UTILIZATION OF THERMAL DENATURATION OF SOY PROTEIN IN THE RECOVERY OF CAROTENOIDS FROM NATURAL SOURCES

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ABSTRACT OF THE THESIS

UTILIZATION OF THERMAL DENATURATION OF SOY PROTEIN IN THE RECOVERY OF CAROTENOIDS FROM NATURAL SOURCES

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Carotenoids are associated with several health benefits, mainly as antioxidants. The consumption of carotenoid as mean of prevention and correction of health conditions is in sync with the growing functional foods shift in the world of food and wellness. However, carotenoids are conventionally extracted using solvent based methods which is facing a number of challenges, the greatest being the regulatory restrictions on the use, storage, waste management and percentage of contamination in final product. Social, environmental and economical reasoning have triggered a wide spread interest in green, convenient and economical alternatives.

SPI is a GRAS material that is widely used in the food industry for functional and nutritional purposes. Thermal treatment of SPI results in denaturation of native protein structure exposing the hydrophobic core that has the ability to act as a site for adsorption of molecules or ions. DSC measurement demonstrated two endothermic peaks at 42^{o}C and 72^{o}C corresponding to the two major protein fraction, β-conglycinin and Glycinin respectively. Rheological measurements of SPI during thermal treatments at concentration below heat-gelation critical limit revealed details on the molecular level regarding protein-protein interaction during phase transition. UV/visible spectroscopy of SPI

suspension coincided with DSC results and rheological measurements where protein hydrophobicity increases at or above the denaturation point of the least heat stable fraction.

Thermal denaturation of SPI in mango juice solution was the proposed treatment required to achieve unfolding of protein, exposing the hydrophobic core and allowing adsorption of mango carotenoids. Examined thermal treatment of heating to 95^{o}C for 30 min was confirmed safe as it resulted statistically insignificant ($p<0.05$) reduction. Adsorption measurements of β-carotene, α-carotene, β-cryptoxanthin, Zeaxanthin, and Violaxanthin as a function of SPI concentration demonstrated similar magnitude of adsorption. Adsorption maxima were reached at 15 g/L of SPI for all carotenoids with the exception of violaxanthin where maximum adsorption capacity is reached at 20 g/L of SPI with 98.06% retention. Overall results suggest that SPI has the ability to adsorb carotenoids present in aqueous medium during thermal denaturation treatment, this solvent-free method results in a nutrient dense functional ingredient.

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Prophet Mohammed (peace be upon him) said: {Those who do not thank people, do not thank Allah} Al-Tirmidhi 1878.

As I complete this rite of passage I am filled with gratitude to those who have enriched my experience and engraved a lasting impact on my life. Few deserving words for some of the substantial figures in my Rutgers journey…

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List of abbreviations

FOSHU	Foods for Specified Health Use
FDA	United States Food and Drug Administration
SPI	Soy Protein Isolate
7S	β-conglycinin
11S	Glycinin
a.a.	amino acids
SC	Sedimentation Coefficient
DV	Daily Value
DSC	Differential Scanning Calorimetry
RT	Room Temperature
DLS	Dynamic Light Scattering
c.d.b.	conjugated double B=bonds
USDA	United States Department of Agriculture
GRAS	Generally Recognized As Safe

Chapter 1: Literature review, research motivation, problem statement, and research objectives

Functional foods

History, definition and overview

Nearly 2,500 years ago, Hippocrates spoken words that we still live by "Let food be thy medicine and medicine be thy food". Over centuries and as far as history have been recorded food and medicines were inextricable. But it seems that in recent history, no more than few decades ago, we have been showing greater interest in food as medicine rather than the pure medicine supplied by pharmaceutical industry. We are concerned with food that can provide more than pleasure and satisfaction of our hunger, food that can provide additional physiological benefit and promote health and wellbeing. Such foods is termed *functional foods*.

The term functional foods first became popular in Japan in the mid-1980s [1, 2], often descripting food stuff containing ingredients with specific function in the human body beside its nutritious value. In realization of soaring cost of health care associated with sizable aging demographic in Japan, a wholesome strategy easily-modeled into everyday life was placed using foods that have been approved by a regulatory system put in place by the Ministry of Health and Welfare. In Japan such food items are known as Foods for Specified Health Use (FOSHU), more than 300 food items bear FOSHU status [2].

While some define functional foods with extreme precision and notion to

updated scientific causation and correlation between food compounds and physiological benefits. Some have a more holistic approach including all food matter under the functional food category since all food matter have a function, whether it be sensory and organoleptic properties, or nutritive and health-promoting qualities. Thus the term is ever confusing and ever controversial. There is no universally accepted definition; defining this relatively new and emerging category of food have been the task for health, nutrition and medicinal organization. Following is number of definitions that are receiving wide acceptance in scientific literature:

> "Any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains", The Institute of Medicine's Food and Nutrition Board. [1]
>
> “Foods that, by virtue of the presence of physiologically-active components, provide a health benefit beyond basic nutrition”, International Life Sciences Institute. [3]
>
> “Whole, fortified, enriched, or enhanced food,…..as part of a varied diet on a regular basis, at effective levels”, American Dietetic Association. [4]
>
> “Any modified food or food ingredient that may provide a health benefit beyond that of the traditional nutrients it contains”, The Food and Nutrition Board of the National Academy of Sciences. [5]

These relatively similar definitions clearly distinguish functional food from nutraceuticals, a term created by the Foundation for Innovation in Medicine in early 1990s including all bioactive component that delivers a health benefit. Zeisel (1999) [6] defined nutraceuticals as “those diet supplements that deliver a concentrated form of a presumed bioactive agent from a food, presented in a nonfood matrix, and used to enhance health in dosages that exceed those that could be obtained from normal food”.

Though the concept of functional foods is clear enough in previously mentioned definitions; functional foods in the United States lack a regulatory identity [2]. It is crucial for both the industry and consumers that health claims and exact definition of this food category is well defined and approved via a well established system. Such legal definition would help accurately identify the market, consumers and technologies associated with such food.

Many food items can be considered functional foods due to its fortification with doses of nutrients. One of the earliest examples is Vitamin B-enriched flour that was introduced in the 1940s to battle pellagra, other examples include iodine-fortified salt to decrease goiter cases, and vitamin D-enriched milk that have eradicated rickets, table (1). All these initiatives have been generated and endorsed by governmental actions. This is no longer the case, health-conscious consumers are the driving force for increasingly expanding market of functional foods. These initiatives include probiotic, omega-3, and phytonutrients containing or enriched foods. [7]

Table 1: Examples of Functional Components. [8]

Class/component	Source	Potential benefit
Carotenoids		
α-carotene	Carrots	Neutralizes free radicals which may cause damage to cells
β-carotene	Various fruits and vegetable	Neutralizes free radicals
lutein	Green vegetables	Contributes to maintenance of healthy vision
lycopene	Tomato and tomato product	May reduce the risk of prostate cancer
Dietary Fiber		
Insoluble fiber	Wheat bran	May reduce risk of breast and/or colon cancer
β-glucan	Oats	Reduces risk of cardiovascular disease

		(CVD)
Soluble fiber	Psyllium	Reduces risk of CVD
Whole Grains	Cereal grains	Reduces risk of CVD
Soy Protein Soy protein	Soybeans & soy-based foods	25 grams per day may reduce risk of heart disease
Plant Sterols Stanol ester	Corn, soy, wheat, wood oils	Lowers blood cholesterol levels by inhibiting cholesterol absorption

Trend motivation

The interest in functional food by producers and consumers alike is caused by several interrelated factors that work synergically. Starting with health-conscious consumers that created the self-care phenomena where they take charge of their own well being, focusing on everyday practices starting with their diet with a life philosophy of “kitchen cabinet as the medicine cabinet”. This created a demand for products that supports health and longevity. [2, 7, 8]

In the era of aging demographics, age-related chronic diseases (e.g. heart disease, cancer, osteoporosis, Alzheimer’s disease and age-related macular degeneration) creates enormous economical stress on the health care system, evidence by the souring cost of the health care services needed for chronic diseases of aging. In the U.S. the total annual costs have been estimated at $659 billion. Preventative healthcare strategies have been adapted globally as a cost saving holistic and alluring alternative for both customers and healthcare service providers, especially with the indisputable scientific evidence connecting dietary habits among other factors with poor health. Promoting functional foods as part of a strategy to combat aging diseases and promote healthy long life; is a

trending worldwide. [2, 5, 8, 9]

By recognizing the growing trend, and strong and ever increasing consumer gravitation toward the concept of “kitchen cabinet as the medicine cabinet”; regulatory agencies are continuously implementing new regulations to control and standardize this segment’s practices. Such regulations might have increased consumer’s trust in products quality and effectiveness, evidence in increase demand, production and consumption of food under this category [2, 5, 8]. An example of the impact of such seal of approval can be seen in the increase in the sale of oatmeal in 1997 after the Food and Drug Administration (FDA) have approved oatmeal-related health claim, similarly soy-containing product gained increased popularity and are featured on more food labels after 1999 where some health claims were approved [8].

In addition, continuous scientific discoveries in the fields of food and science are the fuel for growing consumers demand, acceptance and adaptation of functional food. Subsequently the industry has responded to fill consumer’s need. The major food companies in the world are becoming key players in the development as well as the manufacturing of functional food ingredients and food items. Nestlé declared it was “moving from an agrifood business to an R&D-driven nutrition, health and wellness company” [7]. The trend now is moving toward creating food stuff that would promote optimal health and wellness, as well as reduce the risk of future chronic disease, rather than correcting deficiencies (conventionally fortified food items). Table (2) includes some of the

world leading food manufacturers and some of their key functional food items. [2, 7, 8]

Company	Key functional brands
PepsiCo	Quaker, Gatorade
Coca-Cola	Vitamin Water, Odwalla
General Mills	Cheerios, Yoplait
Kellogg	Special K, Kashi
Kraft	Capri Sun, Balance Bar
Nestlé	Nesquick, Power Bar
Danone	Activia, Essensis
Unilever	Slim-Fast, Blue Band
Yakult Honsha	Yakult 400, Jole

Table 2: World-renowned food companies and their top functional foods. Ranking of companies is based on US functional food sale in 2006. [7]

Functional foods market

Growing numbers of health-conscious consumers caused the health and wellness market segment to grow tremendously, however, there are no clear estimation of the size of the market world wide and specifically in the US since a legally binding definition of what constitutes a functional food does not exist to date [1]. However, the ability to make health claims on food labels have triggered an interest amongst manufacturers to invest in the research and production of functional foods [2]. Nevertheless segments of the scientific community is concerned regarding this growing trend in fear of increased consumption due to marketing strategies rather than solid scientific understanding on proper consumption habits [5].

In term of research trend, The Institute of Food Technologists published a survey in 2000 ranking research in area of food and health, well above other segments such as food safety, organic foods, and reduced fat foods [10]. A year

later, a survey by the Food Processing Magazine revealed that functional foods/nutraceuticals is the leading category concerning research and development in the food industry [2]. Though exact figures of the size of the market in the united states cannot be identified for reasons stated above, an estimation of $18.5 billion was generated by the Nutrition Business Journal in 2002 [11], with more recent estimates of $20 to $30 billion in annual sales [7].

Leading food manufacturers and Ingredient makers are collaborating to maintain their share of the market by modifying formulation and developing new food items to bear additional health benefits. Most of the concern and focus is on beverages, particularly soft drinks (enhanced water as alternative to sugary carbonated beverages) due to these item's convenience of consumption and wide range of choices due to ease of formula modification (e.g. creating new flavors). In addition, a large segment of the functional food market is dedicated to dairy products (pro and prebiotics). [7]

Regulation of functional foods

To date, Japan, the birth place of functional foods concept; is the only country with specifically established regulatory practice regulating all aspects of Foods for Specified Health Use (FOSHU) via the Japanese Ministry of Health and Welfare [1]. Legally, the United States does not recognize functional food as a distinct category of food [1]. However a number of health, nutrition, and wellness organizations have recognized that consumers need some guidance on the consumption of functional foods. For example the American Dietetic

Association states that functional foods should be “consumed as part of a varied diet on a regular basis, at effective levels” [4], which differentiate functional foods dietary recommendations from nutracitucals [4].

Though there are no regulations that explicitly regulates functional foods, previously enforced legislations such as the Nutrition Labeling and Education Act of 1990 by the FDA requires most food items to show a nutritional label, and abide to the specific regulations regarding health claims and disease prevention. The Dietary Supplement Health and Education Act of 1994 that defines dietary supplements have established a mechanism to verify the safety of what can be defined as functional food, and regulates labeling issues. The FDA Modernization Act of 1997 of Federal Food, Drug, and Cosmetic Act allowed health claims that are not preauthorized by FDA if the claims are based on “authoritative statements” of government agencies such as the National Academy of Sciences or the National Institutes of Health. These are minor efforts that do not realistically match the size of the growing market of functional foods, further improvement on existing regulations as well as generation of new guidelines will become a necessity in the near future as the trend transforms to common practice in manufacturing and consumption.

One of the most concerning issues is safety of functional foods. Optimization of the level of the biologically active components in the food matter and in the human body are key, adverse effect such as carcinogenicity at high levels is possible for some compounds. The fact that the active compounds are natural and present in a food matrix might be misleading to some consumers,

that would otherwise assume safety at all levels. Determination of a compound toxicity is critical before proceeding to offer these items to the public (balance the risk : benefit ratio), specially that unlike medicinal foods or drugs, functional foods are meant to be consumed freely as part of a balanced diet [7]. Table (3) summarizes the difference between functional foods, medical foods, and drugs in term of uses, methods of obtainment, distribution channels, regulatory body, and amount consumed.

Difference	Functional foods	Medical foods	Prescription drugs
Uses	Energy enhancement; weight management; bolster gut, bone or heart health; disease risk reduction; memory improvement.	Dietary management of a disease or condition with distinctive nutritional requirements (e.g. difficulty swallowing, loss of appetite, nutrition repletion post surgery).	Treatment of disease, symptom, or condition.
Methods of obtainment	No prescription or supervision needed; consumer selects.	Used with medical supervision	Prescribed by health provider.
Distribution channels	Supermarkets, drugstores, online, major retailers.	Hospitals, pharmacies, drugstores, online.	Pharmacies, hospitals.
Regulatory body	No specific body, but is considered food and is therefore subject to FDA regulation, FDA regulates any specific health claims that might be made.	No additional FDA review/ approval needed, but must abide by regulations concerning foods, e.g., labeling. FDA regulates any specific health claims that might be made.	FDA approval needed, a multiyear, multistage review process.
Amount consumer	As desired	As needed	As prescribed

Table 3: Comparing functional foods with medical foods and drugs. [7]

One of the most pressing needs for a regulatory mechanism of functional foods is the establishment of these compounds effectiveness via thorough research. Such research is complex and time consuming, thus not often carried

by manufacturing companies unless enforced by law. Figure (1) is a schematic representation of the steps required to assess the physiological response to the bioactive component in functional foods.

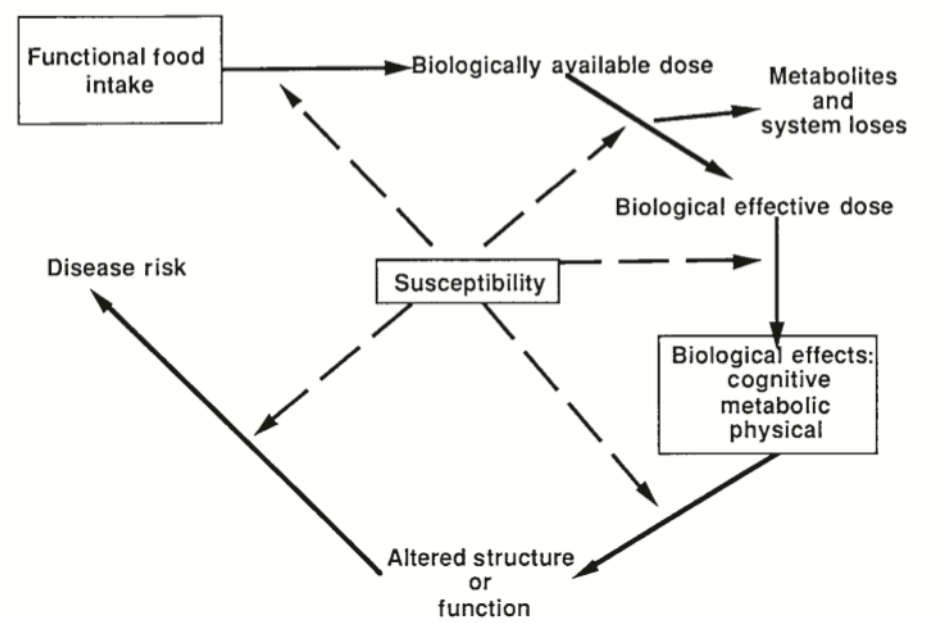


Figure 1: Assessment of the response to functional foods' bioactive component via changes in biomarkers. Broken lines indicate the influence of susceptibility on uptake, metabolism, and biological response to functional foods. [5]

Evaluation of the scientific evidence linking the bioactive component of functional foods to a the prevention of diseases and support of optimal health, is the primary function of the FDA. FDA have issued an outline in 1999 to help manufacturers produce the required scientific documentations that support their health claims, hypothetically by following this scheme, a "significant scientific agreement" on the health benefits can be proclaimed. Figure (2), is a simplified version of FDA's schematic of significant scientific agreement, the steps are used to asses the strength and consistency of the scientific evidence in order to generate a significant scientific agreement. The scheme clearly shows that a "consistent, relevant evidence from well-designed clinical, epidemiologic and

laboratory studies, and expert opinions from a body of independent scientists" [2] is curtail for FDA approval of any health claim.

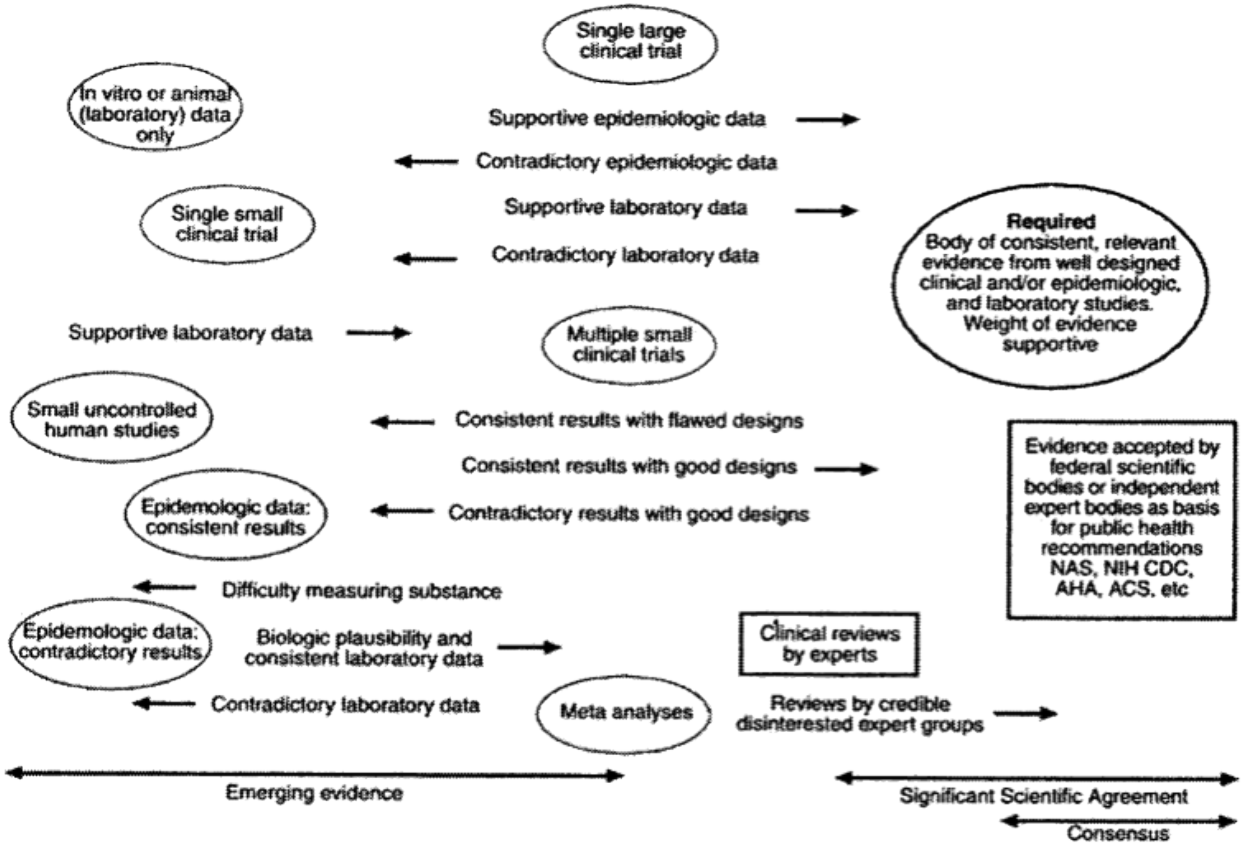


Figure 2: The FDA's schematic significant scientific agreement released in December 22, 1999 guidance document titled, Guidance for Industry: Significant Scientific Agreement in the Review of Health Claims for Conventional Foods and Dietary Supplements.

Carotenoids

The focus of this research is on one group of natural compounds that are widely distributed in nature, these compounds are responsible for the yellow, orange, and red colors of fruits, roots, flowers, fish, invertebrates, and birds. Following is an overview of carotenoids, our compounds of interest.

Chemical structure

About 750 different carotenoids identified, about 60-100 are consumed by humans directly from plant sources or indirectly from animal sources that it obtains from feed. Carotenoids are a linear conjugated hydrocarbon chain, makes carotenoids highly hydrophobic molecules, these polyenes consisting of 3 to 13 conjugated double bonds and up to 6 carbon ring structures at one or both ends of the molecule. Those that are made of carbon and hydrogen (non-oxygenated) *only* are termed carotenes and those that contain oxygen as well are termed xanthophylls. [12-15]

Economical value of carotenoids

Carotenoids posses high economic value and there is an increase demand for these compounds to be used in applications such as dying, food, and nutraceutical compounds. Individual carotenoids such as β-carotene and lycopene are certified to be used as pigments in the food industry, carotenoids-contacting products such as paprika have been used as food colorants thereby affecting consumer acceptability by altering food aesthetic quality. These

pigments also have possible applications as colorants in the pharmaceutical and cosmetic industry. Categorization of carotenoids as by the Generally Recognized As Safe (GRAS) by FDA is another cause of its wide use in cosmetics, drugs, and food.

The food industry is going through a great shift toward natural additives that potentially possess some health benefits. β-carotene and lycopene have been proven to have beneficial effect on human health, mainly as vitamin A precursor and antioxidant activity. These various applications are the driving forces for increase interest in research related to extraction methods and characterization of these compounds for possible applications as colorants, functional ingredients and health supporting compounds. To give you an idea of how valuable these compounds are, 1 kilogram of astaxanthin can cost as much as $2000 [14]. Figure (3) is the global carotenoids market value according to BCC Research, a market-forecasting firm.

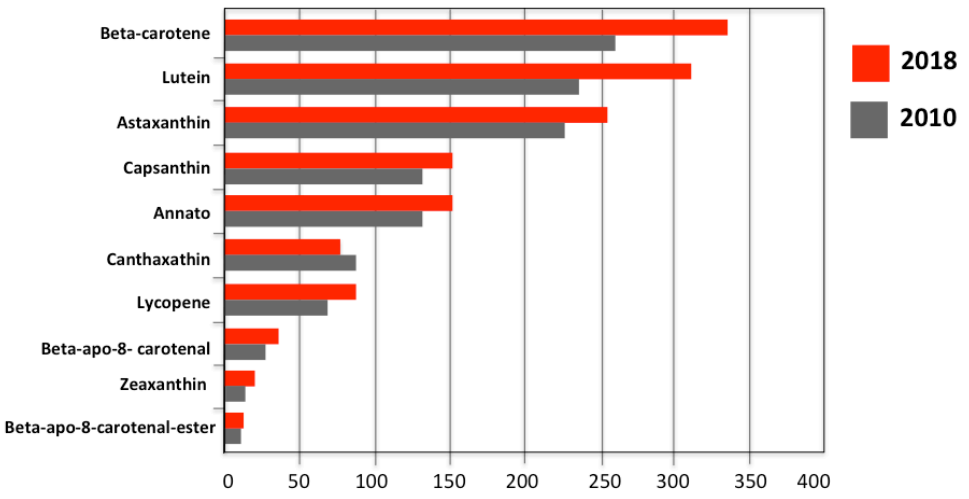


Figure 3: Global carotenoids market value, 2010 market value and expected values in 2018 ($Millions). [16]

Health benefits associated with carotenoids

Most of the health benefits of carotenoids are attributed to their antioxidant activity, figure (4); oxidation is attributed to a large number of chronic diseases (figure (5)), thus carotenoids are braised for their singlet oxygen quenching abilities. Some other functions are related to their: bioactivity as in the ability to absorb light (i.e. eye health), anti-inflammatory activity, in vivo metabolism into active compounds. [15]

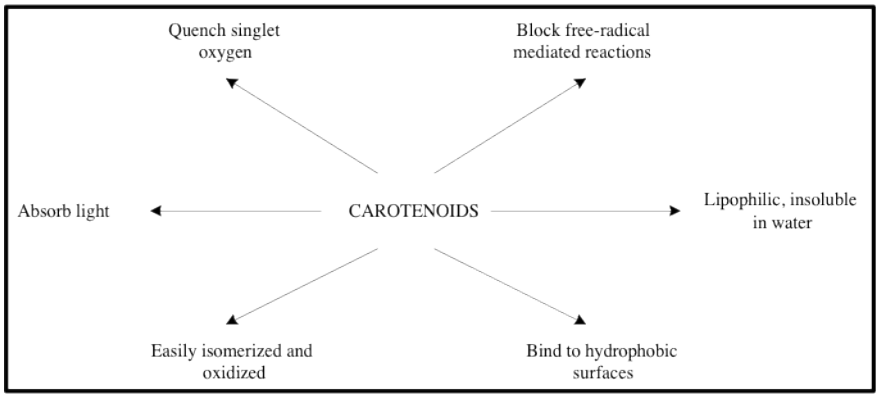


Figure 4: Physical and chemical properties of carotenoids. [17]

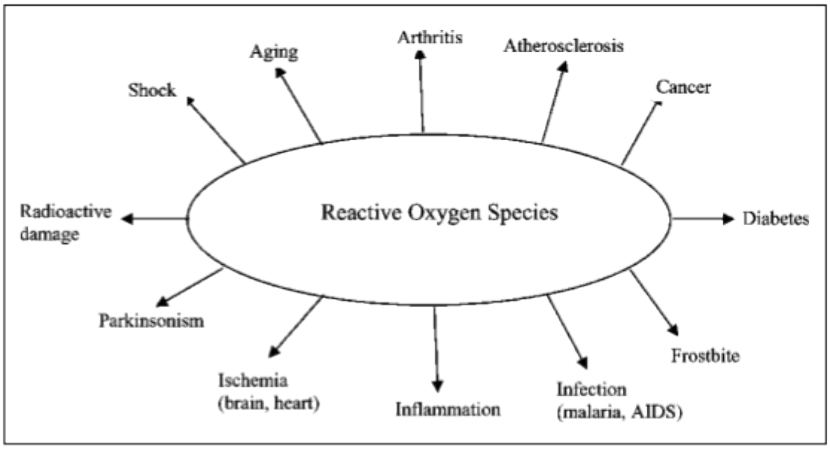


Figure 5: Health benefits of dietary antioxidants are often associated with its preventative role in controlling reactive oxygen species that causes chronic diseases and other oxidative damages. [18]

Following we explore some of the health benefits of carotenoids in term of specific health conditions.

1. Brain and neurodegenerative degenerative diseases

Oxidative stress of brain cells which causes the production of high levels of peroxynitrite and reactive oxygen species, destroys neurons and trophic cells and may ultimately results in death. Thus neurodegenerative diseases are associated with antioxidant deficiency, accordingly consumption of such compound can be used as possible preventative method to combat these oxidation-related illnesses. A positive relation was found between carotenoid and tocopherol and the maintenance of memory capacity. Carotenoids are showing promising results in reducing risk of dementia better than some major flavonoids. [9, 19]

2- Cataract

Cataract it is a disease associated with oxidative damage often occurring at older age, but other risk factors such as smoking, air pollution, and a decrease in protective carotenoids and antioxidants in the diet could damage eyes and trigger such macular degeneration. Prevention using carotenoids, riboflavin, tocopherol and ascorbate supplements; is a common practice. Cartenoids function through several proposed modes of action mainly by acting as antioxidant and the “free radical scavenging; modulation of carcinogen metabolism; inhibition of cellular proliferation; enhancing of cellular differentiation (retinoids); activation o intercellular communication; increasing of immunological responses; and ultraviolet light filtration” [19]. Lutein and zeaxanthin were found

to be more consistently correlated with these functions than other carotenoids. [9, 19] [15]

3- Anticarcinogens

Most of carotenoids functionality in the prevention and reduction of the prevalence of cancer such as breast and ovarian cancers specially in post-menopausal period [9]. Most of the anti-cancer activity have been associated with carotenoids' free radical and singlet oxygen removal capabilities, as well as abrogating DNA oxidative damages. New mechanisms of action have been proposed, such actions can actually cause the death of tumor cells suggesting the use as a cure and not just prevention. [19]

A great number of research have been focused on β-carotene as a cancer preventive element, such a large sum of research activity on this subject have created public interest in β-carotene, and β-carotene-containing foods. Consequently FDA title 21 section 101.78, have regulated health-claims on labeled fruits and vegetables, or fruit and vegetable-containing food; allowing producers to make cancer claims using the terms "Some types of cancer" or "Some cancers" giving that these crops are low fat and a good source of vitamin A *"beta-carotene",* vitamin C, or dietary fiber [20]. However many other carotenoids are showing promising results. For example α-carotene showed higher activity than β-carotene in suppressing the tumorigenesis in skin, lung, liver and colon. Another carotenoid is zeaxanthin, few data are available regarding its health benefits, nevertheless the new discoveries regarding its anti-carcinogenic activity against liver carcinogenesis as well as anti-metastatic

activity; have triggered great research interest. [8]

β-cryptoxanthin have the highest potency among carotenoids tested for their effect on Epstein–Barr virus, which have been linked to several types of cancers such as Hodgkin's lymphoma. However other carotenoids, such as zeaxanthin; have also been show to be effective in suppressing TPA-induced expression of early antigen of Epstein–Barr virus. Zeaxanthin and β-cryptoxanthin have also shown other defense mechanism against this virus by inhibition of pi-incorporation into phospholipids of cultured cells. Greater interest have been devoted in this research area toward β-cryptoxanthin since it is one of the most widely distributed carotenoid in daily foodstuff, thus it is the major detectable carotenoids in blood circulation. [8]

From our preceding review of the updated status of medical research on the health benefits of carotenoids, it is clear that many of these carotenoids have either an overlapping effect or a synergic effect. This theory is not new, since the production of such great number of carotenoids in nature by one single plant must surly be related to the diversity of functionality of these carotenoids. It is now clear that various natural carotenoids are valuable to apply for cancer prevention. These carotenoids may be suitable in combinational use, as well as single use. In fact, it have been recently found that multi-carotenoids (i.e., mixture of various carotenoids, such as β-carotene, α-carotene, lutein, lycopene and so on) show potent anti-carcinogenic activity. In addition combining carotenoids with other micronutrients such as tocopherols and vitamin C yield better over all results in many of published studies that are beyond the scope of this review.

However, these remarks indicate that, undoubtedly carotenoids are crucial micronutrients that need to be administrated in a holistic vehicle that is easily accessible by the general public. Functional foods presents itself as the perfect mean for this endeavor.

Common extraction methods of carotenoids

Carotenoids can be extracted from both animal and plant sources, extracted compounds are used for their aesthetic appeal as well as nutritional and health benefits. Carotenoids extraction methods can be divided into three categories, physical, chemical, and enzymatic extraction.

A- Chemical:

A relatively large number of chemical methods are available for carotenoids extraction, the bases for these methods differ, some are based on specific affinity, other on crystallization, or solubilization (in organic solvents). However organic solvent extraction is the most common method. To enhance solubilisation without the use of organic solvents, the use of lipids and oils is a common substitute. These alternatives are receiving a wide interest in the world of research and experimentation as a natural and environmentally friendly material. Shanbrom (1999) [21] proposed the use of material such as chlolestyramine, starch, cross-linked starch, carboxymethyl cellulose, and cross-linked polyvinylpyrollidone as binding material; with fruit juices such as cranberry and blueberry and juicing by-products (press cake) serving as sources of carotenoids. Carotenoid-containing material are allowed to come into contact

with the binding material which is nontoxic, then washed to remove water soluble compounds. He reported good affinity using those binding material.

