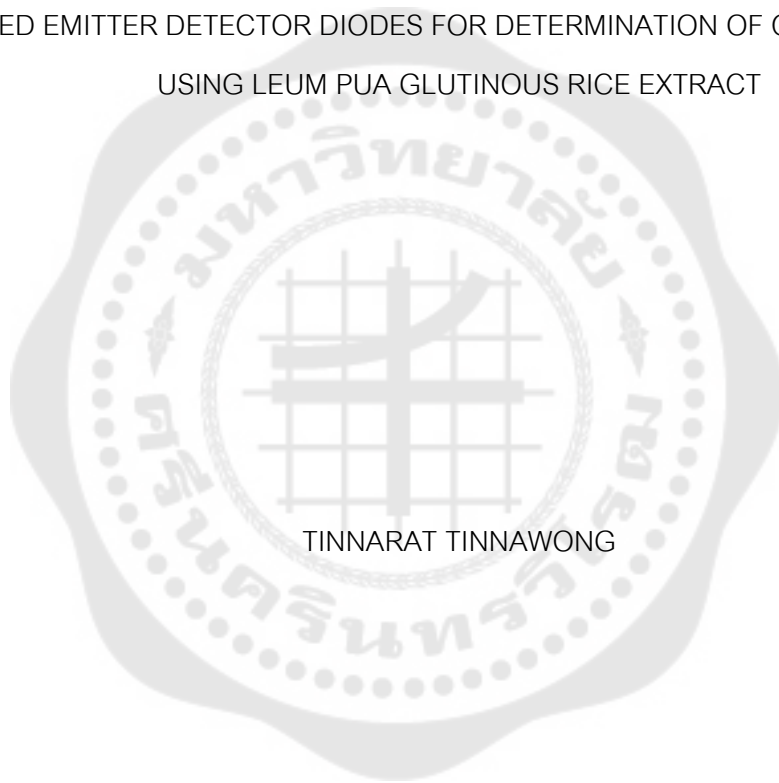




DEVELOPMENT OF PAPER-BASED ANALYTICAL DEVICE WITH  
PAIRED EMITTER DETECTOR DIODES FOR DETERMINATION OF COPPER (II) ION  
USING LEUM PUA GLUTINOUS RICE EXTRACT



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2020

การพัฒนาอุปกรณ์ตรวจวัดแบบกระดาษร่วมกับตัวตรวจวัดชนิดพีอีดีดีสำหรับการวิเคราะห์คอปเปอร์ (II) ไอออน โดยใช้สารสกัดจากข้าวลิ้มผั่ว



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A Thesis Submitted in Partial Fulfillment of the Requirements  
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(Chemistry)

Faculty of Science, Srinakharinwirot University

2020

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BY  
TINNARAT TINNAWONG

HAS BEEN APPROVED BY THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR THE MASTER OF SCIENCE  
IN CHEMISTRY AT SRINAKHARINWIROT UNIVERSITY

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Title	DEVELOPMENT OF PAPER-BASED ANALYTICAL DEVICE WITH PAIRED EMITTER DETECTOR DIODES FOR DETERMINATION OF COPPER (II) ION USING LEUM PUA GLUTINOUS RICE EXTRACT
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This work presents the use of a paper-based analytical device (PAD) with paired emitter detector diodes (PEDD) and a natural reagent extracted from Leum Pua glutinous rice for the detection of Cu (II). The natural reagent contained anthocyanin compounds, which react with copper (II) ion in a buffer solution (pH 5.7), forming a blue complex. This blue product was detected on PAD with a PEDD device in two minutes. In the optimum conditions, the linearity was in the concentration range of 0.50-10.00 mg/L ( $R^2 = 0.9973$ ). The relative standard deviations (RSD) were less than 1.85 and 2.12% for intraday and interday precision, respectively. The limit of detection (LOD) was 0.15 mg/L and the limit of quantification (LOQ) was 0.42 mg/L. The developed method successfully applied the analysis of copper (II) in water samples. The percentage recovery was in the range of 96.87-99.64. This developed method is simple, rapid and environmentally friendly.

Keyword : Paper-based Analytical Device, Paired Emitter Detector Diodes, Copper (II), Leum Pua Glutinous Rice, Natural reagent

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## TABLE OF CONTENTS

	Page
ABSTRACT .....	D
ACKNOWLEDGEMENTS.....	E
TABLE OF CONTENTS.....	F
LIST OF TABLES.....	I
LIST OF FIGURES .....	J
CHAPTER 1 INTRODUCTION .....	1
Background.....	1
Objectives of This Study .....	2
Scopes of This Study .....	3
Expected Outputs .....	3
CHAPTER 2 LITERATURE REVIEW.....	4
1. Basic chemistry of copper .....	4
2. Paper-based analytical device (PAD) .....	5
3. Paired emitter detector diodes (PEDD) .....	7
4. Leum Pua glutinous rice .....	8
5. Literature reviews .....	11
5.1 Analytical method for the determination of copper (II) ions. ....	11
5.2 Applications of paper-based analytical devices (PAD).....	13
5.3 Applications of natural reagents for the analysis of metal ions .....	15
CHAPTER 3 RESEARCH METHODOLOGY .....	17
1. Instrumentation .....	17

1.1 Instrumentation .....	17
1.2 Paired emitter detector diodes (PEDD) device.....	18
2. Chemicals and reagents .....	18
3. Experimental method.....	19
3.1 Fabrication of the PAD .....	19
3.2 Preparation of crude anthocyanin-rich extracted from Leum Pua glutinous rice .....	20
3.3 The effect of pH on the selectivity of a crude anthocyanin-rich extract.....	21
3.4 Study of optimum conditions for determination of copper (II) ion using the PAD with PEDD and natural reagent extracted from Leum Pua glutinous rice....	22
3.5 Study of interfering metal ions for determination of copper (II) ion using Leum Pua glutinous rice extract with PAD-PEDD.....	23
3.6 Analytical characteristics of the developed method .....	23
3.7 Application of the PAD with PEDD device for determination of copper (II) in water samples .....	24
CHAPTER 4 RESULTS.....	25
1. Characterization of crude anthocyanin-rich reagent extracted from Leum Pua glutinous rice .....	25
2. The effect of pH on the selectivity of a crude anthocyanin-rich extract.....	27
3. Study of optimum conditions for determination of copper (II) ion using the PAD with PEDD and natural reagent extracted from Leum Pua glutinous rice.....	31
3.1 Effect of the total solution volume .....	31
3.2 Effect of the sequence of reaction .....	31
3.3 Effect of natural reagent extract concentration.....	33



3.4 Effect of equilibration time.....	34
4. Effects of interfering metal ions for determination of copper (II) ion using Leum Pua glutinous rice extract with PAD-PEDD .....	35
5. Analytical characteristics of the developed method .....	36
5.1 Linearity range.....	36
5.2 The limit of detection (LOD) and the limit of quantification (LOQ) .....	37
5.3 The method precision.....	37
6. Application of the PAD with PEDD device for determination of copper (II) in water samples .....	38
CHAPTER 5 SUMMARY DISCUSSION AND SUGGESTION .....	39
REFERENCES.....	41
VITA .....	48

## LIST OF TABLES

	Page
Table 1. The results of the nutritional analysis of the Leum Pua glutinous rice and the Hom Nilin rice. ....	9
Table 2. The form of anthocyanidin in plants. ....	10
Table 3. Optimization of the sequence of reactions on the paper-based analytical device ....	22
Table 4. The precisions of the developed method for the determination of copper (II) ion. ....	37
Table 5. The percentage of recovery was analyzed using the developed method in various water samples. ....	38

## LIST OF FIGURES

	Page
Figure 1. Various fabrication methods of the hydrophobic barrier on $\mu$ PAD. ....	6
Figure 2. Scheme and photo of prototypes of PAD compatible PEDD-based detectors dedicated to transmittance (A) and reflectance (B) optical measurements. ....	8
Figure 3. The structure of anthocyanidin. ....	10
Figure 4. The PAD with PEDD-based photometer setup in this work. ....	18
Figure 5. The PAD is used in this work. ....	20
Figure 6. (A) Color of anthocyanin-rich reagent from Leum Pua glutinous rice in methanol (B) UV-Vis spectrum of reagent obtained from 200.0-800.0 nm. ....	26
Figure 7. $^1\text{H}$ NMR spectrum of anthocyanin-rich extracted from Leum Pua glutinous rice. ....	27
Figure 8. (A) The color of complexes between natural reagent extract from Leum Pua glutinous rice and each metal solution at pH 3.0-8.0 on PAD. (B) The spectra of complexes between natural reagent extract from Leum Pua glutinous rice and various metal solutions at pH 6.0. ....	28
Figure 9. Structural forms of anthocyanins at different pH. ....	29
Figure 10. The structure of the anthocyanin-rich extract-Cu (II) complex. ....	30
Figure 11. The signal from PAD with PEDD measurement for detection of copper (II) ion in buffer solution at pH 5.0-6.0. ....	30
Figure 12. The total volume of solution loaded onto the PAD. ....	31
Figure 13. Effect of the sequence of reaction on PAD. ....	32
Figure 14. The effect of loading steps of the reaction mixture on the PEDD signal of Cu (II) solution in a concentration range of 1.0-10.0 mg/L. (A) Three steps; reagent-buffer (pH5.7)-Cu (II) and (B) two steps; reagent-Cu (II) in buffer (pH5.7). ....	33

Figure 15. Effect of the natural reagent extract concentration on PEDD signal.....	34
Figure 16. Effect of the equilibration time in 60-120 seconds on PEDD signal using copper (II) solution at a concentration range of 1.0-10.0 mg/L. ....	35
Figure 17. Interfering effects of metal ions for determination of copper (II) ion using Leum Pua glutinous rice extract with PAD-PEDD detection. Detection data are expressed as means $\pm$ SD (n=3). ....	36
Figure 18. The calibration curve is obtained by this proposed method.....	36



## CHAPTER 1

### INTRODUCTION

#### Background

Copper (Cu) is an important element for all plants, animals and industries. Copper has many good properties, including electrical conductivity, resistance to corrosion and bacterial growth inhibition. It is widely used in various industries, such as electroplating, metal finishing, electrical, fertilizer and pigment industries. However, it can be toxic to human health and environments when they receive in excessive amounts. An excess amount of copper can induce various diseases such as anemia, kidney and liver damage, intestinal and stomach irritation. Copper is also a common hazardous pollutant in wastewater and releases metallurgical, plating, fertilizer and refining industries. Therefore, it is important to monitor copper (II) content to decrease the potential risk of high copper (II) contents in the environment.

Several methods have been reported for copper (II) determination based on atomic absorption spectrometry (AAS) (Özzybek et al., 2017; Sousa et al., 2018; Tobiasz & Walas, 2014), inductively coupled plasma atomic emission spectrometry (ICP-AES) (Nunes et al., 2011) and UV-visible spectrophotometry (Babayeva et al., 2017; Niu et al., 2016; Paluch et al., 2018). These methods are excellent in selectivity and sensitivity. However, they still have some disadvantages, such as expensive instruments, time-consuming, unable to be portable and trained operators. The analytical method for an environmental application using the rapid and low-cost instrument is widely interesting.

In the last decade, many research groups have developed a paper-based microfluidic analytical device. This device has many advantages, including ease of use, low cost, simple fabrication and on-site detection (Almeida et al., 2018; A. W. Martinez et al., 2007). Moreover,  $\mu$ PADs can be used with a small and portable detector, namely a paired emitter detector diode (PEDD). The PEDD-based photometric device is a simple optical sensor that consists of two light-emitting diodes (LEDs). One LED used as an emitter source and a second LED as a light detector comprises a novel photometric device. PEDD detector is low-cost, small in size, and consumes a small amount of power.

In addition, PEDD has a simple operating principle and is easily customized for particular kinds of analysis (Lau et al., 2006; Tymecki & Koncki, 2009; Tymecki et al., 2008).

