รายงานการวิจัยฉบับสมบูรณ์ ประจำปีงบประมาณ 2557

การพัฒนาอุปกรณ์ตรวจวัดแบบกระดาษเพื่อวิเคราะห์ปริมาณยาฆ่าแมลงในตัวอย่างอาหารทะเลแห้งโดยใช้อนุภาคนาโนซีเรียเป็นโพรบวัดสี

Development of Paper-Based Microfluidic Device for Pesticide Analysis in Dried Seafood using Nanoceria as Colorimetric Probes

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กันยายน 2558

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สนับสนุนโดย สำนักบริหารโครงการส่งเสริมการวิจัย
ในอุดมศึกษาและพัฒนามหาวิทยาลัยวิจัยแห่งชาติ

สำนักงานคณะกรรมการการอุดมศึกษา

กิตติกรรมประกาศ

งานวิจัยเรื่องการพัฒนาอุปกรณ์ตรวจวัดแบบกระดาษเพื่อวิเคราะห์ปริมาณยาฆ่าแมลงในตัวอย่างอาหารทะเลแห้งโดยใช้อนุภาคนาโนซีเรียเป็นโพรบวัดสี ได้รับทุนสนับสนุนการวิจัยจากสำนักบริหารโครงการส่งเสริมการวิจัยในอุดมศึกษาและพัฒนามหาวิทยาลัยวิจัยแห่งชาติ สำนักงานคณะกรรมการการอุดมศึกษา นอกจากนี้ผู้วิจัยขอขอบพระคุณ Prof. Charles S. Henry ที่ปรึกษาโครงการวิจัยที่ให้คำปรึกษาที่ดีตลอดมา ขอขอบคุณนิสิตทุกคนที่ช่วยทำการทดลองให้สำเร็จลุล่วง ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยบูรพา ที่ให้ความสนับสนุนด้านสถานที่และเครื่องมือต่างๆ ในการทำการทดลอง

ผู้วิจัย

บทคัดย่อ

งานวิจัยนี้ได้รายงานเป็นครั้งแรกในการพัฒนาอุปกรณ์แบบกระดาษที่เคลือบด้วยอนุภาคนาโนซีเรียที่ใช้เป็นบริเวณตรวจวัดในการวิเคราะห์ยาฆ่าแมลงออแกโนฟอสฟอรัสโดยอาศัยปฏิกิริยาการยับยั้งเอนไซม์ เพื่อให้ได้การวิเคราะห์ที่ง่าย ราคาถูก และรวดเร็วในการวิเคราะห์ออแกโนฟอสฟอรัสในตัวอย่าง การตรวจวัดประกอบด้วยอนุภาคนาโนซีเรีย อะเซติลโคลีนเอสเตอเรส และโคลีนออกซิเดส ในสภาวะที่มีอะซิติลโคลีน อะเซติลโคลีนเอสเตอเรสและโคลีนออกซิเดสเร่งปฏิกิริยาการเกิดไฮโดรเจนเปอร์ออกไซด์ ซึ่งถูกตรวจวัดด้วยอนุภาคนาโนซีเรียที่เคลือบอยู่บนอุปกรณ์ตรวจวัดแบบกระดาษและปรากฏสีเหลืองเกิดขึ้น เมื่อเติมยาฆ่าแมลงออแกโนฟอสฟอรัสการเร่งปฏิกิริยาของอะเซติลโคลีนเอสเตอเรสจะถูกยับยั้งและผลิตไฮโดรเจนเปอร์ออกไซด์ได้น้อยลงทำให้สีเหลืองที่เกิดขึ้นมีความเข้มน้อยลงด้วย ความเข้มสีสามารถวิเคราะห์ได้ด้วยโปรแกรมอิมเมจเจ วิธีที่พัฒนาขึ้นสามารถวิเคราะห์ออแกโนฟอสฟอรัสได้โดยไม่จำเป็นต้องใช้อุปกรณ์ในการวิเคราะห์ที่ยุ่งยาก และให้ขีดจำกัดการตรวจวัดที่ดีโดยสามารถวิเคราะห์ออแกโนฟอสฟอรัสมาตรฐานได้ความเข้มข้นต่ำถึง 18.3 ng/mL และ 5.2 ng/mL สำหรับการวิเคราะห์เมธิลพาราออกซอน และคลอร์ไพริฟอสออกซอน ตามลำดับ นอกจากนี้วิธีที่พัฒนาขึ้นสามารถประยุกต์ใช้ในการวิเคราะห์เมธิลพาราออกซอนในตัวอย่างผักและอาหารทะเลแห้งที่ถูกสไปค์ พบว่าให้การวิเคราะห์มีร้อยละการได้กลับคืนประมาณ 95 ในการวิเคราะห์ตัวอย่างทั้งสองชนิด ตัวอย่างที่ถูกสไปค์ยังถูกตรวจวัดด้วยเทคนิค LC-MS/MS เพื่อเป็นการเปรียบเทียบการวิเคราะห์กับวิธีที่พัฒนาขึ้น พบว่าค่าที่ได้จากการวิเคราะห์ด้วยสองวิธีมีค่าใกล้เคียงกัน แสดงให้เห็นว่าวิธีที่พัฒนาขึ้นให้การวิเคราะห์ที่มีความแม่นยำที่ดีและเหมาะสมที่จะนำไปวิเคราะห์ออแกโนฟอสฟอรัสในตัวอย่างได้ ดังนั้นวิธีที่พัฒนาขึ้นมีแนวโน้มที่สามารถใช้เป็นวิธีทางเลือกใหม่ในการวิเคราะห์ออแกโนฟอสฟอรัสที่ให้การวิเคราะห์ที่ราคาถูก รวดเร็ว สามารถพกพาไปตรวจวัดภาคสนามได้

Abstract

We report the first use of paper-based device coated with nanoceria as a detection platform for the analysis of organophosphorus (OPs) pesticides using enzyme inhibition assay to allow for simple, low-cost and rapid analysis OPs in the samples. The detection assay consisted of nanoceria, acetylcholinesterase (AChE), and choline oxidase (ChOX). In the presence of acetylcholine, the enzyme AChE and ChOX catalyze the formation of H_2O_2, which is then detected colorimetically by nanoceria-coated paper-based device producing yellow color. After incubation with OP pesticides, the activity of AChE was inhibited and produced less H_2O_2, hence, causing a drop of yellow color intensity. Color intensity was analyzed on the scanned picture using ImageJ software. This developed assay is able to analyze OP pesticides without the use of any external sophisticated instruments with excellent detection limits of two model OP pesticides where as low as 18.3 ng/mL of methyl-paraoxon and 5.2 ng/mL of chlorpyrifos-oxon were observed. The developed method has been successfully applied to detect methyl-paraoxon in spiked vegetables (cabbage) and dried seafood product (dried green mussel), obtaining recovery values about 95% for both types of samples. The spiked samples were also analyzed using LC-MS/MS as a comparison to the developed method and similar values were obtained indicating that the developed method is validated and suitable for OP analysis in real samples. Therefore, the developed assay is promising method to use as a new alternative method for analysis of OP pesticides that provides low-cost, rapid, and onsite OPs analysis.

CHAPTER 1
INTRODUCTION

1.1 Statement and significance of the problems

Dried seafood products (DSPs) are popular among Asian countries as they are a source of preservative proteins and give specific component of food. DSPs are high nutritional value food because they have proteins, polyunsaturated fatty acids, a wide range of vitamins (B, A and D) and minerals for instance; calcium, phosphorus and iron (Huss, 1995). Export of DSPs worldwide could provide massive incomes for people in Southeast Asia (Agusa et al., 2007). Mass production of DSPs in the region is carried out by several manufacturing processes such as braise, baking and sundry (Cao et al., 2009). Of these methods, sundry is the most commonly used for DSP production in the coastal area. After that, the products are generally stored in a warehouse at the production site prior to export. During sundry process and the storage period, the DSPs were exposed to pesticides used to prevent the DSPs from pets/insects. One of the most commonly used pesticides in DSPs manufacturing process is organophosphates (OP) and carbamates (Sun, Wong, Li, & Chen, 2006).

OP and carbamate pesticide poisoning is a global health problem with over 250,000 deaths per year (Carey, Dunn, & Gaspari, 2013). The acute toxicity of OPs and carbamate is associated with their capacity to irreversibly inhibit the activity of acetylcholinesterase (AChE) in the central and peripheral nervous system, resulting in the accumulation of the neurotransmitter acetylcholine in the body, which can lead to organ failure and eventual death(Chowdhary, Bhattacharyya, & Banerjee, 2014; Sultatos, 1994). Their toxicity is based on specificity differences in the AChE targets(Chowdhary et al., 2014). In human, acute poisoning displays symptoms within minutes that include headache, dizziness, nausea, vomiting, pupillary constriction, and excessive sweating, tearing, and salivation. Moreover, poisoning also cause muscle weakness and twitches, change in heart rate and can progress to convulsions and coma (Kamel & Hoppin, 2004). The dose of pesticides in human are depended on how much they have with each pathway of exposure, which in turn is affected by the individual's lifestyle, such as living places, and socioeconomic status or job-exposure such as agricultural spray drift, dietary exposure, municipal, medicinal and home pesticide

use as shown Figure 1-1 (Ambroise et al., 2005; Bouvier, Blanchard, Momas, & Seta, 2006; McKinlay, Plant, Bell, & Voulvoulis, 2008; Tongeren et al., 2002).

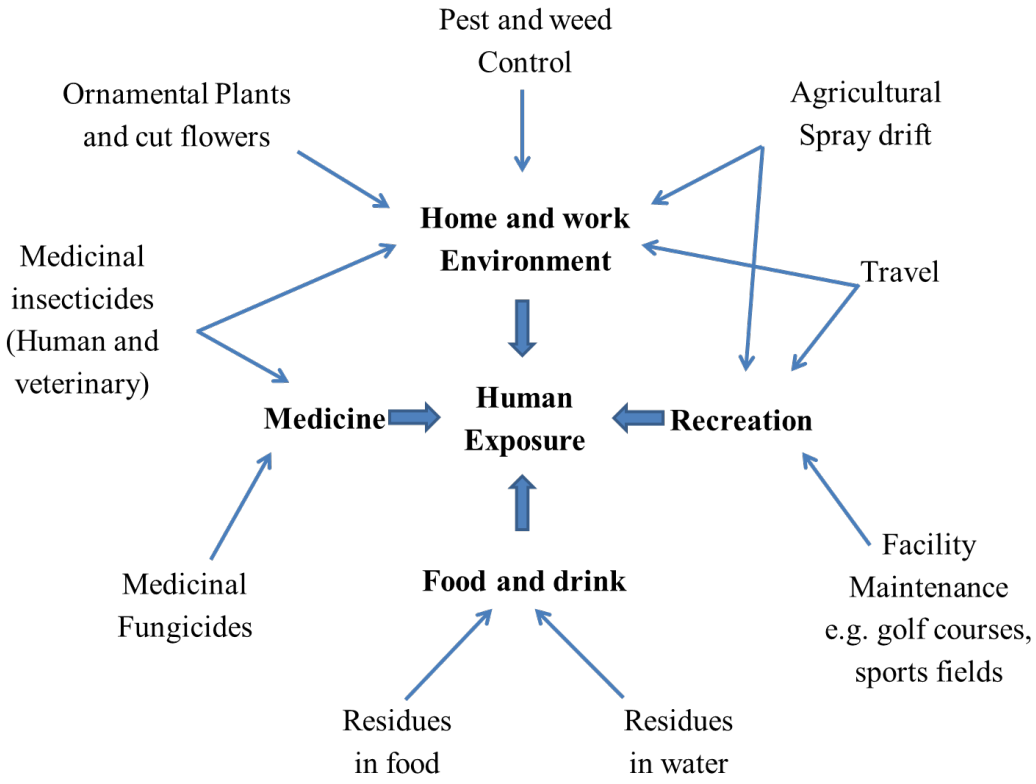


Figure 1-1 Routes of pathway of human exposure to pesticides

Many analytical techniques are used for OP and carbamate pesticides determination, namely liquid/gas chromatography-mass spectrometry (LC-MS/GC-MS)(M. Liu, Hashi, Song, & Lin, 2005; Wong et al., 2007), high-performance liquid chromatography (HPLC)(Dzuman, Zachariasova, Veprikova, Godula, & Hajslova, 2015; Seebunrueng, Santaladchaiyakit, & Srijaranai, 2014), enzyme activity inhibition methods (Amine, Mohammadi, Bourais, & Palleschi, 2006; Tekaya et al., 2013) and enzyme-linked immunosorbent assays (ELISAs)(Lee, Kim, Park, Chung, & Lee, 2005; Nunes, Toscano, & Barceló, 1998). These methods provide low-level detection limits in the nanomolar concentration range for OP analysis. However, they are time-consuming, expensive, have to be performed by a highly trained technician, require expensive laboratory-based instruments, and not suitable for on-site analysis.

An ideal method for detection of pesticides in DSPs should provide on-site and fast, ease-of-use analysis to allow people capable of testing the product prior to consume for their safety. So far, some test kits have been developed for field-testing of pesticides(Hossain, Luckham, McFadden, & Brennan, 2009; Q. Liu et al., 2015). However, these test kits have some drawbacks as they could provide only yes/no answer of pesticide safety level in the samples (semi-quantitative method), cannot detect low level concentration, and require large volumes of reagents and sample.

In an effort to solve the problem of the existing test kits that already had and to reach the goal of an ideal method for pesticide analysis in DSPs, this work proposes to develop a microfluidic paper-based analytical device (μPAD) and CeO_2 nanoparticles (CeO_2 NPs) as colorimetric probes for quantitative analysis of OP and carbamate pesticides in dried seafood. First introduced in 2007 (A. W. Martinez, Phillips, Butte, & Whitesides, 2007), μPADs consist of a patterned, hydrophilic paper substrate that serves as a platform for both single and multiplexed analyte detection. Paper functions as a three-dimensional microfluidic substrate because its cellulosefiber network acts as capillaries, wicking aqueous solutions without the need for active pumping. Channels/detection zone/ analysis zones are created in paper by depositing a hydrophobic material, such as polymer or wax to confine and direct microliter volumes of liquid flow to perform the analysis. The developed μPADs with CeO_2 NPs have the following attractive features that could eventually used as the on-site device for pesticide analysis.

1. The device is made from paper which is inexpensive and widely available resulting in reduction of analysis cost.
2. The analysis on μPADs requires only microliter level of reagent and samples therefore only small amount of sample need to be prepared.
3. The device is easy-to-use and does not require high trained personal to run the assay.
4. CeO_2 NPs can be used as colorimetric probes on the paper and the color is easy to observe making them possible to detect pesticides.
5. The paper is thin and light weight so that the developed μPAD is possible to use on-site.

Pesticide analysis using the developed μPAD was based on enzyme inhibition assay (Figure 1-2). In aqueous solution, acetylcholine is catalyzed by acetylcholineesterase (AChE)

producing choline and acetate and choline is subsequently converted to an aldehyde and hydrogen peroxide (H_2O_2) by choline oxidase (ChOX). The H_2O_2 product is detected by CeO_2 NPs coated on paper changing from colorless to yellowish-orange (Figure 1-2B). In a presence of OP and carbamate pesticides, AChE activity is inhibited, less acetylcholine is converted to the products and hence less H_2O_2 produced resulting in lower color intensity observed on the CeO_2 NPs coated paper.

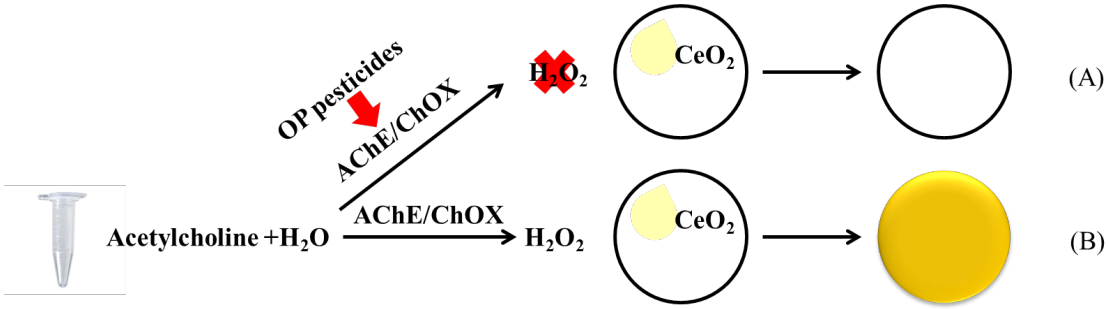


Figure 1-2 Typical procedures for the analysis of pesticide using OP pesticides on the developed μPAD. (A) In a presence of OP pesticides, AChE will be inhibited and hence less H_2O_2 produced resulting in low color intensity observed on the device. (B) In an absence of pesticide, AChE and ChOX can catalytically oxidize acetylcholine and produce H_2O_2 resulting in the color development on the CeO_2 paper.

