

CHAPTER 3 – RESEARCH METHODS

3.1 Time and Venue

This research was conducted at the Life Sciences and Technology Laboratory of the Swiss German University, Alam Sutera, South Tangerang, from February 2025 to June 2025. Kelakai samples (*Stenochlaena palustris*), distinguished by their medium-sized green and red leaves (Figure 3.1(b)), were obtained from six different locations in Palangkaraya, Central Kalimantan (Figure 3.1(a)). All experiments and analyses are conducted on-site at Swiss German University's laboratory. Additionally, GC-MS analysis was performed at Osaka University, Osaka, Japan, and PT. Ditek Jaya (DIJ), Jakarta, Indonesia. For the proximate analysis conducted at PT. Saraswanti Indo Genetech (SIG), Bogor, Indonesia.

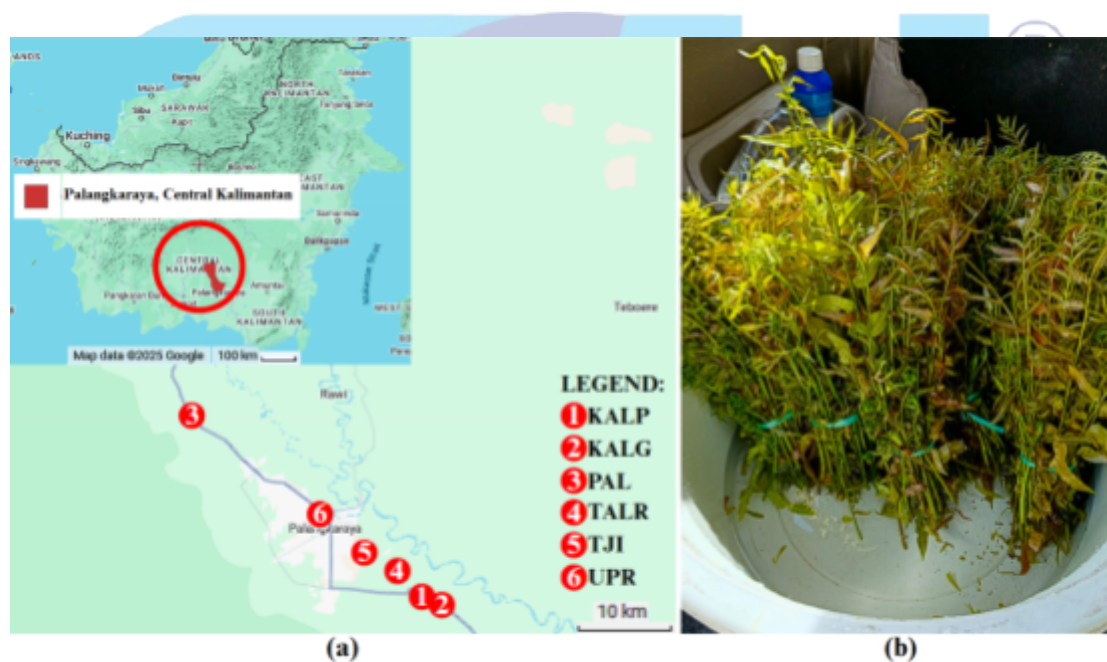


Figure 3.1 (a) Kalimantan Map with kelakai harvesting area; (b) Kelakai (*Stenochlaena palustris*)

3.2 Materials and Equipment

3.2.1 Materials

Kelakai samples were harvested from Palangkaraya, Central Kalimantan, representing a range of soil types (Figure 3.1(a)): Kalampangan 1, ex-rice paddy field peat soil