Nowadays, the concept of green chemistry is highly required to minimize or avoid producing harmful solvents, reagents and waste. Some synthetic reagents are harmful to the body and affect the environment due to a lack of appropriate treatment or disposal systems. One approach to acquire an environmentally friendly analysis method is utilizing natural compounds in plants as alternative reagents. Various natural compounds have been widely used as natural reagents for metal analysis, such as Butterfly pea flower (Grudpan et al., 2011), *Hibiscus rosa-sinensis* Linn flower (Porrawatkul et al., 2018), *Morinda citrifolia* (Tontrong et al., 2012), Ginkgo leaves (Yang et al., 2016), Red cabbage (Khaodee et al., 2014), *Phyllanthus emblica* (Jaikrajang et al., 2018), Turmeric (Supharoek et al., 2018) and Pumpkin (Supharoek et al., 2017).

In this work, anthocyanin-rich extract from Leum Pua glutinous rice was used as a reagent to determine copper (II). Leum Pua is in traditional Thai glutinous rice that contains a high nutritional value. It has also been reporting that the Leum Pua glutinous rice contains a high amount of bioactive compounds, namely phenolic acids, flavonoids and anthocyanin substances (Peanparkdee et al., 2019). Anthocyanin can react with some metal ions and produce a colored product, which is detected by the naked-eye method. (Khaodee et al., 2014; Kondo et al., 1998; Makris & Rossiter, 2002; Yoshida et al., 2006). To the best of our knowledge, there is no report on the use of PAD with an in-house PEDD-based photometer to determine copper (II) in water samples using natural reagent extract from Leum Pua glutinous rice.

### Objectives of This Study

1. Develop a rapid and environmentally friendly method using PAD with an in-house PEDD-based photometer to determine copper (II) employing natural reagent extract from Leum Pua glutinous rice.
2. To apply the developed method for quantitative analysis of copper (II) in water samples.

### Scopes of This Study

1. The extraction and characterization of crude anthocyanin-rich reagent from Leum Pua glutinous rice by UV-Vis spectrophotometry and nuclear magnetic resonance spectrometry.
2. The development of PAD with a PEDD-based photometer for the determination of copper (II) by optimization of various parameters including pH, the total volume of solution, sequence of reaction, reagent concentration, and equilibration time.
3. The validation of the analytical performance of the developed method in terms of linearity, limits of detection, intra- and inter-day precisions and recovery.
4. The application of developed PAD with a PEDD-based photometric method to detect copper (II) in water samples.

### Expected Outputs

1. An environmentally friendly method using PAD with in-house PEDD detection for copper (II) measurement was obtained.
2. The developed method was successfully applied to determine copper (II) in various water samples.

## CHAPTER 2

### LITERATURE REVIEW

In this work, the related concept and literature review of analytical methodologies have been introduced on the following topics:

1. Basic chemistry of copper
2. Microfluidic paper-based analytical device ( $\mu$ PAD)
3. Paired emitter detector diodes (PEDDs)
4. Leum Pua glutinous rice
5. Analytical methods for copper (II) determination

#### 1. Basic chemistry of copper

Copper is a chemical element with the symbol Cu and atomic number 29. Copper has many beneficial properties, including good thermal and electrical conductivity, good corrosion resistance, hygienic, low chemical reactivity, recyclable and attractive color. Its compounds exist in predominantly two oxidation states: cuprous, Cu (I) and cupric, Cu (II). The aqueous solution of copper (II) ions is more stable than copper(I) ions. Copper is a common substance widely released into the environment by natural sources, agriculture, mining and industries. It is widely used in many industries such as electroplating, electrical power, fertilizer and pigment industries. It has many good properties, but it is also toxic. Soluble copper (II) compounds are pollutants that are harmful to human health. Long-term exposure to copper can irritate the respiratory system. High uptakes of copper may cause kidney and liver damage, Wilson's disease, and even death. (U.S. Department of Health & Human Services, 2020)

Therefore, it is imperative to monitor the level of copper (II) contaminated in water samples to decrease the potential risk of high copper (II) contents released into the environment. The Pollution Control Department, Ministry of Natural Resources and Environment has controlled the amount of copper ions in natural water not exceeded 2.0 mg/L and the permissible limit of copper ions in drinking water to be 1.0 mg/L. (The Pollution Control Department, 2016)



## 2. Paper-based analytical device (PAD)

Microfluidic devices are an innovative platform technology for manipulating and controlling small amounts of fluids, usually in microliters to nanoliters, through microchannel with dimensions from 10-100 micrometers (Lisowski & Zarzycki, 2013). Microfluidic devices are used in many applications because they have displayed attractive advantages, including less sample volume, cost-effectiveness, portability and in-situ analysis. Many materials are used to fabricate microfluidic platforms such as plastics, glass, silicone, polydimethylsiloxane (PDMS) and other polymers. Each material has particular advantages and disadvantages. In the last decade, a new generation of analytical devices has emerged based on the cellulose paper material called paper-based analytical device (PAD).

Paper is now considered an attractive substrate material for microfluidic applications because of its inexpensiveness, flexibility, lightness, ease of safe handling and disposal, and inherent capillary flow. Compared with conventional analytical devices made of glass and polymer substrates, PAD possesses many unique advantages, easy-to-fabricate, including low-cost, strong capillary action and using a low sample and reagent consumption. (Andres W. Martinez et al., 2010)

Various fabrication methods of a hydrophobic barrier on PAD have been developed, as shown in Figure. 1 (Cate et al., 2015). Fabrication of paper-based analytical devices can be divided into four general categories: handcrafted, masks, printing and cutting/shaping.

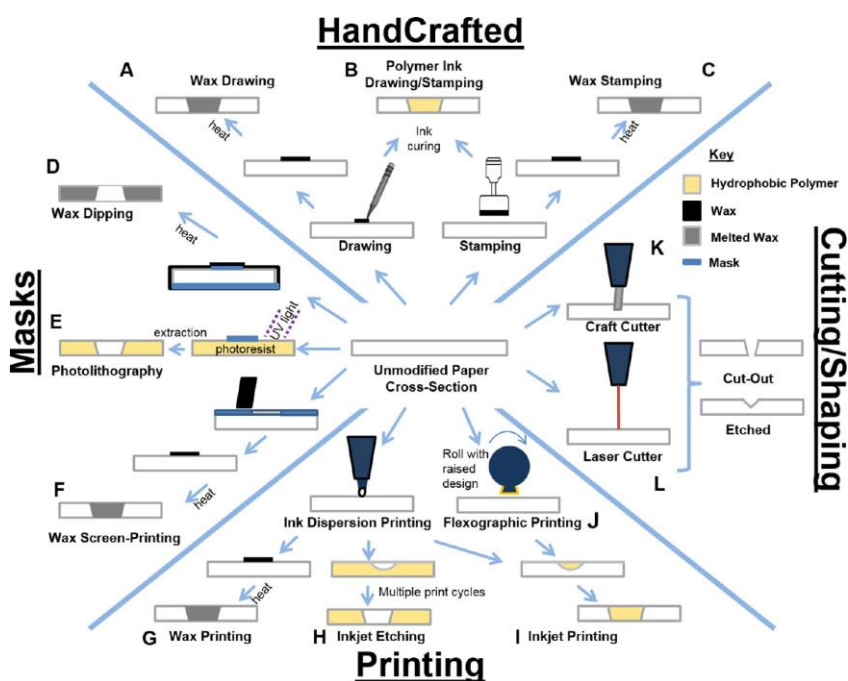


Figure 1. Various fabrication methods of the hydrophobic barrier on  $\mu$ PAD.

Source: Cate, D. M., Adkins, J. A., Mettakoonpitak, J., & Henry, C. S. (2015). Recent developments in paper-based microfluidic devices. *Anal Chem*, 87(1), 19-41.

The paper channels are generally obtained in the cutting category by cutting the paper with a cutter or laser cutting. The main disadvantage of this method is that it is difficult to control the channel size and requirements of special equipment precisely. The photolithography is based on entire hydrophobization, followed by select dehydrophobization of the paper. Nevertheless, the necessity of multiple steps, the high cost of the equipment needed and limits its wide use. The PAD fabrication by printing technique is one of the low-cost device fabrication methods. Permanent ink is printed on the paper and penetrates the depth of the paper to form a hydrophobic wall. However, ink formulation depends on inkjet printing and requires an extra heating step. The wax impregnation technology was introduced in 2009. The wax-based fabrication techniques are based on selective hydrophobization using a non-toxic reagent. The drawback of the wax-based method is melting the wax during long term-storage at ambient air conditions and the samples were possible cross-contamination. Recently, a method of stamping ink has been proposed as an alternative production method based on forming a hydrophobic

barrier on paper using a stamp and permanent ink (Mathaweensurn et al., 2020). This method is low-cost, simple and convenient to patterns on the paper.

In this work, the PAD device was fabricated using the stamping method. The designed hydrophobic structure will be patterned by contact stamping of permanent ink on filter paper using a rubber stamp to form a hydrophobic barrier. The PAD will be dry within a few minutes at ambient temperature and ready to use.

### 3. Paired emitter detector diodes (PEDD)

Paired emitter detector diodes (PEDD) is a simple and inexpensive optical detector consisting of a pair of light-emitting diodes (LEDs) that has been developed by Diamond's group (Lau et al., 2006). One LED is used as a LED light source or light emitter, while the other LED is a light detector. Diamond's group proposed PEDD detectors that showed accurate and precise photocurrent using the threshold detector and timer circuit. The detection principle is based on measuring the time taken for the photon-induced current to discharge the reversed bias LED from an initial 5 V to 1.7 V.

Tymecki, Pokrzywnicka and Koncki developed a convenient and simple PEDD detector by employing a common pH-meter (Tymecki et al., 2008) or a digital multimeter with high input impedance (Tymecki & Koncki, 2009). The PEDD detector is based on a potential measurement generated at the LED detector, which varies with the logarithm of the incident light intensity. The measured voltage is therefore reduced linearly to the absorbance of the solution.

The PEDD detector was successfully applied as a detector for photometric measurement of absorbing or non-absorbing solution in the batch-wise method (Lau et al., 2006; Mieczkowska et al., 2011) and flow-based analysis method (Manthong et al., 2019; Strzelak et al., 2016). In 2018, Fiedoruk-Pogrebniak, Granica and Koncki proposed compact PEDD detectors for photometric and fluorometric measurements on microfluidic paper-based analytical devices or PAD, as shown in Figure. 2 (Fiedoruk-Pogrebniak et al., 2018)

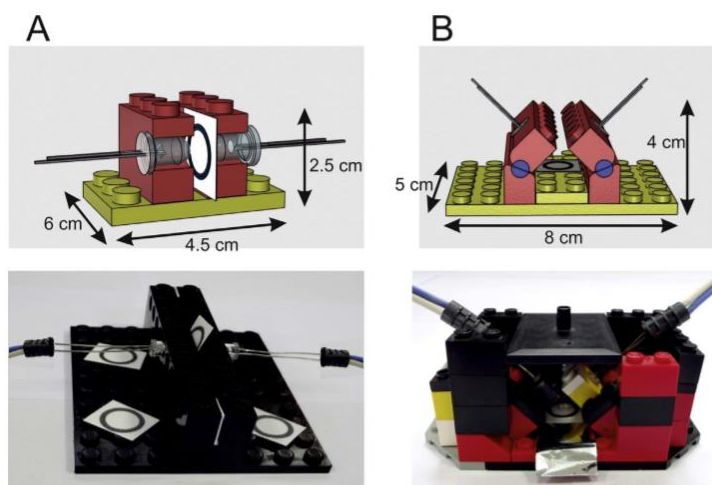


Figure 2. Scheme and photo of prototypes of PAD compatible PEDD-based detectors dedicated to transmittance (A) and reflectance (B) optical measurements.

Source: Fiedoruk-Pogrebniak, M., Granica, M., & Koncki, R. (2018). Compact detectors are made of paired LEDs for photometric and fluorometric measurements on paper. *Talanta*, 178, 31-36.

In this work, the PEDD detector was designed and developed to measure a color change of PAD for analysis of copper(II) ion using a natural anthocyanin reagent.

