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Effects of Chitosan Concentrations in the Chitosan-Alginate Composite on the Quality of Mulberry Caviar during Storage

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Abstract

This research aimed to study the effect of chitosan concentration in the chitosan-alginate composite on the texture profile analysis and sensory properties, the correlation between instrumental and sensory properties, and the qualitative change of mulberry juice caviar (MJC) during storage. MJC with chitosan-alginate composite containing chitosan concentrations of 0, 0.5, 1.0 and 1.5% by-weight (ALG 1.5, CHI 0.5, CHI 1.0 and CHI 1.5, respectively) were prepared by spherification process. The results showed that the MJC formed with CHI 1.5 had the best sensory acceptance scores, while which of CHI 0.5 was easier consumed with lower texture profile analysis (TPA) parameters. Meanwhile, the high positive correlation (> 0.7) between the instrumental textures and sensory scores implied that most consumers favoured MJCs with high springiness, gumminess, and chewiness. In addition, chitosan-alginate composite prolonged the shelf-life of MJCs better than alginate alone, especially refrigeration temperature. CHI 1.5 was the most effective against changes in MJC size and total phenolic content, while CHI 1.0 was the best for preserving total anthocyanin content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and microbiological quality. This product gives a special texture during chewing when adding it into jelly or similar products and it can also use to decorate foods.

Introduction

Mulberries (*Morus* spp.) derive from a fast-growing commercial plant and are cultivated around the world (Khan et al., 2013). There are many countries that cultivate the mulberry tree to focus on leaf production as a food source for silkworms (Butkhup et al., 2013). Based on its pleasant taste and nutritional value, mulberry fruit consumption has rapidly increased in recent years (Yang et al., 2016). The mulberry fruit contains various

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antioxidants, e.g. phenolics, flavonoids, and anthocyanins (Donno et al., 2015; Sánchez-Salcedo et al., 2015; Tchabo et al., 2015; Gültekin-Özgüven et al., 2015). Several studies have reported that mulberry fruit extract possesses a wide range of biological activities including neuroprotective, antioxidant, and antiobesity effects, as well as the potential to prevent cardiovascular disease, immunomodulation, and antitumor activity (Chen et al., 2005; Lim et al., 2013; Sarikaphuti et al., 2013; Seo et al., 2015; Raman et al., 2016).

It is well known that mulberry fruits can be consumed as both fresh and processed fruits. For the fresh fruit, its thin skin contributes to rot and loss during transportation and storage, mainly due to scratching and postharvest diseases. Freezing is commercially available for extending the shelf-life of mulberry fruits. However, the formation of ice in frozen fruits negatively affects the texture and disruption of cell compartments, leading to the release of chemically reactive components (Lim et al., 2004; Sirijariyawat & Charoenrein, 2012). This leads to a change in the natural texture of the mulberry fruit. Even after the texture of a frozen mulberry fruit has changed, it remains useful as an ingredient in various products including jam and other sweet recipes. One interesting alternative method to use the mulberry fruit is the creation of a new food product by transforming frozen fruits into alginate bead called fruit caviar. This leads to a new form of food. Moreover, fruit caviar can be an interesting food in order to promote essential nutrition for elderly people in the future.

Spherification is one of the culinary processes as a basic method used in recent years that is suitable for fruit caviar production. Fruit juice can be formed into the spherical shapes of caviar through the interfacial polymerization of alginate (polymers consist of 1,4-linked β-D-mannuronic and α-L-guluronic acid residues) solution after exposure to the solution of divalent ions like calcium, i.e., calcium chloride, calcium lactate, etc (Lee & Rogers, 2012; Comaposada et al., 2015; Rodríguez-Sánchez et al., 2017). Finally, a gradual gelation of edible membrane around fruit caviars occurs (Anal & Singh, 2007). Fruit caviar can be made in many sizes depending on the method, application, and consumer acceptance. Noticeably, most fruit caviar is prepared in small sizes of approximately 2 to 4 mm because of its function as a topping on sweet dishes or to add piquancy to a favourite dish. Fruit caviars can also create a special texture during chewing, to the products that added them into, such as jelly, gel, or other similar products. Clearly, the edible membrane formed by alginate helps to protect the storage loss of antioxidants existing in the fruit juice. Moreover, the addition of functions into the alginate edible membrane is increasingly challenging, especially in terms of how to improve the antimicrobial properties in order to raise the potential for extending the shelf-life of fruit caviar.

Chitosan is a natural linear polymer produced by the deacetylation of chitin, which is a major component of the exoskeleton of crustaceans, i.e., shrimp, lobsters, and crabs (Xia et al., 2011). Chitosan is non-toxic and possesses a positive charge under a low pH solution. It has been proven by prior research to possess the potential to extend the shelf-life of certain kinds of foods (Farajzadeh et al., 2016; Aquino et al., 2015; Vieira et al., 2016). Likewise, this polymer has been reported for the activity of inhibiting and killing *Staphylococcus aureus* at concentrations of 16 and 32 μg/mL, respectively (Laokuldilok et al., 2017). In addition to its antimicrobial activity, chitosan has been used as a coating agent for reducing the porosity of alginate gel surface (Gombotz & Wee, 1998). Low concentrations (2-4 g/m^3) of chitosan coating agent have also been used for shell-making on alginate and gelatin capsules (Abbaszadeh et al., 2014; Sashiwa & Aiba, 2004; Zhou et al., 1998). However, the combination of chitosan and alginate could lead to ionic crosslinking between the two polymers, which may be better for the aspect of structural bead stability.

The challenge faced by this research was to produce mulberry juice caviar (MJC) from chitosan-alginate composite. Based on the highest scores for sensory overall acceptance, one of the suitable alginate concentrations would be selected for preparing the chitosan-alginate composite with different chitosan concentrations for further use to prepare MJCs. The objectives of this research were to study the effect of chitosan concentration in the chitosan-alginate composite on the texture profile analysis and sensory properties, the correlation between instrumental and sensory properties, and the qualitative change of mulberry juice caviar (MJC) during storage. Additionally, the correlation and regression of the instrumental texture and sensory properties are discussed in this paper.

