UNDERSTANDING AND MODELING HIGH PRESSURE ASSISTED INFUSION OF
CALCIUM IN FRUITS AND VEGETABLES

By

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A dissertation submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Food Science

Written under the direction of

Mukund V. Karwe

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New Brunswick, New Jersey

May 2021
ABSTRACT OF THE DISSERTATION

Understanding and Modeling High Pressure Assisted Infusion of Calcium in Fruits and Vegetables

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High Pressure Processing (HPP) is a widely commercialized non-thermal batch processing technique in the food industry, that is aimed at improving the shelf-life and quality of foods through microbial and enzyme inactivation. Beyond this traditional application of HPP in foods, some researchers have explored its application to infuse nutrients (sugar, salt, etc.) and micronutrients (calcium, zinc, iron, anthocyanins, etc.) into food matrices with an aim to improve the textural and / or nutritional properties of those foods. The research reported in this dissertation explored whether HPP can infuse significant amounts of calcium in
fruits and vegetables. If so, can we develop a mathematical model to quantify the effect of microstructure of the foods and pressure on the infused amount?

Calcium lactate gluconate (CLG) was used as the infusing medium. All high pressure experiments were performed in the 10 L HPP vessel at Rutgers. Baby carrots were pre-treated with pectin methylesterase (PME) to enhance the infusion of calcium. Among pressure, time, and CLG solution concentration, CLG concentration was the most important parameter, followed by pressure, that affected the extent of calcium infusion in baby carrots, with increasing concentration leading to increased infusion. Processing the PME-pretreated baby carrots at 350 MPa for 15 min, using 9% CLG solution led to highest amount of calcium infusion (134 mg/85 g serving) in the baby carrots, which was >10 % of the daily requirement of calcium for average adults.

HPP infusion experiments were performed using baby carrots, celery, and mango – root, stem, and fruit, respectively. It was found that the transport tubes in baby carrots and celery were mainly responsible for the uptake of the infusate solution during HPP assisted infusion.

In order to quantify the effect of microstructure of different food matrices, a protocol to stain and visualize the fruit and vegetable tissue under a light microscope was developed and the distributions of cell diameters (CDs) and cell wall thicknesses (CWT) were obtained. No statistical differences were observed in
the CWT values across the different food matrices; however, the CD values were significantly different, within and across each system. It was observed that higher cell diameter in mango (76 ± 8 μm) led to highest infusion (111.02 ±14.03 mg/100 g fruit), followed by celery and baby carrots. Tagging the calcium with a fluorescent dye (Fluo-8) showed the infused calcium concentrated around the transport tubes in baby carrots and celery, while it was evenly distributed throughout the tissue in mangoes.

To develop a mathematical model for infusion of calcium, experiments were performed using pre-permeabilized food matrices. Principal component analysis (PCA) and response surface analysis (RSA) showed that the microstructure of these foods was the primary factor in governing cell permeabilization and consequently the calcium infusion. A new effective microstructure term was defined such that it encompassed the effects of cell diameter and transport tubes which is a unique aspect of this study.
Acknowledgements

My journey in graduate school at Rutgers Food Science did not start with me joining the program, but it started when I was an impressionable 11-year-old who was encouraged by her teachers and family alike to pursue this path. To all who have crossed my path in this journey and in unknowing ways influenced me to be where I am, I say ‘thank you!’

Joining the Food Science department at Rutgers in September 2013, fresh out of undergrad, and coming from a country 8500 miles away, I found the much-needed support in the Food Science family. My research advisor, Dr. Mukund Karwe, took me under his wing with as much care and support as he would have extended to his own family. As an advisor, he pushed me to do things outside of my comfort zone, exposed me to numerous research projects and works that would have been rare for me to experience otherwise, and ultimately developed the rigorous researcher in me. Above all, he helped rekindle my love for the mountains by taking our lab on multiple hikes, and I will be forever grateful to him for that and more.

Before I continue with a long list of thanks you-s, I would like to take a moment and express my heartfelt gratitude to the rest of my committee members – Dr. Karen Schaich, Dr. Paul Takhistov, and Dr. Paulo Santos. Thank you for taking time out of your overwhelmingly busy schedules to read my
dissertation and provide constructive feedback. With the unusual circumstances of the COVID-19 pandemic and the excess load it has put onto your teaching and work schedules, I appreciate your time and efforts even more. It is imperative that I express gratitude to these journals – Reference Module in Food Science, Food & Bioprocessing Technology, Food Engineering Reviews, which published our research papers that comprise Chapters 1, 2, and 3 of this dissertation.

Many people at the Rutgers Food Science department were crucial in my journey as a first-year graduate student. Right from the administrative staff (special mention: Irene Weston) to my fellow lab-mates – I found no shortage of comfort and support here. I found my role model for teaching in Dr. Karen Schaich, to whom I will be always grateful. She believed in my abilities right from day one and honed my skills as a teacher by being an exemplary example herself.

My first introduction to the food industry as an intern at Ingredion Inc. was made even better with the mentorship of Dr. Paulo Santos. Thank you, Paulo, for changing my outlook towards the industry while also showing that you can keep the researcher and the teacher in you alive. Your guidance and mentorship have made a huge impact on me and my career.

While the HPP unit at Rutgers was out of commission for 16 months, Dave Petrenka and Bill Sumal in the Food Science department worked tirelessly to fix it. Not only that, but they both have fixed almost any instrument that has ever
broken in our laboratory and saved our research projects from being discarded. They are nothing short of magicians at the department. Dr. Brendan Neimera, Dr. Allen Sheen, Rommel Ramos, and Joe Sites at the USDA-ARS Eastern Regional Research Center in Wyndmoor, PA, stepped in to help me with the HPP experiments when my research was severely affected by the unavailability of the HPP at Rutgers. A huge thank you to all of them for saving my research! While I was about to defend my PhD proposal on a short notice, Dr. Deepti Salvi kindly stepped in as a committee member and offered valuable inputs.

I was fortunate to have been financially supported throughout my graduate studies, which was made possible by the Excellence Fellowship offered to me by Rutgers Food Science and the Teaching Assistantship from the Division of Life Sciences. My research was also partially facilitated by the support we received from New Jersey Agricultural Experiment Station (NJAES).

My list of acknowledgements would be incomplete and dishonest if I did not mention these individuals – Dr. Bhas Bhamre, who shaped my interest in science during my school years, Dr. Gregg Transue and Dr. Daniel Stern Cardinale in General Biology for providing me with a wonderful and supportive family in GB, and Dr. Racquel San Juan and Dr. Robert Grabelsky for ensuring that my mental health was always taken care of.
Throughout my time at Rutgers and even before that, I have been blessed to have had friends who have stood by me through thick and thin. There are too many of them for me to name here, so I want all my friends to know that I see you and I thank you for being there. Special thanks to – Neeta who unconditionally believed in me, Karthik who never let me feel alone, Morgan who has showered me with insurmountable amounts of love, Parwani who never gave up on me despite my poor communication skills, Gaival who filled my time with the most interesting and weird conversations, and my first friends at Rutgers (Parikshit, Mary, Bhushan, Dharmesh, Siddhi, Isha, Soham, & the whole AMRU family) who made it easy for me to acclimatize to a new country and place. To the other protagonist in my life, my partner – Kartheik, you are my best friend and no single day with you is ever boring. Thank you for holding my hand through all the ups and downs.

It would be ungrateful if I ended this without mentioning my family – my parents and my brother. Thank you, mummy and papa, for giving me the wings to fly on my own, for always supporting my decisions and endeavors, and for helping me morph into the strong and independent individual that I am today. As I mention my family, I must mention my feline family – Troy, Abed, and Crowley, whose presence has been a tremendous support to my mental health. Lastly, a huge thanks to the self-critical voice in my head – you help me be better.
Dedication

To my late grandfather, Anna (अण्णा), for telling me what a Ph.D. is when I was 11 and sowing the seeds of my future.
# Table of contents

Abstract...................................................................................................................................................... ii

Acknowledgements........................................................................................................................................ v

Dedication.................................................................................................................................................... ix

Table of contents.......................................................................................................................................... x

List of tables.................................................................................................................................................. xiv

List of illustrations........................................................................................................................................ xvi

1. **Introduction** ......................................................................................................................................... 1
   1.1. Research description......................................................................................................................... 1
   1.2. Rationale and significance............................................................................................................... 2
   1.3. Need to explore HPP infusion........................................................................................................ 7
   1.4. Background on HPP......................................................................................................................... 11
   1.5. Existing knowledge about HPP assisted infusion.......................................................................... 18
   1.6. Gaps in the existing literature......................................................................................................... 26
   1.7. Research objectives.......................................................................................................................... 27

2. **Influence of HPP parameters on infusion of calcium in PME pre-treated baby carrots** ..................... 29
   2.1. Brief introduction.......................................................................................................................... 29
   2.2. Materials and methods............................................................................................................... 31
2.3. Experimental design........................................................................................................... 41

2.4. Analysis of controls............................................................................................................... 47

2.5. Effect of HPP parameters on extent of calcium infusion................................................. 48

2.6. Effect of HPP parameters on beta-carotene extractability, color, and

texture of the calcium infused baby carrots............................................................................. 54

2.7. Effect of PME pre-treatment on calcium infusion in baby carrots under

HPP..................................................................................................................................................... 60

2.8. Effect of pressure cycling on calcium infusion in baby carrots under

HPP..................................................................................................................................................... 61

2.9. Key takeaways for this study................................................................................................. 64

3. Effect of food microstructure on the extent of high pressure assisted

infusion of calcium in selected fruits and vegetables................................................................. 66

3.1. Brief introduction...................................................................................................................... 66

3.2. Materials and methods........................................................................................................... 67

3.3. Microstructure analysis.......................................................................................................... 74

3.4. Microstructure quantification................................................................................................. 78

3.5. Mapping of infused calcium in processed samples................................................................. 93

3.6. Effect of fruit/vegetable microstructure on determining the extent of

infusion............................................................................................................................................... 102
3.7. Cell-permeabilization index (Zp) and its effect on calcium infusion ................................................................. 107

3.8. Effect of HPP assisted calcium infusion on texture and color of the samples ......................................................................................................................... 113

3.9. Key takeaways for this study ........................................................................................................................................ 119

4. Development of a mathematical model for high pressure assisted calcium infusion in fruits and vegetables ................................................................. 122

4.1. Brief introduction ..................................................................................................................................................... 122

4.2. Existing models by other researchers ....................................................................................................................... 124

4.3. Need for a new model .............................................................................................................................................. 129

4.4. Experimental design ............................................................................................................................................ 130

4.5. Materials and methods ......................................................................................................................................... 132

4.6. Results ................................................................................................................................................................. 135

4.7. Principal component analysis .............................................................................................................................. 144

4.8. Response surface analysis .................................................................................................................................... 151

4.9. Proposed mathematical model ............................................................................................................................ 159

4.10. Advantages and drawbacks of the proposed model ......................................................................................... 172

4.11. Key takeaways for this study ................................................................................................................................ 174

5. Conclusions ......................................................................................................................................................... 176

6. Future work ............................................................................................................................................................ 179
7. Bibliography ................................................................................................................. 180

8. Acknowledgment of previous publications ................................................................ 200
List of tables

Table 2.1 Experimental points according to the BBD ........................................ 42

Table 2.2 Summary of analysis of all controls ...................................................... 47

Table 2.3 Summary of model coefficients for the effects of the processing variables along with their associated p-values ................................................... 49

Table 3.1 Comparison of characteristic lengths (µm) of baby carrots, celery, and mango ................................................................. 81

Table 3.2 Values of CD and CWT in different regions of a baby carrot sample .... 91

Table 3.3 Values of CD and CWT in different regions of a celery sample .......... 92

Table 3.4 Values of CD and CWT in a mango sample ........................................ 92

Table 3.5 Variation between the extent of calcium infusion under high pressure with changes in the microstructure of fruit or vegetable ......................... 103

Table 3.6 Differences between the color parameters (L*, a*, and b*) of raw baby carrots, celery, and mango and HPP processed samples at 500 MPa for 15 min using 6% CLG solution ................................................................. 118

Table 4.1 Summary of experimental design for developing mathematical model for HPP assisted calcium infusion in different food matrices .............. 132

Table 4.2 Coefficients corresponding to each principal component ................. 149
Table 4.3 Regression coefficients for P, CD, and Zp for calcium infusion in case 1

Table 4.4 Regression coefficients for P and CD for cell permeabilization in case 1

Table 4.5 Regression coefficients for P, CD, and Zp for calcium infusion in case 2

Table 4.6 Regression coefficients for P and CD for cell permeabilization in case 2

Table 4.7 Regression coefficients for CD and Zp for calcium infusion in case 3

Table 4.8 Variation of $\frac{CWT}{CD}$ across different regions in baby carrots, celery, and mango. Higher value of $\frac{CWT}{CD}$ indicates higher resistance to infusion.

Table 4.9 Effect of transport tubes ($f_{tt}$) in different food systems

Table 4.10 Values of model constants obtained after fitting the model to the calcium infusion data from case 1 using non-linear least squares regression.
List of illustrations

Figure 1.1 Number of industrial scale HPP equipments operating worldwide and across different food sectors. Figure adapted from Pinto et al. (2020) and courtesy of Hiperbaric S.A. (Burgos, Spain).................................................3

Figure 1.2 (a) Direct versus (b) indirect pressurization........................................13

Figure 1.3 Variation of pressure (MPa) and temperature (°C) during a typical HPP cycle..................................................................................................................................................13

Figure 1.4 Hiperbaric 525 by Hiperbaric™.................................................................14

Figure 1.5 Steps involved in the operation of an HPP unit......................................15

Figure 1.6 A schematic representation of mass transfer during HPP assisted infusion (based on a figure from Karwe et al. 2016).................................22

Figure 1.7 Enhanced infusion of quercetin in cranberries by HPP, as compared to osmotic and vacuum infusion (from Mahadevan et al. 2015)..........24

Figure 1.8 Infusion of quercetin in fresh cranberries by HPP and at atmospheric pressure (from Mahadevan et al. 2016).........................................................24

Figure 1.9 High pressure assisted infusion of quercetin in fresh and frozen-thawed cranberries, a comparative graph (from Mahadevan et al. 2016).............................................................................................................25
Figure 2.1 Schematic representation of the high pressure processing (HPP) unit at the Department of Food Science, Rutgers University.................34

Figure 2.2 Pressure and temperature variation during a single HPP cycle at 500 MPa for 10 min; recorded using LabVIEW™.................................35

Figure 2.3 Standard curve for calcium analysis at 317.99 nm...............................37

Figure 2.4 Munsell color system...........................................................................41

Figure 2.5 Experimental design to evaluate the effect of PME pre-treatment on high pressure assisted infusion of calcium in baby carrots..................45

Figure 2.6 Pressure and temperature variation during the pressure cycling experiments..........................................................................................46

Figure 2.7 Effect of pressure and CLG concentration on the amount of calcium infused in PME pre-treated baby carrots using HPP assisted infusion (t=10 min)......................................................................................51

Figure 2.8 Effect of time and CLG concentration on the amount of calcium infused in PME pre-treated baby carrots using HPP assisted infusion (P= 350 MPa)......................................................................................53

Figure 2.9 Effect of pressure (MPa) and time (min) on the amount of β-carotene extracted from HPP – calcium infused baby carrots (CLG=6% w/v)....55
Figure 2.10 Color difference between the raw unprocessed control (L) and HPP – calcium infused baby carrots (R) processed at the BBD center-point (350 MPa/10 min/6% CLG). .......................................................... 59

Figure 2.11 Effect of PME pre-treatment on the amount of calcium infused in baby carrots using high pressure-assisted infusion at 550 MPa for 15 min. ........................................................................................................................................... 61

Figure 2.12 Effect of pressure cycling at 550 MPa on the amount of calcium infused in PME pre-treated baby carrots using high pressure-assisted infusion of calcium (1 cycle: 15 min, 3 cycles: 5 min each, 5 cycles: 3 min each). ...................................................................................................................................... 63

Figure 2.13 Effect of pressure cycling on hardness of high pressure-assisted calcium infused baby carrots (1 cycle: 15 min, 3 cycles: 5 min each, 5 cycles: 3 min each). ........................................................................................................................................... 64

Figure 3.1 (a) Carrot cross-section (b) celery cross-section, and (c) mango cross-section. All samples were imaged at positions depicted with an ‘x’. ........................................................................................................................................... 76

Figure 3.2 Cell structure of celery (a) before and (b) after using Canny Edge Detection method, (c) 8-bit binary image of the well-defined cells, and (d) outlines of the cells showing the ‘particles’ that were analyzed
for area and perimeter. (All images are to the same scale: 850.19 µm x 850.19 µm)

**Figure 3.3** (Top) Qualitative differences between the cell structure of (a) baby carrots, (b) celery, and (c) mango (all images to the same scale (850.19 µm x 850.19 µm)). (Bottom) A histogram of the CLs obtained for (a) baby carrots, (b) celery, and (c) mango.................................81

**Figure 3.4** Cell structure differences within a baby carrot sample as observed at its (a) central region (b) around central region (c) periphery of central region (d) inner cells, and (e) outer edge..............................................85

**Figure 3.5** Cell structure differences within a celery sample as observed around its (a) transport tubes (b) outer edge, and (c) inner cells.........................86

**Figure 3.6** Cell structure in a mango sample.........................................................86

**Figure 3.7** (a) Representative image of the cell structure in baby carrots (b) Corresponding graph for variation in the intensity of pixels along a given horizontal line drawn on the image (a). Peaks below the threshold represent cell walls, with distance between peaks representing cell diameter (CD) and size of the peak at the threshold representing the cell wall thickness (CWT)..................................................89
Figure 3.8 (a) Locus of the infused calcium in a cross-section of a baby carrot tissue obtained by tagging the calcium with a fluorescent Fluo-8 dye and observing under a laser microscope. Intensity of each pixel is directly proportional to the amount of calcium present in that area; brighter the point, higher the calcium. (b) Variation in the intensity of Fluo-8 fluorescence from the center of the baby carrots (x = 0) to the outer edge (x = 1) shown by red box, indicating the distribution of infused calcium in the tissue. x is normalized radial distance in a baby carrot sample.

Figure 3.9 (a) Locus of the infused calcium in a cross-section of a celery tissue obtained by tagging the calcium with a fluorescent Fluo-8 dye and observing under a laser microscope. Intensity of each pixel is directly proportional to the amount of calcium present in that area; brighter the point, higher the calcium. (b) Variation in the intensity of Fluo-8 fluorescence from the inner edge of celery (x = 0) to its outer edge (x = 1), indicating the distribution of infused calcium in the tissue. x is normalized distance in a celery sample.

Figure 3.10 (a) Locus of the infused calcium in a cross-section of a mango tissue obtained by tagging the calcium with a fluorescent Fluo-8 dye and observing under a laser microscope. Intensity of each pixel is directly
proportional to the amount of calcium present in that area; brighter
the point, higher the calcium. (b) Variation in the intensity of Fluo-8
fluorescence from one edge of the mango cuboid (x = 0) to the other
edge (x = 1), indicating the distribution of infused calcium in the
tissue. x is normalized distance in a mango sample........................................99

**Figure 3.11** Histograms of cell diameter distribution in (a) baby carrots –
periphery of central region (b) celery – transport tubes, and (c)
mango..................................................................................................................102

**Figure 3.12** Amounts of calcium infused in baby carrots, celery, and mango with
increasing pressure. Bars that do not share a letter have significantly
different values from each other (p-value < 0.05). Calcium amounts
for HPP data points show infused calcium (total – inherent from
raw)...... ........................................................................................................104

**Figure 3.13** (a) Changes in the cell permeabilization index ($Z_P$) of baby carrots
with increasing pressure. Higher values of $Z_P$ indicated higher cell
breakage. (b) Effect of increasing pressure on the amount of calcium
infusion in baby carrots. Differences in letters indicate statistical
significance (p-value < 0.05).................................................................109
Figure 3.14 (a) Changes in the cell permeabilization index ($Z_p$) of celery with increasing pressure. Higher values of $Z_p$ indicated higher cell breakage. (b) Effect of increasing pressure on the amount of calcium infusion in celery. Differences in letters indicate statistical significance (p-value < 0.05).

Figure 3.15 (a) Changes in the cell permeabilization index ($Z_p$) of mango with increasing pressure. Higher values of $Z_p$ indicated higher cell breakage. (b) Effect of increasing pressure on the amount of calcium infusion in mango. Differences in letters indicate statistical significance (p-value < 0.05).

Figure 3.16 Variation between the extent of calcium infusion and the cell permeabilization index ($Z_p$) across all food samples, without taking into consideration the food matrix that was infused ($R^2 = 0.19$).

Figure 3.17 Change in the energy ($J$) required to cut a sample with increasing pressure (MPa) for baby carrots, celery, and mango. Bars that do not share a letter have significantly different value from each other (p-value < 0.05).

Figure 3.18 Change in the cutting peak force (N) with increasing pressure (MPa) for baby carrots, celery, and mango. Bars that do not share a letter
have significantly different value from each other (p-value < 0.05)………………………………………………………………………………………………114

**Figure 3.19** Color of (a) baby carrots, (b) celery, and (c) mango before (left) and after HPP processing (right) at 500 MPa for 15 min using 6% CLG solution………………………………………………………………………………………………117

**Figure 4.1a** Calcium infusion in baby carrots across the pressure range in the three cases…………………………………………………………………………………………………………………………………………..137

**Figure 4.1b** Cell permeabilization ($Z_P$) in baby carrots across the pressure range in the three cases…………………………………………………………………………………………………………………………………………..137

**Figure 4.2a** Calcium infusion in celery across the pressure range in the three cases…………………………………………………………………………………………………………………………………………..139

**Figure 4.2b** Cell permeabilization ($Z_P$) in celery across the pressure range in the three cases…………………………………………………………………………………………………………………………………………..140

**Figure 4.3a** Calcium infusion in mango across the pressure range in the three cases…………………………………………………………………………………………………………………………………………..142

**Figure 4.3b** Cell permeabilization ($Z_P$) in mangoes across the pressure range in the three cases…………………………………………………………………………………………………………………………………………..142
**Figure 4.4** Infusion of calcium in baby carrots, celery, and mango via osmosis, using 9% CLG solution, observed over a period of 24 h. The dashed lined indicate amount of calcium infusion obtained under HPP within 15 min of processing. 144

**Figure 4.5** (Left) Original data. (Center) Zero-centered data obtained by mean subtraction. (Right) Normalized data obtained by additional scaling of the standard deviation of zero-centered data. The red lines indicate the range of the data (Figure adapted from Stanford University's course materials (CS231n: Convolutional Neural Networks for Visual Recognition)). 146

**Figure 4.6** Representation of principal components (PC1 and PC2) with a model dataset. First PC accounts for the maximum variance in the data. Second PC accounts for maximum of the remaining unexplained variance, while being orthogonal to the first PC. 147

**Figure 4.7** Variance in the original data explained by each principal component (PC). The first PC explains the most variance, and the percentage of variance explained decreases with each principal component. (Figure adapted from Jaadi 2020 and Holland 2008). 148
Figure 4.8 Data distribution across the first two principal components. Orange markers indicate data for baby carrots, green markers for celery, and yellow markers for mango.

Figure 4.9 Contour plot visualizing the change in calcium infusion with cell diameter (CD) and pressure in case 1, based on regression analysis.

Figure 4.10 Contour plot visualizing the change in cell permeabilization (Zp) with cell diameter (CD) and pressure in case 1, based on regression analysis.

Figure 4.11 Contour plot visualizing the change in calcium infusion with cell diameter (CD) and pressure in case 2, based on regression analysis.

Figure 4.12 Contour plot visualizing the change in cell permeabilization (Zp) with cell diameter (CD) and pressure in case 2, based on regression analysis.

Figure 4.13 Contour plot visualizing the change in calcium infusion with cell diameter (CD) and pressure in case 3, based on regression analysis.
Figure 4.14 (Left) Cross section of a carrot with transport tubes at the center (image adapted from Adams et al. 2014) and (Right) the corresponding direction of calcium infusion throughout the cross section. Calcium molecule is primarily transported through the transport tubes (vertical direction) into neighboring cells (horizontal direction) that pose a barrier to calcium movement. Thicker lines depict higher resistance to calcium movement.

Figure 4.15 Variation of $\frac{CWT_{CD}}{CD}$ in baby carrots, celery, and mango with increasing distance from the site of infusion. Higher $\frac{CWT}{CD}$ indicates higher resistance to infusion.

Figure 4.16 Proposed model fit to observed data for calcium infusion in case 1, shown with respect to pressure (MPa) and effective microstructure $(M_{C_{eff}})$.

