HIGH PRESSURE ASSISTED INFUSION OF PHYTOCHEMICAL ANTIOXIDANTS INTO FRUITS: INFLUENCE OF PROCESS PARAMETERS AND MECHANISTIC INSIGHTS

By

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ABSTRACT OF THE DISSERTATION

High Pressure Assisted Infusion of Phytochemical Antioxidants Into Fruits:
Influence of Process Parameters and Mechanistic Insights

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Professor Mukund V. Karwe

High hydrostatic pressure (HHP) processing has been shown to infuse small size molecules into food materials much faster than does osmosis-based transport. The accepted mechanism is that HHP permeabilizes cells which increases the mass diffusivity of the food matrix. Hence, infusion is enhanced when foods are treated under high pressure.

In this study we investigated, using a model system, HHP driven infusion of quercetin into cranberries. The main objectives of the study were to test whether enhanced infusion of quercetin can be achieved using HHP and to test whether the commonly accepted mechanism of cell permeabilization is operative in this model system. Two systems were used in this study - fresh cranberries, cells of which are intact and frozen-thawed cranberries, cells of which get permeabilized during freeze-thawing process.
Under HHP, infusion of quercetin was enhanced, compared to infusion at ambient conditions (control), in both fresh and frozen-thawed cranberries. While the amount of quercetin infused under pressure was 3-5 times that in control, it was independent of the applied pressure in a broad range (5-551 MPa). Low pressure of 5 MPa was sufficient to cause enhanced infusion in frozen-thawed cranberries. Furthermore, pressure cycling treatment (2 cycles and 5 cycles) significantly increased the amount of quercetin infused. Unlike other studies, no additional cell permeabilization was observed in frozen-thawed cranberries after HHP, although amount infused was higher. This result suggested that the commonly accepted mechanism of cell permeabilization may not be the only cause of enhanced infusion. Additionally, while both fresh and frozen-thawed cranberries showed similar cell-membrane permeability after HHP, fresh cranberries had about two times greater amount of infused-quercetin than in frozen-thawed cranberries after HHP. If cell-membrane rupture were the only mechanism of infusion, then the amount infused into both, fresh and frozen-thawed cranberries, should have been equal.

These results suggest that HHP infusion is not just cell-permeabilization based but may be caused by pressure driven flow (Darcy flow in porous media). Understanding the actual mechanisms of transport under pressure may enable to develop process guidelines that will help the food industry to develop value-added foods.
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CHAPTER 1

INTRODUCTION

1.1 MOTIVATION

High hydrostatic pressure (HHP) processing is a food processing method, which has shown great potential as a food preservation method. The applications of HHP include microbial inactivation and shelf-life extension through influencing biochemical reactions, enzymatic reactions and structural changes in the food product. HHP techniques have also gained momentum in areas of food preservation outside of sterilization and pasteurization. These applications include possibilities offered by combining high pressure with low temperatures (HPLT) for pressure-supported freezing, thawing and subzero storage, again, to extend shelf life of food products (Urrutia-Benet et al., 2004). There is, however, limited to no research conducted to explore applications of HHP to carry out mass transport under pressure, specifically to infuse selected substances into food matrices in order to enhance their nutritional and sensorial quality.

Application of high pressures causes permeabilization of the cell structure (Dornenburg and Knorr, 1993; Eshtiaghi, Stute, and Knorr, 1994; Farr, 1990; Rastogi, Subramanian, and Raghavarao, 1994). Researchers have exploited this phenomenon to enhance mass transfer rates during osmotic dehydration (Sopanangkul et al., 2002) and extract substances (Corrales et al., 2008; Bi et al., 2009; Jun, 2009) from fruits and vegetables. Cell-membranes permeabilize under pressure that reduces resistance to mass transport and therefore enhances mass transfer rates during extraction or osmotic dehydration. In most of these studies HHP was merely used as a pre-treatment to osmotic dehydration. The scope of infusion under pressure and the underlying mechanisms of
pressure assisted mass transport have not been investigated so far. The motivation behind studying high pressure assisted infusion is to investigate its potential to enhance and accelerate infusion compared to conventional osmotic diffusion based processes.

Moreover, pressure assisted infusion will help develop nutritionally enhanced “super food” products. In response to U.S. Army’s combat rations program’s call to improve the antioxidant value of their existing berry rations, the research study was designed to understand infusion under pressure of phytochemical antioxidant molecules into berries. Specifically, we studied high pressure assisted infusion of quercetin (an antioxidant) into cranberries. Cranberry-quercetin system served as a model system. The quercetin-cranberry system was selected as the system of study as per suggestions from the U.S. Army’s Natick labs. Based on their research quercetin was a highly potent antioxidant and cranberry was one of the popular berries consumed by soldiers (USARIEM report, September 2008).

In this thesis, experimental techniques were used to analyze systematically the influence of HHP on infusion. Specifically, the effect of HHP process variables on extent of quercetin infusion and cell-membrane structure of cranberries is analyzed. The fundamental mechanisms driving pressure assisted infusion in cranberries are explained. Additionally, a proof of concept study was conducted using different infusing molecules, food substrate matrices and methods of infusion in order to understand the potential of HHP assisted infusion.
1.2 BACKGROUND

Osmotic dehydration has been traditionally used in the food industry for partial removal of water from fruits and vegetables and infusion of small molecular size solute molecules such as sugar and salt, when immersed in a concentrated solution of sugar or salt. Due to difference in the osmotic pressures between the inside of fruit and the solution outside, water diffuses out from the fruit into the solution while the solute molecules from the solution diffuse into the food matrix. Diffusion, however, is usually a slow mass transfer process. The time of diffusion ranges from several hours to days depending on the transport species and the substrate matrix and is reflected in very low diffusion coefficient values. For example, diffusion coefficient value for sucrose ranges from 0.5 to 1.0 x 10^{-10} m^2/s for sugarcane with hard cell-wall and 1.5 to 4.5 x 10^{-10} m^2/s for materials with soft cell-wall (potatoes, sugar-beets, apples and tomatoes) (Schwartzberg and Chao, 1982). As a result, assuming unidirectional diffusion of sucrose in a piece of potato (infinite plane geometry) with a diffusion coefficient value of 1.5 to 4.5 x 10^{-10} m^2/s, based on Fickian diffusion, it will take approximately 1 day to 4 days to diffuse through 1 cm of potato tissue. The relation used to approximate time of diffusion was 

\[ t = \frac{x^2}{2D} \]

where \( t \) is the time of infusion, \( x \) is the dimension along which diffusion occurs and \( D \) is the diffusion coefficient (Crank, 1975). Therefore, there is a need for additional methods that can accelerate mass transport in and out of food products.

A number of techniques have been explored to accelerate mass transport rates of solutes into food materials. These techniques include: using high intensity pulsed electric field (HIPEF) prior to osmotic treatment, vacuum impregnation (VI), power ultrasound
during atmospheric pressure diffusion (Rastogi et al., 2002; Andres et al., 2001; Simal et al., 1998). In HIPEF and VI methods, electric field pulses, or vacuum is applied to the system that affects mechanical properties of the product being processed, decreasing resistance to infusion and hence processing time. During vacuum impregnation, the external liquid flows into capillary pores of plant food and meat tissues due to expansion and partial outflow of internal gases during vacuum step. The vacuum step is followed by compression of internal gases during restoration to atmospheric pressure (Andres et al., 2001). In the HIPEF processing, the applied electric field causes electroporation of biological tissue increasing their porosity and reducing resistance to mass transport (Rastogi et al., 2002). Application of ultrasound results in a phenomenon known as cavitation that increases diffusion and osmotic processes. During sonication, cavitation occurs when bubbles of liquid are formed that burst and generates localized high pressure regions. The bubbles implode on the substrate surface causing cell damage resulting in rapid mass transport (Simal et al., 1998). These processing techniques, however, have technical challenges related to scale up and cost for commercialization (Rastogi et al., 2002; Rosa and Giroux, 2001). Alternatively, high hydrostatic pressure (HHP) processing is another method that can be used to accelerate diffusion based mass transport processes. HHP processing poses lesser challenges of commercialization compared to HIPEF, VI and ultrasound due to its already existing application in the food industry as a pasteurization technique.
1.3. BRIEF HISTORY OF DEVELOPMENT OF HIGH HYDROSTATIC PRESSURE PROCESSING OF FOODS

HHP processing is defined as a non-thermal process where food materials are subjected to isostatic pressures from 40 MPa (5 kpsi) to 1000 MPa (145 kpsi) for a period of 1 to 20 min. A pictorial representation of the magnitude of pressures used in foods is shown in Figure 1.1. The key advantages of this technology are:

(i) it enables processing at ambient and lower temperatures, on most occasions
(ii) it enables instantaneous transmittance of pressure to the food product, irrespective of the size and shape of the product
(iii) it causes microbial inactivation in foods without application of heat and use of chemical preservatives, thus eliminating heat damage and improving overall quality of food
(iv) it can be used to design ingredients and products with novel functional properties due to its minimal effects on nutritional compounds.

The HHP technology was adopted from the materials and process-engineering industry where materials were subjected to cold isostatic processing for sheet metal forming and pressing of advanced materials used in turbines and ceramics (Rastogi et al., 2007). The technology has been explored even to make artificial diamonds and in powder metallurgy.
HHP processing in foods was first demonstrated in 1899 by Bert H. Hite at West Virginia Agricultural Experimental Station as a possible food preservation process (Hoover et al., 1989; Knorr, 1999). Hite showed that high pressure treatment could delay the souring of milk at ambient temperatures (Knorr, 2003). Bridgman (1914) observed coagulation of egg white with high pressure treatment. The technology did not attract wide recognition in this period due to non-availability of suitable high pressure equipment (Rastogi et al. 2007) for processing at larger scale. The technology re-surfaced in the food industry in the late 1980s. Between 1982-1988, Daniel Farkas, Dallas Hoover, and Dietrich Knorr at the University of Delaware attempted to repeat Hite’s work using a cold isostatic press and showed that pressures of 350 MPa (50,000 psi) can inactivate a wide range of pathogenic and spoilage microbes. During the same period, studies were undertaken in Japan on preservation of foods by high pressure. In 1992, commercialized high pressure processed products (high acid products including apple, strawberry, and pineapple jams) were marketed in Japan (Hayashi, 2002). Since then, high pressure
processing has also been applied to fruit preserves, raw squid, grape juice, and mandarin orange juice in Japan (Hayakawa et al., 1996; Rastogi et al., 2007). High pressure processed foods are available in the markets of Japan since 1992 (Suzuki, 2002), and in Europe and in the United States since 1996 (Knorr, 1999; Knorr et al. 2002). In the U.S., the impetus for high pressure technology came from the U.S. Army research center in Natick, Massachusetts, in order to develop better quality MREs (Meal Ready to Eat) for the troops.

The first commercial high pressure product in the U.S. was Avo Classic Guacamole (a heat sensitive product) with extended refrigerated shelf life. It is manufactured by Avomex, Inc., Keller, TX. Recently, a leading Mexican manufacturer of juices and nectars, Grupo Jumex, utilized HHP for their juice and smoothie product lines. Also, growing concerns for seafood safety led seafood manufacturers to explore this technology and show many benefits, such as, almost 100% meat separation from shell of clams, crabs, lobsters, and oysters, with increased yield without any mechanical damage to the meat, in addition to reduction in microbial load. Other, commercially available high pressure processed products in Australia, Europe and the U.S. include juice, tomato salsa, smoothies, fruit & vegetable purees, and ready to eat meats. Examples of HHP processed products available in the food markets of the world are shown in Figure 1.2. Recently Starbucks invested $70 M for using HHP to process their “evolution” juices. Other U.S. based mid-size companies that have recently invested in HHP processed beverages include Suja Juice Co., Hain Celestial, and Forager Project. (http://news.starbucks.com/news/evolution-fresh-opens-70-million-state-of-the-art-
The high pressure technology is expanding rapidly for new product development and product improvements in other segments of food industry, such as, the dairy sector (cheese, yogurt, and mayonnaise). Since HHP processing has minimal detrimental impact on thermally labile bioactive ingredients, the technology is becoming a topic of major interest for cosmetic, nutraceutical and pharmaceutical industry.

1.4. PRINCIPLES OF HHP PROCESSING

There are two general scientific principles that play a major role in high pressure processing of foods. They are Le Chatelier’s principle and Pascal’s isostatic principle.
The physico-chemical changes that occur during HHP processing follow Le Chatelier’s principle, which states that when a system at equilibrium is disturbed, the system responds in a way to minimize the disturbance. This means that any phenomena (reaction, conformational change, or phase transition) that are accompanied by decrease in volume are enhanced by pressure and reactions involving increase in volume are not favored at high pressure. Thus, under pressure, reaction equilibriums are shifted towards the compact or low specific volume state. Further, high pressure affects only non-covalent bonds (hydrogen, ionic, and hydrophobic bonds). This property causes protein chain unfolding under high pressure but has little effect on chemical constituents associated with desirable food quality components, such as flavor, color and nutritional compounds (Hogan et al., 2005). The low compression energy levels associated with pressure processing may explain why covalent bonds in food constituents are less affected than the weak non-covalent interactions. For example, the compression energy of 1 liter of water at 400 MPa is 19.2 kJ which is close to 20.9 kJ required for heating 1 liter of water from 20 °C to 25 °C (Rastogi et al., 2007). This example illustrates that energy associated with heating substances to low temperatures of 25 °C is equivalent to compression energy associated with HHP processing of similar composition substances and is not sufficient to affect covalent bonds.

According to Pascal’s isostatic principle, high pressure acts uniformly and instantly throughout liquid and soft solids. The time necessary for high pressure processing is therefore independent of sample size, in contrast to thermal processing. Therefore, the process time is independent of the volume and shape of the food product. Recently, however, the concept of pressure uniformity has been challenged especially in
heterogeneous food products (Minerich and Labuza, 2003). The principle of isostaticity, i.e., uniform pressure distribution, in a food product subjected to HHP processing is valid for homogeneous isotropic foods, but it may not hold for heterogeneous and non-isotropic food products such as, meat with bones and muscle fibers. This hypothesis is still under investigation by a few researchers (Karwe et al., 2011).

Pressurization is accompanied by other effects, such as, electrostriction and adiabatic compression heating of foods. Electrostriction observed during HHP results in pH change. Ions are formed under pressure from molecules that are associated with volume contraction. For example, self-ionization of water molecules is promoted under pressure. The change in partial molal volume for ionization of water in H3O+ and OH– is -22.2 cm³/mol at 25 °C. Similar changes in ionization volume associated with volume contraction in inorganic and organic acids. In large ions and in ions characterized by charge delocalization contraction is lower. The volume contraction is brought about by strong electrostriction around the ions formed. This results in negative and reversible pH shifts depending on the chemical nature of the system (Tauscher, 1995).

The increase in temperature of food materials during pressurization can be explained by compression heating. Heat of compression is a thermodynamic outcome of high pressure processing. Although many food and non-food materials are considered as incompressible at atmospheric and low pressures, they do get compressed substantially under very high pressures (water can be compressed by 4 % at 100 MPa and 15 % at 600 MPa and 22 °C) (Tauscher, 1995). The work done to compress a material under pressure gets converted into heat, which increases the temperature of the substance. This is called as adiabatic compression heating and is usually expressed as temperature rise per 100
MPa increase in pressure. Typical values of adiabatic compression heating are: 3 °C for water, 6-9 °C for cooking oils and fatty foods, and 30 °C for synthetic chemicals such as hexane. For most foods containing water, carbohydrates, fats, protein, the range of values for adiabatic compression heating value ranged between is 3 °C to 6 °C. Therefore, although HHP is considered as a non-thermal process, in some situations pressure increase will result in significant temperature rise. Since the compressibility values of materials are different, the associated compression heating values also vary considerably (Rasanayagam et al., 2003). This could potentially generate non-uniformity of temperature during HHP processing that could consequentially impact extent of microbial and enzyme inactivation (Khurana and Karwe, 2009; Khurana, 2012). The aspect associated with adiabatic temperature rise of products and temperature uniformity was neglected in this study. All infusion using HHP was carried out at an initial temperature near 22 °C (room temperature). The associated adiabatic temperature rise was never more than 15 °C where infusion in fruits was unaffected by temperature rise. All the observed changes on the extent of infusion were contributed by high pressure. The negligible effect of temperature (< 40 °C) on infusion was confirmed experimentally which is discussed in Chapter 3.

1.5. HHP PROCESSING EQUIPMENT AND PROCESS

High pressure food processing is a batch operation wherein food products in packages with very little head space or gas are processed at a desired pressure and time. Although the effect of high pressure on foods up to 1000 MPa (145,000 psi) has been investigated in laboratory scale vessels, for the commercial applications the equipment
design limitations restrict the pressure levels to 690 MPa (100,000 psi). High pressure food processing equipment with pressures up to 690 MPa and temperatures in the range of 5 °C to 120 °C are currently available to the food industry for large scale processing (Henry and Chapman, 2002).

A high pressure food processing unit operation consists of a cylindrical steel vessel with high tensile strength, two end closures, a yoke for restraining end closures, direct or indirect pressure pumps for pressure generation, pressure and temperature controls, and loading and unloading equipment. Commercial high pressure units are available in vertical or horizontal vessel orientations. The vertical vessels are more common for medium capacity (up to 320 L) pressure vessels. As the demand for high pressure processed products increased and the technology was more commercialized, there was a need for higher capacity vessels. From high pressure vessel design, safety and mechanical strength point of view, it is always recommended to increase length of the cylinder instead of radius for higher capacity vessels. Longer vessels in vertical orientation can pose various problems such instability, difficulty in loading, and high ceiling. Today most high capacity (~1000 liters) vessels come in horizontal orientation. In addition, in horizontal orientation, loading can be done from one end and unloading from the other end, which allows ease of separation of processed and unprocessed product streams. Examples of processing vessels used in the industry are shown in Figure 1.3.
In a typical HHP process operation, food products are vacuum packed (some products such as shell fish are placed in a mesh bag and tied with rubber bands to prevent meat falling out of the shells after HHP) in flexible pouches or containers and loaded in the vessel. HHP processing requires airtight packages that can withstand a change in volume corresponding to the compressibility of the product. Generally, the packaging must be able to accommodate up to 15% reduction in volume and return to its original volume without loss of seal integrity or barrier properties. Vacuum packed products in flexible packages are ideally suitable for HHP processing. After loading the packages, the vessel is filled with a pressure transmitting medium. Water is the most commonly used pressure-transmitting medium. Cost, process temperature range used, viscosity of the media under pressure, its ability to corrode inner walls of the vessel are some factors involved in selection of the pressure-transmitting medium. HHP process is used as stand-alone processing method or in combination with thermal (commercially used) or other non-thermal processing (lab scale) methods to achieve synergistic effects on pathogenic microorganism inactivation in foods (Hogan et al., 2005).
The high pressure process is accomplished in three stages: compression (pressurization) stage where pressure is increased from atmospheric pressure to the desired high pressure. During pressurization, the temperature of water and food products inside the vessel may increase due to adiabatic compression heating. The pressurization stage is followed by pressure holding stage. During pressure-hold, pressure is held constant for several minutes. Finally, pressure is reduced to atmospheric pressure during depressurization stage. One of the end closures is removed and the product is unloaded for further handling. These steps involved in carrying out a HHP run are shown in Figure 1.4. The three stages of the process can be represented on a pressure-time plot as shown in Figure 1.5.

![Figure 1.4 Process steps involved in the operation of high pressure process](http://www.hiperbaric.com/Cold-Pasteurization/, June 28, 2011)
High pressure can be generated in two ways: direct compression and indirect compression. Figure 1.6 shows a schematic of the direct compression using a piston and indirect pumping pressurization method.

In direct compression, a piston with a large end and a small end is used. The large end of the piston is connected to a low pressure pump and small end is used to apply pressure on the transmitting medium. Indirect compression uses a high pressure...
intensifier pump to pump the pressurizing medium from a reservoir directly into a closed
and de-aerated vessel until desired pressure is reached.

Two of the major manufacturers of high pressure equipment are (i) Avure
Technologies Incorporated (USA) and (ii) NC Hiperbaric (Spain). Some of the other high
pressure equipment manufactures include Elmhurst Research, Inc. (Albany, NY, United
States), Engineered Pressure Systems International (Belgium)/ Engineered Pressure
Systems Incorporated (Boston, MA, United States), UHDE Hockdrucktechnik
(Germany), Stansted Fluid Power (United Kingdom), Resato International (Netherlands),
Kobe Steel (Japan), ACB Pressure System-Alstom (France), and UNIPRESS (Poland).

1.6. APPLICATIONS OF HHP PROCESSING IN FOODS

HHP processing is known to inhibit and inactivate vegetative microorganisms
rendering food products safe to consume. This minimal processing technique has limited
adverse effects on nutritional and sensory attributes. By applying high pressure instead of
heat, foods can be pasteurized and preserved to retain fresher and healthier characteristics
desired by the consumer. Another major application of HHP processing is for
activation/inactivation of enzymes in foods. Application of HHP to inactivate enzymes is
advantageously used to prevent texture, color and flavor loss in food products and
prolong their shelf-life. For example, polygalacturonase (PG) enzyme found in Chinese
cabbage can be inactivated to render firm and fresh like structure of cabbage and prevent
spoilage of plant based foods such as kimchies (Eun et al., 1999). Polyphenol oxidases
(PPO) and peroxidases (PO), responsible for color and flavor loss, can be inactivated in
mushrooms, potatoes and apples (Gomes and Ledward, 1996). Other specific applications
of HHP processing include protein denaturation, pressure assisted freezing and thawing, solid-liquid extraction of high value substances from foods, and infusion of substances into food materials (Rastogi et al., 2007). Each of these applications and their use in food products has been discussed in several book chapters and review papers (Rastogi et al., 2007). We have limited the discussion in this thesis to use of HHP processing to infuse selective biomolecules into food products in order to enhance their nutritional quality. The following section discusses previously published literature that has been carried out in the area of high pressure assisted infusion.

1.7. PREVIOUS RESEARCH ON HHP ASSISTED INFUSION

Traditionally, HHP processing has been used as a pre-treatment to disrupt cell-membranes of a substrate, thereby, making the cells less resistant to diffusion during subsequent osmosis (Rastogi and Niranjan, 1998; Rastogi et al., 2000; Fraeye et al., 2010). Researchers have explored the effect of HHP pre-treatment to enhance mass transfer rates of small size molecules such as sucrose and NaCl in fruits, vegetables and meat during osmosis. Some examples of such findings are summarized in Table 1.1. For example, Rastogi and Niranjan (1998) observed three to eight times higher diffusivity of sucrose in pineapple slices while Rastogi et al. (2000) observed similar enhancements in diffusivity of NaCl in potato cylinders after high pressure pre-treatment. Some studies have shown increased diffusivities of the same small molecules into turkey breasts and potato where infusion was carried out under high pressure (Villacis et al., 2008). Ahromrit et al. (2006) studied pressure treatment in Thai glutinous rice. They found an increase in water uptake and equilibrium moisture content with increasing pressure (up to
600 MPa). HHP processing of Thai glutinous rice in water below 300 MPa and 60 °C gave high effective diffusion coefficients. Above 300 MPa, the effective diffusion was not much dependent on temperature. This was attributed to starch gelatinization that could limit water transport. It is to be noted here that most studies report the effective diffusion coefficients ($D_{\text{eff}}$) computed based on Fickian diffusion model. Effective diffusion coefficient is a lumped value that may account for other mass transport processes (e.g. pressure driven flow, capillary flow, etc.) in addition to mass diffusion. Hence, $D_{\text{eff}}$ does not elucidate the actual mechanism of mass transport (Rastogi and Niranjan, 1998; Rastogi et al., 2000; Villacis et al., 2008).