Materials and methods

1. Materials and chemicals

Frozen mulberry fruit was purchased from a local

supermarket in Chiang Mai Province, Thailand. The powders of sodium alginate and chitosan (90% deacetylation degree) were bought from Union Science, Thailand. 2,2-Diphenyl-1-picrylhydrazyl (DPPH radicals), tartaric acid, Folin–Ciocalteu's reagent, and gallic acid were purchased from Sigma-Aldrich Chemie GmbH, Germany. Potato dextrose agar, plate count agar, and peptone were purchased from Merck KGaA, Germany. Potassium chloride, sodium acetate, calcium chloride, sodium carbonate, glacial acetic acid, distilled water, and ethanol were purchased from RCI Labscan, Thailand.

2. Preparation of mulberry juice

Frozen mulberry fruits were used for preparing the mulberry juice. Firstly, the ice in mulberry fruits was left to thaw for 60 min at room temperature (25-30°C). Subsequently, the thawed fruits were blended into puree using a blender. The fraction of juice and residue was separated by filtering through a white cloth. The obtained juice was diluted to 50% using water before being investigated for physicochemical properties as follows: moisture, 88.86%; total solid content, 11.14 degree brix; total acidity, 5.5% citric acid equivalent; pH, 3.0; total phenolic content, 260.59 mg gallic acid equivalent (GAE)/L; total anthocyanin content, 120.26 mg/L; DPPH radical scavenging activity, IC_{50} = 2.25 µg/mL. The natural color of the mulberry juice was deep red, while its color parameters for L^* (lightness), C^* (chroma), $h°$ (hue angle) were 20.25, 4.20, and 80.59 respectively. The mulberry juice was used for preparing MJCs

3. Preparation of MJCs with chitosan-alginate composite

Preparation of MJCs with chitosan-alginate composite is shown in Fig. 1. Firstly, 1.5% (w/v) of alginate solution was prepared by dissolving 1.5 g of sodium alginate with distilled water (50 mL) under magnetic agitation at 80ºC for 15 min. After that, the alginate solution was left to cool (below 40ºC). Meanwhile, chitosan solutions with different concentrations were also prepared. Different amounts of chitosan powders (0.5, 1.0, and 1.5 g) were dissolved using 1.0% (v/v) acetic acid solution (25 mL) together with heating at 80°C for 15 min. The chitosan solutions were mixed with 1.5% alginate solution, after which the chitosan-alginate mixtures were stirred using an overhead stirrer (RW20, IKA Works, NC, USA) at 250 rpm for 5 min. According to the preliminary results for preparing the chitosan-alginate composite, the by-weight ratio of alginate to chitosan solution as 2:1 (w/w) was considered as the most suitable ratio based on the gel-forming properties and the mixture's viscosity. After that, mulberry juice (25 g) was added into the chitosan-alginate solutions and the weight of the mixtures were adjusted to 100 g using distilled water. These mixtures were homogenized at 12,000 rpm for 3 min using a homogenizer (Ultra-Turrax T25, IKA, Malaysia). The homogenized mixture was sonicated for 20 min to remove air bubbles prior to loading into a burette having an orifice size of 2 mm. The distance between the orifice end and the surface of 1.0% (w/v) calcium chloride solution was set at 5 cm. The mixture was allowed to form a liquid drop and dripped by gravity force for exposure with the calcium chloride solution. At this point, the MJCs were formed and immersed in a high-calcium ion environment for 30 min before being sieved through a 20-mesh sieve. The MJCs were washed with drinking water at least 5 times before being analyzed. All the obtained MJCs consisted of chitosan-alginate composite containing 1.5% (w/w) alginate and three different concentrations of chitosan (0.5, 1.0, and 1.5%, w/w). These MJCs were identified as CHI 0.5, CHI 1.0, and CHI 1.5, respectively. Several properties of the MJCs formed with chitosan-alginate composite were studied and compared to the MJC containing only 1.5% (w/w) alginate, called ALG 1.5.

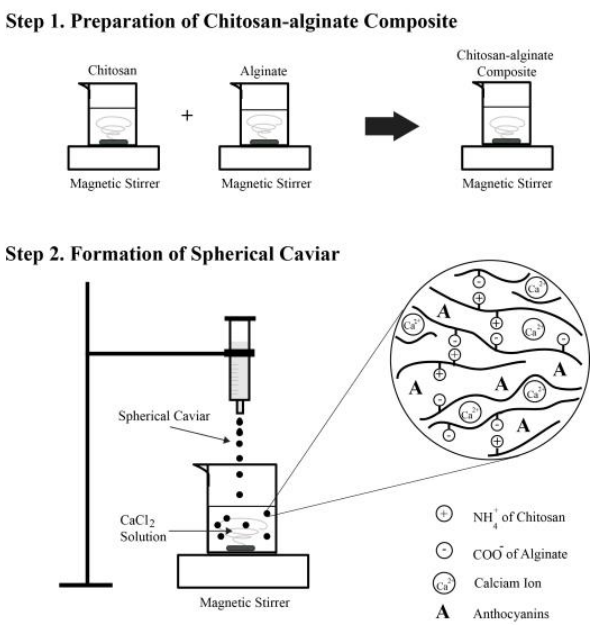

Fig. 1 The scheme shows the preparation of MJCs with chitosan-alginate composite

4. Production yield

This parameter was calculated as the ratio of the mass of collected beads (m_s) per the mass of all raw materials (m_i), as given in below equation.

$$\text{Production yield (\%)} = \left(\frac{m_s}{m_i}\right) \times 100$$

5. Determination of total phenolic content

Total phenolic content was determined using a Folin–Ciocalteu assay according to the method of Xu & Chang (2008) with some modification, while pure, authentic garlic acid was used as a standard phenolic for creating the calibration curve. Sample (50 µL), distilled water (3,000 µL), 250 µL of Folin–Ciocalteu's reagents, and 7% $NaCO_3$ (750 µL) were mixed into a 15-mL test tube and incubated for 10 min at ambient temperature. After that, distilled water (950 µL) was immediately added followed by vigorous mixing. The mixture was placed in a dark room for 2 h under ambient temperature before the absorbance of each sample was measured at 765 nm against a blank. Finally, total phenolic content was reported as milligram gallic acid equivalent per 100 g dry basis (mg GAE/100 g d.b.) by calibrating with a standard linear plot of gallic acid with linearity range of 50 to 1000 µg/mL ($R^2 = 0.992$).

