SOLVENT-FREE EXTRACTION OF ANTHOCYANINS BY SOY GLOBULAR PROTEINS

By

YUXIN WANG

A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Food Science

written under the direction of

Paul Takhistov

and approved by

_____

_____

_____

New Brunswick, New Jersey

[May, 2012]

ABSTRACT OF THE THESIS

Solvent-Free Extraction of Anthocyanins by Soy Globular Proteins

By YUXIN WANG

Thesis Director:
Paul Takhistov

According to the global health risks report（2009）, insufficient intake of fruit and vegetables was estimated to cause around 14% of gastrointestinal cancer deaths, about 11% of ischemic heart disease deaths and about 9% of stroke deaths worldwide (Mathers, Stevens et al. 2009). Anthocyanins, a major type of phytonutrient in berry, are considered to be able to provide the positive effect on health and it has been drawn great attention by institution and food industry. Thereby, it is significant to concentrate the anthocyanins into food matrix by an efficient method.

This study covers the adsorption process of the cranberry juice anthocyanins by soy protein isolate (SPI). Provided results indicate that the soy protein isolate can effectively adsorb the anthocyanins and can be used for successful extraction of it from the low acid juice matrix. The adsorption capabilities of the SPI are tested at various temperature and pH values. It is found that the optimal processing condition for the adsorption is with 45℃ at pH 7. Particle size and zeta potential of the SPI are investigated, and the results of conformational changes in soy protein as the

processing conditions vary describe the mechanism of adsorption. Chemical changes in vibrational spectra of soy protein and anthocyanins are monitored. Mathematical modeling of the adsorption equilibrium reveals that both Langmuir and Dubinin–Radushkevich isotherms well describe the adsorption equilibrium of anthocyanins onto soy protein isolate, and process kinetics follows the pseudo-first order. The mean free energy of adsorption per molecule of adsorbate (i.e. anthocyanins) is equal to 1.96 kJ/mol, which corresponds to an electrostatic physicosorption nature of anthocyanins–soy protein interaction. Proposed processing method for the extraction of health-promoting phytonutrients from cranberry juice by plant protein matrix can be applied for wide range of juices and extracts and used for development of new high value food products.

ACKNOWLEDGEMENT

I would like to show my heartily gratitude to my advisor, Paul Takhistov, whose encouragement, guidance and support in every stage enabled me to develop an understanding of the subject and accomplishments of my thesis. My sincere appreciation goes to Dr. Yam and Dr. Daun for all their kindness and help as well.

I also would give special thanks to my parents, who have supported me throughout my years of education, both morally and financially. Last but not least, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

TABLE OF CONTENTS

LISTS OF TABLES

LISTS OF FIGURES

1. INTRODUCTION

Consuming food is an indispensable part in human daily life. Each food intake provides different nutrients, which plays a key role in regulating the metabolism and maintaining body's function at the normal level. Food selection could make a potential influence on people's health. Diet with too much salt or alcohol could lead to raising blood pressure; Diets high in saturated fat can increase cholesterol levels. WHO global health risks report (2009), even predicted that, due to changes in diet, the number of overweight people and obesity will increase in most countries, with 1.5 billion people in 2015. On the other hand, a good eating habit is beneficial for people by reducing the chance for being ill.

In 2011, the dietary guidelines for Americans were released and emphasized three major goals for Americans. One of them is consuming more certain foods and nutrients such as fruits, vegetables, whole grains, fat-free and low-fat dairy products, and seafood. The evidence demonstrates that increasing intake of high fiber foods that rich in folacin and other B-complex vitamins can reduce heart attack risk by 80%. According to the global health risks report（2009）: The top risks leading to mortality around the world are high blood pressure, which is responsible for 13% of deaths globally, tobacco usage (accounts for 9%), high blood glucose (6%), physical inactivity (6%), and overweight and obesity (5%). These risks highly bring about raising the possibility of chronic diseases such as heart disease, diabetes and cancer. Fruit and vegetable consumption is broadly recognized as one element of healthy diet. Insufficient intake of fruit and vegetables is estimated to cause around 14% of

gastrointestinal cancer deaths, about 11% of ischemic heart disease deaths and about 9% of stroke deaths worldwide (Mathers, Stevens et al. 2009). Most health benefits of fruits and vegetables come from reduction in cardiovascular disease, prevention cancer; meanwhile keeping the figures and decreasing the rate of obesity (Fund 1997; Ness and Powles 1997).

Food products compose of variety of mixtures containing many complex chemical entities. There are three terms-functional food, nutraceuticals and dietary supplements – relevant with nutrient-rich food are always confused by people. Functional food is a very popular term and has attracted people's attention, not only in food industries but also the general public. The term functional foods were first introduced in Japan in the mid-1980s and usually refer to the processed foods containing ingredients that aid specific bodily functions. From the literal aspect, when food is being cooked or prepared using "scientific intelligence" with or without knowledge of how or why it is being used, the food is called "functional food." A food can be said to be functional if it contains a component that affects one or a limited number of functions in the body in an expected way, leading to positive effects that may justify functional or even health claims (Haschke, Firmansyah et al. 2001). Thus, the active component in functional food covers up a broad realm, such as vitamins, fats, proteins, carbohydrates, etc,, providing the body with the required amount mutrition for the healthy survival. The functional food has either higher amounts of a biologically active substance than the conventional food or has been enriched with a bioactive ingredient not usually present. When functional food aids in the prevention or treatment of disease or disorders other than anemia, it is called a nutraceutical. At the meanwhile, nutraceuticals differ from

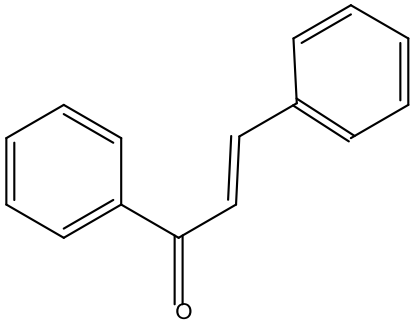
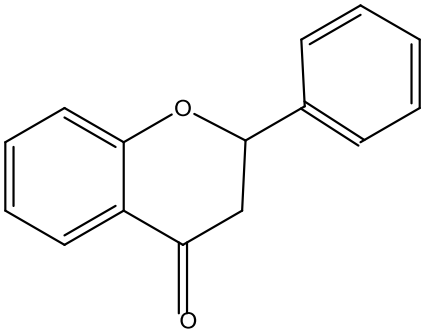
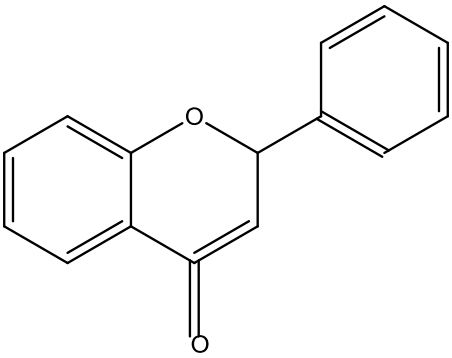
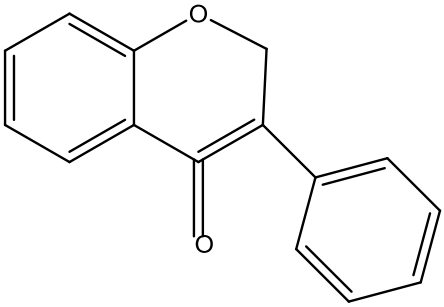
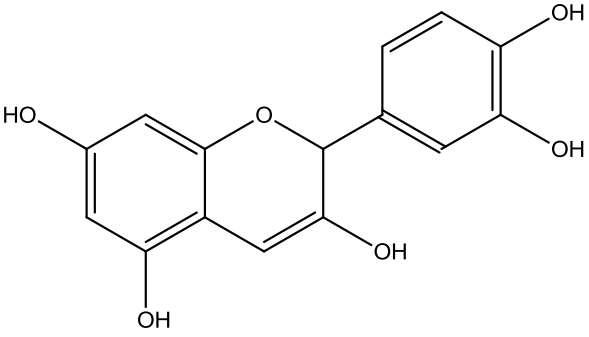
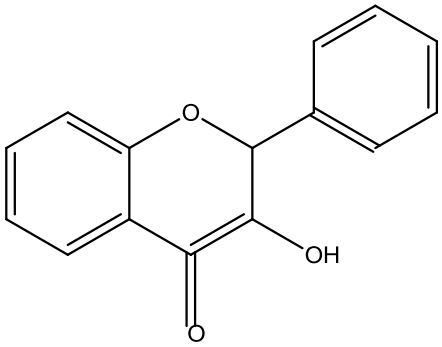
dietary supplements in that nutraceuticals must not only supplement the diet but should also aid in the prevention or treatment of disease or disorder (Kalra 2003).

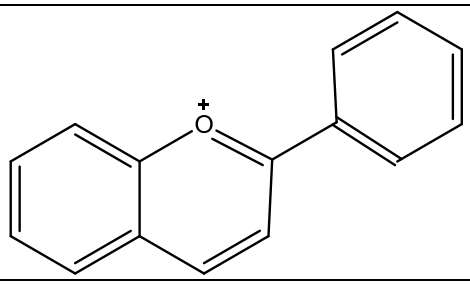
Phytochemicals are biologically effective chemical compounds that occur naturally in plants, this term is generally used to name those chemicals that may affect health, but are not necessarily essential nutrients. And phytochemicals are usually regarded to as either primary or secondary metabolites. Primary metabolites could be used mainly as industrial raw materials, foods, or food additives; Secondary metabolites are derived from primary metabolites and often have an ecological role (Morris 2003).

Flavonoids, considerable type of phytochemical, constitute one of the largest groups of plant secondary metabolites that originate from the phenylpropanoid and polyketide pathways. So far, more than 8,000 different flavonoid compounds have been discovered from vascular plants and bryophytes (Andersen and Markham 2006). According to the oxidation level of ring C, flavonoids are categorized into different groups which include the chalcones, flavanones, flavones, isoflavones, flavonols, proanthocyanidins and anthocyanidins (See Table 1). They are widely distributed in foods, such as fruits and vegetables, and beverages of plant origin (Zhou and Ibrahim 2010). In addition, they are considered an important part of the human diet and act as the active composites of many medicinal plants as well. For example, several plant flavonoids are epidemiologically associated with apoptotic pathways leading to lower cancer incidence, and cancer chemoprevention that yielded encouraging results both in vitro and in vivo (Wong and Lemoine 2009). There are already plenty of research showing that consumption of flavonoids is usually correlated with health benefits due to

their antioxidant, antiviral, antitum origenic, and cancer preventive activities (Kuo 1997).

Table 1 Chemical structure of flavonoids

	Chalcones	Flavanones
Structure		
	Flavones	Isoflavones
Structure		
	Proanthocyanidins	Flavonols
Structure		

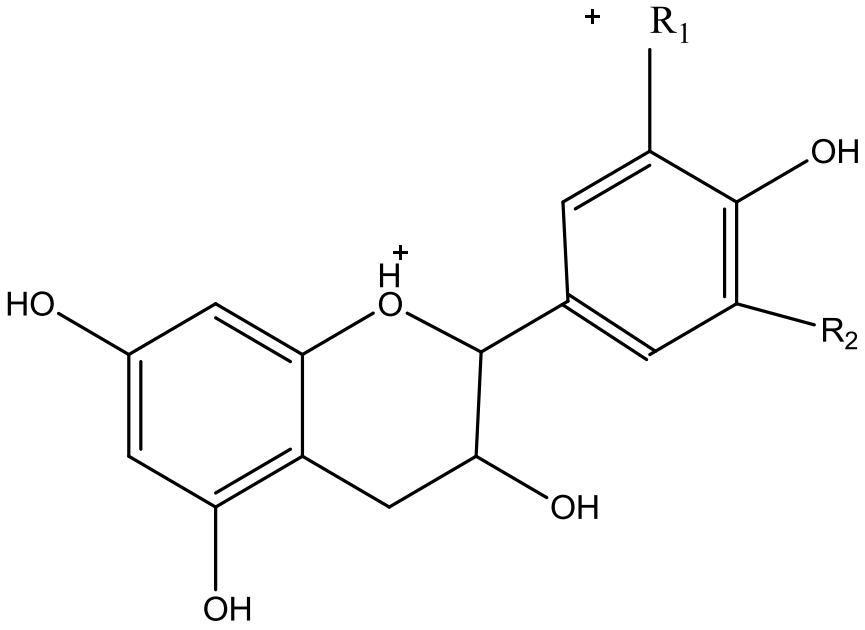
	Anthocyanidins
Structure	

Anthocyanins, a proanthocyanindin-type of flavonoid, are stabilized by modification of the aglycone forms (anthocyanindins) by glycosylation, methylation, and acylation. They contain a large quantity of functional phytochemicals and mostly occur in fruits such as cranberry, blueberry, orange, apple and in vegetables such as tomato, sweet pepper, spinach, and radishes. Consistently consuming anthocyanins comes to the positive effect on health. Anthocyanins have higher antioxidant capacity against oxidative stress induced by excess reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide, and thus the human body might be protected from oxidative injury by anthocyanins. Specifically, they inhibit release of reactive oxygen species from activated human granulocytes (Gsiorowski, Szyba et al. 1997) and suppress free-radical mediated lipid peroxidation and cell death in cultured aortic endothelial cells (Youdim, Martin et al. 2000; Youdim, McDonald et al. 2002). Moreover, anthocyanins aglycones and glycosides are effective inhibitors of oxidant-induced DNA damage in immortalized normal human colon cells (Duthie, Gardner et al. 2005) and potent inhibitors of tumour cell growth (Kamei, Koide et al. 1996; Hakimuddin, Paliyath et al. 2004; Duthie, Gardner et al. 2005). It could provide health benefits for age-related diseases as well as other diseases, and have been

clinically utilized in numerous medicines worldwide (Clifford 2000; Motohashi and Sakagami 2009).

The berry is regarded as a natural high-value food raw material and a source of many essential nutritional components such as anthocyanins, flavonols, flavan-3-ols, and phenolic acids. The studies show that cranberry is abundance of flavonoids (anthocyanidins, flavonols) and hydroxycinnamic acid (Heinonen, Meyer et al. 1998; Hakkinen, Karenlampi et al. 1999), and cyanidin and peonidin glycosides (see in Table 2) comprised the major anthocyanins content (Duthie, Jenkinson et al. 2006). From Table 3, in cranberry, 39.8mg/100g of the anthocyanidins are in form of cyanidin and 36.8mg/100g in the form of peonidins. There is around 600-2000 mg anthocyanins per kilogram of cranberries (Fulcrand, Benabdeljalil et al. 1998).

Table 2 Structural and spectral characteristics of the major anthocyanins.



Anthocyanins	R_1	R_2	Λ_{max}(nm) Visible spectra
Pelargonidin	H	H	494
Cyanidin	OH	H	506
Delphinidin	OH	OH	507
Peonidin	OCH_3	H	506
petunidin	OCH_3	OH	508
malvidin	OCH_3	OCH_3	510

Table 3 The major phytonutrients in cranberry

Major Phytonutrients in Cranberry (Milligrams per 100g of Fresh Weight)			
Major Phytonutrients	Sub-Class 1	Sub-Class 2	Quantity
Flavonoids (polyphenols)	Flavonols	Quercetin	19.4[a]
		Myricetin	16.6[a]
	Flavan-3-ols	Epicatechin	4.5[a]
	Anthocyanidins	Cyanidin	39.8[a]
		Peonidin	36.8[a]
		Delphinidin	9.5[a]
Hydroxycinnamic acids	*p*-Coumaric		25[b]
	Sinapinic		21[b]
	Caffeic		16[b]

a The data are from "Flavonoid Content of U.S. Fruits, Vegetables, and Nuts" written by James M. Harnly etc..b The data are from "Separation, Characterization, and Quantitation of Benzoic and Phenolic Antioxidants in American Cranberry Fruit by GC−MS" written by Yuegang Zuo etc..

The high levels of anthocyanins in berries contribute to their powerful antioxidant activity with the potential to prevent oxidative damage caused by reactive oxygen species (Prior and Lazarus 2001). Cranberry is generally recognized as "superfruit" because of its significant antioxidant function and anti-inflammatory properties (Watson 2007).

With the consistency with the US dietary Guidelines and current emphasis on cost-effective health care, the food company is responding to consumer's demands for

more healthful food supply. Producing the berries or their by-products, such as berry juices and berry jams, would be the basic and traditional way for the industries to provide anthocyanins-contained food. For instant, the juice of cultivated black currants is used as a raw material in the food industry for drinks and jam production (Häkkinena, Heinonenc et al. 1999; Määttä-Riihinen, Kamal-Eldin et al. 2004; Koponen, Happonen et al. 2008; Sandell, Laaksonen et al. 2009). However, it is highly encouraging and promising to develop anthocyanins-rich food products satisfying people's need for high level of phytonutrients without limiting to fruit products. Extraction photochemical from berry and then putting it in the new food matrix are naturally targeted as two significant procedures to accomplish this task.

In order to isolate anthocyanins, the factors to be taken into consideration include the solubility properties of the flavonoids. Solubility of flavonoids depend to a large degree on whether they occur bound to one or more sugar residues, which renders them highly polar or whether they occur in the free form in which case they are much less polar, and in the case of highly alkylated flavonoids, quite lipid soluble. Anthocyanins are phenolic molecules that preferably soluble in polar solvents, such as methanol, ethanol, acetone (Metiever, Francis et al. 1980). Methonal is the solvent most commonly employed. Extraction of anthocyanins are usually carried out under cold conditions with methanol containing a small amount of acid. For industrial principles, ethanol would probably be better than methanol seeing that eventual solvent residues are less toxic (Suhaj 2006). These solvents can be used as single carrier or in varying mixtures with water. Experience has shown that two or three extractions with 80% aqueous methanol serve to remove the bulk of soluble materials (Bohm 1998). To isolate

anthocyanins, the mixed carrier that recommended are hydroalcoholic solutions containing ethanol or methanol, and it could also consist of n-butanol, cold acetone, propylene glycol, methanol/acetone/water mixtures or boiling water. The addition of some organic acids has become useful for the extraction of complex polyacylated anthocyanins (Strack and Wray 1989; Garcia-Viguera, Zafrilla et al. 1998). Still, there is no best system. Moreover, some people even use the heating device to accelerate extraction rate, but thermal extraction with a long time could cause the degradation of anthocyanins and decrease the antioxidant activity of the extracts (Camel 2000; Lapornik, Prošek et al. 2005).

Recently, there have been many reports on the application of MAE on the extraction of some natural products, such as artemisinin, ginseng saponins (Kwon, Belanger et al. 2003). Microwave-assisted extraction is a new extraction technique that combines microwave and traditional solvent extraction with advantages include shorter time, less solvent used, or higher extraction rate (Sun, Liao et al. 2007). Microwave assisted extraction of anthocyanins from red raspberry was investigated by Hao and Han et al.. Comparison with the conventional solvent extraction, MAE utilizes the energy of microwaves to cause molecular movement and rotation of liquids with a permanent dipole leading to a very fast heating of the solvent and the sample. In the study, because of the strong disruption of fruit tissue structure under microwave irradiation, MAE is more efficient and rapid to extract anthocyanins (Hao, Han et al. 2002).

General speaking, current solvent based extraction processes are ineffective, the berries were about to went through the process-fractionated using juice pressing, ehanol

extraction and ethanol evaporation. But also the extracts are not safe for human consumption as a result of potential toxic effects from the residual solvents.

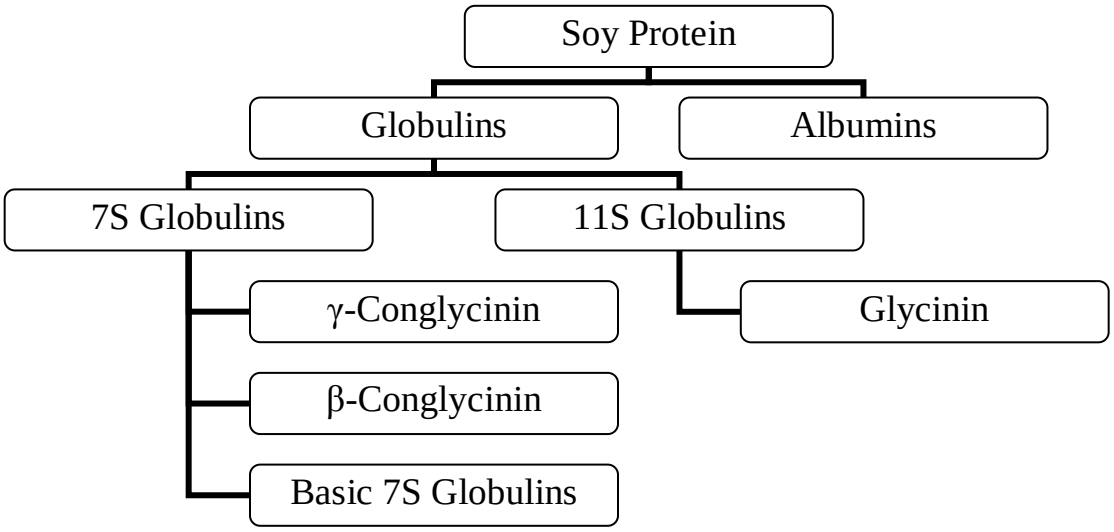
Supercritical fluid extraction (SFE) is an environmental friendly substitute to traditional organic solvent extraction of these composites. SFE methods are rapid, automatable and avoid making use of great amounts of toxic solvents. Supercritical carbon dioxide (SC-CO_2) method seems to be a good way for the extraction of natural products from plant materials. However SC-CO_2 process requires high pressure and the presence of an organic co-solvent in high percentage due to the polarity of anthocyanins (Mantell, Rodriguez et al. 2003; Hun, Heon et al. 2005). These factors limit application of SC-CO_2 technology for the purification of the health-promoting compounds for foods. There is a strong need for the environmentally friendly, "green" and GRAS type processes for polyphenol extraction from the natural sources. Except the good side mentioned above, this method still takes weakness of complicated procedure that stimulates the researcher to develop a better technique.

Soybeans are the only vegetable food that contains all eight essential amino acids and can be used to replace animal proteins in human's diet (Morrison and Hark 1999; Dudek 2001). The seed proteins of legumes are albumins and globulins. Most soy proteins are globulins, which account for about 50-90% of seed protein. Storage globulins are grouped into two types according to their sedimentation coefficients: 7S globulins (vicilin) and 11S globulins (legumin). The classification of soy protein can be seen from Figure 1. The ratio of 11S to 7S globulins is about 0.5-1.7 in soybean. Glycinin belongs to 11S globulins group and accounts for 40% of the total soy protein.

Its majority in soy protein renders it be a interest to food scientist. Glycinin is comprised of two hexagonal rings loaded one on top of the other, and each ring is made of three acidic and three basic units rotating around the ring. The hydrophobic polypeptide forms the core of the subunits (Lakemond, Jongh et al. 2000). Vicilin can be classified into three major fractions, and β-conglycinin (85% of 7S globulins) is the most abundant among them (Damodaran and Paraf 1997). Molecular properties of proteins, like hydrophobicity, conformational stability, charge and molar mass have a large impact on the functionality of proteins. There are numerous reports on SPI functionality (Luck, Lanier et al. 2002) concluding that compact tertiary and quaternary structures of the protein components lead to high emulsifying properties and formation of continuous network through hydrophobic, covalent and/or intermolecular hydrogen bonding (Liu, Lee et al. 1999). The aqueous suspension of soy protein exhibits a shear thinning (Berli, Deiber et al. 1999) and thixotropic behavior (Wagner, Sorgentini et al. 1992).

The functionality of proteins mainly depends on the structures and be affected by pH and anions present in solvent. In an aqueous environment, native globular proteins (such as those found in soy) maintain a compact three-dimensional structure. However, during denaturation, the native conformation undergoes structural changes by breaking and reforming of the intermolecular and intramolecular bonds without altering the amino acid sequence.

Figure 1 The major protein in soy



Adsorption is prevalent phenomenon which refers to the procedure of attraction the adsorbate molecules to an adsorbent surface. The preferential concentration of molecules beyond the surface arises as the forces on the adsorbent surface are unsaturated. Once adsorption occurs, both attractive and repulsive forces become balanced. Therefore we might be able to utilize the adsorption principle to extract anthocyanins by soy protein isolate.

Adsorption process is classified into to two types, either physical adsorption or chemisorption, depending on the type of forces between the adsorbate and the adsorbent. Generally speaking, the forces involved in physical adsorption are the relatively weak intermolecular forces (Van der Waal's interaction forces) between the solid surface and the adsorbate-a physical attraction; whereas the chemisorption can result in the sharing of electrons between the adsorbate and the solid surface-a chemical bond.

Some characteristic could be used to distinguish between physical adsorption and chemisorption. Firstly, under proper conditions, physical adsorption happens on all surfaces. However, only between certain adsorbents species and adsorbed molecules, does chemisorption procedure occur. It requires activation energy to initiate the reaction. Secondly, unlike physisorption, adsorbed molecule is strongly bound to the surface and chemical bond generated between adsorbate and adsorbent when chemical adsorption takes place, so the chemisorption process is irreversible. But physical adsorption is easily reversed. Heating accelerates desorption because it makes readily available to the adsorbed molecules the energy necessary to escape the adsorption site. As the features of both adsorptions, physisorption tends to occur below the boiling point at a relative low temperature. It is not the case with chemisorpiton, which often requires a significant quantity of energy -high temperatures well above the boiling point- to activate the reaction (Webb 2003).

The feasibility of adsorption method for phytonutrients has been verified by Liu and Xiao et al. They found that it is to be an efficient potential method for extracting anthocyanins in mulberry anthocyanins (Liu, Xiao et al. 2004). So, based on the information, we present interest in studying the adsorption of anthocyanin by soy protein through changing its structure under industrial processing condition. Proposed processing method for one-step adsorption process can be applied for wide range of juices and extracts, and used for development of new high value food products.

In order to study the issue stated, three primary objectives need to be identified. The first one step is to design an extraction process and assess the feasibility of extraction of

anthocyanin by soy protein. After that, the mechanism of soy protein-anthocyanin interaction can be determined. Finally, the optimal processing conditions, where the adsorption capability is the largest, can be found out.

In the study, we choose cranberry juice with a considerable amount of anthocyanin as a model juice matrix, and soy protein isolate is used as sorbent to carry out the experiment.

2. MATERIALS AND METHODS

2.1. Materials

Model cranberry juice matrix (27% cranberry juice, Ocean Spray, NJ and 12.5 ^{o}Brix sucrose), which contains a significant amount of anthocyanins, is used as a model juice matrix. Soy protein isolate (SPI) (Supro 500E, Protein Technologies International, Inc., St. Louis, MO) has been used as sorbent to extract bio-active compounds from the juice matrix. Additionally, glucose and citric acid are used to simulate berry juice matrix in control experiments. Glucose is purchased from the Sigma Chemical Company (St. Louis, MO) and citric acid (10% w/v) is from VWR International, Inc. (West Chester, PA). To investigate pH influence on adsorption of anthocyanins, the pH of the juice matrix is adjusted with small amount of calcium hydroxide (VWR International, Inc.,West Chester, PA)

2.2. Methods

2.2.1. Solubility of SPI

NaOH solution is brought to pH 8 with double distilled water through dilution method. Then, Soy Protein Isolate (SPI) dispersion is prepared by stirring 5 g of SPI in 500 mL of defined NaOH solution. The pH value of protein dispersions are adjusted by 1 M HCL, from pH 8 to pH 2, through controlling the flow rate of HCL (1.72 g/min) by mini pump. We have utilized a fiber-optic 20mm pathlength transmission immersion probe attached to S200 spectrometer (OceanOptics, FL) to monitor SPI solubility.

Fiber optic probe of spectrophotometer is dipped into SPI dispersion. Then, the transmission spectra of SPI dispersion changing with pH are collected and evaluated with OOIBase and MATLAB software. Obtained data are in good correspondence with the data reported (Fuleki and Francis 2006).

2.2.2. Zeta Potential and Particle Size Measurement of SPI

SPI is comprised the mixture of globular proteins that exist in form of colloidal particles and aggregates. The formation of a colloidal suspension has been observed when soy protein isolates are dispersed in water (Puppo and Anon 1998; Puppo and Anon 1998; Puppo and Anon 1999). Colloidal particles accumulate charges at their surface that can be expressed as a zeta potential. Surface potential is an important indicator for determining the magnitude of charged-based colloidal interactions in particulate systems including proteins. It signifies the level of repulsion between similarly charged particles in dispersion, and can be related to the stability of colloidal dispersions. Figure 2 illustrates the schematic representation of zeta potential. The liquid layer surrounding the particle exists as two parts: an inner region (Stern layer) where the ions are firmly bound to the particle and an outer (diffuse) region where they are less strongly associated. Within the diffuse layer, there is a hypothetical boundary inside where the ions and particles form a stable entity. The potential at the boundary, the ions beyond which stay with the bulk dispersant, is the zeta potential. For molecules and particles that are small enough, a high zeta potential will confer stability. When the potential is low, attraction force surpasses repulsion and the dispersion will break and flocculate. Measurement of size and surface charge of such formations would provide good information regarding dispersion and interactions between protein molecules.

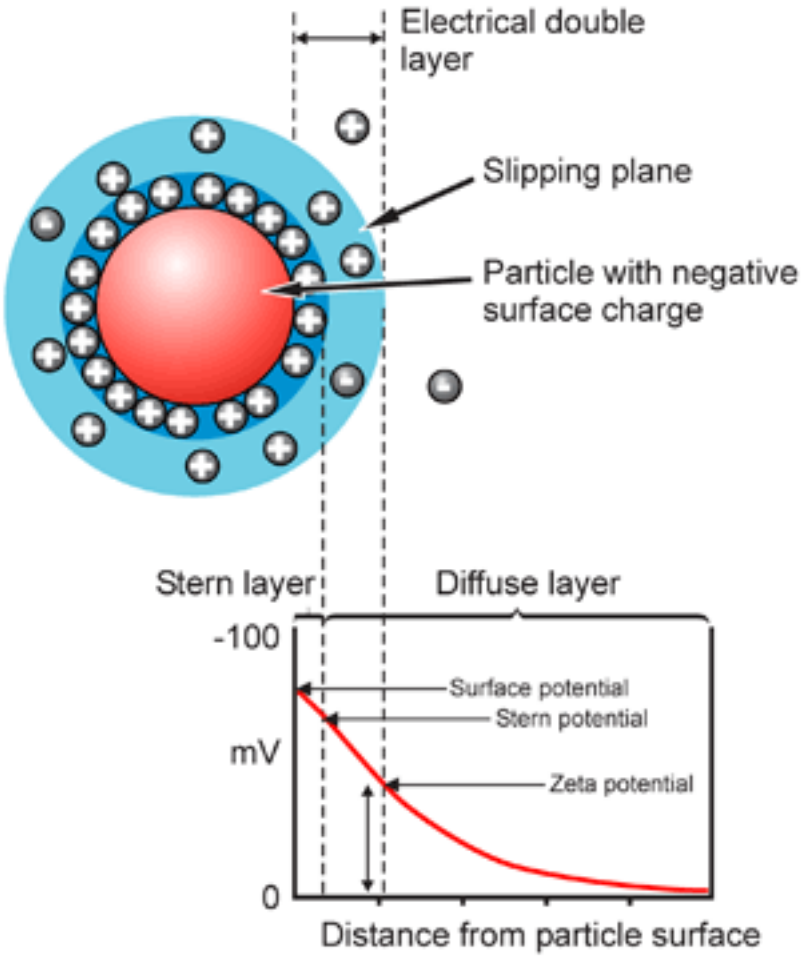


Figure 2 Particle and zeta potential (the scheme is obtatined from Technical Note, Malvern Instruments Ltd., 2012)

The zeta potential of particles in suspension is obtained by measuring electrophoretic mobility of the particles. The zeta potential can be calculated from the Smoluchowski equation:

$$\xi = \frac{\eta}{\varepsilon_o \varepsilon_r} U \qquad 1$$

where ε_0 and ε_r are dielectric constants in vacuum and of the solvent respectively, and *U* is an electrophoretic mobility of the particles. Particles move toward an electrode opposite to its surface charge, when the electric field is applied to charged particles in the suspension. For the reason that the velocity is proportional to the amount of charge of the particles, zeta potential can be estimated by measuring the electric field induced displacement of the particles.

In order to exclude effect of the phytophenols and evaluate effect of juice matrix (sucrose and pH) on the surface properties of soy proteins, 6 solutions of citric acid with various pH values (pH 2.2, 3.4, 4.22, 5, 6, 7) are prepared by diluting citric acid pH 1.7 with double distilled water. 2.2 is the pH value of canberry juice, pH 3.4 and pH 4.22 are the points where SPI could get the minimum solubility. Then SPI (5 g), sucrose (concentration of sucrose in the sample is 4.3 g/100 g) and citric acid solution (25 mL) are mixed together and homogenized by sonication (550 Sonic Dismembrate, Fisher Scientific Inc.) for 1 minute at 25℃. The mean particle size and zeta potential of the SPI in the solutions are determined by Dynamic Light Scattering (Beckman Coulter Delsa™ Nano C, Inc., Brea, Ca). To study to impact of sugar on the zeta potential and particle size, the solutions are prepared as described above, but without sucrose added. Protocol of testing zeta potential and SPI particle size in the cranberry juice matrix with different pH are similar. Additionally, for assessment of heat SPI stability, samples are tested at 25 ℃, 37℃, 45℃ and 60℃. The range of temperatures selected is guaranteed that there is no significant degradation of anthocyanins. Beyond the degree of 65, the anthocyanins starts to suffer degradation of oxidation (Stanciu, Lupsor et al. 2010). The data of zeta

potential and particle size were calculated by Delsa Nano 2.31 software (Beckman-Coulter).

2.2.3. Determination of Sugar Content

Sugar concentration is evaluated by measuring changes in refracting index of tested samples (10450 Abbe Refractometer, American Optical Corporation, Buffalo, NY). The refractometer has both refractive index (n_d) and Brix ° scales, with the prism temperature being monitored electronically and displayed on the integral digital display. Sugar concentrations are determined by measuring how light is refracted in a sugar solution. Brix ° value is the total sugar concentration in % (w/v) of a solution with the same refractive index. One measurement takes only a few seconds.

2.2.4. Quantitative Characterization of Adsorption Process and Conformation Changes in Anthocyanins Molecule

To evaluate an efficacy of the adsorption process, we use a VIS optical spectroscopy Through Beer's Law, the concentration of analyte (*i.e.* anthocyanins) can be determined by adsorbance:

$$A = \varepsilon b C \qquad 2$$

where ε indicates molar absorptivity, b represents the optical path length and C is the concentration of the analyte. The total anthocyanins content is calculated from adsorbance at 510 nm (Wrolstad, Decker et al. 2005) by using 1 cm path length quartz

cell, and the molar absorptivity value established for cyanidin 3-galactoside (ε =26.9 L/mol·cm) as suggested by Jurd and Asen (Jurd and Asen 1966). Thus, the concentration of anthocyanins could be determined through the equation 2. Then, the adsorption of anthocyanins can be obtained by plotting concentration of anthocyanins as a function of the concentration of SPI.

Raman spectroscopy has been successfully applied to investigate various phytochemicals. In order to monitor chemical changes in vibrational spectra of soy protein and anthocyanins in the study, we have used a RXN1 Raman spectrometer (Kaiser Optical Systems, Ann Arbor, MI) that consists of two instrument parts: a coherent stabilized light source (Invicta laser 685 nm) for producing light, and a spectrograph for measuring the intensity of light at various wavelength coupled with Raman filters. The amount of light passing through the tube is measured by the photomultiplier. The spectrograph delivers a signal proportional to the scattered light. A Raman spectrum is obtained by plotting the intensity of anti-Stokes scattering as a function of the wavenumber, and provides an information based on stretching and bending vibration modes of the adsorption or transmission of energy as a function of the light frequency (Chan 1996).

Totally six concentrations (0, 20, 40, 60, 80, 100 g/L) of SPI in cranberry juice matrix are prepared by mixing. Then SPI and cranberry juice mixtures are separated via centrifugation process. Supernatant solutions are analyzed by means of Raman spectroscopy and VIS spectroscopy respectively to determine the content of phytophenols in supernatant solution. To evaluate the effect of pH on the efficiency

of the adsorption process, calcium hydroxide is utilized in the SPI-cranberry juice mixtures to bring up the pH value to 3.4, 4.2 and 7. To evaluate the effect of residence time on the efficiency of the adsorption process, the residence time of mixing is changed from 15 second to 35 minutes.

3. RESULTS AND DISCUSSIONS

3.1. Anthocyanins Extraction Process

Proposed process for anthocyanins extraction consist of four stages as depicted in Figure 3. The first step of the process is mixing of cranberry juice matrix and SPI. Mixing is a common operation in food processing, which involves mixing of two materials of the same or different phases. The goal of mixing is to get a homogenous final mixture, and mechanical force is usually used in mixing to cause one material to randomly distribute in another. Mixing time is an significant indicator for this procedure, which is not only determined by mixer configuration (impeller type and pumping number) but also requirs residence time for the individual particles and total energy input. Then, the next process stage is decanting, which refers to separation of mixture by transfer the mixed solution to another container in order to leave the sediment in the bottom of the original container. After this, the samples ae separated efficiently to liquid (supernatant) and solid phases via centrifugation process. The centrifugal force acting on a particle is directly related to and dependent on the distance of the particle from the center of rotation, the centrifugal speed of rotation, and the mass of the particle (Singh and Heldman 2009). Centrifugation separation allows highly effective collection of protein-polyphenol fraction and obtaining naturally sweetened supernatant solution that can be used for beverages.

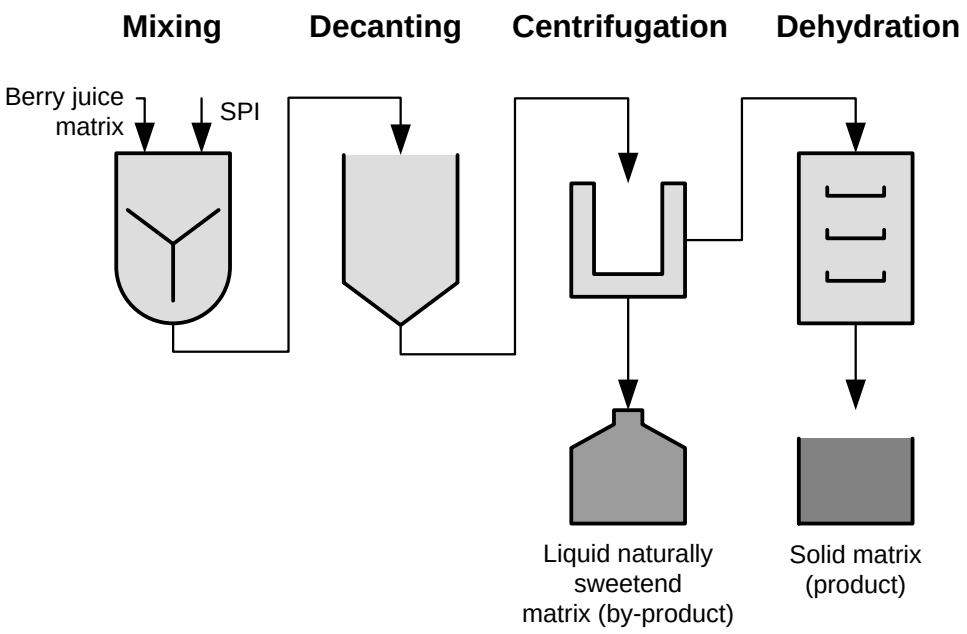


Figure 3 Process flow diagram for the anthocyanins extraction processing

We would like to emphasize the fact that there is no waste stream or unused by-products in the proposed technological process. Obtained after centrifugal separation, protein-polyphenolic slurry can be used directly in baking products. However, further dehydration allows obtaining more versatile product. It is possible to utilize a freeze-drying technology in order to preserve all natural polyphenolic compounds and obtain product of highest quality to satisfy industry needs and consumer demands. Described processing flow uses only food grade GRAS materials and does not require any additional solvent.

3.2. Anthocyanins Adsorption onto SPI

Red fruit extracts are frequently used as natural colorants for sugar confectionary, dairy products, ice creams, etc. which is owing to the existence of ancythocyanins. Ancythocyanins are the largest group of water-soluble pigments that widespread in the plant kingdom. The efficiency of the soy protein absorption process is evident from the changes in the appearance of the juice supernatant solution as result of the SPI adsorption processing (see Figure 4). As the quantity of SPI used in the study increases from 0 to 100g/L, the color of the supernatant tends to become more transparent. It is obvious to see that, with the concentration of 100g/L SPI, dark pink color of cranberry juice (associated with anthocyaninss) almost completely disappears from the liquid phase. This phenomenon can be initially identified as the result of formation of dark purple/black protein fraction that has been easily separated via centrifugation.

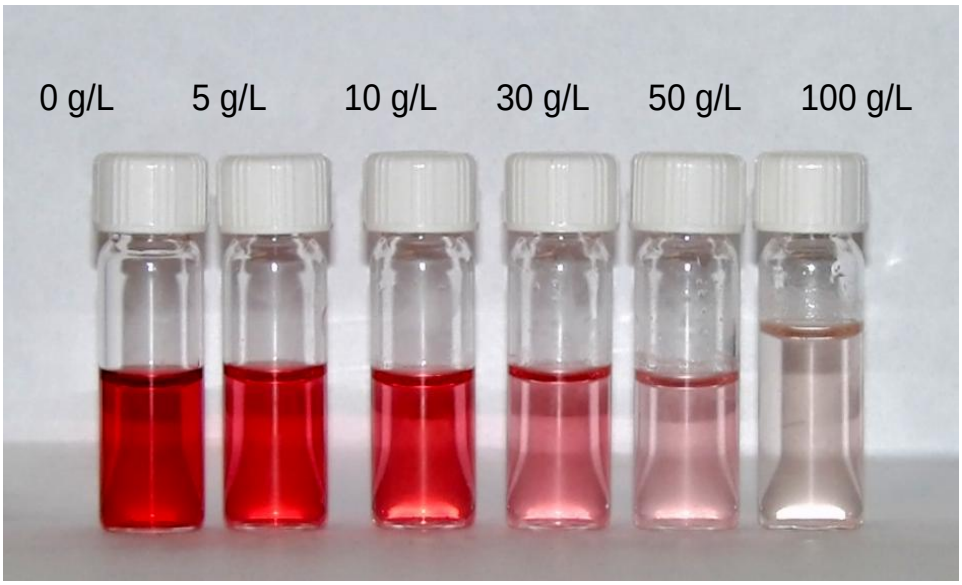


Figure 4 Photographs of soy protein induced extraction process: cranberry juice before (left) and after treatment with various amount of isolated soy protein. Color is indicative of the presence of anthocyanins.

Since anthocyanins have very specific adsorption behavior in "red" region of the VIS spectrum, we utilize an optical to quantify an extraction process. Figure 5 and Figure 6 summarize the quantitative analysis of anthocyanins' content in supernatant solution after centrifugation of the mixture with different amount of soy protein isolate. At the meanwhile, the effects of temperatures and pHs on the adsorption are also evaluated. The concentration of SPI utilized in the research ranges between 20 and 100g/L by 20g/L. From the data presented, we can see that under various temperatures and pH values, the amount of anthocyanins in supernatant solution reduces with concentration of SPI increases, which means, that reversibly amount of anthocyanins adsorbed by soy protein is proportional to the concentration of SPI. Figure 5 illustrates the influence

of temperature on content of phytochemicals in supernatant. In comparison, SPI could be able to extract more anthocyanins at 45℃. In Figure 6, the optimal pH value for adsorbing the phytonutrient from cranberry juice can be obtained, is 7. In both figures, the curves show a general tendency of decrease of the anthocyanins until concentration of SPI reaches 60 g/L, after which point there is little change in the adsorption of anthocyanins.

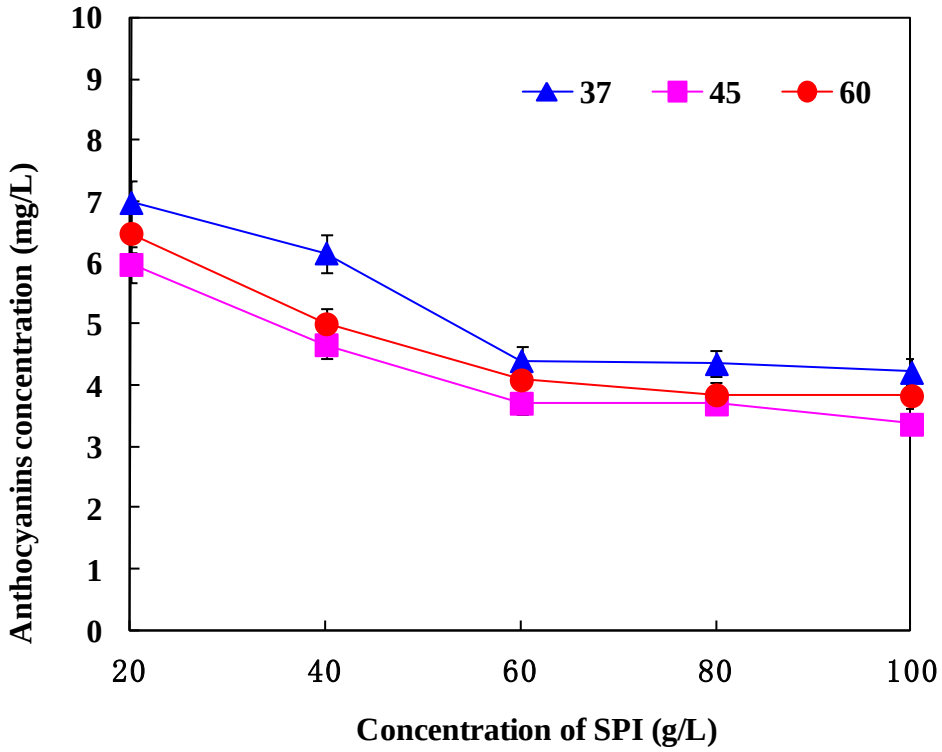


Figure 5 The content of anthocyanins in supernatant solution at different temperature and pH 7.

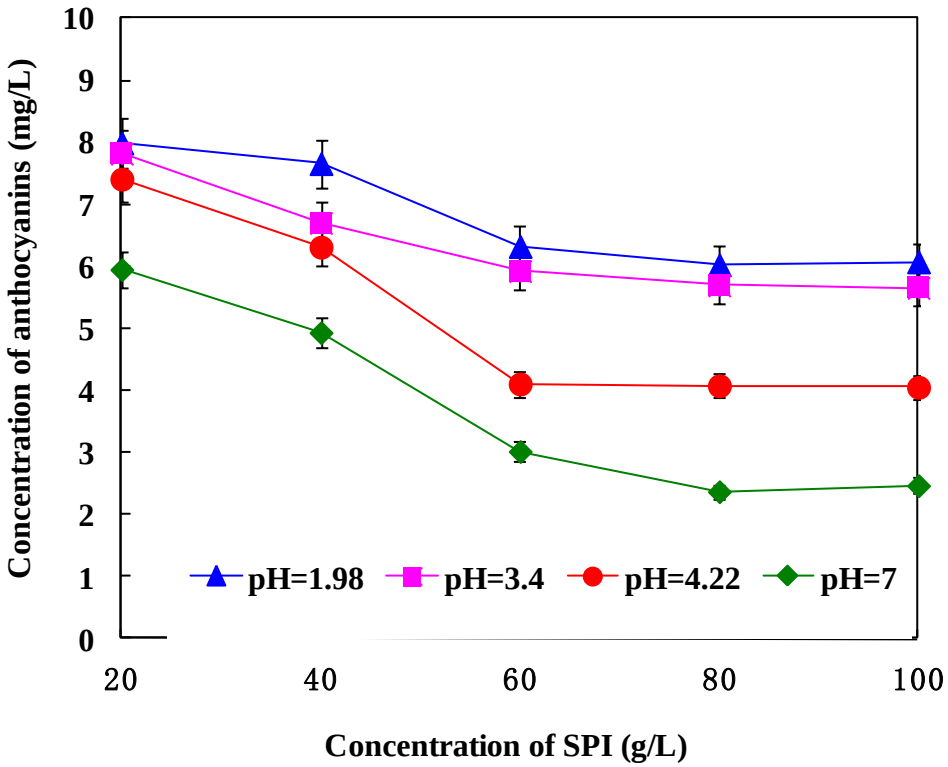


Figure 6 Content of phytochemicals in supernatant solution at various pH values

The adsorption phenomena prevail in nature. In this study, the level of anthocyanins in the supernatant does not change with time once adsorption density has been saturated, thus we assume it as the equilibrium procedure. With VIS optical spectroscopy method, the initial anthocyanins content in cranberry juice is calculated to be 8.42mg/L by Beer's Law. At the equilibrium state, the amount of anthocyanins adsorbed by SPI could be obtained via mass balance, once phytonutrients concentration in the supernatant is known.

$$q_e(mg/g) = \left|\frac{c_i - c_e}{M}\right| \times V \qquad 3$$

Impact of SPI concentration on equilibrium adsorption density of anthocyanins (q_e) can be evaluated via the equation above and is represented as function of SPI concentrations in Figure 7.

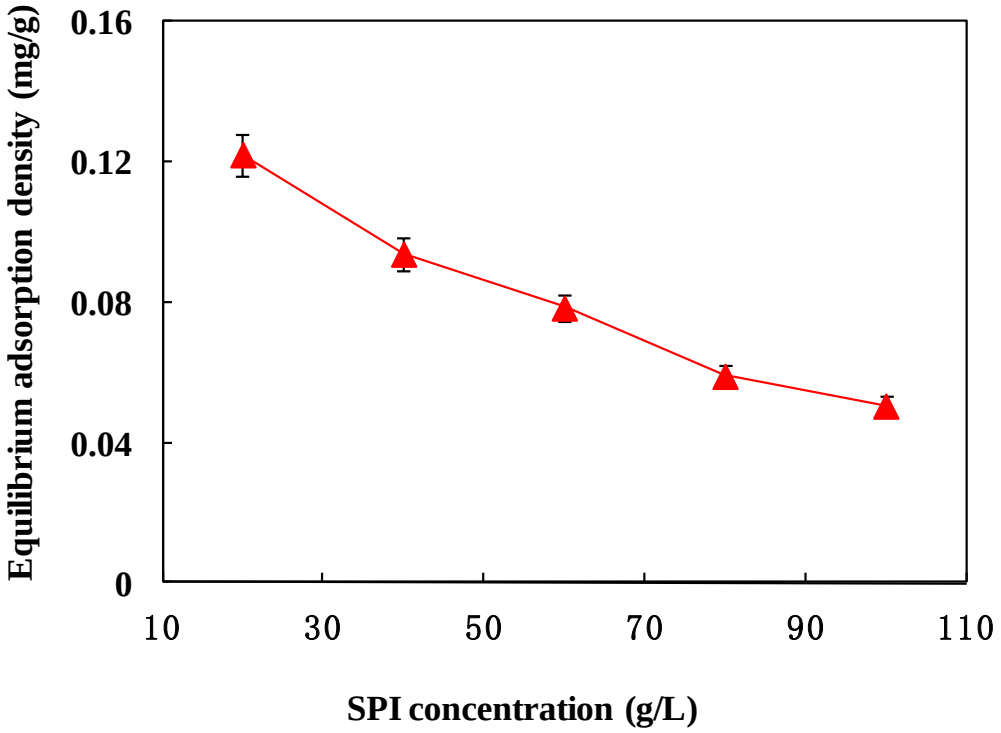


Figure 7 Effects of SPI concentration on the adsorption density of anthocyanins at room temperature

At a lower SPI concentration, the equilibrium anthocyanins adsorption density could reach at relatively higher level. With the increase of SPI concentration from 20 to 100 g/L, the curve decreases gradually. For practical purpose, this dependence can be well

represented (R^2>0.99) by second order empirical model

$q_e = 6 \cdot 10^{-6} C_s^2 - 0.0017 C_s + 0.1521$, where C_s is a SPI concentration.

After verifying the SPI's anthocyanins adsorption capability, we check the sugar content in supernatant to figure out whether SPI extract the sugar at the same time. The total sugar content of supernatant is quantified by measuring changes in refractive index of supernatant solution by the Abbe refractometer. Figure 8 shows the relationship between the concentration of SPI and sugar content (Brix-value). The original sugar content in cranberry juice matrix is 12.5^{o} Brix. The average Brix-value is still around 12.5 in supernatant after being treated by mixing with soy protein isolate and removal of protein fraction. Performed ANOVA F-test indicates that there is no statistically significant difference between the processed samples and control ones ($F \leq F_{crit}$, p-value$\geq$0.05, at α=0.05 level). Therefore, it is clear that soy protein isolate is capable to extract anthocyanins from cranberry juice without adsorption of the sugars, which provides the densed, health-promoted compounds but with lower sugar comparing with fresh berry fruit. Also obtained data is good indicator that there is no protein glycation in the tested system.

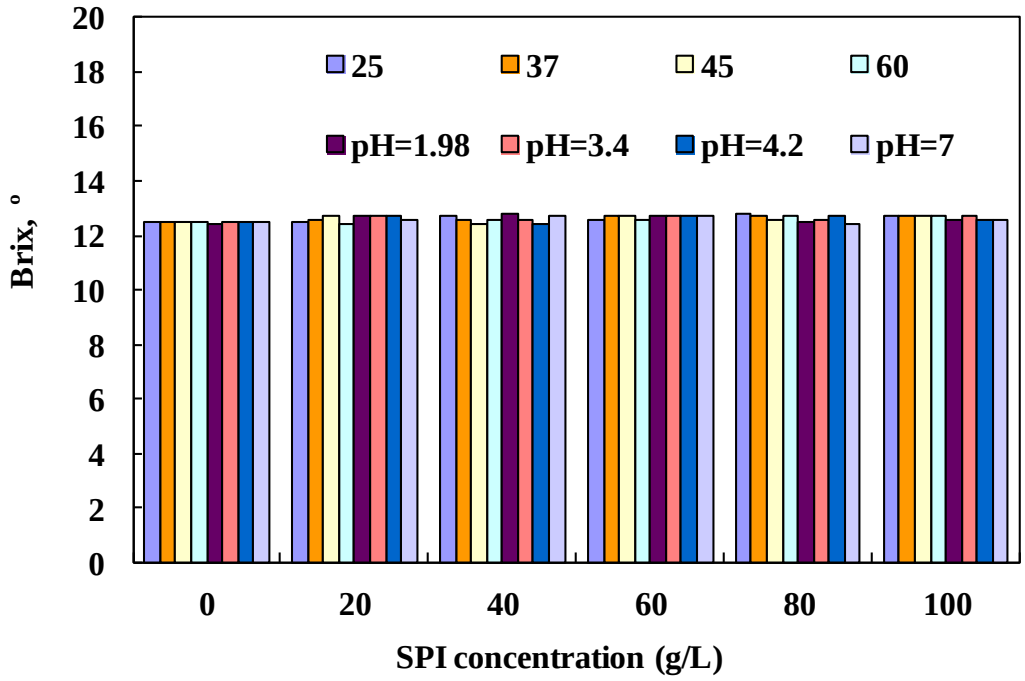


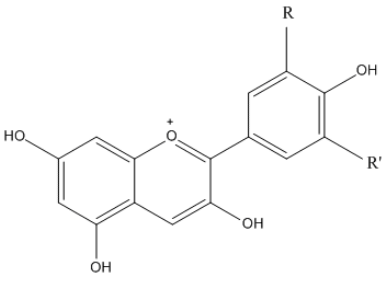
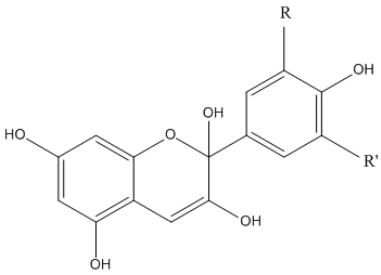
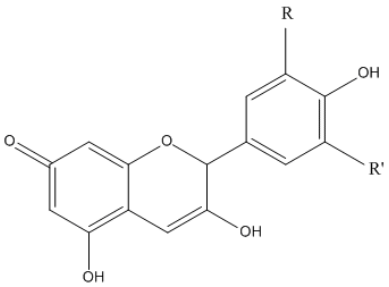
Figure 8 Sugar content of the supernatant solution at different temperatures and pH

3.3. Effect of Processing Conditions on Anthocyanins Conformations by Means of Raman Spectroscopy

Raman spectroscopy allows obtaining spectra that represent characteristic bands of individual components, through which way, the SPI adsorption of anthocyanins is confirmed and its molecular structure is testified. The laser light interacts with molecular vibrations, phonons or other excitation in the system, and causes that energy of the photons being shifted. The shift in energy provides the information about the vibration modes in the system, which is specific to the chemical bonds and symmetry of molecules.

Anthocyanins are widely used as nature colorant and the color displayed is strictly related to the pH-dependent environment. In fact, they co-exist in aqueous solution in equilibrium between several species, demonstrated inTable 4, depending on pH. At pH 1-3 the flavylium cation is red, at pH 4-5 the carbinol pseudo-base (*pb*) is colorless, and at pH 7-8 the quinoidal-base (*qb*) formed is blue-purple (Lapidot, Harel et al. 1999). Anthocyanins at pH 7 exist in the form of the quinoidal-base with C=O bonds at position 7, and has its characteristic bonds at about 1623 cm^{-1}. The other pH values have no obvious peak around this wavenumber that can be used for identification purposes.

Table 4 Structure of anthocyanins under different pH.

pH < 4	4 < pH < 5	pH > 5
red	colorless	purple
		

Chemically anthocyanins are based on a C15 skeleton with a chromane ring bearing a second aromatic ring B in position 2 and with one sugar molecules bonded at different

hydroxylated positions of the basic structure. The distinctive characteristic of cyanidin and peonidin glycosides structure, which comprising the major anthocyanins in cranberry, is monoglycosides in position 3 of the C ring, and the close electronic and geometrical configurations of benzopyrylium moieties. It is a light-adsorbing chromophore that responsible for the color of these compounds, explaining the great similarity in the spectra of the anthocyanidins (Baranska, Schulz et al. 2006).

Table 5 Raman assignment to molecular vibrations (Merlin, Statoua et al. 1993; Lapidot, Harel et al. 1999)

Peaks (cm^{-1})	Assignment	Indication
1623	C=O bonds	Quinoidal-base
1602	Aromatic ring	Anthocyanins specific (C15 skeleton)
1585	Aromatic ring	Anthocyanins specific (C15 skeleton)
1455	Aromatic ring	Anthocyanins specific (C15 skeleton)
1335	C-O-H, $(R)_3C$-OH	C-OH, Pseudo-base
1194	Aromatic ring, in-plane C-O-C	Anthocyanins specific (C ring)
1157	Aromatic ring, in-plane C-O-C	Anthocyanins specific (C ring)
1033	Aromatic ring	Anthocyanins specific (C15 skeleton)
1002	Aromatic ring	Anthocyanins specific (C15 skeleton)
801	-OH group	Anthocyanins specific (stretching attached to aromatic ring)
623	C-H bond	Anthocyanins specific (stretching on aromatic ring)
530	Monoglycosylation	Glucose moiety to carbon-5 on A ring

The Raman intensity of a normal mode is as function of the concentration of the scattering species (Liang, Noda et al. 2000). It can therefore be assumed that the intensities increase with higher concentrations of anthocyanins in supernatant. The major bands of the anthocyanins at 530, 623, 801, 1002, 1033, 1157, 1194, 1455, 1585, and 1602 cm^{-1} dominate the spectrum. As being analyzed in Table 5, the spectral range between 1585 and 1620 cm^{-1}, 1430 and1465 cm^{-1}, as well as the strong Raman peaks at 1002 and 1033 cm^{-1}, depend on the aromatic ring. Signal at 623 cm^{-1} can be assigned to the C-H bond on aromatic ring, which comprised the C15 skeleton of the anthocyanins. The other characteristic band between the region 1180-1215 cm^{-1} and 1130-1200 cm^{-1} in spectrum correspond to in-plane C-O-C stretching in second aromatic ring B (Baranska, Schulz et al. 2007). Additionally, -OH groups attached to the aromatic ring are seen at 801 cm-1. Merlin et al. (Merlin, Statoua et al. 1993) have investigated anthocyanins using resonance Raman spectroscopy and showed a strong influence of glycosylation upon the benzopyrylium part of the flavonoid molecule (ring A and C ring). Some characteristic spectral features of this phenomenon were provided, for example, monoglycosides, in position 3 of the C ring, exhibit a strong RR signal close to 540 cm^{-1}. In this experiment, monoglycosylation corresponds to carbon-5 of the A ring of anthocyanins. The characteristic band at 530 cm^{-1} can therefore be associated with the presence of the glucose moiety of the anthcyanin molecule.

Supernatant solutions are analyzed with Raman spectroscopy to determine the content of anthocyanins after centrifugal separation. Figure 9 depicts the concentration of anthocyanins is highest at room temperature, and then followed by the temperature at 60 ^{o}C, whereas at 45 ^{o}C the concentration of anthocyanins is lowest. The content of

phytochemicals in supernatant solution tested by Raman spectroscopy corresponds with the result obtained by spectrophotometer (Figure 9). At 45^{o}C, the content of anthocyanins in supernatant is least, which implies that, conversely, the soy protein isolate could adsorb the largest amount of anthocyanins. The tendency of capability of the SPI adsorption increases with temperature rises until 45℃. However, it drops significantly at 65℃. This can be explained as the onset of protein denaturation process. The denaturation of soy protein is studied by differential scanning calorimetry (DSC). The endothermic peaks in DSC thermograms and the peak area (enthalpy) represents a parameter of protein denaturaion degree (Arnfield and Murray 1981). At 55℃ and 61℃, β-conglycinin had onset and peak denaturation temperature respectively (Renkema, Gruppen et al. 2002). Denaturation is a prerequisite for gel formation by soy protein. Heat-induced gel formation is a complex process which involves various reactions, such as dissociation of globulins, and the bonds broken between protein molecules (Van Vliet, Van Dijk et al. 1991). The alternation of protein structure might be the reason that lowers the ability for SPI adsorption above 60^{o}C, which will be discussed later.

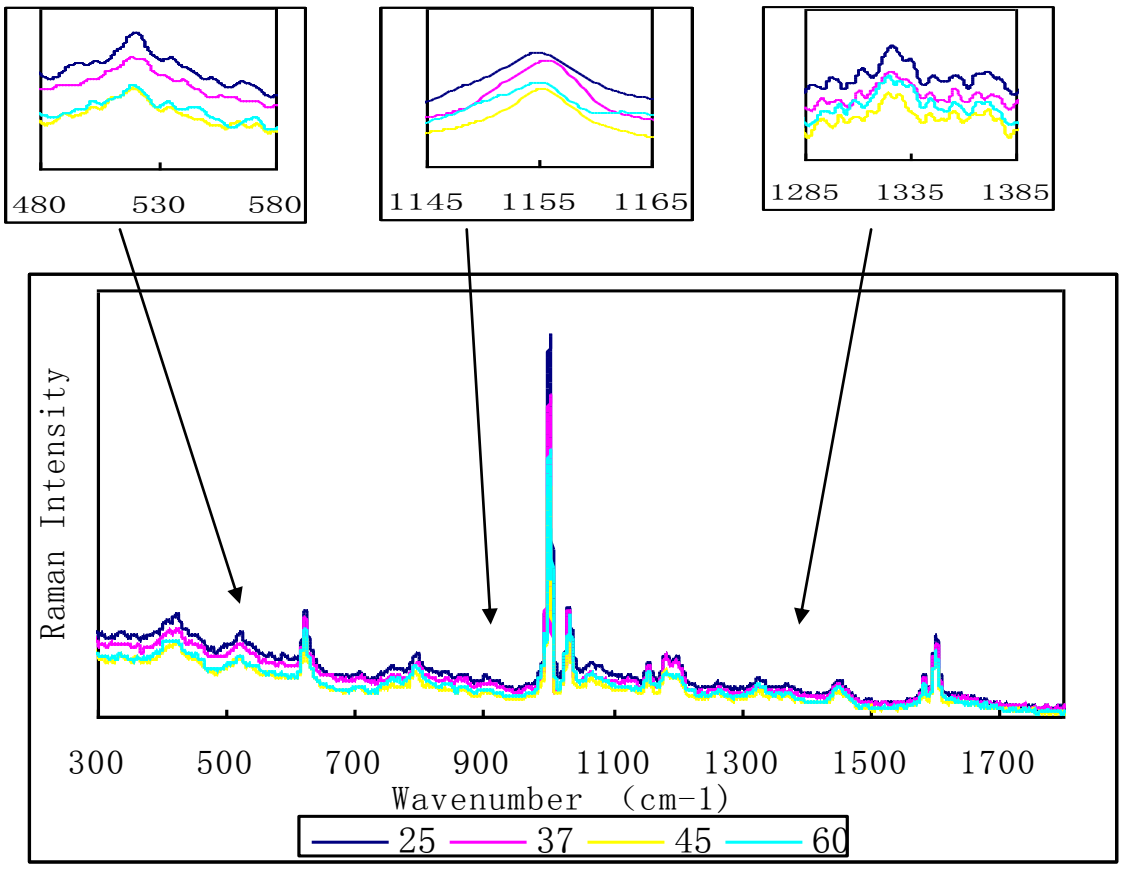


Figure 9 Raman shift of the supernatant solution with pH 4.2 at different temperatures.

The influence of pH value on atomic interactions in anthocyanins is depicted in Figure 10. As the pH increases, the structure of anthocyanins change to pseudo-base (pb) form and then, at pH 7, quinoidal-base (qb) form is generated. It can be seen from Figure 10, well recognizable Raman peak at pH 4.2 (pb) located near 1335cm^{-1} line, which is related to the chemical bond of $(R)_3C$-OH on pseudo-base. Moreover, the peak located at 1623 cm^{-1}, which attributes to C=O bonds in quinoidal-base, is only shown at pH 7. Under different pH values, the appearance of peak corresponding with specific chemical bond confirms that the molecular structure of anthocyanins alters in acidic condition.

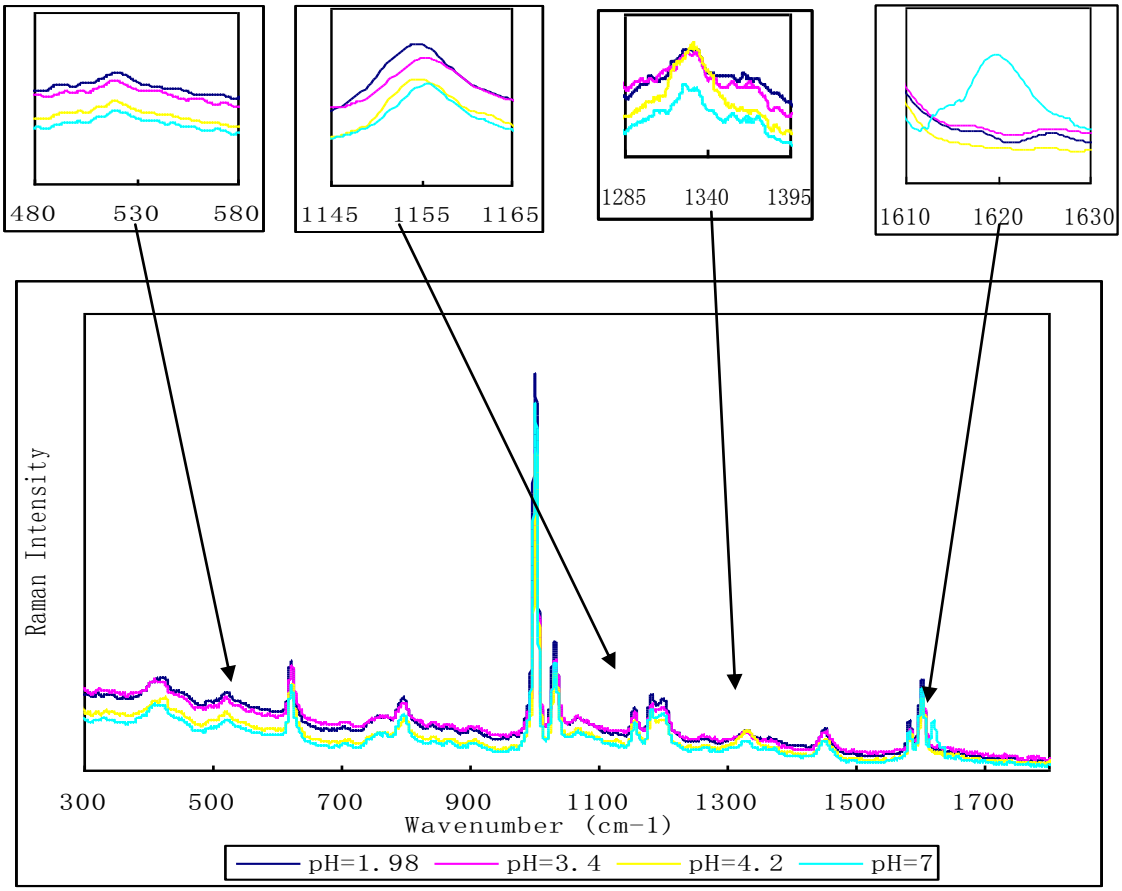


Figure 10 Raman shift of the supernatant solution at room temperature with different pH values.

3.4. Anthocyanins Adsorption and Corresponding Changes of SPI Surface Charge

Since the functional properties of proteins are often affected by protein solubility, the mechanism of the adsorption might be found out through researching solubility, surface charge and particle size. The occurrence of minimum solubility near the isoelectric pH, at which a particular molecule or surface carries no net electrical charge, is primarily due to lack of electrostatic repulsion, which promotes aggregation and precipitation via

hydrophobic interaction. Then the protein will be insoluble at the pI. With changing pH, the patern of intensity for soy protein isolate in solution (See Figure 11) is similar with the results (See Figure 12) of the solubility of SPI (Lee, Ryu et al. 2003). It can be seen that the solubility gradually increases from 2% to 14% when the pH is ascended from 2 to 7. It showed a typical U-shaped pH-solubility profile, with a minimum around pH 4.2.

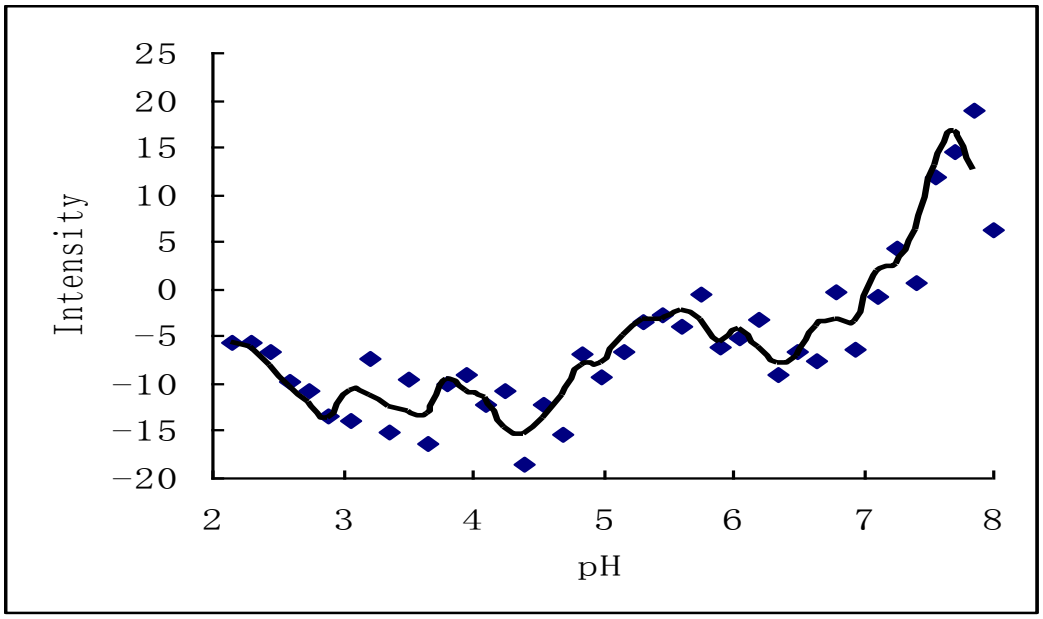


Figure 11 Intensities of soy protein isolate in transmission mode varies with pH changing from 2 to 8.

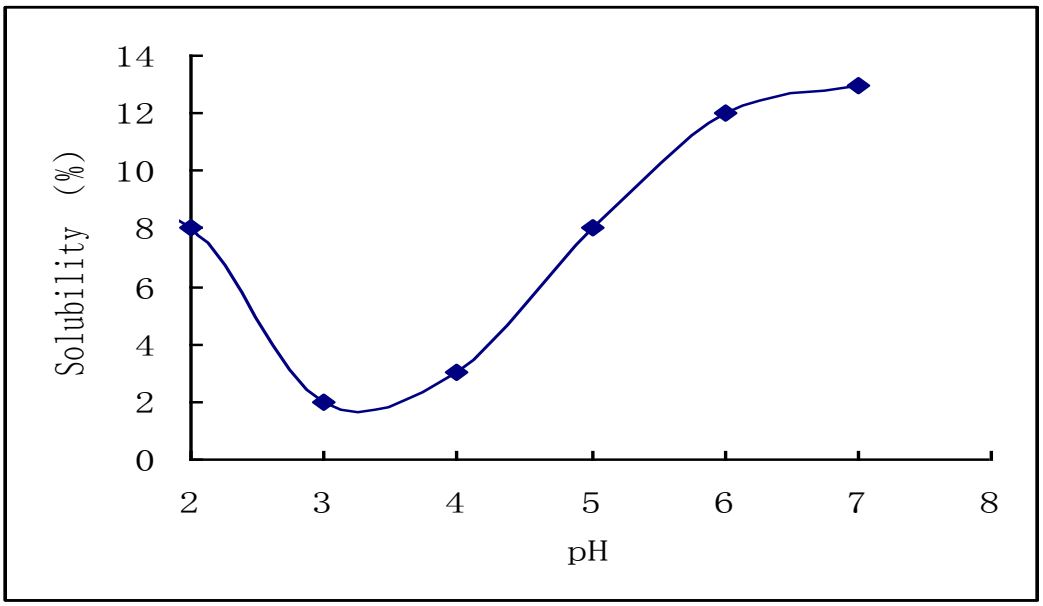


Figure 12 Effect of pH on protein solubility of soy protein isolate (Lee, Ryu et al. 2003).

The ξ-potentials of SPI are shown as a function of pH in Figure 13. The ξ-potentials of SPI are negative at high pH values and become positive as the pH was decreased. The charge is to be neutral point corresponding to the isoelectric point around pH 4.2, which matches up the minimum solubility tested. The phenomenon of charges from positive to negative with increasing pH could be explained by using the Henderson-Hasselbach activity theory, which can estimate the charge on a protein, from the sum of the charges on the individual amino acid residues. Over the tested pH range, the net charge is dominated by a positive contribution from lysine and histidine and increasingly negative contributions from aspartic and glutamic acid. Other amino acids carry charge but are not present on an adequate mass basis to influence the total charge (Malhotra and Coupland 2004).

Based on size separation by ultracentrifugation, the soy protein fractions are designated as 7S and 11S. Glycinin, the major protein in the soy bean, belong to the 11S type protein family, it is characterized by a hexameric structure, organised in a close packed globular confirmation. The quaternary structure of this protein, consisted of six subunits, is stabilized by electrostatic and hydrophobic interactions, as well as by disulfide bridges between polypeptides. Jiang, Xiong, et al. (Jiang, Xiong et al. 2010) presented results indicating that 11S globulins were the most significant contributors to the overall aggregation of SPI. Similarly, Wagner and Guluen (Wagnert and Gueguen 1995) found out that 11S globulins were more sensitive than 7S globulins to pH-shifting treatment.

From Figure 13, we can see that the particle size increases as pH changes from 2.2 to 4.2, and then the curve reaches a plateau after that till pH 7. The mechanism is complicated, but it could be explained by the reason that the quaternary structure of glycinin is effectively modulated by pH. Previous study illustrated at pH 7.6, glycinin is mainly present in a hexameric form of 360kDa (Badley, Atkinson et al. 1975), but lowering the pH might cause glycinin to partly dissociate from the 11S form into the 7S (β-conglycinin) form. In addition, as demonstrated by solubility and gel electrophoretic experiments (Lakemond, Jongh et al. 2000), this disruption does not take place decreasing pH at 5.2. Thus, it maintains the larger diameter between pH 5 to 7 in 11S form. Furthermore, it is found that in the range of pH 3.8-2.2, the glycinin complex is, to a degree, present in the 7S form (Wolf and Briggs 1958). The dissocation from 11S to 7S causes the reduction in particle size. But around pH 4, the soy protein with the lowest solubility still gets the large diameter. Tertiary unfolding and secondary unfolding at low pH value (<2.5) have been reported (Koshiyama 1972). It is believed to shape a more nonstructured conformation and result in a large particle size this condition (Utsumi, Nakamura et al. 1987). The changes of diameter below pH 2 could be accounted for this reason.

Figure 13 (a) and (b) show the zeta potential and particle size of the soy protein isolate in the solution with sucrose and without sucrose. The relative data of SPI in cranberry juice are also shown in Figure 13 (c). In cranberry juice, the total sugar is ranging from 4.8-6.4g/100g, and sucrose ranging from 3.7-5.0g/100g. Data reported in Figure 13 (a) (b) show a shift in the isoelectric point from around pH 4 to about 3.7 after adding sucrose, which could be explained by the decrease of the pK value; the net charge of

the molecules at this pH slightly decreases (Courthaudon, Colas et al. 1989). The single sucrose is electronically neural molecule, therefore the adsorbance mechanism of SPI is based on electrostatic ground instead of chemical binding, and otherwise the protein will interact with sugar.

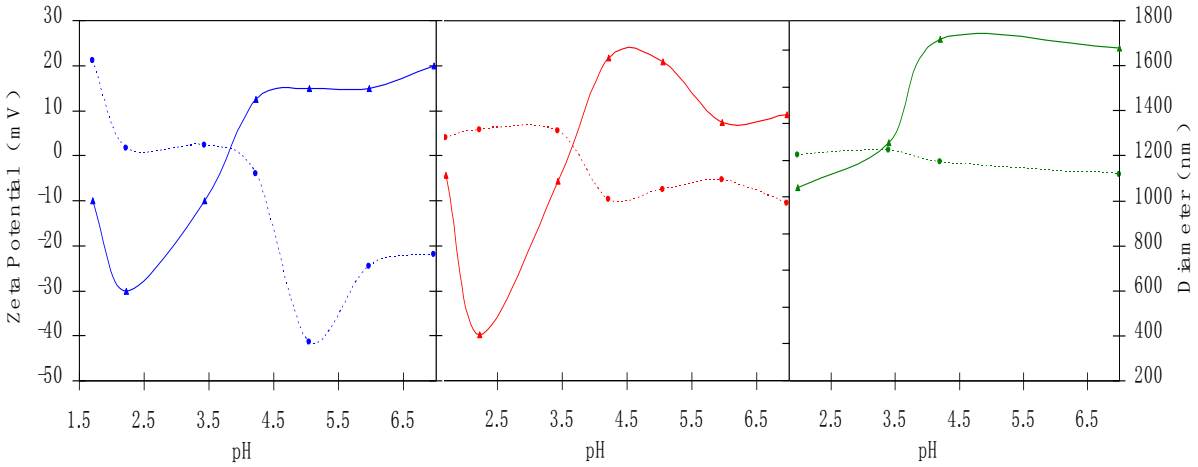


Figure 13 (a) Effect of pH on zeta potential (⬬) and particle size (▲) of SPI in solution without sucrose. (b) Effect of pH on zeta potential and particle size of SPI in solution with sucrose. (c) Effect of pH on zeta potential and particle size of SPI in cranberry juice matrix

Denaturation of globular proteins due to extreme condition leads to unfolding of the polypeptide chain, hydrophobic groups located in the interior of the molecule exposing outside for interaction (Arrese, Sorgentini et al. 1991). Thus increase of surface hydrophobicity indicates that the hydrophobic regions are exposed at the molecular surface as a result of the conformational changes caused by acid-induced or heat-induced protein denaturation. The modified soy protein leads to a reduction of surface free energy and in inverse proportion to the increase in surface hydrophobicity.

Figure 14 shows the influence of temperature on zeta potential and particle size of SPI in cranberry juice. At constant pH, an increase of diameter is observed when the solution are heated from room temperature to 45℃, while heating at higher temperatures has negative effect on the particle size. The similar relationship between the diameter of droplet and temperature has been found by others. Several explanations for the decline of the particle size upon heating at higher temperatures have been presented. Soy protein is globular proteins which unfold and aggregate once heated above their denaturation temperature. Some (Monohan, McClements et al. 1996) propose that there is balance between inter-and intra- droplet interactions which affects the degree of droplet aggregation. As temperature increases, inter-droplet interactions are replaced with intra-droplet interactions. Above 40 ℃, the increase in thermal kinetic energy of atomic vibrations causes protein unfolding (denaturation) (Rennema, Damodaran et al. 2007). The degree of unfolding of proteins at the interface affects the hydrophobicity of the droplet surface (Demetriades, Coupland et al. 1997). In the temperature range of 37-45℃, SPI are partial unfolding. Consequently, not all hydrophobic side chains exposed towards the aqueous phase leading to a relatively high hydrophobicity on the surface, thereby making it susceptible to aggregation. On the contrary, at higher heating temperatures intra-droplet protein-protein interactions are favored, complete unfolding of protein makes its hydrophobic groups rearranging themselves in order to reduce the surface hydrophobicity by decreasing particle size. The reason for the subsequent decrease in droplet size at higher temperatures is consented by the other researchers. Kulmyrzaev etal. (Kulmyrzaev, Bryant et al. 2000) stated that, in the whey protein system, the weakening of hydrophobic interactions occur when temperature exceeds a certain value. Another system in which

thermodynamically heat unfolding leads to an increase in the particle size is lipoproteins (Benjwal, Verma et al. 2006). Another reason to explain this phenomenon would be heating accelerates desorption because it makes readily available to the adsorbed molecules the energy necessary to escape the adsorption site. Thus the diameter decreases.

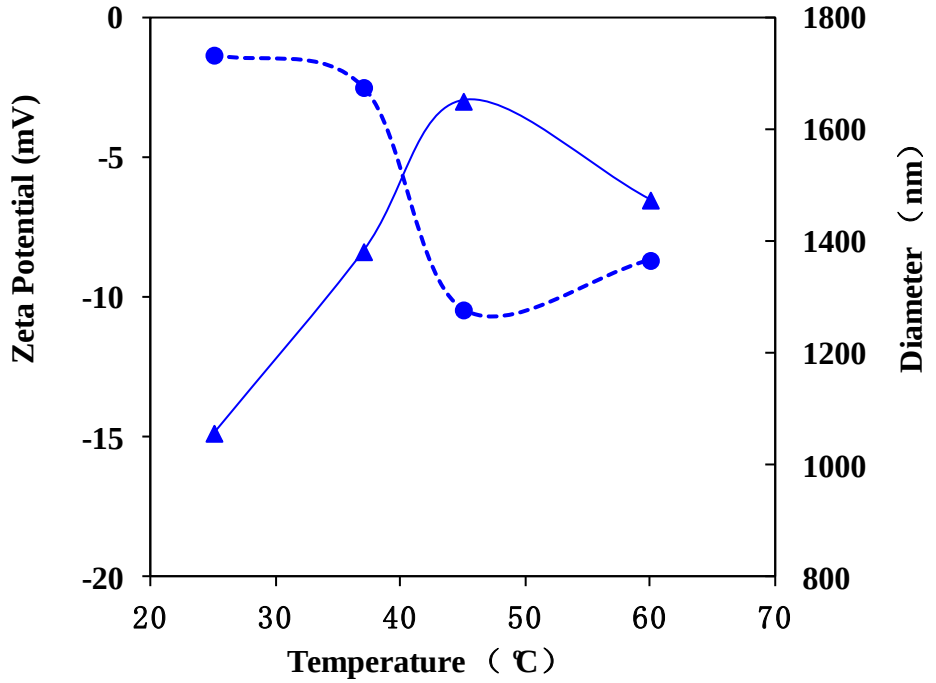


Figure 14 Effect of temperature on zeta potential（●) and particle size（▲) of SPI in cranberry juice at pH 4.2.

McClements (McClements 2001) and Mora-Gutierrez etc (Mora-Gutierrez and Farrel 2000) pointed out that cosolvent in the surrounding aqueous phase influence thermal stability, conformation and aggregation of globular proteins because of protein transfer free energies in cosolvent solutions. The transfer of a protein molecule from water to

the sugar solution requires free energy to maintain the concentration gradient between the sugar-depleted local-domain surrounding the protein molecule and the bulk aqueous solution, which is thermodynamically unfavorable process. Thus, sucrose molecules in cranberry juice are excluded from the soy proteins, which might be due to a combined result of excluded volume effect, caused by steric exclusion of larger in size sucrose molecules and differential interaction effect. In addition, it is suggested that sugars is able to stabilize globular protein and decrease protein surface activity at the steady state. Prior work has documented that in the presence of sucrose, the steady-state values of surface tension of protein are found to increase both at pH 7 and 5.5 (Antipova, Semenova et al. 1999). This is consistent with the data of zeta potential tend to be neutral under respondent pH in Figure 13 (b, c), compared with Figure 13 (a).

3.5. Adsorption Equilibrium Models

Adsorption equilibrium data which expresses the relationship between mass of adsorbate per unit weight of adsorbent and liquid phase equilibrium concentration of adsorbate are represented by adsorption isotherms and provide important design data for extraction process. The adsorption isotherms provide information on the adsorption capacity at various concentrations for a given adsorbate on a given adsorbent. Therefore, they play a crucial role in the choice and in the utilization of adsorbents. In this part of the study, the parameters of Langmuir and Dubinin–Radushkevich (D-R) models were evaluated by fitting the theoretical isotherm to experimental data

The Langmuir adsorption model assumes that molecules are adsorbed at a fixed number of well-defined sites, each of which can only hold one molecule and no transmigration of adsorbate happening in the plane of the surface. These sites are also assumed to be energetically equivalent to each other so that there are no interactions between molecules adsorbed to adjacent sites.

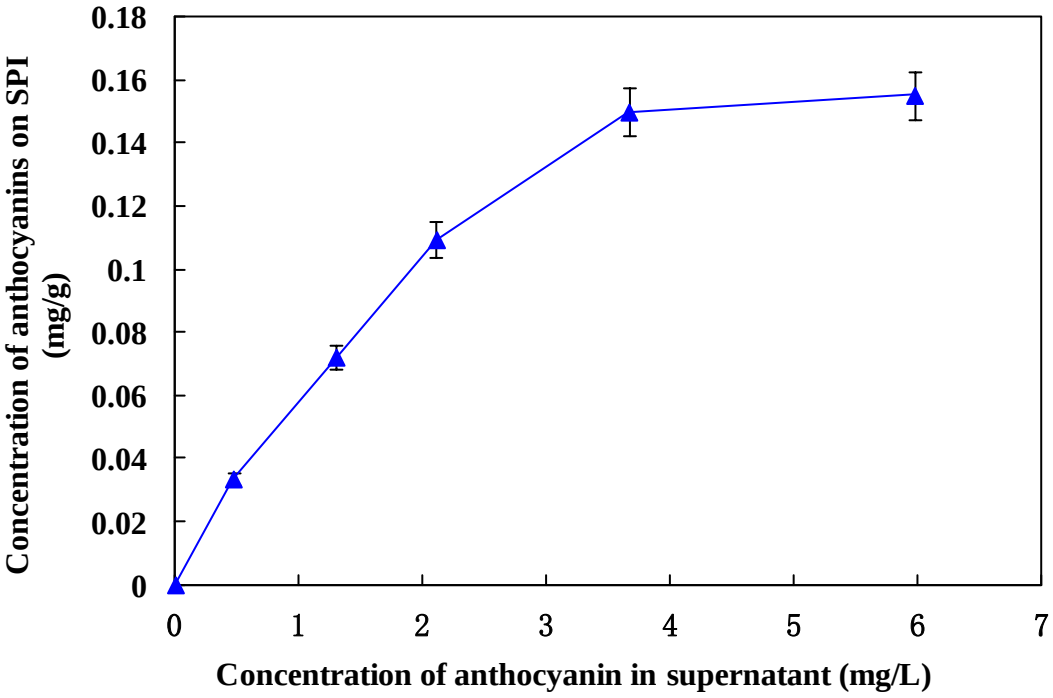


Figure 15 An equilibrium concentration of anthocyanins in supernatant solution

The adsorption density increases with increasing of c_e. Linearized form of Langmuir model can be written in the form (Jain, Garg et al. 2009):

$$\frac{c_e}{q_e} = \frac{c_e}{Q_o} + \frac{1}{Q_o b} \qquad 4$$

where Q_0 represents the maximum sorption capacity and b was a model parameter (Yao 2000).

Now one can use plot of c_e/q_e versus c_e to determine Q_0 and b values from slope and intercept of the graph. The values obtained are Q_0 =0.23 mg/g, b =2.59 L/mg and the regression correlation coefficients R^2=0.96 support our hypothesis that Langmuir isotherm model can be used to describe the adsorption process of anthocyanins by SPI. The essential characteristics of Langmuir isotherm can be explained in terms of dimensionless constant separation factor (R_{L}) which is expressed as (Kadirvelu, Thamaraiselvi et al. 2001):

$$R_L = \frac{1}{1 + bC_i} \qquad 5$$

where b is the Langmuir constant and C_i (mg/L) is the initial concentration of anthocyanins. Thus, R_L is a positive number whose magnitude determines the feasibility of the adsorption process. The R_L value for the initial concentration (C_i =8.42026 mg/L) used is 0.04 indicating favorable adsorption of anthocyanins onto SPI.

However, Langmuir isotherm is insufficient to explain the physical and chemical characteristics of adsorption. Dubinin–Radushkevich isotherm is commonly used to

describe the sorption isotherms of single solute systems. The D–R isotherm, apart from being analog of Langmuir isotherm, is more general than Langmuir isotherm as it rejects the homogeneous surface or constant adsorption potential The Dubinin–Radushkevich model is often used to estimate an apparent free energy of adsorption. Thus, for evaluating this parameter for the adsorbent, we test obtained adsorption data with Dubinin–Radushkevich model represented as follows (Hall, Eagleton et al. 1996):

$$\log q_e = \log Q_0 - 2B_D R^2 T^2 \log\left(\frac{C_e + 1}{C_e}\right) \qquad 6$$

where B_D is a constant related to adsorption energy (mol^2 kJ^{-2}), R is the gas constant (kJ mol^{-1} K^{-1}) and T is the temperature (K). The slope of $\log(q_e)$ versus $\log(1+1/C_e)$ gives B_D. The constant B_D is related with the mean free energy E_D (kJ mol-1) of adsorption per molecule of adsorbate and can be calculated through the equation below (Hasany and Chaudhary 1996):

$$\mathrm{E}_D = \frac{1}{\sqrt{2B_D}} \qquad 7$$

This energy gives information about the sorption mechanism. If E_D value is between 8 and 20 kJ/mol, the sorption process follows by chemical ion exchange and if E_D < 8 kJ/mol, the sorption process is of a physical nature. The calculated E_D value is equal to 1.96 kJ/mol for anthocyanins adsorption onto SPI (Table 6).

Table 6 D-R model constants for adsorption of anthocyanins on SPI

Adsorbent	Q_O (mg/g)	B_D (mol^2/kJ^2)	E_D (kJ/mol)	R^2
SPI	0.20	0.13	1.96	0.98

E_D value less than 8 kJ/mol indicating that the adsorption of anthocyanins is the physical process. According to the charcteristics of physical adsorption, we can get better knowledge on the anthocyanins adsorption process. Reversely, the observed phenomenon verifies the fact of its physisorption procedure. For example, unlike chemisorption, it happens below the boiling point which does not require a large amount of energy to activate the reaction. Physical nature of the anthocyanins adsorption means that electrostatic force played a significant role as a sorption mechanism for the sorption of phytophelos onto soy protein. This founding is also harmonized with our data on changes of SPI surface potential during adsorption process.

3.6. Modeling of Adsorption Kinetics

To some extend, when the resident time equals to zero, SPI does not have time to bind any chemicals, then SPI and supernatant solution are totally separated. Therefore, it can be assumed that the adsorption of anthocyanins in cranberry cocktail represented the adsorption of supernatant with the resident time zero. As mentioned above, the adsorbance at 510 nm indicates the anthocyanins, the original absorbance of anthocyanins is calculated to be 0.509, and then the content of anthocyanins in

supernatant solution droppes off as time increasing till around 20 minutes, where the sorption reaches equilibrium.

The adsorption process of phytonutrients onto soy protein could be assumed to occur in three steps.(Santiago, Maldonado-Valderrama et al. 2008) Firstly, the anthocyanins diffuse from the solution to the surface. In 10 minutes, content of anthocyanins increases rapidly and reached about 70% of the saturated adsorbance. The adsorption density of phytochemicals as a function of time is shown in Figure 16.

Then, the second step begins at a minimum coverage of phytochemicals reaching at the interface. The procedures described above dependent on the structural confirmation of protein. Finally, the surface of soy protein is saturated with anthocyanins, the adsorption of SPI arrives at equilibrium around 25 minutes. Figure 16 is depicts the concentration of anthocyanins as the function of time.

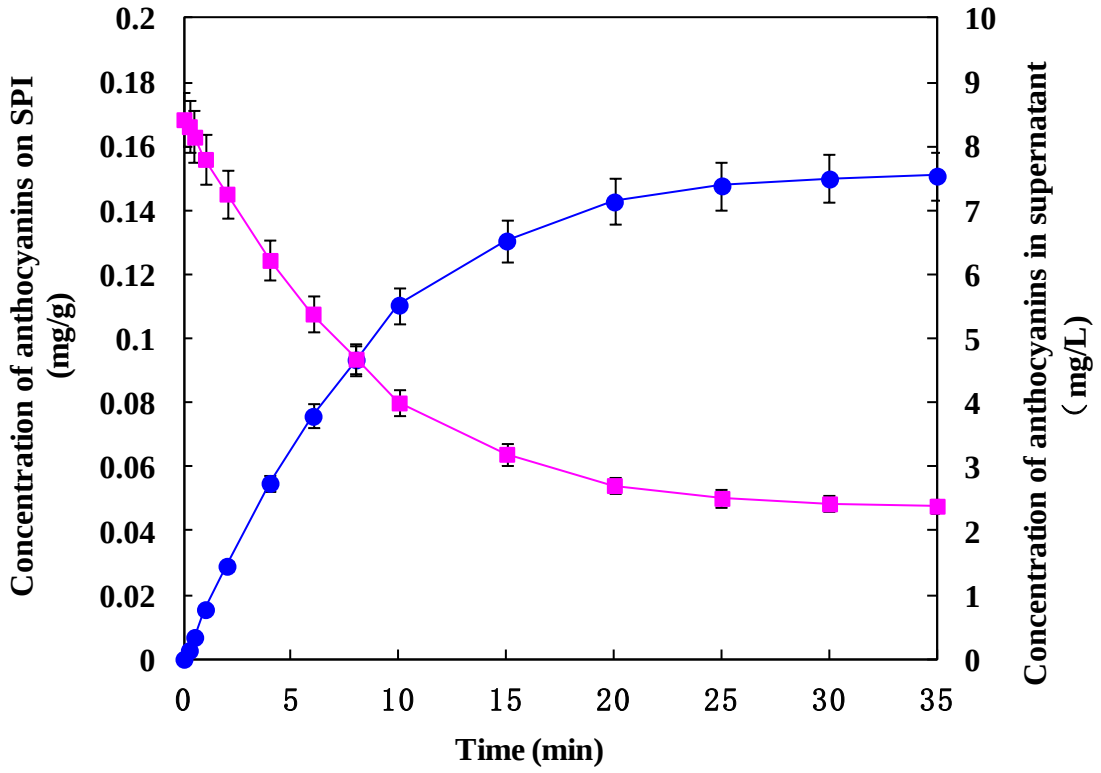


Figure 16 Concentration profiles of anthocyanins on SPI and supernatant solution as a function of time. Concentration with the unit of mg/g(■), concentration with the unit of mg/L(●).

Pseudo first order reaction is commonly used for the mathematical modeling describing adsorption kinetics (Aksu 2000). The pseudo-first order model assumes that the rate of an adsorption process is a chemical reaction process with the driving force being the difference between the averages adsorbent i.e. SPI concentration and the equilibrium concentration with the overall adsorption rate proportional to the driving force. The pseudo first order rate kinetics can be expressed as follows:

$$\frac{dq}{dt} = k_1(q_e - q) \qquad 8$$

Using method of separation of variables, a linearized form of Eqn.9 can be obtained in the following form:

$$\log(q_e - q) = \log q_e - \frac{k_1 t}{2.303} \qquad 9$$

Kinetics parameter k_1 can be estimated from the linear plot of $\log(q_e - q)$ versus t. In order to quantify the adsorption kinetics and investigate the mechanism of the adsorption of anthocyanins onto the soy protein, the pseudo first order model is applied to the experimental data. Obtained values k_1=0.0481 and q_e=0.8 are well fitted the model. The correlation coefficients for the first order model is R^2=0.987. It is also found that the calculated value of the equilibrium adsorption capacity q_e shows good agreement with the experimental data. This analysis suggests that the model adequately describes the experimental data, which may indicate that the pseudo first order adsorption mechanism is predominant and the rate-limiting step may be physisorption involving electrostatic interactions.

3.7. Optimal Processing Conditions in Terms of Maximum Yield

Figure 17 summarizes the influence of pH and temperature on the adsorption. It is obvious to find the optimal condition (45℃ and pH 7) for adsorbing anthocyanins using soy protein isolate.

As the temperature ascends to 45℃, the kinetic energy of small anthocyanins particle in solution increases and tends to interact with soy protein molecules. Thus, the SPI has relatively better adsorption ability at higher temperature. The adsorption capability also depends on the available sorption site on SPI. At constant temperature, the solubility of soy protein increases with pH value change from 4.2 (isoelectric point) to 7, and soy protein isolate is able to provide more free surface area to attract anthocyanins adsorb to it.

The amount of anthocyanins binding on the SPI increases result in the increasing the surface area, and vice versa. From Figure 13, the particle size is smaller at pH 1.7 compared with those at the other pH points. Besides, denaturation at extreme acidity, causing a significant conformational change and hence inhibits affinity for anthocyanins, has been demonstrated by the other researchers (Folch and Toner 2000).

In addition, through the analysis of conformation change under various condition (in 3.3.4), we could give the explanation of observation （in Figure 17）that the adsorbed anthocyanins decrease when temperature beyond 45℃. The extreme temperature condition could lead to formation of gel and protein denaturation. Then, the denaturation alteres the surface properties of SPI through changing the conformation (folding form to unfolding form). Specifically, at higher heating temperatures (beyond 45℃), complete unfolding of protein makes its hydrophobic groups rearranging themselves in order to reduce the surface hydrophobicity by decreasing particle size. Consequently, free surface area for anthocyanins adsorption shrinks. Thus, the denaturation of SPI beyond 45℃ causes alteration in the surface properties and

decreasing in the SPI binding efficacy. Another reason would be heating accelerates desorption of anthocyanins to escape from the SPI.

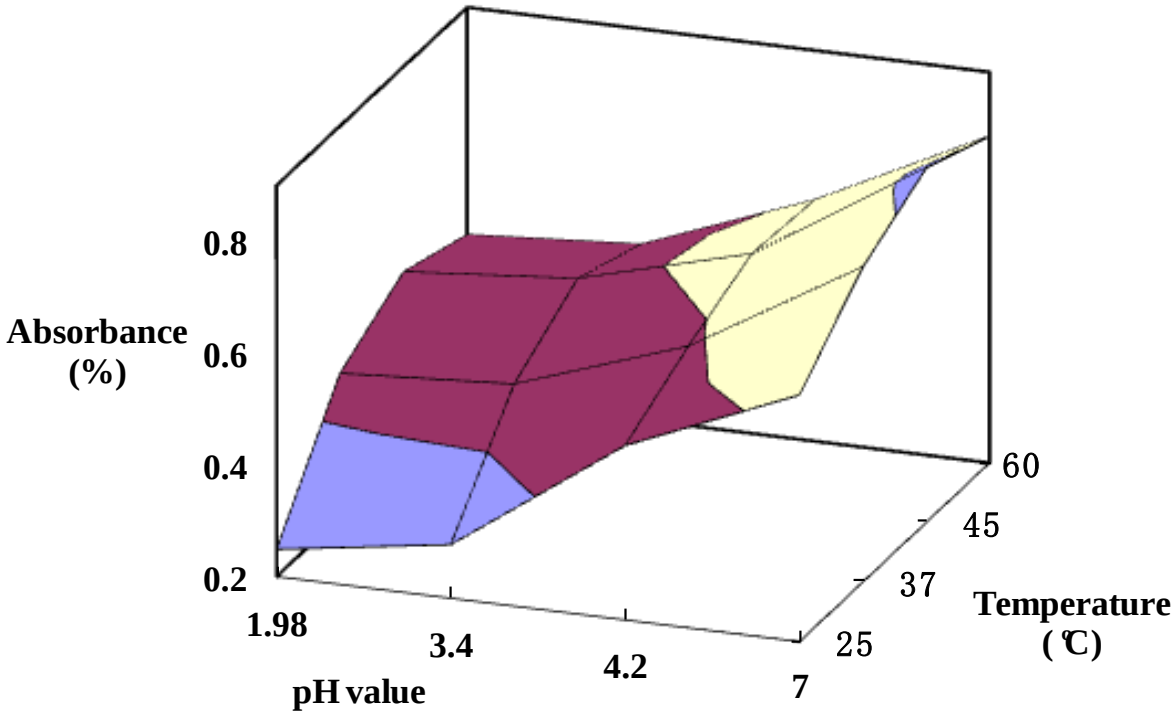


Figure 17 The profile of the relationship among pH, temperature and the adsorbance (%) when the concentration of SPI is 60 g/L.

4. CONCLUSION

We have designed a simple adsorption batch-type process, where soy protein isolate is used as a model system and cranberry juice matrix is used as a model berry juice system with significant amount of anthocyanins. This study shows that the soy protein isolate can successfully adsorb anthocyanins from the juice matrix without binding sugar. We systematically investigated the major processes that control adsorption process of anthocyanins within cranberry juice matrix under industrial processing relevant conditions. Obtained data on conformational changes in anthocyanins during adsorption process as function of pH and temperature; changes in protein surface potential and aggregative behavior will lead to better understanding of polyphenols, anthocyanins and protein interface interactions at the microscopic level. Obtained adsorption equilibrium data demonstrates good fit to Langmuir and Dubinin–Radushkevich isotherms. Based on calculated values of mean free energy of adsorption it is clear that the mechanism of soy protein-anthocyanins interaction is based on electrostatic ground instead of chemical binding or ion exchange. The effect of resident time on adsorption illustrated that it takes about 25 minutes for the adsorption of SPI to reach the equilibrium situation and pseudo-first order can be applied to describe the adsorpiton kinetics. Finally, the optimal processing conditions are summarized with the temperature 45℃ and pH 7, where the adsorption capability for IPS is largest.

Proposed technological process enables a simple concentration and separation of beneficial functional compounds from sugars, liquids, and other major components of

berry juices and their immobilization within the healthy, nutritious, and low-sugar food matrix provided by soy proteins. A promise to deliver bioactive natural products with potential anti-obesity and anti-diabetic effects from fruits and vegetables in a highly concentrated form without sugar and water represents a major interest for the functional food industry.

REFERENCES

Aksu, Z. (2000). "Equilibrium and kinetic modelling of cadmium(II) sorption by C. vulgaris in a batch system: effect of temperature." Separation and Purification Technology **21**: 285-294.

Andersen, O. M. and K. R. Markham, Eds. (2006). Flavonoids Chemistry, Biochemistry and Applications. Boca Raton, CRC Press.

Antipova, A., M. Semenova, et al. (1999). "Effect of sucrose on the thermodynamic properties of ovalbumin and sodium caseinate in bulk solution and at air-water interface." Colloids and Surfaces B: Biointerfaces **12**(3-6): 261-270.

Arnfield, S. D. and E. D. Murray (1981). "The influence of Processing Parameters on Food Protein Functionality. I. Differential Scanning Calorimetry as an Indicator of Prtein Denaturation." Can. Inst. Food Sci. Technol. J. **14**: 289-294.

Arrese, E. L., D. A. Sorgentini, et al. (1991). "Electrophoretic, solubility and functional properties of commercial soy protein isolates." J. Agric. Food Chem **39**(6): 1029-1032.

Badley, R. A., D. Atkinson, et al. (1975). "The structure, physical and chemical properties of the soy bean protein glycinin." Biochimica et Biophusica Acta **412**(2): 214-228.

Baranska, M., H. Schulz, et al. (2006). "In situ flavonoid analysis by FT-Raman Spectroscopy: Identification, distribution, and quantication of aspalathin in green rooibos." Anal. Chem. **78**: 7716-7721.

Baranska, M., H. Schulz, et al. (2007). "Identification and quantiication of valuable plant substances by IR and Raman spectroscopy." Vibrational Spectroscopy **43**: 13-25.

Benjwal, S., S. Verma, et al. (2006). "Monitoring protein aggregation during thermal unfolding incircular dichroism experiments." Protein Science **15**(3): 635-639.

Berli, C. L., J. A. Deiber, et al. (1999). "Connection between rheological parameters and colloidal interactions of a soy protein suspension " Food Hydrocolloids **13**(6): 507-515.

Bohm, B. A., Ed. (1998). Introduction to flavonoids. Chemistry and Biochemistry of Organic Natural Products. Amsterdam, Harwood Academic Publisher.

Camel, V. (2000). "Microwave-assisted solvent extraction of environmental sample." Trends in Analytical Chemistry **19**(4): 229-248.

Chan, L. (1996). "The applications of Raman spectroscopy in food science." Trends in Food Science and Technology **7**: 361-370.

Clifford, M. N. (2000). "Anthocyanins-nature, occurrence and dietary burden." Journal of the Science of Food and Agriculture **80**(7): 1063-1072.

Courthaudon, J. L., B. Colas, et al. (1989). "Covalent bonding of glucosyl residues to bovine casein: effect on solubility and viscosity." J. Agric. Food Chem. **37**: 32-36.

Damodaran, S. and A. Paraf (1997). Structure-function relationships of soy proteins. Food Proteins and Their Applications. New York**:** 257-258, CRC Press.

Demetriades, K., J. N. Coupland, et al. (1997). "Physicochemical properties of whey protein-stabilised emulsions as affected by heating and ionic strength." Journal of Food Science **62**(3): 462-467.

Dudek, S. G. (2001). Nutrition essentials for nursing practice. Philadelphia, Lippincott Williams & Wilkins.

Duthie, S. J., P. T. Gardner, et al. (2005). "DNA stability and lipid peroxidation in vitamin E-deficient rats in vivo and colon cells in vitro: modulation by the dietary anthocyanin, cyanidin-3-glycoside." European Journal of Nutrition **44**(4): 195-204.

Duthie, S. J., A. M. Jenkinson, et al. (2006). "The effects of cranberry juice consumption on antioxidant status and biomarkers relating to heart disease and cancer in healthy human volunteers." Eur. J. Nutr. **45**(2): 113-122.

Folch, A. and M. Toner (2000). "Microengineering of cellular interatcions." Annual Review of Biomedical Engineering **02**: 227-256.

Fulcrand, H., C. Benabdeljalil, et al. (1998). "A new class of wine pigments yielded by reaction between pyruvic acid and the grape anthocyanins." Phytochemistry **47**: 1401-1407.

Fuleki, T. and F. J. Francis (2006). "Quantitative methods for anthocyanins. 4. determination of individual anthocyanins in cranberry and cranberry products." Journal of Food Science **33**(5): 471-478.

Fund, W. C. R. (1997). "Food, nutrition and the prevention of cancer: a global perspective." Nutrition **15**(6): 523-6.

Garcia-Viguera, C., P. Zafrilla, et al. (1998). "The use of acetone as an extraction solvent for anthocyanins from strawberry fruit." Phytochemicl Analysis **9**(6): 274-277.

Gsiorowski, K., K. Szyba, et al. (1997). "Antimutagenic activity of anthocyanins isolated from aronia melanocarpa fruits." Cancer Letters **119**(1): 37-46.

Hä kkinena, S., M. Heinonenc, et al. (1999). "Screening of selected flavonoids and phenolic acids in 19 berries." Food Research International **32**(5): 345-353.

Hakimuddin, F., G. Paliyath, et al. (2004). "Selective cytotoxicity of a red grape wine flavonoid fraction against MCF-7 cells." Breast Cancer Res Treat **85**: 65-79.

Hakkinen, S. H., S. O. Karenlampi, et al. (1999). " Content of the flavonols quercetin, myricetin, and kaempferol in 25 edible berries." J. Agric. Food Chem. **47**: 2274-2279.

Hall, K. E., L. C. Eagleton, et al. (1996). "Pore and solid diffusion kinetics in fixed bed adsorption under constant pattern conditions." Industrial & Engineering Chemistry Fundementals **5**: 212-223.

Hao, J., W. Han, et al. (2002). "Microwave-assisted extraction of artemisinin from Artemisia annua L." Separation and Purification Technology **28**(3): 191-196.

Hasany, S. M. and M. H. Chaudhary (1996). "Sorption potential of Hare River sand for the removal of antimony from acidic aqueous solution." Applied Radiation and Isotopes **47**: 467-471.

Haschke, F., A. Firmansyah, M. Meng, P. Steenhout and A. L. Carrie (2001). "Functional food for infants and children." Monatsschrift Kinderheilkunde **149**: 66-70.

Heinonen, I. M., A. S. Meyer, et al. (1998). "Antioxidant activity of berry phenolics on human low-density lipo protein and liposome oxidation." J. Agric. Food Chem. **46**(10): 4107-4112.

Jain, M., V. K. Garg, et al. (2009). "Chromium(VI) removal from aqueous system using *Helianthus annuus*(sunflower) stem waste." Journal of Hazardous Materials **162**: 365-372.

Jiang, J., Y. L. Xiong, et al. (2010). "pH shifting alters solubility characteristics and thermal stability of soy protein isolate and its globulin fractions in different pH, salt concentration, and temperature conditions" J. Agric. Food Chem. **58**: 8035-8042.

Jurd, L. and S. Asen (1966). "The formation of metal and "co-pigment" complexes of cyanidin 3-glucoside." Phytochemistry **5**: 1263-1271.

Kadirvelu, K., K. Thamaraiselvi, et al. (2001). "Adsorption of nickel(II) from aqueous solution onto activated carbon prepared from coir pith." Separation and purification technology **24**: 497-505.

Kalra, E. K. (2003). "Nutraceutical - Definition and Introduction." AAPS PharmSci **5**(3): 25.

Kamei, H., T. Koide, et al. (1996). "Flavonoid-mediated tumour growth suppression demonstrated by in vivo study." Cancer Biother Radiopharm **11**: 193-196.

Koponen, J. M., A. M. Happonen, et al. (2008). "Characterization and fate of black currant and bilberry flavonols in enzyme-aided processing." Journal of Agricultural and Food Chemistry **56**(9): 3136-3144.

Koshiyama, I. (1972). "Comparison of acid induced conformation changes between 7S and 11S globulin in soybean seeds." Journal of the Science of Food and Agriculture **23**: 853-859.

Kulmyrzaev, A., C. Bryant, et al. (2000). "Influence of sucrose on the thermal denaturation, gelation and emulsion stabilization of whey proteins." Journal of Agricultural and Food Chemistry **48**: 1593-1597.

Kuo, S. M. (1997). "Dietary flavonoid and cancer prevention: evidence and potentialmechanism." Critical Reviews in Oncogenesis **8**(1): 47-69.

Kwon, J.-H., J. M. R. Belanger, et al. (2003). "Application of the microwave-assisted process (MAPTM) to the fast extraction of ginseng saponins." Food Research International **36**(5): 491-498.

Lakemond, C. M. M., H. H. J. d. Jongh, et al. (2000). "Heat denaturation of soy glycinin: influence of pH and ionic strength on molecular structure." Journal of Agricultural and Food Chemistry **48**: 1991-1995.

Lapidot, T., S. Harel, et al. (1999). "pH-Dependent forms of red wine anthocyanins as antioxidants." J. Agric. Food Chem. **47**: 67-70.

Lapornik, B., M. Prošek, et al. (2005). "Comparison of extracts prepared from plant by-products using different solvent and extraction time." Journal of Food Engineering **71**: 214-228.

Lee, K. H., H. S. Ryu, et al. (2003). "Protein solubility characteristics of commercial soy protein products." JAOCS **80**(1): 85-90.

Liang, Y., L. K. Noda, et al. (2000). "Polarizability and concentration effects on the Raman spectra of picolinic acid species in aqueous solution." Journal of Molecular Structure **554**: 271-277.

Liu, M., D. S. Lee, et al. (1999). "Emulsifying properties of acidic subunits of soy 11s globulin." J. Agric. Food Chem. **47**(12): 4970-4975.

Liu, X., G. Xiao, et al. (2004). "Quantification and purification of mulberry anthocyanins with macroporous resins." Journal of Biomedicine and Biotechnology **5**: 326-331.

Luck, P. J., T. C. Lanier, et al. (2002). "Viscoelastic behavior of commercially processed soy isolate pastes during heating and cooling." Journal of Food Science **67**(4): 1379-1382.

Määttä-Riihinen, K. R., A. Kamal-Eldin, et al. (2004). "Distribution and contents of phenolic compounds in eighteen Scandinavian berry species. ." Journal of Agricultural and Food Chemistry **2**(14): 4477-4486.

Malhotra, A. and J. N. Coupland (2004). "The effect of surfactants on the solubility, zeta potential, and viscosity of soy protein isolates." Food Hydrocolloids **18**: 1001-1008.

Mantell, C., M. Rodriguez, et al. (2003). "A screening analysis of the high-pressure extraction of anthocyanins from red grape pomace with carbon dioxide and cosolvent." Engineering in Life Sciences **3**: 38-42.

Mathers, C., G. Stevens, et al. (2009). Mortality and burden of disease attributable to selected major risks. Global Health Risks. Geneva, World Health Organization**:** 16-18.

McClements, D. (2001). "Estimation of steric exclusion and idfferential interaction contributions to protein transfer free energies in aqueous cosolvent solutions." Food Hydrocolloids **15**: 355-363.

Merlin, J.-C., A. Statoua, et al. (1993). "Resonance raman spectroscopic studies of anthocyanins and anthocyanidins in aqueous solutions " Phytochemistry **35**(1): 227-232.

Metiever, R. P., F. J. Francis, et al. (1980). "Solvent extraction of anthocyanins from wine pomace." Journal of Food Science **45**: 1099-1100.

Monohan, F. J., D. J. McClements, et al. (1996). "Disulfide mediated polymerisation reactions and physical properties of heated WPI-stabilised emulsions." Journal of Food Science **61**(3): 504-540.

Mora-Gutierrez, A. and J. H. Farrel (2000). "Sugar-casein interaction in deuterated solutions of bovine and caprine casein as determined by oxygen-17 and carbon-13 nuclear magnetic resonance: a case of preferential interactions." J. Agric. Food Chem **48**: 3245-3255.

Morris, J. B. (2003). "Biofunctional legumes with nutraceutical, pharmaceutical, and industrial uses." Economic botany **57**(2): 254-261.

Morrison, G. and L. Hark (1999). Medical nutrition and disease. Malden, Blackwell Science.

Motohashi, N. and M. Sakagami (2009). "Anthocyanins as functional food colors." Topics in Heterocyclic Chemistry **16**: 1-40.

Ness, A. R. and J. W. Powles (1997). "Fruit and vegetables, and cardiovascular disease: a review. ." International Journal of Epidemiology **26**: 1-13.

Prior, R. L. and S. A. Lazarus (2001). "Identification of procyanidins and anthocyanins in blueberries and cranberries (vaccinium spp.) using high-performance liquid chromatography/ mass spectrometry." J. Agric. Food Chem **49**(3): 1270-1276.

Puppo, M. C. and M. C. Anon (1998). "Effect of pH and protein concentration on rheological behavior of acidic soy bean protein gels." J. Agric. Food Chem. **46**(8): 3039-3046.

Puppo, M. C. and M. C. Anon (1998). "Structural properties of heat-induced soy protein gels as affected by ionic strength and pH." J. Agric. Food Chem. **46**: 3583-3589.

Puppo, M. C. and M. C. Anon (1999). "Soy bean protein dispersions at acid pH.Thermal and rheological properties." Journal of Food Science **64**: 50-56.

Renkema, J. M. S., H. Gruppen, et al. (2002). "Influence of pH and Ionic Strength on Heat-Induced Formation and Rheological Properties of Soy Protein Gels in Relation to Denaturation and Their Protein Compositions." J. Agric. Food Chem **50**: 6064-6071.

Rennema, O. R., S. Damodaran, et al. (2007). Amino acids, peptides, and proteins. Food Chemistry. S. Damodaran. Boca Raton, FL, CRC Press**:** 373.

Sandell, M., O. Laaksonen, et al. (2009). "Orosensory profiles and chemical composition of black currant (Ribes nigrum) juice and fractions of press residue." Journal of Agricultural and Food Chemistry **57**(9): 3718-3728.

Santiago, L. G., J. Maldonado-Valderrama, et al. (2008). "Adsorption of soy protein isolate at air-water and oil-water interfaces." Colloids and Surfaces **223**: 155-162.

Singh, R. P. and D. R. Heldman (2009). Supplemental Process. Introduction to Food Engineering. S. L. Taylor. London, Elsevier Inc.

Stanciu, G., S. Lupsor, et al. (2010). "Spectrophotometric study on stability of anthocyanins extracts from black grapes skins." Ovidius University Annals of Chemistry **21**(1): 101-104.

Strack, D. and V. Wray, Eds. (1989). Anthocyanins. Methods in Plant. New York, Academic Press.

Suhaj, M. (2006). "Spice antioxidants isolation and their antiradical activity:a review." Journal of Food Composition and Analysis **19**: 531-537.

Sun, Y., X. Liao, et al. (2007). "Optimization of microwave-assisted extraction of anthocyanins in red raspberries and identification of anthocyanin of extracts using high-performance liquid chromatography–mass spectrometry." European Food Research and Technology **225**: 511-523.

Utsumi, S., T. Nakamura, et al. (1987). "Occurrence of dissociable and undissociable soybean glycinin." Agricultural and Biological Chemistry **51**(8): 2139-2144.

Van Vliet, T., H. J. M. Van Dijk, et al. (1991). "Relation between syneresis and rheological properties of particle gels." Colloid Polymer Science **269**: 620-627.

Wagner, J. R., D. A. Sorgentini, et al. (1992). "Effect of physical and chemical factors on rheological behavior of commercial soy protein isolates: protein concentration, water imbibing capacity, salt addition, and thermal treatment." J. Agric. Food Chem. **40**(10): 1930-1937.

Wagnert, J. R. and J. Gueguen (1995). "Effects of dissociation, deamidation, and reducing treatment on structural and surface active properties of soy glycinin." J. Agric. Food Chem **43**(8): 1993-2000.

Watson, E. (2007). "Super fruits." Food Ingredients: Health and Nutrition **4**: 45-47.

Webb, P. A. (2003). "Introduction to chemical adsorption analytical techniques and their applications to catalysis." Micromeritics **13**: 1-4.

Wolf, W. J. and D. R. Briggs (1958). "Studies on the cold insoluble fraction of the water extractable soybean proteins. Factors influencing conformation changes in the 11S component." Archives of Biochemistry and Biophysics **76**: 337-393.

Wong, H. H. and N. R. Lemoine (2009). "Pancreatic cancer: molecular pathogenesis and new therapeutic targets." Nature Reviews Gastroenterology and Hepatology **6**(7): 412-422.

Wrolstad, R. E., E. A. Decker, et al. (2005). Characterization and measurement of anthocyanins by UV-visible spectroscopy. Handbook of Food Analytical Chemistry, Water, Proteins, Enzymes, Lipids, and Carbohydrates. Hoboken, John Wiley & Sons, Inc.

Yao, C. (2000). "Extended and improved Langmuir equation for correlating adsorption equilibrium data." Separation and Purification Technology **19**(3): 237-242.

Youdim, K. A., A. Martin, et al. (2000). "Incorporation of the elder berry anthocyanins by endothelial cells increases protection against oxidative stress." Free Radical Biology & Medicine **29**: 51-60.

Youdim, K. A., J. McDonald, et al. (2002). "Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults." Journal of Nutritional Biochemistry **13**: 282-288.

Zhou, J.-M. and R. K. Ibrahim (2010). "Tricin—a potential multifunctional nutraceutical." Phytochemistry Reviews **9**(3): 413-424.