DEVELOPMENT AND EVALUATION OF PROTEIN-RICH FRUIT BASED BEVERAGES

Thesis

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By

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CERTIFICATE

This is to certify that the thesis entitled **"DEVELOPMENT AND EVALUATION OF PROTEIN-RICH FRUIT BASED BEVERAGES"** submitted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** with major in **Food Technology** minor in **Process and Food Engineering** of the College of Post-graduate Studies, G.B.Pant University of Agriculture and Technology, Pantnagar, is a record of *bona fide* research carried out by **Mr. Ashish Kumar Singh**, **Id. No.21348** under my supervision and no part of the thesis has been submitted for any degree or diploma.

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07.8.02.

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We, the undersigned, members of the Advisory Committee of **Mr. Ashish Kumar Singh, Id.No. 21348**, a candidate for the degree of **Doctor of Philosophy** in **Food Technology** with minor in **Process and Food Engineering**, agree that the thesis entitled **"DEVELOPMENT AND EVALUATION OF PROTEIN-RICH FRUIT BASED BEVERAGES"** may be submitted in partial fulfillment of the requirements for the degree.

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Introduction

1.INTRODUCTION

Beverages are an integral part of human diet. The cycle starts with the infant formulas- highly complex drink, rich in many key nutrients. As human ages and their nutritional requirements change, product designer keeps pace by developing new and innovative beverages to meet these needs. In India, traditional cuisine includes drinks, which were developed primarily to provide aesthetic appeal, though they also contained certain components having nutritional and therapeutic values. In the course of time these traditional health drinks vanished and for a long period the Indian beverage industry was dominated by aerated synthetic drinks. However, the situation has changed dramatically, the aerated soft drinks, which had registered a whopping 20% growth during late 90's, could manage its present share in market against possible slide. In contrary to this last few years have witnessed a significant development in fruit based beverages. Newly introduced fruit beverages fall into the category of functional foods or neutraceuticals. Energy drinks, isotonic (sport) beverages, herbal and green teas, fortified waters, caffienatted drinks, and recreational soft drinks are some of the functional beverages, which have gained popularity in recent years.

Fruit juices are excellent source of carbohydrates, vitamins, and minerals but they lack in certain nutrients like proteins and quality fats. Hence, they are not considered as nutritionally rich and have to compete with others as thirst quencher in the market. Growing health consciousness among the

consumers, availability of new flavours and blends, innovations into packaging and other technological developments are expected to push up the per capita consumption of fruit based beverages (Epeson and Bhowmik, 1992). Fortification of fruit juices with proteins may increase their nutritive value and consumer appeal. A number of sources namely, vegetable proteins, milk proteins, their derivatives and hydrolyzates may serve the purpose. Among them, whey proteins are capable of promoting rapid growth of infants and children due to their high protein efficiency ratio (PER of 3.0).They also support the maintenance of body tissues among adults, as they possess high protein digestibility corrected amino acid score (PDCAAS) (Sharma *et al.*, 1998; Sizer and Whitney, 1994).

In late 90's India was the top producer of milk in the world. This has accelerated the pace of value addition in dairy sector, and now a wide variety of products like cheese, casein and indigenous coagulated dairy products are being produced in larger quantity. Large quantity of milk is being utilized for the production of these products, generating substantial quantity of whey as a by-product. According to an estimate more than, 80,000 tonnes of cheese whey is produced annually to India. This whey contains about 5000 tonnes of valuable nutrients including lactose and whey proteins. Their utilization as by - product will increase the value of milk and reduce environmental problems arising out of its disposal.

Concerted efforts have been made for utilizing whey in preparation of products such as drinks, soups. Individual whey protein fractions and lactose have been isolated and purified for food and pharmaceutical industries. Biological role of whey proteins and other minor components, present in whey is well documented. Its role in prevention of cancer, coronary heart diseases, enhancement of natural antioxidant level had made whey proteins as an essential component in diet therapy. But demand for lactose and whey proteins is limited and there are cyclical variations in their price. Production of lactic acid and vinegar is not economical due to the low amount of fermentable substances (Nelson *et al.*, 1972). Maximizing the recovery of available whey solids has been considered alternative for whey management. Whey solids may be utilized in production of beverages, which are light, refreshing, healthful and nutritious. Moreover, whey proteins have a unique solubility profile at acidic pH, where most other proteins are insoluble (Jelen and Buccheim, 1984).

Utilization of whey proteins in fortifying fruit juices or beverages has vast potential. One of the major problem encountered in this direction is large variations observed in the composition and functional properties of most of the commercially available whey protein preparations. In addition to this, certain processing operations like forewarming and pasteurization during cheese or casein preparations, freezing or drying in whey protein concentrate (WPC) manufacturing process, cause partial denaturation of whey proteins. Solubility behaviour and thermal stability of these partially denatured whey proteins is

also not known. Initial studies indicated that whey proteins have poor thermal stability and formed gel under acidic conditions. To avoid sedimentation in these beverages various attempts had been made, but most of these methods were found to increase the cost of production. But 'colloidal stabilization' of whey proteins in acidic fruit beverage by applying appropriate hydrocolloid seems to be more economical. Complex formed between partially denatured whey proteins and hydrocolloids increases the functionality of these proteins over a wide range of pH and under various processing conditions. These techniques can be applied to fortify fruit juices with proteins and improve their nutritional and therapeutic values.

Citrus juices are compatible with whey and can be used for developing thirst quenching drinks. Sour varieties of citrus fruits specially mandarins, are not considered fit for table purpose. Hence their processing in the form of value added products, require utmost attention.

Revitalized interests among the consumers about the healthy image of indigenous minor fruits like "aonla", "phalsa", "bael" etc. has diverted the R&D efforts for development of new products based on them for domestic and overseas markets. Phytonutrients present in 'bael' have been known to improve normal metabolism of human being. Value addition for these fruits by developing protein-enriched beverage using whey proteins is an attractive opportunity, to boost their production and efficient utilization.

Information on utilization of whey proteins in fruit juice is scanty. A suitable processing technology with appropriate type of ingredients is essential for its successful commercial scale up. Further, no information is available on nature of spoilage of protein-rich fruit based beverage at elevated temperatures. Therefore the present investigations were undertaken with following specific objectives:

(i) To investigate the thermal behaviour of whey proteins obtained from freeze or spray drying process.

(ii) To investigate the effect of polysaccharides on the thermal stability of whey proteins in acidic fruit beverages.

(iii) To develop whey protein-enriched fruit based beverages using optimum level of ingredients.

(iv) To study the storage stability of fruit based whey protein-enriched beverage.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Nutritional Beverages

Most nutritional beverages fall into the category of functional foods or nutraceuticals. A number of fruit based beverages have been developed with specific nutrients in mind. They offer a ready and unique delivery system for proteins, vitamins, minerals, dietary fibres and other food ingredients (Sharma et al., 1998). Among the functional drinks protein based beverages such as in sports drinks or health promoting drinks (Jayaprakash and Bruenker, 1999) are becoming popular day by day. Protein and amino acid drinks are formulated to help muscle recover after exercise and to build muscle mass.

2.1.1 Protein-rich Beverages

Proteins are integral components of foods, both nutritionally and functionally (Giese, 1994). Commercially proteins are obtained from a range of animal and plant sources (Rakosky, 1988; Lawson, 1993). These sources include oilseed-processing wastes, animal proteins from slaughterhouse waste and whey proteins as by-products in dairy industry.

In 1970's popularization of soybean as protein ingredient led to the development of various products like snack foods, extruded products, dairy analogues and beverages. Initially a process was developed to prepare soy beverage base powder using full fat soy flour. It contained 33% protein, 30% fat and 28% carbohydrate and the product, when dispersed in appropriate

quantity of water, has a composition similar to cows milk. This product was developed primarily for children suffering from malnutrition in underdeveloped nations (Anon, 1970). But most of the soy beverages suffered from the problem of off-flavour during storage, mainly due to the activity of lipoxygenase (Cowan et al., 1973). To improve the flavour of the product and also to enhance the acceptability of the beverage, soy protein supplementation was attempted in fruit juices. But all these products suffered from the problem of sedimentation. One of the approach, which was attempted to increase the solubility and thermal stability of soy proteins, was hydrolysis of proteins. Hydrolysis of soy proteins with two commercial enzyme preparations namely amyloxizin P10x and Prototerrizin P10x, resulted in production of protein product with all essential amino acids and its flavour profile was similar to milk (Polyachenko, 1973). This product was added to enrich fruit products with proteins. Holsinger et al. (1974) developed a nutritional beverage base from soy products (full fat soy flour, soybean oil), sweet cheese whey, and corn syrup solids. They spray-dried the product and its nutritional quality was further improved by adding vitamin pre-mix. Kikkoman Shoya Co. Ltd. patented a process for the production of nutritious, transparent, acidic soft drinks (Japanese Patent, 1975). Insolubility of soy proteins at or below their isoelectric point was a major obstacle in the preparation of acidic or fruit juice based beverages. They subjected denatured, de-fatted soybeans to the action of acid protease to form soluble peptides and used the clear portion in the

preparation of beverages. But soluble peptides imparted bitterness to fruit beverages. Increasing bitterness of hydrolyzates has been correlated with the degree of hydrolysis and content of hydrophobic amino acids and release of carbonyl compounds bound to soy protein as a result of proteolysis. Hence, controlled hydrolysis with appropriate type of enzyme was important for manufacturing peptides. Olsen and Adler-Nissen (1979) produced alcalase (commercial proteolytic enzyme) hydrolyzed soyprotein concentrate by stepwise aqueous washing of defatted soy flour at pH 4.5. After 30 min the enzyme was inactivated by heating at 50°C and pH was adjusted to 4.0-4.2. It was the centrifuged or filtered and hydrolyzate was added directly to acidic fruit based beverages.

Morris (1982) developed a product named Calpro 70. It was a powdered composition containing soy protein concentrate and lecithin. The product dispersed readily in water and remained suspended in hot or cold beverages like fruit juices even at 70% concentration. Calpro 70 was reported to contain all essential amino acids and could substitute soy protein isolate (SPI) and caseinates in beverages. Another protein enriched fruit juice beverage was prepared by suspending a protein rich vegetable material, e.g., soy protein, in fruit juice and/or water, adding calcium salts at pH 3-3.5 and removing insoluble solids (U.K. Patent, 1982).

Patil and Gupta (1982) prepared protein rich beverage by blending whey and soy slurry in the ratio of 3:1. The beverage had 4% protein and 7% sugar.

The beverage with pineapple or strawberry flavour was liked most by the consumers. The beverage was spray dried successfully after adding the sugar and the powder was fortified with water-soluble vitamins and methionine.

Soymeal and sunflower seed meal (8:2 by weight) were subjected to successive extraction of soluble proteins with $Ca(OH)_2$ and water. It provided a base containing 84% protein (Taha *et al.*, 1986). This protein base was cream or gray coloured and could be used in the preparation of nutritious beverages.

Schenz and Trumbetas, (1986) observed that addition of 1-2% lecithin to soyprotein hydrolyzates improved mouth feel of RTS beverage and almost resembles to natural fruit juices, in consistency.

Singh *et al.* (1991) investigated the chemical composition and functional properties of jack fruit (*Artocarpus heterophyllus*) seed flour, a by-product, which contain moderate amount (16.3%) of proteins. These proteins are comprised mainly of albumins and globulins (56.4%) and in vitro digestibility of the protein was very high 89%. At isoelectric point (4.0 pH) about 20% proteins remained soluble. According to them, these proteins can be utilized in the formulation of acidic foods such as protein rich carbonated beverages.

Fruit juices are generally fortified with proteins from plant sources. But Vyas and Joshi (1982) developed, a new protein fortified beverage using apple juice and egg yolk. The beverage contained 90% apple juice, 10% egg yolk, had final T.S.S. of 15° Brix and acidity 0.34% as malic acid. In the same

study, egg white and whole egg were found to be unacceptable for protein fortification.

2.1.2 Milk based fruit juice beverage

Many milk based fruit juice beverages have been developed in order to increase the protein content of fruit juices. Some of the milk and fruit based products, popularly called shakes, are relished by consumers, both for their aesthetic appeal and nutritional quality. However, to achieve longer shelf life, acidification and thermal treatment are must (Pagote, 2001). Acidification of milk leads to the destabilization of milk proteins due to acidic conditions. It has been viewed as a major obstacle in developing milk based fruit juice beverages. Several approaches, therefore, have been adopted by different workers to develop a process for the manufacture of acidified milk based beverages. Doesburg and Vos (1959) developed a milk-fruit juice sour drink by mixing sugar, high ester pectin powder, and milk, allowing to age for 10 min, and adding fruit juice to bring down the pH within the range of 3-4.2 (optimum being 3.8). They pasteurized the product even at higher temperature without curdling. Stabilized milk-orange juice beverage was patented by Shenkenberg *et al.* (1971) in USA. The product was developed by a process in which a sugar-stabilizer mixture was added to milk at a temperature below 32.2^0 C (90°F) and the mixture was allowed to stand for 10 min. Orange juice was added to the mixture and the resulting milk orange juice mix was aged, pasteurized and homogenized. They also observed that CMC concentration was

critical from stability point of view. A refreshing and nutritious drink with a pH of less than 5.0 has been developed from a combination of milk and grapefruit juice. The formulation consisted of 56.8% milk, 38.0 % grapefruit juice, 5.0% sugar and 0.2% stabilizer. The preheated, homogenized (double stage) product was pasteurized at 76°C for 15 sec (Proffit and Moore, 1974).

Nishiyama (1976) patented a process for apple juice flavoured milk beverage. The apple-juice preparation (beverage base) contained 4.2-6.0% (w/v) sodium carboxymethyl cellulose, 10-15% (w/v) fruit juice, and 3.1-5.0% (w/v) acid. For product manufacture, 11.2% sugar, 30% milk, and 5.5% apple juice preparation was mixed, heated to 80°C and sterilized water was finally added to make up the volume. The finished product had a pH of 4.4.

Casein derivatives like calcium caseinate, calcium sodium caseinate are often used in nutritional and diet beverage, but their application is hampered in acidic fruit beverage (Marsili, 1993). Milk protein isolate or total milk protein (TMP), which contains about 90% protein, was unstable in fruit juice beverage at pH 4.0 because the presence of casein fraction. TMP is quite stable in meal replacer drinks of pH 6.0 to 7.0 (Cantor, 1997). Mango-milk beverage was developed by adding 15% milk with 30-40%mango pulp and adjusting the T.S.S. to 20°Brix with sugar. Four stabilizers, i.e., CMC, pectin, sodium, alginate, and starch were tried, but none of them were effective in improving quality of the product (Hassan and Ahmed, 1998).

2.1.2.1 Whey as base for fruit juice mixes

Use of cheese whey as a beverage in human nutrition, especially for therapeutic purpose can be traced back to 460 BC. Hippocrates, the legendary Greek physician, is reported to have prescribed whey for an assortment of human ailments. Wagner *et al.* (1975) reported that whey drinks could stabilize the osmolar system in the body and had a thirst quenching effect. Processing, physico- chemical and nutritional aspects of whey beverages have been well documented in the literature (Kosikowski, 1979; Mann, 1987; Krarvchenko, 1988; Gandhi, 1989; Driersen and Vanden berg, 1990, Jelen, 1992; Mann, 1994; Puranik, 1999).

The simplest product of ready-to-serve beverage type may be prepared by mixing an appropriate fruit juice or concentrate and minimally processed whey. Despite its relative simplicity, finding a successful flavour combination to mask the unpleasant whey taste is difficult. The whey flavour was observed to be most compatible with citrus flavour (Holsinger et al. 1978; Anon, 1978).

Development of whey beverages is an attractive possibility from the nutritional and therapeutic point of view. Several attempts have been made to increase protein content of whey based beverages. Fortification with soy proteins has been discussed in Sec 2.1.1.

Holsinger et al. (1973) added dried cottage cheese whey to concentrate soft drinks to improve their nutritional value without any detectable changes in their flavour or appearance. In another experiment, orange juice was combined

with dried cheese whey which contained 74 % protein by weight and had a bland flavour with high PER (3.1-3.2). The resultant beverage was found to have appearance and flavour similar to orange juice with a protein content approximately equal to that of milk. A British patent (1975) described a method for preparation of a concentrate with 40-50% whey proteins in dry matter and the product contained citric acid, natural orange flavour, sorbitol, sodium saccharin and colouring matter as other ingredients. Nazare et al., (1979) added dried cheese whey (12.06% protein) at 4.2-20.8% levels to passion fruit juice to obtain a final protein concentration of 0.5-2.5% in finished product. The product was processed at 85-90°C for 30 min, bottled and stored at room temperature upto 60 days. A refreshing beverage could be prepared by adding the concentrate with 2-3 volumes of water. Swedish Dairy Cooperative developed and marketed a protein rich fruit drink called "Nature's Wonder". It was produced by mixing a high-grade whey protein, hydrolyzed lactose and pineapple, orange and passion fruit juices (Hakansson, 1983).

Visco-de-Velez (1986) filed a patent for a process for preparing a nutritional drink from whey. This process consisted of concentration of whey 5-fold by ultrafilteration, inoculating of the retentate with 1-5% lactic acid bacteria and incubating at 35-50°C for 8-18 hours. Stabilizers and pasteurized cream were added to give 0.5-5% fat content in finished product. The product with or without fruit juice concentrates preservatives, and 7-12% sugar was heat treated to partially denature the soluble proteins and homogenized at 50-

300 kg/cm^2 and at 60-80°C. The drink had a shelf life of upto 45 days at refrigerated temperature

Bangert (1976) developed a nutritious orange drink concentrate of about 10% protein content by using whey protein concentrate. A whey protein enriched juice beverage was made from 19.48% orange juice concentrate, 5.85% sugar, 0.53% starch and 74% cheese whey protein concentrate (Anon, 1978). They reported a good shelf-life and were able to concentrate and dry the beverage. Benea and Contarelli (1984) worked on the development of a beverage based on frozen mandarin concentrate and milk proteins. They found whey proteins to be the most stable at low pH. The formulations contained 2.5 or 5% whey protein, 15% sucrose, 33% orange concentrate, and 0.2% guar meal. The spray-dried formulations showed better solubility, dispersibility and organoleptic quality than vacuum dried formulations. But the dried product did not have as good flavour as liquid formulation. Most consumers preferred pasteurized beverage to sterilized formulation. Whey protein fortified beverage was developed by Sharma et al. (1998) using 4% whey protein isolate, 3% hydrolyzed guar gum, 11% orange juice concentrate, 10% sucrose, 1.2% calcium gluconate and 0.2% citric acid. The pH of beverage was adjusted to 3.75 and other minor ingredients were also added. Beverage was processed in a pulsed electric field (PEF). The PEF treated samples were microbiologically less stable than conventional heat treatment.

2.1.2.2 Problems in processing of whey protein-enriched fruit based beverages

Major problems reported in whey beverages are turbidity and sedimentation after heat treatment and during subsequent storage. During thermal treatment whey proteins coagulate near their isoelectric point (at acidic pH) which causes development of turbidity and subsequent sedimentation in whey-fruit juice beverage (Gagrani *et al.*, 1987). Shekilango *et al.* (1997) observed that the other major problems often encountered in whey-fruit beverage are cloudiness and high viscosity. They are caused by the interaction between whey components (protein and calcium) and fruit components (pectin, tannins and starch).

According to Jelen (1992), such quality problems often lead to failure of these products in market. Several methods have been tried to minimise the problems. In one method whey was first passed through a centrifugal clarifier to remove residual fats and casein fines (Jelen *et al.,* 1987). Another approach adopted was to homogenize the product after pasteurization (Barabas and Albrecht, 1988). According to them whey drinks processed in this way were rated as homogenous with excellent flavour quality. De-proteination of whey (Holsinger *et al.*, 1974, Reddy *et al.*, 1987; Mathur *et al.*, 1988; Jayaprakasha *et al.*, 1986; Krishnaiah *et al.*, 1989) and protein coagulation by addition of 0.5% CMC and 0.08% pectin in UHT-sterilized product (D'yachenko and Suarez, 1984) were the other methods adopted to obtain sparkling clear beverages. Tuohy et al. (1988) reported that pH less than 4 were necessary to prevent

protein coagulation during pasteurization. Sharma et al. (1998) evaluated the feasibility of pulsed electric field (PEP) treatment, as an alternative to conventional heat treatment for to check/retard tendency of sedimentation. PEF treated protein fortified fruit beverages had less protein denaturation and no problem of sedimentation during storage but microbiologically less stable as compared to the heat-treated product and had shorter shelf- life.

2.2 Whey protein as a protein source

Whey proteins are globular, smaller in size, and heat denaturable. They are not coagulated by rennet or precipitated by acid. Whey proteins are one of the few proteins that are soluble at low ionic strength over the entire pH (2-8) range encountered in food applications (Jayaprakash and Brueckner, 1999). In undenatured from whey proteins remain soluble at their isoelectric point (Kinsella and Whitehead, 1989). The unique solubility of whey proteins enable them to be used in fruit juices, milk-based beverages, soy drinks, fermented dairy beverages, fruit yams, jellies, carbonated beverages, etc. (Hoogstraten, 1987; Mulvihil 1991). Whey proteins can be used in various forms in protein rich beverages such as concentrated whey, lactose hydrolyzed dried whey, UF-retentate, whey protein concentrates, whey protein isolates.

2.2.1 Nutritional and therapeutic properties of whey proteins

Dairy whey is a yellow-green liquid that results from the transformation of milk into cheese or casein or other coagulated dairy products. Whey contains 40-50% of total solids present in milk. It includes proteins, lactose,

mineral matters and water-soluble vitamins. Proteins can be separated from whey using ultrafilteration or diafilteration technologies and during this process low molecular weight compounds (lactose, non-protein nitrogen, vitamins and minerals) are removed from whey into the permeate. The remaining proteins are, in turn, concentrated in the retentate.

The permeate is used in the production of lactose, alcohol, single cell protein, galactose for yeast fermentation, glucose, cattle feed and a variety of pharmaceutical products. Whey proteins, in the form whey protein concentrate (WPC) and whey protein isolate (WPI) have high nutritional and functional properties and are capable of fulfilling the diverse attributes to satisfy different forms of utilization (de Wit, 1998).

2.2.2 Composition and quality of whey proteins

The major whey proteins are β-lactoglobulin and α-lactalbumin; minor whey proteins include proteose- peptones, blood proteins and lactoferrin (Table 2.1). All these proteins perform specific biological function in human body (Table 2.1).

Whey proteins are rich source of all essential amino-acids (Regester, 1996). Biological value (104) and protein digested corrected amino - acid score (PDCAS) of whey protein is 1.00 and therefore it superior to other dietary proteins. The sulphur containing amino-acids i.e. cysteine and methionine is also reported to be on the higher side than meat, soy and casein. Whey proteins provides more than 100% of the requirement for sulphur amino

Table 2.1: Whey proteins and their biological function

Sl. No.	Whey proteins	Concentration (g/l)	Percent	Biological activity
1.	Beta-lactoglobulin	2-4	50%	Resistant to acid and proteolytic enzymes. Act as a resistant carrier of retinol. Rich in Cystein.
2.	Alpha-lactalbumin	1-1.5	20%	Susceptible to low pH and pepsin in stomach. Biosynthesis of lactose. 41% protein in human milk is α-lactalbumin. Add to "humanize" products.
3.	Bovine serum albumin	0.1-0.4	5.7%	Help in absorption of fatty acids.
4.	Immunoglobulins	0.6-1.0	14%	Provide passive immunity
5.	Lactoperoxidase	0.03	0.5%	Nutraceutical component of milk and whey. Active against a number of enteric bacterial strains.
6.	Lactoferin (LF)	0.02	0.3%	Iron containing protein. Improve bioavailability of iron and show antibacterial properties.
7.	Protease - Peptones	0.6-1.2	10%	Not specific function. Glycomacropeptide (GMP) in cheese whey. Acts as prebiotic and immunomodulatory.

acids in the growing human being, whereas soy protein is limiting in these amino acids (Sanwar et al., 1985). Tryptophan, which acts as building block for niacin, is present in higher amount in whey proteins.

2.2.3 Therapeutic value of whey proteins

Dietary whey proteins have a number of putative, biological effects when ingested (Horton, 1995). The ability of whey proteins to increase the level of natural anti-oxidants within the body and possibly in stabilizing DNA during cell division, is emerging as premier contribution to population health (Bonous *et al.*, 1989). Growth factors present cheese whey have attracted increasing interest since Howarth and co-workers (1996) showed that oral administration of this extracted growth factors from cheese whey could reduce small bowl damage in methotrexate-treated rats. Some of the growth factors that have been identified in whey include: transforming growth factor (beta and basic fibroblast growth factors) and an inhibitor (mammary derived growth inhibitor) with an anticarcinogenic activity at extremely low concentrations (Steijns, 2001). These compounds may survive digestion in sufficient quantities to generate a physiological response. Experimental animals fed with four different foods (name them), as sources of protein, were administered with injection of the carcinogen- dimethylhydrazine. Whey protein-fed animals showed the lowest incidence of colon cancer (McIntosh *et al.*, 1995). Experiments in rodents indicate that the antitumor activity of the dairy products

lies with protein fraction and more specifically in the whey protein component of milk.

Possible modes of action maybe biochemical, including levels of sulphur containing peptide-glulathione, and the influence of protein on fat metabolites generated in gut, or immunological or a combination of both (Regester *et al.*, 1995). The anticarcinogenic properties of whey proteins are related to compounds rich in sulphur containing amino acids, methionine and cysteine. They contain γ– glutamyl-cysteine residue, which makes cysteine readily available for synthesis of glutathion, (γ– glutamyl- cysteinyl- glycine) a strong xenobiotic deactivating and anti-neoplastic agent (Parodi, 1998). Methionine is utilized for glutathion synthesis in times of cysteine deficiency and it also acts as methyl donor. Hypomethylation of a DNA is an important risk factor for cancer at number of sites. Glutathion is, believed to act as an antioxidant, anticarcinogenic and in stabilisation and repair of DNA. Evidences indicate that its level increased after receiving the whey protein diet (Bonous *et al.*, 1991; Bonous and Gold, 1991). The whey proteins act as precursor glutathion, this at least in part, explains the ability of whey protein diet to enhance humoral and cell-mediated immune responses in laboratory animals.

Another explanation that has been put forward for anticarcinogenic activity of whey proteins is its activity to folic acid, vitamin B_{12}, riboflavin, retinol and vitamin D (Parodi, 1998). Binding of iron by lactoferrin makes this

potential pro-carcinogenic unavailable for intestinal damage. Binding of vitamin B to proteins make them more bioavailable and protect them for being utilized by intestinal microorganisms.

Probiotic cultured milk products, have been demonstrated to lower the total and LDL cholesterol in experimental animals (Beena and Prasad, 1997). In another experiment, the serum total cholesterol level for the group of rat fed on whey protein concentrate containing milk fermented with both *Lacto bacillus casei* TMC 1543, was significantly lower than that of control group (Kawase *et al.*, 2000). They also conducted similar long-term intake (4weeks) trials with twenty healthy adult men and found a significant rise in HDL cholesterol level and decrease in triglyceride level. The atherogenic index for the fermented milk group decreased from 4.24 to 3.52. The systolic blood pressure was found to be lowered significantly. Whey proteins and peptides may act as prebiotics and they may be effective particularly in situations where host nutrition or intestinal competence is compromised.

The α-La contains 2-3 times more tryptophan than an average protein. In body, tryptophan is converted into 5-hydroxytryptophan and then to 3-hydroxytryptamine (sorotonin). Inadequate level of serotonin in the brain has been linked to depression, obesity, insomnia and chronic headache (Welzem, 2001). Whey protein isolates (WPI) has been used to treat HIV patients because immunoglobulins and bovine serum albumins present in it, may stave off this disease (Horton, 1995; Welzem, 2001).

2.2.4 Thermal denaturation of whey proteins

Whey proteins including possess well developed secondary and tertiary structures and therefore, they are susceptible to protein denaturation. (Rattray and Jelen, 1993). Tanford (1968) defined protein denaturation as a major change from the highly ordered native protein structure without breakage of primary covalent bonds in the peptide backbone. Denaturation of proteins may be caused by heat, freezing, pressure, extremes of pH, chaotrophic agents, urea, guandium chloride, sodium dodecyl sulphate and organic solvents such as ethanol and mercaptoethanol (Paulsson, 1990). These factors have a major impact on the functionality of whey proteins. Heat induced denaturation may have beneficial impact on some of the desired functionality such as gelation, whereas in liquid foods, sediment formation is viewed negative. The factors affecting whey protein denaturation are summerised here.

2.2.4.1 Temperature

deWit and Klarenbeck (1981) used Differential Scanning Colorimetry (DSC) and evaluate effect of temperature on denaturation by subjecting a 10% aqueous solution of individual whey proteins at pH 6.5 to a heating rate of 5K/min. The temperature at which endothermic heat uptake was maximum (T_d) was designated as denaturation temperature. However, considerable variations occur with other experiments, particularly involving more complex systems.

The thermal behaviour of whey and whey protein products is probably dominated by β-lactoglobulin (β-lg) which is the most abundant whey protein.

At room temperature (20°C) and pH 7.0, the β-lg exists in dynamic equilibrium between its dimeric and monomeric forms and very mild heating (> 30°C) initiates conversion of dimeric form to its monomeric form (IDF,1994). This transition further increases exposure of histidine, tyrosine and tryptophan residues to polar solvents like water (Townend *et al.*, 1969) and enhances thiol reactivity (Dunnill and Green, 1966). Upon further heating, i.e., above 40°C, the monomers undergo small reversible conformational changes (Kella and Kinsella, 1988) and continuous heating induces extensive and irreversible protein denaturation, which is regarded as classical denaturation step.

Earlier workers established that heat induced cleavage of hydrogen and hydrophobic bonds results in subsequent loss of secondary and tertiary structure and it also exposes the apolar residues and thiol groups to the aqueous environment (Dupont, 1965; Timasheff *et al.*, 1967). But DSC experiments yielded two endothermic peaks at ~ 80°C and 140°C for β-lg and it was accompanied by increased thiol reactivity. Intra and inter-molecular sulphdryl-disulphide interchange reactions led to the formation of an intermediate transition state, which temporarily restricted further protein unfolding. Further heating to ~ 140°C caused breakage of the stabilizing thiol bands and a second smaller endothermic peak was observed (de Wit, 1981; de Wit and Klarenbeck, 1981).

α-Lactalbumin is one of the most heat-sensitive milk proteins. At pH 6.7, this protein denatures at about 65°C. Despite its susceptibility to heat

denaturation, α-lactalbumin is quite resistant to heat coagulation (Shukla, 1973). According to a study, 80-90% reversible denaturation occurs during heating from 20 to 110°C (Ruegg *et al.*, 1977) and most probably it was manifested as high apparent thermal stability of α-1a, when loss of solubility in heated protein solution is determined. Renaturation of protein solution is believed to be owing to its small size and the presence of four disulphide bonds, which restricts the number of conformational changes that the protein can assume (Rattaray and Jelen, 1993). However, this reversibility decreases with increasing temperature and prolonged heating at 100°C for 10-30 min resulted in irreversible denaturation and aggregation (Chaplin and Lyster, 1986).

Thermal denaturation of bovine serum albumin (BSA) is a complex phenomenon, attributed to its higher molecular weight and numerous disulphide bonds. Sulphydryl- disulphide interchange reactions during heating are likely to occur, and it permitted the formation of numerous intermediates (Ruegg *et al.*, 1977; Hillar and Lyster, 1979). Thermal analysis revealed that T_d is in the range of 71-74°C (Bernal and Jelen, 1985).

Immunoglobulin fraction was reported to be most heat resistant among the whey proteins. A T_d of 72°C for IgG was reported by deWit and Klarenbeek (1984), whereas Ruegg *et al.* (1977) observed a single endothermic peak for γ-globulin at about 80°C, when sample heating rate was of 10°C/min.

Iron binding ability of lactoterrin molecules has important influence on its thermal behaviour. Iron-saturated lactoferrin exhibited a complex denaturation thermoprofile, with maximum heat uptake at 69°C and 83°C (Ruegg *et al.*, 1977) and at 74°C and 86.5°C (Sanchez *et al.,* 1992). Thermal denaturation of lactoferrin and immunoglobulins does not appear to be reversible.

Other minor whey proteins such as protease - peptone (pp) fraction exhibit high heat resistance probably due to their relatively small size and non-globular nature.

The apparent order of denaturation of individual whey proteins is Ig > BSA > β-lg > α-La > protease - peptone. Their rates of denaturation are strongly influenced by pH, ionic composition and the concentration of total solids (Morr and Ha, 1993).

2.2.4.2 pH

Whey proteins are susceptible to heat-induced denaturation and Ca^{+2} protein aggregation at alkaline pH, but their susceptibility to electrostatic aggregation and precipitation is maximum at pH 4.5-4.6 (Hidalgo and Gampler, 1977; Oysum and Alpkent, 1989). Varunsation *et al.* (1983) found that acid whey, which contains a higher concentration of Ca^{+2} and PO^{-3} than sweet whey, was more prone to heat denaturation.

Heat denatured α-La and β-lg interact by ionic and disulphide binding to form insoluble aggregates at pH 4.5 to 4.6. The reduced stability at higher pH

values has been attributed in part to increased thiol reactivity (Watanabe and Klostermeyer, 1976). But Harwalker (1979) found that the interaction activity of salt groups increased on the alkaline side of pH. Since such conditions caused a reduction of the electrostatic repulsion between heated protein molecules, consequently more denaturation was observed. The pH affected the distribution of electrostatic charges along the respective protein polypeptide chains, which control their tertiary conformational structures (Morr and Ha, 1993). Jelen and Buchheim (1984) demonstrated that whey protein solutions were highly heat stable below pH 3.9 and they concluded that stabilizing effect appeared to be due to higher heat resistance and not to acid - heat disruption of existing structure. The β-lg dimer reversibly dissociates below pH3.3 and the monomerization of native β-lg molecules is due to repulsive electrostatic forces developed as the pH is decreased (Swaisgood, 1982).

2.2.4.3 Compositional factors

Compositional factors such as presence of sugar, fatty acids, amount of cations influence the thermal behaviour of whey proteins.

2.2.4.3.1 Calcium ions

Calcium is present in an appreciable quantity in whey and whey products. However, their relative concentration differs because of the method of preparation. Ca^{+2} ions have been demonstrated to protect the tertiary structure of α-lactalbumin during thermal treatment (Hiroka *et al.*, 1980, Kronman *et al.*, 1981). Bernal and Jelen (1984) studied the role of ca^{+2} during

heating of model solutions of α-lactalbumin in the region of pH 2.5-6.5 by DSC. The model solutions simulated milk ultrafilterate. They found that chelation of calcium in model solution with 0.2 M EDTA solution reduced $T_d$by 20°C. However, Ibrahim *et al.* (1995) noticed that decreasing the Ca^{+2} concentration in whey through dialysis, significantly reduce denaturation of proteins in whey of pH 6.5 heated at 90°C for 10 min. Heat induced aggregation of whey proteins involve a multistage set of reaction including sulphydryl-disculphide interchange and Ca^{+2} binding. Zittle *et al.* (1957) suggested that bound Ca^{+2} neutralized the charge on whey proteins, resulting in isoelectric protein precipitation.

2.2.4.3.2 Sugars

Sugars have stabilizing effect on whey proteins against thermal transition and the disaccharides, such as sucrose and lactose, improve thermal stability of whey proteins (Morr and Ha, 1993). Ibrahim *et al.* (1995) reported that sugars particularly lactose extend stabilizing effect on whey proteins at all pH (3-8) and but the effect was more pronounced at pH 4.5-6.5. The presence of lactose caused slight inhibition of thermal denaturation of BSA but it had no effect on α-lactalbumin. Hydrolysis of lactose enhanced stability of β-lg (Gaafar and Gaber, 1992).

Garrett *et al.* (1988) observed that though sucrose promoted the denaturation of both native and individual whey proteins, but prevented their subsequent coagulation. The effect of sucrose in the heat denaturation of 0.2%

(w/v) WPI solution (pH 7.0) was measured using DSC. Sucrose increased Td (Kulmyrzaev *et al.*, 2000).

2.2.4.3.3 Fatty acids

Presence of fatty acids in whey proteins, particularly BSA, was reported to increase in Td (Bernal and Jelen, 1985). The enhanced stability was attributed to binding of fatty acid with BSA. This binding has lot of physiological significance (Brown and Shockley, 1982).

During the course of review of available literatures on thermal behaviour of whey proteins, it was found that most of the studies were performed with untreated whey or isolated individual whey proteins. Not much information was available on thermalstability of partially denatured whey proteins, and effect on manufacturing procedure on their stability in a complex food system.

2.3 Protein-polysaccharide interactions

Beverage processing technology nowadays is involving greater use of nutrients, minerals and herbs without any loss of flavor, appearance or consistency of the product and new beverage formulations are being developed continuously to suit the changing needs of consumer .The selection of appropriate and economical beverage stabilization systems, has become a crucial to accomplish this goal.

One of the problems faced in developing milk protein rich acidic drinks, is the poor heat stability of milk proteins below their isoelectric point.

Denatured protein particles tend to agglomerate sediments, giving product an undesirable appearance. Denaturation of proteins adversely affects the flavor of the product. Various substances, especially the polysaccharides may help in stabilization of protein suspension. The stabilization processes involve a number of interactions between polysaccharides and protein molecules. Complexes so formed occur throughout the biological systems such as the lipoprotein complex in animal tissues. These interactions fall into two major categories (Hansen, 1982; Samant *et al.*, 1993).

a) Generalized electrostatic association which is primarily governed by the availability of ionizable groups of opposite charge.

b) Formation of micelle structures between specific hydrocolloids and calcium- sensitive proteins.

These two interactions generally results in the formation of three types of complexes: (a) insoluble complexes; (b) the reversible soluble complexes; and (c) irreversible soluble complexes. The three possible type of complexes formed depends upon the nature of polysaccharide, which may be acidic, sulphated or neutral. Most of the reported work has been done on systems involving acidic polysaccharides as well as sulphated, because there is listtle or no interaction between proteins and neutral polysaccharides (Ganz, 1974).

2.3.1 Interaction of milk proteins and ionic polysaccharides

Doesburg and Devos (1959) described a process for stabilization of milk proteins with pectin below their isoelectric point. Another patent was granted

to Exler (1969) for a process of stabilizing a fermented milk product at approximately pH 4.0 with high methoxyl pectin. Commonly used anionic polysaccharides for the stabilization of milk protein includes pectin, CMC and PGA (Propylene Glycol alginate): Protection against heat coagulation can be obtained by stabilizing with one of three acid polysaccharides. However, the forces by which the hydrocolloids are bound to the casein particles are not known. Both hydrophobic and electrostatic forces can be of importance, and it is possible that above mentioned three stabilizers are not bound by the same mechanism (Glahn, 1982). High methoxyl pectin, carrageenans or modified starches can be used in systems, where milk proteins have to be stabilized below their isoelectric point. Pectin can stabilize acidic juice and milk or soy beverage by complexing with protein (Gerlat, 2000). This complex prevents precipitation of protein below isoelectric point. The unique stabilizing ability of acidic polysaccharides is attributed to the negative charge the casein particles acquire. Casein particles probably acquire negative charge in the presence of acidic polysaccharides and cause coulombic repulsion between the particles, which prevents the tendency of the particles to adhere to each other and protect against gravitational pull. According to Glahn (1982) size of casein particle has also influence over the interaction with polysaccharides and ultimately obtaining long shelf-life.

Milk proteins form complex with ionic polysaccharides and this principle was used to recover proteins from cheese whey (Hidalgo *et al.*, 1971).

Protein -polysaccharide complexes so formed are least soluble when the two constituents are mixed in calculated amounts and the pH is adjusted below isoelectric point to form isoelectric aggregates. The soluble complexes are produced by alkali treatment, and they exhibit excellent functional properties like whipping characteristics (Hidalgo and Hansen, 1969).

Formation of complexes is consistent with the nature of electrostatic interactions and the carboxyl groups of polysaccharides interact with positive groups on proteins like amides to form insoluble complex. Electrostatic interactions also occur between CMC and casein, but the mechanism of complex formation is more difficult to explain. However, studied conducted with CMC and $\alpha-s_1$ casein showed that formation of complex lowered down the isoelectric point (Valaris and Hansen, 1972).

Zaleska (2000) reported electrochemical complexation of apple pectin and whey protein mixture. Aqueous solutions of apple pectin (65% esterified group) and WPI (85% protein) at ratios of 1:0.5 to 1:50 were complexed using an electrolytic cell. They observed that in complex formation, interactions between amino group and hydroxyl group of both components were involved.

Co-precipitation phenomenon commonly observed in milk systems by the addition of hydrophilic colloids. According to Grindord and Nickerson (1968), the wheying-off and coprecipitation, which occurs in milk upon the addition of hydrocolloiods, in some instances can be reversed merely by

dilution. Such interactions could be weak and become disrupted under the altered conditions of pH, and ionic strength.

However, interaction of partially denatured proteins with charged polysaccharides is another approach to form soluble complex (Dickinson, 1998; Mishra, 1999). Two biopolymers form complexes through covalent coupling and electrostatic interactions. Since the major advantage of a covalent protein-polysaccharide hybrid over a non-covalent complex is the retention of molecular integrity and solubility over a wide range of soluble conditions. Strong dipole effects and local interactions are responsible in some cases for electrostatic interactions at pH values, where both colloids carry the same overall charge (Hansen, 1982).

2.3.2 Interaction of milk proteins with sulphated hydrocolloids

Strongly sulphated hydrocolloids, including carrageenan and locust bean gum, are frequently used in fluid milk products like puddings, flan, evaporated milk etc. to provide a smoother creamy consistency. Carrageenan is known to prevent age gelation and sedimentation problems in milk products like chocolate milk (Thomas, 1992). In these applications k-carrageenan form a weak gel in the water portion of milk system, further it interacts with the surface area of the casein micelles. Model experiments have shown that k and I (iota) Carrageenan, and to a lesser extent- λ- carrageenan, can stabilize αS_1 - and β-casein against precipitation by calcium ions (Hansen 1968, Lin and Hansen 1970).

Table 2.2: Structural and functional properties of food hydrocolloids

Hydrocolloid	Source	Structural unit	Properties and application
CMC	Chemical modification of cellulose	Sodium salt of CMC	Water soluble compatible with a wide range of food ingredients including protein sugar, starch, protein reactive and form soluble complex with casein near its isoelectric pH. Used in acidic milk drinks (Keller, 1984).
Carrageenan	Red sea weeds *Conducrus crispus*	Galactose and 3.6-anhydrogalactose units, both sulphated and non-sulphated, joined by altering α-(1,3) and B-C (1,4)_ bonds	Hot water soluble acid stable, form gel even at low concentrations, protein reactive, especially with milk proteins. Prevent coagulation during treatment. Used in milk gels, evaporated milk and ice cram mixes (Thomas, 1992).
Guar gum	Seeds of *Cyromopsis tetragonolobus*	Straight chain of D- mannopyranose with D-galactopyranose unit at side chain.	Water soluble, neutral polysaccharide, form viscous solution. Compatible with salt and water soluble proteins. Stable at acidic pH-used in juices, dairy products, sauces (Glicksman, 1969).
Pectin	Citrus peel / apple pomace	Galacturonic acid (β 1,4-linkage) esterified side chain	Water soluble, stable at low pH, anionic polysaccharide. Protein reactive. Form soluble complexes with milk protein near/below their isoelectric point.
Alginate	Brown sea weed (*Laminaria hyperborea*)	Linear copolymer of D-mannuronic acid and L-gluronic acid	Water soluble, sodium alginate unstable at low pH, whereas PGA is stable at low pH. Acid stability depends upon degree of esterification. Interaction with protein under acidic conditions (Sime, 1990).

Carrageenan mimics the action of k-casein and is effective at about the same weight ratio as required for k-casein. In contrast to k casein, αS_1 - casein interaction, does not form complex with k-carrageenan in absence of Ca^{+2} (Sakura and Nakai, 1981). Efficacy of sulphated polysaccharides depends upon molecular weight, presence of sulphate groups, location of the sulphate ions and primary structure (Lin and Hansen, 1970). The exceptions are non-gelling λ–carrageenan, which is still somewhat effective and locust bean gum which is very effective (Lin, 1977). A model based on sol-gel transition was used to explain casein stabilization by carrageenan (Snoeren, 1976). Another model put forward by Chakraborty and Hansen (1971), through electron microscopic studies, indicate micelle formation by α-S_1 casein and carrageenan. This model suggests that stabilization is achieved by entrapping small, calcium aggregated protein bodies in carrageenan structure before such bodies can agglomerate further into large colloidally unstable particles.

Various hydrocolloids have been presented in Table (2.2) for their reactivity and nature of interaction with proteins.

2.4 'Bael' fruit

'Bael' fruit (*Aegle marmelos*) belongs to the family rutaceae and it occupies an important place among the indigenous fruits of India. It has been known in India from pre-historic period and commonly referred as "Bengal quince". The bael has great mythological and religious significance. It is regarded as a sacred tree and widely quoted in ancient literatures including

vedas, Ramayana, and early Buddhist and Jain literatures. The tree thrives well in semi-arid and arid zones and grows wild throughout the deciduous forests of India. Attempts have been made to characterize its varieties as well as develop new cultivars for organized orcharding of this fruit in the country. It is also found in large members of South Asian and Southeast Asian countries.

'Bael' fruits, because of its hard shell, mucilaginous pulp and seeds, requires a lot of preparatory operations. Hence it is not much popular as a dessert fruit. However, some of the processed products of 'bael', specially the delicious cooling drink, are relished because of their excellent flavour and therapeutic value. Drinks are prepared by mixing bael pulp with tamarind pulp or curd at home and used as refreshing drink. Bael fruit has an untapped potential for processing into products, which can attract both the internal and export market.

2.4.1 Chemical characteristics

There are no standard names for 'bael' fruit cultivars. They are generally named after the locally where they are available.

The fruit is possess considerable amount of dry matter. Most of the dry matter is comprised of mucilage (Roy and Singh, 1978).They contribute up to 40% of dry matter. Non-reducing sugars are the predominant sugars. The transparent slimy mucilage is mainly confined around the seeds and is water soluble. The 'bael' fruit is non-acid fruit and the pH generally varied in the rage of 5.0-3.3. Hence, higher processing temperatures are required to ensure

product safety. The size of the fruits also have pronounced effect on the chemical composition (Roy and Singh, 1978), such as smaller fruits had higher TSS and higher mucilage. Gopalan et al. (1978) reported that 'bael' fruits contain 0.13 mg of thiamine, 55μg of carotene, 1.19 mg of riboflavin, 1.1 mg of niacin and 8 mg of ascorbic acid per 100 gm of edible portion. Amount of riboflavin is comparable to other good sources like legumes. Mukherjee and Ahmed (1954) also reported a high riboflavin content. Roy and Singh (1978) reported that ascorbic acid content in different cultivars ranged 7.68 – 18.79 mg/100 gm. Phenolics are another group of chemicals that have pronounced effect on its organoleptic quality. Tannic acid is the only polyphenolic substance detected in 'bael' fruit (Siddappa, 1978). The phenolic content (1755-2650 mg/100 gm as tannic acid) of the 'bael' fruit varied considerably among the cultivars and in general smaller fruits have higher phenolics as compared to bigger fruits (Roy and Singh, 1978). Marmelosin ($C_{13}H_{12}O_3$) is the active principle identified in 'bael' fruit and responsible for therapeutic attributes of the fruit. It was isolated as a colourless crystalline compound and the content varied from 0.03-0.37% among different cultivars (Dixit and Dutt, 1932). Tokitomo *et al.* (1982) has identified 32 flavour compounds in bael fruit and the major components were terpenes, alcohols and α-ionone. Various chemical constituents namely alkaloids, coumarins and steroids have been isolated and identified from different parts of 'bael'. The varieties with high pulp to peel ratio, lesser number of seeds, low mucilage, high sugar particularly

non-reducing sugars, and low phenolics, are preferred both for table and processing purposes.