Table 1.1 Effective diffusion coefficients of solutes after HHP at different applied pressures

<table>
<thead>
<tr>
<th>HHP pretreatment followed by osmotic dehydration in potato cylinders (Rastogi et al. 2000)</th>
<th>$D_{\text{eff}}$ for HHP infusion in pineapple slices (Rastogi and Niranjan 1998)</th>
<th>HHP infusion in turkey breast cubes (Villacis et al. 2008)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressure (MPa)</td>
<td>$D_{\text{eff}}$ of sucrose ($10^{-9} \text{ m}^2/\text{s}$)</td>
<td>Pressure (MPa)</td>
</tr>
<tr>
<td>0.1</td>
<td>0.31</td>
<td>0.1</td>
</tr>
<tr>
<td>200</td>
<td>0.41</td>
<td>300</td>
</tr>
<tr>
<td>400</td>
<td>0.67</td>
<td>500</td>
</tr>
</tbody>
</table>

More importantly, while numerous studies have been reported infusion of very small molecules into plant and meat tissues, there are no studies on the infusion of larger nutrient molecules, such as bioactive polyphenols (~ [300 – 480] Da) that have been reported. In order to test whether infusion of large size molecules (> 300 Da) in fruit and vegetable matrices can be enhanced under high pressure, multiple infusion experiments using different sizes of infusants (> 300 Da) and substrates were carried out in this research as proof of concept. The details of these experiments are discussed in Chapter 2.
Furthermore, the primary cause for the observed enhanced infusion under high pressure has been attributed to cell-membrane disruption in plant and animal tissues (Rastogi et al. 2000). Such disruption in the tissues provides less obstructive paths for the infusing molecule to travel and thus leads to increased infusion. The observed correlation between cell membrane rupture and enhanced infusion may not be the actual or only cause for the observed phenomenon. Other mechanisms may also be contributing to enhanced diffusion. For example, it may be possible that the dominant effect of higher pressure is to increase driving force for infusion (chemical potential) compared to decrease resistance to infusion, seen as decrease in intact cell-membrane structure.

In order to test the role of cell membrane rupture one needs to decouple the effect of cell-membrane permeabilization from high pressure treatment. The objective of this work was to study infusion in a fruit system that does and does not permeabilize further upon HHP. Frozen-thawed and fresh cranberries were selected for this purpose. Cells of fresh cranberries were intact and that of frozen-thawed cranberries get ruptured in the freeze-thawing process. Preliminary results indicated that cell-membranes of fresh cranberries permeabilized and that of frozen-thawed cranberries did not permeabilize further upon HHP. The selected model system of cranberries was infused with quercetin (302 Da), an antioxidant molecule. We investigated influence of HHP on extent of infusion, microstructure of infused fruit tissues and mapped infusant loci. The details of this study are discussed in Chapter 3. The main advantage of this process of high pressure assisted infusion is the shorter times of processing (Rastogi et al., 2000). The shorter times (few minutes) of processing not only help retain nutritional and sensory characteristics of the original product but can also be cost effective to the food industry.
These advantages make HHP a promising alternative to osmotic dehydration, which is a rather slow process.

1.8. RATIONALE AND SIGNIFICANCE OF THIS RESEARCH

HHP processing has been shown to accelerate mass transport of components into food materials. Additionally, HHP has limited adverse effects on nutritional and sensory attributes of food components, and therefore, allows to retain fresher and nutritional properties of foods desired by the consumer (Rastogi et al., 2007). HHP may therefore be employed to infuse higher quantities of antioxidant compounds such as flavonoids to produce value added food products from relatively common and inexpensive fruits.

There is evidence showing enhanced infusion of few small-size molecules, such as salt and sugar into fruits, vegetables and meat tissues under high pressure (Rastogi and Niranjan, 1998; Rastogi et al., 2000; Villacis et al., 2008). To demonstrate this infusion phenomenon for larger-size molecules, a model system was used consisting of a fruit and a phytochemical antioxidant (> 300 Da), as the substrate and infusing molecule (infusant), respectively. Preliminary studies, carried out as proof of concept, used different sizes of molecules (150 Da to 30 kDa) as the infusant that were infused into commonly available soft (grapes and cranberries) and hard plant tissues (cut apples and carrots). The main study used quercetin (flavonoid antioxidant) as the infusant that was infused into cranberries under high pressure. The choice of cranberry-quercetin system as the system of study was in response to the RFP from the U.S. Army Natick laboratory to improve the nutrient and nutraceutical quality of their existing cranberry rations.

Quercetin was selected as the model infusant because it is a prominent dietary
antioxidant, extensively reported in literature for its anti-inflammatory and antioxidant properties (Nijveldt et al., 2001). Numerous studies have shown evidence for its beneficial health effects due to its radical scavenging activity of reactive oxygen species (Boots et al. 2008) As of May 26, 2010, the U.S. FDA has granted quercetin GRAS status (GRN 000341, U.S. FDA, 2010). Quercetin is a flavonol that belongs to the family of flavonoids with the three base carbon ring flavylium cation structure. The molecular formula of quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one) is C_{15}H_{10}O_{7} and has a molecular weight of 302 Da. The structure of quercetin is shown in Figure 1.7. It is commonly found in onions, cranberries, apple skin, broccoli, and other berries (Nijveldt et al., 2001). It is known to exert beneficial health effects by providing protection against cardiovascular and pulmonary diseases, some forms of cancer, and osteoporosis. Its radical scavenging property of quenching reactive oxygen species such as peroxynitrite and hydroxy radical is suggested to be reasons for its health beneficial effects (Nijveldt et al., 2001).

Although stable at high temperatures (up to 100 °C) at pH 5 (Buchner et al., 2006), little is known about quercetin stability to high pressure. Other flavonoids, such as anthocyanins have been tested under high pressure. There is little or no effect on anthocyanin stability at pressures ranging from 200 MPa to 500 MPa at 22 °C (Oey et al., 2008). Since quercetin and anthocyanins belong to the same family of flavonoids and share their backbone structure, the likelihood of quercetin being stable under high pressure is high. The pressure stability of these flavonoids makes them suitable
candidates for infusion into fruits under high pressure.

In our exploratory studies, isoquercetin was used as another representative antioxidant with slightly higher molecular weight and higher solubility than quercetin. Isoquercetin or quercetin-3-glucoside is a naturally occurring form of quercetin with a molecular weight of 464.37 Da. The structure of isoquercetin is shown in Figure 1.8. It is a potent antioxidant and is known to have high bioavailability. The sugar moiety attached to the quercetin molecules gives isoquercetin greater solubility in water compared to quercetin (Appleton, 2010). The main study with used quercetin, the aglycone form of isoquercetin, susbstance. The main reason to discontinue use of isoquercetin was based on U.S. Army labs’ sugges that quercetin was a more potent antioxidant than isoquercetin.

Cranberry was selected as the substrate into which quercetin was infused. Cranberry is a commonly consumed fruit in United States after processing. They are not consumed directly as a fruit due to their extremely tart taste. Most cranberries are processed into products such as juice, sauce, and sweetened dried cranberries. The fruit is most popular for its high nutrient content and antioxidant properties. Cranberries have shown to prevent urinary tract infections and anticancer effects. These benefits have been attributed to polyphenolic compounds such as anthocyanins, flavonols and procyanidins (Howell et al., 2001; Neto, 2007). Polyphenol antioxidants in cranberries are found to have health benefits as anticancer agents and anti-inflammatory agents (Cote et al., 2010). Dried cranberries are a part of MRE military rations and are commonly consumed
by soldiers. To counter oxidative stress brought on by extreme exercise, and to boost war
fighter immune systems, the natural phytochemical content, such as antioxidants and/or
other performance enhancing ingredients, need to be enhanced in the rations. According
to Harnly et al. (2006), cranberry naturally contains about 200 ppm (fresh weight basis)
of quercetin. The recommended daily intake of quercetin is 500-2000 mg per 70 kg body
weight individual (USARIEM report, 2008). Based on the recommended dietary intakes
(RDI), an individual would have to consume about 2.5 kg to 10 kg per day of fresh
cranberries or 1 kg to 3 kg of dried cranberries (at 15 % moisture [w/w]) per day to meet
the RDI. These are very high amounts of cranberries that need to be consumed by an
individual, practically not possible. Hence, this research was focused on developing
quercetin infused cranberries that can potentially make the berries an “excellent” source
(as per U.S. FDA definition 21 CFR 101.54(b)) of quercetin, in other words a “super
fruit”. Infused fruits can provide the war fighters with essential nutrients that can only be
found in fruits. These products will be lightweight, nutrient dense, convenient to
consume, and ideal for MRE and the new FSR (First Strike Ration). However, currently
only standard raisins and dried cranberries are in the MRE. Hence, in response to
CORANET program’s call to improve the health value of their cranberry rations, this
study proposed to infuse quercetin into cranberries. Furthermore, whole cranberries, as a
model system, is a good representation of mass transport barrier properties of those found
in other berries.

The understanding from this study can possibly be implemented for a broad class
of fruits and infusing molecules to develop value-added nutrient rich foods.
1.9. HYPOTHESES OF THIS RESEARCH

The hypotheses of this study are:

1. HHP processing will enhance and accelerate infusion of quercetin into cranberries.
2. Fruit cell-membrane permeabilization will assist in pressure-enhanced infusion.
3. Pressure driven flow in addition to cell-membrane permeabilization, causes pressure enhanced infusion.

1.10. OBJECTIVES OF THIS RESEARCH AND THESIS OUTLINE

The aim of this study was to demonstrate whether it is possible to enhance and accelerate infusion of large size phytochemical antioxidant molecules (300 Da - 500 Da) into food materials using high hydrostatic pressure (HHP) processing. The studies in this thesis concentrate on two aspects: (i) influence of processing conditions on the extent of infusion, and (ii) mechanisms operative during high pressure assisted infusion.

Preliminary studies were carried out as a proof of concept to test whether enhanced infusion of various bioactive compounds can be achieved under high pressure into soft and hard fruits, and vegetables. Specifically, the preliminary studies investigated the effect on HHP infusion of bioactive compounds of (a) varying size of molecules (150 Da to 30 kDa) and (b) varying water solubility into soft and hard fruits (cranberries, grapes and apples) and vegetables (carrots). The results from the preliminary studies are discussed in Chapter 2.

A significant part of this thesis used a model system of cranberries as the substrate that was infused with quercetin (an antioxidant molecule). Using this model system, the specific objectives undertaken were to experimentally: (i) Demonstrate enhanced infusion
of quercetin under high pressure (100 MPa – 600 MPa), (ii) Measure the influence of experimental parameters (fruit pre-treatment, pressure, time of infusion, pressure cycling, infusate concentration, fruit-infusate ratio) on extent of infusion and cell structure changes in cranberries, (iii) Gain insights into fruit tissue permeability, microstructure changes and distribution of infused compounds into fruit matrix and, (iv) Gain understanding of the mechanisms of enhanced infusion under high pressure to elucidate transport and distribution of infused compounds. The details of this study are discussed in Chapters 3 and 4 covering design of experiments, methodologies, experimental procedures, results, discussion and conclusions.

Chapter 5 summarizes the key findings and conclusions from this research study. Finally, future directions for research on elucidating mechanisms of pressure assisted infusion are discussed in a concise manner in Chapter 6.

This thesis also includes an appendix discussing the fundamentals of impedance spectroscopy technique used to measure changes in cell-membrane permeability of cranberries.

This research was published in the conference proceedings of XVIII Brazilian Congress of Chemical Engineering and 11th International Congress on Engineering and Food, Athens, Greece (Mahadevan and Karwe, 2010 and 2011).
CHAPTER 2

PRESSURE ENHANCED INFUSION IN FRUITS AND VEGETABLES:
PROOF OF CONCEPT

2.1 PRELIMINARY STUDIES

Earlier published literature has shown that high hydrostatic pressure (HHP) processing can infuse small size molecules, such as sucrose (342.3 Da) and NaCl (58.4 Da), into fruits, vegetables, and meat tissues much faster than osmosis-based diffusion. To the best of our knowledge, there is no published research on infusion of bioactive compounds of varying molecular sizes under pressure into fruit and vegetable tissues. The present study was carried out as a proof of concept to test whether enhanced infusion of bioactive compounds can be achieved under high pressure into different fruits and vegetables. The overall goal was to explore potential of HHP assisted infusion to develop fruit and vegetable infused products with improved nutritional and quality aspects. The applications of HHP assisted infusion explored here include its use to develop antioxidant rich fruit products and its use to improve/maintain texture of nutrient infused fruit and vegetable products.

The preliminary studies investigated influence of infusing molecule and substrate matrix on infusion under pressure. Specifically, the preliminary studies investigated the effect on HHP infusion of bioactive compounds of (a) varying size of molecules (150 Da to 30 kDa) and (b) varying water solubility into soft and hard fruits (cranberries, grapes and apples) and vegetables (carrots). The understanding from these studies helped design
a model system to carry out a detailed study on HHP infusion - process development, mechanistic understanding and product development. The following infusants and substrates were selected.

I. Pomegranate concentrate and muscadine grape concentrate was infused separately into green grapes. The objective of this study is to test whether enhanced infusion of polyphenol molecules from fruit concentrates containing mixture of polyphenols of molecular sizes between 200 Da and 500 Da can be achieved in grapes under high pressure.

II. Calcium chloride (CaCl$_2$ anhydrous; mol wt. 110 Da) and calcium lactate gluconate (CLG; mol wt. 324.3 Da) were infused separately into apple wedges and carrot discs. The objective of this study is to test whether enhanced infusion of calcium salts of varying molecular sizes can be achieved in hard plant tissues such as, apple wedges and baby carrots under pressure. Another aim was to understand if enhanced infusion of calcium salts influenced texture and helped maintain fresh-like firmness of apple wedges and carrot discs.

III. Nanoemulsion of canola oil stained with Nile red dye (particle size 150 nm), fluorescently tagged whey protein (mol wt. ~ 30 kDa) and fluorescein (mol wt. 150 Da) were infused into carrot discs. The objective of this study is to test whether enhanced infusion of molecules with varying sizes (150 Da to 30 kDa) and in different forms in solution (nanoemulsions or dispersion of WPI or soluble solution of fluorescein) could be achieved under high pressure in carrot discs. Additionally, the goal was to understand the loci of the infusants inside carrot tissue. Hence, fluorescently tagged molecules were infused into carrot discs and their loci were mapped using fluorescence microscopy. This
study was carried out in collaboration with Dr. Nitin’s group at University of California, Davis.

IV. Sugar (sucrose; mol. wt. 342.30 Da), muscadine grape juice and muscadine grape concentrate (mixture of polyphenols ranging from 200 Da to 500 Da) and isoquercetin (quercetin-3-glucoside; mol wt. 464.37) were infused separately into cranberries. This study was conducted to test whether enhanced infusion of varying size of molecules (200 Da to 500 Da) and varying solubility (water soluble sucrose, water soluble polyphenols, water insoluble isoquercetin) could be achieved in cranberries under high pressure.

In each system, the extent of infusion of selected substance was measured after HHP processing and after atmospheric pressure infusion (control). The experimental set up and results from each study system are discussed in the following sections.

2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

Thompson seedless green grapes, carrots, baby carrots, granny smith apples and frozen-thawed cranberries were used as substrates for infusion. They were purchased from a local supermarket (Edison, NJ) except cranberries. Cranberries (Vaccinium macrocarpon var. Stevens) were purchased from J.J. White Farms (Brown Mills, NJ) and stored in a freezer at -20 °C until further use.

In the study focused on testing whether infusion of polyphenol molecules (300 Da – 500 Da) was enhanced in grapes under pressure, Thompson seedless green grapes were infused with purple muscadine grape juice, muscadine grape concentrate, and
pomegranate concentrate. Purple muscadine grape juice was procured from Paulk’s Vineyard (Wray, GA), and purple muscadine grape concentrate and pomegranate concentrate were procured from Stiebs Inc. (Madera, CA).

In the study focused on testing whether enhanced infusion of calcium salts into apples and carrots improved their texture (hardness) after HHP, granny smith apples (cut into wedges) and baby carrots were infused with calcium chloride and calcium lactate gluconate. Calcium chloride was purchased from Sigma Aldrich (St. Louis, MO) and calcium lactate gluconate was purchased from Jungbunzlauer (Newton, MA).

In the study focused on testing whether infusion of varied size of fluorescently tagged molecules (300 Da – 30 kDa) was enhanced in carrots under pressure, carrot discs were infused with nano emulsion (150 nm) of canola oil stained with Nile red at 4% oil concentration, 0.2 % whey protein isolate tagged with fluorescein isothiocyanate (30 kDa) in sodium phosphate buffer (pH = 7), 10 mg/L fluorescein (150 Da) in sodium phosphate buffer (pH = 7). Dr. Nitin’s group at the Department of Food Science in University of California Davis provided all three infusate solutions (nanoemulsion, WPI-FTIC and fluorescein). Fluorescently tagged molecules were selected as infusants so that their location inside carrot tissue could be mapped using fluorescent microscopy.

In the study focused on testing whether enhanced infusion of water soluble and water insoluble molecules can be achieved in cranberries, frozen-thawed cranberries were infused with Domino sugar (water soluble) and isoquercetin (water insoluble polyphenol). Domino sugar was purchased from a local supermarket (Edison, NJ). Isoquercetin (powder 98.9 % pure) was procured from Natick Labs (Natick, MA). Isoquercetin, being water insoluble, was dissolved in food grade ethanol and glycerin (1:1
w/w) for use as infusate. Glycerin (99.7%) was purchased from Qualichem Technologies Inc. (Woodstock, GA). Ethanol used was 200 proof absolute anhydrous alcohol of ACS/USP grade manufactured by Pharmco Products Inc. (Brookfield, CT).

For analytical testing, all chemicals and reagents used were HPLC grade. Folin-Ciocalteau’s phenol reagent (2M) and gallic acid (3,4,5 – trihydroxy benzoic acid) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). HPLC mobile phase of acetonitrile, phosphoric acid and HPLC water were purchased from Fisher Scientific.

2.2.2 METHODS

2.2.2.1 HIGH PRESSURE PROCESSING

*Pilot-scale high pressure vessel*

![HHP processing unit](image)

*Figure 2.1* HHP processing unit used in this study at Rutgers University

The high pressure processing unit (Elmhurst Research Inc., Albany, NY) used in this study is shown in Figures 2.1 and 2.2. This unit is a vertically oriented vessel having
a working capacity of 10 liters operating between pressures of 100 MPa and 690 MPa and operating temperatures between 5 °C and 90 °C. The pressure transmitting medium used was filtered tap water. The vessel, made of multiple layers of steel (SA-705 forging XM 16 stainless steel and FA-723 carbon steel), has a bore diameter of 142 mm and wall thickness of 150 mm. The external length is 1090 mm and the pressure cavity is 800 mm long. A pneumatically controlled top closure is used for loading and unloading the samples. The vessel can be manually tilted up to 100 degrees from the vertical for loading/unloading. The maximum working pressure is 620 MPa (90,000 psi). A 20 HP pump is used to pressurize the unit using filtered tap water. The come-up time to reach 600 MPa is 180 s. The high pressure can be maintained (dwell time) for up to 60 min. Depressurization time is 10 s maximum, and cannot be controlled. The pressure vessel was equipped with three thermocouple probes that were threaded to the inside of the top closure. These thermocouples facilitated measurement of temperature of water (pressure transmitting medium) in the vessel during process. Type K thermocouple probes used were enclosed in high pressure tubing (inner diameter 2.1 mm). The temperature range for the type K thermocouple is -200 °C to 1200 °C. Often type K thermocouples are used in high pressure vessels because their calibration is least sensitive to pressure. The tips of the thermocouples are located at three different depths (203 mm, 318 mm and 546 mm) from the top of the pressure cavity and 10 mm away from the inner wall of the vessel. The operation of the unit is controlled through a PLC control unit. The data on pressure and temperature as a function of time can be recorded using a data acquisition system from National Instruments (Labview™ 7 Express Version) on a PC.
Figure 2.2 Schematic of the pilot scale HHP processing unit

The fruit samples immersed in the infusate and packaged in vacuum-sealed polyethylene pouches were pressure treated between 100 MPa (15000 psi) and 551 MPa (80000 psi) for 1 min to 60 min at room temperature (~21 °C). There was small rise in temperature due to compression heating during pressurization. The temperature rise recorded for the pressure transmitting medium did not exceed 14 °C (at 551 MPa, water temperature rose from 21 °C to 35 °C) and its effect was found to be insignificant in this temperature range; extent of infusion measured at 21 °C and 35 °C were not significantly different. Therefore, all infusion experiments were carried out at initial temperature of about 21 °C. Figure 2.3 shows the pressure and temperature data recorded as a function of time for a pressure cycle of 551 MPa for 10 min. In addition, experiments were also carried out at atmospheric pressure and room temperature, which formed the control.
2.2.2.2 SAMPLE PREPARATION

2.2.2.2.1 Study on HHP infusion in green grapes

In this study, Thompson seedless green grape halves were infused with pomegranate concentrate and muscadine grape concentrate, separately.

Thompson seedless green grapes were used as a model substrate system for carrying out high pressure assisted infusion. Grapes were sliced into halves along their longer axis as shown in Figure 2.4. Slicing of grapes was necessary since whole fruit with intact grape skin was found to be impermeable even under high pressure (600 MPa).

The infusate used was muscadine grape fruit concentrate and pomegranate fruit concentrate at 60 ºBrix. ºBrix of infusate was adjusted from 65 ºBrix of fruit concentrate to 60 ºBrix with addition of distilled water. Dilution of fruit concentrates was necessary
to decrease the viscosity and for ease in handling. Consistent with the goal of the study to test infusion of phytochemical antioxidants into fruit substrates, fruit concentrates that are rich in polyphenols were chosen. Polyphenols such as anthocyanins and other flavonoids present in pomegranate and muscadine grapes are potent antioxidants (Gil et al., 2000; Pastrana-Bonilla et al., 2003). Grape slices were immersed in the infusate, packaged in vacuum-sealed polyethylene pouches, and transferred to the high pressure unit (described in earlier section 2.2.2.1) for processing. A parallel process of infusion at atmospheric pressure was carried out in grape slices (control). The fruit to infusate ratio was maintained at 1:2 (w/w). Each experiment was performed in triplicates, i.e., process treatment and post-process analyses were carried out in triplicates.

### 2.2.2.2 Study on HHP infusion in Granny Smith apples and baby carrots

In this study, Granny Smith apple wedges and baby carrots were each infused with calcium chloride and calcium lactate gluconate.

Granny Smith apples and baby carrots were used as another model substrate system (Figure 2.5). The focus of this study was to understand whether enhanced infusion of calcium salts under pressure could influence texture of baby carrots and apple wedges. The hypothesis for this study was that infused calcium salts (CaCl$_2$ and calcium lactate gluconate) would help preserve the firm fresh-like texture that softens during HHP. Apples were sliced into wedges (20 degree wedge) and baby carrots were washed and graded by size (~ 43 mm long and 7 mm base diameter).
The infusates used were 10% (w/v) calcium chloride (CaCl₂) and 30% (w/v) calcium lactate gluconate (CLG) solution in water. The concentration of CLG was selected to match the calcium equivalents from CaCl₂.

Apple wedges and baby carrots were immersed in the infusate, packaged in vacuum-sealed polyethylene pouches, and transferred to the high pressure unit (described in earlier section 2.2.2.1) for processing. A parallel process of infusion at atmospheric pressure was carried out in apple wedges and baby carrots (control). The fruit to infusate ratio was maintained at 1:2 (w/w). Each experiment was performed in triplicates, i.e., process treatment and post-process analyses were carried out in triplicates.

2.2.2.3 **Study on HHP infusion in carrots discs**

In this study, carrot discs were infused with fluorescently tagged molecules of different molecular sizes (150 Da to 30 kDa).

Carrots were purchased from the local supermarket and cut into discs as shown in Figure 2.6. Carrots were sliced into discs of 2.5 cm diameter x 1 cm thickness.

Carrot discs were immersed in three different infusates: (i) o/w nanoemulsion of canola oil stained with Nile red at 4% oil concentration (avg. particle size 150 nm), (ii) 0.2% whey protein isolate (WPI) conjugated with fluorescein isothiocyanate (FITC) in sodium phosphate buffer (pH = 7) and, (iii) 10 mg/L fluorescein in sodium phosphate buffer (pH = 7). Infusates were prepared by Dr. Nitin’s group at UC Davis and shipped overnight to Rutgers for HHP processing. In this study, WPI (30 kDa) was selected to model infusion.
of large molecules and fluorescein (150 Da) was selected to model infusion of small molecules. The fluorescent tags enabled evaluation of carrot tissue using fluorescence and confocal microscopy. Carrot discs were immersed in infusate, packaged in vacuum-sealed polyethylene pouches, and transferred to the high pressure unit (described in earlier section 2.2.2.1) for processing. A parallel process of infusion at atmospheric pressure was carried out in carrot discs (control). The vegetable to infusate ratio was maintained at 1:2 (w/w). Each experiment was performed in triplicates, i.e., process treatment and post-process analyses were carried out in triplicates.

