Faculty of Engineering and Science

Enhanced solubility and stability of antioxidants from *Mangifera pajang* in natural deep eutectic solvent of choline chloride/ascorbic acid

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DECLARATION

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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Date : 25/5/2020

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ABSTRACT

Mangifera pajang (M. pajang) or best known as bambangan or mawang (in local word) is an indigenous fruit widely found on Borneo Island. Recent studies have shown that *M. pajang*'s fruit wastes (peels and kernels) contain large quantities of antioxidant compounds which demonstrates noteworthy health beneficial effects. Nonetheless, the application of these antioxidant extracts is often impeded by its poor solubility and stability. For that, numerous approaches such as nanoemulsion, liposomes, hydrogels, ionic liquid-based formulations and natural deep eutectic solvent-based formulations have been designed to improve the solubility and stability of these antioxidant compounds. Amongst, natural deep eutectic solvent-based formulations receive tremendous attentions as they demonstrated great potential in the food and pharmaceutical application. For this reason, the search for effective natural deep eutectic solvent has become a major research endeavor.

Recently, natural deep eutectic solvent which composed of choline chloride and ascorbic acid was synthesized for improving the solubility of drug (dexamethasone) and this formulation path the direction of this present work as the components used to synthesize the natural deep eutectic solvent are green from the point of view of green chemistry. Moreover, these two components are often used in food, nutraceutical and pharmaceutical industry, such that choline chloride is used as nutrients and dietary supplements and ascorbic acid is well-known vitamin. Given these advantages, the application of the choline chloride/ascorbic acid natural deep eutectic solvent (CHCL/AA NADES) in food, nutraceutical and pharmaceutical industry is of great interest and seems promising. However, the application of this novel CHCL/AA NADES are rather little. Particularly, the role of CHCL/AA NADES as a solubilizing and stabilizing agent of antioxidant compounds remain unknown and the information on the complexation of this natural deep eutectic solvent on the antioxidant compounds is strongly desired. Therefore, this study addresses the problem of poor solubility and stability of antioxidant compounds by incorporating them in a green CHCL/AA NADES. This study aims to (1) optimize the extraction parameters in the ethanol extraction of antioxidant compounds from the fruit wastes of *M. pajang*, (2) prepare

and characterize the choline chloride/ascorbic acid natural deep eutectic solvent, (3) investigate the solubilization ability of choline chloride/ascorbic acid natural deep eutectic solvent and (4) evaluate the stabilizing capacity of choline chloride/ascorbic acid natural deep eutectic solvent under combined effects of pH and temperature by modelling the degradation kinetics.

This research was performed through experimental works and development of degradation kinetic models. The experiment started with the ethanol extraction of antioxidant compounds from the fruit wastes of *M. pajang*. The effects of ethanol solvent concentration (0 - 100%), extraction temperature (30 - 60℃) and shaking speed (100 - 300 rpm) were studied and the optimum extraction conditions were obtained by using the centered composite design (CCD). The model obtained was highly significant with high coefficients of determination (R^2) of 0.9714. The optimum extraction parameter were 54% ethanol concentration, 51℃ extraction temperature and 178 rpm of extraction shaking speed. Under these optimum conditions, the extract with maximum antioxidant activity (11.11 ± 0.06 mg AEAC/g, DPPH free radical scavenging effect of 91.09%) was obtained and this was comparable to the predicted value by the model (11.38 ± 0.00 mg AEAC/g, DPPH free radical scavenging effect of 91.57%).

Following that, CHCL/AA NADES was prepared and characterized by using polarized optical microscopy (POM), proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and Fourier transform infrared spectroscopy (FTIR). These analyzes confirmed the formation of CHCL/AA NADES, where the POM analysis suggeted that the formation of the CHCL/AA NADES at molar ratio of 2:1 was completely liquid. On the other hand, ^{1}H NMR, which is often used to identify the distribution of protons manifested the the high purity of mixture and the FTIR assay, which is used to identify the functional groups of mixture, confirmed the interaction of hydrogen bonding between the choline chloride and ascorbic acid. The solubility of antioxidant extracts in water and choline chloride/ascorbic acid natural deep eutectic solvent with different water contents (10 - 50 wt%) were also investigated. It was observed that the antioxidant extracts were most soluble in CHCL/AA NADES with 10 wt% of water

and the concentration of antioxidant extracts was found to be approximately 15% and 4% more as compared to water and pure CHCL/AA NADES, respectively. The positive effect of water on NADES can be related to the reduced viscosity of NADES, as the viscosity of CHCL/AA NADES system with 10 wt% of water decreased up to 74% as compared to pure NADES system. Moreover, it was also found that all the tested CHCL/AA NADES enhanced the antioxidant capacity of antioxidant extracts by 1.3 – 14.64% as compared to the antioxidant extracts in aqueous form. This finding highlights the role of natural deep eutectic solvent as an antioxidant capacity enhancer.

The degradation of antioxidant extracts in its neat form and in the choline chloride/ascorbic acid natural deep eutectic solvent were studied under combined effects of selected temperature (25℃, 40℃, 60℃ and 80℃) and pH (3.0 - 8.0). The concentration of the antioxidant extracts was found to decrease gradually with increasing pH and temperature. For the antioxidant extracts in aqueous system, the lowest (4×10^{-4} min^{-1}) and highest degradation rate constant (6×10^{-3} min^{-1}) were obtained at pH 3.0, pH 4.0 at 25℃ , and pH 8.0 at 80℃, respectively, whereas the lowest and highest degradation rate constant for antioxidant extracts in the CHCL/AA NADES system at similar condition were 3×10^{-4} min^{-1} and 5.5×10^{-3} min^{-1}, respectively. This finding suggests the ability of CHCL/AA NADES in protecting the antioxidant extracts against extreme temperature and pH. Furthermore, the degradation of antioxidant extracts followed the first-order kinetics, which is in accordance with numerous previous studies. The kinetic parameters for both systems were modelled using the Arrhenius equation. The largest activation energy value and pre-exponential factor for both systems are determined at pH 8.0, suggesting that the degradation of antioxidant extracts are more likely to occur at higher pH. The half-life value for both systems were also determined and the result suggests that CHCL/AA NADES system exhibited higher half-life value (4.17 – 25%) than the aqueous system. Aside from that, a new multi-term degradation model was proposed to estimate the degradation profile and it was found to have a better fit between the model predictions and the experimental results, as compared to the first-order kinetic profile. Besides, the proposed multi-term degradation model allows better estimation of half-life value, as clear deviation of half-life value for both aqueous and CHCL/AA NADES system was observed. Remarkably, the proposed model confirms that the antioxidants in

CHCL/AA NADES have longer half-life duration at all tested conditions. Such finding once again supports the potential of CHCL/AA NADES in protecting the antioxidant compounds. The protecting effect of CHCL/AA NADES system can be ascribed to the formation of multiple hydrogen bonding interactions between the antioxidant extracts and the CHCL/AA NADES, as proven by the shifting vibration absoprion bands observed from FTIR assay. Additionally, it is interesting to find out that the antioxidant extracts that solubilized in the CHCL/AA NADES system formed a nano-scale cluster structure, as depicted by the transmission electron micrographs (TEM). The antioxidants are found to aggregate and well-rounded in the center of CHCL/AA NADES, which denotes that CHCL/AA NADES could potentially act as a nanocarrier that provides protection to the antioxidant extracts. Moreover, the antioxidant capacity analysis was also performed on the antioxidant extracts in CHCL/AA NADES using DPPH assay. It was found that the antioxidant activity fluctuated throughout the treatment period and the changes of antioxidant activity under combined effect of pH and temperature were insignificant.

PUBLICATION

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
ANOVA	Analysis of variance
API	Active pharmaceutical ingredient
CAT	Catalase
CCD	Centered composite design
CD	Cyclodextrin
CHCL	Choline chloride
CHCL/AA NADES	Choline chloride/ascorbic acid natural deep eutectic solvent
DES	Deep eutectic solvent
DPPH	2,2-diphenyl-1-picrylhydrazyl
EFSA	European Food Safety Authority
FRAP	Ferric reducing activities power
FTIR	Fourier transform infrared spectroscopy
GRAS	Generally Recognized as Safe
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
LUV	Large unilamellar vesicle
MARDI	Malaysia Agriculture Research and Development Institute
MLV	Multilamellar vesicle
O/W	Oil-in-water
POD	Peroxidase
POM	Polarized optical microscopy

ROS	Reactive oxygen species
RSM	Response surface methodology
SLN	Solid lipid nanoparticles
SOD	Superoxide dismutase
SSE	Sum of square
SUV	Small unilamellar vesicle
TEM	Transmission electron microscopy
THEDES	Therapeutic deep eutectic solvent
W/O	Water-in-oil
^{1}H NMR	Proton nuclear magnetic resonance

LIST OF SYMBOLS

A	Frequency factor of reaction/ pre-exponential factor
C	Concentration of compounds
C_0	Initial concentration of compounds
$\tilde{C}$	Logarithm of concentration and initial concentration of compounds
D	Decimal reduction time
$\frac{dC}{dt}$	Rate of degradation of compound
E_A	Activation energy (kJ/mol)
g	Gram
kg	Kilogram
kJ	Kilojoule
K	Rate constant
k'	Pseudo first-order rate constant
L	Liter
ℓ	Number of degradation term
ln k	Logarithm of rate constant
mg	Milligram
min	Minutes
ml	Milliliter
nm	Nanometer
n	Order of the degradation
Q_{10}	Temperature coefficient
R	Universal gas constant (8.314 J/mol. K)

R^2	Coefficient of determination
τ_j	Time constant
t	Time
$t_{1/2}$	Half-life
T	Absolute temperature (K)
1/T	Reciprocal of absolute temperature
Y	Dependent variable
β_0	Intercept coefficients
β_1, β_2 and β_3	Linear coefficients
β_{11}, β_{22} and β_{33}	Quadratic coefficient
β_{12}, β_{13} and β_{23}	Interaction coefficients
℃	Degree Celsius

CHAPTER 1
INTRODUCTION

1.1 Background

Oxidation reactions are essential to many living organisms in biological system from which free radicals are produced. These free radicals are derived from oxygen which are commonly known as reactive oxygen species (ROS). ROS such as superoxide anion radical, hydroxyl radical and hydrogen peroxide are important in regulating the growth, proliferation and differentiation of cells. They also help to control the immune responses (Yang et al. 2013). Nonetheless, ROS can be overproduced in human body. Excessive ROS can cause oxidative stress which results in oxidative damage to the biomolecules (Patlevič et al. 2016). They can also disrupt the cellular structures and their functionality. Critically, excess ROS lead to cell death, tissues damages and even development of cancers and other human diseases (Halliwell and Gutteridge 2015, Bergamini et al. 2004, Chatterjee, Jungraithmayr, and Bagchi 2017, Liou and Storz 2010). Fortunately, researchers have discovered that the consumption of dietary antioxidants can reduce the oxidative stress in human body (Neha et al. 2019, Halliwell and Gutteridge 1995, Poljsak, Šuput, and Milisav 2013, Sarangarajan et al. 2017).

Antioxidants are defined as molecules that are able to stabilize, inhibit, deactivate and scavenge free radicals, thus protecting the human body against oxidative damage (Oroian and Escriche 2015). Generally, antioxidants can be synthesized through either enzymatic or non-enzymatic pathway. Examples of enzymatic antioxidants are superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) while examples of non-enzymatic antioxidants are ascorbic acid (vitamin C), polyphenols and carotenoids (Zang et al. 2017, Shabana et al. 2017, Bahorun et al. 2006). Some researchers have pointed out that a high intake of antioxidant compounds can reduce the risk of developing numerous chronic diseases such as inflammation (Chapple 1997), cancer (Singh et al. 2018), cardiovascular diseases (Mangge et al. 2014) and neurodegenerative diseases (Gelain et al. 2012), which are due to over expression of free radicals through cellular metabolism (Neha et al. 2019, Sarangarajan et al. 2017).

For instance, in a pilot, 30 subjects with colorectal tumours consumed 60g of freeze-dried black raspberries (one of the highest-ranked fruits with excellence antioxidant properties) until their scheduled surgery date. Analysis demonstrated the proliferation and angiogenesis biomarkers were greatly reduced (Stoner, Wang, and Casto 2008).

After discovering of the role of antioxidants in reducing or preventing the oxidative damage, numerous antioxidant sources have been studied (Oroian and Escriche 2015). In particular, Agudo et al. (2007) pointed out that many of the underutilized indigenous fruits possess good antioxidant properties that might be useful as phytomedicine. Also, indigenous fruits exhibit important economic and nutritional value since they are part of the diet for local people (Hegazy et al. 2019). This brings to more varieties of tropical fruit species being studied and it was found that some underutilized indigenous fruit such as dabai (*Canarium odontophyllum*), cerapu (*Garcinia prainiana*), jambu mawar (*Syzygium jambos*) and rambutan (*Nephelium lappaceum*) previously unknown and unavailable has drawn great attention and become available in the local markets (Shakirin et al. 2010, Palanisamy et al. 2008, Ikram et al. 2009). However, there are still a lot of potential underutilized tropical fruit has not been explored. Particularly, *M. pajang* or commonly known as bambangan or mawang (in Malay) is found to be one notable underutilized fruit by the fact that they are locally available but universally erratic. They are abundant and growing wildly in the backyards, orchards and rain forest of Borneo Island (Sabah and Sarawak in Malaysia, Brunei, and Kalimantan in Indonesia) (Hassan, Ismail, Hamid, et al. 2011). Recently, the phytochemical properties of *M. pajang* have been studied (Abu Bakar et al. 2009). Research has shown that the fruit wastes of the *M. pajang* (kernels and peels) gave highest phytochemical contents (mainly phenolic acids) per fruit. The phytochemical contents of the fruit wastes are found to be superior to other commercialized fruits such as mango, banana and dabai (Olivas-Aguirre et al. 2017). For that, Prasad et al. (2011) studied the effects of solvent extraction parameters on the recovery of antioxidant compounds from the peels of *M. pajang* fruit and found that at optimum extraction parameters (68% of ethanol concentration, 56℃ and 31.8 mL/g of liquid-to-solid ratio), the extracts with maximum antioxidant capacity can be obtained. Moreover, a recent review paper highlighted the antioxidant properties and economic potential of *M. pajang* (Jahurul et al. 2019). Despite their potential to be processed as a valuable

nutraceutical or food product, the fruit wastes of *M. pajang* have been scarcely exploited commercially, i.e., they are often discarded. To-date, there is still no report on research to exploit these cheap fruit wastes to nutraceutical or antioxidant product.

Prior to the development of nutraceutical or antioxidant product, extraction of the antioxidant products is required. The extraction step is important as the yield and bioactivity of extract are often correlated with the applied extraction methods (Castro-López et al. 2017). For this reason, various extraction methods have been developed in order to achieve a few objectives: 1) isolate the antioxidant compounds that are potentially beneficial to human health; 2) improve the yield of these desired antioxidant compounds; 3) increase the bioactivity of crude extracts (Azmir et al. 2013). Nevertheless, the solubility and stability of raw antioxidant extracts often impede their functionality. Poor solubility of antioxidant compounds can lead to limited absorption at the target site, which results in low therapeutic effects (Morry, Ngamcherdtrakul, and Yantasee 2017). On the other hand, the extracted antioxidant compounds are very sensitive to environmental conditions such as heat and can be easily degraded during storage or food processing (Aguiar, Estevinho, and Santos 2016, Davidov-Pardo and McClements 2015). For these reasons, the extracted natural antioxidant compounds are often encapsulated or protected through various formulation approaches such as nanoemulsion, hydrogel, solid lipid nanoparticles, liposomes and others (Aditya, Espinosa, and Norton 2017, Gleeson, Ryan, and Brayden 2016). Aside from these formulation approaches, solubilisation of bioactive compounds in the ionic liquid and deep eutectic solvent are also found to be promising approaches as they successfully enhanced the bioavailability of the bioactive compounds (Egorova and Ananikov 2018, Pedro et al. 2019).

Among these formulation strategies, deep eutectic solvent (DES) has recently emerged rapidly in diverse fields including pharmaceutical and biomedical applications, owning to their green characters such as high biodegradability, negligible toxicity, low cost and can be easily prepared without any purification step. Moreover, the physicochemical properties of DES could be easily altered to suit vast applications (Tomé et al. 2018, Cunha and Fernandes 2018, Mbous et al. 2017). DES was first

introduced by Abbott et al. (2003), as a mixture composed of hydrogen donor (HBD) and hydrogen acceptor compounds (HBA). Following that, Choi et al. (2011) prepared the eutectic mixtures of two or more natural compounds and denominated them as natural deep eutectic solvent (NADES). Since NADES are synthesized by natural compounds, they have no adverse effects and compatible with food, pharmaceutical and cosmetic formulations (Benvenutti, Zielinski, and Ferreira 2019). Owning to these properties, the number of publications adopting these solvents as a solubilization vehicle for bioactive compounds was exponentially growing (Faggian et al. 2016, Liu, Zhang, Chen, Friesen, et al. 2018, Dai et al. 2013, Morrison, Sun, and Neervannan 2009, Sut et al. 2017, Shamseddin et al. 2017, Silva et al. 2018). These reports confirmed the effectiveness of NADES in improving the solubility of bioactive compounds and can be used as solubilization enhancers in the development of drug delivery system. Among these reports, the NADES formulation designed by Silva et al. (2018) is found to be interesting as the components used to synthesize the NADES, choline chloride and ascorbic acid are green from the point of view of green chemistry. Such that choline chloride is a non-toxic nutrition additive which is approved under Council Directive 70/524/EEC8 (Radošević et al. 2015), where the benefits of ascorbic acid (which is also referred to as Vitamin C) as dietary antioxidant are universally recognized. Predominantly, ascorbic acid readily scavenges reactive oxygen species (ROS), modulate neurological functions and prevent scurvy (Cheng et al. 2018).

It is evident that the NADES composed of choline chloride and ascorbic acid offer exciting possibility for solubilizing vehicle for antioxidant compounds. Nevertheless, in a period when much attention is focused on the solubility of bioactive compounds, approaches to improve the stability of antioxidant compounds has been sometime overlooked. To-date, the information reporting the viability of NADES to enhance the stability of bioactive compounds remain scarce. In this context, the evaluation of the feasibility of choline chloride/ascorbic acid natural deep eutectic solvent (CHCL/AA NADES) to improve the stability of antioxidant compounds during exposure to environmental conditions or food processing is desired, and such is the motivation of this project.

1.2 Research gaps

Several research gaps can be summarized as follows:

1. The fruit wastes of *M. pajang* (kernels and peels) contain higher level of total antioxidant concentration compared to its flesh (Abu Bakar et al. 2009). Nevertheless, to-date, the potential of the fruit wastes of *M. pajang* are rarely exploited for economic purposes (Jahurul et al. 2019). In view of the remarkable antioxidant properties of fruit wastes of *M. pajang*, there is a bright prospect to utilize the fruit wastes as natural sources of antioxidant products. For an economical utilization of *M. pajang's* antioxidant compound in nutraceutical industry, an effective extraction of the phytochemicals involved is crucial before formulating the extract into a desired product. Previous study performed by Prasad et al. (2011) focused only on the effect of ethanol concentration, extraction temperature and liquid-to-solid ratio on the recovery of antioxidant compounds from the peels of *M. pajang*. However, the kernels of fruits which exhibit higher antioxidant capacity is not utilized in their study. Moreover, the effect of extraction shaking speed on the extraction performance is also overlooked. Therefore, it is significant to perform subsequent research on the effect of shaking speed on the extraction of antioxidant compounds from both kernels and peels of *M. pajang*.

2. The synthesis of CHCL/AA NADES successfully enhanced the solubility of dexamethasone by several order, as compared to the powder form of dexamethasone (Silva et al. 2018). The designation of this NADES system is of great intrest as the components used are green, but the application of this novel NADES system is rather limited. No study to-date has provided information regarding the effect of this NADES system on the solubility of antioxidant compounds.

3. The stability of extracted antioxidant compounds is greatly affected by the environmental conditions and industrial processing and questions addressing the stability received very little attention. Dai, Verpoorte, and Choi (2014) found that sugar-based NADES successfully stabilized the carthamin (pigment with antioxidant properties) under various conditions, including high

temperature, exposed light and storage time. Their finding paths a research direction for present study, stabilizing the antioxidant compounds by using NADES system. Nonetheless, the application of NADES on stabilizing the antioxidant compounds are limited. Particularly, there is no study reported on the effect of complexation of the CHCL/AA NADES on the stability of antioxidants. The effects of processing conditions on the stability of antioxidants in CHCL/AA NADES remain unclear. Such information is crucial for the assessment of health benefits of antioxidant compounds, and their lack could hinder the design of effective antioxidant product that can provide maximum benefits upon consumption.

4. Modeling of the degradation kinetics is relevant to evaluate the influence of processing on the loss of antioxidant activity. It was realized that most of the study evaluated the degradation kinetics on the extracted antioxidant compounds, but the investigations on the antioxidant-loaded formulations are rather limited. Therefore, accurate knowledge on the kinetic parameters is essential to predict the stability of antioxidants in CHCL/AA NADES system during food processing, especially under the combined effect of pH and temperature. This finding could aid in the designation of optimum food process and provides desired storage conditions for maximum preservation of the antioxidant compounds.

1.3 Research Questions

Several research questions arise when addressing the research gaps mentioned above:

1. What are the effects of extraction shaking speed on the recovery of antioxidant compounds from the fruit wastes of *M. pajang*? Is there any interactive effect of shaking speed with the other extraction parameters on the antioxidant activity?
2. What are the optimum conditions to develop a stable CHCL/AA NADES? What is the right molar ratio of components to formulate the CHCL/AA NADES? Is there any hydrogen bonding interaction between the choline chloride and ascorbic acid? What is the ionization state of proton of the

CHCL/AA NADES mixture?

3. How does the incorporation of CHCL/AA NADES improve the solubility and antioxidant activity of antioxidant compounds from *M. pajang*? What is the effect of addition of water to the CHCL/AA NADES on the solubility and antioxidant properties of antioxidant extracts?
4. What is the influence of pH, temperature and their combined effects on the stability of antioxidants in the CHCL/AA NADES? What is the degradation pattern of antioxidants in the aqueous and CHCL/AA NADES under combined pH and temperature? How does the degradation profile look like as compared to the degradation profile of crude antioxidant compounds? What are the kinetic parameters for the degradation process?

1.4 Aim and Objectives of the study

The main scope of this present study is to enhance the solubility and stability of antioxidant compounds from the fruit wastes of *M. pajang* in choline chloride/ascorbic acid natural deep eutectic solvent. In order to address the aim of the study, the following objectives are developed:

i. Determine the optimum parameters in the ethanol extraction of antioxidant compounds from the fruit wastes (peels and kernels) of *M. pajang*.
ii. Synthesize and characterize the choline chloride/ascorbic acid natural deep eutectic solvent.
iii. Evaluate the solubility of antioxidant extracts in the aqueous form and the choline chloride/ascorbic acid natural deep eutectic solvent and explore the effect of the water inclusion in the choline chloride/ascorbic acid natural deep eutectic solvent on its solubilization capacity.
iv. Evaluate the stability of antioxidant extracts in the aqueous form and choline chloride/ascorbic acid natural deep eutectic solvent under combined effects of pH and temperature by modelling the degradation kinetics.

1.5 Significances of the study

The significances of this research can be viewed in both scientific research and practical application. For the scientific research contribution, the dissolution of antioxidant extracts in CHCL/AANADES could help to pave some new research directions or insights on improving or overcoming the limitations of some bioactive compounds, namely solubility and stability. The novel CHCL/AA NADES can be potentially use to carry the antioxidant compounds for pharmaceutical and nutraceutical applications. Furthermore, this study also brings attentions on a low-value underutilized indigenous *M. pajang* fruit from which it is able to produce products of high-value antioxidants from non-edible fruit part. Such utilization can inspire other researchers in exploiting many other indigenous plants in the whole of Borneo which possess all sort of pharmacological properties that are beneficial to both human and animal.

For the practical applications, this study helps to enhance the bioaccessibility of antioxidant compounds by improving its solubility and stability through utilizing CHCL/AA NADES. Besides that, the establishment of kinetic model to describe the stability of antioxidant compounds in the synthesized NADES allows derivation of kinetic parameters and hence, predict the loss of antioxidant activity. The obtained data enable design of optimized food processing conditions which could lead to maximized preservation of antioxidant compounds.

Additionally, a successful commercialization of fruit wastes of *M. pajang* as an antioxidant product will helps the local community to acquire self-sufficiency and also enhance the economic in the local region. For example, in rural community, those who have these fruit trees can enjoy greater economic benefits as both edible and nonedible parts have values. Moreover, it provides sufficient nutrient and can be used as an excellent compact food for people of all ages and lifestyles. Also, the availability of *M. pajang*'s antioxidant product in the market will provide more choices of nutraceutical products to the consumers and bring about improved health.

1.6 Layout of the thesis

This thesis consists of five chapters. The layout of this thesis is illustrated as Figure 1.1. Chapter 1 provides a brief introduction of the project and emphasize the research gaps, research questions, objectives and the significances of the project.

Chapter 2 reviews the recent experimental reports, findings and knowledge from other researchers. It covers the backgrounds of the fruit *M. pajang*, numerous types of extraction methods, limitations of extracted antioxidant compounds and the common formulation strategies for enhancing the solubility and stability of antioxidant compounds. Following that, the kinetic model equations and the recent modelling of degradation kinetics of antioxidant compounds are scrutinize.

Chapter 3 describes the research methodologies to achieve the objectives of this study. The experiment procedures are written in details.

Chapter 4 presents the results and discussions. The chapter starts with the reports on the optimization of solvent extraction of antioxidant compounds from fruit wastes of *M. pajang*. In particular, the effects of solvent extraction parameters on the antioxidant activity is discussed. Following that, the synthesis and characterization of CHCL/AA NADES and the solubility testing of antioxidant compounds in the aqueous and CHCL/AA NADES are performed. Aside from that, the stability testing of antioxidant compounds in aqueous and CHCL/AA NADES system under combined effects of temperature and pH are studied. Using the obtained data, a kinetic model is established and the kinetic parameters are determined.

Chapter 5 gives conclusions and a few recommendations for this project. The main findings of the objectives are summarized and the scopes of future works are recommended.

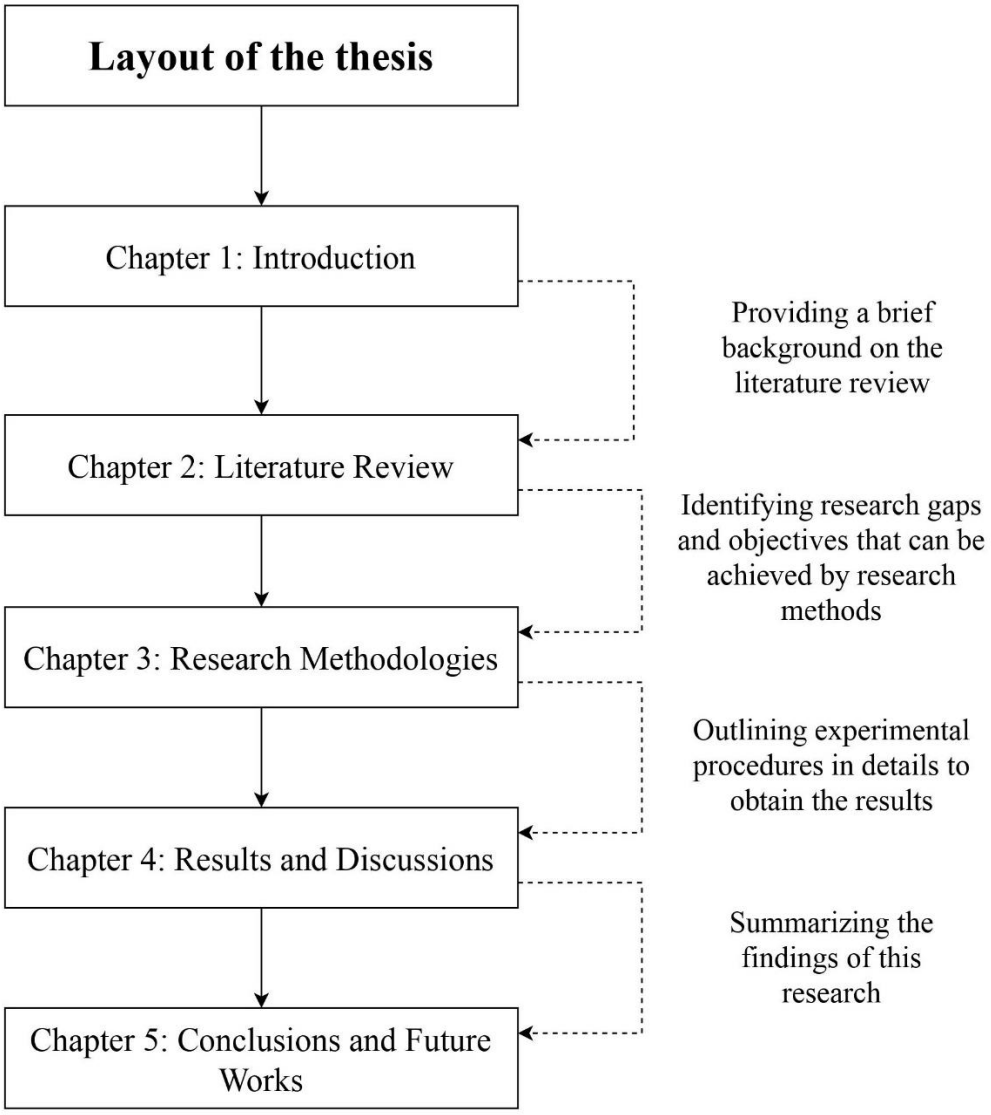


Figure 1.1 Layout of the thesis

CHAPTER 2
LITERATURE REVIEW

2.1 Overview

In today's world, there is a huge interest among consumers on antioxidant that can promote health and well beings. Substantial research have been carried out and evidently confer on the health-promoting and disease-preventing properties of the antioxidant (Neha et al. 2019, Shashirekha, Mallikarjuna, and Rajarathnam 2015, Zou et al. 2016). In this context, the advantages of dietary antioxidants have caused a renewed interest to discover under-utilized fruit species with high antioxidant activity. Particularly, *M. pajang*, an under-utilized fruit was found to contain high antioxidant compounds, bringing about a bright prospect to exploit as a natural source of antioxidant products (Jahurul et al. 2019). However, it is known that the application of extracted antioxidant compounds are often hinder by its low solubility and stability (Tapia-Hernández et al. 2018). Therefore, it is extremely significant to develop a prospective approach to enhance the solubility and stability of antioxidant compounds from *M. pajang* for maximum therapeutic effects.

The aim of this chapter is to summarize the current knowledge on improving the solubility and stability of antioxidant compounds. The literature review starts with descriptions of the *M. pajang* fruit and its antioxidant properties, follows by recent advancement of solvent extraction method. Aside from that, the challenges of extracted antioxidant compounds are also discussed. In addition, the common formulation approaches to enhance the solubility and stability of antioxidant compounds are also reviewed. Thereafter, the model describing the degradation of antioxidant compounds and recent studies on the degradation kinetics are presented.

2.2 Background of *M. pajang* (Bambangan)

M. pajang or commonly known as ‘bambangan’, ‘mawang’, ‘embang’, ‘buah pangin’ and ‘membangan’ (local names) is found to contain high level of antioxidant compounds which are known to confer health benefits (Abu Bakar et al. 2011, Bakar and Fry 2013). Its fruit size is three times larger, has thicker peel and bigger seed compared to commercial mango (*Mangifera indica*). *M. pajang* weighs about 0.5 - 1 kg or more, and is the largest known in the *Mangifera* fruits. The fruits are brown in colour, ovoid in shape, juicy and possess pleasant aromatic flavour and smell. It can be grown up in many types of soil while the tree can grow up to 35 m tall and bear up to hundreds of fruits during fruiting season (October to February each year) (Khoo et al. 2010, Ibrahim, Prasad, and Hamid 2010, Ibrahim et al. 2013). The proximate nutrient composition of the *M. pajang* per 100 g of edible portion was reviewed by Ibrahim, Prasad, and Hamid (2010), where they found that the fruit contain 1.13% protein, 1.98% fat, 21.02% carbohydrate and 5.26% total fibre. A whole fruit of *M. pajang* is illustrated in Figure 2.1.

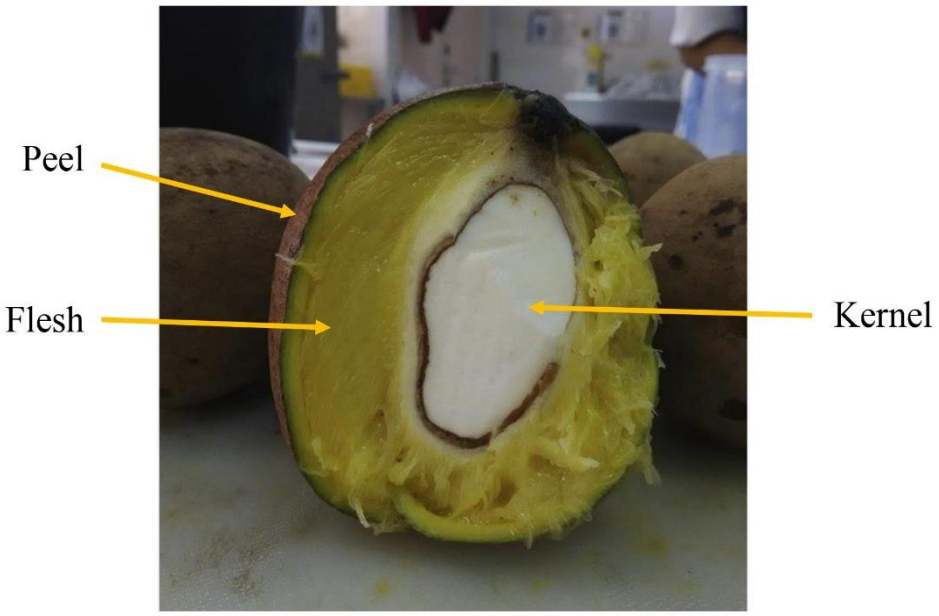


Figure 2.1. Whole fruit of *M. pajang* (Jahurul et al. 2019)
(Reproduced with permission – Appendix H)

2.2.1 Antioxidant capacities

Few studies were conducted to examine the antioxidant capacities of *M. pajang*. The antioxidant compounds in the kernel, peel and flesh of *M. pajang* are summarized in Table 2.1.

Table 2.1 Phytochemical content in different part of *M. pajang* (Abu Bakar et al. 2009) (Reproduced with permission – Appendix H)

Sample (Parts of fruit)	DPPH Free radical scavenging (mg AEAC/g)	FRAP (Ferric reducing antioxidant power) (μM/g)	Total phenolic (mg GAE/g)	Total flavonoid (mg RE/g)	Total anthocyanins (mg c-3-Ge/100g)
Flesh	9.94±0.22	150.00±1.44	5.96±0.34	0.07±0.00	1.47±0.28
Peel	20.32±0.10	343.17±7.25	22.93±0.36	7.50±0.09	28.29±0.70
Kernel	23.23±0.03	3130.00±35.47	103.30±0.63	10.98±0.16	Not detected

The results from their study indicated that the wastes of the fruits (kernel and peel) contain superior antioxidant capacity and displayed higher level of total antioxidant properties than the flesh. Particularly, the kernel presented highest free radical scavenging (DPPH), ferric reducing activities (FRAP), total phenolic content and total flavonoid content, followed by the peel and flesh extracts. Another interesting finding to note, is they found that approximately 90% of the antioxidant compounds content in the fruit wastes are phenolic acid (non-flavonoid). The amounts of polyphenols (phenolic acids, flavonoid and anthocyanin) profiling in the kernel, peel and flesh of *M. pajang* was also studied by Fadzelly Abu Bakar et al. (2010), where they have identified the presence of gallic acid, p-comaric acid, sinapic acid, caffeic acid, ferulic acid, chlorogenic acid, naringin, hesperidin, quercetin, kaempferol, rutin, luteolin and diosmin in the *M. pajang*. The polyphenol content was confirmed by Hassan, Ismail, Abdulhamid, et al. (2011).

In addition, the diversity of carotenoids present in *M. pajang* were also evaluated. Ibrahim, Prasad, and Hamid (2010) carried out a study to review the carotenoid contents of the *M. pajang*'s flesh. The ascorbic acid (vitamin C) and the β-carotene (vitamin A) were examined to be 46.31 ± 5.84 mg/g and 42.21 ± 1.80 mg/g, respectively. The presence of carotenoid in *M. pajang* was validated by Khoo et al. (2010). They evaluated various carotenoids found in *M. pajang*'s peel and pulp extracts. It was reported that the *M. pajang*'s pulp contains higher α- and β-carotene (7.96 ± 1.53 and 20.04 ± 1.01 mg/g) than its peel (4.2 ± 0.14 and 13.09 ± 0.27 mg/g). Besides that, cryptoxanthin (carotenoid pigment) content of *M. pajang*'s pulp and peel were identified to be 1.18 and 0.60 mg/g, respectively. The presence of these phytochemicals in different parts of *M. pajang* provides diverse choice of prospective antioxidants.

2.2.2 Comparison of total phenolic content of *M. pajang* with other fruits

Total phenolic acid appear to function primarily in disease resistance and defence system against pathogens, resulting in the high phytochemical and antioxidant properties (Abu Bakar et al. 2009). In this context, a comparison of total phenolic content of *M. pajang* with other fruits is tabulated and shown in Table 2.2. The total phenolic content of *M. pajang* fruit displays superior total phenolic content compared to other tropical fruits listed in Table 2.2. The high level of total phenolic content suggest a high antioxidant potential in protecting human body from free radical damage. Today, natural antioxidants from tropical fruit have received tremendous attention as source of bioactive constituents. For example, mango and its wastes (from the same *Mangifera* species, similar to *M. pajang*) are used as nutraceutical products (Asif et al. 2016). However, it is surprising that exploitation of *M. pajang* which contain rich level of total phenolic content are rather limited.

Table 2.2 Comparison of total phenolic content of *M. pajang* with other fruits

Scientific Name of fruit	Common name of fruit	Part of fruit	Total phenol content (mg GAE/g of dry sample)	Reference
M. pajang	Bambangan /mawang	Flesh	5.96 ± 0.34	Abu Bakar et al. (2009)
		Peel	22.93 ± 0.36	
		Kernel	103.30 ± 0.63	
Artocarpus integer	Cempedak	Flesh	4.40 ± 0.20	Bakar et al. (2015)
		Peel	21.29 ± 0.43	
		Seed	11.87 ± 0.30	
Artocarpus kemando	Pudu	Flesh	6.57 ± 0.60	
		Peel	8.46 ± 0.76	
		Seed	11.67 ± 0.55	
Artocarpus odoratissimus	Tarap	Flesh	3.53 ± 0.33	
		Peel	42.38 ± 0.20	
		Seed	13.72 ± 0.87	
Anana comosus	Pineapple	Flesh	2.70 ± 0.11	Olivas-Aguirre et al. (2017)
Carica papaya	Papaya	Flesh	2.96 ± 0.56	
Mangifera indica	Mango	Flesh	9.87 ± 0.76	
Musa sapientum	Banana (Raja)	Pulp	76.37 ± 1.79	Sulaiman et al. (2011)
		Peel	12.27 ± 1.05	
Dimocarpus longan Lour.	Longan	Pericarp	20.80 ± 1.60	Prasad et al. (2009)
Nephelium lappaceum	Rambutan	Peel	42.30 ± 0.10	Maisuthisakul, Suttajit, and Pongsawatmanit (2007)
		Seed	43.50 ± 0.40	

Garcinia mangostana L.	Mangosteen	Flesh	9.30 ± 0.80	Naczk et al. (2011)
Canarium Odontophyllum	Dabai	Skin	25.68 ± 1.02	Shakirin et al. (2010)
		Skin + Flesh	6.13 ± 0.74	
		Flesh	3.79 ± 0.69	
		Kernel	2.38 ± 0.32	
Litsea garciae	Pengolaban	Flesh	2.65 ± 0.11	Hassan, Fry, and Bakar (2013)
		Stem cap	8.29 ± 0.70	
		Seed	8.09 ± 0.60	
Baccaurea lanceolate	Liposu	Flesh	4.81 ± 0.14	Bakar et al. (2014)
		Pericarp	3.31 ± 0.48	
		Seed	3.29 ± 0.33	
Baccaurea macrocarpa	Tampoi	Flesh	4.60 ± 0.10	
		Pericarp	60.04 ± 0.53	
		Seed	2.74 ± 0.24	

2.2.3 Cultivation and Usage

In Malaysia, there are about 168 species of underutilized fruit, including *M. pajang*. *M. pajang* has been conserved in the field genebank of Malaysia Agriculture Research and Development Institute (MARDI) for future germplasm enhancement (Nordin 2015). Currently, *M. pajang* trees are widely grow in the forest and were planted in farms and homes (Tangah et al. 2017). It was reported that the cultivation of bambangan is increasing and the production increased from 115.3 to 121.6 metric tons in Sabah from 2013 to 2015 (Jahurul et al. 2019). The area of distribution of *M. pajang* include Sandakan, Sipitang, Beauford and Kota Belud in Sabah; Kapit, Long Silat and Ulu Dapoi in Sarawak and West Kutei and Sangkaruling in Kalimantan (Bakar and Fry 2013). The flesh of the *M. pajang* which represents 60%-65% of the total weight is normally made into pickles, juices or cooked with fish or meat. On the other hand, the kernel (15-20% of the total weight) and the peel (10-15% of total weight) of the *M. pajang* are normally disposed (Abu Bakar et al. 2011).

2.3 Solvent extraction of antioxidant compounds

Extraction is the initial step in recovering the valuable antioxidant compounds from plant materials before rendering them into wide range of industries (Castro-López et al. 2017). There are various extraction methods and they are categorized into conventional and non-conventional type. Examples of conventional extraction methods are solvent extraction and accelerated solvent extraction while examples of non-conventional extraction methods or so-called green technologies are supercritical fluid extraction, microwave-assisted extraction, ultrasound-assisted extraction and others (Toubane et al. 2017, Kehili et al. 2017, Alara, Abdurahman, and Olalere 2018, Vu, Scarlett, and Vuong 2017, Heng et al. 2017, de Camargo et al. 2016, Boussetta et al. 2011, Mushtaq et al. 2015, Renard 2018).

Amongst, solvent extraction is a separation technique which employs a solvent to separate a desired solute from the solid food (Oroian and Escriche 2015). This technique is relatively low in processing cost and easy to operate (Kislik 2012). For these, it is effectively implemented as separations process in pharmaceutical, food and waste treatment industries (Płotka-Wasylka et al. 2017). The driving force for solvent extraction is the concentration difference of components between the solid matrix and solvent phases (Oroian and Escriche 2015). In this context, the selection of solvent is of paramount important as the polarity of solvent is found to be very influential to the extraction performancem where polar solvent facilitates the extraction of polar antioxidant compounds and non-polar solvent can only dissolve the non-polar antioxidant compounds in the plant (Abarca-Vargas, Pena Malacara, and Petricevich 2016). Among the variety of solvents, ethanol is the most prefer polar solvent because it is “GRAS” (Generally Recognized as Safe) and the extracts are accepted in different industries, particularly in food and pharmaceutical industries (Alzeer and Abou Hadeed 2016, Sajedi-Amin et al. 2017).

Numerous studies reported on the application of solvent extraction in isolating the antioxidant compounds from different plant origin (Gullón et al. 2017, Dent et al. 2013, Prasad et al. 2011, Anbalagan et al. 2019, Sousa, Júnior, and de Souza Buarque 2019, Mohamad et al. 2013, Singh, Sharma, and Sarkar 2012, Andrade et al. 2015).

Particularly, it was conceded that a mixture of solvent and water could significantly enhance the extraction efficiency. Gullón et al. (2017) successfully extracted 92.9 mg GAE/g dried leaf and 53.7 mg RE/g dried leaf for total phenolic content and total flavonoid content, respectively from the leaves of *Eucalyptus globulus* by using solvent extraction method at 56% ethanol concentration. Nevertheless, the study carried out by Dent et al. (2013) found a different behavior in the extraction of antioxidant compounds from sage. They observed that maximum yield of antioxidant compounds was attained at 30% aqueous ethanol and the increment of volume fraction of ethanol decreased the extracted amounts of antioxidant compounds. The differences could be explained by the variation in polarity of solvent, which selectively extract different polar or non-polar antioxidant compounds in the plant sample.

In addition to the solvent concentration, the recovery of antioxidant compounds is also affected by other extraction parameters such as extraction temperature, ratio of sample and solvent and other factors. For that, a plethora of studies reported on the optimization of the extraction parameters for maximum extraction efficiency (Prasad et al. 2011, Anbalagan et al. 2019, Sousa, Júnior, and de Souza Buarque 2019, Singh, Sharma, and Sarkar 2012, Mohamad et al. 2013, Andrade et al. 2015, Gullón et al. 2017). Prasad et al. (2011) performed an optimization study to study the effects of solvent extraction parameters on the recovery of antioxidant compounds from the peels of *M. pajang* fruit. In particular, they evaluated the effects of ethanol concentration, temperature and liquid-to-solid ratio and found that at 68% of ethanol concentration, 56℃ and 31.8 mL/g of liquid-to-solid ratio, maximum antioxidant capacity can be obtained. Anbalagan et al. (2019) learned that at 6 hours, 70℃ and volume ratio of sample to solvent at 1:15, the yield of mangiferin from mango leaves (*Mangifera indica L.*) is at maximum level. Additionally, Sousa, Júnior, and de Souza Buarque (2019) also optimized the extraction conditions for isolating antioxidants from murici (*Byrssonima crassifolia L. Kunth*). They found that at extraction temperature of 40℃, 53 minutes of extraction time and 45% of solvent concentration, the DPPH radical scavenging radical is at greatest level. On the other hand, they realized different optimum extraction conditions (29℃, 51minutes and 44% solvent concentration) are required for maximum total phenolic content. From their study, it was observed that the different extraction conditions varied with different responses.

Singh, Sharma, and Sarkar (2012) has also investigated the effects of solvent extraction, extraction temperature and time on the total phenolic content, total flavonoid content and total antioxidant activity of antioxidants from hard winter wheat bran (*Triticum spp.*). Their results showed that the optimal conditions for extraction is 85% v/v solvent concentration, extraction temperature of 75℃ and 45 minutes of extraction time. Interestingly, they observed that the total phenolic content and total flavonoid content increased with the increasing temperature at constant time while different behaviour was found for the total antioxidant activity, such that the antioxidant activity increased up to 60℃ and followed by a decline. The former could be probable that higher temperature enhances the diffusion rate of phenolic and flavonoid compounds to the extracting medium which results in higher extraction performance. The authors explained the trend of total antioxidant activity by ascribing that the mobilization of antioxidants could only happen up to a certain level, whereas the decline was due to the decomposition of antioxidants at high temperature. Similar observations with Gullón et al. (2017) that at higher temperature, the total phenolic content and total flavonoid content retained while the antioxidant activity decreased. Their studies highlighted the importance in investigating the impact of extraction parameters on the extraction efficiency.

Apart from that, an investigation of extraction shaking speed could possibly enhance the extraction performance. For instance, shaking allows rapid diffusion of sample to the solvent and this could significantly improve the extraction efficiency (Azmir et al. 2013). However, the studies evaluating the effect of shaking speed on the extraction process are comparatively scarce. To-date, only a few researchers have considered the effect of shaking speed on the extraction (Andrade et al. 2015, Mohamad et al. 2013). Andrade et al. (2015) extracted the polyphenol compounds from cashew apples agro-industrial residues and performed an optimization study using central composite rotational design to investigate the effects of extraction parameters on the antioxidant capacity. They obtained the extracts with maximum polyphenol level at 55% solvent concentration, 30℃, 150 rpm and 30 minutes. Mohamad et al. (2013) also evaluated the effects of different extraction parameters on the extraction of antioxidant

compounds from the roots of *Eurycoma Logifolia*. They pointed out that the optimum extraction conditions for obtaining highest yields were 45-minute extraction time, 400 rpm extraction shaking speed and solvent to solid ratio of 20 to 1. Remarkably, both studies did not discuss the interactive effects of shaking speed with other extraction parameters. The extraction shaking speed may possibly develop a synergistic or antagonistic interactive effect with other extraction parameters, which are yet to be tested. A brief summary of the review on solvent extraction is tabulated in Table 2.3.

