Liquid Impregnation into Plant Food Materials Promoted by High Hydrostatic Pressure

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Abstract

Liquid impregnation can enhance sensory and nutritional qualities of plant food products, and conventional liquid impregnations improve mass transfer of water and solute into plant food materials. However, impregnation processes may damage cell membrane and tissue, which deteriorate the quality of products. It is necessary to find an alternative process to promote impregnation, while minimizing quality loss. Besides, quantitative methods are necessary to evaluate impregnation efficacy, cell membrane, and tissue damages for optimization of the impregnation processes.

In this study, high hydrostatic pressure (HHP) treatment was applied to promote the liquid impregnation into plant food materials. Apple and carrot were selected as typical fruit and vegetable materials with porous and nonporous structures, respectively. The quantitative evaluations of impregnated apple and carrot have been optimized. Furthermore, the effect of HHP treatment, and combined use of vacuum heat sealing (VHS), subsequent storage, and HHP treatment on the apple and carrot impregnations was investigated to explore their feasibilities regarding sufficient impregnation and suppression of the cell membrane and tissue damages. The results can be summarized as follows.

(1). Quantitative methods for impregnated plant food materials were established. Impregnation efficacy was quantified as impregnation ratio by image analysis, color (yellow, green, blue, or red) and concentration (apple: 0-0.15 %; carrot: 0-5.0 %) of pigment solutions have been optimized for distinguishing color difference before and after impregnation. It was recommended to use 0.05 % red and 3.0 % blue pigment solutions for apple and carrot, respectively. Cell membrane damage was quantitatively evaluated as size of Cole-Cole circular arc (SCA) by electrical impedance spectroscopy (EIS). Water mobility and distribution before and after impregnation have been quantified as relaxation time and area fraction by low-field nuclear magnetic resonance (LF-NMR). Tissue damage was evaluated by texture analysis, and a plunger of 5 mm diameter was adopted to obtain reproducible data among all tested plungers. The methods established in this study were applied thereafter.

(3). Apple was impregnated with 0.05 % red pigment solution by vacuum heat sealing (VHS) and/or HHP treatment (100-600 MPa, 25 °C, 5 min). Effect of storage after VHS on the impregnation efficacy was also studied. Impregnation of apple was sufficiently achieved by a combination of VHS and 100 MPa treatment, while minimizing the cell membrane and texture damages. It was demonstrated that the impregnation via VHS was time-dependent and insufficient. On the other hand, HHP treatment achieved 100 % impregnation regardless of storage time after VHS. VHS and subsequent HHP treatments significantly damaged the cell membrane of apple. Meanwhile, the cell membrane

damages between VHS and VHS+100 MPa samples were comparable, and it was aggravated by HHP treatment at 200 MPa and higher. Probably due to water uptake after impregnation, relaxation times of semibound water (T_{22}) and free water (T_{23}) were significantly increased after VHS and VHS+100 MPa. As for the texture analysis, breaking stress (hardness) decreased comparably after VHS and VHS+HHP treatment (100-600 MPa). Meanwhile, breaking strain (deformability) significantly increased after VHS+HHP treatment (200-600 MPa), indicating further damage resulting in increased deformability as compared with that after VHS and VHS+100 MPa treatment.

(4). Carrot was impregnated with 3.0 % blue pigment solution by vacuum heat sealing (VHS) and/or HHP treatment (100-600 MPa, 25 °C, 5 min). After optimal impregnation (i.e., VHS, subsequent storage for 120 min, and HHP treatment at 100 MPa), the impregnation ratio reached 100 %, while the parameters for damages of cell membrane and tissue were comparable with those of fresh carrot. It can be demonstrated that HHP treatment significantly assisted VHS as for improving the impregnation ratio, where the impregnation ratio of VHS+S_0+100 MPa (98.6 ± 0.9 %) was drastically higher than that of VHS+S_0 (28.9 ± 6.6 %). The SCA_n of VHS+100 MPa sample was almost comparable with that of fresh control samples, and the SCA_n was significantly reduced by HHP treatments at 150-600 MPa. It was demonstrated that the cell membrane of the carrot was slightly affected by 100 MPa, while HHP treatment at 150 MPa or higher significantly damaged the cell membrane. As for the texture analysis, normalized breaking stress and strain of 100 MPa-treated samples were almost comparable with those of control samples. Further severe texture damages were observed at 200 MPa and higher.

These results suggest that a combination of VHS, storage time for 120 min, and HHP treatment can be applied to process high-quality apple and carrot for sufficient impregnation, with cell membrane and tissue damages being minimized. In addition, quantitative methods for the impregnated products quality were established and can be applied to apple and carrot, which have distinct structural features. It may be of further interest to study HHP-assisted liquid impregnation by using other fruits and vegetables for optimizing their impregnation efficacy, cell membrane damage, and texture deteriorations.

Key words: Plant food materials; Impregnation; High hydrostatic pressure; Image analysis; Electrical impedance spectroscopy

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Abbreviations

AHS	ambient heat sealing
EIS	electrical impedance spectroscopy
HHP	high hydrostatic pressure
LF-NMR	low-field nuclear magnetic resonance
SN	scan numbers
RGB	red, green, and blue
RO	reverse osmosis
S_t	storage after vacuum heat sealing for *t* min
SCA	size of circular arc in Cole-Cole plot
SCA_n	normalized SCA by the SCA of fresh apple
VHS	vacuum heat sealing

Chapter 1 Background

1.1 Liquid impregnation

With the increasing demands for high quality processed fruits and vegetables in the food industry, it is required for food processors to enhance sensory and nutritional qualities of fruit and vegetable products.

Liquid impregnation can enhance the sensory quality of plant food products (Lin *et al*., 2006) and extend their shelf lives (Dermesonlouoglou *et al*., 2017; Moraga *et al*., 2009). Liquid can be impregnated into plant food materials to produce plant food products such as compote, pickles, and canned fruits and vegetables (Mendoncla *et al*., 2001; Zhang *et al*., 2020; Hidalgo *et al*., 2019). Heating (Moreno *et al*., 2012), vacuum impregnation (Fito *et al*., 2001; Perez-Cabrera *et al*., 2011), and high hydrostatic pressure (HHP) treatments (Rastogi *et al*., 2000; Villacís *et al*., 2008; Yamamoto, 2017) have been applied to promote the liquid impregnation into plant food materials.

Among common plant food materials, apple and carrot have intensively been studied for liquid impregnation via heating (Allali *et al*., 2010; Rastogi *et al*., 1997), vacuum impregnation (Neri *et al*., 2016; Vargas *et al*., 2009), and HHP treatment (Vatankhah *et al*., 2019; Sila *et al*., 2004). Apple and carrot have longer shelf lives, and they do not show significant tissue damage during cold storage (Johnston *et al*., 2001; Alvo *et al*., 2004). Therefore, taking into account of stability for reproducible experiments, apple and carrot were adopted as experimental plant food materials in this study.

Quantitative evaluations of impregnated apple and carrot may contribute to optimizing their impregnation processes. The liquid impregnation in food processing has qualitatively been evaluated by microscopic observations (Assis *et al*., 2019) while quantitatively evaluated by impregnated amount of chemicals (Schulze *et al*., 2012) and texture analysis (Allali *et al*., 2010). However, quantitative evaluations for the impregnation efficacy and damages of plant food materials have been limitedly studied for impregnation effect of HHP treatment.

1.2 Conventional impregnation methods

1.2.1 Heating

Heating is a conventional method to enhance liquid impregnation. Raised temperature (30 to 50 ºC) increases mass transfer of water and solute (sucrose) for plant tissues such as apples, carrots, and pineapples (Allali *et al*., 2010; Rastogi *et al*., 1997;

Rastogi *et al*., 2004). Heating may promote the mass transfer by increasing the molecular motion, and overheating can further increase cell permeability duo to cell membrane lysis (Allali *et al*., 2010).

1.2.2 Vacuum heat sealing (VHS)

Vacuum heat sealing (VHS) or vacuum impregnation is recognized as an alternative method to effectively introduce liquids into porous structures in foods, while minimizing changes in the texture, nutritional quality, and color of fruits and vegetables (Santarelli *et al*., 2021). Pressure reduction in the vacuum process induces expansion of inter-/intracellular gas and removal of the gas from the apple cells (Neri *et al*., 2016). After the process, pressure rises to the atmospheric pressure, and the residual gas is again compressed, while external liquid rapidly flows into the tissues. This gas-liquid exchange phenomenon has been named the hydrodynamic mechanisms (HDM) in vacuum impregnation (Fito *et al*., 1994; Zhao *et al*., 2004; Atarés *et al*., 2008). Mass transfer of water in the process depends on the vacuum pressure from 5 to 85.7 kPa and processing time from 10 to 1000 sec (Neri *et al*., 2016).

1.3 Quality deteriorations due to improper impregnation methods

Although impregnation methods can effectively improve the nutritional properties of plant food materials by inducing functional liquid, improper processing conditions such as excessive high temperature or pressure differences can lead to severe damages to the cell membrane or cell wall of the plant food materials and result in quality deteriorations.

1.3.1 Softening

Texture is an essential aspect of food sensory quality, and it has been reported that texture changes of plant food materials were related to the integrity of plant cell membranes (Gonzalez *et al*., 2010). Overheating may soften carrot tissues immediately, because it significantly increases the water-soluble pectin of carrots and results in texture degradation (Sila *et al*., 2006). As for the vacuum impregnation, the excessive pressure differences between vacuum and ambient pressures may lead to physical stress to cell membrane and resulting membrane damages and texture alteration (often softening) (Neri *et al*., 2016; Biegańska-Marecik *et al*., 2007).

1.3.2 Intracellular fluid leakage and color change

Cell membrane, a semi-permeable membrane, is a boundary of the intracellular and extracellular fluids. Thus, when the integrity of the cell membrane reduced, the intracellular fluid can leak from the cell through the membrane. Intracellular fluid contains polyphenol oxidase which reacts with polyphenols and oxygen to cause browning, especially for apple with white fleshes (Toivonen *et al*., 2008). Color is also an indicator of

food sensory evaluation, quality deterioration caused by the browning reaction will reduce consumer acceptance.

1.4 Liquid impregnation by high hydrostatic pressure

1.4.1 High hydrostatic pressure technology

High hydrostatic pressure (HHP) treatment is a nonthermal process, which suppresses chemical reactions while inactivating microbes and enhancing liquid impregnation (Yamamoto *et al*., 2017). It has wildly been applied to process various food materials. For instance, apples, carrots, potatoes, and turkey breasts were HHP-treated to improve their sensory qualities by enhancing the impregnation of food additives such as ascorbic acid, calcium lactate gluconate, and calcium chloride (Vatankhah *et al*., 2019; Gosavi *et al*., 2019; Rastogi *et al*., 2008; Villacís *et al*., 2008; Mahadevan *et al*., 2016; Yagiz *et al*., 2009; Denoya *et al*., 2015; George *et al*., 2016). Therefore, HHP treatment is expected to enhance the efficacy of liquid impregnation, which has conventionally been carried out often by VHS (Biegańska-Marecik *et al*., 2007). Furthermore, HHP treatment of pouched foods has mostly been carried out after VHS (Gosavi *et al*., 2019; Rastogi *et al*., 2008; Villacís *et al*., 2008; Mahadevan *et al*., 2016; Yagiz *et al*., 2009; Denoya *et al*., 2015). However, the effect of HHP treatment with/without VHS on impregnation damages to plant food materials has only been limitedly clarified.

1.4.2 Effect of HHP treatment on the tissue of fruits and vegetables

The effect of HHP treatment on fruits and vegetables tissues depends on processing parameters such as pressure levels and holding time as well as the structural features of the plant food materials. As for the processing parameters, with an increase of pressure levels or holding time under the target pressure can intensify the textural alterations due to the physical disruption of plant food materials, and it even causes damages to the texture when the critical value is exceeded (George *et al*., 2016; Araya *et al*., 2007). On the other hand, resistance to pressure varies depending on plants. As for the firm plant food materials such as carrots, 100 MPa did not induce the hardness (firmness) loss (Basak *et al*., 1998), while microscopic results revealed that carrots treated at 300 MPa lost their intact cellular structure and compact cell arrangement of untreated carrots with nonporous structure (Srikiatden *et al*., 2008), the tissues were significantly damaged with broken cellular structure and increased intercellular spaces (Araya *et al*., 2007). On the other hand, for apples and strawberries with porous structures (Van Buggenhout *et al*., 2009), HHP treatment at 100 MPa causes a reduction in firmness (Basak *et al*., 1998; Núñez-Mancilla *et al*., 2014).

1.5 Evaluations of liquid impregnation into plant food materials

1.5.1 Conventional methods

Microscopic observations by scanning electron microscopy demonstrate microstructural changes before and after treatments. However, samples should be preliminarily subjected to freeze drying and then coated with gold (Assis *et al*., 2019). Although the freezing damage caused by freeze drying was lower than those of conventional freezing methods, it still may lead some damages to the structure of the tissue. Therefore, the observed tissue changes may be caused by the impregnation and/or subsequent freeze drying, which affects the accuracy of the results; **Quantification of increases in solutes** reveals mass transfer of the solutes during impregnation, but it requires multi-step pretreatment such as grinding, extraction, filtration, and analysis of HPLC (Schulze *et al*., 2012); **Texture analysis** is a widely used method to measure textural properties of samples, reflecting hardness and deformability, respectively (Imai *et al*., 2008; Oey *et al*., 2007). Texture analysis has been carried out to evaluate tissue damages of plant food materials caused by heating (Leca *et al*., 2021; Peña *et al*., 2004), freezing (Paciulli *et al*., 2015; Van Buggenhout *et al*., 2006), vacuum impregnation (Neri *et al*., 2016) and HHP treatment (Basak *et al*., 1998; Araya *et al*., 2007; Núñez-Mancilla *et al*., 2014). Since texture is a crucial sensory characteristic of food products, it was applied to judge the textural qualities of impregnated products in this study.

The pretreatment of the scanning electron microscopy causes extra damage, and the quantification of solutes is a laborious and complex method. It is necessary to find some alternative quantitative methods for liquid impregnation into plant food materials without extra damages to the samples nor laborious works.

1.5.2 Image analysis

Image analysis has been applied to study the effect of vacuum impregnation on the quality of potatoes, and the impregnation efficacy has been visualized by red areas of potatoes which were vacuum impregnated with red ink (Hironaka *et al*., 2011), and the impregnated areas were further quantitatively evaluated by using a software ImageJ (Mashkour *et al*. 2014). Besides, image analysis has been used to quantify apple maturity via the content of starch which was stained with iodine (Jakab *et al*., 2018). However, image analysis has not been sufficiently studied as a tool for quantitative analysis of HHP-enhanced liquid impregnation.

1.5.3 Electrical impedance spectroscopy (EIS)

As a quantitative method, EIS has widely been used to analyze physiological and electrical properties of various biological tissues, it has also been applied to evaluate tissue changes during the processing including bruise, heating, and freezing-thawing (Jackson *et*

al., 2000; Watanabe *et al*., 2019; Ohnishi *et al*., 2004). As for the application of EIS to HHP-treated foods, HHP-treated apples have been analyzed by EIS for quantitative evaluation of cell membrane damages (Lee *et al*., 2019), indicating that EIS has a potential to evaluate cell membrane damages of apple and carrot which are caused by HHP treatment with/without preliminary VHS.

1.5.4 Low-field nuclear magnetic resonance (LF-NMR)

LF-NMR has become an alternative and effective method to quantify the changes in water mobility and visualize the internal water distribution of plant food materials. The potential of NMR to detect the changes in plant tissues during homogenization (Yang *et al*., 2018), storage (Yang *et al*., 2021), hot air drying (Kamal *et al*., 2019), blanching (Chitrakar *et al*., 2019), and freezing (Hills *et al*., 1997) has been reviewed. The redistribution of water in the plant tissues may reflect the cell membrane damage during processing. However, the effect of HHP treatment on apple and carrot has not been clarified by LF-NMR.

The applications of image analysis, EIS, LF-NMR, and texture analysis during storage and processing of apple and carrot are summarized in the Table 1-1.

1.6 Objectives

In this study, the objectives were

1) To study the applicability of image analysis, EIS, LF-NMR, and texture analysis as quantitative methods to evaluate liquid impregnation of plant food materials

2) To investigate the effect of vacuum heat sealing, HHP treatment, and their combinations on the impregnation

3) To improve the efficacy of liquid impregnation while minimizing the damages of cell membrane and tissue in plant food materials

1.7 Scheme of this study

There were 3 main experiments to achieve the above objectives, and the scheme figure of this study is shown in Fig. 1-1. There are three main aspects in this study.

(1) Quantitative methods for liquid impregnation into plant food materials

In this part, the parameters of quantitative evaluations have been optimized to increase the reproducibility of experimental results. As for image analysis, color of pigments (yellow, green, blue, or red) and concentration of pigments (0, 0.01, 0.05, 0.10, and 0.15 % for apple; 0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 % for carrot) have been optimized. Furthermore, selection of prepared samples (for EIS), scan number (for LF-NMR), and

plungers (for texture analysis) have been optimized.

(2) Vacuum impregnation of liquid into apple assisted by high hydrostatic pressure

In this part, the effect of VHS (0.1 kPa for 1 min), storage after VHS (0, 10, 30, 60, and 120 min), HHP treatment (100, 200, 400, and 600 MPa for 5 min), and their combinations on the apple have been investigated. Apple piece was put into a pouch with 0.05 % red pigment solution then subjected to VHS or AHS. After the impregnation, sample pieces were cut into slices for further quantitative analyses, including EIS, LF-NMR, and texture analysis.

(3) Vacuum impregnation of liquid into carrot assisted by high hydrostatic pressure

The same experimental system as for the apple was used to study the effect of VHS (0.1 kPa for 1 min), storage after VHS (0, 10, 30, 60, and 120 min), HHP treatment (100, 150, 200, 400, and 600 MPa for 5 min), and their combinations on the carrot. Cylindrical carrot was put into pouch with 3.0 % blue pigment solution. Cubic or cuboidal samples were put into pouch with RO water. The pouches were subject to VHS or AHS. After impregnation processes, samples were drained and prepared for further quantitative analyses.

Table 1-1 Summary of published quantitative methods for apple and carrot during storage and processing.

Materials	Processing	Image analysis	EIS	LF-NMR	Texture analysis
apple	storage	√	√	√	√
	freezing-thawing	-	√	√	√
	heating	-	√	√	√
	vacuum impregnation	-	-	-	√
	HHP treatment	-	√	-	√
carrot	storage	-	-	-	√
	freezing-thawing	-	√	√	√
	heating	-	-	√	√
	vacuum impregnation	-	-	-	√
	HHP treatment	-	-	-	√

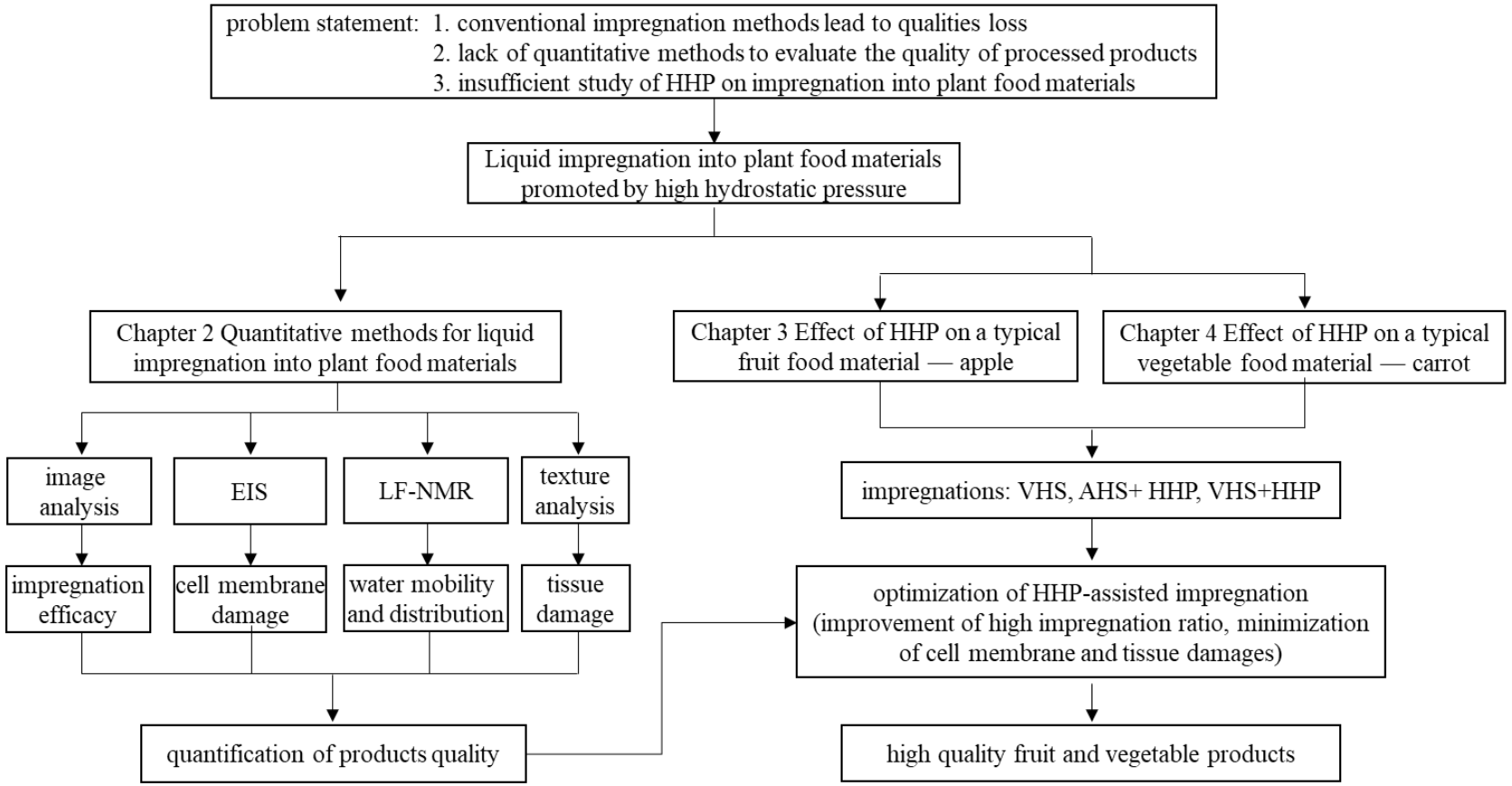

Fig. 1-1 The scheme of this study

Chapter 2 Quantitative methods for liquid impregnation into plant food materials

2.1 Introduction

Quantitative methods including image analysis, EIS, and LF-NMR were adopted to evaluate the impregnation efficacy, cell membrane damages, and water mobility and distribution. Texture analysis as a conventional method was also applied to evaluate the tissue damages of samples quantitatively. However, since there are interindividual difference among individual plants of one cultivar as well as intraindividual difference in one piece of plant food material such as apple and carrot, there were many factors to be optimized for data reproducibility: pigment selection and its concentration for image analysis, preparation of apple and carrot samples for EIS, scan number for LF-NMR, and plunger size for texture analysis.

