CHAPTER III
METHODOLOGY

This research was to determine the influence of shade on the physical characteristics, biochemical composition and organoleptic quality of coffee (*Coffea arabica* L. cv. Catimor) produced at Chulabhorn Dam Agricultural Research and Training Station (CRTS), Faculty of Agriculture, Khon Kean University, Chaiyaphum Province, Thailand. The effects of roasting conditions on total antioxidant activity of coffee were determined. In addition, determination of phenolic acids compositions and antioxidant activities from coffee bean grown under different shade conditions were also investigated.

Experimental plan

This research was divided into two experiments including (1) determination of the physiological characters of coffee grown under shading conditions (2) determination of quality of coffee: determination of antioxidant activity and cup test of coffee grown under shading conditions and roasting levels. All experiments were done in triplicate.

1.1 Experiment 1 was to determine physiological characters, morphology characters, yields and yield components of coffee grown under shading conditions.

1.2 Experiment 2 was to determine phenolic compounds including total phenolic contents, phenolic acids and cup test of coffee beans grown under five shading conditions and three roasting conditions and evaluate their antioxidant activities.

For experimental plans used in this research were completely randomized design (CBD) in experiment 1 and split plot experiment in a randomized complete block design in experiment 2. Analysis of variance was used to test any difference resulting from these methods and was compared by the Bonferroni test to determine significant differences at $p < 0.05$.

1. **Experiment 1** was to determine physiological characters of coffee grown under shading conditions.

1.1 Field experiment

The plants of *Coffea arabica* L. cv. Catimor were cultivated at Chulabhorn Dam Agricultural Research and Training Station, Faculty of Agriculture, Khon Kean University, Chaiyaphum Province, Thailand. (16° 32′ 9.6″ N, 101° 38′ 49.2″ E: 780 m above sea level). The plants were divided and grown in the field and covered with black saran shade cloth. Five different shading conditions are described in Table 11. A Completely Randomized Design (CRD) with four replications was used for experiment. The rainfall and average daytime temperature during growth under different treatments are shown in Table 11. Average temperature was measured from the period between sunrise to sunset at different six time points (08.00, 10.00, 12.00, 14.00, 16.00 and 18.00). The average yearly annual rainfall was 284 mm, without a marked dry season. The climatic conditions at the experimental site were suboptimal for coffee (Kumar and Tieszen, 1980). During winter months, relatively cold nights (minimum temperatures typically ranging from 20 to 34°C) followed by warm, sunny days (maximum temperature exceeding 34°C) are frequently observed. The average yearly annual temperature was 27 °C. The plans of coffee management was intensive with two fertilizations per year of 300 kg ha^{-1} of fertilizer (15-15-15; N-P-K) and 250 kg ha^{-1} of ammonium nitrate (33%N). Weed and diseases were controlled with a minimum of herbicides and fungicides. Five shade treatments (Table 11) were repeated in four blocks. The sizes of each plot measured between 10 x 20 m^2. Netted plots consisted of 20 trees each. Light intensity was measured by Luxmeter. Coffee samples used in this study were collected from different shading conditions.

Table 11 Light intensity and temperature of coffee grown under different shading conditions

Shading conditions	Light intensity (lux)	Temperature (^{0}C)
full sunlight	58,800	24-34
50% shade with saran covering	11,050	23-33
60% shade with saran covering	10,700	20-31
lychee shade	9,980	20-31
70% shade with saran covering	9,080	20-31

1.2 Plant materials

The coffee beans were harvested from five year - old trees. Coffee beans were sampled in 15 plots for harvest. In each plot, a total of 500 fresh cherry samples were taken. Coffee cherry samples were harvested at the maturation stage (231 day after flowering). Coffee-fruit weight was measured as the fresh weight (g) of 300 randomly selected ripe fruits from each plot, taken from the collection. After harvesting, the ripe cherries were dipped in water to soften the skin and then the soft ripe cherries were put in the pulping machine to remove pulp and mucilage. After that, green beans were separated from the husks and ripe cherries.

1.3 Harvesting and postharvest processing

In each plot, coffee was harvested at the maturity peak when coffee is supposedly of higher quality (Guyot et al., 1996). The green beans were fermented by natural microflora contamination. Fermentation of green coffee was done under water at room temperature (36 hours). The green beans were removed from the fermented plastic bags and dried on a cement floor by solar drying until the moisture content was about 12% or even lower.