(KALP), Kalampangan 2, peat soil (KALG), PAL20 (20 kilometres away from city), sandy soil (PAL), Talawang Raya, peat soil (TALR), Tjilik Riwut, sand (TJI), and Palangkaraya University, sand (UPR). Other primary ingredients for the “kelakai nori sprinkles” included roasted sesame seeds (Javasuperfood, Jakarta, Indonesia), sugar (PT. Sweet Indolampung, Indonesia), and a commercial nori sprinkle product (GimBori Original, Mamasuka, Indonesia), such as fried shallots, dried nori, vegetable oil, sesame seeds, sugar, savoury salt, *monosodium glutamate* (MSG), and antioxidant (*α -tocopherol*). All the chemicals and reagents were analytical or GC-MS grade. For analyses conducted in Indonesia, materials included a Spectroquant® Iron Test kit (1.14761.0001), methanol, chloroform (Supelco), nitric acid (HNO₃, 65%, Supelco), and filter paper (15-20 μ m, Beimu, China). Ultrapure water was sourced from PT. Ikapharmindo Putramas, Indonesia. Liquid nitrogen, used for preserving raw materials, was obtained from PT. Putra Sinar Gas, Indonesia. The following reagents were sourced: ribitol (internal standard) and pyridine (Fujifilm Wako Pure Chemical Industries, Ltd., Osaka); methanol (Kanto Chemical Co., Inc., Tokyo); chloroform (Kishida Chemical Co., Ltd., Osaka); methoxyamine hydrochloride (Sigma-Aldrich, Tokyo); N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and an alkane standard mixture (C₉-C₄₀) (GL Sciences, Tokyo). For GC-MS metabolomics analysis conducted at Osaka University, Japan, ultrapure water was generated using a Genpure system (Thermo Scientific). Helium was used as the carrier gas for all GC-MS analyses, locally sourced for each instrument (DIJ and Osaka University).

3.2.2 Equipment

Kelakai production and formulation equipment includes a chest freezer to store raw kelakai, liquid nitrogen for quenching, a Memmert 490L oven with trays, as well as a food dehydrator (LocknLock FD990-A/B 8L, Indonesia). Mixer grinder (MX-AC400 Panasonic) for size reduction. Moisture content analyzer (i-Thermo G Moisture Analyzer, Italy), a UV-Vis spectrophotometer (T60 PG Instrument, United Kingdom) for iron content analysis, a centrifuge (PrO-Research Centurion Scientific Ltd, UK), Memmert water bath and shaker, shaker incubator (Eppendorf Ltd., Hamburg, Germany), freeze dryer (Operon Co. Ltd, South Korea) for lyophilization and a GC-MS QP2010 Ultra (Shimadzu, Japan) equipped with an InertCap 5 MS/NP

column (GL Sciences) and a GCMS-TQ8040 NX (Shimadzu, Japan) equipped with a SH-I-5SiI MS column (Shimadzu, Japan) for metabolomic profiling and AOC-6000 Plus Multifunctional Autosampler (Shimadzu, Japan).

3.3 Method and Design of Experiments

3.3.1 Phase 1: Kelakai Selection as Raw Material

In Figure 3.3.1, phase 1, kelakai is processed into a sprinkle through several structured stages. Kelakai is harvested from six different sites spread across Kalimantan and then brought to the SGU laboratory for further processing. In the laboratory, the kelakai undergoes a series of preparation steps, which include washing, quenching, drying, and grinding the fern to achieve the desired texture. Each treatment applied to the kelakai will utilize the exact amounts of ingredients to maintain consistency across different treatments. This standardization is crucial for effectively comparing the nutritional and sensory outcomes of each treatment.

