SPECTROMETRIC DETERMINATION OF NITRITE USING 1,1'-DIETHYL-2,2'-CYANINE IODIDE AS A REAGENT

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การหาปริมาณไนไตรท์ด้วยวิธีการวัดทางสเปกโทรสโกปีโดยใช้ 1,1'-ไดเอทิล-2,2'-ไซยาไนน์ ไอโอไดด์ เป็นรีเอเจนต์

นางสาวเบญจกาญจน์ บุญวร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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เบญจกาญจน์ บุญวร : การหาปริมาณไนไตรท์ด้วยวิธีการวัดทางสเปกโทรสโกปี โดยใช้ 1,1'-ไดเอทิล-2,2'-ไซยาไนน์ ไอโอไดด์ เป็นรีเอเจนต์ (SPECTROMETRIC DETERMINATION OF NITRITE USING 1,1'-DIETHYL-2,2'-CYANINE IODIDE AS A REAGENT) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.สัญชัย ประยูรโภคราช, 68 หน้า.

ไนไตรท์ (NO_2^-) ความเข้มข้นสูงที่เจือปนในน้ำดื่ม แหล่งน้ำธรรมชาติและอาหาร เป็นอันตรายต่อสุขภาพของมนุษย์และสิ่งแวดล้อม โดยไนไตรท์สามารถเกิดปฏิกิริยากับเอมีนทุติยภูมิหรือ เอมีนตติยภูมิ เกิดเป็น เอ็น-ไนโตรซามีน ซึ่งถือว่าเป็นสารก่อมะเร็ง ดังนั้น การวิเคราะห์ปริมาณไนไตรท์ในน้ำและอาหารเป็นประจำถือเป็นเรื่องสำคัญ งานวิจัยนี้ใช้ 1,1'-ไดเอทิล-2,2'-ไซยาไนน์ ไอโอไดด์ เป็นรีเอเจนต์เพื่อวิเคราะห์ปริมาณของไนไตรท์โดยวิธีวัดทางสเปกโทรเมตรี โดยไนไตรท์สามารถเกิดปฏิกิริยากับรีเอเจนต์ และทำให้ค่าการดูดกลืนแสงที่ความยาวคลื่น 522 นาโนเมตร มีค่าลดลง นอกจากนี้ยังได้ใช้เทคนิค HPLC และ LC-MS ศึกษาปฏิกิริยาระหว่าง 1,1'-ไดเอทิล-2,2'-ไซยาไนน์ ไอโอไดด์ กับไนไตรท์

มีการศึกษาตัวแปรที่มีผลต่อสัญญาณการตอบสนองและศึกษาหาสภาวะที่เหมาะสม เช่น พีเอช เวลาในการทำปฏิกิริยาและความเข้มข้นของสารละลาย 1,1'-ไดเอทิล-2,2'-ไซยาไนน์ ไอโอไดด์ การตอบสนองที่ดีที่สุดของสารละลาย 1,1'-ไดเอทิล-2,2'-ไซยาไนน์ ไอโอไดด์ ต่อไนไตรท์เกิดในสารละลาย อะซิเตตบัฟเฟอร์ พีเอช 4.0 ที่เวลาในการทำปฏิกิริยา 5 นาที กราฟการเทียบมาตรฐานเป็นเส้นตรงที่ความเข้มข้นในช่วง 2.5-60 มิลลิกรัมต่อลิตร ซึ่งมีสมการเชิงเส้น $\Delta A = 0.0143C + 0.0075$ และ R^2 มีค่าเท่ากับ 0.9989 ขีดจำกัดการตรวจวัดมีค่า 1.0 มิลลิกรัมต่อลิตร วิธีการวิเคราะห์นี้ได้นำไปประยุกต์สำหรับการวิเคราะห์ปริมาณไนไตรท์ในตัวอย่างน้ำดื่มและอาหาร

ได้ศึกษาการวิเคราะห์ปริมาณไนไตรท์โดยใช้วิธีการตรึงรีเอเจนต์บนตัวรองรับ รีเอเจนต์ถูกตรึงบนพอลิเมอร์หลากชนิด เช่น ไคโตซาน อะการ์โรส ไทรอะซิติล เซลลูโลสและแนฟฟีออน อะมิโนซิลิกากระจายบนแนฟฟีออนร้อยละ 0.25 โดยน้ำหนักต่อปริมาตร เป็นตัวรองรับที่เหมาะสมที่สุดสำหรับการตรึงรีเอเจนต์ สภาวะที่เหมาะสมในการทดลองสำหรับการวิเคราะห์ไนไตรท์ คือ สารละลายพีเอช 4.0 และเวลาในการทำปฏิกิริยา 35 นาที กราฟเส้นตรงสำหรับการเทียบมาตรฐานในช่วงความเข้มข้น 50-500 มิลลิกรัมต่อลิตร มีสมการเส้นตรง $\Delta A = 0.0009C - 0.0088$ และ R^2 มีค่าเท่ากับ 0.9933 ขีดจำกัดของการตรวจวัดมีค่า 1.85 มิลลิกรัมต่อลิตร วิธีที่พัฒนานำไปประยุกต์ใน

การวิเคราะห์ปริมาณไนไตรท์ในตัวอย่างจริงและให้ผลการวิเคราะห์ที่สอดคล้องกันดีกับผลการวิเคราะห์ที่ได้จากวิธีมาตรฐาน

สาขาวิชาเคมี ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา _____

BENJAKARN BOONWORN : SPECTROMETRIC DETERMINATION OF NITRITE USING 1,1'-DIETHYL-2,2'-CYANINE IODIDE AS A REAGENT. THESIS ADVISOR : ASST. PROF. SANCHAI PRAYOONPOKARACH, Ph.D. 68 PP.

NITRITE, SPECTROPHOTOMETRY, 1,1'-DIETHYL-2,2'-CYANINE IODIDE, NAFION FILM

High concentration of nitrite (NO_2^-) in drinking water, natural water and food are harmful to human health and environment. Nitrite can react with secondary or tertiary amines to form N-nitrosamines which are regarded as carcinogens. Therefore, it is important to regularly determine the amount of nitrite in water and food samples. In this study, 1,1'-diethyl-2,2'-cyanine iodide was used as a reagent for the spectrophotometric determination of nitrite. The reagent reacted with nitrite causing a decrease in the absorbance at 522 nm. The reaction between 1,1'-diethyl-2,2'-cyanine iodide and nitrite in the solution were also investigated by HPLC and LC-MS.

Parameters affecting response signals such as pH, reaction time and concentration of 1,1'-diethyl-2,2'-cyanine iodide solution were investigated and optimized. The optimum response of 1,1'-diethyl-2,2'-cyanine iodide to nitrite in solutions was obtained in acetate buffer pH 4.0 with the response time of 5 min. A calibration graph was linear over the concentration range of 2.5-60.0 mg/L with a linear equation, $\Delta A = 0.0143C + 0.0075$, $R^2 = 0.9989$. A limit of detection was 1.0 mg/L. The method was also applied for the determination of nitrite in drinking water and food samples.

Determination of nitrite using the method based on immobilized reagent on a support was investigated. The reagent was immobilized onto different polymeric supports including chitosan, agarose, triacetyl cellulose and Nafion. Amino silica dispersed in Nafion with 0.25%wt/v was found to be the most suitable matrix for the reagent immobilization. With the developed sensing films the optimized experimental condition for the analysis of nitrite was solution pH 4.0 and 35 min reaction time. A linear calibration graph in nitrite concentration range of 50-500 mg/L was $\Delta A = 0.0009C - 0.0088$, $R^2 = 0.9933$. A limit of detection was 1.85 mg/L. The developed method was applied for the determination of nitrite in real samples and the results were in good agreement with those obtained with the standard method.

School of Chemistry

Academic Year 2016

Student's Signature _____

Advisor's Signature _____

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First of all, I would like to thank my advisor Asst. Prof. Dr. Sanchai Prayoonpokarach, for giving me the opportunity to learn something new, advice, supporting, sharing me his knowledge and his experience that I have never seen before. Beside my advisor, I gratefully to thank the committee, Prof. Dr. Jatuporn Wittayakun, Asst. Prof. Dr. Patcharin Chaisuwan and Asst. Prof. Dr. Theeranun Siritanon for their comments and suggestion. I would like to thank all lecturers and group members of the School of Chemistry for advice and friendship, all staffs at the Center for Scientific and Technological Equipment for their assistance with the equipment, help the chemicals and experience in laboratory teaching. Finally, thank to my family for their supports and endless love.

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CHAPTER I

INTRODUCTION

1.1 Significance of the study

Cancer is caused by an abnormal growth of cells which can damage nearby tissues and spread to the other organs. There are different kinds of cancer, for example, lung, stomach, colon and liver cancer. Cancer can be caused by many factors including smoking, infections, radiation exposure and dietary. Substances that can cause cancer are called carcinogens and these are, for example, pyrolysates, polycyclic aromatic hydrocarbons and N-nitrosamines.

N-nitrosamines are produced by chemical reactions between nitrosating agents (nitrite or nitrate) and secondary or tertiary amines. Nitrosamines can be found in many cured meats because nitrites or sometimes nitrates are added in meats as a preservative to keep the red color of meats and prevent the outgrowth of bacteria.

Besides applications for curing meat products, nitrite is also used as fertilizing agent and these can contaminate fruits, vegetables and water. This contamination is a threat to human health and environment. Exposure to high nitrite level related to methemoglobinemia or blue baby syndrome in infants and birth defects. In addition, high concentration of nitrate or nitrite in natural water can enhance the growth of aquatic plants and consequently, cause eutrophication, which will affect the water quality.

The U.S. Environmental Protection Agency (U.S. EPA) recommended a maximum contaminant level (MCL) of nitrite in drinking water to be 1 mg/L (EPA, 2006). The US Food and Drug Administration (U.S. FDA) suggests that the level of nitrite as an additive to food should not exceed 200 mg/kg. Therefore, it is important to regularly determine the amount of nitrite in water and food.

This work aims to develop spectrophotometric methods for the determination of nitrite using 1,1'-diethyl-2,2'-cyanine iodide which has never been reported as a reagent for the determination of nitrite. The methods based on the reaction of nitrite with the dye in solutions and with the dye immobilized on supports were studied. Parameters that affect the reaction of nitrite and the reagent were investigated and optimized. The developed methods were applied for the determination of nitrite in real samples.

1.2 Optical chemical sensors

One class of chemical sensors is optical chemical sensors. This type of sensors is classified based on a type of the transducer. In this case, phototransducers are used for signal generation. Typically an optical chemical sensor contains two units which are a receptor part and a transducer part as shown in Figure 1.1. The receptor part contains two main components including sensing reagent which interacts with the analyte usually an analyte-sensitive dye molecule and a supporting matrix which holds a sensing reagent. Polymers are commonly used as a supporting matrix. When a receptor part responds to an analyte, a chemical signal such as color change or emission of radiation is produced and transformed into electrical signal by a transducer.

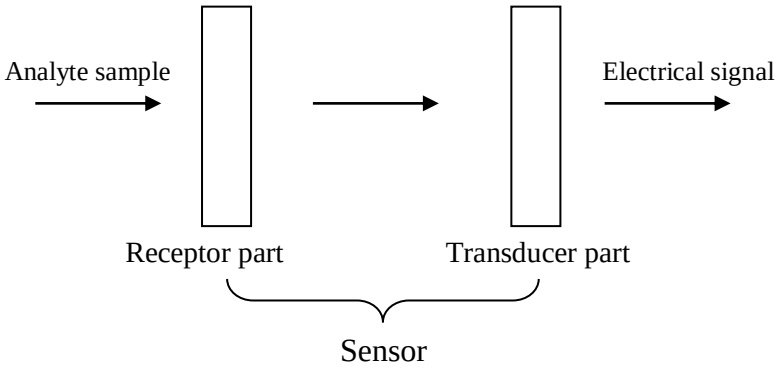

Figure 1.1 Components of an optical chemical sensor.

1.2.1 Supporting matrix

Supporting matrices used in optical chemical sensors should be compatible with sensing reagents, optically transparent and physically and chemically stable. In this work chitosan, agarose, triacetyl cellulose and Nafion were investigated as supporting matrix for 1,1'-diethyl-2,2'-cyanine iodide. Chitosan was investigated as a supporting matrix because it is non-toxic, hydrophilic biopolymer and optically transparent. The presence of free amino and hydroxyl groups on its polysaccharide chain as shown in Figure 1.2 provides active reaction sites for coupling of various ligands.

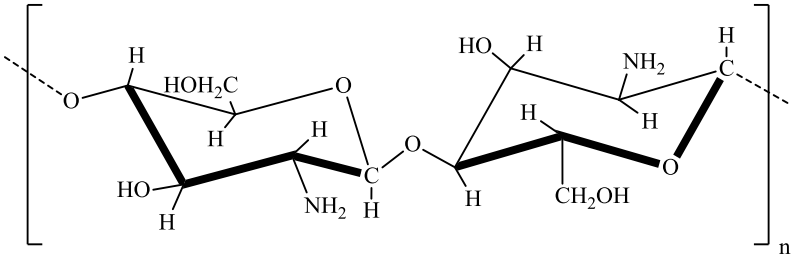

Figure 1.2 A structure of chitosan (López-Garcia, Lehochý, Humpolíćek and Sáha, 2014).

Agarose is a polysaccharide polymer material consisting of alternating D-galactose and 3,6-anhydro-L-galactose units as shown in Figure 1.3. It can be dissolved in boiling water, and forms a gel when it cools. The gels have large pore sizes. Agarose is a nontoxic gel-type hydrophilic support, chemically inert and microbiologically resistant material that is stable in a pH range of 0-14 (Abolgfasemi, Sobhia and Piryaeib, 2016).

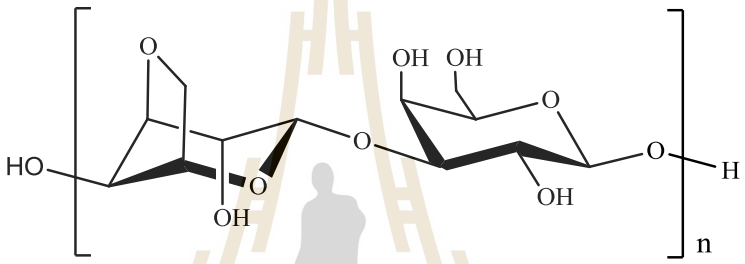

Figure 1.3 A structure of agarose (https://en.wikipedia.org/wiki/Agarose).

Triacetyl cellulose has a structure as shown in Figure 1.4. Triacetyl cellulose possess high optical clarity and relatively hydrophobic. Porosity of the membrane can be increased by simply treating in potassium hydroxide solution. This makes triactyl cellulose an interesting support.

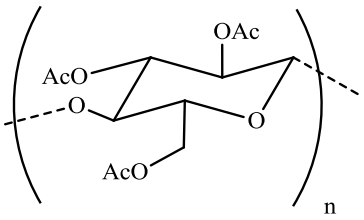

Figure 1.4 A structure of triacetyl cellulose membrane where OAc represents –O–CO–CH_3 (https://en.wikipedia.org/wiki/Cellulose_triacetate).

Nafion is an ionic polymer. Its structure as shown in Figure 1.5 contains of a polytetrafluoroethylene backbone and a side chain with sulfonic acid group. The cationic species could be incorporated in Nafion by ion-exchange process. Nafion is hydrophobic and stable at high temperature, resistant to most solvent, oxidants and bases. These properties make Nafion a good support for many chemical sensors.

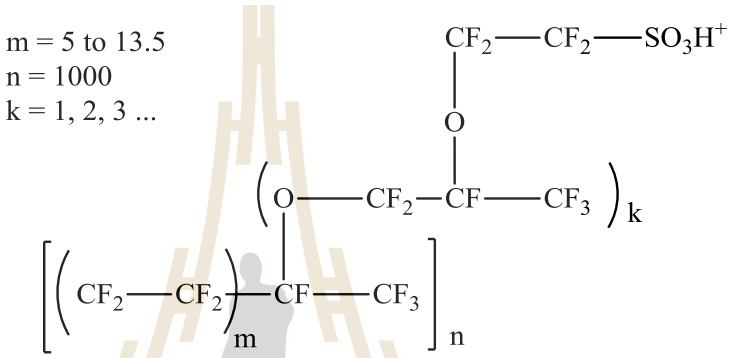

Figure 1.5 A structure of Nafion (Misra, Mishra, Joshi and Pant, 2002).

