Bacopa monnieri Linn. (Scrophulariaceae family), commonly known in Sanskrit and Hindi as *Brahmi*, is a perennial creeping plant found throughout tropical India in wet, damp and marshy areas. *Brahmi* is a Sanskrit word derived from "Brahma", the Lord of Creation in Hindu cosmology, suggesting that the herb has direct ability to facilitate divine consciousness. *Brahmi* literally means the energy (or "Shakti") of Brahma (Warrier *et al.*,

1994; Srikanthamurthy, 2001; Dash, 2002). It has been used in the Ayurvedic system of medicine for centuries and is classified as a *medhyarasayana*, a drug used to improve memory and intellect (*medhya*) (Bhattacharya *et al*., 2000). It has bitter taste and provides a cooling and laxative effect. The compounds responsible for the pharmacological effects of *Brahmi* are alkaloids, saponins and sterols. The major chemical entity responsible for neuropharmacological effects and the nootropic activity of *Brahmi* is Bacoside A which is a saponin (Chatterji *et al*., 1965). The triterpenoid saponins and their bacosides are responsible for the ability of *Brahmi* to enhance nerve impulse transmission, suggesting that bacosides induce membrane dephosphorylation, with a concomitant increase in protein and RNA turnover in specific brain areas (Singh *et al*., 1988). Singh and Dhawan (1997) reported that the nootropic action of *Brahmi* may also be due to the increase in protein kinase activity and protein in the hippocampus.

Brahmi has been reported to have various pharmacological effects such as acetylcholinesterase activity, anti-dementia (Das *et al*., 2002), calcium antagonist (Dar and Channa, 1999), anti-spasmodic (Dar and Channa, 1999), anti-epileptic (Vohora *et al*., 2000), thyrostimulant (Kar *et al*., 2002), antioxidant (Tripathi *et al*., 1996; Bhattacharya *et al*., 2000; Chowdhuri *et al*., 2002), hepatoprotective (Sumathy *et al*., 2001), anti-cancer (Elangovan *et al*., 1995), vasodilatory (Channa *et al*., 2003), anti-addictive (Sumathi *et al*., 2007), anti-ulcerogenic (Sairam *et al*., 2001), analgesic (Vohora *et al*., 1997), anxiolytic (Calabrese *et al*., 2008), anti-depressant (Sairam *et al*., 2002), anti-inflammatory (Channa *et al*., 2006) and memory vitalizing properties (Uabundit *et al*., 2010). Singh and Dhawan (1997) studied the pharmacological and toxicological effects of bacosides on normal healthy male human subjects for 4 weeks and found that a single dose (20-300 mg) and multiple doses (100 and 200 mg) administered for the given period were safe and well tolerated by the subjects without any side effects. *Brahmi* significantly improved the speed of visual information processing, learning rate and memory consolidation, and reduced anxiety in healthy human subjects (Stough *et al*., 2001). The LD_{50} of aqueous and alcoholic extracts of *Brahmi* in rats were 1000 mg and 15 g/kg by the intraperitoneal route, respectively (Martis *et al*., 1992). No toxicity of the aqueous extract has been reported at a dose of 5 g/kg given orally and the LD_{50} of the alcoholic extract was 17 g/kg given orally. The daily dosage of *Brahmi* extract

standardized to 20% bacosides A and B was reported to be 200-400 mg for adults and 100-200 mg for children, in divided doses (Anonymous, 2004).

2.5 Emulsion based delivery system

Emulsions are composed of two immiscible phases (usually oil and water) wherein one phase is dispersed as fine spherical droplets into the other continuous phase. The droplet sizes in most of the conventional emulsions usually range from 100 nm to 100 µm, however, those in nano-emulsions range from 20-200 nm (Dickinson and Stainsby, 1982). If oil droplets are dispersed in an aqueous phase, then the system is called an oil-in-water or O/W emulsion, whereas a system in which water droplets are dispersed in an oil phase is called a water-in-oil or W/O emulsion. Apart from these simple emulsions, it is also possible to prepare multiple emulsions of oil-in-water-in-oil (O/W/O) and water-in-oil-in-water (W/O/W) type. Multiple emulsions, also referred to as double emulsions, are complex systems in which the dispersed phase is itself an emulsion present as fine droplets (Evison *et al.*, 1995; Garti, 1997; Garti and Bisperink, 1998). Fig. 2.1 shows the schematic representation of the different types of W/O/W double emulsion, as classified by Florence and Whitehill (1981). Double emulsions are potentially useful for producing low calorie and reduced fat products, masking flavours, preventing oxidation, and improving sensory characteristics of foods, or as a delivery system for controlled release and protection of sensitive ingredients (Benichou *et al*., 2004; McClements *et al*., 2007; Muschiolik, 2007; Dickinson, 2011). W/O/W emulsions are most suitable for encapsulation of hydrophilic bioactive components, though they may also be used as delivery systems for both lipophilic and hydrophilic bioactive components in the same system (Cournarie *et al*., 2004). Hence, double emulsions offer great possibilities for use as food ingredients in new food systems such as functional foods.

Double emulsions have been usually prepared by two-step emulsification technique by using ultrasonicator, high pressure homogeniser, high shear mixer (e.g. Ultraturrax), microfluidiser and membrane emulsification. The first step involves preparation of primary W/O emulsion by using lipophilic or low hydrophilic-lipophilic balance (HLB) emulsifier in case of W/O/W emulsion. In the second step, the primary W/O emulsion thus formed is dispersed in an aqueous continuous phase containing hydrophilic or high HLB emulsifier to

form a W/O/W emulsion (Kumar *et al*., 2012). Fig. 2.2 shows the schematic representation of the two-step emulsification technique used for preparation of W/O/W double emulsion.

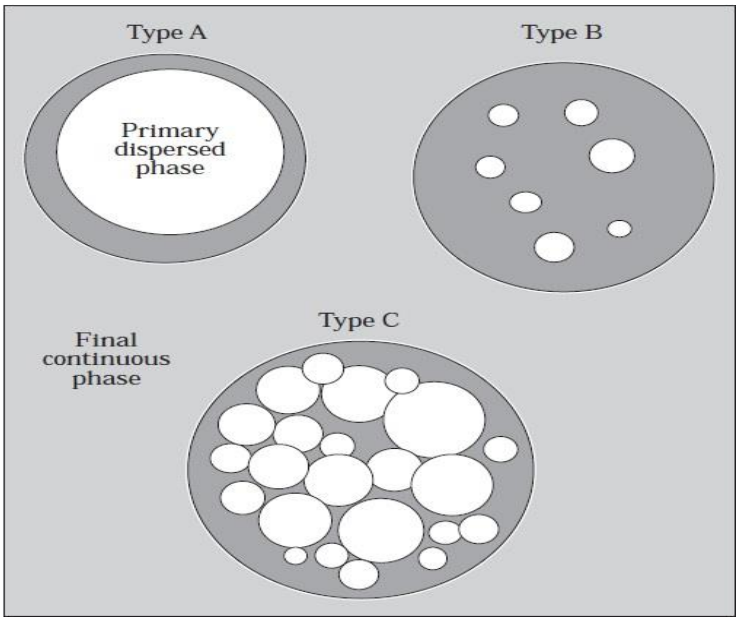


Fig. 2.1 Schematic representation of different types of W/O/W double emulsion

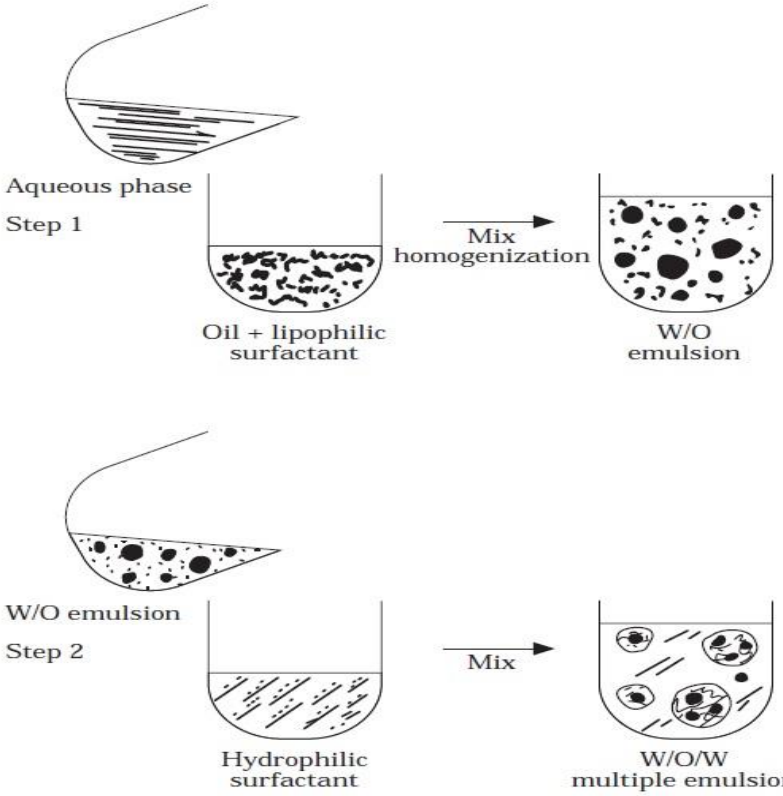


Fig. 2.2 Schematic representation of the two-step emulsification of W/O/W double emulsion

2.6 Composition of W/O/W emulsions

W/O/W emulsions are mainly composed of internal aqueous phase, hydrophobic emulsifiers, oil phase, hydrophilic emulsifiers and external aqueous phase.

2.6.1 Aqueous phase

The aqueous phase is the dispersed phase in W/O emulsion and the continuous phase in W/O/W emulsion. Often, distilled water is used as the aqueous phase. The internal aqueous phases are often solutions of encapsulated compounds such as nutrients, electrolytes (e.g. salt), and gelling or thickening agents (e.g. gelatin) (Garti and Aserin, 1996). The external aqueous phases are solutions of emulsifiers (e.g. proteins) and stabilizers (e.g. polysaccharides). The volume fraction of the aqueous phases and the additives used has a large effect on the stability of W/O/W emulsions.

2.6.2 Oil phase

In order to form stable W/O/W emulsions, the oil phase should ideally have low viscosity and low water solubility (Garti, 1997). Commercially available vegetable oils usually have higher viscosity and higher water solubility than mineral oils (Hamilton, 1993). But mineral oil is not advisable to be used in food applications since it is difficult to excrete from the body. Hence, vegetable oil has been used as oil phase in double emulsions intended for food formulations. However, vegetable oils require suitable high molecular weight hydrophobic emulsifiers to form stable double emulsions (Garti and Benichou, 2001; Kanouni *et al.*, 2002). Canola oil (Sapei *et al.*, 2012), medium chain triglyceride (MCT)-rich oil (Fechner *et al.*, 2007; O'Regan and Mulvihill, 2009a; 2010), rice bran oil (Kumar, 2011; Wankhade, 2012), soybean oil (Su *et al.*, 2008) and rapeseed oil (Frank *et al*., 2012; Kaimainen *et al*., 2014) have been tried successfully as oil phase in double emulsions.

2.6.3 Emulsifiers

Emulsifiers are amphiphilic compounds that consist of two distinct groups (hydrophilic and lipophilic) in the same molecule. Being amphiphilic in nature, they have affinity for both oil and water phase while residing at the interface. Emulsifiers lower the interfacial tension and facilitate droplet disruption, resulting in smaller droplets. Emulsifiers are classified as hydrophilic or hydrophobic, based on their hydrophilic/lipophilic balance (HLB). A low

HLB indicates a strongly lipophilic or hydrophobic emulsifier, whereas a high HLB indicates a strongly hydrophilic emulsifier. The HLB is also useful as a general indicator of emulsifier solubility in oil and water (Fennema, 1996). Both hydrophilic and hydrophobic emulsifiers are required for stabilization of the two interfaces in double emulsion.

Various ionic and non-ionic monomeric emulsifiers were most widely used during earlier studies on double emulsion. But monomeric emulsifiers could not provide long term stability to W/O/W emulsions because they have tendency to migrate from one phase to another. Progressively, polymeric emulsifiers were found to provide long term stability and longer shelf life to double emulsions compared to monomeric emulsifiers (Tadros *et al*., 1998; Muschiolik *et al*., 2006). As polymeric emulsifiers are complex molecules having high molecular weight, their tendency to migrate is very slow. They form strong and rigid film at the interface, preventing release of encapsulated material and also provide steric stabilization which prevents coalescence of internal droplets, thus improving stability (Dickinson, 2011). Table 2.1 depicts the various matrix materials used for preparation of W/O/W emulsion.

2.6.3.1 Hydrophobic emulsifiers

Hydrophobic emulsifiers with HLB value ranging from 3-6 are often used in W/O emulsions. Polyglycerol polyricinoleate (PGPR) derived from castor beans is the most commonly used hydrophobic emulsifier for food applications. PGPR has an HLB value of 4, similar to that of Span 80 and molecular weight of 4400 g/mol, about 10 times that of Span 80. PGPR provides the most stable W/O/W emulsions when vegetable oil is used as oil phase. Muschiolik *et al*. (2006) reported that minimum 4% PGPR was necessary to produce double emulsions with limited release of bioactive substances. They also tried partial substitution of PGPR with phosphatidyl choline (PC) depleted lecithin to produce stable W/O emulsions. PC-depleted lecithin produced similar sized internal droplets as those produced by PGPR. Unlike PGPR, the properties of emulsions stabilized by PC-depleted lecithin depend strongly on the lipid composition of the oil phase. Such lecithin-stabilized emulsions showed less emulsion stability due to formation of large aggregates of water droplets when long chain fatty acid lipids were used. Su *et al.* (2006) reduced the concentration of PGPR to 2% by partially replacing PGPR with 0.5% sodium caseinate (SC) without affecting the yield and stability of the W/O/W emulsion. They had concluded that the synergistic effects of PGPR and SC

Table 2.1 Various matrix materials used for preparation of W/O/W emulsions

Inner aqueous phase (W_1)	Oil phase (O)	Outer aqueous phase (W_2)	Ratio of phases	Encapsulant (in W_1 phase)	Reference
Gelatin (5%) and NaCl (0.1 mol/L)	PGPR (2 or 8%)	SC (0.7%) or SC-dextran conjugate (2.1%)	8:32:60	Vit. B_{12} (1%)	Fechner *et al*. (2007)
NaCl (1, 1.5 or 4.4%) or Sodium ascorbate (1, 5, 15 or 30%)	PGPR (10%)	WPI (4%) and pectin (0.5%) mixture	9:21:70	-	Lutz *et al*. (2009)
Gelatin (5%) and NaCl (0.6%)	PGPR 90 (2%)	SC (1%) or SC-Md 40 conjugate (1% w/w protein) or SC-Md 100 conjugate (1% w/w protein)	8:32:60	Vit. B_{12} (0.03%)	O'Regan and Mulvihill (2010)
Gelatin (0, 3 or 10 %), NaCl (0-8%)	PGPR (6%)	Polysorbate 80 (1%)	8:12:80	-	Sapei *et al.* (2012)
Deionized water	PGPR 90 (8%)	WPI-LMP or WPI-KCG mixture (0.2, 0.4, 0.6, 0.8 and 1%)	1.5:3.5:95	Vit. B_2 (50% in W_1) and Vit. E (20 % of oil phase)	Li *et al*. (2012)
(a) SC (8%) (b) SC (2%) and NaCl (2%) (c) WPC 70 (6%) and NaCl (2%)	PGPR 90 (4%)	Tween 80 (1%)	9:21:70	Potential nutraceuticals	Wankhade (2012)

NaCl- Sodium chloride, SC- Sodium caseinate, WPC-Whey protein concentrate, PGPR- Polyglycerol Polyricinoleate, WPI- Whey protein isolate, Md- Maltodextrin, LMP- Low methoxyl pectin, KCG- κ-carrageenan

produced stable W/O emulsions with reduced amount of emulsifier. Other hydrophobic emulsifiers used for the formation and stabilization of double emulsions include sorbitan mono-oleate or Span 80 (Sapei *et al.*, 2012), mixture of glycerol mono-oleate and lecithin in equal amounts (Garti *et al*., 1999), and α-form of fat microcrystals along with PGPR (Garti *et al*., 1999).

