อุปกรณ์แบบกระดาษสำหรับการวิเคราะห์ฟอร์มัลดีไฮด์

PAPER-BASED DEVICES FOR FORMALDEHYDE ANALYSIS

NATCHANON TAPRAB

Burapha University

2018

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ณัฐชนน ตาปราบ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเคมีศึกษา
คณะวิทยาศาสตร์ มหาวิทยาลัยบูรพา
2561

PAPER-BASED DEVICES FOR FORMALDEHYDE ANALYSIS

NATCHANON TAPRAB

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR MASTER OF SCIENCE
IN CHEMICAL EDUCATION
FACULTY OF SCIENCE
BURAPHA UNIVERSITY
2018

The Thesis of Natchanon Taprab has been approved by the examining committee to be partial fulfillment of the requirements for the Master of Science in Chemical Education of Burapha University

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NATCHANON TAPRAB : PAPER-BASED DEVICES FOR FORMALDEHYDE ANALYSIS. ADVISORY COMMITTEE: YUPAPORN SAMEENOI, PH.D. 2018.

A rapid and simple paper-based analytical device (PAD) for quantitative analysis of formaldehyde in food samples has been developed. The analysis was based on sulfite assay where the formaldehyde (FA) was reacted with excess amount of sulfite to generate sodium hydroxide and the product consequently quantified using acid-based titration on the PAD. The acid-based titration PAD consisted of a central sample zone connected to ten reaction and detection zones. Polyethylene glycol and phenolphthalein (indicator) were deposited to the detection zones, and different amounts of titrant, potassium hydrogen phthalate (KHP), were added to the reaction zones. The formaldehyde concentration can be quantified from the number of color-changed detection zone(s) which are correlated to known amount of KHP on reaction zone. The areas of each zone and volumes of the reagents added to the corresponding zones were optimized. The PAD gave range of FA analysis from 100 to 1000 ppm and the LOD was 100 ppm. The PADs were stable for up to a month under dark and cold conditions. The analysis of formaldehyde in food samples was demonstrated using the PAD can be completed within 5 minutes, and the results agreed well with those obtained from the classical titration.

ACKNOWLEDGEMENTS

I would first like to express my sincere gratitude and deep appreciation to my advisor Asst. Prof. Dr.Yupaporn Sameenoi for her patience, enthusiasm, motivated thought, and valuable supports and advices through my study and research. She consistently allowed this research to be my own work, but steered me in the right the direction whenever I needed it. Also great appreciation is warmly brought to all committee members for thesis defense, including Dr.Sirirat Choosakoonkriang and Dr.Nawasit Rakbamrung, and Dr.Anan Athipornchai and Dr.Piyaporn Na Nongkhai for proposal defense for their encouragement, insightful comments.

I would also like to acknowledge the Project for The Promotion of Science and Mathematics Talent Teacher (PSMT) scholarships from the Institute for the Promotion of Teaching Science and Technology (IPST). I'm deeply grateful to Department of Chemistry, Faculty of Science, Burapha University where I conducted my wonderful experiment and all staff members and instructors of the department for your kindness and encouragement.

Last but not the least, I would like to thank my wonderful family for grateful support and encouragement which push and promote to achieve my master degree. My colleagues for nice thought and encouragement.

NATCHANON TAPRAB

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

CHAPTER 1
INTRODUCTION

1.1. Statement and significance of the problems

Formaldehyde (FA) (CH_2O) is one of volatile organic compounds (VOCs) containing aldehyde group in form of colorless gas at room temperature which is easily soluble in water and alcohol. FA in aqueous solution is known as formalin, which contains FA up to 55% by weight (Agency for Toxic Substance and Disease Registry, 2004; Changsap, 2015).

FA has been widely used in many areas. In medical, FA is used for cadaver and anatomical specimens preservations (Brenner, 2014) and medical instruments sterilisation (Environmental Health & Safety, 2008; Lubbe & Henton, 1997). Futhermore, it has been used in industry including textiles, wood, furniture, cosmetic, paper, sanitary, and carpet, etc (Geddie, 2013), and also in agriculture and photography (Environmental Protection Agency, 2000).

The department of Health and Human Service (HHS) considered FA as a carcinogen since 2011 (Program, 2016). When in contact to the body, FA can cause body irritation, risk of asthma neurological effect, and allergy (Agency for Toxic Substance and Disease Registry, 2015). Therefore, FA is classified as Type 2 hazardous substance according to Hazardous substance act in 1992 (Department of Industrial Works, 2010). Despite these toxicities, FA has been found to contaminate in several types of food, especially seafood, at significant-level. In 2013, Food Safety News of the U.S. declared that about 25% fish samples imported from China and Vietnam were contaminated with FA (Andrews, 2013). Department of Medical Sciences disclosed that FA was contaminated in many plastic food containers from roadside markets (Auwanichaya, 2013). Notification of the Ministry of Public Health (No. 151) in 1993 regulated that the use of FA is prohibited in food (Ministry of Public Health, 1993). The US Environmental Protection Agency (US EPA) specified that the oral reference dose (RfD) of FA is 0.2 mg/kg/day (US Environmental Protection Agency, 1989).

Several methods have been reported for FA determination including gas chromatography (GC) (Dojahn, Wentworth, & Stearns, 2001), high performance liquid

chromatography (HPLC) (Jian-Rong Li, Jun-Li Zhu, & Li-Fang Ye, 2007) as well as enzymatic method using FA dehydrogenase with nicotinamide adenine dinucleotide (Ho & Richards, 1990). Spectroscopic methods using chromotropic acid (Georghiou & Ho, 1989), actylacetone (Nash, 1953), and sulfite with dye (Carrico, 2002) have been proposed for simple colorimetric detection. In addition, titration method using sulfuric acid as a titrant and thymolphthalein as an indicator (American Chemical Society, 2017; Walker, 1964) have been widely used set as a standard method for FA analysis in many organization (Ministry of Industry, 2014; TAPPI, 2011). These methods offered effective analysis of FA, however, they suffered from complicated analysis steps, expensive detection system, large amount of reagent consumption and long analysis time. To be more rapid and simple, several FA test kits have been developed and are commercially available in Thailand (Appealing Products Inc. Company, 2018; Institution of Medical Sciences; Mahidol University, 2013; Prince of Songkla University, 2012) to allow consumer to easily test FA contamination. However, these kits can allow for only semi-quantitative analysis, have high limit of detection, require toxic chemicals for the assay and need large amount of reagents and samples and most importantly, they suffered from color background of the samples for colorimetric measurement.

Paper-based devices have been developed for a decade as an alternative technology for chemical analysis in various fields such as medical diagnosis and environmental monitoring (de Oliveira, Camargo, Pesquero, & Faria, 2017). Moreover, many paper-based devices are also available for school use, for example, litmus papers, turmeric paper, and universal indicator paper. The devices allow for low-cost analysis with simple operation, low regent and sample consumption.

This research proposed to develop a simple, rapid, low-reagent consumption, inexpensive and green colorimetric paper-based method for FA analysis. The analysis is based on titration method using the reaction shown in Figure 1-1. In the presence of FA, sulfite is consumed and NaOH is produced. The NaOH is quantified by acid-base titration with a primary standard, potassium hydrogen phthalate (KHP). Amount of hydroxide is related with amount of FA in the samples.
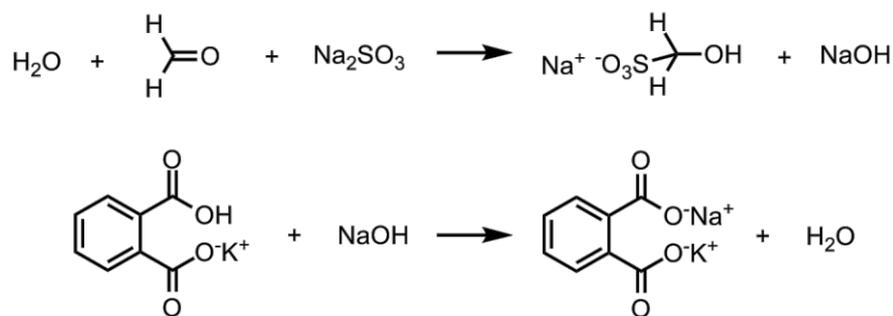
Figure 1-1   Reaction associated with the titration method for FA analysis. FA react with sodium sulfite giving NaOH (Top) and NaOH was undergone acid-base titration with KHP (Bottom).

Here, all the acid-base titration steps were performed in a paper-based titration card containing a central sample zone connected to ten reaction and detection zones. Various amounts of KHP were deposited on the reaction zones and constant amount of phenolphthalein indicator deposited on the detection zones. The product solution containing NaOH, from the reaction of FA and excess amount of sulfite, was applied to the sample zone and the reagent spreading to reaction zone where various amount KHP was deposited. NaOH would be neutralized by KHP before the remaining NaOH reach the detection zone. When NaOH amount is higher than KHP, unneutralized NaOH would reach the detection zone, then caused color change of pH indicator in. On the other hand, if amount of NaOH were less than KHP in the reaction zone, the KHP would reach the detection zone and cause different color change of indicator. The numbers of detection zones at which giving color change of indicator would allow for quantitative measurement of FA without using any additional instrument or software. To the best of our knowledge, this work is the first time that paper-based titration method is employed for quantitative analysis of FA. Moreover, the developed method would allow for simple, fast, low-reagent consumption, low cost and green analytical method.

1.2 Objectives

To develop a paper-based titration method for the simple, rapid, instrument-free and inexpensive quantitative analysis of FA in food samples using sulfite assay.

1.3 Contribution to knowledge

The proposed paper-based titration method of FA analysis using sulfite assay can be used to determine FA content in food samples with rapid, simple and low-cost analysis with low-reagent consumption.

1.4 Scope of Study

1. Study optimal conditions of FA analysis including types of indicator, concentration of indicator, size of detection zone, distance between detection zone and sample zone, and volume of reagent.

2. Study analytical features including detection range, limit of detection, reproducibility, stability and tolerance limits of interferences for FA analysis using the optimal conditions.

3. Study the method validation by comparing FA content in the food samples obtained from the proposed method and those obtained from the standard method.

4. Study the optimal conditions of sample preparation and device platform for on-site analysis.

CHAPTER 2
LITERATURE REVIEWS

2.1 Formaldehyde: General information, application and toxicity

Formaldehyde (FA) is a gas-phase compound classified as aldehyde compounds. Thus, it is a reducing agent. FA is normally prepared as FA solution as known as formalin. Nowadays, FA is widely used in many areas, for instance, industrial, medical, cosmetic, agricultural, and etc. According to its usefulness, FA could be contaminated in many everyday-life products. Other than its benefits, many risks and toxicology are discussed.

FA is a pungent, flammable, and colorless gas at room temperature. As its instability, FA is prepared in form of methylene glycol solution called formalin, which could be prepared up to 55% by weight. Methanol is mostly used up to 15% to stabilize the solution. In addition, FA would be transformed to be formic acid at high temperature, while polymer-paraformaldehyde could be formed at low temperature (Agency for Toxic Substances and Disease Registry (ATSDR), 2008; Changsap, 2015).
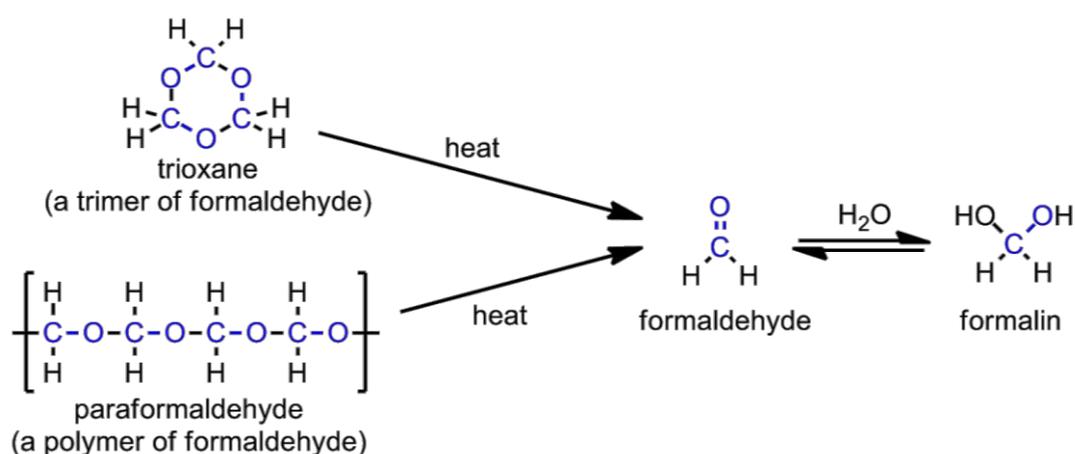
Figure 2-1.   Transformation of formaldehyde (Wade, 2006c)

2.1.1 Physical and Chemical Properties

Molecular formula   : CH_2O

Structural formula   :

$$\underset{H \qquad H}{\overset{\displaystyle \overset{O}{\parallel}}{C}}$$

IUPAC name   :   Methanal

Other name   :   Formalin; Methylene oxide;

                                            Oxymethane; Formic aldehyde;

                                            Methyl aldehyde.

Molecular weight   :   30.03 g/mol

Melting point   :   -92 °C

Boiling point   :   -21 °C

Density (at -20 °C)   :   0.815 g/mL

2.1.2 Definition

Department of health and ageing defined "free FA" as all non-hydrated and hydrated forms of FA in aqueous solution including methylene glycol (Department of Health and Ageing, 2012), while, "total FA" refers to the sum of both free FA and bound FA (Rosen & McFarland, 1983). However, Meson from Central Science Laboratory of the United Kingdom Government stated that only free FA should be determined in order to get accurately result (Mason, Sykes, Panton, & Rippon, 2004; Yeh, Lin, Chen, & Wen, 2013).

2.1.3 Sources

FA could be produced by the oxidation process of methanol or methane. Therefore, it is considered to be produced by both natural species such as human activity, and manufacture process. Photochemical oxidation of organic

compounds is reported as secondary source of FA released (Liteplo, Beauchamp, Meek, & Chénier, 2002).

Directly and indirectly biomass combustion process is considered to be most of the FA entering the environment. As a metabolic intermediate, small amount of FA are found in most living things e.g. plants, animals, and human (Agency for Toxic Substances and Disease Registry (ATSDR), 2008). In the United States, there is large amount production of FA to use in many industries (Liteplo et al., 2002). FA is also found in the air both outdoor e.g. vehicle or industrial exhausts and indoor e.g. consumer product. Water is also believed to be contaminated by FA from irradiation of humic substances by sunlight (Liteplo et al., 2002) and the uses and disposal FA-based products (Agency for Toxic Substances and Disease Registry (ATSDR), 2008).

In the nature, methane is produced by the microbial degradation, conversely, methane is consumed by another microorganism. Methanotroph is a group of microorganism found to be able to transform methane to FA by their oxidation process (Hütsch, 2001).

Methane oxidation in methanotrophs
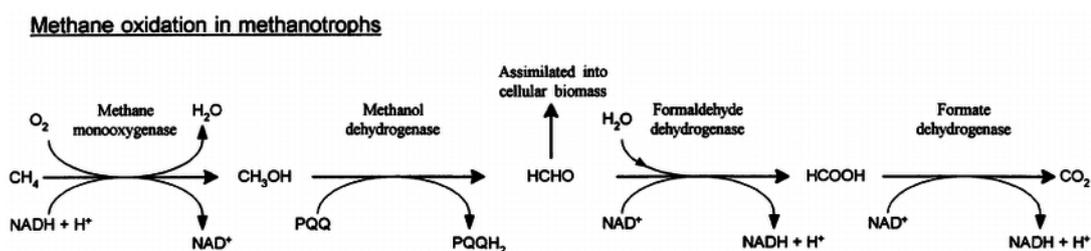
Figure 2-2   Pathway of methane oxidation in methanotrophs to produce formadehyde (Hütsch, 2001)

In addition, incomplete combustion of both on-road and aircraft vehicles, burning including cooking and cigarette, off-gassing of the polymeric produce e.g. latex paint, resin, and textile product, and etc. are considered as anthropogenic source (Liteplo et al., 2002).