In order to evaluate properly the effects of various treatments in this study on the treated materials, the parameters of quantitative methods for the impregnated products have been optimized in the chapter 2.

2.2 Materials and methods

2.2.1 Preparation

Mature apples (*Malus domestica*, var. Sun Fuji) were purchased at a local market and then stored at 4 °C. Apples were washed, peeled, and cut into 6 equals. Core portions of each equal were removed (Fig.2-1). After impregnation, apple piece was died by paper and prepared for image analysis. For further quantitative analysis, apple piece was cut into slices of 10- or 15-mm thickness. The slices samples of 10 mm thickness were prepared for EIS and texture analysis. The slices samples of 15 mm thickness were further cut into cylinders with a diameter of 5 mm and a height of 15 mm, and the cylindrical samples were prepared for LF-NMR.

Carrots (*Daucus carota* L. Gosun) were purchased from a local market. Carrots were thereafter stored at 4 °C. The maximum storage period was two weeks. Carrot was cut along the longitudinal axis into a cylinder with a weight of 25 g or a square log with a section of 15 mm × 15 mm (Fig. 2-2). The carrot square log was cut into cuboids (15 mm × 15 mm × 5 mm) for texture analysis and cubes (15 mm × 15 mm × 15 mm) for EIS.

Pigment solutions were prepared by using four kinds of commercial food-grade pigments (Kyoritsu-foods Co., Ltd, Tokyo, Japan): yellow, 14 % tartrazine ($C_{16}H_9N_4Na_3O_9S_2$) and 86 % dextrin; green, 8.4 % tartrazine, 3.6 % Brilliant Blue FCF, and 88.0 % dextrin; blue, 8 % Brilliant Blue FCF ($C_{37}H_{34}N_2Na_2O_9S_3$) and 92 % dextrin; red 15.0 % new coccine (ponceau 4R) and 85 % dextrin.

2.2.2 Vacuum heat sealing (VHS)

A portion (25 g) of the fresh-cut apple piece (Fig. 2-1) was put in a translucent plastic retort pouch (BRS-1624S, Meiwa pax Co., Ltd, Osaka, Japan) containing 0.01, 0.05, 0.10, or 0.15 % (w/w) aqueous solution (25 g) of food-grade yellow, green, blue, or red pigment (Kyoritsu-foods Co., Ltd, Tokyo, Japan). A portion (25 g) of fresh-cut cylindrical carrot (Fig. 2-2) was soaked in an equivalent weight of reverse osmosis water or 0.5, 1.0, 2.0, 3.0, 4.0, or 5.0 % (w/w) aqueous solution of food-grade yellow, green, blue, or red pigment (Kyoritsu-foods Co., Ltd, Tokyo, Japan) in the plastic pouch. Pouches were subjected to VHS using a vacuum heat sealer (SV-300G, TOSEI Co., Ltd, Shizuoka, Japan) at 1.0 kPa and 25 °C for 1 min and then stored for maximum 120 min.

2.2.3 HHP treatment

The pouches after VHS were subjected to HHP treatment by using an HHP food processor (TFS2-50, Toyo Koatsu Co., Ltd., Hiroshima, Japan). Cylindrical pressure vessel was filled with water as pressure transmitting medium. Water temperature in the vessel was controlled at 65 °C. The sample pouches were immersed in the vessel and pressurized up to 100 MPa and held for 30 min and then released within 5 sec.

2.2.4 Image analysis

Apple or carrot samples after paper draining of excess liquid were placed on a white plastic weighing dish. Digital color photographs as RGB (red, green, and blue) images were taken using a digital camera (PowerShot S120, Canon Inc., Tokyo, Japan). The digital color images were processed and analyzed by ImageJ (ver.1.52a, National Institutes of Health, USA). Apple or carrot image was trimmed, and its background was removed. The trimmed color image was converted into an 8-bit gray image and further binarized into a 2-bit (black-and-white) image by adjusting the binarization threshold value. The threshold value was determined by visually matching each 2-bit image to its color/8-bit image so that black and white areas in the 2-bit image should maximally correspond to the impregnated and unimpregnated areas in the color/gray images. The impregnation ratio (%) was calculated as follows:

$$\text{impregnation ratio (\%)} = A_i / A_t \times 100 \qquad (2\text{-}1)$$

, where A_t and A_i are total and impregnated areas in a trimmed image, respectively.

Since it has been qualitatively demonstrated that liquid impregnation of various fruits was enhanced by combining VHS, 100 MPa treatment, and heat treatment at 65 °C for 30 min (Nakuura 2017; Uddin *et al*., 2019). Apple pieces and cylindrical carrots after the combined treatment of VHS, 100 MPa treatment, and heat treatment at 65 °C for 30 min were adopted as positive controls of full impregnation. Untreated apple pieces and

cylindrical carrots were taken as negative unimpregnated controls of no impregnation.

2.2.5 Electrical impedance spectroscopy (EIS)

Electrical impedances of the samples were analyzed by using an impedance analyzer (E4990A, Keysight Technologies Inc., USA; Fig.2-3) with two 5 mm length stainless needle electrodes, the electrodes were inserted into the slice or cube samples with a depth of 5 mm from the surface and a distance of 10 mm. Electrical impedance Z (Ω) and phase angle difference (rad) of the samples were obtained at 81 points over the frequency from 10^2 Hz to 10^7 Hz. The measurements were repeated 6 times. Complex electrical impedance (Z) is defined as:

$$Z = R + jX \tag{2-2}$$

where j is the imaginary unit, R (Ω) is the real part of impedance (resistance), and X (Ω) is the imaginary part of the impedance (reactance). R and X can be expressed as functions of Z by the following equations, respectively:

$$R = |Z| \cos\theta \tag{2-3}$$

$$X = |Z| \sin\theta \tag{2-4}$$

The relationship between resistance R and reactance X can be visualized by Cole-Cole plot analysis, which can be used to evaluate structural damage of plant tissues (Cole 1932; Ando *et al*., 2014). Intact plant tissues may give a circular arc in the plot, while the arc may shrink or disappear in damaged tissues (Watanabe *et al*., 2019). Therefore, the size of circular arc (SCA) in the Cole-Cole plot was defined as a parameter for cell membrane damage of apple and carrot in this study. It was defined as a graphical distance between the top coordinate and the left-endpoint coordinate of the circular arc in the Cole-Cole plot as follows:

$$SCA = \sqrt{(R_{\text{top}} - R_{\text{left}})^2 + (X_{\text{top}} - X_{\text{left}})^2} \tag{2-5}$$

where R_{top} and X_{top} are the resistance and reactance at top coordinate of the circular arc and R_{left} and X_{left} are the resistance and reactance at left-endpoint coordinate of the circular arc in the Cole-Cole plot as depicted in Fig. 2-4.

2.2.6 Low-field Nuclear Magnetic Resonance (LF-NMR)

LF-NMR measurements were carried out by using a benchtop NMR analyzer (MQC+, Oxford instruments Co., Ltd., Oxon, UK) with a magnet cabinet (AM4000 MQC, Oxford instruments Co., Ltd., Oxon, UK) at 23.4 MHz. Each cylindrical sample was put in

a glass tube (10 mm in diameter and 18 mm in height) and the spin-spin (T_2) relaxation times of samples were measured using the Carr-Purcell-Meiboom-Gill (CMPG) pulse sequence at 25 °C. The number of echoes was 512, scan number (SN) was set at 4, 8, 16, 32, and 64 scans, tau (τ) time and repetition delay were 3 ms and 30 s, respectively. The relaxation curves were fitted as a continuous distribution of exponentials with WinDXP software (Oxford Instruments Co., Ltd.,). The measurements were replicated 3 times.

2.2.7 Texture analysis

Textural properties of the samples were measured by a creep meter (RE-3305C, Yamaden Co., Ltd, Tokyo, Japan; Fig. 2-5). Apple samples were measured with a load cell (LC2-3305B-20N) of 20 N at 25 °C, and carrot samples were measured with a load cell (LC2-3305B-200N) of 200 N at 25 °C. Apple or carrot sample was placed on its plastic platform and compressed to a strain of 80 % with a plunger of 3, 5, or 8 mmϕ (No.4, 5, or 6, Yamaden Co., Ltd, Tokyo, Japan) and a plunger extender L50 of 50 mm length at a constant speed of 0.5 mm/s. Textural properties of samples were evaluated by the stress and strain of breaking points in the compression tests (Fig. 2-6). The measurements were repeated 6 times. To minimize the interindividual and intraindividual effects on the data reproducibility, the parameters of breaking stress and strain were normalized by using those of untreated fresh-cut apple or carrot.

2.2.8 Statistical analysis

Data was expressed as mean ± standard deviation (SD). The results of SCA were analyzed by one-way analysis of variance using Excel 2016 (Office 365, Microsoft Co., Ltd, Redmond, USA). For each analysis, significant difference was statistically judged at a confidence level of 95 %.

2.3 Results and discussion

2.3.1 Pigments selection for the image analysis

Apple samples were impregnated with RO water or 4 kinds of 0.05 % solution of yellow, green, blue, and red pigments by a combination of VHS and subsequent 100 MPa HHP treatment. After impregnation, the images of samples were photographed and processed into binary images by using ImageJ. The binary images consist of pixels with different gray shades, and it is shown in 8 bit gray scale distribution. As for the RO water samples and yellow samples, their gray scale distributions were overlapped with that of control samples (Fig. 2-7), indicating the undistinguishable color difference between the RO (or yellow) sample and the control sample. On the other hand, gray scale distributions of green, blue, and red samples were separated from those of control samples, which indicated that the color differences of the green, blue, and red samples were easier than RO and yellow samples to distinguish before and after impregnations. Moreover, the threshold

value for binarization to determine whether each pixel in the binary image was white or black was carried out by visually matching each 2-bit image to its color/8-bit image and comparing the color differences. It can be suggested that the color after impregnation was more homogenous and better when the gray scale distribution range of impregnated sample was narrower. Since the gray scale distribution range of red sample was the narrowest among all the samples, it was adopted for the subsequent image analysis.

A combination of VHS, 100MPa treatment, and heating at 65 °C (denoted by VHS+100 MPa+65 ºC) has been used in food processing, which has been reported to impregnate fully various types of fruits and vegetables (Nakuura 2017; Uddin *et al*., 2019). In this study, apple samples treated with VHS+100 MPa+65 ºC for 30 min were identified as fully impregnated. Meanwhile, untreated apple samples were regarded as negative unimpregnated control of no impregnation. As shown in Fig. 2-8, the samples were impregnated with RO water, 0.01 %, 0.05 %, 0.10 %, and 0.15 % red pigment aqueous solution. The gray scale distribution of RO water or 0.01 % red sample were overlapped with that of control sample, it can be assumed that the color difference between samples impregnated with RO water (or 0.01 % red pigment solution) was not enough to distinguish impregnated and unimpregnated areas. When the concentration of the pigment solution was equal and higher than 0.05 %, the color difference was sufficiently large to distinguish unimpregnated and impregnated areas. It facilitated the matching the impregnated areas of binary images and color images. Since the color difference between 0.05 % and control samples was already distinguishable, and the higher concentration of the pigment solution only slightly increased the color difference. Red pigment solution of 0.05 % was adopted and used for the image analysis of apple in chapter 3.

Carrot samples were impregnated with RO water and yellow, green, blue, and red pigment solutions. Since carrot was much less stained by impregnation than apple, the concentration of pigment solution was set at o 3 %. As shown in Fig. 2-9, the gray scale distribution of RO water, yellow, or red sample was overlapped with that of control sample (Fig. 2-9), indicating the undistinguishable color difference between RO (or yellow or red) sample and the control sample. On the contrary, the gray scale distribution of blue or green sample was separated with that of the control sample, indicating that the color separations of impregnated with blue and green pigments were much more distinguished than before and after impregnations than the other impregnated samples. In addition, the gray scale distribution range of blue sample was narrower than that of green sample, indicating that the color of samples impregnated with blue pigment was more homogenous. Therefore, blue pigment was adopted for image analysis of carrot.

In the current study, carrot samples subjected with VHS+100 MPa+65 ºC for 30 min were identified as fully impregnated samples, and untreated carrot samples were

regarded as negative unimpregnated controls of no impregnation. As shown in Fig. 2-10 the samples were impregnated with RO water, 0.5 %, 1.0 %, 2.0 %, 3.0 %, 4.0 % and 5.0 % of blue pigment solution. The pigment for 5.0 % preparation did not dissolve completely. The color of the samples impregnated with 0.5 %, 1.0 % and 2.0 % blue solutions was stained heterogenous. The carrot section is composed of central xylem and outer cortex (phloem), and the boundary is the vascular cambium (Ahmad *et al*., 2019). The central xylem of carrot samples was more homogenously impregnated than the outer cortex. When the concentration of the pigment solution was equal or higher than 3.0 %, the color of the impregnated samples was stained more homogenous, and the homogeneity facilitated image analysis. Therefore, blue solution of 3.0 % was used for image analysis of impregnated carrots in the chapter 4.

2.3.2 Optimization of sample preparations for EIS

In this study, cell membrane damage of apple was quantified as SCA in EIS using three block samples (10 mm thick) taken from the middle part of apple piece which was cut into 6 equals (blocks A, B and C in Fig. 2-11). At the last step of VHS to prepare pouched samples, reduced pressure restores to atmospheric pressure instantaneously, and the pouch with expanded volume due to expanded gas by the reduced pressure shrinks rapidly. The shrinkage process might squeeze the edges and corners of the samples in the pouch and cause unnecessary damages to the samples. It has been reported that physical damage led to a decrease in SCA (Watanabe *et al*., 2018). To avoid the influence of packaging damage on the cut samples, the edges and corners of the cut sample were removed and only its middle part (3 blocks) were used for EIS measurement. SCA values of 3 block samples were shown in Fig. 2-11. There was no significant difference among the blocks A, B, and C. It was suggested that the sample selection among the 3 blocks did not affect the SCA. Thereafter, the middle three blocks were used for EIS in Chapter 3.

As for carrot, the central xylem seems to be impregnated more homogenously than that of outer cortex, so the carrot samples for EIS were mainly cut as a square log from central xylem of carrot. Since the distance between two stainless needle electrodes was 10 mm (Fig. 2-3), the diameter of carrot xylem had to be larger than the distance. Therefore, the side of cubic samples for EIS was set at 15 mm. A maximum of 4 cubic samples per one carrot square log was used for EIS, and the SCA results were shown in Fig. 2-12. SCA values of the samples A, B, C, and D were not significantly different with each other. It was suggested that the sample selection among the 4 cubic samples did not significantly affect the SCA.

Furthermore, the SCA of carrot was significantly lower than that of apple. This may be related to the air contents in the carrot and apple. It has been reported that the density of

carrot was about 1.040 to 1.210 kg/m^3 (Lozano *et al*., 1983), which was higher than that of apple (0.837 to 0.900 kg/m^3; O'Neill *et al*., 1998; Karathanos *et al*., 1996). In addition, apple tissue structure is porous, comprising of intercellular spaces filled with gas which account for 25 % of total volume (Van Buggenhout *et al*., 2009). On the contrary, carrot tissue structure is nonporous (Srikiatden *et al*., 2008), consisting of tightly arranged small cells of about 50 μm in diameter which are approximately by one-sixth smaller than apple cells (Nielsen *et al*., 1998; Khan *et al*., 1993; Zdunek *et al*., 2005), it can be assumed that apples may contain more internal gas than carrots. Since the electrical resistance of gas is higher than that of internal liquid or cellular tissue, higher gas content may result in higher SCA.

2.3.3 Optimization of scan numbers for the LF-NMR

Water status of apple samples was quantified by LF-NMR, it was reported (Li *et al*., 2021), that the relaxation time curves of apple consisted of three peaks (Fig. 2-13), representing bound (T_{21}), semibound (T_{22}), and free water (T_{23}) Free water is the most abundant and mainly distributed in vesicles, semibound water is mainly distributed with cytosol to intercellular fluid, and bound water is in the cell wall (Hills *et al*., 1997). The relaxation times of three peaks (T_{21}, T_{22}, and T_{23}) were used to evaluate the water mobility, and the area ratio of three peaks (A_1, A_2, and A_3) were used to evaluate the water distribution in the sample.

Scan number (SN) is an important parameter in LF-NMR. It was reported that an increase in SN from 2 to 24, enhanced the detection performance of LF-NMR during carrot drying, and decreased the standard deviation of peak area fractions (Sun *et al*., 2020). In this study, SN was set to 4, 8, 16, 32, and 64 for measuring relaxation times to optimize the measurement condition by using fresh apple samples (Table 2-1). When SN was set at 16, the standard deviation of relaxation times and area fractions were relatively lower than the cases where SN was set at 4, 8, 32, or 64. Considering the detection time and data accuracy, it was adopted to set the SN at 16 to obtain the most reproducible data in following experiments.

2.3.4 Optimization of plungers' size for texture analysis

The textural parameters (breaking stress and breaking strain) of apple and carrot are shown in Table 2-2. Data was normalized by the fresh samples and shown in parentheses and red font. Breaking stress and strain were expressed as average ± standard deviation (n=5). The breaking forces of apple were lower than 20 N, and it was difficult to accurately measure the texture changes of apple with the load cell of 200 N. To increase the data accuracy, the load cell of 20 N was selected for the mass structure analysis of apple. Based on Table 2-2, the results measured by No. 4 probe had larger standard deviations than those

measured by the other probes. During the compression test, puncture damage and compression damage may lead to sample rupture. As a probe of small diameter was used, it occasionally punctured into the sample surface causing sample rupture after contacting the sample. Meanwhile, compression also caused a rupture to the samples. Whereas the rupture caused by compression may require higher breaking stress than by puncture, the different rupture patterns may increase the standard deviation of data. On the other hand, measurement with a probe of relatively large diameter may cause compression damage to the samples, resulting in smaller standard deviations as compared with the measurements by using the probe No.4. When using the No. 6 probe, the breaking stress exceeded the maximum range. Therefore, the No. 5 probe was adopted for the texture analysis of apples.

As for carrot, the breaking forces were all higher than 20 N. Therefore, a load cell of 200 N was selected for the textural analysis of carrots. As shown in Table 2-2, results measured by Probe No. 5 had the smallest standard deviation among all the results. To minimize the variances in breaking stress and strain in apple and carrot, probe No. 5 was adopted for the texture analysis of carrot in chapter 4.

Table 2-1 Effect of scan number on the relaxation time (T_2) and area fraction of apple samples.

Scan number	Relaxation time (ms)						Area fraction (%)					
	T_{21} (peak 1)		T_{22} (peak 2)		T_{23} (peak 3)		A_1 (peak 1)		A_2 (peak 2)		A_3 (peak 3)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
4 scans	30.0	14.7	166.2	8.5	1,019.0	52.2	7.3	0.7	9.6	4.9	83.1	4.2
8 scans	22.0	3.3	109.3	8.0	1,016.5	55.8	5.8	0.8	9.6	0.5	84.5	1.3
16 scans	26.4	2.9	143.3	33.2	1,064.1	11.6	5.4	0.5	8.7	1.2	85.9	1.6
32 scans	25.1	2.1	163.5	39.9	1,037.3	85.2	6.7	1.3	7.5	2.7	85.8	3.9
64 scans	22.6	8.3	108.6	33.4	1,024.6	67.4	5.4	1.1	6.0	1.4	88.6	0.2

Table 2-2 Effect of plunger size on the breaking stress and breaking strain of untreated apple and carrot samples. The data in parentheses are the normalized values of breaking stress and breaking strain.