Figure 4.17a Proposed model fit to observed data for calcium infusion in case 1, shown with respect to factor of pressure (MPa) and factor of transport tubes $(f_{tt})$.

Figure 4.17b Proposed model fit to observed data for calcium infusion in case 1, shown with respect to pressure (MPa) and cell diameter ($\mu$m).
CHAPTER 1

INTRODUCTION

1.1. Research description

High pressure processing (HPP) is a well-known non-thermal technology that has been extensively researched and explored as a food processing technique. It has also been widely commercialized since it is known to improve the quality and safety of foods by inactivating spoilage microorganisms and many deteriorative enzymes which consequently leads to an extended shelf-life. However, beyond these applications, HPP also has the potential to be employed as a technique to make functional foods by enhancing the micronutritional profile of fruits and vegetables. In the past, a few researchers have demonstrated the use of HPP to enhance the mass transfer of biomolecules such as salt, sugar, and polyphenols in and out of different food matrices. Motivated by these studies, our work was intended to evaluate if HPP would be a suitable technique for food fortification and enrichment.

The research presented in this dissertation was aimed at exploring High Pressure Processing (HPP) as a technique to infuse calcium, a short-fall micronutrient, into commonly consumed fruits and vegetables. The main focus of this research was to understand the factors that govern the mass transfer of
calcium under high pressure and propose a mathematical model that could explain this process at mechanistic level, if not completely, at least partially. The two unique aspects of this study were (1) the development of a new methodology to quantify the microstructure of fruits and vegetables, and (2) the development of a mathematical model that takes into account the level of pressure applied and the nature of the food matrix that is being infused.

1.2. Rationale and significance

The consumer demand for fresh and minimally processed fruits and vegetables has greatly increased over the last decade (Patrignani et al. 2015). Minimally processed fruit and vegetable products are made from fresh fruits and vegetables that have been processed to improve their functionality without significantly altering their fresh-like properties. Examples of such products include but are not limited to fresh-cut produce (lettuce, apple, pear, peach, and celery) immersed in hot water to reduce browning as well as fruits (pears, strawberries, kiwi, honeydew, nectarines, peaches, and melons) dipped in calcium solution to retain firmness of the fruit tissues. Technologies such as refrigeration, chemical preservatives and additives, mild heat treatments, microwave processing, high pressure processing, high intensity pulsed electric field, pulsed light, ozone technology, edible coatings, treatments using electrolyzed oxidizing water, and dipping in chemical solutions (ascorbic acid or calcium) are some examples of
technologies that are and can be used to develop minimally processed fruits and vegetables (Siddiqui et al. 2011). Among all the technologies mentioned above, high pressure processing is the only technology that has been widely commercialized. Over the past three decades, there has been a drastic increase in the number of HPP equipment used across different food sectors around the world (Fig. 1.1). Majority of these machines are used for improving the shelf-life and quality of fresh fruits and vegetables, processed fruits and vegetables in the form of juices and beverages, and meat products (Pinto et al. 2020).

![Figure 1.1](image)

**Figure 1.1** Number of industrial scale HPP equipments operating worldwide and across different food sectors. Figure adapted from Pinto et al. (2020) and courtesy of Hiperbaric S.A. (Burgos, Spain).
In addition to the minimally processed foods, there has been an increasing interest for developing functional foods that have health promoting properties beyond the basic function of supplying nutrients (Bellary and Rastogi 2016). A functional food is any food that can beneficially affect one or more target functions in the body, in addition to its inherent nutritional effects, in a way that it is relevant to either improved stage of health or wellbeing and/or reduction of the risk of diseases (Diplock et al. 1999). Since fruits and vegetables play a significant role in the human diet and are widely consumed across all populations, developing strategies to enhance the nutritional composition of fruit and vegetable products as functional foods can be of great interest (Gras et al. 2003).

The 2020 Dietary Guidelines Advisory Committee (DGAC) of the United States Department of Agriculture identified calcium as a nutrient of public health concern based on low intakes and prevalence of low bone mass and osteoporosis in the U.S. population. According to the report, about 44% of the U.S. population is at the risk of calcium inadequacy, with the number being higher for women across different age groups (68% in girls in school, 80% in adolescent girls, and 76% in women above 51 years). Although calcium supplementation is an option, due to concerns about the safety of these supplements and a relative lack of data about their health benefits, the strategy of supplementation to meet the
recommended daily intake (RDI) for calcium has limited recommendation. Alternatively, the report advises that calcium intakes can be improved by increasing the consumption of dairy and fortified foods. Consumption of dairy, however, has limited outreach owing to the occurrence of lactose intolerance. More than 36% of the American population has lactose malabsorption (NIH, 2018) which leads to lactose intolerance. Food fortification, hence, seems to be a promising alternative to supplementation. In 2005, FAO/WHO identified food fortification as a valid technology to tackle micronutrient malnutrition. The technique of food fortification when combined with existing technologies, such as HPP, has the potential to lead rapid improvements in the micronutrient status of a population at a reasonable cost. Hence, it is important to look into fortification of commonly eaten fruits and vegetables, especially the ones that are eaten raw and are easily available across the US.

Foods fortified with minerals can be developed by adopting various impregnation techniques (Bellary and Rastogi 2016). Fortification of liquid foods is a fairly straightforward process and is a developed technology. However, enhancing the amounts of micronutrients in solid foods, especially fresh fruits and vegetables, using various impregnation techniques entails an added complexity of maintaining the cellular and structural integrity of the food matrix (Fito et al. 2001). Most widely explored infusion techniques for developing
functional foods have been osmotic and vacuum impregnation. A large number of studies have investigated the roles of osmosis and vacuum in enhancing the mass transfer in foods (details about these studies are included in section 1.3). However, a major challenge with osmotic and vacuum impregnation is the long processing times (order of hours) required to achieve significant levels of infusion (Karwe et al. 2016). In that regard, HPP has been shown to enhance the infusion levels in much shorter times (order of minutes). It was therefore necessary to explore a novel aspect of HPP as a food fortification technique to make nutritionally enhanced functional foods that are minimally processed.

An important consideration with respect to improving the micronutritional profile of fruits and vegetables by HPP assisted calcium infusion, is the choice of these foods such that they are widely consumed and are most commonly eaten raw. This choice would ensure that the HPP – calcium infused fruits and vegetables would be convenient to consume. Based on this understanding, the choices of fruit and vegetables for this research were – baby carrots, celery, and mango. Baby carrots (also called fresh-cut carrots) are one of the most popular vegetables in the U.S. and account for more than half of the supermarket sales of fresh-cut vegetables (ERS, 2007). Similarly, fresh celery and cut-mango are popular in the US and their per capita consumption in pounds has been steadily increasing over the last decade (USDA-ERS survey, 2017). Baby carrots, celery,
and mango are popular for being convenient and healthy snacks/lunchbox items but lack substantial amounts of inherent calcium. The focus of this study was, therefore, to investigate the application of HPP to infuse calcium in baby carrots, celery, and mango.

1.3. Need to explore HPP infusion

The process of infusion of a solute or nutrient molecule into a food matrix has been traditionally performed by osmosis. Osmotic dehydration has been used to partially remove water from and simultaneously infuse small solute molecules, such as salt and sugar, into cut fruits and vegetables. Because of the osmotic pressure difference between fruit/vegetable matrix and the surrounding hypertonic solution, water in the food diffuses out into the solution, while the solute simultaneously diffuses (infuses) into the food. This movement of molecules in and out of the food matrix takes place through the semi-permeable cell membrane in the food matrix that resists the mass transfer. The state of the cell membrane (completely intact to completely damaged/permeable) is vital in determining the efficiency of diffusion into and out of the food matrix (Rastogi et al. 2002). The effective diffusivity values for soft plant tissues (fruits and vegetables) at a temperature of 25 °C are estimated to be in the order of magnitude of 10^{-10} m^2/s, while that for hard structured foods (ex. nuts, rice) are estimated in the order of magnitude of 10^{-11} m^2/s (Doulia et al. 2000). Moreover,
these values vary depending on the strength of the cell wall of fruits/vegetables being used. The diffusion coefficient for sucrose in soft cell walled fruits and vegetables, such as apples and potatoes, is two to four times higher than that for hard cell walled sugarcane (Schwartzberg and Chao 1982).

This process of moving molecules in and out of a food matrix simply by osmosis is extremely slow (hours to days depending on the target amount of infusion) and hence, other treatments that have better efficiency and performance have been explored. These include vacuum impregnation, exposure to high intensity pulsed electric field (HIPEF) prior to osmotic treatment, ultrasound treatment during osmotic diffusion, and high pressure processing.

In vacuum impregnation, solid food is immersed in a liquid and vacuum is applied in pulses to this solid-liquid system. While the vacuum is applied, the internal gas in the pores of the product expands and partially flows out. This is coupled with inflow of the external immersed liquid through the capillary pores, during the vacuum release step (Andres et al. 2001) which leads to infusion of nutrient molecules into the food matrix. In HIPEF, rapid pulses of the electric field increase the permeability of the plant cells facilitating faster movement of solute and water in and out of the cells. Application of ultrasound leads to the phenomenon of cavitation (formation of gaseous bubbles that burst and generate localized pressure) inside the fruit/vegetable matrix that results in faster
diffusion during osmotic processes (Simal et al. 1998). Numerous studies are available that explore these techniques in detail (to name a few - *Osmotic infusion*: Barrera et al. 2004; Barrera et al. 2009; de Escalada Pla et al. 2009; Rózek et al. 2009; Rastogi and Raghavrao 1996; Santacruz-vazquez et al. 2008; *Vacuum impregnation*: Hironaka et al. 2014; Yilmaz et al. 2018; Xie and Zhao 2003; Zhao and Xie 2004; Torreggiani and Bertolo 2001; Alzamora et al. 2005; Anino et al. 2006; Deng and Zhao 2008; Moraga et al. 2009; *HIPEF*: Phoon et al. 2008; Ade-Omowaye, et al. 2002; Taiwo, K. A. et al. 2003; *Ultrasound*: Hamedi et al. 2018; Mashkour et al. 2018; Yilmaz et al. 2018; Stojanovic and Silva 2007; Carcel et al. 2007; Deng and Zhao 2008) and their comprehensive review is available in more recent publications by de Escalada Pla et al. (2020) and Bellary and Rastogi (2016).

A major hurdle with these techniques is that their applications are limited to pre-treatments to osmotic infusion. Moreover, they have been primarily explored on a laboratory scale only and have technical difficulties related to commercialization, due to the small batch size of foods that can be processed, and the disproportionate cost associated with their operation. Alternatively, high pressure processing (HPP), which has also been shown to enhance and accelerate the mass transfer of solutes into a food matrix, has been commercialized in the past decade as a food processing technique for pasteurization and enzyme
inactivation to improve food quality. Some commercially available HPP food products in the US are vegetable and fruit products, such as juice, salsa, salad dressing and guacamole; meat products such as ready-to-eat meats and poultry; and seafood such as shellfish and fish products (Frestl and Frestl 2013). In June 2016, the New South Wales Food Authority in Australia approved cold pasteurization of raw milk, making cold-pressed milk the latest HPP product in the market (The Food Authority in New South Wales 2016b). Similar to the techniques mentioned earlier, HPP has been shown to enhance the amount of nutrient molecules in a food matrix via infusion. However, a major difference between HPP and the other techniques is that HPP not only enhances infusion, but it also accelerates the mass transfer i.e., it is a faster process. HPP infusion typically lasts for 10 min to 30 min, which is significantly lesser processing time as compared to the other techniques. Given its commercialization and its known effect of significantly improving the nutritional profile of fruits and vegetables through enhanced and accelerated mass transfer, HPP has the potential to be food fortification technique. However, the mechanisms of mass transfer under high pressure are poorly understood and hence this application of HPP has not been researched very well. It is therefore important to investigate this novel application of the technology further and study the mechanisms involved to
better understand its potential and limitations, from future product development point of view.

1.4. Background on HPP

High pressure processing (HPP) is a non-thermal, batch processing technology wherein food products are subjected to extremely high pressures in the range of 100 MPa to 900 MPa, for a period of a few minutes up to 30 min. The first application of HPP on a food system was investigated by Hite (1899) who demonstrated the effectivity of the process in preserving milk while also further exploring the technology to preserve fruits and vegetables (Hite et al. 1914). The technology remained dormant for a long time until it was rediscovered by a Japanese company, Meidi-ya, who introduced the first ever high pressure processed food products (jams and jellies) in the commercial market (Thakur and Nelson 1998). Since then, a variety of high pressure processed food products have been introduced in the US as well as in other countries.

In a typical HPP run, the food product is packed in a flexible pouch and placed inside the pressure vessel which is built to withstand the extreme high pressures. The vessel is then filled with a pressure transmitting fluid, which in most cases is filtered water. The vessel is sealed and pressurized through direct (using a piston) or indirect pressurization (pumping excess water) until the
desired pressure is reached (Fig. 1.2). The pressure come-up time (CUT) ranges between 1 min to 4 min, depending on the target pressure, the size of the vessel, and the pump. At the end of the hold period, the pressure is released within a few seconds. Ethylene glycol is used as the pressure transmitting fluid when the HPP run has to be performed at sub-zero (°C) temperatures. Usually, HPP is performed at room temperatures, but can also be performed at higher temperatures ranging from 50 °C to 120 °C. Higher temperatures during an HPP process are typically used for inactivation of pressure resistant microbes and spores. was approved by the USFDA for low acid foods in 2009. In 2009 and 2015, USFDA approved two industrial petitions for preserving low-acid foods (mashed potato and seafood) by Pressure assisted thermal processing (PATP). However, PATP treated low acid shelf-stable products are not commercially available yet.

During pressurization, the temperature of the pressure transmitting fluid and the food product rises by 8 °C to 15 °C depending on the type of the fluid and the food composition. During the pressure hold period the temperature of the systems decreases slightly as the fluid loses its heat to the vessel. Figure 1.3 represents a typical HPP cycle with corresponding variation of the pressure and temperature with processing time.
**Figure 1.2** Direct (a) versus indirect (b) pressurization


**Figure 1.3** Variation of pressure (MPa) and temperature (°C) during an HPP cycle
All high pressure systems used industrially are batch processing systems. Large capacity HPP vessels are horizontally oriented, with current maximum capacity of 525 liters for in-package processing. In September 2017, Hiperbaric™ in Spain launched a new HPP equipment, Hiperbaric 1050Bulk, with a capacity of 1050 liter that can process beverages in bulk before bottling. Figure 1.4 shows an industrial scale 525 liter high pressure unit by Hiperbaric™, while Fig. 1.5 shows a schematic of the steps involved in the operation of an HPP unit.

**Figure 1.4** Hiperbaric 525 by Hiperbaric™.

Key advantages of HPP are:

1. Instantaneous transmittance of pressure to the food product, irrespective of its shape or size; thus, process time is not dependent on the amount of food product being processed,

2. Processing at or near room temperatures, in most cases

3. Microbial and enzyme inactivation in food at ambient conditions without the use of preservatives,

4. Changes induced in ingredients and products that provide novel functionality

5. Retention of nutrients, flavor, aroma, and color, unlike in thermal treatment
The energy required to compress 1 liter of water to 400 MPa is 19.2 kJ, as compared to 20.9 kJ for heating 1 liter of water from 20 °C to 25 °C. Owing to this difference, during HPP, the covalent bonds in a food molecule are less affected than the weak interactions (H-bond, Van der Waal’s forces) (Rastogi et al. 2007). As a result, HPP causes unfolding of protein chains that alters their functionality but does not affect the chemical constituents associated with desirable food quality components such as color, flavor, aroma, and nutritional compounds (Tao et al. 2015) in most cases. The change in functionality after HPP processing, thus, does not come at the cost of compromising on the sensorial attributes, unlike the case in thermal processing. However, HPP does not work favorably for all food systems. For example, whole pears and onions develop a strong pungent smell as well off-flavor.

During an HPP cycle, increase in pressure is accompanied by a decrease in volume of the food product as well as the pressure transmitting fluid. Thus, reactions that are favored by a decrease in volume are enhanced by pressure, and (Le Chatelier’s principle). Consequently, the reaction equilibrium shifts towards the most compact state and the reaction rate constant is altered depending on the activation volume ($\Sigma$ partial molar volume of activation complex – $\Sigma$ partial molar volume of reactants) of the reaction.
Another thermodynamic effect of decrease in volume at high pressure is the adiabatic heating associated with an increase in pressure. The thermodynamic relationship for this rise in temperature due to an increase in the pressure is given by Eq. 1.1, where $T$ is process temperature (K), $P$ is the applied pressure (Pa), $\nu$ is specific volume of the material (m$^3$/kg), $\beta$ is coefficient of thermal expansion (K$^{-1}$), and $C_p$ is the heat capacity of the material (J/kg·K).

$$\frac{dT}{dP} = \frac{T\nu\beta}{C_p}$$

when expressed as temperature rise per 100 MPa increase in pressures is also called as the compression heating value of the substance. Water has the lower compression heating value of ~3 K/100 MPa while oils and fats have higher values 6 K/100 MPa to 8.7 K/100 MPa (Rasanayagam et al. 2003). Since the compressibility of different food materials is different this can lead to non-uniformity in temperature in the food material as well as in the pressurizing medium, and potentially impact the microbial and enzyme inactivation (Khurana and Karwe 2009).

**Effect of HPP on food safety and food quality**

Vast literature is available on the use of HPP to improve food quality through enzyme and microbial inactivation. Pressures above 300 MPa can cause
irreversible protein denaturation and hence can be effective to inactivate
deteriorative enzymes in foods (Knorr et al. 2006). Several studies have shown
application of HPP to inactivate polygalacturonase (PG), polyphenol oxidase
(PPO), peroxidase (POD), β-glucosidase, lipoxygenase (LOX), and many other
enzymes that are responsible for degradation of color, flavor, texture, and
nutritional properties of food (Terefe et al. 2014). Similarly, many studies are
available that have shown microbial inactivation efficacy of HPP (Salvi et al. 2016).
Since the effect of HPP on food safety and food quality was not the focus of this
dissertation, these studies have not been discussed in detail. More information
about the effect of HPP on food related enzymes and microbes can be found in
the above-mentioned references as well as in Salvi et al. (2016).

1.5. Existing knowledge about HPP assisted infusion

**HPP as a pre-treatment to osmotic infusion**

Rastogi and Niranjan (1998) conducted one of the pioneering studies
involving application of HPP to enhance mass transfer. They evaluated the effect
of HPP pre-treatment to osmotic dehydration of pineapple slices, to enhance
mass transfer during the dehydration step. They observed that the diffusivity
values of water \( (D_{ew}) \) and solute \( (D_{es}) \) increased from \( 0.54 \times 10^{-9} \text{ m}^2/\text{s} \) and \( 0.71 \times 10^{-9} \text{ m}^2/\text{s} \) in untreated pineapple slices to \( 2.2 \times 10^{-9} \text{ m}^2/\text{s} \) and \( 1.4 \times 10^{-9} \text{ m}^2/\text{s} \),
respectively, after application of HPP (700 MPa for 5 min). In a similar study by
Ahromit et al. (2006), HPP pre-treatment was shown to enhance the water uptake of Thai glutinous rice during subsequent soaking. The enhanced mass transfer following HPP pre-treatment was attributed to the structural damage caused to the cell membrane as a result of HPP, thus increasing the cell permeability and resulting in increased mass transfer rate. In both the studies, the mass transfer facilitated by HPP was assumed to follow Fickian diffusion i.e., mass transfer due to a concentration gradient. Crank’s diffusion models (Crank, 1979) for respective geometries were used to calculate the diffusion coefficient. In a recent study by George et al. (2016), HPP pre-treatment was employed to increase the anthocyanin infusion during osmotic treatment of apple pieces. In another study by Rastogi et al. (2008), HPP was employed as a pre-treatment to carrot discs along with other pre-treatments of mild heat and calcium chloride followed by pressure-assisted-thermal-processing (PATP). It was observed that HPP pre-treatment in combination with calcium chloride pre-treatment improved the hardness of carrot discs after pressure-assisted-thermal-processing (PATP) at 700 MPa, 105 °C, for 15 min. The authors proposed that the combined high pressure and calcium treatment may have led to an increased amount of calcium in carrots tissues (demonstrated by slightly higher calcium content in the samples), which contributed to increased hardness by forming linkages with the demethylated pectin in the carrot tissue.
**HPP as an infusion technique**

High pressure processing (HPP) as an infusion technique by itself (i.e., not as a pre-treatment) was initially evaluated by Sopanangkul et al. (2002), who demonstrated an eight-fold increase in sucrose infusion in potato cylinders under high pressures (200 MPa to 600 MPa; 30 min to 180 min). They observed that the potato cells appeared to be progressively more permeabilized after HPP and the tissue structure opened up to diffusion. On similar lines, Villacis et al. (2008) evaluated the effect of varying pressures on the infusion of NaCl into turkey breasts subjected to HPP. Their study demonstrated increased diffusivity coefficients of NaCl under high pressure (50 MPa to 300 MPa), with highest diffusivity values at 150 MPa. HPP treatment at 150 MPa for 15 min was observed to have the highest values of the diffusivity coefficient for NaCl ($5 \times 10^{-8}$ m$^2$/s), while also providing meat samples with minimum hardness, gumminess, and chewiness. Fraeye et al. (2010) demonstrated a synergistic effect of HPP, pectinmethylesterase (PME), and calcium chloride, resulting in increased firmness of strawberry tissue during frozen storage. This increased molecular rigidity of the strawberry tissue was attributed to the crosslinking of the demethylated pectin (caused by the action of PME) with calcium, forming an egg-box structure (Grant et al. 1976), which improved the structural rigidity.
Until now, researchers have attributed the increase in mass transfer under HPP to disruption of cell structure and consequent cell permeabilization (Dörnenburg & Knorr 1993; Rastogi and Niranjan 1998; Rastogi et al. 2000; Sopanangakul et al. 2002; Taiwo et al. 2003; Fraeye et al. 2010; Landl et al. 2010; Nuñez-Mancilla et al. 2011). It has been hypothesized that a concentration gradient between the constituents of the cell cytoplasm and the surrounding hypertonic infusing solution outside the cell, coupled with increased cell permeability, leads to an increased rate of transfer of infusate molecules into the cell, and concomitant loss of some water-soluble cell constituents. A schematic representation of this phenomenon is shown in Fig. 1.6.
Before HPP, the infusate molecules ('I') dissolved in a solution are outside the undamaged plant cell membrane. The water-soluble ions and molecules ('S') are inside the cell along with cytoplasmic water ('W').

During HPP, the volume is compressed and the cell membrane is permeabilized. The infusate molecules ('I') are pushed into the cells and the water-soluble molecules ('S') and cytoplasmic water ('W') are squeezed out.

After HPP, the concentration of infusate molecules ('I') inside the cell is higher.

**Figure 1.6** A schematic representation of mass transfer during HPP assisted infusion (based on a figure from Karwe et al. 2016)
Mahadevan et al. (2015 and 2016) evaluated the role of cell permeabilization in enhanced infusion during HPP. They studied high pressure assisted infusion of quercetin (>300 kDa) into scarified fresh and scarified frozen-thawed cranberries to evaluate the enhanced infusion and the role of cell permeabilization during HPP. HPP at 100 MPa for 10 min led to two to three times higher amount of quercetin in frozen-thawed cranberries, as compared to vacuum and osmotic infusion (Fig. 1.7). Moreover, HPP was shown to result in ~144 times faster infusion compared to atmospheric pressure diffusion. The amount of quercetin infused in fresh cranberries after HPP treatment at 100 MPa for 10 min was shown to be equivalent to the amount infused after 24 h at atmospheric pressure (osmotic infusion) (Fig. 1.8). However, unlike the observations by other researchers (Rastogi and Niranjan 1998; Rastogi et al. 2000; Villacis et al. 2008), Mahadevan et al. (2015) did not observe significant increase in the amount of infused quercetin with increase in pressure beyond 100 MPa (Fig. 1.9). They showed that even at low pressures up to 20 MPa, the amount of quercetin infused was twice as much when compared to vacuum and osmotic infusion.
**Figure 1.7** Enhanced infusion of quercetin in cranberries by HPP, as compared to osmotic and vacuum infusion (from Mahadevan et al 2015)

**Figure 1.8** Infusion of quercetin in fresh cranberries by HPP and at atmospheric pressure (from Mahadevan et al. 2016)
Mahadevan et al. (2016) evaluated possible mass transfer mechanisms related to enhanced infusion under pressure. In order to decouple the effect of cell permeabilization, HPP assisted quercetin infusion was carried out in scarified fresh and frozen-thawed cranberries. The freeze-thawing process caused cell permeabilization in cranberry tissue prior to HPP treatment thus ensuring that the cells were permeabilized before HPP assisted infusion. The extent of cell permeabilization caused by freeze-thawing was similar to that of HPP processed fresh cranberries (cell permeability index ($Z_P$) = 0.5, for both frozen-thawed and HPP cranberries). However, it was observed that the amount of quercetin infused in fresh cranberries under HPP was higher as compared to the frozen-thawed HPP infused cranberries (Fig. 1.9). If cell permeabilization was the sole factor

Figure 1.9 High pressure assisted infusion of quercetin in fresh and frozen-thawed cranberries, a comparative graph (from Mahadevan et al. 2016)
driving infusion, the amount of infusion in both fresh and frozen-thawed cranberries would have been the same after HPP. Mahadevan et al. (2016) suggested that the enhanced infusion under high pressure may be a combination of the effect of concentration driven diffusion due to cell permeabilization and the instantaneous pressure-driven mass transport. Their work showed that the pressure-driven transport contributed to 60% of the infusion while concentration-driven diffusion (as a result of cell permeabilization) contributed to 40% of the infusion. The difference in the quercetin amounts infused in fresh and frozen-thawed cranberries after HPP indicated that more factors beyond cell permeabilization are involved in the process and significantly affect the extent of infusion. In addition to cell permeabilization, we think that other factors such as the microstructure of the food matrix, the size of the infusing molecule, and the processing conditions play an important role in enhancing the mass transfer of molecules under HPP. However, these aspects remained unexplored.