2.4.2 Therapeutic properties

Its medicinal properties had been described in "Charak Samhita" (Aiyer, 1956). The unripe or partially ripe fruit is regarded as astringent, digestive and stomachic. The fruit is used in the treatment of chronic diarrhoea and dysentery and acts as the tonic for brain and heart. Clinical traits of unripe fruits showed anti-viral activity, against Ranikhet disease virus, hypoglycemic activity and significant results against intestinal parasites (Anon, 2001).

Besides the fruits, the root, bark, leaf and seed of 'bael' are valued in the indigenous system of medicine. The roots as well as bark are used in the form of decoction as a remedy in melancholia, intermittent fevers and palpitation of the heart. Fresh leaves are used in West Bengal as a remedy for dropsy and beriberi associated with weakness of heart.

2.4.3 Storage of fruits

'Bael' fruits have a high shelf-life, due to the presence of hard shell. However, breakage of shell during harvesting, improper handling, and application of ripening accelerator may initiate the activity of browning enzymes and cause substantial loss in product quality. 'Bael' fruits were found to be vulnerable to the fungal attack and invasion, generally starts from stem end portion. The storage life of ripe 'bael' fruit is 2 weeks at 30°C. It can be stored upto 12 weeks at 9°C. But lower storage temperature results in

physiological breakdown (Roy, 1975).

2.4.4 Processing of 'bael'

Processing of 'bael' into various product starts right from the green tender stage, where it is used for making candy or preserve. 'Bael' preserves or candy is usually recommended for control of stomach ailment. Lal *etal.* (1960) described the method of preparing 'bael' preserve by slicing the peeled fruits crosswise into pieces of 2.5 inch thickness, pricking them with stainless steel fork and steeping the pieces overnight in cold water. Next day fruits were blanched in boiling water, containing edible food grade red colour and then mixed with half its quantity of sugar. After, a few days pieces were cooked in syrup of 60° Brix and the syrup strength was finally maintained at 70° Brix and were left pieces in syrup for two weeks. For candy making the final Brix of syrup was raised to 75° Brix. However, prolong storage has been reported to cause browning in some of the commercial samples, probably due to the insufficient blanching treatment. Singh and Dutt (1941) observed that though 'bael' contains sufficient quantity of pectin, but jelly formation was not possible, due to the presence of higher mucilaginous content. Agnihotri (1950) prepared syrup from ripe fruit. A process for preparing 'bael' squash and jam was described by Roy and Verma (1950). Extraction of pulp is a major obstacle in processing of pulp. Roy and Singh (`1979) developed a process to obtain pulp. Their process involved mixing of pulp with equal quantity of water, adjusting the pH to 4.2 and heating at 80°C for 2 min to inactivate the

polyphenol oxidase enzyme, and filtration to remove seeds and fibres. They further standardized the process for nectar, squash, and fruit slab, toffee and bael powder. 'Bael' fruit powder has a long shelf life and is used by some of the pharmaceutical companies. Rai and Mishra (2001) studied the effect of clones and drying methods on the quality of 'bael' fruit powder. In this experiment six clones of bael viz., Pant bael1, Pant-bael 2, Pant bael 3, Pant bael 7, Pant bael 10 and Pant bael 11 were pulped and the pulp was dried separately in the sun and cabinet drier. Pant bael 7 dried in cabinet drier was found to give the best pulp powder.

2.5 Citrus fruit

Citrus are the world's largest sub-tropical and tropical fruit crop with an estimated annual production of 98.6 million tonnes (FAO, 1998). Among citrus fruits, orange accounts for 70% of the total production. In India area under citrus fruits, especially mandarin (*Citrus reticulata* Blanco), sweet orange (*Citrus sinensis* Osbeck) and acid lime (*Citrus auranlifolia* Swingle), have increased from 90 to 386 thousand hectare and production from 8.23 to 28.20 lakh tonnes during a period 1961 to 1992-93 (NHB, 1994). In India, citrus production rank's third after mango and banana.

A number of mandarin cultivars are grown in different parts of India but Nagpur mandarin, Coorg orange as well as Kashi orange of Assam are the commercial ones (Ranganna *et al.*, 1985). Oranges grown in Coorg region contain 10.0-12.5% TSS, 0.37 to 0.75% acidity, and TSS to acid ratio ranges

from 17.2 to 28.1 (Ranganna *et al.*, 1985). Due to wide variations in TSS: acid ratio, TSS alone is considered as a reliable index for fixing the maturity (Ramana *et al.*, 1979). Two types of extractors commercially used for juice extraction are the FMC citrus juice extractor and the Brown extractor (Girard and Mazza, 1998). In FMC machine, juice is extracted from the whole fruit without halving, whereas in Brown extractor fruits, are cut into halves and the two halves are oriented and picked up by synthetic rubber cups. Subsequently, serrated plastic reamer penetrates the fruit with a rotary movement and juice is collected at the bottom (Chen *et al.*, 1993).

Yield of juice from mandarins is reported to be lower as compared to sweet orange (Patil and Pai, 2001). Italian mandarins yield 35.58-39.3% juice containing 8.78 to 10.5°Brix, 0.92 - 1.38% acidity and sugar : acid ratio of 6.76 to 12.6 (Schachtr, 1977). However, care should be taken to avoid mixing of excessive peel oil in juice during extraction.

2.5.1 Problems in commercial processing of mandarin oranges

Citrus juices though refreshing, and rich in minerals and vitamins, suffer from the drawback of bitterness. Excessive bitterness lowers the quality and it has hindered the widespread processing of citrus fruits in India (Ranganna *et al.*, 1983). The bitterness of citrus juices is attributed mostly to limonin, naringin and nomilin (Roy, 1990). Except grapefruits, fresh juices of other citrus fruits are not bitter and experience delayed bitterness upon prolonged storage at room temperature (enzyme induced) and upon thermal processing

(acid catalyzed) (Maier *et al.*, 1977). Berry (2001) has reviewed the literature pertaining to bitterness in citrus fruits and their solutions. He pointed out that masking bitterness through altering the sugar level and pH might be one of the most economic ways though it can be done only in case of sweetened juices.

The UHT processed and aseptically packaged orange juice recently launched in the Indian market is largely produced from imported concentrate. In citrus juices most of the flavour and colour is associated with particulate material. Therefore, it is extremely important to preserve the cloud stability, Sensory properties of citrus juices such as flavour, colour, texture and aroma are closely or partly attributed to cloud (Kalvons *et al.*, 1994). Loss of cloud stability are due to the activity of pectic enzymes in general and pectin esterases in particular (Lea, 1991). Thermal inactivation of PE is essential to maintain product quality (Wicker and Temelli, 1988). Nath and Ranganna (1977) found the values of thermal inactivation of PE in mandarin juice to be $F^{10.8}_{91.94} = 1.00$ and $D^{11.4}_{9.94} = 0.54$ at pH 3.6 and $F^{9.4}_{91.94} = 1.0$ and $D^{10.1}_{91.94} = 0.44$ at pH 4.0. They recommended 2.0 D process at pH 3.6 and 2.5 D process at pH 4.0 for maintaining cloud stability. In canning of mandarin segments in syrup, the segments are acid peeled, packed in cans, covered with syrup and processed to 3.67 and 4.67 D at 3.5 and 4.0 pH, to inactivate PE (Nath and Ranganna, 1977).

Cloud stabilization and improvement in juice characteristics can be achieved by sonication (Patil and Pai, 2001). Utilization of whey with orange

juices has been suggested by some worker to develop RTS beverage, with no apparent change stability of cloud.

2.6 Non-enzymatic browning in food products

Browning in fruit products may be caused by (i) loss of natural colour, degradation and formation of new compounds from natural pigments, (ii) interaction between macromolecules specially sugar and proteins or (iii) caramelization of sugars (iv) oxidation of ascorbic acid or a combination of all these reactions. Discolouration and associated formation of brown pigments, is often accompanied by undesirable changes in flavour, odour and nutritive value (Stadlman, 1948; Hodge, 1953). Interaction between sugars and amino acids form basis of Maillard condensation theory. According to this theory sugar-amine condensation products are formed,, which undergo Amadori rearrangement and a variety of secondary reactions take place yielding ultimately to dark coloured melanoidin compounds. Examples of such reactions include the browning of bread crust and roasting of coffee beans. The Amadari rearrangement occurs near neutral or slightly alkaline pH, therefore the possibility of this reaction as major contributor in browning of acidic products like fruit juices is unlikely. However, Hass and Stadtman (1949) working with apricot showed that nitrogenous compounds contributed to browning their role must be considered in acidic products.

According to another theory (ascorbic acid theory) the most important precursors to browning are ascorbic acid and related compounds. Their

oxidation compounds polymerize and react with nitrogenous constituents to form brown pigments. This reaction is widely accepted as major pathway of browning in citrus products (Clegg, 1964).

The third theory, i.e., active aldehyde theory considers that the browning involves the decomposition of sugars and sugar acids to furfuraldehyde or similar compounds characterized by an active carbonyl group. These compounds condense with nitrogenous compounds/polymerize to form brown resinous materials.

All three theories of browning have been reviewed by a number of workers (Hodge, 1953, Dworschak, 1980; Mauron, 1981; Saltmarch and Labuza, 1982). Heyns and Klier (1968) described difference between Maillard reaction and caramalization.

2.6.1 Browning reactions in milk and milk products

Complex browning reactions occur in milk and milk products when it is heat processed or during storage. Milk undergoes maillard reaction and caramalization At higher temperatures, lactose caramalization is the major cause of browning (Gothwal, 1998). One of the first reaction products of this change is 5-hydroxymethyl furural (HMF). Its formation kinetics is used to predict the severity of heat treatment.

Interaction of lactose with milk proteins cause low protein stability with consequences of heat coagulation and age gelation of milk specially in sterilized and condensed milk, and browning reactions.

In whey powders, maillard reaction is one of the important modes of deterioration (Labuza and Saltmarch, 1981). Whey powders contain relatively high concentrations of lactose and proteins with higher lysine content. Thus in the presence of moisture, these components may readily undergo maillard reaction (Finot and Furniss, 1986).

Davis *et al.* (1998) stored lactose hydrolyzed whey protein concentrate (WPC) at water activities of 0.22, 0.33, 0.55 and at temperatures of 22,30 and 45°C with or without glucose (5% w/w) and monitored browning reactionsin them. They found that in absence of glucose, WPC samples did not form browning compounds.

2.6.1.1 Consequence of Browning Reactions

Browning reactions milk and milk products cause 'discolouration, whereas development of cooked flavour during heating is a result of release of sulphhydryl groups from serum proteins (Fink and Kessler, 1984). Prolong heating may result in development of caramel flavour.

Maillard reaction reduces availability of lysine through formation of lysino-alanine involving β-elimination reaction (Fritsch, 1984). Decrease in nutritive value is also attributed to the formation of antinutritive and toxic compounds (Finot *et al.*, 1989). They showed that maillard reaction products interfere in bioavailability of minerals. Formation of cross-linkages due to reaction with lactose may further reduce the digestibility of milk proteins. Some of the low molecular weight maillard reaction products have been shown

to inhibit trypsin carboxypeptidase-A, Carboxypeptidase-B and amino peptidase - N (Oste *et al.*1986). Immunological properties of these products remain unclear and lactosylation of β-lg a major whey protein, was considerably increased specific allerginicity.

2.6.2 Reaction Kinetics of Browning Reaction

Browning reactions are measured generally as O.D. at 420 nm and by determining HMF formation. Number of studies have been carried out over the years to monitor the kinetics of browning reactions in model system and in food products (Ibarz et al., 2000; Patel *et al.*, 1996, Morales *et al.*, 1997).

Kessler and Fink (1986) studied the kinetics of HMF formation in stored UHT milk. They found that the amount of HMF formed was extremely small in comparison to the amount of the compounds from which it is formed. Therefore, they described HMF formation as zero order reaction and obtained activation energy equivalent to 139 KJ/mol.. Sweetened condensed milk stored at 7°, 15 °, 30 °, 45 ° and 55°C showed that HMF formation followed first-order kinetics whereas reflectance values which indicated change in colour of product followed zero order reaction. This reaction kinetics was utilized to develop shelf-life prediction models (Patel *et al.*, 1996).

Morales *et al.* (1995) studied the kinetics of HMF formation and loss of available lysine in milk and model systems i.e. SMUF (Synthetic milk ultrafelterate) and found activation energies in the range of 66.67-112.41 KJ for available lysine and 93.04 KJ-118.5 KJ/mol for HMF formation.

Ibraz *et al.* (1993) investigated NEB and HMF formation in clarified peach juice subjected to high temperature (100-108°C for 90 min.). Zero order and first order kinetic models were applied to NEB, whereas HMF formation was described by a zero order equation. Exponential type of equations were also obtained which explained the combined effect of temperature and soluble solids concentration on these two reactions.

Gogus *et al.* (1998) observed that in acidic media fructose caused formation of more brown pigment as compared to glucose and that glutamine is more reactive than arginine when fructose is used as substrate.

Arena *et al.* (2001) described thermal damage in blood orange juice through reaction kinetics of HMF formation in presence of sugars at elevated temperatures at 70°C - 90°C upto 11 hours. They concluded that HMF formation in blood orange juice depends only on sugar concentration.

The above literature survey shows that kinetics of browning reactions have not been studied so far in thermal processed products containing whey proteins, sugar and fruit juice and even though these reactions are bound to occur in such products during processing and subsequent storage and exert profound effect on the shelf life of products.

Materials and Methods

3.MATERIALS AND METHODS

3.1 Plan of Work

Present study was carried out in four stages: (i) establishment of thermal behaviour of partially denatured whey proteins manufactured by different methods, (ii) development of whey protein- polysaccharide complexes that could be utilized to enrich fruit beverages and also investigation of suspension stability of hydrocolloids at acidic pH, (iii) development of protein-rich beverage using response surface methodology, and (iii) evaluation of quality and shelf life of developed beverages.

3.2 Raw materials

3.2.1 Bael pulp

Ripe 'bael' fruits obtained from orchards in Lucknow, were washed in running water to remove adhered dust, dirt and mucilaginous substances. Pulp was extracted with slight modification in the method described by Roy and Singh (1979). Bael fruit was broken and pulp along with seeds and fibers was scooped out, mixed with water in the ratio of 1:1(Extract I) or 1:1.25(Extract II) and blended in a warring blender at low speed. The pulp was passed through 120 mesh sieve, pH of extracted pulp was adjusted to 4.2 with 50% citric acid solution, heated at 90°C for 2 min in a steam jacketed kettle and cooled to room temperature by circulating chilled water. Pulp was mixed with 600 ppm

sodium benzoate and 250 ppm KMS, packed in HDPE container of 5 L capacity and stored under frozen conditions at -22°C till further use.

Pulp extracts was analyzed for pH, acidity, T.S.S., reducing and total sugars, crude fiber, mucilage, polyphenols, pectin, ash and proteins. Their rheological behaviour was studied at 10°C, 20°C , 30°C, 39°C, 52°C, and 59°C.

3.2.2 Orange juice

Nagpur mandarins (*Citrus reticulata* Blanco) were purchased from local fruit market, washed thoroughly, cut into two halves and the juice was extracted using bowl type citrus juice extractor (Sumeet Electronics, SP-16). Precaution was taken to minimize grinding of rind portion and crushing of seeds. Extracted juice was passed through a finisher having 0.11mm perforations and filled in glass bottles (200 ml). Bottled juice was placed in a boiling water bath for 11.5 min. These processing conditions allowed orange juice at the geometric centre of bottle to reach the temperature of 90°C for 10 sec, which has been reported to be sufficient to inactivate pectin esterase (Chen *et.al.*,1990) Bottles were cooled immediately to room temperature by immersing in chilled water tank. Subsequently orange juice was stored in refrigerator at 4± 1°C until used.

Orange juice was analysed for T.S.S., pH, acidity, reducing and total sugars, ascorbic acid, polyphenols and protein content.

3.2.3 UF- Retentate and Freeze Dried WPC

Buffalo milk cheddar cheese whey (pH 6.8, T.S.S, 6° Brix and protein content 0.77%) was collected from the Experimental Dairy of NDRI, Karnal and passed through a centrifugal separator to remove casein fines and residual fat. Clarified whey was heated at 74°C for 15 seconds and readily cooled to 4°C in a plate heat exchanger. The pH of whey was adjusted to 7.0 with food grade 1 N NaOH and subjected to ultrafilteration process, using a pilot scale module hallow fibre membrane unit (Ramicon membrane type PM 50). Inlet and outlet pressures in the UF unit were 1.80 bar and 0.5 bar respectively. Temperature of circulating whey was maintained at 48 ± 1°C and average flux rate of permeate was 9.80 L/hour. The process was continued till the 80% volume reduction was obtained. Equal amount of dist. water (50°C) was added to UF-whey retentate and first diafilteration was carried out. Process was stopped when the T.S.S. of retentate reached 12.5°Brix.

For the preparations of freeze dried whey protein concentrate (WPC), second diafilteration of ultrafiltered whey was executed when 85% volume reduction had achieved. UF-retentate was subjected to second and third diafilteration so as to achieve 90% reduction in its volume. UF- retentate so obtained contained 65% (approximately) protein on dry matter basis. The UF-retentate was freeze dried at 55°C plate temperature of and absolute pressure of 4.3 torr in a table top freeze drier (Feeze Mobile II VIRTIS Gardiner, NY,

Table 3.1: List of Grade Stabilizers used in the study, their Suppliers and Properties.

Hydrocolloid	Supplier	Properties
Carboxy methyl cellulose	CDH (Bombay)	Low viscosity (1% solution; 30-70 CP), degree of substitution > 0.4
Carrageenan	Sigma Chemicals (USA)	80% kappa and 20% lambda Carrageenan
Guar gum LVR-1	Sarda Gums & Chemicals (pali-Marwar)	Viscosity (1% solution; 500-1000 CP), compatible with acidic emulsion, agglomerated guar gum.
Pectin	Sisco Research Lab. (Bombay)	Methoxyl content < 7%.
Propylene glycol alginate	Burzin & Leons Agenturen Pvt. Ltd. (Bombay)	Brand name Manucol Ester- ER/K, Kelcoloid ® LVF
Sodium alginate	CDH (Bombay)	
Stab – PX	Sarda Gums & Chemicals (Pali- Marwar)	No chemical modification, compatible with acidic juice, user level 0.15-0.25%.
Xanthan gum	Burzin & Leons Agenturen Pvt. Ltd. (Bombay)	Keltrol Xanthan gum, approved by Chemical Codex, viscosity (1% solution, 1200-1600 CP).

Model 10 MR -1R) make for about 36 hours to reduce the moisture content to 7±1%.

The UF-Retentate and freeze-dried WPC were analysed for proteins, total solids, ash, lactose and calcium content.

3.2.4 Spray Dried WPC

Spray dried WPC was gifted by Mahan Proteins Ltd. (Kosikalan) and product was analyzed for proteins, total solids, ash, lactose and calcium content..

3.2.5 Stabilizers

Food grade commercial stabilizers were procured from different manufacturers (Table 3.1).

3.3 Thermal behaviour of whey proteins

3.3.1 Thermal behaviour of whey proteins at acidic pH

Whey proteins prepared by three different methods (Sec 3.2.3 and 3.2.4) were dissolved in dist. water to obtain a 2% protein solution. The solution was divided into five lots of 50 ml, and their pH was adjusted to 3.5, 3.75, 4.0, 4.25 land 4.5 using 50% lactic acid solution. Samples (10 ml) were poured in screw capped test tubes (15 x 176 mm). The tubes were placed in temperature controlled water bath set at 95 ± 0.5°C, heated to a centre temperature of 92.5°C, held for 5 min, and quickly cooled to room temperature by placing them in an ice bath.

The degree of denaturation of WPC, their soluble proteins content and Heat Coagulation Time (HCT) profile were estimated. The soluble protein was determined by centrifuging the sample at 5000 rpm for 20 min in a Remi centrifuge and the protein content of supernatant was estimated by micro-Kjeldahl method using a conversion factor of 6.38.

3.3.2 Effect of acidulants on thermal stability of whey protein concentrate

A whey protein solution (1.5% protein) was prepared by dissolving calculated quantity of freeze dried WPC, solutions were divided into three lots and their pH were adjusted to 3.5, 4.0 and 4.5 using 50% solution of four food grade acidulants, i.e., citric acid, lactic acid, glucono-δ-lactone or tartaric acid. Acidified samples were heated and degree of denaturation and amount of soluble proteins in them were determined as per procedure described in Sec 3.3.1.

3.3.3 Effect of whey protein supplementation on quality of model beverage

This experiment was performed. Model beverages were prepared by adding WPC (spray dried) to ascertain the efficacy of whey protein enrichment using spray dried WPC in model beverages @ of 4,5, and 6% level to dist water (control), TSS was raised to 16°Brix and 100 ppm lemon flavour was added. Its pH was adjusted to 3.5, 3.75 and 4.0 with 50% glucono-δ-lactone. Model beverage using diluted (1:1) cheese and acid whey were also prepared by the procedure described above for dist. water. The beverages were taken in separate Erlenmeyer flasks, covered with aluminum foil and held in a boiling

water bath about 10-11 min. Typically, 6-7 min were needed to bring the samples to desired processing temperature and it was processed at 92.5°C for 5 min.. Flasks were immediately cooled in an ice-bath to about 20°C. Samples were analysed for soluble proteins using micro-Kjeldahl method and subjected to sensory evaluation for overall acceptability on a 5-point scale.

3.3.4 Kinetic studies in model whey protein beverage

3.3.4.1 Preparation of model beverage

Model beverages were prepared by dissolving freeze dried WPC citrate-phosphate buffer of pH 3.5 or 4.0. Pectin was added @ 0.2% (w/v), T.S.S. of the beverage was raised to 15° Brix using sucrose (Nice Chemicals, Bombay). The beverage was mixed thoroughly and final pH, if required was adjusted using 1N NaOH or 1 N HCl to pH 3.5 or 4.0. Samples were centrifuged at 4000 rpm for 10 min in order to remove suspended particles of minerals and denatured proteins

3.3.4.2 Kinetic studies

Beverage (70 ml) was taken in glass stoppered Erlenmeyer flasks of 250-ml capacity and placed in a circulating water bath maintained at 75°, 85° or 95°C (Wurtf. Elecktromotoroen G.m.b.h., Balingen). Samples took 5-7 minutes to reach the desired bath temperature. Samples were removed at intervals of 0, 5, 10 and 15 min interval, quickly cooled to 10°C by placing them in ice bath. Heated samples were analyzed for degree of denaturation and soluble protein content.

3.4 Interaction between protein and polysaccharides

Whey protein-polysaccharide interaction was assessed under two sub-headings.

3.4.1 Development of heat stable proteins - polysaccharide complex for fortification in acidic beverages

Complex formation between partially denatured WPC (spray dried) and two anionic polysaccharides, i.e., CMC and pectin and one sulphated polysaccharide i.e. carrageenan, was carried out as described by Mishra (1999). Twenty per cent WPC solution (w/v) distilled water and 10% solution of polysaccharide were mixed in the ratio of 2:1, kept for 30 min to equilibrate and pH of mix was adjusted to 7 using 1 N NaOH or 50% citric acid. The resulting complex was air dried in a cabinet drier at 50° ± 1°C till moisture content reached to 8±1% content, ground in a mixer and passed through 20 mesh sieve.

In subsequent part of investigation the complexes were developed only between denatured WPC and two acidic polysaccharides, because sulphated polysaccharide formed unstable complex. To investigate the effect of heating on complex formation, resulting complex was heated in a water bath at 50°C for 1-hour (T_2), at 80°C for 1-hour (T_3). Another complex was not given any heat treatment (T_1) and served as control. Further drying of complex was carried out as described above.

To study the effect of pH on complex formation, pH of mix was adjusted to three levels, i.e., 3.5, 5.5 and 7.0 1 N NaOH or 50% citric acid solution. Dried complexes were obtained following treatment T_2 for Pectin-WPC complex and T_3 for CMC-WPC complex described in the above paragraph.

3.4.2 Evaluation of protein-polysaccharide complex

To confirm the complex formation, 1% dispersion of complex was prepared by suspending in dist water. The solution was centrifuged at 16,000 g at 20°C for 30 min and the protein content in supernatant was determined by micro-Kjeldahl method, using 6.38 as conversion factor (Mishra, 1999).

Protein content of complex was determined following standard method (AOAC, 1997). The solubility of complex was estimated by ISI method (ISI, 1981).

To check the heat stability, 2% dispersion of complex was made and the pH was adjusted to 4.0. Sample (10 ml) was poured in a screw- capped test tube and heated in a water bath at 95°C for 5 min, cooled rapidly to room temperature by placing them in ice bath and observed for precipitation/coagulation.

3.5 Product development

3.5.1 Development of whey-protein enriched 'bael' beverage using Complex

Various ingredients used to develop an acceptable beverage, namely 'bael' pulp, sugar and WPC-polysaccharide complex, exert different effects on

sensory quality of beverage. Therefore, their selection and the optimization of the selected ingredient were carried out during product development studies. The ingredients and their levels tried during the course of product development are listed in Table 3.1

3.5.1.1 Optimization of pulp level

Bael pulp (16.2°C Brix) was added to water @ 15, 20, 25, 30 and 35%, sugar was added to raise the refractometeric solids to 16° Brix. The pH of the beverage was adjusted to 3.9 with 50% citric acid solution. The beverage was pre-heated to 85°C and filled hot into glass bottles (200ml), capped and processed in boiling water for 20 min. Bottles was cooled quickly to room temperature and sensory quality of beverage was compared on the next day using a modified sensory evaluation card (Appendix-I).

3.5.1.2 Optimization of sugar level

The standardized pulp (25%, 3.9 pH) was divided into three lots of 500 ml, crystalline sugar was added to raise the T.S.S. of beverage to 15, 16 and 17° Brix and it was processed in similar way as described in Sec 3.5.1.1. Sensory quality of beverages was evaluated.

3.5.1.3 Optimization of WPC-polysaccharide complex level

CMC-protein and pectin - protein complexes were added to the standardized beverage (16°Brix, 25% 'bael' pulp, 3.9 pH) to adjust their protein content to 1.75, 2.75 and 3.75%. Complexes were dissolved separately in minimum quantity of water, allowed to hydrate and then mixed with other

ingredients in a blender and processed as before. These products were evaluated for sensory characteristics and acceptable products were analysed for T.S.S., pH, total solids, acidity, sugars (reducing and total), mucilage content, total phenolics, protein content and viscosity.

3.5.2 Hydrocolloid stabilization of whey proteins in acidic beverage

3.5.2.1 Screening of hydrocolloids for their reactivity at low pH

Food grade stabilizers (Table 3.1) were evaluated for their ability to form soluble complexes with whey proteins at pH 3.5 and 4.0. One percent solution of whey protein was prepared by dissolving calculated quantity of freeze dried WPC in de-ionised water. Stabilizers (0.1% w/v) were mixed with equal quantity of ground sugar, dissolved in 100 ml of whey protein solution and allowed to hydrate for 1 hour at room temperature. Then this solution was passed through a laboratory model hand homogenizer to form a uniform suspension. The pH of the solution was adjusted to 3.5 and 4.0 using 1 N NaOH or 50% lactic acid solution. Samples (50 ml) in duplicate were poured in graduated beakers (100 ml), covered with silver foil, heated on a water bath at 92.5°C for 5 min and immediately cooled to room temperature by placing them in an ice bath. Samples were mixed again and left undisturbed overnight. Next day samples were assessed for their reactivity with whey proteins.

3.5.2.2 Stabilization of whey proteins in model beverage

A model beverage was prepared by dissolving freeze dried WPC in de-ionised water to get a solution of 3.01 % protein content, sucrose was added to

raise the T.S.S. to 15°Brix, 100 ppm lemon flavour was also added. The required quantity of stabilizers namely carboxy methyl cellulose (CMC), pectin, propylene glycol alginate (PGA) and guar gum (Table 3.2) were added in 50 ml quantity of beverage, allowed to hydrate for 1 hours, temperature was raised to 45°C and passed through a laboratory model hand homogenizer. Samples were cooled to room temperature and their pH was adjusted to 3.5 and 4.0 using 50% GDL solution. Samples were processed as described in Sec 3.5.2.1.

Heat-treated samples were evaluated for suspension stability, sediment content and viscosity.

3.5.3 Development of whey protein enriched orange beverage

Ingredients used for the development of whey protein-enriched orange beverage were orange juice, UF- whey retentate, sugar, acid and stabilizer. Additional ingredients used were colouring material (Sunset yellow) and orange juice flavour base. Levels of ingredients were selected on the basis of FPO specifications (FPO, 1955) for RTS beverage, values cited in the literature and preliminary studies.

In preliminary studies critical factors that exerted pronounced effect on sensory quality of beverage were identified. Juice level, level of protein in UF-whey retentate, pH and types of acidulant (Table 3.3) were investigated for their effect on beverage quality. During preliminary investigations, beverage

with a TSS of 17°Brix was rated as the best. Hence in subsequent studies T.S.S. of the orange beverages were adjusted to 17°Brix with cane sugar.

Levels of critical factors identified in the preliminary investigations were used in determining their optimum level using response surface methodology (RSM) (Table 3.4).

3.5.3.1 Application of response surface methodology for optimization

Response surface methodology is an effective tool for optimizing a process when independent variables have a combined effect on a desired response. Approach, which is generally followed for formula optimization, is the so-called one variable at-a-time method. Although this approach is simple to plan and execute, its main drawback is its inability, in majority of the cases, to determine the true optima, as it does not consider interaction among factors. It is, therefore, better to use factorial experiments rather than one variable at-a-time method.

Because of its comprehensive theory, reasonably high efficiency and simplicity (Artega *et al.*, 1994), RSM technique was adopted for experimental design in this investigation. Hoke's response surface design (Thompson, 1982), consisting of 4 variables with 3 levels was adopted to optimize the level of ingredients (Table 3.4). It involved fewer trials than CCRD and complete factorial design, but still checked and located the presence of an optimum condition in the process and defined its nature without sacrificing the accuracy of design. The coded form of design matrix for the experiment and uncoded

Table 3.3: Selection parameters for optimization process

Factor	Level	Parameters evaluated
Orange juice	20, 30 and 40%	Flavour, overall acceptability.
UF- retentate	2.5, 3.5, 4.5%	Flavour, colour and appearance, mouth feel, overall acceptability.
pH	3.5, 3.8, 4.0, 4.25	Flavour, colour and appearance, Mouthfeel, overall acceptability.
Acidulant	Citric acid tartaric acid, GDL	Flavour, overall acceptability
Stabilizer	Pectin, PGA, Guar gum	Flavour, mouth feel overall acceptability

Table 3.4 : Experiment variables and their coded and uncoded values

Ingredients	Code	Coded levels		
		-1	0	+1
Orange juice (%)	X_1	30.0	32.5	35.0
UF-retentate (protein level %)	X_2	1.4	2.4	3.4
Sugar (%)	X_3	10	11	12
Stabilizer (%)	X_4	0.1	0.2	0.3

Table 3.5: Experimental design matrix for ingredients level in coded and uncoded form

Experiment No.	Fruit juice (X_1)	Protein in UF-Retentate (X_2)	Sugar (X_3)	Stabilizer (X_4)
1	1(30)	0(2.4)	0(11)	0(0.2)
2	0(32.5)	-1(1.4)	0(11)	0(0.2)
3	0(32.5)	-(2.4)	-1(10)	0(0.2)
4	0(32.5)	0(2.4)	0(11)	-1(0.1)
5	-1(30.0)	-1(1.4)	-1(10)	-1(0.1)
6	-1(30.0)	1(3.4)	1(12)	1(0.3)
7	1(35)	-1(1.4)	1(12)	1(0.3)
8	1(35)	1(3.4)	-1(10)	1(0.3)
9	1(35)	1(3.4)	1(12)	-1(0.1)
10	1(35)	1(3.4)	-1(11)	-1(0.1)
11	1(35)	-1(1.4)	1(12)	-1(0.1)
12	1(35)	-1(1.4)	-1(10)	1(0.3)
13	-1(30)	1(34.)	1(12)	-1(0.1)
14	-1(30)	1(3.4)	-1(10)	1(0.3)
15	-1(30)	-1(1.4)	1(12)	1(0.3)
16	0(32.5)	1(3.4)	1(12)	1(0.3)
17	1(35)	0(2.4)	1(12)	1(0.3)
18	1(35)	1(3.4)	0(11)	1(0.3)
19	1(35)	1(34)	1(12)	0(0.2)

values are given in Table (3.4). The plan consisted of 19 experiments for the preparation of beverages and these experiments were performed in random order.

Beverage samples (50 ml) were subjected to sensory evaluation on a 9 point hedonic scale for flavour, colour and appearance, mouthfeel and overall acceptability. Evaluation card used for this purpose is given in Appendix II. The sensory data was analysed using Design-Expt. Statistical Package using generalized polynomial form given in Eq.3.1.

$$Y = b_0 + b_i X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 \quad \text{.....(3.1)}$$

where y is dependent variables (Flavour, Colour & appearance, Mouth feel or overall acceptability), X_1 (Fruit juice level), X_2 (Protein content in UF-retentate), X_3 (Sugar level) and X_4 (Stabilizer level) X_1,X_2,X_3 and X_4 are independent variables.

Adequacy of model was evaluated using F-ratio and co-efficient of determination (R^2). Model was considered adequate when F-calculated was more than table-F and R^2 was more than 80% (Henika, 1982, Flores and Chinnan, 1987). The effect of variables at linear, quadratic, and interactive level on the individual response was described using significance at 1, 5 and 10% levels of confidence. The magnitude and sign of coefficients in the model indicated the effects of variables on response. The magnitude of co-efficient described the extent of dependency of variables on increasing or decreasing the response depending on positive or negative sign of coefficient terms. In the

case of negative interaction, level of one ingredient could be increased while decreasing the level of other ingredient variable. All negative coefficients of quadratic terms indicate maximum response at stationary point, all positive coefficients of quadratic terms indicate minimum response at origin of stationary point, whereas mixed sign of quadratic terms indicate mini-max response (middle point) at origin of stationary point.

Contour plots were developed using second order polynomial models for sensory responses, using Surfer package. Contour was used to determine the interaction between two variables on sensory responses.

3.6 Storage studies

Whey-protein enriched orange beverage was prepared by using optimized level of ingredients (orange juice, whey protein level in UF-retentate, sugar and stabilizer).

Beverages were packed in 200 ml glass bottles and pasteurized in boiling water bath at 92.5°C for 10 min. Bottles were air cooled to room temperature. The beverage was stored at elevated temperatures of 36°, 42°, 52° alongwith a control at refrigeration temperature (9°C) following the guidelines of Singh *et al.* (1998). Samples were analysed for changes in TSS, total solids, pH, acidity, sugars (reducing and total sugars), protein content, proteolysis, hydroxymethyl furfural formation (HMF), proteolysis, non enzymatic browning (NEB), colour and viscosity. Microbiological and sensory attributes of the beverage were also evaluated.

The reaction kinetics of various physico-chemical changes during storage was also computed. The data so obtained at different time intervals at 9° (control), 36°, 42° and 52°C were substituted in equations of zero, first, second and third order (Eq. 3.2 - 3.5).

Zero order reaction

$$-dN/dt = k \qquad (3.2)$$

First order reaction

$$-dN/dt = kN \qquad (3.3)$$

Second order reaction

$$-dN/dt = kN^2 \qquad (3.4)$$

Third order reaction

$$-dN/dt = kN^3 \qquad (3.5)$$

Where, dN/dt is rate of reaction; N is concentration of reactants and k is reaction rate constant.

The reaction rate constant (k) obtained as slope of regression equations were plotted on semilog paper against inverse of absolute temperature ($^{o}K^{-1}$). The slope of lines on Arrhenius plot was measured and the activation energy was calculated for various chemical reactions (acidity, total and reducing sugars, non enzymatic browning, reflectance, HMF, proteolysis, protein, in-vitro-protein digestibility).

3.7 Analytical methods

3.7.1 Physical analysis

3.7.1.1 Viscosity

Viscosity of 'bael' pulp, protein-polysaccharide complexes, 'Bael' beverage and whey protein- enriched orange beverage was measured using Rheomat 108 E/R programmable Co-axial cylinder viscometer (Metler-Toledo, Switzerland). The viscosity was measured at eight different shear rates ranging from 100 to 1000 S^{-1} using system 33 for 'bael' pulp and 11 for whey protein-polysaccharide complex, polysaccharide stabilized model beverages, 'bael' beverages and whey protein-enriched orange beverages. Viscosity of all the products except 'bael' pulp was determined at 20 ± 2°C. The viscosity was expressed as apparent viscosity (μa). The rheological constants of the power law model were determined for the flow behaviour and consistency. Effect of temperature on apparent viscosity of 'bael' pulp was also studied.

The above products exhibited non-newtonian behaviour, therefore, their consistency is expressed by power law equation (Rao *et al.*, 1981; Ahmed *et al.*, 1999) and given by following expression (Eq. 3.6).

$$\tau = K(\gamma)^n \quad \ldots\ldots\ldots\ldots \quad (3.6)$$

Where τ is shear stress, γ is the shear rate, K is the consistency index and n is the flow behaviour index.

To investigate the effect of temperature on viscosity, 'bael' pulp was maintained at 10°, 20°,32°, 39°, 50° or 59°C using a water circulating

precision water bath (Lauda E100 ecoline RE104). Samples were allowed to equilibrate to reach the bath temperature. The value of rheological constants n and K were calculated by regression analysis of shear rate and shear stress data on log-log paper. However, power law model does not consider yield stress, but the later has been related to the strength of the coherent network structure, followed by a rupture of the network bonds or linkages connecting the flow units. Hence Herschel-Bulkely Model (Eq.3.7) and modified Casson model (Eq. 3.8) were also employed for calculating the rheological parameters (Rao et al.,1984; Harnanan et al., 2001)

$$\tau = \tau_{OH} + K_H (\gamma)^n_H \quad \text{.....(Eq. 3.7)}$$

$$\tau^{0.5} = K_{om} + K_m y^n_m \quad \text{.....(Eq. 3..8)}$$

Where K_H and K_m are consistency indices as per Herschel-Bulkely and modified Casson model, τ_{OH} is the Herschel-Bulkely yield stress. K_{om} is yield stress as obtained by the modified Casson model.

The Herschel-Bulkely yield stress (τ_{OH}) was obtained as the intercept of the regression line between shear stress and shear rate data. The Casson yield stress (K_{om}) was determined from the linear regression analysis of square root of shear rate and shear stress.

The effect of temperature on the rheological behaviour of 'bael' pulp can be described by the Arrhenius relationship (Saravacos 1970; Roy *et al.*, 1997; Gujral *et al.*, 2001).

$$K = Ko \, Exp \, (Ea/RT) \text{.....(3.9)}$$

Where Ko is Arrhenius constant, Ea is activation energy (KJ/gmol) of flow, R is gas constant, and T is absolute temperature in Kelvin.

3.7.1.2 Degree of protein denaturation

Degree of denaturation was measured as turbidimetric measurements of Absorbance Index (A.I.) using a spectrophotometer (Specord 200, Analytikjena, Version 2.1E) at 900 nm. (Jelen and Buchheim, 1984).

3.7.1.3 Heat coagulation time profile

To study the heat coagulation time (HCT) profile, the protein samples were prepared as in previous experiment (Sec. 3.3.1), pH was adjusted, samples were allowed to equilibrate for 10 min. and centrifuged at 3000 rpm for 5 min to remove any turbidity (Remi Centrifuge). Two ml of sample (duplicate) was taken in heat stability tubes and were heated in an oil bath maintained at 95 ± 1°C. The time taken by each sample to coagulate, which was judged by the formation of white flakes, was recorded using stopwatch.

3.7.1.4 Suspension stability

Suspension stability of hydrocolloid-whey protein solution at low pH i.e. 3.5 or 4.0 (Sec. 3.5.2.2) was determined by the method of Glahn (1982) with slight modification. A sample was diluted with equal quantity of 0.1 M solution of sodium phosphate/citric acid buffer (3.5 or 4.0 pH) for whey protein-hydrocolloid solutions and heated at 92.5^0 C for 5 min. Samples were centrifuged for 10 min at 1400 g. and the optical density (OD) of the supernatant was measured at 660 nm.

3.7.1.5 Sediment formation

Sediment content was determined using BIS method (BIS, 1969) with slight modification Samples (10ml) was taken in 15ml capacity graduated centrifuge tubes (0.1 ml graduation), centrifuged at 2000 rpm for 10 min. The supernatant was carefully discarded and replaced with equal amount of dist water and centrifuged again for 10 min at 2000 rpm. The amount of solids in ml remaining in the centrifuged tube was taken as sediment.

3.7.1.6 Solubility

Solubility of whey protein concentrates, and protein-polysaccharide complex was determined by BIS method (BIS, 1981). Sample (500 mg) was taken in 10-ml tube. To this 3 ml of water (50 ± 1°C) was added and final volume was kept in a water bath at 50 ± 1°Cfor 5 min. About 2 ml of homogenous liquid was transferred to a previously weighed tared aluminum dish. The tube was then centrifuged at 700 g for 10 min. Supernatant (2 ml) was pipetted out in another aluminum dish. Both dishes were uncovered and placed in a boiling bath until dried. Then the dishes were dried at 100 ±2°C for 90 min.

$$\text{Solubility} = \frac{W_4 \times W_1}{W_3 \times W_2} \times 100$$

Where W_1, weights in gm of the liquid taken in dish No.1,W_2, supernatant liquid in dish 1, W_3, solids in No.1 and W_4, are total solids in dish number 2, respectively

3.7.1.7 Colour

The colour of the whey protein-enriched orange beverage was measured in terms of reflectance using a reflactometer (ICL-28, Elico Pvt Ltd. Hyderabad). Beverage sample (tempered at 25°C for 3 hours) was taken into a rectangular glass bottle and placed below the search unit. The reflectivity was measured at 450 nm wavelength at several places on the bottle surface. Average value of the measured reflectivity was expressed as percent reflectance.

3.7.1.8 Microscopic examination of whey protein suspension

To ascertain the nature of interaction between protein and hydrocolloids in model beverage, two ml of heated sample was placed on a slide, dried using hot air and stained with saffranine. The slides were examined in phase contrast microscope.

3.7.2 Chemical analysis

3.7.2.1 Moisture content and total soluble solids

Moisture content of WPC samples was determined by AOAC (1997) method using an oven at 100 ±1°C. Moisture content of the pulp and beverage was determined by oven drying method at 70± 1°Ctill constant weight obtained.

Total soluble solid (T.S.S.) of the pulp and beverages were determined using a hand refractometer (range 0-32°Brix, Erma). Refractometer reading were corrected to 20°C(Ranganna, 1986).

3.7.2.2 pH and Acidity

The pH of pulp, protein-polysaccharide complex, and beverage was determined using a microprocessor controlled pH meter (LABINDIA) at 20°C.

Samples of juices, pulp or beverages were titrated against 0.1 N NaOH using pH meter till the pH reached to 8.1. Acidity of the samples were expressed as per cent anhydrous citric acid.

3.7.2.3 Fat

Fat content of the sample was determined by Majonnier method (Anon, 1959). Test sample (about 1 gm) was weighed accurately in 100 ml beaker. It was mixed with 5 ml of conc. HCl and heated to 100°C. After cooling 10 ml ethanol (95%) was added to it in installments and contents were transferred to Majonnier flask. First extraction of fat was done with 25 ml each of diethyl ether and petroleum ether (60-80°C), and second and third extractions were done with 15 ml each of diethyl ether and petroleum ether. The extracts were collected in flasks, ether was evaporated over a water bath and contents were dried to constant weight at 100°C ± 2°C.

Fat was determined as follows :

$$\text{Fat (\%)} = \frac{\text{Weight of extracted}}{\text{Weight of sample}} \times 100$$

3.7.2.4 Protein

Micro-Kjeldahl method (AOAC, 1997) was used to estimate the protein content in the samples. The protein content was calculated by multiplying the nitrogen percentage to 6.38 (whey protein) and 6.25 (plant protein).

3.7.2.5 Ash and calcium

AOAC (1997) method was used to determine ash content. The sample were weighed in an oven dried silica dish (102°C for 6 hours), ignited on flame till fuming ceased, ashed in muffle furnace at 55°C for 6 hours. Dish was cooled in a desiccator and weighed. Ash content was obtained by difference. Calcium content was determined by titrametric method (Ranganna, 1986).

3.7.2.6 Sugar

Reducing and total sugar (as % invert sugar) were determined following the method of Lane and Eynon (1923). However, there was slight variation in preparation of sample for 'bael' pulp and 'bael' beverage.

3.7.2.6.1 Sample preparation

In 'bael' samples, complete extraction of sugars without dissolving polysaccharides and solubilizing other interfering substances was accomplished by repeated extraction with 90% ethanol on a water bath maintained at 80°C and the process continued till the washings were clear and odourless. Sugar solution was deproteinized with 45% neutral lead acetate solution and excess of lead was removed by treating with 22% potassium oxalate solution.

For estimation of total sugars, to a 50 ml clarified sugar solution, 5 gm of citric acid and 50 ml dist water were added and boiled over a hot plate for 10 min. to ensure inversion of sugars. It was cooled to room temperature, neutralized with 1 N NaOH and the volume was made upto ____ ml with dist water.

Sugar solutions were titrated against 10 ml of mixed Fehling's solution, using methylene blue as indicator until brick red colour was observed. The procedure of titration was carried out over hot plate.

Percentages of reducing sugars or total sugars were calculated using Eq. 3.6. Fehling's factor was determined by titrating mixed Fehling's solution with standard invert sugar solution.

$$\text{Reducing sugar or total sugar (\% invest sugar)} = \frac{\text{Fehling's factor} \times \text{Dilution} \times 100}{\text{Titre value} \times \frac{\text{Sample}}{\text{Weight}} \times 1000} \quad \ldots\ldots\ldots\ldots\ldots\ldots (3.6)$$

3.7.2.7 Mucilage

Mucilage content in 'bael' product was determined by the method of Roy and Singh (1979). The sample was extracted out with cold water from the residue obtained after sugar extraction. It was hydrolyzed with conc. HCl followed by neutralization with 1 N NaOH and rest of the process was similar as in case of sugar determination. Mucilage content was expressed as per cent reducing sugar.

3.7.2.8 Lactose

Modified Lane and Eynon method as described by Ranganna (1986) was adopted. Five grams of samples were dispersed in 100 ml of water, deprotenized by adding 20 ml of 2.7%, barium hydroxide slowly with continuous stirring, allowed to rest for 10 min, transferred to a 250 ml volumetric flask and volume was made upto 250 ml with dist water. The contents were then filtered through Whatman No.4 Filter paper and the lactose content was determined as in case of sugar.