1.2 Objective

To develop paper-based microfluidic device based on ceria nanoparticles as colorimetric probes for detection of pesticides in dried seafood.

1.3 Contribution to knowledge

1. The developed μPAD can be used for detection of pesticides in dried seafood effectively.
2. The local people can afford to buy and used it because the developed device is low cost and easy to use.

1.4 Scope of study

1. To develop the paper-based microfluidic device for OP pesticide analysis based on enzyme inhibition assay using nanoceria as colorimetric probes.
2. To study optimal conditions of enzyme inhibition assay for the analysis of pesticides using the developed μPAD.
3. To study the analytical features including linear range, reproducibility and detection limit of the developed method for pesticide analysis using methyl-paraoxon and chlorpyrifos-oxon as a standard OP pesticides.
4. To study the method validation by comparing pesticide amounts in spiked samples analyzed using the developed method and compared with those obtained from the conventional method (LC-MS/MS).

CHAPTER 2
LITERATURE REVIEW

2.1 Organophosphate and carbamate pesticide poisoning

Pesticides are substances or mixtures of substances used in agriculture to protect crops against destructive pests and thereby increase food supply both in the field and during storage (Blasco et al., 2003; Pareja et al., 2012; Sharma, Nagpal, Pakade, & Katnoria, 2010). There are many different groups of pesticides but the most widely used pesticides are OP and carbamates groups (Sharma et al., 2010). The structures of commonly used OPs (diazinon, parathion-methyl, Profenofos, Chlorpyrifos-oxon, malathion, and Methyl-paraoxon) are shown in Figure 2-1, and carbamates (Carbaryl, Carbofuran, Formetanate, Methiocarb, Methomyl, and Oxamyl) are shown in Figure 2-2 (Albero, Sánchez-Brunete, & Tadeo, 2003; Nogueira, Sandra, & Sandra, 2004; Padilla, Marshall, Hunter, & Lowit, 2007).

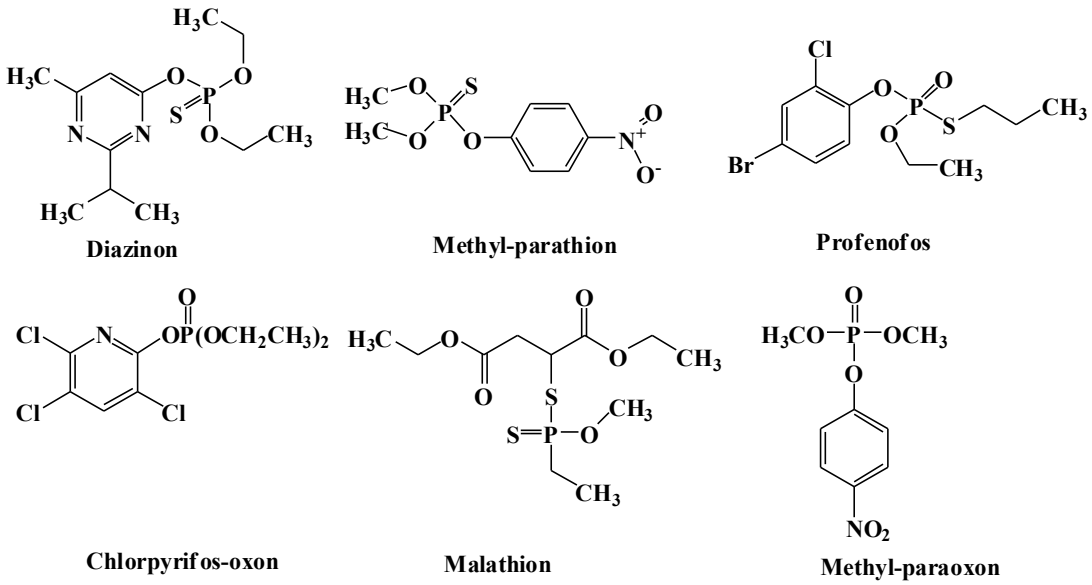


Figure 2-1 Examples of organophosphate pesticides

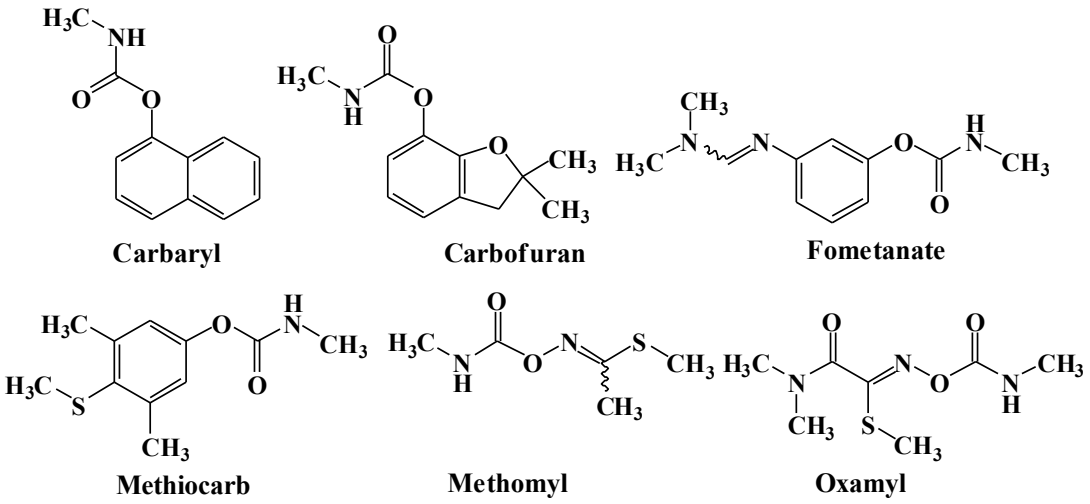


Figure 2-2 Examples of carbamate pesticides

Worldwide, over 5.2 billion pounds of pesticides is applied each year, and roughly 85% of the pesticides currently used are devoted to the agricultural sector (Arthur Grube, David Donaldson, Timothy Kiely, & Wu, 2011). Exposure to pesticides can occur through multiple pathways (e.g. food, drinking water, residential, occupational) and multiple routes (e.g., inhalation, ingestion, and dermal absorption). Exposure to even small amounts of an OP can be fatal and death usually caused by respiratory failure, and pulmonary dysfunction (Carey et al., 2013). The mechanism of OP poisoning involves inhibition of acetyl cholinesterase (AChE) leading to inactivation of the enzyme which has an important role in neurotransmission (Casida & Quistad, 2004; Jokanović, 2009; T. Liu et al., 2011). The inhibition of AChE may cause accumulation of acetylcholine in the synaptic cleft, leading to over-stimulation and the disruption of nerve impulses and ultimately causing symptoms such as ataxia, central respiratory paralysis, seizures, coma and death (Costa, 2006; Holth & Tollefsen, 2012).

2.2 Chlorpyrifos-oxon

Chorpyrifos-oxon (CPO) is an OP insecticide, which was transformed from CPS (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate). The metabolism of chlorpyrifos (Figure 2-3) shows that it undergoes CYP450-mediated oxidative desulfation or dearylation to form chlorpyrifos-oxon (the neurotoxic moiety) or 3,5,6-trichloro-2-pyridinol (TCP) and diethylthiophosphate, respectively (Amitai, Moorad, Adani, & Doctor, 1998; Barry, Lin, Wang, Liu, & Timchalk, 2008; Tang et al., 2001). Like other OP insecticides, CPO is toxic to the

nervous system by inhibiting an enzyme that is important in the transmission of nerve impulses. Symptoms of acute poisoning include headache, nausea, muscle twitching, and convulsions (Barry et al., 2008; Kousba, Sultatos, Poet, & Timchalk, 2004).

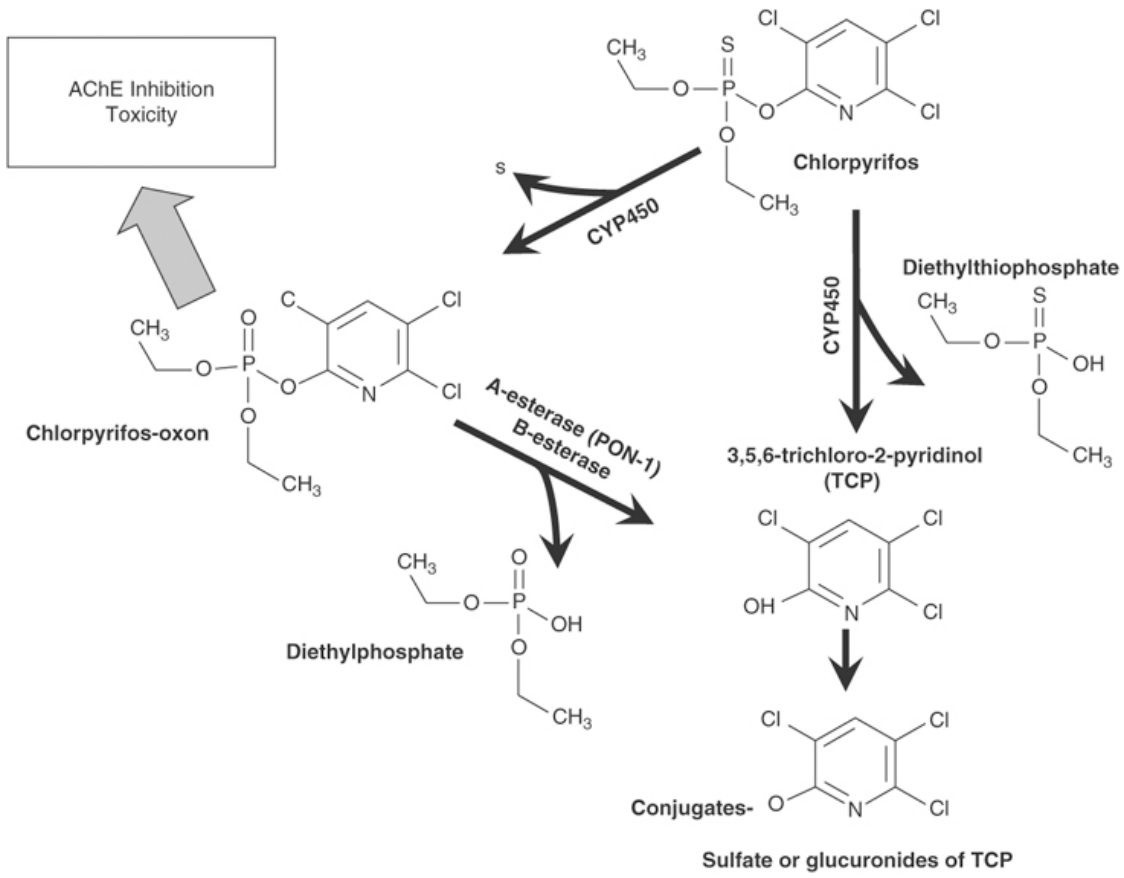


Figure 2-3 Metabolic scheme for the metabolism of chlorpyrifos and the major metabolites chlorpyrifos-oxon, trichloropyridinol (and conjugates), diethylphosphate and diethythiophosphate (Barry et al., 2008).

2.3 Methyl-paraoxon

Methyl-paraoxon (MPO) is also an OP pesticide, which was transformed from methyl-parathion associating with cytochrome P450 (CYP450) and flavin-containing monooxygenase (FMO) as shown in Figure 2.4. In pure condition, OPs having structure of phosphorothioates such as parathion and phosphorodithioates such as malathion are weak inhibitors of AChE due to the poor electron-withdrawing ability of the sulfur atom, which is directly bound to phosphorus (P=S)(Jokanović, 2001). However, corresponding oxidative

analogs of these compounds are very toxic to acetylcholinesterase similar to other OP compounds.

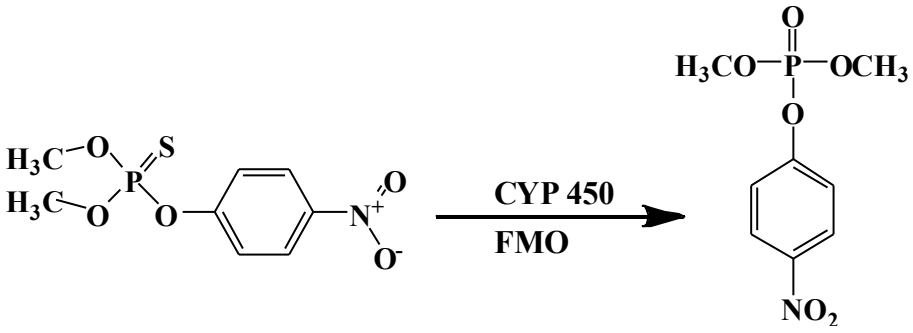


Figure 2-4 Transformation of methyl-parathion to methyl-paraoxon by oxidative desulfation reaction.

2.4 Pesticides in dried seafood products

Recently, the use of chemicals in agricultural and fishery productivity increases significantly. These chemicals include pesticides (Kan-atireklap, Subramanian, & Tanabe, 2007; Sun et al., 2006). The use of pesticides is mainly to protect the products from insects or destructive pests both in the manufacturing process and during storages (Jaikanlaya, Settachan, Denison, Ruchirawat, & van den Berg, 2009; Moon, Kim, Choi, Yu, & Choi, 2009). The contamination of food with agricultural pesticide residues is an obvious pathway of human exposure and it is strongly influenced by age and dietary preferences (McKinlay et al., 2008). The recent studies have found that a large number of seafood products from the coastal region in Asia were contaminated with pesticides. Studies in China reported that a 65.7 ng/g of dichlorodiphenyltrichloroethane (DDT) was found in mussels (Guo, Wu, Shen, & Zeng, 2010). In Korea, a dietary intakes of DDT in seafood for the general population was estimated as 69 ng/kg body weight/week (Moon et al., 2009). In Vietnam, 220–34,000 ng/g lipid wt concentration of DDTs were found in mussels (Monirith et al., 2003). And in Thailand, 0.19–4.2 ng g^{-1} wet wt of DDT residues in bivalve was found mollusks from coastal water (Tanabe, Prudente, Kan-atireklap, & Subramanian, 2000).

2.5 Current methods for pesticide determination

A variety of methods have been developed for determination of OPs and carbamate pesticides in biological and environmental samples including chromate-graphic methods such as gas chromatography (GC) (Jardim & Caldas, 2012; Lozowicka et al., 2014), high performance liquid chromatography (HPLC)(Dzuman et al., 2015; Seebunrueng et al., 2014), gas chromatography mass spectrometry (GC–MS) (Camino-Sánchez et al., 2011), liquid chromatography mass spectrometry (LC–MS) (Masia et al., 2013; Paradis, Berail, Bonmatin, & Belzunces, 2014), enzyme linked immunosorbent assays (ELISAs) (Lee et al., 2005; Nunes et al., 1998) and enzyme inhibition assays (Amine et al., 2006; Tekaya et al., 2013). Of these methods, chromatography-mass spectrometry (LC-MS, GC-MS) is the most effective method as it can detect nanomolar-level of the pesticides, provides reproducible analysis, and has high sensitivity. The chromatography-based methods, however, have some limitations including expensive analysis cost per sample, complicate and expensive instruments, long analysis time as well as laboratory-based analysis. These drawbacks make the chromatography inappropriate for screening test for food safety.

Traditional ELISA and enzyme inhibition assay are an alternative pesticide analysis to GC/LC-MS as they provide faster analysis time and cheaper analysis cost. The traditional enzyme methods, however, requires sophisticated techniques for detection including UV-visible, fluorescence and chemiluminescence spectrometry and electrochemistry. These detection methods require expensive instrument for operation includes spectrophotometer (for spectrophotometry) and potentiostat (for electrochemistry) making them unsuitable for field-testing of pesticides.

