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Stability study of royal jelly in alginate-pectin beads

Muhammad Fitri Azhar[1], Nurul Ain Mohammad Hamdi[1], Muhammad Salahuddin Haris[1,2,*]

ABSTRACT

Introduction: The stability of royal jelly (RJ) beads is a critical aspect to ensure the product is safe, efficacious, and possesses an acceptable quality for consumers. This study aims to establish storage duration and condition to ensure the stability of RJ in alginate-pectin beads.

Methods: In this study, two types of packaging material have been chosen, namely polyethylene (PET) opaque bottles and glass containers. Samples of RJ beads were stored in four different storage conditions that include freezer, laboratory environment, real-time (30 °C, 75% RH) and accelerated (40 °C, 75% RH) stability chambers at different sampling points (0, 14 days, 1 month, 3 months). The RJ beads were characterised for physicochemical properties and 10-hydroxy-2-decenoic acid (10-HDA) content in the RJ-encapsulated beads.

Results and discussion: The colour of RJ beads in the refrigerator remained whitish grey throughout the study but colour change in room temperature (laboratory) is observable starting from 1-month time point. The particle size of RJ beads stored in accelerated stability chamber had a decreasing pattern with significance ($p < 0.05$) for both different types of storage container. No significant difference ($p > 0.05$) between sphericity coefficient values of RJ beads stored in glass and PET container in refrigerator, room temperature and real-time stability chamber at 0 month and 14-day time point. Constant peaks of 10-HDA appeared for RJ samples stored in all storage conditions at 14-day time point. Nonetheless, at 1-month and 3-months, peak area starts to show decreasing trend for beads stored in room temperature, real time and accelerated stability chambers.

Conclusion: The study showed that the RJ beads exhibited convincing stability for 3 months.

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Introduction

Stability can be defined as the ability of a pharmaceutical or nutraceutical product to withstand physical, chemical, or microbiological changes or decomposition when exposed to various environmental conditions (Association of Southeast Asian Nations, 2013). In general, the purpose of stability testing is to provide the evidence on the effect of time on the product under the influence of a variety of environmental factors, such as temperature, humidity, and light to establish a shelf life or expiry date for the pharmaceutical product hence recommending appropriate storage conditions (Association of Southeast Asian Nations, 2005).

The stability of a pharmaceutical product is complex, often being dependent on multiple physical, chemical, and microbiological factors that may or may not interact with each other (Aulton, 2018). In this stability study, two types of packaging material have been chosen, namely polyethylene (PET) opaque bottles and glass containers. Plastic and glass are the most used primary packaging materials. Glass is widely used for packaging pharmaceuticals because of its excellent barrier properties, relative inertness, and compatibility with pharmaceuticals (Polshettiwar, 2021). On the other hand, the growing use of plastics as a pharmaceutical packaging material is because of the significant advantages and consumer preference. Plastics are lightweight and shatterproof. Plastics are also easily shaped and sealed, which gives great versatility in the design of the pack (Andjelković et al., 2021).

Stability study was executed to investigate the characteristics of royal jelly (RJ) encapsulated in alginate-pectin beads in different storage conditions with varying temperatures and relative humidity (RH). In general, a drug product should be evaluated under distinctive storage conditions that test its thermal stability and, if applicable, its sensitivity to moisture or potential for solvent loss (Association of South East Asian Nations, 2005).

Alginate-pectin beads filled in PET opaque plastic bottles and glass containers were randomly sampled and later were kept in refrigerator (2-8 °C), room temperature (25 °C), a real time (30 °C and 75% RH) and an accelerated (40 °C and 75% RH) stability chambers. At different stability time points (0-day, 14-days, 1-month and 3-months), samples were taken out from each different storage condition for later characterisation. In this stability study, all the sample were characterised for physical appearance, particle size, sphericity coefficient, microscopic morphology, 10-HDA content and compression testing.

A suitable storage condition is crucial to ensure the quality of RJ as its bioactivities are primarily influenced by its storage condition. The physical and chemical constituent of RJ can change when it is stored improperly resulting in the loss of its functional properties. Therefore, it is important to evaluate the effect of storage conditions on the quality of RJ.

Materials

The RJ beads filled in PET opaque bottle and clear glass bottle were randomly sampled and stored (n=30) into four different storage conditions: in refrigerator, laboratory, accelerated and real time stability chambers. The KBF 240 accelerated and Max 1400 real time stability chambers were provided from Capromax (Selangor, Malaysia).

Methodology

The stability study of RJ beads was executed based on the pharmaceutical product guideline written in the ASEAN Guideline on Stability Study of Drug Product (2013). As mentioned earlier, RJ bead samples were stored in refrigerator (2-8 °C), laboratory (25 °C), real time (30 °C, 75% RH) and accelerated (40 °C, 75% RH) stability chambers. At predetermined stability time point: 0-day, 14-days, 1-month and 3-months, samples were taken out (n=30) from each different storage condition. The stability samples were observed and characterised for physical appearances, particle size, sphericity coefficient, microscopic morphology, 10-HDA content and compression testing. The physical appearance of RJ beads, namely under its organoleptic properties at different time points was compared to the appearance of the beads at 0-month stability time point.

1. Determination of the diameter and sphericity coefficient of RJ beads

To achieve statistical result, thirty RJ beads were randomly chosen and rinsed with distilled water after thirty minutes of gelation time. Image analysis software (Image J, National Institute of Health, USA) was used to measure the diameter of each of the RJ beads taken. Statistical data such as mean, median, and mode were generated automatically.

The sphericity coefficient (SC) of RJ beads was calculated using the following equation by Houghton & Amidon (1992) (Equation 1):

$$SC=d_{min}/d_{max} \quad \text{(Eq. 1)}$$

where, dmin and dmax are minimum and maximum Ferret's diameters of the RJ beads, respectively (Shaiqah et al., 2020). Beads with a SC value approaching 1 are considered ideal and spherical (Azhar et al., 2021).

