CHAPTER III

RESEARCH METHODOLOGY

This chapter gives an overview of the research design used and was conducted as a research and development (R&D) methodology to evaluate the efficacy of capsaicin transdermal nanofibers patches made from capsaicin extract for pain relief. This chapter gives a brief description of the study design and treatment, the study design, the study are, materials and methods, statistical analysis, and the methodology used to determine the setting recruitment strategy, the data collection, data analysis, ethical statement, research plan, and budgets for this study.

3.1 Study design

This study is to investigate of research and development (R&D) that will be conducted at Suranaree University of Technology, Thailand.

3.2 Study area

This study was conducted at Nakhon Ratchasima, Thailand

3.3 Materials and methods

3.3.1 Materials

1. Reagents and Materials

1) Poly (vinyl alcohol) PVA (MW = 360,000, Sigma Aldrich)

2) Polyvinylpyrrolidone average (Mw ~1,300,000, Sigma Aldrich)

3) Capsaicin analytical standard

4) Deionized water

5) Ethanol absolute ≥99.8%, AnalaR NORMAPUR® ACS, Reag. Ph. Eur. analytical reagent

2. Apparatus

1) Nipro Syringe 10 ml

2) Aluminum foil (Diamond brand)

3) Magnetic stirrer and magnetic bar (size 40x0.8 mm.)

4) Electrospinning Machine

5) Start-M membranes Transdermal diffusion Test Model (Sigma Aldrich, Merk KGaA, Darmstadt, Germany)

6) The scanning electron microscope (SEM) (JEOL JSM-6010LV InTouchScope)

7) Fourier transform infrared (FTIR)

8) Franz diffusion cells (FDC)

9) High-performance liquid chromatography (HPLC)

MTT assay

DPBS: Dulbecco's Phosphate Buffered Saline, CORNING, USA

DMSO: DIMETHYL SULPHOXIDE (CH3)2SO, Sigma-Aldrich Co. (St. Louis, MO)

MTT: 3-4,5-dimethyl-2-thiazolyl-2,5-diphenyl-2H-tetrazolium bromide, Sigma-Aldrich Co. (St. Louis, MO)

DMEM/ high glucose > Dulbecco's Modified Eagle Medium (High glucose), HyClone (Logan, UT)

Penicillin-Streptomycin Solution, HyClone (Logan, UT)

FBS: Fetal bovine serum, HyClone (Logan, UT)

Real time PCR

Trypsin: 25% Trypsin EDTA (1x), gibco, CA, USA

qPCR: 2x qPCRBIO SyGreen Mix Lo-ROX, PCR BIOSYSTEMS

RNA: NucleoSpin® RNA Plus

ReverTra Ace™ qPCR RT Master Mix with gDNA Remover, TOYOBO CO., LTD. (2-8 Dojima Hama 2-Chome Kita-ku Osaka 530-8230 JAPAN

3.3.2. Methods

1) The Fabrication of CAP/PVA/PVP Nanofibers: To prepare the polymer solutions, 20 g of polyvinyl alcohol (PVA) was dissolved in a solvent mixture consisting of 45 mL of distilled water and 45 mL of absolute ethanol, achieving a 20% w/v concentration. The solution was continuously stirred at 80 °C for 3 hours using an electromagnetic stirrer to obtain a homogeneous and transparent solution. Similarly, 20 g of polyvinylpyrrolidone (PVP) was dissolved in the same solvent mixture and stirred at 60 °C for 3 hours until a clear and uniform solution was achieved. The two polymer solutions were then combined to yield a final polymeric blend containing 10% w/v of each polymer (PVA/PVP). Subsequently, capsaicin (CAP) powder at a concentration of 0.1 mg/mL was incorporated into the PVA/PVP blend at 10% v/w. The resulting drug-polymer mixture was allowed to cool to room temperature for several hours prior to electrospinning as shown in Figure 3.1 (Hindi et al., 2021).

