THIN-LAYER CHROMATOGRAPHY FOR DETERMINATION OF PHENOLIC COMPOUND PROFILE AND ANTIOXIDANT ACTIVITY OF THAI HONEY

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ทินเลเยอร์โครมาโตกราฟีสำหรับการหาโพรไฟล์ของสารประกอบฟีนอลและฤทธิ์ต้านอนุมูลอิสระของน้ำผึ้งไทย

น.ส.ภัทรภร ชอบประดิถ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ภัทรภร ชอบประดิถ : ทินเลเยอร์โครมาโตกราฟีสำหรับการหาโพรไฟล์ของสารประกอบฟีนอลและฤทธิ์ต้านอนุมูลอิสระของน้ำผึ้งไทย. (THIN-LAYER CHROMATOGRAPHY FOR DETERMINATION OF PHENOLIC COMPOUND PROFILE AND ANTIOXIDANT ACTIVITY OF THAI HONEY) อ.ที่ปรึกษาหลัก : รศ. ดร.ธรรมนูญ หนูจักร, อ.ที่ปรึกษาร่วม : ผศ. ดร.ชฎิล กุลสิงห์

ในงานวิจัยนี้เป็นการพัฒนาวิธีการวิเคราะห์สเกลขนาดเล็กโดยใช้ทินเลเยอร์โครมาโตกราฟี (TLC) เพื่อหารูปแบบและค่าการต้านอนุมูลอิสระของตัวอย่างน้ำผึ้งไทยที่ได้จากดอกไม้ต่างชนิดกัน ได้เตรียมตัวอย่างโดยการสกัดด้วยไดคลอโรมีเทนก่อนการวิเคราะห์ด้วย TLC จากการใช้อนุมูลอิสระ 2, 2-diphenyl-1-picrylhydrazy (DPPH•) ทำปฏิกิริยากับส่วนสกัดตัวอย่าง ก่อนการหยดลงบนแผ่น TLC และการตรวจวัดสีด้วยโปรแกรม imageJ พบว่าค่าความเข้มข้นที่ยับยั้งอนุมูลอิสระได้ 50% (IC_{50}) ของน้ำผึ้งทั้ง 7 ชนิด อยู่ในช่วง 9 ถึง 22 mg/mL ตามลำดับ เทียบกับ *L*-ascorbic acid (LA) ที่มีค่า IC_{50} เท่ากับ 0.13 mg/mL และด้วยค่าขีดจำกัดของการตรวจวัดและค่าขีดจำกัดการวัดเชิงปริมาณเท่ากับ 0.010 และ 0.030 mg/mL ตามลำดับ โดยใช้กราฟสอบเทียบเชิงเส้นตรงระหว่างเปอร์เซ็นการยับยั้งและความเข้มข้นของ LA ทั้งนี้วิธี TLC-DPPH• และวิธี UV-Vis สำหรับการหาเปอร์เซ็นการยับยั้งอนุมูลอิสระ ให้ความสัมพันธ์เป็นเส้นตรง y = 2.2073x - 76.864 ด้วย R^2 ที่ 0.98 สำหรับ LA และ y = 4.0683x - 135.22 ด้วย R^2 ที่ 0.95 สำหรับตัวอย่างส่วนสกัดน้ำผึ้งดอกลำไย โดยที่ตัวแปร x และ y เป็นค่าเปอร์เซ็นการยับยั้งที่ได้จากวิธี UV-Vis และ วิธี TLC-DPPH• ตามลำดับ สำหรับการหาปริมาณสารประกอบฟีนอลทั้งหมดด้วย TLC โดยใช้ปฏิกิริยาระหว่าง Folin–Ciocâlteu (FCR) กับgallic acid (GA) หรือตัวอย่างส่วนสกัดน้ำผึ้งก่อนหยดลงบนแผ่น TLC และตรวจวัดสีด้วยโปรแกรม imageJ ได้กราฟสอบเทียบเชิงเส้นตรง y = 0.2024x + 4.2946 ด้วย R^2 ที่ 0.96 ระหว่างค่าสี (y) และความเข้มข้น (x) ของ GA และด้วยค่าขีดจำกัดของการตรวจวัดและค่าขีดจำกัดการวัดเชิงปริมาณเท่ากับ 0.02 และ 0.05 mg/mL ตามลำดับ นอกจากนี้พบว่าปริมาณสารประกอบฟีนอลทั้งหมดในตัวอย่างส่วนสกัดน้ำผึ้งทั้ง 7 ชนิด อยู่ในช่วง 1 ถึง 14 mg GA/g extract

สาขาวิชา เคมี ลายมือชื่อนิสิต .....

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In this study, a small-scale method using thin-layer chromatography (TLC) was developed to determine profile and antioxidant activity value of Thai honey samples obtained from different flora sources. Prior to TLC analysis, the samples were prepared by solvent extraction with dichloromethane. Using 2, 2-diphenyl-1-picrylhydrazy radical (DPPH•) reacted with the samples extract prior to TLC spot and color detection with imageJ, half-maximal inhibitory concentration (IC_{50}) values of seven honey samples was obtained in a range of 9 to 22 mg/mL in comparison with IC_{50} values of 0.13 mg/mL for L-ascorbic acid (LA), along with limit of detection (LOD) and limit of quantitation (LOQ) for antioxidant activity of 0.010 and 0.030 mg/mL, respectively, using a linear calibration plot between %inhibition and concentration of LA. Note that TLC-DPPH• and UV-Vis methods for %inhibition determination give linear correlations of y = 2.2073x - 76.864 with R^2 of 0.98 for LA and y = 4.0683x - 135.22 with R^2 of 0.95 for Longan honey sample where x and y are the %inhibition obtained from UV-Vis and TLC-DPPH•, respectively. For TLC determination of total phenolic compounds using Folin–Ciocâlteu (FCR) reagent reacted with gallic acid (GA) or samples extract prior to TLC spot and color detection with imageJ, a linear calibration plot of y = 0.2024x + 4.2946 with R^2 of 0.96 for GA were obtained between the color value (y) and GA concentration (x), along with LOD and LOQ for total phenolic compounds of 0.016 and 0.049 mg/mL, respectively. In addition, the total phenolic compounds of seven honey samples were found in a range of 1 to 14 mg GA/g extract.

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Student's Signature .....
Advisor's Signature .....
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Lastly, one day you will thank yourself for not giving up, and today I would like to thank myself for not giving up, having the patience and strength to get through every problem that made me today.

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LIST OF ABBREVIATUINS

%	percentage
µm	microliter
AUC	area under curve
C18	octadecylsilane
ACN	acetronitile
CH_2Cl_2	dichloromethane
CH_3COOH	acetic acid
cm	centrimeter
DPPH•	2, 2-diphenyl-2-picrylhydrazyl radical
EtOH	ethanol
FCR	Folin–Ciocâlteu
g	gram
GA	gallic acid
h	hour
HPLC	high-performance liquid chromatography
IC_{50}	half maximal inhibitory concentration
IUPAC	International Union of Pure and Applied Chemistry
LA	*L*-ascorbic acid
LLE	liquid-liquid extraction
LOD	limit od detection
LOQ	limit of quantitation

MeOH	methanol
mg	milligram
mg/mL	milligram per milliliter
$MgSO_4$	magnesium sulfate
min	minute
N_2	nitrogen
Na_2CO_3	sodium carbonate
nm	nanometer
°C	degree celsius
R^2	correlation coefficient
R_f	retention factor
R_t	retention time
RSD	relative standard deviation
S.D.	standard deviation
TLC	thin-layer chromatography
UV-Vis	Ultraviolet-Visible
v/v/v	volume per volume per volume
v/v	volume per volume

CHARPTER I

INTRODUCTION

1.1. Problem Definition

Honey bees and plants have mutual benefits resulting in the characteristic types of the honey. Honey is a source of energy containing a wide range of chemical compounds such as fructose, glucose, gallic acid, cinnamic acid, ferulic acid, kaempferol, etc. that provide positive health effects (White, 1996). The unique chemical and biochemical compounds of each honey depend on nectar and pollen of the flower (Jennifer, 2013). The main components of honey include sugar and water, with lower contents of organic acids, enzymes, amino acids, proteins, minerals, elements, aroma compounds, and phenolic compounds. Phenolic compounds in honey are transferred to honey via the different parts of plants such as nectar or pollen, and they were mostly studied in the biological, chemical, agricultural, and medical aspects (Erejuwa et al., 2012; Santos-Buelga, 2017). They have also been used as chemotaxonomic markers in plant systems and provided various pharmacological benefits such as antioxidant, antidiabetic, antibacterial, anticancer, anti-inflammatory, gastroprotective, cardioprotective, and wound healing properties (Baruah, 2011; Pasupuleti et al., 2017). On the other hand, the honey has been established as a potential therapeutic antioxidant agent for various biodiverse ailments (Ahmed et al., 2018).

High-performance liquid chromatography (HPLC) is one of the powerful analytical methods typically used for honey characterization of different floral origins. However, HPLC requires specialists to operate, costly instrument and high solvent consumption. Therefore, thin-layer chromatography (TLC) is an alternative method for authentication of honey samples because it provides simpler operation, faster analysis time and lower cost of chemicals and equipment. In addition, another advantage of TLC over HPLC is that TLC can be used for determination of both phenolic compounds and antioxidant activity by reacting with Folin–Ciocâlteu

reagents (FCR) and 2, 2-diphenyl-1-picrylhydrazy radical (DPPH•), respectively. Therefore, it is interesting in this work to develop small-scale method using TLC for determination of profile and antioxidant activity of phenolic compounds in Thai honey from different flora sources.

1.2. Literature Review

TLC can be applied to separate and identify phenolic and other chemical components in honey samples with different botanical origins by comparing the characteristic retention factors (R_f) after TLC separation. The results are normally obtained under UV light at wavelengths of 254 and 366 nm or can be observed by naked eyes after derivatization of phenolic compounds with different reagents. These processes result in spot/band characteristics of each compound. Phytochemical compositions of honey are varied with different geographic origins, time of harvest, processing approaches, storage conditions, collecting bee species, and potential interactions between different constituents and enzymes.

In previous work on TLC analysis of honey, the following separation mechanism approaches were reported, C_{18}-reverse phase TLC (RP-TLC) for determination of flavonoids in sunflower honeys [4], silica gel-normal phase high-performance TLC (NP-HPTLC) for fingerprint and identification of typical bands of non-sugar honey constituents of different floral origins (Locher et al., 2017), and NP-HPTLC for determination of polyphenols for differentiation of various honey and detection of the adulterations (Stanek & Jasicka-Misiak, 2018).

Prior to TLC or HPTLC analysis, sample preparation is required to extract target compounds and remove non-target compounds that may interfere with detection of target compounds. For fingerprint and identification of typical bands of non-sugar honey constituents (Locher et al., 2017), direct solvent extraction was reported using dichloromethane to extract and concentrate the target analyte amounts with the polar species (sugar) separated out of the honey into the aqueous phase. For the work on identification of flavonoids from sunflower honey (Sabatier,

1992), alkaline hydrolysis and solvent extraction was used by the following steps: (i) alkaline hydrolysis with a NaOH solution, under nitrogen gas, adjusted to pH 2 with HCl, (ii) extraction of hydrolysates with ethyl acetate and treat the resulting solution with a 0.5 M $NaHCO_3$ solution, and (iii) separation of ethyl acetate extract and evaporation of ethyl acetate to obtain residual flavonoids prior to TLC analysis. In addition, solid-phase extraction (SPE) with Amberlite XAD-2 polymeric adsorbent (Stanek & Jasicka-Misiak, 2018) was reported for sample preparation of honey to determine polyphenols using (i) conditioning an SPE column with methanol and acidified water, (ii) loading the honey sample diluted with acidified water, (iii) removing all sugar and other polar compounds in the honey sample with acidified water and then neutral distilled water, and (iv) eluting polyphenol from the sorbent with methanol.

Sillica gel HPTLC separation for fingerprint and identification of typical bands of non-sugar honey constituents was performed using 6:5:1 v/v toluene:ethyl-acetate:formic acid as mobile phase (Locher et al., 2017). Then the separated compounds were detected with UV wavelength at 254 and 366 nm, and the vanillin was sprayed in order to obtain derivatized compounds with different colors for fingerprint of honey sample. The characteristic bands of Jarrah honey were observed with R_f of 0.54, 0.61, and 0.68 at 254 nm, R_f of 0.51 at 366 nm and R_f of 0.55 and 0.75 at 254 nm as the characteristic bands of Manuka honey.

RP-TLC separation for flavonoids in sunflower honey (Sabatier, 1992) was carried out using 1.0 M acetic acid solution in 60%v/v methanol as the mobile phase. After spraying the TLC plate with 5% aluminum chloride in ethanol, the flavonoid detection was obtained using UV light at a wavelength of 366 nm. Based on different R_f and colors of the TLC spots, seven flavonoids could be separated and analyzed as shown in **Table 1.1.**

Sillica gel HPTLC separation of polyphenols in honey samples was performed using 5:4:1 v/v/v chloroform: ethyl-acetate: formic acid as a mobile phase. Visualization of polyphenols was obtained under the UV light at wavelengths of 254

and 366 nm before and after spraying the TLC plate with 1% methanolic 2-aminoethyl diphenyl borate. By comparison of the band colors and R_f with that of the available standards, the following polyphenols were identified, chlorogenic acid, gallic acid, caffeic acid, ferulic acid, *p*-coumaric acid, rosmarinic acid, ellagic acid, 3,4-dihydroxybenzoic acid, abscisic acid, myricetin, and chrysin. The high performance thin-layer chromatography (HPTLC) results showed differences in the chemical compositions of the honey extracts with different origins.

Table 1. 1 Identification of sunflower honey flavonoids by RP-TLC**.**

R_f	**Spot appearance UV light (366 nm) after spaying with $AlCl_3$**	**Compound identified (class)**
0.4	Yellow	quercetin (flavonol)
0.36	green-yellow	pinobanksin (flavanonol)
0.26	Yellow	kaempferol (flavonol)
0.19	Green	pinocembrin (flavanone)
0.15	dark-yellow	chrysin (flavone)
0.12	Yellow	galangin (flavonol)
0.06	dark-yellow	tectochrysin (flavane)

In additional pervious work on detection of phenolic compounds after TLC separation and spraying with a 20% sodium carbonate solution (Dinakaran et al., 2019), direct derivatization of phenolic compounds in marketed polyherbal products with the FCR reagent on a TLC plate was reported. The blue zones of the phenolic compounds were observed on the TLC plate. Each spot concentration was determined according to calculated peak area using a photo-documentation chamber (Camag Reprostar 3). Phenolic compound profiles of polyherbal products were also correlated with the anti-diabetic properties. Thus, HPTLC fingerprints are useful for quality control of prepared formulation as shown in **Table 1.2.**

Table 1. 2 HPTLC data profiles of DBC, DMV.

Sample	R_f value of the characteristic spot	Peak area	Identified compound
DBC (Alcoholic extract)	0.54 & 0.73	7176.8 & 21693.4	Gallic acid & Kaempferol derivatives
DMV (Alcoholic extract)	0.56 & 0.74	3084.4 & 21883.6	Gallic acid & Kaempferol derivatives
DBC (Aqueous extract)	0.53	1380.8	Gallic acid derivatives
DMV (Aqueous extract)	0.55 & 0.73	7469.7 & 7461.5	Gallic acid & Kaempferol derivatives

DBC = Poly herbal tablet formulation 1

DMV = Poly herbal tablet formulation 2

Furthermore, TLC was used to investigate antioxidant activity via the reaction between the antioxidant compound and DPPH• on a TLC plate (Jesionek et al., 2015; Wang et al., 2012). Antioxidant properties of the component of plant extracts were assessed using TLC-DPPH• (Jesionek et al., 2015). R_f values of the spots obtained from the TLC-DPPH• analysis of antioxidant components in plant extracts were compared with polyphenolic standards (quercetin, apigenin, luteolin, apigetrin, cynaroside, rutin, hyperoside, chlorogenic acid, caffeic acid, and rosmarinic acid). In addition, an approach for evaluation of antioxidant activity of bamboo leaves extracts was developed using the TLC-DPPH• method combined with image processing (Wang et al., 2012). The antioxidant activity of bamboo leaves can be compared based on their total peak areas obtained from the image processing method. In addition, TLC has been used for quantitative analysis of the incubated reagents and sample mixtures loaded onto a TLC plate. The half-maximal inhibitory concentration (IC_{50}) result was then obtained according to the concentration of sample which contributes to half of the maximum valve at complete DPPH• reduction (CSC_{50}) as observed via

the increasing color intensity of yellow color. The CSC_{50} can then be approximated from the color intensity vs concentration graph at the sample concentration showing the highest $\Delta_{colour\ value}/\ \Delta_{concentration}$. (Akar et al., 2017). However, the method still applied considerably large sample volumes and long incubation period before the analysis.

Thus, this work provided the developed small-scale method to determine profile and antioxidant activity of Thai honey samples obtained from different flora sources. The experiments consist of 1) TLC separation approach for characterization of the samples and qualitative analysis of antioxidant and phenolic compounds and 2) colorimetric approach on a TLC plate for quantitative analysis of antioxidant activity and total phenolic compound content has not been reported for determination of the analytes in honey samples.

1.3. Aim and Scope

This work is aimed to develop and validate thin-layer chromatography for determination of phenolic compound profile and antioxidant activity of Thai honey from different flora sources.

The possible phenolic compounds in Thai honey from different floras and sources with solvent extraction will be identified using HPLC, and then TLC separation approach will be used for characterization of the samples and qualitative analysis of antioxidant and phenolic compounds. In addition, a developed colorimetric approach on a TLC plate will be performed for quantitative analysis of antioxidant activity and total phenolic compound content. Their antioxidant activities and phenolic content will also be investigated using the two different chemical testing methods (DPPH• and FCR reagents) on TLC plates. The results will be photographed and analyzed with Image processing software (ImageJ) in order to perform qualitative and quantitative analysis. Finally, the developed TLC based techniques will be validated with UV-Vis spectrophotometer.

CHAPTER II

THEORY

2.1 Honey

In general, honey is divided into two categories; floral and honeydew honeys. Floral honey is made mostly from flower nectar, whereas honeydew honey is made by bees after collecting aphid or other plant sap-sucking insects' secretions, which pierce plant cells, absorb plant sap, and then emit it again.

Honey is a naturally sweet product that is one of the most widespread use as an ingredient in food, cosmetics, medicinal products, and nutritionals. Honey, as well known, is a source of energy that contains macro- and microelements and several other compounds that exhibit properties favorable for human health (White, 1996). The main components of honey are show in **Table 2.1.**

Table 2. 1 Chemical composition of Honey.