1.6. Gaps in the existing literature

Although high pressure processing has been shown by many researchers to have enhanced the diffusion of different biomolecules, such as salt, sugar, and polyphenols, the technique has not been further explored for the purpose of food fortification. Owing to the enhanced mass transfer under HPP, HPP has the potential as a food fortification method to improve the nutritional content of
common fruits and vegetables. Moreover, although the diffusivities of the studied biomolecules have been calculated by researchers in their studies, no mechanistic insight has yet been provided into the process of mass transfer under high pressure. All earlier studies have assumed that the mass transfer under high pressure follows Fickian diffusion laws i.e., driven by concentration gradient only. However, these models and calculations do not account for the effect of pressure at all. As mentioned earlier, factors such as the microstructure of the food matrix, the size of the infusing molecule, and the processing conditions, still remain poorly understood. It is, therefore, important that this phenomenon of high pressure assisted infusion be studied in more detail at mechanistic level and evaluated for its application as a food fortification technique.

1.7. Research objectives

The overall objective of this research was to demonstrate enhanced infusion of calcium, an important micronutrient, into selected fruits and vegetables while evaluating the role of fruit/vegetable microstructure in determining the extent of infusion and providing quantitative insights into the process of mass transfer under high pressure. To ensure that this research addressed the aforementioned research gaps, specific objectives of this study were developed accordingly. The specific objectives were:
1. To evaluate the role of HPP as a technique to infuse significant amounts of calcium, by studying the influence of processing parameters on the extent of calcium infusion in baby carrots and the consequent effect on other nutritional and physical properties of baby carrots.

2. To evaluate the role of food microstructure on determining the extent of infusion by infusing calcium in baby carrots, celery, and mango using HPP.

3. To develop a mathematical model that describes the effect of pressure, microstructure, and cell permeabilization, all together on the extent of calcium infusion under HPP.
CHAPTER 2

INFLUENCE OF HPP PARAMETERS ON INFUSION OF CALCIUM IN PME PRE-TREATED BABY CARROTS

This chapter has been reproduced from two research papers titled “High pressure-assisted infusion of calcium into baby carrots part I: influence of process variables on calcium infusion and hardness of the baby carrots” and “High pressure-assisted infusion of calcium into baby carrots part II: Influence of process variables on β-carotene extraction and color of the baby carrots” authored by Noopur Gosavi, Deepti Salvi, and Mukund V Karwe and published in the Food and Bioprocess Technology journal in 2019.

2.1. Brief introduction

The aim of this study was to understand the potential of HPP to infuse significant amount of calcium (> 10% RDI ~ 100 mg per serving) in baby carrots and to evaluate the effect of processing parameters on the extent of calcium infusion. As previously discussed in section 1.2, the choice of calcium as an infusing micronutrient was motivated by the scientific report by USDA’s DGAC (2015) that categorized calcium as a nutrient of public health concern, and the choice of baby carrots was based on findings by Lucier and Lin (2007) that
showed that baby carrots are one of the most popular vegetables in the US accounting for more than half the supermarket sales of fresh-cut vegetables. Earlier studies (Guillemin et al. 2008; Fraeye et al. 2010) have demonstrated the synergistic effect of PME (pectinmethylesterase) and calcium on preserving the structural rigidity of plant tissue. Hence, PME pre-treatment was used on the baby carrots before HPP infusion. PME pre-treatment would cause the pectin bonds in the cell membranes of the baby carrot tissue to demethylate, making them available for calcium to bind. The effect of cross-linking calcium to demethylated pectin chain causes the formation of an “egg-box” structure (Grant et al. 1973) which contributes to improving / preserving cellular rigidity.

The primary objective of this research was to evaluate the effect of high pressure processing (HPP) variables (pressure and holding time) and the concentration of infusate solution (Calcium Lactate Gluconate (CLG)) on the amount of calcium infused in the PME pre-treated baby carrots, along with the effect on the baby carrot hardness, beta-carotene extraction, and color. The secondary objectives of this research were to evaluate the effect of PME pre-treatment and the effect of pressure cycling on the extent of infused calcium along with the other variables mentioned above.
2.2. Materials and methods

**Materials**

Bolthouse® Farms Premium Sweet Petites™ baby carrots were purchased from a local retail store. For the purpose of this study, one serving size of baby carrots was defined as per the nutritional label on the purchased baby carrots, which was 85 g of baby carrots per serving. FirmSEB® (Specialty Enzymes and Biotechnologies Co., California) PME enzyme was used for enzyme pretreatment of the 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 = 16.67×10⁻⁹ kat = 16.67 nkat). Calcium Lactate Gluconate (CLG, Jungbunzlauer Suisse Ag, Switzerland) in anhydrous form, containing 13 % calcium, was used as a source of calcium for the infusion experiments. Different concentrations of the CLG solution (% w/v) (also referred to “infusate solution” in text) were prepared by mixing the anhydrous CLG in DI water at room temperature. Calcium analysis was performed using ICP-OES (Inductively Coupled Plasma – Optical Emission Spectroscopy) (Varian Vista-Pro™ (Illinois, USA) and a 1000 ppm standard solution of calcium (1000 µg/mL of CaCO₃ in 2% HNO₃)
procured from High Purity Standards™ (Charleston, South Carolina, USA) was used to prepare the standard solutions. All other chemicals for the study were purchased from Sigma Aldrich®, St. Louis, Missouri, USA. Texture analysis was performed on Brookfield Ametek® (Massachusetts, USA) CT3 Texture Analyzer, while color analysis was performed using Konica-Minolta CR-410 handheld colorimeter. Dionex Ultimate 3000 series reverse phase HPLC was used for the estimation of β-carotene in the control and processed baby carrot samples.

**Methods**

**Enzyme pre-treatment**

The manufacturer of the PME enzyme recommended a usage of 200 g to 500 g of the PME solution (1000 U) per ton of produce. Based on these recommendations, the PME solution (1000 U) was diluted to 0.0175 % (w/v) for experimental purposes. 0.0175 % PME solution was kept in a water bath to maintain the enzyme solution at 37 °C. Baby carrots were graded to have uniform size (~50 mm long and 10 mm to 15 mm diameter) with no physical defects or surface contamination. Washed and dried baby carrots were soaked in the PME solution at 37 °C for 45 min. The ratio of baby carrots to the PME enzyme solution was kept constant at 1:2 (w/w) based on previous research by Mahadevan et al. (2015). Post PME treatment, the PME enzyme solution was
drained off and the baby carrots were thoroughly washed with DI water to remove any PME solution adhered to their surface.

**Sample preparation for infusion experiments**

Different concentrations of the CLG solution (3 % to 9 % (w/v)) were prepared by dissolving the anhydrous CLG in DI water at room temperature. PME pre-treated baby carrots were immersed in the infusate solution of CLG and vacuum-packed in flexible polyethylene pouches for subsequent HPP. The ratio of baby carrots to infusate solution was maintained at 1:2 (w/w).

**High pressure assisted infusion**

The Rutgers HPP unit (Elmhurst Research Inc., Albany, NY, USA) was used for all HPP experiments. The unit has a vertically oriented vessel with a working capacity of 10 liters and operating over a range of 100 MPa to 700 MPa. For all experiments, filtered water at room temperature was used as the pressure transmitting fluid. A schematic of the unit is shown in Fig. 2.1.
Figure 2.1 Schematic representation of the high pressure processing (HPP) unit at Department of Food Science, Rutgers University

Samples were loaded and unloaded through a pneumatically controlled top closure. The vessel can be titled up to 100 degrees from the vertical position for the loading and unloading. Vacuum packed samples were loaded into the vessel along with water as the pressure transmitting fluid, and a 20 HP pump was used to pressurize the unit. The come-up time for all experiments varied between 45 s to 180 s. Once the desired pressure was reached, the pressure was maintained for a specified hold time and the depressurization at the end of the cycle occurred in less than 10 s. The operation of the unit was controlled through a PLC control unit and the pressure and temperature data was recorded for each HPP cycle using a LabVIEW™ 7 Express Version (National Instruments, Austin, Texas, USA). An increase in temperature due to adiabatic compression heating during each
A pressure cycle was noted. The temperature rise recorded was in the range of 8 °C to 15 °C. During the hold period, the temperature dropped by 2 °C to 5 °C, due to loss of heat to the vessel. Figure 2.2 shows the pressure and temperature data recorded during a pressure cycle at 550 MPa for 15 min.

![Graph showing pressure and temperature variation during a single HPP cycle at 500 MPa for 10 min](image)

**Figure 2.2** Pressure and temperature variation during a single HPP cycle at 500 MPa for 10 min; recorded using LabVIEW™.

At the end of each pressure cycle, the samples were unloaded and the vacuum-packed bags were cut open immediately to drain the infusate solution. The processed baby carrots were washed thoroughly with DI water and dried by gently blotting on paper towels to remove surface adhered infusate solution. Samples were immediately analyzed for texture and color, and then stored at –20 °C until further analysis.
Analysis of calcium

Baby carrot samples were ground in a home blender (Osterizer®, Sunbeam products Inc., Florida, USA). Ground baby carrots (30 g) were weighed in a ceramic crucible and subjected to ashing in a muffle furnace (Thermolyne corporation, Iowa, USA) at 600 °C for 6 h. The ash from each sample was dissolved in 5 mL of conc. HNO₃ (15.6 M). Concentrated acid solution (0.1 mL) containing the dissolved ash was diluted with 4.9 mL of DI water to make a final concentration of 2 % HNO₃ in the samples. These samples were used to analyze the amount of calcium infused.

ICP-OES at the Department of Marine Sciences, Rutgers University, was used for analysis of calcium in the processed baby carrots. The samples were analyzed for calcium at five different wavelengths of 315.887 nm, 317.99 nm, 393.366 nm, 396.847 nm, and 422.673 nm, to find which wavelength gave the best correlation. Based on literature, calcium emission is known to occur at these five wavelengths. Standard calcium solutions in the range of 0 ppm Ca to 500 ppm Ca were analyzed at the five different wavelengths, and linear standard curves were obtained at each wavelength. Based on the R² values of the linear standard curves obtained at each wavelength, the wavelength of 317.99 nm (R² = 0.9996) was selected for further experiments. Figure 2.3 represents the standard curve at 317.99 nm that was used to determine the calcium content of the processed...
samples as well as of the controls. Calcium content was quantified in terms of mg of calcium/85 g of baby carrots.

![Graph of Standard Curve for Calcium Analysis at 317.99 nm using ICP-OES](image)

**Figure 2.3** Standard curve for calcium analysis at 317.99 nm using ICP-OES

### Analysis of β-carotene

β-carotene being a photosensitive compound, its extraction was performed under dark conditions, and each processed sample was extracted in triplicates. Baby carrots were ground in a home blender and extracted for β-carotene following a slightly modified version of a procedure by Rodriguez-Amaya (2001). Ground sample (0.5 g) was mixed with 50 mL of cold acetone along with 0.05 g of butylated hydroxytoluene (BHT) to avoid carotenoid degradation. This mixture was subjected to various extraction steps in the order given below:
1. Shear mixing for 20 min using a Polytron® PT 1600 E bench top homogenizer (Kinematica Inc., NY, USA).

2. Ultrasonication for 2 min.

3. Vacuum filtration using 50 mL of cold acetone and Whatman filter paper #1 to remove the residual calcium tissues.

4. Partitioning to petroleum ether through decantation: Decantation was performed in a separatory funnel to dissolve the extracted β-carotene in 100 mL of petroleum ether. The solution was washed with 100 mL of DI water multiple times to remove the acetone.

5. Rotary evaporation of the petroleum ether at 37 °C until a yellow residue of β-carotene remained at the bottom of the flask.

6. The β-carotene residue was dissolved in 15 mL of the mobile phase (70% acetonitrile, 15% methanol, 15% methyleneschloride).

7. Samples were stored at −20 °C until further HPLC analysis.

Quantification of extracted β-carotene was performed in a reverse phase HPLC using the method described by Kim and Quadro (2010). Dionex Ultimate 3000 Series HPLC coupled with a photodiode array (PDA) detector was used. β-carotene was separated on a Beckman Ultrasphere C18 (5 µm; 4.6 mm x 250 mm) column along with a C18 (7 µm; 3.2 mm x 15 mm) guard column and detected
using a PDA detector at 325 nm. The mobile phase consisted of acetonitrile, methanol, and methylene chloride in the ratio of 70:15:15. Total run time for each sample injected at 1.8 ml/min was 35 min, and the peaks at 14.5 min were identified as β-carotene. External standards (0 ng β-carotene/μL to 20 ng β-carotene/μL) were used for obtaining a standard curve ($R^2 = 0.99$) and β-carotene extracted from the baby carrots was quantified as mg β-carotene per serving (85 g) of baby carrots.

**Analysis of texture**

A CT3 Brookfield Texture Analyzer equipped with a TA-BT fixture was used for texture analysis. One cm long cylindrical baby carrot piece was cut from the middle portion of the processed baby carrot. The cylindrical piece was compressed axially to 50% using a TA 3-100 flat base cylindrical probe (25.4 mm diameter) at a test speed of 1 mm/s. The maximum force (N) required to axially compress a cylindrical sample by 50% was quantified as the hardness of the baby carrot.

**Analysis of color**

Color analysis was performed using Konica-Minolta CR-410 handheld colorimeter that 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 their color was
recorded in terms of L*, a*, b* values (CIE Lab color space). Based on these values, the color of the samples was quantified according to the Munsell color system (Fig. 2.4) in terms of L* (also called VALUE), Chroma (C), and Hue (h) using Eq. 2.1 and Eq. 2.2. 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 as degrees (°), starting from Red at 0° to Yellow at 90°, Green at 180° to Blue at 270°. The change in the color of the processed samples was quantified as ∆E*, calculated using Eq. 2.3

\[
\text{Chroma} = \sqrt{(a^* + b^*)^2} \quad \text{...Eq. 2.1}
\]

\[
\text{Hue} = h_{ab} = \tan^{-1} \left( \frac{a^*}{b^*} \right) \quad \text{...Eq. 2.2}
\]

\[
\Delta E^* = \sqrt{(L_{\text{sample}}^* - L_{\text{unprocessed}}^*)^2 + (a_{\text{sample}}^* - a_{\text{unprocessed}}^*)^2 + (b_{\text{sample}}^* - b_{\text{unprocessed}}^*)^2}
\]

...Eq. 2.3
Statistical analysis

Regression analysis was performed on the data obtained from the experimental design (section 2.3) using Design-Expert® Version 9.0 software and the data analysis was performed according to Tukey’s Honest Significance Difference test ($\alpha = 0.05$) in MATLAB®.

2.3. Experimental design

Evaluating the effect of processing variables

Fifteen HPP experiments (Table 2.1) were performed, in a randomized order, following a $3^3$ Box-Behnken Design (BBD) of experiments to evaluate the effect of three independent factors – pressure (150 MPa to 550 MPa), hold time (5 min to 15 min), and CLG solution concentration (3% to 9% (w/v)) on high pressure infusion of calcium in PME pre-treated baby carrots. The center point (350 MPa,
10 min, 6 % CLG solution concentration) was performed in triplicates (1+2). A response surface methodology (RSM) was used to evaluate the main effects (pressure, hold time, and CLG solution concentration), the quadratic effects (pressure*pressure, hold time*hold time, and CLG solution concentration*CLG solution concentration), and the interaction effects (pressure*time, pressure*CLG solution concentration, and time*CLG solution concentration) on calcium infusion, β-carotene extractability, texture and color of the processed baby carrots. A quadratic regression equation (Eq. 2.4) was used, where ‘y’ is the predicted response (ex. amount of calcium infused), β_0 is the model constant, β_1, β_2, β_3 are linear coefficients, β_{11}, β_{22}, β_{33} are the quadratic coefficients, and β_{12}, β_{13}, β_{23} are cross product coefficients (Aslan and Cebeci, 2007). The coded variables P, t, and C in Table 2.1 represent the pressure (MPa), hold time (min), and CLG solution concentration (% w/v), respectively.

\[ y = \beta_0 + \beta_1 P + \beta_2 t + \beta_3 C + \beta_{11} P^2 + \beta_{22} t^2 + \beta_{33} C^2 + \beta_{12} P \times t + \beta_{13} P \times C + \beta_{23} t \times C \]

...Eq. 2.4
Three processing controls were used to compare the effect of high pressure assisted infusion of calcium in PME pre-treated baby carrots and each control experiment was performed in triplicates:

- Unprocessed raw baby carrots
- Osmotically infused baby carrots:

<table>
<thead>
<tr>
<th>Run #</th>
<th>Coded values</th>
<th>Uncoded values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P  t  C</td>
<td>Pressure (MPa)</td>
</tr>
<tr>
<td>1</td>
<td>-1 0 -1</td>
<td>150</td>
</tr>
<tr>
<td>2</td>
<td>1 0 1</td>
<td>550</td>
</tr>
<tr>
<td>3</td>
<td>-1 1 0</td>
<td>150</td>
</tr>
<tr>
<td>4</td>
<td>0 0 0</td>
<td>350</td>
</tr>
<tr>
<td>5</td>
<td>1 -1 0</td>
<td>550</td>
</tr>
<tr>
<td>6</td>
<td>-1 0 1</td>
<td>150</td>
</tr>
<tr>
<td>7</td>
<td>1 0 -1</td>
<td>550</td>
</tr>
<tr>
<td>8</td>
<td>0 1 1</td>
<td>350</td>
</tr>
<tr>
<td>9</td>
<td>0 -1 -1</td>
<td>350</td>
</tr>
<tr>
<td>10</td>
<td>0 -1 1</td>
<td>350</td>
</tr>
<tr>
<td>11</td>
<td>1 1 0</td>
<td>550</td>
</tr>
<tr>
<td>12</td>
<td>-1 -1 0</td>
<td>150</td>
</tr>
<tr>
<td>13</td>
<td>0 1 -1</td>
<td>350</td>
</tr>
<tr>
<td>14</td>
<td>0 0 0</td>
<td>350</td>
</tr>
<tr>
<td>15</td>
<td>0 0 0</td>
<td>350</td>
</tr>
</tbody>
</table>

*Table 2.1* Experimental points according to the Box-Behnken Design (BBD)
PME pre-treated and baby carrots were osmotically infused with calcium by soaking in 9 % (w/v) CLG solution for 15 min.

c. Vacuum infused baby carrots:

PME pre-treated and vacuum-packed baby carrots were infused with calcium under vacuum using 9 % (w/v) CLG solution. Infusion was carried out for 15 min under a vacuum of 40 mm Hg (= 52 mBar).

_Evaluating the effect of PME pre-treatment_

To evaluate the effect of PME pre-treatment on the amount of calcium infused, baby carrots were divided into two batches — one batch was pre-treated with PME and the other was not treated with the enzyme. Experiments were performed according to the workflow diagram shown in Fig. 2.5. The aim was to determine if the PME pre-treatment had any effect on the calcium content in baby carrots, in their natural state (atmospheric pressure), simply pressurized under HPP, and pressurized in presence of CLG. The highest concentration of CLG used in this study was 9% and hence, the same was used for evaluating the effect of PME pre-treatment. Apart from the raw and the PME control, baby carrots were vacuum packed in
Figure 2.5 Experimental design to evaluate the effect of PME pre-treatment on high pressure-assisted infusion of calcium in baby carrots

**Pressure cycling experiments**

To evaluate the effect of pressure cycling, all the three variables 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. Experiments were carried out with 3 different pressure cycles, i.e., single cycle (1 cycle, 15 min), 3 cycles (5 min/cycle), and 5 cycles (3 min/cycle), to have a total holding time of 15 min for all samples. Figure 2.6 shows the variation of pressure and temperature during the pressure cycles. The come-up time (CUT) for each cycle was ignored. It must be noted that the cycles were carried out consecutively without unloading the samples between each cycle. Pressure cycling experiments were performed in triplicates.
Figure 2.6 Pressure and temperature during the pressure cycling experiments.
2.4. Analysis of controls

The controls were analyzed for the total amount of calcium (mg calcium per serving of baby carrots), extracted β-carotene (mg β-carotene per serving of baby carrots), hardness (N), and color (L*, Chroma, Hue (°), color difference (ΔE*)).

Table 2.2 summarizes these results:

<table>
<thead>
<tr>
<th>Samples</th>
<th>Calcium (mg/serving)</th>
<th>Extracted beta-carotene (mg/serving)</th>
<th>Hardness (N)</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unprocessed raw</td>
<td>19.54 ± 0.32</td>
<td>4.64 ± 0.72</td>
<td>141.46 ± 14.8</td>
<td>L* 56.5 ± 0.3</td>
</tr>
<tr>
<td>Osmotically infused</td>
<td>38.66 ± 9.36</td>
<td>14.15 ± 4.47</td>
<td>186.62 ± 34.24</td>
<td>L* 54.9 ± 0.5</td>
</tr>
<tr>
<td>Vacuum infused</td>
<td>38.86 ± 4.74</td>
<td>16.42 ± 3.38</td>
<td>198.85 ± 27.65</td>
<td>L* 55.3 ± 0.8</td>
</tr>
</tbody>
</table>

*Table 2.2 Summary of analysis of all controls*

Infusion of calcium by both 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 our 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).

2.5. Effect of HPP parameters on extent of calcium infusion

The influence of processing variables on the extent of calcium infusion and the other parameters was evaluated by performing regression analysis as described in section 2.3. A summary of the coefficients for each of the effects (linear and quadratic) along with their associated p-values is given in Table 2.3.
Table 2.3 Summary of model coefficients for the effects of the processing variables along with their associated p-values (table adapted from Gosavi et al. 2018)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Effect on total amount of calcium in baby carrots after HPP infusion</th>
<th>Effect on hardness of baby carrots after HPP infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressure (P)</td>
<td>8.32(^b)</td>
<td>-11.37</td>
</tr>
<tr>
<td>Time (t)</td>
<td>7.86(^b)</td>
<td>11.78</td>
</tr>
<tr>
<td>CLG conc. (C)</td>
<td>26.11(^b)</td>
<td>1.94</td>
</tr>
<tr>
<td>P(^t)</td>
<td>7.44</td>
<td>-9.86</td>
</tr>
<tr>
<td>P(^t)C</td>
<td>10.63(^b)</td>
<td>18.55</td>
</tr>
<tr>
<td>t(^t)C</td>
<td>10.50(^b)</td>
<td>-0.26</td>
</tr>
<tr>
<td>P(^2)</td>
<td>-13.47(^b)</td>
<td>-0.49</td>
</tr>
<tr>
<td>t(^2)</td>
<td>4.91</td>
<td>8.72</td>
</tr>
<tr>
<td>C(^2)</td>
<td>6.03</td>
<td>-4.91</td>
</tr>
</tbody>
</table>

\(^b\) p<0.05 (significant effect)

The total amount of calcium in baby carrots increased by two- to six-folds after infusion under high pressure as compared to the raw unprocessed baby carrots. The highest amount of calcium infusion was obtained at processing conditions of 350 MPa-15 min-9% CLG resulting in the total calcium content of the baby carrots to be 134.73 mg/serving which was up to 7 times that of the raw unprocessed baby carrots. Based on all the 15 BBD experiments, an average of 77.4 mg of total calcium was obtained per serving of baby carrots. The amount of calcium infused in the baby carrots was significantly affected by all the three processing variables (processing, holding time, and CLG concentration) as well as
their interactions. Eq. 2.5 represents the quadratic equation obtained for modeling the effects of the processing variables on the amount of calcium infused. It should be noted that the equation contains terms in coded variables, and hence P, t, and C do not have units but range between -1 to 1.

\[
Ca = 77.4 + (8.32)P + (7.86)t + (26.11)C + (10.63)P*C + (10.5)t*C - (13.47)P^2
\]

...Eq. 2.5 \((R^2 = 0.92)\)

The concentration of CLG solution played the most important role in determining the amount of infusion, also denoted by the large value of its coefficient \((=26.11)\) in Eq. 2.5. Higher concentration of CLG would create a larger concentration gradient of calcium between the solution and the carrot cells, thus facilitating more infusion of calcium into the carrot cells. Additionally, the application of pressure also significantly influenced the extent of infusion. Since higher pressure treatments correlate to higher breakdown of cell structure (Mahadevan et al. 2015 and Rastogi et al. 2008), the resistance of the cell walls to infusion is reduced leading to increased infusion (Rastogi and Niranjan 1998; Rastogi 2000; Sopanangkul et al. 2002; and Villacís et al. 2008). A similar trend of increase in calcium infusion with increased pressure was observed. This increase, however, was not linear and was affected by the CLG concentration. The interaction effect of pressure and CLG concentration on calcium infusion is shown
as a contour plot in Fig. 2.7 that was constructed using Eq. 2.5. Contours were generated using MATLAB®.

