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DEVELOPMENT OF DISTANCE PAPER-BASED DEVICES AND ELECTROCHEMICAL SENSORS  
FOR FOOD AND COSMETIC SAFETY ASSESSMENT



A Dissertation Submitted in Partial Fulfillment of the Requirements  
for the Degree of Doctor of Philosophy in Biomedical Sciences  
Inter-Department of Biomedical Sciences  
GRADUATE SCHOOL  
Chulalongkorn University  
Academic Year 2020  
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การพัฒนาอุปกรณ์ฐานกระดาษแบบวัดระยะทางและตัวรับรู้ทางเคมีไฟฟ้าสำหรับประเมินความ  
ปลอดภัยในอาหารและเครื่องสำอาง



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต  
สาขาวิชาชีวเวชศาสตร์ สหสาขาวิชาชีวเวชศาสตร์  
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Thesis Title	DEVELOPMENT OF DISTANCE PAPER-BASED DEVICES AND ELECTROCHEMICAL SENSORS FOR FOOD AND COSMETIC SAFETY ASSESSMENT
By	Miss Kasinee Katelakha
Field of Study	Biomedical Sciences
Thesis Advisor	Associate Professor Wanida Laiwattanapaisa, Ph.D.
Thesis Co Advisor	Assistant Professor VANIDA NOPPONPUNTH, Ph.D.

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Accepted by the GRADUATE SCHOOL, Chulalongkorn University in Partial  
Fulfillment of the Requirement for the Doctor of Philosophy

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เกษิณี เกตุเลขา : การพัฒนาอุปกรณ์ฐานกระดาษแบบวัดระยะทางและตัวรับรู้ทางเคมีไฟฟ้าสำหรับประเมินความปลอดภัยในอาหารและเครื่องสำอาง. ( DEVELOPMENT OF DISTANCE PAPER-BASED DEVICES AND ELECTROCHEMICAL SENSORS FOR FOOD AND COSMETIC SAFETY ASSESSMENT) อ.ที่ปรึกษาหลัก : รศ. ดร.วนิดา หลายวัฒนไพศาล, อ.ที่ปรึกษาร่วม : ผศ.วนิดา นพพรพันธุ์

