CHAPTER 3
METHODOLOGY

3.1 Formulations and properties of chitosan/cellulose derivative based films

3.1.1 Film formulations

Chitosan with 90% deacetylation and purity of more than 99.75% (Bannawach Bio-line Co., Ltd, Thailand) was prepared by dissolving chitosan (Table 3.1) in 100 ml of a 1% acetic acid solution during continuous agitation. Methylcellulose (M-043, BENECEL®) or hydroxypropyl cellulose (Type LF, KLUCEL®) was also dissolved in 100 ml of a 50% aqueous ethanol solution. Polyethylene glycol (PEG400), 0.66 g, was used as a plasticizer. Chitosan and methylcellulose or hydroxypropyl cellulose solutions were mixed and heated to 72°C. Stearic acid was added (15% of cellulose derivatives) to improve water barrier (Ayranci and Tunc, 2001). The film-forming solutions were filtered to remove particles and degassed. The film-forming solutions, 30 g were poured onto glass plates (13.5 cm diameter), and dried at 40°C for 42 or 48 hours for chitosan/methylcellulose or chitosan/hydroxypropyl cellulose, respectively. Dried films were peeled off from the glass plates and conditioned at 25±2°C, 50±5%RH for at least 48 hours prior to evaluation.

Table 3.1 The ratio of chitosan, methylcellulose (MC) and hydroxypropyl cellulose (HPC) in film-forming solution.

Formulation	Chitosan (g)	MC (g)	HPC (g)
Control	1.5	-	-
A	1.5	0.5	-
B	1.5	1.0	-
C	2.0	0.5	-
D	2.0	1.0	-
E	1.5	-	2.0
F	1.5	-	3.0
G	2.0	-	2.0
H	2.0	-	3.0

3.1.2 Film properties

3.1.2.1 Determination of film thickness

Film thickness was measured using a gauge micrometer model GT-313-A (Gotech testing machines Inc., Taiwan) with an accuracy of 0.01 mm. The reported thickness values were the average of at least 30 measurements.

3.1.2.2 Determination of mechanical properties

Tensile strength and percent elongation were tested by using the Instron Universal Testing Instrument model 5565 (Instron, Canton, MA, USA) following ASTM D638M (ASTM, 1993). Film specimens were cut into rectangular strips, 1 cm x 10 cm. The initial grip separation was 5 cm and the cross-head speed was 25 mm/min. Tensile strength was calculated by dividing the peak load by the cross-sectional area (average thickness × 1 cm) of the initial specimen. The percentage elongation was defined as the percent change in the length (ΔL) of the specimen to its original length (L) between the grips (5 cm). Tensile strength and percent elongation results were obtained from 8 sample replications.

3.2 Effect of vanillin and plasticizer on chitosan/methylcellulose films

3.2.1 Film formulations

Chitosan 1.5 g was dissolved in 100 ml of 1% acetic acid solution. One half gram of methylcellulose was dissolved in a 50% aqueous ethanol solution. Polyethylene glycol 400 (PEG) was used as a plasticizer (Table 3.2). Film processing was performed according to 3.1.1. Vanillin (Sigma, St. Louis, USA) was incorporated into the chitosan/methylcellulose solution after its temperature reached 83°C (after stearic acid). Dried films were shown in Figure 3.1.

