

Calcium from Pacific White Shrimp (*Litopenaeus vannamei*) Shells: Properties and Function as Fortificant in Soy Milk

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Abstract

Shrimp shells are the waste from shrimp processing industry that can be a potential source of calcium. This study aimed to determine the chemical and functional properties of calcium gluconate (SCG) and calcium lactate (SCL) prepared from shrimp shells and their application as food fortificants in soy milk. Calcium extraction was performed at 70°C for 2 h by mixing the enzymatically-deproteinized shrimp shell with the solutions of gluconic or lactic acid containing equivalent number dissociated carboxyl group to calcium available in the shells. The obtained calcium salts were characterized using Fourier-transformed infrared spectroscopy. Both gluconic and lactic acids gave >80% calcium extraction yield. The obtained SCG and SCL had calcium solubility of >70% across the pH range of 3–8. Dialyzability of SCG and SCL, evaluated by *in vitro* equilibrium, and continuous flow dialysis methods, was 30–40% and 60–70%, respectively. Soy milks fortified with SCG and SCL to obtain a final calcium concentration of 125 mg/100 mL had slightly different color and slightly higher apparent viscosity than the unfortified one. However, such differences did not affect the sensory acceptability scores of the product in all attributes. Although the solubility and dialyzability of SCG and SCL were lowered by the presence of food matrix of soy milk, the prepared calcium salts could still increase total calcium and dialyzable calcium contents of the fortified samples. Therefore, Pacific white shrimp shells can be used to prepare different forms of calcium salts with potential application as fortificants in food.

Keywords: shrimp shells, calcium, solubility, bioaccessibility, fortificant

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1. Introduction

Calcium is a major mineral in human body, accounting for 1–2% of an adult's body weight. However, a survey in Thai population revealed that 67% of male and 87% of female, who lived in rural area, had daily amount of dietary calcium intake less than half of the recommended level, and only 6% of men and 3% of women had an intake of higher than 800 mg/d (Pongchaiyakul *et al.*, 2008). Moreover, Thai school-aged children also did not achieve the recommended daily allowance for calcium, which is 800–1,000 mg/d (Yamborisut *et al.*, 2015). Nowadays, there are many calcium-fortified food products available in the market. Organic and inorganic salts of calcium as well as their mixtures have been widely used for food fortification. However, solubility and bioavailability of different calcium salts are different, which affect their potentials for food application and their effects on health (Kressel, 2010). Most of the calcium fortificants, especially the highly soluble and bioavailable ones, have to be imported and thus are expensive.

Pacific white shrimp (*Litopenaeus vannamei*) has the highest market value among other seafood in Thailand. The annual production of Pacific white shrimp in 2014 reached 200,000 metric tons and increased to be 220,000 metric tons in 2015, of which 10% of the produces was for domestic consumption and the rest was exported (FAO, 2016). Shrimp shells, which account for about 35% of the whole shrimp weight, are the major production waste from shrimp processing industry. The major mineral components in shrimp shells are calcium and phosphorus, which account for about 44.75 and 7.06% of the total mineral content, respectively (Mahmoud *et al.*, 2007). Therefore, shrimp shells is an underutilized natural source of calcium that can be a suitable raw material for a local production of calcium. Calcium in shrimp shells is naturally presented as calcium carbonate that is closely associated with the mineralized proteinous matrix of the chitin complex; and is normally removed from shrimp shell during the production of chitin. In such process, strong acid, i.e., HCl, is used under harsh conditions to maximize the solubilization of minerals from the shells. Hence, the removed calcium is poor in functional properties and usually discarded. Several studies have demonstrated that demineralization of shrimp shells with organic acids, e.g., acetic, citric, formic, and lactic acids, as well as their combinations, could be optimized to provide satisfactory calcium removal of up to 90%, which was comparable to that of HCl (Charoenvuttitham *et al.*, 2006; Mahmoud *et al.*, 2007; Wang *et al.*, 2014); but the properties of such removed calcium have not been studied. Therefore, demineralization of shrimp shells using weak acids would be an alternative for the more environmental-friendly production of calcium salts with better functional properties than the conventional method.

This study aimed to determine the properties of calcium salts prepared from Pacific white shrimp shells by solubilizing with weak organic acids. To determine their potential as calcium fortificants in food, the effect of the obtained calcium salts on the quality of calcium-fortified soy milk and the properties of calcium salts in the food matrix of soy milk were also investigated.

2. Materials and Methods

2.1 Materials

Shells of Pacific white shrimp (*Litopenaeus vannamei*) aged 70–80 d were donated by Charoen Pokphand Foods (Public) Co., Ltd. (Samut Sakhon, Thailand) from three production batches. The shells were collected and washed with 100 mg/kg chlorinated water, packed in low-density polyethylene (LDPE) bags and kept frozen at $-18\pm 3^{\circ}\text{C}$. The composition of shrimp shells, analyzed according to the AOAC Official Methods (AOAC 2016), was 78.80% moisture, 12.30% total nitrogen, 1.32% crude fat, 6.65% dietary fiber, 5.76% total ash, and 2.31% calcium. Lactic acid (88% w/w) was purchased from Purac (Thailand) Co., Ltd. (Rayong, Thailand). Tri-potassium citrate was obtained from Merck KGaA (Darmstadt, Germany). Pasteurized unsweetened soy milk (OHAYO™, Booncharoen Pokphand, Pathum Thani, Thailand) and sugar (Mitr Phol Pure Refined Sugar, Mitr Phol Sugar Corp., Bangkok, Thailand) were purchased from a local supermarket. D-gluconic acid (50% w/v) and all other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, Missouri, U.S.A.).