**Figure 2.7** Effect of pressure and CLG concentration on the amount of calcium infused in PME pre-treated baby carrots using HPP assisted infusion (t=10 min)

For a given CLG concentration the amount of calcium infused increased until moderate pressures of 350 MPa but the amount decreased beyond that pressure. This effect was more prominent at lower CLG concentration of 3%. As the CLG concentration increased to 9% the influence of this interaction effect was not as significant as that of the CLG concentration alone. During the pressurization of baby carrots, the baby carrot cells are permeabilized due to the high pressure which contributes to enhancing the infusion through concentration driven flow, but at the same time, these cells also reduce in volume due to compression under high pressures. At a given pressure, the extent of infusion is thus
influenced by two competing effects – number of cells permeabilized and the extent of volume reduction in cells. Mahadevan et al. (2016) observed that beyond 100 MPa, the amount of cell permeabilization in frozen-thawed cranberries did not change. Based on this observation by Mahadevan et al. (2016), assuming that cell permeabilization in our samples was the same for all processing conditions, the extent of calcium infusion was thus governed by the extent of volume reduction under high pressure. At higher pressures (550 MPa), the volume of the cells was significantly reduced and hence, the infusion at that pressure would be reduced. This effect was more prominent at lower CLG concentration of 3% and reduced as the CLG concentration increased. At high CLG concentration of 9%, the concentration driven infusion was significantly high and hence the reduction in infusion at high pressure was not as pronounced as it was for 3% CLG concentration.

The extent of calcium infusion was also significantly influenced by the holding time of the process. The amount of calcium infused in baby carrots increased with increase in the holding time. Longer holding times would allow the process of calcium diffusion into the cells to be longer and thus lead to more calcium infusion into the baby carrots. Figure 2.8 shows a contour plot that depicts the effect of the interaction between time and CLG concentration on the amount of calcium infusion. At high concentrations of CLG, the driving force due
to concentration difference was high and in combination with longer infusion times, the amount of calcium infused is significantly higher. Hence, longer infusion time of 15 min at a moderate pressure of 350 MPa using high concentration (9%) of CLG solution was able to achieve calcium infusion into baby carrots to provide more than 10% of the daily RDI of calcium.

Figure 2.8 Effect of time and CLG concentration on the calcium infused in PME pre-treated baby carrots using HPP assisted infusion (P = 350 MPa)
2.6. Effect of HPP parameters on beta-carotene extractability, texture, and color

\textit{β-carotene extractability}

The amount of β-carotene extracted from baby carrots increased by three to five times after high pressure processing, with an average β-carotene extraction of 13.86±3.87 mg/serving of baby carrots, based on all the 15 experiments in BBD. Highest extraction of β-carotene (22.72 mg/serving) was obtained from baby carrots treated at 150 MPa for 5 min in 6% CLG solution. This increase in β-carotene extraction from HPP – calcium infused baby carrots can be associated with a breakdown of cell structure under HPP which increased the accessibility of carotenoids in the cell structure causing increased extraction. De Ancos et al. (2002) hypothesized that increase in carotenoid extraction under high pressure is due to the destruction of chormoplasts, where carotenoids are located. A similar reason has been cited by other researchers (Sánchez-Moreno et al. 2005 & 2006; Jacobo-Velázquez et al. 2012) for enhanced extraction of carotenoids after HPP treatment.

A regression analysis performed on the data obtained from the BBD experiments indicated that the extraction of β-carotene from processed carrots was significantly affected by the interaction of pressure and time only. Figure 2.9 depicts a contour plot to summarize the effect of pressure and time on the
amount of β-carotene extracted from processed baby carrots. The contours in Fig. 2.9 were plotted using Eq. 2.6, which represents the quadratic regression equation obtained for modeling the effects of processing parameters on amount of β-carotene extraction, without dropping the insignificant effects. Although Eq. 2.6 may be a poor model ($R^2 = 0.50$) for prediction, it can be used to gain a general idea of the effects of processing parameters on the amount of β-carotene extracted from high pressure – calcium infused baby carrots.

\[
B = 13.86 - (0.85)P - (2.07)t - (0.40)C + (5.61)P^t + (0.71)P^C - (3.32)t^C - (2.70)P^2 + (4.48)t^2 - (2.54)C^2
\]

...Eq. 2.6

**Figure 2.9** Effect of pressure (MPa) and time (min) on the amount of β-carotene extracted from HPP – calcium infused baby carrots (CLG=6% w/v)
A high amount of β-carotene (~23 mg/serving) was extracted from baby carrots processed at low pressure (150 MPa) – low time (5 min) combinations. The extractability of β-carotene decreased with an increase in pressure – time combination. During high pressure processing of baby carrots, it was observed that the infusate solution, which was a clear solution before processing, had a yellow color after processing, which was more prominent after processing at higher pressures and for longer times. The decrease in β-carotene extractability with increasing pressure – time combination (Fig. 2.9) may have been due to extraction of β-carotene from the baby carrot cells into the infusate solution, which was discarded after processing. However, this would only explain the lower extraction of β-carotene at pressure – time combinations below 350 MPa – 10 min but would contradict the increase in β-carotene extraction at very high pressure – long time (550 MPa – 15 min) combinations (as seen from Fig. 2.9). This increase in β-carotene extraction at pressure – time combinations beyond 350 MPa – 10 min can be explained by a phenomenon described by De Ancos et al. (2002). Although high pressures (50 MPa to 100 MPa) destruct the chromoplasts causing increased extraction of carotenoids, these pressures do not affect the protein – carotenoids complex. However, pressures above 350 MPa can denature the protein – carotenoid complex, thus releasing more carotenoids from the cellular matrix.
**Texture**

The texture of the baby carrots, measured in terms of hardness, was observed to have a similar or a higher value (146 N to 226 N) than that of the raw unprocessed control (141±15) N. The hardness of the calcium infused baby carrots was thus similar or better than the control. Based on the 15 BBD experiments, average hardness of the calcium infused baby carrots was 193 N. The process of calcium infusion under high pressure thus resulted in an overall increase in the hardness of the baby carrots, however, the resulting hardness could not be attributed as being a result of any one particular processing parameter or the interactions between the parameters.

The overall hardness of the baby carrots improved after calcium infusion under high pressure. It can be attributed to binding of the excess calcium with the demethylated pectin bonds in the cell wall forming ‘egg-box structures’ that improve the mechanical rigidity of the cells (Guillemin et al. 2008). Increasing the pressure reduced the hardness of the baby carrots, most probably due to higher breakdown of cell structure at higher pressures and a consequent loss of hardness. Longer hold times during infusion, even at high pressures, showed a general trend to increase the hardness. Longer processing times imply higher calcium infusion, especially at higher CLG concentrations, thus making more calcium available to bind to the pectin to strengthen the cellular matrix. Thus,
longer infusion times at low or moderate pressures have the potential to give maximum hardness to the baby carrots. However, it must be noted that these are just trends in the data and further investigation is needed to make conclusive quantitative statements.

**Color**

Figure 2.10 shows the visual color difference observed between raw baby carrots and the HPP – calcium infused baby carrots (350 MPa-10 min-6% CLG). Regression analysis of the data obtained for color measurements from BBD experiments, did not yield significant results. No particular processing parameter was seen to have a significant effect on determining the change in color of the processed baby carrots. However, it was observed that the color of the HPP – calcium infused baby carrots significantly changed after high pressure processing. The L* value for processed carrots was lower than that of the control, indicating that the processed carrots were less bright compared to the control. On similar lines, lower a*, b*, and chroma values suggested that the baby carrots had lower intensity of red – yellow color. A higher hue value of the processed baby carrots indicated that the baby carrots were slightly pale, i.e. more yellow than orange, compared to the control. The average ΔE* value of the HPP – calcium infused baby carrots was 11.29±2.20, which indicated a significant change in color after processing. Thus, the process of HPP – calcium infusion caused significant color
changes in the baby carrots; however, this change could not be attributed to a single processing parameter.

**Figure 2.10** Color difference between the raw unprocessed control (L) and HPP – calcium infused baby carrots (R) processed at the BBD center-point (350 MPa/10 min/6% CLG)

Paciulli et al. (2016) reported similar results for beetroots treated with HPP which had L*, a*, and b* values lower than that of the blanched as well as that of the raw beetroots, indicating a blue-shift (red to red-violet) in color. Oey et al. (2008) suggest an interaction between structure and pigmentation of food that affects the color and the opacity of the food. HPP causes changes in cellular structure and consequent texture modification that can potentially change the nature and extent of internally scattered light resulting in a perceived change of color (Oey et al. 2008). Another possible reason for the darkening of baby carrots after HPP can be the activation of latent enzymes like peroxidase and
polyphenoloxidase under HPP (Júnior et al. 2017) and an increased interaction of these enzymes with the substrates in the cellular matrix due to loss of cell integrity (Techakanon et al. 2016).

2.7. Effect of PME pre-treatment on calcium infusion in baby carrots under HPP

The batch of baby carrots used to evaluate the effect of PME pre-treatment had inherent calcium content of (32.06 ± 1.59) mg/serving of baby carrots. These data are represented by the ‘No PME – No HPP’ bar in Fig. 2.11. PME pre-treatment on the baby carrots did not have a significant effect on the amount of calcium in the baby carrots, showing that demethylation of pectin bonds in baby carrots does not alter the calcium content in baby carrots. Additionally, Fig. 2.11 indicates that only HPP treatment on baby carrots (without PME pre-treatment) enhanced the infusion of calcium threefold ((101.65 ± 17.77) mg/serving of baby carrots). Such increase in infusion under high pressure as compared to infusion under vacuum or by osmosis has been reported in literature (George et al. 2016; Mahadevan et al. 2015). A further twofold increase in the extent of calcium infusion under high pressure was observed in baby carrots pre-treated with PME ((231.07 ± 125.61) mg/serving of baby carrots). Thus, PME pre-treatment followed by HPP resulted in significantly higher infusion of calcium as compared
to no PME pre-treatment. Hence, all further experiments included PME pre-treatment prior to HPP.

Figure 2.11 Effect of PME pre-treatment on the amount of calcium infused in baby carrots using high pressure-assisted infusion at 550 MPa for 15 min.

2.8. **Effect of pressure cycling on calcium infusion in baby carrots under HPP**

*Calcium infusion*

The amount of calcium infused increased with an increase in the number of pressure cycles during the processing. A similar finding has been reported by Mahadevan et al. (2015) on the infusion of quercetin in fresh cranberries. Figure 2.12 shows that the amount of infused calcium increased with increasing the
number of pressure cycles. Calcium infusion of up to 150 mg/serving of baby
carrots (equivalent to 15% of the RDA) was achieved with five pressure cycles (of
3 min each) at the highest pressure of 550 MPa. The increased infusion with more
number of pressure cycles can be attributed to breaking up of more cell walls,
due to the repetition of pressurization-depressurization cycles, facilitating
multiple cycles of infusion leading to higher amount of total calcium in the baby
carrots. The calcium infused after 5 cycles of pressurization-depressurization was
1.5–2 times (156.7 ± 43.0 mg/serving) higher than that infused in a single cycle
(91.9 ± 4.2 mg/serving), and 4 times higher than that infused under vacuum and
by osmosis (38.8 ± 4.7mg/serving and 38.6 ± 9.3 mg/serving, respectively). Three
pressure cycles for 5min each (total holding time of 15min) at 550 MPa resulted
in calcium infusion of 127 ± 21 mg/serving.
Figure 2.12 Effect of pressure cycling at 550 MPa on the amount of calcium infused in PME pre-treated baby carrots using high pressure-assisted infusion of calcium (1 cycle: 15 min, 3 cycles: 5 min each, 5 cycles: 3 min each)

Hardness

Similar to the observations of the BBD experiments, calcium infusion due to pressure cycling led to an overall increase in the hardness of the baby carrots, as compared to the raw baby carrots. The increased calcium infusion due to pressure cycling (Fig. 2.12) can potentially contribute to increasing the hardness of baby carrots due to higher cross-linking of pectin and calcium. The mean hardness of the baby carrots increased from (197 ± 13) N for a single cycle (15 min) treatment to (228 ± 23) N for a treatment of three cycles (5 min each),
although the increase was statistically insignificant. Similarly, further increase to 5 cycles (3 min each) did not show a statistically significant increase in the hardness, as can be seen from Fig. 2.13.

![Figure 2.13 Effect of pressure cycling on hardness of high pressure-assisted calcium infused baby carrots (1 cycle: 15 min, 3 cycles: 5 min each, 5 cycles: 3 min each)](image)

**Figure 2.13** Effect of pressure cycling on hardness of high pressure-assisted calcium infused baby carrots (1 cycle: 15 min, 3 cycles: 5 min each, 5 cycles: 3 min each)

### 2.9. Key takeaways for this study

- High pressure processing (HPP) was shown to enhance infusion of calcium in PME pre-treated baby carrots to levels that could provide 10–14% of the RDI of calcium, which was significantly higher in comparison to the commonly used techniques of osmosis and vacuum infusion that resulted in no more than 4% of the RDA of total calcium.
A detailed regression analysis on the influence of pressure (MPa), holding 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 significantly affected the infusion of calcium in the PME treated baby carrots.

Higher concentration of CLG solution (9% w/v), and pressure treatment at moderate pressure (350 MPa) and longer holding time (15 min), were found to result in the highest calcium infusion (134 mg/serving).

Amount of calcium infused in the baby carrots reached a maximum at mid-pressure levels (~350 MPa) and did not further increase with increasing pressure beyond 350 MPa.

The extraction of β-carotene from the HPP – calcium infused baby carrots increased by three to five times (8.2 to 22.7 mg/85 g).

The texture of the baby carrots, measured as hardness (N), improved after HPP assisted calcium infusion, while their color became darker. However, due to the lack of sensory analysis which was out of scope for this research, it is difficult to say if the changes in texture and color of the baby carrots have practical significance for human perception.
CHAPTER 3

EFFECT OF FOOD MICROSTRUCTURE ON THE EXTENT OF HIGH PRESSURE ASSISTED INFUSION OF CALCIUM IN SELECTED FRUITS AND VEGETABLES

This chapter has been reproduced from a research paper titled “Effect of Food Microstructure on Calcium Infusion Under High Pressure” authored by Noopur Gosavi, Marianne Polunas, Daniel Martin, and Mukund V Karwe and published in the Food Engineering Reviews journal in 2020.

3.1. Brief introduction

The work discussed in the previous chapter showed that HPP could be used to infused high amount of calcium in baby carrots. However, it would have been difficult to extrapolate the conclusions and results of this work to other fruits and vegetables. Each fruit/vegetable has a different cellular structure that makes the mass transfer in these different matrices vastly different. There was therefore an interest to extend this technology of HPP assisted calcium infusion to other fruits and vegetables while studying how their microstructures would play a role in determining the extent of infusion. Moreover, the different microstructures would also respond differently to pressure and have varying degrees of cell permeabilization which would in turn affect the level of infusion. It was therefore
important to explore these aspects of microstructure and cell permeabilization in the context of HPP assisted infusion. A major challenge in this study was designing and developing an entire new methodology to quantitatively distinguish between different food matrices. The development of this process required a multi-disciplinary effort between Food Science, Histology, and Biomedical Engineering departments at Rutgers. Section 3.3 will discuss two different approaches that were developed to quantify microstructure, however, only one of them was further used for consideration.

The main objective of this research was to understand how the microstructure of selected fruits and vegetables and their cell permeabilization under high pressure affected the HPP assisted infusion of calcium. Specifically, we selected baby carrots, celery, and mango (peeled) representing a root, a stem, and a fruit, respectively.

3.2. Materials and methods

Bolthouse® Farms baby carrots, Dandy® celery sticks, and Mexican mangoes (variety #4959) were purchased from a local supermarket 24 h prior to processing. Visually damaged and abnormally small or large samples were discarded. Celery sticks were cut into pieces of 50 mm length and mangoes were peeled and cut into 50 mm x 15 mm x 15 mm blocks (cuboids). Store bought baby carrots were not further cut into smaller pieces, but were graded by size to
ensure that the pieces were approximately 50 mm long and 15 mm – 20 mm in diameter. These samples were stored in refrigerated conditions overnight until further processing.

Calcium Lactate Gluconate (CLG, Jungbunzlauer Suisse Ag, Switzerland) in anhydrous form, containing 13% calcium and solubility of 20% (w/w) in water, was used as a source of calcium for the infusion experiments. Hematoxylin and Eosin (H&E) stain was used for staining the cells of the fruit and vegetable samples. Nikon® TE200-S (NY, USA) widefield microscope was used for visualizing the cells. Abcam Fluo-8 Calcium Flux Assay Kit - No Wash (ab112129) was used as a fluorescent tag for the infused calcium. Zeiss LSM 780 (NY, USA) confocal laser scanning microscope was used for fluorescence imaging. Image analysis was performed using MATLAB® and ImageJ®. Varian Vista-Pro (IL, USA) CCD Simultaneous ICP-OES (Inductively Coupled Plasma–Optical Emission Spectroscopy) was used for analyzing the amount of infused calcium. Cell permeabilization was measured using Bode 100 Vector network Analyzer (Omicron Electronics Corp., Houston, TX, USA). Brookfield Ametek® (MA, USA) CT3 Texture Analyzer and Konica-Minolta (NJ, USA) CR-410 handheld colorimeter were used for texture and color analysis, respectively.
Infusion experiments were performed in triplicates at 5 pressures levels (100 MPa, 200 MPa, 300 MPa, 400 MPa, and 500 MPa) using 6% CLG (w/w) solution. In the previous study (Chapter 2; Gosavi et al. 2019), the concentration of CLG was varied from 3% to 9% (w/w) and it was observed that at 9% CLG the calcium solution concentration dominated other factors of pressure and time. In order to ensure that no one parameter was dominant, in this research 6% CLG concentration was used. Hold time for all pressures was kept constant at 15 mins based on our previous findings in which the highest amount of calcium infusion occurred at 15 min hold period. For each experiment, 30 g of sample (baby carrots or cut celery or peeled and cut mango) and 60 g of 6% (w/w) CLG solution were vacuum packed together in flexible polyethylene pouches. Vacuum packed samples (sealed under 93% vacuum, i.e., 69 mBar) were subjected to HPP at five different pressure levels – 100 MPa, 200 MPa, 300 MPa, 400 MPa, and 500 MPa. Each experiment was performed three times, and each run had three samples.

All HPP experiments were performed at the United States Department of Agriculture – Agricultural Research Service, Eastern Regional Research Center (USDA-ARS ERRC) in Wyndmoor, PA, USA. HPP was performed using a laboratory scale pressure unit (Mini Food lab FPG5620, Stansted Fluid Power Ltd., Essex, UK), comprised of a double-jacketed thick-wall stainless steel cylinder (0.3 L) with an
internal stainless steel sample holder of 25.4 mm × 254 mm (diameter × length) (Hsu et al. 2015). The thick-wall cylinder was maintained at a set temperature by continuously circulating fluid from a refrigerated chiller (Proline RP 855, Lauda, Germany). The refrigerated chiller was set at 4 °C which indirectly cooled the pressure transmitting medium (a mixture of ethanol and castor oil). The pressure come-up rate was 100 MPa per 15 s and the release rate was 100 MPa per 9 s. The initial temperature in the processing chamber with samples was ~6 °C and reached a maximum 35 °C when the highest pressure (500 MPa) was applied. The chamber temperature was monitored by a built-in sensor (a T-type thermocouple). Immediately after processing, the vacuum pouches were removed from the chamber, CLG solution was drained, and the samples were lightly washed with deionized water to remove any CLG solution adhering to their surface.

**Calcium analysis**

The details of this analysis have been discussed earlier in section 2.2. In this study, Calcium content was quantified as mg calcium/100 g initial fruit or vegetable.
Cell permeabilization index ($Z_P$)

Cell damage in the processed samples was quantified as cell permeabilization index ($Z_P$) using electrical impedance spectroscopy. This method is based on the electrical properties of living cells with varying ratios of ruptured and intact cells that can be represented as an equivalent circuit model (Angersbach et al. 1999). The electrical conductivity of the cells is frequency dependent and can be expressed in terms of a cell permeabilization index, $Z_P$ (Rastogi et al. 2000). $Z_P$ is a ratio of electrical conductivities of processed and unprocessed samples, that can be determined using Eq. 1, where $\sigma$ is the electrical conductivity, superscripts $i$ and $t$ indicate conductivities before and after treatment, respectively, and subscripts $h$ and $l$ represent conductivities at low (1 kHz) and high (1500 kHz) frequencies, respectively. The value of $Z_P$ ranges between 0 to 1; $Z_P = 0$ when all the cells are intact and $Z_P = 1$ when all the cells are ruptured. For the purpose of experiments in presented research, $\sigma^i$ was the electrical conductivity of the respective unprocessed samples. A detailed description of this method has been presented by Angersbach et al. (1999).

$$Z_P = \frac{\left(\frac{\sigma^i}{\sigma^t}\right) \times \sigma^i - \sigma^i}{\sigma^h - \sigma^l} \quad \ldots \text{Eq. 3.1}$$
**Calcium mapping**

Fluo-8 Calcium Flux Assay Kit (ab112129) by Abcam was used to visualize the infused calcium in the processed samples. It is a no-wash, fluorescence-based assay for detecting intracellular calcium mobilization in cells. Fluo-8 can cross the cell membrane and once inside the cell, the lipophilic blocking groups of Fluo-8 are cleaved by an esterase, resulting in a negatively charged fluorescent dye that stays inside the cell. Its fluorescence is greatly enhanced upon binding to calcium. If intracellular calcium levels increase, the fluorescence of Fluo-8 significantly increases and can be observed using a laser microscope (Abcam 2019).

Fluo-8 dye (4 µM) was made by diluting 1 mg anhydrous Fluo-8 in 500 µL DMSO (dimethyl sulfoxide) and 250 mL HHBS (Hank’s buffer with 20 mM HEPES). Processed samples were cut into thin sections (250 µm to 300 µm) using a custom-made slicer and stained with 1 mL of 4 µM Fluo-8 dye. After 30 min of staining under dark at room temperature, the dye was discarded and samples were immediately observed on the Zeiss LSM 780 confocal microscope using the 10X objective and a 514 nm laser. Quantitative analysis of the calcium distribution in the sample tissue has been discussed in details in section 3.5.
Texture measurement

A CT3 Brookfield Texture analyzer equipped with a TA-BT base fixture was used to measure the texture of the processed and unprocessed samples. A TA-7 Knife edge probe (clear acrylic, 8 g, 60 mm wide) was used for the measurement. Processed samples were placed horizontally on the base fixture while aligning them perpendicular to the sharp edge of the probe. Samples were cut in the middle with the knife probe moving at a test speed of 1 mm/s. As the probe cut through the sample, force (N) required to cut was recorded as a function of distance (mm). Sample texture was quantified in two ways – (a) the energy (J) required to cut the sample, which was calculated as area under the force (N) vs distance curve (mm), and (b) the peak force (N) required to cut the sample.