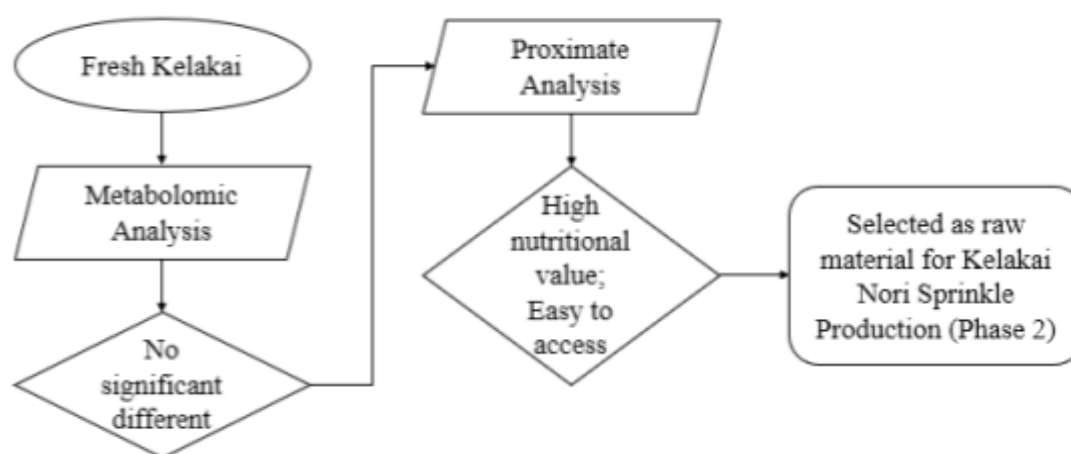


Figure 3.3.1 DoE Phase 1 Kelakai Selection as Raw Material Selection

The selection of kelakai harvesting sites was based on a previous study (Rahmawati, D., *et al.*, 2015) and practical considerations, such as ease of harvesting, which also played a role in selecting optimal locations. Locations yielding better quality and higher quantities are prioritized for harvesting. Kelakai (*Stenochlaena palustris*) was grown in various media types in Kalimantan, affecting its growth and quality. After

arriving at the laboratory, Kelakai is immediately washed and quenched to maintain its freshness, wrapped in aluminum foil.

3.3.1.1 Kelakai Pre-treatment

The quenching process was conducted to maintain the quality and freshness of the raw materials. Liquid nitrogen was utilized to preserve kelakai raw materials after harvesting from various areas in Kalimantan.

3.3.1.2 Kelakai Analysis for Raw Material Selection

Then, frozen kelakai underwent metabolomic analysis at Osaka University to assess the metabolite profile. If no significant differences were noted among the samples, proximate analysis was performed at the SIG Laboratory. A representative sample was selected from various soil types (peat, sandy soil, and sand). Three samples were then analyzed to determine their nutritional composition. If the chosen kelakai source exhibits high nutritional value and is readily accessible, it moves to phase 2: dried kelakai production.

3.3.2 Phase 2: Dried Kelakai Production

Fresh kelakai samples from Phase 1 were thoroughly washed with running water to remove soil, stones, and impurities. After washing, samples followed their designated pre-treatment protocol. Kelakai underwent quenching immediately after washing for quenched samples to reduce enzymatic activity and retain quality. The samples were tightly wrapped in aluminum foil and quenched with liquid nitrogen until frozen. Afterward, the samples were stored in the freezer for later processing. Non-quenched samples were directly frozen after washing for preservation without quenching.

Continuing to Phase 2 (Figure 3.3.2), all frozen Kelakai samples were chopped into 1–2 cm pieces after freezing. Preparing dried Kelakai involved pre-treatments (soaking, grinding) and oven-drying at 70°C until the moisture content was $\leq 8\%$. Based on the experimental design, samples underwent various pre-treatments. The KQW sample, quenched and derived from frozen stock, was soaked in tap water for 12–24 hours, then blended with a 1:1 ratio of Kelakai to drinking water to make a puree. This

mixture was spread onto a baking sheet and oven-dried, following a modified method from Tianasari, E., *et al.* (2018).