2.2 Deproteinization of shrimp shells

Shrimp shells were deproteinized enzymatically according to Chumputong et al. (2013) by mixing the thawed shells with deionized water at a weight-to-volume ratio of 1:10. Then, alcalase enzyme was added at the concentration of 0.1% (v/w) of the shells and the hydrolysis was performed for 2 h at $50\pm 2^{\circ}\text{C}$ and pH 9.0, which is the enzyme's optimal condition. After hydrolysis, the mixture was heated at $90\pm 2^{\circ}\text{C}$ for 10 min in a water bath to inactivate the enzyme followed by cooling down immediately in an ice bath. The deproteinized shells were separated from the mixture using a strainer and dried in a hot-air oven at $50\pm 2^{\circ}\text{C}$ for 6 h, ground with an electric grinder to obtain the size of smaller than 35 mesh, sealed in LDPE bags and kept in a desiccator. The dried deproteinized shells contained 9.96% moisture, 41.79% total nitrogen, 30.38% dietary fiber, 25.49% ash and 8.37% calcium, as analyzed according to the AOAC Official Methods (AOAC, 2016).

2.3 Preparation of calcium salts

Calcium was extracted from deproteinized shrimp shell by acid solubilization. The deproteinized shell was mixed with appropriate solution of gluconic or lactic acid at a weight-to-volume ratio of 1:40. Concentration of acid solutions was varied, based on their dissociation constants (pKa), to obtain the molar ratio of acid and calcium of 2:1. Calcium extraction was performed by heating the mixture at $70\pm 5^\circ\text{C}$ for 2 h, which was the optimized condition obtained from our preliminary experiments. After completion, the mixture was cooled immediately to room temperature prior to removal of the remaining shells using a strainer. The obtained solutions were collected and adjusted their pH to be 7.0 with NaOH to neutralize the excess acid. The precipitate was washed twice with deionized water, and desiccated to $>5\%$ moisture in a hot air oven at $60\pm 2^\circ\text{C}$ for 12 h. The obtained pellets, i.e., calcium gluconate (SCG) and calcium lactate (SCL), were kept in glass vials and placed in a desiccator. The obtained salts were weighed to determine production yield from 100 g shrimp shells.

2.4 Determination of calcium salt properties

2.4.1 Calcium content

Calcium content in the obtained salts was analyzed to determine their purity using atomic absorption spectrometry (AAS; S-Series 711906 v1.26, Thermo Electron, Cambridge, U.K.) according to AOAC Official Method 985.35 (AOAC, 2016). Calcium extraction yield was calculated as the percentage of calcium content in the obtained salt to that presented in the deproteinized shells.

2.4.2 Molecular structure

In order to confirm the molecular structure, Fourier transform infrared (FTIR) spectra of calcium salts were obtained using KBr pellet method. Desiccated samples were mixed with dry KBr fine powder to obtain a final concentration of approximately 1 g sample/100 g. The mixture was then finely pulverized and put into a pellet-forming die. A force of approximately 7 tons was applied under atmospheric pressure for several minutes to form transparent pellets. Measurements were performed by placing the sample-KBr discs on a pellet holder inserted into the sample chamber of FTIR spectrometer (Nicolet 6700, Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.) equipped with a single bounce diamond crystal. The spectral value of the sample was obtained by co-adding 64 scans at the resolution of 4 cm^{-1} in mid-infrared region ($4,000\text{--}400\text{ cm}^{-1}$). The obtained spectra was subtracted with a blank spectra collected from a pellet of KBr only. Spectra of SCG and SCL was compared with the Spectral Database for Organic Compounds of the National Institute of Advanced Industrial Science and Technology of Japan (SDBSWeb, 1999).

2.4.3 Color

Color values (L^* , a^* , b^*) were measured using a spectrophotometer (ColorFlex EZ, Hunter Associates Laboratory, Reston, Virginia, U.S.A.). The L^* , a^* , and b^* values represent lightness, redness, and yellowness, respectively.

2.4.4 Calcium solubility

Solubility of calcium salts was determined by adjusting the pH of 2% (w/v) solutions of SCG and SCL with NaOH or HCl to be 3.0–8.0. The solution was centrifuged at 3,120 xg for 15 min at 25°C and calcium content in the supernatant was analyzed as described earlier. Solubility of calcium salt at each pH was calculated as the percentage of calcium content in the supernatant to calcium content in the solution (Yazici *et al.*, 1997).

2.4.5 Calcium dialyzability

Total calcium dialyzability of calcium salts was determined using equilibrium dialysis system according to methods described by Miller *et al.* (1981). Samples were digested *in vitro* under simulated gastro-intestinal condition. Gastric digestion was performed by pepsin-HCl (500 mg pepsin/mL food sample-buffer mixture) at pH 2.0 for 2 h at 37±1°C in a shaking water bath, followed by intestinal digestion with pancreatin-bile extract mixture (100 mg pancreatin and 625 mg bile extract/mL gastric digesta) at pH 7.5 for 2 h at 37±1°C in a regenerated cellulose tubular membrane with 6-8 kDa molecular weight cut-off. Dialysis was performed against sodium bicarbonate buffer pH 7.5. At the end of pancreatic digestion, the dialysate was weighed and determined for its calcium content by AAS as described earlier. Total calcium dialyzability was expressed as the percentage of amount of calcium in the dialysate to the total amount of calcium present in sample, as follows:

$$\text{Total calcium dialyzability (\%)} = \frac{100 \times D}{A \times W}$$

where D represents the total amount of dialyzed calcium during gastro-intestinal digestion (mg); A represents the concentration of calcium in samples (mg/mL); and W represents the volume of sample used for pancreatin digestion (mL), respectively.

