HIGH PRESSURE ASSISTED INFUSION OF CALCIUM IN PME TREATED BABY CARROTS

By

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ABSTRACT OF THE THESIS

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High pressure processing (HPP) is a technology in which foods are subjected to high pressures in the range 100 MPa – 800 MPa. HPP has been greatly explored as a process technology for ensuring food safety in the past. Recently, this technology has shown a great promise to enhance the infusion of biomolecules into food systems. The aim of this project was to explore and evaluate HPP as a technique for infusion of calcium in a food matrix, specifically baby carrots.

HPP was employed in infusing calcium in PME (pectin methylesterase) treated baby carrots. Calcium lactate gluconate (CLG) salt was used as the infusate solution. The effect of HPP was evaluated on the extent of calcium infusion in baby carrots along with its effect on other important attributes of baby carrots, such as beta-carotene extractability, hardness (N), and color. In addition, the effect of pressure cycling on calcium infusion were also studied.

Fifteen HPP experiments were performed following a Box-Behnken design of experiments. The three independent factors evaluated were pressure (MPa), time (min), and CLG solution concentration (% w/v). To evaluate the effect of pressure cycling, high

pressure infusion was carried out by varying the number of cycles as 1, 3, and 5 keeping the maximum pressure level, the total hold time at maximum pressure, and the maximum CLG solution concentration fixed. Unprocessed baby carrots, osmotically infused baby carrots, and vacuum infused baby carrots were used as controls.

It was observed that HPP enhanced the infusion of calcium in PME treated baby carrots from 19.5 mg/serving to up to 134 mg/serving of baby carrots, which is more than 10 % of the RDA of calcium, and was more than 3 times the amount of calcium infused in osmotically and vacuum infused controls. The processing variables of pressure, time, and CLG concentration, all played a significant role in determining the amount of calcium infused. The amount of infused calcium increased with the number of pressure cycles (1 to 5). The extractability of beta-carotene from the HPP calcium infused baby carrots was 4-5 times higher than the unprocessed control. The hardness of the baby carrots was observed to increase after calcium infusion, but the color of the carrots became darker. Microbial shelf life of the calcium infused baby carrots was observed to be only 4 days, indicating that further research is needed to optimize the handling and post-processing techniques.

In conclusion, HPP was found to enhance the infusion of calcium in PME treated baby carrots while also improving the extractability of beta-carotene and increasing the hardness, but darkening the color.

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1. INTRODUCTION

High pressure processing (HPP) technology has been widely explored for extending the shelf life of food products and improving their sensory quality. Recently, there has been an interest in exploring this technology to infuse nutrient molecules in the food matrices. In this project our aim was to explore and evaluate the effect of HPP on infusion of an important micronutrient, calcium, in enzyme treated baby carrots. If successful, HPP can be further explored to fortify a variety of fruits and vegetables with necessary micronutrients they inherently lack.

1.1. Calcium

Calcium is an important mineral in the human body, primarily responsible for maintaining the bone and teeth health. Deficiency of calcium in the human body has been associated to osteoporosis, weak bones, and has been linked to higher risk of cardiovascular diseases. Hence, consumption of calcium in amounts adequate for the body to function well is of primary importance.

1.1.1. Role of calcium in the human body

Calcium is the fifth most abundant element in the human body after oxygen, carbon, hydrogen, and nitrogen, and accounts for almost 2 % of the total bodyweight (~1200 g of calcium). About 99 % of the calcium in the body is found in the skeleton and the teeth. It is present in the inorganic crystalline form of hydroxyapatite

[$Ca_{10}(PO_4)_2(OH)_2$], which provides skeletal rigidity. The remaining 1 % of calcium is found in soft tissues and body fluids. Along with being an essential nutrient for bone and teeth health, calcium is also responsible for regulating intracellular functions in body tissues and physiological functions such as muscle contraction and nerve function (Theobald, 2005).

1.1.1.1. Skeletal functions

Bone is essentially a collagen-protein matrix, with deposits of mineral salts within. Calcium, phosphate, and magnesium are the most important minerals in the bone, calcium being the most abundant. Calcium and phosphate are present in the form of hydroxyapatite [$Ca_{10}(PO_4)_2(OH)_2$].

The process of bone formation and bone resorption occurs continuously throughout the life cycle at different rates depending on the age. A number of specialized cells in the bone along with the mineral salts are responsible for the production of collagen and other organic compounds of the protein matrix, bone mineralization, and bone resorption (Theobald, 2005). Abundance of mineral salts in the body is important for the bone mineralization to occur efficiently. Bone mineralization is the deposition of minerals in the protein matrix during bone formation. On the other hand, during bone resorption, the minerals from the bone are released into the blood.

Bone mineralization occurs on the outer surface of the bone, while bone resorption occurs at the inner surface. Depending on the relative rates of bone formation and bone resorption, thickening or thinning of the bone cortex takes place. After the body

reaches its peak bone mass (PBM) and with increasing age, the rate of bone resorption increases relative to bone formation, thus contributing to bone loss. During periods of rapid growth, such as in infants and adolescents, the calcium deposited in the skeleton is more than when the growth is slower. Table 1, adopted from Theobald (2005), depicts the amount calcium deposition during the lifespan of an individual.

Group	Age (years)	Weight (kg)	Bone calcium deposited (mg/kg/day)	Total bone calcium deposited (mg/day)
Premature infants	-	1.34	160	214
Infants	0.8	8	90	720
Pre-pubertal	8.3	28	52	1456
Pubertal	10.2	65	53	1960
Post-pubertal	15.4	59	21	1240
Adult	30-60	60	5-10	300-600

Table 1. Rate of calcium deposition in bones during different life stages. Adopted from Theobald (2005).

1.1.1.2. Regulatory role

Calcium is an important mineral for the living cells to remain viable and perform their functions in the body. It is involved in maintaining the structural integrity of the intracellular organelles, while in the biological membranes, like the cell plasma, calcium bound to phospholipids and proteins is necessary to maintain the permeability and the integrity of these membranes. The concentration of intracellular calcium varies from a small amount of 0.02 mmol/L cell water in red blood cells with no organelles to larger amounts of 5 mmol/L – 15 mmol/L cell water in muscle cells, depending on the cell type (Theobald, 2005).

Role of calcium in blood clotting: Calcium triggers the activation of the inactive blood clotting factors, which consequently results in the production of thrombin from prothrombin, which catalyzes the formation of a stable fibrin clot, thus preventing premature dissolution of the blood clot.

Role of calcium in digestion: Calcium aids in the optimal activity of several digestive enzymes, such as the proteases, and phospholipases. It has been postulated that the expression of the calcium ion sensing receptor along the gastrointestinal tract plays an important role in gastric acid secretion (Theobald, 2005).

Role of calcium in muscle function: Muscle fibers are composed of myosin (thick) and actin (thin) filaments. Calcium plays an important role in controlling the interaction of these filaments which cause muscle contraction.

1.1.1.3. Calcium absorption in the human body

On an average, 20 % - 30 % of the total calcium consumed is absorbed by the human body. Absorption of calcium mainly occurs in the small intestine. At low calcium intakes, absorption occurs by transcellular transport (active transport), while at higher calcium intakes, the absorption occurs by simple paracellular diffusion (passive transport). Thus, with higher calcium intake the net amount of calcium absorbed in the body increases, but the fractional amount of calcium absorbed (i.e., amount absorbed in proportion to amount consumed) decreases. Owing to the inverse relation of calcium absorption at higher levels of calcium intakes it is suggested to have less than 500 mg of calcium at once (Office of Dietary Supplements (ODS), NIH). Unabsorbed calcium is excreted from the body through urine, feces, and to some extent through sweat.

A lot of factors influence the absorption of calcium, such as the estrogen level, intensity of physical activity, dietary choices and restriction, and most importantly, levels of Vitamin D consumed. Vitamin D interacts with calcium to generate the hormone calcitrol (hormonally active metabolite of vitamin D), which aids in calcium absorption. The recommended daily intake of Vitamin D for adults for optimum calcium absorption is 600 IU (~15 µg).

1.1.2. Dietary reference values of calcium in the U.S.

The Food and Nutrition Board (FNB) at the Institute of Medicine (now the National Academy of Medicine) has suggested recommended daily intakes (RDA) for

calcium in the Dietary Reference Intakes (DRI) of 2010. RDA is defined as the average daily level of intake sufficient to meet the nutrient requirements of nearly all (97 % - 98 %) healthy individuals. Table 2 lists the FNB established RDAs for calcium (mg per day) for different age groups.

Age	Male	Female	Pregnant	Lactating
0 – 6 months*	200 mg	200 mg		
7 – 12 months*	260 mg	260 mg		
1 – 3 years	700 mg	700 mg		
4 – 8 years	1000 mg	1000 mg		
9 – 13 years	1300 mg	1300 mg		
14 – 18 years	1300 mg	1300 mg	1300 mg	1300 mg
19 – 50 years	1000 mg	1000 mg	1000 mg	1000 mg
51 – 70 years	1000 mg	1200 mg		
71+ years	1200 mg	1200 mg		

Table 2. Recommended Dietary Allowances for calcium as recommended by the FNB. (DRI, 2010)

*Adequate intake (AI). AIs are established when the evidence is insufficient to develop an RDA and is set to a level assumed to achieve nutritional adequacy.

For the purpose of this study, the RDA of calcium has been considered to be 1000 mg per day for an average healthy individual.

1.1.3. Sources of calcium

Human body does not internally produce calcium, and hence, the requirement for calcium by an individual can only be satisfied through diet or supplements. Dairy food products, predominantly milk, yogurt, and cheese, are the richest natural sources of calcium. Vegetables such as Chinese cabbage, kale, broccoli, and spinach, are also rich sources of calcium. However, bioavailability of calcium in spinach is very low (ODS, NIH). Grains do not have high calcium content, however, their widespread and large scale consumption contributes to small amounts of calcium in the diet. Quinoa is a good source (8 % RDA in 1 cup cooked quinoa) of calcium and so are some nuts such as almonds (24 % RDA in 1 cup sliced almonds). Calcium fortified fruits juices, tofu, and manufactured breakfast cereals, are also important contributors to the daily calcium requirement. Table 3 shows the calcium content of selected common foods.

Food	Serving size	mg calcium per serving	% Daily value (DV)
Yogurt, plain, low fat	8 ounces	415	42
Mozzarella, part skim	1.5 ounces	333	33
Sardines, canned in oil, with bones	3 ounces	325	32
Milk, non-fat	8 ounces	299	30

Milk, reduced-fat (2 % milk fat)	8 ounces	293	29
Milk, whole (3.25 % milk fat)	8 ounces	276	28
Orange juice, calcium fortified	6 ounces	261	26
Tofu, firm, made with calcium sulfate	½ cup	253	25
Almonds, sliced	1 cup	243	24
Kale, fresh, cooked	1 cup	94	9
Quinoa, cooked	1 cup	80	8
Broccoli, raw	½ cup	21	2

Table 3. Calcium content of foods commonly consumed by the humans (ODS, NIH)

Dietary supplements for calcium, primarily in the form of carbonates or citrates, are widely consumed in the U.S. to fulfill the RDA for calcium. On an average, one pill of the calcium supplement provides around 500 mg calcium.

1.1.4. Calcium intake in the U.S.

The National Health And Nutritional Examination Survey (NHANES) of 2003-2006 evaluated the estimated calcium intakes of the U.S. population from both food sources and dietary supplements. According to NHANES (2003-2006), half of the of the

U.S. population did not meet the Estimated Average Requirements as recommended (~1000 mg/day) by the Institute of Medicine, while more than 43 % of the population used calcium supplements that contributed to an average increase of 331 mg/day in calcium intake. Mean dietary intake of calcium in males was observed to be between 871 mg/day – 1266 mg/day, depending on the life stage, while that for females was between 748 mg/day – 768 mg/day.

Table 4 shows the % population with calcium intake below the recommended intake (refer to Table 2) based on the NHANES 2003-2006 data.

Age group	% population with calcium intake below RDA	
	Males	Females
1 – 3 years	4 ± 1.0	3 ± 0.9
4 – 8 years	17 ± 2.5	33 ± 4.9
9 – 13 years	77 ± 4.2	85 ± 4.4
14 – 18 years	58 ± 3.2	87 ± 2.8
19 – 30 years	35 ± 3.2	62 ± 3.3
31 – 50 years	36 ± 2.9	56 ± 2.6
51 – 70 years	68 ± 2.0	61 ± 2.6
71+ years	69 ± 2.7	61 ± 1.7

Table 4. Population with calcium intakes below the recommended intake. (NHANES, 2003-2006)

Table 4 includes the data for calcium intake from all sources, including the dietary supplements. However, it is seen that the population above the age group of 4 years is deficient in calcium, in spite of consumption of calcium supplements.

1.1.5. Calcium deficiency and correlation to diseases

Calcium deficiency arises when an individual is unable to achieve the recommended intake of calcium according to the RDA. Low intake of calcium reduces the availability of calcium for bone formation, and consequently can lead to loss of bone mass. Loss of bone mass over a period of time due to calcium deficiency along with age related factors can lead to osteoporosis. Osteoporosis is defined as 'a disease characterized by low bone mass and micro-architectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk' (WHO, 1994). Owing to an increased rate of bone resorption with increasing age, older adults are more prone to osteoporosis as a result of the loss of bone mass. Vulnerability of an individual to osteoporosis is calculated based on a T-score, that is calculated by comparing the bone mineral density (BMD) of an individual to the optimal BMD (of a healthy 30-year-old adult). A T-score of -1 or above indicates normal bone density, -1 to -2.5 indicates low bone mass (osteopenia), and < -2.5 indicates osteoporosis. In the U.S. the estimated number of population suffering from osteoporosis is about 5-6 million in older (> 45 years) women and 1-2 million in older (> 45 years) men, while about 1.5 million fractures have been estimated to have occurred as a consequence of osteoporosis

(ODS, NIH). Adequate consumption of calcium throughout the life may reduce the risk of osteoporosis.

Calcium has been proposed to aid in reducing the lipid absorption, lowering the cholesterol, and thus lowering the risk of cardiovascular diseases. High intakes of calcium have also been thought to reduce the risk of colon and prostate cancer (ODS, NIH).

1.2. Food fortification

As defined by the U.S. Food and Drug Administration (FDA), food fortification is the deliberate addition of one or more essential nutrients to a food, whether or not it is normally contained in the food. Fortification may be used to prevent or correct a demonstrated deficiency in the population or specific population groups; restore naturally occurring nutrients lost during processing, storage, or handling. On the other hand, food enrichment is the addition of specific nutrients (i.e., iron, thiamin, riboflavin, and niacin) to refined grain products in order to replace losses of the nutrients that occur during processing. Enrichment of refined grains is not mandatory; however, those that are labeled as enriched (e.g., enriched flour) must meet the standard of identity for enrichment set by the FDA. When cereal grains are labeled as enriched, it is mandatory that they be fortified with folic acid.

In 2005, FAO/WHO identified food fortification as a valid technology to tackle micronutrient malnutrition. The technique of food fortification, especially when combined with existing technology, has the potential to lead to rapid improvements in the micronutrient status of a population at a reasonable cost. An obvious requirement, however, is that the food must be consumed in adequate amounts by a large population. Though the impact of food fortification may not be immediate, it is much wider and sustainable. The benefits associated with food fortification are large and cost effective.

Food fortification has been in practice for long to prevent the deficiencies of various micronutrients. Iodized salt, fortified grain flours, and cereals, calcium fortified fruit juices, are examples of successful and existing practices of food fortification.

Figure 1. Examples of fortified food products available in the market

1.3. Baby carrots

According to USDA (2007), carrots are one of the most popular vegetables in the U.S. The consumption fresh carrots has been on a rise over the past few decades. About 80 % of the fresh carrots are bought at a retail store and majority of them are baby carrots.

Baby carrots are small pieces cut out from a larger carrot. The invention of baby carrots dates back to 1986 and attributed to a California farm grower, Mike Yurosek. The larger carrots are washed with chlorine water, cut into 2 inch pieces, peeled and packaged as baby carrots. The videos in the following links

https://www.youtube.com/watch?v=lNy948H2ta4

and

https://www.youtube.com/watch?v=stCKRExcRBA

explain the manufacturing process of baby carrots briefly.

Baby carrots can be eaten raw or cooked, as a plain snack or as an appetizer, or added to salads, salad dressings, soups, stews, etc. Thus, baby carrots are consumed in a variety of forms and provide a healthy and convenient snack option for the population.

According to the USDA, one serving of baby carrots is defined as ½ a cup, equivalent to 6 baby carrots, which provides more than the daily requirement of Vitamin

A. The recommended daily intake of vegetables is 2 ½ cups. Table 5 shows the nutritional content of baby carrots as determined by the USDA.

NUTRITION FACTS Serving size: ½ cup (70 g) baby carrots			
Amount Per Serving			
Calories	25	Calories from fat	0
			% Daily Value*
Total Fat 0 g Saturated fat 0 g *Trans* fat 0 g			**0 %** **0 %**
Cholesterol 0 mg			**0 %**
Sodium 55 mg			**2 %**
Total Carbohydrate 6 g Dietary fibers 2 g Sugars 3 g			**2 %** **8 %**
Protein 0 g			
Vitamin A	190 %	Vitamin C	4 %
Calcium	2 %	Iron	4 %
*Percent daily values as based on a 2000 calorie diet.			

Table 5. Nutritional content of baby carrots (USDA, 2012).

1.4. High pressure processing (HPP)

High pressure processing (HPP), also known as high hydrostatic pressure processing (HHPP), is a non-thermal batch processing technique, wherein food products are subjected to high pressures in the range of 20 MPa – 700 MPa, for a time period ranging between 2 min – 30 min. HPP offers the advantage of making minimally

processed foods, which are wholesome, convenient, and contain minimal to no preservatives (Balasubramaniam, et al., 2015).

1.4.1. History and background

The earliest research on HPP in foods has been reported by Hite in 1882 and Bridgman in 1912 (Mahadevan, 2015; Balasubramaniam, et al., 2015). Hite observed the delay in souring of milk at ambient temperatures after HPP, and reported the possible use of the technique as a food preservation technique. Later in 1912, Bridgman observed the coagulation of egg white protein under high pressure, thus reporting structural changes in proteins under pressure. However, the technology remained dormant until late 1900s. In 1992, pressure-treated jams and jellies were introduced in Japan, followed by introduction of high pressure processed guacamole in the U.S. in 1997. Since then HPP has been applied to food products such as fruits preserves, fruit juices, shell fish, packaged meats, deli salad dips and dressings, and salsa.

Few of the recent applications of HPP are the commercialization of the high pressure processed fruit juices by Starbucks™ (Evolution Fresh™ juices), Coke™ (invested in Suja™) and Pepsico™ (Naked™ Pressed).

Figure 2. Currently available high pressure processed juice products

The main advantages of HPP are:

- Low or ambient temperature processing
- Instantaneous transmittance of pressure to and within the food product, irrespective of the shape and size of the product
- Uniform application of pressure throughout the food matrix, for most foods
- Significant microbial and enzyme inactivation without the application of heat or use of chemical preservatives
- Retention of the nutrients and fresh-like properties of the food
- Processing in packages

High pressure processing has been demonstrated to bring about significant microbial reduction in food matrices. In addition, HPP has been shown to enhance the sensory appeal of some food products, for example - improved shucking of oysters, improved meat tenderness of pre-rigor meat, and increased firmness and elasticity of gels prepared from HPP treated fish (Balasubramaniam, et al., 2015; Cadesky, 2015.).

A few limitations associated with HPP are:

- Only liquid foods and very few solid foods that contain minimal amounts of air and dissolved gases can be processed under HPP. The presence of air pockets in the food product may cause an irreversible damage to the food structure (such as collapsing) after pressurization.
- The food product to be processed under high pressure must be vacuum packed in flexible pouches or containers that will allow the transmission of pressure.

- HPP is a batch process with maximum batch volume of 525 liters at present
- *Clostridium botulinum* spores cannot be inactivated by HPP and hence, it cannot be used as a sterilization process for low-acid foods
- The equipment cost ($2.5 million) and capital cost ($5 million) involved are high (assuming the equipment capacity of 420 L)

1.4.2. Equipment and processing

HPP is a batch processing technique in which the food products vacuum packed in flexible pouches/containers are subjected to pressurization of up to 700 MPa using a pressure transmitting fluid, most commonly water, in a large container of fixed volume.

Figure 3 shows a schematic of a typical HPP unit and the ancillary equipment.