2.6.3.2 Hydrophilic emulsifiers

A number of monomeric and polymeric hydrophilic emulsifiers have been used in the aqueous phase of double emulsion. Polymeric emulsifiers, mainly proteins and polysaccharides, are known to provide better encapsulation and stability to emulsions. The main mechanisms behind the stabilization of emulsions by polymeric emulsifiers are electrostatic repulsions between two droplets carrying same charge, and steric stabilization resulting from hydrophobic interactions between adsorbed polymers (Benichou *et al*., 2004). Commonly used hydrophilic emulsifiers include whey or soy protein isolates (Frank *et al*., 2011; 2012), sodium caseinate (O'Regan and Mulvihill 2009a; Frank *et al*., 2012), bile acid (Frank *et al*., 2012), Tween 80 (Sapei *et al*., 2012), Tween 20 (Frank *et al*., 2011), SUPER GUM^{TM} which is modified gum arabic (Su *et al*., 2008), Panodan SDK which are esters of monoglycerides and diglycerides of diacetyl tartaric acid (Rodriguez-Huezo *et al*., 2004), and β-lactoglobulin isolated from whey protein isolates (WPI) (Frank *et al*., 2011). In order to reduce the amount of emulsifiers without compromising the stability of the emulsion, researchers have used modified emulsifiers such as SUPER GUM^{TM}, Panodan SDK, complex mixtures and conjugates of protein and polysaccharides.

2.6.3.2.1 Protein-polysaccharide conjugates as emulsifiers

Conjugation of protein to polysaccharides can improve the functional properties such as enhanced emulsifying properties (Shepherd *et al*., 2000; Kato, 2002; Neirynck *et al*., 2004; Einhorn-Stoll *et al*., 2005; Akhtar and Dickinson, 2007; O'Regan and Mulvihill, 2009b) and increased solubility around the iso-electric pH of the protein (Chevalier *et al*., 2001; Kato, 2002; Einhorn-Stoll *et al*., 2005; Oliver *et al*., 2006; O'Regan and Mulvihill, 2009b). Protein-polysaccharide conjugates formed through Maillard-type reactions are suitable for food applications. It allows formation of covalent linkages between the ε-amino group of protein and the reducing end of polysaccharide, which are very stable to changes in pH, ionic

strength and temperature (Dickinson and Euston, 1991; Schmitt *et al*., 1998). Dry heating has been reported to be the most suitable method for carrying out Maillard reaction (Einhorn-Stoll *et al*., 2005). Such conjugates have high molecular weights and when used as emulsifiers show combined properties of both proteins and polysaccharides. In conjugates, proteins constituting the hydrophobic part, assist in binding the oil portion and provide better emulsification and solubility, particularly around their isoelectric point. Polysaccharides on the other hand, contribute to the hydrophilic part of the conjugates and provide resistance against acidic conditions. They produce stable emulsions preventing creaming, flocculation and coalescence by steric stabilization (Dickinson 1993). Fig. 2.3 depicts a W/O/W emulsion stabilized by protein-polysaccharide conjugate in the outer phase.

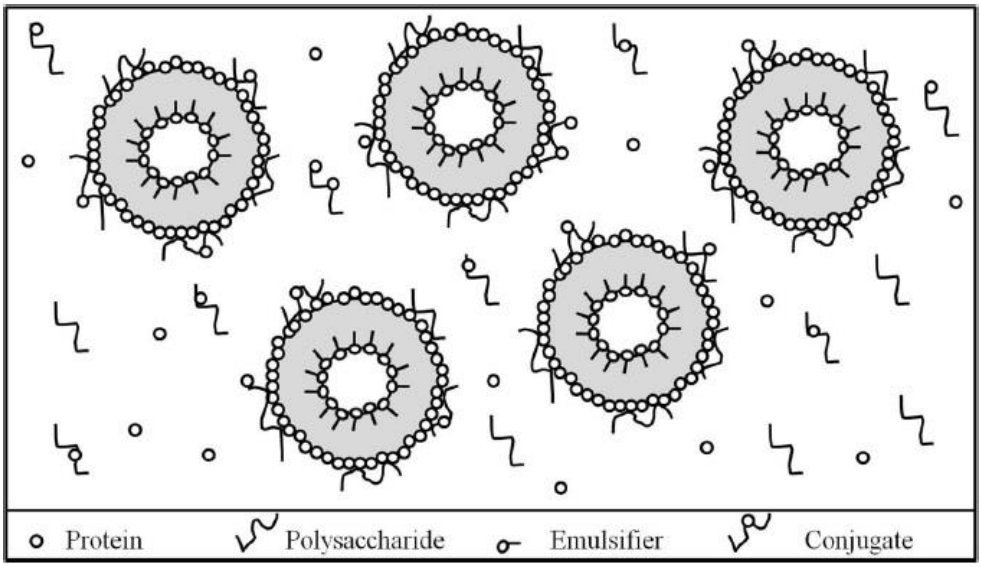


Fig. 2.3 W/O/W emulsion stabilized by protein-polysaccharide conjugate in the outer phase

Several proteins and polysaccharides have been used by researchers to prepare conjugates for use in food grade double emulsions. These include ovalbumin-dextran (Kato *et al*., 1990), ultrafiltered WPC-pectin (Mishra *et al*., 2001), whey proteins-dextran (Akhtar and Dickinson, 2003; Sun *et al*., 2011), milk protein-pectin (Einhorn-Stoll *et al*., 2005) and caseinate-dextran (Shepherd *et al*., 2000; Fechner *et al*., 2007; O' Regan and Mulvihill, 2009b). Table 2.2 depicts the various constituents, their ratio and incubation conditions used for conjugates.

Table 2.2 Various constituents, their ratios and incubation conditions used for conjugates

Protein Source	Carbohydrate Source	Ratio (protein: carbohydrate)	Incubation conditions			Reference
			Temperature	Relative humidity	Time	
Ovalbumin	Dextran (60-90 kDa)	1:1 or 1:5	60°C	65%RH	3 weeks	Kato *et al*. (1990)
Ultrafiltered WPC	Pectin (6% methoxyl content)	1:1	60°C	-	5,10 and 15 days	Mishra *et al*. (2001)
WPI	Dextran (488 kDa)	1:3	80°C	79%RH	2 h	Akhtar and Dickinson (2003)
WPI	LMP (33-38%DE) or HMP (69-74%DE)	1:0, 4:1, 2:1, 1:1, 1:2 and 0:1	60°C	75%RH	14 days	Neirynck *et al*. (2004)
WPI or SC	LMP (34.7%DE) or HMP (74.7%DE)	1:1, 1:3 and 1:5	50°C and 60°C	65%RH and 80%RH	5, 10 and 15 days	Einhorn-Stoll *et al*. (2005)
WPI or SC	Glucose, lactose, pectin, or dextran	1:2	70°C	65%	0-240 h	Hiller and Lorenzen (2010)
WPI	Dextran (150kDa)	1:1	60°C	79%RH	7 days	Sun *et al*. (2011)
Soy whey protein isolate	Fenugreek gum	1:3	60°C	75%RH	3 days	Kasran *et al*. (2013)

HMP- high methoxyl pectin

2.7 Properties of protein-polysaccharide conjugates

2.7.1 Emulsifying properties

Protein-polysaccharide conjugates exhibit excellent emulsifying properties combining the emulsifying nature of proteins and stabilizing effect of polysaccharides. The emulsifying properties can be evaluated by several methods such as size distribution of oil droplets formed, emulsifying activity, emulsion capacity and emulsion stability. Mishra *et al*. (2001) showed 98.56% emulsifying activity (EA) at 0.10% concentration and 99.65% emulsion stability (ES) at 0.25% concentration of ultra-filtered WPC-pectin conjugates. Akhtar and Dickinson (2003) reported that WPI-dextran conjugates produced fine emulsions with much better stability at lower pH compared to WPI and gum arabic alone under similar conditions. Conjugation of WPI with LMP, improved the emulsifying properties at pH 5.5 which is near the iso-electric pH of β-lactoglobulin (Neirynck *et al.*, 2004). WPI-pectin conjugates showed better emulsifying properties compared to those made with sodium caseinate (SC). Conjugates made with WPI had better EA and ES in combination with HMP, whereas conjugates made with SC had a higher EA in combination with LMP (Einhorn-Stoll *et al*., 2005). Xu *et al*. (2012) measured the stability of emulsions using LUMiSizer (L.U.M. GmbH, Berlin, Germany), a novel instrument employing centrifugal sedimentation to accelerate the occurrence of instability phenomena such as sedimentation, flocculation or creaming. They found that WPI-beet pectin conjugate stabilized emulsions had improved physical stability compared with mixture and WPI alone.

2.7.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) can be carried out to verify the covalent linkage between protein and polysaccharide due to conjugation process. Researchers have used discontinuous gel system following the method of Laemmli (1970). Conjugates show polydispersed bands along the gel with diminished characteristic bands of native protein. This suggests that high molecular weight conjugates have been formed by Maillard reaction during conjugation process.

2.7.3 Degree of glycation

Degree of glycation gives the concentration of free amines that remains unreacted with the polysaccharide during the conjugation process. Thus, it gives the extent of conjugation that occurred between protein and polysaccharide. The degree of glycation was measured using the 2,4,6-trinitro benzene sulfonic acid (TNBS) method based on the reaction of free amines with TNBS (O'Regan and Mulvihill, 2009b; Wang and Zhong, 2014). Higher absorbance values indicate a greater amount of unreacted amines and thus a lower degree of glycation.

2.7.4 Protein solubility

Protein solubility affects the functional properties of proteins such as emulsification, thickening, foaming and gelling. Solubility plays a role in emulsifying properties of proteins, but 100% solubility is not an absolute requirement. Some degree of solubility is likely to be necessary for favourable interactions with both the oil and aqueous phase to form stable protein film at the oil-water interface. The improved solubility of conjugates compared to protein near the iso-electric point of protein was attributed to the change in net charge of the protein and also hydration due to the attached sugar residues. The solubility of UF WPC-pectin conjugate at pH 4.6 increased from 78.91% to 89.30% when incubated at 60°C for 0 and 15 days respectively, while that of UF WPC alone decreased from 74.25% (0 day) to 36.09% (15 day) (Mishra *et al*., 2001). Kasran *et al*. (2013) reported that soy whey protein isolate-fenugreek gum conjugate were almost completely soluble (>90%) over the pH range of 3.0-8.0, including around the iso-electric pH of protein. For solubility, the soluble protein and total protein content in the conjugates and native protein were determined by various methods such as macro-Kjeldahl method (Mishra *et al.*, 2001; Neirynck *et al.*, 2004), Lowry method (Kasran *et al.*, 2013) and Bradford method (Shepherd *et al.*, 2000; Mao and Hua, 2012).

2.7.5 Colour

Colour intensity is a measure of extent of Maillard reaction that occurs during the conjugation process. Einhorn-Stoll *et al.* (2005) measured *L* a* b** colour coordinates of powdered sample of conjugates using Minolta chromameter. They reported that whey protein and caseinate conjugate with pectin had the highest *b*-value when incubated at high

temperature (65°C) and high humidity (80% RH) for 15 days compared to other lower temperatures and humidities.

2.8 Properties of double emulsion

2.8.1 Zeta potential

Zeta potential is a measure of the magnitude of the electrostatic or charge repulsion/attraction between particles, and is one of the fundamental parameters known to affect stability. Its measurement gives brief insight about the causes of dispersion, aggregation or flocculation, and can be applied to improve the formulation of dispersions, emulsions and suspensions. Emulsions with zeta potential greater than absolute value of 25 mV are normally considered stable (Malvern Instruments, 2011). Lower value shows higher van der Waal attraction, which causes aggregation and thus instability in the emulsion (Nanocomposix, 2012). The higher the zeta potential, the higher will be the stability of particles against flocculation because of greater prevalence of repulsive forces on its surface. Stability of emulsion as affected by any surface modification and/or alteration in processing step can be evaluated using zeta potential. Hence, its determination becomes even more important in case of double emulsions, which depend on surface polarities and ionic structures for its stability. Kumar (2011) reported a significant increase in the zeta potential of the particles subjected to higher pressure of microfluidiser (30,000 psi). This increase had been attributed to sudden decrease in particle size.

Although zeta potential gives a measure of surface modification, it is unable to describe complex phenomenon like charge neutralisation. Charge neutralisation is the phenomenon that stabilizes emulsion through the interaction of positively charged proteins and negatively charged polysaccharides. Charge neutralization being dependant on conformation of the molecule and charge distribution, cannot be explained by zeta potential alone. Li *et al*. (2012) reported that charge neutralisation on the surface of WPI-LMP complex stabilized emulsion was faster than that of WPI-KCG, even though KCG (−32.9 mV at pH 3, 0.1 % concentration) had higher charge amount than LMP (−13.6 mV at pH 3, 0.1 % concentration). WPI-LMP complex was able to decrease zeta potential from 20.7 to 3.6 mV when used in the ratio of 5:0 to 5:0.5 and corresponding decrease by WPI-KCG complex was

from 20.7 to 6.9 mV. This property was attributed to the lower affinity of KCG's surface charge with that of WPI.

2.8.2 Sedimentation stability

Sedimentation stability is an easy, reliable and important parameter to determine the stability of emulsion but its evaluation is time consuming. The stability of emulsion system is influenced by a number of factors such as emulsion composition, droplet size, viscosity, phase volume, pH, etc (Florence and Whitehill, 1982). Sapei *et al*. (2012) prepared double emulsions containing both NaCl and gelatin with control as either no NaCl or no gelatin. They found that the double emulsions containing both NaCl and gelatin were stable against sedimentation for one month while control emulsions were unstable immediately after preparation. The double emulsions containing only NaCl had higher sedimentation stability (~90% on day one and ~80% on day 29) compared to that containing gelatine alone.

Instability of multiple emulsions has been a major concern for use in encapsulation of nutraceuticals and commercializing the technique. The stability of double emulsions is expected to be improved remarkably with the use of conjugates as emulsifiers in the present study. Double emulsions have a great potential for use in functional foods, provided they are made stable to the processing conditions like high heat treatment, high shear, freezing, drying, storage, etc. Hence, further studies are required for their application in dairy and food products as a delivery system for nutraceuticals. However, shelf-life stability and effect on organoleptic properties need to be ascertained before use in the dairy or food industry.

Chapter-3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

This chapter deals with the materials and methods including equipments used during the present investigation relating to the technological, analytical and statistical aspects.

3.1 Materials

3.1.1 Whey Protein Concentrate-80

Whey protein concentrate (WPC) having 79.86% protein and 8.09% lactose content used for preparation of conjugates was purchased from Mahaan Proteins Ltd., New Delhi.

3.1.2 Maltodextrin

Maltodextrin of brand name RISIMALDEX and having dextrose equivalent of 11.07% on dry matter basis was purchased from Riddhi Siddhi Corn Processing Pvt. Ltd., Gokak, Belgaum, Karnataka.

3.1.3 High Methoxyl Pectin

High methoxyl pectin (GENU® pectin type YM-115-L) extracted from citrus peel and with degree of esterification of about 72% was purchased from CPKelco, Lille Skensved, Denmark.