In this work, 1,1'-diethyl-2,2'-cyanine iodide was used as a sensing reagent. It is a cationic dye which has strong electronic absorption bands in the visible spectral range and high molar absorption coefficient of 7.5×10^{4} M^{-1} cm^{-1} in methanol (Sheppard and Gedde, 1994). Its spectroscopic properties were extensively investigated (Struganova, Wallner and Pazos, 2008 and Zarow and Shin, 2009). This reagent, to our knowledge, has never before been used for the analysis of nitrite.

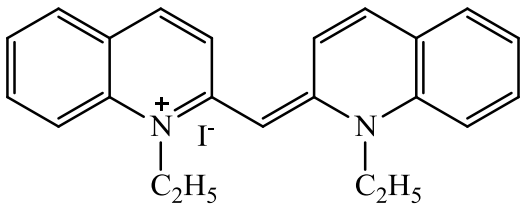

Figure 1.6 A structure of 1,1'-diethyl-2,2'-cyanine iodide.

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CHAPTER II

LITERATURE REVIEWS

2.1 Determination of nitrite based on spectrometric methods

Many spectrometric methods for the determination of nitrite have been reported in recent years. Commercial reagents and synthesized reagents were used in the analyses as summarized in Table 2.1 and 2.2, respectively. The modes of signal measurements are based mainly on absorption and fluorescence.

The advantages of spectrometric method are simple to use, high sensitive and selective, wide concentration range and low detection limit. Therefore, in this work, spectrometric determination of nitrite of environmental relevant levels is proposed using 1,1'-diethyl-2,2'-cyanine iodide as a reagent in typical solutions or in immobilized supports. It is noted that the development of a method are usually based on the seeking of new reagent and improvement of sample treatment and detection strategies.

2.2 Optical chemical sensor

In the recent years, many researchers have developed one of the detection strategies by immobilizing reagents in suitable supports and fabricated as a thin layer membrane. The advantages of this strategy compared to typical solution analysis are reduction of the amount of a sensing reagent used, lower waste generation, preconcentration of an analyte on the support, and reusability of the membrane.

Table 2.1 Spectrometric determination of nitrite using commercial reagents.

Researchers[a]	Reagent	Signal mode[b]	Concentration range (µg/L)	Detection limit (µg/L)
1	indole	F	10-600	2.5
2	ammonium purpurate (murexide)	F	5.0-1000	0.6
3	barbituric	A	10-250	5.0

[a] 1: Jie, Yang, Jiang and Wei, 1999.

2: Biswas, Chowdhury and Ray, 2004.

3: Aydin, Ercan and Taşcıoğlu, 2005.

[b] F: fluorescence and A: absorbance.

Table 2.2 Spectrometric determination of nitrite using synthesized reagents.

Researchers[a]	Reagent	Signal mode[b]	Concentration range (µg/L)	Detection limit (µg/L)
1	rhodamine 110	F	0.46-13.8	0.03
2	mono[6-(2-carboxy-phenyl)]-β-cyclodextrin (OACCD)	F	0.92-78.2	0.0092

[a] 1: Zhang, Wang, Fu and Zhang, 2003.

2: Gao, Zhang, Wang, She and Zhu, 2005.

[b] F: fluorescence.

Table 2.2 Spectrometric determination of nitrite using synthesized reagents (Continued).

Researchers[a]	Reagent	Signal mode[b]	Concentration range (µg/L)	Detection limit (µg/L)
3	2-amino-5,7-dimethyl-1,8-naphthyridine (ADMND)	F	4.6-115	1.87
4	6-(2-carboxyphenyl)-9-(dimethylamino)-3,4-dihydro-2H-chromenol [3,2-g]quinolin-1-ium	F	0.46-16.1	0.0092
5	1-((Z)-(naphthalene-4-ylimino)naphthalene-2-ol	F	460-4600	2.62
6	1-butyl-3-methylimidazonium-modified methyl red ([BMIM]MR)	A	4.0-191	0.75

[a] 3: Chen, Tong and Zhou, 2007.

4: Liu, Yan, Guo, Wang, Li, Yan and Chen, 2009.

5: Sahana, Banerjee, Lohar, Das, Hauli, Mukhopadhyay, Matalobos and Das, 2013.

6: Zhang, Qi, Dong, Chen, Xu, Ma and Chen, 2014.

[b] F: fluorescence and A: absorbance.

There are many supporting matrices that were demonstrated to be successfully used in optical chemical sensors. This includes chitosan (Yusof and Ahmad, 2002; Fen, Yunus, Yusof, Ishak, Omar and Zainudin, 2015 and Sombatsri, Wittayakun, Sanai, Kajsanthia and Prayoonpokarach, 2012), agarose (Hashemi, Abolghasemi, Alizadeh and Zarjani, 2008; Alizadeh, Parooi, Hashemi, Rezaei and Ganjali, 2011 and Zargoosh and Babadi, 2015), triacetyl cellulose membrane (Ensafi and Kazemzadeh, 2002; Afkhami, Madrakian and Aleseyyed, 2012 and Ensafi and Amini, 2012) and Nafion (Zinger and Shier, 1999; Coo and Martinez, 2004 and Iwamori, Nishiyama and Oya, 2016).

Ensafi and Kazemzadeh (2002) determined nitrite with an optical sensor based on Safranine O immobilized on triacetyl cellulose membrane. Nitrite reacted with the reagent to form diazonium salt in acidic media which caused a decrease in the absorbance at 520 nm. The sensor was applied to determine nitrite in meat products and environmental water samples.

Ensafi and Amini (2012) fabricated an optical chemical sensor by using lauth's violet immobilizing on triacetyl cellulose membrane for the determination of nitrite. Nitrite acted as a catalyst for the oxidation of lauth's violet by bromate in acidic media. This reaction caused the change of absorbance at 617 nm. The sensor was applied for the analysis of nitrite in vegetable and food samples.

In this work, polymeric supports such as chitosan, agarose, triacetyl cellulose membrane and Nafion were investigated as a support for immobilization 1,1'-diethyl-2,2'-cyanine iodide. This dye has never been reported as a reagent for the determination of nitrite.

2.3 References

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CHAPTER III

EXPERIMENTAL

3.1 Chemicals and materials

Chemicals used in this research are shown in Table 3.1.

Table 3.1 List of chemicals used in this work.

Chemicals	Supplier	Content (%)
1,1'-diethyl-2,2'-cyanine iodide	Sigma-Aldrich	97.0
agarose	Fluka	-
acetic acid	J.T. BAKER	99.9
ammonium fluoride	Merck	98.0
ammonium lauryl sulfate	Aldrich	~30% in water
cetyltrimethylammonium bromide	Unilab	-
chitosan high viscous	Fluka	99.9
chitosan low molecular weight	Aldrich	-
chloroform	Carlo Erba	99
copper sulphate heptahydrate	UNIVER	98.0
deuterium oxide	Sigma-Aldich	97.0
disodium hydrogen phosphate	QRëC	99.0
ethyl alcohol	Carlo Erba	99.9
ethylenediamine	Merck	99.0

Table 3.1 List of chemicals used in this work (Continued).

Chemicals	Supplier	Content (%)
glutaraldehyde	Unilab	25
methyl alcohol	Carlo Erba	99.0
methyl alcohol-d4	Wilmad Labglass	99.8
N-1-naphthylethylene diamine dihydrochloride	Carlo Erba Fluka	99.0 5.0
Nafion® 117 solution	Carlo Erba	98.5
nickel nitrate hexahydrate	QRëc	99.8
N,N-Dimethylformamide	Fluka	85.0
phosphoric acid	Sigma-Aldich	99.9
potassium bromide	Carlo Erba	99
potassium hydroxide	Merck	85.0
silicon dioxide	BDH	99.5
sodium acetate trihydrate	Carlo Erba	99.0
sodium chloride	Fluka	99.0
sodium dihydrogen phosphate	Fluka	99.9
sodium hypochlorite	Haiter	6
sodium nitrite	Fluka	99.0
sulfanilamine	BDH	99.0
sulphuric acid	Carlo Erba	96
tetraethyl-orthosilicate	Fluka	98.0

3.2 Preparation of aqueous solutions

All chemicals were analytical reagent grade and deionized water was used for the preparation of all aqueous solutions.

A stock nitrite solution (1000 mg/L) was prepared by dissolving an appropriate amount of sodium nitrite in deionized water with 2 mL of chloroform added in order to prevent the outgrowth of bacteria in the solution. Solutions of nitrite with lower concentrations were prepared by dilution of the stock nitrite solution with a suitable buffer.

Solutions of 1,1'-diethyl-2,2'-cyanine iodide were prepared by dissolving appropriate amount of the chemical in methanol and kept in brown glass reagent bottles.

Acetate buffer solutions with pH values in the range of 3.0-7.0 were prepared by mixing appropriate volumes of 0.1 M acetic acid and 0.1 M sodium acetate solution. The pH of the solutions was adjusted to a required value using a solution of sodium hydroxide or hydrochloric acid. A pH meter (DELTA 320, Mettler Toledo) was used to monitor the pH of the solutions.

Solutions of Cu^{2+}, Ni^{2+}, NO_3^-, Cl^-, Br^- and F^- were prepared using copper sulphate heptahydrate, nickel nitrate hexahydrate, sodium nitrate, sodium chloride, potassium bromide and ammonium fluoride, respectively. The salts were dissolved in deionized water and diluted to the required concentration by acetate buffer pH 4.0.

3.3 Study of the reaction of 1,1'-diethyl-2,2'-cyanine iodide and nitrite in aqueous solutions

3.3.1 Spectrometric study

A 2.0 mL of 1,1'-diethyl-2,2'-cyanine iodide in buffer solution was put in a cuvette and the cuvette was placed into a sample holder of a fiber optic spectrometer (USB 4000, Ocean Optics) with stirring provided. A spectrum of the solution was acquired within the wavelength range of 350-650 nm. After that a solution of nitrite was pipetted into the dye reagent. Spectra of the solution were monitored at various times. The concentration of the dye solution was fixed at 2.0×10^{-5} M. The nitrite concentration was also varied from 1-100 mg/L.

3.3.2 High performance liquid chromatographic analysis

A high performance liquid chromatograph (HPLC) with a diode array detector (1260, Agilent Technology) was used for the characterization of the reaction between 1,1'-diethyl-2,2'-cyanine iodide and nitrite. A column was Zorbax SB C-18 (4.6×250 mm, 3.5 µm). A mobile phase consisted of 60:40 volume ratio of acetonitrile: 0.2 M acetate buffer (pH 4.0). The flow rate of the mobile phase was 0.5 mL/min. A liquid injection volume was 30 µL. The absorbance of eluates was monitored at 254 nm. The sample solutions were 2.0×10^{-5} M 1,1'-diethyl-2,2'-cyanine iodide in acetate buffer pH 4.0 and in 0.1 M HCl and 2.0×10^{-5} M 1,1'-diethyl-2,2'-cyanine iodide mixed with nitrite. The solutions of dye mixed with nitrite were allowed to react for 10 min before the injection.

3.3.3 Liquid chromatography- mass spectrometric analysis

A liquid chromatography tandem mass spectrometry triple quadrupole with a diode array detector (6490, Agilent Technology) was used to characterize the

reaction of 1,1'-diethyl-2,2'-cyanine iodide before and after reacting with nitrite in acetate buffer pH 4.0. The column was Zorbax SB C-18 (2.1×150 mm, 1.8 µm). The column temperature was controlled at 40℃. A mobile phase was 60:40 volume ratio of acetonitrile: 1.0×10^{-4} M HCl. The flow rate of the mobile phase was 0.5 mL/min. A liquid injection volume was 3 µL. The electrospray ionization in the positive (ESI+) mode was used. Parameters for electrospray ionization were chamber current; 0.59 µA, nitrogen gas flow; 14.0 L/min, gas temperature; 200℃, sheath gas (nitrogen) flow; 11.0 L/min and sheath gas temperature; 350℃. For the detection with the diode array detector, the absorbance of the eluates was monitored at 254 nm.

Sample solutions were 1.0×10^{-5} M 1,1'-diethyl-2,2'-cyanine iodide in acetate buffer pH 4.0 and 1.0×10^{-5} M 1,1'-diethyl-2,2'-cyanine iodide mixed with 25 mg/L nitrite. The solutions of the dye mixed with nitrite were allowed to react about 10 min before first injection and about 40 min for the second injection.

3.3.4 Effect of pH on the reaction of 1,1'-diethyl-2,2'-cyanine iodide and nitrite

Influence of pH on the reaction was investigated. A 2.0 mL of a dye solution was placed into a cuvette and nitrite solution was spiked into the dye solution. Absorbance measurements of the solutions were made at 522 nm. The concentration of 1,1'-diethyl-2,2'-cyanine iodide was fixed at 2.0×10^{-5} M and nitrite concentration was fixed at 5 mg/L. The pH of the solutions was varied within the range of 3.0-7.0 controlled by acetate buffer.

3.3.5 Effect of 1,1'-diethyl-2,2'-cyanine iodide concentration

The concentration of dye solutions in the range of 1.0×10^{-5} - 6.0×10^{-5} M were investigated. A dye solution with the concentration of 4.5×10^{-4} M was diluted to

a required concentration with acetate buffer pH 4.0. A 2.00 mL solution of the diluted dye solution was put in a cuvette which was placed in a spectrometer with stirring provided and then a solution of nitrite was spiked into the dye solution to obtain the in-cell concentration of nitrite of 10 mg/L. The absorbance change was recorded at 522 nm.

3.3.6 Calibration study

A calibration curve for nitrite was constructed in the concentration range of 2.5-60 mg/L. The optimized condition for spectrometric measurement of nitrite was employed. A 2.0 mL of 2.0×10^{-5} M dye in acetate buffer solution was put in a cuvette and nitrite solution was added to obtain the required concentration. The absorbance of the solutions was recorded at 522 nm.

3.3.7 Interference study

The effect of foreign ions on the reaction between 1,1'-diethyl-2,2'-cyanine iodide and nitrite was studied at the optimum reaction condition for nitrite. Solutions of the dye mixed with nitrite and foreign ions were prepared to obtain 1:1 molar ratio of nitrite to a foreign ion. The concentration of nitrite and a foreign ion in a solution was 1.0×10^{-4} M. The ions investigated were NO_3^-, Br^-, F^-, Cl^-, Cu^{2+} and Ni^{2+}. Absorption spectra of solutions containing nitrite and nitrite mixed with the investigated ions were recorded in the wavelength range of 350-650 nm.

3.3.8 Analysis of samples

Drinking water and pork samples were collected from a local market in Nakhon Ratchasima, Thailand. Water samples were filtered through a Whatman No.1 filter paper and the filtrate was stored in polypropylene bottles at room temperature. An aliquot of 4.0 mL of the filtrate was diluted with acetate buffer in a 10-mL

volumetric flask and the solution was used for further analysis. A similar solution was also prepared as previously described, but with the addition of 4.0 mL of 500 mg/L nitrite solution. To make absorbance measurements, a 2.0 mL of 2.0×10^{-5} M dye solution was placed into a cuvette with stirring provided and a sample solution was spiked into the dye solution. The volumes of the spiked sample solution were 25, 55 and 80 µL. The absorbance of a solution was recorded at 522 nm for 5 min.

To extract nitrite from pork samples, a method from the literature was used (Siddiqui, Wabaidur, Alothman and Rafiquee, 2015). A pork sample was ground and 10 g of the sample was mixed with 100 mL of deionized water at 80℃ with stirring provided for 15 min. After the mixture was cooled down to room temperature, it was centrifuged for 10 min at 6000 rpm and the supernatant was filtered through a Whatman No.1 filter paper. The filtrate was diluted to 100 mL with deionized water. The sample solution was further used for the spectrometric analysis with the same method used for the water samples.

An AOAC method no. 973.31 (Horwitz, 2005) based on Griess reaction was also used to determine the concentration of nitrite for a comparison purpose. Solutions of two reagents were prepared. A solution of sulfanilamide was prepared by dissolving 0.0833 g sulfanilamide in 25 mL of 15% v/v CH_3COOH. A solution of N-(1-naphthyl)ethylenediamine (NED) was prepared by dissolving 0.033 g NED in 25 mL of 15% v/v CH_3COOH. A 0.5 mL of sulfanilamide solution was mixed with a suitable volume of a sample solution in a 10-mL volumetric flask for 5 min and then 0.5 mL of NED solution was added. The volume of the solution was made up to 10 mL with deionized water. After 15 min, a pink compound was produced and

absorbance measurements of the solution were made at 540 nm. A calibration curve for nitrite was constructed in the concentration range of 0.25-2.5 mg/L.