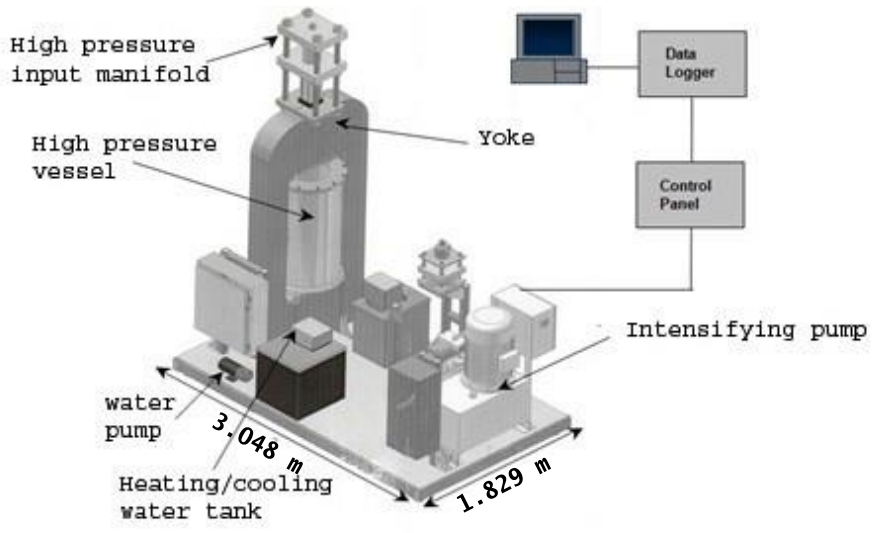


Figure 3. Schematic representation of high pressure processing unit at the Department of Food Science, Rutgers University.

HPP processing unit typically consists of the following components:

- Pressure vessel: Pilot scale pressure vessels have vessel volume in the range of 1 L – 35 L, while commercial pressure vessel volumes range between 35 L – 525 L, with processing capacities up to 30 million kg per year.
- Closures: Both the ends of the pressure vessel are sealed by means of two closures, which contribute to the mechanical and processing safety of the vessel.
- Yoke: The yoke retains high axial forces that act on the closures when under pressure.
- Pressure pump: A pressure pump is used to achieve the desired pressures in the vessel through direct pressurization (using a piston) or indirect pressurization (through a pressure transmitting fluid, like water).
- Process control system: A computer control system is used to regulate the operating pressure and time conditions.

In a typical HPP cycle, the pressure vessel (with the food products to be processed) is completely filled with water, the pressure transmitting fluid, before the start of a cycle. After the vessel is completely filled, it is sealed with end closures. A high intensity pressure pump pumps water into the vessel until desired pressure is achieved. The time required for the pressure vessel to achieve the desired pressure is known as the come-up time (CUT). The come-up time generally ranges between 2 min – 5 min, depending on the desired pressure, and degree of product fill in the vessel. Once the vessel reaches the desired pressure, it is held at that pressure for a pre-determined time

period, known as the hold time. After holding the sample under desired pressure and desired time period, the pressure is released. The depressurization takes 2 s – 5 s.

The temperature of the fluid and the food product varies minimally during the HPP cycle. The temperature rises by 8 °C – 15 °C during the pressurization of the vessel depending upon the composition of the product and the maximum pressure. However, the temperature drops a little during the pressure hold due to loss of heat to the HPP vessel walls.

Figure 4 represents a typical HPP cycle along with changes in pressure (MPa) and temperature (°C) as a function of time.

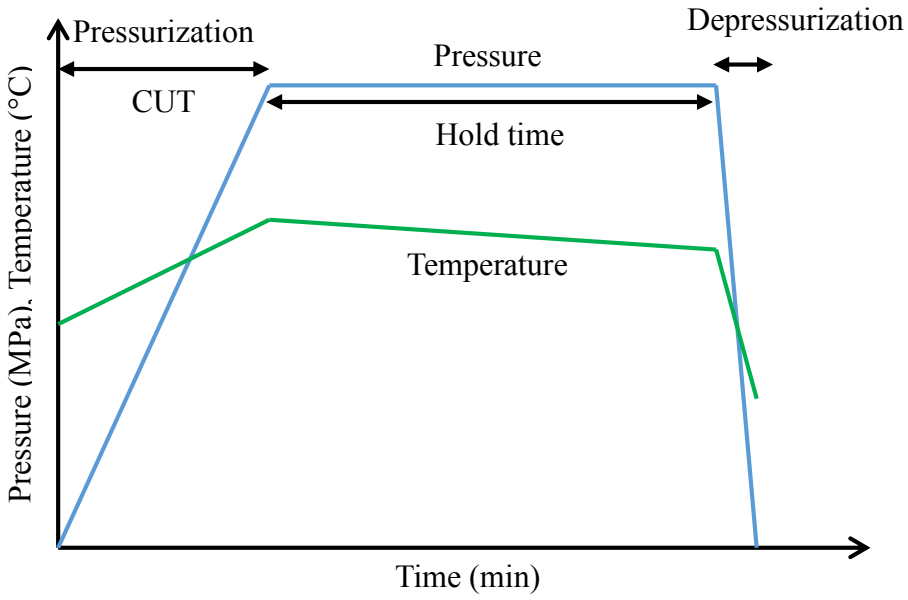


Figure 4. Variation of pressure (MPa) and temperature (°C) during the HPP cycle

1.4.3. High pressure processing of fruits and vegetables and its effect on infusion

Traditionally, HPP has been used as a pre-treatment for osmotic dehydration, thereby reducing the resistance of the cells during subsequent dehydration (Rastogi, et al., 1998; Rastogi, et al., 2000; Fraeya, 2010). The effect of HPP to enhance the mass transfer rates of molecules into food matrices has also been explored. The mass diffusivity values of sucrose in pineapple slices were observed to increase two-fold after high pressure treatment (Rastogi and Niranjan, 1998). This increase in the rate of transfer of molecules into the food matrix was attributed to breaking up of the cell walls. Similarly, enhanced mass transfer of NaCl was observed in potato cylinders under high pressure of up to 400 MPa (Rastogi, 2000). Villacis et al. (2008) had similar observations when they evaluated the effect of high pressure (0.1 MPa – 300 MPa) on the diffusion of NaCl and water into turkey breasts. At the processing pressures of 150 MPa, the meat samples with minimum hardness, gumminess, and chewiness, along with maximum infusion of NaCl were obtained.

In a more recent study, Mahadevan et al. (2014 and 2015) reported enhanced infusion of antioxidant quercetin in scarified fresh as well as frozen-thawed cranberries. Fig. 5 compares high pressure infusion of quercetin in cranberries to vacuum and osmotic infusion. A two-fold and three-fold increase in infusion was observed under high pressure, in comparison to vacuum and osmotic infusion, respectively.

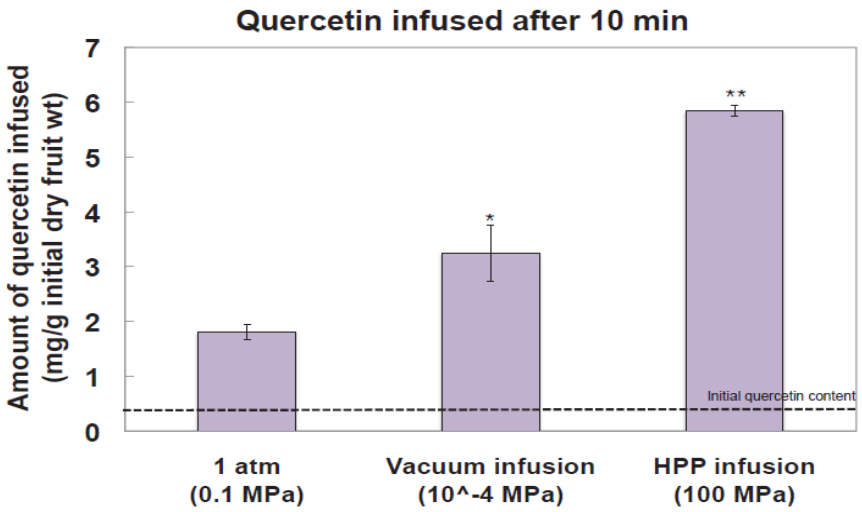


Figure 5. Infusion of quercetin in frozen-thawed cranberries, under osmotic, vacuum, and high pressure conditions (Mahadevan, 2014).

Since HPP has been proven to enhance infusion of molecules in food matrices, it has the promise to be used as a food fortification technique.

HPP has shown minimal effects on color and flavor components of fruits and vegetables. High pressure changes the cell permeability and enables movement of water, enzymes, and other molecules across the cell. Application of pressure can also enhance the desired action of pectin methyl esterase (PME) which causes demethylation of pectin bonds in the cell membrane.

Rastogi et al. (2008), studied the effect of high pressure treatment in combination with temperature and calcium treatment on the texture of carrots. A combination of pressure (200 MPa for 15 min), temperature (60 °C), and calcium chloride solution (1 %) was observed to have increased the hardness of carrots from 14.08 N (raw, unprocessed

carrots) to 129.07 N after pressure assisted thermal processing (PATP) at 700 MPa for 15 min at 105 °C.

In another study, simultaneous vacuum infusion of PME and calcium chloride resulted in an increased hardness of the apples tissues. It was proposed that the PME causes demethylation of the pectin chains in the call wall, thus exposing the carboxylic groups to the action of calcium. Calcium binds with these carboxylic groups to form an 'egg-box model' (Grant, et al., 1973) which increases the mechanical rigidity of fruit tissues (Guillemin, et al., 2008).

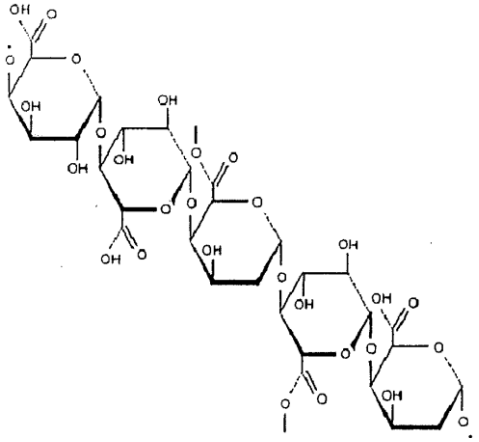


Figure 6a. Structure of the pectin molecule

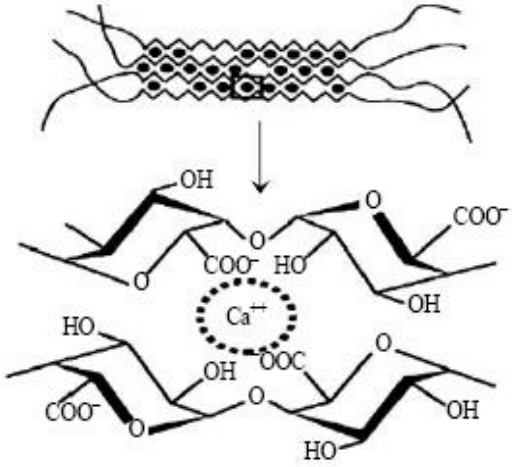


Figure 6b. Egg-box model of calcium and pectin complex

In addition to enhanced infusion and increased hardness of fruit and vegetable matrices, HPP has been also shown to increase the extraction of compounds from these matrices. The total concentration of carotenoids extracted from avocados was observed to

be 56 % higher after high pressure treatment (600 MPa, 3 min), while the extraction of beta-carotene increased by 107 % (Jacobo-Velázquez, et al., 2012).

1.5. Rationale and significance of this research

Previous research in high pressure processing has reported enhanced infusion of biomolecules in food matrices, along with increasing the mechanical rigidity of the fruit and vegetable matrices. Therefore, HPP can possibly be employed as a technique to fortify food products.

Although Rastogi et al. (2008) evaluated the effect of calcium and high pressure pre-treatment on the hardness of carrots, their research did not focus on infusing higher amounts of calcium but on improving the hardness of the carrots. The same study evaluated the effect of HPP as a pretreatment to PATP, but the effect of only HPP on the infusion of calcium and hardness was not evaluated. Hence, we propose to evaluate the effect of HPP on the infusion of calcium in PME treated baby carrots.

In an effort to provide the U.S. population with a healthier and convenient option to fulfill the daily calcium requirement, it was thought to explore the technique of HPP as a food fortification technique, by fortifying the most commonly eaten vegetable snack – baby carrots – with calcium.

Since baby carrots are consumed widely, fortifying them with calcium would help in providing a large population with a healthy and nutritive source of calcium along with the inherent nutrition of baby carrots.

Research has also shown a synergistic effect of PME treatment and calcium treatment on the textural properties fruit and vegetable tissues. PME treatment on baby carrots would cause the pectin bonds to demethylate, opening up more carboxylic sites for calcium to bind, thus retaining their texture after processing.

In order to infuse calcium in baby carrots, calcium salt of calcium lactate gluconate (CLG), containing 13 % calcium, was proposed to be used in this study. Commonly used calcium salts for fortification, such as calcium chloride, calcium citrate, and calcium malate, have been known to contribute to bitter taste to the food product. Moreover, at higher concentrations of these salts, the bitterness would be more pronounced.

Baby carrots are rich in beta-carotene (precursor to Vitamin A) content. Based on previous research, high pressure processing may increase the amount of beta-carotene that can be extracted from baby carrots.

A significant aim of this project was to explore a new technique of food fortification to tackle the increasing deficiency of an important mineral, without deteriorating the existing nutritional and physical properties. A successful outcome from

this research would provide with a potential food fortification technique to fortify commonly eaten fruits and vegetable with micronutrients that they inherently lack.

1.6. Hypothesis

It was hypothesized that high pressure processing would enhance the infusion of calcium in PME treated baby carrots, along with an increase in the extractability of beta-carotene and will cause minimal to no effect on the physical properties of texture and color.

1.7. Research objectives

The primary objective of this project is to evaluate the effect of high pressure processing parameters (pressure (MPa) and hold time (min)) and CLG solution concentration (% w/v) on:

- Calcium infusion (mg calcium per serving of baby carrots)
- Beta-carotene extractability (mg beta-carotene per serving of baby carrots)
- Texture (N) of baby carrots
- Color (L*, Chroma (C), Hue (h), color difference (ΔE)) of baby carrots

Secondary objectives of this project were:

- To evaluate the effect of pressure cycling on:
  - Calcium infusion (mg calcium per serving of baby carrots)
  - Beta-carotene extractability (mg beta-carotene per serving of baby carrots)
  - Texture (N)
  - Color (L*, Chroma (C), Hue (h), color difference (ΔE))

- To evaluate the effect of calcium infusion and extent of high pressure, independently, on the color (L*, Chroma (C), Hue (h), color difference (ΔE)) of the high pressured processed-calcium infused baby carrots

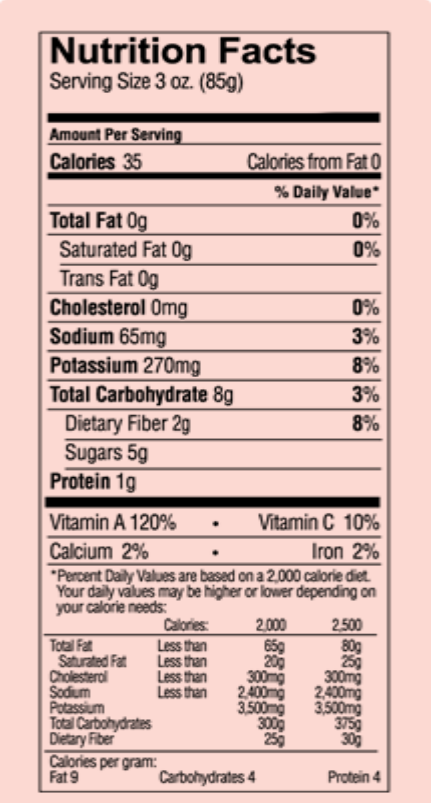
2. MATERIALS AND METHODS

2.1 Materials

2.1.1. Baby carrots

Bolthouse® Farms Premium Sweet Petites™ baby carrots were purchased from a local retail store. One serving size of these baby carrots has been defined by the manufacturer as 85 g of baby carrots on the nutritional label. All calculations in this dissertation have been performed using this serving size.

Figure 7a. Bolthouse® Farms Premium Sweet Petites™ baby carrots used in the study



Nutrition Facts
Serving Size 3 oz. (85g)

Amount Per Serving	
Calories 35	Calories from Fat 0
	% Daily Value*
Total Fat 0g	**0%**
Saturated Fat 0g	**0%**
Trans Fat 0g	
Cholesterol 0mg	**0%**
Sodium 65mg	**3%**
Potassium 270mg	**8%**
Total Carbohydrate 8g	**3%**
Dietary Fiber 2g	**8%**
Sugars 5g	
Protein 1g	

Vitamin A 120% • Vitamin C 10%
Calcium 2% • Iron 2%

*Percent Daily Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs:

	Calories:	2,000	2,500
Total Fat	Less than	65g	80g
Saturated Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Potassium		3,500mg	3,500mg
Total Carbohydrates		300g	375g
Dietary Fiber		25g	30g

Calories per gram:
Fat 9 Carbohydrates 4 Protein 4

Figure 7b. Nutritional label of Bolthouse® Farms Premium Sweet Petites™ baby carrots

2.1.2. Pectin methylesterase (PME) enzyme solution

FirmSEB® (Specialty Enzymes and Biotechnologies Co., California) PME liquid enzyme was used for enzyme treatment of baby carrots. The enzyme activity of this enzyme solution was declared by the manufacturer as 1000 U/g. (1 enzyme unit (1 U) is defined as the amount of enzyme that catalyzes the conversion of 1 micro mole of substrate per minute. The SI unit of enzyme activity is defined as katal (kat) which is the amount of enzyme required that catalyzes the conversion of 1 mole of substrate per second. ($1\ U = 16.67 \times 10^{-9}$ kat = 16.69 nkat).

The manufacturer recommended usage of the enzyme was 200 g – 500 g of the FirmSEB® PME solution per ton of produce. As per this recommendation, the FirmSEB® PME solution was diluted to 0.0175 % of the original solution for experimental purposes.

2.1.3. Calcium Lactate Gluconate (CLG)

Calcium lactate gluconate by Jungbunzlauer Suisse Ag (Switzerland) was procured from Univar USA (Edison, NJ) in anhydrous form. Different concentrations of the CLG solution (w/v) were made by mixing the anhydrous CLG in DI water at room temperature.

2.1.4. Peptone water

Peptone water was used as a buffer solution for dilutions during microbiological studies in the shelf life analysis. Peptone water was prepared by dissolving 1.5 g of Difco™ peptone powder in 1 L of DI water. The solution was divided into aliquots of 9 mL in glass test tubes for serial dilution. These test tubes were sterilized by autoclaving at 121 °C for 15 min.

2.1.5. Total plate count (TPC) agar

The total plate count (TPC) agar was used as the plating media for the microbiological studies in the shelf life analysis. TPC agar prepared by dissolving 23.5 g of Difco™ TPC agar powder in 1 L of DI water. The solution was mixed on a hot plate at 400 °C with a magnetic stirrer until a clear solution was obtained. This solution was sterilized by autoclaving at 121 °C for 15 min. The autoclaved solution was allowed to cool down to 50 °C. 20 mL of the sterilized TPC agar was poured onto sterile petri dishes and allowed to solidify.

2.1.6. Instruments used for measurement of calcium and beta-carotene extractability, texture (hardness), and color

The amount of calcium infused was measured using an ICP-OES, beta-carotene extractability was measured using a reverse phase HPLC, the texture was measured using a CT3 Brookfield Texture Analyzer, and a Konica Minolta CR-410 colorimeter was used

to measure the color. These instruments and their respective working principle has been discussed in detail in the sections 2.4.1.2., 2.4.2.2., 2.4.3.2., and 2.4.4., respectively.

2.2 Experimental design

2.2.1 Box-Behnken design

A 3^3 Box-Behnken deisgn (BBD) of experiments (see Fig. 8) was used to evaluate the effect of three independent factors – pressure (MPa), time (min), and CLG solution concentration (% w/v) on high pressure infusion of calcium in PME treated baby carrots. According to the BBD, each factor was varied at three levels in the following intervals:

	Pressure (MPa)	Time (min)	CLG solution concentration (% w/v)
Lower level	150	5	3
Upper level	550	15	9

Table 6. Lower and upper levels of pressure (MPa), time (min), and CLG solution concentration used in the BBD experimental design.

Instead of performing 81 runs for a 3^3 full factorial design (with each point 3 times), BBD allows to obtain Response Surface Models (RSM) with 15 experimental runs (12 + 3 center points). The number of runs is determined by the equation $N = 2p (p-1) + C_p$ (p = number of factors; C_p = number of center points).

Each axis in Fig. 8 represents an independent factor. The center points of each of the cube edges (12) and the center point of the cuboid (3, performed in triplicate), as depicted in Fig. 8, are the 15 experimental points performed according to the BBD.

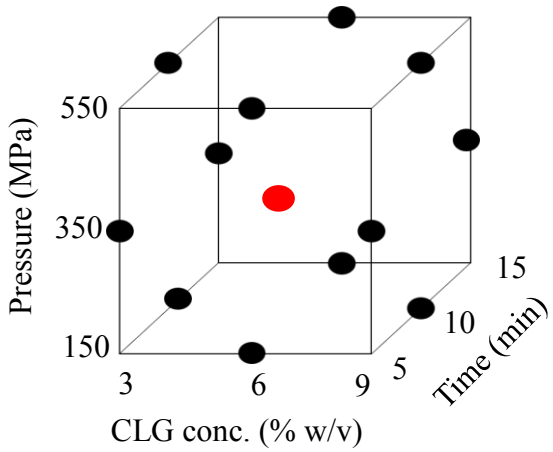


Figure 8. Experimental points according to the BBD used in the present study.