Color measurement

Color measurements were performed using Konica-Minolta CR-410 colorimeter calibrated to D65 standards (Y = 94.7, x = 0.3156, and y = 0.33199). Processed samples were placed on an opaque white base and their color was recorded in terms of L*, a*, b* values (CIE Lab color space). Samples were arranged to ensure that the white base was entirely covered and color measurements were taken at 3 to 5 randomly selected points. The change in the color of the processed samples was quantified as ΔE*.
**Statistical analysis**

Data obtained from all the experiments were first analyzed using Shapiro-Wilk & Shapiro-Francia parametric hypothesis test of composite normality (α = 0.05). This test was performed to ensure that the data obtained could be described as a Gaussian distribution (normal distribution). Following the normality test, statistical analysis was performed using one-way Analysis of Variance (ANOVA) to analyze the differences in the variances of the data. If the differences in the variances of the data were significant (p < 0.05), Tukey’s Honest Significant Difference (HSD) test (α = 0.05) was performed to understand the differences in the sample means. All data analyses were performed using MATLAB®.

### 3.3. Microstructure analysis

Two different methodologies were used to stain the fruit/vegetable tissue and visualize it under different microscopes. The first method involved the use of propidium iodide dye and observation under a confocal laser scanning microscope, while the second method used an H&E staining dye that involved an exhaustive staining process and observation under a widefield microscope.
**Method 1: Using propidium iodide staining**

Thin slices (200 µm to 300 µm) of each fruit/vegetable were cut using a custom-made slicer (courtesy of Bill Sumal, Food Science). For each sample, ~10 slices were soaked in ~20 mL of 1:250 propidium iodide (PI) solution (40X PI solution) and incubated in dark conditions at RT for 30 min. After the incubation period, the PI solution was drained and stained slices were washed multiple times with DI water to remove excess PI adhering to the surface. Stained slices were immediately visualized using the Zeiss confocal laser scanning microscope. For each fruit, 5 to 7 different slices were imaged at 7 to 10 different positions each. Imaging positions for each sample were chosen such as to not be too close to the edge or any other characteristic feature of the samples (ex. central part of the carrot, transport tubes of the celery, and fibers in the mango). Figure 3.1 shows the different positions at which each sample was imaged. Thus, for each sample, ~50 images were obtained. Microstructure of the samples was quantified in terms of the characteristic length (µm) of cells in each sample, details of which have been discussed in the next section.
Figure 3.1 (a) Carrot cross-section (b) celery cross-section, and (c) mango cross-section as seen under CLSM. All samples were imaged for more details using CLSM at positions shown by ‘x’ on each cross-section.

Method 2: Using H&E staining

[Note: This methodology was performed at the Rutgers Research Pathology Services by Dr. Marianne Polunas]

Raw and unprocessed baby carrot, celery, and mango samples were trimmed into 3 mm thick slices and treated according to a modification of a plant processing program developed by Dr. Oliver Leroux at Ghent University, Belgium (personal communication, 12/20/2018). Samples were fixed in 10% neutral buffered formalin for 4 h at room temperature, washed 3 x 20 minutes in PBS (phosphate buffered saline) and rinsed in double distilled water (ddH₂O). Samples then underwent a series of slow dehydrations, starting with 10% EtOH and
increasing by 10% each hour until reaching 90% EtOH. This was followed by 2 x 45 min changes of 95% EtOH, 3 x 1-hour changes of 100% EtOH, 2 x 1-hour changes of xylene, and 4 x 1-hour changes of melted paraffin. All steps prior to 95% EtOH were performed manually; subsequent steps were carried out in a Sakura Tissue Tek VIP® processor.

Samples were embedded in paraffin blocks and cut at 5 µm thickness on a Thermo Scientific™ Microm 355S microtome, placed in a 40 °C water bath, then picked up on Platinum Line charged slides (Mercedes Medical, FL, USA). Slides were air dried and excess paraffin was melted off in a 60 °C oven for 1 h.

Slides were H&E stained using the Optik Type 1 H&E stain system (Avantik Biogroup, NJ, USA) on a standard schedule on Leica Autostainer XL (IL, USA). Briefly, the remaining paraffin was removed in 3 changes of xylene, slides were rehydrated through 2 changes each of 100% and 95% EtOH, then rinsed in water for 1 min. They were then incubated for 5 min in hematoxylin solution (Hematoxylin DK), rinsed in clarifier for 30 s, water for 1 min, blueing agent for 30 s, and water for 1 min. This was followed by a rinse in 95% EtOH and incubation in eosin solution (alcohol-based Eosin-PX, with phloxine) for 1 min 15 s. They were then dehydrated through 2 changes each of 95% and 100% EtOH and 3 changes of xylene before coverslipping in Permount™ mounting media.
Stained samples were observed under the Nikon TE200-S widefield microscope using the 10X objective. Microstructure of the samples was quantified in terms of their average cell diameter (CD) and average cell wall thickness (CWT). Calculations for CD and CWT have been discussed in detail in the next section.

3.4. Microstructure quantification

Method 1: Using propidium iodide staining

Image analysis was performed using a combination of ImageJ® and MATLAB®. All the images obtained from the CLSM were in the .czi format, which were converted to .png images using the batch converter in ImageJ®. A MATLAB® code was drafted to identify the cell edges in each of these images, using the Canny Edge Detection Method. Figure 3.2 shows an image on a celery before and after being processed in MATLAB® for edge detection. It can be seen from Fig. 3.2 that the edge detection method was able to identify the overall structure of the cells, however, it also identified other high intensity cell areas as ‘edges’. Since the edge detection is not 100% accurate, the images obtained after applying Canny edge detection i.e. Fig. 3.2(b) were manually edited in ImageJ® (using a Surface Pen) to exclude the ambiguous cell areas and an 8-bit binary image was created for well-defined cells (Fig. 3.2(c)). Using the ‘Analyze Particles’ command in ImageJ®, the binary image was analyzed for area and perimeter of each particle i.e. cell. All images obtained for each sample (50 images per sample)
were processed according to this method and data for each cell of each image and its corresponding area and perimeter were obtained. For each sample, data was obtained for 300 to 500 cells.

![Figure 3.2](image)

**Figure 3.2** Cell structure of celery (a) before and (b) after using Canny Edge Detection method, (c) 8-bit binary image of the well-defined cells, and (d) outlines of the cells showing the ‘particles’ that were analyzed for area and perimeter. (All images are to the same scale: 850.19 µm x 850.19 µm).

Figures 3.2(a)-(c) show the qualitative differences between baby carrots, celery, and mango, as observed under CLSM. Based on the cell area and the cell perimeter for each cell of each image (Fig. 3.2(d)) for each sample, characteristic
length (µm; CL) was calculated as 4*(Cell Area)/(Cell Perimeter). Histograms were plotted based on 300 – 500 values of characteristic lengths (CLs) obtained for each sample (Fig. 3.3). The Shapiro-Wilk & Shapiro-Francia parametric hypothesis test of composite normality (α = 0.05) was performed on these data sets using MATLAB® to analyze if the data was normally distributed. A p-value of 0.16 (>0.05) indicated that the null hypothesis of normal distribution of data was true. The mean characteristic length of each sample was compared using Tukey’s HSD test in MATLAB®. The means of characteristic lengths for each sample were significantly different (Table 3.1). Thus, a method to quantify the fruit/vegetable cell structure was developed and the chosen samples had cell structures significantly different from each other. However, a drawback with this method was that it involved a step where boundaries for cells were physically drawn and hence this step would be potentially different for each researcher leaving a component of human error. Moreover, the use of a custom-made slicer is not ideal as it involves little control over the sample thickness and can therefore lead to uneven staining between samples. Hence, it was important to look into other staining techniques that would have precise control over the thickness of samples (using something similar to a microtome or a vibratome) and would use a dye that selectively stains cell walls.
Figure 3.3  (Top) Qualitative differences between the cell structure of (a) baby carrots, (b) celery, and (c) mango (all images to the same scale (850.19 µm x 850.19 µm)). (Bottom) A histogram of the CLs obtained for (a) baby carrots, (b) celery, and (c) mango

<table>
<thead>
<tr>
<th></th>
<th>Baby carrots</th>
<th>Celery</th>
<th>Mango</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (µm)</td>
<td>51.12 ± 10.12</td>
<td>116.76 ± 28.76</td>
<td>90.32 ± 17.31</td>
</tr>
<tr>
<td>Median (µm)</td>
<td>50.57</td>
<td>113.36</td>
<td>89.04</td>
</tr>
</tbody>
</table>

Table 3.1 Comparison of characteristic lengths (µm) of baby carrots, celery, and mango (different letters as superscripts indicate statistically significant difference between the means according to the Tukey’s HSD test at α = 0.05)
Method 2: Using H&E staining

Figures 3.4, 3.5, and 3.6 show the microstructure of baby carrots, celery, and mango, respectively, as observed after H&E staining. The five different regions observed in a baby carrot sample are shown in Fig. 3.4 as (a) central region, (b) around the central region, (c) periphery of the central region, (d) inner cells, and (e) outer edge. The central region of the baby carrot (Fig. 3.4a) was observed to have structures that looked different from regular cells. These were identified as the transport tubes (circled and shown in Fig. 3.4a) – the xylem and the phloem, that are present in baby carrots and are used by the root for transport of water and nutrients. Moving further away radially from the transport tubes but still around the central region (Fig. 3.4b), the cell sizes were found to be more uniform. Further away, on the periphery of the central region (Fig. 3.4c), the cell size decreased significantly and was less uniform. Radially halfway between the center and the outer edge of the baby carrot, the cells, referred to here as the “inner cells” (Fig. 3.4d) were uniform. At the outer edge of the baby carrot (Fig. 3.4e), the cells were uniform in size but were elongated along the edge and were larger than the inner cells. It was thus observed that the cell structure varied significantly within the carrot.

Similar differences in microstructure were also observed within celery samples (Fig. 3.5). Towards the outer edge of celery, the microstructure displayed
a cluster of cells (Fig. 3.5a) which were different from the rest. These were similar to the structures observed in the central region of the baby carrot and were identified as the transport tubes of the celery (circled and shown in Fig. 3.5a). Based on the description of the celery tissue by Leroux (2012), it was confirmed that the celery cells in Fig. 3.5a were indeed the xylem and the phloem, i.e., the transport tubes. The outer edge of the celery (Fig. 3.5b) displayed cells that were significantly smaller than the rest of the celery cells and were densely packed. For the vast majority of the celery tissue, between the transport tubes and the inner edge, the cell size was uniform as observed in Fig. 3.5c. This region is referred to here as the inner cells. The cells in Fig. 3.5b and Fig. 3.5c were identified as collenchyma and parenchyma cells, respectively, by comparing these images to those from Leroux (2012). The collenchyma cells provide structural support to the tissue while the parenchyma cells make up a majority of the tissue, also called as ground tissue, that is mainly involved in storage of nutrients and in photosynthesis. These cells do not contribute significantly to nutrient transport. Comparisons with the images from Leroux (2012) confirmed the appropriate identification of transport tubes in baby carrots as well (Fig. 3.4a). Similar to baby carrots, the differences in microstructure within a celery sample were significant.

Mango, unlike baby carrots and celery, was observed to have a uniform cell size (Fig. 3.6) throughout the sample. Mango is a fruit and does not have
transport tubes. The lack of transport tubes in mango, made their cell structure more uniform, while the presence of transport tubes in baby carrots and celery samples, made their cell structure less uniform.
**Figure 3.4** Cell structure differences within a baby carrot sample as observed at its (a) central region (b) around central region (c) periphery of central region (d) inner cells, and (e) outer edge.
Figure 3.5 Cell structure differences within a celery sample as observed around its (a) transport tubes (b) outer edge, and (c) inner cells.

Figure 3.6 Cell structure in a mango sample.
There are a few studies that have looked at the quantification of plant cell structure, but none of them from a food product perspective. Some have quantified the plant cell structure in terms of “stiffness” of the cells by calculating the Young’s Modulus (Nezhad et al. 2013; Geitmann 2006). However, to our knowledge, there has been no information on the quantification of cell size, cell size distribution, cell structure, and cell wall thickness. Therefore, the methods used to quantify the cell structures in this study have been developed entirely independently and constitute a unique and novel aspect of this study. The details of microstructure quantification are discussed below.

For an image (Fig. 3.7a) that was observed in the widefield microscope after H&E staining, image analysis was performed using MATLAB®. One hundred horizontal lines were drawn randomly across the image. For each line that was drawn, the variation in the intensity of each pixel at points along the horizontal line (= breadth of the image in µm) was plotted. A representative plot for one such image has been shown in Fig. 3.7b. Lower intensity values indicate darker spots which is why the stained cell walls are represented by the sharp valleys seen in Fig. 3.7b. However, there can be some noise in this image because of which only certain valleys in Fig. 3.7b represent cell walls. It was therefore important to establish a threshold intensity value to identify cell walls. A large number of images were analyzed and intensity values were compared to visual observations
of the respective images. After analyzing a large number of images, it was found that if the cut off was set anywhere between mean threshold intensity value ± 20, the number of cell walls identified remained the same and corresponded to the number of cell walls recognized visually in an image. Based on this observation, a threshold value of [mean intensity – 10] was used to characterize cell walls. This threshold was observed to work well for all the images in this work, however, may change for other researchers attempting a similar technique. Fig. 3.7b displays a horizontal orange line across the plot that represents the threshold used for that plot. All the valleys below this threshold were characterized as cell walls.
Figure 3.7 (a) Representative image of the cell structure in baby carrots (b)

Corresponding graph for variation in the intensity of pixels along a given horizontal line drawn on the image (a). Peaks below the threshold represent cell walls, with distance between peaks representing cell diameter (CD) and size of the peak at the threshold representing the cell wall thickness (CWT).
The cell structure was quantified in two terms – the average cell diameter (CD) and the average cell wall thickness (CWT). Distance between two valleys was quantified as the cell diameter (µm), while the width of a valley (µm) at the threshold was quantified as the cell wall thickness (CWT).

Tables 3.2, 3.3, and 3.4 show the cell diameter (CD) and cell wall thickness (CWT) values obtained for different regions in baby carrots, celery, and mango, respectively. Within a baby carrot sample (Table 3.2), it was observed that CWT of cells observed in different regions was the same irrespective of the region of interest. This observation was also true for CWT in celery and mango samples (Tables 3.3 and 3.4). Although, it must be noted that the CWT of celery inner cells was slightly but significantly different from the rest of the celery cells. However, when the average values of CWT were compared across all the samples, no significant difference was observed. This implied that the cell wall thickness did not change significantly regardless of the sample type and the position of cells within that sample. On the other hand, the cell diameter (CD), which is representative of the cell size, varied significantly within a given food sample and across different food samples. Consistent with the visual observations in Fig. 3.4, the cell diameter decreased radially away from the center and increased again. The smallest CD of 22.84 µm ± 1.74 µm was observed at the periphery of the central region (Table 3.2 and Fig. 3.4c) which was significantly different than that
of the rest of the baby carrot which showed similar values of average CD. For celery, the average CD values were significantly different within the celery sample (Table 3.3). The outer edge (Fig. 3.5b) had small cells (average CD of 14.24 µm ± 0.93 µm), while the cells increased in size around the transport tubes (Fig. 3.5a; 38.96 µm ± 7.57 µm), and the rest of the celery (referred to here as the “inner cells”, Fig. 3.5c) had significantly larger cells with average CD of 86.40 µm ± 12.92 µm (Table 3.3). Mango, having uniform structure, was quantified by a single value of CD (76.30 µm ± 8.65 µm) and was observed to have largest cells among all the samples.

<table>
<thead>
<tr>
<th>Baby carrot</th>
<th>Cell wall thickness (µm)</th>
<th>Cell diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central region</td>
<td>5.26 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.59 ± 1.72&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Around central region</td>
<td>5.49 ± 0.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.65 ± 2.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Periphery of central region</td>
<td>4.74 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.84 ± 1.74&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inner cells</td>
<td>5.38 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.08 ± 1.93&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Outer edge</td>
<td>5.77 ± 0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.02 ± 3.63&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Table 3.2** Values of CD and CWT in different regions of a baby carrot sample.
Table 3.3 Values of CD and CWT in different regions of a celery sample.

<table>
<thead>
<tr>
<th></th>
<th>Cell wall thickness (µm)</th>
<th>Cell diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transport tubes</td>
<td>5.88 ± 0.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.96 ± 7.57&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Outer edge</td>
<td>5.82 ± 0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.24 ± 0.93&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inner cells</td>
<td>4.07 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86.40 ± 12.92&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 3.4 Values of CD and CWT in a mango sample.

Since the cell wall thickness did not vary significantly between all the samples, the values of cell diameters were used to quantify the tissue microstructure. However, since the CD varied significantly within the baby carrot and celery samples, it was difficult to quantify their microstructure using just a single value of CD. It was thus necessary to understand the location of calcium infusion within the samples after HPP, and use the findings to decide which regions and respective cell diameter values were to be used.
3.5. Mapping of infused calcium in processed samples

As has been previously discussed in section 3.2, calcium infused in the samples was tagged with Fluo-8 fluorescent dye after HPP and observed under the confocal laser microscope to visualize the fluorescence of the dye, which was intensified in presence of calcium. Thus, areas that showed higher fluorescence represented areas with high calcium. This was done to understand the location of infused calcium in the samples. It would have been ideal to tag the calcium before infusion and observe the changes under pressure. However, the dye used in this study was sensitive to time, light, and pressure, and hence difficult to use during high pressure calcium infusion.

Figure 3.8a shows an image of a baby carrot infused under high pressure with 6% CLG at 200 MPa for 15 min. It can be observed that most of the calcium infused was concentrated in and around the central region of the baby carrot. It was our hypothesis earlier that the infusion of calcium would occur from the outside edge to the center, however, that was not supported by these observations. It was observed that the calcium diffused from the center of the baby carrot towards the outer edge. This observation, in hindsight, seems consistent with the microstructure data that was reported earlier. The transport tubes for the baby carrots are located in the central region and are responsible for water and nutrient transport. During HPP, the calcium was infused via the
transport tubes and further diffused in the surrounding cells, thus causing the infusion from center of the baby carrots to the outer cells. Most of the calcium was concentrated along the periphery of the central region (corresponding to Fig. 3.4c), indicating that the infused calcium was located around those cells. To understand if any calcium was located in the rest of the baby carrots, all images were subjected to image analysis in ImageJ™ to obtain the data for variation in the intensity of the Fluo-8 fluorescence starting from the center to the outer edge. Due to the large data set that was obtained from image analysis, it was important to represent data for each pressure with a single curve. To achieve this, Lowess (Locally Weighted Scatter Plot Smooth) regression was used in MATLAB®, which uses locally weighted linear regression to smooth the data in a narrow range. Figure 3.8b shows the Lowess curves obtained for baby carrots processed at each pressure (100 MPa – dark blue; 200 MPa – orange; 300 MPa – yellow; 400 MPa – purple; and 500 MPa - green) and their comparison to the unprocessed samples (light blue curve). It can be seen that for the high pressure processed – calcium infused samples, the calcium was primarily concentrated at the periphery of the central region (0.2 to 0.3 normalized units away from the center, where 1 normalized unit away from center is the outer edge; region represented in Fig. 3.4c). The unprocessed sample (light blue curve) had no variation in the amount of calcium throughout the sample, and so did the sample processed at 100 MPa,
most likely due to the low pressure that was not enough to enhance the infusion significantly. It must be noted that the spike in the intensity data on the outer edge (Fig. 3.8b) is due to the peeled skin of the baby carrot that was in contact with the CLG solution throughout processing. Since no gradient of calcium can be seen from the outer edge towards the center, it was concluded that the infusion did not take place from outside to inside, but from inside to the outside due to the presence of the transport tubes in the central region.
**Figure 3.8** (a) Locus of the infused calcium in a cross-section of a baby carrot tissue obtained by tagging the calcium with a fluorescent Fluo-8 dye and observing under a laser microscope. Intensity of each pixel is directly proportional to the amount of calcium present in that area; brighter the point, higher the calcium. (b) Variation in the intensity of Fluo-8 fluorescence from the center of the baby carrots ($x = 0$) to the outer edge ($x = 1$) shown by red box, indicating the distribution of infused calcium in the tissue. $x$ is normalized radial distance in a baby carrot sample.
Similar to baby carrots, the infused calcium in celery (Fig. 3.9a) was concentrated around the transport tubes (represented in Fig. 3.5a). This further validated our previous observation that the calcium uptake during infusion took place via transport tubes and further diffused in the surrounding cells. The location of infused calcium around transport tubes can also be seen from the intensity plots in Fig. 3.9b (100 MPa – dark blue; 200 MPa – orange; 300 MPa – yellow; 400 MPa – purple; 500 MPa – green; and unprocessed samples – light blue).
Figure 3.9 (a) Locus of the infused calcium in a cross-section of a celery tissue obtained by tagging the calcium with a fluorescent Fluo-8 dye and observing under a laser microscope. Intensity of each pixel is directly proportional to the amount of calcium present in that area; brighter the point, higher the calcium. (b) Variation in the intensity of Fluo-8 fluorescence from the inner edge of celery (x = 0) to its outer edge (x = 1), indicating the distribution of infused calcium in the tissue. x is normalized distance in a celery sample.
**Figure 3.10** (a) Locus of the infused calcium in a cross-section of a mango tissue obtained by tagging the calcium with a fluorescent Fluo-8 dye and observing under a laser microscope. Intensity of each pixel is directly proportional to the amount of calcium present in that area; brighter the point, higher the calcium. (b) Variation in the intensity of Fluo-8 fluorescence from one edge of the mango cuboid ($x = 0$) to the other edge ($x = 1$), indicating the distribution of infused calcium in the tissue. $x$ is normalized distance in a mango sample.
In case of mango (Fig. 3.10a), which does not have transport tubes, it was observed that the infused calcium was uniformly distributed in the entire tissue and the infusion occurred from the edge to the inside. This trend is also seen from the flat nature of the curves in Fig. 3.10b (100 MPa – dark blue; 200 MPa – orange; 300 MPa – yellow; 400 MPa – purple; 500 MPa – green; and unprocessed samples – light blue). From all the graphs (Figs. 6b, 7b, and 8b), it was observed that the highest amount of calcium was present in the samples processed at 500 MPa, and all the high pressure processed – calcium infused samples had significantly higher levels of calcium as compared to the raw, unprocessed samples.

In a study by Gras et al. (2003), where calcium was infused in different vegetables via vacuum impregnation, calcium distribution was analyzed by depressive X-ray microanalysis. They observed that calcium incorporation occurred in the xylem of the carrots, consistent with the findings in our study, and proposed that the transport tubes played an important role in impregnation of calcium in carrots. On the other hand, calcium incorporation in eggplant and oyster mushroom occurred in the intercellular spaces due to lack of transport tubes. Gras et al. (2003) also noted a marked difference in the amount of calcium infused in these vegetables and its influence on the tissue characteristics.
The fluorescence tagging of the infused calcium and the quantitative analysis of the intensity of fluorescence throughout the fruit / vegetable tissue is an aspect of this study that is unique and provides additional insights into high pressure assisted infusion of calcium. Based on these insights, it was decided that the areas that showed concentration of calcium in each sample would be used to represent the quantitative microstructure of that sample. Hence, baby carrots were represented by the CD values of the region represented in Fig. 3.4c (periphery of the central region), celery was represented by the CD values of the area around the transport tubes (represented in Fig. 3.5a), and mango was quantified as the average CD obtained for all the images. Cell diameter (CD) distributions for each of these samples and their respective regions of interest are shown in Fig. 3.11. The cell diameter distributions followed a Gaussian trend (normal distribution) based on the normality test.
3.6. Effect of fruit/vegetable microstructure on determining the extent of infusion

The values of cell diameters for each of the samples at their chosen locations are shown in Table 3.5. Mangoes had the highest CD of 76.30 µm ± 8.65 µm while baby carrots had the smallest CD of 22.84 µm ± 1.74. Infusion in each of these food systems at 500 MPa is also shown in Table 3.5, and it was observed
that mangoes, which had the highest CD also had highest level of infusion under high pressure.