Continuous calcium dialyzability of calcium salts was determined using continuous-flow dialysis system described by Judprasong *et al.* (2005). Simulated gastro-intestinal digestion was performed in the same manner as equilibrium dialysis system, except that the dialysis was conducted in a regenerated cellulose tubular membrane with 12-14 kDa molecular weight cut-off against sodium bicarbonate buffer pH 7.5 that flowed through the dialysis chamber at 1 mL/min. Fractions of dialysate was collected at 10, 20, 30, 40, 50, 60, 90 and 120 min and

analyzed for their calcium content with AAS. The continuous calcium dialyzability was expressed as:

$$\text{Continuous calcium dialyzability (\%)} = \frac{100 \times D}{C}$$

where D represents dialyzed calcium content (mg/g sample) and C represents the total calcium content (mg/g sample), respectively.

2.5 Preparation of calcium-fortified soy milk

Soy milk, which contains 4 g protein, 2.25 g fat, 1.5 g carbohydrate, and 0.024 g calcium in 100 ml, was heated to $50 \pm 1^\circ\text{C}$ prior to adding tri-potassium citrate (0.67 g/100 ml). Such concentration of tri-potassium citrate provides an equivalent mole number with calcium, and thus was proven to prevent calcium-induced coagulation of soy protein upon heating in our preliminary experiment (data not presented). The mixture was mixed with sugar (3 g/100 ml), and appropriate amount of SCG or SCL was subsequently dissolved into the soy milk at the concentration of 100 mg Ca/100 ml in order to obtain the fortified soy milk with a calcium concentration of about 120 mg Ca/100 ml, which is similar to that in cow's milk. The fortified soy milks, i.e., SCGM and SCLM, were pasteurized at $75 \pm 1^\circ\text{C}$ for 30 s in a double-boiler, hot-filled in glass bottles, immediately cooled in an ice bath, and kept under refrigeration. The control formula was prepared without the addition of calcium salt.

2.6 Quality determination of calcium-fortified soy milk

2.6.1 pH and color

The pH value of sample at room temperature was measured using a digital pH meter (EcoMet P 25, Istek, Seoul, Korea). Color values were measured as mentioned in 2.4.3 and color difference (ΔE^*) between each of samples and control formula was calculated as:

$$\Delta E^* = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2}$$

where L_0^* , a_0^* , and b_0^* are color values of the control sample.

2.6.2 Rheological properties

Viscosities of calcium-fortified soy milk were measured at room temperature ($28 \pm 2^\circ\text{C}$) using a Brookfield digital viscometer (RVTDV-II, Brookfield Engineering Laboratories, Stoughton, Massachusetts, U.S.A.) fitted with a UL Adapter 304 S/S (part number: ULA-Y), that consisted of a cylindrical spindle rotating inside a sample tube. Sixteen milliliters of sample was added to the sample tube and allowed to equilibrate for 5 min prior to analysis. Apparent viscosity was recorded every 2 min while the shear rate was increased from 0.61 to 122.4 s^{-1} over 16 min. Flow behaviour index and consistency index were determined by fitting the obtained flow curves using the Power Law equation:

$$\sigma = K\dot{\gamma}^n$$

where σ is shear stress, $\dot{\gamma}$ is shear rate, K is consistency index ($\text{Pa}\cdot\text{s}^n$), and n is the flow behavior index (Rao, 2014).

2.6.3 Sensory acceptability

Sensory acceptability of the fortified and unfortified soy milks was evaluated by 50 untrained panelists (32% male, 68% female aged 20-58 years). All samples were prepared on the day before the test day and kept in a refrigerator (4-5°C). Samples were labeled with three-digit random code numbers and served cold in 20-mL clear disposable plastic cups in random orders, one at a time, to avoid comparison between samples. Panelists were asked to rinse their mouths with drinking water between samples. Nine-point hedonic scale was used to evaluate the acceptability for general appearance, color, odor, viscosity, taste, mouthfeel, and overall acceptability of samples. The scale ranged from 1 to 9, where 1 means “dislike extremely”, 5 means “neither like nor dislike”, and 9 means “like extremely”.

2.6.4 Fortificant properties

The content and solubility of calcium in the samples were analyzed as described earlier. Calcium dialyzability was also analyzed according to *in vitro* equilibrium dialysis method aforementioned. Solubility and dialyzability of the fortificant were calculated by subtracting those of the control sample from the fortified samples and expressed as the percentage of the amount of fortified calcium in the sample.

2.7 Statistical analysis

All experiments, except sensory evaluation, were performed in Completely Randomized Design with triplicate of separate sets of experiment. Sensory evaluation was performed in Randomized Complete Block Design. All results were presented as means \pm standard deviations. Statistical software (IBM SPSS Statistics for Windows Version 19.0, IBM Corp., Armonk, New York, U.S.A.) was used to analyze data. Differences between mean values were analyzed by Student's t-test or one-way Analysis of Variance using Duncan's New Multiple Range Test with a confidence level of 95%.