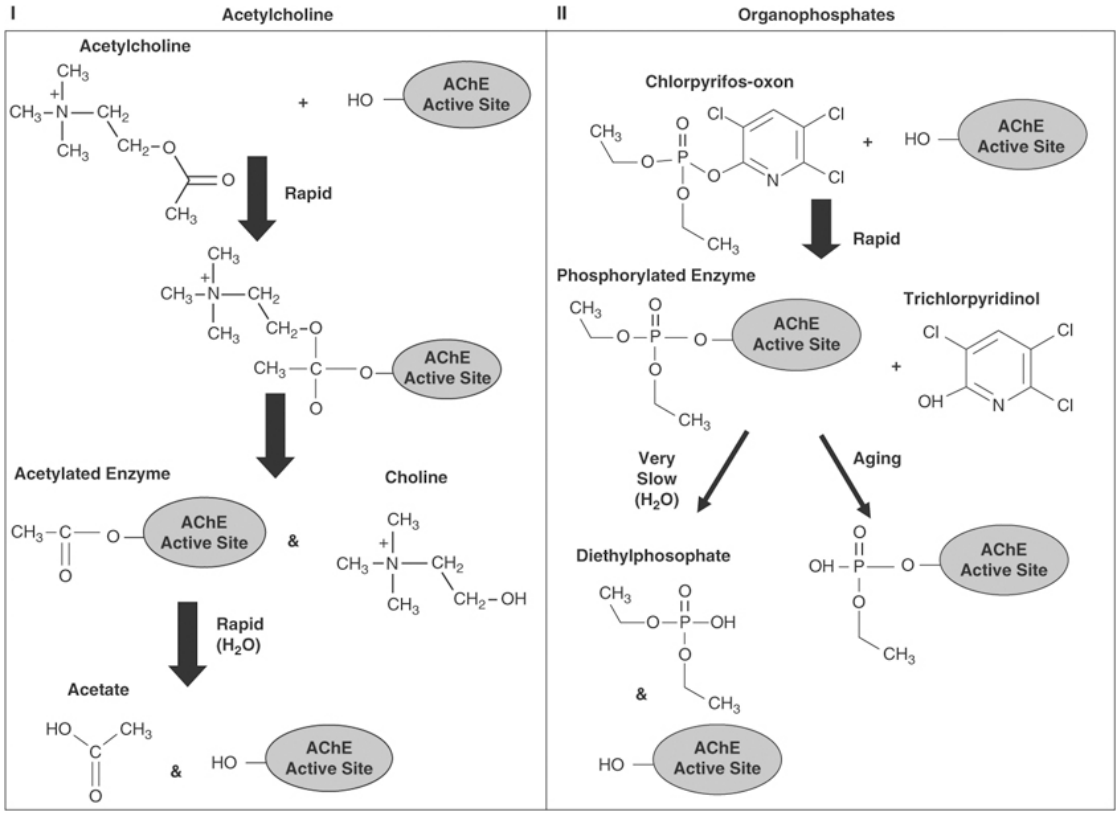
2.6 Enzymes inhibition assay

OPs and carbamate pesticides analysis using enzyme inhibition assay is based on the inhibition of AChE activities by OPs and carbamates *in vivo*. AChE is the enzyme that catalytically cleaves the neurotransmitter acetylcholine *in vivo* into acetate and choline as shown in Figure 2-5 A. In a presence of OPs and carbamate pesticides, the AChE is inhibited and less acetylcholine can be converted to the products (Figure 2-5 B). Using this *in vivo* toxicity of pesticides, the enzyme inhibition assay is carried out by detecting the product associated with this assay. The most widely used method is detection of H_2O_2 produced by the reaction of acetylcholine and H_2O that is enzymatically catalyzed by the AChE and

choline oxidase (ChOX). In aqueous solution, acetylcholine is catalyzed by AChE producing choline and acetate (reaction 1). Choline is subsequently converted to an aldehyde and hydrogen peroxide (H_2O_2) by choline oxidase (ChOX) (reaction 2). H_2O_2 key product can be detected by electrochemistry (G. Liu & Lin, 2006) and colorimetry (Liang et al., 2013). In a presence of OPs and carbamate pesticides, AChE is inhibited and less H_2O_2 is produced. Detection signal of H_2O_2 is inversely proportional to amount of OPs and carbamate pesticides in the samples.

$$\text{Achetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{choline} + \text{Acetate} \qquad (1)$$

$$\text{Choline} + O_2 \xrightarrow{\text{ChOX}} \text{betaine aldehyde} + H_2O_2 \qquad (2)$$



(A) (B)

Figure 2-5 Schematic illustrating the interaction of acetylcholine (A), and the organo-phosphate chorpyrifos-oxon (B) with the active site of AChE (Barry et al., 2008).

2.7 Microfluidic paper-based analytical devices

Microfluidic paper-based analytical devices (μPADs) are very attractive because they are portable, inexpensive, and easy to use and known as a novel alternative technology for

diagnostic assays. μPADs have been applied in many fields such as biochemical analysis (e.g., enzyme activity), environment monitoring, food quality control, and health diagnostic (e.g., urinalysis, pregnancy test) (de Souza, Alves, & Coltro, 2012; Hu et al., 2014; X. Li, Ballerini, & Shen, 2012; Z. Nie, Deiss, Liu, Akbulut, & Whitesides, 2010). μPADs are produced by patterning hydrophilic paper with hydrophobic barriers(Cate, Dungchai, Cunningham, Volckens, & Henry, 2013; Sameenoi et al., 2013). The hydrophilic fiber network of paper provides a self-priming capillary channel allowing wicking of solution without the need of external pumps. The pattern is generated by depositing a hydrophobic material on the paper serving as a barrier to guide the liquid wicking to wanted directions. Unlike traditional analytical methods using cellulosic substrates like litmus paper, μPADs are suitable for multiplexed chemical analysis as well as more advanced sample pretreatment methods due to the ability to store reagents on the device (Andres W. Martinez, Phillips, Whitesides, & Carrilho, 2009). Various procedures have been reported for making the devices, including, wax printing (Carrilho, Martinez, & Whitesides, 2009), photolithography (Andres W. Martinez et al., 2008), polydimethylsiloxane printing (Bruzewicz, Reches, & Whitesides, 2008), plasma etching (Xu Li, Tian, Nguyen, & Shen, 2008) and permanent marker plotting (J. Nie et al., 2012). Normally, two types of patterned, lateral flow format and well-shaped format were used for chemical analysis. The versatility of μPADs has also been extended to the detection step, where a variety of detection methods have been implemented such as colorimetric (Ornatska, Sharpe, Andreescu, & Andreescu, 2011), electrochemical (Del Carlo, Mascini, Pepe, Compagnone, & Mascini, 2002) and fluorescence (He & Liu, 2013).

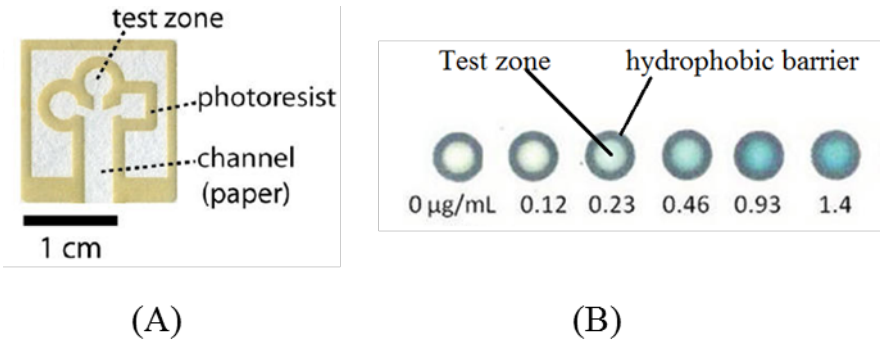


Figure 2-6 Patterns of μPADs: (A) lateral flow format (Andres W. Martinez et al., 2009), (A) Well-shaped format (Jokerst et al., 2012)

2.8 Cerium (IV) oxide nanoparticles

Cerium (IV) oxide Nanoparticle (CeO_2 NPs) are currently used in variety industrial applications, such as catalysis (Das et al., 2007), gas sensors (Jasinski, Suzuki, & Anderson, 2003), UV filters (El-Toni, Yin, & Sato, 2008), and solid oxide fuel cell (Adijanto et al., 2013). CeO_2 NPs has been found to protect cardiac progenitor cells from oxidative stress (Pagliari et al., 2012). CeO_2 NPs have oxidase-like activity in aqueous environments, making it suitable to use an oxidation catalyst (Asati, Santra, Kaittanis, Nath, & Perez, 2009). Ceria exists as mixed valence state oxides of Ce^{3+} and Ce^{4+} in which the two states can interchange in a redox environment. For example, hydrogen peroxide (H_2O_2) can act as both an oxidation and reduction agent and induce a switch between Ce^{3+}/Ce^{4+} states (Yu, Hayes, O'Keefe, O'Keefe, & Stoffer, 2006). According to the recent study, ceria nanoparticles can be used for as colorimetric probes for H_2O_2 in paper-based device (Ornatska et al., 2011). So, it is possible to apply ceria nanoparticles as probes for detection of H_2O_2, which were produced from analysis of pesticides by enzyme inhibition methods.

(A) (B)

Figure 2-7 The detection of glucose using (A) the ceria-based assay, (B) the traditional method (Ornatska et al., 2011)

2.9 Related literature review

Hossain et al (2009) reported the use of paper-based devices for determination of pesticides in beverage and food samples (Hossain, Luckham, McFadden, et al., 2009). In this studied, a reagentless bioactive paper-based biosensor was developed for detection of AChE inhibitors, organophosphate pesticides. In this assay, an indophenyl acetate (IPA) was used as a substrate. AChE hydrolyzes the red-yellow colored substrate IPA under slightly basic

conditions (pH 8.0) to forms blue-purple color of indophenoxide anion (IDO^-). Decreasing of blue-purple color indicated the presence of OPs pesticides as shown in the reaction in Figure 2-8

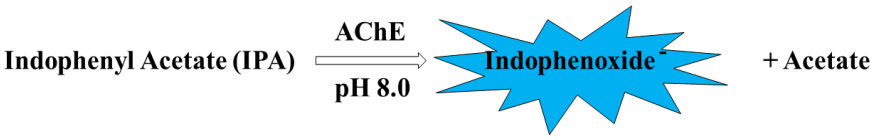


Figure 2-8 Reaction of the detection principle of the indophenyl acetate (IPA) based colorimetric assay

Fabrication of this device was carried out as follows. A section of Whatman filter paper number 1 was cut into small pieces (1 × 10 cm). Using either inkjet printing or over spotting, AChE and IPA were entrapped on the paper in two different regions (e.g., sensing and substrate regions). The sensor can be used two different ways, one directly (normal lateral flow-based assay) without incubating the contaminated sample and other inverted lateral flow-based assay with incubation of the sample over the sensing area.

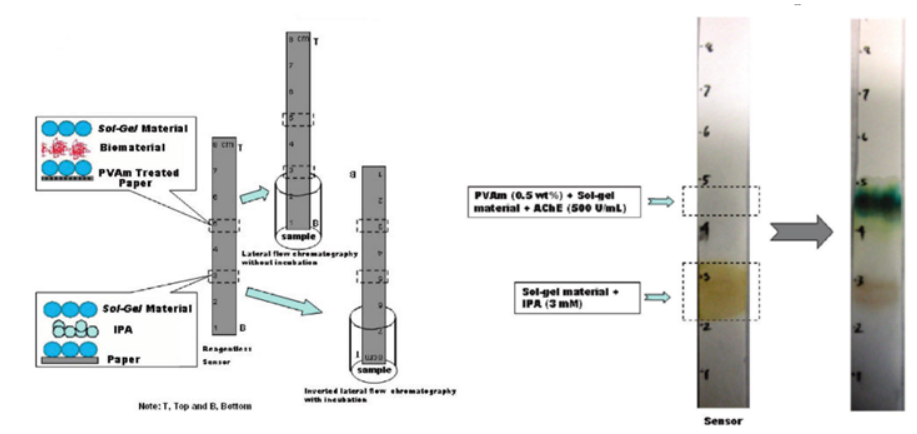


Figure 2-9 (A) Schemes of fabrication device and analysis steps, (B) Picture of device was shown the color before and after detection.

In this assay, the determination of pesticides was conducted without the use of any external reagents with excellent detection limits (bendiocarb~1 nM; carbaryl ~10 nM; paraoxon ~1 nM; malathion ~10 nM) and sort time (5 min). However, the method has some

disadvantages as well. Detection of pesticides in spiked milk and apple juice samples had matrix effects problem. The method also required of in milliliters volume of samples to flow and reach to the sensing region on the paper.

Hossain et al. (2009) studied the development of a bioactive paper sensor for detection of neurotoxins using piezoelectric inkjet printing of sol-gel - derived bioinks (Hossain, Luckham, Smith, et al., 2009). In this study, a novel paper-based solid-phase biosensor was utilized piezoelectric inkjet printing of biocompatible, enzyme-doped, sol-gel-based inks to create colorimetric sensor strips for the detection of AChE substrates and inhibitors, based on Ellman's colorimetric assay. Herein, AChE hydrolyzes the acetylethiocholine (ATCh) and forms thiocholine (TCh), which then reacts with dithiobisnitrobenzoate (DTNB) to generate yellow color of 5-thio-2-nitrobenzoate (TNB, an anion) (Figure 2-10)

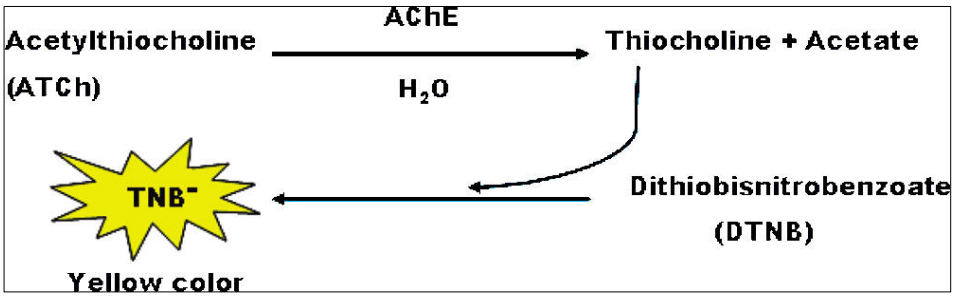


Figure 2-10 Schematic representation of detection principle of the Ellman assay (Hossain, Luckham, Smith, et al., 2009)

Fabrication of devices was done by coated papers with three different materials in a specific sequence as follows; (1) printing a PVAm underlayer directly onto the paper surface; (2) printing a silica sol intermediate layer; (3) printing a buffered enzyme solution containing AChE and DTNB; and (4) printing a silica sol overlayer (Figure 2-11).

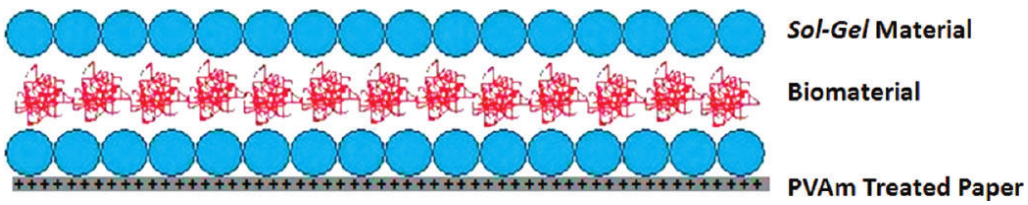


Figure 2-11 Layers of PVAm, biomaterial and sol-gel material were coated on paper (Hossain, Luckham, Smith, et al., 2009)

The result of this studied showed a good detection limits (paraoxon, ~100 nM; aflatoxin B1,~30 nM) and rapid response times (<5 min). The use of sol-gel-based entrapment produced a sensor that retained enzyme activity and gave reproducible results after storage at 4 °C for at least 60 days, making the system suitable for storage. On the other hand, this assay required an expensive piezoelectric inkjet printer and mead brand cardboard paper substrate making the method inappropriate to use in developing countries.