6. Determination of total anthocyanin content

Total anthocyanin content was determined using the pH differential method described by Giusti & Wrolstad (2001). Briefly, the sample was diluted using potassium chloride buffer (pH 1.0) and sodium acetate buffer (pH 4.5). The absorbance of both sample-buffer mixtures was read at 510 nm (A_{510}) and 700 nm (A_{700}) by a UV-visible spectrophotometer (Genesys 10, Thermo Scientific, NY, USA), which required calculating the absorbance difference (A_{diff}). The total anthocyanin content was calculated as milligram cyanidin-3-*O*-glucoside per liter (mg C3G/L) using the below equation and reported as mg C3G per 100 g d.b.

Where, MW is the molecular weight of C3G (449.39 g/mol). DF is the dilution factor, while the molar absorptivity (ε) is 26,900 l/mol.cm and the path length (l) is 1.0 cm.

7. DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method described by Gülçin et al. (2006) with some modifications. Firstly, the sample (1.0 mL) was added into 99% ethanol solution (3.0 mL) of 0.1 mM DPPH solution (the absorbance at 517 nm is 0.40 ± 0.02). After that, the mixture was shaken vigorously for 30 sec and left to stand in the dark for 30 min at ambient temperature. Then, the absorbance of the sample (A_{sam}) was read at 517 nm against a blank by using a UV-visible spectrophotometer (Genesys 10, Thermo Scientific, NY, USA). For the control solution, the sample was replaced by distilled water and the absorbance of control (A_{con}) was measured against a blank. The percentage of DPPH radical discoloration of the sample was calculated using the following equation:

Finally, the antioxidant concentration inhibiting 50% radicals (EC_{50}) was calculated and reported as the radical scavenging activity

8. Microbiological analysis

Serial dilutions (10^{-1},10^{-2}, and 10^{-3}) of the sample were prepared with sterile peptone water. Volumes of each dilution sample (1.0 mL) were poured into 3 plates. Total plate counts were enumerated by using the pour plate technique on plate count agar (PCA). Incubation was done at 37°C for 48 h. Total yeast and moulds were enumerated using the surface plate technique on potato dextrose agar (PDA), plus 10% tartaric acid. Incubation for total yeast and mould counts was done at 37°C for 5 days. Each test was performed in triplicate. The results were expressed as colony-forming units (CFU) per mL.

9. Texture profile analysis

Texture profile analysis was done according the method described by Suebsaen et al. (2019) with slight modification. A texture analyser (TA-XT.Plus, Stable Micro Systems, Surrey, UK) connected to a compression probe of P/50 with 50 mm diameter was used, in which 75% strain and 2 mm/sec speed were set to determine the texture properties. The sample was stored in a cool and dry place at ambient temperature (25 ± 1°C) for 30 min before being measured. Fifty replicates were

$$A_{diff} = (A_{510} - A_{700})\ \text{pH 1.0} - (A_{510} - A_{700})\ \text{pH 4.5}$$

$$\text{Total anthocyanin content (mg C3G/L)} = \left(\frac{\text{Adiff} \times \text{MW} \times \text{DF} \times 1000}{\varepsilon \times l}\right)$$

investigated for each sample and the data for 6 texture profiles was collected, including hardness (N), adhesiveness (g.sec), cohesiveness, springiness, chewiness (g), and gumminess (g).

10. Sensory evaluation

Sensory properties were evaluated by 50 consumers using a 9-point hedonic scaling test. The samples were tasted by the consumers, who scored them using 4 parameters including color, flavor, taste, and overall acceptability.

11. Physicochemical analysis

Moisture and titratable acidity were determined according to the method described by AOAC (2000). The *L*C*h°* color space was used to describe the color properties, which were measured using a colorimeter (CR-410, Konica Minolta, Japan). Fruit caviar size was measured using a digital Vernier caliper. Bulk density was investigated according to the method of Jinapong et al. (2008), in which samples were loaded into a 10-mL cylinder to the 10-mL mark and weighed. Bulk density was calculated by dividing the weight with the volume of the sample at the 10-mL mark.

12. Changes in fruit caviar quality under different storage temperatures

To mimic the actual storage conditions, a sample (20 g) was placed into a glass clear bottle, which was stored in a refrigerator and at room temperature. All samples were stored for 12 days and analysed for their physico-chemical properties (size, total phenolic content, total anthocyanin content, and DPPH free radical scavenging activity) every 3 days. Importantly, changes in the growth of bacteria and fungi as yeasts and moulds were also investigated.

13. Statistical analysis

All analyses were performed in triplicate. Data was analysed using IBM SPSS Statistics for Windows version 20 (IBM, Armonk, NY, USA.). The differences between values were considered significant at $p \leq 0.05$. The averages were calculated by Duncan's new multiple range test. Pearson's correlation coefficients were applied to explain the relationship between parameters. The analysis was also executed using IBM SPSS Statistic version 20 software.