	Plunger	Breaking stress (kPa)		Breaking strain (%)	
		Mean	SD	Mean	SD
apple	No.4 (3 mm)	464.97 (1.00)	128.18 (0.28)	5.27 (1.00)	1.38 (0.26)
	No.5 (5 mm)	667.69 (1.00)	20.22 (0.03)	9.68 (1.00)	1.68 (0.17)
	No.6 (8 mm)	exceed range	-	exceed range	-
carrot	No.4 (3 mm)	4225.27 (1.00)	311.84 (0.07)	22.08 (1.00)	2.43 (0.11)
	No.5 (5 mm)	3679.66 (1.00)	171.01 (0.05)	28.40 (1.00)	1.65 (0.06)
	No.6 (8 mm)	3000.07 (1.00)	206.95 (0.07)	32.94 (1.00)	2.04 (0.09)

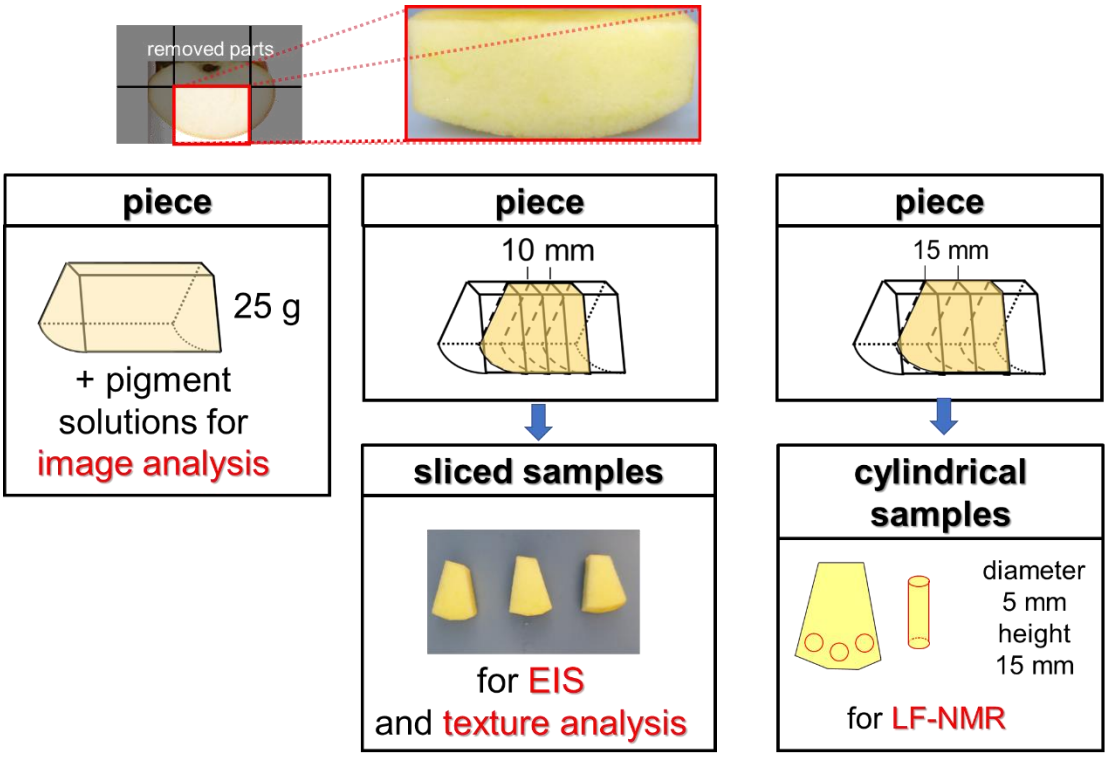

Fig. 2-1 Preparation of apple samples

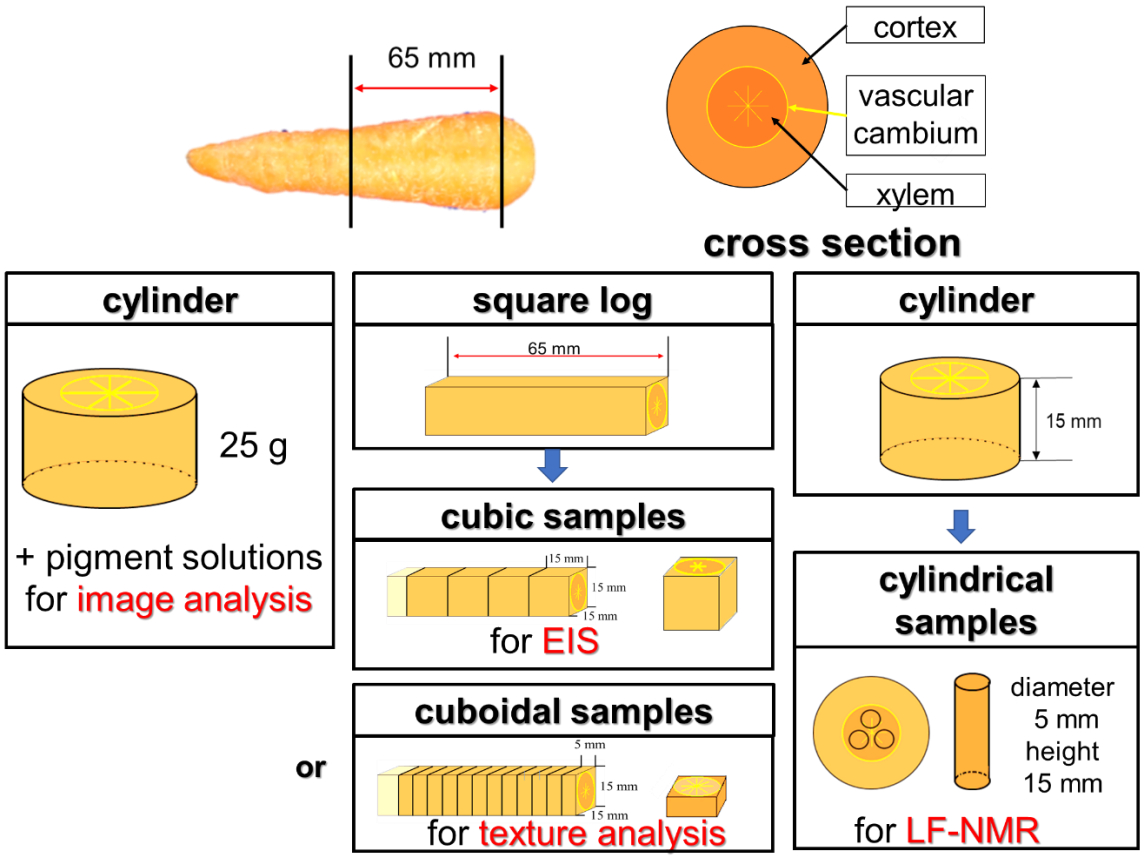

Fig. 2-2 Preparation of carrot samples

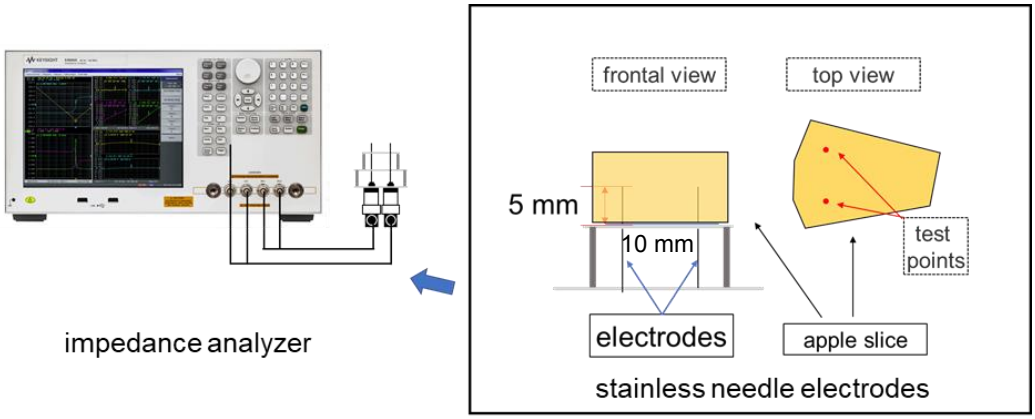

Fig. 2-3 Electrical impedance measurement

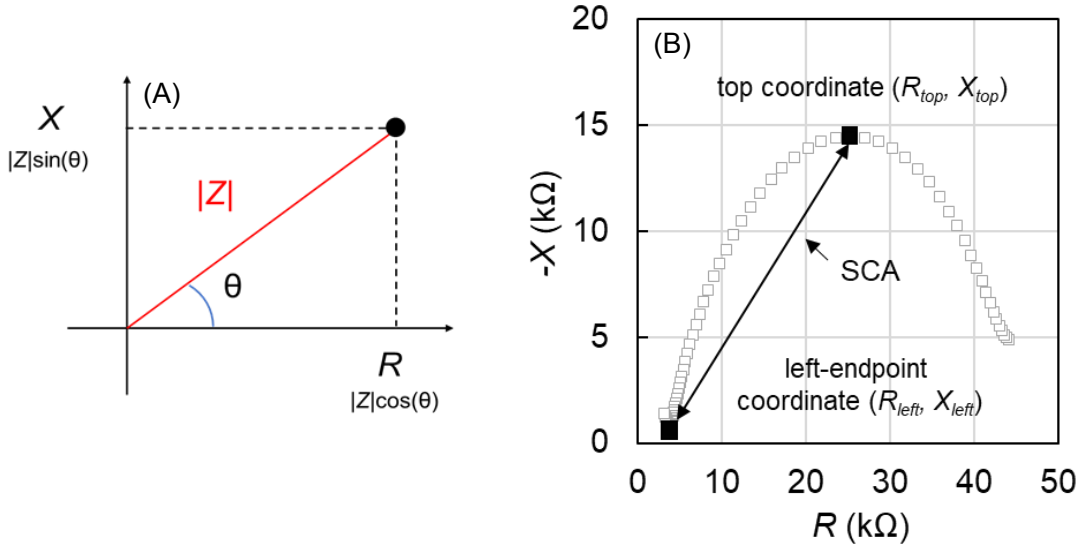

Fig. 2-4 Diagram (A) of resistance *R* and reactance *X* in the impedance magnitude *Z* and (B) Cole-Cole plot of fresh apple.

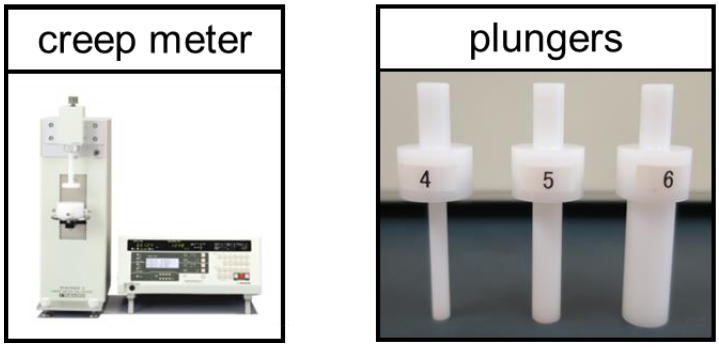

Fig. 2-5 Texture measurement: creep meter (RE-3305C) and plungers (No.4, No.5, and No.6)

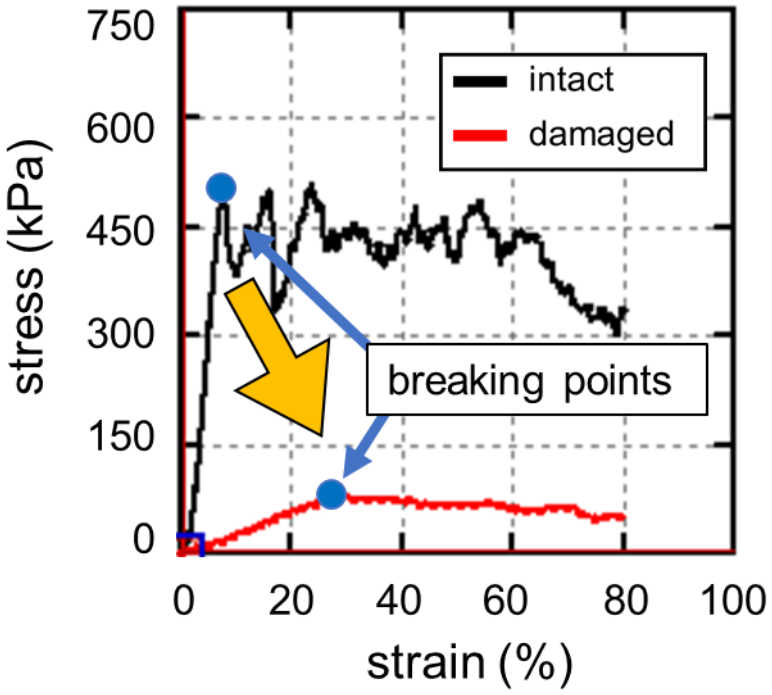

Fig. 2-6 Stress-strain curves of intact (fresh) and damaged apples

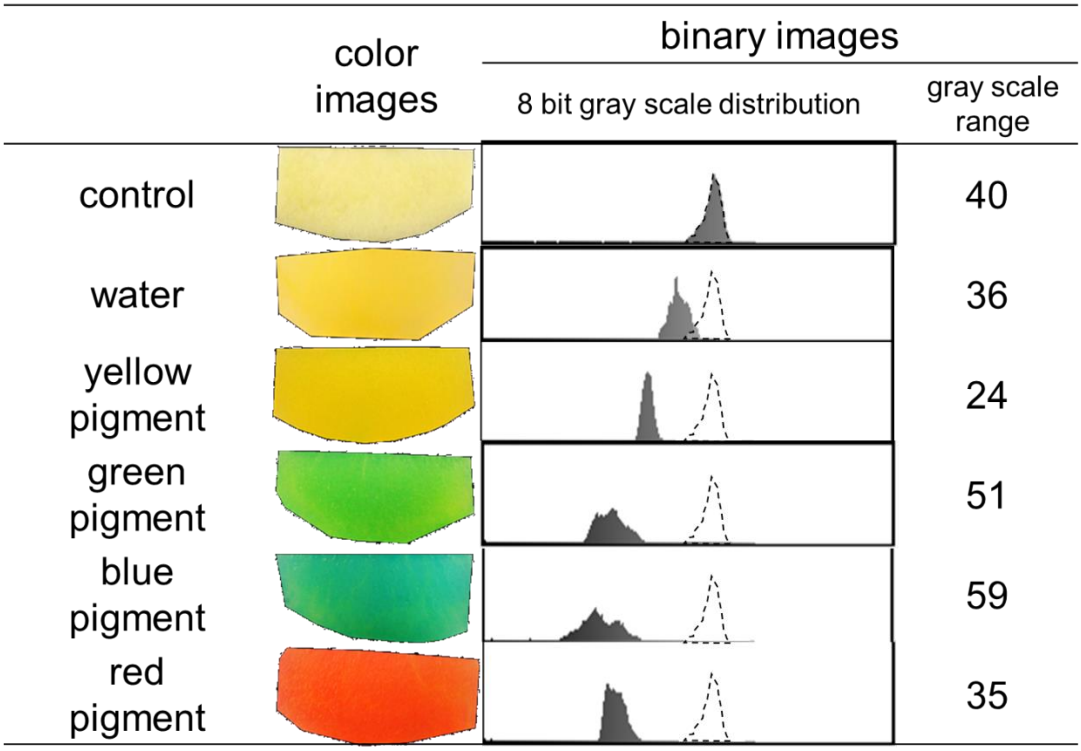

Fig. 2-7 Gray scale distributions and ranges of apple pieces impregnated with water and yellow, green, blue, and red pigment solutions. The horizontal axis of gray scale distribution is pixel gray shade, and vertical axis is pixel number.

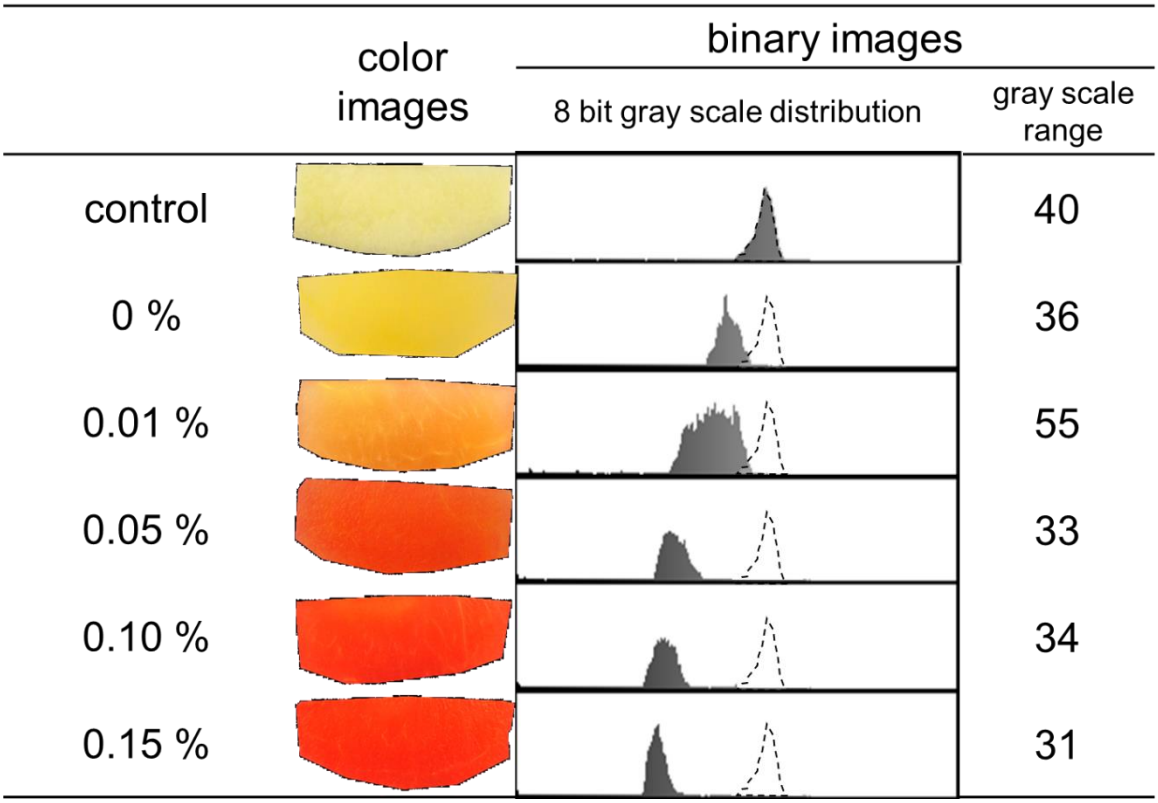

Fig. 2-8 Gray scale distributions and ranges of apple pieces impregnated with RO water and 0.01 %, 0.05 %, 0.10 %, and 0.15 % red pigment solutions. The horizontal axis of gray scale distribution is pixel gray shade, and vertical axis is pixel number.

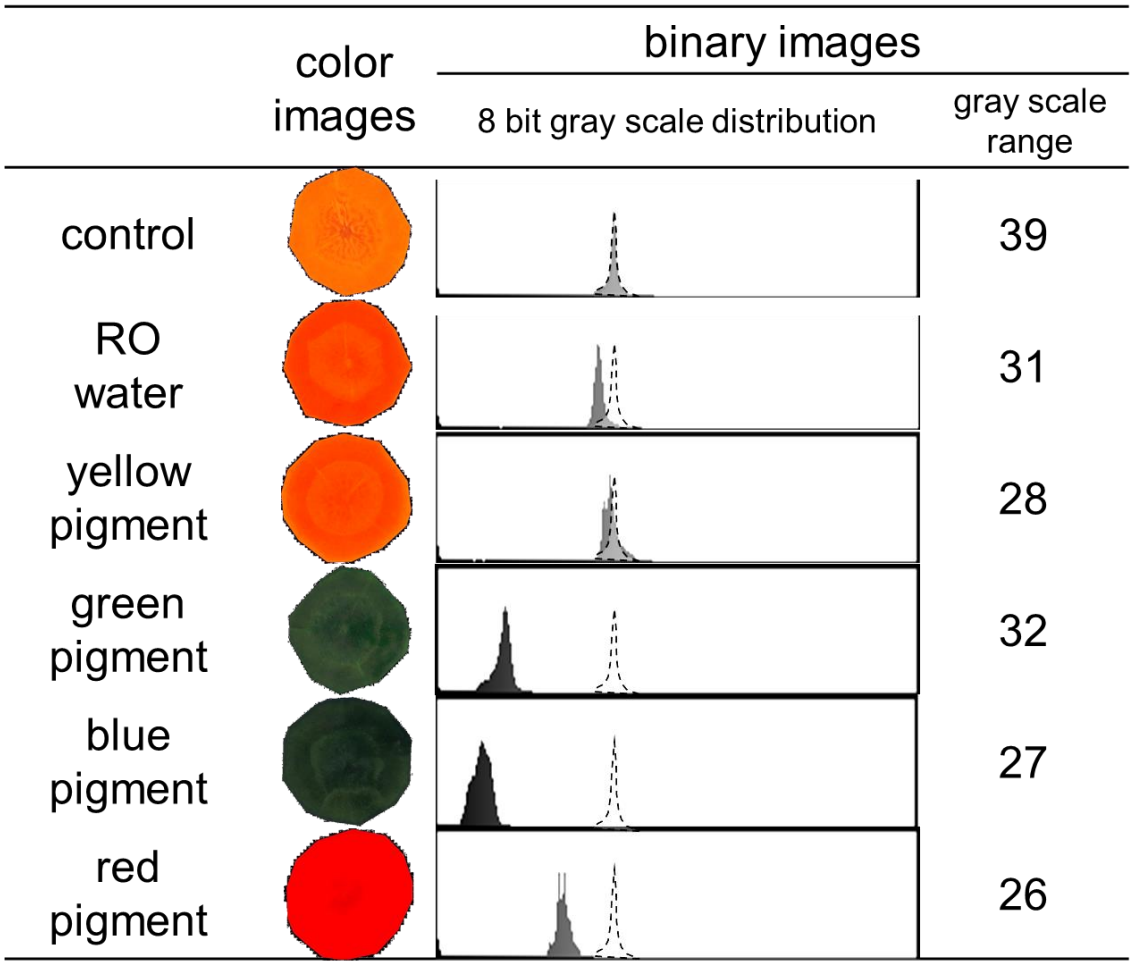

Fig. 2-9 Gray scale distributions and ranges of carrot cylinders impregnated with RO water and 3.0 % yellow, green, blue, and red pigment solutions. The horizontal axis of gray scale distribution is pixel gray shade, and vertical axis is pixel number.