2.1.4 Uses of Formaldehyde

In industrial area, FA is used as a substrate or intermediate for many productions (Pollution Control Department, 1998) as following;

- Resin and plastic manufacturing e.g. Urea-FA, Phenol-FA, Melamine-FA, etc.

- Synthesis of urotropire, propagyl alcohol, drug, explosive agent, and dye e.g. indigo, rose mary, or acrylic dyes.

- Bleaching, printing, and dying

- Waterproof paper manufacturing

- Anti-oxidation in metal

- Textile industry; anti-wrinkle, texture adjusting, etc.

- Material fumigation in leather or fur for insect protection

In medical area, FA is used in direct way and indirect way as intermediate or substrate as following;

- Cadaver and anatomical specimens preservations

- Cleaning and sterilization medical instrument

- Synthesis drug and vaccine

In agricultural area, FA is used for fertilizer production as urea-FA (Covey, Koch, Larsen, & Haglund, 1984; Ikeda, Suzuki, Kawahara, Noshiro, & Takahashi, 2014; Yamamoto, Pereira, Mattoso, Matsunaka, & Ribeiro, 2016), soil sterilization (Stroetmann, Kämpfer, & Dott, 1994), and application of FA to soil is reported to alleviate an apple replant disease (Covey et al., 1984).

In many countries, FA in a specific limit of concentration under the law, is also applied to cosmetic product as an antimicrobial and preservative agent (Liteplo et al., 2002).

2.1.5 Hazards of Formaldehyde

FA is highly reactive species and cause irritating to tissue. Human and animal would occasionally receive FA from the products or workplace. There are many reports claim about how could FA affect health and estimate of exposure level by inhalation and ingestion as shown in Table 2-1 and 2-2. In

addition, FA was known to be human carcinogen, which also first listed in the Second Annual Report on Carcinogens 1981 (National Toxicology Program, 2016). Nasal tumor rate in rats can be increased by inhalation exposure of FA (Monticello et al., 1996). The rats were exposed with drinking-water contaminating FA, and then were observed that the increase concentration of FA the increase in tumors of the hematopoietic system (Soffritti, Maltoni, Maffei, & Biagi, 1989).

Table 2-1.　　Health Effects of FA Ingestion (Agency for Toxic Substances and Disease Registry (ATSDR), 2008)

Dose (mg/kg/day)	Effect in Animals
251 to 300	- Erosions and ulcers, - Histopathological changes
201 to 250	No study
151 to 200	- Testicular effect (altered sperm morphology)
101 to 150	- Decreased food and water intake - Decreased body weight - Gastrointestinal effects - Liver effects - Kidney effects
50 to 100	- Decreased food intake and body weight - Gastrointestinal effects - Liver effects - Kidney effects
0 to 49	No effect
0.3 mg/kg/day	Intermediate MRL
0.2 mg/kg/day	Chronic MRL

*Exposure levels posing minimal risk to humans (MRLs)

Table 2-2   Health Effects of FA Inhalation (Agency for Toxic Substances and
Disease Registry (ATSDR), 2008)

Concentration in Air (ppm)	Effects in Humans	Effect in Animals
More than 50	No study	- Pulmonary edema - Bloody nasal discharge
11 - 50	No study	- Effect as lower concentration may also present - Neurological effects as listlessness, hunched appearance, uncoordinated movement, ataxia
6.0 - 10.9	- Nasal, eye, throat, and skin irritation - Headache and nausea - Discomfort in breathing	- Nasal and eye irritation - Nasal ulceration - Nasal tumors
2.0 - 5.9	- Nasal and eye irritation - Eczema (skin inflammatory) - Pulmonary function change	- Liver effect - Testicular effect - Effects as lower concentration level may also present
0.6 - 1.9	- Nasal and eye irritation - Eczema (skin inflammatory) - Pulmonary function change	- Change in pulmonary functions - Effects as lower concentration level may also present
0.1 - 0.5	- Nasal and eye irritation - Neurological effects - Increased risk of asthma and/or allergies	- Decrease breathing rate and/or increased airway resistance (change in pulmonary functions) - Increased allergic responds - Neurological effects
0.04		Acute MRL

Table 2-2   Health Effects of FA Inhalation (Agency for Toxic Substances and Disease Registry (ATSDR), 2008) (cont.)

Concentration in Air (ppm)	Effects in Humans	Effect in Animals
0.03		Intermediate MRL
0.02		Chronic MRL

*Exposure levels posing minimal risk to humans (MRLs)

2.1.6 Information and Regulations about FA Uses

2.1.6.1 United States Food and Drug Administration (FDA)

According to the title 21 of food and drug administration part 175 of indirect food additives: adhesives and components of coatings section 105 adhesives (21CFR 175.105 FDA 1977), FA is allowed for adhesives and components of coatings (United States Food & Drug Administration, 2017).

2.1.6.2 United States Environmental Protection Agency (EPA)

According to the Clean Water Act (CWA) on title of Designation of hazardous substances, FA is registered as a hazardous substance. This Act is under the implementation of pollution control programs by United States Environmental Protection Agency (EPA) (United States Environmental Protection Agency, 2013). FA is also registered on The Clean Air Act Amendments of 1990 List of Hazardous Air Pollutants (United States Environmental Protection Agency, 1990).

2.1.6.3 Thai Organization

a) Ministry of Public Health

According to the Notification of the Ministry of Public Health (No.151) 1993 on topic of Prescribed prohibited substances used in food, FA, FA solution, and ParaFA are prohibited to use in food (Ministry of Public Health, 1993).

b) Ministry of Industry

According to the Notification of the Ministry of Industry Health 2015 on topic of Hazardous substances list (No.2), FA is listed on no. 296 (Ministry of Industry, 2015).

2.2 Methods of Formaldehyde Analysis

2.2.1 Titration method

The acid-base titration which using sodium sulfite (Na_2SO_3), sulfuric acid (H_2SO_4), and thymolphthalein as an indicator have been proposed for FA analysis (American Chemical Society, 2017). FA react with sodium sulfite results NaOH as a product (Andrews, Reinhardt, & Harris, 1983; Chin, 1976), and then thymolphthalein is added and following by titration with standardized sulfuric acid until reaching the end point. Moreover, several organization have been using the techniques as the standard method for FA analysis (Ministry of Industry, 2014; TAPPI, 2011).

$$HCHO\ (aq)\ +\ H_2O\ (l)\ +\ Na_2SO_3\ (aq)\ \rightarrow\ HOCH_2SO_3Na\ (aq)\ +\ NaOH\ (aq) \qquad (a)$$

$$H_2SO_4\ (aq)\ +\ 2NaOH\ (aq)\ \rightarrow\ Na_2SO_4\ (aq)\ +\ 2H_2O\ (l) \qquad\qquad\qquad (b)$$
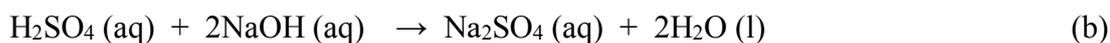
Figure 2-3   Reaction between FA and sodium sulfite (a) (Andrews et al., 1983).
                Reaction between NaOH and sulfuric acid (b)

2.2.2 Chromatography

According to (Yeh et al., 2013), FA in squid and its products samples was derivatized from 2,4-dinitrophynylhydrazine (DNPH) resulting FA-DNPH as shown in Figure 2-4 and analyzed using gas chromatography-mass spectrometry. The supernatant of prepared sample was mixed with DNPH and left in dark for 6 hours. The solution was mixed with CH_2Cl_2, and the bottom layer was taken for analysis.
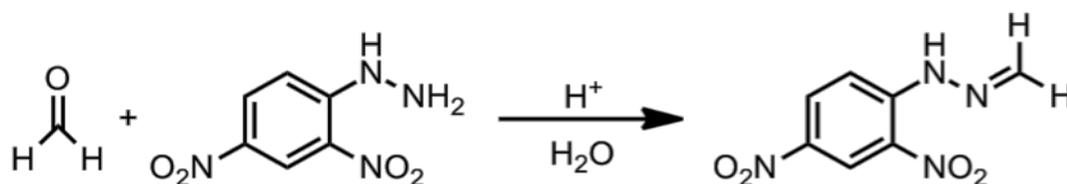
Figure 2-4   Reaction between FA and 2,4-dinitrophynylhydrazine results FA-DNPH.

2.2.3 Spectrophotometry

2.2.3.1 *Phenylhydrazine colorimetric method* (Tanenbaum & Bricker, 1951)

In this method, FA reacted with phenylhydrazine hydrochloride, NaOH, and potassium hexacyanoferrate as shown in figure 2-5, which give off radish pink rapidly. However, aliphatic aldehyde species are considered as interferences (Rosen & McFarland, 1983). In addition, the FA test kit of department of medical sciences is based on this method (Chantara, 2011).
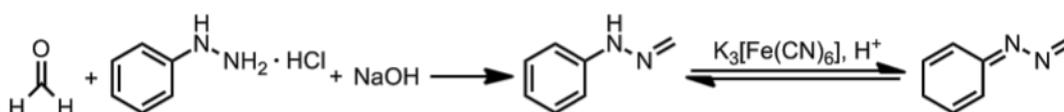
Figure 2-5   Reaction of FA and phenylhydrazine hydrochloride, NaOH, and potassium hexacyanoferrate.

2.2.3.2 *Chromotropic acid*

Chromotropic acid or 4,5-dihydroxy-2,7-napthalenedisulphonic acid is a chromogen-forming reagent which is specifically formed with FA. This method is recommended by National Institute for Occupational Safety and Health (NIOSH) (Georghiou & Ho, 1989). Prepared sample is mixed with solution of chromotropic acid followed by sulfuric acid. The purple color indicates presence of FA.

Chromotropic acid   Formaldehyde
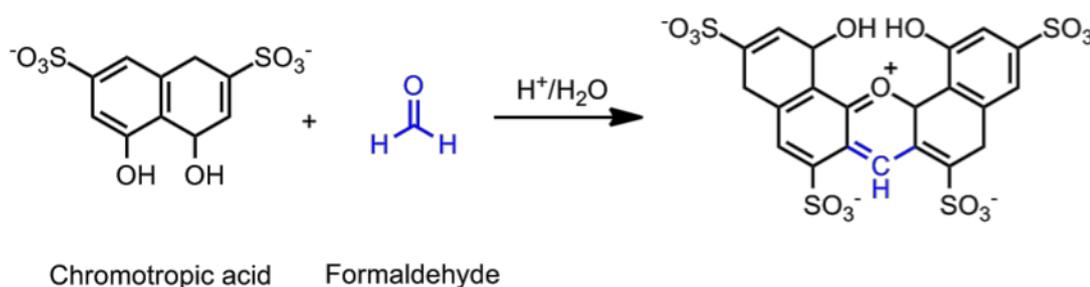
Figure 2-6   Reaction of FA and chromotropic acid in presence of acid (Jendral, Monakhova, & Lachenmeier, 2011)

2.3 Microfluidic paper-based analytical devices (µPADs)

Microfluidic technology emphasizes on management of small amount of fluids to flow in microchannel (Lisowski & Zarzycki, 2013). This technology has been developed as an alternative way for chemical analysis. The main purpose of the development is to complete the analysis on one device, use small amount of reagent, and to be portable, and cheap. Many materials are chosen for this development which filter paper is the typical one.

For a decade, microfluidic paper-based analytical devices or as known as µPADs have been intensively researched and developed. µPADs are considered as a novel and alternative technology used in many areas such as medicine, clinical chemistry, environment monitoring (Sameenoi, Nongkai, Nouanthavong, Henry, & Nacapricha, 2014). The first microfluidic paper-based analytical device was developed in 1950 for semi-quantitative glucose assay in urine. Thus, µPAD allowed for the analysis with simple to use, cheap, and portable which could be served as a device for point-of-care (POC), since it fulfills the criteria stated by World Health Organization (WHO) as "ASSURED" (Nilghaz, Guan, Tan, & Shen, 2016).

µPADs consist of hydrophilic (allowed solution to flow) and hydrophobic (forced the solution to flow to given direction) areas as shown in Figure 2-7. Several methods of µPADs fabrication for creating hydrophobic barrier have been researched and developed such as wax printing, polydimethylsiloxane (PDMS) printing, alkyl ketene dimer (AKD) printing, inkjet etching, photolithography, and cutting (Martinez, Phillips, Whitesides, & Carrilho, 2010; Yetisen, Akram, & Lowe, 2013). Various patterns of fabrication have been proposed such as single and multi analyte analysis (Kudo, Yamada, Watanabe, Suzuki, & Citterio, 2017; Li, Ballerini, & Shen, 2012), two-dimensional and three-dimensional µPADs (de Oliveira et al., 2017). Moreover, many detection methods have been used such as colorimetric, electrochemical, and chemiluminescence (Ge et al., 2012; Hong & Chang, 2014; Wu, Xue, Kang, & Hui, 2013).

Figure 2-7   Pattern of μPADs: (A) Single analyte analysis (Kudo et al., 2017), (B) multi-analyte analysis (Ratnarathorn, Chailapakul, Henry, & Dungchai, 2012), and (C) three-dimension μPADs (Jeong, Kim, Nam, Song, & Lee, 2013).

2.4 Related Literature Reviews

Carrico (2000) proposed the colorimetric method of FA as a patent. FA in a sample is neutralized with solution containing sulfite ion such as sodium sulfite, potassium sulfite, sodium bisulfite, or potassium bisulfite. Dyes such as 2,6-dichlorophenol-indophenol, Thionin, or Indigo carmine, are used as the indicator to exhibit color change on over amount of sulfite. However, the reaction needs to be completed under buffer condition of pH 6.0 – 8.0 (Carrico, 2002).

$$CH_2O(aq) + 2Na^+(aq) + SO_3^{2-}(aq) \rightleftharpoons HOCH_2KSO_3(aq) + Na^+(aq) + OH^-(aq)$$

(blue)   (colorless)

Figure 2-8   (Top) Reaction of FA and sulfite solution, (Bottom) the left over sulfite react with thionin giving change of color.

Ministry of Industry of Thailand proposed the method of FA determination in sample using titration method. FA in sample is reacted with sodium sulfite then resulting in NaOH (Andrews et al., 1983). The presence of NaOH is titrated with standardized sulfuric acid using thymolphthalein in ethanol as an indicator. However, sulfuric acid has to be standardized with NaOH which has been standardized with KHP (Ministry of Industry, 2014).

Rapid and simple acid based titration on microfluidic paper-based analytical devices (µPADs) has been developed in 2014. The µPAD was fabricated by wax printing to create hydrophobic barrier for sample, reaction, and detection zones. In order to titrate NaOH, various concentration of KHP (KHP) is used as reagent and deposited on reaction zone. Specific amount of Phenolphthalein, which is used as an indicator, was deposited on detection zone. NaOH-containing sample was applied on sample zone, and then mobilized to neutralize with KHP. The exceed of NaOH would reach the detection zone resulting pink color. Numbers of pink detection zones to colorless detection zones allow determining concentration of NaOH. The range of detection of NaOH is between 0.01 to 1 M. Hence, acid-containing sample, such as nitric acid, sulfuric acid, or acetic acid, is applicable for this µPADs by using sodium carbonate in reaction zone. However, any acid and base could be applicable with this µPADs as change the indicators observed (Karita & Kaneta, 2014).
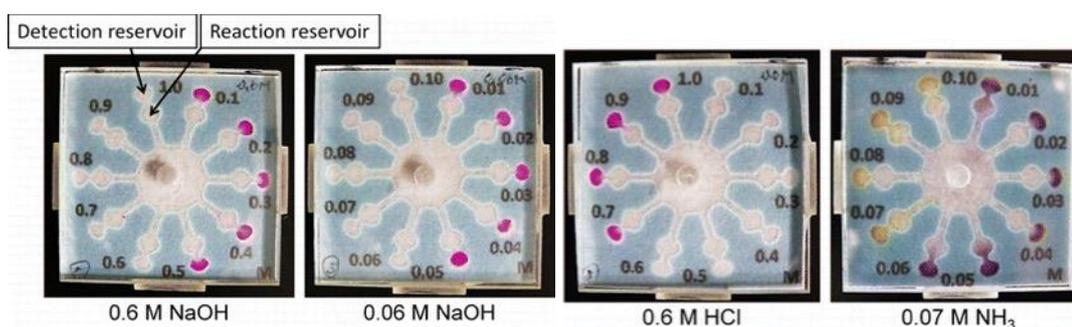
Figure 2-9   µPADs for acid-base titration, titrant is deposited on reaction zone, while indicator was deposited on detection zone (Karita & Kaneta, 2014).