3. Results and Discussion

3.1 Properties of calcium salts prepared from shrimp shells

Total production yields of both SCG and SCL were about 9.9 and 6.6 g/100 g shells, respectively (Table 1). The content of calcium in different salt forms varied due to the difference in molecular weights of calcium gluconate and calcium lactate, i.e., 430.37 and 218.22 g/mol, respectively. It was found that the contents of calcium in SCG and SCL were closed to the theoretical calcium content in calcium gluconate and calcium lactate, i.e., 9 and

13 g/100g, respectively, which were obtained from calculation based on their molecular weights (Table 1). In this study, weak organic acids are used as the more environmental-friendly method for demineralization of shell waste in alternative to HCl, which is the strong acid usually used for maximizing the demineralization yield. The extractable calcium achieved by gluconic and lactic acids was about 80 and 87% of that present in the shell, respectively, which was considerably high. Decalcification of shrimp shell using organic acids has been proved to give a comparable calcium extraction yield with HCl elsewhere (Charoenvuttitham *et al.*, 2006; Mahmoud *et al.*, 2007). In this study, it was probably due to the fact that gluconic and lactic acids were completely ionized during extraction since the pH values of shell-acid mixtures before and after extraction, i.e., 4.27–4.75, were higher than 3.86, which is the pKa of both acids (Serjeant and Dempsey, 1979).

Both SCG and SCL were burnt sienna-colored powder, because they might contain astaxanthin, a carotenoid that is the natural orange pigment in shrimp shells (Wade *et al.*, 2005). The color values indicated that SCG had lower L* values but higher a* and b* values than SCL, suggesting that the color of SCG was darker, redder and yellower (Table 1). The molecular structure of the obtained calcium salts was characterized using FTIR spectroscopy which is an analytical technique for classification of organic compounds and chemical bonds in the molecules (Griffiths and de Haseth, 2007). Spectra of SCG and SCL (Figure 1) matched well with those available in the database (SDBSWeb, 1999). The broad peak at around 3,200–3,550 cm^{-1} corresponds to the symmetric stretching of –OH group. The stretching and bending of C–H bands appear around 3,000 and 1,000 cm^{-1} . The stretching of the COO^- group occurs at wavenumbers 1,400–1,600 cm^{-1} . The C–C stretching band is revealed around 900 cm^{-1} (Nakamoto, 2009). Metal oxygen stretching is represented by the medium band centered at 700–900 cm^{-1} (Jini *et al.*, 2005). The results confirmed that different forms of calcium salt could be prepared from shrimp shells using different acids under the suitable acid concentration, temperature and time.

Table 1 Properties of calcium salts prepared from shrimp shells

Parameters	SCG	SCL
Production yield (g/100 g shells)	9.87±0.01 ^{&}	6.63±0.01 ^{&}
Calcium content (g/100 g salt)	13.25±0.01 ^{&}	18.23±0.01 ^{&}
Calcium extraction yield (g/100 g Ca in shells)	86.88±0.36 ^{&}	80.28±0.37 ^{&}
Color values		
L*	69.77±0.89 ^{&}	74.93±0.55 ^{&}
a*	7.77±0.36 ^{&}	3.56±0.09 ^{&}
b*	20.45±0.30 ^{&}	13.74±0.17 ^{&}
Calcium dialyzability (%)		
Total calcium dialyzability	35.97±4.10	36.91±3.80
Continuous calcium dialyzability	70.12±9.47	59.48±3.92

Note: Values are means ± standard deviations of triplicates.

SCG: calcium gluconate prepared from shrimp shells; SCL: calcium lactate prepared from shrimp shells

[&] Means with ampersands within the same row are significantly different ($p \leq 0.05$).

Solubility of SCG and SCL was determined across the pH region of 3-8, which covers the pH values of most food products. It was found that SCG and SCL were >90% soluble at ant acidic pH value of ≤ 5 (Figure 2). Calcium solubility of both calcium salts significantly decreased to 70–80% at less acidic, and alkali pH regions ($p \leq 0.05$). The solubility of SCG and SCL were not significantly different ($p > 0.05$) at any pH value except pH 6 where SCG showed higher solubility than SCL. The high solubility of both SCG and SCL across the wide pH range suggests their potential applications as an ingredient in a wide range of food products without precipitation that might affect the product's appearance. The solubility of prepared calcium salts was consistent to that reported previously that calcium lactate and calcium gluconate showed moderate solubility and stability in aqueous solution, but dissolved more quickly at higher temperatures, as compared among different forms of calcium salt (Gerstner, 2003; Oort, 2004).