2) Production of the Electrospun PVA/PVP Nanofiber: The electrospinning will be performed at room temperature (Salles et al., 2015) using a 10 ml disposable syringe with a metal needle, 0.55 mm internal diameter, is a common misunderstanding. The resulting polymer solution was loaded into syringe. The solution was powered by a syringe pump with a feeding rate of 10 µL/min. The solution was electrospun under an applied voltage of 15 kV at a 17-cm tip-to-collector distance as well as a room temperature. The fibers were put on aluminum foil that was stuck to a drum that was moving cylindrical collector that represented in Figure 3.1.

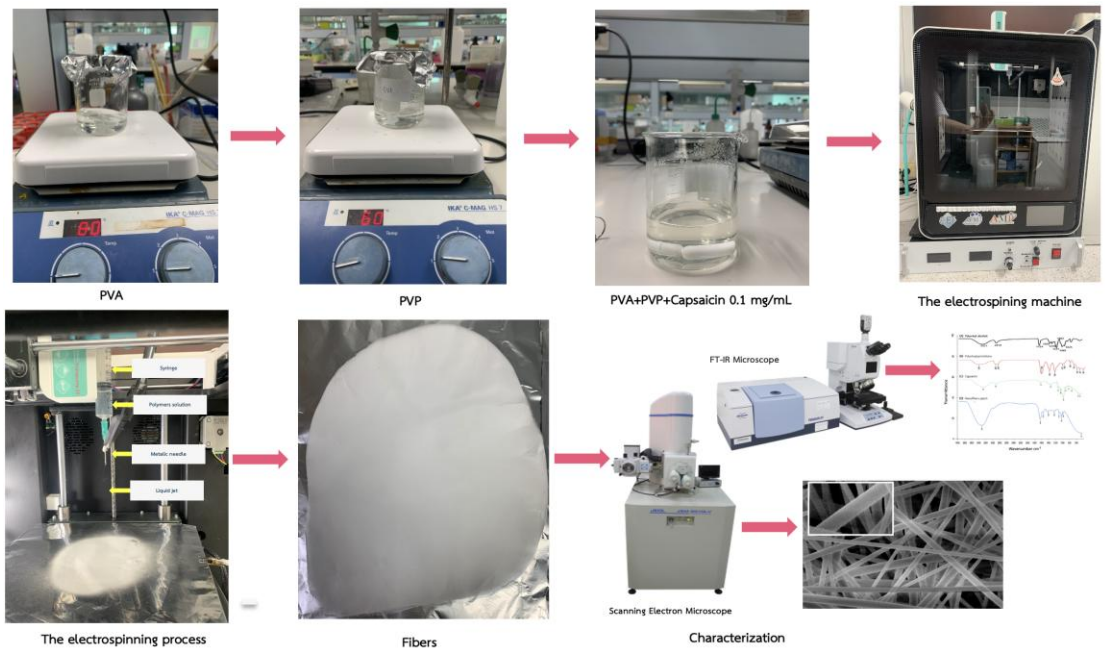

Figure 3.1 Schematic representation of the electrospinning process used to fabricate CAP/PVA/PVP nanofibers

3) Physicochemical Characterizations:

3.1) A Fourier transform infrared spectroscopy (FT-IR) was employed to analyze the chemical composition of the capsaicin-loaded nanofiber patches. The spectra were recorded over a wavelength range of 4000–600 cm^{-1} at a controlled ambient temperature of 25 °C using the Attenuated Total Reflectance (ATR) mode. Each sample was analyzed using a resolution of 4 cm^{-1}, with both the sample and background scanned 64 times to enhance signal-to-noise ratio. All measurements were performed in triplicate to ensure reproducibility. The data acquisition and spectral processing were conducted using OPUS software (Bruker Optics) as shown the workflow in Figure 3.2, enabling detailed evaluation of functional group interactions and confirmation of capsaicin encapsulation within the polymeric nanofiber matrix. (Hindi et al., 2021). The patch sample was analyzed using a Bruker VERTEX 70 FTIR spectrometer, followed by spectral analysis with OPUS software. The spectra show the characteristic transmittance peaks of polyvinyl alcohol (A), polyvinylpyrrolidone (B), capsaicin (C), and the final nanofiber patch formulation (D), confirming successful component incorporation.