3.4 Preparation of sensing films

3.4.1 Preparation of chitosan sensing films

A 1% chitosan solution was prepared by dissolving 1.0 g of high viscosity chitosan or low molecular weight chitosan in 100 mL of 1% acetic acid solution. The mixture solution was stirred for 3 h and then filtered through a glass frit. The filtrate was sonicated for 30 min to remove air bubbles. The obtained solution was further used for the preparation of sensing films. Chitosan sensing films were prepared using two crosslinking methods that are homogeneous and heterogeneous crosslinking.

For a homogeneous crosslinking method, 6.25 mL of 1% chitosan solution was mixed with 5 mL of 1.0×10^{-4} M dye solution and 2.0 mL of 0.25% glutaraldehyde solution as a crosslinking agent. The mixture solution was stirred for 30 min. After that a 200 µL of the solution was cast onto a transparent film in the size of 0.9×4.0 cm and dried in a hot air oven at 60℃ overnight.

For a heterogeneous crosslinking method, 6.25 mL of 1% chitosan solution was mixed with 5.0 mL of 1.0×10^{-4} M dye solution and stirred for 30 min. A 200 µL of the mixture solution was cast onto a transparent film in the size of 0.9×4.0 cm and dried in the hot air oven at 60℃ overnight. The obtained films were soaked in a 50 mL of 0.25% glutaraldehyde solution for 5 min and dried at room temperature for 120 min. The films were washed with deionized water several times before used.

3.4.2 Preparation of agarose sensing films

Agarose sensing films were prepared by dissolving 0.5 g of agarose in 50 mL of hot water. The mixture was stirred for 5 min and 25 mL of the solution was poured into a petri dish which had an i.d. of 9 cm and left for 30 min. The obtained films were cut into the size of 0.9×4.0 cm and placed into 1.15×10^{-3} M dye solution for 2, 4 h and overnight. After the immobilization process, the films were washed with deionized water several times to remove the unimmobilized dye.

3.4.3 Preparation of triacetyl cellulose sensing films

Transparent triacetyl cellulose films were produced from waste photographic film tapes that were previously treated with commercial sodium hypochlorite in order to remove colored gelatinous layers and washed with water several times. The membranes were treated with 1.0 M KOH for 4 h to increase the porosity of the membranes.

Various strategies were used to immobilization 1,1'-diethyl-2,2'-cyanine iodide onto triacetyl cellulose films. In the first method, the membranes were soaked in 10.0 mL of ethylenediamine (EDA) mixed with 0.0035 g of 1,1'-diethyl-2,2'-cyanine iodide for 3 min. Then the obtained films were washed with deionized water several times to remove unbound dye and cut into the size of 0.9×4.0 cm. Finally, the obtained films were kept in deionized water.

In the second method, the membranes were soaked in 10.0 mL of EDA for 3 min and then immersed in 5.0×10^{-4} M dye solution for 4 h. After immobilization, the obtained films were washed several times with water, cut to the size of 0.9×0.4 cm and kept in deionized water.

Prevention of dye leaching from sensing films was studied by coating films with either Nafion or chitosan. The third method was Nafion coating, 20 µL Nafion solution was spread onto a sensing film prepared from the 1st method and the film was washed with water several times and dried in the air at room temperature. The fourth method was chitosan coating, the sensing films was coated with 20 µL of chitosan solution mixed with 1% glutaraldehyde solution. Similar treatment processes were made after the coating.

In the fifth method, ~0.1 g of activated membrane was dissolved in 2.0 mL of the mixture of EDA and 0.0035 g of dye for 60 min. Then a 200 µL of mixture was dropped onto a transparent film and dried in an oven at 60℃ overnight.

In the sixth method, the activated membrane was dissolved in 2.0 mL of the mixture of EDA and 0.0035 g of dye for 60 min and 40 µL of chitosan solution and 1% or 2% v/v of glutaraldehyde solution were added to the mixture. Then a 200 µL of mixture was dropped onto a transparent film and dried as in the fifth method.

3.4.4 Preparation of sol-gel based films

A 0.5 mg of 1,1'-diethyl-2,2'-cyanine iodide was dissolved in 1.25 mL of ethanol and the solution was stirred for 30 min. A 1.0 mL of tetraethyl orthosilicate (TEOS) and 0.5 mL of 2×10^{-3} M NaOH were added into the dye solution. The mixture was stirred for 30 min and aged at room temperature at various times (1-3 days) before it was coated onto activated cover glass slides. Cover glass slides were activated by soaking in 1.0 M NaOH for 1 day and after that washed with water several times. After a given aging time, activated cover glass slides were dipped into the dye-TEOS mixture and then slowly removed. The coated glass slides were dried at

room temperature in the dark environment. At day 4 of aging, the dye-TEOS mixture turned into powder, which was later used to mix with Nafion to form sensing films.

3.4.5 Preparation of Nafion sensing films

A 0.5 mg of 1,1'-diethyl-2,2'-cyanine iodide was dissolved in a 1.0 mL of 5% Nafion solution. The solution was sonicated for 30 min. Films were formed using two methods which were manual casting and spin coating. The first method was manual casting, a 20 µL of the dye-Nafion mixture was cast onto a transparent film in the size of 0.9×1.0 cm. The spread mixture was dried at room temperature overnight, or dried in an oven at 40℃ for 10 min and later dried under a vacuum condition for 10 min. The vacuum condition was setup by connecting a desiccator with a suction pump and before the pump was initiated, the spread mixture was placed inside the desiccator and the pump was turned on for 10 min.

The second method was spin coating, a 50 or 100 µL of the mixture was dropped onto a transparent film in a spin coater (KW-4A, Chemat technology). The spin rates were 500 rpm for 10 s and then 600 rpm for 50 s. The obtained films were dried at room temperature overnight.

Incorporation of silica particles in Nafion films was also investigated. Fume silica and silica particles obtained from gelation process were added into the dye-Nafion solution at the concentration of 0.5%wt/v. Amino silica particles added into the dye-Nafion solution in a concentration range of 0.1-1.0%wt/v were studied. When silica particles were added into the dye-Nafion solution, the mixture was sonicated for 20 min. Manual casting method as previously described was used to form sensing films. The films were dried at room temperature and kept in a desiccator before use.

Silica particles obtained from gelation process were from the experiment in section 3.4.4 and amino silica particles were prepared by Stöber method (Liang, Xue, Xu, Li, Zhang and Yang, 2013) and modified the particles surface with aminopropyltetriethoxysilane (APTES). All of silica particles were sieved to obtain the particles with the size <63 μm before mixed with dye-Nafion solution.

3.5 Characterization of sensing films

3.5.1 Stability test

All the prepared sensing films were tested for their stability in aqueous solutions. A film was placed against the opaque side of a cuvette that did not face the optical path. The cuvette contained 2.0 mL of acetate buffer pH 4.0. Absorbance at 522 nm was monitored for 60 min to observe the leaching of the dye.

3.5.2 Response of silica particles/Nafion sensing films to nitrite

Nafion mixed with silica particles films were tested for their reactivity with nitrite in aqueous solutions. A film was placed into a cuvette containing 2.0 mL of acetate buffer pH 4.0 and the absorbance was recorded at 518 nm for ~5 min or until the signal was relatively stable. After that, the buffer was replaced by 2.0 mL of 100 mg/L nitrite solution and the change of absorbance was monitored at 518 nm for 60 min.

3.5.3 Calibration study

A calibration curve for nitrite was made with amino silica particles/Nafion films (0.25%wt/v amino silica particles). A film was dipped into 2.0 mL of acetate buffer pH 4.0 in a cuvette. Absorbance at 518 nm was recorded for ~5 min. After that,

the film was placed into 2.0 mL of nitrite solution and the absorbance was monitored. Nitrite solutions in concentration range of 50-500 mg/L were used in the study.

3.5.4 Interference study

Responses of sensing films to foreign ions were evaluated at the optimum condition for nitrite. Solutions of nitrite mixed with an investigated ion were made to obtain 1:1 molar ratio of nitrite to the investigated ion. The concentration of nitrite and a foreign ion in a solution was 3.2×10^{-3} M. Ions investigated were NO_3^-, Br^-, F^-, Cl^-, Cu^{2+} and Ni^{2+}. The solutions of nitrite mixed a foreign ion were prepared in a 10.0-mL volumetric flask. The pH of the solutions was controlled using acetate buffer pH 4.0. Absorbance measurements condition was similar to that used in the calibration study.

3.5.5 Regeneration

Solution of 0.1 M NaOH was used for regeneration study. A sensing film was dipped in acetate buffer pH 4.0 and the absorbance at 518 nm was recorded for ~5 min. Then the film was placed into 200 mg/L of nitrite solution and the absorbance was monitored for 40 min. After that, the sensing film was washed with deionized water several times and soaked into 6.0 mL of 0.1 M NaOH in a beaker for 40 min. The sensing film was washed with deionized water several times before monitored the absorbance of a sensing film in acetate buffer pH 4.0 for 5 min.

3.6 References

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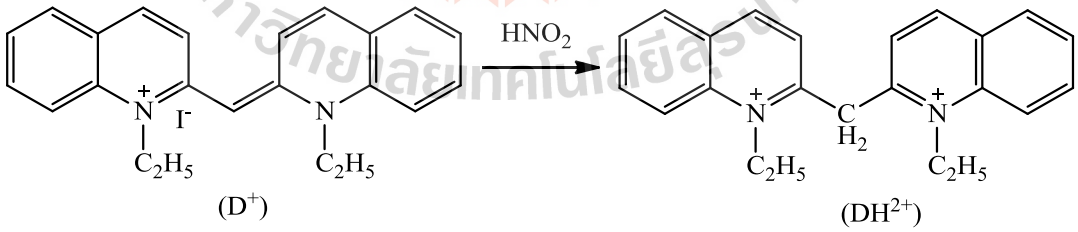
CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Spectrometric study of 1,1'-diethyl-2,2'-cyanine iodide in aqueous solutions

Spectra of 2.0×10^{-5} M 1,1'-diethyl-2,2'-cyanine iodide solution and the dye solution mixed with nitrite are shown in Figure 4.1. The dye has two absorption peaks at 490 and 522 nm and a shoulder at 457 nm. When nitrite was added into the dye solution at pH 4.0, a decrease in absorbance was observed. This could be resulted from the protonation of NO_2^- in the solution to form HNO_2 in the acidic solution (Ormerod, Copeland, Hey, Husain and Ewen, 1999 and Lundberg, Weilzberg and Gladwin, 2008) and HNO_2 could then react with the dye via protonation as follow,



The reaction of the dye with proton in acidic solutions was also reported by a group of researchers (Feldman, Herz and Regan, 1968). In further study, the change in the absorbance (ΔA) at 522 nm was further used for the analysis of nitrite.

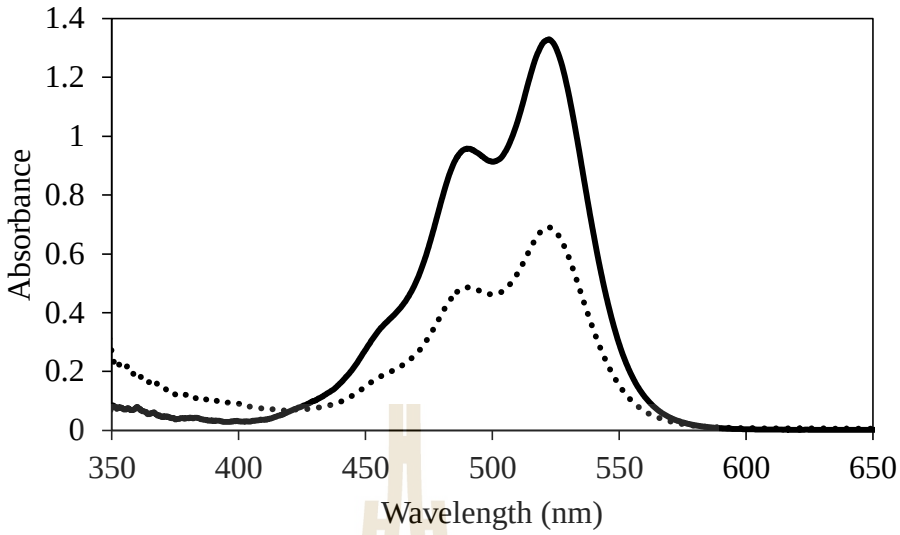


Figure 4.1 Absorption spectra of 1,1'-diethyl-2,2'-cyanine iodide solution (-) and 1,1'-diethyl-2,2'-cyanine iodide solution in the presence of 10 mg/L nitrite (···).

4.1.1 HPLC analysis

HPLC was used to characterize the reaction between the dye and nitrite. Figure 4.2 shows chromatograms of the dye and the dye mixed with nitrite at different concentrations. The retention time of the dye was 15.4 min as shown in chromatogram (a). In the presence of nitrite, peaks in the time range of 4-6 min and 8.3 min were observed as shown in chromatogram (b) and (c). However, two peaks of the protonated and unprotonated dye were expected. When the concentration of nitrite was increased from 10 to 50 mg/L the signal at 15.4 min decreased with the increase of the signal time range of 4-6 min and 8.3 min. A shorter retention time of the protonated species is expected because the nonrigid planar structure would have lower interaction with the stationary phase compared to the unprotonated species.

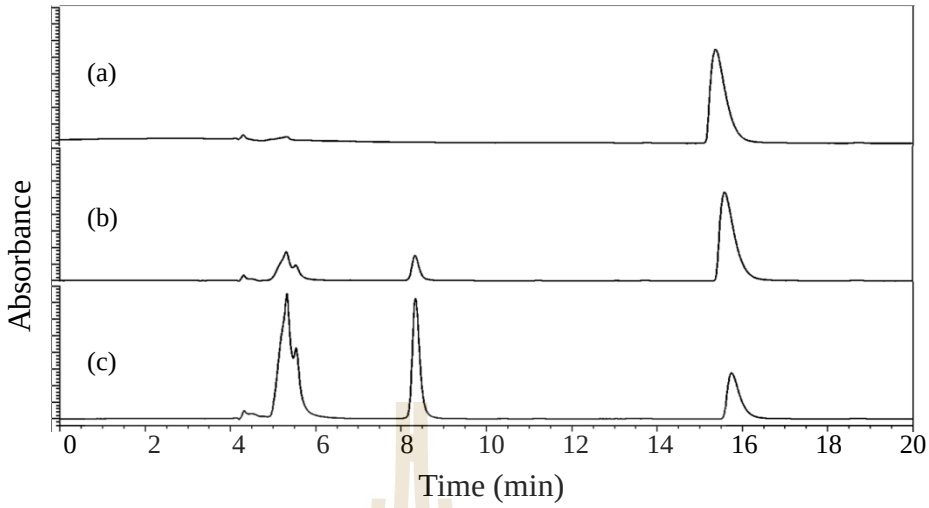


Figure 4.2 Chromatograms of 2.0×10^{-5} M 1,1'-diethyl-2,2'-cyanine iodide solution in acetate buffer pH 4.0 (a), dye solution with 10 mg/L nitrite (b) and dye solution with 50 mg/L nitrite (c).

4.1.2 LC-MS analysis

Further investigation of the reaction was conducted with LC-MS. A solution of 1,1'-diethyl-2,2'-cyanine iodide mixed with nitrite was prepared in acetate buffer solution pH 4.0. The concentration of the dye in the solution was 1.0×10^{-5} M and that of nitrite was 25 mg/L. Two injections of the sample solution were made after the preparation. The first injection was made right after the solution preparation about 10 min while the solution still had observable pink color. The second injection was about 40 min after the solution preparation and the pink color disappeared.

The total ion current (TIC) chromatograms in Figure 4.3 show a peak of 1,1'-diethyl-2,2'-cyanine iodide at 6.2 min. When nitrite was added to the dye solution another peak appeared at 4.1 min. In addition, as the reaction proceeded from the first

to the second injection, the signal at 4.1 min increased whereas that at 6.2 min decreased. This suggested the formation of the protonated dye.

From a mass spectrum of the dye solution at the retention time 6.2 min, mass to charge ratio (m/z) of 327.2 was found, this m/z corresponded to the unprotonated dye. At the retention time of 4.1 min, a different pattern of the mass spectrum was obtained. This indicated a change in the dye structure after the reaction, although the mass of the suspected species was not successfully elucidated. Detail information of the mass spectra are shown in Appendix A.