Chemical composition	**Amount min-max %(w/w)**
Carbohydrates	
Monosaccharides	70-80%
Disaccharides	7-8%
Oligosaccharides	1-2.0%
Water	13–26%
Phenolic compounds	
Phenolic acids	1-4%
Flavonoids	1-2%
Proteins, vitamins, amino acid, minerals, Etc	Trace amounts

2.1.1 Phenolic compound

Phenolic compounds in honey (including volatile phenols, phenolic acids, and polyphenols) can be classified into two main families: phenolic acids and flavonoids **(Figure 2.1).** These compounds are transferred to honey via the different parts of plants such as nectar or pollen, and they were mostly studied in the biological, chemical, agricultural, and medical aspects (Erejuwa et al., 2012; Santos-Buelga, 2017).

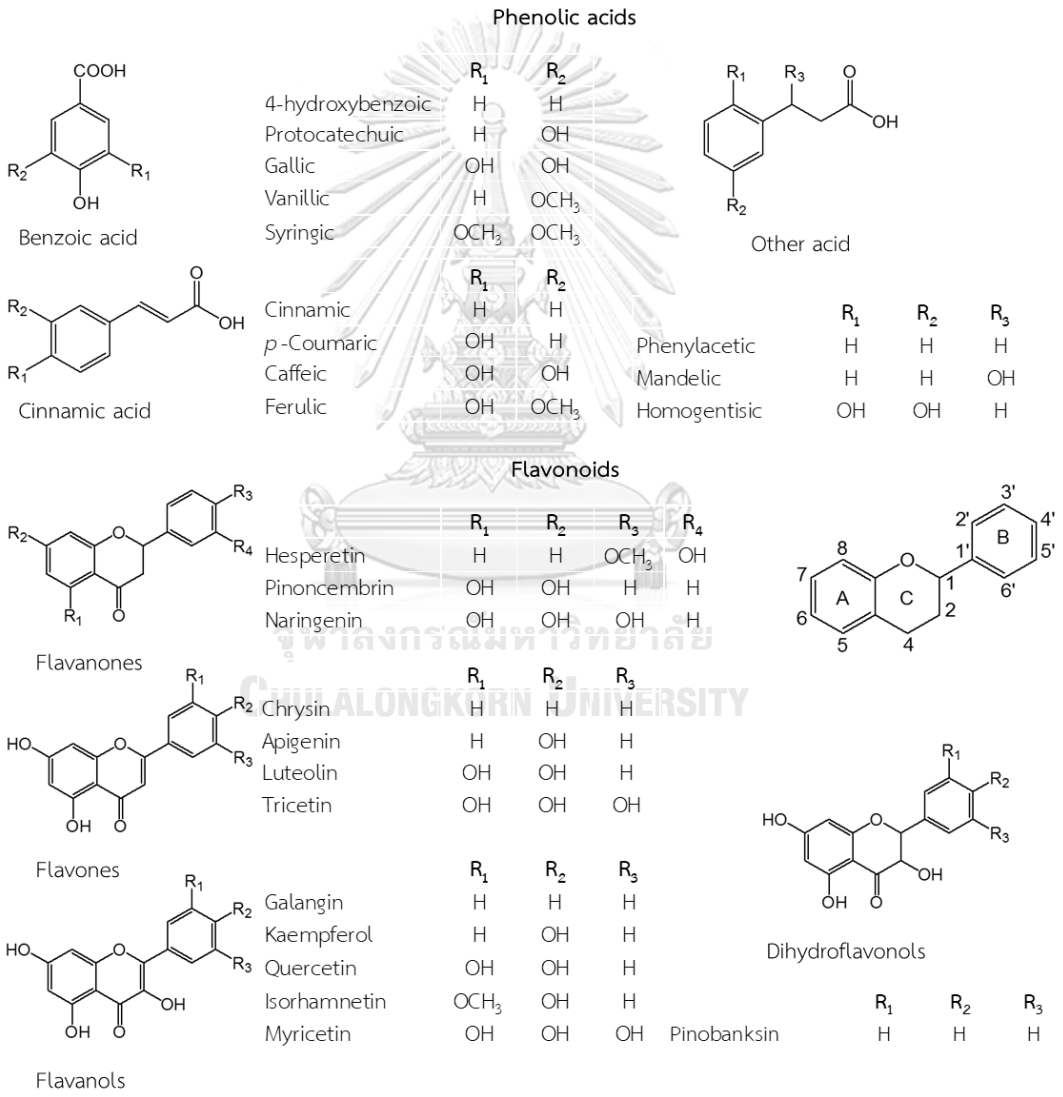


Figure 2. 1 Structures of the principal phenolic acids and flavonoids described in honey**.** Adapted from Erejuwa et al., 2012; Santos-Buelga, 2017.

Phenolic compounds in honey have been used as chemotaxonomic markers in plant systems as well as providing the benefits of human health (*e.g.* anti-bacterial, anti-cancer and antioxidant).

Anti-bacterial: Honey provides antibacterial effect which is stemmed from its methylglyoxal components. In addition, these compounds greatly help to alleviate halitosis. Honey can thus help to heal damaged cells by stimulating the formation of granulation tissue.

Anti-cancer: Honey exhibits anticancer properties via antioxidant or pro-oxidant mechanisms which are selectively dependent on the state of oxidative stress in the cancer cells. When cancer growth is rapid under high levels of reactive oxygen species (ROS), honey acts as an antioxidant to prevent cancer cell growth by minimizing oxidative stress and scavenging the ROS. On the other hand, at low levels of ROS, it will act as a pro-oxidant and promotes cancer cell growth by further generation of ROS and maximizing oxidative stress. Thus, the effects of honey on cancer cell death are different under various conditions.

Anti-inflammatory: Honey can heal damaged cells by stimulating the formation of granulation tissue. Honey coats inside the lining of the throat, killing harmful germs, as well as relaxing the throat at the same time.

Metabolism and Cardioprotective: Health of the metabolic and cardiovascular systems against epinephrine-induced heart abnormalities and vasomotor dysfunctions. Natural wild honey also has cardioprotective and therapeutic effects. Honey consumption was linked to a considerable reduction in metabolic and cardiovascular disease risk variables. Honey has cardioprotective properties such as vasodilation, vascular homeostasis, and lipid profile improvements.

Antioxidant: Honey's antioxidant and antibacterial qualities aided in the reduction of persistent cough and improved sleep for both children and adults after honey consumption (2.5 ml). In addition, a study found that honey has been used remedy for pneumonia 82.4% when compared with other natural products.

Furthermore, the antioxidant activities of honey can also promote good effects on human health such as soothing pain, balancing liver systems, and neutralizing toxins in liver, inhibiting cancer cell growth and etc**.** (Pasupuleti et al., 2017)

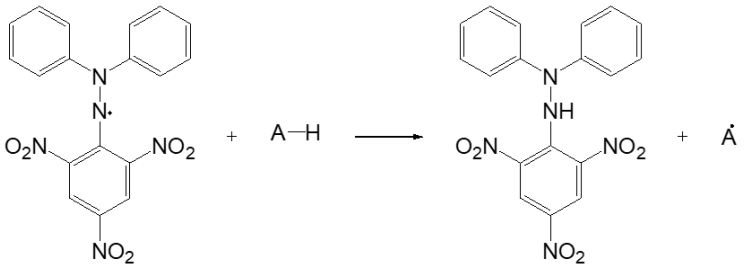
Wound healing: Honey is a safe natural substance, effective in the inhibition of bacterial growth and the treatment of a broad range of wound types, including burns, scratches, diabetic boils (skin abscesses associated with diabetes), malignancies, leprosy, fistulas, leg ulcers, traumatic boils, cervical and varicose ulcers, amputation, burst abdominal wounds, septic and surgical wounds, cracked nipples, and wounds in the abdominal wall (Tashkandi, 2021).

2.1.2 Thai honey

Thailand is the 2nd largest honey producer in Southeast Asia and the 36th in the world. Honey has been one of the main agricultural products exports. Longang, wild flower and lychee honey are the three main varieties of floral honey produced in Thailand. The northern part of Thailand, such as Chiang Mai, Lamphun and Lampang, is where the majority of Thailand's beekeeping takes place (Duangphakdee & Rod-im, 2022).

2.2 Determination antioxidants

Antioxidants are molecules with free radical scavenging property donating electrons to neutralize rampaging free radicals. These antioxidants delay or inhibit cellular damage (Lobo et al., 2010). DPPH• assay is one of the most popular and frequently employed antioxidant assays. As seen in **Figure 2.2**, the reaction can be performed by mixing a sample with DPPH• reagent, and then the A-H sample acts as an antioxidant donating electrons or hydrogen atoms. The DPPH• color changes from purple to yellow of the reduced form. The method is rapid, simple, efficient and considerably inexpensive. However, it requires a UV–Vis spectrophotometer or a HPLC instrument (Edwards & Alexander, 2017) for determination of color change at the wavelength of 517 nm, and antioxidant activities are reported as IC_{50}. Such techniques require specialists, costly instrument and high solvent consumption.



Purple **Yellow**

Figure 2. 2 DPPH• free radical inhibition reaction with a sample (A-H).
Reproduce from Pisoschi et al., 2009

2.3 Determination of total phenolic compounds

Total phenolic compounds can be determined using phenolic compounds in a complex sample interaction with phosphomolybdate (Mo) and phosphotungstate (W) in the FCR reagent (Blainski et al., 2013). As shown in Equation 2.1, phenolic compounds reduce the Mo and W ions, and a color change from yellow to blue is observed by measuring absorption of blue color at a wavelength of 760 nm.

$$Mo^{6+}, W^{6+} + e^{-} \text{ (Phenolic compound)} \rightarrow Mo^{5+}, W^{5+} \text{ (Complex)} \quad (2.1)$$

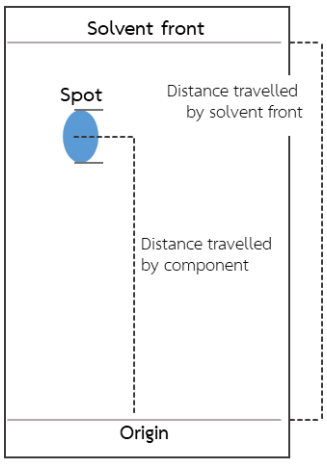
Yellow Blue (λ_{max} = 760 nm)

2.4 Thin-Layer Chromatography (TLC)

TLC is one of the basic chromatography methods for both the identification and determination of chemical components in various samples. It is possible to handle both pure and crude extracts, and the equipment is simple and inexpensive. Additionally, results can be acquired quickly. The general detection method in TLC is based on the unique hue of chemicals contained in samples that emerge on the spot. In case of a colorless spot, a TLC plate is sprayed with a detecting reagent to

generates visible spot after chemically reacting with the component to be identified (Lau, 2001).

TLC results are reported in form of a R_f value, calculated by Equation 2.2, that depends on solubility and absorbed of solvent (mobile phase) and absorbent (TLC plate). The R_f value can be used to identify an unknow compound by comparing with a standard compound.



$$R_f = \frac{\text{Distance travelled by solvent front (cm)}}{\text{Distance travelled by component (cm)}} \quad (2.2)$$

2.5 High Performance Liquid Chromatography (HPLC)

HPLC has been widely used for separation of non-volatile compounds in various samples such as pharmaceutical, food, and environmental samples (Gika et al., 2016). **Figure 2.3** shows a diagram of HPLC which consists of mobile phase, pump, column, sample injector, detector and data processing.

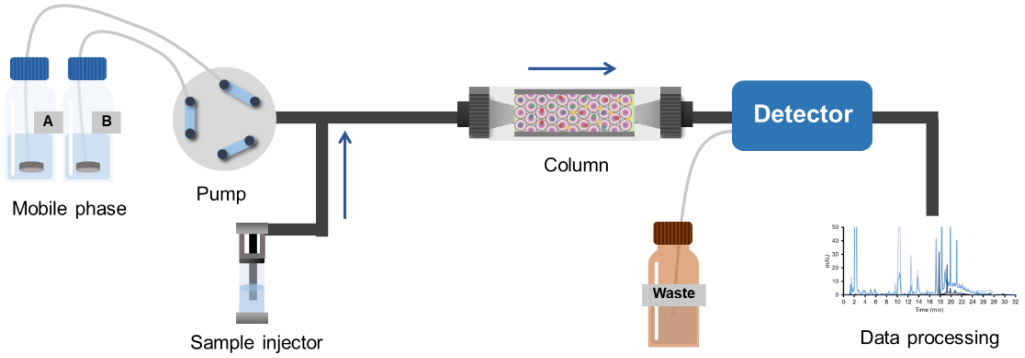


Figure 2. 3 Schematic diagram of HPLC. Adapted from Czaplicki, 2013.

The injector is a part of HPLC instruments to transport a sample solution to an HPLC column inlet. The mobile phase is a solution (single or constantly mixed solution) that transports analytes to a separation column and then delivers the separated compounds to a detector. Pumps are used to mix solvents from mobile phase reservoirs and drive a mobile phase into a column to reach the detector. The HPLC column, an important component, is used to separate constituent compounds of a sample before going to the detector. The analytes are separated by selective distribution between mobile phase and stationary phase coated on packing material inside the column. The concept of HPLC detector is used to detect and identify separated compounds eluted from an HPLC column outlet. The electrical signal represents the amount of each analyte. In addition, detection sensitivity depends on concentration and compatibility of analytes. Traditional HPLC detectors popularly used include UV-Visible, fluorescence and mass spectrometer (Choudhary, 2008).

2.6 UV-Visible spectrophotometer

UV-Vis spectrophotometer, a type of absorption spectrophotometer, is used for a sample containing compounds that absorb light in ultraviolet to visible region. This absorption is related to the species' transition from a ground to an excited state. The wavelength of absorption is higher when less energy is required for this transition, and lower when more energy is required (Holmes-Hampton et al., 2014). **Figure 2.4** shows a diagram of UV-Vis spectrophotometer and the mechanism by which it separates light absorbed from a sample.

The radiation source for this optical system is a deuterium-discharge lamp (emits light from 190-800 nm range) for ultraviolet wavelengths and a tungsten lamp (emits light from 370-1100 nm range) for visible and short wave near-infrared wavelengths. Both light sources are optically combined and utilize a common axis to the source lens, which forms a single collimated light beam. This beam travels through the shutter/stray light filter, then through the sample and finally reaches the

spectrograph lens and slit. The lens disperses light onto the diode array by a holographic grating, allowing access to all wavelength data (Djomehri, 2012).

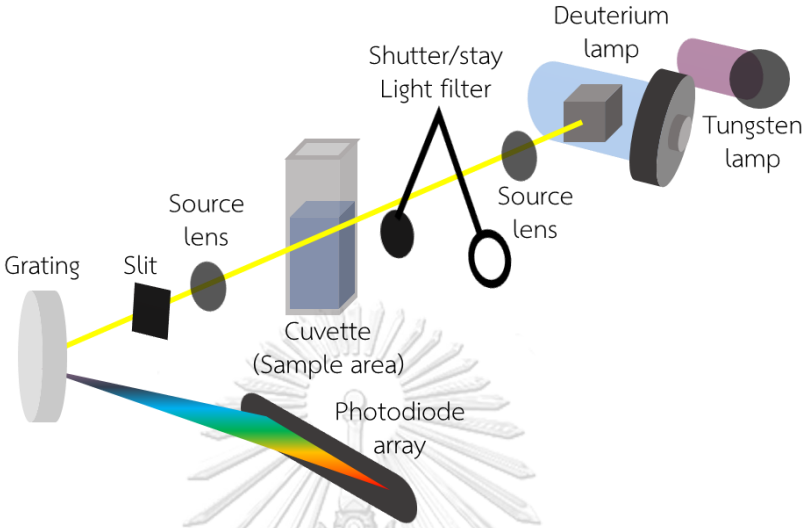


Figure 2. 4 Schematic diagram of UV-Vis spectrophotometer. Adapt from Djomehri, 2012

2.7 Limit of detection (LOD) and Limit of quantification (LOQ)

Limit of detection (LOD) is defined as the lowest concentration of a compounds that can be measured (detected) with statistical significance by employing a given analytical procedure.

Limit of quantification, LOQ stands for the smallest amount or the lowest concentration of a substance that is possible to be determined by utilizing a given analytical procedure with the established accuracy, precision, and uncertainty.

CHAPTER III

EXPERIMENTAL

3.1 Chemical

Formic acid, dichloromethane (CH_2Cl_2), ethanol (EtOH), methanol (MeOH), acetic acid (CH_3COOH) was sourced from Merck (Darmstadt, Germany) with acetonitrile (ACN) and toluene from J.T. Baker (New Jersey, USA), magnesium sulfate anhydrous ($MgSO_4$) from Panreac (Barcelona, Spain), 2, 2-diphenyl-1-picrylhydrazyl (DPPH•) from TCL (Tokyo, Japan), and vanillin, *L*-ascorbic acid (LA), sodium carbonate (Na_2CO_3), gallic acid (GA) and Folin–Ciocâlteu (FCR) from Sigma-Aldrich (St. Louis, MO, USA).

Standards for HPLC analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA): caftaric acid, gallic acid, homovanillic acid, hydrocaffeic acid, kaemferol, protocatechuic acid, ethyl 3, 4-dihydroxybenzoate, quercetin, resveratrol, sinapic acid, syringic acid, *trans*-cinnamic acid, vanillic acid. The following standards were purchased TCI (Tokyo, Japan): (-)-epicatechin, (+)-catechin hydrate, 2, 5-dihydroxybenzoic acid, benzoic acid, caffeic acid, caffeine, chlorogenic acid, ferulic acid, ethyl gallate, *m*-hydroxybenzoic acid, myricetin, nicotinic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, rutin trihydrate.

TLC silica gel 60 F_{254} aluminum sheets (20 x 20) cm were purchased from Merck (Darmstadt, Germany). The vanillin reagent was prepared by dissolving 1 g of vanillin in 200.0 mL of ethanol, followed by the dropwise addition of 5.0 mL of concentrated sulfuric acid in ice bucket (E.A. Bell, 1980). The DPPH• spraying reagent was prepared by dissolving 0.2 g of DPPH• in 100.0 mL of ethanol (Cieśla et al., 2012).

Botanical origins of honey samples used in this work include longan, lychee, wild flower, and coffee. The honey samples are located at Fora bee farm and Shupa farm, Chiangmai. **Table 3.1** shown information of honey samples including botanical origin, supplier origin, manufactured date and sample ID.