Results and discussion

1. Physicochemical properties of MJCs with chitosan-alginate composite

Mulberry juice was mixed with chitosan-alginate composite in order to create the MJC. All MJCs exhibited a spherical shape with different sizes depending on chitosan concentration. It is well known that a spherical shape is normal for alginate bead products produced by using the extrusion-dipping technique (Lee et al., 2006; Chan et al., 2011)

As given in Table 1, the yield of MJCs was in the range of 73.99% to 82.75%, in which CHI 1.5 was the chitosan-alginate composite providing the highest yield ($p \leq 0.05$). All of the MJCs were similar in size between 3.27 and 3.82 mm and tended to be smaller with higher chitosan concentrations. All the MJCs had bulk density in the range of 0.68 to 0.76 g/cm^3, which was higher in bulk density with smaller MJC size. These MJCs were very juicy with high moisture contents (95.28-96.50%). For analysis of total phenolic and total anthocyanin contents, 1 mg/mL aqueous MJC extract was used for this section. The result was found that these antioxidants tended to have lower content when the chitosan concentration was higher. In the case of antioxidant activity, DPPH radical scavenging activity of all MJCs (1 mg/mL aqueous MJC extract) was in the range of 110.97 to 119.30 µg/mL, which was related to the total phenolic and total anthocyanin contents for all. In addition, it was obvious that the difference in the MJC's color parameters depended on the chitosan concentrations. Noticeably, the *L** and *C** values were significantly enhanced with increasing chitosan concentrations ($p \leq 0.05$). Meanwhile, the h values of MJCs varied depending on each concentration of chitosan ($p \leq 0.05$) and expressed the different shades of red to dark red. According to the results, it was probable that the network structures of MJCs prepared with CHI 1.0 and CHI 1.5 were stronger when compared to those of CHI 0.5 and ALG 1.5.

With the stronger structure, less water inside MJCs was released due to the dense crosslinking between the interactions of both alginate-Ca^{2+} and alginate-chitosan. As a result, the strength of these interactions held each of near polymer chains together, becoming MJCs with smaller size, which also resulted in greater bulk density. However, the increased strength of the MJC structure could cause difficulty for the antioxidants in order to be leached away by the solvent during extraction, leading to lower contents of phenolics and anthocyanins as well as lower antioxidant activity.

2. Instrumental texture properties and sensory score of MJCs with chitosan-alginate composite

In this study, the texture profile analysis (TPA) of

MJCs was investigated in order to mimic human mastication and performed using a two-bite compression test to explain the textural properties during oral processing (Chen, 2009; Sharma et al., 2017). The results of the instrumental texture investigation for all MJCs are summarised in Table 2. A significant difference was clearly observed between ALG 1.5 and CHI 0.5 ($p \leq 0.05$). Most TPA parameters tended to have a lower value, except for adhesiveness. For hardness, springiness, gumminess, and chewiness, all parameters tended to have higher magnitudes when the percentage of chitosan concentrations increased from 0.5 to 1.5. Meanwhile, the adhesiveness of MJCs showed a higher negative value depending on the increasing concentrations of chitosan. A significant difference in cohesiveness was found in the MJC formed with CHI 1.5, showing the lowest value ($p \leq 0.05$).

When the switching of ALG 1.5 towards CHI 0.5 was performed, the negligible chitosan fractions might interfere with the network structure formation through the crosslinking of alginate and divalent calcium ions, leading to the significant soft-shell structure of spherical MJCs. For the hardness value, it mimics the highest force used to bite down into food during the first bite. With greater fractions of chitosan, MJCs having greater hardness were formed. Similarly, a strong network structure was created through electrostatic interaction between the residue of the chitosan's ammonium ion (NH_4^+) and the opposite alginate's carboxylate (COO^-), which were more intensified with increasing chitosan fractions (Lin et al., 2005; Pasparakis & Bouropoulos, 2006). As an interval of the first bite, the work required to overcome the attractive forces between the food and instrumental probe (mimicking the human teeth) is defined as adhesiveness (Tunick, 2000). According to the adhesiveness results, more adhesive MJCs were observed with those of greater chitosan concentrations, but still lower than the MCJ without chitosan. After the first bite, the springiness values for all MJCs with chitosan combination below the MJC without chitosan implied that the chitosan-alginate composite could strongly contribute to the ease of structural deformation during consumption. Surprisingly, the MJC formed with CHI 1.5 had the lowest cohesiveness, indicating the decreased strength of the MJC's internal bonds making it easier for disintegration into fragments while swallowing (Rosenthal, 1999). In contrast, MJCs formed with CHI 1.5 exhibited the highest values for both gumminess and chewiness, which implied that more energy was needed to chew the MJCs and make them ready for swallowing compared to that of ALG 1.5, CHI 0.5, and CHI 1.0. The lowest values for both gumminess and chewiness were found in the MJC formed with CHI 0.5, indicating that it was easily disintegrated with minimal chewing effort.

The sensory evaluation results for prepared MJCs are shown in Table 2. It was apparent that different chitosan concentrations did not affect the sensory score in terms of sourness and overall acceptance ($p > 0.05$). For appearance, color, sweetness, and texture, all samples tended to have similar scores. Noticeably, the color and texture scores for the MJC formed with CHI 0.5 were significantly lower than other concentrations ($p \leq 0.05$). This might be attributable to the instability of the bead structure (Huang & Lin, 2017; Orive et al., 2006), leading to the release of liquid to the exterior of beads in the interval of being weighed for testing a sensory attribute. As a result, the bead structure collapsed and the shriveled texture was more intensified for a darker color. This result was not acceptable among the consumers.