The experiments were performed in a randomized order according to the Box-Behnken design as shown above. The red center point was performed 1+2 times. The responses from each experiment were recorded as the parameter to be analyzed (infused calcium, extractability of beta-carotene, texture, and color). A response surface methodology (RSM) was used to study, optimize and evaluate the main effects (ex. CLG solution concentration), the quadratic effects (ex. Pressure^2), and the interaction effects (ex. Pressure * CLG solution concentration). RSM is a statistical and mathematical technique that helps in quantifying and determining the functional relationship between a response and the independent experimental factor. The measured response y (y can be either amount of calcium infused or extractability of beta-carotene or texture measured in

terms of hardness or color) for a nonlinear quadratic RSM is given by the following model equation (Eq. 1)

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad \text{(Eq. 1)}$$

where, β_0 is the model constant, $\beta_1, \beta_2, \beta_3$ are linear coefficients, $\beta_{11}, \beta_{22}, \beta_{33}$ are the quadratic coefficients and $\beta_{12}, \beta_{13}, \beta_{23}$ are cross product coefficients (Aslan and Cebeci, 2007).

The three independent factors in a BBD were expressed as coded or uncoded values in an experimental design. The uncoded values were the actual values of the independent factors (ex. 350 MPa, 5 min, 9 % CLG concentration). In terms of coded values, a value of -1 represents the lowest level of the factor, a value of +1 represents the highest level of the factor, and a value of 0 represents the midpoint value of the factor. The nomenclature described in Table 7 was used for the representation of coded and uncoded values.

Factor	Coded representation	Uncoded representation
Pressure	X_1	P
Time	X_2	t
CLG solution concentration	X_3	C

Table 7. Nomenclature of the coded and uncoded values for the independent factors

Table 8 denotes the coded values for each value of the independent factors.

Independent factor	**Coded value**		
	-1	**0**	**1**
Pressure (MPa)	150	350	550
Time (min)	5	10	15
CLG solution concentration (% w/v)	3	6	9

Table 8. Coded values and uncoded variables of the BBD

Table 9 shows all the 15 BBD experiments that were performed.

Run #	Coded values			Uncoded values		
	X_1	X_2	X_3	P (MPa)	t (min)	C (% w/v)
1	-1	0	-1	150	10	3
2	1	0	1	550	10	9
3	-1	1	0	150	15	6
4	0	0	0	350	10	6
5	1	-1	0	550	5	6
6	-1	0	1	150	10	9
7	1	0	-1	550	10	3
8	0	1	1	350	15	9
9	0	-1	-1	350	5	3
10	0	-1	1	350	5	9

11	1	1	0	550	15	6
12	-1	-1	0	150	5	6
13	0	1	-1	350	15	3
14	0	0	0	350	10	6
15	0	0	0	350	10	6

Table 9. BBD experimental design.
Highlighted experimental points represent the center point.

For each point in the BBD, a total of seven responses were measured – amount of calcium absorbed (mg calcium per serving size of 85 g of baby carrots), beta-carotene extracted (mg beta-carotene per serving size of 85 g of baby carrots), texture (N), and four responses for color (L*, Chroma (C), Hue (h), color difference (ΔE, calculated value)). Measurement of these responses is discussed in detail in section 2.4.

Using the data obtained from the experimental design, response surface analysis was performed to evaluate the effect of processing parameters (pressure and time) and CLG solution concentration on each of the responses.

2.2.2 Pressure cycling

In a typical high pressure process, a cycle of pressurization-pressure hold-depressurization occurs only once as shown in Fig. 4. However, repetition of this cycle of pressurization-pressure hold-depressurization, with no time gap between two cycles, may have a significant role in the extent of infusion in food products.

In order to evaluate the effect of pressure cycling, all the three parameters of pressure, time, and CLG solution concentration were kept constant at their highest values of 550 MPa, 15 min, and 9 % (w/v), with a change only in the number of pressure cycles. However, the hold time during each cycle changes with the number of pressure cycles involved so as to have a total hold time of 15 min.

Experiments were carried out with 3 different pressure cycles, i.e., single cycle (1 cycle), 3 cycles, and 5 cycles. For single cycle of HPP, the pressure hold time during the cycle was 15 min. When 3 pressure cycles were employed, each pressure cycle had a hold time of 5 min, thus leading to a total hold time of 15 min from all the three cycles. Similarly, the hold time for each cycle during 5 pressure cycling processing was 3 min each, contributing to a total of 15 min hold time after 5 cycles. It must be noted that the cycles were carried out consecutively without unloading the samples between each cycle. The come-up time (CUT) for each cycle was ignored. Fig. 9 shows a simple representation of pressure cycling.

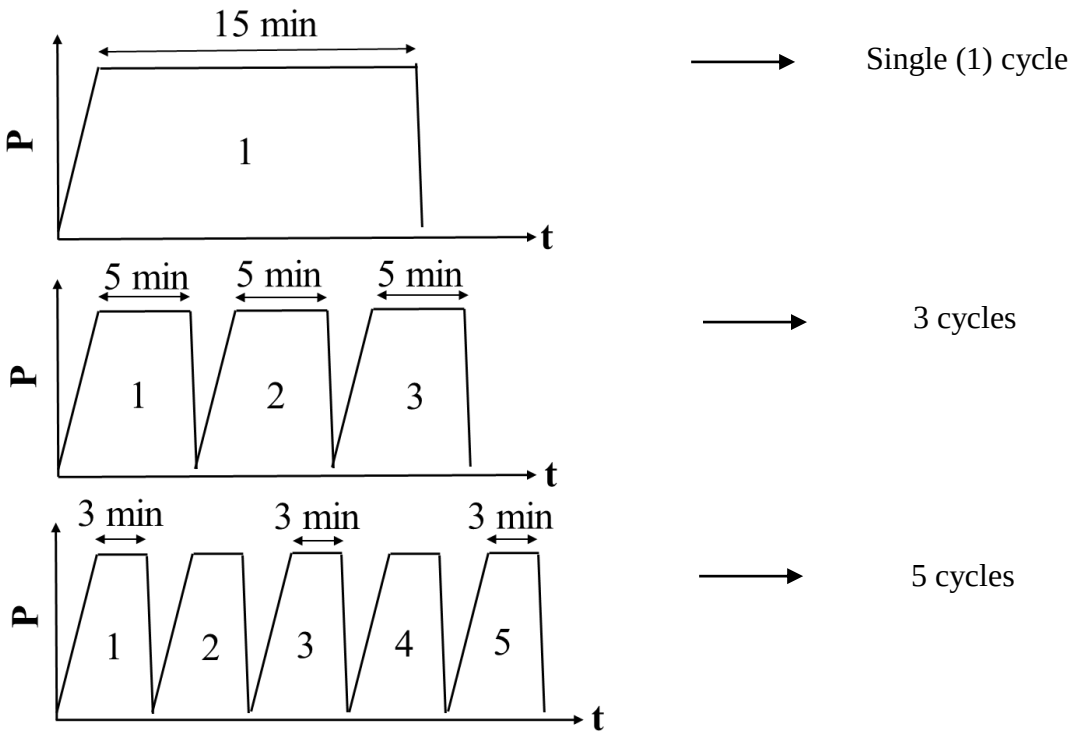


Figure 9. Pressure cycling and hold time during each cycle.

Note: All three have the same hold time of 15 min.

Pressure cycling experiments were performed in duplicates, with duplicate analysis of each replicate. Analysis was performed for the amount of calcium absorbed (mg calcium per serving size of 85 g of baby carrots), beta-carotene extracted (mg beta-carotene per serving size of 85 g of baby carrots), texture (N), and color (L*, Chroma (C), Hue (h), color difference (ΔE)).

2.2.3 Controls

Three types of controls were used to compare the effect of high pressure assisted infusion:

- Unprocessed raw control: Unprocessed fresh baby carrots bought from the retail store.
- Osmotically infused baby carrots: Baby carrots were osmotically infused with calcium by simply soaking them in 9 % (w/v) solution of CLG for 15 min.
- Vacuum infused baby carrots: Baby carrots were infused with calcium under vacuum using a 9 % (w/v) CLG solution. Infusion was carried out for 15 min under a vacuum of 40 mm Hg (= 0.052 atm).

The control samples were processed in duplicates, with duplicate analysis of each replicate. Analysis was performed for the amount of calcium absorbed (mg calcium per serving size of 85 g of baby carrots), beta-carotene extracted (mg beta-carotene per serving size of 85 g of baby carrots), texture (N), and color (L*, Chroma, Hue (°), ΔE).

2.2.4 Experiments to evaluate the effect of calcium infusion and extent of high pressure

In order to understand the effect of calcium infusion and the extent of high pressure on the texture and the color of the high pressure processed-calcium infused baby carrots, PME treated baby carrots were high pressure processed in absence of infusate solution, with only DI water (absence of calcium), and with 6 % CLG solution. The hold

time for these set of experiments was kept constant at 15 min. Experiments were performed in duplicates according to Table 10, with duplicate analysis of each replicate. Analysis was performed to evaluate the color (L*, Chroma, Hue (°), ΔE).

Infusate solution	High pressure extremes	
	100 MPa	600 MPa
No infusate	×	×
DI water	×	×
6 % CLG solution	×	×

Table 10. Experiments performed at extreme high pressure conditions with different infusate solutions

2.3 Processing

2.3.1 PME treatment

PME enzyme solution of concentration 0.0175 % was first heated up to a temperature of 37 °C in a water bath. Once the temperature was reached, baby carrots were soaked in the PME solution (0.0175 %) in the proportion of 1:2 baby carrots:enzyme solution ratio (w/w) for 45 min at 37 °C. The PME solution was drained off after the treatment and carrots were thoroughly washed in deionized water to remove residual PME solution.

2.3.2 Preparation of CLG solution

CLG solution was prepared by mixing the anhydrous CLG powder in deionized water at room temperature. Solutions of 3 %, 6 %, and 9 % (w/v) were made for experimental purposes.

2.3.3 Sample preparation for HPP

PME treated baby carrots were vacuum packed in their respective CLG solutions (according to the BBD experimental design) in the ratio of 1:2 baby carrots:CLG solution.

Figure 10. PME treated baby carrots vacuum packed in CLG solution.

2.3.4 High pressure processing (HPP)

HPP was carried out under the pressure-time conditions according to the BBD experimental design and according to Table 10. Pressure cycling was carried out at 550 MPa, 9 % CLG solution concentration, and according to the cycles described previously in section 2.2.2.

Figs. 11a, 11b, and 11c show the pressure and temperature data recorded as a function of time during the pressure cycling experiments.

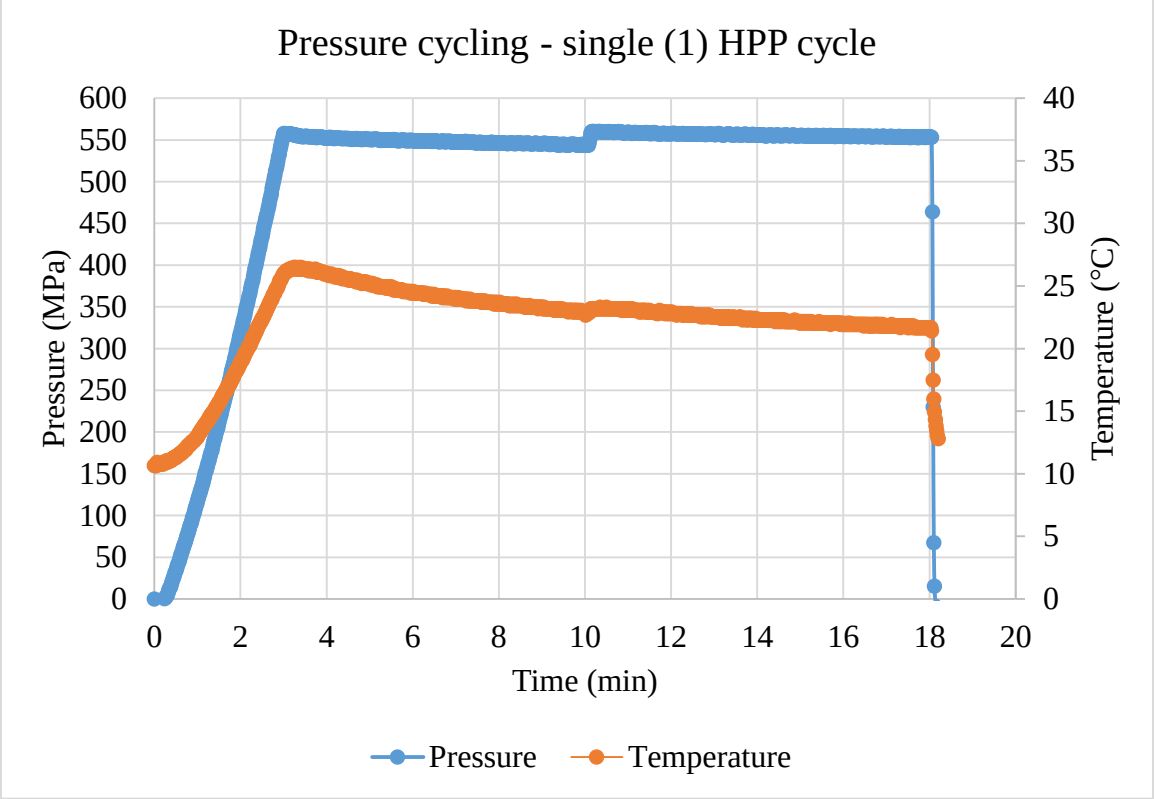


Figure 11a. Single pressure cycle as recorded in the LabVIEW™ software.

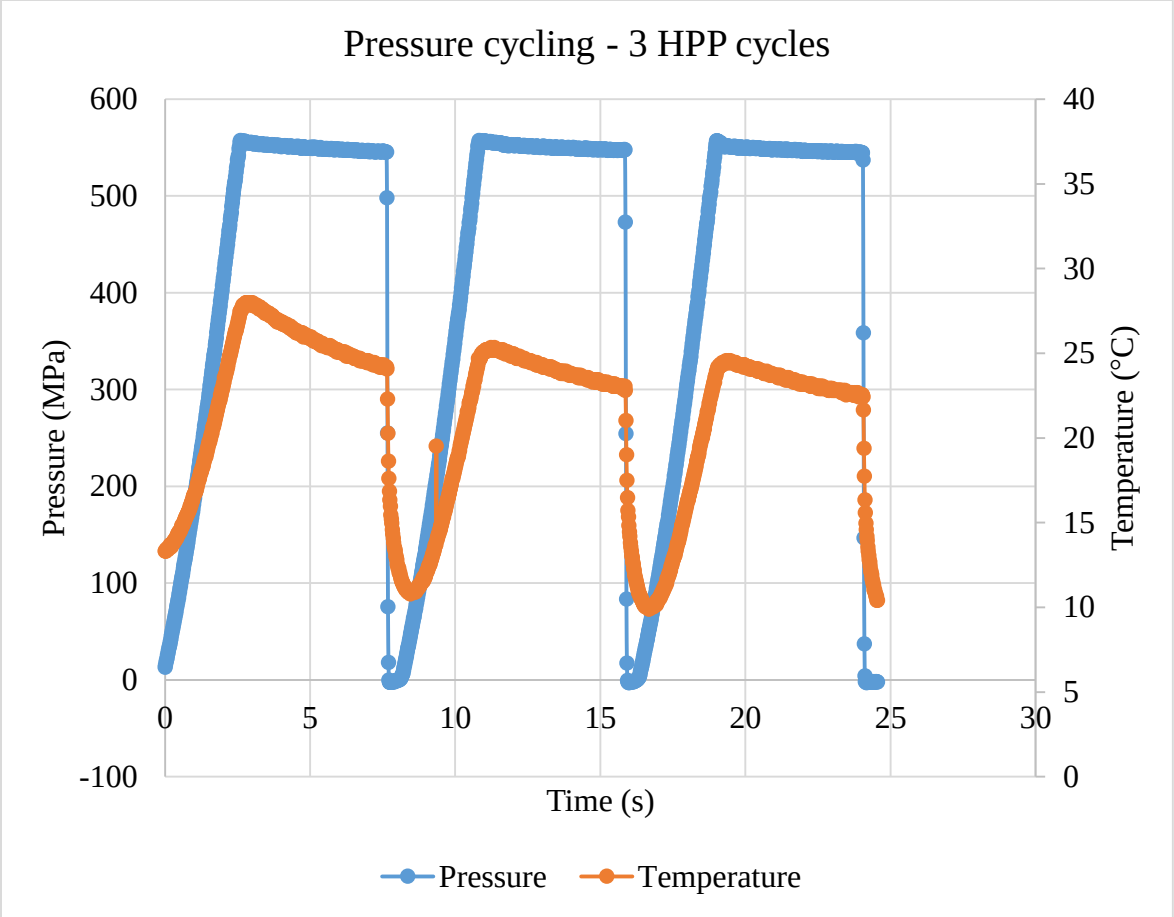


Figure 11b. Pressure cycling – 3 cycles, as recorded in the LabVIEW™ software.

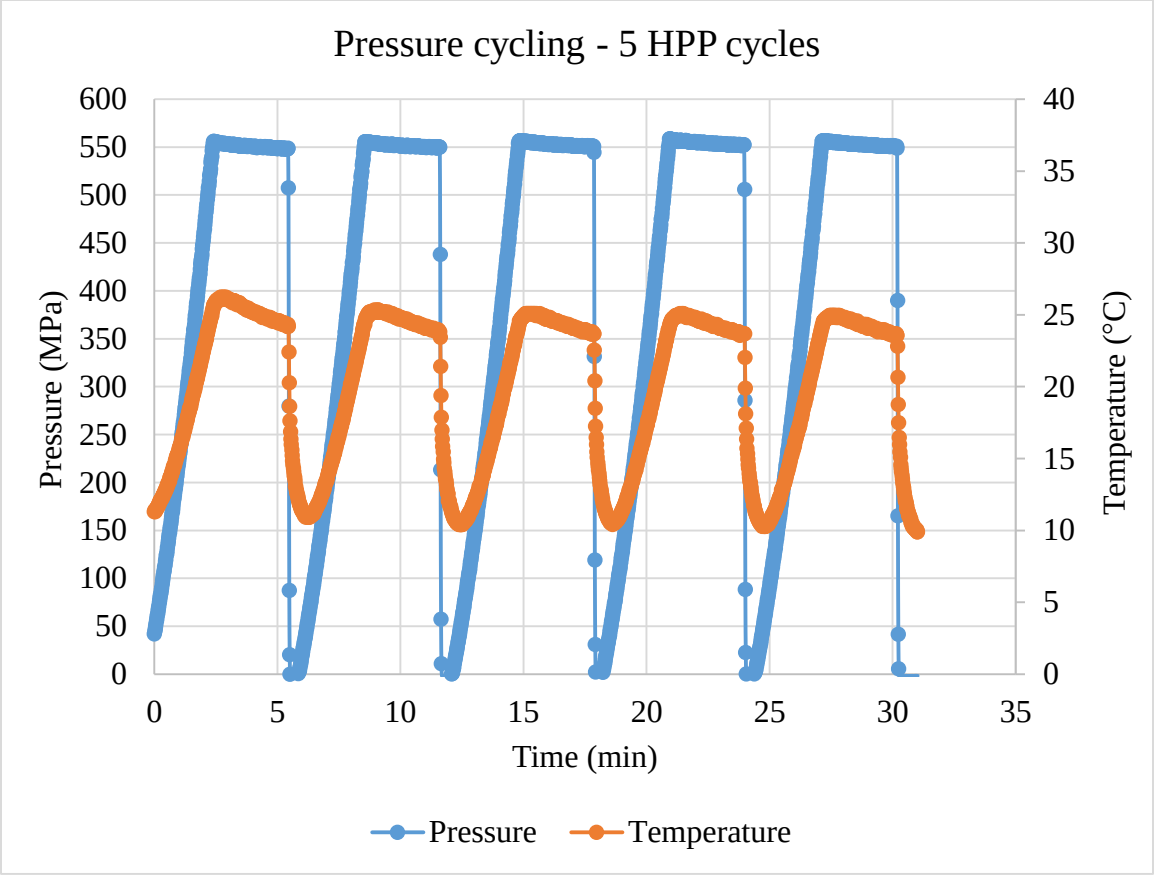


Figure 11c. Pressure cycling – 5 cycles, as recorded in the LabVIEW™ software.

2.3.5 Post-processing and storage of samples

After high pressure processing of the baby carrots, the CLG solution was drained and carrots were washed with deionized water. Half of the carrots from each of the processed samples were stored at – 20 °C to be analyzed for calcium and beta-carotene later, while the other half were immediately analyzed for texture and color.

2.4 Analysis

2.4.1 Calcium analysis

The amount of calcium in the baby carrot samples was measured using ICP-OES (Inductively Coupled Plasma – Optical Emission Spectroscopy) in the Department of Marine Sciences at Rutgers University.