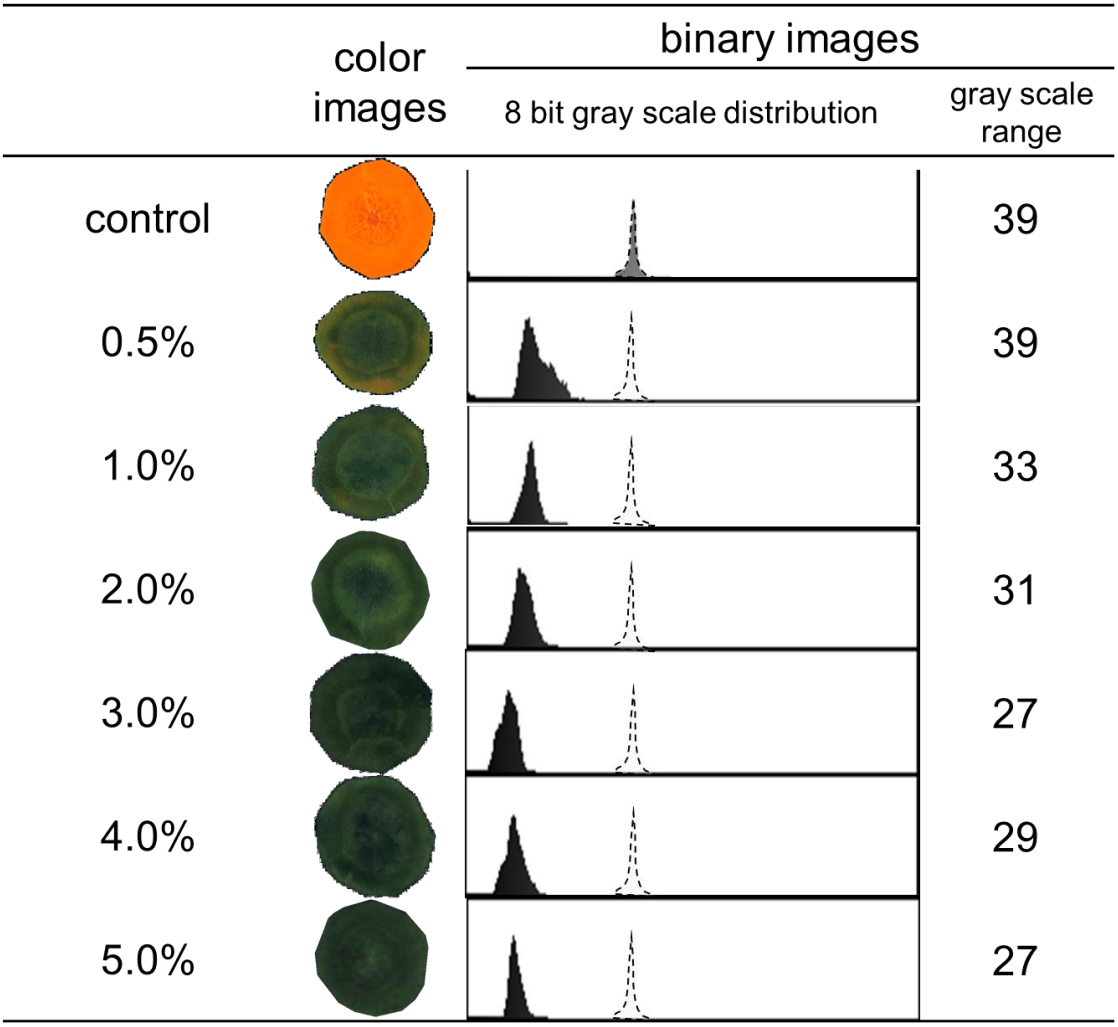

Fig. 2-10 Gray scale distributions and ranges of carrot cylinders impregnated with RO water and 0.5 %, 1.0 %, 2.0 %, 3.0 %, 4.0 %, and 5.0 % blue pigment solutions. The horizontal axis of gray scale distribution is pixel gray shade, and vertical axis is pixel number.

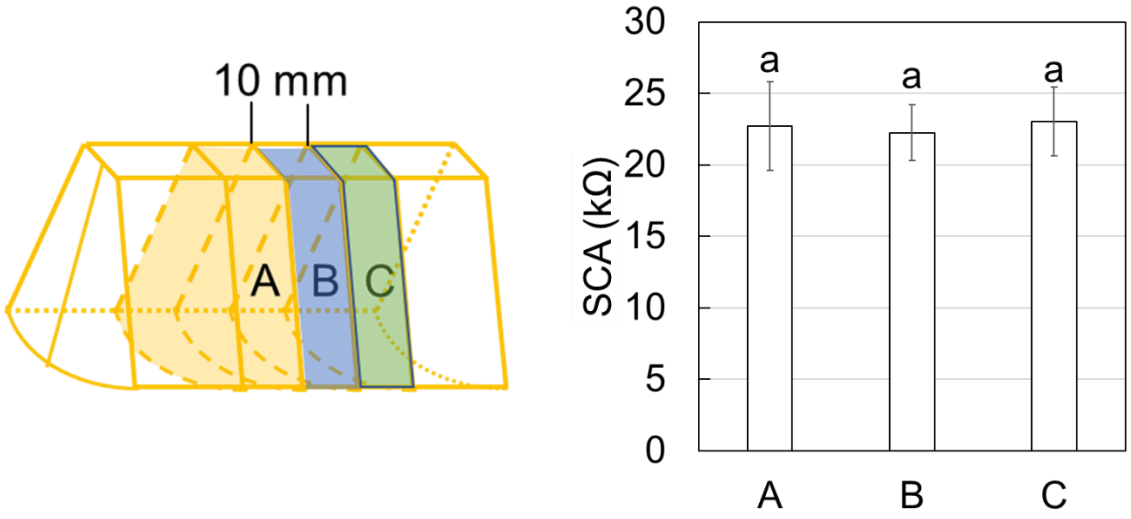

Fig. 2-11 Equivalence of SCA among the untreated apple samples A, B and C prepared for EIS measurement. Values with different alphabets are significantly different (one-way analysis of variance, $p < 0.05$).

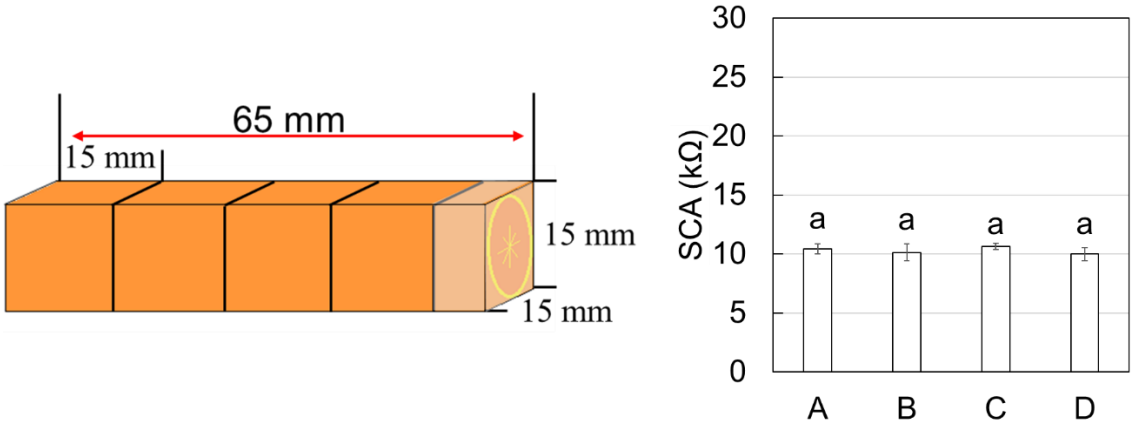

Fig. 2-12 Equivalence of SCA among the untreated carrot samples A, B, C, and D prepared for EIS measurement. Values with different alphabets are significantly different (one-way analysis of variance, $p < 0.05$).

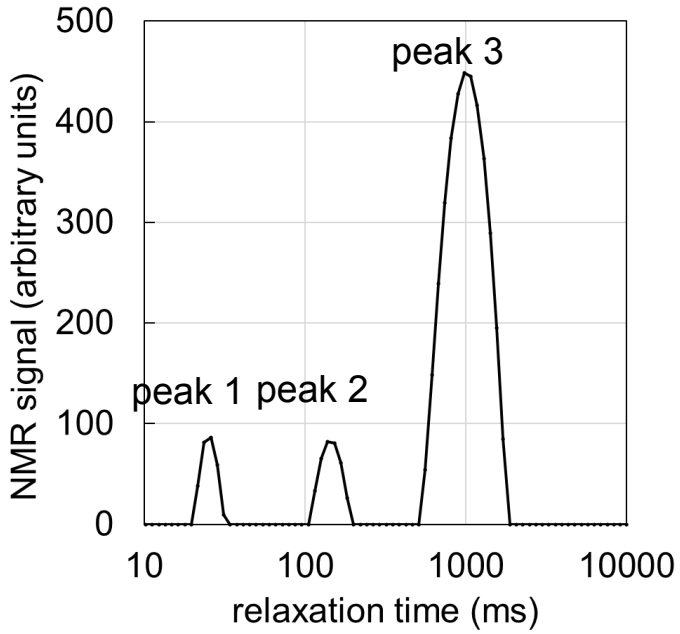

Fig. 2-13 Distribution curve of relaxation time of apples fitted by WinDXP.

2.4 Summary

In this chapter, a quantitative evaluation system for apple and carrot has been established. Differences in parameters of quantitative methods were optimized. The following conclusion can be drawn:

(1) For image analysis of apple and carrot, 0.05% red and 3.0 % blue pigment solutions were adopted, respectively.

(2) Sample preparation was optimized for SCA in EIS of apple and carrot samples. SCA values for the optimized sample portions show no significant difference among the prepared samples.

(3) For LF-NMR, the scan number of 16 was adopted, showing relatively low standard deviations for 3 relaxation times and relevant area fractions.

(4) Plunger No. 5 of 5 mmϕ thick was adopted to measure the textural parameters (breaking stress and breaking strain) of apple and carrot to obtain the most reproducible results.

Chapter 3 Vacuum impregnation of liquid into apple assisted by high hydrostatic pressure

3.1 Introduction

Apple (*Malus domestica*) is consumed worldwide, and its tissue has porous structures, comprising of intercellular spaces filled with gas which accounts for 25 % of total volume (Van Buggenhout *et al*., 2009). Vacuum impregnation can replace the gas in the intercellular spaces with infusion liquids such as high fructose corn syrup, sucrose solution, and sodium erythorbate solution, which may improve sensory and nutritional properties and/or resistance to freezing damages of apples (Park *et al*., 2005; Ursachi *et al*., 2009).

HHP treatment has wildly been applied to process various food materials. For instance, apples, carrots, potatoes, and turkey breasts were HHP-treated to improve their sensory qualities by enhancing the impregnation of food. Therefore, HHP treatment is expected to enhance the efficacy of liquid impregnation, which has been conventionally carried out often by vacuum heat sealing (VHS). However, the effect of HHP treatment as well as VHS and subsequent HHP treatment on impregnation damages to plants including apple has only been limitedly clarified. For instance, EIS has been used for quantitative evaluation of cell membrane damages in HHP-treated apples (Lee *et al*., 2019).

The Chapter 3 focused on liquid impregnation of apple which was adopted as a representative of plant food materials with porous structure, aiming at quantitative evaluation of HHP-impregnated apples from viewpoints of damages in cell membrane and texture as well as impregnation efficacy. The damage was quantified by EIS and texture analysis, respectively. The efficacy of liquid impregnation was quantified by image analysis. Liquid impregnation was carried out by VHS, HHP treatment (100–600 MPa), and their combinations with/without storage after VHS.

3.2 Materials and methods

3.2.1 Materials

Mature apples (*M. domestica*, var. Sun Fuji) harvested in Aomori prefecture of Japan were purchased at a local market and then stored at 4°C. The apples were washed, peeled, and cut into 6 equal portions, and their core parts were removed. The weight of the fresh-cut apple portion was adjusted to 25 g (25.4 ± 0.5 g; n = 90). The cut apple portions were put into pouches and vacuum- or ambient-heat-sealed (VHS or AHS) for further treatments. Samples after the treatment were cut into slices or cylinders for quality

evaluations as shown in Fig. 2-1.

3.2.2 Vacuum heat sealing (VHS)

One portion of the fresh-cut apple (25 g) was put in a translucent plastic retort pouch (BRS-1624S, Meiwa pax Co., Ltd, Osaka, Japan) containing 0.05 % (w/w) aqueous solution (25 g) of a food-grade red pigment (Kyoritsu-foods Co., Ltd, Tokyo, Japan). The pouch was subjected to VHS using a vacuum heat sealer (SV-300G, TOSEI Co., Ltd, Shizuoka, Japan) at 1.0 kPa and 25 °C for 1 min and then stored for 0, 10, 30, 60, or 120 min. In addition, some samples were heat-sealed without vacuum (ambient heat sealing; AHS), while the air in the pouch was minimized by manual removal.

3.2.3 High hydrostatic pressure (HHP) treatment

Samples after VHS or AHS were subjected to HHP treatment by using an HHP food processor (TFS6-50, Toyo Koatsu Co., Ltd., Hiroshima, Japan). A cylindrical pressure vessel (120 mm in diameter × 450 mm) was filled with water as a pressure transmitting medium. Water temperature in the vessel was controlled at 25 °C using a water circulator. The sample pouches were immersed in the vessel and pressurized up to 100, 200, 400, or 600 MPa at a come-up rate of about 130 MPa/min. The target pressure was held for 5 min and released within 5 sec.

3.2.4 Image analysis

The image analysis method was the same as that in section 2.2.4.

3.2.5 Electrical impedance spectroscopy (EIS)

The image analysis method was the same as that in section 2.2.5. As necessary, the SCA of the samples after treatment was normalized as SCA_n by the SCA of unimpregnated fresh apple, of which SCA varies due to interindividual differences.

3.2.6 Low-field Nuclear Magnetic Resonance (LF-NMR)

The LF-NMR method was the same as that in 2.2.6, with a modification. Briefly, the optimal scan number was set at 16 scans.

3.2.7 Textural analysis

The texture analysis method was the same as that in 2.2.7, with a modification. Briefly, the optimal compression test was performed by using the plunger No. 5 of 5 mm in diameter.

3.2.8 Statistical analysis

The data of impregnation ratio, normalized SCA, and normalized values for breaking stress and breaking strain were analyzed by Tukey–Kramer test using Excel 2016 (Office 365, Microsoft Co., Ltd, Redmond, USA). For each analysis, a significant

difference was statistically judged at a confidence level of 95 %.

3.3 Results and discussion

3.3.1 Impregnation ratio

As visual qualitative evaluations, Fig. 3-1 shows 2-bit and 8-bit images of apple samples after vacuum heat sealing (VHS) and after a combination of VHS and HHP treatment at 100 MPa (denoted by VHS+100 MPa) . The black area in all the 2-bit images after VHS+100 MPa was equal to the total area of the samples, indicating full liquid impregnation (Fig. 3-1(B)). On the other hand, the unimpregnated area (white area in 2-bit image) of the VHS samples tended to decrease with extended storage (Fig. 3-1(A)). It was likely that the samples after VHS were further impregnated by the subsequent storage. As a quantitative evaluation of the impregnation efficacy, the impregnation ratio was evaluated for each sample based on the 2-bit images, and the effect of the storage time (up to 120 min) after VHS on the impregnation ratio was demonstrated in Fig. 3-2. As observed visually and qualitatively, regardless of the storage time, the impregnation ratio after the combination process (VHS+100 MPa) reached 100%. On the other hand, with increased storage time (S_0 to S_{120}), the impregnation ratio after VHS increased from 80.0 ± 10.9% (VHS+S_0) to approximately 98%: 97.7 ± 3.7% (VHS+S_{30}), 98.4 ± 2.3% (VHS+S_{60}), and 98.9 ± 1.8% (VHS+S_{120}). It was indicated that the newly defined impregnation ratio evaluated the efficacy of liquid impregnation quantitatively.

Image analysis was adopted to quantify the impregnation efficacy into apple as impregnation ratio (Fig.3-2). As compared with other conventional quantitative methods, such as the quantification of increases in volume, weight, and solute (Atarés *et al*., 2008; Schulze *et al*., 2012; Gras *et al*., 2003), the image analysis evaluating impregnation ratio may also be available as an alternative quantitative method, since image analysis using a pigment solution gives visual evidence of liquid impregnation as well as a quantitative parameter ‘impregnation ratio’ without too much laborious works. As shown in Fig.3-2, VHS impregnation of apple did not reach an equilibrium within 30 min of storage after VHS. Furthermore, impregnation ratio of VHS apples did not achieve 100% even after storage for 120 min. Meanwhile, a combination of VHS and 100 MPa treatment achieved 100% impregnation regardless of the storage. It was indicated that impregnation efficacy of VHS was time-dependent, and subsequent 100 MPa treatment supplemented the efficacy of VHS impregnation. Since apple has porous structures holding gas and liquid (Allali *et al*., 2010), the porous structures may provide spaces for the external liquid to fill in when apple is impregnated (Fito *et al*., 2001). In general, as for gas–liquid exchange in vacuum impregnation of fruits and vegetables, the impregnation reaches an equilibrium when the interior pressure becomes equal to the exterior pressure after VHS (Zhao *et al*., 2004). This

study revealed that a small amount of the internal gas remained in VHS samples as white area in 2-bit images even after the storage of 120 min (Fig. 3-1(A)), indicating possible equilibria at impregnation ratios below 100%. The residual internal gas might have been compressed by hydrodynamic actions under ambient pressure (Fito *et al*., 1996), possibly contributing to the increasing trend of impregnation ratio during storage after VHS. Meanwhile, a combination of VHS and 100 MPa treatment achieved an impregnation ratio of 100% (Fig. 3-2), indicating that internal gas of apple could be replaced with external liquid partly by VHS and then supplementally by 100 MPa treatment. It has been reported that gas solubility increased with increased pressure in apple juice (Calix *et al*., 2008). Therefore, 100 MPa treatment after VHS might have compressed or dissolved the residual internal gas into liquid.

3.3.2 Effect of HHP level on the SCA of impregnated apples

Solutes in soaking solution may affect the electrical conductivity in EIS, but no significant difference in SCA_n was confirmed between impregnated apple samples soaked in reverse osmosis (RO) water and those soaked in red pigment solution ($p \geq 0.05$; data not shown). Therefore, the samples were prepared by soaking apple pieces in RO water instead of red pigment solution for apple as described in the chapter 2. EIS was carried out by using the samples which were subjected to VHS or AHS, stored for 0 min or 120 min, and HHP-treated at 100 MPa to 600 MPa. As shown in Fig. 3-4, SCA_n decreased from 1.00 ± 0.21 (the control fresh samples) to 0.28 ± 0.10 (VHS+S_0) and 0.15 ± 0.06 (VHS+S_{120}), respectively, indicating cell membrane damages due to VHS. Furthermore, SCA_n decreased with the increased pressure level of the subsequent HHP treatment, indicating more cell membrane damages with a higher level of HHP. After VHS and 120 min storage, SCA_n was comparable ($p \geq 0.05$) between the VHS sample (VHS+S_{120}; 0.15 ± 0.06) and the VHS+100 MPa sample (VHS+S_{120}+100 MPa; 0.13 ± 0.04). Meanwhile, SCA_n was further reduced by HHP treatments at 200-600 MPa ($p < 0.05$): 0.06 ± 0.03 (VHS+S_{120}+200 MPa), 0.07 ± 0.01 (VHS+S_{120}+400 MPa), and 0.06 ± 0.02 (VHS+S_{120}+600 MPa). There was no significant difference in SCA_n among the HHP-treated samples at 200, 400, and 600 MPa with or without storage after VHS (VHS and VHS+S_{120}) ($p \geq 0.05$). In addition, AHS samples showed slightly higher SCA_n than the samples VHS+S_0 or VHS+S_{120}, indicating that cell membrane could be damaged more by VHS than by AHS or HHP treatments at 100–600 MPa.

It was reported that shrinkage of the circular arc in the Cole–Cole plot as an index of cell membrane damage was induced by heating (Watanabe *et al*., 2019), freezing-thawing (Ohnishi *et al*., 2004), and HHP treatment (Villacís *et al*., 2008; Lee *et al*., 2019);. The damage allows intracellular fluid to leak out of the cell and to flow into the extracellular fluid, resulting in reduced resistance of the extracellular fluid (Ando *et al*.,

2017). In this study, SCA as an index of cell membrane damage was significantly decreased by VHS and HHP treatment (Figs. 3-3 and 3-4). It was reported that VHS caused loss of native liquid in plant tissues, indicating that cell membrane damages caused partial leakage of native liquid out of the tissue cells (Denoya *et al*., 2015; Gras *et al*., 2002). This phenomenon would have occurred in the VHS apple samples in this study. Furthermore, cell membrane damages were comparable between the samples after VHS and those after VHS+100 MPa treatment, and the damages were further aggravated by the treatments at 200 to 600 MPa (Figs. 3-4 and 3-8)). It was reported that VHS and subsequent HHP treatment (500 MPa, 20 °C, 5 min) caused softening and translucency of peach samples indicating cell wall and membrane ruptures probably due to the impact of VHS rather than HHP treatment itself (Denoya *et al*., 2015).