3.1.4 Gum Arabic

Gum Arabic powder was purchased from HiMedia Laboratories Pvt. Ltd., Mumbai.

3.1.5 Polyglycerol Polyricinoleate (PGPR)

GRINSTED® PGPR 90 produced from castor oil was supplied by Danisco, Denmark.

3.1.6 Sodium Chloride

Vacuum evaporated iodised edible salt manufactured by Nirma Ltd., Bhavnagar, Gujarat was obtained from Experimental Dairy, National Dairy Research Institute, Karnal, Haryana.

3.1.7 Rice Bran Oil

Refined rice bran oil was purchased from local market of Karnal, Haryana.

3.1.8 *Brahmi* extract

Bacopa thick paste (*Brahmi* extract) having total solids content of 60.0% and saponin content of 22.1% was purchased from Ambe Phytoextracts Pvt. Ltd., New Delhi. Water soluble Bacopa thick paste was diluted to 50% using distilled water and filtered through double layered muslin cloth. The filtrate was then centrifuged at 5000 rpm for 7 min and the supernatant obtained was used for encapsulation.

3.2 Equipments

3.2.1 Hand blender

Lee handy blender (Type: DX-505, Lumix Appliances, India) was used for blending and preparation of pre-emulsion. The hand blender was operated at 10,000 and 12,000 rpm.

3.2.2 Freeze dryer

Labconco FreeZone 2.5 Litre Benchtop Freeze Dryer with collector temperature -50°C and ice holding capacity of 2.5 L was used for drying of protein-polysaccharide mixtures and conjugates.

3.2.3 Mixer grinder

Maxie DX food processor i.e. mixer grinder (INALSA Appliances, New Delhi) was used for fine grinding of freeze dried samples in a 400 mL stainless steel jar.

3.2.4 Ultra-Turrax

T 25 digital Ultra-Turrax® homogenizer (IKA Laboratory Equipment Ltd., Germany) was used for the preparation of the double emulsion. The high shear mixer is equipped with a stainless steel dispersing tool S 25 N-18 G and has maximum speed of 25,000 rpm.

3.3 Preparation of whey protein based conjugates

WPC and polysaccharides (PS) such as maltodextrin, high methoxyl pectin or gum arabic in the ratios 1:2, 1:1 and 2:1 were dissolved in distilled water. The samples were then freeze dried to remove water, and were ground to make powder. A desiccator containing saturated KCl was placed in an oven at 60°C to achieve equilibrium and maintain relative humidity of 80%. Then the samples were placed in desiccator at 60°C temperature and 80% RH for 10 days to induce conjugation (Einhorn-Stoll *et al.*, 2005). The samples were again freeze dried

and stored at 4°C for further analysis. Fig. 3.1 shows the flow diagram for the preparation of whey protein based conjugates.

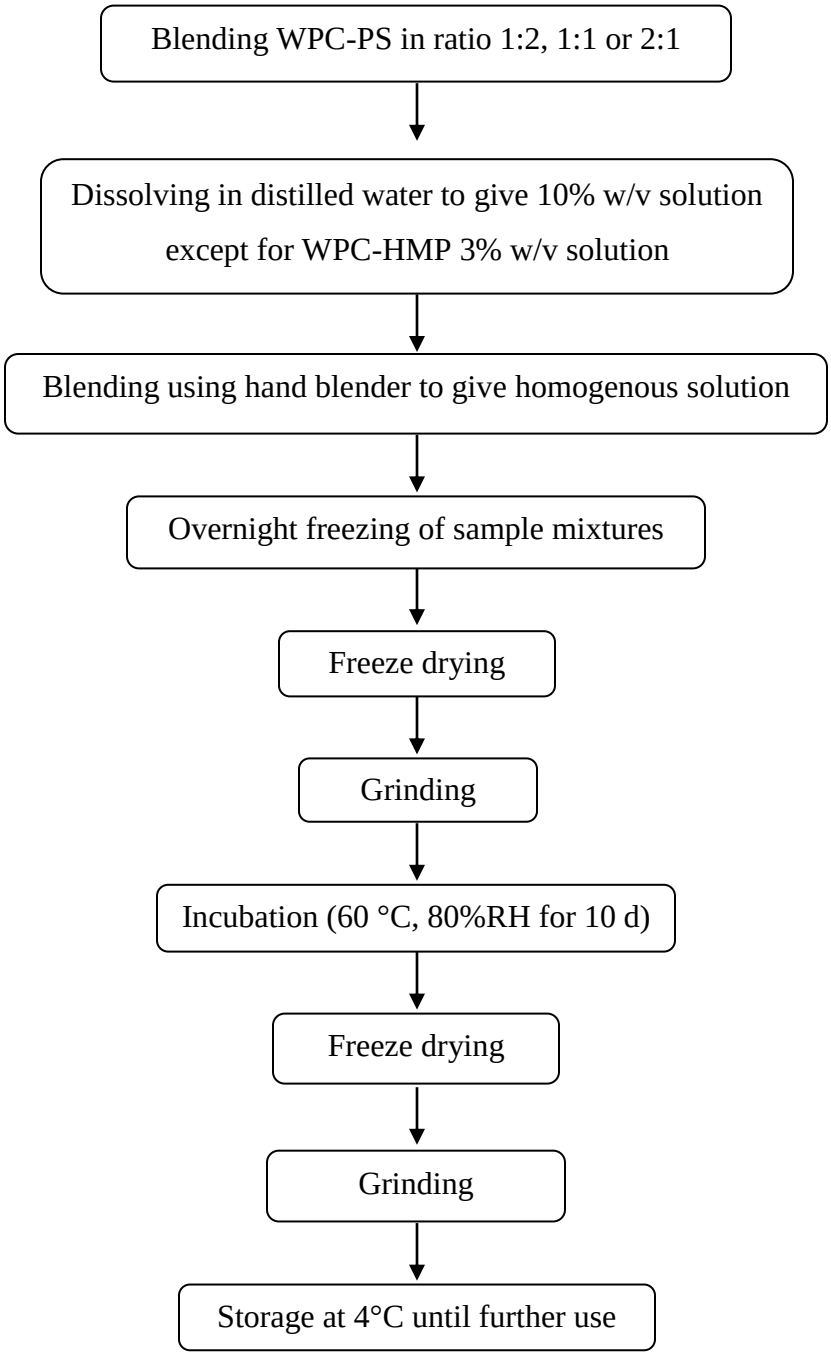


Fig. 3.1 Preparation of whey protein based conjugates

3.4 Analysis of whey protein based conjugates

The whey protein based conjugates were analysed for their emulsification, physical and physico-chemical properties.

3.4.1 Emulsifying properties

The emulsifying properties of the conjugates were determined by the method of Dalev and Simenova (1995) with some modifications. Conjugate (0.7% w/v protein) was dissolved in 90 mL 0.06 M phosphate buffer, pH 7.0, containing 0.01% (w/v) sodium azide (aqueous phase) at ambient temperature under moderate magnetic stirring conditions for 1 h. The emulsions were prepared by mixing the aqueous phase with the 60 mL of oil phase (rice bran oil) and homogenizing the mixture with an Ultra-Turrax at 15,000 rpm for 1 min. For emulsifying activity (EA), the emulsions were centrifuged in graduated tubes at 2600 g for 5 min and the whole volume (WV) of the system and the emulsion phase volume (EPV) were measured. Emulsifying activity was expressed as:

$$\text{EA (\%)} = \frac{EPV}{WV} \times 100$$

For emulsion stability (ES), the emulsions were kept at 80°C for 30 min, then in an ice bath for 15 min and finally centrifuged at 1300 g for 5 min. The emulsion stability was calculated as:

$$\text{ES (\%)} = \frac{EPV}{WV} \times 100$$

3.4.2 Colour measurement

The extent of Maillard reaction that occurred in the conjugates were determined by measuring the Hunter chromaticity coordinates (L^* a^* b^*) with Colorflex® (Hunter lab, Reston, Virginia, USA) loaded with the Universal software (version 10). The instrument was calibrated with a standard white and black tile before measurement. Dry WPC or WPC-PS conjugate, was placed into a petri-dish and the L^* a^* b^* colour coordinates were measured. The light source was dual beam xenon flash lamp. In the L^* a^* b^* colour space system, L^* values (lightness/darkness) ranges from 0 (black) to 100 (white), a^* values (redness) ranges from -60 (green) to +60 (red), and b^* values (yellowness) ranges from -60 (blue) to +60 (yellow). The colour intensity (C^*) was then calculated using the below formula:

$$C^* = \sqrt{a^2 + b^2}$$

3.4.3 Degree of glycation

The degree of glycation was measured for unreacted amines using the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method (Tainturier *et al.*, 1992), with some modifications. A fresh working solution of TNBS was prepared prior to assays by diluting the 5% TNBS solution (Sigma-Aldrich Inc., St. Louis, MO, USA) in 0.1 M sodium bicarbonate aqueous solution to an overall TNBS concentration of 0.01% w/v. Two hundred µg/mL of WPC and each conjugate solution was prepared in 0.1 M sodium bicarbonate solution at pH 8.5. TNBS working solution was then mixed with the sample solutions at a volume ratio of solution: TNBS working solution = 2:1. The mixture was incubated in water bath at 37°C for 2 h and then the reaction was terminated by adding 10% w/v SDS solution and 1N HCl to a volume ratio of mixture: SDS: HCl = 6:2:1. Absorbance of the final mixture solution was measured in triplicates at 335 nm using UV-Vis spectrophotometer with distilled water as blank.

3.4.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using Amersham Biosciences miniVE vertical electrophoresis system. The method was conducted according to the method of Laemmli (1970) with slight modifications.

3.4.4.1 Solutions:

Stock Acrylamide/Bisacrylamide (30%): Approximately 29.2 g acrylamide and 0.8 g bisacrylamide were dissolved in distilled water and total volume was made to 100 mL. The solution was filtered and stored at 5°C in a dark bottle.

1.5 M Tris-HCl (pH 8.8, 4X running gel buffer): Approximately 18.15 g tris was dissolved in 50 mL distilled water. The pH was adjusted to 8.8 with 1 N HCl and total volume was made up to 100 mL with distilled water. The buffer prepared was stored at 5°C.

0.5 M Tris-HCl (pH 6.8, stacking gel buffer): 6.05 g tris dissolve in 50 mL distilled water. The pH was adjusted to 6.8 with 1 N HCl and total volume was made to 100 mL with distilled water. The buffer prepared was stored at 5°C.

10% SDS: 10 g sodium dodecyl sulphate was dissolved in distilled water and the total volume was made to 100 mL. The solution was then stored at room temperature.

5X Electrode buffer (25 mM tris, 192mM glycine, 0.1% SDS, pH 8.3): 15 g Tris, 72 g Glycine and 5 g SDS were dissolved in distilled water and the total volume was made upto one litre with distilled water. The buffer was diluted five times with distilled water before use.

2X Sample buffer (0.125 M tris, 4% SDS, 20% glycerol, 0.2 M dithiothreitol (DDT), 0.02% bromophenol blue, pH 6.8): The following were mixed to prepare 2X buffer: 4X stacking gel buffer- 2.5 mL, glycerol- 2.0 mL, 10% SDS- 4.0 mL, bromophenol blue- 2.0 mg, DDT- 0.31 g, and distilled water- 1.5 mL.

10% Ammonium per Sulphate (APS): This was prepared fresh each time by dissolving 100 mg APS in 1.0 mL of distilled water.

Stacking gel (5%, 10 mL): Distilled water- 5.7 mL, 30% acrylamide/bisacrylamide mixture- 1.7 mL, 0.5 M Tris-HCl (pH 6.8)- 2.5 mL, 10% SDS- 0.10 mL, 10% APS- 0.050 mL and TEMED- 0.01 mL.

Separating gel (12%, 15 mL): Distilled water: 5 mL, 30% acrylamide/bisacrylamide mixture- 6 mL, 4X running gel buffer- 3.8 mL, 10% SDS- 150 µL, 10% APS- 150 µL, TEMED- 9 µL.

Staining solution: 0.25% Coomassie brilliant blue (CBB) R-250 in methanol / glacial acetic acid / water (2.5:1:6.5) (v/v).

Destaining solution: Methanol / glacial acetic acid / water (1:1:8) (v/v).

3.4.4.2 Gel Casting:

A pair of dry glass rectangular plates were taken and a spacer was placed between both sides of the plates. A tape was applied along the bottom and sides of the plates. The gel casting assembly was secured by clamping the sides with casting clamps. The assembly was allowed to stand on the side clamps. The separating gel after addition of APS, was immediately deposited between the assembled glass plates of the gel equipment such that the level remains slightly below the comb. Water about 200 µL was carefully overlaid by pipetting down the side of the glass plates. The gel was allowed to polymerize at room temperature for at least 30 min. The stacking gel was prepared and was carefully deposited on top of the

polymerized separating gel, after removing water from the top of the separating gel, until the cavity was full. Comb was then inserted into the stacking gel solution, avoiding trapping of air bubbles underneath the comb. The stacking gel was allowed to polymerize for at least 1 h at room temperature. Comb was removed and sample wells were overlaid with enough water to fill completely. Clamps were then removed and the plates were fixed into the electrophoretic unit.

3.4.4.3 Sample preparation:

Sample solution (2 mg protein/mL) was mixed with equal volume of sample buffer. The resultant solution was boiled for about 3 min. The molecular weight marker was also prepared in the similar way.

3.4.4.4 Electrophoresis:

Diluted and chilled electrode buffer was poured appropriately into the electrophoretic unit. The overlaid water was removed from the polymerized stacking gel and the upper reservoir filled with electrode buffer. Ten µL of sample, control and marker were loaded per well using a micropipette. After loading of samples and marker, the reservoir buffer was poured carefully. The electrophoretic unit was connected with the power supply and the electrophoresis was carried out at constant voltage of 100 V at refrigeration temperature. The electrophoresis was stopped when the tracking dye reached the bottom end of the separating gel.

3.4.4.5 Staining and Destaining:

At the end of electrophoresis, the gel was removed from the electrophoretic unit and kept for staining in the staining solution for 1 h. After staining, the gels were transferred to destaining solution at room temperature till protein bands are completely visible. The protein bands from the sample were compared with that from the control and marker.

3.4.5 Protein solubility

The solubility of WPC and whey protein conjugates as a function of pH was determined according to the method of Mohanty *et al.* (1988). Two hundred mg sample was accurately weighed and dispersed (1%, w/v, protein) into 20 mL distilled water contained in a 250 mL glass beaker. Then pH of the dispersion was adjusted in the range of 3-7 (with 0.5 unit

interval) using 0.1 N HCl or 0.1 N NaOH as required. Moderate magnetic stirring of the dispersion was carried out for 1 h. After 30 min of moderate magnetic stirring, the pH of each sample was rechecked and re-adjusted, if necessary. Fourteen mL of the dispersion was measured in 15 mL centrifuge tubes and centrifuged at 1000 g for 20 min. After centrifugation, the supernatant was decanted and filtered through Whatman No. 1 filter paper. The soluble and total protein content were determined using filtered supernatant and initial dispersion respectively by Bradford method (Bradford, 1976).

3.4.5.1 Preparation of Standard curve: A vial of Standard Protein Bovine Serum Albumin (BSA) (5 mg/mL) was reconstituted with 1 mL of distilled water to get concentration of 5 mg/mL. Then 1 mg/mL of BSA was prepared by diluting 0.2 mL of 5 mg/mL BSA with 0.8 mL of distilled water. Various concentrations of the standard protein (BSA @ 0, 10, 20, 40, 60 and 80 µg/0.2 mL) were prepared in a glass test tube by further diluting 1 mg/mL of BSA with distilled water. Two mL of Bradford reagent was added to each tube, mixed and kept at room temperature for 10 min. Absorbance of the standard tubes was taken at 595 nm with the first tube taken as blank. A standard curve was derived by plotting a graph of absorbance (nm) versus concentration (µg) (Fig. 3.2).