#### 4. Leum Pua glutinous rice

“Leum Pua” Rice or “Forget husband” rice is Thai purple-black glutinous rice known over 20 years ago by Hmong people in Phop Phra District, Tak province. Nowadays, it is trendy for the consumer due to its high nutritional value. It is known as a source of omega-3, omega-6, omega-9, vitamin E,  $\gamma$ -oryzanol, calcium, zinc, manganese and lipid compositions, as shown in Table 1, reported by Rice Research and Development Division (Rice knowledge Bank, 2016). The nutrients, antioxidants and anthocyanin in Leum Pua glutinous rice were found than other colored rice. Table 1 demonstrated that Leum Pua glutinous rice contains 40 folds more levels of anthocyanins than Hom Nilin rice. Anthocyanins are naturally occurring pigments belonging to the group of flavonoids or polyphenol compounds. The major source of anthocyanin is color rice bran. Anthocyanin is also found in the number of plants, flowers and fruits. It appears in red,

purple, blue, or black color, depending on its pH. In acidic conditions ( $\text{pH} < 7$ ), anthocyanins give red color. Anthocyanins appear as purple color in neutral ( $\text{pH} = 7$ ) while the color changes to blue in alkaline condition ( $\text{pH} > 7$ ). Anthocyanins in plants exist as *o*-glycosides and acylglycosides of anthocyanidins. Anthocyanidin is the water-soluble glycoside of 2-phenylbenzopyrylium or flavylium cation (Figure. 3). Sugar such as glucose, galactose, rhamnose, xylose and arabinose may be substituted at the  $R_3$  position. The significant anthocyanin forms found in nature consist of six anthocyanidins, namely delphinidin, cyanidin, petunidin, peonidin, malvidin and pelargonidin. (Rice knowledge Bank, 2016) Each form has different substitution groups at  $R_1$  and  $R_2$  positions, as shown in Table 2.

Table 1. The results of the nutritional analysis of the Leum Pua glutinous rice and the Hom Nilin rice.

Nutrient	Leum Pua glutinous rice*	Hom Nil Rice**	Unit
Monounsaturated fatty acid	1.16	0.91	g/100g
Polyunsaturated fatty acid	1.19	0.88	g/100g
Unsaturated fat	2.35	1.76	g/100g
Omega 3	33.9	31.5	mg/100g
Omega 6	1,160	850	mg/100g
Omega 9	1,146	901	mg/100g
Niacin	6.48	5.78	mg/100g
Vitamin E (Alpha-Tocopherol)	16.8	7.78	mg/kg
Gamma-oryzanol	491	412	mg/kg
Calcium (Ca)	170	122	mg/kg
Zinc (Zn)	23.6	23.8	mg/kg
Manganese (Mn)	35.4	22.3	mg/kg
Anthocyanin(Cyanidin-3- <i>o</i> -glucoside)	46.6	1.4	mg/100g
Total antioxidant	834	193	mg Ascorbic acid/100g

\*Planted at a farm condition in Khao Kho District, Phetchabun, 2009.

\*\*Planted in rice fields. Naresuan University, Phitsanulok, 2009.

Source: Phitsanulok Rice Research Center. Rice Research and Development Division. (2012). Leum Pua glutinous rice.

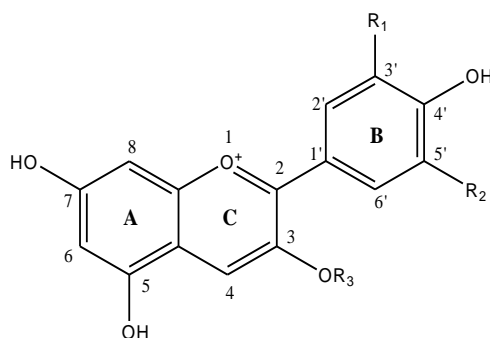


Figure 3. The structure of anthocyanidin.

Table 2. The form of anthocyanidin in plants.

Compounds	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
Delphinidin (Dp)	OH	OH	H
Cyanidin (Cy)	OH	H	H
Petunidin (Pt)	OCH <sub>3</sub>	OH	H
Peonidin (Pn)	OCH <sub>3</sub>	H	H
Malvidin (Mv)	OCH <sub>3</sub>	OCH <sub>3</sub>	H
Pelargonidin (Pg)	H	H	H

Source: Bureau of Science and Technology Information. Ministry of Science and Technology. (2010). Anthocyanin.

The hydroxyl groups (-OH) and methoxyl groups (-OCH<sub>3</sub>) substitution will cause different anthocyanidin colors. The increase in the number of hydroxyl groups substitution gives bluish shade while increasing the number of methoxy groups causes reddish color.

## 5. Literature reviews

### 5.1 Analytical method for the determination of copper (II) ions.

#### 5.1.1 Atomic absorption spectroscopic methods

In 2017, Özzeybek et al. (Özzeybek et al., 2017) developed a trace amount of copper (II) ions analysis in environmental samples, including tap water, seawater and wastewater. Dispersive liquid-liquid microextraction (DLLME) coupled with a slotted quartz tube (SQT) was used to enhance the signals of copper measurement using flame atomic absorption spectrometry (FAAS). The results showed that the DLLME-SQT-FAAS method provided higher sensitivity than the standard FAAS method. The detection and quantitation limits were calculated as 0.7 and 2.2  $\mu\text{g/L}$ , respectively. The sample throughput was 79 samples/hour. Percentages of recoveries for all tested samples were in the range 78-99.

In 2018, Sousa et al. (Sousa et al., 2018) developed a method for Cu (II) and Cd (II) ions determination in the river and swimming pool water samples using the FAAS method with online preconcentration on polyurethane foam functionalized with phenylfluorone. The detection limits were that the quantification limits were 0.8 and 2.6  $\mu\text{g/L}$  for Cu (II) ion and 0.3 and 0.8  $\mu\text{g/L}$  for Cd (II) ion. The precision of the procedure was 7.6% at the concentration of 25  $\mu\text{g/L}$  Cu(II) and 4.0% at a concentration of 10  $\mu\text{g/L}$  Cd(II). The percentages of recovery varied in the range of 83-109.

In 2019, Suquila and Tarley (Suquila & Tarley, 2019) had been reported an analytical method based on simultaneous online sample preparation and preconcentration of Cu (II) ions from milk samples using copper imprinted poly(allylthiourea) modified with HEMA and BSA detection by FAAS. The method provided a calibration curve in the range 3.6 to 100.0  $\mu\text{g/L}$ . The preconcentration factor was 24-fold. The limit of detection was 1.1  $\mu\text{g/L}$ . The sample throughput was 20 samples/hour. This method is low-cost and good sensitivity for copper ions detection in milk samples.

#### 5.1.2 Electrochemical methods

In 2015, Oliveira et al. (Oliveira et al., 2015) used biochar as an electrode modifier for the determination of copper (II) ion in drinking water using differential pulse

adsorptive stripping voltammetric (DPAdSV) technique. The linearity response for copper ions was in the range concentration of  $1.5 \times 10^{-6}$  to  $3.1 \times 10^{-5}$  mol/L with a limit of detection of  $4.0 \times 10^{-7}$  mol/L. The precision of the method, as shown in % RSD, was 6.6.

In 2018, Deshmukh et al. (Deshmukh et al., 2018) studied and developed the differential pulse voltammetry (DPV) method for the measurement of copper(II), lead(II) and mercury(II). Single-walled carbon nanotubes (SWCNTs) and EDTA chelating ligand were exploited to modify the electrode surface. The limit of detection of copper (II), lead (II) and mercury (II) ions was 0.08  $\mu$ M, 1.65  $\mu$ M and 0.68  $\mu$ M, respectively.

In 2019, Frag et al. (Frag & Abdel Hameed, 2019) developed CuNiO nanoparticles on graphite sheets [CuNiO/Gt] coupled with the potentiometric method using modified carbon paste electrode (MCPE) for the determination of copper ions in water samples. The effects of CuNiO content, pH and response time on the measured signal were optimized. The detection limit was  $1.0 \times 10^{-6}$  mol/L. The percentages of recovery varied in the range of 102-103.

#### 5.1.3 Spectrophotometric methods

In 2017, Gonzalez et al. (Gonzalez et al., 2017) studied and developed a microsequential Injection Lab-on-Valve coupled with online spectrophotometric detection to determine copper (II) ions. The sample and reagent volumes were only 150.0 microliters and 120.0 microliters, respectively. The limit of detection was 12.0  $\mu$ g/L. The precision of the developed method showed in %RSD was 2. The percentage recovery was in the range of 90-107.

Niu et al. (Niu et al., 2016) developed microplate technology with colorimetric detection to measure copper (II) ions in animal feed samples. The copper (II) ions from the animal feed were extracted with trichloroacetic acid and then formed a complex with 2,20-bicinchoninic acid (BCA) before measurement absorbance values at wavelength 354.0 nm. The analysis can be within 20 minutes. The limit of detection was 0.33  $\mu$ g/ml. The percentages of recovery varied in the range of 96.44-109.79. The developed method was successfully applied to analyze copper (II) ions in a real sample. The obtained results were in agreement with atomic absorption spectroscopic results.



Babayeva et al. (Babayeva et al., 2018) developed a method for analysis of copper (II) ions in natural water and wastewater samples using N, N'-bissalicylidene-2,3-diaminopyridine ( $H_2IF$ ) as complexing agent and detection the complexed product at a wavelength of 414.0 nm. The standard curve was linear in the concentration range of 6.35-318  $\mu\text{g/L}$ . The limit of detection was 6.38  $\mu\text{g/L}$ . The proposed method is precision in terms of % RSD was less than 0.62 for copper (II) ions at a concentration of 63.5  $\mu\text{g/L}$ . The developed method was successfully used for the analysis of copper (II) ions in the samples.

In 2018, Paluch et al. (Paluch et al., 2018) studied and developed sequential injection (SI) systems coupled with solid-phase extraction for analysis of zinc (II) ions and copper (II) ions in rain and soil samples. The complex of 1-(2-pyridylazo)-2-naphthol (PAN) complex and metal ions was detected at 560.0 nm. The calibration curve provided linearity in the concentration range of 0.005-0.3 and 0.011-0.2 mg/L for zinc (II) and copper (II) ions. The precisions of the proposed method shown that in% RSD was in the range of 5.1-6.0. The detection limits were 1.4 and 3.0  $\mu\text{g/L}$  for zinc (II) ions and copper (II) ions.

## 5.2 Applications of paper-based analytical devices (PAD)

In 2016, Karita and Kaneta (Karita & Kaneta, 2016) developed  $\mu\text{PAD}$  for chelate titrations of calcium (II) and magnesium (II) in water samples (mineral water, river water and seawater). The designed  $\mu\text{PAD}$  had a sample zone at the center on paper and ten reaction zones and was detected radially. The sample was added into the middle of  $\mu\text{PAD}$  and moved through the channel to react with different EDTA concentrations at reaction zones. The number of uncomplexed metal ions penetrated to react with Eriochrome Black T or Calcon at the detection zone. The developed method was successfully applied for the determination of calcium (II) and magnesium (II) concentrations in mineral water, river water and seawater. The results determined by  $\mu\text{PAD}$  were in good agreement with those obtained by classical titrations.

Ortiz-Gomez et al. (Ortiz-Gomez et al., 2016) fabricated the  $\mu\text{PAD}$  for the determination of nitrites content in water samples. The  $\mu\text{PAD}$  consisted of a sample zone

connected with three detection zone. The s-dihydropyridazine solution was added into the detection zone to observe color change color from colorless to pink when reacting with nitrite. A digital camera and smartphone measured the color of the detection zone. The limit of detection was 1.30  $\mu\text{M}$ .

Chaiyo et al. (Chaiyo et al., 2016) described the method for the detection of lead, copper and cadmium ions using  $\mu\text{PADs}$  with electrochemical colorimetric detections. In work, the analysis of lead and cadmium was simultaneously detected using diamond electrodes. For copper detection, color change of AgNPIs to purple was observed with the naked eye. The detection limit was 0.1 ng/mL for lead and cadmium and 5.0 ng/mL for copper.