3.3.3 Effect of HHP level on the relaxation time and area fraction of impregnated apples

Water mobility and distribution of impregnated apples were evaluated by relaxation time and area fraction of peak 1 (bound water), peak 2 (semibound water), and peak 3 (free water) as shown in Fig.3-5. The three relaxation time peaks located between 20 to 80, 100 to 200, and 800 to 1200 ms, and it can be assigned to water located in the vacuolar, cytoplasm and cell wall compartments (Hills *et al*., 1997), respectively. The values of T_{21} or A_1 of all tested samples were comparable with each other, indicating impregnations via VHS and/or HHP treatment slightly affected the bound water. As for semibound water, T_{22} increased from 134.79 ± 14.90 (the control fresh samples) to 224.34 ± 58.36 (VHS) and 187.78 ± 41.01 (VHS+100 MPa), respectively. Furthermore, T_{22} decreased by HHP treatments at 200-600 MPa: 95.94 ± 9.19 (VHS+200 MPa), 118.71 ± 6.47 (VHS+400 MPa), and 136.61 ± 11.22 (VHS+600 MPa). indicating severe water loss after the impregnation. As for the free water, T_{23} has a similar tendency of T_{22}, it significantly increased from 1072.27 ± 0.01 (the control fresh samples) to 1152.06 ± 112.84 (VHS) and 1141.96 ± 60.36 (VHS+100 MPa), respectively, it was further decreased with the increased pressure level of the subsequent HHP treatment, indicating more water loss with a higher level of HHP (Fig. 3-6).

It has been reported that water loss of strawberry caused by HHP treatment (300 MPa for 5 min) leads to a significant decrease in relaxation time and area fraction of semibound water and free water (Marigheto *et al*., 2004), HHP treatments at 200 MPa and higher may severely damage the cell membrane of apples, and internal fluid may leak out of the cell and tissue then resulted in the reduction of T_{22} and T_{23}.

As shown in Fig. 3-5(B), A_2 significantly increased by VHS and VHS+100 MPa from 8.4 ± 1.4 % (the control samples) to 19.7 ± 2.2 % and 20.7 ± 5.5 %, respectively,

while A_3 significantly decreased by VHS and VHS+100 MPa from 85.6 ± 1.4 % (the control fresh samples) to 73.3 ± 2.0 % and 73.3 ± 5.9 %.

Increase in the area fractions of semibound water after a freezing-thawing process may be ascribed to water flow from the vacuole into the cytoplasm and intercellular spaces due to cell membrane rupture caused by the process (Xu *et al*., 2015). In this study, external liquid might be impregnated into the cytoplasm or intercellular spaces after VHS or VHS+100 MPa rather than vacuole of samples, resulting in the increase in A_2.

3.3.4 Effect of HHP level on the texture of impregnated apples

In texture analysis, breaking stress and breaking strain, which reflected hardness and deformability, respectively (Imai *et al*., 2008; Oey *et al*., 2007). The parameters were normalized by those of fresh control samples to minimize interindividual and intraindividual differences of apple. Normalized breaking stress significantly decreased below 0.4 after VHS and/or HHP treatments ($p < 0.05$) (Fig. 3-7(A)). HHP-treated samples after AHS tended to show higher breaking stress than those after VHS, indicating aggravated texture damages by VHS. Furthermore, HHP-treated apples after AHS showed higher breaking stress, indicating slightly better hardness. Storage for 120 min after VHS did not affect the subsequent HHP treatment at any HHP levels significantly ($p \geq 0.05$).

Normalized breaking strain increased with increased pressure levels, and the increase was notable with the samples treated at 200, 400, and 600 MPa (Fig. 3-7(B)). It should be noted that the normalized breaking strains of AHS/VHS+100 MPa samples were almost comparable or a little higher than those of AHS/VHS samples without HHP treatment. Furthermore, normalized breaking strain was comparable between AHS and VHS+S_{120} for those samples treated with 100 or 200 MPa. VHS significantly increased the normalized breaking strain of the samples treated at 400 and 600 MPa, indicating aggravated texture damage.

The HHP-treated samples after AHS showed relatively higher breaking stress (hardness) and lower breaking strain (deformability) than HHP-treated samples after VHS (Fig. 3-7). In addition, as visually observed, insufficient impregnation was obvious among all the HHP-treated samples after AHS (data not shown). It was indicated that a synergy of VHS and HHP treatment caused more texture damage on apple than the other treatments.

HHP treatment without VHS (denoted by AHS+HHP) is probably due to insufficient liquid impregnation. It was reported that HHP-treated (250 MPa, 35 °C, 10 min) apple tissue showed a loose and irregular microstructure because of cellular rupture and turgor loss (George *et al*., 2016). Turgor pressure is considered as an indispensable factor contributing to tissue turgidity and rigidity (Oey *et al*., 2007). Depending on intracellular osmotic pressure, turgor pressure is exerted by intracellular liquids on the cell

membrane and cell wall (Oey *et al*., 2007), and it positively impacts the hardness to fruits such as apple (Iwanami *et al*., 2008). Turgor pressure of HHP-treated radish samples decreased with increased HHP levels from 150 to 200 MPa (Rux *et al*., 2020). Meanwhile, it was reported that HHP treatment at 200 MPa induced irreversible texture loss in apple samples and 100 MPa treatment did not induce serious damages in cellular structures, suggesting that the initial texture loss by 100 MPa treatment might have recovered during pressure holding (15–60 min; Basak *et al*., 1998). In this study, although the breaking stress (hardness) among all the VHS samples (VHS+S_0 and VHS+S_{120} in Fig. 3-7(A)) were comparable with each other, the breaking strain (deformability) of the samples significantly increased with increased pressure level from 200 to 600 MPa (Fig. 3-7(B)) while SCA_n decreased (Fig. 3-4). It can be suggested that the factors causing the additional increase in breaking strain could be ascribed to cell membrane damages induced by HHP treatments at 200 MPa and higher.

3.3.5 Effect of storage time after VHS on the SCA and texture of apples

Since the impregnation ratio increased with extended storage time (Fig. 3-2), it was speculated that the storage time after VHS may have also affected the quality of impregnated apples by VHS+HHP. Therefore, apple pieces after VHS were stored for 0 min to 120 min (S_0 to S_{120}) before HHP treatment at 100 MPa, and the impregnated samples were subjected to EIS (Fig. 3-8) and texture analysis (Fig. 3-9). As can be seen from Fig. 3-8, VHS reduced SCA_n of all tested samples, depending on the storage time. It was likely that storage for 30 min or 60 min resulted in an equilibrated low SCA_n or a maximum of cell membrane damage. As for the VHS samples after 100 MPa treatment, SCA_n increased as storage time was extended, while it reached a plateau after 30 min or 60 min. The difference in SCA_n between the samples VHS and VHS+100 MPa was not significant for the samples stored for 60 and 120 min ($p \geq 0.05$).

As for the texture analysis, VHS reduced the breaking stress, which was further slightly reduced by subsequent 100 MPa treatment (Fig. 3-9(A)). Storage time (0–120 min) did not affect its reductions. Breaking strain was comparable among the control and VHS samples. Furthermore, it was significantly higher for VHS+100 MPa samples with no / short storage (VHS+S_0 and VHS+S_{10}) than VHS samples, and it decreased as storage time extended.

VHS enhances liquid impregnation of fruits and vegetables (Santarelli *et al*., 2021), whereas it damages plant tissues (Neri *et al*., 2016; Biegańska-Marecik *et al*., 2007). The tissue damage solely due to VHS has not been evaluated by EIS, although the damage has been studied by texture analysis (Biegańska-Marecik *et al*., 2007). In this study, EIS was adopted to evaluate cell membrane damage quantitatively as SCA. However, it should be

noted that EIS may reflect electrical resistance of the plant tissue as well as cell membrane damage (Ando *et al*., 2014). Since the electrical resistance of external dilute pigment aqueous solution is lower than that of internal gas, the electrical resistance of apple sample could be reduced by gas exclusion from and/or liquid uptake into the cells due to VHS. This might contribute to the decrease in SCA_n after VHS (VHS+S_0/S_{120} at 0.1 MPa in Fig. 3-4). In addition, reduced breaking stress due to VHS at 0.1 MPa (Fig. 3-7(A)) indicated texture deterioration due to apple tissue damage, although the breaking strain was not significantly affected (Fig. 3-7(B)). The tissue damage can be ascribed to cell membrane damage as evaluated by SCA_n (Fig. 3-4). In this study, although the contributions of electrical resistance and cell membrane damage to SCA data cannot be distinguished, SCA evaluated both contributions as a whole.

Combinations of VHS and HHP treatment at 100 MPa and higher achieved an impregnation ratio of 100% (Fig. 3-2; data not shown for 200–600 MPa), whereas the combination of VHS and 100 MPa treatment minimized cell membrane damage (Fig. 3-4) and texture damage (Fig. 3-7(B)). Furthermore, storage time after VHS seemed to play an important role to equilibrate the impregnation (Fig. 3-2). For further clarifying the impregnation damages during storage after VHS, the effect of storage time after VHS on the cell membrane damage (Fig. 3-8) and texture (Fig. 3-9) was studied after the combination of VHS and 100 MPa treatment. Storage after VHS negatively affected the quality of VHS samples (VHS): SCA_n was the highest and breaking stress was the lowest at VHS+S_0, while breaking strain was comparable (Figs. 3-8 and 3-9). In contrast, it was positive for VHS+100 MPa samples: SCA_n was the lowest and breaking strain was the highest at VHS+S_0, while breaking stress was comparable. As can be seen from Fig. 3-2, the impregnation was mostly (approximately 80%) achieved by VHS+S_0 and thereafter proceeded to an equilibrium at slightly below 100% (VHS+S_{30}, VHS+S_{60}, and VHS+S_{120}). It can be speculated that the storage after VHS reduced SCA_n via replacement of remaining gas with liquid in the tissue during storage and that the replacement might have hardened the unimpregnated gas-containing soft tissue part and resulted in increased breaking stress during storage. In addition, it can also be speculated that 100 MPa treatment might have compressed the gas-containing soft tissue and resulted in the aggravated damages of the cell membrane and texture for unequilibrated samples (VHS+S_0 and VHS+S_{10}).

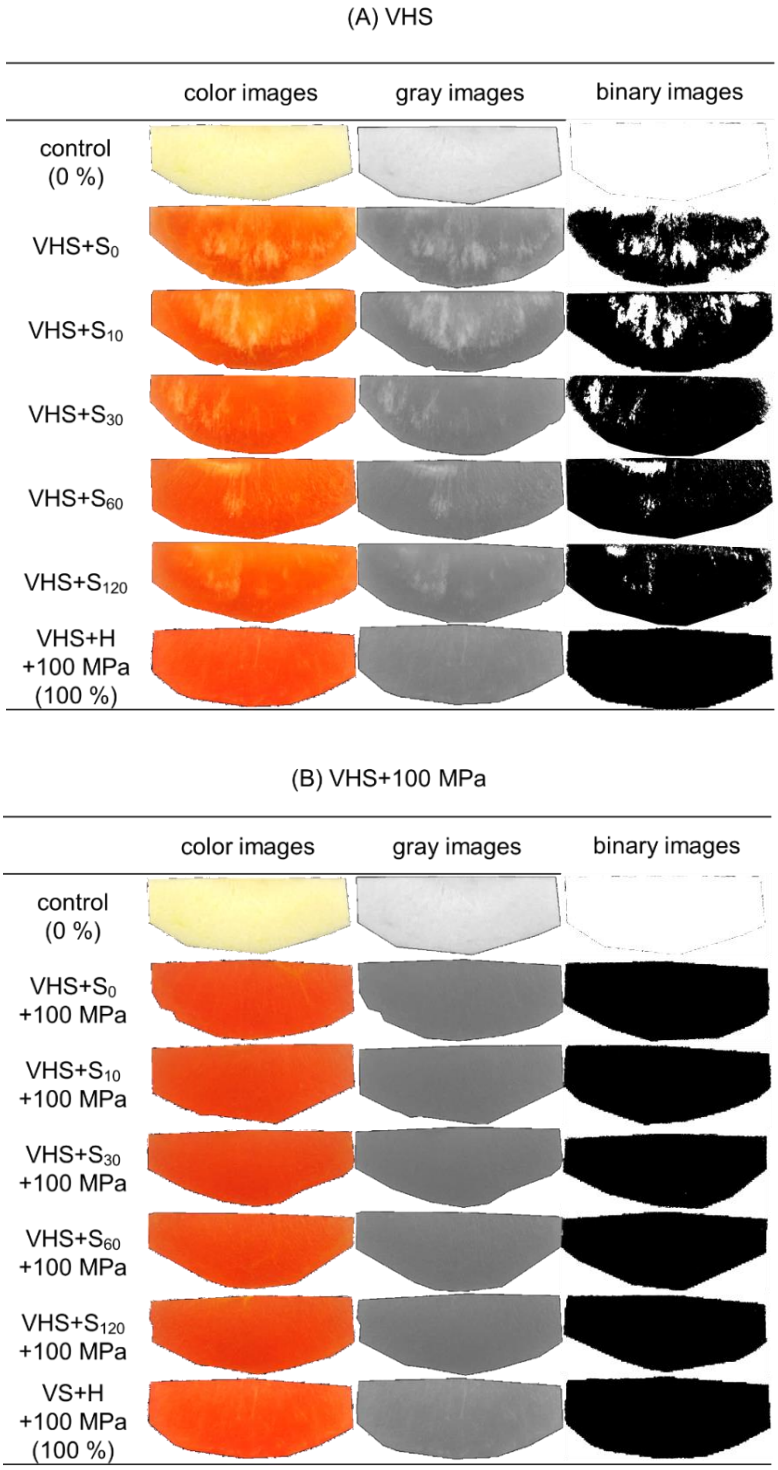

Fig. 3-1 Color (RGB bit), gray (8 bit), and binary (2 bit) images of apple pieces impregnated with 0.05 % red pigment solution. A, vacuum heat sealing (VHS) at 1.0 kPa and 25 ºC for 1 min; B, VHS followed by HHP treatment at 100 MPa and 25 ºC for 5 min (VHS+100 MPa). S_t, storage after VHS at 4 ºC for t min (t = 0, 10, 30, 60, and 120). Impregnation ratio of the sample treated at 100 MPa and 65 ºC for 30 min (VHS+H+100 MPa) was defined as 100 %. Each color image was a representative from independent trials repeated at least 4 times.

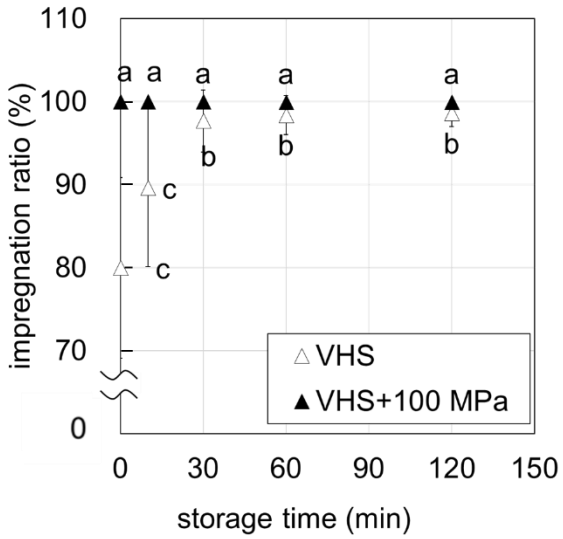

Fig. 3-2 Effect of storage time after vacuum heat sealing on the impregnation ratio of carrots. VHS, vacuum heat sealing (1.0 kPa and 25 ºC for 1 min); VHS+100 MPa, VHS followed by 100 MPa treatment at 25 ºC for 5 min. Values with different alphabets are significantly different (Tukey-Kramer test, $p < 0.05$). Impregnation ratio was expressed as average and standard deviation of independent trials repeated at least 4 times.

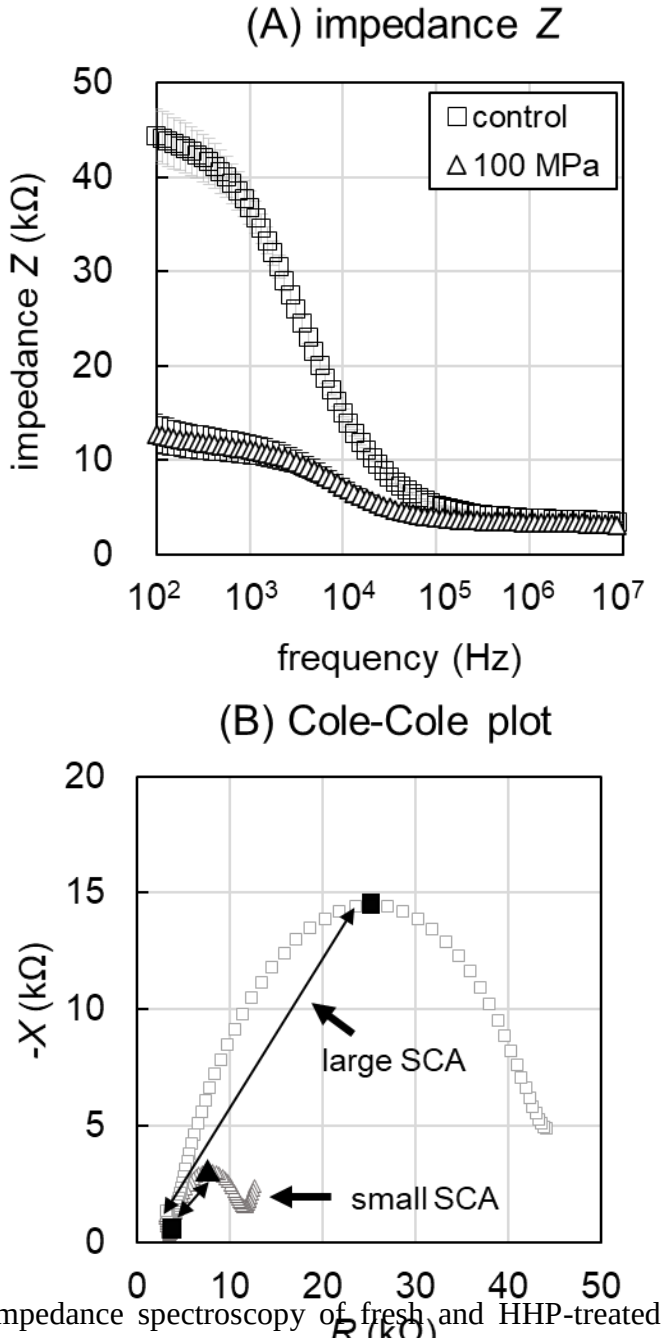

Fig. 3-3 Electrical impedance spectroscopy of fresh and HHP-treated apples as typical examples. A, impedance magnitude; B, Cole-Cole plots. HHP-treatment was carried out at 100 MPa and 25 ºC for 5 min. Values with different alphabets are significantly different (Tukey-Kramer test, $p < 0.05$). EIS measurement was repeated for each sample at least 6 times.

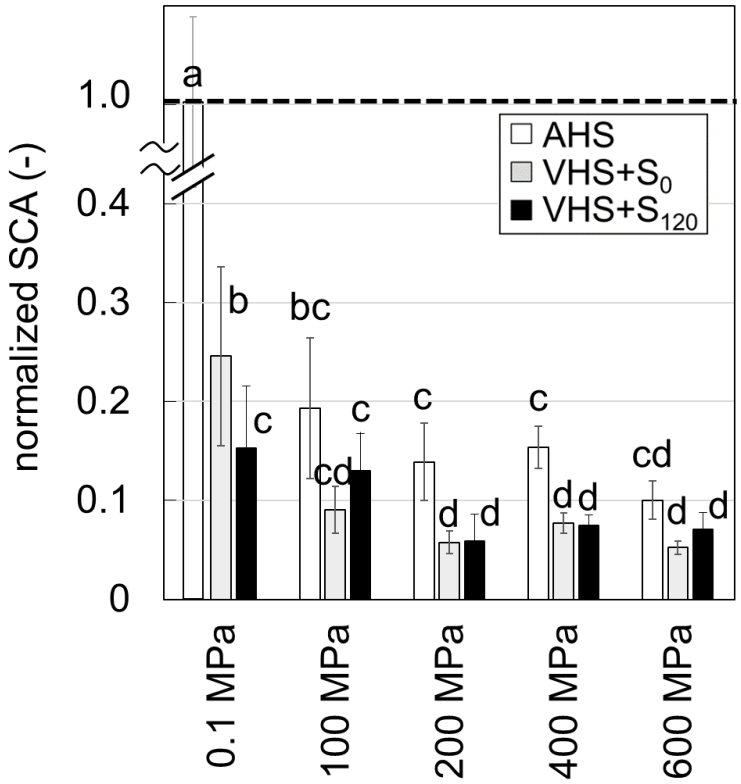

Fig. 3-4 Effect of vacuum heat sealing and subsequent storage on the SCA_n of HHP-treated apples. AHS, ambient heat sealing (0.1 MPa and 25 °C) followed by HHP treatment; VHS+S_0, vacuum heat sealing (VHS; 1.0 kPa and 25 °C for 1 min) immediately followed by HHP treatment; VHS+S_{120}, VHS and subsequent storage at 4 °C for 120 min followed by HHP treatment. HHP treatment was carried out at 100, 200, 400, or 600 MPa and 25 °C for 5 min. Values with different alphabets are significantly different (Tukey–Kramer test, $p < 0.05$). EIS measurement was repeated for each sample at least 6 times.

