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STABILITY OF CHITOSAN-TRIPOLYPHOSPHATE COMPLEX-ENCAPSULATED ANTHOCYANIN AT HIGH WATER ACTIVITY

[Stabilitas Antosianin yang Terenkapsulasi melalui Komplek Kitosan-Tripolifosfat pada Kondisi Aktivitas Air yang Tinggi]

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ABSTRACT

Previous study successfully conducted encapsulation of the purple-fleshed sweet potato's anthocyanin but the study has yet to reveal the stability of encapsulated anthocyanin. Therefore, this research aims to observe the stability of encapsulated anthocyanin regarding the characteristic of low anthocyanin stability, which depends on environmental factors, such as temperature, pH, humidity, and water activity. The kinetic parameters of stability, including kinetic constant (k), reaction order, and half-life ($t_{1/2}$), were also studied. Stability testing was conducted in high water activity of 0.75 and various incubation temperatures at 16, 25, 35, and 45°C. Un-encapsulated anthocyanin extract was also tested for its stability in the same condition in order to be compared with encapsulated anthocyanin. This study revealed that the encapsulated anthocyanin had lower stability than un-encapsulated anthocyanin extract. It was proven by higher kinetic constant and lower half-life of encapsulated anthocyanin for every incubation temperature which was induced by higher pH of encapsulated anthocyanin compared with anthocyanin extract. Besides, high water activity reduced glass transition temperature (Tg), in which encapsulated anthocyanin was in rubbery state. Both encapsulated anthocyanin and anthocyanin extract were degraded following the first order kinetic. Using the Arrhenius equation, it was obtained that the degradation kinetic constant of encapsulated anthocyanin was stated as k= 420.44 exp (-23.33/RT). Meanwhile, k= $1.12x10^6$ exp (-46.70/RT) described degradation of kinetic constant of anthocyanin extract. The stability test revealed that the application of encapsulated anthocyanin was not suitable for wet-type food product.

Keywords: anthocyanin, chitosan, encapsulation, stability, tripolyphosphate

ABSTRAK

Penelitian mengenai enkapsulasi antosianin ubi jalar ungu telah berhasil dilakukan, tetapi penelitian tersebut belum mengungkap stabilitas antosianin terenkapsulasi. Oleh karena itu, penelitian ini bertujuan untuk mempelajari stabilitas antosianin terenkapsulasi berkaitan dengan karakteristik stabilitas antosianin yang rendah yaitu tergantung pada faktor-faktor lingkungan, seperti suhu, pH, kelembapan, dan aktivitas air. Parameter kinetika stabilitas yang mencakup konstanta kinetika (k), orde reaksi, waktu paruh ($t_{1/2}$) juga perlu dipelajari. Pengujian stabilitas dilakukan pada aktivitas air yang tinggi sebesar 0,75 dan suhu inkubasi yang divariasikan pada 16, 25, 35, dan 45°C. Ekstrak antosianin yang tidak dienkapulasi juga diuji stabilitasnya pada kondisi yang sama, dengan tujuan untuk dibandingkan stabilitasnya terhadap antosianin terenkapsulasi. Uji stabilitas menunjukkan bahwa antosianin terenkapsulasi memiliki stabilitas yang lebih rendah daripada antosianin yang tidak terenkapsulasi. Hal tersebut terbukti dengan konstanta kinetika yang lebih tinggi dan waktu paruh yang lebih rendah pada antosianin terenkapsulasi untuk masing-masing suhu inkubasi. Hasil tersebut dikarenakan faktor pH antosianin terenkapsulasi yang lebih tinggi daripada pH ekstrak antosianin. Selain itu, aktivitas air yang tinggi menurunkan suhu gelas transisi (Tg), dengan antosianin terenkapsulasi berada pada kondisi bentuk karet (rubbery). Antosianin terenkapsulasi dan ekstrak antosianin mengalami degradasi menurut kinetika orde 1. Melalui persamaan Arrhenius, didapatkan bahwa konstanta kinetika degradasi antosianin terenkapsulasi dinyatakan sebagai k= 420,44 exp (-23,33/RT). Sementara itu, k= $1,12x10^6$ exp (-46,70/RT) menggambarkan konstanta kinetika degradasi ekstrak antosianin. Berdasarkan uji stabilitas, antosianin terenkapsulasi tidak layak untuk diaplikasikan pada produk pangan bertipe basah.