Table 1 Properties of MJCs prepared with chitosan-alginate composite

Chitosan concentrations (%, w/w)	Yield (%)	Size (mm)	Bulk density (g/cm^3)	Physicochemical properties						
				MC^{ns} (%)	TPC (mg GAE/100 g d.b.)	TAC (mg C3G/100 g d.b.)	EC50 (µg/mL)	*L**	*C**	*h°*
ALG 1.5	75.45^{bc}±2.42	3.82^{a}±0.04	0.68^{b}±0.03	95.35±0.00	3718.64^{a}±77.60	440.70^{a}±43.31	110.97^{b}±6.53	24.63^{c}±0.27	4.53^{c}±0.20	35.67^{a}±0.79
CHI 0.5	73.99^{c}±0.64	3.63^{b}±0.15	0.70^{b}±0.02	95.47±0.51	3360.22^{ab}±267.82	383.22^{b}±10.98	109.28^{b}±2.96	30.56^{b}±0.29	7.01^{b}±0.36	4.52^{d}±0.77
CHI 1.0	78.19^{b}±0.62	3.28^{c}±0.16	0.74^{a}±0.01	95.30±0.65	3333.33^{ab}±229.68	370.76^{b}±21.17	119.25^{a}±2.98	33.64^{a}±0.93	8.90^{a}±0.70	9.79^{b}±0.76
CHI 1.5	82.75^{a}±2.48	3.27^{c}±0.24	0.76^{a}±0.01	94.99±0.04	2912.19^{b}±388.00	342.50^{b}±16.59	119.30^{a}±2.43	33.74^{a}±0.42	8.51^{a}±0.93	7.11^{c}±0.80

Remark: MC; Moisture content, TPC; Total phenolic content, TAC; Total anthocyanin content, EC_{50}; the effective concentration of antioxidants that can scavenge 50% of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals when tested by DPPH radical scavenging activity.
Data indicate the average (standard deviation) of three replicates (n = 3), except for size (n = 30).
Different superscript letters in the same column indicate significant difference at $p \leq 0.05$.
^{ns} indicates non-significant difference ($p > 0.05$).

Table 2 The instrumental texture properties and sensory attributes of MJCs with chitosan-alginate composite

	ALG 1.5	CHI 0.5	CHI 1.0	CHI 1.5
Instrumental texture properties				
Hardness (N)	1.45^{c} ±0.05	1.23^{d}±0.05	1.75^{b}±0.06	3.23^{a}±0.08
Adhesiveness (g.sec)	-1.38^{d} ±0.19	-0.35^{a}±0.04	-0.63^{b}±0.03	-1.15^{c}±0.05
Springiness	0.47^{a}±0.02	0.31^{d}±0.01	0.37^{c}±0.01	0.40^{b}±0.01
Cohesiveness	0.51^{a}±0.00	0.47^{a}±0.04	0.50^{a}±0.01	0.31^{b}±0.01
Gumminess (g)	75.59^{c}±2.98	59.31^{d}±7.21	89.14^{b}±1.38	102.99^{a}±2.49
Chewiness (g)	35.16^{b}±2.03	18.37^{c}±2.23	32.39^{b}±0.75	41.18^{a}±0.94
Sensory attributes				
Appearance	7.94^{ab}±0.59	7.11^{b}±0.38	8.06^{a}±0.42	8.44^{a}±0.35
Color	7.39^{a}±0.72	5.94^{b}±0.59	7.50^{a}±0.29	7.67^{a}±0.17
Sweetness	4.67^{b}±0.17	5.22^{ab}±0.25	5.28^{ab}±0.38	5.44^{a}±0.59
Sourness^{ns}	5.50±0.87	6.33±0.17	5.61±0.69	5.94±0.77
Texture	8.28^{a}±0.51	6.33^{b}±0.33	8.11^{a}±0.63	8.17^{a}0.29
Overall acceptance^{ns}	7.06±0.25	6.56±0.42	7.06±0.42	7.33±0.44

Remark: Data indicate the average standard deviation of fifty replicates. Different superscript letters in the same row indicate significant difference at $p \leq 0.05$.
^{ns} indicates non-significant difference ($p > 0.05$).

3. Correlation for the relationship between the instrumental texture and sensory properties of MJCs with chitosan-alginate composite prepared using different chitosan concentrations

As seen in Table 3, the instrumental texture properties including hardness, adhesiveness, springiness, gumminess, and chewiness were correlated with the sensory properties including appearance, color, texture, and overall acceptance. In this work, the correlation coefficient (r) equal to 0.7 or higher was considered a high relationship. The correlation results of all the MJCs showed that higher springiness contributed to a higher texture score ($r = 0.711$, $p \leq 0.01$). Meanwhile, high correlation coefficients were found between the gumminess and sensory scores of both appearance ($r = 0.798$, $p \leq 0.01$) and color ($r = 0.824$, $p \leq 0.01$). Likewise, chewiness was highly correlated with appearance ($r = 0.762$, $p \leq 0.01$), colour ($r = 0.863$, $p \leq 0.01$), and texture ($r = 0.825$, $p \leq 0.01$). According to the results, the high positive correlation between the instrumental textures and sensory scores implied that most consumers favoured MJCs with high springiness, gumminess, and chewiness. Apparently, small-size MJCs with high springiness, gumminess, and chewiness enhanced the joyful feeling when eating, although MJCs could be swallowed easily. Additionally, more chewing was required to be confident about safe swallowing. However, food with greater springiness and chewiness requires more chewing (Wee et al., 2018). Thus, this product may not be appropriate for older adults if considering the aspect of energy usage for chewing to get the bolus for safe swallowing. It is suggested that MJCs are of greater benefit as toppings for a favourite dish because they are rich sources of antioxidants.

Table 3 Pearson's correlation coefficients for the relationship between the instrumental texture properties and sensory attributes of MJCs with chitosan-alginate composite

Instrumental Texture profiles	Sensory attributes					
	Appearance	Color	Sweetness	Sourness	Texture	Overall
Hardness	0.640*	ns	ns	ns	ns	ns
Adhesiveness	ns	-0.644*	ns	ns	-0.662*	ns
Springiness	ns	0.595*	ns	ns	0.711**	ns
Cohesiveness	ns	ns	ns	ns	ns	ns
Gumminess	0.798**	0.824**	ns	ns	0.698*	0.635*
Chewiness	0.762**	0.863**	ns	ns	0.825**	0.642*

Remark: * Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed). ns is not significant ($p > 0.05$).