2.2.2.2.4 Study on HHP infusion in cranberries

In this study, cranberries were either infused with water soluble molecules, such as, sugar, muscadine grape juice or muscadine grape concentrate, or with water insoluble molecule, such as, isoquercetin (quercetin-3-glucoside).

Frozen cranberries purchased from J.J. White Farms (Brown Mills, NJ) were sorted based on size, shape and color (Figure 2.7). Berries used were bright red colored, ovoid in shape and ranging in size between 14 mm to 18 mm for minor diameter and 22 mm to 28 mm for major diameter. These cranberries were then scarified to cause partial damage to the cranberry surface since cranberry skin was impermeable even under high pressure. To scarify, the berries were rolled under a bed of pins (bed length x width = 500 mm x 80 mm, distance between pins = 2 mm, pin diameter = 0.5 mm) over a gritty sand paper belt.
(Craftsman®36 grit, very coarse) to make sub-millimeter pinholes on their surface. This apparatus was built in-house as shown in Figure 2.8.

![Figure 2.8 Schematic diagram and photograph of the in-house built scarifier](image)

Multiple passes of cranberries through the scarifier belt were carried out to achieve uniformity in surface damage. The number of passes through the scarifier belt was optimized to six passes. The number of passes was fixed to six in order to achieve optimum amount of infusion achieved while maintaining the fruit integrity (details of this experiment is explained in the following section, Figure 2.17). Cranberries were scarified while frozen; the berries got softer on thawing and thawed berries could not hold up to the process of scarification. These scarified-frozen cranberries were then thawed at room temperature before HHP processing.

Infusates carrying water soluble molecules included 50 °Brix solution of sugar, muscadine grape juice at 50 °Brix and muscadine grape concentrate at 60 °Brix. °Brix of muscadine grape juice was adjusted from 10 °Brix to 50 °Brix by adding sugar. Fruit concentrates initially at 65 °Brix were adjusted

![Figure 2.9 Structure of isoquercetin (quercetin-3-glucoside)](image)
to 60 °Brix with addition of distilled water. The fruit concentrates were chosen since they are rich sources of polyphenolic antioxidants in the form of anthocyanins. Dilution of fruit concentrates was necessary to decrease the viscosity and for ease of handling. The other infusate carrying water insoluble molecules was a 5 % (wt) solution of isoquercetin in 1:1 (w/w) glycerin-ethanol mixture. Isoquercetin (structure shown in Figure 2.9) was solubilized in alcohols (food grade glycerin and ethanol) since it has very poor water solubility. Isoquercetin was selected as a model infusant to help understand the phenomenon of enhanced infusion of water insoluble molecules into cranberries under pressure.

Scarified cranberries were immersed in the infusate, packaged in vacuum-sealed polyethylene pouches, and transferred to the high pressure unit (described in earlier section 2.2.2.1) for processing. A parallel process of infusion at atmospheric pressure was carried out in frozen-scarified-thawed cranberries (control). The fruit to infusate ratio was maintained fixed at 1:2 (w/w). Each experiment was performed in triplicates, i.e., process treatment and post-process analyses were carried out in triplicates.

2.2.2.3 POST-INFUSION ANALYSES

Following infusion (HHP and control), the polyethylene pouches with samples were cut open and the infusate was drained. The processed fruits were dipped in lukewarm water (~ 40 °C) and removed immediately, then blotted with a paper towel to remove any surface adhered solvent and infusant. Water at 40 °C helped remove surface adhering infusate faster than water at room temperature (22 °C). The samples were
immediately frozen and stored at -20 °C until further analysis. All analyses were performed in triplicates and an average concentration value for the amount infused was measured at each treatment condition.

2.2.2.3.1 Extraction of polyphenols

Following infusion, the extent of polyphenol infusion was measured in grape halves and cranberries infused with fruit juices and fruit concentrates. The method of extraction of polyphenols used prior to their quantification was as follows. Processed fruits were mashed to pulp using mortar and pestle prior to extraction of polyphenols. The method used for extraction was similar to the method discussed by Asami et al. (2003) with some modifications. The fruit pulp (25 g) was mixed with 80 ml of acetone/water/acetic acid (70:29.5:0.5 v/v/v), homogenized with Polytron PT1600E high shear homogenizer (Kinematica Inc., Bohemia, NY) for 7 min, and stirred at room temperature for 1 hour on a magnetic stirrer. Two more extractions were performed, with 20 ml portions of extracting solvent, each, for 15 min. After each extraction stage samples were centrifuged in IEC Clinical centrifuge at 7100 rpm (Thermo IEC Inc., Milford, MA) and the supernatants were pooled. The final volume of the extract was recorded.

2.2.2.3.2 Polyphenol content measurement

The polyphenol content, in infused grape halves and cranberries, was determined colorimetrically using Folin-Ciocalteau’s assay (Sensoy et al., 2006). 250 µl of fruit extract (prepared following extraction method described in section 2.2.2.3.1) or standard,
250 µl of Folin-Ciocalteau reagent (diluted with water 1:1 v/v), 500 µl of saturated Na₂CO₃ (200 g/l), and 4 ml of distilled water were mixed in test tubes. The mixture was incubated at room temperature for 25 min. Absorbance of the reacted mixtures was read at 725 nm. Gallic acid standard curve (25 µg/ml to 500 µg/ml) was used to quantify free phenols. Interferences caused by other reducing compounds (ascorbic acid, reducing sugars, and proteins) in the assay were corrected using polyvinylpolypyrrolidone (PVPP) binding method; PVPP selectively binds to polyphenols (Vinson et al., 1999). Phenol bound PVPP settled to the bottom on overnight standing. The clear supernatant was used to measure phenol content. The difference in the measured value before and after PVPP binding gave the corrected polyphenol content in the samples. Results were expressed as mg gallic acid equivalents (GAE)/ g initial dry fruit weight.

2.2.2.3.3 Texture analyses

Following infusion of calcium salts in apple wedges and baby carrots, the extent of calcium infusion was measured indirectly in terms of change in the texture (hardness) of samples. If calcium infused were higher, the change in hardness before and after HHP would be small as compared to a system where in which no calcium was infused. Calcium can bind with demethylated pectin in the carrot matrix and maintain hardness of the plant tissue. Texture analyses was carried out using a Brookfield CT3 texture analyzer (Brookfield Engineering Laboratories Inc, Middleboro, MA) with a 25 kg load cell and a 2 mm diameter cylindrical probe, which was set up to penetrate through the baby carrot or apple wedge at two locations within each sample. The probe speed was set to 5 mm/s.
The peak force of the test, defined as hardness, was used for comparison between processing treatments.

2.2.2.3.4 Color analyses (redness, a*, measurement)

In order to gain insights into spatial distribution of infusing molecule after HHP, apple wedges were infused with calcium solution mixed with red food color. Spatial distribution of this red color was measured in apple wedges using a Konica Minolta CR-410 handheld colorimeter (Konica Minolta, Tokyo, Japan). D65 standard illuminant and 2° observer angle were used, calibration was done with a white standard Y = 94.7, x = 0.3156 and y = 0.3319. CIE LAB a* values were recorded.

2.2.2.3.5 Fluorescence microscopy

Carrots infused with fluorescently tagged molecules (o/w nano emulsion of canola oil stained with Nile red, 0.2 % whey protein isolate tagged with fluorescein isothiocyanate and fluorescein) were transversely sectioned to make discs (1 mm thick). Each disk was placed on a glass slide, covered with glass cover slip on the top. The cover slip was taped to the glass slide on either side to keep the slip from falling off the sample. Fluorescence and white light (differential interference contrast microscopy) images were acquired using a laser scanning confocal fluorescence microscope.

2.2.2.3.6 °Brix measurement

Extent of sugar infusion in cranberries and grape halves was recorded using a °Brix meter. °Brix was measured using Reichert Analytical Instruments (Depew, NY) r².
mini handheld digital refractometer. Infused fruit samples and control were homogenized using mortar and pestle. The homogenate was then filtered through a coffee filter. The filtrate was used for measurement.

2.2.2.3.7 RP-HPLC determination of isoquercetin

Cranberry polyphenol extracts (prepared as described in section 2.2.2.3.1) were filtered through 0.45 µm filters prior to HPLC analysis. A 5 µm, 4.6 mm x 250 mm Ascentis C18, RP Amide column was attached to a Waters HPLC equipped with 486 UV-Vis Tunable detector and Peak Simple Chromatography data system. The mobile phase consisted of a gradient of 0.085 % phosphoric acid (A) and acetonitrile (B). The flow rate was set at 1.0 ml/min and the sample injection volume was 10 µl. The gradient used was as follows: 15 % B at 0 min, 35 % B at 30 min and 85 % B at 35 min. Isoquercetin was detected at 366 nm and quantified using external calibration curves of quercetin standard. Results were expressed as milligrams of isoquercetin per gram initial dry fruit wt. Figure 2.10 shows a representative chromatogram of HHP treated cranberry extracts showing quercetin and isoquercetin peak.
2.2.2.3.8 Statistical analyses

Statistical analyses were performed using SAS version 9.2 software for Windows (SAS Institute, Cary, NC). Analyses of variance and separation of the means were carried out using PROC GLM. When effects were significant, comparisons between the means were performed using least square difference (LSD) comparison test at $\alpha = 0.05$.

2.3 RESULTS AND DISCUSSION

2.3.1 Infusion of pomegranate and muscadine grape concentrates into grape halves

Higher amounts of purple muscadine grape concentrate and pomegranate concentrate were found in grape halves infused under high pressure. This was reflected in

Figure 2.10 Chromatogram for frozen-thawed cranberry (Stevens var.) extract
quantitative measurement of °Brix and polyphenol content of grape halves after HHP processing.

Table 2.1 shows the °Brix of grape halves before and after infusion under pressure. The extent of infusion under HHP was compared to control samples that were infused under similar conditions at atmospheric pressure and room temperature (22 °C). In HHP infused grape halves, °Brix value almost doubled after infusion of pomegranate concentrate (from [17.7 ± 0.3] °Brix before infusion to [37.8 ± 3.0] °Brix after infusion) and muscadine grape concentrate (from [17.7 ± 0.3] °Brix before infusion to [30.0 ± 4.1] °Brix after infusion). Infusion at ambient conditions (control), however, showed no significant increase (p<0.05) in °Brix before and after infusion. The results suggest that HHP enhanced infusion of soluble solids from pomegranate and muscadine grape concentrates.

Table 2.1: °Brix of grape halves before and after infusion. Numbers indicate mean ± sd. Superscript letters indicate statistical significance. Different letters indicate statistical significance at p<0.05

<table>
<thead>
<tr>
<th>Infusate</th>
<th>°Brix of grape halves</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HHP infusion</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(100 MPa, 10 min)</td>
<td>(0.1 MPa or 1 atm, 10 min)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Before infusion</td>
<td>After infusion</td>
<td>Before infusion</td>
</tr>
<tr>
<td>Pomegranate concentrate</td>
<td>17.7 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.8 ± 3.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.7 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Muscadine grape</td>
<td>17.7 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.0 ± 4.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.7 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>concentrate</td>
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</table>
Polyphenol content of grape halves, another measure of amount of fruit concentrate infused, is shown in Figure 2.11. This experiment used lower ends of pressure and time, 100 MPa and 10 min, for the HHP unit as a proof of concept.

![Infusion for 10 min in grape halves](image)

**Figure 2.11** Phenolic content of grape halves infused with pomegranate and muscadine grape concentrates for 10 min. Different letters indicate statistical significance compared to control at p<0.05

Grape halves infused with pomegranate concentrate after HHP processing (100 MPa, 10 min) showed 23 % increase in polyphenol content compared to control (0.1 MPa, 10 min). Muscadine grape concentrate infused grape halves, however, showed no significant difference in their polyphenol content after HHP and atmospheric pressure infusion. This observation of no change in polyphenol content after HHP processing could be attributed to simultaneous transport of muscadine grape polyphenols into (seen as change in color of green grapes halves to faint purple color) and green grape polyphenols out of grape halves. If the rate of diffusion of the polyphenols in and out of the substrate were similar, there would be no net change in their polyphenol content.
Thus the results from this study suggest that HHP (100 MPa) can enhance infusion of pomegranate and muscadine grape molecules into green grape halves.

### 2.3.2 Infusion of calcium salts into baby carrots and apple wedges

The aim of this study was to test whether enhanced infusion of calcium ions can be achieved in baby carrots and apple wedges using HHP in order to preserve their fresh like texture that softens during HHP. Higher levels of calcium chloride (CaCl$_2$) and calcium lactate gluconate (CLG) were infused into baby carrots and apple wedges after HHP processing. This was reflected as an increase in hardness of the fruit and vegetable tissues after HHP infusion. The hypothesis is that infused CaCl$_2$ and CLG will help cross-link with pectin in plant tissues (Rosenthal, 1999) thus increasing their hardness that is affected during HHP.

HHP processed baby carrots (450 MPa, 15 min, 23 °C initial temperature), not infused with any substance, showed a significant drop in hardness (from hardness values before infusion in raw baby carrots) suggesting that HHP causes cell rupture and tissue softening. Contrastingly, carrots infused with CaCl$_2$ and CLG showed a smaller decrease in hardness compared to raw baby carrots, which could be attributed to infusion of calcium that bound with demethylated pectin in the carrot matrix (Figure 2.12).
Figure 2.12 Hardness of baby carrots infused with CaCl₂ and CLG before and after HHP at 450 MPa for 15 min. Different letters indicate statistical significance compared to control at p<0.05

Pectin, in carrot tissue, could be demethylated by pectin methyl esterase (PME) enzyme that may have been released from their cellular compartments in carrot tissue during HHP processing. Similar release of PME from potato tissue has been observed in earlier research (Rastogi et al., 2000). This study provides preliminary evidence that calcium salts (CaCl₂ and CLG) could be infused into baby carrots under pressure. Moreover, calcium also helps in recovery of texture damage after HHP and can help obtain fresh like texture characteristics.

In the case of HHP infusion into apple wedges, enhanced infusion of red food color + CaCl₂ was observed from increase in redness (a*) values (Figure 2.13) of apple wedges and an increase in their hardness (Figure 2.14). HHP infusion at 100 MPa for 30 min increased redness (a*) values by twice on the surface of apple wedges (0 mm distance from surface) compared to those in control (1 atm). Furthermore, unlike the
apple pieces infused at 1 atm, the red food dye in HHP treated apple wedges infused deep into the apple tissue (beyond 3 mm from surface).

![Enhanced infusion of red food dye under pressure](image)

**Figure 2.13** Enhanced infusion of CaCl$_2$ + red food dye ($a^*$ values) after HHP at 100 MPa for 30 min in apple wedges and spatial distribution of red color in apple tissue

Hardness value measurements showed that, apple wedges softened significantly immediately after HHP as shown in **Figure 2.14**. Moreover, CaCl$_2$ infusion did not significantly increase their hardness after HHP. These results suggest that CaCl$_2$ infusion was not significantly enhanced at 100 MPa after 30 min in apple wedges. The amount of CaCl$_2$ infused may not be significant to bind all the demethylated pectin and hence did not reflect as significant increase in hardness. Precise measurement of the actual amount of calcium infused and PME released may help explain the observed phenomenon better.
2.3.3 Infusion of fluorescent molecules into carrot discs

The objective of this study was to test whether enhanced infusion of varying size of molecules (150 Da to 30 kDa) into carrots can be achieved under high pressure. Carrot discs were infused with fluorescently tagged or fluorescent molecules of varying molecular sizes. Fluorescent microscopy was used to record changes in extent of infusion in carrot discs. In addition, microscopy images were used to map the loci of infused substances inside carrot tissue.

Carrot discs (2.5 cm diameter x 1 cm) were immersed in three different infusates: (i) o/w nano emulsion of canola oil stained with Nile red at 4% oil concentration (Avg. particle size 150 nm), (ii) 0.2 % whey protein isolate tagged with fluorescein isothiocyanate (mol wt ~ 30 kDa) in sodium phosphate buffer (pH = 7) and (ii) 10 mg/L fluorescein in sodium phosphate buffer (pH = 7). Figure 2.15 shows the transverse section of a carrot tissue at approximately 2 mm from the top surface of the carrot.

Figure 2.14 Texture (hardness) changes in apple wedges infused with CaCl$_2$ before and after HHP at 100 MPa for 30 min
The results show that molecules of varying sizes can be infused under pressure. The nanoemulsions were predominantly distributed along the cell walls (red outline around cells), while the fluorescein molecules were dispersed throughout carrot tissue.

2.3.4 Infusion of sugar, muscadine grape concentrate and isoquercetin into frozen-thawed cranberries

The objective of this study was to test whether infusion of molecules of varying sizes (200 Da to 500 Da) and solubility can be enhanced under pressure. This section discusses pre-treatment methods developed and influence of process variables (pressure, pressure hold-time and pressure cycling).
2.3.4.1 Infusion of sugar

These experiments on sugar infusion were carried out on scarified cranberries packaged in pouches containing 50 °Brix sugar solution.

2.3.4.1.1 Selection of method of pre-treatment for cranberries

Cranberry skin is a mass transfer barrier. Diffusion through cranberry skin was found to be negligible even under high pressure (600 MPa). Fruit skin, made of an outermost layer of epidermal cells, excretes cuticular wax that makes the skin impermeable to most molecules, including small molecules such as water (Bourne, 1979). Mass transport barrier characteristics, however, was not limited to the presence of the cuticular wax. Even after the removal of cuticular wax by washing in water (Bourne, 1979), diffusion through cranberry skin was negligible under pressure. This observation suggested that the cranberry skin cell structure was unaffected under pressure and did not allow for diffusion of substances through them. Therefore, in order to reduce the mass transfer barrier caused by cranberry skin other physical methods of pre-treatments were tested. The method of pre-treatment selected was limited to physical methods to reduce cranberry skin barrier properties. The methods used were peeling of fruit skin, slicing of fruit into halves, making sub-millimeter pores on fruit surface, vacuum packing of fruit (use of vacuum provides a pressure differential that can disrupt some fruit tissues). Frozen-thawed cranberries were used in this study since cranberry is a seasonal fruit and it is primarily available frozen. However, few studies were conducted in season using fresh cranberries for comparison to understand if fruit tissue structure before freezing (fresh cranberries) and after freezing (frozen-thawed cranberries) influenced the extent of
infusion under pressure. Whole fruits were used as the pre-treatment method control. Pre-treated cranberries were infused at high pressure (551 MPa) and at atmospheric pressure (0.1 MPa, process treatment control). The selection of the pre-treatment method was based on obtaining high amounts of infusion in fruit while maintaining its physical integrity.

The effect of pre-treatment method on the extent of infusion of sucrose in frozen-thawed cranberries is shown in Figure 2.16. Peeled fruit infused under high pressure showed the highest amount of sugar infused. HHP infused cranberry halves and cranberries with surface pores also showed significant increase in °Brix compared to those infused at 1 atm. Whole fruits and vacuum packed fruits showed much lower levels of infusion (measured as change in °Brix) compared to other pre-treatment methods tested.

![Figure 2.16: Effect of different pre-treatment methods on sugar infusion in frozen-thawed cranberries. Infusion time was 60 min. Different letters indicates significant difference (p<0.05) comparing within each pre-treatment method.](image-url)
Although peeled fruit showed higher infusion than cranberry halves and cranberries with surface pores, it was not a practical option. Hence, with the goal to carry out infusion in cranberries that were as close in appearance to whole fresh cranberries, the pre-treatment method where pores were made on cranberry surface was selected for future experiments. This pre-treatment resulted in only partial surface damage of the berries. In order to make the process of making surface pores less labor-intensive, an equivalent process unit, called as scarifier, was designed in-house. In the scarifier, cranberries were rolled under a bed of pins to make sub-millimeter pinholes on the fruit surface. The scarifier design and its working is discussed in section 2.2.2.2.4.

Optimization of scarifying process

To achieve uniformity of surface damage per berry during cranberry scarification, they were rolled under the bed of pins multiple times. In order to optimize the number of passes under the bed of pins required to achieve maximum infusion with minimum loss in fruit intactness, the extent of sugar infusion was measured as a function of number of passes. Figure 2.17 shows the effect of number of passes through the scarifier on sugar infusion at 1 atm for 10 min. The amount of sugar infused increased with increasing number of passes. Although sugar infused after 10 passes through the scarifier belt was the highest, the cranberry had lost its integrity and did not hold its shape. The same was the case after 8 passes. Hence, the optimum number of passes through the scarifier belt was decided as six passes.
2.3.4.1.2 Effect of applied pressure on extent of infusion

Higher level of infusion of sugar under high pressure was observed in frozen-scarified-thawed cranberries compared to those infused at atmospheric pressure (Figure 2.18). Similar results of enhanced infusion of sugar in fresh cranberries were observed after HHP processing (Figure 2.19). °Brix of cranberries, both fresh-scarified and frozen-scarified-thawed, after HHP were about 2 to 2.5 times higher than in those infused at ambient conditions. Increase in pressure from 200 MPa to 551 MPa did not have a significant effect on °Brix of fresh cranberries but showed statistical significance in frozen-scarified-thawed cranberries. It is not clear at this point why infusion in frozen-scarified-thawed cranberries shows pressure dependence and in fresh cranberries is pressure independent. A point to note is that °Brix is indicative yet approximate.
measurement of sugar infused. The light refraction based method of °Brix measures total soluble solids and not just the sucrose infused.

Figure 2.18 Variation of sugar infused in frozen-scarified-thawed cranberries with applied pressure for 10 min. Means with different letters indicate statistical significance at p<0.05

Figure 2.19 Variation of sugar infused in fresh-scarified cranberries with applied pressure for 10 min. Means with different letters indicate statistical significance at p<0.05
These results suggest that infusion of water soluble sucrose molecules is significantly enhanced and accelerated under high pressure compared to infusion at atmospheric pressure for similar times.

2.3.4.1.3 Effect of pressure hold time on extent of infusion

Figure 2.20 shows the variation in amount of sugar infused at 0.1 MPa (1 atm) and at 400 MPa after 10 min and 60 min of infusion in frozen-scarified-thawed cranberries. For same time of infusion, HHP infused cranberries showed a significant increase in the relative °Brix compared to control (0.1 MPa). Interestingly, at 400 MPa for 10 min and 60 min infusion time, °Brix of infused cranberries was not significantly different. This minimal effect of pressure hold time on infusion allowed reduction in processing time to 10 min for our following studies.

Figure 2.20 Variation of sugar infused in frozen-scarified-thawed cranberries with pressure hold time. * indicates statistical significance at p<0.05 compared to control
2.3.4.1.4 Effect of pressure cycling on extent of infusion

Pressure cycling was used to test whether infusion of sugar can be further enhanced into cranberries when more than one pressure cycle is used. Figure 2.22 shows the variation of °Brix infused after 1 cycle (10 min), 2 cycles (5 min each) and 5 cycles (2 min each) at 100 MPa. The total pressure hold time in all treatment samples was fixed to 10 min. The come-up time for each cycle was less than a minute (as shown in Figure 2.21). With pressure cycling the amount of sugar infused was marginally higher (Figure 2.22) compared to that after one HHP cycle. After 2 and 5 cycles, the amount infused was 13 % and 21 % higher, respectively, compared to 1 cycle pressure treatment.