Parchment was removed after a rest period of at least two months. Coffee beans with defects were eliminated. Chemical analysis and roasting were carried out on beans retained in the size 16 screen (larger than 16/64 of an inch).

1.4 Data collection

1.4.1 Environment data

Temperature was measured at different six periods (08.00, 10.00, 12.00, 14.00, 16.00 and 18.00). The average yearly annual rainfall was 284 mm, without a marked dry season.

1.4.2 Physiological characters

1) Percentage of Nitrogen (N), Phosphorus (P) and Potassium (K) in leaf

10 lateral branches were randomly selected per experimental unit. To estimate lateral growth, nodes supporting fruit or unopened flowers were counted on each branch. 10 pairs of the most recently matured leaves from each experimental unit in *Kunia* were collected and analyzed for nutrient concentrations according to Jones et al. (1991) for N and Kalra (1998) for all other nutrients.

2) Chlorophyll contents

Contents of chlorophyll a and b were determined with a spectrophotometer at the vegetative phase. Chlorophyll contents were determined by the method described by AOAC (1990).

3) Caffeic acid content

e data were counted on each branch using 4 pairs of matured leaves from each experimental unit. Contents of caffeic acid were determined with HPLC analysis was performed using Shimadzu LC-20AC pumps, SPD-M20A with diode array detector and chromatographic separations were performed on a LUNA C-18 column (4.6-250 mm i.d., 5 lm). The composition of solvents and the gradient elution conditions used were described previously by Schenker et al. (2002); Butsat et al. (2009).

1.4.3 Morphological character

Plant height (cm), stem diameter (cm), number of branch, node per branch, node length were collected at the maturation stage (231 day after flowering).

1.4.4 Yields and yield component data

Total ripening fruit per branch, total ripening fruit per plant and total coffee bean per plant were collected at the maturation stage (231 day after flowering).

1.4.5 Quality data

1) DPPH radical-scavenging activity

The hydrogen atom or electron-donation ability of the corresponding extracts and some pure compounds was measured from the bleaching of a purple-colored methanol solution of DPPH (Gulluce et al., 2007). The anti-oxidant activity of the extracts, on the basis of the scavenging activity of the stable 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical, was determined by the method described by Braca et al. (2001). Aqueous extract (0.1 ml) was added to 3 ml of a solution of 0.001 M DPPH in methanol. Absorbance at 517 nm was determined after 30 min, and the percent inhibition of activity was calculated as [(Ao - Ae)/Ao]/100 (Ao = absorbance without extract; Ae = absorbance with extract).

2) Determination of Total Phenolic Content (TPC)

The total phenolic content was measured using the Folin-Ciocalteu method. In this procedure, 1 ml of appropriately diluted samples and a standard solution of gallic acid were added to a 25 ml volumetric flask containing 9 ml of double-distilled water. Reagent blank using double-distilled water was prepared. One milliliter of Folin-Ciocalteu phenol reagent was added to the mixture and shaken. After 5 min, 10 ml of a 7% sodium carbonate solution was added with mixing. The solution was then immediately diluted to a volume of 25 ml with double-distilled water and mixed thoroughly. After incubation for 90 min at room temperature, the absorbance at 765 nm was measured. The total phenolic contents of the samples are expressed in milligrams of gallic acid equivalents (GAE) (Braca et al., 2001; Shimada et al., 1992).

1.4.6 Identification and quantification of phenolic compounds

1) Phenolics extraction

The phenolic compounds in the coffee beans were extracted using a modification of the procedure described by Bencoechea et al. (1997), as adapted from Uzelac et al. (2005). All extractions were performed in triplicates. Each

sample (5 g) was mixed with 50 ml of methanol/HCl (100:1, v/v) which contained 2% tert-butylhydroquinone, in inert atmosphere (N_2) during 12 h at 35 ^{0}C in the dark. The extract was then centrifuged at 4000 rpm/min, and the supernatant was evaporated to dryness under reduced pressure (35-40 ^{o}C). The residue was redissolved in 25 ml of water/ethanol (80:20, v/v) and extracted four times with 25 ml of ethyl acetate. The organic fractions were combined, dried for 30-40 min with anhydrous sodium sulphate, filtered through the Whatman-40 filter, and then evaporated to dryness under vacuum (35-40 ^{0}C). The residue was redissolved in 5 ml of methanol/water (50:50, v/v) and filtered through a 0.45 ml filter before injection (20 ml) into the HPLC aperture.