2. 10-HDA analysis

RJ beads were immersed in the phosphate-buffer solution (PBS) with concentration 0.1 M and pH 6.8. The samples were subjected under vigorous stirring for 30 minutes until the alginate-pectin coating disintegrated. Then, 25 mL of water and methanol (1:1, v/v) were added and the suspension formed was centrifuged at 4000 rpm for 10 minutes using Rotofix 32 from Andreas Hettich GmbH & Co. (Tuttlingen, Baden-Württemberg). The 10-HDA content in the RJ beads was obtained by analysing the supernatant solution spectrophotometrically at 215 nm using HPLC (waters e2695, Waters Corporation, Milford, USA).

3. Surface morphology

Prior to SEM imaging, 10 to 15 RJ beads were rinsed with an increasing gradient of ethanol concentration of 10%, 50%, 70%, 90%, and 100% and left air-dried for 30 minutes at room temperature (25-30 °C) for proper sample dehydration. The surface morphology of the beads was evaluated by using a scanning electron microscope at 100 and 500 times of magnification (SEM, Fei, Quanta 450, ThermoFisher Scientific, Oregon, USA).

4. Compression testing

Brookfield CT3 Texture Analyser (Middleboro, USA) was used for uniaxial compression of a single RJ bead. For statistically significant results, 30 RJ beads were randomly selected from the samples that had been dipped previously in simulated gastric fluid or simulated intestinal fluid for 30 minutes. Cylindrical aluminium probe with 6 mm diameter was attached to compress the bead at 1.0 mm/s. The trigger load of 0.05 N and peak deformation of up to 50% of the initial bead diameter were set. Equation 2 was utilised to calculate Young's modulus, E (Pa).

$$\boldsymbol{E = \frac{3 \times (1 - v^2) \times F}{\sqrt{d \times H^3}}} \qquad \text{(Eq. 2)}$$

where,

d: diameter of the bead (m)
F: trigger load (N)
H: deformation of the bead (m)
v: Poisson's ratio

Results and Discussion

1. Physical appearance

RJ is whitish grey colour. It is a complex compound that consist of water (60%-70%), proteins (27%-41%), carbohydrates (30%), lipids (8%-19%), free amino acids, trace mineral, and water-soluble vitamin (Maghsoudlou, Sadeghi Mahoonak, Mohebodini, & Toldra, 2019). The colour of RJ is a critical parameter as it acts as indicator of its freshness and suitability (Zheng, Wei, Wu, Hu, & Dietemann, 2012). Figures 1, 2, 3 and 4 display the appearance of RJ beads at 0-day, 14-days, 1-month and 3-months stored in refrigerator, room temperature, real time, and accelerated stability chambers. The colour of RJ beads in both type of packaging stored in the refrigerator remained whitish grey throughout this study (Figure 1). In comparison, at 1-month and 3-months stability time points, the colour of the beads stored at room temperature turned purplish grey (Figure 2). Changing of RJ beads colour also occurred in both accelerated and real time stability chambers at 1-month and 3-months stability time point for both packaging, where the beads changed from whitish grey to dark brownish yellow (Figure 3 and Figure 4). This result is in agreement with previous studies that reported browning reaction of RJ during storage at room temperature as early 1-month storage (Chen & Chen, 1995; Qiao, Wang, Liu, & Zhang, 2018).

This effect is attributed to the Millard reaction or also known as non-enzymatic reaction where a chemical reaction occurs between amino acids present in RJ and reducing sugar that produce brown colour. Chen & Chen, (1995) proposed that browning reaction was stimulated by the higher temperature in which this reaction was sensitive to the ambient temperature and higher temperature stimulate the reaction rates. During Maillard reaction, a wide range of reaction products is formed, leading to significant alteration that affect nutritional value of nutraceuticals (Starowicz & Zieliński, 2019). In contrast, the colour change of RJ beads at room temperature (laboratory) is observable starting at 3-months. At room temperature, PET bottle is believed to reduce and delay the change of colour of the RJ beads. The opaqueness of the bottle aids in protecting the beads from direct sunlight and its subsequent heat changes. Hence, at room temperature, PET bottle can serve as the best potential candidate for the storage of RJ beads and can be considered for future recommendation in the market.