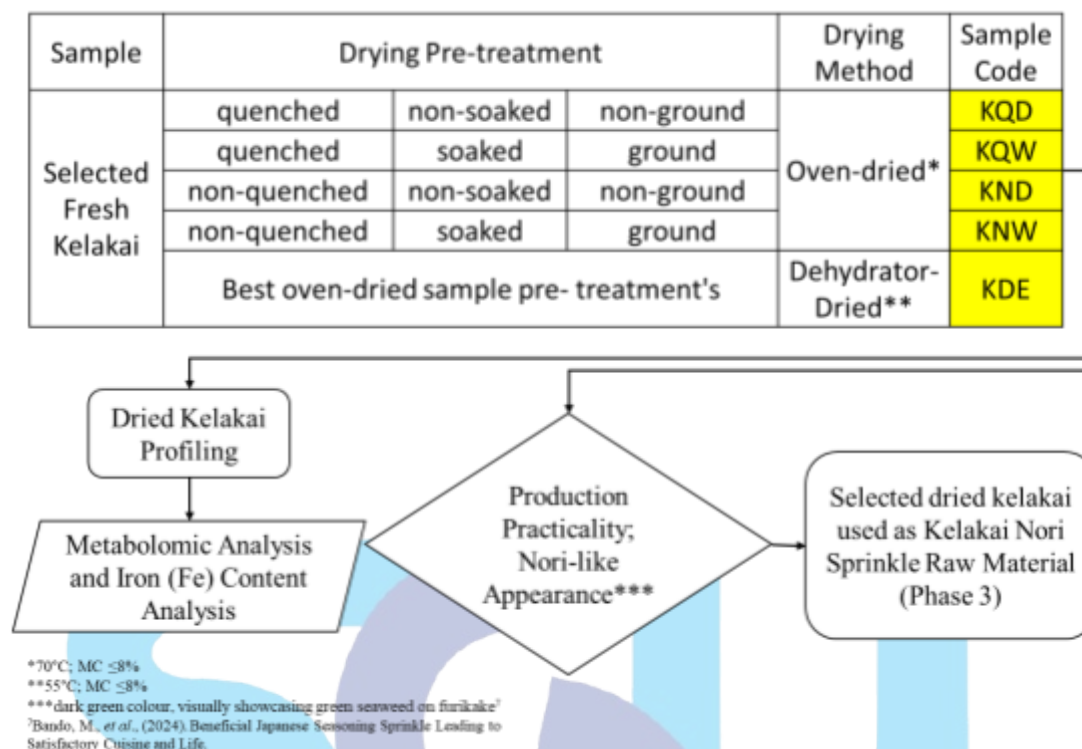


Figure 3.3.2 DoE Phase 2 Dried Kelakai Production

The KQD sample was directly dried in an oven without soaking or grinding, adapted from Orilda, R., *et al.* (2021). The KNW sample from the non-quenched batch underwent soaking, blending with water (1:1), and molding, while the KND sample was directly oven-dried. A Dehydrator (LocknLock 8L) was used to dry vegetables at 55°C (Shodikin, M., *et al.*, 2024). Following oven drying, all four oven-dried samples (KQW, KQD, KNW, KND) were initially evaluated for sensory quality. Based on the evaluation, the drying protocol for the best oven-dried sample was adapted for dehydrator-drying, yielding the KDE sample.

Five dried kelakai samples underwent a selection process based on production practicality, loading density of the drying equipment, nori-like appearance, and nutritional profiling, which included metabolomic analysis and iron (Fe) content analysis. The dried samples were evaluated for their dark green colour, visually

showcasing green seaweed in furikake (Bando, M., *et al.*, 2024). Only samples that passed the sensory evaluation moved to phase 3, Kelakai Nori Sprinkles production.

Table 3.1 Dried Kelakai Sample Code

Sample Code	Drying Pre-treatment			Drying Method
KDE	quenched	non-soaked	non-ground	dehydrator-dried
KQD	quenched	non-soaked	non-ground	oven-dried
KQW	quenched	soaked	ground	oven-dried
KNW	non-quenched	soaked	ground	oven-dried
KND	non-quenched	non-soaked	non-ground	oven-dried

3.3.3 Phase 3: Kelakai Nori Sprinkle Formulation and Sensory Evaluation

In this phase, the final Kelakai Nori Sprinkle was formulated for consumer acceptance testing (Figure 3.3.3), using the selected dried kelakai from the previous sensory evaluation. The formulation was designed to achieve high consumer acceptance by striking a balance between nutritional value and sensory appeal. The sprinkle was prepared by combining the dried kelakai with a mixture of roasted sesame seeds, fried shallots, nori, vegetable oil, sugar, savoury salt, *monosodium glutamate* (MSG), and an antioxidant (*alpha-tocopherol*). A fixed 1:1 mass ratio of kelakai to nori was maintained in the formulation, while all other seasoning ingredients were kept in constant proportions. A negative control of the sprinkle mixture without kelakai was also prepared for comparison.

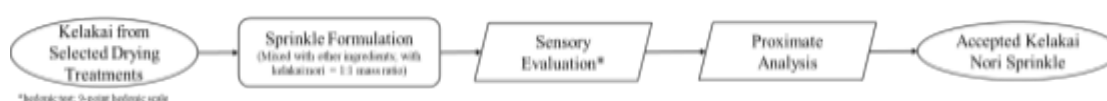


Figure 3.3.3 DoE Phase 3 Kelakai Nori Sprinkle Production and Acceptance Study

3.4 Data Analysis

Data analysis was performed using several software packages. Raw data were acquired and processed for metabolomics using GC-MS Solution, followed by alignment and annotation with MS-DIAL and the GL-Science DB Library. Multivariate analysis (Principal Component Analysis) was performed with SIMCA. Statistical analysis for proximate analysis, Sensory evaluation data scores, and Iron

(Fe) content analysis, including one-way ANOVA, Friedman's test, and Tukey's HSD, was performed using Microsoft Excel Analysis-ToolPak add-ins and SGU internally developed programs (courtesy of Abdullah Muzi Marpaung).