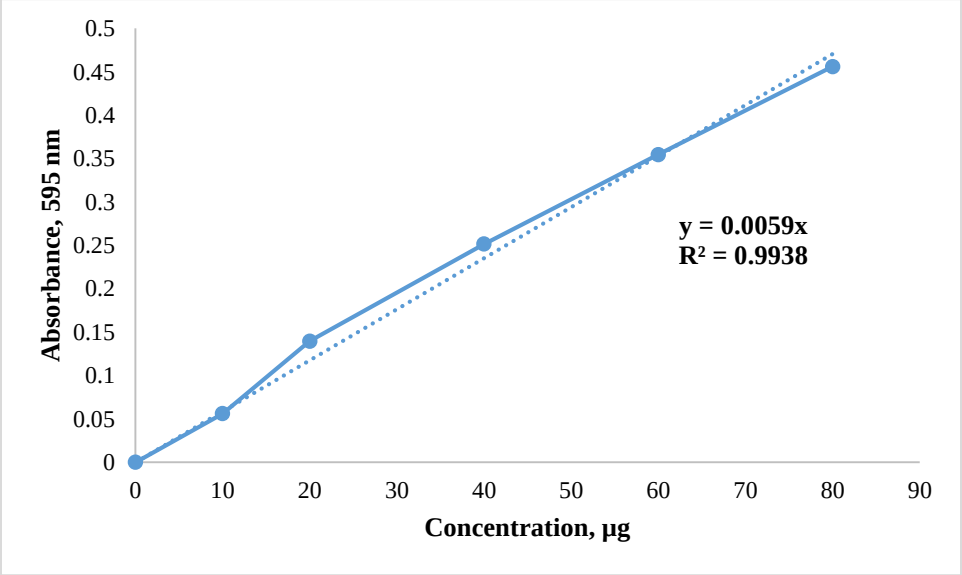


Fig. 3.2 BSA standard curve for protein estimation

3.4.5.2 Sample Procedure: Ten µL (v) of sample (filtered supernatant or initial dispersion) was pipetted in a glass test tube and diluted to 0.2 mL volume with distilled water so that the

concentration of the sample fell in the range of Bradford assay (10 µg to 150 µg). Two mL of Bradford reagent was added to each tube, mixed and kept at room temperature for 10 min. Absorbance of the sample tubes were taken at 595 nm with the same blank as above and the protein content of the sample was determined using the standard curve (a).

$$Protein\ concentration\ per\ mL = \frac{Amount\ of\ sample\ in\ \mu g\ (a)}{v\ (\mu L)} \times 1000$$

Solubility was calculated using the formula given below:

$$\text{Solubility}\ (\%) = \frac{Protein\ content\ in\ supernatant}{Protein\ content\ in\ initial\ dispersion} \times 100$$

3.5 Preparation of water-in-oil-in-water emulsions

Water-in-oil-in-water ($W_1/O/W_2$) emulsions were prepared using the two-step emulsification method described by Fechner *et al*. (2007) with some modifications. The inner aqueous phase (W_1) was prepared with NaCl (0.4-1.2%, w/w) and a water soluble/oil insoluble marker (*Brahmi* extract, 50%, w/w) in RO water, at 60°C for 5 min using moderate magnetic stirring. The oil phase (O) i.e. rice bran oil containing 2-4% (w/w) of the oil soluble emulsifier PGPR, was also mixed at 60°C for 5 min with moderate magnetic stirring. The outer aqueous phase (W_2) was prepared by hydrating the optimized whey protein based conjugate (0.33-0.67%, w/w, protein) in RO water containing sodium azide (0.02%, w/w) as preservative and blending using hand blender to .

A water-in-oil (W_1/O) pre-mix was prepared by mixing the inner aqueous phase (W_1) (30%, w/w) with the oil phase (O) (70%, w/w) to room temperature, using a magnetic stirrer. The mixture was then homogenized using an Ultra-Turrax operating at 18,000-22,000 rpm for 5 min to form primary W_1/O emulsion. The W_1/O emulsion (30%, w/w) was gradually added to the outer aqueous phase (W_2) (70%, w/w) and moderately mixed using magnetic stirring. The pre-mix was finally homogenized using Ultra-Turrax at 12,000 rpm for 5 min to produce final $W_1/O/W_2$ (9:21:70::$W_1/O/W_2$) double emulsion. The preparation of $W_1/O/W_2$ emulsion for *Brahmi* extract has been shown in Fig.3.3.

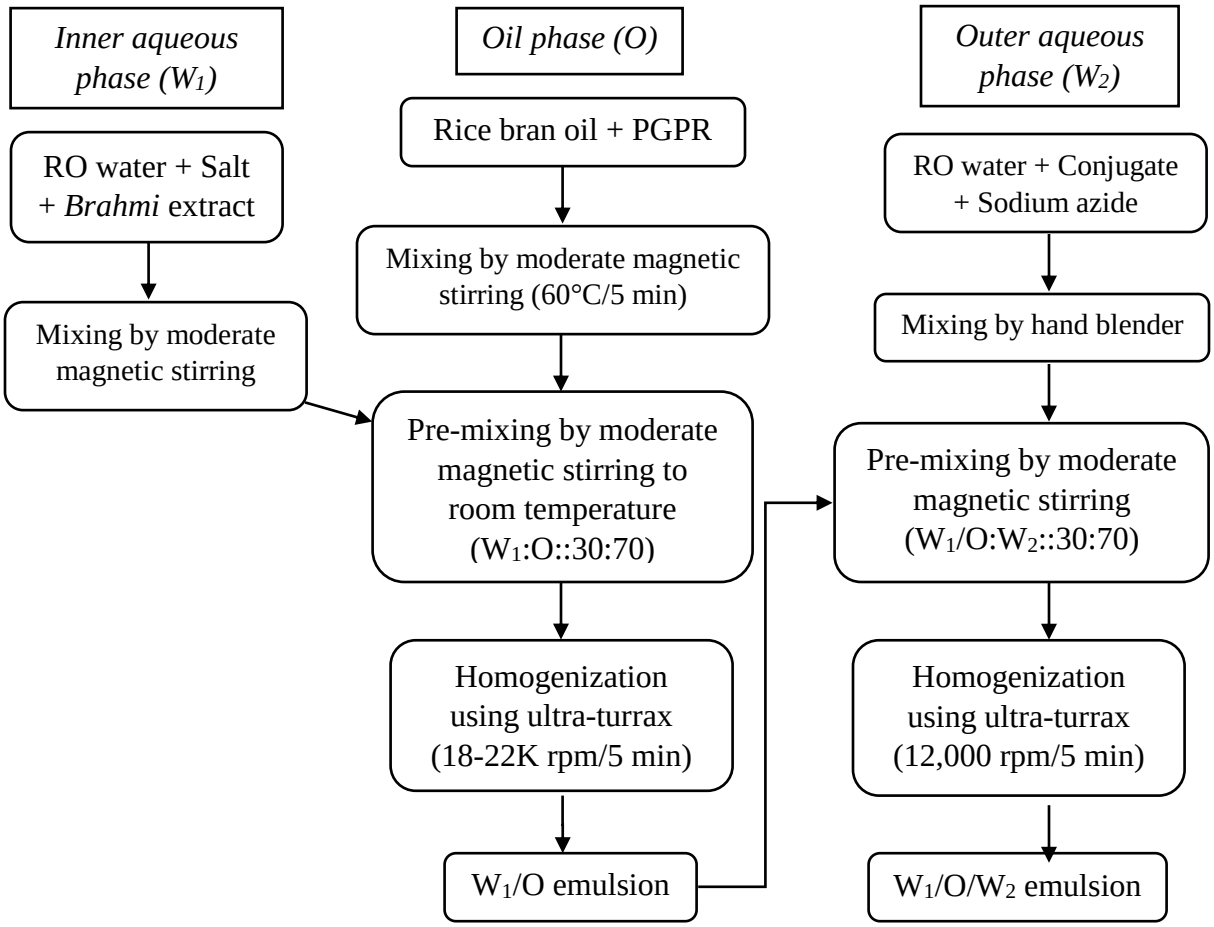


Fig. 3.2 Preparation of W_1/O/W_2 double emulsion

3.6 Analysis of W_1/O/W_2 emulsion

3.6.1 Zeta potential

The zeta potential of the double emulsion was measured using Zetasizer Nano-ZS90 (Malvern Instrument Ltd., Malvern, Worcestershire, UK) to determine the stability of the emulsion. The emulsions were diluted 100 times with RO water and the experiment was carried out at 25°C temperature as per the method of Kumar (2011). Zeta potential measurements were carried out in triplicate for each emulsion and the results were expressed in mV.

3.6.2 Sedimentation stability

The sedimentation stability of the double emulsion was determined in triplicates according to Sapei *et al*. (2012). Freshly-made double emulsions were poured into 40 ml glass vials (ID =

21 mm; length = 90 mm; Corning India, Gurgaon) to a height of ~6 cm and stored at 4ºC and 37ºC. The height of the opaque emulsion phase was measured daily for one month (h_t) and compared to the initial emulsion height (h_o) to determine sedimentation stability (S):

$$S\ (\%) = \frac{h_t}{h_0} \times 100$$

3.7 Statistical analysis

The data generated in objective 1 were statistically analysed using Analysis of Variance (ANOVA) in SPSS software (IBM SPSS Statistics Version 21) by applying Duncan Post Hoc Test for optimization of whey protein based conjugate. Two-way ANOVA was performed for protein solubility at given pH range while the other responses were analysed using One-way ANOVA. Further, for objective 2, Central Composite Rotatable Design (CCRD) of RSM Design Expert (9.0.4.1) was used to optimize $W_1/O/W_2$ double emulsion. The validation of the optimized solution given by Design Expert software was carried out by comparing the predicted and observed values using paired t-test in Microsoft Excel.

Chapter-4

RESULTS AND DISCUSSION

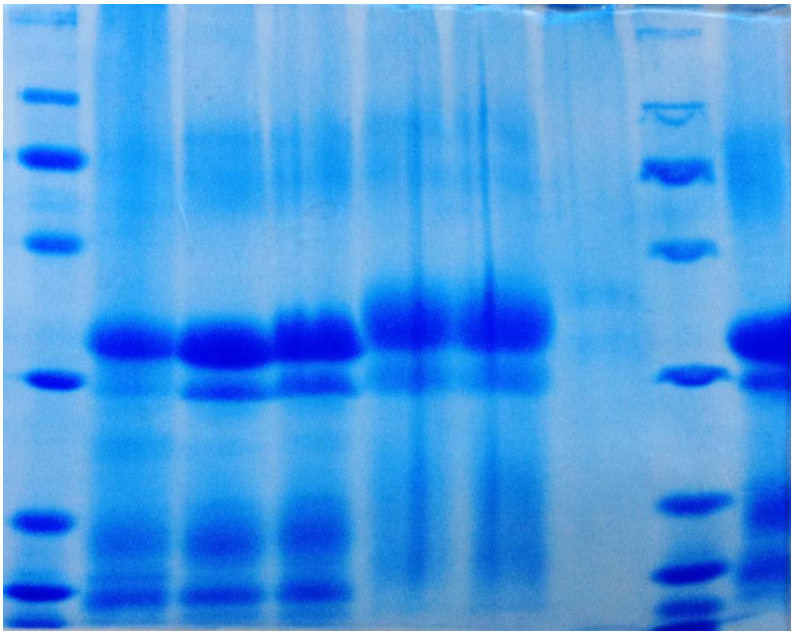
4. RESULTS AND DISCUSSION

This chapter contains the information generated during the present study 'Preparation of whey protein based conjugates and evaluation of their efficacy in w/o/w double emulsion' directed to achieve both the objectives listed in the first chapter. The results obtained are presented in this chapter in a tabulated form, figures and graphs along with relevant discussion. The study was done in a stepwise manner as presented below:

1) Preliminary studies for selection of incubation conditions for conjugation process
2) Characterization of whey protein based conjugates for their efficacy to provide stable emulsions
3) Optimization of w/o/w double emulsion formulation using Response Surface Methodology to obtain maximum stability.

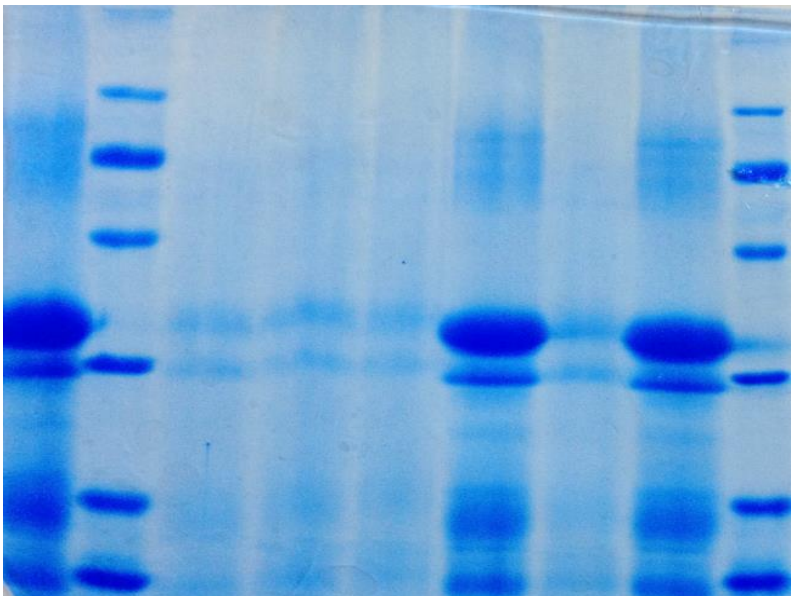
4.1 Preliminary studies for selection of incubation conditions for conjugation process

Preliminary studies were conducted to select the incubation conditions to be used for conjugation process. Since, incubation conditions play an important role in conjugation of proteins with polysaccharides, these conditions were to be selected to yield conjugate with excellent properties. Based on previous studies, two incubation conditions 80°C/79% RH for 2 h and 60°C/80% RH for 10 days were studied for conjugation process. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to confirm the conjugation reaction between protein and polysaccharides at both the incubation conditions. Fig. 4.1 shows the SDS-PAGE analysis of conjugates made with incubation at 80°C/79% RH for 2 h, while Fig. 4.2 shows the SDS-PAGE analysis of conjugates made with incubation at 60°C/80% RH for 10 days. It can be seen from Fig. 4.1 that there is no change in the characteristic whey protein band pattern in all the conjugates at incubation of 80°C/79% RH for 2 h. The characteristic band in conjugates of whey protein concentrate with high methoxyl pectin and gum arabic disappeared slightly (lanes 5 and 6 in Fig.4.1). This may be due to slight denaturation of whey proteins in both the conjugates. In contrast, slight disappearance in the characteristic band pattern for the protein was observed in all the conjugate samples incubated at 60°C/80% RH for 10 days, which indicated that conjugation had occurred between protein and polysaccharide. Thus, 60°C/80% RH for 10 days incubation was selected for preparation of whey protein based conjugates.