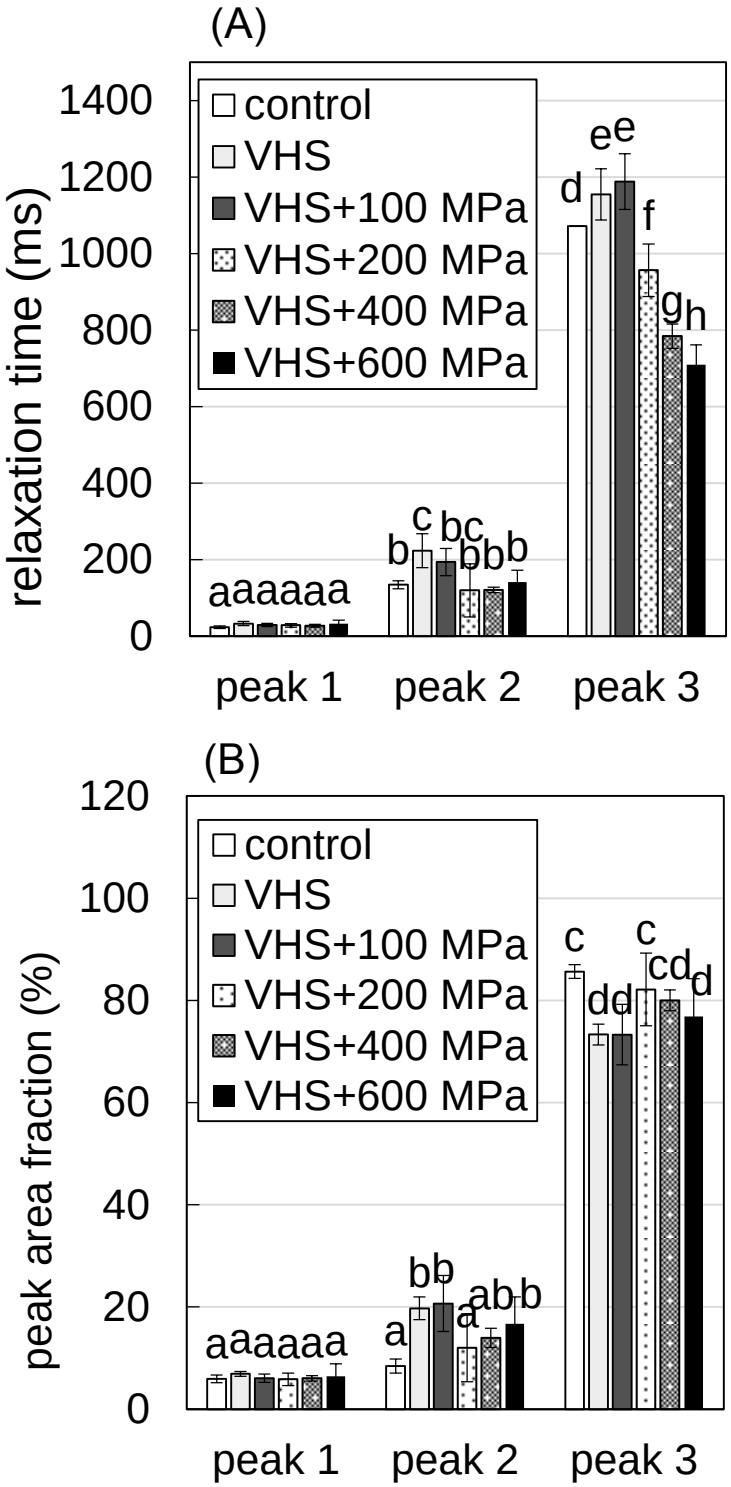

Fig. 3-5 Effect of vacuum heat sealing on the water mobility and distribution (A, relaxation time; B, area fraction) of HHP-treated apples. VHS, vacuum heat sealing (VHS; 1.0 kPa and 25 °C for 1 min) and subsequent storage at 4 ºC for 120 min; VHS+100 to 600 MPa, VHS and subsequent storage at 4 ºC for 120 min, then followed by 100 MPa treatment at 25 °C for 5 min followed by HHP treatment. HHP treatment was carried out at 100, 200, 400, or 600 MPa and 25 ºC for 5 min. Values with different alphabets are significantly different (Tukey-Kramer test, $p < 0.05$). EIS measurement was repeated for each sample at least 6 times.

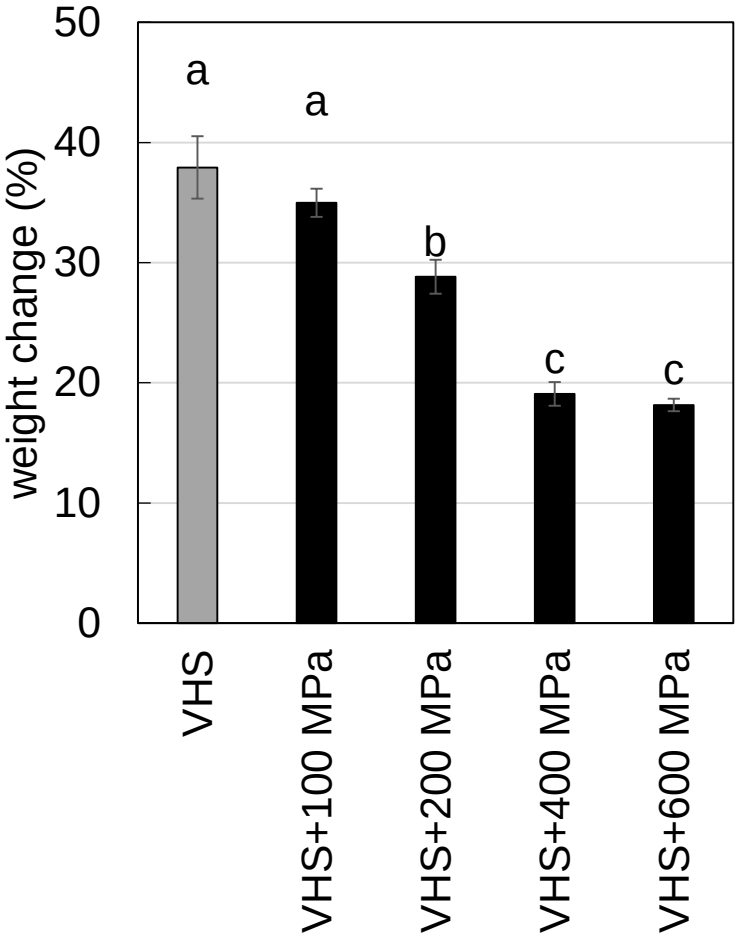

Fig. 3-6 Effect of vacuum heat sealing on the weight change of HHP-treated apples. VHS, vacuum heat sealing (1.0 kPa and 25 ºC for 1 min), stored for 120 min. HHP treatment was carried out at 100, 200, 400, or 600 MPa and 25 ºC for 5 min. Values with different alphabets are significantly different (Tukey-Kramer test, $p < 0.05$).

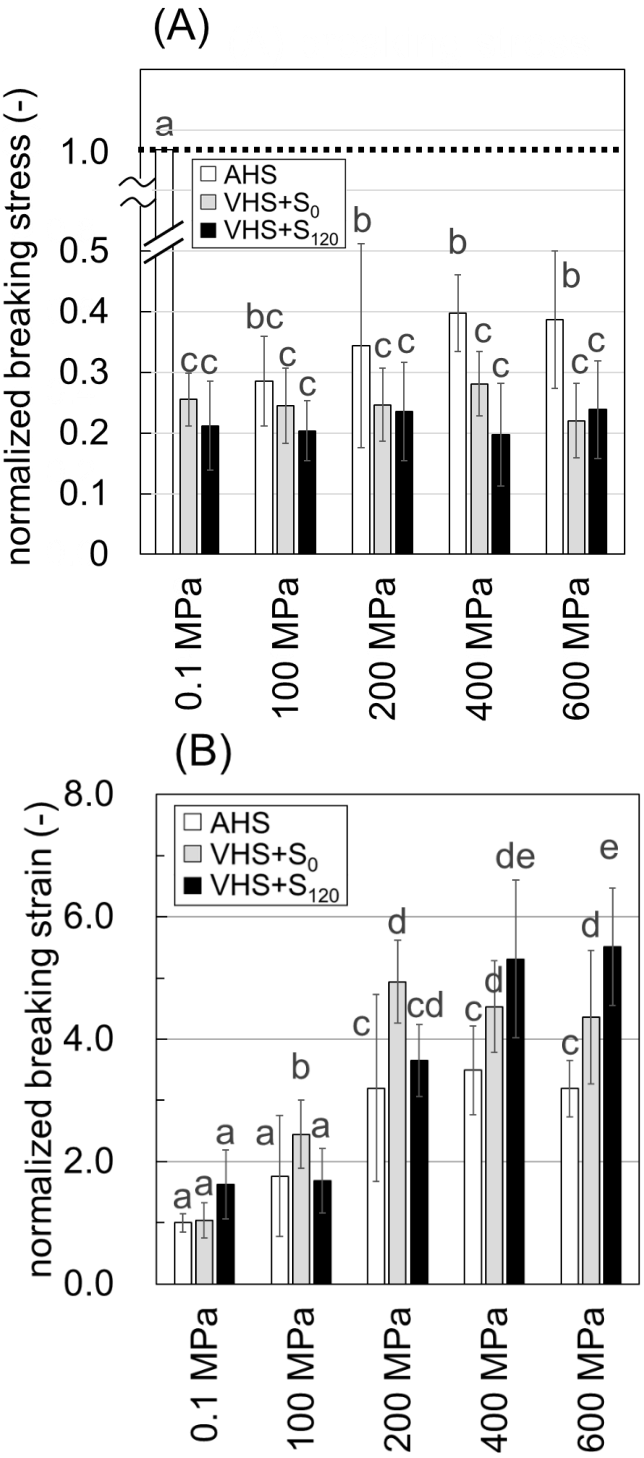

Fig. 3-7 Effect of vacuum heat sealing and subsequent storage on the textural parameters (A, breaking stress; B, breaking strain) of HHP-treated apples. AHS, ambient heat sealing (0.1 MPa and 25 ºC) followed by HHP treatment; VHS+S_0, vacuum heat sealing (VHS; 1.0 kPa and 25 ºC for 1 min) immediately followed by HHP treatment; VHS+S_{120}, VHS and subsequent storage at 4 ºC for 120 min followed by HHP treatment. HHP treatment was carried out at 100, 200, 400, or 600 MPa and 25 ºC for 5 min. Values with different alphabets are significantly different (Tukey-Kramer test, $p < 0.05$). Texture measurement was repeated at least 6 times for each HHP-treated sample.

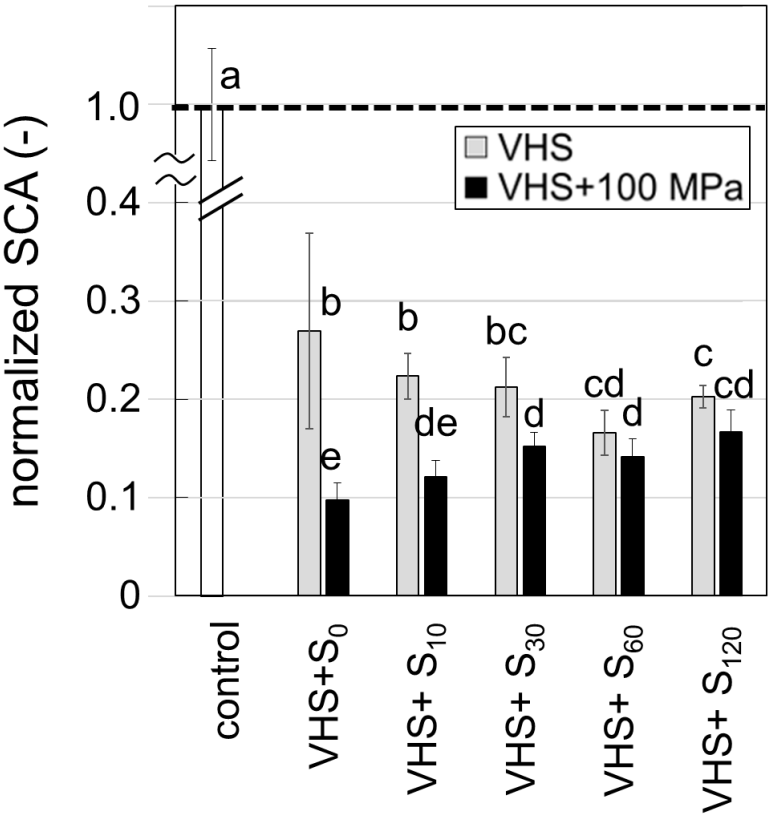

Fig. 3-8 Effect of storage time after vacuum heat sealing on the SCA_n of 100 MPa-treated apples. VHS, vacuum heat sealing; VHS+100 MPa, VHS followed by 100 MPa treatment at 25 °C for 5 min; S_t, storage after VHS at 4 ºC for t min (t = 0, 10, 30, 60, and 120). Values with different alphabets are significantly different (Tukey-Kramer test, $p < 0.05$). Each test was repeated at least 6 times.

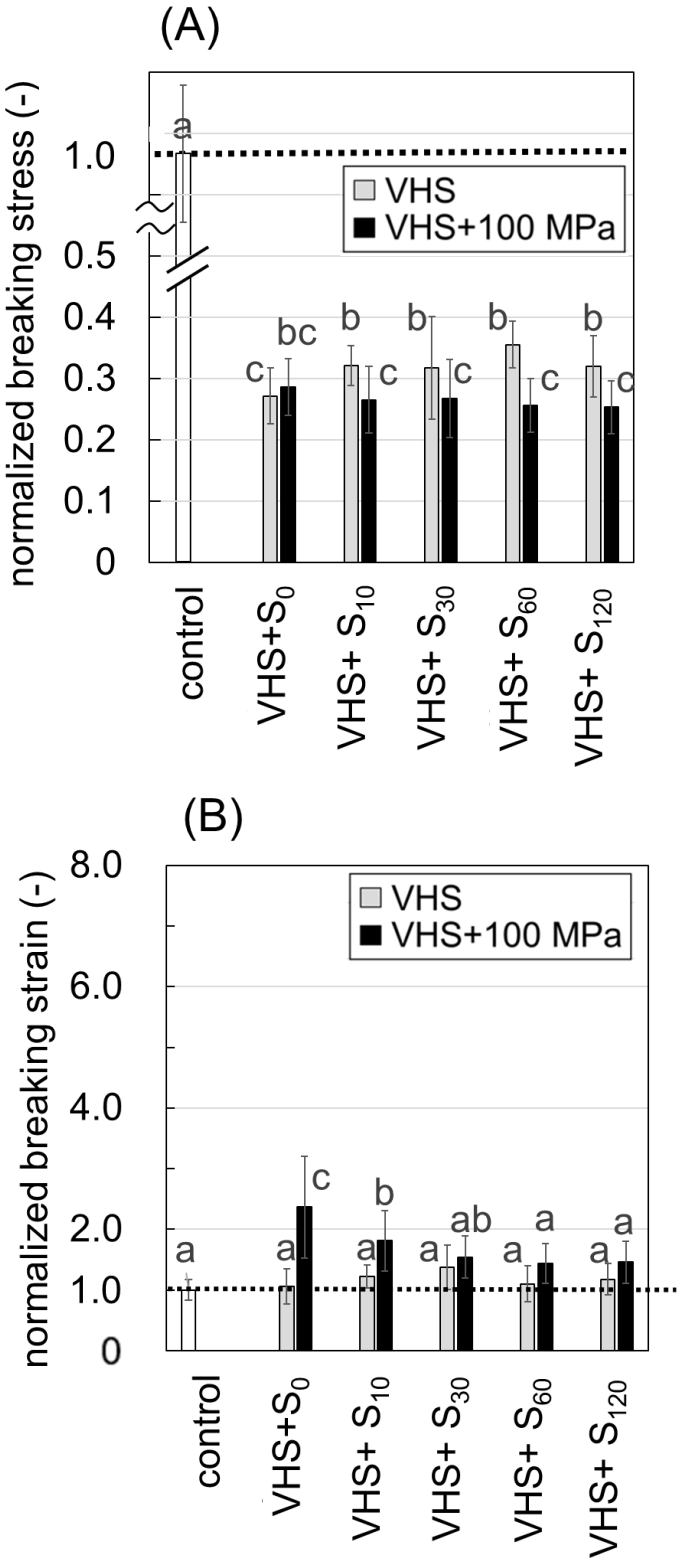

Fig. 3-9 Effect of storage time on the textural parameters (A, breaking stress; B, breaking strain) of vacuum-sealed and 100 MPa-treated apples. VHS, vacuum heat sealing; VHS+100 MPa, VHS followed by 100 MPa treatment at 25 °C for 5 min; S_t, storage after VHS at 4 ºC for t min (t = 0, 10, 30, 60, and 120). Values with different alphabets are significantly different (Tukey-Kramer test, $p < 0.05$). Each test was repeated at least 6 times.

3.4 Summary

The quantitative analyses revealed the effect of VHS, HHP treatment, and their combinations with/without storage after VHS on the impregnated apple. It can be concluded that the optimized condition maximized the impregnation ratio, while suppressing cell membrane damage and texture deteriorations. According to the results, following conclusions can be drawn:

(1) Damages of cell membrane and tissue after VHS were comparable with those after 100 MPa treatment. Damages were enhanced by HHP treatments at 200, 400, and 600 MPa.

(2) Efficacy of liquid impregnation into apple, cell membrane damage, water mobility and distribution, and tissue damage have been quantitatively evaluated.

(3) The combination of VHS, and subsequent storage for 30 min followed by HHP treatment at 100 MPa maximized the impregnation ratio, while minimizing cell membrane damage as reduction in SCA_n and texture deteriorations as reduction in breaking stress and increment in breaking strain.

(4) As for apples, HHP treatment at 100 MPa after VHS can be applied to process high quality apple products for sufficient impregnation, while the damages were minimized.

Chapter 4 Vacuum impregnation of liquid into carrot assisted by high hydrostatic pressure

4.1 Introduction

Carrot is a widely consumed root vegetable, which is a good resource of carotenoids, minerals, and vitamins, and minerals (Singh *et al*., 2021). It has nonporous structures (Srikiatden *et al*., 2008), which are observed as its tightly arranged cells (Nielsen *et al*., 1998) and are related to its sensory crispness (Liu *et al*., 2015). Liquid impregnation is an effective method to provide nutrients, increase the flavor, and extent the shelf lives for fruit and vegetable productions.

The changes in chemical compounds, microstructure, and texture of impregnated carrots have been widely investigated by HPLC (Tumer *et al*., 2021), scanning electron microscopy/SEM (Vargas *et al*., 2009), and texture analysis (Rico *et al*., 2007). However, the effect of HHP treatment on impregnation efficacy, water mobility and distribution, and cell membranes damage has not been quantitatively evaluated.

In the Chapter 4, liquid impregnation was carried out by VHS, HHP treatments (100 – 600 MPa), and their combinations with/without storage after VHS. The applicability of image analysis, EIS, LF-NMR, and texture analysis as quantitative methods to evaluate liquid impregnation of carrot was studied. In addition, the effect of VHS and/or HHP treatment was investigated by the quantitative methods.

4.2 Materials and methods

4.2.1 Materials

Carrots (*Daucus carota* L. Gosun, July 2020 to August 2021) were purchased from local market in Ibaraki prefecture, Japan. Carrots were then stored at 4 °C. The maximum of storage period was 2 weeks. Carrots were cut along the longitudinal axis into square logs with a section of 15 mm × 15 mm or cylinders with a height of 15 mm. As for textural profile analysis (TPA), the logs were cut into cuboids (15 mm × 15 mm × 5 mm); As for the EIS, the logs were cut into cubes (15 mm × 15 mm × 15 mm).

4.2.2 Vacuum heat sealing

Cylindrical carrot (25 g) was put in a translucent plastic retort pouch (BRS-1624S, Meiwa pax Co., Ltd, Osaka, Japan) containing 3.0 % (w/w) aqueous solution (25 g) of a food-grade blue pigment (Kyoritsu-foods Co., Ltd, Tokyo, Japan) consisting of 8 % FD&C Blue No.1 and 92 % dextrin. The pouch was subjected to vacuum heat sealing (VHS) using a vacuum heat sealer (SV-300G, TOSEI Co., Ltd, Shizuoka, Japan) at 1.0 kPa and 25 °C for 1 min and then stored for 0, 10, 30, 60, or 120 min. In addition, some samples were

heat-sealed without vacuum (ambient heat sealing; AHS), while air in the pouch was minimized by manual removal.

4.2.3 High hydrostatic pressure (HHP) treatment

HHP treatment was the same as that in 3.2.3, with a modification: HHP treatment was conducted at 100, 150, 200, 400, and 600 MPa for 5 min.

4.2.4 Image analysis

The image analysis method was the same as that in section 3.2.4.

4.2.5 Electrical impedance spectroscopy (EIS)

The image analysis method was the same as that in section 2.2.5. The SCA of the samples after treatment was normalized as SCA_n by the SCA of unimpregnated fresh carrot, of which SCA varies due to interindividual difference.

4.2.6 Low-field Nuclear Magnetic Resonance (LF-NMR)

The method for LF-NMR was the same as that in 3.2.6.

4.2.7 Textural analysis

The texture analysis method was the same as that in 3.2.7, with a modification: the compression test was performed by using a load cell of 200 N instead of 20 N.

4.2.7 Statistical analysis

The data of impregnation ratio, normalized SCA, and normalized values for breaking stress and breaking strain were analyzed by Tukey-Kramer test using Excel 2016 (Office 365, Microsoft Co., Ltd, Redmond, USA). For each analysis, significant difference was statistically judged at a confidence level of 95 %.

4.3 Results and discussion

4.3.1 Impregnation ratio

Figure 4-1 shows the binary and color image of carrot sample after vacuum heat sealing (VHS) and combinations of VHS and HHP treatments at 100, 150, 200, 400, and 600 MPa (VHS+100-600 MPa) with/without storage (S*t*; $t = 0 – 120$ (min)).