In 2017, Pratiwi et al. (Pratiwi et al., 2017) developed  $\mu\text{PAD}$  for the detection of copper (II) in drinking water using distance-based quantification. The copper (II) was detected using a porphyrin derivative as a colorimetric reagent under pH 2.0-4.0. After adding copper (II) to the channel, the naked eye noticed a change in color from green to pink. The length of the pink band increases as the concentration of copper (II) increases. Under the optimized condition, the lowest concentration of copper (II) was 1.0 mg/L. This method is rapid, simple, instrument-free, cost-effective and selective for on-site copper (II) analysis.

In 2019, Peters et al. (Peters et al., 2019) developed a 3D  $\mu\text{PAD}$  for the determination of ammonia in samples. The  $\mu\text{PAD}$  device consisted of two layers, sample zone and detection zone with hydrophilic circular shapes. A sample zone impregnated with sodium hydroxide converts ammonium in samples into ammonia gas and reacts with an acid-base indicator (nitrazine yellow or bromothymol blue) in the detection zone. The limit of detection were 0.32 and 0.47 mg N/L, respectively. The linearities were 0.5-3.0 mg N/L and 2.0-10 mg N/L when using nitrazine yellow and bromothymol indicators. The inter- and intra- repeatabilities of 7.6% and 9.0% were obtained for the nitrazine yellow and bromothymol blue-based  $\mu\text{PAD}$ , respectively.

Wu et al. (Wu et al., 2019) developed a method for the detection of copper in water samples by solid-phase extraction (SPE) column combined with  $\mu\text{PAD}$ . The

colorimetric reagent in this method was 1-(2-pyridylazo)-2-naphthol in polyvinyl butyral used for complexation with copper (II) ion to form colorless products. The detection limit was 0.340  $\mu\text{M}$ , which was less than the limit amount of copper (II) ion in drinking water. This method is a convenient, easy, cheap, rapid and on-site assessment of copper.

### 5.3 Applications of natural reagents for the analysis of metal ions

In 2012, Tontrong et al. (Tontrong et al., 2012) studied and developed a flow injection technique to determine aluminium (III) ion in tea samples using natural reagent extract from *Morinda citrifolia* root. The anthraquinone complex extracted from *Morinda citrifolia* root and aluminium (III) ion at pH 4.5 appeared red color. The wavelength for detection was 499.0 nm. The linearity was in the concentration of 0.1-1.0 mg/L with the limit of detection of 0.05 mg/L. The relative standard deviations at 0.1 and 0.6 mg/L of aluminium (III) ion were 1.2 and 1.7, respectively. The sample throughput of this proposed method was 35 samples/hour.

In 2014, Khaodee et al. (Khaodee et al., 2014) used cyanidin extracted from red cabbage as a reagent for the determination of Cu(II), Al (III), Fe (III) and Pb (II) in water samples by the naked-eye method. Cyanidin extracted can react with Cu (II), Al (III), Pb (II) and Fe (III) to form a colored complex at pH 7, pH 5, pH 6 and pH 4, respectively. The limit of detection were 50  $\mu\text{M}$  for Cu (II) and Al (II), 80  $\mu\text{M}$  for Pb (II) and 200  $\mu\text{M}$  for Fe (III).

In 2016, Watsaka et al. (Watsaka et al., 2016) developed a flow injection spectrophotometric method with natural reagent extract from Heartwood of *Caesalpinia sappan Linn* quantification of aluminium (III) ion. The natural reagent can react with aluminium (III) ion at pH 5.5 to form a red color complex with the maximum absorbance at 530.0 nm. The linear range was in the concentration of 0.075-1.0 mg/L. The limit of quantification and the limit of detection were 0.072 and 0.021 mg/L, respectively. The relative standard deviations of 0.1 and 0.25 mg/L aluminium (III) ion were 3.2 and 2.4, respectively. The sample throughput was 128 samples/hour. The percentage recovery was in the range of 87-104.

Yang et al. (Yang et al., 2016) used natural isorhamnetin extract from Ginkgo leaves as a fluorescent sensor to detect copper(II) ion. The reaction between natural isorhamnetin with copper (II) ion at pH 7.4 generated fluorescence quenching. The developed method was applied to quantify copper (II) ion in water samples, vegetables and fruits. The calibration curve was linear in the concentration range  $1.0 \times 10^{-8}$  -  $1.9 \times 10^{-6}$  mol/L with the limit of detection of  $4.0 \times 10^{-9}$  mol/L.

In 2018, Jaikrajang et al. (Jaikrajang et al., 2018) studied and developed the flow injection spectrophotometric method to determine copper (II) ion using natural reagent extract from *Phyllanthus emblica* Linn. The natural reagent and copper(II) ion reaction gave the dark-purple complex at pH 5.6 with maximum absorbance at wavelength 570.0 nm. The limit of detection and the limit of quantification were 0.31 and 0.50 mg/L, respectively. The linearity was in the concentration range of 0.50-20.0 mg/L, with a correlation coefficient of 0.9996. The precision presented in terms of relative standard deviation was 2.5.

Porrawatkul et al. (Porrawatkul et al., 2018) studied and developed the naked-eye method for the detection of copper (II) and aluminium (III) ions in water using natural reagent extract from *Hibiscus rosa-sinensis* Linn. The natural reagent can react with copper (II) ion and aluminium (III) ion at pH7 to give a purple complex. The masking agents, namely NaF and DMG, were also used to determine copper (II) ion and aluminium (III) ion, respectively. The limit of detection was 0.5 and 1.0 mg/L for copper (II) ion and aluminium (III) ion, respectively. The results of the developed method were compared with the standard method (ICP-AES), and no significant differences were noticed.

Khaodee et al. (Khaodee et al., 2018) studied reagent extract from the purple sweet potato method to detect copper (II) ion in water by the naked-eye method. The natural reagent can react with copper (II) ion at pH7. It was found that the complex showed color differences from the blank. The detection limit was  $3 \times 10^{-4}$  M. The results of the developed method were compared with the standard method (AAS) and the two techniques were similar.

## CHAPTER 3

### RESEARCH METHODOLOGY

The following steps describe all instruments, reagents and experimental methods used in this research:

1. Instrumentation
2. Chemicals and reagents
3. Fabrication of the PAD
4. Preparation of crude anthocyanin-rich extract from Leum Pua glutinous rice
5. The effect of pH on the selectivity of a crude anthocyanin-rich extract
6. Study of optimum conditions for determination of copper (II) ion using the PAD with PEDD and natural reagent extracted from Leum Pua glutinous rice
7. Study of interfering metal ions for determination of copper (II) ion using Leum Pua glutinous rice extract with PAD-PEDD
8. Analytical characteristics of the developed method
9. Application of the PAD with PEDD for determination of copper (II) ion in water samples

#### 1. Instrumentation

##### 1.1 Instrumentation

- UV-Visible spectrophotometer (Model V-750, Jasco, Japan)
- Nuclear magnetic resonance spectrometer (Model Avance II300 MHz, Bruker, USA)
- Analytical balance (Model ME 2041, Mettler-Toledo, USA)
- Electric rice polisher (Model HOM-157102, Homemade, Thailand)
- pH meter (Model Five Easy Plus, Mettler-Toledo, USA)
- Rotary evaporator (Model R114, BÜCHI, Switzerland)
- Ultrasonic bath (Model GT SONIC-D13, GT SONIC, China)
- Vacuum filter aspirator (Model A-3S, Eyela, Japan)

- Water purification system (Model Direct-Q® 3 UV, MERCK MILLIPORE, USA)

### 1.2 Paired emitter detector diodes (PEDD) device

Paired emitter detector diodes (PEDD) based photometric device used in this research (Figure. 4) consisted of a pair of amber light emitting diodes (LED,  $\lambda_D$  590.0 nm,  $\phi$  5.0 mm). One LED uses as a LED emitter, with another LED serving as a LED detector. Two LEDs were aligned on the same axis facing each other into black acrylic sheets serving as holders. The LED emitter is connected in series to a 100-ohm resistor and a DC power supply with a constant 8 volts (BK-1502DD, Baku, China). LED detector is connected with a digital multimeter (5-1/2 Digit Multimeter, FLUKE 8808A to measure voltage signal. The voltage signal is directly proportional to light transmission intensity through PAD and inversely proportional to the copper(II) ion concentration.

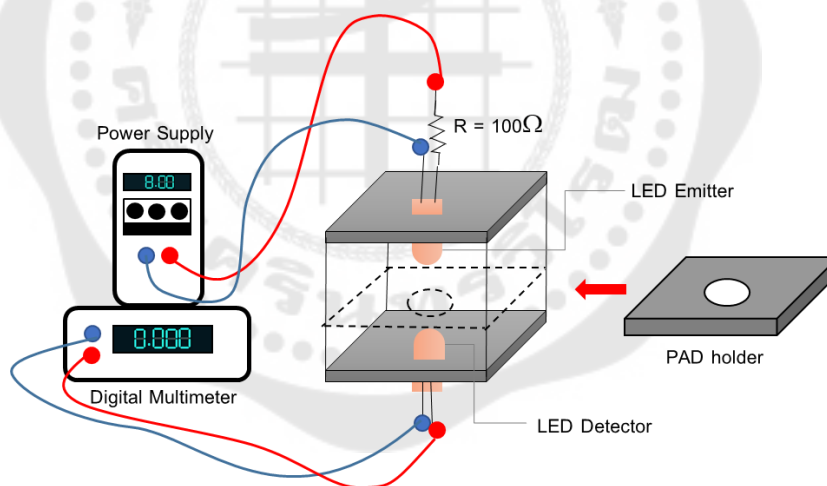


Figure 4. The PAD with PEDD-based photometer setup in this work.

## 2. Chemicals and reagents

- Barium nitrate ( $\text{Ba}(\text{NO}_3)_2$ ) from Merck (Germany)
- Cadmium (II) nitrate tetrahydrate ( $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ) from Carlo Erba (Italy)
- Chromium (III) nitrate nonahydrate ( $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ ) from Ajax Finechem (Australia)
- Cobalt (II) nitrate hexahydrate ( $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ) from Carlo Erba (Italy)

- Copper (II) nitrate trihydrate ( $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ ) from Carlo Erba (Italy)
- Iron (III) nitrate nonahydrate ( $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ ) from Ajax Finechem (Australia)
- Lead (II) nitrate ( $\text{Pb}(\text{NO}_3)_2$ ) from Carlo Erba (Italy)
- Manganese sulfate tetrahydrate ( $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ ) from Merck (Germany)
- Nickel (II) nitrate hexahydrate ( $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ) from Ajax Finechem (Australia)
- Zinc nitrate hexahydrate ( $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ) from Ajax Finechem (Australia)
- Acetic acid ( $\text{CH}_3\text{COOH}$ ) from Merck (Germany)
- Dichloromethane ( $\text{CH}_2\text{Cl}_2$ ) from RCI Labscan (Thailand)
- Hydrochloric acid (HCl) from Carlo Erba (Italy)
- Methanol ( $\text{CH}_3\text{OH}$ ) from RCI Labscan (Thailand)
- Potassium chloride (KCl) from Carlo Erba (Italy)
- Sodium acetate ( $\text{CH}_3\text{COONa}$ ) from Ajax Finechem (Australia)

### 3. Experimental method

#### 3.1 Fabrication of the PAD

In this work, a handheld stamping method was used to fabricate PAD based on forming hydrophobic ink regions on paper using a rubber stamp, which was manufactured by a local shop (Bangkok, Thailand). Indelible inks (HORSE Stamp Pad Ink, Industry NAN MEE Co., Ltd., Thailand), was added to the rubber stamp. The inked stamp was pressed on Whatman filter paper No. 4 to produce a hydrophobic barrier of the PAD. The embossed paper was dried at ambient temperature ( $\sim 25^\circ\text{C}$ ). The backside of the device was sealed with packing tape to prevent the solution leak and provide rigidity. As shown in Figure. 5, the final dimensions of the PAD were 2.5 cm x 2.5 cm with a circular hydrophilic barrier of 1.0 cm. diameter. The solutions were added onto the circular shape. The PAD was then put into a PAD holder (Figure. 4) to measure a signal using a PEDD-based photometer.

