CHAPTER 3

Materials and methods

3.1 Plant material

Baby Cos lettuce (*Lactuce sativa* L. var. *longifolia*) cv. Baby star were cultivated under commercial maturity and harvested in the morning. The plant area located at the upland area near Chom Thong district, Chiang Mai province and transport to laboratory within 2 hours by pick-up truck. The plants were selected for uniformity of color and size and pre-trimming from the field. The initial produce temperature before precooling was generally 16-25^{o}C.

3.2 Research Methods

The research was divided into 4 parts. First experiment was an experiment on determining optimum parameters of vacuum cooling process of baby cos lettuce. The second experiment, artificial neural network (ANNs) and respond surface methodology were used to predict the optimum parameters for vacuum cooling. The third experiment investigated the effect of precooling method on physico-chemical qualities of baby cos lettuce during storage and the forth experiment measure the antioxidant capacity of lettuce base on flow injection Coularray detector.

3.2.1 Experiment 1: The study of optimum parameter conditions for baby cos lettuce under vacuum cooling

The trimming baby cos lettuce were sorted into perforated plastic basket (0.5x0.36x0.31m). Then arranged the basket in a vacuum chamber to reduce temperature from initial temperature of produce until final temperature at 4±1 °C with following vary parameter set of vacuum cooling systems.

- **Reserving pressure**: Requiring the final pressure of vacuum chamber is lower than the atmospheric pressure was vary from 5-10 mbar

- **Reserving time**: The duration requiring for holding the produce under the final pressure set varied from 10-30 min.
- **Initial produce temperature** of produce were between 13.40 to 26.13 °C
- **Initial weight** of produce was varied from 79-330 kg.
- **Chamber temperatures** were between 14.20-24.90°C

The following data during the vacuum cooling process were collected in order to study the changes in atmospheric conditions and the relationship between the pressure, air temperature, produce temperature and time of cooling process. Then selected the best optimum parameters from the following criteria :

- Weight loss percentage
- Relative humidity (%RH)
- Core temperature of baby cos lettuce (°C)
- Chamber pressure reduction (mbar)
- Cooling rate (°C/min)
- Total cycle time (min)
- Electrical energy used during the process of vacuum cooling.

The optimum parameters of vacuum cooling baby cos lettuce were collected from parameters resulted core temperature of baby cos lettuce got to the final temperature of 4±1 °C with lower weight loss percentage and electrical energy used.

3.2.2 Experiment 2: Prediction of Baby cos lettuce final temperature and weight loss percentage using Artificial Neural Network (ANNs) and Multiple linear regression (MLR)

The Artificial Neural Networks : ANNs model developed in this study by using Matlab 11.0 software (The Mathworks, Inc., Natick, MA, USA). In this experiment one hidden-layer Feed-forward Back Propagation (FFBP) is used to predict two performance indices consisted of produce final temperature and weight loss percentage after vacuum cooling process based on 5 input variables included initial temperature, final pressure, reserving time, initial produce weight and chamber temperature.

Generally, the architecture of the ANNs consists of three layers: an input layer, a hidden layer and an output layer (Figure 3.1).