Kata kunci: *antosianin, enkapsulasi, kitosan, stabilitas, tripolifosfat*

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INTRODUCTION

Anthocyanins giving rise to various natural pigment or color from red, blue, and violet to plant parts, such as leaf, root, fruit, and tuber, are secondary metabolites dominantly produced by plants (Diniyah *et al.,* 2010). They belong to flavonoid compounds with a chemical structure composed of two aromatic ring connected to each other by oxygen-attached heterocyclic ring. One of the aromatic ring is bonded to sugar group through glycosidic bond (Bueno *et al.,* 2012). Just like other flavonoid compounds, anthocyanins are known to have beneficial bioactivities and functionalities. Compared with other antioxidants, such as (+)-catechin, vitamin E, BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene), anthocyanins have higher antioxidant capacity (Justino, 2017). Anthocyanins play key roles in antidiabetic and anti-inflammatory activities, improve visions (Khoo *et al.,* 2017), and maintain cardiovascular activity (Whitson, 2010) and heparin system (Hou *et al.,* 2013).

The superiority of anthocyanin's bioactivities are opposed to their stability, since anthocyanins are very sensitive to environmental factors, *i.e*. pH, temperature, light, oxygen, water activity, and others. In particular, anthocyanins are different from other flavonoid in terms of their pH change since their structure undergoes different conformations. The conformations are flavylium cation, quinoidal base, carbinol base, and chalcone that arise in pH 1-3, 3-4, and 5, 6 respectively (Faria *et al.,* 2013). Anthocyanin stability will increase along with the decreasing pH so that flavylium cation conformation can achieve the highest stability. The high stability is achieved by the conformation capability to avoid hydrolysis reaction of glycosidic bond (Turumtay *et al.,* 2015). Meanwhile, the increasing temperature plays an important role in influencing anthocyanins' stability by shifting the conformation to carbinol and chalcone. Alteration of conformation is induced by hydrolysis of glycosidic bond by which more opened and unstable anthocyanin structures, namely carbinol and chalcone, are formed (Sun *et al.,* 2011). The decrease in anthocyanin stability by oxygen is resulted from either direct oxidation or oxidator enzyme aid (Patras *et al.,* 2010). The oxidation results in brown-coloured or colorless substance (Martynenko and Chen, 2016). The lowering of anthocyanin stability can also be induced by the presence of light (Askar *et al.,* 2015).

Several studies mentioned that encapsulation is the way to reduce sensitivity of anthocyanin toward environmental factors that lower anthocyanin stability (Parisi *et al.,* 2014) to allow the encapsulation to improve anthocyanin stability (Kanokpanont *et al.,* 2018). Our previous study reported that anthocyanin was successfully encapsulated by chitosan-sodium tripolyphosphate complex using emulsification-crosslinking technique (Laila *et al.,* 2019). The research studied and optimized parameters influencing encapsulation in which the best microcapsule was obtained by the stirring intensity of 1600 rpm, chitosan concentration of 3% (w/v), pH of chitosan, NaTPP solution of 3.0, and ratio of core to chitosan concentration of 150% (w/w). The optimization was focused to parameters, *i.e.* efficiency of encapsulation, particle size distribution, and antioxidant activity. The use of NaTPP as crosslinker was considered by its availability in food application due to low toxicity and immediate gel formation.

It had been mentioned that dry products, including microcapsule tend to have slow chemical changes. However, some conditions, like storage at temperature above their glass-transition temperature (Tg) will accelerate deteriorative process (Slade and Levine, 1991). It is known that glass-transition temperature (Tg) will be affected by water activity. Alpizar-Reyes *et al.* (2017) said that increasing water activity will reduce Tg because water plays the role as plasticizer which will reduce viscosity and increase free volume, so that active (solid) molecule can move intensely. In other words, it will enlarge molecule structure to change from glassy state towards rubbery state. In this regard and given the possibility or impossibility to apply encapsulated anthocyanin in high humidity or wet-type food product like yogurt, pasta, or drink, the present study focused on observing stability of the encapsulated anthocyanin at critical condition, namely the condition with water activity of 0.75 and various incubation temperature at 16, 25, 35, and 45°C. The kinetic parameters of stability including kinetic constant reaction order, and half-life were also needed to study. Furthermore, Arrhenius equation was used to determine kinetic constant as the function of temperature. As a comparison, un-encapsulated anthocyanin extract was also tested for its stability using the same condition.

MATERIALS AND METHODS

Materials

The study used Chitosan of medical grade (PT. Biotech Surindo) with deacetylation degree of > 85% and viscosity of 130 cps, and sodium tripolyphosphate (NaTPP) with technical grade of 85% (Sigma-Aldrich, US). Purple-fleshed sweet potatoes (*Ipomoe batatas* L.) were supplied from Ngawi, East Java and obtained from *Pasar Telo*, Karangkajen Yogyakarta.