Some of the more effective method is supercritical fluid extraction, but the cost remains the biggest hurdle preventing wider industrial implementation. CO_2 is one of the popular supercritical fluids, it have been tested on crab shell waste preceded by solvent extraction using ethanol [22]. To concentrate the extract silica gel column chromatography have been used on laboratory scale [23]. This method is superior to solvent extraction because: A. it eliminates or at least limit the use of organic solvents, B. it results in carotenoids in form of particles, the size and form of these particles can be manipulated by adjusting temperature and pressure [24]. A large number of patents have utilized this method, differing in processing conditions and carotenoid source. Some of the limitations of this method is that it is mostly a batch process, although some patents [25] have proposed the possibility of a continuous process, but industrial applicability is yet to be weight in. One of the factors affecting the efficiency of the process is moisture content of carotenoid-containing material, presence of moisture have been demonstrated to reduce the efficiency of the process, but drying can destroy some carotenoids such as lycopene [26]. Nobrea et al. (2009) [26] explained the effect by stating that water acts as a barrier preventing the contact between CO_2 and the fat soluble hydrophobic compound (i.e. lycopene or trans-lycopene), in addition solubility of CO_2 in water is higher than in those hydrophobic compounds introducing an issue of competitive availability of CO2. Nobrea et al. (2009) [26] tested an alternative drying method, freeze drying using

tomatoes as test material. The results were 15% higher yield of lycopene in freeze dried samples (4.6 % moisture content) compared to fresh wet samples (58% moisture content). This increase might not be enough to justify the added step (increase in time) and the cost associated with freeze drying treatment when considering industrial application.

2- Physical:

Physical extraction methods tend to concentrate the targeted compound then extract/separate using fatty carriers rather than organic solvents. One of the methods that have been patented and applied is by Helgason (2001) [27] the method involves the use of fish scale as an adsorbent of cpmpounds including carotenoids such as astaxanthin, zeaxathin, canthaxathin, violaxathin, carotene, and retinol; that remains suspended in the wastewater generated from the precooking process of arctic shrimp, this water has reddish hue which is an indication of the presence of these compounds. The innovation of this process is in the use of two by-products, fish scale and the runoff water of arctic shrimp. The process not only recovers carotenoids but also makes it possible to reuse the runoff water, thus reducing water usage. The binding mechanism though not very clear, it is most probably a combination of several physical interactions. Most probably lipophilic interactions between pigments and collagen, hydrophobic protein present in ctenoid scales. Coagulation mechanism is also a proposed adsorption mechanism. The limitation of this process is in the fact that organic solvents are needed to desorb the bounded carotenes since fish scale has limited use.

Hyoung et al. (2004) [28] proposed another methods that involves several steps that aims to concentrate carotenoids. Through steps of filtration, centrifugation, homogenization, washing and pelletization; a powdered material can be obtained. This martial can be used directly for esthetic and nutritional purposes. Solid filtration have also been proposed by Shivashankara et al. (2004) [29] as a physical mean of recovering carotenoids. Cremades et al. (2003) [30] used solid filtration to separate Crayfish (Procambarus clarkii), the material were filtered over a stainless steal filter with 1-mm pore size hence two different fractions of carotenoproteins with the aid of enzymes, but the process was not sufficient to separate carotenoids only, on the other hand, carotenoids are more stable when they are present in a complex with proteins via covalent bonds forming caroteno-proteins. Several supporting techniques are usually coupled to achieve the recovery of caretoproteins, Meenata et al. (2011) [31] used pH shift technique to isolate carotenoids from shrimp waste. The use of ethylenediaminetetraacetic acid (EDTA) and proteolytic enzyme trypsin have also been used as chelating agents for the extraction of caroteno-proteins from shrimp waste ([32], [33]).

3- Enzymatic:

Enzymatic degradation of tissues and cells can be used as a pretreatment of samples to facilitate the extraction process. Enzymes such as cellulose and pectinases are used to treat plant material, while proteinases are used for animal tissues. The process require precise control of temperature and pH for optimal

enzymatic activity. Most of enzymatic treatment are required prior to the recovery of carotenoids using methods such as solvent extraction. [14]

Green Extraction Technologies

Sustainability as a concept

The Brundtland Commission, one of the United Nations commissions; have defined sustainable development back in 1987 as “development that meets the needs of the present without compromising the ability of future generations to meet their own needs", for an operation or a product to be labeled as sustainable it must fit this definition [34]. The terms sustainable and sustainability became widely used after the Earth Summit in Rio de Janeiro Brazil 1992. This concept have grown over time to surpass the conventional direct ecosystem reservation to include sectors such as economic market, health care systems, and chemical engineering and design [35]. Sustainability for any industry have to successfully and equally meet its economic prosperity, social well being and environmental protection [35].

Sustainability in the food industry

The food industry is not an exception of the green revolution, green models reached almost all aspects of the industry (processing, manufacturing, packaging..etc). Green technologies in the food industry are meant to achieve various goals such reduction of energy usage, waste generation, and overall reduction of carbon footprint in ways beyond green food-agricultural practices [36]. Retrieval of valuable compounds from food can be achieved using harvesting processes such as extraction, concentration, purification, and

fractionation, the end products can be used as food ingredient that serve multiple purposes or used as functional ingredients in nutraceuticals.

Extraction in food processing is defined as: “the transfer of one or more components of a biological feed from its source material into a fluid phase, followed by a separation of the fluid phase and recovery of the component(s) from the fluid” [37]. Extraction of nutraceutical is a relatively costly process for food manufacturers [7], research and development in the area is critical for the development of new technologies. Within the concept of sustainability in the food industry extraction practices, green separation process is defined as: “separation processes that are environmentally friendly and result in less air pollution and industrial waste” [36]. Such practices are know as green chemistry, it is defined as “pollution prevention on the molecular level by efficiently using raw material, eliminating waste and avoiding the use of toxic and/or hazardous reagents and solvents in the manufacture and application of chemical products” [35]. Chemists working on developing such green separation technologies are refereed to as green chemists; their ultimate goal is to create a technology that “minimizes the number of steps, separation and protection groups for product synthesis and limiting the use of solvents” [35] . Such technology will ultimately result in smaller to non existing solvent waste, consequently eliminating the need for solvent removal and handling steps, naturally reducing the overall cost and energy [35].

Motives behind green changes in the food industry

Legislations and manufacturing regulations demand environmentally sound manufacturing procedures. A host of regulatory legislation in the United States, such as Environmental Protection Agency (EPA) Pollution Prevention Act, the Superfund Amendments and Reauthorization Act (SARA); as well as The Resource Conservation and Recovery Act (RCRA) of 1976, and The Montreal Protocols of 1987 by the United Nations on Substances that Deplete the Ozone Layer; are used to regulate the use of organic solvents in all aspects (handling, waste management, storage…etc). [38]

Even though the use of organic solvents is a common extraction practice in the food industry (i.e. hydrophobic compounds) [37], after all, solvent extraction is a practice that dates back to the Palaeolithic age [39].These solvents are viewed as atmospheric pollutants and might contaminate the extract event if present in trace amounts, lessening it's purity [37]. In term of extraction of valuable compounds, the food industry regulations limit the use of organic solvents for safety, health and environmental causes [14]. Such continuously changing and stringent regulations, places pressure on researchers to satisfy the new industry regulations thru innovations that uses alternative non-solvent based separation methods [36].

Beside the obligatory need to change in order to comply with industrial laws and regulations, green change can mean profit for manufacturer. The economical gain from switching to green manufacturing process extend from: lower overhead and production costs via reduction in processing time, and

possibly lower facility cost if the process is switched form batch to continuous, safer working environment which translated to fewer injuries, and fewer precautionary steps that consume time and requires trained personal. Eliminating the use of hazardous material vanishes the need for special storage and waste removal thus reducing the chances of possible injuries and capitalize on facility and human resources for better use. [35, 38]

Another important concept that can be categorized as part of the green revolution is utilization of the food industry by-product in a way that adds value. Extraction of valuable compounds from such waste (e.g. press cake) means greater profit margin [14], a universal incentive for change. Items such as carotenoids (including b-carotene and lycopene) have created a $1 billion world market with a 3% annual growth rate, hence another reason for the increased interest in utilizing industrial by-products to generate highly valuable materials [36]. Extraction of carotenoids from the food-agricultural industry (tomato processing industry) and the seafood industry (shrimp and crustaceans) by-products adds value to waste, and minimizes waste processing. Strict waste management regulations promotes the generation of solvent-free waste material; thus encouraging the use of green technologies, therefore greater environmental and economical benefits are achieved [14].

One of the most powerful motivations behind the widespread of any trend is consumer demand and interest. Consumers around the world have showed great sense of responsibility toward making sustainable product purchase and are willing-despite the tough economical market- to pay more for such products.

Environmental as well as social responsibilities in industrial practices are compelling reasons for product selection. This is particularly true for consumers in developing markets such as Brazil, China, and India and are willing to pay for the added cost associated with such out of the norm practices. Consumers very well understand the shared responsibilities of all sectors and parties to achieve and establish such practices, thus holding their governments, and businesses responsible for making such changes. [40]

The most important issue that consumers frequently emphasize on when defining environmentally cautious industrial practices is the safety of the drinking water by avoiding the pollution of water sources. “Safe drinking water is consumer’s top priority” as much as 93% of consumers in developing market and 90% in developed markets that have been surveyed in a consumer trends and sustainability survey published in 2012. Surprisingly this issue ranks higher in the list of consumer demands of environmental and social demands, ranking higher than fair wages and working conditions. [40]

Consumers are savvy and highly demanding, they demand that not only that these sustainable products be produced in a manner that does not harm the environment, it is key that these products improve their lives and serve their needs as well as alternative products. The matter of fact is consumers are more skeptical now that they have tried and purchased products over the years that did not deliver on personal and product performance level, and environmental and social responsibility level. Nevertheless, consumers in emerging markets such as china and India -markets that are setting world wide trends- provide great

business opportunity for producers to fill a market gap with sustainable products that fit into the new emerging lifestyle that people all over the world are adopting. However, companies seeking to thrive in the new emerging markets and maintain their position in established markets needs to highlight the mutual benefit of adapting any changes throughout the production chain in a way that sustainability and social purpose are integrated in their business strategy. [40]

Efforts of green extraction of carotenoids

Conventionally, the recovery of carotenoids is accomplished using solvent extraction methods. Vegetable oil such as soybean oil, cotton seed, and even salmon oil have been used as a hydrophobic medium in many of the these trials. To improve the efficiency of recovery additional processes have been used to work synergistically to improve the recovery of carotenoids, enzymatic hydrolysis have been used to aid the recovery process [41].

Currently a number of green techniques have been tested, and are implemented on small industrial scale; these techniques are the first step toward a safe, energy-saving, and sustainable extraction methods. Green sustainable technologies include those that uses fewer amounts of solvent and reduces energy use, most commonly by the use of an aid such as ultrasound and microwave to assists the conventional solvent extraction process. Under this category lies a number of technologies such as supercritical fluid extraction, pulsed electrical field, solvent-free microwave extraction, instant controlled pressure drop technology, microwave hydrodiffusion and gravity, ultrasound

assisted extraction, supercritical water extraction, high pressure-assisted extraction, aqueous two-phase and enzyme-assisted aqueous extraction. All of these technologies seem to be lacking in certain aspects to be declared as both environmentally and economically sound application, hence it is suggested to combine one or two of these techniques together to achieve best results [37].

The use of alternative solvents such as in supercritical fluid extraction and supercritical water extraction have also been proposed as possible alternatives of supporting methods [42]. These technologies have plenty of hurdles to overcome, the biggest being the added cost for reformation of existing facilities, hence the idea of continuing to use solvent extraction as the method of choice but replacing the petroleum solvent with biosolvent is comforting for investors. Consequently the use of green solvents in the extraction of valuable compounds from complex food matrices has presented itself another green alterative. Compared with petroleum-based solvents that often result in loss of material, low extraction efficiency, time and energy consumption; green solvents seems to overcome some or almost all of these shortcomings. A green extraction technique and a green solvent are two different things, green separation process is defined as: “separation processes that are environmentally friendly and result in less air pollution and industrial waste” [36]. Such technology will ultimately result in smaller to non existing solvent waste, consequently eliminating the need for solvent removal and handling steps, naturally reducing the overall cost and energy [35]. However technique such as microwave assisted extraction would most likely still rely on solvents as a carrier of targeted material, combining these

technologies with green extractant have been tested and is showing promising results, comparable to conventional methods with some advantages such as time and energy reservation.

One of the biosolvents that is showing promising results is *d*-Limonene; an essential oil found in citrus peels, the peel being a by-product of the juice industry. *d*-limonene holds a GRAS status by US Food and Drug Administration (FDA). However *d*-limonene has its shortcomings, it has low viscosity and high energy consumption compared to *n*-hexane making its boiling point around 175℃ (5-15% concentration mixed with surfactant) thus it has low recovery rate and higher energy consuming than common solvents. Table (4) compares some physical properties of *d*-Limonene with more common solvents. [42]

Properties	***n*-Hexane**	**Toluene**	**Dichloromethane**	***d*-Limonene**
Empirical formula	C_6H_{14}	$C_6H_5CH_3$	CH_2Cl_2	$C_{10}H_{16}$
Molecular weight	86.18	2.14	84.93	136.23
Boiling point	68.7	110.6	39.8-40.0	175.5
Heat of vaporization	334	351	28.6	353
Density	0.6603	0.8669	1.325	0.8411
Toxic	Yes	Yes	Yes	No
Environmental impact	High	High	High	Low

Table 4: Physical properties of d-limonene compared to n-hexane. [42]

Challenges and limitations of green extraction technologies

Designing a green industrial technology is a complex process due to the involvement of heat, mass and phase transfer beside chemical reactions. Hence full understanding of the thermodynamics on a molecular level is necessary to reach optimal processing operations. This requires in-depth knowledge of the system and process coupled with experimental data on thermodynamic and phase behavior, which can be used to build initial mathematical model to be used

for heat and mass transfer under changing variables. This is only the beginning, much more work needs to be completed before a new process is considered for commercialization. All this aside, there is the issue of safety and bioavailability of extracted material. [36]

Designing a separation process requires an understating of the type of separation, properties of material used, targeted bioactive component, and most importantly the purity requirement of the end product. Reasonable level of purity should be coupled with the economic value of the selected process and end value of separated material [36]. A favored holistic approach to such issue making sustainability an inherent part of the design, is applying the concept “beginning by design” [35]. New green technologies should be able to provide the same efficiency, time, energy and money savings as conventional methods, if not improve on these aspects.

Unfortunately most of the new green technologies have limited industrial scale application and are used on small laboratory scale isolation and purification purposes. The greatest hurdle is the cost of switching to new practices, changes in the overall operation procedure all the way from the floor plan to the day-to-day practices [36, 43]. The future relies on innovative and sustainable extraction that combines several green technologies, moreover employing green extractants gives greater hope for wide spread industrial applications [37, 42].

In general, most of the green extraction methods rarely uses any chemicals in the extraction steps, but some methods like that by Helgason (2001) [27] and Shanbrom (1999) [21] mentioned before; result in minimizing the use of

solvents as extractants but still fail to separate the adsorbed hydrophobic compounds from the non-edible extractant; without the use of solvents. In a recent paper, Riggi (2010) [44] reviewed a large number of green extraction technologies of carotenoids, non of the methods were 100% effective and solvent free at the same time, so he concluded that combining two or more of the processes together would be best. [14]

Conclusions of literature review

Problem statement

Carotenoids are commonly extracted using solvent-based methods, however environmental laws and some of the food industry regulations limit the use of organic solvents for safety, health and environmental causes. In addition, consumers are demanding sustainable products that are obtained through environmentally friendly practices. This has triggered a wide interest in developing greener technology that limit or avoid the use of organic solvents. However, inefficiency, cost and impracticality of most of the modern green extraction technologies means that the search must continue for a green technology that meets industrial and consumer standards. As a green solvent-free approach, we will be assessing soy protein ability to adsorb carotenoids present in mango juice.

Hypothesis

It is possible to utilize the exposure of the hydrophobic core of proteins during thermal denaturation to adsorb hydrophobic compounds present in an aqueous medium.

Research aim

Develop a process that utilizes Soy Protein Isolate (SPI) as an adsorbent of carotenoids resulting in nutritious functional ingredient that harvest the cumulative nutritional value of soy proteins and carotenoids via solvent-free green extraction technology.

Objectives

1- Study the thermal denaturation behavior of SPI in suspension.

2- Establish the thermal safety of the proposed treatment of carotenoids content in mango juice solution.

3- Determine the feasibility of carotenoid sorption using SPI using optical measurements.

Chapter 2: Materials and Methods

Materials

Soy Protein Isolate

Soybean is a small yellow legume, soybean seed is made of seed coat, embryo and cotyledons that accounts for the majority of the weight of the seed containing most of the seed's oil and protein content. As with all plants, chemical composition varies among varieties, geographic locations, and climatic conditions [45]. Protein content in soybean ranges between 48% and 50%, proteins in soy are categorized into: metabolism-related proteins, structural proteins like ribosomal and chromosomal proteins, and storage proteins that have no biological activity. Storage proteins are the predominate proteins in soy; they are globulins, slightly soluble in water with increasing solubility in salt solutions. [46]

The processing from seed to soy protein for human consumption involves: cleaning, cracking, dehulling, tempering, flaking, and extracting with organic solvents, typically hexane. The proceeding steps depends on the intended end product as illustrated in figure (6) [45]. Treatment of soy flour in the production of soy protein isolate depends in concept on protein solubility (minimum around 4.5-4.8 PI) that is maximum around high and low pH values. The first step would be to defat the soy sample by the removal of oil content via the use of organic solvents. This is followed by the dispersion of the fat-free contents in water where solubility in water can be used as a mean to separate the different fractions. This is achieved by adjusting the pH of the aqueous medium to near the isoelectric point (pI) of the proteins fractions (pH 4.5). Precipitated fractions are separated

by centrifugal force. Neutralization of the wet precipitate is the final step before drying. Discrepancies in the details of the processing method specifically precipitating agent (sulfuric acids, sodium hydroxide, etc), pH (maximize precipitation at pH 4.5, pI of proteins), and temperature; affects the degree of protein recovery and changes product composition. Processes such as grinding, drying and heating can alter protein's functional properties and bioavailability. [46]

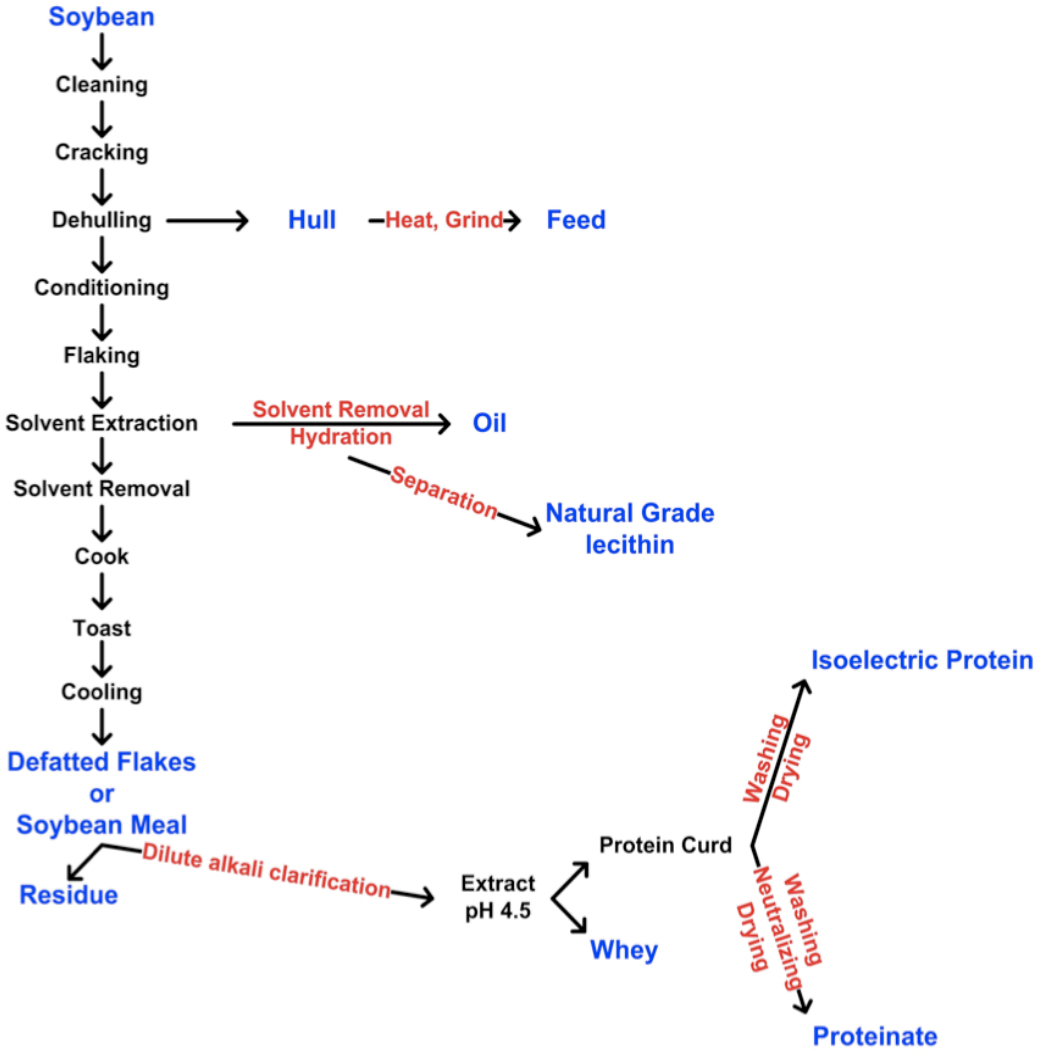


Figure 6: A schematic representation of commercial production of isolated soybean protein that starts with contiguous solvent extraction of soybeans. Adapted from Horan (1974). [45]

Processing affect protein fractions proportions and other qualities hence affecting its functionality and possible applications [46]. Soy protein constitutes of mainly (80-90% [46, 47]) two globulin fraction β-conglycinin and glycinin, they are frequently referred to as 7S and 11S globulins respectively, based on the Sedimentation Coefficient (SC) during water-extraction [47]. The other less abundant fractions are narbonin 2S, 9S, and 15S globulins [46].

The difference in molecular structure between β-conglycinin and Glycinin results in their distinct functional properties [47]. β-conglycinin (7S) is a trimer that consists of three subunits (α', α , β) with molecular weight 72, 68 and 54 kDa respectively, in addition to a minor γ subunit. The presence of β3 causes the association or disassociation upon changes in pH and ionic strength in surrounding medium. 7S lacks the ability to form disulfide bonds, it dimerizes at 0.1 ionic strength producing a 9S sedimentation form. β-conglycinin thermal instability is advantageous over glycinin in its function as emulsifying and emulsion-stabilizing abilities. Association and disassociation without the presence of disulfide bonds assists the formation of transparent, soft and elastic gel by β-conglycinin. [47]

Glycinin (11S) is a hexamer of total molecular weight 300-380 kDa [47]. It is composed of 12 subunits linked together by a disulfide bond, forming a dimer of two identical hexamers. Three of the hexamer are acidic making 40-50% of the total structure, and the remainders are basic making 50-60%. The acidic and basic subunits are linked by disulfide bonds, hence glycinin have the ability to associate (increasing the molecular weight) by forming more of these disulfide

bonds, and subsequently disassociate into its subunit via the breakage of these bonds [46]. 11S disassociate under pH, ionic strength and heat treatments. The presence of disulfide bonds makes 11S more stable thermally than 7S. Formation of disulfide bond results in a turbid, hard and brittle gel. [47]

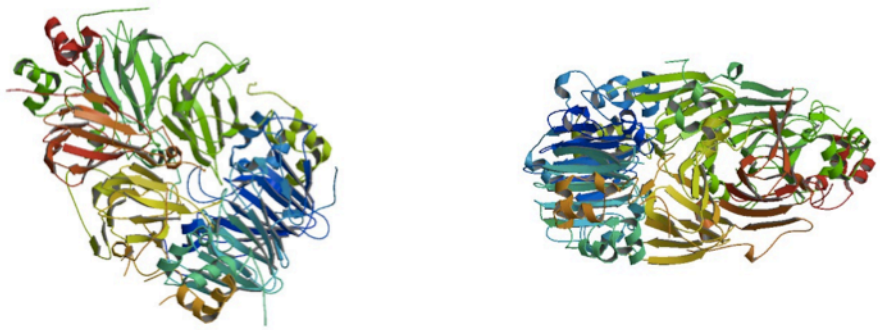


Figure 7: Ribbon diagrams of A. the crystal structure of soybean proglycinin A1aB1b homotrimer [48], B. crystal structure of soybean beta-conglycinin beta homotrimer [49].

Soy globulin globular proteins [50] in the form of soy protein isolate are widely used in various industries such as the food and pharmaceutical industries for their functional properties that enables a wide range of applications that are summarized below: [46]

- Hydration properties ➔ swelling, solubility, and viscosity.
- Protein-protein interactions ➔ precipitation and gelling.
- Interface properties ➔ surface tension, foaming and emulsion capacities.

Soy proteins have relatively high solubility in water or dilute salt solutions at pH far form its isoelectric point [50]. It is used to stabilize emulsions, foams and gels in beverages, mayonnaise, sausages, soups and salad dressings [46].

Its gelation capabilities have enabled it to be used in the making of imitation cheese, yogurt, and sausage production. [46]

Due to the effect of processing on the molecular structure of soy protein, subsequently its functional abilities, we opted to start with a commercial grade soy protein isolate that is being used in the food industry in applications that require heat treatment such as gelation. Commercial processing of soy protein results in partial hydrolysis that increase the number of charged groups, decrease molecular weight, increasing overall solubility, and cause the exposure of protein hydrophobic core via partial denaturation using heat or chemical treatment, or a combination of both. This process facilitates the formation of protein aggregates that during protein gelation can for a space-filling gel network [51], unlike SPI prepared on lab scale that is often unhydrolyzed and does not support the formation of protein aggregates.

This will enable uniformity of material and simplify the processing steps involved in this project. Sample used in our experimental design is SUPRO 500E without sulfite, Isolated Soy Protein (Solae LLC, St. Louis, Missouri). Protein content comprises 90% of the fat-free, dry weight [52]. It is characterized by high gelling and emulsion abilities, and strong water retention. Used in meat paste, sausage and vegetarian sausages and burgers [53-55]. In addition, commercial SPI (Bob's Red Mill, Milwaukie, OR, USA), and unhydrolyzed laboratory prepared SPI (Raskin laboratory research group, Rutgers University, NJ, USA) were used for better understanding of the relation of commercial processing that results in partial denaturation, on the rheological behavior of SPI.

Mango concentrate

Mango (Mangifera indica L. Anacardiacea) is a tropical climacteric fruit, India is the number one producer of this fruit in the world [56, 57]. Mango is the 5th fruit in world production of major fruit crops, only 20% of harvested mango is processed and the remainder is consumed fresh [57]. This seasonal fruit is highly perishable once fully ripen, more than 50% of the harvest is lost due to postharvest spoilage of the fruit [58]. Mango pulp, the major by-product of the mango industry; consists mostly of water and carbohydrates with small amounts of fiber, protein, fat and minerals, respectively [56, 57].

Carotenoids give mango its distinctive orange color, mango's nutritional value is closely related to its being a good source of provitamin A [59], ripe mangoes are 10 times richer in carotenoids than partially ripe ones [56]. Based on the mean value of vitamin A (in form of its precursor b-carotene), mango is considered a good source of vitamin A [59, 60], figure (8), being able to provide +10% of the Daily Value (DV) of vitamin A which is 5000 IU [61]. Estimation of total carotenoid content in plant tissues can vary depending on methods used [57], and sample variation that can be attributed to variations in growth, ripening, and storage conditions as well as postharvest conditions [59, 62, 63].

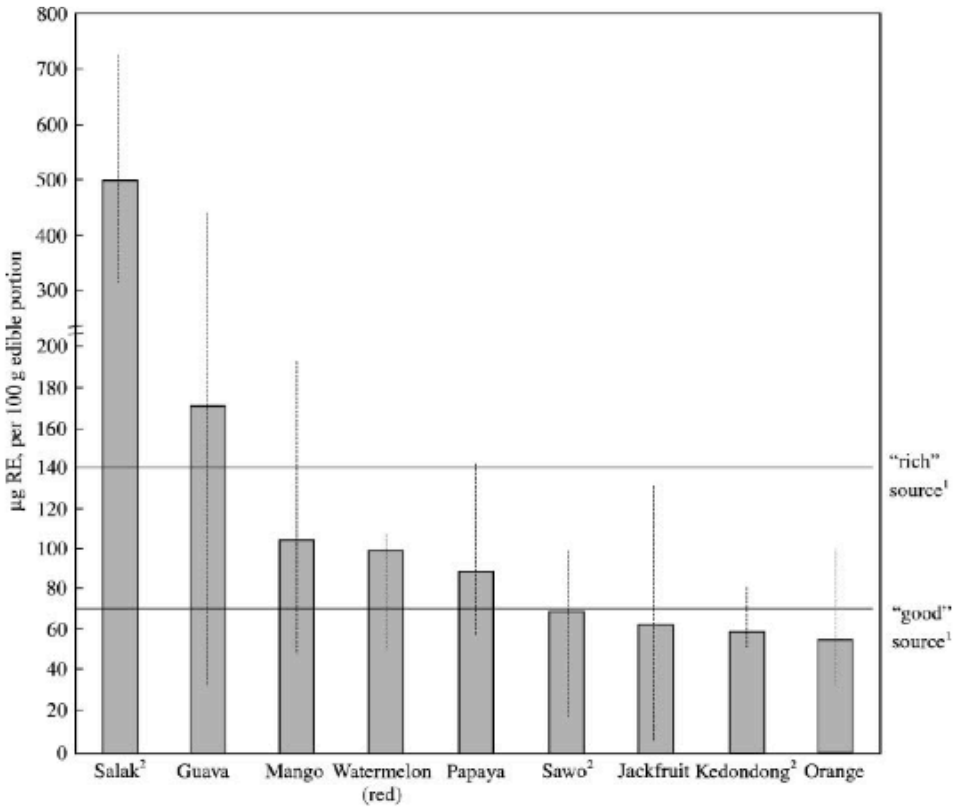


Figure 8: Comparison of some fruits (specifically popular in Indonesia) according to Vitamin A content, estimated as retinol equivalent (μg RE). Bars represent the means of the three values with the dotted lines connecting the minimum and maximum values. [59]

Fully ripened mangoes are the preferred choice for most of mango products due to their well developed flavor profile, desired color and soft texture [64]. Mango puree is the starting material for the making of mango concentrate. To make mango puree, fully ripen mangos are first washed to remove dirt and impurities from the surface. To soften the fruit making pulp extraction easier; fruits are steamed briefly. Whole fruits are passed through a pulper that consists of a series of sieves and paddle to move and push the fruit pulp through the sieves while eliminating the skin and pit [65].

Mango concentrate is made of mango puree that is pasteurized (167° C for 1 min), then cooled and centrifuged, the supernatant/serum is then

concentrated either by: a) evaporation at low temperature under reduced pressure, or b) freeze concentration where water ice crystals are removed leaving concentrate behind [64]. Aseptic mango concentrate typically have a Brix value of 28° and is shelf stable [64]. A concentrate power can be 4.5-6 folds meaning one volume of concentrate can be diluted to obtain 4.5-6 volumes of single strength juice [65]. According to United States Food and Drug Administration (FDA) 21 CFR 101.30 (h) a single strength mango juice (100% juice made from concentrate) should have a minimum of 13° Brix value. [20]

The quality of products made from mango puree concentrate is comparable to those prepared from fresh fruit. Mango concentrate is used in the making of host of products including beverages such as ready-to-serve, nectars, juices, and squash. These products can be made by adjusting sugar to acids ratios, and pulp concentration. [66]

Acids and sugars are directly related to flavor, hence consumer's acceptability that favors an increase in sugar content and decrease in acidity for consumption of fresh mango. Discrepancies in the sugar and acid content have been reported between varieties of mango, depending on analytical measures and ripening conditions. The main sugars in mango are glucose, fructose, and sucrose. Overall sugar content increases with ripening and can be quantifies as the total soluble solids. In a fully ripened mango, sucrose makes up the greatest concentrations of all sugars, while fructose is the highest reducing sugar, and glucose to fructose ratio remains constant through the ripening process. Citric and malic acids are the main acids in mango, succinic and tartaric acid are

present in small amounts. During ripening acidity decreases while pH value increases, this is attributed to the decrease of the available citric acid in addition to small reduction of malic acid. [67]

Commercially produced mango concentrate was selected as model to avoid sample-to-sample variations that would occur as a result of fresh sample preparations starting using fresh mango. In addition, preparation of mango concentrate to simulate that available commercially would be time consuming and adds complexity to experimental design and flow. Mango concentrate of Totapuri mango variety was klindly donated by iTi Tropicals (iTi Tropicals, Lawrenceville, NJ) and used through the project. The sample is described as follow on the company's website: "Mango concentrate is manufactured using mature fruit, free of insects and diseases. The fruit pulp is extracted, followed by an enzymatic deactivation, evaporation, enzyme addition and puree concentration and sterilization. Mango concentrate can be used in beverages, ice-cream, jams, jellies, sauces and cereal bars". The material is free of any additives or preservatives, refer to appendix (1 and 2) for material safety data sheet and statement of compliance. Concentrate reported a minimum of 28° Brix, pH 3.6-4.2, and maximum acidity of 1.3% as citric acid. Aseptically packed concentrate is stable at room temperature for 24 months, sample was delivered frozen with instructions to be kept frozen since it has been removed from the large aseptic steel drums to smaller sample containers. Sample was thawed by storage in fridge (4°C) over night. Due to sample's photosensitivity, samples

were shield from direct light exposure at all times, opaque containers where used.