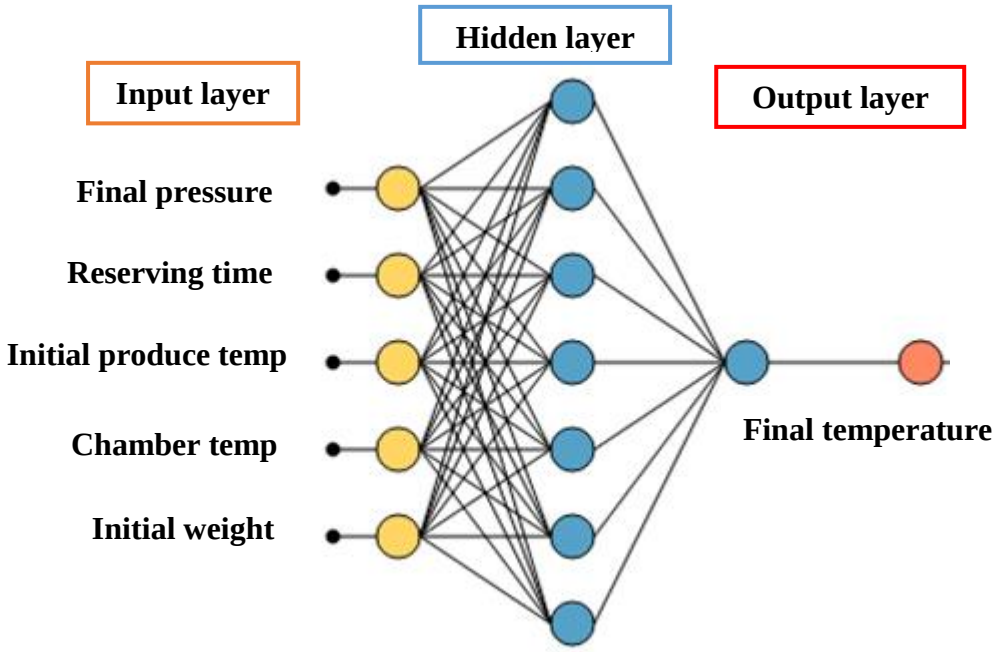

Figure 3.1 Architecture of ANNs selected for prediction of vacuum cooling process

3.2.2.1 Network Training

The optimized configurations selected from actual 52 data records. Total of 36 data records were used for training set (70%), data records for testing set (15%) and 8 data records for validated (15%) the performance of ANNs to predict final produce temperature and % weight loss. Before training, the network inputs and target were normalized in order to facilitate generalization of network and to avoid over-fitting that may appear due to very large or very small weights (42). Both input variables and targets were normalized ranging from 0-1 as shown in equation 1

$$X_{norm} = \frac{X - X_{\min}}{X_{\max} - X_{\min}} \qquad (1)$$

Where, X_{norm} is the normalized value

X is the actual value

X_{min} is the minimum value

X_{max} is the maximum value

A back propagation algorithm was used to implement supervised training of the network. Hyperbolic tangent sigmoid was used as the transfer function in hidden layer, and a linear transfer function was used in the output layer. Levenberg-Marquardt (LM) algorithm (Levenberg, 1944; Marquardt, 1963) optimization procedure was used to minimize error. Training was finished when the mean square error (MSE) converged and was less than 0.001 or training was completed after 1000 epochs, where an epoch represents one complete sweep through all the data in the training set.

3.2.2.2 ANNs and MLR performance determinations

The competent ANNs model for predicting vacuum cooling parameters of baby cos lettuce was selected based on the performance of the ANNs networks architecture and RSM model that presented the minimum error from the training process. The mean relative error (MRE), mean absolute error (MAE), root mean square error (RMSE) and adjusted coefficient of determination (R^2_{adjust}) were calculated following by the equation 2-5. The better predictability were used to compare the performance of various ANNs configurations which considered as a competent model for final temperature and weight loss percentage prediction (50-52).

$$\text{Mean Relative Error} = \frac{\sum_{i=1}^{N}\left(\frac{y_{predicted,i} - y_{observed,i}}{y_{observed,i}}\right)}{N} \times 100 \quad (2)$$

$$\text{Mean Absolute Error} = \frac{\sum_{i=1}^{N}\left|y_{predicted,i} - y_{observed,i}\right|}{N} \quad (3)$$

$$\text{Root Mean Square Error} = \sqrt{\frac{\sum_{i=1}^{N}\left(y_{predicte,i} - y_{observed,i}\right)}{N}} \quad (4)$$

$$R^2_{adjust} = 1 - \left[\frac{\left(1 - R^2\right) \times \left(N - 1\right)}{N - K - 1}\right] \quad (5)$$

Where; R^2 = Coefficient of Determination

N = Number of observation

K = Number of Independent Variables

3.2.3 Experiment 3 Effect of vacuum cooling on qualities of baby cos lettuce during storage compare with the forced-air cooling and room cooling

This experiment was conducted to determine the effect of difference precooling methods on the physical, chemical and microbiological qualities of baby cos lettuce after cooling and during storage. The experimental design was completely random (Completely Randomized Design: CRD) with 4 sets of experimental treatments and 3 replications with the following treatments;

Treatment 1 : Control (non-precool)