Table 2.3 Summary of experimental works on solvent extraction

Sample origin	Extraction parameters	Extraction response	Reference
Eucalyptus (*Eucalyptus globulus*)	Ethanol concentration: 56% Temperature: 50℃ Extraction time: 225 minutes	Total phenolic content: 92.9 mg GAE/g dried leaf Total flavonoid content: 53.7 mg RE/g dried leaf DPPH radical scavenging activity: 205.4 mg TE/g	Gullón et al. (2017)
Sage (*Salvia officinalis L.)*	Ethanol concentration: 30% Temperature: 60℃ Extraction time: 30 minutes	Mass fraction of phenolic compound (rosamrinic acid): 3634.12 mg / 100 g Mass fraction of flavonoid (luteolin-3-glucuronide): 998.12 mg/ 100 g	Dent et al. (2013)
Bambangan (*M. pajang*)	<u>For total phenolic content:</u> Ethanol concentration: 68% Temperature: 55℃ Solvent to solid ratio: 32.7 mL/g <u>For antioxidant capacity:</u> Ethanol concentration: 68% Temperature: 56℃ Solvent to solid ratio: 31.8 mL/g	Total phenolic content: 14.6 mg GAE/g Antioxidant capacity (phosphomolgybdenum method): 0.2065	Prasad et al. (2011)

Mango leaves (*Mangifera indica L.*)	Solvent (Ethanol) to solid ratio: 15:1 Temperature: 70℃ Extraction time: 6 hours	Yield for young leaves: 22.28% Yield for old leaves: 10.74%	Anbalagan et al. (2019)
Murici (*Byrssonima crassifolia L. Kunth*)	<u>For total phenolic content:</u> Acetone concentration: 45% Temperature: 40℃ Extraction time: 53 minutes <u>For DPPH radical scavenging activity:</u> Acetone concentration: 45% Temperature: 40℃ Extraction time: 53 minutes	Total phenolic content:28 mg GAE/100 mg DPPH radical scavenging activity: 139 µg/ mL	Sousa, Júnior, and de Souza Buarque (2019
Hard winter wheat bran (*Triticum spp.*)	Methanol concentration: 85% Temperature: 75℃ Extraction time: 45 minutes	Total phenolic content:0.921 mg GAE/g bran Total flavonoid content:0.4588 mg CE/g bran Total antioxidant activity: 0.01408 mM AAE/g bran	Singh, Sharma, and Sarkar (2012)
Cashew apples (*Anacardium occidental L.*)	Acetone concentration: 55% Temperature: 30℃ Time: 30 minutes Shaking speed: 150 rpm	Total phenolic content:1975.64 mg/ 100 g DPPH radical scavenging activity: >80%	Andrade et al. (2015)
Tongkat Ali (*Eurycoma Logifolia*)	Solvent (Ethanol) to solid ratio: 20:1 Extraction time: 45 mins Shaking speed: 400 rpm	Yield: ~1.2 mg/g	Mohamad et al. (2013)

2.4 Limitations of extracted antioxidant compounds

There are few noticeable hurdles with the extracted antioxidant compounds. The main challenges include the solubility, inadequate absorption and stability. These limitations impede the bioaccessibility and bioavailability of antioxidant compounds and subsequently restrict their health functions (Rouge, Buri, and Doelker 1996, Gleeson, Ryan, and Brayden 2016).

2.4.1 Solubility and inadequate absorption

Problem regarding solubility is always a challenging aspect. The solubility of antioxidant compounds is an intrinsic parameter to ensure adequate amount of bioactivity in the systemic circulation for health benefit effects. In other words, the antioxidant compounds must reach certain level of solubility to be dissolved at the absorption site (Stegemann et al. 2007). In general, aqueous solubility act as indicator for the solubility in the intestinal fluid, this is because any bioactive compounds to be absorb shall stay in the form of aqueous at the absorption site (Savjani, Gajjar, and Savjani 2012). The antioxidant compounds with poor aqueous solubility have low absorption rate and can thereby affect the therapeutic efficacy (Recharla et al. 2017).

Oral dosage is one of the most convenient and prominent routes for intake of antioxidant compounds. Upon oral consumption, the antioxidant compounds undergo a series of complex process as they pass through various segments of gastrointestinal tract. Briefly, the digestive process starts with the mouth by mastication (breaking down ingested foods) where interaction of antioxidant compounds and salivary proteins such as mucin occurs and leads to digestion of only very little antioxidant compounds. However, the mastication process is not significant as the antioxidant compounds are not present in the mouth for any length of time and traverse very quickly to the stomach (Sultatos 2007). Swallowed complex or termed as bolus enters the highly acidic stomach with pH lie between 1.0 and 3.0. The residence time at this phase is normally 1 to 3 hours (Acosta 2009). In the stomach, the antioxidant compounds encounter high concentration of monovalent ions which may lead to changes in the electrical characteristics of the ionisable groups, which in turn affect the integrity and permeability of antioxidant compounds. Also, they are exposed to

gastric enzymes and gastric juice where gastric enzymes may hydrolyse some components of the antioxidant compounds, and the gastric juice possesses active substance that can alter the surface characteristics of antioxidant compounds (Gonçalves et al. 2018). Notably, the short and insufficient gastric residence time often affect the antioxidant compounds that are preferentially absorbed in the stomach and results in low bioavailability (Ting et al. 2014).

On leaving the stomach, the partially digested complex (chyme) enters small intestine. The gall bladder contracts upon the arrival of antioxidant compounds and induces the secretion of sodium bicarbonate, digestive pancreatic enzymes, bile salts, phospholipids and other salts. At this phase, the acidic chyme is mixed with sodium bicarbonate and the alkaline juices neutralises the stomach acids, increasing the pH to 5.4 - 7.4. The retention time in small intestine is about 3 to 5 hours (Acosta 2009). The solubilisation, transport and absorption of the antioxidant compounds are facilitated by the formation of mixed micelles which transport the solubilized compounds across the mucus layer and allow them to be absorbed by enterocytes. The uptake of antioxidant compounds can be achieved through two main mechanisms namely passive and active transports. Eventually, these antioxidant compounds are absorbed into the portal blood or intestinal lymphatic system (Yao, McClements, and Xiao 2015).

Along the gastrointestinal tract, the extracted antioxidant compounds are often affected by several factors that can reduce their therapeutic efficacy. These physiological barriers include gastrointestinal transit time, enzyme activities and gastrointestinal pH. The wide ranges of pH and enzymes could greatly affect the bioactivity of natural antioxidant compounds. Remarkably, the antioxidant compounds might be metabolized either by the enzymes along the gastrointestinal tract. This metabolism is well known as first-pass metabolism. Additionally, some antioxidant compounds might experience physicochemical interaction with other foods or drugs and causes inadequate uptakes of antioxidant compounds before excreted out of the body (Chillistone and Hardman 2017). Therefore, the antioxidant compounds need to be solubilized at the targeted site in order to effectively increase the bioavailability.

2.4.2 Stability

The extracted antioxidant compounds are prone to degradation or oxidation during exposure to environmental or processing factors like heat, light, oxygen, metal ions and pH (Aguiar, Estevinho, and Santos 2016, Cerqueira et al. 2017). In particular, thermal degradation is one of the most common phenomena in antioxidant compounds as most of the foods undergo thermal treatment at numerous stages such as pre-treatment, processing and storage. Thermal treatment is usually used for preservation, kills microorganism and to improve the shelf life (Nunes and Tavares 2019, Putnik et al. 2019). The heat treatment temperature for food processing usually ranged from 50 to 150℃ (Patras et al. 2010). However, regardless of how minimum the heat energy is, this process can still potentially cause the loss of antioxidant compounds. Therefore, it is very important to maintain a safe temperature range to protect the antioxidant compounds from degradation. Subjecting the food product containing antioxidant compounds to heat treatment under presence of oxygen can potentially lead to oxidation and isomerization which results in degradation of the antioxidant compounds (Martínez-Delgado, Khandual, and Villanueva–Rodríguez 2017). It was found that the formation of *cis* isomers of β-carotene increased upon the rising of temperature (Chen and Huang 1998). Noticeably, relatively little detail is known about the degradation mechanism of antioxidant compounds, probably due to the diversity of antioxidant compounds.

Different heating methods like drying, blanching, roasting inevitably emit heat that can compromise the antioxidant compounds and results in degradation of antioxidant compounds (Ioannou et al. 2012). Zhang, Chen, et al. (2010) studied the effect of roasting on the antioxidant capacity of tartary buckwheat flour extracts. A total loss of 33% in total flavonoids content was observed when the extract was roasted at 120℃ for 40 mins. Moreover, Zarei, Fazlara, and Tulabifard (2019) found that the total phenolic content in lotus, thyme and multifloral honey reduced significantly from 609, 538 and 462 mg tannic acid/kg to 482, 447 and 404 mg tannic acid/kg, respectively after 30 min of heating, confirming the impact of thermal treatment on the stability of antioxidant compounds.

However, the degradation of antioxidants is not restricted to the function of temperature, it may also depend on other parameter, especially pH. For example, Sui, Dong, and Zhou (2014) evaluated the combined effect of pH and temperature on the stability of two anthocyanins (Cyanidin-3-rutinoside and cyanidin-3-glucoside). The results showed that pH helps to stabilizing the anthocyanins under high temperature treatment. Their findings were confirmed by the study conducted by Xu, Yu, and Zhou (2019). Similarly, they also found that at lower pH, the influence of heat treatment on the stability of epigallocatechin gallate is reduced, suggesting that there is an interaction effect between the pH and temperature on the stability of antioxidant compounds. These studies highlighted the importance on stabilizing the antioxidant compounds under the combined effect of pH and temperature, which has to be considered to reduce the loss of antioxidant activity, extend the shelf life and improve the bioavailability of bioactive compounds (Volf et al. 2014).

2.5 Formulation strategies for enhanced solubility and stability

In a fast-growing market of functional food products and nutraceutical, solubility and stability of the bioactive compounds must be enhanced to ensure their biological effects. In term of solubility, solubilization of antioxidant compounds are unlikely to happen without the use of an appropriate delivery system as the absorption behavior of antioxidant compounds is greatly attributed to the composition of delivery system (Gonçalves et al. 2018). For instance, lipophilic compounds, which have low solubility in the intestinal fluid, tends to precipitate and could be excreted without being absorbed. In the case that the lipophilic compounds are encapsulated in the delivery system, lipolysis process could digest the oil component of delivery system and transform them into di-glycerides, mono-glycerides and free fatty acids. At that stage, the antioxidant compounds that release in cavity can be incorporated into mixed micelles formed by the liberated oil fraction or bile salts. Thereafter, the mixed micelles will further solubilize the antioxidant compounds and transport them to the absorption site (Singh et al. 2017). On the other hand, stability of antioxidant compounds is equally important and shall be taken into account. In the food matrix in which the antioxidant compounds are to be incorporated with, it is of paramount importance to select a suitable method to preserve their stability because the antioxidant compounds are susceptible to degradation. Degradation can be prevented by choosing a formulation that can protect

the antioxidant compounds against the atmosphere and other elements and ensure high retention rate upon consumption (Martínez-Delgado, Khandual, and Villanueva–Rodríguez 2017).

In this regard, numerous formulations have been developed to augment the bioavailability of antioxidant compounds, and yet act as a stabilizing agent against physicochemical factors. These formulation strategies include nanoemulsion, liposome-based system, hydrogel beads, cyclodextrin complexation and solid lipid nanoparticles (Braithwaite et al. 2014, Ting et al. 2014). These delivery systems provide several advantages over traditional forms such as enhancement of water solubility, improvement of physicochemical stability and facilitation of controlled release (Summerlin et al. 2015). Additionally, these delivery systems could be engineered for optimal stability (Boon et al. 2010). Aside from these delivery systems, in recent years, numerous researches suggest incorporation of bioactive compounds in the ionic liquid and deep eutectic solvent-based delivery system. These novel approaches demonstrated great ability in improving the bioavailability of bioactive compounds (Egorova and Ananikov 2018, Pedro et al. 2019). A trivial illustration of some of the delivery systems are drawn and depicted as Figure 2.2.

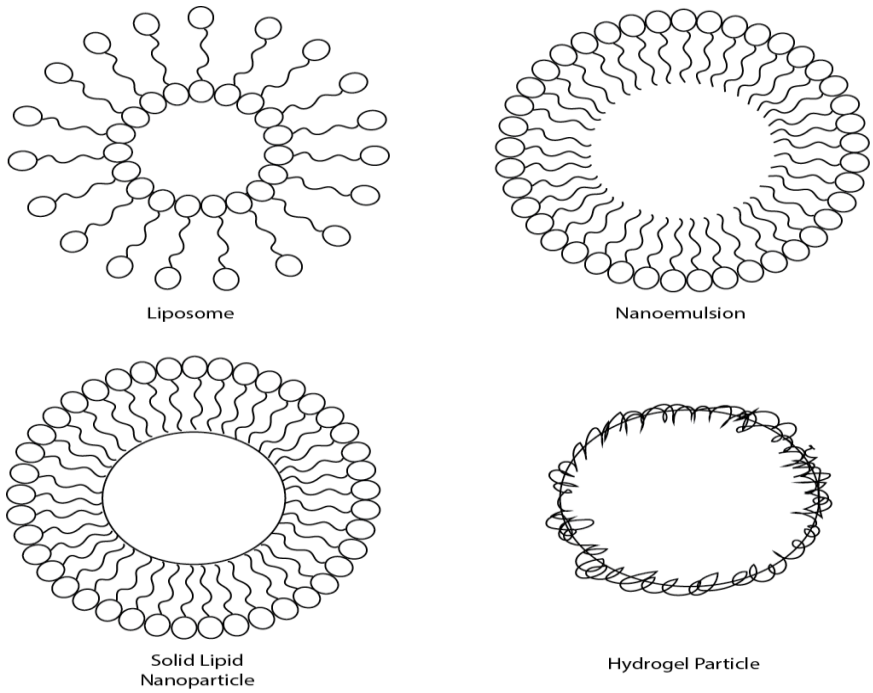


Figure 2.2. Examples of nutraceutical-based delivery system

2.5.1 Nanoemulsion

Nanoemulsion is an isotropic and colloidal dispersion which made up of two different immiscible liquid (typically oil and water), with droplets scaled between 20-200 nm (Montes de Oca-Ávalos, Candal, and Herrera 2017, Acevedo-Fani, Soliva-Fortuny, and Martín-Belloso 2017). The major difference between nanoemulsion and conventional emulsion (macroemulsion or microemulsion) lies in their droplet size ranges and stability characteristics. With smaller particle size, nanoemulsion have greater stability to gravitational separation, flocculation, aggregation and coalescence compared to the conventional emulsion (McClements and Rao 2011).

A typical nanoemulsion is made up of oil phase, aqueous phase and surfactants (emulsifiers) (McClements and Rao 2011). A surfactant is used as an emulsifier to stabilize the nanoemulsion by reducing the interfacial tension between the two immiscible liquid and prevent the occurrence of droplet coalescence. Some common emulsifiers are polyoxyethlene sorbitan monolaurate (Tweens), sorbitan monooleate (Spans) and proteins (Lam and Nickerson 2013, Harwansh, Deshmukh, and Rahman 2019). Nanoemulsion can be classified based on the spatial organization of aqueous and oil phase, such that it can be categorized into oil-in-water (O/W) nanoemulsion, and water-in-oil (W/O) nanoemulsion. Sometime, nanoemulsion can also be prepared in the form of double emulsion or also referred as multiple emulsion (Lamba, Sathish, and Sabikhi 2015, McClements et al. 2009). In fact, O/W nanoemulsion is currently the most widely used emulsion-based delivery system as most of the antioxidant compounds and drugs are poorly water-soluble in nature (Rai et al. 2018).

Nanoemulsion possess ability to encapsulate and protect bioactive components while simultaneously preserves the stability of bioactive components, hence slowing down the degradation processes and enhances the bioavailability (Harwansh, Mukherjee, and Biswas 2017, Campani et al. 2016, Sivakumar, Tang, and Tan 2014). Nanoemulsion also improves the solubility of bioactive compounds and can be rendered into numerous dosage forms such as creams, gels, sprays and liquids which can be administered via various routes, including oral, intranasal, pulmonary and intravenous

pathway (Singh et al. 2017). Additionally, nanoemulsion is often used to deliver fragrance, drug and nutraceutical compounds (Rai et al. 2018).

For these remarkable advantages, a nanoemulsion-based system has been widely studied to enhance the efficacy of antioxidant compounds. Sari et al. (2015) encapsulated highly unstable curcumin compounds inside nanoemulsions with an encapsulation efficiency of 90.56%. The research group performed an in *vitro* release study and found out that the curcumin-loaded nanoemulsion can resist simulated gastric digestion and ensures slow release of curcumin compound and thus improving its bioavailability. For that reason, the research group concluded that nanoencapsulation of unstable compounds can be a potential approach to protect them from premature degradation. In another study, Ha et al. (2015) protected lycopene compound from tomato extract which are highly sensitive and has poor bioavailability by entrapping it into nanoemulsion. From their study, lycopene-loaded nanoemulsion with droplet size less than 100 nm displayed great *in vitro* bioaccessibility. Moreover, Rabelo et al. (2018) formulated anthocyanins-loaded W/O nanoemulsion to protect the unstable anthocyanins compound from açaí berry. Rabelo and his research team prepared the nanoemulsion samples with different concentrations of acai berry extract and realized that all these samples demonstrated great antioxidant activity and high retention rate of anthocyanins compound after a storage duration of 30 days. In particular, sample with 2% açaí berry extract had an estimated half-life of 385 days, indicating a relative low degradation rate of anthocyanin. The results suggested that the nanoemulsion system is effective in protecting the unstable antioxidant compounds. Nonetheless, the stability of nanoemulsion remains a critical challenge that hinders its wide applications. Nanoemulsion tends to be degraded and separated into different phases after certain period of storage time, i.e., via destabilization processes. The well-known destabilization processes are coalescence and Ostwald ripening (McClements and Rao 2011).

2.5.2 Liposomes-based system

Liposomes are formed from phospholipids and structured as one or more phospholipid bilayer shell with an aqueous core. The size of liposomes ranges from small vesicles

(0.025 μm) to large vesicles (2.5 μm). Vesicles is a measure to define the circulation half-life of liposomes, while both the size of liposome and number of bilayer structure are parameters that can affect amount of the drug loading (Akbarzadeh et al. 2013).

Liposomes are commonly categorized into two broad categories: 1) multilamellar vesicles (MLV); 2) unilamellar vesicles. The MLV are formed by more than one bilayer and can be prepared by thin-film hydration method or hydration of lipids in the presence of an organic solvent. On the other hand, the unilamellar vesicles has only single bilayer structure and can be further classified into large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV). The size of LUV is more than 0.1 μm while the SUV has size lesser than 0.1 μm. LUV can be prepared by several methods, including detergent dialysis, reverse-phase evaporation, ether injection, active loading techniques and others (Sharma and Sharma 1997). For the SUV, it can be produced by reducing the size of MLV or LUV. The reduction of size is generally achieved by mechanical devices such as sonication technology, high pressure homogenization and microfluidization (Shin, Kim, and Park 2015). However, these conventional technologies have notable drawbacks, which may lead to the production of large particle size of liposomes, high amount of organic solvent in the final product and limitations to scale-up for industrial production. Because of these drawbacks, many green technologies have been developed to prepare liposomes. These technologies comprise of supercritical fluid technology, microfluidics-based technology, freeze drying technology, curvature-tuning, modified electroformation methods and membrane contactor technology (Huang et al. 2014, Patil and Jadhav 2014).

Liposomes present distinct advantages, such that the bilayer structure allows liposomes to be a good delivery vehicle for both hydrophilic and lipophilic bioactive compounds. This ability makes them useful in various applications such as cosmetics, food, pharmaceutics and others (Li et al. 2017). Particularly, liposomes have been extensively explored and found to be ideal for skin delivery of drugs (Shah et al. 2015). Also, liposomes possess other great characteristics including biocompatibility, biodegradability, ability to load large payloads, low toxicity, ability to improve solubility of poor soluble core material, non-immunogenicity and ability to enhance

the circulation half-life at target sites, thus enhancing the bioavailability of encapsulated bioactive compounds (Zununi Vahed et al. 2017). However, liposome usually has shorter half-life and poor physical and chemical stabilities, where the phospholipids may undergo oxidation and hydrolysis-like reaction leading to leakages and fusions of encapsulated bioactive compounds (Akbarzadeh et al. 2013, Park, Jo, and Jeon 2014).

Many studies have been performed to demonstrate the potential of liposome-based method in encapsulating antioxidant compounds which exhibit poor bioavailability. Paini et al. (2015) encapsulated apigenin which displayed preventive activity against cancer and cardiovascular disorders with liposomes. In this study, the apigenin-loaded liposomes are prepared by adding food-grade rapeseed lecithin into saturated apigenin solution and subsequently subjected to proposed sonication treatment technique. The results displayed a noteworthy encapsulation efficiency of more than 92% which indicates the potential of apigenin formulation. Recently, Caddeo et al. (2018) overcame the physicochemical and pharmacokinetic limitations of resveratrol by synthesizing the compound with PEG-modified liposomes. The research group evaluated the product stability in simulated body fluids and discovered that the resveratrol formulation is able to preserve its structure against osmotic stress and protein adsorption after incubation of 24 hours. Additionally, they realized that the resveratrol formulation did not affect its antioxidant activity. The formulation ensured a protection against the oxidative stress induced by *ex vivo* human erythrocytes-based model. Their results assured the potential of liposomes carrier system in delivering antioxidant compounds with enhanced stability and biocompatibility. Besides that, Zhou et al. (2018) improved the stability of acteoside (polyphenolic compounds) by incorporating them in liposome and chitosan-coated liposome. The stability test results were presented as Figure 2.3, it clearly showed that the retention rates of acteoside-liposome and acteoside-chitosan-coated liposome were significantly higher than the pure acteoside at different storage conditions, suggesting the ability of liposome in reducing the degradation of antioxidant compounds. Remarkably, the authors also found that the half-life values of acetoside, acteoside-liposome and acteoside-chitosan-coated liposome were 31.5, 37.1 and 48.5 days, respectively. This finding highlighted

the potential of chitosan-coated liposome in protecting the antioxidant compounds from degradation.

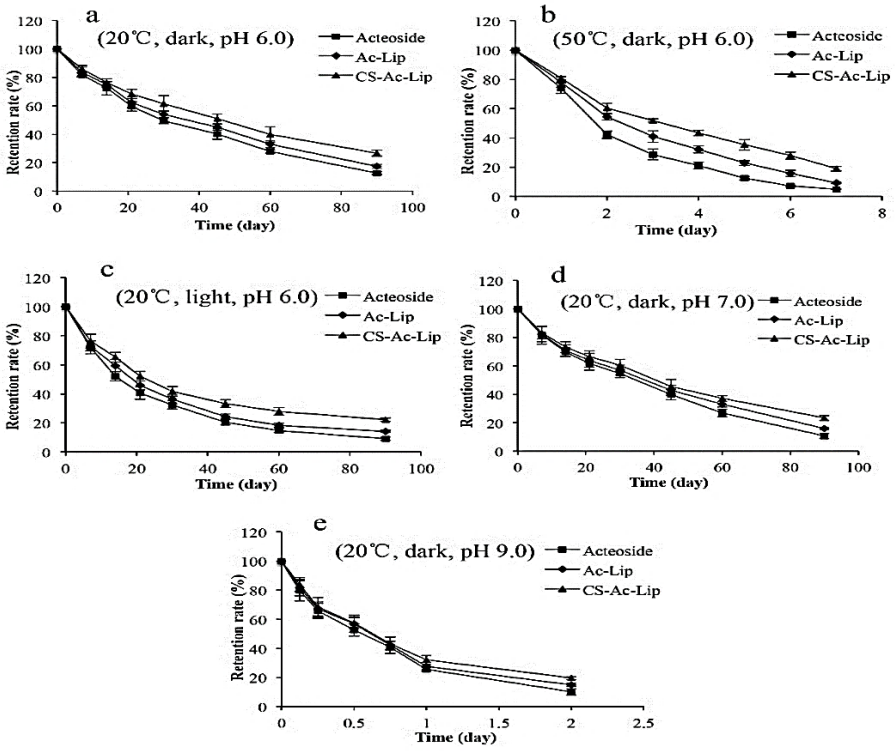


Figure 2.3 Stability of acteoside, acteoside-liposome (Ac-Lip) and acteoside-chitosan-coated liposome (CS-Ac-Lip) at different storage conditions (Zhou et al. 2018) (Reprinted with permission – Appendix H)

2.5.3 Hydrogel-based system

Hydrogel are three-dimensional solid network which can imbibe a considerable amount of fluids. Hydrogel exhibits remarkable characteristics such as swelling ability, biomimetic, biocompatibility, high drug loading, low surface tension, stimuli-responsive properties and amenability of physical/chemical properties and ensures prolonged drug release (Chillistone and Hardman 2017, Lai and He 2016). These properties have made hydrogel a potentially suitable approach for delivery of nutraceutical compounds. For an example, owning to its swelling ability and stimuli-responsive properties, the release of nutraceutical compounds may be controlled by external stimuli such as pH, temperature and light, and subsequently ensure controlled release of nutraceuticals compounds (Braithwaite et al. 2014). However, the utilization

of hydrogel beads is limited to applications associated with pH-sensitive bioactive compounds as they are highly porous. In this regard, the small ions such as H^+ or OH^- can easily diffuse in or out of the hydrogel beads which consequently induce deactivation of bioactive compounds when exposed to gastric conditions (Zhang et al. 2017).

The hydrogel structure can be synthesized through both physically and chemically cross-linking of natural or synthetic hydrophilic polymer. Hydrophilic functional groups such as hydroxyl, amine and amide allow the penetration of water that lead to expansion of hydrogel. This phenomenon is commonly known as swelling process. Notably, during the swelling process. the hydrogels is able to resist and prevent dissolution and disintegration due to its cross-links between polymer chains (Hamedi et al. 2018). The physical cross-linking of polymer chain can be prepared via physical interactions such as hydrogen bonding, complexation, charge interactions and chain aggregation. They can also be triggered by environmental parameters which include pH, temperature and ionic strength. The examples of method that relies on physical interactions are electrospraying and ionically cross-link polymer chains under rapid mixing. On the other hand, hydrogel can also be prepared by chemical methods that depend on chemical cross-linking. During the chemical cross-linking, covalent bonds are formed between the polymer chains (Mahinroosta et al. 2018). The commonly used chemical methods are polymerization and grafting monomers with cross-linkers (Lai and He 2016). Nonetheless, attentions are required on cleaving the cross-linker which are sometimes toxic and can reduce the biocompatibility (Pellá et al. 2018).

Several reports have been made on the capability of hydrogel in protecting easily degrade antioxidant compounds. For example, Wang et al. (2015) incorporated carotenoid-enriched lipid droplets into hydrogel beads consisting of starch and sodium trimetaphosphate. The research team conducted *in vitro* release study to investigate the release behavior of carotenoid-loaded hydrogel. Their revealed that the carotenoid-loaded hydrogel is able to preserve and retain the compound desirable characteristics under simulated gastric conditions in addition to ensuring a controlled release of carotenoid-enriched lipid droplets in simulated intestinal fluid. Another study involved

the loading of the superoxide dismutase into hydrogel made up of chitosan, heparin and poly (γ-glutamic acid) for healing diabetic wounds (Zhang et al. 2018). In particular, the hydrogel formulation possesses great swelling ability and biocompatibility. The hydrogel formulation displayed controlled release of superoxide dismutase and was able to enhance formation of collagen which accelerate wound healing. Spizzirri et al. (2013) coated folic acid and thiamine (B complex vitamins) into catechin-based crosslink hydrogels to protect the compounds against damages. The authors subjected the folic acid-loaded catechin-based hydrogel to UV irradiation while treating the thiamine-loaded catechin-based hydrogel with chemical oxidizing agent. The results successfully proved the ability of hydrogel in protecting both folic acid and thiamine from oxidative degradation with the preservation value of 96% (after 24 h) and 92% (after 2 h) for folic acid and thiamine, respectively. Apart from that, Zhang, Zhang, and McClements (2016) found that β-carotene-loaded lipid droplet in alginate hydrogel bead exhibit greater stability as compared to nanoemulsion. It was observed that the concentration of β-carotene decreased to 0.2%, 38% and 55% for the nanoemulsion, 1% alginate hydrogel bead and 0.5% alginate hydrogel bead system, respectively after 12 days at 55℃ (Figure 2.4). The research team gave an explanation for their findings, stating that hydrogel bead provides a physical barrier that restrict molecular diffusion and the interaction of pro-oxidant or free radical with the carotenoids and results in a higher retention of β-carotene. Thus, it can be concluded that incorporation of antioxidant compounds in hydrogel bead is feasible as it enhanced both the solubility and stability of antioxidant compounds.

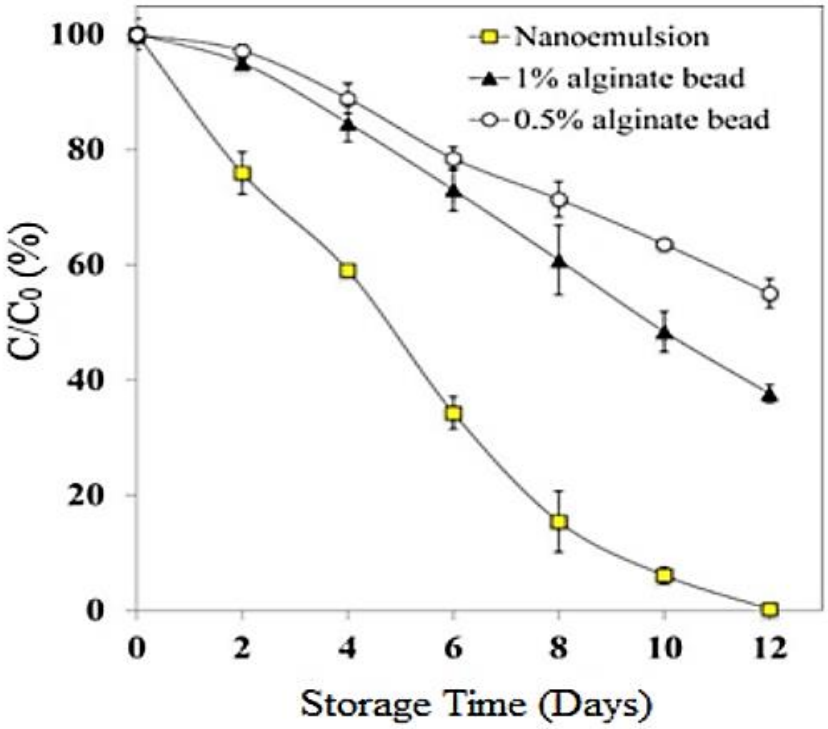


Figure 2.4 Degradation rates of beta-carotene in nanoemulsion and hydrogel beads during storage at 55℃ (Zhang, Zhang, and McClements 2016)
(Reprinted with permission – Appendix H)

2.5.4 Cyclodextrin complexation

Cyclodextrin (CD) consists of cyclic oligosaccharides which derived from starch degradation via cyclodextrin glycosyltransferase. Classically, CDs are classified into α, β and γ, which contain 6, 7 and 8 glucopyranose units, respectively. CD are structured as truncated cones with a hydrophobic internal cavity and hydrophilic external surface. This special structure allows them to form complexes with different bioactive compounds such as polyphenols or drug and bring about the inclusion complex formation (Matencio, García-Carmona, and López-Nicolás 2017). An illustration of encapsulation of drug in cyclodextrin is presented as Figure 2.5.

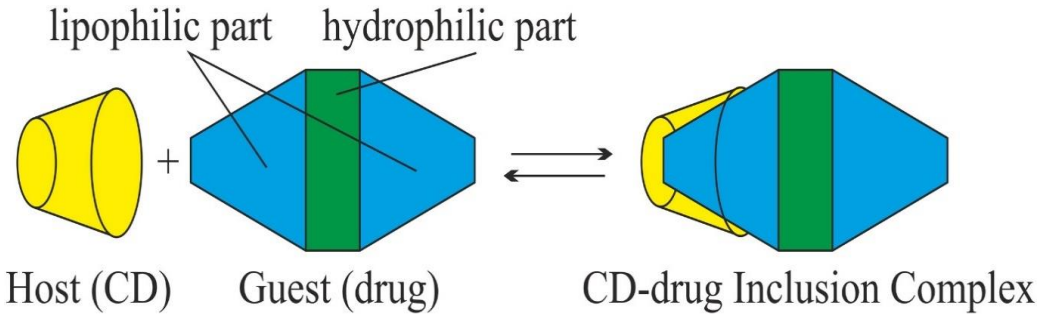


Figure 2.5 Encapsulation of drug in cyclodextrin (Radu, Parteni, and Ochiuz 2016) (Reprinted with permission – Appendix H)

The inclusion complex can be defined by a host-guest relationship, where the hydrophobic cavity of CDs act as host and the encapsulated bioactive compounds act as the guest (Sherje et al. 2017). The main driving force can be described as the replacement of the polar-apolar interactions between the host and the guest by the apolar-apolar interactions between the guest and cavity (Mura 2014). The types of forces that occur during the complexation process are hydrogen bonding, hydrophobic interactions, dipole-dipole interactions, van der Waals, dispersion forces, reduction of conformational strain and electrostatic interactions. Specifically, there is no formation of covalent bonds during the complexation process (Radu, Parteni, and Ochiuz 2016).

CDs complexation present several advantages. Predominantly, the CDs complexation displays the ability to increase the water solubility, dissolution, bioavailability and physicochemical stability. Additionally, CDs complexation can greatly reduce an adverse drug reaction in gastrointestinal tract such as topical irritation phenomena and avoid drug-drug and drug-excipient interactions. Moreover, they can modify the release site and ensure controlled release (Conceição et al. 2018). Among CDs,β-CD is commonly selected as carrier compounds to enhance the bioavailability of nutraceuticals in the body due to its suitable cavity size (Pérez-Abril et al. 2017). Nevertheless, β-CD possesses strong intramolecular hydrogen bonds which results in poor solubility in water. Many β-CD derivatives have been formulated through chemical modifications to overcome this problem (Pérez-Abril et al. 2017).

Several studies have utilized the β-CD system to protect the antioxidant compounds. For example, Ferreira et al. (2013) studied the antioxidant activity of mangiferin-loaded β-CD complex and evaluated its protective effect against lipid peroxidation. The results showed that the mangiferin-loaded β-CD complex has great inhibition effect which is comparable to the positive control (Trolox) used in the assay. Interestingly, they also observed the enhancement of antioxidant activity of mangiferin in β-CD complex when compared to free mangiferin. Aside from that, Ho et al. (2017b) evaluated the effects of temperature, light and oxygen on catechin-β-CD. The authors found that the inclusion complexation of catechin in β-CD and successfully protected the catechin against these environmental conditions and significantly enhanced the solubility of catechin in water by two-fold. In the same year, Ho et al. (2017a) compared the degradation of free catechin and catechin-loaded β-CD in protein, oil and sugar models and realized that the catechin-loaded in β -CD gave higher percentage of antioxidant retention. Their study reinforced the ability of HP- β-CD in protecting antioxidant compounds. A recent study also reported the ability of β-CD and hydroxyl-propyl-beta-cyclodextrins (HP- β-CD) in enhancing the solubility and stability of flavanones (naringenin and hesperetin) (Lucas-Abellán et al. 2019). Particularly, the inclusion of β -CD improved the solubility of naringenin and hesperetin by 9.3-fold and 30-fold, respectively. Whereas, the HP- β-CD enhanced the solubility of both flavanones to a greater extent than conventional β-CD, increment of 143-fold and 467-fold were observed for the solubility of naringenin and hesperetin, respectively, highlighting the great ability of HP- β-CD. Additionally, the authors also discovered the inclusion of both CD increase the stability of flavanones at different pH (3.5, 6.5 and 8.5), suggesting their applications as functional foods.

2.5.5 Solid lipid nanoparticles

Solid lipid nanoparticles (SLN) are a class of colloidal emulsion system which contain solid or semi-solid lipid core structure. The main excipients of SLNs are solid lipid, emulsifier and water. The solid lipid serves as the matrix material and remains in the solid state at both room and body temperature. The commonly employed solid lipid include triglycerides, fatty acids, steroids and wax (Rostami et al. 2014). Interestingly, the employment of solid lipids are suggested by researchers to replace the conventional

liquid oil because solid lipid matrix can give a lower mobility of drug and ensure a controlled release profile (Geszke-Moritz and Moritz 2016).

SLN presents distinct advantages over the other commercial nutraceutical formulation methods. These include enhanced stability of bioactive compounds, avoid the usage of organic solvents, high entrapment efficiency and ensure controlled release (Mehnert and Mäder 2012). Apart from that, SLN is feasible in embedding both labile lipophilic and hydrophobic bioactive compounds and protecting them from degradation (Lin et al. 2017). For these reasons, SLN are widely used in the pharmaceutical, food and supplement industries (Gao and McClements 2016).

SLNs are normally fabricated in two stages. The first stage involves the incorporation of bioactive compounds in the melted lipid then mixed with surfactant solution. Numerous techniques have been exploited for the SLN production which include high pressure homogenization, ultrasonication, supercritical fluid extraction, solvent evaporation, solvent emulsification-diffusion, microemulsion and double emulsion (Lin et al. 2017, Teixeira, Carbone, and Souto 2017). Subsequently, the SLN mixture will be cooled to induce crystallization and nanoparticle formation. The crystallization can help reduce the molecular diffusion within the nanoparticle interior and therefore results in better chemical stability of entrapped bioactive compound (Qian et al. 2013). Nonetheless, the SLN is less physically stable than the liquid lipid particle. The instability of SLN is caused by the crystallization of lipids where the lipid molecules in a crystal rearrange to assume a different packing. During the transition, the shape of crystal formed is altered from spherical (α-form) to needle or amorphous shaped (β-form). As a result, the surface area increases. In the case of insufficient surfactant, the newly created surfaces cannot be fully covered. The resulting hydrophobic interaction between the non-polar regions of particles induces aggregation which can lead to gelation (Qian et al. 2013, Helgason et al. 2009).

There are numerous literatures reporting on the application of SLN in protecting antioxidant compounds. A study investigated by Gaber, Nafee, and Abdallah (2017)

presented the protection effect of SLN. In their study, they encapsulated myricetin into Gelucire-based SLN and studied the protection effect of SLN against degradation. The *in vitro* release study showed a sustained release profile of myricetin from the SLN, indicating the ability of SLN in protecting antioxidant compounds from degradation in human gastrointestinal fluid. In another study, Wang et al. (2016) employed natural biopolymers (casinate and pectin) to coat SLN with biopolymeric double layer. They used this complex to encapsulate curcumin and found that such formulation gives higher antioxidant activity than free curcumin. Additionally, a recent study has also emphasized on the ability of SLN in protecting antioxidant compounds, which Mehrad et al. (2018) protected β-carotene by engulfing them into SLN that was stabilized by whey protein isolate (surfactant). The results indicated there is no significant changes on the chemical properties of β-carotene when the β-carotene-loaded SLN is treated at both 75^{o}C and 85^{o}C for storage duration of 18 days. Thus, this study has confirmed the protective effect of SLN against adverse environmental conditions.

2.5.6 Ionic liquid-based formulation

Ionic liquids are molten salts comprise of cations and anions. They have been regarded as 'green' replacement for traditional volatile organic solvents due to their remarkable properties such as high thermal stability, low toxicity, high conductivity, low melting point and low vapour pressure (Goindi, Kaur, and Kaur 2015). The desired physicochemical properties of ionic liquids can be easily tuned and tailored by modifying the anion/cation combination (Mahamat Nor, Woi, and Ng 2017). Besides that, ionic liquids can also be of interest as a versatile character in solubilizing bioactive compounds with poor water solubility (Dobler et al. 2013). Remarkably, ionic liquid can solubilize both hydrophilic and hydrophobic bioactive ingredients (Jesus et al. 2019). Considering these unique properties, the application of ionic liquid in pharmaceutical and food industry are expanding (Vraneš et al. 2017, Egorova, Gordeev, and Ananikov 2017).

Particularly, ionic liquid-based formulation has gained more attention as a new approach to improve solubility and as a bioactive compounds delivery system. For example, Alevizou and Voutsas (2013) examined the solubility of *p*-coumaric acid and

caffeic acid in few ionic liquids and organic solvents. The research team found that both phenolic compounds are more soluble in ionic liquid with BF_4^- and TFO^- hydrophilic anion than organic solvents (*t*-pentanol and ethyl acetate) and other ionic liquid which based on hydrophobic anions. This ranking is in agreement with the polarity of solvent, such that hydrophilic solvent has higher interaction with the hydrophilic antioxidant compounds. An interesting study performed by Jesus et al. (2019) also successfully proved the ability of ionic liquid in enhancing the solubility of poor soluble drugs. It was found that *N*-acetyl amino acid *N*-alkyl cholinium-based ionic liquids increased the aqueous solubility of paracetamol and sodium diclofenac by 3.2-fold as compared to pure water and inorganic salt solution. Aside from that, Wu et al. (2019) prepared ionic liquid based on two food additives, choline and malic acid and evaluate its potential in delivering hydrophilic compounds, dextran. The results showed that the amount of dextran delivered to the skin by incorporating in the ionic liquid was improved by two folds when compared to the pure dextran solution.

Apart from that, the combination of ionic liquid with other micro- or nanocarrier has also become another emergent strategy. In this context, Moniruzzaman et al. (2010) reported a fascinating formulation of a novel ionic liquid-in-oil (IL/O) microemulsion system for transdermal delivery of acyclovir. They successfully proved that the new ionic liquid-based microemulsion can be used to solubilize pharmaceuticals compounds which has poor aqueous solubility. Their successful innovation has led to a few publications on the formation of microemulsion and nanoemulsion based on ionic liquids (Li et al. 2012, Dobler et al. 2013, Goindi, Kaur, and Kaur 2015, Goindi et al. 2014, Nor, Woi, and Ng 2017). These researches showed that the utilization of ionic liquids could enhance the solubility of drugs and facilitate the delivery of bioactive compounds to the targeted site. Lately, Safdar et al. (2019) found that the addition of ammonium based ionic liquids enhance the thermal stability of chitosan-tetramethylammonium hydroxide-sodium tripolyphosphate microparticles. Additionally, they also observed that the addition of ionic liquid did not affect the particle size of microparticles, suggesting the ability of ionic liquid in preparing microparticles with enhanced stability. Nevertheless, the low sustainability properties, toxicity and high cost of ionic liquids often limits their applications and shall be overcome for vast applications (Kudłak, Owczarek, and Namieśnik 2015).

2.5.7 Deep eutectic solvent-based formulation

Deep Eutectic Solvent (DES) has recently attracted considerable attentions for its potential to replace the conventional organic solvents (Cunha and Fernandes 2018, Cao et al. 2018). The DES is a mixture consisting of 2 or 3 hydrogen-bond donor (HBD) and hydrogen-bond acceptor (HBA) components. Upon mixing, the HBD and HBA components will interact with each other through hydrogen bonding (Zhang et al. 2012, Jiang et al. 2019, Abbott et al. 2003). The most commonly employed hydrogen bond acceptor is choline chloride while the hydrogen bond donors are amino acids, alcohol, carboxylic acids and sugars (Dai et al. 2013). One of the unique features of the DES solution is that it has a lower melting point than that of its individual HBD and HBA components (Meng et al. 2018). Note that, the DES solution has several advantages: (a) highly biodegradable, (b) negligible toxicity, (c) low cost, (d) simple preparation, (e) no requirement for subsequent purification steps (Cunha and Fernandes 2018). Furthermore, the desired physicochemical properties of the DES solution can be easily adjusted by modifying the compositions of the HBD and HBA components (Smith, Abbott, and Ryder 2014). Because of these attractive properties of the DES, several researchers have studied the applications of DES as extraction media and successfully proved that it is suitable for extracting bioactive compounds from different plants (Cao et al. 2018, Chanioti and Tzia 2018, García et al. 2016, Ozturk, Parkinson, and Gonzalez-Miquel 2018, Meng et al. 2018, Jancheva et al. 2017, Bakirtzi, Triantafyllidou, and Makris 2016).

With increasing effort devoted to study DES, designation such as natural deep eutectic solvent (NADES) and therapeutic deep eutectic solvent (THEDES) are also synthesized, where NADES is made of naturally occurring molecules like sugars and organic acid. On the other hand, THEDES is defined when contain an active pharmaceutical ingredient (API) as one of the eutectic components (Choi et al. 2011, Dai et al. 2013, Tuntarawongsa and Phaechamud 2012, Cherukuvada and Nangia 2014, Aroso et al. 2015, Mbous et al. 2017).

Numerous studies demonstrated that these designations successfully enhanced the solubility and the bioavailability of the both polar and non-polar bioactive compounds

which indicated the potential of these eutectic system as dissolution enhancers in the development of drug delivery system (Faggian et al. 2016, Liu, Zhang, Chen, Friesen, et al. 2018). Duarte et al. (2017) prepared novel THEDES designation based on choline chloride, menthol and three different APIs (ibuprofen, phenylacetic acid and benzoic acid) and evaluated their solubility in an isotonic solution. The most evident result is the increment of solubility of ibuprofen, such that a total of 12-fold improvement was observed as compared to the solubility of pure ibuprofen in the isotonic solution. Aside from that, Silva et al. (2018) prepared a novel DES system based on choline chloride and ascorbic acid. According to their research, the synthesized DES successfully improved the solubility of a poor soluble drug (dexamethasone) by several order as compared to the powder form of dexamethasone.

Moreover, Dai, Verpoorte, and Choi (2014) highlighted the potential of DES as a stabilization media for the bioactive compounds. The research team investigated the ability of NADES in stabilizing carthamin with antioxidant properties, a red pigment in safflower. The research team found that xylitol-choline chloride NADES significantly enhanced the stability of the carthamin under various conditions, including high temperature, exposed light and storage time, as compared to water. Particularly, the degradation rate of carthamin in water and xylitol-choline chloride NADES at different temperature was depicted as Figure 2.6. It was observed that the degradation rate of carthamin in the NADES was much slower than water which clearly showed that NADES exhibit ability to protect the antioxidant compounds against thermal degradation. Besides that, the authors also estimate the half-life of carthamin in both media and found that the half-life ($t_{1/2}$) of carthamin in the NADES at 60℃ was more than twice than in water. Their finding paths a research direction for the application of NADES in stabilizing bioactive compounds with poor stability against environmental conditions. It is also important to highlight that information reporting the ability of DES as a stabilizing agent and solubility enhancers remain scarce. This may due to their high viscosity. Nonetheless, researchers have suggested to overcome this problem by inclusion of water. Remarkably, the physicochemical properties of DES system changed upon the dilution of water (Khodaverdian et al. 2018).

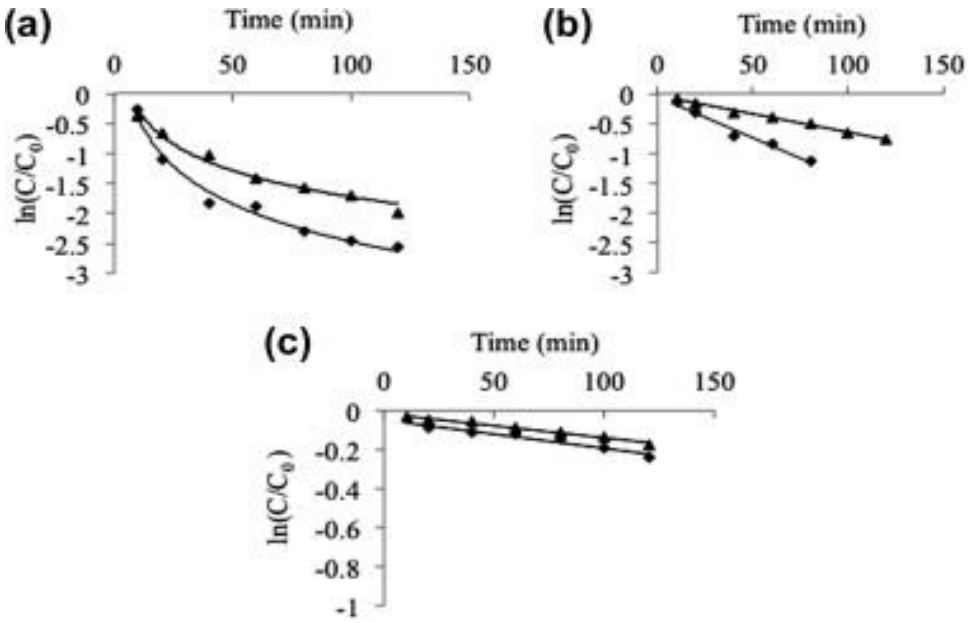


Figure 2.6 Degradation rate of carthamin in xylitol-choline chloride (▲) and water (♦) at different temperature: (a) 80 °C, (b) 60 °C and (c) 40 °C) (Dai, Verpoorte, and Choi 2014) (Reprinted with permission – Appendix H)

2.5.8 Summary of Section

All of the formulation methods described earlier were shown to enhance the solubility and stability of antioxidant compounds. A summary of the advantages and disadvantages of these methods are tabulated in Table 2.4. Amongst, the advantages of deep eutectic solvent-based formulation clearly shows that it has the potential to improve the solubility and stability issues of antioxidant compounds. The versatility of DES is comparable to other formulation methods with their great advantages, such that they are formulated by natural primary metabolites. Nonetheless, clear efforts are still required to design the DES system suitable for the application as the information on this field remains limited.

Table 2.4 Summary of advantages and disadvantages of the formulation methods

Formulation Techniques	Advantages	Disadvantages	References
Nanoemulsion-based system	• Able to encapsulate lipophilic bioactive compounds • Enhance the solubility of hydrophobic bioactive compounds • Kinetically stable • Allow interactions with cells • Enhance permeability of biological membranes • Sustained gastric retention time • Improve oral bioavailability	• Destabilization process occur after certain period of storage time	Lin et al. (2017), Sivakumar, Tang, and Tan (2014), Rai et al. (2018)
Liposome-based system	• Able to incorporate both hydrophilic and lipophilic bioactive compounds • Biocompatible • Biodegradable • Low toxicity • Improve solubility of poor soluble core material • Enhance the circulation half-life at target sites	• Short half-life • Poor physical and chemical stability	Zununi Vahed et al. (2017), Patil and Jadhav (2014), Park, Jo, and Jeon (2014)

Hydrogel-based system	• Possess swelling ability • Stimuli-responsive properties • Biocompatible • Biomimetic • Ensure controlled release	• Not able to encapsulate pH-sensitive bioactive compounds • The usage of cross-linker during chemical method are sometimes toxic	Pellá et al. (2018), Lai and He (2016), Zhang et al. (2017)
Cyclodextrin complexation	• Increase oral bioavailability of bioactive compounds • Improve physicochemical stability • Reduce adverse drug reaction in gastrointestinal tract • Modify the release site • Ensure controlled release	• Poor solubility in water • Require chemical modification	Pérez-Abril et al. (2017), Conceição et al. (2018), Matencio, García-Carmona, and López-Nicolás (2017)
Solid lipid nanoparticles	• Able to encapsulate both lipophilic and hydrophobic bioactive compounds • Enhance stability of bioactive compounds • Eliminate the usage of organic solvent • Ensure controlled release	• Less physical stable • Often undergo polymorphic transition	Mehnert and Mäder (2012), Qian et al. (2013), Lin et al. (2017)
Ionic liquid-based formulation	• Able to solubilize both hydrophilic and hydrophobic bioactive	• Low sustainability • Low toxicity • High cost	Mahamat Nor, Woi, and Ng (2017), Goindi, Kaur, and Kaur

	compounds • High thermal stability • High conductivity • Easily tailored by modifying anion/cation combination		(2015), Dobler et al. (2013), Kudłak, Owczarek, and Namieśnik (2015)
Deep eutectic solvent-based formulation	• Able to improve solubility and bioavailability of bioactive compounds • Synthesized by natural primary metabolites • Highly biodegradable • Negligible toxicity • Low cost • Simple preparation • Physiochemical properties can be easily tailored	• High viscosity • Limited information	Meng et al. (2018), Cunha and Fernandes (2018), Smith, Abbott, and Ryder (2014), Duarte et al. (2017), Dai, Verpoorte, and Choi (2014), Khodaverdian et al. (2018).