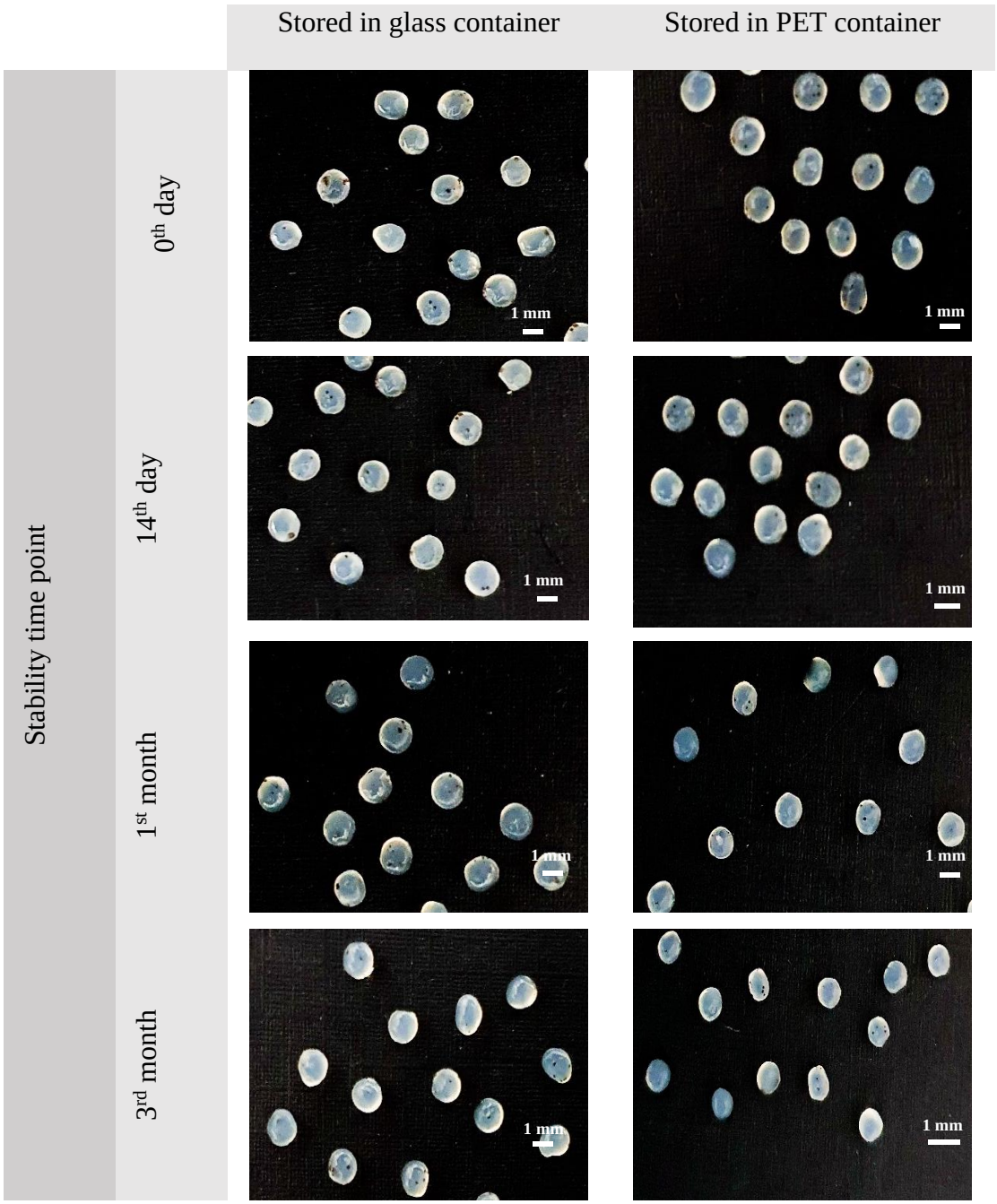


Figure 1. The physical appearance of RJ beads stored in refrigerator at four stability time points (0-day, 14-day, 1-month and 3-month)

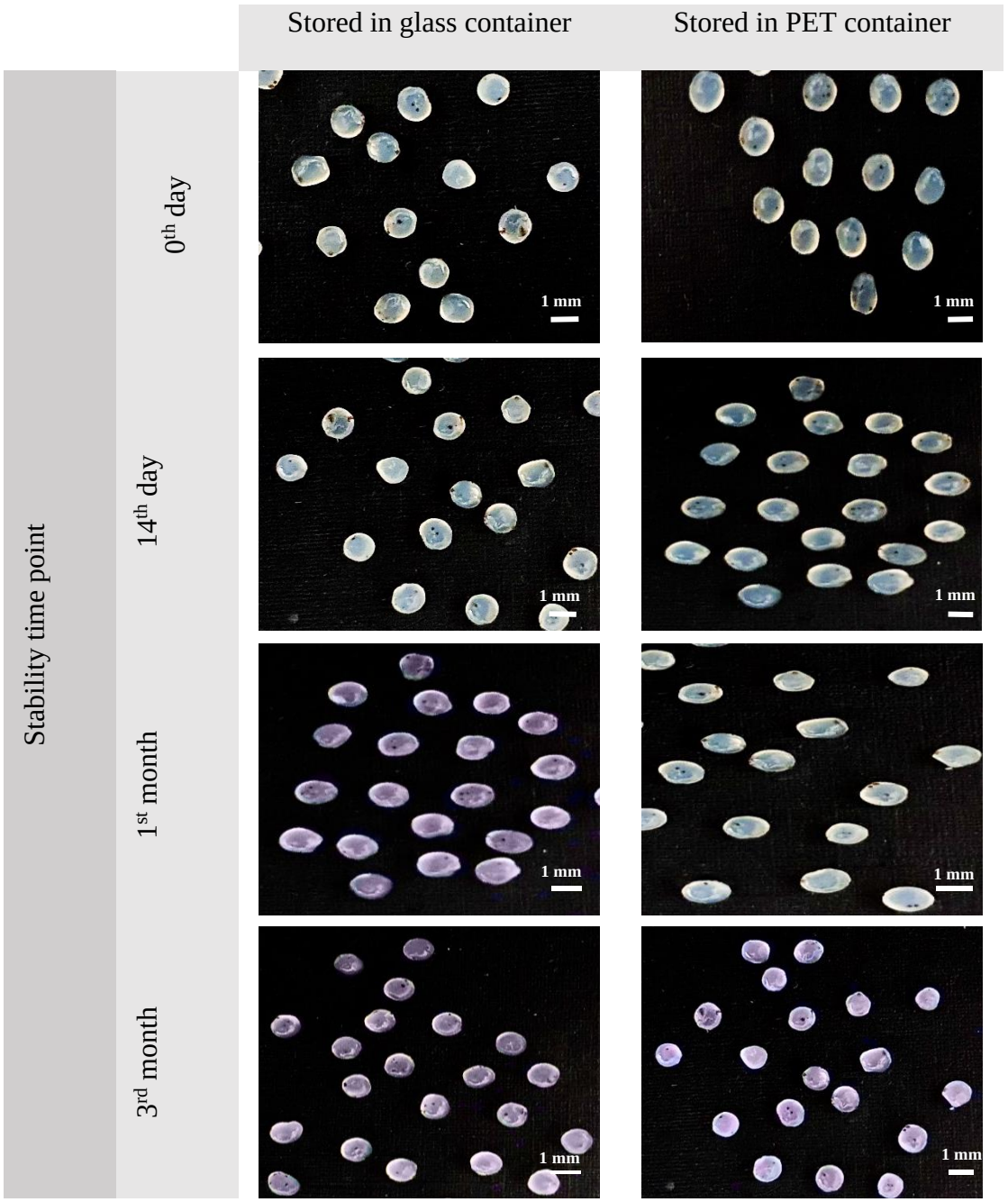


Figure 2. The physical appearance of RJ beads stored in room temperature at four stability time points (0-day, 14-day, 1-month and 3-month)