Table 3. 1 Honey samples origin and sample ID

Botanical origin	Supplier origin, manufactured date	Sample ID
Longan honey	Fora bee farm, Chiangmai, 26th Feb 2018	LON1
Longan honey	Supha farm, Chiangmai, 15th Mar 2018	LON2
Lychee honey	Fora bee farm, Chiangmai, 19th Feb 2018	LYN1
Lychee honey	Supha farm, Chiangmai, 26th Feb 2018	LYN2
Wild flower honey	Fora bee farm, Chiangmai, 16th Jan 2018	WIF1
Wild flower honey	Supha farm, Chiangmai, 12th Feb 2018	WIF2
Coffee honey	Fora bee farm, Chiangmai, 16th May 2017	COF

3.2 Honey Extraction

In order to remove sugar causing complicated matrix and concentrating the analytes, 10 g honey was dissolved in 10.0 mL of deionized water and extracted three times with 25.0 mL of CH_2Cl_2. The organic extract was dried with $MgSO_4$ anhydrous and then filtered (Locher et al., 2017). The solvent was evaporated under N_2 gas using an automated evaporator (TurboVap® Classic LV automated evaporation system, Biotage).

3.3 Identification of possible phenolic compounds crude honey using HPLC

Crude honey extracts were taken up in 1.0 mL of 50%v/v ACN/milli Q water for HPLC analysis, and then 0.5 g of honey was dissolved in 0.5 mL of milli Q water used as the control sample.

HPLC analysis was performed using Agilent Technologies 1260 Infinity series. The chromatographic separation was performed using an Agilent poroshell 120 SB-C18 column, 4.6 mm x 100 mm, 1.8 µm particle size. The mobile phase consisted of 0.1%v/v CH_3COOH/deionized water (A) and 0.1%v/v CH_3COOH/ACN (B). The gradient

was from 8 to 100 %v/v B over 32 min. The flow rate of mobile phase was set at 1.0 mL min^{-1} and the injection volume of 10 µL was used. UV-Vis detector (DAD WR) at wavelength of 254, 270 and 365 nm were used for detection of phenolic compounds. Results are demonstrated in **Section 4.1.**

3.4 Separation and characterization of crude honey using TLC

3.4.1 Study of suitable mobile phase ratio for TLC separation

Crude honey extracts were dissolved in dichloromethane 100.00 µL. 2 µL of the sample was loaded onto the TLC plate (10x6.6 cm) and separated by using different mixtures of toluene-ethyl acetate-formic acid; 3:8:1, 5:6:1, 10:1:1 (v/v/v), as the mobile phases. Results are given in **Section 4.2.1**.

3.4.2 Characterization

Crude honey extracts 8 mg were dissolved in dichloromethane 100.00 µL. After that, 2 µL of the sample was loaded onto the TLC plate (10x6.6 cm) and separated by using mobile phase toluene-ethyl acetate-formic acid, 5:6:1 (v/v/v). The results are obtained under the UV wavelengths of 254 nm and 366 nm, respectively. After photographing the results, each plate was derivatized by spraying with the vanillin reagent using a TLC glass sprayer (CT laboratory). The plate was heated at 110 °C for 5 to 10 min and then detected by naked eyes and photographed using the smartphone's camera (12 megapixels resolution). Results are depicted in **Section 4.2.1.**

3.5 Qualitative analysis

3.5.1 TLC-DPPH• assay

Crude honey extracts around 3-4 mg were dissolved in CH_2Cl_2 100.00 µL, and then 2 µL of sample was loaded onto the TLC plate (10x6.6 cm) and separated using toluene-ethyl acetate-formic acid, 5:6:1 (v/v/v), as the mobile phase. Each plate was derivatized with 0.2%v/v DPPH•/EtOH. After spraying, the plate was

kept in darkness and photographed for a period of time at 0.5, 1 and 2 h. Any antioxidant compound is shown as a yellow spot on a purple background. Results are shown in **Section 4.2.2.**

3.5.2 TLC-FCR reagent test

Crude honey extracts around 5 mg was dissolved in 100.00 µL of CH_2Cl_2. After that, 2 µL of sample was spot onto a TLC plate (10x6.6 cm). Toluene-ethyl acetate-formic acid, 5:6:1 (v/v/v), was used as the mobile phase. Each plate was derivatized with 7% Na_2CO_3, let it dry and then the plate was sprayed with FCR reagent, finally, the plate was heated at 110 ^{o}C for 5 min. Any phenolic compound is shown as a dark blue spot. Results are given **in Section 4.2.3.**

3.6 Quantitative analysis

3.6.1 Antioxidant activity

LA and DPPH• reagent were separately weighted and dissolved these in 50% ACN/EtOH to give each standard stock solution at 1.00 mg/mL of LA and 0.79 mg/mL of DPPH• reagent.

Appropriate amounts of the LA stock solution at 1.00 mg/mL were pipetted and diluted in 50% ACN/EtOH to give working standard solutions of LA at 0.05, 0.10, 0.15, 0.20, 0.25 and 0,30 mg/mL.

Appropriate amounts of the DPPH• stock solution at 0.79 mg/mL were pipetted and diluted in 50% ACN/EtOH to give working solutions of DPPH• at 0.02, 0.05, 0.10, 0.20, 0.39 and 0.79 mg/mL.

3.6.1.1 TLC-DPPH• assay

Each LA working standard solution of 16 µL was mixed with 24 µL of 0.79 mg/mL DPPH• reagent. The mixed solution was kept in darkness for 15 min. Then 2 µL of each mixed solution in triplicate spots were separately loaded on

a TLC plate (7x12 cm), and then each DPPH• working solution at 2 µL in triplicate spots were separately loaded on the same TLC plate. The TLC plate was detected by photographing using the smartphone's camera (12 megapixels resolution) under a portable photography light box.

3.6.1.2 Data analysis

From experiment in **Section 3.6.1.1**, after the TLC plate was photographed, a picture was processed by using imageJ software. The results were optimized by measuring 4 colors including red, green, blue and grey. The color intensity value was obtained by averaging area of each spot. A linear calibration plot was established by plotting between the color intensity values and the concentrations of working DPPH• solution spots (0.02 to 0.79 mg/mL). This is performed in order to obtain the amount of DPPH• remaining in each sample. This concentration was converted into percent inhibition for each sample. Relationship between the percent inhibition and the concentrations of samples was then established to the IC_{50} value. Results are shown in **Section 4.3.1.2.**

3.6.1.3 Time effect

Time effect of solution stability was optimized by mixing 16 µL of each LA working standard solution with 24 µL of 0.79 mg/mL DPPH• reagent. The mixed solution was kept in darkness for 5 to 30 min. Then 2 µL of each mixed solution in triplicate spots were separately loaded on a TLC plate (7x12 cm) and then each DPPH• working solution at 2 µL in triplicate spots were separately loaded on the same TLC plate. TLC plate was detected by photographing using the smartphone's camera for 0 to 30 min after the latest spot was spotted on TLC plate. The red value was used to compare the different periods to study the time effect. Results are demonstrated in **Section 4.3.1.3.**

3.6.1.4.Method validation

Due to the small amount of honey sample LON1, UV-Vis analysis of antioxidant activity of LON1 and LA was determined using HPLC

instrument (Agilent 1260 infinity) coupled with a UV-Vis detector (1260 DAD WR) without a column (Edwards & Alexander, 2017), along with applying 50% ACN/EtOH as HPLC mobile phase with a flow rate of 0.200 ml/min for 1 min and DAD WR detection at wavelength of 517±4 nm.

Appropriated amounts of honey sample LON1 were separately weighted and dissolved these in 50% ACN/EtOH to given working solutions at 1, 3, 5, 10, 15 and 20 mg/mL. Each honey sample working solution or LA working standard solution of 160 µL was mixed with 240 µL of 0.79 mg/mL DPPH• reagent. The mixed solution was kept in darkness for 30 min. Then 2 µL of each mixed solution in triplicate was injected into HPLC. The DPPH• working solution (0.02 to 0.79 mg/mL) of 2 µL in triplicate was injected into HPLC.

A linear calibration plot was established by plotting between the area under curve and the concentration of DPPH• working solution (0.02 to 0.79 mg/mL). This is performed in order to obtain the amount of DPPH• remaining in each sample. This concentration was converted into percent inhibition for each sample. In addition, the relative standard deviation value (RSD) for %Inhibition of LA and LON1 at different concentration were calculated to compare between TLC- DPPH• and the UV-Vis method. Results are given in **Section 4.3.1.4.**

3.6.1.5.Application and real sample

Honey sample working solutions were separately weighted and dissolved these in 50% ACN/EtOH to give each solution at 1, 3, 5, 10, 15 and 20 mg/mL of LON, 1, 5, 10, 15, 20 and 25 mg/mL of LYN, WIF and 5, 10, 15, 20, 25 and 30 mg/mL of COF.

Each honey samples working solution or LA working standard solution of 16 µL was mixed with 24 µL of 0.79 mg/mL DPPH• reagent. The mixed solution was kept in darkness for 15 min. Then 2 µL of each mixed solution in triplicate spots were separately loaded on a TLC plate (7x12 cm), and then each DPPH• working standard solution at 2 µL in triplicate spots were also separately

loaded on the same TLC plate. The TLC plate was detected by photographing using the smartphone's camera under a portable photography light box. Results are depicted in **Section 4.3.1.5.**

3.6.2 Determination of total phenolic compounds

GA was separately weighted and dissolved these in 50% ACN/EtOH to give each standard stock solution at 1.00 mg/mL.

Appropriate amounts of the GA stock solution at 1.00 mg/mL were pipetted and diluted these in 50% ACN/EtOH to give working standard solutions of GA at 0.03, 0.05, ,0.07, 0.10, 0.15, and 0.20 mg/mL.

3.6.2.1 TLC-FCR reagent test

Each GA working standard solution of 40 µL was mixed with 5 µL of FCR reagent. Then 10 µL of 7% Na_2CO_3 was added into the mixed solution before 25 µL of MeOH was added into the mixed solution. After that the resulting solution was centrifuged using mini centrifuge (hi-speed vortex mini centrifuge AMC-V1, ACT Gene, Inc.) for 5 min. Then 2 µL of each mixed solution in triplicate spots were separately loaded on a TLC plate (7x3.5 cm). The TLC plate was detected by photographing using the smartphone's camera (12 megapixels resolution) under a portable photography light box.

3.6.2.2 Data analysis

From **experiment 3.6.2.1**, after the TLC plate was photographed, a picture was processed by using imageJ software. The results were optimized by measuring the blue value. The color intensity value was obtained by averaging area of each spot. A linear calibration plot was generated by plotting between the concentration (0.03 to 0.20 mg/mL) and the color value of mixed solution spots. This is performed in order to obtain amount of Mo (VI) reduced to Mo (V) and provide the blue spot. Results are revealed in **Section 4.3.2.2.**

3.6.2.3 Time effect

Time effect of color value of solution stability on TLC plate was optimized by mixed 40 µL of each GA working standard solution with 5 µL of FCR reagent. Then 10 µL of 7% Na_2CO_3 was added into the mixed solution before 25 µL of MeOH was added into the mixed solution, after that centrifuged for 5 min. Then 2 µL of each mixed solution in triplicate spots were separately loaded on a TLC plate (7x3.5 cm). After that photographed for 0-30 min after the latest spot was spotted on the TLC plate. The blue value was used to compare the different periods to present the time effect. Results are shown in **Section 4.3.2.3.**

3.6.2.4 Method validation

Honey sample working solutions were separately weighted and dissolved these in 50% ACN/EtOH to give 70 mg/mL of each honey sample.

Each GA working standard solution or honey sample working solution of 40 µL of was mixed with 5 µL of FCR reagent, the solution was mixed with 10 µL of 7% Na_2CO_3 and then 500 µL of MeOH was added into the mixed solution, after that centrifuged using mini centrifuge for 5 min. The solution was detected by using UV-Vis spectrophotometer (Agilent/HP 8453) at a wavelength of 760 nm. Relative standard deviation value (RSD) of total phenolic compounds of each honey sample were calculated to compare between TLC-FCR and the UV-Vis method. Results are demonstrated in **Section 4.3.2.4.**

3.6.2.5 Application in the real samples

Honey sample working solutions were separately weighted and dissolved these in 50% ACN/EtOH to give 7 mg/mL of each honey sample.

Each GA working standard solution (0.03 to 0.20 mg/mL) was mixed with was mixed with 5 µL of FCR reagent, the solution was mixed with 10 µL of 7% Na_2CO_3, next added 25 µL of MeOH and centrifuged for 5 min. Then 2 µL of each mixed solution in triplicate spots were separately loaded on a TLC plate

(7x3.5 cm). Each honey sample working solution (7 mg/mL) was done in an experiment as same as GA working standard solution. The resulting spots were photographed and their blue color intensity values were recorded. The value of each sample was converted to the concentration of total phenolic compounds in each honey sample that related to amount of GA, total phenolic compounds in samples were presented in form of mg GA/g sample. Results are given in **Section 4.3.2.5.**

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Identification of possible phenolic compounds crude honey using HPLC

From **Section 3.3**, HPLC was used for separation analysis of each honey sample after the LLE extraction in order to investigated the possible phenolic compounds in the samples by comparing with standard phenolic compounds. The peaks in HPLC chromatogram of each honey samples were identified according to comparison of their retention time and UV spectra with those of the available 28 standards analyzed under the same conditions. The results are summarized in **Table 4.1.** The HPLC chromatograms of each extracted honey sample are shown in Figure **B-1-28** in **Appendix-B**).

Table 4. 1 Results of phenolic compounds in crude honey obtained from HPLC.

Standard compounds		RT (min)	Honey extract						
			LON1	LON2	LYN1	LYN2	WIF1	WIF2	COF
1	Nicotinic acid	1.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	Gallic acid	1.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	Caftaric acid	2.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4	Protocatechuic acid	3.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5	2,5-dihydroxybenzoic acid	3.7	n.d.	n.d.	n.d.	n.d.	Match	Match	n.d.
6	Chlorogenic acid	4.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	*p*-Hydroxybenzoic acid	4.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	(+)-Catechin hydrate	5.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9	Hydrocaffeic acid	5.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	Caffeine	5.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
11	Vanillic acid	6.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	*m*-Hydroxybenzoic acid	6.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
13	Caffeic acid	6.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
14	Homovanillic acid	6.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15	Syringic acid	7.0	n.d.	n.d.	n.d.	n.d.	n.d.	Match	n.d.
16	(-)-epicatechin	8.2	Match	Match	Match	Match	n.d.	n.d.	n.d.
17	Ethyl gallate	10.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18	*p*-Coumaric acid	10.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
19	Ferulic acid	12.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20	Sinapic acid	13.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
21	Benzoic acid	13.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22	Rutin trihydrate	14.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
23	Ethyl 3, 4-dihydroxybenzoate	15.8	n.d.	n.d.	n.d.	n.d.	Match	Match	n.d.
24	Myricetin	16.8	n.d.	n.d.	Match	Match	n.d.	n.d.	Match
25	Resveratrol	17.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
26	*trans*-Cinnamic acid	18.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
27	Quercetin	18.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
28	Kaemferol	20.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

4.2 TLC separation approach for characterization of crude honey

4.2.1 The conventional TLC method

According to **Section 3.4.2**, **Figure B-29 in Appendix-B** shows typical TLC fingerprints of the honey extracts obtained from different botanical origins using

different mobile phase compositions. Toluene-ethyl acetate-formic acid, 5:6:1 (v/v/v) was found to provide better separation with good distribution of the spots along with the TLC plate based on all the detection approaches. Major bands of these honey samples are shown in **Table 4.2.**

This mobile phase was thus selected for further uses as well as for the TLC-DPPH• assay and TLC-FCR methods below.

Table 4. 2 R_f of honey samples after TLC separation using the conventional detection approaches.

Sample	Spot position		
	Under 254 nm	Under 366 nm	After derivatized with vanillin
LON1	R_f 0.45, 0.53 and 0.62	R_f 0.13 (blue)*, 0.42 (yellow), 0.48 (yellow)*, 0.61 (blue) and 0.85 (blue)	R_f 0.40 (orange), 0.47 (green), 0.53 (purple), 0.58 (purple), 0.64 (yellow)* and 0.92 (purple)
LON2	R_f 0.45, 0.53 and 0.62	R_f 0.13 (blue)*, 0.42 (yellow), 0.48 (yellow)*, 0.61 (blue) and 0.85 (blue)	R_f 0.40 (orange), 0.47 (green), 0.53 (purple), 0.58 (purple), 0.64 (yellow)* and 0.92 (purple)
LYN1	R_f 0.45, 0.53 and 0.62	R_f 0.42 (yellow), 0.51 (yellow), 0.61 (blue) and 0.85 (blue)	R_f 0.40 (orange), 0.47 (green), 0.52 (brown), 0.60 (purple) and 0.92 (purple)
LYN2	R_f 0.45, 0.53 and 0.62	R_f 0.42 (yellow), 0.51 (yellow), 0.61 (blue) and 0.85 (blue)	R_f 0.40 (orange), 0.47 (green), 0.52 (purple), 0.58 (green) *, 0.60 (purple) and 0.92 (purple)
WIF1	R_f 0.45, 0.53 and 0.62	R_f 0.30 (yellow)*, 0.51 (yellow), 0.61 (blue) and 0.85 (blue)	R_f 0.47 (green), 0.51 (yellow)* and 0.92 (purple)
WIF2	R_f 0.45, 0.53 and 0.62	R_f 0.30 (yellow)*, 0.51 (yellow), 0.61 (blue) and 0.85 (blue)	R_f 0.47 (green), 0.51 (yellow)* and 0.92 (purple)
COF	R_f 0.45, 0.53 and 0.62	R_f 0.28 (yellow)*, 0.41 (yellow), 0.51 (yellow), (yellow), 0.61 (blue) and 0.85 (blue)	R_f 0.40 (orange), 0.47 (green), 0.51 (purple), 0.60 (purple) and 0.92 (purple)

* characteristic spots of each honey sample.

* LON = longan, LYN = lychee, WIF = wild flower, and COF = coffee.

From **Table 4.2**, All honey sample fingerprints cloud not be differentiated under 254 nm, while the fingerprints were clearly different with the characteristic spots observed under 366 nm or by naked eyes after the vanillin derivatization. The sample obtained from the same floral showed similar fingerprints *e.g.* LON1 and LON2 regardless of the supplier sources

After separation, Longan honey samples (LON1 and LON2) showed the characteristic spots at R_f of 0.13 (blue) and 0.48 (yellow) (also detected at 366 nm without the derivatization) and at R_f of 0.64 (yellow). Lychee honey samples (LYN1 and LYN2) showed the characteristic yellow spots at R_f of 0.58. Wild flower honey samples (WIF1 and WIF2) showed the characteristic yellow spots at R_f of 0.30 (yellow) and 0.51 (yellow). The first spot could also be detected at 366 nm without the derivatization. Coffee honey samples (COF) showed the characteristic yellow spots at R_f 0.28 (also detected at 366 nm without the derivatization). This study showed the TLC capability to visualize and classify the honey samples with different botanical origins and to identify the common spots among these samples.