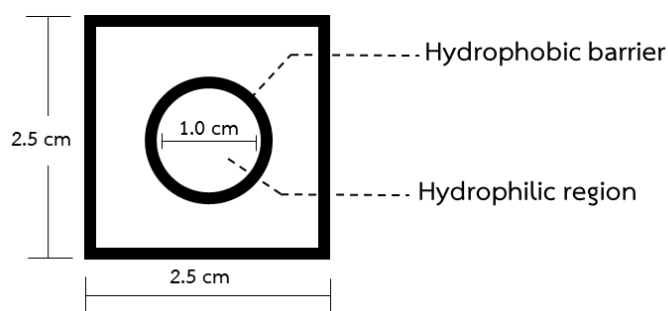


Figure 5. The PAD is used in this work.

### 3.2 Preparation of crude anthocyanin-rich extracted from Leum Pua glutinous rice

In this work, natural reagent extracted from Leum Pua glutinous rice, purple-black sticky rice from Phetchabun province, Thailand, was extracted using ultrasonic and liquid-liquid extraction methods. The extraction process was modified from the previous report (Ukwueze et al., 2009; Warangkhan et al., 2014). First, Leum Pua glutinous rice (1.0 kg) was polished to separate rice bran using an electric rice polisher in milled embryo mode. Next, 50g of rice bran of Leum Pua glutinous rice was placed in 600.0 mL of a mixed solution of methanol and 2 M HCl (85:15 v/v). The solution was extracted using an ultrasonic iced bath at 50 kHz for 3-4 hours. The extracted solution was filtered through a Whatman No.1 filter paper. A rotary evaporator had evaporated the filtrate at 35-38 °C for 3 hours to remove methanol. The solution was then extracted using 15.0 mL of chloroform as an extraction solvent in a separation funnel. The extraction was repeated two times to remove other non-polar interference. The chloroform layer was removed and 20.0 mL of concentrated HCl was added to the aqueous solution. The solution was then refluxed at 80 °C for 2 hours to obtain the deep-red violet solution. The reagent extracted was precipitated in the dark for 24 hours. The reagent powder was filtered by a vacuum aspirator and stored in a brown bottle at -10 °C in the refrigerator. The reagent powder from Leum Pua glutinous rice was characterized by the UV-Visible spectrophotometry and NMR.

The measurement of total anthocyanin content was carried out by the pH-differential method (Durst & Wrolstad, 2005). Crude anthocyanin-rich extracted from Leum Pua glutinous rice (3.2 mg) was added to 5.0 mL of methanol and stored in the refrigerator



for 1 hour. Then, 400.0  $\mu\text{l}$  of the extracted solution was mixed with 1.6 ml of 0.025 M potassium chloride buffer (pH 1.0), and the other 400.0  $\mu\text{l}$  of the extracted solution was mixed with 1.6 ml of 0.4 M sodium acetate buffer (pH 4.5). The absorbance of both solutions was measured at 520.0 and 700.0 nm. The total anthocyanin content was calculated by the following Equation (1):

$$\text{Anthocyanin content (mg/100g)} = \frac{(A \times \text{Mw} \times \text{DF} \times V \times 100)}{\epsilon \times l \times m} \quad (1)$$

- A =  $(A_{520 \text{ nm}} - A_{700 \text{ nm}})_{\text{pH 1.0}} - (A_{520 \text{ nm}} - A_{700 \text{ nm}})_{\text{pH 4.5}}$   
 MW = molecular weight of cyanidin (449.2 g/mol)  
 DF = dilution factor of solution  
 V = the solvent volume in mL  
 l = path length in cm  
 $\epsilon$  = molar extinction coefficient of cyaniding ( $26900 \text{ M}^{-1} \text{ cm}^{-1}$ )  
 m = weight of crude anthocyanin extract in g

### 3.3 The effect of pH on the selectivity of a crude anthocyanin-rich extract

The effect of pH on the selectivity of natural reagent extract from Leum Pua glutinous rice was investigated for the detect ion of various metal ions at pH 3.0-8.0. Firstly, 6.0  $\mu\text{L}$  of natural reagent extract from Leum Pua glutinous rice (640 mg/L) was added to the PAD. Then, 6.0  $\mu\text{L}$  of all 0.10 M buffer solution pH range 3.0-8.0 was added into each of the PAD followed by 6.0  $\mu\text{L}$  of 20.0 mg/L metal solution (Cu (II), Co (II), Cd (III), Cr (II), Fe (III), Ni (II) and Pb (II)). The color change of each metal complex at various pH was observed and compared. Moreover, the wavelength of maximum absorbance ( $\lambda_{\text{max}}$ ) for detecting anthocyanin-rich extract-Cu (II) ion complex at the desired pH was studied using a UV-Visible spectrophotometer.

3.4 Study of optimum conditions for determination of copper (II) ion using the PAD with PEDD and natural reagent extracted from Leum Pua glutinous rice

#### 3.4.1 Effect of the total solution volume

The total volume of solution on the PAD measurement was tested in the range of 6-30  $\mu\text{L}$ . The volume ratio of anthocyanin reagent extract from Leum Pua glutinous rice, buffer solution and deionized water was fixed at 1: 1: 1. The optimal total volume of solutions that produce color uniformity and spread within the designed circular shape was selected.

#### 3.4.2 Effect of the sequence of reaction

The PAD with a PEDD-based photometer was used to detect copper(II) ion based on the reaction between anthocyanin-rich extract from Leum Pua glutinous rice and copper(II) ion in a buffer solution yielded the blue complex. The reaction mixture contained 6.0  $\mu\text{L}$  of 640 mg/L natural reagent, 6.0  $\mu\text{L}$  of 0.10 M buffer (pH 5.7) and 6.0  $\mu\text{L}$  of 20.0 mg/L copper(II) ion. The various sequence of the reaction mixture on PAD (Table 3) were studied and compared the color uniformity production onto the designed circular shape.

Table 3. Optimization of the sequence of reactions on the paper-based analytical device

Condition	The sequence of reaction
1	Natural reagent, buffer and Cu (II) ion
2	Natural reagent, Cu (II) ion and buffer
3	Buffer, natural reagent and Cu (II) ion
4	Buffer, Cu (II) ion and natural reagent
5	Cu (II) ion, natural reagent and buffer
6	Cu (II) ion, buffer and natural reagent

### 3.4.3 Effect of natural reagent extract concentration.

The natural reagent extract from Leum Pua glutinous rice in 400.0-1020.0 mg/L concentrations was studied. After the addition of 6.0  $\mu$ L of natural reagent extract, the buffer solution (6.0  $\mu$ L, 0.10 M) and copper(II) solution (6.0  $\mu$ L, 20 mg/L) consecutively was added onto the PAD. The potential signal of anthocyanin-rich extract-Cu (II) complex at each natural reagent concentration was measured and compared using a PEDD-based photometer at wavelength 590.0 nm.

### 3.4.4 Effect of equilibration time

The natural reagent extracted from Leum Pua glutinous rice (640.0 mg/L, 6.0  $\mu$ L) and a buffer solution (0.10 M, pH 5.7, 6.0  $\mu$ L) was serially added onto PAD. The potential signal of anthocyanin-rich extract-Cu (II) complex was measured after the addition of 6  $\mu$ L copper (II) solution (1.0-10.0 mg/L) from 0-120 seconds using a PEDD-based photometer at wavelength 590.0 nm.

### 3.5 Study of interfering metal ions for determination of copper (II) ion using Leum Pua glutinous rice extract with PAD-PEDD

The effect of interfering metal ions including Ba (II), Cd (II), Co (II), Cr (III), Mn (II), Ni (II), Pb (II), Fe (III) and Zn (II) for determination of copper (II) ion using Leum Pua glutinous rice extract with PAD-PEDD detection was studied. The reaction mixture contained 6.0  $\mu$ L of 640.0 mg/L natural reagent, 6.0  $\mu$ L of 0.10 M buffer (pH 5.7) and 6.0  $\mu$ L of 2.0 mg/L solutions containing copper (II) ion and other metal. The potential signal of anthocyanin-rich extract-Cu (II) complex was measured using a PEDD-based photometer at wavelength 590.0 nm.

### 3.6 Analytical characteristics of the developed method

#### 3.6.1 Linearity range

The standard solution of copper(II) in the concentration range of 0.5-50.0 mg/L was analyzed three times by the PAD with a PEDD-based photometer. The calibration curve was established by plotting the potential signal difference ( $\Delta$ PEDD signal) versus the copper (II) concentrations. The signal difference was calculated from a Cu (II) complex signal with a blank anthocyanin reagent signal.

### 3.6.2 The limit of detection (LOD) and the limit of quantification (LOQ)

In this research, the limit of detection and the limit of quantification was calculated from the calibration curve (Shrivastava, 2011) using Equation (2) and (3), respectively.

$$\text{LOD} = 3(\text{Sy}/\text{S}) \quad (2)$$

$$\text{LOQ} = 10(\text{Sy}/\text{S}) \quad (3)$$

$\text{S}_y$  is the standard deviation of the responses or y Intercept of a regression line

$\text{S}$  is the slope of the calibration curve

### 3.6.3 The method precision

The precisions of the developed method were determined at three concentrations of copper(II) (1.0, 5.0 and 10.0 mg/L) covering the linearity range. The precision was expressed as the percentage of relative standard deviation (% RSD) of five replicate (n=5) analyses of each working standard copper(II) solution. Intra-day experiments were performed within 1 day, whereas inter-day experiments were performed over five consecutive days.

### 3.7 Application of the PAD with PEDD device for determination of copper (II) in water samples

The developed method was applied to determine copper(II) content in water samples, including tap water and river water. River water samples were collected from the Tha Chin River in Samut Sakhon province. All water samples were filtered to remove all suspended particles prior to loading onto PAD. A PEDD-based photometer was used to detect the anthocyanin-rich extract-Cu (II) complex that appeared on the PAD device at 590.0 nm. The analysis was carried out in triplicate.

For recovery of the method, the experiment was performed by spiking 2.0 mg/L of standard copper(II) solution into each water sample. Then, samples were analyzed with a PAD with a PEDD-based photometer in triplicate analyses. The percentage of recovery was calculated in each spiked sample.

## CHAPTER 4

### RESULTS

The chapter presents the results and discussion of this study. The findings are presented under the following sections:

1. Characterization of crude anthocyanin-rich reagent from Leum Pua glutinous rice
2. The effect of pH on the selectivity of a crude anthocyanin-rich extract
3. Study of optimum conditions for determination of copper (II) ion using the PAD with PEDD and natural reagent extracted from Leum Pua glutinous rice
4. Effects of interfering metal ions for determination of copper (II) ion using Leum Pua glutinous rice extract with PAD-PEDD
5. Analytical characteristics of the developed method
6. Application of the PAD with PEDD for determination of copper (II) ion in water samples

#### **1.Characterization of crude anthocyanin-rich reagent extracted from Leum Pua glutinous rice**

The crude anthocyanin-rich reagent was extracted from Leum Pua purple-black sticky rice using ultrasonic and liquid-liquid extraction methods. The percentage of extraction yield was 2.52 based on the dry extract weight and dry Leum Pua glutinous rice. The crude reagent powder in methanol showed a purple-red color (Figure 6A). The absorption spectrum of the natural reagent was characterized by UV-Visible spectrophotometry at 200.0-800.0 nm. It was found three maximum absorption peaks ( $\lambda_{\max}$ ) at 277.6, 428.2 and 540.0 nm corresponding to the flavonoid and phenolic compounds of anthocyanin, as shown in Figure 6B.