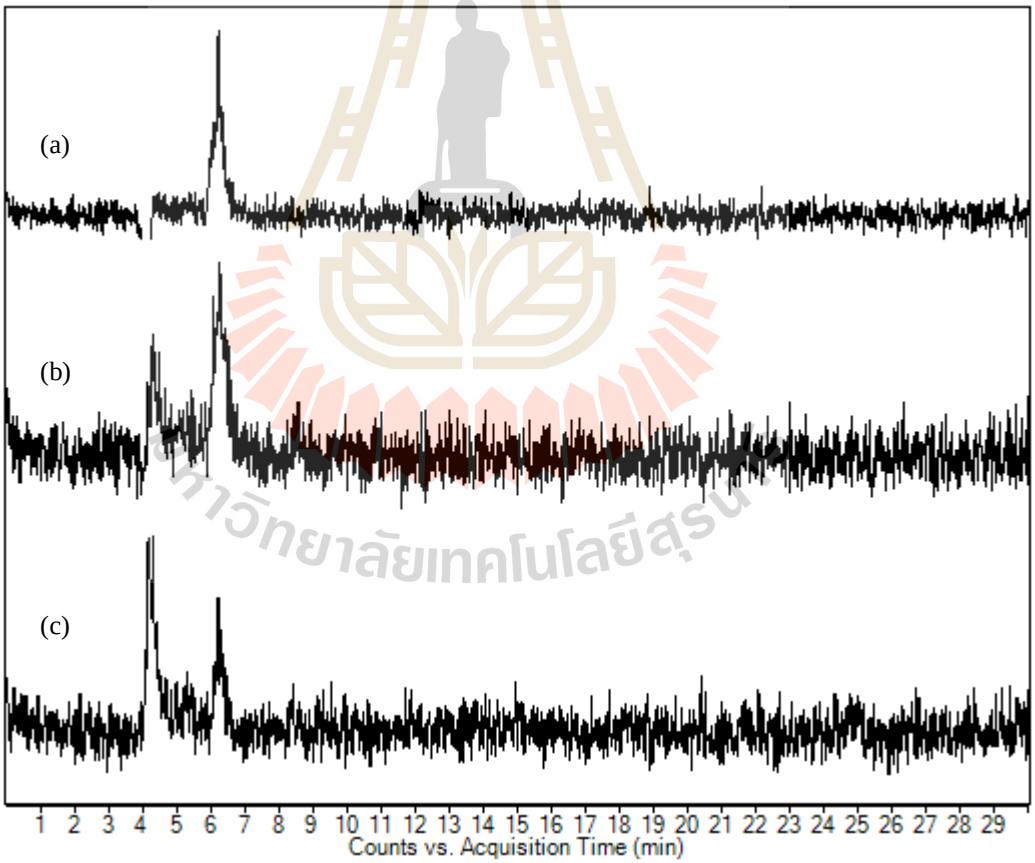


Figure 4.3 TIC chromatograms of the dye solution (a), the dye solution mixed with nitrite first (b) and second injection (c).

4.1.3 Effect of pH

The influence of pH on the reaction between the dye and nitrite was investigated in solutions using fixed concentration of dye at 2.0×10^{-5} M and nitrite at 5 mg/L. The pH of the solutions was varied within the range of 3.0-7.0. The results are shown in Figure 4.4. A larger change of the signals (ΔA) was observed at pH 3.0 and 4.0, but the signal at pH 3.0 tends to reach a plateau faster. Slight change of the signals was observed in the solutions pH 5.0, 6.0 and 7.0. This could be due to lower concentration of H^+ to protonate nitrite to form nitrous acid. At the pH 3.0 and lower, the acid solutions could cause the reduction of the initial absorbance signal due to the

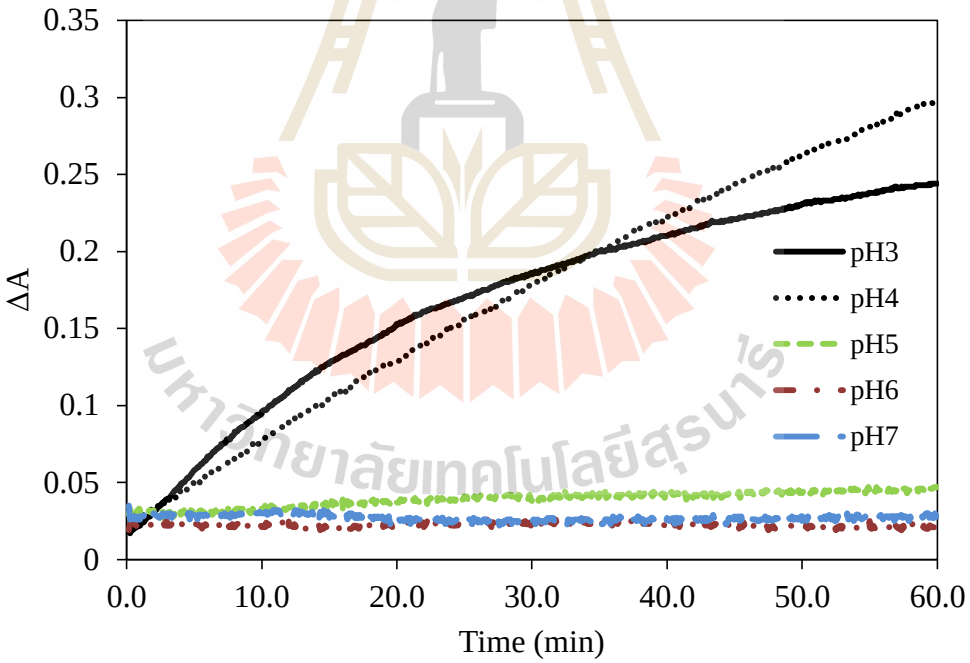


Figure 4.4 Time profiles of the signal change obtained from the reaction of 2.0×10^{-5} M 1,1'-diethyl-2,2'-cyanine iodide solution with nitrite concentration in solutions of 5 mg/L.

reaction of H^+ with the dye and consequently, the concentration of the dye in the active form, D^+, available for the reaction with nitrite would be too low. Therefore, in this work we chose to control the pH of the studied solutions at 4.0.

4.1.4 Effect of 1,1'-diethyl-2,2'-cyanine iodide concentration

The effect of the dye concentration on the reaction was studied in solutions pH 4.0 with the fixed concentration of nitrite at 10 mg/L and the dye concentration varied from 1.0×10^{-5} - 6.0×10^{-5} M. The absorbance change was monitored for ~1 h. In Figure 4.5, a relative absorbance change which was calculated from the absorbance change due to nitrite (ΔA) divided by the initial absorbance of the dye solution with respected to the dye concentration (A_{dye}), was plotted against the reaction time. The results show that the dye concentration of 2.0×10^{-5} M produced the largest relative signal change. At the dye concentration lower than 2.0×10^{-5} M, lower signal change

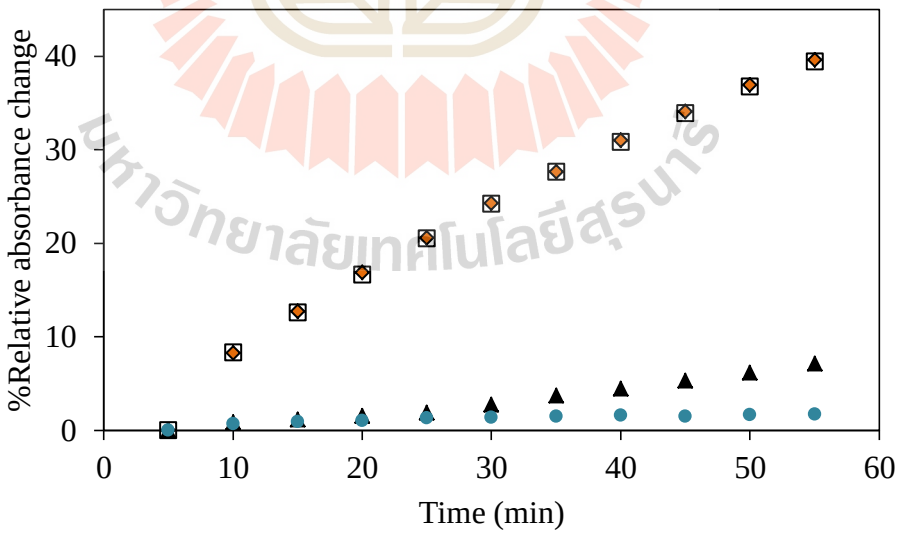


Figure 4.5 Optimization of the dye concentration. The studied dye concentrations were 1.0×10^{-5} M (♦), 2.0×10^{-5} M (□), 4.0×10^{-5} M (▲) and 6.0×10^{-5} M (•).

was obtained. This could be from a lower rate of the reaction. When the concentration of the dye was higher than 2.0×10^{-5} M, the signal change was also lower because the initial absorbance of the dye was relatively high, consequently, the slight change in the signal produced the lower relative signal change. Therefore, the dye concentration of 2.0×10^{-5} M was used for further study.

4.1.5 Calibration study

A linear calibration curve as shown in Figure 4.6 was obtained with the nitrite concentration range of 2.5-60.0 mg/L under the optimum condition which the concentration of the dye was 2.0×10^{-5} M, the pH of the solutions was 4.0 and the reaction time was 5 min. The linear equation was $\Delta A = 0.0143C + 0.0075$ with the R^2 of 0.9989, where C is the concentration of a nitrite solution. The limit of detection was 1.0 mg/L calculated based on three times the standard deviation of the blank signals.

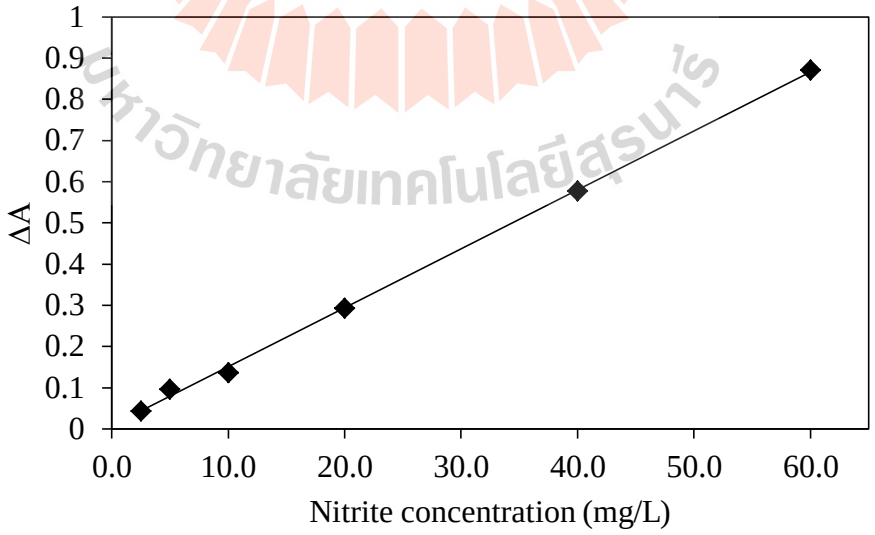


Figure 4.6 Calibration curve for nitrite in aqueous solutions.

4.1.6 Interference study

The effect of interference ions on the determination of nitrite was investigated at a 1:1 molar ratio of a studied ion and NO_2^-. A change of absorbance signal at 522 nm obtained from a solution containing NO_2^- and that obtained from a solution with both NO_2^- and the studied ion were used to calculate %error as shown in equation (4.1),

$$\%\text{Error} = \frac{(\Delta A_{\text{nitrite,ion}} - \Delta A_{\text{nitrite}})}{\Delta A_{\text{nitrite}}} \times 100 \qquad (4.1)$$

Where $\Delta A_{\text{nitrite,ion}}$ is the absorbance change obtained from a solution containing nitrite and a foreign ion. $\Delta A_{\text{nitrite}}$ is the absorbance change obtained from a solution containing nitrite only.

The results are summarized in Table 4.1. A tested ion that exhibits an error of less than 5.0% is considered as tolerable for the analysis. Therefore, in this study the tested ions are considered not interfere with the determination of NO_2^- at the studied concentration.

Table 4.1 Interference study in solutions at 1:1 molar ratio of the ions.

Ion	%Error
Cu^{2+}	1.01
Ni^{2+}	-1.04
NO_3^-	-0.28
Cl^-	-0.58
Br^-	-0.76
F^-	-0.71

4.1.7 Analysis of samples

To evaluate the applicability of the proposed method, determination of nitrite in drinking water and pork samples was carried out. Samples were collected from a local market in Nakhon Ratchasima, Thailand. An AOAC method no. 973.31 based on Griess reaction was also used for a comparison purpose. The results obtained from the two methods were in good agreement as shown in Table 4.2. The recoveries of nitrite in the spiked samples ranged from 100% to 113% and the relative standard deviations below 5% were obtained. This indicating that the proposed method could be alternatively used for the determination of nitrite in real samples.

Table 4.2 Determination of nitrite in pork and drinking water samples.

	Nitrite added (mg/L)	Nitrite found[a] (mg/L) ± SD	Recovery (%)	RSD (%)
Pork sample				
Proposed method	0	Not detected	-	-
	2.50	2.71±0.04	108	1.21
	5.50	5.52±0.03	100	0.51
	8.00	8.07±0.19	101	2.20
AOAC method	0	Not detected	-	-
	0.50	0.52±0.01	104	0.88
	1.00	1.10±0.00	110	0.08
	2.00	2.07±0.01	103	0.38
Drinking water sample				
Proposed method	0	Not detected	-	-
	2.50	2.57±0.10	103	3.87
	5.50	5.69±0.08	103	1.39
	8.00	9.01±0.11	113	1.26
AOAC method	0	Not detected	-	-
	0.50	0.52±0.01	103	2.15
	1.00	1.09±0.01	109	0.67
	2.00	2.18±0.02	109	0.81

[a]The reported values are means of 3 replicate measurements ± standard deviation.

4.2 Immobilization of 1,1'-diethyl-2,2'-cyanine iodide

4.2.1 Supporting matrices

Various materials were used to immobilize 1,1'-diethyl-2,2'-cyanine iodide. They were chitosan, agarose, triacetyl cellulose membrane, Nafion and siliceous materials synthesized via a sol-gel method. Suitable supports should be compatible with the dye, optically transparent and physically and chemically stable in aqueous solutions. After the dye was immobilized, the obtained materials were physically examined and some of them were tested for dye leaching in solutions where absorbance at 522 nm was monitored.

For the immobilization using chitosan as a support, the obtained films were pink color the dye was inhomogeneously distributed. When the films were soaked in 2.0 mL of acetate buffer pH 4.0 in a cuvette, the films swelled and dye leaching occurred. Therefore, chitosan was not a suitable support for the dye. Pictures of chitosan films are shown in Appendix B.

To immobilize the dye in agrose, agarose films were first formed and the films were soaked into a dye solution. The obtained films were reddish pink color with the dye distributed evenly in the films. When the films were soaked in acetate buffer solution, the dye continuously leached into the solution within 3 h of absorbance monitoring. Therefore, agarose was not used as the dye support.

Immobilization of the dye in triacetyl cellulose membranes were done using six methods. In the first method, membranes were soaked in a solution of ethylenediamine (EDA) mixed with the dye for 3 min. In the second method, membranes were soaked in EDA for 3 min and then immersed in a dye solution for 4 h. The obtained films from both methods were pink color with evenly dye

distribution. However, when the membranes were soaked in acetate buffer solution, dye leaching occurred in 1 h.

Due to dye leaching, coating triacetyl cellulose membranes with Nafion or chitosan were investigated. In the third method, membranes obtained from the first method were coated with Nafion. In the fourth method, membranes from the first method were coated with chitosan and chitosan was crosslinked with glutaraldehyde. The membranes obtained from both methods were tested in acetate buffer solutions. Leaching was still observed.

In the fifth method, the activated films were dissolved in EDA mixed with dye. An aliquot of 200 µL of the solution was dropped on a transparent film and placed into an oven at 60℃ overnight. With this procedure, films could not be reconstructed. In the sixth method, the solution mixture obtained from the fifth method was combined with the mixture of chitosan solution and glutaraldehyde solution. An aliquot of 200 µL solution mixture was placed on a transparent film and dried in an oven. The obtained films were reddish pink color with homogeneously distributed dye. Leaching was observed when the films were soaked in acetate buffer solution.

The sol-gel based films were prepared by dipping activated cover glass slides in a dye-TEOS mixture. The obtained product was solid fine pink particles which were not strongly sticked to the cover glass slides. When the films were tested in acetate buffer solution, no leaching of the dye was observed. No further investigation of sol-gel based films was conducted. Pictures of the films are shown in Appendix B.