2.6 Degradation kinetic models

Physical, chemical and biological changes often cause the food product deteriorate and these changes might in time compromise the nutritional effects. Therefore, it is important to evaluate and predict the degradation of antioxidant compounds and their kinetics according to the process conditions. The degradation of antioxidant compounds usually obeys zero- and first-order kinetics as pioneered by Saguy and Karel (1980) and Labuza (1984). Figure 2.7 presented a deterioration of compound for a reaction at various order. Considering these models can reduce the necessity of bio-studies, able to describe the degradation pattern in function of time and enable quantitative interpretation of the values from the degradation rate, these mathematical descriptions must be thoroughly studied.

The general rate law describing the rate of degradation of a food compound is given by Equation 2.1:

$$r = -\frac{dC}{dt} = k.C^n \quad (2.1)$$

where r and $\frac{dC}{dt}$ is the rate of degradation of compound, k is the rate constant and n is the order of the degradation.

Degradation at zero-order reaction are often used when only a small amount of food compounds present or only small fraction of product is formed from the reactant, such that the reactant is in large excess that the concentration remain constant throughout the testing period. In other word, the rate is independent on the concentration.

At n = 0, the zero-order rate of degradation can be rewrite as:

$$r = -\frac{dC}{dt} = k \quad (2.2)$$

Integration of equation 2.2 leads to:

$$C = C_0 - kt \quad (2.3)$$

where C_0 is the initial concentration of compounds at time, t = 0.

The first-order kinetics model defines the changes in concentration as a function of time. At n=1, the first-order rate of degradation can be present as:

$$r = -\frac{dC}{dt} = kC \quad (2.4)$$

Equation 2.4 can be integrated with respect to time to predict the concentration profile:

$$C = C_0\, e^{-kt} \quad (2.5)$$

It can also be rewritten in the logarithmic form:

$$\ln C = \ln C_0 - kt \tag{2.6}$$

Notably, the second-order kinetics is not usually reported in the food science because the experimentally observed kinetics of a reaction may not correspond to the prediction. For example, considering the reaction between compound A and B, and the compound B is present in excess:

$$-\frac{d[A]}{dt} = k[A][B] = k'[A] \tag{2.7}$$

$$k' = k[B] \tag{2.8}$$

Based on equation 2.7 and Equation 2.8, it is obvious that the pseudo first-order rate constant (k') remains constant as long the concentration of compound B does not change (Xu, Yu, and Zhou 2019).

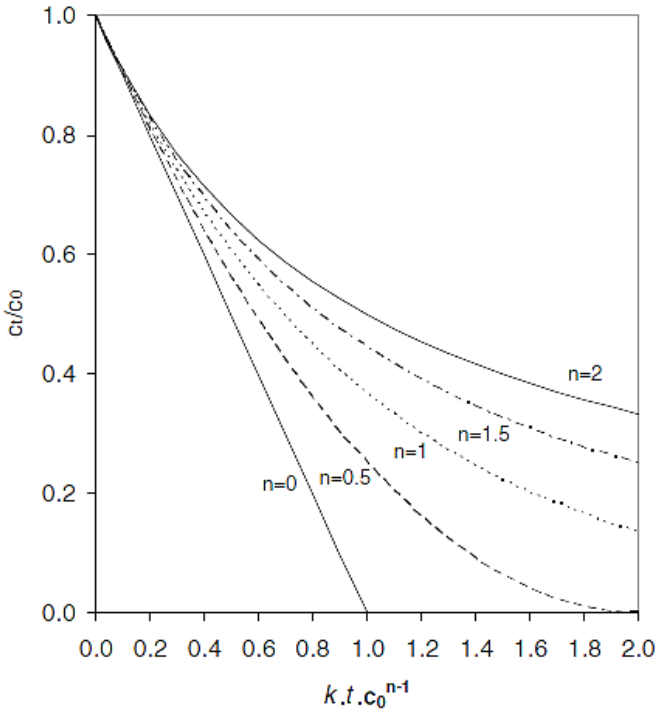


Figure 2.7 Degradation of compounds at same initial concentration and rate constant but different order n (Van Boekel 2008) (Reprinted with permission – Appendix H)

2.6.1 Kinetic parameters

The temperature dependence of the rate constant can be described by using the simple exponential form of Arrhenius equation (Equation 2.9). Arrhenius equation allows the extrapolation of kinetic data over a wide range of temperature and help define the food deterioration kinetic model. The degradation kinetic parameters including degradation rate constant, activation energy and frequency factor can be calculated and used to estimate the rate of degradation of bioactive compounds during storage or during exposure to environmental factors (Xu, Yu, and Zhou 2019).

$$k = Ae^{-\left(\frac{E_A}{RT}\right)} \tag{2.9}$$

where A is the frequency factor of reaction, also known as pre-exponential factor, E_A is the activation energy (kJ/mol), R represents the universal gas constant (8.314 J/mol. K) and T is the absolute temperature (K).

The activation energy and frequency factor can be obtained by plotting natural logarithm of rate constant ($\ln k$) against reciprocal of absolute temperature (1/T):

$$\ln k = \ln A - \frac{E_a}{RT} \tag{2.10}$$

$$\ln k = -\frac{E_a}{R}\left(\frac{1}{T}\right) + \ln A \tag{2.11}$$

The activation energy can be described as the energy require for the molecules to cross the barrier in order to react. The frequency factor represent the rate constant at which all the compounds have sufficient energy to react ($E_a = 0$) (Van Boekel 2008).

Other kinetic parameters such as decimal reduction time (D), half-life ($t_{1/2}$) and temperature coefficient (Q_{10}) are also often employed to predict the degradation profile. The decimal reduction time refers to the required time to reduce the concentration of bioactive compounds by 90%, whereas the half-life is the duration corresponding to the concentration of bioactive compounds diminishing to 50% of the

initial concentration. The temperature coefficient is used to characterize the influence of the temperature on the degradation rate of bioactive compounds. These parameters can be calculated using the following equations (Peron, Fraga, and Antelo 2017, Mercali et al. 2013):

$$D = \frac{\ln 10}{k} \tag{2.12}$$

$$t_{\frac{1}{2}} = \frac{\ln(2)}{k} \tag{2.13}$$

$$Q_{10} = \frac{k_2}{k_1}^{\left(\frac{10}{T_2 - T_1}\right)} \tag{2.14}$$

where k_1 and k_2 are the rate constant corresponding at temperature T_1 and T_2.

2.6.2 Modelling of degradation kinetics of antioxidant compounds

It is relevant to study the deterioration of antioxidant compounds and then model their kinetics. Knowledge of degradation kinetics, including reaction order, rate constant and activation energy are very important to estimate the loss of antioxidant compounds. Recent studies focus on the modelling of degradation kinetics of antioxidant compounds during heat process and their findings were summarized in Table 2.5. According to the studies presented in Table 2.5, it was realized that the degradation of antioxidant compounds generally exhibited a first order kinetics. This finding was found to be in accordance with numerous previous studies (Henríquez et al. 2014, Fischer, Carle, and Kammerer 2013, Sadilova, Carle, and Stintzing 2007, Reyes and Cisneros-Zevallos 2007). However, some reserachers also pointed out that the simple first-order kinetics may not be sufficient to estimate the degradation pattern (Ibarz, Pagan, and Garza 1999, Peleg et al. 2018). Thereby, it is of great interest to propose alternative kinetic model to accurately predict the degradation of antioxidant compounds.

Aside from that, it was found that the rate constant showed a strong temperature dependence, in which k value increased with temperature, indicating that higher degradation rate occurs at higher temperature. It is important to highlight that the half-life value reduced with faster degradation reaction, accompanied by higher k values. In terms of activation energy, different values were reported. In general, higher activation energy revealed the temperature dependence of the degradation rate constants. The activation energy is associated with the high temperature; such that higher activation energy values are observed at higher temperature. The observed difference of activation energy reported in Table 2.5 might be due to the differences in processing and the composition of antioxidant sources studied. In light of the scarce data, further research in the kinetic modelling to describe these basic kinetic parameters and to predict the changes in food system during processing is of paramount importance and desirable.

Table 2.5 Thermal degradation kinetics of different antioxidant compounds

Antioxidant sources or product	Operating conditions	Reaction kinetic order	Kinetic parameters	References
Plum (*prunus domestica*)	Temperature ranged from 70 to 110℃ for 20 mins	First-order kinetic model	Degradation kinetics on DPPH radical scavenging activity: k = 0.004 – 0.021 min^{-1} E_a = 47.22 kJ/mol $t_{1/2}$ = 33 – 173.28 min Degradation kinetics on total phenolic content: k = 0.007 – 0.029 min^{-1} E_a = 35.50 kJ/mol $t_{1/2}$ = 23.90 – 99.02 min Degradation kinetics on total anthocyanin content: k = 0.013 – 0.049 min^{-1} E_a = 36.42 kJ/mol $t_{1/2}$ = 14.14 – 53.31 min Degradation kinetics on total flavonoid content: k = 0.012 – 0.025 min^{-1}	Turturică et al. (2016)

			E_a = 17.99 kJ/mol $t_{1/2}$ = 30.12 – 57.76 min	
Black rice flour (*Oryza sativa L.*)	Temperature ranged from 60 to 100℃ for 20 mins	First-order kinetic model	Degradation kinetics on DPPH radical scavenging activity: k = 0.64 – 0.85 × 10^{-2} min^{-1} E_a = 6.71 kJ/mol Degradation kinetics on total phenolic content: k = 7.61 – 11.05 × 10^{-2} min^{-1} E_a = 7.26 kJ/mol Degradation kinetics on total anthocyanin content: k = 0.92 – 1.22 × 10^{-2} min^{-1} E_a = 10.07 kJ/mol	Bolea et al. (2016)
Honeybush (*Cyclopia genistoides*)	Temperature ranged from 60 to 140℃	First-order kinetic model	Degradation kinetics of benzophenones: k = 0.0141 – 3.41 h^{-1} E_a = 99.8 - 125 kJ/mol Degradation kinetics of xanthones: k = 0.0313 – 1.87 h^{-1} E_a = 106 - 107 kJ/mol	Beelders et al. (2017)

Apricots (*Prunus armeniaca L.*)	Temperature ranged from 50 to 70℃	First-order kinetic model	Degradation kinetics of total carotenoid: k = 0.0208 – 0.1710 min^{-1} E_a = 96.95 kJ/mol	Fratianni et al. (2017)
Sour cherries (*Prunus cerasus*)	Temperature ranged from 100 to 160 ℃ for 120 mins	First-order kinetic model	Degradation kinetics on DPPH radical scavenging activity: k = 0.09 – 8.12 × 10^{-2} min^{-1} E_a = 31.47 kJ/mol $t_{1/2}$ = 8.52 – 752.43 min Degradation kinetics on total anthocyanin content: K = 0.43 – 6.05 × 10^{-2} min^{-1} E_a = 54.19 kJ/mol $t_{1/2}$ = 11.44 – 158.40 min Degradation kinetics on total flavonoid content: k = 0.11 – 0.25 × 10^{-2} min^{-1} E_a = 38.38 kJ/mol $t_{1/2}$ = 273.61 – 601.95 min	Oancea et al. (2017)

Juçara (*Euterpe edulis Martius*) and "Italia" grapes (*Vitis vinifera L*)	Temperature ranged from 50 to 90℃	First-order kinetic model	Degradation kinetics of Juçara: $k = 2.2 – 125 \times 10^{-5}$ min^{-1} E_a = 99.77 kJ/mol $t_{1/2}$ = 9 – 516 hours Degradation kinetics of "Italia" grapes: $k = 12.3 – 545 \times 10^{-5}$ min^{-1} E_a = 93.62 kJ/mol $t_{1/2}$ = 2 – 94 hours	Peron, Fraga, and Antelo (2017)
Hardy kiwifruit (*Actinidia argute*)	Temperature at 5, 15, 25 and 45℃ for 72 h	First-order kinetic model	Degradation kinetics on DPPH radical scavenging activity: $k = 1.90 – 7.92 \times 10^{-3}$ h^{-1} E_a = 29.07 kJ/mol $t_{1/2}$ = 3.65 – 15.19 days Degradation kinetics on total phenolic content: $k = 1.59 – 6.54 \times 10^{-3}$ h^{-1} E_a = 28.15 kJ/mol $t_{1/2}$ = 3.65 – 15.19 days	Kim et al. (2018)
Açai-berry (*Euterpe oleoracea*)	Temperature ranged from 40 to 80℃	First-order kinetic model	Degradation kinetics: $k = 4.04\times 10^{-4} – 1.08\times 10^{-3}$ min^{-1}	Costa, Silva, and Vieira (2018)

			E_a = 24.16 kJ/mol $t_{1/2}$ = 10.7 – 28.6 hours	
Trans-carotenoids	Temperature ranged from 25 to 45℃ for 10 hours	First-order kinetic model	Degradation kinetics of lutein: k = 0.1672 – 0.4462 h^{-1} E_a = 38.8958 kJ/mol $t_{1/2}$ = 1.5534 – 4.1456 hours Degradation kinetics of β-carotene: k = 1.1223 – 1.3964 h^{-1} E_a = 8.6324 kJ/mol $t_{1/2}$ = 0.4964 – 0.6176 hours Degradation kinetics of cis-lutein: k = 0.1679 – 0.3067 h^{-1} E_a = 23.9784 kJ/mol $t_{1/2}$ = 2.26 – 4.1283 hours Degradation kinetics of cis-β-carotene: k = 0.8497 – 1.5963 h^{-1} E_a = 24.9112 kJ/mol $t_{1/2}$ = 0.4342 – 0.8158 hours	Xiao et al. (2018)

Epigallocatechin gallate	Temperature ranged from 25 to 165℃ at pH 2.2 – 8 for 1440 min	First-order kinetic model	Degradation kinetics: k =1.06× 10^{-7} – 8.83× 10^{-2} s^{-1} E_a = 56.3 – 72.4 kJ/mol	Xu, Yu, and Zhou (2019)

2.7 Summary of Chapter 2

In view of the remarkable antioxidant properties of *M. pajang*, there is a bright prospect to utilize the fruit wastes as natural sources of antioxidant products. For an economical utilization of *M. pajang's* antioxidant compounds in nutraceutical industry, an effective extraction of the phytochemicals involved is crucial before formulating the extract into a desired product. However, antioxidant extracts are often limited by its poor solubility and stability. Specifically, the problem regarding the stability is often overlooked. It is known that the antioxidant formulation inevitably degrades during food processing or storage, mainly due to the impact of heat and pH. Therefore, it is of great interest to develop a suitable formulation that enhances the solubility and preserve their stability in providing maximum therapeutic benefits. Numerous approaches were proposed and amongst, CHCL/AA NADES show great promising as solubility and stability enhancers. Nonetheless, the information on the solubilization and stabilizing capacity of NADES remain obscure, which forms the motivation for this work. The goal of this work is to enhance the solubility of stability of antioxidant compounds from the fruit wastes of *M. pajang* in CHCL/AA NADES. The practical applicability of the formulated CHCL/AA NADES system were investigated by systematic testing as evident in subsequent chapters to address the problems regarding the solubility and stability of antioxidant extracts from *M. pajang*.

CHAPTER 3
RESEARCH METHODOLOGIES

3.1 Overview

This chapter presents the research materials and research methodologies to achieve the objectives stated in Chapter 1. An overall research methodology with the methodology description is illustrated as Figure 3.1. This study starts with the extraction of antioxidants from fruit wastes of *M. pajang* and the preparation and characterization of choline chloride/ascorbic acid natural deep eutectic solvent (CHCL/AA NADES). Following that, the solubilization and stabilization capacity of CHCL/AA NADES were evaluated. The methodologies for these experiments are detailed in this chapter.

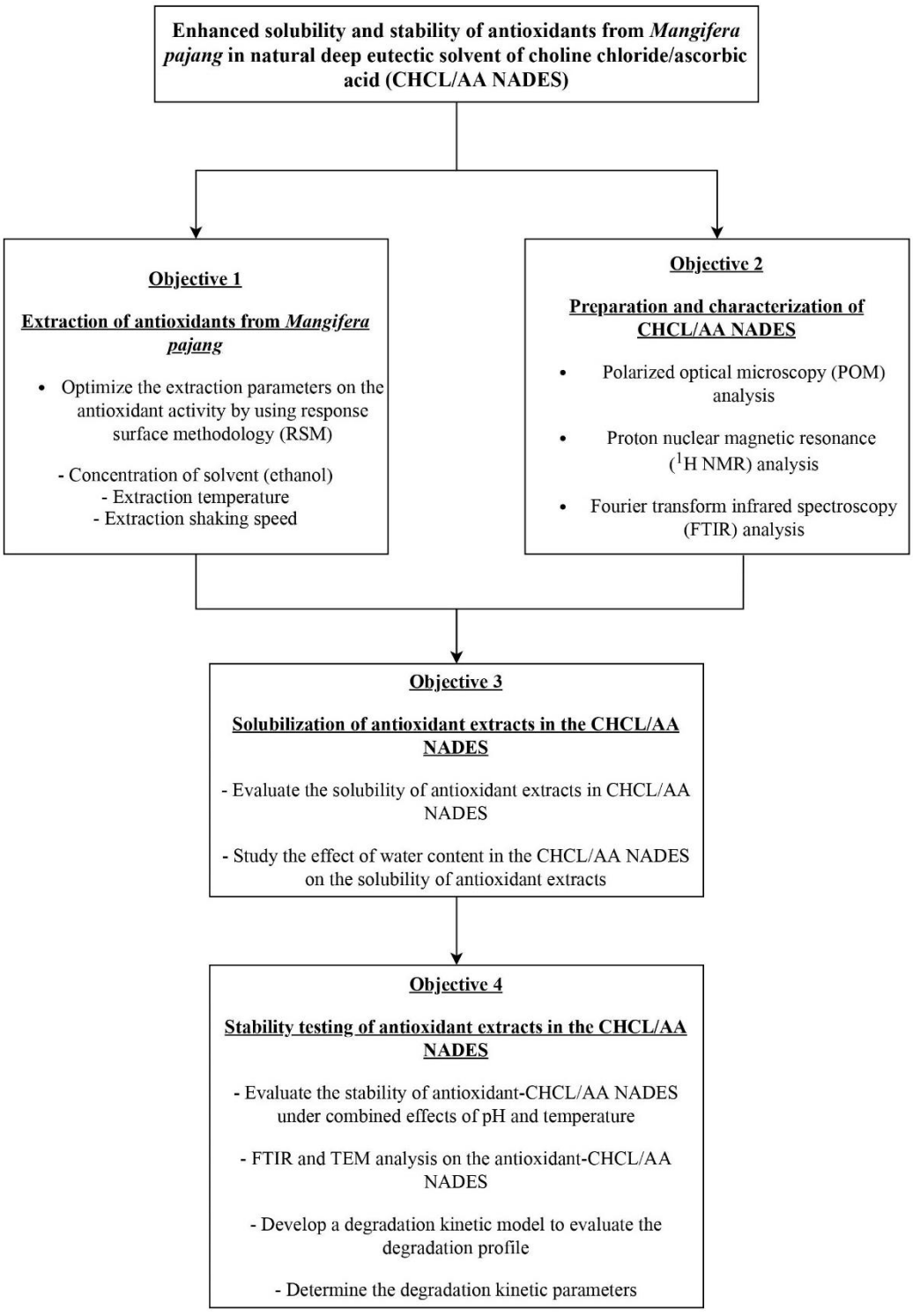


Figure 3.1 Flow chart of research methodology

3.2 Research material and chemicals

3.2.1 Plant material and sample preparation

The fresh ripe fruits of *M. pajang* were collected from the local market in Sarawak, Malaysia. The fruits were cleaned and manually separated into the flesh, kernel and peel. The kernels and peels were dried at 60 ℃ for 24 h and were grounded into fine powders using a mill grinder (A11, IKA). The sample were screened through stainless-steel sieves with 1.18 mm size to get uniform particle size, kept in amber storage bottle to avoid contact with light and stored in a freezer (-20 ℃) until further analysis (Abu Bakar et al. 2009).

3.2.2 Chemicals

The experimental works in this study mainly utilized a few chemicals, including ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent, choline chloride and ascorbic acid. The descriptions of these chemicals are summarized as following:

1) Ethanol

Ethanol is a common solvent that can be used for extraction of bioactive compounds (Shi et al. 2005). Aside from that, it is considered as generally recognized as safe (GRAS) and used in different industries such as beverage and pharmaceutical industry (Renard 2018, Sajedi-Amin et al. 2017, Alzeer and Abou Hadeed 2016). Absolute ethanol was purchased from Merck (USA). It was used to prepare aqueous ethanol by mixing with deionized water for the extraction of antioxidant compounds from the fruit wastes of *M. pajang* and also used to prepare DPPH solution.

2) 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent

The DPPH assay is low-cost approach to determine the antioxidant activity of natural products. DPPH is a stable free radical and has a strong absorption band at 517 nm (deep violet colour). The chemical compound of DPPH is presented as Figure 3.2. In the presence of antioxidant compounds which can donate a hydrogen atom, the electron become paired, and this reaction induces the loss of the violet colour and reduce the absorbance. The changes in absorbance indicates the antioxidant capacity

of the tested sample (Elbadrawy and Sello 2016, Ajila et al. 2007). The DPPH reagent powder used in this study was obtained from Merck (USA).

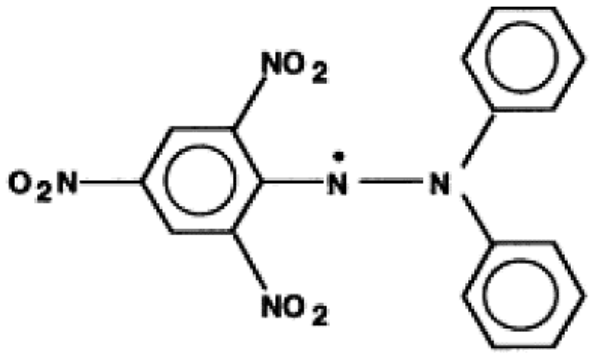


Figure 3.2 Chemical structure of DPPH free radical

3) Choline chloride

Choline is a biodegradable, low cost and water-soluble salt which used as a source of Vitamin B. It is always associated with chloride anion and choline chloride can be produced through gas phase reaction between ethylene oxide, trimethylamine and hydrochloric acid. Choline chloride is non-toxic and is often used as a poultry feed additive (Gadilohar and Shankarling 2017, Fu et al. 2017). Moreover, it was listed in the Code of Federal Regulations as nutrients or dietary supplement that are generally recognized as safe, thus, can be used in food application (Zeisel 1994). The chemical structure of choline chloride is illustrated as Figure 3.3. Choline chloride with purity ≥98% was purchased from Sigma-Aldrich (USA) and was used as a hydrogen bond acceptor in the synthesis of natural deep eutectic solvent.

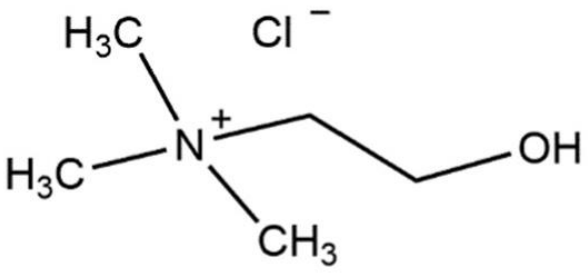


Figure 3.3 Chemical structure of choline chloride

4) Ascorbic acid

Ascorbic acid or often known as Vitamin C is an essential antioxidant. It has been widely used in pharmaceutical and food industry due to its remarkable role in inhibiting the free radicals, preventing scurvy and detoxifying (Wang et al. 2018, Kesinger and Stevens 2009). The chemical structure of ascorbic acid is given in Figure 3.4. L-Ascorbic acid was purchased from the Merck (USA) and was used in the preparation of natural deep eutectic solvent and also as a standard for the DPPH assay.

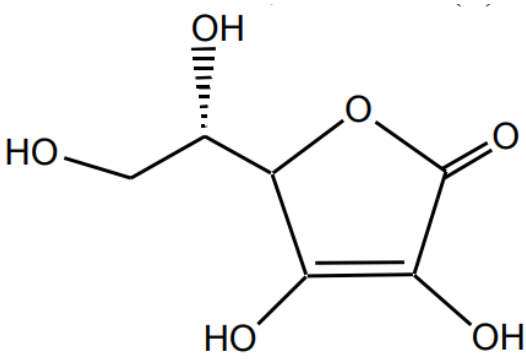


Figure 3.4 Chemical structure of ascorbic acid

3.3 Extraction of antioxidant from peels and kernels of *M. pajang*

3.3.1 Experimental procedures

The extraction of antioxidant from the peels and kernels of *M. pajang* was performed using solvent extraction method. Ethanol was chosen as the extraction media. Prior to the extraction process, aqueous ethanol at desired concentrations (20% - 100%) were prepared by mixing absolute ethanol and deionized water at the right composition.

The extraction procedures were based on the study reported by Abu Bakar et al. (2009) with modifications. 0.5 g of peel powders and 0.5 g of kernel powders were mixed with 30 ml of aqueous ethanol at desired concentrations and the mixture was loaded in an incubator shaker (SASTEC ST-200R) which set at speed ranged from 100 rpm to 300 rpm and temperature ranged from 30℃ to 60℃. The extraction process was performed for 2 hours.

After the extraction process, the mixture was dispersed into 50 ml centrifuge tube and subjected to centrifuge. The centrifugation was performed at 4000 rpm and 25℃ for 20 min. The supernatant was decanted into a 30 ml amber vial tube and the remaining pellet was re-extracted under identical conditions. The supernatant was combined and then subjected to rotary evaporator (1100S-WD, EYELA) to remove excess solvent. Finally, the remaining antioxidant suspension was stored in the freezer (-20℃) for overnight and freeze dried using freeze dryer at -50℃ and 0.133 mbar (Fisher 1.5L, Labconco). The antioxidant powders were stored in amber storage bottle and kept in the freezer for further analysis.

3.3.2 DPPH radical scavenging assay

The antioxidant activity of the extracts were estimated by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as free radical model which adapted from Magalhães et al. (2006) and Brand-Williams, Cuvelier, and Berset (1995). An aliquot of 0.1 ml of extract or control (absolute ethanol) was mixed with 3.9 ml of 6×10^{-5} mol/L DPPH in absolute ethanol. The mixture was shaken vigorously and left to stand at room temperature for 30 min in the dark. The mixture was measured using a UV-visible spectrophotometer (LAMBDA Bio, PerkinElmer, USA) at 517 nm. The free radical scavenging activity was calculated using following equation:

$$\text{Scavenging effect (\%)} = [1 - (\frac{\text{Absorbance of sample}}{\text{Absorbance of control}})] \times 100 \quad (3.1)$$

A calibration curve using ascorbic acid as standard was prepared according to method described by Abu Bakar et al. (2009). A standard curve was obtained by plotting the logarithm of absorbance of control/absorbance of sample versus its concentration ranging from 0.02 to 0.5 mg/ml. The slope and intercept of the line provide a relationship between the absorbance and concentration. The final result was expressed as mg ascorbic acid equivalent antioxidant capacity in 1 g of sample (mg AEAC/g).

3.3.3 Optimization of solvent extraction parameters by using research surface methodology (RSM)

3.3.3.1 Experimental design

Response surface methodology (RSM) was employed to evaluated the effects of independent variables, including concentration of ethanol solution (A), extraction temperature (B) and extraction shaking speed (C) on the antioxidant activity (Y). A face-centered composite design (CCD) along with quadratic model was used. 20 experimental runs including 8 factorial points, 6 axial points and 6 replicates of center points were performed according to CCD and tabulated in Table 3.1.

Table 3.1 Independent variables and their corresponding coded levels

Variables	Unit	Symbol	Coded levels		
Independent Variable			-1	0	+1
Ethanol concentration	%	A	20	60	100
Extraction Temperature	℃	B	30	45	60
Extraction Shaking Speed	rpm	C	100	200	300

3.3.3.2 Statistical analysis

The Design Expert 11.0.3 Software (Star-Ease Inc., Minneapolis, USA) was used to perform all the experimental design and response surface analysis. The experimental data were subjected to statistical analysis through regressions to fit second order polynomial equation (Equation 3.2) for the independent variables (Mehmood et al. 2018):

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 \quad (3.2)$$

where Y is the dependent variable (antioxidant activity), A, B and C represent the ethanol concentration (%), extraction temperature (℃) and extraction shaking speed (rpm), respectively. β_0 is the intercept coefficients. β_1, β_2 and β_3 are the linear coefficients. β_{11}, β_{22} and β_{33} represent the quadratic coefficient. β_{12}, β_{13} and β_{23} are the interaction coefficients.

The analysis of variance (ANOVA) was applied to determine the regression coefficient, to investigate the interaction between the dependent and independent variables and to select the best fit polynomial model for the experimental data. The statistical parameters, including lack of fit, coefficient of determination, the F-test value, adequate precision and coefficient of variation were analyzed to determine the adequacy of the quadratic response surface model. The relationship between the independent variables and the dependent variables was presented by the response surface plots (Jang et al. 2017). The numerical optimization was performed by using the desirability function method to determine the optimum conditions that obtain extracts with maximum antioxidant activity. All the experiments were performed in triplicate.

3.3.3.3 Validation of the model

The model validation was performed by carried out experiments at optimal extraction conditions. The experimental and predicted value by model were compared to determine the validity of the model. Values with a confidence level higher than 95% were considered as significant.

3.4 Preparation and characterization of choline chloride/ascorbic acid natural deep eutectic solvent (CHCL/AA NADES) and evaluation of its solubilization and antioxidant capacity

3.4.1 Preparation of CHCL/AA NADES

The CHCL/AA NADES was prepared by using the method of Silva et al. (2018) with modifications. The choline chloride and ascorbic acid were weighted at molar ratio of 1:1. 1:2 and 2:1 by using analytical balance (Sartorius balance QUINTIX 613-1S, Germany). The mixture was heated at 40℃ under constant stirring of 100 rpm by using a heating plate (Bibby Scientific, UK) until the formation of a homogenous clear liquid. The mixture was allowed to cool to room temperature before storing in the storage bottle for further analysis.

3.4.2 Characterization of CHCL/AA NADES

The CHCL/AA NADES at different molar ratio were first analyzed using polarized optical microscopy (POM) to observe the presence of crystal. The natural deep eutectic solvent with no crystal presence indicates formation of a stable natural deep eutectic solvent and was further characterized by using proton nuclear magnetic resonance (^{1}H NMR) and Fourier transform infrared spectroscopy (FTIR) analysis.

3.4.2.1 Polarized optical microscopy (POM) analysis

POM analysis is a promising approach to tentatively detect the single crystals and define the formation of liquid mixture. It allows identification of crystal even in the presence of liquid mixture (Aroso et al. 2017). A small droplet of natural deep eutectic solvent at molar ratio of 1:1, 1:2 and 2:1 was deposited on a microscopic slide for observation at a magnification of 10x. The polarized light image was observed at room temperature and captured using a Nikon ECLIPSE LV100N polarizing microscope (Nikon Corporation, Japan) coupled with Nikon DS-Fi2 camera and the analyzer software (Silva et al. 2018). When there is no presence of solid crystalline structure, the polarized light image is uniformly black (Aroso et al. 2016).

3.4.2.2 Proton nuclear magnetic resonance (^{1}H NMR) analysis

The ^{1}H NMR analysis has been used to study the ionization states of proton and determine the structure of the molecules. It quantitatively determines the distribution of protons by detecting the proton spectra (Tan, Chin, et al. 2016, Delso et al. 2019). The ^{1}H spectra were recorded at 500 MHz on a JEOL. ECA 500 spectrometer (JEOL, USA). The solvent used was dimethyl sulfoxide-d6 (DMSO-D_6) and tetramethylsilance (TMS) was used as a reference with the chemical shift values (δ) are quoted in part per million (ppm).

3.4.2.3 Fourier transform infrared spectroscopy (FTIR) analysis

FTIR analysis is often used to analyze the molecular interactions of the formulation components and to characterize the sample by confirming the presence of functional

groups within the formulation components (Shafie, Yusof, and Gan 2019). The experimental procedures were referred to method described by Telange et al. (2017) with modifications. Approximately 0.1-1% of sample was uniformly mixed with potassium bromide (KBR, ~200 mg) and compressed into pellets using a vacuum pelletizer. The samples were dried in a hot air oven at 50 ℃ for 2 h to remove the influence of any residual moisture. The sample was then scanned by using FTIR spectroscopy (Agilent Technologies Cary 630, USA), at a resolution of 4 cm^{-1} in the wavelength range of 4000 to 400 cm^{-1}. The functional group present in the sample was analysed using IRsolution FTIR control software associated with the instrument.

3.4.3 Solubility capacity of CHCL/AA NADES

The solubility testing was carried out according to the method described by Soares et al. (2017) with modifications. An excess amounts of antioxidant extracts were added to the aqueous and CHCL/AA NADES. The mixture was shaken for 24 h using an orbital shaker (KS 501 digital shaker, IKA WERKE, Germany) at 100 rpm to ensure homogeneity. After the saturation, the samples were subjected to centrifugation for 25 min at 1500 rpm. The supernatant was subjected to dilution and assayed by using a UV-visible spectrophotometer (LAMBDA Bio, PerkinElmer, USA) at the optimum wavelength. The testing was conducted at room temperature. Each experiment was repeated three times and the mean of triplicates were taken. A calibration curve using the antioxidant in water and CHCL/AA NADES as standard were prepared for quantification. The effect of water inclusion in the CHCL/AA NADES on the solubility was performed by adding different weight percentage of water in the mixture. The viscosity of CHCL/AA NADES at different water level were measured using a viscometer (Fann Model 36SA) at a speed of 6 rpm.

3.4.4 DPPH radical scavenging assay

The determination of antioxidant activity was carried out by using DPPH radical scavenging assay as detailed in Section 3.3.2.

3.5 Stability testing of antioxidant in aqueous and CHCL/AA NADES system

3.5.1 Experimental procedures

The degradation experiments were conducted based on the experimental procedures described by Beelders et al. (2018) and Xu, Yu, and Zhou (2019) at four temperatures for 6 different pH value. The heating temperature (25 – 80℃) and durations (0 – 360 minutes) under different pH levels (3.0 – 8.0) were tabulated as Table 3.2. The phosphate buffer saline solution (PBS) at desired pH were prepared using 0.1 M HCl and 0.1 M NaOH. The pH of the buffer solution was measured using a pH meter (SensION + pH1, HACH). The antioxidants were dispersed in aqueous and CHCL/AA NADES and later dissolved in the prepared buffer solution to obtain a working solution with a concentration of 500 mg/L. The working solutions were placed in Memmert WNB14L thermostatic water bath (Germany) at desired temperature. For reaction at 25℃, the working solutions were kept at ambient condition. The samples were periodically removed at specified durations and allowed to cool to room temperature. The samples were assayed by using a UV-visible spectrophotometer (LAMBDA Bio, PerkinElmer, USA) at the optimum wavelength. A standard curve using the antioxidant suspension and antioxidants in CHCL/AA NADES was prepared for quantification. The antioxidant assay was also performed using DPPH radical scavenging assay described in Section 3.3.2. All the experiments were performed in triplicates and the mean of triplicates were taken.

Table 3.2 Designated experiment conditions at every pH (pH 3.0 – 8.0)

Temperature (℃)	Heating durations (minutes)
25	0, 80, 160, 240, 320, 400, 480
40	0, 60, 120, 180, 240, 320, 360
60	0, 40, 80, 120, 160, 200, 240
80	0, 30, 60, 90, 120, 150, 180

3.5.2 Characterization of antioxidants in CHCL/AA NADES system

The antioxidants were dissolved in CHCL/AA NADES and the sample was subjected to FTIR as mentioned in earlier section and also scanned at transmission electron microscopy (TEM) analysis. The morphology and size of the sample was visualized by using TEM. 1 ml of the sample was dispersed in 20 ml of absolute ethanol and

sonicated before dropped on the formvar-coated copper grid. The TEM micrograph was observed via transmission electron microscopy (JEOL Model 1230).

3.5.3 Degradation kinetics model development

The degradations kinetics of the antioxidants in aqueous and CHCL/AA NADES system were defined by a regression of the experimental data from the initial concentration with increasing time. The degradation of antioxidant compounds usually obeys zero- and first-order kinetics as pioneered by Saguy and Karel (1980) and Labuza (1984). The experimental degradation data were fitted to two kinetic models, including zero- and first-order model, as these models best described the degradation of bioactive compounds (Labuza 1984). These models are described as:

1. Zero-order kinetic

$$r = -\frac{dC}{dt} = k \tag{3.3}$$

2. First-order kinetic

$$r = -\frac{dC}{dt} = kC \tag{3.4}$$

where r and $\frac{dC}{dt}$ is the rate of degradation of compound, k is the rate constant and C is the concentration.

The goodness-of-fit was obtained by evaluating the coefficient of determination value (R^2). For each pH value, the rate constant was determined and were compared over the different temperatures. The effect of temperature on the degradation rate constant at different pH level were investigated by calculating the activation energy by using Arrhenius law. The activation energy was determined by estimating the slope and y-intercept of plot of logarithm of rate constant (ln k) against reciprocal of temperature (1/T). The Arrhenius equation is written as (Xu, Yu, and Zhou 2019):

$$k = Ae^{-\left(\frac{E_A}{RT}\right)} \tag{3.5}$$

where A is the frequency factor of reaction, also known as pre-exponential factor, E_A is the activation energy (kJ/mol), R represents the universal gas constant (8.314 J/mol.K) and T is the absolute temperature (K).

The estimation of half-life value is important as it determines the time taken for the concentration of antioxidant compounds to diminish to half of its original concentration. The half-life of sample was calculated using the following equation (Peron, Fraga, and Antelo 2017):

$$t_{\frac{1}{2}} = \frac{\ln(2)}{k} \tag{3.6}$$

Aside from fitting the degradation experimental data into the zero- and first-order kinetic models, a new alternative degradation model was proposed to account for the description of degradation profile. It is assumed that over the entire degradation period, several terms of kinetics could exist, however, only a certain term is deemed to predominant while the rest may contribute in lesser proportion to the degradation. Based on this assumption, It is proposed that the degradation dynamics of the antioxidant follows a general form of the proposed multi-term degradation model:

$$\frac{dC(t)}{dt} = \sum_{j=1}^{\ell} \{-\kappa_i C(t)^{n_j}\} \tag{3.7}$$

where κ_i denote the degradation kinetics constant, n_j the order of degradation for j-th term of kinetics, and ℓ the number of terms to describing the degradation over a given period of time.

The degradation model above can also be written in the Laplace Domain as follows:

$$P(s) = \frac{\tilde{C}(s)}{U(s)} = \frac{k_1}{(\tau_1 s + 1)^{n_1}} + \frac{k_2}{(\tau_2 s + 1)^{n_2}} + \cdots + \frac{k_\ell}{(\tau_\ell s + 1)^{n_\ell}} \qquad (3.8)$$

Where $\tilde{C} = \ln(C/C_o)$ where C is the concentration of the antioxidant at any time, $t \neq 0$ and C_o is the initial concentration of antioxidant at $t = 0$, τ_j represents the time constant for $j = 1, 2, \ldots, \ell$ represent the time-constant of the j-th term of kinetics.

In the present study, for simplicity the general degradation model shown in (3.8) is simplified to only 3 terms (1 short-term, 1 medium-term and 1 long-term) given as follows:

$$P(s) = \frac{\tilde{C}(s)}{U(s)} = \underbrace{\frac{k_1}{(\tau_1 s + 1)^{n_1}}}_{\text{short-term}} + \underbrace{\frac{k_2}{(\tau_2 s + 1)^{n_2}}}_{\text{medium-term}} + \underbrace{\frac{k_3}{(\tau_3 s + 1)^{n_3}}}_{\text{long-term}} \qquad (3.9)$$

Depending on the degree of degradation to be covered by the model, the number of the degradation terms may be further reduced to only two terms – a short-term and a medium-term kinetics are required if the degree of degradation is up to about 0.5 (half-life). Here, the degree of degradation D is given as $D = C(t)/C_0$, where $C(t)$ is the concentration for $t > 0$ and C_0 is the initial concentration.

Because in the present study, the modeling goal is to be able to predict up to degradation degree of 0.5, only 1 short term and 1 medium term kinetics were adopted. Furthermore, the following specific model structure can sufficiently fit the experimental data:

$$P(s) = \frac{\tilde{C}(s)}{U(s)} = \underbrace{\frac{k_1}{(\tau_1 s + 1)^{1}}}_{\text{short-term}} + \underbrace{\frac{k_2}{(\tau_2 s + 1)^{3}}}_{\text{medium-term}} \qquad (3.10)$$

The identification of the above model parameter can be represented as an optimization problem given as follows:

$$J = \min_{k_1,k_2,\tau_1,\tau_2} \left\{ \sum_{i=1}^{n} (C_{ex}(i) - C(i))^2 \right\}$$

s.t:

$$\begin{cases} -0.5 \leq k_1 < 0 \\ -1.0 \leq k_2 < 0 \\ 0 < \tau_1 < 1000 \\ 0 < \tau_2 < 1000 \end{cases}$$

Another word, the optimization was used to find the values of k_1, k_2, τ_1 and τ_2 that minimize the sum of square error (SSE), where the error is difference between experimental concentration $C_{ex}(i)$ and predicted concentration $C(i)$ at time $t = t_i, \forall i \in \{1,2,\dots n\}$ where n is the number samples. The range of parameters are set based on various simulation trial. The optimization problem above was solved using the Matlab *fmincon* function.

CHAPTER 4
RESULTS AND DISCUSSIONS

4.1 Solvent extraction of antioxidants from fruit wastes of *M. pajang*

Fruit wastes of *M. pajang* were selected as the source of antioxidant compounds as they were reported to possess superior amount of antioxidant compounds (Abu Bakar et al. 2009). Extraction of the antioxidants is an important step in recovering these valuable compounds. For that, this section focuses on investigating the optimum conditions of extraction parameters, including concentration of ethanol, extraction temperature and extraction shaking speed by using response surface methodology (RSM).

Remarkably, it is important to point out that the extracts are not further purified in this study even it is generally known that a higher purity would give a higher value to the extract. This is because separating and purifiying specific antioxidant compounds from the mixture form of extract is time-consuming as they involves multiple fractionation steps and they requires expensive chromatographic matrices that results in high costs of operation (Zhang, Yue, et al. 2010). On top of that, purification process often leads to loss of antioxidant activity (Li et al. 2013). Moreover, it was reported that around 90% of the total polyphenols in the fruit wastes of *M. pajang* are phenolic acids (aromatic ring with one carboxylic acid functional group) (Jahurul et al. 2019, Hassan, Ismail, Abdulhamid, et al. 2011). Since these phytochemicals are in same sub-group of phenolic compounds and considering the mixture form of extract might exhibit synergistic effect which may contributed to higher free radical scavenging properties, so the further purification of the extracts mixture to specific phenolic compound is not required (Hsieh et al. 2018).

4.1.1 Optimization of extraction parameters

A face-centred composite design (CCD) was used to identify the optimum conditions and investigate the effects of independent variables which include concentration of ethanol solution (A), extraction temperature (B) and extraction shaking speed (C) on the antioxidant activity (Y). CCD was chosen because it provides clear trends about the effects of independent variables on the dependent variables and their interactive effect could be achieved and elucidated (Verseput 2001). Also, it has been used for process optimization of extraction of antioxidant compounds from different plant sources (Belwal et al. 2016, Prasad et al. 2011, Gan and Latiff 2011).

In this study, ethanol was selected due to its economic adffordability and it has been generally recognized as safe (GRAS) in accordance with the European Food Safety Authority (EFSA) (Gullón et al. 2017). Numerous studies stated that the concentration of solvent plays a key role in extraction and strongly impact the extraction performance (Prasad et al. 2011, Anbalagan et al. 2019, Sousa, Júnior, and de Souza Buarque 2019). Interestingly, it was highlighted that the mixture of organic solvent and water present a better extract quality and efficiency than the corresponding mono-component solvent system (Mota et al. 2012). In this regard, the effect of aqueous ethanol concentration ranged from 20% to 100% on the antioxidant activity was investigated in this study. In particular, 100% ethanol was included in our study to represent a mono-component solvent system.

On the other hand, the extraction temperature is also a critical parameter that should be taken into account as it greatly affects the antioxidant activity of extract. Although higher extraction temperature is normally preferred as it results in greater diffusivity of the solutes, but a high temperature could also causes oxidation and degradation of antioxidant compounds. Specifically, Sulaiman et al. (2017) found that when the extraction temperature exceed 60℃, a lower extraction effiencyiy was obtained. In addition, costs are expected to increase with the increase of extraction temperature (Zhang et al. 2007). For that, the extraction temperature for the optimization design was set in the range of 30℃ to 60℃ to evaluate its impact on the antioxidant activity.

Besides that, it was reported that an increase of shaking speed could enhance the mass transfer rate, leading to greater extraction performance (Mohamad et al. 2013). Nevertheless, the effects of shaking speed is often overlooked and neglected. Therefore, study of the effect of shaking speed on the antioxidant activity was undertaken in this study. The extraction shaking speeds from 100 rpm to 300 rpm were chosen based on the study performed by Andrade et al. (2015).

4.1.2 Experimental design and statistical analysis

The antioxidant activity of extracts under different extraction conditions are tabulated in Table 4.1. The experimental values were relatively close to the predicted values, indicating a satisfactory model. The experimental data were used in regression analysis to compute the coefficients of polynomial equation which predicted the antioxidant activity. The regression coefficient of different response is summarized in Table 4.2. The regression equation obtained from the response surface methodology is as follows:

$$\begin{aligned}&\text{Antioxidant activity (Y)}\\&= 11.28 - 0.3804A + 0.2026B - 0.3237C + 0.1635AB\\&+ 0.1592AC - 0.5781BC - 1.11A^2 - 0.3886B^2 - 1.31C^2\end{aligned} \tag{4.1}$$

The analysis of variance (ANOVA) suggested that the experimental data could be represented with a quadratic polynomial model. The ANOVA showed that the experimental data gave high coefficient of determination (R^2) of 0.9714 which is near to unity, i.e., $R^2 \approx 1$, indicating a high level of correlation between model and experimental data (Jang et al. 2017). The proximity to unity R^2 demonstrates that the effect of independent variables which include ethanol concentration (A), extraction temperature (B) and the extraction shaking speed (C) can be described through the model (4.1). Additionally, a large F-value of 37.74 and p-value less than 0.0001 were obtained, implying that the model is statistically significant (Table 4.3). On the other hand, the lack of fit p-value was calculated as 0.0995, showing that the adequacy of model to precisely estimate the variation (Prasad et al. 2012). The coefficient of variation (CV%) was 3.28% for the response which indicates satisfactory reproducibility of the model. The model also displayed high adequate precision value (17.279) which indicates an adequate signal of model (Bilgin et al. 2018).

Table 4.1 Experimental design for extraction, experimental and predicted values of response

STD	Factor 1: Ethanol Concentration (%)	Factor 2: Extraction Temperature (℃)	Factor 3: Shaking Speed (rpm)	Response: Antioxidant activity (mg AEAC/g)	
				Actual value	Predicted value
1	20	30	100	8.93	8.72
2	100	30	100	7.36	7.32
3	20	60	100	9.87	9.96
4	100	60	100	9.40	9.20
5	20	30	300	8.67	8.91
6	100	30	300	8.18	8.14
7	20	60	300	7.74	7.83
8	100	60	300	7.46	7.72
9	20	45	200	10.77	10.55
10	100	45	200	9.76	9.79
11	60	30	200	10.64	10.69
12	60	60	200	11.33	11.09
13	60	45	100	9.93	10.30
14	60	45	300	10.21	9.65
15	60	45	200	11.48	11.28
16	60	45	200	11.26	11.28
17	60	45	200	10.91	11.28
18	60	45	200	11.04	11.28
19	60	45	200	11.41	11.28
20	60	45	200	11.19	11.28

Table 4.2 Regression coefficient values for antioxidant activity

Regression Coefficient	Antioxidant Activity (Y)
Intercept (β_0)	11.28
A - Ethanol Concentration (β_1)	-0.3804
B - Extraction Temperature (β_2)	+0.2026
C - Shaking Speed (β_3)	-0.3237
AB (β_{12})	+0.1635
AC (β_{13})	+0.1592
BC (β_{23})	-0.5781
A^2 (β_{11})	-1.11
B^2 (β_{22})	-0.3886
C^2 (β_{33})	-1.31

4.1.3 Effects of independent variables on the antioxidant activity

Antioxidant activity is the measure of ability of bioactive compounds to scavenge the free radicals and inhibit the chain reaction of oxidation process through different mechanisms of action (Bravo 1998, Shahidi and Zhong 2015). It was reported that the radical scavenging ability of the phenolic antioxidant compounds are due to the delocalization on the aromatic ring of the phenolic compounds (Mohanan, Nickerson, and Ghosh 2018). In other word, evaluating the free radical scavenging capacity is a promising way to indicate the presence of phenolic compounds. For that, DPPH scavenging activity was used in this study to evaluate the antioxidant activity of the extracts and a standard curve of ascorbic acid was prepared and the standard curve is attached in Appendix A (Figure A.1). ANOVA was used to evaluate the influence of independent variables on the antioxidant activity. Specifically, independent variable with large F-value and p-value less than 0.05 have significant impact on the response variable (Tan, Masoumi, et al. 2016). The F-value and p-value obtained from ANOVA are tabulated in Table 4.3.