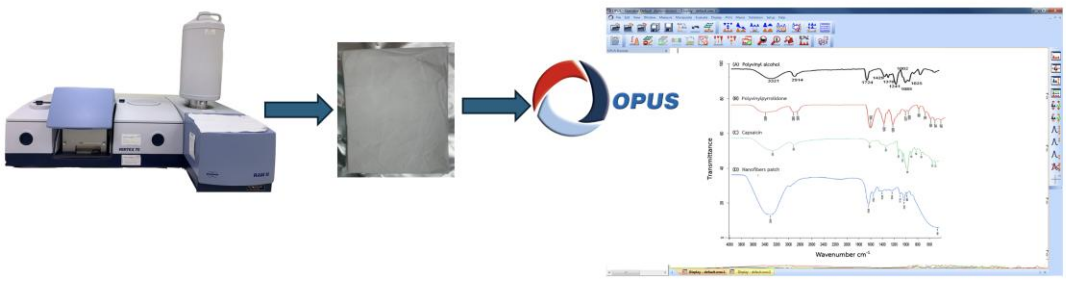

Figure 3.2 FTIR characterization workflow of capsaicin-loaded PVA/PVP nanofiber patch

3.2) Surface Morphology Characterizations (SEM) is the surface morphology of the electrospun nanofibers was examined using scanning electron microscopy (SEM). Prior to imaging, the nanofiber samples were sputter-coated with a thin layer of platinum to enhance conductivity as shown in Figure 3.3. The average fiber diameter and its distribution were quantitatively analyzed using image analysis software based on the SEM micrographs (Hindi et al., 2021).

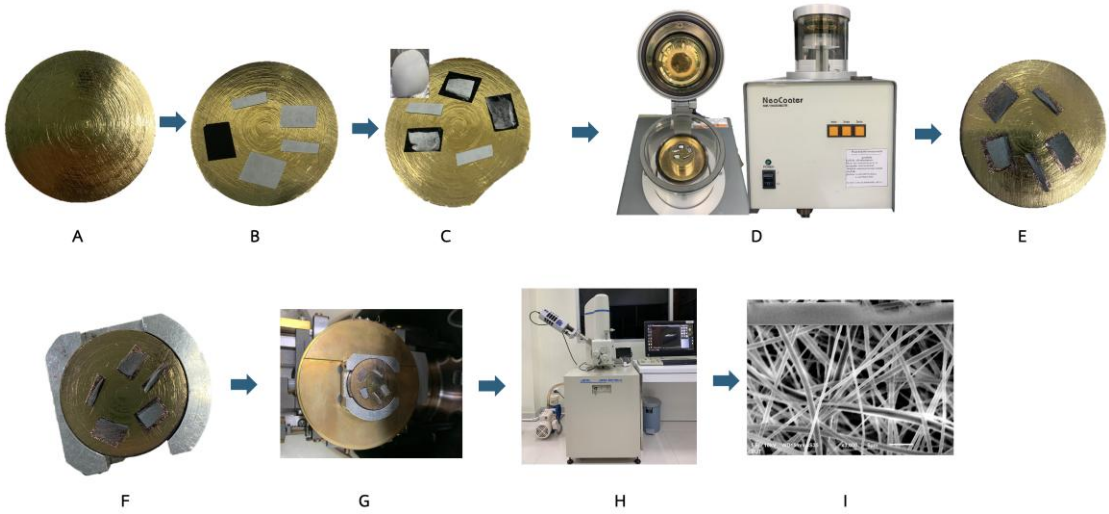

Figure 3.3 Workflow of SEM sample preparation and imaging of electrospun nanofiber patches were prepared for SEM observation by first cutting into square sections (1 × 1 cm^2).

(A) Clean bare aluminum stub surface.

(B) Placement of carbon tape on the stub surface.

(C) Mounting nanofiber samples onto carbon tape.

(D) Sputter coater (Neo Coater/MP-19020NCTR) used for gold coating to enhance conductivity.

(E) Gold-coated nanofiber samples post-coating.

(F) Stub secured into the SEM holder.

(G) Mounted holder being positioned into the SEM chamber.

(H) Scanning Electron Microscope (JEOL JSM-6010LV) system setup.

(I) SEM micrograph of electrospun nanofiber mat, revealing uniform, interconnected fibrous morphology.