<table>
<thead>
<tr>
<th></th>
<th>Cell diameter (μm)</th>
<th>Calcium infusion at 500 MPa (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baby carrot (periphery of central region)</td>
<td>22.84 ± 1.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.89 ± 4.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Celery (transport tubes)</td>
<td>38.96 ± 7.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.05 ± 2.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mango</td>
<td>76.30 ± 8.65&lt;sup&gt;c&lt;/sup&gt;</td>
<td>111.02 ± 14.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Table 3.5** Variation between the extent of calcium infusion under high pressure with changes in the microstructure of fruit or vegetable. (n=6)

Figure 3.12 shows the amount of calcium infused in baby carrots, celery, and mango at all the pressures and their comparison to the inherent calcium that was present in them before processing. It must be noted that the calcium infusion data presented here represents infused calcium, not the total calcium, i.e., total calcium = inherent calcium + infused calcium. Additionally, it is important to point out the data labels used to indicate statistical differences. Upper case letters have been used to compare baby carrot samples across the pressure range, lower case letters have been used to compare celery samples across the pressure range, while italicized letters have been used to compare mango
samples across the pressure range. Difference between two samples (across the pressure range or at that pressure) should be considered statistically significant only if they do not share a letter despite the differences between the letter cases. For example, in Fig. 3.12, there is no significant difference between baby carrots at 200 MPa and celery at 200 MPa because they share the letter ‘B/b’; however, there is significant difference between baby carrots and celery processed at 400 MPa since they do not share a letter.

**Figure 3.12** Amounts of calcium infused in baby carrots, celery, and mango with increasing pressure. Bars that do not share a letter have significantly different value from each other (p-value < 0.05).

Across the entire pressure range, mangoes were observed to have the highest level of infused calcium. The amount of infused calcium was significantly
different in baby carrots, celery, and mango at every pressure. For all the three food systems, highest infusion was observed at 500 MPa. Celery exhibited significantly higher levels of infused calcium at all pressures as compared to baby carrots. At any given pressure, mango showed higher infusion, followed by celery and baby carrots in that order. This trend was also seen in their cell diameters, where mango had significantly high CD, and celery baby carrots had smaller CDs, although significantly different from each other. It appeared that the microstructure of the samples, quantified as their cell diameter (CD), was correlated to the extent of high pressure assisted calcium infusion in those samples. It was also observed that in case of baby carrots and celery, both of which have lower CDs, the level of infused calcium either plateaued off after 300 MPa or decreased at 400 MPa and increased again at 500 MPa. Since this was not observed in mangoes, the microstructure may have played a role in this effect as well. A similar trend of calcium infusion was observed in the previous study, where it was postulated that the slight reduction in the amount of infusion after mid pressure levels (300 MPa) may be due to the shrinkage of cells under pressure, reducing the cell volume, and making the inflow of calcium more difficult. However, at higher pressures (500 MPa), the effect of pressure to push the calcium molecules in the matrix can be significantly higher than the resistance of cells to the inflow due to shrinkage, and hence, the level of infusion can be
observed to increase again at these high pressures. Baby carrots and celery, having smaller cells as seen by their small CDs, exhibit this effect strongly. Mango, on the other hand, had larger cells that may have been affected by shrinkage under pressure, however this shrinkage did not affect the levels of infusion because the resultant volume of cells after shrinkage may still have been enough to allow inflow of calcium. It was thus evident that there exists a correlation between the microstructure, quantified as cell diameter, and extent of calcium infusion under high pressure. Larger cells caused more infusion due to their larger cell volume that allowed for more calcium to be infused. This lends support to the hypothesis that food microstructure is an important parameter driving infusion under high pressure.

In case of baby carrots and celery, the observation of lower amount of infusion at mid pressure levels has not been reported in works from other researchers (Mahadevan et al. 2016; Rastogi et al. 2008; Sopanangkul et al. 2002). However, direct comparison between previously reported research in literature and ours is not feasible, since the concentration of infusate (calcium) solution used in our study was significantly higher than that used in those studies. Moreover, we are not aware of any study in published literature that has addressed the quantification of microstructure in terms of cell and diameter cell
wall thickness, therefore direct comparison of our results with those published is not possible.

Infusion at atmospheric pressure (osmosis) was not performed for all the samples in this study, as there is sufficient evidence from the previous study as well as from the works of other researchers (Mahadevan et al. 2016; Sopanangkul et al. 2002; Rastogi et al. 2000) that infusion under high pressure is significantly higher than that at atmospheric pressure. In the present study, total calcium in the samples after HPP assisted calcium infusion accounted for 6% (in celery) to 10% (in mangoes) of RDI of calcium, in 100 g of sample.

3.7. Cell-permeabilization index (Zp) and its effect on calcium infusion

Researchers who have explored the effect of high pressure on mass transfer of biomolecules in food matrices have all unanimously claimed that cell disintegration in a food material due to high pressure is an important parameter influencing the mass transfer (Sopanangkul et al. 2002; Rastogi et al. 2000; Rastogi and Niranjan 1998). As explained earlier, cell disintegration in plant tissues can be quantified as cell permeabilization index (Zp). It has been reported by previous researchers that increase in the Zp values correlated with increased mass transfer coefficients under high pressure.
Figures 3.13a, 3.14a, and 3.15a, show the change in $Z_P$ values with increasing pressure for baby carrots, celery, and mango, respectively. Figures 3.13a, 3.14b, and 3.15c show the corresponding amounts of calcium infused in baby carrots, celery, and mango, respectively, at different pressures. The high standard deviation in the $Z_P$ values for celery as well as for mango was due to the significant amount of water that oozed out of the samples during $Z_P$ measurements, because the samples had to be slightly squeezed between the two probes. For baby carrots, the $Z_P$ values increased gradually with an increase in pressure (Fig. 3.13a), so did the amount of infused calcium (Fig. 3.13b). The calcium infused decreased at 400 MPa before increasing again at 500 MPa, reason for which has been discussed in an earlier section. For celery, the $Z_P$ values increased with increasing pressure (Fig. 3.14a) although the increase was statistically insignificant. The amount of calcium infused also showed gradual increase (Fig. 3.14b), similar to the $Z_P$, but also showed decrease at 400 MPa and increase again at 500 MPa due to the role of microstructure as discussed in an earlier section. In case of mangoes, the $Z_P$ values did not change with an increase in pressure (Fig. 3.15a), and the amount of infused calcium also showed little to no increase with pressure until 400 MPa (Fig. 3.15b), after which the amount was significantly higher. Similar trends were observed by Rastogi et al. (2000), where the $Z_P$ of potato slices increased with an increase in pressure from 100 MPa to
400 MPa and continued to increase during the period of osmotic dehydration (up to 6h) that followed the pressure treatment. On the contrary, Mahadevan et al. (2015) did not observe any change in the $Z_P$ of scarified cranberries beyond 100 MPa. It must be noted that the food systems used in the studies by Rastogi et al. (2000) and by Mahadevan et al. (2015) were different and hence, the observations for the change in $Z_P$ are different as well.

**Figure 3.13** (a) Changes in the cell permeabilization index ($Z_P$) of baby carrots with increasing pressure. Higher values of $Z_P$ indicated higher cell breakage. (b) Effect of increasing pressure on the amount of calcium infusion in baby carrots. Differences in letters indicate statistical significance (p-value < 0.05).
Figure 3.14 (a) Changes in the cell permeabilization index ($Z_P$) of celery with increasing pressure. Higher values of $Z_P$ indicated higher cell breakage. (b) Effect of increasing pressure on the amount of calcium infusion in celery. Differences in letters indicate statistical significance (p-value < 0.05).
Figure 3.15 (a) Changes in the cell permeabilization index ($Z_P$) of mango with increasing pressure. Higher values of $Z_P$ indicated higher cell breakage. (b) Effect of increasing pressure on the amount of calcium infusion in mango. Differences in letters indicate statistical significance (p-value < 0.05).

Based on these insights, there appears to be an effect of cell permeabilization on the extent of calcium infusion under high pressure, however, the effect is also dependent on the microstructure of the sample. If $Z_P$ was the
only factor affecting the extent of HPP assisted calcium infusion, completely independent of the microstructure, there would have been a strong correlation between the $Z_P$ values and the amount of calcium infused. However, as shown in Fig. 3.16, there was no correlation between the $Z_P$ values and the calcium infused, when the differences in samples were ignored, i.e., all three food systems were pooled together and no distinction was made between baby carrots, celery, and mango. Thus, it is evident that cell permeabilization has an effect on the amount of high pressure assisted infusion in context of a given food material, but is not the only factor driving infusion.

**Figure 3.16** Variation between the extent of calcium infusion and the cell permeabilization index ($Z_P$) across all food samples, without taking into consideration the food matrix that was infused ($R^2 = 0.19$).
3.8. Effect of HPP assisted calcium infusion on texture and color of the samples

As described in section 3.2, the texture for each sample was quantified as the energy (J) and the peak force (N) required for to cut the sample. Figure 3.17 and Figure 3.18 show the texture data obtained for high pressure processed – calcium infused baby carrots, celery, and mango at varying pressures and their comparison to their respective unprocessed (raw) samples.

**Figure 3.17** Change in the energy (J) required to cut a sample with increasing pressure (MPa) for baby carrots, celery, and mango. Bars that do not share a letter have significantly different value from each other (p-value < 0.05).
Figure 3.18 Change in the cutting peak force (N) with increasing pressure (MPa) for baby carrots, celery, and mango. Bars that do not share a letter have significantly different value from each other (p-value < 0.05).

It was observed that the energy required to cut the processed baby carrots did not significantly change with increasing pressure and was comparable to that of the unprocessed sample. On the other hand, the energy required to cut processed celery samples reduced with increasing pressure until 300 MPa, indicating that HPP made celery samples softer and easier to cut. Similar to the observations in baby carrots, energy required to cut the processed mango samples did not change significantly with pressure and was comparable to the unprocessed mango sample (Fig. 3.17). It is a well-known fact that calcium ions
contribute to improving the structural rigidity of plant cells by binding with the pectin present in fruit and vegetable tissues (Gosavi et al. 2019; Bellary and Rastogi 2016; Rastogi et al. 2008; Gras et al. 2003). Gras et al. (2003) suggested that the calcium ions interacted with plant tissues and resulted in the modification of its mechanical properties. They reported that mechanical behavior of eggplant and carrot were markedly affected by calcium, and no significant effects were observed in oyster mushroom due to the absence of pectin in their cell architecture. In a study by Rastogi et al. (2008), carrot texture improved during thermal and pressure-assisted thermal processing due to a combined pre-treatment of calcium, mild heat, and high pressure. The hardness of thermally processed samples and pressure-assisted thermally processed samples increased by about nine times (from 14 N to 129 N) and by about thirteen times (from 4 N to 57 N), respectively, after the combined pre-treatment of calcium, high pressure, and mild heat. The enhanced diffusion of calcium due to combined effect of pressure and temperature resulted in firmer plant tissue by binding of calcium to pectin carboxyl groups that were exposed through the action of PME.

The comparable values of energy required for cutting the raw unprocessed samples and the HPP – calcium infused samples for both baby carrots and mango can thus be explained by the binding of infused calcium with the high level of
pectin present in both samples. Celery, on the other hand, has negligible amount of pectin and hence the energy required to cut the celery samples decreased with pressure due to the lack of pectin for the infused calcium to bind to.

The peak force (N) required to cut the calcium infused baby carrots was significantly affected after processing at 100 MPa and 500 MPa, however, remained unaffected for the other pressures. The values of peak cutting force (N), although statistically different, are close to each other and the differences in the values may be the result of variation in individual samples. The peak cutting force did not change for calcium infused celery until processing at 500 MPa, while it was significantly different for mango samples processed at 200 MPa and 400 MPa (Fig. 3.18). Between the samples, mangoes were observed to have the softest tissue based on the low values of energy required (J) and peak force (N) for all the samples, while celery had the toughest tissue given the high values of energy required and peak force. The differences observed here were statistically significant but sensory studies are needed to evaluate their practical significance.

The color of the samples was significantly affected by the application of HPP. Figure 3.19 shows comparisons between unprocessed samples and samples processed at 500 MPa (pressure for highest infusion in all samples) while Table 3.6 shows the corresponding L*, a*, b*, and ΔE* values. It was observed that after HPP, the L* values of all the sample decreased, indicating that the samples
became darker. A possible reason for the darkening of the samples after HPP could be the activation of latent isoenzymes after HPP (Júnior et al. 2017) and an increased interaction of these isoenzymes with the substrates in cellular matrix due to loss of cell integrity (Techakanon et al. 2016) after HPP.

**Figure 3.19** Color of (a) baby carrots, (b) celery, and (c) mango before (left) and after HPP processing (right) at 500 MPa for 15 min using 6% CLG solution.
Table 3.6 Differences between the color parameters (L*, a*, and b*) of raw baby carrots, celery, and mango and HPP processed samples at 500 MPa for 15 min using 6% CLG solution. (n=6)

<table>
<thead>
<tr>
<th></th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
<th>ΔE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw baby carrots</td>
<td>57.97</td>
<td>31.59</td>
<td>41.03</td>
<td>-</td>
</tr>
<tr>
<td>HPP baby carrots</td>
<td>52.49</td>
<td>26.34</td>
<td>34.01</td>
<td>9.12</td>
</tr>
<tr>
<td>Raw celery</td>
<td>53.09</td>
<td>-17.67</td>
<td>29.68</td>
<td>-</td>
</tr>
<tr>
<td>HPP celery</td>
<td>45.34</td>
<td>-15.37</td>
<td>23.79</td>
<td>20.22</td>
</tr>
<tr>
<td>Raw mango</td>
<td>74.86</td>
<td>4.49</td>
<td>63.21</td>
<td>-</td>
</tr>
<tr>
<td>HPP mango</td>
<td>61.22</td>
<td>5.12</td>
<td>50.16</td>
<td>19.35</td>
</tr>
</tbody>
</table>

Additionally, for celery and mango samples, it was observed that the processed samples were significantly greener (more negative a* values) and yellower (more negative b* values), respectively, as compared to their unprocessed controls. This observation is consistent with results from Paciulli et al. (2016) where beetroots treated with HPP had L*, a*, and b* values lower than those of the raw beetroots, indicating a blue-shift in color. Application of HPP caused significant color differences in the processed samples (ΔE* > 2) to be perceptible to human eye, but the color difference did not change with
increasing pressure after 100 MPa. HPP causes changes in cellular structure and consequent texture modification that can potentially change the nature and extent of internally scattered light resulting in a perceived change of color (Oey et al. 2008). If the increase in darkness of the samples and the appearance of greener celery and yellower mango are of practical significance, can only be evaluated after detailed sensory studies. However, sensory evaluation of these fruits and vegetables was out of scope of our research, since our focus was only on understanding the role of microstructure and cell permeabilization on extent of calcium infusion.

3.9. Key takeaways for this study

- Efficacy of HPP to infuse calcium in fruits and vegetables – specifically baby carrots, celery, and mango, was evaluated.

- A novel and unique method was developed to quantify fruit/vegetable microstructure in terms of cell diameter (CD) and cell wall thickness (CWT) using H&E staining.

- Baby carrots, celery, and mango displayed significantly different microstructures. Moreover, there were significant internal microstructural differences within baby carrots and celery, due to the presence of transport tubes.
- All the three food matrices had similar CWT (4 µm to 5.5 µm) but had significantly different CDs – mango having the largest cells (76.30 ± 8.65) µm, followed by celery (38.96 ± 7.57) µm, and baby carrots (22.84 ± 1.74) µm.

- The amount of calcium infused was significantly affected by the pressure level employed for all the three food systems. At all pressures, mango showed the highest amount of calcium infusion, followed by celery and baby carrots – which correlated to their microstructures (CD values).

- A unique method was developed to visualize the infused calcium in the fruit and vegetable matrix using a fluorescent dye, which provided important insights in terms of the loci of calcium uptake during HPP. Calcium uptake due to externally applied high pressure took place via the transport tubes in baby carrots and celery, and via diffusion from the outer surface to the inner regions in peeled mangoes.

- Application of high pressure also caused cell permeabilization in all the samples, which influenced the extent of calcium infusion under HPP. However, cell permeabilization was correlated to the level of calcium infusion only within a given food system, but not across all the three food systems considered here, indicating that the microstructure of the fruits and
vegetables influenced the cell permeabilization as well as the level of calcium infusion under high pressure.

- The process of high pressure assisted calcium infusion in baby carrots, celery, and mango influenced their instrumentally measured texture and color, however, the practical significance of these effects in terms of sensory perception was not evaluated.
CHAPTER 4

DEVELOPMENT OF A MATHEMATICAL MODEL FOR HIGH PRESSURE ASSISTED CALCIUM INFUSION IN FRUITS AND VEGETABLES

4.1. Brief introduction

As has been extensively discussed in the previous chapters, the application of High Pressure Processing to enhance the nutritional value of foods through infusion of nutrient molecules has been of significant research interest. However, despite the high interest and the novelty of its application beyond food safety and food quality improvement, studies in this area remain limited and have a narrow focus. One of the major reasons for this dissonance has been the inadequate understanding of the nature and the process of mass transfer (i.e., infusion) under high pressure in biological materials. This difficulty in mechanistic understanding the process arises from two confounding effects that play a role in driving the infusion process –

(1) Concentration driven infusion due to the concentration difference between the isotonic infusate solution and the food matrix, and

(2) Pressure driven infusion as a result of the high pressure that is applied in the process. Additionally, these effects also work together to enhance the infusion
even further. The application of pressure leads to permeabilization of the cell membranes reducing the resistance to concentration driven flow and enhancing the amount of infusion in the food matrix. Owing to the complexity of this phenomenon, limited research exists that has been aimed towards understanding the mechanics of this process.

Currently available research aimed at understanding infusion under high pressure is based on one common theory that the infusion is driven solely due to the concentration difference between the infusate solution and the food material which is enhanced by permeabilization of cells under high pressure and hence follows the laws of Fickian diffusion. More information about these models has been discussed in section 4.2. However, a major drawback with this assumption of Fickian diffusion is that the models do not consider the direct effect of pressure driven transfer, since Fickian diffusion only describes mass transfer due to concentration difference. As a result, the mechanisms described by these models are not limited to infusion under pressure but describe any infusion process into a food matrix with the same level of cell permeabilization due to the concentration gradient. In simpler terms, the assumption of Fickian diffusion implies that cell permeabilization is important and not how the cell permeabilization occurs (i.e., by pressure or by cooking). Since these models do not explain the concomitant role of high pressure in infusion under HPP, there is
a need to develop mathematical models that can explain the pressure driven
effect along with concentration driven effect. Moreover, these models should also
be able to explain the effect of food microstructure in determining the extent of
infusion (as discussed in Chapter 3).

4.2. Existing models by other researchers

Over the past two decades, multiple studies have been published that have
evaluated HPP as a technique to enhance mass transfer either as a pretreatment
to subsequent osmotic infusion/dehydration or as a direct treatment for infusion.
Many of these studies have attempted to explain infusion under high pressure. A
common theme between these studies has been that they assumed mass transfer
under high pressure to be driven by the concentration gradient between the food
matrix and the infusate solution, which is further enhanced by the cell
permeabilization caused by high pressure. As a result, the infusion process under
high pressure has been modeled based on Fick’s second law, which describes the
rate of infusion in a food matrix as being proportional to the concentration
gradient. Mathematically, Fick’s second law can be written as

\[
\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} \quad \text{... Eq. 4.1}
\]

where, \( C \) is the infusate concentration (mol/m\(^3\)), \( t \) is the time (s), \( x \) is the
distance (m) in the direction of infusion, and \( D \) is the mass diffusivity coefficient
To calculate the diffusivity coefficient \( D, \text{m}^2/\text{s} \) under high pressure based on concentration difference, the solution to Fick’s second law (derived by Crank, 1979) has been used. Crank (1979) proposed this solution for diffusion in plane sheet surrounded by a stirred solution of limited volume. The solution was thus assumed to be isotropic in concentration (i.e., a constant solute concentration throughout), and this concentration declined as the solute diffused into the sheet. The partition coefficient at the sheet-solute surface was assumed to be one, which implied that the solute concentration within the surface of the sheet and that in the solution was the same. Another important assumption was that the mass transfer properties of the sheet i.e., the material being infused, remained constant and did not change with time. Thus, the solution to Fick’s second law for a rectangular sample with dimensions of \( 2a \times 2b \times 2c \) is given by Eq. 4.2a.

\[
S_r = \frac{S_t - S_\infty}{S_0 - S_\infty} = \sum_{n=1}^{\infty} C_n^2 \exp \left\{ \left( -D_e \right) (t) \left( q_n \right) \left( \frac{1}{a^2} + \frac{1}{b^2} + \frac{1}{c^2} \right) \right\} \quad \text{... Eq 4.2a}
\]

where, \( S_r \) is the solute ratio; \( S \) is the solute concentration and the subscripts ‘0’, ‘\( \infty \)’, and ‘t’, and represent the solute concentrations initially, at equilibrium, and at any given time; \( D_e \) represents the effective diffusivity of the solute (\( \text{m}^2/\text{s} \)); \( C_n = 2\alpha'(1 + \alpha')/(1 + \alpha' + \alpha'^2 q_n^2) \), where \( q_n \)'s are the non-zero positive roots of Eq. 4.2b

\[
\tan(q_n) = -\alpha' q_n \quad \text{... Eq 4.2b}
\]
and $\alpha'$ is the ratio of the volume of the solution to the volume of each piece. In case of calcium infusion in mango pieces of dimensions $50 \text{ mm} \times 15 \text{ mm} \times 15 \text{ mm}$ surrounded by $100 \text{ mL}$ of solution, and assuming that each side of each mango piece was in contact with the solution, the value of $\alpha'$ would be 8.8. When the Fourier number $F_0 (= -D_e t (1/A^2)) > 0.1$, only the first term is significant and the rest can be dropped, condensing Eq. 4.2 into the following form, which on further rearranging leads to Eq. 4.4.

$$S_r = \frac{S_t - S_\omega}{S_0 - S_\omega} = C_1^3 \exp \left[ ( -D_e) (t) (q_1) \left( \frac{1}{A^2} \right) \right] \quad \text{...Eq 4.3}$$

$$- \ln \left( \frac{S_r}{C_1^3} \right) = q_1 \left( \frac{D_e \ t}{A^2} \right) \quad \text{...Eq 4.4}$$

Based on $\alpha'$, $q_1 = 1.64$ (obtained from Table 4.1 in Crank 1979). By fitting Eq. 4.4 to data obtained from high pressure assisted infusion / dehydration, diffusivity values ($D_e$) can be calculated from the slope of the $\ln(S_r)$ vs $t$ curve. This method of calculating diffusivity has been used in many of the previously mentioned studies to show accelerated rate of infusion. Rastogi and Niranjan (1998), who explored HPP as a pre-treatment to osmotic dehydration of pineapple slices, observed that the increase in calculated effective diffusivities was pronounced at lower pressures, but flattened at higher pressures. Based on this observation, they proposed a correlation (Eq. 4.5) similar to the Arrhenius equation to explain the effect of high pressure on effective diffusivities.
where $B$ is a constant (MPa) similar to activation energy in the Arrhenius equation, and $P$ is the pressure applied (MPa). Other researchers have also reported a similar trend in diffusivity values of the solute where the increase has been evident until certain pressures but flattened or even reduced slightly on further increasing the pressure. Although Eq. 4.5 explains the data observed by Rastogi and Niranjan (1998), there is no physical explanation of the process or why mass transfer under high pressure would follow Arrhenius kinetics. The mechanism of pressure driven mass transfer under high pressure remains unexplained. Moreover, Eq. 4.5 cannot sufficiently explain how the diffusivities would vary for different food materials of different microstructures and permeabilities.

To better explain the role of pressure driven flow during HPP of porous biological materials confined in a fluid phase, Vatankhah et al. (2018) proposed a hybrid Fickian-Darcian approach. This approach combined the two laws – Fick’s law of mass transport driven by concentration difference and Darcy’s law of mass transport driven by pressure difference. According to Darcy’s law (Eq. 4.6), the volumetric flow rate ‘$Q$’ (m$^3$/s) of a liquid of viscosity ‘$\mu$’ (Pa.s) into a porous material of cross-section $A$ (m$^2$) is driven by the pressure difference ‘$\Delta P$’ (Pa) within the material and the intrinsic permeability ‘$k$’ (m$^2$) of the material.
\[
Q = \frac{-kA}{\mu} \Delta P
\] ... Eq. 4.6

In addition to the calculation of diffusivities using Crank’s solution to Fick’s second law (Eq. 4.3), Vatankhah et al. (2018) proposed incorporation of pressure driven flow through use of another coefficient – the lumped permeability of the porous medium. The lumped permeability was calculated based on the intrinsic permeability of the porous biomaterial (apples) and the relative permeability at various stages of saturation of the biomaterial during HPP impregnation. Using numerical modeling, they also demonstrated the existence of a low-pressure zone at the center of the biomaterial. The pressure difference between the outside of the material and its core ranged from 10 MPa for a 5 min holding period to 4 MPa for a 15 min holding period. It was proposed that this pressure difference along with the lumped permeability of the material contributed to mass transfer during HPP impregnation. This was an innovative approach that further highlighted the need to understand pressure driven HPP infused even further. The Fickian-Darcian approach accounted for the change in permeability during HPP, however, it did not explain how the microstructure of the biomaterial would govern the process. Moreover, it was assumed that the concentration and pressure driven flows contributed independently to the impregnation process. However, based on Mahadevan et al. (2015b) study, these processes are not only
dependent but work synergistically to enhance the impregnation of materials under HPP.