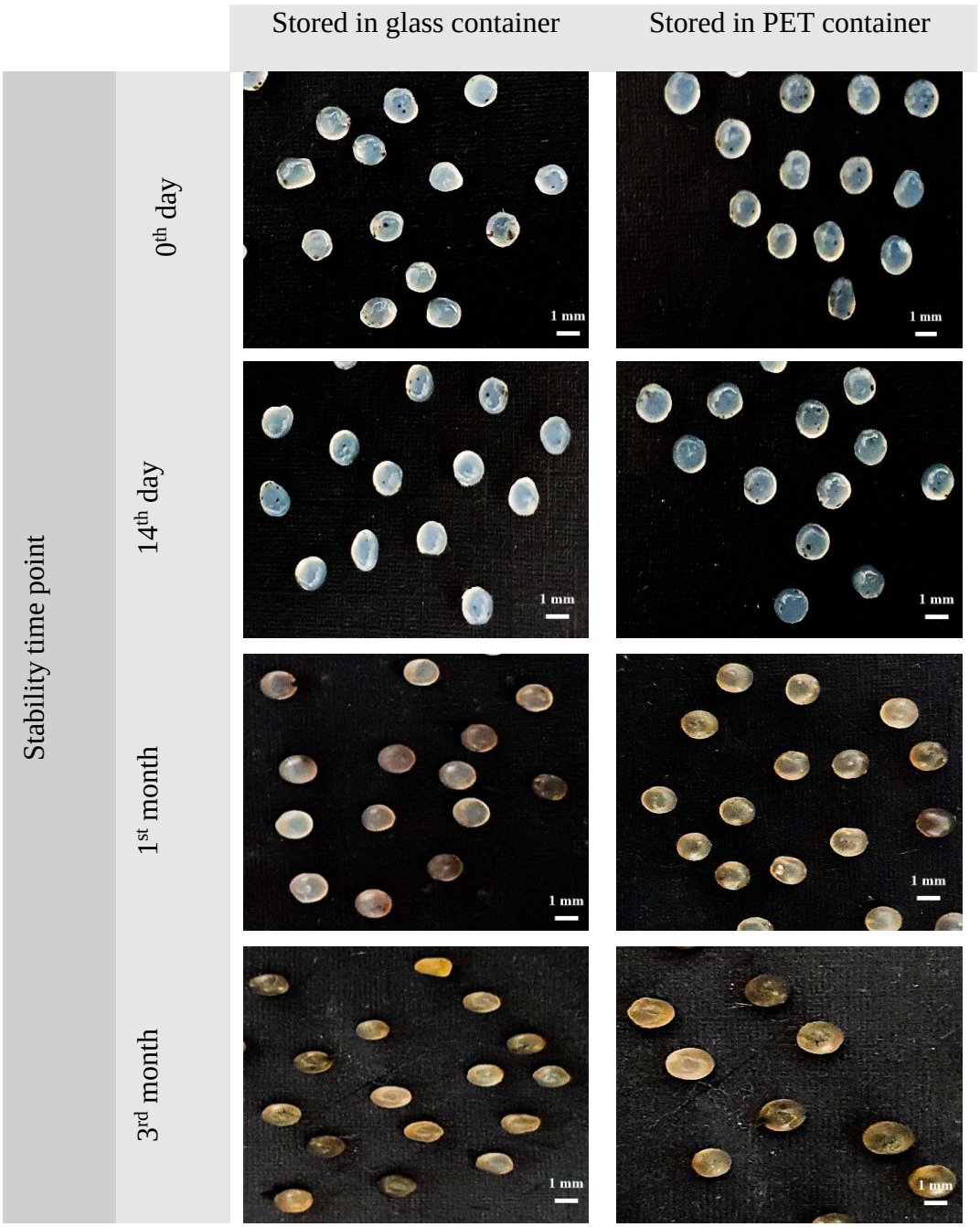


Figure 3. The physical appearance of RJ beads stored in real-time stability chamber at four stability time points (0-day, 14-day, 1-month and 3-month)