Treatment 2 : Precooled with fast cooling rate by vacuum cooling

Treatment 3 : Precooled with medium cooling rate by forced-air cooling

Treatment 4 : Precooled with slow cooling rate by room cooling

Vacuum cooling was carried out in a vacuum cooler with a capacity for maximum load 300 kg, with a power of 500 kW and a final reserving pressure of 6.0 mbar for 25 min (optimum parameter collected from experiment 1). Forced air cooling was carried out with blower fan with a power of 1.5 kW and air velocity was vary from 0.8-2.1 m/s depending on the position of product, air temperature was 4±2 °C and 80-85% RH. And room cooling was conducted in a cold room storage size 4.86 m x 9.10 m x 3.26 m. with air temperature 2±2 °C and 65% RH (Figure 3.2). After precooling, baby cos lettuce outer leaves was trimmed and packed in commercial plastic bag (perforate polyethylene, ∅ 0.5 cm and 18 holds) for 250-300 gram/pack. The sample from three different precooling treatment subsequently stored at 4±1 ºC with 85% RH. Physico-chemical qualities were analyzed throughout the storage period.

Figure 3.2 Different precooling methods for baby cos lettuce

3.2.3.1 Weight loss percentage

All samples were weighed using a balance before and after treatment. The percentage of weight loss were calculated from the difference between the initial and the final weight (53).

3.2.3.2 Color changes

The change of baby cos lettuce leaf color was measured by Minolta Chroma Meter. Produce were measured on subsequent occasion at the same spot (as far as possible) to assess changes in baby cos lettuce color using the CIELAB system (54).

3.2.3.3 Texture

Determine texture properties of the samples using texture analyzer. Texture values were expressed as the coefficient (max load−min load)/max load) which was defined as the Crispiness Coefficient (CC) (54).

3.2.3.4 Total soluble solids (TSS)

Total soluble solids was measure from fresh juice of baby cos lettuce with a digital refractometer (53).

3.2.3.5 Chlorophyll and Carotenoid contents

Chlorophylls were conducted by extracted homogenized of 1 g frozen leave in 20 ml of 80% acetone. The extract was filter through Whatman® filter paper No.1 and fill up to 25 ml in volume, the chlorophyll content were determined via a spectrophotometer from the acetone extract at the respective wavelengths of 663 and 645 nm. Concentrations of chlorophylls *a*, chlorophylls *b* and total chlorophyll were determined from the following equations and expressed in µg/g FW (55).

Chlorophyll *a* = [12.7(Abs. at 663 nm) – 2.69(Abs. at 645 nm)] × (V / 1 0 0 0 W)

Chlorophyll *b* = [22.9(Abs. at 645 nm) – 4.68(Abs. at 663 nm)] × (V/1000W)

Total chlorophyll = [20.2(Abs. at 645 nm)+8.02(Abs.at 663 nm)]×(V/1000W)

By mean; V = final volume of solute

W = sample weight (g)

Carotenoid contents evaluated by dimethylsulphoxide tonoplast hydrolysis follow by Pawelzik, 2005 with some modifications. Homogenized of 1 g frozen leave were extracted with 10 ml of dimethylsulphoxide solution and stirred for 5 minutes. Then left sample in dark condition for 16 h. After this, the extract were filter through Whatman® filter paper No.4 and measured the extract with the spectrophotometer at the respective wavelengths of absorbance of 665, 649 and 480 nm. Total carotenoid content were determined from the following equations and expressed in µg/g FW (56).

Chlorophyll a = [(12.19 × Abs. at 665) – (3.45 × Abs. at 649)]

Chlorophyll b = [(21.99 × Abs. at 649) – (5.32 × Abs. at 665)]

Total chlorophyll = Chlorophyll a + b

Total carotenoid = [(1000 × Abs. at 480)-(2.14 × Chlo. a)-(70.16 × Chlo. b)]

220

3.2.3.6 Total phenolic content

The total phenolic content was measured using the Folin–Ciocalteu procedure based on the method of Singleton *et al*. (1999) with some modifications. Methanolic plant extract 250 µl mixed with 1,000 µl of Folin–Ciocalteu reagent (diluted 1:10 in DI water). After standing for 3 min, 2,000 µl of 7.5% sodium carbonate/water (w/v) was added and the contents of the tubes were thoroughly mixed before incubation at room

temperature for 120 min. The absorbance at 765 nm wavelength was read and calculated by using gallic acid as standard and expressed as mg of gallic acid equivalent (57).