4.2.2 TLC-DPPH• assay

From **Section 3.5.1**, the antioxidant compounds in the honey extracts after TLC separation were detected according to 0.2%v/v DPPH•/EtOH dying. As shown in **Figure B-30** in **Appendix-B**, the result showed the color change from purple (background) to yellow spots of expected antioxidant compounds obtained using different reaction time. The result in **Figure 4.1** and **Table 4.3** showed that 2 h reaction provided the brighter yellow spots of the antioxidant compounds. This implies the possibility of qualitative examination of antioxidant spots of the honey extracts. According to the optimal reaction time, the spot R_f values in **Table 4.3** were calculated for each sample as indicated by the yellow circles on the TLC plate in **Figure 4.1**.

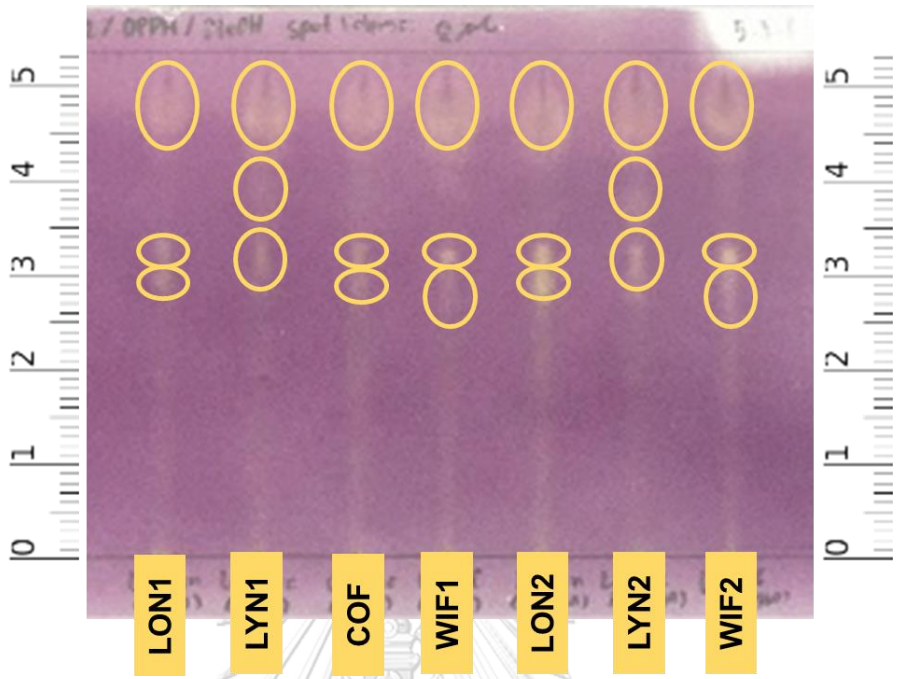


Figure 4. 1 TLC photographs of the crude honey extracts after separation and dying with 0.2%v/v DPPH•; LON = longan, LYN = lychee, WIF = wild flower and COF = coffee; where 1 and 2 come from Foral bee and Supha farms, respectively.

Table 4. 3 R_f of antioxidant spot of each honey sample.

Sample	Antioxidant spot position
LON1	R_f 0.55, 0.61 and 0.90
LON2	R_f 0.55, 0.61 and 0.90
LYN1	R_f 0.60, 0.74 and 0.90
LYN2	R_f 0.60, 0.74 and 0.90
WIF1	R_f 0.52, 0.61 and 0.90
WIF2	R_f 0.52, 0.61 and 0.90
COF	R_f 0.55, 0.61 and 0.90

LON = longan, LYN = lychee, WIF = wild flower, and COF = coffee.

4.2.3 TLC-FCR reagent test

According to **Section 3.5.2**, the FCR reagent was used to screen for the phenolic compounds in the honey extracts. After TLC separation, the reagent was sprayed on a TLC plate. The colors of the phenolic compound spots were observed in dark blue as indicated by the circles in **Figure 4.2**.

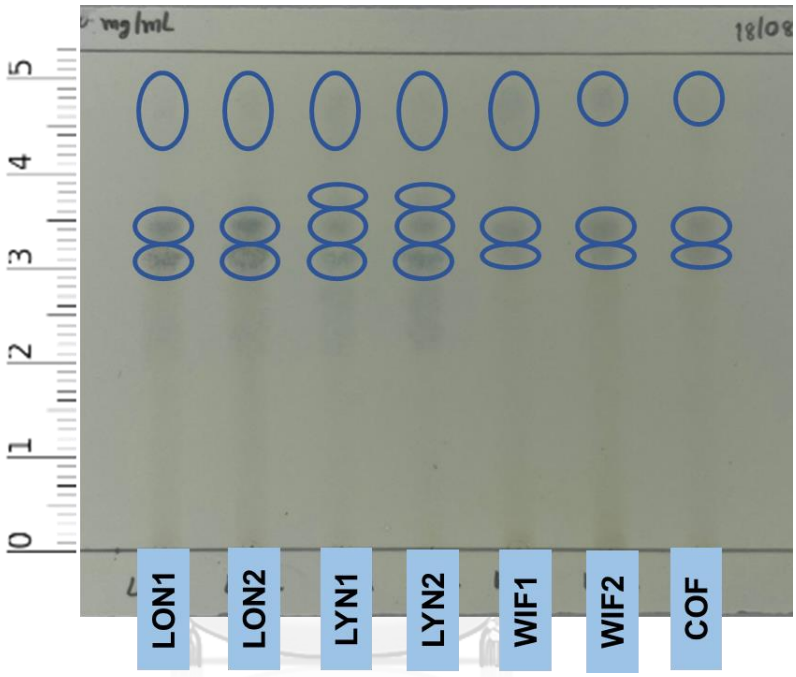


Figure 4. 2 TLC photograph of the crude honey extracts after separation and dying with the FCR reagent, LON = longan, LYN = lychee, WIF = wild flower and COF = coffee; where 1 and 2 come from Foral bee and Supha farms, respectively.

The R_f values of phenolic compound spots of each sample were calculated and documented in **Table 4.4.** According to these qualitative results, the developed approach showed an alternative technique for differentiation of phenolic compounds in the samples especially for LYN1 (3) and LYN2 with the additional spots with R_f of 0.71.

Table 4. 4 R_f of phenolic compound spots of each honey sample.

Sample	Phenolic compound spot
LON1	R_f 0.58, 0.64 and 0.89
LON2	R_f 0.58, 0.64 and 0.89
LYN1	R_f 0.58, 0.64, 0.71 and 0.89
LYN2	R_f 0.58, 0.64, 0.71 and 0.89
WIF1	R_f 0.59, 0.64 and 0.89
WIF2	R_f 0.59, 0.64 and 0.89
COF	R_f 0.59 0.64 and 0.90

LON = longan, LYN = lychee, WIF = wild flower, and COF = coffee.

4.3 Colorimetric approach on a TLC plate

4.3.1 Determination of antioxidant activity

4.3.1.1 TLC-DPPH• assay

This study develops small scale TLC-DPPH• method for analysis of antioxidant activities of the samples on a TLC plate. As mentioned in **Section 3.6.1.1**, the method was performed without TLC separation, fastening the analysis time with the capability to load many samples within each analysis and requiring only 2 µL for each sample. The same sample could be loaded several times to repeat the analysis.

4.3.1.2 Data analysis

From experimental in **Section 3.6.1.2** and results in **Section 4.3.1.1**, data analysis was evaluated by using ImageJ software. The four-color modes of grey, blue, green, and red were investigated for the antioxidant compounds with different concentrations as shown in **Figure 4.3**. It can be seen in **Figure 4.3** that all the color scales, especially, red and green scales showed similar signal of yellow spots of LA working standard solution reacted with DPPH•. The red scale was chosen in this study.

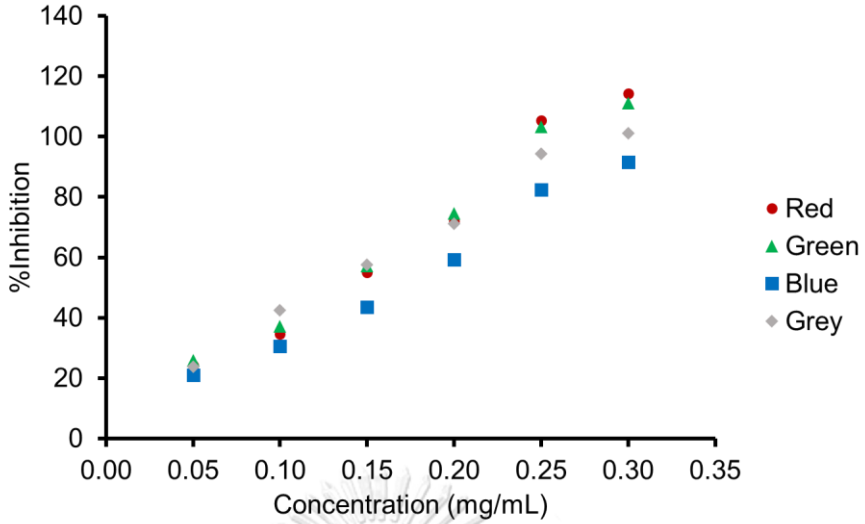


Figure 4. 3 Corresponding color mode plots of %inhibition vs concentration of LA.

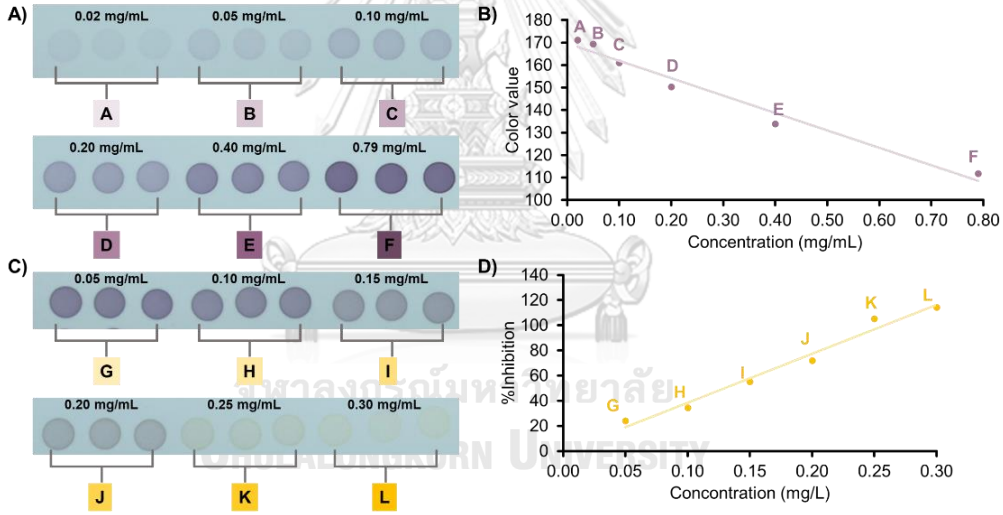


Figure 4. 4 Experimental results of 3 repeated spots of (A) each DPPH• working solution on TLC plate (B) linear calibration plot between color value and concentration of DPPH• working solution (C) spots of each LA working standard solution and the DPPH• reagent on TLC plate and (D) linear calibration plot between %inhibition and concentration of LA working standard solution. The letters A to F in (B) indicate the corresponding average color values in (A) and G to L in (D) indicate %inhibition obtained from the corresponding average color values in (C).

As shown in **Figure 4.4A**, average color values of DPPH• working solution on TLC plate (A to F) was converted a linear calibration plot of y = -76.811x + 169.06 with R^2 of 0.97 for determination of %Inhibition in **Figure 4.4B**. This was used for calculation of the average color values of DPPH• remaining in the mixture after the reaction with LA in **Figure 4.4C.** From **Figure 4.4D** and **Equation 4.1**, %Inhibition was then calculated from the remaining DPPH• described in **Equation 4.2**, compared with the starting amount of y = 388.21x - 0.376 with R^2 of 0.98. IC_{50} can then be calculated from **Equation 4.3**, obtained from the plot in **Figure 4.4D** and approximating the concentration at inhibition of 50%.

$$\%\text{Inhibition} = \frac{\text{DPPH remaining}}{0.79\frac{\text{mg}}{\text{L}}\text{DPPH•}} \times 100 \quad (4.1)$$

$$\text{DPPH remaining} = \frac{y_{sample} - C_{DPPH•}}{m_{DPPH•}} \quad (4.2)$$

$$IC_{50} = \frac{50 - C_{sample}}{m_{sample}} \quad (4.3)$$

y, c, and m in **Equation 4.1-4.4**, obtained from linear calibration plot of DPPH• or sample.

4.3.1.3 Effect of reaction time

According to experimental in **Section 3.6.1.3** and results in **Section 4.3.1.1**, **Figure 4.5 shows the effect of** reaction time of LA reacted with DPPH• reagent and stability of mixed solution on a TLC plate. Although reaction time of 5 min was sufficient for the reaction time between each sample and DPPH• (*e.g.* see the color value data for LA/DPPH• solutions at different concentrations in **Figure 4.5A**), longer reaction time of 15 min was applied in this study. This allowed more samples to react with DPPH• and could be analyzed within the same TLC plate. It is

noted that the loading time also increased with more samples. In addition, the starting reaction times progressively increased with the number of samples and all the samples experienced the same total reaction time of 15 min before loading onto the plate. However, the maximum sample number to be analyzed within the same plate was limited by stability of DPPH• on the TLC plate shown in **Figure 4.5B** represented by the color intensity after loading each sample on to the plate. This was experimentally observed for DPPH•/L-ascorbic acid solutions at different concentrations loaded on a TLC plate with the significantly increasing color intensity value (decreasing DPPH• concentration) after 15 min.

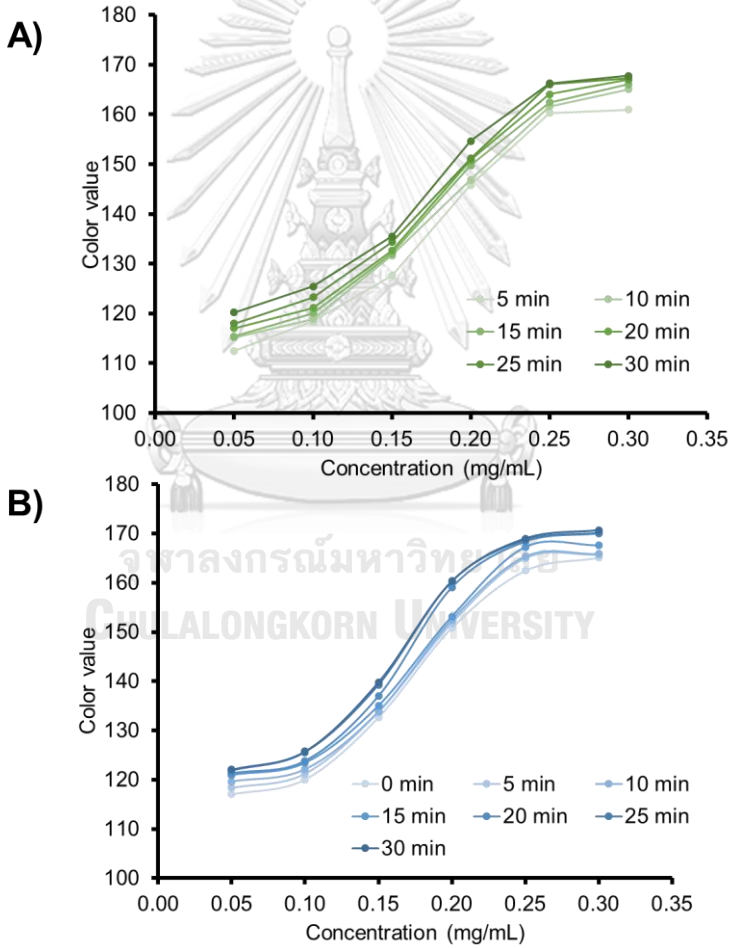


Figure 4. 5 Plots of color intensity value vs concentration of LA reacted with DPPH• reagent with the solutions left at different time before loading onto a TLC plate and being photographed (A) and the solutions left onto the TLC plate at different time before being photographed (B).

4.3.1.4 Method validation

The TLC-DPPH• results were validated by comparison with direct UV-Vis analysis using the HPLC instrument with the UV-Vis detector at wavelength 517 nm but without a separating column as mention in **Section 3.6.1.4**. The HPLC instrument was used due to the limited amount of sample available in this study, which allowed automatic injection of 2 µL of sample solution per each analysis. An example of the HPLC chromatogram of DPPH• is shown in **Figure 4.6,** while that of each sample at different concentrations **Figure B-31-33 in Appendix-B**.

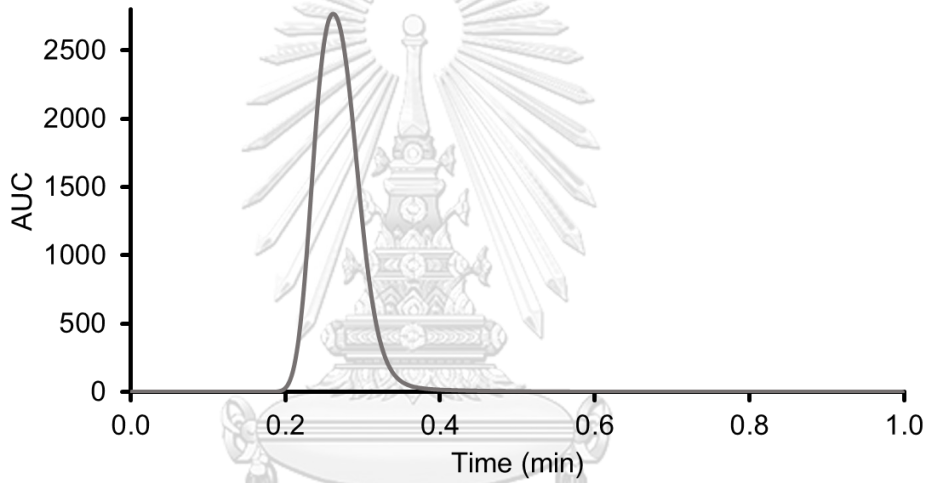


Figure 4. 6 Chromatogram of DPPH• reagent.

The developed TLC-DPPH• method was found to correlate well with the HPLC result. LA and LON1 were used for validation of the results. The correlation plots for %inhibition results obtained from TLC-DPPH• and HPLC methods showed the R^2 values of 0.98 for LA in **Figure 4.7A** and 0.95 for LON1 sample in **Figure 4.7B** at different concentrations. The correlation in **Figure 4.7B** was further used to correct the %inhibition below.

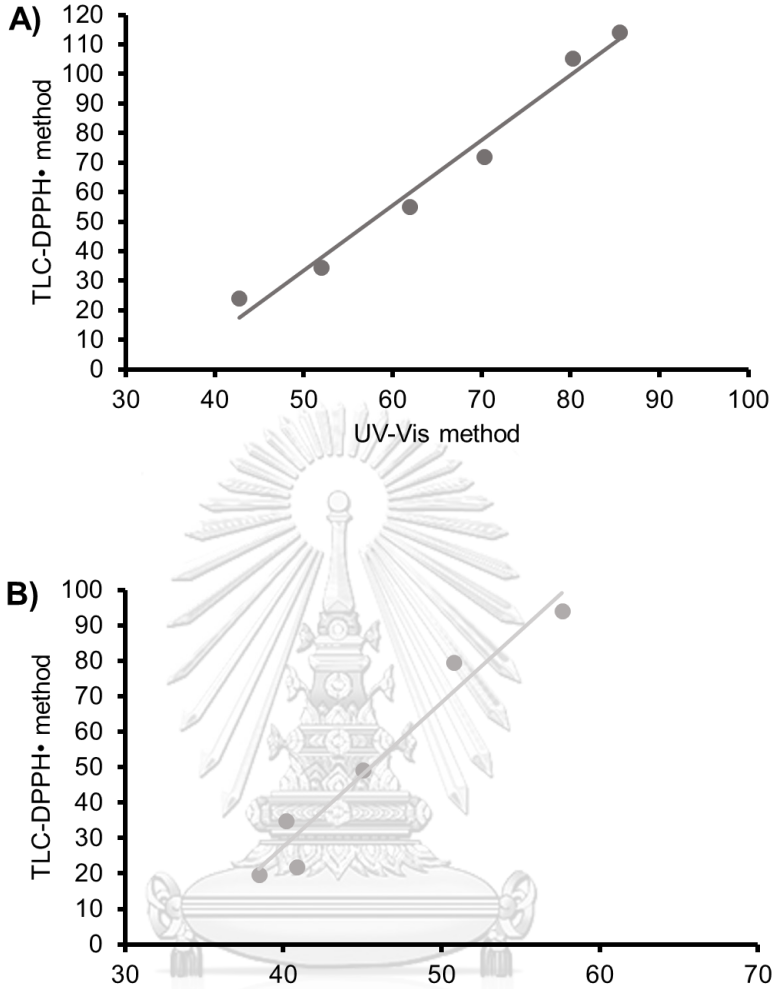


Figure 4. 7 Correlation between %inhibition obtained with the developed TLC-DPPH• and HPLC methods for (A) LA (B) LON1 at different concentration. The linear equations in (A) and (B) are y = 2.2073x - 76.864 (R^2 0.98) and y = 4.0683x - 135.22 (R^2 0.95) respectively.

From TLC-DPPH• and UV-Vis methods in **Section 3.6.1.2** and linear calibration plots as shown in **Figure 4.4**, **Table 4.5** shows an RSD comparison of TLC-DPPH• and UV-Vis methods. Experimental RSD values from TLC-DPPH• were obtained in a range of 0.80-3.1% for 0.05-0.25 mg/mL LA, 1.3-3.9% for 3-20 mg/mL

LON1 and 9.0% for 1 mg/mL LON1. Although the worse RSD result of TLC-DPPH• than UV-Vis was obtained, all experimental RSD values from TLC-DPPH• falls in the acceptable criteria value of $2^{(1-0.5\log C)}$, where C in a Horwitz equation is the concentration analyte expressed as dimensionless mass fraction (Horwitz & Albert, 2019).

Table 4. 5 Validation parameter of %inhibition of LA and LON1 using UV-Vis and TLC-DPPH• methods of RSD (*n* = 3)

Concentration of LA (mg/mL)	%RSD		Acceptable RSD (%)
	UV-Vis	TLC-DPPH•	
0.05	0.41	0.80	25
0.10	0.35	1.0	23
0.15	0.50	1.3	21
0.20	0.14	2.2	20
0.25	0.40	3.1	20
0.30	0.32	1.5	19

Concentration of LON1 (mg/mL)	RSD (%)		Acceptable RSD (%)
	UV-Vis	TLC-DPPH•	
1	0.57	9.0	16
3	0.47	3.5	14
5	0.13	1.4	13
10	0.17	1.2	11
15	0.24	3.9	11
20	0.25	1.3	10

As experiment previously mentioned in **Section 4.3.1.1**, linear calibration plot of LA was plotted, LOD and LOQ were calculated from the **Equation 4.4** and **4.6**. This LOD definition is now abandoned by IUPAC due to resulting in an underestimation. According to the modern IUPAC recommendation, LOD is defined as the minimum analyte concentration that can be discriminated from the blank,

controlling the risks of false positive and false negative, and therefore, may be expressed by **Equation 4.4.**

$$LOD = \frac{3.3\ S_{x/y}}{A}\sqrt{1+h_0+\frac{1}{I}} \quad (4.4)$$

A is the slope of a linear plot between the signal against the analyte concentration, $S_{x/y}$ is the residual standard deviation, *I* is the number of calibration samples, and h_0 is the leverage for the blank sample as described in **Equation 4.5.**

$$h_0 = \frac{c_{cal}^{-2}}{\sum_{i=1}^{I}(c_i - \bar{c}_{cal})^2} \quad (4.5)$$

Where $\bar{c}_{cal}$ is the mean calibration concentration, and c_i is the calibration concentration i.

Similar concepts also apply to LOQ calculation with the factor of 10 instead of 3.3 in **Equation 4.4**, to ensure a maximum relative prediction uncertainty of 10 as given in **Equation 4.6** (Olivieri, 2015).

$$LOQ = \frac{10\ S_{x/y}}{A}\sqrt{1+h_0+\frac{1}{I}} \quad (4.6)$$

the LOD and LOQ data obtained from the analysis of different concentration levels of LA (*n* = 3) are 0.010 and 0.030 mg/mL, respectively.

4.3.1.5 Application for the honey sample analysis

From **Section 3.6.1.5**, the developed method was used for determination of %inhibition of the honey extract samples at different concentrations. The %inhibition vs concentrations of each sample is shown in **Figure 4.8A** with the plot of 3 repeating spots of each sample at different concentrations provided in **Figure A1-A9** in **Appendix-A** and the corresponding experimental result provided in **Figure 4.8B**. Good linearity ranges were observed with the R^2 values of > 0.94 for all the sample curves. As shown in **Table 4.5,** the result of each sample was converted to IC_{50}. There are 4 types of honey samples investigated in this study (longan (LON), lychee (LYN), wild flower (WIF) and coffee (COF)).

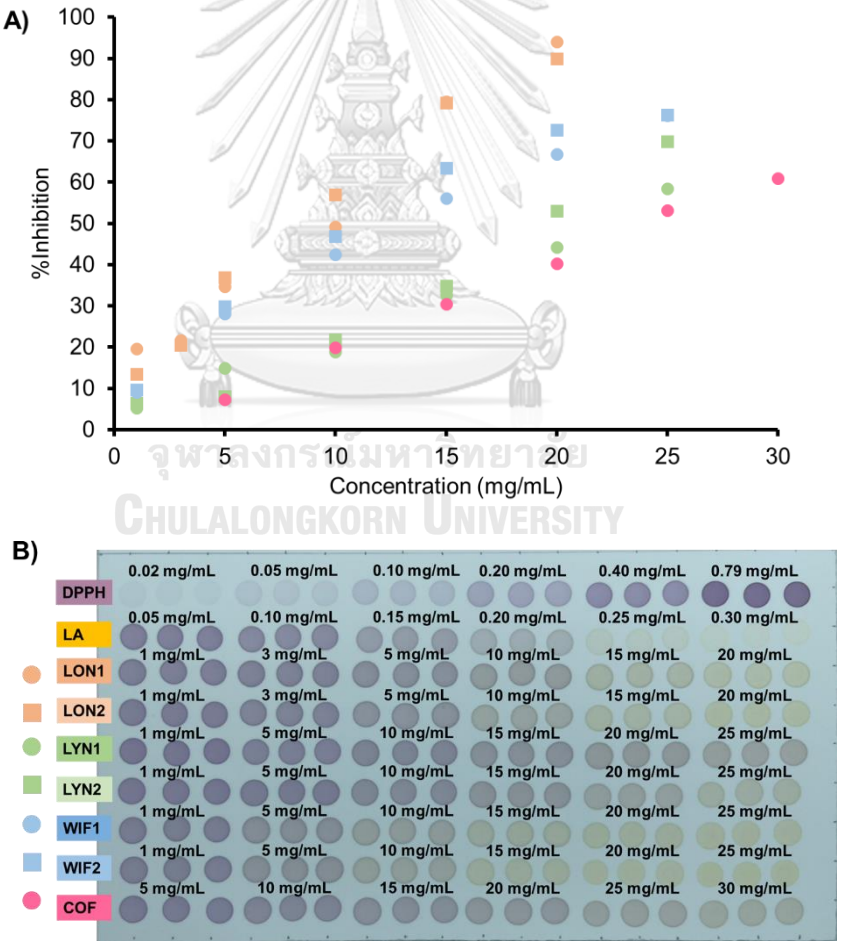


Figure 4. 8 Corresponding plots of %inhibition vs concentration of each sample (A) was converted from spots of honey extracts reacted with DPPH• reagent (B).

All the samples were produced in the same province. The antioxidant properties appear to be more strongly affected by the botanical origin than the supplier sources. For the same manufactured date, the antioxidant activities were in the order of Longan > Lychee > Wild flower. Coffee honey showed the lowest IC_{50} probably due to the coffee flower origin or the oldest shelf life.

Table 4. 6 IC_{50} detected by the developed TLC- DPPH• method with standard deviation (*n* = 3) in the honey samples.

Sample	IC_{50} (mg/mL)
LA	0.13 ± 0.01
LON1	8.9 ± 0.2
LON2	9.0 ± 0.2
LYN1	22.1 ± 0.6
LYN2	18.9 ± 0.4
WIF1	12.6 ± 0.2
WIF2	13.7 ± 0.2
COF	22.6 ± 2.4

LON = longan, LYN = lychee, WIF = wild flower, and COF = coffee.

4.3.2 Determination of total phenolic compounds

4.3.2.1 TLC-FCR

This study develops small scale TLC-FCR method for analysis of total phenolic compounds of the samples on a TLC plate. As mentioned in **Section 3.6.2.1,** the method was performed without TLC separation, fastening the analysis time with the capability to load many samples within each analysis and requiring only 2 µL for each sample. The same sample could be loaded several times to repeat the analysis. According to **Figure 4.9A,** the spots of phenolic compounds reacted with FCR were observed with the blue color as shown in **Figure 4.9A**

4.3.2.1 Data analysis

From **Figure 4.9B,** according to ImageJ software, the blue scale was chosen for detection of the blue color of Mo (V) which is the reaction marker as mention in **Section 3.6.2.2**. The example calibration curve plotted between color value and concentration (mg/mL) of GA, as a representative of total phenolic compounds.

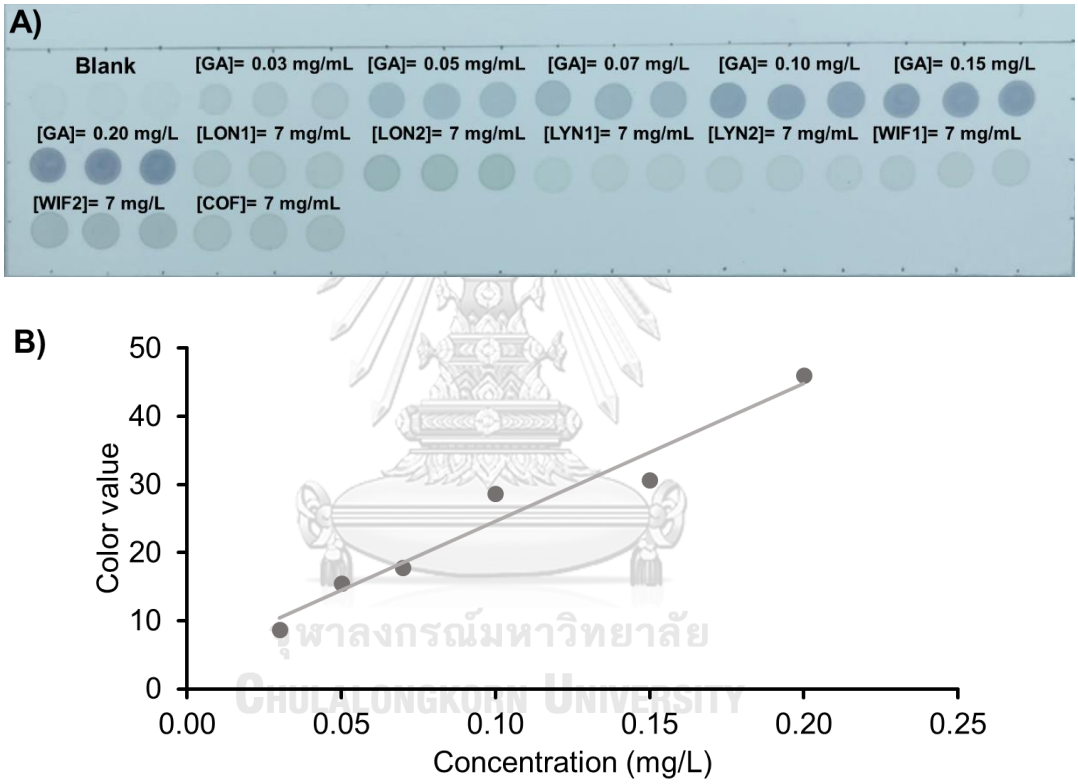


Figure 4. 9 Experimental results of 3 repeated spots of (A) honey samples (7 mg/mL) and GA at different concentrations with the corresponding plot of (B) color value vs concentration of GA at different concentrations. The linear equation is $y = 0.2024x + 4.2946$ (R^2 of 0.96).

4.3.2.1 Effect of reaction time

Figure 4.10 shows the intensity color of mixed solution between GA and FCR at various concentrations and times after loading onto the TLC plate as mention in **Section 3.6.2.3**. The intensity color represented the instable signal of each mixed solution because FCR reagent are highly sensitive to light. Therefore, it should be tested in a dark room and immediately collected the color after loading the last spot on the TLC plate to prevent signal loss.

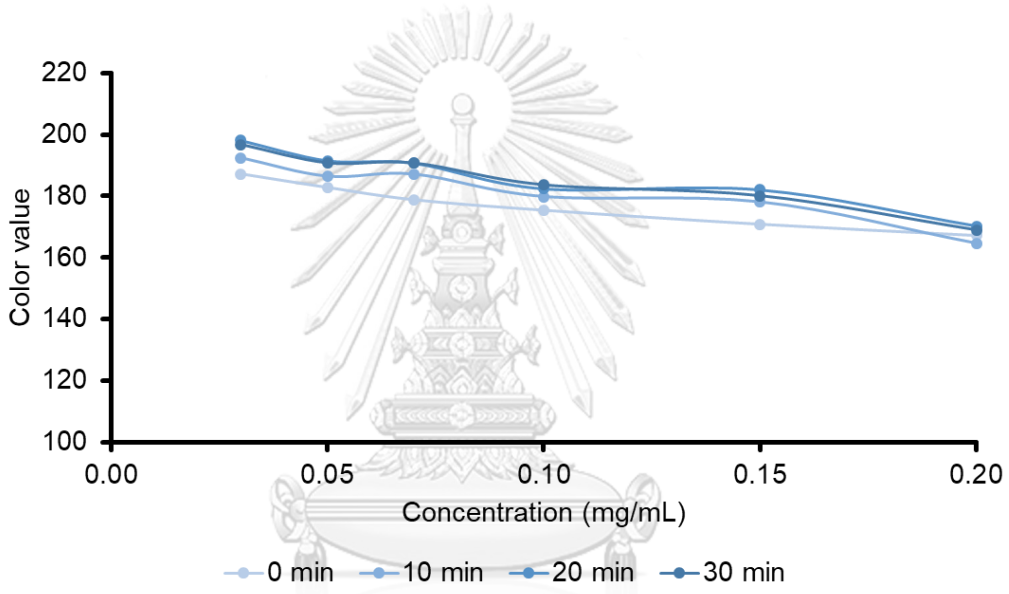


Figure 4. 10 Plots of color intensity value vs concentration of GA reacted with FCR reagent with the solutions left after loading onto a TLC at different time before being photographed.

4.3.2.2 Method validation

The method was validated by using UV-Vis spectrophotometer. Each GA standard solution in triplicate were detected at a specific wavelength of 760 nm as mention in **Section 3.6.2.4**. The developed TLC-FCR method was found to correlates well with the UV-Vis methods result. GA was used for validation of the results. The correlation between color value and absorbance results of GA solutions

at the same concentrations obtained from the developed TLC-FCR (**Figure 4.9B**) and UV-Vis methods were plotted in **Figure 4.11**. The result showed the correlation with the R^2 of 0.98. It should be noted that the developed approach enables the effective detection of total phenolic compounds.

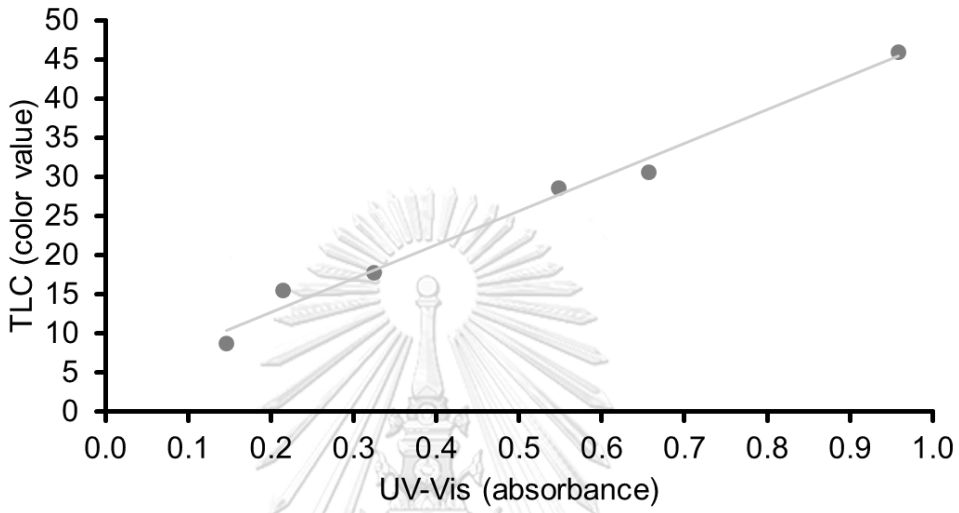


Figure 4. 11 Correlation between color value and absorbance obtained from the developed TLC-FCR and UV-Vis methods of GA at different concentrations with the linear equation of y = 43.193x + 4.0502 (R^2 of 0.99).

Table 4. 7 Validation parameter of absorbance and color value of GA using UV-Vis and TLC-FCR methods of RSD (*n* = 3)

Concentration of GA (mg/mL)	UV-Vis	TLC-FCR	Acceptable RSD (%)
	Absorbance	Color value	
0.03	25	0.23	27
0.05	16	0.43	25
0.07	6.9	0.21	24
0.10	7.8	0.52	23
0.15	4.3	0.42	21
0.20	7.4	0.65	20

From experimental in **Section 3.6.2.4**, and linear calibration plots as shown in **Figure 4.9B**, **Table 4.7** shows an RSD comparison of TLC-FCR and UV-Vis methods. Experimental RSD values from TLC-FCR were obtained in a range of 0.21-0.65%. RSD results of TLC-FCR showed better than UV-Vis method in the acceptable criteria value of a Horwitz equation.

As experiment previously mentioned in **Section 4.3.1.1**, linear calibration plot of GA was plotted, LOD and LOQ were calculated from the **equation 4.4** and **4.6**. The LOD and LOQ data obtained from the analysis of different concentration levels of GA (n = 3) are 0.016 and 0.049 mg/mL, respectively.

4.3.2.3 Application in the real samples

From **Section 3.6.2.5**, the developed method was used for determination of total phenolic compound contents of the honey extract samples at concentration of 7 mg/mL. Color value vs concentrations of GA is shown in **Figure 4.9** with good linearity range was observed with the R^2 0.95. The result of each sample was converted to mg GA/ g extract as shown in **Table 4.6.** There are 4 types of honey samples investigated in this study (longan (LON), lychee (LYN), wild flower (WIF) and coffee (COF)). All the samples were produced in the same province. The total phenolic compound content appears to be more strongly affected by the botanical origin than the supplier sources. According to the result, total phenolic compound contents were the highest in wild flower 2 and followed by longan 2, coffee, longan 1, wild flower 1, and lychee2, respectively (WIF2>LON2>COF>LON1>LYN1>WIF1>LYN2). The developed approach allows sufficient quantitative analysis of total phenolic compounds in the real samples.

Table 4. 8 Total phenolic compounds detected by the developed TLC-FCR method with standard deviation (*n* = 3) in the honey samples.

Sample	Total phenolic compounds (mg GA/g extract)
LON1	6.3 ± 0.7
LON2	12.7 ± 0.2
LYN1	1.2 ± 0.4
LYN2	0.9 ± 0.2
WIF1	1.2 ± 0.4
WIF2	14 ± 1
COF	9.6 ± 1.0

LON = longan, LYN = lychee, WIF = wild flower, and COF = coffee.

CHAPTER V

CONCLUSION

This work is aimed to develop and validate thin layer-chromatography based approach for separation of the chemical components and determination of antioxidant activity and total phenolic compound contents of Thai honey samples obtained from different flora sources (LON1, 2 = longan1, 2, LYN1, 2 = lychee1, 2 WIF1, 2 = wild flower1, 2, and COF1 = coffee; where 1 and 2 come from Foral bee and Supha farms, respectively). All the samples were extracted with dichloromethane followed by the solvent evaporation in order to enrich the extracted compound concentrations. Then, the extracts were investigated with TLC separation detected with different approaches to obtain the sample fingerprints which could differentiate the samples from different sources. Furthermore, the colorimetric approach on TLC plates was performed to quantify antioxidant activity and total phenolic compound contents. The developed TLC based techniques were then validated with the UV-Vis spectrophotometer.

For the TLC separation analysis, the honey extracts showed the characteristic spots for different botanical origins with the suitable mobile phase solution of toluene-ethyl acetate-formic acid, 5:6:1 (v/v/v) and then detected by naked eyes under the wavelength of 254 and 366 nm or use of the vanillin, DPPH• and FCR reagents. The R_f analysis could differentiate the samples especially for LYN samples with the disappeared antioxidant spots with R_f < 0.6 and the additional antioxidant and phenolic compound spots with R_f of 0.7.

The colorimetric approaches on TLC plates were successfully applied for determination of antioxidant activity and total phenolic compound contents of the extracted samples using ImageJ software for the data processing. The analyses consume <100 µL of the sample, reagent and solvent with the total analysis time of 30 min in order to obtain the antioxidant activity or total phenolic compound content for each sample. The analyses reveal that the antioxidant activities were

observed to be more strongly affected by the botanical origins. With the same manufactured date, the antioxidant activities were in the order of Longan > Lychee > Wild flower. In addition, total phenolic compound contents were the highest in wild flower 2 followed by longan 2, coffee, longan 1, wild flower 1, and lychee 2, respectively.

Finally, this work successfully demonstrated simple, small-scale, and low-cost approaches for analysis of chemical profile, antioxidant activity, and total phenolic compound contents of Thai honey samples with different flora sources. The developed approach is expected to be applicable as an alternative “green” analytical process for other food analyses in the future.

APPENDIX-A

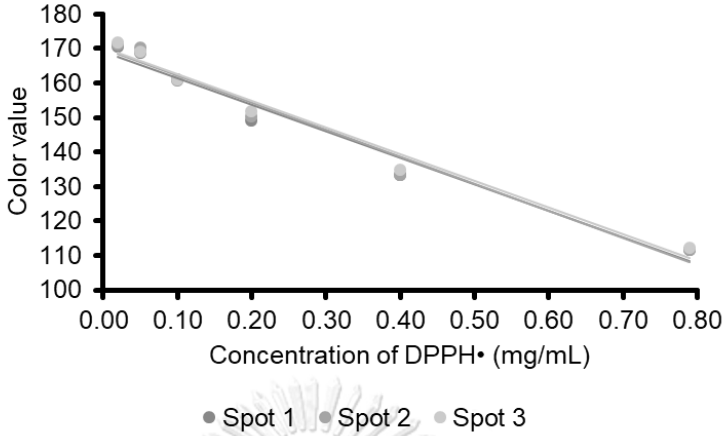


Figure A- 1 Corresponding plots of color value vs concentration of DPPH• reagent at different concentration was converted from repeated spot. The linear equations in spot 1, 2 and 3 are y = y = -76.811x + 169.06 (R^2 0.97), y = -78.201x + 169.96 (R^2 0.97) and y = -77.096x + 170.19 (R^2 0.98), respectively.

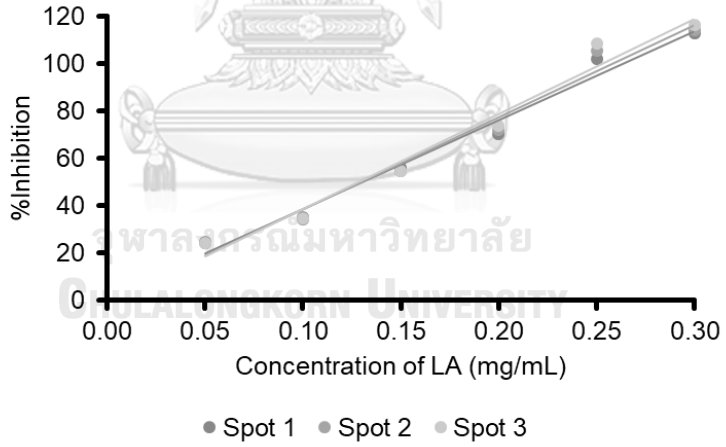


Figure A- 2 Corresponding plots of %inhibition vs concentration of LA was converted from spots of LA reacted with DPPH• reagent at different concentration. The linear equations in spot 1, 2 and 3 are y = 375.11x + 1.0485 (R^2 0.98), y = 389.08x - 0.6851 (R^2 0.98) and y = 400.85x - 1.5922 (R^2 0.97), respectively

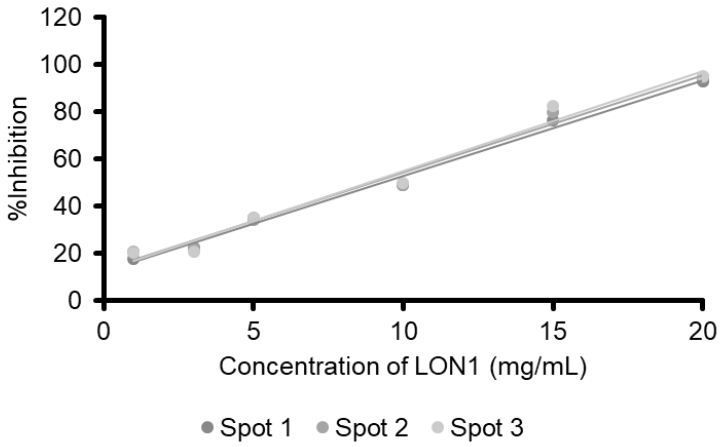


Figure A- 3 Corresponding plots of %inhibition vs concentration of LON1 was converted from spots of LON1 reacted with DPPH• reagent at different concentration. The linear equations in spot 1, 2 and 3 are y = 4.0487x + 12.31 (R^2 0.99), y = 4.134x + 12.85 (R^2 0.98) and y = 4.2433x + 12.353 (R^2 0.98), respectively.

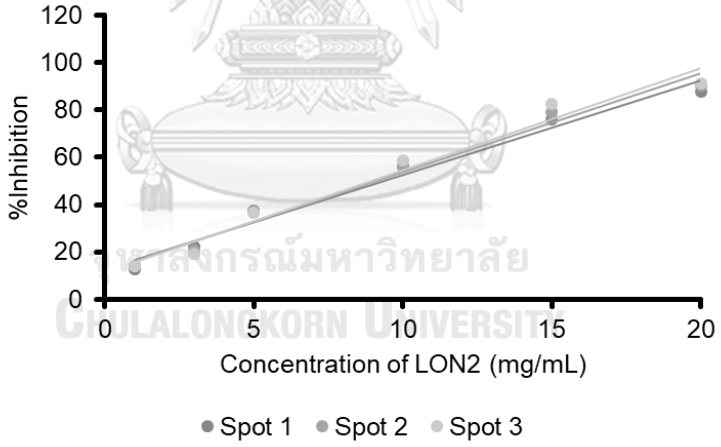


Figure A- 4 Corresponding plots of %inhibition vs concentration of LON2 was converted from spots of LON2 reacted with DPPH• reagent at different concentration. The linear equations in spot 1, 2 and 3 are y = 3.9984x + 12.767 (R^2 0.98), y = 4.1849x + 11.817 (R^2 0.98) and y = 4.3255x + 11.337 (R^2 0.97), respectively.

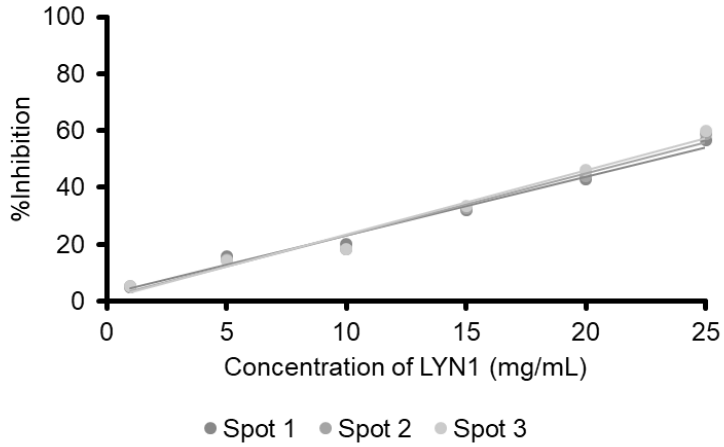


Figure A- 5 Corresponding plots of %inhibition vs concentration of LYN1 was converted from spots of LYN1 reacted with DPPH• reagent at different concentration. The linear equations in spot 1, 2 and 3 are y = 2.0674x + 2.4376 (R^2 0.98), y = 2.1755x + 1.3178 (R^2 0.98) and y = 2.2585x + 0.9666 (R^2 0.98), respectively.

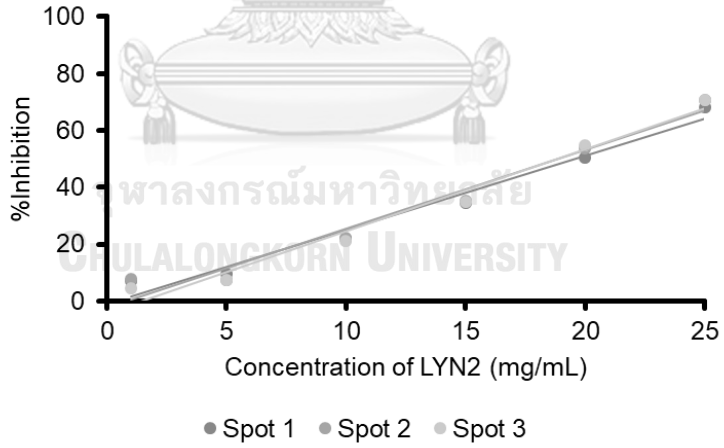


Figure A- 6 Corresponding plots of %inhibition vs concentration of LYN2 was converted from spots of LYN2 reacted with DPPH• reagent at different concentration. The linear equations in spot 1, 2 and 3 are y = 2.608x - 1.1002 (R^2 0.97), y = 2.7625x - 2.1664 (R^2 0.96) and y = 2.8721x - 4.1267 (R^2 0.98), respectively.

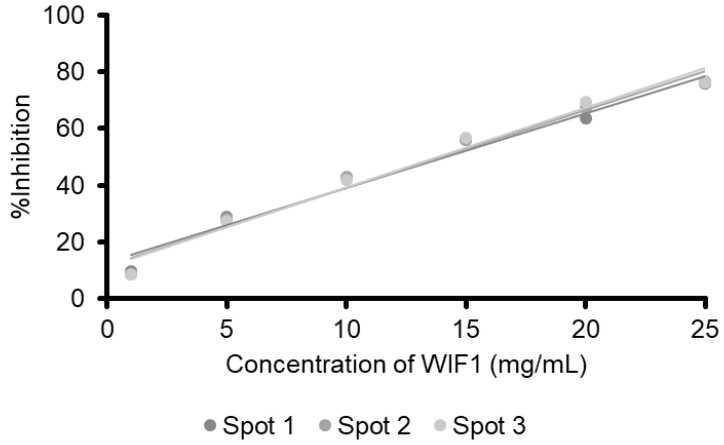


Figure A- 7 Corresponding plots of %inhibition vs concentration of WIF1 was converted from spots of WIF1 reacted with DPPH• reagent at different concentration. The linear equations in spot 1, 2 and 3 are y = 2.6248x + 12.851 (R^2 0.97), y = 2.7465x + 11.73 (R^2 0.97) and y = 2.8017x + 11.197 (R^2 0.98), respectively.

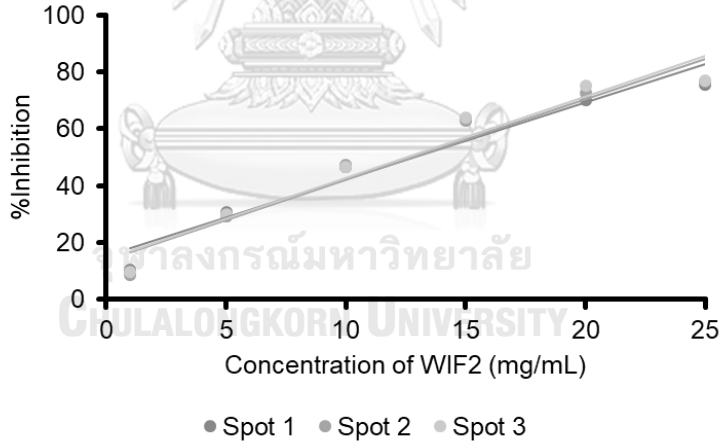


Figure A- 8 Corresponding plots of %inhibition vs concentration of WIF2 was converted from spots of WIF2 reacted with DPPH• reagent at different concentration. The linear equations in spot 1, 2 and 3 are y = 2.6888x + 15.42 (R^2 0.94), y = 2.8405x + 13.605 (R^2 0.94) and y = 2.8551x + 14.235 (R^2 0.94), respectively.

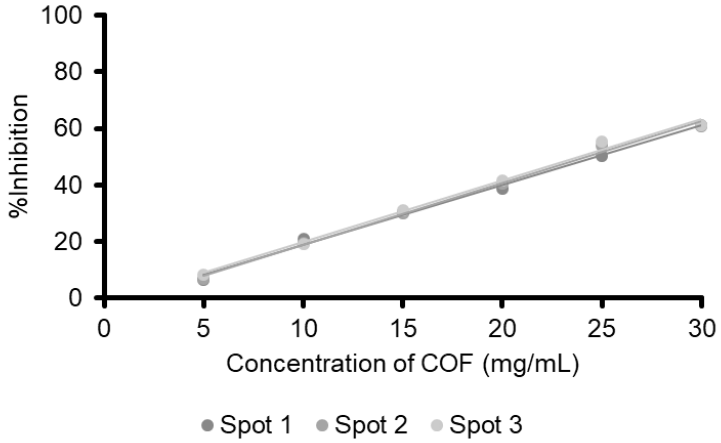


Figure A- 9 Corresponding plots of %inhibition vs concentration of COF was converted from spots of COF reacted with DPPH• reagent at different concentration. The linear equations in spot 1, 2 and 3 are y = 2.1102x - 2.2439 (R^2 0.99), y = 2.1927x - 3.062 (R^2 0.99) and y = 2.1752x - 1.9658 (R^2 0.99), respectively.

APPENDIX-B

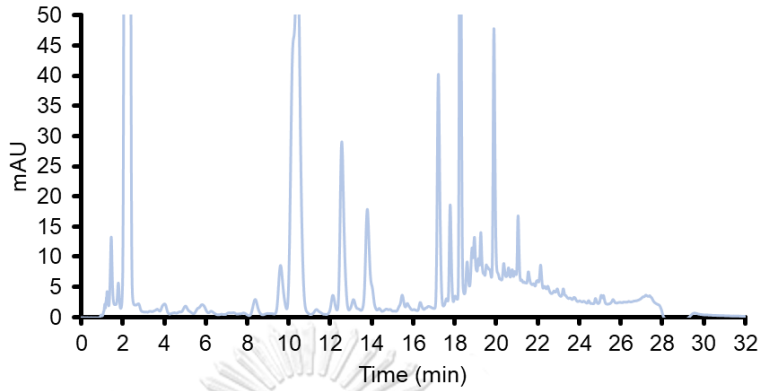


Figure B- 1 HPLC chromatogram of LON1 at wavelength 254 nm.

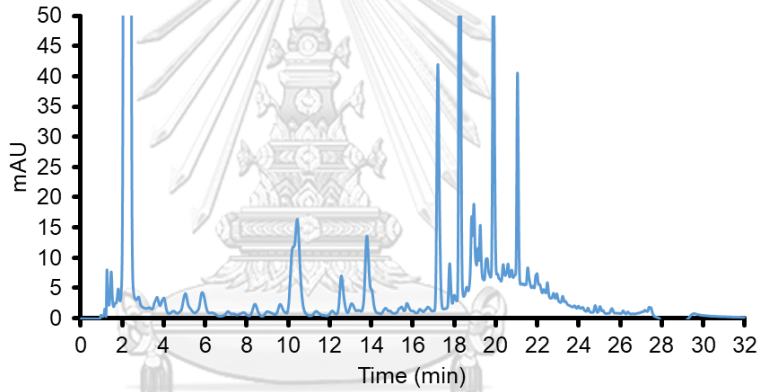


Figure B- 2 HPLC chromatogram of LON1 at wavelength 270 nm.

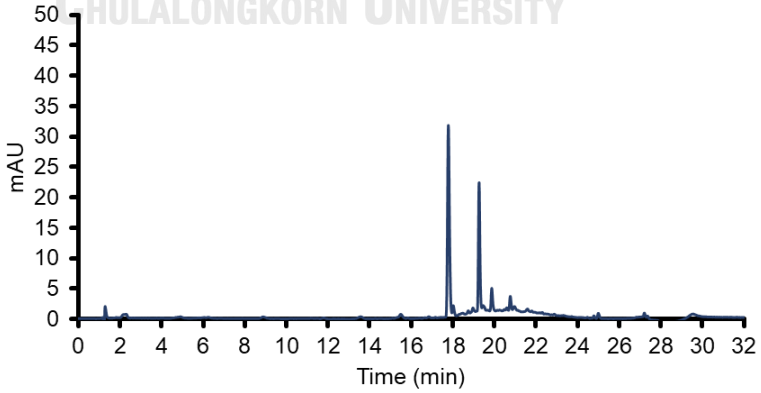


Figure B- 3 HPLC chromatogram of LON1 at wavelength 365 nm.

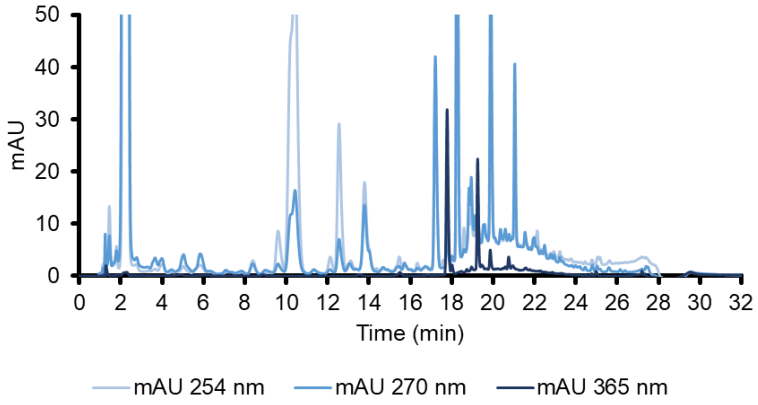


Figure B- 4 Overlay HPLC chromatogram of LON1 at 3 wavelengths.

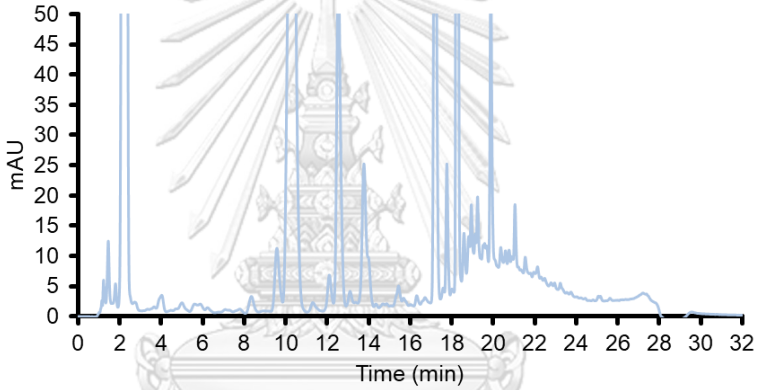


Figure B- 5 HPLC chromatogram of LON2 at wavelength 254 nm.

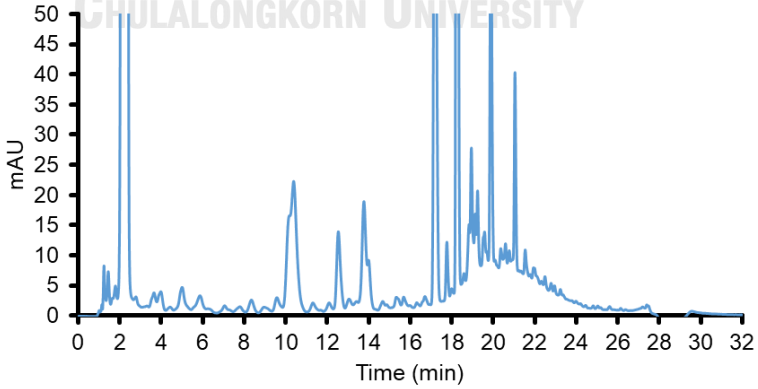


Figure B- 6 HPLC chromatogram of LON2 at wavelength 270 nm.

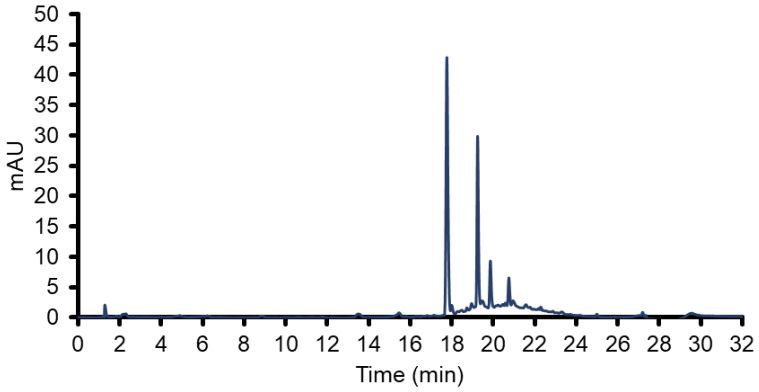


Figure B- 7 HPLC chromatogram of LON2 at wavelength 365 nm.

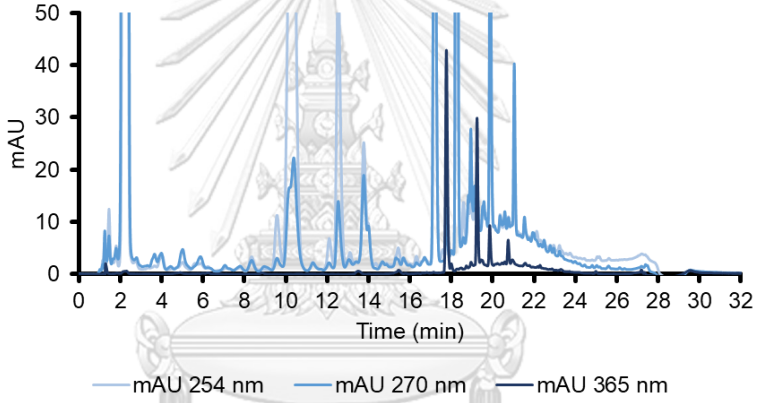


Figure B- 8 Overlay HPLC chromatogram of LON2 at 3 wavelengths.

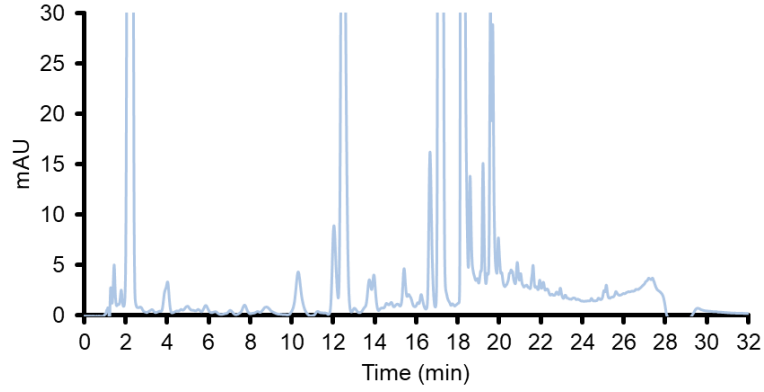


Figure B- 9 HPLC chromatogram of LYN1 at wavelength 254 nm.

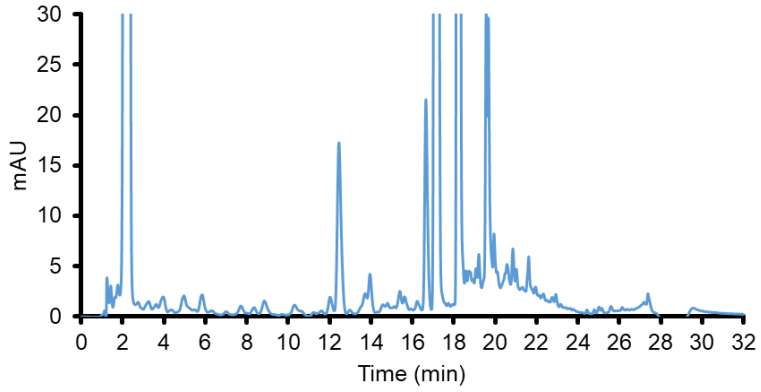


Figure B- 10 HPLC chromatogram of LYN1 at wavelength 270 nm.

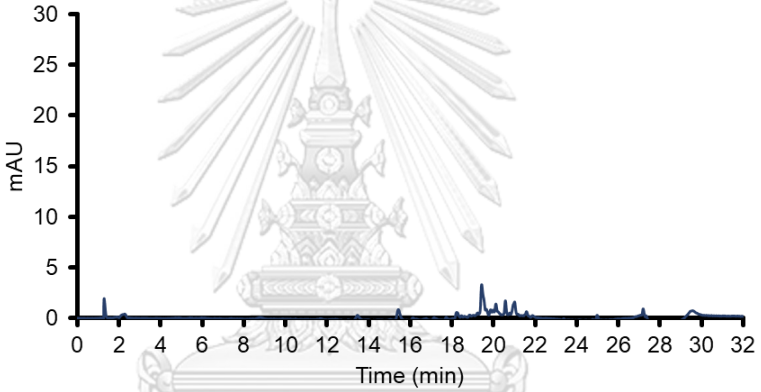


Figure B- 11 HPLC chromatogram of LYN1 at wavelength 365 nm.

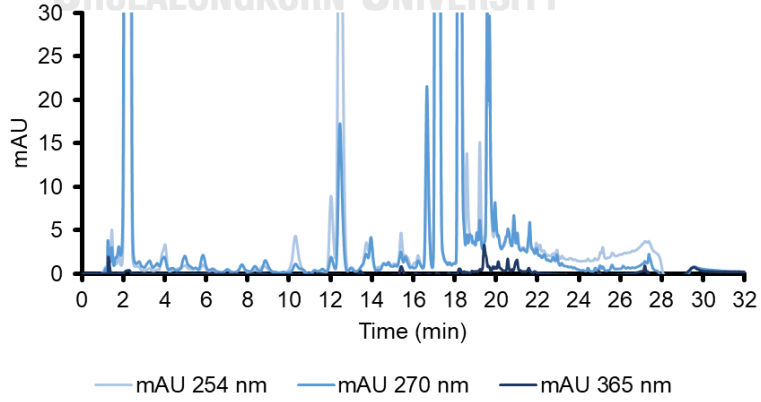


Figure B- 12 Overlay HPLC chromatogram of LYN1 at 3 wavelengths.

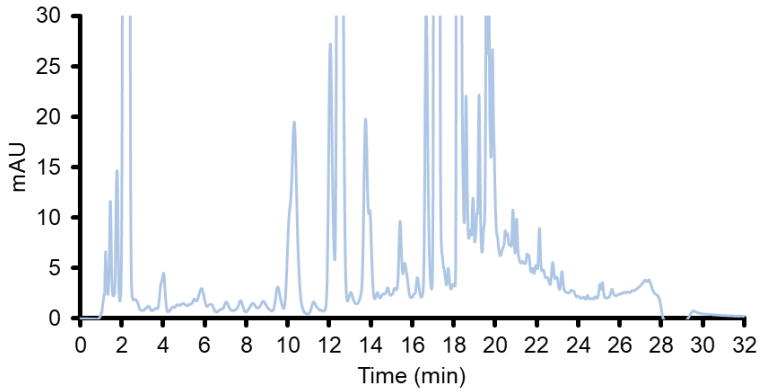


Figure B- 13 HPLC chromatogram of LYN2 at wavelength 254 nm.

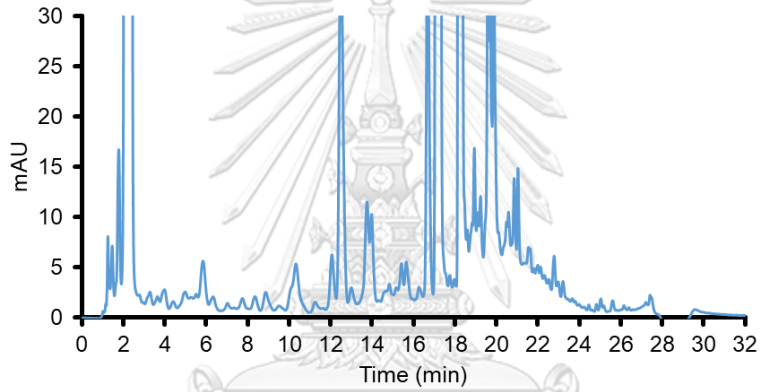


Figure B- 14 HPLC chromatogram of LYN2 at wavelength 270 nm.

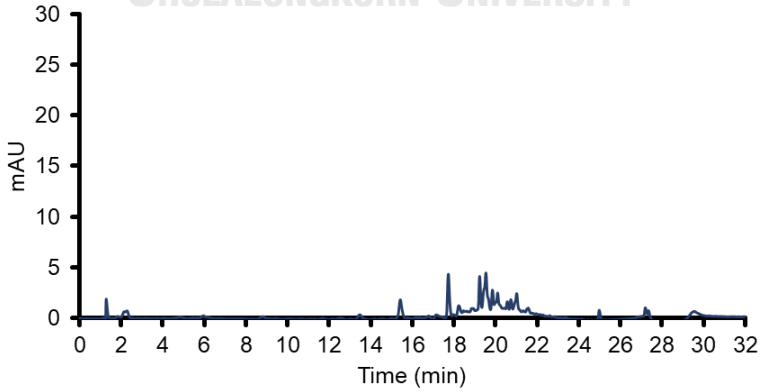


Figure B- 15 HPLC chromatogram of LYN2 at wavelength 365 nm.

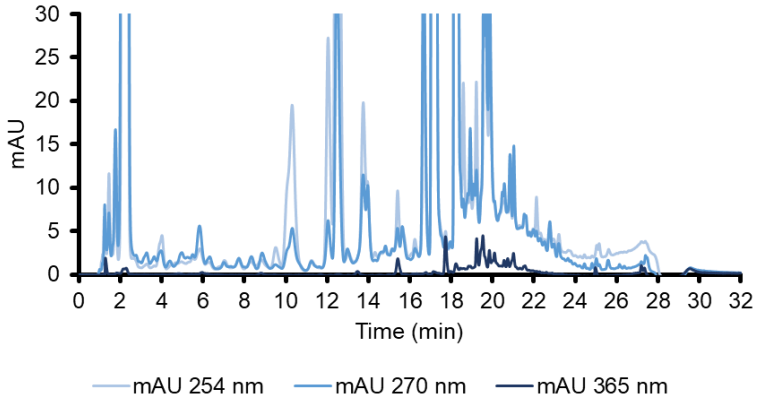


Figure B- 16 Overlay HPLC chromatogram of LYN2 at 3 wavelengths.

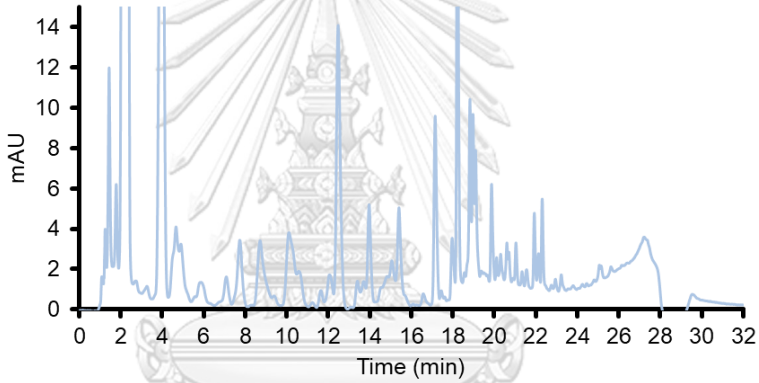


Figure B- 17 HPLC chromatogram of WIF1 at wavelength 254 nm.

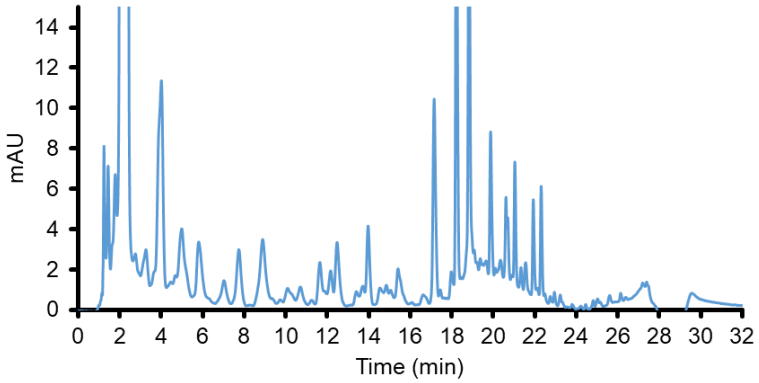


Figure B- 18 HPLC chromatogram of WIF1 at wavelength 270 nm.