Carrot section is composed of xylem and cortex (phloem), and the boundary is vascular cambium as shown in Fig. 2-2 (Ahmad *et al*., 2019). The effect of storage time on the efficacies of VHS and HHP-assisted VHS impregnation of carrot was shown in Figs. 4-1(A) and 4-1(B), and the effect of HHP level on the impregnation efficacy of carrot was shown in Fig. 4-1(C). The white areas in all binary images corresponded to the unimpregnated areas. As for the effect of storage time on the VHS samples, the outer

cortex was impregnated, while the inner cortex was not impregnated sufficiently. The unimpregnated area of cortex obviously decreased with extended storage time from 0 to 120 min (Fig. 4-1(A)), indicating that the impregnation direction of carrot cortex was from outside to inside, and this impregnation was time-dependent. On the other side, the xylem of carrot was not impregnated sufficiently by VHS without storage (S_0 in Fig. 4-1(A)), and it was mostly impregnated by VHS and storage for 10 min and longer ($S_{10\text{-}120}$ in (Fig. 4-1(A)). Subsequent HHP treatment significantly reduced the unimpregnated areas in the cortex (Fig. 4-1(B)), and impregnated areas were almost equal to the total areas of carrot samples (Fig. 4-1(B) and Fig. 4-1(C)). It was indicated that sufficient impregnation was achieved by combining VHS, storage for 120 min, and HHP treatment. However, the impregnations were heterogenous with the samples which were stored after VHS for 0, 10, and 30 min (S_0, S_{10}, and S_{30} in Fig. 4-1 (B)). As compared with the gray images of VHS+S_0+100 MPa treatment and VHS+S_{120}+100 MPa treatment, the gray area of the cortex was slightly lighter than that of the xylem, indicating that the homogenous impregnations were achieved by VHS+S_{120}+100 MPa treatment and VHS+S_{120}+150 to 600 MPa treatments (Fig. 4-1(C)).

The impregnation efficacy of carrot after VHS was quantified as the impregnation ratio (Fig. 4-2). With the increase in storage time (S_0 to S_{120}), the impregnation ratio after VHS increased from 28.9 ± 6.6 % (VHS+S_0) to 92.9 ± 4.5% (VHS+S_{120}), indicating impregnation efficacy after VHS was improved by the storage depending on the storage time. In contract, subsequent 100 MPa treatment significantly increased the impregnation ratio regardless of the storage ($p < 0.05$), where the impregnation ratio of VHS+S_0+100 MPa was higher than that of VHS+S_0 (Fig.4-2). Furthermore, the impregnation ratio of a combination of VHS, storage for 120 min, and 100 treatment (100, 150, 200, 400, or 600 MPa) all reached 100% (Figs. 4-1(C) and 4-2).

As the carrot was impregnated with the blue pigment solution by VHS, the central xylem was impregnated more thoroughly than the cortex (Fig. 4-1(A)). Plant can absorb and transport water as well as compounds through the xylem (Bollard, 1960). Therefore, liquid impregnation into the central xylem could be easier than the cortex.

Pressure reduction in VHS process induces expansion of inter-/intracellular gas and removal of the gas from the cells (Neri *et al*., 2016). After the process, the pressure goes back to the atmospheric pressure, and the residual expanded gas is again compressed, while external liquid rapidly flows into the tissues. This gas-liquid exchange phenomenon was named as hydrodynamic mechanisms (Fito *et al*., 1996). The impregnation efficacy of VHS carrot without storage (VHS+S_0) was approximately 29% (Fig. 4-2), which was significantly lower that of apple after VHS+S_0 (80 %, in Fig.3-2). Intercellular spaces (internal gas spaces) have been quantitatively described as porosity (Visser *et al*., 2003),

and the porosity of carrot (0.04) is lower than that of apple (0.21) (Karathanos *et al*., 1996), indicating nonporous structure of carrots does not contain as many intercellular spaces as apples. Since the porosity of carrot is lower than that of apple, it can be assumed that lack of intracellular spaces in carrot limited the efficacy of VHS impregnation. In this study, sufficient impregnation of carrot was achieved by a combination of VHS, storage for 120 min, and 100 MPa treatment. It was demonstrated that HHP treatment at 100 MPa can effectively improve the vacuum impregnation into the nonporous carrot as well as the porous apple.

HHP treatment (400 MPa, 5 ºC for 30 min) increased the cell permeability of cauliflower, which allowed the intercellular water to flow into the outside of the cell, resulting in a 'soaked' appearance (Préstamo *et al*., 1998), and the cell permeability was dependent on the integrity of the cell membrane (Picchioni *et al*., 1994). Therefore, it can be speculated that carrots were partly impregnated by VHS, and subsequent HHP treatments might modify the cell membrane integrity of carrots and enhance its permeability, resulting in the improved impregnation.

4.3.2 Effect of storage time after VHS on the SCA and texture of carrots

Since VHS and subsequent storage significantly increased the impregnation ratio of carrot, the cell membrane of carrots after VHS and subsequent storage were evaluated by EIS. Carrots were subjected to VHS with reverse osmosis (RO) water, then stored at 4 ºC for 0, 10, 30, 60, or 120 min. As shown in Fig. 4-3, the SCA_n of VHS samples was significantly higher than fresh carrot ($p < 0.05$). In addition, weight of carrot increased by 8 % after VHS (Fig. 4-7). On the other hand, weight of apple increased by 38% after VHS (Fig. 3-6), and the SCA_n of apple significantly lower than that of fresh apple (Fig. 3-8). Size of Cole-Cole arc was dependent on the electrical resistance of intra-/extracellular fluids of plant food materials (Ando *et al*., 2017). Therefore, it can be assumed the cell membrane of carrot was not damaged by VHS, but the electrical resistance of internal fluids could be diluted by the impregnated water, resulting in increased electrical resistance which could contribute to the increment in the SCA_n after VHS.

Furthermore, the differences in SCA_n among all the VHS samples after storage (VHS+$S_{10\text{-}120}$) were not significant ($p \geq 0.05$), indicating storage time did not affect the SCA_n of carrot. As shown in Chapter 3, the SCA_n of apple was significantly decreased by VHS due to the cell membrane damage, indicating cell membrane of carrot may not be significantly damaged by VHS.

As for the texture analysis, the normalized values of breaking stress and strain of carrot samples slightly increased after VHS, and further storage did not affect the values (Fig. 4-4). It has been reported that pressure reduction in the vacuum process induces

expansion of inter-/intracellular gas and removal of the gas from the cells (Neri *et al*., 2016). Since there were a few intercellular spaces in carrot tissue (Nielsen *et al*., 1998), the effect of VHS and subsequent storage time on the carrot tissue could be negligible.

4.3.3 Effect of pressure level on the SCA of impregnated carrots

Figure 4-5 shows SCA_n of impregnated samples subjected to VHS or AHS, stored for 120 min, and HHP-treated at 100 to 600 MPa. At each pressure level, the SCA_n of VHS samples was slightly higher than that of AHS samples. The SCA_n of AHS+100 MPa (0.94 ± 0.07) or VHS+100 MPa (1.13 ± 0.16) was comparable with that of fresh carrot ($p \geq 0.05$). Meanwhile, SCA_n was significantly reduced by HHP treatment at 150-600 MPa ($p < 0.05$, indicating cell membrane after VHS was significantly damaged by the combined processes of 150 MPa and 200 MPa treatments and further damaged by 400 and 600 MPa treatments.

The SCA_n of VHS+100 MPa was comparable with that of fresh carrot ($p \geq 0.05$), indicating that cell membrane was not damaged by VHS+100 MPa. The reduction of SCA_n after VHS+150 MPa (Fig. 4-5) may suggest that intracellular fluid could leak out of the cell due to the partial cell membrane damages during the impregnation. It was indicated that the critical pressure threshold of cell membrane damage was between 100 MPa and 150 MPa. Furthermore, cell membrane damages were further aggravated by HHP treatments at 400 MPa and 600 MPa (Fig. 4-5). It was reported that HHP treatments at 400 MPa and higher significantly decreased hardness and chewiness of vacuum-packed (or VHS in this study) carrots, indicating texture of carrot were severely damaged (Sun *et al*., 2019). Severe cell membrane damages allowed intracellular fluid to leak out of the cell and flow into the extracellular fluid, resulting in reduced resistance of the extracellular fluid and decrease in the size of Cole-Cole arc (Ando *et al*., 2017).

Turgor pressure is another indicator for evaluating cell membrane damage, since turgor pressure reflects on the integrity of the cell membrane (Oey *et al*., 2007). It has been reported that HHP treatment damaged the cell membrane of radish and reducing the turgor pressure, and HHP treatments over 200 MPa caused irreversible damage to the cell membrane (Rux *et al*., 2020). Probably, this phenomenon also occurred in carrots, while the pressure of HHP exceeds the pressure threshold, the cell membrane might be damaged in different levels, which were positively correlated to the pressure level of HHP treatment (Fig. 4-5).

4.3.4 Effect of pressure level on the relaxation time and area fraction of impregnated carrots

LF-NMR was carried out by the combinations of VHS and HHP treatment at 100, 200, 400, and 600 MPa for 5 min. Water mobility and distribution of impregnated carrots

were evaluated by relaxation times (T_{21}, T_{22}, and T_{23}) and area fractions (A_{21}, A_{22}, and A_{23}) of the peaks 1, 2, and 3 (Fig. 4-6). As for the carrot, three peaks for relaxation time (peaks 1, 2, and 3) were observed at relaxation times of 20 - 40 ms, 20 - 80 ms, and 290 - 800 ms, respectively. The relaxation time of free water (T_{23}) in carrot was shorter than that of apple, indicating that the exchange of water in carrot might be slow due to its tightly arranged cells and small cell size.

The values of T_{21} and A_1 reflecting bound water for all impregnated samples were comparable with each other, indicating impregnations via VHS and/or HHP treatment little affected the bound water. As for semibound water (peak 2), it should be noted that the T_{22} peak for HHP-treated carrot was hardly observed at VHS+400 and 600 MPa treatments, and similar phenomenon was occasionally appeared in other HHP-treated samples (VHS+100 and 200 MPa treatments). It was reported that the number of peaks for relaxation time in tomato seeds was reduced by HHP treatment (500 MPa for 15 min; Unal *et al*., 2020) probably because cell compartments were disrupted at 500 MPa. Besides, A_2 of all impregnated carrot samples were below 4 % (Fig. 4-6(B)), indicating the amount of semibound water in carrot may be lower than that of apple after VHS or VHS+100 MPa treatment (20 %) as shown in Fig. 3-5(B). As for the free water, T_{23} was significantly increased by VHS from 382.1 ± 53.3 ms (control sample) to 550.4 ± 105.5 ms. With the increase in pressure level of HHP treatment (100 to 600 MPa), T_{23} after VHS decreased from 705.3 ± 134.4 % (VHS+100 MPa treatment) to 294.0 ± 42.2 ms (VHS+600 MPa treatment): 624.4 ± 69.7 ms (VHS+200 MPa treatment) and 340.4 ± 65.1 ms (VHS+400 MPa treatment), indicating the mobility of free water was significantly increased by VHS and VHS+100 MPa treatment. The increased mobility of free water may be related to the water uptake during the impregnation processes (Fig. 4-7). In fact, severe water loss was caused by HHP treatments at 400 MPa and 600 MPa and resulted in the reduction of T_{23}.

4.3.5 Effect of pressure level on the texture of impregnated carrots

Texture of impregnated samples was evaluated as normalized breaking stress and strain (in Fig.4-8), which reflected hardness and deformability, respectively. Normalized breaking stress of the carrot sample after each HHP treatment at 100 to 600 MPa was comparable ($p \geq 0.05$) with that of the fresh carrot regardless of sealing methods (AHS and VHS) as shown in Fig. 4-8(A). On the other hand, breaking strain did not differ significantly ($p \geq 0.05$) between AHS/VHS and AHS/VHS+100 MPa treatment, indicating no significant change in the deformability. However, the pressure levels higher than or equal to 150 MPa resulted in significant increases in breaking strain ($p < 0.05$). Besides, it should be noted that the normalized breaking stress and strain of 100 MPa-treated samples were comparable with those of control samples ($p \geq 0.05$), indicating that 100 MPa treatment did not damage both the cell membrane and tissue structure of carrot.

In both cases of AHS and VHS, the breaking stress of the carrot samples after 100 to 600 MPa treatment were comparable with that of the fresh carrot (Figure 8(A)). On the other hand, there is no significant difference between the normalized breaking strain of AHS/VHS+100 MPa and AHS/VHS (Fig. 4-8(B)). However, the normalized breaking strain of the AHS/VHS samples significantly increased by the pressure levels higher than 150 MPa. It was indicated that the critical pressure of carrot tissue was between 100 MPa and 150 MPa. Furthermore, HHP treatments at 150 MPa or higher aggravated tissue damages on the carrot samples. Since the normalized breaking stress and strain of 100 MPa-treated samples were almost comparable with those of control samples, it might be indicated that 100 MPa treatment did not damage the cell membrane and tissue of carrot significantly.

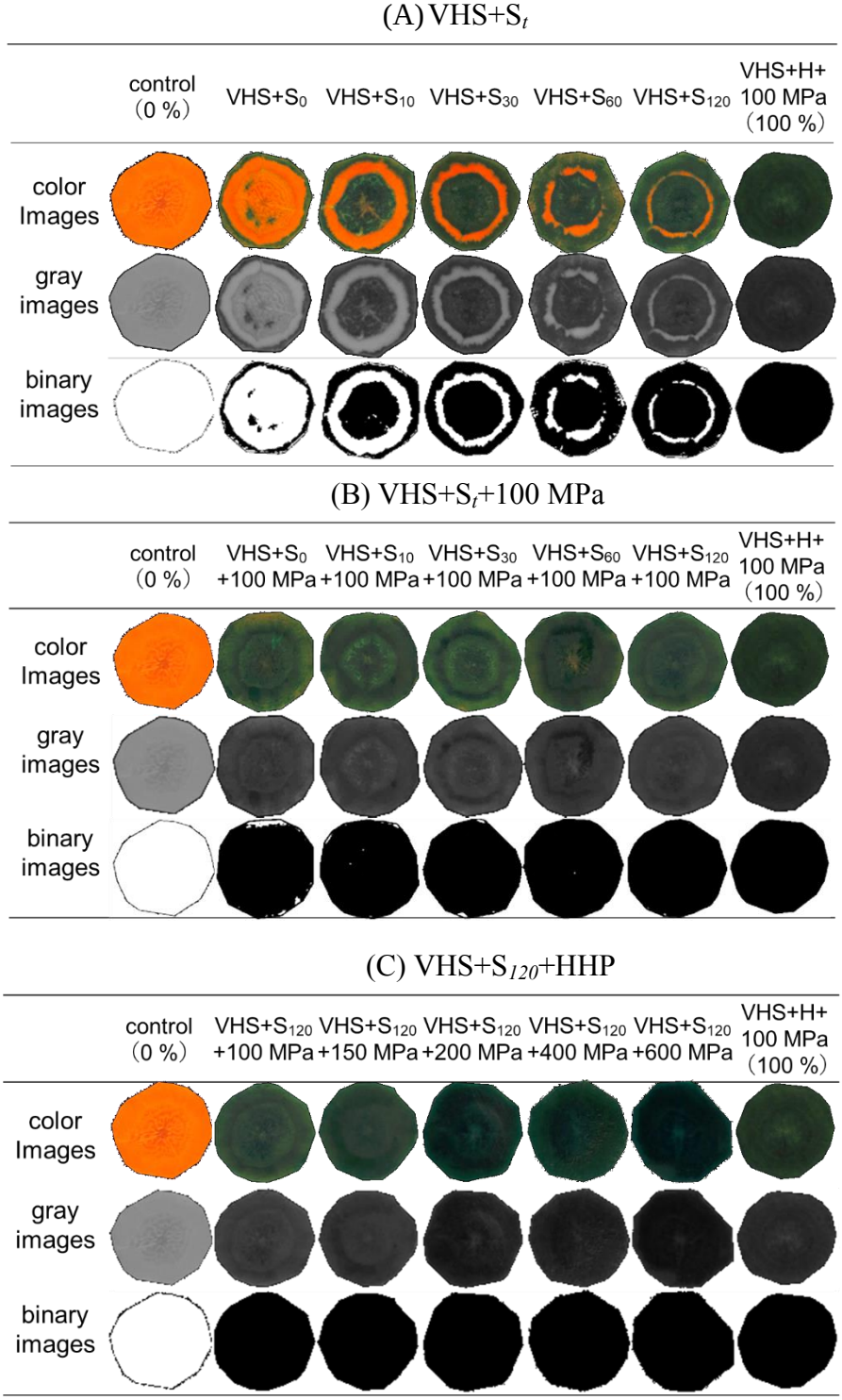

Fig. 4-1 Color (RGB bit), gray (8 bit), and binary (2 bit) images of carrot cylinders impregnated with blue pigment solution. A, vacuum heat sealing (VHS+S_t) at 1.0 kPa and 25 ºC for 1 min; B, VHS followed by HHP treatment at 100 MPa and 25 ºC for 5 min (VHS+S_t+100 MPa). C, VHS followed by HHP treatment at 100, 150, 200, 400, and 600 MPa, 25 ºC for 5 min (VHS+S_{120}+HHP). S_t, storage after VHS at 4 ºC for t min (t = 0, 10, 30, 60, and 120). Impregnation ratio of the sample treated at 100 MPa and 65 ºC for 30 min (VHS+H+100 MPa) was defined as 100 %. Each color image was a representative from independent trials repeated at least 4 times.

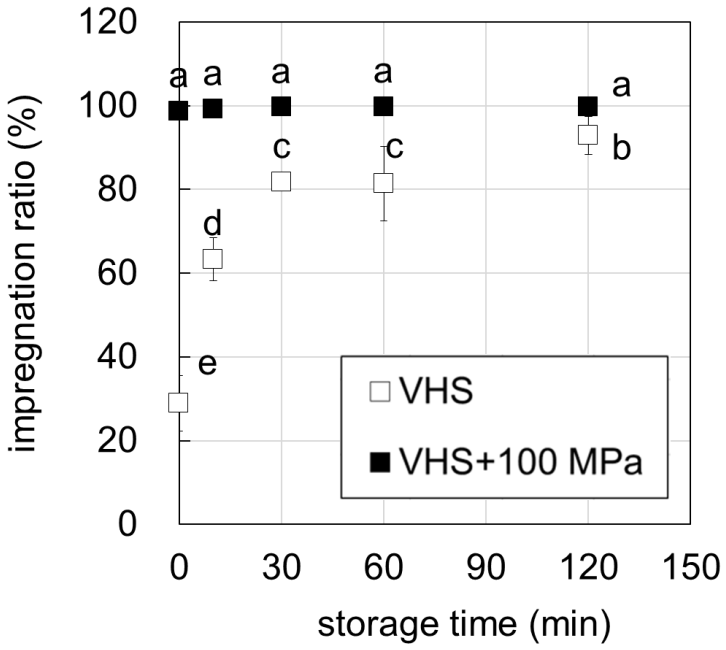

Fig. 4-2 Effect of storage time after vacuum heat sealing on the impregnation ratio of carrots. VHS, vacuum heat sealing (1.0 kPa and 25 ºC for 1 min); VHS+100 MPa, VHS followed by 100 MPa treatment at 25 ºC for 5 min. Values with different alphabets are significantly different (Tukey-Kramer test, $p < 0.05$). Impregnation ratio was expressed as average and standard deviation of independent trials repeated at least 4 times.

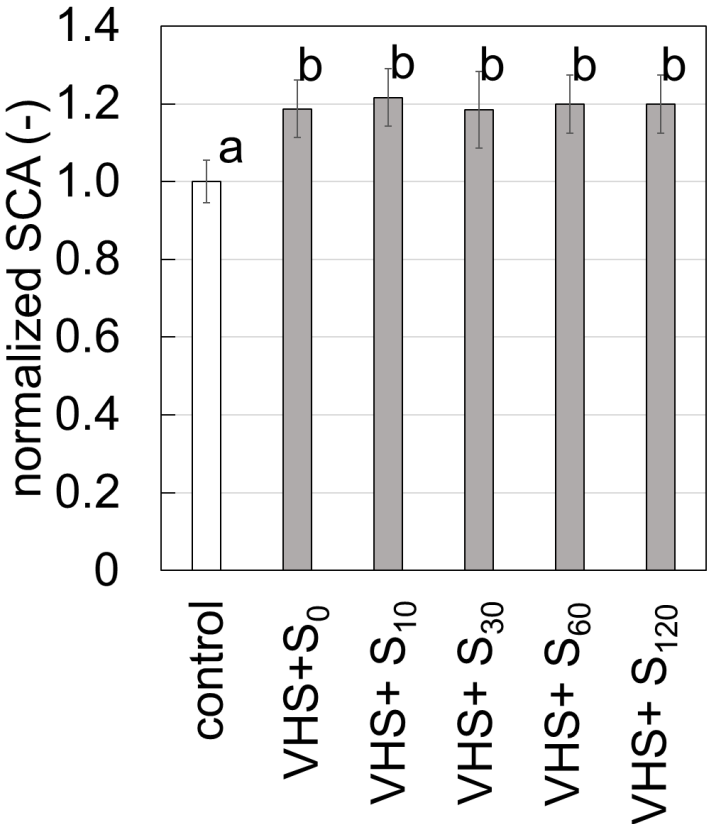

Fig. 4-3 Effect of Effect of vacuum heat sealing and subsequent storage on the SCA of carrots. VHS, vacuum heat sealing; S_t, storage after VHS at 4 ºC for t min (t = 0, 10, 30, 60, and 120). Values with different alphabets are significantly different ($p < 0.05$).