4.3. Need for a new model

The process of high pressure infusion is not simple. In addition to the driving forces of applied pressure and concentration gradient between the infusate and the food matrix, two other major factors play a role – cell permeabilization and food microstructure. The integrity of cells is significantly reduced as shown in earlier sections. This extent of cell permeabilization can significantly accelerate the process of infusion. However, the extent of cell permeabilization is in turn affected by pressure level and the type of food matrix being infused. The microstructure of the food matrix is the most important factor in this process, which has an added complexity associated. Food matrices, such as baby carrots and celery, which are the root and the stem of their respective plants, have structures known as transport tubes that specifically function for water and mineral uptake. The presence of these transport tubes significantly changes the extent of infusion, but since they are not present in every food matrix, their effect is difficult to model. Given these multiple factors that are also interdependent, the process of mass transfer under HPP does not seem to follow simple Fickian diffusion only. These factors need to be further explored and modeled to understand this process better.
Current models do not describe the direct effect of pressurization on mass transfer, nor do they describe if the model can be applied to any food material that is being infused. As a result, there exists a gap in our understanding of this process of infusion under high pressure.

4.4. Experimental design

Concise descriptions of the experimental design have been included in Table 4.1. In order to decouple the effect of pressure and cell permeabilization during high pressure infusion of calcium in different food matrices, three sets of experiments were performed to evaluate three separate cases. The first case was designed to evaluate concomitant effect of pressure and permeabilization. The second case was designed to evaluate the effect of pressure alone under high pressure, while eliminating the effect of cell permeabilization beforehand. This was achieved by performing high pressure sequentially. In the first cycle of high pressure, samples (baby carrots, celery, and mango) were subjected to 5 different pressure levels (100 MPa to 500 MPa) to achieve permeabilization at respective pressures. It must be noted that the first cycle of pressurization did not involve use of CLG solution and samples were not infused with any liquid medium. The second cycle of HPP was performed to infuse calcium into the permeabilized samples (from the first cycle) at the same pressure levels that the samples were permeabilized at. For example, samples permeabilized at 100 MPa in cycle one
were infused with calcium at 100 MPa in cycle two using 9% CLG. The third case of experiments was designed to evaluate the effect of cell permeabilization alone without the effect of pressure. Samples were permeabilized under high pressure at different pressure levels (similar to cycle one in previous case of experiments) followed by infusion of calcium at osmotic pressure. These particular cases of experiments were designed with an aim to separate the effect of pressure from the effect of permeabilization during HPP assisted calcium infusion. Since case 2 involved permeabilization of cells prior to HPP infusion, while case 1 involved simultaneous effect of permeabilization and high pressure on infusion, it would be possible to understand the effect of pressure only by looking at the differences in infusion between the two cases. However, the phenomenon of HPP infusion is not that simple, since effect of pressure and permeabilization during HPP infusion is synergistic rather than simply additive. However, these experimental cases would still be useful in understanding the mechanics of infusion better. Similarly, case 3 was designed to understand the effect of permeabilization only by eliminating the effect of pressure during calcium infusion. However, as was observed in our experiments, cells are further permeabilized under the second HPP cycle during infusion which led to higher infusion. As a result, differences in case 3 and case 1 would not give a complete understanding of effect of permeabilization only while eliminating effect of
pressure, but this experimental design would be helpful in our understanding of
the HPP assisted infusion process, nonetheless. As a control to the high pressure
infusion experiments, osmotic infusion was performed on raw, unprocessed baby
carrots, celery, and mango using 9 % CLG solution for a hold period up to 24 h,
with time intervals at 15 min, 180 min (3 h), 360 min (6 h), 720 min (12 h), and
1440 min (24 h).

<table>
<thead>
<tr>
<th>Experiment</th>
<th># of HPP cycles</th>
<th>9% CLG solution used?</th>
<th>Pressures applied (MPa)</th>
<th>Purpose of experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>1</td>
<td>Yes</td>
<td>100, 200, 300, 400, 500</td>
<td>Concomitant effect of pressure and permeabilization</td>
</tr>
<tr>
<td>Case 2</td>
<td>2</td>
<td>Cycle 1 (HPP) – No</td>
<td>100, 200, 300, 400, 500 (both cycles)</td>
<td>Effect of pressure only, by permeabilizing cells before HPP infusion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cycle 2 (HPP) – Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 3</td>
<td>1</td>
<td>Cycle 1 (HPP) – No</td>
<td>HPP – 100, 200, 300, 400, 500</td>
<td>Effect of permeabilization only, no effect of high pressure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cycle 2 (osmosis) – Yes</td>
<td>Osmosis – 0.1</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1 Summary of experimental design for developing mathematical model for HPP assisted calcium infusion in different food matrices.

4.5. Materials and methods

Bolthouse® Farms baby carrots, Dandy® celery sticks, and Mexican mangoes (variety #4959) were purchased from a local supermarket 24 h prior to processing. Visually damaged and abnormally small or large samples were discarded. Celery sticks were cut into pieces of 50 mm length and mangoes were
peeled and cut into 50 mm x 15 mm x 15 mm blocks (cuboids). Store bought baby carrots were not further cut into smaller pieces but were graded by size to ensure that the pieces were approximately 50 mm long and 15 mm to 20 mm in diameter. These samples were stored in refrigerated conditions overnight until further processing.

Calcium Lactate Gluconate (CLG, Jungbunzlauer Suisse Ag, Switzerland) in anhydrous form, containing 13% calcium and solubility of 20% (w/w) in water, was used as a source of calcium for the infusion experiments. Hematoxylin and Eosin (H&E) stain was used for staining the cells of the fruit and vegetable samples. Nikon® TE200-S (NY, USA) widefield microscope was used for visualizing the cells. Image analysis was performed using MATLAB® and ImageJ®. Varian Vista-Pro (IL, USA) CCD Simultaneous ICP-OES (Inductively Coupled Plasma–Optical Emission Spectroscopy) was used for analyzing the amount of infused calcium. Cell permeabilization was measured using Bode 100 Vector network Analyzer (Omnichron Electronics Corp., Houston, TX, USA).

**High pressure assisted infusion**

Infusion experiments were performed in triplicates at 5 pressures levels (100 MPa, 200 MPa, 300 MPa, 400 MPa, and 500 MPa) using 9% CLG (w/w) solution. In the previous study (Chapter 2; Gosavi et al. 2019), the concentration of CLG was varied from 3% to 9% (w/w) and it was observed that at 9% CLG the calcium
solution concentration dominated other factors of pressure and time. In order to ensure that maximum infusion occurred, 9% CLG concentration was used for these experiments. The hold time for all pressures was kept constant at 15 mins based on our previous findings in which the highest amount of calcium infusion occurred at 15 min hold period. For each experiment, 30 g of sample (baby carrots or cut celery or peeled and cut mango) and 60 g of 6% (w/w) CLG solution were vacuum packed together in flexible polyethylene pouches. Vacuum packed samples (sealed under 93% vacuum, i.e., 69 mBar) were subjected to HPP at five different pressure levels – 100 MPa, 200 MPa, 300 MPa, 400 MPa, and 500 MPa. Each experiment was performed three times, and each run had three samples. High pressure unit with a working capacity of 10 L at Rutgers University was used for these experiments. Details of the unit have been discussed in section 2.2.

**Analysis of samples**

Samples were analyzed for the amount of calcium infused after each set of experiments according to the methodology described in section 2.2. Calcium content was quantified as mg calcium/100 g initial fruit or vegetable. Cell permeabilization index ($Z_P$) of samples after each set of experiments was analyzed using electrical impedance spectroscopy (described in section 3.3). Frequency sweep measurements were performed by passing an AC electrical current through the samples sandwiched between two electrodes. The admittance (S/m) across the frequency range (10 Hz to 40 MHz) was recorded for
all samples and $Z_p$ was calculated using Eq. 3.1. Details of cell permeabilization measurements have been discussed in section 3.3. Measurements for microstructures of baby carrots, celery, and mango were performed using H&E staining and analyzed using ImageJ and MATLAB. Details of these measurements has been extensively discussed in section 3.4. The cell diameter (CD) values calculated for baby carrots, celery, and mango in section 3.4 were used while developing the mathematical model.

*Microstructure analysis and quantification*

Microstructures of baby carrots, celery, and mango were quantified using the H&E staining method described in section 3.3. Staining of the tissues was performed at the Rutgers Research Pathology Services by Dr. Marianne Polunas.

**4.6. Results**

Figures 4.1a and 4.1b depict the amount of calcium infusion achieved in baby carrots and the corresponding levels of cell permeabilization, respectively. Figures 4.2a & 4.2b and figs. 4.3a & 4.3b show the calcium infusion and cell permeabilization observed in celery and mangoes, respectively. Different letters on the figures indicate significant statistical difference between the values. The aim of performing these experiments was to understand how cell permeabilization and the calcium infusion was controlled by cell microstructure and high pressure in conjunction.
As can be seen from Fig. 4.1a, the extent of calcium infusion obtained in cases 2 and 3 was significantly higher than that in case 1 across the pressure range. Since the difference between case 1 and the other two cases was the pre-permeabilization of the microstructure through HPP, it was evident that the change in microstructure in the first cycle led to an increased infusion of calcium in baby carrots during the second cycle of infusion. The permeabilization achieved in the first cycle, thus, had more influence on the extent of infusion in baby carrots than the concurrent permeabilization under pressure as in case 1. The cell permeabilization was comparable across all the three cases until 200 MPa (Fig. 4.1b), after which the difference between $Z_P$ values for case 1 and other two cases increased significantly. Cell permeabilization was enhanced during the second pressure cycle at 300 MPa and above. This increase in $Z_P$ during the second cycle, however, did not contribute to further increase in calcium infusion in cases 2 and 3 beyond 200 MPa (Figs. 4.1a and 4.1b). Moreover, the amount of calcium infused in case 2 was significantly higher than in case 3, despite the comparable $Z_P$ values at and beyond 200 MPa. Thus, the application of high pressure during the second cycle in case 2 along with increased cell permeabilization led to higher infusion than application of simple osmosis in case 3. These observations highlighted that the cell permeabilization does not entirely explain the increased infusion under HPP and there are more factors at play,
namely, the pressure and the cell microstructure. The microstructure and pressure, together, influence the cell permeabilization.

**Figure 4.1a** Calcium infusion in baby carrots across the pressure range in the three cases (see Table 4.1; n=9).

**Figure 4.1b** Cell permeabilization ($Z_P$) in baby carrots across the pressure range in the three cases (see Table 4.1; n=9).
Similar trends of infusion and cell permeabilization were observed in celery (Figs. 4.2a and 4.2b). Calcium infusion obtained in cases 2 and 3 was significantly higher than that obtained in case 1 at 100 MPa and 300 MPa, underlining the effect of change in microstructure in cycle one in increasing the infusion of calcium during the second cycle of infusion. It is important to note that the extent of calcium infusion observed in celery was significantly lower than that in baby carrots, further highlighting the influence of microstructure. This effect of microstructure was also observed when calcium infusion values in celery and baby carrots were compared at 400 MPa and 500 MPa. Unlike in baby carrots, the extent of calcium infusion in celery at 400 MPa and 500 MPa was comparable across the three cases. This can be explained by the presence transport tubes in both baby carrots and celery. While baby carrots have transport tubes present only at the center (Fig. 3), celery has transport present in multiple pockets around the outer edge (Fig. 4). At higher levels of pressures, more calcium could be infused through the large number of transport tubes in celery in a single pressure cycle (case 1). It is still important to note, that infusion in celery was consistently lower than in baby carrots due to differences in cell diameter values. The $Z_p$ values in celery, however, are comparable across the three cases as well as across the pressure range, unlike baby carrots. However, these observations also have a higher standard deviation and therefore, were not used to make conclusive
statements. During the cell permeabilization measurements, excessive water leakage was observed due to the samples being pressed between the two electrodes, which may have led to a higher variation in the data. It is important to note though, that the ZP values in celery are significantly lower than those in baby carrots, indicating the permeabilization obtained in celery at similar pressure levels was lower than obtained in baby carrots. This once more emphasizes the role of microstructure in conjunction with pressure in determining permeabilization and calcium infusion.

**Figure 4.2a** Calcium infusion in celery across the pressure range in the three cases (see Table 4.1; n=9).
Figure 4.2b Cell permeabilization ($Z_P$) in celery across the pressure range in the three cases (see Table 4.1; $n=9$).

The trends observed in mango for calcium infusion and cell permeabilization were different than those observed for baby carrots and celery. The extent of calcium infusion obtained in case 2 was higher than that in case 1 across the pressure range (Fig. 4.3a), emphasizing the significant effect of change in microstructure in the first pressure cycle on determining the level of calcium infusion under pressure. However, unlike as observed in baby carrots, the level of calcium infusion in mango for cases 1 and 3 was comparable until 300 MPa, after which the infusion in case 1 was higher than that in case 3. This indicated that at higher pressures (400 MPa and 500 MPa), the change in microstructure in cycle 1 did not play as a significant role as it did at lower pressures. Thus, in mangoes,
the application of pressure had more effect on determining infusion. The cell permeabilization obtained in mangoes across the three cases was similar until 300 MPa, but the calcium infusion obtained was different. Similarly, at 400 MPa and 500 MPa, the $Z_P$ obtained in case 1 was significantly lower than in the other two cases, yet the calcium infusion in case 1 was higher than that in case 3. As a result, in mangoes, the application of pressure was seen to have more influence than the cell permeabilization. As previously indicated, the trend observed in mangoes was different than that observed in baby carrots and celery. Unlike baby carrots and celery, mangoes do not have transport tubes and the infusion therefore takes from outside to inside. This difference in microstructure not only led to a different trend in calcium infusion but also higher levels of infusion. This signifies that the microstructure of a food system being infused was the primary parameter determining the extent on infusion. The process of infusion under high pressure is a complex process driven by the microstructure of the food material being infused (cell diameter and the transport tubes) and the processing pressure.
**Figure 4.3a** Calcium infusion in mango across the pressure range in the three cases (see Table 4.1; n=9).

**Figure 4.3b** Cell permeabilization ($Z_P$) in mangoes across the pressure range in the three cases (see Table 4.1; n=9).
As controls for the high pressure infusion experiments, baby carrots, celery, and mango were osmotically infused with calcium using 9% CLG solution over a period of 24 h (Fig. 4.4). Similar to the observations in infusion using HPP, the calcium infusion was highest for mango and increased throughout the 24 h period. The difference between infused calcium between mango and the other two food systems increased with longer times of infusion. This showed that absence of transport tubes in mango led to more infusion overall. Infusion increased in baby carrots and celery throughout the time period, but to a lesser extent that in mango. The lines on Fig. 4.4 indicate infusion obtained under HPP (500 MPa) after 15 min of processing. The amount of calcium infused in mango under HPP after 15 min was higher than that infused by osmosis even after 12 h. Similarly, for celery, 24 h of osmotic infusion was needed to reach the same level of calcium infusion obtained after 15 min of HPP processing; and for baby carrots, 6 h of osmotic infusion infused the same amount as that infused in 15 min under HPP. Enhanced and accelerated infusion is thus an important aspect of HPP (as observed by Mahadevan et al. 2016 too), but it is also important to note the changes in osmotic infusion due to the fruit and vegetable microstructure and due to the presence of transport tubes.
**Figure 4.4** Infusion of calcium in baby carrots, celery, and mango via osmosis, using 9% CLG solution, observed over a period of 24 h (n=9). The dashed lines indicate amount of calcium infused under HPP within 15 min of processing.

### 4.7. Principal component analysis

To better understand any underlying trends and correlations in the obtained data, Principal Component Analysis (PCA) was performed. PCA is a statistical technique typically used in machine learning where datasets with a large number of inter-correlated variables are used (Tharwat 2016). PCA is used in such cases to reduce the dimensionality of the dataset such that the new dimensions (principal components), while being uncorrelated, explain maximum variance in the data thus preserving as much information in the original data as possible (Holland...
Principal components are new variables that are constructed such that they are linear combinations of the original variables. The original data when plotted in the PCA space which has each PC as the axis, can show important data trends and clusters that can be helpful for further data analysis. The individual PCs are orthogonal to each other in the PCA space and are calculated by solving the covariance matrix (Tharwat 2016). It is important to note that PCA is more of a descriptive technique, meant for easy and simple data visualization, than an inferential one (Jolliffe & Cadima 2016).

Data standardization is crucial to PCA since it ensures that all the initial variables are within the same range and no single variable is dominating the analysis. Most commonly, data standardization is performed by either zero-centering or normalizing the data. Zero-centering the data involves subtracting the mean of individual variables from the respective variables \((x_i - \bar{x}, y_i - \bar{y})\) so that the data is now clustered around the origin for every variable. The mean-subtracted data can be further divided by the standard deviations in each variable such that the standard deviation of the zero-centered data is 1 for all variables. Normalization, on the other hand, simply ensures that the range of the data in each variable is \([-1,1]\). Figure 4.5 below, adapted from Stanford University’s course materials (CS231n: Convolutional Neural Networks for Visual Recognition), represents the differences between the two standardization techniques. For PCA
analysis of the data obtained from calcium infusion in this study, zero-centered
data scaled to a standard deviation of 1 was used.

**Figure 4.5** (Left) Original data. (Center) Zero-centered data obtained by mean
subtraction. (Right) Normalized data obtained by additional scaling of the
standard deviation of zero-centered data. The red lines indicate the range of the
data. (Figure adapted from Stanford University’s course materials (CS231n:
Convolutional Neural Networks for Visual Recognition))

For a dataset with $n$ dimensions, $n$ principal components can be calculated.
Each principal component is a linear equation in the original variables. The first
principal component calculated contains the most information i.e., explains the
majority of the variance in the data; the second PC explains the majority of the
remaining variance; and so on and so forth. Given an original dataset as shown in
Fig. 4.6, the first PC is the solid line on the graph that stretches across the
dimension with maximum scatter in data. Second PC is the dotted line that is
orthogonal to PC-1 and explains maximum scatter of data in that direction. PCs equal to the number of variables are calculated in this way until all the variance in the original data has been explained. However, the first PC is the most important since it contains the most information, followed by the second, and so on. Figure 4.7, adapted from Jaadi (2020) and Holland (2008), shows how the amount of variance explained decreases with each principal component. Discarding the PCs with low information i.e., explaining low variance, and considering only the remaining PCs, the data can now be projected in the PCA space with each PC as an axis.

**Figure 4.6** Representation of principal components (PC1 and PC2) with toy data. First PC accounts for the maximum variance in the data. Second PC accounts for maximum of the remaining unexplained variance, while being orthogonal to the first PC.
Figure 4.7 Variance in the original data explained by each principal component (PC). The first PC explains the most variance, and the percentage of variance explained decreases with each principal component. (Figure adapted from Jaadi 2020 and Holland 2008)

Principal component analysis was used in this study to understand any underlying trends in the data by projecting the data onto the PCA space and observing for data clustering. Although the infusion data obtained in this study is not large enough to demand the use of PCA, the analysis was performed primarily to identify trends. The inbuilt pca function in MATLAB was used to obtain four principal components for the infusion data.
Principal component 1 (PC-1) explained 40.8% of the variance in the data, principal component 2 (PC-2) explained 31.6%, principal component 3 (PC-3) explained 22.8%, and principal component 4 (PC-4) explained 4.9% of the variance. Each PC, as discussed earlier, was a linear combination of the individual variables in the original data. Corresponding linear coefficients for each of the PCs have been shown in Table 4.2.

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>PC-1</th>
<th>PC-2</th>
<th>PC-3</th>
<th>PC-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell diameter (CD)</td>
<td>-0.1618</td>
<td>0.8397</td>
<td>-0.1393</td>
<td>-0.4992</td>
</tr>
<tr>
<td>Pressure (P)</td>
<td>0.3323</td>
<td>0.1885</td>
<td>0.9229</td>
<td>0.0482</td>
</tr>
<tr>
<td>Z\textsubscript{P}</td>
<td>0.6911</td>
<td>-0.2776</td>
<td>-0.2250</td>
<td>0.6282</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
<td>0.6211</td>
<td>0.4269</td>
<td>-0.2797</td>
<td>-0.5948</td>
</tr>
</tbody>
</table>

Table 4.2 Linear coefficients corresponding to each principal component.

Based on the coefficients, each principal component can be written as:

PC-1 = -0.1618*CD + 0.3323*P + 0.6911*Z\textsubscript{P} + 0.6211*Ca

PC-2 = 0.8397*CD + 0.1885*P - 0.2776*Z\textsubscript{P} + 0.4269*Ca

PC-3 = -0.1393*CD + 0.9229*P - 0.2250*Z\textsubscript{P} - 0.2797*Ca

PC-4 = -0.4992*CD + 0.0482*P + 0.6282*Z\textsubscript{P} - 0.5948*Ca

Since the first two principal components described >70% of the variance, data was plotted in the PC-1 – PC-2 coordinate space (Fig. 4.8). Figure 4.8 showed
three distinct data clusters, as highlighted. Each data cluster was specific to the food system used for infusion i.e., baby carrots, celery, and mango. The orange cluster corresponds to baby carrots, the green cluster corresponds to celery, while the yellow cluster corresponds to mangoes. The observations for baby carrots were clustered in the fourth quadrant while those for celery were in the third quadrant. Little to no overlap between the baby carrots and celery data indicated that the differences in their microstructure significantly influence the infusion of calcium.

![Figure 4.8](image)

**Figure 4.8** Data distribution across the first two principal components. Orange markers indicate data for baby carrots, green markers for celery, and yellow markers for mango.
4.8. **Response surface analysis**

Unlike PCA, which is a data visualization and descriptive technique, response surface analysis (RSA) can provide more quantitative insights into the data that can be inferential. Regression analysis was performed on calcium infusion data obtained from the three cases. The aim was to find how calcium infusion was affected by pressure, microstructure (defined as a cell diameter (CD) here), and cell permeabilization ($Z_p$). Since cell permeabilization itself was affected by the pressure and the microstructure, regression analysis was also performed to find how $Z_p$ changed with pressure and microstructure. Regression analysis was performed in MATLAB® using the `regstats` function for regression diagnostics ($\alpha = 0.05$) to obtain a quadratic function that best fit the data. All analysis was performed on normalized data. Equation 2.4 in Chapter 2 shows a generic form of quadratic equation obtained when performing RSA on a dataset with 3 input variables and one output variable. More information on RSA can be found in Chapter 2 which discussed the methodology of RSA in significant detail.

Tables 4.3, 4.5, and 4.7 show the coefficients obtained for each parameter as well as parameter interactions for calcium infusion in each of the cases, while Tables 4.4, 4.6, and 4.8 show the coefficients for cell permeabilization for the three cases, respectively. Based on the significance of the coefficients, contours were generated for each case to depict the effect of individual parameters and
their interactions on predicting the calcium infusion under high pressure (Figs. 4.9, 4.11, and 4.13) and the cell permeabilization (Figs. 4.10 and 4.12).