งานวิจัยนี้มีวัตถุประสงค์เพื่อผนวกการใช้วิธีวิเคราะห์ในการสนับสนุนการตรวจประเมินความปลอดภัยในอาหารและเครื่องสำอาง วิธีวิเคราะห์ 2 วิธี ได้ถูกนำเสนอในงานวิจัยนี้ ประกอบด้วย 1). การพัฒนาอุปกรณ์ฐานบนกระดาษสำหรับการตรวจตะกั่วในตัวอย่างอาหาร 2). การพัฒนาการตรวจวัดกรดคาร์บอนิกในผลิตภัณฑ์ฮาลาลโดยใช้วิธีเคมีวิเคราะห์เชิงไฟฟ้าบนขั้วพิมพ์สกรีนชนิดใช้ครั้งเดียว (SPE) สำหรับวิธีการแรก อาศัยหลักการจับแบบแข่งขันระหว่างกรดคาร์บอนิก (CA) และโพลีเอทิลีนเอมีน (PEI) กับตะกั่วในตัวอย่างอาหาร โดยที่การลดลงของระยะทางของ PEI-CA บนกระดาษ เป็นสัดส่วนกับปริมาณตะกั่ว สามารถทดสอบการจำแนกอาหารที่มีตะกั่วมากกว่าหรือน้อยกว่า  $2 \mu\text{g}\cdot\text{mL}^{-1}$  ได้โดยอาศัยวิธีเติมสารมาตรฐานตะกั่ว (standard addition) ความเข้มข้น 8.0, 9.0, และ  $10.0 \mu\text{g}\cdot\text{mL}^{-1}$  ลงไปในตัวอย่างอาหารและทดสอบบน dPAD การอ่านผลใช้หลักการวาดเส้นเชื่อมต่อจากระยะทางที่เกิดสียบนกระดาษ (drawing PAD) ได้ผลสอดคล้องกับปริมาณตะกั่วที่วัดได้โดยวิธีมาตรฐาน Atomic Absorption spectroscopy (AAS) สำหรับผลการทดลองที่ได้จากวิธีการที่สอง ซึ่งเป็นการแสดงการตรวจวัดกรดคาร์บอนิกและคาร์โมอีซินแบบพร้อมกันได้ภายใต้สภาวะกรด (100 mM sodium citrate buffer, pH 3.0) โดยให้ความต่างศักย์แบบ differential pulse voltammetry (DPV) ในช่วงความต่างศักย์  $-1.5 - +1.5$  โวลต์ บนขั้วไฟฟ้าที่ไม่ได้ผ่านการปรับปรุงพื้นผิว (unmodified electrode) พบว่ามีขีดต่ำสุดของการวัดคือ  $33.68 \mu\text{M}$  ความแม่นยำของวิธีวิเคราะห์แสดงโดยค่าการเบี่ยงเบนสัมพัทธ์ระหว่าง 2.78 – 9.52 เปอร์เซ็นต์ การทดสอบความใช้ได้ของวิธีวิเคราะห์ในตัวอย่างลิปสติก พบค่าเปอร์เซ็นต์การคืนกลับ (recovery) มากกว่า 80 เปอร์เซ็นต์ สอดคล้องกับปริมาณที่คำนวณได้จากวิธีเทคนิคโครมาโทกราฟีแบบของเหลวสมรรถนะสูงยิ่งยวด (ultra-high performance liquid chromatography) ค่าเปอร์เซ็นต์การคืนกลับเมื่อทดสอบในตัวอย่างน้ำผลไม้และสารสัณฐานดิบตั้งต้นเกรดเครื่องสำอาง พบว่าอยู่ในช่วง 101.9 – 123.6 เปอร์เซ็นต์ และ 93.2 – 109.0 เปอร์เซ็นต์ตามลำดับ นอกจากนี้เพื่อเพิ่มความไวของการวิเคราะห์ให้เพียงพอต่อปริมาณกรดคาร์บอนิกในอาหาร จึงได้ปรับปรุงพื้นผิวของขั้วนำไฟฟ้าด้วยอนุภาคทองคำนาโนและโพลีเอทิลีนเอมีน (AuNP/PEI) พบว่าความไวของการวิเคราะห์เพิ่มขึ้น 24 เท่าเมื่อเทียบกับการวัดบนขั้วไฟฟ้าที่ไม่ได้ปรับปรุงพื้นผิว และมีช่วงความเป็นเส้นตรงระหว่าง 10– 100 ไมโครโมลาร์ ( $R^2 = 0.986$ ) เมื่อพิจารณาที่ความต่างศักย์ 0.297 โวลต์ กล่าวโดยสรุป วิธีวิเคราะห์เชิงไฟฟ้าเคมีบนขั้วพิมพ์สกรีนที่ปรับปรุงพื้นผิวด้วยอนุภาค AuNP/PEI เป็นวิธีการที่มีศักยภาพในการวิเคราะห์กรดคาร์บอนิกเพื่อสนับสนุนกระบวนการตรวจประเมินความปลอดภัยในผลิตภัณฑ์ฮาลาล

CHULALONGKORN UNIVERSITY

สาขาวิชา ชีวเวชศาสตร์  
ปีการศึกษา 2563

ลายมือชื่อนิสิต .....  
ลายมือชื่อ อ.ที่ปรึกษาหลัก .....  
ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

# # 5887818620 : MAJOR BIOMEDICAL SCIENCES

KEYWORD: carminic acid-lead complex, halal safety assessment, electrochemistry of carminic acid,  
distance-based paper device, portable paper-based device

Kasinee Katelakha : DEVELOPMENT OF DISTANCE PAPER-BASED DEVICES AND ELECTROCHEMICAL  
SENSORS FOR FOOD AND COSMETIC SAFETY ASSESSMENT. Advisor: Assoc. Prof. Wanida  
Laiwattanapaisa, Ph.D. Co-advisor: Asst. Prof. VANIDA NOPPONPUNTH, Ph.D.