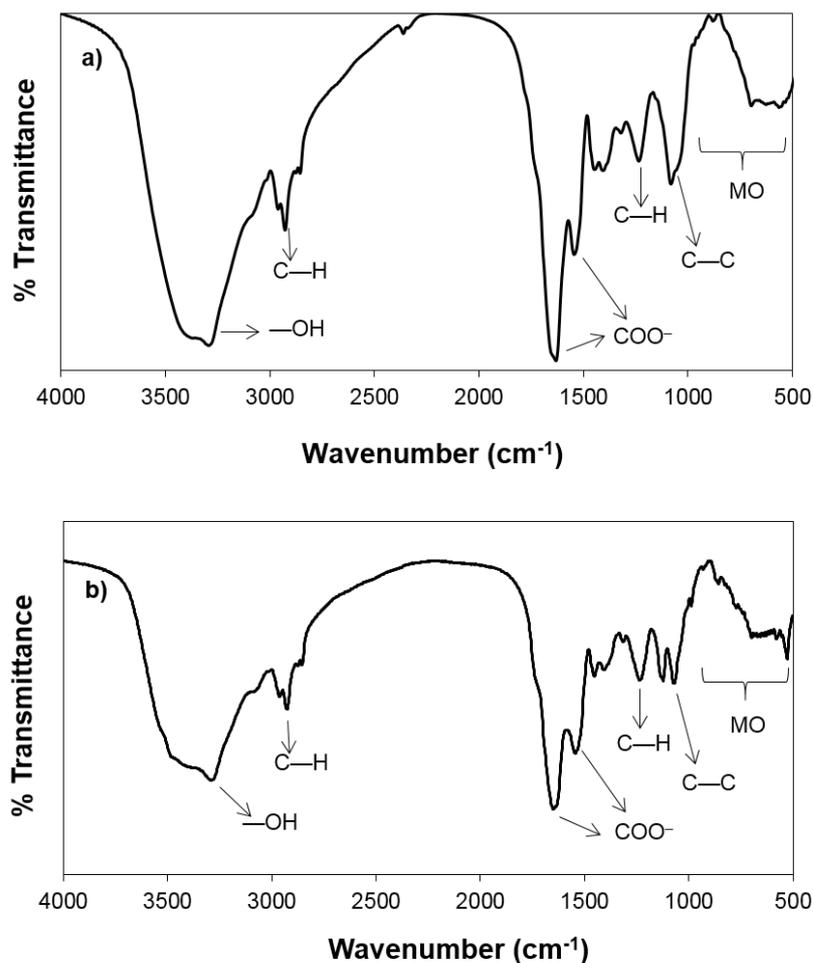


Figure 1 Typical FTIR spectra of calcium gluconate (a) and calcium lactate (b) prepared from shrimp shells.

—OH: hydroxyl group; C—H: alkane band; COO⁻: carboxyl group; C—C: alkene band;
MO: metal oxygen band.

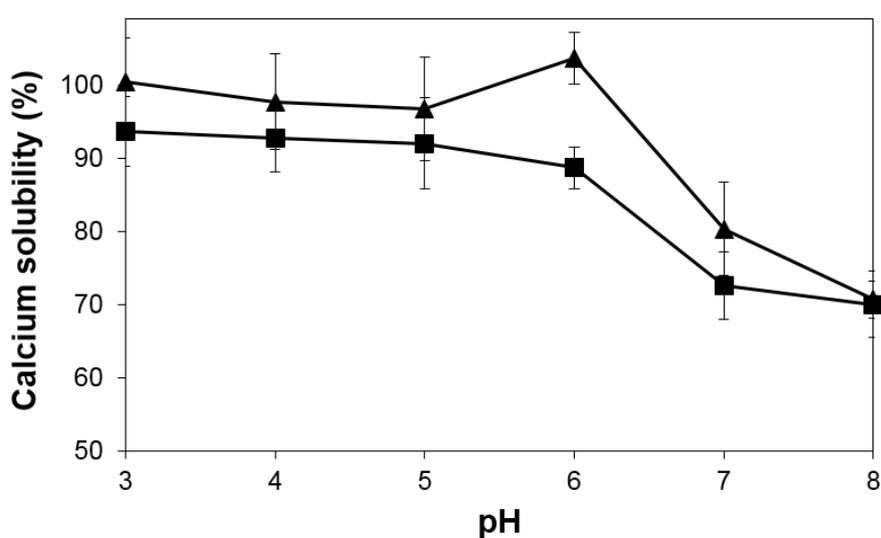


Figure 2 Calcium solubility across pH region of (▲) calcium gluconate and (■) calcium lactate prepared from shrimp shells

Bioavailability is the important factor in selection of micronutrient fortificant in food. The *in vitro* method involves a simulated gastro-intestinal digestion conditions and measuring the dialyzability of minerals through a semi-permeable membrane during the intestinal stage. The calcium bioaccessibility of SCG and SCL obtained from equilibrium dialysis were about 36.0 and 37%, respectively (Table 1). Dialysis profiles obtained from the continuous flow dialysis, presented in Figure 3, reveals that the bioaccessibility were about 70 and 60% for SCG and SCL, respectively (Table 1), which did not significantly differed ($p>0.05$). The *in vitro* continuous flow dialysis method is believed to be more representing the *in vivo* physiological condition than that based on equilibrium dialysis because dialyzable components are continuously removed from the simulated intestinal digestion system during dialysis (Shiowatana *et al.*, 2006). Therefore, the reverse osmosis of dialyzed components is minimized, resulting in the higher dialyzability obtained from the continuous flow dialysis than that determined by the equilibrium system of both calcium forms (Table 1). A similar trend of difference in values obtained from these two methods has also been reported previously (Shiowatana *et al.*, 2006). It could also be due to the fact that the pore size of dialysis membranes used in the continuous flow dialysis method is larger than that of equilibrium method, i.e., 12–14 and 6–8 kDa, respectively. Therefore, in the former system, the greater amount of calcium could permeate through the membrane. Previous studies have proven that organic calcium salts are more bioavailable than the inorganic salts (Gerstner, 2003; Sakhaee *et al.*, 1999; Singh *et al.*, 2007), and the solubility of calcium salt is relative to calcium binding strength in its complex (Vavrusova and Skibsted, 2014). Calcium lactate and calcium gluconate are highly soluble and are able to form supersaturated calcium salt solutions. In particular, gluconic acid is a good ligand for calcium ion so its respective calcium salt has high solubility and also low precipitation rate (Skibsted and Kilde, 1971). In addition, kinetic factors such as rate of precipitation, rates of complex formation and dissociation, and the forms of calcium complex formed throughout the digestion system, also affect the calcium bioavailability (Vavrusova and Skibsted, 2014). However, several studies have reported that there was no difference in bioaccessibility of organic and inorganic calcium salts, and weak correlation between solubility of calcium salts and their bioavailability (Hansen *et al.*, 1996; Heaney *et al.*, 1999; Heaney *et al.*, 1990); but the physical state of the fortificant while in the food and its interaction with the food matrix are more important determinants of absorbability than its actual chemical character (Heaney *et al.*, 2005).