Preparation of encapsulated anthocyanin (Laila *et al.,* 2019)

Encapsulated anthocyanin was prepared according to our previous study (Laila *et al.,* 2019). Preparation was initiated by anthocyanin extraction from purple fleshed sweet potatoes. Extraction was conducted using 3% (w/v) citric acid (PT. Bratachem, Indonesia) in ethanol 96% (CV. General Labora, Indonesia) as solvent. Purple fleshed sweet potatoes were extracted in chips with the ratio to solvent of 1:1 (w/v). Extraction was done by maceration at the temperature of 35°C, extraction time of 4 hours and with no light (dark) condition. Afterward, the extract was separated from solid residue using double filter cloth and the filtration was accomplished using Buchner funnel-vacuum filtration apparatus. The filtrate was concentrated in rotary evaporator (BUCHI R-114, Switzerland) at temperature of 50°C to obtain final concentrated anthocyanin extract with concentration of 70-80°Brix.

Preparation of encapsulated anthocyanin was done using emulsification-crosslinking technique by applying paraffin liquid (CV. Alfa Kimia, Indonesia) as continuous phase and anthocyanin-chitosan liquid as dispersed phase (aqueous phase). Initially, anthocyanin extract was dissolved and homogenized in chitosan solution 3% (w/v) using hot plate stirrer (Thermolyne Cimarec 2, USA). The chitosan solution was previously made by dissolving chitosan in 0.2 M acetate buffer solution with pH of 3.0. Anthocyanin extract was added and mixed to chitosan solution in a concentration of 150% (w/w) at a room temperature (28-30°C) and the mixed solution was further named as aqueous solution (aqueous phase). Aqueous phase was dropped gradually using drop pipette to continuous phase with volume ratio of aqueous phase to continuous phase of 10%. Previously, continuous phase was prepared by mixing sorbitan monooleate (Sigma-Aldrich, US) and paraffin liquid with a concentration of 2% (w/v). While aqueous phase was dropped, stirring was carried out using mechanical agitation with flat 2-blade impeller in liquid system at stirring intensity of 1600 rpm for getting water in oil (W/O) emulsion. Stable emulsion was obtained after emulsification was conducted for 1 hour. Furthermore, solidification of emulsion droplet was done by dropping 7.5% (w/v) of sodium tripolyphosphate (NaTPP) aqueous solution to emulsion system. The stirring intensity was maintained at 400 rpm during solidification. Solidification process was conducted for 4 hours until stronger encapsulated anthocyanin was gained. Stronger encapsulated anthocyanin was filtered and then washed using N-hexane (CV. General Labora, Indonesia) to eliminate paraffin (oil) residue by Buchner funnel-vacuum filtration apparatus. Encapsulated anthocyanin was further rinsed by ethanol, dried, and stored in vacuum desiccator at ambient temperature for 24 hours under dark condition before stability testing was conducted.

Stability test (Jie *et al.,* 2013)

Stability test was aimed to investigate anthocyanin degradation during storage with a parameter to interpret the anthocyanin degradation of total anthocyanin content. Stability test was conducted under dark condition with water activity of 0.75. The water activity of 0.75 was obtained by putting saturated NaCl (RAFINA, Indonesia) solution in closed container or desiccator. Equilibrium water activity was obtained by letting the condition for one week. After equilibrium was achieved, samples was put in the desiccator, exactly above NaCl solution without having a direct contact to the solution. In short, 100 mg of encapsulated anthocyanin was packed in polyethylene–based wrapping plastic, and it was laid in desiccator providing the equilibrium water activity of 0.75. Desiccators were incubated at temperature of 16, 25, 35, and 45°C. The stability test was conducted for 35 days followed by regular sampling for further analysis of the total anthocyanin content. As a control and comparison, the same experiment and procedure was applied to anthocyanin extract with the concentration of 83°Brix and the pH of 2.21.

Analysis of total anthocyanins content (Giusti and Wrolstad, 2001)

Determination of total anthocyanins content (TAC) in encapsulated anthocyanin and anthocyanin extract was conducted according to pH-differential method (Giusti and Wrolstad, 2001). Analysis of TAC in encapsulated anthocyanin was initiated by anthocyanin extraction. Approximately, 100 mg of encapsulated anthocyanin was mixed by 3 mL of HCl 2M (Merck Millipore, US)-ethanol (Merck Millipore, US) of 70% solvent with HCl to ethanol volume ratio of 2/3. Extraction was conducted at temperature of 4°C under dark condition for 12 hours. Afterwards, centrifugation was conducted to separate extract liquid from encapsulant at 5000 rpm for 10 minutes at room temperature using benchtop centrifuge (Centrifuge GEMMY PLC-05, Taiwan). Sample extract was added within extract to buffer volume ratio of 1/6 into two kind of buffer, which are potassium chloride buffer of 0.025 M (Merck Millipore, US) with a pH of 1.0, and sodium acetate buffer of 0.4 M (Merck Millipore, US) with a pH of 4.5. Furthermore, the liquid was incubated under dark condition for 30 minutes. The absorbance of mixture liquid was measured by UV-Vis Spectrophotometer (Dynamica HALO RB-10 Spectrophotometer, UK) at wavelengths number of 530 and 700 nm. Total anthocyanin content (TAC) expressed as cyanidin-3-glucoside was determined using the following formula:

$$TAC\left(mg/L\right) = \frac{A \times MW \times DF}{E \times L} \times 1000 \quad \text{..... (1)}$$

$A = (Abs_{530} - Abs_{700})_{pH\ 1.0} - (Abs_{530} - Abs_{700})_{pH\ 4.5}$; Molecular weight of cyanidin-3-glucoside (MW)= 449.2 g/mole; Dilution factor (DF)= 7; Molar absorptivity of cyanidin-3-glucoside (E)= 26900 L/mole.cm; Cell path length (L)= 1 cm.