2) HPLC system for analysis of phenolic compounds

HPLC analysis was performed using Shimadzu LC-20AC pumps, SPD-M20A with diode array detector and chromatographic separations were performed on a LUNA C-18 column (4.6-250 mm i.d., 5 lm). The composition of solvents and the gradient elution conditions used were described previously by Bengoechea et al. (1997) and Butsat et al. (2009), with slight modifications. The mobile phase consisted of purified water with acetic acid (pH 2.74) (solvent A) and acetonitrile (solvent B) at a flow rate of 0.8 ml/min. Gradient elution was performed as follows: from 0 to 5 min, linear gradient from 5% to 9% solvent B; from 5 to 15 min, 9% solvent B; from 15 to 22 min, linear gradient from 9% to 11% solvent B; from 22 to 38 min, linear gradient from 11% to 18% solvent B; from 38 to 43 min, from 18% to 23% solvent B; from 43 to 44 min, from 23% to 90% solvent B; from 44 to 45 min, linear gradient from 90% to 80% solvent B; from 45 to 55 min, isocratic at 80% solvent B; from 55 to 60 min, linear gradient from 80% to 5% solvent B and a equilibration period of 5 min with 5% solvent B used between individual runs. Operating conditions were as follows: column temperature, 38 ^{0}C, injection volume, 20 ll, and UV-diode array detection at 280 nm (hydroxybenzoic acids), 320 nm (hydroxycinnamic acids) and 370 nm (flavonols) at a flow-rate of 0.8 ml/min. Spectra were recorded from 200 to 600 nm. Phenolic compounds in the samples were identified by comparing their relative retention times and UV spectra with those of authentic compounds and were detected using an external standard method.

1.4.7 Determination of sugar content by HPLC

1) Sugar extraction

For sucrose evaluation, samples (1 g) of coffee powders were mixed with 20 ml of deionized water at an extraction temperature of 80 ^{0}C distilled water and clarified with activated charcoal. Sugars were extracted using a modification of the procedure described by Trugo (2001). Coffee sample (200 g) was homogenized in a blender. 1 g of the homogenate sample was dissolved with 1 ml solvent containing 80% ethanol and 20% water at 80°C. The sample was then centrifuged at 13,000 rpm for 10 min at 20 ^{0}C. The supernatant was collected in a container. The extraction procedure was repeated four times for complete extraction of sugar from the sample. The supernatant was added with the solvent to give a sample of 5 ml, and the sample was further centrifuged and filtered thought 0.45 μm nylon syringe filter and the final sample was stored in a freezer at -20°C until the sugar analysis.

2) HPLC system for analysis of sugar

The HPLC consisted of a Shimadzu™ LC10AT pump, a Shimadzu™ RID 10A reflextive index detector, a Shimadzu™ SIL 10AD$_{VP}$ auto injector, a Water™ Temperate Control Module II and a Water™ Column Heater Module. The column is 300 x 6.5 mm, Sugar Pak™ with Guard Pak Holder and Guard Column insert. The mobile phase was deionized water previously filtered through 0.45 μm nylon membrane filter and degassed prior to use. Chromatography was carried out at 90 °C with a flow rate of 0.5 ml/min.

Standard solution of sucrose, glucose, and fructose were prepared and filtered as described above and injected into the HPLC instrument to determine the standard curve. A 20 μl portion of sample extract was injected to determine the amount of each sugar. The total sugar content was estimated by summing the amount of each sugar (Braca et al., 2001; Alcázar et al., 2005).

1.4.8 Color analysis

Color analysis was carried out on ground roasted coffee using a tristimulus colorimeter (Chromameter-2 CR-300, Minolta, Osaka, Japan). The instrument was standardized on a white tile before each sample measurement. The color parameters corresponding to the uniform color space CIELAB (1986) were

obtained directly from the apparatus. Within the approximately uniform space CIELAB, 2 chromaticity coordinates a^* and b^* as well as lightness L^* are defined. Coordinate a^* takes positive values for reddish colors and negative values for greenish ones whereas b^* takes positive values for yellowish colors and negative values for bluish ones. L^* parameter is an approximate measurement of lightness taking values within the range of 0 (black) and 100 (white).

2. **Experiment 2** was to determine phenolic compounds including total phenolic contents, phenolic acids and cup test of coffee beans grown under five shading conditions and evaluate their antioxidant activities. A split-plot experiment in a randomized complete block design with four replications was used. Five shading conditions treatments were assigned as main plots and three roasting levels as sub-plots.