Acidified water

Acidified water was prepared in the pH range 2-7. Citric acid 10% w/v aqueous solution (BDH, PA,) and dilutions of 2N Sodium Hydroxide (J.T.Baker, PA, USA) were used for this purpose.

Simulated juice solution

Acidified water and sugar were used to prepare a simulated juice solution to be used as a control. Sucrose (D(+)-Sucrose, Fluka Biochemika, Buchs, Switzerland) was used in the preparation giving that it is the main sugar in ripe mango. Citric acid 10% w/v aqueous solution (BDH chemicals Ltd, UK) was used as the predominant acid in fully ripe mango.

Methodology

Preparation of soy protein isolate suspension

A desired amount of SPI powder was weighed and mixed with solution using VWR Fixed Speed Vortex Mixer, touch mini vortexer (VWR International, Inc., West Chester, PA, USA); for approximately 30 seconds until fully dispersed. Suspension was allowed to stand at room temperature for 30 min prior to any measurements to assure particle hydration.

Differential scanning calorimetry

Identification of changes in material qualities and characteristics during chemical reactions or physical transitions are important for process precision and quality control. In the pharmaceutical industry for example, such information are needed to ensure purity of formulations and starting material, and in the polymer industry to determine melting or degradation behavior of material caused by treatments such as extrusion-related heating [68]. Such data is feasible via Differential Scanning Calorimetry (DSC) that measures heat generated or consumed during chemical reactions or physical transitions.

DSC can be defined as the measurement of heat flow rate difference between sample and reference material due to change in sample temperature; while being subjected to a controlled temperature program. Therefore the objective of calorimetry is the measurement of heat, more precisely heat exchange that is identified as heat flow. The amount of heat exchanged cause a change in material temperature compared to that of reference sample. This

fluctuation in heat flow occur at characteristic temperatures of the reaction or transition such as glass transition temperature, melting points, and protein denaturation. [69]

DSC measurements are widely used as the measurements are relatively quick, small amount of sample -in milligram range- is needed, can operate over a wide temperature range, and highly accurate. Hence DSC is used to characterize material (specially polymers), quality control and substance identification, stability investigations, evaluation of phase diagrams, purity determination, kinetic investigation, safety investigation to name a few. [69]

Perkin Elmer DSC-7 (Perkin–Elmer, Norwalk, CT, USA) power compensation DSC is used in this study. In a power compensated DSC, the same heating power is supplied to both microfurnaces holding the control/reference and sample in order to change the mean temperature in accordance with the present heat rate. Once there is a difference in the temperatures of both furnaces that corresponds to sample temperature change (increase or decrease), the difference is compensated by the heating power supply to the furnace. The compensated power (delta P) is proportional to the remaining temperature difference (ΔT). Asymmetry of heat exchange cause a differential signal that is recorded. The relationship between the heat flow rate Φm and rate temp signal ΔT (factory calibration) [69] :

$$\Delta P = -k_1 \cdot \Delta T \qquad \text{Eq. 1}$$

$$\Phi m = -k_2 \cdot \Delta T \qquad \text{Eq. 2}$$

k_1 is factory set fixed quantity of the proportional controller. And k_2 change in the instrument with calibration. Figure (9) is a block diagram of a the functional principle of a power compensated DSC [69].

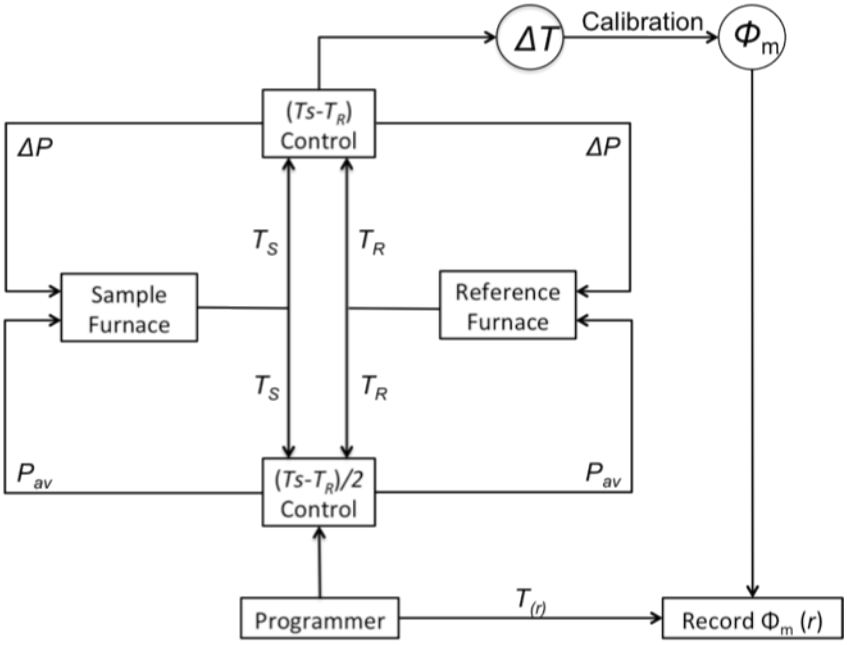


Figure 9: Block diagram of the functional principle of a power compensation DSC (Perkin-Elmer Instruments). [69]

Advantages of power compensated DSC over the more common heat flux DSC include shorter heat conduction path allows almost instantaneous response to a sample reaction. In addition only small temp difference ΔT remain between the microfurnace holding sample and reference material, which means that the calibration factor KΦ is practically independent of the intensity and kinetics of the samples reaction. At the end the total compensated energy is equal to the reaction heat or heat of transition, therefore easily related.

To identify the denaturation points of SPI that correspond to the two major protein fractions 7S and 11S, DSC measurements were carried using Perkin Elmer DSC7 in conjunction with Perkin Elmer Thermal Analysis Controller TAC

7/DX and a refrigeration unit, along with a Perkin Elmer Pyris software for thermal analysis version 7.0.0.0105, was used to acquire data and complete further analysis.

A 8% (w/w) suspension of soy protein in distilled water was prepared fresh prior to each run as described previously [70]. A maximum amount of 10 mg of the suspension was weighed directly into a Perkin Elmer stainless steal pan that was later hermetically sealed.

The test program was designed to hold the sample at RT (25C) for 5 min, heat the sample to 95°C at a rate of 2 °C/min, holding at this temperature for 30 min, the sample was then cooled back to RT at the same rate and held at Room Temperature (RT) for 5 min.

Rheological measurements

In order to study the denaturation and subsequent aggregation behavior of soy protein we attempted to replicate the processing steps used in the making of cold-set gels with the exemption of the final step of gelation induction. In the making of cold-set gel, a relatively lower protein concentration is used compared to that of heat-set gel. Heat treatment of protein suspension at low concentration results in the formation of soluble aggregates rather that three-dimensional network that forms at higher concentration, therefore processing at low protein concentration allows the study of the progression of aggregates formation that results from protein denaturation. Subsequent changes in viscosity therefore can be attributed to aggregate formation rather than three-dimensional gel network.

In order to form a pregel solution of soluble aggregates, the initial protein concentration must be below the critical values required for the formation of three-dimensional network during heating (heat-set gel). The critical value for soy protein have been established by Maltais et al. (2005) and others [71-73] as below 10% w/w.

Morales and Kokini (1997, 1998) used dynamic rheological measurements as a method for studying the glass transition temperatures and phase transition of the main protein fractions of soy protein [74, 75]. The concept of using rheological measurements to identify changes in protein structure is based on the fact that changes in the molecular structure and intermolecular interactions affect suspension's rheology [76]. Study of the rheology of SPI gives an insight into the conformational changes in protein structure, degree of denaturation, and aggregation under defined experimental conditions [77]. However, the use of dynamic rheological measurements where the storage modulus (G′) and the loss modulus (G″) can be determined; might be flawed.

The use of dynamic rheological measurement to study the progress of soy protein gelation can be misleading considering the conventional methods used to minimize volume reduction. Studying the progression of gel formation is often accomplished by monitoring the change in the storage modulus (G') and modulus (G") using dynamic oscillatory rheometer where a sample is between placed between two parallel plates or a plate/cone space. To minimize water evaporation from samples during prolonged testing at low temperature or elevated testing temperature, oil coating is used (silicon or mineral oil, paraffin oil

for example), the amount and method of addition varies. This is associated with many concerns such as contaminating sample with oil [78, 79], and in case of protein gels, during heating protein denaturation causes exposure of the hydrophobic core causing diffusion of protein into the interface between the suspension hydrophilic medium and the hydrophobic medium of the coating oil. Therefore we believe that the use of rotational cylinder rheometer at low shear will provide an accurate representation of the molecular changes during heat treatment of soy protein suspensions without emphasizing on the changes in storage modulus (G') and modulus (G") since the ultimate goal is not the formation of SPI gel, rather the formation of a progel solution of denatured SPI via the use of heat as a denaturant.

Effective denaturation is necessary for the formation of pre-denatured SPI solution; this can be accomplished using different combinations of temperature and time. Maltais et. al. 2005 [71] for example opted heating SPI suspension at 105°C for 30 min, while in the case of another globular protein, why protein; heating to 68.5°C for 2 h [80] was sufficient. The goal is to achieve a 95-100% denaturation of protein, later this percentage is diluted by the addition of a gelling solution (salt or acid solution) [80]. For our goal the temperature was set sufficiently above the denaturation point of the most heat stable protein fraction (glycinin) that reported a denaturation temperature as high as 90°C [81, 82] to achieve a high degree of denaturation. Zhu et al. (2011) [83] were able to achieve adequate denaturation required to make soy glycinin cold-set gel by treatment at 95°C for 30 min.

Giving that soy protein and carotenoids are selected as models to study the feasibility of efficient adsorption of hydrophobic molecules to proteins hydrophobic core, heat treatment was designed not to cause excessive degradation and loss of carotenoids.

Rotary rheometer is used to report absolute viscosity by measuring fluid parameters of shear stress and viscosity at given shear rate. Rheometer function by immersing a spindle in a test fluid, the spindle rotates thru a calibrated spring, deviation from the calibrated zero values are deflection caused by viscous drag of the fluid against the spindle [84]. Rheometrical studies are used to correlate material behavior with rheometrical function making it an effective method of quality and process control [85].

Brookfield DV-III ULTRA programmable rheometer with Rheocalc version 3.2 software (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) were used. Speed range for viscosity test 0.01-250 rpm, with viscosity and torque accuracy of ± 1.0% for a specific spindle running at a specific speed. Temperature monitoring in also possible in the range of - 100°C to 300°C. [84]

9% (w/w) suspension of SPI was prepared fresh prior to each run as described previously. Sample was placed in an adaptor (2-16 ml sample capacity) that fits in the rheometer's water jacket. Spindle SC4-31 was selected as the appropriate spindle for the adaptor and sample. Temperature control in the water jacket was achieved using Thermo Scientific HAAK DC30 water bath (Thermo Fisher Scientific, Waltham, MA, USA) and LabView software (National Instruments, Austin, TX, USA).

Appropriate rotational speed was determined by running a speed ramp test (data not reported). Speeds ranging between 0.01-250 rpm were used, sample was held for 30 sec at each speed. Shear rate and viscosity were recorded after each speed change. Rotational speed that resulted in the lowest viscosity measurement was selected to avoid error in obtaining viscosity measurements during testing. This value was found to be 91.84 rpm, we approximated this value to 90 rpm for simplicity. This speed was used throughout each run and for all runs.

An equilibration period of 1 h at 25°C was determined for a 9% (w/w) SPI suspension. This equilibration period is necessary for our suspension (inherent time dependency) sample to reach a constant viscosity before applying heat treatment to avoid shearing effect on viscosity as processing is progressed. [78, 79, 86, 87]

At the end of the equilibration period, sample was heated to 95°C at a rate of 2 °C/min. When the mixture temperature reached 95°C, it was maintained at that temperature for various amount of time 30 min. Mixture was allowed to cool down gradually by setting the water bath temperature back to 25°C at a rate of 1 °C/min, and maintained at this temperature for the remainder of the test time. Humidity cover was used to prevent dehydration during analysis. It is possible to minimize moisture loss but not completely eliminating the issue [79], thus approximately 1 ml reduction was detected at the end of 6 h run (data not reported).

UV/Visible spectroscopy of SPI

UV/Visible spectroscopy have been chosen as a method for the assessment of protein conformational changes due to its rapidness, convenience, requirement of a small amount of sample, and it is a nondestructive method [88]. Spectroscopic measurements are widely used to obtain quantitative and qualitative information about proteins in solution without the need for calibrations with standards [89]. It is a useful tool in monitoring changes in tertiary structure associated with protein unfolding.

Protein's native structure contains a hydrophobic core that is shielded from any aqueous medium. This native structure is maintained under certain conditions of temperature, pH, ionic strength…etc. Due to the disruptive effect of heat -and medium pH- on protein structure, the hydrophobic core is exposed when the structure uncoil during heat denaturation. Thus changes in the spectra of individual a.a. reflects the enviromental impact on protein molecule conformation, it can also be attributed to intermolecular bonding and electrostatic interactions between nearby residues. [88]

Conformational disturbance caused by thermal treatment results in exposure of buried hydrophobic a.a., thus enabling their detection using UV/VIS spectroscopic measurements [90, 91]. Table (5) includes the amino acid composition of soybean protein, 11 of the 18 a.a. present in soy protein are hydrophobic amino acids.

Table 5: Amino Acid Composition of Soybean. [46]

Amino Acid	Three letter code	Molecular weight (g/mol)	Empirical formula	Content (g/100 g protein)	Side chain polarity*
Glutamic acid	Glu	146	$C_5H_{10}O_3N_2$	16.4	Negatively charged
Aspartic acid	Asp	133	C4H6O4N	10.2	Negatively charged
Leucine	Leu	131	$C_6H_{13}O_2N$	7.2	Hydrophobic
Arginine	Arg	174	$C_6H_{15}O_2N_4$	6.7	Positively charged
Lysine	Lys	146	$C_6H_{15}O_2N_2$	5.5	Positively charged
Proline	Pro	115	$C_5H_8O_2N$	4.5	Hydrophobic
Phenylalanine	Phe	165	$C_9H_{12}O_2N$	4.6	Hydrophobic
Serine	Ser	105	$C_4H_7O_3N$	4.6	Polar without charge
Valine	Val	117	$C_5H_{11}O_2N$	4.4	Hydrophobic
Isoleucine	Ile	131	$C_6H_{13}O_2N$	4.3	Hydrophobic
Alanine	Ala	89	$C_3H_7O_2N$	3.8	Hydrophobic
Glycine	Gly	75	$C_2H_5O_2N$	3.7	Polar without charge
Threonine	Thr	119	$C_4H_9O_3N$	3.3	Polar without charge
Tyrosine	Tyr	181	$C_9H_{13}O_3N$	3.3	Polar without charge
Histidine	His	155	$C_6H_{10}O_2N_3$	2.3	Positively charged
Cysteine	Cys	121	$C_3H_7O_2NS$	1.1	Polar without charge
Methionine	Met	149	$C_5H_{11}O_2NS$	1.2	Hydrophobic
Tryptophan	Trp	204	$C_{11}H_{12}O_2N_2$	1.1	Hydrophobic

***Based on the polarity of the side chain, amino acids are categorized as nonpolar or hydrophobic, neutral (uncharged) but polar, acidic (negative charge at pH 7), and basic (positive at ph7) [92]**

The UV/Visible spectrum is divided into three regions, far-UV below 210 nm, UV region between 200-400 nm, and visible region 400-750 nm. At each wavelength, one chemical group dominated the absorbance; this chemical group is called chromophore. In proteins, chromophors absorb light at wavelength less than 300 nm, while water the most common solvent, absorb very strongly below 170 nm restricting useful study to wavelengths above this value [93]. Absorption spectra of protein extends from 175 nm up to 350 nm divided into region each corresponding to different chromophores [89].

In proteins, three different groups of chromophore respond to electronic excitation within UV range, first peptide bond that absorb in the far UV region between 210-220 nm with 190 nm being a characteristic wavelength [88, 93]. Second is the presence of disulfide bonds that absorb in the 250-300 nm region [89]. Third, amino acids side chain groups which absorb at wavelength longer than 200 nm with moderate to high intensity [94], their absorbance is affected by changes in medium acidity and polarity. Out of the third group, aromatic amino acids side chains of tryptophan, tyrosine, phenylalanine absorb in the 230-300 nm region which is above that corresponding to peptide bond [93]. Other chromophores that absorb in the UV region are the amino, carboxyl and amide groups, but these groups are transparent and do not absorb significantly, and the same is true to aliphatic side chains [94].

Evaluation and estimation of degree of unfolding/folding of globular proteins is accomplished by measuring the spectral changing of the three aromatic amino acids phenylalanine tyrosine and tryptophan, such procedure is reported frequently in literature, table (6) [88, 89, 91, 95-98]. Tryptophan is the major chromophore in the UV region with λ_{max} at 280 nm [90, 91, 93], compared to tryptophan; phenylalanine and tyrosine molar absorptivity is only about one sixth the of that of tryptophan [89]. Though tryptophan content in soy protein is low, any protein with at least one tryptophan residue can be detected by UV spec at concentration of 0.1 mg/ml or more. [89]

Aromatic amino acid side chains absorb at long wavelength that is defined by different limits by different reference sources [89-91, 93], this range therefore

can extent from 230 nm as defined by Pattabhi et al. 2002 [93], to as high as 320 as defined by Bang 2005 [89] with radiation maximal at 280 nm where the molar absorbance of proteins can be determined by the number of aromatic residues [88]. Variations of solvent, overall medium conditions, and the presence of other molecules in solution that absorb in the same region; are possible reasons for the slight discrepancies in reported absorbance wavelength. The useful near UV region is represented in figure (10).

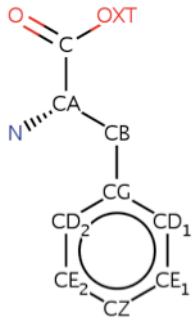
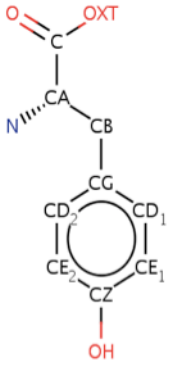
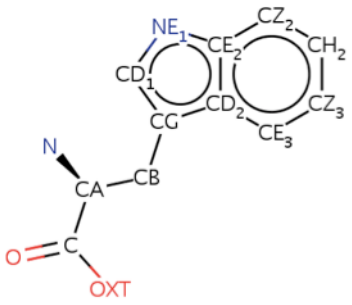
Amino Acid Molecular Structure	pK_1 α-COOH	pK_2 α-NH_3	pK R group	Isoelectric pH
Phe[99] 	2.16	9.18	-	5.67
Tyr[100] 	2.20	9.11	10.13	5.66
Trp[101] 	2.43	9.44	-	5.94

Table 6: Molecular structure, macroscopic dissociation constant (pK_1, pK_2, pK of R group) in water at 25°C and isoelectric pH of phenylalanine tyrosine and tryptophan. [102]

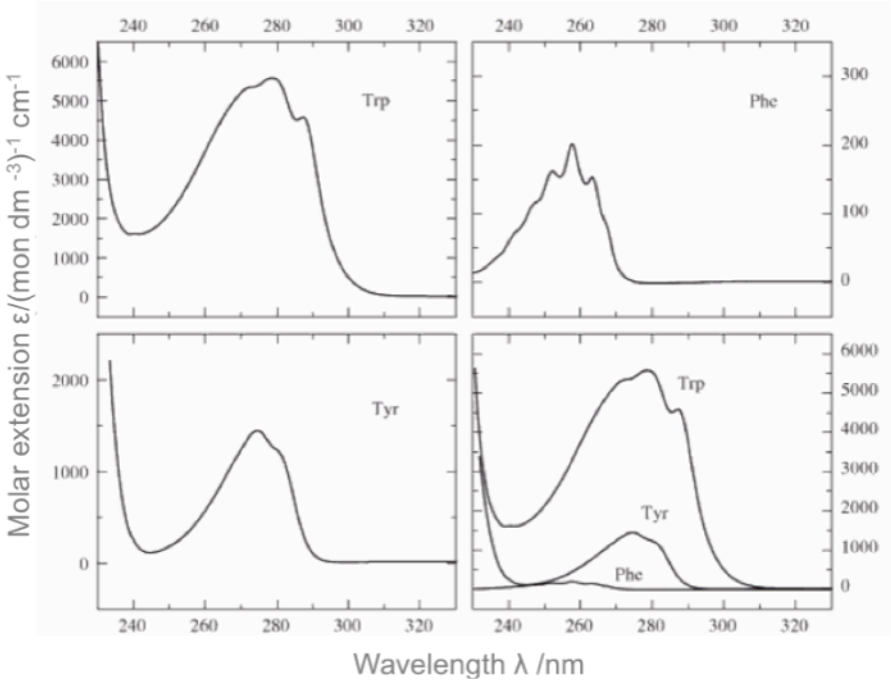


Figure 10: Near UV absorbance spectra for aromatic amino acid side chains. In a proteins containing all three amino acids, Tryptophan and Tyrosine residues dominate the spectral contribution, bottom right diagram. [103]

Shift and change in intensity of absorbance is an indication of the level of energy required to excite valence electrons to higher energy levels from ground state. Simple electrostatic understanding of the system of chromophore and solvent (polarity or dielectric constant) can explain the shift. For example the presence of electrons in a polar environment (water for example) results in lower energy levels required to excite these electrons to a higher energy transition state. Therefore the difference in the energy required to cause excitation of valance electrons translated into a shift in spectrum and change in intensity. These changes can be used to follow conformational changes or binding processes in bio-macromolecules. [103]

Spectroscopy functions by excitation of molecules, transition of electrons between orbits is responsible for the registered spectrum, and each spectrum characterizes the quantum mechanical state of the molecules or atoms. Common transitions observed (1) between ground state orbital π and excited orbital π^* and (2) between nonbonding orbital n and the excited orbital π^*. When a light beam with initial intensity I_0 pass throught a cell containing a sample, the emergent radiation intensity I decreases due to absorption by a portion of the sample, specifically the chromophores. Absorption differs at each wavelength which is characteristic to the sample. This characteristic quantity is called the absorbance, calculated form Beer-Lambert principle. [93]

According to Beer-Lambert principle, the fraction of the light absorbed is proportional to the number of absorbing molecules [93]:

$$-\mathbf{dl}/\mathbf{I} = \mathrm{C}\boldsymbol{\varepsilon}'\mathbf{dI} \qquad \text{Eq. 3}$$

Where $d\mathrm{l}$ is a thin slice of sample, C is the concentration of molecules in mol/L. The negative sign indicates a diminution of the intensity of the radiation as it travels the sample. ε' is proportionality constant called molar extinction coefficient and is constant for a wavelength for a given molecules and independent of concentration. Previous equation was for a thin layer of sample, for the entire sample thickness I: [93]

$$\mathbf{A} = \mathbf{log}\left(\mathbf{I_0}/\mathbf{I}\right) = \mathbf{C}\boldsymbol{\varepsilon}(\boldsymbol{\lambda})\mathbf{l} \qquad \text{Eq. 4}$$

$\varepsilon = \varepsilon'/2.303$ is the molar extinction coefficient converted to log base 10, expressed as function of the wave length λ. $log\left(\mathrm{I}_0/\mathrm{I}\right)$ is the absorbance A. λ_{max} at maximal extinction and the extinction ε_{max} at λ_{max} are both characteristic values

of each chromophore. For biopolymers the extinction coefficient is difficult to measure, therefore average extinction per residue is calculated [93]. λ_{max} depends *primarily* on “available electronic molecular energy levels for a particular chromophore” and ε is determined by the absorption cross section σ. Although both λ_{max} and ε are intrinsic quantum mechanical properties of molecular orbits, they can be affected by surrounding environment. [103]

A possible source of errors and inaccuracy in measurements is the presence of molecules that absorb at the same wavelength as targeted chromophore. In the UV range of 210-240 nm sulfur-containing a.a. such as cysteine, cystine, methionine, and the histidine imidazole chain adsorb strongly, so do aromatic residues [88]. However, all the previous are not of a concern to our measurements that target aromatic a.a. that absorb in the range 230-320 nm. Some problems associated with the effect of solvent and path length etc. can be easily eliminated by using the difference in adsorption between cell containing sample and other containing a the solvent (lacking targeted chromophore) is measured. [93]

The goal of these spectrophotometric measurements is to verify conformational changes of protein structure as function of temperature and medium pH, via changes in the spectral position and intensity. An important concern is aggregate formation induced by conformational changes of protein structure, aggregates can result in errors in UV spectroscopy measurements due to light scattering. [89]

Measurements were carried using Ocean Optics USB4000-XR1-ES (Ocean Optics, Inc. Dunedin, FL, USA) features a 3648-element Toshiba linear CCD (Charge Coupled Device) array detector for increased signal-to-noise performance. The device covers the range of 194-1061nm. Light energy transmitted through single-strand optical fiber is dispersed via fixed grating across the linear CCD array detector. The model is market as Enhanced Sensitivity (ES) as a result of installed detector collection lens that increases light collection efficiency.

Spectroscopic measurements require the use of clear solutions that allow light to pass through the sample, therefore an appropriate concentration of SPI solution needs to be selected for the measurements. 0.05% w/w SPI suspension was selected after testing several concentrations starting with the maximum SPI suspension concentration of 9% w/w. Since selected concentration is very low, there was no need to centrifuge the sample and remove solid particles as suggested by Gauglitz et al. (2003) [104].

Sugar content

Change in sugar content of mango juice solution is quantifies using by Abbe refractometer 10450 Series, (American Optical Corporation, Buffalo, NY, USA). Sugar concentration is determined by measuring the light refracted in sample solution. Critical angel is adjusted using a light indicator through an eyepiece. Brix value is the total sugar concentration in % (w/w) where 1° Brix

value is equivalent to 1 gram of sugar per 100 grams of solution. All measurements are conducted at room temperature.

Visible spectroscopy of carotenoids

Pigment present in solution subtract part of the visible radiation received and allow the measurement of the remainder (transmitted or reflected). The absorbance of a sample is proportional to the number of absorbing molecules in the path of the spectrometer light beam. In carotenoids, the structural feature responsible for the absorption of visible light, are the conjugated double bonds (c.d.b.) Carotenoids absorb violet and bluish light at range 400-500 nm of the spectrum [14, 105], thus they appear either yellowish, orange or reddish. When carotenoids form complexes with other molecules (proteins in marine invertebrates animals for example) they can project a wide range of colors such as blue, purple and green [14]. Not all carotenoids are colored or contribute to the color of food, thus not all absorb in the visible wavelength; for a carotenoid to be colored it must contain at least 7 c.d.b., thus phytoene for example with 3 c.d.b. is colorless and report maximum absorbance in the UV region [105].

Beer-Lambert law is used to quantify compounds in solution using information collected by spectroscopic measurements. Concentration of carotenoids can be quantified using Beer's law of analytical absorption spectroscopy $A = \varepsilon bC$. Determination of absorption coefficient (ε) is accomplished experimentally by dissolving 1-2 mg (0.001mg accuracy) of pure pigment in appropriate solvent [14]. In addition the absorption coefficient for cis-isomers is

lower than all-trans counterparts, adding to the level of inaccuracy. Luckily absorption coefficient tables exist for carotenoids at defined wavelength at various solvents. But the values in these tables differ between sources for the reasons mentioned above. If no experimental value is available an arbitrary value of 2500 is used, this often cause an underestimation. We will be using the arbitrary value of 2500 $M^{-1}cm^{-1}$ due to the lack of reporting of ε value of carotenoids in water.

Extraction of carotenoids is *often* considered necessary for identification and quantification of carotenoid from natural sources (complex system) prior to further testing such as spectrophotometric measurements. Extraction of these compounds from their complex systems is accomplished using any of several analytical methods including High-Performance Liquid Chromatography (HPLC) or Reverse Phase Liquid Chromatography RPLC [59], Open-Column Chromatography (OCC) [17]. These extraction procedures can cause some destruction to carotenoids and even increase sensitivity toward light and pH changes. Although extraction is required for accurate quantification of carotenoids content, measurements conducted on a mixture of carotenoids (food, pharmaceuticals, food colorants…etc.) can produce a reasonably accurate estimation, however it is never as accurate as in using individual isolated carotenoids that are often used to construct calibration curves. [105]

We will attempt to *estimate* the concentration of a number of carotenoids in a juice solution without prior extraction using spectroscopic absorption. Spectrophotometry is a time saving procedure with minimum to no sample

preparation, and is relatively less damaging to sample [14]. Since such practice - direct absorbance- is uncommon, several precautions were taken to ensure maximum accuracy. First sample was clarified enough to ensure not to over saturate the signal by the presence of other components such as pulp. Secondly simulated juice was used as a reference thereby eliminating the effect of the presence of sugars and acids. Consequently the measurements are not as precise in quantification of carotenoids as those coupled with isolation and extraction, but do meet our goal in detecting the reduction of carotenoid content in solution. Quantification of adsorbed amounts of specific carotenoids will be reported as a percentage of original sample rather than conventional µg/L concentration.

It is very unlikely that individual carotenoid contents can be *accurately measured* in a complex system of several carotenoids using the coefficient of individual isolated carotenoids, therefore it is advised in scientific literature to *estimate* total carotenoids content in a complex system (several carotenoids) [105]. For single wavelength quantification of carotenoids, 450 nm is frequently used as the results can be expressed as total β-carotene equivalents. This approach is reasonably accurate and convenient as estimation due to the use of adsorption at 450 nm *only*, and the arbitrary values of (ε) 2500 $M^{-1}cm^{-1}$. Though this method is widely used for *total* carotenoids estimation and construction of calibration curves, it frequently underestimates other carotenoids content due to the wide range of absorption wavelength of these compounds, specially when other specific carotenoids are the predominant ones such as lycopene in tomato

(ε = 3450 $M^{-1}cm^{-1}$ is preferred) [105]. The use of spectrophotometric measurements for quantification of *individual carotenoids* in a complex system can only *reasonably estimate* the concentration of analyte due to the slight inaccuracy of the ε due to interfering compounds (those that adsorb at the same spectral region as our analyte, those that influence absorbance and those that react with reagents specific for the analyte) [106]. Estimations were based on calculating *C* using equation (5) where ε is 2500 $M^{-1}cm^{-1}$. However due to estimation of content, values are reported as percentage of original sample rather than concentration.

Carotenoids composition of mango varies tremendously depending on variety, ripening stage, growth, harvest and post harvest conditions amongst a long list of possible causes that extend to the sensitivity of the analytical method. For this reason we have chosen to monitor the change in the content of the carotenoids most commonly found in mango and those used by United States Department of Agriculture (USDA) in evaluation of provitamin A potential of food items. β-carotene, specifically all-trans-β-carotene, is frequently present in high quantities in all mango varieties, therefore the most common carotene [62, 107]. The most abundant xanthophyll in mango is violaxanthin and its isomers [107]. Some carotenoids such as β-crypoxanthin are primarily found in tropical fruits as mango and papaya [108]. The USDA National Nutrient Database of carotenoids of 1998 [109] reports five carotenoids: α-carotene, β-carotene, β-cryptoxanthin, lycopene, and (lutein and zeaxanthin) in 215 food items. These data are later added to the USDA National Nutrient Database for Standard Reference [110]. In

100 g of raw mango (edible portion only) vitamin A is quantified as 54 µg (1082 IU), β-carotene 640 µg, α-carotene 9 µg, β-cryptoxanthin 10 µg., lycopene 3 µg, Lutein + zeaxanthin 23 µg. Therefore *estimation* of individual carotenoids content will include the following:

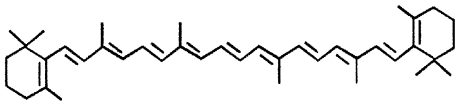
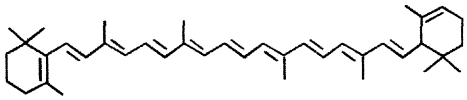
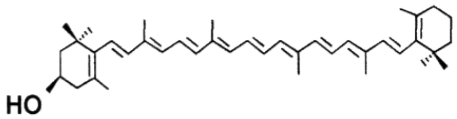
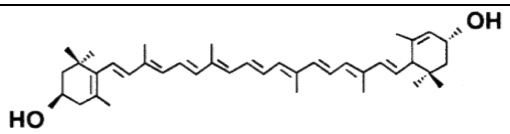
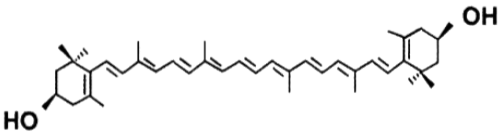
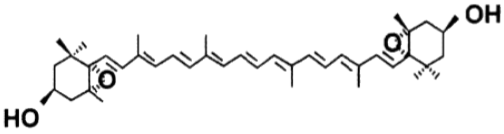
Carotenoids	Chemical structure[111]	Classification	Vitamin A activity	λ_{max}
β-carotene		carotene	yes	479 [112]
α-carotene		carotene	yes	474 [112]
β-cryptoxanthin		xanthophyll	yes	478[105, 113, 114]
Lutein		xanthophyll	no	475 [112]
Zeaxanthin		xanthophyll	no	476 [113]
Violaxanthin		xanthophyll	yes	440 [114, 115]

Table 7: Six major carotenoid in mango to be evaluated, λ_{max} was selected by cross referencing solution spectra in the 400-500 nm range with values reported values in literature while keeping in mind that carotenes absorb at longer wavelength than xanophylls [116].