Similar μPADs using for chelate titration of calcium ion and magnesium ion or water hardness have been developed later in 2016. pH-13 buffer was deposited on all hydrophilic area. Different amounts of ethylenediaminetetraacetic acid (EDTA) and a consistent amount of a metal indicator (Eriochrome Black T) were added to the reaction zones and the detection zones respectively. This μPAD was claimed as rapid, simple, and low-reagent consumption analysis of water hardness (Karita & Kaneta, 2016).
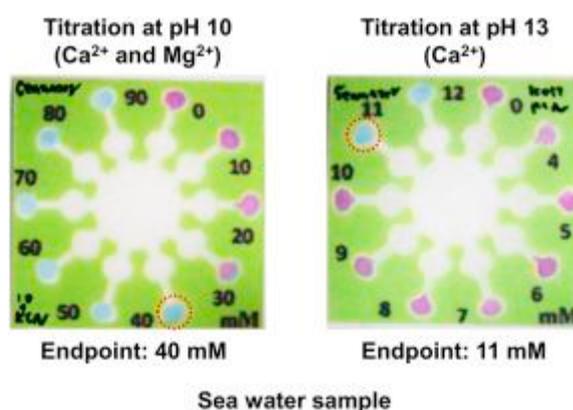
Figure 2-10   μPADs for chelate titration of calcium ion and magnesium ion in water sample (Karita & Kaneta, 2016)

Indictors are considered as dyes or compounds that exhibit color change to respond pH change. Therefore, indicator is the reagent that needs to be added for titration to identify end point. However, matching pK_a of indicator with pH of the titration system is essential to complete the quantitative analysis (Invitrogen, 2010).

Table 2-3   Some of pH indicator with pH responded on color change (Merck).

Indicator	Color change (pH)	
Bromothymol blue	Yellow (6.0)	Blue (7.6)
Cresol Red	Yellow (7.2)	Reddish-purple (8.8)
Phenolphthalein	Colorless (8.2)	Red-Violet (9.8)
Thymolphthalein	Colorless (9.3)	Blue (10.5)

CHAPTER 3
METHODOLOGY

3.1. Materials and Chemicals

3.1.1 Materials

1. Analytical Balance (MS semi-micro models, Mettler Toledo, USA)
2. Automatic pipette (Eppendorf Research®, Eppendorf, Germany)
3. Blender (Phillips HR2115, Phillips, Netherlands)
4. Filter papers no.1 (Whatman, GE Healthcare company, China)
5. Hotplate (Stirring Hot Plate type, Cole-Parmer, USA)
6. pH-indicator strips (non-bleeding, MColorpHast^{TM}, Germany)
7. Sonicator Bath (Elmasonic S30H, Elma Electronic, Switzerland)
8. Wax printer (Xerox ColoQube 8870-13, Flextronics Technology, Malaysia)

3.1.2 Chemicals (All AR grade)

1. Acetaldehyde; CH_3CHO, MW: 44.05 g/mol, CAS: 75-07-0 (Sigma Aldrich, USA)
2. Acetone; $(CH_3)COCH_3$ MW: 58.08 g/mol, CAS: 67-64-1 (RCI Labscan, Thailand)
3. Acetophenone; $C_6H_5COCH_3$ MW: 120.15 g/mol, CAS: 98-86-2 (Thermo Fisher Scientific, USA)
4. Benzaldehyde; C_6H_5CHO, MW: 106.12 g/mol, CAS: 100-52-7 (Sigma Aldrich, USA)
5. Bromothymol Blue; $C_{27}H_{28}Br_2O_5S$, MW: 624.38 g/mol, CAS: 76-59-5 (Sigma Aldrich, USA)
6. Butyraldehyde; C_3H_7CHO, MW: 72.11 g/mol, CAS: 123-72-8 (Sigma Aldrich, USA)
7. o-Cresolsulfonphthalein; $C_{21}H_{18}O_5S$, MW: 382.43 g/mol, CAS: 1733-12-6 (Sigma Aldrich, USA)

8. D-(+)-Glucose; $C_6H_{12}O_6$, MW: 180.16 g/mol, CAS: 50-99-7 (Merck, Austria)

9. Ethanol; C_2H_5OH, MW: 46.07 g/mol, CAS: 64-17-5 (RCI Labscan, Thailand)

10. Formaldehyde (FA) 1000 µg/mL in H_2O; CH_2O, MW: 30.031 g/mol, CAS: 50-00-0 (Analytical Standard, Sigma Aldrich, USA)

11. 3-methyl-2-butanone; $(CH_3)_2CHCOCH_3$, MW: 86.13 g/mol, CAS: 563-80-4 (Fluka, Germany)

12. Phenolphthalein; $C_{20}H_{14}O_4$, MW: 318.32 g/mol, CAS: 77-09-8 (Ajax Finechem, Australia)

13. Polyethylene glycol (PEG), MW: 6000 g/mol, CAS 25322-68-3 (Sigma Aldrich, USA)

14. Potassium Hydrogen Phthalate (KHP); $C_8H_5KO_4$, MW: 204.22 g/mol, CAS: 877-24-7 (Ajax Finechem, Australia)

15. Salicylaldehyde; $2-(HO)C_6H_4CHO$, MW 122.12 g/mol, CAS: 90-02-8 (TCI, USA)

16. Sodium Hydroxide; NaOH, MW: 40.00 g/mol, CAS: 1310-73-2 (Loba Chemie, India)

17. Sodium Sulfite; Na_2SO_3, MW: 126.04 g/mol, CAS: 7757-83-7 (Sigma Aldrich, USA)

18. Sulfuric acid; H_2SO_4, MW: 98.08 g/mol, CAS: 664-93-9 (RCI Labscan, Thailand)

19. Thymolphthalein; $C_{28}H_{30}O_4$, MW: 430.54 g/mol, CAS: 125-20-2 (Ajax Finechem, Australia)

3.1.3 Samples

Samples including foods and other FA containing products were purchased from local markets in Chon Buri province area.