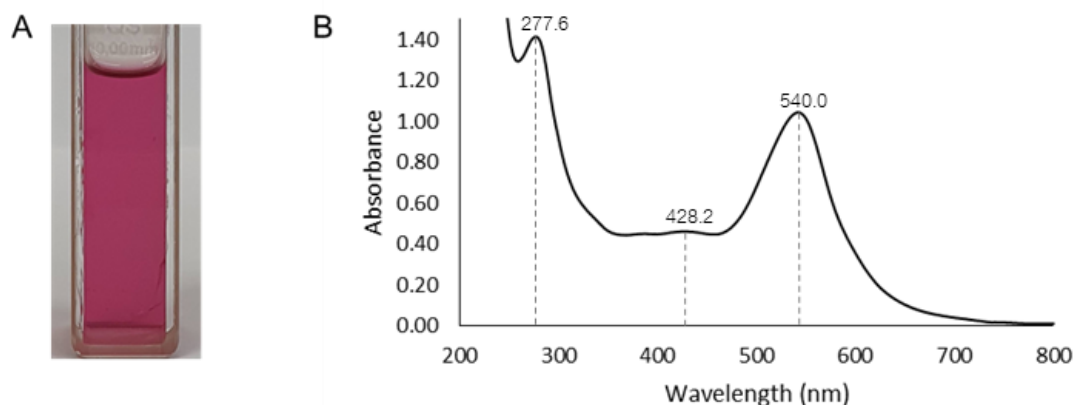


Figure 6. (A) Color of anthocyanin-rich reagent from Leum Pua glutinous rice in methanol (B) UV-Vis spectrum of reagent obtained from 200.0-800.0 nm.

The purification of crude anthocyanin-rich reagent from Leum Pua glutinous rice using a Sephadex LH-20 method was carried out prior to the structural characterization of purified reagent powder by NMR.  $^1\text{H}$  NMR spectroscopic data demonstrated signals corresponding to cyanidin molecule. A singlet at  $\delta$  8.87 ppm was a tropical signal of H-4. A doublet of a doublet at  $\delta$  8.22 ppm ( $J = 8.7, 2.2$  Hz) corresponded to H-6'. Four doublets at  $\delta$  7.99 ( $J = 2.2$  Hz), 7.03 ( $J = 8.8$  Hz), 6.93 (brs) and 6.88 ( $J = 2.1$  Hz) ppm corresponded to H-2', H-5', H-8 and H-6, respectively. The spectrum results were agreed with Warangkhan et al. (Khaodee et al., 2014). Therefore, it can be concluded that the anthocyanin-rich extracted from Leum Pua rice contains cyanidin as the major pigment.

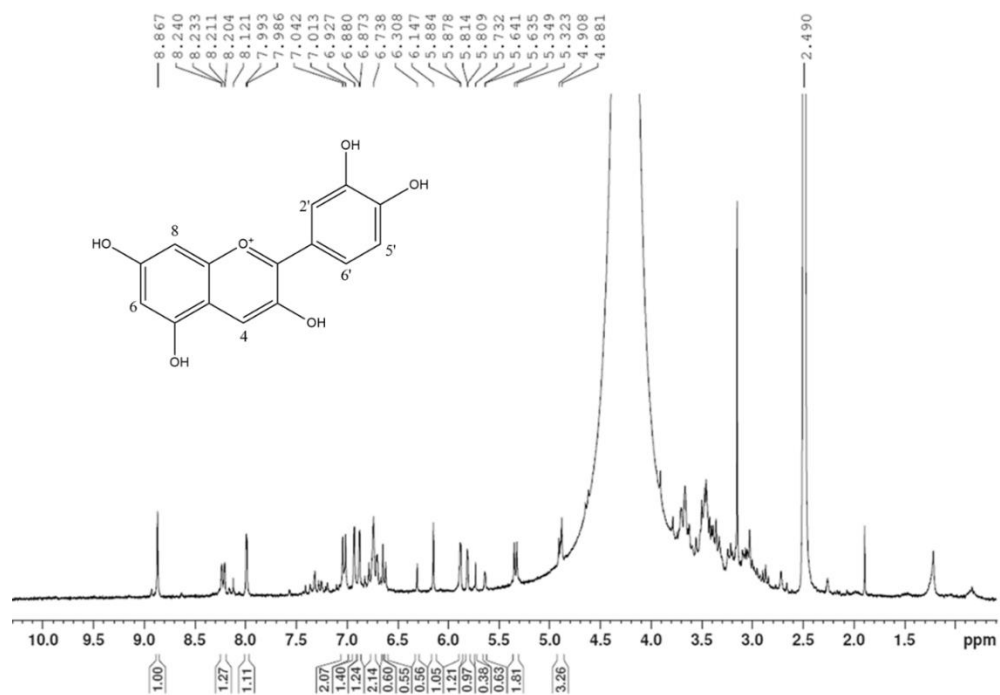


Figure 7.  $^1\text{H}$  NMR spectrum of anthocyanin-rich extracted from Leum Pua glutinous rice.

The measurement of total anthocyanin content in natural reagent extract was performed using the pH-differential method. The method can be used to determine total anthocyanin content based on the structural change of the anthocyanin between pH 1.0 (flavylium cation (oxonium form)) and 4.5 (carbinol pseudo-base (hemiketal form)). The difference of absorbance at 520.0 nm of both pH solutions was deducted to eliminate the absorption signal from other substances. Based on this method, total anthocyanin content was calculated and expressed as mg of cyaniding-3-glucoside equivalents per 100g of crude extract, as shown in Equation (1). Anthocyanin content in crude extract from Leum Pua glutinous rice in this work was 1,813 mg/100g. It was found that the anthocyanin content extracted from Leum Pua glutinous rice in this research was higher than other Thai pigmented rice (Ratseewoa et al., 2019)

## 2. The effect of pH on the selectivity of a crude anthocyanin-rich extract

The effect of pH on the selectivity of natural reagent extracted from Leum Pua glutinous rice was investigated for the detection of various metal ions, including copper (II), cobalt (II), cadmium (II), chromium (III), iron (III), nickel (II) and lead (II) ions at pH

range 3.0-8.0. The color of metal complexes on the PAD and the absorption spectrum of each metal complex at pH 6.0 using a spectrophotometer were presented in Figure 8. The results indicated that natural reagent extracted from Leum Pua glutinous rice appeared in pink color at pH 3.0-6.0 and purple color at pH 7.0-8.0 (Figure 8A). In different pH conditions, the structure of anthocyanins can change in four forms: flavylium cation, carbinol, chalcone and quinonoidal (Figure 9). In the acidic condition ( $\text{pH} < 3.0$ ), the pink color predominates as flavylium cation form and changes to purple and blue colors of quinonoidal species in neutral and basic conditions. At pH 4.0-5.0 colorless carbinol pseudo base and chalcone are formed. Thus, at this pH range four molecular structures of anthocyanins, including flavylium cation, carbinol, chalcone and quinonoidal are formed.

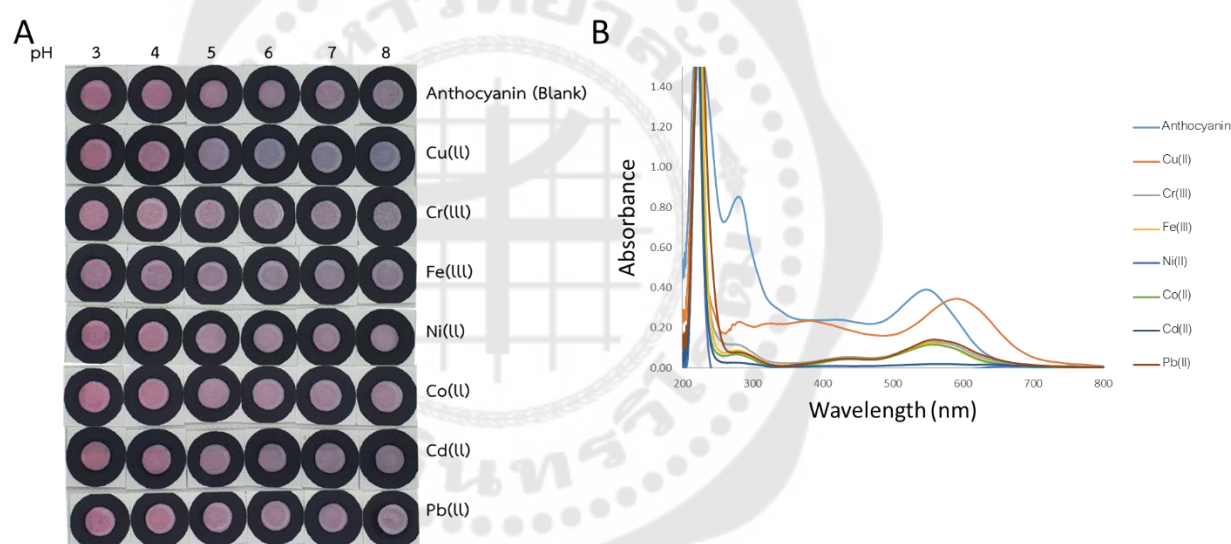


Figure 8. (A) The color of complexes between natural reagent extracted from Leum Pua glutinous rice and each metal solution at pH 3.0-8.0 on PAD. (B) The spectra of complexes between natural reagent extracted from Leum Pua glutinous rice and various metal solutions at pH 6.0.

It can be seen from Figure 8A that the natural reagent extracted from Leum Pua glutinous rice could react selectively with copper (II) ion at pH 5.0-6.0 and color changed clearly from pink to blue-purple. Figure 8B showed the absorption spectra of each metal complex at pH 6.0 using anthocyanin-rich extract. The Cu (II) complex gave the highest



absorbance compared with other complexes and showed two wavelengths of maximum absorption at 393.0 and 590.0 nm. The results showed that the wavelength of maximum absorption ( $\lambda_{\text{max}}$ ) was changed from 540.0 nm to 590.0 nm when adding copper(II) ion to a reagent solution. The bathochromic shift occurred due to the formation of copper (II) ion with the ortho-dihydroxyl groups of anthocyanin-rich extracted at the B ring, as shown in Figure 10. The spectral results in agreement with the previous reported that anthocyanin-Cu (II) complex exhibited two maximum absorbances in the range of 390.0-440.0 and 475.0-605.0 nm and The  $\lambda_{\text{max}}$  of anthocyanin-Cu (II) complex was bathochromic shifted ranged 10.0-66.0 nm (Fedenko et al., 2017). In this work, LED at wavelength 590.0 nm was used as a LED emitter and LED detector in a PEDD device to detect Cu (II).

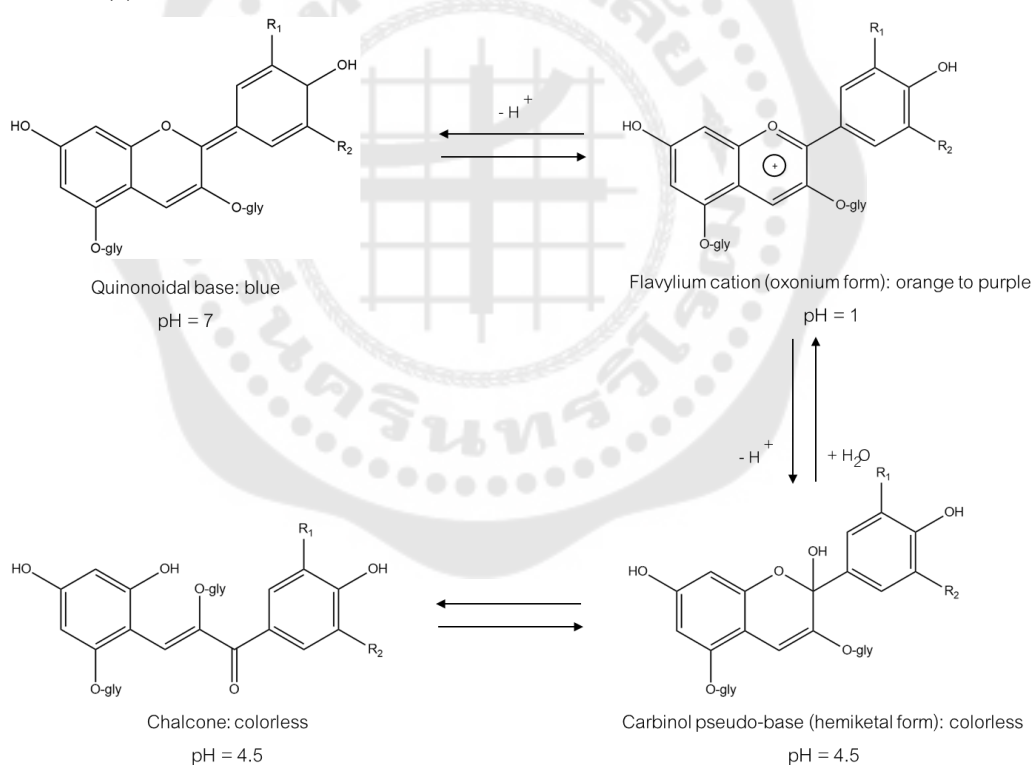


Figure 9. Structural forms of anthocyanins at different pH.