Mango concentrate (28 °Bx) was allowed to thaw gradually in the fridge over night, as recommended by the providing company, the sample was first mixed using a blender (Vita-Mix Total Nutrition Center, Whole Food Machine, OH, USA) to produce a uniform sample. Concentrate was then used in the

preparation of a single strength mango juice, according to FDA regulations of specific food labeling requirements (21 CFR 101.30) single strength mango juice has a minimum 13°Bx using dilution factor of 1.3 according to sample provider instructions. Juice was then ultrasonicated (Fisher Scientific 550 Sonic Dismembrator, Pittsburgh, PA, USA) at a 30 s on/off pulse interval for 3 min at power setting number 3 to achieve proper mixing; sample was placed in a ice bath during sonication to prevent over heating. To clarify sample of pulp and reduce turbidity, mango juice was centrifuged at 14,000 rpm for 60 min using Avanti® J-E centrifuge (Beckman Coulter, Fullerton, CA, USA). No further dilution was required since clarified sample produced distinctive spectra in the 400-500 nm range. The clarification process caused a decrease in the Brix value, mango juice solution recorded 12.8°Bx and pH 3.89±0.01.

To confirm the safety of the thermal process on the concentration of carotenoids, samples of mango juice solution were treated thermally to 40-50-60-70-80-90-95°C and carotenoids concentration was compared to that of original solution at RT. After confirming our hypothesis of the safety of the thermal process on the nutritive quality of the sample (refer to chapter 4 for details of the results), adsorption process was carried on. Figure (11) is the flow diagram of the experimental design. First, mango juice solution and SPI were combined at a 0.5-2-5-10-15-20-25-30-40-50-100 g/L concentration with the aid of a touch mini vortexer (VWR Fixed Speed Vortex Mixer, VWR International, Inc., West Chester, PA, USA). The mixture was subjected to thermal treatment of heating to 95°C and holding at this temperature for 30 min, mixture was then cooled rapidly

in a ice bath and centrifuged for 30 min at 14,000 rpm (Avanti® J-E centrifuge, Beckman Coulter, Fullerton, CA, USA). Supernatant was collected as subjected to absorption measurements where simulated juice (12.8°Bx and pH 3.89±0.01) served as a reference. Measurements were carried using Ocean Optics USB4000-XR1-ES (Ocean Optics, Inc. Dunedin, FL, USA) features a 3648-element Toshiba linear CCD (Charge Coupled Device) array detector for increased signal-to-noise performance.

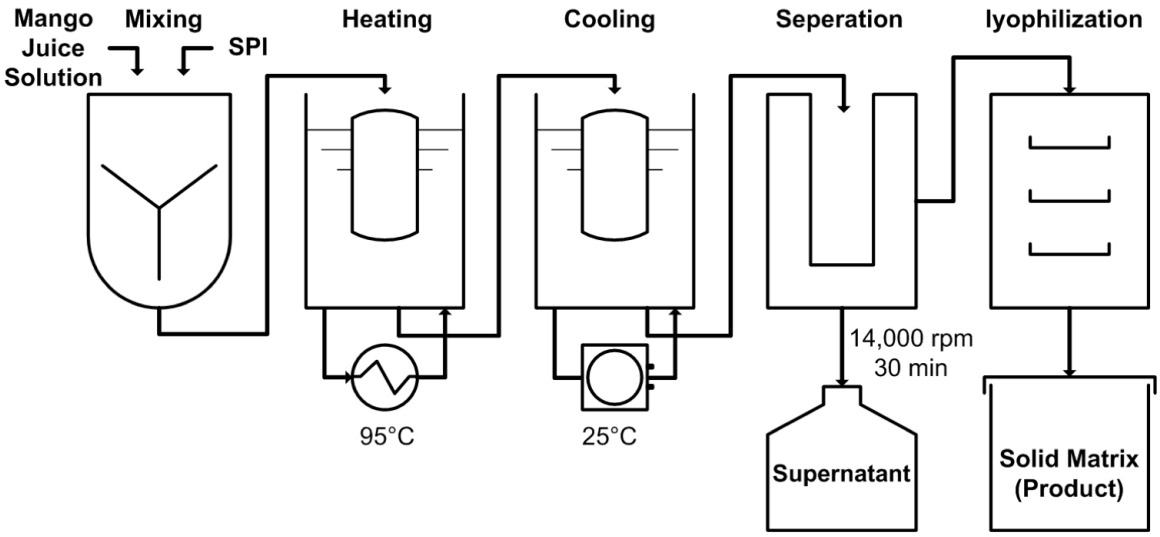


Figure 11: Flow diagram of the experimental design during carotenoids adsorption process.

Good laboratory practices were maintained through the experimentation time. All carotenoid-containing material/solutions were handled under dimmed light and stored in opaque containers. Statistical analysis of data was carried to evaluate the significance of change in the major 6 carotenoids selects as a response to treatments. Single factor Analysis of variance (ANOVA) and T-test were utilized for this purpose. Analysis ToolPack of Microsoft® Excel 2007 (Microsoft Corporation, Redwood, WA, USA) was used for statistical analysis throughout.

Lyophilization of SPI

A 9% w/w SPI suspension was prepared by combining SPI with acidified water. Suspension was heat processed in similar manner used in the adsorption study. Later the cooled mixture centrifuged at 10,000 rpm for 30 min at RT using Labnet Herrmle Z400K refrigerated centrifuge unit (Labnet, Woodbride, NJ, USA). The centrifuged mixtures separated into slurry of SPI solids, slurry was spread forming a layer approximately 1 cm in thickness. Samples were placed into a deep freezer for 1 h then into a freeze dryer for 14 h.

Lyophilized sample were manually reduced into powder and resuspended in distilled water at 1.25 %w/w concentration for DLC measurements of particle size and zeta potential measurements. Images of the surface of lyophilized samples are taken using Nikon SMZ-10A Stereo Microscope (Nikon Instruments Inc., Melville, NY, USA) fitted with Canon Rebel XS digital camera (Canon, Melville, NY, USA).

Dynamic light scattering

Geometrical structure and state of motion of particles can be determined by measuring the light scattered by these particles, flocculation of particles cause an increase of scattering. Particles in a suspension move following the theory of Brownian motion in both laminar and turbulent flow. [117]

The motion of particles in a suspension is described as *diffusion* through the medium, as a result of the Brownian motion. The diffusion coefficient (D) is inversely proportional to particle size based on Stokes-Einstein equation. [118]

$$\mathbf{D} = \frac{\mathbf{k_B T}}{\mathbf{3\pi\eta_0 d}} \qquad \text{Eq. 5}$$

Where D is the diffusion coefficient, k_B is Boltzmann's constant, and T is absolute temperature, η_0 is viscosity, and d is the hydrodynamic diameter. From this equation it can be inferred that for a large particle, the value of D is small indicating a slower movement than that for smaller particles with larger D value. Since the speed at which particles move -diffuse- is determined by their size, particle diffusion coefficient can be used to determine its size. [118] Light scattering technique operates by imposing a laser light on a samples, the light source is scattered in all directions as it comes into contact with particles, the scattered beam is measured by a detector that is placed at an angle [119]. Scattered light is then monitored by a collection of scattering elements within a scattering volume that is defined by the scattering angel and detection apparatus. The intensity of the scattered light is a results of the interface of light scattered by each element, therefore it depends on the relative positions of the elements [118]. Diagram of the optical path of the Delsa™ Nano C Particle Analyzer (Beckman Coulter Inc., CA, USA) is illustrated in figure (12).

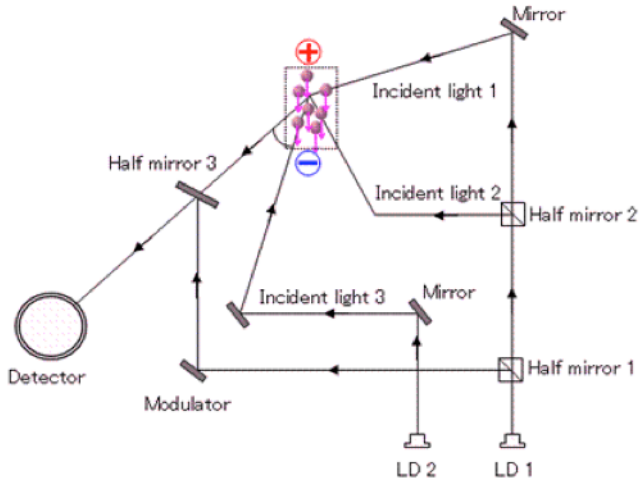


Figure 12: Diagram of the optical path of the Delsa™Nano C. [118]

The fluctuations in time of scattered light intensity from particles in Brownian motion are attributed to the change in the relative position of particles with time. Those changes are random just as the Brownian motion. Referring back to Stokes-Einstein equation, a smaller particle that moves faster will result in rapid fluctuations while the opposite is true for a larger, slower moving particle. The fluctuations are analyzed using the autocorrelation function. [118]

Modern fully automated particle analyzer that operates on the principle of photon correlation spectroscopy, are able to accommodate the measurement of particle size of both small (rapidly diffusing) and large (slowly diffusing) particles. The scattered light intensity is collected as a series of photon pulses either per sampling time using the Time Domain (TD) method, or sampling time between two photon pulses using the Time Interval (TI) method depending on particle s. TD method is used for large particle (slowly decaying correlation coefficient due to slow movement) with strong scattering levels that require large sampling time. On the contrary is the TI method that is used for small (fast diffusing) that require shorter sampling time. In TI method the time between photon pulses is also accounted for in data for more effective determinations of the autocorrelation function [118]. Particle size measurement using Dynamic Light Scattering (DLS) is used in studies of emulsion stability and phase transition amongst a number of other applications in the fields of biology, chemistry and physics [120].

Change of particle size was used as an indication of denaturation, aggregation, and refolding of proteins suspended in solution as function of temperature and acidity. A fresh suspension of SPI was prepared prior to each

measurement, described in soy protein isolate suspension section of this chapter. Suspension concentration was adjusted according to the signal intensity monitor of Delsa^{TM} Nano C, hence a 1.25% w/w concentrated suspension was used for measurement accuracy. Sample was loaded in a flow cell with 1 mm wide optical surface. Humidity cover was used to seal both ends of the cell to prevent evaporation loss during testing.

DLS method is used to determine zeta potential of charged particles such as proteins. Zeta potential is used to describe the electrokinetic potential in colloidal systems [121]. Delsa^{TM}Nano C uses forward scattering through Transparent Electrode (FST) method to obtain zeta potential values. When applying an electrical field to charged particles, the motion vector is the sum of the random Brownian motion of particles in solution, and charge-dependent motion in the direction of the opposite charged electrode [122]. Hence it is a measure of the magnitude of the repulsion or attraction between particles. Charge is a result of contact of particle with liquid forming a charge at surface, in addition of the permanent charges on ionizable groups [123]. The measure of potential of the charge at the interface and not at particle surface, thus absolute value of zeta potential is slightly lower than absolute value at particle surface [122]. Zeta potential charge and magnitude is used to predict and control stability of colloidal suspension [123]. Similar charges between particles indicate repulsive forces, thus maintain dispersion in solvent. Most particle in polar solvents such as water have negative potential [124].

Fully automated Beckman Coulter Delsa^{TM} Nano C Particle Analyzer and Beckman Coulter Delsa^{TM} Nano version 2.31 / 2.03 software (Beckman Coulter Inc., Brea, CA, USA) were used to measure particle size and zeta potential, the instrument is able to measure particle size in suspension in a range from 0.6 nm to 7 mm and molecular weight 10^3 – 3×10^7 Daltons. Measurements can be performed in the temperature range of 15°C ± 0.3°C below ambient to 90°C ± 0.3°C. It operates on both photon correlation spectroscopy and electrophoretic light scattering principles using two laser diodes. [118]

Chapter 3: Soy protein denaturation and aggregation

Introduction to protein denaturation

Functional properties of individual food ingredients are receiving great attention due to the increase demand for fabricated food such as meat alternatives for vegetarian diets. Functionality of food ingredients is defined as “any property aside from nutritional attributes that influence an ingredient’s usefulness in foods” [125]. The wide range of functional properties (textural, sensory, and nutritional) of proteins places it in the center of attention for a possible research interest and manufacturing alternative.

Functional properties of proteins are related to their physical, chemical, and conformational properties and attributes, which reflect the microstructural details of the molecule’s size, shape, charge distribution, a.a. composition and sequence [125]. All in which affects the molecular geometry of protein, hence the way proteins are folded is extremely important [126, 127]. Some of the most important conformational properties of a protein molecule that can alter its functionality are the hydrophobic/hydrophilic ratio, secondary structure content and distribution, tertiary and quaternary arrangements of polypeptides, inter- and intrasubunit cross-links, and the rigidity and flexibility of protein in response to external conditions. These properties affect the physical behavior of proteins during processing, therefore changing the textural and sensory attributes of protein-containing food item. [125]

Stability of the conformational state of protein molecules is a major determinant of its functional properties since its inability to assume certain

desirable conformation will affect or even demolish its functional qualities. The most prominent example would be alteration of protein molecule conformation via denaturation with appropriate denaturant. Heat is frequently employed as a mean of denaturation of proteins in food-processing operations. Damodaran et al. (1997) [125] have summed the importance of protein denaturation in relation to functionality by stating that "denaturation of food proteins is a prerequisite in the exhibition of any functional properties".

Since the exact conformational state of protein is reflected on the functional property, denaturation of food proteins has to be a precise and controlled process. A well established and comprehensive understanding of the link of state of proteins denaturation and its corresponding functional property is key in food ingredient manipulation and utilization. [125]

Denaturation of food proteins is defined as "a process or a sequence of process in which the spatial arrangement of polypeptide chains within the molecules is changes from the typical of the native protein to a more disordered arrangement that causes any modification in conformation (secondary, tertiary, or quaternary) not accompanied by the rupture of peptide bonds involved in primary structure"[125]. This general definition though highly accurate, is too general. Defining protein denaturation requires limiting the levels of conformational changes, this is specially important in when denaturation is used to introduce a functional property to a protein, since some minute conformational changes can occur *without* introducing any functional properties. [125]

Transition from native to denatured state

Denaturation results in destruction of the native state, denatured proteins exist in several nonspecific structures according to the type (partial or complete) and extent of denaturation. Various levels of denaturation can be distinguished according to the structural level that was distrusted (secondary, tertiary, or quaternary). Transition from the native (N) to denatured (D) state is temperature dependent, hence degree of order of the protein molecule decreases as the temperature increases [125],

$$\mathbf{N} \xrightarrow[\mathbf{K}]{\mathbf{Heat}} \mathbf{D}$$

Where K is the equilibrium constant: K=D/N. The thermodynamic parameters involved in in protein denaturation are [125],

$$\Delta \mathbf{G}^\circ = -\mathbf{RT}\ln\mathbf{K} \qquad \text{Eq. 6}$$

$$\mathbf{\Delta H}^\circ = \mathbf{R}\,\frac{\ln \mathbf{K}}{\mathbf{1/T}} \qquad \text{Eq. 7}$$

$$\Delta \boldsymbol{S}^\circ = \frac{\mathbf{\Delta H}^\circ - \mathbf{\Delta G}^\circ}{\mathbf{T}} \qquad \text{Eq. 8}$$

$$\Delta \mathbf{Cp}^\circ = \mathbf{T}(\Delta \mathbf{S}^\circ / \Delta \mathbf{T})_{\mathbf{P}} \qquad \text{Eq. 9}$$

ΔG° is the change in standard free energy, the difference between the free energy of the native system containing all reactants at equimolar concentration and the free energy of the system at equilibrium after denaturation. ΔG° is the fundamental measure of the stability of protein, stable proteins are those who achieve a minimal free energy. R is the gas constant, T absolute temperature. ΔH° is the change in the enthalpy at constant pressure, ΔS° is the change in entropy (degree of order), ΔCp° is the change in heat capacity at constant pressure. [125]

Stability in term of thermal denaturation is associated with changes in $\Delta H°$ and $\Delta S°$, both temperature dependent [125]:

$$\Delta \mathbf{H_T} = \Delta \mathbf{H}_\circ + \Delta \mathbf{C_P}\,(\mathbf{T} - \mathbf{T}_\circ) \qquad \text{Eq. 10}$$

$$\Delta \mathbf{S_T} = \Delta \mathbf{S}_\circ + \Delta \mathbf{C_P}\,\ln(\mathbf{T} - \mathbf{T}_\circ) \qquad \text{Eq. 11}$$

ΔH_T ΔS_T are at temperature T, while ΔH and ΔS are at reference temperature $T_\circ$. ΔC_P, ΔH_T, and ΔS_T are strongly dependent temperature dependent, a temperature change of 1℃ cause changes in ΔH_T and in ΔS_T of about 1-2 kcal mol^{-1}. ΔH and ΔS have large compensating values at temperature which proteins denature, however compensation mechanism is not well understood. [125]

When protein structure changes from N → D, buried aliphatic or aromatic non-polar groups are transferred to an aqueous environment causing a disruption of water structure. Heat absorbed by the water of hydrophobic hydration is expressed as:

$$\Delta \mathbf{C_P}(\Delta \mathbf{H}/\boldsymbol{\delta}\mathbf{T}) \qquad \text{Eq. 12}$$

This value is about 1-2 kcal mol^{-1}k^{-1}. ΔC_P is the difference in heat capacity between N and D states, ΔC_P it is more heat stable than the other thermodynamic parameters, it is constant or nearly constant in the temperature range of 0-80℃. While ΔH increase consequent to denaturation indicating a lower energy state than native conformation, hence enthalpy decreases with increasing temperature in the denaturation range. [125]

Entropy δS of protein denaturation is also temperature dependent. As the transition between N and D states progresses, entropy increases indicating disorder associated with unfolding. Entropy of denatured protein is highly

temperature dependent subsequent to temperature dependency of the salvation effect. At high temperature water molecules are present in a random orientation around non-polar residues causing disorganized solvation interaction, while at low temperature water is oriented into structures of solvation around non-polar group . [125]

Kinetic considerations of protein denaturation

At any given instant in time, each protein that is undergoing unfolding would have a unique conformation that is constantly changing by rapid rotation around single bonds. The kinetic pathways of unfolding and refolding are determined by which and how many intermediate conformational state the molecules passes through [125]. The possible number of conformations for a polypeptide chain is incredibly large, this is due to the flexibility of the polypeptide backbone around the N-C_α (Φ) and Cα-C (Ψ) angels introducing a large number of possible H-bond between peptide groups [103]. For unfolded protein to explore all these possibilities in its search for a low energy conformation would require infinite length of time. However native proteins fold rapidly and into a specific *functional structure* with very few errors using the information stored in the a.a. sequence with very little help from other control systems. Therefore the refolding process must satisfies the kinetic (rate) and thermodynamic (minimum free energy) requirements of the system [128].

Proteins fold in compliance with the thermodynamic hypothesis that states that “the 3D structure of native protein in its normal favorable physiological

environment (solvent, pH, ionic strength, temp…etc.) is the one in which the Gibbs free energy of the whole system is lowest" meaning a more stable conformation, thus favorable from a thermodynamic point of view [129]. Several forces have been recognized as stabilizing of the native folded structure, the entropy gain due to release of ordered water from nonpolar and polar residues, and by van der Waals interactions. Surface ion pairs are only involved in stabilization, while intramolecular hydrogen bonds contribute significantly to protein stability in addition to conferring structural specificity. [130]

Fast folding of proteins implies it is not a random search, specific mechanism, control systems and defined laws have been identified [131]. By utilizing the physical, biological, chemical, and physiochemical understanding of a.a., polypeptide chains and proteins scientists were able to use computational statistics to - at least partially - solve this complex issue. Several models have been developed based on the physiochemical understanding of protein residue interaction and the forces governing the mechanisms of protein folding; such as the lattice model.

Accordingly, the previously mentioned denaturation reaction ($\mathrm{N} \xrightarrow[\mathrm{K}]{\mathrm{Heat}} \mathrm{D}$) can be more accurately written as $\mathrm{N} \leftrightarrows \mathrm{I}_1 \rightleftarrows \mathrm{I}_2 \leftrightarrows \cdots \leftrightarrows \mathrm{I}_{n-1} \rightleftarrows \mathrm{I}_n \rightleftarrows \mathrm{D}$, where I_x represents molecules with intermediate structure [125]. And the kinetic parameter for unfolding can be written as:

$$\frac{-\mathbf{dA}}{\mathbf{dt}} = \frac{\mathbf{dB}}{\mathbf{dt}} = \mathbf{kA} \qquad \text{Eq. 13}$$

Where k is the reaction rate constant s^{-1}, t is time of reaction, A and B are the concentrations of native state and the intermediate or final form, respectively.

The equation can be rewritten as: [125]

$$\frac{\ln A}{A_{\circ}} = -\mathbf{kt} \qquad \text{Eq. 14}$$

Where $A_{\circ}$ is the initial concentration. Rate constant can be calculated from the slope of a logarithmic plot of the time-dependence change in A (or B) relative to $A_{\circ}$, giving it is a linear plot. A non-linear multiphase curve represents a greater number of reaction steps (and species), Figure (13). [125]

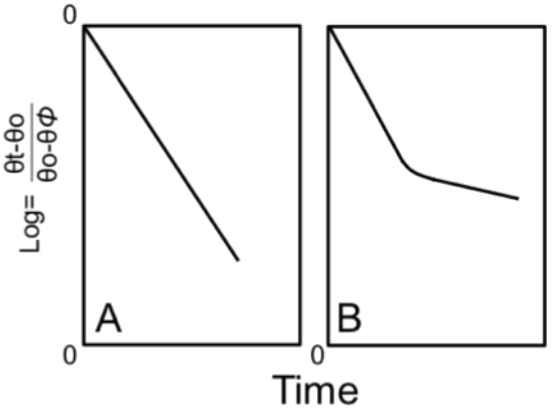


Figure 13: (A) one-step and (B) two-step reaction kinetics. θt is a parameter of the extent of the reaction, θo is initial value of the parameter and $\theta \phi$ is the final value. Apparent rate constant is calculated from the slope. [125]

Mechanism of thermal denaturation and aggregate formation

Denaturation can be confined to region of protein, or involve the whole protein in a “all-or-non” style change. Due to unfolding/disassociation, the original inward configuration of the secondary and tertiary orders is restructured exposing the hydrophobic a.a. residues buired in the core of proteins (i.e. globular proteins). These new intermediate conformation of proteins are short-lived, rapidly proteins try to minimize the exposure of ractionve groups, specially the hydrophobic groups. Thus hydrophobic protein-protein interaction occures ultemitly resulting in aggregate formation (soluble or insoluble); giving that protein

concetration, thermodynamic condiction, and conditions related the medium are favorable to the fromation of tertiary structure. Thermal coagulation differs from thermal gelation which involves the formation of three-dimensional ordered network. [125]

Extent of heat treatment required to achieve thermal coagulation of protein solution differs depending and not limited to, protein structure. Monomeric proteins are first denatured before they are able to coagulate. Oligomeric proteins -such as our protein of interest, soy protein (breakage of the disulfide bonds between the acidic and basic polypeptide units [132]- are composed of several monomeric units with a complex quaternary structure. Heat coagualtion of oligomeric proteins in an aqouse medium starts with reversible dissassociation of the quaternary strucutre (oligomers) into subunits or monomers followed by irreversible denaturation of the secondary and tertiary structure. Generally loss of these strucutures is only partial and not complete. So the mecahnism of coagulum formation from pratially denature prtoeins is: nP_N->nP_D_>[P_D]n, n is the number of protein molecules, P_N native protein, P_D is denatured protein. [125]

Protein Aggregation forms high molecular weight complxes with denautured proteins as the building blocks. Association of unfolded proteins occure via intermolecular interaction- similar to those that determines the native structure- to form aggreagtes. Proteins involved in aggegate formation are premenantly denatured and can’t exit this association to regain its native structure. Interaction of denautred proteins may result in precipitable aggreagets, soluble aggregates (coagulum) as in the case a low ceoncctration SPI

suspension, or form an ordered gel matrix in a three-steps process starting with denaturation followed by aggreagtion and finally cross-linking. [125]

Arakawa el al. (2006) [133] have suggested three mechanism of aggregation mostly related to extrinsic factors such as the presence of a contaminate (protein and non protein contaminant) that associate with the protein via ionic of hydrophobic interactions leading to aggregation. The second mechanism is based on an assumption that a protein solution contains both folded and unfolded proteins in an equilibrium, however environmental conditions (temp, shaking, physical or chemical stress) cause additional unfolding of proteins that may aggregate with preexisting unfolded molecules or aggregate nucleus forming larger/visible aggregates [133]. The third proposed mechanism attributes aggregation to high protein concentration, which facilitate the formation of self-association of the native proteins to form oligomers. These associations can be reversible or irreversible [133, 134].

Aggregation rate and final protein morphology of aggregates is also related to thermodynamics parameters of the protein, solution conditions, and protein concentration [125, 126]. The rate of aggregate formation is partially and not strictly related to the physiochemical properties of the a.a. where reactive a.a. in an unfolded chain interact with other a.a. in another unfolded chain [126].

Reversibility of thermal denaturation

Unfolding of proteins increases entropy, an unfavorable state due to increased free energy [135]. When proteins unfold, they lose their native 3D

structure, subsequently their biological functionality but denaturation is a reversible process [129]. To maintain the 3D structure of folded proteins, living cells uses chaperones, however cells do not contain enough number of chaperones to control all protein molecules in a cell. This suggests the theory that proteins in a living cell are always spontaneously unfolding and re-folding [136]. This is also true even in an in vitro setting due to the fact that the a.a. sequence is a guide for the correct way a protein should fold via physiochemical interaction [136]. Folding reduces the degree of freedom that a chain of a.a. has where the chain becomes confined into a specific form related to it a.a. sequence and other external factors [127]. It is generally assumed that a protein solution contains an equilibrium of folded and unfolded proteins ([136],[126]). Depending on environmental conditions (in a test tube for example), unfolded/denatured state last only milliseconds to hours as long as the conditions are favorable for unfolding to last [136].

Most thermal denaturation is irreversible, however several proteins have demonstrated the ability to partially or completely regain of the entire native structure when the denaturing agent is diluted or removed. In general the new structure though resembles that of the original, is still not identical. Degree and extent of renaturation depends on the extent of damage of the original structure which is defined by the level of denaturation and the severity of the denaturation treatment. [125]

Intentional carefully planned refolding of protein is a concept understood and practiced in the world of microbiology, genetics and pharmaceutical industry

by methods and techniques many of which are patented. Genetically engineered proteins are harvested form microbial cells that are used as a host; once the production is complete, extraction of the protein is possible through methods that won't only destroy the host microbial cell but also the target protein that often accumulates in a form of insoluble inclusion bodies ([137],[138]).Two common methods used to refold extracted proteins are refolding by dilution (e.g. urea, glycerol, sucrose, and salts such NaCl, Na_2SO4, K_2SO_4 solution) and column refolding (e.g. oxidative column chromatography) [137]. Other techniques have been used to force renaturation of proteins, for example human serum albumin denatured by heat can be renatured by exposure to a pressure of 2000kg/cm^2.[125]

It was logical to assume that heat denatured storage proteins that are biologically inactive, in a solution remain in the distributed state. But Damodaran (1988) [70] suggested the possibility of "partial refolding and regaining of secondary structures during the cooling regime of the gelation process"[70]. For example heat gelled collagen composed of random coiled molecules can be reversed in a spontaneously refolding action by cooling the to a temperature well below the melting temperature of collagen [125]. The refolding of proteins in a progel solution is undesirable since it decreases the number of protein molecules with exposed functional groups that are available for intermolecular interactions that will ultimately affect the gelation process (i.e. enthalpy of gelation) and gel properties [70]. Unfortunately the careful folding and unfolding of biologically active proteins that was discussed earlier does not apply to progel solution of

globular proteins. When heat is used to produce a progel solution, the structural state of the proteins changes along the heating and cooling process [70].

It is not quit clear when and how was the refolding phenomenon of soy proteins upon cooling was discovered. One of the few published researches dedicated to study this phenomenon in progels is by Damodaran (1988) [70], he showed that partial refolding of proteins might occur during cooling. When the unfolded proteins refold upon cooling, the number of possible conformations is endless, but according to the Levinthal's paradox, the protein folds in areas of the surface that will achieve a greater overall decrease in free energy aiming toward a more stable conformation or state [135].

Several factors can control the extent of refolding and level of structure that can be recaptured, these factors are similar to those that can potentially denture a native protein including solution condition such as pH, ionic strength, presence of salt, type of salt and rate of solution cooling. Damodaran (1988) [70] hypothesized that "by controlling the extent of refolding of the protein during the cooling regime, it is possible to improve the gelation of globular proteins even at suboptimum protein concentrations" [70].

Factors affecting thermal denaturation

Alteration of the 3-D structure of food proteins can be achieved by altering the external environment, traditionally by increasing temperature, but physical (e.g. increase pressure) or chemical (e.g acidification, enzymatic cross-linking, using salts and urea) treatments that achieve similar results are also used [139].

Since heat treatment is most commonly used denaturing agent of food proteins[125], in this section factors related to heat treatment conditions and heating medium composition will be discussed.

A. Heat

The most common method of protein denaturation is heating in solution which increases protein thermal motion causing a rupture of some intermolecular and intarmolecular bonds that stabilizes the native structure including the balance of electrostatic inteactions, hydrogen bonding, disulfied bonds, and hydrophobic inteactions. Heat is widely used as a denaturing agent in the food industry due to its common use as a processing method for various reasons and applications. Our understanding of the relationship between the microstructural changes and macrostructural properties of food proteins leads to the intuitive conclusion that heating temperature, rate and time will collectively affect the extent of protein denaturation due to the tangible effect it has on food qualities. [125].

Denaturation rate is temperature-dependent, protein denaturation rate increases 600 times with a 10^{o}C increase in the denaturation temperate range for the specific protein [125]. This extends from the low energy associated with each of the interactions stabilizing the secondary, tertiary and quaternary structures. Thus a higher degree of denaturation can be achieved within the same time using a higher temperature, Mulvihill and Donovan (1987) [140] reported a 10-folds reduction in the time required to achieve a fixed level of denaturation by increasing heating temperature by 7.5^{o}C.

Denaturation has frequently been reported as a gradual process that occurs in stages. Harwalker (1980) [141] detected denaturation of β-lactoglobulin at 75 °C with two distinct stages of progression of denaturation, at the first 5 min of heating time at 75 °C; denaturation appeared to occur at a higher rate than that in later stages. That led to the conclusion of progressive and frequently incomplete unfolding and loss of native structure. Many proteins do not achieve complete denaturation even if heated above denaturation temperature for relatively long time. When C-actin is heated to 30°C above its denaturation temperature at pH 8, about 60% of the native helical structure was retained. [125]

However extreme heating conditions results in rapid aggregate formation where aggregates (or precipitates, depending on protein solubility) are poorly hydrated, thus lacks a continuous matrix [125]. Excessive heating of protein dispersions at temperature far above the denaturation point (125℃ of 1-8% of soy globulins [142]) can cause the formation of a metasol state which is a progel containing partially refolded proteins, thus does not set into a gel upon cooling as expected from heat-set gels [125], figure (14).