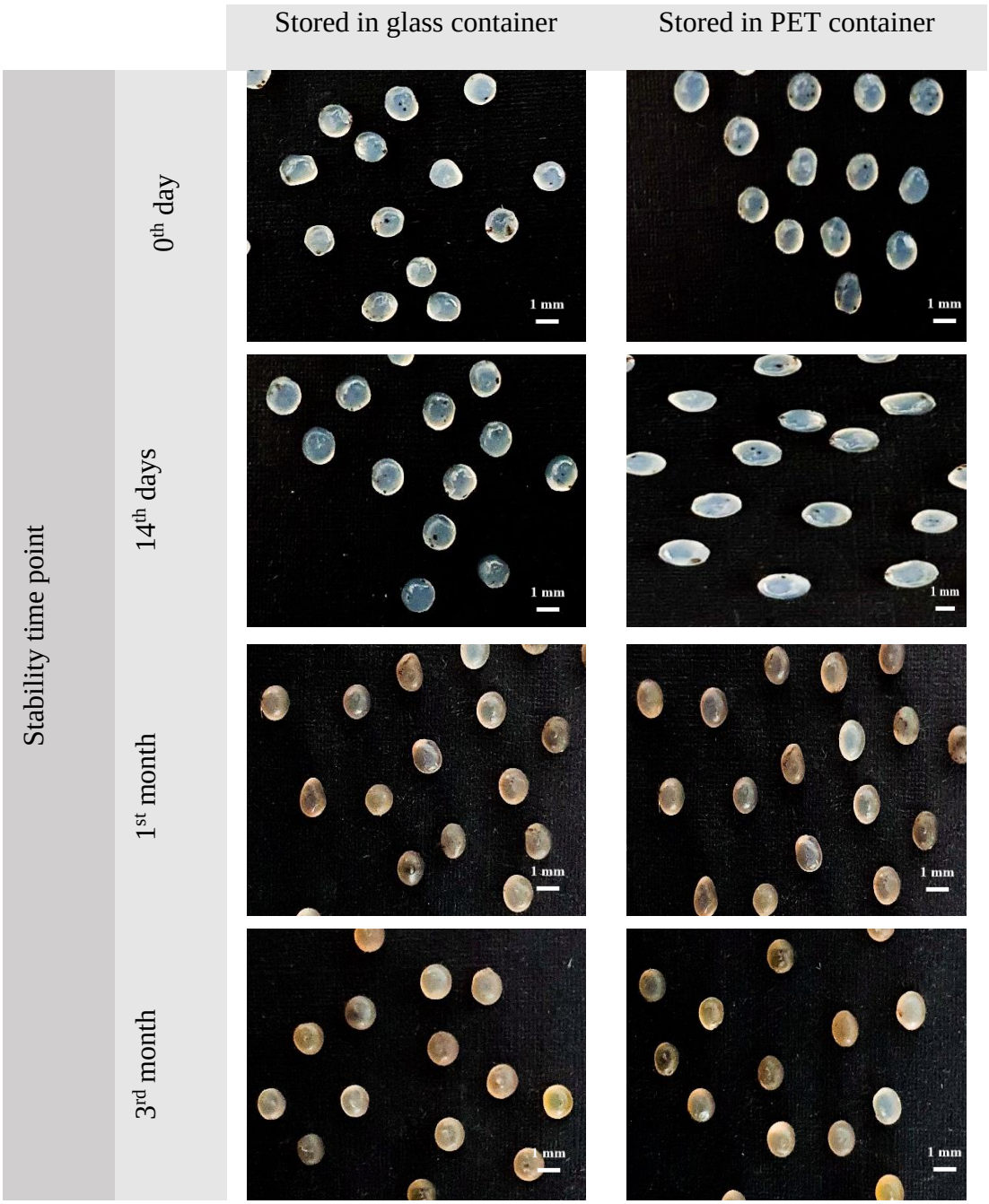
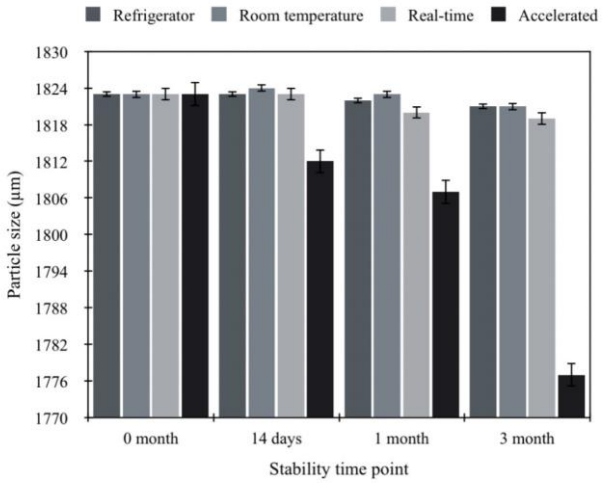


Figure 4. The physical appearance of RJ beads stored in accelerated stability chamber at four stability time points (0-day, 14-day, 1-month and 3-month)

2. Particle size

The particle size of the RJ beads was measured and analysed in four different storage conditions that include refrigerator, laboratory, real time, and accelerated stability chambers. For better overview, Figure 5 descriptively illustrates particle size of the randomly selected RJ beads (n=30) at different stability study points. The mean bead diameter ranges between 1777 ± 121 µm to 1823 ± 199 µm. Reduction of particle size was observed in all storage conditions for both types of beads container. However, only the particle size of RJ beads stored in accelerated stability chamber had a decreasing pattern with significance ($p<0.05$) for both types of storage container. The reduction in particle size of RJ beads stored in accelerated stability chambers across all stability time points might be an evident sign that 10-HDA undergoes chemical and physical degradation when exposed to higher temperature (40 °C). On top of that, occurrence of total water loss in the beads via evaporation and disruption of polymeric cross-linking of the biopolymer by high temperature explains the further reduction of the bead diameter over time (Bannikova, Rasumova, Evteev, Evdokimov, & Kasapis, 2017; Vargas, Pereira, Guimarães, Waldman, & Pereira, 2018). Nevertheless, RJ beads stored in refrigerator, room temperature and real-time stability chamber manifests insignificant size reduction ($p>0.1$) from 0-month till 3-months for both types of storage container. The statistical analysis using two-factorial ANOVA is also insignificant between all groups.

(A)



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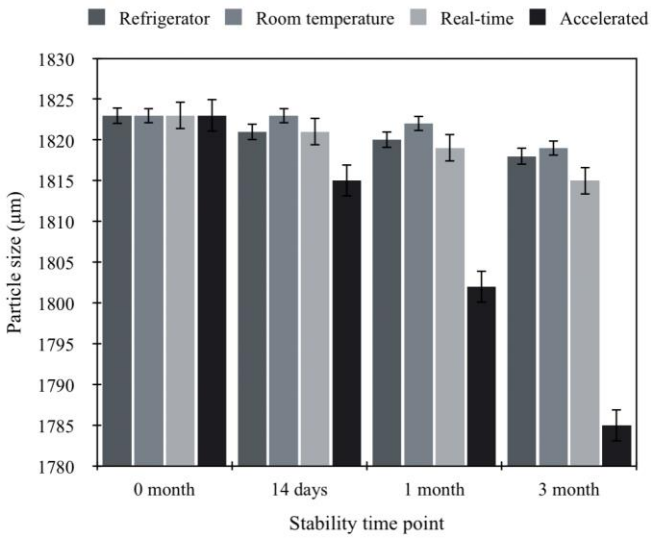