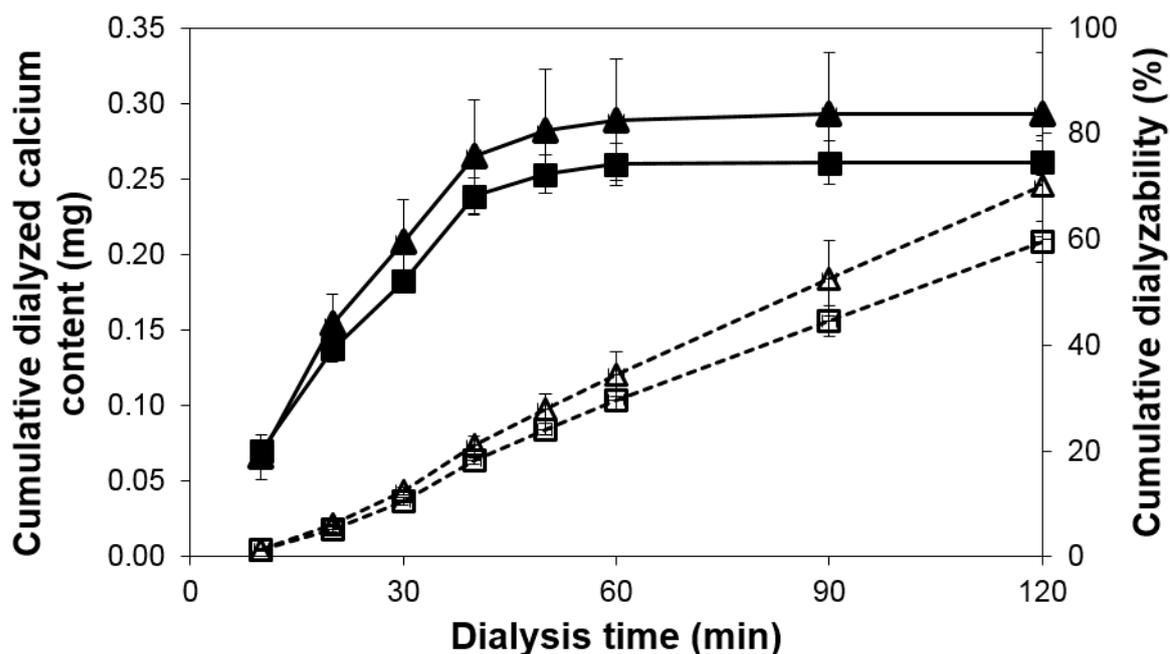


Figure 3 Cumulative (▲, ■) dialyzed calcium content and (△, □) calcium dialyzability obtained from continuous flow dialysis of (▲, △) calcium gluconate and (■, □) calcium lactate prepared from shrimp shells

3.2 Effect of calcium-fortification on the quality of soy milk

The physical properties of control, unfortified soy milk and the samples fortified with calcium salts from shrimp shells, i.e., SCGM and SCLM, are presented in Table 2. Unfortified soy milk had a pH value of 6.67. Fortification with calcium salts prepared from shrimp shells significantly increased the pH of soy milk to be slightly above 7, with no significant difference between the pH value of SCGM and SCLM ($p > 0.05$). The higher pH value of calcium-fortified soy milks than was due to the presence of calcium salt, as well as tri-potassium citrate, which is a calcium-chelating agent added to prevent calcium-induced coagulation of soy protein (Pathomrungruangsriyounggul *et al.*, 2010). It has been reported previously that the addition of tri-calcium phosphate at a concentration of 25 mmol Ca/L caused an increase in pH of soy milk from 7.36 to 7.55 (Weingartner *et al.*, 1983).

L^* value of soy milk containing calcium salts from shrimp shells was significantly lower than the control sample (Table 2). The fortified-soy milks had lower lightness when SCG and SCL were used as fortificants. Considering a^* value, the sample fortified with SCG had the highest a^* value, followed by SCL-fortified soy milk, indicating its greatest redness among all samples. For yellowness, fortification with SCG significantly increased the b^* value of soy milk; while SCL did not affect the yellowness of the product. The color difference value (ΔE^*) revealed that the color of both calcium-fortified soy milk samples differed from the unfortified one; while the two fortified samples was not significantly different in their color (Table 2).

Fortification of soy milk using SCG and SCL caused the change in color of the final product to be darker and redder, which arose by the color of the calcium salts (Table 1).

There was no significant difference among the apparent viscosities of SCGM and SCLM, but both fortified samples were slightly more viscous than the control sample (Table 2). Such increase in viscosity caused by calcium fortification indicated that protein coagulation might be occurred in a small extent although tri-potassium citrate was added to prevent the coagulation of protein in soy milk due to calcium (Saeidy *et al.*, 2013; Yazici *et al.*, 1997). Flow behavior and consistency indices of soy milk samples were determined by fitting their flow curves (Figure 4) with the Power Law Model ($R^2 > 0.99$). Flow behavior index of SCGM, and SCLM was slightly lower than 1, indicating that they are non-Newtonian fluids with minimal shear-thinning (pseudoplastic) behavior. Viscosity of both SCGM and SCLM decreased with increasing shear rate while the control sample exhibited Newtonian fluid behavior of which the viscosity remained unchanged at about 6 mPa·s across the range of shear rate used for measurement. Addition of SCG and SCL significantly reduced the flow behavior index of soy milk, meaning that the products had more non-Newtonian behavior. Unfortified soy milk had the lower consistency index than the both calcium-fortified samples, which was consistent with their apparent viscosity (Table 2). The slightly higher apparent viscosity and the more shear-thinning behavior caused by calcium fortification indicated that protein coagulation might occurred to a small extent. However, it can be stated that application of calcium salts prepared from shrimp shells in soy milk did not majorly alter the rheological properties of the product.