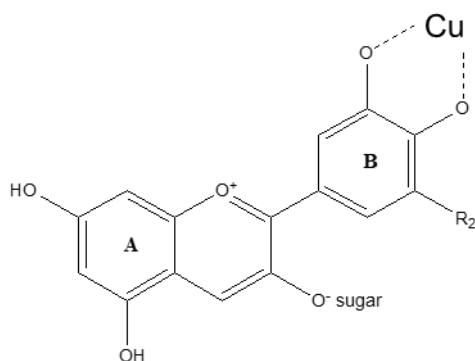


Figure 10. The structure of the anthocyanin-rich extract-Cu (II) complex.

Therefore, the pH ranges 5.0-6.0 were selected for further investigation using PAD with PEDD detection. The results in Figure 11 illustrated that pH 5.7, and the highest gave PEDD signal and the signal varied with Cu (II) concentrations. In this research, a buffer solution at pH 5.7 was used to measure Cu (II) using PAD with PEDD and natural reagent extract.

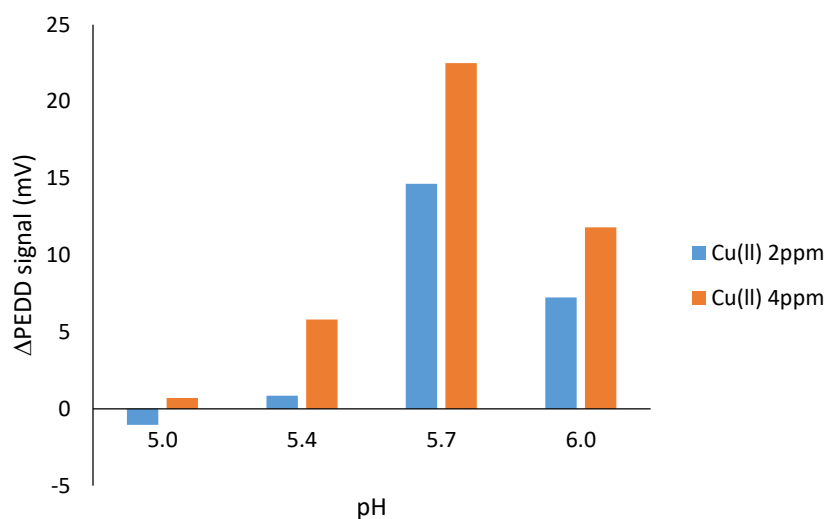


Figure 11. The signal from PAD with PEDD measurement for detection of copper (II) ion in buffer solution at pH 5.0-6.0.

### 3. Study of optimum conditions for determination of copper (II) ion using the PAD with PEDD and natural reagent extracted from Leum Pua glutinous rice

#### 3.1 Effect of the total solution volume

In this work, the PAD with a diameter of 1.0 cm. was used to study the optimal total volume of solutions that could spread within the full circular shape. The total volume of solution in the range of 6.0-30.0  $\mu\text{L}$  on the PAD measurement was studied using the volume ratio of anthocyanin reagent extract from Leum Pua glutinous rice, buffer solution (pH 5.7) and deionized water at 1: 1: 1. The results showed in Figure 12 demonstrated that a total volume of 18.0  $\mu\text{L}$  (6:6:6 volume ratio) produced the color uniformity and spread completely in the circular zone. When the volume increased, the solutions spread outside the detection zones. Consequently, the total volume of 18.0  $\mu\text{L}$  of natural reagent, buffer solution (pH 5.7) and standard or sample solution was chosen as the optimal condition for loading onto the PAD.

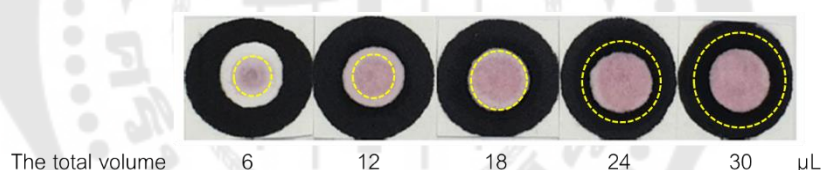


Figure 12. The total volume of solution loaded onto the PAD.

#### 3.2 Effect of the sequence of reaction

The PAD with PEDD-based photometer was evaluated for the detection of copper (II) ion based on the formation of a blue complex between anthocyanin-rich extract from Leum Pua glutinous rice and copper (II) ion at pH 5.7. The reaction mixture contained of 6.0  $\mu\text{L}$  of 640.0 mg/L natural reagent, 6.0  $\mu\text{L}$  of 0.10 M acetate buffer (pH 5.7) and 6.0  $\mu\text{L}$  of 20.0 mg/L copper (II) solution. The results of various sequences of the reaction mixture on PAD was presented in Figure 13. The results indicated that the sequence in condition 1 produced color uniformity and reproducibility onto the designed circular shape.

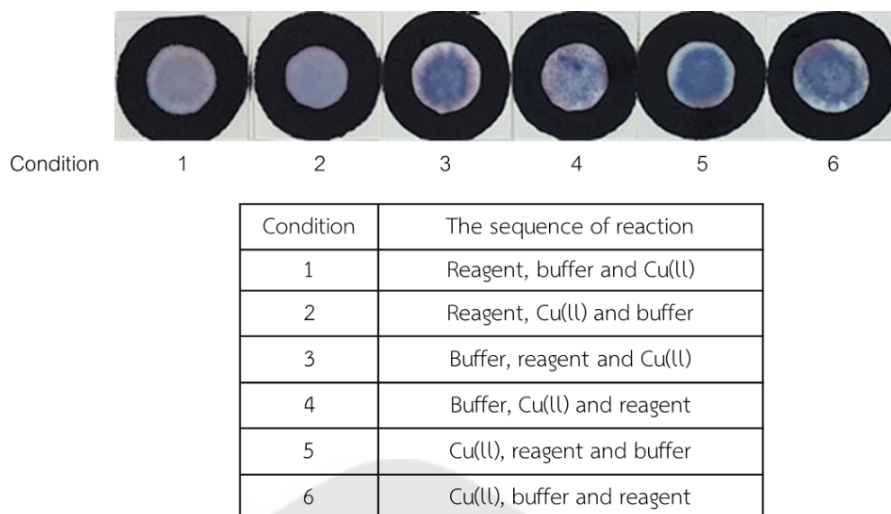


Figure 13. Effect of the sequence of reaction on PAD.

Furthermore, a sequence in condition 1 was studied to compare the steps for loading solutions on PAD in the copper concentrations range of 1.0-10.0 mg/L using PEDD detection. Figure 14A presented the PEDD signal by adding 6.0  $\mu\text{L}$  of 640.0 mg/L natural reagent, 6.0  $\mu\text{L}$  of 0.10 M acetate buffer (pH 5.7) and 6.0  $\mu\text{L}$  copper (II) solution serially (three steps) on the PAD. Figure 14B showed the PEDD signal by adding 6.0  $\mu\text{L}$  of 640.0 mg/L natural reagent and 12  $\mu\text{L}$  of copper (II) solution in 0.10M buffer (pH5.7) serially (two steps) on the PAD. The results clearly showed that the signal increased as the concentration of Cu (II) solution increased when using three steps of loading solution on the PAD. Moreover, the sensitivity of this condition was higher than the other. Therefore, in this research, loading the solution using three steps was used for the analysis of copper (II) content in water samples.

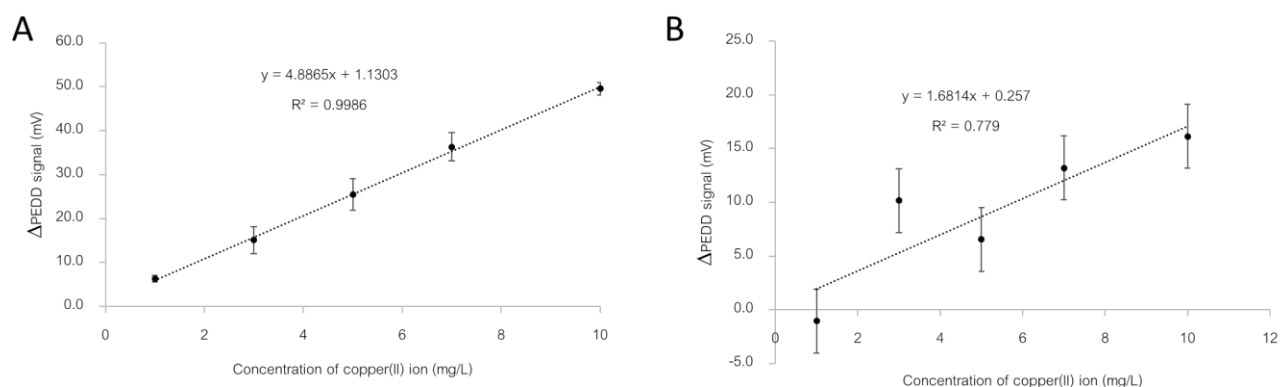


Figure 14. The effect of loading steps of the reaction mixture on the PEDD signal of Cu (II) solution in a concentration range of 1.0-10.0 mg/L. (A) Three steps; reagent-buffer (pH5.7)-Cu (II) and (B) two steps; reagent-Cu (II) in buffer (pH5.7)

### 3.3 Effect of natural reagent extract concentration.

The color intensity of the natural reagent might effect the blue color of a complex on the PAD. Thus, the concentration of anthocyanin-rich extract should be considered. In this work, concentration in the range of 400.0–1020.0 mg/L of natural reagent extracted from Leum Pua glutinous rice was investigated. Figure 15 shows the effect of anthocyanin-rich extract concentrations on the blue color of the PAD and the potential signal of copper (II) complexes from a PEDD-based photometer. It was found that the potential signal increased with anthocyanin-rich extract concentrations from 400.0 to 640.0 mg/L, but not significant increased above 640.0 mg/L. When a high concentration of the anthocyanin-rich extract was used, low signal repeatability was obtained. This result might be caused from the reddish color of the anthocyanin reagent. Therefore, anthocyanin-rich extract at a concentration of 640.0 mg/L was selected as the optimal concentration for copper (II) determination.

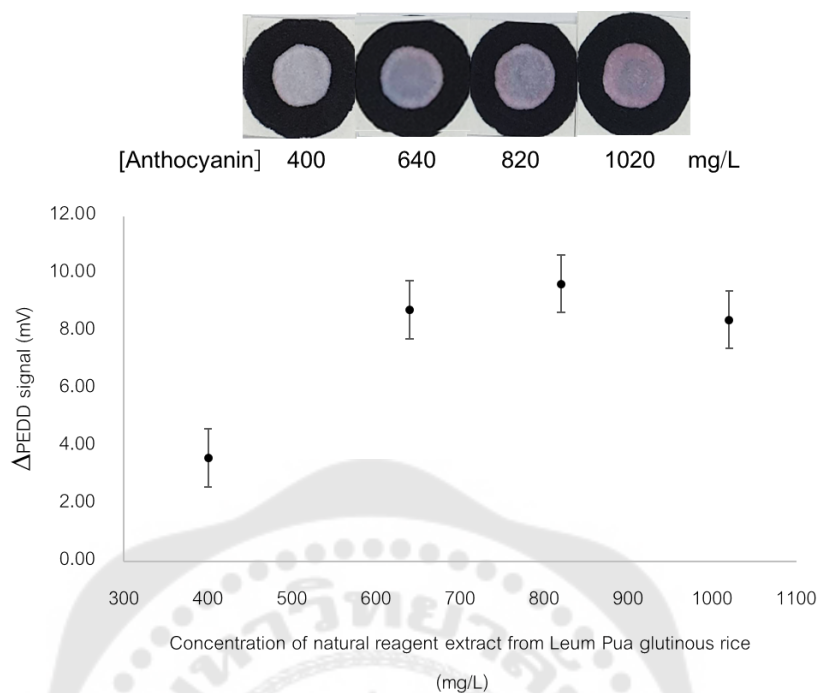


Figure 15. Effect of the natural reagent extract concentration on PEDD signal.