1 2 3 4 5 6 7 8 9

Fig. 4.1 SDS-PAGE analysis of conjugates prepared by incubation of 80°C/79% RH for 2 h. The labelled lanes are: (1) Marker, (2) Spray dried WPC-MD, (3) WPC-MD, (4) WPC-LMP, (5) WPC-HMP, (6) WPC-GA, (7) WPC-MD (60°C/80%RH for 10 d), (8) Marker, (9) WPC (control)



1 2 3 4 5 6 7 8 9

Fig. 4.2 SDS-PAGE analysis of conjugates at 60°C/ 80% RH for 10 d and mixture (no conjugation). The labelled lanes are: (1) Control, (2) Marker, (3) WPC-LMP (2:1), (4) WPC-LMP (1:2), (5) WPC-HMP (1:2), (6) WPC-HMP mixture (1:2), (7) WPC-HMP (2:1), (8) WPC-HMP mixture (2:1), (9) Marker

4.2 Characterization of whey protein based conjugates

The characterization of whey protein based conjugates was based on their efficacy in w/o/w double emulsion. Characteristics such as emulsifying properties, colour, degree of glycation, SDS-PAGE and protein solubility were studied to select the best combination of whey protein and polysaccharide and their ratio. Each property is discussed below in detail. The ANOVA table of characteristics of whey protein based conjugates are presented in Appendix I and II. The emulsifying properties, colour intensity and degree of glycation of whey protein based conjugates are presented in Table 4.1.

Table 4.1 Emulsifying, physical and physico-chemical properties of whey protein based conjugates

Combinations	Emulsifying properties		Colour intensity*	Degree of glycation
	Emulsifying activity (%)	Emulsion stability (%)		
Control (WPC)	46.67±0.00^{g}	48.33±0.00^{h}	37.89±0.02^{e}	0.430±0.001^{a}
WPC-MD (1:2)	50.56±0.56^{e}	50.56±0.56^{f}	35.00±0.05^{f}	0.273±0.002^{cde}
WPC-MD (1:1)	47.78±0.56^{fg}	53.33±0.00^{d}	38.94±0.07^{c}	0.287±0.016^{cde}
WPC-MD (2:1)	47.22±0.55^{fg}	49.44±0.56^{g}	42.69±0.05^{b}	0.252±0.011^{e}
WPC-HMP (1:2)	84.44±0.56^{a}	78.33±0.00^{a}	25.53±0.04^{i}	0.291±0.001^{cde}
WPC-HMP (1:1)	63.33±0.00^{b}	60.00±0.00^{b}	30.33±0.01^{h}	0.309±0.015^{c}
WPC-HMP (2:1)	57.22±0.55^{c}	54.44±0.56^{c}	32.59±0.04^{g}	0.381±0.027^{b}
WPC-GA (1:2)	53.33±0.00^{d}	60.00±0.00^{b}	35.02±0.00^{f}	0.296±0.006^{cd}
WPC-GA (1:1)	48.33±0.00^{f}	51.67±0.00^{e}	38.66±0.10^{d}	0.267±0.010^{de}
WPC-GA (2:1)	48.33±0.00^{f}	53.33±0.00^{d}	43.24±0.04^{a}	0.275±0.007^{cde}

*Control (WPC) was incubated at 60°C/80% RH for 10 days
Values are Mean±SE (n=3)
a, b, c - Means with different superscript within the column differ significantly ($p \leq 0.05$)
WPC – whey protein concentrate, MD – maltodextrin, HMP – high methoxyl pectin, GA – gum arabic

4.2.1 Emulsifying properties

The emulsifying properties of WPC alone and its conjugates with polysaccharides were investigated via the formulation of 40% (v/v) oil-in-water emulsions. The present investigation was carried out using different polysaccharides such as maltodextrin (MD), high methoxyl pectin (HMP), and gum arabic (GA) at various protein/polysaccharide weight ratios. The emulsifying properties were assessed in terms of emulsifying activity and emulsion stability. The emulsifying activity and emulsion stability of WPC alone and its conjugates have been presented in Table 4.1.

4.2.1.1 Emulsifying activity

It is obvious from the Fig. 4.3 that the emulsifying activity of WPC based conjugates were significantly ($p \leq 0.05$) higher than WPC alone and that the emulsifying activity increased by decreasing the ratio of protein/polysaccharide. The conjugate of WPC with HMP at weight ratio of 1:2 showed the highest emulsifying activity (84.44%) among the other polysaccharides and protein/polysaccharide ratios. A possible explanation for the poorer emulsifying characteristics of WPC-MD and WPC-GA conjugates compared to WPC-HMP conjugate is the low molecular size of MD and GA required for optimum steric stabilization. Further, Einhorn-Stoll *et al*. (2005) showed that whey proteins are highly compatible with HMP in formation of conjugates with excellent emulsifying properties. In all the cases, the protein/polysaccharide ratio of 1:2 showed higher emulsifying activity compared to 1:1 and 2:1 ratios which commemorates the findings of Xie and Hettiarachchy (1977). Though the presence of some unreacted proteins in 1:1 and 2:1 ratio conjugate may be slightly beneficial to its emulsifying capacity, the higher polysaccharide concentrations provide better steric stabilization and thus higher emulsifying activity.

4.2.1.2 Emulsion stability

The emulsion stability of WPC based conjugates were also significantly ($p \leq 0.05$) higher than WPC alone, as in the case of emulsifying activity. Emulsion stability increased by increasing the polysaccharide contribution in the protein/polysaccharide ratio as shown in Fig. 4.4. In emulsion system containing both proteins and polysaccharides, proteins form an adsorbed coherent viscoelastic layer at the oil-water interface, while polysaccharides confer stability

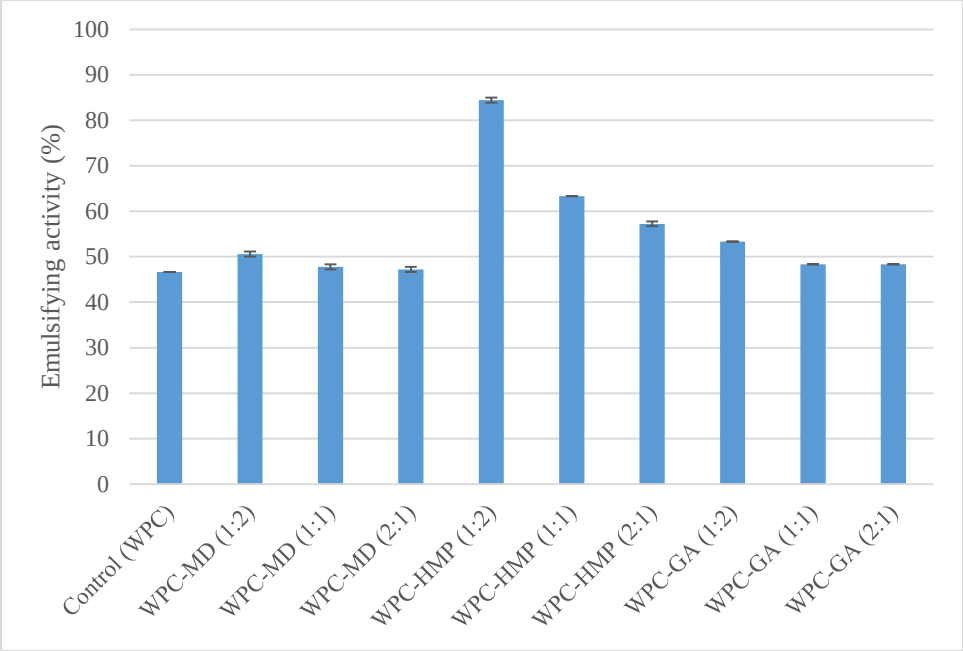


Fig. 4.3 Emulsifying activity of whey protein based conjugates

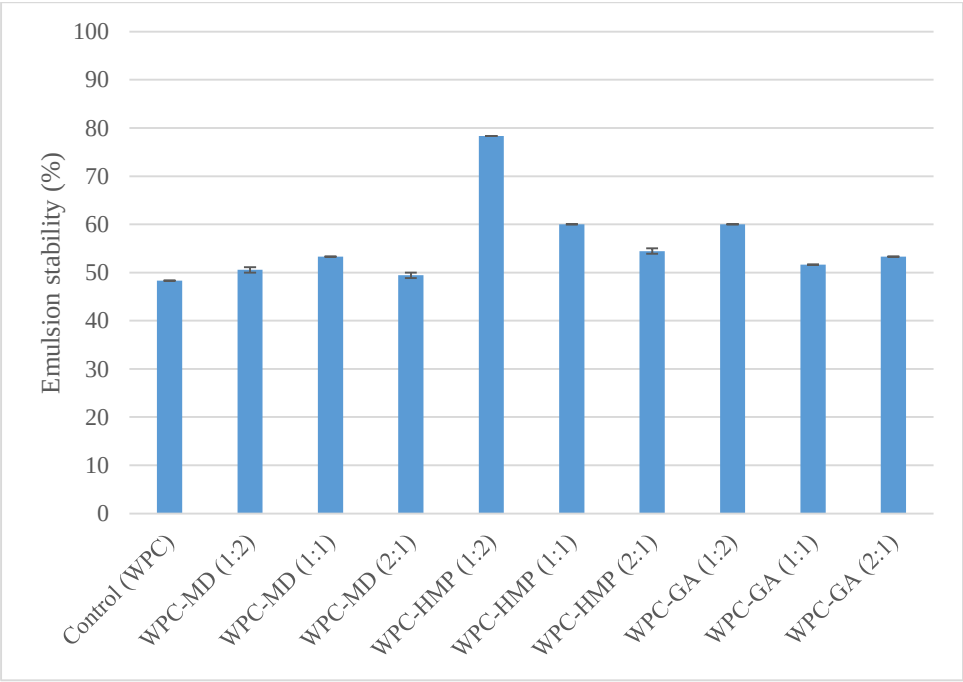


Fig. 4.4 Emulsion stability of whey protein based conjugates

through their thickening and gelation behaviour in the aqueous phase (Kato, 2002). The results revealed that WPC-HMP conjugate at weight ratio of 1:2 had the highest emulsion stability. The high emulsion stability of WPC-HMP conjugate may be due to higher viscosity of the continuous phase. Burova *et al*. (1992) reported increased emulsion stability in legumin-pectin complexes due to increased viscosity of the continuous phase. Xie and Hettiarachchy (1997) also reported that improved emulsion stability of soy protein isolate-xanthan gum complexes was related with the increase in viscosity.

4.2.2 Colour

Colour measurement is an indicator of the extent of advanced Maillard reaction (brown colour) that occurs during conjugation of protein and polysaccharides. In the present study, the colour coordinates (L^* a^* b^*) of dry powders of incubated WPC and its conjugates were measured and their colour intensity (C^*) was calculated. The results of colour intensity of incubated WPC and its conjugates are presented in Table 4.1 and Fig. 4.5. It is obvious from Fig. 4.5 that *C** value increased with the increase in protein/polysaccharide ratio and only those conjugates with higher ratio had higher colour intensity compared to incubated WPC alone. Conjugates with protein/polysaccharide ratio of 1:2 showed even lower colour intensity than WPC. This high colour intensity of WPC after incubation at 60°C/80% RH for 10 days may be due to lactose content of 8.09% present in WPC. Maillard browning between whey proteins and lactose in WPC may have occurred during incubation. Low molecular saccharides such as lactose form intensively coloured complexes with proteins (Kato *et al*., 1992; Aoki *et al.*, 2001). The low colour intensity of WPC-HMP conjugates may be because of non-reducing sugar sucrose present in HMP. The results indicate that Maillard reaction had occurred in all the conjugates.

4.2.3 Degree of glycation

In the present investigation, the degree of glycation was measured based on the reaction of free amines with 2,4,6-trinitrobenzene sulfonic acid (TNBS). The higher absorbance value at 335 nm (Abs_{335}) indicates greater amount of free amines that remained unreacted with the polysaccharide and thus a lower degree of glycation. As shown in Fig. 4.6 and Table 4.1, all the conjugates had significantly ($p \leq 0.05$) lower absorbance values compared to control

(WPC). The results indicate that conjugation had occurred between all protein-polysaccharide conjugates studied.

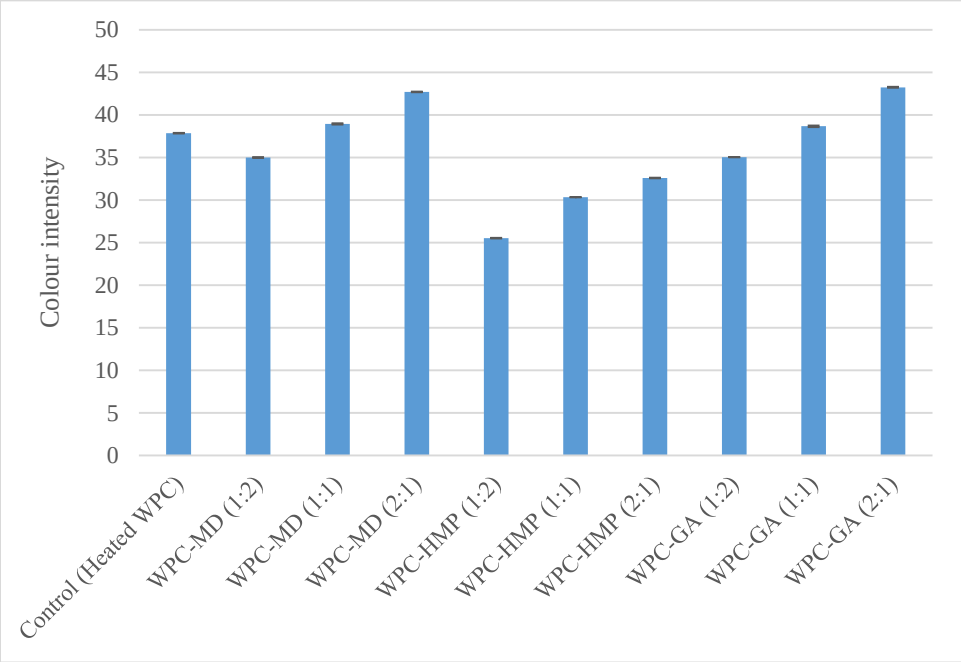


Fig. 4.5 Colour intensity of whey protein based conjugates

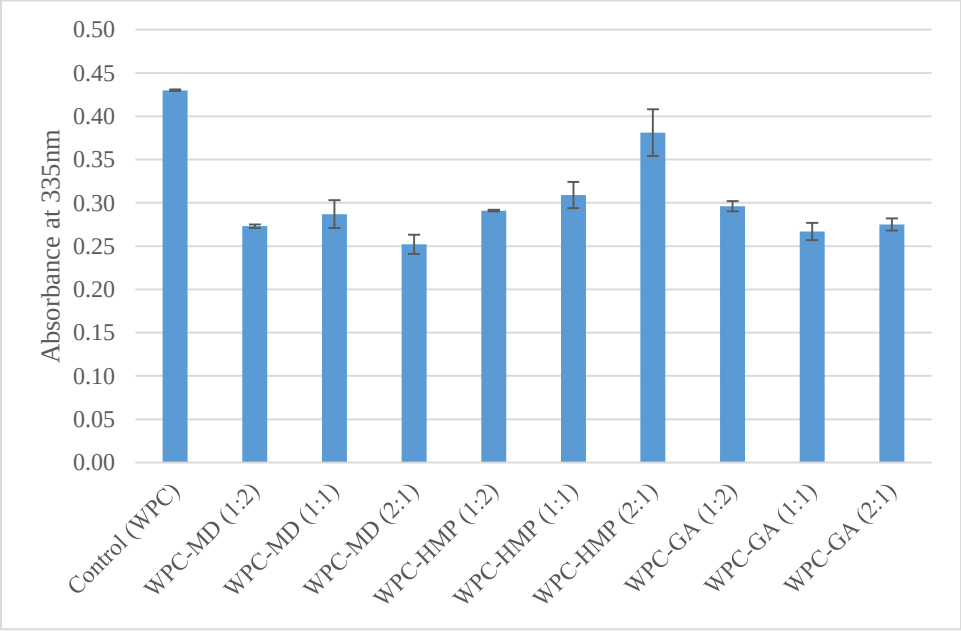


Fig. 4.6 Degree of glycation of whey protein based conjugates

4.2.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The covalent linking of whey protein to polysaccharides was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as shown in Fig. 4.7, which illustrates patterns of native whey protein, broad range protein marker and protein-polysaccharide conjugates. The pattern of the untreated whey protein (lane 1, 10 and 11) showed five bands, which could be α-lactalbumin, β-lactoglobulin, α-lactalbumin dimer, β-lactoglobulin dimer and bovine serum albumin (BSA), starting from the lowest molecular weight protein. The slight disappearance in the characteristic band pattern for the protein was observed in all the conjugate samples (lanes 3-8 and 12-14). The residual presence of the characteristic whey protein bands in the conjugate samples indicated that some protein remained unreacted with the polysaccharide, as reported by Akhtar and Dickinson (2007).