Total anthocyanin content of anthocyanin extract was also analyzed by pH-differential method, but anthocyanin extract did not need to be extracted. In this case, an aliquot of anthocyanin extract was mixed directly by solvent consisting of HCl 2M: ethanol 70% with volume ratio of 2/3.

Anthocyanin's destabilization kinetic (Jie *et al.*, 2013)

Anthocyanin degradation may be due to deglycolisation reaction and is followed by cleavage of anthocyanidin resulted from deglycolisation that will turn into aldehyde and phenolic acid. The kinetics of anthocyanin stability reduction (destabilization) during storage were simplified through the chemical reaction kinetics.

Anthocyanin → Degraded anthocyanin ..... (2)

The above reaction can be stated as:

A → C ..... (3)

The reaction rate can be written as follows:

$$(-r_A) = kC_A^{\ n} \quad \text{..... (4)}$$

By a batch system reaction approach, the destabilization kinetics can be evaluated as:

$$-\frac{dC_A}{dt} = kC_A^{\ n} \quad \text{..... (5)}$$

By integral method, the equation (5) can be changed to:

$$C_{A0}^{\ 1-n} - C_A^{\ 1-n} = (1-n)kt \quad \text{..... (6)}$$

C_{A0} was initial concentration of anthocyanin, t was reaction time, C_A was concentration of anthocyanin at time t, and n was reaction order. Especially for the first reaction order (n=1), the equation (5) can be changed to:

$$\ln C_A = \ln C_{A0} - kt \quad \text{..... (7)}$$

To get the value of the reaction rate constant (k) and to find out whether the value of the reaction rate constant can function as the temperature or not, we used the following Arrhenius equation:

$$k = Ae^{-\frac{E}{RT}} \quad \text{..... (8)}$$

By linearization, equation (8) can be changed to:

$$\ln k = \ln A - \left(E/RT\right) \quad \text{..... (9)}$$

A= frequency factor, T=temperature, E= activation energy.