3.2. Research Plans

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                            ┌─────────────────────┐
                            │   Scope of Study    │
                            └─────────────────────┘
                                      │
        ┌─────────────────────────────┼─────────────────────────────┐
        │                             │                             │
┌───────────────┐           ┌───────────────────────┐     ┌───────────────┐
│  Optimization │           │ Analytical performance│     │  Application  │
└───────────────┘           └───────────────────────┘     └───────────────┘
        │                             │                             │
┌───────────────┐           ┌───────────────────────┐     ┌───────────────┐
│ - Types of    │           │ - Detection range     │     │ Analysis of   │
│   Indicator   │           │                       │     │ Sample        │
│               │           │ - Limit of Detection  │     └───────────────┘
│ - Indicator   │           │                       │             │
│   Concentrations          │ - Reproducibility     │     ┌───────────────┐
│               │           │                       │     │   Accuracy    │
│ - Size of Detection       │ - Stability           │     └───────────────┘
│   zone        │           │                       │     ┌───────────────┐
│               │           │ - Tolerance limits of │     │ Using standard│
│ - Distance between        │   interferences       │     │    method     │
│   detection zone and      │                       │     └───────────────┘
│   sample zone │           └───────────────────────┘     ┌───────────────┐
│               │                                         │ Using proposed│
│ - Volumes of reagent                                    │ paper-based device
└───────────────┘                                         └───────────────┘
```

3.3. Experiment Procedures

3.3.1 Preparation of solutions

3.3.1.1 Preparation of acetaldehyde solution

Stock solution of 1000 ppm of acetaldehyde was prepared by diluting a 6.5 µL of 99 % acetaldehyde using DI water to have a final volume of 5000 µL. Working solutions of acetaldehyde were prepared by diluting the stock solution to the desired concentration using DI water. (Density of acetaldehyde = 0.785 g/mL)

3.3.1.2 Preparation of acetone solution

Stock solution of 1000 ppm of acetone was prepared by diluting a 6.3 µL of 99.5 % acetone using DI water to have a final volume of 5000 µL. Working solutions of acetone were prepared by diluting the stock solution to the desired concentration using DI water.

3.3.1.3 Preparation of acetophenone solution

Stock solution of 1000 ppm of acetophenone was prepared by diluting a 4.8 µL of 99 % acetophenone using DI water to have a final volume of 5000 µL. Working solutions of acetophenone were prepared by diluting the stock solution of acetophenone to desire concentration using DI water.

3.3.1.4 Preparation of benzaldehyde solution

Stock solution of 1000 ppm of benzaldehyde was prepared by diluting a 4.8 µL of 97 % benzaldehyde using DI water to have a final volume of 5000 µL. Working solutions of benzaldehyde were prepared by diluting the stock solution of benzaldehyde to the desired concentration using DI water.

3.3.1.5 Preparation of bromothymol blue solution

Stock solution of 0.1% w/v of bromothymol blue was prepared by dissolving a 5 mg of bromothymol blue in 5 mL of 99% ethanol. Working solutions of bromothymol blue were diluted the stock solution using DI water to the desired concentration.

3.3.1.6 Preparation of butyraldehyde solution

Stock solution of 1000 ppm of butyraldehyde was prepared by diluting a 6.3 µL of 99% butyraldehyde using DI water to have a final volume of 5000 µL. Working solutions of butyraldehyde were prepared by diluting the stock solution of acetone to the desired concentration using DI water.

3.3.1.7 Preparation of o-cresolsulfonphthelein solution

Stock solution of 0.1% w/v of o-cresolsulfonphthalein was prepared by dissolving a 5 mg of o-cresolsulfonphthalein in 5 mL of 99% ethanol. Working solutions of o-cresolsulfonphthalein were diluted the stock solution using DI water to the desired concentration.

3.3.1.8 Preparation of D-glucose

Stock solution of 1000 ppm d-glucose solution was prepared by weighing out 0.100 g of D-glucose powder and then adjusting to 100 mL with deionized water.

3.3.1.9 Preparation of standard FA solution

Working solution of FA solution was weekly prepared by diluting 1000 ppm (33.3 mM) FA solution with deionized water to the desired concentrations. Standardization of FA with standardized sulfuric acid was conducted in order to get the accurate concentration of FA.

3.3.1.10 Preparation of 3-methyl-2-butanone solution

Stock solution of 1000 ppm of 3-methyl-2-butanone was prepared by diluting a 6.3 µL 99 % of 3-methyl-2-butanone using DI water to have a final volume of 5000 µL. Working solutions of 3-methyl-2-butanone were prepared by diluting the stock solution of 3-methyl-2-butanone to the desired concentration using DI water.

3.3.1.11 Preparation of phenolphthalein

Stock solution of 1% (w/v) phenolphthalein solution was prepared by dissolving a 0.010 g of phenolphthalein with 1000 µL of 99 % ethanol.

3.3.1.12 Preparation of polyethylene glycol (PEG) solution

Stock solution of 1% (w/v) polyethylene glycol solution was prepared by dissolving a 0.010 g of polyethylene glycol with 1000 µL of DI water.

3.3.1.13 Preparation of Potassium Hydrogen Phthalate (KHP)

Stock solution 0.033 M KHP was prepared by weighing out 0.034 g of KHP powder and then adjusting volume to 5 mL by deionized water. Working solution was prepared by diluting the stock solution using deionized water.

3.3.1.14 Preparation of salicylaldehyde solution

Stock solution of 1000 ppm of salicylaldehyde was prepared by diluting a 4.4 µL of 98% salicylaldehyde using DI water to have a final volume of 5000 µL. Working solutions of salicylaldehyde were prepared by diluting the stock solution of salicylaldehyde to the desired concentration using DI water.

3.3.1.15 Preparation of NaOH

Stock solution of 0.5 M NaOH was prepared by weighing out 5.000 g of NaOH pellet and then adjusting volume to 250 mL by deionized water. Working solution was prepared by diluting the stock solution using deionized water.

3.3.1.16 Preparation of sodium sulfite (Na_2SO_3)

Stock solution 1.0 M sodium sulfite was prepared by weighing out 12.6043 g of sodium sulfite powder and then adjusting volume to 100 mL by deionized water.

3.3.1.17 Preparation of sulfuric acid (H_2SO_4)

Stock solution of 0.25 M sulfuric acid was prepared by transferring approximately 14 mL of 98% sulfuric acid into a beaker and then adjusting volume to 1000 mL by deionized water.

3.3.1.18 Preparation of thymolphthalein

Stock solution of 1% (w/w) thymolphthalein solution was prepared by dissolving 1.00 g thymolphthalein with 99.0 g ethanol.

3.3.2 Fabrication and design of paper-based device

3.3.2.1 Design of paper-based device

A paper-based device was designed *via* Adobe Illustrator program to fabricate on the letter-size filter paper. The designed pattern has a radial shape with 7-mm-diameter sample zone connected to 10 channels that have 2-mm long and 1.5-mm width where each of which is linked to 4-mm-diameter reaction zones and 5-mm-diameter detection zones, respectively (Figure 3-1). The black color was used to print a wax and to define the white analysis zone.
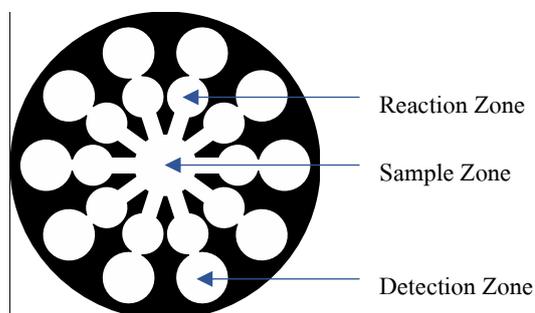
Figure 3-1   Paper-based device pattern designed by Adobe Illustrator

3.3.2.2 Fabrication of paper-based device

The paper-based device was fabricated using wax-printing technique. The designed pattern was printed onto the filter paper using a wax printer as shown in Figure 3-2.

Figure 3-2   The printing process of paper-based device using a wax printer.

The printed filter paper was then heated on the hotplate with temperature setting of 150 degrees Celsius for 30 seconds to allow the wax melt through the paper creating 3D hydrophobic barrier as shown in Figure 3-3. After this step, the device dimension was slightly changed from the original design due to wax spreading.
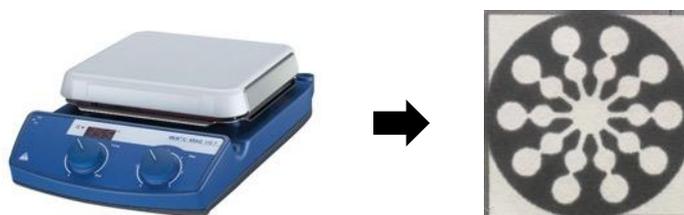
Figure 3-3   The paper-based device after heating.

After heating, the clear adhesive tape was used to cover the backside of device to prevent leaking during analysis. Polyethylene glycol (PEG) was first deposited for 1.5 µL into the detection zones before deposition of 1.5 µL of 1% phenolphthalein, and followed by deposition of 0.75 µL of various concentration (3.33 – 29.9 mM) of KHP to each reaction zone twice as shown in figure 3-4. The device was allowed to dry after each reagent deposition. Sodium sulfite powder of 9.0 mg was filled in the pretreatment vial.
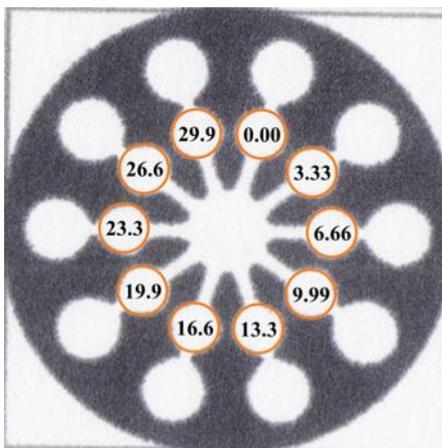
Figure 3-4   Given concentration (mmol/L) of KHP as a primary standard solution deposited to the corresponding reaction zones.

3.3.3 General procedure for FA analysis using developed paper-based titration method.

The testing method was designed to meet two types of applications including in-lab and on-site analysis. The differences between these applications are the samples preparation process where no sample preparation was required for on-site analysis and a traditional sample preparation process was used for in-lab analysis.

For on-site analysis, known-weight sample was added into sodium-sulfite-containing vial, followed by adding deionized water for 2 mL and mix thoroughly. A 30 µL of the mixed solution was dropped onto sample zone of the prepared paper-based device to allow flow to the reaction and detection zones containing KHP and phenolphthalein, respectively. The number of color change at detection zone indicates range of FA concentration as written on the device which is correlated to KHP concentration in reaction zone. For in-lab analysis, sample solution was prepared differently as described below (Section 3.3.4) and a 2-mL prepared sample solution was added to the pretreated vial. After that, the analysis of FA content was similar to the process described above for on-site analysis.

3.3.4 Sample Preparation

Food samples purchasing from market were kept in Ziploc bag and stored in ice box before the experiment. A weighed sample was mixed with deionized water and then homogenized using a blender. The mixture was transferred to an Erlenmeyer flask, sealed with parafilm and sonicated for 20 minutes. The mixture was then centrifuged and the supernatant gravitational filtered through Whatman No. 1 filter paper before keeping in the polyethylene bottle in cold (6 °C) until the analysis.

3.3.5 Optimization of the proposed assay

3.3.5.1 Types of Indicator

Four types of pH indicators including bromothymol blue, cresol red, phenolphthalein, and thymolphthalein were studied to obtain the most suitable indicator. The optimization was carried out both in solution vial and in the paper-based devices. The experimental design as well as the results are discussed in Chapter 4.

3.3.5.2 Indicator Concentrations

Various concentrations of the selected indicator obtained from the optimization condition in section 3.3.4.1 in the range of 0.1 - 2.0 % w/v were deposited on each of the detection zone. The color change was observed using the solution from reaction with 500 (16.5 mM) and 100 (3.33 mM) ppm FA to obtain the clearest color change at the detection zone of the PAD.

3.3.6 Analytical features for amount of FA

3.3.6.1 Limit of Detection (LOD)

The limit of detection defined as the lowest analyte concentration that device give a distinguishable signal from blank signal was determined by decreasing the concentration of FA until apparent color on the detection zone could not be obtained. Ten replicates were conducted in order to calculate standard deviation.

3.3.6.2 Detection Range

Ability of the proposed device to effectively distinguishably detect small different amount of FA was investigated to determine the sensitivity of the developed assay. Concentration resolutions of a hundred-ppm to one-ppm were evaluated.

3.3.6.3 Reproducibility

Different concentrations of standard FA solution were evaluated for five replicates at each concentration to determine standard deviation. Percent relative standard deviation (%RSD) was then calculated to determine reproducibility of the developed method.

3.3.6.4 Stability

