Influence of Preparation and Isolation Methods on the Measurement of Calcium Levels in Dried Freshwater Shrimp (Macrobrachium sp.)

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Abstract. In Indonesia, shrimp are an important natural resource. They are easily obtained and are assumed to contain high levels of calcium. This research aims to determine the calcium levels in Macrobrachium sp. as well as the effects of preparation and isolation methods on such measurements. Ebi shrimp were divided into two preparation groups: oven-dried and nonheated. Oven drying was performed at 80°C for 20 min. The samples in each preparation group were divided according to three isolation methods: dilution, acid digestion, and dry ashing. In the dilution method, a sample was shaken in demineralized water. The acid digestion method used microwave-assisted digestion with nitric acid and hydrogen peroxide. In the dry ashing method, a sample was heated from 26°C to 550°C to produce ash before nitric acid and hydrochloric acid were added. Measurements were performed using atomic absorption spectrometry and an ion-selective electrode. The results showed that the highest calcium levels were obtained by acid digestion isolation: 7749 ppm (oven-dried) and 8853 ppm (nonheated). There were differences in the calcium level between the preparation methods and among the isolation methods. The preparation methods were found to have a strong correlation with calcium level measurement ($r^2 = 0.878$, $p < 0.05$).

1. Introduction

Calcium is the most abundant mineral in the human body, reaching up to 40% of the total mineral mass or 1.5–2% of the body mass [1,2]. Ninety-nine percent of the calcium in the body is found in the form of hydroxyapatite calcium ($Ca_{10}(PO_4)_6(OH)_2$) in the bones and teeth [3]. The remaining 1% is found in soft tissues and bodily fluids [4]. The calcium needs of individuals differ depending on age, and the daily optimum amount needed by an adult human is 1000 mg, with a tolerance limit of 2500 mg [5]. Calcium deficiency may cause a decrease in bone density (bone loss), which can lead to osteoporosis [2].

Calcium is essential for bone and dental health, and it is necessary for a number of bodily processes such as blood clotting, muscle contraction, blood pressure regulation, nerve transmission, fluid balance in the cell, and hormone secretion [2]. In the oral cavity, calcium ions specifically contribute to increasing the remineralization rate, decreasing the risk of caries, and preventing alveolar bone resorption [6]. The importance of calcium to several bodily functions highlights the need for adequate calcium consumption through the intake of foods such as milk, vegetables, soy, eggs, fish, and shrimp [4].
Shrimp are a source of nutrition and are easily accessible. In Indonesia, they are one of the main water-based natural resources. Shrimp are usually consumed fresh or dried, known as ebi. Ebi usually consist of relatively smaller shrimp [7]. Shrimp are believed to contain high levels of calcium and can be a source of calcium for the human body. A study by Maharani and Julshamn in 1977 found that ebi without shells contain 5080 ppm calcium [8]. Indonesian data on food composition indicate that headless and tailless ebi contain 7600 ppm calcium [9]. However, there are no data on calcium levels in whole ebi. This is an important area of research because calcium in shrimp is found not only in the meat but also in the shells and head.

To measure the calcium levels in ebi, a number of standard laboratory methods for preparation and isolation are used to yield calcium in its free form. The preparation methods require the separation of organic materials from the shrimp with or without heat, followed by isolation methods such as dilution, acid digestion, and dry ashing. Next, the calcium levels are measured using atomic absorption spectrometry (AAS) and ion-selective electrode (ISE) methods. The AAS method is used to examine the concentration of a specific element by measuring the light absorption of a free atom at its lowest energy level. The ISE method is used to quantify the concentration of a specific ion on the basis of the relationship between electrode potential and ionic activity [10,11]. The preparation and isolation methods can serve as the basis for observing the effects of the methods on calcium measurements. Freshwater shrimp cultivation is very common in Indonesia; therefore, we opted to study Macrobrachium sp. (freshwater ebi) in this work. The results of this study can be used to inform the public as to the potential of freshwater ebi as a source of calcium. Furthermore, the methods used for preparation and isolation may be of interest to other researchers attempting to obtain maximum calcium yields from other types of samples.