Liu, Kou, Xing, and Li (2014) have developed a paper-based chromatographic chemiluminescence chip for the detection of dichlorvos (DDV) in vegetables without complicated sample pretreatment (W. Liu, Kou, Xing, & Li, 2014). The analysis is based on the reaction of luminol with H_2O_2 to produce chemiluminesence signal (CL). In a presence of DDV, H_2O_2 can also react with DDV and hence lower amount of H_2O_2 remained to react with luminol causing lower CL intensity (Wang, Zhang, Wang, Yang, & Zhang, 2001). The reactions are shown in Figure 2-12. The paper chromatography separation procedure can be accomplished in 12 min on a paper support (0.8×7.0 cm^2) by using 5 μL sample spotted on it. After sample developing, the detection area was cut and inserted between two layers of water impermeable single sided adhesive tapes. The paper-based chip was made by attaching the middle layer of paper onto the bottom layer. Then it was covered by another tape layer, which was patterned by the cutting method to form a square hole in it. The mixed solution of luminol and H_2O_2 was dropped on the detection area to produce CL. The CL intensity was inverted to the concentrations of DDV.

$$\text{Luminol} + H_2O_2 \longrightarrow \text{CL} \qquad (1)$$

$$\text{DDV} + H_2O_2 \longrightarrow \text{peroxophosphonate} \qquad (2)$$

Figure 2-12 Reaction of DDV in CL method (1) the reaction of luminol and H_2O_2 to form CL signals, (2) DDV was oxidized by H_2O_2 to produce peroxophosphonate

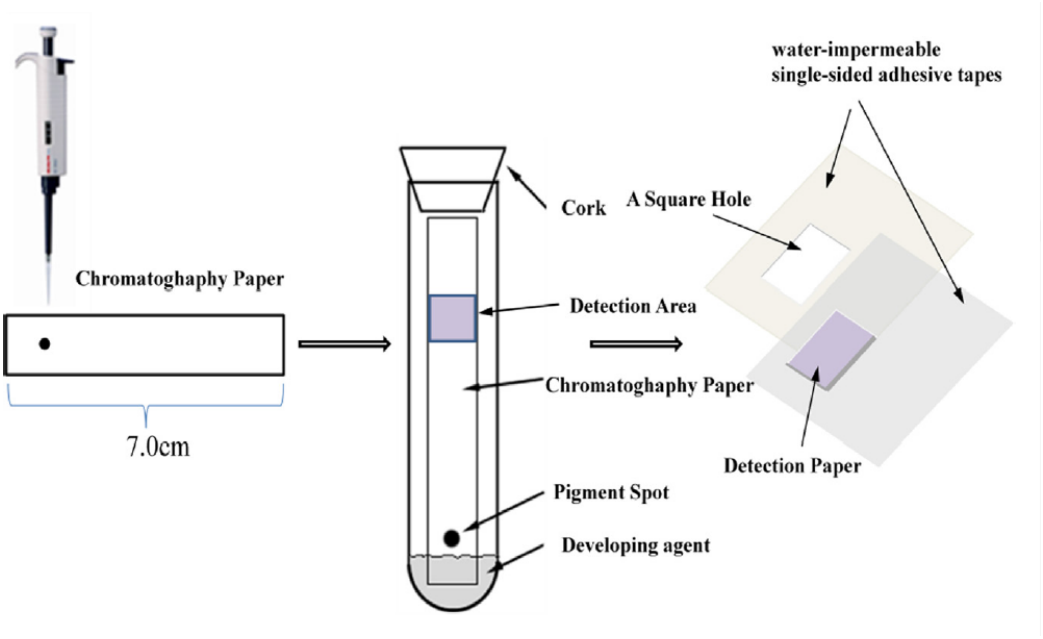
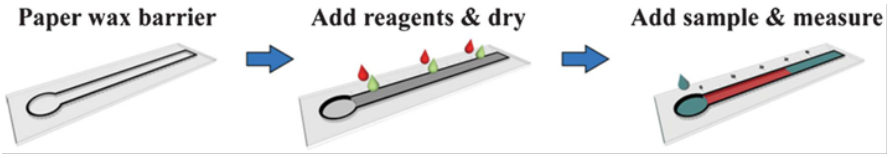


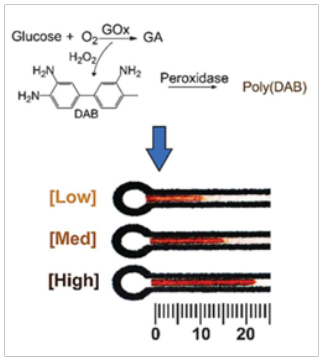
Figure 2-13 Diagram of paper chromatography and paper-based chip (W. Liu et al., 2014).

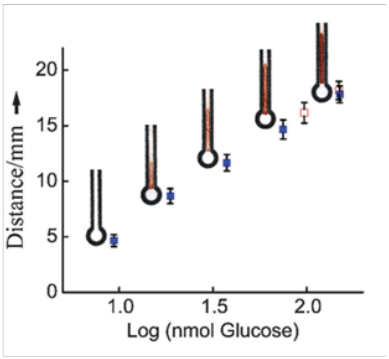
The detection limit for DDV using the developed method was 3.6 ng mL^{-1}. This study should, therefore, be suitable for rapid and sensitive detection of trace levels of organophosphate pesticides in environmental and food samples. However, the fabrication of devices requires the expensive equipment and need a large amount of reagents and samples.

Recently, Cate, et al (2013) reported a new method for measuring the signal on the paper-based device without any external supported tools such as camera and imaging program (Cate et al., 2013). It is called distance-based measurement paper device. The pattern was designed as a thermometer shape (Figure 2-14). The analytical procedures were carried out as follow. The flow circuit was first printed on filter paper by wax ink. A circular reservoir at the bottom accommodates sample addition (Figure 2-14 A). Colorimetric reagents are deposited along the flow channel by spraying or pipetting. After dry, the sample is added to the sample reservoir and flow along the channel by capillary action. Then the analyte reacts with the reagent and the color develops along detection zones. Analyte quantification is performed by measuring the length of the coloured region in the flow channel, typically with a ruler that can either be held up to or printed directly along the channel for user friendliness and portability. The length of coloured channel depended on the concentration of samples as shown in the Figure 2-14 B.



(A)





(B)

Figure 2-14 (A) chemical analysis based on distance-based measurement on paper devices, (B) the length of colored channel is directly proportional to glucose concentration in the sample.

CHAPTER 3
METHODOLOGY

3.1 Materials and chemicals

3.1.1 Materials

1. Whatman No. 4 Filter paper (Whatman International Ltd , China)
2. Micropipettes 100-1000 µL, 10-100µL, 0.5-10 µL (Eppendorf Research Plus, Germany)
3. Scanner (Canon, LiDE110)
4. Microcentrifuge tubes with size of 250 µL and 1500 µL
5. Hair dryer (Mamaru, MR-7502)
6. Patterned screen

3.1.2 Chemicals

1. Acetylcholine esterase (AChE), 518 Units/mg Solid, 3.9 mg solid, CAS: 9000-18-1, Sigma-Aldrich, USA

2. Acetylcholine chloride ($C_7H_{16}ClNO_2$), (ACh), MW: 181.66 g/mol, CAS: 60-31-1, Sigma-Aldrich, Switzerland
3. Choline oxidase (ChOX), 8 mg solid, CAS: 9028-67-5, Sigma-Aldrich, Japan
4. Cerium (IV) oxide Nanoparticles (CeO_2 NPs), colloidal dispersion in 2.5% acetic acid with 10-20 nm particle sizes, MW: 172.11 g/mol, CAS: 1306-38-3, Sigma-Aldrich, USA
5. Polyethylene glycol (PEG), MW: 6000 g/mol, Sigma-Aldrich, USA
6. Bovine Serum Albumin (BSA), CAS: 9048-46-8, Sigma-Aldrich, USA
7. Tris (hydroxymethyl) aminomethane ($NH_2C(CH_2OH)_3$), MW: 121.14 g/mol, CAS: 77-86-1, Omnipur, China
8. Methanol (CH_4O), MW: 32.04 g/mol, CAS: 67-56-1, Sigma-Aldrich, Venezuela
9. Chlorpyrifos ($C_9H_{11}Cl_3NO_3PS$), MW: 350.59 g/mol, CAS: 2921-88-2, Sigma-Aldrich, Germany
10. Chlorpyrifos-oxon ($C_9H_{11}Cl_3NO_4P$), MW: 334.52 g/mol, CAS: 5598-15-2, Sigma-Aldrich, Singapore
11. Methyl-paraoxon ($C_8H_{10}NO_6P$), MW: 247.14 g/mol, CAS: 950-35-6, Sigma-Aldrich, Singapore
12. Hydrogen peroxide (H_2O_2), MW: 34.01 g/mol, CAS: 7722-84-1, Sigma-Aldrich, Singapore
13. Polystyrene (PS), local stationary shop, Chon Buri, Thailand
14. Toluene ($C_6H_5CH_3$), MW: 92.14 g/mol, CAS: 108-88-3, RCI Labscan, Thailand

3.1.3 Samples

Dried seafood products such as dried green mussel was purchased from local markets in Chon Buri province area, and cabbage was purchased from supermarket, in Chon Buri province area.

3.2 Research plan

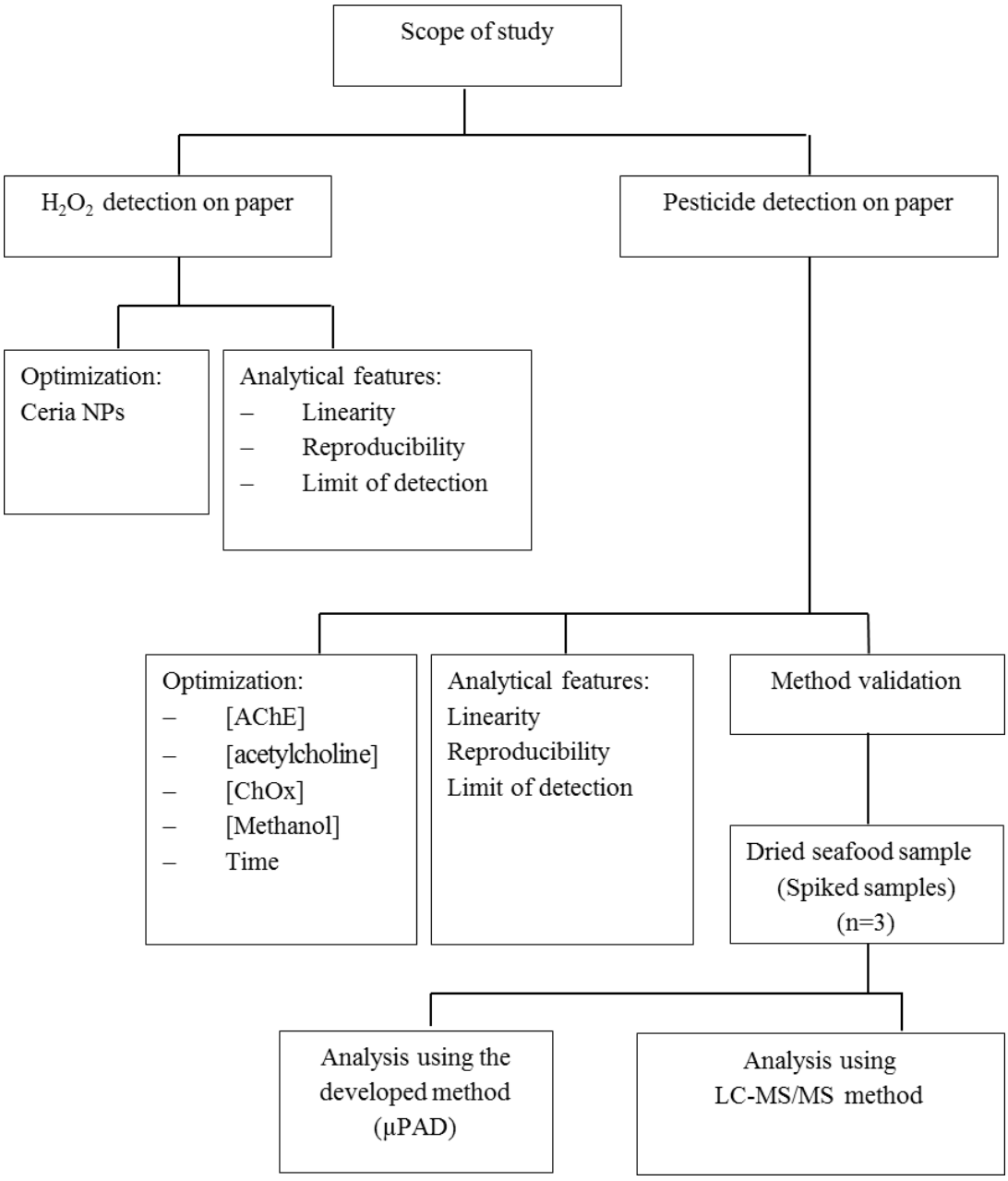


Figure 3-1 Research Plan

3.3 Experimental

3.3.1 Preparation of solution

3.3.1.1 Tris-buffer pH 7.4, 10 mM

10 mM of Tris-buffer pH 7.4 was prepared by dissolving 0.1211 g of tris (hydroxymethyl) aminomethane in deionized water. The buffer pH was adjusted to pH 7.4

using 1M HCl. The solution was then made up to 250 mL by deionized water. The buffer solution was stored at 2-8°C until use.

3.3.1.2 Acetylcholinesterase solution

1000 Units/mL stock solution of acetylcholinesterase (AChE) was prepared by dissolving 3.9 mg solid (518 Units/mg Solid) of AChE in 2000 µL of Tris-buffer pH 7.4. The stock solution was stored at -20°C until use. Working AChE solutions were daily prepared by diluting the stock solution using BSA solution (1 mg/mL in Tris-buffer pH 7.4).

3.3.1.3 Choline oxidase solution

100 Units/mL stock solution of choline oxidase (ChOX) was prepared by dissolving 8 mg solid (13 Units/mg Solid) of ChOX in 1000 µL of Tris-buffer pH 7.4. The stock solution was stored at -20 °C until use. Working ChOX solutions were daily prepared by diluting the stock solution using BSA solution (1 mg/mL in Tris-buffer pH 7.4).

3.3.1.4 Acetylcholine chloride solution

100 mM stock solution of acetylcholine (ACh) 1000 µL was prepared by dissolving 0.181.66 g of acetylcholine in Tris-buffer pH 7.4 and adjusted the volume to 1000 µL by tris-buffer pH 7.4. Working acetylcholine solutions were daily prepared by diluting the stock solution using tris-buffer pH 7.4. The stock solution was stored at 2 - 8 °C

3.3.1.5 Bovine serum albumin

1 mg/mL solution of bovine serum albumin (BSA) was prepared by dissolving 5 mg of BSA in Tris-buffer pH 7.4 and adjusted the volume of 5000 µL by tris-buffer pH 7.4. The solution of BSA was stored at 2 - 8 ℃ until use.

3.3.1.6 Cerium (IV) oxide solution

3% w/v cerium (IV) oxide (CeO_2) solution was prepared by mixing 750 µL of 20% w/v CeO_2 NPs solution with 4250 µL deionized water. The solution was stored at 2 - 8 ℃.

3.3.1.7 Polyethylene glycol solution

10 mg/mL of polyethylene glycol (PEG) solution was prepared by dissolving 10 mg of PEG in 1 mL deionized water then stored at 2 - 8 ℃.

3.3.1.8 Pesticide solution

Stock solution of 50 mg/mL of methyl-paraoxon and 10 mg/mL of chlorpyrifos-oxon were dissolved in methanol and stored at 2-8 °C until use. Working pesticide solutions were daily prepared by dilution of the stock solution using 4%(v/v) methanol.

3.4 Fabrication of patterned paper-based device

Paper-based device fabrication was based on the polymer screen-printing method (Sameenoi, Nongkai, Nouanthavong, Henry, & Nacapricha, 2014) shown in Figure 3-2 below. Every step of fabrication was performed in the fume hood. In this work, the screen pattern was created as a well shape with diameter of 5 mm serving as a detection zone on the paper. Firstly, the patterned screen was placed onto a Whatman No.4 filter paper. The polystyrene solution obtained from dissolving 1.25 g polystyrene in 5 mL toluene was squeezed through the screen. The polystyrene solution passed through the screen and the paper creating three-dimensional hydrophobic barrier on the paper. The patterned paper was allowed to dry and ready to use. Prior to use, clear packing tape was put on the backside of the patterned paper to prevent leaking during assay analysis.

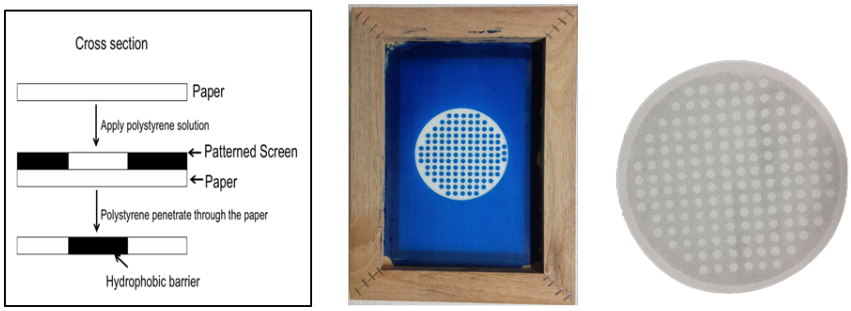
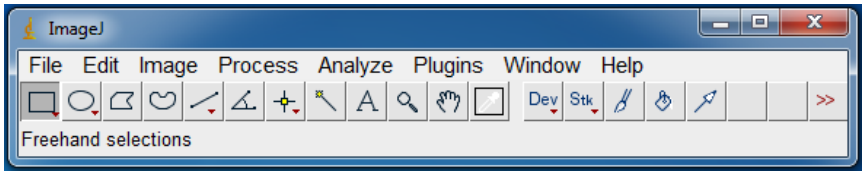


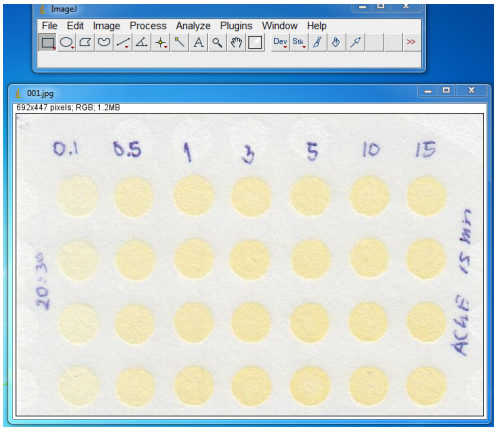
Figure 3-2 (A) step to perform the paper fabrication (B) actual patterned screen (C) obtained patterned paper device.