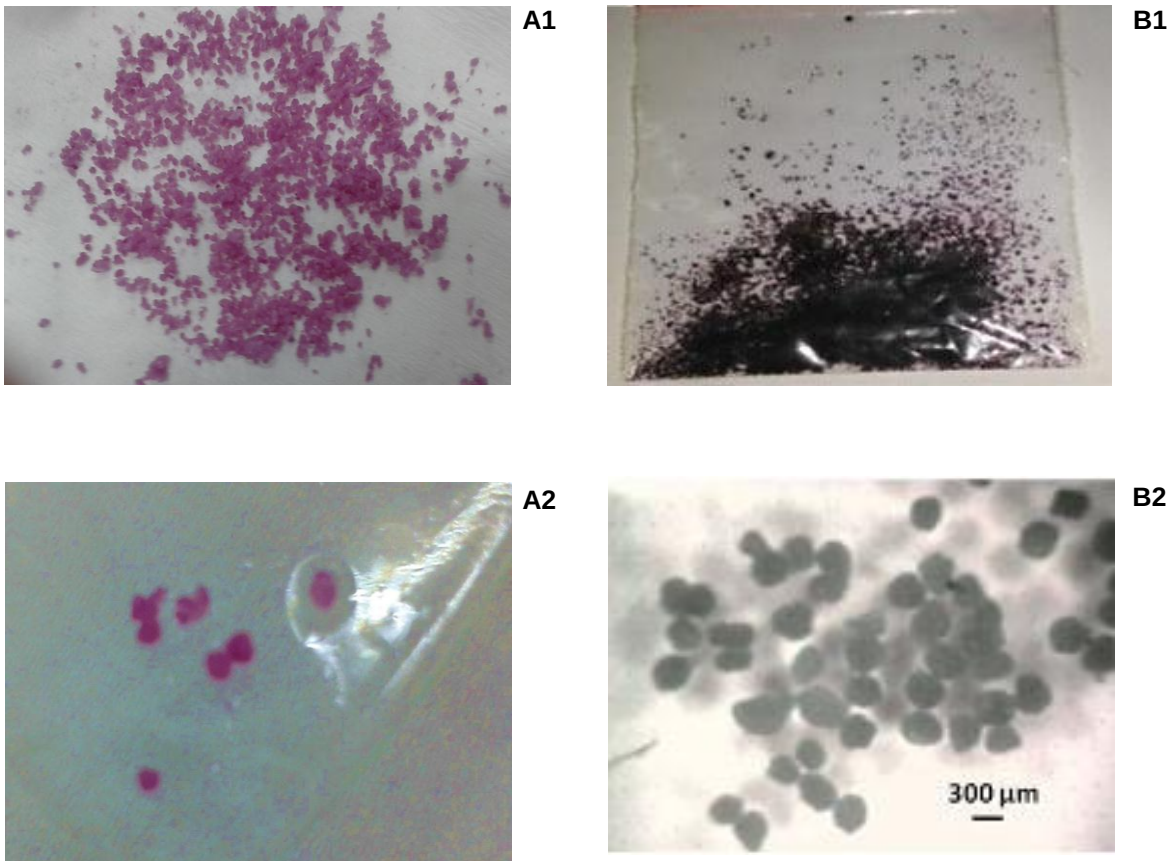
RESULTS AND DISCUSSION

Stability profile of encapsulated anthocyanin and anthocyanin extract

The appearance of encapsulated anthocyanin in wet and dried condition can be seen in Figure 1. It was clear that anthocyanin was encapsulated in a solid matrix. Meanwhile, the stability of encapsulated anthocyanin and also anthocyanin extract are shown at Figure 2. It can be seen at Figure 2 that at various storage temperature, anthocyanin concentration was found to faster decrease in encapsulated anthocyanin compared with anthocyanin extract. It indicated that encapsulated anthocyanin had smaller stability than anthocyanin extract in the high water activity of storage.

Acidity (pH) of system essentially influenced anthocyanin stability. Anthocyanin extract which had higher anthocyanin retention was possible due to the lower pH of extract *i.e*. 2.21 compared with encapsulated anthocyanin prepared at pH of 3.0. The low pH made the anthocyanin species to be in flavylium cation conformation which had the highest stability. Meanwhile, encapsulated anthocyanin had structure in both flavylium cations and quinoidal bases, which was likely to be the cause of lower stability.

The second factor affecting lower stability of encapsulated anthocyanin was the high water activity (a_w), which was 0.75. The high water activity which was supported by storage temperatures of 16, 25, 35, and 45°C reduced transition glass temperature (Tg) of the encapsulated anthocyanin. Therefore, encapsulated anthocyanin was shifted to rubbery state. Under these circumstances, the mobility of anthocyanin molecules (reactants) would increase and lead to the increase of anthocyanin degradation through several mechanism, *i.e.* hydrolysis of chemical, hydrolysis by microbes, the release of sugar moiety, or other possibilities (Garzón and Wrolstad, 2001).



Note: In wet condition (A1); In wet condition with magnification of 4x by digital camera (A2); In dried condition (B1); In dried condition with magnification of 65x by digital microscope (B2)