2. Methods
Freshwater ebi (Macrobrachium sp.) from North Sumatra were used in this study. Verification of the shrimp genus was performed by the Department of Marine Science and Technology, Faculty of Fisheries and Marine Science, Institute Pertanian Bogor. This research used a descriptive laboratory research design. The independent variables were the preparation and isolation methods, and the dependent variable was the calcium content (ppm) measured by ISE and AAS. The data obtained in this research were numeric in nature, and comparisons and correlations between the groups were assessed using a parametric test at a 5% significance level (p ≤ 0.05). The equipment used in this research consisted of a blender, an oven, a measuring balance, aluminum foil, a timer, beakers, volumetric flasks, 1 and 10 mL volumetric pipettes, Pasteur pipettes, glass spatulas, stainless steel spatulas, a porcelain basin, a stopwatch, an electric heater, funnels, filter papers, a burette and burette clamp, a watch glass, and a furnace. The materials used were freshwater ebi, demineralized water, HCl (37%), HNO₃ (30%), KCl (4 M), and CaCl₂ (50, 100, 150, 200, and 250 ppm).

The samples were prepared and isolated before measurement using AAS and ISE. They were divided into two groups for preparation: nonheated and oven-dried (heated at 80°C for 20 min). The samples from each group were pureed with a blender to a sandlike consistency.

In the dilution isolation method, 0.5 g of sample was placed inside a beaker and 30 mL of demineralized water was added, followed by shaking for 15 min. The samples were then filtered into a 50 mL beaker and 1 mL of ISAB KCl (4 M) was added, followed by the addition of demineralized water to the 50 mL mark. The solution was then shaken until it became homogeneous.

In the acid digestion isolation method, 0.5 g of sample was placed in a digestion tube, and then 8 mL of HNO₃ (65%) and 1 mL of H₂O₂ (30%) were added. The digestion tube was sealed, placed inside a cartridge, and subjected to microwave digestion. The temperature regime was set to 130°C for 10 min, 150°C for 5 min, and 180°C for 15 min. Then, the solution was filtered into a beaker using a filter paper and a glass funnel. Next, 1 mL of ISAB KCl (4 M) was added followed by demineralized water to the 50 mL mark. The solution was shaken until it became homogeneous.

In the dry ashing method, 2 g of sample was placed inside a porcelain crucible and then set inside the furnace. The sample was heated from 26°C to 450°C. The temperature was then held for 15 min.
every 50°C until it reached 550°C, at which temperature the samples were held for 5 h. The ash produced was white-grayish in color. The furnace was then cooled, before the porcelain crucible was removed and left to further cool to room temperature. The ashes were weighed and transferred to a beaker. Next, 2 mL of 65% HNO₃ was added to the beaker and stirred on a heater to dryness. Later, 2 mL of 37% HCl was added to the sample and stirred on a heater until boiling. The beaker was then removed from the heater and left to cool to room temperature. De-mineralized water was added to the sample until it reached the 30 mL mark. The sample was then filtered into a 50 mL beaker before 1 mL of ISAB KCl (4 M) was added, followed by adding de-mineralized water to the 50 mL mark. The solution was shaken until it became homogeneous.

A standard solution was made by diluting a 1000 ppm CaCl₂ solution to 50 ppm (2.5 mL), 100 ppm (5 mL), 150 ppm (7.5 mL), 200 ppm (10 mL), and 250 ppm (12.5 mL). Next, 1 mL of ISAB KCl (4 M) was added to each solution followed by de-mineralized water to the 50 mL mark. The potential difference of each solution was measured using the ISE, and the absorbancy was measured using AAS; thus, calibration curves were prepared as a basis for determining sample concentrations. In the ISE method, the equation of the line relates the potential difference and the logarithm of the standard solution molarity. In the AAS method, the equation of the line relates absorbancy and concentration (ppm). The equations were then used to convert the absorbancy and potential difference values of the samples to concentrations (ppm).

