

CHAPTER 3 – RESEARCH METHODS

3.1. Materials and Equipment

The materials used in this research were Robusta coffee silverskin, coffee cascara, and coffee pixel originated from Lampung, supplied by PT. Cahya Aroma Inti, Tangerang. Earl grey black tea was obtained from Bensons Tea purchased online. Chemicals used for analysis were Folin Ciocalteu reagent (Merck, Germany), Na_2CO_3 (Merck, Germany), DPPH (Sigma-Aldrich, USA), gallic acid (Sigma-Aldrich, USA), ascorbic acid (Merck, Germany), NaOH (Merck, Germany), methanol (Merck, Germany), and distilled water.

The equipment used in this research were analytical balance 0.0001 mg (Ohaus, Germany), digital kitchen scale 0.01 g (Joil, Indonesia), oven dryer (Mettler, ULM-700, Germany), UV-Vis spectrophotometer (AELAB, AE-S60 U2, China), pH meter (Thermo Fisher, Eutech 1710, USA), Brix refractometer (ATAGO, PAL-1, Japan), colorimeter (PCE, CSM-3, Germany), and Design Expert 13.0[®] software (Stat-Ease Inc., Minneapolis, USA).

3.2. Research Design

This research followed a mixed approach (quantitative and qualitative) where numerical data obtained from lab experiments and survey (sensory evaluation) was used to analyse its result. This research was conducted in two phases, namely characterization of CBP as kombucha ingredients, followed by optimization of ingredients and fermentation duration of CBP kombucha (Fig. 4).