Nafion sensing films were formed using two methods which were manual casting and spin coating. The obtained films from the first method were prepared by casting 20 µL of dye-Nafion mixture onto a transparent film and drying at room temperature. The dried films were colorless, but pink color was recovered when the films were soaked in acetate buffer solution. Dye leaching was not observed. It was observed that the prepared films were thinner around the center, but thicker at the edges. Consequently, more intense dye color was found about the edges of the films. This might be due to the rate of solvent evaporation was not the same throughout the films. A picture of the film is shown in Appendix B. When measurements of the initial absorbance of the prepared films were made, the results were not quite reproducible. Therefore, a different drying process was used. The dye-Nafion mixture after casting was dried in oven at 40℃ for 10 min and then dried under a vacuum condition for 10 min. Similar results were still obtained.

Another method used for the film preparation was spin coating. A dye-Nafion mixture was dropped on a transparent film inside a spin coater. The obtained films were relatively thin with unevenly distribution of the dye. Therefore, the spin coating was not suitable for the dye-Nafion film preparation.

The absorption spectra of various sensing films were investigated. The sensing films were dipped in a 2.0 mL acetate buffer solution pH 4.0 in a cuvette and the absorbance in the wavelength range of 350-650 nm was acquired. Figure 4.7 shows the absorption spectra of dye immobilized in chitosan, agarose, triacetyl cellulose and Nafion films before dye leaching was tested. The chitosan, triacetyl cellulose and Nafion sensing films show maximum absorption ~519 nm. In the absorption spectrum of the agarose film, there is an additional absorption peak at 580 nm.

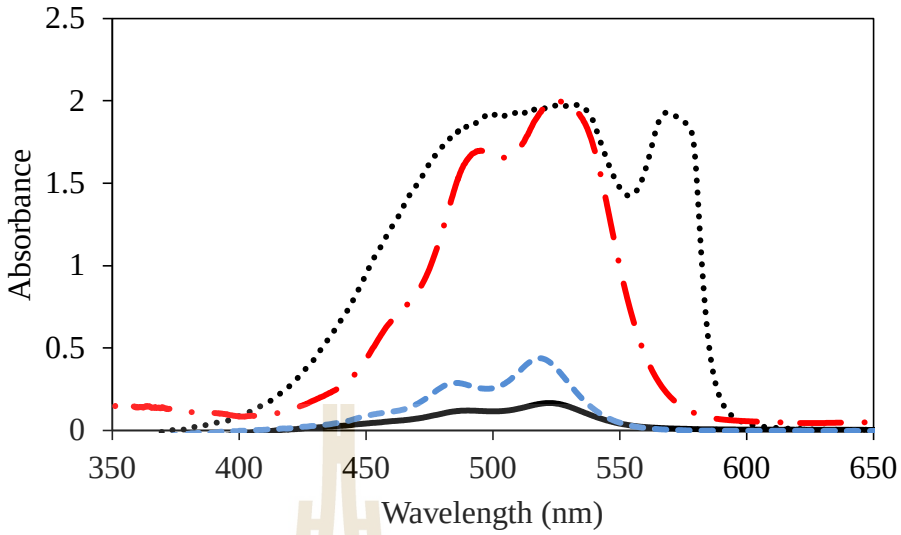


Figure 4.7 Absorption spectra of the dye immobilized on chitosan (□), agarose (•••), triacetyl cellulose (-•-) and Nafion (---) in acetate buffer solution at 5 min.

This spectrum indicates that the dye aggregated in the film (Yao, Morita and Kimura, 2003 and Brito de Baros and Ilharco, 2001). The aggregation of 1,1'-diethyl-2,2'-cyanine iodide (J-aggregration) was observed in solutions with a concentration higher than 2×10^{-4} M (Struganova, 2000). Absorbance signals for agarose and triacetyl cellulose films are higher than those of chitosan and Nafion films because the films were much thicker. In addition, the signals also depend on the solubility of the dye in the supports.

4.2.2 Silica particles/Nafion sensing films

The dye immobilized in Nafion films was unevenly distributed. In addition, Nafion films after dye immobilization tended to be thinner around the middle part of the films and thicker about the edges. The amount of the dye immobilized was much concentrated about the edges of the films which were not the part that absorbance signals were monitored. Various types of silica particles including fume silica, silica

particles obtained from gelation process and amino silica particles were incorporated into Nafion films at the concentration of 0.5%wt/v. The liquid-particles interaction could assist the dispersion of the dye/Nafion solution evenly. Silica particles from the gelation process were prepared by the same method used to prepared the sol-gel based films. The particles were obtained after day 4 of the experiment. Amino silica particles were prepared using tetraethyl orthosilicate (TEOS) as a silica source and the particles were later modified with 3-aminopropyltriethoxysilane (APS). The obtained particles were called amino silica particles. The synthesized particles have nanosize range as shown in Figure 4.8.

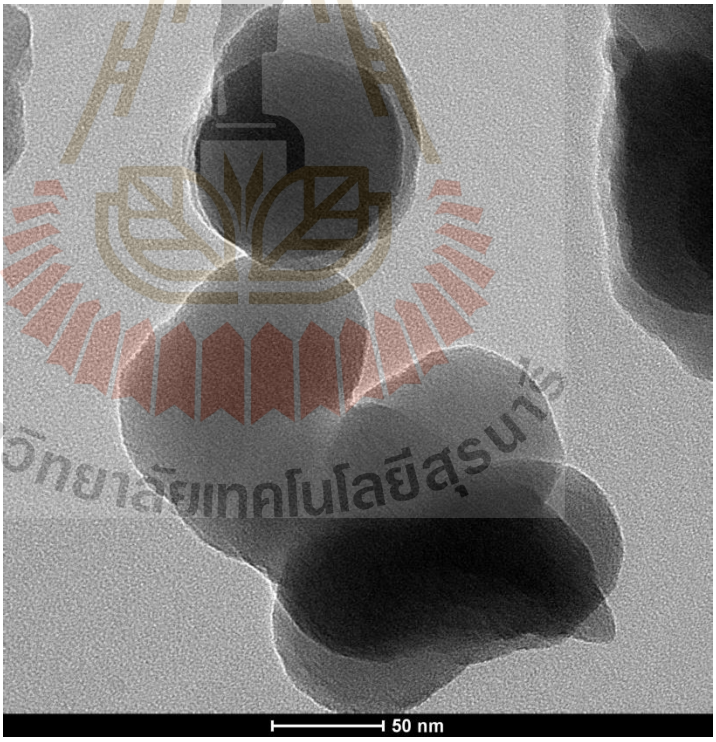


Figure 4.8 A TEM image of amino silica particles.

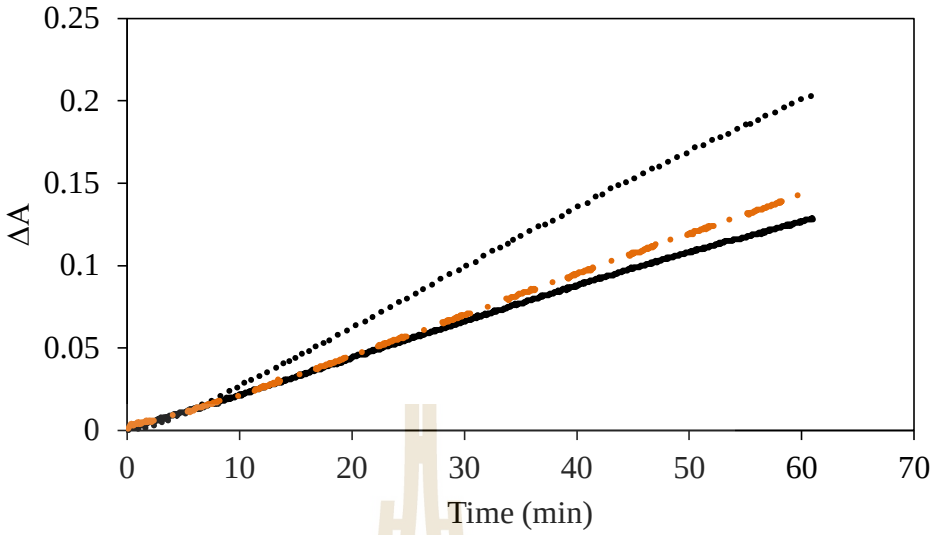


Figure 4.9 Time profiles response of Nafion sensing films mixed with amino silica (••••), fume silica (□) and silica from gelation process (-•-). The concentration of nitrite in the solutions was 100 mg/L. The pH of the solutions was controlled to 4.0 with acetate buffer.

The obtained sensing films were tested in solutions with 100 mg/L of nitrite. The results are shown in Figure 4.9. Films with amino silica particles provided the highest signal. The amino silica particles were nano size particles which could increase the surface area of the films and consequently, increase the amount of the dye exposed to the analyte on the film surface. In addition, the dye was homogeneously distributed. Therefore, the amino silica particles/Nafion films were used for further study.

4.2.3 Optimization of the amount of amino silica particles in Nafion films

Sensing films were prepared with different amount of amino silica particles in the range of 0.1-1.0%wt/v. The obtained films were tested in 100 mg/L nitrite solutions pH 4.0. The results are shown in Figure 4.10. The signals tended to increase

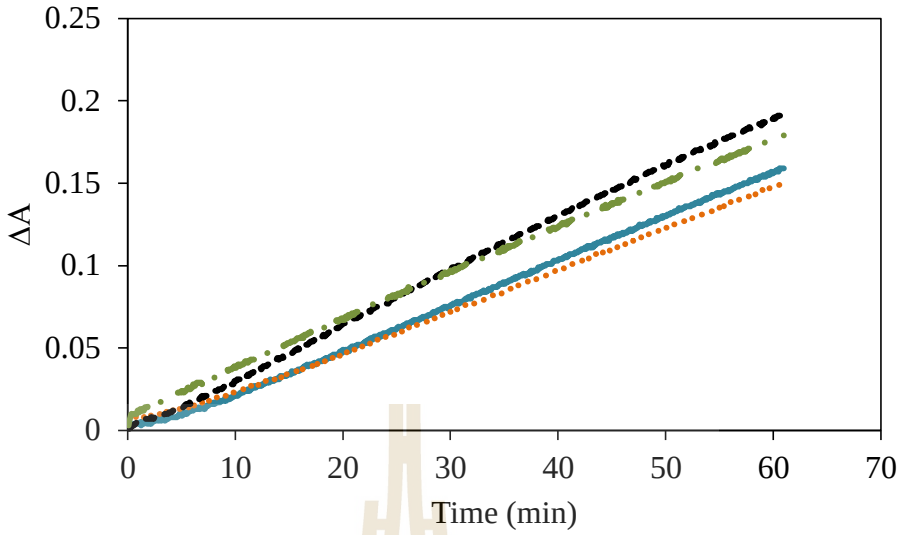


Figure 4.10 Time profiles response to nitrite of Nafion sensing films mixed with amino silica paricles amount 0.1% (□), 0.25% (••••), 0.5% (----) and 1.0% (-•-). The concentration of nitrite was 100 mg/L in acetate buffer pH 4.0.

with the amount of particles added, although the signals did not seem to be much different from each other. The films fabricated with 0.25% particles provided the highest reproducibility. Therefore, further study was conducted with films with 0.25% amino silica particles.

4.2.4 Calibration study

Nitrite solutions in the concentration range of 20-800 mg/L were used to construct calibration graphs. Response time profiles of sensing films in nitrite solutions are shown in Figure 4.11. Signals at various response times were used to plot calibration graphs as shown in Appendix C. Response time of 35 min provided the best fit and the linear equation was $\Delta A = 0.0009C - 0.0088$ with the R^2 of 0.9933. The limit of detection was 1.85 mg/L calculated based on three times the standard deviation of the blank signals.

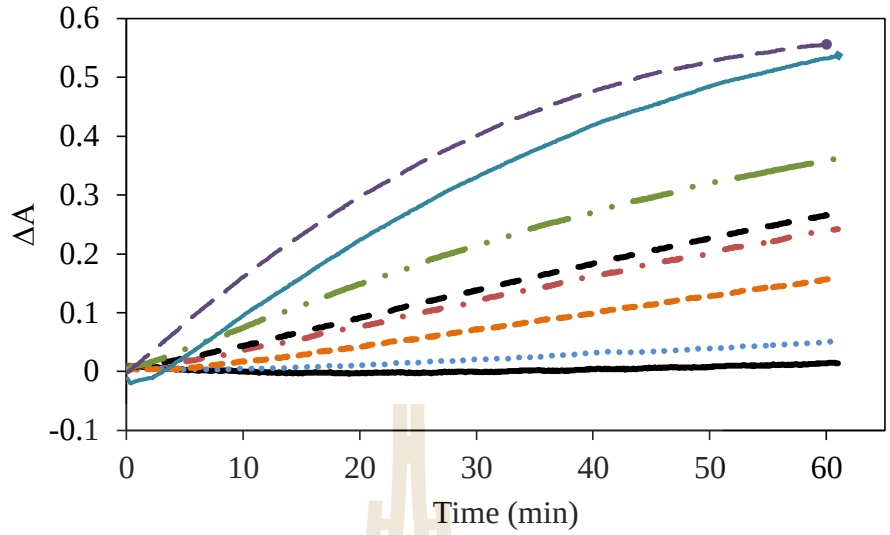


Figure 4.11 Time profiles response of 0.25% amino silica particles on Nafion films to nitrite at 20 (□), 50 (••••), 100 (----), 150 (-•-), 200 (---), 300 (□••□), 500 (□•) and 800 mg/L (□♦) for 60 min in acetate buffer pH 4.0.

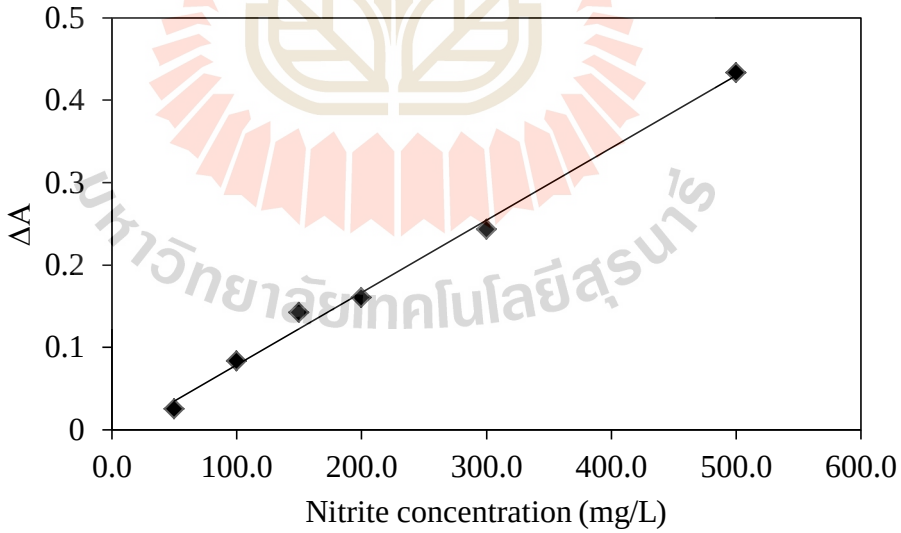


Figure 4.12 Calibration curve for nitrite with amino silica particles/Nafion films.

4.2.5 Interference study

The effect of interference ions on the response of sensing films were studied with solutions of an investigated ion mixed with nitrite at a molar ratio of 1:1. The obtained results were compared with those obtained from the measurements in nitrite solutions with no addition of the investigated ions. The signals were used to calculated %error as defined in equation (4.1) and %errors are shown in Table 4.3. All ions were considered not interfered with the determination of nitrite since as the %error was lower than 5%.

Table 4.3 Interference study with sensing film.

Ion	%Error
Cu^{2+}	-3.81
Ni^{2+}	-4.45
NO_3^-	-2.69
Cl^-	-3.93
Br^-	-4.82
F^-	-2.83

4.2.6 Reproducibility and regeneration of sensing films

The reproducibility of the sensing film was investigated by measuring the absorbance at 518 nm of nine sensing films. Sensing films were reacted with 100, 200 and 300 mg/L nitrite in acetate buffer pH 4.0 for 35 min. For each nitrite concentration, three sensing films were used in the study. From Table 4.7, the initial absorbance of nine films in acetate buffer solution are about the same and the calculated % relative standard deviation (%RSD) was 0.60%. In addition, %RSDs calculated from the signal responses of the sensing films in nitrite solutions were

<5%. This indicated that the method used for the preparation of sensing films was highly reproducible.

Table 4.4 Absorbance of films in acetate buffer pH 4.0 and 100, 200 and 300 mg/L of nitrite.