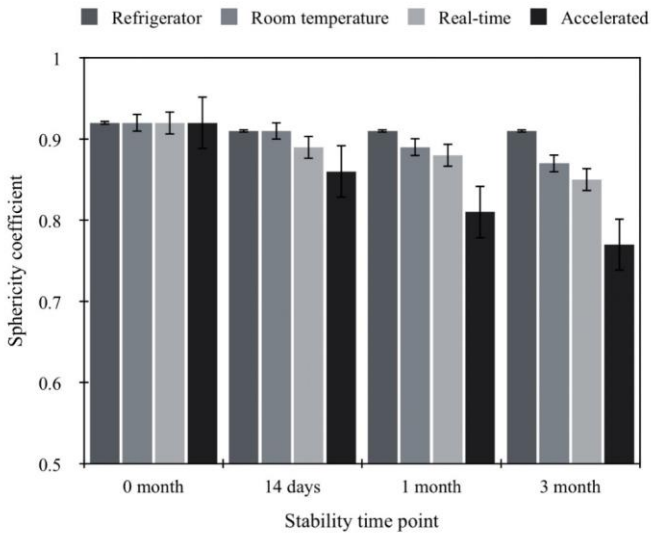
Figure 5: (A) Particle size (µm) of the RJ beads at different stability time point stored in PET container (n=30) (B) Particle size (µm) of the RJ beads at different stability time point stored in glass container (n=30)

3. Sphericity coefficient

The sphericity coefficient of RJ beads should be near to 1.0 as indication of perfect circular shape is attained during electrospraying process (Azhar et al., 2021). Figure 6 illustrates sphericity coefficient values of the randomly selected RJ beads (n = 30) in four storage conditions. All the sphericity coefficient values did not reach below 0.75. It shows that the spherical shape of the beads is maintained regardless of the storage conditions and time points. On top of that, there was no significant difference ($p > 0.05$) between sphericity coefficient values of RJ beads stored in glass and PET bottle in refrigerator, room temperature and real-time stability chamber at 0 month and 14-day time point.

However, the sphericity coefficient values of RJ beads decreased in significant manner ($p<0.05$) for both containers inside accelerated stability chambers. It ranged from 0.81 at 1 month to 0.77 at 3 months in glass container and from 0.81 at 1 month to 0.76 at 3 months in PET container. These results are in agreement with the findings that increasing storage temperature of calcium-alginate beads yields more irregular internal structure, hence decreasing the rupture strength of the beads (Jeong, Kim, Lee, Cho, & Kim, 2020). Nevertheless, the sphericity coefficient of RJ beads in both type of storage container stored in refrigerator manifests insignificant changes for 3-months stability time point indicating good physical stability of RJ beads.

(A)



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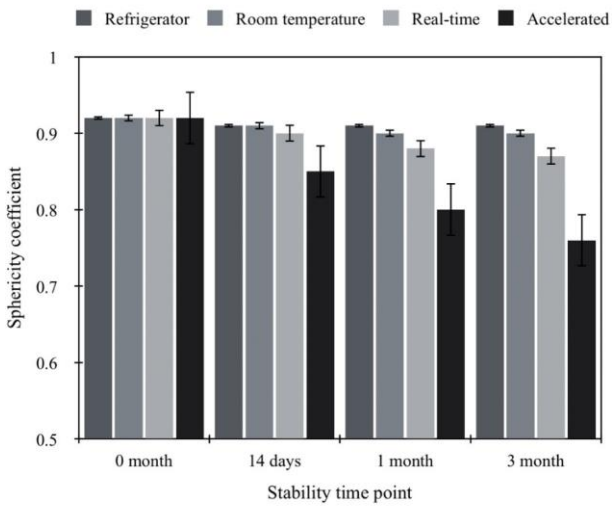


Figure 6: (A) Sphericity coefficient of the RJ beads at different stability time point stored in glass container (n=30) (B) Sphericity coefficient of the RJ beads at different stability time point stored in PET container (n=30)

4. Microscopic morphology

Figures 7 and 8 manifest the microscopic morphology of random RJ beads sample under different magnification of SEM at 0 and 3-month storage in refrigerator, room temperature, real time, and accelerated stability chambers. It is observed that microscopic morphology of the beads remained relatively indifferent after 3 months of storing in both glass and PET container.

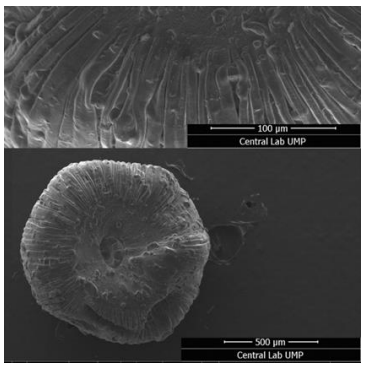


Figure 7: SEM photographs of RJ beads of selected samples at 0-month time-point (A: Surface at magnification 100 µm; B: Surface at magnification 500 µm)

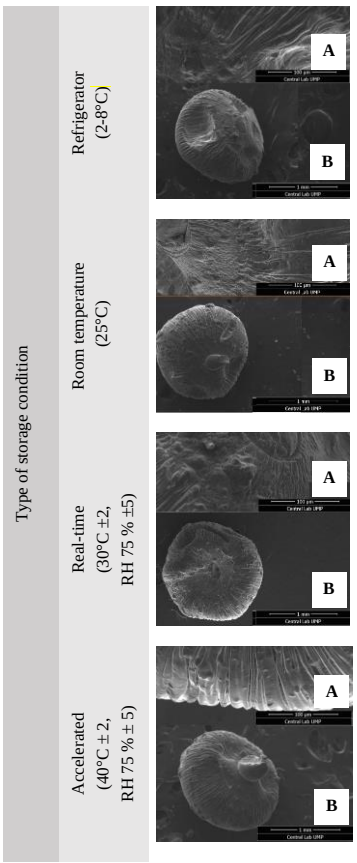


Figure 8: SEM photographs of RJ beads of selected samples at 3-month time point (A: Surface at magnification 100 µm; B: Surface at magnification 1 mm)

5. 10-HDA assay content

10-HDA is one of the main bioactive compounds of RJ that is only found in RJ in nature (Khazaei, Ansarian, & Ghanbari, 2018). Thus, the present of 10-HDA can be used as a marker to validate the freshness and quality of RJ (Antinelli et al., 2003; kim & Lee, 2010). Table 1 summarises the variation of 10-HDA content in four different conditions.