The stability of device was investigated over a period of time when stored at different temperatures and light exposure. Temperature conditions including at ambient and low temperature at 6 °C (refrigerator). Light exposure including normal day light and dark (under aluminum-foil wrap). However, there is no light condition observed for low temperature at 6 °C (refrigerator). The devices from ambient temperature and day light conditions were tested every day, while the devices in dark condition were tested every 3 days for a month.

3.3.6.5 Tolerance limit of interference

Tolerance limit of interference is the ability of device with proposed assay to maintain the interference at high concentration. The interferences are the species containing similar structure, functional group to FA or found in the samples. The experiment was performed in similar manner to the method described in (Section 3.3.4) except that the interference was used instead of FA.

The tested interferences in this work included: (1) aldehyde group; acetaldehyde, D-glucose, salicylaldehyde, and benzaldehyde, and (2) ketone group; acetone, 3-methyl-2butanone, and acetophenone.

3.3.7 Accuracy of FA analysis between the developed PAD titration assay and classical titration

To determine the accuracy, the developed assay was validated against the traditional assay for FA analysis in various samples. The traditional assay is based on the titration with sulfuric acid and thymolphthalein as an indicator after FA sulfite reaction (American Chemical Society, 2017). Firstly, a 20-mL sample solution prepared as described in Section 3.3.4 was added into an Erlenmeyer flask and followed by 20 mL of 1.0 M sodium sulfite solution and about 3 – 5 drops of thymolphthalein. The mixture was titrated with standardized sulfuric acid until reach the end point (blue color disappeared). The measurement was quintuplicated. FA content in the samples was calculated and compared with those obtained from the developed PAD assay. The result between the developed and classical assay were compared.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Principle of the proposed assay

This work presents a rapid, simple, instrument-free, paper-based device for FA analysis using sulfite assay. The analysis is based on the reaction between FA and excess amount of sodium sulfite to generate NaOH in a one-to-one mole ratio of FA (Walker, 1964). The NaOH is then quantified by acid-base paper-based titration method. The procedure for FA analysis consisted of two parts which are sample pretreatment carried out in a pretreatment vial containing sodium sulfite and the titration on the PAD to quantify the generated NaOH. Sodium sulfite is less stable in solution phase (The International Agency for Research on Cancer, 1992), therefore, its powder form was filled in the pretreatment vial. The paper-based device for acid-base titration of NaOH was fabricated on no.1 Whatman filter paper by a wax-printing technique. The PAD consists of a central sample zone connected through ten narrow channels radially to reaction and detection zones, respectively. Polyethylene glycol and phenolphthalein were deposited to the detection zones and different amounts of KHP deposited on the reaction zones. For the analysis, FA containing solution was added into the sample pretreatment vial containing sodium sulfite which allow NaOH to be generated as shown in reaction Figure 4-1A (Carrico, 2002). After thoroughly mixed, a 30 μL the pretreated sample solution containing NaOH product was applied to the sample zone. Once the solution spreading out through the reaction zone, NaOH was neutralized by KHP as shown in Figure 4-1B. In case that NaOH is less than KHP, no unneutralized NaOH gets to detection zone, therefore, the phenolphthalein remained colorless. On the other hand, unneutralized NaOH remaining from the reaction zone reached the detection zone and turned phenolphthalein from colorless to pink as shown in Figure 4-1C (Takano, Kikkawa, Suzuki, & Kohno, 2015).

$$HCHO \ (aq) \ + \ Na_2SO_3 \ (aq) \ \rightarrow \ HOCH_2NaSO_3 \ + \ NaOH \ (aq) \qquad (A)$$

$$KHP \ (aq) \ + \ NaOH \ (aq) \ \rightarrow \ KNaP \ (aq) \ + \ H_2O \ (l) \qquad (B)$$

$$H_2P \ (aq) \ + \ 2OH^- \ (aq) \ \rightarrow \ P^{2-} \ (aq) \ + \ 2H_2O \ (l) \qquad (C)$$

Colorless   Pink

Figure 4-1   Chemical equations on the proposed assay

4.2 Optimization of the proposed assay

4.2.1 Paper-Based Device Pattern

In preliminary study, single spot pattern has been employed for the titration on PAD. The paper-based device was designed to have 10 circular shapes line up together with diameter of 5 mm test zones. The black color was used to define the analysis zone. Constant amount of phenolphthalein was deposited to the zone, and followed by deposition of different amounts of KHP which coresponding to mole ratio of NaOH generated from FA and sulfite. After the paper-based devices were dried, 2 mL of 500 ppm standard solution of FA was added to the pretreatment vial containing excess amount of sodium sulfite and mixed throughtly. The pretreated sample solution was applied to every test zone. The expected results are demonstrated in Figure 4-2A where the pink color should be observed in the test zones containing KHP lower than 16.5 mM (equal mol to 500 ppm FA). However, the experimental results provided in Figure 4-2B showed unclear color change at 13.2 mM (400 ppm) to 16.5 mM (500 ppm). Moreover, the pink color were observed in all tested zones although the amount of KHP was higher than 16.5 mM indicating that the reaction of NaOH and phenolphthalein occurred as a results of incomplete of nutralization reaction of NaOH and KHP prior to react with phenolphthalein. Therefore, the design of the PAD was changed to the patern that can allow the nutralization reaction to complete and subsequently lead the excess amount of NaOH or KHP to react with the phenolphthalein.

Figure 4-2   Paper-based device tested with 500 ppm (16.5 mM) standardized FA solution. (A) Expected results and (B) Experimental results.

The new PADs were designed to separate the storage reagents into different positions which were reaction zones and detection zones. FA in the range of 100-1000 ppm with an interval of 100 ppm were set as a target level of detection. The new design was a radial pattern consists of a central sample zone connected through ten narrow channels to the reaction and detection zones, respectively, with the device dimension as same as described previously (Karita & Kaneta, 2014). With this pattern, the paper-based devices were used with acrylic plate holder to accelerate sample flow radially to the detection zones. Volume of the reagent deposition per unit area was calculated using imageJ software. Constant amount of phenolphthalein was applied to the detection zones and different amounts of KHP applied to the reaction zones. The PADs were tested with 500 ppm FA solution. The result showed distinguishable endpoint, however, unexpected partial color change at the detection zone were observed as a results of incomplete neutralization reaction at the reaction reservoir prior to reach the detection zones as shown in Figure 4-3. Using acrylic plate creates the pressure which could result the sample flow too fast to the detection zones with insufficient reaction time for neutralization at the reaction zones (Giokas, Tsogas, & Vlessidis, 2014). Moreover, the paper-based titration reported by Karita and Kaneta (Karita & Kaneta, 2014) gave the range of NaOH analysis in between 10 – 1000 mM which is much higher concentration range than this experiment. Therefore, the PAD design was improved for the low-level analysis of NaOH generated from low-level FA contaminated in the samples with are in the range of of 3.33 to 33.3 mM (100 – 1000 ppm FA).
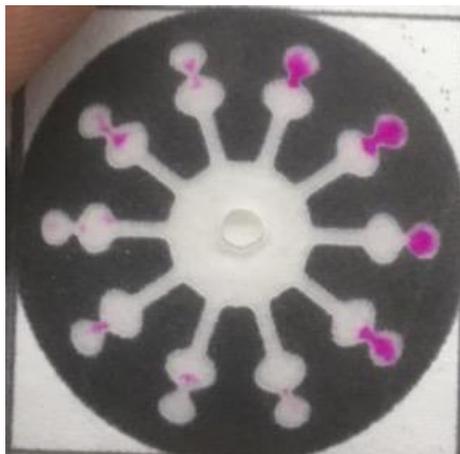
Figure 4-3   Kaneta's pattern with 500 ppm FA analysis. Device dimension: 30×30 mm^2. Sample zone with diameter of 10.0 mm connected with a narrow channel of 1.5 mm wide and 3.0 mm long to reaction zone of 4.0 mm by 3.0 mm oval shape next to 3.0 mm by 2.8 mm oval shape.

4.2.2 Improvement the device designs

The paper-based device designed by Kaneta (Karita & Kaneta, 2014) was modified for FA analysis in the range of 3.33 to 33.3 mM (100 – 1000 ppm FA). Acrylic plate was removed and the paper-based device was tested with 500 and 100 ppm FA standard solution. The paper-based device was prepared in similar manner as the above design except that KHP was not deposited to the reaction zone. The results shown in Figure 4-4A that small pale color change at the detection zone for 500 ppm FA measurement while no color change was observed for 100 ppm FA. Moreover, the detection zones were found to be hydrophobic due to the hydrophobicity of the phenolphthalein. Therefore, polyethylene glycol (PEG) was used to increase hydrophilicity of the detection zones (Piyanan, Athipornchai, Henry, & Sameenoi, 2018). The result of using PEG in detection zones have shown in Figure 4-4B which almost similar comparing between with and without PEG treatment. However, without PEG, the detection zone showed white film due to hydrophilicity of the phenolphthalein. Therefore, the detection zone was treated with PEG for further experiments.

500 ppm FA	100 ppm FA	Deionized water
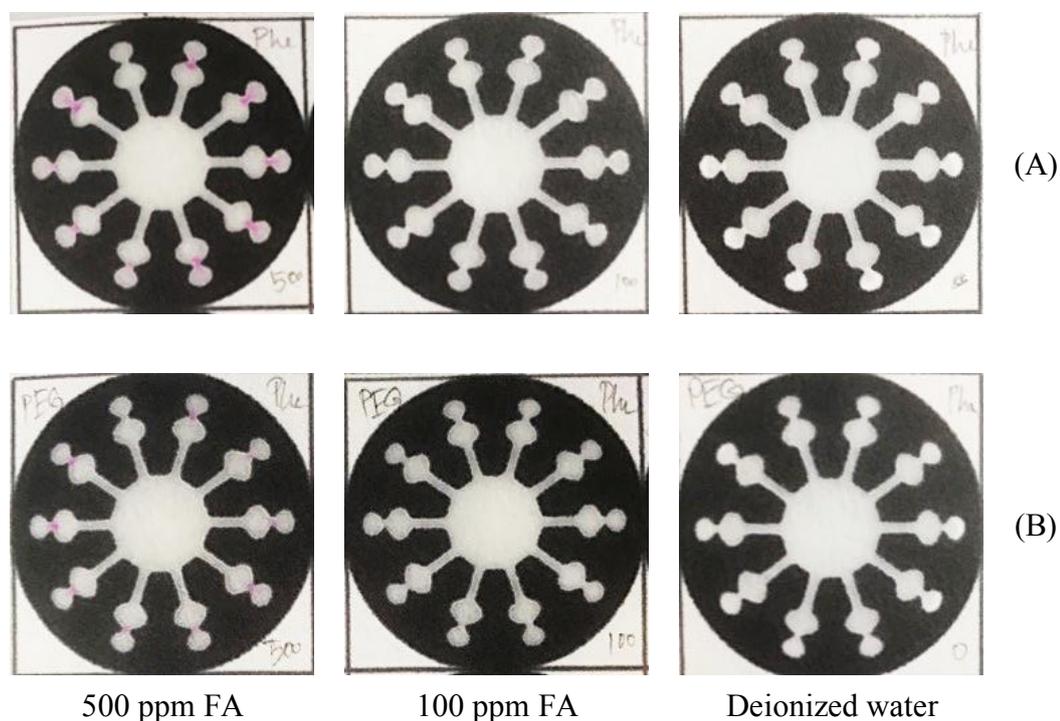
Figure 4-4   The paper-based device without deposition of KHP at the reaction zone for the analysis of FA standard solution of 500 ppm, 100 ppm, and deionized water respectively. (A) The detection zones were not pretreated with PEG and (B) The detection zones were pretreated with PEG. 0.5 μL of 1% w/v PEG and 0.5 μL of 1% w/v phenolphthalein were deposited to the detection zone respectively. After all reagent dried, 30 μL the pretreated FA solution was deposited to the sample zone.

As a result of using PEG to increase detection zone's hydrophobicity, 100 ppm FA still could not give color change on detection zone. Therefore, the design was further modified by investigation the effect of shape and size of the reaction and detection zones. The shape of reaction and detection zones were changed from oval in above design to be circular (Figure 4-5). The reaction zone diameter was changed to be constant at 4 mm. The detection zone diameters in the range of 3-5 mm were also

investigated. By this modification, the device dimension was expanded up to 40×40 mm^2. The paper-based devices were tested with 500 ppm FA. The reaction zones were leave without deposition of KHP in order to observe color change at the detection zone.

| 3 mm | 4 mm | 5 mm |
| (A) | (B) | (C) |
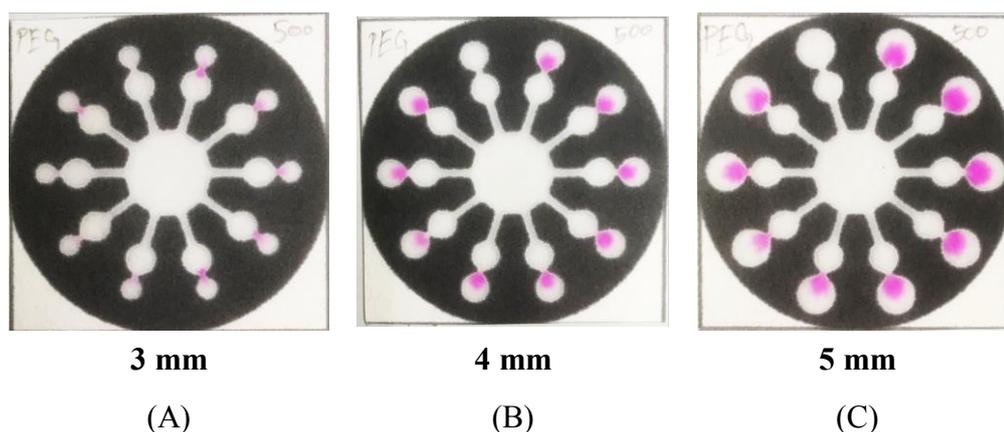
Figure 4-5   Color change observed from optimization of detection zone diameter ranging from (A) 3 mm, (B) 4mm, and (C) 5mm. The length between sample and detection zone are constant at 7 mm. Analysis of 500 ppm FA standard solution without the deposition of KHP at the reaction zones. All designs were deposited with 30 μL of pretreated 500 ppm FA standard solution at the sample zone.

From the analysis of 500 ppm FA, the results showed that the larger the detection zone was used, the clearer the color change can be observed. Larger detection zones provided higher capillary pressure that can drive higher amount of sample to flow to the detection zones (Hofstetter et al., 2018). However, no color change can be observed for 100 ppm FA. This might be the results from the adsorption at the sample zone of all hydroxide ions with low concentrations. To prove this hypothesis, the paper-based devices were prepared to investigate whether NaOH product from the reaction of FA and sulfite spreading from the sample zone radially to the detection zones. Polyethylene glycol and phenolphthalein solutions were deposited to all area of the hydrophilic zone of the device and allowed to dry. Different concentrations of FA

standard solution were mixed with sulfite to generate NaOH. The solution (30 µL) was then added to the sample zones.

100 ppm	**200 ppm**	**300 ppm**	**400 ppm**	**500 ppm**
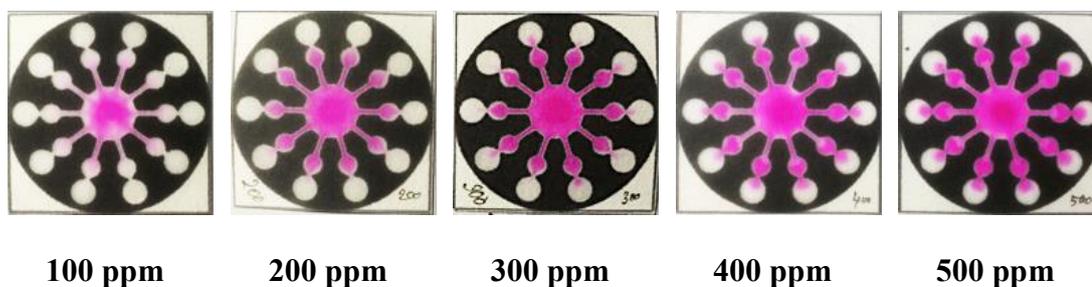
Figure 4-6   Observation on how NaOH generated from different concentration of FA standard solution spreading through the test zone. 30 µL of 1% w/v PEG solution and 1% w/v phenolphthalein were deposited at the sample zone and allow the reagent spreading through all test zones. 30 µL of the pretreated solution of different concentration ranging from 100 to 500 ppm FA was added to the sample zone of each device.

In Figure 4-6, the results obtained from 100 and 200 ppm FA standard solution tests showed that amount of generated NaOH could not reach the detection zone even the reaction zone contained no KHP which was probably due to free hydroxide ions tended to interact with hydroxyl group in cellulose (Jiang et al., 2014). Therefore, the paper-based devices dimension was changed by decreasing the channel length in order to move the detection zones closer to the sample zone. The length between detection zone and sample zone was optimized in the range of 4-7 mm and the obtained devices were tested for 100 ppm FA standard solution analysis.