The samples were mounted on aluminum stubs using double-sided conductive carbon tape (Figures 3.3 (B-C). To reduce charging during imaging, a thin layer of gold was sputter-coated onto the sample surface using a Gold (Au) Sputter Coater (Neo Coater/MP-19020NCTR) (Figure 3.3 (D–E). The coated stubs were then loaded into a JEOL JSM-6010LV SEM system (Figure XG–H), and surface morphology was observed under an accelerating voltage. A representative SEM image of the capsaicin-loaded nanofiber mat is shown in Figure 3.3 (I). capsaicin nanofiber patches.

Cytotoxicity assay

1. Evaluation of Anti-inflammatory Activity on Human Dermal Fibroblasts (HDF) Using the MTT Assay

The experiment was conducted as represented in Figure 3.4 as follows:

1.1 Human dermal fibroblast (HDF) cells were seeded at a density of 6,000 cells/well in 96-well plates using DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were incubated for 24 hours in a CO_2 incubator maintained at 37°C with 5% CO_2.

1.2 The test extract was prepared at various concentrations by dilution in DMEM medium. Each concentration (0.0001, 0.001, 0.01, 0.1, 1, 10, 50, and 100 mg/mL) was added to the wells at a volume of 100 µL and incubated for 24 hours.

1.3 After 24 hours, 100 µL of MTT solution (0.5 mg/mL) was added to each well and incubated in the dark for 3 hours. Subsequently, the supernatant was removed, and the resulting formazan crystals were dissolved in 100 µL of DMSO.

1.4 The absorbance was measured at 570 nm using a microplate reader.

1.5 The percentage of cell viability was calculated using the following formula (Jaiboonma et al., 2020):

$$\% \text{ cell viability} = \frac{\text{Absorbance of treated cell}}{\text{Absorbance of control cell}} \times 100 \quad (1)$$

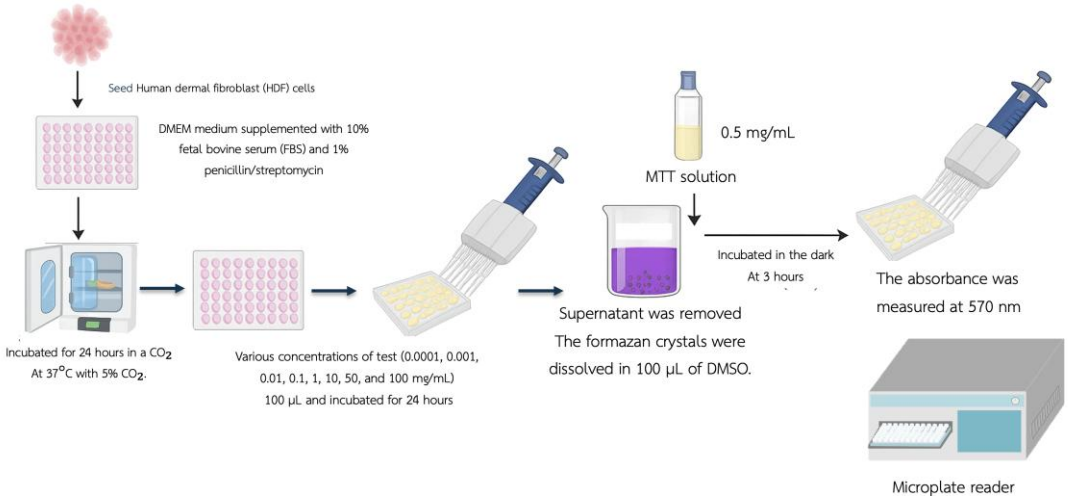

Figure 3.4 Evaluation of Anti-inflammatory Activity on Human Dermal Fibroblasts (HDFs) Using the MTT Assay

2. Investigation of Cyclooxygenase-2 (*COX-2*) Inhibitory Activity for Analgesic Properties

To assess the *COX-2* inhibitory activity of capsaicin at different concentrations, the experiment was performed in Figure 3.5 as follows:

2.1 HDF cells were cultured in 6-well plates at a density of 1×10^6 cells/well and incubated for 24 hours in a CO_2 incubator at 37°C with 5% CO_2.