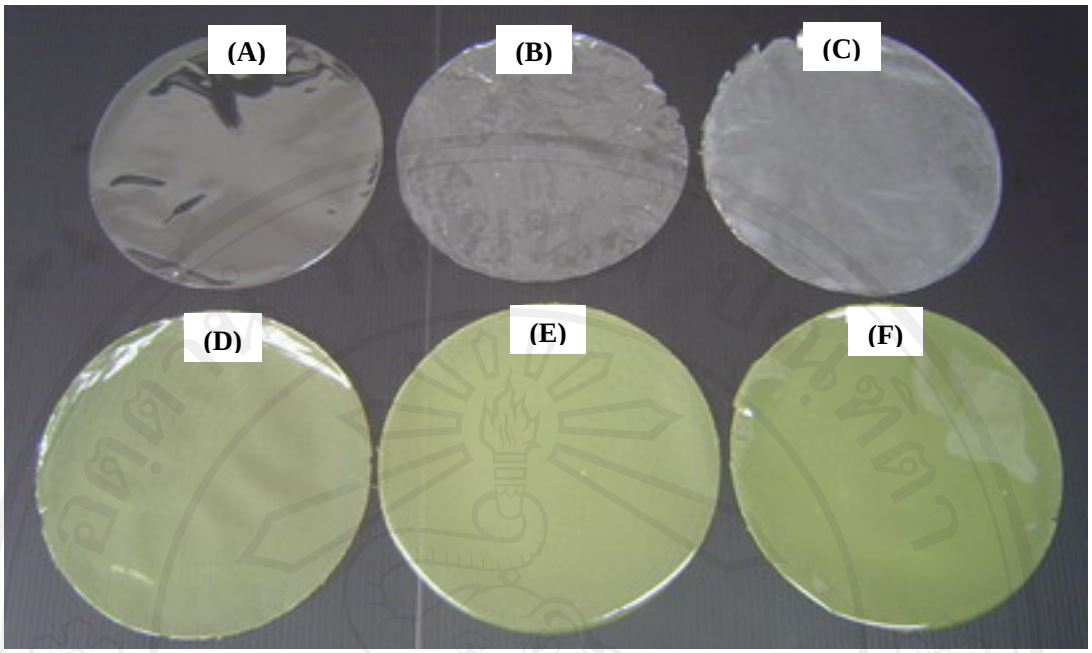

Figure 3.1 **Dried films of (A) chitosan film, (B) and (C) low and high plasticized chitosan/methylcellulose films, (D), (E) and (F) low, medium and high vanillin containing films.**

Table 3.2 Film formulations with varying PEG and vanillin concentration.

Film sample with	%PEG	%Vanillin
Low plasticizer		
- No vanillin	0.17	0
- Low vanillin	0.17	0.15
- Medium vanillin	0.17	0.30
- High vanillin	0.17	0.45
High plasticizer		
- No vanillin	0.50	0
- Low vanillin	0.50	0.15
- Medium vanillin	0.50	0.30
- High vanillin	0.50	0.45

* % expressed as %w/v of film forming solution.

3.2.2 Film properties

3.2.2.1 Determination of film thickness.

Detail had been written in 3.1.1.

3.2.2.2 Determination of mechanical properties.

Detail had been written in 3.1.2.

3.2.2.3 Determination of water vapor permeability (WVP)

WVP was determined gravimetrically according to ASTM E96-95 (ASTM, 1995). Film specimens, approximately 8 cm diameter, were mounted on the aluminum cups containing 10 ml of distilled water. Paraffin was used to fix a film specimen to the wide rim of an aluminum cup (Figure 3.2). The cups were weighed and then placed in a desiccator containing saturated magnesium nitrate solution. The relative humidity of the chamber was kept at 53±1% and 23±1^{o}C throughout the experiment. The water vapor which passed through the film was determined by recording the weight loss daily for 5 days. The test was performed in triplicate. WVP was calculated using the following equation.

$$\text{WVP} = \left(\frac{w}{t}\right)\left(\frac{x}{A \cdot \Delta p}\right) \quad (3.1)$$

where $\frac{w}{t}$ = the slope of the plot between weight loss and time

x = the average thickness of the films

A = the permeation area

Δp = the partial pressure difference of distilled water in the cup and the atmosphere inside the desiccator.

3.2.2.4 Determination of oxygen permeability (OP)

OP was tested using a Gas Permeability Tester VAC-V1 (M&E Instruments, Jinan, China) according to ASTM D1434-82 (ASTM, 2003). A film specimen with 10 cm diameter was fixed between the upper and lower chambers. Oxygen in both chambers was removed under vacuum for 8 hours. After 8 hours, oxygen was flowed

into the upper chamber. The amount of oxygen that permeated through the film in the lower chamber was then determined. The test was done in duplicated at 23±1°C, 0%RH.

Figure 3.2 Film specimen fix on aluminum cup containing distilled water.

3.2.2.5 Determination of opacity and color

Opacity and color (L, a*, b*, chroma and hue) of film were measured using the Hunterlab color meter ColorQuest XE (The Color Management Company, Reston, Virginia, USA) calibrated with a white tile. Absolute measurements were displayed as tristimulus color values which closely represents human sensitivity. Readings were taken on 8 samples for each treatment.

3.2.2.6 Determination of film thermal properties

Evaluation of the film thermal properties was accomplished by differential scanning calorimetry, using a DSCQ100 (TA Instruments, New Castle, DE, USA). Approximately 10 mg film was weighed in a precision balance (±0.01 mg) model XS205 (Mettler-Toledo, Menlo Park, CA, USA) and placed into an aluminum pan with a sealed cover. The aluminum pan containing the film was heated at a rate of 10°C/min from -50 to 350°C in an inert atmosphere (50 ml/min of N_2).

3.2.2.7 Determination of film solubility

Films were cut into square 1 cm x 1 cm pieces and dried to constant weight in a vacuum oven at 60°C for 24 hours to obtain the initial film dry weight (W_d). Each film was placed in a bottle containing 20 ml distilled water for 24 hours under gentle agitation and controlled temperature at 25°C (Zactiti, 2006). Films were dried at the same condition to obtain the dry weight of water-leached film (W_{ws}). Film solubility was calculated as

$$\%\text{Solubility} = \frac{W_d - W_{ws}}{W_d} \times 100 \quad (3.2)$$

3.2.2.8 Sorption behavior

Sorption isotherms of films were determined at 23°C using a VTI SGA-100 Symmetric Water Sorption Analyzer (VTI Corporation, Hialeah, FL, USA). Each sample (25-30 mg) was hung in the microbalance sample holder and dried at 40°C for 6 hours. The sample was then exposed to a sequence of relative humidities, 10, 30, 50, 60, 70, 80 and 90%RH. Water sorption was measured by the change in mass over the range of the programmed relative humidities.

3.2.2.9 Morphology observation

The surface and cross-sectional morphologies of chitosan/methylcellulose film with and without vanillin were examined using scanning electron microscopy model JSM-5019LV (JEOL, Tokyo, Japan). Cross-sectional samples were prepared by fracturing films in liquid nitrogen. Samples were mounted on metal grids and coated with gold under vacuum prior to observation.

3.3 Release of vanillin and migration test

3.3.1 Factors affecting release of vanillin from the films

3.3.1.1 Effect of temperature

Films containing high vanillin concentrations (Table 3.2B) were cut into square 1 cm x 1 cm pieces. Thickness and weight were recorded. They were then

placed in 8 ml amber glass vials. Three milliliters of water, cantaloupe juice or pineapple juice were then added. Vials were closed with caps and placed in controlled temperature chambers at 10, 25, 35^{o}C. Release of vanillin was determined at time intervals of 1, 2, 4, 8, 16, 24, 48, 96, 144, 192, 288, 384 and 480 hours.

3.3.1.2 Effect of initial vanillin concentration in film

Films containing low, medium and high vanillin concentrations (Table 3.2B) were cut into square 1 cm x 1 cm pieces. Thickness and weight were recorded. They were placed in 8 ml amber glass vials and 3 ml of water was added to each vial. Vials were closed with caps and placed in a controlled temperature chamber at 10^{o}C. Release of vanillin was determined at time intervals of 1, 2, 4, 8, 16, 24, 48, 96, 144, 192, 288, 384 and 480 hours.

3.3.1.3 Effect of pH

Films containing high vanillin concentration (Table 3.2B) were cut into square 1 cm x 1 cm pieces. Thickness and weight were recorded. They were then placed into 8 ml amber glass vials. Three ml of citrate buffer pH 3.5, 5.0 and 6.5 were added to different vials. Vials were closed with caps and placed in a controlled temperature chamber at 10^{o}C. Release of vanillin was determined at time intervals of 1, 2, 4, 8, 16, 24, 48, 96, 144, 192, 288, 384 and 480 hours.

3.3.1.4 Swelling behavior

Films were cut into square 1 cm x 1 cm pieces. Thickness and weight were recorded. The solvents used in this study were water, cantaloupe juice, pineapple juice and citrate buffer pH 3.5, 5.0 and 6.5. A piece of film and 3 ml solvent were placed in 8 ml amber glass vials. Vials were closed with caps and placed in controlled temperature chambers of 10, 25 and 35^{o}C according to 3.3.1.1 – 3.3.1.3. Wet films were removed from solvents after specific time intervals, and blotted dry between filter papers to remove the access surface liquid. They were reweighed using an analytical balance and then the thickness were determined using a micrometer.

3.3.1.5 Solvent preparation

Six solvents were used in this study; HPLC grade water (Sigma-Aldrich), cantaloupe juice, pineapple juice and citrate buffer pH 3.5, 5.0 and 6.5. Cantaloupe juice was prepared by blending the cantaloupe flesh using a blender (Moulinex, French) and filtered through cheesecloth. The pineapple juice was 100% pineapple juice, canned, (Dole, Thailand). The total soluble solids and pH of cantaloupe and pineapple juices were 9%, 15%, 6.2 and 3.7, respectively. Citrate buffer was prepared from 0.1 M citric acid (Fisher Scientific) and adjusting the pH by 0.1 N NaOH.

3.3.1.6 Vanillin determination

Vanillin was determined using a Waters HPLC model 2695 equipped with a quaternary pump system, an autosampler and a UV detector at 280 nm (Waters Corporation, Milford, MA, USA). The analytical column was a Nova-Pak® C18 (3.9 x 150 mm, 4 µm particle size). The sample was filtered using a 0.45 µm syringe filter. The filtrate, 5 µl, was injected into the HPLC system. A gradient solvent system consisting of HPLC water (Sigma-Aldrich) with 0.015% sulfuric acid (pH 2.3) and acetonitrile (Sigma-Aldrich) was used as follows: initially 10% acetonitrile (ACN), at 3 and 4 minutes 40% ACN, at 6 minutes 80% ACN and at 7 minutes 90% ACN. The eluent flow rate was 0.8 ml/min and the column temperature was 30°C (Herrmann *et al*., 1982). The calibration curve was created by diluting standard vanillin (Sigma, St. Louis, USA) in the concentration range from 5-400 mg/L. Calibration curve had a $R^2 = 0.9999$. The retention time was 4.4 minutes.

3.3.2 Migration of vanillin from film

Evaluation of the migration of vanillin from films was done according to ASTM D4754-98 (ASTM, 1998). Film specimens were cut into a disk shape with a 15 mm diameter. Eight film disks were threaded onto a stainless steel wire with alternating glass bead spacers and placed in a 35 ml amber glass vial (Figure 3.3). Three FDA food stimulant solvents; 10% ethanol representing acid foods, 50% ethanol in water representing high alcoholic foods and corn oil representing oily foods, were added into the vials (US FDA, 2002). Vials were capped and placed in controlled temperature chambers at 20 and 40°C. Solvents were withdrawn from the

vial at specific time intervals. Vanillin concentration in each solvent was determined according to the method in 3.3.1.6.

Figure 3.3 Film disks threaded onto a stainless steel wire.

3.3.3 Release of vanillin on fruit pieces

Cantaloupe (*Cucumis melo*) and pineapple (*Ananas comosus)* fruit were purchased from a wholesale market in Chiang Mai province, Thailand from the same period. Total soluble solids were determined to indicate the maturity of the fruit. The cantaloupe and pineapple used in this study had total soluble solids in the range of 7.0-8.2 and 15.0-17.4%, respectively. Both fruit were washed with 500 mg/L chlorine solution (Aquino *et al*., 1998) and cut into 2.5 cm x 2.5 cm x 0.5 cm pieces. Films containing a high vanillin concentration were cut into 1.5 cm x 1.5 cm pieces. The weight and thickness were measured. A film piece was placed underneath each fruit piece on a glass plate to ensure complete contact between film and fruit piece. They were then stored at 10^{o}C. After a period of time, the film was removed from under the fruit piece and dissolved in 50% acetic acid. The amount of vanillin left in the film was determined using a HPLC following the method in 3.3.1.6.

3.4 Inhibition effect of chitosan/methylcellulose film and chitosan/methyl cellulose film incorporating vanillin as an antimicrobial agent.

3.4.1 Standard disk diffusion technique.

All film formulations were tested for antimicrobial activity against *Escherichia coli* (TISTR 780) and *Saccharomyces cerevisiae* (TISTR 5240) at 10°C which is a storage temperature typical for fresh-cut, 25°C which is an appropriate temperature for growth of *Saccharomyces cerevisiae* and 35°C which is an appropriate temperature for growth of *Escherichia coli*. A one day old *Escherichia coli* culture (10^5-10^6 colony-forming units (CFU)/ml) was spread on to nutrient agar plates (0.1 ml/plate). A two day old culture of *Saccharomyces cerevisiae* was spread on to Sabouraud agar plates. Films were cut into 1 cm x 1 cm pieces, and 5 film pieces were then placed in contact with the inoculated agar. Chitosan/methylcellulose film without vanillin and a polypropylene film (PP) served as controls. The plates were incubated at 10 and 37°C for *Escherichia coli* and 10 and 27°C for *Saccharomyces cerevisiae*. Inhibition effect was determined by observing the inhibition zones at the contact area as well as around the films. Area of inhibition was observed for approximately 20 days (adapted from Zivanovic *et al*., 2005).

3.4.2 *Escherichia coli* and *Saccharomyces cerevisiae* inoculation and determination number of *Escherichia coli* and *Saccharomyces cerevisiae*.

Prior to use, cantaloupe and pineapple were washed with 500 mg/L chlorine solution. The blossom and stem ends were discarded. Cantaloupe and pineapple flesh were sliced into 2.5 x 2.5 x 0.5 cm pieces with a sanitized sharp knife and cutting board. Fruit pieces were then inoculated with 20 µl of approximately 10^5 CFU/ml *Escherichia coli* and *Saccharomyces cerevisiae* suspensions on the top surface of each piece (Zivanovic *et al*., 2005). Then, commercial stretch film, M wrap®, chitosan/methylcellulose film and chitosan/methylcellulose film containing vanillin (Vanillin film) were wrapped around each piece. Wrapped fruit pieces were placed in polystyrene trays and stored at 10°C up to 20 days. Inoculated fruit without wrapping served as control. After set time intervals, fruit pieces and films were washed with sterile 0.1% peptone. The numbers of *Escherichia coli* on the samples

were enumerated by using violet red bile agar with Methylumbelliferyl Glucuronide (MUG, Criterion, USA) as a selective media. MUG generally permits the rapid detection of *Escherichia coli* when the medium is observed for fluorescence under long wavelength UV light. The samples were incubated at 37°C for 48 hours and the number of organisms was counted under long wavelength UV. The numbers of yeast cells were determined by surface plating on Sabouraud agar (MERCK, Germany) containing 1% yeast extract. They were incubated at 25°C for 48 hours prior to counting.

3.5 Application of chitosan/methylcellulose film and chitosan/methylcellulose film incorporating vanillin on fresh-cut cantaloupe and pineapple.

3.5.1 Fruit preparation

The blossom and stem ends of cantaloupe and pineapple (according to section 3.3.3) were discarded. Cantaloupe and pineapple fruit were sliced longitudinally into 12 wedges and 8 wedges, respectively using a sanitized sharp knife and cutting board. Then, the seeds or core and peel were removed. All knives, cutting boards and other equipments which came into contact with the fruit were sanitized by immersion into 1,000 mg/L chlorine solution for 30 minutes prior to cutting.

3.5.2 Quality evaluation of fruit wrapped with antimicrobial films

Fresh-cut cantaloupe and pineapple wedges were wrapped with the 3 films (stretch film, chitosan/methylcellulose film and vanillin film), and unwrapped fruit served as control (Figure 3.4 and 3.5). All fruit pieces were placed into polystyrene trays and stored at 10°C for up to 20 days. Since Thailand is a tropical country and fresh-cut fruit is stored in open chiller displays or even placed on ice-cubes, 10°C was selected as an appropriate storage temperature for this study. Measurement of all attributes was done every 2 to 4 days until the end of storage.

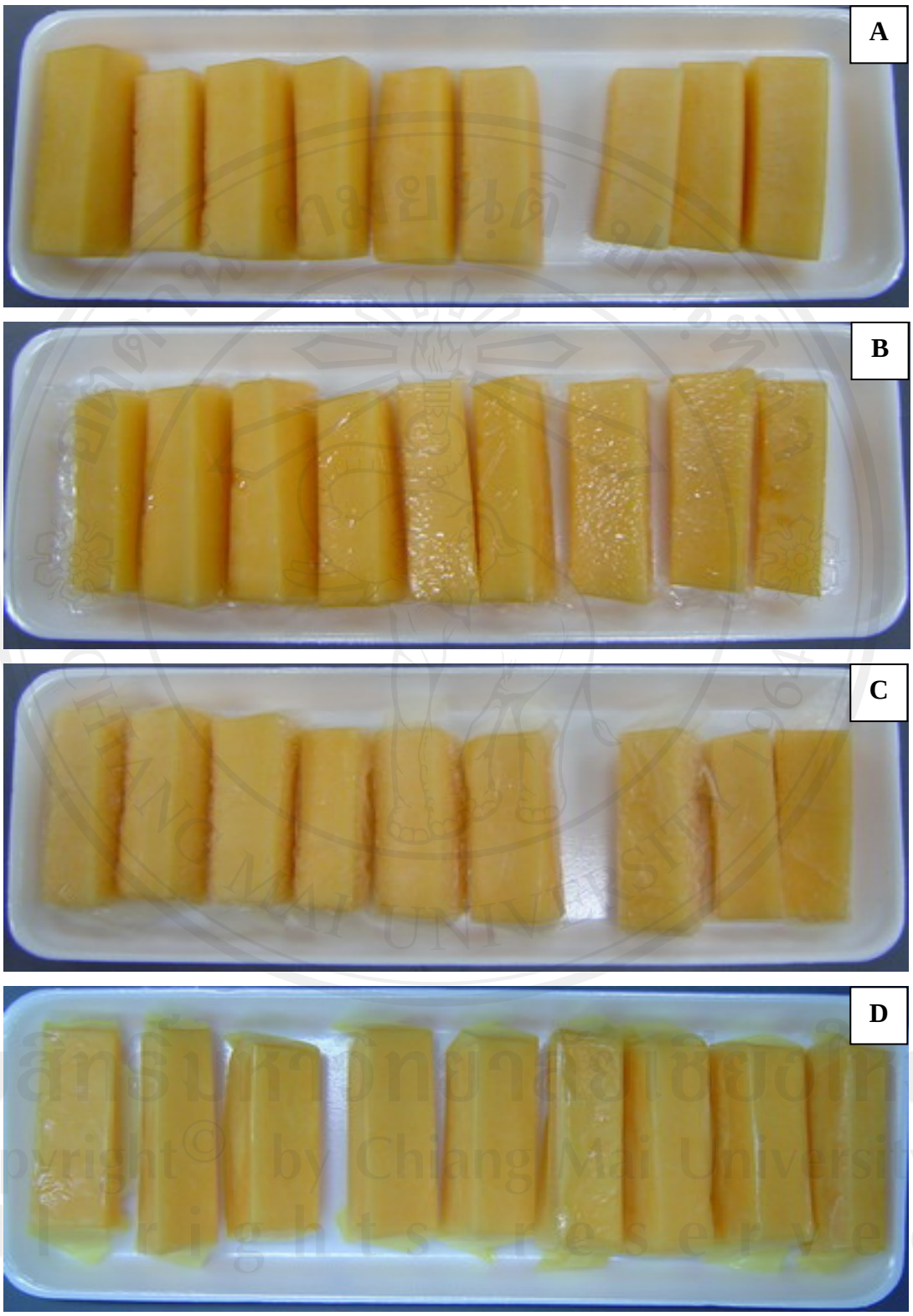

Figure 3.4 **Fresh-cut cantaloupe without wrapping (A), wrapped with stretch film (B), chitosan/methylcellulose film (C) and vanillin film (D).**

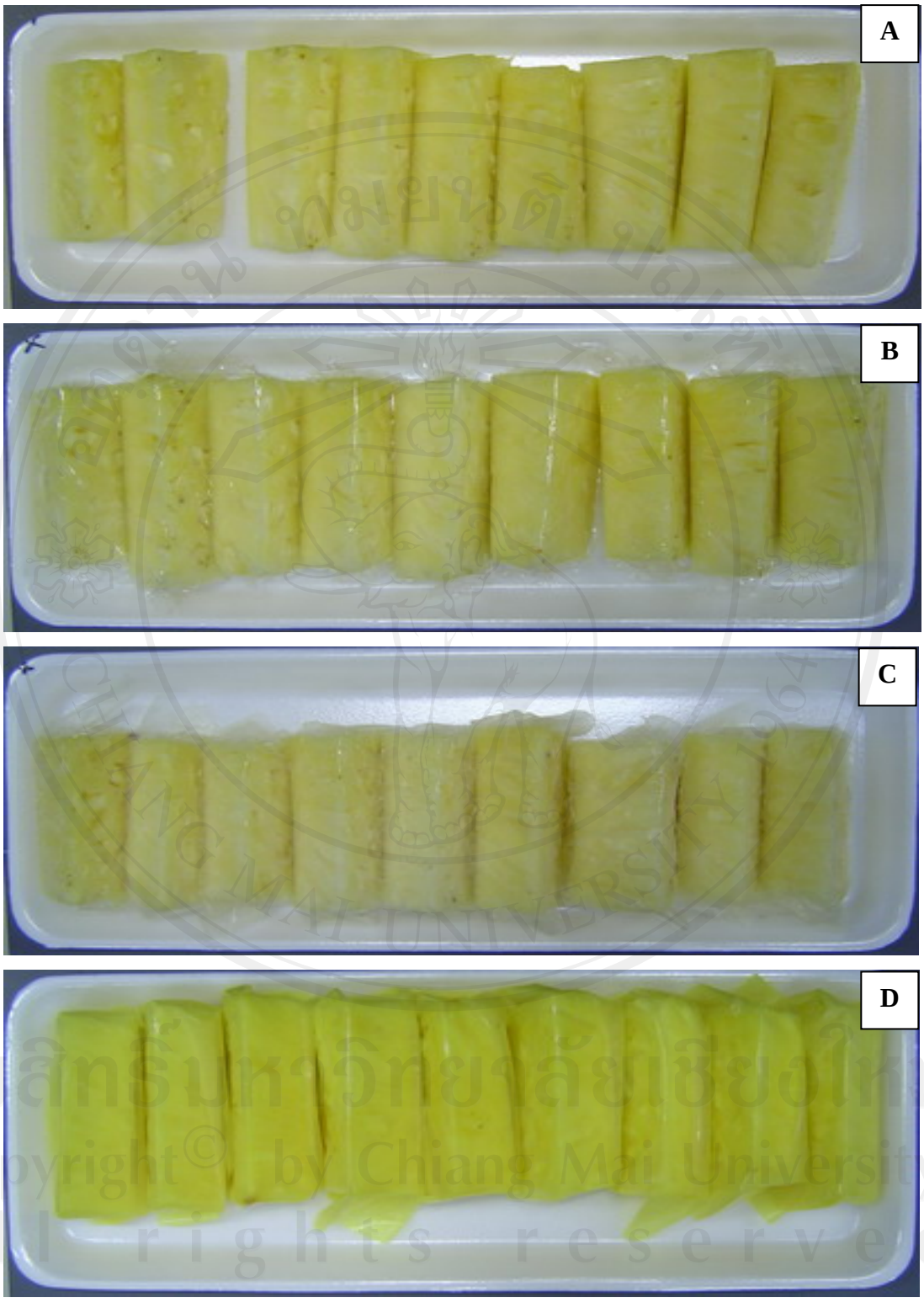

Figure 3.5 **Fresh-cut pineapple without wrapping (A), wrapped with stretch film (B), chitosan/methylcellulose film (C) and vanillin film (D).**

3.5.2.1 Flesh color

The flesh color (L*, a*, b*, chroma and hue angle) of both fruit was measured on both longitudinally cut surfaces after removal from the wrap using the Hunterlab color meter ColorQuest XE (The Color Management Company, Reston, Virginia, USA) calibrated with a white tile. Multiple readings were taken on the fruit surface from 6 wedges from each treatment.

3.5.2.2 Firmness

Firmness was measured as the maximum force required to penetrate into the fruit wedges using a Texture Analyzer TA.XT2i (Texture Technologies Corp., Scarsdale, NY, USA) equipped with a 50 kg load cell. A 6 mm diameter flat head stainless steel cylindrical probe was set to penetrate 7.5 mm into the fruit at a speed of 0.5 mm/min.

3.5.2.3 L-ascorbic acid

L-ascorbic acid (AA) in the fruit was determined using an Agilent 1100 HPLC equipped with a quaternary pump system, an autosampler and a UV detector set at 254 nm (Agilent Techologies, Palo Alto, CA, USA). The analytical column was a Restek Ultra Aqueous C18 (150 mm x 4.6 mm, 5 µm particle size). The sample preparation was done under cold temperature (15^{o}C) and reduced lighting. Fifteen grams of each fruit flesh was blended with cold 0.4% oxalic acid solution and adjusted to a final volume of 50 ml. The homogenate was filtered with Whatman no. 1 filter paper and then pushed through a 0.45 µm syringe filter. The filtrate, 20 µl, was injected into the HPLC system. Isocratic separation was carried out using a mobile phase of Milli-Q water with 0.1% v/v acetic acid. The eluent flow rate was 0.7 ml/min and the column temperature was 25^{o}C (Romeu-Nadal *et al*, 2006). HPLC grade L-ascorbic acid (MERCK, Germany) was used to make the calibration curve. The calibration curve was created by diluting L-ascorbic acid in concentration ranges of 5-100 mg/L. Standard solutions were prepared fresh under cold and dark conditions to avoid L-ascorbic acid degradation in the samples. The determination of linearity (R^2) of the standard curve was 0.9993. The retention time was 4.5 minutes. Triplicate determinations of different samples were carried out in this study.

3.5.2.4 Respiration rate

Fruit respiration rate was determined using a static method. Each wrapped cantaloupe or pineapple wedge was put into a 24 ounce (710 ml) air-tight glass container. The CO_2 which was given off by the products was absorbed by a calibrated 0.01 N NaOH solution for 1 hour at 10°C. The solution was then titrated with 0.005 N oxalic acid. The respiration rate was expressed as mg CO_2/kg.hr (Zhang *et al*., 2005).

3.5.2.5 Ethanol content

The ethanol content of the fruit was determined by gas chromatographic analysis of head space according to a method developed by Davis and Chace (1969) with some modification. Five grams of the fruit flesh were placed in a 10 ml amber glass bottle with rubber cap and incubated in a water bath at 60°C for 45 minutes. Headspace gas was withdrawn using a 1 ml syringe and injected into a TRACE GC gas chromatograph (ThermoQuest Italia SpA., Italy) equipped with a flame ionization detector. The temperature of the oven, injector and detector were 150, 175 and 200°C, respectively. The column used was a 30 m x 0.53 mm i.d. x 1 μm OV-1 (100% dimethylpolysiloxane) capillary column. Retention time and standard curve of absolute ethanol in water solutions (31 to 2000 mg/L) were used for peak identification and quantification.

3.5.2.6 Total soluble solids (TSS), titratable acidity (TA) and pH

TSS was determined using a digital refractometer (Pocket PAL-1, Japan). Ten grams of the fruit flesh was blended with 40 ml distilled water in a blender. The solution was titrated with standardized 0.1 N NaOH to pH 8.1. The results were expressed as percent succinic acid (g succinic acid/100 g dry weight) for cantaloupe and percent citric acid (g citric acid/100 g dry weight) for pineapple. The pH was measured at 25°C with a pH meter (Consort C831, Belgium).

3.5.2.7 Weight loss

Weight loss of fresh-cut cantaloupe and pineapple wedges were determined by weighing the samples at specific time intervals and plotting weight loss against time.

Weight of fruit pieces were measured using a balance (±0.01 g) model PB1502-S (Mettler-Toledo, Menlo Park, CA, USA).

3.5.2.8 Sensory evaluation

Each cantaloupe and pineapple wedge (3 cm x 6 cm x 2 cm) was wrapped in stretch film, chitosan/methylcellulose film and vanillin film and stored at 10^{o}C for 2 and 8 days for cantaloupe and 2 and 6 days for pineapple. To avoid sensory fatigue in panelists, sensory testing of the different fruit were done on separate days. Evaluations were tested under ambient conditions at about 25^{o}C in a sensory evaluation room. Fifty panelists of both genders (10-12 male and 38-40 female) and of ages from 15 to 60 were given 4 samples at a time with a break between each set (different storage time). The acceptability of color, odor, flavor, taste, texture and overall quality were evaluated using a nine-point hedonic scale where 9=like extremely, 8=like very much, 7=like moderately, 6=like slightly, 5=neither like nor dislike, 4=dislike slightly, 3=dislike moderately, 2=dislike very much, and 1=dislike extremely. The variability of acceptance was analyzed by ANOVA and Tukey's-b test ($p<0.05$). Fruit samples were removed from the refrigerator before serving to allow them to equilibrate to room temperature. Samples were labeled with three-digit random codes and placed in small white plastic cups. The order of presentation of the samples was randomized. Panelists were given verbal instructions and an evaluation sheet. Panelists were told to rinse their palates with water and low salt saltine cracker between samples.

3.5.2.9 Statistical analysis

Data were subjected to analysis of variance and Tukey's-b Multiple Range ($P<0.05$) test.