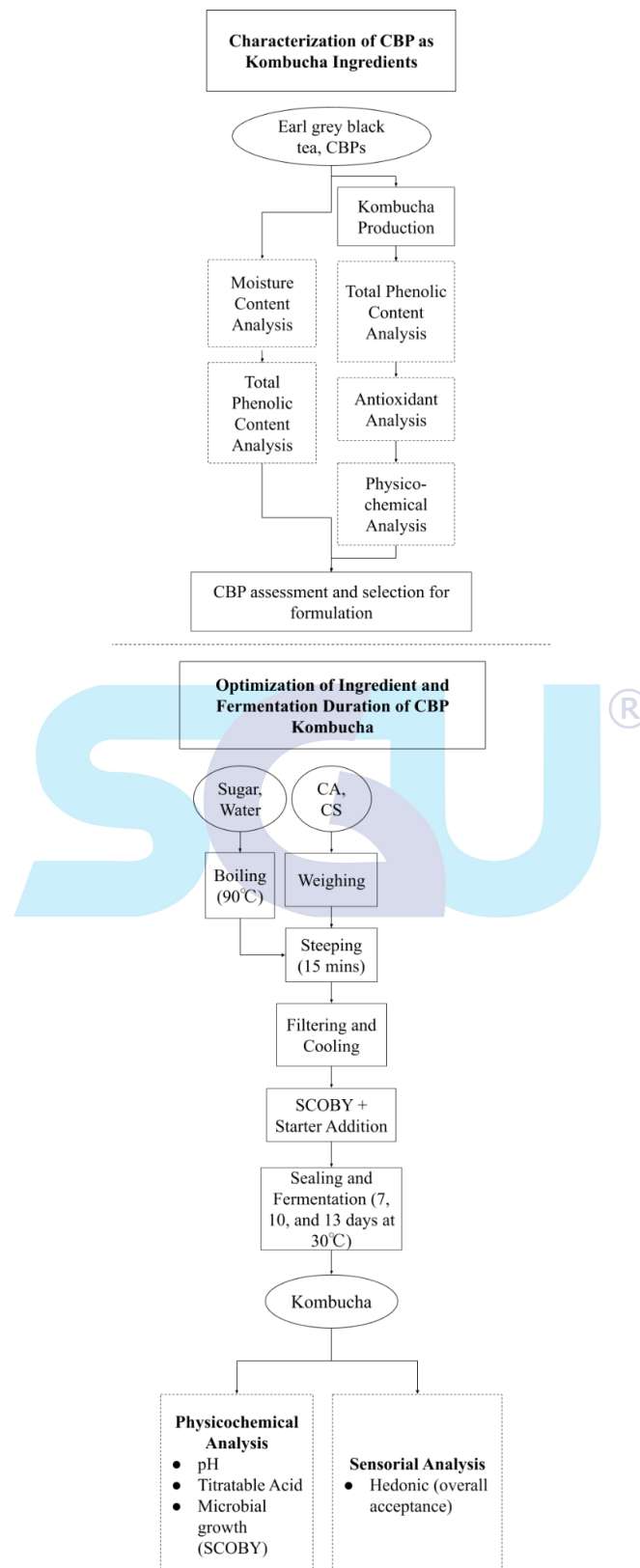


Fig. 4 Phases of Research Design of CBP Kombucha

3.2.1. Characterization of CBP as Kombucha Ingredients

As the raw materials used in this research possessed different characteristics, several initial analyses to determine the characteristics of each material were conducted. Raw materials, such as earl grey black tea, coffee silverskin, coffee cascara, and coffee pixel were subjected to analysis of moisture content and total phenolics content. Moisture content was measured in triplicates using the oven-drying method (AOAC, 2000), where 2 grams of raw material sample was dried in a convection oven at $105 \pm 5^\circ\text{C}$ for approximately 4 hours until the dry weight measured was constant. Total phenolic content (TPC) was performed following methods used by Muzaifa et al. (2023) with modifications. The raw materials were brewed at $90 \pm 5^\circ\text{C}$ for 15 minutes before filtered and cooled down (until approx. 30°C). Afterwards, 0.1 mL tea sample was added with 7.9 mL distilled water, 0.5 mL Folin-Ciocalteu (1:9) reagent, and 1.5 mL Na_2CO_3 (7.5%) before vortexed and incubated in a dark chamber for 2 hours. Absorbance of the samples were measured using a UV-Vis spectrophotometer at a wavelength of 765 nm. Results were expressed as milligram gallic acid equivalent per gram (mg GAE/g).

Following the two initial analyses of the raw materials, several kombuchas made using CBPs were also conducted. As shown in Fig. 4, production of the kombucha began from weighing the raw materials (earl grey black tea, coffee silverskin, coffee cascara, and coffee pixel) before steeping them with hot water containing dissolved sugar in it for around 15 minutes. Once the solution had cooled down to approximately 30°C , the tea liquids were transferred to glass containers and added with the SCOBY and liquid starter (10% v/v). A breathable cloth was used to seal the opening and the kombucha were left to ferment in room temperature of 30°C for 13 days. Once all formulations of kombucha had completed fermentation, samples from each of them were taken for analysis, including total phenolic content, antioxidant analysis, and physicochemical analysis. Total phenolic content measurements were identical to the procedure for the raw materials. Antioxidant analysis was performed using a modified DPPH assay method according to Thongbai et al. (2025), where 200 μL of diluted samples (1:100) was mixed with 1800 μL of methanolic solution of DPPH (0.1mM). A mixture of methanol and DPPH solution was used as control, whereas pure methanol was used as blank. The mixture was incubated in a dark chamber for 30 minutes and its absorbance was

measured at 517 nm. Results were expressed as milligram ascorbic acid equivalent per liter (mg AAE/L). Physicochemical analysis included pH, sugar concentration, microbial growth, and color measurements. pH measurement was conducted using a calibrated pH meter (calibrated in pH 4, pH 7, and pH 10 buffer solutions before usage). A Brix refractometer was used to calculate the total soluble solids (TSS) of the samples. SCOBY growth was measured by calculating the weight difference between SCOBY from before and after fermentation using a calibrated scale. Color measurements were conducted using a calibrated colorimeter (alternatively, using a color intensity chart). Once all analyses were complete, statistical analyses were performed (using one-way ANOVA for raw materials, Tukey HSD test for post-hoc analysis) for all data obtained to determine which parameters were significant to be considered for ingredient selection of kombucha optimization.

3.2.2. Optimization of Ingredient and Fermentation Duration of CBP Kombucha

In order to optimize CBP kombucha, several formulations of CBP kombucha were made based on boundaries in Table 2. This was conducted by designing several formulations with varying ratios between CBPs, sugar, and fermentation duration using Design Expert 13.0[®] software. The upper and lower limits of ingredients used in the CBP kombucha are shown in Table 4 below.

Table 4 Limits of CBP Kombucha Formulation Variables

Limit	CA (g)	CS (g)	Sugar (g)	Fermentation Duration (days)*
Bottom	0	0	180	7
Upper	10	10	200	13
Additional				10

Note: CA = coffee cascara, CS = coffee silverskin.

*Fermentation duration is not set as an independent variable, but as a categorical factor.

Utilizing the Optimal (Combined) Design in Design Expert 13.0[®] software, cascara, silverskin, and sugar were inputted as components, where the formulation was set to a total of 200 grams. Cascara, silverskin, and sugar were regarded as numerical factors. Additionally, three types of fermentation duration (7, 10, and 13 days) were added as categorical factors. The combination of the aforementioned components and factors resulted in a set of 16

randomized designs (Table 8). Lack of fit points were set to 3 and replicate points were set to 2. Furthermore, a control kombucha made from earl grey black tea was also made as control against CBP kombuchas. Once all formulations of kombucha had completed fermentation, samples from each of them were taken for physicochemical and sensorial analysis.