2.4.1.1 Sample preparation

Processed baby carrots along with controls were first ground in a home blender (Osterizer®, Sunbeam products Inc., Florida), until carrot pieces broke down into small pieces and a coarse mixture of baby carrots pieces was obtained. 30 g of ground baby carrots was weighed in a ceramic crucible and subjected to ashing in a muffle furnace (Thermolyne corporation, Iowa) at 600 °C for 6 h.

Figure 12. Carrot samples before (left) and after (right) ashing.

The ashed samples were dissolved in 5 mL of conc. HNO_3 (15.8 M HNO_3). 0.1 mL of the concentrated acid containing the dissolved ash was mixed with 4.9 mL of deionized water to make a final concentration of 2 % HNO_3 in the samples. These samples were then analyzed in the ICP-OES for quantification of calcium infused.

2.4.1.2 ICP-OES

Varian Vista-Pro CCD Simultaneous ICP-OES in the Department of Marine Sciences at Rutgers was used for analysis of calcium in the baby carrots. ICP-OES is a technique to determine the elemental composition (commonly in water) in samples using plasma and a spectrophotometer.

Figure 13. ICP-OES at the Department of Marine Sciences, Rutgers University.

The solution to be analyzed is carried by a peristaltic pump through a nebulizer into a spray chamber. The aerosol produced by the sample is lead into an argon plasma. The temperature of this plasma is between 6000 K – 7000 K. As a result of the interaction with the hot plasma, the elements in the solution get ionized. Due to the thermal energy absorbed by the electrons, they reach an excited state. When these excited electrons come back to the ground energy level they release energy in the form of light (photons). Using an Echelle grating (a type of diffraction grating), a prism, and a focusing mirror, the emitted photons are captured on a CCD (charged coupled device) chip, and detected using a UV-Vis spectrophotometer. Each element has its own characteristic emission spectrum (Radboud University (Netherlands), retrieved on 2016).

Calcium was analyzed at five different wavelengths of 315.887 nm, 317.993 nm, 393.366 nm, 396.847 nm, and 422.673 nm. Based on the R^2 values of the linear standard curves obtained at each wavelength, samples were chosen to be analyzed at 317.993 nm ($R^2 = 0.9996$).

2.4.1.3 Sample analysis

Each of the processed as well as the control baby carrot samples was analyzed in triplicates in the ICP-OES. A standard curve was obtained by analyzing standard solutions of calcium in the range of 0 ppm – 500 ppm with an increment of 100 ppm for each standard solution. A 1000 ppm standard solution (1000 µg/mL of $CaCO_3$ in 2 % HNO_3) procured from High Purity Standards™ (Charleston, South Carolina) (Catalog number: 10009-1) was used to prepare the standard solutions of desired concentrations (0 ppm – 500 ppm).

The ICP-OES gives the data for calcium content in arbitrary units of intensity. Based on the data obtained from the standard solutions, a standard curve at 317.993 nm was obtained which is shown in Fig. 14.

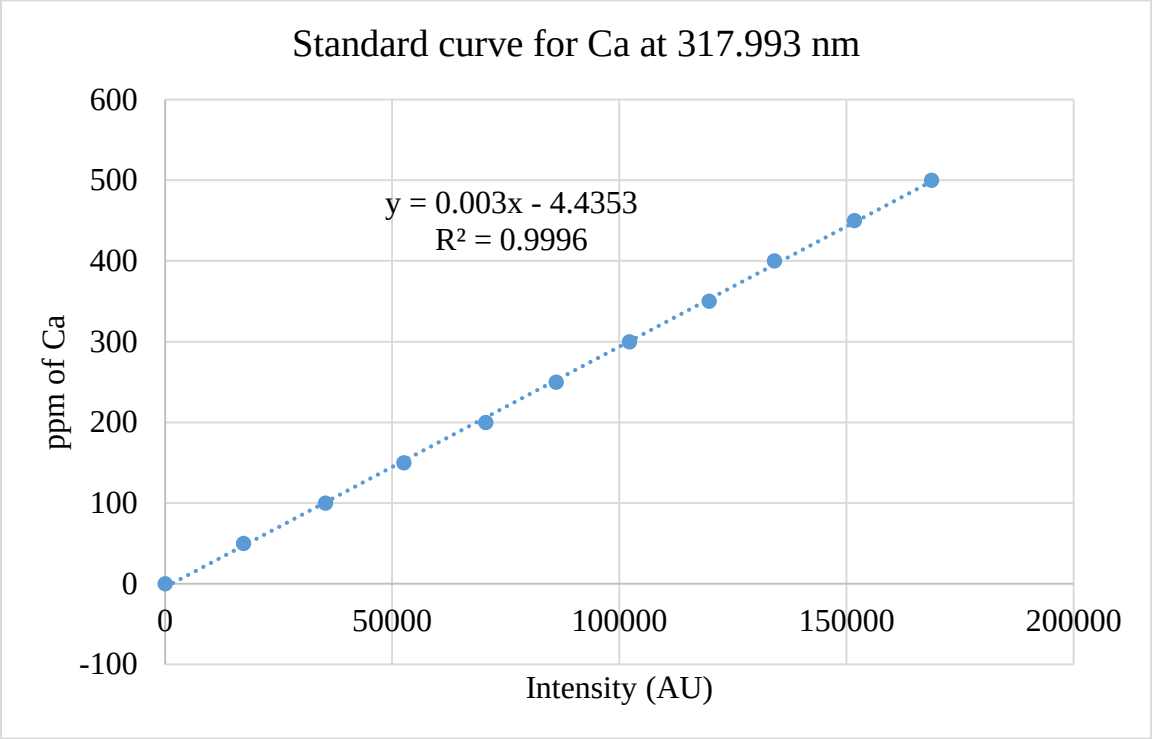


Figure 14. Standard curve for estimation of calcium at 317.993 nm

Using the standard curve, calcium content in samples can be calculated in terms of ppm. In order to convert the value obtained in ppm to amount of calcium (mg) per 85 g of carrots, following set of calculations were performed. It is important to refer to the preparation of sample for calcium analysis to understand the following calculations. The preparation of sample for calcium analysis has been summarized in the flow chart in Fig. 15 for reference.

30 g carrots

↓ Ash in the muffle furnace (600 °C for 6 h)

Ash

↓ Dissolve in 5 mL conc. HNO_3

5 mL conc. HNO_3 with dissolved ash

↓ Dilute to 2 % HNO_3

0.1 mL conc. HNO_3 + 4.9 mL DI water

↓ Analyze in ICP-OES

Intensity reading

↓ Standard curve

'x' ppm

Figure 15. Flow chart for sample preparation for calcium analysis

Calculations:

Assume a value of 'x' ppm was obtained for one of the samples.

x ppm = x mg calcium/L sample = (0.001)*x mg calcium/mL of sample

➟ 5 mL of analyzed sample ➔ 5*0.001*x mg calcium = 0.005*x mg calcium

➟ 0.1 mL of sample in conc. HNO_3 ➔ 0.005*x mg calcium

➟ 5 mL of sample in conc. HNO_3 ➔ 50*0.005*x mg calcium = 0.25*x mg calcium

➟ ash from the sample ➔ 0.25*x mg calcium

➟ 30 g carrots ➔ 0.25*x mg calcium

➟ one serving of baby carrots ➔ (0.25*x)*(85/30) mg calcium

2.4.2 Beta-carotene analysis

2.4.2.1 Extraction of beta-carotene

Baby carrots were ground in a home blender (Osterizer®, Sunbeam products Inc., Florida) and then extracted for beta-carotene following a procedure suggested by Rodriguez-Amaya (2001). Beta-carotene, being a photosensitive compound, the extraction was carried out under dark conditions.

1 g ground sample was mixed with 50 mL of cold acetone along with 0.05 g of BHT (butylated hydroxytoluene) in a test-tube. Beta-carotene is prone to degradation at ambient temperatures and hence, cold acetone is used. This mixture was then subjected to various extraction techniques as below, in that order:

1. Shear mixing for 20 min: A Polytron® PT 1600 E bench top homogenizer was used.
2. Ultrasonication for 2 min: The sample was subjected to ultrasonication in an ultrasonic water bath (CPXH series, purchased from Fisher Scientific) at 25 °C.
3. Vacuum filtration using a Whatman filter paper #1 to remove the residual calcium tissues. Vacuum filtration was carried out using 50 mL of cold acetone.
4. Partitioning to petroleum ether using decantation: The filtrate which contains beta-carotene dissolved in acetone was subjected to decantation with petroleum ether to change the solvent from acetone to petroleum ether. Beta-carotene can degrade in acetone if kept for long, and hence is extracted in a

less polar organic solvent, petroleum ether. The decantation was carried in a separatory funnel using water. The filtrate is poured in the separatory funnel containing 100 mL of petroleum ether. This mixture is washed with 100 mL of deionized water five times to get rid of the acetone. Acetone being slightly polar, dissolves in water and is separated from petroleum ether which does not dissolve in water. The mixture thus gets separated into two layers – the lower transparent layer of water and acetone, and the upper yellowish layer of petroleum ether that contains beta-carotene. The lower layer is discarded and the mixture is washed with deionized water again, repeating the procedure. After five washings, the upper layer of petroleum ether containing beta-carotene is collected in a round bottom flask.

5. Rotary evaporation: The petroleum ether mixture collected in the round bottom flask is subjected to rotary evaporation at 37 °C until all the solvent is evaporated and only the yellow residue of beta carotene remains.
6. Dissolution in mobile phase: The residual beta-carotene is dissolved in 15 mL of the mobile phase (70 % acetonitrile, 15 % methanol, 15 % methyleneschloride).
7. Storage at -20 °C: The beta-carotene sample dissolved in the mobile phase is stored in amber colored glass bottles at -20 °C until analysis in the HPLC, to prevent the degradation of beta-carotene.

Each sample was extracted 3 times.

2.4.2.2 HPLC

Beta-carotene analysis was performed in a reverse phase HPLC according to the specifications described by Kim and Quadro (2010). The mobile phase used was a combination of acetonitrile, methanol, and methylene chloride in the ratio of 70:15:15. The chromatography conditions used were as below:

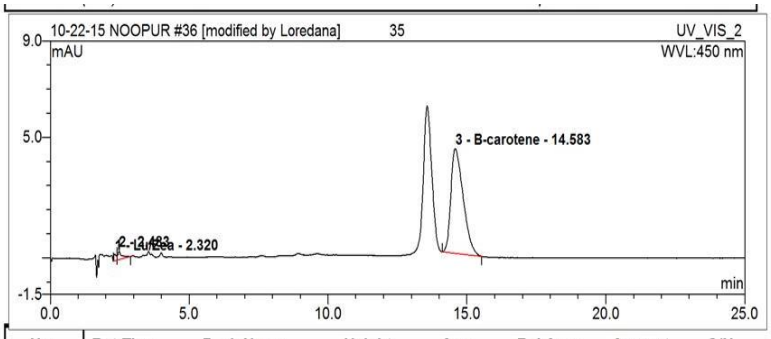
Column	Beckman Ultrasphere C18 (5 μm), 4.6 mm × 250 mm
Guard column	C18 (7 μm), 15 mm × 3.2 mm
Flow rate	1.8 mL/min
Run time	35 min
Injection volume	20 μL
PDA detection wavelength	325 nm

Table 11. Specifications of the HPLC conditions used for beta-carotene analysis

2.4.2.3 Sample analysis

Samples stored at -20 °C were transferred into small glass vials with septa and placed in the auto-sampler of the HPLC. Along with the samples of high pressure processed baby carrots and controls, external standards in the concentration range of (0 ng beta-carotene/μL – 20 ng beta-carotene/μL) were also analyzed. Beta-carotene standard was purchased from Sigma Aldrich® (Catalog number: C4582-5MG). A PDA (photo-diode array) detector was used to measure the absorbance of beta-carotene at 325 nm.

The beta-carotene component in the sample had a retention time of 14.5 min (See Fig. 16). A characteristic absorption curve as observed in the HPLC is shown in Fig. 16. Using the area under the beta-carotene curve (mAU*min) a standard curve was plotted as shown in Fig. 17. Using this standard curve and the area under the curve of beta-carotene (mAU*min), concentration of beta-carotene extracted from the samples was calculated.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	S/N
1	2.32	Lu/Zea	0.280	0.026	1.13	n.a.	n.a.
2	2.48	n.a.	0.356	0.060	2.61	n.a.	2.7
3	14.58	B-carotene	4.332	2.227	96.26	n.a.	1.4
Total:			4.968	2.313	100.00	0.000	

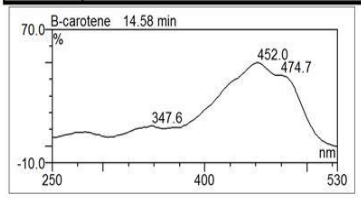


Figure 16. HPLC chromatogram of beta-carotene analysis

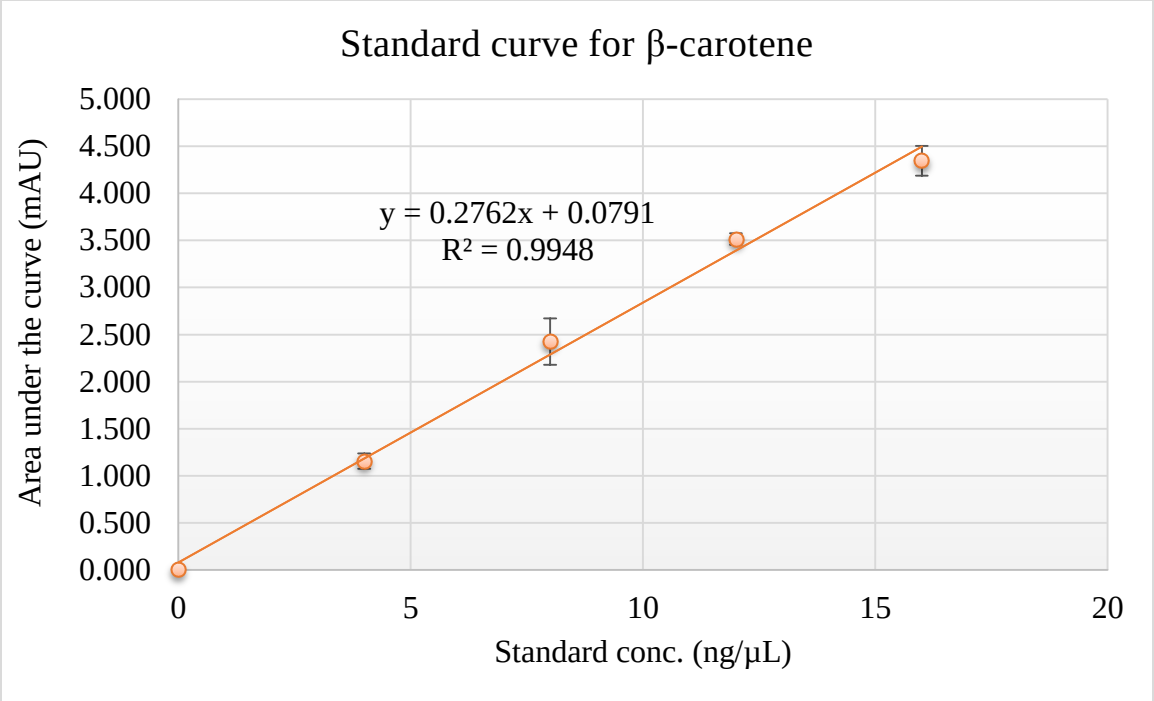


Figure 17. Standard curve for estimation of beta-carotene in baby carrots

In order to calculate the amount of beta-carotene extracted from one serving (= 85 g) of baby carrots, it is important to remember that the beta-carotene extracted from 0.5 g of ground baby carrots was dissolved in 15 mL of the mobile phase, which was analyzed in the HPLC. Using the standard curve, beta-carotene content in the sample is obtained in terms on ng/µL.

Calculations:

Assume, for a given sample, 'a' ng/µL as calculated from the standard curve.

a ng/µL = (0.001)*a mg beta-carotene/mL

➡ 15 mL of mobile phase ➔ (15*0.001*a) mg beta-carotene

➟ 0.5 g baby carrots ➔ (15*0.001*a) mg beta-carotene

➟ 85 g baby carrots ➔ (85/0.5)*(15*0.001*a) mg beta-carotene = (2.55*a) mg beta-carotene

2.4.3 Texture analysis

The texture of high pressure processed and control baby carrots was analyzed as hardness (N) using a Texture Analyzer.

2.4.3.1 Sample preparation

Baby carrots were cut into cylinders of 1 cm height to be analyzed for texture (see Fig. 19a). Measurement for each sample was performed in triplicates.

2.4.3.2 Texture analyzer

A CT3 Brookfield Texture Analyzer equipped with a TA-BT fixture was used for texture analysis. A 25.4 mm diameter flat base cylindrical probe (TA 3-100) was used to perform the measurements.

Figure 18. CT3 Brookfield Texture Analyzer used for texture analysis of baby carrots

2.4.3.3 Sample analysis

A 1 cm long cylindrical baby carrot piece was compressed axially to 50 % using a TA 3-100 cylindrical probe. According to Bourne (2002), the maximum force required to axially compress a cylindrical sample of carrot by 50 % is quantified/defined as the hardness of the carrot. The test speed for the probe was set as 1 mm/s. The characteristic curve obtained during compression of the carrot cylinder is shown in Fig. 20.

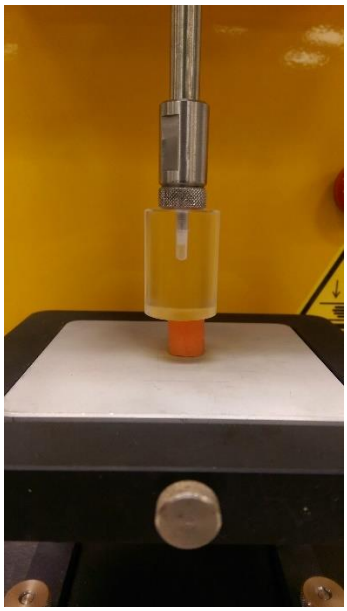


Figure 19a. 1 cm long baby carrot cylinder before compression

Figure 19b. Baby carrot cylinder after compression

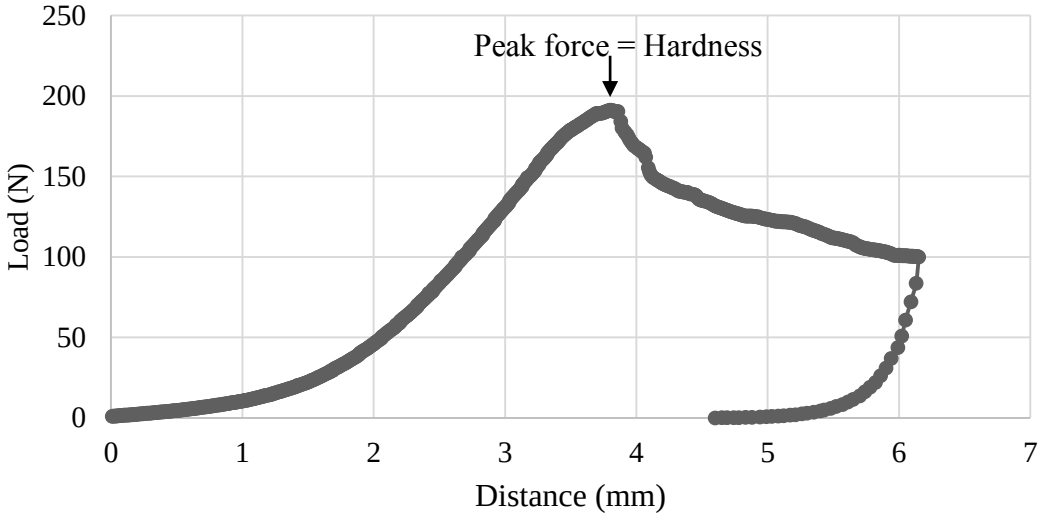


Figure 20. Texture analysis graph for compression of a baby carrot cylinder by 50 % (Test speed = 1 mm/s)

2.4.4 Color analysis

A Konica Minolta CR-410 colorimeter was used to measure the color of high pressure processed and control baby carrots. The colorimeter was calibrated to D_{65} standards (Y = 94.7, x = 0.3156, and y = 0.33199). Baby carrots were placed on an opaque white base and the color was measured using the colorimeter in terms of L*, a*, b* values (CIE Lab color space). L* value represents the brightness of the sample, with lower values indicating darker samples and higher values indicating brighter samples. The a* value represents the greenness (-ve value)/redness (+ve value) of the samples while the b* value represents the blueness (-ve value)/yellowness (+ve value) of the samples. Color measurement for each sample was performed in triplicates.

In the Munsell color system, the color of the samples is quantified in terms of L* (also called VALUE), Chroma (C), and Hue (h). L* is the vertical (y) axis that represents the lightness or darkness of the samples. Chroma is defined as the amount of saturation or purity of a particular color, and Hue is the angular representation of color and is expressed in terms of degrees, starting from Red at 0° to Yellow at 90°, Green at 180° to Blue at 270° as explained in Fig. 21.