Nitrite concentration (mg/L)	Sensing film no.	Absorbance		ΔA	%RSD
		Film in acetate buffer pH 4	Film in nitrite		
100	1	0.553	0.467	0.086	3.45
	2	0.555	0.471	0.084	
	3	0.549	0.469	0.080	
200	4	0.555	0.395	0.160	0.48
	5	0.553	0.392	0.161	
	6	0.551	0.390	0.161	
300	7	0.560	0.319	0.241	0.86
	8	0.554	0.309	0.245	
	9	0.558	0.315	0.243	

Regeneration of sensing films after the reaction with nitrite was studied in a solution of 0.1 M NaOH. After the reaction in nitrite solution, sensing films were soaked in 0.1 M NaOH for 40 min to deprotonate the dye on the support. Then the films were washed by deionized water several times and reactivated in acetate buffer pH 4.0. The absorbance of the films was recorded at 518 nm and the results are shown in Figure 4.13. After the treatment process, the absorbance of the film could not be recovered to the initial value; therefore, the film could not be regenerated.

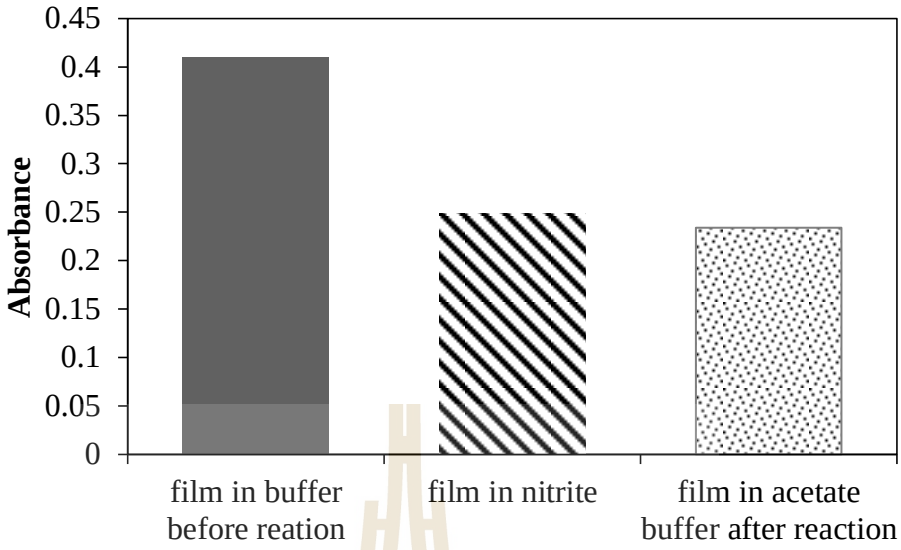


Figure 4.13 Absorbance signals of a sensing film before and after regenerated by 0.1 M NaOH for 40 min.

4.3 References

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CHAPTER V

CONCLUSIONS

In this research, 1,1'-diethyl-2,2'-cyanine iodide was used as a reagent for the determination of nitrite. The reaction was assumed to occur via the protonation of the reagent by nitrous acid which was generated from nitrite in acid solutions. The reaction species were confirmed by HPLC and LC-MS. The protonated reagent product resulted in the change of the absorbance at 522 nm which was used for quantitative measurements of nitrite concentration.

Method development for spectrometric determination of nitrite was conducted based on the reagent dissolved in solutions and the reagent immobilized in Nafion films. For the measurements based on dissolved reagent in solutions, the optimized reaction condition was 2.0×10^{-5} M dye concentration, solution pH 4.0 controlled by acetate buffer solution, and 5 min reaction time. The linear calibration equation obtained with the nitrite concentration range of 2.5-60 mg/L was $\Delta A = 0.0143C + 0.0075$, $R^2 = 0.9989$. The limit of detection was 1.0 mg/L.

For the method based on immobilized reagent, various supporting matrices were investigated and the best one was amino silica particles dispersed in Nafion films. The optimized reagent concentration used for the immobilization in this support was 1.1×10^{-3} M in 1.0 mL Nafion solution. The optimized condition for the determination of nitrite was in acetate solutions pH 4.0 and 35 min reaction time. The linear calibration equation was $\Delta A = 0.0009C - 0.0088$, $R^2 = 0.9933$ obtained with

nitrite concentration in the range of 50-500 mg/L. The limit of detection was 1.85 mg/L. The developed method exhibited good reproducibility in the determination of nitrite and could be applied for the analysis of real samples contaminated with high concentration of nitrite. Although the developed sensing films could not be reused, the cost of the films was relatively low.

Improvement of the detection limit should be of concern for the detection of nitrite with Nafion sensing films so that the analysis at part per billion concentration level could be performed. One possibility is to increase the amount of the reagent in the films so that the signal could be enhanced.

APPENDICES

APPENDIX A

TOTAL ION CURRENT CHROMATOGRAMS AND MASS SPECTRA

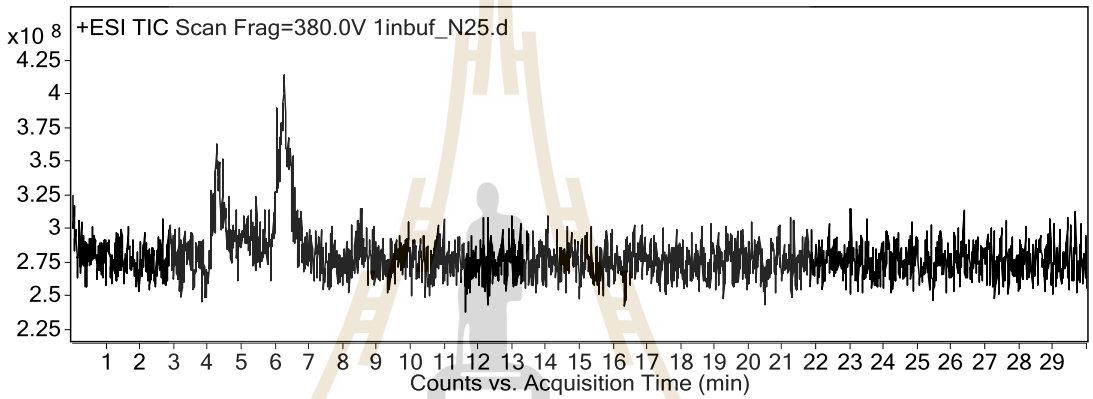

Figure A.1 TIC chromatogram of 1.0 ×10^{-5} M dye solution mixed with 25 mg/L nitrite solution (first injection).

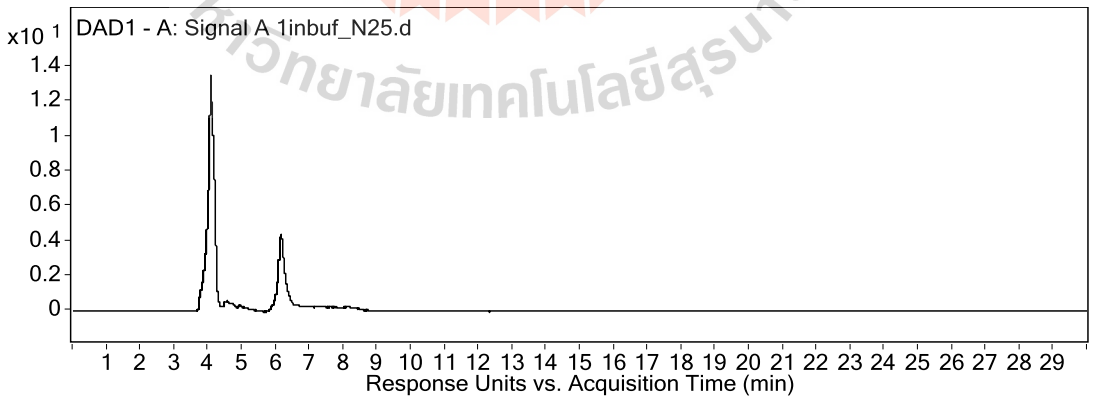

Figure A.2 DAD signal at 254 nm of 1.0 ×10^{-5} M dye solution mixed with 25 mg/L nitrite solution (first injection).

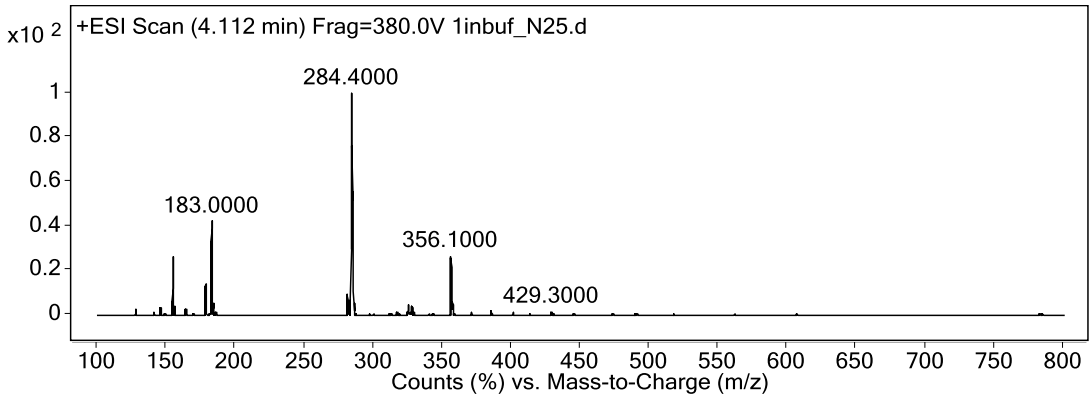

Figure A.3 Mass spectrum of 1.0 $\times 10^{-5}$ M dye solution mixed with 25 mg/L nitrite solution at retention time 4.112 min (first injection).

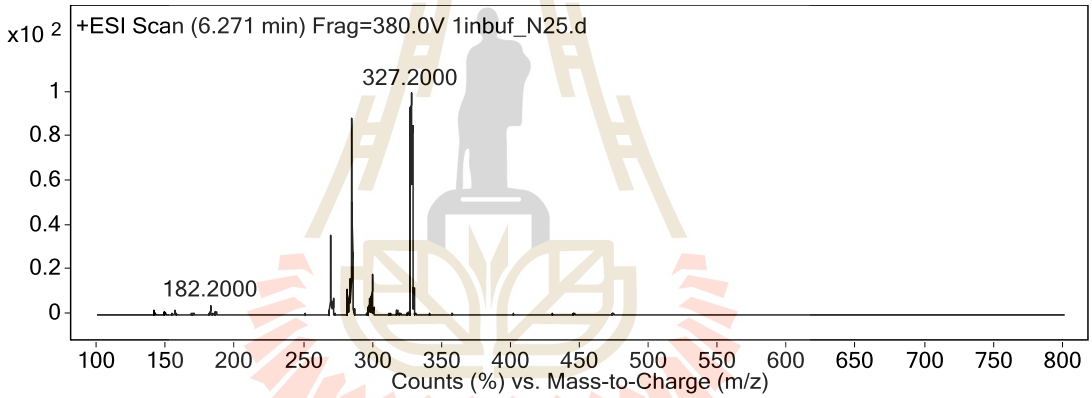

Figure A.4 Mass spectrum of 1.0 $\times 10^{-5}$ M dye solution mixed with 25 mg/L nitrite solution at retention time 6.271 min (first injection).

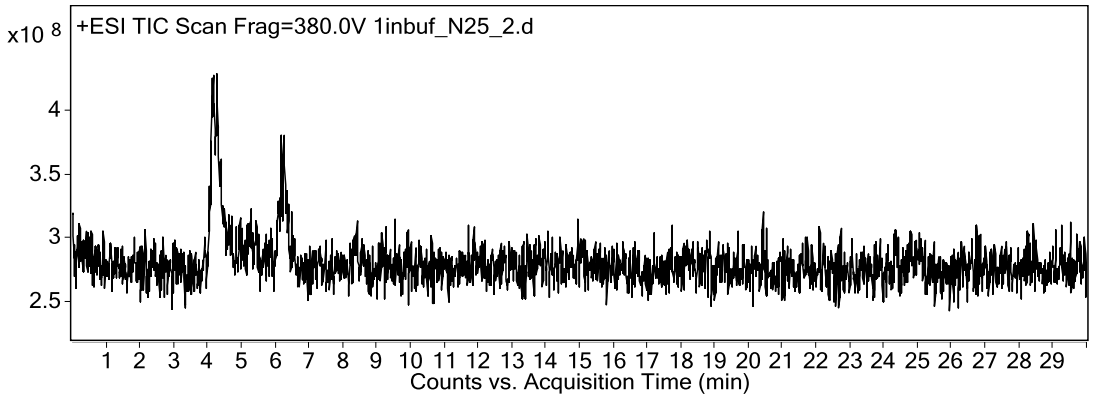

Figure A.5 TIC chromatogram of 1.0 $\times 10^{-5}$ M dye solution mixed with 25 mg/L nitrite solution (second injection).

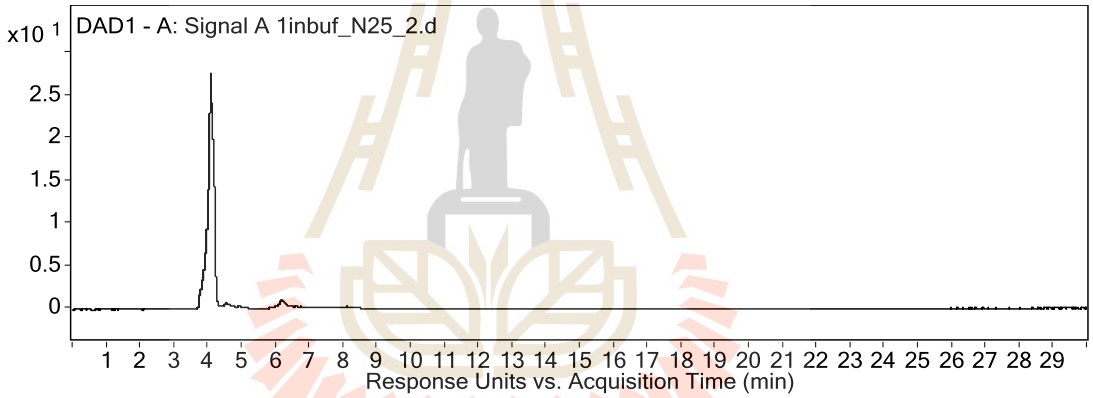

Figure A.6 DAD signal at 254 nm of 1.0 $\times 10^{-5}$ M dye solution mixed with 25 mg/L nitrite solution (second injection).

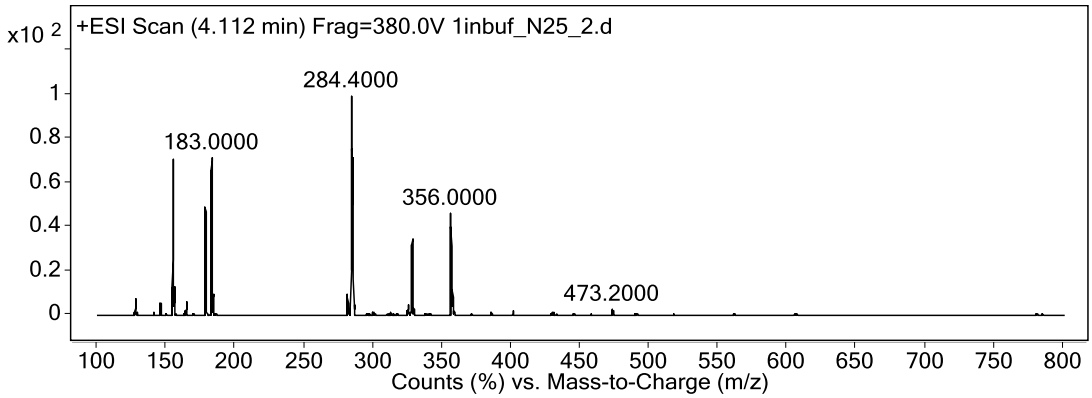

Figure A.7 Mass spectrum of 1.0 $\times 10^{-5}$ M dye solution mixed with 25 mg/L nitrite solution at retention time 4.112 min (second injection).