Figure 2.21 Real-time pressure-temperature recorded as a function of pressure hold time
2.3.4.2 Infusion of muscadine grape juice and muscadine grape concentrate

Phenolic content of frozen-scarified-thawed cranberries measured after high pressure assisted infusion (100 MPa, 10 min) significantly increased compared to in cranberries infused at ambient conditions. Phenolic content increased by 100% and 54% for muscadine grape concentrate infusion and muscadine grape juice infusion, respectively (Figure 2.23). Pictures shown in Figure 2.23 show that high pressure infused cranberries remain intact after HHP. Cranberries infused with purple muscadine grape concentrate changed to a dark purple color from its natural red color.
2.3.4.3 Infusion of isoquercetin

In these experiments, isoquercetin or quercetin-3-glucoside was infused into frozen-scarified-thawed cranberries. The objective of the study was to investigate the extent of infusion of isoquercetin in cranberries. Isoquercetin was first tested for its ability to dissolve in food grade solvents and make a stable infusate since it is water insoluble.

2.3.4.3.1 Selection of solvent to solubilize isoquercetin

Figure 2.23 Polyphenol content of infused cranberries at 100 MPa for 10 min. Different letters indicate statistical significance at p<0.05 compared to control

**Figure 2.24a** Solubility of isoquercetin in water at 22 °C

**Figure 2.24b** Solubility of isoquercetin in 1:1 glycerin-ethanol mixture at 22 °C
Isoquercetin was found to be soluble in water at 0.017 g/l or 0.0017 % at 22 °C. **Figure 2.24a** shows a picture of isoquercetin solution clarity/turbidity at different levels of solute concentration (0.1 %, 0.005 % and 0.0017 %). At concentrations of 0.1 % and 0.005 %, isoquercetin is not completely soluble in water and forms dispersion. Although more soluble in water than quercetin, 0.0017 % solution could not be used as an infusate since it did not make a hypertonic solution. To enhance solubility, other food grade solvents were tested. Isoquercetin was soluble up to 5 % level in 1:1 glycerin-ethanol mixture. Isoquercetin solubility in 1:1 glycerin-ethanol mixture is ten times higher than solubility of quercetin in the same solvent. **Figure 2.24b** shows a picture of isoquercetin solution clarity/turbidity in 1:1 glycerin-ethanol mixture at 0.498 %, 1.96 %, 4.76 % and 9.09 %. At 9.09 % concentration, isoquercetin was not completely soluble and the solution turned cloudy. Hence, the selected concentration to be used as infusate was 5 % isoquercetin in 1:1 glycerin-ethanol mixture.

### 2.3.4.3.2 Effect of applied pressure and concentration of isoquercetin in infusate on extent of infusion

High pressure showed significantly higher levels of infusion after HHP at 100 MPa for 10 min compared to infusion at ambient conditions (0.1 MPa, 10 min). For the two different concentrations of isoquercetin in the infusate, there was about two to four times increase, in the amount infused under high pressure compared to control (0.1 MPa).
Isoquercetin levels measured in frozen-scarified-thawed cranberries after infusion are shown in Figure 2.25.

**Figure 2.25** Isoquercetin levels in frozen-scarified-thawed cranberries after infusion for 10 min. * indicates statistical significance at p<0.05 comparing within each pressure treatment.

There is a linear correlation between the concentration of solute in infusate and the amount infused under high pressure. Comparing red and blue columns at 100 MPa shown in the plot in Figure 2.25, it is seen that when concentration of infusate was increased by ten times (from 0.5 % to 5 %) the amount of isoquercetin infused also increased by ten times (from 5.7 mg/g dry wt to 57 mg/g dry wt) in the corresponding samples.

### 2.4 SUMMARY

The results from the exploratory studies provided evidence that infusion is enhanced under high pressure in different kinds of fruits and vegetables. Soft and hard fruits such as grapes, apples and cranberries, and hard vegetables, such as carrots showed promise in which infusion could be enhanced after HHP. Specifically, the results from
the study on infusion using the following infusant-substrate systems - fruit concentrate-grapes, fruit concentrate-cranberries, sucrose-cranberries, isoquercetin-cranberries, fluorescent molecules-cranberries, calcium salts-apples, and calcium salts-baby carrots - can be summarized as follows:

(a) Infusion of varying size of molecules (150 Da to 30 kDa) can be achieved during HHP

(b) Infusion of substances with varying solubility can be achieved during HHP.

From the cranberry study, we observed that cranberry skin was impermeable to sucrose, polyphenols and isoquercetin infusion even under pressure. Scarification in the form of sub millimeter pinholes with six passes on the scarifier reduced the mass transfer barrier. Among the process variables tested, applied pressure, pressure cycling and concentration of infusate influenced the amount of substance infused. Pressure hold time did not have significant effect on the amount infused between 10 min and 60 min.

The present research conducted as a proof of concept study showed strong evidence that enhanced infusion of bioactive compounds into fruits and vegetables can be achieved under high pressure. The understanding from this study helped carry out a detailed study on HHP infusion using cranberries - process development and mechanistic understanding. The results from the study on infusion of quercetin into cranberries are discussed in Chapters 3 and 4.
CHAPTER 3

PRESSURE ENHANCED INFUSION OF QUERCETIN INTO CRANBERRIES

PART I: INFLUENCE OF PROCESS VARIABLES

3.1. INTRODUCTION

In the previous chapter, we discussed studies showing preliminary evidence of enhanced infusion of molecules of varied sizes and solubility under high pressure. Furthermore, earlier research on HHP assisted infusion also reported that the primary cause for enhanced infusion is cell permeabilization, expressed as increased effective diffusion coefficients (Rastogi and Niranjan, 1998; Rastogi et al., 2000; Villacis et al., 2008). These effective diffusion coefficients ($D_{\text{eff}}$) were computed based on Fickian diffusion model. Effective diffusion coefficient is a lumped up value that may account for other mass transport processes (e.g., pressure driven flow, capillary flow, etc.) in addition to mass diffusion. Hence, $D_{\text{eff}}$ does not elucidate the actual mechanism of mass transport. In the case of high pressure assisted infusion, the observed correlation between cell membrane rupture and enhanced infusion may not be the actual or only cause for the observed phenomenon. Other mechanisms may also be contributing to enhanced diffusion. In order to test the role of cell membrane rupture one needs to decouple the effect of cell-membrane permeabilization from high pressure treatment. One method to decouple these effects was to use a system that does not permeabilize further upon HHP processing. Frozen-scarified-thawed cranberries were selected for this purpose. Cells of frozen-thawed cranberries were already ruptured in the freeze-thawing process and preliminary results indicated that they do not permeabilize further upon HHP. Thus, using
frozen-thawed cranberries, it was possible to decouple cell-membrane permeabilization from the effect of HHP on infusion. In addition to frozen-thawed cranberries, fresh cranberries, cells of which are intact before HHP and ruptured after HHP, were selected as a second system of study. The use of fresh cranberries as a second system of study helped compare the differences in HHP infusion in an intact and permeabilized system. Further, comparison of infusion in fresh and frozen-thawed cranberries helped gain insights into possible mechanisms of infusion under high pressure. The current chapter discusses the influence of HHP process variables on extent of infusion and cell-structure of frozen-thawed cranberries. Part II is discussed in the next chapter (chapter 4) that delves deeper into the mechanism and dynamics of HHP infusion using fresh and frozen-thawed cranberries.

The objective of this research was to study the influence of process variables on HHP assisted infusion and test if cell permeabilization is the only factor responsible for enhanced infusion under high pressure, using two systems: (i) fresh cranberries, cells of which are intact and (ii) frozen-thawed cranberries, cells of which are already permeabilized during freeze-thaw process before HHP. The specific objectives of this study were, using frozen-thawed cranberries, to (a) test whether enhanced infusion can be achieved using HHP, (b) determine the dependence of enhanced infusion on applied pressure, hold time, concentration of infusate and ratio of fruit to infusate, and (c) test whether there are other mechanisms in addition to just cell-permeabilization that cause enhanced infusion under high pressure.

Frozen-thawed and fresh cranberries were infused with quercetin. The reason for interest in using cranberry-quercetin as the substrate-infusant system was because U.S.
Army, who were sponsors of this research study, were interested in developing cranberry super-fruit with high antioxidant value. Although, cranberry is inherently rich in quercetin, their level is not sufficient to make them an “excellent” or “good” source as per U.S. FDA Food labeling guide, 21 CFR 101.54(b), January, 2013. According to Harnly et al. (2006), cranberry naturally contains about 200 ppm (fresh weight basis) of quercetin. The recommended daily intake of quercetin is 500-2000 mg per 70 kg body weight individual (USARIEM Status-of-the-science review, 2008). Based on the recommended dietary intakes (RDI), an individual would have to consume about 2.5 kg to 10 kg per day of fresh cranberries or 1 kg to 3 kg of dried cranberries (at 15% moisture) per day to meet the RDI. These are very high amounts of cranberries that can be consumed by an individual on a daily basis. Hence, this research is focused on developing quercetin infused cranberries that can potentially make it an “excellent” or “good” source of quercetin, in other words a “super fruit”. The selected fruit-antioxidant system served as a model system. Mass transfer barrier in cranberries is a good representation of those found in other berries.

The results from this research are discussed in two parts. Part I discusses the influence of process variables on infusion in cranberries and Part II sheds light on the potential mechanisms of mass transport.
3.2. MATERIALS AND METHODS

3.2.1. MATERIALS

Cranberries (*Vaccinium macrocarpon* var. *Stevens*) were purchased from J.J. White Farms (Brown Mills, NJ). Fresh cranberries were used for the study within a week of harvest. Frozen-thawed cranberries were frozen and stored at -20 °C. They were thawed at ambient conditions (22 °C) and then used for HHP processing. Quercetin used was QU995 food grade QP FA manufactured by Merck S.A. (Rio de Janeiro, Brazil). Glycerin (99.7 %) was purchased from Qualichem Technologies Inc. (Woodstock, GA). Ethanol used was 200 proof absolute, anhydrous alcohol of ACS/USP grade manufactured by Pharmco Products Inc. (Brookfield, CT). All chemicals and reagents used were HPLC grade. Fisetin, used for fluorescent microscopy was purchased from Sigma-Aldrich (St Louis, MO). Other fixatives and stains used for light microscopy and SEM were purchased from Electron Microscopy Sciences (Hatfield, PA).

For analytical testing, all chemicals and reagents used were HPLC grade. Folin-Ciocalteau’s phenol reagent (2 M) and gallic acid (3,4,5 – trihydroxy benzoic acid) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). HPLC mobile phase of acetonitrile, phosphoric acid and HPLC water were purchased from Fisher Scientific.

3.2.2. METHODS

In this research, cranberries were used as a model system to understand the influence of process variables and mechanisms of HHP assisted infusion. Cranberries were infused with quercetin, a water insoluble antioxidant that belongs to the family of
flavonoids. The following sections discuss the experimental setup and procedures used in the study. Some methods discussed here are similar to those used in the preliminary study described in Chapter 2, section 2.2.2.

3.2.2.1. **Sample preparation**

Cranberries purchased from J.J. White Farms were either frozen-thawed or used fresh depending on the focus of the study. In the experiment to test the influence of method of freezing on infusion, cranberries used were either slow frozen or fast frozen. Cranberries were slow frozen by storing in freezer at -20 °C and fast frozen by immersing in liquid nitrogen and then stored at -20 °C (individually quick freezing cranberries). All other experiments used slow frozen cranberries or fresh cranberries. Fresh cranberries were stored refrigerated and used within 7 days of harvest.

Since, cranberry skin was impermeable to quercetin even under high pressure (600 MPa), they were surface scarified to reduce the mass transfer barrier. Method of scarification is discussed in details in Chapter 2, section 2.2.2.1.4. Briefly, bright red colored cranberries of uniform size and shape were rolled under a bed of pins to make sub-millimeter pinholes on their surface. Cranberries were passed six times through the scarifier belt. Multiple passes of cranberries through the scarifier belt were carried out to achieve uniformity in surface damage. The number of passes was optimized to six passes based on achieving maximum extent of infusion while maintaining fruit integrity (results from this experiment is discussed in Chapter 2, section 2.3.4.1.1.).

For experiments using frozen-thawed cranberries, they were scarified while frozen; the berries got softer on thawing and thawed berries could not hold up to the
process of scarification. Fresh cranberries were scarified and used immediately for HHP processing.

3.2.2.2. **Infusate development and its preparation**

![Figure 3.1 Structure of quercetin and quercetin chalcone (U.S. patent 5977184)](image)

The infusate used was a hypertonic solution of quercetin in which the fruits were immersed. Quercetin is almost insoluble in water. Hence, to develop an infusate containing quercetin other food grade solvents were considered.

In our preliminary experiments, an emulsion was formed with medium chain fatty acids containing oil and polyglyceryl-6-dioleate as the emulsifier. Here, quercetin did not stay dispersed and settled to the bottom almost immediately. In the next trial a dispersion using only emulsifier (polyglyceryl-6-dioleate) in water was formed. This dispersion too was not stable at concentrations of 0.1 % (minimum concentration targeted in the infusate). At very low concentrations of 60 ppm, this emulsion was stable for a week. Another form of quercetin, known as quercetin chalcone
(US patent 5977184) is found to be water soluble. Quercetin chalcone is a chemically modified form of quercetin as shown in Figure 3.1. The additional OH group is attributed to the higher water solubility observed in quercetin chalcone. This compound is sold in the market in the form of dietary supplements under the brand name of Quercetone® manufactured by Thorne research Inc (Sandpoint, ID). In our experiment, quercetin chalcone was soluble in water, however, there is very limited research discussing its toxicity and bioavailability. Hence, the use of this compound was discontinued.

Quercetin was found to be soluble in glycerin (up to 1%) and in ethanol. Use of alcohol at a food manufacturing plant may have handling issues due to its inflammability and environmental problems. Hence alcohol was partially replaced by glycerin. In addition, use of glycerin in combination with ethanol would impart some sweet taste to the cranberry (glycerin is 0.6 times as sweet as sucrose). Quercetin was found to be soluble up to 1% (by wt) in 1:1 mixture of glycerin-ethanol. Quercetin is also soluble in water at alkaline pH > 9.5 between 0 to 1% concentrations. Quercetin solutions prepared in glycerin-ethanol mixture and at alkaline pH are shown in Figure 3.2. Quercetin stability was monitored at room temperature (22 °C) in the glycerin-ethanol mixture and in the alkaline system. Figure 3.3 shows the UV-Visible spectrum of quercetin dissolved in glycerin-ethanol mixture and in alkaline solution (pH 10.13). Quercetin at pH 10.13 was highly unstable as seen by the decrease in the absorbance at 280 nm within 10 min and 20 min. Similar observation of instability of quercetin in alkaline pH was reported by Kitson and Kitson (2000). On the contrary, quercetin in 1:1 glycerin-ethanol solution was stable for up to 20 days. HPLC analysis of quercetin in glycerin-ethanol system after 0 and 20 days had the same retention times (36 min). This further confirmed quercetin
stability in 1:1 glycerin-ethanol solution. It was soluble up to 1 % (by wt.) in 1:1 glycerin-ethanol mixture. Quercetin in glycerin-ethanol mixture was also found to be stable to pH changes between 4 and 7, which translate to conditions during infusion process in cranberries during HHP. Only at pH < 3.29, quercetin precipitates in solution. In this study, 0.5 % quercetin dissolved in 1:1 (w/w) glycerin-ethanol solution was used to conduct all experiments except when concentration of quercetin in solution was the test variable.

To prepare the infusate, 1 % (wt) of quercetin was dispersed in glycerin and heated to 95 °C. The solution was then cooled to room temperature and measured amount of alcohol was added to the cooled mixture with stirring to make 0.5% solution of quercetin.

Scarified cranberries (fresh and frozen-thawed) were immersed in the infusate, packaged in vacuum-sealed polyethylene pouches, and transferred to the high pressure unit for processing. The effect of vacuum packing step on the extent of infusion was significantly smaller (< 10 %) compared to the actual treatment with HHP. The fruit to
infusate ratio was maintained fixed at 1:2 (w/w) except when fruit to infusate ratio was the test variable.

3.2.2.3. HHP assisted infusion

Pilot scale hydrostatic pressure processing vessel

The pilot scale hydrostatic pressure vessel used in this study is a vertically oriented vessel having a working capacity of 10 liters. Details of the design and operation of this high pressure vessel are described in Chapter 2, section 2.2.2.1. The operating pressures of this vessel are in the range 100 MPa to 690 MPa. The pressure transmitting medium used was filtered tap water. The samples were pressure treated between 100 MPa and 551 MPa for 1 min to 60 min at room temperature (19 °C - 23 °C). The come up time for pressurization step ranged between 1 min and 3 min. There was some rise in temperature due to adiabatic compression heating during HHP. The temperature rise recorded for the pressure transmitting medium did not exceed 13 °C (at 551 MPa, water temperature rose from 22 °C to 35 °C) and its effect was found to be insignificant in this temperature range as shown in Figure 3.4. Comparison of extent of infusion at 0.1 MPa (or 1 atm) for 10 min at the initial temperature (21 °C) and at the highest temperature of 35 °C reached after HHP showed no significant difference in the amount of quercetin infused. Therefore, the contribution of adiabatic compression heating on extent of quercetin was considered negligible even during HHP. Therefore, it would be safe to assume that any difference observed in the extent of infusion in cranberries was solely due to the effect of the test variable.
Figure 3.4 Influence of temperature of pressure transmitting medium on the extent of quercetin infusion in frozen-scarified-thawed cranberries at 0.1 MPa (or 1 atm) for 10 min. Different letters indicate statistical significance at $p<0.05$.

Scarified cranberries (fresh and frozen-thawed) immersed in the infusate were packaged in vacuum-sealed polyethylene pouches. The pouches were then put inside the sample cavity of the high pressure vessel. Each pouch contained about 25 g of scarified berries immersed in 50 g infusate. Three pouches of sample were used per batch HHP treatment. The vessel was pressurized to the desired pressure and held for a fixed time before depressurization. After processing, the pouches were cut open and the infusate was drained. The processed berries were dipped in lukewarm water (~ 40 °C) and removed immediately, then dried with a paper towel to remove any surface adhered infusate. The samples were immediately frozen and stored at -20 °C until further analysis.

Control experiments on infusion were carried out at atmospheric pressure for similar time-temperature conditions as in HHP processing. In the experiment comparing different infusion treatments, besides HHP and atmospheric infusion, vacuum infusion
was used as another treatment variable. Infusion under vacuum was carried out at final pressure of 2 torr in a vacuum oven operated at room temperature.

Each experiment was performed in triplicates, i.e., process treatment and post-process analyses were carried out in triplicates. An average concentration value for the amount infused per batch of fruit was measured after each treatment condition. The moisture content of fruit before and after infusion was measured using a Sartorius MA30 Moisture Analyzer.

**Pressure Cycling**: The high pressure vessel was pressurized to 100 MPa and pressure cycling was performed for up to 5 cycles with pressure hold time ranging between 2 min and 5 min. **Figure 3.5** shows real-time pressure-temperature data for 1, 2 and 5 pressure cycles at 100 MPa for 10 min hold time, not including the come-up time.

**Figure 3.5** Real time pressure-temperature data for a pressure cycle of 100 MPa for hold time of 10 min
**Bench top hydrostatic pressure processing vessel**

The bench top hydrostatic pressure vessel (Elmhurst Systems, LLC., Albany, NY) used in this study is shown in Figure 3.6. This unit is a vertically oriented vessel having a working capacity of 60 ml operating between pressures of 5 MPa and 137 MPa. The pressure transmitting medium used was water. The vessel has internal bore diameter of 25.4 mm and wall thickness of 96.5 mm. Overall length is 203.2 mm and the sample-loading cavity is 76.2 mm long. The vessel volume is 75 ml with a working volume of 60 ml. The vessel was pressurized using a 40,000 lb-f hydraulic press. The hydraulic press is used to push a piston inserted in the vessel downward. The internal pressure and temperature was measured using a pressure transducer and thermocouple attached to the unit. Operating temperature range is 1 °C to 90 °C. Pressure and temperature were read manually from the displays connected to the pressure transducer and thermocouple, respectively.

![Figure 3.6 Bench top scale hydrostatic pressure processing unit at Rutgers University used in this study](image)

**Figure 3.6** Bench top scale hydrostatic pressure processing unit at Rutgers University used in this study
In the bench scale vessel, about 6 g of scarified cranberries (three berries) was immersed in 12 g of infusate and packaged in vacuum-sealed polyethylene pouches. Each pressure treatment was repeated six times. The pouches were cut to shape to fit in the sample cavity (60 ml) of the pressure vessel. The cavity could accommodate one pouch at a time. The control sample was cranberries infused at ambient conditions for similar infusion times.

### 3.2.2.4. Post-infusion analyses

Following infusion, the pouches were cut open and the infusate was drained. The processed berries were dipped in lukewarm water (~ 40 °C) and removed immediately, then dried with a paper towel to remove any surface adhered infusate. The samples were immediately frozen and stored at -20 °C until further analysis.

### 3.2.4.1. Polyphenol content measurement

Folin-Ciocalteau’s spectrophotometric method was used to quantify the polyphenol content of infused cranberries. This method was used as an indirect measure of extent of infusion. Following HHP assisted infusion, extent of polyphenol infusion was measured in quercetin infused cranberries using Folin-Ciocalteau’s spectrophotometric method. The method of measurement is discussed in details in Chapter 2, section 2.2.2.3.2.
**Validation using Spike and recovery method**

The accuracy of Folin-Ciocalteau’s spectrophotometric method was established by determining the recovery of gallic-acid spiked cranberry extract. **Table 3.1** shows the analyzed results for recovery of quercetin by the proposed method. This method showed almost 100 % recovery in all the spiked samples.

<table>
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<th>Amount initially present in sample</th>
<th>Amount added to sample</th>
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<th>Experimental value</th>
<th>% Recovery</th>
<th>% RSD</th>
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<td>mg GAE/100 g fresh fruit</td>
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<td>1128.7</td>
<td>97.3</td>
<td>4.0</td>
</tr>
</tbody>
</table>

**3.2.2.4.2. RP-HPLC quantification of quercetin**

RP- HPLC was used to quantify the amount of quercetin infused in cranberries. Cranberries were solvent extracted using the method discussed in Chapter 2, section 2.2.2.3.1. The extracts were filtered through 0.45 m filters prior to HPLC analysis. A 5 µm, 4.6 mm x 250 mm Ascentis C_{18}, RP Amide column was attached to a Waters HPLC equipped with 486 UV-Vis tunable detector and Peak Simple Chromatography data system. The mobile phase consisted of a gradient of 0.085 % phosphoric acid (A) and acetonitrile (B). The flow rate was set at 1.0 ml/min and the sample injection volume was 10 ml. The gradient used was as follows: 15 % B at 0 min, 35 % B at 30 min and 85 % B at 35 min. Quercetin was detected at 366 nm and quantified using an external calibration curve of quercetin standard. Results were expressed as mg of quercetin/g initial fruit weight, on dry basis.
**Validation using spike and recovery method**

Figure 3.7 shows the calibration curve for quercetin measurement. The external standard calibration curve showed good linearity in the range tested.

![HPLC calibration curve for standard quercetin](image)

**Figure 3.7 HPLC calibration curve for standard quercetin**

Calibration curve yielded the following equation $y = 1724x - 117.53 \ (R^2 = 0.9979)$, $y$ representing peak area and $x$ representing amount of std quercetin. The limit of detection (LOD) was found to be 2.6 μg/ml for quercetin (S/N = 3) and quantification limit (LOQ) was 8.9 μg/ml (S/N = 10). The accuracy of the method was established by determining the recovery of quercetin spiked to the cranberry extract.
Table 3.2 shows the analyzed results for recovery of quercetin by the proposed method. Observed percent recovery of over 100% can be explained by possible variation in the initial amount of quercetin present in cranberries.

### 3.2.2.4.3. Cell membrane permeabilization

Cell membrane permeabilization changes were recorded using impedance spectroscopy technique. The method is based on the electrical properties of cells with different ratios of ruptured and intact cells that can be represented as an equivalent circuit model as discussed by Angersbach *et al.* (1999). The frequency-dependent electrical conductivity spectrum recorded can be expressed in terms of a permeabilization index, $Z_p$ (Rastogi *et al.*, 2000). $Z_p$ is a ratio of electrical conductivities of processed and unprocessed samples and was determined using Equation (3.1).

$$\frac{\sigma_{hi}^i - \sigma_{li}^i}{\sigma_{hi}^i - \sigma_{li}^i} \quad \text{......(3.1)}$$

where $\sigma$ is the electrical conductivity, superscripts $i$ and $t$ are conductivities before and after infusion treatment, respectively, and subscripts $l$ and $h$ are conductivities at low and high frequency, respectively. The theory behind the use of the technique for cell-permeability measurement is discussed in Appendix I.
In this study, \( \sigma \) before treatment meant that for unprocessed cranberries. \( Z_p \) value ranges between 0 and 1. \( Z_p \) equals zero when all cells are intact and \( Z_p \) equals 1 when all cells are completely permeabilized. Characteristic low and high frequency for cranberry cells were recorded from the two ends of the spectrum.

The measurement set-up for the electrical conductivity \( \sigma \) (S) included a sample holder connected using coaxial cables to Bode 100 Vector network Analyzer (Omicron Electronics Corp., Houston, TX). **Figure 3.8** shows a schematic of the set-up for electrical conductivity measurements. Cranberry samples were sandwiched between the 2 parallel plate disc electrodes across which an input signal ranging from -27dBm to 13 dBm was applied. The electrical conductivity spectra were recorded between 1 kHz and 40 MHz using CD Bode Analyzer Suite v. 2.23 (Omicron Electronics Corp., Houston, TX). For cranberry cells, the characteristic low and high frequencies were 1 kHz and 5 MHz, respectively. All electrical conductivity measurements were performed immediately after treatment (HHP and control).

**Figure 3.8** Set up for impedance spectroscopy and its circuit representation
3.2.2.4.4. Light microscopy

Cranberries were cut into 1 mm x 1 mm x 1 mm cubes with sharp single edge stainless steel blades, stained with 0.1 % toluidine blue and observed under the microscope for cell membrane rupture in cranberry samples before and after HHP.

3.2.2.4.5. Fluorescence microscopy

For mapping the distribution of the infused molecule, fisetin was used as the infusing molecule. Fisetin was selected as a surrogate for quercetin (structure shown in Figure 3.9), since it is a fluorescent molecule with comparable molecular weights of 286 g/mol for fisetin and to 302 g/mol for quercetin, and partition coefficient log P values of 2.53 ± 0.7 for fisetin and 2.88 ± 0.32 for quercetin (Moridani et al., 2003).

![Figure 3.9 Structures of quercetin and fisetin showing their molecular weights and log P values](image)

Post infusion, cranberries were transversely sectioned to make discs (1 mm thick). Each disk was placed on a glass slide, covered with glass cover slip on the top. The cover slip was taped to the glass slide on either side to keep the slip from falling off the sample. Fluorescence and white light (differential interference contrast microscopy) images were acquired using a laser scanning confocal fluorescence microscope (Zeiss 180 LSM 510). Multiphoton fluorescence images were obtained using 780 nm laser excitation and a 400 nm - 440 nm bandpass emission filter using a 25 oil objective (numerical aperture=1.4).
The mean fluorescence intensity of each image was calculated using ImageJ software (NIH, Bethesda, MD).

### 3.2.2.4.6. Scanning electron microscopy

The microstructure changes in cranberry tissue were evaluated using scanning electron microscopy (SEM). Six blocks of control and pressure treated tissue, each measuring 3 mm x 2 mm x 1 mm, were cut from the fruit. Sample cross sections were kept immersed in a fixative solution of 1% p-formaldehyde and 1% glutaraldehyde in 0.1 M acetic acid buffer (pH 2.8) for 4 h. The fixed tissue was washed 4 times with 0.1 M acetic acid buffer. The samples were then immersed in a solution containing 1% osmium tetraoxide (OsO₄) and 0.5% uranyl acetate made in 0.1 M acetic acid buffer overnight. The samples were then rinsed three times with water and dehydrated by immersing in increasing concentration of ethanol (40%, 60%, 80%, 90%, 100%) for about 1 h each. 100% ethanol changes were made three times. The last change of 100% ethanol was left overnight. Ethanol dehydrated cranberry samples was then subjected to critical point drying (CPD) before microscopic examination. During CPD, at the critical point, there is a phase change from liquid to gas without any deleterious effects caused by surface tension of liquid. Hence, CPD is suitable for drying of delicate biological specimens without any structural distortion (Bozzola and Russell, 1999). Ladd critical point dryer (Williston, VT) was used. CO₂ was the transitional fluid medium (critical point of 31.1 °C and 1072 psi), which is not miscible with water but is miscible with ethanol (intermediate fluid). The specimen was transferred to the pressure chamber with intermediate fluid and flushed several times with CO₂ to replace all the ethanol. The
chamber was then heated to the critical point of CO₂. Pressure was obtained by the effect of applying heat. The chamber was heated to temperature slightly above the critical point of CO₂ and gas CO₂ is vented. Equal density of liquid and gas phase at the critical point causes drying with minimum distortion of specimen structure. The CPD dried specimen was then sputter coated with platinum using Balzers sputter coater.

The specimens were examined under JEOL JSM 35C scanning electron microscope operated at 20 kV. Images captured showed the changes in the tissue under a specific treatment condition.

3.2.2.4.7. **Statistical analyses**

Statistical analysis was performed using SAS version 9.2 software for Windows (SAS Institute, Cary, NC). Analysis of variance and separation of the means were carried out using PROC GLM. When effects were significant, comparisons between the means were performed using least square difference (LSD) comparison test at \( \alpha = 0.05 \).

3.3. **RESULTS AND DISCUSSION**

In this section, the influence of process variables during HHP processing on the extent of infusion of quercetin and on cell structure of frozen-scarified-thawed cranberries and fresh-scarified cranberries are discussed. The section begins with a proof of concept study on use of HHP for enhanced infusion of quercetin in cranberries. This is followed by a detailed discussion of effect of each HHP process variable on infusion in cranberries. The process variables tested were method of freezing of cranberries prior to HHP, applied pressure, pressure hold time, concentration of infusate, ratio of fruit to
infusate, and pressure cycling. All process variables were selected based on common knowledge of physical parameters that influence mass transport in biological systems.

3.3.1. Proof of concept study: Comparison of HHP infusion and traditional infusion processes

To test whether pressure assisted infusion is a promising method to carry out infusion in cranberries, HHP infusion was compared to traditional methods, such as vacuum infusion and osmotic diffusion at 1 atm, currently used in the food industry. Figure 3.10 shows the comparison of extent of infusion between different process treatments. HHP treated frozen-scarified-thawed cranberries showed 2 to 3 times higher levels of quercetin infused compared to those infused under vacuum and at ambient conditions (0.1 MPa or 1 atm).

![Image of Figure 3.10](image_url)

**Figure 3.10** Comparing quercetin infusion in frozen-scarified-thawed cranberries at 1 atm, under vacuum and under HHP (100 MPa). * and ** indicate statistical significance at p < 0.05.
These results suggested that HHP processing is a promising alternative to enhance infusion compared to atmospheric pressure infusion and vacuum infusion.

3.3.2. Influence of process parameters on extent of infusion

This part of the study is focused on understanding the influence of process variables on the extent of quercetin infusion in frozen-scarified-thawed cranberries. Frozen-thawed cranberries were used in this study since freezing helped extend their shelf-life and ensured their availability all through the year; cranberry is a seasonal crop harvested between October and December in U.S.A. Additionally, the berries were scarified to reduce the mass transfer barrier offered by the fruit skin and to make them permeable to quercetin infusion. The process of scarification was discussed in Chapter 2. The process variables tested were method of freezing of cranberries prior to HHP, applied pressure, pressure hold time, concentration of infusate, ratio of fruit to infusate, and pressure cycling.

3.3.2.1. Method of freezing: fast freezing and slow freezing

Although freezing ensured consistent cranberry supply through the year, it is a well-known fact that freezing causes change in the cell structure of fruit and vegetables tissues. Ice crystals formed can disrupt or partially damage the cell wall and the cell membrane structure of biological tissues. In addition, the method of freezing influences the size of ice crystals and hence the extent of tissue damage that will occur. Slow freezing (or conventional freezing) results in large ice crystal formation and more damage
to the cell structure compared to fast freezing that is associated with smaller size ice crystals and lesser cell damage of biological tissues. Further, earlier research showed that the extent of infusion was influenced by cell-membrane permeability, in other words higher the cell damage higher the amount of substance infused in the substrate (Rastogi et al., 2000; Villacís et al., 2008). It was therefore important to understand if and how freezing and the method of freezing affect the extent of infusion. In the present section the effect of method of freezing on extent of infusion in frozen-scarified-thawed cranberries is discussed.

![Influence of slow and fast freezing of cranberries on extent of infusion of quercetin as function of applied pressure. Different letters indicate statistical significance at p<0.05](image)

**Figure 3.11** Influence of slow and fast freezing of cranberries on extent of infusion of quercetin as function of applied pressure. Different letters indicate statistical significance at p<0.05

Higher level of quercetin was infused in cranberries after HHP compared to infusion at ambient conditions (0.1 MPa). However, method of freezing (slow or fast) did not show a significant difference in the amount of quercetin infused after HHP between pressures of 100 MPa and 551 MPa (**Figure 3.11**). Since the method of freezing did not significantly influence the amount of quercetin infused, despite differences observed in
extent of cell damage (data shown later in Figures 3.22 and 3.27), the more economical method of slow freezing was chosen for this research study. However, if more intact texture of fruit after HHP was an important criteria during product development, fast freezing may be an alternative based on the feasibility analysis.

### 3.3.2.2. Applied pressure

High pressure treated cranberries showed significantly higher levels of quercetin compared to control. The amount of quercetin infused was measured using RP-HPLC method. Figure 3.12 shows the HPLC chromatograms for high pressure infused cranberries (100 MPa, 10 min) and control (0.1 MPa, 10 min). Higher levels of quercetin infused after HHP was seen as increase in quercetin peak height and area in the HPLC chromatograms.

![Figure 3.12](image)

**Figure 3.12** Chromatograms of extracts of cranberries infused with quercetin at 0.1 MPa (control) and 100 MPa for 10 min

Figure 3.13 shows the variation in the amount of quercetin infused with applied pressure for infusion time of 10 min. The amount of quercetin infused in HHP treated cranberries ([5.44 ± 0.2] mg/g initial fruit wt. dry basis) was found to be three times more
than that in control ([1.81 ± 0.15] mg/g initial fruit wt. dry basis). The moisture content of unprocessed cranberries was [87.04 ± 1.01] % (wet basis) which was used to calculate the quercetin infused on fruit weight dry basis. In addition, amount of quercetin infused was found to be independent of the applied pressure between 100 MPa and 551 MPa. This observation was unlike earlier studies, which have shown increase in the infused amounts of sugar and salt with increase in applied pressure (Rastogi et al., 2000; Sopanangkul et al., 2002).

![Figure 3.13](image)

**Figure 3.13** Variation of quercetin in frozen-scarified-thawed cranberries with applied pressure. Different letters indicate statistical significance at p<0.05

The initial amount of quercetin in frozen-scarified-thawed cranberries was determined to be [0.45 ± 0.02] mg/g initial fruit wt. dry basis. This amount was lower than those reported in literature (Bilyk and Sapers, 1986; Zheng and Wang, 2003). This could have been due to varietal and fruit maturity differences.

Polyphenol content, another indicator of the amount of quercetin infused, was measured before and after HHP treatment of cranberries. These measurements showed a
similar trend as seen in quercetin measurements. The results are expressed as mg of gallic acid equivalents (GAE)/ g of initial fruit dry weight. The initial polyphenolic content of cranberries, prior to infusion was $[11.07 \pm 0.62]$ mg GAE/ g initial dry wt. Polyphenols content in HHP treated cranberries was $[51 \pm 6.1]$ % higher than in control (Figure 3.14).

The percent increase in polyphenols (51 %) was lower than observed in quercetin measurement (200 %). This could be due to simultaneous transport of quercetin into and water soluble polyphenols out of cranberries. The diffusion of water soluble polyphenols out of cranberries can reduce the total polyphenols measured in cranberries.

![Figure 3.14 Polyphenol levels in infused frozen-scarified-thawed cranberries as a function of pressure applied for 10 min. * indicates statistical significance at p<0.05](image)

The observation that beyond a lower limit of pressure (100 MPa), infusion into cranberries is independent of the applied pressure can be advantageous from a commercial standpoint. Energy requirements to pressurize to 100 MPa is significantly lower than that required to pressurize to 200 MPa [according to thermodynamics, Work done or Energy stored is proportional to $(\text{pressure})^2$]. Additionally, operating at lower
pressures can reduce the mechanical stress and wear on the equipment thus lowering cost of maintenance and extending the life of the equipment. It is possible that pressures lower than 100 MPa will be sufficient to enhance infusion in cranberries. To identify the critical pressure required to enhance infusion by 3 times (as observed at 100 MPa) under HHP, the influence of low pressures (<100 MPa) on the extent of infusion was also investigated. The results from the low pressure study are discussed in section 3.3.2.7.

3.3.2.3. Pressure hold-time

![Graph showing the amount of quercetin infused as a function of pressure hold time at 0.1 and 100 MPa in frozen-scarified-thawed cranberries.

Figure 3.15 Quercetin infused as a function of pressure hold time at 0.1 and 100 MPa in frozen-scarified-thawed cranberries (in legend, insert space before MPa)

The transient variation of quercetin concentration in cranberries at pressures of 100 MPa and 0.1 MPa (control) are shown in Figure 3.15. The quercetin content of cranberries after HHP was always higher than in cranberries that were infused at ambient conditions. At 100 MPa, there was a significant increase in quercetin infused for process time up to 5 min. Further, as the pressure hold time was increased beyond 5 min there
was no significant change in the amount infused. Pressure hold time (greater than 5 min) did not have significant effect on the amount of quercetin infused. Amount of quercetin that could be infused in 10 min under high pressure needed at least 3 h in control (1 atm) indicated by an arrow in Figure 3.15. The approximate mass transfer rates in cranberries after 10 min was 0.83 μg/s at 0.1 MPa and 3.81 μg/s at 100 MPa. These results suggest infusion under high pressure in cranberries was not only enhanced but was also accelerated. In order to understand the transport mechanism, a more detailed analysis of the phenomenon is discussed in the next chapter where Fickian diffusion principles are applied to extract effective diffusion coefficients values for quercetin in cranberries before and after HHP.

3.3.2.4. Concentration of quercetin in infusate

![Figure 3.16](image.png)

Figure 3.16 Quercetin infused as a function of concentration of quercetin at 100 MPa in frozen-scarified-thawed cranberries. Different letters indicate statistical significance at p<0.05.
Varying quercetin concentration in infusate between 0.05 % and 0.5 % (w/w) showed a dose dependent response on extent of infusion (Figure 3.16). We found maximum quercetin infused in cranberries ([7.4 ± 0.01] mg/g dry fruit wt) when 0.5 % (w/w) quercetin containing solution was used as infusate. Concentration of 0.5 % quercetin infusate is close to the solubility limit of quercetin in 1:1 glycerin-ethanol solution. This concentration was kept constant for all the experiments.

3.3.2.5. Ratio of fruit to infusate

Figure 3.17 shows the variation of quercetin infused as a function of ratio of fruit to infusate. The objective of this experiment was to minimize the amount of infusate required to achieve desired levels of infusion. This process parameter was of importance since use of large amounts of infusate adds to processing cost and is a challenge faced by the food industry that uses traditional processes, such as osmotic dehydration of fruits (ratio of fruit to infusate is 1:25; Rosa and Giroux, 2001). It was found that there was significant different in the amount of quercetin infused between 1:1 and 1:3 but no significant difference in the quercetin infused at 1:2, and 1:3 ratios (w/w) of fruit to infusate. All were, however, significantly higher than that infused at ratio of 1:0.5. Therefore, a minimum ratio of 1:1 of fruit to infusate is required to achieve significant levels of infusion. Although infusion was enhanced at ratio of 1:1, it was challenging to have the berries physically immersed in the infusate solution for effective mass transfer. Therefore, a minimum ratio of 1:2 was used for all experiments.
Figure 3.17 Variation of quercetin infused as a function of fruit to infusate ratio. Different letters indicate statistical significance at p<0.05

3.3.2.6. Pressure cycling

Pressure cycling was used to test whether infusion of quercetin can be further enhanced into cranberries when more than one pressure cycle is used. Figure 3.18 shows the variation of amount of quercetin infused after 1 cycle (10 min), 2 cycles (5 min each) and 5 cycles (2 min each) at 100 MPa. The total pressure hold time in all treatment samples was fixed to 10 min. The come-up time for each cycle was less than a minute (as shown in Figure 3.5). With increase in the number of cycles, the amount of quercetin significantly increased (Figure 3.18). After 2 and 5 cycles, the amount infused was 57 % and 84 % higher, respectively, compared to single cycle of pressure treatment.
3.3.2.7. **Low pressure (5 MPa to 100 MPa)**

Earlier experiment discussed in section 3.3.2.2 showed that amount of quercetin infused was found to be independent of the applied pressure between 100 MPa and 551 MPa. It is possible that pressures lower than 100 MPa may be sufficient to enhance infusion in cranberries. The goal of the current experiment was to identify critical pressure below 100 MPa to achieve enhanced infusion in cranberries. Low pressure infusion can make the process less energy-intensive and more cost-effective. Pressure treatment between 5 MPa to 100 MPa was carried out in the bench scale pressure vessel.

Infusion was enhanced under low pressure (5 MPa to 100 MPa), when compared to infusion under ambient conditions (control) in frozen-scarified-thawed cranberries (Figure 3.19). The amount infused under lower range of high pressure (5 MPa to 100 MPa) was 1.6 times to 2 times higher than that infused at 0.1 MPa. Additionally, the amount infused was independent of applied pressure between 20 MPa to 100 MPa.
Enhanced infusion in frozen-scarified-thawed cranberries at low pressure of 5 MPa suggests that very low pressure is sufficient to cause enhanced infusion in frozen-scarified-thawed cranberries.

**Figure 3.19** Variation of amount of quercetin infused as a function of low pressures in frozen-scarified-thawed cranberries. Different letters indicate statistical significance at p<0.05

A point to note was that the magnitude of quercetin infused at 100 MPa in the bench-scale pressure vessel was lower than that infused at the same pressure using the pilot scale vessel for similar test conditions. **Figure 3.20** shows that the variation in the quercetin infused amount using bench-scale and pilot scale pressure vessels. The reasons for the observed differences are not clearly understood.
Figure 3.20 Variation in amount of quercetin infused as function of size of pressure vessel in frozen-scarified-thawed cranberries. Different letters indicate statistical significance at p<0.05

Although, pressure as low as 5 MPa was sufficient to enhance infusion in cranberries, most studies in this research were conducted at pressures between 100 MPa and 551 MPa. This was because the pilot scale pressure vessel used for pressurization had more control (temperature, rate of pressurization, rate of depressurization) on the process conditions compared to the manually operated bench scale vessel. Also on the pilot scale vessel, larger batch size could be processed as opposed to few cranberries on the bench scale vessel. The results from the experiments using the pilot scale vessel were also more reproducible than while using the bench scale vessel, perhaps due to small sample size.

3.3.3. Influence of HHP assisted infusion process on cranberry microstructure

In order to gain further insights into changes taking place in the cranberry tissue architecture, light microscopy and scanning electron microscopy experiments were carried out on pressurized and unpressurized cranberries.
Light microscopy

**Figures 3.21a and 3.21b** show the cell structure of a section of cranberry skin before and after HHP, respectively. On qualitative evaluation, no significant differences were observed in their structure before and after HHP. Cranberry skin cells remained intact even after HHP. These observations corroborate with the earlier observation that cranberry skin is impermeable to water and quercetin even under pressure. **Figures 3.21c and 3.21d** show corresponding cranberry flesh structure before and after HHP. No observable differences were noticed in the cranberry flesh structure before HHP and after HHP. These observations are in agreement with the results from cell-membrane permeabilization measurements discussed in the following section. Thus, light microscopy showed that cranberry cells were already ruptured before HHP, during freeze-thawing, and did not show significant change in cell rupture after HHP.

![Figure 3.21](image-url) *Figure 3.21* Frozen-scarified-thawed cranberry microstructure images from light microscopy of (a) skin before HHP (b) skin after HHP at 100 MPa (c) flesh before HHP and (d) flesh after HHP at 100 MPa
Scanning electron microscopy (SEM)

Using SEM, changes in cranberry cell structure before and after HHP in slow and fast frozen-thawed cranberries were recorded. **Figure 3.22** shows microscopic images of slow and fast frozen-scarified-thawed cranberries before HHP (0.1 MPa or 1 atm) and after HHP (at 100 MPa and 551 MPa). In slow frozen-scarified-thawed cranberries, no observable differences were noticed in the specimen before and after HHP. These observations are in agreement with the observations from light microscopy (shown in Figure 3.21). Cell damage in fast-frozen-scarified-thawed cranberries was lower than in slow frozen-scarified-thawed cranberries. Moreover, comparing effect of HHP before and after HHP in fast frozen-scarified-thawed cranberries, cell damage after HHP (at 100 MPa and 551 MPa) was more than that before HHP (Figure 3.22). These observations suggest that (a) fast freezing causes less cell damage in cranberries compared to slow freezing. The observed difference in cell damage was due to the differences in size of ice crystals formed during freezing which is a well known fact about freezing of biological tissues. Large ice crystals formed during slow freezing causes more cell damage than small size ice crystals formed during fast freezing and, (b) HHP did not cause further cell damage in slow frozen-scarified-thawed cranberries, however, it did partially rupture cell-membranes in fast-frozen-scarified-thawed cranberries.
Slow frozen-thawed cranberries

Fast frozen-thawed cranberries

**Figure 3.22** SEM microstructure of slow frozen-scarified-thawed and fast frozen-scarified-thawed cranberries before and after HHP

**Figure 3.23** shows SEM image of frozen-scarified-thawed cranberry flesh structure before and after HHP at 30 x and 270 x magnifications.

**Figure 3.23** SEM microstructure of frozen-scarified-thawed cranberry tissue before (a & b) and after HHP (c & d)
While infusion of quercetin was enhanced and similar in slow and fast frozen-scarified-thawed cranberries, microscopy results showed that there were differences in their cell structure. In case of slow frozen-scarified-thawed cranberries, while infusion was increased after HHP, there were no observable differences in their cell structure after HHP. These results provided preliminary evidence that, unlike earlier studies, tissue structure may not play a primary role of enhancing infusion in cranberries during HHP. The understanding in the research community, based on previously published literature, is that HHP disrupts and permeabilizes cell membranes resulting in less obstructive paths for the infusing molecule to travel and thus leads to increased infusion. In order to test this hypothesis that cell-membrane permeabilization causes enhanced infusion under high pressure in the slow frozen-scarified-thawed cranberry-quercetin system of study, cell-permeability was measured. The following section discusses the results of the influence of process variables on cell-membrane permeability.

3.3.4. Influence of process parameters on cranberry cell-membrane permeabilization

In this section the influence of HHP process variables on cranberry cell-membrane permeabilization in frozen-scarified-thawed cranberries is discussed. The process variables discussed include applied high pressure, pressure hold-time, pressure cycling, low range of pressures and method of freezing.
3.3.4.1. Applied pressure

Variation in cranberry cell-membrane permeabilization as a function of applied pressure is shown in Figure 3.24. The conductivity measurements were recorded immediately after HHP using impedance spectroscopy technique. To compute the permeabilization index ($Z_p$) using equation 3.1, the electrical conductivity was recorded as a function of frequency. Figure 3.24a shows a representation of characteristic electrical conductivity response in the frequency range of 1 kHz to 40 MHz before HHP (0.1 MPa or 1 atm) and after HHP (400 MPa). In the low frequency range, conductivity of untreated cranberry tissue (fresh cranberry) was significantly lower than of 0.1 MPa and 440 MPa treated frozen-scarified-thawed cranberries. This is because cranberry tissue of fresh or untreated cranberry is more intact with low electrical conductivity than that of frozen-scarified-thawed cranberry tissue at 0.1 MPa and 400 MPa. High conductivity in processed samples can be attributed to cell-membrane disintegration. This phenomenon of change in electrical conductivity associated with cell-membrane disruption is discussed in details in Appendix I. Untreated/fresh cranberries have intact tissue and exhibit high resistance and reactance in the low frequency range (Angersbach et al., 1999). These results were consistent with observations made by Angersbach et al. (1999) in high pressure processed apple tissue (50 MPa for 10 min). At higher frequencies, the intact and processed tissues have negligible impedance and hence show high conductivities (Angersbach et al., 1999).
Figure 3.24 shows that there was no significant difference in cell-membrane permeability between control samples infused at 0.1 MPa ($Z_p = 0.48 \pm 0.01$) and HHP treated cranberries ($Z_p = 0.48 \pm 0.04$). These results suggest that freeze-thawing, carried out before HHP, affected cranberry tissue structure and permeabilized their cell membranes. HHP did not further permeabilize cranberry cell membranes. These results are unlike earlier studies that have reported increase in cell permeabilization with applied pressure (Rastogi et al., 2000). If the cell-membrane permeabilization were the primary cause for infusion, HHP should not have caused additional infusion in cranberries that were already freeze-thaw ruptured without any further cell permeabilization.

Results of similar degree of permeabilization in all samples (HHP and control) and observed enhanced infusion under high pressure suggest that cell-membrane permeabilization is not the only cause for high pressure assisted infusion.
3.3.4.2. Pressure hold time

Preliminary results showed there was no difference in cell permeability of frozen-scarified-thawed cranberries before and after HHP when held at applied pressure for the maximum time the vessel was rated (60 min). Hence, a detailed study of the effect of pressure hold time on frozen-scarified-thawed cranberry cell permeability was not carried out. However, cell permeability of fresh cranberries changed with pressure hold time at a given pressure. The results on effect of HHP infusion in fresh cranberries are beyond the scope of this chapter. All results pertaining to fresh cranberries are discussed in the next chapter.

3.3.4.3. Pressure cycling

Figure 3.25 shows the variation of cell permeability of frozen-scarified-thawed cranberries after 1 cycle (10 min), 2 cycles (5 min each) and 5 cycles (2 min each) at 100 MPa. The total pressure hold time in all treatment samples was fixed to 10 min. The come-up time for each cycle was less than a minute (as shown previously in Figure 3.5). Pressure cycling caused no significant changes in the cell-membrane permeability compared to those not pressure cycled (0 cycles or not HHP treated). Interestingly, even though there was no change in cell-membrane permeability, the amount of quercetin infused increased with an increase in the number of pressure cycles as was shown in Figure 3.18. If cell-membrane permeabilization were the primary cause for enhanced infusion, for similar degree of permeabilization the amount infused should also be the same. This further substantiates our earlier hypothesis that pressure assisted infusion is more involved than cell-permeabilization based mechanisms. Enhanced infusion under
high pressure may be due to increase in driving force favoring accelerated transport under HHP. Further studies to elucidate possible mechanisms operating under high pressure are warranted.

![Figure 3.25 Zp as a function of number of pressure cycles at 100 MPa for 10 min in frozen-scarified-thawed cranberries. Same letters indicate no statistical significance at p<0.05](image)

### 3.3.4.4. Low pressure (5 MPa to 100 MPa)

While the amount infused was enhanced after pressure treatment when infused between 5 MPa and 100 MPa for 10 min at 22 °C compared to control (0.1 MPa, 10 min), there was no significant difference in cranberry cell permeability before and after pressure treatment. Average $Z_p$ value after HHP was 0.54 ± 0.02 and $Z_p$ of control was 0.51 ± 0.03 (Figure 3.26). Similar to the results from pressure treatment between 100 MPa and 551 MPa, these results also suggest that HHP did not further permeabilize cranberry cell membranes. These results are unlike earlier studies that have reported increase in cell permeabilization with applied pressure (Rastogi et al., 2000). If cell-membrane permeabilization were the primary cause for enhanced infusion, for similar degree of permeabilization, the amount infused should also be the same.
Figure 3.26 Changes in cell permeability of frozen-scarified-thawed cranberry tissue as a function of applied pressure (5 MPa to 100 MPa). Same letter on each bar indicates no statistical significance at p<0.05

### 3.3.4.5. Method of freezing: fast freezing and slow freezing

Figure 3.27 shows the variation of cell permeability of slow and fast frozen-scarified-thawed cranberries as a function of applied pressure. Consistent with the observations from SEM images shown in Figure 3.22, cell permeability of slow frozen-scarified-thawed cranberry tissue was similar before and after HHP and was dissimilar in fast frozen-scarified-thawed cranberry. Cell permeability before HHP of fast frozen-scarified-thawed cranberries ($Z_p = 0.21 \pm 0.05$) is lower than that of slow frozen-scarified-thawed cranberries ($Z_p = 0.44 \pm 0.11$). These results suggest that fast freezing permeabilized cell membranes to a smaller degree compared to slow freezing. The results are consistent with the common knowledge that fast freezing damages cell structure to a smaller extent due to the formation of smaller size ice crystals compared to slow or conventional freezing. Further, while comparing cell permeability before ($Z_p = 0.21 \pm$
0.05) and after HHP (Zp = 0.40 ± 0.06) of fast frozen-scarified-thawed cranberries, Zp increases after HHP. In slow frozen-scarified-thawed cranberries, Zp before (Zp = 0.44 ± 0.11) and after HHP (Zp = 0.48 ± 0.04) does not change with pressure.

![Infusion for 10 min](attachment:infusion.png)

**Figure 3.27** Variation of cell permeability in fast and slow frozen-scarified-thawed cranberries as function of applied pressure. Different letters indicate statistical significance at p<0.05.

### 3.3.5. Mapping distribution and loci of infused-quercetin in cranberry tissue

Investigating the distribution and loci of the infused molecule (quercetin) inside the cranberry tissue would enable us to gain insights into the possible mechanism of pressure assisted infusion. Preliminary SEM work showed that much of the infused quercetin was deposited in the intercellular spaces or apoplasts (**Figure 3.28**). Quercetin was mainly found in the apoplasts around the sub-epidermal cells close to the skin.
In order to understand the exact loci of the infused quercetin inside cranberry tissue, a fluorescent surrogate molecule was used to map their distribution using fluorescence microscopy. Fisetin was chosen as the fluorescent surrogate molecule of quercetin since their molecular sizes and partition coefficients were comparable. **Figure 3.29** shows the fluorescence microscopy images of fisetin infused across the cranberry tissue from the surface (near the skin) to the center of the berry at 0.1 MPa (1 atm) and at 551 MPa. The images clearly show increased fluorescence intensity (blue lines indicate fisetin infused) after HHP compared to their atmospheric pressure infused counterparts. There is also a gradient of fluorescence intensity observed from surface of fruit to the center. **Figure 3.30** shows a quantitative representation of the fluorescence intensity across the distance from fruit surface to center. The peak fluorescence intensity, representing the highest amount of fisetin infused, was observed at a distance of 2 mm from the surface. Fluorescence intensity at the surface was negligible since the cranberry surface was wiped clean of fisetin containing solution.
Figure 3.29 Fluorescent microscopy images of fisetin infused (a fluorescent surrogate of quercetin) across the cranberry tissue from the surface (near the skin) to the center of the berry at 1 atm and at 551 MPa.

Figure 3.30 Fluorescence intensity from fisetin infused in cranberry tissue
3.4. SUMMARY

Cranberry skin was almost impermeable to water and quercetin even under high pressure. Pretreatment of cranberry surface by partially scarifying the fruit skin was required to aid in the process of infusion. HHP treated cranberries showed a significant increase in the amount of quercetin infused over those treated at ambient conditions. Under high pressure, mass transfer rates were enhanced and processing time was shortened substantially. It was found that infusion into frozen-scarified-thawed cranberries was independent of the applied pressure (5 MPa to 551 MPa). Very low pressure (~ 5 MPa) was sufficient to cause enhanced infusion in frozen-scarified-thawed cranberries. Further, pressure cycling increased the amount of quercetin infused in frozen-scarified-thawed cranberries. The optimum conditions to achieve maximum infusion of quercetin into frozen-scarified-thawed cranberries were pressure of 100 MPa, pressure hold time of 10 min, two pressure cycles of 5 min each, infusate of 0.5 % quercetin (w/w) and 1:2 (w/w) ratio of fruit to infusate.

From a structural standpoint, the frozen-scarified-thawed cranberry cell-membrane was permeabilized by freeze-thawing prior to HHP processing. HHP had no additional effect on cell-membrane permeabilization in frozen-scarified-thawed cranberries. Results of similar degree of permeabilization in all samples (HHP and control) and observed enhanced infusion under high pressure suggest that cell-permeabilization may not be the only cause for high pressure induced infusion. Results from this study suggested that understanding of the mechanisms of high pressure assisted infusion is warranted. The next chapter is focused on gaining further insights into pressure assisted infusion mechanism.
CHAPTER 4

PRESSURE ENHANCED INFUSION OF QUERCETIN INTO CRANBERRIES

PART II: MECHANISTIC INSIGHTS

4.1. INTRODUCTION

In this chapter, the focus of the study was to gather further mechanistic insights into the transport mechanisms that enhance infusion of quercetin in cranberries under high pressure. Part I of this study, discussed in the previous chapter, showed evidence that HHP assisted infusion in cranberries was more involved than just permeabilization based mechanisms. The results suggested that transport mechanisms in addition to cell permeabilization might contribute to enhanced and accelerated infusion under pressure. In addition, the extent of quercetin infusion was independent of the applied pressure (5 MPa to 551 MPa). Pressure as low as 5 MPa was sufficient to enhance infusion in cranberries. Moreover, pressure cycling further increased the amount of quercetin infused in cranberries. These results demonstrate the potential of HHP assisted infusion process to be a low cost and commercially feasible process.

Other transport mechanisms that may contribute to enhanced infusion include pressure driven flow. It may be possible that higher pressure increases driving force contributed by pressure gradients in addition to reducing resistance (cell rupture) to infusion. Direct measurements of changes in pressure during HHP are not possible at the moment. Therefore, in order to test the role of multiple transport mechanisms, one needs to be able to decouple the individual effects of cell permeability and HHP. One approach
was to extend understanding from the frozen-scarified-thawed cranberry system (cells of which were already permeabilized during freeze-thaw process) to fresh-scarified cranberry system (cells of which are intact).

The goal of this work was to test if cell permeabilization is the only factor responsible for enhanced infusion under high pressure, in two systems: (i) fresh cranberries, cells of which are intact and (ii) frozen-thawed cranberries, cells of which are already permeabilized during freeze-thaw process before HHP. Frozen-thawed and fresh cranberries were used as substrate matrix to illustrate infusion mechanisms beyond cell-permeabilization.

4.2. MATERIALS AND METHODS

All materials and methods used in this study were the same as discussed in Chapter 3, section 3.2. A point to note is that all experiments in this study were carried out in the pilot scale pressure vessel described in Chapter 2, section 2.2.2.1. Although, bench top pressure vessel that operated at low pressure (<100 MPa) was sufficient to achieve enhanced infusion and that testing at low pressure would be more commercially meaningful, the vessel had limited process control and could accommodate only small sample sizes. Therefore, experiments were carried out with pilot scale pressure vessel that operated at high pressure.

The pressures treatments were carried out between 100 MPa and 551 MPa at 22 °C initial temperature for 1 min to 60 min, and with or without pressure cycling.
Determination of diffusion coefficient of quercetin in cranberries

In order to determine the diffusivity of quercetin in cranberries, a mass transport model is necessary. The first approach was to use Fick's diffusion model to estimate effective diffusion coefficients of quercetin in cranberries. We model our cranberry system as hollow spheres (Figure 4.1). In this model, a and b are the inner and outer radii of the cranberry. Even though cranberries are hollow ellipsoids, we restrict our model to that of a hollow sphere, as we are interested only in comparing diffusivity with and without the application of pressure. In this model, the assumptions made were:

(a) Cranberries are isotropic, i.e., concentration of the quercetin only varies radially.
(b) Initial concentration of quercetin was uniform throughout the cranberry and a constant ($C_0$).
(c) Concentration at the surface of the cranberry is a constant ($C_\infty$). Measurements of quercetin concentration in the solution before and after the experiment suggest that quercetin concentration remains constant throughout the process.
(d) At the boundary of the inner sphere (hollow sphere), we notice that quercetin precipitates at long times. We, therefore, assume that the flux at $r = a$ is zero.
(e) Cranberries in the solution are sufficiently far from each other that there is no quercetin depletion at parts of cranberry surface due to crowding of multiple cranberries.

Under these conditions, the mass transport into cranberries is governed by the
constitutive equation 4.1 where C is the concentration of the quercetin which varies with time t and radial distance r (a < r < b) and D is the diffusivity of the quercetin in cranberries at room temperature. In using this equation, based on Ficks law, diffusivity remains constant over all values of temperatures and pressures.

\[
\frac{\partial C(r,t)}{\partial t} = D \left[ \frac{1}{r^2} \frac{\partial}{\partial r} \left( r^2 \frac{\partial C(r,t)}{\partial r} \right) \right] \quad \text{……..(4.1)}
\]

where the boundary conditions used were

\[
\frac{\partial C}{\partial r} = 0 \text{ at } r = a, \ t > 0 \quad \text{…………………..(4.2)}
\]

\[
C = C_\infty \text{ at } r = b, \ t > 0 \quad \text{…………………..(4.3)}
\]

\[
C = C_0 \text{ at } t = 0, \ a \leq r \leq b \quad \text{…………………..(4.4)}
\]

On solving the constitutive equation, the concentration of quercetin in the hollow sphere can be expressed using equation 5.5.

\[
\frac{C - C_0}{C_\infty - C_0} = -2(b - a) \sum_{n=1}^{\infty} \frac{-1^n \sin(n\pi(n/r-a))}{n\pi} \frac{r-a}{b-a} \left[ 1 - \exp \left( -n^2\pi^2Dt \frac{r-a}{b-a} \right) \right] \quad \text{……..(4.5)}
\]

and the total amount of quercetin, which is obtained by integrating the concentration of quercetin over the volume of the sphere, is given by equation 5.6 as follows:

\[
\frac{M_t}{M_\infty} = \frac{6(b-a)^2}{\pi^3(a^2 + ab + b^2)} \sum_{n=1}^{\infty} \frac{1}{n^3} \exp \left( -n^2\pi^2Dt \frac{r-a}{b-a} \right) \quad \text{……………………………..(4.6)}
\]

where \( M_\infty = \frac{4\pi(b^3 - a^3)(C_\infty - C_0)}{3} \quad \text{……………………………..(4.7)}
\]

The experimentally obtained \( M_t \) at various times were fitted to equation 4.6 using Microsoft excel goal seek in order to compute the values of D before and after pressure.
4.3. RESULTS AND DISCUSSION

In this section, influence of two process variables - applied pressure and pressure hold-time on extent of infusion and structure of fresh-scarified and frozen-scarified-thawed cranberries, are discussed.

4.3.1 Influence of process parameters on extent of infusion: comparative study

Here the focus was on comparing the effects of applied pressure and pressure hold time on the extent of quercetin infusion in fresh-scarified cranberries and frozen-scarified-thawed cranberries.

4.3.1.1 Effect of applied pressure on fresh-scarified and frozen-scarified-thawed cranberries

On comparison of amount of quercetin infused in fresh-scarified and frozen-scarified-thawed cranberries, the results recorded were (a) infusion was enhanced under HHP (100 MPa – 551 MPa) in both fresh-scarified and frozen-scarified-thawed cranberries when compared to infusion at ambient conditions, and (b) infusion in fresh-scarified cranberries was 2 times higher than in frozen-scarified-thawed cranberries after HHP. Figure 4.2 shows the variation in the amount of quercetin infused with applied pressure for infusion time of 10 min. The initial amount of quercetin in fresh-scarified and frozen-scarified-thawed cranberries was found to be [0.51 ± 0.04] mg/g initial fruit wt. dry basis and [0.45 ± 0.02] mg/g initial fruit wt. dry basis, respectively.
Figure 4.2 Quercetin infused in cranberries as a function of pressure applied for 10 min. Different letter indicate statistical significance at p<0.05

Frozen-scarified-thawed cranberries showed three times higher levels of infusion after HHP ([5.44 ± 0.2] mg/g initial fruit wt. dry basis) compared to its control ([1.81 ± 0.15] mg/g initial fruit wt. dry basis) whereas fresh-scarified cranberries after HHP showed five times ([10.55 ± 0.57] mg/g initial fruit wt. dry basis) more than in its control ([1.78 ± 0.20] mg/g initial fruit wt. dry basis). Further, while comparing amount of quercetin infused in fresh-scarified and frozen-scarified-thawed cranberries after HHP, it was found that fresh-scarified cranberries had twice the amount infused than in frozen-scarified-thawed cranberries under similar conditions. Based on earlier reported mechanism, if cell-membrane rupture were the only cause for enhanced infusion (Rastogi et al., 2000), the amount infused in fresh-scarified and frozen-scarified-thawed cranberries should have been the same. The differences measured in the extent of infusion between the fresh-scarified and the frozen-scarified-thawed cranberries when studied with cell-permeability data help gain better understanding of the physics of HHP.
infusion. The combined interpretation of influence of HHP on extent of infusion and cell permeability is discussed in section 4.3.5.

Furthermore, the amount of quercetin infused was found to be independent of the applied pressure between 100 MPa and 551 MPa. This observation was unlike earlier studies, which have shown increase in diffusion with increase in applied pressure (Rastogi et al., 2000; Sopanangkul et al., 2002).

Polyphenol content, another indicator of the amount of quercetin infused, was measured before and after HHP in cranberries. These measurements showed a similar trend as seen in quercetin measurements. The results are represented as mg of gallic acid equivalents (GAE)/g of initial fruit dry weight. The initial polyphenolic content of frozen-scarified-thawed cranberries, prior to infusion was [11.07 ± 0.62] mg GAE/g initial dry wt and in fresh-scarified cranberries was [19.28 ± 0.57] mg GAE/g initial dry wt.

Polyphenols in HHP treated frozen-scarified-thawed cranberries ([16.7 ± 0.6] mg GAE/g initial fruit dry wt) was [51 ± 6.1] % higher than in control ([11.07 ± 0.62] mg GAE/g initial dry wt) as shown in Figure 4.3. Polyphenols in fresh-scarified cranberries showed [22 ± 6.4] % increase in infusion under high pressure ([24.9 ± 0.2] mg GAE/g initial fruit dry wt) compared to control infused at ambient conditions ([19.28 ± 0.57] mg GAE/g initial dry wt.).
Figure 4.3 Polyphenol levels in infused cranberries as a function of pressure applied for 10 min. Means with different number of stars (*) indicate statistical significance at p<0.05

The percent increase in polyphenols (22 % - 51 %) was lower than that observed in quercetin measurements (200 % - 500 %). This could be due to simultaneous transport of quercetin into and water soluble polyphenols out of cranberries. The diffusion of water soluble polyphenols out of cranberries can reduce the total polyphenols measured in cranberries. While the percent change (+22 %) in polyphenol content in fresh-scarified cranberries after HHP was lower than that in frozen-scarified-thawed cranberries (+51 %), the percent change in amount of quercetin infused after HHP in fresh-scarified cranberries was higher. This difference could be attributed to the limitation of the Folin-Ciocalteau assay that measures the total phenolic content of a sample. Therefore, phenol content measurement is only an indirect indicator of extent of infusion and is not an absolute measure of the quercetin infused and hence not comparable. Polyphenol levels of samples processed between 200 MPa and 551 MPa were not significantly different.
(p<0.05). This may be indicative of the pressure independent effect of amount infused (100 MPa - 551 MPa) as observed in quercetin infusion plots (Figure 4.2).

4.3.1.2 Effect of pressure hold time on fresh-scarified and frozen-scarified-thawed cranberries

The variation of quercetin concentration in fresh-scarified and frozen-scarified-thawed cranberries as a function of pressure hold time at 100 MPa and 0.1 MPa (control) are shown in Figure 4.4. Pressure condition of 100 MPa was chosen since it is the lowest pressure required to infuse similar amounts of quercetin as achieved at highest pressure of 551 MPa for the given test pressure vessel (amount infused was independent of the applied pressure between 100 MPa and 551 MPa). The quercetin content in the berries after HHP for both, fresh-scarified and frozen-scarified-thawed cranberries was always higher than in cranberries that were infused at ambient conditions (0.1 MPa). After HHP infusion, amount infused in fresh-scarified cranberries was higher than that in frozen-scarified-thawed cranberries. In both, fresh-scarified and frozen-scarified-thawed cranberries, the amount of quercetin infused increased with process time up to 5 min at 100 MPa. Holding pressure beyond 5 min had no significant effect on the amount of
quercetin infused. Furthermore, not only was infusion enhanced under pressure but was also accelerated. The amount of quercetin that could be infused in 10 min under high pressure needed at least 24 h at ambient conditions in fresh-scarified cranberries and at least 3 h at ambient conditions in frozen-scarified-thawed cranberries. These results suggest that process time can be considerably shortened during HHP assisted infusion, which may make the process economically viable.

4.3.2 Influence of HHP assisted infusion on cranberry microstructure: comparative study

In order to compare changes taking place after HHP in the two cranberry systems, one where freeze-thawing had partially damaged the cell structure (frozen-scarified-thawed cranberries) and another where cells were intact (fresh-scarified cranberries), light microscopy and scanning electron microscopy was carried out on pressurized and unpressurized cranberries.

Light microscopy

Figures 4.5a and 4.5b show frozen-scarified-thawed cranberry flesh structure before and after HHP. On qualitative evaluation, no significant differences were observed in their structure before and after HHP as explained in the previous chapter (Chapter 3, section 3.3.3). Figures 4.5c, 4.5d and 4.5e show fresh-scarified cranberry flesh cell structure before and after HHP.
Fresh unprocessed cranberries (0.1 MPa or 1 atm) had an intact cell structure compared to high pressure treated cranberries. HHP at 100 MPa and 551 MPa showed cell membrane disruption. These observations will be correlated to data from cell permeability measurements discussed in the following section. Qualitatively, tissue treated at 551 MPa appeared more permeabilized than tissue treated at 100 MPa. The dark black spots seen in pictures of Figures 4.5d and 4.5e that are close to the edges of a cell represent infused quercetin. Qualitative observations of loci of quercetin in cranberry tissue are also in agreement with results from mapping of quercetin distribution as discussed in the previous chapter (Chapter 3, section 3.3.5).
Scanning electron microscopy

![SEM images](image)

Figure 4.6 SEM microstructure of frozen-scarified-thawed (a & b) fresh-scarified (c, d & e) cranberry tissue before and after HHP

Scanning electron microscopy (SEM) images were also used to evaluate changes in fresh-scarified and frozen-scarified-thawed cranberry microstructure of pressurized and unpressurized cranberries. Figures 4.6a and 4.6b show the cell structure of frozen-scarified-thawed cranberry parenchymatic tissue before (at 0.1 MPa or 1 atm) and after HHP (100 MPa), respectively. Cells of frozen-scarified-thawed cranberries appear to be ruptured with no significantly observable differences, before and after HHP. Figures 4.6c, 4.6d and 4.6e show the cell structure of fresh-scarified cranberry parenchymatic tissue before (0.1 MPa) and after HHP (100 MPa and 551 MPa). Cranberry cell structure was seen as intact and organized before HHP (at 0.1 MPa) as in most fresh fruits. The berry cells, however, appeared partially ruptured after HHP at 100 MPa and 551 MPa. Cells after 551 MPa treatment looked more disintegrated than after 100 MPa treatment. Moreover, comparing images of frozen-scarified-thawed and fresh-scarified cranberry
tissue after HHP (Figures 4.6b, 4.6d and 4.6e), the cranberry cells appear disintegrated with no noticeable differences. The results suggest that in frozen-scarified-thawed system, HHP did not significantly affect the cell structure. However, in fresh-scarified cranberry system, HHP caused noticeable damage to the cells. These results are consistent with the cell permeability measurements discussed in the following section.

4.3.3 Influence of process parameters on cell permeability of cranberry tissue: comparative study

In order to gain quantitative information on degree of cell-membrane rupture, also called as cell permeability, impedance spectroscopy technique was used to measure cell permeability.