3.4.1 Metabolite Profiling on Kelakai

Metabolite profiling of kelakai collected from different harvesting sites was conducted. The GC-MS conditions were adapted and modified from Putri, S. P., *et al.* (2022), Ikram, M., *et al.* (2020), and Rahmawati, D., *et al.* (2021). Metabolomic analysis was done twice on fresh kelakai (M1) and dried kelakai (M2).

3.4.1.1 Metabolomics 1 for Kelakai Selection as Raw Material (M1)

Kelakai samples were collected from six different areas, allowing a comparative study to select the most favorable source based on metabolite profiles. The samples were: 1. Kalampangan 1 (KALP), 2. Kalampangan 2 (KALG), 3. Palangkaraya (PAL), 4. Talawang Raya (TALR), 5. Tjilik Riwt (TJI), and 6. Palangkaraya University (UPR). Metabolomics-based GC-MS was used to identify key metabolites and compare the chemical composition among six distinct sampling locations to determine the most advantageous harvesting site.

3.4.1.1.1 Sample Preparation (M1 and M2)

Kelakai samples were collected from six different locations based on preliminary studies conducted at the Swiss German University (SGU). Quenched kelakai samples were lyophilized overnight using a freeze dryer. The freeze-dried samples were immediately packed into ice-chilled insulated bags and transported to Japan for further analysis. Upon arrival, the samples were ground into a fine powder using a multi-bead shocker for 10 seconds, twice, and stored at -30°C until further processing at Osaka University, Osaka, Japan (M1) and PT. Ditek Jaya (DIJ), Jakarta, Indonesia (M2).

3.4.1.1.2 Sample Extraction and Derivatization (M1)

Ten mg of powdered kelakai sample from each of the six sites was weighed into a 2 mL microtube. Each tube added one mL of a mixed solvent (methanol: ultrapure

water: chloroform in a 5:2:2 v/v/v ratio) containing 200 µg/mL ribitol (used as an internal standard). Each mixture sample, blank, and internal standard were thoroughly vortexed and incubated (1200 rpm, 37°C for 30 min), then centrifuged for 3 minutes at 4°C and 10,000 rpm. Subsequently, 400 µL of supernatant was transferred to a 1.5 mL microtube, and 400 µL of ultrapure water was added, followed by vortexing. Centrifuged samples for 3 minutes at 4°C, 10,000 rpm. Separated supernatants into two parts. Collected 200 µL from each sample for making QC samples. Transferred 200 µL of supernatant into a new 1.5 mL microtube, closed all samples with holed caps, and centrifuged at room temperature for two hours. Samples were stored in the freezer. After drying, 100 µL of methoxyamine hydrochloride in pyridine (20 mg/mL) was added to the sample. The mixture was incubated in a shaker incubator at 30°C for 90 minutes to allow oximization. Following this, 50 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added for silylation, and the sample was incubated at 37°C for 30 minutes. It was then centrifuged at 10,000 rpm and 25°C for 3 minutes. Finally, the derivatized solution was transferred to a GC-MS vial for analysis. Each region's biological replicate (n = 3) was processed in parallel.

3.4.1.1.3 GC-MS Conditions (M1)

GC-MS analysis was performed using a GC-MS-QP2010 Ultra system (Shimadzu, Japan) equipped with an InertCap 5MS/NP capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; GL Sciences, Japan). As a carrier, helium gas flowed at a constant rate of 1.12 mL/min, with a linear velocity of 39 cm/s. The sample was injected in split mode at a ratio of 25:1 (v/v) with an injector temperature of 230°C. The oven temperature program started at 80°C (held for 2 minutes), increased at a rate of 15°C/min to 330°C, and was maintained at this final temperature for 6 minutes. The ion source and transfer line temperatures were set at 200°C and 250°C, respectively. Electron ionization (EI) was performed at 70 eV with a scan range of m/z 85–500 and a scan rate of 6.67 scans per second. An alkane standard mixture (C₉–C₄₀) was injected before sample analysis to calculate retention indices (RIs) and facilitate metabolite identification.