Analysis using one-way ANOVA inferred that the difference in 10-HDA content (%, w/v) in RJ beads stored in accelerated stability chamber was the only one that is statistically significant ($p<0.05$) for 1-month and 3-months compared to 0-month time point (Table 1). Both types of storage container, PET and glass type manifest similar findings with no significance ($p> 0.05$). Nonetheless, the 10-HDA content of RJ in alginate-pectin beads store in refrigerator remain the same throughout the study for both type of storage container.

This result is in accordance with the colour changes reported in this study. Changing of RJ beads colour is correlated with degradation of 10-HDA content. Maintaining required amount of 10-HDA is encouraged as a low 10-HDA content implies a low RJ activity (Muñoz, Decap, Ruiz, Arbildua, & Monasterio, 2011). The variation of 10-HDA content (mg/mL) between stability time points and type of container can be optimised by proper standardisation of the procedure in preparing the samples. In addition to the above-mentioned measure, the declining pattern of 10-HDA content occurred in the beads stored in room temperature and both real-time and accelerated stability chambers signals RJ deterioration.

Nutraceutical products, which include the formulation of RJ beads contain numerous phytoconstituents of different chemical classes (Maghsoudlou et al., 2019). These constituents may undergo various inter- as well as intra-molecular reactions under the influence of varied environmental conditions, such as heat, humidity, air and/or light during processing, formulation and storage of the material (Maghsoudlou et al., 2019; Ramadan & Al-Ghamdi, 2012).

10-HDA is a major fatty acid in RJ can degrade under the influence of heat and light which may, in turn, alter the actual content, shorten shelf-life and reduce therapeutic efficacy of the final products. Recent studies propose that 10-HDA is the leading indicator in the determination of the freshness, however, inconsistent stability of this fatty acid will cause as long-term challenge for the standardisation of RJ formulation (Shen et al., 2015). In this study, 10-HDA represents an adulteration indicator and should be above 1.4% for fresh RJ (Abdulqader Yaslam Bazeyad, Ahmad

Abdullah Al-Ghamdi, & Yehya Zaki Alattal, 2022). The values for the analysed RJ samples were within the limits proposed by the ISO RJ International Standard (International Organization for Standardization, 2016), which sets the minimum concentration of 10-HDA is 1.4% for pure RJ.

In brief, the best storage conditions to preserve critical quality attributes (CQA) of RJ beads especially the 10-HDA content itself is the refrigerator (2-8 °C) with the most practical and versatile packaging of PET. The low temperature of storage condition reduces the phenomenon of oxidation and hydrolysis reaction subsequently minimising physical and chemical degradation of RJ beads and later extending its shelf-life (Muresan et al., 2016).

6. Compression testing

Determination of degree of deformation in compression testing is crucial to assess the physical stability of the RJ beads during storage and transportation as well as its bioavailability when exposed to human gastric and intestinal environment (Rayment et al., 2009). The higher the Young's modulus being expressed in the compression testing, the higher the force needed for the beads to resist deformation phenomenon (Lee, Zhang, & Ryu, 2018).

Figures 9, 10 and 11 summarise all the Young's modulus values of RJ beads being stored in two types of containers (glass and PET) with four different storage conditions; refrigerator, room temperature, real-time and accelerated stability chambers. All the Young's modulus values were above 7 x 1000 Pa at all stability time points when the beads were tested air-dried as well as when being dipped into simulated gastric solution for 2 hours regardless the type of container used (Figure 9 and Figure 10). This proves that the encapsulation of RJ beads using alginate and pectin confers marked improvement of mechanical protection to the formulation in these both conditions. However, when the beads were subjected under simulated intestinal fluid for 2 hours, the values of Young's modulus exhibited substantial decreasing trends from 7.7 x 1000 Pa at 0 month to 2.32 x 1000 Pa at 3 month with statistical significance ($p<0.05$) (Figure 11). It was inferred that the decreasing pattern of Young's modulus is due to the reduction

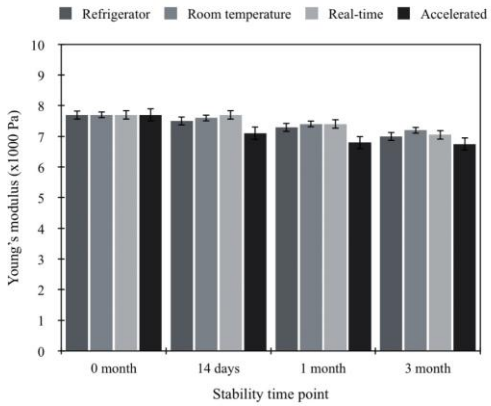
RJ beads rigidity in alkaline environment as compared to in acidic condition (Abu, Rasel, & Hasan, 2012). More open and porous polymeric network formed during rigorous swelling in alkaline environment. By increasing the dipping time of the beads in the simulated intestinal fluid, the activity contributes to the gradual reduction of the deformation and its corresponding Young's modulus values. The reason for this is that mannuronic and guluronic acids residue of alginate have pka values of 3.38 and 3.65 respectively.

The beads are stabilised by intermolecular hydrogen bonding network in a gastric environment where the pH value (1.5 – 3.5) is lower than the pka of the uronic acid (Pawar & Edgar, 2012). Meanwhile in simulated intestinal condition as the pH rises above the pka of the polysaccharides, the beads are expected to disintegrate due to the deprotonation of the polysaccharides of alginate beads leading to electrostatic repulsion and eventually disintegration of the beads (Chuang et al., 2017; Marciani et al., 2019).