Table 4.3 ANOVA of regression coefficient of the fitted quadratic equation from all the independent variables

Parameter	Antioxidant Activity (Y)	
	F-value	p-value
Model	37.74	< 0.0001
A - Ethanol Concentration	13.75	0.0041
B - Extraction Temperature	3.90	0.0765
C - Shaking Speed	9.95	0.0102
AB	2.03	0.1845
AC	1.93	0.1953
BC	25.41	0.0005
A^2	32.09	0.0002
B^2	3.95	0.0750
C^2	44.62	< 0.0001

Table 4.3 shows that the first-order linear and quadratic effect of ethanol concentration (A and A^2) was at the level of $p < 0.05$, manifesting the ethanol concentration greatly influence the antioxidant activity of extracts. A one factor plot evaluating the effect of ethanol concentraation is presented as Figure 4.1. As shown in Figure 4.1, the antioxidant activity increased when the concentration of ethanol decreased from 100% to 60% and when the concentration of ethanol increased from 20% to 60%, inferring that the concentration of ethanol greatly influence the antioxidant activity. This behavior may be attributed to the affinity and interactions between the polarity of solvent and antioxidant compounds in the plant (Vega et al. 2017). Generally, polar antioxidant compounds in the plant matrix are easier to be isolated by using high polar solvent due to its thermodynamically favorable for the solvent-solute forces whereas the non-polar solvent can only dissolve the non-polar compounds (Abarca-Vargas, Pena Malacara, and Petricevich 2016). In this context, it can be said that the polarity of antioxidants is similar to the polarity of ~60% ethanol-water mixture. This finding confirms that antioxidant compounds in the fruit wastes of *M.pajang* has high polarity and this is reasonable since approximately 90% of the major antioxidant compounds

content in the fruit wastes of *M. pajang* are phenolic acids, which is hydrophilic in nature (Jahurul et al. 2019, El Riachy et al. 2011, Materska 2010). The addition of water (polarity index of 9.0) to the ethanol solvent (polarity index of 5.2) might create a high-polar medium that allows the solvent system extract antioxidant compounds of *M. pajang* from both ends of the polarity range (Uma, Ho, and Wan Aida 2010, Abarca-Vargas, Pena Malacara, and Petricevich 2016). A similar relationship of ethanol concentration and the antioxidant activity was observed in study performed by Liyana-Pathirana and Shahidi (2005), where ethanol at concentration around 50% yielded higher amount of phenolic compounds from wheat as compared to other solvent composition. Additionally, Gullón et al. (2017) found that the optimum concentration of ethanol for the extraction of antioxidant compounds from *Eucalyptus globulus* is 56%, which is in accordance to our findings. Their studies confirm that extraction with binary solvent or aqueous ethanol mixture contributes to higher antioxidant capacities.

Interestingly, the combinative effects of the ethanol concentration with both extraction temperature (AB) and extraction shaking speed (AC) were insignificant in our study ($p > 0.05$). As depicted in Figure 4.2A, changes in ethanol concentration and temperature revealed that maximum antioxidant activity was obtained when the ethanol concentration was around 60% and the temperature was around 50℃. It is worth to highlight that at ethanol concentration of around 60%, an increase in temperature did not show any profound effect and this suggests that the antioxidant activity might relies on its individual effect and their interactive effect is less significant. For the interactions between ethanol concentration and shaking speed (AC), Figure 4.2B shows that both parameters presented a linear relationship, where elevating both parameters up to a certain level lead to decomposition of antioxidant compounds. This suggests that the interactive effect of AC on the antioxidant activities of extract is less significant. Therefore, it can be presumed that the antioxidant activity is solely depending on the effects of each parameter.

Aside from the interactive effects of AB and AC, the linear and quadratic effect of extraction temperature did not significantly affect the antioxidant activity of extracts

($p > 0.05$). Even so, it was observed that at increasing temperature until a certain temperature, the antioxidant activity significantly increased (Figure 4.2C). On top of that, the antioxidant activity of extracts also depended on the interactive effect of extraction temperature and shaking speed (BC), with p-value of 0.0005 and the first-order linear and quadratic effect of extraction shaking speed (C and C^2) at the level of $p < 0.05$.

The interactive effect of extraction temperature and shaking speed may relate to the mass transfer theory which describe the solubility, diffusion and mass transfer of the antioxidant compound. It is known that in the extraction process, the solutes (antioxidant compounds) must first diffuse to reach the surface of solid matrix that is affected by convection before transferring to the bulk solvent. In other words, the transportation of solutes to the bulk of solvent solution is limited by convective mass transfer (Mohamad, Ali, and Ahmad 2010, Pawliszyn 2003). From Figure 4.2C, it was observed that the antioxidant activity of extracts increased with increased temperature and shaking speed. This is reasonable as the elevated temperature facilitates the breakdown of cellular constituents of plant cells which increases the solubility of solutes (antioxidant compounds) and diffusion rate of solutes to the bulk solvent (Thoo et al. 2010, Mokrani and Madani 2016). In respect to the elevated extraction temperature, accelerating shaking speed can creates more turbulence which enhances the mass transfer coefficient. The mass transfer coefficient refers to the diffusuion rate constant. In this context, increasing of the diffusion rate constant enhances the convective mass transfer. The increment of convective mass transfer then allows rapid diffusion and solubilization of antioxidants and subsequently, produce extracts with high antioxidant activity (Mohamad et al. 2013).

However, it was realized that the antioxidant activity of extracts started to decrease when the temperature and shaking speed beyond certain values. This suggests that further increment of temperature likely cause thermal degradation of phenolic compounds present in the extracts, where the phenolic compounds are thermaly destructed and consequently, reduced the antioxidant capabilities of extract (Chew et al. 2011). Similar finding was reported by Sarkar and Ghosh (2017), where the further

increase of extraction temperature reduced the antioxidant activity of extract. With regard of the shaking speed, it can be said that optimum shaking speed is sufficient to overcome the mass transfer limitations (Panchal, Deshmukh, and Sharma 2014). In this context, it is important to highlight that the linear and quadratic effect of shaking speed were also found to significantly affect the antioxidant activity of extracts. The one factor plot illustrating its impact is presented as Figure 4.3. This trend clearly showed that the antioxidant activity increased with the shaking speed. The positive effect of shaking speed can be coherent with above-mentioned effect. Wheras, further increase of shaking speed beyond the optimal value is found to affect the extraction process. This is possible as high shaking speed may create a shear force that cause damage to the antioxidant structures and consequently obtain extracts with lower antioxidant activity (Mustafa, Chua, and El-Enshasy 2019). This finding is in good agreement with previous study conducted by Andrade et al. (2015).

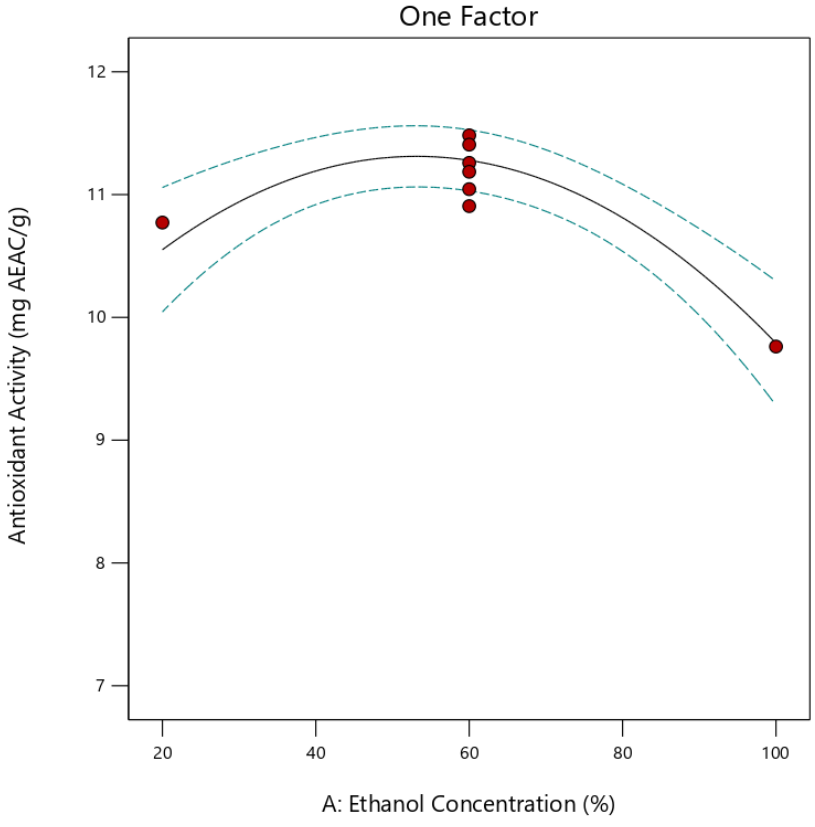


Figure 4.1 One factor plot – Effect of ethanol concentration on antioxidant activity

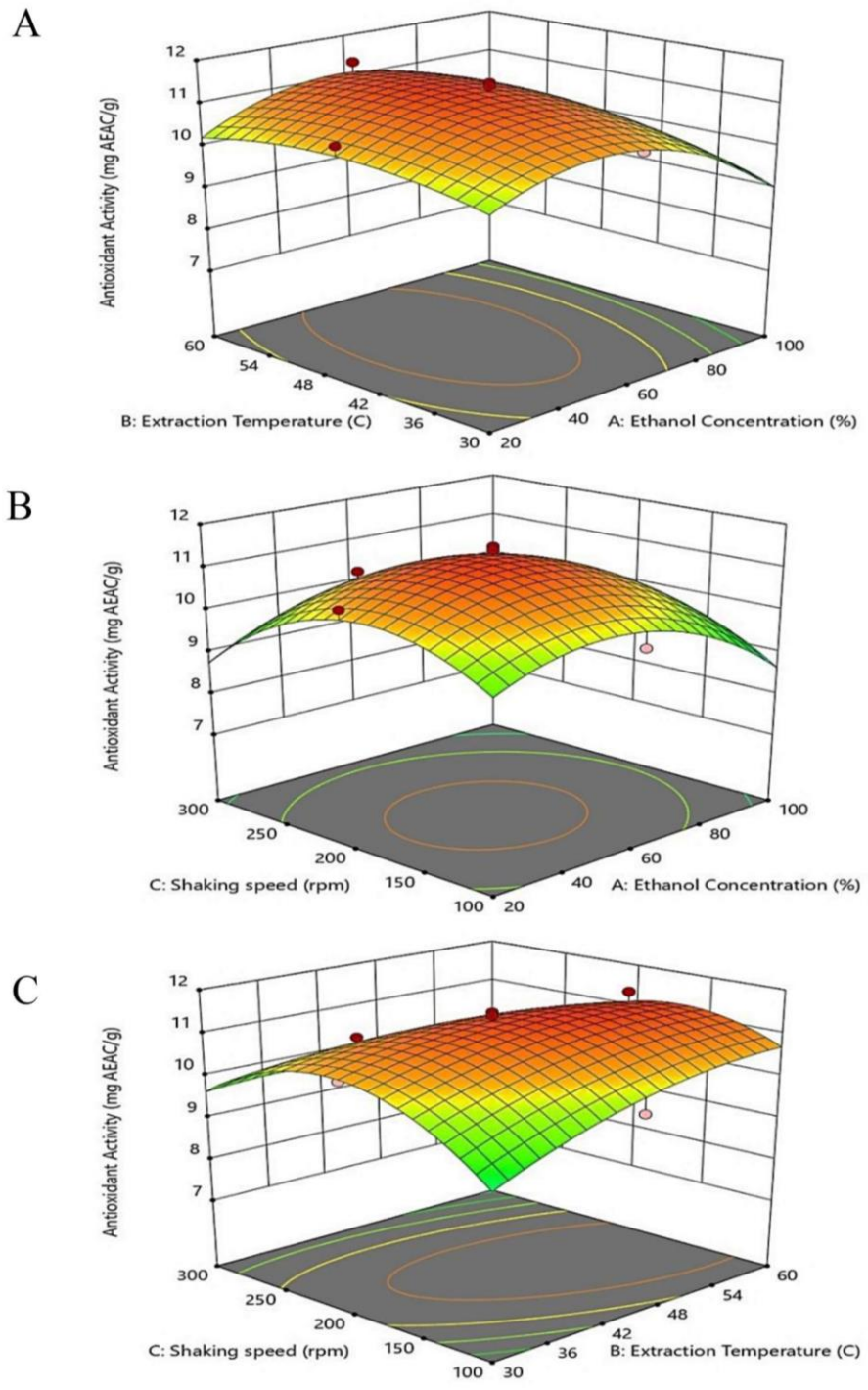


Figure 4.2 Response surface plots showing (a) Effect of interaction between ethanol concentration and extraction temperature on antioxidant activity (AB); (b) Effect of interaction between ethanol concentration and extraction shaking speed on antioxidant activity (AC); (c) Effect of interaction between extraction temperature and extraction shaking speed on antioxidant activity (BC)

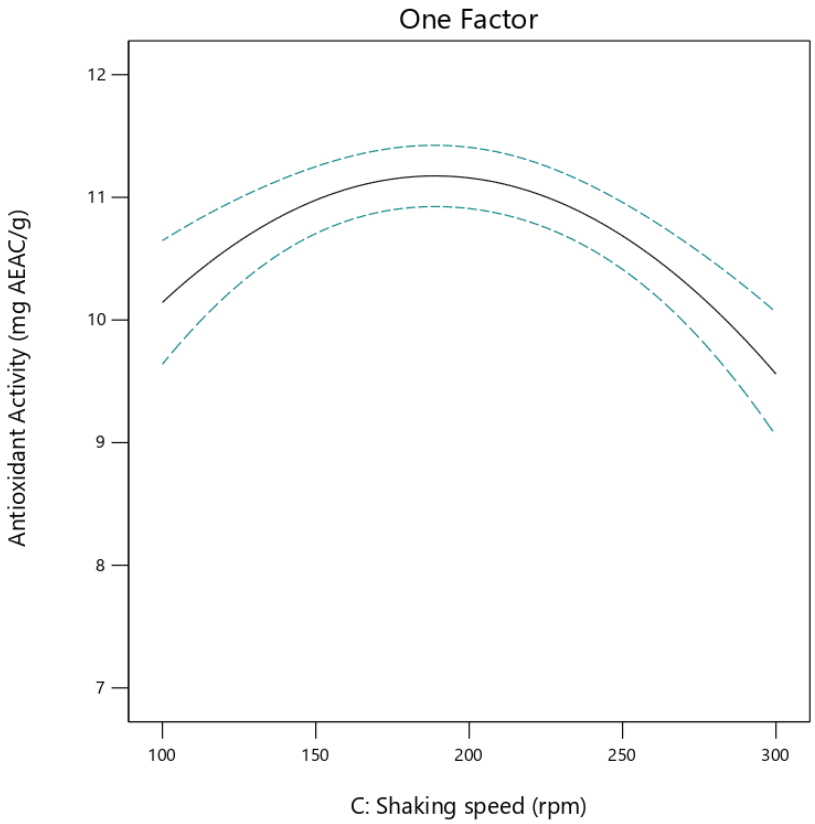


Figure 4.3 One factor plot – Effect of ethanol concentration on antioxidant activity

4.1.4 Optimization of independent variables and verification of the results

The numerical optimization was performed by using the desirability function method to determine the optimum conditions that obtain extracts with maximum antioxidant activity. The numerical optimization is depicted as Appendix A (Figure A.2). According to the numerical optimization study, the optimized conditions for this study were 54% ethanol concentration, 51℃ extraction temperature and 178 rpm of shaking speed. The predicted response value by model was 11.38 mg AEAC/g (DPPH free radical scavenging effect of 91.57%). The suitability and adequacy of model was verified by conducting experiment at optimum conditions obtained from the model. Table 4.4 presents the results of experiment in triplicate. Under these optimum conditions, the average experimental value was 11.11 mg AEAC/g (DPPH free radical scavenging effect of 91.09%). The experimental value was in close context with the predicted value with differences of 2.37% (within the 95% confidence interval of the fitted model), proving that the model is well fitted for the extraction of antioxidant compounds from *M. pajang* under the optimal extraction conditions.

Table 4.4 Predicted and actual values for the optimized extraction parameters

Experiment	Predicted Antioxidant activity (mg AEAC/g)	Experimental Antioxidant activity (mg AEAC/g)	Percentage Error (%)
1	11.38	11.11	2.37
2	11.38	11.19	1.67
3	11.38	11.04	2.99
Average	11.38	11.11 ± 0.06	2.37

4.2 Preparation and characterization of choline chloride/ascorbic acid natural deep eutectic solvent (CHCL/AA NADES) and evaluation of its solubilization capacity and antioxidant properties

It was reviewed that one of the interesting applications of NADES is to improve the solubility of bioactive compounds (Faggian et al. 2016, Liu, Zhang, Chen, Friesen, et al. 2018). In this regard, we prepared CHCL/AA NADES by mixing the choline chloride and ascorbic acid at different molar ratio. The choline chloride was used as a hydrogen bond donor in this study as it is non-toxic and is listed in the Code of Federal Regulations as nutrients and dietary supplementary that are GRAS (Gadilohar and Shankarling 2017, Fu et al. 2017, Zeisel 1994). Ascorbic acid or often known as Vitamin C is a well-known essential antioxidant and it was used as the hydrogen bond donor in this formulation (Wang et al. 2018, Kesinger and Stevens 2009). In this section, the characterization of CHCL/AA NADES is performed by using polarized optical microscopy (POM), proton nuclear magnetic resonance (^{1}H NMR) and Fourier transform infrared spectroscopy (FTIR) analysis. Additionally, the role of CHCL/AA NADES as a liquid solvent for solubilizing the antioxidant extracts obtained from preceding section is investigated. Additionally, the role of CHCL/AA NADES as antioxidant activity enhancer is also studied. It is hypothesized that the solubility and antioxidant activity of antioxidant extracts can be enhanced by incorporating them in this NADES designation.

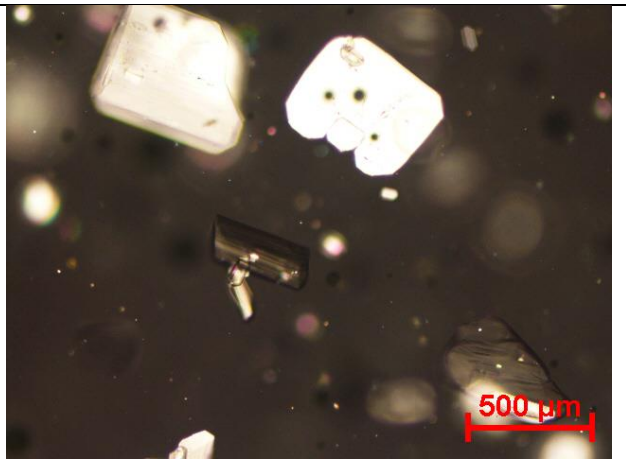
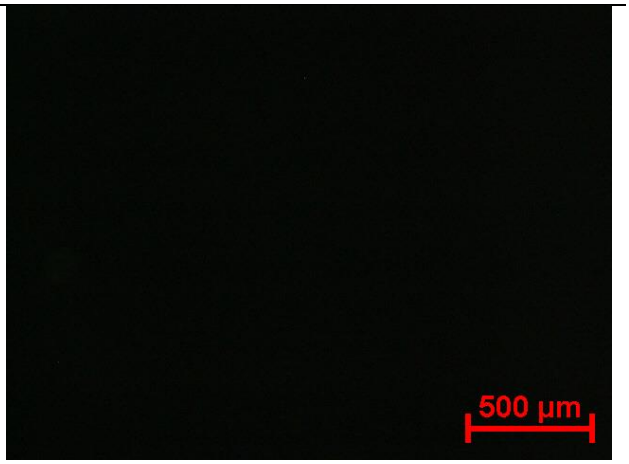
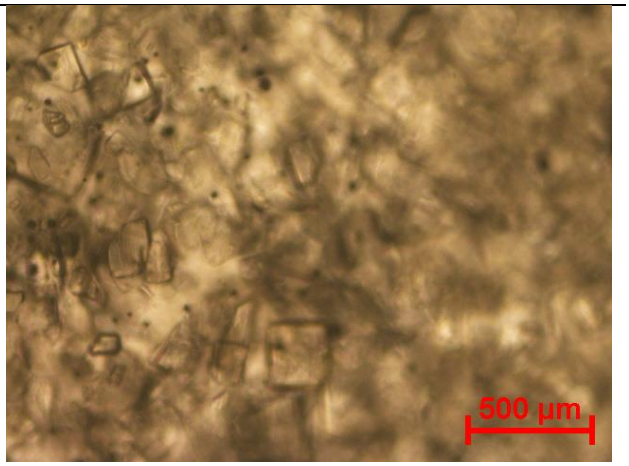
4.2.1 Characterization of CHCL/AA NADES

4.2.1.1 Polarized optical microscopy (POM) analysis

In this work, CHCL/AA NADES at different molar ratios (1:1, 1:2 and 2:1) were prepared and the formation of a liquid at room temperature was assessed. According to the visual observation, a clear and transparent liquid without any visible crystals was obtained when molar ratio of CHCL and AA at 2:1 was used. There were noticeable small amounts of crystals present in the CHCL/AA NADES (1:1), nonetheless the NADES still behaved like a liquid even after 24 hours. In the case of CHCL/AA NADES (1:2), a viscous pasty-like solid was obtained. The mixture was very viscous, making them very difficult to manipulate. These observations show that the molar ratio of the NADES components define the physical structure of NADES. In fact, in a general 'eutectic' solvent, at the eutectic ratio, the intermolecular hydrogen forces between the two components are balanced. The formation of hydrogen bonds, which generate significant intermolecular order prevents the mixture from crystallize and forms a stable deep eutectic mixture (Hammond 2019). That said, an excess or lack of either one of the NADES component can potentially makes the mixture metastable and results in the formation of crystalline particles.

All of the three mixtures were subjected to POM analysis for clarification. POM allows the observation of the presence of crystals, where the POM image is uniformly black if there is no crystal-like structure. The POM images obtained are illustrated in Table 4.5. The result of the POM analysis corroborates with the visual observations where the POM image for CHCL/AA NADES (1:1) CHCL/AA NADES (1:2) denoted the presence of crystals. For CHCL/AA NADES (2:1), the POM image was entirely black, as evidenced in Table 4.5, indicating that the mixture is completely liquid. The POM images are in good agreement with previous study conducted by Silva et al. (2018) and therefore, the CHCL/AA NADES at the molar ratio of 2:1 were prepared for subsequent studies.

Table 4.5 Polarized optical image CHCL/AA NADES system at different molar ratio of choline chloride/ascorbic acid

Molar ratio of choline chloride: ascorbic acid	Polarized optical microscopy
1:1	
2:1	
1:2	

4.2.1.2 Proton nuclear magnetic resonance (^{1}H NMR) analysis

The ^{1}H NMR analysis has been used to study the ionization states of proton and determine the structure of the molecules. It quantitatively determines the distribution of protons by detecting the proton spectra (Tan, Chin, et al. 2016, Delso et al. 2019). The ^{1}H NMR analysis on the synthesized CHCL/AA NADES and its neat components, choline chloride and ascorbic acid were performed and obtained from Dimethyl sulfoxide (DMSO-d_6). Figure 4.4 presents the proton spectra of CHCL/AA NADES. As shown in Figure 4.4, the signals can be basically assigned to the molecular groups of choline chloride and ascorbic acid, manifesting the high purity of CHCL/AA NADES. However, it was realzed that the signal for hydroxymethyl groups (R-CH_2-OH) of both choline chloride (Appendix B, Figure B.1) and ascorbic acid (Appendix B, Figure B.2) were overlapping, The chemical shift for choline chloride, appeared at δ (ppm) 3.093 (s, 9H, CHCL-$H_{1,2,3}$), 3.394 – 3.428 (m, 2H, CHCL-H_4) and 3.737 – 3.749 (m, 2H, CHCL-H_5). On the other hand, the chemical shift of ascorbic acid was observed at δ (ppm) 3.737 - 3.749 (m, 3H, AA-$H_{6,7}$), was overlapped with CHCL and 4.718 (s, 1H, AA-H_8). These findings explain that the OH groups of ascorbic acid may interact with choline chloride and then form intermolecular hydrogen bonds which enhances the solvation capacity of NADES (Huang et al., 2018). Considering the formation of hydrogen bonds, the possible hydrogen bonding interactions between choline chloride and ascorbic acid are also depicted in Figure 4.4 along with the ^{1}H NMR spectra.

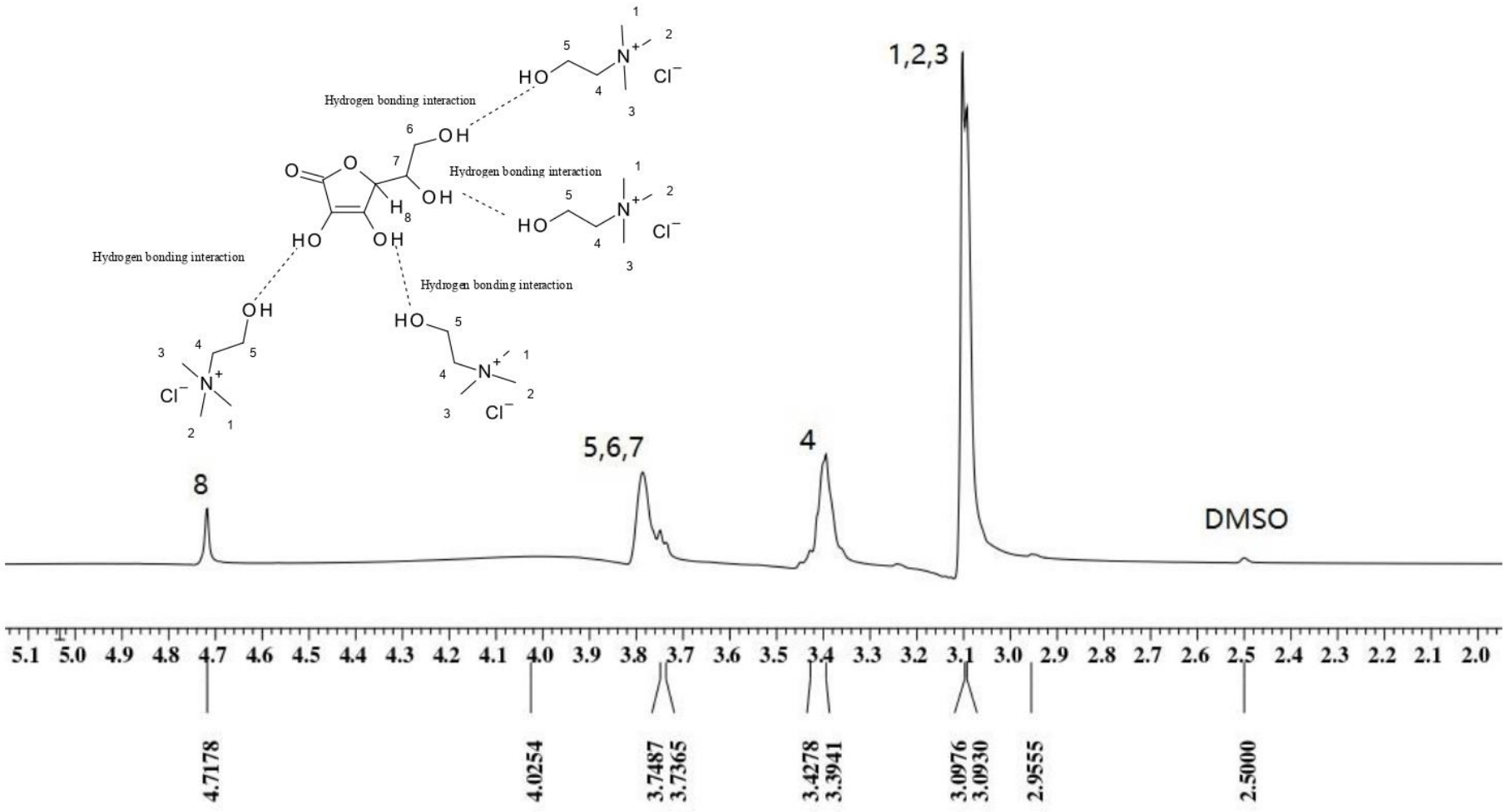


Figure 4.4 ^{1}H NMR spectra of choline chloride/ascorbic acid NADES (X: parts per Million: 1H)

4.2.1.3 Fourier transform infrared spectroscopy (FTIR) analysis

The formation of CHCL/AA NADES system and the type of interaction between the two neat components can be observed by using FTIR spectroscopy. The FTIR spectra of pure choline chloride, ascorbic acid and their eutectic mixture are illustrated in Figure 4.5 and the functional groups corresponded to the wavelength are tabulated in Table 4.6. Figure 4.5A shows the spectra of pure choline chloride. Predominantly, a broad band at 3220 cm^{-1}, which belongs to O-H stretching vibration and a vibration band at 3011 cm^{-1}, which can be referred to the stretching mode of C-H stretching vibration were observed, confirming the characteristic of choline chloride (Delgado-Mellado et al. 2018). On the other hand, a strong absorption at 1751 cm^{-1} and intense band at 1654 cm^{-1}, which can be ascribed to the C=O stretching and C=C stretching vibrations, respectively were noticed in Figure 4.5B, corresponding to the functional groups of the ascorbic acid (Yohannan Panicker, Tresa Varghese, and Philip 2006).

It was reported that the formation of NADES are relies on hydrogen bonding (Vanda et al. 2018, Wang et al. 2019). In infrared spectroscopy, the changes of bond length and corresponding vibrations of hydrogen bonds can depict the establishment of hydrogen bond (Maréchal 2007). It can be noticed that the spectrum of choline chloride/ascorbic acid NADES which presented in Figure 4.5C was roughly an overlap of the choline chloride and ascorbic acid. As can be seen, the vibration bands which appeared at 3220 cm^{-1}, the stretching vibration of hydroxyl group (-OH) in choline chloride was shifted to 3250 cm^{-1} in the choline chloride/ascorbic acid NADES. The shifting of the -OH stretching vibration suggests the formation of hydrogen bonding between the pure choline chloride and ascorbic acid when the NADES is formed. Moreover, the C-H stretching vibration which was observed at 3011 cm^{-1} disappeared when the NADES is formed, indicating the existence of hydrogen bonding (Liu, Zhang, Chen, and Yu 2018). In addition, by comparing Figure 4.5B and Figure 4.5C, it can be noticed that the absorption bands of ascorbic acid in the range of 3200 cm^{-1} to 3600 cm^{-1} became broader and wider bands in Figure 4.5C. This could once again ascribe to the formation of more hydrogen bonds between ascorbic acid and choline chloride (Yue et al. 2012). Owing the extensive hydrogen-bonding network between the choline chloride and ascorbic acid, the synthesized CHCL/AA NADES can be considered as super-molecules.

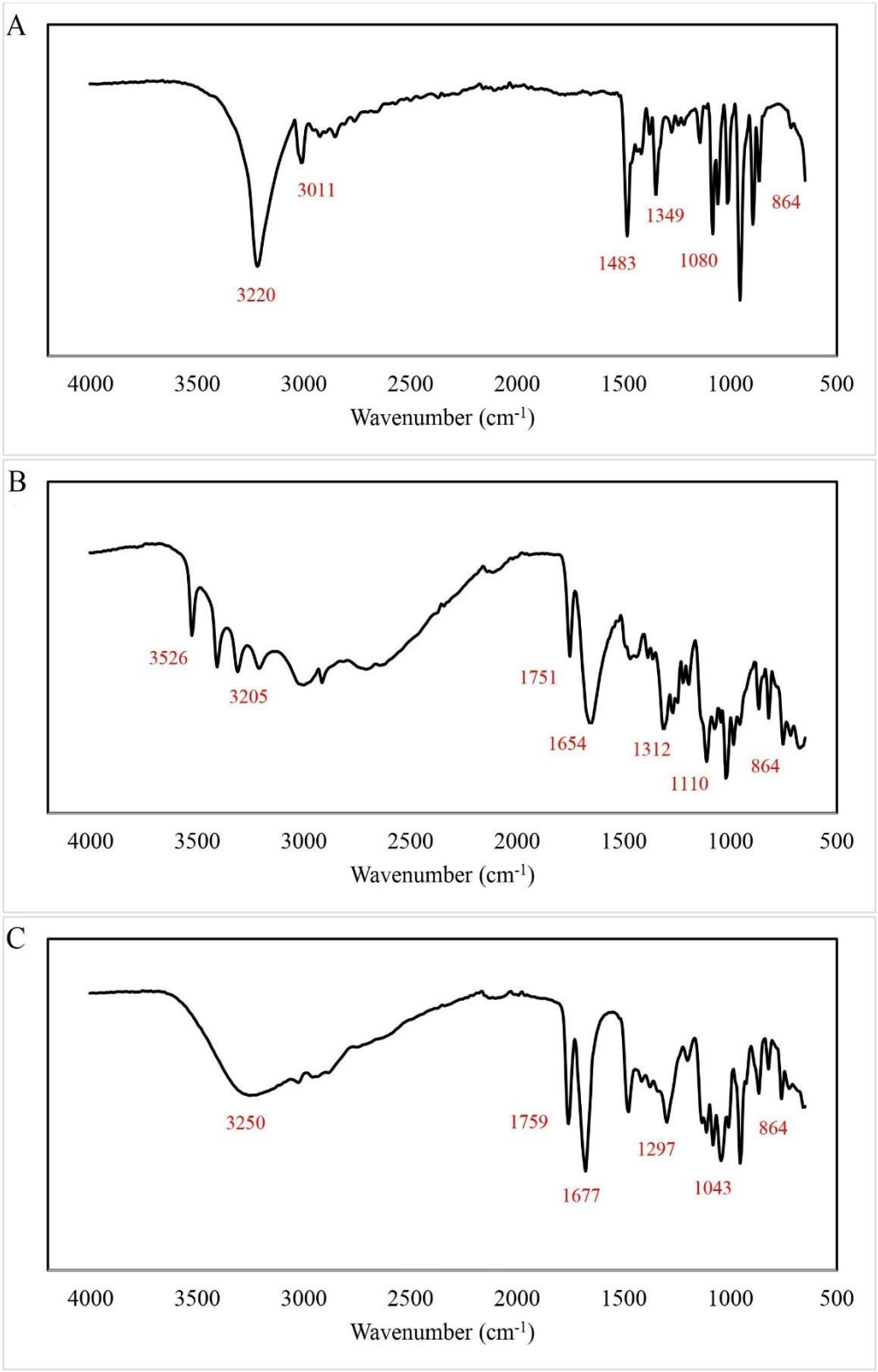


Figure 4.5 FTIR spectrum of A) Choline chloride, B) Ascorbic acid, C) Choline chloride/ascorbic acid natural deep eutectic solvent

Table 4.6 FTIR Spectrum showing functional groups

Functional groups	Wavelength (cm^{-1})		
	Choline chloride	Ascorbic acid	CHCL/AA NADES
O-H	3220	3200 - 3600	3250
C-H	3011	-	-
C=O	-	1751	1759
C=C	-	1654	1677
$C-H_2$	1483	1476	-
C-O	1080 - 1349	1110 - 1312	1043 – 1297
$C-N^+$	864	864	864

4.2.2 Solubilization capacity of CHCL/AA NADES

4.2.2.1 Ultraviolet spectra of antioxidant extracts

Phenolic rings exhibit strong ability to absorb UV light, so it can be easily quantified by using UV-visible spectrophotometer (Aleixandre-Tudo and Du Toit 2018). Prior to the solubility testing, the optimum UV-visible spectra region of the diluted antioxidant extracts (100-fold) was obtained by measured in the 200 - 800 nm region. It was found that the antioxidant extracts have two excited absorption bands, located at 212 nm and 277 nm, as illustrated in Figure 4.6. It is known that the phenolic compounds usually show two bands ranged between 200 to 360 nm, where the band with shorter wavelength is commonly known as B-band and the band with longer wavelength is called C-band, which correspond to the structure of antioxidant compounds (Friedman and Jürgens 2000). For example, Friedman and Jürgens (2000) found that the optimal UV spectra of gallic acid at pH 6 were recorded at 212 nm and 270 nm, respectively. The result is found to corroborate to theirs, suggesting that the antioxidant extracts are primarily responsible for the observed two bands.

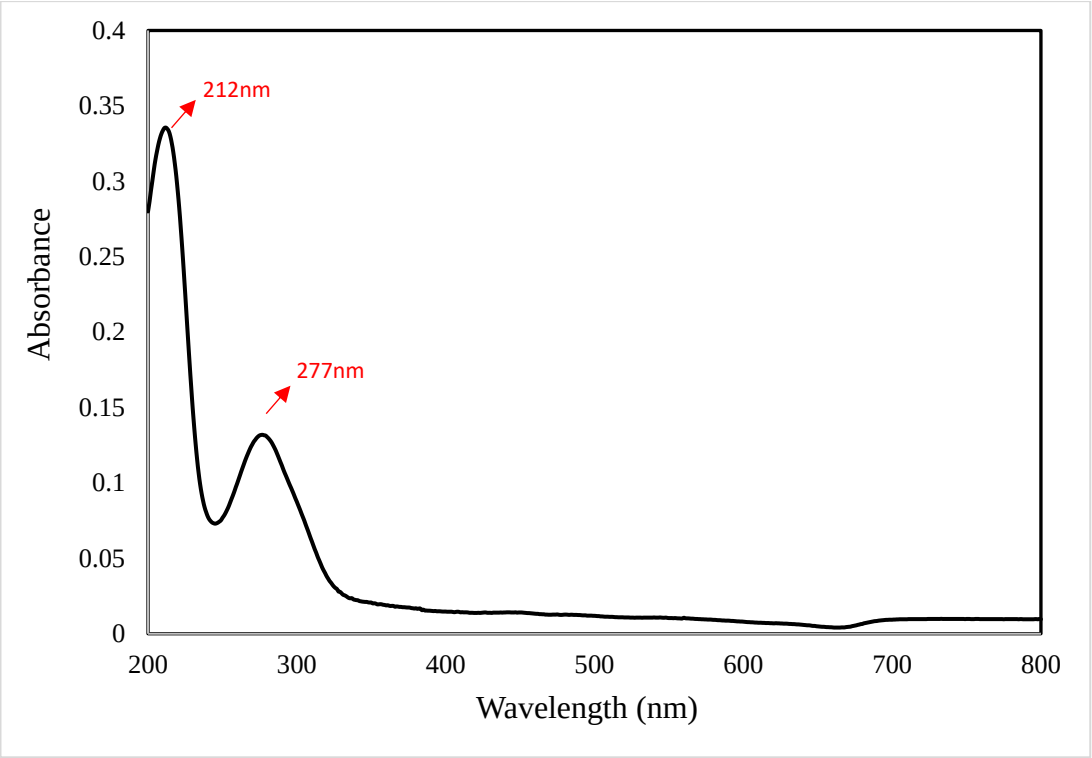


Figure 4.6 UV spectra of *M. pajang*'s antioxidant extracts

4.2.2.2 Solubility testing of antioxidant extracts in aqueous and CHCL/AA NADES system

Considering the solubility of bioactive compounds is a key parameter to ensure sufficient bioactivity level for health benefit effects, a model experiment was carried out to explore the solubilization capacity of CHCL/AA NADES system. Prior to the test, standard curves were prepared by dissolving antioxidants in both aqueous and CHCL/AA NADES system, as presented in Appendix C (Figure C.1 and Figure C.2). The solubility results are tabulated in Table 4.7 and illustrated in Figure 4.7. It was found that the solubility of antioxidant extracts in CHCL/AA NADES were increased by approximately 11% as compared to water. This finding indicates that the CHCL/AA NADES system exhibit outstanding solubilization capacity. With respect to this finding, the solvation capacity deep eutectic solvent was also evidenced in the study reported by Duarte et al. (2017), where the solubility of ibuprofen was increased up to 12-fold when incorporated in a choline chloride-based THEDES. Additionally, Li and Lee (2016) found that DES based on choline chloride, glycolic acid and oxalic acid significantly improved the solubility of itraconazole up to 53600-fold, as compared to

the aqueous solubility. Remarkably, the increment of solubility in this study is rather low as compared to these studies, which is reasonable, considering that the bioactive compounds they studied are hydrophobic and have relatively low solubility. In our case, the antioxidant extracts used in this study are mainly phenolic acid which is hydrophilic in nature (generally favors the hydrophilic interaction with water) (Jahurul et al. 2019, El Riachy et al. 2011, Materska 2010). Since the antioxidant extracts can interact well with the water, the magnitude of increment is smaller. Even so, the solubility of the antioxidant extracts in the CHCL/AA NADES is still substantially higher compared to the solubility in water. This suggests that the CHCL/AA NADES plays an important role as a liquid phase for solubilizing the hydrophilic bioactive compounds.

It was reported that the addition of water could increase the solubilization capacity of the NADES (Dai et al. 2013). So, as a further step, the effects of different percentage of water (10 - 50 wt%) on the solubilizing capacity of CHCL/AA NADES system was also studied. As it can be observed from Table 4.7 and Figure 4.7, the solubility of antioxidant extracts in aqueous CHCL/AA NADES displayed same behavior at both optimum wavelengths. It was found that the antioxidant extracts are most soluble in CHCL/AA NADES system with 10 wt% of water, where the solubility was increased by approximately 15% and 4% as compared to water and pure CHCL/AA NADES, respectively. This finding is in good accordance to the study reported by Dai et al. (2015), where they found that addition of water improved the solvation ability of DES. However, it was observed that the solubility decreased dramatically when the water content increased from 20% to 50%, reaching nearly the same solubility values in water. It is known that the formation of NADES relies on intermolecular hydrogen bonds between the constituent components. As water is introduced into the system, hydrogen bonds between the components are broken and new hydrogen bonds are established between them and water. In this condition, the physicochemical property of the NADES change (Gabriele et al. 2019). In this context, upon dilution with water, the hydrogen bonding between the components of NADES broken and this may lead to disappearance of NADES supermolecular structure. The hydrogen bonds between the choline chloride and ascorbic acid are likely to break and the dilution of water creates an aqueous solution of the free forms of the individual components (Meng et

al. 2018). At this stage, the CHCL/AA NADES system losses its unique properties and exists like a liquid with individual choline chloride and ascorbic acid. This is in agreement with previously reported experiments (Dai et al. 2013, Gutiérrez et al. 2010). A simple illustration of the rupture of hydrogen bonds is presented as Figure 4.8.

Another interesting finding is that the solubility of antioxidant compounds in the CHCL/AA NADES system with 10 and 20 wt% water was much higher than that in the pure CHCL/AA NADES system. The positive effect of water on NADES can be related to the reduced viscosity of NADES. Referring to Figure 4.9, the viscosity of CHCL/AA NADES system is affected by the water percentage. It was observed that the viscosity reduced from 51570 cP to 8.92 cP with the dilution of water. It was reported that high viscosity of NADES is correlated to the presence of extensive hydrogen bonding interaction between the components of NADES (Dai et al. 2015). Clearly, pure CHCL/AA NADES which possess extensive hydrogen bonds network are relatively viscous (51570 cP). High viscosity then hinders the solubilization capacity as there is no space to dissolve the solutes. When 10 wt% of water added to the CHCL/AA NADES system, the viscosity decreased up to 74% as compared to pure NADES. The reduction of viscosity then causes changes in the structure of NADES, where the H-bonded structure is loosened, this provides more spaces for the solubilization of the antioxidant extracts and consequently increase the solubility (Dai et al. 2015).

Another possible explanation is attributed to the polarity of antioxidant extracts and the CHCL/AA NADES system. It was showed that the CHCL/AA NADES system with 10 wt% of water resulted in highest solubility of antioxidant extract, whereas the antioxidant extracts that solubilized in CHCL/AA NADES system with 50 wt% of water and in aqueous solubility exhibited a similar solubility result. This finding suggests that the solubility of antioxidant extracts is influenced by the polarity of CHCL/AA NADES system, where the polarity can be affected by the addition of water. From this finding, one conclusion can be drawn, the polarity of *M. pajang*'s antioxidant compounds are identical to the polarity of CHCL/AA NADES system with 10 wt% of water. It is also interesting to point out that upon dilution of water, the

polarity of CHCL/AA NADES increases to a polarity index similar to the polarity of water, hence, results in similar solubility value in water. The influence of polarity on the solubility is supported by the study reported by Dai et al. (2013), where they found that the addition of 5% of water to the DES increase the polarity and further enhance the solubility of rutin. As demonstrated in this section, it is noteworthy that CHCL/AA NADES system with optimal water content can indeed enhances the solubility of bioactive compounds which makes the nutraceutical and pharmaceutical applications of CHCL/AA NADES system more feasible.

Table 4.7 Solubility and viscosity of pure NADES and NADES with different water content

Sample	Percentage of water (wt%)	Solubility (mg/ml)		Viscosity (cP)
		Absorbance at 212 nm	Absorbance at 277nm	
Water	-	0.8041 ± 0.0111	0.8017± 0.0130	-
NADES	0	0.9035 ± 0.0064	0.8935± 0.0142	51570.00 ± 9.4281
	10	0.9428 ± 0.0128	0.9535± 0.0071	13290.00 ± 4.0824
	20	0.9271 ± 0.0064	0.9235± 0.0142	588.50 ± 5.3385
	30	0.8669 ± 0.0098	0.8535± 0.0071	53.50 ± 0.4082
	40	0.8118 ± 0.0098	0.8185± 0.0187	17.83 ± 0.1443
	50	0.8065 ± 0.0037	0.7934± 0.0187	8.92 ± 0.9622

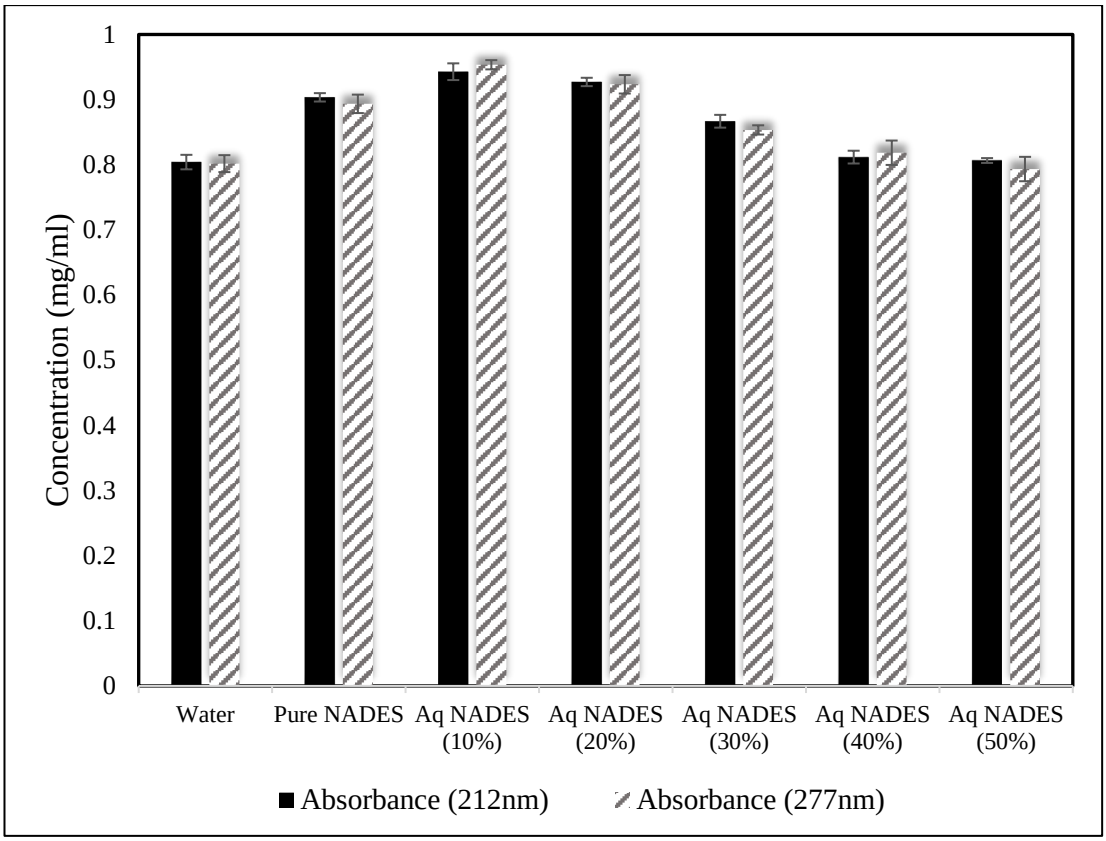


Figure 4.7 Effect of water inclusion in the choline chloride/ascorbic acid NADES on the solubility of antioxidant compounds

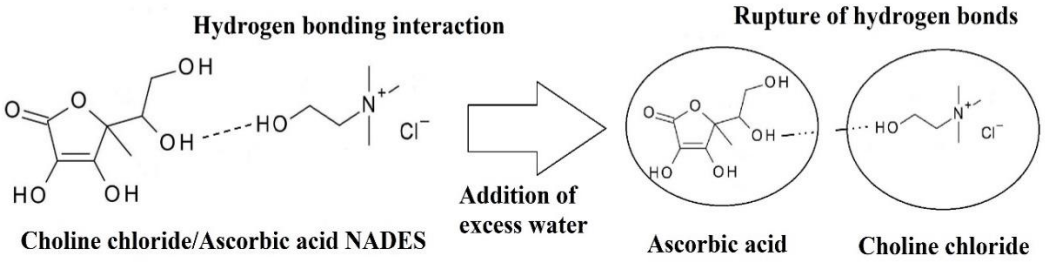


Figure 4.8 Schematic diagram illustrating the rupture of hydrogen bond between choline chloride and ascorbic acid upon addition of water

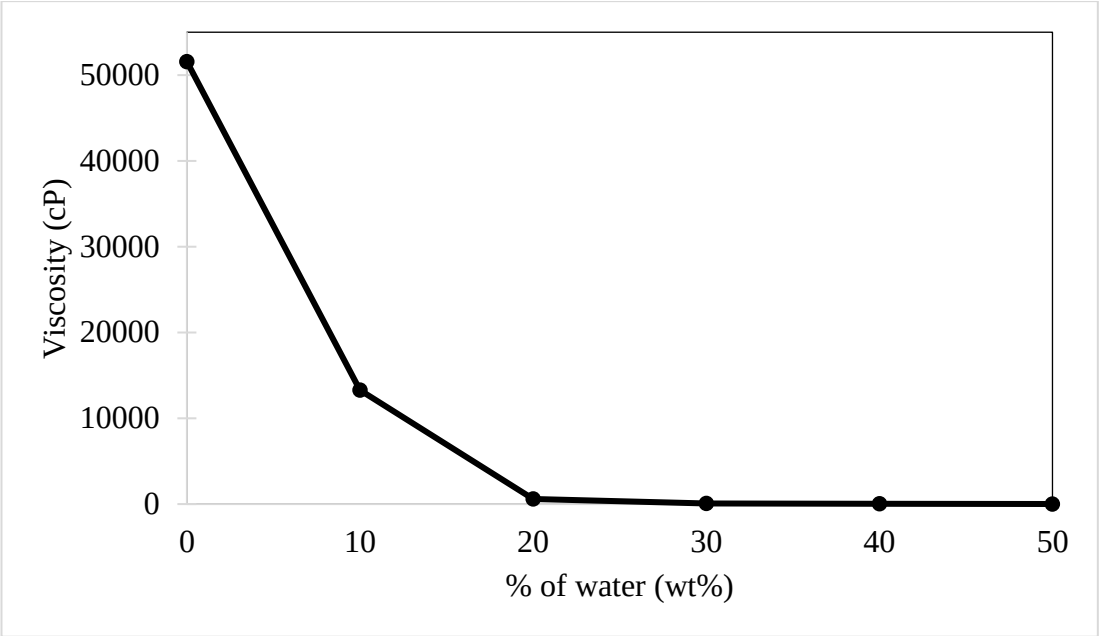


Figure 4.9 Viscosity of pure choline chloride/ascorbic acid NADES and added water percentage (wt %)

4.2.3 Antioxidant properties of antioxidants in CHCL/AA NADES system

The antioxidant extracts that solubilized in water and CHCL/AA NADES system with different percentage of water (10 – 50 wt%) were also prepared and subjected to DPPH radical scavenging assay to investigate the antioxidant capacity. The DPPH assay results are illustrated in Figure 4.10. The DPPH assay showed that the highest antioxidant activity was obtained at aqueous CHCL/AA NADES system with water inclusion of 10 wt% (12.53 mg/g). Remarkably, all the tested antioxidant-loaded CHCL/AA NADES solution exhibited a higher antioxidant potential (1.3 - 14.64%) than the antioxidant in aqueous form. Greater antioxidant activity can be attributed to one of the components of CHCL/AA NADES system, ascorbic acid. Ascorbic acid is well known natural antioxidant due to its capability in scavenging free radical species (ROS) (Putchala et al. 2013). The inclusion of ascorbic acid may contribute to the increased antioxidant activity of antioxidant-CHCL/AA NADES system. This fits in with the hypothesis of the role of NADES. Another notable finding is that the antioxidant activity of CHCL/AA NADES system with 10 wt%, 20 wt% and 30 wt% of water content slightly outperformed the pure CHCL/AA NADES system. This phenomenon is coherent with the above-mentioned effect of viscosity on the solubility of antioxidant compounds. It implies that the physicochemical properties of NADES

may exert a big effect on the antioxidant activity. Given this information, it can be concluded that CHCL/AA NADES system with 10 wt% water shows an excellent ability in improving the antioxidant activity, which once again highlights its potential in food, nutraceutical and pharmaceutical industry.