Sensory evaluation was performed to investigate the effect of SCG and SCL on the sensory acceptability of soy milk. There was no significant difference in the scores on appearance, color, odor, viscosity, mouthfeel, and overall acceptability of unfortified sample, and sample fortified with either SCG or SCL (Table 3). The scores ranged between 6 and 7, which means the samples were rated to be “like slightly” to “like moderately”. The sensory acceptability scores for color, viscosity, and mouthfeel were inconsistent with the differences in color values and rheological properties between unfortified sample and calcium-fortified samples, based on apparatus measurements (Tables 2 and 3). This suggested that although the samples were different in their color and rheological parameters, they were still acceptable by the consumer at the similar extent. It might also be that such differences were undetectable by the panelists. For taste, unfortified sample had the lowest acceptability score among the three samples, which was rated between “neither like nor dislike” and “like slightly”. While both calcium-fortified samples were more acceptable by the panelists with ratings between “like slightly” and “like moderately”. It is likely that the more acidic pH of unfortified sample accounted for its lower acceptability score on taste than the fortified samples, of which the pH

was about 7 (Tables 2 and 3). Therefore, it can be stated that the addition of calcium salts prepared from shrimp shells to soy milk did not affect the sensory quality of the product.

Table 2 Properties of soy milks fortified with calcium salts prepared from shrimp shells

Parameters	Control	SCGM	SCLM
pH	6.67±0.03 ^B	7.23±0.07 ^A	7.15±0.07 ^A
Color values			
L*	82.12±0.05 ^A	78.22±1.44 ^B	79.89±0.95 ^B
a*	1.55±0.01 ^B	4.93±1.55 ^A	3.29±0.8 ^{AB}
b*	18.45±0.27 ^B	22.86±2.64 ^A	19.45±1.39 ^{AB}
ΔE	-	6.83±3.35 ^A	3.23±0.84 ^A
Apparent viscosity at 24.48 s ⁻¹ (mPa·s)	5.65±0.17 ^B	7.15±0.17 ^A	7.32±0.04 ^A
Flow behavior index	1.01±0.01 ^A	0.93±0.01 ^B	0.94±0.01 ^B
Consistency index (Pa·s ⁿ)	0.005±0.01 ^B	0.009±0.01 ^A	0.009±0.01 ^A

Note: Values are means ± standard deviations of triplicates.

SCGM: soy milk fortified with calcium gluconate prepared from shrimp shells; SCLM: soy milk fortified with calcium lactate prepared from shrimp shells

^{A,B} Means with different superscripts within the same row are significantly different ($p \leq 0.05$).

Table 3 Sensory acceptability scores of soy milks fortified with calcium salts prepared from shrimp shells

Attributes	Acceptability scores ¹		
	Control	SCGM	SCLM
Appearance	6.86±1.05	6.82±1.04	6.74±1.16
Color	6.92±1.18	6.88±1.07	7.00±1.01
Odor	6.56±1.15	6.58±1.07	6.66±1.00
Viscosity	6.68±0.98	6.52±1.17	6.64±1.12
Taste	5.60±1.60 ^B	6.50±1.87 ^A	6.06±1.75 ^{AB}
Mouthfeel	6.00±1.34	6.40±1.43	6.32±1.39
Overall acceptability	6.00±1.30	6.56±1.30	6.34±1.52

Note: Values are means ± standard deviations from 50 panelists.

SCGM: soy milk fortified with calcium gluconate prepared from shrimp shells; SCLM: soy milk fortified with calcium lactate prepared from shrimp shells

¹ Rated on 9-point hedonic scale: 1=dislike extremely, 5=neither like nor dislike, 9=like extremely

^{A,B} Means with different superscripts within the same row are significantly different ($p \leq 0.05$).