4. Physicochemical and microbiological changes in MJCs under different storage temperatures

4.1 Changes in physicochemical quality

Change in the size of MJCs is one of the most important indicators for determining the stability of alginate bead product, which can influence the texture and sensory properties. As seen in Fig. 2, MJCs stored under both temperature ranges tended to be smaller in size throughout the storage period. After storage, the storage of MJCs formed with ALG 1.5, CHI 0.5, CHI 1.0, and CHI 1.5 under a refrigeration temperature ranging between 5 and 10°C showed they were shrunken by 5.2, 4.3, 3.2, and 2.1%, respectively, when compared with the initial size. Storage under ambient temperature ranging between 25 and 30°C resulted in greater shrinkage of the spherical shape by 10, 6.4, 4.7, and 3.4%, respectively. Apparently, the higher storage temperatures negatively affected the MJC size through the encouragement of mass transfer of water. MJCs stored in a refrigerator were more stable than those stored at ambient temperature. Interestingly, using a greater proportion of chitosan than alginate in the chitosan-alginate composite contributed to the stability of the MJC size via less shrinkage of the sphere, likely as the result of releasing water to the exterior of beads. With the high chitosan fraction, chitosan and alginate film layer could reduce the diffusion rate of water through the crosslinking of chitosan with alginate by intermolecular bonds (e.g., electrostatic and hydrogen bonding). The network of this bonding might be increasingly dense with the increasing of chitosan concentrations, leading to fewer changes in MJC size.

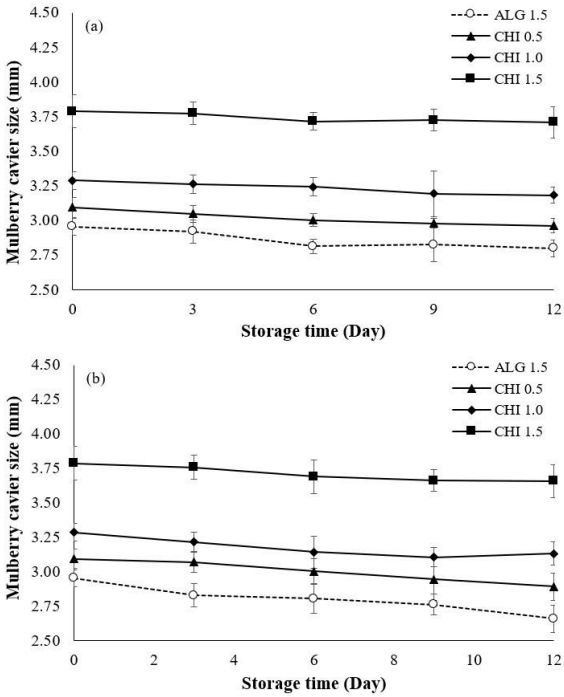

Fig. 2 Changes in size of MJCs formed with chitosan-alginate composite under (a) refrigerator (5–10°C) and (b) ambient temperatures (25–30°C)

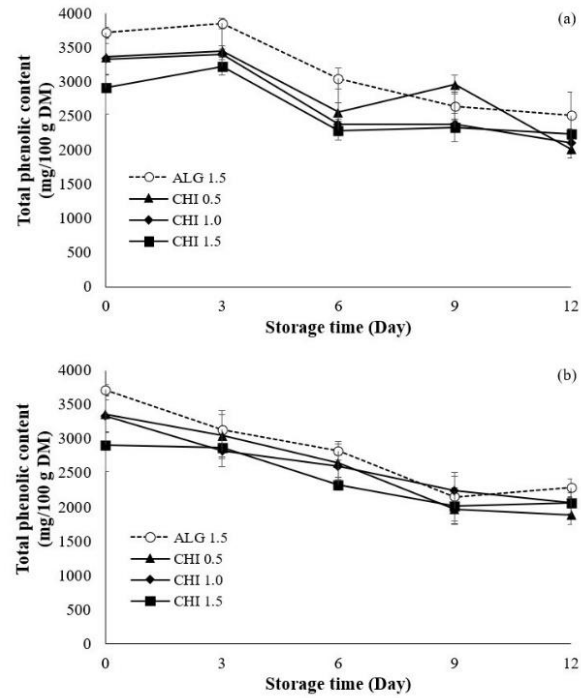

Fig. 3 Changes in total phenolic content of MJCs formed with chitosan-alginate composite under (a) refrigerator (5–10°C) and (b) ambient temperatures (25–30°C)

Total phenolic content is one of the most important indicators for explaining the content of hydrophilic antioxidants in the example. As seen in Fig 3, total phenolic contents decreased throughout the storage time. Under chilling temperature, total phenolic contents of all MJCs were reduced by 32.53, 40.00, 36.83 and 23.08%, respectively. A greater loss in percentage of phenolics was found when MJCs were stored under ambient temperature by 38.54, 44.00, 38.17, and 29.23%, respectively. Seemingly, the higher storage temperatures negatively affected the MJC size through the encouragement of the mass transfer of water. MJCs stored in a refrigerator were more stable than those stored at ambient temperature, which could activate phenolic degradation, as proven by several previous research (Serea et al., 2014; Moldovan et al., 2016; Galani et al., 2017). The MJC formed with CHI 1.5 could retard the change of total phenolic content greater than others. In this case, the strong network structure formed by high chitosan concentrations might have an important role as a barrier for reducing the negative effects associated with the temperature on phenolic compounds.