3.7.2.9 Pectin

Pectin content in bael pulp was estimated as calcium pectate (Ranganna 1986).

3.7.2.10 Crude fibre

Crude fibre was estimated by gravimetric method as suggested by Ranganna (1986).

3.7.2.11 Tannins

Tannins were estimated as per cent tannic acid by the colorimetric method of AOAC (1997) employing Folin-Denis reagentas described by Ranganna (1986).

3.7.2.11.1 Preparation of standard curve

A series of aliquot ranging from 0.2 ml to 1 ml of std (100 ppm) tannic acid solution with blank were taken in 6 volumetric flasks (100 ml). Five milliliters of Folin-Denis reagent and 10 ml of saturated Na_2CO_3 were added to each

volumetric flask. The volume was made up with dist water and kept for 30 min. The colour was measured at 760 nm in UV-spectrophotometer (Specord 200, Analytik Jena) against experimental blank at zero absorbency.

3.7.2.11.2 Determination

Sample (5-10 g) was dissolved in 200 ml of dist water and placed in a boiling water bath for 3 0 min. The volume was made upto 500 ml with dist water. A known volume of sample representing about 0.1 mg of tannic acid was taken in a 100 ml volumetric flask, 5 ml of Folin-Denis reagent and 10 ml of saturated Na_2CO_3 were added, volume was made upto the mark and allowed to stand for 30 min. Optical density of sample (X) was measured at 760 nm as in case of standard curve and tannic acid (Y, % mg) was calculated using Eq. 3.10.

$$Y = 0.359\,X + 0.0944 \quad \ldots\ldots\ldots \quad (3.10)$$

3.7.2.12 Ascorbic acid

Ascorbic acid content was determined by visual titration method using 2,6-dichlorophenol indophenol (Ranganna, 1986) and the results were expressed as mg ascorbic acid per 100 gm sample.

3.7.2.13 Non enzymatic browning

Non enzymatic browning was measured using method suggested by Ranganna (1986). To 20 ml of sample, 30 ml of 65% of aqueous ethanol were added and thoroughly mixed, kept overnight, and filtered through whatman No.1 filter paper. The OD of filtrate was measured at 440 nm in Specord 200 (Analytik

Jena). Sixty five percent ethanol was used as blank and results were reported as absorbance value.

3.7.2.14 HMF content

Both free and total HMF contents were determined by the method developed by Keeney and Bassette (1959).

3.7.2.14.1 Total HMF

Sample representing 3 mg of protein was taken in a 5 ml of glass stoppered tube and the volume was made upto 10 ml with dist water. To this 5 ml of 0.3 N oxalic acid was added, content were mixed properly, the tube was stoppered properly and placed in a boiling water bath for 1 hr. The tube was then removed and cooled to room temperature with tap water. Five ml of 40% TCA was added to the tube. The contents were mixed thoroughly and filtered through whatman No.42 filter paper. Four ml of the filtrate was pipetted into a test tube and 1 ml of 0.05 M aqueous solution of TBA (Sigma, USA) was added. After gentle mixing, the tubes were placed in a thermostatically controlled water bath maintained at 40°C for 40 min. At the end of incubation period, tubes were removed from the water bath, cooled to room temperature, and OD of the content was measured in a spectrophotometer (Specord 200, Analytik Jena) at 443 nm against blank prepared in similar manner, substituting dist water for sample.

3.7.2.14.2 Free HMF

Free HMF was measured in similar manner as for total HMF, with only omission of the step involving heating of sample with 0.3 N oxalic acid.

3.7.2.14.3 Preparation of standard curve

A stock solution (0.2 µml) of HMF (Sigma, USA) was prepared in dist water. HMF stock solution was added in different tubes to give 0.005-0.1 µml/ml HMF content on dilution representing 5-100 µ mol/L of HMF. The contents were treated as the sample for free HMF. A standard curve (Appendix-IV) was drawn and the regression equations (3.11-3.13) were obtained.

Y = 61.7078 x + 4.3438 for 5-35 µmol/L .....(3.11)

Y = 85.2961X - 5.4384 for 40-70 µmol/L .....(3.12)

Y = 111.6412 X - 28.9790 for 70-100µmol/L ..... (3.13)

Where, X is observed optical density at 443 mm and Y is µmol of HMF per litre.

3.7.2.15 Proteolysis

Proteolysis was measured in terms of free amino groups reactive to 2, 4, 6- trinitrobenzene sulphonic acid (TNBS). The method of Fields (1971) as modified by Spadaro *et al.* (1979) and described by Mckellar (1981) was followed.

One milliliter of sample and 1 ml of distilled water were treated with 4 ml of 0.72 N trichloroacetic acid (TCA) for 20 min at 25°C and filtered through Whatman No.1 filter paper. Duplicate samples (0.2 ml) aliquots of the filtrate

were transferred to test tubes and mixed with 2 ml of 1 N potassium borate buffer (pH 9.2) and 0.8 ml of 5 mM TNBS (Sigma, USA) solution. Tubes were incubated at 25°C for 30 min. Reaction was terminated by addition of 0.8 ml of 2 M monobasic sodium phosphate containing 18 mM sodium sulphite to each of the tubes. Optical density (OD) was measured at 420 nm in a UV-spectrophotometer (Specord 200, Analytik Jena). Reagent blank was prepared by replacing sample with dist water.

3.7.2.15.1 Preparation of standard curve

Standard glycine solution (100 µmol/ml) was prepared in dist water and volumes measuring 0.5-6.0 ml were transferred to a set of test tubes. The final volume of these samples was adjusted to 10 ml with dist water. One ml of glycine solution form each of the test tube was transferred to another set of test tubes and mixed with 1.0 ml of dist water. Rest of the procedure was performed in similar manner as in the case of product. The regression equations (3.14 & 3.15) were obtained for the standard curve (Appendix-V) of glycine.

$$Y = 64.5324\,X - 0.5691 \text{ for 5-30 µmol/ml. .....(3.14)}$$

$$Y = 73.8067\,X - 5.1481 \text{ for 35-60 µmol/ml. .....(3.15)}$$

Where, Y is µmol of glycine /ml, X is observed OD at 420 nm.

3.7.2.16 *In vitro* protein digestibility

In vitro protein digestibility was determined using multi-enzyme technique of Hsu *et al.* (1977), using trypsin, chymotrypsin and peptidase (Sigma Chemicals, U.S.A.).

A sample sufficient to give a suspension of 6.25 mg protein /ml was dissolved in 50 ml dist water for 1 hour at 5°C. The suspension was transferred to a water bath at 37°C and its pH adjusted to 8.0 by adding 0.1 N NaOH /0.1 N HCl with continuous. The multi-enzyme solution (1.6 mg trypsin, 3.1 mg chymotrypsin and 1.3 mg peptidase/ml) was maintained in an ice-bath and adjusted to pH 8.0 with 0.1 NHCl /0.1 N NaOH. Five millilitre of multi-enzyme solution was then added to the protein suspension, which was being stirred during incubation at 37°C. Due to the hydrolysis and release of amino acids, a rapid decrease in pH occurred. The pH drop was recorded at 2 min interval over a period of 10 min and the value was used in Eq. 3.16 to obtain the digestability.

$$Y = 210.464 - 18.103\ X \qquad \text{.....(3.16)}$$

Where, X is the pH of suspension after 10 min digestion and Y is % digestibility of protein.

3.7.3 Microbiological analysis

Total plate count, coliform count, and yeast and mold count of fresh and stored beverage samples were determined as per the standard methods given in APHA (1992). Different dilutions of samples were prepared from aseptically drawn 1 ml of sample and 9 ml of sterile dist water.

Samples were inoculated in duplicate plates of suitable media and incubated at the recommended temperature (Table 3.6). At the end of incubation period, the plates were counted for number of colonies.

Table 3.6: Media and incubation conditions for microbial examination

Determination	Medium		Incubation	
	Type	PH	Temperature (°C)	Period (hr)
Total plate count	Plate count agar	7.0	37	24-48
Yeast and mold count	Potato dextrose agar	3.0	22	72-110
Coliform count	Violet red bile agar	7.0	37	24-48

3.7.4 Sensory analysis

A panel consisting of at least 10 semitrained members drawn from the faculty and students of the department carried out sensory analysis of products. The panelist were served with 30-40 ml of chilled beverage. They evaluated samples for various attributes, namely flavour, colour and appearance, mouthfeel and overall acceptability using a 9-point hedonic scale rating (Amerine, 1965) on the given performa (Appendix-I,II). A score of 7 was considered as minimum acceptability score.

3.7.5 Statistical analysis

The statistical analysis of data were carried out by using two way ANOVA (Snedecor and Cochran, 1968). The critical difference between samples/treatments were determined by critical difference at 1% or 5% level.

Results and Discussion

4. RESULTS AND DISCUSSION

4.1 Chemical constituents of whey protein concentrates

4.1.1 Moisture

UF-retentate contained 87.21% moisture (Table 4.1). Moisture content of freeze dried WPC and spray dried WPC were 7.43 and 4.01%, respectively.

4.1.2 Protein

Chemical composition of freeze-dried whey protein concentrate (WPC), spray dried whey protein concentrate (WPC) and UF-retentate is presented in Table 4.1. Their protein content varied from 58.93 to 73.05% (on db). The variations may be due to the differences in procedure involved in their preparation. The spray dried and freeze dried WPC had higher (68.90-73.03%) protein levels because they were subjected to more than two stages of diafilteration, which has been reported to increase the protein level in the resulting WPC (Jayprakasha, 1992; Morr and Foegeding, 1990). UF- retentate, which was diafiltered once, had the lowest protein content. Protein content was reported to range from 72.0 to 76.6% for 8 commercial samples from different manufacturers in Europe (Morr and Foegeding, 1990), whereas it was found to vary from 28 to 80% for WPC available in Germany (Jayaprakasha, 1992). These variations may be because of the differences in manufacturing processes, source of raw material and also on the basis of desired functionality in finished product.

Table 4.1: Chemical constituents of whey protein preparations.

Whey protein preparation	Moisture (%)	Protein (% db)	Lactose (% db)	Fat (% db)	Ash (% db)	Calcium (% db)
UF-Retentate	87.21	58.92	27.70	8.18	5.11	0.462
Freeze dried WPC	7.43	68.90	18.32	7.20	4.95	0.392
Spray dried WPC	4.01	73.05	14.48	5.12	5.01	1.14
S.E.M. ±	0.079	0.126	0.16	0.028	0.035	0.0071
CD at P≤0.01	0.415	0.659	0.83	0.15	0.18	0.037

4.1.3 Lactose

Lactose was found to be 14.48, 18.32 and 27.70% in spray dried WPC, freeze dried WPC and UF retentate, respectively (Table 4.1). All the three samples differed significantly in their lactose content. As the protein content of samples increased its lactose content decreased because of its removal in permeate stream. Similar results were reported by Tratnik and Kersev (I991), who found that upon diafilteration lactose and ash content of WPC reduced to minimum values of 32.8 and 4.7% with corresponding increase in protein level to 52.4%, from initial value of 52, 5.2 and 35 % for lactose, ash and protein, respectively.

4.1.4 Fat

Fat content in three WPC samples ranged between 5.12 to 8.18% (db) being lowest in spray dried WPC and highest in UF-retentate (Table 4.1). Lower fat content in spray dried WPC is due to the raw material i.e. skim milk, used for making casein whey, whereas for manufacture of freeze dried and UF-retentate mixed milk cheddar cheese whey was utilized. Jayaprakasha (1992) and Vijaykumar and Sangwan, (2000) have reported 5.2-5.95% fat in WPC samples prepared by using ultrafilteration conditions, with mixed milk cheddar cheese whey. This difference in reported values and those found in this study may be due the differences in clarification process. In previous case cream separator was used. Whereas in the present investigation clarifier was used. The higher lipid content is due to the fact that the residual lipids in whey get concentrated along with protein during ultrafilteration/diafilteration process

(Harper, 1984). Presence of residual lipid adversely affects the functional properties of WPC (Kinsella and Whitehead, 1989).

4.1.5 Ash and calcium content

Ash content of freeze dried WPC, spray dried WPC and UF-retentate ranged between 4.95 and 5.12% (Table 4.1), which did not differ significantly. As previously reported, ultrafilteration process rejected minerals in permeate stream, and there was continuous reduction in ash content on subsequent diafilteration. Wide variations (3.36-15.03%) were reported in ash content of four commercial samples (Jayaprakasha, 1992). There was apparent difference in the calcium content of the two WPCs and Uf-retentate. Spray dried WPC contained highest amount of calcium (1.14% db). Spray dried WPC was manufactured from acid whey which are known to contain higher amount of calcium as compared to sweet whey. Calcium content in freeze dried WPC is almost similar to one prepared by Vijaykumar and Sangwan (2000).

The US specifications for WPC are : Protein (min) 25%, fat 0.2-10%, ash 2-15%, lactose (max) 60% and moisture 1-6%. Spray dried SPC used in this study fulfill all the above criteria.

4.2 Thermal behaviour of whey protein preparations

4.2.1 Thermal denaturation index

Thermal denaturation index (AI), is a measurement of protein denaturation caused by thermal treatment. All the three whey protein solutions exhibited an increase in AI with increase in pH from 3.5 to 4.5 (Table 4.2).

Table 4.2: Thermal denaturation index (A.I.)[1] of three whey protein solutions[2] at different pH.

pH	Thermal denaturation index of		
	Spray dried WPC solution	Freeze dried WPC solution	UF-Retentate solution
3.50	0.27	0.14	0.09
3.75	1.40	0.47	0.40
4.00	1.76	0.79	0.73
4.25	2.42	1.07	1.05
4.50	2.56	1.21	1.11

	F value	±S.E.M.	C.D. at 5%
A(pH)	**	0.014	0.04
B(WPC)	**	0.011	0.03
A x B	**	0.025	0.07

[1] O.D. at 900 nm

[2] 2% protein level in the whey protein solutions

Table 4.3: Soluble protein in heat treated whey protein solutions[1] at different pH.

PH	Soluble protein content of		
	Spray dried whey protein solutions (%)	Freeze dried whey protein solutions (%)	UF-Retentate Solution (%)
3.50	1.37	1.50	1.78
3.75	1.09	1.24	1.41
4.00	0.86	1.03	1.22
4.25	0.40	0.62	0.66
4.50	0.35	0.59	0.61

	F value	±S.E.M.	CD at 5%
A (pH)	**	0.02	0.05
B (WPC)	**	0.01	0.04
A x B	*	0.03	0.09

[1] 2% protein level whey protein solutions.

Among the three WPC solutions, spray dried WPC showed maximum A.I. value at all the pH followed by freeze dried WPC and UF-retentate. This indicates that spray drying process commonly employed to manufacture commercial WPC has a significant effect on the protein denaturation. There was significant difference among A.I. of WPC samples and also pH affected thermal denaturation significantly. Their interaction (WPC and pH) differed significantly at 5% level. However, all WPC solutions heated at pH 3.5, showed no visible aggregation and sedimentation during the course of heating. But upon overnight storage slight sedimentation was observed in spray dried WPC solution. High thermal stability of whey protein solutions at pH $\leq$3.9 was reported by several workers (Bernal and Jelen, 1985; Ibrahim *et al.*, 1995; Jelen and Buchheim, 1984; Patocka and Jelen, 1986). They further reported that the stabilizing effect against heat denaturation was maximum at or below pH 3.5. Both α-la and β-lg undergo structural changes leading to thermal behaviour, which may be different from that at pH $\geq$4.0. The dimer configuration of β-lg fraction dissociates at or below 3.5 pH due to electrostatic repulsion and the resulting monomers are resistant to coagulation (Harwalkar *et al.*, 1985). In addition to these reports Bernal and Jelen (1985) sighted the possible effects of minor contaminants in WPC also for high thermal stability at pH $\leq$3.5. All the WPC samples, showed coagulation and aggregation at pH above 4.0. In UF-retentate there was slimy aggregate formation and these aggregates floated loosely in the liquid. The low denaturation index of UF-retentate may be

attributed to the less thermal treatment (batch type pasteurization 63°C for 30 min.) given to mixed milk during cheese processing. Spray dried WPC had highest AI (0.27-2.56) because of the application of high air temperature. Hall and Iglesias (1998) had reported similar results and showed that the degree of denaturation increased with an increase in air temperature. Freezing process is also reported to denature proteins if not carefully controlled because of salting out effect. Hence, freeze dried WPC showed intermediate denaturation. UF-retentate, which was stored under deep frozen conditions (approximately -20°C) for 15-20 days, exhibited extensive denaturation and coagulation upon thawing at room temperature. It shows that slating out effect caused the protein denaturation. In addition, different thermal behaviour of three WPC's was because of differences in their constituents (Table 4.1) specially lactose content. Lactose has been reported to be most effective in preventing the thermal aggregation of whey proteins (Moor and Ha, 1993, Ibrahim *et al.*, 1995, Spiegel, 1999). The stabilization of the native protein structure by sugars is explained by a preferential hydration of the protein molecules in an aqueous sugar solution (Arakawa and Timasheffs, 1982).

4.2.2 Soluble proteins

The soluble protein content of heated whey protein solutions ranged from 1.37to 1.78, 1.09 to 1.41, 0.86 to 1.22, 0.40 to 0. 66, and 0.35 to 0.61% at pH 3.5, 3.75, 4.00, 4.25 and 4.50 respectively (Table 4.3). The maximum soluble protein content was observed in heated samples of pH 3.5 and above pH 3.75 there was appreciable loss of soluble proteins in all the samples. At pH

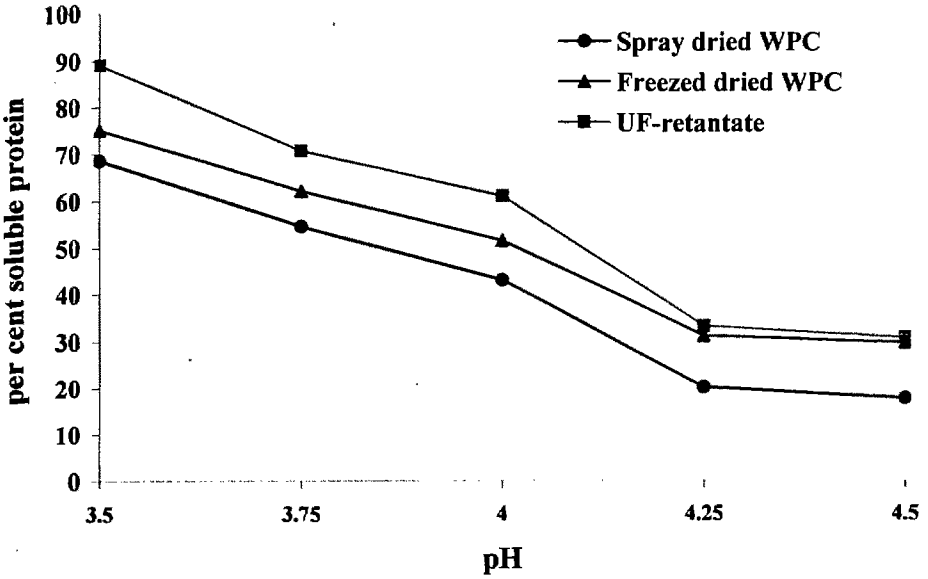


Fig. 4.1 : Effect of pH on soluble protein content of whey protein preparation upon heating.

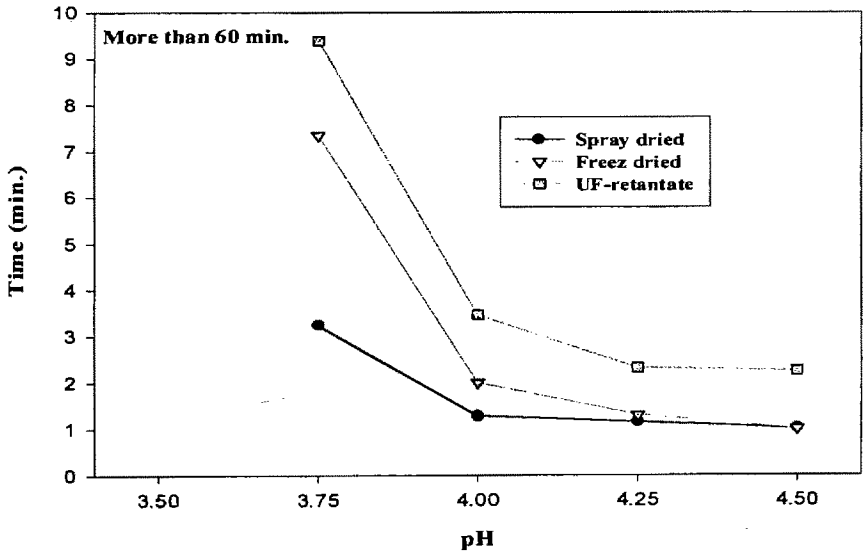


Fig. 4.2: Heat coagulation time (HCT) profile of whey protein preparations.

3.5, 68.5-89% proteins remained soluble, though the corresponding turbidity measurement values varied between 0.09 to 0.27. (Fig 4.1) Except spray dried whey protein solution, no visible coagulation and aggregation was observed in the other two WPC solutions at pH 3.5. Soluble protein content was found to decrease with an increase in AI (Table 4.2 &4.3). The overall results showed that although the whey proteins are quite resistant to precipitation during at or below pH 3.5, they are still denatured by heat. Soluble protein content of WPC solutions, were found to differ significantly with pH and method of preparations. But their interaction is significant only at higher level. Further, the manufacturing process and the composition of whey protein concentrates exerted a pronounced effect on their thermal behaviour. Pasteurization of whey did not have a significant effect on protein solubility, but heating of UF-retentates caused a significant reduction in solubility (Morr, 1987). Ibrahim *et al.* (1995) reported a recovery of 90% for soluble proteins from whey protein samples heated at 90°C for 10 min pH $\leq$ 4.5 but above this pH only 50% recovery was obtained.

According to Bernal and Jelen (1985) approximately 50% whey proteins get precipitated at pH $\geq$ 3.8. In this experiment also a heavy precipitation was observed at or above pH 4.0. Above pH 3.9, the repulsive action becomes very strong and protein-protein interaction are more likely to occur, resulting precipitation of proteins. Hidalgo and Gamper (1977) reported 47-51% protein denaturation in 2% WPC solution of 3.4-4.9 during heating at 90°C.

4.2.3 Heat stability

Heat stability, measured as time taken by whey protein solutions to coagulate, varied with pH and WPC sample. At pH 3.5, there was no coagulation or flake formation even after heating for more than one hour (Fig 4.2). UF-retentate showed maximum thermal stability followed by freeze dried and spray dried WPC solutions. At pH 4.0 or above, there was no apparent difference between heat stability of UF-retentate and freeze dried WPC.

4.3 Effect of acidulants on thermal behaviour of whey protein solutions

Present investigation was carried out to elucidate the role of food grade acids on thermal stability and heat solubility of whey proteins.

4.3.1 Denaturation index

Denaturation index of whey protein solutions was lowest (0.23-0.28) at pH 3.5 and highest (2.18-1.61) at pH 4.5 (Table 4.4). The effect of acidulants on protein denaturation, at acidic pH was found to differ significantly (Table 4.4). Similar observation was recorded in previous experiment (Table 4.2). Whey protein solutions containing glucono-δ-lactone showed lowest A.I. values at all pH, whereas higher A.I. values are observed with other three acidulants (Table 4.4).

One of the important factors affecting the thermal behaviour of whey proteins is the amount and the state of calcium present in the system. Ca^{+2} ions are known to have stabilizing effect on whey proteins particularly α-la and β-lg (Bernal and Jelen, 1984; Ibrahim *et al.* 1995). Under acidic condition Ca^{+2} ions

Table 4.4: Effect of acidulants and pH on denaturation index (A.I.) of whey protein solution[1]

pH of solution	Denaturation index (A.I.) of solution contaning			
	Citric acid	Glucono-δ-Lactone	Lactic acid	Tartaric acid
3.5	0.28	0.23	0.27	0.390
4.0	2.17	1.04	2.15	2.24
4.5	2.61	2.18	2.58	2.56

	F value	±S.E.M.	CD at 5%
A (pH)	**	0.01	0.03
B (Acidulants)	**	0.01	0.03
A x B	**	0.02	0.06

[1] 1.5% protein in whey protein solutions.

Table 4.5: Effect of acidulants and pH on soluble protein in whey protein solution[1] ants

pH of solution	Citric acid (%)	Glucono-δ-Lactone (%)	Lactic acid (%)	Tartaric acid (%)
3.5	0.93	1.13	0.96	0.88
4.0	0.75	0.97	0.77	0.67
4.5	0.67	0.86	0.64	0.60

	F value	±S.E.M.	CD at 5%
A (pH)	**		0.02
B (Acidulants)	**		0.03
A x B	**		0.05

[1] 1.5% protein in whey protein solutions.

are chelated by citric acid, which reduces the thermal stability of α-la (Patocka *et al.*, 1986). However, at very low pH i.e. 3.5, the chelation of Ca^{+} ions may not be complete and some of the calcium may still be available for stabilization of the protein against heat induced precipitation. Polycarboxylic acids like citric and tartaric acids showed similar denaturation index (Table 4.4). GDL is neutral ester of gluconic acid. It hydrolyzes in aqueous solutions to form gluconic acid and increase in temperature favours hydrolysis (Dzieyzak, 1990). Gluconic acid and delta-and gamma-lactones exist as equilibrium mixture in solution at the end of hydrolysis. Among the four acids tested, gluconic acid contains maximum number of hydroxyl groups and hydrogen bonding between these hydroxyl groups and peptide carboxyl groups may have contributed towards structural stability. This stability may also be due to localized electrostatic interactions between ionized groups of gluconic acid and charged groups partially burried in protein interior. This interaction may be similar to that observed between charged groups present on denatured protein (β-lactoglobulin) and phosphate ions (Mcphail and Holt, 1999). Such interactions could inhibit aggregation at elevated temperatures.

4.3.2 Soluble protein

There was a significant effect of acidulant and pH of on soluble protein content of whey protein solutions (Table 4.5). Glucono-δ-lactone samples showed maximum soluble protein contents at all the three pH, whereas tartaric acid exhibited lowest soluble protein content (Fig. 4.3). Lactic acid and citric

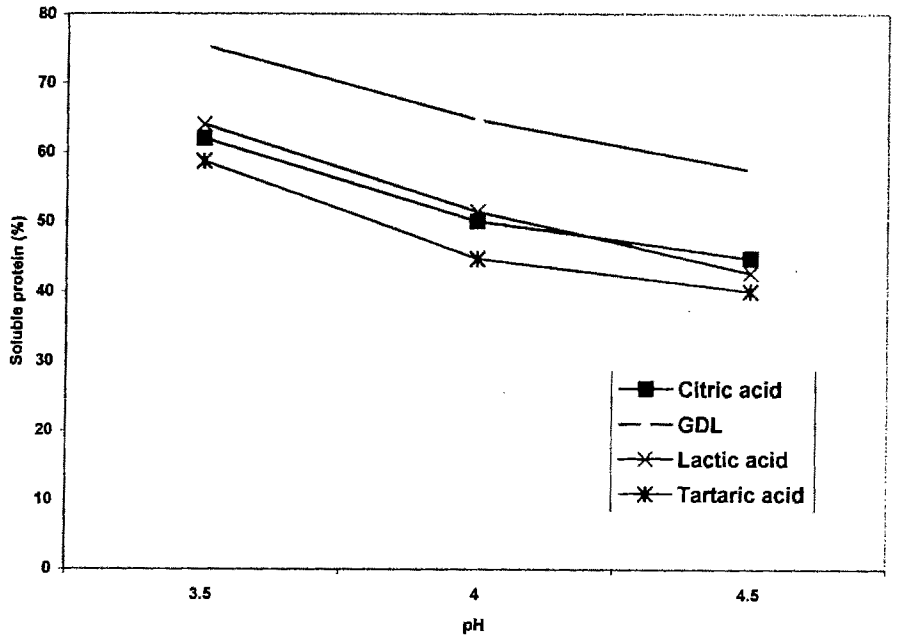


Fig 4.3 Effect of acidulant on soluble whey proteins upon heating

acid exhibited intermediary effects and there was no significant difference among these two. There was difference in the nature of precipitation and aggregation among the samples. Among acids, only tartaric acid caused turbidity at pH 3.5, where whey proteins are known to be quite heat stable. Glucono-δ-lactone samples exhibited coagulation of whey proteins at pH 4.0 and 4.5, but these aggregates were slimy and uniformly distributed throughout the solution. Glucon-δ-lactone has been reported to form soft curd with soymilk at elevated temperature and is generally used for production of in - package- sterilized silken tofu (Berry, 2001). Lactic acid and citric acid formed loose aggregates that slowly settled down at the bottom of test tubes. However, tartaric acid formed very compact, aggregates which stuck to the bottom of test tubes.

Differences in soluble protein contents at different pH as already explained in Sec 4.3.2. It is mainly related to the effect of these acids on two whey proteins i.e. α-la and β-lg. Most of the whey protein fractions which undergo denaturation during heating under acidic pH, get reversed upon cooling (Damodaran 1996). α-la undergo renaturation after heating and Ca^{+2} promote renaturation. Therefore, chelation of Ca^{+2} ion by citric acid or tartaric acid favours aggregation.

4.4 Effect of whey protein supplementation on quality of model beverage

4.4.1 Protein content of model beverage

Model beverages prepared with 4 , 5, or 6% spray dried WPC in dist water (control), acid whey (1:1 dilution) or cheese whey (1:1 dilution) contained 2.14-4.10% protein (Table 4.6). Protein content of control sample was the lowest and it was highest in sweet whey samples. The difference in protein level of model beverages was because of variations in protein content of acid and cheese whey samples. Sugar required to raise the refractometeric solids of model beverages also differed, as there was variations in TSS of the acid and cheese whey. Cheese whey has been reported to contain more soluble solids and proteins as compared to acid whey (Jayaprakasha, 1992).

4.4.2 Effect of Heating on protein content

Protein precipitation upon heating was less in model beverage of pH 3.5, and moderate to heavy in samples of pH 3.75 to 4.00. The soluble protein content of these beverages is an indicator of protein denaturation and it also indicates the effect of protein level on beverage quality. The soluble protein content of heated samples varied between 0.96 to 2.87 per cent (Table 4.7). Soluble protein content was highest at pH 3.5 and lowest at pH 4.0 in all the samples. This confirmed the observation of previous experiments (Sec. 4.2), that whey proteins are more stable at pH$\leq$ 3.5. However, gel formed in acid and cheese whey beverages at higher levels of proteins at pH 3.5 or pH 3.75 were very soft. The gel formed in acid whey beverage of pH 3.5 and 6% WPC level

Table 4.6 : Protein content of model beverages supplemented with spray dried whey protein concentrate.

Model beverage[1] based on	Protein content of beverage with		
	4% WPC	5% WPC	6%WPC
Dist. water (Control)	2.14	2.66	3.24
Acid whey (1:1)	2.58	2.95	3.84
Cheese whey (1:1)	2.72	3.15	4.10

A (Protein level)	**	CD at 5%	0.034
B (Model beverage)	**	CD at 5%	0.034
A*B(Protein x Model beverage)	**	CD at 5%	0.059

[1] T.S.S. 15%

Table 4.7 : Effect of heating and pH on soluble protein content of model beverages[1]

Beverage pH	Spray dried WPC level (%)	Soluble protein content of beverage based on		
		Dist. water (Control) (%)	Acid whey (%)	Cheese whey (%)
3.50	4	1.88	2.22	2.33
	5	2.34	2.61	2.72
	6	2.33	Gelling	Gelling
3.75	4	1.32	1.72	1.81
	5	1.83	2.08	2.13
	6	2.18	Gelling	Gelling
4.00	4	0.96	1.01	1.08
	5	1.18	1.19	1.24
	6	1.37	1.35	1.51

T.S.S.15^0 Brix, pH adjusted with 50% GDL.

was compact and transparent . Jelen (1992), had also observed that addition of dried WPC to fluid milk (pH 6.5) at 5% level caused gel formation during heating at 90°C for 5 min. In acidic conditions gelation was reported at about 2.5-3.0% whey protein level (El-Etriby, 1996; Abd-El-Salam and El-Etriby, 1996). They also observed that Ca^{+2} ions play an important role in gelation process. The variation in Ca^{+2} content of acid and sweet whey might have affected the firmness of gel. Hongsprabhas and Barbat (1996) reported that pre-heating of whey protein above 80°C resulted in higher strength of Ca^{+2} induced gelation of whey protein isolate. In the present investigation since WPC was prepared by spray drying process, protein molecules were partially denatured during manufacturing process. They cause gel formation under acidic conditions and in the presence of sugar. Sucrose has been shown to possess unique ability to increase the protein-protein interaction in unfolded (i.e. denatured) protein molecules that produce stronger gel (Kulmyrzaev *et al.*, 2000).

4.4.3 Sensory quality

All the nine beverages, containing 4% WPC level were acceptable (Overall acceptability score $\geq$ 3.0 out of 5.0), but scores (3.4 to 4.6) were higher at pH 3.75. Samples prepared with 5 or 6% WPC levels were not liked by panelists because of the presence of predominant whey flavour .

Samples having pH 4.0 were also not acceptable because of sedimentation problem. Samples with pH 3.5 were sparkling clear and its score was at par with samples of pH 3.75. Control samples scored maximum (2.1-

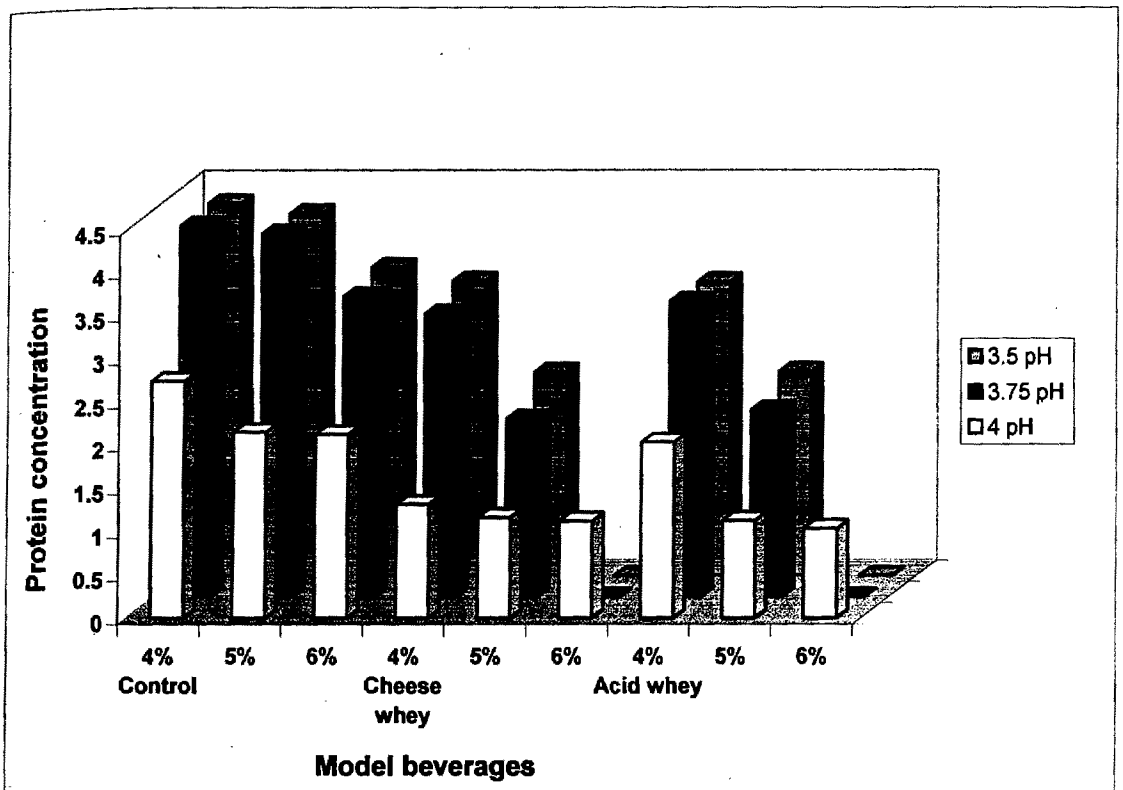


Fig 4.4 Effect of spray dried whey protein suplementation on sensory score of model beverages

4.6) at all WPC levels and pH. Model beverages containing 6% WPC and pH 3.5 or 3.75 formed gel upon heating, hence their sensory score was taken as zero. (Fig 4.4).

4.5 Kinetics of protein denaturation in model beverages

4.5.1 Denaturation index

Denaturation of whey proteins in model beverage having pH 3.5 or 4.0 during heating at 75°, 85° and 95° were observed. Substitution of data into zero and first order reaction models yielded coefficient of regression (R^2) values ranging from 0.978 to 0.999 and 0.989 to 0.999, respectively (Table 4.8). The standard error of estimates (S.E.E.) values for zero order reaction (0.0008 to 0.0130) was lower than first order model (0.064 to 0.0130) which shows that AI followed zero order reaction at pH 3.5 as well as 4.0 at pH (Table 4.8).

Plot between reaction rate constants (ln k) and inverse of absolute temperature yielded a straight line at pH 3.5, but at pH 4.0 nature of curve was different (Fig. 4.5). There were two straight lines between 75°-85° and 85-95°C. The activation energy (Ea) obtained from Arrhenius equation was 53.41 KJ/mole of protein, and the value of Arrhenius constant (K_o) was 8.29×10^6 (MW/K) (Table 4.8). The activation energies for the curve between 75°-85° and 85°-95° were 534.71 and 142.65KJ/mole of proteins with corresponding Arrhenius constants of 8.29×10^6 and 1.45×10^{19} (M W/k). The activation energy at pH 4.0 was 75.62 (75-95°C) KJ/mole of protein, having arrhenius constant value of 1.55×10^9 (M W/k), however, the r^2 value was much less i.e.

Table 4.8: Reaction kinetics parameters for denaturation index of whey proteins in model beverage

Model beverage	Temperature	Rate constant K^{-1}	R^2	S.E.E.	Rate Constant K^{-1}	R^2	S.E.E.	Activation energy kJ/mol.	Arrhenius constant	R^2
pH 3.5	75	0.0082	0.999	0.0024	0.01743	0.990	0.0121	53.41	8.29×10^6	0.99
	85	0.0126	0.978	0.0130	0.01434	0.989	0.010			
	95	0.0224	0.999	0.004	0.0121	0.999	0.0007			
pH 4.0	75	0.0056	0.999	0.0008	0.009247	0.999	0.0006	75.62 (75-95°C)	1.55×10^9	0.78
	85	0.0222	0.999	0.0032	0.02081	0.992	0.0130	534.71 (75-85°C)	8.29×10^9	1.00
	95	0.0229	0.995	0.0110	0.002178	0.997	0.0064	142.65 (85-95°C)	1.45×19^{19}	1.00

Table 4.9 : Reaction kinetics parameters for denaturation index of whey proteins in model beverage

Model beverage	Temperature (°C)	Zero order reaction			First order reaction			Arrhenious Parameters		
		Rate constant (min^{-1})	R^2	S.E.E.	Rate Constant (min^{-1})	R^2	S.E.E.	Activation energy (KJ/mol.)	Arrhenious constant	R^2
pH 3.5	75	-0.0180	1.00	0	-0.00981	0.998	0.0022	44.51	8.85×10^4	0.946
	85	-0.0210	0.993	0.0122	-0.01285	0.998	0.00365			
	95	-0.0243	0.975	0.0273	0.02358	0.993	0.0139			
pH 4.0	75	-0.0152	0.999	0.00249	-0.00743	0.997	0.0024	31.84	8.27×10^6	0.988
	85	-0.0088	0.962	0.01224	-0.00513	0.968	0.0065			
	95	-0.0069	0.903	0.0159	0.02358	0.908	0.0091			

0.78. Hence, arrhenius type of equation did not predict thermal kinetics at pH 4.0.

The difference in the nature of curve at two pH may be attributed to the relatively high thermal stability of whey proteins at pH 3.5. Moreover, temperature also has influence on denaturation, and whey protein denaturation starts at around 70°C and complete denaturation and aggregation occur only at very high temperature. At lower temperature, in acidic conditions most of the whey protein undergo renaturation (Bernal and Jelen, 1985). Thermal kinetics of individual whey proteins has been reported to follow different orders of reaction because of the wide variation in methodology and conditions in which experiments were performed (Harwalker, 1986; Park and Lund, 1984; Anema, 2001).

4.5.2 Soluble protein

Heating of model beverage resulted in protein denaturation and consequent loss of soluble proteins. The loss of soluble proteins was more at pH 4.0 and at higher temperature. The coefficient of regression for zero order reaction values varied from 0.903 to 0.999 and 0.975 to 1 at pH 4.0 and pH 3.5 respectively. The corresponding values for first order reactions were 0.908-0.997 and 0.993 to 0.998, however, later gave a much lower standard error of estimates (S.E.E) values (Table 4.8). Hence protein loss followed a first order reaction.

The activation energy (Ea) values were 46.51 KJ and 31.84 KJ/mole of protein and Arrhenius constant was 8.27×10^6 and 8.83×10^4 at pH 4.0 and pH

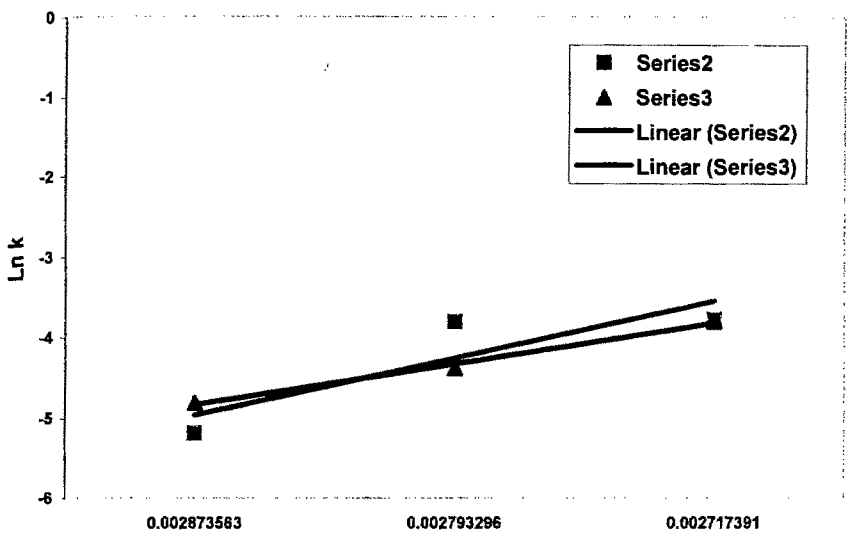


Fig 4.5 Arrhenious Plot for Protein Denaturation in Model Whey protein Beverage

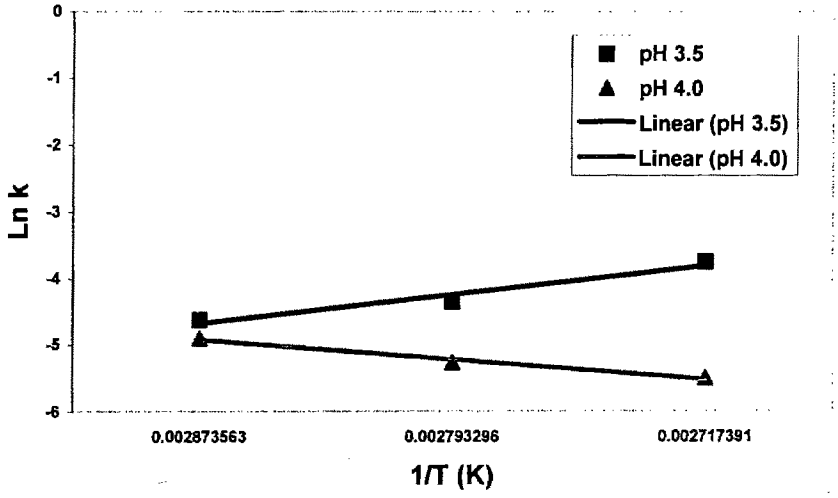


Fig 4.6 Arrhenious Plot for Loss of Soluble Protein in Model Whey Beverage

3.5 respectively. A lower Ea indicates less thermal resistance of proteins at pH 4.0 as compared to pH 3.5 (Fig 4.6).

High thermal stability of whey proteins has already been demonstrated at low pH i.e. 3.5. Thermal denaturation and aggregation are two different phenomenon and aggregation occurred only at very high temperatures, resulting in complete loss of protein solubility. Denaturation of β-lactglobulin has been reported to follow first order reaction kinetics (Harwalkar, 1980, de Wit and Swinkles,1980), with a relatively higher Ea of 341 KJ/mol. α-lactalbumin is also reported to follow first order reaction kinetics (Harwalker, 1986, Dannenberg and Kersler, 1998; Anema 2001).

4.6 Protein - Polysaccharide complex formation

4.6.1 Complex formation between partially denatured whey proteins and polysaccharides

During preliminary experiments, complex formation between partially denatured whey proteins and two acidic polysaccharides, namely carboxymethyl cellulose (CMC) and pectin and one sulphated polysaccharides i.e. carrageenan was explored by taking WPC and polysaccharides in 1:1 ratio in a 10% WPC solution.

4.6.1.1 Protein content of complex

Protein contents of the protein-polysaccharide complex were 44.82, 45.41 and 48.72% with pectin, CMC and carrageenan respectively (Table 4.10). Both acidic polysaccharides gave complexes with lower protein content than sulphated polysaccharide. The variations in protein contents may be due to

Table 4.10: Properties of Protein- Polysaccharide Complex

Complex	Protein content (%)	Soluble protein (%)[2]	Thermal stability[1]
CMC- WPC Complex	45.81	87.53	Fair
Carrageenan- WPC Complex	48.72	59.93	Poor
Pectin – WPC Complex	44.82	75.02	Fair

[1] 2% complex solution at pH 4.0

[2] Percentage of whole protien

the differences in their reactivity and interaction with partially denatured whey proteins. At neutral pH, weak attractive interactions occurs between acidic polysaccharides and proteins carrying a net negative charge (Ferandes, 1995). Even, if both polymers carried the same net charge, there is still a possibility for localized electrostatic attractions between the protein and polysaccharide molecules. Snoeren *et al.* (1975) had shown that electrostatic interaction between k-carrageenan and k-casein occurs even on the alkaline side of the isoelectric point of the proteins due to non-random distribution of anionic and cationic groups on the polypeptide chains. Similar is the case of pectin (Samant *et al.* , 1993). Chen *et al.* (1989) observed that for optimum complexation between whey proteins and polysaccharide, the molecular weight of polysaccharide should be higher than 100 K Da. For this, degree of substitution of CMC should be close to 0.9, but the degree of substitution of CMC used for this experiment was about 0.4. Mann and Malik (1996) reported protein contents of CMC-WPC complex in the range of 57.70-64.27%, which is higher than those obtained in the present investigation. This variation attributed to the differences in methodology of complexation.

4.6.1.2 Soluble protein content

Protein content of supernatant indicates the protein not complexed by polysaccharides and is present in soluble form. The amount of protein in supernatant of 1% complex solutions were 59.93%, 75.02%, and 87.53% for WPC-carrageenan, WPC-pectin and WPC-CMC complexes respectively (Table 4.10). Mishra (1999) had reported 90.34 and 70.73% soluble protein for

WPC-Pectin and WPC-CMC complexes. These values are higher than those obtained in present study because slightly modified method was used for complexing and polysaccharides used had different properties.