Figure 1. Appearance of encapsulated anthocyanin

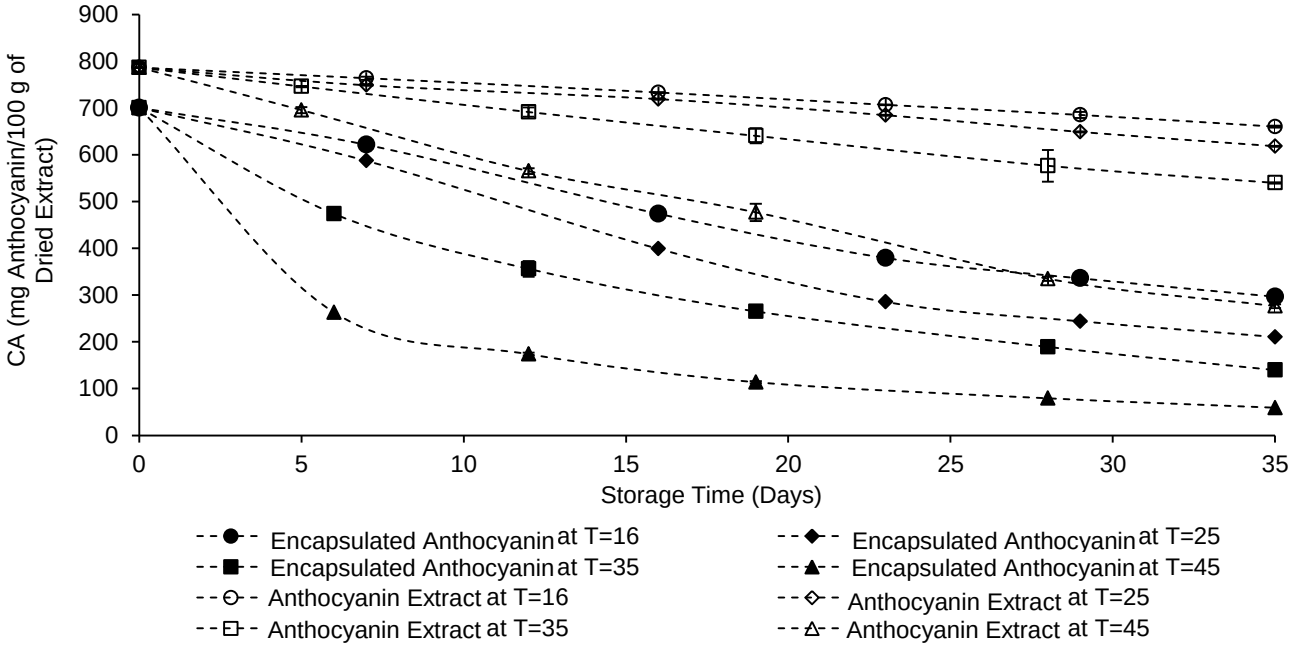


Figure 2. Stability of encapsulated anthocyanin and anthocyanin extract during storage at various temperatures

The other aspect was the possibility of an interaction between anthocyanin and NaTPP through the formation of a complex between the cation of flavylium and multivalent anion TPP. It was likely that the stability of anthocyanin in complex form differ from anthocyanin in the structure of the flavylium cation.

Kinetic of anthocyanin degradation

The degradation of kinetic of anthocyanin both in encapsulation system and extract are figured out on Figure 3 and 4 respectively. The figures correlate anthocyanin concentration (C_A) and time in first order kinetic which is stated by -ln (C_A/C_{A0}) vs time. It can be seen that first order reaction gives a good regression coefficient ($0,92<r^2<0,99$). Therefore, first order (n=1) was available for determining anthocyanin degradation. In the graph of -ln (C_A/C_{A0}) vs time, the reaction speed constant value (k) is obtained as a slope of graph. The greater k value was obtained in encapsulated anthocyanin. All the more so, the k value of encapsulated anthocyanin at 16°C was higher than that of anthocyanin extract at 45°C. Therefore, it is clear that the rate of anthocyanin degradation reaction in encapsulated anthocyanin was greater than that of in anthocyanin extract stored in water activity of 0.75.