3.5 Color intensity analysis by ImageJ

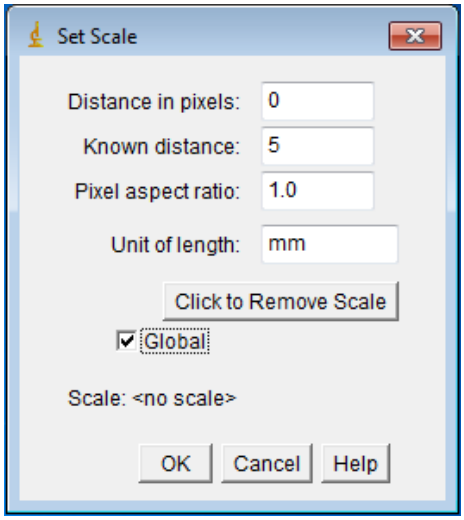
1. Starting ImageJ program: Start →ImageJ/or double click at icon of program on desktop of computer. Then the box below will appear.



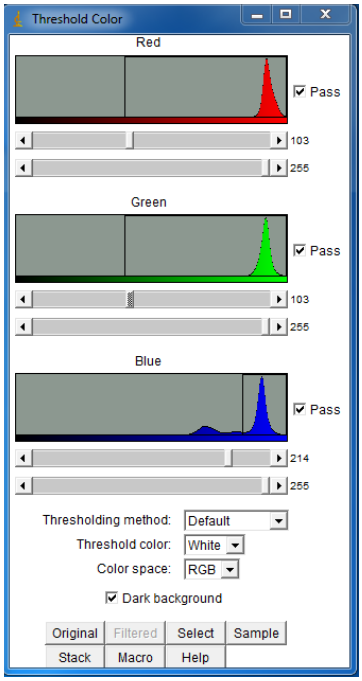
2. Choosing the image: File→Open, and choose the image.



3. Setting scale: Analyze→Set Scale, as show as picture below then clicks OK

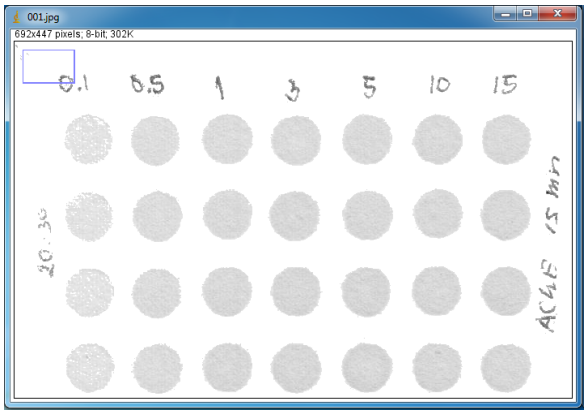


4. Adjust the color: Image →Adjust →Color Threshold, then Threshold Color's Box appears.

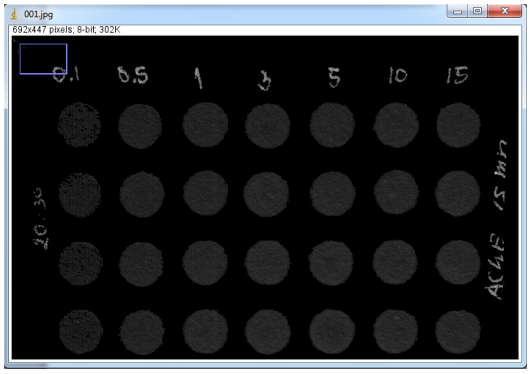


All hue adjusts, selects “pass”, Color space: RGB, Threshold color: White, Thesholding method: Default

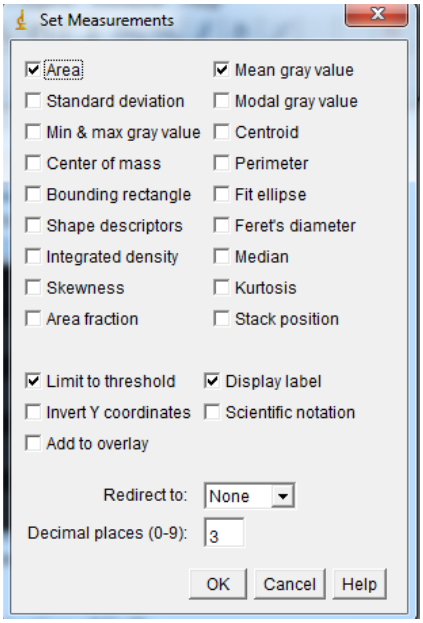
5. Setting gray scale: Image →Type →8-bit, then image is converted to the picture below.



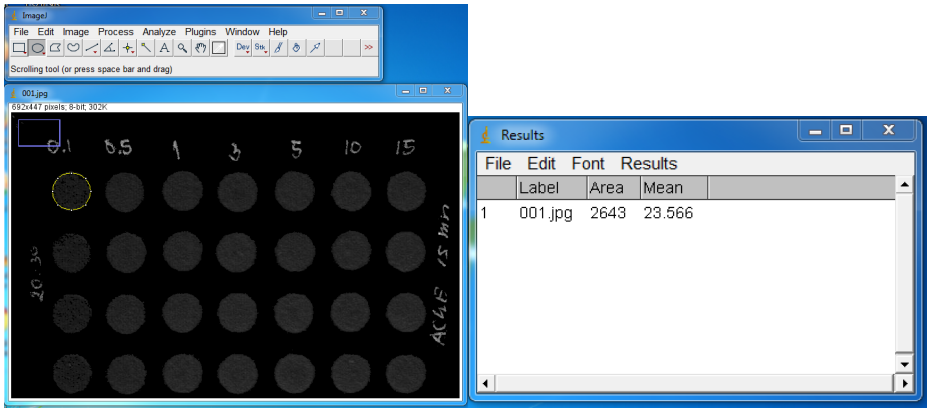
6. Adjust to gray intensity: Edit →Invert



7. Set measurements: Analyze →Set measurements, then select Area, Mean gray value, Limit to threshold, display label as shown in the picture, then click OK



8. Measuring the color intensity: click “Oval selection”. Then drag at the wanted area for measurement. Next, go to Analyze →Measure or Ctrl+M. The mean gray intensity appears in the box’s result.



Oval selection Mean intensity of the selected area

3.6 Hydrogen Peroxide Detection on Paper Devices

3.6.1 General procedure

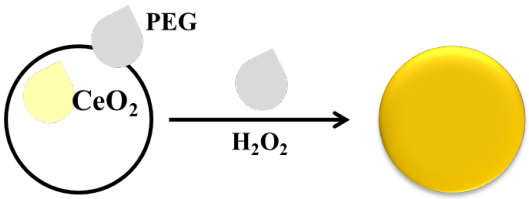


Figure 3-3 Hydrogen peroxide analysis using paper-based device with CeO_2 NPs as colorimetric probes

As shown in the reaction in section 2.6, H_2O_2 is a key product to indicate amount of the pesticide presented in the sample. The efficiency of the fabricated paper device using CeO_2 NPs as colorimetric probes for H_2O_2 was first investigated. General procedure for the analysis of H_2O_2 is shown in Figure 3-3. Briefly, CeO_2 NPs was deposited on the fabricated paper, followed by PEG solution. The device was allowed to dry. H_2O_2 was added and the yellowish-orange color developed. The picture of the device was obtained using a scanner and the color intensity was analyzed the using ImageJ as described in section 3.5. To study the performance of the paper device for H_2O_2 analysis, amount of CeO_2 NPs were first optimized. Analytical features for the analysis of hydrogen peroxide using the developed paper device were evaluated.

3.6.2 Optimization of CeO_2 NPs

The effect of CeO_2 NPs concentration on H_2O_2 detection on paper device was studied. CeO_2 NPs in the concentration range of 1 to 5 %w/v was evaluated. A 5 µL of an investigated concentration of CeO_2 NPs was dropped onto a paper-based device (n=3). Another 5 µL of PEG solution (10 mg/mL) was pipetted onto the device. After dried, a 5 µL of 100 mM H_2O_2 was added and the yellow color product formed. Color intensity was measured as the method described above. A graph plotted between concentration of CeO_2 and mean gray intensity were constructed to find optimal CeO_2 NPs concentration which would be the amount that give highest color intensity at the investigated H_2O_2 concentration.

3.6.3 Analytical features for the analysis of H_2O_2

3.6.3.1 Linearity

To study linearity, H_2O_2 concentrations in the range of 0 to 100 mM was evaluated. A 5 µL of CeO_2 NPs (3% w/v) was dropped onto a paper-based device (n=4). Another 5 µL of PEG solution (10 mg/mL) was pipetted onto the device. After dried, a 5 µL of each concentration of H_2O_2 was added and the yellow color product formed. Color intensity was measured as the method described in section 3.5. A graph plotted between concentration of H_2O_2 and mean gray intensity were constructed to find the linearity.

3.6.3.2 Limit of Detection

The LOD is the lowest analyte concentration likely to be reliably distinguished from the blank and at which detection is feasible. According to initial experiment, blank solution gave zero intensity. In this work, experimental LOD was evaluated as the lowest H_2O_2 concentration that can give measurable intensity.

3.6.3.3 Reproducibility

Reproducibility for analysis of using the developed paper device was evaluated by analyzing ten replicates of three different concentrations of H_2O_2 in the linear range. The relative standard deviation (%RSD) was calculated to indicate the reproducibility of the developed paper device.

3.7 Analysis of Pesticide on a Paper-Based Device

3.7.1 General procedure

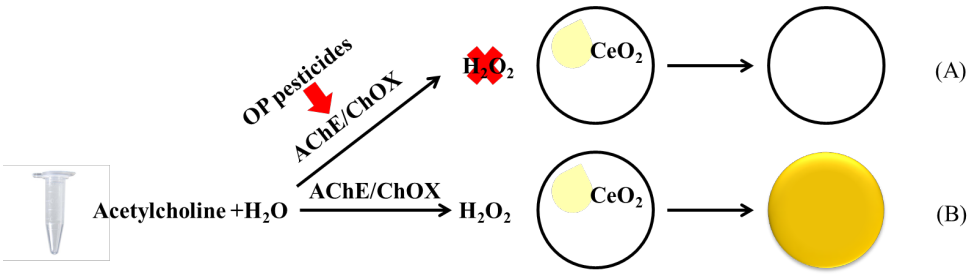


Figure 3-4 General procedure for the analysis of pesticide using OPs as a model standard pesticides. (A) In a presence of OP pesticide, AChE was inhibited and hence less H_2O_2 produced resulting in low color intensity observed on the device. (B) In an absence of pesticide, AChE and ChOX can catalytically oxidize acetylcholine and produce H_2O_2 resulting in the color development on the CeO_2 paper.

The proposed procedure for the analysis of pesticide in dried seafood using a paper-based device using ceria nanoparticle as colorimetric probes is as follows. 1 U/mL of AChE was mixed with chlorpyrifos-oxon pesticide (0-200ng/mL) in a microcentrifuge tube and incubated for 15 minutes. Then, 5 U/mL of ChOX and 3 mM of acetylcholine were added to the mixture and incubated for another 15 minutes. The mixture was dropped onto CeO_2 NPs coated paper-based device prepared as described (section 3.6.1). The yellowish-orange color produced on paper can be observed with the naked eye. For quantitative analysis of pesticides, the color intensity was analyzed using the method described in section 3.5. The obtained intensity was used to generate the calibration curve which is the plot of intensity as a function of chorpyrifos – oxon concentration. To get optimal conditions for pesticide analysis, the effect of parameters associated with the enzymatic assay on the color intensity were first studied including reagents (i.e. concentration of AChE, acetylcholine, ChOX), reaction time and reaction pH. Using optimized parameters, the analytical features for pesticide analysis were investigated. Finally, the pesticide analysis in some dried seafood and

vegetable samples were carried out and validated with the traditional method for pesticide analysis such as LC-MS/MS.

3.7.2 Optimizations

3.7.2.1 Concentration of AChE

Starting amount of AChE used for the enzymatic assay plays a crucial role on the assay sensitivity for pesticide analysis. Suitable starting AChE concentration would provide high signal intensity in an absence of pesticide. At this AChE concentration, when the pesticide is added to the assay, the decreasing of initial signal should be clearly observed as the AChE activity is inhibited. To find the appropriate amount of AChE used in the assay, a dose-response curve plotted between AChE concentration and intensity was studied. This experiment was performed without the addition of pesticide. AChE in the concentration range of 0.1 to 15 U/mL was evaluated. The assay was carried out by mixing 5 µL of each concentration of AChE with 5 µL of 20 U/mL ChOX, and 5 µL of 5 mM acetylcholine in a microcentrifuge tube. After 15 minutes incubation time, 15 µL of the mixtures in tubes were dropped onto test zones of CeO_2 NPs-coated paper devices (n=4) for H_2O_2 detection. The device was allowed to dry and the color intensity was measured as the method described above.

3.7.2.2 Concentration of ChOX

The optimization of ChOX was evaluated similar to methods of AChE optimization (in 3.7.2.1) where the concentration of ChOX was varied in the range 0.1 to 20 U/mL at 5 mM acetylcholine and 1 U/mL AChE.

3.7.2.3 Concentration of acetylcholine

Concentration of acetylcholine used in the assay is another key parameter that affects the enzymatic assay as it is an initial substrate for the reaction. acetylcholine in the concentration range of 1 to 10 mM was studied. This experiment was conducted without pesticide. The experiment was performed by adding 5 µL of each acetylcholine concentration into the mixture of 5 µL of 1 U/mL AChE and 5 µL of 5 U/mL ChOX. After 15 minutes incubation time, 15 µL of the mixtures in each tube was dropped onto test zones (n=4) and the product was analyzed using the method described above.

3.7.2.4 Methanol concentration as solvent for pesticide

Most of organophosphate pesticide is insoluble in water. In this study, methanol was used as a solvent to prepare stock solution of pesticide. However, high concentration of some organic solvents including methanol has been found to inhibit the AChE activity.

Therefore, suitable concentration of methanol that could dissolve pesticide at a concentration in working range and could not inhibit enzyme activity was studied. The optimization was done by mixing 5µL of 1 U/mL AChE with 5 µL of each concentration of methanol, ranging from 0% to 10% v/v in a microcentrifuge tube. The mixture was allowed to react for 15 minutes. Then, 5 µL of 5 U/mL ChOX and 5 µL of 3 mM acetylcholine were added to a tube and allowed to react for another 15 minutes. Lastly, the mixed solution in tubes were dropped onto the test zones (n=3) and the color intensity was analyzed as described above.

3.7.2.5 Reaction time

The reaction time for reaction of acetylcholine to produce H_2O_2 catalyzed by AChE and ChOX play an important role on the sensitivity to observe the change of AChE. Reaction time of 15, 20, and 30 minutes were evaluated. The assay was carried out by mixing 5 µL of 1U/mL AChE with 5 µL of 5 U/mL ChOX, and 5 µL of 3 mM acetylcholine in a microcentrifuge tube. After 15, 20, and 30 minutes incubation times, 15 µL of the mixtures in tubes were dropped onto test zones of CeO_2 NPs-coated paper devices (n=4). The device was allowed to dry and the color intensity was measured as the method described above. The graph plotted between intensity as a function of reaction times were constructed and compared. The lowest reaction time that gives high intensity was selected as optimized reaction time.

3.7.3 Analytical Features

The analytical features for the development of a paper-based microfluidic device for pesticide analysis in dried seafood and vegetable samples using ceria nanoparticle as colorimetric probes was investigated under the optimized conditions. The studied parameters are linearity, limit of detection (LOD), limit of quantification (LOQ), and reproducibility.

3.7.3.1 Linearity

AChE and ChOX were fist prepared in BSA, and stock of OP pesticides such as methyl-paraoxon and chlorpyrifos-oxon solutions were diluted to concentration in the range of 0- 200 ng/mL and 0-5 µg/mL using 4% methanol, respectively. For evaluating linear range of methyl-paraoxon, the experiment was done by mixing 5 µL of each concentration of AChE (0.1, 0.5 and 1 U/mL) with 5 µL of different concentration of methyl-paraoxon in a microcentrifuge tubes and incubated for 15 minutes. Then, 5 µL of 5 U/mL ChOX and 5 µL of 3 mM acetylcholine were added to the tube and allowed to react for another 15 minutes. Lastly, 20 µL of the mixture was pipetted onto the CeO_2-treated paper-based device (n=5). Paper-based devices were scanned, and the color intensity was analyzed by ImageJ as described

above. The calibration curve was constructed by plotting concentration of mean gray intensity as a function of methyl-paraoxon concentrations to find the linear range. For examining linear range of chlorpyrifos-oxon, the experimental were performed in the same manner as determining linear range of methyl-paraoxon.