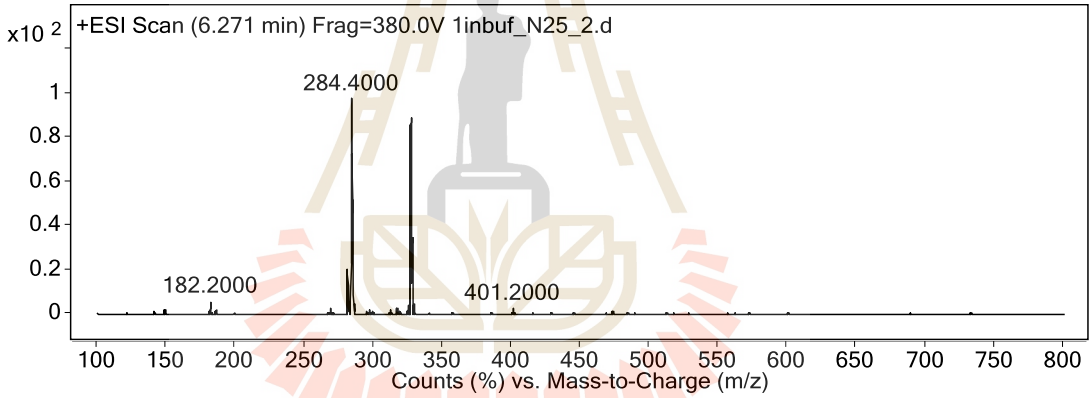

Figure A.8 Mass spectrum of 1.0 $\times 10^{-5}$ M dye solution mixed with 25 mg/L nitrite solution at retention time 6.271 min (second injection).

APPENDIX B

DYE ON VARIOUS SUPPORTS

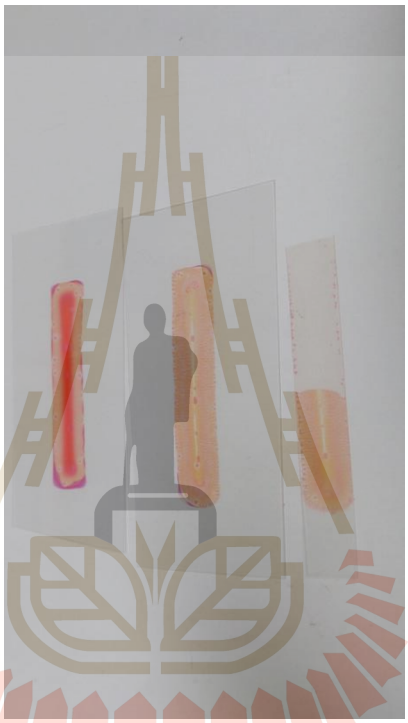

Figure B.1 Chitosan films prepared from homogeneous crosslinking method.

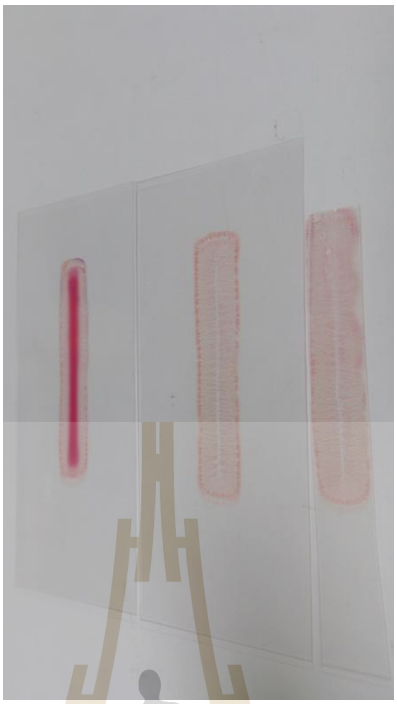

Figure B.2 Chitosan films prepared from heterogeneous crosslinking method.

Figure B.3 Sol-gel based films.

Figure B.4 Nafion film prepared from manual casting method.

APPENDIX C

CALIBRATION CURVES OBTAINED FROM VARIOUS RESPONSE TIMES IN SOLUTIONS

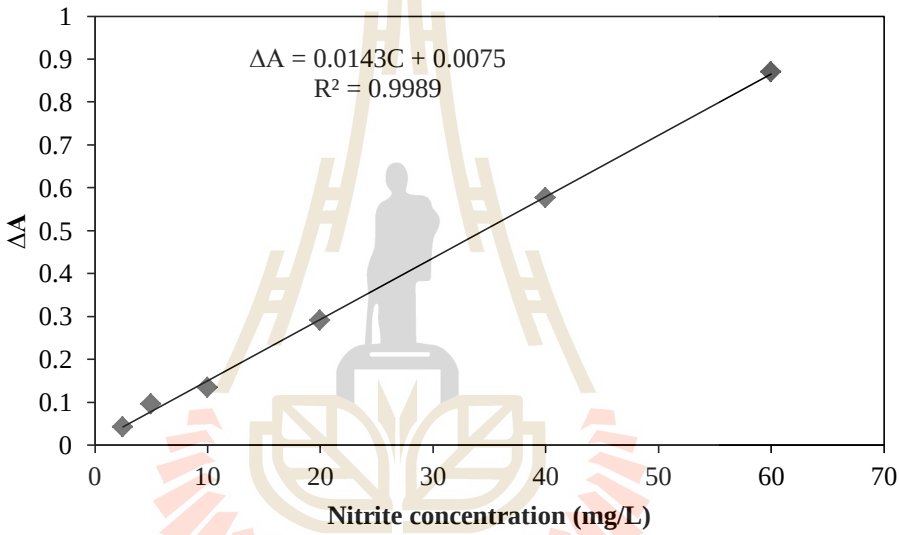

Figure C.1 Calibration curve of 1,1'-diethyl-2,2'-cyanine iodide solution and nitrite at 5 min of response time.

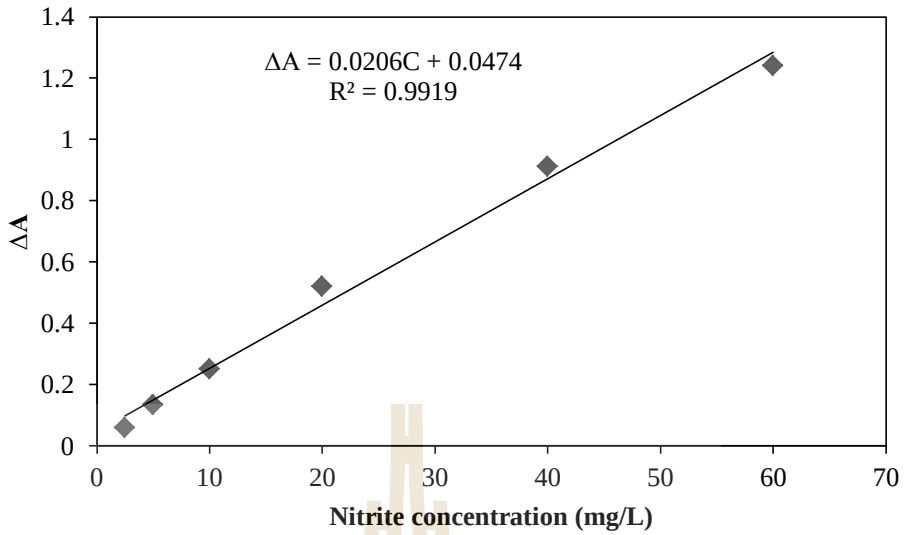

Figure C.2 Calibration curve of 1,1'-diethyl-2,2'-cyanine iodide solution and nitrite at 10 min of response time.

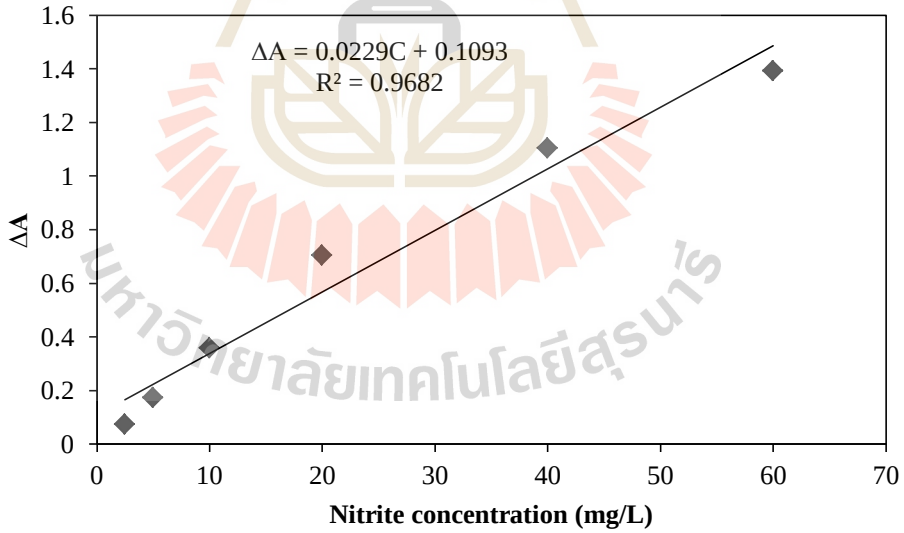

Figure C.3 Calibration curve of 1,1'-diethyl-2,2'-cyanine iodide solution and nitrite at 15 min of response time.

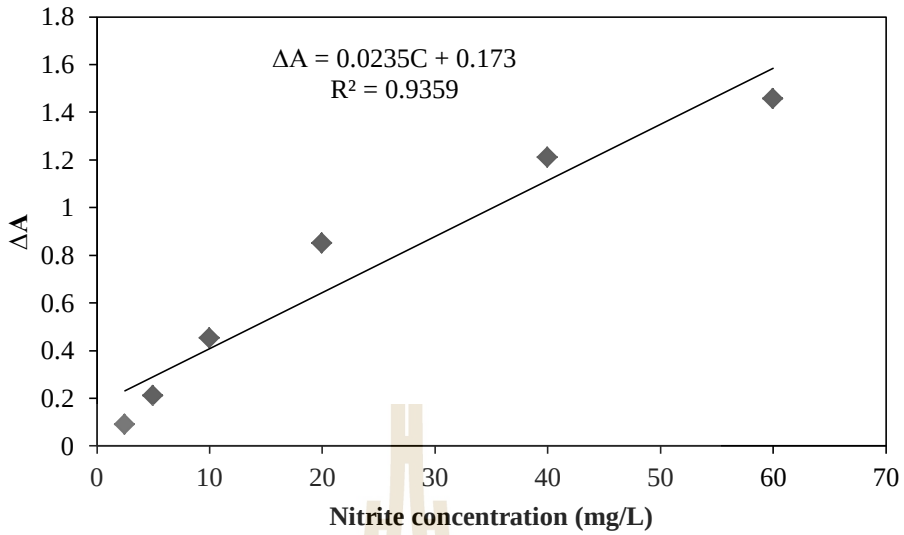

Figure C.4 Calibration curve of 1,1'-diethyl-2,2'-cyanine iodide solution and nitrite at 20 min of response time.

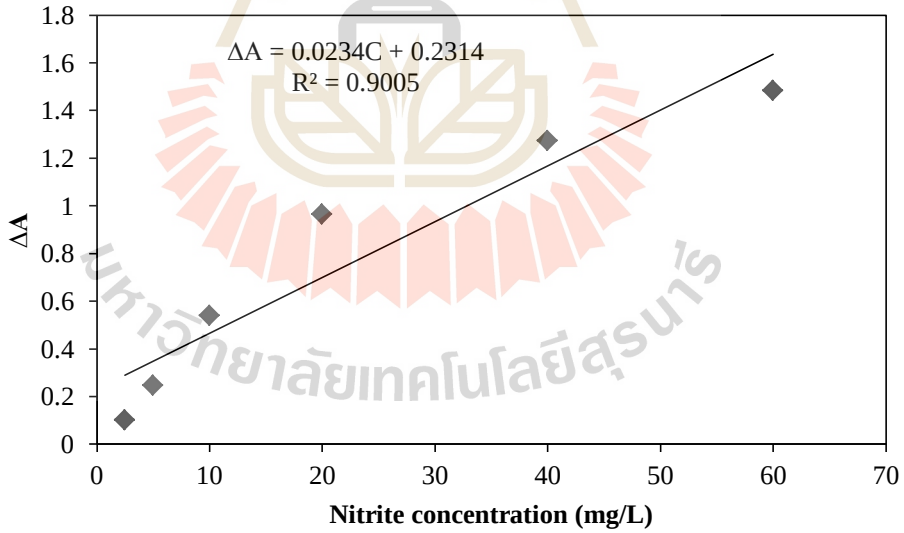

Figure C.5 Calibration curve of 1,1'-diethyl-2,2'-cyanine iodide solution and nitrite at 25 min of response time.

APPENDIX D

CALIBRATION CURVES OBTAINED FROM VARIOUS RESPONSE TIMES IN AMINO SILICA PARTICLES/NAFION FILMS

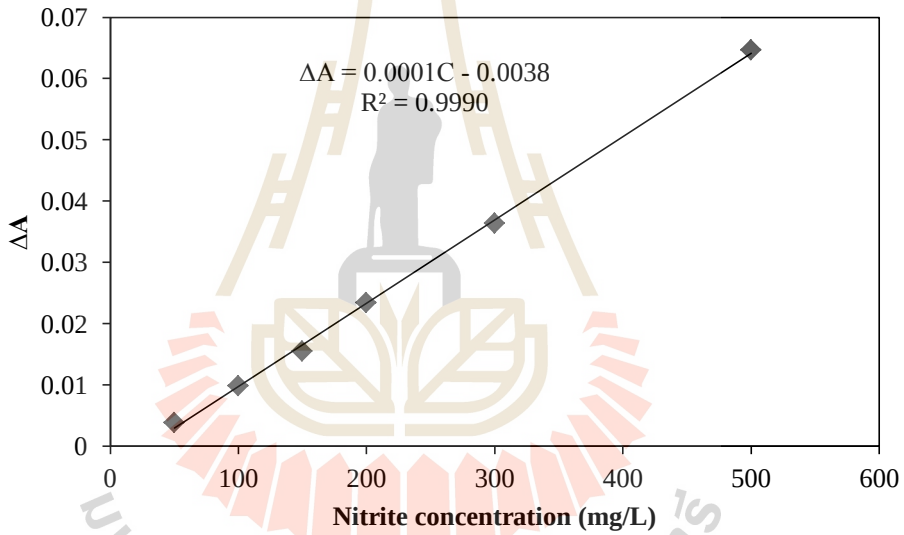

Figure D.1 Calibration curve of sensing films and nitrite at 5 min of response time.

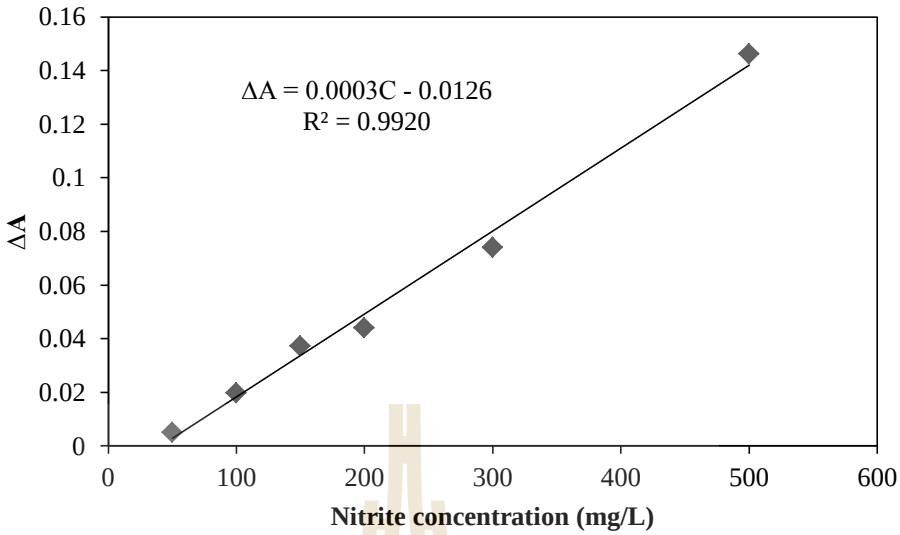

Figure D.2 Calibration curve of sensing films and nitrite at 10 min of response time.