3.2.3.7 Ascorbic acid content

Ascorbic acid content was determined by 2,6-dichlorophenol-indolphenol titration method by standardizing 0.04% 2,6-dichlorophenol-indolphenol dye solution against 0.1% ascorbic acid solution according to Ranganna (1986). Ascorbic acid was estimated by diluting 10 g of homogenized sample with 0.4% oxalic acid and adjust volume to 100 ml. Mix thoroughly by shaking to ensure uniform test portion, and filtered through filter paper Whatman® No.1. After filtered, 10 ml solution aliquots of each treatment were titrated with dye solution until sample's color change to rose pink color solution > 15 s. The results were expressed in mg of ascorbic acid per 100 g FW (58).

3.2.3.8 Determination of total antioxidant capacity (TAC)

The antioxidant capacity were estimated using DPPH (2,2-diphenyl-1-picrylhydrazyl) assays. The antioxidant assay carried out using a spectrophotometer at 515 nm and the results expressed as Trolox equivalent antioxidant activity (TEAC) in µm (Trolox)/g fresh weight (59).

3.2.3.9 Visual quality evaluation

Heads of lettuce was examine in the laboratory at the beginning of the experiment and during cold storage by a sensory panel following a 9 point hedonic scale show in Table 3.1. Rating for individual heads are based on presumed consumer acceptance and are related to quality factor such as decay, wilting, mechanical damage and other defects (33).

Table 3.1 Rating scale for visual quality

Score	***Visual quality description***
9	Excellent, essentially free from defects
7	Good, minor defects; not objectionable
6	Fair, slightly to moderately objectionable defects; lower limit of sale appeal
3	Poor, excessive defects, limit of salability
1	Extremely poor, not usable

3.2.3.10 Transmission Electron Microscope

Transmission electron microscopy (TEM) was used to evaluate the ultrastructure of precooled cos lettuce. Following the difference cooling methods and cold storage for 2 weeks, one representative sample of every replicate from each treatment. Tissue samples were cut into sections of approximately 1 mm thick from the center of the leaf lamina by hand and chosen from the same area for each treatment. Samples were immediately fixed in 3% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), for 24 h at room temperature and rinsed three times in the same buffer and then, the samples were post fixed with 1% (w/v) osmium in the phosphate buffer, for 1.30 h at room temperature. After aqueous washing (twice) in phosphate buffer, tissues were dehydrated twice for 10 min through a graded series of ethanol/water (50%, 70%, 85%, 95% and 100% v/v) and embedded in pure epon–araldite (Embed-812) over a period of 24 h, and polymerized at 60 °C for 48 h. Following polymerization, thin sections (approx. 83 nm) were cut by ultra-microtome (Leica EM UC7, Germany) and then the sections were placed on copper sieves and post-stained with uranyl acetate (ethanolic) and lead citrate, for 30 min and viewed under JEM-2200FS FIELD EMISSION ELECTRON MICROSCOPE (JEOL Japan Electronics Co., Ltd., Japan) (60).

3.2.4 Experiment 4: Rapid Determination of Lettuces Antioxidant Capacity by e-Tongue Based on Flow Injection Coulometry.

This study was evaluated a simple flow-injection protocol based on Coularray profiling to compare the stability of lettuce samples treated with different cooling procedures by determined the correlation of antioxidant capacity in precooled lettuce sample obtained from a Coularray detector system and those obtained from the simple test method for detected the antioxidant capacity using DPPH assay.

3.2.4.1 Lettuce samples

Fresh lettuce samples were obtained from commercial sources and stored at 4 °C until used**.** Wrapper leaves were discarded. Lettuce leaves were sheadded with a stailess steel knife before extraction.

3.2.4.2 Sample extraction

Before extraction, fresh lettuce tissues were dried by **(**1**)** liquid nitrogen or **(**2**)** lyophilization**.** Lyophilization was performed for 48 h. Two different cycles were performed**:** 1**)** with a primary stage at 35 °C and 0.37 mbar for 26 h, followed by a secondary stage at 40 °C and 0.05 mbar for 22 h**;** 2) with a primary stage at 40 °C and 0.37 mbar for 26 h followed by a secondary stage at 45 °C and 0.05 mbar for 22 h. After drying, the extraction was performed with one of the following solvents: methanol, ethanol or acetonitrile. Briefly, 10 g of dried sample was mixed with 10 ml of the solvent and left for 1 h at 20 °C. Finally, the suspension was filtered through a filter paper No.1 and 0.45 μm Nylon filter discs **(**Millipore, Billerica, Mass., USA**)**. The extracts was finally injected into the Coularray Detector system or used for analysis with the DPPH method.