3.7.3.2 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD is the lowest analyte concentration likely to be reliably distinguished from the blank and at which detection is feasible. In this work, limit of detection (LOD) and limit of quantitation (LOQ) were evaluated from MPO and CPO analysis at different starting amount of AChE used in the assay. LOD was obtained by calculating the concentration of pesticide giving the intensity equal to I_0 – 3 blank standard deviation of I_0 and LOQ was equal to I_0 – 10 blank standard deviation of I_0 where I_0 was the intensity of the blank as represented in the following equations;

$$I_{LOD} = I_0 - 3SD_{blank} \quad (1)$$

$$I_{LOQ} = I_0 - 10SD_{blank} \quad (2)$$

$$I_{LOD} = -mx_{LOD} + C \quad (3)$$

$$I_{LOQ} = -mx_{LOQ} + C \quad (4)$$

- I_{LOD} and I_{LOQ} are the signal of color intensity of the assay, which is exposed to pesticide at LOD value
- m and C is a slope and y-intercept of calibration curve for pesticide analysis, respectively
- SD_{blank} is a standard deviation of the intensity of the blank from seven replicates.
- X_{LOD} and X_{LOQ} are the LOD and LOQ from the pesticide analysis using developed assay.

3.7.3.3 Reproducibility

Reproducibility for the pesticide analysis using the developed assay was evaluated by analyzing seven replicates of three different concentrations of pesticide in the linear range. The relative standard deviation (%RSD) was calculated to indicate the reproducibility of the developed method.

3.7.4 Method Validation

The developed paper-based assay for pesticide analysis was validated with the standard LC-MS/MS method using dried seafood and vegetable samples collected from local

markets in Chon Buri area. LC-MS/MS analysis was provided by the Central Laboratory (Thailand) Co., Ltd. Chachoengsao branch.

3.7.4.1 Sample preparation without the use of dispersive device

First, dried green mussel and cabbage samples were chopped and homogenized using the blender. A 5 g of each sample was put into a 50 mL centrifuge tube and spiked with OP standard pesticides (MPO and CPO). The mixture was vortexed for 1 min and left in dark for 15 min to create homogeneous samples. A 20 mL of methanol was added to the mixture and shaked for 30 min using vortex mixer for extraction. The sample tube was then centrifuged at 4000 rmp for 10 min. A 1 mL of supernatant was added into a vial and allowed to dry in the hood. Finally, the extracted residue was redissolved in 1 mL of 4% methanol for the analysis using the developed paper-based device assay without the use of dispersive kit.

3.7.4.2 Sample preparation using dispersive device

First, samples, dried green mussel and cabbage were homogenized and spiked with MPO and CPO in the same manner as the method described for sample preparation without the use of dispersive device. Then, 1.5 mL of supernatant was added into a 2 mL micro-centrifuge tube of dispersive SPE containing 25 mg C18 and 150 mg $MgSO_4$. The mixture was vortexed for 30 min and centrifuged for 10 min at 4000 rpm and the supernatant collected and dried in the hood. Finally, the extracted residue was redissolved in 1 mL of 4% and 100% methanol for the analysis using the developed paper-based device assay and traditional LC-MS/MS, respectively.

3.7.4.3 LC-MS/MS analysis conditions

In order to verify the results of developed method, both samples dried green mussel and cabbage were analyzed using LC-MS/MS, which performed by Central laboratory (Thailand) Co.,Ltd. Chachoengsao branch. The LC-MS/MS operating conditions were as follows: the HPLC WATERS, 269 model equipped with the MS/MS (Micromass, Quattro Ultima FS) was used with C18 2.0x150 mm, 3μm column. Injection volume was 10 μL. Isocratic elution was used with mobile phase mixture of 0.1% formic acid in H_2O and acetonitrile in the ratio of 40:60 with the flow rate of 0.2 mL/min. The mass selective detector was operated in electrospray ionization (ES) mode with cone voltage and temperature of 55 kV and 120 °C, respectively. The desolvation temperature of 350 °C was used.

3.7.4.4. Analysis of DSP sample using the developed paper based assay

To measure pesticide in the samples, dried seafood (dried green mussel) and vegetable (cabbage) sample sample were spiked with MPO and CPO for this study. The samples were prepared as method described in section 3.7.4.1 and 3.7.4.2 were analysis using developed nanoceria-coated paper device.

For OP pesticides analysis in dried green mussel, the concentration in the range of 0-0.7 μg/mL MPO and 0-80 ng/mL CPO were used to construct the calibration curves using the procedure described for linear range study for the two pesticide. For sample measurement, the analysis was performed as follows; 5 μL sample was incubated with 5 μL of 0.5 U/mL AChE in microcentifuge tube for 15 minutes. Then 5 μL of 5 U/mL ChOX and 3 mM Acetylcholine were added and waited for another 15 minutes. A 20 μL of the mixture was dropped onto nanoceria-coated paper based device to generate yellow color reaction and device was allowed to dry. The image of device was obtained using scanner and analyzed for mean gray color intensity using ImageJ as described above.

To verify the effective analytical performance of developed method, the nanoceria-coated paper based device was also applied to determine OP pesticides in vegetable sample (cabbage). The MPO and CPO residue in cabbage were determined in similar manner as the analysis of OPs in dried green mussel.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 The principle of the assay

This study presented the new alternative technique for organophosphorus pesticides, which are well-known as the irreversibly inhibtors of AChE activity in central and peripheral nervous system of both insects and humans, causing variety of toxicological effects on human health(Gupta & Milatovic, 2012). The principle of the assay is based on enzymatic inhibition of AChE by the pesticides using nanoceria as a colorimetric agent as described in equations 1 to 3. Normally, ACh is catalyzed by AChE to form choline as a product (equation 1) which is then catalytically oxidized by ChOX to generate H_2O_2 (Ivanov et al., 2011) (equation 2). Amount of H_2O_2 product is measured colorimetrically by nanoceria as its surface oxygen vacancies is oxidized from cerium(III) to cerium(IV) by H_2O_2 resulting in the color change from colorless to orange (Das et al., 2007). The intensity of orange color developed on nanoceria surface is directly proportional to H_2O_2 yield. In a presence of OP pesticides, AChE activity is inhibited and hence produces less H_2O_2 yielding less orange color intensity of nanoceria. The nanoceria as colorimetric agent here was coated on paper-based device to allow for easy, inexpensive and potable detection format.

$$\text{Achetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{Choline} + \text{Acetate} \qquad (1)$$

$$\text{Choline} + O_2 \xrightarrow{\text{ChOx}} H_2O_2 \qquad (2)$$

$$CeO_2 + H_2O_2 \longrightarrow CeO_{2\ (ox)} \qquad (3)$$

4.2 Fabrication of paper-based device

The pattern of paper-based device was designed by the computer program Illustrator CC. The pattern was designed as a well shape with diameter of 5 mm serving as a detection zone on paper, and 125 detection zones per one Whatman no 4 filter paper that has diameter

of 11.5 cm were obtained. The fabrication process was based on one-step polymer screen-printing method where all steps were performed in the fume hood as described in section 3.4 (Sameenoi et al., 2014). Briefly, the patterned screen was placed on to Whatman no4 filter paper. The solution of polystyrene was squeezed through the screen. Through this fabrication method, three-dimensional hydrophobic barrier was created on filter paper as shown in Figure 4-1. The patterned paper was allowed to dry and ready to use. Prior to use, clear packing tape was put on the backside of the patterned paper to prevent leaking during assay analysis.

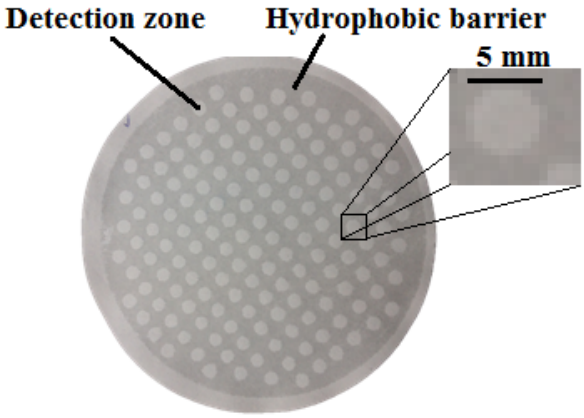


Figure 4-1 The pattern of the paper-based device fabricated by one-step polymer screen-printing method.

4.3 Study of optimal nanoceria concentration coated on paper-based devices

In this work, the effect of the concentration of nanoceria-coated onto paper-based device in the range of 1-5%w/v were examined for the detection of 100 mM H_2O_2 on paper device. The results are shown in Figure 4-2. The visible color change of nanoceria-coated paper from colorless to yellow in the presence of H_2O_2 was observed. The color intensity increased as concentration of nanoceria increased (Figure 4-2A). Color intensity was plotted against nanoceria concentration giving the graph in Figure 4-2B. Changes of color intensity were observed until at 4 % w/v of nanoceria where the intensity became steady. At 4%w/v or higher, the detection zone become very hydrophobic because of the aggregation of nanoceria on surface of paper (Andreescu et al., 2014). The concentration of 3%w/v was then selected to use in further experiments which is in similar amount reported previously (Ornatska et al., 2011).

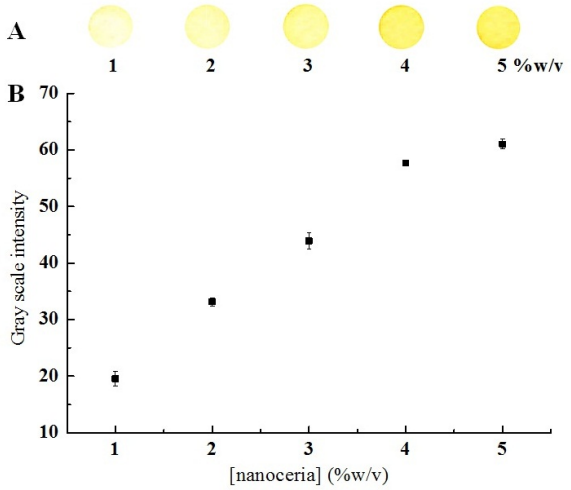


Figure 4-2 (A) Colorimetric response of nanoceria-coated paper-based device with concentration range of 1 to 5 % (w/v) for the analysis of 100 mM H_2O_2.(B) Plot of mean grey intensity as a function of nanoceria concentrations.

4.4 Hydrogen peroxide analysis using nanoceria-coated paper-based device

Hydrogen peroxide is a key product from the enzymatic assay that is related to amount of the residue OP pesticides in real sample. Under the optimum condition, the determination of H_2O_2 was carried out using nanoceria-coated paper device to obtain the linearity, reproducibility, sensitivity and limit of detection.

4.4.1 Linearity

The linearity of hydrogen peroxide was evaluated in the range of 0 to 100 mM using nanoceria-coated paper-based device. The color response on nanoceria-coated paper-based device is dependent on H_2O_2 concentration presented in Figure 4-3A. The color intensity increased at higher H_2O_2 concentration. The mean gray scale intensity of color was plotted versus the concentration of H_2O_2. This plot has two linear portions which are in the range of 1 to 2.5 mM ($y=4.7651x-3.8942$, $R^2=0.998$) and 3 to 12 mM ($y=1.1584x+5.6286$, $R^2= 0.992$) as shown in Figure 4-3 (inset). The images and the linearity provide evidences that quantitative colorimetric detection of H_2O_2 is possible using nanoceria-coated paper device with the developed assay.

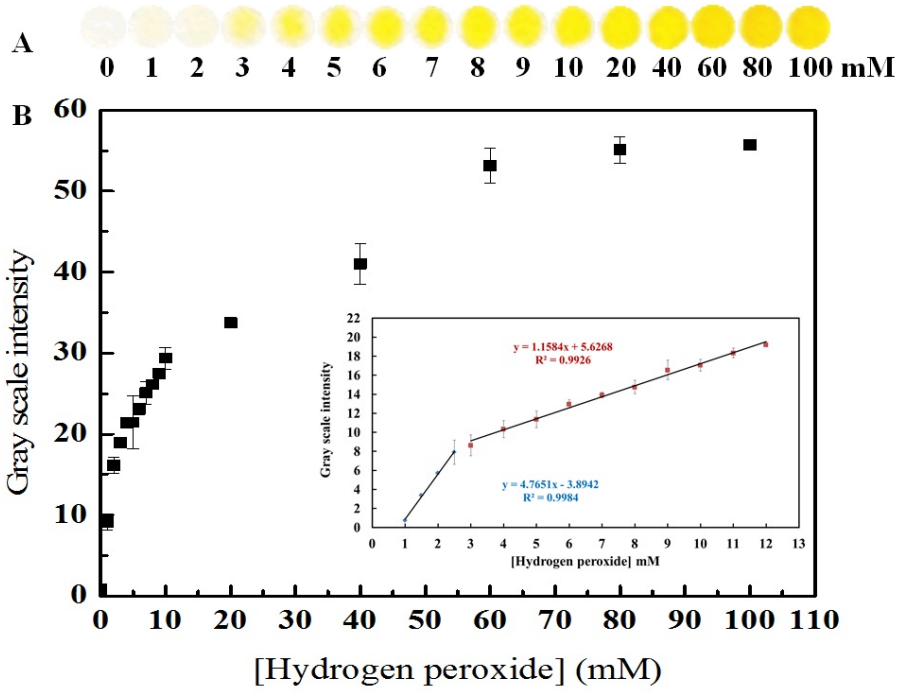


Figure 4-3 The study of linear range of nanoceria-coated paper-based device for H_2O_2 analysis in the range of 0-100 mM. Inset; the linear ranges of H_2O_2 analysis in the range of 1 to 2.5 mM and 3 to 12 mm using nanoceria-coated paper-based device.

4.4.2 Limit of detection

Limit of detection (LOD) is defined as the lowest concentration of analyte that give a signal three times higher than the standard deviation (SD) of the blank analysis. In this work, the LOD was obtained from the experiment because the blank analysis gave zero mean gray intensity. The lowest concentration of H_2O_2 that the method could detected was 0.5 mM which is lower than that reported previously (Ornatska et al., 2011).

4.4.3 Reproducibility

In this work, the percent of relative standard deviation (%RSD) was determined to indicate the reproducibility of the developed paper-based device. The experiment was performed by measuring mean gray intensity of three different concentrations of H_2O_2 in the linear range which were 4, 7 and 12 mM (n=10) and the %RSD were 2.21, 2.48 and 5.50, respectively. These results indicated that the developed paper-based device could be effectively used to determine H_2O_2 with high reproducibility.

4.5 Optimization of the enzymatic assay

4.5.1 Concentration of acetylcholine esterase

Acetylcholine esterase (AChE) is a key catalyst for OP pesticides analysis based on enzyme inhibition assay. Proper starting concentration of AChE affects the assay sensitivity as decreasing of initial signal should be obviously observed after addition of OP pesticide since the AChE activity is inhibited. To obtain appropriate concentration of AChE, the experiment was done without addition of pesticide and the results are shown in Figure 4-4

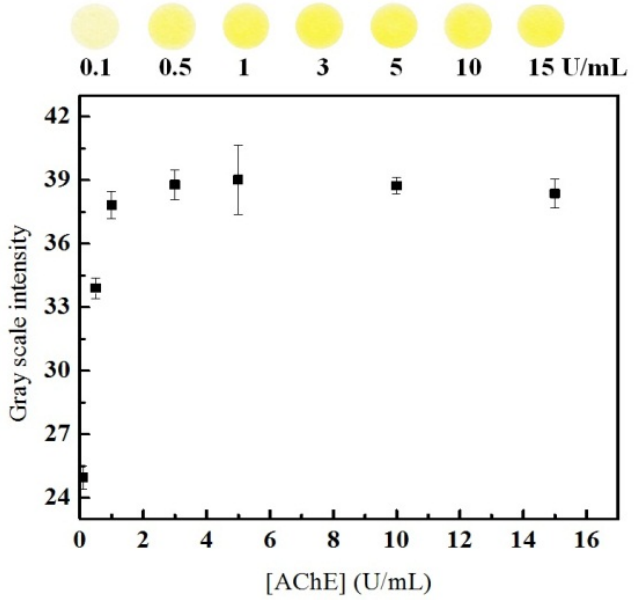


Figure4-4 The color responses of concentration of AChE

The results showed that when amount of AChE increased from 0.1-1 U/mL, the color intensity increased indicated that higher AChE acitivity generated more H_2O_2. However, when AChE concentration at above 1 U/mL, saturated color intensity was obtined. In this work, AChE at the concentration less than 1 U/mL were selected for the assays to allow for sensitive-response analysis of OP pesticide. The result demonstrates that it is possible to measure AChE activity and thus OP pesticides that inhibit AChE activity in a dose-response manner.

4.5.2 Concentration of choline oxidase

The optimum concentration of choline oxidase (ChOX) was examined at the concentration in the range of 0.1 U/mL - 20 U/mL using the method similar to that of AChE optimization described above. The results shown in Figure 4-5 indicated that the intensity of

the color is dependent upon the concentration of ChOX. The color intensity becomes saturated at ChOX concentration of 5 U/mL or higher. Therefore, the concentration of 0.5 U/mL of ChOX was selected for further experiment in this work.