2.2 The study was divided into three groups: 1) Control group (untreated), 2) Positive control group, where inflammation was induced using 1 mM H_2O_2 for 30 minutes, and 3) Treatment group, where 1 mM H_2O_2 (Kar et al., 2021) was applied for 30 minutes followed by treatment with capsaicin patch extract at 0.1 mg/mL, then incubated for 24 hours.

2.3 After 24 hours, the cells were harvested using trypsinization.

2.4 Total RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel, Dueren, Germany). RNA concentration was quantified using a microplate reader (BMG LABTECH, Ortenberg, Germany). Subsequently, 1 µg of total RNA was reverse-transcribed into complementary DNA (cDNA) using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd). The cDNA samples were stored at −20°C for subsequent *COX-2* gene expression analysis by quantitative real-time PCR (qRT-PCR). All experiments were performed in triplicate.

2.5 qRT-PCR analysis was conducted using the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, MA, USA). The PCR reactions were prepared using SYBR® Green Master Mix (Thermo Fisher Scientific) with primers specific for *COX-2* and GAPDH (used as an internal control). Thermal cycling conditions included initial denaturation at 95°C for 1 minute, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. Melting curve analysis was performed with the following steps: 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds.

2.6 Gene expression was analyzed using the 2^ (-delta delta C(T)) method (Rao et al., 2013), with *COX-2* expression normalized to GAPDH as the housping gene.

2.7 Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using the Student's t-test. Statistical significance was indicated as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (compared to control group); #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 (compared to the positive control group).

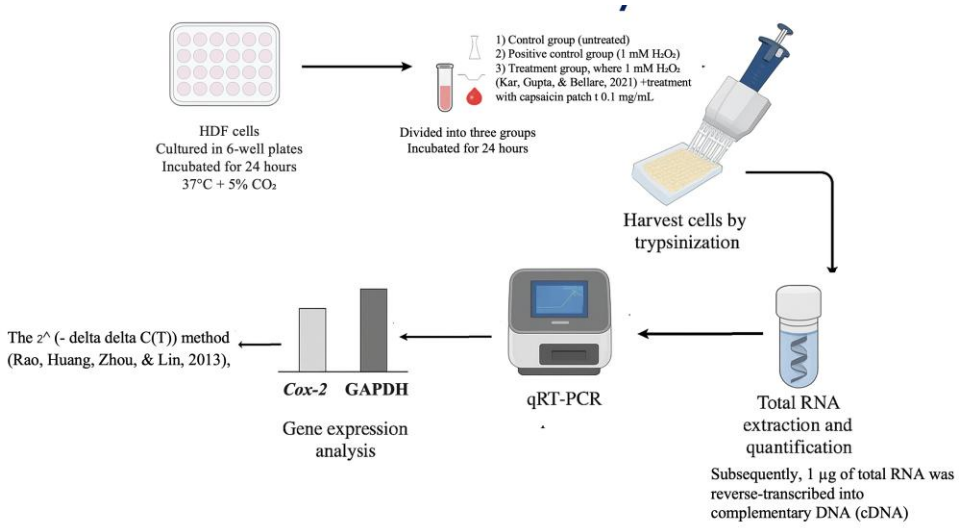

Figure 3.5 *Cox-2* Inhibition assay

The *in vitro* skin permeation

1) Franz diffusion cells: The *in vitro* permeation experiments were carried out using Franz diffusion cells with Strat-M™ Skin permeation as shown in Figure 3.6. The release medium was maintained at 37 °C and the experiment was carried out for 24 h. The Strat-M experiment was done three times, and at different times (1, 2, 4, 6, 8, 12 hours), the circulating solution in the receiver compartment was collected (Pulsoni et al., 2022). After the permeation study was completed, the skin was removed from the Franz cell, then washed with a sodium chloride 0.9%, and dried with a paper towel. (Klebeko et al., 2021).