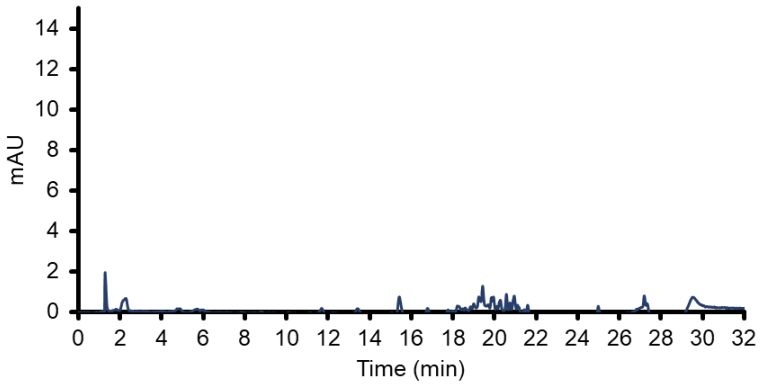


Figure B- 19 HPLC chromatogram of WIF1 at wavelength 365 nm.

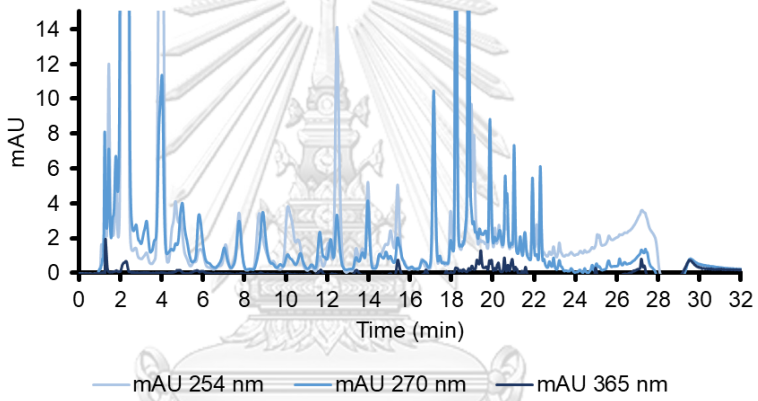


Figure B- 20 Overlay HPLC chromatogram of WIF1 at 3 wavelengths.

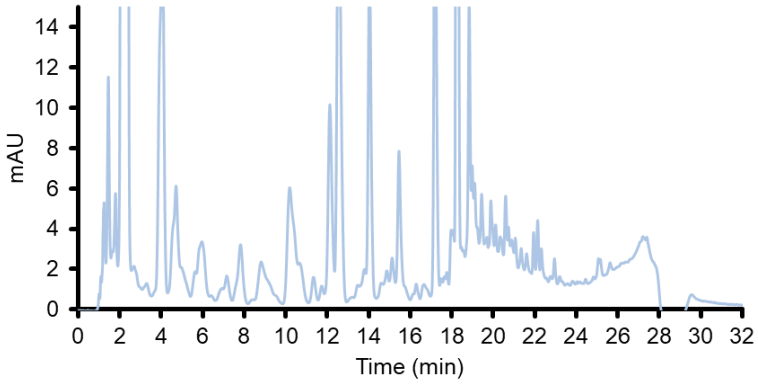


Figure B- 21 HPLC chromatogram of WIF2 at wavelength 254 nm.

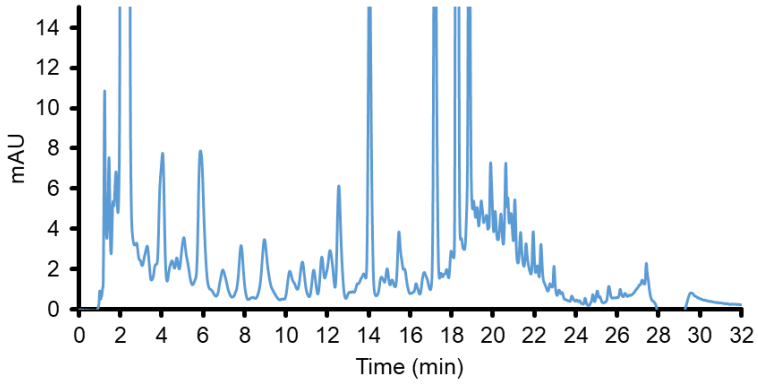


Figure B- 22 HPLC chromatogram of WIF2 at wavelength 270 nm.

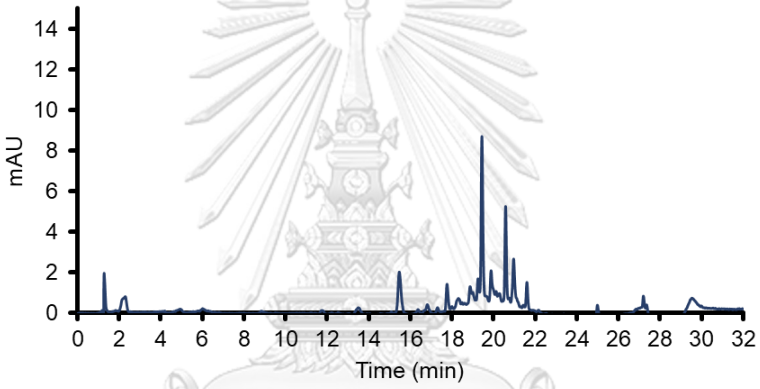


Figure B- 23 HPLC chromatogram of WIF2 at wavelength 365 nm.

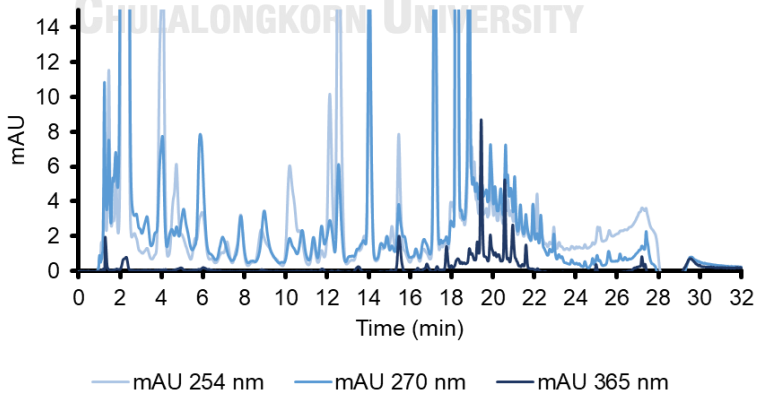


Figure B- 24 Overlay HPLC chromatogram of WIF2 at 3 wavelengths.

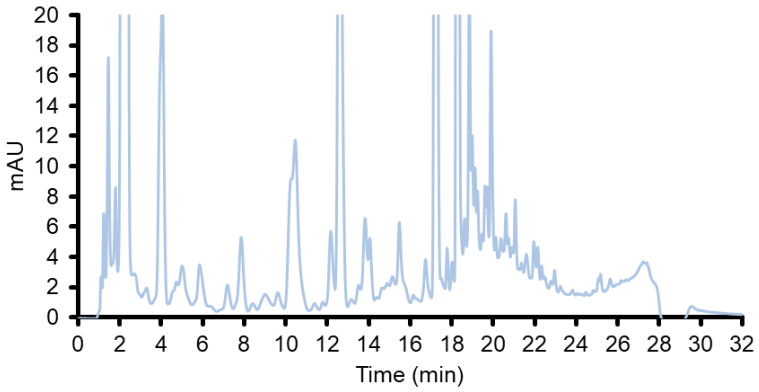


Figure B- 25 HPLC chromatogram of COF at wavelength 254 nm.

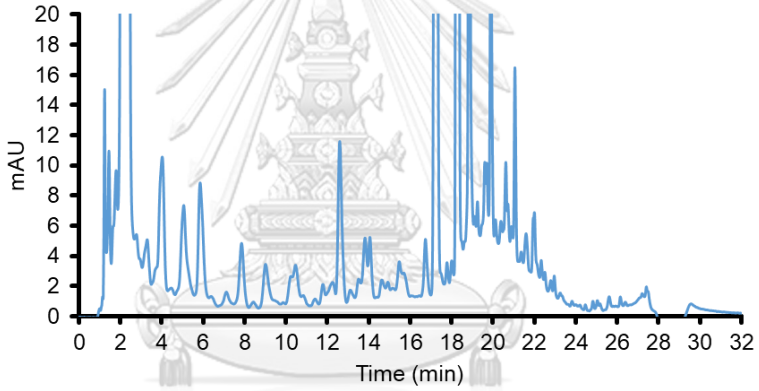


Figure B- 26 HPLC chromatogram of COF at wavelength 270 nm.

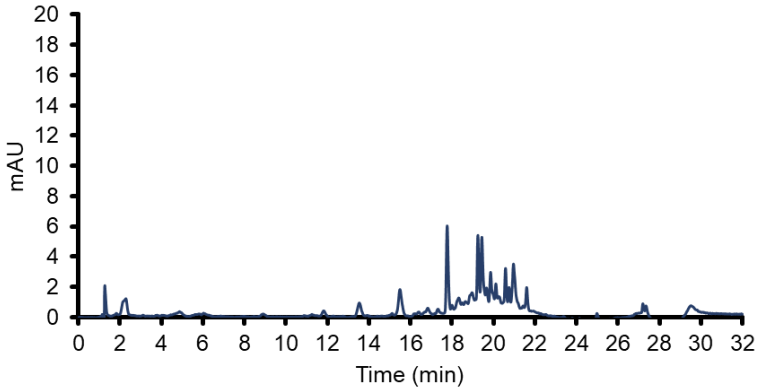


Figure B- 27 HPLC chromatogram of COF at wavelength 365 nm.

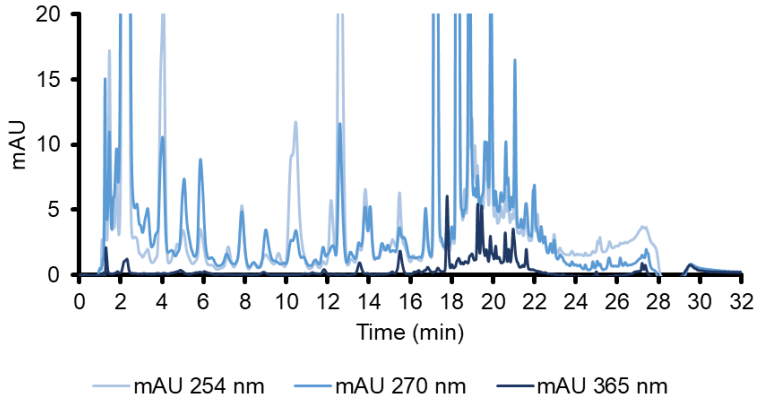


Figure B- 28 Overlay HPLC chromatogram of COF at 3 wavelengths.

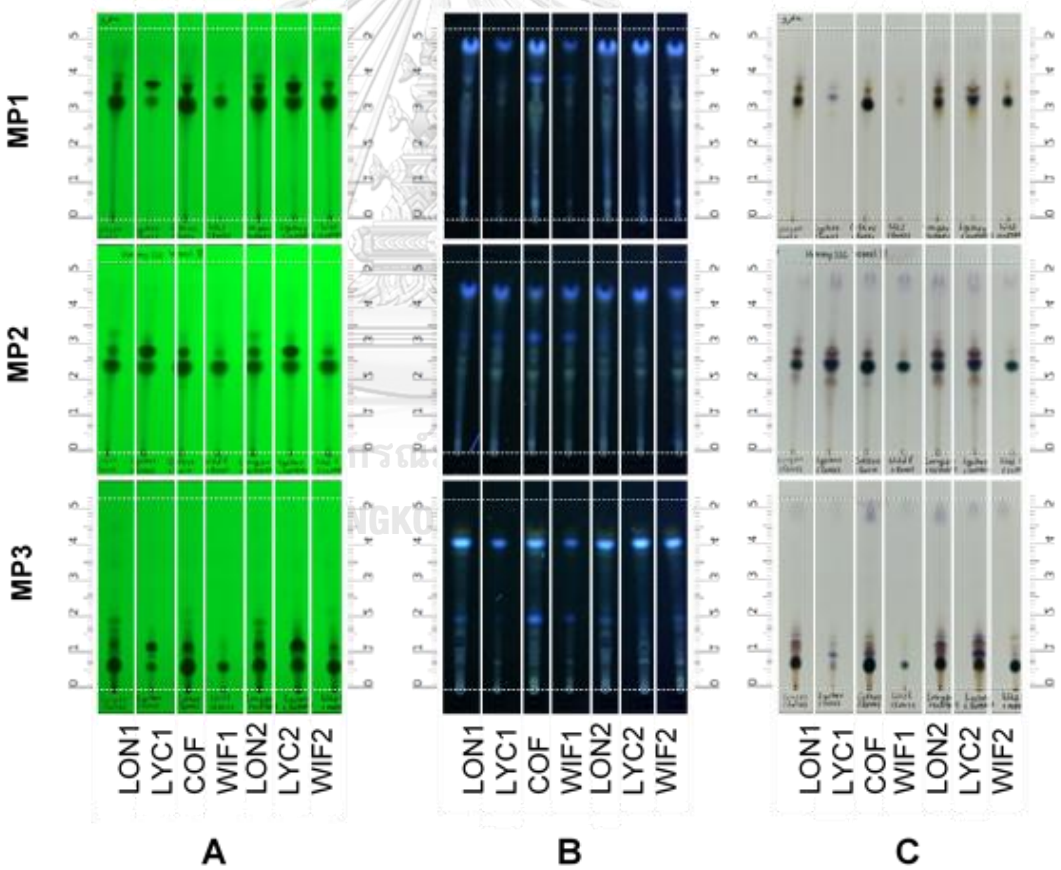


Figure B- 29 TLC results of the crude honey extracts detected at 254 nm (A), 366 nm (B), and the photograph after the vanillin derivatization (C). Mobile phase: toluene-ethyl acetate-formic acid, 3:8:1 (MP1), 5:6:1 (MP2), 10:1:1 (v/v/v) (MP3).

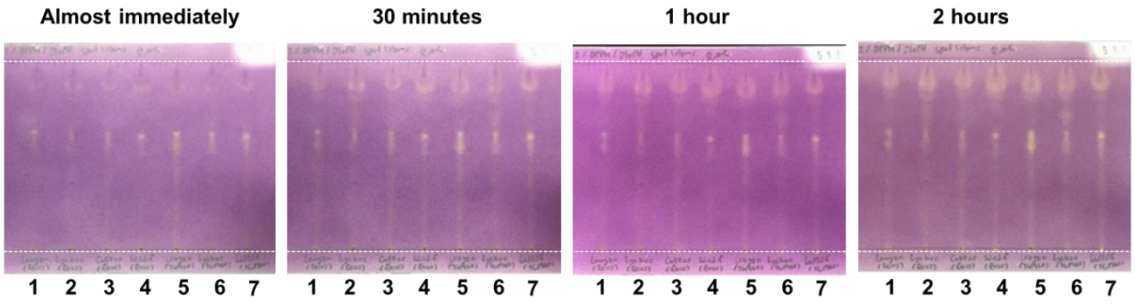


Figure B- 30 TLC photographs of the crude honey extracts with 0.2%v/v DPPH; LON1 (1), LYN1 (2), COF (3), WIF1 (4), LON2 (5), LYN2 (6) and WIF1 (7) at different reaction time.

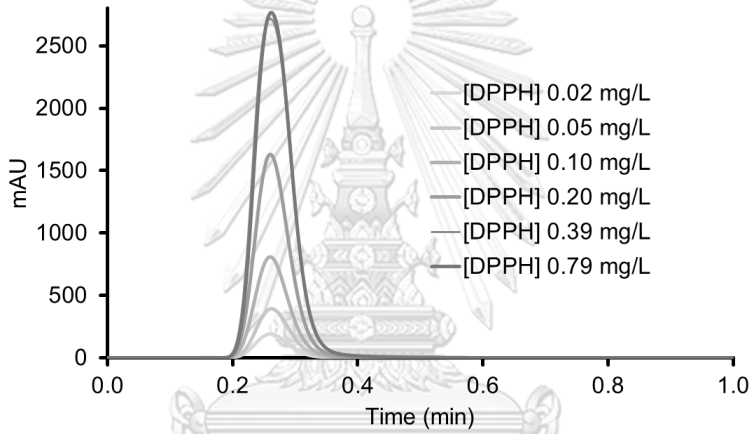


Figure B- 31 HPLC chromatogram of DPPH• reagent at different concentration.

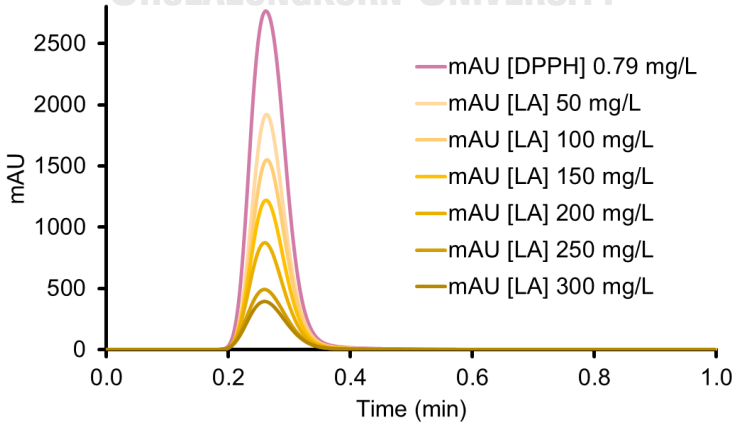


Figure B- 32 HPLC chromatogram of LA react with DPPH• at different concentration.

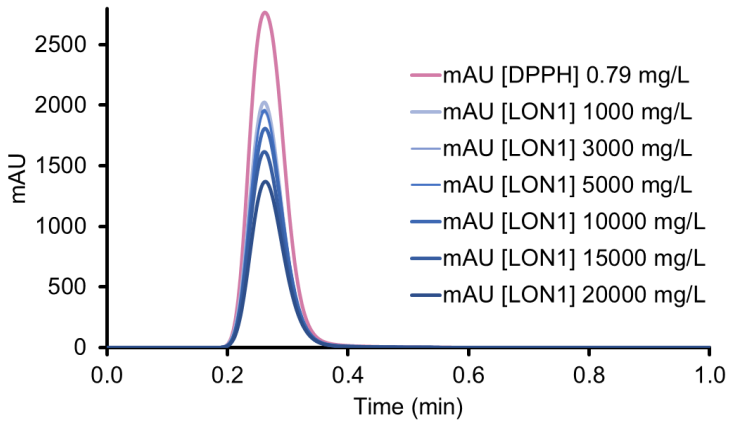


Figure B- 33 HPLC chromatogram of LON1 react with DPPH• at different concentration.

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