4.6.1.2 Thermal stability

Thermal stability of 2% solution of acidic polysaccharides complex (CMC and Pectin) was fair as compared to poor stability of carrageenan complex at pH 4.0 (Table 4.10). The carrageenan-whey protein complex gets coagulated rapidly during heating. Carrageenan complex, when dispersed in water and allowed to hydrate, formed a gel even at room temperature. Fernandes (1998) had reported similar observation. Since, in the present study a acid stable complex was not observed with carrageenan, hence further experiments were carried out with only acidic polysaccharides.

4.6.2 Effect of heating on interaction between whey proteins and polysaccharides

4.6.2.1 Soluble protein content

Soluble protein content for pectin-WPC complex was 67.52, 47.26 and 60.18% for T_1 (Control), T_2 (heating at 50°C for one hour) and T_3 (heating at 80°C for one hour) treatments, respectively (Table 4.11). The corresponding values for CMC-WPC complex were 72.35, 57.85 and 54.60%, respectively. There was significant ($P \leq 0.01$) difference among the treatments and also between the two types of complexes. Soluble protein content of pectin-WPC complex was low which shows that complexation between the two biopolymers were strong in samples heated for 1 hour at 50°C. Similar was results when

Table 4.11 : Properties of CMC- WPC and Pectin- CMC complex produced by heating

Complex	Soluble protein content (%)	Solubility (%)	Thermal stability[1]
CMC- WPC complex			
T_1	72.85	63.59	Poor
T_2	57.85	72.25	Average
T_3	54.60	93.41	Average
Pectin- CMC complex			
T_1	67.52	58.70	Poor
T_2	47.26	82.10	Good
T_3	60.18	80.10	Excellent
CD at $P \leq 0.01$	2.47	0.68	

[1] 2% solution of complex

T_1: Unheated (Control sample),T_2: Heating at 52^0 C for 1 hour,T_3: Heating at 80^0 C for 1 hour

CMC was complexed by heating at 80°C for one hour. Results also indicate that heating of whey protein concentrate and acidic polysaccharide solutions favours interaction between them and complex formed were stable. During heating the interaction of the carboxyl groups of polysaccharides takes place with some or all of the positively charged protein residues. Strength of such interactions is related to the number and distribution of these sites as well as the overall charge on the protein molecules (Samant *et al.*, 1993). Heating denatured proteins exposed the buried basic groups. Flexibility of random coil in denatured state permits configurational adjustment to maximise the interaction. As a result of above changes, complex formed with denatured proteins are more stable than the ones formed with native protein. The differences in degree of coplexation between these two polysaccharides may be because of differences in the number of groups involved in complex formation. The CMC contains more carboxyl groups than high-methoxyl pectin. Mishra (1999), reported that heating favoured complex formation between CMC as well as pectin with whey protein concentrate. Pectin showed maximum interaction upon heating. Heating of pectin in aqueous conditions might have adverse effect on their structural integrity.

4.6.2.2 Solubility of polysaccharide- protein complexes

Solubility of WPC-pectin complex was 58.70, 82.10 and 80.10% for treatment T_1, T_2 and T_3 (Table 4.11). The corresponding values for WPC- CMC complex were 63.59, 72.25 and 93.41%. The solubility differed significantly among heat treatments and also between two types of complexes. Heating was

found to increase the solubility of pectin-WPC complex and there was slight difference in solubility between T_2 and T_3. However, complex formed in treatment T_2 had maximum solubility. Similar solubility of CMC-WPC complex increased with heating. CMC-WPC complex formed by T_3 was most soluble. Mishra (1999) had also reported that heating improved solubility and WPC-CMC complexes were more soluble than WPC-pectin complexes at acidic as well as at neutral pH.

It shows that protein-anionic polysaccharide complex formation increases solubility of protein and stability of partially denatured protein. Similar results have reported other workers (Payens, 1971; Ledward 1979; Xie and Hettiarachdy, 1997).

Soluble complex formed between acidic polysaccharides and proteins, which are further stabilized by electrostatic interactions and hydrogen bonding, due to interaction between macromolecules carrying same charges. Soluble protein- anionic polysaccharide complexes generated as a result of interaction between those carrying the same or opposite charges (Tolstoguzor, 1974; Sorenson *et al.*, 1974).

4.6.2.3 Viscosity

Viscosity of WPC-CMC complex and WPC-pectin is presented in Fig. 4.7. In both the cases there was gradual increase in viscosity upon heating. The viscosity of CMC-WPC complex is lower than pectin-WPC complex. Viscosity of CMC depends upon degree of substitution (DS) and the CMC, which was used, had DS of 0.4, and it corresponds to high viscosity. Heating of

protein-polysaccharide mixture might have resulted in partial unfolding of protein molecules with a corresponding increase in viscosity (Mann and Malik, 1996).

4.6.2.4 Thermal stability

Heating had a pronounced effect on the thermal stability of complexes at acidic pH (pH 4.0). Control samples of both complexes were least heat stable and got precipitated on heating. WPC-pectin complex produced by heating showed no coagulation during heating, but sedimentation was observed on cooling and subsequent storage (Table 4.11). CMC-WPC complexes produced by different treatments were more heat stable than pectin-WPC complexes. Mishra (1999) had also reported that CMC-WPC complex were more heat stable than pectin-WPC complex. Chen *et al.* (1989) patented a process, where heating of complex at 80°C to produce a stable complex.

4.6.3 Effect of pH on complex formation between whey proteins and acidic polysaccharides

4.6.3.1 Soluble protein content

Percentages of soluble proteins in pectin-WPC complex were 49.91, 59.27 and 48.76% at pH values of 3.5, 5.5 and 7.0 respectively. (Table 4.12). The values for CMC-WPC complex ranged between 42.18 to 68.21%t. pH was found to exert significant effect on complex formation and both polysaccharides and their protein content differed significantly. High soluble protein content shows less complexing. Therefore, pectin-WPC complex formation was maximum at pH 7.0 and it was followed by at pH 3.5. There was

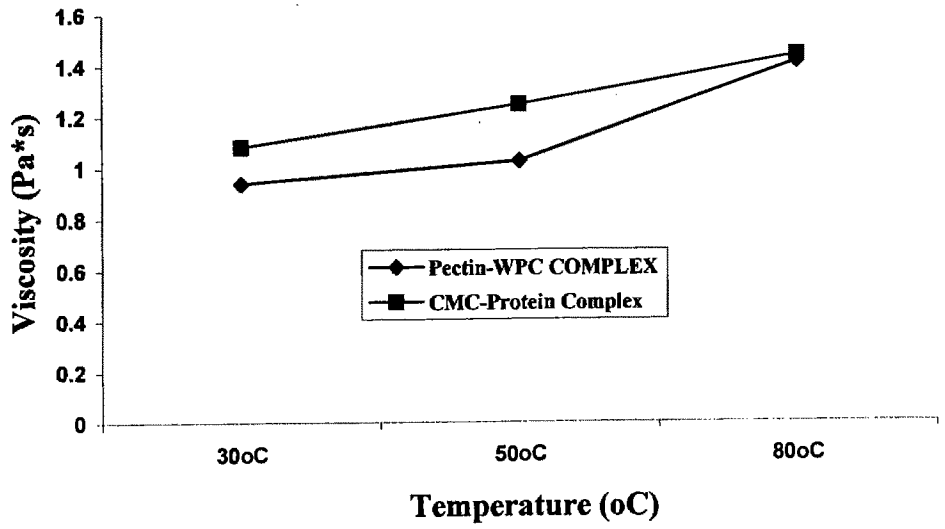


Fig. 4.7: Effect of Heating on Rheological behaviour of Whey protein-Polysaccharide Complex

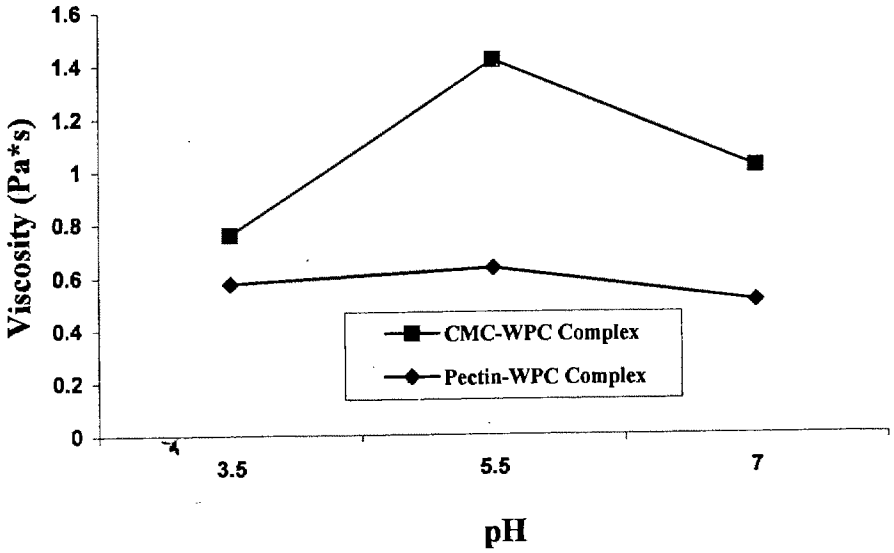


Fig. 4.8: Effect of pH on viscosity of Whey protein-Polysaccharide Complex

Table 4.12 : Properties of CMC- WPC and Pectin- WPC complexes as affected by pH of complexation

Complex	pH	Soluble protein content (%)	Solubility (%)	Thermal stability
CMC-WPC complex	3.5	42.18	36.89	Very poor
	5.5	68.21	58.23	Good
	7.0	58.95	93.14	Excellent
Pectin-WPC complex	3.5	49.91	71.92	Average
	5.5	59.27	82.14	Average
	7.0	48.76^{ab}	81.19^{a}	Good
CD at 1%		6.01	1.05	

no significant difference between these two treatments. There was least complex formation at pH 5.5. The maximum interaction of CMC with whjey proteins occurred at acidic pH i.e. 3.5 followed by at neutral pH.

Pectin exhibit affinity with bovine serum albumin over a wide range of pH values (3-8) and ionic strength, which breaks down only on addition of urea. Hence, the affinity between the two polymers is believed to be non-electrostatic (Samant *et al.*, 1993). It is difficult to ascertain the exact nature of interaction, which was involved in complexation over such a wide range. pH of solution was reported to affect the interaction between the two biopolymers and also to the nature of resulting complex (Samant *et al.*, 1993, Dickinson, 1998).

4.6.3.2 Solubility

Solubility of pectin-whey protein complex was 71.92, 82.14, 81.19% at pH 3.5, 5.5 and 7.0 respectively (Table 4.12). These values differed significantly. Solubility of CMC-WPC complex was 36.89, 58.23, and 93.14% (Table 4.12), which differed significantly. Least soluble complex was formed at acidic pH in both the cases. However, the solubility of CMC-WPC complex formed at acidic pH was lower that of pectin-WPC complex. This unique property of complex formation with desired solubility by varying pH has been reported by several workers (Grinrod and Nickerson, 1968; Hill and Zadow, 1978). Solubility of complex usually depends upon the structure of polymers involved and on the solution conditions, i.e, pH, and ionic strength (Dickinson, 1998). Whey proteins have been reported to form insoluble complex at acidic

pH and this interaction was utilized to recover whey proteins from dilute solutions of cheese whey (Hansen *et al.*, 1971; Morr *et al.* 1973; Sternberg *et al.*, 1976; Mann, 1992).

Solubility of Polysaccharide-WPC complex was found to increase with an increase in pH (Table 4.12). The enhanced solubility of complex at higher pH may be because net negative charges formed on both polymers above the isoelectric point of proteins. This caused interaction between positively charged local patches on the proteins and acidic polysaccharides. The residual amounts of complexing agents, which are perhaps present as anionic species, had no effect on the solubility of WPC at pH above the isoelectric point, where these proteins were also carrying negative charge (Mann and Malik, 1996).

4.6.3.3 Viscosity

Viscosity of pectin-WPC complex was 0.57, 0.63 and 0.50 Pa*s at pH 3.5, 5.5 and 7.0 respectively (Fig 4.8). The maximum viscosity was observed at pH 5.5 followed by acidic pH. Complex was least viscous at neutral pH, the pH at which it showed highest thermal stability (Table 4.12). Viscosity of CMC-WPC complex varied from 0.55 Pa*s to 1.418 Pa*s. Minimum viscosity for CMC-WPC was observed at acidic pH i.e, 3.5 (Fig. 4.8). At this pH, maximum complexation occurred, where interaction was minimum. This supports the hypothesis that viscosity of protein- polysaccharide is mainly governed by availability of free polysaccharide. The viscosity of pectin-WPC complex was less as compared to CMC-WPC complex. Glahn (1982) had noticed that viscosity was minimum at the point of maximum stabilization because of

strong complexation between casein and acidic polysaccharides. Ganz (1974) reported similar results for protein-CMC system.

4.6.3.4 Thermal stability

Both CMC-WPC and Pectin-WPC complex formed at neutral pH were highly heat stable (Table 4.12). The stability of CMC-WPC complex formed at pH 5.5 was fair and very poor at pH 3.5. However, pectin-WPC complex formed at pH 5.5 and 3.5 were heat stable, but get sedimented upon cooling. Covalent complexes formed over the iso-electric point of proteins are very strong and essentially permanent (Dickinson, 1998).

4.7 Hydrocolloid stabilization of whey protein suspension of low pH

4.7.1 Screening of stabilizers

In the first part of this experiment various types of stabilizers were screened for their stability at pH 4.0, where whey proteins were reported to be less stable and pH 3.5, the point of high protein stability. The reactivity of whey proteins at two acidic pH is summarized in Table 4.12. Carboxymethyl cellulose (CMC) showed different kind of reactivity at these two pH. It formed a soluble, heat stable complex at pH 4.0, whereas an insoluble complex was formed at pH 3.5 (Plate 4.1) which underwent heavy denaturation upon heating. In a similar study Hidalgo and Hansen (1971) had found that complexes were least soluble, when the proteins and polyanions are mixed in appropriate quantity and pH was adjusted to below their isoelectric points.

Table 4.13: Nature of interaction between hydrocolloids and 1% whey protein solution at two acidic pH

Hydrocolloid	Type of interaction at	
	pH 3.5	pH 4.0
Carboxyl methyl cellulose (CMC)	Insoluble complex	Soluble complex
Carrageenan	Phase separation	Heavy precipitation
Guar gum	Soluble complex	Slight precipitation
Pectin	Soluble complex	Soluble complex
Propylene glycol alginate (PGA)		
a. Manucol	Soluble complex	Soluble complex
b. Kelkoid	Soluble complex	Soluble complex
STAB PX	Phase separation	Phase separation
Modified starch	Precipitation	Precipitation
Sodium alginate	Insoluble complex	Precipitation
Xanthan gum	Insoluble complex	Precipitation

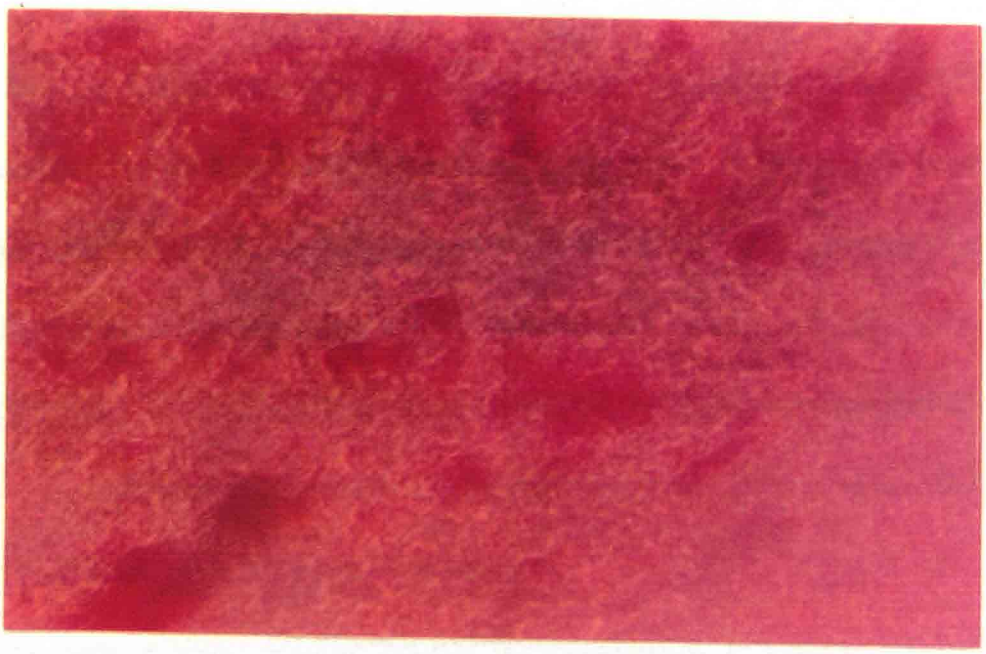


Plate 4.3 : **Interaction between pectin and whey proteins in model beverage**

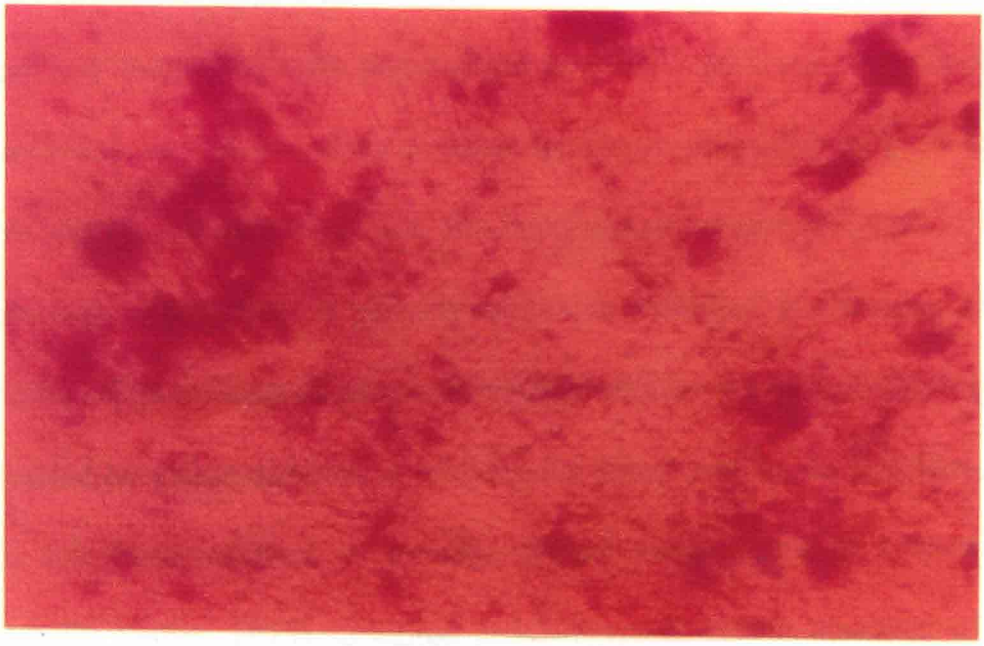


Plate 4.4 : **Interaction between PGA and whey proteins in model beverage**

In this study, carrageenan exhibited phase separation at pH 3.5 and further heating led to the formation of heavy aggregates (Plate 4.2). Carrageenan and whey protein solutions were quite miscible at pH 4.0, but it also precipitated heavily. Carrageenan specially k-form had been reported to be very much effective in preventing the precipitation of casein and other plant proteins (Hansen, 1968, Chakraborty and Randolph, 1972). K-carrageenan and whey proteins form complex through electrostatic attraction at neutral pH (Sec. 4.5.1) but high ionic strength breaks the complex.

Guar gum, a water soluble neutral polysaccharide, is compatible with salt and water soluble proteins (Glicksman, 1969). The guar gum whey protein solutions remained soluble and heat stable at pH 3.5. But slight precipitation was observed during heating at pH 4.0. Being neutral polysaccharide, it should not undergo any interaction with proteins but possibility of local interactions may not be ruled out. Since, whey proteins itself are very stable at pH 3.5, hence there was no precipitation at this pH. Denaturation of whey proteins and their subsequent aggregation at pH 4.0 might get inhibited in presence of polysaccharide. This stability as observed visually may be because of physical action imparted by galactomannan in keeping the whey protein in solutions.. Guar gum has been reported to promote partial denaturation at a lower temperature (Fernandes, 1998). Grindrod and Nickerson, (1968) observed co-precipitate formation on addition of guar gum, which resisted sedimentation by centrifugation. They, through polyacrylamide gel electrophoresis revealed no electrostatic/ionic interaction between any milk proteins and guar gum.

Pectin formed soluble complex with whey proteins at pH 3.5 and 4.0, indicating its protective colloidal, action. The product was clear and with no coagulate or aggregates (Plate 4.3). However, degree of esterification has pronounced effect on the nature of interaction (Tolstoguzov *et al.*, 1981).

Among the alginates, propylene glycol alginate (Mannucol and Kelcoid) were found to be more effective than sodium alginate, in preventing the whey protein precipitation and in their ability to form soluble complex (Table 4.13). No coagulation or sedimentation was observed with propylene glycol alginate (Plate 4.4). However, samples treated with kelkoid LVF (Table 3.1) showed sediment formation and milky appearance of resulting whey when kept at refrigeration temperature overnight. The apparent difference between two commercial PGA may be because of differences in their esterification.

PGA interacts with whey protein like other anionic polysaccharides and form soluble complex. On contrary to this, sodium alginate under acidic conditions formed insoluble complex with proteins (Imeson, 1984) and the interaction is considered to be electrostatic in nature (Ganz, 1974). The strength of interaction between proteins and alginate increases to maximum as the pH is reduced and under these conditions alginate was even more effective than CMC and pectates in removal of whey proteins from cheese whey (Imeson *et al.*, 1978). The poor stability of sodium alginate-protein complex most probably occurred because of destabilization effect of alginate. Whey proteins have been reported to form stable high molecular weight complexes with sodium alginates during low temperature under acidic conditions (Sime, 1990).

Modified starch and a commercial fruit juice stabilizer (STAB PX), essentially a guar gum and gum arabic based hydrocolloids were ineffective in preventing the protein precipitation (Table 4.13). Starch might have undergone acid induced hydrolysis during heating causing breakdown of polymer, while gum arabic has been shown to be incompatible with milk proteins (Grindrod and Nickerson, 1968).

Xantham gum formed insoluble complexes at pH below isoelctric point (i.e.5.2) of whey protein (Table 4.13). Though xanthan gum is compatible with proteins at neutral pH, but heating under acidic conditions it has been reported to cause precipitation with some proteins, such as dairy proteins (Urlacher and Dalbe, 1992).

4.7.2 Suspension stability and sedimentation

High suspension stability and low sedimentation values indicate the tendency of soluble and heat stable complex formation between whey proteins and polysaccharides in acidic solutions.

OD value of pectin stabilized solution increased linearly with an increase in pectin concentration in the range of 0.5 to 1.5% concentration at pH 3.5, (Fig. 4.9). But at pH 4.0 this trend was observed only upto 1.0% concentration; any further increase in pectin concentration decreased suspension stability. Reverse trend was observed in case of sediment formation. Beverage of pH 4.0 having, 1% pectin concentration had same OD as the beverage of pH 3.5 with 1.5% pectin. It shows that low pH of beverage necessitated use of higher amount of stabilizer to achieve same degree of

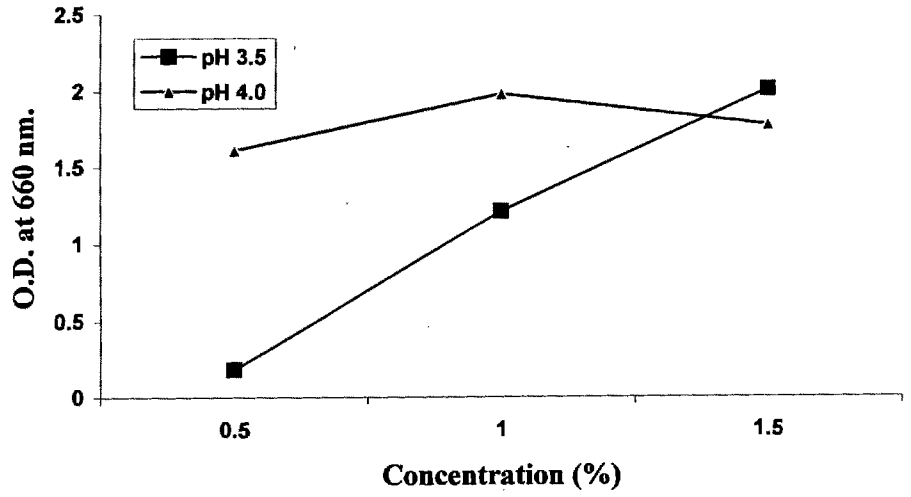


Fig. 4.9: Effect on Pectin Concentration on Suspension Stability of Model Whey Protein Beverage.

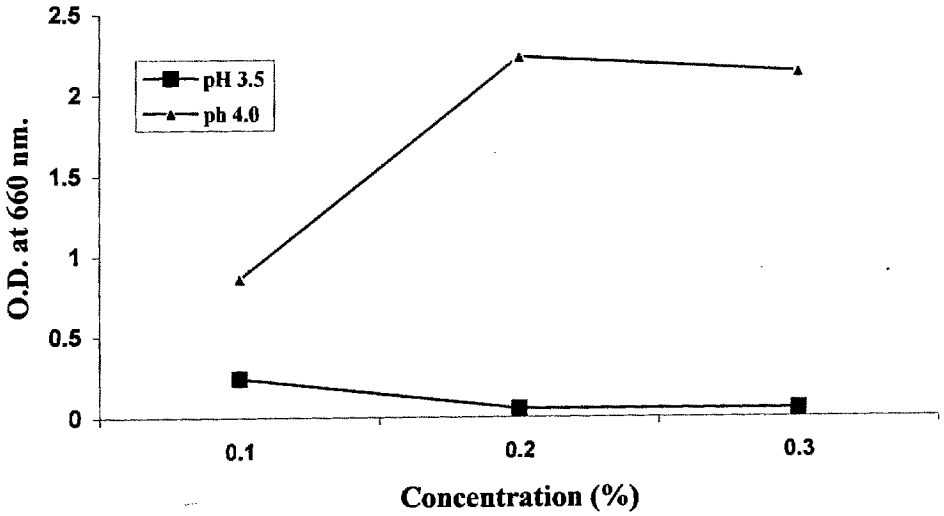


Fig. 4.10: Effect of PGA concentration on Suspension Stability of Model Whey Protein Beverage.

thermal stability. Glahn (1982) had also reported shifting of stability value towards higher pectin concentration at extremely low pH i.e. 3.5. He explained this phenomenon on the basis of partial suppression of dissociation of galacturonic acid molecules in pectin chain and higher positive charges on protein molecules. To neutralize this positive charge a higher pectin concentration is accordingly necessary.

A decrease in OD of beverage of pH 4.0 upon addition of more than 1.0% pectin (Fig 4.9) and a corresponding increase in sedimentation value (Fig 4.13). This can be explained by disturbed equilibrium between charged particles that was found to favours denaturation of proteins and subsequent aggregation.

PGA unlike pectin was effective in preventing the precipitation of whey proteins only at pH 4.0. At pH 3.5, there was insoluble complex formation and heavy sedimentation upon centrifugation (Fig. 4.13). At pH 4.0, the protective action of PGA was pronounced even at low (0.2-0.3%) concentrations in comparison to 1.0% of pectin. The distinguishing feature between two polysaccharides can only be explained on the basis of their configuration and amount of charged group. PGA molecules contain more ester groups and self-association of polymer chains becomes harder in presence of relatively high concentrations of hydrogen ions and calcium ions (Gerlat, 2000). PGA was not able to stabilize whey protein suspension at pH 3.5 and a very heavy precipitation was observed as in case on sodium alginate. Under acidic conditions alginate chain might have undergone depolymerization and this also

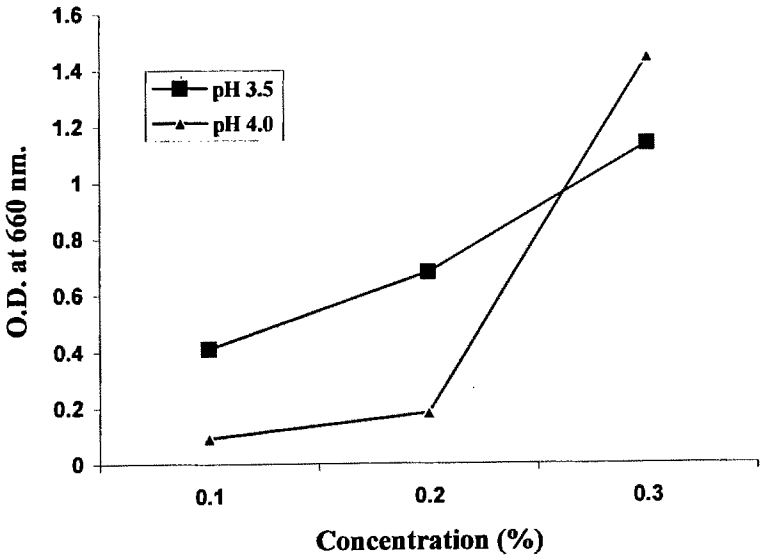


Fig. 4.11: Effect of Guar gum Concentration on Suspension Stability of Model Whey protein Beverage.

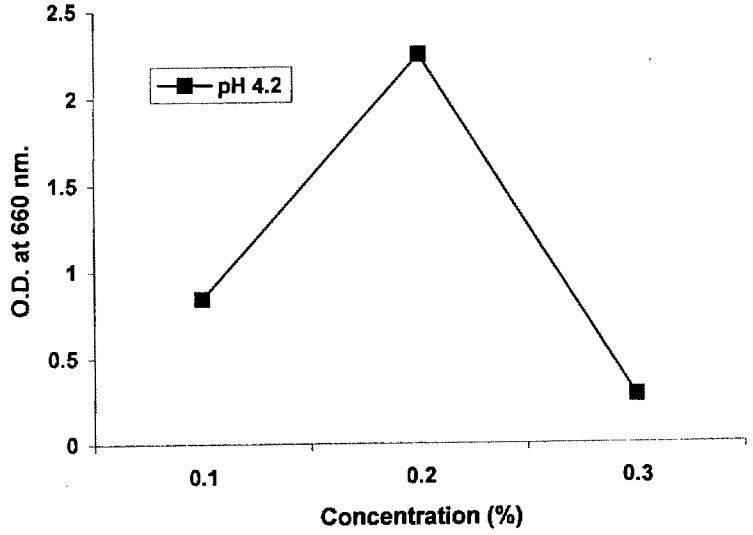


Fig.4.12: Effect of CMC concentration on Suspension Stability of Model Whey Protein Beverage.

disturbed the charge balance causing protein precipitation or PGA might have formed insoluble complex as sodium alginate do at extremely low pH (Sime, 2000).

CMC at 0.1-0.3% level did not stabilized whey protein solution at any of the two pH i.e. 4.0 and 3.5 (Fig 4.12). At pH 4.2, concentration at 0.1% did not stabilise the suspension and a sediment was formed. Further increase in CMC level to 0.2 was found to solubilise proteins and at this pH (4.2), CMC was as effective as pectin or PGA in preventing the whey proteins sedimentation during heating. In beverages at pH 4.0, initially there was very less coagulation on thermal treatment, but as temperature was lowered sedimentation took place. This phenomenon commonly referred as "peptization" occurred when concentration of stabilizer was increased from the optimum concentration required to develop insoluble complex (Hansen, 1982). This activity involved redistribution of protein molecules on the polysaccharide chain, giving rise to increased hydration and thus solubilization (Hidalgo and Hansen, 1969).

Guar gum was not effective in preventing the precipitation and aggregation at pH 4.0, which was reflected by low O.D. value and large amount of sediment (Fig.4.11). Though, suspension stability was poor at pH 3.5 also; there was no visual precipitation. Actually, both whey proteins and guar gum formed translucent solution as compared to milky opaque solutions formed by other polysaccharides.

4.7.3 Rheological behaviour of model whey protein beverage

The flow properties of model beverages, where stabilizers were utilized to prevent protein precipitation, were determined using 11 system and applying shear rate in the range of 700-1000 s^{-1} (Table 4.14). Their viscosity was found to be influenced by nature and concentration of stabilizer. All the beverages exhibited non-newtonion behaviour (Fig 4.14-4.17)except the one's where stabilizer used were PGA of 0.2 and 0.3% at pH 4.0 and 0.3% at pH 3.5, CMC of 0.1-0.3% at 4.2 or gaur gum of 0.3% at pH 3.5 or 0.1-0.3% at pH 4.0. In these cases, values of flow behaviour index were 1 or very close to it (Table 4.14). In all other beverages value of flow behaviour index values varied between 0.526 to 0.968 (Table 4.14). No correlation was observed between stabilizer concentration and flow behaviour index (n). But higher suspension stability corresponded with higher flow behaviour index and lower apparent viscosity of that concentration (Table 4.14).

In this study, the viscosity was higher in the beverages having stabilizer concentration was insufficient to stabilize proteins or where no interaction took place between the two polymers. Higher temperature caused protein denaturation which resulted in unfolding of polypeptide chains and hence in an increase of viscosity (Mann, 1991, Damodaran, 1998). Free stabilizer, which had not formed complex with whey proteins, may also contributed towards increased viscosity. Glahn (1982) observed that sour milk drinks without hydrocolloid or insufficient concentrations of stabilizer were rather viscous.

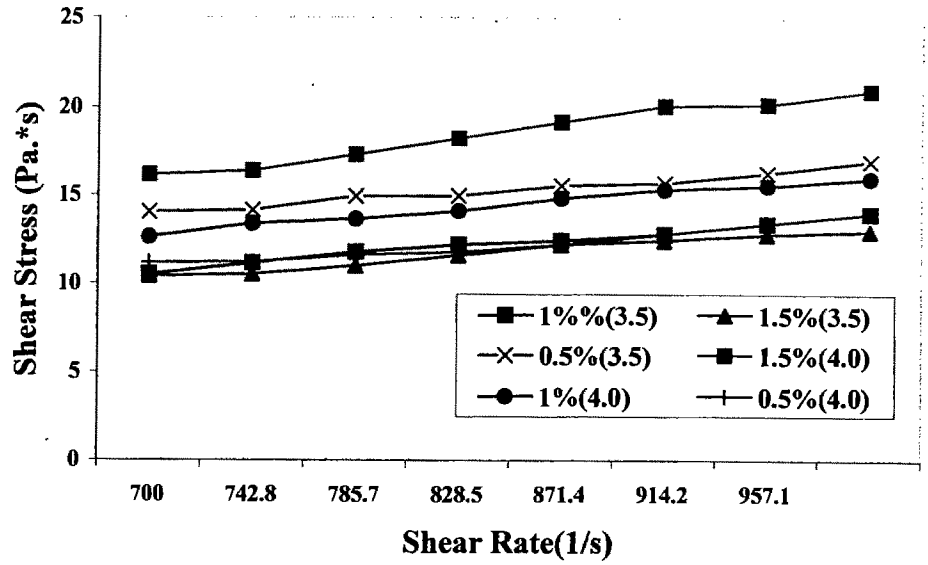


Fig. 4.14: Effect of pectin concentration and pH on rheological behaviour of model whey protein beverage

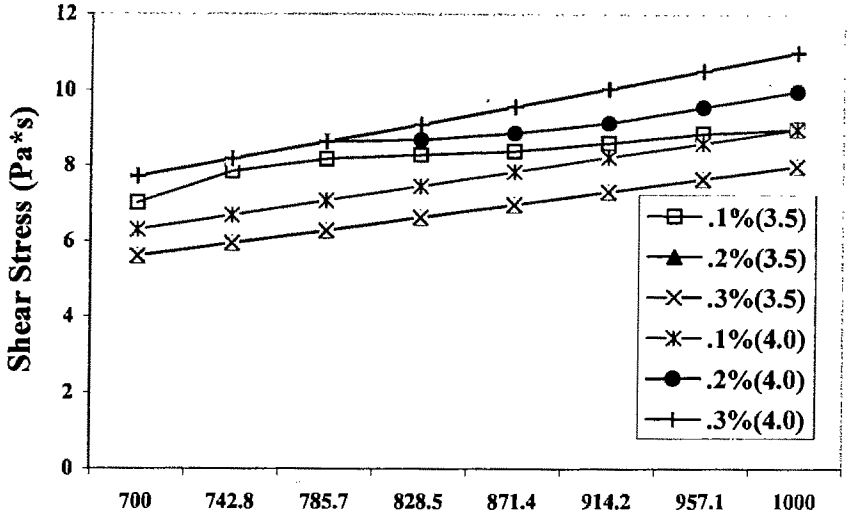


Fig. 4.15: Effect of PGA concentration and pH on rheological behaviour of model whey protein beverage

Table 4.14: Effect of nature of hydrocolloids and their concentration on rheological properties of model whey protein- enriched beverage.

Hydrocolloid	pH 3.5		pH 4.0	
	Consistency index (K)	Flow behaviour index (n)	Consistency index (K)	Flow behaviour index (n)
Pectin (%) Concentration				
0.5%	0.44	0.526	0.15	0.652
1.0%	0.11	0.698	0.16	0.664
1.5%	0.07	0.751	0.08	0.80
PGA (%)				
0.1	0.011	0.968	0.140	0.60
0.2	0.105	0.656	0.008	1.00
0.3	0.011	1.00	0.11	1.00
Guar gum				
0.1%	0.005	0.999	0.007	1.000
0.2%	0.045	0.713	0.008	1.015
0.3%	0.007	1.00	0.011	0.988
CMC				
0.1	-	-	0.100	1.001
0.2	-	-	0.009	1.00
0.3	-	-	0.012	0.99

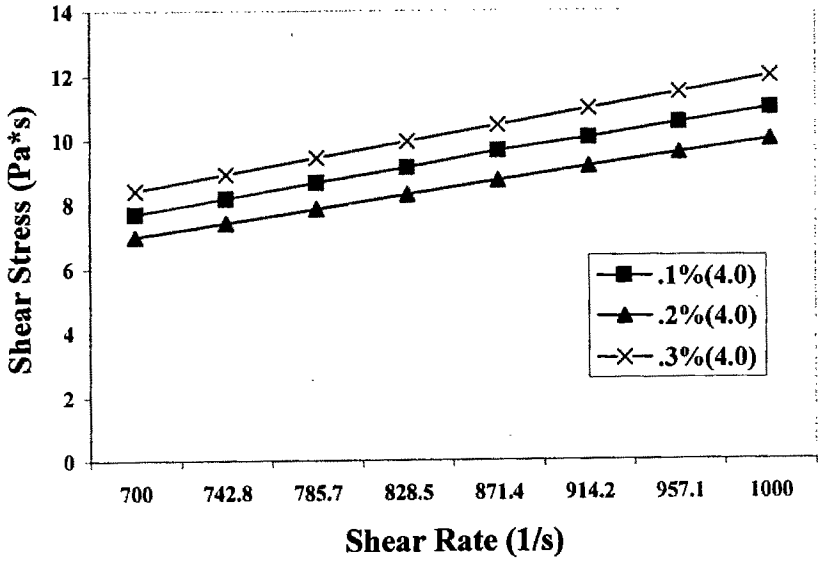
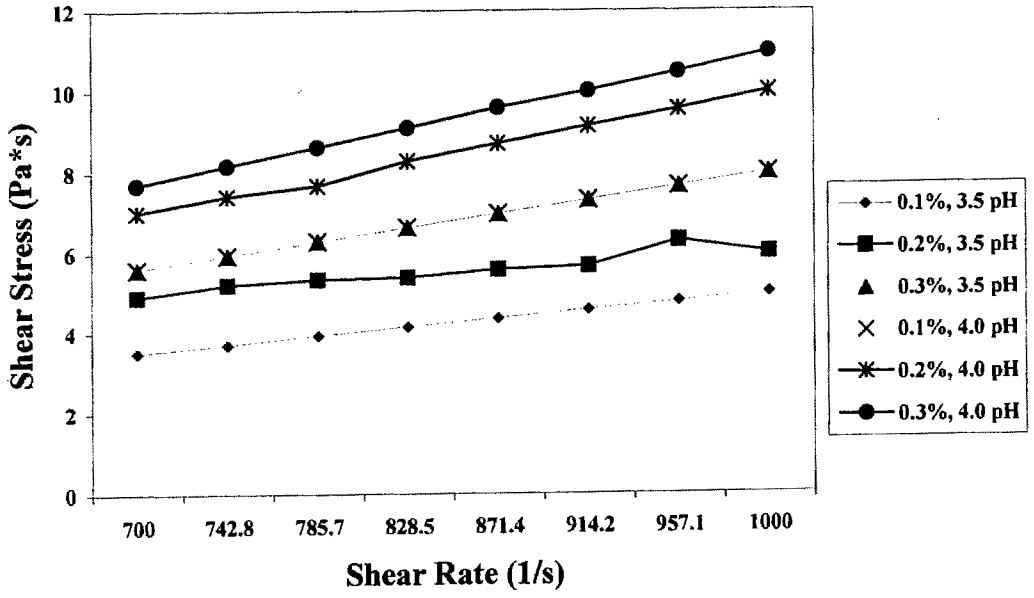


Fig. 4.16: Effect of CMC concentration and pH on rheological behaviour of model whey protein beverage



Fif. 4.17: Effect of guar gum concentration on rheological behavior of model whey protein beverage.

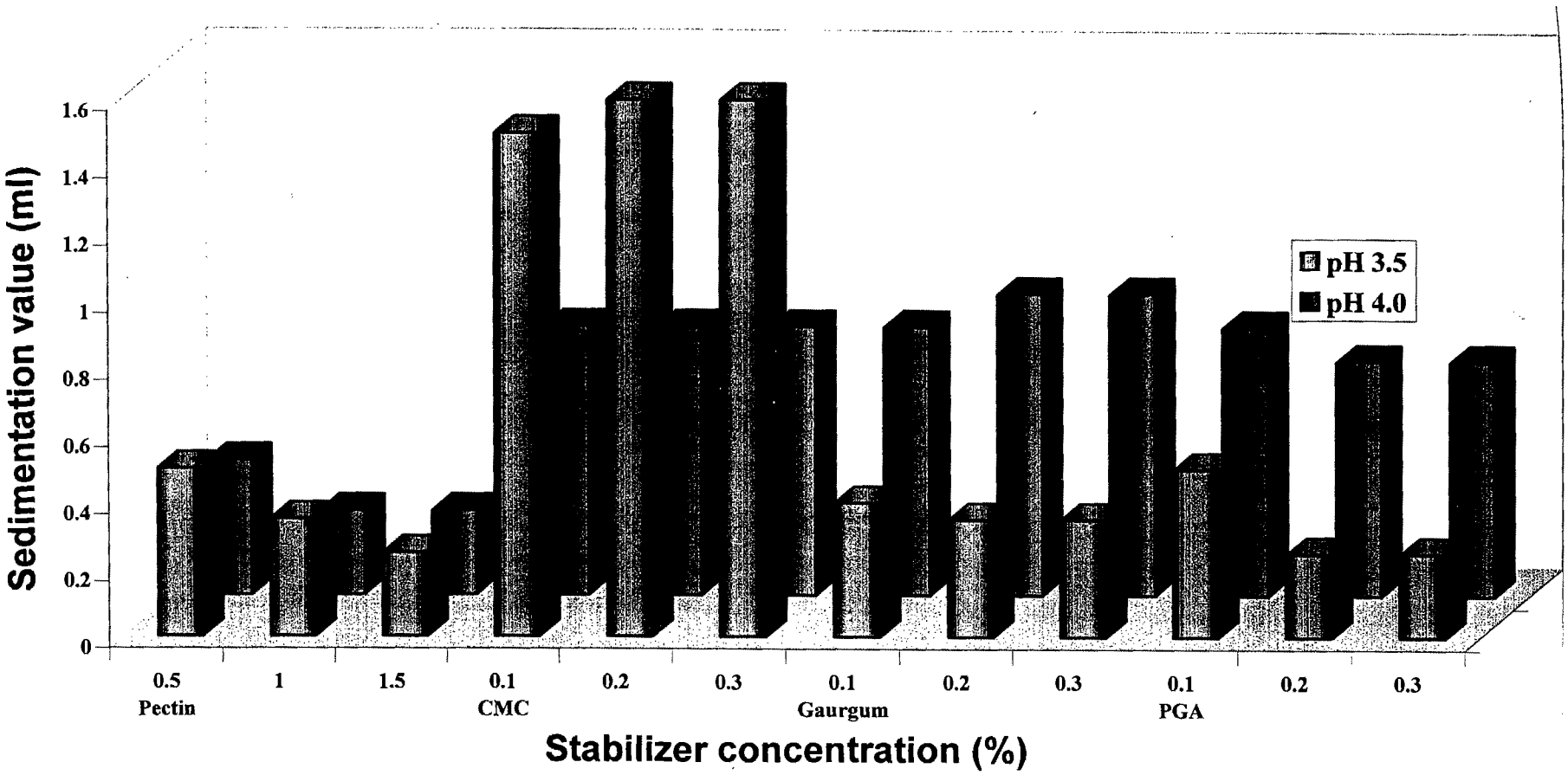


Fig 4.13 : Effect of stbilizer concentration and pH on sedimentation values of model whey protein beverages,

With increasing stabilizer concentration, the drink viscosity drops till full stability was obtained.

Anionic polysaccharides, i.e. pectin, CMC or PGA, adhere to the protein particles to give bridging flocculation at low concentration, accompanied by higher viscosity, but more desirable steric stabilization with reduced viscosity, was achieved at optimum concentrations (Dickinson, 1998). Colloidal stabilization induced by pectin is sometimes attributed directly to hydrocolloid-induced viscosity changes (Haylok *et al.*, 1995).

Among polysaccharides minimum consistency index values were observed in case of guar gum (0.005-0.045 Pa*s) and highest for pectin (0.07-0.44) (Table 4.14). Like flow behaviour index, the values of consistency index correlated very well with stability of suspension and values of consistency index were high at the point of maximum stability. This can again be explained on the basis of relative interaction between proteins and hydrocolloids. Polysaccharides are bound at the surface of protein molecules, become unavailable in solution and do not contribute to the viscosity.

Being neutral polysaccharide, guar gum did not interact with proteins. Hence such samples were clear and without coagulation and with low viscosity of solution (Table 4.14). Heating may cause the low consistency index and high flow behaviour index values of solutions. For CMC solution when heated at 121°C in a still retort for 5-10 min, the magnitude of the consistency index (K), decreased from 0.45 to 0.16 Pa*s and flow behaviour index (n) increased from 0.77 to 0.89, indicating that with heating, the

solutions became less shear thinning and approached the behaviour of a Newtonian solution (Rao *et al,* 1981).

4.8 Chemical composition of Bael pulp

Extraction of 'bael" pulp with different ratios (1 or 1.25 part of water) of water had a significant effect on the chemical constituents. The total soluble solids and total solid content were 21.93°Brix and 23.58% and 16.20°Brix and 16.52% for extract I and II respectively. There was significant difference for the values for TSS and total solids between the two extraction methods (Table 4.15). Their total sugar contents were 6.55 and 5.14 per cent and reducing sugar content were 2.62 and 1.85 extract I and II, respectively (Table 4.15). Roy and Singh (1978) have reported total sugars and reducing sugars in the range of 13.65-17.62 and 2.35-5.68% respectively for 6 cultivars of 'bael' grown in Varanasi region of Uttar Pradesh (India).