4.3.3.1 Effect of applied pressure on fresh-scarified and frozen-scarified-thawed cranberries

Variation in cranberry cell-permeability (expressed as $Z_p$) as a function of applied pressure is shown in Figure 4.7. Before HHP (at 0.1 MPa or 1 atm), fresh-scarified cranberries showed low $Z_p$ value of $0.28 \pm 0.02$ indicating that their cell membranes were relatively intact. After HHP (200 – 551 MPa) of fresh-scarified cranberries, $Z_p$ further increased ($Z_p = 0.49 \pm 0.01$) indicating that more cell membranes were permeabilized. This result suggests that HHP promotes cell permeabilization in fresh-scarified cranberry tissue (cells of which were intact to begin with) similar to what was observed in the images from SEM and light microscopy. In case of frozen-scarified-thawed cranberries, however, cell permeability before HHP ($Z_p = 0.48 \pm 0.01$) and after HHP ($Z_p = 0.48 \pm$
0.04) was similar. While $Z_p$ of frozen-scarified-thawed cranberries before and after HHP was similar they were also comparable to $Z_p$ of fresh-scarified cranberries after HHP. These results suggest that HHP had no additional effect on cell permeability in frozen-scarified-thawed cranberries (cells of which were already permeabilized to begin with). Results of similar degree of permeabilization in frozen-scarified-thawed samples before and after HHP and enhanced infusion under high pressure suggest that cell-membrane permeabilization may not be the only cause for high pressure assisted infusion.

![Graph](image)

**Figure 4.7** Variation in cranberry cell-permeability (expressed as $Z_p$) as a function of applied pressure

**4.3.3.2 Effect of pressure hold time on fresh-scarified and frozen-scarified-thawed cranberries**

**Figure 4.8** shows the change in cranberry cell permeability with infusion time. At 100 MPa, $Z_p$ of fresh-scarified cranberries did not change between 5 min and 60 min infusion time. This indicates that cranberry cells permeabilize either during pressurization or during depressurization but not during pressure hold time.
Cranberry cell permeability is independent of pressure hold time up to 60 min at 100 MPa. However, cell-permeability of unpressurized cranberries (0.1 MPa or 1 atm) showed a gradual increase with increase in infusion time. This increase in permeability at 1 atm could be attributed pectinase enzyme, pectin methyl esterase (PME). PME could be released during scarification process and can result in tissue softening through de-esterification of pectin (Basak and Ramaswamy, 1998).

Furthermore, cell permeability ($Z_p$) of HHP cranberries is always higher than that of unpressurized (1 atm) cranberries. These results indicate much of the change in cell permeability of HHP cranberries was caused due to cell rupture under pressure and due to enzyme influenced tissue softening in cranberries at 1 atm.
4.3.4 Comparing effect of freeze-thawing and HHP as a pre-treatment on extent of quercetin infusion at atmospheric pressure

As discussed in the previous section 4.3.3.1 on the effect of applied pressure on cell permeability, the results suggested that freeze-thawing permeabilized cranberry cells to a similar extent, as did HHP. If cell-permeability of frozen-scarified-thawed unprocessed cranberries was similar to that of HHP treated fresh-scarified cranberries, the amount infused in these HHP treated fresh-scarified cranberries should also be similar to the amount infused in frozen-scarified-thawed berries. This hypothesis was tested by comparison of amount of quercetin infused as a function of infusion time in two systems: (A) frozen-scarified-thawed unpressurized cranberries infused at 0.1 MPa, and (B) HHP treated (at 100 MPa) fresh-scarified cranberries infused at 0.1 MPa. In system A, quercetin was infused at 0.1 MPa in cranberries whose cells were permeabilized by freeze-thawing. In system B, quercetin was infused at 0.1 MPa in cranberries whose cells were permeabilized by HHP. Infusion was carried out at ambient conditions (0.1 MPa and 22 °C). Figure 4.9 shows that quercetin infused at 0.1 MPa in frozen-scarified-thawed cranberries (not pressure processed) and HHP (100 MPa) pre-treated fresh-scarified cranberries was comparable.
The results support our hypothesis that, if cell rupture caused by freeze-thawing is similar to the cell rupture caused by HHP pre-treatment in fresh-scarified cranberries, the amount infused in them at 0.1 MPa must be comparable. The results further substantiate that freeze-thawing and HHP treatment produce similar levels of cell permeability in cranberries.

4.3.5 Combined interpretation of results from effect of HHP on extent of infusion and cell permeability

What does it mean to have higher levels of infusion in fresh-scarified cranberries compared to frozen-scarified-thawed cranberries, while their cell permeability is comparable? The amount infused in fresh-scarified cranberries was about 2 times higher than in frozen-scarified-thawed cranberries (Figure 4.10a). Cell permeability of both, fresh-scarified and frozen-scarified-thawed cranberries was comparable after HHP
The difference in the two systems was seen in their cell permeability before HHP. Fresh-scarified cranberries had lower cell permeability (intact cells) before HHP that were permeabilized during HHP. On the other hand, frozen-scarified-thawed cranberries had pre-permeabilized cells (due to freeze-thawing) that did not further permeabilize after HHP. These results suggest that cell permeability is not the only factor that contributed to enhanced infusion under pressure. If cell permeabilization were the only cause, the amount infused in fresh-scarified and frozen-scarified-thawed cranberries should have been comparable, which, however, was not observed. Therefore, pressure enhanced transport may be more involved than just permeabilization based mechanisms.

**Figure 4.10** Comparison of extent of infusion (a) and cell permeability (b) in fresh-scarified and frozen-scarified-thawed cranberries. * and ** indicate statistical significance at p<0.05

Multiple transport mechanisms may be operative during HHP. They can be classified as mechanisms based on cell permeabilization and those based on pressure driven mass transport. In fresh-scarified cranberries where the cells are intact, HHP not only assists in cell rupture but also contributes to concurrent (concomitant) mass transport. In frozen-scarified-thawed cranberries, cells of which are already ruptured...
during the freeze-thawing process, HHP does not further rupture the cells (Figure 4.10b) and it only contributes to mass transport. In other words, during HHP treatment of frozen-scarified-thawed cranberries, the two processes, cell rupture and mass transport occur sequentially before and after HHP, respectively. In fresh-scarified cranberries, they occur concurrently. Hence, amount infused in frozen-scarified-thawed cranberries is lower than that infused in fresh-scarified cranberries. Therefore, infusion in frozen-scarified-thawed cranberries signifies transport based on pressure driven mass transport and not on cell permeabilization. The amount attributed to pressure driven transport is 60 % of the total amount infused in fresh-scarified cranberries. The remaining 40 % can be attributed to transport based on in-situ cell permeabilization.

4.3.6 Concurrent versus sequential cell rupture and mass transport

In the previous section, the result showed that pressure enhanced infusion was more involved than just permeabilization mechanisms. In order to gather further insights, extent of infusion in two systems were tested: concurrent system: fresh-scarified cranberries were infused under pressure of 100 MPa (the process of cell rupture and mass transport happens concurrently inside the pressure vessel) and, sequential system: fresh-scarified cranberries were pre-treated at 100 MPa and then infused under pressure of 100 MPa (the process of cell rupture due to pre-treatment at 100 MPa followed by mass transport due to infusion at 100 MPa). Figure 4.11 shows the comparison in terms of the extent of infusion in these two systems of study.
It was observed that cranberries from concurrent system showed higher infusion compared to cranberries from sequential system. These results suggest that concurrent mechanisms of cell rupture and transport resulted in higher amount of infusion compared to sequential cell rupture and transport. Therefore, pressure-enhanced infusion process is much more involved than previously postulated permeabilization-based mechanisms and is perhaps caused by both pressure-driven mass transport and cell-permeabilization assisted mass transport. At this point, it is not clear what could be other possible mechanisms that may cause enhanced infusion under high pressure. It may be due to increase in driving force such as pressure gradient favoring accelerated transport under HHP. One of the hypothesized mechanisms in addition to diffusion under pressure is pressure driven convective flow (Darcy flow) commonly observed in porous materials. The Darcy law relates linearly the flow velocity and pressure gradient across the porous medium. Cranberry tissue can be treated as a porous medium consisting of dispersed cells separated by connective voids, which allow for flow of quercetin and other nutrients. The
driving force in Darcy flow is pressure gradient (Khaled and Vafai, 2003). Small pressure gradients that may be created across the fruit during pressurization cycle of HHP could be causing enhanced infusion of quercetin into cranberries. Further experiments to test for pressure driven flow mechanisms operating under high pressure are warranted.

4.3.7 Preliminary model development

Estimate of quercetin infused using a physical model

In order to estimate the amount of quercetin infused, a physical model was used. Based on the results from light microscopy and SEM, quercetin infusion occurred through the scarified pinholes into the inter-cellular spaces inside cranberry tissue (parenchymal cells). A schematic of this cranberry model is shown in Figure 4.12. Therefore, knowledge of the volume of scarified pinholes and volume of intercellular spaces per gram of berry will help approximate the amount of quercetin infused.
In the physical model, total quercetin infused is sum of quercetin initially present before infusion, quercetin in scarified pinholes, quercetin in intercellular spaces and the quercetin in the permeabilized cells (Equation 4.8).

\[
Q_{\text{total}} = Q_{\text{initial}} + Q_{\text{pinhole}} + Q_{\text{intercellular}} + Q_{\text{permeabilized}}
\]

(4.8)

The initial amount of quercetin \(Q_{\text{initial}}\) was measured experimentally and found to be 60 \(\mu g/g\) of fruit. Each of the scarified pin-hole was approximated as a cylinder. The dimensions of each pin-hole was 2 mm in height and 0.5 mm in diameter. Therefore, the total volume of the scarified pinholes/g of cranberry is a product of number of pin-holes and volume of each pin-hole. To compute the total number of pin-holes/g of fruit an indirect measurement of impedance spectroscopy technique was used. Details of the measurements are discussed in the following paragraph. The intercellular space volume was computed based on the knowledge that up to 20 % of the total volume of fruit could be occupied by intercellular spaces (Lewicki and Lenart, 2007). Details of the measurements are discussed in the following paragraph.

To compute the number of pin holes/g of fruit was computed change in cell permeability \(Z_p\) in fresh-scarified cranberries was used as an indirect measure of pin-hole scarification. Change in cell permeability was using impedance spectroscopy technique. The hypothesis for this experiment was that during scarification the cranberry skin was punctured to make pin-holes that will be reflected as an increase in cell permeability (increase in electrical conductivity). Therefore, a change in electrical conductivity (represented as \(Z_p\)) can be positively correlated to a proportional change in degree of scarification. From the percentage degree of scarification \(Z_p\) for a given
surface area of cranberry and known size of pin hole, the number of pin holes can be estimated. Figure 4.13 shows variation of $Z_p$ with the number of passes through the scarifier bed.

There was a strong positive linear correlation ($R^2 = 0.90$) observed between $Z_p$ and the number of passes through the scarifier bed. According to Schwan (1963), the $\beta$-dispersion theory explains that $Z_p$ of 0.15 corresponding to six passes through the scarifier signifies that 15% of the cranberry surface area has punctured holes. The total cranberry surface area ($S.A._{\text{cranberry}}$) ranged between 860 to 1410 sq. mm assuming cranberry to be an ellipsoid. The area of each punctured hole ($A_{\text{hole}}$) was about 0.19 sq. mm, which was calculated using the pin diameter of 0.5 mm. Thus, 15% scarified surface corresponds to 650 to 1050 number of pin holes ($N$) made during scarification using Equation 4.9.
\[ N = \frac{(S \cdot A.)_{cranberry} \cdot Zp}{A_{hole}} \]  
..........................(4.9)

So, the total volume of the scarified pin holes cylinder was about 255 mm\(^3\) to 392 mm\(^3\) per berry (1 berry \(\sim\) 2 g) given by Equation 4.10.

\[ V_{pinhole} = N \times \pi r_{pinhole}^2 h_{pinhole} \]  
...............(4.10)

Therefore, the amount of quercetin infused in the scarified pinholes/g of cranberry ranged between 65 \(\mu\)g and 100 \(\mu\)g given by Equation 4.11. The concentration of quercetin in infusant (\(\text{Conc}_{\text{infusate}}\)) was 0.5 % w/w.

\[ \text{Quercetin}_{\text{pinhole} / \text{gofcranberry}} = \frac{\text{Conc}_{\text{infusate}} \times V_{\text{pinhole} / \text{berry}}}{\text{Weight}_{\text{cranberry}}} \]  
...............(4.11)

To estimate the amount of quercetin infused in the intercellular spaces/g of cranberry, we first compute the volume of the intercellular space as a percentage of the total volume of a berry. According to Lewicki and Lenart (2007), up to 20 % of the total volume of the fruit can be occupied by intercellular spaces. Hence, volume of intercellular space of one berry was approximately 85 mm\(^3\) assuming cranberry as an ellipsoid of 9 mm x 14 mm radii. The amount of quercetin infused in the intercellular spaces/g of cranberry was about 211 \(\mu\)g /g of fruit given by Equation 4.12. This is assuming 100 % of the intercellular space was filled with infusate. If this were true, there will be no spatial gradient across the radii of the cranberry. However, section 3.3.5 on fluorescence microscopy in Chapter 3 showed that gradient of the infusing molecule is present.

\[ \text{Quercetin}_{\text{intercellular} / \text{gofcranberry}} = \frac{\text{Conc}_{\text{infusate}} \times V_{\text{intercellular} \text{berry}}}{\text{Weight}_{\text{cranberry}}} \]  
...............(4.12)
Assuming only 50 % of the intercellular spaces were filled with infusate, the amount of quercetin/g of fruit was estimated as 105 µg.

The quercetin infused in the permeabilized cells was estimated just as discussed for that in intercellular spaces. Here, the cell permeability (Zp) of the permeabilized cranberry parenchymal cells is 35 % (measured using impedance spectroscopy). Therefore, the amount of quercetin infused in permeabilized cells/g of fruit was 278 µg.

Using Equation 4.8, the total quercetin infused was about 523 µg/g fruit (60 µg +80 µg + 105 µg +278 µg). This estimate does not match the measure value of 800 µg of quercetin/g of frozen-scarified-thawed fruit. The explanation for the difference between the model estimates and the experimental values is that potentially quercetin precipitates inside the cranberry tissue. This precipitation accounts for the higher measured quercetin.

**Diffusion coefficient calculations**

Based on Fick’s diffusion model, diffusivity \( (D_{\text{eff}}) \) of quercetin in cranberries was computed before and after HHP using Eqn 4.6. In order, to compute diffusivity values, higher order terms in Equation 4.6 were neglected and made linear as shown in Equation 4.13. Higher order terms had less than < 5% influence on the \( D_{\text{eff}} \) computed.

\[
-\ln(1 - \frac{M_t}{M_\infty}) = \ln\left(\frac{6(b-a)^2}{\Pi^2(a^2 + ab + b^2)}\right) + \left\{\frac{D\Pi^2}{(b-a)^2}\right\}t \quad \ldots \ldots \ldots (4.13)
\]

The slope of plot of \(-\ln \left(1-(M_t/M_\infty)\right)\) and time (Figure 4.14) was used to compute \( D_{\text{eff}} \) values. The \( M_\infty \) value was chosen as the maximum amount infused we had observed in cranberries of 17.43 mg quercetin/g of fruit wt dry basis. Quercetin infused in cranberries after 2 days at 1 atm was found to be 17.51 mg/g of fruit wt dry basis and
after 5 days was 17.37 mg/g of fruit wt dry basis. Diffusivity (D_{eff}) of quercetin in cranberries after HHP (100 MPa) was higher than before HHP (1 atm) as shown in Table 4.1. These results were consistent with earlier published literature that diffusivity increases after application of HHP (Rastogi et al., 2000; Sopanangkul et al., 2002). Among HHP treated cranberries, diffusivity of frozen-scarified-thawed berries was higher than that of fresh-scarified cranberries.

Table 4.1 Diffusivity of quercetin in cranberries

<table>
<thead>
<tr>
<th></th>
<th>D_{eff} fresh-scarified cranberries (10^{-10} m^2/s)</th>
<th>D_{eff} frozen-scarified-thawed cranberries (10^{-10} m^2/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 MPa</td>
<td>0.593 ± 0.107</td>
<td>0.923 ± 0.47</td>
</tr>
<tr>
<td>100 MPa</td>
<td>5.56 ± 4.51</td>
<td>1.62 ± 0.10</td>
</tr>
</tbody>
</table>

However, plot of –Ln (1-(M_t/M_0)) and time for fresh-scarified and frozen-scarified-thawed cranberries after HHP, was a very poor fit to the present model (R^2 < 0.5). If the model were a good fit, we would expect to see a strong linear relation between –Ln (1-(M_t/M_0)) and time as seen with cranberries infused at 0.1 MPa.
Fresh cranberries (1 atm)

\[ y = 3 \times 10^{-5}x + 0.1188 \]
\[ R^2 = 0.98262 \]

\[ \text{Slope} = \frac{D \pi^2}{(b - a)^2} \]
\[ D = 0.593 \times 10^{-10} \text{ m}^2 / \text{s} \]

Frozen-thawed cranberries (1 atm)

\[ y = 4 \times 10^{-5}x + 0.1553 \]
\[ R^2 = 0.9342 \]

\[ \text{Slope} = \frac{D \pi^2}{(b - a)^2} \]
\[ D = 0.923 \times 10^{-10} \text{ m}^2 / \text{s} \]
Figure 4.14 Plot of $-\ln \left(1 - \left(\frac{M_t}{M_\infty}\right)\right)$ and time for fresh-scarified and frozen-scarified-thawed cranberries before and after HHP.
These results suggest that diffusion model does not represent the physical mass transport mechanism occurring during HHP. This exercise further illustrated that cell-permeabilization based diffusion may not be the only mechanism driving enhanced infusion during HHP. Further studies to investigate other phenomenological models and transport models need to be explored.

4.4. SUMMARY

HHP enhanced and accelerated the infusion of quercetin in fresh-scarified and frozen-scarified-thawed cranberries compared to infusion carried out under ambient conditions. In fresh-scarified cranberries where cells were intact initially, HHP permeabilized their cell membranes. However, in frozen-scarified-thawed cranberries where cells were permeabilized by freeze-thawing, HHP caused no further permeabilization. The cell permeability of fresh-scarified and frozen-scarified-thawed cranberries after HHP was comparable. These results summarized as (a) comparable degree of permeabilization after HHP, (b) no additional permeabilization in an already permeabilized system, and (c) observed enhanced infusion under high pressure, strongly suggest that cell-permeabilization may not be the only cause for HHP assisted infusion. Moreover, infusion was always higher in fresh-scarified cranberries compared to in frozen-scarified-thawed cranberries. This suggested that pressure enhanced infusion is more involved than just cell permeabilization based mechanisms. Concurrent action of cell rupture and transport, as demonstrated in fresh-scarified cranberries, resulted in greater extent of infusion than the sequential action of cell rupture and transport, as demonstrated in frozen-scarified-thawed cranberries. Comparison of the extent of
infusion in fresh-scarified and frozen-scarified-thawed cranberries in light of their cell permeability, suggested that 60% of infusion could be attributed to pressure driven transport and remaining 40% was caused due to cell permeabilization based mechanisms. Preliminary model development showed that diffusion models are not a good fit and do not explain the transport mechanisms occurring during HHP. Further studies to investigate these transport mechanisms operating during high pressure assisted infusion are needed.
CHAPTER 5

CONCLUSIONS AND POTENTIAL APPLICATIONS OF THIS RESEARCH

5.1 CONCLUSIONS FROM PRELIMINARY STUDIES

The results from the exploratory studies provided evidence that infusion is enhanced under high pressure in different kinds of fruits and vegetables. Soft and hard fruits such as grapes, apples and cranberries, and hard vegetables such as carrots showed promise wherein infusion was enhanced after HHP. In addition, small size molecules from 300 Da to large size proteins up to 30 kDa, could be infused into fruit and vegetable matrices during HHP. Further, water soluble and water insoluble molecules could be infused during HHP by selecting appropriate infusate medium. The understanding from the preliminary study helped provide a proof of concept for enhanced infusion using HHP. This study was followed by a detailed study on HHP infusion in cranberries that was focused on process optimization and understanding the transport mechanisms driving infusion during HHP.

5.2. CONCLUSIONS FROM PRESSURE ENHANCED INFUSION IN CRANBERRIES

This study demonstrated that HHP processing can enhance infusion of water-insoluble bioactive quercetin molecules in frozen-scarified-thawed and fresh-scarified cranberries using ethyl alcohol and glycerin mixture as infusate medium. HHP processing also permeabilized cell-membranes of fresh cranberries. However, unlike earlier studies, we observed that in an already permeabilized system, such as in frozen-scarified-thawed
cranberries, HHP did not further permeabilize their cell membranes. Additionally, concurrent rupture of cell-membrane and quercetin transport into cranberry tissue showed higher amount of quercetin infused compared to sequential cell rupture followed by quercetin transport. These results suggested that HHP assisted infusion is more involved than just permeabilization based mechanisms. This research showed evidence that transport mechanisms in addition to those based on cell-permeability caused enhanced infusion of quercetin in cranberries. The specific conclusions from this study are summarized as follows.

**Effect of HHP on extent of infusion in frozen-scarified-thawed and fresh-scarified cranberries**

- Cranberry skin was almost impermeable to water and quercetin even under high pressure.
- Pretreatment of cranberry surface by partially scarifying the fruit skin was required to aid in the process of infusion.
- Compared to vacuum infusion and osmotic diffusion, the traditional infusion methods presently used in the food industry, HHP processing resulted in about two times increase in quercetin infused. Thus, HHP processing showed promise as an alternative mass transport process.
- In fresh-scarified and frozen-scarified-thawed cranberries, HHP processing enhanced and accelerated infusion of quercetin compared to infusion carried out under ambient conditions. HHP treated cranberries showed about 5 times higher infusion in fresh cranberries and about 3 times higher infusion in frozen-thawed...
cranberries over those treated at ambient conditions. Amount of quercetin infused in frozen-scarified-thawed cranberries after HHP was \([5.44 \pm 0.2]\) mg/g initial fruit wt. dry basis and \([10.55 \pm 0.57]\) mg/g initial fruit wt. dry basis in fresh-scarified cranberries.

- Under high pressure, mass transfer rates were accelerated and processing time was shortened substantially. In frozen-scarified-thawed cranberries, amount infused in only 10 min at 100 MPa took 3-6 h at atmospheric pressure. In fresh-scarified cranberries amount infused in only 10 min at 100 MPa took 24 h at atmospheric pressure. It was found that infusion in cranberries was independent of the applied pressure in the range 5 MPa to 551 MPa.

- Furthermore, very low pressure (~ 5 MPa) was sufficient to cause enhanced infusion in frozen-scarified-thawed cranberries. This was, however, a preliminary trial and optimization of infusion in the low pressure range (5 MPa to 100 MPa) requires further experimentation.

- Pressure cycling of 2 and 5 cycles (at 100 MPa) increased the amount of quercetin infused by two to three times compared a single cycle of pressure treatment.

- Therefore, from our experiments the optimum conditions to achieve enhanced infusion of quercetin into frozen-scarified-thawed cranberries were pressure of 100 MPa, pressure hold time of 10 min, two pressure cycles of 5 min each, infusate of 0.5 % quercetin (w/w) and 1:2 (w/w) ratio of fruit to infusate.

Through this research, we demonstrated that HHP processing could significantly enhance and accelerate infusion of quercetin in frozen-scarified-thawed cranberries and fresh-scarified cranberries. Low pressures (5 MPa to 100 MPa) and short times of
processing (5 min to 10 min) were sufficient to enhance infusion in the selected cranberry-quercetin system.

**Effect of HHP on cell permeability of frozen-scarified-thawed and fresh-scarified cranberries**

In order to understand the mechanism of HHP infusion and test if the current understanding that cell permeabilization is the primary cause for enhanced infusion under high pressure two cranberry systems were used in this study. Fresh and frozen-thawed cranberries of different cell permeabilities were used as test systems. Cell membranes of frozen-thawed cranberries were permeabilized by freeze-thawing prior to HHP processing. HHP had no additional effect on cell-membrane permeabilization in frozen-scarified-thawed cranberries.