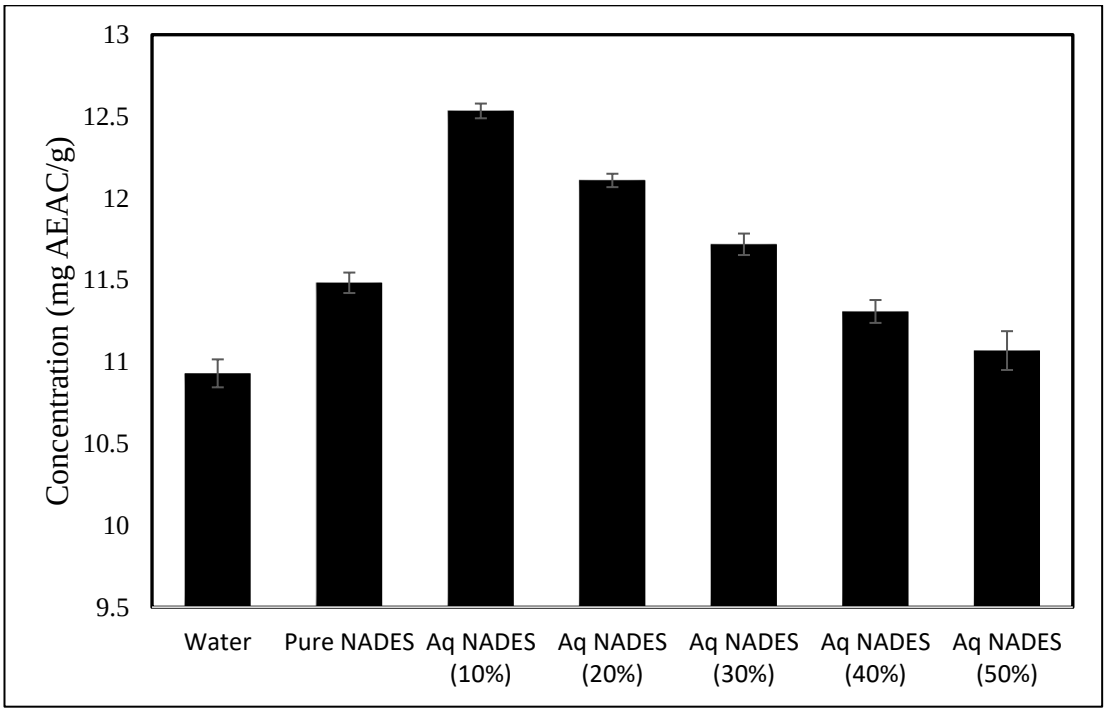


Figure 4.10 Effects of water inclusion in the antioxidant-choline chloride/ascorbic acid NADES on the antioxidant activity

4.3 Stability testing of antioxidant extracts in aqueous and CHCL/AA NADES system

High temperature use in thermal treatment often deteriorate the antioxidant compounds present in the foods. However, the deterioration is not restricted to the function of temperature, it may also rely on the pH of the food. In fact, for acidic foods, lower temperature (~80 ℃) are used for preservation purpose (McGlynn 2003). Therefore, knowledge of temperature and pH effects and their control during preservation process must be taken into account to produce value-added product. Following the previous section, the ability of CHCL/AA NADES system in stabilizing the antioxidant extracts was also investigated. Considering the antioxidant extracts has highest solubility in CHCL/AA NADES with 10 wt% water, the antioxidant extracts were prepared in it and in aqueous form. The samples were then subjected to thermal treatment under combined effects of pH (pH 3.0 – 8.0) and temperature (25℃, 40℃,

60℃ and 80℃). It is known that the majority of food are naturally acidic (less than 7.0), therefore, the pH ranged from 3.0 to 6.0 were chosen to represent these foods. In addition, the shift of the pH value of a single food ingredient can give a strong impact on the stability of product (Reineke, Mathys, and Knorr 2011). In this context, the behavior of antioxidant compounds at basic (pH 7.0) and alkali (pH 8.0) conditions were also studied. The degradation experimental data were fitted to the zero-order and first-order kinetic model and the kinetic parameters were determined. Additionally, a multi-term degradation model, described via Laplace Domain was proposed to predict the degradation profile. Furthermore, the DPPH scavenging activity of antioxidant extracts prepared in both systems were monitored throughout a period of time. Remarkably, since similar behavior and data were found at both the optimum wavelength of antioxidant extracts (212 nm and 277 nm), the data obtained at 212 nm was reported herein and the data assayed at 277 nm is summarized in Appendix.

4.3.1 Retention rate of antioxidants in aqueous and CHCL/AA NADES system at different pH level

The effects of pH (pH 3.0 - 8.0) on the retention rate of antioxidants in both aqueous and CHCL/AA NADES system after being heated at room temperature are illustrated as Figure 4.11 and the data is presented in Appendix D (Table D.1 and Table D.2). As presented in Figure 4.11A, the concentration of antioxidants reduced rapidly with time when the pH level of aqueous system was altered to pH 8.0 at 25℃ . It was realized that about 37% of total antioxidant level deteriorated after 480 minutes. In comparison, the antioxidants tend to retain more at lowest pH (pH 3.0), where only around 19% of total antioxidant level disappeared after 480 minutes. In other word, at lowest pH, more than 81% of antioxidants were retained; Whereas, at highest pH, only about 63% of antioxidants were retained. A similar observation can be found from Figure 4.11B. The reduction of antioxidant concentration in CHCL/AA NADES system was most pronounced at the highest pH value (pH 8.0), where only 66% of antioxidants were retained after 480 minutes. In contrast, the decrease in concentration of antioxidants was much slower in lower pH system. The antioxidants concentration still remained at about 83% of its initial value.

From the results, it is remarkable that increasing of the pH level from 3.0 to 8.0 hasten the deterioration of antioxidants in both systems and this finding suggests that the antioxidant extracts are much more stable in acidic environment. This should be related to the phenolic hydroxyl structure of antioxidant extracts. The increment of pH value results in dissociation of the hydroxyl groups and led to destruction of the antioxidant structures (Sun, Yan, and Lin 2018). Moreover, at higher pH environment, less protons are existing. Thus, greater amounts of antioxidants are ionized. The ionization state of antioxidants may facilitate the oxidative degradation and results in the decrease of antioxidant concentration (Zeng et al. 2017, Proniuk, Liederer, and Blanchard 2002). A schematic diagram showing the process of ionization is depicted as Figure 4.12. Notably, there is no significant difference in the changes of antioxidant concentration between pH 3.0 and pH 4.0, indicating that the optimum stability of antioxidant extracts falls between this range.

Our results are in agreement with those previous studies, where Buchner et al. (2006) reported that the concentration of antioxidant (quercetin) in solution of pH 5.0 decreased to 75% after 300 minutes, whereas the amount of quercetin in solution of pH 8.0 depleted to around 20%, their results suggested that the antioxidants were more stable in acidic condition (pH 5.0) as compared to alkaline condition (pH 8.0). Similarly, Kırca, Özkan, and Cemeroğlu (2007) observed that the degradation rate of black carrot anthocyanins was much rapid at pH larger than 5.0. Thus, it can be concluded that pH significantly affects the stability of antioxidant extracts of *M. pajang* and shall be taken into account in the industry process.

Notably, an interesting finding is that the stability of antioxidants was promoted in CHCL/AA NADES system as compared to aqueous form. For example, at pH 8.0, the retention rate of antioxidants in CHCL/AA NADES were 3% higher after 480 minutes. Although the increment is remarkably low, the CHCL//AA NADES system still demonstrated a protective effect against pH degradation at ambient condition. Such information shows that CHCL/AA NADES has the potential to retain more antioxidant compounds during exposure to alkaline environment.

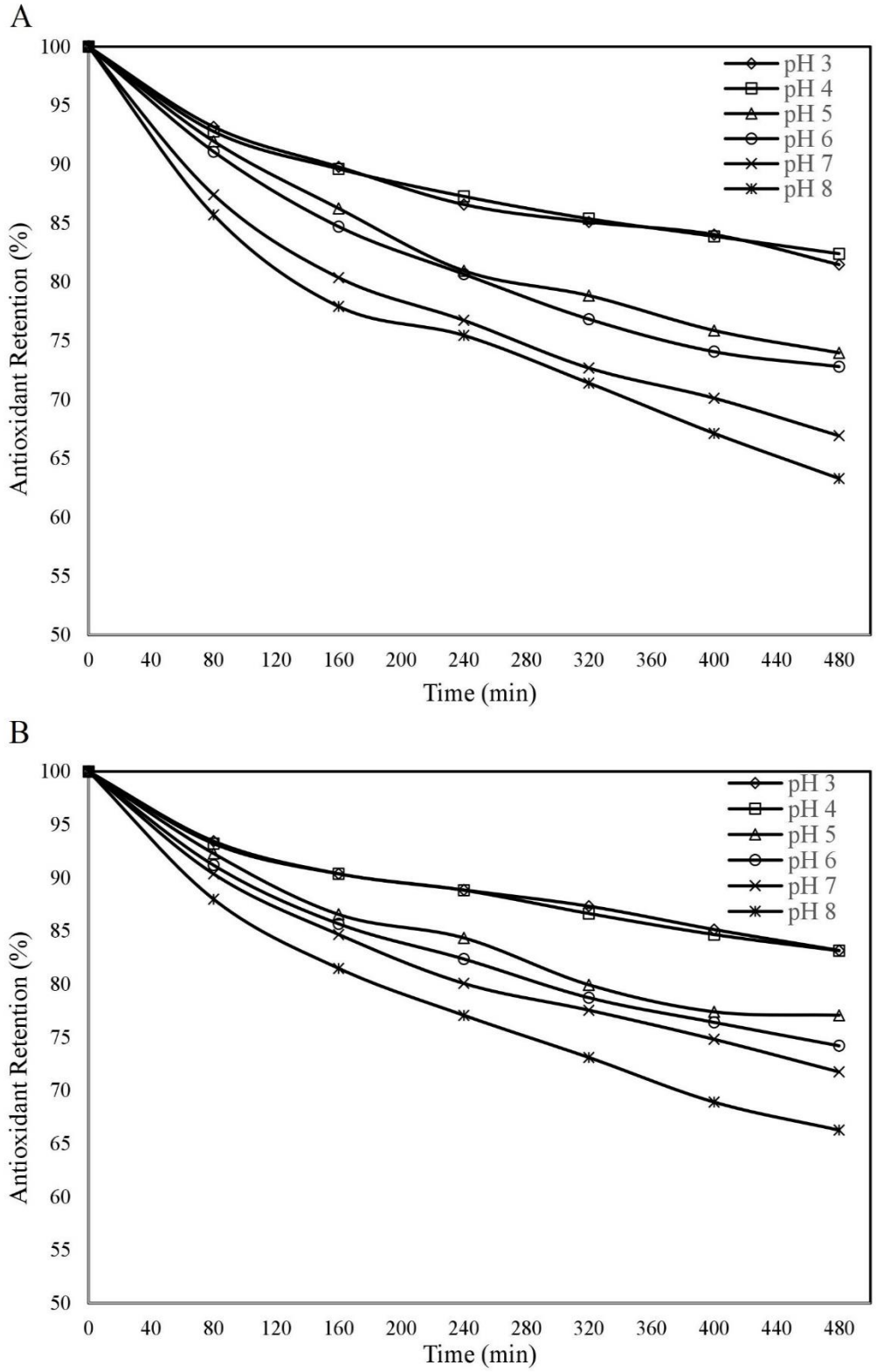


Figure 4.11 Retention percentage (%) of antioxidant at a period of heating time; at A) Antioxidants in aqueous system, B) Antioxidants in CHCL/AA NADES system

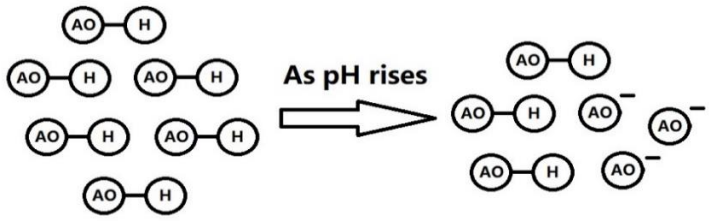


Figure 4.12 Schematic diagram illustrating the ionization of antioxidant compounds at high pH condition

4.3.2 Degradation kinetics

4.3.2.1 Estimation of the order of degradation

It is known that degradation kinetics is useful to estimate the decrease of antioxidant concentration during food processing, which is important for assuring the quality of the food (Sun et al. 2016). In this regard, the stability of antioxidants in the aqueous and CHCL/AA NADES system were assessed kinetically. Prior to the evaluation of degradation kinetics, a set of experiment designed to cover a wide range of conditions was conducted to study the degradation of antioxidants in the aqueous and CHCL/AA NADES system. The experimental results are tabulated in Appendix E (Table E.1 – E.8). The degradation experimental data were fitted to zero-order kinetic model and first-order kinetic model. The order of degradation of antioxidant extracts in both system with heating at different temperature (25℃, 40℃, 60℃ and 80℃) and pH (pH 3.0 - 8.0) were estimated by examining the coefficient of determination (R^2) that obtained from the plots of antioxidant concentration (at 212 nm) against treatment time (Figure 4.13 - 4.14). The mean R^2 of the degradation process (at 212 nm) were tabulated in Table 4.8 - 4.9, whereas the first-order plot along with the data obtained at 277 nm are presented in Appendix F (Table F.1 - F.2 and Figure F.1 - F.2). Based on Figure 4.13 - 4.14, the high R^2 values of the range of 0.89 - 0.96 for the degradation of antioxidants in aqueous and CHCL/AA NADES indicate a good data fit to first-order kinetic model. This finding is found to be in accordance to numerous previous studies that report on the degradation of antioxidants (Bolea et al. 2016, Turturică et al. 2016, Xu, Yu, and Zhou 2019, Sui, Dong, and Zhou 2014, Fratianni et al. 2017, Oancea et al. 2017). Since the degradation of antioxidant extracts can be well-described by the first-order kinetic, the degradation rate constants were calculated and given in Table 4.10 – 4.11.

Table 4.8 Estimation of coefficient of determination (R^2) from the plot of zero and first order reactions: Degradation of antioxidants in aqueous system at wavelength 212 nm (n=3)

pH	25℃				40℃				60℃				80℃			
	Zero order	RMSE	First order	RMSE	Zero order	RMSE	First order	RMSE	Zero order	RMSE	First order	RMSE	Zero order	RMSE	First order	RMSE
3	0.9172	0.022	0.9324	0.017	0.9032	0.021	0.9241	0.021	0.9136	0.026	0.9380	0.025	0.9405	0.024	0.9613	0.023
4	0.9045	0.022	0.9212	0.023	0.8863	0.022	0.9084	0.023	0.8875	0.028	0.9167	0.029	0.9176	0.028	0.9459	0.027
5	0.9384	0.023	0.9565	0.021	0.9191	0.029	0.9455	0.026	0.8924	0.037	0.9286	0.037	0.8637	0.044	0.9094	0.045
6	0.9320	0.028	0.9522	0.027	0.9032	0.031	0.9342	0.032	0.8716	0.048	0.9214	0.048	0.8132	0.073	0.8958	0.077
7	0.9131	0.033	0.9440	0.031	0.8757	0.040	0.9151	0.040	0.8670	0.053	0.9253	0.052	0.8228	0.075	0.9098	0.079
8	0.9139	0.034	0.9481	0.034	0.8801	0.048	0.9231	0.049	0.8315	0.082	0.9289	0.084	0.7584	0.095	0.9015	0.91

Table 4.9 Estimation of coefficient of determination (R^2) from the plot of zero and first order reactions: Degradation of antioxidants in CHCL/AA NADES system at wavelength 212 nm (n=3)

pH	25℃				40℃				60℃				80℃			
	Zero order	RMSE	First order	RMSE	Zero order	RMSE	First order	RMSE	Zero order	RMSE	First order	RMSE	Zero order	RMSE	First order	RMSE
3	0.9164	0.015	0.9320	0.066	0.8895	0.020	0.9112	0.078	0.9139	0.028	0.9383	0.027	0.9383	0.024	0.9598	0.023
4	0.9155	0.016	0.9314	0.020	0.8938	0.019	0.9151	0.018	0.8885	0.026	0.9176	0.026	0.9158	0.028	0.9444	0.027
5	0.9366	0.023	0.9548	0.024	0.9209	0.027	0.9469	0.028	0.8932	0.024	0.9287	0.035	0.8616	0.044	0.9072	0.045
6	0.9391	0.021	0.9586	0.020	0.9024	0.030	0.9332	0.028	0.8735	0.027	0.9224	0.037	0.8155	0.050	0.8973	0.050
7	0.9166	0.027	0.9465	0.026	0.8761	0.028	0.9157	0.028	0.8685	0.045	0.9264	0.044	0.8264	0.062	0.9131	0.065
8	0.9106	0.029	0.9455	0.025	0.8846	0.043	0.9286	0.045	0.8326	0.068	0.9300	0.064	0.7572	0.073	0.8998	0.076

Table 4.10 Degradation rate constants (min^{-1}) of antioxidants in aqueous system under different pH-temperature conditions

pH	25℃	40℃	60℃	80℃
3.0	4.0×10^{-4}	6.0×10^{-4}	1.2×10^{-3}	1.9×10^{-3}
4.0	4.0×10^{-4}	6.0×10^{-4}	1.2×10^{-3}	1.9×10^{-3}
5.0	6.0×10^{-4}	9.0×10^{-4}	1.7×10^{-3}	2.4×10^{-3}
6.0	7.0×10^{-4}	1.0×10^{-3}	2.0×10^{-3}	3.8×10^{-3}
7.0	8.0×10^{-4}	1.1×10^{-3}	2.3×10^{-3}	4.2×10^{-3}
8.0	9.0×10^{-4}	1.4×10^{-3}	3.8×10^{-3}	6.6×10^{-3}

Table 4.11 Degradation rate constants (min^{-1}) of antioxidants in CHCL/AA NADES system different pH-temperature conditions

pH	25℃	40℃	60℃	80℃
3.0	3.0×10^{-4}	5.0×10^{-4}	1.1×10^{-3}	1.8×10^{-3}
4.0	4.0×10^{-4}	5.0×10^{-4}	1.2×10^{-3}	1.8×10^{-3}
5.0	5.0×10^{-4}	8.0×10^{-4}	1.6×10^{-3}	2.3×10^{-3}
6.0	6.0×10^{-4}	9.0×10^{-4}	1.9×10^{-3}	3.0×10^{-3}
7.0	7.0×10^{-4}	1.0×10^{-3}	2.2×10^{-3}	3.8×10^{-3}
8.0	8.0×10^{-4}	1.3×10^{-3}	3.0×10^{-3}	5.5×10^{-3}

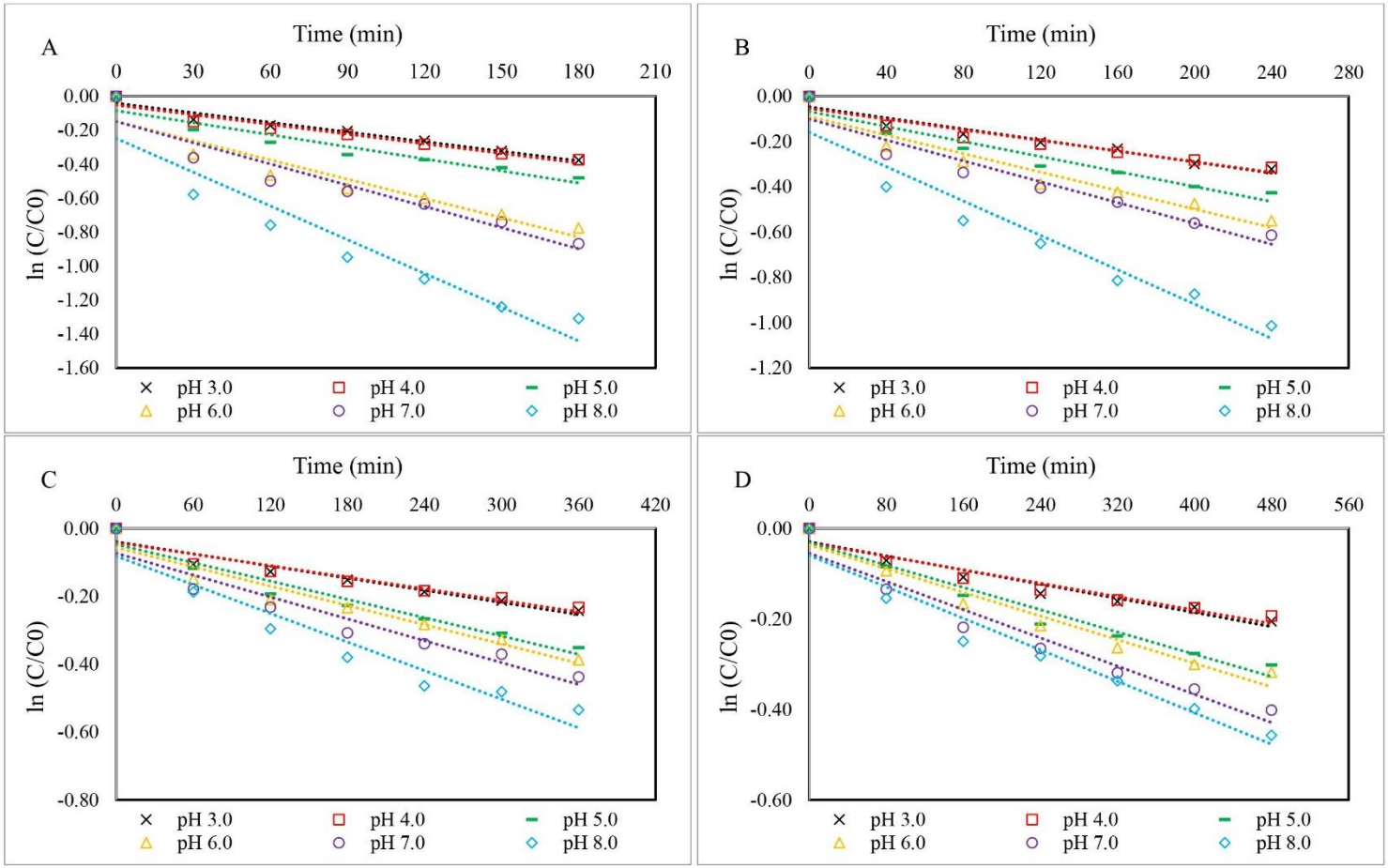


Figure 4.13 ln (C/C_0) value of antioxidants in aqueous system at wavelength 212 nm; Degradation at A) 80℃, B) 60℃, C) 40℃ and D) 25℃

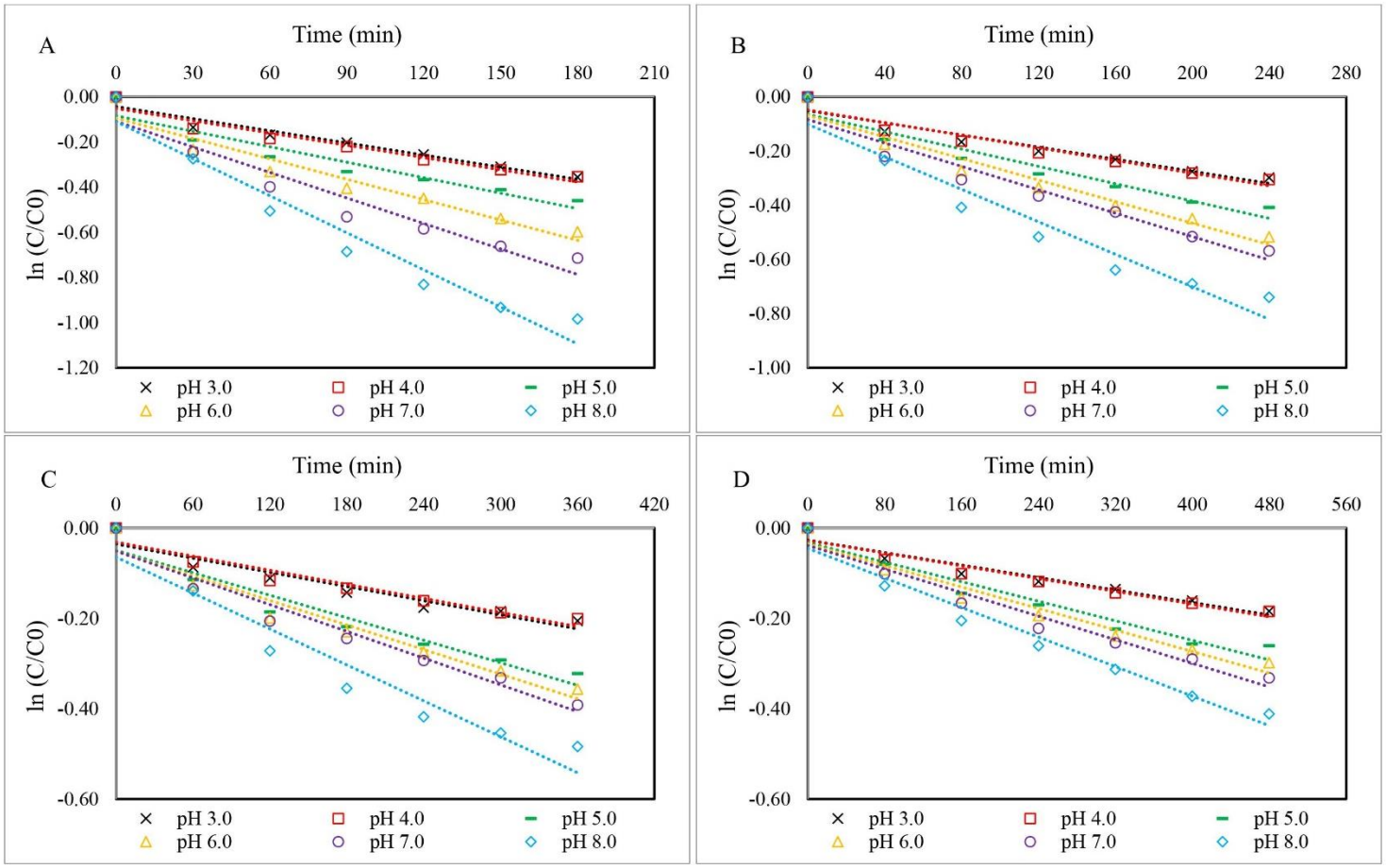


Figure 4.14 ln (C/C_0) value of antioxidants in CHCL/AA NADES system at wavelength 212 nm; Degradation at A) 80℃, B) 60℃, C) 40℃ and D) 25℃

4.3.3 Effect of temperature

The stability of antioxidant compounds was studied by estimating the changes in the concentration of antioxidants in aqueous and CHCL/AA NADES system over different heating time (0 - 480 minutes) at different temperature (25℃, 40℃, 60℃ and 80℃). The first-order plots are depicted as Figure 4.13 and Figure 4.14. From Figure 4.13 and 4.14, the line equations were generated and the degradation rate constant are summarized in Table 4.10 and 4.11. The degradation rate of antioxidants was reported to increase at high temperature by numerous researchers (Peron, Fraga, and Antelo 2017, Xu, Yu, and Zhou 2019, Chaaban et al. 2017). This was also observed in this study, in which the concentration of antioxidants in both systems continuously decrease with time at all temperature tested, indicating temperature has a significant effect on the degradation of antioxidants.

For both systems, the degradation rate constants, given in Table 4.10 - 4.11 were found to increase with accelerated temperature under all the pH conditions (pH 3.0 - 8.0). In the case of antioxidants in aqueous system, the highest degradation rate constant was determined to be 6.6×10^{-3} min^{-1} when the temperature was heated up to 80℃ and the system was altered to pH 8.0. While the lowest degradation rate constant (4.0×10^{-4} min^{-1}) was obtained at 25℃ and pH 3.0 and pH 4.0. An increment of temperature from 25 to 40℃, 40℃ to 60℃ and 60℃ to 80℃ enhanced average degradation rate constant by 1.38 - 1.56, 1.89 - 2.71 and 1.41 - 1.9 times, respectively. The highest magnitude was observed when the temperature heated up from 40℃ to 60℃, suggesting that this temperature range exert greatest impact on the deterioration of antioxidants.

For the antioxidants in CHCL/AA NADES system, the highest degradation rate constant was estimated to be 5.5×10^{-3} min^{-1} at 80℃ and pH 8.0. In contrast, the lowest degradation rate constant was calculated to be 3.0×10^{-4} min^{-1} at 25℃ and pH 3.0. Similarly, elevated temperature increased the degradation rate constant, where the degradation rate constant was enhanced by 1.25 - 1.67, 2 - 2.4 and 1.44 - 1.83 times when the temperature increased from 25 to 40℃, 40℃ to 60℃ and 60℃ to 80℃, respectively. The largest magnitude was observed at temperature range of 40℃ to 60℃, indicating highest level of antioxidants concentration was degraded at this temperature range. This finding clearly points out that temperature strongly affects the stability of antioxidants.

By comparing the degradation rate constant obtained from both systems, it was realized that the degradation rate constant determined from aqueous system at all conditions were comparatively larger (4.17% - 25%) than the one obtained in CHCL/AA NADES system. For example, at pH 8 and 80℃, the degradation rate constant obtained from aqueous system was about 16.67% higher than the degradation rate constant obtained for the CHCL/AA NADES system, implying that the degradation is much stronger in the aqueous system. This finding also indicates the ability of CHCL/AA NADES system as a protective system against thermal treatment. This finding is in accordance to the case reported by Dai, Verpoorte, and Choi (2014), where they also found that the degradation rate of carthamin in the xylitol-choline chloride NADES was much slower than in water.

4.3.4 Effect of pH

The changes in concentration of antioxidants in aqueous and CHCL/AA NADES system over different heating duration (0 - 480 minutes) at different pH (pH 3.0 - 8.0) can also be observed from Figure 4.13 and 4.14. As shown in Figure 4.13 and 4.14, the decreasing trend of ln (C/C_0) value with pH suggest that the antioxidants in both systems are relatively more stable at lower pH environment and tend to degrade as the pH of system rises.

The degradation rate constant of antioxidants in both systems (Table 4.10 - 4.11) exhibited a gradual increase at a pH level above 4.0, indicating the degradation of antioxidants occurs at higher pH level of system. For antioxidants in aqueous system, an increase in pH values from pH 3.0 to pH 4.0, pH 4.0 to pH 5.0, pH 5.0 to pH 6.0, pH 6.0 to pH 7.0 and pH 7.0 to pH 8.0 increased the degradation rate constant by 1, 1.26 - 1.5, 1.11 - 1.58, 1.1 - 1.15 and 1.13 - 1.65 times, respectively. Highest increment of degradation rate constants was noticed when the pH values was increased from pH 7.0 to 8.0, indicating that the antioxidant is relatively unstable at neutral and alkali environments. A good agreement in the results obtained in another study, where Xu, Yu, and Zhou (2019) found that the highest increase of degradation rate constant for the degradation of catechin (antioxidant) also occurred from pH 7.0 to 8.0. Notably, it is worth to mention that there is no significant difference in the concentration of antioxidants between pH 3.0 and 4.0, indicating that the optimum pH for antioxidant stability possibly fall between pH 3.0 and 4.0.

On the other side, as the pH of CHCL/AA NADES system increased from pH 3.0 to pH 4.0, pH 4.0 to pH 5.0, pH 5.0 to pH 6.0, pH 6.0 to pH 7.0 and pH 7.0 to pH 8.0, the antioxidant degradation rate constant was found to increase by 1 - 1.33, 1.25 - 1.6, 1.19 - 1.3, 1.11 - 1.27, and 1.14 - 1.45 times. Remarkably, the highest increment of degradation rate constant was found to be different from the antioxidant in aqueous system. The highest increment of rate constant in CHCL/AA NADES system was observed when the pH was adjusted from pH 4.0 to 5.0. This suggests that the CHCL/AA NADES system exhibit excellence ability in slowing the degradation of antioxidants at higher pH medium. Nonetheless, it is important to note that even the highest increment of degradation rate constant was observed at pH ranged from 4.0 to 5.0, the degradation rate constant estimated for CHCL/AA NADES system was still smaller as compared to the degradation rate constant obtained for aqueous system at similar pH range. Not only that, it is remarkable that the rest of degradation rate constant obtained from aqueous system were significantly higher than the degradation rate constant obtained from CHCL/AA NADES system.

Moreover, it was realized that the antioxidants in both systems exhibited differences in their stability under different combination of pH and temperature. In general, it can be seen that pH plays an important role in stabilizing the antioxidants under heat treatment, where antioxidant system with lower pH value significantly reduced the degradation of antioxidants. For example, the degradation rate constant of antioxidant in aqueous system at 60℃ was increased from 1.2×10^{-3} min^{-1} to 3.8×10^{-3} min^{-1} when the pH of system adjusted from pH 3.0 to 8.0. An increment of 68.42% was observed and this observation showed that by lowering down the pH of the system reduced the degradation rate of antioxidants. Therefore, it is of priority to reduce the pH of food during food processing to ensure maximum retention of antioxidants.

4.3.5 Kinetic parameters

Arrhenius equation (Equation 3.5) allows the extrapolation of kinetic data and was applied to quantify the effects of pH and temperature on the degradation of antioxidants in aqueous and CHCL/AA NADES systems described in the preceding section. The first-order kinetic constants were fitted to the Arrhenius plot (ln k against 1/T) and the coefficients of determination (R^2) for all cases were found to be greater than 0.95 (linear regression). The Arrhenius plots were illustrated in Figure 4.15. A clear negative linear relationship was found in Figure 4.15 and their corresponding Arrhenius activation energies (E_a) and pre-exponential factor (A) for the thermal degradation of antioxidants in both systems at different pH (pH 3.0 – 8.0) were determined and given in Table 4.12. In particular, the activation energy is vital to predict the loss of food quality in food processing (Xu, Yu, and Zhou 2019).

From Table 4.12, it can be seen that the E_a values for both systems ranged from 22.71 to 33.10 kJ/mol. There were no systematic differences among the E_a values for the degradation of antioxidants in all cases. However, it is interesting to point out that antioxidants in system at pH 8.0 exhibited highest activation energy. For thermal treatment, a higher activation energy suggests that the antioxidant has higher tendency to degrade at a small temperature change, is to be less stable (Jaiswal, Gupta, and Abu-Ghannam 2012). Hence, it can be said that when the pH of aqueous and CHCL/AA NADES system were adjusted to higher pH value, the antioxidants are more susceptible to the degradation, as mentioned in earlier section. In addition to the E_a value, pre-exponential factors were also found to increase with pH and the highest peak was found at highest pH. This clear trend once again implies that the degradation of antioxidants is likely to occur at higher pH level.

It is found that the calculated E_a value are similar to the E_a reported for the degradation of other antioxidant source. Kim et al. (2018) found that the E_a value for the deterioration of phenolic compounds from hardy kiwi was 28.15 kJ/mol. Additionally, Costa, Silva, and Vieira (2018) obtained an E_a value of 24.16 kJ/mol for the degradation of anthocyanin from açai pulp. On the other hand, a slightly lower E_a value (10.07 kJ/mol) for the deterioration of phenolic compounds from flour extract was reported by Bolea et al. (2016). These reported E_a values are either slightly lower or slightly higher. In fact, these slight differences in the calculated E_a value can be attributed to the source and nature of the sample studied. Since the nature of the sample

are different, they may undergo different degradation mechanism, consequently, the activation energy obtained are different (Lal et al. 2019).

Another way to express the degradation of antioxidants is the use of the half-life ($t_{1/2}$), estimation of the time taken for the concentration of antioxidant compounds to diminish to half of its original concentration (Peron, Fraga, and Antelo 2017). It is useful to estimate the storage time and helps to optimize the quality of antioxidants. The half-life of antioxidants in both systems are expressed using Equation 3.6 and presented in Table 4.13. As shown in Table 4.13, it was noticed that an increase in temperature and pH caused a drastic decrease in the $t_{1/2}$ value, given the shortest $t_{1/2}$ of 105.02 and 126.03 min for aqueous and CHCL/AA NADES system, respectively at the highest temperature (80℃) and highest pH (pH 8.0). This trend is in good agreement with other literatures, such that Dai, Verpoorte, and Choi (2014), Costa, Silva, and Vieira (2018), Kim et al. (2018), Kırca, Özkan, and Cemeroğlu (2007) and Turturică et al. (2016). However, the $t_{1/2}$ value reported by these researchers are found significantly different compared to the antioxidant analysed in this study, probably due to the difference in the source of sample and other external factors such as pH and solid contents. Remarkably, it was noticed that most of the antioxidants in CHCL/AA NADES system exhibited significantly higher half-life value as compared to aqueous system, indicating that the CHCL/AA NADES system can act as a protection system that stabilize the antioxidants and extend the shelf-life duration. The protective effect of NADES can be supported by Dai, Verpoorte, and Choi (2014), where they found that the $t_{1/2}$ value of carthamin (colorant) in the xylitol-choline chloride NADES is about two times larger than in water, confirming the stabilizing effect of NADES.

The good performance of CHCL/AA NADES shows great promising to be fortified into various food matrix such as probiotic product. It is generally known that the probiotic product must be kept under refrigerated conditions to maintain the viability of probiotic bacteria for beneficial health effects, but the probiotic products inevitably stored at room temperature for hours during transportation, distribution or storage, and the quality changes during storage at room temperature limits their large-scale production (Mohammadi and Mortazavian 2011, Bhattarai, Pradhananga, and Mishra 2015, Fenster et al. 2019). For instance, common probiotic strain, *B. Lactis* used in probiotic yogurt product showed low resistance to be stored at 20℃, where it was able to maintain its survival higher than 10^7 cfu mL^{-1} (minimum amount for

therapeutic effects) only for 12 hours, indicating that room temperature has substantial impact on maintaining viability of probiotic bacteria (Ferdousi et al. 2013). Fortification of antioxidants-CHCL/AA NADES (which has high $t_{1/2}$ value at room temperature) into the probiotic products can be a viable approach to improve the stability of probiotic products at room temperature. Apart from that, it is worth to mention that even though probiotic products contains various health-promoting ingredients, the amount of phenolic compounds in probiotic product like yoghurt is comparatively low (Raikos et al. 2019). The incorporation of antioxidant-CHCL/AA NADES likewise increase their antioxidant profile. In this context, the possibility of finding an optimized formulation of probiotic product supplements with antioxidants-CHCL/AA NADES seems interesting. Nevertheless, the processing should be evaluated to ensure maintenance of the desired shelf-life.

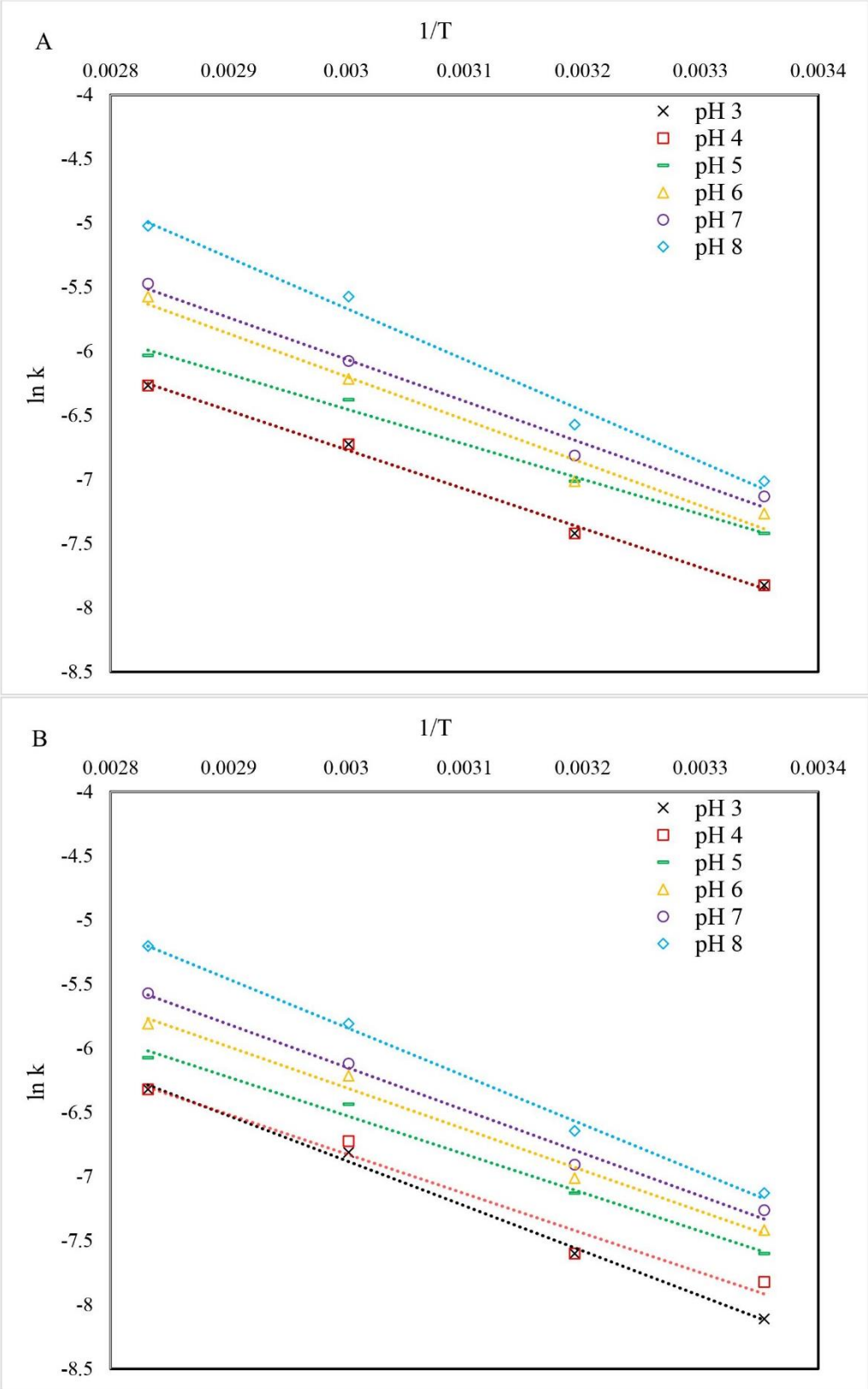


Figure 4.15 Arrhenius plot (1/T against ln k); Antioxidants in A) Aqueous system; B) CHCL/AA NADES system

Table 4.12 Activation energy (E_a) and pre-exponential factor (A) for the degradation of antioxidants in aqueous and CHCL/AA NADES system

pH	Antioxidants in aqueous system		Antioxidants in CHCL/AA NADES system	
	E_a (kJ/mol)	A (min^{-1})	E_a (kJ/mol)	A (min^{-1})
3.0	25.43	11.11	29.19	38.63
4.0	25.43	11.11	25.62	11.26
5.0	22.71	5.72	24.96	11.94
6.0	27.89	47.69	26.68	27.65
7.0	27.09	40.94	27.81	48.74
8.0	33.10	532.24	31.36	239.13

Table 4.13 Half-life value for the degradation of antioxidants in aqueous and CHCL/AA NADES system, $t_{1/2}$ (min)

pH	Antioxidants in aqueous system				Antioxidants in CHCL/AA NADES system			
	25℃	40℃	60℃	80℃	25℃	40℃	60℃	80℃
3.0	1732.87	1155.25	577.62	364.81	2310.49	1386.29	630.13	385.08
4.0	1732.87	1155.25	577.62	364.81	1732.87	1386.29	577.62	385.08
5.0	1155.25	770.16	407.73	288.81	1386.29	866.43	433.22	301.37
6.0	990.21	770.16	346.57	182.41	1155.25	770.16	346.57	231.05
7.0	866.43	630.13	301.37	165.04	990.21	693.15	315.07	182.41
8.0	770.16	495.11	182.41	105.02	866.43	533.19	231.05	126.03

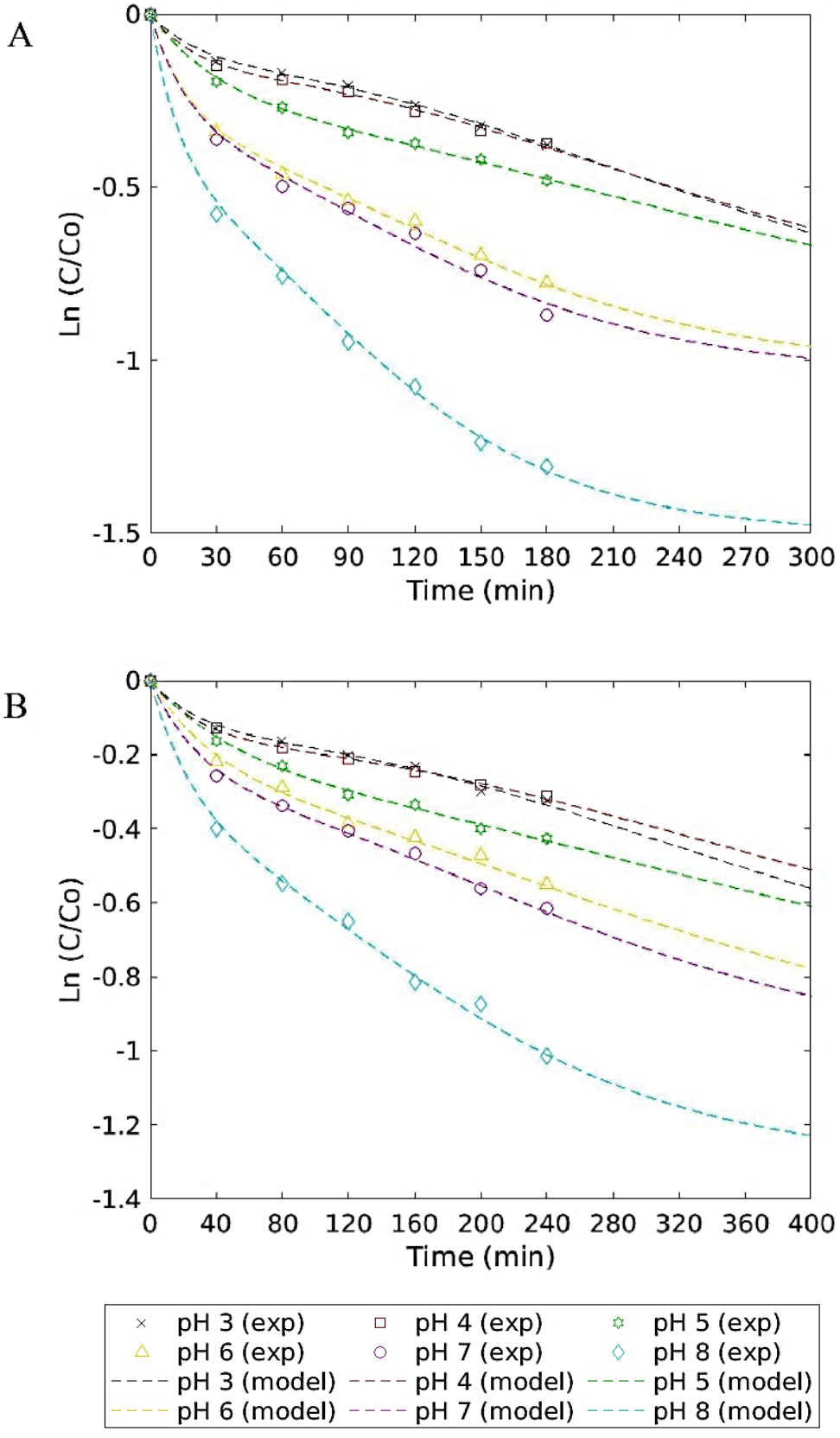
4.3.6 Multi-term degradation model

In previous section, it was found the degradation pattern of antioxidants in both aqueous and CHCL/AA NADES follows the first-order kinetics. The profile (Figure 4.13 - 4.14) show how the apparent first-order dependency with the concentration of the antioxidants at different pH and temperature condition. The first-order model and kinetic parameters obtained should be able to successfully fit the entire range of degradation experimental data. Nevertheless, only a part of the experimental data in Figure 4.13 - 4.14 were fitted with the line, indicating that it is not always possible to apply kinetics as simple as first-order kinetic model for the description of the degradation of antioxidant compounds (Ibarz, Pagan, and Garza 1999, Peleg et al. 2018). The complexity of first-order kinetic model is not sufficient to estimate the degradation profile. On the basis of this supposition, a rather complex multi-term degradation model, in the form of Laplace Domain was developed to describe the degradation profile. The model is given as (Equation 3.10):

$$P(s) = \frac{\tilde{C}(s)}{U(s)} = \underbrace{\frac{k_1}{(\tau_1 s + 1)^1}}_{\text{short-term}} + \underbrace{\frac{k_2}{(\tau_2 s + 1)^3}}_{\text{medium-term}}$$

In this study, the degree of degradation was assumed up to about 0.5, considering the evaluation of half-life value (the time taken for the concentration to reduce to half (50%) of its initial concentration). Under this assumption, the number of degradation kinetics terms were categorized to only two terms, namely a short-term and a medium-term kinetics. In Laplace Domain, the short-term kinetics is first-order while the medium-term kinetic is third-order, suggesting that the medium-term kinetic effect is rather delay.