The L*, a*, and b* values measured were converted to Munsell color system using the Eq. 2 and Eq. 3.

$$Chroma = \sqrt{(a^{*2} + b^{*2})} \quad \text{Eq. 2}$$

$$Hue = h^{\circ}_{ab} = \tan^{-1}\frac{a^*}{b^*} \quad \text{Eq. 3}$$

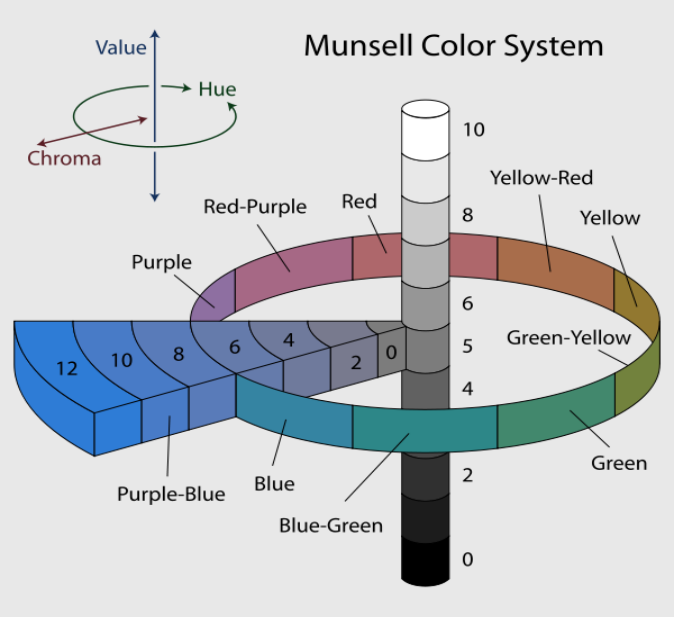


Figure 21. Munsell color system

In order to analyze the change in color of the sample after calcium infusion under high pressure, color difference (ΔE) was calculated as follows:

$$\Delta E = \sqrt{\left(L^*_{sample} - L^*_{unprocessed}\right)^2 + \left(a^*_{sample} - a^*_{unprocessed}\right)^2 + \left(b^*_{sample} - b^*_{unprocessed}\right)^2}$$

Eq. 4

2.4.5 Shelf life analysis

Shelf life analysis (measured in terms of microbial growth and changes in texture and color over a storage of 10 days) was performed on the baby carrots that were infused with maximum calcium. Maximum infusion of calcium in baby carrots was obtained when the baby carrots were infused using 9 % CLG solution at 350 MPa for 15 min.

The calcium infused baby carrots were stored in a refrigerator during the course of analysis. Analysis was performed immediately after processing and 2, 5, and 10 days after storage.

The analyses performed to evaluate the shelf life of the high pressure processed – calcium infused baby carrots were:

a. Texture analysis (as described in 2.4.3.)
b. Color analysis (as described in 2.4.4.)
c. Microbial analysis

For microbial analysis, 10 g – 15 g of baby carrot samples were homogenized in 9 times the amount of peptone water (90 mL – 135 mL) for 5 min in sterilized filter bags. Homogenization was carried out for 5 replicates of each sample. The homogenized samples (10^1 dilution) were serially diluted 4 times to achieve the dilutions of 10^2, 10^3, 10^4, and 10^5, by mixing 1 mL of the previous dilution in 9 mL of sterilized peptone water.

0.1 mL of each of the serial dilutions for each replicate was plated on the TPC agar in duplicates. The plated samples were incubated at 37 °C for 24 h and the colonies were counted after the incubation period. Samples with colony counts between 30 – 300 were considered. Based on the colony count and the dilution factor, CFU (colony forming units)/g of the sample was calculated.

CFU/g baby carrots = (no. of colonies) x (dilution factor) / (volume plated = 0.1 mL)

CFU/g baby carrots = (no. of colonies) x (dilution factor) x 10

The data obtained from the microbial shelf life analysis was analyzed using DMFit by ComBase in Microsoft Excel 2016, to analyze the growth curve and determine the microbial lag time and growth rate.

3. RESULTS AND DISCUSSION

3.1. Analysis of controls

The controls - unprocessed raw baby carrots, baby carrots infused with calcium under osmosis, and baby carrots infused with calcium under vacuum – were analyzed for calcium infused (mg calcium per 85 g serving size of baby carrots), extracted beta-carotene (mg beta-carotene per 85 g serving size of baby carrots, hardness (N), color (L*, Chroma, Hue (°), color difference (ΔE)). Table 12 summarizes these results:

Samples	Calcium (mg/85 g baby carrots	Extracted beta-carotene (mg per 85 g baby carrots)	Hardness (N)	Color			
				L*	Chroma	Hue (°)	ΔE
Unprocessed raw baby carrots	19.54 ± 0.32	4.64 ± 0.72	141.46 ± 14.8	56.59 ± 0.29	44.84 ± 2.51	46.83 ± 3.17	0
Baby carrots osmotically infused with calcium	38.66 ± 9.36	14.15 ± 4.47	186.62 ± 34.24	54.94 ± 0.5	56.59 ± 0.7	52.52 ± 0.08	13.31 ± 0.93
Baby carrots vacuum infused with calcium	38.86 ± 4.74	16.42 ± 3.38	198.85 ± 27.65	55.38 ± 0.79	53.7 ± 0.69	51.98 ± 0.07	10.90 ± 1.44

Table 12. Analysis of controls

Infusion of calcium either by osmosis or vacuum impregnation doubled the amount of calcium in the baby carrots. However, no significant difference ($p > 0.05$) in the calcium content was observed between osmotically infused calcium and vacuum

infused calcium, contrary to expectations. Although, high standard deviation of osmotically infused baby carrots must be noted. Similarly, the hardness of baby carrots increased with infusion of calcium, but no significant difference ($p > 0.05$) between the two infused controls was observed.

The L* value of calcium infused controls did not show a significant difference ($p > 0.05$) from the unprocessed control; however, high values of Chroma and hue in the infused control indicated darker yellow color in comparison to unprocessed control.

The ΔE value for the infused control clearly showed that they were different ($p < 0.05$) from the unprocessed raw baby carrots. The difference was observed mainly in terms of brightness (infused carrots are darker i.e., less bright) and hue (infused carrots are more yellow and less orange).

3.2. Response surface methodology

A response surface analysis looks at the effect of independent factors (pressure, time, and CLG solution concentration) on the recorded responses (infused calcium, extracted beta-carotene, hardness, L*, Chroma, Hue, ΔE), by fitting a quadratic model which evaluates the linear, quadratic and interaction effects of the independent factors. The quadratic model calculates the coefficients (known as ‘model coefficients) for each of these linear, quadratic, and interaction effects, and the p-value of these coefficients determines the significance of their effect on the responses. Table 13 summarizes the

model coefficients calculated for the independent factors, their quadratic effects, and their quadratic interactions. For a given response, a model equation can be constructed from the coefficients and the intercept (mean value, also called as the model constant) of the response. When the insignificant terms ($p>0.05$) in the model equation are dropped, a predictive equation for determining the response is obtained. The coefficients and predictive equations have been calculated using the coded variables.

	X_1	X_2	X_3	$X_1 * X_2$	$X_1 * X_3$	$X_2 * X_3$	X_1^2	X_2^2	X_3^2
C	8.32[b]	7.86[b]	26.11[a]	7.44	10.63[b]	10.50[b]	-13.47[b]	4.91	6.03
B	-0.85	-2.07	-0.40	5.61[b]	0.71	-3.32	-2.70	4.48	-2.54
H	-11.37	11.78	1.94	-9.86	18.55	-0.26	-0.49	8.72	-4.91
L	0.50	0.63	-0.64	-0.57	-0.84	0.33	0.67	0.98	0.44
c	0.40	1.88	-1.72	-2.72	-0.76	0.22	1.85	2.79	-0.60
h	-0.13	-0.38	0.69	0.07	0.20	0.006	-0.55	-0.26	-0.33
E	-0.62	-1.04	1.27	0.78	1.00	-0.65	-1.19	-1.25	-0.68

Table 13. Model coefficients of the independent factors, their quadratic effects and interactions.

[a] $p < 0.01$ (very significant) [b] $0.01 \leq p < 0.05$ (significant)

Legend for Table 13:

X_1: Pressure (MPa); X_2: time (min); X_3: CLG solution concentration (%w/v); C: calcium content (mg per serving); B: extracted beta-carotene (mg per serving); H: hardness (N); L: L* value; c: Chroma; h: hue (°), E: color difference.

3.3. Effect of processing parameters

3.3.1. Calcium infusion

It can be seen from Table 13, based on the p-values that the amount of calcium infused in baby carrots was significantly affected by pressure, hold time, CLG solution concentration, pressure*CLG solution concentration, time*CLG solution concentration, and square of pressure. The predictive equation for determining the effect of pressure, hold time, and CLG solution concentration can be written as follows: (Note: the values are in coded variables)

Calcium content of high pressure-calcium infused baby carrots =

$$77.4 + 8.32^{*} X_1 + 7.86^{*} X_2 + 26.11^{*} X_3 + 10.64^{*} X_1^{*} X_3 + 10.5^{*} X_2^{*} X_3 - 13.47^{*} X_1^2$$

$$\ldots R^2 = 0.92 \qquad \text{Eq. 5}$$

The positive coefficients of pressure (X_1), time (X_2), and CLG solution concentration (X_3) in Eq. 5 indicate a positive effect of higher values of these variables on the infusion of calcium in baby carrots.

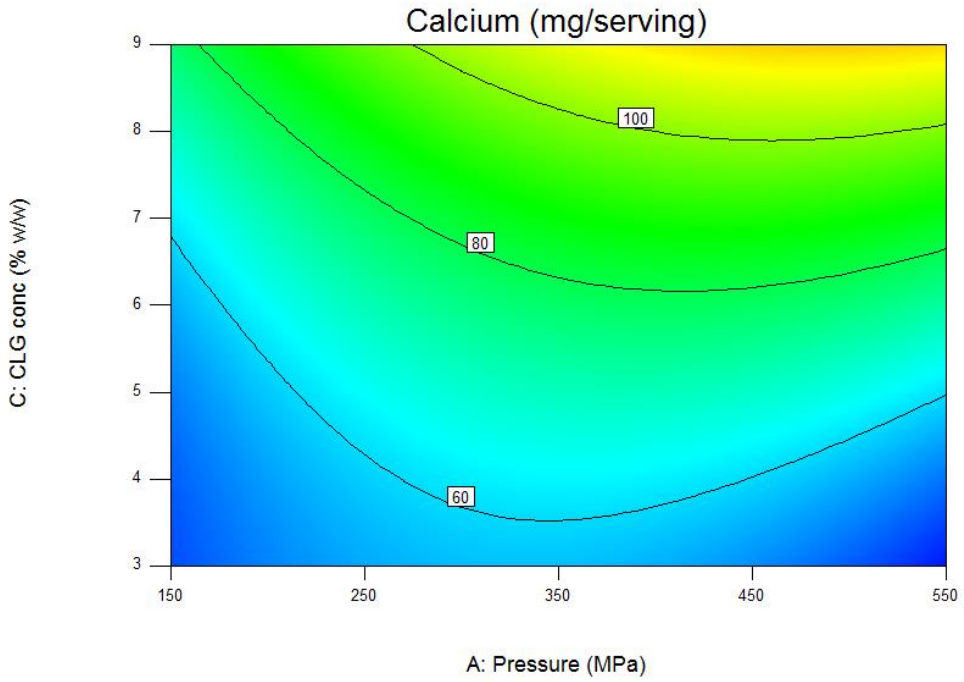


Figure 22. Effect of pressure-CLG solution concentration interaction on the infusion of calcium at a constant processing time of 10 min.

Fig. 22 shows a positive effect of interaction of pressure and CLG solution concentration on calcium infusion i.e., at higher pressures and higher CLG solution concentrations, infusion of calcium was higher. Higher concentration of CLG solution would mean that the difference between the calcium concentration in CLG solution and the calcium concentration in the baby carrots was higher. This higher difference of calcium concentration with higher CLG solution concentration was thought to contribute to a higher driving force for the infusion of calcium from the CLG solution to the baby carrots. The high concentration of CLG solution coupled with higher pressure showed a

positive effect on calcium infusion, as higher pressure would further contribute to a pressure driving force to infuse more calcium.

However, as seen in Fig. 22, at a constant value of CLG solution concentration, the infusion of calcium in baby carrots increased until moderate pressures of 300 MPa – 400 MPa, above which the effect of high pressure became negative. Thus, contrary to expectations, very high pressures (>350 MPa) decreased the infusion of calcium in the baby carrots. A possible explanation to this could be that at very high pressures, the system became too dense (for ex. too crowded) to allow an efficient flow of calcium into the food matrix, thus decreasing the amount infused. This negative effect on calcium infusion at higher pressures can also be seen in terms of the negative coefficient value of the quadratic pressure term in Eq. 5.

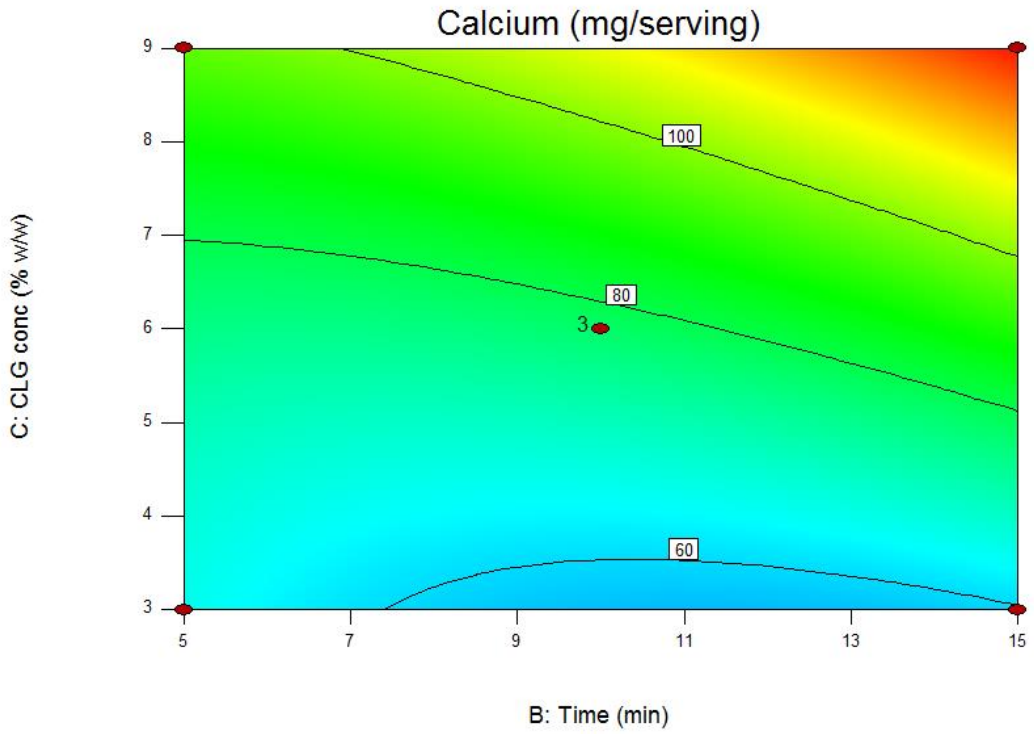


Figure 23. Effect of time-CLG solution concentration interaction on the infusion of calcium at 350 MPa

In Fig. 23, a synergistic effect of higher values of time and CLG concentration solution was observed on the higher infusion of calcium in baby carrots, as was expected from Eq. 5. Since, a synergistic effect of high CLG concentrations and moderate pressures is previously seen, carrying out the process for longer times can only have a positive effect on the infusion process.

The least amount of calcium infusion (39.30 mg calcium/serving of baby carrots) was obtained in the BBD at the processing parameters of 550 MPa, 10 min, and 3 % CLG solution concentration, while the highest infusion obtained was 134 mg calcium/serving

of baby carrots, at 350 MPa using 9 % CLG concentration solution, and processed for 15 min.

Thus, high pressure processing was successfully employed to enhance the infusion calcium in baby carrots to levels of approximately 100 mg calcium/serving (~10 % RDA).

3.3.2. Beta-carotene extractability

Pressure and time interaction was found to significantly affect the extraction of beta-carotene from the high pressure processed – calcium infused baby carrots. Fig. 24 shows the effect of interaction of pressure and time on the extractability of beta-carotene.

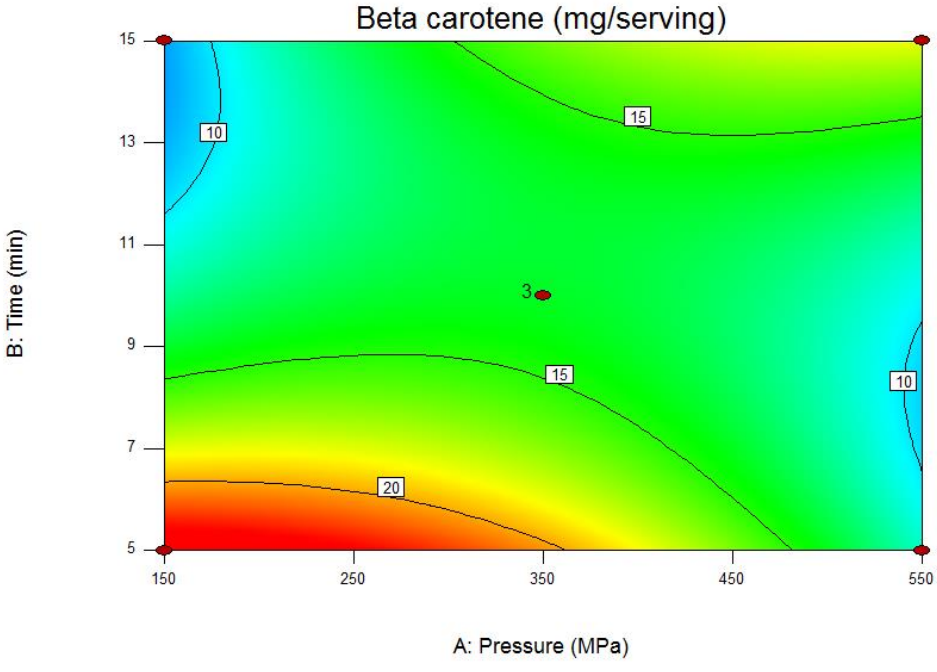


Figure 24. Effect of pressure-time interaction on the extractability of beta-carotene at 6 % CLG solution concentration

Highest extractability of beta-carotene is observed at lower pressure-time combinations, while high pressure-low time and low pressure-high time combinations showed lower beta-carotene extractability. Table 13 shows negative coefficients for X_1, X_2, and X_3, for B, but not significant.

Higher extractability of beta-carotene can be related to higher number of broken cells during the process. Since HPP causes breaking of cell structure, the higher extractability of beta-carotene is expected. However, with higher processing times or higher pressures, the beta-carotene may get extracted out of the baby carrots into the infusate solution, leading to a decrease in the total amount of beta-carotene extracted from the processed carrots. It was observed at all times after HPP that the infusate solution that was drained off of the processed carrots was slightly yellow in color, suggesting the presence of carotenoids, which in this case was beta-carotene.

An increase in the extractability of beta-carotene at high values of pressure-time combination can be seen (see Fig. 24), as a result of the significant effect of the pressure-time interaction and the quadratic model considered (quadratic models are symmetric). This increase in the beta-carotene extractability at high pressure-time combinations may be a result of the cell structure damage caused at these extreme conditions, thus releasing the beta-carotene from the cells and increasing it accessibility during the extraction process.

No other factor was observed to have caused the increased extraction of beta-carotene from high pressure processed-calcium infused baby carrots. Consequently, no mathematical model (linear or quadratic) was found to predict the equation for extraction of beta-carotene after HPP.

The master model for predicting the extractability of beta-carotene from high pressure processed calcium infused baby carrots is given by Eq. 6, however, owing to the very low R^2 value (0.50), Eq. 6 is not a good predictive model.

Beta-carotene extracted from one serving of high pressure processed-calcium infused baby carrots = $13.86 - 0.85*X_1 - 2.07*X_2 - 0.4*X_3 + 5.61*X_1*X_2 + 0.71*X_1*X_3 - 3.32*X_2*X_3 - 2.70*X_1^2 + 4.48*X_2^2 - 2.54*X_3^2$ ….$R^2 = 0.50$…. Eq. 6.

However, the average value of beta-carotene extracted from high pressure processed baby carrots (13. 86 ± 3.87 mg beta-carotene per serving of baby carrots) was approximately three times higher than that of the raw unprocessed control, thus potentially being a richer source of Vitamin A.

3.3.3. Hardness

The hardness of baby carrots after calcium infusion under high pressure was significantly affected by the pressure and the hold time, although not linearly. Higher pressures showed a negative effect on the hardness of the baby carrots, indicating loss of

texture at higher pressures, which can be correlated to the higher breakdown of the cell structure and lower calcium infusion at high pressures (as was seen in Fig 22). Since the calcium infusion is lower at higher pressures, the amount of calcium available to bind to the broken pectin chains (as a result of cell damage) is less, thus forming less 'egg-box' structures, which may contribute to improving the hardness of the baby carrots.