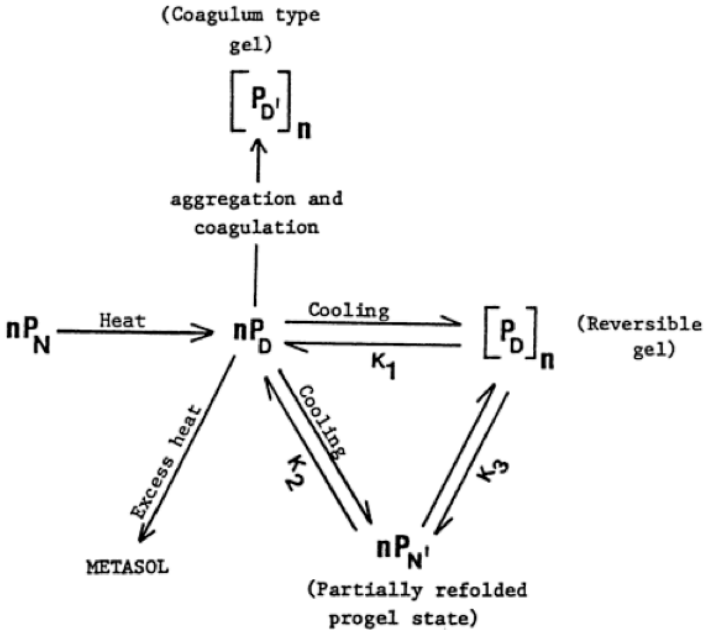


Figure 14: A proposed mechanism for heat-induced gelation of globular proteins. n is the number of protein molecules, P_N, P_D, and $[P_{D'}]_n$ are protein in native, denatures (progel), and gel states, respectively. $P_{N'}$ is protein in partially refolded state. $[P_D]_n$ is coagulum-type gel state, K_1, K_2, and K_3 are equilibrium constant. [143]

B. pH

As long as proteins are dispersed/present in a solution, environment pH has to be considered as a contributing factor in thermal stability. Not only does pH affect the denaturation temperature of proteins but it also affect the functional characteristics such as gelation, foaming and emulsification [125]. Proteins are most stable at their Isoelectric Point (pI) and least stable at either pH extremes below or above pI. In addition at pI proteins are least soluble and the opposite is true at pH values far from pI. pH adjustment is a common practice in the manufacturing of commercial proteins, subjecting proteins to extremer pH conditions for a short length of time followed by neutralization results in partial

denaturation and exposure of sulfhydryl groups, thus improving protein functionality. [125]

C- Salts

Similar to pH effect, the presence of ions in the medium can alter the protein net charge, hence affecting it thermal susceptibility. As a rule of thump, increase protein solubility renders proteins more sensitive and susceptible to thermal denaturation. Therefore, in the presence of any stabilizing force, the denaturation temperature of protein is higher. [125]

Aggregation of globular proteins is affected by ionic strength, which can be altered in the presence of certain salts. In the case of cold-set gels, a gelation-inducing salt solution is used because salts enhance protein-proteins interactions by the formation of bridges between molecules resulting in a stronger network [125]. Salts affect protein stability in a solution by affecting surface charge or in higher concentration can act as structure stabilizers (kosmotropic salts) or as de-structuring ions (chaotropic) [125]. Protein hydrophobic interactions is affected in the presence of salt ions due to the ability of these ions to alter the structure of water around proteins [125]. Subsequently the presence of salts affects the denaturation temperature of proteins. By taking a deeper look into Damodaran (1988) [70] key research where he aimed to “understand the relationship between the extent of formation of gel network and the state of protein structure in the gel”. He started by exploring the contribution of non-covalent bonding on the stability of the major protein reactions of soy protein in the form of isolate (7S and 11S) by studying its denaturation behavior in the presence of salts.

It has long been suggested that non-covalent bonds are the major stabilizing forces in globular protein gels. Starting from the previous statement, Damodaran (1988) [70] repeated the calorimetric measurements in the presence of neutral salts at various concentrations, NaCl used as an example of a promoter and NaSCN as an example of a destabilizer of hydrophobic interactions. Both the type and concentration of salt had an effect on T_D of SPI, promoter salts such as NaCl caused an increase in T_D of both 7S and 11s with increased concentration and the opposite was true for the destabilizing salt such as NaSCN. But it should be noted that the destabilizing salts caused an unexpected increase in T_D (stabilizing effect) at low concentration, this can be attributed to the neutralizing effect that salt would have, at low concentration; to the surface charge of the protein [144]. Similar solutions used in DSC measurements where then used to make heat set gels. Gels could not be formed in the presence of promotor salts (NaCl) up to protein concentration of 9.2%. This can be attributed *to some extent* to the stabilizing effect of these salts that results in an increase in T_D (T_D of 11S in the presence of 0.5M NaCl is 100 ^oC) making the progel treatment at 90 ^oC insufficient.

To test the effect of destabilizing salts on gelation and gel properties, gel melting point was used as a key characteristic to compare because it depends on the gelation enthalpy, which in turn depends on the number of non-covalent cross-links present in a gel. Inverse of the melting point ($1/T_m$) increased with decrease concentration in both the presence and absence of salts. Lower protein concentration were needed to achieve similar T_m in the presence of destabilizing

salts, meaning a similar number of cross-links can be formed in the presence of salt using fewer molecules. Damodaran (1988) [70] attributes this phenomenon to the electrostatic forces involved in gel formation, the presence of salts reduces or even neutralizes electrostatic repulsion increasing the possible number of non-covalent cross-links that can be formed. Another reason could be due to that fact that these salts are destabilizing salts, which means protein fractions might undergo a further denaturation from its native structure during progel treatment compared to the absence of these salts, hence exposing more interactions sites, increasing the possibility of forming a gel. [70]

Far-UV-Circular dichroic measurements were used to determine the conformational stage of the proteins in the *gel* structure formed with different compositions (salts). Centrifuged supernatant of the gels was used in these measurements. All samples including control (no salts are added) and progel solutions produced in the presence of promoter salts showed negative ellipticities, an indication of the presence of a “significant amount of secondary structures” [70]. This is the single most important piece of data that supports Damodaran (1988) theory of partial refolding of thermally unfolded proteins upon cooling. The spectra also shows a smaller number of β-sheet conformation is lower in the gel prepared in the presence of an destabilizing salt (i.e. NaClO) hence a grater number of functional groups are exposed and available for cross-linking which stabilizes the gel. The opposite was observed in gels prepared in the absence of salts, a larger number β-sheet structure was detected. Even though no gels were formed in the presence of promoter salts (NaCl) CD

measurements were carried on, it showed the presence of α-helix and some β-sheet structure. This is an indication of a further refolding of protein molecules explaining the inability to gel [70]. In a later research Damodaran et al. (1991) [145] showed that increasing the protein concentration increases tendency of formation of intermolecular interactions and formation of aggregates, thus limiting the refolding of proteins [145]. Protein refolding cannot be viewed as the sole reason of failed gelation of protein. Using a high concentration of a protein with high levels of a polar amino acid residues to make a gel will result in the formation of a insoluble aggregates that can form an irreversible, coagulum-type gel. Hence protein concentration is the limiting factor of gelation and not protein refolding. [70]

Other globular proteins also showed similar response to the presence of salts, Whey Protein Concentrate (WPC) denaturation temperature decreased in the presence of $CaCl_2$ or $MgCl_{2+}$ nonetheless increase in the presence of NaCl. In addition the ability of some proteins to bind to metal ions due to the presence of a binding site can help stabilize protein structure against thermal denaturation as well as regain of native structure after extensive thermal treatment (i.e. α-lactalbumin). [125]

D. Sugars

The effect of any solutes present in the medium on the structure and conformational stability of proteins can be attributed to one of two mechanisms. First direct interaction with proteins, second indirect interaction through modification of the solvent environment by influencing protein conformation thru

affecting the extent of hydrophobic interactions. And most probably it's a combination of both direct and indirect interactions. But sugars, like salts, affect protein conformational stability by indirectly affecting the hydrophobic interactions. [125]

Increase in denaturation temperature of some proteins (e.g. β-lactoglobulin) have been reported in the presence of glucose and/or sucrose, with sucrose causing the greatest increase, Table (8). At low levels, sugars increase the number of hydrophobic associations, which can be detected by decrease in surface hydrophobicity. As sugar levels increase, these hydrophobic interactions are strengthened. The extent of stabilization of sugars depends upon the type and concentration as well as the nature of proteins in question. [125]

Table 8: Effect of sugars and polyols on DSC characteristics of *β*-lactoglobulin. Reproduced by Damodaran et al. (1997)[125], adapted from Hegg el al. (1974)[146].

Polyol conc. (%)	Sucrose		Glucose		Glycerol		Ethylene Glycol	
	$\Delta T_d(°C)^a$	$\Delta H\left(J/g\right)^b$	$\Delta T_d(°C)^a$	$\Delta H\left(J/g\right)^b$	$\Delta T_d(°C)^a$	$\Delta H\left(J/g\right)^b$	$\Delta T_d(°C)^a$	$\Delta H\left(J/g\right)^b$
10	0.9	10.6	1.05	11.35	0.05	16.0	-2.1	13.5
20	1.15	12.3	1.55	7.9	1.1	16.6	-4.2	14.1
30	2.9	10.7	4.4	9.9	1.6	15.4	-7.6	10.9
40	3.9	11.8	4.0	11.5	2.8	14.7	-11.5	9.5
50	5.0	13.0	9.3	-	3.6	16.5	-14.9	11.0

[a] $\Delta T_d(°C) = T_d(\beta\text{-LG}) - T_d\ (\beta 0\text{-LG})$
[b] Enthalpy

Sugars may inhibit coagulation of proteins by preventing the loss of native structure associated with aggregate formation, with sucrose having greater inhibition effect than glucose. As the level of sugar increase so does the solvent cohesive forces, which translated into an increase in the energy required to break the hydrophobic associations in protein. The energy required for aggregate

formation is therefore greater, hence higher denaturation temperatures. Partially denatured proteins (i.e. β-lactoglobulin) are stabilized in the presence of sugars, inhibiting aggregation. [125]

E. Protein modifiers

Certain chemicals such as urea, guanidine hydrochloride, and anionic detergents can modify protein structure thus influencing its thermal stability. These chemicals can cause denaturation of proteins at certain concentration without the involvement of heat by destruction of quaternary structure or subunit orientation of some protein leading to aggregation. Addition of heat can cause acceleration of these changes. [125]

On the other hand some chemicals such as sodium dodecyl sulfate (SDS) at low concentrations have been reported to stabilize the conformation structure of some proteins (i.e. β-lactoglobulin and oat globulin) causing an increase in denaturation temperature. Stabilization is attributed to formation of bridges between positively charged groups in one loop of the polypeptide chin and hydrophobic region in another loop. However at higher concentrations, SDS reduces the thermal stability of some proteins (i.e. conalbumin and ovalbumin) without significant shift in denaturation temperature**.** A reduced endothermic curve of DSC graph indicates prior compromise of the conformational integrity inducing protein denaturation. [125]

Methods of assessing thermal denaturation and subsequent aggregation of soy protein isolate

Diffrential scanning calorimetry

Alteration of protein native strucutre is associate with energy uptake in from of heat absorbance causing an endothermic peak on the DSC thermogram. Enthalpy of denaturation (ΔH) , peak of denaturaiton temprature (T_d) and width of the calorimetric transtion at half peak hight (ΔT_w) are all information that can be inferred from a DSC thermogram. (ΔH) is an estimate of the thermal energy required to denature the protein and is measured from the preak area of the thrmogram. T_d is a measure of the thermal stability of the proteins, the differnce in Gibbs free enrgy (ΔG) (between folded and unfolded states) at T_d is zero. If denaturation occure within narrow range of tempratature presented with a narrow-edged peak, the transition is considered highly cooperative. Reversability of protein denaturation can be evaluated by scanning sample after denaturation. Additional kinetic parameters of protein denaturation can be obtained using DSC data. For example the basic equation for rate of change of a scpecies (S) with time (t) can be expressed: [125]

$$\pm \frac{dA}{dt} = kA^n \qquad \text{Eq. 15}$$

Where k is rate constant (sec^{-1}), n apparent reaction order. The temperature dependence of the rate constant can be determined from Aarrhenius equation: [125]

$$k = Ze^{-Ea/RT} \qquad \text{Eq. 16}$$

Where *Z* is the pre-exponential factor (sec^{-1}), and *Ea* is the activation energy (J/mol). [125]

At 8% (w/w) SPI, two endothermic denaturation points were recorded. The first peak corresponds the least stable subunit 7S and the second corresponds to the more heat stable fraction 11S, recording 42°C and 70°C consecutively, figure (15). These points were determined using the conventional manual method of extrapolating the portions of the curve before and after the peak as a straight line and using the left side line intercepts (beginning of the peak) as the denaturation temperature.

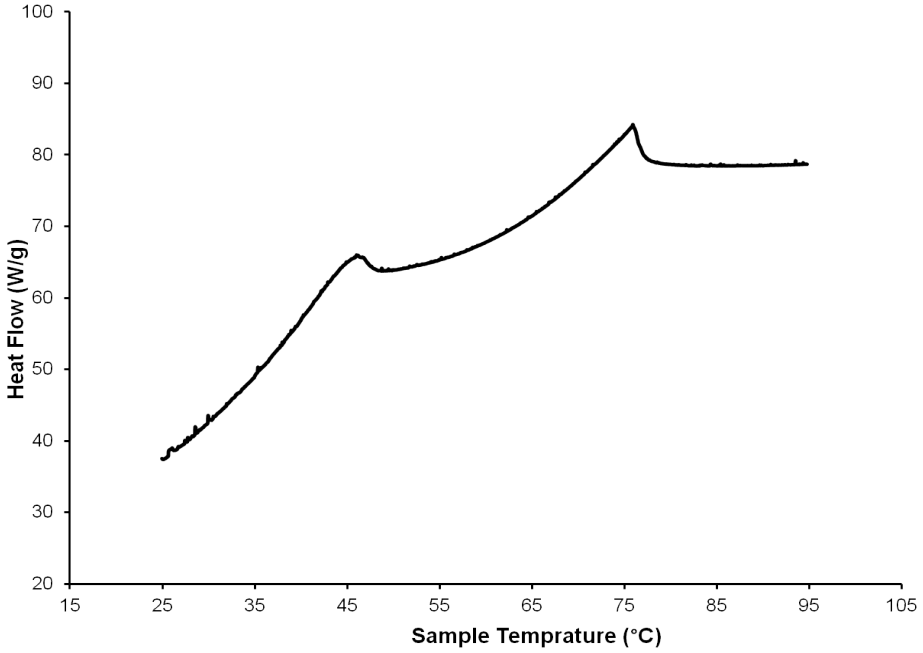


Figure 15: DSC thermogram of 8%(w/w) SPI suspension. Heating rate 2°C per min from 25-98 °C.

Cramp et al. (2008) [147] found that denaturation by thermal treatment used to produce a progel solution was found to cause irreversable denaturation of protein, similar results were observed for our sample where a repetition of

heating and cooling cycles reported no peaks on a previosly heated sample, data not shown.

Rheological measurements

Our objective is to monitor the progression of aggregate formation as an indicator of denaturation; therefore we opted to use a method that can evaluate changes in the viscous behavior of protein suspension using rotational rheometer. However we will frequently refer to information that were obtained routinely using dynamic viscosity measurements during gel formation, though these information were rarely focused on during data discussion and analysis in the original publications since the tendency is toward analysis of the resulting gels rather than intermediate viscous solution.

The complexity of the system behavior during thermal processing requires dividing the viscosity curve into five sections and addressing each section separately. Following is a dissection of the general behavior of 9% w/w SPI in distilled water as illustrated in figure (20).

Rheological behavior of SPI suspension at room temperature

SPI dispersed in water form a suspension that consists of a continuous phase, water, and a dispersed phase, SPI particles. The flow of particles in suspension is governed by three forces, those of colloidal origin that arise from interactions between particles. These forces can cause overall repulsion or attraction between particles. Repulsive forces such as electrostatic repulsion

cause particles stay in suspension. Attraction forces on the other hand such as Van der Waals or electrostatic attraction cause flocculation of particles, which might result in sedimentation of these larger bodies. The balance between these forces is exemplified in the general form of the potential energy curves for the interaction of a pair of colloidal particles developed by Derjaguin, Landau, Verwey, and Overbeek in the 1940s, giving it the name DLVO figure (16).

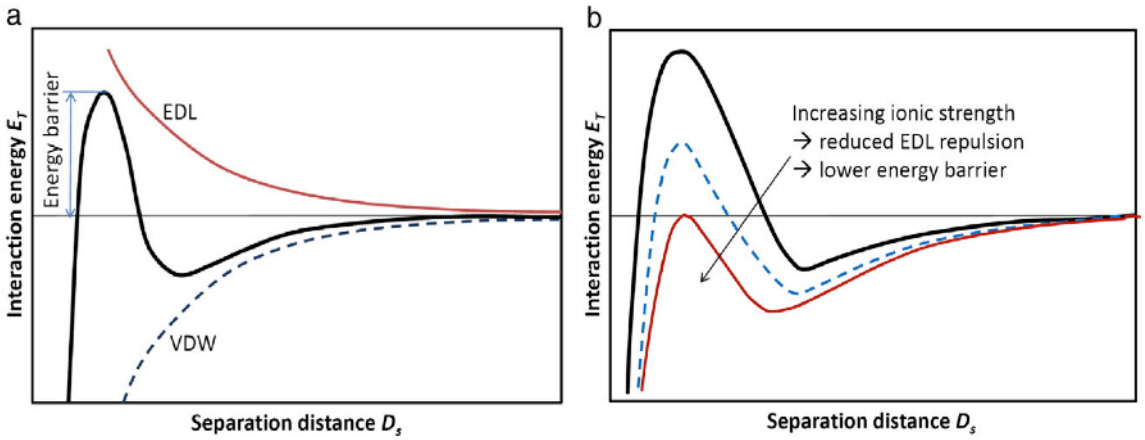


Figure 16: Potential energy for the particle–particle (or particle–surface) interaction based on the DLVO theory (assuming repulsive electrical double layer force). (a) The interactional energy as a function of separation distance; (b) effect of ionic strength on energy barrier.[148]

The second factor affecting particles in suspension is the randomized Brownian motion which is strongly size dependent. Thirdly the viscous forces acting on the particles, viscous forces depend on the local velocity difference between the particle and the surrounding fluid. Therefore higher protein concentration cause increased resistance to flow, increasing suspension viscosity. [85]

Suspension such as polymeric liquids exhibit a wide range of rheological phenomena, the general behavior is illustrated in figure (17). The behavior is divided into three regions. At the beginning the suspension goes through a

Newtonian plateau at a low shear rate. This is followed by a shear thinning region before the line flattens out again with a second Newtonian plateau at high shear rate [85]. According to Tropea at al. (2007) [149], the increase of viscosity at higher shear rate can be attributed to changes in particles structure caused by high shear. This behavior was noticed in our SPI suspension with the exception of the second plateau due to experimental limitations where a high enough shear is not obtainable, see figure (18). This behavior strongly depends on the solid volume concentration as illustrated in figure (19).

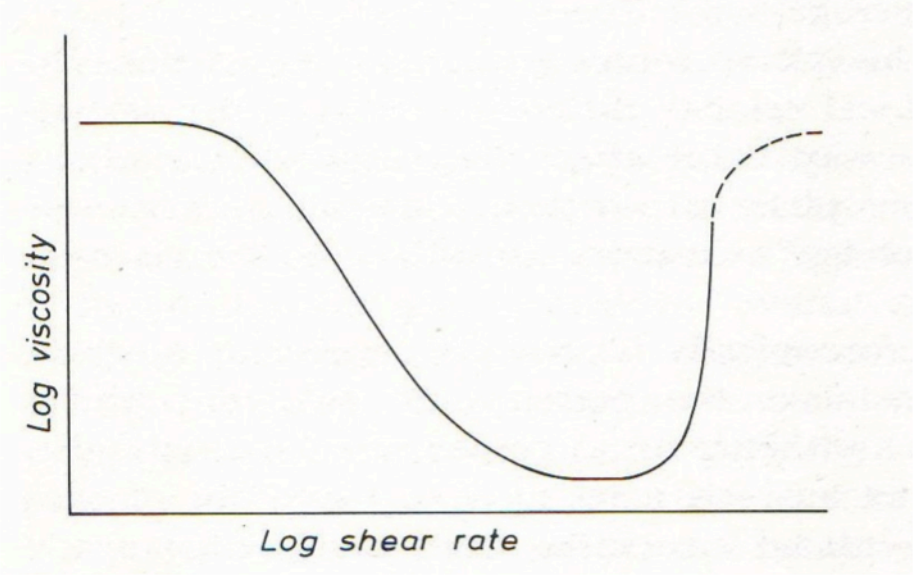


Figure 17: Schematic representation of the flow behavior of a concentrated suspension. [85]

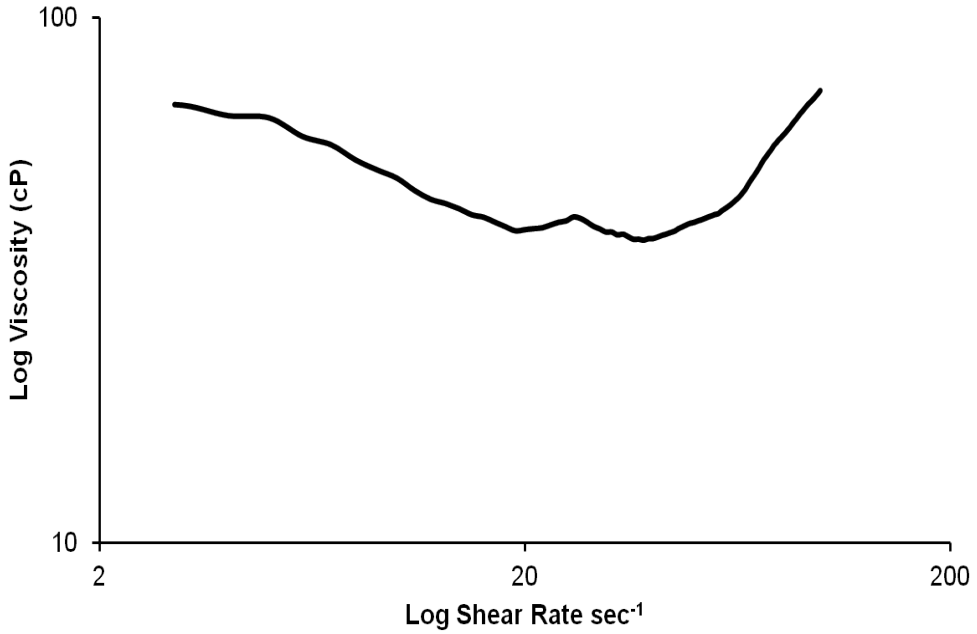


Figure 18: Flow behavior of 9% (w/w) SPI suspension at 25°C. reported results are of average of three samples.

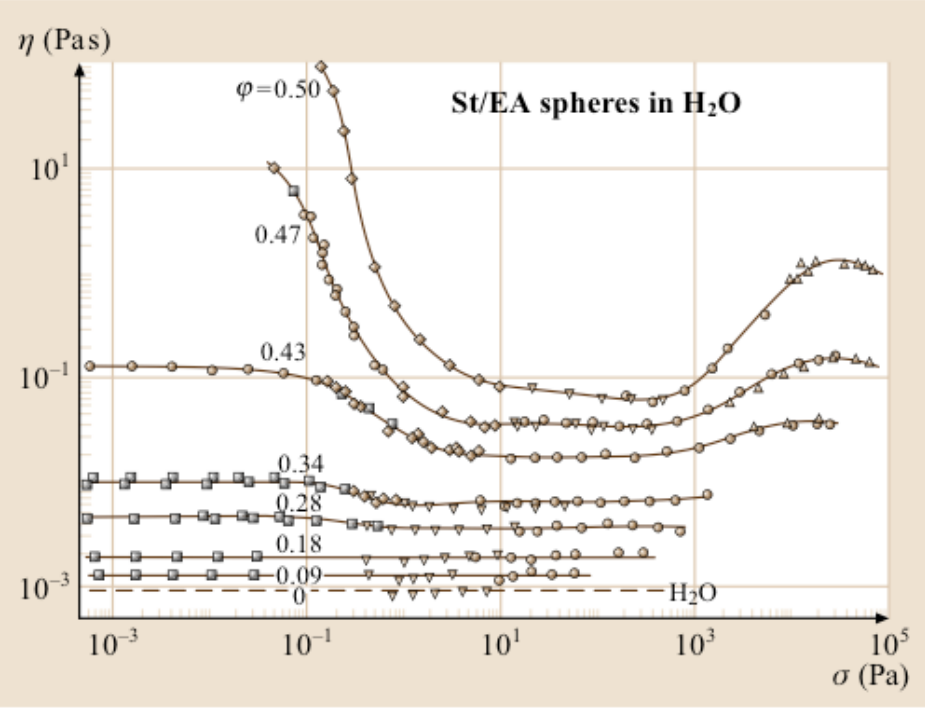


Figure 19: Shear viscosity versus shear stress of a colloidal latex suspension with 250nm particles at different solid volume concentrations at nearly the same pH values. [149]

This general behavior is sustainable for our suspension as long as proteins molecules are intact. But the introduction of heat treatment results in deformation of the microstructure in a way that is tangible and measurable on a macrostructure level.

The viscosity response of SPI suspension is monitored during a thermal treatment that consists of a cycle of heating followed by holding at constant maximum temperature. This is followed by a gradual cooling cycle before returning to RT. The general behavior was similar to that reported by Farhat et al. (2012) [150] of a 2% xanthan gum and soy flour mixture, figure (20). Under similar heat treatment regime, the mixture reported an increase in viscosity that seized during the holding time at 95°C. This increase in viscosity was followed by a decrease in viscosity. Even though Farhat et al. (2012) did not provide any explanation of this viscosity change, we can speculate that it is associated with the gelation properties of hydrolyzed soy proteins. By comparing the gelation power of hydrolyzed versus un-hydrolyzesd soy protein isolates we notice the lack of viscosity increase in response to temperature treatment further confirming our speculations, figure (21).

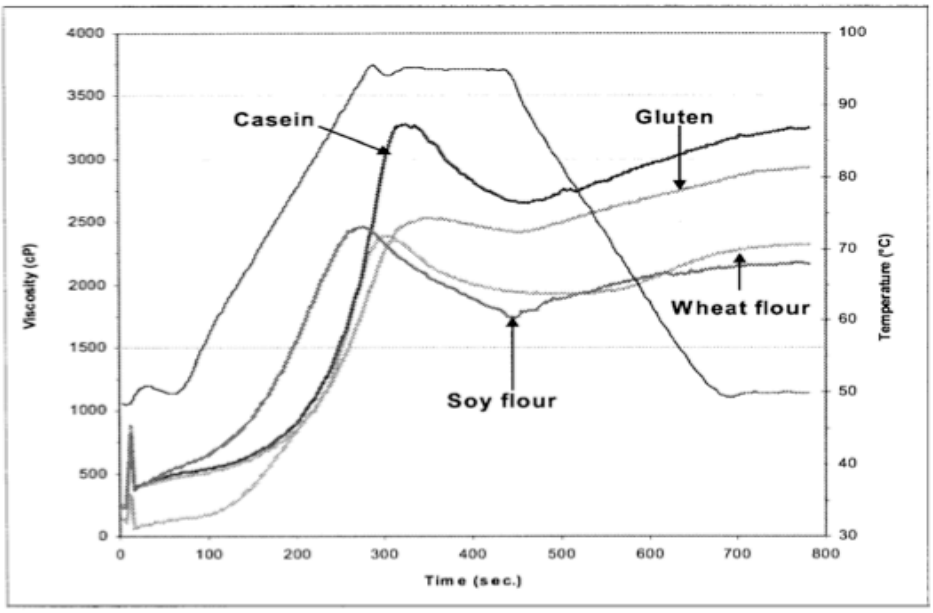


Figure 20: Viscosity and temperature profile of 1:1 Coextruded blends of Xanthan with other biopolymers. [150]

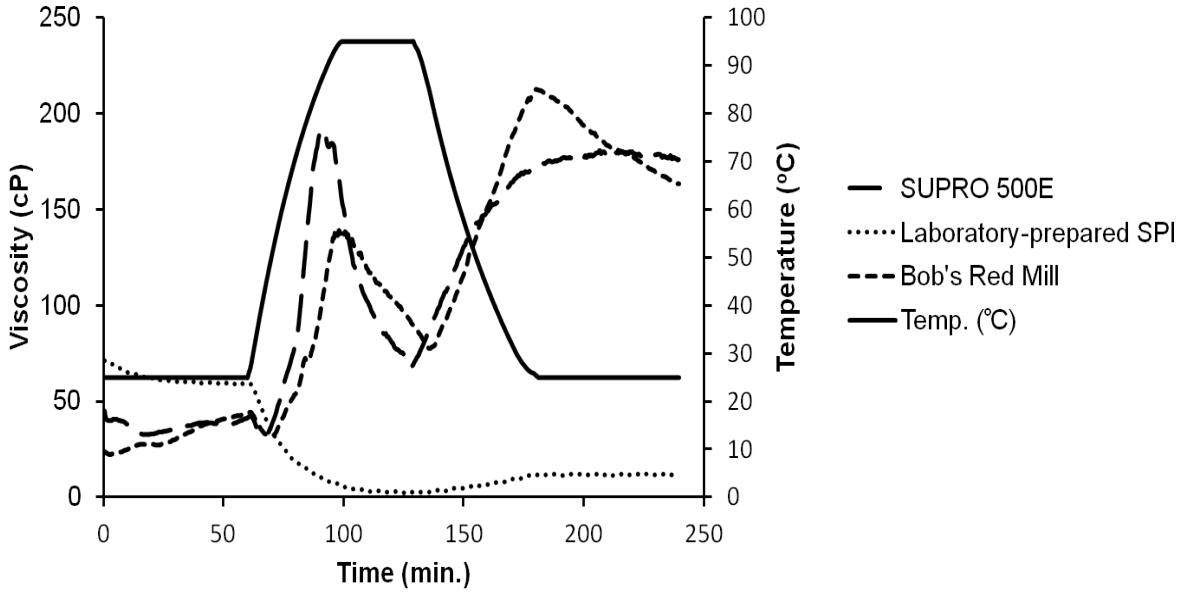


Figure 21: Viscosity and temperature profile of 9% (w/w) of two commercially processed SPI (SUPRO 500E and Bob's Red Mill SPI), and one unhydrolyzed laboratory prepared SPI.

A. Momentary viscosity decline at the beginning of heating cycle

At the beginning of the heating cycle viscosity decreased from 42 cP at 25 °C to 32 cP at approximately 40°C before resuming to increase, figure (22).

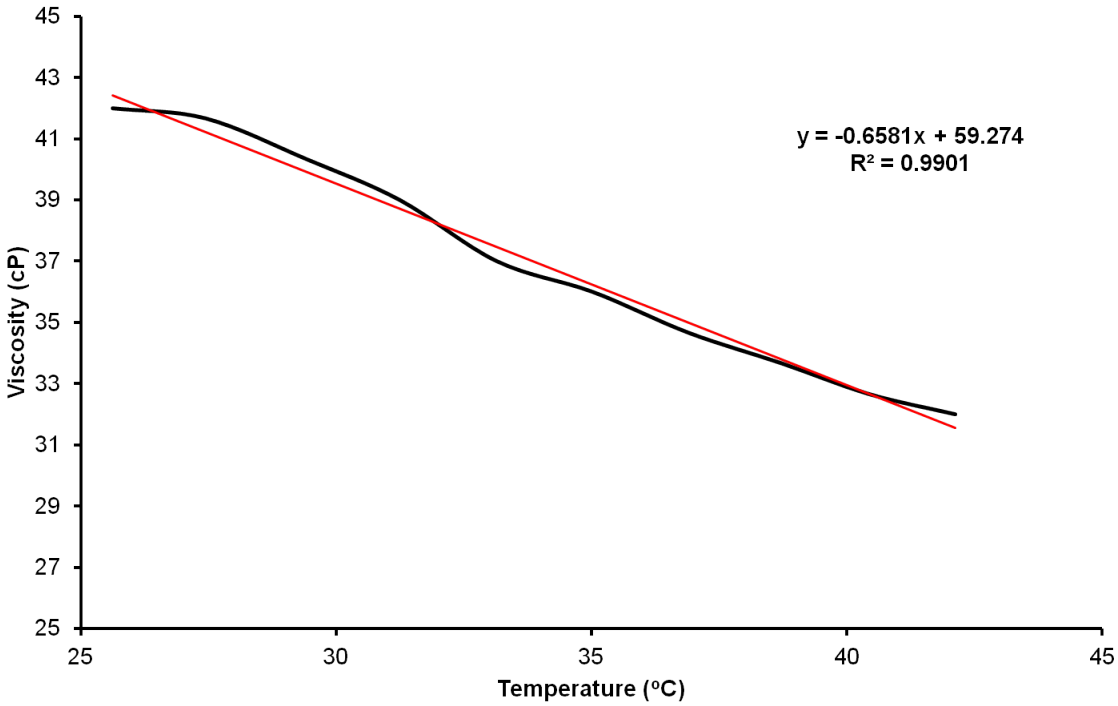


Figure 22: Decline in suspension viscosity at the beginning of the heating cycle.

In this temperature range where proteins are still to a certain extent intact and unaffected structurally by the thermal treatment, the Brownian motion is the predominant force. Increasing temperature prior to protein molecules denaturation results in increase Brownian motion of protein particles in solution resulting in decrease in recorded viscosity.