| 7 mm | 6 mm | 5 mm | 4 mm |
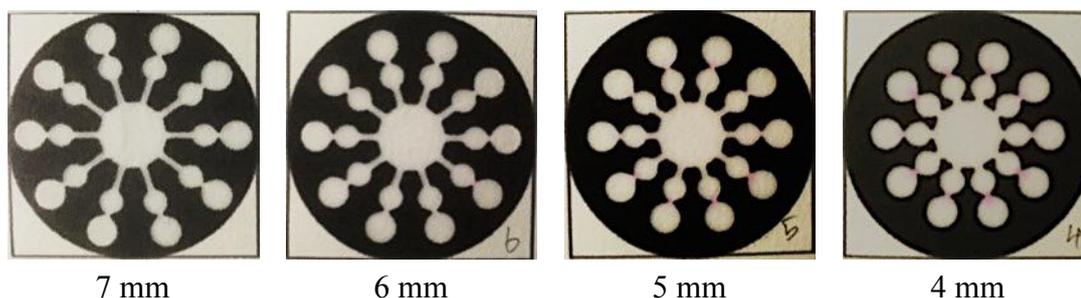
Figure 4-7   Color change observed from optimization of length between sample and detection zone tested with 100 ppm FA standard solution. Without deposition of KHP to the reaction zones, 1.5 µL of 1% w/v PEG and 1.5 µL of 1% w/v phenolphthalein were deposited to the detection zone respectively. After all reagent dried, a proper volume of the pretreated FA solution was deposited to the sample zone.

The result from Figure 4-7 showed that, the 4-mm-length between the sample zone to the detection zone gave best observable color change comparing to the other investigated lengths. Therefore, the paper-based device was decreased to the dimension of 30 x 30 mm^2 containing a 4-mm length between sample zone and the detection zones. Using this design, however, when the optimal volume of 0.75 µL KHP was deposited to the reaction zone, it can spread into the sample zone resulting in incorrect result of analysis. Therefore, the sample zone diameter was decreased from 10 mm to 7 mm to add some length between sample and reaction zones and hence, prevent leaking of KHP into the sample zone, as shown in Figure 4-8 below, which is the final design for FA analysis.

| 10 mm | 7 mm |
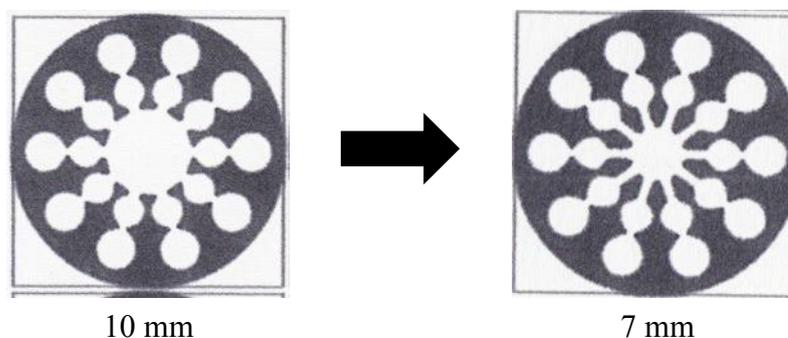
Figure 4-8   The paper-based device pattern was changed to obtain the most accurate result.
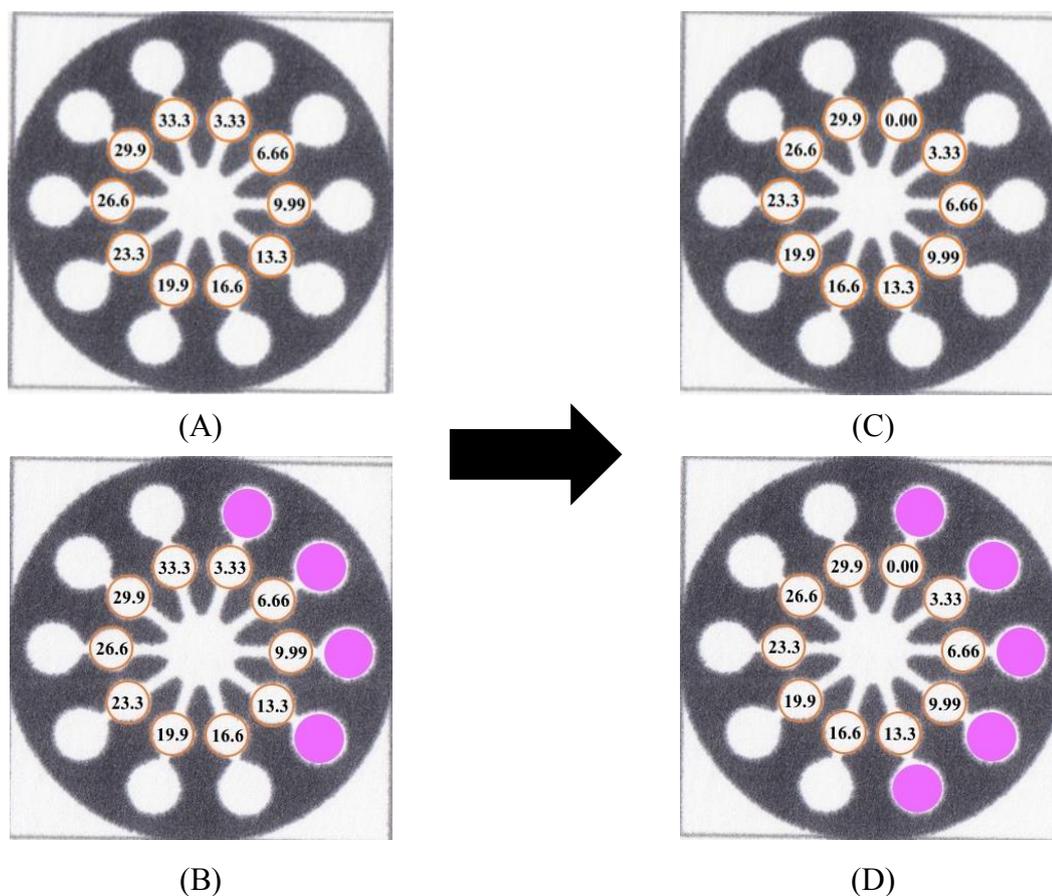
Figure 4-9   Concentrations deposition pattern of KHP deposited to the reaction zone for 500 ppm FA. (A) The pattern of KHP concentration deposition (B) The expected result should be observed from 500 ppm FA (16.6 mM) (C) The adjust pattern of KHP concentration deposition (D) The expected result should be observed from 500 ppm FA (16.6 mM) using the adjust pattern.

The concentration of KHP deposited at the reaction reservoir was also modified to get reasonable interpretation for the users. Since the target concentration of FA for the analysis was in the range of 100 – 1000 ppm (3.33 – 33.3 mM) FA in ten detection zones with an interval of 100 ppm. The reaction zones were initially deposited by KHP with the concentration corresponding to the target FA concentration, starting at 3.33 mM KHP for the first reaction zone with the increment of 3.33 mM for each reaction

zone to 33.3 mM for the tenth reaction zone (Figure 4-9A). Using this deposition pattern, however, the number of color appeared on the detection zones might lead to wrong interpretation of FA concentration for the user even though the result of analysis was correctly obtained. For example, four pink color detection zones were observed for the analysis of 500 ppm (16.6 mM) FA since the end point at the fifth as well as the remaining detection zone gave colorless (Figure 4-9B). With this result, some users might interpret the concentration of the FA to be 400 ppm which is incorrect concentration. Therefore, the KHP concentration deposition pattern on the PAD was modified to be more reasonable for interpretation, for example, 5 color-changed zones would be observed for 500 ppm (16.6 mM) FA analysis. The pattern of KHP concentration deposited to the reaction zone was adjusted according to Figure 4-9C and the expected result is shown in Figure 4-9D where 5 pink zones are obtained for 500 ppm FA analysis because the endpoint of titration has moved to the 6th zone.

Volumes of reagent deposited to each zone were calculated using imageJ software to get accurate titration results. Firstly, dye solution was applied to the sample zone in order to investigate total volume filled all the hydrophilic zone. After that, the paper-based device was scanned and imported to the software for calculation. The table 4-1 show the calculated volume for each zone. The result showed that, the detection zone can hold approximately two times higher volume than the reaction zone.

Table 4-1   Calculations for volume of reagents deposited in each zone
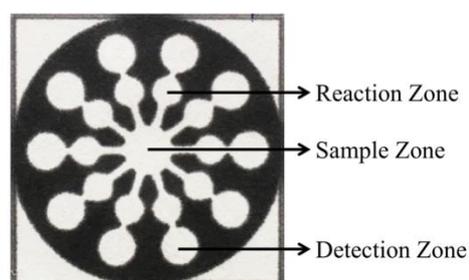
	Area (mm^2)	Volume (μL)
All hydrophilic Zone	270.26	30
Reaction Zone	6.76 ± 0.15	0.75 ± 0.02
Detection Zone	13.67 ± 0.26	1.52 ± 0.03

Table 4-2   Different volumes of a given concentration of KHP deposited by drop-dried process to the reaction zone for the analysis of 500 ppm FA standard solution and the obtained results.

Total Volume of KHP	Result
0.75 µL × 1	
0.75 µL × 2	
0.75 µL × 3	
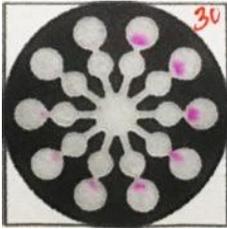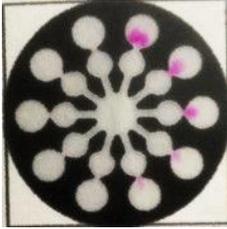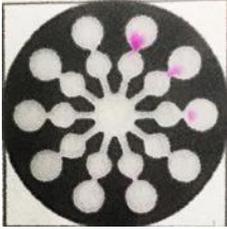
The paper-based device was prepared by deposition of the reagent to each zone with corresponding calculated volumes per unit area. Using this calculated volume deposition, the result showed higher concentration than expected for the 500 FA analysis as the color change at the detection zones showed 8 instead of 5 zones (Table 4.3-first row). This might be the results of higher amount of NaOH flowing through the reaction zones than expected. As mentioned earlier that the detection zones can hold two times higher volume than that of the reaction zones, amount of NaOH flowed through the reaction zones to reach the detection zones should be two times higher volume than that of the reaction zones as well. Therefore, the volume of KHP deposited at the reaction zones was increased by two times of the calculated volume to get complete neutralization of flowing NaOH. However, the reaction zone cannot hold any greater volume than that obtained from the calculation, drop-dried technique was

employed to deposit two times amount of KHP at the reaction zones by dropping 0.75 µL of each concentration of KHP to the reaction zone and waiting until dry, followed by another deposition of the same volume of KHP. Using this concept, the results was observed as expected where the accurate result was obtained when KHP was applied by two times the calculated volume.

4.2.3 Types of Indicator

As the acid-base titration PAD was used to detect NaOH generated from the reaction of sulfite and FA, indicator used is a key reagent in order to get accurate results. Even thymolphthalein has set to be used as indicator for the standard method for FA analysis(American Chemical Society, 2017; Ministry of Industry, 2014; TAPPI, 2011), four types of pH indicators including bromothymol blue, cresol red, phenolphthalein, and thymolphthalein were studied to obtain the most suitable indicator used. For this study, the experiment was preliminary carried out in both in solution phase and on the PAD.

4.2.3.1 In solution phase

Each indicator was tested for its ability to indicate the pH change when the titration occurred. Standard solution of 500 ppm FA was used as a concentration of analyte to be titrated. All tubes were firstly filled with 10 µL of 1% w/v of a given indicator. The blank tube was added with another 400 µL of DI water. The negative control tube was added with 200 µL sulfite solution and another 200 µL DI water. The titrant test tubes were added with 200 µL of various concentration of KHP (19.98, 16.65, 13.32, 9.99, 6.66, and 3.33 mM). Standard solution of 500 ppm (16.65 mM) FA was firstly mixed with sodium sulfite in the separated vial to generate NaOH. A 200-µL of the produced NaOH solution was then transferred to each titrant test tube and color change observed.

Table 4-3   Color change of each indicator from different concerning condition.

[FA]	500 ppm (16.65 mM)						DI water	DI water
[KHP]	19.98 mM	16.65 mM	13.32 mM	9.99 mM	6.66 mM	3.33 mM	0.00 mM	
BTB								
CR								
PHPH								
THY								
	Excess KHP	Endpoint	Excess NaOH	Excess NaOH	Excess NaOH	Excess NaOH	Negative Control	Blank
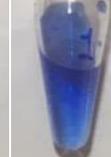
The Table 4-3 showed color change for all indicators. The appropriate indicator would give the clearest color difference at the end point and negative control. Bromothymol blue (BTB) (pK_{In} = 7.3) (Daniel L. Reger, Scott R. Goode, & David W. Ball, 2010) and cresol red (CR) (pK_{In} = 8.3) (Kahlert, Meyer, & Albrecht, 2016) gave similar color for negative control and all the titration test with excess NaOH, and hence, they could not be used as they would gave false negative results. On the other hand, thymolphthalein (THY) (pK_{In} = 9.7) (Kahlert et al., 2016) and phenolphthalein (PHPH) (pK_{In} = 8.7) (Daniel L. Reger et al., 2010) gave distinguishable color between negative control and all the excess NaOH titration test. Therefore, both thymolphthalein (pK_{In} = 9.7) and phenolphthalein (pK_{In} = 8.7) were appropriate as indicators for further experiments. The negative control solution containing only sodium sulfite has K_b of 1.6×10^{-7} (John Kotz, Paul Treichel, & John Townsend, 2009) and the pH of saturated

sodium sulfite aqueous solution is about 9 . Bromothymol blue and cresol red would give the same color since the pK_{In} of the indicator was lower than 9. Therefore, they could not differentiate the solution pH of negative control (only sodium sulfite) or that of NaOH (generated from the reaction of FA and sulfite). However, thymolphthalein gave the clearly distinguished color change between the solutions with or without FA as the pK_{In} of the indicator was higher than 9. Phenolphthalein gave slightly different color between the two types of solutions since the pK_{In} of the indicator was close to 9.

4.2.3.2 On PADs

According to the result above, only thymolphthalein and phenolphthalein were investigated to obtain the more suitable indicator to be used on the PAD. The PADs were prepared in order to investigate the most suitable indicator that gave best distinguished color change at the endpoint and negative control. Polyethylene glycol (PEG) was also employed in order to improve the intensity of the color. The results were shown in Figure 4-9. From the analysis of NaOH product from the reaction of 100 ppm (3.33 mM) FA standard solution with sodium sulfite, no any color from thymolphthalein was observed for all tested conditions whereas phenolphthalein gave observable color for some condition concentrations only when used together with PEG modification.

4.2.4 Indicator Concentration

Various concentrations of indicator (0.1, 0.5, 1.0, 1.5, and 2.0 % w/v) were deposited on each of the detection zone. The color change was observed using the solution of NaOH product from the reaction of 100 ppm (3.33 mM) FA standard solution and excess of sodium sulfite to observed color change at the detection zone of the PAD. The results showed in Figure 4-10 demonstrated that no color change was observed using thymolphthalein at any concentrations. Conversely, phenolphthalein gives color change at concentration of 1.0, 1.5, and 2.0% w/v. Therefore, 1.0% w/v phenolphthalein was used for further experiments.

2.0% Phph + No PEG   2.0% Phph + 1% PEG

1.5% Phph + No PEG   1.5% Phph + 1% PEG

1.0% Phph + No PEG   1.0% Phph + 1% PEG

0.5% Phph + No PEG   0.5% Phph + 1% PEG

0.1% Phph + No PEG   0.1% Phph + 1% PEG

(A)

2.0% Thy + No PEG   2.0% Thy + 1% PEG

1.5% Thy + No PEG   1.5% Thy + 1% PEG

1.0% Thy + No PEG   1.0% Thy + 1% PEG

0.5% Thy + No PEG   0.5% Thy + 1% PEG

0.1% Thy + No PEG   0.1% Thy + 1% PEG
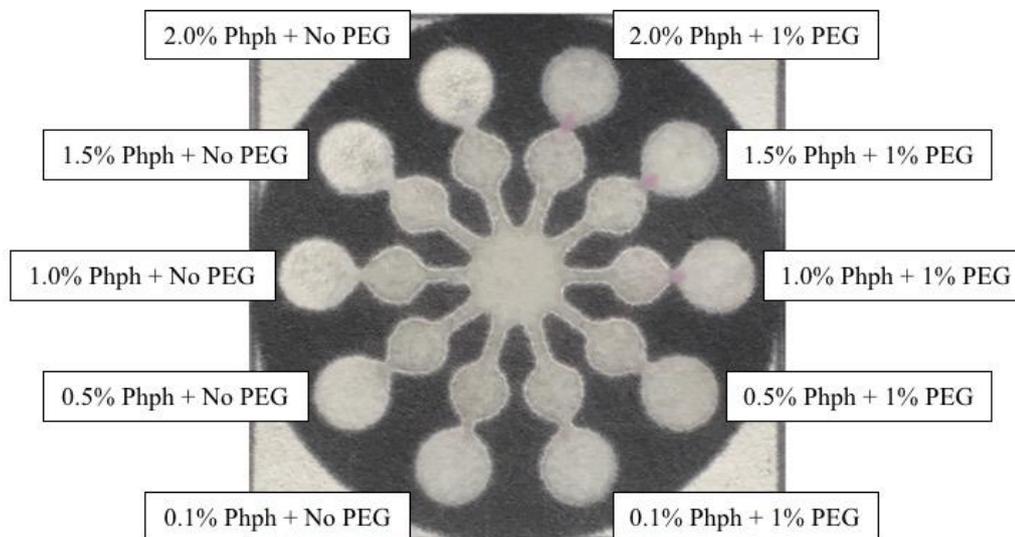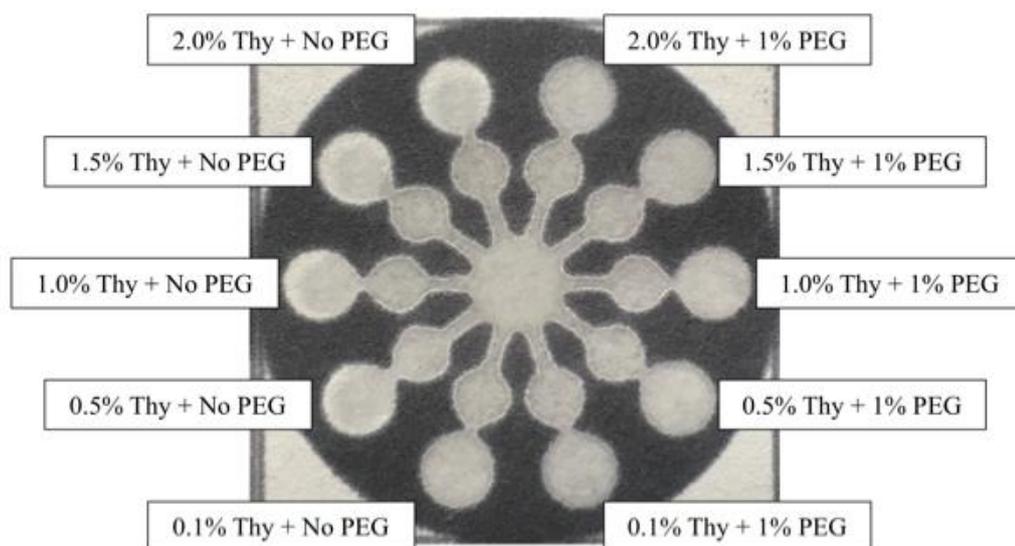
(B)

Figure 4-10   Types of indicator for FA analysis on the PAD. Five different concentrations of a given indicator were deposited to each different detection zone with/without applying PEG for the analysis of FA at 100 ppm. (A) Phenolphthalein (Phph) and (B) Thymolphthalein (Thy).

With all the optimization conditions, the Table 4-4 and Figure 4-11 summarize the optimal condition for device preparation in term of device dimension and reagent deposition at each analysis zones.

Before Heat   After Heat
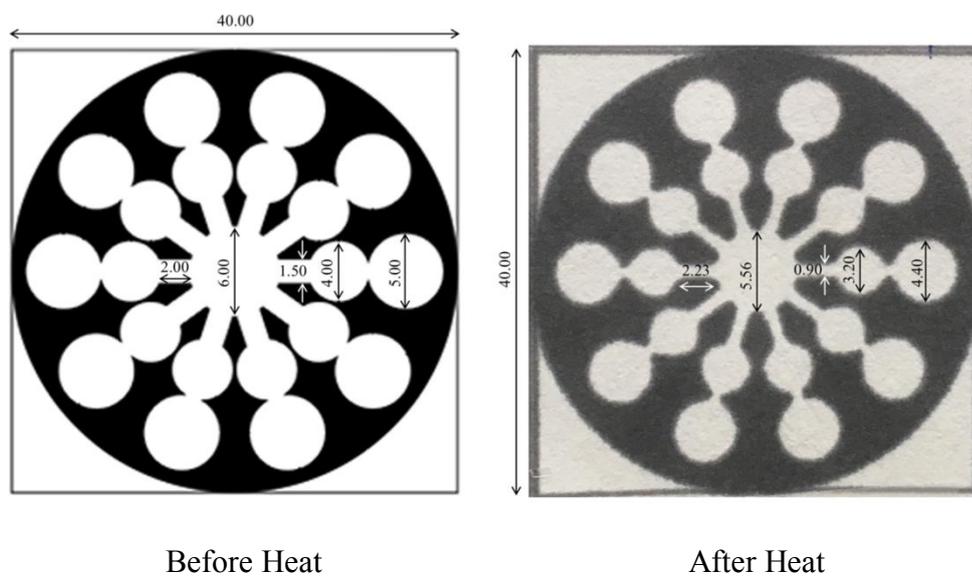
Figure 4-11   The dimension of the design PAD. The lengths are indicated in millimeters.

Table 4-4   Summaries of optimal conditions for device fabrication (concentrations and volumes of reagents)

Reagent	Zones	Concentration	Volume
Polyethylene Glycol (PEG)	Detection	1.0 % w/v	1.50 μL
Phenolphthalein (Phph)	Detection	1.0 % w/v	1.50 μL
KHP	Reaction	3.33 -29.9 mM	0.75 μL × 2

4.4 Method Validation

Once optimal conditions were obtained, the performance of the developed paper-based devices was investigated for analysis of FA in terms of detection range, reproducibility and limit of detection.

4.4.1 Detection Range