#### 3.4 Effect of equilibration time

The equilibration time in the range of 60-120 seconds was investigated for measurement of the complex between natural reagent extracted from Leum Pua glutinous rice and copper (II) in a concentration range of 1.0-10.0 mg/L. It was found that the potential signal from PEDD detection was directly proportional to Cu (II) concentrations in all equilibration times, as presented in Figure 16. The highest sensitivity and linearity were achieved at an equilibration time of 120 seconds. In this work, the reaction was allowed to proceed for 120 seconds before measurement the copper (II) with a PEDD.

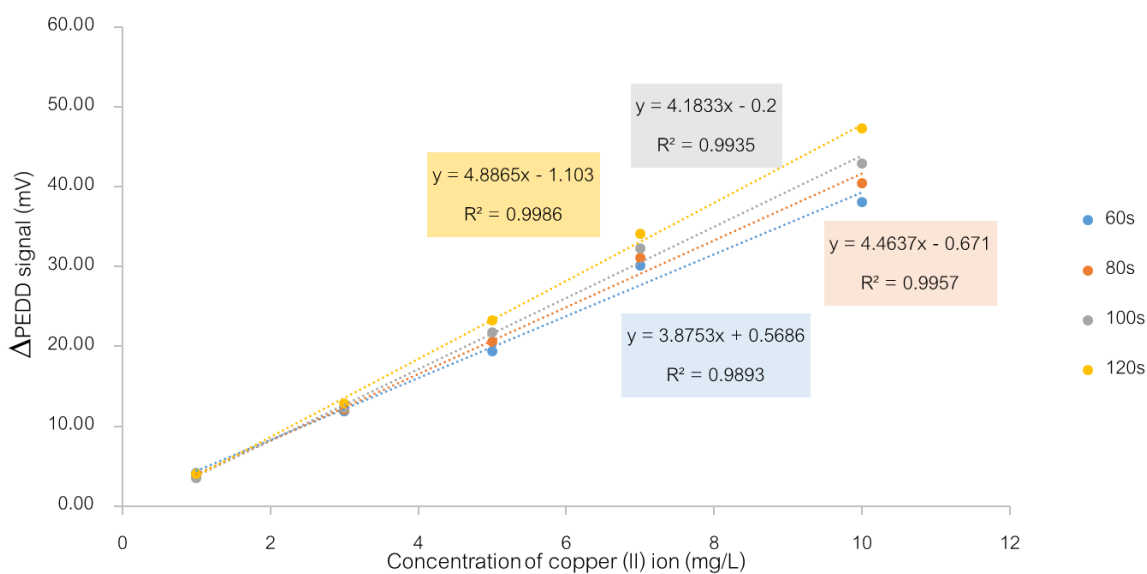


Figure 16. Effect of the equilibration time in 60-120 seconds on PEDD signal using copper (II) solution at a concentration range of 1.0-10.0 mg/L.

#### 4. Effects of interfering metal ions for determination of copper (II) ion using Leum Pua glutinous rice extract with PAD-PEDD

The effect of interfering metal ions including Ba (II), Cd (II), Co (II), Cr (III), Mn (II), Ni (II), Pb (II), Fe (III) and Zn (II) for determination of copper(II) was investigated. Each foreign ion at a concentration of 2.0 mg/L was prepared in the buffer solution (pH 5.7) and spiked into a standard Cu (II) solution (2.0 mg/L) prior to react with a natural reagent on PAD. The results from the PEDD-based photometer are presented in Figure 17. show that no significant difference (<5%) between the potential signal of pure Cu (II) complex and those of Cu (II) complex in the presence of interfering ions was observed.

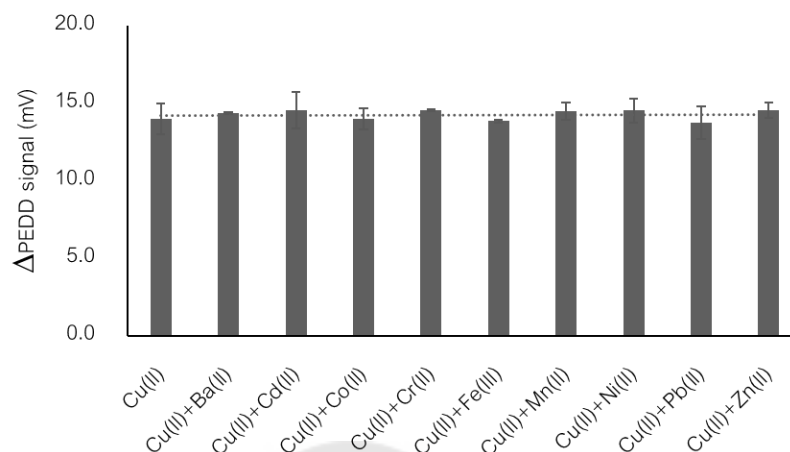


Figure 17. Interfering effects of metal ions for determination of copper (II) ion using Leum Pua glutinous rice extract with PAD-PEDD detection. Detection data are expressed as means  $\pm$  SD (n=3).

## 5. Analytical characteristics of the developed method

### 5.1 Linearity range

The linearity range of the copper (II) analysis was studied using a natural reagent solution extracted from Leum Pua glutinous rice with the PAD-PEDD device under optimal conditions. As shown in Figure 18, the calibration was linear over the range of copper (II) concentration range from 0.5-10.0 mg/L with a correlation coefficient ( $R^2$ ) to be 0.9973.

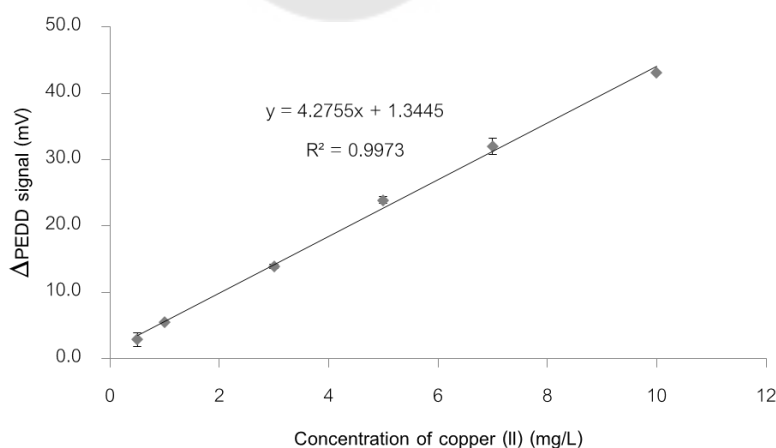


Figure 18. The calibration curve is obtained by this proposed method.



## 5.2 The limit of detection (LOD) and the limit of quantification (LOQ)

In this study, the limit of detection and the limit of quantification was calculated based on calibration data. The results showed that the limit of detection and the limit of quantification of the developed analytical method were 0.11 mg/L and 0.42 mg/L, respectively.

## 5.3 The method precision

The precision of the developed method was evaluated using copper (II) standard solution with three concentrations, 1.0, 5.0 and 10.0 mg/L. The analysis was repeated five replicates (n=5) for both intra-day and inter-day. As shown in Table 4, the precision results were expressed as the relative standard deviation (% RSD). The developed method showed good reliability, with %RSD of intra-day and inter-day in the range of 0.33-1.85 and 0.98-2.12, respectively. Thus, the results showed that Leum Pua glutinous rice extract could be used as a natural reagent to determine copper (II) by the PAD with PEDD detection.

Table 4. The precisions of the developed method for the determination of copper (II) ion.

Concentration of copper(II) (mg/L)	The percentage of relative standard deviation (% RSD)	
	Intra-day (n=5)	Inter-day (n=5)
1.00	1.85	2.12
5.00	1.78	1.99
10.00	0.33	0.98

## 6. Application of the PAD with PEDD device for determination of copper (II) in water samples

In this research, the developed method was applied to analyze copper (II) content in four samples from tap and river water. The results showed that copper (II) was not detected in all of the analyzed samples. The recovery of the method was evaluated in four water samples by spiking 2.0 mg/L of copper (II) standard solution to each sample and analyzed by the developed method. As a result, the percentage recovery values in the range of 96.87-99.64 were obtained, indicating that the developed method could well be used to analyze copper (II) in various water samples, as shown in Table 5.

Table 5. The percentage of recovery was analyzed using the developed method in various water samples.

Sample	Concentration (mg/L)		% Recovery (n=3)
	Spiked sample	PAD-PEDD detection	
Tap water	2.00	1.94±0.49	96.87±0.57
River water 1	2.00	2.00±0.69	99.64±1.28
River water 2	2.00	2.00±0.49	99.54±0.76
River water 3	2.00	1.97±0.62	98.30±0.84

## CHAPTER 5

### SUMMARY DISCUSSION AND SUGGESTION

In this research, an analytical method using simple and small PAD with an in-house PEDD-based photometer and natural reagents extracted from Leum Pua glutinous rice was developed for application in the analysis of copper (II) in water samples. The reaction between anthocyanins in natural reagents extracted from Leum Pua glutinous rice and copper(II) solution produced a colored complex product on the PAD. The signal was then measured by a PEDD-based photometer.

The anthocyanin-based natural reagent was extracted from Leum Pua purple-black sticky rice using ultrasonic and liquid-liquid extraction methods. The extraction yield was 2.52% (1.30 g of crude extract/51.53 g of rice bran). The results from the NMR spectrum indicated that major anthocyanin in Leum Pua glutinous rice was cyaniding. The total anthocyanin content extracted from Leum Pua glutinous rice was measured using the standard pH differential method. The result showed that total anthocyanin content expressed as cyanidin-3-o-glucoside equivalent in Leum Pua glutinous rice extract was 1,813 mg/100g. The anthocyanin concentration was higher than other plants such as Hom Nil rice (Pitija et al., 2013), red cabbage (Junka et al., 2017), purple waxy corn (Kapcum et al., 2021) and red sweet potato (Cevallos-Casals & Cisneros-Zevallos, 2003). Moreover, Leum Pua glutinous rice is easily available, inexpensive and in large quantities in Thailand.

In the development of analytical methods, the PAD device was designed in a circular hydrophilic region with a diameter of 1.0 cm and fabricated using the ink stamping method. PEDD-based photometer was created for optical detection of copper (II). The PEDD detector using LED emitter and LED detector at a wavelength of 590.0 nm. was designed and developed to measure a color change of PAD for analysis of the blue complex between copper (II) and a natural anthocyanin reagent extracted from Leum Pua glutinous rice. Various parameters affecting the detection of Cu (II) complex on PAD with a PEDD-based photometer were investigated. The results indicated that the optimum pH of reaction at 5.7 was obtained. This pH condition showed high selectivity and sensitivity for copper (II) detection. The maximum PEDD signals were achieved by consecutive dropping of each 6.0  $\mu$ L of 640.0 mg/L of anthocyanin reagent extract from Leum Pua

glutinous rice, 0.10 M of buffer solution (pH 5.7) and copper (II) solution. Under the optimum condition, the efficiency of this developed method provides a linearity range in the concentration range of 0.50-10.00 mg/L ( $R^2 = 0.9973$ ). The limit of detection was found to be 0.11 mg/L. The precision of the developed method, expressed in terms of the relative standard deviation (RSD), were lower than 1.85 and 2.12 for intraday and interday, respectively. The percentage recoveries of copper (II) in spiked water were in the range of 96.87-99.64.

This research aimed to develop a green method for copper (II) analysis using natural reagent extracts from Leum Pua glutinous rice and measured on PAD with a PEDD-based photometer. It was found that the developed method was simple, environmentally friendly and inexpensive: More, this proposed device is easy to operate, portable and suitable for on-site analysis. In addition, the developed method can be used for quantitative analysis of copper (II) in water samples to control the amount of copper in natural waters according to the Pollution Control Department, Ministry of Natural Resources and Environment.

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