Mucilage content of 'bael' was 9.86 to 6.32 % for extract I and II respectively, whereas corresponding values of crude fiber were 3.91 and 2.51% (Table 4.15). Mucilage content was reported to vary with cultivars and stage of maturity (Roy and Singh, 1978).

The protein content of bael pulp was 1.54 and 1.44 % for extract I and II. Gopalan *et al.*(1995) reported protein content of 1.8 per cent in 'bael' fruit but Rai and Mishra (2001) have reported a very high crude protein (4.00-5.96%) content in six clones of 'bael' grown at Pantnagar. The phenolic contents of pulps were 0.54 and 0.37%. These values are much less than those reported by Roy and Singh (1978). The variation may be because of difference

Table 4.15: Physico-chemical constituents of 'bael' pulp extracted with 1 (Extract I) or 1.25 (Extract II) parts of water

'Bael' pulp	T.S.S. (°Brix)	Moisture (%)	Sugar		Mucilage (%)	Crude fiber (%)	Protein (%)	Tannins (%)
			Total (%)	Reducing (%)				
Extract I	21.93	76.42	6.55	2.62	9.86	3.91	1.54	0.54
Extract II	16.20	83.48	5.14	1.85	6.32	2.51	1.43	0.37
S.E.M. ±	0.24	0.36	0.066	0.033	0.124	0.076	0.058	0.040
CD at P≤0.05	0.062	0.076	0.016	0.002	0.032	0.019	0.015	0.011

in variety. The pulp content of these two extracts was 24.68 and 18.86 per cent. Extract I contained more 'bael' constituents compared to Extract II because the low quantity of water used in the former.

4.8.1 Rheological properties of 'bael' pulp

4.8.1.1 Flow Properties

Plot of shear stress at different shear rate indicated that 'bael' pulp extracted with 1.0 (extract I) or 1.25 (extract II) parts of water exhibited non-Newtonian behaviour as their viscosity decreased with an increase in shear rate and it also indicated shear thinning behaviour (Fig. 4.18 and 4.19). This plot also exhibited non-newtonian pseudoplastic behaviour with slight yield stress as the curve intersected (Y-axis) at lower shear stress. Plant food dispersions (PFD) are known to behave as non-Newtonian shear thinning (pseudoplastic) (Rao, 1986). PFD's contain a continuous phase made up of an aqueous solution of sugars, pectins, flavour compounds, and other water soluble chemical compounds and a dispersed phase made up plant particles from the cell walls and other parts of fruits and vegetables. Method of extraction exerted pronounced effect on rheological properties. The viscosity of 'bael' pulp decreased with an increase in temperature of processing. 'Bael' pulp extract II had a lower viscosity profile at all temperature than that prepared with I extraction (Fig. 4.18 and 4.19). Decrease in viscosity on heating for PFD was reported by many workers (Ahmed, 1999, Gujral et al., 2001)

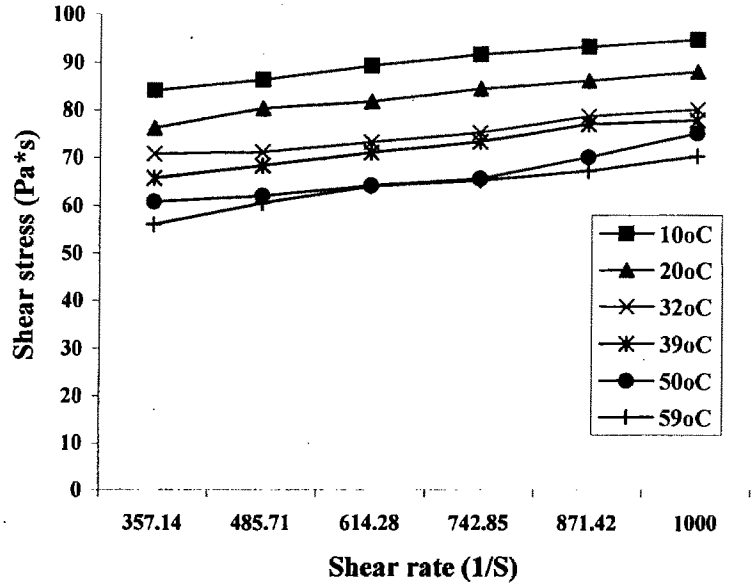


Fig. 4.18: Shear rate Vs shear stress curve for bael pulp extract I

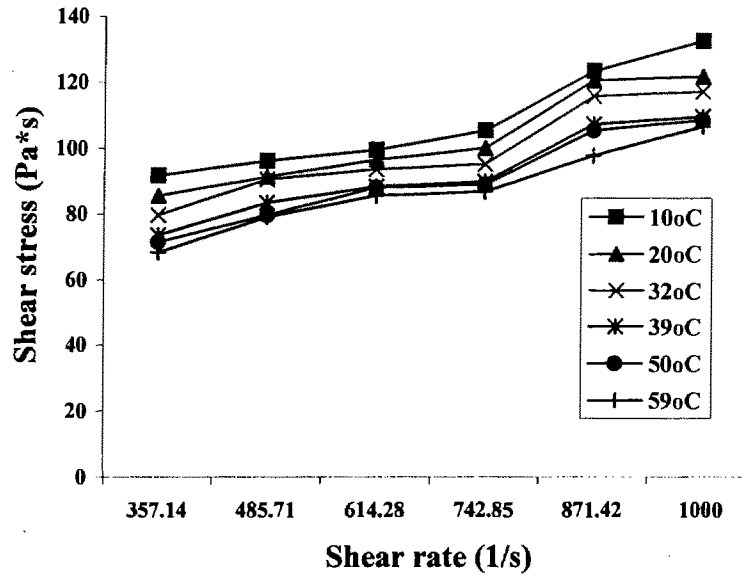


Fig. 4.19: Shear rate Vs shear stress curve for bael pulp extract II

4.8.1.2 Model Adequacy

Flow behaviour data of 'bael' pulp prepared by two different extraction method were computed for checking the fitness of the equations i.e. power law model, Herschel-Bulkely equation and modified Casson model (Sec.3.7.1.1). Among all the rheological equations, simple power law equation gave a higher correlation coefficient (0.980-0.998) for extract I and slightly lower r^2 (0.861-0.969) for extract II (Table 4.16).

The Herschel-Bulkley equation, which considers yield stress, gave a higher correlation coefficient in the range of 0.941-0.999 extract I and 0.902-0.969 for extract II (Table 4.17). The flow curves intercepting the stress ordinate at low shear rate (Fig.4.18 and 4.19) demonstrated the presence of a yield stress.

Modified Casson model when applied to the experimental data gave high correlation coefficient (0.883-0.999) (Table 4.18). The good fit of all the rheological models confirmed the linearity of the curves as well as magnitudes of the co-efficients of correlation, which was more than 0.8 for 'bael' pulp (Fig. 4.20 to 4.25).

4.8.1.3 Yield stress

Results for the effect of method of extraction and temperature on the magnitude of yield stress of the 'bael' pulps are presented in Table 4.19. The comparable values of yield stress obtained by using Herschel-Bulkely model and modified Casson's model revealed satisfactory representation of the rheological behaviour of the 'bael' pulp in the range of shear rates between

Table 4.16 : Effect of temperature on power law model components of bael pulp extracts

Temperature (^{0} C)	Bael Pulp Extract I			Bael Pulp Extract II		
	Consistency-Coefficient (K)	Flow behaviour index (n)	R^2	Consistency-Coefficient (K)	Flow behaviour index (n)	R^2
10	24.27	0.346	0.993	10.73	0.356	0.861
20	18.99	0.342	0.984	9.91	0.360	0.890
30	15.95	0.332	0.991	8.83	0.372	0.899
39	14.19	0.312	0.985	7.49	0.387	0.933
52	13.26	0.314	0.980	6.25	0.412	0.952
59	12.91	0.313	0.998	5.71	0.419	0.969

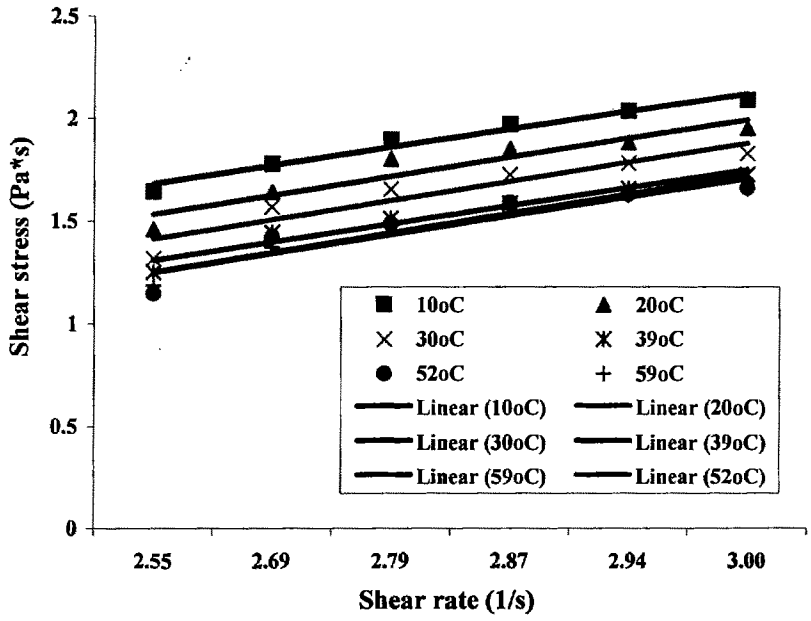


Fig. 4.20: Power law plot for bael pulp extract I

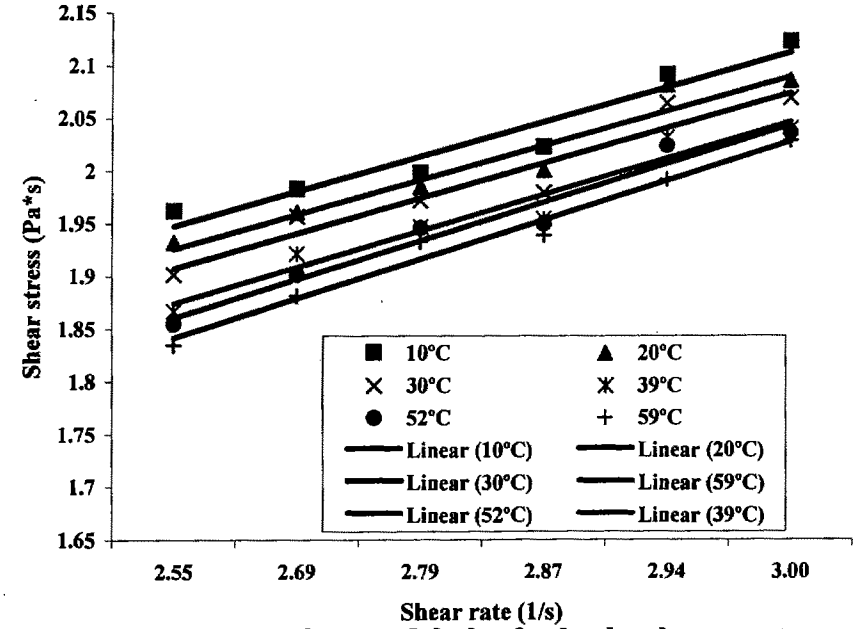


Fig. 4.21: Power law model plot for bael pulp extract II

357.14 to 1000 S^{-1}. The yield stress values obtained by Herschel-Bulkley model were much higher than those by the modified Casson model. Similar observations were reported by Harnanan *et al.* (2001) for guava pulp. However, Rao and Cooley (1983), and Vitali and Rao (1984) reported higher yield stress values by the Casson model than by the Herschel-Bulkley model for tomato concentrate and concentrated orange juice.

The extract I had higher values for yield stress than those of extract II (Table 4.19). This apparent difference may be attributed to the relative differences in their pectin and mucilage contents. Mucilages surrounding the seeds of 'bael' fruit and are soluble in water and get extracted into pulp extract during extraction process. They have significant effect on flow behaviour of 'bael' pulp.

The magnitude of yield stress was found to decrease with an increase in the temperature. Harnanan *et al.* (2001) observed lower yield stress values for white guava pulp prepared by hot extraction than by the cold extraction. They explained this difference on the basis of heat-induced hydrolysis of pectin molecules at acidic pH. Heating causes break down of pectin network in acidic conditions due to decarboxylation of carboxyl groups.

4.8.1.4 Flow behaviour index

Flow behaviour index obtained by power law model varied from 0.37 to 0.34 for extract I and 0.35 to 0.41 for extract II (Table 4.16) at different temperatures, which indicate their non-Newtonian behaviour (Rao, 1986; Gunjal and Waghmare, 1997). The pseudoplasticity of extract I was slightly

Table 4.17: Effect of temperature on Herschel- Bulkley model components of bael pulp extracts

Temperature (^{0} C)	Bael Pulp Extract I			Bael Pulp Extract II		
	Consistency-Coefficient (K)	Flow behaviour index (n)	R^2	Consistency-Coefficient (K)	Flow behaviour index (n)	R^2
10	0.116	1.01	0.999	0.130	0.883	0.902
20	0.053	1.08	0.966	0.086	0.927	0.932
30	0.036	1.09	0.957	0.072	0.969	0.926
39	0.047	1.01	0.987	0.062	0.985	0.953
52	0.023	1.11	0.941	0.063	0.989	0.969
59	0.031	1.06	0.987	0.053	1.01	0.967

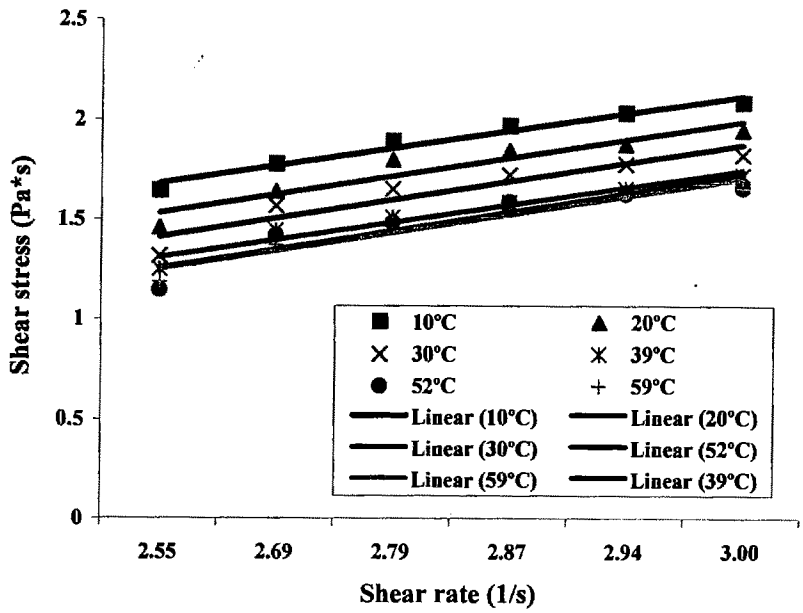


Fig. 4.22: Hearschel-Bulkely plot for bael pulp extract I

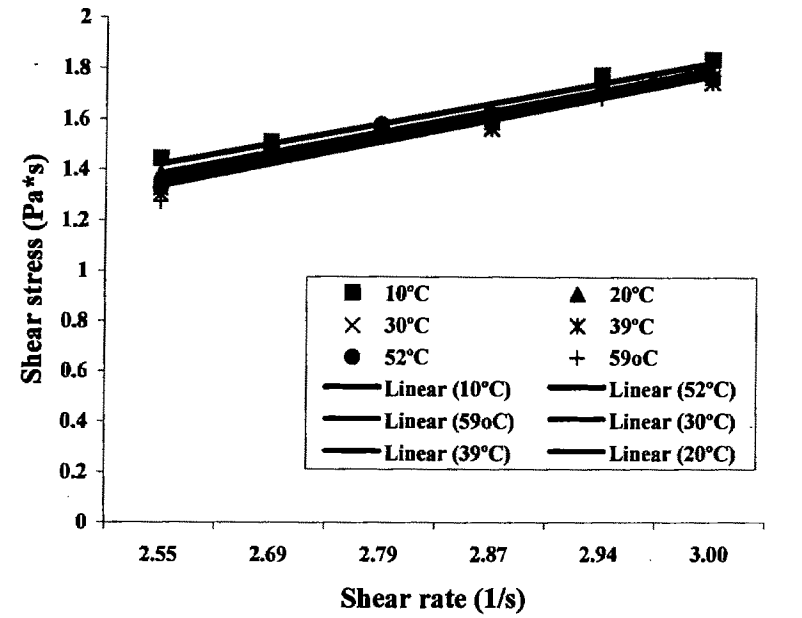


Fig. 4.23: Herschel-Bulkely plot for bael pulp extract II

Table 4.18: Effect of temperature on rheological parameters of modified Casson model for bael pulp extracts

Temperature (^{0} C)	Bael Pulp Extract I			Bael Pulp Extract II		
	Consistency-Coefficient (K)	Flow behaviour index (n)	R^2	Consistency-Coefficient (K)	Flow behaviour index (n)	R^2
10	0.214	0.497	0.999	0.193	0.465	0.883
20	0.166	0.511	0.979	0.174	0.476	0.913
30	0.139	0.514	0.982	0.165	0.485	0.915
39	0.132	0.497	0.990	0.159	0.490	0.946
52	0.112	0.517	0.968	0.166	0.492	0.962
59	0.118	0.506	0.995	0.156	0.495	0.969

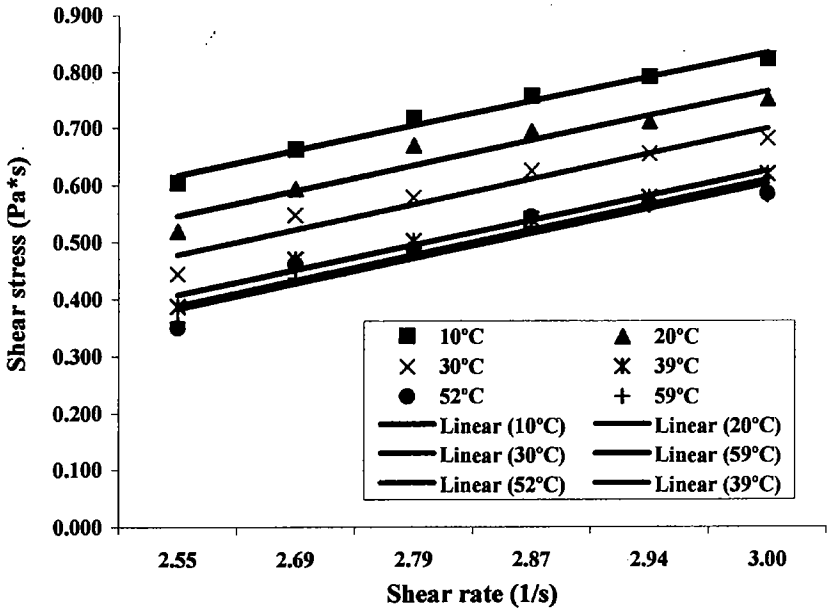


Fig. 4.24: Modified Casson model plot for bael pulp extract I

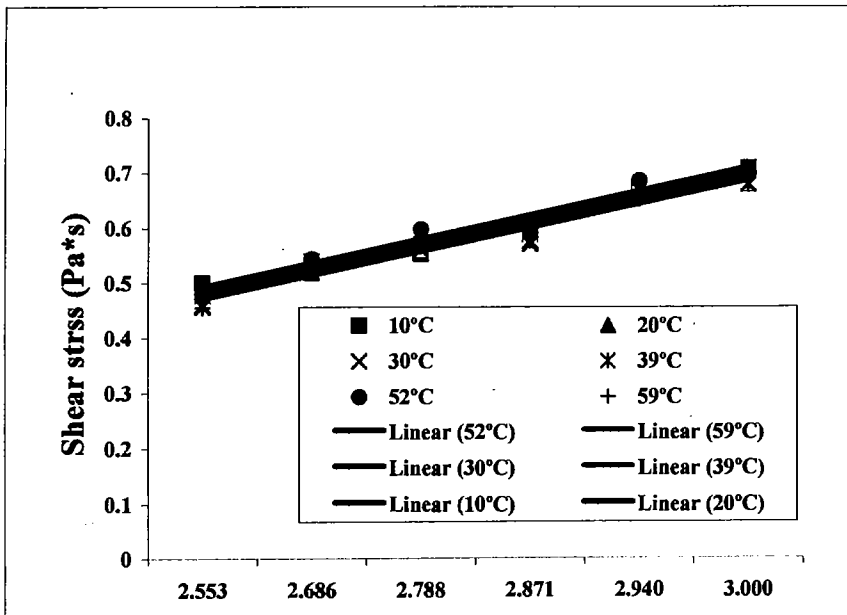


Fig. 4.25: Modified Casson model for bael pulp extract II

lower than the extract II . Flow behaviour index varied from 0.285 and 0.299 for unhomogenized and from 0.178 to 0.250 for homogenized mango pulp at various temperatures (Roy *et al.*, 1997). There was an increase in flow behaviour index with increase in temperature, but at higher temperature, no trend was observed. Ahmed (1999) made similar observation. They reported that magnitude of flow behaviour index showed no definite trends for pastes, as flow behaviour index value of garlic paste decreased with increase in temperature, while the reverse was true for onion paste. Harnanan *et al.* (2001) observed that processing (hot or cold pulping) and preservation techniques caused 33 to 44% increase in the pseudoplasticity of guava pulp. In present experiment, addition of water, that resulted in decrease of TSS of 'bael' pulp, caused increased pseudoplasticity (Table 4.16). Roy *et al.* (1987) observed similar decrease pseudoplasticity with reduction in pulp content and TSS of mango products. The values of flow behaviour index obtained from Herschel Bulkley equation were much higher than those obtained using power law (Table 4.17). The Herschel - Bulkley flow behaviour index (K_H) of extract I was slightly higher than extract II and in the former case temperature had no effect on it. However, K_H increased linearly with an increase in temperature. Harnanan *et al.* (2001) reported significant higher values of K_H than flow behaviour index obtained by power law model. Crandall and Davis (1991) calculated K_H of concentrated juice of various orange cultivars using Herschel- Bulkley model and found the values varied from 0.836 to 1.055.

Table 4.19: Effect of temperature on yield stress value of 'Bael' pulps extracted with 1 (Extract I) or 1.25 (Extract II) of water

Temperature (^{0} C)	'Bael' Pulp Extract I		'Bael' Pulp Extract II	
	Herschel- Bulkley yield stress (σ_{0H})	Modified Casson yield stress (K_{0m})	Herschel- Bulkley yield stress (σ_{0H})	Modified Casson yield stress (K_{0m})
10	144.47	9.71	63.88	6.41
20	112.71	8.59	61.42	6.26
32	90.22	7.75	58.55	6.06
39	71.33	7.00	53.53	5.72
52	68.82	6.86	50.34	5.42
59	65.87	6.72	49.53	5.42

4.8.1.5 Consistency index

Consistency indices of 'bael' pulp extracted by two procedures and at different temperatures are presented in Table 4.16 to 4.19. The consistent index calculated by power law model ranged from 12.91 to 24.27 NS m^{-2} for extract I and from 5.71 to 10.73 NSm^{-2} for extract II at various temperature. The variation between the values for pulp extracted with two different ratios of water may be because of differences in their TSS, pulp content, mucilage and pectin content. Roy (1992) reported consistency coefficients of unhomogenized and homogenized mango pulp to range between 125.46 to 149.46 dyne sec/ cm^2. Gujral *et al.* (2001) observed that consistency index of tamarind sauce varied from 1.19 to 3.11 NS m^{-2} at different temperatures and there was significant increase in the magnitude of consistency index upon addition of hydrocolloids. Harnanan *et al.* (2001) observed a decrease in consistency index values for fresh guava pulps upon processing and preservation and there was variation among varieties as well as pulping procedure. Generally hot break method resulted in higher consistency coefficient.

The values of consistency coefficient obtained by Herschel-Bulkley model, were much lower than power law consistency index (Table 4.16 to 4.17). Similar observation was reported by Harnanan *et al.* (2001) with guava pulp, Roy *et al.* (1997) for mango pulp and Crandall and Davis (1991) with concentrated orange juice.

The values of consistency index calculated by using modified Casson model were lower than power law model values, but higher than Herschel-

Bulkley model values (Table 4.16-4.18). The values of consistency index decreased with increase in temperature. But no definite trend was observed with other two models. Similar values were reported for concentrated Shamuti orange juice when this model was applied and the pulp content was found to exert significant effect on consistency index (Mizrahi and Berk, 1972 and Mizrahi and Firstenberg, 1975). Consistency index has been reported to be highly dependent on temperature (Ahmed, 1999 , Rao *et al.*, 1984; Gujral *et al.*, 2001).

4.8.1.6 Effect of temperature on Rheological properties of bael pulp

Arrhenius equation was plotted between in K (consistency index) against inverse of absolute temperature and the curve were found to be straight line for both pulp extracts i.e. extract I or II (Fig 4.26). The Arrhenius type of equation predicted the effect of temperature on rheological properties of bael pulp extract I more accurately than bael pulp extract II as indicated by higher r^2 value in case of former (Table 4.20). The activation energy was 8.314 KJ/mole and 7.98 KJ/mole for extract I and II respectively. The values of Arrhenius constant were 0.081 and 1.82. Ahmed (1999) reported activation range of 9.16-14.02 KJ/mole. Saravacos (1970) had reported activation energies of pseudoplastic fruit products in the range of 1.2 to 11.2 Kcal/g mole

4.8.2 Development of whey protein-fortified 'bael' beverage

4.8.2.1 Optimization of level of ingredients

In the preliminary investigations, RTS beverage of 15°Brix were prepared using 20% pulp of "bael" pulp extracted with different ratio of water (Sec 3.1.1)

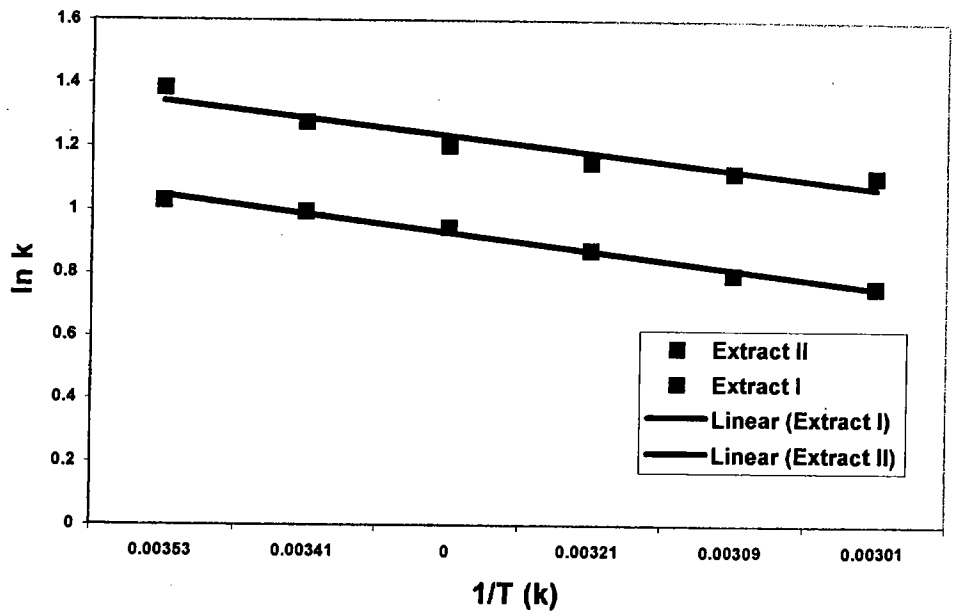


Fig 4.26 : Effect of temperature on cosistency index of bael pulp extracts

Table 4.20 : Effect of temperature on consistency index of 'beal' pulp extract I & II.

Bael Pulp	Equation	Activation energy KJ/mole	Arrhenius constant	R^2
Extract I	ln k= 1000X-5.9314	8.314	0.001	1
Extract II	ln k=960.64- 0.599	7.98	1.82	0.857

and the 'bael' pulp extracted with water in ratio of 1:1.25 (Extract II) was preferred. 'Bael' pulp extracted with this procedure contained lesser amount of suspended particles and particles were smaller in size and did not sediment. Therefore panelists liked its beverage. Hence, pulp obtained as extract II was used in subsequent studies.

Among the samples with various pulp content, 'bael' beverage with 25% pulp was rated the best for flavour (Table4.21) and was rated significantly ($P \leq 0.05$) superior over other samples. Samples below this level of pulp were reported to be less sweet and flat and enhanced pulp level gave the product with astringent taste. The astringency reported by panelists may be because of its higher total phenolic levels.

The colour and appearance scores ranged from 6.50-7.75 and the highest score was obtained for beverage prepared with 25% pulp content. The lowest score was for beverage of 15% pulp level. Increasing the pulp content though improved the colour of the product, but appearance of product was adversely affected. At higher pulp level there was sedimentation of pulp and it was negatively evaluated.

There was significant difference among beverage samples for their colour and appearance score (Table 4.21)

The sensory scores for mouthfeel of beverages ranged from 6.33-8.00 (Table 4.21). Beverages with 25% pulp content was found superior to other levels for mouthfeel, lower pulp levels made product thin and watery. Though the consistency of the products improved by adding higher level of pulp, but at

Table 4.21: Effect of pulp content on sensory qualities of 'bael' beverage

Pulp content (%)	Sensory scores (maximum 9.0)			
	Flavour	Colour/ appearance	Mouthfeel	Overall acceptability
15	6.41	6.50	6.33	6.41
20	7.16	7.00	7.20	7.12
25	7.83	7.75	8.00	7.86
30	6.91	7.12	7.25	7.09
35	6.16	7.02	7.12	6.76
S.E.M.	0.214	0.157	0.131	0.170
CD at P≤0.05	0.62	0.45	0.38	0.48

Table 4.22 : Effect of sugar level on sensory qualities of 'bael' beverage

Sugar level (°Brix)	Sensory scores (maximum 9.0)			
	Flavour	Colour/ Appearance	Mouthfeel	Overall acceptability
15	5.75	6.41	6.66	6.27
16	7.91	7.66	7.45	7.67
17	7.41	7.50	7.29	7.40
S.E.M.	0.242	0.150	0.228	0.257
CD at P≤0.05	0.73	0.46	0.68	0.62

high level slight gumminess and mealiness was reported due to the presence of suspended pulp particles.

Since, beverage with 25% pulp content scored highest for all sensory attributes, it scored maximum for overall acceptability and it was significantly different from other samples at 5% level. 'Bael' beverage with 25% pulp content was found to be optimum. Roy and Singh (1979) developed a nectar (25° Brix) and had found 35% pulp content as optimum. They also reported that pulp content above this level made the product thicker and below it lighter.

The TSS of 'bael' beverage was adjusted to 15°, 16° and 17° Brix using sugar and their flavour scores were 5.75, 7.91 and 7.41 respectively. Sample of 16°Brix scored highest for flavour (Table 4.22). These was no significant difference among 'bael' beverages of 16°and 17°Brix. However, both samples differed significantly with 'bael' beverage of 15°Brix. Sample was not liked by panelists because of its lesser sugar content. 'Bael' beverage of 16° and 17°Brix did not differ statistically for other sensory scores i.e. colour and appearance, mouthfeel and overall acceptability. Therefore beverage with 16°Brix was selected for further studies. Keeping the sugar content at optimum level will increase product acceptability and made product economical.

CMC-WPC complex (61.06% protein) (Plate 4.5) was added to 'bael' beverage (25.0% pulp content, 16°Brix TSS 3.9 pH) to give products 1.75, 2.75 and 3.75% proteins and subjected to sensory evaluations. Flavour scores for the three beverages ranged from 6.08 to 7.91 and the sample with 1.75% protein level scored maximum, whereas beverage with maximum protein level i.e.

Table 4.23: Effect of CMC-WPC complex level on sensory scores of fortified 'bael' beverages[1].

Protein content (%)	Sensory scores (maximum 9.0)			
	Flavour	Colour/ appearance	Mouthfeel	Overall acceptability
1.75	7.91	7.91	7.92	7.91
2.75	7.25	7.16	7.42	7.28
3.75	6.08	6.75	7.16	6.67
S.E.M±	0.294	0.163	0.253	0.266
CD at P≤0.05	0.88	0.49	0.76	0.71

Table 4.24: Effect of pectin - WPC complex level on sensory scores of 'bael' beverages[1].

Protein content (%)	Sensory scores (maximum 9.0)			
	Flavour	Colour/ appearance	Mouthfeel	Overall acceptability
1.75	7.50	7.41	7.50	7.47
2.75	6.50	6.33	6.78	6.54
3.75	6.08	5.58	5.33	5.67
SEM ±	0.36	0.31	0.23	0.30
CD at P≤0.05	1.11	0.94	0.69	0.91

[1] 25% pulp ; 16° Brix TSS

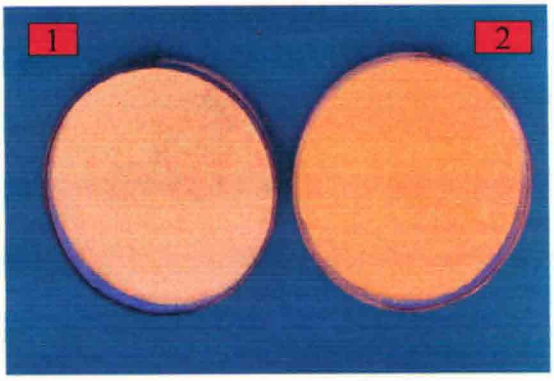


Plate 4.6 : Whey Protein-Polysaccharide Complex
1. CMC-Protein Complex
2. Pectin-Protein Complex

Plate 4.7 : Whey Protein-enriched Bael Beverage

3.75% was lowest (Table 4.23). The colour and appearance scores were 7.91, 7.16 and 6.75 for beverage of 1.75, 2.75 and 3.75% protein level respectively. There was significant difference among samples for their colour and appearance scores at $P \leq 0.05$. Increasing the level of complex resulted in more milky appearance of the samples hence they scored less. The mouthfeel score of the beverage ranged from 7.16 to7.92. Maximum score was at lowest protein level, whereas minimum score was obtained at highest protein level (Table 4.23). There was significant difference between these beverages for mouthfeel attribute, because at highest protein level the relative concentration of stabilizer was also more and it contributed towards viscosity of beverage. Overall acceptability scores for three beverages varied from 6.67 to 7.91 (Table 4.23). The sensory attributes of sample with 3.75% proteins differed significantly ($P \leq 0.05$) from other two samples in all respects except mouthfeel. Incorporation of complex improved the consistency of the beverage. The lowest sensory scores for sample with 3.75% protein were because there was slight precipitation of complex upon heating. Increasing the protein level of beverages is often associated with development of off-flaovur and chalky flavour (Marchio, 1995).

Pectin-WPC complex (46.02% protein) (Plate 4.5) was added to fortify 'bael' beverage to adjust their protein level to 1.75, 2.75 and 3.75%. The flavour score of the beverages ranged from 6.08 to 7.50 and the sample with minimum protein content scored highest (Table 4.24). However further increase in protein level resulted in decrease of their flavour score. The increase in level of

protein caused off-flavour development and panelists did not like it. The colour and appearance scores varied from 5.58 to 7.41, being highest for minimum protein level and lowest for maximum protein level. There was significant difference between the samples both for flavour and colour & appearance. The mouthfeel and overall acceptability scores of three beverages varied from 5.33 to 7.50 and 5.67 to 7.47 respectively. Sample with lowest protein content scored maximum for all sensory attributes (Table 4.23). The poor sensory scores at elevated protein content reflected the poor thermal stability of pectin-WPC complex at higher protein level. In all the samples, there was sediment formation, except the one with lowest protein level.

4.8.2.2 Physico-chemical characteristics of 'bael' Beverage

'Bael' beverages (Plate 4.6), which were developed using CMC-WPC and pectin-WPC complexes with overall acceptability scores of 7 or more were analysed for various physico-chemical properties. The beverage developed with 1.75 (A), and 2.75% (B) protein level of CMC-WPC complex and 1.75% (C) protein with pectin-WPC complex were acceptable on this criteria (Table 4.25).

Viscosity of bael beverages decreased with an increase in shear rate which indicated its non-Newtonian behaviour (Fig. 4.26)

pH of the three beverages varied from 3.93 to 3.95 and there was no significant difference among samples (Table 4.17). pH of the samples was adjusted with 50% admixture of glucono-δ-Lactone, and citric acid to pH 3.90 and slight variation can be expected from processing induced changes.

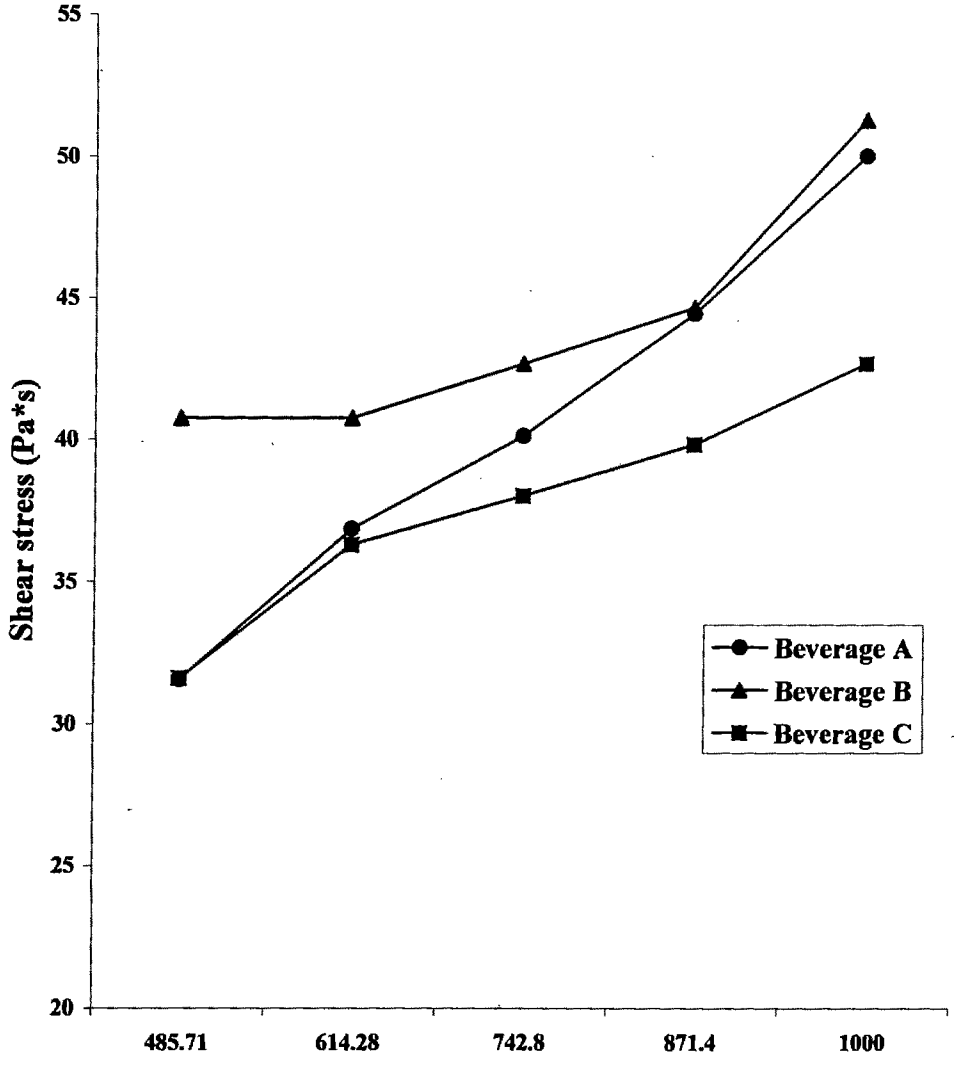


Fig. 4.27: Flow charecteristics of whey protein-enriched bael beverages

Table 4.25: Chemical components of whey protein - enriched 'Bael' beverages.

Beverages	pH	Total acidity (% citric acid)	Total solids (%)	Sugars		Protein (%)	Phenols mg/100 gm
				Total (%)	Reducing (%)		
A	3.95	0.35	16.77	14.45	5.42	1.94	161.93
B	3.94	0.47	18.41	14.81	5.68	2.59	159.10
C	3.93	0.54	18.80	14.87	5.73	3.03	157.06
SEM ±	ND	0.0027	0.181	0.023	0.025	0.016	0.37
CD at P≤ 0.05	ND	0.009	0.62	0.081	0.087	0.057	1.29

A Pectin-WPC complex (1.75% protein)
B CMC-WRC complex (1.75% protein)
C CMC-WPC complex (2.75% protein)
ND Not determined

The acidity of these beverage samples were 0.35, 0.47 and 0.54 per cent (Table 4.25). Acidity of these samples differed significantly at $P \leq 0.01$ level. In contrast to pH the difference in their acidity may be because of their protein content. Proteins and their derivatives are known to have good buffering capacity and they resists change in pH of solution, hence a higher concentration of acidulant was required to bring down pH to desired level.

The refractometric solids in beverage samples were 16.23, 17.03 and 17.20°Brix with corresponding total solid content of 16.77, 18.41 and 18.80% (Table 4.25). These values for pectin-WPC complex added beverage and CMC WPC complex added beverage differed significantly (Table 4.25). However, there was no statistical difference among CMC-WPC added beverages. The variations in TSS and total solid content among beverage samples is because the amount of ingredients used in them were different in their amount of soluble contents.

The total sugars (as % invert sugar) of beverages ranged between 14.45 to 14.87 per cent and reducing sugar content varied from 5.42 to 5.73% (Table 4.25). As observed with TSS and total solids, total sugar and reducing content of beverage differed significantly between beverages with two type of complex, however, sugar contents differed statistically even at $P \leq 0.05$. The sugar to acid ratio for 'bael' beverages ranged between 27.53 to 41.28 and higher sugar to acid ratio was observed for beverage with 2.75% protein level (B).

The soluble protein content of 'bael' beverage was 1.94, 2.59 and 3.03 per cent for C, A and B respectively. Protein content of all the three beverage differed significantly. It was interesting observation that protein content in pectin-WPC complex added beverage was much less than the CMC-WPC complex added beverage at same protein level. It arised because of relative stability of complexes during thermal treatment and though there was no visible precipitation in C beverage, but some protein denaturation might have occurred and these proteins get sedimented on centrifugation.

The phenolic content of 'bael' beverages were 161.93, 159.10 and 157.06 mg/100 gm for C, A and B respectively (Table 4.25). There was significant difference among beverages and decrease in total polyphenols was observed with corresponding increase in protein content and a protein- polyphenol interaction to a limited extent can not be ruled out.

4.9 Composition of mandarin orange juice

Chemical constituents of mandarin orange juice are presented in Table 4.26. The total soluble solid content was 10.2°Brix, whereas total solids was 12.44 %. The pH of the juice was 3.8, that corresponded to 0.81 % acidity as citric acid. The TSS and total solid content in various Indian orange juice were reported to be in the range of 7.2-14.4°Brix and 2.3 to 14 % (Mehta and Bajaj, 1983; Ranganna *et al.* 1983; Sahani *et al.,* 1994). Similarly, pH values varied from 2.8 to 4.3 and acidity (% citric acid) as low as 0.4 and as high as 2.4%. The maturity indices for harvesting of Ambia crop of Nagpur mandarin have

Table 4.26: Physico- chemical constituents of orange juice

Constituents	Value
T.S.S. (^{0} Brix)	10.2
Total solids (%)	12.44
pH	3.8
Acidity (% Citric acid)	0.81
T.S.S. to Acid Ratio	12.59
Total Sugars (%)	7.57
Reducing Sugar (%)	4.94
Ascorbic acid (mg/100 gm)	24.57
Protein (%) (N%X6.25)	0.93
Total Polyphenols (mg/100 gm)	72

been standardized as minimum of 10% TSS and minimum TSS to acid ratio of 14 for acceptable flavour. The juice obtained in this experiment had a TSS to acid ratio of 12.59 and higher acidity of certain mandarin orange cultivars make them unfit for table purpose. The total sugar content of orange juice was 7.57 %, more than 65% of which were reducing sugars (4.94 %). Total sugar and reducing sugars in mandarin orange were reported to vary in the range of 6.74 to 8.5 % and 3.2 to 5.1 % respectively (Sandhu and Bhatia, 1985, Patil and Pai, 2001). Higher reducing sugar content might be because of sugar inversion during thermal treatment.

The ascorbic acid content of orange juice was 24.57 mg/100 gm, which was slightly above than those reported by Sandhu and Bhatia (1985) and below those observed by Patil and Pai (2001).

Protein content in orange juice was 0.93% as compared to the reported value 0.2 to 1.29% (Arlin, 1977; Southgate *et al.*, 1989; Gopalan *et al.*, 1995). The total polyphenolic content was 72 mg/100 gm.

4.9.1 Critical factors and ingredients for whey protein orange beverage

In first part of experiment orange juice mixed with diluted UF-retentate (1% protein) and sugar to raise the TSS to 15°Brix, pasteurized at 90°C for 10 sec., cooled and its sensory quality was evaluated. The flavour, colour and appearance, mouth feel and overall acceptability scores were from 6.00-7.64, 5.79-7.64, 6.42 -7.36 and 6.21-7.71 respectively (Table 4.27). There was significant difference between the samples for flavour and overall acceptability score. The sample prepared with 20% orange juice scored minimum for all

Table 4.27: Effect of juice level and protein content on the sensory characteristics of whey protein - enriched orange beverage[1]

Juice (%)	Level of protein (%)	Flavour	Consistency	Colour and appearance	Overall Acceptability
20	**1.00**	6.00	5.79	6.43	6.21
30	**1.00**	7.64	7.64	7.21	7.71
40	**1.00**	6.36	7.29	7.36	6.64
SEM ±		0.239	0.203	0.259	0.298
CD at P≤0.05		0.24	0.20	0.77	0.89

Table 4.28 : Effect of protein level UF- retenante on the sensory characteristics of whey protein - enriched orange beverage

Juice (%)	Protein level (%)	Flavour	Consistency	Colour and appearance	Overall Acceptability
30	**2.5**	7.86	7.71	7.26	7.50
30	**3.5**	7.24	6.71	6.36	6.82
30	**4.5**	5.93	5.79	6.07	5.71
SEM ±		0.221	0.149	0.188	0.161
CD at P≤0.05		0.66	0.44	0.56	0.48

[1] pH of beverage 3.8; TSS 15° Brix

Table 4.29: Effect of pH on flavour and overall acceptability of whey protein- enriched orange beverage[1]

pH	Flavour	Overall acceptability
3.50	7.00	7.14
3.80	8.00	7.93
4.00	7.11	6.82
4.25	6.89	6.71
SEM ±	0.158	0.174
CD at P≤0.05	0.462	0.508

[1] protein level 1.5% ; juice content 30%

sensory attributes. The samples were termed as bland due to improper sugar-to-acid ratio, flat flavour, thin consistency, dull appearance and possessing distinct "whey taint". Product prepared with 40% orange juice was at par with that prepared with 30% orange juice in consistency and colour and appearance (Table 4.27). However, former beverage was rated low due to poor flavour and low overall acceptability. Some panelists reported slight bitter after taste. Hence, orange juice at 30% level was selected for further trials.

In next trial, beverages were prepared with 30% orange juice and UF-retentate to give 2.5, 3.5 and 4.5 % protein content and subjected to sensory evaluation. The scores for flavour, colour and appearance, mouthfeel and overall acceptibility varied from 5.93-7.86, 5.79-7.71, 6.07-7.26 and 5.71-7.50 respectively (Table 4.28). Except flavour, all other sensory attributes differed significantly at 5% level and the score was found to decrease with an increase in the protein level. At higher protein level and in absence of a proper stabilization system some of the whey protein denatured and formed sediment which, affected their mouthfeel as well as colour and appearance. The beverage prepared with 2.5% protein level was quite acceptable (sensory score >7.50), despite slight denaturation of proteins.

pH had a pronounced effect on stability of whey protein during thermal treatment. (Sec 2.2.4.2). Flavour and overall acceptability scores of beverages ranged from 6.89-8.00 and 6.71-7.97 respectively (Table 4.29). The beverage with pH 3.8 scored highest for flavour and overall acceptability. It was followed by beverage of pH 3.5. The scores for beverages differed

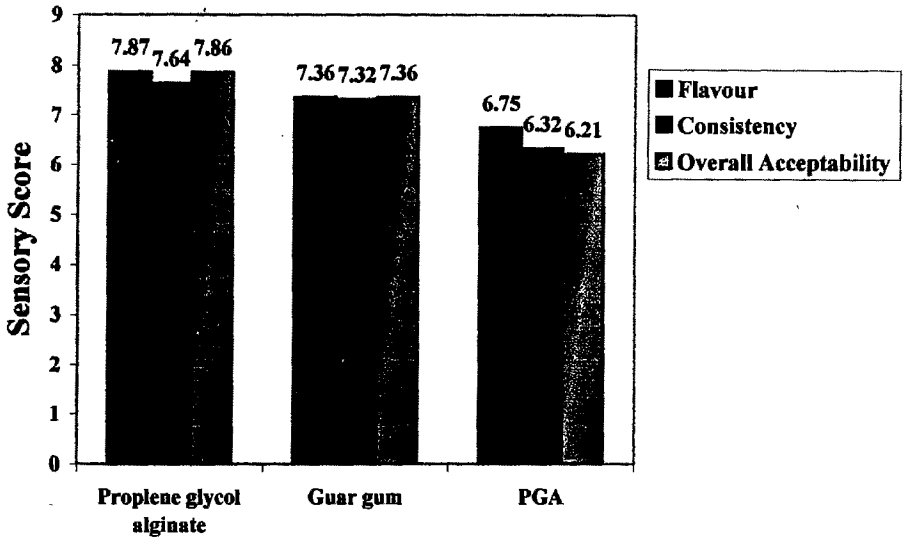


Fig. 4.28: Effect of stabilizer on sensory scores of whey protein enriched orange beverage

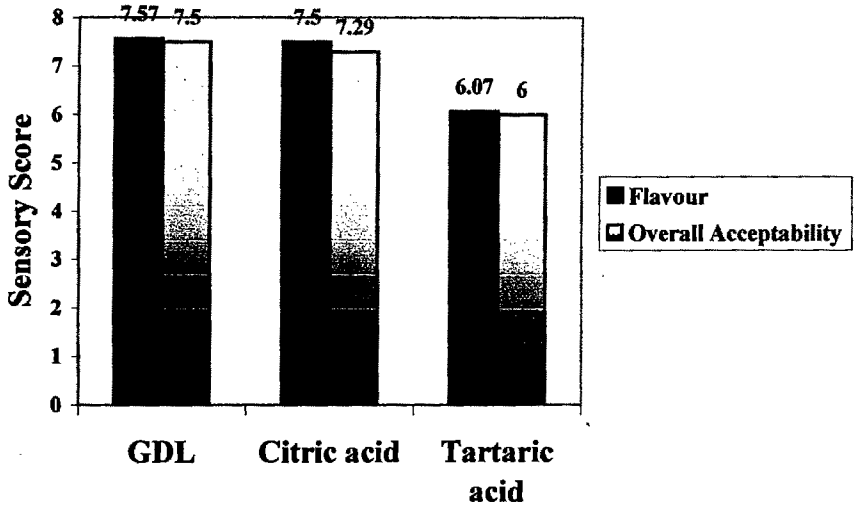


Fig. 4.29: Effect of acidulant on flavour & overall acceptability of whey protein enriched orange beverage

significantly. The natural pH of orange juice was 3.8 and most probably the flavour profile of beverage at this pH was most compatible with orange juice. Moreover, whey proteins have been reported to be heat stable below pH 3.8 (Ibrahim *et al.*, 1995). Despite their reported stability, slight coagulation was observed at this pH and product was clear at pH 3.5. Panelists were of the opinion that increasing the sugar content of beverage with pH 3.5 may improve its overall acceptability.

Acidulants were found to exert significant effect on stability of whey protein (sec 4.2). But their sensory acceptability in beverage systems is equally important. The flavour score of three beverages containing citric acid, GDL or tartaric acid, ranged between 6.07-7.57 and overall acceptability values varied from 6.00-7.50 (Fig 4.28). Beverage prepared with citric acid and GDL did not differ significantly though citric acid scored maximum for both the attributes. Beverages based on tartaric acid were rated as "very sour", and there was crystal formation during heating and subsequent cooling. Samples with GDL were less acidic acid with very little coagulation, whereas precipitation with citric acid was appreciable. Application of GDL or its derivatives has been reported to yield good whey beverages (Watine, 1995). A mixture of 50% GDL and citric acid in 1:1 ratio was used in further study.

In beverage, three stabilizers namely pectin, propylene glycol alginate and guar gum were used. The flavour, consistency and overall acceptability score for the beverage prepared with three stabilizers varied from 6.75-7.78, 6.32-7.64 and 6.21-7.85 respectively (Fig 4.29). Beverages containing pectin

and guar gum did not differ significantly for consistency and overall acceptability score but their flavour scores differed significantly. Samples with PGA scored least for all the attributes because it was not able to prevent protein precipitation. Guar gum added beverages had stale after-taste, hence scored least but it was rated best sample because of its appearance and mouthfeel.

4.9.2 Optimization of level of ingredients by RSM for whey protein-enriched orange beverage

4.9.2.1 Quality attributes of beverage

Beverage samples were prepared by using different levels of ingredients and evaluated for their sensory characteristics (Table 4.30). Sensory data were fitted into second order polynomial model and the results of regression analysis are given in Table (4.31).

Flavour scores of the beverages varied from 6.00 to 8.40 (Table 4.30). The minimum score was obtained for samples 8 and 9 which had 35% orange juice, 3.4 % whey protein level, 10 or 12% sugars and 0.3 or 0.1% stabilizer respectively (Table 3.5). The maximum score was obtained in experiment number 16, which had 35, 3.4, 12 and 0.3 % orange juice, whey protein, sugar and stabilizer, respectively. The minimum scores were obtained for samples where whey protein was maximum and sugar content minimum and scores were maximum when reverse was the case. Low scores were also obtained for beverages having higher protein and lower stabilizer levels. During experiments, it was observed that at higher levels of whey protein, a large quantity of acidulant was required to adjust the pH to desired level, i.e., 3.5

Table 4.30: Effect of ingredient level on sensory characteristics of whey protein - enriched orange beverage.

Experiment number[1]	Flavour	Mouthfeel	Colour and appearance	Overall acceptability
1	6.80	6.70	7.10	7.05
2	6.10	6.20	6.03	6.10
3	6.45	6.90	6.45	6.60
4	8.10	8.10	8.20	8.45
5	6.50	6.60	6.80	6.60
6	8.35	8.40	8.45	8.23
7	6.20	6.20	6.35	6.10
8	6.00	6.30	6.35	6.60
9	6.00	6.30	6.35	6.10
10	6.57	6.54	6.60	6.85
11	7.90	7.80	7.20	7.50
12	8.20	8.05	8.40	8.60
13	6.40	6.15	6.08	6.35
14	6.45	6.50	6.55	6.50
15	6.50	6.85	6.25	6.80
16	8.40	8.30	8.80	8.65
17	8.15	8.35	8.30	8.40
18	7.05	7.20	7.50	7.62
19	6.25	6.20	6.45	6.24

[1] cf table 3.5 for details

because of high buffering capacity of whey proteins. Moreover, a lower level of sugar caused reduced sugar to acid ratio which lowered their sensory scores (Table 4.30).

Coefficients of full second order polynomial model are presented in (Table 4.31). The equation for flavour score was significant at 10% level only. The coefficient of determination R^2 for flavour was 0.918, which is higher than 0.8 considered to be good by Filmore *et al.* (1976) and Joglekar and May (1991), or 0.85 suggested as minimum value by Henika (1982) for developing a model for sensory data. A high value of experimental R^2 (0.918) shows that the model developed was satisfactory.

The partial coefficients of the flavour models show that level of stabilizer ($P \leq 0.1$) at linear level and quadratic terms of whey protein level ($P \leq 0.05$) and stabilizer concentration ($P \leq 0.05$) affected the flavour scores. There was significant effect of fruit juice and sugar content interactively at $P \leq 0.05$, whereas protein content and sugar content interact at $P \leq 0.1$. If the protein content is increased then sugar content should also increased to improve flavour scores. This confirms the observation discussed above.

The positive sign indicates increase in response with an increase in the level of parameters, while, negative sign indicates decrease in response. So, by increasing the fruit juice level, sugar level and stabilizer concentration simultaneously reducing whey protein level flavour scores can be improved.

Table 4.31 : Coefficients of full second order polynomial model for sensory responses to different levels of various ingredients in beverage.

Partial coefficient	Flavour	Colour and appearance	Mouthfeel	Overall acceptability
b_0	+7.057	+7.003	+7.207	+7.278
b_1	+0.248	+0.246	+0.273	+0.277
b_2	+0.120	-0.018	-0.004	-0.041
b_3	-0.130	-0.160	-0.095	-0.185
b_4	-0.395*	-0.477*	-0.394	-0.354
b_{11}	+0.454	+0.388	+0.586	+0.455
b_{22}	+0.979*	+2.113**	+0.886	+1.031*
b_{33}	-0.231	-0.088	+0.080	-0.116
b_{44}	-1.529**	-1.496**	-1.863**	-1.765**
b_{12}	-0.0282	+0.051	-0.112	-0.101
b_{13}	-0.600**	-0.562*	-0.650*	-0.541*
b_{14}	-0.163	-0.119	-0.050	-0.158
b_{23}	+0.487*	+0.575**	+0.462	+0.491*
b_{24}	-0.293	-0.214	-0.188	-0.203
b_{34}	-0.050	-0.037	+0.075	-0.091
R^2	0.918	0.905	0.882	0.892
Adeq. Precision	5.001	5.387	5.012	5.008
Equation significance	*	NS	NS	*

$$Y = b_0 + b_1 x_1 + b_2 x_2 + b_3x_{3+}b_4x_4+bb_{11}x_{11}+bb_{22}x_{22}+bb_{33}x_{33}+bb_{44}x_{44}+b_1b_2x_1x_2+b_1b_3x_1x_3+b_1b_4x_1x_4+b_2b_3x_2x_3+b_2b_4x_2x_4+b_3b_4x_3x_4$$

Where Y is sensory score, X_1 (Fruit juice level), X_2 (Whey protein level), X_3 (Sugar level), X_4 (Stabilizer level).

4.9.2.2 Mouthfeel

Mouthfeel scores for beverages varied from 6.15 to 8.40 (Table 4.30). Highest consistency score was for beverage prepared by 30% orange juice, 3.4% whey protein level, 12% sugar level and 0.3% stabilizer level (Experiment number 6), whereas lowest was for experiment number (30% fruit juice level, 3.4% whey protein level, 12% sugar and 0.1% stabilizers). Beverages prepared with maximum level of whey proteins and lowest level of stabilizer generally scored low. In absence of sufficient amount of stabilizer some of whey proteins underwent heat induced denaturation which caused sedimentation in samples, and hence their sensory scores were low.

The coefficients of regression are presented in (Table 4.31). The model was non-significant, because F calculated was less than F-tabulated, but a higher co-efficient of correlation (0.905) for the equation may be interpreted as an adequate indicator for fitness of model. Moreover calculated adeq. precision, value of 5.387 was higher than table value of 4.00 and hence this model can be used to navigate the design.

The partial coefficients indicates that linear terms of stabilizer level ($P \leq 0.1$) and quadratic terms of protein and stabilizer concentrations had a significant effect over sensory scores at $P \leq 0.05$. Significant effect was also exerted by interaction terms. Decreasing the level of protein as well as stabilizer improved the colour and appearance score, as depicted by negative sign of co-efficient. Higher stabilizer concentration, in some experiments was not liked by penalist specially where protein level was at minimum level (Table

3.5). The fruit juice content and sugar content ($P \leq 0.1$) affected sensory score through interaction at $P<0.1$ level, protein and sugar interaction was also significant (at $P \leq 0.05$) (Table 4.31). Decreasing the fruit juice level and sugar improves the mouthfeel, whereas increasing protein level with concomitant increase in sugar level improved mouthfeel. Though sweetness are primarily used to improve the flavour of beverages, they can also contribute to the fullness or mouthfeel of the beverage (Hicks, 1989). Sugars have been reported to enhance the thermal stability of whey (Ibrahim *et al.*, 1995) and thus prevented thermal denaturation of proteins which otherwise cause chalkiness in protein rich beverages (Anantha Narayanan, 1993).

4.9.2.3 Colour and appearance

Colour and appearance scores for whey protein enriched orange beverage ranged from 6.05-8.80 (Table 4.30). It was highest in experiment number 16 (35% orange juice, 3.4% whey protein,12%sugar and 0.3% stabilizer) and lowest for experiment number 2 (32.5% fruit juice,1.4 % protein, 11% sugar and 0.3% stabilizer). In general higher protein level in beverages produced opaqueness and hence such formulations scored low for colour and appearance. Moreover these formulations contained lower level of stabilizer and sugar which caused slight denaturation and sedimentation of whey protein and adversely affected colour and appearance of the beverage.

On the basis of calculated F value, it can be concluded that this model is non-significant, but coefficient of correlation value of 0.881 and adeq. Precision value of 5.012 (Table 4.31) indicates a good fitness of the equation

and model to describe the colour and appearance of the product. In this study, the stabilizer level in quadratic terms was found to have significant effect on colour and appearance at $P \leq 0.05$, while fruit juice content and sugar level had significant effect interactively at $P \leq 0.1$(Table 4.31).

4.9.2.4 Overall acceptability scores

Overall acceptability score of whey protein-enriched orange beverage varied from 6.10 to 8.65 (Table 4.30). Beverage prepared in experiment number 16, scored maximum because of its better flavour and colour and appearance. Beverages developed in experiment number 7 and 9 scored minimum owing to their poor scores for all the sensory attributes.

The model terms were significant at $P \leq 0.1$(Table 4.31). The coefficient of correlation was 0.892 with Adeq. precision, value of 5.008, which was good enough to indicate the adequacy of equation and model developed for overall acceptability. An analysis of partial coefficients shows that stabilizer concentration exerted significant effect on overall acceptability of the product quadratically at $P \geq 0.05$, whereas protein level was significant at $P \leq 0.1$. The fruit juice content and sugar content had significant effect at $P \leq 0.1$ in interactive terms. Similar effect was observed for protein and sugar level

4.9.3 Experimental varification of optimum condition

The response optimization was done by selecting a minimum score of 7 for each sensory attribute i.e. flavour, colour and appearance, mouthfeel and overall acceptability. The package state-Ease, provided ten set of solutions for compromise optimum response out of which. the four solutions were randomly

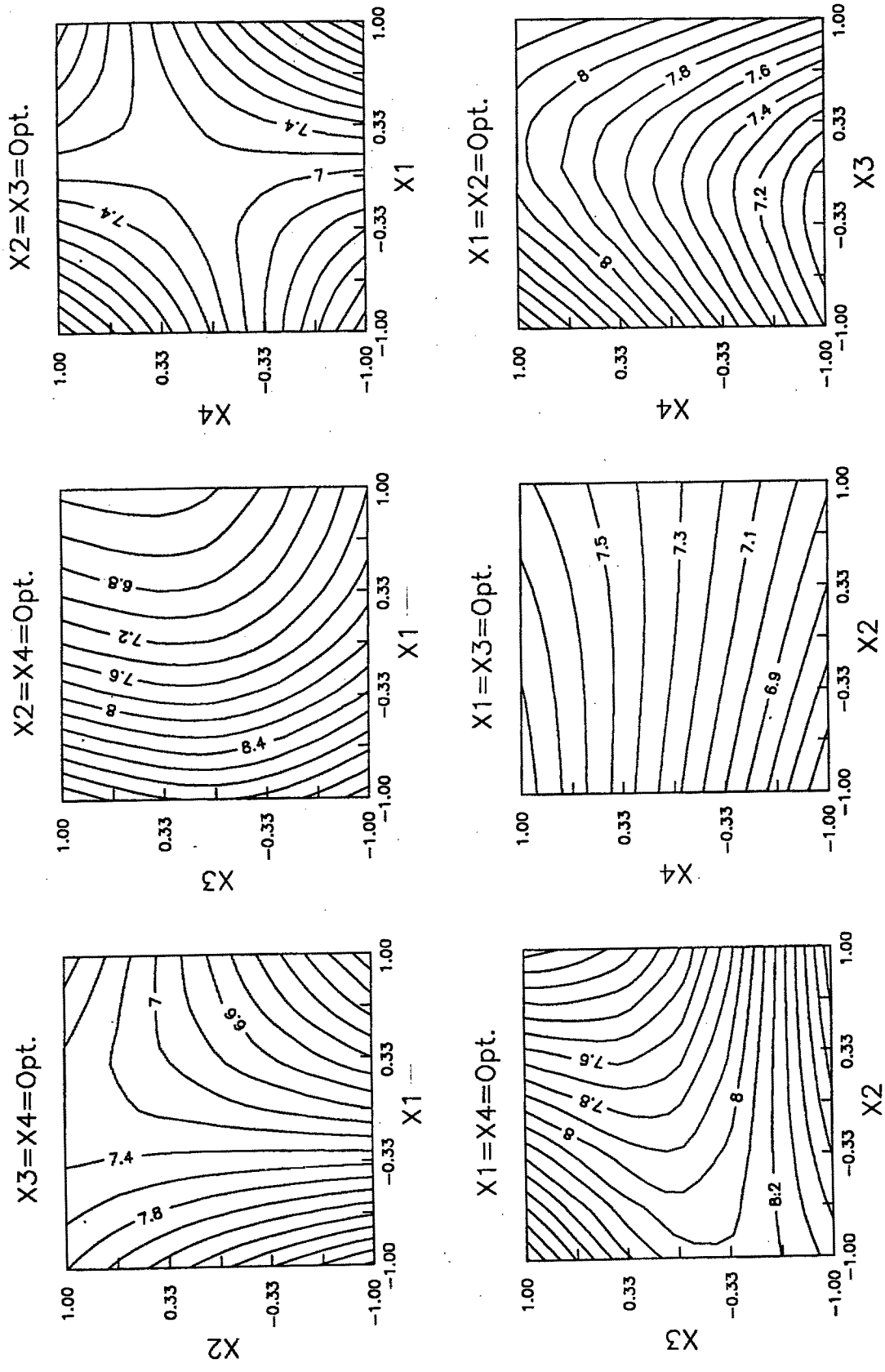


Figure 4.30 Contour plots for flavour attribute of whey protein-enriched orange beverage at optimum point

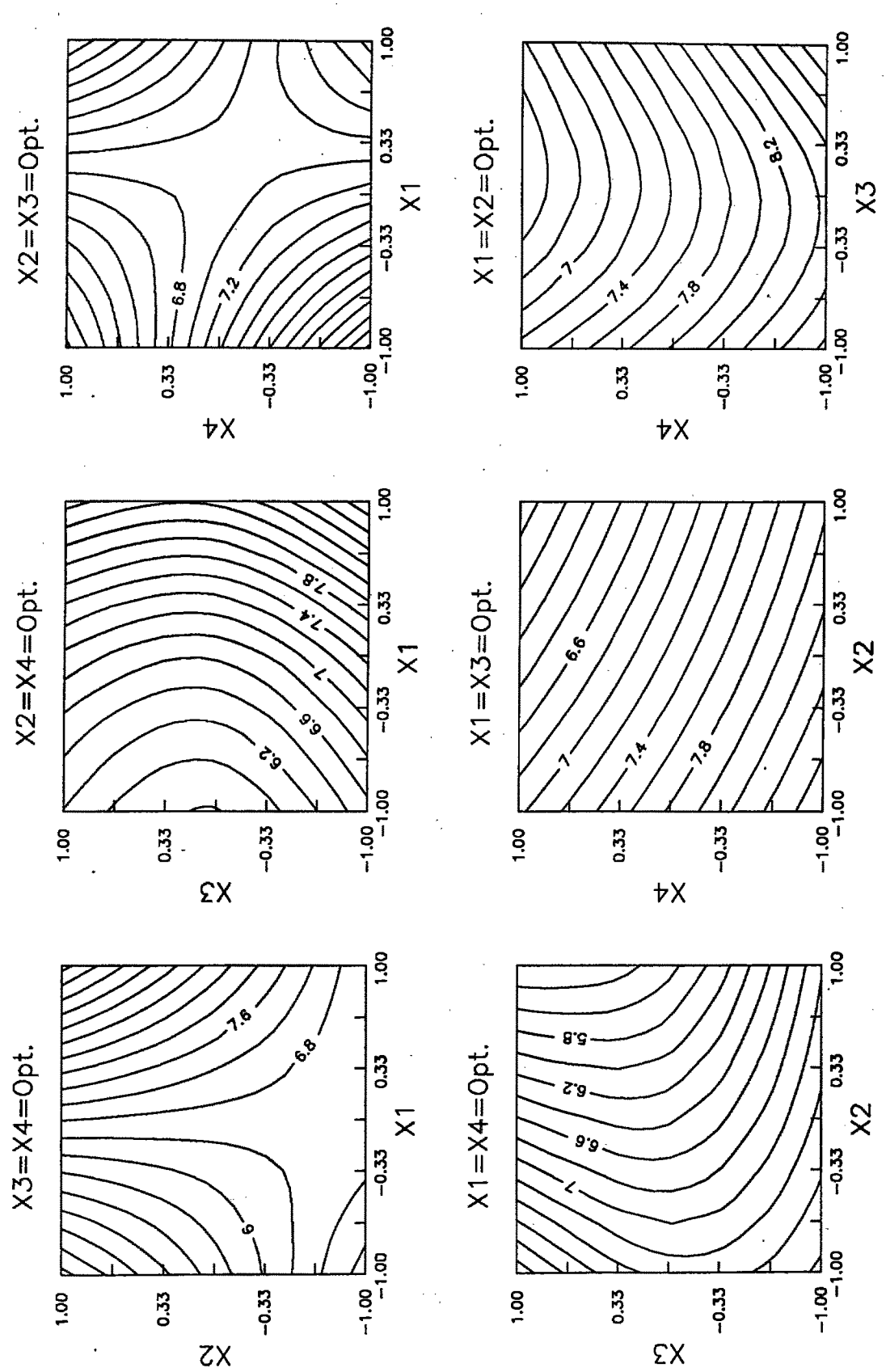


Figure 4.31 Contour plots for mouthfeel attribute of whey protein-enriched orange beverage at optimum point

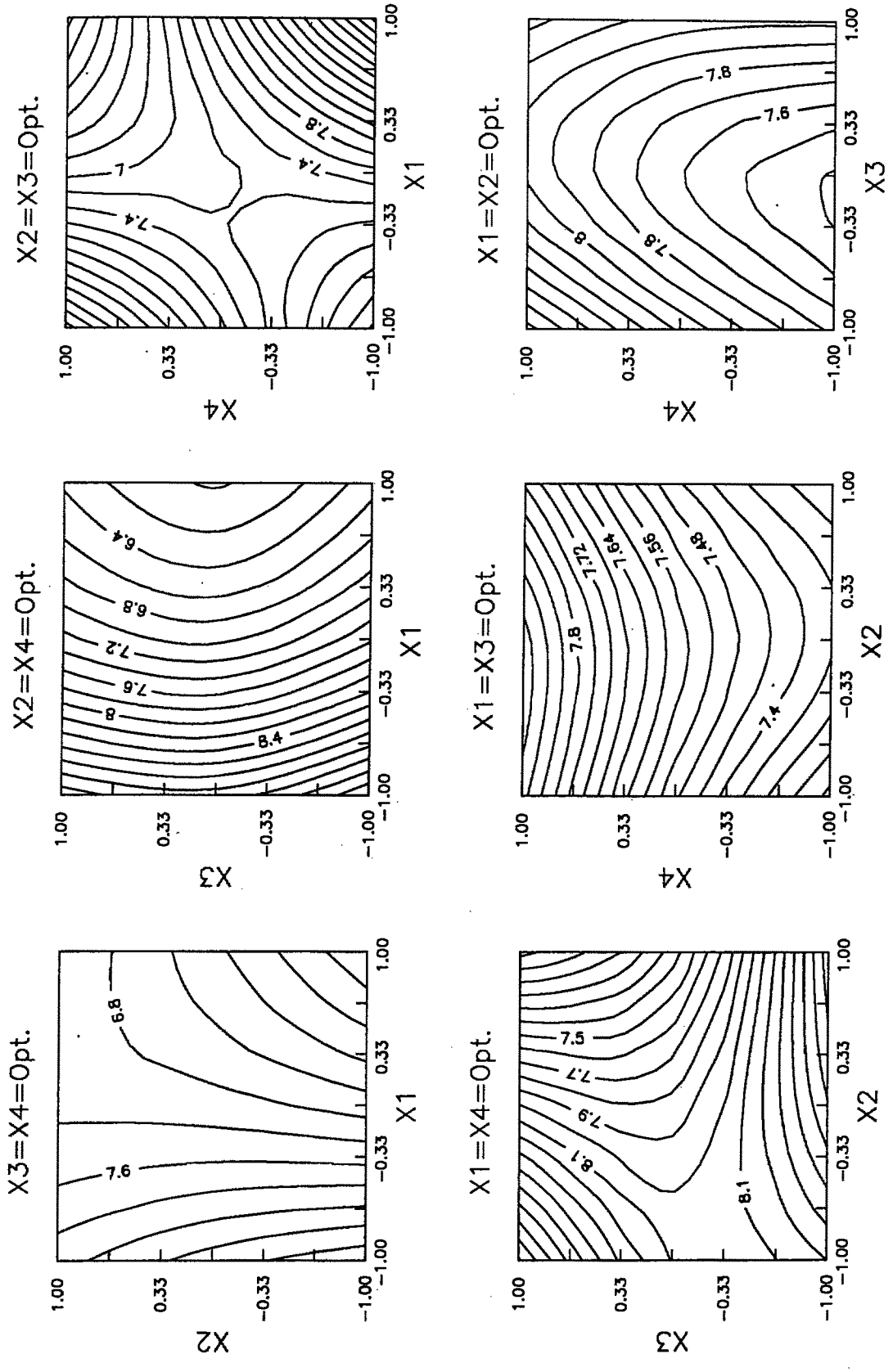


Figure 4.32 Contour plots for colour & appearance of whey protein-enriched orange beverage at optimum point

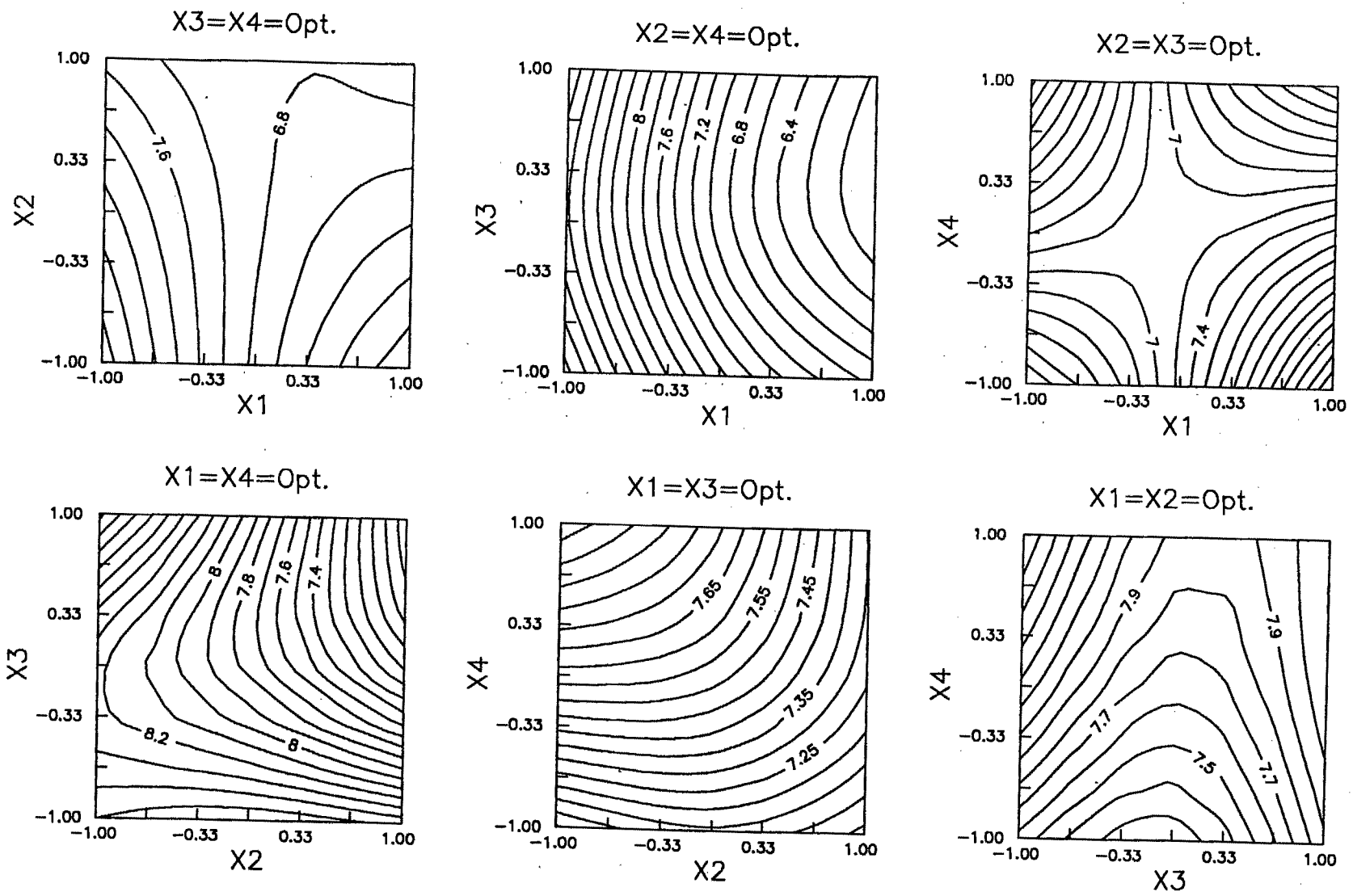


Figure 4.33 Contour plots for overall acceptability of whey protein-enriched orange beverage at optimum point

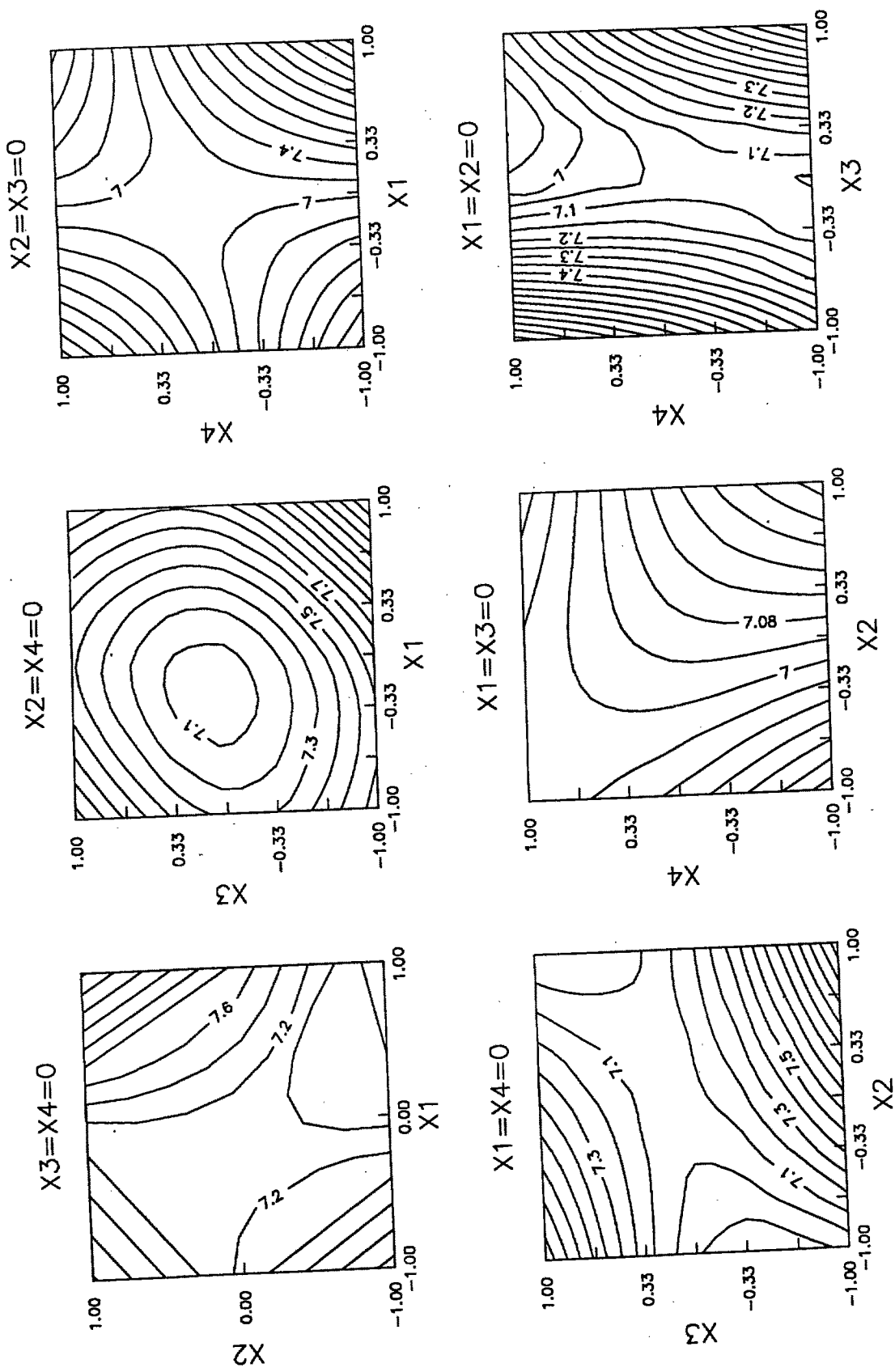


Figure 4.34 Contour plots for flavour attribute of whey protein-enriched orange beverage at center point

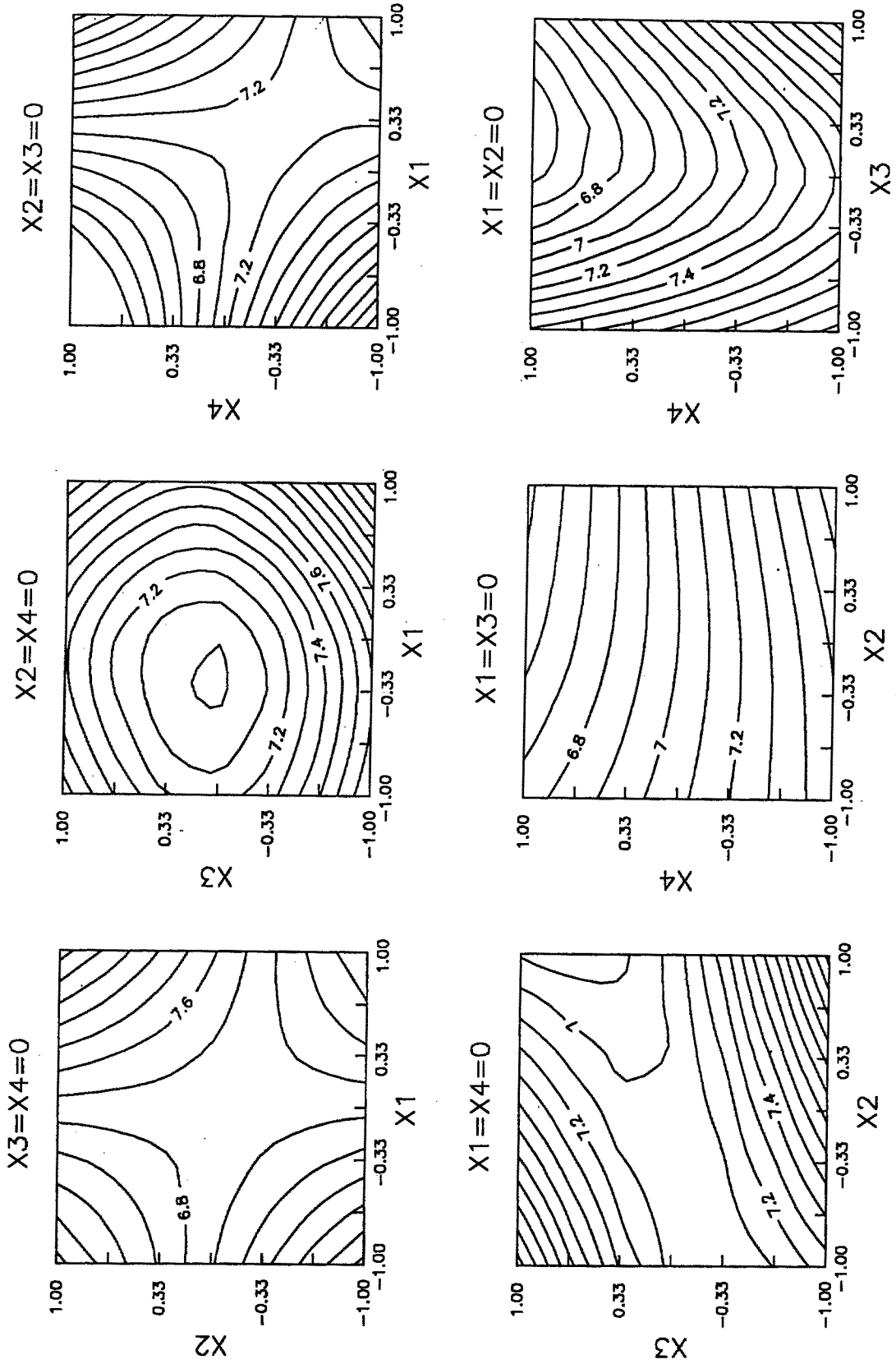


Figure 4.35 Contour plots for mouthfeel of whey protein-enriched orange beverage at center point

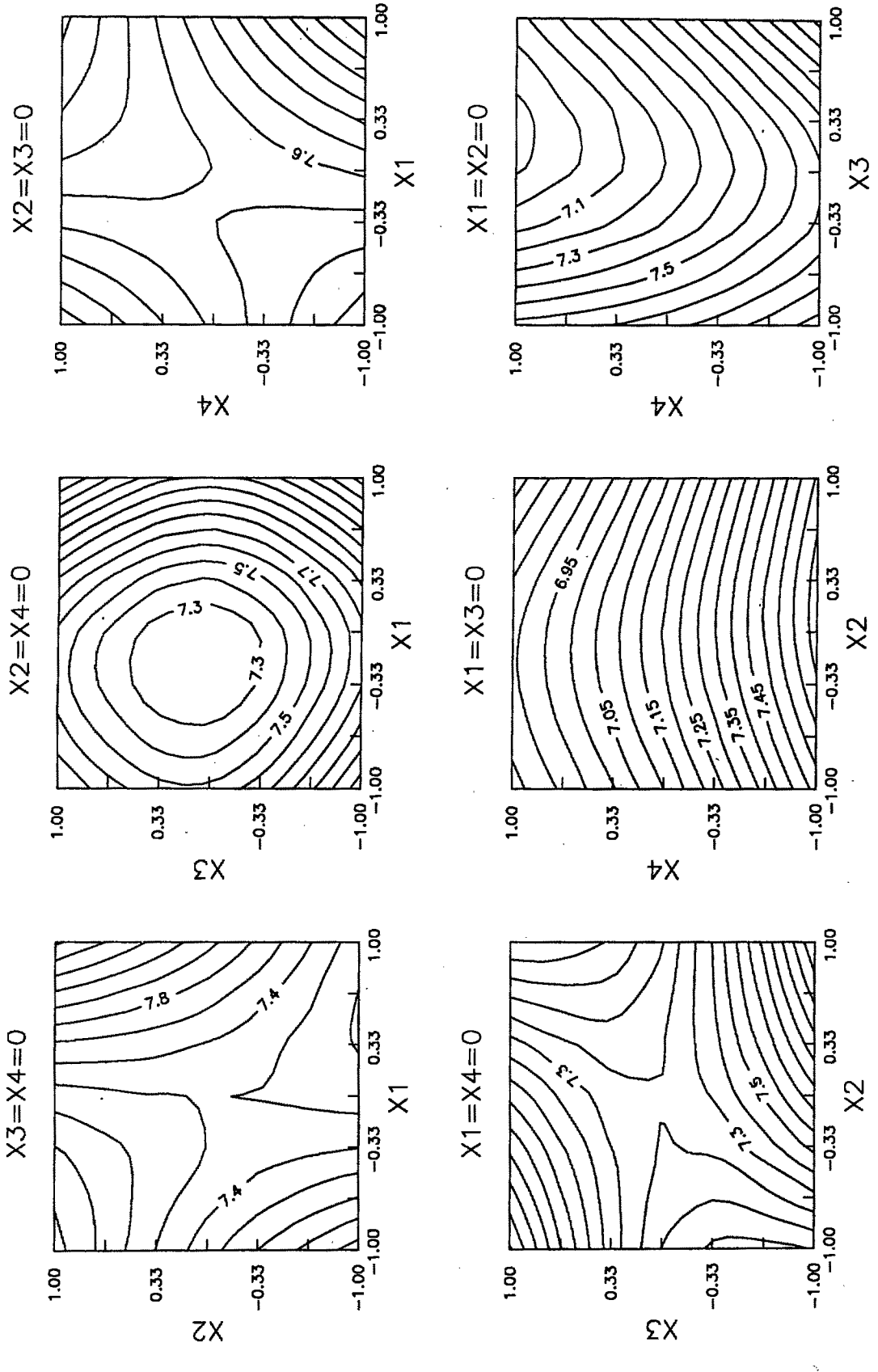


Figure 4.36 Contour plots for colour & appearance of whey protein-enriched orange beverage at center point

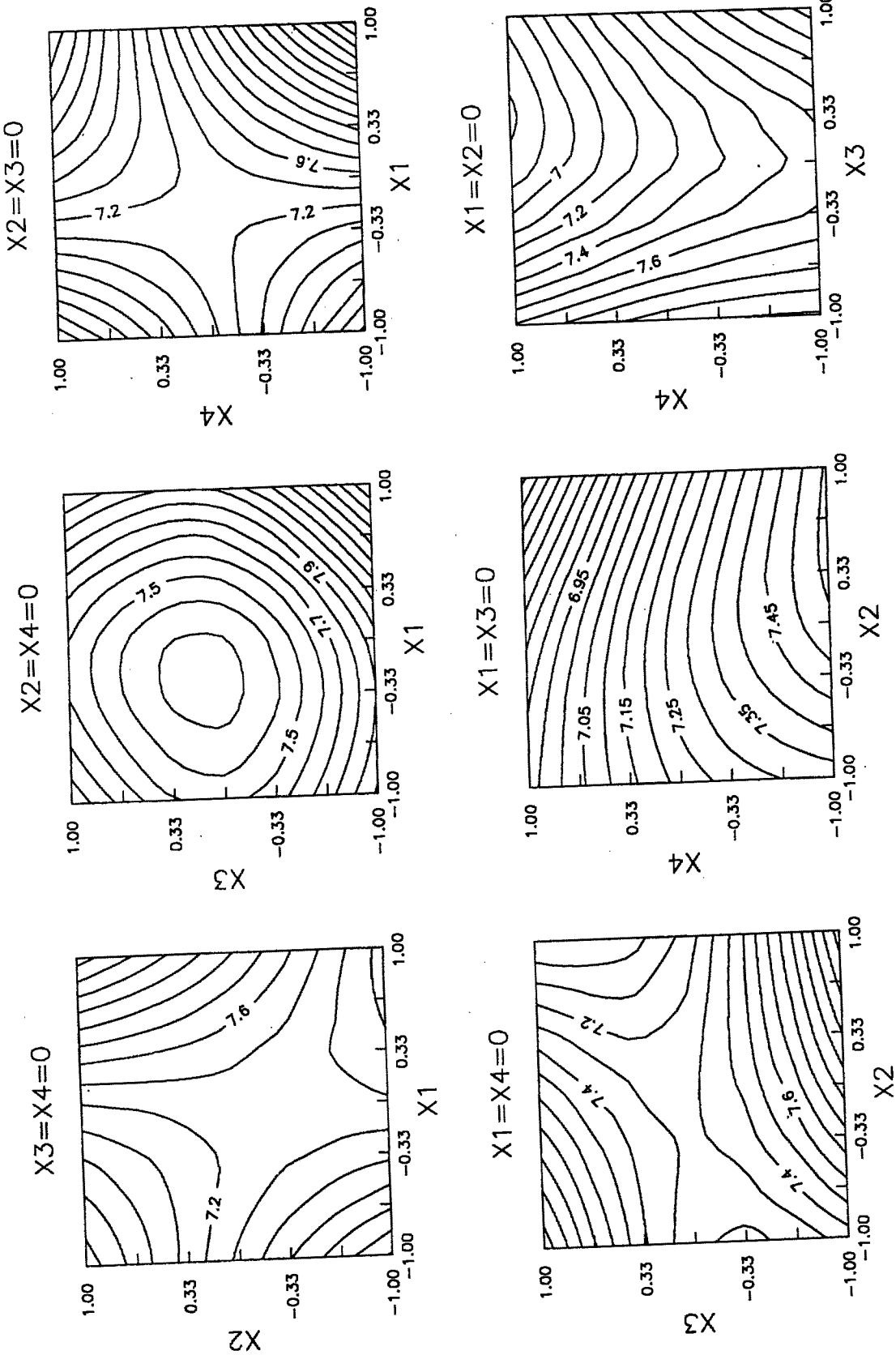


Figure 4.37 Contour plots for overall acceptability of whey protein-enriched orange beverage at center point

selected (Table 4.32). To check the best combination products were prepared using the optimum level of ingredients (Table 4.32) and subjected to sensory evaluation. Amiri (2001) had reported that compromise optimum conditions provided better results than individual optimum

The scores were 6.2- 7.15, 7.3-7.42, 7.4-7.76 and 7.1-7.67 for flavour, colour and appearance, mouthfeel and overall acceptability (Table 4.33). The second set of optimum levels yielded the highest scores and there was only slight variation between predicted and actual sensory scores. All other three combinations differed significantly from second combination and their was difference between actual and predicted scores. The first and second combination did not differ statistically for colour and appearance and mouthfeel, but there was significant between them on flavour. Since optimization was carried out for overall acceptability of the product and instead of wide variations in predicted and actual sensory scores of other responses there was not much difference for overall acceptability (Table 4.33).

The difference in sensory scores may be due to the difference in protein level, which was earlier noticed to affect the acidity of the product. In absence of proper sugar to acid ratio samples were observed to be too sour or lack proper orange flavour. The second and third combination did not differ on overall acceptability score, but former scored maximum for flavour and contained higher protein level. Hence second combination was selected as optimum compromise level. The beverage formulation included 31.59% fruit juice, 2.45%whey protein in UF-retentate, 11.12% sugar and 0.29 %stabilizer.

Table 4.32: Levels of ingredients (Fruit juice, Whey proteins in UF –retentate, Sugars and Stabilizers) yielding optimum response.

Combinations	Fruit Juice (%)	Whey Protein (%)	Sugar (%)	Stabilizer (%)
I	30.02 (-0.992)[a]	1.76 (-0.64)	10.04 (-0.96)	0.22 (0.2)
II	31.59 (-0.364)	2.23 (-0.17)	11.12 (0.12)	0.29 (0.9)
III	32.20 (-0.12)	1.85 (-0.55)	11.86 (0.86)	0.20 (0)
IV	33.20 (0.29)	3.38 (0.98)	10.21 (-0.79)	0.27 (0.7)

[a] values in parenthesis are in coded terms.

Table 4.33: Sensory scores of combinations at optimum compromise conditions.

Combination	Flavour	Colour & appearance	Mouthfeel	Overall acceptability
I	6.20 (7.25)[a]	7.30 (7.46)	7.65 (8.04)	7.05 (7.16)
II	7.85 (7.74)	7.40 (7.79)	7.76 (7.84)	7.67 (7.76)
III	6.85 (8.40)	7.42 (8.13)	7.40 (8.42)	7.22 (7.91)
IV	6.60 (7.81)	7.30 (7.88)	7.40 (7.92)	7.10 (7.67)
C.D. at P≤0.05 level	0.66	0.72	0.35	0.66

[a] values in parenthesis indicate predicated score.

4.9.4 Selection of range of ingredients for whey protein –enriched orange beverage

To prepare an acceptable product at low cost, it is desirable to have formulation that could be used for the product. This could be achieved by plotting contours of different responses and then overlapping of them on each other at corresponding conditions and then find out the common range of acceptable responses. The contours were plotted between two variables, while keeping the other variables constant at centre point or at optimum point (Fig 4.30-4.37). The contour equations at optimum point can be developed by simplifying the full second order equation after substituting the optimum values of other variables. These contour plots showed the effect of two variables at one time on responses. Since the overall acceptability scores of the product indicate its relative likeness for product's all sensory attributes, hence final range of product was selected by considering common range of all attributes. The criteria for range selection (Table 4.34) were that the beverage would have maximum sensory scores. By observing the contour plots it was decided that the entire lower limit for sensory score should be kept at 7.0 on nine-point hedonic scale.

4.9.5 Physico-chemical properties whey protein-enriched orange beverage

Whey protein-enriched orange beverage prepared using optimized levels of ingredients, i.e., 31.59 % orange juice, 2.45% whey proteins in UF-retentate,

Table 4.34: Range of ingredients required to develop acceptable whey protein-enriched orange beverage

Interaction	Orange juice (%)	Whey protein (%)	Sugar (%)	Stabilizer (%)
	X_1	X_2	X_3	X_4
X_1X_2	30-32.125 (-1 to -0.15)[1]	1.4-3.4 (-1 to +1)	11.86* (0.86)	0.25* (0.5)
X_1X_3	30-32.5 (-1 to 0)	2.45* (0.05)	10-12 (-1 to +1	0.25* (0.5)
X_1X_4	-	-	-	-
X_2X_3	31.59* (-0.36)	1.4-1.74 (-1 to -0.66)	10-12 (-1 to +1)	0.25* (0.5)
X_2X_4	31.59* (-0.36)	7.4- 2.55 (-1 to 0.15)	11.86* (0.86)	0.167 to 0.225 (-0.33 to 0.25)
X_3X_4	31.59 * (-0.36)	2.45* (0.05)	10-12 (-1 to +1)	0.1-0.24 (-1 to 0.4)

[1] value in parentheses are in coded terms.
* values at optimum point.

Table 4.35: Chemical constituents of whey protein enriched orange beverage

Constituents	Value
T.S.S. (^{0} Brix)	18.2
Total solids (%)	18.76
pH	3.5
Acidity (% Citric acid)	0.60
T.S.S. to Acid Ratio	30.33
Total Sugars (%)	16.21
Reducing Sugar (%)	4.28
Ascorbic acid (mg/100 gm)	2.75
Protein (%) (N% x 6.38)	2.75

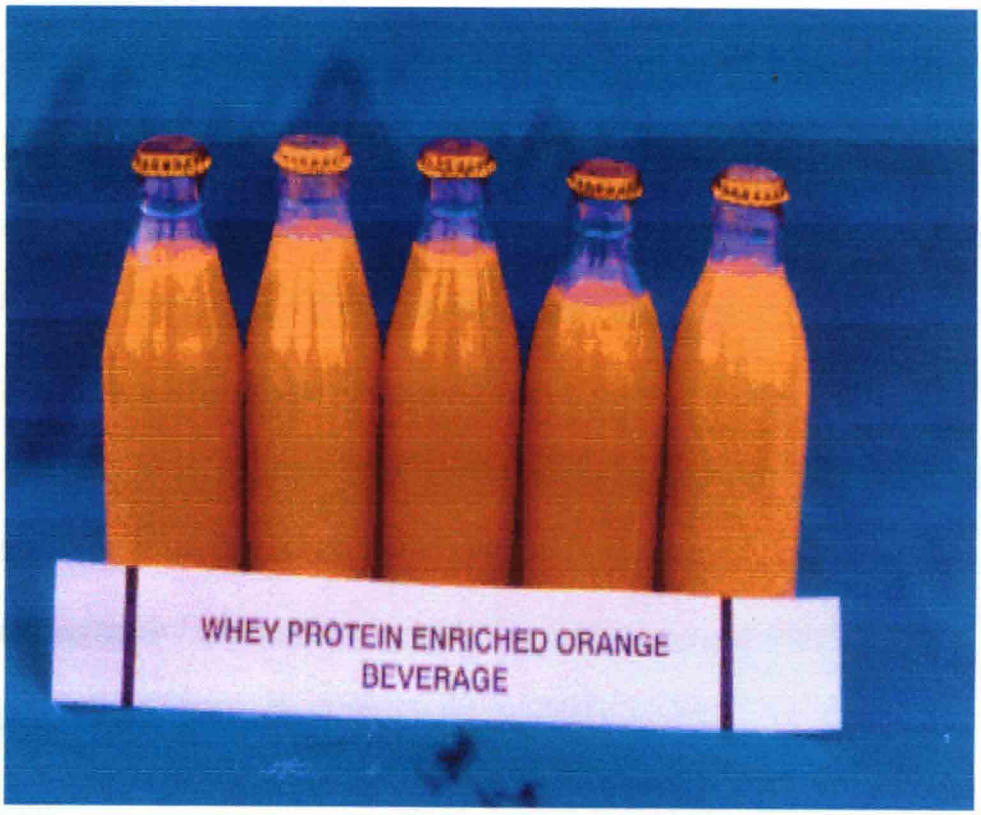


Plate 4.8 :Whey Protein-enriched Orange Beverage

11.12% sugar and 0.25 % stabilizer was analyzed for physico-chemical characteristics (Table 4.35)

It had a pH of 3.5 acidity of 0.60 % as citric acid, TSS was 18.2° Brix and total solids of 18.76 %. Sharma *et al.* (1998) had developed a similar beverage and beverage but its pH was 3.75. Total sugar content of beverage was 16.21% and protein content was 2.75 %. The reducing sugar content was 4.28% .The ascorbic acid content was only 2.78 mg/100 gm, which was much lower than reported value in orange beverages. Initial ascorbic acid content was 27.4 mg in orange juice. Low ascorbic acid content may be attributed to use of only approx. 32% of orange juice.

According to Jelen *et al.* (1987) commercially available whey drinks in European market contained 11.88-14.47% TS, 0.06-0.71% protein, 1.00-4.30%, lactose, 0.10-7.7% sucrose, 0.04-1.36% lactic acid and 3.00-3.93 pH.

4.10 Storage studies

Changes in whey protein-enriched orange beverages during storage at 36°, 42° and 52°C alongwith control (7°C) were monitored.

4.10.1 Total soluble solids and total solids

Total soluble solids of whey protein-enriched orange beverage were decreased from the initial value of 18.2°Brix to 18.0, 17.25, 16.8 and 15.8°Brix during storage at 7°Cfor 124 days, 36°C for 124 days, 42°C for 50 days and 52°C for 15 days (Fig.4.38). Per centage of total solids increased from a initial value of 18.45 to 18.68 in control samples and to 18.96, 19.43 and 19.89 for

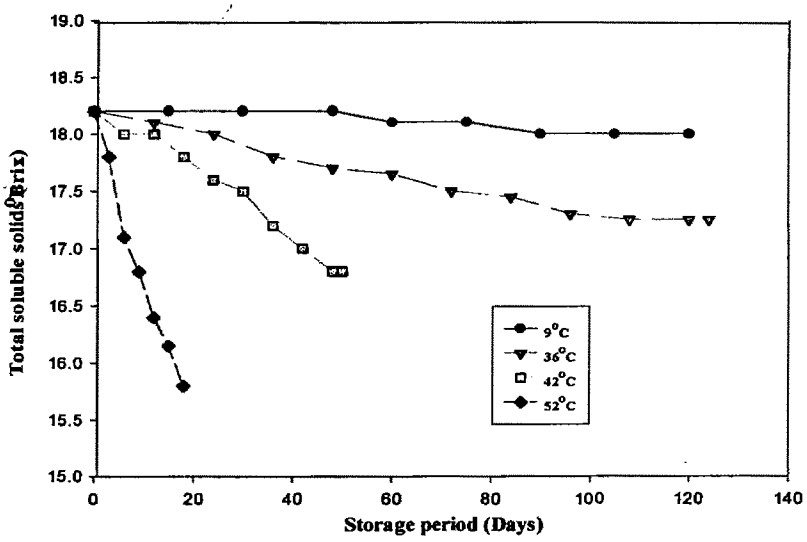


Fig 4.38 : Change in total soluble solids in whey protein-enriched orange beverage during storage.

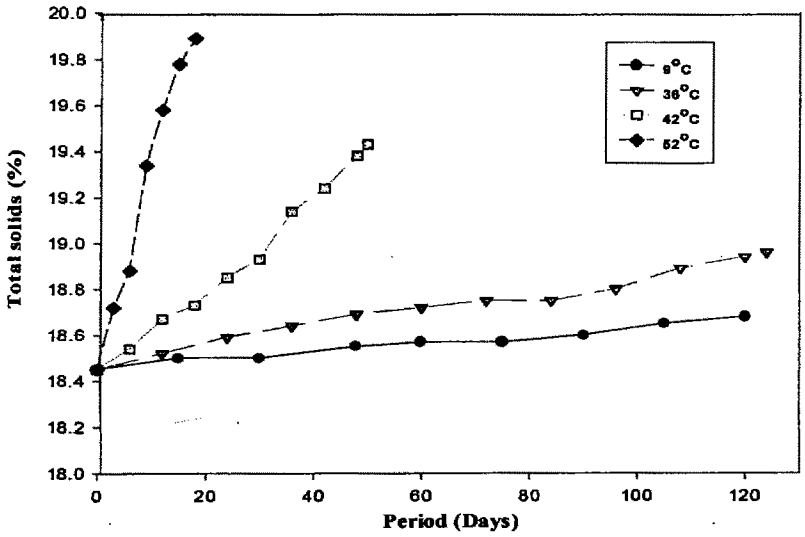


Fig. 4.39 : Change in total solids in whey protein-enriched orange beverage during storage.

beverages stored at 36°, 42° and 52°C respectively (Fig 4.39). The loss in total soluble solids was caused by interaction of sugars with other macromolecules specially proteins at elevated temperatures. Roy (1992) reported no change in T.S.S. of RTS beverages during 30 day storage at 4°C, 30°C and 40°C whereas, Prasad (2000) found a slight increase in T.S.S. of beverages stored at 37°C for 90 days but this samples contained very small amount (~2.75%) of protein.

Sharma *et.al* (2001) had reported increase in total solid content and attributed this increase in various chemical reactions, which utilize water. Storage of beverage samples at elevated temperature might cause dissolution of polysaccharide molecules resulting in increased hydration.

4.10.2 pH and Acidity

The pH of the beverage decreased during storage from an initial value of 3.5 to 3.44 (control), 3.38 (36°C), 3.36 (42°C) and 3.29 (52°C). The results show that decrease in pH was greater at higher temperatures (Fig 4.40). The initial acidity of 0.60 % as citric acid increased to 0.69 (control), 0.94 (36°C), 0.92 (42°C) and 0.94% (52°C) at the end of storage period (Fig 4.41). Decrease in pH with subsequent increase in acidity of the fruit beverages has been observed by several workers (Roy, 1992, Prasad, 2000). pH drop can be attributed to a decrease of positive charges on protein molecules caused by a loss of free -amino group of lysine in Maillard reaction, Storage at higher temperatures favor Maillard reaction resulting in production of organic acids and net increase in product acidity.

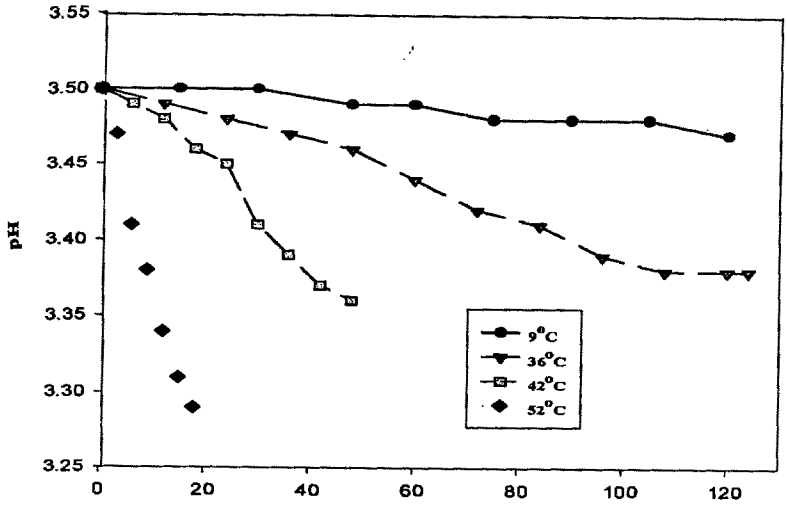


Fig 4.40 : Change in pH during storage of whey protein-enriched orange beverage

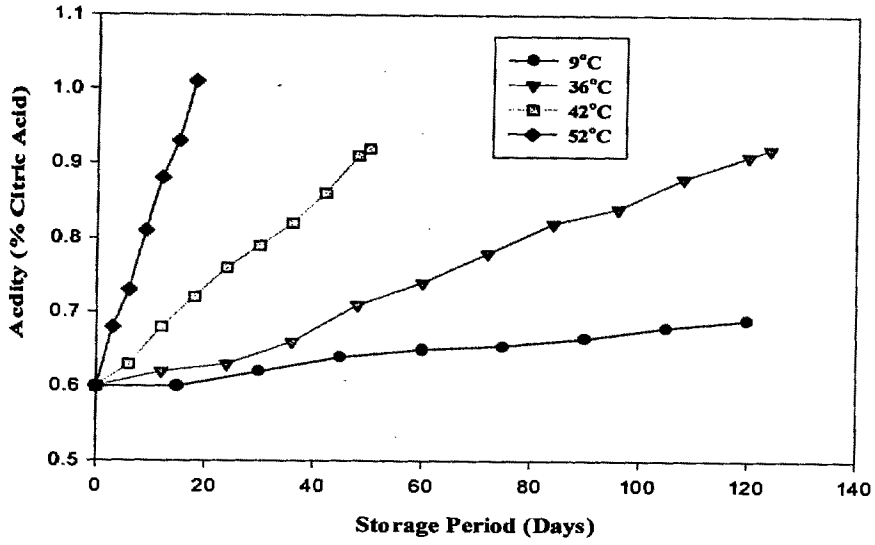


Fig 4.41 : Change in acidity of whey protein-enriched orange beverage during storage.

4.10.3 Total sugars and reducing sugars

The total sugars of the beverage decreased from 16.21 % to 15.96 (control), 15.36 (36°C), 15.02 (42°C) and 14.16 (52°C) %, after 120, 124, 50 and 15 days of storage respectively (Fig 4.42). During the same period, reducing sugars increased from a initial value of 4.28% to 4.99% (control), 6.28% (36°C), 7.86% (42°C) and 8.61% (52°C) respectively (Fig .4.43). The above changes were more prominent at elevated temperatures of storage. Similar observations were reported in products like protein-enriched mango bar (Mir, 1990), mango beverage (Roy, 1992). The increase in reducing sugars during storage has been attributed to inversion of disaccharide under acidic conditions (Labuza, 1970). Acid may have also induced hydrolysis of polysaccharides, i.e,. stabilizers under highly acidic conditions and at higher storage temperature. The decrease in total sugar content during storage was observed in number of beverages, (Roy ,1992, Dhaliwal and Heera, 2001 and Sharma *et.al*, 2001). They attributed participation of sugars in Maillard reactions. Gogus *et.al* (1998) had reported similar observation while working on non-enzymatic browning reactions.

4.10.4 Protein content and in-vitro digestability

Protein content of the beverage was initially 2.75 % (Table 4.35). During storage this value decrease to 2.60% (control), 2.18 (36°C), 2.03 (42°C) and 2.00%(52°C) (Fig 4.44). There was a reduction in *in vitro* digestibility of beverage protein from its initial value of 84.83% to 82.16%, 76.38, 74.96, and 69.54 % for the samples stored at control, 36°, 42°C, and 52°C respectively

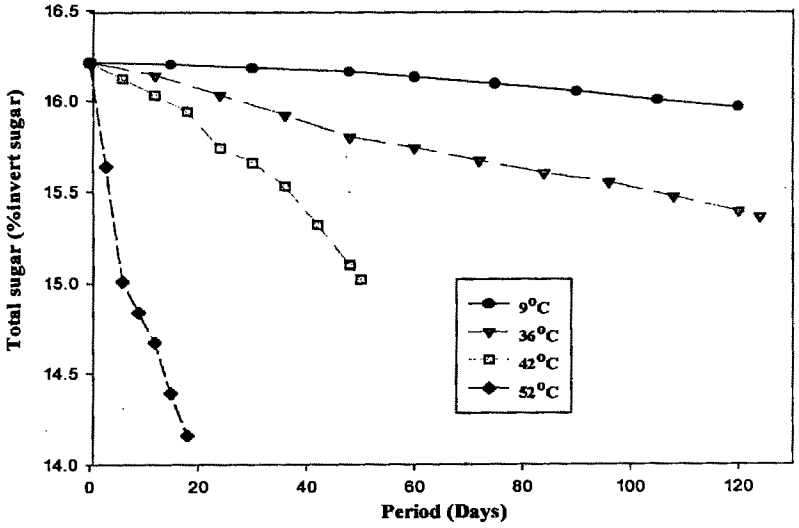


Fig. 4.42 : Change in total sugar content in whey protein-enriched orange beverage during storage

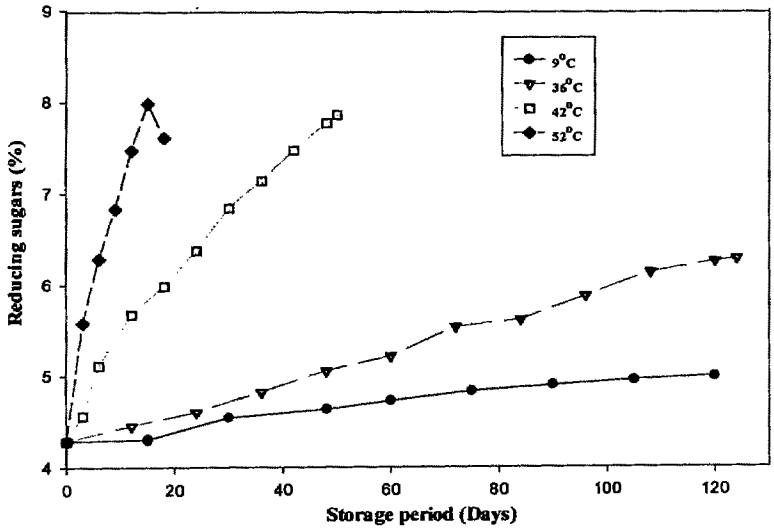


Fig. 4.43 : Change in reducing sugar contents in whey protein-enriched orange beverage during storage

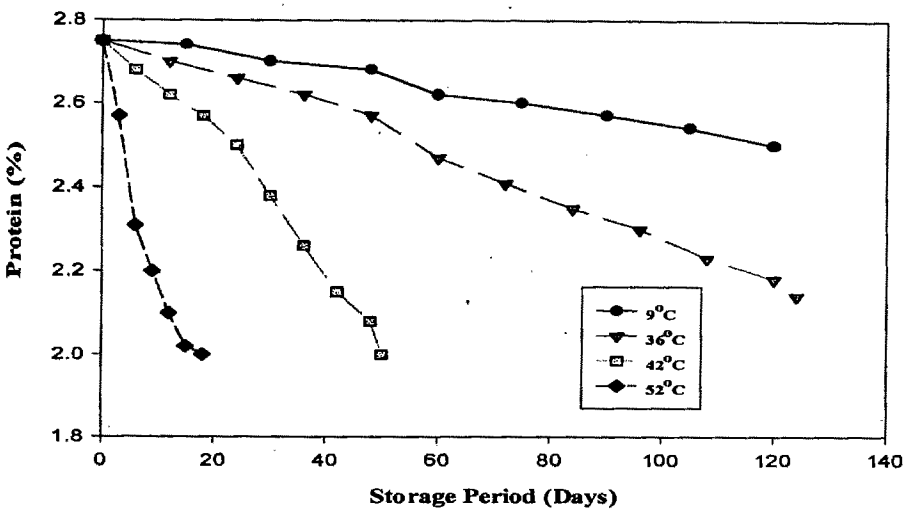


Fig 4.44 : Effect of storage tempertature on loss of soluble proteins in whey protein-enriched orange beverage

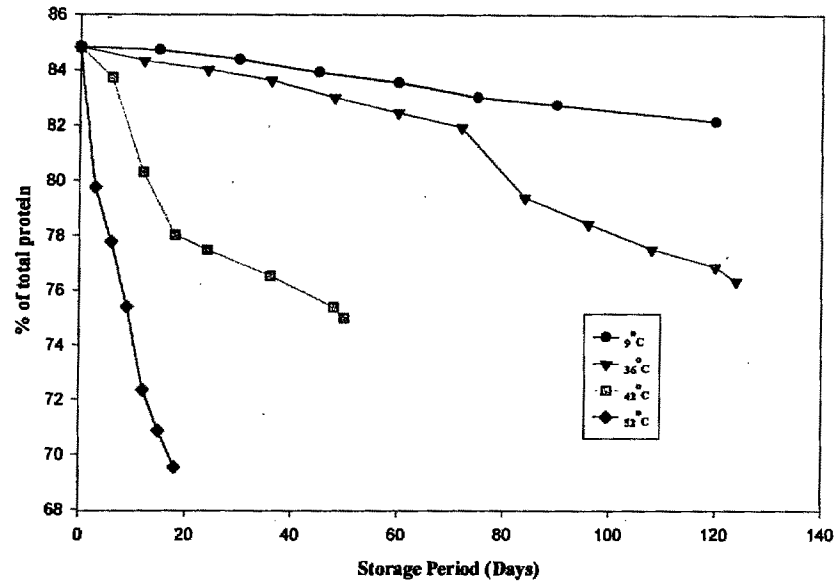


Fig.4.45 : Effect of storage temperature on *Invitro* protein digestability of whey protein enriched orange beverage

(Fig. 4.45). The changes were pronounced at higher temperatures. Increased protein-protein interaction at low pH of beverage, as a result of decrease of net charge at higher temperature decreased protein content. This explanation is well supported by sedimentation problem encountered in beverages stored at higher temperature. Decrease in protein content and *in vitro* digestibility was caused by reaction between free amino group of amino acids, peptides and protein with sugars i.e Maillard reaction. Similar results were reported earlier by Friedman, (1996).

4.10.5 Proteolysis

Proteolysis, measured as μmol of glycine/ml of whey protein-enriched orange beverage, increased during storage from an initial value of 0.12 to 0.20, 0.48, 0.55 and 1.07 at storage of temperature of 9° (control), 36°, 42° and 52°C at the end of 120, 124, 50 and 15 days (Fig 4.46). It shows that increase in proteolysis was greater at 52°C. Solanky (1987) and Anantha Narayanan (1993) have reported similar type of proteolysis in UHT milk and UHT soy beverage during storage even though they did not observe presence of any proteolytic enzyme or microorganism in samples.

4.10.6 Hydroxymethyl Furfural content

Total HMF content at the beginning of storage period was 27.78 μmol /L of beverage. This value increased to 31.26, 39.62, 43.64, and 66.67 μmol/L at respective storage temperatures of 9°, 36°, 42° and 52°C after 120, 124, 50 and 15 days (Fig 4.48). A simultaneous increase was observed in free HMF content. Its level increased from initial value of 7.30 μ mol/L to 12.34, 18.26,

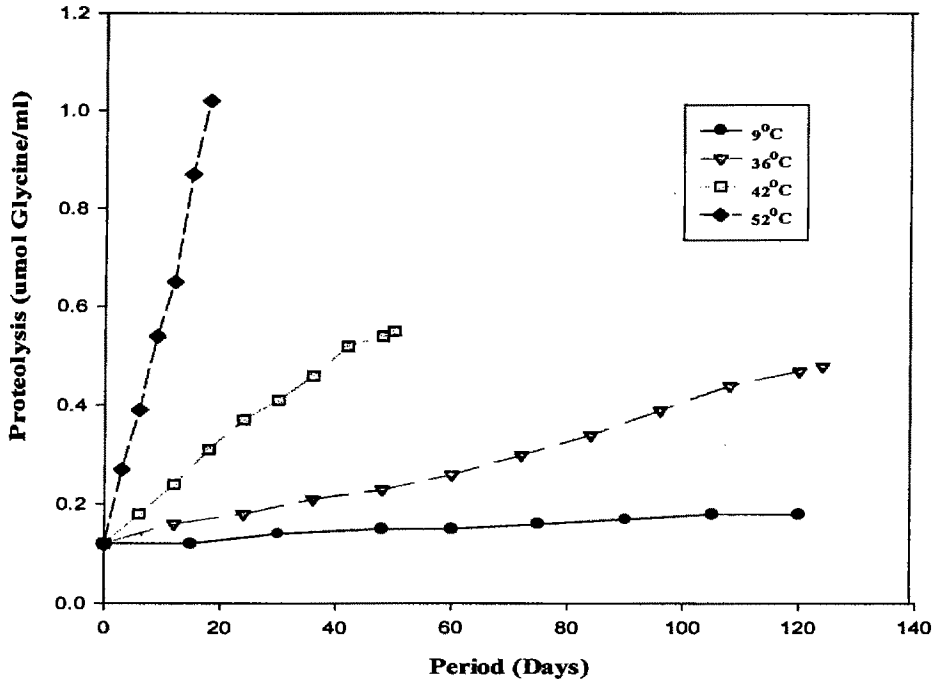


Fig 4.46: Effect of storage temperature on proteolysis whey protein-enriched orange beverage

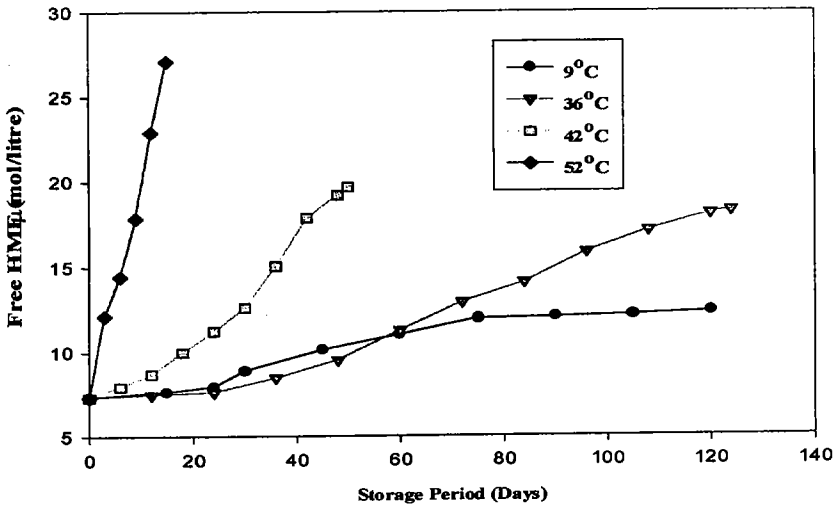


Fig. 4.47 : Changes in free HMF in whey protein-enriched Orange beverage during storage

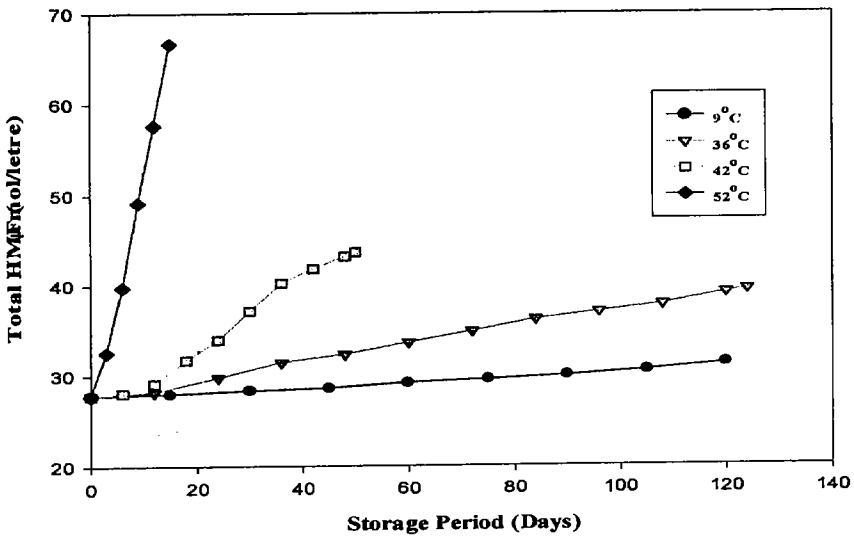


Fig.4.48 : Changes in total HMF in whey protein-enriched orange beverage during storage

19.66 and 27.08 μmol/L for the samples stored of 9° (control), 33°, 42°, and 52°C (Fig 4.47). The results show that there was rise in free and total HMF content during the storage and the increase in their formation was faster at higher temperatures. A number of workers have reported such an increase in HMF contents of food products at elevated temperatures. (Lee and Negy, 1988, Singh, 1991, Rassis and Sagay, 1990, Anantha Naryanan, 1995).

4.10.7 Colour and non-enzymatic browning

Colour measured as reflectance at 450 μm, decreased from 62 % to 57.11(control) 38.86,39.58 and 36.73% for whey protein-enriched orange beverages stored at 36,42 and 52°C, for 124, 50 and 15 days respectively(Fig 4.49). There was a simultaneous increase in non-enzymatic browning, of samples from 0.068 to 0.099 (control), 0.231 (36°C), 0.283 (42°C) and 0.268 (52°C) during storage (Fig.4.50). The decrease in colour of the beverage with simultaneous increase in NEB values has been reported by several other workers (Anantha Narayanan, 1993; Singh, 1991; Prasad 2000). The change in colour and increase in NEB is mainly attributed to formation of brown coloured products as a result of Maillard reactions and accumulation of hydroxy methyl furfural (HMF) during storage

4.11 Kinetics of deteraiorative reactions during storage

4.11.1 Acidity

The change in acidity of beverage samples followed zero order reaction kinetics. The reaction rate constants were 2.89 x 10^{-3}, 6.37 x 10^{-3}, and 2.29 x

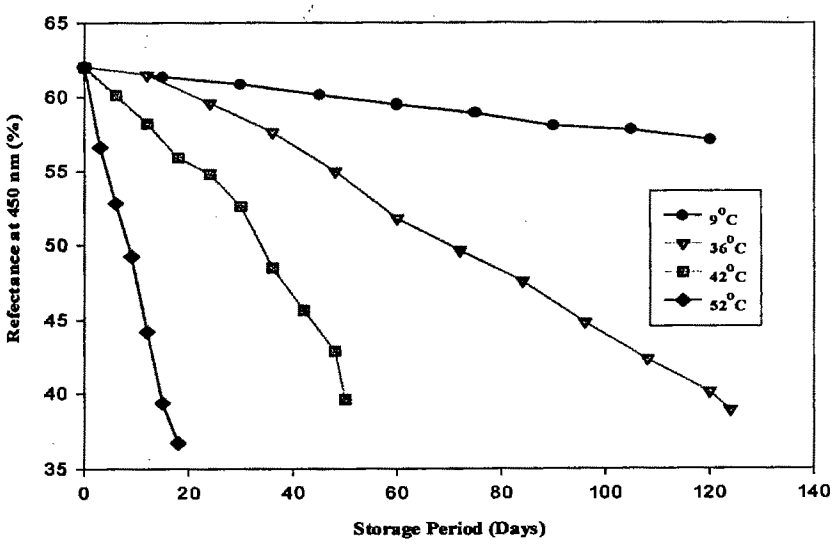


Fig. 4.49 :Effect of storage temperature on colour of whey protein enriched orenge beverage

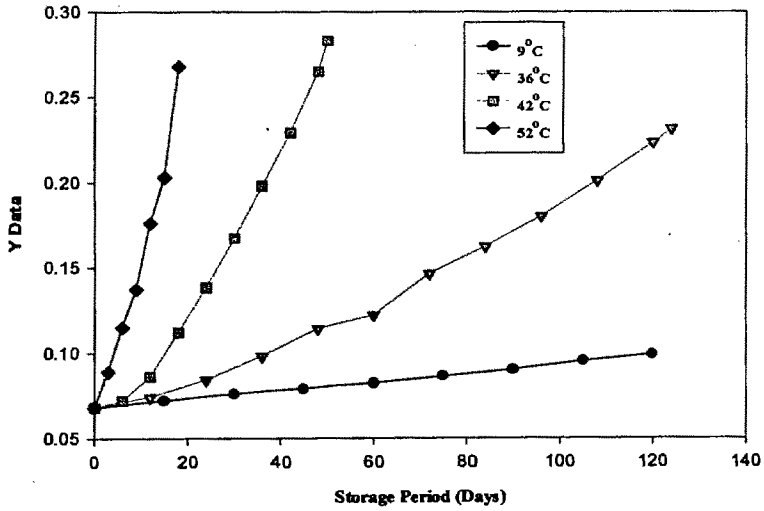


Fig. 4.50: Changes in NEB during storage of whey protein enriched Orenge beverage

Table 4.36: Kinetic parameters for change in acidity, reducing sugar, total sugar during storage of whey protein-enriched whey beverage

Chemical reaction	Temperature (^{0}C)	Rate constant k (day^{-1})	R^2	Activation energy (KJ/mol)	Arrhenius constant Ko
Acidity	9^{0}C	9.05 x 10^{-4}	0.966	31.19 (9-36^0 C)	5.44 x 10^2
	36^{0}C	2.80 x 10^{-3}	0.993		
	42^{0}C	6.37 X10^{-3}	0.994	108.13 (36-52^{0}C)	5.48 X101^5
	52^{0}C	2.22 X10^{-2}	0.995		
Reducing Sugar	9^{0}C	6.75X10^{-3}	0.963	6.17(9-36^0 C), 68.93(42-52^0 C)	2.06X10^3
	36^{0}C	1.69X10^{-3}	0.994	80.06 (36-52^0 C)	4.73X10^{15}
	42^{0}C	8.43 X10^{-2}	0.922		
	52^{0}C	4.68 X10^{-2}	0.862		1.95X10^{18}
Total Sugar	9^{0}C	1.5 X10^{-4}	0.979	26.98 (9-36^0 C)	1.49X10^2
	36^{0}C	4.1 X10^{-4}	0.987		
	42^{0}C	5.04 X10^{-3}	0.941	142.83 (36-52^0 C)	5.79X10^{20}
	52^{0}C	6.53 X10^{-3}	0.954		

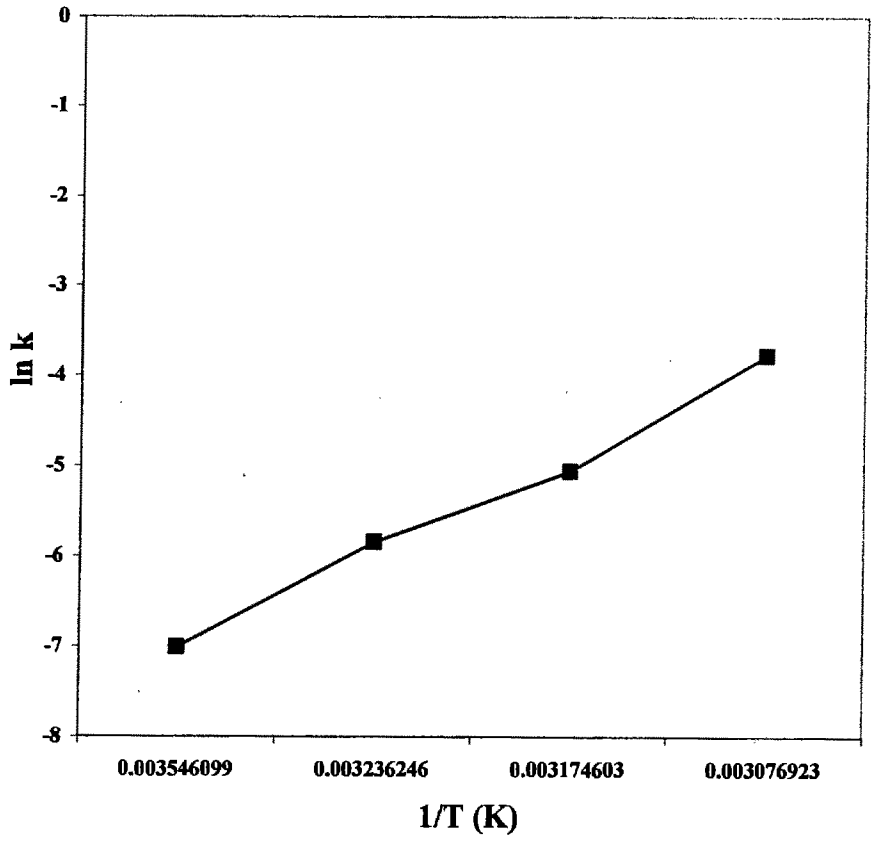


Fig. 4.51: Effect of temperature on acidity of whey protein-enriched orange juice during storage

10^{-2} at the storage temperature of 9°, 36, 42 and 52°C and there was an increase in reaction rate on increasing storage temperature (Table 4.36). There were two straight line portions in Arrhenius curve (Fig 4.51) and similar observations was reported by Ananthaarayan *et al.* (1993). Change in acidity of the beverage was very high at higher temperature as compared to lower storage temperature, because at elevated temperature, resulting in formation of certain organic acids . The activation energy (Ea) for two straight line portions were 31.9 (9-36°C) and 108.13 KJ/mole with corresponding values of (36-52°C) Arrhenius constant of 5.44 x 10^2 and 5.49 x 10^{15}. Singh (1991) reported that changes in acidity follow zero order reaction kinetics with five straight line portions on Arrhenius curve.

4.11.2 Sugar and reducing sugars

The decrease in total sugars during storage was very much apparent at higher temperatures and reaction rate constants (k) increased with increase in temperature (Table 4.36). The Arrhenius plot was not a straight line and it indicate two straight line portions between 9° to 36°C and 36°-52°C (Fig 4.53). The activation energy was higher i.e.. 142.83 KJ/mole in the temperature zone of 36-52°C in comparisons to 26.98 KM/mole at lower temperature range (9-36°C). The corresponding values for Arrhenius constant were 5.79 x 10^2. Decrease in sucrose the major sugar in fruit juices has been reported to follow first order reaction kinetics (Garza *et al.* 1996).

The reducing sugar content was found to increase during storage period and their formation followed first order reaction rate. The values of rate

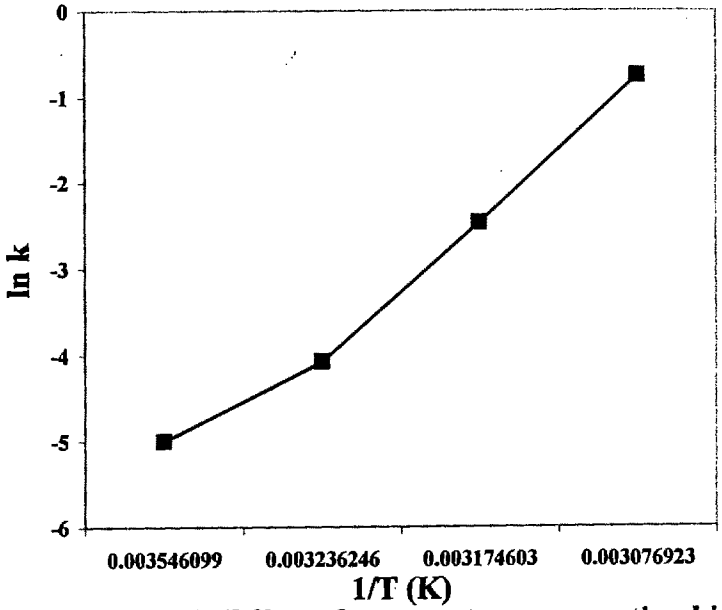


Fig. 4.52: Effect of temperature on reaction kinetics of reducing sugar during storage

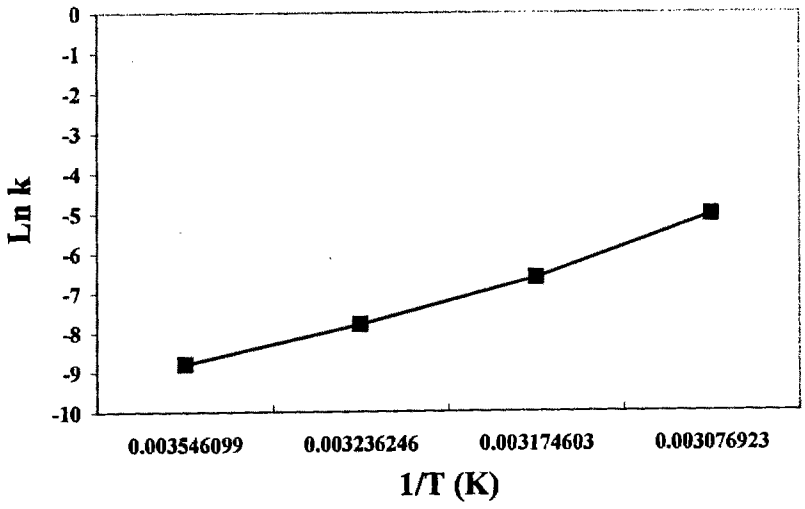


Fig. 4.53: Effect of temperature on reaction kinetics of total sugar loss in whey protein-enriched orange beverage

constants increased with increase in temperature from1.51 x 10^{-3} to 0.129 per day(Table 4.36) This indicate that at higher temperature rate of reaction also get increases. However, Arrhenius type equation was not able predict temperature induced effect on reducing sugar formation. The curve had three straight line portions (Fig.4.52).

The activation energy for these straight line portions were 6.17, 68.93 and 80.06 KJ/mole, with corresponding values for Arrhenius constants were 2.06 x 10^{3}, 4.73 x 10^{15} and 1.95 x 10^{18} respectively (Fig 4.36). The increase in activation energy with an increase in storage temperature suggesting increased solution of reactants in these reaction at higher temperature.

4.11.3 Soluble protein loss and proteolysis

The soluble protein content loss in beverage followed zero order reaction kinetics. The reaction rate constants varied from 7.2 x 10^{-4} to 1.23 x 10^{-2} in the temperature range of 9-52°C. The rate of reaction increased with an increase in storage temperature (Table 4.37). The Arrhenius curve was not a single straight line and there were three straight line portion (Fig 4.54). The activation energies for these straight line portions were 22.24, 167.19, 209.74 KJ/mole (Table4.37). The corresponding values of Arrhenius constant were 9.52, 3.038 x 10^{25}, 3.44 x 10^{32}. Higher activation energies were obtained at higher temperature.

The proteolysis reaction also followed zero order with reaction rate constant vary in the range of 4.52 x 10^{-4} to 5.14 x 10^{-2} (Table 4.37). The Arrhenius curve has two straight portion with activation energies of 60.75 (9-

Table 4.37: Kinetic parameters for the loss of soluble proteins and proteolysis during storage of whey protein-enriched whey beverage

Chemical reaction	Temperature (^{0}C)	Rate constant k (day^{-1})	R^2	Activation energy (KJ/mol)	Arrhenius constant Ko
Protein	9^{0}C (control)	7.2X10^{-4}	0.995	22.24 (9-42^0 C)	9.52
	36^{0}C	1.65 X10^{-3}	0.989	167.19 (36-42^0 C)	3.03 X10^{25}
	42^{0}C	5.7 X10^{-3}	0.982		
	52^{0}C	1.23 X10^{-2}	0.898	99.45 (42-52^0 C)	3.44 X10^{32}
Proteolysis	9^{0}C (control)	4.52 X10^{-4}	0.940	60.75 (9- 42^0 C)	7.7 X10^7
	36^{0}C	3.04 X10^{-3}	0.984		4.05 X10^{22}
	42^{0}C	8.44 X10^{-3}	0.986	148.72 (36- 52^0 C)	
	52^{0}C	5.12 X10^{-2}	0.988		

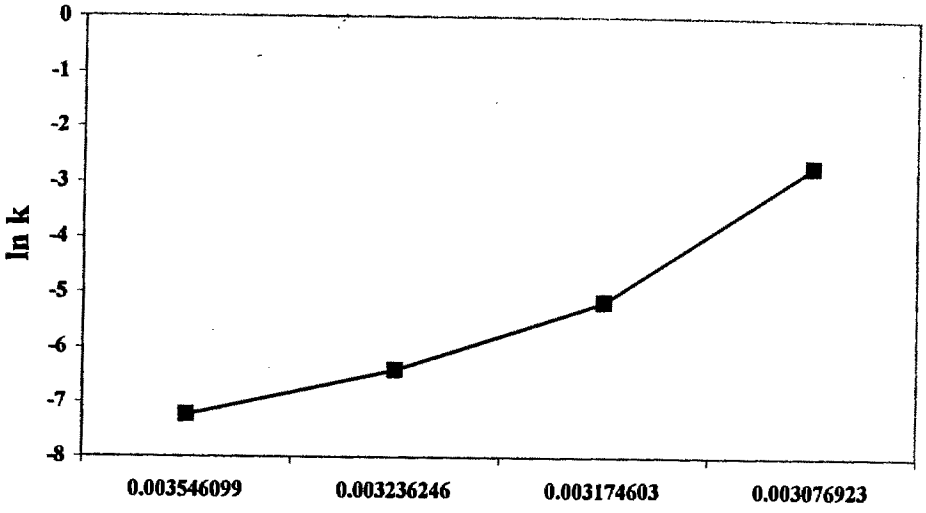


Fig 4.54 Effect of temperature on loss of soluble protein in whey protein-enriched orange beverage during storage

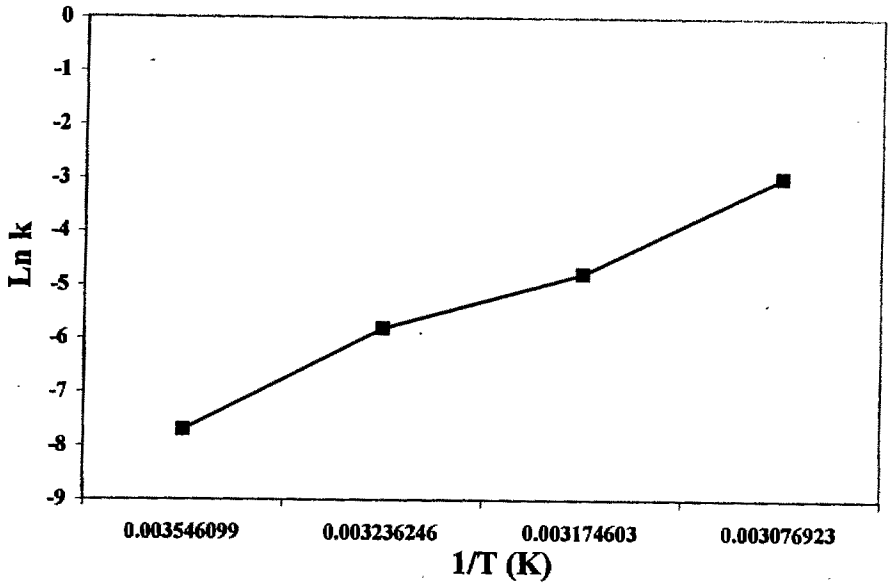


Fig. 4.55: Effect of temperature on kinetics of proteolysis in whey Protein-enriched ornage beverage

42°C) and 148.72 KJ/mole (36-52°C) (Fig 4.55). The values of Arrhenius constants were 7.70 x 10^7 and 4.05 x 10^{22} for these temperature range. The arrhenius equation filled well over a narrow range of temperature. Anantha Naryanan *et al.* (1993) reported proteolysis as first order reaction in stored UHT-milk.