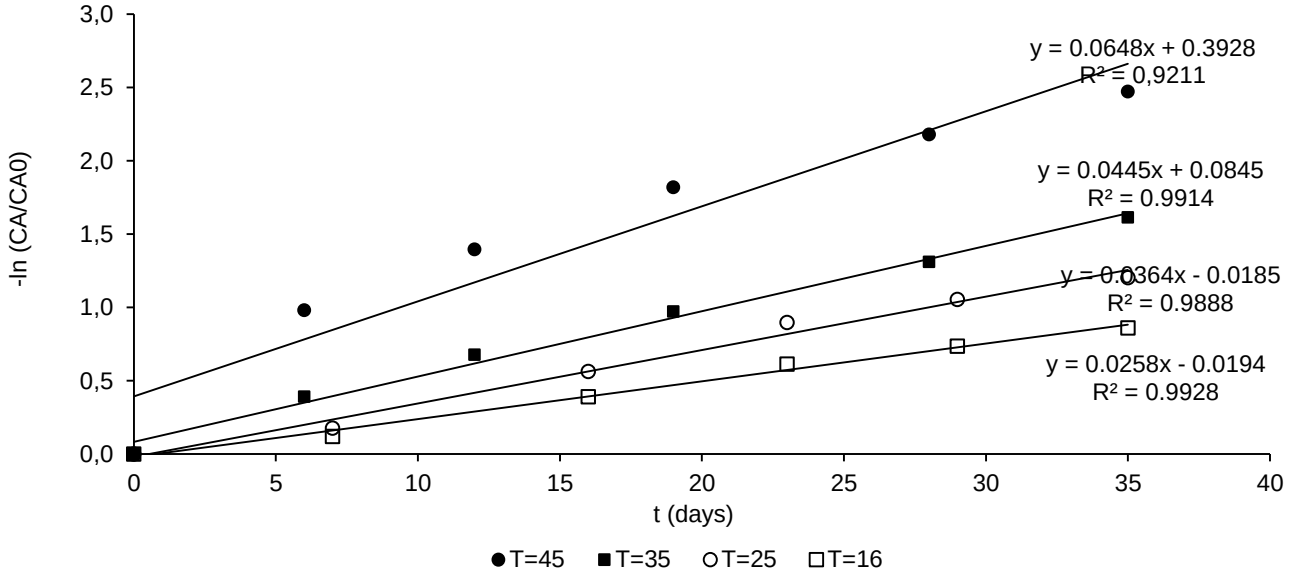


Figure 3. The kinetics of encapsulated anthocyanin degradation

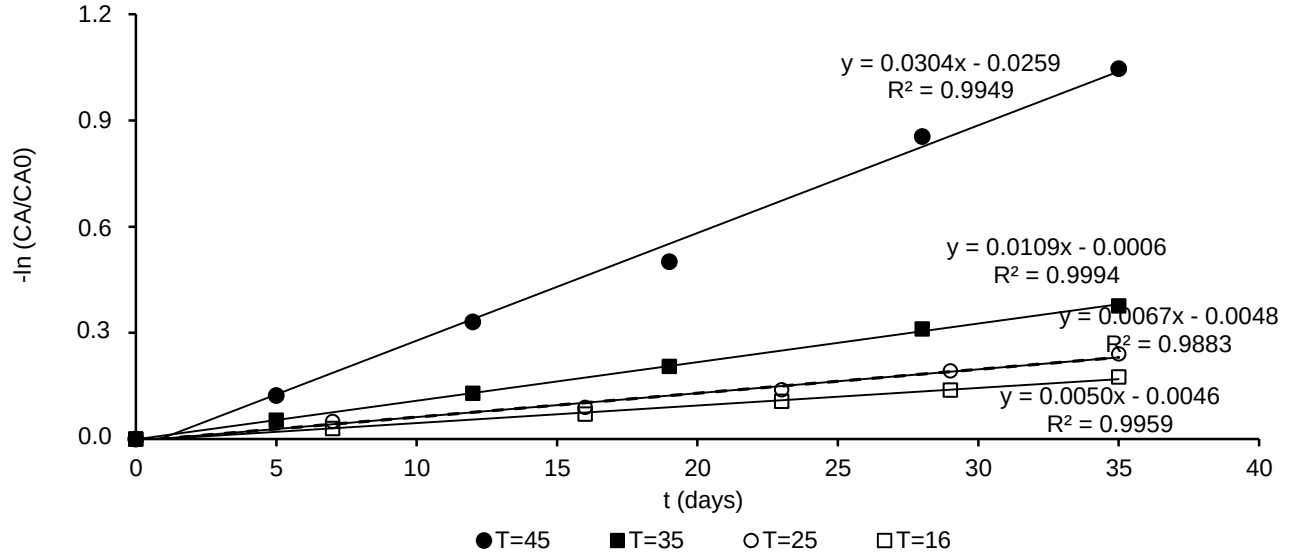


Figure 4. The kinetics of anthocyanin extract degradation

The greater k value of encapsulated anthocyanin might be due to pH factor in which encapsulated anthocyanin had higher pH than anthocyanin extract. It was agreed with the research of Oancea and Draghici (2013) that the increase of pH would improve anthocyanin degradation of kinetic constant value (k). Loypimai *et al.* (2016) also obtained that k value would be higher with increasing temperature and pH. The other factor was high water activity that allows the presence of encapsulated anthocyanin in rubbery state as had been explained before.

Anthocyanin degradation in encapsulated anthocyanin and anthocyanin extracts following first order kinetic were consistent with other studies. Jie *et al.* (2013) observed anthocyanin stability in purple sweet potato solution. Based on the study, it was found that anthocyanin stability followed first order kinetic. Nayak *et al.* (2011) obtained anthocyanin degradation in purple-fleshed potato following first order reaction kinetics model. Figure 3 and 4 indicate that the higher the temperature, the greater the rate of anthocyanin degradation. The reaction speed constant (k) increased with increasing temperature for encapsulated anthocyanin and anthocyanin extract. This means that k is a function of temperature (T) as is figured out at Figure 5.

The kinetic parameters of anthocyanin degradation in encapsulated and extracts can be seen at Table 1. The activation energy of anthocyanin degradation in encapsulated anthocyanin was 23.33 kJ/mole and the activation energy of anthocyanin degradation in the extract was 46.70 kJ/mole still in the same range as other studies. Jie *et al.* (2013) obtained that activation energy of anthocyanin degradation for purple sweet potato juice with pH of 2-6 was 66.5-111.57 kJ/mole. Chen *et al.* (2019) got almost the same results, namely the activation energy for purple sweet potato extract degradation with a pH of 3 that was 59.55 kJ/mole.

The activation energy in anthocyanin degradation of encapsulated anthocyanin was smaller than that of the anthocyanin extract. This indicates that only less energy was needed by encapsulated anthocyanin to initiate anthocyanin degradation. Meanwhile, the activation energy of anthocyanin extract obtained was greater, thus kinetic of anthocyanin degradation of extract was more sensitive to temperature compared with encapsulated anthocyanin. Another parameter that can be reviewed was the half-life ($t_{1/2}$), which is the time needed for anthocyanin degradation to occur by 50%. It can be seen from Table 1 that half-life of encapsulated anthocyanin was smaller than anthocyanin extract by a factor of 3 to 6 in the temperature range of 16-45°C. This convinces that the rate of anthocyanin degradation in encapsulated anthocyanin was greater than that in anthocyanin extract.