As seen from Table 4.3, pressure, microstructure, and cell permeabilization significantly influenced the infusion of calcium in case 1, where permeabilization and calcium infusion occurred simultaneously. However, as denoted by the negative value of the quadratic Z_p term, simultaneous permeabilization during infusion in case 1 was deterrent to infusion. At a constant value of cell permeabilization, increasing pressure and cell diameter led to increased infusion (Fig. 4.9). Although, the permeabilization in case 1 was significantly affected by both pressure and microstructure (Table 4.4, Fig. 4.10). At lower pressures, increase in cell diameter from baby carrots to celery led to reduced cell permeabilization, but it increased with further increase in cell diameter (celery to mango). This trend in permeabilization was observed with increasing pressures but was less evident. As pointed out earlier, it is important to note that cell diameter is one representation of cell microstructure but may not accurately represent the presence of transport tubes in baby carrots and celery. As a result, this predicted trend in cell permeabilization affected by pressure and microstructure, may be a result of the transport tubes present in baby carrots and celery. However, it is difficult to justify a physical explanation of why the infusion of calcium and the effect of transport tubes would follow a quadratic form. The
analysis from RSA is, therefore, best to be used to identify dominating parameters that affect the infusion rather than make conclusive statements about the physical nature of calcium infusion under high pressure.

\[ \text{Ca} = f(P, \text{CD}, Z_p) \]

<table>
<thead>
<tr>
<th>CASE 1</th>
<th>K</th>
<th>P</th>
<th>CD</th>
<th>Z_P</th>
<th>P*CD</th>
<th>P*Z_P</th>
<th>CD*Z_P</th>
<th>P^2</th>
<th>CD^2</th>
<th>Z_P^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>0.69</td>
<td>0.28^a</td>
<td>0.77^a</td>
<td>0.55^a</td>
<td>0.42^a</td>
<td>0.25</td>
<td>-0.45</td>
<td>-0.18</td>
<td>-0.32</td>
<td>-0.50^a</td>
</tr>
</tbody>
</table>

R^2 = 0.67

**Table 4.3** Regression coefficients for pressure, CD, and Z_P for calcium infusion in case 1. Coefficients in red indicate statistical significance (p<0.05).
Figure 4.9 Contour plot visualizing the changes in calcium infusion with cell diameter (CD) and pressure in case 1, based on regression analysis.

\[ Z_P = f(P, CD) \]

<table>
<thead>
<tr>
<th>CASE 1</th>
<th>k</th>
<th>P</th>
<th>CD</th>
<th>P* CD</th>
<th>P^2</th>
<th>CD^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z_P</td>
<td>-0.08</td>
<td>0</td>
<td>-0.30^a</td>
<td>-0.06^a</td>
<td>-0.03</td>
<td>0.28^a</td>
</tr>
</tbody>
</table>

\[ R^2 = 0.65 \]

Table 4.4 Regression coefficients for pressure and CD for cell permeabilization in case 1. Coefficients in red indicate statistical significance (p<0.05).
Figure 4.10 Contour plot visualizing the change in cell permeabilization ($Z_p$) with cell diameter (CD) and pressure in case 1, based on regression analysis.

Unlike case 1, the pre-permeabilization of microstructure during the first HPP cycle in case 2, significantly influenced the infusion in cycle 2. The microstructure and cell permeabilization played a significant role, while pressure in conjunction with CD and $Z_p$ was significant (Table 4.5). Thus, highlighting that the effect of pressure alone was not significant. At a constant cell permeabilization, the infusion of calcium was primarily governed by the cell diameter than the applied pressure (Fig. 4.11). The cell permeabilization too was only affected by the microstructure, with no significant effect of applied pressure.
Thus, the cell permeabilization as well as the calcium infusion in the second cycle were significantly affected by the food microstructure.

Since no pressure was applied during the osmotic infusion in second cycle in case 3, the calcium infusion was modeled based on effect of microstructure and cell permeabilization only. As expected, based on previous observations, the infusion was significantly governed by the food microstructure (Table 4.7 and Fig. 4.13).

\[
Ca = f(P, CD, Z_P)
\]

<table>
<thead>
<tr>
<th>CASE 2</th>
<th>k</th>
<th>P</th>
<th>CD</th>
<th>ZP</th>
<th>P* CD</th>
<th>P*ZP</th>
<th>CD*ZP</th>
<th>P^2</th>
<th>CD^2</th>
<th>ZP^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>-0.08</td>
<td>-0.05</td>
<td>0.15</td>
<td>(0.71^a)</td>
<td>0.09</td>
<td>0.13</td>
<td>-0.45^a</td>
<td>-0.09</td>
<td>0.11</td>
<td>-0.1</td>
</tr>
</tbody>
</table>

\[R^2 = 0.92\]

**Table 4.5** Regression coefficients for pressure, CD, and \(Z_P\) for calcium infusion in case 2. Coefficients in red indicate statistical significance (p<0.05).
Figure 4.11 Contour plot visualizing the changes in calcium infusion with cell diameter (CD) and pressure in case 2, based on regression analysis.

\[ Z_P = f(P, CD) \]

<table>
<thead>
<tr>
<th>CASE 2</th>
<th>k</th>
<th>P</th>
<th>CD</th>
<th>P*CD</th>
<th>P^2</th>
<th>CD^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZP</td>
<td>0.29^a</td>
<td>0.06</td>
<td>-0.23^a</td>
<td>-0.01</td>
<td>-0.08</td>
<td>0.27^a</td>
</tr>
</tbody>
</table>

R^2 = 0.49

Table 4.6 Regression coefficients for pressure and CD for cell permeabilization in case 2. Coefficients in red indicate statistical significance (p<0.05).
**Figure 4.12** Contour plot visualizing the change in cell permeabilization ($Z_P$) with cell diameter (CD) and pressure in case 2, based on regression analysis.

\[ Z_P = f(P, CD) \]

**Table 4.7** Regression coefficients for CD and $Z_P$ for calcium infusion in case 3.

Coefficients in red indicate statistical significance ($p<0.05$).
Figure 4.13 Contour plot visualizing the change in calcium infusion with cell diameter (CD) and pressure in case 3, based on regression analysis.

4.9. Proposed mathematical model

Until now, our understanding has been that the effect of cell microstructure on driving infusion was due to cell permeabilization. However, based on the insights from PCA and RSM, it is evident that cell permeabilization alone (even as an effect of cell microstructure) is not enough to explain the variation in calcium infusion under high pressure. A major consideration in the cell microstructure of these specific food systems (baby carrots, celery, and mango) that has been ignored up until now has been the effect of presence transport tubes in infusion.
The images in Chapter 3 clearly show the presence of transport tubes in baby carrots (Fig. 3.3a) and celery (Fig. 3.4a), and their absence in mango (Fig. 3.5). The infusion in baby carrots and celery has also been observed to be consistently lower than that in mango, indicating that the presence of transport tubes affects infusion. Our hypothesis is that the movement of calcium molecules that can happen from the outside edges of mango to the inside, is slowed in baby carrots and celery. Instead, the calcium solution is taken up by the transport tubes in these foods and further distributed into the cells from the transport tubes to the cells surrounding them and further into the tissue (Fig. 4.14). The cells around transport tubes are significantly smaller than the cells in the rest of the tissue (Figs. 3.3 and 3.4) which may affect the travel of calcium through the tissue. A calcium molecule that moves from the transport tubes to nearing cells thus encounters a higher number of cell walls, which pose a resistance to mass transport. It is therefore important to consider the effect of these transport tubes and incorporate it into building a mathematical model to describe the governing factors that drive of calcium infusion under high pressure.
Figure 4.14 (Left) Longitudinal cross section of a carrot with transport tubes at the center (image adapted from Adams et al. 2014) and (Right) the corresponding direction of calcium infusion throughout the cross section. Calcium molecule is primarily transported through the transport tubes (vertical direction) into neighboring cells (horizontal direction) that pose a barrier to calcium movement. Thicker lines depict higher resistance to calcium movement.

Since pressure and cell microstructure have been shown to have independent as well as interactive effects in driving infusion (case 1, Table 4.3 Fig. 4.9), we propose a multiplicative model with pressure and microstructure terms such that:

$$I_{ca} \propto f(P) \times g(Me_{eff})$$

...Eq. 4.7
where, $I_{Ca}$ is the estimated amount of calcium infused, and $f(P)$ and $g(MC_{eff})$ are functions defined in pressure (P, MPa) and effective microstructure ($MC_{eff}$, µm), respectively. Effective microstructure is a term developed to incorporate the effect of transport tubes on the cell microstructure. We define $MC_{eff}$ as:

$$MC_{eff} = CD - \gamma \times f_{tt}$$

...Eq. 4.8

where, $CD$ is the cell diameter (µm) for each food system as calculated in Chapter 3 (Table 3.4), $f_{tt}$ is a term for ‘factor of transport tubes, and $\gamma$ (µm) is a weighted constant. From our previous findings (Chapter 3 and Gosavi et al. 2019), increasing cell diameter led to an increase in infused amount of calcium. However, the presence of transport tubes in baby carrots and celery has been observed to reduce the extent of calcium infusion, since their presence alters the pathway of infusion and forces the calcium to go through the transport tubes (orthogonal to the normal nutrient flow direction) and nearby cells before reaching the rest of the cells in the tissue. In order to incorporate this effect, the effective microstructure term ($MC_{eff}$) denotes a positive effect of increasing cell diameter which is dampened to some extent by the transport tubes ($f_{tt}$).

The factor of transport tubes, $f_{tt}$, is calculated based on the ratio of cell wall thickness (CWT) to cell diameter (CD) at different locations in a food system. For baby carrots, values of $\frac{CWT}{CD}$ ratio were calculated at five different regions in the
carrot tissues (regions shown in Figs. 3.3a – 3.3e), while for celery, the ratios were calculated at three different regions (Figs. 3.4a – 3.4c). For mango, the ratio was calculated across the entire tissue. Table 4.8 and Fig. 4.15 show the variation in $\frac{\text{CWT}}{\text{CD}}$ across different regions in the three food systems. Values of have been plotted against normalized radial distance ($r_n$) from the site of infusion. For baby carrots and celery, the site of infusion is the transport tubes. Distance from the transport tubes to the edge of the tissue farthest from the tubes has been scaled to 1, so that $r_n = 0$ at the transport tubes and $r_n = 1$ at the farthest edge.

<table>
<thead>
<tr>
<th>Normalized radial distance ($r_n$) from site of infusion/transport tubes</th>
<th>Baby carrots (Fig. 3.4)</th>
<th>Celery (Fig. 3.5)</th>
<th>Mango (Fig. 3.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (center)</td>
<td>0.34</td>
<td>0.70</td>
<td>0.16</td>
</tr>
<tr>
<td>0.25 (around center)</td>
<td>0.32</td>
<td>0.88</td>
<td>0.16</td>
</tr>
<tr>
<td>0.50 (periphery of center)</td>
<td>0.47</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>0.75 (inner cells)</td>
<td>0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00 (edge)</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.8 Variation of $\frac{\text{CWT}}{\text{CD}}$ across different regions in baby carrots, celery, and mango. Higher value of $\frac{\text{CWT}}{\text{CD}}$ indicates higher resistance to infusion.
Figure 4.15 Variation of $\frac{CWT}{CD}$ in baby carrots, celery, and mango with increasing distance from the site of infusion. Higher $\frac{CWT}{CD}$ indicates higher resistance to infusion.

It was evident that $\frac{CWT}{CD}$ did not change throughout the mango tissue (depicted by a horizontal line across the radial distance), while for baby carrots and celery, the change in $\frac{CWT}{CD}$ is significant across the tissue. Higher value of $\frac{CWT}{CD}$ indicates higher resistance to mass transfer, since it implies that the calcium molecule must travel through more region of the cell wall which poses a higher
resistance than through the cell cytoplasm within the cell that does not restrict the calcium movement. Thus, in celery, the infusing calcium molecule has to travel through significantly thicker cell walls near the transport tubes which would lead to slower infusion. Baby carrots also showed the same trend of high \( \frac{CWT}{CD} \) near the transport tubes, however, this ratio was lower in baby carrots than that in celery. The variation in \( \frac{CWT}{CD} \) was also significantly higher in celery than in baby carrots, while the ratio did not vary at all in mango since it does not have transport tubes. To account for this variation in \( \frac{CWT}{CD} \), \( f_{tt} \) was calculated as the difference between the highest and lowest \( \frac{CWT}{CD} \) values in each food system. Thus, higher the \( f_{tt} \) value, more the difference between the \( \frac{CWT}{CD} \) across the tissue indicating that a greater number of cells are affected by the presence of transport tubes, and hence, more the effect of transport tubes. Higher \( f_{tt} \) value would consequently lead to lower \( MC_{eff} \) and lower infusion. Table 4.9 shows the \( f_{tt} \) values for baby carrots, celery, and mango.
Table 4.9 Effect of transport tubes \((f_{tt})\) in foods with different microstructures (baby carrots, celery, and mango)

<table>
<thead>
<tr>
<th></th>
<th>(f_{tt})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baby carrots</td>
<td>0.73</td>
</tr>
<tr>
<td>Celery</td>
<td>1.40</td>
</tr>
<tr>
<td>Mango</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Based on our observations in this study as well as the works of researchers in the area of high pressure assisted mass transfer (Rastogi and Niranjan 1998, Knorr et al. 2000, Rastogi et al. 2002, Sopanangkul et al. 2002, Rastogi et al. 2008, Mahadevan et al. 2015a, George et al. 2016, etc.), it is well known that the amount of infusion under high pressure follows an exponential rise with an increase in pressure but saturates at mid pressure levels and remains unchanged with any further pressure increase. Based on this knowledge, the effect of pressure was proposed to have an exponential form approaching saturation, as denoted by Eq. 4.9, where ‘\(\alpha\)’ is a constant (MPa\(^{-1}\)).

\[
f(P) = 1 - e^{-\alpha P} \quad \text{...Eq. 4.9}
\]

We had previously observed higher infusion with increasing cell diameter. Therefore, we propose the effect of microstructure on infusion as a linear function (Eq. 4.10), where \(\beta\) (\(\mu\text{m}\)^{-1}) is a constant
\[ g(MC_{eff}) = \beta \times MC_{eff} + c \quad \text{...Eq. 4.10} \]

Substituting for \( MC_{eff} \) from Eq. 4.8 and defining a new constant \( \gamma' \) such that

\[ \gamma' = \frac{\gamma}{\beta} \]

\[ g(MC_{eff}) = \beta \times CD - \gamma' \times f_{tt} + c \quad \text{...Eq. 4.11} \]

Substituting the terms for \( f(P) \) and \( g(MC_{eff}) \) in Eq. 4.7 from Eqs. 4.9 and 4.11, respectively, our proposed model has the following form:

\[ I_{Ca} = k \times (1 - e^{-\alpha P}) \times (\beta \times CD - \gamma' \times f_{tt} + c) \quad \text{...Eq. 4.12} \]

where \( k \) is the minimum amount of calcium infusion obtained under high pressure (mg Ca / 100 g). In order to reduce the number of constants, we assumed \( k = 1 \). Equation 4.12 therefore becomes:

\[ I_{Ca} = (1 - e^{-\alpha P}) \times (\beta \times CD - \gamma' \times f_{tt} + c) \quad \text{...Eq. 4.13} \]

The proposed model (Eq. 4.13) was evaluated by least squares regression using calcium infusion data from case 1, where calcium was infused in fresh baby carrots, celery, and mango in a single HPP cycle using 9% CLG solution with a hold time of 15 minutes. Values of the constants obtained through least squares regression have been listed in Table 4.10.
Table 4.10 Model constant values for Eq. 4.13 obtained after validating the model with calcium infusion data from case 1.

<table>
<thead>
<tr>
<th>Model constant</th>
<th>Value after fitting ($R^2 = 0.72$)</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>0.004</td>
<td>MPa$^{-1}$</td>
</tr>
<tr>
<td>$\beta$</td>
<td>0.76</td>
<td>$\mu$m$^{-1}$</td>
</tr>
<tr>
<td>$\gamma'$</td>
<td>88.22</td>
<td>-</td>
</tr>
<tr>
<td>$c$</td>
<td>135.54</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 4.16 shows the model fit to observed data with respect to pressure and $MC_{eff}$. Both the data and the model show an increase in infusion with increase in effective microstructure. This effect is more pronounced at higher pressures, at which the pressure and microstructure have a synergistic effect. The effective microstructure term indicates the resistance to movement of calcium molecules within a tissue of a given food system. Higher the term, lesser the resistance which would lead to higher amounts of calcium infused and vice versa. Since effective microstructure is a combination of cell size and the factor of transport tubes, higher values may indicate that either the cells are large or that the transport tubes have little to no role to play, which would be the case for fruits like mango which do not have transport tubes. Similarly, lower values indicate a significant role of transport tubes that pose higher resistance to mass transfer due to comparatively thicker cell walls and smaller cells. A strong physical
explanation of the process of infusion using the proposed mathematical model along with a reasonable $R^2$ value of 0.72, indicates that this model is a step in the right direction to improve our understanding of the process, although it is not the final step.

![Graph](image)

**Figure 4.16** Proposed model fit to observed data for calcium infusion in case 1, shown with respect to pressure (MPa) and effective microstructure ($MC_{eff}$).

To highlight how the transport tubes affect infusion, the proposed model and observed data were plot against $f_{tt}$ and pressure (Fig. 4.17a). As can be seen from the figure, the infused amount decreases with an increase in $f_{tt}$. Higher values of $f_{tt}$ indicate a larger role of transport tubes which pose a higher
resistance to mass transfer due to increased cell wall thickness and smaller cell diameters. Mango, which does not have transport tubes and hence a low value of \( f_{tt} \), has the higher infusion under high pressure. With a higher \( f_{tt} \), the infusion is affected more by the food microstructure at lower pressure, but the effect of increasing pressure is more evident in food systems with no transport tubes i.e., low \( f_{tt} \) values, which was observed in mango.

**Figure 4.17a** Proposed model fit to observed data for calcium infusion in case 1, shown with respect to factor of pressure (MPa) and factor of transport tubes (\( f_{tt} \))
To show the effect of CD only, the proposed model and observed data were plot against CD and pressure (Fig. 4.12b). The sudden drop in infusion value observed at lower levels of CD is a partial picture of the process of infusion. When Fig. 4.12a is combined with Fig. 4.12b, a complete picture of the proposed mathematical model and high pressure infusion can be obtained. If Fig. 4.11 were broken down into two parts – one showing the effect of transport tubes only and the other the effect of cell diameter (CD) only, Figs. 4.12a and 4.12b would represent those two parts, respectively.

Figure 4.17b Proposed model fit to observed data for calcium infusion in case 1, shown with respect to pressure (MPa) and cell diameter (µm)
If the proposed model is fit to the calcium infusion data obtained from case 2, where calcium infusion occurred in pre-permeabilized cells (i.e., altered microstructure), the value of $\alpha$ increases indicating higher effect of pressure, and the values of $\beta'$ and $\gamma''$ decrease indicating lesser effect of microstructure, and that of $c$ increases showing that it proportionately increases the effect of pressure. This explains the higher calcium infusion obtained in case 2.

Our model, given by eq. 4.13, thus explained the data obtained during calcium infusion of baby carrots, celery, and mango under high pressure. Proposed model tries to take into account not only the effect of pressure but also that of food microstructure on infusion under high pressure. A novel aspect of the model is the incorporation of a microstructure term that explains the role of transport tubes in driving infusion along with the cell sizes.

4.10. Advantages and drawbacks of the proposed model

Proposed model is the first attempt of its kind to have been developed for high pressure assisted infusion in food materials that explains the role of food microstructure on infusion along with that of applied pressure. This model is the first step in understanding the mechanism of infusion under high pressure beyond simple cell permeabilization. As has been discussed and shown extensively in this chapter, cell permeabilization alone does not account for the increased infusion under high pressure. While it explains the total
permeabilization in the sample (at macro level), it does not capture specific cell
breakage or the difficulty in breaking cells in certain regions in the tissue (at
micro level). The proposal of a new term – effective microstructure, is an
advancement in that direction since it accounts for both micro and macro level
cell properties. Using this model to further develop and validate nutrient infusion
in a variety of food systems under high pressure will lead to the first ever
mathematical explanation of high pressure assisted infusion, which can be used
by researchers and industry alike to further explore this technique to develop
nutritionally enhanced commonly eaten fruit and vegetable snacks.

A major drawback of the proposed model is that it has been modeled
specifically for the infusion of calcium using only calcium lactate gluconate
solution at a fixed concentration of 9% w/w. As a result, there is no term for the
effect of infusate concentration or molecular size of the infusant/infusate in the
model or the effect of choice of calcium infusate. The current format of the model
incorporates a linear effect of the effective microstructure. However, without
further validation with a larger dataset involving high pressure assisted calcium
infusion into a variety of fruits and vegetables, it is difficult to rule out higher
order effects of effective microstructure and pressure. The model can further be
improved by incorporating the effect of infusing molecule, so as to make it
applicable to high pressure infusion of a range of nutrient molecules.
4.11. Key takeaways for this study

- Calcium infusion under high pressure was performed in different ways with an aim to decouple the effect of pressure and cell permeabilization. However, cell permeabilization was observed to be influenced by pressure itself in conjunction with the cell microstructure.

- Principal component analysis and response surface methodology were performed to identify trends in calcium infusion under high pressure.

- Food microstructure, defined here as cell diameter (CD), was the primary factor in determining the extent of calcium infusion under high pressure.

- Application of pressure led to increase in infusion until mid-pressure levels, after which the infusion remained unaffected by pressure.

- Cell diameter and pressure alone were not enough to describe high pressure assisted infusion of calcium in baby carrots, celery, and mango.

- Cell microstructure is a complex parameter to quantify due to the presence of transport tubes in baby carrots and celery, but their absence in fruits like mango.

- To improve on the mathematical definition of food microstructure, a new term was developed – effective microstructure – which incorporated the effect of cell sizes through CD as well the effect of presence of transport tubes in each food system.
- A novel method was developed to quantify the effect of transport tubes by calculating the deviation in the ratio of CWT/CD across a tissue.

- Based on the insights gained through these experiments, a mathematical model was proposed describing the effect of pressure and effective microstructure on calcium infusion under high pressure.

- Proposed mathematical model was fit to the obtained data using least squares regression with an $R^2$ of 0.72.

- The mathematical model proposed here is the first model for high pressure assisted infusion that describes the effect of food microstructure on infusion.
CHAPTER 5

CONCLUSIONS

High pressure processing (HPP) has been widely explored and commercialized as well to improve food safety and quality through inactivation of spoilage microorganisms and deteriorative enzyme. This non-thermal technique, however, has potential to be explored beyond food safety and quality, to nutritionally enhance food materials through infusion of nutrient molecules. Although research exists that has been aimed at evaluating the application of HPP to enhance and accelerate mass transfer of molecules in food matrices, the mechanics behind the process and the factors driving infusion under pressure are not well understood. Presented research was aimed at addressing these gaps in the existing literature.

HPP was first evaluated to understand if significant amounts of any nutrient (>10% daily RDI of that nutrient) could be infused in any food system. For this purpose, high pressure infusion was performed on baby carrots using calcium lactate gluconate solution to infuse calcium into the baby carrot matrix. Calcium infusion of 100 mg to 134 mg, equivalent to 10% to 13% of the RDI for calcium was achieved at moderate pressure (350 MPa) with a 15 min process hold time. Subsequently, calcium infusion under high pressure was performed in baby carrots, celery, and mango, to evaluate how the extent of calcium infusion varied
in different food matrices. Microstructure analysis was performed to quantify the differences between these fruits and vegetables in terms of cell diameter (CD, \( \mu m \)). Additionally, fluorescence microscopy was used to detect the location of infused calcium within the tissue. These microscopy techniques are unique aspects of this study, since they have not been explored in the context of high pressure assisted infusion. Increase calcium infusion was observed with increasing cell diameter of the fruit / vegetable. The cell permeabilization value (ZP) indicated higher cell breakage in infused samples which enhanced the infusion along with the application of high pressure.

To better understand the factors that drive calcium infusion in these food materials under high pressure, a mathematical model was developed. Food microstructure, as defined earlier in terms of CD, did not explain the data observed. It was noted that the food microstructure of these fruits and vegetables do not only have different cell sizes, but also have different biological structure. Baby carrots and celery display transport tubes that function as the primary mechanism for mineral uptake, while these tubes are absent in mango. The presence of these transport tubes significantly affected the calcium infusion. Therefore, a newer term to explain their role was developed. Food microstructure was redefined in terms of cell diameter and a factor of transport tubes that better explained the data. A mathematical model which had multiplicative terms in
pressure and effective microstructure was developed, that was validated by the observed data with an $R^2$ of 0.72. This mathematical model is the first developed in this research area to explain the governing factors during high pressure infusion. This model will serve as the first step towards understanding the process of infusion under high pressure in more detail and can be evaluated and used by researchers to further validate the model and their data.
CHAPTER 6

FUTURE WORK

To continue the exploration and understanding of high pressure assisted infusion, more studies need to be performed to explore infusion of different micronutrients in different fruit and vegetable systems. This can be done by replacing the calcium in this research with another micronutrient of interest and performing high pressure infusion experiments in the same food matrices. This would help to understand how changing the infusing molecule would change the infusion.

Furthermore, more food matrices need to be evaluated for infusion along with more microstructure analysis to quantify their microstructure. It would be important to apply the quantification methods developed in this study to other food materials. This would also help detect the merits and the demerits of the methods and help to further develop these quantification methods. The proposed mathematical model also should be validated using multiple data sets to evaluate its robustness and modify it further, if needed. Lastly, more work is needed to understand the role of infusate concentration and infusate molecule, along with pressure and microstructure. This can be done by tagging the infusate molecule with a fluorescent dye that can be tracked in-situ during the process of infusion under HPP.


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CHAPTER 8

ACKNOWLEDGMENT OF PREVIOUS PUBLICATIONS

Five research papers/articles were published during the course of this dissertation, and one research paper is in preparation. Following is the list of publications:

