CHAPTER 3

Methodology

3.1 Preparation and Properties of Active-Coated Papers

3.1.1 Preparation of Active Coating Solutions

1) Vanillin-chitosan coating solution

The vanillin-chitosan coating solution was prepared by according to the method of Sangsuwan et al. (2008) with some modification. Low molecular weight chitosan (1.5 g) with 85 % degree of deacetylation (DD85) (Ta Ming Enterprises, Samutsakorn, Thailand) was dissolved in 100 mL of 1.0 % (v/v) acetic acid (RCI Labscans, Thailand) solution and heated at 72 °C for 30 min under continuously stirred by a magnetic stirrer (C-Mag HS 7, IKA®-Werke GmbH & Co. KG, Germany). Then, various amounts of vanillin (0, 0.5, 1.0, 2.0, and 4.0 g) (Sigma-Aldrich, St. Louis, USA) were incorporated into chitosan solutions after theirs temperature reached 83 °C and stirred for another 30 min.

2) Vanillin-carboxymethyl cellulose (CMC) coating solution

This active coating solution was prepared according to the method of Sayanjali et al. (2011) with slight modification. One gram of CMC (Union Science, Thailand) was dissolved in 100 mL of distilled water (RCI Labscans, Thailand) and then heated at 80 °C for 10 min under continuously stirred by a magnetic stirrer. Similarly to vanillin-chitosan coating solution, various amounts of vanillin (0, 0.5, 1.0, 2.0, and 4.0 g) were incorporated into CMC solutions after theirs temperature reached 83 °C and stirred for another 30 min.

3.1.2 Preparation of Active-Coated Papers

The active coating solutions prepared from Section 3.1.1 were used for preparing the active-coated papers. The 15 mL of active coating solutions was dropped on a standard bleached paper (A4 size, 21.0 cm × 29.7 cm) with 39 gsm and coated on the paper surface using the multicoater (model K303, RK Print Coat Instruments, UK). Active-coated papers were dried at room temperature (25 ± 2 °C) and coated again with the same coating solutions to obtain as two coating layer. All coated papers were stored in re-sealable zipper storage bags at room temperature (25 ± 2 °C) under 50 % relative humidity (RH) in a desiccator until being analyzed.

3.1.3 Properties of Active-Coated Papers

Properties of active-coated papers were identified by the following properties:

1) Thickness

Paper thickness was measured using a gauge micrometer model GT-313-A (Gotech testing machines, Taiwan) with a 0.01 mm accuracy. Five thickness measurements were taken on each sample along the length of the strip, mean values was reported.

2) Moisture content

The moisture content of paper was determined by according to the method of ASTM D644-99. The paper was cut into a small piece before drying to the hot air oven and dried at 105 ºC for 24 h. Then, dried paper was weighed. Moisture content of paper was expressed as a percentage of dry weight, which could be calculated by following Equation (3.1).

$$\text{Moisture content } (\%, \text{dry weight}) = \left(\frac{W_1 - W_2}{W_2} \right) \times 100 \qquad (3.1)$$

Where W_1 is the weight of paper before drying, and W_2 is the weight of dried paper.

3) Color

Color properties of paper were expressed as the CIE $L^*a^*b^*$ color space, which was measured by the colorimeter (CR-410, Konica Minolta, Japan) using a standard white calibration tile for calibration. L^*, $+a^*$ or $-a^*$, and $+b^*$ or $-b^*$ were a color parameters described the lightness, redness or greenness, and yellowness or blueness, respectively. Readings were taken on 5 samples for each treatment.

4) Water vapor permeability (WVP)

The WVP was evaluated in accordance with ASTM E96-95. Paper with 8 cm diameter was mounted on the aluminum cups containing 10 mL of distilled water. Paraffin was used to fix a sample with an aluminum cup. The cup was weighed and then placed in a desiccator containing saturated magnesium nitrate solution providing relative humidity at 53 ± 1 % and stored at 25 ± 1 °C throughout the experiment. The water vapor which passed through the paper was recorded the loss of weight every 5 days. The WVP was calculated as follows Equation (3.2):

$$WVP = \left(\frac{w}{t}\right)\left(\frac{X}{A \times \Delta P}\right) \quad (3.2)$$

Where, w/t is the slope of the plot between weight loss and time, x is the average thickness of the films, A is the permeation area, and ΔP is difference of the partial water vapor pressure of distilled water in the cup (100 % RH) and the atmosphere inside the desiccator (53 % RH).

5) Mechanical properties

Tensile strength (TS) and percentage elongation at break (E) of each paper were evaluated in accordance with ASTM D882-12 by using the universal testing machine (model H1KS, Tinius Olsen, Horsham, PA, USA). The active coated papers were cut into rectangular strip, 1.5 cm × 15 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.

6) Morphology observation

The surface and cross-sectional morphologies of active-coated papers were evaluated using a scanning electron microscopy. Cross-sectional samples were prepared by fracturing the active-coated papers in liquid nitrogen. Each sample was mounted on metal grids and coated with gold under vacuum prior to observation.

7) Statistical analysis

All the analyses were performed in three replicates. The data obtained were analyzed statistically by using the statistic software program (IBM SPSS statistic 20, IBM, Armonk, NY, USA.). Differences of data considered significant at $p \leq 0.05$ by using Duncan's new multiple range test.

3.2 Efficacy of Active Coating Solutions and Active-Coated Papers against Mango Anthracnose Fungi

3.2.1 Isolation of *Colletotrichum* spp. from Anthracnose Infected Mango Fruit

Colletotrichum spp. were isolated from the infected mango fruit by using a tissue transplanting method. Inoculum was prepared by using a cork borer (5 mm diameter). Fungal plugs (3 – 5 mm) were removed with a knife disinfected by 70 % (v/v) ethanol and dissolved in sterile distilled water for 1 min. Small portions of symptomatic tissue were placed on a petri dish containing potato dextrose agar (PDA) and incubated at an ambient temperature (25 – 30 °C) for 72 h. Colony was removed again with a cork borer from the growing margin of each fungus colony and placed on PDA for re-isolation.

3.2.2 Efficacy of Active Coating Solution against Mycelial Growth of *Colletotrichum* spp. on PDA

Colletotrichum spp. isolated from Section 3.2.1 was then cultured on a center of petri dish containing PDA medium under room temperature (25 ± 2 °C) for 24 h. Then, coating solutions (20 µL) prepared from Section 3.1.1 were added into above petri dish that filled with *Colletotrichum* spp.'s colonies and incubated at room temperature for 7 days (Figure 3.1). The antifungal activity was expressed as the percent inhibition of radial growth (PIRG). The experiments were done in triplicate and comparing with the control without addition of coating solution.

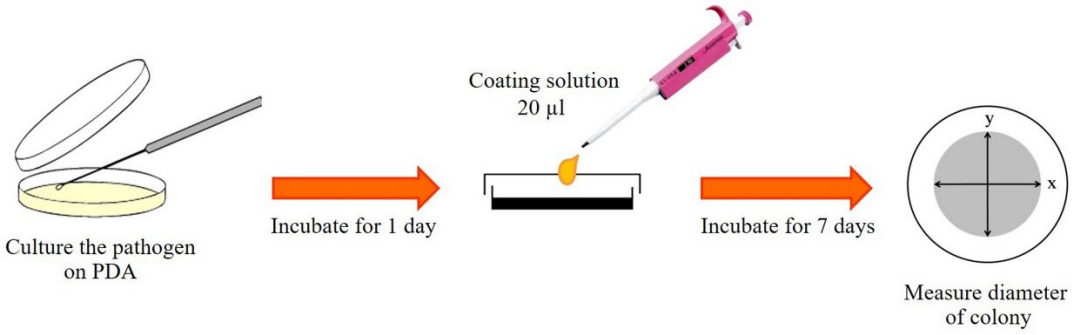

Figure 3.1 The test method for determining the efficacy of active coating solutions against mycelial growth of *Colletotrichum* spp.

3.2.3 Efficacy of Active Coating Solution against Conidiospores Germination of *Colletotrichum* spp.

Colletotrichum spp. isolated from Section 3.2.1 were then cultured on a Petri dish containing PDA medium under a room temperature (25 ± 2 °C) until conidiospores germinated. To prepare the conidiospores suspension, *Colletotrichum* spp.'s conidiospores was leached with distilled water and filtered by a white cloth. As the concentration of conidiospores was adjusted as 10^6 spore/mL using a haemacytometer. Afterward, the conidiospores suspension (30 µL) and active coating solutions (30 µL) prepared from Section 3.1.1 were mixed in the ratio of 1:1 and vigorously shaken. The mixture (20 µL) was dropped into a piece of PDA medium (1 cm × 1 cm) placing on a microscope slide and then incubated at room temperature (25 ± 2 °C) for 6 h. After incubation, the conidiospores were

dyed using lactophenol cotton blue and observed the germination of fungal conidiospores under a light microscope with 400× magnification (Figure 3.2).

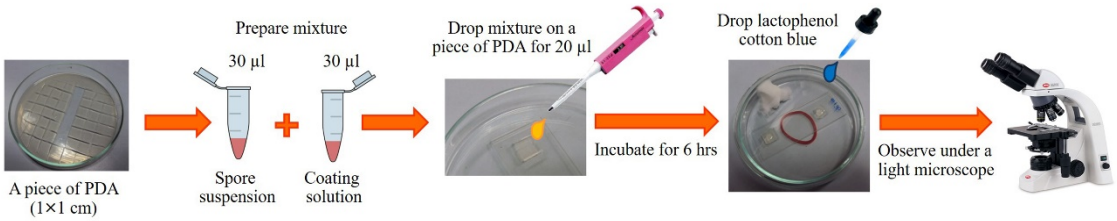

Figure 3.2 The slide culture technique for determining the efficacy of active coating solutions against conidiospores germination of *Colletotrichum* spp.

3.2.4 Efficacy of Active-Coated Paper against Mycelial Growth of *Colletotrichum* spp. by the Dual Culture Technique

This experiment was done by using a dual culture technique. The mycelial plugs of *Colletotrichum* spp. were transplanted to PDA by using a cork borer far from the one side of a Petri dish for 2 cm and then incubated on PDA at 25 °C for 24 h. Then, active-coated papers were cut as a small piece (1 cm × 1 cm) and placed on the same dish far from mycelium for 3.5 cm (Figure 3.3). All dishes of both treatments (UNC, VCS 0.0, VCS 0.5, VCS 1.0, VCS 2.0, VCS 4.0, VCMC 0.0, VCMC 0.5, VCMC 1.0, VCMC 2.0, and VCMC 4.0) and control (without any papers) were stored at 25 °C until the mycelial growth of the fungus in the control dish was fully grown over the petri dish (approximately 7 days). Afterward, the antifungal activity of all dishes (UNC, VCS 0.0, VCS 0.5, VCS 1.0, VCS 2.0, VCS 4.0, VCMC 0.0, VCMC 0.5, VCMC 1.0, VCMC 2.0, and VCMC 4.0) were measured and expressed as the percent inhibition of radial growth (PIRG).

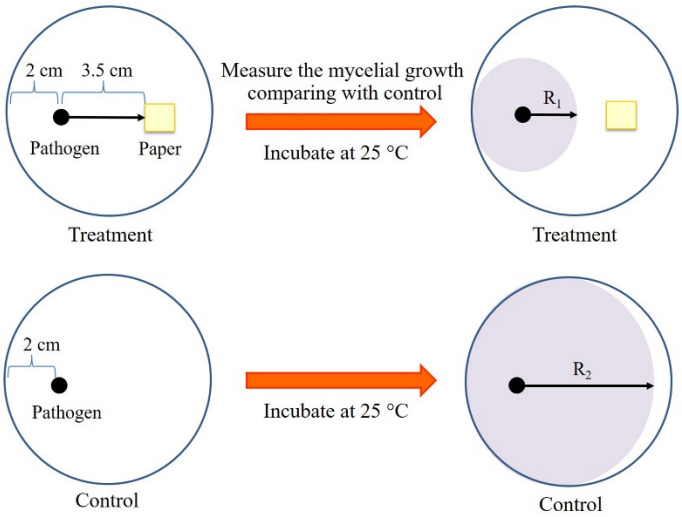

Figure 3.3 The dual culture technique for determining the efficacy of active-coated paper against mycelial growth of *Colletotrichum* spp.

3.2.5 Efficacy of Active-Coated Paper against Mycelial Growth of *Colletotrichum* spp. on PDA by the Vapor-Diffusion Technique

This experiment was done by a vapor-diffusion technique described by Sanla-Ead, Jangchud, Chonhenchob, and Suppakul (2006). The mycelial plugs of *Colletotrichum* spp. were transplanted to PDA by using a cork borer and incubated on PDA at 25 °C for 24 h. Active-coated papers were cut into a diameter of Petri dish cover (8.7 cm). Each active-coated paper underneath a cover was sterilized with UV light for 2 min prior to being placed on a Petri dish (Figure 3.4). The antifungal activity of all dishes were measured and expressed as the percent inhibition of radial growth.

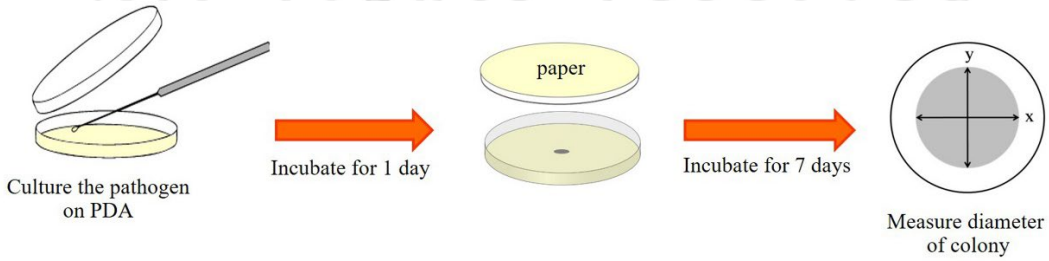

Figure 3.4 The vapor-diffusion technique for determining the efficacy of active-coated paper against mycelial growth of *Colletotrichum* spp.

3.2.6 Efficacy of Active-Coated Paper against Conidiospores Germination of *Colletotrichum* spp.

The inhibition of conidiospores germination of *Colletotrichum* spp. was evaluated by a slide culture technique. The 20 µL of conidiospores suspension was dropped onto the active-coated paper (1 cm × 1 cm), which was placed on a slide. The microscope slide was then incubated at 25 °C for 6 h. After incubation, the conidiospores were dyed using lactophenol cotton blue and observed the germination of fungal conidiospores under a light microscope with 400× magnification (Figure 3.5).

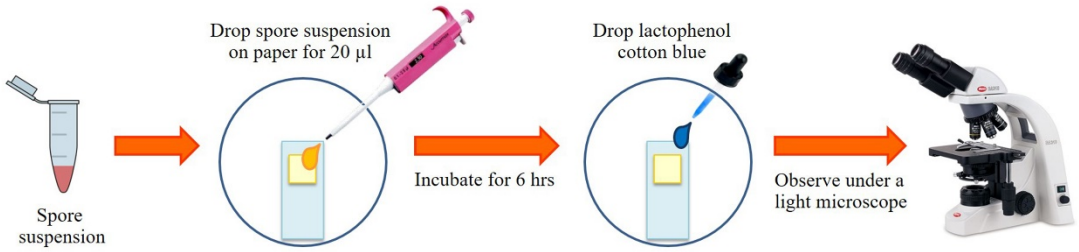

Figure 3.5 The slide culture technique for determining the efficacy of active-coated paper against conidiospores germination of *Colletotrichum* spp.

3.2.7 Statistical Analysis

All the analyses were performed in triplicate. The data obtained was subjected to analysis of variance (ANOVA) using the statistic program (IBM SPSS statistic 20, IBM, USA.). The differences of averages considered significant at $p \leq 0.05$ using Duncan's new multiple range test.

3.3 Efficacy of Active-Coated Paper on Ethylene Removal

3.3.1 Preparation of Active-Coated Paper Incorporating Ethylene Adsorbents

The formula of coating solution that provided the best result of active-coated paper from Section 3.2 was incorporated with different ethylene adsorbents including either activated carbon (NORIT® E SUPRA, Alpharetta, GA, USA) or zeolite (Sigma-Aldrich,

St. Louis, MO, USA). Such coating solution was individually added with various amounts of ethylene adsorbents (1.0, 2.0, and 4.0 %, w/v) and continuously stirred until the whole mixture became homogenous. Afterwards, active-coated papers were formed by using the coating solutions incorporating with ethylene adsorbents prepared. The process for preparing the active-coated paper has been described in Section 3.1.2.

3.3.2 Sample Preparation for Investigating the Ethylene Removal

Active-coated papers (21.0 cm × 29.7 cm) were cut as a small piece and placed in a glass container (720 mL), which was tightly closed with a lug cap and sealed with a silicon septum. Thirty milliliter (approximately 4.17 µg/mL) of ethylene gas (Scott® Mini-Mix®, Restek Corporation, Bellefonte, PA, USA.) was fed into the container. Afterwards, all the glass containers were stored under 25 ºC for 35 days. The amount of ethylene in each glass container was sampling every 7 days.

3.3.3 Quantification of Ethylene using Gas Chromatography

The determination of ethylene gas in a glass bottle was performed by using the gas chromatography instrument (model 7820A GC system, Santa Clara, CA, USA). Each bottle was vigorously shaken to increase the dispersion of gas. Then, 5 mL of ethylene gas in such bottle was sucked by a syringe prior to be injected to which was installed with a flame ionization detector (FID) and equipped with Agilent column 19095P-K25 model having diameter, length, and film thickness of 530 µm, 50 m, and 15 µm, respectively. Helium gas (He) used as a carrier gas that its flow rate maintained at 5 mL/min. The GC front detector was heated at 300 ºC. The flow rate of hydrogen gas (H_2) as a fuel gas was set at 30 mL/min to maintain the flame. While, the flow rates of air and nitrogen gas (N_2) were set at 400 and 25 mL/min, respectively. Total GC run time was approximately 6 min and the result of all GC measurement was analyzed by using the Agilent ChemStation software (Santa Clara, CA, USA.). The standard graph (Appendix B) was plotted by using the area under the GC peaks, which obtained from various ethylene concentrations (0.7, 1.4, 2.1, 2.8, 3.5, and 4.2 µg/mL).

3.3.4 Statistical Analysis

All the analyses were performed in triplicate. The data obtained was subjected to analysis of variance (ANOVA) using the statistic program (IBM SPSS statistic 20, IBM, USA.). The differences of data averages considered significant at $p \leq 0.05$ using Duncan's new multiple range test.

3.4 Anthracnose Incidence and Physicochemical Properties of Mango Fruits Wrapped by Active-Coated Papers

3.4.1 Disease Incidence on the Inoculated Mango Fruits

Mango fruits *cv* Nam Dok Mai used in this study were purchased from a local market in Chiang Mai, Thailand. A small lesion (~1 cm length) was cut on the mango peel using a sterile knife. Then, one-loop of mycelia *Colletotrichum* spp. was inoculated into that lesion. The inoculated mango fruits were wrapped with active coated papers which exhibit the best result against mango anthracnose and ethylene removal from Section 3.3, active-coated papers which the best result from Section 3.2, and uncoated papers. Mango fruits without wrapping was used as control. All samples were incubated at 25 °C for 3 days.

3.4.2 Severity Index of Disease of the Wrapped Mango Fruits

Mango fruits were wrapped with the best active-coated papers from Section 3.3, active-coated papers which were the best from Section 3.2, and uncoated papers. All of the wrapped mango fruits were kept in the corrugated boxes with 40 cm × 55 cm × 10 cm size and stored in the refrigerated room under 13 ± 1 °C and 90 ± 5 % RH for 35 days. The wrapped mango fruit was randomly sampled every 3 days of storage to evaluate its disease incidence. Fruits without wrapping were set to be a control treatment for the comparison test. Disease index was assessed according to the method described by Zheng et al. (2007) with slight modification. The percent of disease index was calculated by Equation (3.3):

$$\text{Severity index of disease (\%)} = \frac{\sum\left(DS \times N_d\right)}{N_t \times DS_h} \times 100 \quad (3.3)$$

Where, DS is disease scale, N_d is number of fruit in each class, N_t is number of total fruit, and DS_h is highest disease scale.

Fruit with scores of 0 and 1 had commercial value. The total percentage of fruit with these scores was defined as marketable fruit. The extent of total decay area on each fruit surface was determined using the following disease scale: 0 = no visible decay, 1 = < 1 % decay spots, 2 = 1 – 20 % decay, 3 = 20 – 50 % decay, and 4 = > 50 % decay.

3.4.3 Physiochemical Properties of the Wrapped Mango Fruits

Mango fruits were wrapped with the best active-coated papers from Section 3.3, active-coated papers which the best from Section 3.2, and uncoated papers. All of the wrapped mango fruits were kept in the corrugated boxes with 40 cm × 55 cm × 10 cm size and stored in the refrigerated room under 13 ± 1 °C and 90 ± 5 % RH for 35 days. The wrapped mango fruit was randomly sampled every 3 days of storage time to evaluate its weight loss. Likewise, the physicochemical properties of mango fruits wrapped were also investigated every 7 days of storage. The physicochemical properties are evaluated as follows:

1) Weight loss

Weight loss of mango fruit during storage was determined by weighing the samples at specific time intervals using a digital analytical balance with two decimal (Cub is model 225S, Sartorius AG, Germany) and plotting weight losses against time.

2) Firmness

Firmness defined as the maximum force which required to penetrate into the mango flesh, which was measured by using a Texture Analyser TA.XT2i (Texture Technologies Corp., Scarsdale, NY, USA) with a 50-kg load cell. The stainless steel needle probe (P/2N) was used and set to penetrate 5 mm into the fruit at a speed of 2 mm/sec.

3) Color

Color properties of mango fruit's peel and flesh were expressed as the CIE $L^*a^*b^*$ color space, which was measured by the colorimeter (CR-410, Konica Minolta, Japan) by using a standard white calibration tile for calibration. L^*, $+a^*$ or $-a^*$, and $+b^*$ or $-b^*$ were a color parameters described the lightness, redness or greenness, and yellowness or blueness, respectively.

4) Total soluble solid (TSS)

Mango pulps were blended and then pulp solutions were filtrated through a cheese-cloth. Total soluble solids (TSS) were determined by a hand refractometer (Master, Atago, Japan).

5) Titratable acidity

Titratable acidity was determined by titration with NaOH solutions (0.1 N) using phenolphthalein as an indicator. The percentage of titratable acidity was calculated by the below Equation (3.4) and citric acid was used as predominant acid.

$$\text{Titratable acidity}(\%) = \frac{N \times V_1 \times Eq.\ wt.}{V_2 \times 1000} \times 100 \qquad (3.4)$$

Where, N is the normality of NaOH, V_1 is the volume of NaOH, $Eq.\ wt.$ is the equivalent weight of predominant acid, and V_2 is the volume of sample.

3.4.4 Statistical Analysis

All the analyses were performed in 3 replicates. The data obtained was subjected to analysis of variance (ANOVA) using the statistic program (IBM SPSS statistic 20, IBM, USA.). The differences of data averages considered significant at $p \leq 0.05$ using Duncan's new multiple range test.

3.5 Factors Affecting Release Properties of Vanillin from Active-Coated Papers

3.5.1 Release of Vanillin from Active-Coated Paper under Different Conditions

The release of vanillin from the active wrapping paper which gave the best results against mycelial growth and conidiospores germination was investigated in this experiment under three different temperatures, relative humidity (RH), and pH. For temperature effect, an active wrapping papers (1×1 cm) were placed in the incubator (IB-05G, Biotechnical Services, CA, USA) where the temperatures were individually controlled at 13, 25 and 37 ºC for 35 days. For RH effect, active wrapping papers (1×1 cm) were placed in the containers with three different RH (75.7, 86.0, and 95.0 %) generated by various types of saturated salts. These containers were subjected to the incubator controlled at 13°C for 35 days. At time intervals of investigation, the piece of paper was sampled and extracted with 50 % (v/v) ethanol solution to quantify vanillin left in active wrapping paper using HPLC. Under different pH citrate buffers (pH 3.8, 5.2 and 6.2), the vanillin releases were investigated by placing the active wrapping papers (1×1 cm) in amber glass vials, tightly closed with a cap and incubated under 13 ºC and 86.8 % RH for 35 days. The amounts of released vanillin in the buffer solutions during storage were determined by HPLC (refer to Section 3.5.4).

3.5.2 Release of Vanillin in Food Stimulants

HPLC grade water, 3 % (v/v) acetic acid, 10 % (v/v) ethanol, and olive oil were prepared to apply as food stimulants mimicking aqueous foodstuffs, acidic foodstuffs (pH < 4.5), alcoholic foodstuffs, and fatty foodstuffs, respectively. To evaluate the release of vanillin from active-coated paper, the experiment was done according to ASTM D4754-98 with some modification. Samples of active coated paper were cut as a disk shape with a 15 mm diameter. Eight paper disks were threaded onto a stainless steel wire with alternating glass bead spacers and inserted in a 35-mL amber glass vial. Four FDA food stimulant solvents were added into different vials (US FDA, 2002). Vials were capped and placed in controlled temperature chambers at 13, 25 and 37 ºC. Solvents (30 mL) were withdrawn from the 35-mL vial at specific time intervals of 1, 2, 4, 8, 16, 24, and 72 h. The released vanillin in each solvent was quantified by using a HPLC system (refer to Section 3.5.4).

3.5.3 Release of Vanillin from Active-Coated Paper to the Surface of Mango Fruit

The release of vanillin on mango fruit was evaluated in this experiment. Each fruit was disinfected by 70 % (v/v) ethanol solution and immediately wrapped with active-coated paper. The wrapped mango fruits were stored under simulated transportation condition (13 °C and 90 % RH) for 35 days. At time interval of investigation, active-coated papers was removed from mango fruit and then cut as a small piece (5 cm × 5 cm). The piece of paper was extracted with 50 % (v/v) ethanol solution. The vanillin content in the extract obtained was determined using HPLC and expressed as the amount of vanillin left in the active-coated paper.

3.5.4 Quantification of Vanillin

The released vanillin was quantified by according to the method described by Sangsuwan et al. (2009) with slight modification using a high performance liquid chromatography (HPLC) equipped with a quaternary pump system, an autosampler and a UV detector at 280 nm (Agilent 1200 series HPLC system, Agilent Technologies, Santa Clara, California, USA). The analytical column was a Nova-Pak® C18 (3.9 mm ×150 mm, 4 µm particle size). The sample was filtered through a 0.45 µm syringe filter. The filtrate, 5 µL, was injected into the HPLC system. A gradient solvent system consisted of water with 0.015 % (v/v) sulfuric acid and acetonitrile. They were mixed in the ratio of 70:30. The eluent flow rate was 0.8 mL/min and the column temperature was 30 ºC. The calibration curve was created by diluting the standard vanillin in various concentration ranges from 5 to 400 mg/L. The calibration curve with $R^2 = 0.9999$ was plotted. The retention time was 4.2 minutes.

3.5.5 Statistical Analysis

All the analyses were performed in triplicate. The data obtained was subjected to analysis of variance (ANOVA) using the statistic program (IBM SPSS statistic 20, IBM, USA.). The differences of data averages considered significant at $p \leq 0.05$ using Duncan's new multiple range test. In the part of vanillin release, results were analyzed by multifactor analysis of variance with significance at $p < 0.05$. All the analyses were determined using the statistic program (IBM SPSS statistic 20, IBM, NY, USA.).