3.3. Experimental Method

All 16 formulations generated by Design Expert 13.0[®] software were made according to the procedure in Figure 4. Cascara, silverskin, and sugar were weighed according to their respective formulas using a digital kitchen scale. Approximately 1500 mL of water was heated until 90°C before the heat was turned off and sugar was added. Once the sugar dissolved, CBPs were added and stirred, then the mixture was left to steep for 15 minutes. The mixture was then sieved and cooled to room temperature (30°C) before 1350 mL of the filtered liquid was transferred to a glass container. 150 mL of starter liquid and one SCOBY culture were added before the container was covered using a cheesecloth. Three types of batches were made on three separate days to maximize time efficiency of fermentation completion (formulas that have the same fermentation duration were made at the same day). The 16 formulations were analyzed for their physicochemical (pH, acid value, and SCOBY growth) and sensorial properties (hedonic overall acceptance). Responses from all the formulations were analyzed using ANOVA statistical analysis. An optimal formula was generated by the software based on adjusted criteria and desirability scores.

3.4. Analytical Methods

Moisture content and total phenolic content (TPC) analysis were measured for CBP raw materials, whereas antioxidant (TPC and DPPH) and physicochemical (pH, TSS, SCOBY growth, and color) properties were measured for pre-formulation CBP kombucha. Two types of evaluations were conducted on the formulated CBP kombuchas: physicochemical analysis and sensorial analysis. The former included evaluating the kombucha in terms of its pH, titratable acid, and microbial (SCOBY) growth. The latter included conducting hedonic sensory evaluation to measure the consumer acceptance of the samples and color profiling using a color intensity chart.

3.4.1. Acidity Analysis

A calibrated pH meter was used to determine the pH reduction between samples from before

and after fermentation. Approximately 40 mL of sample was poured into a 50 mL beaker glass, the probe of the pH meter was positioned until its indicator was submerged in the liquid. The pH meter was left to calculate the pH until it showed a stable number. This method was also applied for formulated CBP kombuchas with 7, 10, and 13 days of fermentation.

Titrateable acid content of formulated CBP kombucha was measured using a titration method with 0.1M NaOH. 10 mL of samples were poured into erlenmeyer flasks and added 2-3 drops of phenolphthalein indicator. Titration was done until the sample's color turned from yellow to slightly red (indicates end point). The amount of NaOH used was regarded as equivalent to acetic acid in the samples. Results were expressed as percentage of acid value (%).

3.4.2. Antioxidant Analysis

Antioxidant potential was measured using TPC and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) methods. TPC analysis followed methods used by Muzaifa et al. (2023) with modifications. The CBPs were brewed at $90 \pm 5^{\circ}\text{C}$ for 15 minutes before filtered and cooled down (until approx. 30°C). Afterwards, 0.1 mL of sample was added with 7.9 mL distilled water, 0.5 mL Folin-Ciocalteu (1:9) reagent, and 1.5 mL Na_2CO_3 (7.5%) before vortexed and incubated in a dark chamber for 2 hours. Absorbance of the samples were measured using a UV-Vis spectrophotometer at a wavelength of 765 nm. Results were expressed as milligram gallic acid equivalent per gram (mg GAE/g).

Radical scavenging activity of CBP kombucha was determined using a modified DPPH assay method according to Thongbai et al. (2025), where 200 μL of diluted samples (1:100) was mixed with 1800 μL of methanolic solution of DPPH (0.1 mM). A mixture of methanol and DPPH solution was used as control, whereas pure methanol was used as blank. The mixture was incubated in a dark chamber for 30 minutes and its absorbance was measured at 517 nm. Results were expressed as milligram ascorbic acid equivalent per liter (mg AAE/L).

3.4.3. SCOBY Growth

SCOBY growth was measured by calculating the weight difference between SCOBY cultures from before and after fermentation of CBP kombucha using a calibrated scale. The scale used was a digital kitchen scale with 0.01 g precision.

3.4.4. Total Soluble Solids (TSS) and Color

A Brix refractometer was used to calculate the total soluble solids (TSS) of the CBP kombucha samples. The refractometer was calibrated using distilled water for 0% TSS reference. A few drops of samples were placed on the sensor of the refractometer for measurement. The sensor was cleaned for every change of samples. Measurements were done in triplicates.

Color measurements were conducted using a calibrated colorimeter. An additional glass container was used to hold the sample while the colorimeter was placed directly against the container. The container was cleaned for every change of samples. Measurements were done in triplicates.