Longer hold times at lower pressures increased the hardness of baby carrots, as shown in Fig. 25. This increase in hardness can be explained by the higher amounts of calcium infused with longer times at lower pressures. Higher calcium infusion is hypothesized to bind to the pectin molecules, thus improving the hardness of the baby carrots, similar to what was observed by Guillemin et al. (2008).

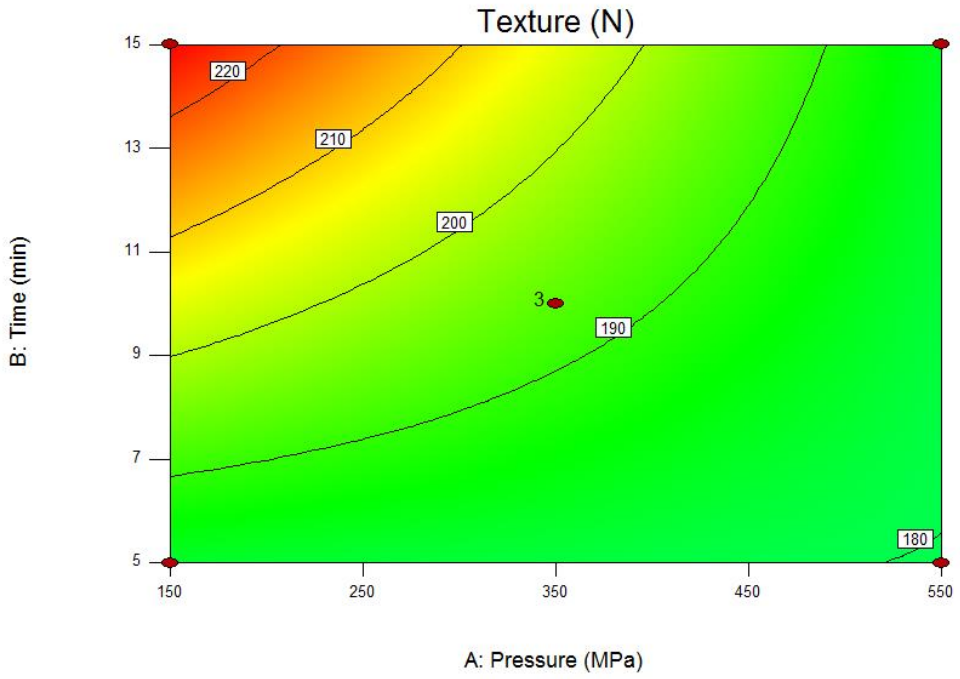


Figure 25. Effect of pressure and time on hardness of the calcium infused baby carrots using 6 % CLG solution concentration

The predicted equation for the hardness of baby carrots as affected by the high pressure processing parameters is as below:

$$\text{Hardness (N)} = 193.07 - 11.37*X_1 + 11.78*X_2 + 1.95*X_3 - 9.86*X_1*X_2 + 18.55*X_1*X_3 - 0.26*X_2*X_3 \quad \ldots R^2 = 0.52 \quad \text{Eq. 7}$$

The low R^2 value indicates the poor prediction of the hardness of high pressure processed-calcium infused baby carrots by Eq. 7.

It was observed that the hardness of the high pressure processed – calcium infused baby carrots was comparable (146.0 N – 226.2 N) or higher than that of the unprocessed control (141.4 ± 14.8), indicating that the texture of baby carrots after high pressure infusion of calcium was maintained as before or improved.

3.3.4. Color

The L* (lightness) value of the high pressure processed baby carrots was not significantly affected by any specific factor nor was the effect predictable by a linear or a quadratic model. However, the mean value of L* of the high pressure processed baby carrots (46.24 ± 1.31) was lower than the value of the controls (see Table 12). This indicates that the baby carrots became darker after high pressure infusion of calcium. This, however, is not desirable.

A similar observation was made in terms of effect of high pressure infusion on the Chroma values of baby carrots. No particular factor was found to significantly affect the Chroma value, however, the mean Chroma value of high pressure calcium infused baby carrots (39.24 ± 2.7) was significantly different from that of the controls (see Table 12), indicating that the high pressure processed samples were less bright.

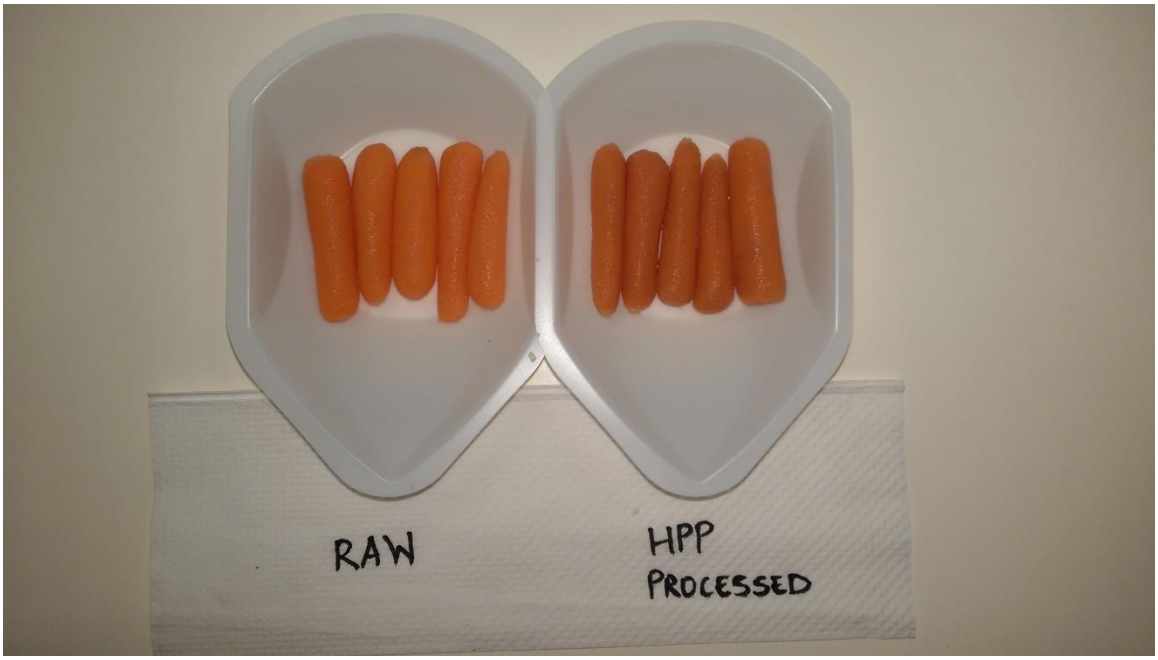


Figure 26. Baby carrots before and after high pressure-calcium infusion. The processed baby carrots in this figure were processed at the center point (350 MPa, 10 min, 6 % CLG solution concentration).

Fig. 26 shows the difference between unprocessed and high pressure processed-calcium infused baby carrots.

Following equations are the predicted equations for the L* and the Chroma values after dropping the insignificant terms in the model equation.

$L^* = 47.36 + 0.5*X_1 + 0.63*X_2 - 0.64*X_3$ ….$R^2 = 0.13$…. Eq. 8

Chroma (C) = $39.24 + 0.4*X_1 + 1.88*X_2 - 1.72*X_3 - 2.73*X_1*X_2 - 0.76*X_1*X_3 + 0.22*X_2*X_3 + 1.85*X_1 + 2.79*X_2 - 0.60*X_3$ ….$R^2 = 0.37$…. Eq. 9

The CLG solution concentration was found to have a significant and linear effect on the Hue (°) values. However, the small value of the R^2 value (= 0.50) for the predicted linear equation (Eq. 10) suggested that the equation cannot accurately predict the effect of processing parameters in the hue values.

Hue (°) = $53.15 - 0.13*X_1 - 0.38*X_2 + 0.69*X_3$ …. $R^2 = 0.50$… Eq. 10

From the ΔE values, it was clearly observed that the color of the baby carrots changed after high pressure infusion of calcium. However, no specific processing factor or the interaction of factors had a significant effect on the change on color. Eq. 11 is the quadratic equation predicted for the change in color of baby carrots after high pressure-calcium infusion.

$\Delta E = 12.96 - 0.62*X_1 - 1.04*X_2 - 1.27*X_3 + 0.79*X_1*X_2 + 1.01*X_1*X_3 - 0.65*X_2*X_3 - 1.19*X_1^2 - 1.26*X_2^2 - 0.68*X_3^2$ ….$R^2 = 0.69$…. Eq. 11

However, the ΔE values of the high pressure processed-calcium infused baby carrots were comparable to that of osmotically and vacuum infused control, indicating the calcium infusion may have a greater effect on the color of the baby carrots than the high pressure processing. Color degradation of carrots after a combination of thermal and high pressure treatment has been extensively reported in the literature (Nguyen et al., 2007; Patras et al., 2009; Araya et al., 2009; Goncalves et al., 2010; Nguyen et al., 2010; and Patras et al., 2010). However, the causes of this color degradation have not been reported. In order to gain better insight, further experiments need to be performed in order to decouple the effect of HPP and calcium infusion on the change in color of the baby carrots.

3.4. Summary of results for BBD experiments

Table 14. includes the results obtained from the BBD experiments.

Run #	Calcium infused (mg/serving)	Beta-carotene extracted (mg/serving)	Texture (N)	Color			
				L*	Chroma (C)	Hue (°)	ΔE
1	48.99	10.39	212.70	47.18	40.7433	53.17	11.08
2	112.22	8.24	196.19	45.84	38.7197	52.98	13.11
3	56.83	9.10	226.22	47.78	47.57	52.33	10.21
4	78.70	7.44	210.23	46.06	38.79	54.48	13.30
5	65.99	10.94	192.54	49.16	45.64	53.42	9.24
6	79.34	9.66	188.70	48.36	41.11	53.12	10.14
7	39.31	6.14	145.99	48.04	41.41	52.24	10.03
8	134.73	8.40	206.85	47.5	40.34	54.14	11.52
9	62.97	16.54	182.84	48.5	42.98	52.22	9.22
10	94.80	20.91	178.05	45.78	36.80	54.60	14.53
11	93.39	19.76	190.63	49.48	44.6	52.50	8.34
12	59.18	22.72	188.69	45.17	37.71	53.54	14.25
13	60.89	17.31	212.69	48.87	45.62	51.73	8.82
14	67.87	16.89	192.69	45.81	36.30	53.46	14.48
15	85.64	17.25	170.98	46.87	42.64	53.34	11.09

Table 14. Results for the BBD experiments.

3.5. Effect of pressure cycling

3.5.1.Calcium infusion

Figure 27 shows the comparison between calcium infused when different pressure cycles are employed.

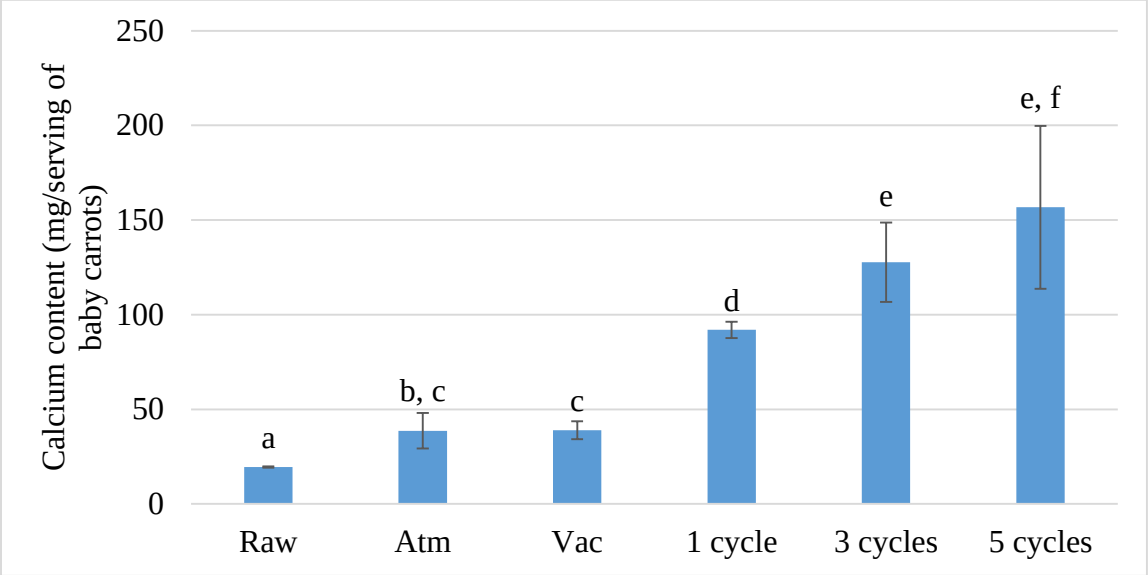


Figure 27. Calcium infused during pressure cycling and comparison to controls*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

It can be observed from Fig. 27 that more number of pressure cycles during HPP lead to higher infusion of calcium, as predicted. The higher infusion with higher number of pressure cycles can be attributed to more breaking up of the cell walls, due to the repetition of pressurization-depressurization cycles, causing higher calcium to infuse into the matrix. The calcium infused by 5 cycles of pressurization was 1.5-2 times higher than that infused in a single cycle.

3.5.2.Beta-carotene extractability

More number of pressure cycles increased the beta-carotene extracted from the high pressure processed-calcium infused baby carrots, however, the increase in extractability was not significantly ($p > 0.05$) different between 3 and 5 cycles. The extractability increased from single cycle to 5 cycles. Fig. 28 shows the effect of pressure cycling on the beta-carotene extractability.

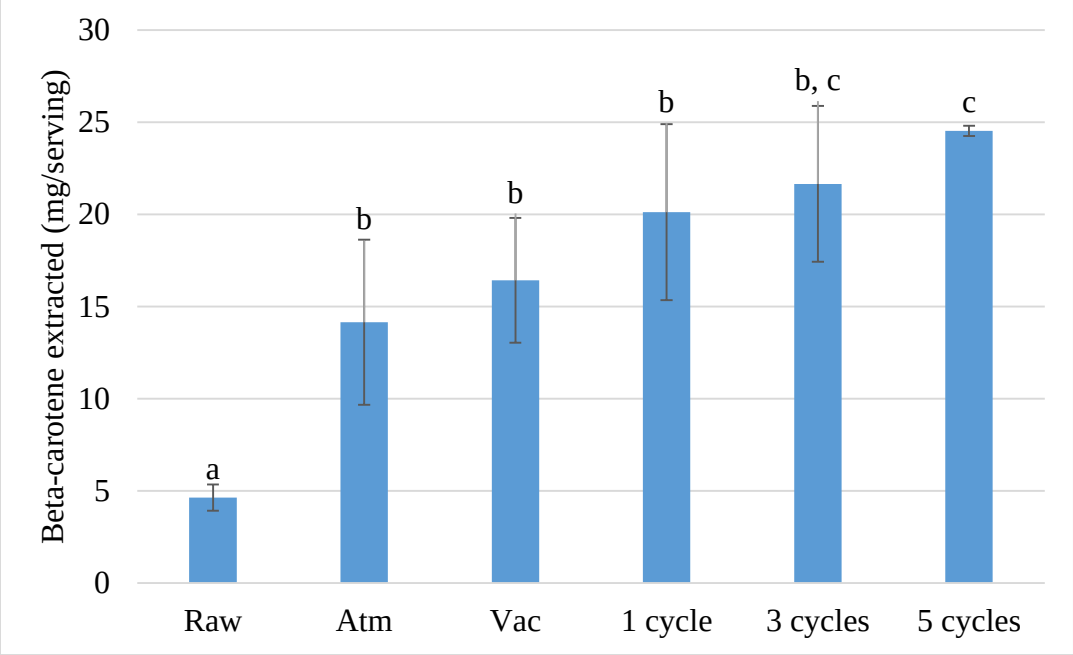


Figure 28. Effect of pressure cycling on the beta-carotene extractability of high pressure processed-calcium infused baby carrots.*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the

A surprising observation was that the beta-carotene extractability of the osmotically infused and vacuum infused controls was comparable to that of the single cycle and 3 cycles HPP. However, this observation can be explained by the duration of frozen storage of the samples before they were analyzed in the HPLC for beta-carotene. The osmotically infused and vacuum infused samples were stored for 5 days more in the

frozen storage as they were extracted last among all the samples. The details of the effect of frozen storage are explained later in this discussion.

As was mentioned in the section 2.2.2. and 2.2.3., the samples for pressure cycling and the controls were performed in duplicates and each replicate was analyzed in duplicates. In an effort to have a triplicate set of data, all of these samples were processed one more time and analyzed for beta-carotene. However, the difference between the previously processed and the newly processed samples was the time they were stored in frozen conditions before being extracted for beta-carotene, and the number of freeze thaw cycles they went through during the process. The previously processed samples were stored frozen for 2 weeks before being extracted for beta-carotene, and went through only one freeze-thaw cycle during their extraction process. The newly processed samples were accidently stored for 4 months (November 2015 to February 2016) before being extracted for beta-carotene, and went through two to three freeze thaw cycles during grinding and the extraction process (refer to section 2.4.2.1.).

Fig. 29 shows the difference in the beta-carotene extractability of the previously and newly processed samples which went through the same processing but different storage durations.

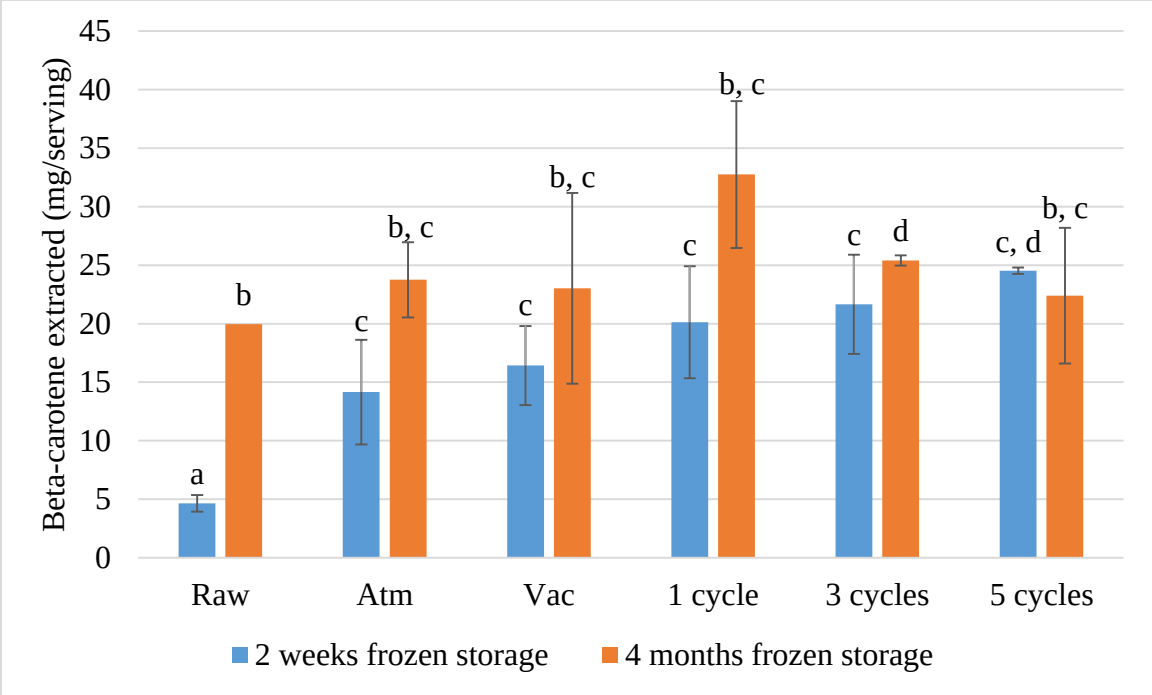


Figure 29. Effect of duration of frozen storage on the extractability of beta-carotene*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

The raw samples (as shown in Fig. 29) showed a large difference between the extractability of the beta-carotene according to their storage times in the freezer. The raw sample being an unprocessed (no HPP, hence, intact cells) sample showed a great difference in the beta-carotene extractability after 4 months frozen storage. This indicates that owing to the long duration of frozen storage, the cell structure of the raw samples was damaged to a large extent, increasing the extractability of the beta-carotene from them.

However, the difference in the extractability of beta-carotene in the HPP samples was not as large as the one in the raw samples. Nevertheless, the extractability of beta-carotene was higher (except for the sample processed in 5 HPP cycles) in the samples stored for 4 months in frozen storage, again, indication that the freezing causes cell

damage which could consequently increase the extractability of beta-carotene. The difference observed in the HPP samples may not be as large as that of the unprocessed sample, since during HPP the cell structure of the baby carrots was already damaged and hence, the damage caused during freezing was proportionately less. This also, in a way, demonstrates the cell damage caused during HPP. If the same hypothesis is applied to the sample processed in 5 HPP cycles, it would imply that the 5 HPP cycles caused cell damage equivalent to the cell damage caused by 4 months of frozen storage.