Table 4: Results of intraday and interday precision for the simultaneous quantification of INH and PYR in pure form

Storage conditions	Stability time point	10-HDA content (%, w/v) in glass container	10-HDA content (%, w/v) in PET container
Refrigerator (2-8 °C)	0 month	1.83 ± 0.1	1.83 ± 0.1
	14 days	1.83 ± 0.7	1.83 ± 0.7
	1 months	1.83 ± 0.3	1.83 ± 0.9
	3 months	1.83 ± 0.1	1.83 ± 0.2
Laboratory (25 °C)	0 month	1.83 ± 0.1	1.83 ± 0.1
	14 days	1.83 ± 0.6	1.83 ± 0.5
	1 months	1.79 ± 0.4	1.79 ± 0.7
	3 months	1.77 ± 0.6	1.77 ± 0.1
Real time (30 °C ± 2, RH 75 % ± 5)	0 month	1.83 ± 0.1	1.83 ± 0.1
	14 days	1.83 ± 0.9	1.83 ± 0.2
	1 months	1.78 ± 0.6	1.78 ± 0.3
	3 months	1.75 ± 0.2	1.75 ± 0.7
Accelerated (40°C + 2, RH 75 % ± 5)	0 month	1.83 ± 0.1	1.83 ± 0.1
	14 days	1.83 ± 0.2	1.83 ± 0.6
	1 months	1.74 ± 0.8	1.74 ± 0.1
	3 months	1.71 ± 0.1	1.71 ± 0.1

(A)



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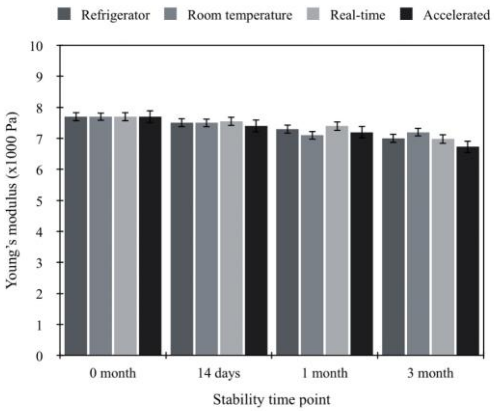
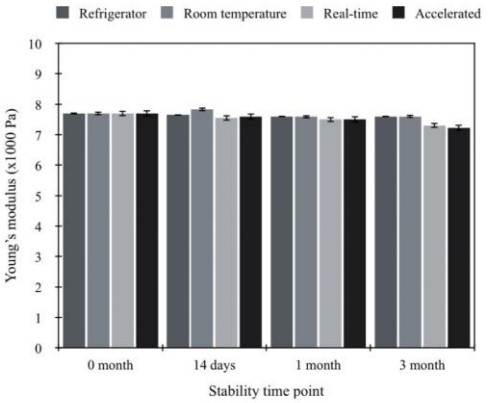


Figure 9: (A) Young's modulus of the RJ beads (air-dried) at different time point stored in glass container (B) Young's modulus of the RJ beads (air-dried) at different time point stored in PET container.

(A)



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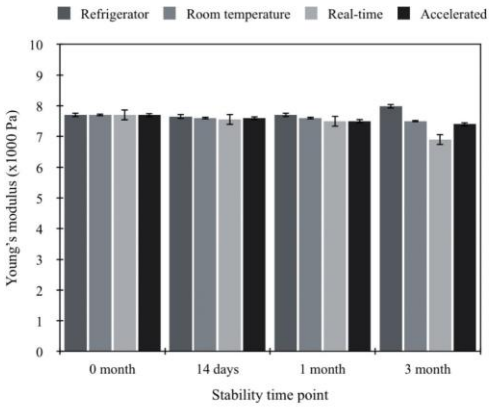
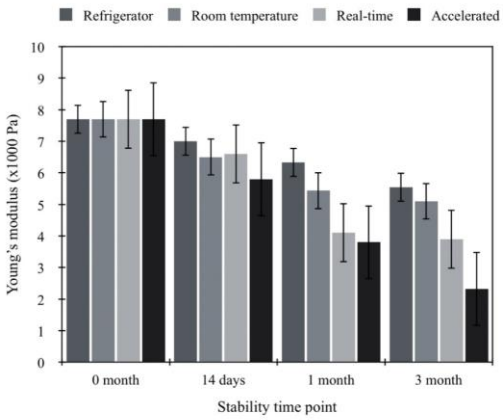


Figure 10: (A) Young's modulus of the RJ beads (dipped in simulated gastric fluid) at different time point stored in glass container (B) Young's modulus of the RJ beads (dipped in simulated gastric fluid) at different time point stored in PET container.

(A)



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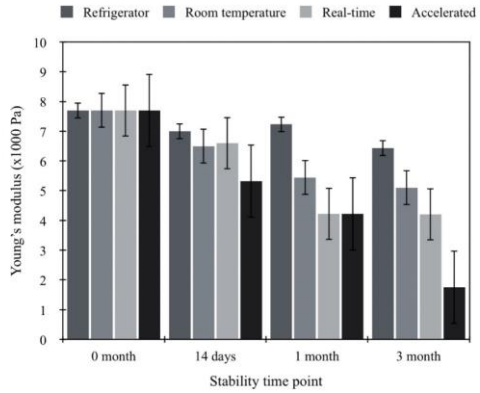

Figure 11: (A) Young's modulus of the RJ beads (dipped in simulated intestinal fluid) at different time point stored in glass container (B) Young's modulus of the RJ beads (dipped in simulated intestinal fluid) at different time point stored in PET container.

Conclusion

In conclusion, the study showed that the RJ beads exhibited convincing stability for 3 months when it is stored at low temperature. Analysis of 10-HDA content in the RJ-encapsulated beads as well as observation of its physicochemical properties that includes physical appearances, particle size, sphericity coefficient, microscopic morphology, and compression testing showed that the results are highly consistent across all stability time points. Environmental conditions especially temperature must be considered during beads preparation and storage since room temperature will accelerate physicochemical changes of RJ beads. Therefore, RJ beads must be stored at low temperature (refrigerator) to maintain the stability of formulation and provide a longer shelf life. Besides, this study also exhibits that colour changes of RJ beads is well correlated with the degradation of the 10-HDA compound. The investigation using different types of container substance proves similar results regardless of the storage conditions. In practicality, PET container offers higher superiority to the consumers compared to glass container as packaging for RJ beads due to its convenience in terms of low cost and lightweight. On top of that, opaqueness and inertness of PET container serve as additional advantages to protect the RJ beads from gradual deterioration to sunlight, heat and moisture.

Conflict of Interest

The authors declare that there is no conflict of interest.

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