2.1 Roasting process

The coffee beans of 400 g for each replication were sampled and then the beans were divided into four sub samples of 100 g for each sub sample. The roasting process was carried out in a roaster which could roast 100 g of coffee beans for each batch. The roasting conditions were set up at 230 °C for 12 min (light), at 240 °C for 14 min (medium), and 250 °C for 17 min (dark), and 100 g of un-roasted coffee for each replication was also available as control. The roasted coffee beans were ground into powder by coffee grinding (Princess silver, coffee grinder, 2194, USA.) before analysis.

2.2 Sample extractions

One gram of ground coffee sample was placed in a test tube; 40 ml methanol/water (50:50) plus HCl were added to obtain a final pH of 2.0. The tube was thoroughly shaken at room temperature for 1 h. The tube was centrifuged at 2500g for 10 min and the supernatant was recovered. After 40 ml of acetone/water (70:30) were added to the residue, shaking and centrifugation were repeated. Both extracts were mixed. Extracts or brewed coffee were produced in triplicate, used to estimate the total phenolic content and the antioxidant capacity and used for analyzing of antioxidant activity in vitro. All analyses were performed in triplicates.

3. Quality data

3.1 DPPH radical-scavenging activity

The hydrogen atom or electron-donation ability of the corresponding extracts and some pure compounds was measured from the bleaching of a purple-colored methanol solution of DPPH (Gulluce et al., 2007).

3.2 Determination of Total Phenolic Content (TPC)

The total phenolic content was measured using the Folin-Ciocalteu method (Braca et al., 2001; Shimada et al., 1992).

3.3 Identification and quantification of phenolic compounds

3.3.1 Phenolics extraction

The phenolic compounds in the coffee beans were extracted using a modification of the procedure described by Ky et al., (2001) and Bengoechea et al. (1997), as adapted from Stalmach et al. (2006).

3.3.2 HPLC system for analysis of phenolic compounds

HPLC analysis was performed using Shimadzu LC-20AC pumps, SPD-M20A with diode array detector and chromatographic separations were performed on a LUNA C-18 column (4.6-250 mm i.d., 5 lm). The composition of solvents and the gradient elution conditions used were described previously by Bengoechea et al. (1997), Ky et al. (2001) and Butsat et al. (2009).

3.4 Determination of sugar content by HPLC

3.4.1 Sugar extraction

For sucrose evaluation, samples (1 g) of coffee powders were mixed with 20 ml of deionized water at an extraction temperature of 80 ^{0}C distilled water and clarified with activated charcoal. Sugars were extracted using a modification of the procedure described by Trugo (2001).

3.4.2 HPLC system for analysis of sugar

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was carried out at 90 °C with a flow rate of 0.5 ml/min. Standard solution of sucrose, glucose, and fructose were prepared and filtered as described above and injected into the HPLC instrument to determine the standard curve. A 20 µl portion of sample extract was injected to determine the amount of each sugar. The total sugar content was estimated by summing the amount of each sugar (Alcázar et al., 2005).

3.5 Color analysis

Color analysis was carried out on ground roasted coffee using a tristimulus colorimeter (Chromameter-2 CR-300, Minolta, Osaka, Japan).

3.5.1 Testing

A group of 10 trained tasters took part in the tasting sessions. An initial group tasting session was used to typify the samples. A taste was identified in the coffees. This typical or distinctive character was sought after in the subsequent tasting sessions. Each sample was roasted and tasted twice. Cup quality was assessed using five criteria: aroma, body, acidity, bitterness and preference taste. Scoring was on an intensity scale of 0 to 5, where 0=nil, 1=very light, 2=light, 3=medium, 4=strong, and 5=very strong. An additional preference score, an hedonic criterion, was used ranging from 0 to 5, where 0=unacceptable, 1=bad, 2=regular, 3=good, 4=very good, and 5=excellent. For the statistical tests, the means of the values attributed by all the tasters were used.

3.5.2 Statistical analysis

In experiment 1, a completely randomized design with four replications was used. In experiment 2, analysis of variance was performed for each character followed a split-plot design (Gomez and Gomez, 1984). When the differences of main effects were significant ($p \leq 0.05$), Bonferroni tests were used to compare means. Pearson correlation test was conducted to determine the correlations amongst means.