The kinetic parameters, k_1, k_2, τ_1 and τ_2 were evaluated through optimization study reported in Chapter 3, Section 3.5.3. The predicted degradation profile by model and the degradation experimental data are presented in Figure 4.16 - 4.17. From the plots, the very good fit obtained for the degradation curve can be noticed, clearly suggesting that the whole range of the degradation experimental data set can be well-described by the proposed kinetic model. The degradation kinetic model parameters that estimate the degradation pattern of antioxidants in both aqueous and CHCL/AA NADES system under combined effect of pH and temperature are given in Table 4.14.


A
B
Ln (C/Co)
Time (min)
0
-0.5
-1
-1.5
0
30
60
90
120
150
180
210
240
270
300
-0.2
-0.4
-0.6
-0.8
-1.2
-1.4
40
80
160
200
280
320
360
400
pH 3 (exp)
pH 4 (exp)
pH 5 (exp)
pH 6 (exp)
pH 7 (exp)
pH 8 (exp)
pH 3 (model)
pH 4 (model)
pH 5 (model)
pH 6 (model)
pH 7 (model)
pH 8 (model)

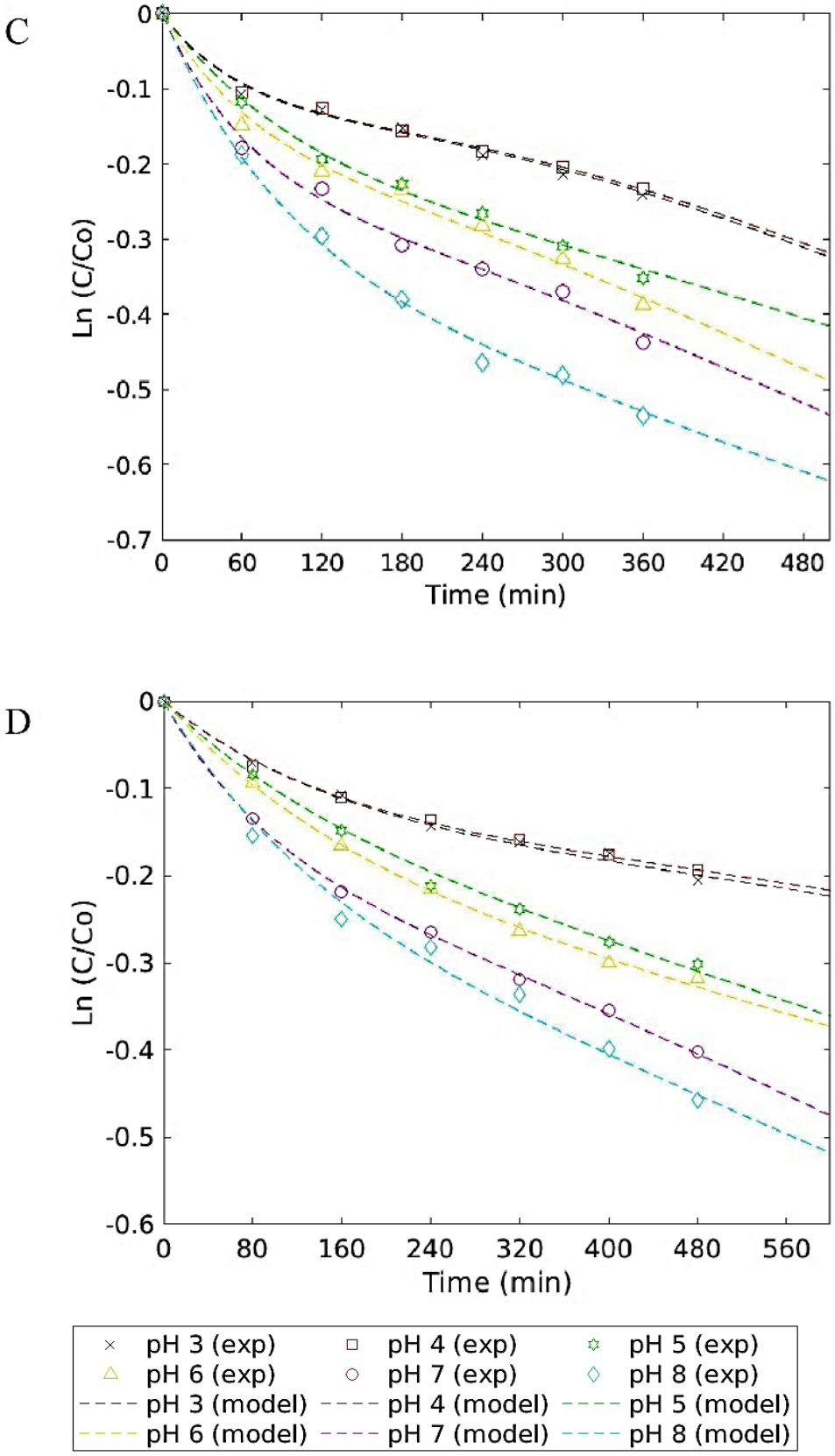
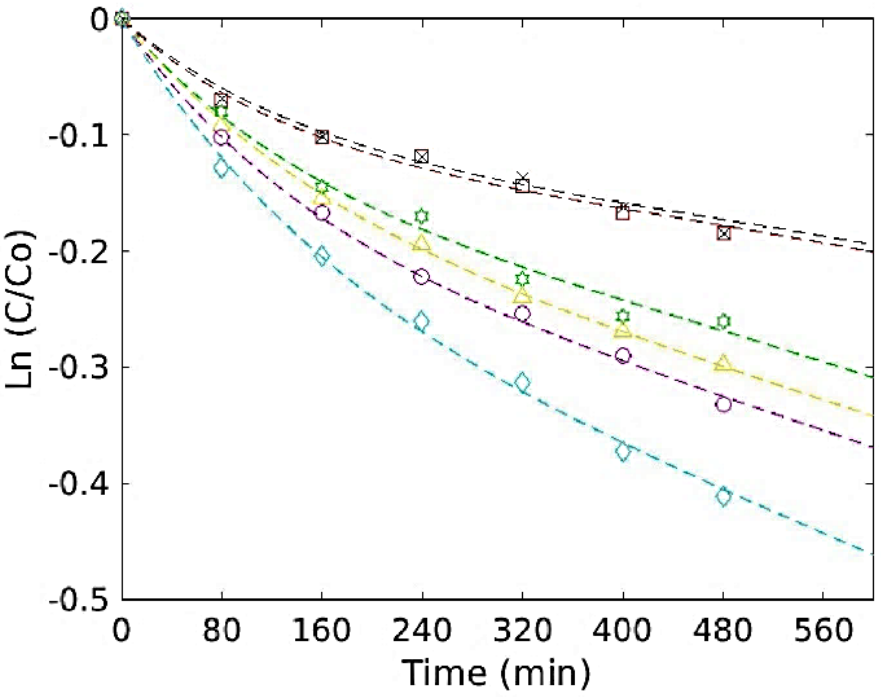
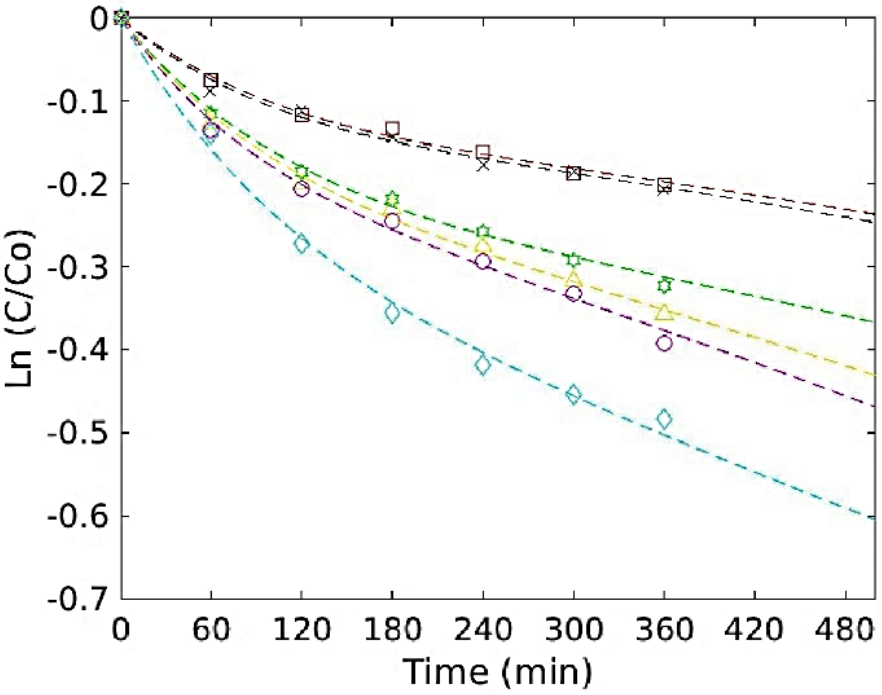


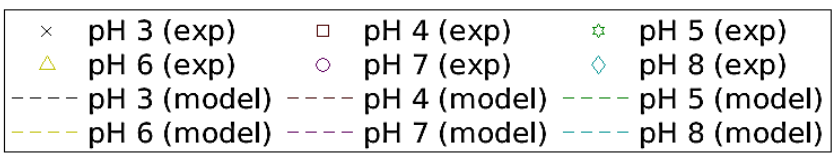
Figure 4.16 Comparison between the experimental data with predicted result of the proposed multi-term degradation model; Degradation of antioxidants in aqueous system at wavelength 212 nm at A) 80℃, B) 60℃, C) 40℃ and D) 25℃

A


0
-0.1
-0.2
-0.3
-0.4
-0.5
Ln (C/Co)
0
80
160
240
320
400
480
560
Time (min)

B


0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
Ln (C/Co)
0
60
120
180
240
300
360
420
480
Time (min)


pH 3 (exp)
pH 4 (exp)
pH 5 (exp)
pH 6 (exp)
pH 7 (exp)
pH 8 (exp)
pH 3 (model)
pH 4 (model)
pH 5 (model)
pH 6 (model)
pH 7 (model)
pH 8 (model)

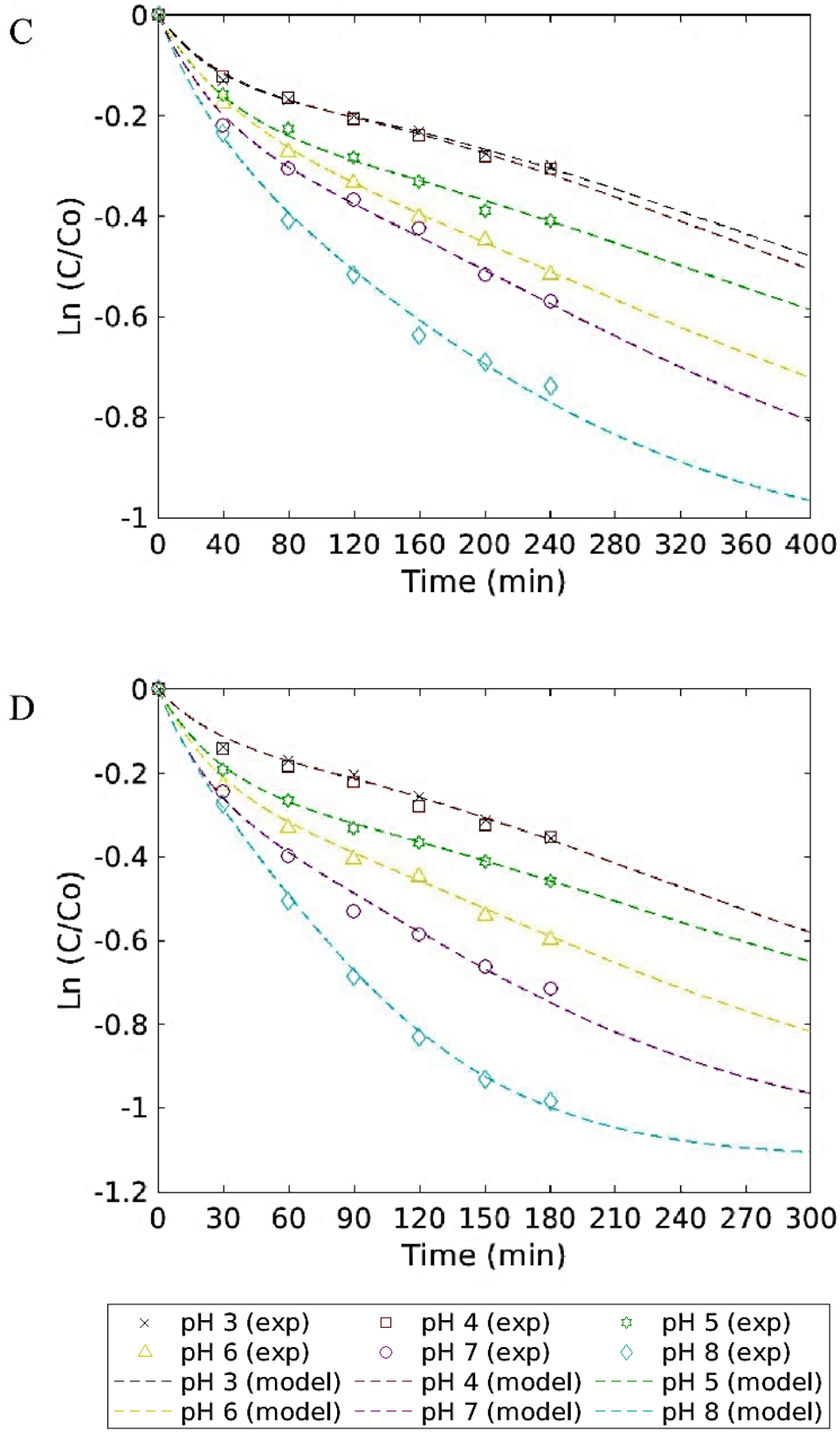


Figure 4.17 Comparison between the experimental data with predicted result of the proposed multi-term degradation model; Degradation of antioxidants in CHCL/AA NADES system at wavelength 212 nm at A) 80℃, B) 60℃, C) 40℃ and D) 25℃

Table 4.14 Multi-term degradation model parameters via Laplace Domain: Degradation of the antioxidants in aqueous and CHCL/AA NADES under combined effect of pH and temperature

pH	Temperature (℃)	Aqueous system				CHCL/AA NADES system			
		k_1	k_2	τ_1	τ_2	k_1	k_2	τ_1	τ_2
3.0	25	-0.19	-0.72	184	699	-0.16	-0.74	174	713
	40	-0.15	-0.82	62	316	-0.18	-0.76	113	475
	60	-0.17	-0.83	34	156	-0.19	-0.73	42	176
	80	-0.17	-0.85	25	105	-0.19	-0.78	34	112
4.0	25	-0.18	-0.82	172	722	-0.16	-0.81	160	704
	40	-0.15	-0.88	64	333	-0.18	-0.78	122	512
	60	-0.19	-0.77	34	170	-0.19	-0.77	44	172
	80	-0.19	-0.78	23	104	-0.19	-0.78	34	112
5.0	25	-0.29	-0.69	236	472	-0.24	-0.73	188	526
	40	-0.29	-0.72	122	342	-0.29	-0.68	126	416
	60	-0.32	-0.66	63	164	-0.29	-0.68	50	165
	80	-0.33	-0.66	38	110	-0.31	-0.68	34	112
6.0	25	-0.32	-0.62	226	497	-0.29	-0.69	223	535
	40	-0.25	-0.72	82	246	-0.30	-0.72	124	335
	60	-0.35	-0.69	48	125	-0.35	-0.69	64	141
	80	-0.41	-0.62	20	58	-0.36	-0.69	35	88
7.0	25	-0.28	-0.69	123	322	-0.30	-0.70	194	504
	40	-0.30	-0.73	76	251	-0.30	-0.72	114	296
	60	-0.35	-0.68	36	104	-0.35	-0.72	49	122
	80	-0.42	-0.62	20	52	-0.40	-0.68	30	66
8.0	25	-0.38	-0.65	180	360	-0.39	-0.79	220	495
	40	-0.45	-0.55	109	251	-0.44	-0.62	135	270
	60	-0.50	-0.80	31	73	-0.48	-0.59	59	89
	80	-0.60	-0.90	16	42	-0.43	-0.69	33	40

Based on Table 4.14, it was noticed that there is significant difference between the kinetic terms (k_1 and k_2). Larger difference in the number of kinetic terms indicate longer degradation period. Largest magnitude of difference in kinetic terms (74 - 82%) was found when the aqueous and CHCL/AA NADES systems were altered to pH 3; On the other hand, the difference of kinetic term in each system reduced (18 - 50%) when the pH was increased to 8.0. This finding suggests that at lower pH, the degradation period tends to be longer, which once again confirms the finding from preceding section, that the antioxidants are relatively more stable at lower pH conditions. Also, for every condition, the time constant of short-term kinetic (τ_1) is

found to be smaller than that of the medium-term constant (τ_2). This means that the short-term kinetics acts immediately and its effect is over a relatively shorter period of time compared to the medium-term kinetics. In other word, the medium-term kinetics which has larger time constant value has a longer effect on the degradation.

The half-life values can also be determined using the established model. The half-life values are tabulated as Table 4.15 and the half-life plots at each experimental condition are illustrated in Appendix G (Figure G.1 - G.12). It can be seen that the half-life duration for the antioxidants in CHCL/AA NADES system was longer (approximately 1.2 - 44.10%) than the antioxidants in aqueous system, where the largest magnitude of increment (44.10%) can be found at pH 8.0 and 25℃. This result once again highlights the ability of CHCL/AA NADES system in slowing down the degradation of antioxidant extracts. Interestingly, it was found that the half-life value obtained using this proposed kinetic model were slightly deviate as compared to the half-life value obtained from the first-order kinetic and Arrhenius plot (Table 4.13). It was noted that under few conditions, there is no difference between the half-life value obtained in both systems. For example, the half-life value for both system at pH 4.0, 25℃ is found to be the same. Nonetheless, in this proposed kinetic model, the estimated half-life value for CHCL/AA NADES system was 11% larger than the half-life value obtained for the aqueous system, this certainly denotes that the first-order kinetic model is not sufficient to fit all the experimental data. From this point of view, improvement on the estimation of the half-life value for the degradation process can be achieved by using this proposed kinetic model.

These results confirm the reliability and validity of the method developed for purpose of determination of kinetic parameters of the degradation process. Nevertheless, examination of more detail kinetic information is still necessary in order to determine how generally this model can be applied. It must be remembered that the model proposed here is simply an alternative approach and the quantitative analysis of the model has not been fully proposed yet. For instance, the differentiation of the antioxidant degradation profile above in the time domain can be obtained by applying the inverse of Laplace Domain. Therefore, the proposed model shall be further

expanded to provide more description of degradation pattern and to account for the variations in the degradation factors.

Table 4.15 Half-life value for the degradation of antioxidants in aqueous and CHCL/AA NADES system via Laplace Domain

pH	Temperature (℃)	Half-life (min)		pH	Temperature (℃)	Half-life (min)	
		Aqueous	NADES			Aqueous	NADES
3.0	25	2525	2676	6.0	25	1561	1623
	40	1079	1655		40	785	959
	60	510	630		60	332	377
	80	334	373		80	146	219
4.0	25	2329	2387	7.0	25	997	1483
	40	1061	1742		40	714	850
	60	573	580		60	282	316
	80	345	385		80	127	160
5.0	25	1435	1680	8.0	25	945	1114
	40	1000	1277		40	612	634
	60	482	507		60	128	200
	80	318	332		80	52	93

4.3.7 Stabilizing capacity of CHCL/AA NADES system

The stabilizing effect of CHCL/AA NADES system could be related to the hydrogen bond interactions between the antioxidants extracts and the components of CHCL/AA NADES. In this regard, FTIR was conducted to analyze the antioxidant dissolved in CHCL/AA NADES system. The FTIR spectra is illustrated in Figure 4.18. Figure 4.18 shows a few absorption bands of the C-O and aromatic ring as well as the carbonyl group from antioxidants in CHCL/AA NADES system and its pure form. The stretching vibration band of the C-OH in *M. pajang* antioxidant extracts, which appear at 1058 cm^{-1}, 1073 cm^{-1}, 1356 cm^{-1} and 1453 cm^{-1} were shifted to 1051 cm^{-1}, 1110 cm^{-1}, 1319 cm^{-1} and 1468 cm^{-1}, respectively in the antioxidant-CHCL/AA NADES system. These stretching vibration absorption band confirms the formation of hydrogen bonds between the antioxidant extracts and molecules of CHCL/AA NADES. Aside from that, the shifting of C=O stretching vibration of antioxidant extracts, from 1625 cm^{-1} to 1677 cm^{-1} and from 1714 cm^{-1} to 1751 cm^{-1} indicate the formation of hydrogen bonds (C=O---HO) between the carbonyl group of antioxidant extracts and the hydroxyl group of CHCL/AA NADES. All these shifting vibration absorption bands suggest the existence of multi hydrogen bonding interaction between the between the antioxidant extracts and CHCL/AA NADES (Dai, Verpoorte, and Choi 2014). It is possible that the formation of hydrogen bonds between the solute and solvent stabilize the structure of antioxidant extracts and provides a protecting effect against external factors such as pH and temperature. This is evidenced by the study reported by Zhang, Mullaney, and Lei (2007), where they successfully improved the thermostability of *Aspergillus niger* PhyA phytase (enzyme) by introducing selected hydrogen bond network from *Aspergillus fumigatus* phytase. They found that all the samples showed greater residual activity after subjected to heat treatment. Moreover, Wang et al. (2017) constructed a mutant pullulanese (enzyme) from *Bacillus naganoensis* by using a new method, namely active hydrogen bond network and found that the mutant exhibit greater thermal and pH stability. This could be attributed to the hydrogen bonding which connect the active and functional residues of enzymes and conservative water molecules. All these studies assure the hydrogen bonding interaction could improve the stability of antioxidant extracts.

To further manifest the protecting or stabilizing effect of CHCL/AA NADES system, the morphology of antioxidants in the CHCL/AA NADES system was observed using transmission electron micrographs (TEM), as depicted in Figure 4.19. From the Figure 4.19A, it was observed that the sample of particles were spherical in shape with average diameter of 50.05 nm and all the particles were within the range diameter of 15.78 – 101.78 nm, indicating that the fabricated system has the potential to be formulated into nano-formulation system (Manuel et al., 2019). Aside from that, Figure 4.19B clearly shows that the antioxidants dissolved in CHCLC/AA NADES system formed cluster structure, where an amount of antioxidant extracts aggregated and well-rounded in the center of CHCL/AA NADES droplet (lighter color). With this observation, it can be said that the CHCL/AA NADES may act as a carrier system which provide an enhanced stabilization effect (Shin, Kim, and Park 2015). The agglomeration is possible when the antioxidant come into contact with the CHCL/AA NADES droplets, where they enter the droplet until the droplet internal volume is saturated with the particles and finally lead to formation of aggregates with spherical shape (Maghsoodi, Derakhshandeh, and Yari 2012). Another explanation for the formation of aggregated clusters of antioxidants could be ascribed to the heterogeneity of mixture, where Häkkinen et al. (2019) stated that the addition of water improved the heterogeneities in the mixture of alcohol-DES and they found that self-aggregated clusters of molecules become more significant at heterogenous mixture. Since 10 wt% of water was added to the CHCL/AA NADES, it is possible that the water act as a liquid solute and distribute the heterogeneity in the CHCL/AA NADES mixture. Consequently, the diffusion of antioxidants become slower and the aggregates are formed. Taking into account that some degree of agglomeration of antioxidants which are not incorporated in the center of CHCL/AA NADES may be attributed to the inadequate amount of CHCL/AA NADES, where the NADES is insufficient to contain all the antioxidant extracts inside. With these observations, CHCL/AA NADES system show a clear protective effect and can be denoted as a potential carrier to protect the antioxidants or other bioactive compounds.

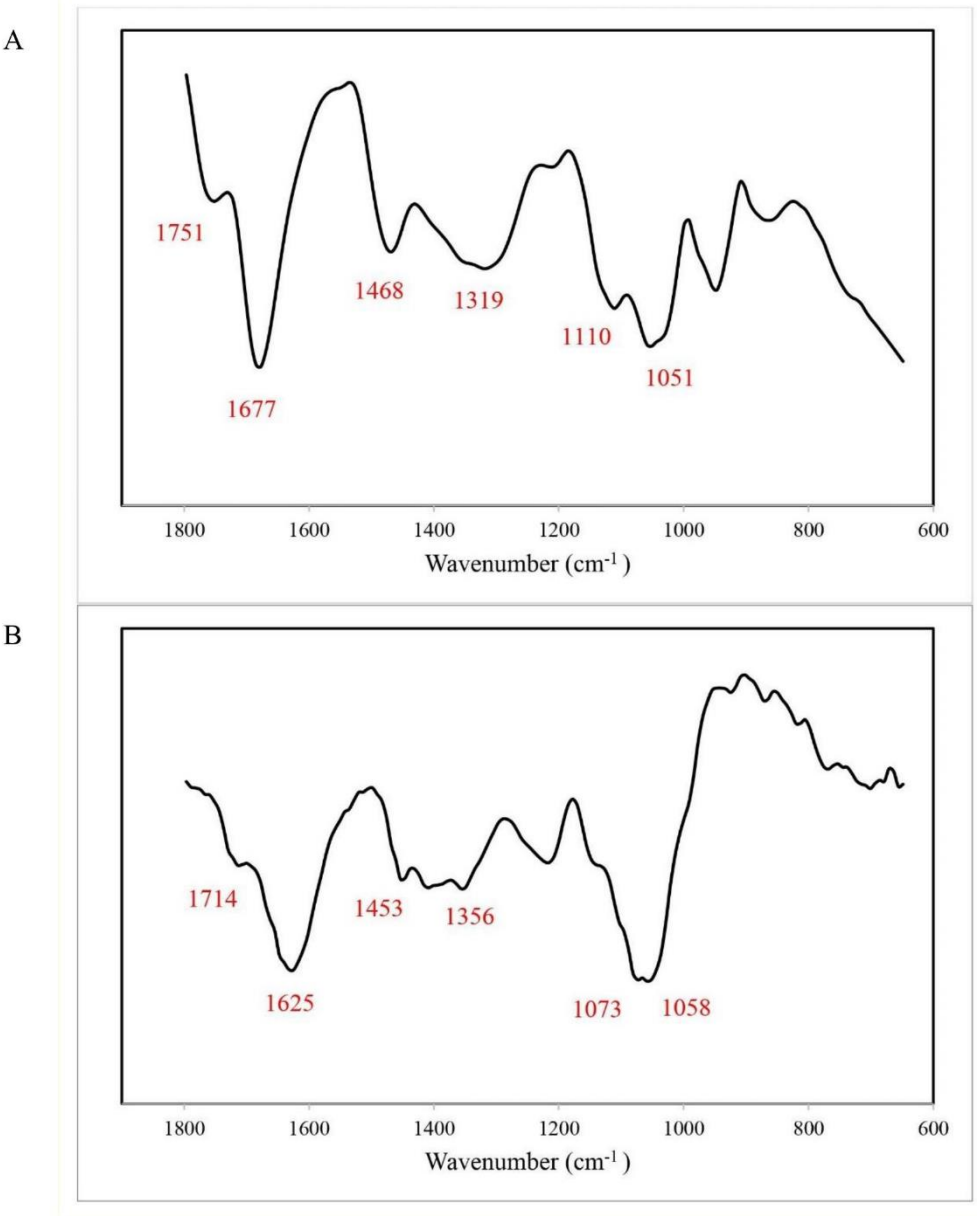


Figure 4.18 FTIR spectrum of A) Antioxidants in CHCL/AA NADES system and B) *M. pajang*'s antioxidant extracts

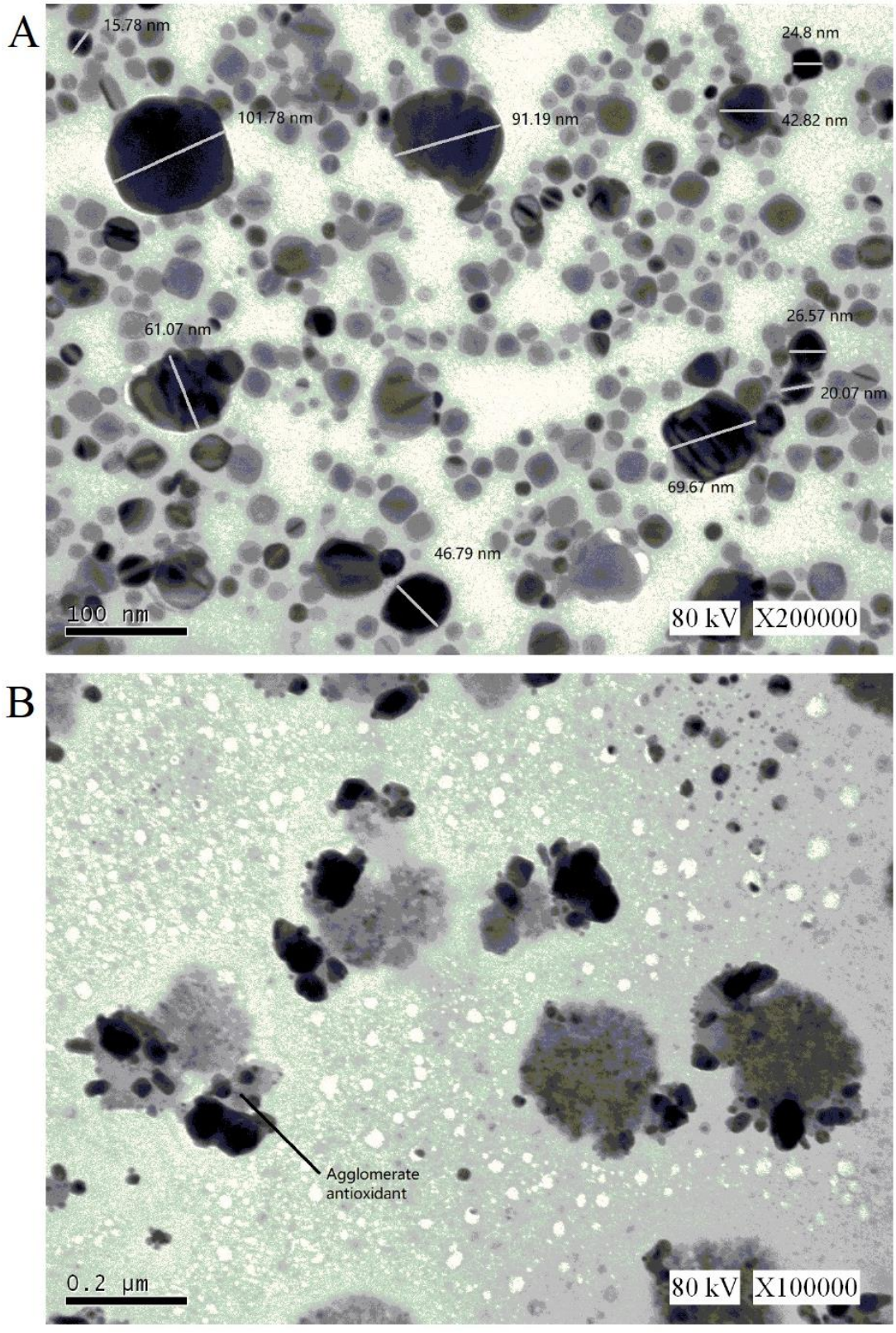


Figure 4.19 TEM images of antioxidant in CHCL/AA NADES system:
A) at magnification x200000; B) at magnification x100000

4.3.8 Antioxidant capacity of antioxidants

The antioxidant activity of antioxidants in both aqueous and CHCL/AA NADES system were determined using DPPH radical scavenging assay and the antioxidant capacity values of the samples are gathered in Table 4.16 and 4.17, respectively. In general, it was realized that although the antioxidants degraded after thermal treatment, the total antioxidant activity was not significantly affected, which is evidenced by the studies reported by Sui, Dong, and Zhou (2014) and Xu, Yu, and Zhou (2019). In other word, the thermal treatment has only a little impact on the antioxidant activity of antioxidants. Nonetheless, it was found that the pH had a greater impact on the antioxidant activity of the sample. In both systems, the antioxidant activity of sample increased with the increased of pH level. For example, the lowest antioxidant activity (6.438 mg/ml) of the control sample in aqueous system was obtained at pH 3.0. In contrast, the antioxidant activity rised up to 7.485 mg/ml when the pH of system was increased to 8.0. Similar trend was observed in the case of CHCL/AA NADES system. This observation is in accordance to the finding reported by Sun, Mu, and Xi (2017). They obtained higher antioxidant activity of sweet potato leaf polyphenols at pH ranging from 5.0 to 7.0, given that highest antioxidant activity was found at neutral solvent system. Another study demonstrated by Di Majo et al. (2011) also showed that the antioxidant activity of different types of antioxidants (flavonoids and phenolic acids) had comparatively higher antioxidant capacity at pH 7.4 as compared to pH 3.5. The effect of pH on the radical scavenging ability of antioxidants may be related to protonation, where in the acidic medium, massive H^+ suppressed ionization and weakened the electron transfer. This phenomenon reduces the interaction of ROO• (DPPH radical) with phenolic antioxidants and consequently results in lower antioxidant action. Deprotonation happens as the pH of media rise up. Upon deprotonation, the interaction of electron become easier and the ability of scavenging radical increases (Xie et al. 2018, Lemańska et al. 2001). Therefore, the antioxidant activity were found to be higher at higher pH level.

From Table 4.16 and 4.17, the antioxidant activity of sample did not follow a particular trend. Noticeably, the antioxidant activity was fluctuating during the thermal treatment in all tested sample. The antioxidant activity may decrease with the loss of bioactive compounds such as phenolic acid and flavonoids during the exposure to external

factors, mainly temperature and pH environment (Kim et al. 2018, Peron, Fraga, and Antelo 2017). In addition, the reduction of antioxidant activity is possible as the antioxidant compounds has a tendency to undergo polymerization reaction, where the polymerization increase the complexity of molecule and reduce the availability of hydroxyl group. Since lesser hydroxyl groups are available, the interaction with DPPH radicals are reduced, and consequently causes a decrease in the antioxidant activity (Pinelo et al. 2004). In contrast, higher antioxidant activity can be due to the hydrolysis of glycoside bonds of phenolic compounds. The hydrolysis increases the available phenolic hydroxy groups and lead to greater antioxidant action (Sun et al. 2018). For the CHCL/AA NADES system, the presence of ascorbic acid are most likely responsible for the increment of antioxidant activity, where the Maillard reaction of ascorbic acid form melanoidins, which could account for the greater antioxidant activity observed (Wang, Qian, and Yao 2011). However, after the thermal treatment, it was realized that all sample experience slight reduction as compared to the initial antioxidant activity. A point to note here, even with this reduction, the antioxidant action of samples was maintained. In this context, it is important to highlight the ability of CHCL/AA NADES system for promoting significantly higher antioxidant capacity, which is potential in the development of functional foods.

Table 4.16 DPPH scavenging activity (mg AEAC/ml) of antioxidants in aqueous system upon treatment at combined temperature and pH (mean ± SD, n=3)

pH	No.[a]	Control	80℃	60℃	40℃	25℃
3.0	1	6.438 ± 0.013	6.411 ± 0.013	6.280 ± 0.053	6.393 ± 0.038	6.376 ± 0.066
	2		6.492 ± 0.046	6.279 ± 0.025	6.420 ± 0.038	6.439 ± 0.091
	3		6.367 ± 0.022	6.067 ± 0.042	6.593 ± 0.047	6.538 ± 0.084
	4		6.193 ± 0.024	6.168 ± 0.012	6.376 ± 0.033	6.547 ± 0.047
	5		6.084 ± 0.054	6.009 ± 0.035	6.253 ± 0.032	6.393 ± 0.038
	6		6.034 ± 0.035	6.060 ± 0.102	6.358 ± 0.033	6.254 ± 0.053
4.0	1	6.621 ± 0.047	6.385 ± 0.033	6.501 ± 0.039	6.474 ± 0.038	6.602 ± 0.047
	2		6.228 ± 0.044	6.547 ± 0.026	6.529 ± 0.022	6.510 ± 0.034
	3		6.263 ± 0.092	6.349 ± 0.033	6.658 ± 0.035	6.411 ± 0.013
	4		6.394 ± 0.058	6.254 ± 0.049	6.465 ± 0.046	6.603 ± 0.073
	5		6.529 ± 0.059	6.402 ± 0.033	6.358 ± 0.033	6.403 ± 0.082
	6		6.323 ± 0.075	6.297 ± 0.086	6.429 ± 0.034	6.447 ± 0.044
5.0	1	6.850 ± 0.073	6.772 ± 0.014	6.811 ± 0.063	6.938 ± 0.014	6.870 ± 0.087
	2		6.763 ± 0.059	6.830 ± 0.028	6.899 ± 0.064	6.869 ± 0.000
	3		6.968 ± 0.028	6.705 ± 0.013	7.059 ± 0.063	7.049 ± 0.025
	4		6.744 ± 0.089	6.791 ± 0.049	6.715 ± 0.049	7.090 ± 0.038
	5		6.603 ± 0.087	6.621 ± 0.047	6.811 ± 0.063	6.850 ± 0.050
	6		6.632 ± 0.131	6.658 ± 0.058	6.763 ± 0.072	6.734 ± 0.027
6.0	1	6.869 ± 0.024	6.919 ± 0.078	6.869 ± 0.064	6.889 ± 0.050	6.801 ± 0.027
	2		6.840 ± 0.000	6.870 ± 0.084	6.900 ± 0.105	6.918 ± 0.037
	3		6.890 ± 0.097	6.869 ± 0.063	7.069 ± 0.014	7.059 ± 0.058

	4		6.989 ± 0.065	7.079 ± 0.025	6.988 ± 0.025	6.998 ± 0.051
	5		6.791 ± 0.049	6.850 ± 0.060	6.908 ± 0.028	6.840 ± 0.000
	6		6.801 ± 0.049	6.841 ± 0.095	6.821 ± 0.049	6.820 ± 0.014
	1	7.419 ± 0.041	7.321 ± 0.040	7.173 ± 0.044	7.332 ± 0.026	7.227 ± 0.157
	2		7.441 ± 0.016	7.100 ± 0.015	7.289 ± 0.060	7.354 ± 0.085
7.0	3		7.194 ± 0.039	7.267 ± 0.026	7.183 ± 0.065	7.300 ± 0.053
	4		7.247 ± 0.079	7.289 ± 0.066	7.141 ± 0.000	7.152 ± 0.053
	5		7.247 ± 0.065	7.321 ± 0.066	7.122 ± 0.106	7.226 ± 0.083
	6		7.069 ± 0.038	7.153 ± 0.115	7.289 ± 0.015	7.246 ± 0.040
	1	7.485 ± 0.032	7.364 ± 0.027	7.386 ± 0.031	7.509 ± 0.110	7.486 ± 0.084
	2		7.463 ± 0.027	7.419 ± 0.062	7.621 ± 0.071	7.441 ± 0.041
8.0	3		7.519 ± 0.063	7.530 ± 0.073	7.508 ± 0.069	7.564 ± 0.028
	4		7.553 ± 0.042	7.508 ± 0.032	7.430 ± 0.027	7.587 ± 0.064
	5		7.576 ± 0.070	7.564 ± 0.000	7.342 ± 0.015	7.397 ± 0.027
	6		7.419 ± 0.016	7.386 ± 0.041	7.419 ± 0.068	7.431 ± 0.094

[a] No. 1 – 6 represents the thermal treatment duration of 30, 60, 90, 120, 150 and 180 minutes for 80℃, 40, 80, 120, 160, 200 and 240 minutes for 60℃, 60, 120, 180, 240, 300 and 360 minutes for 40℃ and 80, 160, 240, 320, 400 and 480 minutes for 25℃, respectively.

Table 4.17 DPPH scavenging activity (mg AEAC/ml) of antioxidants in CHCL/AA NADES system upon treatment at combined temperature and pH (n=3, mean ± SD)

pH	No.[a]	Control	80℃	60℃	40℃	25℃
	1	10.271 ± 0.049	10.173 ± 0.073	9.945 ± 0.070	10.232 ± 0.055	9.873 ± 0.119
	2		10.412 ± 0.029	10.039 ± 0.081	10.394 ± 0.150	10.077 ± 0.027
3.0	3		10.058 ± 0.071	9.781 ± 0.051	10.195 ± 0.168	10.001 ± 0.070
	4		9.853 ± 0.026	9.658 ± 0.114	9.800 ± 0.111	10.312 ± 0.103
	5		9.537 ± 0.064	9.520 ± 0.105	10.001 ± 0.053	10.039 ± 0.081
	6		9.452 ± 0.071	9.855 ± 0.137	10.135 ± 0.099	9.763 ± 0.044
	1	10.729 ± 0.082	10.433 ± 0.029	10.708 ± 0.093	10.372 ± 0.058	10.621 ± 0.061
	2		10.153 ± 0.000	10.312 ± 0.075	10.536 ± 0.030	10.193 ± 0.055
4.0	3		10.579 ± 0.090	11.188 ± 0.118	10.193 ± 0.073	10.474 ± 0.058
	4		10.929 ± 0.032	10.668 ± 0.197	9.964 ± 0.069	10.709 ± 0.142
	5		10.213 ± 0.096	10.478 ± 0.190	10.497 ± 0.157	10.559 ± 0.118
	6		9.983 ± 0.079	10.006 ± 0.231	10.193 ± 0.055	10.413 ± 0.077
	1	11.162 ± 0.034	10.863 ± 0.139	10.685 ± 0.031	10.817 ± 0.031	10.752 ± 0.123
	2		10.643 ± 0.105	10.732 ± 0.189	10.797 ± 0.165	10.708 ± 0.092
5.0	3		11.535 ± 0.096	11.261 ± 0.156	10.626 ± 0.215	11.092 ± 0.067
	4		11.382 ± 0.035	11.563 ± 0.166	11.139 ± 0.089	10.796 ± 0.112
	5		11.045 ± 0.057	11.142 ± 0.190	10.774 ± 0.094	10.999 ± 0.087
	6		10.731 ± 0.135	11.022 ± 0.086	10.795 ± 0.083	10.975 ± 0.056
	1	11.773 ± 0.101	11.484 ± 0.063	11.586 ± 0.037	11.718 ± 0.000	11.410 ± 0.161
	2		11.284 ± 0.069	11.235 ± 0.090	11.410 ± 0.161	11.383 ± 0.071
6.0	3		11.882 ± 0.068	11.910 ± 0.078	11.537 ± 0.158	11.616 ± 0.198

	4		12.197 ± 0.042	12.087 ± 0.263	11.910 ± 0.040	11.590 ± 0.194
	5		11.434 ± 0.128	11.512 ± 0.179	11.459 ± 0.130	11.186 ± 0.000
	6		11.387 ± 0.214	11.433 ± 0.095	11.460 ± 0.156	11.408 ± 0.107
	1	12.142 ± 0.187	11.940 ± 0.144	12.080 ± 0.041	11.969 ± 0.185	11.587 ± 0.073
	2		12.317 ± 0.074	11.613 ± 0.098	12.350 ± 0.154	11.285 ± 0.124
7.0	3		11.666 ± 0.100	11.800 ± 0.067	12.383 ± 0.221	11.719 ± 0.066
	4		12.168 ± 0.042	12.142 ± 0.192	12.023 ± 0.040	12.350 ± 0.154
	5		11.995 ± 0.080	12.290 ± 0.185	12.230 ± 0.195	12.142 ± 0.192
	6		12.026 ± 0.175	11.856 ± 0.142	11.939 ± 0.105	12.024 ± 0.106
	1	12.197 ± 0.042	11.667 ± 0.134	11.943 ± 0.218	11.968 ± 0.138	12.200 ± 0.165
	2		12.081 ± 0.109	12.082 ± 0.146	12.202 ± 0.213	12.138 ± 0.000
8.0	3		12.227 ± 0.073	12.503 ± 0.077	12.261 ± 0.209	11.893 ± 0.605
	4		12.503 ± 0.077	12.441 ± 0.117	12.566 ± 0.045	12.797 ± 0.164
	5		12.598 ± 0.000	12.602 ± 0.206	12.110 ± 0.108	12.604 ± 0.237
	6		12.052 ± 0.070	12.199 ± 0.152	12.200 ± 0.165	12.083 ± 0.165

[a] No. 1 – 6 represents the thermal treatment duration of 30, 60, 90, 120, 150 and 180 minutes for 80℃, 40, 80, 120, 160, 200 and 240 minutes for 60℃, 60, 120, 180, 240, 300 and 360 minutes for 40℃ and 80, 160, 240, 320, 400 and 480 minutes for 25℃, respectively.

CHAPTER 5
CONCLUSIONS AND FUTURE WORKS

5.1 Conclusions

This research focused on the development of a novel antioxidant-choline chloride/ascorbic acid natural deep eutectic solvent system, with the aim of enhancing the solubility and stability of antioxidant extracts obtained from the fruit wastes of *M. pajang*. This research examined the effects of extraction parameters including the concentration of ethanol, extraction temperature and extraction shaking speed by using the response surface methodology to obtain extracts with maximum antioxidant activity. Furthermore, this work studied the characteristics of the synthesized CHCL/AA NADES through the polarized optical microscopy, proton nuclear magnetic resonance analysis and Fourier transform infrared spectroscopy analysis. Following these analyzes, the antioxidant extracts were solubilized in the aqueous and CHCL/AA NADES. In particular, the solvation capacity of the CHCL/AA NADES was evaluated and the effect of water on the solvation capacity was also studied. In addition, the role of the natural deep eutectic solvent as the antioxidant activity enhancer was investigated. Aside from investigating the solubility issue, this study also explored the stabilization capacity of the CHCL/AA NADES. Specifically, the degradation of antioxidant extracts in the aqueous and CHCL/AA NADES systems were analyzed under combined effects of pH (pH 3.0 – 8.0) and temperature (25℃, 40℃, 60℃ and 80℃). The degradation kinetics were curve fitted to the zero-order and first-order kinetic models to quantitatively interpret the loss of antioxidants concentration. Additionally, a new multi-term degradation model was proposed to estimate the degradation profile. Lastly, the antioxidant activity of the antioxidant extracts in both systems were constantly monitored throughout the thermal treatment. The objectives of this project were achieved and some important conclusions were drawn as follow:

- The ethanol extaction parameters were optimized using CCD approach, where the optimum ethanol extraction paramaters for maximum antioxidant activity were 54% ethanol concentration, 51℃ and 178 rpm extraction shaking speed. The ANOVA suggested that the concentration of ethanol, extraction shaking

speed and interactive effect of extraction temperature and shaking speed have significant impact on the antioxidant activity of extracts. Besides that, the experimental value (11.11 mg AEAC/g, DPPH free radical scavenging effect of 91.09%) was in close context with the model predicted value (11.38 mg AEAC/g, DPPH free radical scavenging effect of 91.57%) with differences of 2.37% (within the 95% confidence interval of the fitted model), proving that the model is well fitted for the extraction of antioxidant compounds from *M. pajang* under the optimal extraction conditions.