Since the data collected from the newly processed samples was clearly not comparable to the previous data, it wasn't considered in this study. However, the findings look interesting and may be explored further to get a clear picture.

3.5.3.Hardness

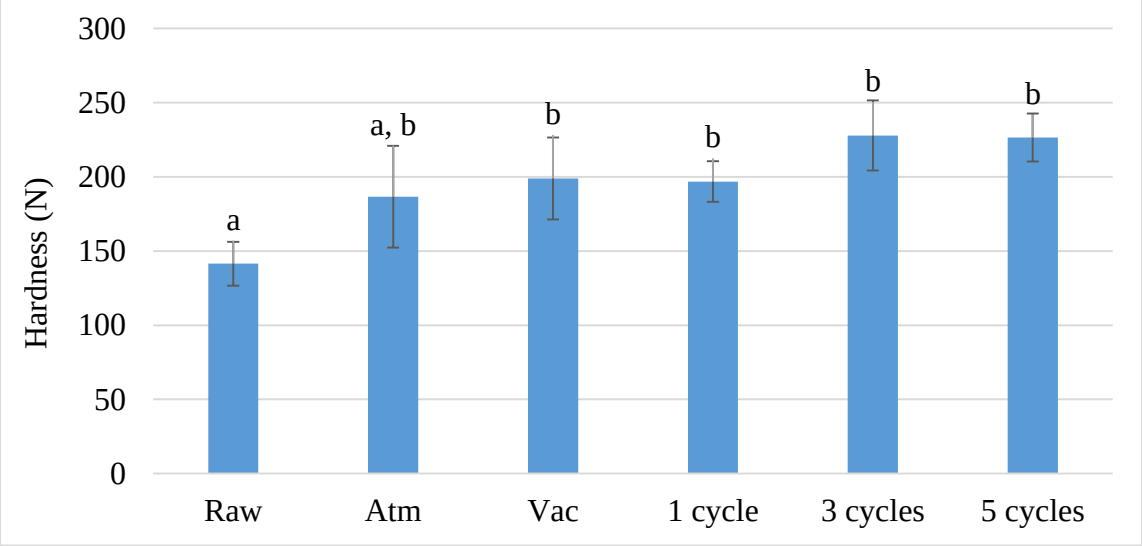


Figure 30. Effect of pressure cycling on the hardness of calcium infused baby carrots and comparison to controls*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

As was observed earlier, high pressure infusion of calcium in baby carrots increased the hardness of baby carrots in comparison to the controls. However, pressure cycling had no significant effect in increasing the hardness of the baby carrots (see Fig. 30).

3.5.4.Color

The following figures (Figs. 31-35) illustrate the effect of pressure cycling on various aspects (L*, Hue, Chroma, ΔE) of the color of the baby carrots.

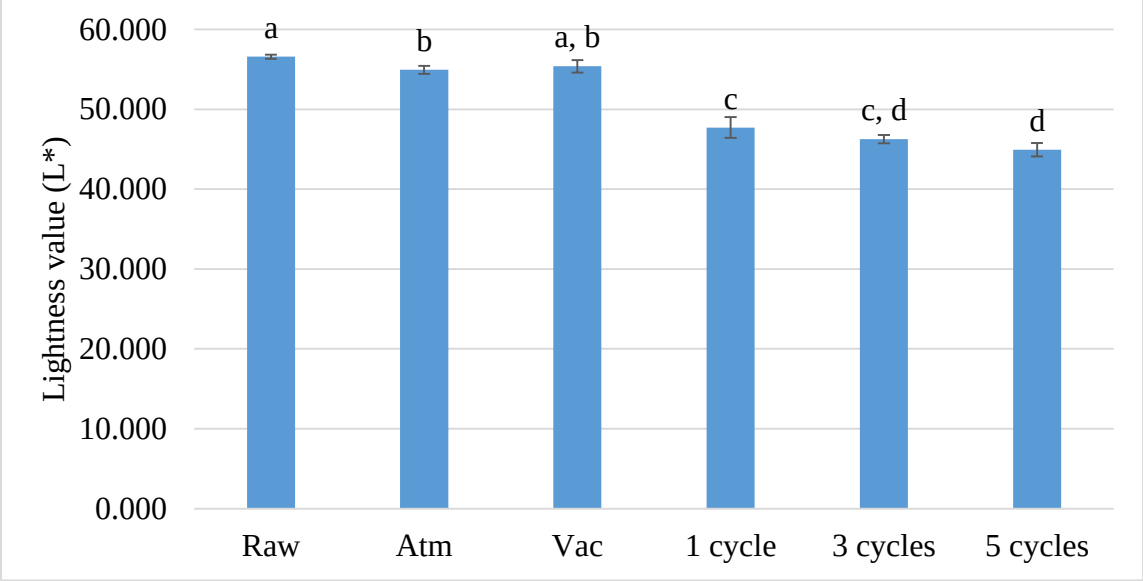


Figure 31. Effect of pressure cycling on the lightness value (L*) of the baby carrots and comparison to the controls*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

As has been previously observed, the lightness value (L*) of the baby carrots decreased after high pressure infusion of calcium. The lightness value further decreased with higher number of pressure cycles. Lower values of L* indicate darker samples (see

Fig. 31). The decrease in the L* value due to pressure cycling may be statistically significant, but the practical significance can be evaluated only through sensory analysis.

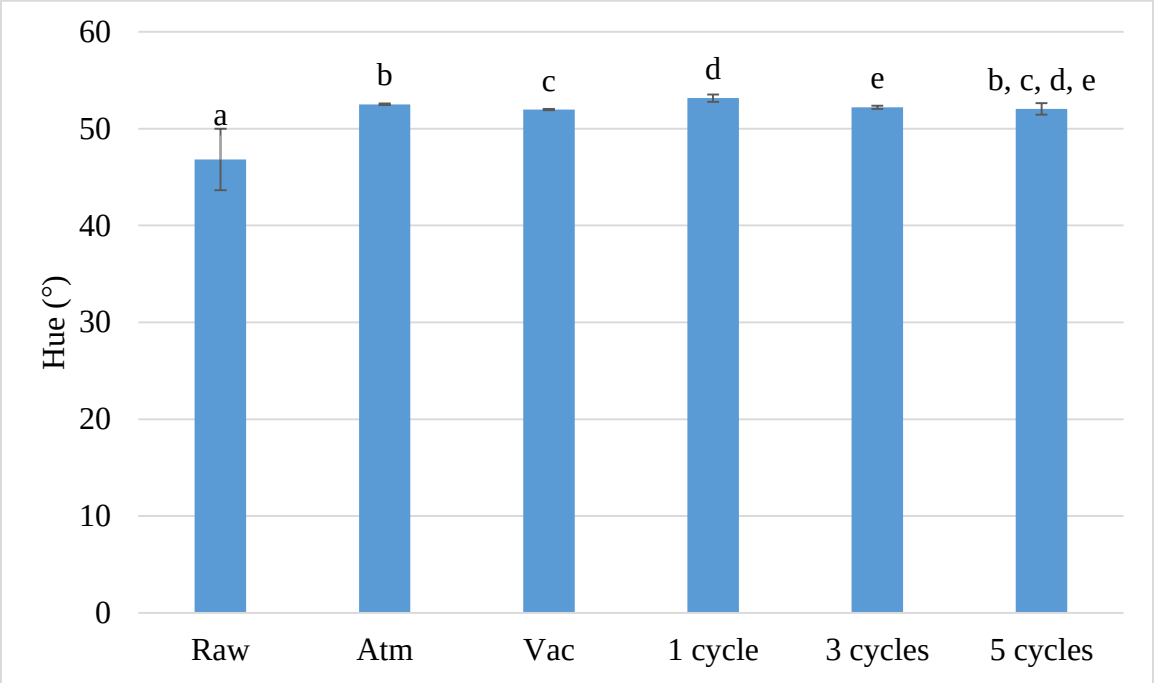


Figure 32. Effect of pressure cycling on the hue (°) values of the baby carrots and comparison to the controls*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

The hue (°) value of the baby carrots suggested that the processed carrots became more yellow and less orange upon high pressure infusion of calcium. However, pressure cycling did not seem to further alter the color in terms of the hue (see Fig. 32).

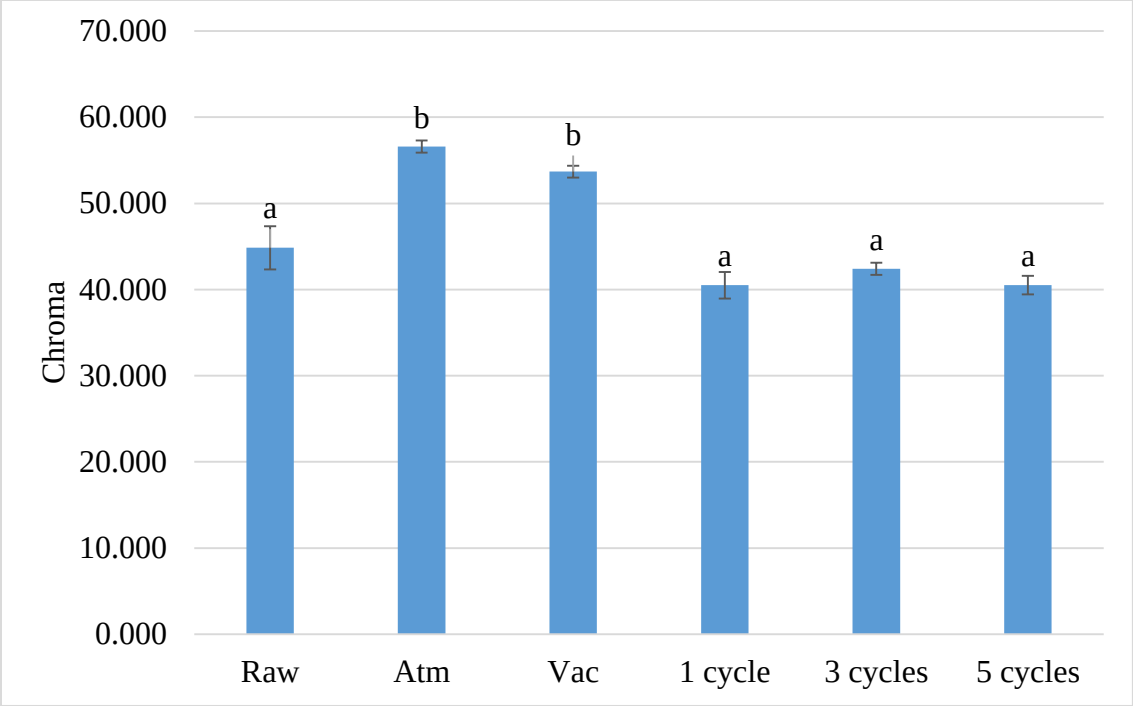


Figure 33. Effect of pressure cycling on the Chroma values of the baby carrots and comparison to the controls*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

Pressure cycling did not seem to alter the Chroma values of the high pressure processed – calcium infused baby carrots, indicating that the saturation of the color remains unaltered even after multiple cycles of pressurization (see Fig. 33).

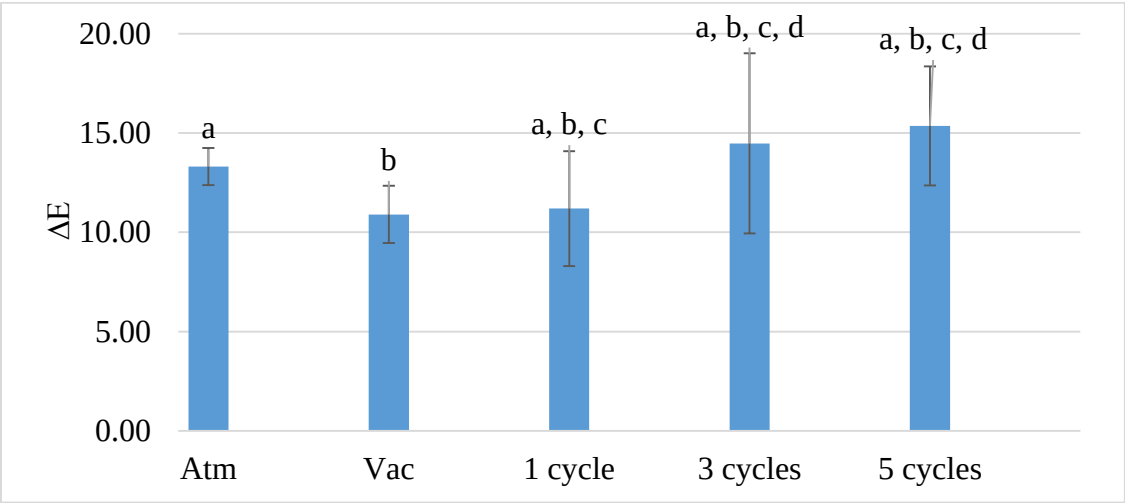


Figure 34. Effect of pressure cycling on the change in color of the high pressure processed-calcium infused baby carrots and comparison to controls*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

According to Fig. 34, pressure cycling did not have a significant effect on the color difference of the high pressure processed-calcium baby carrots and the unprocessed baby carrots.

In order to understand the practical significance of the effect of high pressure assisted calcium infusion on the color of the baby carrots, a sensory evaluation is necessary.

Figure 35. Baby carrots after calcium infusion under different conditions

3.6. Effect of calcium infusion and extent of high pressure, independently, on the color of baby carrots

These experiments were performed in order to understand the reason behind the change in the color of high pressure processed-calcium infused baby carrots.

Baby carrots processed with no infused solution under extreme pressures (100 MPa and 600 MPa) showed no change in the lightness value (L*) of the baby carrots, indicating that high pressure itself does not affect the brightness of the baby carrots. However, in presence of infusate solution, high pressure (600 MPa) significantly affected the brightness of the baby carrots, indicating a darker color (see Fig. 36a). The lightness value decreased to a greater extent in presence of the CLG solution. Low pressure (100 MPa) and presence of CLG solution did not significantly alter the brightness value of the baby carrots, but high pressure (600 MPa) and presence of CLG solution significantly decreased the L* value, indicating darker color of the baby carrots. Thus, high pressure was found to affect the L* value only in presence of an infusate solution, and the effect of more pronounced when the infusate solution was CLG solution. A combination of CLG solution and extreme high pressure was found to significantly lower the brightness of the processed baby carrots.

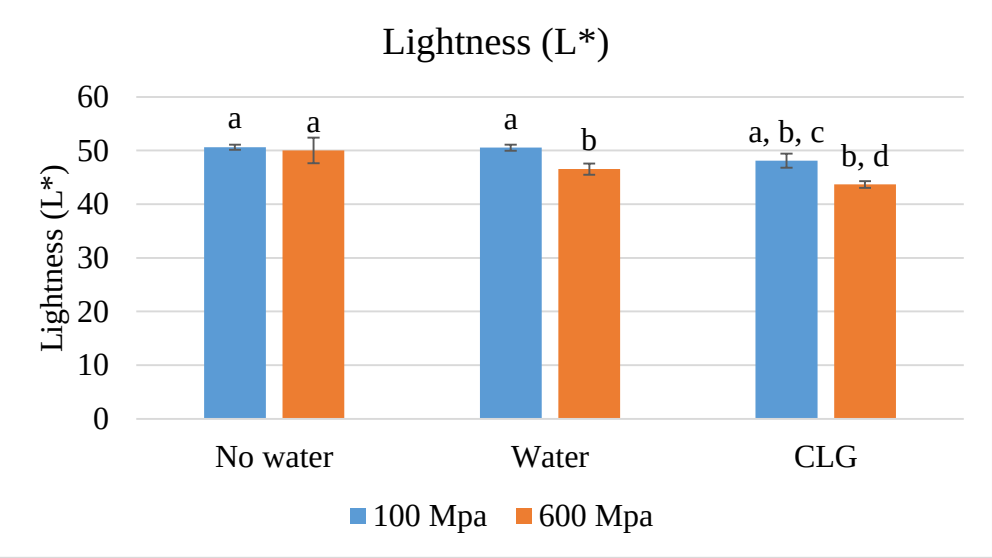


Figure 36a. Effect of calcium infusion and extent of high pressure on the lightness value (L*) of the processed baby carrots*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

Extreme high pressure (600 MPa) was found to significantly decrease the Chroma values, indicating the effect on the intensity of the color of the baby carrots (see Fig 36b). Presence of the CLG solution too decreased the Chroma values, indicating that the carrots appeared pale than the unprocessed controls. CLG solution and increasing high pressure, together, were found to synergistically cause undesirable changes in the Chroma values.

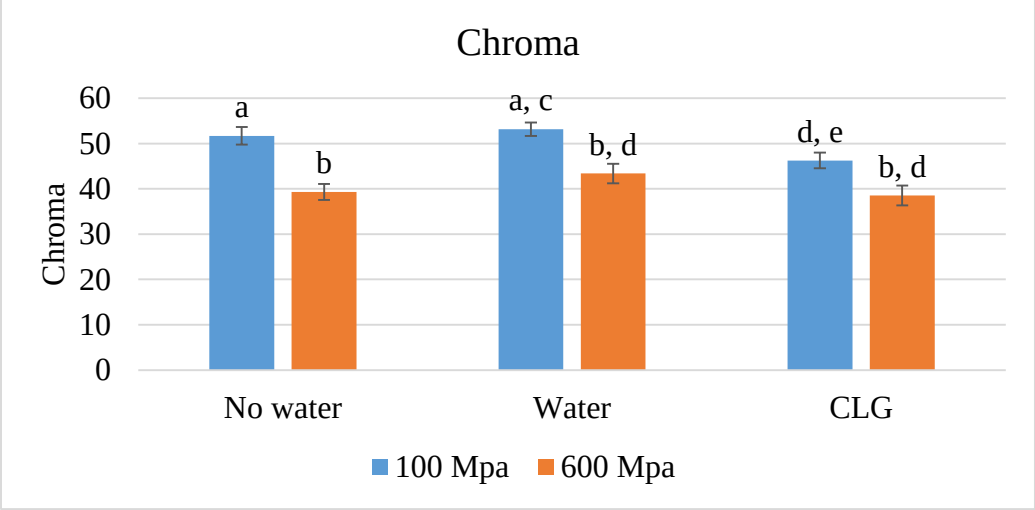


Figure 36b. Effect of calcium infusion and extent of high pressure on the Chroma of the processed baby carrots*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

On the other hand, the Hue values were affected to a lesser extent by the high pressure and calcium infusion, indicating that the color of the baby carrots remained close to yellow-orange. The extent of high pressure did not affect the hue of the baby carrots (as seen in Fig. 36c). Infusion of calcium, however, lowered the hue of the baby carrots (more yellow than orange), but not significantly. The loss of the orange color from the high pressure processed baby carrots under extreme high pressure may be caused by extraction of small amount of carotenoids during the process.

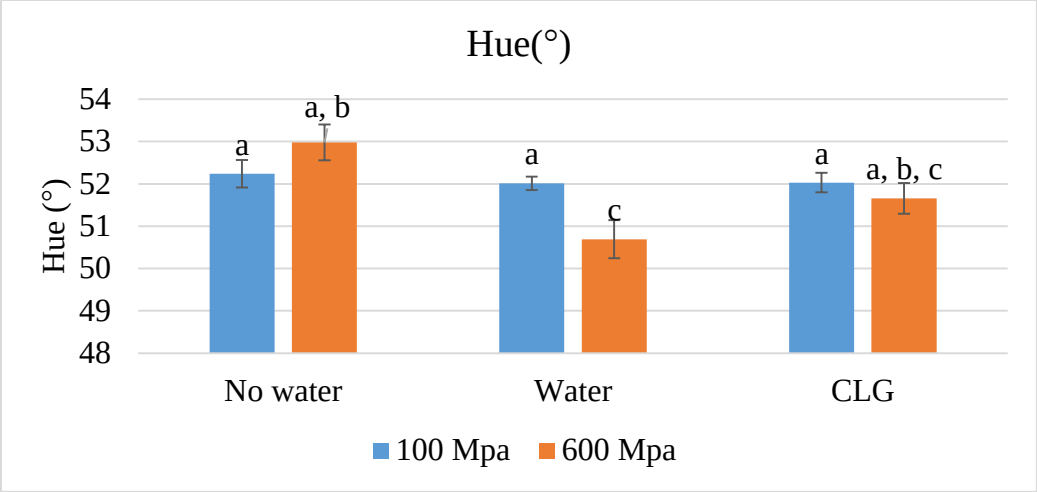


Figure 36c. Effect of calcium infusion and extent of high pressure on the Hue (°) of the processed baby carrots*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

Based on the ΔE values, the overall color of the processed baby carrots was found to be not affected by the extent of high pressure, but by the infusion of calcium (see Fig. 36d).