Total anthocyanin content is an important indicator for food products containing a rich source of anthocyanins, which is an antioxidant found in abundance in black mulberry fruit (Jiang & Nie, 2015). According to Fig. 4, the total anthocyanin contents of all MJCs stored under refrigeration temperature range gradually decreased when increasing the storage time (Fig. 4 (a)). Evidently, an undesirable effect was observed in all MJCs stored under ambient temperature (Fig. 4 (b)). Their total anthocyanin contents were dramatically decreased and disappeared around Day 6 for MJCs formed with all the chitosan-alginate composite of CHI 0.5, CHI 1.0, and CHI 1.5, and Day 9 for the one formed with ALG 1.5 (without chitosan). Degradation under high ranges of storage temperature implied that the storage temperature directly affected the total anthocyanin contents. It is widely recognised that anthocyanins are unstable and extremely sensitive to high temperature conditions (Ersus & Yurdagel, 2007; Kırca et al., 2007; Laokuldilok & Kanha, 2017). In addition, the change of total anthocyanin content in MJCs also depended on chitosan concentration. During storage, the MJC formed without chitosan had lower anthocyanin loss than that containing chitosan. With the ionic interaction between flavylium cations and ammonium ions, it is able to form a repulsive force (Xie et al., 2001), resulting in the instability of the

anthocyanin molecule. Therefore, chitosan might not be suitable for preparing alginate beads containing anthocyanins.

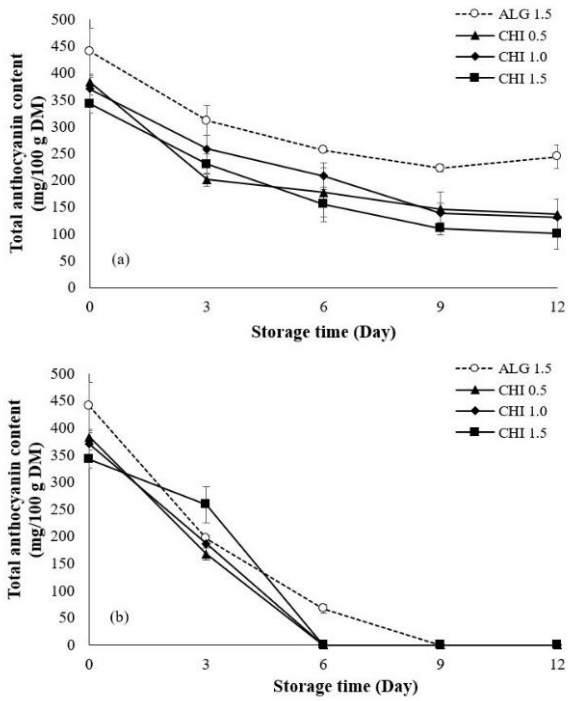

Fig. 4 Changes in total anthocyanin content of MJCs formed with chitosan-alginate composite under (a) refrigerator (5–10°C) and (b) ambient temperatures (25–30°C)

DPPH radical scavenging activity is used to explain the antioxidant activity. The results of the change in DPPH radical scavenging activity of MJCs stored at different storage temperature ranges were reported as the effective concentration of antioxidants able to scavenge 50% of all DPPH radicals, also called the EC_{50} value (Fig. 5). Before storage, MJCs prepared by ALG 1.5, CHI 0.5, CHI 1.0, and CHI 1.5 exhibited EC_{50} values of 112.80, 154.72, 149.38, and 182.19 µg/mL, respectively. After storage, all MJCs tended to have a higher EC_{50} value. The EC_{50} values of all MJCs stored under refrigeration temperature range were 124.01, 155.56, 171.02, and 230.94 µg/mL, increasing by 9.94, 0.54, 14.49 and 26.75%, respectively. Likewise, higher EC_{50} values, e.g. 277.66, 235.44, 342.11, and 331.12 µg/mL, were found for storage at higher temperature, increasing by 146.25, 52.17, 129.02 and 81.74%, respectively. According to the results, CHI 0.5 and CHI 1.0 were the chitosan-alginate composites providing MJCs with the strongest antioxidant activity under ambient and refrigerator temperatures, respectively. Possibly, the porosity and strength of the MJC's surface might provide important contributions. In addition, changes in antioxidant activity were found as a function of storage time and temperature. A higher ambient temperature than refrigeration temperature also contributed to the larger degradation of antioxidants, which could be degraded thermally. With the results of EC_{50} values among the two temperatures, the differences might be attributable to three cases including (1) total phenolic contents, (2) types of phenolics, and (3) the association of antioxidants with other molecules, *i.e.* chelation of metallic ions with anthocyanins, leading to the loss of the proton donation group.

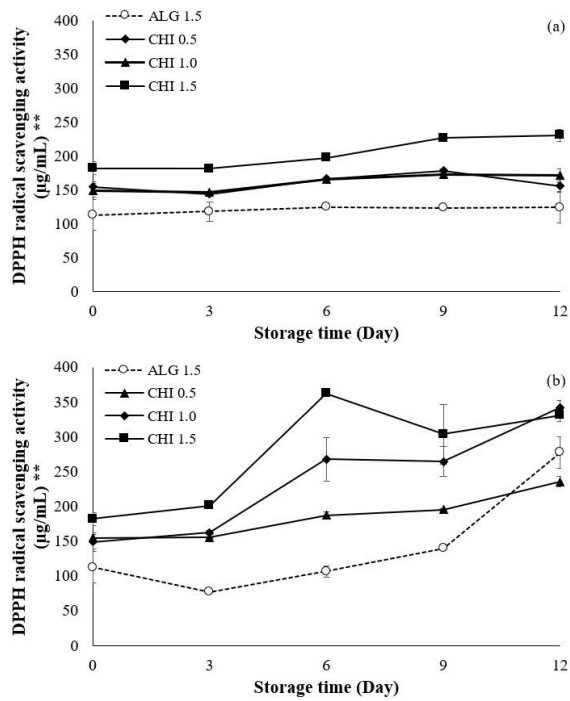

Fig. 5 Changes in DPPH radical scavenging activity of MJCs formed with chitosan-alginate composite under (a) refrigerator (5–10°C) and (b) ambient temperatures (25–30°C). **Reported as EC_{50} in the unit of µg/mL

4.2 Changes in microbiological quality

As given in Table 4, the counts for total bacteria and total yeasts as well as moulds at the initial storage time were lower than 1 CFU/mL, which confirmed that the freshly prepared MJCs were safe for consumption. At the end of storage, the microbiological changes of MJCs could be observed clearly under different storage temperatures. Bacteria could clearly grow under refrigeration temperature less than ambient temperature.