- To facilitate the application of CHCL/AA NADES, the characterstics of the solvents were examined. The best molar ratio of choline chloride and ascorbic acid was found at 2:1 as it allows the formation of a complete liquid solvent, as evidenced in the POM image. The ^{1}H NMR analysis and FTIR analysis indicated a high purity of CHCL/AA NADES was obtained and found that the -OH groups of ascorbic acid may interact with choline chloride and form intermolecular hydrogen-bonding network. Owing to this hydrogen bonding network, the prepared CHCL/AA NADES is considered as a stable liquid solvent.
- The solubility of antioxidant compounds is an important paramter to ensure adequate amount of bioactivity for health benefit effects. The solubility tesing was performed by solubilizing the antioxidant extracts in aqueous and CHCL/AA NADES system. Higher solubility of antioxidant extracts in CHCL/AA NADES (11% as compared to water) was observed, confirming the solubilization ability of the natural deep eutectic solvent. The optimal water content in CHCL/AA NADES was found to relate to the solubility of antioxidant extracts. The addition of water (10 wt%) to the natural deep eutectic solvent reduced the viscosity of CHCL/AA NADES and as a result, further improved its solubilization capacity by 4%. Nevertheless, it is interesting to point out that at further addition of water, the unique property and ability of CHCL/AA NADES disappeared and resulted in the decrease of solubility. Aside from that, the antioxidant extracts that dissolved in CHCL/AA NADES system was found to exhibit a higher antioxidant potential (1.3-14.64%) as compared to that dissolved in the aqueous form. This can be credited to the presence of ascorbic acid as one of the components in

CHCL/AA NADES which contributed to the increased DPPH radical scavenging activity.

- The degradation of antioxidants in both aqueous and CHCL/AA NADES system were investigated under different temperature (25℃, 40℃, 60℃ and 80℃) and pH (pH 3.0 - 8.0). The degradation of antioxidants in both aqueous and CHCL/AA NADES systems obeyed the first-order reaction kinetics. The degradation rate constant was determined from the plot of first-order and it was realized that temperature and pH significantly affected the stability antioxidant extracts. Remarkably, the degradation rate constant determined from aqueous system at all conditions were comparatively larger (4.17% - 25%) than the one obtained in CHCL/AA NADES system, implying that CHCL/AA NADES system can stabilized the antioxidants by reducing the degradation rate of antioxidant under high temperature and high pH. Highest Arrhenius activation energy was found when the pH of both systems was adjusted to pH 8.0, denoting that the antioxidant extracts were more susceptible to degradation at alkali conditions. Also, increasing of temperature and pH caused a drastic decrease in the half-life value, given that the shortest half-life value was obtained at the highest tested temperature (80℃) and highest tested pH level (pH 8.0). It was noticed that antioxidants in CHCL/AA NADES system has a greater half-life value than that in the aqueous system, inferring that the CHCL/AA NADES system possess protecting effect and helps in extending the half-life duration. Moreover, a new multi-term degradation model was proposed as an alternative to the first-order kinetic model to describe the degradation profile. The model developed was able to fit the data obtained from a broad range of experimental conditions. Additionally, by adopting this proposed model, the estimation of half-life value was improved and this finding confirms the reliability of the method developed for the purpose of determination of degradation kinetic parameters. The stabilizing effect of CHCL/AA NADES was proven as revealed in the FTIR analysis, where formation of hydrogen bonds between the antioxidant extracts and the CHCL/AA NADES was observed. It is possible that the formation of hydrogen bonds stabilizes the structure of antioxidant extracts and provides a protecting effect against external factors such as pH and temperature. In addition, the antioxidant extracts were found to agglomerate and well-rounded in the center

of CHCL/AA NADES system, as depicted in the TEM. Based on the TEM, nano-sized clusters were observed, denoting the CHCL/AA NADES shows great promising in protecting the antioxidant extracts and is feasible in the application of nano-sized formulation. Lastly, the antioxidant activity of antioxidant extracts in both aqueous and CHCL/AA NADES system did not follow a particular trend and was constantly fluctuated throughout the testing.

With these observations, it can be concluded that CHCL/AA NADES significantly enhances the solubility and stability of antioxidant compounds or other bioactive compounds. It is of great interest to utilize CHCL/AA NADES in introducing new bioactive products or enhancing the existing drug or antioxidant delivery systems. These remarkable advantages make them fit for numerous applications in, e.g. functional foods, cosmetic, nutraceutical and pharmaceutical products as a neoteric green technology media.

5.2 Future works

Considering the result obtained in the limited time frame of a PhD candidature, several recommendations on future studies can be given:

- The extractability of CHCL/AA NADES for antioxidant compounds from *M. pajang* can be further evaluated. In this context, an optimization study on the extraction parameters to obtain highest recovery of antioxidant extracts can be performed.
- Considering the hydrogen bonding interactions between the choline chloride and ascorbic acid molecules are not detected in ^{1}H NMR analysis, it is of great interest to perform further investigation, probably with heteronuclear overhauser spectroscopy (HOESY) or ^{1}H-^{1}H-nuclear overhauser spectroscopy (NOESY), which reveals the intermolecular and intramolecular interactions between the components.
- The effects of temperature on the solubility capacity of CHCL/AA NADES is not included in this present work. It is substantial to study the relationship between the temperature and solubility.
- The formation of nano-sized cluster of antioxidant-CHCL/AA NADES was observed. It is important to further explore the nanoscale clustering which is useful in designing self-assembly applications.
- The fluctuation of antioxidant activity during the thermal treatment reasonably indicative of a series of reactions taking place. It is important to investigate and study the underlying mechanisms or reaction. Such information would be helpful to establish appropriate processing and storage protocols to reduce the degradation of antioxidants.
- *In vitro* and *in vivo* tests can be considered in the future works to examine the release behavior of the antioxidant compounds from the CHCL/AA NADES. This finding could help in designing a superior formulation of antioxidant delivery system.
- It is reported that designation of ionic liquid-based micro- or nano-emulsion system could significantly improve the solubility of bioactive compounds. It is thought-provoking to replace the ionic liquids by CHCL/AA NADES in the formulation of emulsion system. The novel emulsion system is very interesting

from the point of view of scientific research and practical application, where it would be very useful for the delivery of various kinds of bioactive compounds.

- A more thorough investigation of the new degradation model proposed at Section 4.3.6 should be carried out, considering the model developed was able to fit the data obtained from a broad range of experimental conditions. It shall be further expanded to accurately account for the degradation of antioxidant compounds under different conditions. For instance, the regime of the short-term and medium-term kinetic effects can be idenfitied, if effective, its use could help to design optimized food processing. Moreover, the proposed degradation model in the time domain can be established by applying the inverse of Laplace transform. Once the degradation profile is expressed in the time domain, the kinetic expression involved with respect to time can be determined.

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APPENDIX

Appendix A

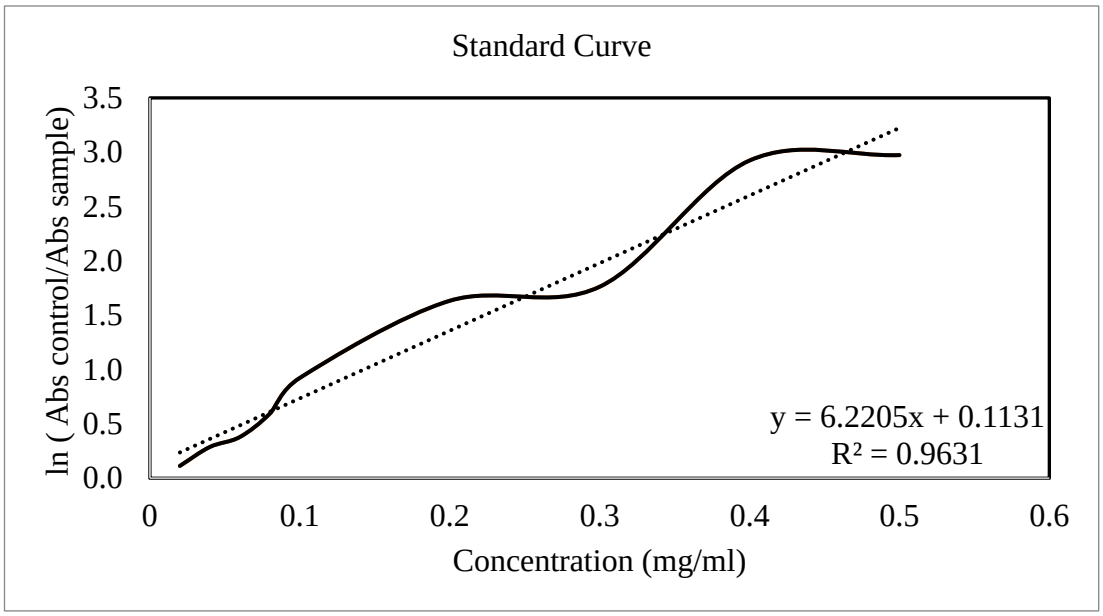


Figure A.1 Standard curve for DPPH assay

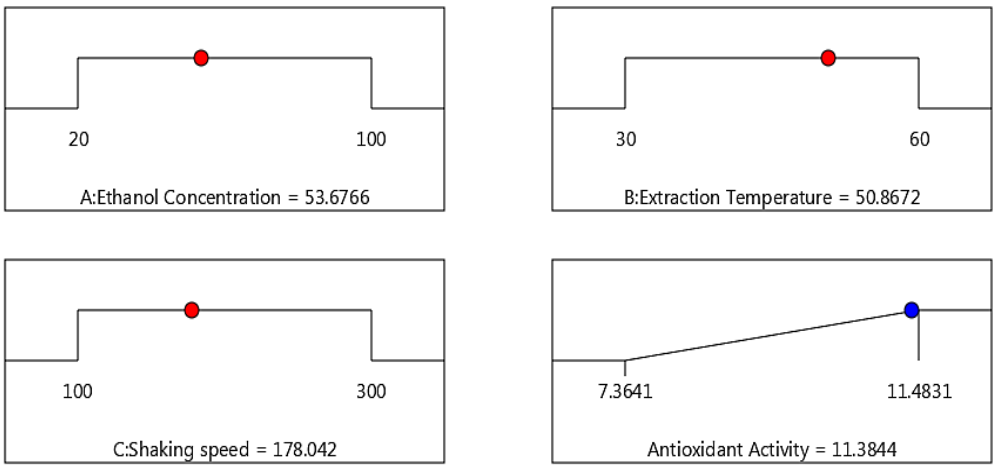


Figure A.2 Numerical optimization for maximum antioxidant activity

Appendix B

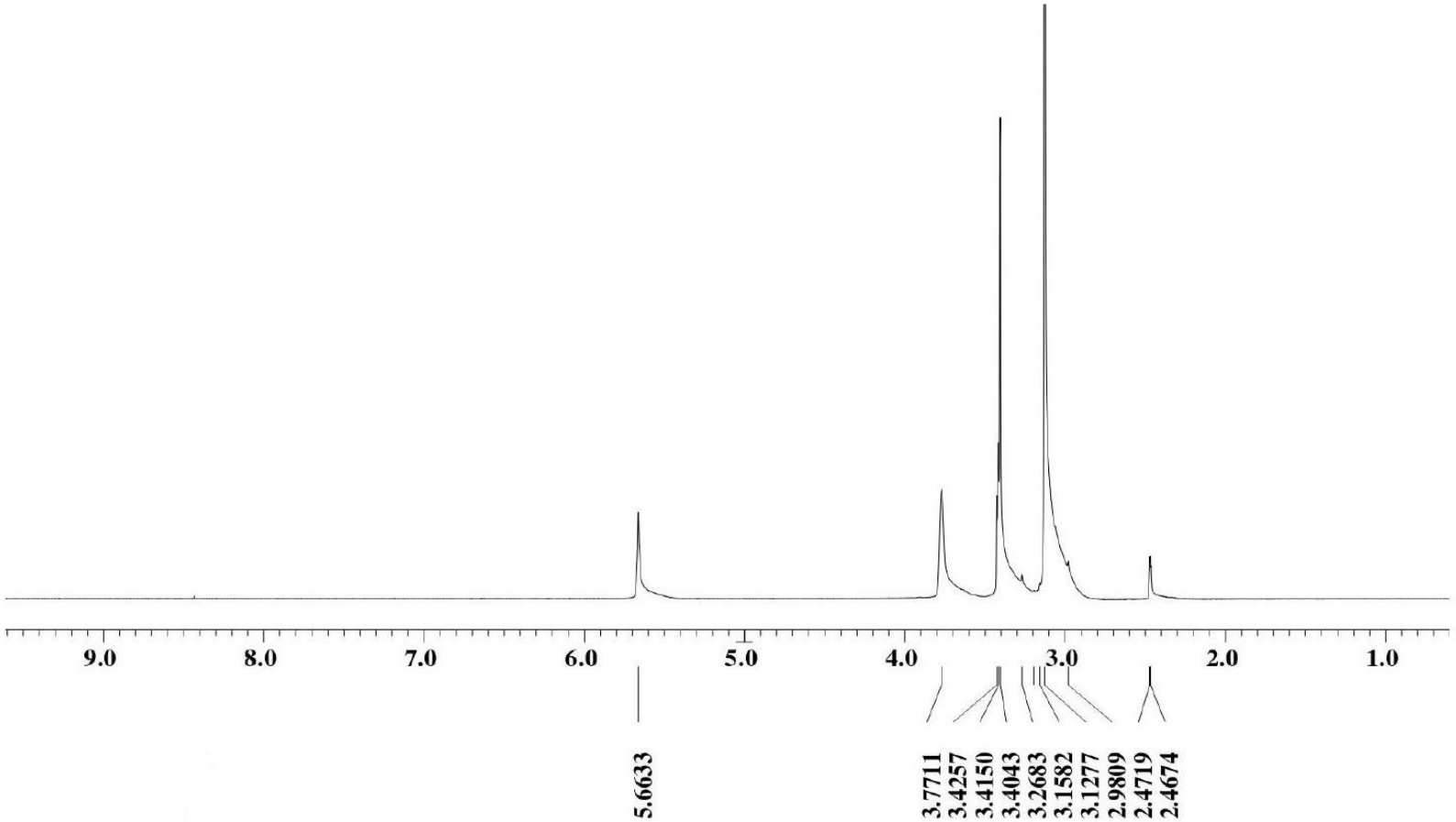


Figure B.1 ^{1}H NMR spectra of choline chloride (X: parts per Million: 1H)

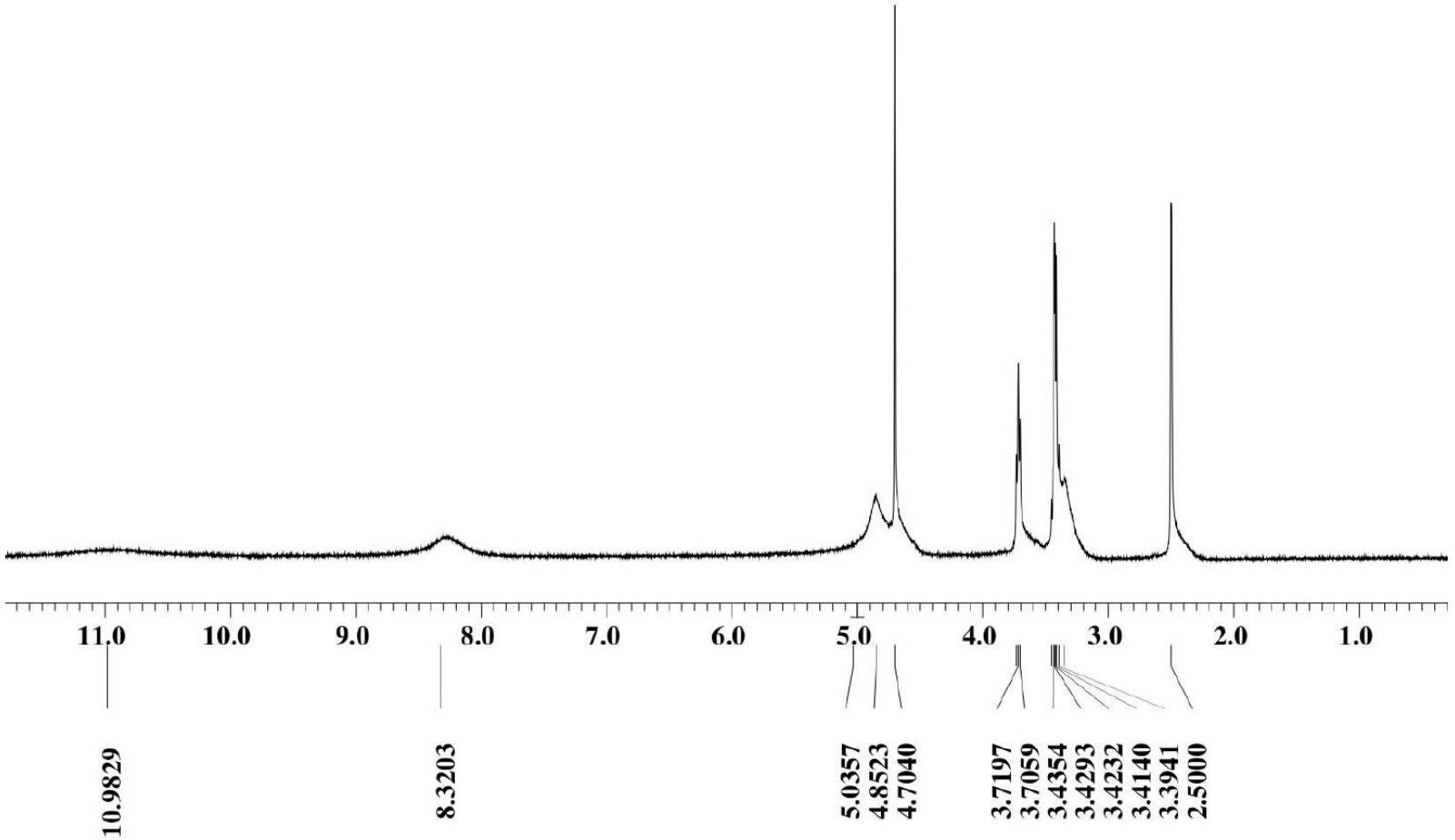
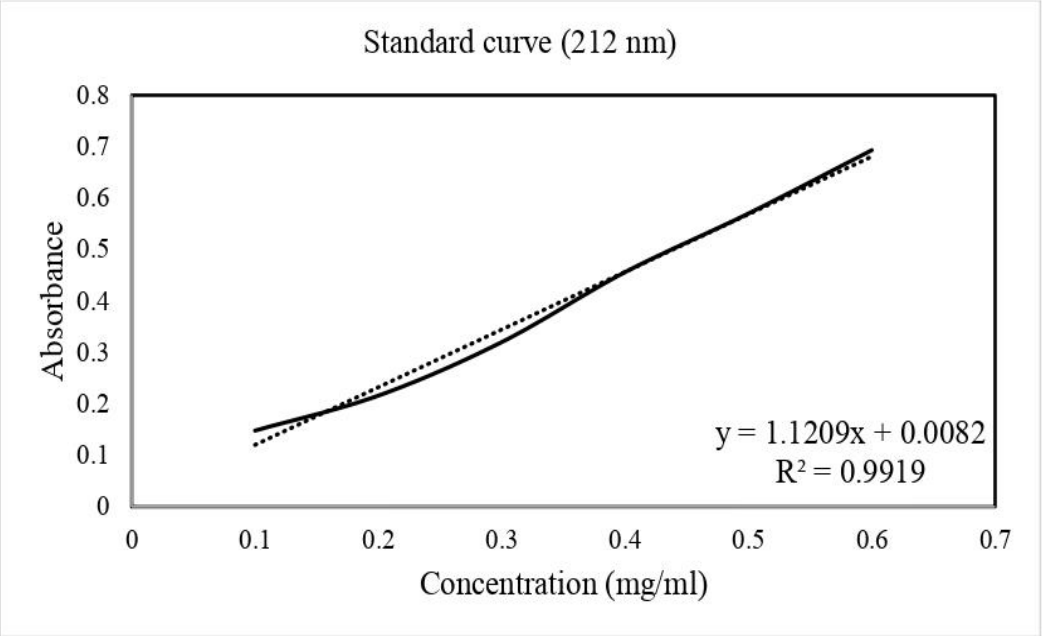


Figure B.2 ^{1}H NMR spectra of ascorbic acid (X: parts per Million: 1H)

Appendix C



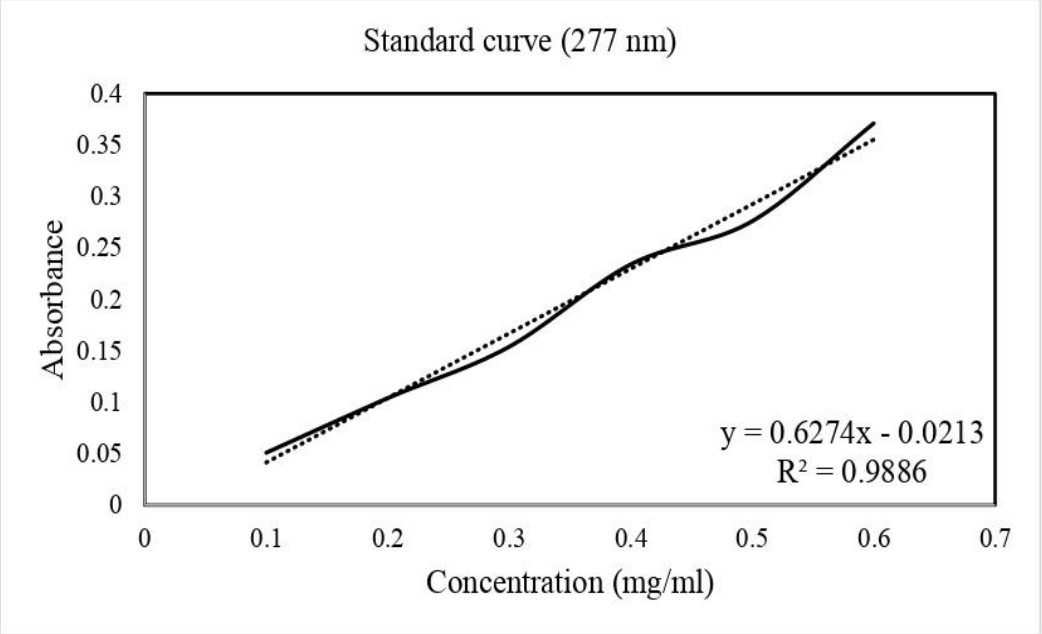
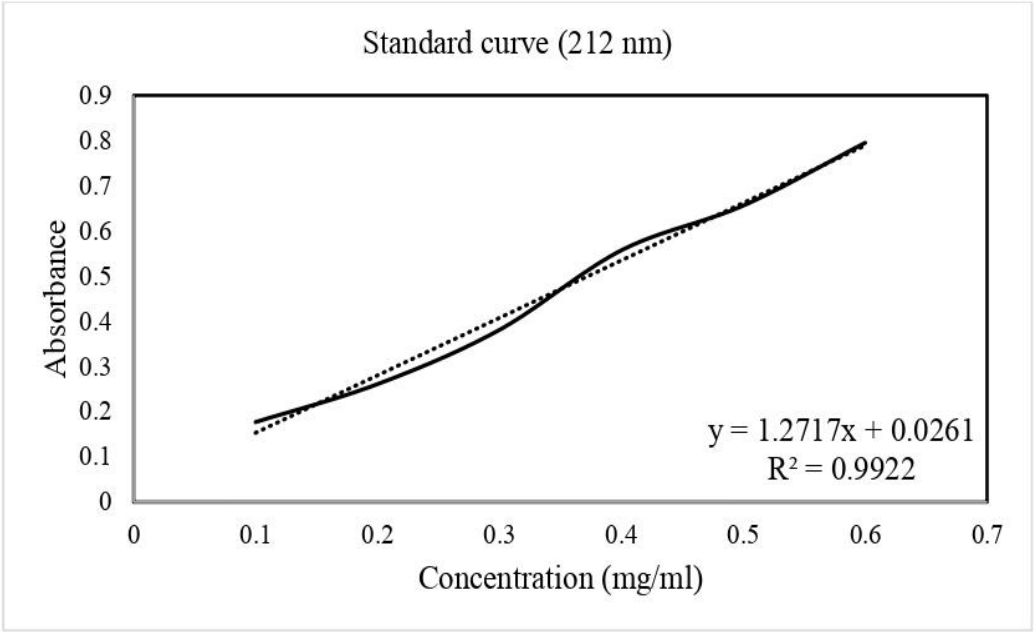


Figure C.1 Standard curve for antioxidants in aqueous form



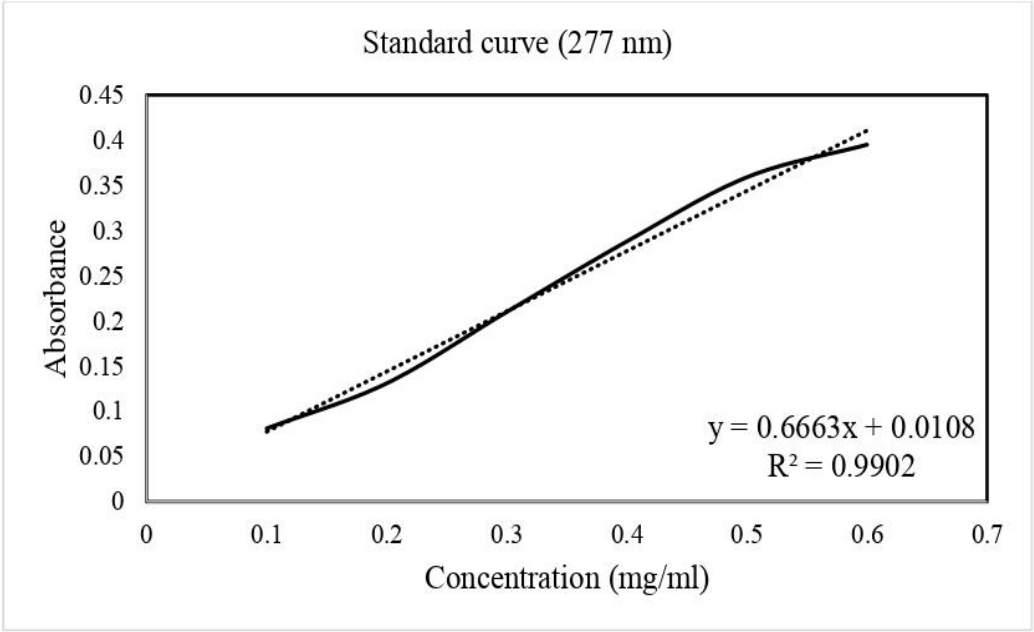


Figure C.2 Standard curve for antioxidants in CHCL/AA NADES

Appendix D

Table D.1 Retention percentage (%) of antioxidant extracts in aqueous system at optimum wavelength (212 nm and 277 nm)

Time	pH 3		pH 4		pH 5		pH 6		pH 7		pH 8	
	212 nm	277 nm	212 nm	277 nm	212 nm	277 nm	212 nm	277 nm	212 nm	277 nm	212 nm	277 nm
0	100	100	100	100	100	100	100	100	100	100	100	100
30	87.22	87.36	86.22	86.21	82.26	82.18	71.21	71.55	69.70	69.77	56.04	56.07
60	84.24	83.91	82.82	82.76	76.30	76.44	62.92	62.93	60.73	61.05	46.86	46.82
90	81.47	81.61	79.86	79.89	71.011	70.69	58.14	58.05	57.11	56.98	38.75	38.83
120	77.00	77.01	75.40	75.29	68.90	68.97	54.95	55.17	53.05	52.91	34.06	34.10
150	72.53	72.41	71.37	71.26	65.72	65.52	49.85	50.00	47.72	47.67	28.94	28.90
180	68.70	68.97	68.83	68.97	61.91	62.07	46.03	45.98	41.95	41.86	27.02	27.17

Table D.2 Retention percentage (%) of antioxidants in CHCL/AA NADES system at optimum wavelength (212 nm and 277 nm)

Time	pH 3		pH 4		pH 5		pH 6		pH 7		pH 8	
	212 nm	277 nm	212 nm	277 nm	212 nm	277 nm	212 nm	277 nm	212 nm	277 nm	212 nm	277 nm
0	100	100	100	100	100	100	100	100	100	100	100	100
30	87.32	87.22	86.86	86.82	82.58	82.75	79.27	79.14	78.31	78.51	75.97	75.93
60	84.48	84.42	83.13	82.82	76.63	76.33	71.78	71.52	67.14	66.97	60.31	60.29
90	81.64	81.62	80.29	80.42	71.78	71.52	66.70	66.31	58.82	58.62	50.39	50.66
120	77.49	77.63	75.69	75.63	69.35	69.11	63.84	63.90	55.75	55.43	43.55	43.44
150	73.33	72.23	72.40	72.43	66.26	66.31	58.32	58.68	51.59	51.45	39.36	38.63
180	70.06	70.04	70.21	70.04	63.18	63.10	55.02	55.07	48.96	49.07	37.38	37.43

Appendix E

Table E.1 Concentration and ln (C/C_0) value of antioxidant in aqueous system; Degradation at 80℃ with respect of time

pH	Time	212 nm		277 nm		pH	Time	212 nm		277 nm	
		Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)			Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)
	0	0.1365 ± 0.0004	0.0000	0.1371 ± 0.0000	0.0000		0	0.1368 ± 0.0004	0.0000	0.1371 ± 0.0000	0.0000
	30	0.1190 ± 0.0008	-0.1367	0.1198 ± 0.0011	-0.1352		30	0.0974 ± 0.0004	-0.3396	0.0981 ± 0.0012	-0.3347
	60	0.1150 ± 0.0004	-0.1715	0.1151 ± 0.0000	-0.1754		60	0.0861 ± 0.0013	-0.4633	0.0863 ± 0.0000	-0.4631
3.0	90	0.1112 ± 0.0011	-0.2049	0.1119 ± 0.0011	-0.2032	6.0	90	0.0795 ± 0.0007	-0.5423	0.0796 ± 0.0011	-0.5439
	120	0.1051 ± 0.0008	-0.2613	0.1056 ± 0.0011	-0.2612		120	0.0752 ± 0.0009	-0.5987	0.0757 ± 0.0024	-0.5947
	150	0.0990 ± 0.0011	-0.3212	0.0993 ± 0.0000	-0.3228		150	0.0682 ± 0.0007	-0.6961	0.0686 ± 0.0000	-0.6931
	180	0.0938 ± 0.0011	-0.3755	0.0946 ± 0.0019	-0.3716		180	0.0630 ± 0.0007	-0.7760	0.0631 ± 0.0011	-0.7770
	0	0.1371 ± 0.0012	0.0000	0.1371 ± 0.0000	0.0000		0	0.1362 ± 0.0012	0.0000	0.1356 ± 0.0011	0.0000
	30	0.1182 ± 0.0004	-0.1483	0.1182 ± 0.0000	-0.1484		30	0.0949 ± 0.0004	-0.3610	0.0946 ± 0.0000	-0.3600
	60	0.1135 ± 0.0000	-0.1884	0.1135 ± 0.0000	-0.1892		60	0.0827 ± 0.0004	-0.4987	0.0828 ± 0.0000	-0.4935
4.0	90	0.1095 ± 0.0015	-0.2249	0.1096 ± 0.0022	-0.2246	7.0	90	0.0778 ± 0.0007	-0.5603	0.0772 ± 0.0011	-0.5625
	120	0.1034 ± 0.0015	-0.2823	0.1033 ± 0.0011	-0.2839		120	0.0723 ± 0.0004	-0.6339	0.0717 ± 0.0011	-0.6366
	150	0.0978 ± 0.0007	-0.3372	0.0977 ± 0.0011	-0.3388		150	0.0650 ± 0.0004	-0.7399	0.0646 ± 0.0011	-0.7408
	180	0.0943 ± 0.0007	-0.3735	0.0946 ± 0.0000	-0.3716		180	0.0571 ± 0.0004	-0.8686	0.0568 ± 0.0004	-0.8708
	0	0.1374 ± 0.0004	0.0000	0.1371 ± 0.0000	0.0000		0	0.1362 ± 0.0014	0.0000	0.1364 ± 0.0011	0.0000
	30	0.1129 ± 0.0004	-0.1957	0.1127 ± 0.0011	-0.1962		30	0.0763 ± 0.0011	-0.5791	0.0765 ± 0.0011	-0.5786
	60	0.1048 ± 0.0007	-0.2705	0.1048 ± 0.0000	-0.2687		60	0.0638 ± 0.0007	-0.7579	0.0638 ± 0.0000	-0.7588
5.0	90	0.0975 ± 0.0004	-0.3423	0.0969 ± 0.0000	-0.3469	8.0	90	0.0528 ± 0.0004	-0.9479	0.0528 ± 0.0011	-0.9486
	120	0.0946 ± 0.0011	-0.3726	0.0946 ± 0.0019	-0.3716		120	0.0464 ± 0.0007	-1.0771	0.0465 ± 0.0011	-1.0758
	150	0.0903 ± 0.0004	-0.4197	0.0899 ± 0.0000	-0.4229		150	0.0394 ± 0.0007	-1.2400	0.0394 ± 0.0011	-1.2413
	180	0.0850 ± 0.0004	-0.4794	0.0851 ± 0.0000	-0.4769		180	0.0368 ± 0.0007	-1.3087	0.0370 ± 0.0011	-1.3031

Table E.2 Concentration and ln (C/C_0) value of antioxidant in aqueous system; Degradation at 60℃ with respect of time

pH	Time	212 nm		277 nm		pH	Time	212 nm		277 nm	
		Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)			Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)
	0	0.1365 ± 0.0004	0.0000	0.1371 ± 0.0000	0.0000		0	0.1368 ± 0.0004	0.0000	0.1371 ± 0.0000	0.0000
	40	0.1199 ± 0.0004	-0.1294	0.1206 ± 0.0000	-0.1286		40	0.1097 ± 0.0011	-0.2202	0.1103 ± 0.0011	-0.2174
	80	0.1156 ± 0.0004	-0.1664	0.1159 ± 0.0000	-0.1686		80	0.1025 ± 0.0004	-0.2887	0.1033 ± 0.0012	-0.2839
3.0	120	0.1115 ± 0.0004	-0.2023	0.1119 ± 0.0011	-0.2032	6.0	120	0.0929 ± 0.0015	-0.3869	0.0930 ± 0.0022	-0.3884
	160	0.1083 ± 0.0007	-0.2314	0.1088 ± 0.0000	-0.2318		160	0.0895 ± 0.0004	-0.4236	0.0899 ± 0.0000	-0.4229
	200	0.1013 ± 0.0000	-0.2980	0.1017 ± 0.0000	-0.2992		200	0.0852 ± 0.0004	-0.4735	0.0851 ± 0.0000	-0.4769
	240	0.0990 ± 0.0004	-0.3212	0.0993 ± 0.0000	-0.3228		240	0.0789 ± 0.0011	-0.5497	0.0796 ± 0.0011	-0.5439
	0	0.1371 ± 0.0012	0.0000	0.1371 ± 0.0000	0.0000		0	0.1362 ± 0.0012	0.0000	0.1356 ± 0.0011	0.0000
	40	0.1205 ± 0.0000	-0.1288	0.1206 ± 0.0000	-0.1286		40	0.1054 ± 0.0008	-0.2564	0.1048 ± 0.0011	-0.2571
	80	0.1144 ± 0.0012	-0.1808	0.1143 ± 0.0012	-0.1823		80	0.0972 ± 0.0004	-0.3368	0.0969 ± 0.0000	-0.3353
4.0	120	0.1109 ± 0.0000	-0.2118	0.1111 ± 0.0000	-0.2103	7.0	120	0.0909 ± 0.0007	-0.4048	0.0906 ± 0.0011	-0.4026
	160	0.1071 ± 0.0008	-0.2464	0.1072 ± 0.0011	-0.2464		160	0.0853 ± 0.0004	-0.4675	0.0851 ± 0.0000	-0.4654
	200	0.1034 ± 0.0004	-0.2823	0.1033 ± 0.0011	-0.2839		200	0.0778 ± 0.0004	-0.5603	0.0772 ± 0.0011	-0.5625
	240	0.1002 ± 0.0008	-0.3137	0.1001 ± 0.0011	-0.3149		240	0.0737 ± 0.0008	-0.6140	0.0733 ± 0.0000	-0.6149
	0	0.1374 ± 0.0004	0.0000	0.1371 ± 0.0000	0.0000		0	0.1362 ± 0.0014	0.0000	0.1364 ± 0.0011	0.0000
	40	0.1167 ± 0.0008	-0.1628	0.1167 ± 0.0011	-0.1618		40	0.0913 ± 0.0004	-0.4000	0.0910 ± 0.0012	-0.4040
	80	0.1092 ± 0.0007	-0.2297	0.1096 ± 0.0012	-0.2246		80	0.0786 ± 0.0009	-0.5491	0.0792 ± 0.0012	-0.5431
5.0	120	0.1010 ± 0.0004	-0.3072	0.1009 ± 0.0011	-0.3070	8.0	120	0.0711 ± 0.0011	-0.6501	0.0717 ± 0.0000	-0.6424
	160	0.0981 ± 0.0004	-0.3364	0.0977 ± 0.0011	-0.3388		160	0.0603 ± 0.0007	-0.8141	0.0607 ± 0.0011	-0.8095
	200	0.0922 ± 0.0004	-0.3990	0.0922 ± 0.0000	-0.3969		200	0.0568 ± 0.0004	-0.8737	0.0568 ± 0.0000	-0.8766
	240	0.0897 ± 0.0004	-0.4262	0.0899 ± 0.0000	-0.4229		240	0.0494 ± 0.0004	-1.0134	0.0497 ± 0.0000	-1.0102

Table E.3 Concentration and ln (C/C_0) value of antioxidant in aqueous system; Degradation at 40℃ with respect of time

pH	Time	212 nm		277 nm		pH	Time	212 nm		277 nm	
		Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)			Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)
	0	0.1365 ± 0.0004	0.0000	0.1371 ± 0.0000	0.0000		0	0.1368 ± 0.0004	0.0000	0.1371 ± 0.0000	0.0000
	60	0.1228 ± 0.0004	-0.1055	0.1230 ± 0.0000	-0.1092		60	0.1179 ± 0.0000	-0.1486	0.1182 ± 0.0000	-0.1484
	120	0.1202 ± 0.0004	-0.1270	0.1206 ± 0.0000	-0.1286		120	0.1109 ± 0.0000	-0.2096	0.1111 ± 0.0000	-0.2103
3.0	180	0.1173 ± 0.0004	-0.1515	0.1182 ± 0.0000	-0.1484	6.0	180	0.1083 ± 0.0007	-0.2335	0.1088 ± 0.0000	-0.2318
	240	0.1132 ± 0.0004	-0.1868	0.1135 ± 0.0000	-0.1892		240	0.1031 ± 0.0009	-0.2830	0.1029 ± 0.0012	-0.2877
	300	0.1103 ± 0.0000	-0.2128	0.1111 ± 0.0000	-0.2103		300	0.0987 ± 0.0000	-0.3262	0.0993 ± 0.0000	-0.3228
	360	0.1071 ± 0.0004	-0.2422	0.1080 ± 0.0011	-0.2391		360	0.0929 ± 0.0004	-0.3869	0.0930 ± 0.0011	-0.3884
	0	0.1371 ± 0.0012	0.0000	0.1371 ± 0.0000	0.0000		0	0.1362 ± 0.0012	0.0000	0.1356 ± 0.0011	0.0000
	60	0.1234 ± 0.0004	-0.1050	0.1237 ± 0.0011	-0.1028		60	0.1140 ± 0.0004	-0.1782	0.1135 ± 0.0000	-0.1777
	120	0.1208 ± 0.0011	-0.1264	0.1214 ± 0.0011	-0.1221		120	0.1080 ± 0.0008	-0.2319	0.1072 ± 0.0011	-0.2348
4.0	180	0.1173 ± 0.0004	-0.1557	0.1174 ± 0.0011	-0.1551	7.0	180	0.1002 ± 0.0004	-0.3074	0.1001 ± 0.0011	-0.3033
	240	0.1141 ± 0.0004	-0.1833	0.1143 ± 0.0011	-0.1823		240	0.0970 ± 0.0000	-0.3398	0.0969 ± 0.0000	-0.3353
	300	0.1118 ± 0.0004	-0.2039	0.1119 ± 0.0011	-0.2032		300	0.0941 ± 0.0004	-0.3702	0.0938 ± 0.0011	-0.3684
	360	0.1086 ± 0.0004	-0.2329	0.1088 ± 0.0000	-0.2318		360	0.0879 ± 0.0009	-0.4373	0.0875 ± 0.0000	-0.4380
	0	0.1374 ± 0.0004	0.0000	0.1371 ± 0.0000	0.0000		0	0.1362 ± 0.0014	0.0000	0.1364 ± 0.0011	0.0000
	60	0.1222 ± 0.0007	-0.1166	0.1222 ± 0.0011	-0.1156		60	0.1129 ± 0.0004	-0.1872	0.1127 ± 0.0011	-0.1904
	120	0.1132 ± 0.0004	-0.1931	0.1127 ± 0.0011	-0.1962		120	0.1013 ± 0.0009	-0.2958	0.1017 ± 0.0000	-0.2935
5.0	180	0.1095 ± 0.0004	-0.2271	0.1096 ± 0.0011	-0.2246	8.0	180	0.0932 ± 0.0015	-0.3796	0.0930 ± 0.0011	-0.3826
	240	0.1052 ± 0.0004	-0.2663	0.1052 ± 0.0012	-0.2650		240	0.0856 ± 0.0009	-0.4641	0.0863 ± 0.0012	-0.4574
	300	0.1009 ± 0.0013	-0.3086	0.1005 ± 0.0012	-0.3109		300	0.0842 ± 0.0000	-0.4812	0.0836 ± 0.0011	-0.4899
	360	0.0967 ± 0.0000	-0.3513	0.0962 ± 0.0011	-0.3550		360	0.0798 ± 0.0017	-0.5344	0.0796 ± 0.0022	-0.5382

Table E.4 Concentration and ln (C/C_0) value of antioxidant in aqueous system; Degradation at 25℃ with respect of time

pH	Time	212 nm		277 nm		pH	Time	212 nm		277 nm	
		Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)			Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)
	0	0.1365 ± 0.0004	0.0000	0.1371 ± 0.0000	0.0000		0	0.1368 ± 0.0004	0.0000	0.1371 ± 0.0000	0.0000
	80	0.1272 ± 0.0008	-0.0706	0.1277 ± 0.0000	-0.0715		80	0.1246 ± 0.0011	-0.0935	0.1245 ± 0.0011	-0.0965
	160	0.1225 ± 0.0004	-0.1078	0.1230 ± 0.0000	-0.1092		160	0.1158 ± 0.0008	-0.1661	0.1167 ± 0.0011	-0.1618
3.0	240	0.1182 ± 0.0004	-0.1441	0.1190 ± 0.0011	-0.1418	6.0	240	0.1103 ± 0.0004	-0.2149	0.1111 ± 0.0000	-0.2103
	320	0.1161 ± 0.0000	-0.1614	0.1167 ± 0.0011	-0.1618		320	0.1051 ± 0.0011	-0.2635	0.1048 ± 0.0011	-0.2687
	400	0.1147 ± 0.0004	-0.1740	0.1151 ± 0.0011	-0.1754		400	0.1013 ± 0.0000	-0.3001	0.1017 ± 0.0000	-0.2992
	480	0.1112 ± 0.0004	-0.2049	0.1119 ± 0.0011	-0.2032		480	0.0996 ± 0.0000	-0.3174	0.0993 ± 0.0000	-0.3228
	0	0.1371 ± 0.0012	0.0000	0.1371 ± 0.0000	0.0000		0	0.1362 ± 0.0012	0.0000	0.1356 ± 0.0011	0.0000
	80	0.1272 ± 0.0004	-0.0748	0.1277 ± 0.0000	-0.0715		80	0.1190 ± 0.0023	-0.1346	0.1190 ± 0.0030	-0.1302
	160	0.1228 ± 0.0004	-0.1097	0.1230 ± 0.0000	-0.1092		160	0.1095 ± 0.0004	-0.2186	0.1088 ± 0.0019	-0.2202
4.0	240	0.1196 ± 0.0007	-0.1361	0.1198 ± 0.0011	-0.1352	7.0	240	0.1045 ± 0.0008	-0.2648	0.1048 ± 0.0011	-0.2571
	320	0.1170 ± 0.0007	-0.1582	0.1174 ± 0.0011	-0.1551		320	0.0990 ± 0.0011	-0.3190	0.0985 ± 0.0011	-0.3192
	400	0.1150 ± 0.0011	-0.1757	0.1151 ± 0.0011	-0.1754		400	0.0955 ± 0.0004	-0.3549	0.0954 ± 0.0011	-0.3517
	480	0.1129 ± 0.0011	-0.1936	0.1127 ± 0.0011	-0.1962		480	0.0911 ± 0.0004	-0.4016	0.0906 ± 0.0011	-0.4026
	0	0.1374 ± 0.0004	0.0000	0.1371 ± 0.0000	0.0000		0	0.1362 ± 0.0014	0.0000	0.1364 ± 0.0011	0.0000
	80	0.1263 ± 0.0011	-0.0838	0.1261 ± 0.0022	-0.0839		80	0.1167 ± 0.0015	-0.1543	0.1167 ± 0.0011	-0.1561
	160	0.1185 ± 0.0004	-0.1480	0.1182 ± 0.0000	-0.1484		160	0.1061 ± 0.0004	-0.2496	0.1064 ± 0.0000	-0.2480
5.0	240	0.1112 ± 0.0004	-0.2113	0.1111 ± 0.0000	-0.2103	8.0	240	0.1028 ± 0.0004	-0.2816	0.1025 ± 0.0011	-0.2858
	320	0.1083 ± 0.0007	-0.2377	0.1080 ± 0.0011	-0.2391		320	0.0972 ± 0.0011	-0.3368	0.0977 ± 0.0011	-0.3330
	400	0.1042 ± 0.0004	-0.2760	0.1040 ± 0.0000	-0.2763		400	0.0914 ± 0.0011	-0.3984	0.0914 ± 0.0011	-0.3997
	480	0.1016 ± 0.0004	-0.3015	0.1017 ± 0.0000	-0.2992		480	0.0862 ± 0.0004	-0.4574	0.0867 ± 0.0011	-0.4528

Table E.5 Concentration and ln (C/C_0) value of antioxidant in CHCL/AA NADES system; Degradation at 80℃ with respect of time

pH	Time	212 nm		277 nm		pH	Time	212 nm		277 nm	
		Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)			Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)
	0	0.1421 ± 0.0000	0.0000	0.1420 ± 0.0008	0.0000		0	0.1409 ± 0.0011	0.0000	0.1415 ± 0.0014	0.0000
	30	0.1241 ± 0.0004	-0.1356	0.1239 ± 0.0008	-0.1368		30	0.1117 ± 0.0004	-0.2323	0.1120 ± 0.0008	-0.2339
	60	0.1201 ± 0.0004	-0.1686	0.1199 ± 0.0008	-0.1694		60	0.1011 ± 0.0008	-0.3316	0.1012 ± 0.0008	-0.3352
3.0	90	0.1160 ± 0.0000	-0.2029	0.1159 ± 0.0000	-0.2031	6.0	90	0.0940 ± 0.0004	-0.4049	0.0938 ± 0.0000	-0.4109
	120	0.1101 ± 0.0004	-0.2551	0.1103 ± 0.0008	-0.2533		120	0.0899 ± 0.0013	-0.4488	0.0904 ± 0.0000	-0.4479
	150	0.1042 ± 0.0009	-0.3102	0.1040 ± 0.0000	-0.3115		150	0.0822 ± 0.0004	-0.5392	0.0830 ± 0.0008	-0.5330
	180	0.0996 ± 0.0004	-0.3559	0.0995 ± 0.0008	-0.3562		180	0.0775 ± 0.0012	-0.5975	0.0779 ± 0.0008	-0.5965
	0	0.1418 ± 0.0004	0.0000	0.1420 ± 0.0008	0.0000		0	0.1418 ± 0.0004	0.0000	0.1426 ± 0.0008	0.0000
	30	0.1232 ± 0.0009	-0.1409	0.1233 ± 0.0008	-0.1414		30	0.1110 ± 0.0004	-0.2445	0.1120 ± 0.0008	-0.2419
	60	0.1179 ± 0.0000	-0.1847	0.1176 ± 0.0000	-0.1885		60	0.0952 ± 0.0004	-0.3984	0.0955 ± 0.0000	-0.4009
4.0	90	0.1138 ± 0.0004	-0.2196	0.1142 ± 0.0000	-0.2179	7.0	90	0.0834 ± 0.0008	-0.5307	0.0836 ± 0.0014	-0.5342
	120	0.1073 ± 0.0012	-0.2786	0.1074 ± 0.0014	-0.2793		120	0.0791 ± 0.0004	-0.5843	0.0790 ± 0.0008	-0.5900
	150	0.1027 ± 0.0009	-0.3230	0.1029 ± 0.0008	-0.3225		150	0.0732 ± 0.0008	-0.6619	0.0734 ± 0.0000	-0.6645
	180	0.0996 ± 0.0004	-0.3537	0.0995 ± 0.0008	-0.3562		180	0.0694 ± 0.0008	-0.7142	0.0700 ± 0.0000	-0.7120
	0	0.1409 ± 0.0012	0.0000	0.1415 ± 0.0014	0.0000		0	0.1409 ± 0.0012	0.0000	0.1415 ± 0.0014	0.0000
	30	0.1163 ± 0.0009	-0.1914	0.1171 ± 0.0008	-0.1893		30	0.1070 ± 0.0004	-0.2749	0.1074 ± 0.0000	-0.2753
	60	0.1079 ± 0.0004	-0.2662	0.1080 ± 0.0008	-0.2701		60	0.0850 ± 0.0012	-0.5057	0.0853 ± 0.0014	-0.5060
5.0	90	0.1011 ± 0.0008	-0.3316	0.1012 ± 0.0008	-0.3352	8.0	90	0.0710 ± 0.0012	-0.6855	0.0717 ± 0.0014	-0.6800
	120	0.0977 ± 0.0004	-0.3660	0.0978 ± 0.0008	-0.3694		120	0.0613 ± 0.0012	-0.8313	0.0615 ± 0.0014	-0.8338
	150	0.0933 ± 0.0012	-0.4115	0.0938 ± 0.0014	-0.4109		150	0.0554 ± 0.0000	-0.9324	0.0546 ± 0.0000	-0.9512
	180	0.0890 ± 0.0008	-0.4593	0.0893 ± 0.0008	-0.4605		180	0.0527 ± 0.0008	-0.9841	0.0529 ± 0.0014	-0.9828