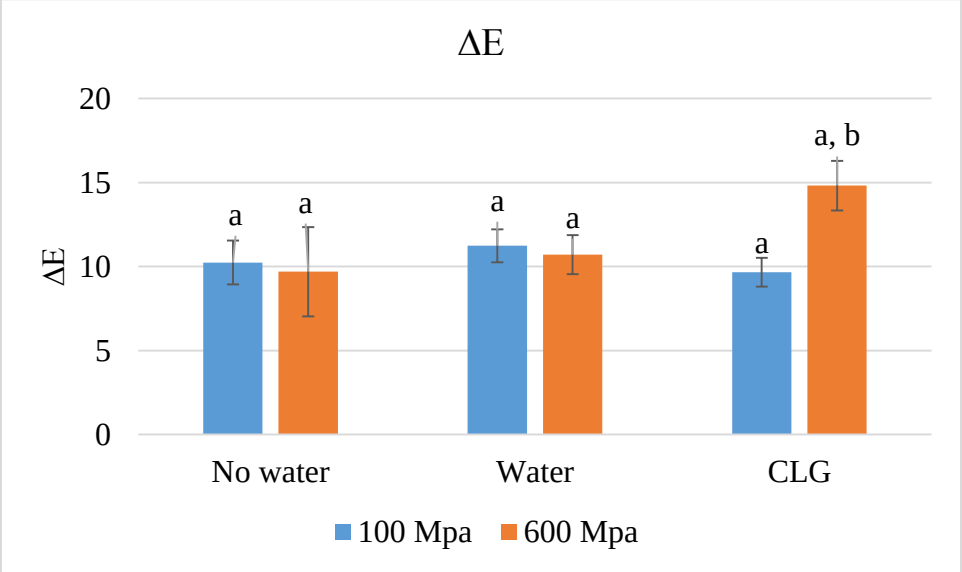


Figure 36d. Effect of calcium infusion and extent of high pressure on the color difference of the processed baby carrots (when compared to the unprocessed control)*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

In order to understand the exact mechanism of color change caused by calcium infusion and high pressure, experiments need to be carried out to evaluate the formation/degradation of specific color compounds.

3.7. Shelf life study

3.7.1. Change in texture during storage

During a storage period of 10 days, the texture of the high pressure processed-calcium infused baby carrots did not show any significant change (Fig. 37). This implied that the texture of the baby carrots remained intact during storage.

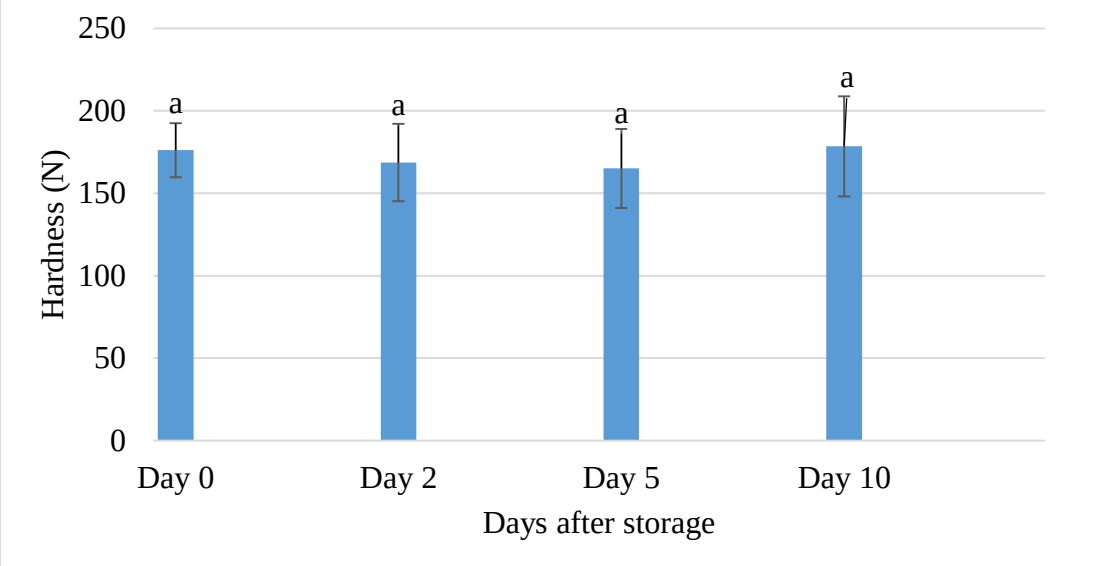


Figure 37. Change in the texture of the baby carrots during storage*

*Different alphabets indicate statistically significant difference in ($p<0.05$) the samples

3.7.2. Change in color during storage

The lightness (L*) (Fig. 38a) and the Chroma value (Fig. 38b) of the baby carrots decreased during the storage period, indicating that the baby carrots became darker during storage. On the other hand, the hue (°) value (Fig. 38c) of the baby carrots increased during storage, indicating that the baby carrots turned pale (more yellow) on storage. The color difference (Fig. 38d) values increased during the storage thus indicating that over an increased storage time, the high pressure processed-calcium infused baby carrots deviated from their natural color more.

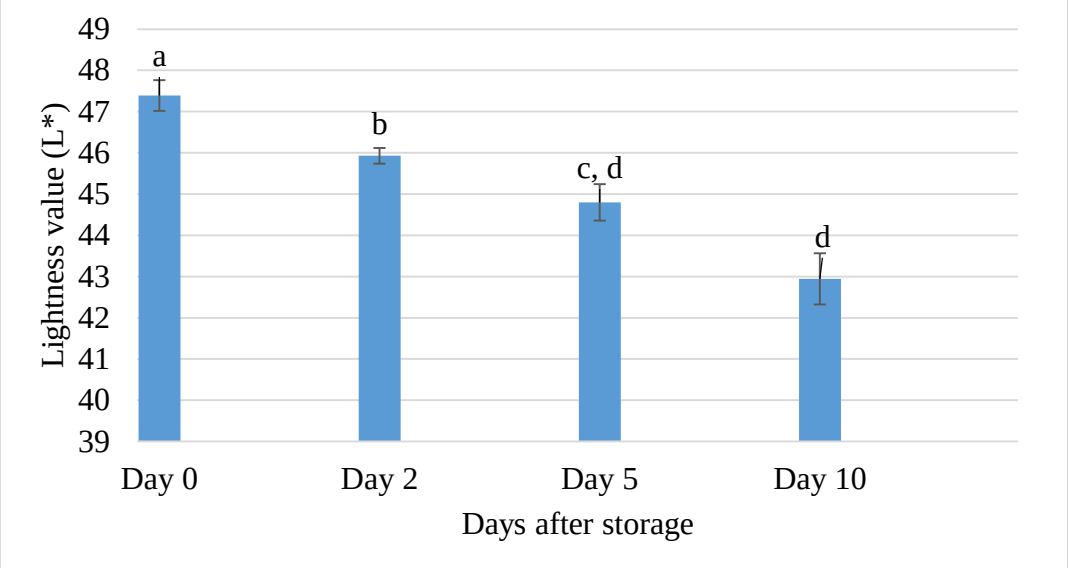


Figure 38a. Change in lightness values (L*) of the baby carrots during the storage*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

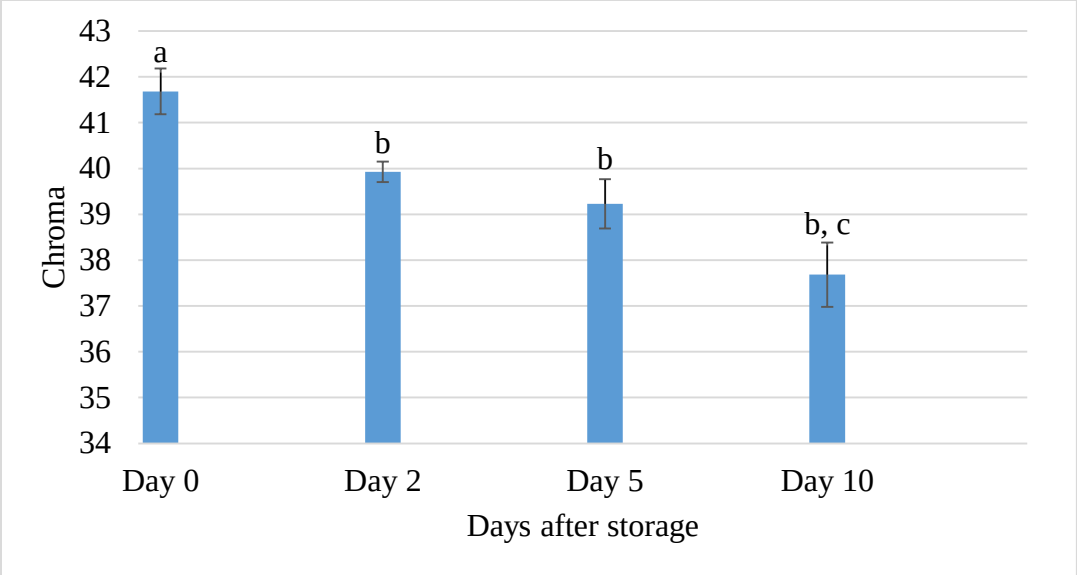


Figure 38b. Change in Chroma (C) values of the baby carrots during the storage.

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

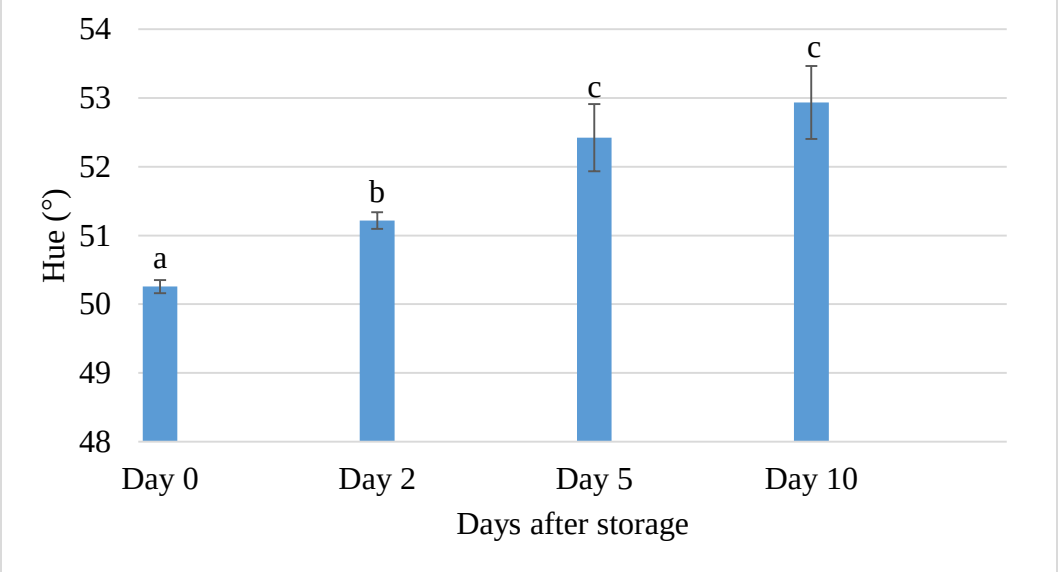


Figure 38c. Change in hue values (°) of the baby carrots during the storage*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

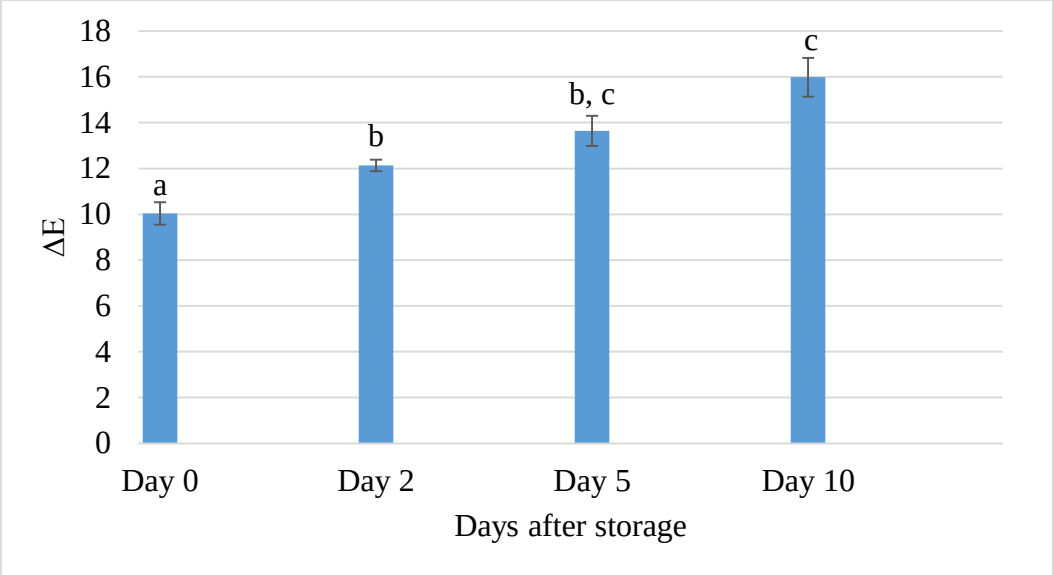


Figure 38d. Change in overall color of the baby carrots during the storage*

*Different alphabets indicate statistically significant difference ($p<0.05$) in the samples

3.7.3. Effect of storage on the microbial quality of the baby carrots

The number of colony forming units (CFU) per gram of the baby carrots increased during storage, as was expected (see Fig. 39).

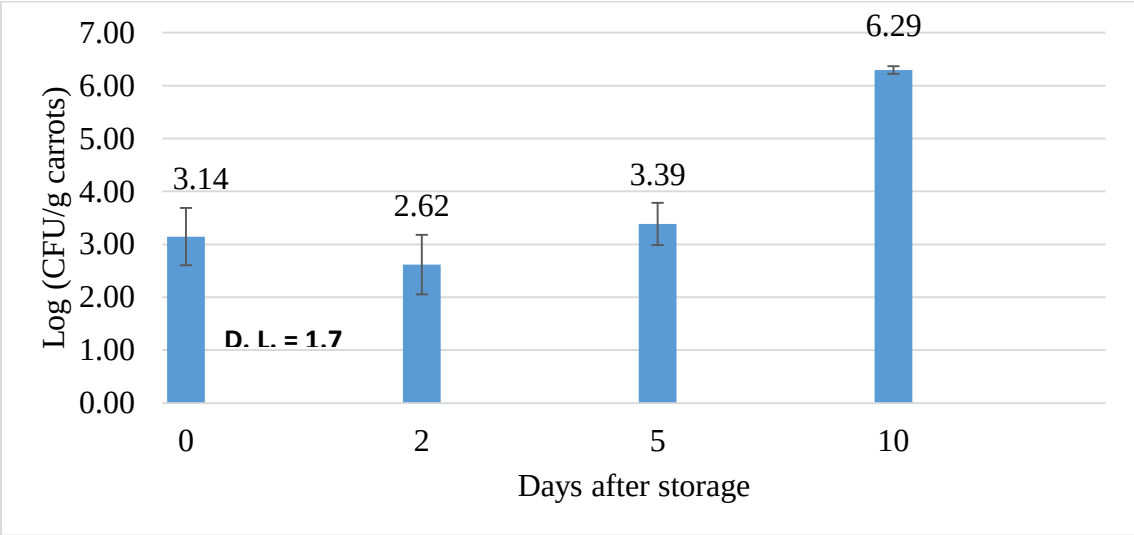


Figure 39. Microbial growth during storage of the high pressure processed-calcium infused baby carrots. (D. L.: detection limit)

Based on this data, a growth curve can be predicted for the microbial spoilage of the high pressure processed – calcium infused baby carrots. Fig. 40 shows a growth curve of the microbial population in the baby carrot samples. However, it must be noted that the log (CFU/g) value on Day 0 was higher than that on Day 2. This may be attributed to human error. Using this value, it was difficult to build a sigmoidal curve using DMFit, as the point would be considered an outlier on a straight line fit was obtained. Hence, the log (CFU/g) value of the microbial load on Day 0 (the day of processing) was assumed to be the original value – standard deviation (Value – 1*σ). To compensate for this, the error bar on the first point of Day 0 is 2*σ above the assumed value.

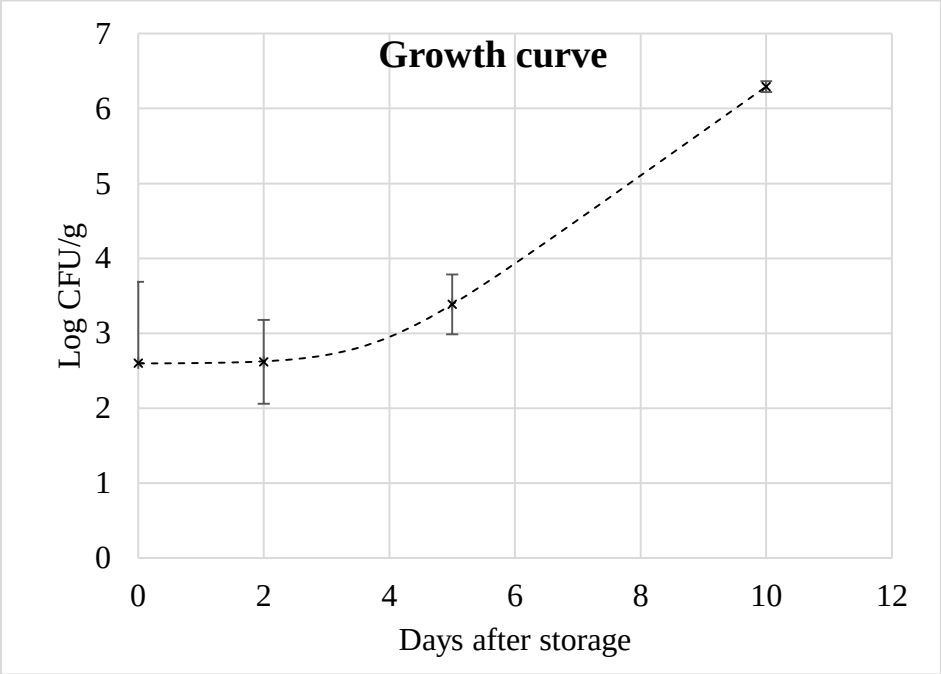


Figure 40. Growth curve for the microbial growth in the high pressure processed-calcium infused baby carrots during storage.

The lag time of the microbial growth curve was calculated to be 3.8 days (~ 4 days) using DMFit and the growth rate was calculated to be 0.6 log (CFU/g) per day. Thus, it was observed that the exponential phase of the microbial growth starts approximately around fourth day after storage, indicating that the product won't be microbiologically shelf stable after four days of storage.

In order to improve the shelf life of the high pressure processed-calcium infused baby carrots, it is necessary to employ more hygienic and time-effective post-processing techniques, such as washing, drying and handling before packaging which could constitute further study.

4. CONCLUSIONS

High pressure processing (HPP) was shown to have enhanced the infusion of calcium in PME treated baby carrots to provide 10 % - 15 % of the RDA of calcium, which was significantly more in comparison to the commonly used infusion techniques of osmotic and vacuum infusion that infused no more than 4 % of the RDA of calcium.

A detailed regression analysis on the influence of pressure (MPa), hold time (min), and CLG solution concentration (% w/v) using BBD experimental design showed that the pressure, time, and the CLG solution concentration, and their interaction effects significantly affect the infusion of calcium in the PME treated baby carrots. Maximum infusion of calcium was 134 mg calcium per serving of baby carrots, when PME treated baby carrots were processed at 350 MPa for 15 min using 9 % CLG solution concentration. High pressure, thus, has a potential to be used as a food fortification technique to tackle the increasing micronutrient deficiency.

In addition to increased infusion of calcium, HPP also enhanced the amount of beta-carotene extracted per serving of baby carrots. Higher extraction of beta-carotene may indicate higher bio-accessibility of the micronutrient, however, more concrete data and studies are necessary to test this hypothesis. The high pressure infusion of calcium in PME treated baby carrots increased the hardness of the baby carrots, but darkened their color.

Pressure cycling was seen to have a significant effect on the infusion of calcium, increasing the amount of infused calcium with higher number of cycles. However, pressure cycling did not significantly alter the extractability of beta-carotene, the hardness, and the color of the calcium infused baby carrots.

Shelf life analysis or storage study of the calcium infused baby carrots revealed that the hardness of the baby carrots was maintained during the storage, but the color darkened. Microbial growth increased during the storage, with an exponential growth starting after a storage of approximately 4 days.

Since, HPP has now been established as an effective technique for infusion of calcium in PME treated baby carrots, further studies to improve the shelf life of the infused baby carrots will increase the potential of this technology to be extended to fortification of wide variety of fruits with necessary micronutrients.

5. FUTURE WORK

In this study the concentration of the PME enzyme used was kept constant. However, the effect of PME treatment with varying concentrations of the enzyme must be evaluated on the infusion of calcium in baby carrots. Hypothesizing that higher concentration of the PME enzyme will demethylate more pectin bonds, increasing the number of sited where the calcium could bind, it may play a role in increasing the infusion of calcium in the baby carrots.

It is important to isolate the effect of high pressure and calcium infusion on the darkening of baby carrots. Experiments can be carried out with baby carrots processed with only water for the same.

A thorough sensory evaluation will give a better insight into the practicality of the project. It is also important to optimize the processing and the post-processing conditions of the process in order to produce a food product that will have a longer microbial shelf life.

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