4.11.4 Hydroxymethyl Furtaral (HMF) content

Both free as well as total HMF increased during storageAND , their formation was found to be affected by storage temperature. The rate of HMF formation (Free and Total) followed first order reaction kinetics. In Maillard reaction, 5-hydroxymethyl furfural (HMF) formation occurs during early stages. The reaction rate constants, for free HMF were 5.03 x 10^{-3} , 8.85 x 10^{-3}, 2.17 x 10^{-2} and 6.88 x 10^{-2} at 9°, 36, 42 and 52°C respectively(Table 4.38). Arrhenius type of curve indicated two straight line portions (Fig 4.56) and the activation energies for these portions were 15.13 (9-36°C) and 106.24 KJ/mol (36-52°C). The Arrhenius constants were for those straight line were 3.20 and 8.43 x 10^{15}. Singh (1991) reported free HMF formation as zero order reaction.

Total HMF also increased during storage and its formation was affected by storage temperature. The reaction of HMF formation followed first order kinetics. The reaction rate constants were 1.01 x 10^{-3}, 2.88 x 10^{-3}, 1.06x 10^{-2} at 9, 36, 42 and 52°C (Table 4.38). The total HMF formation curve had two straight line portions (Fig 4.57).

The activation energies calculated for those straight line portions were 27.87 (9-36°C) and 157.56 KJ/mol (36-52°C). (Table 4.38). HMF formation

Table 4.38: Kinetic parameters of HMF formation in Whey protein - enriched orange beverage during storage.

Chemical reaction	Temperature (^{0}C)	Rate constant k (day^{-1})	R^2	Activation energy (KJ/mol)	Arrhenius constant
Free HMF	9^0C (control)	$5.03X10^{-3}$	0.934	15.13 ($9-36^0$ C)	3.20
	36^0C	$8.88\ X10^{-3}$	0.986		
	42^0C	$2.17\ X10^{-2}$	0.993	106.24	$8.43X10^{15}$
	52^0C	$6.88\ X10^{-2}$	0.996	($36-52^0$ C)	
Total HMF	9^0C (control)	$1.01\ X10^{-3}$	0.986	27.87 ($9-36^0$ C)	$1.48X10^{2}$
	36^0C	$2.88\ X10^{-3}$	0.984		
	42^0C	$1.06\ X10^{-2}$	0.981	157.55	$1.31X10^{24}$
	52^0C	$6.01\ X10^{-2}$	0.997	($36-52^0$ C)	

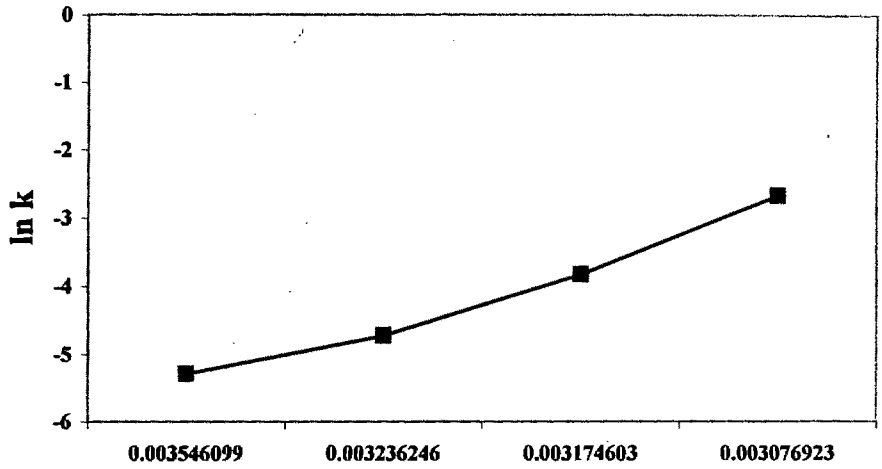


Fig. 4.56: Effect of storage temperature on formation of free HMF in whey protein-enriched orange beverage

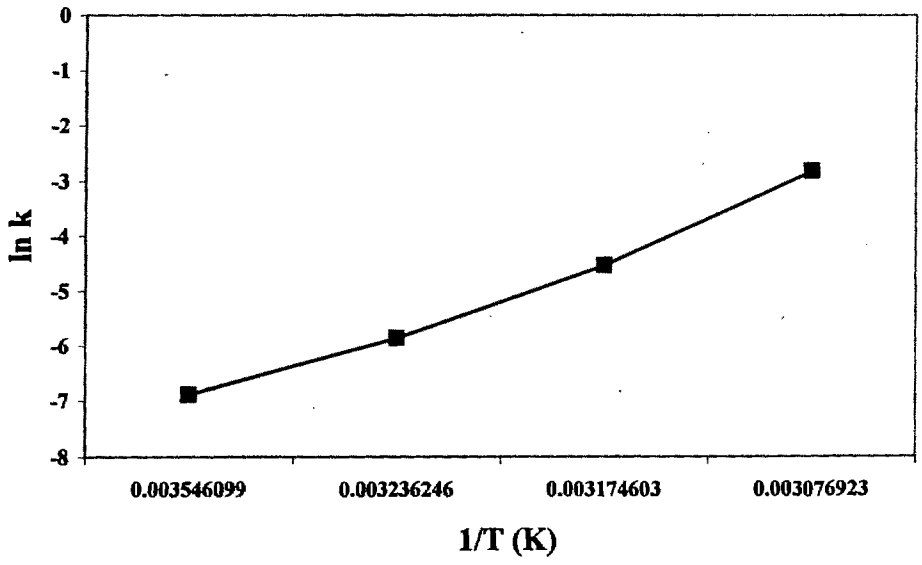


Fig. 4.57: Effect of temperature on formation of total HMF during storage of whey protein-enriched orange beverage

was reported to follow first order (Patel *et al.*, 1996; Pseudo-zero order (Hidalgo and Pompi, 2000) and zero order (Cohen *et al.*, 1998; Morales *et al.*, 1997) reaction kinetics in various food products.

4.11.5 Colour and Non-enzymatic browning

Colour of the whey protein-enriched orange beverage was measured as reflectance and the reflectance values of beverage samples decreased during storage according to zero order reaction kinetics. The reaction rate constants were 6.67 x 10^{-4}, 3.29 x 10^{-3}, 7.2 x10^{-3} and 2.2 x 10^{-2} at 0, 36, 42 and 52°C (Table 4.39). There was a progressive increase in rate of decrease in reflectance at higher temperature storage. The activation energy for two straight line portions on Arrhenius plot (Fig 4.58) indicated that this type of equation did not predict the influence of temperature on product quality over a wide range of storage temperature. The activation energies for two straight line portions were 50.12 and 99.44 KM/ mole, with Arrhenius constant values of 1.21 x 10^{7} and 3.69x 10^{14} (Table 4.39). Change in colour have been reported to follow zero order reactions in soy beverage and sweetened condensed milk (Anatha Narayanan, 1993, Patel *et al.*, 1996). Patel *et al.* (1996) had reported the activation energy (Ea) was 45.2 KM/mol at 7-30°C and 139.9 KM/mol at temperature of storage in the range of 30-55°C.

Non-enzymatic browning was found to increase with increase in temperature and storage period (Fig4.59). This reaction obeyed the zero order reaction kinetics. The rate of reaction was higher at elevated temperature of storage and it is indicated by rate constants (Table 4.39). The Arrhenius plot

Table 4.39: Kinetic parameters for non-enzymatic browning and change in colour during storage.

Chemical reaction	Temperature (^{0}C)	Rate constant k (day^{-1})	R^2	Activation energy (KJ/mol)	Arrhenius constant
Reflectance	9^{0}C (control)	0.00066	0.994	50.12 (9-42^0 C)	1.22X 10^6
	36^{0}C	0.00329	0.998		
	42^{0}C	0.0072	0.970	99.45 (42-52^0 C)	2.17X10^8
	52^{0}C	0.02227	0.994		
Non Enzymatic Browning	9^{0}C (control)	0.000267	0.994	65.75	3.32X10^6
	36^{0}C	0.001414	0.992		
	42^{0}C	0.004844	0.989		
	52^{0}C	0.01141	0.965		

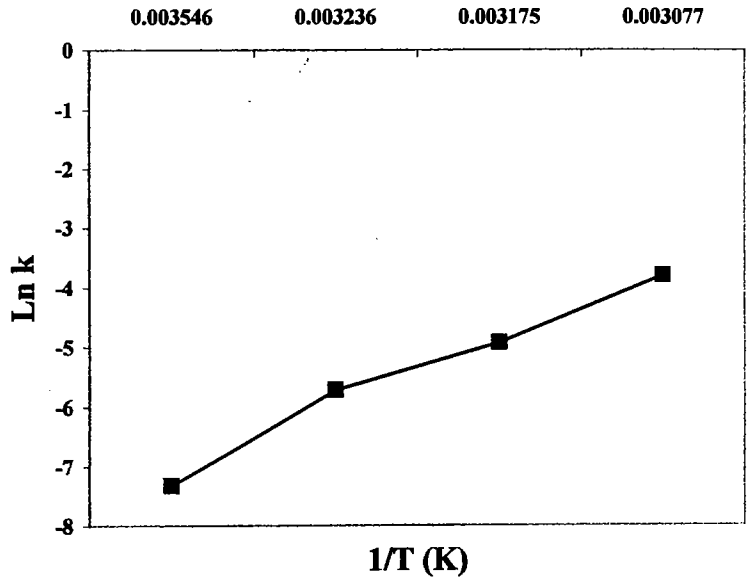


Fig. 4.58: Effect of temperature on reflectance value of whey protein-enriched orange beverage

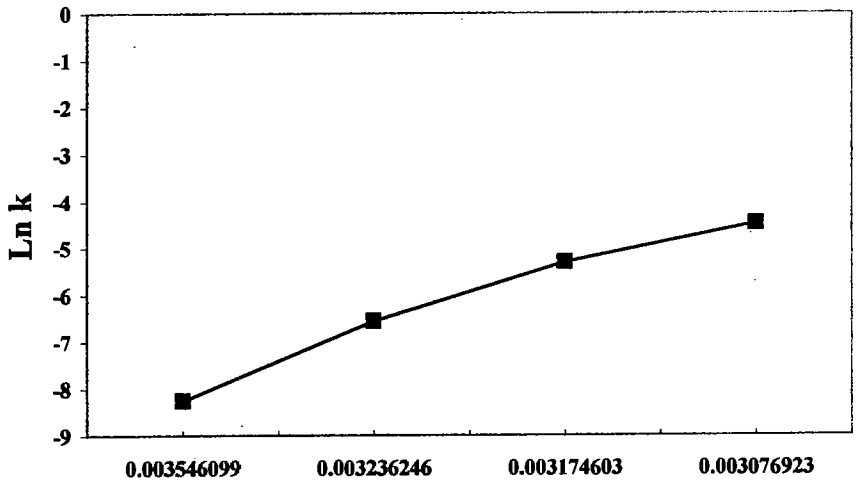


Fig. 4.59: Effect of temperature on reaction kinetics of non-enzymatic browning in whey protein-enriched orange beverage

was almost a straight liner, indicating the fulness of model to predict NEB over a wide activation energy (Ea) was 63.75 KM/mole and Arrhenius constant was 3.31×10^8 (Table 4.39).

Non-enzymatic browning followed zero order reaction kinetics. (Ibaraz *et al.*, 1993; Cohen *et al.* 1998; Lozano. 1991). Gogus *et al.* (1998) on the basis of this experiment concluded that temperature is the most important parameters affecting the rate of non-enzymatic browning in fruit juices

4.12 Microbiological quality

Whey protein-enriched orange beverage did not show any microbial growth on zero day. Further, microbial counts (Total plate, count, coliform count and yeast and mold count) were found to be nil throughout the storage period at 9° (control), 36, 42 and 52°C. This indicated that product remained safe microbiologically during storage.

4.13 Sensory quality

The whey protein-enriched orange beverages were subjected to sensory evaluation during storage. The overall acceptability score was 8.34 on 9 point hedonic scale at zero days, which decreased to 7.85, 7.08, 6.94 and 5.26 after 120, 120, 50 and 16 days of storage at 9° (control), 36, 42 and 52°C respectively. Control sample remained acceptable during storage, whereas those stored at 36°C were also acceptable upto 120 days. However, some panelists indicated deterioration of colour and appearance of the product. The sample stored at 42°C was rejected because of poor colour and appearance as

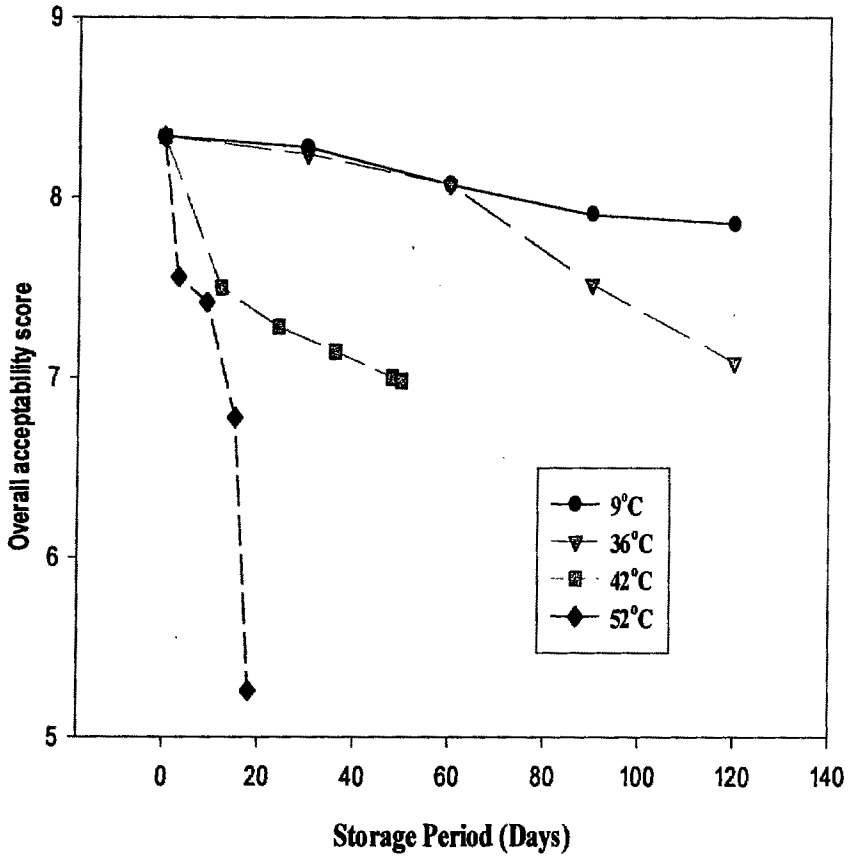


Fig. : Changes in sensory during storage of whey protein enriched Orenge beverage

well as development of slight caramel flavour. On the other hand sample stored at 52°C retained its sensory quality for only 15 days and after which browning, flavour deterioration as well as sedimentation was observed during storage. The major cause of quality loss was browning reactions, which were predominant at elevated storage temperature. The consistency of the product also declined at higher storage temperature because of sedimentation of proteins. Anantha Narayanan (1993) reported a storage life of 70, 42, 24 and 12 days for UHT soy beverage stored at 5, 25, 35 and 45°C. They reported browning reactions as well as oxidative changes as major deteriorative factors.

Summary and Conclusion

5.SUMMARY AND CONCLUSION

The present investigation entitled "Development and Evaluation of protein-rich fruit based beverages" was carried out to develop protein fortified orange and 'bael' beverages using whey proteins. Whey protein preparations, namely UF-retentate, freeze dried whey protein concentrate (WPC) and spray dried WPC, were prepared and investigated for their thermal behaviour. Complex formation using partially denatured whey proteins and acidic polysaccharides, i.e., CMC and pectin, was explored and the effect of heating and pH on complex formation, functionality and heat stability of complex was elucidated. Developed complexes were utilized to prepare a protein-rich bael beverage. Acidic polysaccharides were also studied for suspension stability in model beverage of low pH. Whey protein enriched orange beverage was developed using De Hoke's experimental design and multiple response optimization of response surface methodology. The optimized RTS beverage was subjected to storage studies of 35°, 42° and 52°C alongwith control (9°C).

1. Whey protein preparations obtained by different procedures differed in their chemical constituents. The protein, lactose, fat, ash and calcium content of these preparations were in the range of 58.92-73.05, 14.48-27.70, 5.12-8.18, 4.95-5.1 and 0.392-1.14%, respectively. Ultrafilteration procedure increased the protein content in WPC significantly and simultaneously reduced levels of other constituents.

2. Whey protein solutions (2%) exhibit maximum thermal stability at pH 3.5 and their stability decreased at pH $\geq$ 4.0. Similarly, soluble protein content was maximum at pH 3.5 and minimum at pH 4.5. UF-retentate showed maximum thermal stability followed by freeze dried WPC and spray dried WPC. It showed that care should be taken to minimize whey protein denaturation during freeze or spray drying. Fruit based beverages should be processed at pH below 3.75 to avoid whey protein denaturation.

3. Glucono-δ-lactone (GDL) exhibited stabilizing effect, whereas citric acid, tartaric acid and lactic acid caused aggregation of whey proteins during heating. Therefore, these three acidulants should be avoided in beverages, which require considerable heating to achieve microbiological stability.

4. Protein fortification of cheese and acid whey based model beverage (pH 3.5-4.0) was feasible only at low concentrations (whey protein $\leq$3.25%) Addition of more (>3.25%) whey protein caused gel formation at pH 3.5 and 3.75 or sedimentation at pH 4.0. Moreover, use of higher amount of WPC imparted whey flavour to beverage which resulted in their poor acceptability.

5. Thermal denaturation index (AI) of whey protein in model whey beverage at 75, 85 and 95°C and pH 3.5 or 4.0 followed zero order reaction. However, loss of protein solubility exhibited a first order reaction at both the pH. The reaction rate constants were higher at

elevated temperatures. The Arrhenius plots did not yield a straight line curve over the entire range of temperature and the activation energies were 534.71 and 142.65 KJ/mole of protein at 75-85°C and 85-95°C respectively. The activation energies for soluble protein loss were 46.51 and 31.84 KJ/mole of protein at pH 4.0 and 3.5, respectively.

6. Complex formed between partially denatured WPC and acidic polysaccharides were soluble and heat stable at low pH as compared to those formed between WPC and carrageenan, a sulphated polysaccharide.

7. CMC formed more soluble and stable complex by heating to 80°C, whereas pectin formed thermostable and quite soluble complex by heating to 50°C. Heating at 80°C hydrolyzed pectin, which reduced solubility of its complex. Complexation between acidic polysaccharides and whey proteins was affected by pH. Pectin and CMC formed more soluble and heat stable complexes at pH 7.0 than pH 3.5 and 5.5.

8. CMC-WPC complex was more acceptable than pectin - WPC complex for sensory attributes. Pectin-WPC complex added at 1.75% protein level gave the acceptable 'bael' beverages. Increasing the level of protein further, resulted in sedimentation and reduced acceptability of the beverage. CMC-WPC complex added to raise protein level to 2.75% gave most acceptable 'bael' beverage but higher amount made the beverage viscous and reduced its sensory scores.

9. 'Bael' pulp extracts differ in their chemical composition because of the variation in the amount of water used in their extraction. The total soluble solids and total solids were 21.93°Brix and 23.7% for extract I and 16.20°Brix and 16.52% in extract II respectively. Their total sugar, reducing sugars, mucilage content and protein content were also different.

10. Viscosity of both the extracts exhibited non-Newtonian shear thinning (pseudoplastic) behaviour. The power law model, Herschel-Bulkely model and modified Casson model predicted the behaviour of 'bael' pulp extracts very well and R^2 values for all the three models were more than 0.85. Yield stress for 'bael' extract I was higher than 'bael' extract II. The consistency index decreased with an increase in temperature. The consistency indices obtained by power law model were higher than the other two models.

11. Arrhenius equation described thermal behaviour of 'bael' pulp extracts accurately over the entire range of investigation. The values of activation energies were 8.314 and 7.98 KJ/mol for 'bael' pulp extract I and II, respectively.

12. 'Bael' pulp extracted with water (1:1.25) i.e. extract II was preferred for developing sediment free beverage. Ready to serve (RTS) beverage containing 25% 'bael' pulp, TSS of 16°Brix and pH 3.9 was rated as the best for flavour, colour and appearance, mouthfeel and overall acceptability.

13. 'Bael' beverage enriched with pectin-WPC or CMC-WPC complex exhibited non-Newtonian pseudoplastic behaviour and their flow behaviour index ranged between 0.319-0.565 and with corresponding consistency index values of 1.19-5.3 Pa*s. T.S.S, total solid content, pH and acidity of 'bael' beverages were 16.23 - 17.20°Brix, 16.77-18.80% , 3.93 to 3.95 and 0.35-0.54% (as citric acid), respectively. Higher protein content corresponded to higher acidity in product at similar pH.

14. Among carrageenan, carboxymethyl cellulose (CMC), sodium alginate, propylene glycol alginate (PGA) and gum arabic based commercial juice stabilizers only pectin, PGA, and guar gum formed soluble complexes and stabilized whey protein suspension. CMC provided colloidal stability only at pH 4.2.

15. Pectin required to stabilize whey protein suspension in model beverage was 1.0% at pH 4.0 and 1.5% at pH 3.5, respectively. PGA, though stabilized the whey protein suspension in model beverage at lower concentration of 0.2-0.3% at pH 4.0, was not found effective in preventing the precipitation at pH 3.5.

16. Model beverages exhibited Newtonian behaviour under certain conditions (PGA 0.3% at pH 3.5 and 4.0, CMC at all concentration, and guar gum at 0.3% at pH3.5 and 0.1% at pH 4.0). All other beverage, where stability was not achieved, behaved as non-Newtonian pseudoplastic fluid. The consistency index was maximum for pectin stabilized beverages and minimum for guar gum.

17. The TSS, total solid, pH and acidity of mandarin orange juice were 10.2°Brix, 12.44%, 3.8 and 0.81% respectively. It contained 7.57% total sugars, 4.94% reducing sugars, 24.57mg/100gm ascorbic acid content and 72 mg/100 gm total phenols.

18. Whey protein-enriched orange beverage containing 30% fruit juice, 2.5% whey protein level and pH 3.5 to 3.8 was liked most GDL and citric acid in a combination of 1:1 was selected to adjust the pH. A combination of two stabilizers i.e. pectin and guar gum was selected to develop the product.

19. De Hoke's design comprising of 19 experiments for four factors (X_1 = Fruit juice level, X_2 = whey protein level in UF-retentate, X_3 = Sugar level and X_4 = Stabilizer level) and 3 levels were used to optimize the levels of ingredient in whey protein enriched orange beverage. Their values were 30-35%, 1.4-3.4%, 10-11% and 0.1-0.3%, respectively. The coefficient of regression (R^2) for models of all the sensory attributes ranged from 0.881-0.918, which indicates that models were adequate.

20. Stabilizer level ($P \leq 0.1$) at linear levels, whey proteins and stabilizer ($P \leq 0.05$) in quadratic terms and fruit juice and sugar level ($P < 0.05$) and whey protein level sugar level ($P \leq 0.1$) at interactive terms influenced the flavour of the beverage. Similarly stabilizer level ($P \leq 0.05$) in quadratic terms and fruit juice level and sugar content ($P \leq 0.1$) in interactive terms affected the colour and appearance of the product. The

mouthfeel of the product was affected by stabilizer level ($P \leq 0.1$) linearly, protein content and stabilizer level in quadratic terms, the protein content and sugar ($P \leq 0.05$) and fruit juice and sugar ($P \leq 0.1$) interactively. The stabilizer concentration ($P \leq 0.05$) and protein level ($P \leq 0.1$) affected the overall acceptability of the product in quadratic terms, whereas fruit juice level and sugar content affected in interactive terms at $P \leq 0.1$.

21. Experimental variations of various optimized formulations obtained also indicated accurate predictability of model. A combination of 31.59% orange juice, 2.45% whey protein level in UF-whey retentate, 11.86% sugar and 0.25% stabilizer was found to be the best formulation on the basis of overall acceptability scores.

22. The whey protein-enriched orange beverage had TSS, total solids, pH and acidity of 18.2°Brix, 18.45%, 3.5 and 0.60% (as citric acid), respectively. The total sugars, reducing sugars and protein level in it were 16.21, 4.28% and 2.75%, respectively. Its protein content was 3-4 times higher than those of commercially available fruit beverages.

23. TSS, total sugars, protein in vitro protein digestibility and reflectance values (colour) of the beverage decreased during storage. However, total solids, acidity, reducing sugars, hydroxymethyl furfural (HMF) content, proteolysis, and non-enzymatic browning were found to increase during this period. The changes were much faster at higher storage

temperatures i.e. 42 and 52°C. Its microbial count (total plate count, yeast and mold count and coliform count) remained nil throughout the storage period. The changes in acidity, total sugars, reflectance value, non enzymatic browning, soluble proteins and proteolysis followed zero order reaction kinetics. However, increase in reducing sugars and free and total HMF exhibited first order reaction kinetics. In certain cases the Arrhenius equation was not able to predict the reaction kinetics very well over the entire range of storage temperatures. Activation energies were higher at higher temperature, which indicated increased solvation of reactants at elevated temperatures.

The above study shows that a protein rich fruit beverage can be prepared using 31.59% orange juice, 7.45% whey protein in UF-retentate and 11.86% sugars. It's pH was 3.5 and protein was stabilized with 0.25% stabilizer (3 parts pectin + 1 parts guar gum). Protein content of this beverage was 2.75% as compared to 0.99% of normal beverage. Similarly a good quality beverage based on 'bael' pulp can be obtained by using 25% 'bael' pulp, 16°Brix TSS, pH 3.9 and 1.75% CMC-WPC complex. It further shows that denatured whey protein, in the form of spray or freeze dried WPC can be stabilized in acidic beverage by complexing them with acidic polysaccharide i.e. pectin or CMC.

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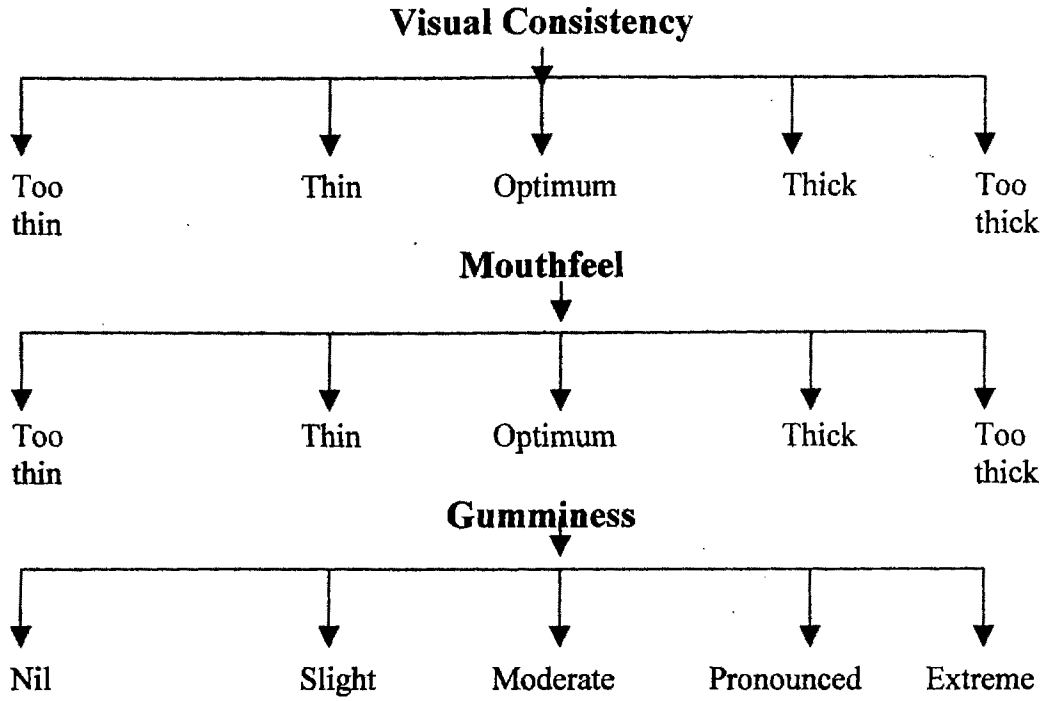
Appendices

APPENDIX - I

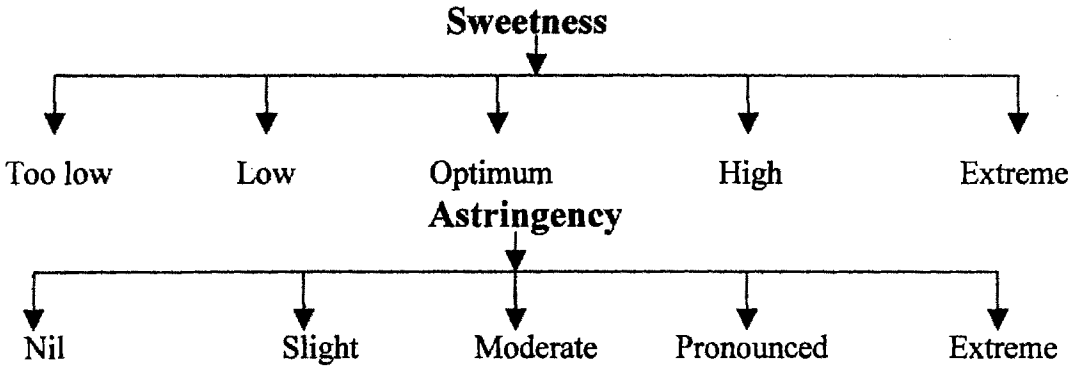
Sensory Evaluation Score Card For Protein – rich 'Bael' Beverage

Please make your assessment by marking a tick- mark along the linear scale by giving the corresponding sample number,

I. Texture



II. Flavour



You are requested to assess the products in terms of following sensory attributes according to the 9- point hedonic scale, as below: -

Sample code/ Attributes	WW	XX	YY	ZZ
Flavour				
Consistency				
Colour & Appearance				
Overall Acceptability				

Remarks, if any_____

Name_____ Date_____

Signature_____

APPENDIX – II

Sensory Evaluation Score Card for Protein- rich Orange Beverage

You are requested to assess the products in terms of following sensory attributes according to the 9- point hedonic scale, as below: -

Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

Sample Code	XX	YY	ZZ
Flavour			
Consistency			
Colour & Appearance			
Overall Acceptability			

Remarks, if any: -

_____

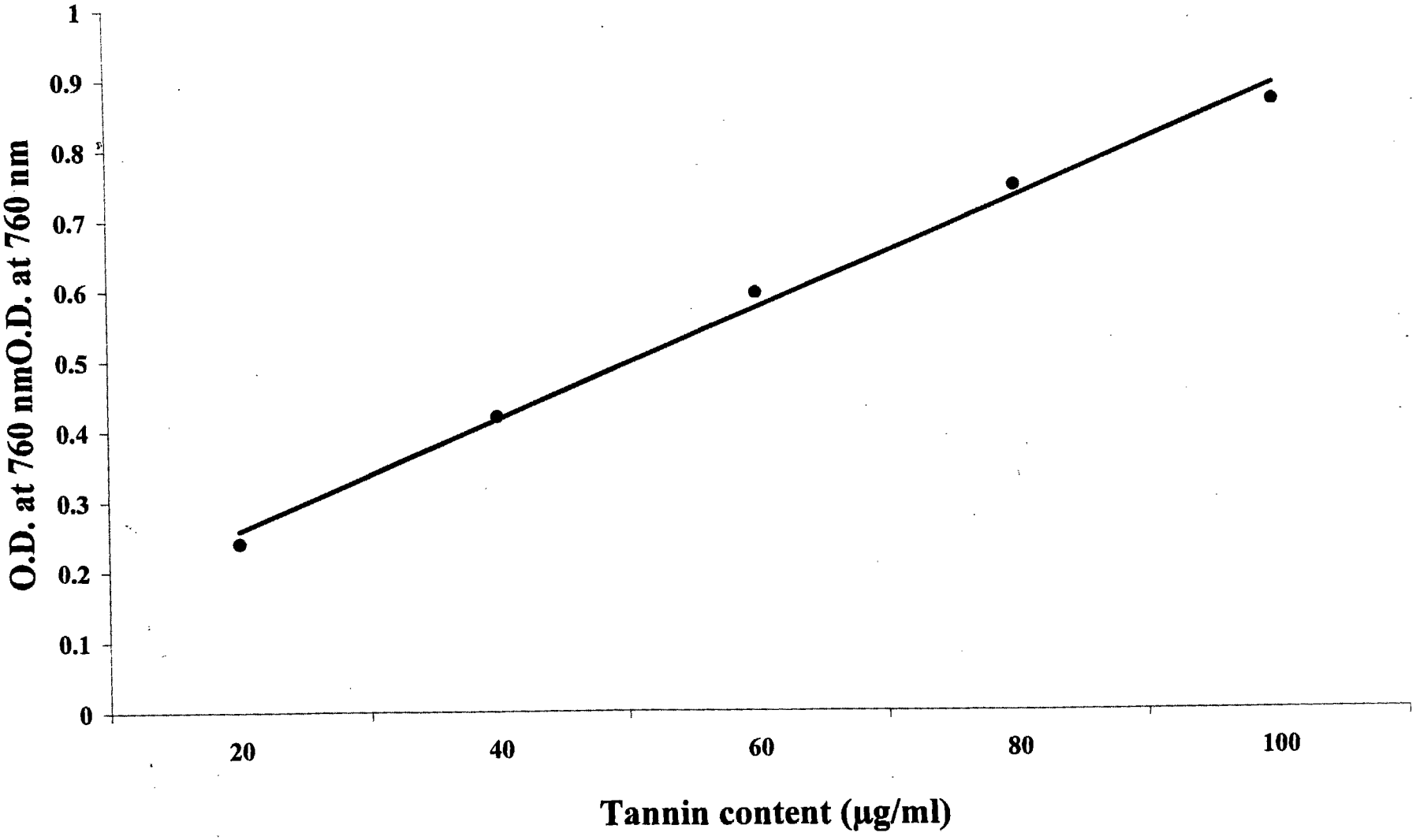
_____

_____

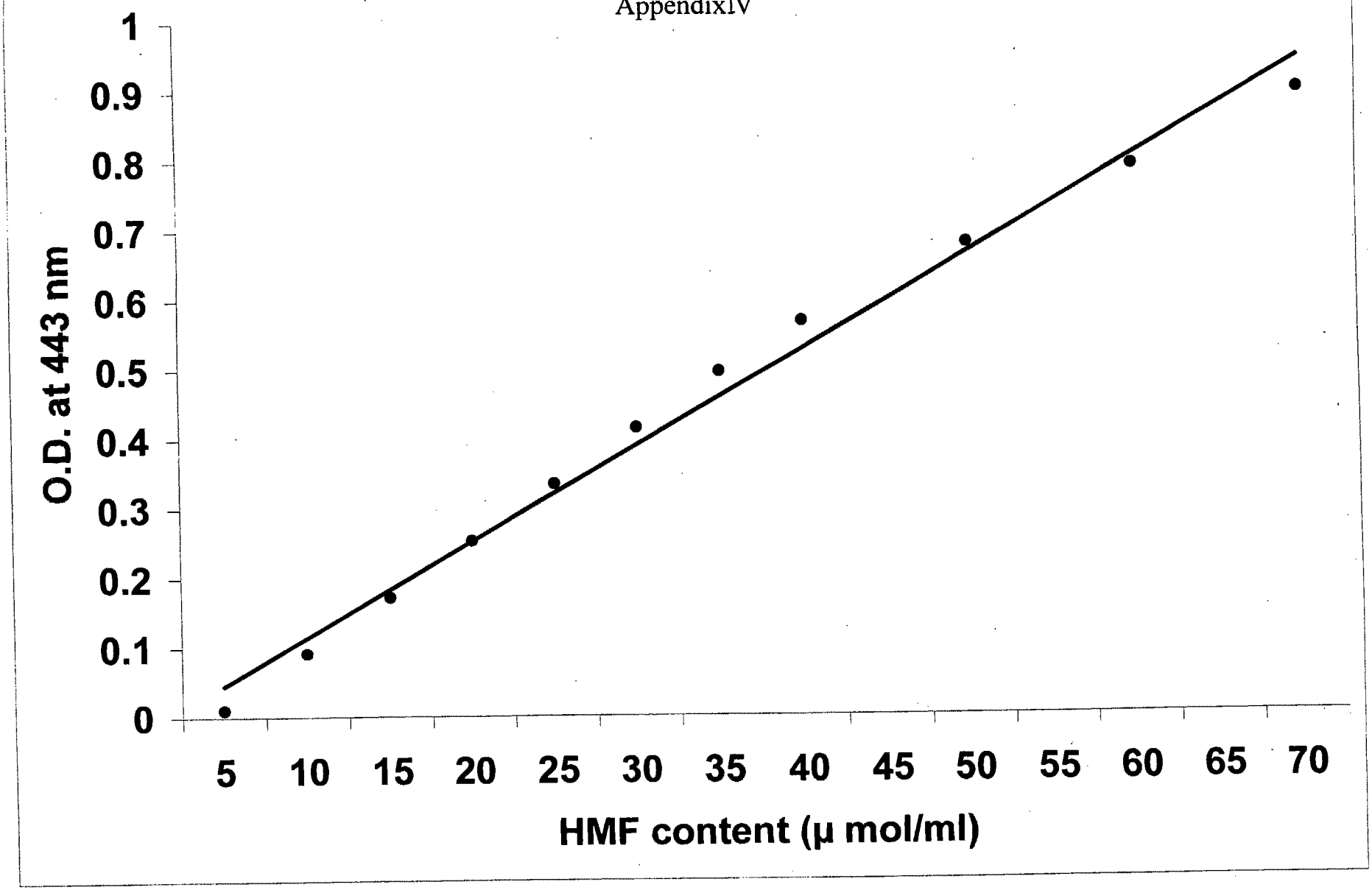
Name_____ Date_____

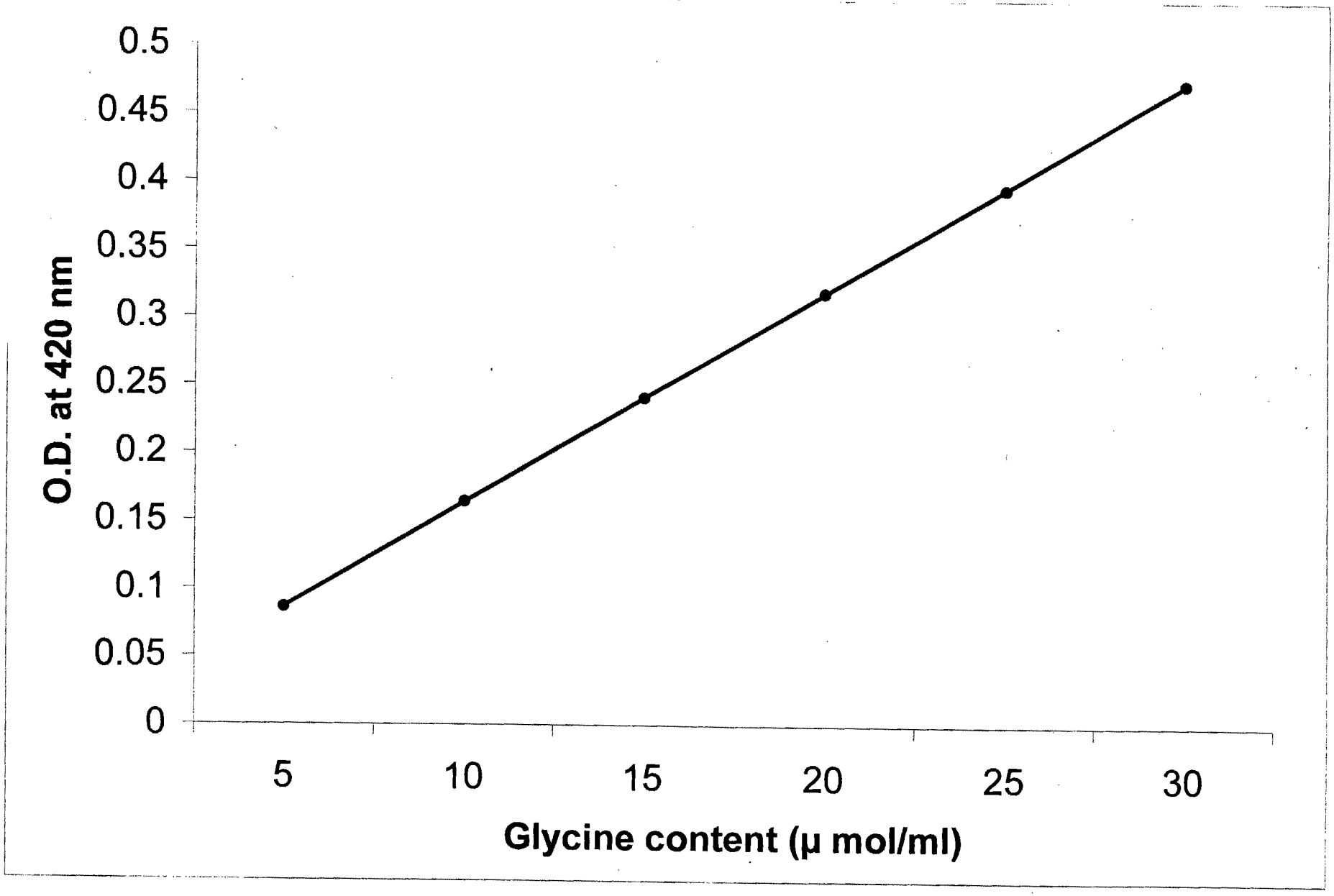
Signature_____

Appendix III



Appendix IV


O.D. at 443 nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
HMF content (µ mol/ml)


0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
O.D. at 420 nm
5
10
15
20
25
30
Glycine content (µ mol/ml)

VITA

The author of this manuscript, Ashish Kumar Singh, son of Shri J.N. Singh was born on 22nd April 1972 at Tulasipur Gonda. He graduated from Lucknow University in Life Sciences in 1993. He joined G.B. Pant University of Agriculture and Technology, Pantnagar for his M.Sc. degree with major in Food Technology in the year 1993-94 and completed in October 1995. Later, he joined for his Doctoral degree in the same university with major in Food Technology and minor in Process and Food Engineering in October 1995. Consequent to his selection for the post of Scientist B, he joined at Defense Food Research Laboratory Mysore and discontinued his degree programme. Presently he is working as Scisentist at National Dairy Research Institute NDRI Karnal.

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ABSTRACT

Name : Ashish Kumar Singh **Id.No.** : 21348
Semester and year of admission : I Semester 1995 **Degree**: Ph.D.
Major : Food Technology **Minor** :Process & Food Engg.
Thesis Title : "Development and evaluation of protein-rich fruit based Beverage"
Advisor : Dr. Nirankar Nath

Beverages provide taste and refreshment to the consumers. They also offer a ready and unique delivery system for various nutrients like protein, vitamins and minerals. Protein-rich beverage has been in demand for quite sometime. Fruit juices may be enriched with various food proteins. Whey proteins, is a by-product of dairy industry. It has excellent amino acid profile and good digestability. Whey proteins have been ascribed with many therapeutic role and proven effective in prevention of cancer, coronary heart diseases and treatment of HIV patient. But steps involved in industrial production of whey protein concentrates (WPC) make them susceptible to thermal denaturation under acidic conditions. Hence, protein fortified fruit juices suffer from sedimentation and off flavour during storage. The present investigation was undertaken to develop whey protein-enriched fruit beverages and to determine their storage stability. Sweet cheese whey UF retentate exhibited better thermal stability under acidic pH (3.5-4.5) conditions than freeze or spray dried WPC. Glucono-δ-lactone (GDL) was found effective in preventing whey protein denaturation than citric acid, actic acid and tartaric acid. Partially denatured whey proteins form acid soluble and heat stable complexes with acidic polysaccharides. Among the complexs, CMC-WPC complex developed at pH 7.0 by heating at 80°C, was most soluble and thermal stable and was found to be most effective in fortification of 'bael' beverage. The 'bael', beverage with 25% pulp, 16°Brix TSS, 3.9 pH and CMC-WPC 1.75% protein level complex, was most acceptable. Among the food hydrocolloids. Pectin, propylene glycol alginate (PGA), CMC, and guar gum were effective in stabilizing whey protein suspension under acidic pH upon heating. Application of response surface methodology provided optimum level of ingredients as 31.59% orange juice, 2.45% whey protein in UF-retentate, 11.86% sugar and 0.25% stabilizer. Non-enzymatic browning was the major cause of spoilage and other storage observed were decrease in pH, total sugar, soluble protein, *in vitro* protein digestability, reflectance and increase in reducing sugar, acidity, HMF content and proteolysis. These reactions followed either zero or first order reaction kinetics and with high activation energies at elevated temperatures. The study shows that whey protein based fruit beverage can be developed using appropriate type of stabilizer or by complexing with polysaccharides that may be used to fortify fruit juices.

(Nirankar Nath)
Advisor

(Ashish Kumar Singh)
Author