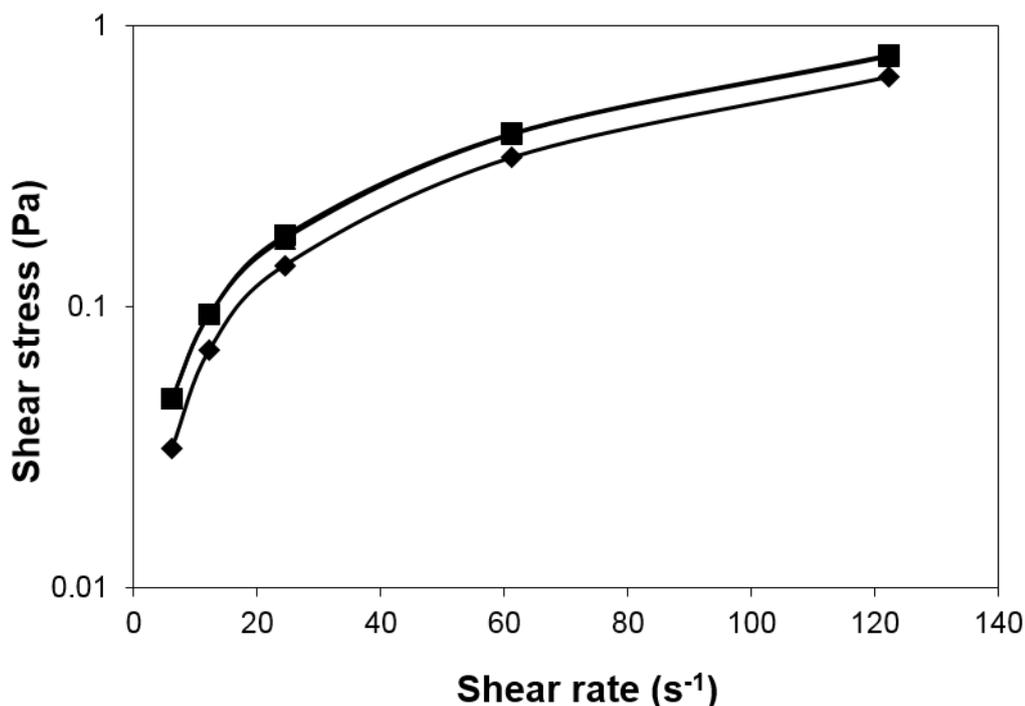


Figure 4 Flow curves of (◆) unfortified soy milk, and soy milks fortified with (▲) calcium gluconate, and (■) calcium lactate prepared from shrimp shells

3.3 Effect of soy milk matrix on the properties of calcium salts prepared from shrimp shells

Table 4 indicates that SCGM and SCLM contained higher calcium content than the control sample and the calcium content in both fortified samples reached the targeted fortification level of 125 mg/100 ml. Calcium solubility in unfortified soy milk was about 86%, indicating the calcium that is naturally present in soy milk is relatively soluble. Such level of solubility of the calcium naturally present in soy milk was higher than solubility of the calcium in fortified samples, which were contributed by calcium from soy and the added calcium salts. Sample fortified with SCG had higher calcium solubility than SCL-fortified sample. When considering only the calcium solubility of fortificant, calcium solubility of SCG and SCL in soy milk was lower than their solubility in buffer solution of the similar pH value of 7.0 (Table 1). Calcium in unfortified soy milk was about 20% dialyzable; while the fortification of calcium salts prepared from shrimp shells increased the total calcium dialyzability of soy milk to be about 31 and 28% for samples fortified with SCG and SCL, respectively (Table 4). However, the calcium dialyzability of both fortified soy milks were not significantly different ($p > 0.05$). It should be noted that, apart from the higher calcium content in the fortified soy milk samples, the addition of tri-potassium citrate, which has been reported to enhance calcium absorption (Moseley *et al.*, 2013; Sellmeyer *et al.*, 2002), could also result in the increase in calcium dialyzability of the calcium-fortified soy milk. When dialyzability of fortificants was calculated, it was found

that calcium dialyzability of both SCG and SCL in soy milk was lower than that analyzed in aqueous solution (Tables 1 and 4). Both calcium salts were about 18% dialyzable in soy milk (Table 4) though they were 36–37% dialyzable in aqueous solution (Table 1). The decrease in dialyzability of SCG and SCL when presented in soy milk was possibly due to the effect of dietary fiber, phytate, and oxalate present in the soy (Al-Wahsh *et al.*, 2005). The presence of soy protein and soluble polysaccharides in soy milk could also lowered the bioaccessibility of calcium salts (Lee and Rha, 1977; Yin *et al.*, 2012). Therefore, it could be stated that the solubility and dialyzability of SCG and SCL were affected by the food matrix of soy milk. Nevertheless, fortification of calcium in soy milk by using calcium salts prepared from shrimp shells could still increase total calcium and dialyzable calcium contents in the final product.

Incorporation of SCG and SCL in soy milk caused slight changes in physicochemical properties of the product but did not affect their sensory acceptability. Moreover, although the properties of both SCG and SCL were diminished by the complex food matrix of soy milk, fortification of SCG and SCL could still improve the total dialyzability of calcium of the product.

Table 4 Properties of calcium salts in calcium-fortified soy milks

Parameters	Control	SCGM	SCLM
Calcium content (mg/100 mL)	27.87±0.37 ^C	134.71±2.12 ^B	147.06±0.89 ^A
Calcium solubility (%)			
Whole sample	85.82±0.30 ^A	49.99±3.83 ^B	26.42±2.94 ^C
Fortificant only	-	41.39±4.74 ^A	12.16±3.65 ^B
Total calcium dialyzability (%)			
Whole sample	20.05±3.44 ^B	31.58±2.29 ^A	28.19±5.09 ^A
Fortificant only	-	18.86±1.06	18.27±0.56

Note: Values are means ± standard deviations of triplicates

SCGM: soy milk fortified with calcium gluconate prepared from shrimp shells; SCLM: soy milk fortified with calcium lactate prepared from shrimp shells

^{A,B,C} Means with different superscript within the same row are significantly different ($p \leq 0.05$).

4. Conclusion

The results from this study showed that calcium salts with appreciable yield and functional properties could be prepared from Pacific white shrimp shells. SCG and SCL exhibited high solubility and dialyzability. The successful application of SCG and SCL in soy milk could imply their great potential in food application. This is due to the fact that soybean

milk is a food system with complex matrix containing protein and substances that can bind with calcium and adversely affect the sensory and nutritional quality of the final product. Moreover, soy milk is a low acid food of which pH is in the range that the solubility of calcium salts is limited. The high solubility of both SCG and SCL across the pH range of food also suggests that it can be applied in liquid foods without causing turbidity or sediment.

Therefore, it is convincing that SCG and SCL can be used as calcium fortificants in a wide range of food products, especially those with more acidic pH, less complex matrix, and more transparent appearance, such as fruit juices, and other beverages. From all the results, it is plausible to conclude that calcium salts prepared from shrimp shells, in particular SCG and SCL, have potential applications as calcium fortificants in food.

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