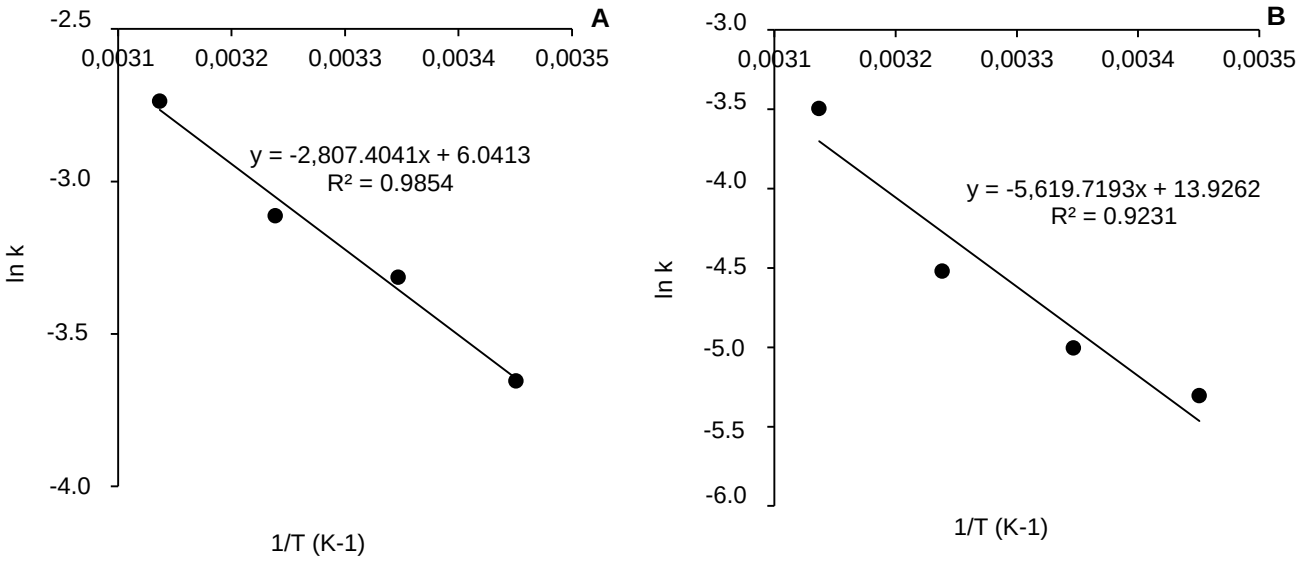


Figure 5. The relationship of k and T at encapsulated anthocyanin (A) and anthocyanin extract (B)

Table 1. The kinetic parameters of anthocyanin degradation in encapsulated anthocyanin and anthocyanin extract

Storage Condition Temperature (°C)	k (1/day)	A (Frequency Factor)	Ea (kJ/mole)	R^2	$t_{1/2}$ (Days)
Encapsulated Anthocyanin					
16	0.0259	420.44	23.33	0.9854	26.54
25	0.0364	420.44	23.33	0.9854	19.82
35	0.0445	420.44	23.33	0.9854	14.62
45	0.0648	420.44	23.33	0.9854	10.99
Anthocyanin Extract					
16	0.0050	$1.12x10^6$	46.70	0.9231	163.86
25	0.0067	$1.12x10^6$	46.70	0.9231	91.37
35	0.0109	$1.12x10^6$	46.70	0.9231	49.69
45	0.0304	$1.12x10^6$	46.70	0.9231	28.08

CONCLUSION

Stability of encapsulated anthocyanin was investigated regarding with lower stability of anthocyanin at high water activity and high temperature. The testing stability was conducted at water activity of 0.75 and various temperature at 16, 25, 35, and 45°C. The kinetic parameters including kinetic constant (k), reaction order, and half-life ($t_{1/2}$) were also studied. For comparison, anthocyanin extract was also tested for its stability using the same condition. This study revealed that encapsulated anthocyanin had lower stability than anthocyanin extract. The result was proven by higher kinetic constant and lower half-life. The lower stability of encapsulated anthocyanin had been induced by higher pH of encapsulated anthocyanin than anthocyanin extract. It was conclusive that the pH affected the amount of flavylium cation conformation. In addition, high water activity also caused lower stability by reducing glass transition temperature (Tg) by which encapsulated anthocyanin was shifted to rubbery state. Anthocyanin degradation in encapsulated anthocyanin and anthocyanin extract followed first order kinetic. The degradation kinetic constant of encapsulated anthocyanin was stated as k= 420.44 exp (-23.33/RT) and in anthocyanin extract was stated as k =$1.12x10^6$ exp (-46.70/RT). Based on the stability test, the application of encapsulated anthocyanin was not suitable for wet-type food product. Further study about stability of the encapsulated anthocyanin in lower water activity is essential to do as a way to investigate the application of encapsulated anthocyanin in dry-type food product.

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