3. Results
3.1. Results of ISE measurements
Table 1 shows the potential differences of the standard solutions and Table 2 shows the calcium concentrations of the samples. Statistical analysis with the independent t-test and ANOVA analysis shows a significant difference in calcium levels between the various preparation and isolation methods. Furthermore, a Pearson correlation test shows a highly positive correlation between the oven-dried and nonheated samples at a significance level of 0.0001 and with a correlation coefficient of 0.993.

Table 1. Standard solutions measured by the ISE.

| Standard solution  | Concentration (ppm) | Potential difference (mV) | Concentration (M) | log Ca²⁺ |
|--------------------|----------------------|----------------------------|-------------------|---------|
| Standard solution 1| 50                   | 278.2                      | 0.00125           | −2.90   |
| Standard solution 2| 100                  | 281.8                      | 0.0025            | −2.60   |
| Standard solution 3| 150                  | 283.3                      | 0.00375           | −2.43   |
| Standard solution 4| 200                  | 284.8                      | 0.005             | −2.30   |
| Standard solution 5| 250                  | 285.1                      | 0.00625           | −2.20   |

Table 2. Calcium levels measured by the ISE.

| Method     | Mean mV | Mean ppm (m/m) | Standard deviation |
|------------|---------|----------------|--------------------|
| Dilution   |         |                |                    |
| Nonheated  | 299.07  | 545,914.64     | 38,352.48          |
| Oven-dried | 300.47  | 747,992.81     | 19,808.97          |
| Digestion  |         |                |                    |
| Nonheated  | 239.73  | 0.74           | 0.02               |
| Oven-dried | 241.37  | 1.07           | 0.03               |
| Ashing     |         |                |                    |
| Nonheated  | 299.93  | 168,711.87     | 34,587.93          |
| Oven-dried | 300.03  | 170,348.57     | 5,954.76           |
3.2. Results of AAS measurements

Table 3 shows the absorbancy measurements of the standard solutions and Table 4 shows the calcium concentrations of the samples. Statistical analysis with the independent t-test and ANOVA analysis shows a significant difference in calcium levels between the various preparation and isolation methods. Furthermore, a Pearson correlation test shows a highly positive correlation between the oven-dried and nonheated samples at a significance level of 0.0001 and with a correlation coefficient of 0.878.

| Standard solution Ca²⁺ Concentration (ppm) | Absorbancy |
|-------------------------------------------|------------|
| Standard solution 1                      | 50         | 0.07  |
| Standard solution 2                      | 100        | 0.21  |
| Standard solution 3                      | 150        | 0.37  |
| Standard solution 4                      | 200        | 0.53  |
| Standard solution 5                      | 250        | 0.66  |

Table 3. Standard solutions measured by AAS.

| Method          | Mean absorbancy | Mean ppm (m/m) | Standard deviation |
|-----------------|-----------------|----------------|--------------------|
| Dilution        |                 |                |                    |
| Nonheated       | 0.04            | 4,109.04       | 11.63              |
| Oven-dried      | 0.03            | 3,814.14       | 8.31               |
| Digestion       |                 |                |                    |
| Nonheated       | 0.18            | 8,583.85       | 116.53             |
| Oven-dried      | 0.15            | 7,749.05       | 67.33              |
| Ashing          |                 |                |                    |
| Nonheated       | 0.45            | 4,415.73       | 46.29              |
| Oven-dried      | 0.63            | 5,910.79       | 58.29              |

Table 4. Calcium levels measured by AAS.