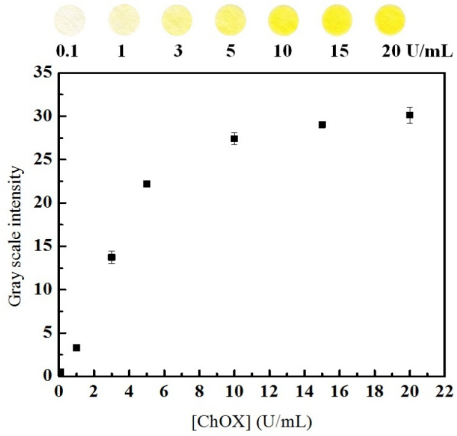


Figure 4-5 The color responses of concentration of ChOX

4.5.3 Concentration of acetylcholine

In this work, the substrate of the assay is acetylcholine which is another key parameter that affects the enzymatic assay. To estimate the appropriate concentration of acetylcholine, the mixture of acetylcholine in the range of concentration 1 to 10 mM, 1 U/mL of AChE and 5 U/mL of ChOX were incubated in microcentrifuge tube for 15 minutes then the mixture was added onto nanoceria-coated paper device to form color reaction. Device was allowed to dry and the color intensity was measured using similar method as described above.

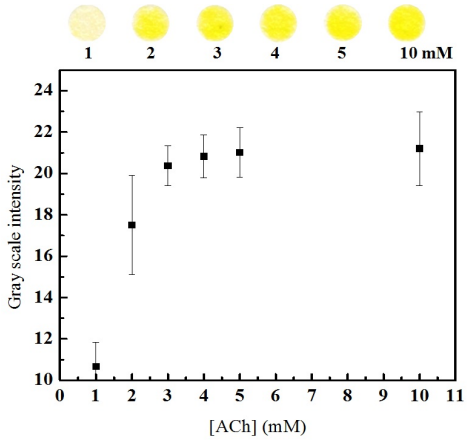


Figure 4-6 The color responses of concentration of acetylcholine

The results are shown in Figure 4-6. The color intensity increased with the increase of acetylcholine concentration and was almost constant at acetylcholine concentration of 4 mM or higher. Therefore, a concentration of 3 mM was selected for subsequent experiments.

4.5.4 Effect of methanol

In this work, a stock solution of OP pesticides was prepared in methanol. However, the activity of AChE was also inhibited by high concentration of organic solvent including methanol (Ingkaninan et al., 2000). For this reason, the suitable concentration of methanol in the range of 0% v/v to 10%v/v was examined and the results are shown in Figure 4-7.

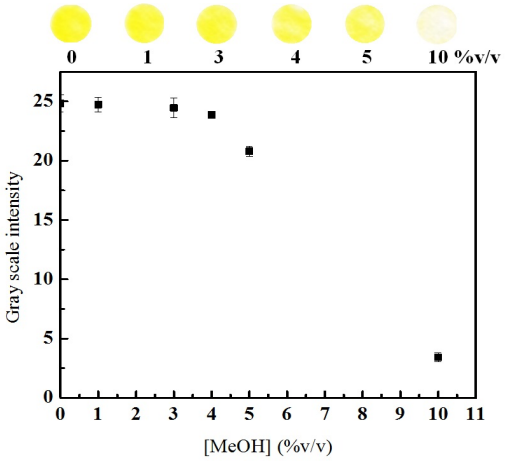


Figure 4-7 The effect of methanol concentration on color intensity.

The results indicated that at 5%v/v or higher concentration of methanol results in the decrease of color intensity because the activity of AChE was inhibited. Therefore methanol concentration of less than 4%v/v was used to dissolve pesticides to prevent false positive for the analysis.

4.5.5 Effect of reaction Time

To demonstrate the feasibility of nanoceria-coated paper based device for OP pesticides analysis as a simpler, faster method, the reaction time of substrate (acetylcholine) and catalysts (AChE and ChOX) to produce H_2O_2 was examined. The experiment was performed by incubating 1 U/mL AChE with 5 U/mL ChOX and 3 mM acetylcholine in a microcentrifuge tube for 15, 20 and 30 minutes. The mixtures were dropped onto devices. After dried, the color intensity was measured as the method described above.

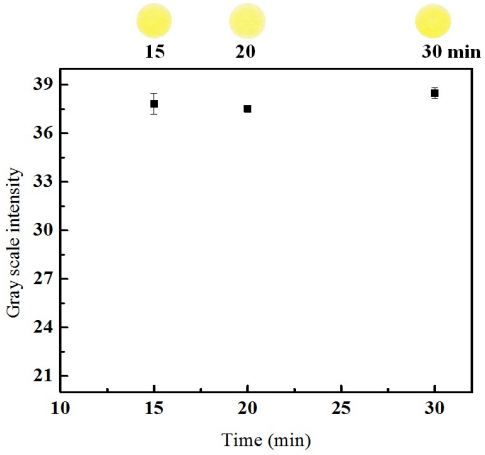
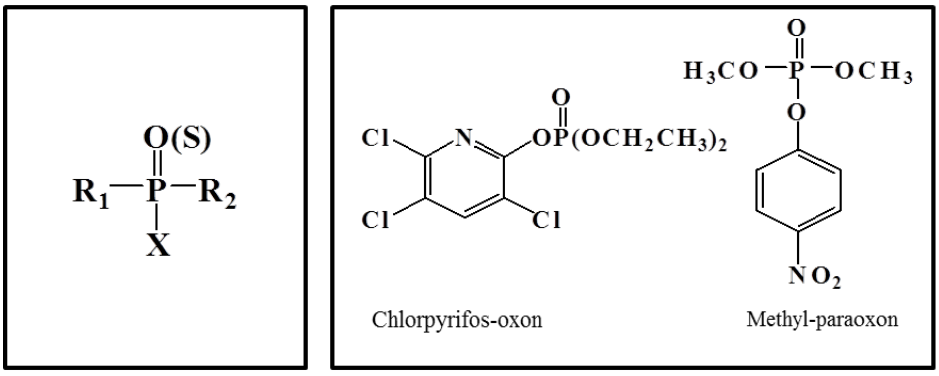


Figure 4-8 Study the effect of reaction times

The results are shown in Figure 4-8 where the color intensity was plotted versus incubation times. It was found that three incubation times provide similar color intensity. Here, for reasonable analysis, 15 minute was selected as reaction time throughout this work.

4.6 Pesticide analysis using a paper-based device

Organophosphorus pesticides play an import role in the improvement of agricultural products. Their universal application in agriculture gives rise to high-level residues of OP pesticides in food and the environment, and this is harmful to human health because of their high toxicities. Therefore, monitoring and determining the levels of these compounds in food are necessary. In this work, methyl-paraoxon and chlorpyrifos-oxon were selected as representative OP pesticides. Both CPO and MPO were examined using different initial amounts of AChE (0.1 U/mL, 0.5 U/mL and 1 U/mL).



(A) (B)

Figure 4-9 (A)General formula of OP compounds. (A) Structure of methyl-paraoxon and chlorpyrifos-oxon used as standard OPs in this assay

4.6.1 Metyl-paraoxon analysis

4.6.1.1 Linearity

Under the optimum condition, the concentration in the range of 0- 5 μg/mL of MPO was investigated to find the linear range. The results for MPO analysis using different concentrations of AChE were shown in Figure 4-10A and the results of linear range study were shown in Figure 4-10B.

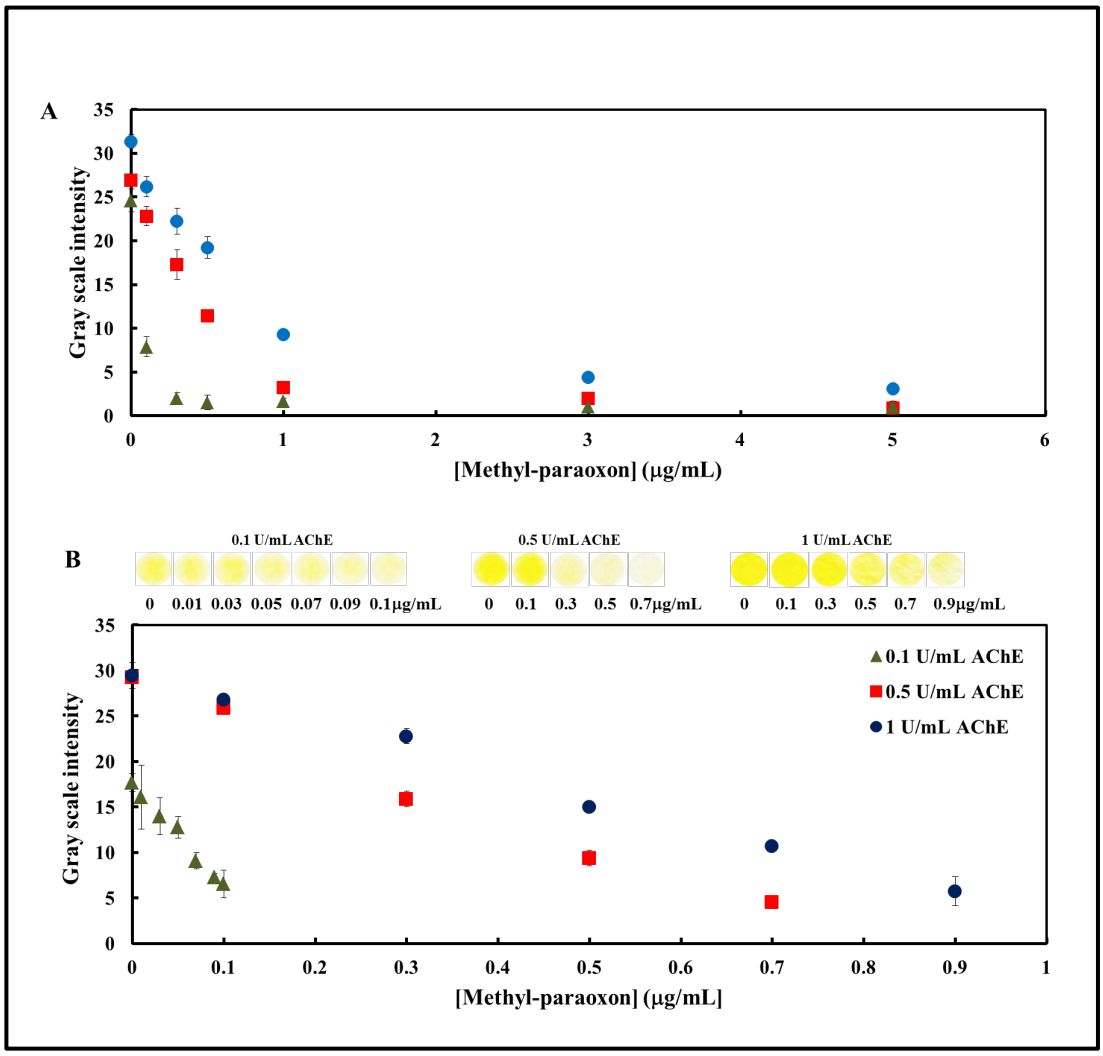


Figure4-10 Nanoceria-coated paper-based device for the detection of MPO with different initial concentration of AChE. (A) Dose-reponse curve for detection of MPO in the range of 0-5 μg/mL. (B) Linear calibration curves for MPO analysis.

The results in Figure 4.10A and B showed that changing initial amount of AChE shifted the dose respone curve. Using low initial concentration of AChE (0.1 U/mL), the signal intensity decreased rapidly as a function of pesticide concentrations and provided highest sensitivity (-111.67 μg/mL) comparing to other conditions. However, this high sensitivity covered a small working range (0-0.1μg/mL). At a higher initial amount of AChE (0.5 U/mL), a larger working range (0-0.7 μg/mL) was obtained. Using highest initial concentration of AChE (1U/mL) gave a largest working range (0-0.9 μg/mL) with a lowest sensitivity (-26.89 μg/mL) of three conditions investigated. The linear range, sensitivity and correlation coefficient (R^2) for MPO analysis are summarized in Table 4-1

Table 4-1 Linear equation, sensitivity, linear range and correlation coefficient (R^2) for MPO analysis using nanoceria-coated paper-based device with different initial concentrations of AChE.

OP pesticide	AChE U/mL	Linear equation	Sensitivity	Linear range μg/mL	R^2
MPO	0.1	$y = -111.67x + 17.493$	-111.67	0-0.1	0.988
	0.5	$y = -36.473x + 28.677$	-36.47	0-0.7	0.988
	1	$y = -26.895x + 29.624$	-26.89	0-0.9	0.998

4.6.1.2 Limit of detection and limit of quantitation

Limit of detection (LOD) and limit of quantitation (LOQ) were evaluated from MPO analysis at different starting amount of AChE used in the assay. The obtained LOD and LOQ are summarized in Table 4-2. A lower concentration of AChE gave a lower LOD. For MPO as low as 0.018 μg/mL detection limit was obtained which is similar to the value reported previously(Liang et al., 2013). The higher concentration of AChE provided high value as 0.050 μg/mL and 0.17 μg/mL of LOD and LOQ, respectively.

Table 4-2 LOD and LOQ of MPO analysis using nanoceria-coated paper based device with various amount of AChE.

OP pesticide	AChE U/mL	LOD μg/mL	LOQ μg/mL
MPO	0.1	0.018	0.061
	0.5	0.031	0.10
	1	0.050	0.17

4.6.1.3 Reproducibility

The reproducibility of the assay was examined at concentration of 0.05, 0.07 and 0.09 μg/mL of MPO, the results in terms of %RSD (relative standard deviation) were presented in table 4-3

Table 4-3 Relative standard deviation(%RSD) of MPO analysis

AChE (U/mL)	MPO (μg/mL)	%RSD (n=10)
0.1	0.05	14.50
	0.07	8.40
	0.09	13.64
0.5	0.1	2.08
	0.5	21.25
	0.7	21.29
1	0.1	1.32
	0.5	8.68
	0.7	6.40

The results showed that, at a high concentration of MPO gave a high %RSD because the low color intensity was obtained. Comparing between initial amount of AChE (0.1, 0.5 and 1 U/mL), a higher concentration of AChE(1 U/mL) provided a lower range of %RSD (1.32-8.68) because the higher amount of AChE produced a higher color intensity. These experiments provides the acceptable relative standard deviation (1.32-21.29) which is

comparable to previous paper based assay(Apilux, Isarankura-Na-Ayudhya, Prachayasittikul, & Tantimongcolwat, 2015).

4.6.2 Chlorpyrifos-oxon analysis

4.6.2.1 Linearity

To examine the linear range for CPO analysis using nanoceria-coated paper-based device, the analysis was performed in the same manner as MPO analysis where different concentrations of CPO were analyzed using three initial concentrations of AChE including 0.1, 0.5 and 1 U/mL. Results are shown in Figure 4-11. The color intensity decreased with the increased standard CPO concentrations (Figure 4-11A). The large working range of 0-120 ng/mL with R^2=0.982 and 0-80 ng/mL with R^2=0.984 was obtained using amount of AChE of 1U/mL and 0.5 U/mL, respectively (Figure 4-11B). The small working range of 0-60 ng/mL with R^2=0.997 was observed using amount of 0.1 U/mL AChE.