B. Increase of viscosity during the heating cycle

After the minor decrease in viscosity a rapid increase was registered during the heating cycle, figure (23). Viscosity started to increase at around 45°C

up to approximately 92°C registering a maximum viscosity value of approximately 190 cP after which viscosity starts to decrease. [125]

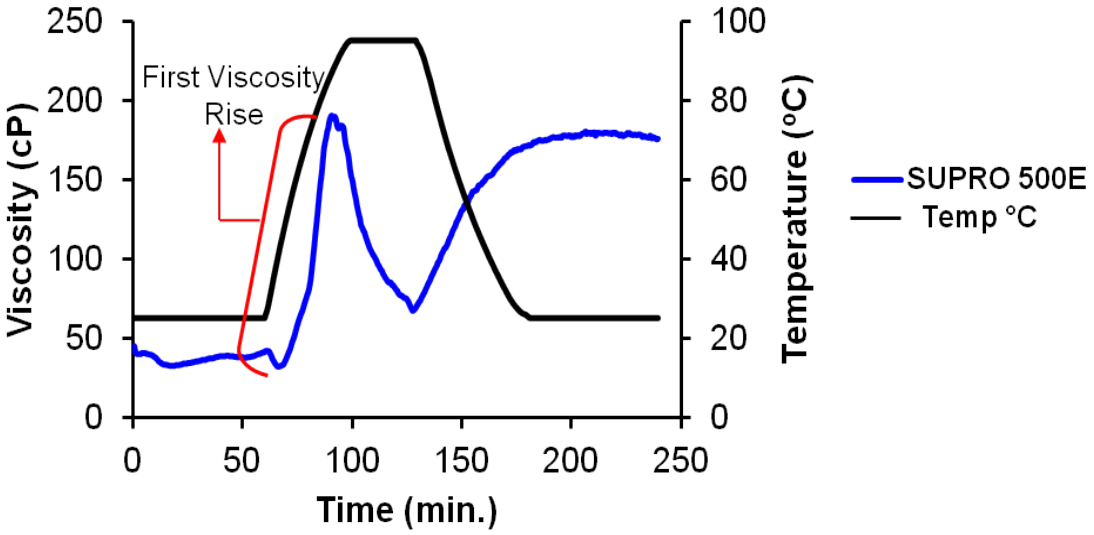


Figure 23: Viscosity response of 9% (w/w) SPI suspension.

In the temperature range between 45-92°C both fractions of soy protein isolate unfold as a result of heat treatment. At selected protein concentration, denatured proteins aggregates as a mean to reduce the portion of hydrophobic a.a. exposed to water and reduce system's free energy. Aggregation is caused by the interaction of highly active a.a. in the a.a. sequence chain [126]. There are several proposed mechanism of aggregate formations, three mechanisms suggested by Arakawa el al. (2006) [133] are mentioned previously in this chapter. Under the preparation conditions of a cold-set gel at which protein concentration is relatively low and at the absence of added ions, aggregates remain soluble.

Aggregation of proteins due to heat causes increase in effective volume fraction that is also referred to as the hydrodynamic volume. [151]

$$\phi_{\text{eff}} = \frac{\text{volume of solid + excluded volume}}{\text{total volume}} \qquad \text{Eq. 17}$$

ϕ_{eff} which is the volume fraction of particles in addition to the volume occupied by the repulsive (soluble aggregates) region around the particles. The effective volume fraction can also be viewed as the volume of the continuous phase that can't be occupied by particles because it is within the region of repulsion forces domination. Unfolding of proteins cause water uptake and swelling, subsequently an increase in the hydrodynamic volume that is manifested in increase in viscosity as a result of increased resistance to flow [125]. The effect of change in ϕ_{eff} can be modeled using the Quemada model, figure (24). [152]

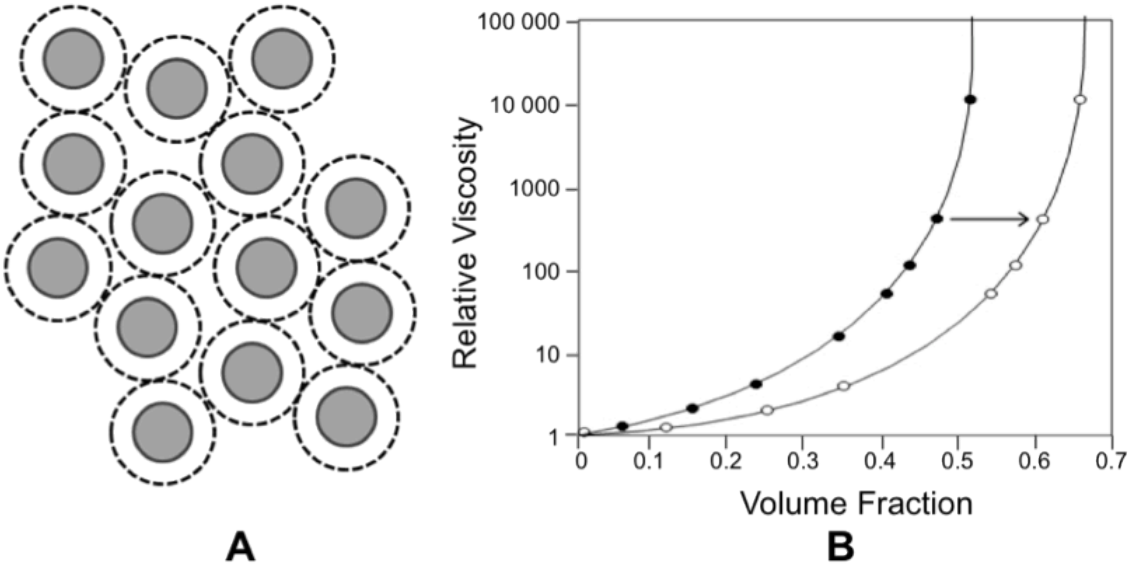


Figure 24: (A) illustration of suspension of particles with volume fraction 0.4 (grey circles) with repulsive interaction extending to the outer line, new phase volume 0.57. (B) relative viscosity of soluble particles with repulsive interaction; as a function of volume fraction (black dots and black line), as a function of effective volume fraction (open dots and black line) that data plotted on Quemada model (solid line of the open dots). [152]

Figure (24) displays aggregates as globular (spherical), which is specific to some protein aggregates such as soy protein. SPI forms particulate aggregates as indicated by the opacity of the progel solution. In comparison filamentous aggregates such as whey protein isolate aggregates solution forms a network often described as string-of-beads, it appears transparent due to the negligible width of the filaments that result in minor to no light scattering [151].

Plotting of changes in viscosity as a function of temperature in the range of 45 and 92°C fits a polynomial model with increasing fitting accuracy up to 6th order polynomial model with R^2 value of 0.9995 (figure (25)), such practice neglects the slight deviation from the polynomial model that can be attributed to a rather important change on the molecular level of the system. Hence, our understanding of the system's behavior under thermal treatment was used to divide the change into two consecutive regions with two distinct rate of viscosity increase in response to temperature at that point. The first section extends from 45-78°C, and the second consecutive section extends to 92°C, the first with smaller rate compared to the second region.

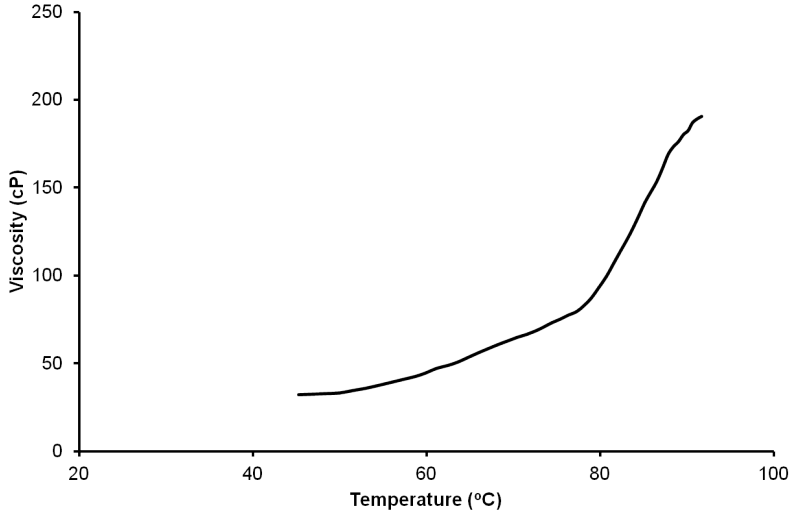


Figure 25: Viscosity increase in the temperature range between 45-92°C.

Although those temperature marks (45 and 78°C) at which the rate of viscosity increase changes do not perfectly match previously reported denaturation points of SPI (by literature as well), it is important from an application point of view since it reflects the macrostructural changes. It also reflects the nature of denaturation, aggregation and floc formation behaviors that are gradual and occur over a range of temperatures and with time at various rates rather than instantaneous. Such behavior was demonstrated by Harwalker (1980) [141] where he detected denaturation of β-lactoglobulin to occur at 75°C with two distinct stages of progression of denaturation, at the first 5 min of heating time at 75°C denaturation appeared to occur at a higher rate than that in later stages. That led to the conclusion of progressive and frequently incomplete unfolding and loss of native structure. The viscosity increase in the temperature range between 45-78°C follows a polynomial order with R^2=0.9907, while the viscosity increase in the temperature range between 78-92°C follows a linear ordered increase with R^2=0.9939, figure (26). These findings could have a potential benefit during system design at which manipulation of heating rate could accelerate the denaturation process, thus saving time and protecting the biologically active compounds from excessive heat damage.

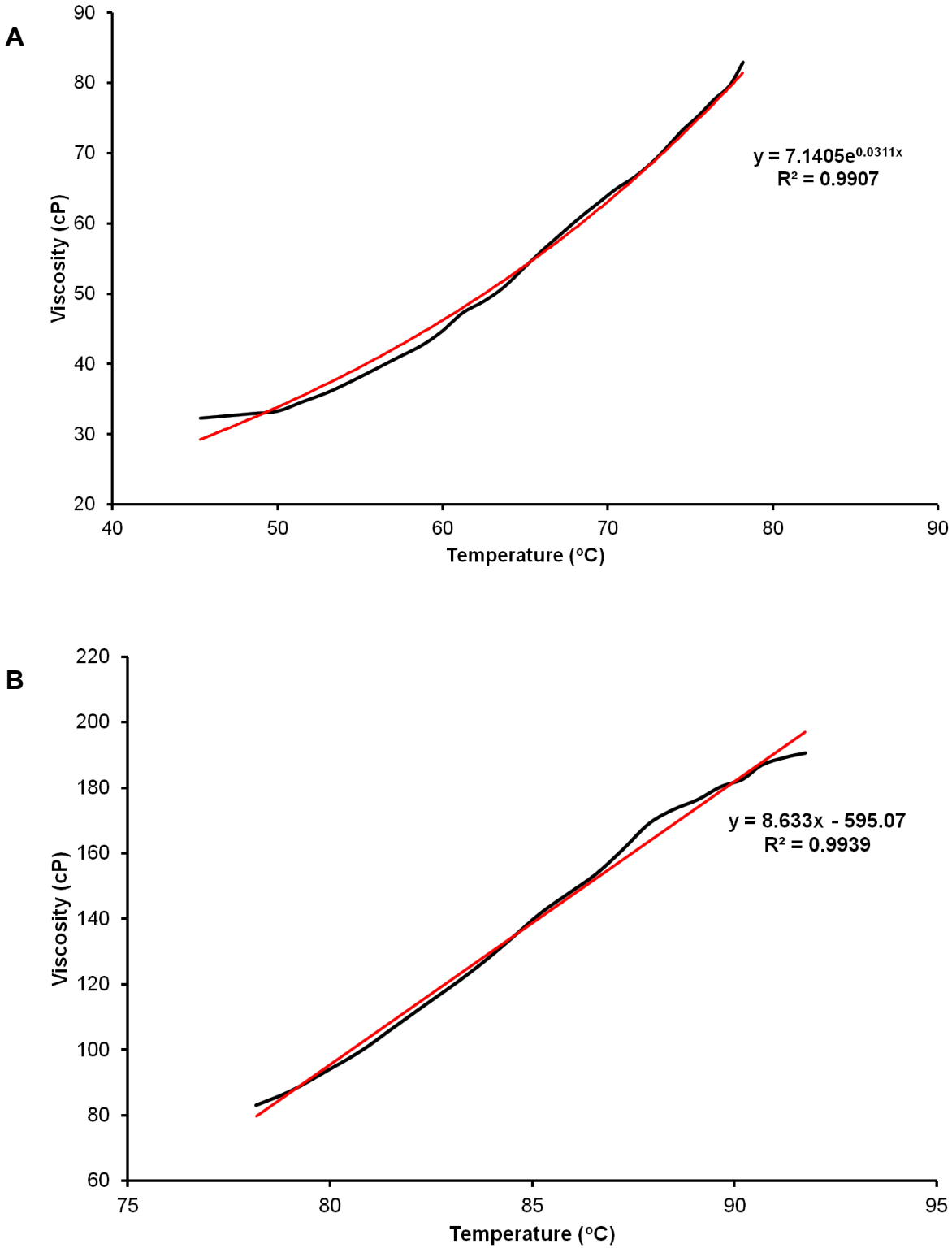


Figure 26: (A) viscosity increase in the temperature range between 45-78°C. (B) viscosity increase in the temperature range between 78-92°C.

C. Decrease of viscosity toward the end of the heating cycle, throughout temperature stagnation, and during a portion of the cooling cycle

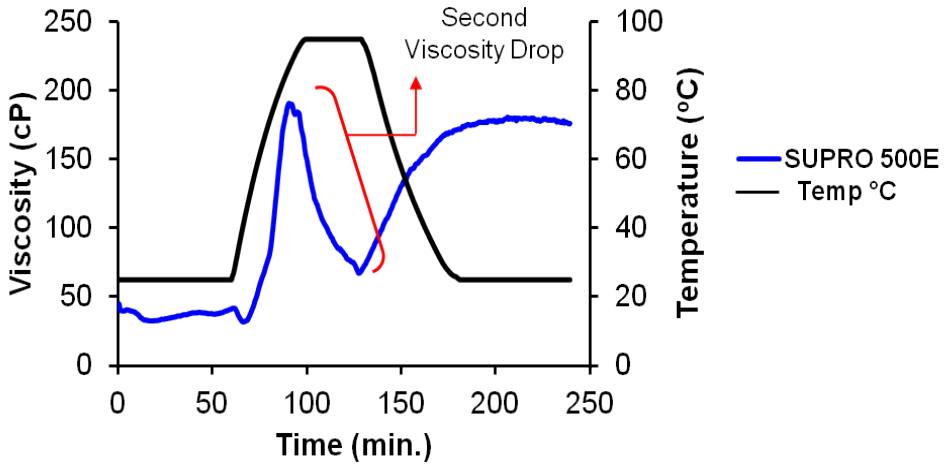


Figure 27: Viscosity decline during temperature stagnation at 95°C and during the cooling cycle between 84-30°C.

As proteins unfold and form aggregates due to thermal denaturation, the total volume that it occupies increases, meaning an increase in phase volume ϕ. Normally this increase is associated with an increase in viscosity as discussed in the previous section. However viscosity did not increase to infinity due to the formation of protein aggregates that immobilized a large portion of the continuous phase, it rather cease momentarily before starting to decrease, figure (27).

A likely point of view that explains the decline in suspension viscosity regardless of heat is the work of Krieger (1972) [153]. He created a variable called the Krieger variable, which is a modified Péclet number:

$$\mathbf{P_e = 6\sigma a^3/kT} \qquad \text{Eq. 18}$$

Where *a* is particle radius, σ is shear stress, *k* is Boltzmann's constant and *T* is absolute temperature. σ and *k* are constant through the run; however as temperature *(T)* increases denaturation occurs resulting in increased particle

size, thus increase *(a).* This results in overall increase in P_e value. The equation was used to build a curve (figure (28)) that can be used to predict the viscosity behavior of suspensions in relation to change in Krieger variable. This model describes same-sized particles dispersed in water.

To explain how changes in particle size, or more specifically (*a)* particle radius cause suspension viscosity to decrease, we need to define a new concept other than phase volume. ϕ_m is the maximum packing fraction defined as the phase volume at which particles form a three-dimensional structure leading viscosity to increase to infinity. In a system with a broad particle-size distribution, small/er particles can fit in the gaps between large/er particles causing the immobilization of a large volume of the continuous phase, hence increasing system viscosity. And the opposite is true for a system of mono-modal particle size. Similarly particle shape affects the extent of particle packing, for instance non-spherical particles have a poorer space-filling ability commanding a lower ϕ_m as a subsequence of the unoccupied space due to poor packing. [85]

As stated before, the main protein fractions of SPI are glycinin (11S) and β-conglycinin (7S) that have distinct physiochemical behavior. 11S is present in higher proportion, ≈ 42% compared to ≈34% for 7S [51]. The difference in the thermal stabilities of 7S and 11S fractions is evident in the DSC determination of the thermal denaturation points of SPI; implying that the contribution to the aggregation of the suspension occur at different stages of the thermal treatment. This is evident in the distinct stages of viscosity increase in response to temperature. At the beginning of the aggregate formation process ϕ increases.

With respect to the particle size distribution, at the first stage at temperature below the denaturation point of the more heat stable fraction 11S, a bi-modal distribution of particle size can be assumed with small particles corresponding to those of 11S and larger to aggregates of 7S.

The shift from a bimodal dispersion to a monomodal or semi-monodal particle distribution of large particles causes the maximum packing fraction ϕ_m to decrease. As overall particle size increases due to denaturation and aggregation, ϕ_m decrease because the system can be “saturated” with fewer particles due to their large size freeing some of the continuous phase that otherwise would be immobilized by the presence of smaller particles present in between larger particles.

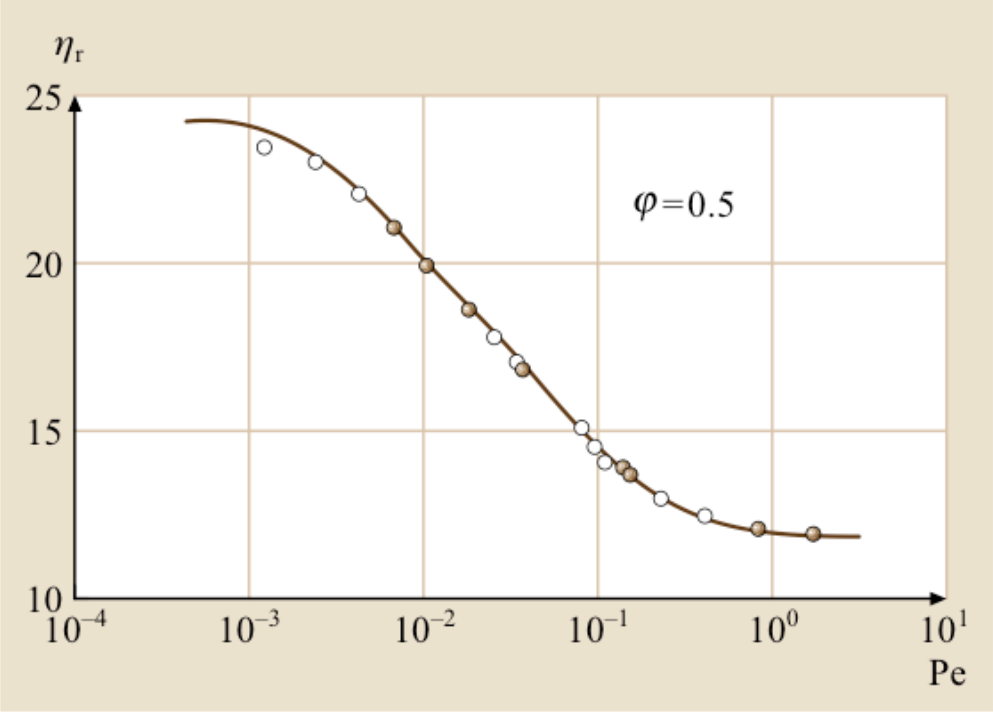


Figure 28: Composite curve of relative viscosity versus modified Péclet number. The solid line is for same-sized particles dispersed in water. Produced by Krieger (1972) [153] published by Tropea et. al. (2007) [149].

Though the justification that we have provided is highly probably, reviewing literature resulted in few findings where decrease in viscosity of protein suspension at high temperature is explained. The behavior was reported by Catsimpoolas et al. (1970) [142] though at slightly higher protein concentration 10%w.v (9.3% w.w), Catsimpoolas et al. attributed the observed drop in viscosity to the formation of metasol which is a state where excess heat cause extensive damage to protein structure that a gel can not be obtained. However that contradicted with his later observation of viscosity increase upon cooling, figure (29). In addition, metasol state is obtainable for protein suspension after excessive heat treatment defined by many, including Wolf (1972) [154] as prolonged heating at 125°C.

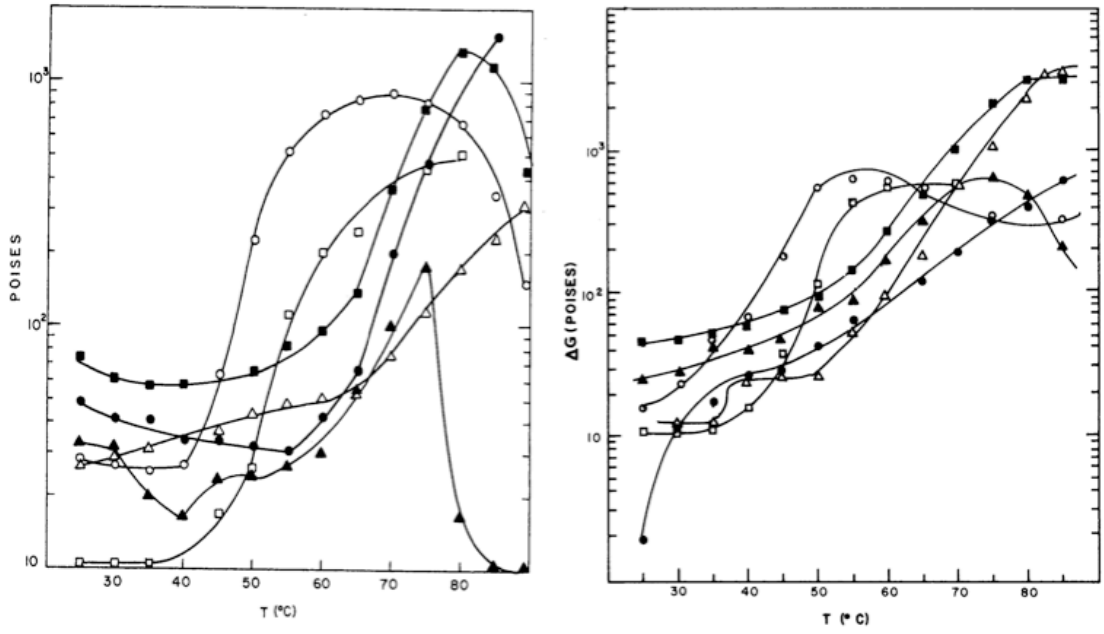


Figure 29: On the left: pH and temperature effect on the apparent viscosity of progel; 10% soybean globulin (w./v.). On the right: pH and temperature effect on the apparent viscosity (ΔG) gained during cooling of the gels from progel state. Open circles (pH 1.0), open squares (pH 2.0), open triangles (pH 6.0), solid circles (pH 7.0), solid squares (pH 8.0), solid triangles (pH 10.0). [142]

D. Increase of viscosity during cooling and second temperature stagnation cycles

The increase in viscosity during cooling and temperature stagnation at 25°C can be attributed to the rapid formation of hydrogen bonds between aggregates at low temperature, which is different from the electrostatic attraction or cross linking the produces a gel matrix, figure (30) [78, 142]. During denaturation, expose CO and NH groups become charged clusters creating a water multilayer surrounding the polypeptide chain, upon cooling these chains interact via formation of H-bonds, thus creating a structure that causes immobilization of surrounding water [155]. Unlike disulfide bond (acidic pH) or non-covalent bonds (alkaline pH) that forms and stabilizes gel matrix increasing it hardness, H-bonds and hydrophobic interactions increase viscosity of the matrix [78, 155, 156]. The extent of viscosity increase seems to be dependent on the maximum heating temperature and time combinations indicating a relation to the degree and extent of denaturation, subsequently amount of aggregates formed in a direct relationship manner. [78]

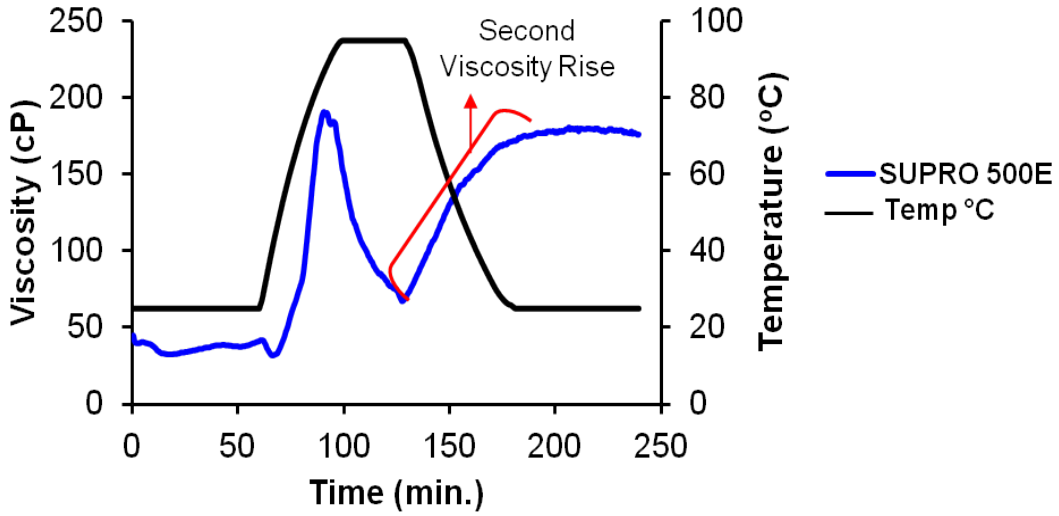


Figure 30: Viscosity increase upon temperature decrease.

However at the end of the run the sample continued to look as a viscous solution rather than a self-supporting gel. Thus the storage modulus would not increase beyond the loss modulus G'' which would indicate gel formation, Maltais et al. (2005) [71] confirmed this concept by comparing the G' values of cooled progel solution with various concentration of calcium salt, a gel-aiding agent at low protein concentration, figure (31) are the results. In the absence of calcium salts, cooled progel solution remained as a viscous solution with G' value remaining below that of a gel a SPI gels prepared with calcium salts. [71]

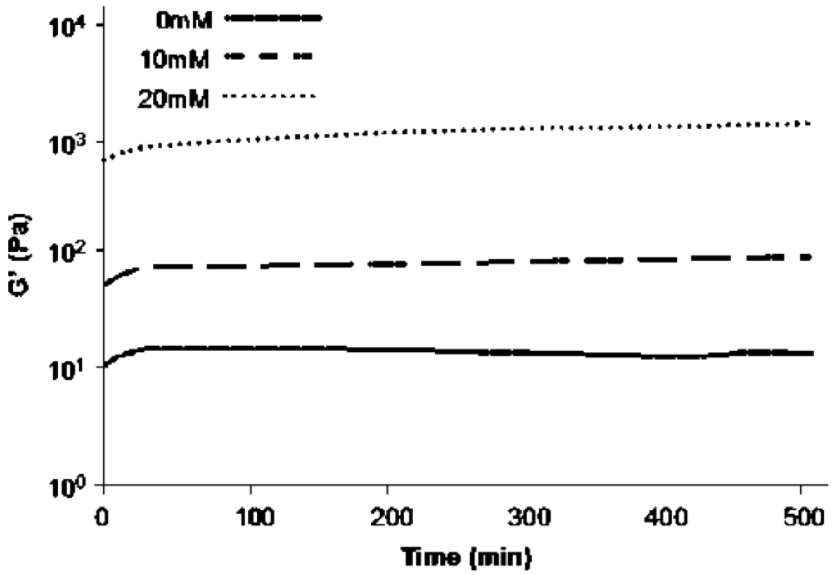


Figure 31: Evolution of the storage modulus (G') of 9% SPI gels with respectively 0, 10, and 20 mM CaCl2 during a 10 h rheological analysis at 25C. [71]

In addition, cooling results in refolding of partially unfolded proteins that have not underwent aggregation. This partial refolding renders these proteins unavailable for aggregate formation or linking necessary to form a self-supporting gel network [139]. It is suggested that the refolding probability is higher in cold-set gels than in is in heat-set gels because at higher protein concentration,

aggregates formation is greater in term of quantity and rate [145]. Hence the proportion of proteins not involved in aggregates is less than that in cold-set gels where the low concentration limits the number of adjacent molecules available for aggregate formation [145]. So in term of possible encapsulation of hydrophobic molecules within the hydrophobic core of a protein molecules, using a lower protein concentration, even lower than what is used in this research or recommended for the formation of cold-set gel will increase the contingency of protein-hydrophobic molecule interaction over protein-protein interaction giving that these hydrophobic molecules are abundant in the medium.

Protein concentration, which affects gel properties, was not much a concern during this study since a tailored formulation can be used for each specific future application. For our adsorption goal, we selected the upper limit of the critical protein concentration required to produce SPI cold-set gel, 9%, since we hypothesized that the number of available hydrophobic cores that acts as adsorption sites would possibly be a limiting factor in the adsorption process. Viscosity was found to increase exponentially with decrease in temperature during the cooling cycle, figure (32).

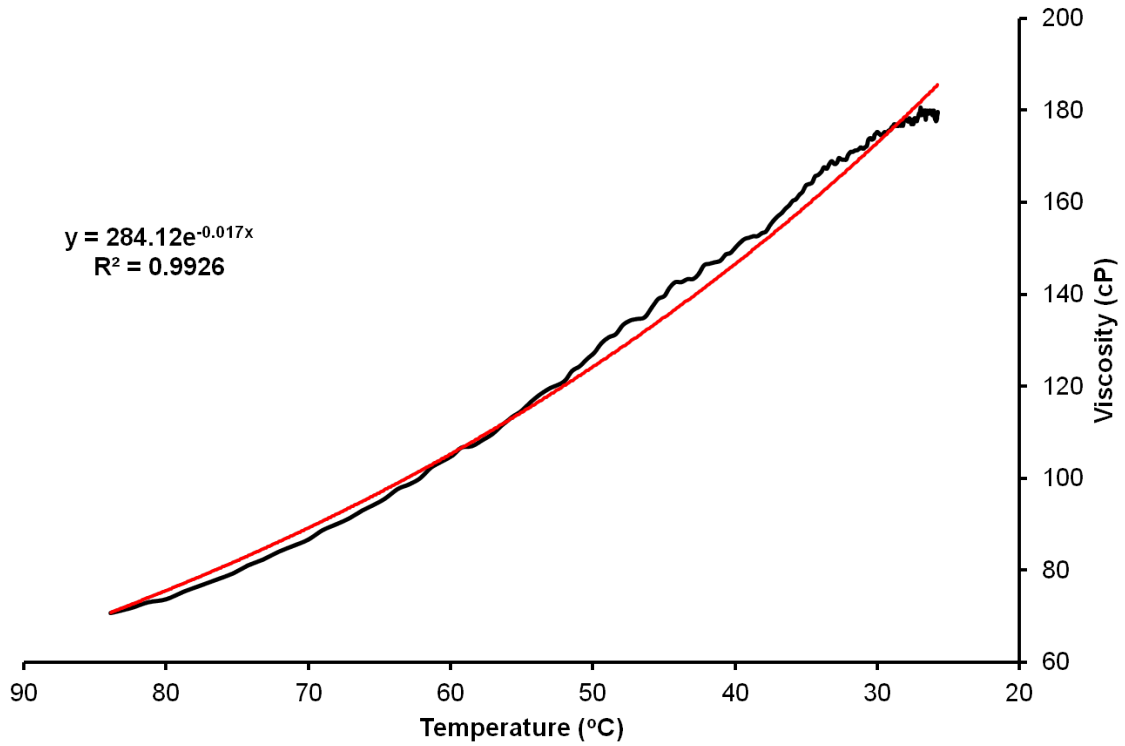


Figure 32: Viscosity increase upon temperature decrease. Minor viscosity stabilization starting minute 226 onwards is not shown.

UV/VIS spectrophotometry

Absorption of the electromagnetic radiation by proteins in the range of 230-320 nm is primarily due to the electronic excitation of the major aromatic a.a. tyrosine, tryptophan, and phenylalanine. Changes in molecular configuration due to thermal denaturation cause changes in the characteristic spectral of proteins, increase in absorbance, and shift in absorption spectrum to shorter wavelength, (blue shift) UV region [98, 125]. Qualitative spectra is show throughout this chapter, changes in overall absorbance in the 230-320 nm is used as a mean of assessing protein denaturation and not quantification of single amino acids.

Direct absorption spectra were produced using water as a reference. We started by monitoring the changes in absorbance throughout the characteristic UV range of 230-320 nm, figure (33 A, B). As temperature progresses, *A*

(absorbance) decreases unexpectedly in the region of 230-320 nm, however when comparing the spectrum at 25°C with that at the end of the heating cycle at 95°C, the expected increase in *A* is witnessed as a result of increased surface hydrophobicity.

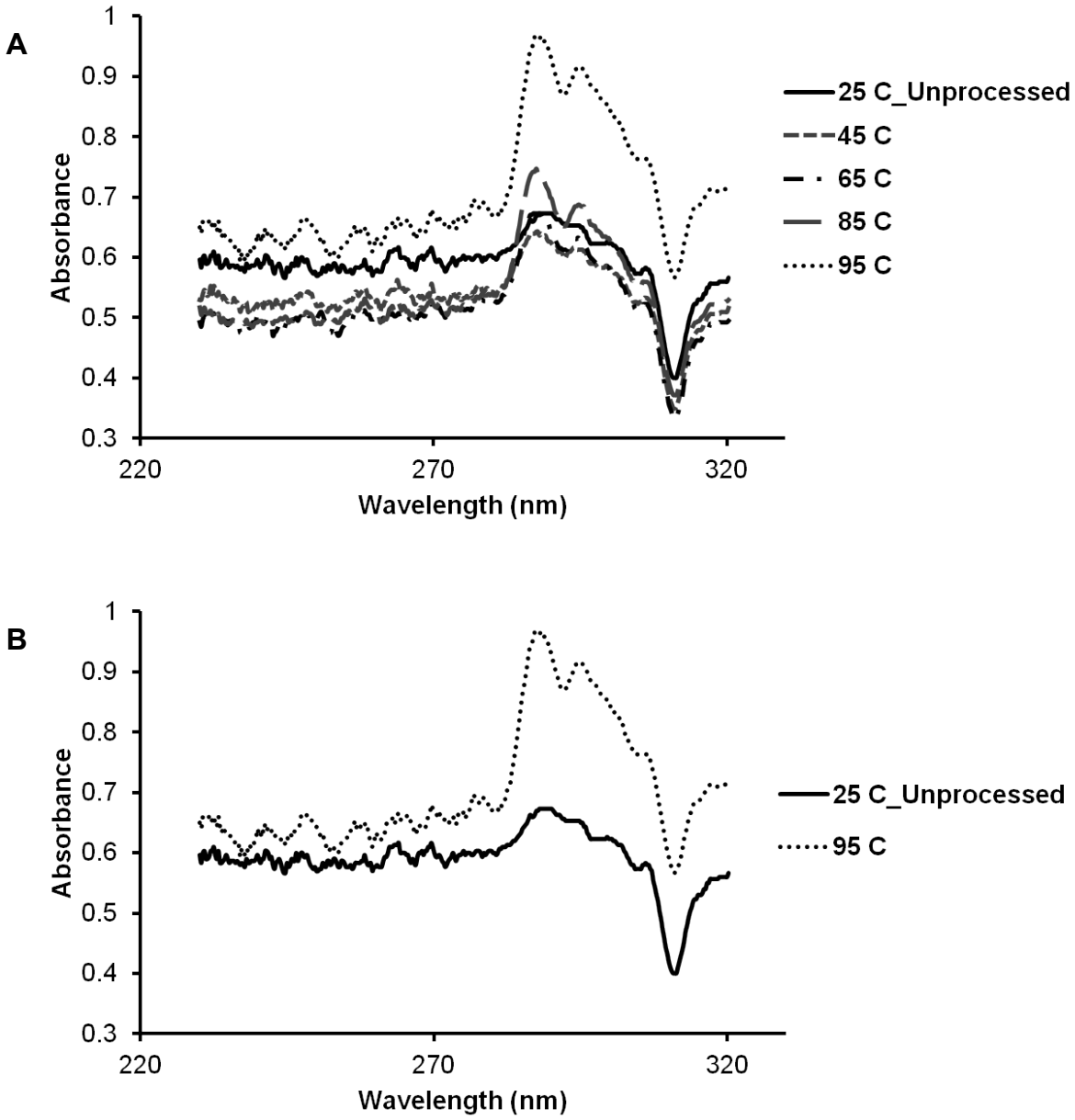


Figure 33: Absorbance in the 230-320 nm range as a function of temperature.

Throughout the experimentation design, samples where held at the maximum temperature (95°C) for 30 min, the effect of the holding time is noticeable when observing the change in A_{280} as a function of time at 95°C.

It is suggested that dilution of denaturation agent result in partial gain of native structure [70]. Reversibility of denaturation was examined using UV spectra of protein before and after thermal treatment. Figure (34) shows spectrum of unprocessed SPI and cooled samples after thermal treatment.

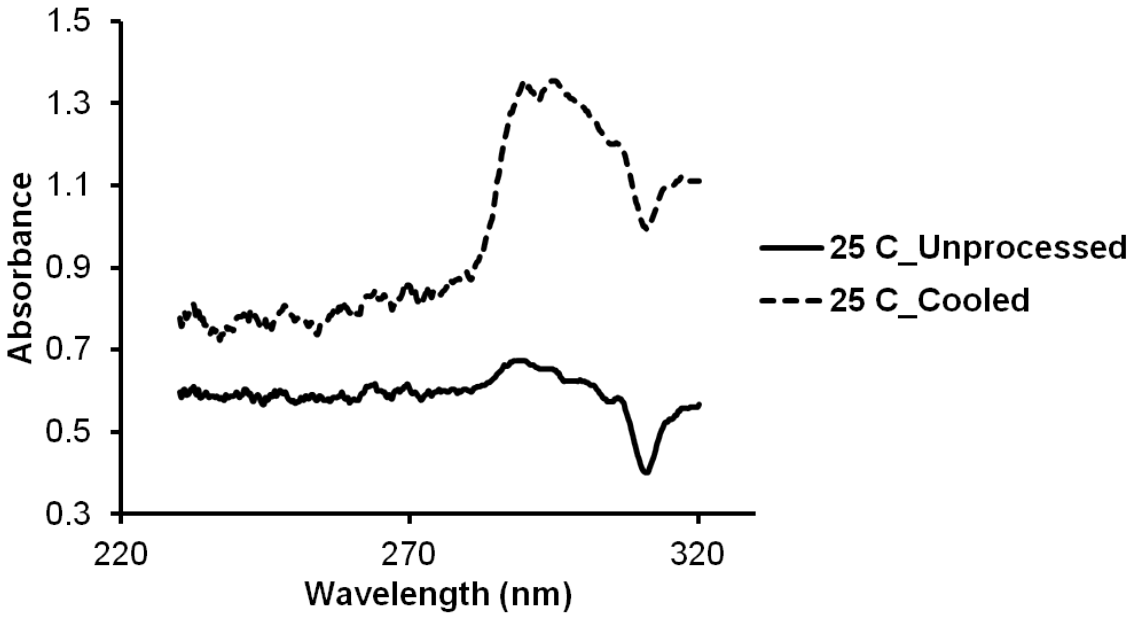


Figure 34: Lack of reversibility of denaturation after heat treatment.

Most of aromatic amino acids absorb strongly Absorbance at 280 nm [88, 125, 157], a characteristic wavelength for proteins, thus it is used in protein quantification using spectroscopic measurements. Figure (35), absorbance (A_{280}) as a function of temperature. The decrease in absorbance at temperature below 45°C coincide with the thermal stability findings of SPI fraction that were reported

in the previous sections. At temperatures below approximately 45°C both fractions are in their native structure and due the nature of this commercially processed SPI, immediate formation of aggregates by the partially denatured fractions decreases the concentration of exposed hydrophobic amino acids. Above 45°C the curve shits direction and an increase in absorbance is recorded, this is due to increase exposure of hydrophobic amino acids in the temperature range from 45-95°C which includes the denaturation point of 7S and 11S.

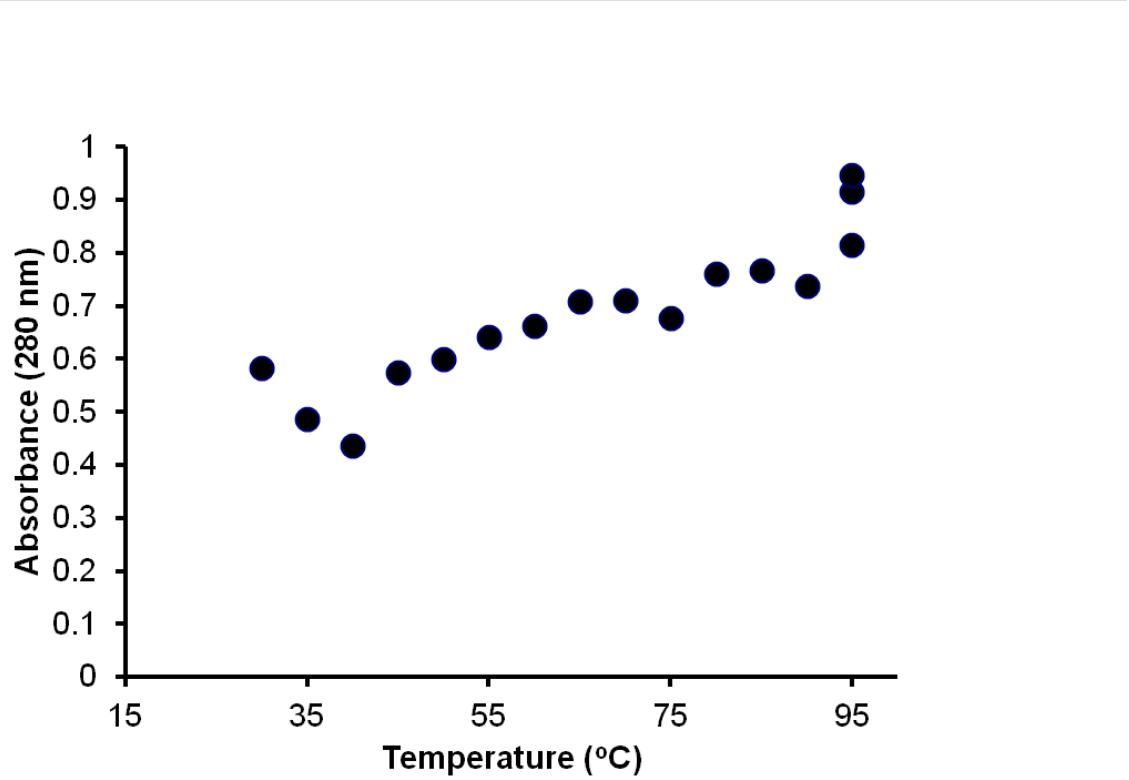


Figure 35: Absobance at 280 nm representing the three main hydrophobic amino acids, Phe, Try, and Tyr. Absorbance is presented as a function of temperature.

Chapter 4: Thermal Adsorption of carotenoids

Carotenoids composition in mango

Mango is a good source of vitamin A precursors as per FDA regulations [60] being able to provide +10% of the Daily Value (DV) of vitamin A which is 5000 IU [61]. Total carotenoids content in fresh mango can range anywhere between 900-9200 µg per 100 g, β-carotene is the major carotenoids making up to 40% of total contend [158]. The composition of mango carotenoids reveals a wide variety of compounds, some function as vitamin A precursor, others have unique health benefits as discussed in chapter (1). Identification of specific carotenoids is commonly completed using spectrophotometric or colorimetric methods [14]. These methods differ in their sensitivity and can be used in combination to achieve greater accuracy. Depending on the method used the number of carotenoids that can be isolated and identifies will vary considerably, method sensitivity and possible transformation and destruction of compounds are probable reasons for such discrepancies. This issue is of a great concern in research focused on carotenoid identification that Rodriguez-Amaya (2000)[159] have addressed it in a paper discussing possible causes and suggesting several precautionary steps to insure reproducibility and consistency of results. For instance while Jungalwala et al. (1963) [160] identified and quantified 16 different carotenoids in alfonso mango, Mercadante et al. (1998) [112] found only 6 in the Keitt variety compared to 5 in the same variety cultivated in different regions in Brazil in previous research [114]. Table (9) includes carotenoids composition in different mango variety as reported in literature.

Carotenoid	Alfonso % of total carotenoids[160]	Cv. Keitt µg/g of total carotenoids 38.0 ±7.7 g[112]	Tommy Atkins µg/g of total carotenoids 51.2±16.8 g[112]	Commercial Juice µg/g of total carotenoids 14.43±3.0 g[112]	Keitt µg/g of total carotenoids 49.9±59.8 g[114]
β-carotene	59.50	6.7±1.6	5.8±2.5	8.6±1.16	13.4±16.2
luteoxanthin	11.25	2.7±0.2	-	-	3.1±4.1
cis-violaxanthin	9.02	-	-	-	-
Phytofluene	6.89	-	-	-	-
Phytoene	3.70	-	-	-	-
auroxanthin	2.71	-	-	5.33±1.86	-
Cis- β-carotene	0.36	-	-	-	-
mutatochrome	1.52	-	-	-	-
γ-carotene	0.01	-	-	-	-
5,6-monoepoxy-β-carotene	0.85	-	-	-	-
violaxanthin	1.25	All-trans 18.0±4.0	-	-	All-trans 8.2±23.9
antheraxanthin	1.01	-	-	-	-
mutatoxathin	0.76	-	-	-	-
Cryptoxanthin	0.66	All-trans-β 0.2±0.0	Cis-β 0.1±0.1 All-trans-β 0.3±0.1	Beta 1.16±0.03	All-trans-0.3±0.3
cis-antheraxanthin	0.50	-	-	-	-
zeaxanthin	0.01	All-trans 0.8±0.3	-	-	All-trans 0.6±0.9
cis-neoxanthin	-	cis 0.3±0.2	All-trans 4.9±4.5	-	-

Table 9: carotenoids composition in different mango variety as reported in literature.

Stability of carotenoids

Stability of carotenoids is a major issue in the food processing industry; loss of carotenoids not only reduces the nutritive value of the food item, but might also affect some of the aesthetic qualities due to color change caused by certain reactions. Due to the hydrophobicity of these compounds, their stability highly depends on the medium matrix. For example, isolated carotenoids present in non-polar solvent are more susceptible to degradation than in a natural food matrix [111]. The major concern is isomerization of all-trans to a cis-form which has a lower vitamin A potency, isomerization can be caused by heat, light and extreme pH conditions [161]. Carotenoids are not considered to be pH-sensitive

in the food industry and are stable in common food pH range 2-7 with the exception of those containing carboxyl groups (e.g. bixin) that solubility is pH-dependent [162]. Oxidation is another cause of degradation of carotenoids, it can be direct through contact with atmospheric oxygen or via hydroperoxide formed during oxidation of lipid present in the medium [163].

The main concern in our experimental design is thermal degradation. Thermal treatment can result in the conversion of trans-isomers to cis-isomers which have lower potencies compared its trans-counterparts in term of vitamin A activity, in addition epoxidation of provitamin A can occur during heat processing [17]. However some thermal treatments are unavoidable and necessary for safety, processing or quality purposes. As mentioned in chapter (2) under the materials section, mango like many other fruits are subjected to some extent to heat processing for safety reasons mainly, and in some cases to ease the extraction of juices. In the making of mango juice, it is heated to 85°C then sealed in cans and further processed at 100°C for 20-30 min [164]. Pasteurization of mango pulp is occasionally achieved by heating cans in boiling water for 10-15 min [65].

The most comparable practice to our thermal treatment is boiling of pieces of fruits or vegetable and retorting of canned puree and juices. Dietz et al. (1988) [165] reported a very good retention of both α and β-carotene in vegetables tested after a relatively harsh thermal treatment by steaming (100°C) for 30 min [17]. Other thermal treatments that are unavoidable, and for the greater good of product quality is blanching, for example steam-blanching is used to inactivates

enzymes and may cause some initial loss of carotenoids content but will result in improve retention during storage. Gomez (1981) [166] gave an example of vegetables that are steam-blanched prior to solar drying, those vegetables displayed greater resistance to photo degradation than those not blanched, however theses results differ depending on plant anatomy that affects its wilting rate and photosensitivity.

In the work of Rodriguez-Amaya (1997) [17] published by U.S. Agency for International Development, the retention of provitamin A carotenoids in food post preparation, processing, and storage is discussed in details using published research as a platform for making processing recommendation for highest carotenoid preservation possible. Severity of thermal processing - both home and industrial practices- were evaluated base on the percentage retention of provitamin A carotenoid. Table (10) exemplifies a number of the studies that were used as references by Rodriguez-Amaya (1997) [17].

Table 10: Retention of provitamin A carotenoids as a result of thermal processing in pilot-plant or industrial settings.

Reference	Processing condition	Food product	Carotenoid	Retention (%)[a]
Dietz et al. (1988)[165]	Water blanching: 10 g sample cooked in 100 ml boiling water 30 minutes	Winged beans Lettuce Carrot Spinach	β-carotene	119 47 60 112[c]
	Steam blanching: 10 g sample steamed in household steamer 30 minutes at 100°C.	Winged beans Lettuce Carrot Spinach		83 104 [c] 99 132 [c]
Ogunlesi et. al (1979)[167]	Retorting conditions of 115.6°C, 30 minutes	Canned carrot	all-trans-α-carotene all-trans-β-carotene	74 65

Godoy et. al (1987)[168]	After hot-filling, sealed cans were immersed in boiling water, 20 minutes	Canned mango slices	β-carotene α-cryptoxanthin	109 [c] 87
	Mango purée heated in open steam-jacketed kettle to 80°C 10 minutes. After hot-filling, sealed cans were immersed in boiling water, 20 minutes	Canned or bottled mango purée	β-carotene α-cryptoxanthin	87 62
Godoy et. al (1991)[169]	Processed as described above for mango purée	Canned or bottled papaya purée	β-carotene β-carotene β-cryptoxanthin	88 100 [c] 74
Chen et al. (1995)[170]	Juice acidified to pH 4.0 with citric acid and heated at 105°C, 30 seconds, with lab. pasteurization system	Carrot juice	all-trans-α-carotene all-trans-β-carotene	92 96
Ramos et. al (1996)[17]	Hot air drying at 70-80°C in a food industry. Frozen at -30°C and lyophilized at -10°C in a food industry	Dehydrated carrot Lyophilized carrot	β-carotene β-carotene	104 [b c] 84

[a] % Retention = (% Yield (product) x % Solid (product) x Carotene content (product)) / (% solid (fresh) x Carotene (fresh))
[b] Retention calculated on the insoluble solid basis
[c] Greater than 100% retention was probably due to greater extractability of carotenoids from processed samples. Prolonged submersion (conned food), or cooking in liquid cause leaching of soluble solids; increasing carotenoid concentration per unit weight of food. Un accounted water loss during drying van also cause overestimation.

As noticed in table (1), some researchers reported more than 100% retention rate, this is caused by the ease of extraction of carotenoids after thermal treatment due to loss of compartmentalization and cellular structure, thus release of carotenoids into solution. In addition, due to the complexity of carotenoids assay, often contradicting results in the retention rates are presented, one example that stands out is sautéing which is often presented as a harmful method of cooking regarding carotenoid retention due to high temperature and associated oxidation, Manorama et al. (1991) [171] viewed

several studies where soybean oil was fortified with vitamin A precursors and then heated (100°C for 20 min) and used in a rat bioassay, the results showed 100% retention of vitamin A palmitate in the oil. He also compared these results to a study on human subjects that showed that vitamin A was well absorbed after using the fortified oil for cooking. It was then concluded that using crude palm oil as a carrier of β-carotene in cooking was an effective method of delivery, being able to retain 70–88% β-carotene in the cooked foods using different methods of cooking (baking, seasoning, deep frying and shallow frying).

In the juicing industry, β-carotene is frequently used not only for the nutritive fortification of fruit juice but also as a natural coloring with an advantage over some dyes due to its stability under the reducing action of ascorbic acid that is frequently present in fruit juices. After the common heat treatment of canned juices, prolonged storage has little effect on added carotenoids to juices, Table (11) shows the results of earlier work of Bunnell (1958) [172].

Sample	**Storage period, months**	**Temp. °F**	**Total carotenoids**	
			Mg per 4 fluid oz.	Retention %
Enamel-lined cans	0		0.66	98
	2	86	0.66	98
	2	98	0.62	92
	6	75	0.60	89
Plain tin cans	0		0.65	96
	2	86	0.69	100
	2	98	0.64	94
	6	75	0.61	91

Table 11: stability of β-carotene in canned citrus beverages. [172]

Despite carotenoids susceptibility to decomposition during thermal industrial practices (juicing, maceration, cutting, thermal treatment), following optimal practices will result in a good retention rate. Maintaining as low of a

temperature and shortest time as possible, and reducing chances of oxidation can all help maximize the retention percentage. In term of thermal treatments and their effect on the retention of provitamin A loss, sautéing is the most harmful and microwaving is the least destructive with steaming and boiling scoring in the middle range. However any of these methods can be severely destructive if applied for prolonged time, at higher temperature, and accompanied with disturbance of tissue (cutting) and cellular compartmentalization (maceration) [17].

Another concern is photodegradation, storage of carotenoid-containing food material in a opaque container to prevent photodegradation might *not* be necessary when carotenoids are present in their natural source and not an extract present in an organic solvent; however favorable. Gil et al. (2006) [158] compared whole mangoes and cut mangoes in term of photodegradation of carotenoids and concluded that transparent containers had “no detrimental effect on nutritional quality” in term of retention of antioxidants in mango, samples stored in the dark for 9 days at 5°C contained 2272.1±164.6 µg per 100g of fresh weight compared to 2789.6±125.6 stored in light.

Thermal loss of carotenoid in mango juice solution

From previous literature review we can expect that the thermal treatment of SPI in mango juice solution (holding at 95°C for 30 min) would be comparable to other common practices in the food industry and should not results in extreme carotenoids loss. In an effort to evaluate the effect of thermal treatment on

carotenoids content in mango juice solution, samples were heated to various temperatures. Holding the sample at 95°C for up to 30 min, caused approximately 6% reduction of total carotenoids (as β-carotene equivalent) compared to original unprocessed sample (method of calculations are explained in the methodology chapter). The loss due to heating is statistically insignificant ($p<0.05$) between all temperatures up to 30 min at 95°C, figure (36). All proceeding calculations of percentage of adsorbed carotenoids are based on the amount present in the thermally processed juice solution.

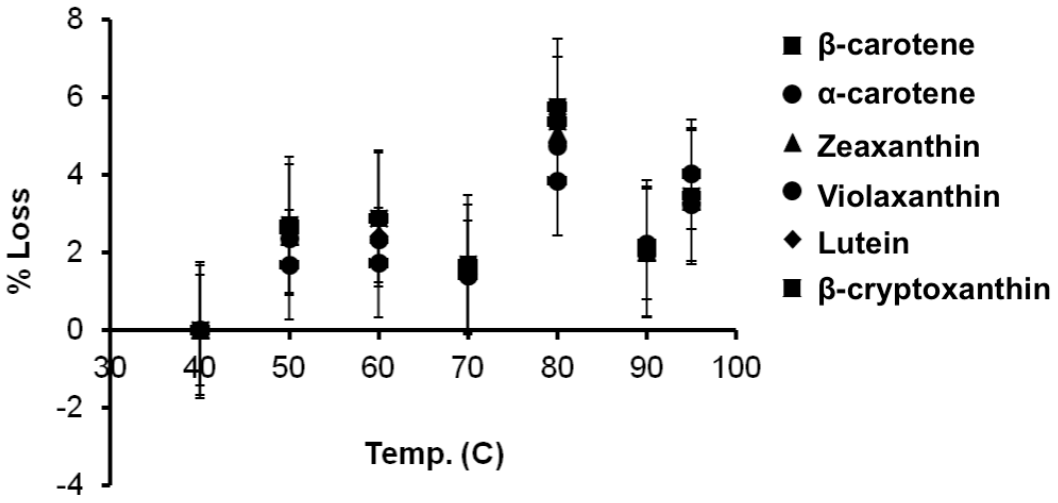


Figure 36: Change in β-carotene concentration in juice solution as function of temperature. Percentage of loss in comparison to original sample at room temperature.

Percentage of loss along the duration (30 min) of holding of sample at 95°C revealed a statistically insignificant change ($p<0.05$). Single sample t-test revealed that the reduction in all major carotenoid of interest suffered a statistically insignificant ($p<0.05$) loss in comparison to original unprocessed sample. Figure (37) shows the results.

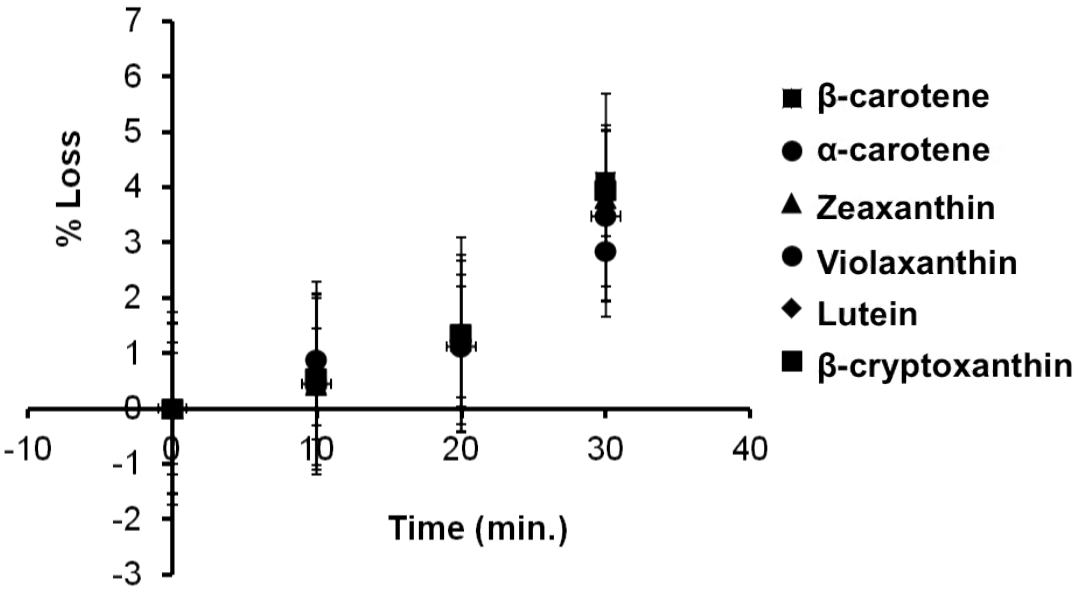


Figure 37: Percentage of loss of major carotenoids in mango juice solution in comparison to original sample at room temperature.

Though the losses are statistically insignificant, it is important to understand what it means in term of nutritive value of the food item. The greatest loss was as it would be expected, at the extreme heat treatment which is 30 min at 95°C resulting in approximately 6% decrease in comparison with original sample. Assuming a mean value of 2854.8 µg provitamin A carotenoids per 100 g of mango juice according to Gouado et al. (2007) [173], which is an equivalent of 2379 IU, thus provides ≅47.6 % of recommended DV of 5000 IU, rendering mango juice as a good source of provitamin A carotenoids. 6% loss reduces vitamin A content to ≅2236.3 IU, ≅44.8 % of DV, thus mango juice after proposed thermal treatment remains a good source of vitamin A [115]. Figure (38) shows color change of sample due to clarification, and thermal treatment.

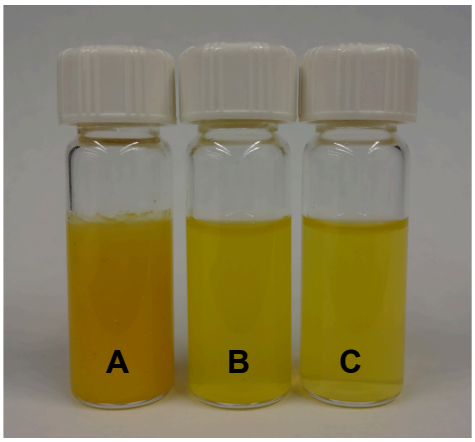


Figure 38: Sample A. 13°Bx mango juice, B. mango juice solution 12.8°Bx, C. sample (B.) after thermal treatment (holding at 95°C for 30 min).

Thermal adsorption of carotenoids

Now that we have established the safety of the thermal treatment in regards to carotenoid content in mango juice solution, we will begin to investigate SPI's ability to adsorb carotenoid from solution. Removal of carotenoids from solution causes a change in the absorption spectra in the 400-500 nm range, figure (39) illustrates a quantitative carotenoid spectra showing a decrease of absorbance in supernatant as SPI concentration increases.

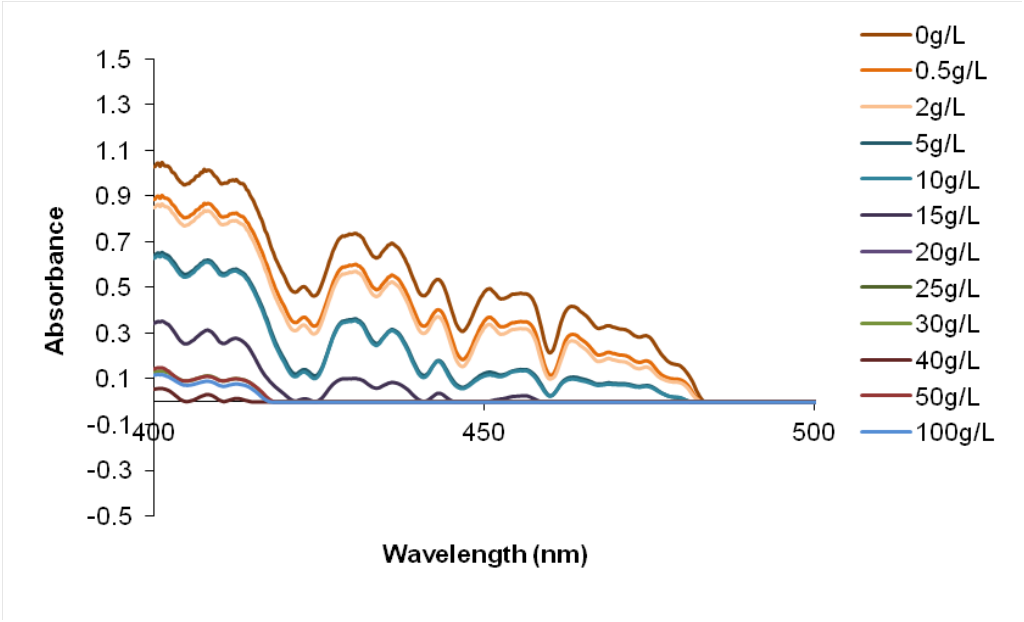


Figure 39: Absorbance spectra of carotenoids in visible range 400-500 nm. Increasing SPI concentration results in decrease absorbance of carotenoids in supernatant.

To examine the relationship between SPI concentration and its adsorption capacity, increasing concentrations of SPI were thermally processed with clarified mango juice. By quantifying the difference in percentage of carotenoids present in solution before and after thermal processing with SPI (as illustrated in the processing scheme in the methodology chapter), the percentage carotenoids adsorbed by SPI can be calculated. Figure (40) demonstrates direct relationship between SPI concentration and percentage of carotenoids adsorbed.

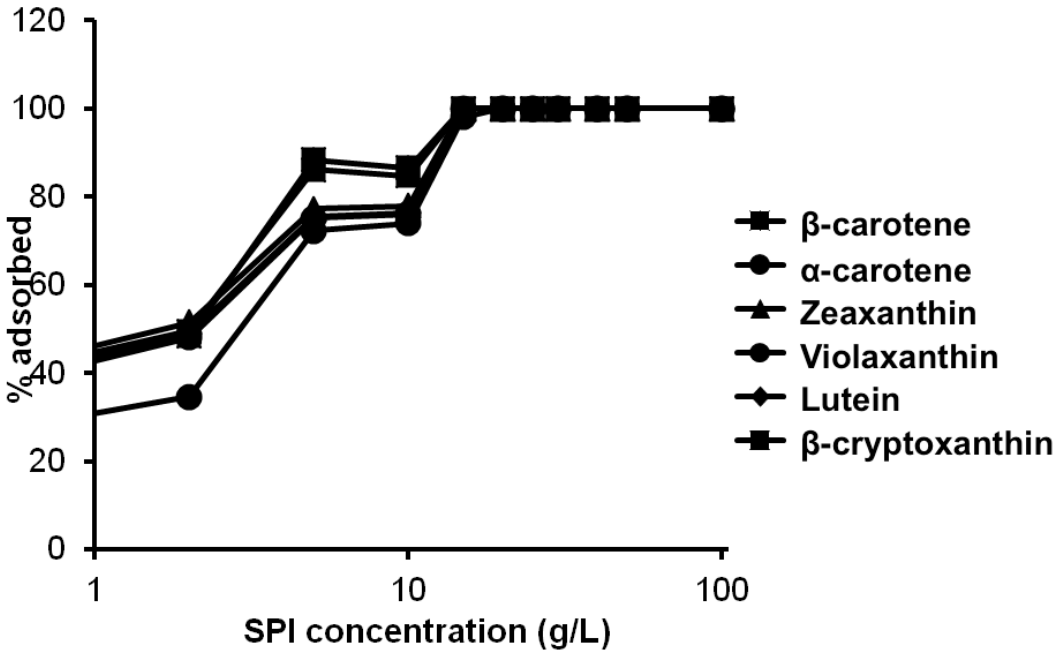


Figure 40: Percentage of major carotenoids adsorbed as function of SPI concentration (g/L).