Total bacteria count below 1 CFU/mL throughout 12 days of storage was found in all MJCs with chitosan-alginate composite stored in a refrigerator. MJCs with chitosan for all concentrations could retard bacterial growth better than MJCs without chitosan under the same storage temperature. The change of total yeast and mild count during storage was found to be different from the total bacteria count, likely due to the greater growth of yeast and mild compared to bacteria (Table 4). Yeast and mild grew under a refrigeration temperature less than ambient temperature. Under the same storage temperature, MJCs with all chitosan concentrations retarded the increase of total yeast and mild count better than MJCs without chitosan. Especially for the MJC formed by CHI 1.0, the best microbiological quality resulted. This result was possible to be relevant to the weak structure of MJC with CHI 0.5 which might lead to the relaxation of the bead structure and opening of the chitosan chain's end. Thus, the growth of yeast and mould were easily inhibited by the action of chitosan during days 6 to 9 of storage before the mechanism of microbial growth would be changed back to be controlled by a function of temperature on days 9 to 12, due to the excessive microbial number. With the higher chitosan concentration, so that MJC with CHI 1.0 had the greater ability to inhibit yeast and mould compared to MJC with CHI 0.5. Meanwhile, dense cross-linking interaction in MJC with CHI 1.5 caused the difficulty of composite hydrocolloid to relax on the day 3 to 6, thus the number of the ammonium cation of chitosan for binding to negatively charged phospholipids of fungi was expected to be less than those of the other chitosan concentrations. When this bead can not inhibit microorganisms effectively, microbial growth is better even though it was considered at the same temperature range. The antimicrobial mechanism of MJCs with chitosan-alginate composite is attributable to the ionic interaction between the chitosan's cation and the bacteria's anion, resulting in increased permeability and intracellular leakage along with the loss of the function of nutrient transportation (Liu et al., 2004). The antibacterial activity of chitosan has been reported by several research (Zhang & Zhu, 2003; Liu et al., 2006; Laokuldilok et al., 2017). With the similar mechanism of chitosan against bacterial growth, the antifungal activity has been reported on by many previous research (Peña et al., 2013; Jaimun et al., 2018).Therefore, chitosan-alginate composite could prolong the shelf-life of MJCs better than using alginate alone.

Table 4 Microbiological change of MJCs with chitosan-alginate composite under two storage temperature ranges

Temperature		Storage time (Day)				
	(ºC)	0	3	6	9	12
Total bacterial counts						
ALG 1.5	4–10	< 1	3.33	3.33	13.33	1833.33
	25–30	< 1	116.67	1100	> 2000	> 2000
CHI 0.5	4–10	< 1	< 1	< 1	< 1	< 1
	25–30	< 1	< 1	10	866.67	> 2000
CHI 1.0	4–10	< 1	< 1	< 1	< 1	< 1
	25–30	< 1	< 1	< 1	10	16.67
CHI 1.5	4–10	< 1	< 1	< 1	< 1	< 1
	25–30	< 1	< 1	6.67	980	> 2000
Total yeast and mould counts						
ALG 1.5	4–10	< 1	560.00	636.67	703.33	2333.33
	25–30	< 1	> 2000	> 2000	> 2000	> 2000
CHI 0.5	4–10	< 1	6.67	33.33	73.33	806.67
	25–30	< 1	3.33	16.67	230	> 2000
CHI 1.0	4–10	< 1	< 1	6.67	20.00	70.00
	25–30	< 1	< 1	16.67	53.33	63.33
CHI 1.5	4–10	< 1	< 1	6.67	46.67	86.67
	25–30	< 1	6.67	10.00	946.67	> 2000

Remark: Data show the change in amounts of microbials (CFU/mL)

Conclusion

Mulberry juice caviar prepared from chitosan-alginate composite with different concentrations of chitosan had different characteristics in terms of physicochemical, textural, and sensory attribute properties. CHI 1.5 was the chitosan-alginate composite providing the highest yield. Chitosan concentrations importantly contributed to all physicochemical properties, which were possibly related to the dense crosslinking between the interactions of both alginate-Ca^{2+} and alginate-chitosan. Such interaction also influenced all TPA parameters. The negligible chitosan fractions might interfere the network structure formation of MJCs with CHI 0.5, leading to the softer shell and spherical structure. MJCs formed with CHI 0.5 were easier for structural deformation while eating based on the lower magnitude of hardness, adhesiveness, springiness, gumminess, and chewiness. However, CHI 1.5 possessed the best sensory acceptance scores. On the other hand, higher instrumental texture properties for springiness, gumminess, and chewiness resulted in higher sensory attributes of texture, appearance, and colour ($r > 0.711$, $p \leq 0.01$), which implied that most consumers favoured MJCs with high springiness, gumminess, and chewiness. Linear regression analysis confirmed the significant linear models ($p \leq 0.001$) with a high R^2 (>0.6) for the gumminess and chewiness as well as the sensory properties of colour and texture. In addition, the storage of MJCs under refrigeration

conditions could greatly preserve their physicochemical and microbiological properties. CHI 1.5 showed the highest effectiveness against changes in MJC size and total phenolic content, while CHI 1.0 was the most effective for total anthocyanin content and DPPH free radical scavenging activity as well as the preservation of microbiological quality. Thus, it could be confirmed that chitosan-alginate composite has the ability to prolong the shelf-life of MJCs better than using only alginate. For commercial applications, MJC is of natural raw material and it can be used to add nutrition to some products such as jelly, gel, or similar products, along with the decoration of foods and giving of a special texture during chewing. Further research should be studied more on the use of the spherification process to produce a nutrient-packing sphere for a culinary application.

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