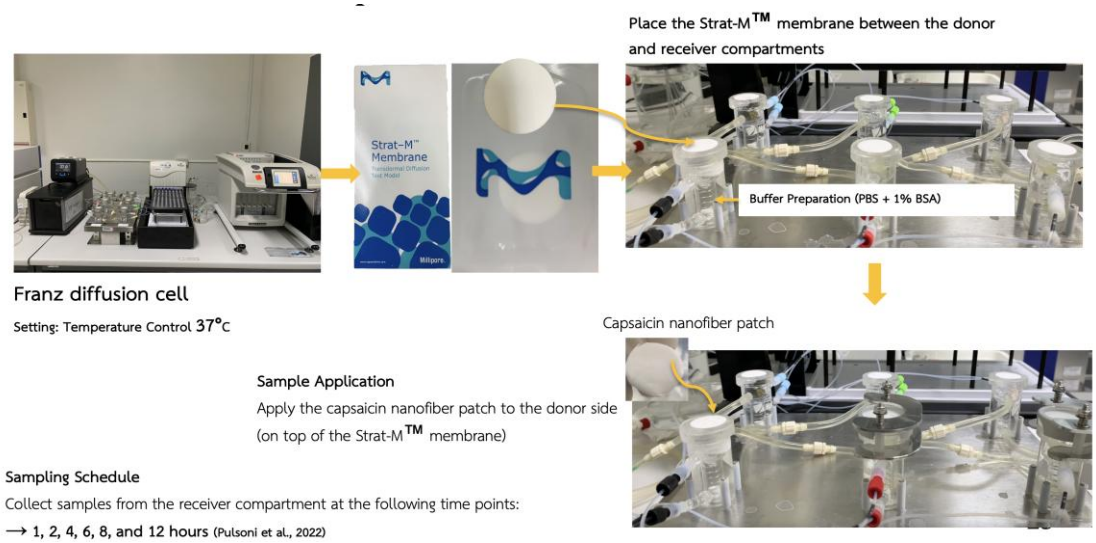

Figure 3.6 Schematic Representation of Capsaicin Permeation Study Using the Franz Diffusion Cell System

The capsaicin-loaded nanofiber patch was applied to the donor compartment of a Franz diffusion cell system, with a Strat-M™ membrane positioned between the donor and receiver chambers to simulate skin permeation. The receiver compartment was filled with PBS containing 1% BSA and maintained at 37 °C. Samples were collected at predetermined time points (1, 2, 4, 6, 8, and 12 hours) to evaluate capsaicin diffusion. This method enables in vitro assessment of transdermal drug delivery and membrane permeation.

2) High-performance liquid chromatography (HPLC) analysis: Following a 12-hour permeation study as represented in Figure 3.7, the skin surface was rinsed with deionized water and air-dried. Capsaicin levels in the stratum corneum (SC) were assessed using a tape-stripping technique with 20 pieces of 3M Scotch Magic™ tape (1 × 1 cm). To determine capsaicin retained within the deeper skin layers, the tape-stripped skin was finely minced. The tape samples and skin fragments were each extracted with a 1:1 (v/v) solution of phosphate-buffered saline (PBS, pH 7.4) and absolute ethanol, using 5 mL and 2 mL volumes, respectively. All samples were subjected to 30 minutes of sonication, followed by filtration. The resulting filtrates were analyzed for capsaicin content by HPLC using a detection wavelength of 280 nm and an injection volume of 10 µL.

High-performance liquid chromatography (HPLC) was carried out using an HP1100 system equipped with a UV detector set at 280 nm (Hewlett-Packard, Waldbronn, Germany). Separation was achieved on a Hypersil ODS column (250 × 4.0 mm i.d., 5 µm; Agilent, CA, USA) using a mobile phase consisting of acetonitrile and 1% acetic acid (1:1, v/v) at a flow rate of 1.0 mL/min. The injection volume was 10 µL. Method validation for capsaicin quantification was assessed based on accuracy, precision, and linearity. Quantification was performed using a calibration curve, which demonstrated strong linearity. All measurements were conducted in triplicate (Anantaworasakul et al., 2020).

The permeation rate of capsaicin across a Strat-M™ membrane was determined from the slope of the plot representing the cumulative amount permeated over time (hours). The lag time was identified as the x-intercept of the linear segment of the curve. The steady-state flux of capsaicin was subsequently calculated according to Equation (2) (Tatke et al., 2018).