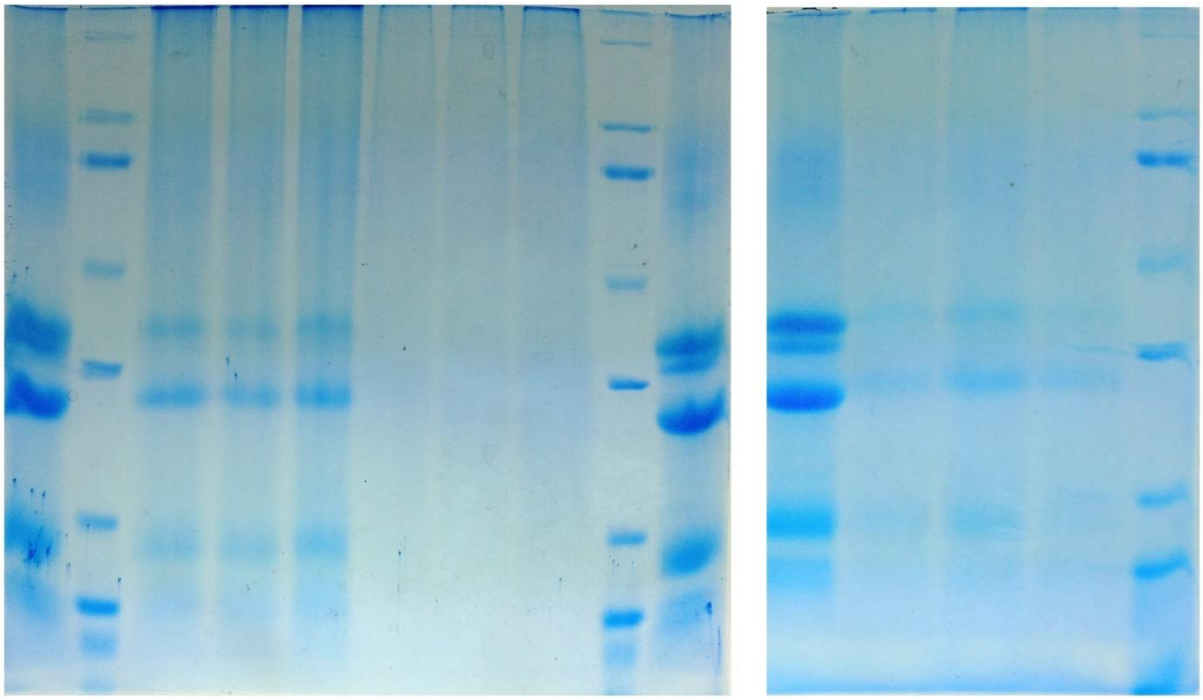


Fig. 4.7 SDS-PAGE of whey protein based conjugates. The labelled lanes are: 1, 10, 11- Control (WPC); 2, 9, 15- Broad range protein marker; 3- WPC-MD (1:2); 4- WPC-MD (1:1); 5- WPC (2:1); 6- WPC-HMP (1:2); 7- WPC-HMP (1:1); 8- WPC-HMP (2:1); 12- WPC-GA (1:2); 13-WPC-GA (1:1) and 14-WPC-GA (2:1).

4.2.5 Protein Solubility

Solubility is an important functional property for proteins, which affects other functionalities such as emulsifying properties and foaming. Protein solubility for WPC and WPC based conjugates as a function of pH from 3.0 to 7.0 is shown in Table 4.2. As the pH of WPC solution was adjusted near to the isoelectric of protein (pH 5.0), the net charge on the proteins was reduced, resulting in precipitation of WPC. It is observed from Table 4.2 that most of the whey protein based conjugates had significantly ($p \leq 0.05$) higher solubility than WPC around the isoelectric pH of whey proteins. The results indicate that conjugation provides a shielding effect against precipitation around isoelectric point of protein (Chevalier *et al*., 2001). As compared with WPC, the solubility of conjugates had a minimum value at pH 4 and increased highly at pH 5, which indicates that conjugation can highly improve the solubility of WPC and shift the minimum to a more acidic pH value. Conjugation shifted pI of WPC to lower pH, which might be caused due to reduction in positive charges and relative increase in net negative charge after covalent attachment of WPC with polysaccharides (Sun *et al*., 2011). WPC-HMP (1:2) conjugate had significantly ($p \leq 0.05$) high solubility over entire pH range of 3.0 to 7.0, compared to other conjugates. The possible explanation for this may be that whey proteins possess open and more reactive structure resulting from intramolecular electrostatic repulsion, making it more favourable at pH above pI, whereas, pectin is more stable in an acidic pH range, particularly at pH 5.8 (Einhorn-Stoll *et al*., 2005).

Based on the above results, it could be concluded that WPC-HMP (1:2) conjugate had the highest emulsifying properties and protein solubility over a range of 3.0 to 7.0 pH. Moreover, degree of glycation and SDS-PAGE confirmed the conjugation of WPC with HMP at the ratio 1:2. Hence, WPC-HMP (1:2) conjugate was selected as optimized combination for use as effective hydrophilic emulsifier in w/o/w double emulsion in further study.

Table 4.2 Protein solubility of whey protein based conjugates

	Protein Solubility (%)								
pH / Combi-nations	**3.0**	**3.5**	**4.0**	**4.5**	**5.0**	**5.5**	**6.0**	**6.5**	**7.0**
Control (WPC)	78.86±0.31^{eE}	78.61±0.59^{eF}	57.80±0.60^{eG}	48.74±0.32^{eF}	47.85±0.52^{eD}	69.76±0.83^{eD}	82.57±0.70^{eC}	83.89±0.48^{eB}	85.33±0.31^{eA}
WPC-MD (1:2)	77.52±1.36^{cE}	61.22±0.95^{cF}	51.52±1.38^{cG}	84.37±0.48^{cF}	82.28±0.96^{cD}	86.72±1.03^{cD}	95.15±0.50^{cC}	92.73±0.47^{cB}	89.37±0.87^{cA}
WPC-MD (1:1)	55.78±0.88^{dE}	48.82±0.96^{dF}	44.74±1.10^{dG}	52.71±0.42^{dF}	84.03±1.05^{dD}	87.38±1.14^{dD}	83.33±0.68^{dC}	92.43±0.39^{dB}	94.60±0.29^{dA}
WPC-MD (2:1)	89.35±0.83^{bE}	83.80±1.14^{bF}	94.07±0.45^{bG}	95.84±0.25^{bF}	92.51±0.77^{bD}	73.58±0.95^{bD}	84.30±1.16^{bC}	73.42±0.76^{bB}	72.21±0.73^{bA}
WPC-HMP (1:2)	94.74±0.49^{aE}	92.44±0.80^{aF}	79.72±0.99^{aG}	78.96±0.65^{aF}	77.78±0.51^{aD}	78.24±0.47^{aD}	83.74±0.49^{aC}	88.37±0.35^{aB}	93.60±0.51^{aA}
WPC-HMP (1:1)	16.62±0.65^{hE}	7.30±0.35^{hF}	7.70±0.27^{hG}	7.39±0.44^{hF}	15.79±0.58^{hD}	35.74±0.61^{hD}	77.52±0.31^{hC}	92.04±0.63^{hB}	92.65±0.72^{hA}
WPC-HMP (2:1)	71.68±0.77^{fE}	44.30±0.76^{fF}	33.24±0.47^{fG}	40.08±0.66^{fF}	72.65±0.84^{fD}	68.56±0.57^{fD}	81.52±1.05^{fC}	87.97±0.63^{fB}	86.04±0.48^{fA}
WPC-GA (1:2)	42.36±0.93^{gE}	43.83±1.03^{gF}	42.08±0.83^{gG}	58.24±0.74^{gF}	62.21±0.85^{gD}	41.56±0.40^{gD}	45.62±0.73^{gC}	48.50±0.64^{gB}	48.98±0.81^{gA}
WPC-GA (1:1)	59.04±1.05^{eE}	56.14±1.02^{eF}	56.85±0.77^{eG}	52.53±0.50^{eF}	69.25±1.08^{eD}	75.86±1.19^{eD}	85.14±0.72^{eC}	87.53±0.92^{eB}	90.10±1.08^{eA}
WPC-GA (2:1)	62.27±0.79^{dE}	66.72±0.71^{dF}	62.80±0.75^{dG}	69.17±0.93^{dF}	77.74±0.68^{dD}	66.12±0.73^{dD}	73.09±0.78^{dC}	80.90±0.53^{dB}	81.86±0.60^{dA}

Values are Mean±Standard Error (n=3)

A, B, C - Means with different superscript in different columns differ significantly ($p \leq 0.05$)

a, b, c - Means with different superscript in different rows differ significantly ($p \leq 0.05$)

WPC – whey protein concentrate, MD – maltodextrin, HMP – high methoxyl pectin, GA – gum arabic

Table 4.3 Coded and actual levels of independent variables for w/o/w double emulsion formulation: RSM experiment (CCRD*)

Coded level / Variable	Axial point (min.)	Factorial point	Centre coordinate	Factorial point	Axial point (max.)
	-2	-1	0	1	2
PGPR	1	2	3	4	5
Salt	0	0.4	0.8	1.2	1.6
Conjugate	0.5	1.0	1.5	2.0	2.5
Speed	16000	18000	20000	22000	24000

*Central Composite Rotatable Design

4.3 Optimization of w/o/w double emulsion formulation: RSM experiment

An RSM experiment was framed on the CCRD (Central Composite Rotatable Design) with four factors viz., PGPR, salt, conjugate and speed at five different levels each (Table 4.3). The 30 experiments so generated by the Design expert software are shown in Table 4.4. The effect of formulation on each response is discussed below in detail.

4.3.1 Effect of formulation on Stability of double emulsion

The formulation of double emulsion can be considered as an important determinant of the stability of double emulsions in terms of zeta potential and sedimentation stability. Quadratic model and 2FI model for zeta potential and sedimentation stability respectively, were obtained through successive regression analysis. The data obtained for these properties with different formulations and dependence of these responses on the four factors (PGPR, salt, conjugate, and speed) are presented in Tables 4.5, 4.6 and 4.7.

Table 4.4 Design matrix of Response Surface Experiment (CCRD) with four independent variables

Run	Independent variables				Coefficient assessed by
	Factor 1	Factor 2	Factor 3	Factor 4	
	PGPR (%)	Salt (%)	Conjugate (%)	Speed (rpm)	
1	4	0.4	1.0	18000	Factorial
2	2	1.2	2.0	22000	Factorial
3	3	0.8	1.5	20000	Centre
4	3	0.8	1.5	20000	Centre
5	3	0.8	1.5	20000	Centre
6	5	0.8	1.5	20000	Axial
7	4	0.4	2.0	18000	Factorial
8	3	0.8	1.5	24000	Axial
9	2	1.2	1.0	22000	Factorial
10	3	0.8	0.5	20000	Axial
11	3	0.8	1.5	16000	Axial
12	4	1.2	2.0	18000	Factorial
13	2	0.4	1.0	22000	Factorial
14	2	0.4	2.0	22000	Factorial
15	1	0.8	1.5	20000	Axial
16	3	0.8	1.5	20000	Centre
17	4	1.2	2.0	22000	Factorial
18	3	0.8	1.5	20000	Centre
19	2	1.2	2.0	18000	Factorial
20	3	0.8	1.5	20000	Centre
21	2	0.4	1.0	18000	Factorial
22	4	1.2	1.0	18000	Factorial
23	3	1.6	1.5	20000	Axial
24	3	0.8	2.5	20000	Axial
25	3	0	1.5	20000	Axial
26	4	0.4	1.0	22000	Factorial
27	4	0.4	2.0	22000	Factorial
28	4	1.2	1.0	22000	Factorial
29	2	0.4	2.0	18000	Factorial
30	2	1.2	1.0	18000	Factorial

Table 4.5 Effect of formulation on stability of w/o/w double emulsion: RSM experiment

Run	Independent variables				Dependent variables		
	PGPR (%)	Salt (%)	Conjugate (%)	Speed (rpm)	Zeta Potential (mV)	Sedimentation Stability at 7°C (%)	Sedimentation Stability at 37°C (%)
1	4	0.4	1.0	18000	-26.8	94.16	81.67
2	2	1.2	2.0	22000	-32.1	56.67	46.67
3	3	0.8	1.5	20000	-29.4	80.83	61.67
4	3	0.8	1.5	20000	-29.0	80.83	61.67
5	3	0.8	1.5	20000	-29.2	80.83	61.67
6	5	0.8	1.5	20000	-28.1	100.00	100.00
7	4	0.4	2.0	18000	-32.0	100.00	72.50
8	3	0.8	1.5	24000	-29.7	95.00	85.83
9	2	1.2	1.0	22000	-27.1	53.33	44.17
10	3	0.8	0.5	20000	-25.9	78.33	59.17
11	3	0.8	1.5	16000	-27.9	69.17	52.50
12	4	1.2	2.0	18000	-31.8	100.00	72.50
13	2	0.4	1.0	22000	-27.2	51.67	44.17
14	2	0.4	2.0	22000	-32.3	56.67	46.67
15	1	0.8	1.5	20000	-27.2	38.33	31.67
16	3	0.8	1.5	20000	-29.8	80.83	61.67
17	4	1.2	2.0	22000	-32.7	96.67	82.50
18	3	0.8	1.5	20000	-29.5	80.83	61.67
19	2	1.2	2.0	18000	-31.3	95.00	88.33
20	3	0.8	1.5	20000	-28.9	80.83	61.67
21	2	0.4	1.0	18000	-26.4	57.50	46.67
22	4	1.2	1.0	18000	-26.7	83.33	72.50
23	3	1.6	1.5	20000	-28.6	83.33	57.50
24	3	0.8	2.5	20000	-33.9	93.33	64.17
25	3	0.0	1.5	20000	-29.0	95.00	77.50
26	4	0.4	1.0	22000	-27.8	100.00	90.00
27	4	0.4	2.0	22000	-33.0	100.00	100.00
28	4	1.2	1.0	22000	-27.6	93.33	73.33
29	2	0.4	2.0	18000	-31.7	100.00	75.83
30	2	1.2	1.0	18000	-26.2	49.16	40.00

4.3.1.1 Zeta Potential

Zeta potential is one of the fundamental parameters known to affect the stability of double emulsions through charge repulsion between the particles. Emulsions with zeta potential greater than absolute value of 25 mV are normally considered stable. The higher the zeta potential, the higher will be the stability of particles against flocculation because of greater prevalence of repulsive forces on its surface. Zeta potential of the w/o/w double emulsion ranged from -25.9 to -33.9 (Table 4.5). Maximum zeta potential was observed in the double emulsion with formulation comprising of 3% PGPR, 0.8% salt, 2.5% conjugate and 20000 rpm speed, while minimum zeta potential was observed with formulation of 3% PGPR, 0.8% salt, 0.5% conjugate and 20000 rpm speed. The coefficient estimates of zeta potential model (Table 4.6) showed that conjugates and speed significantly ($p \leq 0.01$) increased the zeta potential of the emulsion, the increase being linear in both the cases. The interaction between conjugates and speed indicated that increasing both the levels significantly ($p \leq 0.05$) increased the zeta potential of the double emulsion. At the quadratic level, only salt was significant ($p \leq 0.05$), indicating that zeta potential increased with decreasing the level of salt in double emulsion (Fig. 4.8).

The results were in agreement with Kumar (2011) who showed that zeta potential increased with the decrease in particle size attributed to the increase in pressure of microfluidization. Higher zeta potential could also be associated with the higher degree of repulsion between adjacent similarly charged particles caused by increasing the concentration of conjugate in the emulsion, preventing aggregation and flocculation.

The regression analysis of data presented in Table 4.6 shows that the coefficient of determination (R^2) was 0.96 ($p \leq 0.01$) and the 'lack of fit test' was not significant, indicating that the model is sufficiently accurate for predicting the zeta potential of w/o/w double emulsion made with any combination of the factors level within the range evaluated. The adequate precision was 22.502, appreciably higher than the minimum desirable i.e. 4 (for high prediction ability).

Zeta potential in the double emulsion could be predicted by the equation (for actual factors) given below:

Zeta Potential = -18.05417 -1.49583*PGPR +0.23958*Salt -1.90833*Conjugates -1.64583E-004*Speed -0.015625*PGPR*Salt -0.012500*PGPR*Conjugates -2.18750E-005*PGPR*Speed +0.15625*Salt*Conjugates -7.81250E-006*Salt*Speed +1.87500E-005*Conjugates*Speed +0.28646*$PGPR^2$ -6.51042E-003*$Salt^2$ -1.10417*$Conjugates^2$ -2.60417E-010*$Speed^2$

Table 4.6 Regression coefficients and ANOVA of fitted Quadratic model for Zeta Potential w/o/w double emulsion (CCRD)

Factor	Zeta Potential
Intercept	-29.30
PGPR (A)	-0.25^{ns}
Salt (B)	0.1^{ns}
Conjugates (C)	-2.38**
Speed (D)	-0.44**
AB	-0.0063^{ns}
AC	-0.0063^{ns}
AD	-0.044^{ns}
BC	0.031^{ns}
BD	-0.0063^{ns}
CD	0.019*
A^2	0.29^{ns}
B^2	-0.001*
C^2	-0.28^{ns}
D^2	-0.0010^{ns}
R^2	0.96^{ns}
Model F-value	29.41**
Adequate Precision	22.502
Lack of Fit	4.29^{ns}

**Highly significant ($p \leq 0.01$); *Significant ($p \leq 0.05$); ^{ns} Not significant ($p > 0.05$)

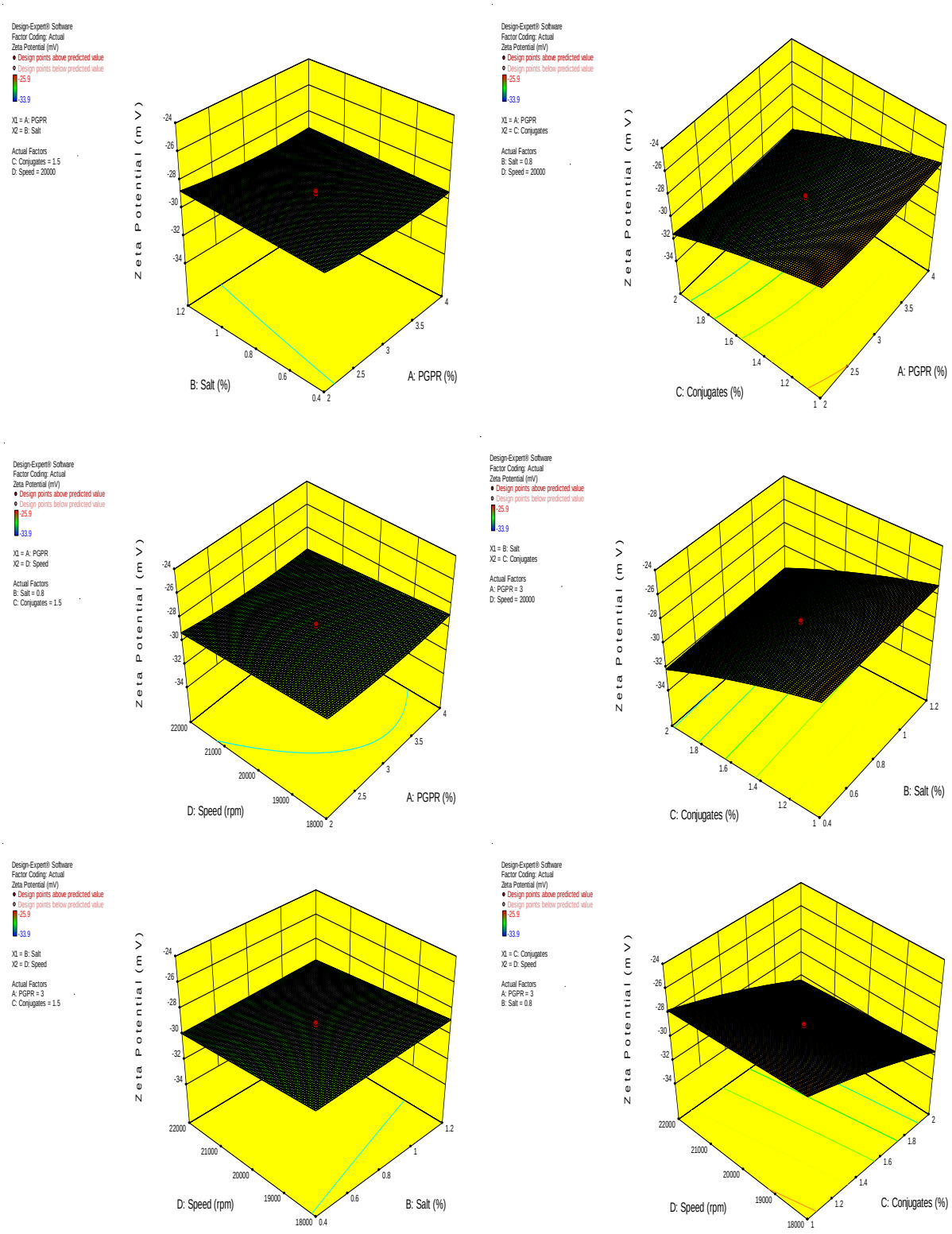


Fig. 4.8 Response surface plots of Zeta Potential as influenced by the levels of PGPR, Salt, Conjugate and Speed

4.3.1.2 Sedimentation Stability

Sedimentation stability is important in determining the stability of emulsion, and was measured as the ratio of the emulsion phase volume to the total volume of the emulsion. Sedimentation stability of the emulsion was measured after storage of the emulsion at 7°C and 37°C temperature for one month. The sedimentation stability of the emulsion as a function of temperature is discussed further.

Table 4.7 Regression coefficients and ANOVA of fitted 2FI model for Sedimentation Stability of w/o/w double emulsion at 7°C and 37°C

Particulars	Sedimentation Stability at 7°C	Sedimentation Stability at 37°C
Intercept	80.83	65.86
PGPR (A)	15.45**	14.55**
Salt (B)	-2.33^{ns}	-3.23 ^{ns}
Conjugates (C)	6.36**	4.27*
Speed (D)	-0.80 ^{ns}	1.84 ^{ns}
AB	-0.57 ^{ns}	-3.07 ^{ns}
AC	-4.43 ^{ns}	-4.53 ^{ns}
AD	5.99*	7.24**
BC	0.99 ^{ns}	1.72 ^{ns}
BD	0.99 ^{ns}	-1.93 ^{ns}
CD	-6.20*	-2.76 ^{ns}
R^2	0.85	0.82
Model F-value	11.00**	8.48**
Adequate Precision	11.694	11.739
Lack of Fit	-	-

**Highly significant ($p \leq 0.01$); *Significant ($p \leq 0.05$); ^{ns} Not significant ($p > 0.05$)

4.3.1.2.1 Sedimentation stability at 7°C

Sedimentation stability of the w/o/w double emulsion at 7°C varied from 38.33% to 100% (Table 4.5). The highest stability at this temperature was observed in six different formulations as shown in Table 4.3, while the least stability was observed in formulation

comprising of 1% PGPR, 0.8% salt, 1.5% conjugate and 20000 rpm speed. The coefficient estimates for sedimentation stability at 7°C (Table 4.7) showed that PGPR and conjugates significantly ($p \leq 0.01$) increased the stability of double emulsion, while salt and speed had no significant effect on sedimentation stability. However, the interaction of speed with PGPR and conjugates showed a significant ($p \leq 0.05$) effect on the sedimentation stability. At lower PGPR levels, increasing the speed decreased the sedimentation stability to a smaller extent, while at higher levels of PGPR, the sedimentation stability increased with increasing speed. The opposite effect was observed on interaction of speed with conjugate but to a smaller extent (Fig. 4.9).

Su *et al.* (2006) reported that increase in viscosity of primary emulsion with increase in PGPR concentration, resulted in increased resistance to shearing during secondary emulsification and subsequent reduction in rate of coalescence of water droplets.

The reduction in viscosity of primary emulsion, due to high shear forces by increasing the speed, was the result of shear thinning behaviour of the primary emulsion. This was noticed practically resulting in lower stability of w/o primary emulsion and subsequent w/o/w double emulsion.

The amount of primary emulsifier must be greater than that of secondary emulsifier. Due to the reduced size of water droplets in primary emulsion with increasing speed, PGPR might not be adsorbed sufficiently on the newly formed droplets. Some of the unadsorbed PGPR might have remained in micellar form in the oil phase which may have migrated to o/w interface of w/o/w double emulsion resulting in destabilization of the emulsion.

The regression analysis of data presented in Table 4.7 shows that the coefficient of determination (R^2) was 0.85 ($p \leq 0.01$), indicating that the model is sufficiently accurate for predicting the sedimentation stability of w/o/w double emulsion at 7°C made with any combination of the factors level within the range evaluated. The 'lack of fit test' was neither significant nor insignificant, because the pure error was zero due to the same value of sedimentation stability of all the centre points at 7°C. The adequate precision was 11.694, appreciably higher than the minimum desirable i.e. 4 (for high prediction ability).

The sedimentation stability of the double emulsion at 7°C could be predicted by the equation (for actual factors) given below:

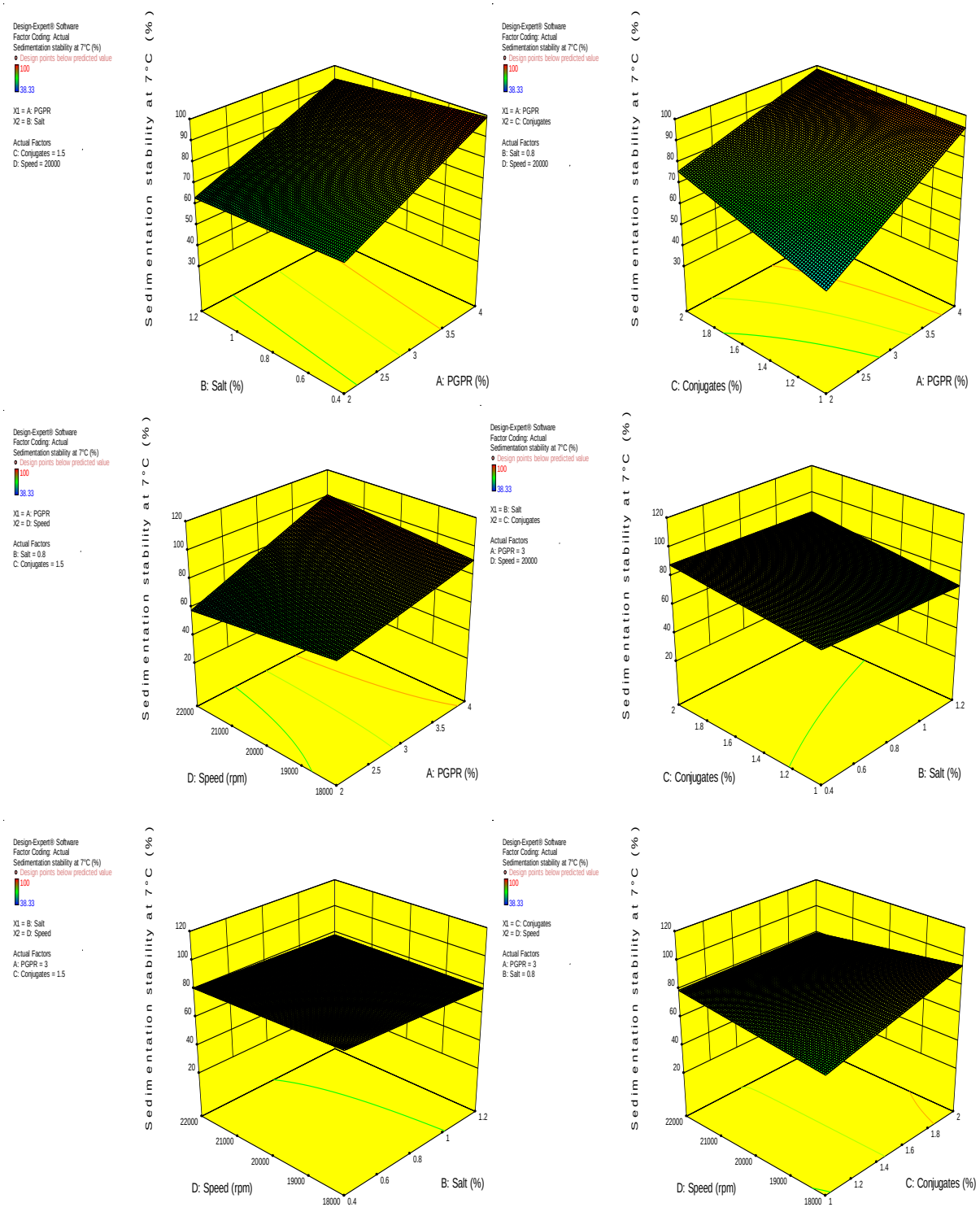


Fig. 4.9 Response surface plots of Sedimentation stability at 7°C as influenced by the levels of PGPR, Salt, Conjugate and Speed

Sedimentation stability at 7°C = 4.24096 -30.01813 * PGPR -33.69271 * Salt +159.27208 * Conjugates -1.07521E-003 * Speed -1.42969 * PGPR * Salt -8.85375 * PGPR * Conjugates +2.99469E-003 * PGPR * Speed +4.95313 * Salt * Conjugates +1.23672E-003 * Salt * Speed -6.19812E-003 * Conjugates * Speed

4.3.1.2.2 Sedimentation Stability at 37°C

Sedimentation stability of the w/o/w double emulsion at 37°C varied from 31.67% to 100% (Table 4.5). The highest stability at this temperature was observed in two formulations comprising of 5% PGPR, 0.8% salt, 1.5% conjugate and 20000 rpm speed, and 4% PGPR, 0.4% salt, 2% conjugate and 22000 rpm speed, whereas, the least sedimentation stability was observed in the formulation of 1% PGPR, 0.8% salt, 1.5% conjugate and 20000 rpm speed. The coefficient estimates for sedimentation stability at 37°C (Table 4.7) showed that PGPR ($p \leq 0.01$) and conjugates ($p \leq 0.05$) significantly increased the stability of double emulsion, while salt and speed had no significant effect on sedimentation stability at this temperature. The interaction of PGPR and speed revealed that, at lower PGPR levels, increasing the speed significantly ($p \leq 0.01$) decreased the sedimentation stability to a smaller extent, while a larger increase in sedimentation stability was observed by increasing the speed at higher levels of PGPR (Fig. 4.10).