3.4.1.2 Metabolomics 2 of Dried Kelakai (M2)

After a fresh kelakai was selected from a specific area based on its metabolite and nutritional profile, dried kelakai samples were treated with different methods, allowing for a comparative study to compare metabolites from the raw material with those from the dried kelakai (See Table 3.1).

3.4.1.2.1 Sample Extraction and Derivatization (M2)

Ten mg of powdered kelakai from each coded sample was weighed into 2 mL microtubes. A mixture of 1000 μ L methanol, ultrapure water, and chloroform (5:2:2, v/v/v) was added to each sample and vortexed. Samples were incubated in a shaking water bath at 37°C for 30 minutes at maximum speed. After incubation, the samples were centrifuged at 10,000 rpm for 3 minutes at 4°C. A 400 μ L portion of the supernatant was transferred into a 1.5 mL microtube. Then, 400 μ L of ultrapure water was added, followed by vortexing. Samples were again centrifuged at 10,000 rpm for 3 minutes at 4°C. The supernatants were divided into 200 μ L for quality control (QC) pooling and 200 μ L for further derivatization. The remaining 200 μ L of supernatant was transferred to new 1.5 mL microtubes. Closed all microtubes with holed caps and centrifuged for 5.5 hours at room temperature. Then, the sample was lyophilized and stored at -30°C until it was derivatized. For derivatization, 100 μ L of methoxyamine hydrochloride in pyridine (20 mg/mL) was added to each dried extract. Samples were incubated in a water bath at 30°C for 90 minutes for oximization. Subsequently, 50 μ L of MSTFA was added for silylation, and samples were incubated at 37°C for 30 minutes. A final centrifugation was performed at 10,000 rpm for 3 minutes at 28°C. The derivatized extracts were transferred to GC-MS vials for metabolomic analysis. Each biological replicate (n = 3) per sample code was processed in parallel.

3.4.1.2.2 GC-MS Conditions (M2)

GC-MS analysis (M2) was conducted using a GC-MS-TQ8040 NX instrument (Shimadzu, Japan) with a SH-I-5Sil MS capillary column (30 m $\times$ 0.25 mm i.d., 0.25 μ m film thickness; Shimadzu, Japan). All other instrumental parameters were specified in subchapter 3.4.1.1.3 GC-MS Conditions (M1).

3.4.1.3 Data Processing and Analysis

The GC-MS solution software converted the raw GC-MS data into the AIA format (Shimadzu). Peak detection, deconvolution, alignment, and annotation were performed using MS-DIAL version 4.60 with the GC/MS-5MP spectral library (RIKEN, Japan). Peak intensities of detected metabolites were normalized against the internal standard ribitol. Metabolites were identified according to Level 1 of the Metabolomics Standards Initiative (MSI), where two or more authentic standards were run under the same analytical conditions as the samples. Additional validation was performed using the GL Sciences InertCap 5MS-NP Kovats RI database, which is available online. Only metabolites with a relative standard deviation (RSD) <30% in the quality control samples were retained for further statistical analysis. Principal Component Analysis (PCA) was performed using SIMCA version 18.0 (Umetrics, Umeå, Sweden) to visualize differences in metabolite profiles among kelakai samples from different collection sites.

3.4.2 Proximate Analysis Approaches

Proximate analysis is a fundamental method used to evaluate the nutritional composition of kelakai (*Stenochlaena palustris*). This analysis typically includes the determination of several key components. Proximate tests are conducted to determine the moisture, ash, protein, fat, and dietary fiber content in harvested kelakai. These parameters are crucial for understanding the nutritional value of kelakai and its potential applications in food products. In detail, the proximate test (AOAC 2005) was conducted in three stages: testing water and ash content using the oven method, fat content using the Soxhlet method, and protein content using the Kjeldahl method. Moisture Content: Samples were weighed (1-2 g) and dried in an oven at 102–105°C for 6 hours. The final weight after cooling in a desiccator was used to calculate moisture content based on weight loss. Ash Content: 1.5 - 2 g of dried sample was placed in an ashing crucible, pre-dried at 105°C, and incinerated in a muffle furnace at 600°C for 3 hours.