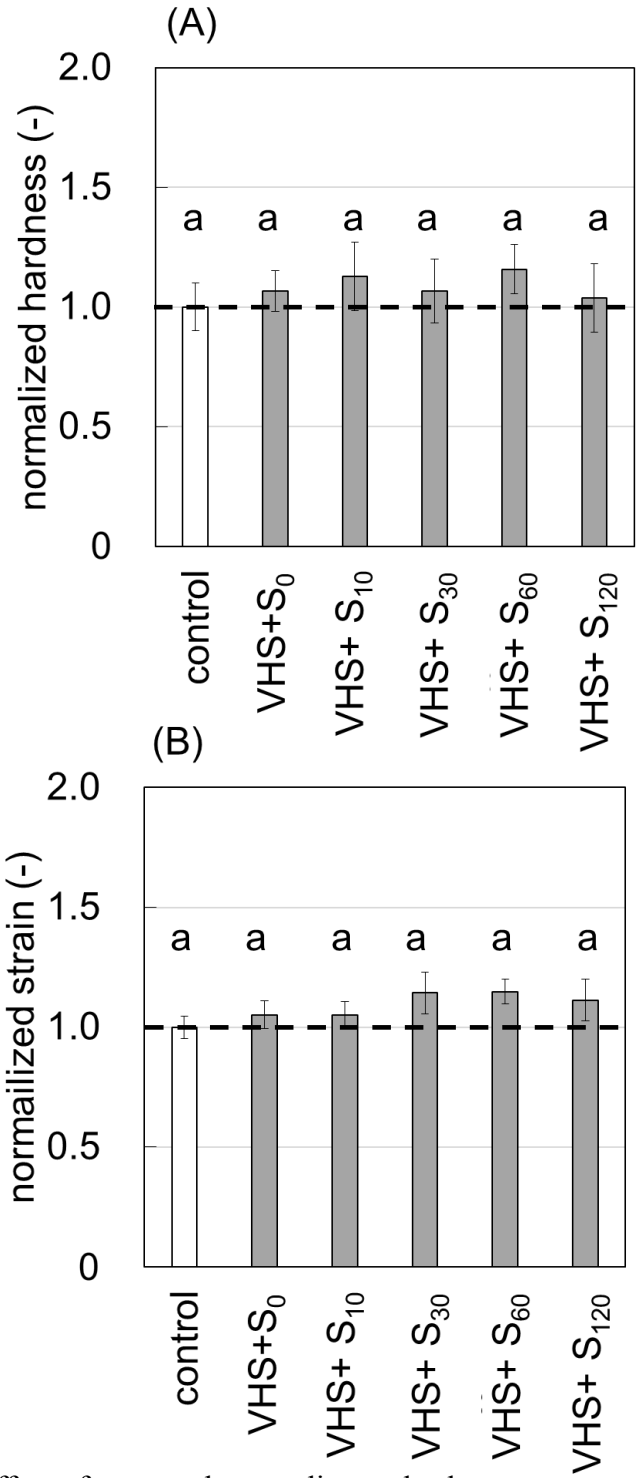

Fig. 4-4 Effect of Effect of vacuum heat sealing and subsequent storage on the textural parameters (A, breaking stress; B, breaking strain) of carrots. VHS, vacuum heat sealing; S_t, storage after VHS at 4 ºC for t min (t = 0, 10, 30, 60, and 120). Values with different alphabets are significantly different ($p < 0.05$).

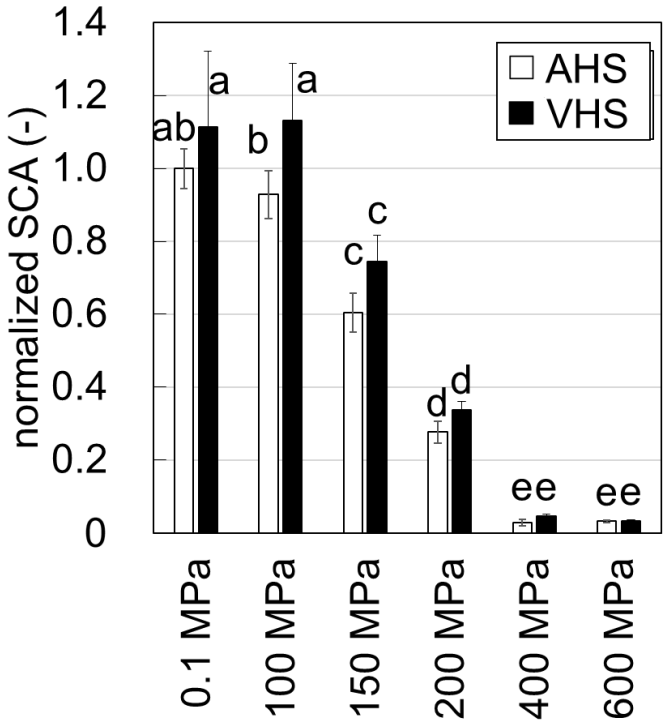

Fig. 4-5 Effect of vacuum heat sealing on the SCA_n of HHP-treated carrots. AHS, ambient heat sealing (0.1 MPa and 25 ºC) followed by HHP treatment; VHS, vacuum heat sealing (1.0 kPa and 25 ºC for 1 min), stored for 120 min then followed by HHP treatment. HHP treatment was carried out at 100, 150, 200, 400, or 600 MPa and 25 ºC for 5 min. Values with different alphabets are significantly different (Tukey-Kramer test, $p < 0.05$).

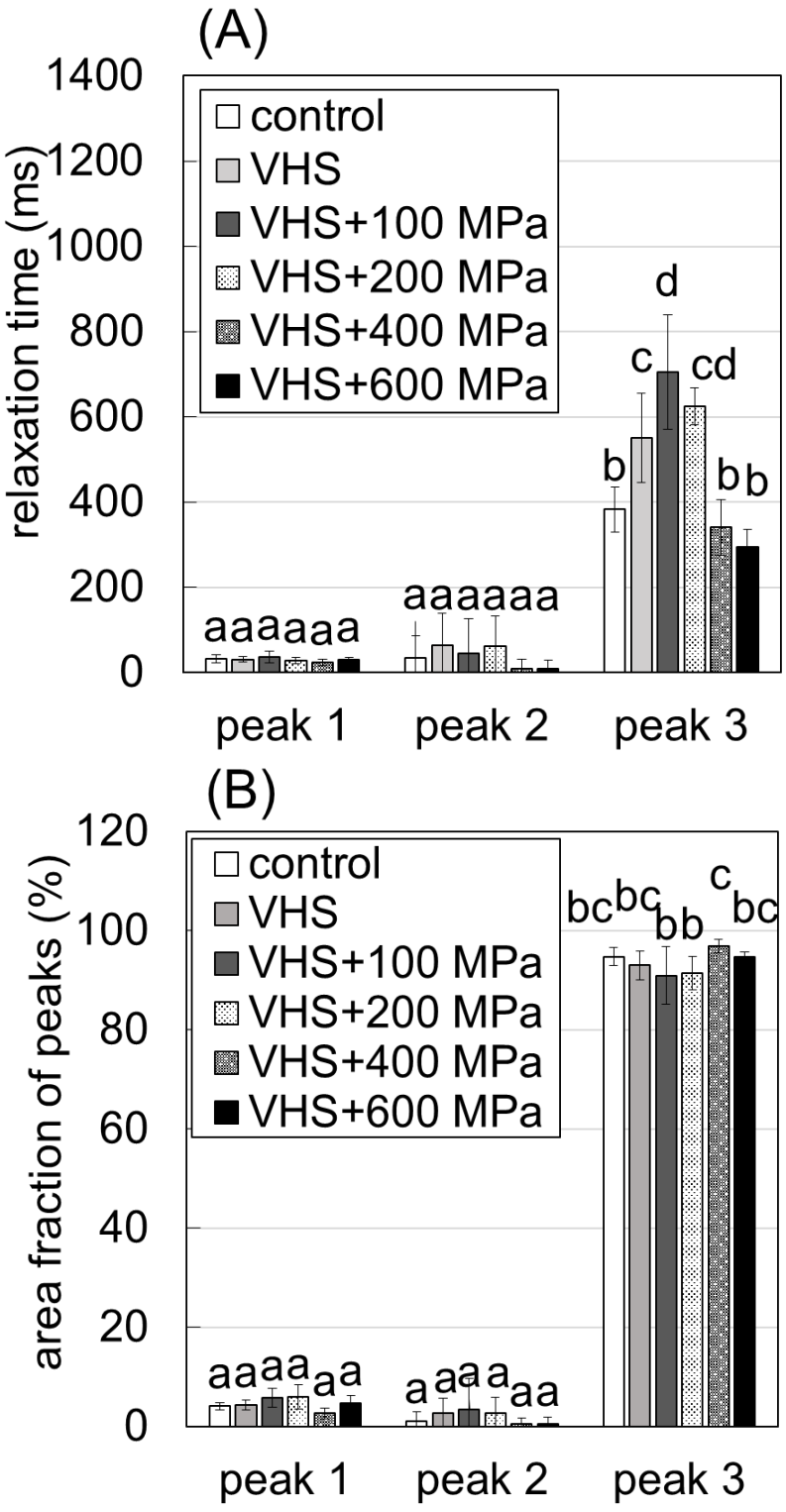

Fig. 4-6 Effect of vacuum heat sealing on the water mobility and distribution (A, relaxation time; B, area fractions) of HHP-treated carrots. VHS: vacuum sealed (with RO water) at 1.0 kPa, at 25 ºC for 5 min, stored for 120 min then followed by HHP treatment. HHP treatment was carried out at 100, 150, 200, 400, or 600 MPa and 25 ºC for 5 min. Values with different alphabets are significantly different (Tukey-Kramer test, $p < 0.05$).

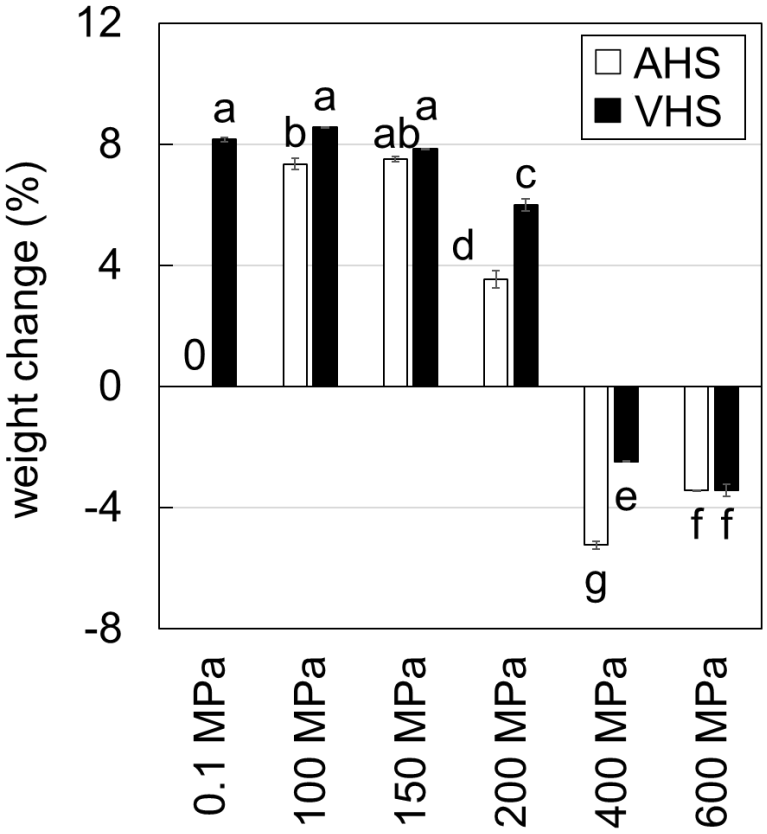

Fig. 4-7 Effect of vacuum heat sealing on the weight change of HHP-treated carrots. AHS, ambient heat sealing (0.1 MPa and 25 ºC) followed by HHP treatment; VHS, vacuum heat sealing (1.0 kPa and 25 ºC for 1 min), stored for 120 min then followed by HHP treatment. HHP treatment was carried out at 100, 150, 200, 400, or 600 MPa and 25 ºC for 5 min. Values with different alphabets are significantly different (Tukey-Kramer test, $p < 0.05$).

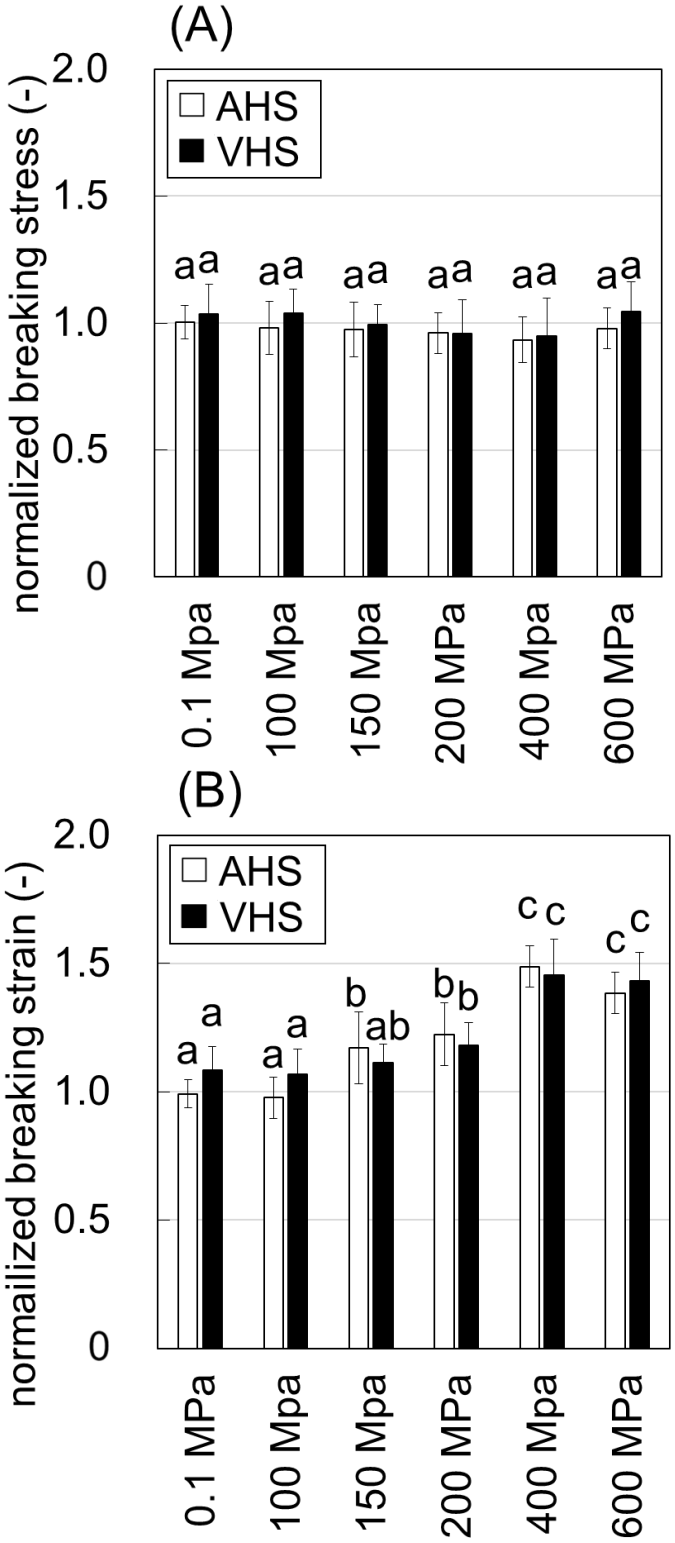

Fig. 4-8 Effect of HHP treatment on the textural parameters (A, breaking stress; B, breaking strain) of HHP-treated carrots. AHS, ambient heat sealing (0.1 MPa and 25 ºC) followed by HHP treatment; VHS: vacuum sealed (with RO water) at 1.0 kPa, at 25 ºC for 5 min, stored for 120 min then followed by HHP treatment. HHP treatment was carried out at 100, 150, 200, 400, or 600 MPa and 25 ºC for 5 min. Values with different alphabets are significantly different (Tukey-Kramer test, $p < 0.05$).

4.4 Summary

The effect of VHS, HHP treatment, and their combinations with/without storage after VHS on the impregnated carrot was investigated. It can be concluded that some combinations successfully minimized the cell membrane and tissue damages of HHP-treated carrots. According to the results, following conclusions can be drawn:

(1) HHP treatment at 150 MPa or higher caused more cell membrane damages on the carrot than at 100 MPa, indicating a critical pressure between 100 MPa and 150 MPa.

(2) The breaking stress of HHP-treated carrots did not significantly change, while breaking strain significantly increased after HHP treatments at 150 MPa and higher, indicating a critical pressure between 100 MPa and 150 MPa.

(3) A maximum liquid impregnation was achieved by a combination of VHS, subsequent storage for 120 min, and HHP treatment at 100 MPa, while damages of cell membrane and tissue were minimized.

(4) As for carrot, HHP treatment at 100 MPa can be applied to process high quality carrot products for sufficient impregnation, while the cell membrane and texture were comparable with that of fresh carrot.

Chapter 5 Conclusions and future research

5.1 Conclusions

Prior to this study, there was limited research focused on the quantitative methods for impregnation efficacy and cell membrane damage of HHP-treated plant food materials. It is the first time that quantitative methods for evaluating impregnation efficacy, cell membrane damage, water mobility and distribution, and tissue damage have been applied to analyze liquid impregnation of apple and carrot as plant food materials with distinct structural features: porous and nonporous structures, respectively. Besides, our study provided a novel impregnation method which achieved 100 % impregnation, while significantly suppressing the cell membrane damage and texture loss of apple and carrot.

5.1.1 Quantitative methods for liquid impregnation into plant food materials

In this chapter, quantitative methods were established and optimized for evaluating impregnation efficacy, cell membrane damage, water mobility and distribution, and tissue damage of plant food materials. Selection of pigments and solution concentrations facilitate the distinguishing the color differences between fresh and impregnated samples and the reproducibility of impregnation ratio. It was recommended to use the 0.05 % red and 3.0 % blue pigment solutions for the image analysis of apple and carrot, respectively. The scan number for LF-NMR was set at 16 to minimize the standard deviation. In the texture analysis, a plunger of 5 mm diameter was adopted to obtain reproducible data among all tested plungers. The methods established were applied thereafter in this study.

5.1.2 Vacuum impregnation of liquid into apple assisted by high hydrostatic pressure

Apple was impregnated with red pigment solution by vacuum heat sealing (VHS) and/or high hydrostatic pressure (HHP) treatment (100-600 MPa, 25 °C, 5 min). VHS was time dependent and insufficient. On the other hand, regardless of storage time after VHS, HHP treatment after VHS exclusively achieved 100% impregnation. The cell membrane and tissue damages of VHS+S_{120}+100 MPa sample were comparable with that of VHS+S_{120} samples. Meanwhile, there was no significant difference in SCA_n among the HHP-treated apples at 200, 400, and 600 MPa with or without storage after VHS (VHS and VHS+S_{120}), indicating that cell membrane could be damaged more by VHS than by HHP treatment at 100–600 MPa. In addition, it was further aggravated by HHP at 200 MPa and higher. Because of water uptake after the impregnation, relaxation time of semibound water and free water were significantly increased after VHS and VHS+100 MPa treatment. As for the texture, breaking stress (hardness) decreased after VHS and VHS+HHP treatments (100-600 MPa) comparably. Meanwhile, breaking strain (deformability) significantly increased after VHS+HHP treatments (200-600 MPa), indicating further damage resulting in increased deformability as compared with that after VHS and VHS+100 MPa treatment. It was

suggested that impregnation of apple was fully achieved by a combination of VHS and 100 MPa treatment, while minimizing the cell membrane and texture damages.

5.1.3 Vacuum impregnation of liquid into carrot assisted by high hydrostatic pressure

HHP treatment significantly promoted the impregnation ratio, where the impregnation ratio of VHS+S_0+100 MPa (98.6 ± 0.9 %) was drastically higher than that of VHS+S_0 (28.9 ± 6.6 %). The relaxation time and area fraction of bound water and semibound were slightly affected among all impregnated carrots. With the increase in pressure level of HHP treatment (100 to 600 MPa), the relaxation time T_{23} of VHS samples decreased. As for the damages of cell membrane and tissue, 100 MPa-treated samples were almost comparable with those of control samples, while HHP treatment at 150 MPa and higher significantly damaged cell membrane and tissue. After optimal impregnation (i.e. VHS, subsequent storage for 120 min, and HHP treatment at 100 MPa), impregnation ratio reached 100 %, while the SCA, breaking stress, and breaking strain were comparable with those of fresh carrot. It was suggested that HHP treatment at 100 MPa can be applied to impregnate carrots for processing high quality carrot products with sufficient impregnation and comparable structures of cell membrane and tissue with fresh carrot.

Based on the results, it can be suggested that cell membrane and tissue of apple are more susceptible to damages caused by impregnation than carrot, probably because of large intercellular spaces of apple. The damages were minimized by the optimal HHP-assisted impregnation (a combination of vacuum heat sealing, storage for 120 min, and 100 MPa treatment) which can be applied to process high quality apple and carrot products with sufficient impregnation and minimized damages of cell membrane and tissue. It was also demonstrated that HHP treatment at 200 MPa and higher induced more damages to cell membrane and texture than 100 MPa treatment.

5.2 Future research

The established quantitative evaluation system can be applied to evaluate other plant food materials. In addition, functional liquids in combination with HHP-assisted impregnation has a potential to further enhance nutritional properties of plant food materials. Therefore, following future research needs to be followed up:

(1). To study HHP-assisted liquid impregnation by using other plant food materials for optimizing their impregnation efficacy and minimizing damages of cell membrane and tissue.

(2). To investigate the effect of combination of trehalose (and other cryoprotectants) and HHP-assisted impregnation on frozen-thawed products.

(3). Further nutritional improvement of plant food materials by impregnating functional liquids.

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