In fresh-scarified cranberries where cells were intact before HHP, HHP processing permeabilized their cell membranes. However, in frozen-scarified-thawed cranberries where internal cells were permeabilized by freeze-thawing before HHP, HHP processing caused no further permeabilization. The cell permeability of fresh-scarified and frozen-scarified-thawed cranberries after HHP was almost the same.

Three key results of (a) comparable degree of cell-permeabilization after HHP, (b) no additional cell-permeabilization in an already permeabilized system, and (c) observed enhanced infusion under high pressure, strongly suggest that cell-permeabilization may not be the only cause for HHP assisted infusion in cranberries. Moreover, infusion was always higher in fresh-scarified cranberries compared to in frozen-scarified-thawed cranberries. This suggested that pressure enhanced infusion is more involved than just
cell permeabilization based mechanisms. Concurrent action of cell rupture and transport, as demonstrated in fresh-scarified cranberries, resulted in greater extent of infusion than the sequential action of cell rupture and transport, as demonstrated in frozen-scarified-thawed cranberries. Comparison of the extent of infusion in fresh-scarified and frozen-scarified-thawed cranberries in light of their cell permeability, suggested that 60 % of infusion could be attributed to pressure driven transport and remaining 40 % was caused due to cell permeabilization based mechanisms. Further studies to investigate these transport mechanisms operating during high pressure assisted infusion are warranted.

At this point it is not clear what could be other possible mechanisms that can cause enhanced infusion under high pressure. It is hypothesized that in addition to cell-membrane permeabilization enhanced infusion may occur due to pressure-driven flow. This could occur due to a transient pressure gradient between the outside and inside of the fruit setting up a convective flow. The possibility of transient pressure gradients could occur due to in-homogeneity of fruit tissue or during pressurization. One of the hypothesized mechanisms in addition to diffusion under pressure is pressure driven convective flow (Darcy flow) commonly observed in porous materials. The Darcy law relates linearly the flow velocity and pressure gradient across the porous medium. Cranberry tissue can be treated as a porous medium consisting of dispersed cells separated by connective voids, which allow for flow of quercetin and other nutrients. The driving force in Darcy flow is pressure gradient. Small pressure gradients that may be created across the fruit during pressurization or depressurization cycle of HHP could be causing enhanced infusion of quercetin into cranberries.
Further studies to investigate other mechanisms operating during high pressure induced infusion are needed. This work elucidates important aspects of the science of pressure-enhanced infusion. In addition, this works demonstrates the potential of HHP to develop nutrient-enriched food products. Since HHP processing results in minimal thermal abuse of the product, it will maintain the stability of the antioxidants and deliver a high quality product. The measurable impacts resulting from the proposed research will be the development of a shelf-stable high-quality product, whose antioxidant/nutraceutical content will be significantly higher than in its natural form. Moreover, the HHP treatment will also substantially reduce the process time, making the overall infusion process economically viable.

5.3 POTENTIAL APPLICATION OF HHP INFUSED QUERCETIN ENRICHED CRANBERRIES

A measurable impact resulting from the current research will be the development of a shelf-stable high-quality product, whose antioxidant/nutraceutical content will be significantly higher than in its natural form. Quercetin infused cranberries could be dried to make products similar to osmotically dehydrated fruits available in the market, such as, Ocean Spray’s Craisins®. Since HHP processing results in minimal thermal abuse of the product, it will maintain the stability of quercetin and deliver a high quality product. Moreover, HHP treatment can also substantially reduce the process time, making the overall infusion process economically viable.

Assuming amount of quercetin infused is retained after drying, dried-infused cranberries can be labeled as “good” source of quercetin that satisfies 15 % of daily value
of quercetin based on a 1 oz serving size. Serving size of 1 oz was selected based on
industry accepted serving size for osmotically dehydrated fruits that recommends 1-2 oz.

Preliminary results from drying of quercetin-infused cranberries using forced air
convection drying, vacuum drying and microwave assisted air drying showed negligible
loss of infused-quercetin after drying. Quercetin infused in cranberries was found to be
thermally stable up to 90 °C over extended drying times (up to 18 h) (Mahadevan et al.,
2010).

Quercetin in HHP infused cranberries (100 MPa, 10 min, 22 °C) was about 800
ppm (wet basis). Therefore, in a 1 oz (28 g) serving size of dried cranberries (~ 15% moisture) the amount of quercetin present is about 75 mg. Based on a RDI of 500 mg/ 70 kg body weight, consuming 1 oz serving size dried-infused cranberries can meet 15 %
daily value of quercetin. These estimates show promise for developing quercetin infused
cranberries as nutrient enriched dried fruit products.
CHAPTER 6
FUTURE WORK

The results from the present study on HHP infusion in cranberries showed that multiple transport mechanisms are involved in enhancing infusion under high pressure. Comparison of infusion in cranberries with different degrees of cell permeability (fresh and frozen-thawed) showed that pressure-enhanced infusion process is much more involved than previously postulated permeabilization-based mechanisms and is perhaps caused by concomitant pressure-enhanced mass transport and cell-permeabilization assisted mass transport. It may be possible that higher pressure increases driving force in addition to reducing resistance to infusion through cell rupture. Increased driving force for infusion during HHP can be explained by possible changes in pressure gradients under high pressure. These pressure gradients may occur during the pressure build up or pressure release phases of a HHP cycle. Direct measurements of changes in pressure gradients during HHP are not possible at the moment. Therefore, in order to test the role of multiple transport mechanisms, it is important to understand if infusion under pressure occurs during pressurization or depressurization. Pressure hold time is not of keen importance since this research showed that HHP infusion in cranberries was independent of pressure hold time between 5 min and 60 min.

One approach to decipher if infusion occurred during pressurization or depressurization would be through real-time tracking of extent of infusion. A test-system of fluorescent surrogate of quercetin, such as fisetin and cranberry can be used as infusant-substrate system. Physical properties of fisetin are comparable to that of quercetin (solubility, molecular size and partition coefficient). Cranberries can be infused
with fisetin in a pressure vessel with a fiber-optic window. Fluorescence spectrophotometer can record fluorescence intensity of the infusing medium containing fisetin. Reduction in fluorescence intensity of the infusing medium can be indicative of increase in extent of infusion. Real-time recording of fluorescence data will indicate whether infusion was dominant during pressure build up or during pressure release.

Effect of change in the rate of pressurization and depressurization on extent of infusion in cranberries could be used to test whether pressurization or depressurization is the dominant phase in which majority of the infusion occurs. Rate of pressurization or depressurization can be controlled by changing the extent of valve opening of the intensifier pump pressurizing the vessel or specially designed equipment. Knowledge of whether pressurization or depressurization contributes to enhanced infusion will help understand physics of quercetin transport under pressure. This knowledge will also help build accurate mathematical models to predict extent of infusion in a given system.

Preliminary model building, discussed in the previous chapter, was inadequate for use as a predictive model. A more rigorous model development exercise is required based on physics of the infusion process. If pressure driven flow is found to be the dominant mechanism, transport models based on Darcy’s flow may be tested.

Among other studies, the next steps include testing HHP infusion in other fruit and infusing molecules with different solubility and optimization of the process. For example, evaluating influence of pressure-assisted-infusion of vitamin C and calcium into apple wedges and baby carrots on their color and texture. Infusion of vitamin C and calcium will not only improve nutritional value but also greatly preserve the color and texture of HHP infused apple wedges and baby carrots. Understanding the actual
mechanisms of transport under pressure may enable to develop process guidelines that will help the food industry to develop value-added foods.
CHAPTER 7

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APPENDIX I

ELECTRICAL IMPEDANCE SPECTROSCOPY

Processing of plant and animal based food products is mostly associated with some degree of tissue disintegration. This tissue disintegration that has been observed during HHP is considered the primary cause for observed enhanced infusion. The degree of tissue disintegration, also called as cell-membrane permeability, can be measured using electrode impedance spectroscopy (EIS). EIS is a technique where complex electrical conductivity of biological samples is measured as a function of frequency when ac field is applied. This is a valuable tool to measure differences in cell-membrane permeability before and after processing of food materials.

AI.1 Theory

Plant tissues and cells are inhomogeneous in structure and exhibit complex electrical conductivity. Each cell unit contains intracellular medium separated from the extracellular medium by highly structured, electrically insulating phospholipids forming the cell membranes. The media inside and outside the cell are electrically conducting. Due to the presence of electrically conducting media present on both sides of a non-conducting surface (cell-membrane), such as a dielectric as in the case of parallel plate capacitor, the macroscopic system shows complex electrical conductivity dependent on the frequency of the alternating current (ac) applied. This phenomenon is called Maxwell and Wagner’s dispersion effect. When a biological cell is placed in an ac field, opposite electric charges accumulate at the opposing interfaces of the cell membrane that separates the cytoplasm (intracellular liquid) from the external media. When
charge carriers are blocked by dielectric boundary layers (cell membrane) there is separation of charges, also called as polarization effect, which can lead to dielectric loss or energy dissipation. The cell thus polarized shows electrical conductivity that changes with frequency. An equivalent circuit can represent this type of dielectric dispersion. The electrical behavior of cell membrane can be represented as a capacitor and a resistor connected in parallel. The liquid media (intracellular and extracellular) can be introduced as resistors in series. When measuring a cell that has cytoplasmic membrane bound organelles, Asami and Yamaguchi (1992) report additional dielectric dispersions due to intracellular organelles. Thus, a plant cell can be represented by a “double-shell model”, which includes extracellular resistance, plasma or cell membrane capacitance and resistance, cytoplasmic resistance, tonoplast or vacuole membrane capacitance and resistance, and vacuole interior resistance. The complete rupture of plasma and tonoplast membranes reduces the equivalent circuit to resistances in parallel connection. Such membrane disruption or permeabilization is frequently caused by physical processing methods, such as freeze-thawing and high pressure processing of plant tissue materials. Equivalent circuit model showing intact and ruptured plant cells are shown in Figure AI.1.
The principal frequency dependence of conductivity can be characterized by three dispersions $\alpha$, $\beta$, $\gamma$, each due to a different mechanism. $\alpha$, $\beta$ and $\gamma$ dispersions are characterized by low, radio and microwave frequencies, respectively. Schwan (1963) shows that $\alpha$ and $\beta$-dispersions are not due to water and electrolyte content of biological samples. $\beta$-dispersion can best explain frequency dependent conductivity coming from plant cell membranes and sub-cellular particles. Within the $\beta$-dispersion frequency band, the dielectric current is small compared to the resistive current, which can be easily determined by electrical conductivity measurements. At higher frequencies ($> 0.1$ GHz; $\gamma$ dispersion), dipole rotation of biological molecules further influences the complex conductivity.
**AI.2 Definitions**

Resistance (R) of an electrical element is a measure of its opposition to the passage of direct electric current. It is defined as a ratio of voltage (V) across it to the current (I) through it. \( R = \frac{V}{I} \)

Reactance (X) is the imaginary part of electrical impedance, where electrical impedance (Z) of an electrical element is a measure of its opposition to alternating current. The real part of electrical impedance is resistance (R). In an only resistor-capacitor circuit (R-C), the electrical impedance can be given as:

\[
Z = R + ( - j ) X_c = R + \frac{1}{j \omega C}
\]

where \( j = \frac{1}{-1} = -j \)

Frequency dependent electrical conductivity of a tissue system is defined as:

\[
\sigma(\omega)^s = \frac{l}{A |Z(j\omega)^s|} \quad \text{............... (AI-1)}
\]

where, \( l \) is length of sample

\( A \) is area perpendicular to the electrical field (H x L)

\( Z(j\omega)^s \) is the system impedance, \( \omega \) is the angular frequency = \( 2\pi f \), \( f \) is the frequency in Hz.
AI.3 Circuit Analysis

Figure AI.1 shows a schematic of a sample of length l composed of n layers of cell tissue in series, each layer of thickness d. In an elementary or single layer of cell tissue, there are m individual cells in parallel. These ‘m’ cells are either intact, ruptured or contains inhomogenieties. The model considered here is based on method discussed by Angersbach et al. (1999).

Model Assumption: The intact cells, ruptured cells and inhomegeneities in the initial and processed fruit samples are regularly distributed.

Using this approximation and Kirchhoff’s circuit rules, the impedance of a tissue sample containing intact, ruptured and non-cellular compartments in parallel can be represented by equation AI-2

\[
Z(j\omega)^s = \frac{n}{m} \left[ \frac{i}{Z(j\omega)^i} + \frac{p}{Z^p} + \frac{g}{Z^g} \right]^{-1}
\]

where i and p are the ratios of number of intact cells and ruptured cells to the total number of cell in the elementary layer, respectively; g is the ratio of inhomogeneous non-cellular units to the total number of cells in an elementary layer. \(Z(j\omega)^i\) is impedance of intact cell in the elementary layer which is a function of \(\omega\). \(Z^p\) is the impedance of ruptured cells of the elementary unit and \(Z^g\) is the impedance of additional intracellular vacuole units. Total number of cells in an elementary layer is, \(i + g + p = 1\).

To find \(Z(j\omega)^i\), \(Z^p\),
Solving for $Z(j\omega)^i$

\[
\frac{1}{Z(j\omega)^i} = \frac{1}{R_6} + \frac{1}{Z(j\omega)_1 + Z(j\omega)_{c+v}} \quad \text{…………………… (AI-3)}
\]

\[
Z(j\omega)^i = \frac{R_6[Z(j\omega)_1 + Z(j\omega)_{c+v}]}{Z(j\omega)_1 + Z(j\omega)_{c+v} + R_6} \quad \text{…………………… (AI-4)}
\]

$Z(j\omega)_1$ is impedance of plasma or cell membrane, where $X_1(j\omega) = (2\pi fC_1)^{-1}$, and $R_1$ and $C_1$ are the resistance and capacitance of plasma membrane.

\[
\frac{1}{Z(j\omega)_1} = \frac{1}{R_1} + \frac{1}{-jX_1(j\omega)}
\]

\[
Z(j\omega)_1 = \frac{R_1[-jX_1(j\omega)]}{R_1 - jX_1(j\omega)} \quad \text{…………………… (AI-5)}
\]

$Z(j\omega)_{c+v}$ is combined impedance of cytoplasm, vacuole and tonoplast, where $R_3$, $R_4$, $R_5$ are the resistances of cytoplasm surrounding the vacuole in the direction of current, cytoplasm surrounding the vacuole in the vacuole direction, and vacuole interior, respectively.
\[
\frac{1}{Z(j\omega)_{c+v}} = \frac{1}{R_3} + \frac{1}{R_4 + R_5 + Z(j\omega)_2}
\]

\[
Z(j\omega)_{c+v} = \frac{R_3[R_4 + R_5 + Z(j\omega)_2]}{R_3 + R_4 + R_5 + Z(j\omega)_2}
\]

\[.......... (AI-6)\]

\(Z(j\omega)_2\) is impedance of tonoplast membrane, where \(X_2(j\omega) = (2\pi f C_2)^{-1}\), and \(R_2\) and \(C_2\) are the resistance and capacitance of tonoplast

\[
\frac{1}{Z(j\omega)_2} = \frac{1}{R_2} + \frac{1}{-jX_2(j\omega)}
\]

\[
Z(j\omega)_2 = \frac{R_2[-jX_2(j\omega)]}{R_2 - jX_2(j\omega)}
\]

\[.......... (AI-7)\]

Substituting equation AI-7 in AI-6

Solving for \(Z_p^0\), impedance of ruptured cells of the elementary unit
Now, for a homogeneous sample with no ruptured cells, $Z_p = 0$ and $Z^g = 0$

Equation AI-2 becomes

$$Z^p = \frac{(R_4 + R_5)R_3 \cdot R_6}{(R_4 + R_5)(R_3 + R_6) + (R_3 \cdot R_6)} \quad \text{........................ (AI-8)}$$

From Equation AI-9, we see that impedance of intact tissue is a linear function of one elementary unit. The slope of this function is influenced by the geometry of the sample. Therefore, we can say that $\sigma(\omega)^i = \sigma(\omega)^i$
Angersbach et al. (1999) showed that in the $\beta$-dispersion region between 1 kHz and 50 MHz, the conductivity-frequency spectra could be divided into 3 characteristic zones. Typical conductivity spectra plots are shown in Figure AI.2.

![Diagram](image)

**Figure AI.2.** Typical conductivity spectra from 1 kHz to 40 MHz of biological tissue for (a) intact cells; (b) intact + ruptured cells and (c) all ruptured cells. (Angersbach et al., 1999)

The general assumption is that at low frequency only extracellular resistance contributed to impedance but at higher frequency both extracellular and intracellular resistance contributed to impedance (Zhang and Willison, 1992). The increase in conductivity of processed samples in the low frequency range ($< 5$ kHz) is a result of cell membrane rupture, which has very high resistance and reactance in this frequency range. Intracellular tonoplast membrane resistance contributes negligibly to overall impedance in the low frequency range. In the high frequency range ($> 5$ kHz), the conductivity of intact and ruptured cells are equal. This can be explained as follows. A point to note is that impedances of ruptured cells and inhomogeneities ($Z^p$ and $Z^g$) are not frequency dependent.
At low frequencies \( (f_l) \), \( f \rightarrow 0 \) and \( X_1(j\omega) \); \( X_2(j\omega) \rightarrow \infty \); \( R_3 << R_1 \); Equation AI-3 becomes:

\[
\frac{1}{Z'_i} = \frac{1}{R_1} + \frac{1}{R_6}
\]

\[
Z'_i = \frac{R_1 \cdot R_6}{R_1 + R_6}
\]

……….. (AI-10)

At high frequencies \( (f_h) \), \( f \rightarrow \infty \) and \( X_1(j\omega) \); \( X_2(j\omega) \rightarrow 0 \); Equation AI-3 becomes:

\[
\frac{1}{Z'_h} = \frac{1}{R_6} + \frac{1}{R_3} + \frac{1}{R_4 + R_5}
\]

\[
Z'_h = \frac{(R_4 + R_5) R_3 \cdot R_6}{(R_4 + R_5)(R_3 + R_6) + R_3 \cdot R_6}
\]

……….. (AI-11)

Thus, from Equations AI-8 and AI-11, \( Z'_h = Z'' \). This shows that the conductivity of intact and ruptured cells are equal.

To assess the effect of process treatments, it is important to know the magnitude of cell that are affected by the treatment. I compute this magnitude using an index, called as permeabilization index or \( Z_p \).

\[
Z_p = \frac{P}{(1 - g)}
\]

……….. (AI-12)

where, \( i + p + g = 1 \); \( i \), \( p \) and \( g \) are the fraction of intact, ruptured and inhomogeneous cells respectively.
Expressing $p$ in terms of conductivity:

$$\sigma_i' = i\sigma_i + p\sigma^p + g\sigma^g$$

Therefore, $p = \frac{\sigma_i' + g(\sigma_i' - \sigma^g) - \sigma_i'}{\sigma^p - \sigma_i'}$ ....................... (AI-13)

Substituting Equation AI-13 in AI-12

$$Z_p = \frac{\sigma_i' - (1 - g)\sigma_i' - g\sigma^g}{(\sigma^p - \sigma_i')(1 - g)}$$  ....................... (AI-14)

When resistance of extracellular compartments is infinitely high, for example gas bubbles produced during mashing of vegetables, then $\sigma^g = 0$. Thus, permeabilization index with no extracellular compartments ($g = 0$ and $Z_p = p$) is

$$Z_p = \frac{\sigma_i' - \sigma_i'}{\sigma^p - \sigma_i'}$$  ....................... (AI-15)

To factor in possible processing effects, which can coincide with cell membrane rupture, for example change in temperature, tissue porosity and electrolyte concentration, a term ‘$k$’ is introduced as correction. Therefore,

$$\sigma_i' = k\sigma_i \quad \text{and} \quad \sigma_h' = k\sigma_h$$

$$\sigma_i' = \sigma_i' \left( \frac{\sigma^g_h}{\sigma_h} \right)$$

Hence, .......................... (AI-16)

$\sigma_h'$ and $\sigma_i'$- electrical conductivity at high and low frequencies before treatment
and electrical conductivity at high and low frequencies after treatment

\( \sigma_h^j = \sigma_l^j \)

Also, as seen in Equations AI-8 and AI-11, at high frequencies,

\[ \sigma^p = \sigma_h^j = \sigma_l^j \]  
\[ \text{.......................... (AI-17)} \]

Substituting, Equations AI-16 and AI-17 in AI-15

\[ Z_p = \frac{[\sigma_h^j / \sigma_l^j \cdot \sigma_h^j] - \sigma_l^j}{\sigma_h^j - \sigma_l^j} \]  
\[ \text{.......................... (AI-18)} \]

The parameter \( Z_p \) ranges from 0 for intact tissues to 1 for fully permeabilized tissue. Determination of \( Z_p \) is carried out by measuring conductivity of initial intact and treated sample at low and high frequencies within the band of \( \beta \)-dispersion.

For cranberry tissue, the characteristic low and high frequencies were 1kHz and 5 MHz. Electrical conductivity measurements were performed immediately after treatment (HHP and control). The impedance measurement method followed in this research is as discussed in Chapter 3, section 3.8.

**AI.4 Nomenclature**

l is length of sample in the current direction

H is height of sample

L is width of sample
A is area perpendicular to the electrical field, where A = HL
b is height of an individual cell in an elementary layer with m cells
a is width of an individual cell in an elementary layer with m cells
d is length of an individual cell in an elementary layer with m cells
A_c is area of an individual cell in an elementary layer, with m cells where A_c = ba
Z is the impedance, where \( Z = R + (-j)X_c = R + \frac{1}{j\omega C} \) in an R-C circuit. Also, \( |Z| = \sqrt{R^2 + X^2} \)
R is the resistance. It is the real part of impedance.
X is reactance (imaginary part of impedance) where \( X(j\omega) = 2\pi fC \)
C is capacitance
\( \omega \) is the angular frequency = \( 2\pi f \),
\( f \) is frequency
\( j \) is \( \sqrt{-1} \). It is a representation of imaginary part of a complex number (Z), where
\[
\frac{1}{j} = \frac{j}{j^2} = \frac{j}{-1} = -j
\]
\( R_1 \) plasma membrane resistance
\( R_2 \) vacuole (tonoplast) membrane resistance
\( R_3 \) cytoplasmic resistance surrounding the vacuole in the direction of current
\( R_4 \) cytoplasmic resistance in vacuole direction
\( R_5 \) resistance of vacuole interior
\( R_6 \) resistance of the extracellular compartment
\( C_1 \) plasma membrane capacitance
\( C_2 \) vacuole membrane capacitance
\( Z(j\omega)^s \) is the sample impedance, which is a function of \( \omega \),
\( \sigma(\omega)^s \) is electrical conductivity of sample, which is a function of \( \omega \), where \( \sigma(\omega)^s = \frac{1}{A|Z(j\omega)^s|} \)
n is total number of layers in series in the sample, where \( n = \frac{l}{d} \)

m is number of cells in parallel in each layer, where \( m = \frac{A}{A_c} \)

i is ratio of number of intact cells to total number of cells in the elementary layer

p is ratio of number of ruptured cells to the total number of cells in the elementary layer

g is ratio of inhomogeneous inclusion units to total number of cells in the elementary layer

\( Z(j\omega)_i \) is the impedance intact cells which is a function of \( \omega \)

\( Z^p \) is impedance of elementary unit with ruptured membranes

\( Z^g \) is impedance of additional intracellular volume elements (vacuoles)

\( Z(j\omega)_1 \) is impedance of plasma or cell membrane

\( Z(j\omega)_{c+v} \) is combined impedance of cytoplasm, vacuole and tonoplast

\( Z(j\omega)_2 \) is impedance of tonoplast membrane

\( \sigma(\omega)_i \) is electrical conductivity of elementary layer with intact cells.

\( \sigma^p \) is electrical conductivity of elementary layer with ruptured cells

\( \sigma^g \) is electrical conductivity of elementary layer with inhomogeneities

\( Z_p \) is permeabilization index

\( \sigma'_h \) - electrical conductivity before treatment at high frequency (S/m)

\( \sigma''_h \) - electrical conductivity after treatment at high frequency (S/m)

\( \sigma'_l \) - electrical conductivity before treatment at low frequency (S/m)

\( \sigma''_l \) - electrical conductivity after treatment at low frequency (S/m)

**AI.5 References**