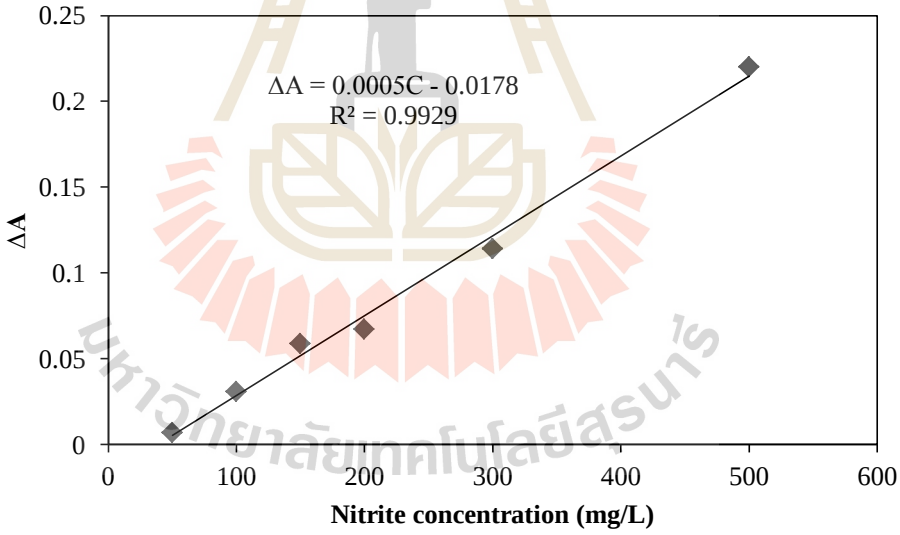

Figure D.3 Calibration curve of sensing films and nitrite at 15 min of response time.

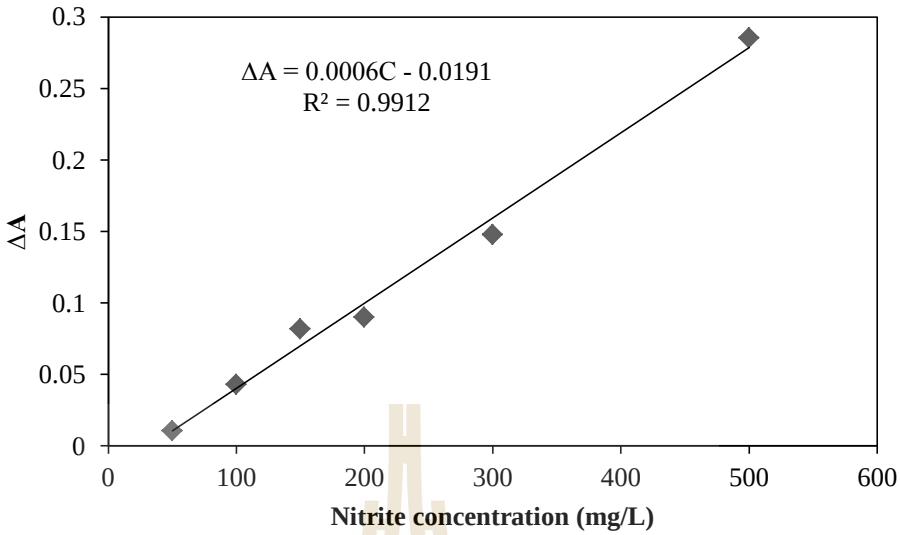

Figure D.4 Calibration curve of sensing films and nitrite at 20 min of response time.

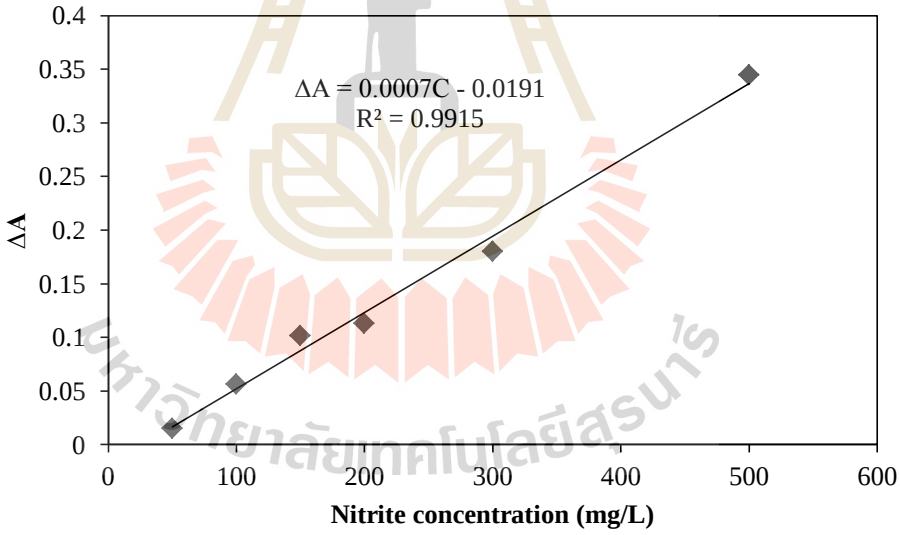

Figure D.5 Calibration curve of sensing films and nitrite at 25 min of response time.

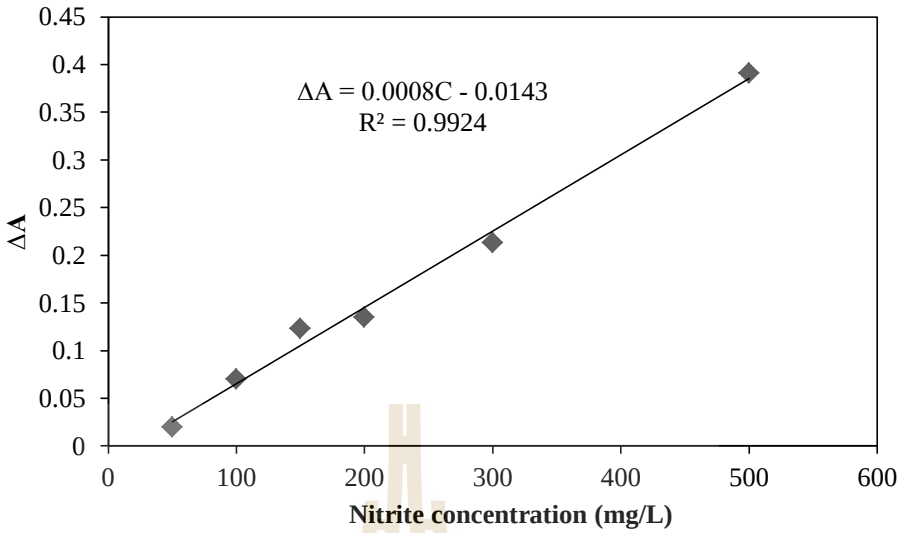

Figure D.6 Calibration curve of sensing films and nitrite at 30 min of response time.

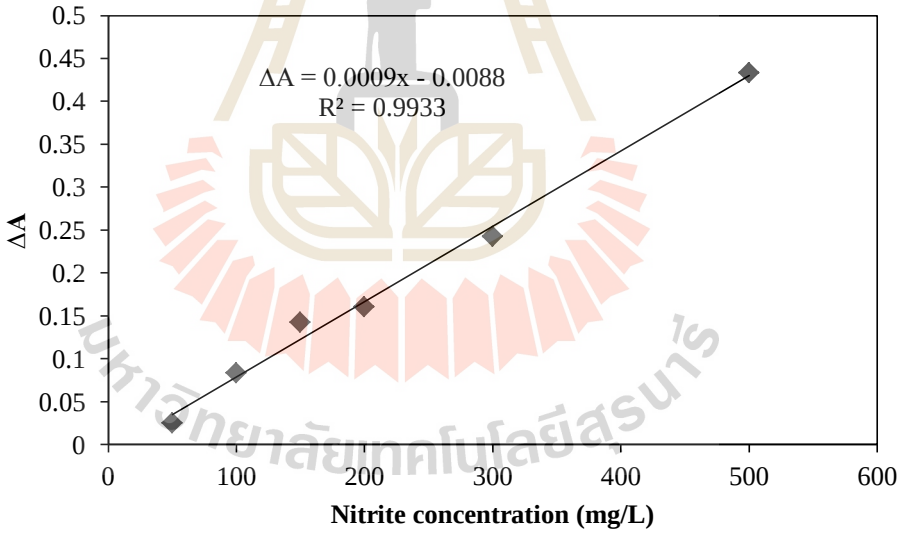

Figure D.7 Calibration curve of sensing films and nitrite at 35 min of response time.

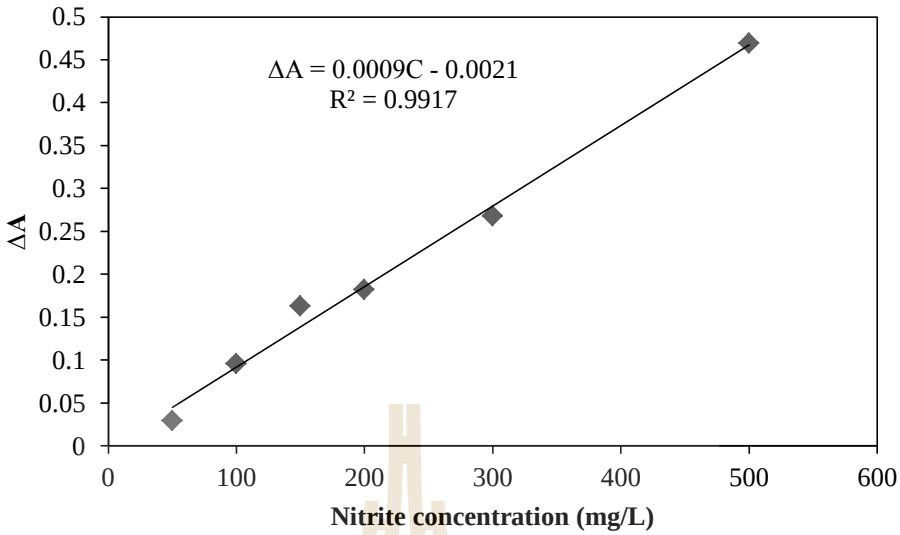

Figure D.8 Calibration curve of sensing films and nitrite at 40 min of response time.

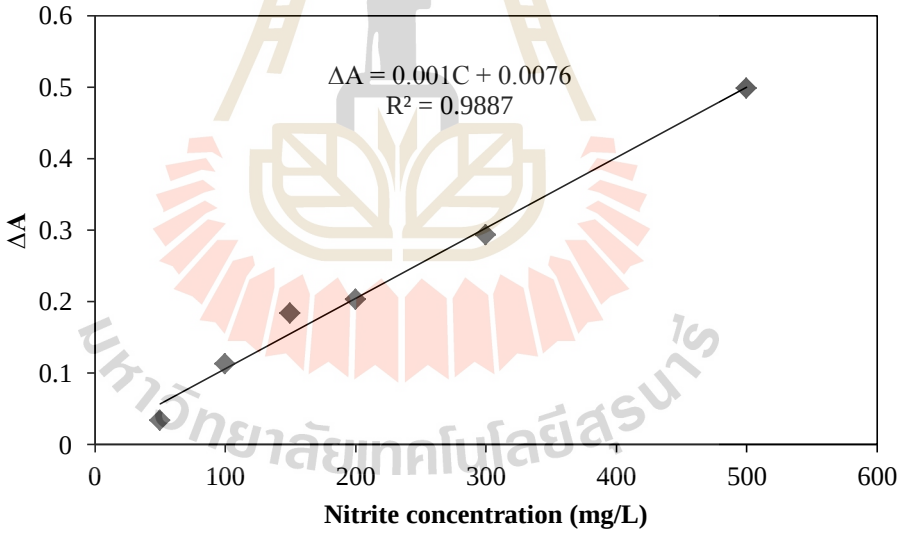

Figure D.9 Calibration curve of sensing films and nitrite at 45 min of response time.

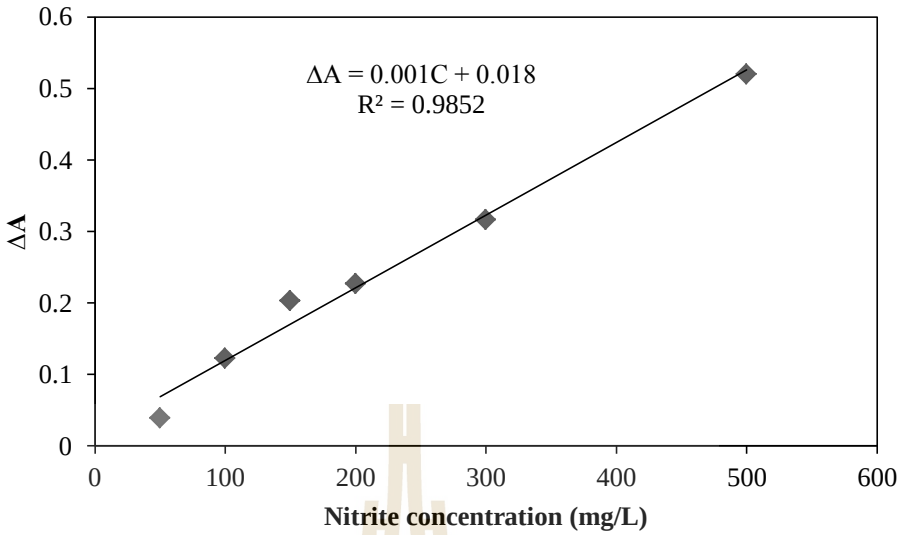

Figure D.10 Calibration curve of sensing films and nitrite at 50 min of response time.

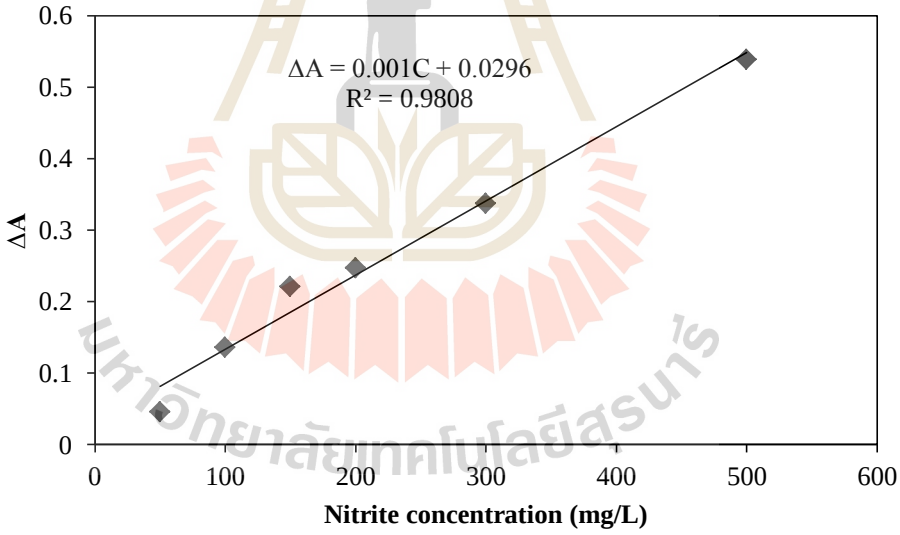

Figure D.11 Calibration curve of sensing films and nitrite at 55 min of response time.

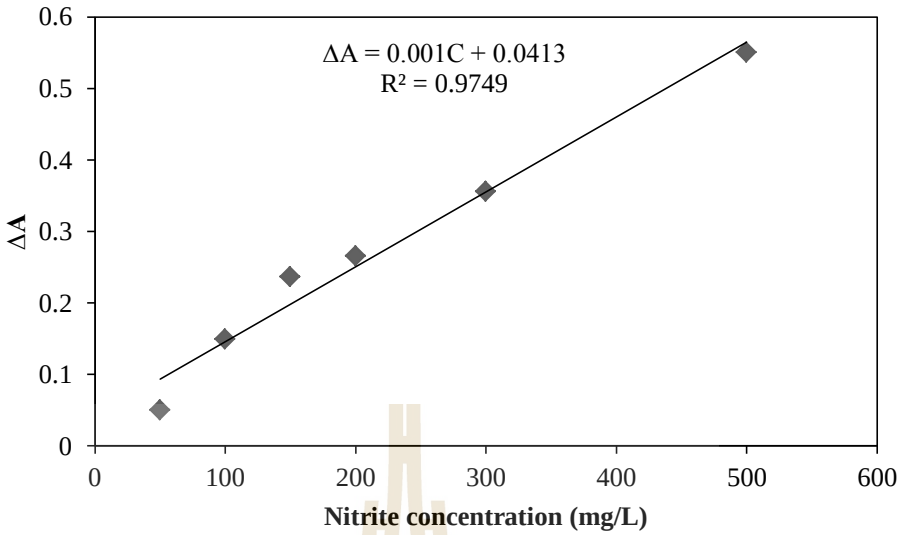

Figure D.12 Calibration curve of sensing films and nitrite at 60 min of response time.

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Publications

"Spectrometric determination of nitrite using 1,1'-diethyl-2,2'-cyanine iodide as a reagent". PACCON 2016: Pure and Applied Chemistry International Conference, 9-11 February 2016, BITEC, Bangkok, Thailand.