The residue was cooled and weighed to determine the total mineral (ash) content. Fat Content: The Soxhlet method was used. About 2 g of dried sample was wrapped in

filter paper and extracted with 150 mL of n-hexane for 6–8 hours. The solvent was evaporated, and the remaining fat was dried and weighed. Protein Content: Protein was measured using the Kjeldahl method. The sample (0.5 g) was digested with sulfuric acid and a selenium catalyst, and then distilled with NaOH. The ammonia was captured in boric acid and titrated with 0.09 N HCl. Nitrogen content was calculated and converted to protein using a factor of 6.25. Total Dietary Fiber: Fiber was analyzed using enzymatic-gravimetric analysis. Samples underwent enzymatic digestion using α -amylase, protease, and amyloglucosidase. The undigested residue was precipitated with ethanol, filtered, dried, and weighed to determine fiber content (AOAC 985.29).

3.4.3 Sensory Evaluation

A sensory evaluation assessed consumer preferences for the Kelakai Nori Sprinkle formulations, focusing on attributes of overall liking, appearance, aroma, and taste. The assessment involved a panel of 43 untrained participants in South Tangerang, Indonesia. Each attribute was rated on a 9-point hedonic scale, ranging from 1 (“dislike extremely”) to 9 (“like extremely”). During the assessment, a 5 g portion of each sprinkle sample was served with 20 g of plain white rice. A token of appreciation was provided to each participant for their contribution.

The collected sensory data were first analyzed using Friedman’s test for all evaluated parameters to determine if any sample was ranked differently. Since the results indicated a statistically significant difference, a further analysis using a one-way Analysis of Variance (ANOVA) was conducted. Subsequently, Tukey’s Honestly Significant Difference (HSD) test was performed as a post-hoc analysis to identify the differences in mean scores between each evaluated sample.

3.4.4 Iron (Fe) Content Determination

The determination of iron content in Kelakai (*Stenochlaena palustris*) was performed in three main stages: (1) peak wavelength identification, (2) calibration curve preparation, and (3) sample digestion and analysis using a spectrophotometric method.

3.4.4.1 Peak Wavelength Determination

The peak absorbance wavelength for iron (Fe) was determined using a UV-Vis spectrophotometer by scanning 5 mL of standard iron solutions reacted with three drops of Reagent Fe-1 (containing ammonium thioglycolate and thioglycolic acid) (Spectroquant), then scanning to identify the peak wavelength. This wavelength was then used for all subsequent measurements of iron content in the samples.

3.4.4.2 Preparation of Iron Standard Curve

A standard calibration curve was prepared using $\text{Fe}(\text{NO}_3)_3$ at 1000 ppm diluted in 0.5 M HNO_3 (65%) to create a range of 5-100 ppm. Iron standard solutions were filtered or centrifuged to obtain a clear solution. 5.0 mL of each dilution was pipetted into the rinsed test tubes. Three drops of Reagent Fe-1 were added. The solution was vortexed and allowed to react for 3 minutes. The reacted solution was transferred into pre-rinsed cuvettes, and absorbance was measured at the previously determined peak wavelength. A standard curve was created by plotting the absorbance values against known iron concentrations, which was then used to calculate the Fe content in the kelakai samples.

3.4.4.3 Extraction of Kelakai Sample for Iron Content Analysis

Iron in the kelakai samples was analyzed using a wet digestion method adapted from Emawati, E., *et al.* (2017). A 0.1 g dried kelakai sample was weighed and transferred to a digestion flask. 20 mL of concentrated nitric acid (HNO_3) was added to the sample. The mixture was heated in a water bath at 80°C for 30 minutes. The digested solution was filtered using filter paper into a 250 mL volumetric flask, then diluted to volume with distilled water. From the diluted solution, 5.0 mL was pipetted into a test tube. Three drops of Reagent Fe-1 were added, vortexed, and allowed to react for 3 minutes. The reacted solution was transferred to a cuvette, and its absorbance was measured using a UV-Vis spectrophotometer. The iron concentration of the kelakai sample was calculated using the established standard curve.