It is remarkable to learn that 0.5 g/L of SPI is able to adsorb 38.26% of β-carotene; the greatest adsorption was recorded for Zeaxanthin with 40.89% retention at just 0.5 g/L SPI concentration. Increasing SPI concentration was accompanied with increasing carotenoids retention. All examined carotenoids adsorbed in similar order of magnitude with the exception of Violaxanthin that

reported lower percentages. The adsorption maximum was reached at 15 g/L for all carotenoids with the exception of violaxanthin where maximum adsorption capacity is reached at 20 g/L of SPI with 98.06% retention.

Full spectrum is not reported here since the shape does not provide much information, the shape can only be useful in the identification of purified carotenoid extracts or pure standard [105]. However, it is important to note that there is a real possibility for misestimation (i.e. underestimation) of carotenoids concentration due to the use of the arbitrary value of $\varepsilon = 2500$ [105].

Since the presence of color correlates well with carotenoids content, the change (i.e. decrease) of color intensity is an evidence of removal of pigments from solution [14, 170] in comparison to thermally processed sample at 0g/L SPI. Figure (41) demonstrated the changes of color due to processing with SPI, the intense opaque color of mango juice is due to the presence of high amount of pulp, removal of pulp by centrifugation renders the sample as a better fit for spectroscopy measurements. The light pigmentation of sample of SPI processed mango solution is an indication of incomplete removal of carotenoids, these carotenoids might be either unavailable for adsorption due to their existence in complexes with some cellular matter, or it is due to the threshold of maximum adsorption capacity by SPI. The reported 100% removal despite the presence of light color in sample supernatant can be attributed to admitted inaccuracy of the estimation process.

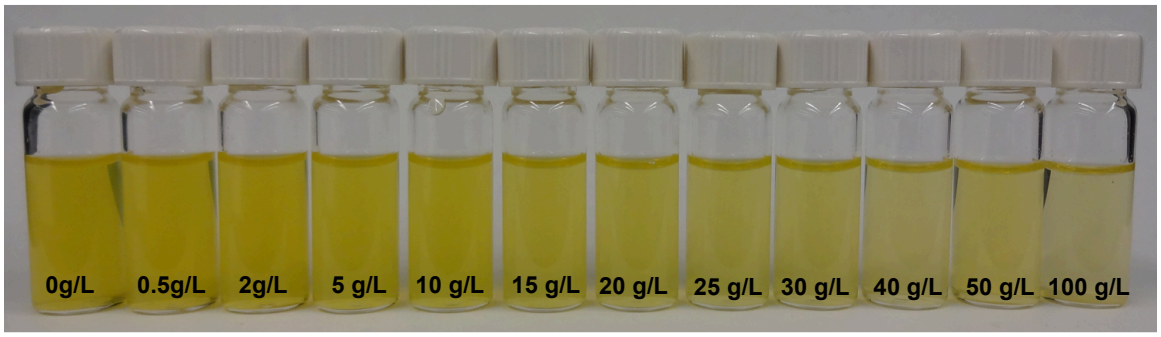


Figure 41: Supernatant of mango juice solution after thermally processing with increasing concentration of SPI. Gradual reduction of color intensity can be observed from left to right, this is a visual indication of reduction of carotenoids content in supernatant.

Evaluation of the efficiency of the adsorbent materials is accomplished via quantitative measurements of the concentration of adsorbable component before and after treatment. Constructing adsorption equilibrium though do not give an insight toward the underlying adsorption mechanism, it does provide information regarding the adsorption equilibrium through the relationship between concentration of adsorbate per unit weight of adsorbent, and concentration in solution. To construct adsorption equilibrium, we based carotenoid content calculations on the findings of Gouado et al. (2007) [173] presented in table (12) below:

Carotenoid	µg/g mango juice	µg/g mango juice after thermal loss
β-carotene	27.970	25.732
α-carotene	0.447	0.411
β-cryptoxanthin	0.131	0.120
Zeaxanthin	0.023	0.021

Table 12: carotenoids content in mango juice according to Gouado et al. (2007) [173]

Based on the 6% overall reductions due to thermal treatment, new values were used for adsorption calculations. The results are presented as an equilibrium isotherm where the y-axis is the amount adsorbed per unit weight of

adsorbent and the x-axis represent the limiting factor, in our case SPI concentration, figure (42).

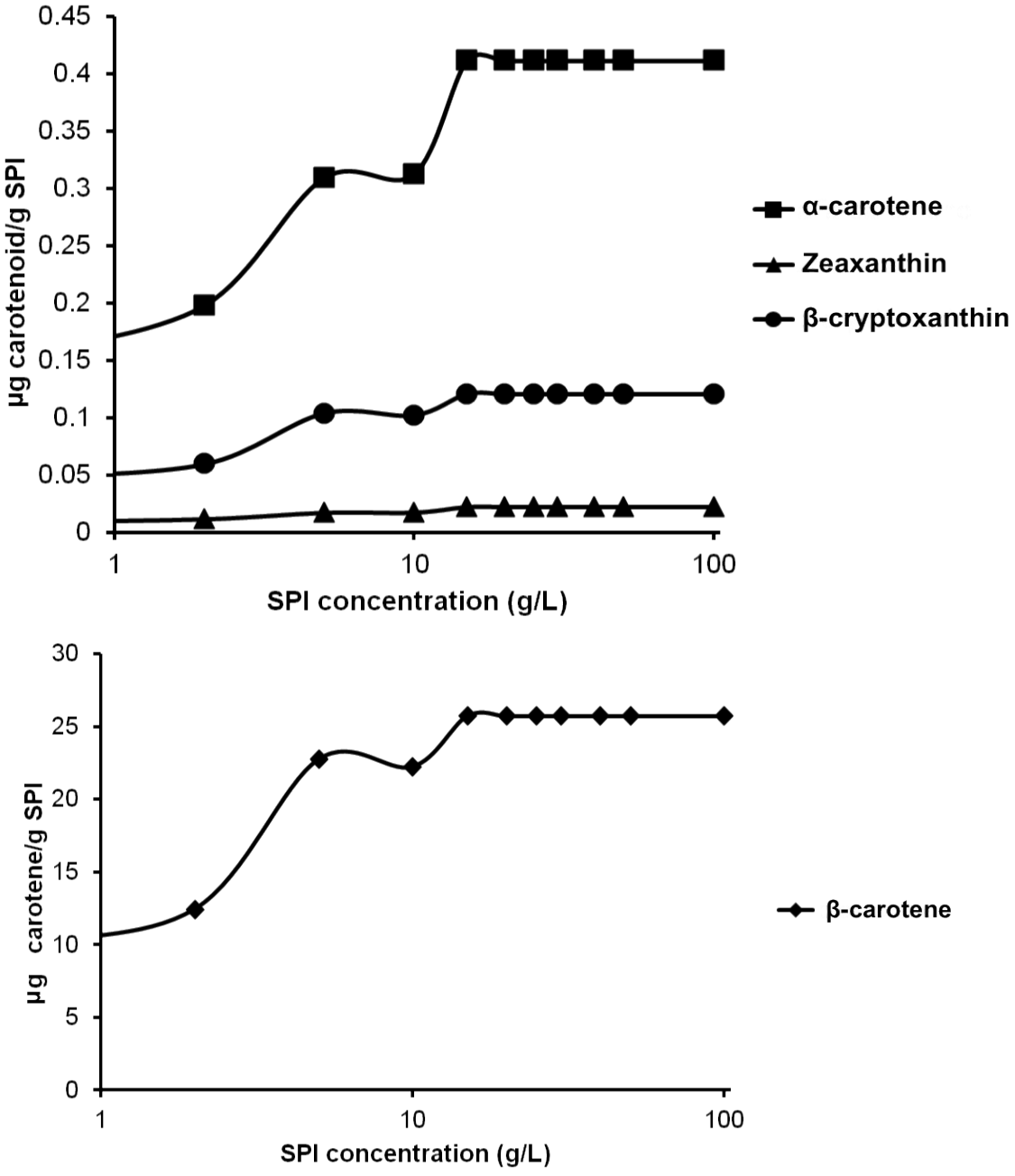


Figure 42: carotenoids content per g of SPI.

To evaluate the nutritive value of, β-carotene values are used for the calculations of IU of vitamin A. At the highest adsorption capacity of 15g/L, 1 g of SPI adsorb 25.73 µg β-carotene an equivalent of ≅42.9 IU, 11.7 g of carotenoids enriched SPI is able to provide 10% of DV (5000 IU) of vitamin A, rendering it a good source of vitamin A. Beside the nutritive value of SPI-carotenoids ingredient, the change of color of SPI might be beneficial for aesthetic appeal of food item, figure (43).

Figure 43: Left, SPI prior to adsorption process. Right, SPI after thermal processing in mango juice solution at 10g/L concentration.

Adsorption of sugars

To determine if sugars were adsorbed by SPI during the mixing and heating process, we measured level of total sugars using a refractometer at RT, results are presents as Brix value. The original sugars content in mango juice solution averaged 12.8°Bx, single-factor ANOVA of all value revealed an insignificantly difference ($p<0.05$) in total sugars content thermally treating the sample with SPI, figure (44) reports average values of total sugars content in supernatant after thermal adsorption processes. T-test of unequal variance also reported insignificant difference ($p<0.05$) between the concentration in each

group, and the averages of concentrations that is represented in figure (44). These results indicate that soy protein did not bind or concentrate the juice's sugar content. These results are consistent with Roopchand et. Al (2012) [174] findings, it was reported that there were no significant difference in the sugar content between treated and untreated juice when defatted soy flour was used as adsorbent of polyphenols in juice at room temperature.

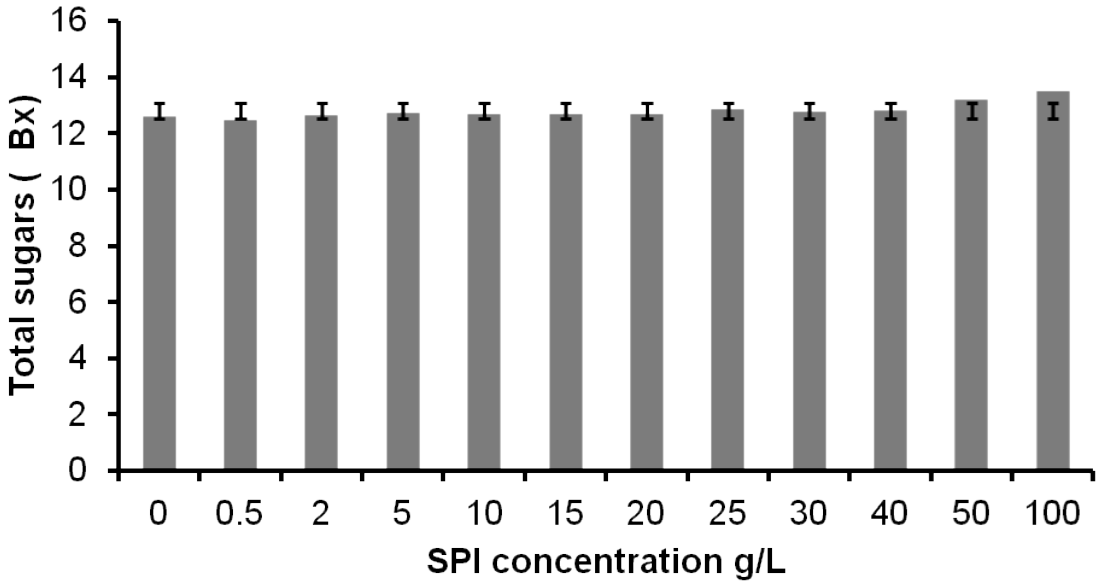


Figure 44: Insignificant changes in sugar content in the supernatant of mango juice solution after the adsorption process. The mean value 12.8 °Bx, SD 0.27, p-value = 0.01.

Lyophilization of SPI

Examination of particle size of re-suspended lyophilized SPI reveals an overall reduction in average particle diameter at all pH condition examined. Since samples have been thermally treated prior to lyophilization, particles size are expected to have increased due to formation soluble aggregates, however reduction of particle size afterwards suggests deaggregation of these clusters which indicates the preservation of original structure possibly due to partial

spontaneous refolding upon cooling refolding as suggested by Damodaran (1988) [70]. Results are illustrated in figure (45). Such results also suggest good dispersibility qualities of lyophilized SPI, thus suitable for many food-related applications that require adequate solubility. Zeta potential measurements supports our speculations, negative zeta potential at all pH values expect pH 2.0 indicates repulsive forces that maintains dispersibility of protein in solution. In term of adsorption process, solubility and adequate dispersion is keen for contact with hydrophobic compounds that are of interest.

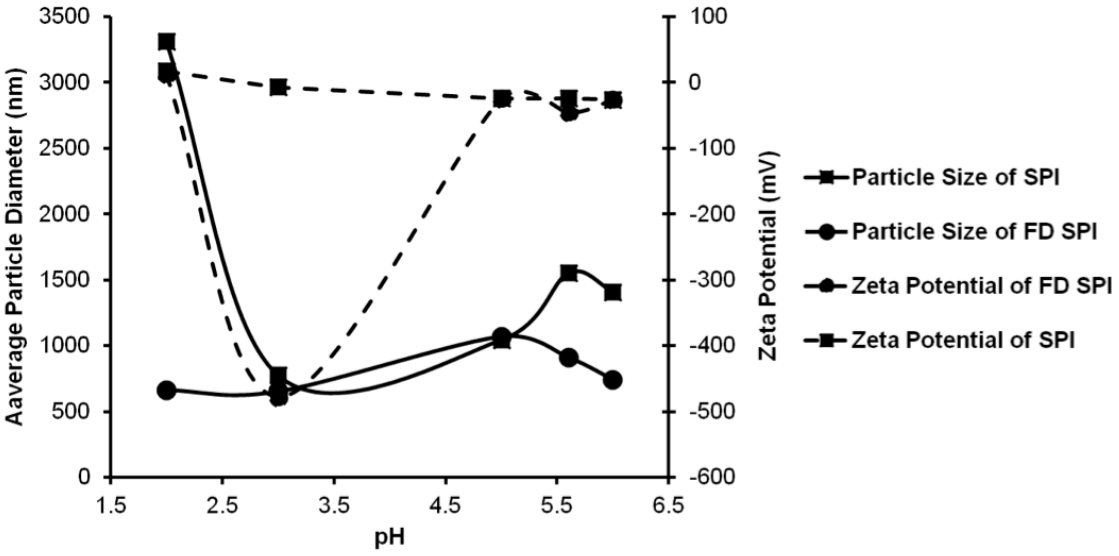


Figure 45: Change in average particle size diameter (nm) and zeta potential (mV) of SPI before and after lyophilization treatment at various pH conditions. Freeze-dried SPI was resuspended in DW.

The practice of freeze-drying thermally denatured SPI might potentially result in the formation of a functional ingredient that not only posses dense nutritive matter, but also introduce a functional quality of gelation at room temperature. In the work of Cramp et al. (2008) [147] where it was attempted to

produce SPI that is able to form a gel at ambient temperatures by rehydration. 8% w/w SPI suspension was first thermally treated by heating to 95±3°C for 3 h followed by lyophilization to produce a powder. The resulting powder showed good dispersion qualities and caused immediate increase in viscosity upon addition of water at room temperature, greater than that caused by control SPI.

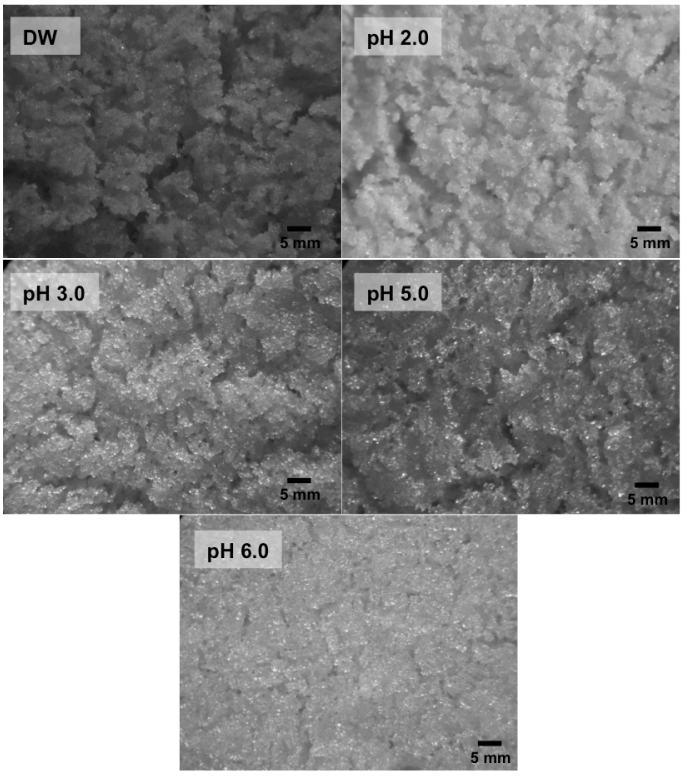


Figure 46: Microscopic imaging of freeze-dried SPI after thermal treatment in acidified water.

Proposed adsorption mechanism

Adsorption is defined as the process producing net accumulation of a substance at the common boundary of two contiguous phases [175]. In the adsorption processes, adsorbate are present in a gas or liquid phase and would adhere to a solid phase (adsorbent) [176], accumulation on the surface

differentiate adsorption from absorption which results in accumulation of concentration within the bulk of the solid or liquid, example of absorption would be the diffusion of carbon dioxide in water, thus present within the bulk and not restricted to the water-air interface.

For centuries some solids have been used to remove contaminating dyes from water, the use of charcol for decolorizing liquids, and bone char in refining sugars continue to the present time. One of the earliest large scale adsobance practices was created in the oil industry where clay is used to refine and decolorize oils and fats. Industrial applications initiated the transition from patch process to continuous flow configuration. Adsorption is employed in chromatographic analysis on laboratory scale and to some extent in the pharmaceutical industry in the preparation of some products. [177]

Experimentation as well as computational modeling have rendered some new natural and synthetic materials as efficient adsorbents. Some of the common natural adsorbents are silica gel, activated alumina, and clay material. Synthetic non-ionic polymers are commonly used for analytical purposes, monomers are prepared in an appropriate solvent that enables the formation of a network. Adsorbent properties can be tailored for application and adsorbate by changes in structure, pore size distribution, surface area and chemical nature of matrix. Properties such as accessibility of high internal volume (porous solids are a common example, high internal surface area decodes higher capacity for adsorption), suitable mechanical properties (strength, resistance to attrition), optimal kinetic properties (adsorption rate), ease of adsorbate separation, and

ability to repurpose and reuse without loss of efficacy; are frequently factored in the design and selection process of adsorbent material. [177]

We are hypothesizing the possible mechanism of adsorption based on our understating of the adsorption of proteins and carotenoids in adsorption chromatography. Two categories of adsorption are frequently employed for analytical as well as manufacturing purposes, chemisorption and physisorption. Chemisorption is cause by strong chemical bonding occuring between adsorbent and adsobate [176]. While physisorption is defined as “a weak interaction characterized by lack of true chemical bonding between adsorbate and surface” [178]. Physisorption immobilizes polymer at surfaces base on physical interaction between particular parts of the polymer with the surface due to the preference of this interaction over that with the bulk of the solvent, the preference is due to formation of a state with lower free energy of the system [179].

Identifying the adsorption mechanism means clearly identifying the interaction occurring at the adsorbent/adsorbate interface. In the case of widely used adsobents such as carbon, the exact and detailed mechanism is still controversial even with the development of several models. The complexity is cause by the large number of of interactions possible that are likely to occur simultaneously [180]. Forces involved in physisorption are categorized into attractive forces and repulsive forces. Attractive forces such as Van der Waals are caused by the formation of dipole of opposite signs on adsorbate and surface [178]. H-bonding is also considered part of the attractive forces despite its chemical nature due to its weakness compared to other chemical bonds.

Repulsive forces are generated on previously attracted particles when the distance between the two species are reduced greatly causing an increase in the kinetic energy of electrons [178]. Hydrophobic effect, electrostatic adsorption, and stacking, are all weak bonding. Samori (2010) [181] have suggested that proteins being large flexible polymers, might be able to form different contacts depending on a.a. composition on exposed surface. This large number of bonds along the polymer structure results in over all strong interactions that is analogous to covalent bonding. Protein's amphiphilic structure results in the formation of solvent polymer interface via hydrophilic shell, while hydrophobic core is excluded from contact with polar solvents [179].

In our system of SPI in carotenoid-containing aqueous solution, heating dispersed proteins causes denaturation exposing protein's hydrophobic core. This void is exposed for as long as the thermal influence is maintained, thus is available for adhering to other hydrophobic molecules present in close approximation. At high protein concentration, protein-protein interaction is favored, however other compounds with similar structural hydrophobic qualities would also adhere to protein's hydrophobic site. The maximum adsorption capacity was reached at 15g/L, an equivalent of 1.5 %w/w SPI-Juice solution, thus, far below the critical limit of 10%w/w required for the formation of SPI heat set gel. Consequently carotenoids that are well dispersed in solution are favored for adsorption. Figure (47) illustrates the hypothesized mechanism of adsorption.

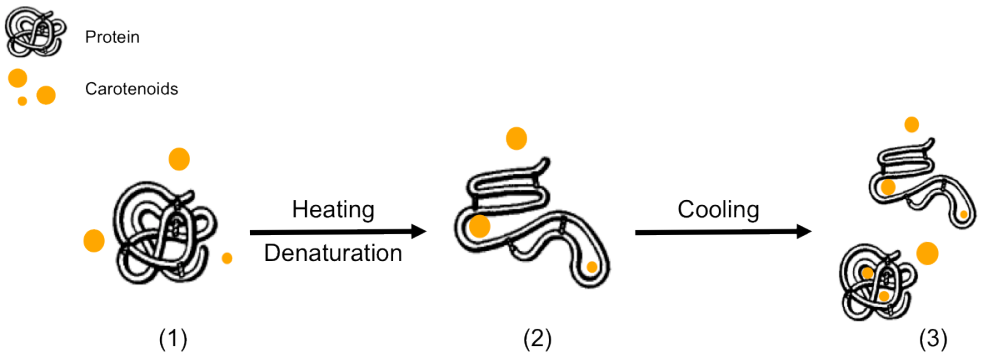


Figure 47: Visualization of possible interaction between denatured proteins and carotenoids present in aqueous medium. (1) Below denaturation temperature of the least heat stable fraction (7S) proteins are intact in carotenoid-containing solution. (2) At temperatures above denaturation point of (7S) and (11S) exposed hydrophobic cores act as adhesion site for hydrophobic carotenoids. (3) Upon cooling some proteins remain in disordered state with adsorbed carotenoids continuing to attach to hydrophobic sites, while others have the ability of partial refolding and regain of original structure causing entrapment of adsorbed carotenoids within protein structure.

The hydrophobic nature of both adsorbate and adsorbent strongly suggests the involvment of one specific physical effect, hydrohobic interaction. Hydrophobic-hydrophobic interactions are attractive in nature, and are stimulated by unfavotable hydrophilic-hydrophobic interaction. These attractive forces originates from Van der Waals-London dispersion forces as well as mutual attraction of hydrophilic solvent molecules in which hydrophobic moelcules are dissolved. Hydrophobic interaction are weak, weaker than electrostatic interaction. [182]

The numbers of variables in the system of SPI/carotenoid-containing solution are large and potentially imperative to the efficiency of the adsorption process. These factors can be divided into three main categories, protein-related factors, carotenoid solution-related factors, and process-related kinetics. First of all, the selection of appropriate protein with desired qualities such as solubility and degree of thermal stability are essential. Solubility of protein in solution

determines its mobility and hence its contact with adsorbate. Thermal stability of protein needs to be balanced with the degree of thermal stability of adsorbate. Both factors are largely governed by solution conditions (pH, ionic strength, presence of contaminants); hence many of these factors are interrelated, and system specific.

Solubility affects mobility of a polymer in the bulk and is related to polymer concentration. At high protein concentration, formation of heat-set gel is feasible under appropriate conditions of heat, pH and ionic strength. Formation of gel could potentially increase the amount of carotenoids removed from solution by entrapment of volumes of solution in gel matrix, and not solely through adsorption by individual proteins. Accordingly protein-protein interactions are a limiting factor and create an upper limit for the concentration that can be used. However maximum adsorption capacity was reached at 15g/L, an equivalent of 1.5 %w/w SPI-Juice solution, thus, far below the critical limit of 10%w/w required for the formation of SPI heat set gel. Adsorption is a concentration dependent process, only if the binding sites have varying affinity, thus sites with greater affinity are populated first, resulting in an inconstant adsorption coefficient [183]. In the case of SPI-mango solution system, formation of SPI-carotenoids complex, presumably by adhering in pairs of protein and carotenoid molecules, the specie with lower concentration would be the determining factor for the final number of complexes that can be achieved. Therefore the process is concentration limited. The cut off limit for SPI concentration was found to be 15g/L at concentration close to single strength mango juice.

The adsorption process is governed by the presence of both elements in solution, contact between adsorbate and adsorbent is essential, solubility and continuous agitation during thermal treatment can ensure adequate contact throught the thermal treatment process. Contact time is another variable frequently addressed in adsorption process, however in our system the adsorbent – hydrophobic core of protein- is available as long as the thermal influence is maintained, after which spontaneous refolding occur during the cooling stage. Consequently holding time at or above the denaturation temperature could potentially affect the adsorption capacity. Sorption is often considered a rapid process, and equilibrium is reached within few hours due to the general consensus on the rapid and extensive occurrence of sorption in the early stages followed by a long and slow sorption before equilibrium is reached [184]. Thermal adsorption however might not be entirely comparable to common sorption process due to the extremely short time span in which protein's hydrophobic core is available for adsorption.

In an ideal solution where only our compound of interest has afiinity to sorb to adsorbent, competitive sorption would not be an issue; however food systems are inherently complex. This issue might require prior treatment of food matrix to eliminate undesired compounds. Competiitve sorption might not be strictly related to hydrophobic compounds; ions and other hydrophilic compounds might sorb to protein hydrophilic shell potentially affecting sorption capacity to its hydophobic core [174, 185]. This rather lack of selectivity might introduce a

number of additional micronutrients, producing even a more nutrient dense compound with wider range of potential health benefits.

This short list of interrelated factors gives a glimpse of complexity of a system compromised of natural materials. Such complexity can be simplified by experimental reasoning and data generated by testing one factor at a time, in addition to computer models that simulate processing condition in order to obtain an optimized sorption process [176]. More detailed understanding of adosption-related amino acids is another area of interest, Chunga et al. 2010 [186] were able to generate more details abuout the nature of protein adsorption in multimodal chromatography system by using mutations of original protein where a group of amino acids are removed and adoption affinity and efficiency is measured in relation to absence of these amino acids. This approach enables the making of recommendation for most appropriate portion depending on a.a. composition and recognition of binding sites.

Conclusion

Our aim was to develop a solvent-free process that utilizes SPI as an adsorbent of carotenoids resulting in nutritious functional ingredient that harvest the cumulative nutritional value of soy proteins and carotenoids. The first goal was to study the thermal denaturation behavior of SPI. DSC measurement demonstrated two endothermic peaks at 42°C and 72°C corresponding to the two major protein fraction, β-conglycinin and glycinin respectively. Rheological measurements of SPI during thermal treatments at concentration below heat-gelation critical limit revealed details on the molecular level regarding protein-protein interaction that occurred at characteristic temperature points. Results confirmed that the upper limit of 9% w/w SPI concentration results in the formation of a solution of solution aggregates rather that a gel network, thus this concentration is a limiting factor at which adsorption of carotenoids is attainable via individual molecules rather than physical entrapment with gel matrix. UV/visible spectroscopy of SPI suspension as a function of temperature at 280 nm clearly coincided with DSC results and rheological measurements where protein hydrophobicity increases at or above the denaturation point of the least heat stable fraction, 7S at 42°C.

Determining the feasibility of carotenoid sorption using SPI was accomplished using optical measurements. The safety of thermal treatment was established prior to adsorption study, proposed thermal treatment resulted in approximately 6% reduction of total carotenoids (as β-carotene equivalent) compared to original unprocessed sample. Loss due to heating was statistically

insignificant ($p<0.05$) between all temperatures tested in the range of 25-95 °C. Adsorption measurements of β-carotene, α-carotene, β-cryptoxanthin, Zeaxanthin, and Violaxanthin demonstrated similar order of adsorption magnitude as a function of SPI concentration with the exception of violaxanthin that reported lower percentages. Adsorption maxima were reached at 15 g/L for all carotenoids with the exception of violaxanthin where maximum adsorption capacity is reached at 20 g/L of SPI with 98.06% retention. Soy protein did not bind or concentrate the juice's sugar content during the adsorption process.

Adsorption included both carotenes and xanthophylls, inclusion of a wide variety of carotenoids into one matrix suggests greater health benefits compared to isolated carotenoids in form of supplements or food additives. In summary, it is possible to utilize the exposure of the hydrophobic core of proteins during thermal denaturation to adsorb hydrophobic compounds present in an aqueous medium resulting in a nutrient dense functional ingredient.

Future work and research opportunities

No research is ever complete; there is always a room for exploration and generation of new findings. Data and conclusions presented in this preliminary research act as a platform for the generation of more detailed research focus.

It is our intention to study the research problem from a feasibility and simplified point of view without introducing the level of complexity associated with two extremely complex food systems. However varying processing history of protein, protein variety, and amino acid composition can all reveal insightful information regarding the adsorption process that can ultimately be used for adsorbent selection.

Testing the efficiency of adsorption from other natural sources is required for validation of the concept on a wider scale. In order to maintain a sustainable green technology -and reduce production cost- like the one proposed in this project it is wise to use material that are available locally to reduce the cost and environmental effect associate with transport. Material such as food-agricultural by-products and biological waste would prevent secondary waste generation.

Adsorption conditions and kinetics in term of temperature-time dependency of the process could uncover details of the nature of the process. Experimenting with possible other denaturants that are food grade, or continuing the use of thermal denaturation under conditions that enable the use of lower temperature and shorter time that could improve the overall carotenoids retention rate.

Bioavailability of carotenoids present in a SPI-carotenoid complex needs to be evaluated before any health claims, dosage, and dietary recommendations can be determined. This complex and long process is an interesting multidisciplinary research opportunity. Shelf life study and product stability are also necessary for the validation of a wide industrial application. In addition, presence of carotenoids in a complex with soy proteins might have an effect on its functional properties an processing qualities, limiting its use to a source of nutrients.

Appendices
Appendix 1

iTi tropicals

11/12/2012

MAHA ALSHEHAB
Rutgers University
65 Dudley Road
Lab 401
New Brunswick, NJ 08901
(732) 668-8013

iTi tropicals Inc.

Tropical Fruit Purees,
Concentrates & IQF Pieces

Dear Customer:

Subject: Sample Request

Product	Code	Quantity
Aseptic Totapuri Mango Concentrate	9450S	4

We take great care to package these products well. However, at times, delays and/or damage in shipping will occur. Please let us know if you're aware of any problems with the samples we have sent, and we will send a replacement shipment.

Please note that many mango varieties when re-packed aseptic and broken down and re-packed into small, frozen sample jars presented to you in this box. The frozen mango samples will typically show seperation after defrosting as a result of the freeze/thaw process. You will need to remix or blend the sample until smooth to obtain an accurate view of what product will look like when delivered in the original aseptic packaging to you.

At iTi Tropicals, Inc., the focus is on Tropicals: Banana, Guava, Mango, Passion Fruit, Papaya and Coconut. For more information about our company and our products we invite you to visit our web sites at:

www.ititropicals.com • www.passionfruitjuice.com • www.tamarindfruit.com • www.acerolajuice.com
www.mangopuree.com • www.bananapuree.com • www.youngcoconutwater.com

Sincerely,

Dalia Lerman
Account Manager
iTi Tropicals, Inc.
Phone: 609-986-2093
Email : dalia.lerman@ititropicals.com

Appendix 2

iTi tropicals

iTi tropicals Inc.

Tropical Fruit Purees,
Concentrates & IQF Pieces

SPECIFICATIONS

PRODUCT:	**ASEPTIC MANGO CONCENTRATE**
ORIGIN:	**INDIA**
CODE:	**9450/9451**
BRIX:	Min. 28°
PH:	3.6 - 4.2
ACIDITY:	Max. 1.3% as citric acid
COLOR:	Yellow Orange
FLAVOR:	Natural Mango Free from off flavor & odor
VARIETY:	**TOTAPURI**
TEXTURE:	Creamy paste No additives or preservatives
HARVEST:	June
STORAGE:	Room temperature (approx. 70°F)
SHELFLIFE:	24 months at room temperature.
PACKING:	Steel Drums at 503 lbs net Cardboard Cartons at 44 lbs net
MICROBIOLOGICAL:	Total Plate Count < 10 cfu/g Yeast and Mold < 10 cfu/g

The specifications of each batch may vary from the specifications above based on a number of factors, including crop conditions. The specifications for each batch are set forth in the certificate of analysis from the producer.
Note: Use product immediately after opening

Depending on crop conditions specifications are subject to variation. *June 29, 2011*

www.iTitropicals.com

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