Table E.6 Concentration and ln (C/C_0) value of antioxidant in CHCL/AA NADES system; Degradation at 60℃ with respect of time

pH	Time	212 nm		277 nm		pH	Time	212 nm		277 nm	
		Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)			Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)
	0	0.1421 ± 0.0000	0.0000	0.1420 ± 0.0008	0.0000		0	0.1409 ± 0.0012	0.0000	0.1415 ± 0.0014	0.0000
	40	0.1250 ± 0.0004	-0.1281	0.1256 ± 0.0008	-0.1231		40	0.1182 ± 0.0004	-0.1755	0.1182 ± 0.0008	-0.1797
	80	0.1204 ± 0.0004	-0.1661	0.1205 ± 0.0008	-0.1647		80	0.1073 ± 0.00012	-0.2720	0.1074 ± 0.0014	-0.2753
3.0	120	0.1163 ± 0.0004	-0.2002	0.1165 ± 0.0008	-0.1982	6.0	120	0.1008 ± 0.0004	-0.3347	0.1006 ± 0.0000	-0.3408
	160	0.1129 ± 0.0004	-0.2300	0.1125 ± 0.0000	-0.2329		160	0.0943 ± 0.0004	-0.4016	0.0949 ± 0.0008	-0.3989
	200	0.1079 ± 0.0004	-0.2750	0.1080 ± 0.0008	-0.2741		200	0.0899 ± 0.0008	-0.4488	0.0904 ± 0.0000	-0.4479
	240	0.1055 ± 0.0004	-0.2983	0.1057 ± 0.0000	-0.2953		240	0.0840 ± 0.0012	-0.5167	0.0842 ± 0.0016	-0.5194
	0	0.1418 ± 0.0004	0.0000	0.1420 ± 0.0008	0.0000		0	0.1418 ± 0.0004	0.0000	0.1426 ± 0.0008	0.0000
	40	0.1253 ± 0.0008	-0.1234	0.1256 ± 0.0008	-0.1231		40	0.1138 ± 0.0012	-0.2196	0.1148 ± 0.0021	-0.2169
	80	0.1204 ± 0.0004	-0.1639	0.1205 ± 0.0008	-0.1647		80	0.1045 ± 0.0012	-0.3050	0.1052 ± 0.0008	-0.3047
4.0	120	0.1154 ± 0.0009	-0.2060	0.1154 ± 0.0008	-0.2080	7.0	120	0.0983 ± 0.0008	-0.3663	0.0989 ± 0.0014	-0.3659
	160	0.1117 ± 0.0009	-0.2389	0.1120 ± 0.0008	-0.2379		160	0.0927 ± 0.0009	-0.4248	0.0930 ± 0.0009	-0.4280
	200	0.1070 ± 0.0009	-0.2815	0.1069 ± 0.0008	-0.2846		200	0.0846 ± 0.0012	-0.5160	0.0853 ± 0.0014	-0.5140
	240	0.1045 ± 0.0004	-0.3050	0.1052 ± 0.0008	-0.3007		240	0.0803 ± 0.0004	-0.5687	0.0808 ± 0.0008	-0.5687
	0	0.1409 ± 0.0012	0.0000	0.1415 ± 0.0014	0.0000		0	0.1409 ± 0.0012	0.0000	0.1415 ± 0.0014	0.0000
	40	0.1204 ± 0.0008	-0.1573	0.1210 ± 0.0000	-0.1560		40	0.1114 ± 0.0008	-0.2351	0.1120 ± 0.0008	-0.2339
	80	0.1123 ± 0.0000	-0.2267	0.1125 ± 0.0000	-0.2289		80	0.0937 ± 0.0000	-0.4082	0.0938 ± 0.0000	-0.4109
5.0	120	0.1061 ± 0.0004	-0.2836	0.1063 ± 0.0008	-0.2859	8.0	120	0.0840 ± 0.0004	-0.5167	0.0842 ± 0.0008	-0.5194
	160	0.1011 ± 0.0008	-0.3316	0.1012 ± 0.0008	-0.3352		160	0.0744 ± 0.0012	-0.6384	0.0745 ± 0.0008	-0.6412
	200	0.0955 ± 0.0008	-0.3885	0.0961 ± 0.0008	-0.3870		200	0.0707 ± 0.0004	-0.6898	0.0705 ± 0.0008	-0.6960
	240	0.0937 ± 0.0009	-0.4082	0.0938 ± 0.0000	-0.4109		240	0.0673 ± 0.0004	-0.7394	0.0671 ± 0.0008	-0.7454

Table E.7 Concentration and ln (C/C_0) value of antioxidant in CHCL/AA NADES system; Degradation at 40℃ with respect of time

pH	Time	212 nm		277 nm		pH	Time	212 nm		277 nm	
		Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)			Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)
	0	0.1421 ± 0.0000	0.0000	0.1420 ± 0.0008	0.0000		0	0.1409 ± 0.0012	0.0000	0.1415 ± 0.0014	0.0000
	60	0.1303 ± 0.0009	-0.0867	0.1307 ± 0.0008	-0.0833		60	0.1241 ± 0.0004	-0.1268	0.1244 ± 0.0000	-0.1282
	120	0.1272 ± 0.0008	-0.1108	0.1273 ± 0.0008	-0.1097		120	0.1154 ± 0.0004	-0.1994	0.1159 ± 0.0000	-0.1991
3.0	180	0.1232 ± 0.0009	-0.1431	0.1233 ± 0.0008	-0.1414	6.0	180	0.1120 ± 0.0004	-0.2295	0.1125 ± 0.0000	-0.2289
	240	0.1191 ± 0.0009	-0.1764	0.1188 ± 0.0008	-0.1789		240	0.1070 ± 0.0009	-0.2749	0.1069 ± 0.0008	-0.2806
	300	0.1182 ± 0.0004	-0.1843	0.1182 ± 0.0008	-0.1837		300	0.1027 ± 0.0012	-0.3164	0.1035 ± 0.0008	-0.3130
	360	0.1157 ± 0.0004	-0.2055	0.1154 ± 0.0008	-0.2080		360	0.0986 ± 0.0004	-0.3565	0.0995 ± 0.0008	-0.3522
	0	0.1418 ± 0.0004	0.0000	0.1420 ± 0.0008	0.0000		0	0.1418 ± 0.0004	0.0000	0.1426 ± 0.0008	0.0000
	60	0.1316 ± 0.0004	-0.0750	0.1313 ± 0.0000	-0.0789		60	0.1239 ± 0.0005	-0.1346	0.1244 ± 0.0000	-0.1362
	120	0.1263 ± 0.0000	-0.1160	0.1261 ± 0.0000	-0.1186		120	0.1154 ± 0.0009	-0.2060	0.1159 ± 0.0000	-0.2071
4.0	180	0.1241 ± 0.0004	-0.1334	0.1244 ± 0.0000	-0.1322	7.0	180	0.1110 ± 0.0004	-0.2445	0.1120 ± 0.0008	-0.2419
	240	0.1207 ± 0.0008	-0.1613	0.1205 ± 0.0008	-0.1647		240	0.1058 ± 0.0008	-0.2932	0.1063 ± 0.0008	-0.2939
	300	0.1176 ± 0.0004	-0.1874	0.1176 ± 0.0000	-0.1885		300	0.1017 ± 0.0004	-0.3321	0.1017 ± 0.0008	-0.3376
	360	0.1160 ± 0.0000	-0.2007	0.1159 ± 0.0000	-0.2031		360	0.0958 ± 0.0004	-0.3919	0.0961 ± 0.0008	-0.3950
	0	0.1409 ± 0.0012	0.0000	0.1415 ± 0.0014	0.0000		0	0.1409 ± 0.0012	0.0000	0.1415 ± 0.0014	0.0000
	60	0.1256 ± 0.0004	-0.1143	0.1261 ± 0.0000	-0.1146		60	0.1225 ± 0.0000	-0.1394	0.1227 ± 0.0000	-0.1420
	120	0.1170 ± 0.0008	-0.1861	0.1171 ± 0.0008	-0.1893		120	0.1073 ± 0.0009	-0.2720	0.1080 ± 0.0008	-0.2701
5.0	180	0.1132 ± 0.0008	-0.2185	0.1137 ± 0.0008	-0.2188	8.0	180	0.0988 ± 0.0005	-0.3549	0.0989 ± 0.0000	-0.3579
	240	0.1089 ± 0.0012	-0.2576	0.1091 ± 0.0014	-0.2596		240	0.0927 ± 0.0008	-0.4182	0.0927 ± 0.0008	-0.4231
	300	0.1051 ± 0.0009	-0.2925	0.1052 ± 0.0008	-0.2967		300	0.0895 ± 0.0005	-0.4540	0.0895 ± 0.0009	-0.4573
	360	0.1020 ± 0.0008	-0.3225	0.1017 ± 0.0008	-0.3296		360	0.0868 ± 0.0004	-0.4840	0.0870 ± 0.0000	-0.4863

Table E.8 Concentration and ln (C/C_0) value of antioxidant in CHCL/AA NADES system; Degradation at 25℃ with respect of time

pH	Time	212 nm		277 nm		pH	Time	212 nm		277 nm	
		Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)			Concentration (mg/ml)	ln (C/C_0)	Concentration (mg/ml)	ln (C/C_0)
	0	0.1421 ± 0.0000	0.0000	0.1420 ± 0.0008	0.0000		0	0.1409 ± 0.0012	0.0000	0.1415 ± 0.0014	0.0000
	80	0.1328 ± 0.0008	-0.0678	0.1330 ± 0.0000	-0.0661		80	0.1284 ± 0.0009	-0.0923	0.1290 ± 0.0008	-0.0924
	160	0.1284 ± 0.0004	-0.1011	0.1284 ± 0.0008	-0.1008		160	0.1207 ± 0.0008	-0.1547	0.1210 ± 0.0000	-0.1560
3.0	240	0.1263 ± 0.0008	-0.1182	0.1261 ± 0.0000	-0.1186	6.0	240	0.1160 ± 0.0000	-0.1941	0.1159 ± 0.0000	-0.1991
	320	0.1241 ± 0.0004	-0.1356	0.1239 ± 0.0008	-0.1368		320	0.1109 ± 0.0005	-0.2393	0.1117 ± 0.0009	-0.2365
	400	0.1210 ± 0.0004	-0.1609	0.1210 ± 0.0000	-0.1600		400	0.1076 ± 0.0008	-0.2691	0.1080 ± 0.0008	-0.2701
	480	0.1182 ± 0.0004	-0.1843	0.1182 ± 0.0008	-0.1837		480	0.1045 ± 0.0009	-0.2984	0.1046 ± 0.0008	-0.3021
	0	0.1418 ± 0.0004	0.0000	0.1420 ± 0.0008	0.0000		0	0.1418 ± 0.0004	0.0000	0.1426 ± 0.0008	0.0000
	80	0.1322 ± 0.0009	-0.0703	0.1324 ± 0.0008	-0.0703		80	0.1281 ± 0.0009	-0.1014	0.1287 ± 0.0009	-0.1026
	160	0.1281 ± 0.0008	-0.1014	0.1284 ± 0.0008	-0.1008		160	0.1201 ± 0.0012	-0.1665	0.1205 ± 0.0008	-0.1686
4.0	240	0.1260 ± 0.0009	-0.1185	0.1261 ± 0.0000	-0.1186	7.0	240	0.1135 ± 0.0012	-0.2223	0.1142 ± 0.0014	-0.2219
	320	0.1229 ± 0.0004	-0.1434	0.1227 ± 0.0000	-0.1460		320	0.1100 ± 0.0005	-0.2543	0.1108 ± 0.0000	-0.2521
	400	0.1201 ± 0.0004	-0.1665	0.1205 ± 0.0008	-0.1647		400	0.1061 ± 0.0004	-0.2902	0.1063 ± 0.0008	-0.2939
	480	0.1179 ± 0.0000	-0.1847	0.1176 ± 0.0000	-0.1885		480	0.1017 ± 0.0004	-0.3321	0.1017 ± 0.0008	-0.3376
	0	0.1409 ± 0.0012	0.0000	0.1415 ± 0.0014	0.0000		0	0.1409 ± 0.0012	0.0000	0.1415 ± 0.0014	0.0000
	80	0.1300 ± 0.0000	-0.0803	0.1296 ± 0.0000	-0.0880		80	0.1239 ± 0.0005	-0.1280	0.1244 ± 0.0000	-0.1282
	160	0.1219 ± 0.0012	-0.1445	0.1222 ± 0.0008	-0.1466		160	0.1148 ± 0.0004	-0.2048	0.1148 ± 0.0008	-0.2089
5.0	240	0.1188 ± 0.0008	-0.1703	0.1188 ± 0.0008	-0.1749	8.0	240	0.1086 ± 0.0008	-0.2605	0.1086 ± 0.0008	-0.2648
	320	0.1126 ± 0.0009	-0.2240	0.1131 ± 0.0008	-0.2238		320	0.1030 ± 0.0000	-0.3134	0.1032 ± 0.0009	-0.3157
	400	0.1090 ± 0.0005	-0.2562	0.1091 ± 0.0000	-0.2596		400	0.0971 ± 0.0012	-0.3724	0.0972 ± 0.0014	-0.3752
	480	0.1086 ± 0.0009	-0.2605	0.1091 ± 0.0000	-0.2596		480	0.0933 ± 0.0004	-0.4115	0.0938 ± 0.0000	-0.4109

Appendix F

Table F.1 Estimation of coefficient of determination (R^2) from the plot of zero and first order reactions: Degradation of antioxidants in aqueous system at wavelength 277 nm (n=3)

pH	25℃				40℃				60℃				80℃			
	Zero order	RMSE	First order	RMSE	Zero order	RMSE	First order	RMSE	Zero order	RMSE	First order	RMSE	Zero order	RMSE	First order	RMSE
3	0.9172	0.022	0.9324	0.017	0.9032	0.022	0.9241	0.022	0.9136	0.026	0.9380	0.025	0.9405	0.024	0.9613	0.023
4	0.9045	0.020	0.9212	0.017	0.8863	0.021	0.9084	0.022	0.8875	0.028	0.9167	0.028	0.9176	0.029	0.9459	0.028
5	0.9384	0.023	0.9565	0.022	0.9191	0.028	0.9455	0.026	0.8924	0.037	0.9286	0.037	0.8637	0.044	0.9094	0.046
6	0.9320	0.026	0.9522	0.025	0.9032	0.031	0.9342	0.031	0.8716	0.047	0.9214	0.048	0.8132	0.072	0.8958	0.077
7	0.9131	0.032	0.9440	0.030	0.8757	0.040	0.9151	0.040	0.8670	0.052	0.9253	0.052	0.8228	0.074	0.9098	0.078
8	0.9139	0.035	0.9481	0.035	0.8801	0.047	0.9231	0.047	0.8315	0.082	0.9289	0.083	0.7584	0.0115	0.9015	0.132

Table F.2 Estimation of coefficient of determination (R2) from the plot of zero and first order reactions: Degradation of antioxidants in CHCL/AA NADES system at wavelength 277 nm (n=3)

pH	25℃				40℃				60℃				80℃			
	Zero order	RMSE	First order	RMSE	Zero order	RMSE	First order	RMSE	Zero order	RMSE	First order	RMSE	Zero order	RMSE	First order	RMSE
3	0.9235	0.015	0.9376	0.019	0.8949	0.019	0.9127	0.020	0.8910	0.027	0.9185	0.026	0.9322	0.024	0.9552	0.024
4	0.9232	0.016	0.9378	0.019	0.9134	0.019	0.9298	0.018	0.9048	0.027	0.9311	0.027	0.9126	0.028	0.9409	0.028
5	0.9269	0.025	0.9429	0.025	0.9035	0.027	0.9303	0.029	0.8949	0.035	0.9298	0.035	0.8589	0.044	0.9040	0.045
6	0.9367	0.021	0.9566	0.020	0.9104	0.031	0.9391	0.029	0.9087	0.027	0.9488	0.038	0.8740	0.051	0.9284	0.052
7	0.9353	0.028	0.9572	0.024	0.9204	0.028	0.9492	0.027	0.8905	0.022	0.9394	0.044	0.8642	0.062	0.9244	0.066
8	0.9392	0.030	0.9656	0.027	0.8932	0.043	0.9274	0.045	0.8776	0.018	0.9348	0.063	0.8804	0.073	0.9496	0.076

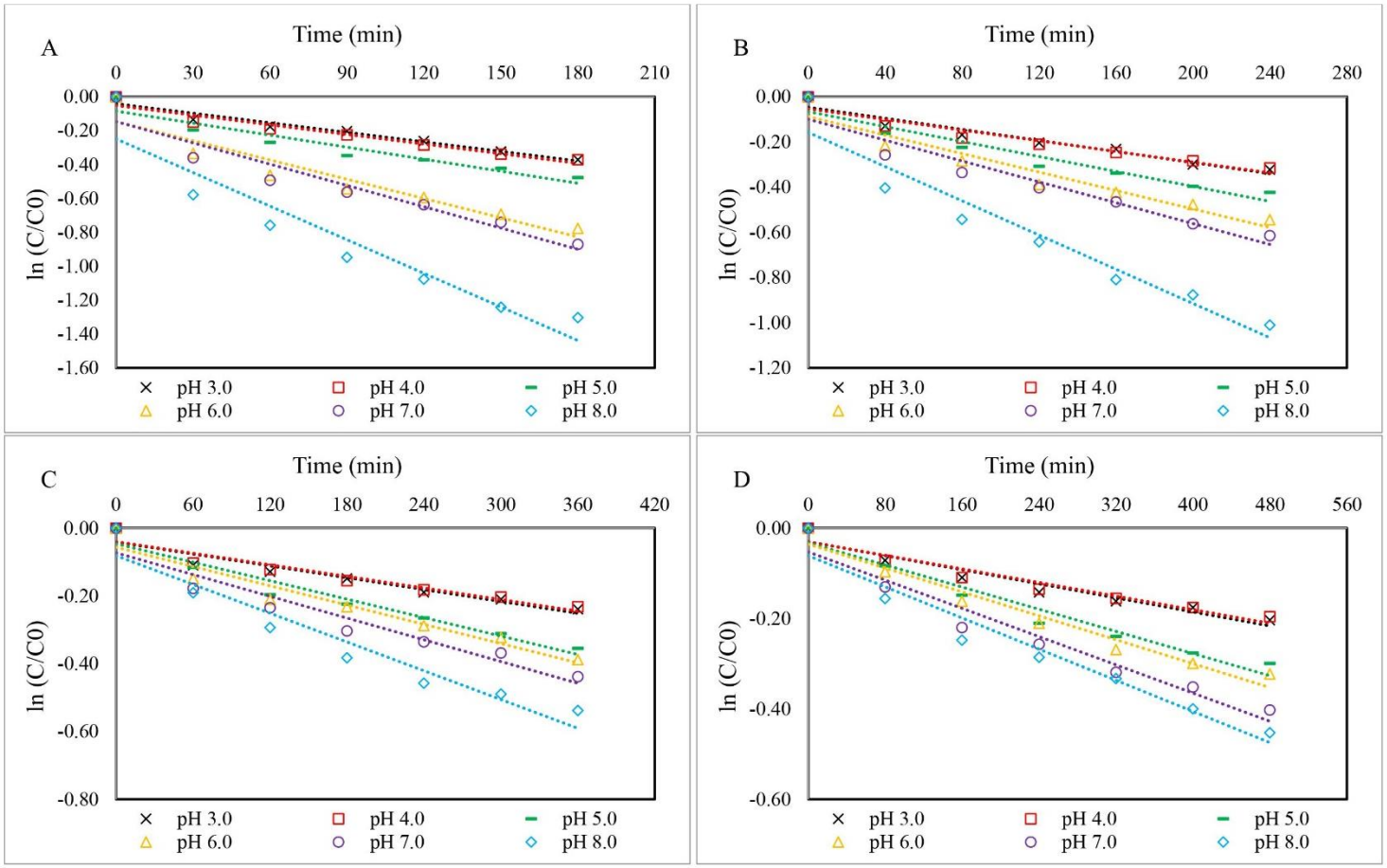


Figure F.1 ln (C/C_0) value of antioxidants in aqueous system at wavelength 277 nm;

Degradation at A) 80℃ ,B) 60℃, C) 40℃ and D) 25℃

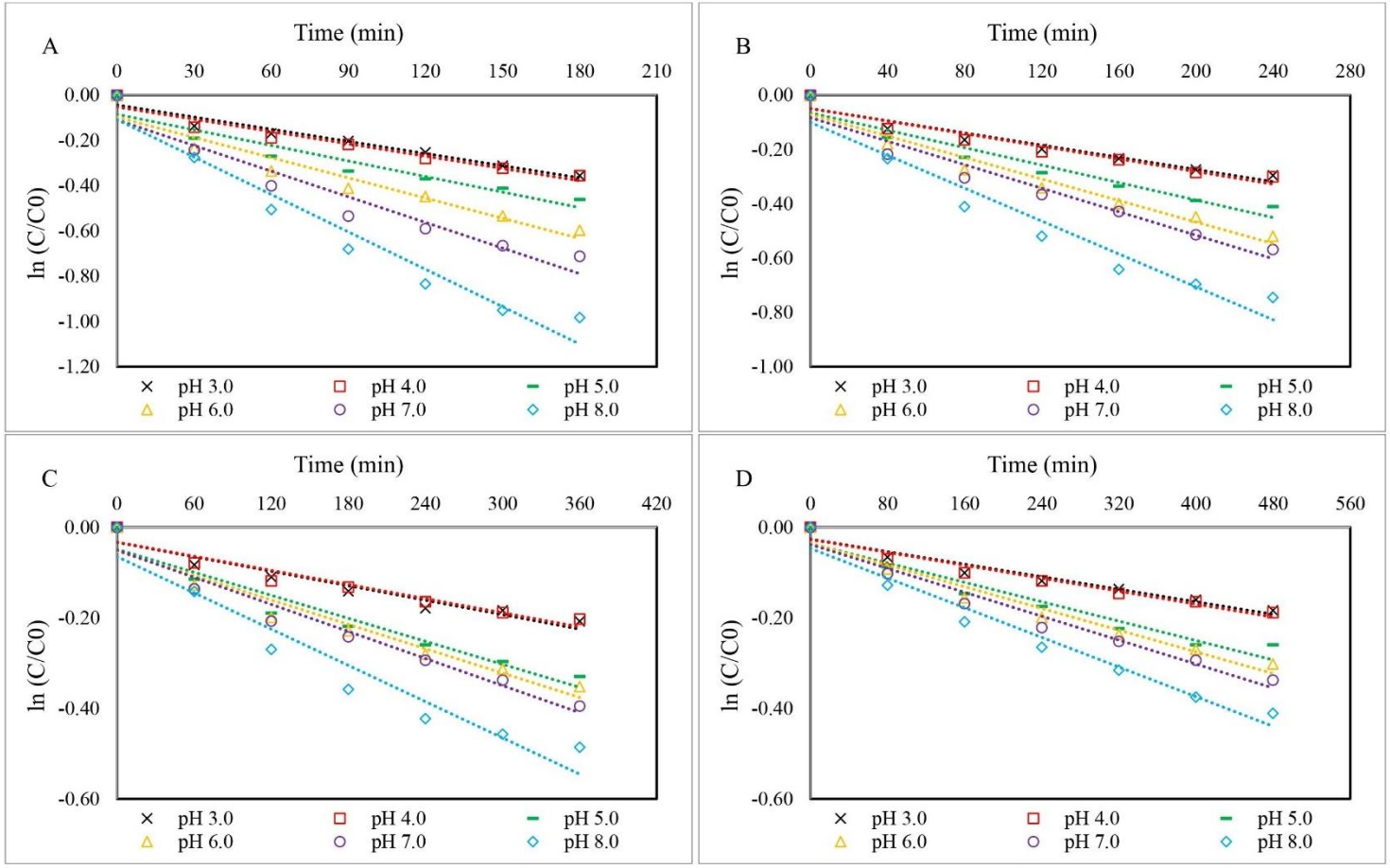


Figure F.2 ln (C/C_0) value of antioxidants in CHCL/AA NADES system at wavelength 277 nm; Degradation at A) 80℃ ,B) 60℃, C) 40℃ and D) 25℃

Appendix G

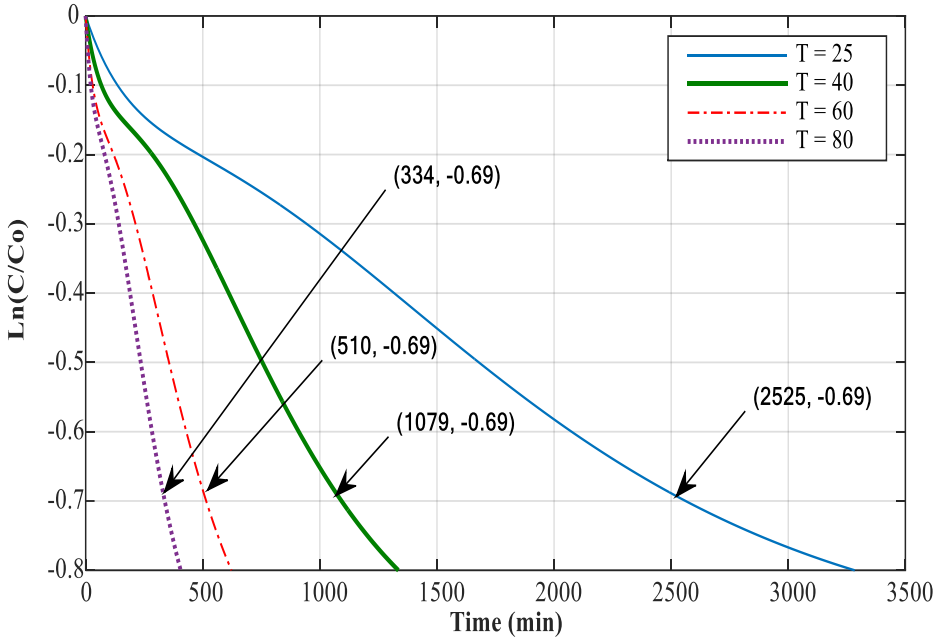


Figure G.1 Degradation profiles and half-life time under different exposure temperature (°C): Degradation of antioxidant in aqueous system at pH 3.0

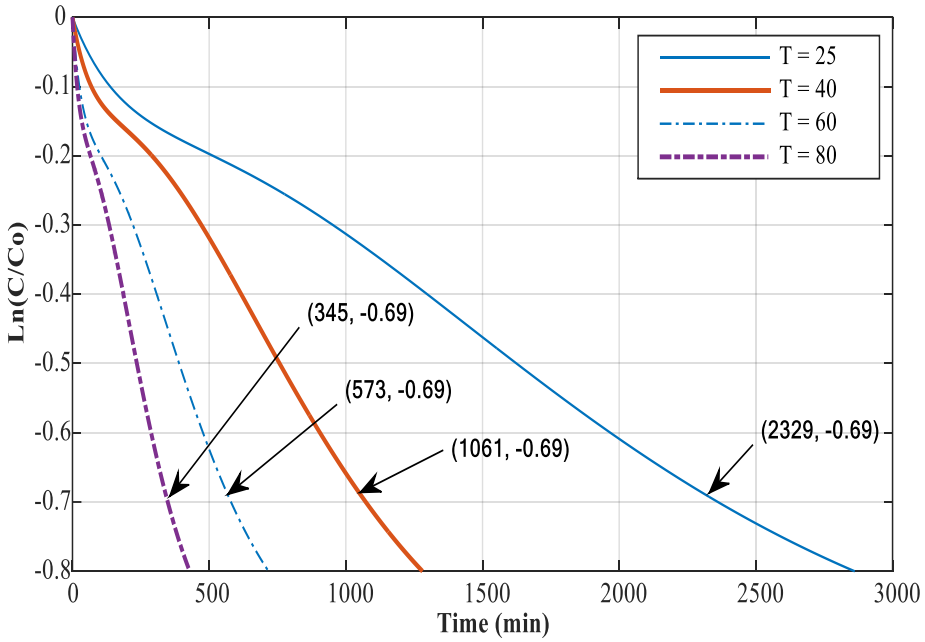


Figure G.2 Degradation profiles and half-life time under different exposure temperature (°C): Degradation of antioxidant in aqueous system at pH 4.0

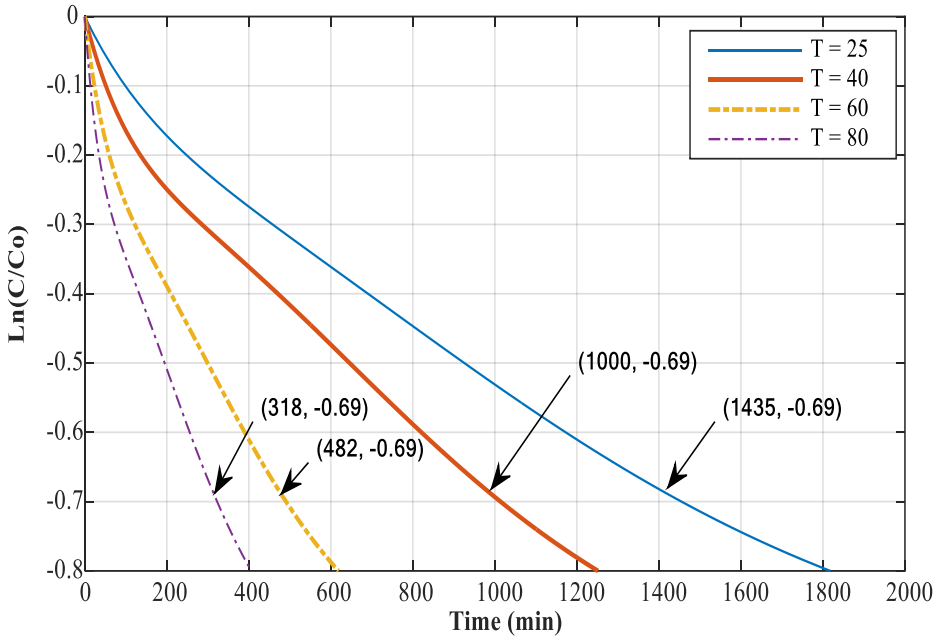


Figure G.3 Degradation profiles and half-life time under different exposure temperature (°C): Degradation of antioxidant in aqueous system at pH 5.0

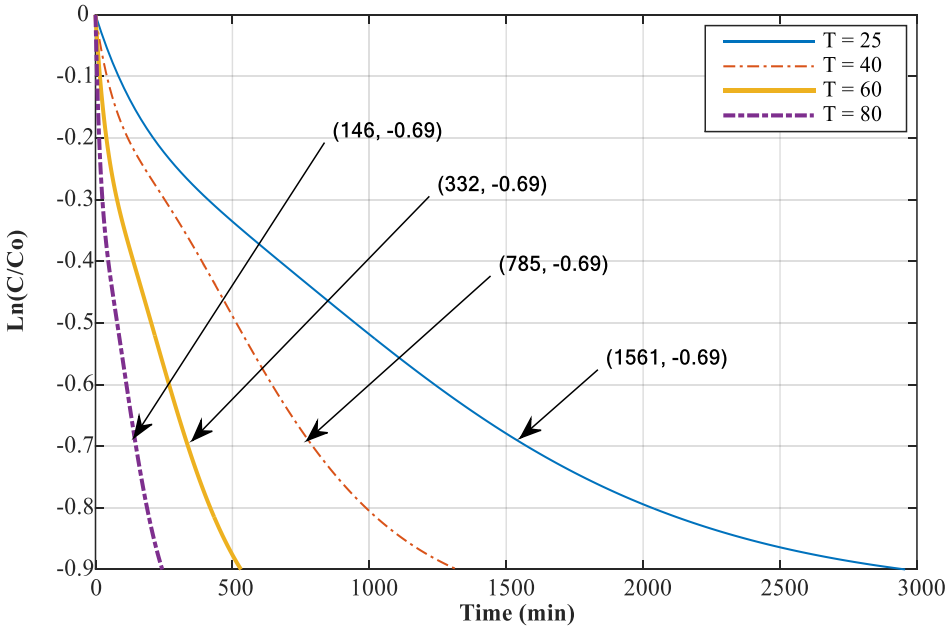


Figure G.4 Degradation profiles and half-life time under different exposure temperature (°C): Degradation of antioxidant in aqueous system at pH 6.0

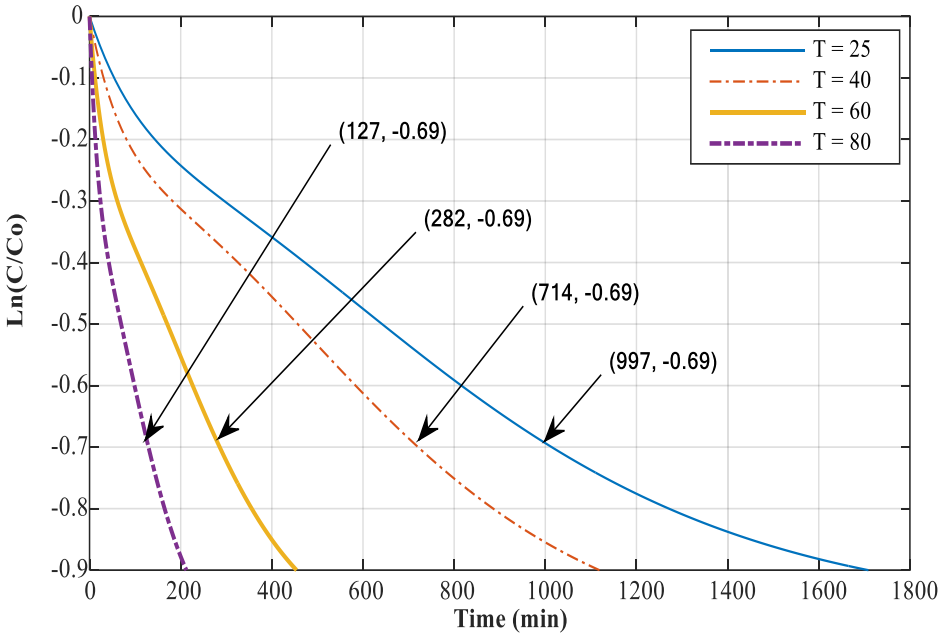


Figure G.5 Degradation profiles and half-life time under different exposure temperature (°C): Degradation of antioxidant in aqueous system at pH 7.0

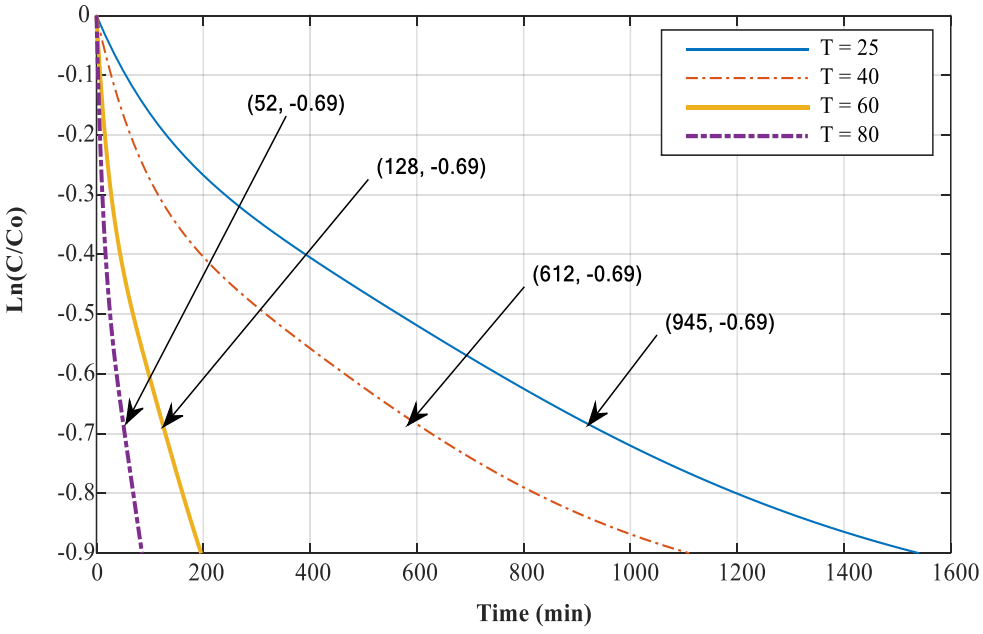
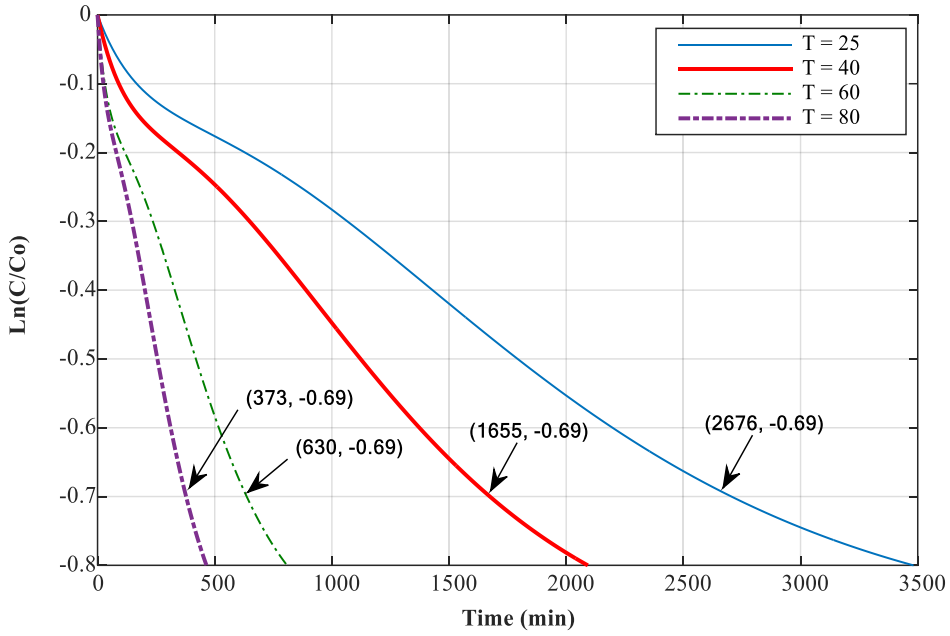


Figure G.6 Degradation profiles and half-life time under different exposure temperature (°C): Degradation of antioxidant in aqueous system at pH 8.0



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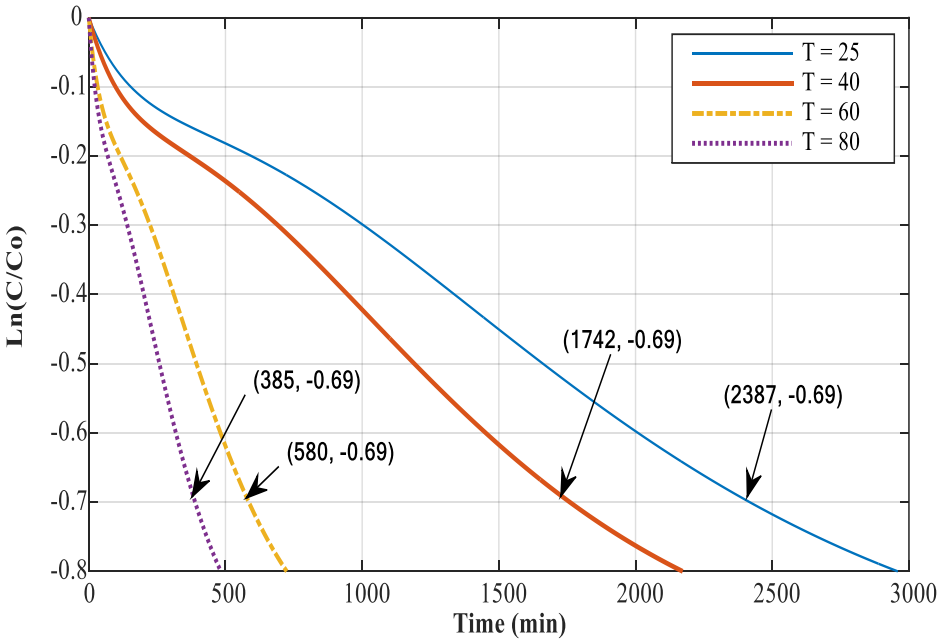

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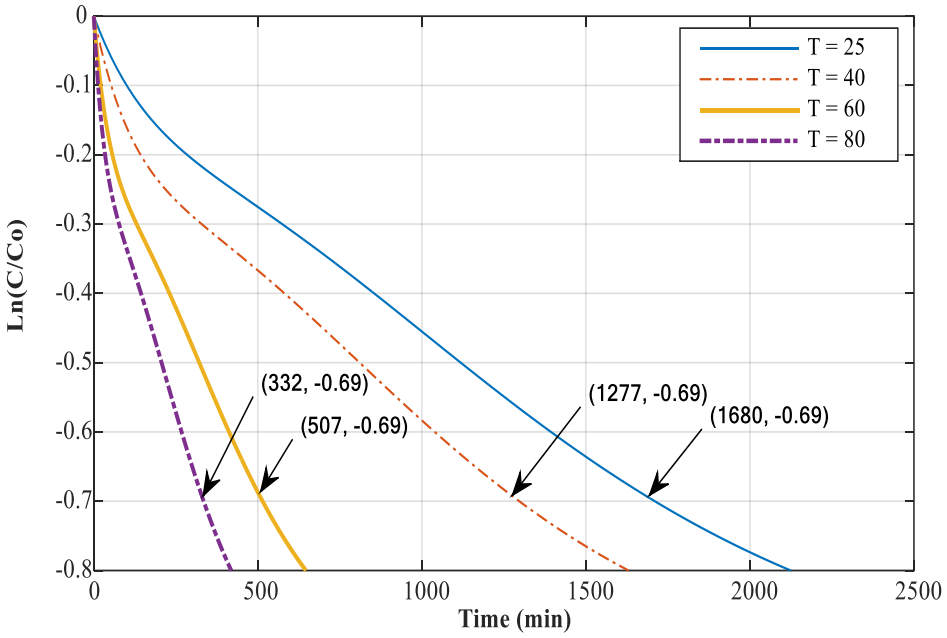

Figure G.9 Degradation profiles and half-life time under different exposure temperature (°C): Degradation of antioxidant in CHCL/AA NADES system at pH 5.0

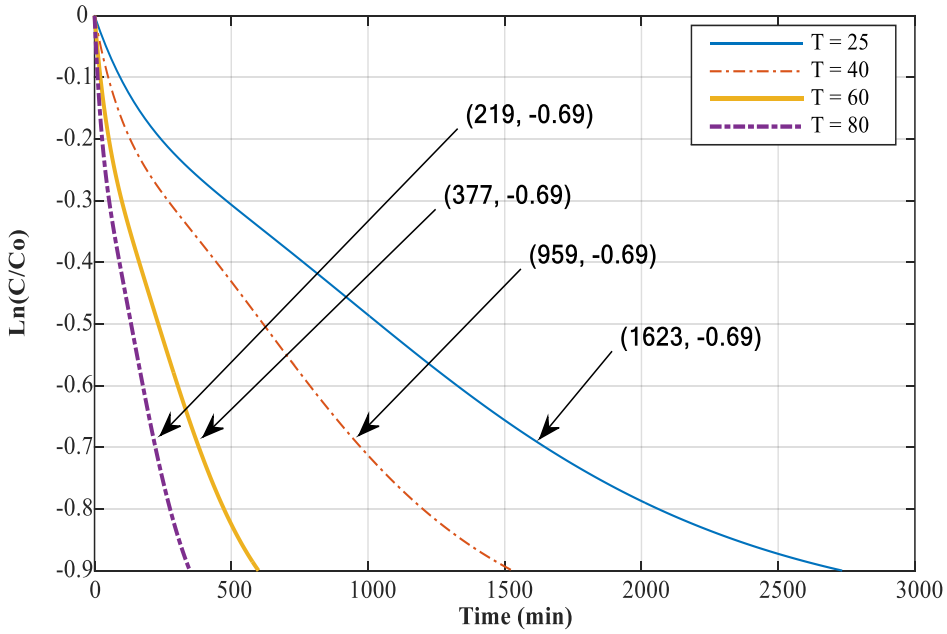

Figure G.10 Degradation profiles and half-life time under different exposure temperature (°C): Degradation of antioxidant in CHCL/AA NADES system at pH 6.0

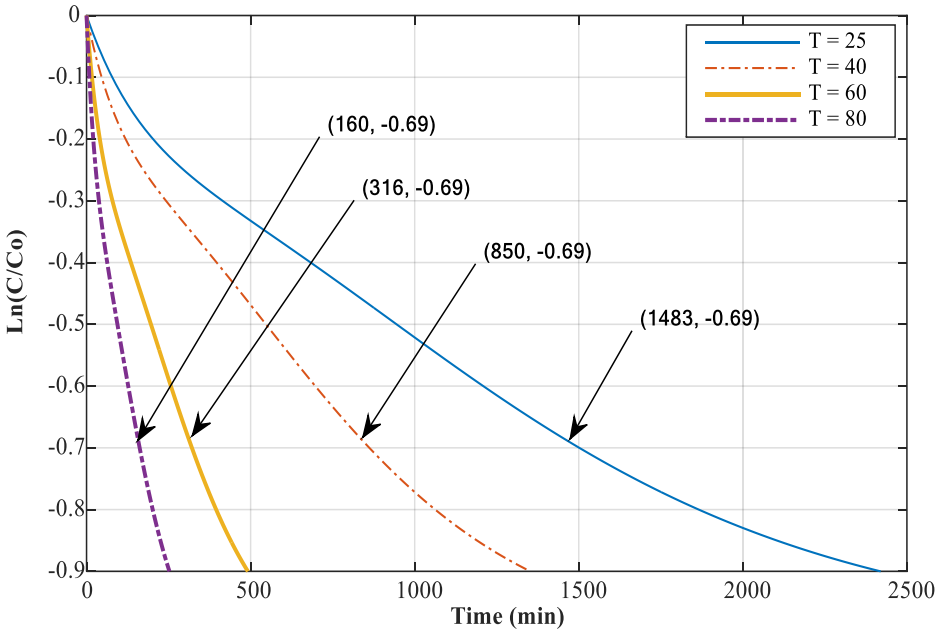

Figure G.11 Degradation profiles and half-life time under different exposure temperature (°C): Degradation of antioxidant in CHCL/AA NADES system at pH 7.0

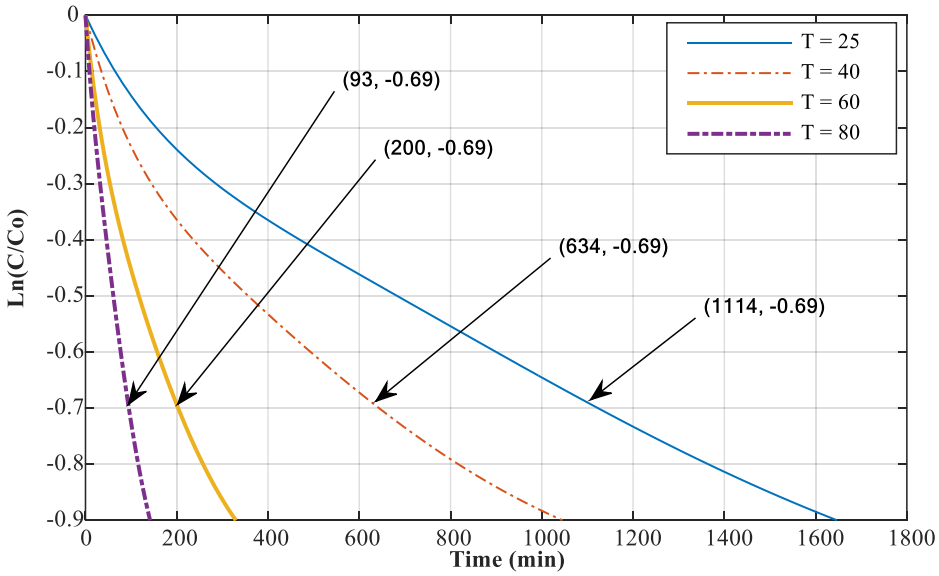

Figure G.12 Degradation profiles and half-life time under different exposure temperature (°C): Degradation of antioxidant in CHCL/AA NADES system at pH 8.0

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