4. Discussion
4.1. Calcium levels measured by the ISE

Calcium levels measured by the ISE are higher for the oven-drying preparation method, as compared with the nonheated method. This is because heating the samples aids in the release of a compound bond into free ions, and the principle of the ISE measurement is the detection of free ions in a solution [10]. The results show higher levels of calcium for samples prepared with the dilution method, as compared with the acid digestion and ashing methods. This is contrary to previous findings in which dilution produced the least amount of calcium ions because the only energy source is kinetic energy from the shaking; there is no contribution from the reagents (such as acids) that could potentially assist in decomposing the compound into free ions [12]. In the method using microwave-assisted acid digestion, the samples are diluted in a strong acid that oxidizes organic materials and extracts elements, resulting in a relatively large amount of calcium ions [13,14]. In the ashing method, the samples are heated, which removes organic matrices in the samples. A strong acid is then added, which helps break the chemical bond into free ions, thus resulting in a higher calcium level [15,16]. The higher calcium level for the dilution method is possibly caused by interference from other ions and a lack of electrode sensitivity toward calcium, resulting in the quantification of other ions in the
solution. Interference may also occur owing to a memory effect during quantification as the same electrode is used across all samples; however, a method aimed at preventing this phenomenon is followed, i.e., washing of the electrode with demineralized water between samples [17].

The highest calcium level is achieved with oven drying and dilution isolation, with a value of 747,992.81 ppm (m/m). This value is not in accordance with Indonesian data on food composition and nutrition or with previous research by Maharani and Julshamn, who found a value of 5080 ppm [9,18]. These results are likely inaccurate because of potential interferences that occurred during measurement.

The inaccuracy of the data can be identified on the basis of the slope value of the linear regression of the standard solutions, which is 10.11. For divalent ions such as calcium, the tolerance level of the slope is around 26 ± 3 [19], and a slope value below the tolerance rate causes measurement errors. The low observed slope value may be caused by contamination of the electrode. Because of the limitations and interferences present in this research, the ISE data are considered an unreliable indicator of calcium concentrations in freshwater ebi (Macrobrachium sp.).

4.2. Calcium levels measured by AAS

AAS measurement requires information on the light wavelength of the element being analyzed. To measure calcium concentrations, the wavelength used is 422.7 nm. The samples undergo atomization to free atoms via supplied heat energy. The oxidant gas and burner used is air–acetylene at a temperature of 2300°C [20,21,22].

By AAS analysis, the calcium levels are highest for the nonheated method with dilution and acid digestion, as compared with the oven-dried method. The ashing method gives higher calcium levels for the oven-dried method, as compared with the nonheated method.

In the dilution method, higher calcium levels are found for the nonheated preparation method, as compared with the oven method. This is because in the nonheated method, the only energy source is kinetic energy from shaking the solution, whereas in the oven-drying method, energy is received as both kinetic energy from shaking and thermal energy from heating, causing the excitation of some atoms [12]. Because AAS measures atoms at the lowest energy level, the measured levels in cases where some of the atoms are excited become lower [23]. Thermal energy from the oven causes ionization interference (excitation and ionization), resulting in a decrease in absorbance and a lower measured concentration [21]. This is also the case for the dilution method, for which calcium levels are higher with the nonheated preparation, as compared with the oven-drying preparation. This is because of the interference occurring in the oven preparation method, which causes excitation of some of the atoms and thus decreases the measured levels [12].

In the nonheated ashing method, organic materials remain in the samples owing to the lack of heating before ashing is undertaken. This causes double combustion during the ashing process, i.e., external burning from the high ashing temperature and internal burning from the organic material in the samples, which may cause some of the calcium ions to also burn. Meanwhile, in the oven ashing method, the content of organic material in the samples is reduced owing to heating, resulting in decreased internal burning as compared with the nonheating method [12].

Among dilution, acid digestion, and dry ashing, the highest calcium levels are achieved by acid digestion. This is because in the microwave-assisted digestion method, the samples are diluted in a strong acid that helps degrade organic materials in the samples and convert the analytes into a form that is easier to determine [13,14].

In this research, the highest AAS-measured calcium levels are for samples prepared with the nonheated method and isolated using acid digestion (8583.85 ppm (m/m)). This level is in accordance with Indonesian data on food composition and nutrition (7600 ppm calcium) and with previous research by Maharani and Julshamn (5080 ppm calcium) [9,18].

5. Conclusions

There were differences in ebi calcium levels between the preparation methods and among the isolation methods. The preparation methods showed a strong correlation with the measured calcium levels. The
acid digestion isolation method produced higher calcium levels, as compared with dry ashing and dilution, when measured by AAS. The ISE measurement of calcium levels for the various preparation and isolation methods did not yield valid results.

6. References

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