The results are in agreement with Su *et al.* (2006), who reported that yields considerably decreased more in emulsions which were stored at 20°C, compared to emulsions stored at 5°C after storage for 4 weeks. At high PGPR concentrations (6 and 8% w/v), yield values remained high (>90%) after storage for 4 weeks at either 5 or 20°C. Further, they reported that higher temperature reduced the viscosity of the oil phase, thereby increasing the rate of coalescence and expulsion of the internal water droplets. In addition, the increase in Brownian motion at higher temperature, increases the rate of flocculation leading to further instability in the double emulsions.

Matos *et al*. (2013) studied the effect of PGPR content on stability of emulsion during storage at room temperature and found that the sedimentation of the aqueous W_1 droplets was less pronounced at higher PGPR concentrations (5%) because of their smaller droplet size and higher viscosity of the oil phase with higher levels of dissolved PGPR polymer.

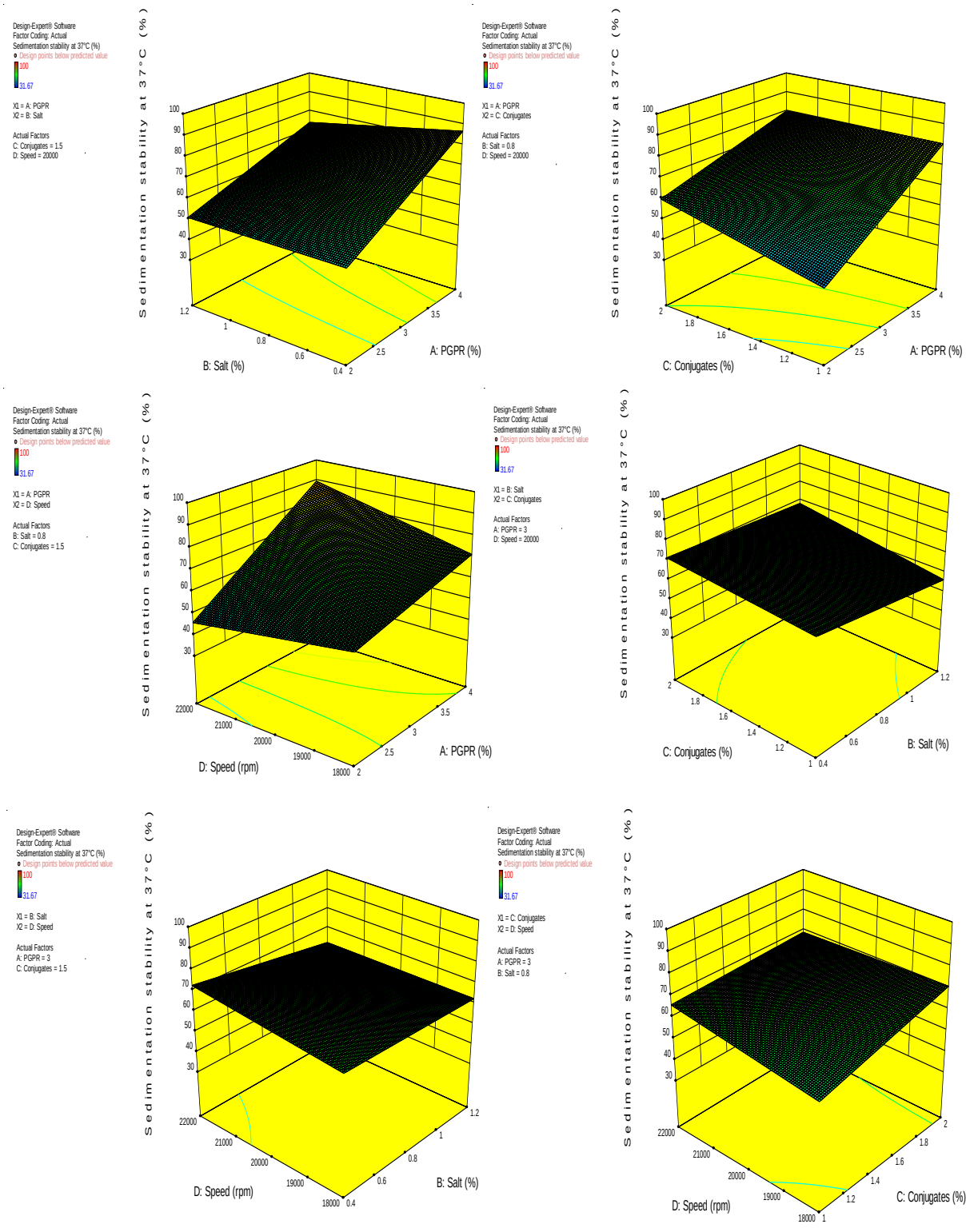


Fig. 4.10 Response surface plots of Sedimentation stability at 37°C as influenced by the levels of PGPR, Salt, Conjugate and Speed

The regression analysis of data presented in Table 4.7 shows that the coefficient of determination (R^2) was 0.82 ($p \leq 0.01$), indicating that the model is sufficiently accurate for predicting the sedimentation stability of w/o/w double emulsion at 37°C made with any combination of the factors level within the range evaluated. The 'lack of fit test' was neither significant nor insignificant, because the pure error was zero due to the same value of sedimentation stability of all the centre points at 37°C. The adequate precision was 11.739, appreciably higher than the minimum desirable i.e. 4 (for high prediction ability).

The sedimentation stability of the double emulsion at 37°C could be predicted by the equation (for actual factors) given below:

Sedimentation stability at 37°C = 44.38921 -38.09521 * PGPR +50.25104 * Salt +84.03458 * Conjugates -3.87104E-003 * Speed -7.68281 * PGPR * Salt -9.06125 * PGPR * Conjugates +3.61906E-003 * PGPR * Speed +8.59688 * Salt * Conjugates -2.40859E-003 * Salt * Speed -2.75937E-003 * Conjugates * Speed

4.3.2 Numerical optimization of w/o/w double emulsion

Based on the characteristics of w/o/w double emulsion obtained with different levels of PGPR, salt, conjugate and speed, optimization was done using Design Expert 9.0.4.1 package to have a highly desirable solution. The main aim was to obtain the best possible combination of the four factors that would result in maximum stability of double emulsion.

The suggested formulations for optimal w/o/w double emulsion were obtained using the 'numerical optimize' command of the Design Expert software. Among the solutions obtained, the one with highest 'desirability' was selected as the optimum solution and was verified by using the suggested levels of PGPR, salt, conjugate and speed in the formulation for actual preparation of w/o/w emulsion and comparing the predicted values with the observed values.

The goals of all the dependent variables were set for optimization purpose keeping 'zeta potential' at 'minimum', and 'sedimentation stability' at 7°C and 37°C 'targeted to 100%', whereas all the independent variables were 'kept in range'. The criteria for numerical

optimization and the optimized solution thus obtained are presented in Table 4.8 and 4.9 respectively.

W/O/W double emulsion was prepared using the first solution from the set of solutions obtained (Table 4.9) and was evaluated for all the responses. The optimized solution obtained had 4% PGPR, 0.4% salt, 2% conjugate and 22000 rpm speed. The validation of the optimized solution was carried out by applying paired t-test to compare the predicted and observed values. It is clear from the Table 4.10 that there was no significant ($p>0.05$) difference between the predicted and observed values for all the responses of the optimized solution.

Table 4.8 Criteria for RSM constraints to optimize the formulation of w/o/w double emulsion

Variables	Goal	Lower limit	Upper limit	Importance
PGPR (%)	In range	2	4	3
Salt (%)	In range	0.4	1.2	3
Conjugates (%)	In range	1	2	3
Speed (rpm)	In range	18000	22000	3
Zeta potential (mV)	Minimize	-33.9	-25.9	2
Sedimentation stability at 7°C (%)	Target	-	100	4
Sedimentation stability at 37°C (%)	Target	-	100	4

Table 4.9 Optimized solutions for w/o/w double emulsion from RSM analysis

Number	Suggested formulation				Desirability
	PGPR (%)	Salt (%)	Conjugate (%)	Speed (rpm)	
1	4.000	0.400	2.000	22000.000	0.910
2	4.000	0.400	1.993	21998.115	0.910
3	4.000	0.404	2.000	21992.325	0.910

Table 4.10 Comparison of predicted and observed values of the responses to validate the optimized solution

Responses	Predicted Values	Observed Values[#]	t_{cal}
Zeta potential (mV)	-32.5	-32.7±0.20	0.34^{ns}
Sedimentation stability at 7°C (%)	98.12	100±0.00	-0.37^{ns}
Sedimentation stability at 37°C (%)	92.98	100±0.00	-1.30^{ns}

[#]Values are Mean±Standard Error (n=3)
[ns]Non-significant (p>0.05)
t_{tab} (one-tail) = 2.92
t_{tab} (two-tail) = 4.30

Thus, based on the results, optimized formulation comprising of 4% PGPR, 0.4% salt, 2% conjugate and 22,000 rpm speed was found to be suitable for producing stable W/O/W double emulsion using protein-polysaccharide conjugates as emulsifiers. The formulation obtained during the present study can be used as potential delivery system for delivery of herbal as well as non-herbal bioactive molecules in dairy and food products.

Chapter-5

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

The drastic change in human lifestyle due to industrialization and changing work cultures have led to increased incidence of lifestyle related and other degenerative diseases over the past few decades. People have moved to better alternatives such as nutraceuticals over pharmaceuticals with increasing health awareness, shift towards preventive health care and increased regulatory clarity in food safety and standards. Herbs have been used as food and as medicine for centuries. *Brahmi* has been used in the Ayurvedic system of medicine for centuries to improve memory and intellect. The present study was conducted to formulate stable W/O/W double emulsion containing *brahmi* extract. Due to the instability problems of double emulsions, whey protein based conjugates were used to improve the stability of w/o/w double emulsion. The salient findings of the study are presented in this chapter.

The present study was conducted in phases following a systematic approach to cover the following aspects:

1) Preliminary studies for selection of incubation conditions for conjugation process
2) Characterization of whey protein based conjugates for their efficacy to provide stable emulsions
3) Optimization of w/o/w double emulsion formulation using Response Surface Methodology to obtain maximum stability.

5.1 Preliminary studies for selection of incubation conditions for conjugation process

The freeze dried mixtures of whey protein concentrate and polysaccharide were kept at two different incubation conditions 80°C/79% RH for 2 h and 60°C/80% RH for 10 days to induce conjugation. SDS-PAGE analysis confirmed that conjugation has occurred in case of incubation at low temperature-long time, whereas slight denaturation was noticed in high temperature short time incubation. Therefore, 60°C/80% RH for 10 days incubation was selected for further studies.

5.2 Characterization of whey protein based conjugates

Emulsifying properties of whey protein concentrate (WPC) increased significantly upon conjugation with polysaccharides. The conjugate of WPC with high methoxyl pectin (HMP)

at weight ratio of 1:2 showed the highest emulsifying properties (84.44% emulsifying activity and 78.33% emulsion stability) of all the other conjugates and WPC alone.

Colour intensity of whey protein increased significantly upon conjugation with polysaccharides. The conjugate of WPC with gum arabic (GA) at the weight ratio of 2:1 had significantly higher colour intensity compared to other conjugates and WPC alone, whereas WPC-HMP conjugates had lower colour intensity. Colour intensity increased by increasing the ratio of whey protein/polysaccharide due to lactose present in WPC which also added to the colour development through Maillard reaction.

SDS-PAGE analysis of all whey protein based conjugates showed slight disappearance in the characteristic whey protein band pattern in all conjugate samples. This confirmed that covalent linkage between whey protein and polysaccharides has occurred in all the conjugates.

Protein solubility of WPC also increased significantly upon conjugation with polysaccharides over a pH range of 3-7. All the whey protein based conjugates had higher protein solubility near the iso-electric pH of whey proteins, except WPC-HMP (1:1) conjugate. WPC-HMP (1:2) conjugate showed the highest solubility of protein over the entire pH range of 3-7.

WPC-HMP (1:2) conjugate with highest emulsifying properties and protein solubility was selected for effective use as hydrophilic emulsifier to produce stable w/o/w double emulsion.

5.3 Optimization of formulation of w/o/w double emulsion

A 4-factor, 5-level Central Composite Rotatable Design (CCRD) of RSM was adopted for optimization purpose using 2-4% PGPR, 0.4-1.2% salt, 1-2% conjugate and 18000-20000 rpm speed. The formulations generated by the design expert software were examined for physico-chemical and physical parameters such as zeta potential and sedimentation stability respectively as responses. The results obtained were analyzed using Design Expert software.

The suggested quadratic and 2FI regression models for various responses and their diagnostic check revealed that all important model parameters (responses) were satisfactory and could be accurately predicted by the four factors (independent variables).

Zeta potential of the W/O/W double emulsion increased with increasing levels of conjugates and speed. This phenomenon was evident with the individual parameters as well as through their interaction.

Sedimentation stability at both 7°C and 37°C increased significantly by increasing the level of PGPR and conjugate. The interaction effect of PGPR and speed showed significant positive effect on sedimentation stability at both temperatures, whereas, the interaction of conjugate and speed showed significant positive effect only on sedimentation stability at 7°C.

5.3.1 Numerical optimization of the formulation

In order to optimize the w/o/w double emulsion formulation, sedimentation stability was targeted to 100%, giving it more importance. The optimized formulation comprising of 4% PGPR, 0.4% salt, 2% conjugate and 22000 rpm speed had the highest desirability. The predicted and observed values of the optimized formulation were compared by using t-test with equal variance. The difference between predicted and observed values was non-significant for all responses.

In the present study, stable W/O/W double emulsion were formed with higher PGPR level along with higher conjugates and higher speed of primary emulsification. Hence, it can be concluded that PGPR (hydrophobic emulsifier) along with whey protein based conjugates (hydrophilic emulsifier) can produce stable W/O emulsions and subsequently W/O/W emulsions, respectively through higher viscosity of the continuous phase provided by both emulsifiers. The optimized formulation obtained during the present study can be used as potential delivery system for delivery of *brahmi* in dairy and food products.

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APPENDICES

Appendix-I

ANOVA of emulsifying, physical and physico-chemical properties of whey protein based conjugates

Dependent Variable		**Sum of Squares**	**df**	**Mean Square**	**F-value**
Emulsifying Activity (%)	Between Groups	3702.367	9	411.374	890.699*
	Within Groups	9.237	20	0.462	
	Total	3711.604	29		
Emulsion Stability (%)	Between Groups	2092.258	9	232.473	834.675*
	Within Groups	5.570	20	0.279	
	Total	2097.828	29		
Colour intensity	Between Groups	815.569	9	90.619	12306.349*
	Within Groups	0.147	20	0.007	
	Total	815.717	29		
Degree of Glycation (Abs at 335nm)	Between Groups	0.084	9	0.009	20.239*
	Within Groups	0.009	20	0.000	
	Total	0.094	29		

*Significant ($p \leq 0.05$)

Appendix-II

ANOVA of protein solubility of whey protein based conjugates

Source	Type III Sum of Squares	df	Mean Square	F-value
Corrected Model	129162.547	89	1451.265	823.628*
Intercept	1269136.415	1	1269136.415	720265.597*
Combinations	53310.308	9	5923.368	3361.654*
pH	29775.122	8	3721.890	2112.263*
Combinations * pH	46077.117	72	639.960	363.193*
Error	317.167	180	1.762	
Total	1398616.129	270		
Corrected Total	129479.714	269		

*Significant ($p \leq 0.05$)