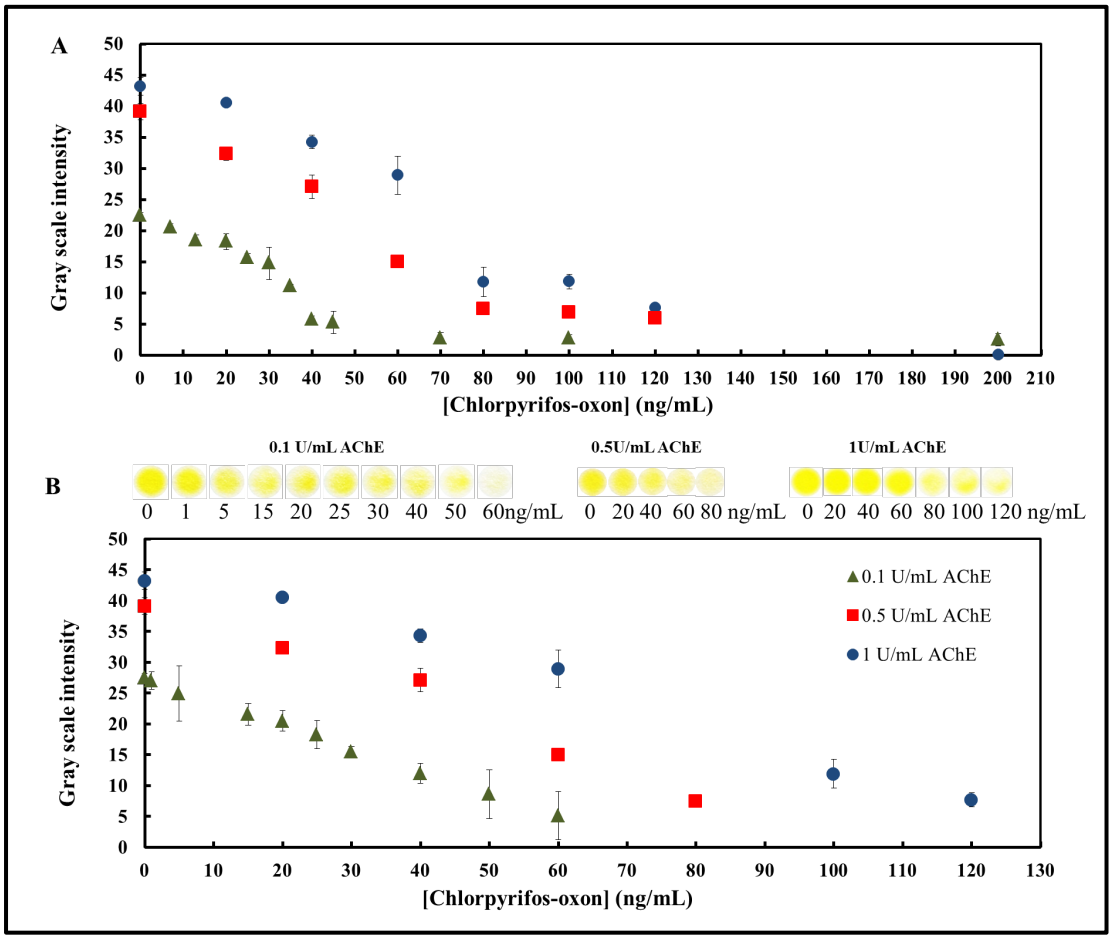


Figure 4-11 Nanoceria-coated paper-based device for the detection of CPO with different initial concentration of AChE. (A) Dose-response curve for detection of MPO in the range of 0-5 μg/mL. (B) Linear calibration curves for MPO analysis

The results of three linear ranges summarized in Table 4-4 illustrated increased amount of AChE gave a high working range. Unfortunately, sensitivity was dropped through the increased amount of AChE.

Table 4-4 Linear equation, sensitivity, linear range and R^2 (correlation coefficient) of CPO analysis using nanoceria-coated paper based device with various amount of AChE

OP pesticide	AChE U/mL	Linear equation	Sensitivity	Linear range ng/mL	R^2
CPO	0.1	$y = -0.3738x + 27.266$	-0.373	0-60	0.997
	0.5	$y = -0.4034x + 40.35$	-0.403	0-80	0.984
	1	$y = -0.3174x + 45.748$	-0.317	0-120	0.982

4.6.2.2 Limit of detection and Limit of quantitation

LOD and LOQ were evaluated in the same manner as described in MPO analysis. The results of LOD and LOQ for CPO analysis at different starting amounts of AChE are shown in Table 4-5. Higher initial concentrations of AChE gave higher LOD and LOQ. A lower concentration of AChE provided a lower value of LOD and as low as 5.24 ng/mL could be detected, which is comparable to that reported with the performance of a portable biosensor prototype assay. (Hildebrandt, Bragós, Lacorte, & Marty, 2008).

Table 4-5 LOD and LOQ of CPO analysis using nanoceria-coated paper based device with various amounts of AChE

OP pesticide	AChE U/mL	LOD ng/mL	LOQ ng/mL
CPO	0.1	5.24	17.48
	0.5	9.80	32.68
	1	13.62	45.40

4.6.2.3 Reproducibility

The reproducibility of the assay was examined with seven replicates of three concentration of CPO using three initial amount of AChE in the same manner as described in MPO analysis above. The results are shown in Table 4-6.

Table 4-6 Relative standard deviation(%RSD) of CPO analysis

AChE (U/mL)	CPO (μg/mL)	%RSD (n=7)
0.1	15	10.17
	20	12.03
	40	19.82
0.5	25	3.73
	60	10.88
	100	18.06
1	25	4.17
	50	8.56
	75	19.02

Results demonstrated that, increasing concentration of CPO was resulted to increase %RSD, because of at a high concentration of CPO results to reduce color intensity of device. In this work, the value of %RSD for CPO analysis was measured as low as 3.73 with a concentration of 0.5 U/mL AChE indicating that high reproducible analysis was obtained using the developed assay.

4.6.3 Determination of OP pesticides in real samples

4.6.3.1 Determination of MPO and CPO in dried seafood samples

To investigate the performance of the nanoceria-coated paper-based device to determine pesticide residues in real samples, the approach was applied to analyze OP pesticides in dried seafood product (dried green mussel). The homogenous of dried seafood was spiked with MPO (0 μg/mL, 0.2 μg/mL, and 0.25 μg/mL) and CPO (0 ng/mL and 50 ng/mL). The sample was prepared by the two methods including dispersive kit and non-dispersive kit for comparison

purpose. The quantity of pesticide residue in samples was quantified by both the developed and LC-MS methods. The results are shown in the Table 4-7

Table 4-7 Determination of spiked 0 µg/mL, 0.2 µg/mL and 0.25 µg/mL of MPO and 0 ng/mL, 50 ng/mL, and 60 ng/mL of CPO in dried green mussel by developed method and compared with LC-MS/MS.

Sample	Spiked MPO (µg/mL) CPO (ng/mL)		Nanoceria-coated paper based device Measured ±SD (n=3)				LC-MS/MS Measured ±SD (n=3)	
			Non-dispersive kit		Dispersive kit			
			Found	%Recovery	Found	%Recovery	Found	%Recovery
Dried green mussel	MPO/CPO	0	-	-	-	-	-	-
	MPO	0.2	-	-	0.19±0.05	95.06	0.23±0.01	113.33
	MPO	0.25	0.17±0.08	69.06	0.20±0.26	80.83	-	-
	CPO	50	34.16±5.02	68.32	39.48±3.56	78.96	13.58±0.10	27.16

The results showed that the analysis of both OP pesticides in spiked sample (dried green mussel) using nanoceria-coated paper-based device without use dispersive kit gave lower percent recovery of 68-69 since dried green mussel contains several matrixes including lipids and proteins which can interfere the analysis (Andreescu et al., 2014). Proteins can bind to particles forming a protein coating that may change the surface chemistry and potentially alter reactivity or induce nanoceria aggregation(Andreescu et al., 2014) . Using dispersive kit to remove matrixes can increase percent recovery of MPO analysis where as high as 80.73-95.06 were obtained. These results are comparable with the results of traditional method (LC-MS/MS). However, for CPO analysis, low percent recovery of 27.16 for LC-MS/MS was obtained. This might be the results of sample instability during shipping and storage prior to analysis as well as matrix effect from the samples.

4.6.3.2 Determination of MPO and CPO in vegetable samples

After successful determined MPO and CPO in dried seafood product using nanoceria-coated paper based device, this developed method was also applied to analysis OP pesticides in vegetables (cabagge) in the same manner as described in determination of OP pesticides residue in dried green mussel. The results are shown in Table 4-8.

Table 4-8 Determination of spiked 0μg/mL, 0.2 μg/mL and 0.25 μg/mL of MPO and 0 ng/mL, 50 ng/mL, and 60 ng/mL of CPO in cabbage by developed method and compared with LC-MS/MS

Sample	Spiked MPO (μg/mL) CPO (ng/mL)		Nanoceria-coated paper based device Measured ±SD (n=3)				LC-MS/MS Measured ±SD (n=3)	
			No dispersive kit		Dispersive kit			
			Found	%Recovery	Found	%Recovery	Found	%Recovery
	MPO/CPO	0	-	-	-	-	-	-
	MPO	0.2	-	-	0.19±0.02	94.80	0.22±0.00	110.00
Cabbage	MPO	0.25	0.21±0.09	83.87	-	-	-	-
	CPO	50	-	-	49.28±1.90	98.56	59.38±0.17	118.76
	CPO	60	54.73±22.17	91.21	-	-	-	-

The results in Table 8 demonstrated the spiked MPO and CPO monitored by the developed method and the traditional method (LC-MS/MS). Using the developed method without dispersive kit for sample preparation recovery of 83.87%for MPO and 91.21%for CPO was obtained. Using dispersive kit, the developed method gave recovery of 94.80%for MPO and 98.56% for CPO. These results confirm that, nanoceria-coated paper-based device can apply to analysis OP pesticides residues in vegetables without using dispersive kit. Comparing to traditional method, this developed method gave comparable results demonstrated that the nanoceria-coated paper-based device gave accurate results and can be applied in real world samples.

CHAPTER 5
CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 Conclusions

This work presents the new method for OP pesticides analysis utilizing enzyme inhibition assay and nanoceria-coated paper-based device as colorimetric probes. The detection assay composed of nanoceria, acetylcholinesterase (AChE) and choline oxidase (ChOX). In the presence of acetylcholine, the enzyme AChE and ChOx catalyze the formation of hydrogen peroxide (H_2O_2) which is detected colorimetrically by nanoceria-coated on the paper producing yellowish-orange color. In the presence of pesticide, the activity of AChE was inhibited and produced less H_2O_2 resulting in decrease of yellow color intensity. Color intensity was analyzed on the scanned picture using ImageJ software. The advantages of this developed method are simple, rapid and inexpensive analysis and required small amount of reagent and sample and the analysis can be performed without well-trained personnel.

The fabrication of device was performed using one-step polymer screen-printing method to create hydrophobic barrier and a well shape with diameter of 5 mm serving as a detection zone on the paper-based device.

The performance of the patterned paper-based device was first studied for H_2O_2 analysis. Suitable concentration of nanoceria coated onto paper-based device was carried out for analysis of H_2O_2. Under the optimum conditions, the limit of detection was 0.5 mM of H_2O_2 analysis. This result confirms that nanoceria-coated paper device can be applied for determination of OP pesticides.

Optimum conditions for OP pesticides analysis was then studied and the results showed that optimal concentration of AChE were 0.1, 0.5 and 1 U/mL. Optimal ChOX concentration was 0.5 U/mL and ACh concentration was 3mM. Solvent of pesticides was 4% v/v of methanol. The analysis of OP pesticides was performed using three initial concentrations of AChE (0.1, 0.5 and 1 U/mL). The results showed that the color intensity decreased as the concentration of OP pesticides increased and the analytical figures of merit for the analysis of OP pesticide standards depended on the starting concentration of AChE used in the assay.

The method was then used to evaluate MPO and CPO in spiked vegetable (cabbage) and dried seafood (dried green mussel). The results of developed method compares favorably with results of traditional method (LC-MS/MS). Therefore, it could be concluded that nanoceria-coated paper-based device associates with enzyme inhibition assay can be used to determine OP pesticides in real samples both in vegetables and dried seafood products in the level of ng/mL. Comparing to traditional method, this developed method provides rapid, sensitive, simple and inexpensive analysis as the reaction was performed on paper, required only small amount of reagent and sample and completed within 60 minutes without expensive instruments as a detection part. The assay is promising to use as an easy test kit for screening and determining OP pesticides in the future.

5.2 Future perspective

The future study will be focus on the development of paper-based microfluidic device based on colorimetric assay without an enzyme assay as a test kits for screening and determining of several types of pesticides/toxic substances residue in environments and verity of food products and also for measuring biological samples. The future device/test kit will be inexpensive, low analysis time, and can be applied for on-site analysis.

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ผลผลิต (Output)

บทความวิจัย

- **Sameenoi Y**, Na Nongkai P, Nouanthavong S, Henry CS, Nacapricha D. “One-step polymer screen-printing for microfluidic paper-based analytical device (μPAD) fabrication”, *Analyst*, 2014, 139, 6580-6588. (impact factor: 4.107)
- Nouanthavong S, **Sameenoi Y**. “Paper-based device for pesticide analysis”, Pure and Applied Chemistry International Conference 2015 (PACCON 2015), 2015, 24-27, Bangkok, Thailand
- Nouanthavong S, Nacapricha D, Henry C, **Sameenoi Y**. “Pesticide analysis using nanoceria-coated paper-based devices as a detection platform”, manuscript in preparation

รายงานสรุปการเงิน ประจำปีงบประมาณ 2557

รหัสโครงการ 2557A10862016

โครงการส่งเสริมการวิจัยในอุดมศึกษาและพัฒนามหาวิทยาลัยแห่งชาติ

สำนักงานคณะกรรมการการอุดมศึกษา

มหาวิทยาลัยบูรพา

การพัฒนาอุปกรณ์ตรวจวัดแบบกระดาษเพื่อวิเคราะห์ปริมาณยาฆ่าแมลงในตัวอย่างอาหารทะเลแห้งโดยใช้อนุภาคนาโนซีเรียเป็นโพรบวัดสี

Development of Paper-Based Microfluidic Device for Pesticide Analysis in Dried Seafood using Nanoceria as Colorimetric Probes

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ชื่อหัวหน้าโครงการผู้รับทุน/ ผู้วิจัย อ.ดร.ยุภาพร สมีน้อย
รายงานในช่วงตั้งแต่วันที่ 1 ตุลาคม 2556 ถึงวันที่ 30 กันยายน 2558
ระยะเวลาดำเนินการ 2 ปี 0 เดือน ตั้งแต่วันที่ 1 ตุลาคม 2556 ถึงวันที่ 30 กันยายน 2558

หมวด	รายจ่าย		คงเหลือ
	งบประมาณทั้งโครงการ	ค่าใช้จ่ายงวดปัจจุบัน	(หรือเกิน)
1. ค่าตอบแทน	70,000	70,000	0
2. ค่าจ้าง	30,000	30,000	0
3. ค่าวัสดุ	65,000	14,646.4	50,353.6
4. ค่าใช้สอย	150,000	220,453.8	-70,453.8
5. ค่าใช้จ่ายอื่นๆ			
เงินทุนอุดหนุนการวิจัยของมหาวิทยาลัย			
เป็นค่าสาธารณูปโภค 10%	35,000	35,000	0
รวม	350,000	370100.2	เกิน 20,100.2

จำนวนเงินที่ได้รับ

จำนวนเงินที่ได้รับ		
งวดที่ 1		157,500 บาท เมื่อ วันที่ 26 มีนาคม 2557
งวดที่ 2		126,000 บาท เมื่อ วันที่ 18 ตุลาคม 2557
	รวม	283,500 บาท

....

นางสาวยุภาพร สมีน้อย
หัวหน้าโครงการวิจัยผู้รับทุน
วันที่ .....

เจ้าหน้าที่การเงินโครงการ
วันที่ .....

ประวัตินักวิจัย

ชื่อ - นามสกุล (ภาษาไทย) นางสาวยุภาพร สมีน้อย

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ตำแหน่งปัจจุบัน อาจารย์ประจำ

หน่วยงานและสถานที่อยู่ที่ติดต่อได้สะดวก ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยบูรพา 169 ถ. ลงหาดบางแสน ต. แสนสุข อ. เมืองฯ จ. ชลบุรี 20131

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ประวัติการศึกษา

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2548	วท.บ.	เคมี	ม.ศิลปากร
2551	วท.ม.	เคมีวิเคราะห์และเคมีอินทรีย์ประยุกต์	ม.มหิดล
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งานวิจัยที่ทำเสร็จแล้ว : ชื่อผลงานวิจัย ปีที่พิมพ์ การเผยแพร่ และแหล่งทุน

ผลงานวิจัยที่ตีพิมพ์ในห้าปีที่ผ่านมา

1. Sameenoi, Y., Na Nongkai, P., Nouanthavong, S., Henry, C.S., Nacapricha, D. “One-Step Polymer Screen-Printing for Microfluidic Paper-Based Analytical Device (**μ**PAD) Fabrication”, *Analyst*, Manuscript under revision. (แหล่งทุน:ทุนสนับสนุนการวิจัยสำหรับ บัณฑิต พสวท แรกบรรจุ 2556, ทุนอุดหนุนการวิจัยงบประมาณโครงการวิจัยภายใต้โครงการส่งเสริมการวิจัยในอุดมศึกษา สกอ. (HERP) ประจำปีงบประมาณ 2557)

2. Koehler, K.A., Shapiro, J., **Sameenoi, Y.,** Henry, C.S., and Volckens, J. “Laboratory Evaluation of a Microfluidic Electrochemical Sensor for Aerosol Oxidative Load”, *Aerosol Sci. Technol.* 2014, 48, 489 (แหล่งทุน National Institute of Environmental Health Science, NIH grant ES019264)

3. Dungchai, W., **Sameenoi, Y.,** Chailapakul, O., Volckens, J., and Henry, C.S., “Determination of aerosol oxidative activity using silver nanoparticle aggregation on paper-based analytical devices”, *Analyst*, 2013, *138*, 6766-6773. (แหล่งทุน National Institute of Environmental Health Science, NIH grant ES019264)

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5. Sameenoi, Y.; Koehler, K.; Shapiro, J.; Boonsong, K.; Sun, Y.; Collett, J.; Volckens, J.; Henry, C. S., "Microfluidic Electrochemical Sensor for On-Line Monitoring of Aerosol Oxidative Activity", *J. Am. Chem. Soc.* 2012, *134*, 10562 (แหล่งทุน National Institute of Environmental Health Science, NIH grant ES019264)

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