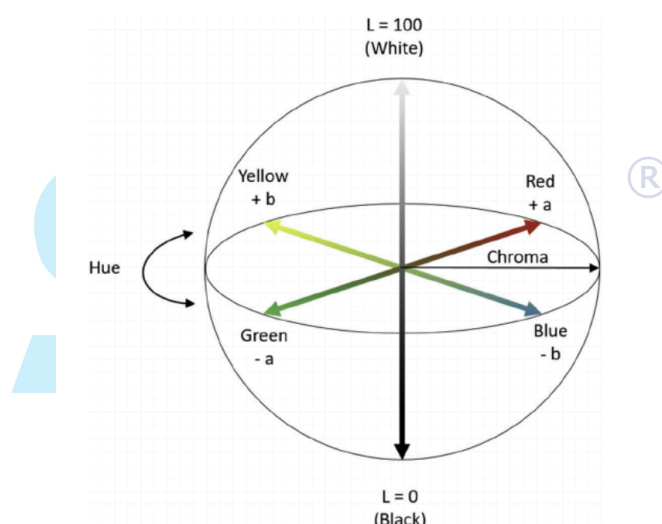


Fig. 5 CIELAB Color Space Diagram (Ly et al., 2020)

The colorimeter expresses results in CIE $L^*a^*b^*$ or CIE $L^*a^*b^*C^*h^\circ$ color space (Fig. 5). L^* value represents lightness (0 = black, 100 = white), whereas a^* and b^* represents chromacity coordinates (Ly et al., 2020). The a^* value represents red-green components of a color, where positive values ($+a^*$) indicate a shift towards red-ness and negative values ($-a^*$) indicate a shift towards green-ness. Similarly, the b^* value represents yellow-blue components of a color, where positive values ($+b^*$) indicate a shift towards yellow-ness and negative values indicate a shift towards blue-ness ($-b^*$). Chroma (C^*) is represented by saturation of color components (distance from central axis) and hue (h°) is represented by the angle on the chromacity axes.

However, upon further considerations of the obtained results, the colors of the samples were

difficult to distinguish using the colorimeter method, therefore the usage of sequential color charts were used to profile the color intensity of each sample.

3.4.5. Sensory Acceptance

Sensory evaluation was conducted on the formulated CBP kombucha samples by using a 9-point hedonic scale (1 = dislike extremely, 9 = like extremely). A total of 50 untrained panelists participated to evaluate the overall acceptance of the samples. A pre-screening was conducted to ensure the panelists did not have any health issues that may have prevented them from testing the samples (e.g. GERD or gastritis) considering kombucha are commonly acidic (see Appendix 1). Panelists also signed on a consent form (google form) prior to the sensory evaluation, ensuring their willingness to have their data collected and used in the study (see Appendix 2).

The sensory evaluation took place in a controlled environment (sensory lab at Swiss German University) with neutral lighting and partition to prevent external disturbances (see Appendix 3). Samples were given to panelists through a small opening from the other side of the wall to minimize interaction that may affect panelists' judgement.

The sensory evaluation was conducted on 2 days, where most panelists tested all 16 samples altogether. The samples were divided into 3 batches of 5-6 samples to avoid sensory fatigue (Albertini, 2016). All kombucha samples were placed in plastic sensory cups and blinded with 3-digit codes. Samples were given in a random order of serving. Demineralized water, coffee powder, and crackers were provided to cleanse the panelists' palate in between sampling. Panelists evaluated the overall liking of the samples through a provided google form (see Appendix 4a, 4b, 4c).

Data from the first research phase (characterization of CBP as kombucha ingredients) was analyzed using one-way ANOVA and Tukey HSD (Honestly Significant Difference) post-hoc analysis. The ANOVA analysis (at significance level 5%) was used to investigate whether each raw materials and pre-formulation CBP kombucha were significantly different from each other in various parameters. Tukey HSD was performed to determine which samples differed significantly. Both ANOVA and Tukey HSD analysis were done by using a custom sensory analytical tool developed in SGU, courtesy of Dr. Ir. Abdullah Muzi Marpaung, MP. The software was made in Microsoft Excel to conduct one-way ANOVA with various types

of post-hoc analysis (e.g. LSD, Tukey, Scheffe, Bonferroni correction). In order to use the software, triplicate data of each sample was inputted into the columns where custom-made formulas automatically calculate the complete ANOVA and post-hoc analysis. On the other hand, data from the second research phase (optimization of ingredients and fermentation duration of CBP kombucha) was analyzed using Design Expert 13.0® which included ANOVA analysis to investigate the significance of the results. Additionally, ANOVA and Tukey HSD tests were conducted on hedonic acceptance results of formulated CBP kombucha using IBM SPSS Statistics software, version 32.