According to Equation (3), the permeability coefficient (Kp) and enhancement ratio (Er) of capsaicin were systematically calculated to assess transdermal performance (Tatke et al., 2018).

$$\text{Steady state flux (Jss)} = (dQ/dt)/A \quad (2)$$

$$\text{Permeability coefficient (Kp)} = \text{Steady state flux/Donor concentration} \quad (3)$$

Where: J is the steady-state flux (μg/cm²/hr)

dQ/dt is the rate of drug permeation (μg/hr)

A is the diffusion area (cm²)

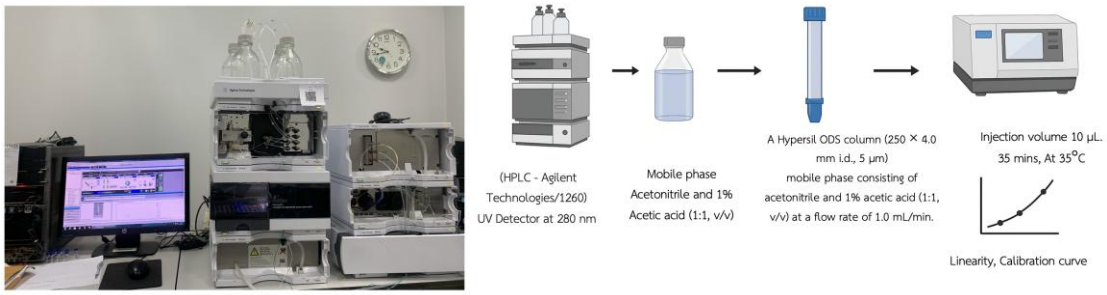

Figure 3.7 High-Performance Liquid Chromatography (HPLC) Setup and Analytical Procedure for Capsaicin Quantification

The quantification of capsaicin was conducted using an HPLC system (Agilent 1260, Agilent Technologies) with a UV detector set at 280 nm. The mobile phase consisted of acetonitrile and 1% acetic acid (1:1, v/v). Chromatographic separation was performed on a Hypersil ODS column (250 × 4.6 mm, 5 μm) at a flow rate of 1.0 mL/min and a column temperature of 35 °C. A sample injection volume of 10 μL was used, with a total run time of 35 minutes. Quantification was based on a linear calibration curve constructed from standard capsaicin solutions (Anantaworasakul et al., 2020).

3) **FTIR microspectroscopy**

Sample preparation for FTIR microspectroscopy analysis: Strat-M membrane samples were prepared by sectioning into small tissue-like pieces, each approximately 1 cm in length and 1 cm in thickness. For each test sample, three pieces were randomly excised from different regions to ensure representative distribution. The tissue fragments were then placed in an aluminum bowl and fully immersed in OCT compound (Tissue-Tek®, Electron Microscopy Sciences, Hatfield, PA, USA). The

bowl was gently placed above liquid nitrogen until the OCT medium was completely solidified. Subsequently, the frozen samples were stored at −80 °C until cryosectioning.

Tissue sections were prepared using a cryostat to obtain slices approximately 5 µm in thickness as shown in Figure 3.8. These sections were carefully mounted onto infrared-transparent (IR) slides. The mounted samples were then dehydrated in a vacuum desiccator for 48 hours to ensure complete drying prior to FTIR microspectroscopic analysis (Thumanu et al., 2017; Thumanu et al., 2015).

FTIR microspectroscopy analysis: FTIR analysis was performed using a Tensor 27 FTIR spectrometer (Bruker Optics, Germany) coupled with a Hyperion 3000 IR microscope (Bruker Optics, Germany) operating in transmission mode. Spectral acquisition and instrument control were carried out using OPUS software version 7.5 (Bruker Optics, Germany). Measurements were conducted at 36× magnification with a spectral resolution of 4 cm^{-1}. The background spectrum was collected using 64 scans. Infrared absorption spectra were acquired in the range of 4000–900 cm^{-1}. Each sample spectrum was recorded using an aperture size of 15 × 15 µm². For spatially resolved chemical imaging, spectral mapping was performed over an area of 22 × 15 µm² (Thumanu et al., 2017; Thumanu et al., 2015)