The assay was first investigated for its ability to determine the standard FA in the target concentration range which is 100-1000 ppm with an interval of 100 ppm. The results are shown in Figure 4-12 from the analysis of standard FA where the observed results on the PAD is related to the concentration of the analysis. By counting the number of detection zones containing pink color, the amount of FA can be semi-quantitatively determined. For example, one pink detection zone was obtained from 100 ppm FA analysis while ten pink detection zones were obtained from 1000 ppm FA analysis and so on. Using this measurement, the user can simply count the pink detection zones to measure FA content in the samples without the need of any other detection instrument. This could allow for point of need measurement for FA analysis with cheap, and simple analysis.

| 100 ppm | 200 ppm | 300 ppm | 400 ppm | 500 ppm |

| 600 ppm | 700 ppm | 800 ppm | 900 ppm | 1000 ppm |
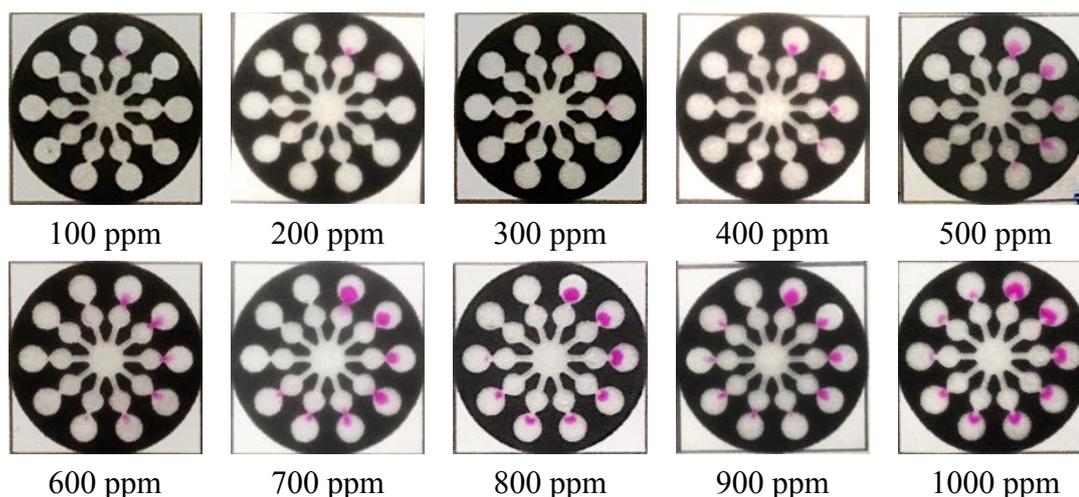
Figure 4-12   Difference concentrations of standard FA analyzed using the developed paper-based devices.

4.4.2 Reproducibility

Reproducibility of the proposed paper-based device was investigated by replicate analysis of standard FA across all tested concentrations in the detection range (n=5). The same concentration was obtained from 5 replicate analyses for all standard FA concentration studied indicating that the proposed method gave high reproducibility for the semi-quantitative analysis of FA (Table 4-5).

Table 4-5   the reproducibility study result of the PAD using standardized FA solution (n=5)

[FA]	1	2	3	4	5
100 ppm					
300 ppm					
500 ppm					
700 ppm					
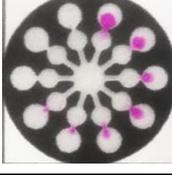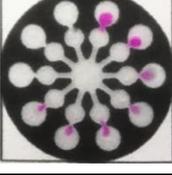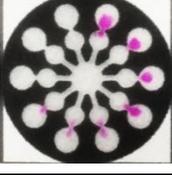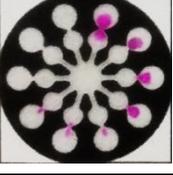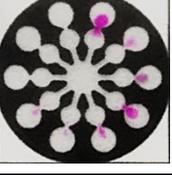
4.4.3. Limit of Detection (LOD)

Experimental limit of detection was investigated by using the standard FA with the lowest concentration that the observable color at the detection zones can be obtained. Figure 4-13 showed that only 3.33 mM of NaOH product generated from the 100 ppm FA solution gave observable color change. On the

other hand, lower FA concentration at 90 and 80 ppm generating 2.99 mM and 2.66 mM NaOH, respectively, could not give any observable pink color at the detection zones. Therefore, the limit of detection and limit of quantification are 100 ppm FA.

100 ppm (3.33 mM)   **90 ppm** (2.99 mM**)**   **80 ppm** (2.66 mM)

(A)   (B)   (C)
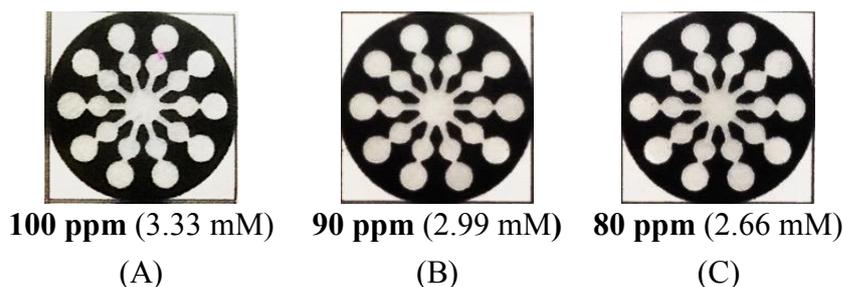
Figure 4-13   Color change at the detection zone from different concentrations of FA.

4.4 Stability of the paper-based device

The stability of the paper-based device was evaluated over a period of time under the different storage conditions such as different light exposures conditions (light and dark) as well as different temperature conditions (ambient and cold temp (6 °C)). All of the paper-based devices were prepared on the same day using the same reagents. The paper-based devices were kept in clear plastic zipper bag. The devices for dark storage condition test were wrapped with aluminum foil. FA standard solution of 100 ppm was freshly prepared for stability test. The obtained results are shown in the Table 4-6 below.

Table 4-6   Stability of the paper-based on different storage conditions

No.	Conditions		Day
	Light	**Temperature**	
1	Light	Ambient Temp	3
2	Dark	Ambient Temp	21
3	Dark	Cold Temp (6 °C)	> 30

The results from table 4-6 showed that storage of the paper-based device in exposure of light gave low stability due to the light sensitive of phenolphthalein deposited at the detection zones of the devices) Karita & Kaneta, 2014; Young, (2001. Without light exposure, storage at low temperature gave better stability than in ambient temperature, as indicated in safety data sheet to be kept in cold (Young, 2001).

For sodium sulfite kept in the vial, sodium sulfite gave less stability in light and oxygen exposure and in solution phase as described by its MSDS (Fisher Scientific, 2018; The International Agency for Research on Cancer, 1992). Therefore, sodium sulfite is kept separately in pretreatment vial in a solid form.

4.5 Interferences and tolerance limits

The effect of potential interferences in FA analysis was investigated using the compounds with similar-functional-group to the FA. The tested interferences included aldehyde compounds (acetaldehyde, buthanal, salicylaldehyde, benzaldehyde, and D-glucose) and carbonyl compounds (acetone, 3-methyl-2-butanone, and acetophenone). Structure of the studied interferences are shown in Figure 4-14.

| Acetaldehyde | Buthanal | Salicylaldehyde | Benzaldehyde |

| D-Glucose | Acetone | 3-methyl-2-butanone | Acetophenone |
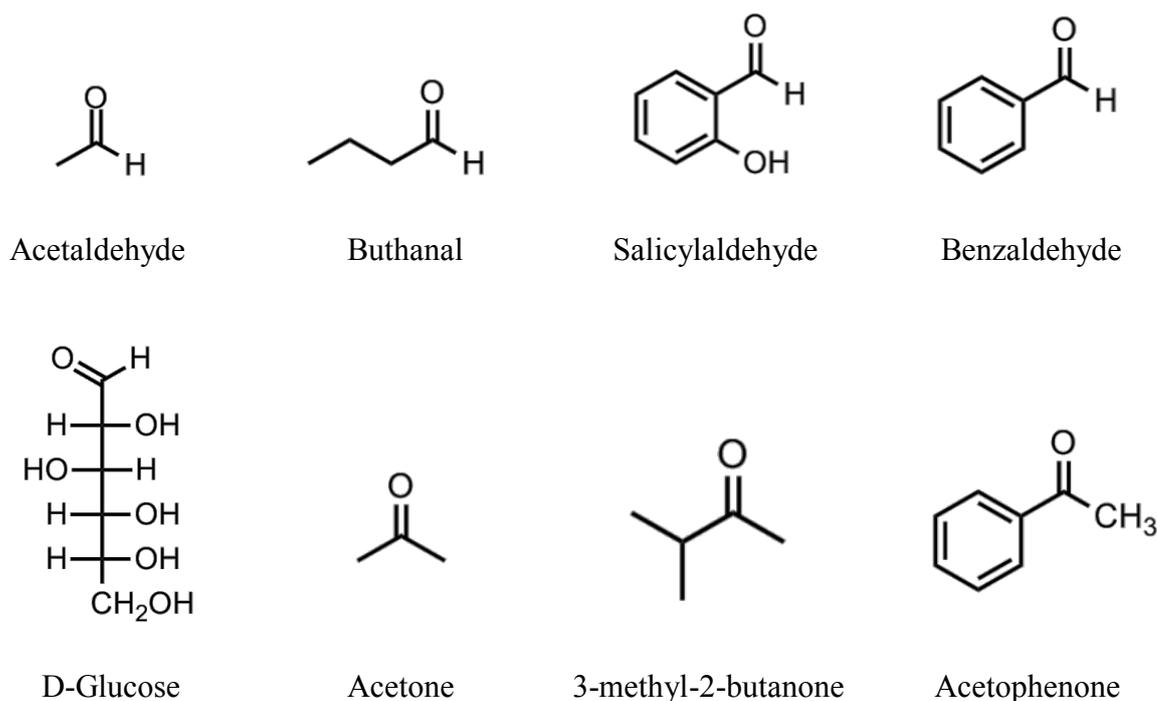
Figure 4-14   Structure of potential interferences.

Interferences and tolerance limit tests were performed using 500 ppm of FA mixed with a given amount of interference. The method was considered to be interfered from interference when color change at the detection zones were observed to be more or less than 5 zones. The result of tolerance limit of potential interferences as shown in Figure 4-15 and Table 4-7. The proposed method has high tolerance limit to carbonyl compounds, while low tolerance limit to short chain aldehyde compounds since they are reactive to sulfite. However, they presented at very low concentration in foods which are lower than the tolerance levels reported here. Acetaldehyde is mostly found in milk product (approximately 17.42 mg/kg), ripping fruits (up to 18.27 mg/kg), vegetables (up to 5.6 mg/kg), and beverages (up to 40.14 mg/kg) (Air Resources Board, 1993c; Uebelacker & Lachenmeier, 2011). Buthanal is reported to be found as essential oil in many plants and also found in may fruits e.g. apples, banana, grapes, pear, and buries etc. ("n-Butyraldehyde," 1979). Salicylaldehyde is reported to be found in many flowers and also in grapes, tomato, and cinnamon etc. ("Salicylaldehyde," 1979). Many plants are reported to be naturally contained benzaldehyde, acetophenone, 3-methyl-2-butanone as an essential oil (U.S. Department of Agriculture & Agricultural Research Service, 2016). Acetone is reported to be found in cabbage up to 800 ppm (U.S. Department of Agriculture & Agricultural Research Service, 2016).

Furthermore, acidity and basicity of the sample solutions are also found to be interfered the method but can be easily overcome by neutralization using acid or base prior to analysis.

| Acetaldehyde | D-glucose | Acetone | Acetophenone |
| 80 ppm | > 500 ppm | > 500 ppm | > 500 ppm |
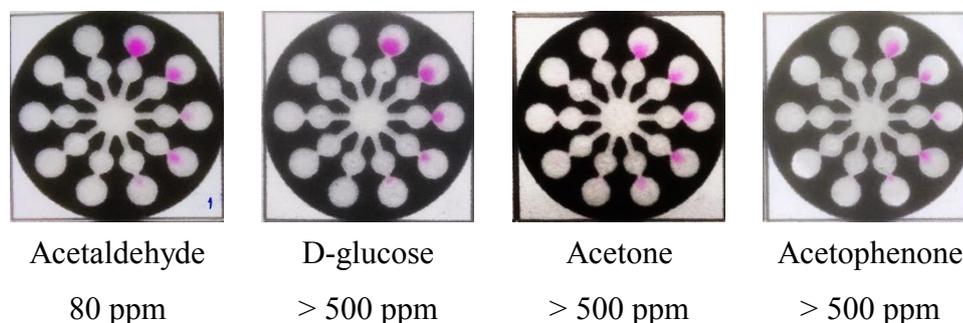
Figure 4-15   The PAD tested with FA solution mixed with given amount of some potential interferences

Table 4-7   Tolerance limit of interferences

Interferences	Tolerance Limit (ppm)
Acetaldehyde	80
Buthanal	300
Salicylaldehyde	300
Benzaldehyde	up to 1000
D-glucose	up to 1000
Acetone	up to 1000
3-methyl-2-butanone	up to 1000
Acetophenone	up to 1000

4.6 Analysis of real samples

The proposed method was used to determine FA content in foods that might contain FA. The proposed method was validated against classical titration in order to obtain the method accuracy by the analysis of FA content in these samples. The samples were prepared by in-lab sample preparation method described in Section 3.3.4. The extracted sample solution was measured for the pH (results in appendix) and neutralized prior to analysis by both classical and paper-based sulfite assay. Figure 4-16 showed some results of FA content from real food sample analysis and Table 4-8 showed the results from FA analysis in real samples obtained from the classical titration and the proposed paper-based titration. The proposed paper-based titration gave good precision (n = 5) for the analysis of real samples. Most importantly, the FA content in the samples obtained from the proposed assay agree well with those obtained from the classical sulfite titration assay. For the samples with FA concentration lower than 100 ppm, they are undetectable using the paper-based device assay that has the limit of detection of 100 ppm.

Table 4-8   Determination of FA content in real samples by the proposed paper-based titration and classical titration (n = 5)

No.	Food Sample	Classical Titration[a] (ppm)	PAD Titration[a] (ppm)
1	Pickled Ginger I	182.22 ± 0.56	100 ± 0
2	Pickled Ginger II	81.48 ± 0.70	Undetectable
3	Pickled Ginger III	Undetectable[b]	Undetectable
4	Shrimp I	271.50 ± 1.02	200 ± 0
5	Shrimp II	154.44 ± 0.48	100 ± 0
6	Shrimp III	51.36 ± 0.84	Undetectable
7	Shrimp IV	Undetectable[b]	Undetectable
8	Squid I	689.16 ± 1.83	600 ± 0
9	Squid II	34.86 ± 1.04	Undetectable
10	Crisp Squid I	458.22 ± 1.19	400 ± 0
11	Crisp Squid II	862.20 ± 2.81	800 ± 0
12	Crisp Squid III	Undetectable[b]	Undetectable
13	Crisp Squid IV	48.84 ± 0.72	Undetectable
14	Cow Tripe I	474.36 ± 1.70	400 ± 0
15	Cow Tripe II	93.87 ± 0.77	Undetectable
16	Bamboo Shoot I	Undetectable[b]	Undetectable
17	Bamboo Shoot II	Undetectable[b]	Undetectable
18	Jasmine Garland I	166.29 ± 0.85	100 ± 0
19	Jasmine Garland II	Undetectable[b]	Undetectable
20	Jasmine Garland III	18.15 ± 0.57	Undetectable

[a] Average concentration ± standard deviation of five titration

[b] There is no color change of Thymolphthalein observed when the sample solution was mixed with sodium sulfite.

The results demonstrated that the proposed paper-based device was suitable as an alternative for FA content analysis in food sample as it gives high accuracy comparing to the standard method classical titration.

Shrimp I	Squid I	Crisp Squid II	Cow Tripe I
[FA] = 200 ppm	[FA] = 600 ppm	[FA] = 800 ppm	[FA] = 400 ppm
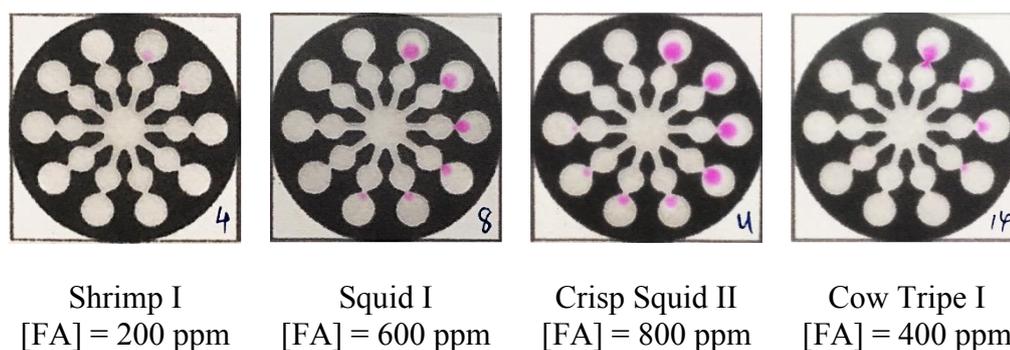
Figure 4-16   The result of FA analysis using real food sample

4.7 On-site analysis

The proposed assay was further investigated for its ability to use on-site. The sample preparation method was studied to offer the on-site preparation without the need of any laboratory-based instruments. Moreover, the device preparation was modified to investigate the possibility to be total analysis system where the sulfite was deposited onto the devices to allow for on-site and one step FA analysis in real samples.

4.7.1 Sample preparation study

The in-lab sample preparation method is complicated and is not applicable for on-site analysis of the proposed method since it required laboratory-based instruments such as vortex and centrifuge machine and also multi-step process. Therefore, another sample preparation to accommodate onsite analysis was proposed. The experiment in this section was performed to investigate the effect of different sample preparations on the analysis. A piece of sample was divided into two pieces for both in-lab and on-site sample preparations. Both sample preparation methods were achieved under the constant sample-solution ratio of 1 g sample to 2 mL solution. The sample

preparation procedure both for in-lab and on-site methods are described in Chapter 3. The extracted solution obtained from both methods were analyzed for FA content using the developed paper-based devices and the results are shown in Table 4-9.

Table 4-9   FA analysis in food sample using different methods of sample preparation

Food Sample	FA concentration (ppm) (n=3)	
	In-Lab	On-Site
Pickled Ginger	100 ± 0	100 ± 0
Cow Tripe	400 ± 0	400 ± 0
Crisp Squid	700 ± 0	600 ± 0

The result showed that, both methods of sample preparation gave the similar range of FA concentration. FA contents in pickled ginger and cow tripe obtained from the PAD analysis using both sample preparation methods were similar. However, laboratory-based sample preparation gave slightly higher FA concentration than on-site sample preparation for the analysis of crisp squid. These variations might be the results of the differences in sample texture. Pickled ginger and cow tripe are sold as thinner and softer pieces than crisp squid and hence, there is more surface area per sample volume available for FA to react with sodium sulfite. It should be noted that if the samples were cut as smaller pieces for on-site testing, the results of FA content would be similar to those obtained from the laboratory-based sample preparation method. Moreover sample preparation method was also investigated for on-site analysis by comparing the FA content obtained from the water extracted samples prior to react with sulfite and the samples that were put directly to the sulfite solution. The solution from both methods were tested with PAD to investigate how this condition affect FA concentration.

Table 4-10　　　Different FA extraction methods in real food sample for on-site analysis

Food Sample	FA concentration (ppm) (n=3)	
	Sulfite in Bottle[a]	Water in Bottle[b]