3.2.4.3 Precooling treatments

Lettuce samples were exposed to four precooling method as follows**:**

1) No treatment **(**control**)**
2) Room cooling**:** lettuce samples were cooled from 20°C to 5.0 °C±0.5 °C in a thermostat**.**

3) Vacuum cooling 1 obtained by placing the samples in the vacuum chamber at final pressure 5.0 mbar and temperature 2.0±1.0 °C, for 21 min.
4) Vacuum cooling 2 obtained by placing the samples in the vacuum chamber at final pressure 10 mbar and temperature 5.0 ± 1.0°C, for 36 min.

After cooling, the samples were packed in perforated polyethylene bag and stored at 5 °C for 7 days. Afterwards, analyzed the qualities of fresh lettuce and antioxidant capacity of the extracts.

3.2.4.4 Flow Injection with Coularray Detector

Ultimate 3000 HPLC system equipped with a membrane degasser, two peristaltic pumps, autosampler with 20 µL injection loop and thermostated column compartment, coupled with 16 channel Coularray Array detector **(**Thermo Fischer Scientific Dionex, USA**)** was used for the analysis of the total antioxidant capacity of oleuropein solutions. The detection system consisted of four packs of four porous grafite working electrodes, poised at growing potentials from from 100 to 850 mV vs Palladium **(**Pd**)** reference electrode, with 50 mV increments, each applied to one of the sixteen serial electrochemical graphite cells. Palladium reference electrode is endowed in the coulometric detector, giving an overall +300 mV in peak voltage than the Ag/AgCl reference in acid environment. Flow injection in isocratic mobile phase consisting of 60 mM $LiClO_4$ in methanol, flow rate of 1 mL/min was used. Samples were diluted with the mobile phase 1:5 before the injection **(**20 µL**)**. The gallic acid solutions and lettuce extracts were analyzed in triplicate. Integrated peak areas of 16 Coularray channels were used as a fingerprint to monitor the antioxidant activity of lettuce extracts. Data acquisition was performed using Coularray ESA software **(**ESA Chelmsford, MA, USA**)**. Integration and export of data was performed in Chromeleon CDS software.

3.2.4.5 Radical scavenging activity of samples by DPPH assay

Antioxidant activity was determined by a free radical scavenging assay using DPPH **(**1,1-diphenyl-2-picrylhydrazyl**)** as the source of the free radicals**.** For the DPPH assay– according to Williams-Brand *et al*. 1995 (61), the 50 µl of methanolic extracts were mixed with 1 mL 6x10-5 M solution of DPPH· in 48% ethanol. Absorbance at 515

nm was measured immediately and after 120 min of incubation. The affinity of the test material to quench DPPH free radicals was evaluated according to the equation**:**

$$DPPH\ scavenging\ capactiy\ (\%) = \frac{A\ control - A\ sample}{A\ control} \times 100$$

The antiradical activity was related to Trolox and expressed as mg of Trolox per gram of dry weight **(**DW**) (**TEAC, Trolox equivalent antioxidant activity**).**

3.3 Statistical Analysis

The experiments 3.2.3 carry out with a completely randomized design and the effect of the precooling methods on qualities of baby cos lettuce were interpreted by analysis of variance (ANOVA) technique. Significance was judged by determining the probability level ($p < 0.05$). The statistic system of the software SPSS Statistics 17.0 and compared means by Duncan's tests. The experiments 3.2.4 were conducted using XLStat**™** 2016**.**02**.**28430 software **(**Addinsoft, Paris, France**)** as an add-in to Microsoft Excel**™** 2010**.**

3.4. Research Locations

1. The Royal Project Produce Center, Mae Hia, Chiang Mai, Thailand.

2. Department of Food Science and Technology, Chiang Mai University, Chiang Mai, Thailand.

3. Faculty of Science and Technology, Free University of Bozen-Bolzano, Bolzano, Italy.