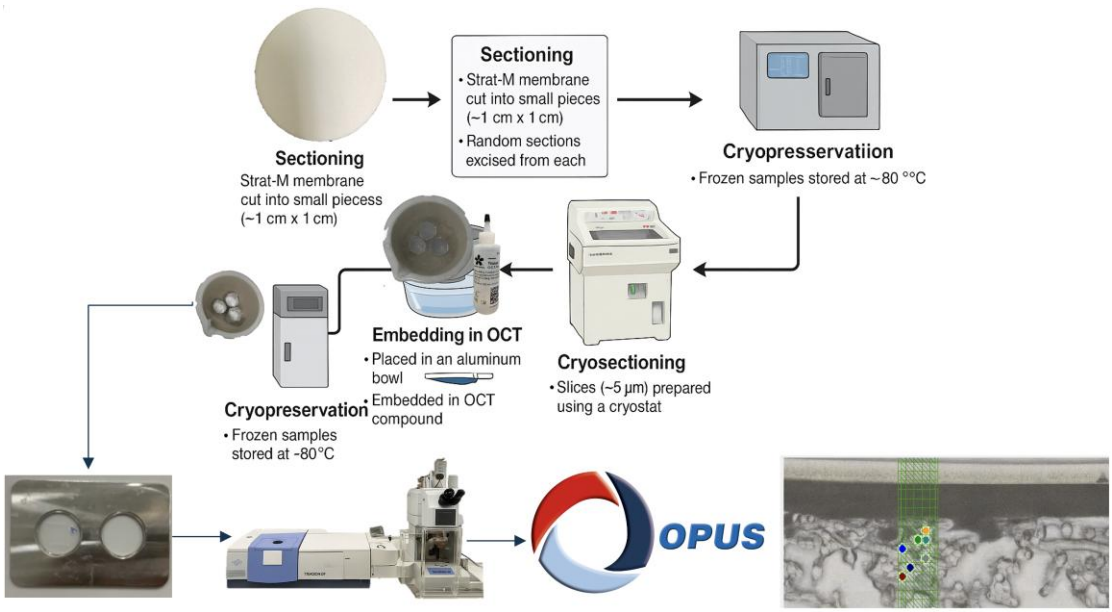

Figure 3.8 Schematic Representation of Strat-M™ Membrane Preparation and FTIR Imaging Workflow

This scheme illustrates the stepwise preparation of Strat-M™ membrane samples for FTIR imaging analysis. The membrane was cut into 1 cm × 1 cm sections and cryopreserved at −80 °C. Selected sections were embedded in optimal cutting temperature (OCT) compound using aluminum molds. The embedded samples were then cryosectioned using a cryostat to obtain thin slices (~5 µm). These slices were subjected to FTIR imaging, and spectral data were analyzed using OPUS software to investigate the spatial distribution of chemical components within the membrane.

3.4 Ethical statement

Ethical consideration: This study does not have any conflicts of interest.

Ethical approval was granted by the Human Research Ethics Committee, Health Research and Development Center of Nakhon Ratchasima Public Health Provincial Office, Nakhon Ratchasima, Thailand, via reference letter No. NRPH 004 dated 01-02-2023.

3.5 Research plan

Table 3.1 Research plan

Descriptions	Month, Year (Thesis started in semester 1/2021)											
	1	2	3	4	5	6	7	8	9	10	11	12
Starts in year 2021												
Meeting committee	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Literature review				✓	✓	✓	✓					
Year 2022												
Proposal & ethic approval											✓	✓
Year 2023												
1. Development of transdermal nanofiber patches - The Fabrication of CAP/PVA/PVP Nanofibers - Electrospinning process (Production of the Electrospun CAP/PVA/PVP Nanofiber) 2. Apparatus and Characterization Characterization its physicals and chemicals properties	✓	✓	✓	✓	✓	✓						
Data collection	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Data and statistical analysis								✓	✓	✓	✓	✓
Discussion and conclusion										✓	✓	✓
Year 2024												
Full report	✓	✓	✓	✓						✓	✓	✓
The *in vitro* skin permeation The Strat-M® experiment was done three times, and at different times for collection the data, the CAP transdermal nanofibers patch prototype	✓	✓	✓	✓	✓							
Conference and publication	✓	✓	✓	✓	✓							
Thesis defense				✓	✓	✓						
PhD completion in 2024					✓	✓						