Cow Tripe	500 ± 0	300 ± 0

[a] A 1-mg piece of sample is added in the preparing bottle containing sodium sulfite powder followed by adding 2 mL of DI water. The final solution is titrated on µPAD.

[b] A 1-mg piece of sample is added in the preparing bottle containing 2 mL of DI water. 2-mL of the solution is transferred to the other preparing bottle containing sodium sulfite powder. The final solution is titrated on µPAD.

The result from table 4-10 showed that higher amount of FA was obtained by directly reacting the FA in the samples with sulfite than that obtained from the reaction of the water extract solution and sulfite. This might be the results of less loss of FA from the sample in case of directly reacting than the loss that might occur during the water extraction step. Therefore, sodium sulfite should be contained in the pretreatment vial and the samples should be put directly to the sulfite power together with some DI water.

4.7.2 Improvement of the paper-based preparation

The proposed method of FA analysis consisted of two-step analyses which are the reaction of FA in sample with sulfite in a pretreatment vial and the acid-based titration on the PAD. Further improvement toward one-step analysis was investigated. To accommodate one-step analysis, sulfite was first deposited on the sample zone of the paper-based device instead of filling in the vial to allow all reaction for FA analysis take place only on the paper-based device. The water extract of FA in the sample reacted with sodium sulfite at the sample zone to generate the NaOH product. The NaOH reached the reaction zone to be neutralized by KHP, and the remaining reached the detection zone.

Table 4-11   One-Step and Two-Step analysis of FA

FA Sources	FA concentration (ppm) (n=3)	
	In-Vial[a]	On-Device[b]
Standard Solution	500	500
	500	400
	500	400
Cow Tripe	500	400
	500	400
	500	400

[a] The pretreatment vial contained sodium sulfite powder. (Two-step analysis)

[b] Sodium sulfite was prepared as a solution and deposited to the sample zone of the PAD. (One-step analysis)

As shown in table 4-11, the FA obtained from the one-step analysis were different from that obtained from two-step analysis. The two-step analysis gave higher amount and more accurate results from standard FA analysis. This might be the result of incomplete reaction between FA and sodium sulfite in the sample zone. In addition, some sodium sulfite could also spread through the reaction zone before reacting with FA. In addition, sodium sulfite is more stable in form of solid rather than solution. Moreover, warm temperature could lead sodium sulfite to be decomposed more easily. Therefore, the two-step analysis was still the most suitable process for FA analysis using PAD-based titration assay.

CHAPTER 5
CONCLUSION AND FUTURE PERSPECTIVES

5.1 Conclusions

This study demonstrated the development alternative FA detection using paper-based titration as a detection platform. The sample-pretreated vial is used together with the paper-based device to achieve more rapid test with low analysis cost. The FA detection is based on sulfite assay where NaOH produced from the reaction of sodium sulfite and FA can cause the distinct color-change of phenolphthalein from colorless to bright pink in the detection zone. Various amounts of KHP in the reaction zone was neutralized NaOH spreading out from sample zone. Different amount of unneutralized NaOH reached the detection zone caused pink color appeared. The number of color change in detection zone is directly proportional to the amount of FA which is also corresponding to the amount of KHP in the reaction zone.

The paper-based device is designed to have a radial shape which consists of a central 7-mm-diameter sample zone connected through ten narrow channels (2-mm long and 1.5-mm width) to the 4-mm-diameter reaction and 5-mm-diameter detection zones, respectively. The device was fabricated on the Whatman no.1 filter paper by wax-printing technique. Moreover, concentration numbers were also added beside the detection zones for more convenient interpretation. After heated and covered the back with clear adhesive tape, the polyethylene glycol and phenolphthalein was added to the detection zone and different amount of KHP was added to the reaction zone of the PAD. The analysis can be simple performed by adding the piece of samples or sample solution containing FA to the vial contained only sodium sulfite powder. The NaOH mixture solution product was measured by applying 30 µL of pretreated sample solution to the sample zone. Two types of sample preparation were studied to demonstrate the applicability of the developed methods including in-lab and on-site sample pretreatments. For in-lab experiment, sample preparation was performed by homogenizing weighed sample in a blender with DI water followed by sonication and centrifugation, and supernatant was kept in cold as a sample solution. A 2-mL-sample solution was added to the sample-pretreated vial containing sodium sulfite and shake

vigorously and analyzed for FA content by the paper-based devices. For on-site analysis sample pretreatment, a 2-mL clean water was added to the sample-pretreated vial containing sodium sulfite followed by a piece of sample, lid and shake vigorously. The mixture solution was analyzed for FA content in the samples. The sample to answer process can be completed within 5 minutes using the on-site sample pretreated system and the developed paper-based method.

The PAD gave range of FA analysis from 100 to 1000 ppm and the limit of detection (LOD) was 100 ppm. Furthermore, potential interfering compounds including acetaldehyde, buthanal, salicylaldehyde, benzaldehyde, D-glucose, acetone, 3-methyl-2-butanone, and acetophenone were tested and only acetaldehyde was found to interfere with low tolerance limit of 80 ppm. Fortunately, this compound is not normally found in FA containing samples. Some sample solution such as pickled ginger, bamboo shoot, and jasmine garland were found to be slightly acidic. NaOH solution was added to neutralize these sample solutions prior to analysis. The PAD and sample-pretreated vial containing sodium sulfite were found to be stable for more than a month when store in cold and dark condition.

The proposed FA analysis method provided good accuracy in 20 samples compared to those obtained from classical titration method indicating that the developed assay is suitable to apply for FA detection with low-cost, rapid, instrument free, and low-reagent-consumption analysis. In addition, problem of background's color of sample solution can be overcome using the proposed device.

5.2 FUTURE PERSPECTIVE

The proposed analysis platform can be improved to be more suitable for on-site analysis. Stability of the paper-based device and pretreated vial are considered to be improved in future study to obtain more suitable condition that allow the paper-based device to be kept longer as it might be used as a commercial test kit in the future. Moreover, the paper-based titration platform can be developed to be available for other substance analysis in different samples using the chemistry of acid-base titration.

APPENDIX

| 20µL | 25µL | 30µL | 35µL |
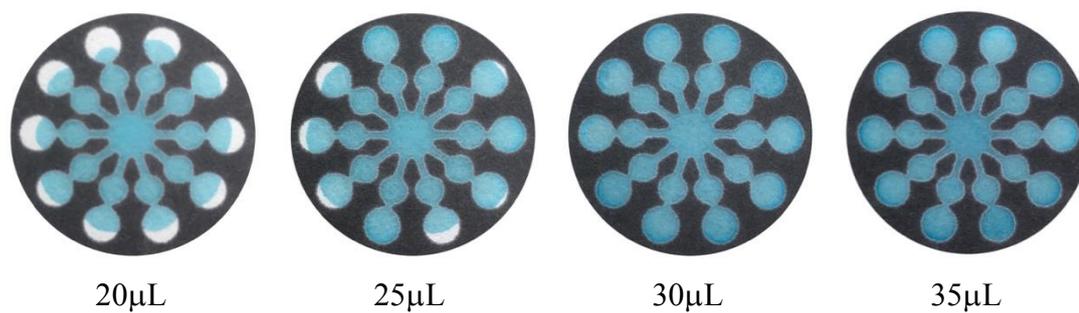
Figure A-1   Optimization of volume occupied the test zone using dye
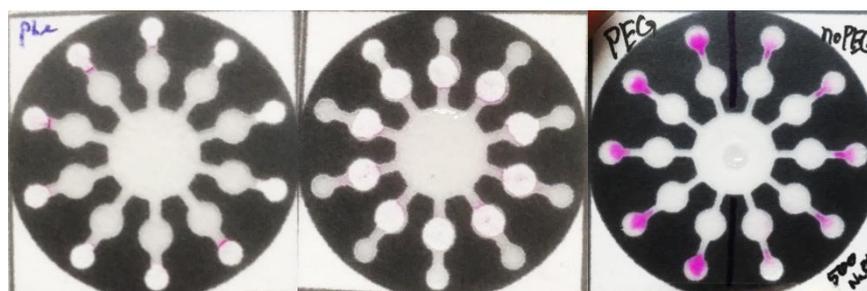
Figure A-2   Confirmation of using Polyethylene Glycol

Table A-1   pH of Sample Solution (Using pH strip)

No.	Food Sample	pH[a]
1	Pickled Ginger I	4
2	Pickled Ginger II	5
3	Pickled Ginger III	4
4	Shrimp I	7
5	Shrimp II	7
6	Shrimp III	7
7	Shrimp IV	7
8	Squid I	7
9	Squid II	7
10	Crisp Squid I	7
11	Crisp Squid II	7
12	Crisp Squid III	7
13	Crisp Squid IV	7
14	Cow Tripe I	7
15	Cow Tripe II	7
16	Bamboo Shoot I	4
17	Bamboo Shoot II	4
18	Jasmine Garland I	4
19	Jasmine Garland II	5
20	Jasmine Garland III	4

[a] Any solution with pH is lower than 7 is neutralized using dilute NaOH

Table A-2   Results of different concentrations of FA tested with the PAD (n=5)

[FA]	1	2	3	4	5
100 ppm					
200 ppm					
300 ppm					
400 ppm					
500 ppm					
600 ppm					
700 ppm					
800 ppm					
900 ppm					

Table A-2   Results of different concentrations of FA tested with the PAD (n=5) (cont.)

[FA]	1	2	3	4	5
1000 ppm					

Table A-3   Results of potential interferences test for determination of 500 ppm FA.

No.	Interferences List	Tolerance Limit (ppm)	µPAD result[a]
1	Acetaldehyde	80	
2	Buthanal	300	
3	Salicylaldehyde	300	
4	Benzaldehyde	> 500	
5	D-glucose	> 500	
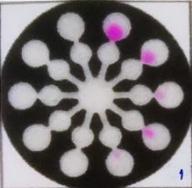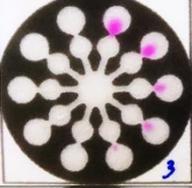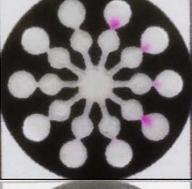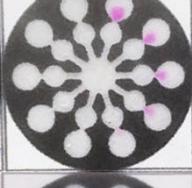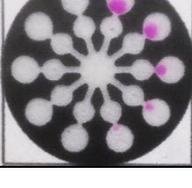
No.	Interferences List	Tolerance Limit (ppm)	μPAD result[a]
6	Acetone	> 500	
7	3-methyl-2-butanone	> 500	
8	Acetophenone	> 500	
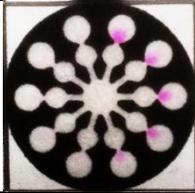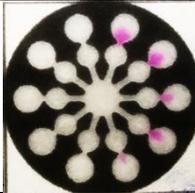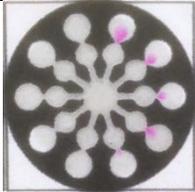
Table A-4   Results of real food samples analysis using the PAD Titration

No.	Sample [FA]	Test Samples (n=5)	Sample Blank
1	Pickled Ginger I [100 ± 0]		
2	Pickled Ginger II [Undetectable]		
3	Pickled Ginger III [Undetectable]		
4	Shrimp I [200 ± 0]		
5	Shrimp II [100 ± 0]		
6	Shrimp III [Undetectable]		
7	Shrimp IV [Undetectable]		
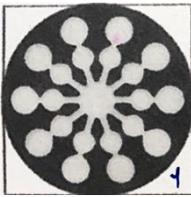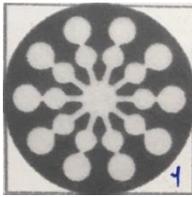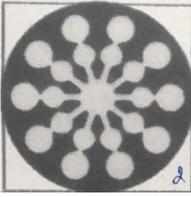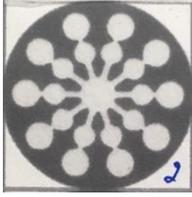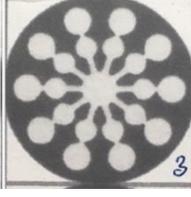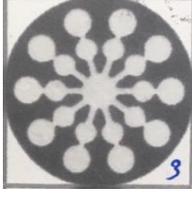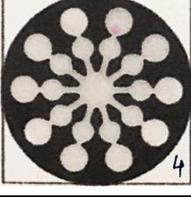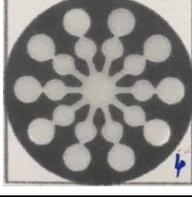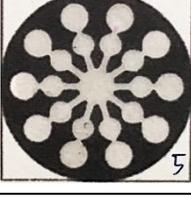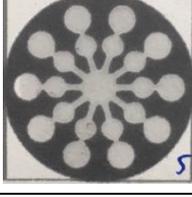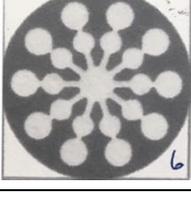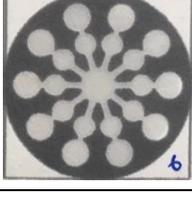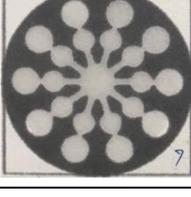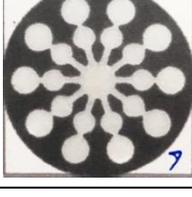
Table A-4   Results of real food samples analysis using the PAD Titration (cont.)

No.	Sample [FA]	Test Samples (n=5)	Sample Blank
8	Squid I [600 ± 0]		
9	Squid II [Undetectable]		
10	Crisp Squid I [400 ± 0]		
11	Crisp Squid II [800 ± 0]		
12	Crisp Squid III [Undetectable]		
13	Crisp Squid IV [Undetectable]		
14	Cow Tripe I [400 ± 0]		
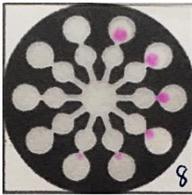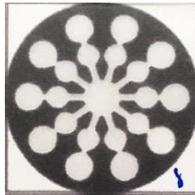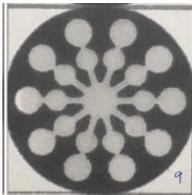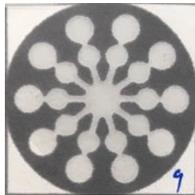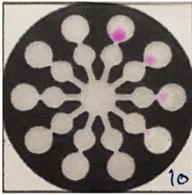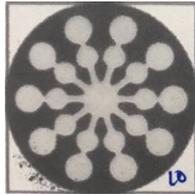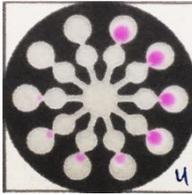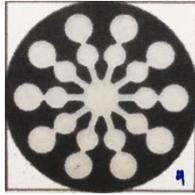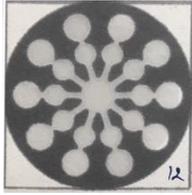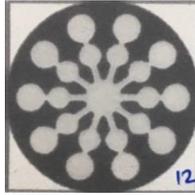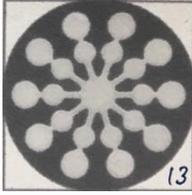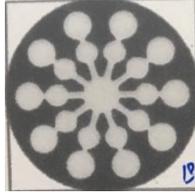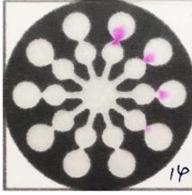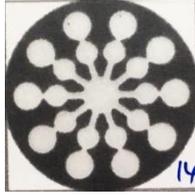
Table A-4   Results of real food samples analysis using the PAD Titration (cont.)

No.	Sample [FA]	Test Samples (n=5)	Sample Blank
15	Cow Tripe I [Undetectable]		
16	Bamboo Shoot I [Undetectable]		
17	Bamboo Shoot II [Undetectable]		
18	Jasmin Garland I [100 ± 0]		
19	Jasmin Garland II [Undetectable]		
20	Jasmin Garland III [Undetectable]		
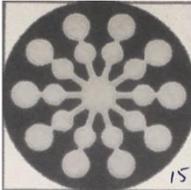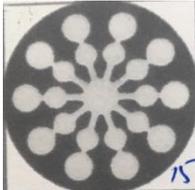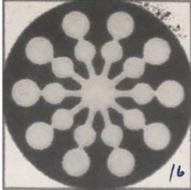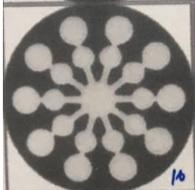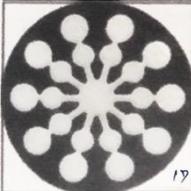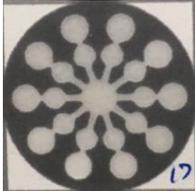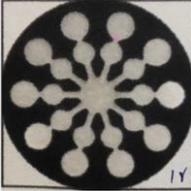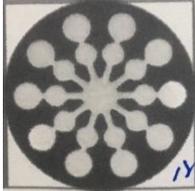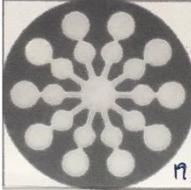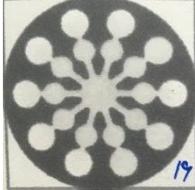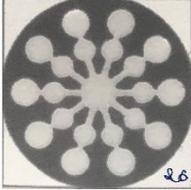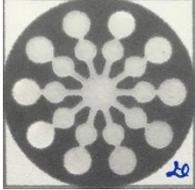
Table A-5   Amount of FA intake for digestive toxicity estimation

No.	Food Sample	FA Content (mg/kg)	Average Consumption[a] (g/p/d)	Intake[c] (mg/kg/day)
1	Pickled Ginger I	362.77	30	0.18
2	Pickled Ginger II	162.70	30	0.08
3	Pickled Ginger III	Undetectable[b]	30	Undetectable[b]
4	Shrimp I	536.99	60	0.54
5	Shrimp II	305.88	60	0.31
6	Shrimp III	102.51	60	0.10
7	Shrimp IV	Undetectable[b]	60	Undetectable[b]
8	Squid I	1361.44	50	1.13
9	Squid II	69.53	50	0.06
10	Crisp Squid I	920.30	20	0.31
11	Crisp Squid II	1725.78	20	0.58
12	Crisp Squid III	Undetectable[b]	20	Undetectable[b]
13	Crisp Squid IV	97.58	20	0.03
14	Cow Tripe I	934.51	80	1.25
15	Cow Tripe II	189.83	80	0.25
16	Bamboo Shoot I	Undetectable[b]	40	Undetectable[b]
17	Bamboo Shoot II	Undetectable[b]	40	Undetectable[b]

[a] Estimation based on amount of Thai people consumption per day (Office of Standard Development, 2016)

[b] There is no color change of Thymolphthalein was observed since the sample solution is mix with sodium sulfite.

[c] The body weight was assumed to be 60 kg

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