This study aimed to integrate analytical techniques for supporting the safety assessment in food and cosmetics. The two analytical methods were proposed, including i) the development of the paper-based device for the screening detection of lead (Pb) in food matrices, ii) development of the disposable screen-printed electrode for the detection of non-permitted color in halal products. For the first approach, the assay principle was based on competitive binding between carminic acid (CA) and polyethyleneimine (PEI) to Pb in food samples. The reduction of the PEI-CA color distance is proportional to the concentration of Pb. To categorize food which higher or lower the cut-off of the Pb concentration ( $2 \mu\text{g}\cdot\text{mL}^{-1}$ ), the standard addition technique combined with the concept of a drawing PAD was integrated using a series of standard addition (8.0, 9.0, and  $10.0 \mu\text{g}\cdot\text{mL}^{-1}$ ). The results obtained from the dPAD are in accordance with the concentration measured by the atomic absorption spectroscopy (AAS). The results from the second approach demonstrated that carminic acid and carmoisine were simultaneously assayed under the acidic condition (100 mM sodium citrate buffer, pH 3.0). The electrode was scanned with differential pulse voltammetry (DPV) from  $-1.5 \text{ V}$  to  $+1.5 \text{ V}$ , and by using a bare unmodified electrode, the detection limit was obtained at  $33.68 \mu\text{M}$ . The method was obtained with acceptable precision with 2.78– 9.52 %CVs. We demonstrate the proof-of-concept for the detection of carminic acid in lipsticks. The recovery of  $> 80\%$  was comparable to those obtained by ultra-high performance liquid chromatography (ultra-HPLC). The recovery study of carminic acid in the beverage and raw material (cosmetic grade) ranged from 101.9% – 123.6% and 93.2% – 109.0%, respectively. Furthermore, to improve the sensitivity of this technique sufficient to detect the CA fortified in actual food samples, the electrode surface was modified with AuNP/PEI composite. Interestingly, the sensitivity was dramatically 24-times improved comparing to the bare electrode. A linear range of  $10 - 100 \mu\text{M}$  ( $R^2 = 0.986$ ) was achieved by using DPV and setting the potential at  $0.297 \text{ V}$ . In conclusion, an electrochemical method based on a screen-printed electrode modified with AuNP/PEI composite was a promising tool for sensitive detection of carminic acid for assisting the safety monitoring in halal products.

Field of Study: Biomedical Sciences

Academic Year: 2020

Student's Signature .....

Advisor's Signature .....

Co-advisor's Signature .....

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Kasinee Katelakha

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## Part 1

### 1. The integrating of the analytical chemistry as an improved approaches to support the safety assessment in food and cosmetic

Safety assessment involves the process of identifying, analyzing, and characterization of product-related health risks. It is a mandatory practical protocol for ensuring that a product does not endanger consumer health. The safety assessment can be included in any food chain management. In this context, the analysis of relevant contaminants is an essential part of food safety assessment, to ensure consumer safety and improve the quality of products. It can be performed to detect or confirm chemical or biological contaminants, in order to assess the authenticity of the products. However, it usually requires sophisticated laboratory infrastructure and expensive reagents, which are critical issues for resource-constrained settings in most developing countries.

Because of its simplicity, portability, disposability, biocompatibility, and inexpensiveness, paper-based analytical devices (PADs) are widely used as POCT devices. Furthermore, the paper substrate shows attractive merit in its absorptive performance and flow-controlled properties due to its hydrophilicity and the capillary force of the fibrous structure. In addition, the hydrophobic parting of the paper substrate can be generated by printing using hydrophobic inks, wax, paraffin, or silicones (Nishat et al., 2021). Although PADs usually offer a limitation in terms of sensitivity, several strategies can be used to enhance the sensitivity on this platform. There are several reports in the literature including, immobilizing of molecules at the solid surface (Hong et al., 2018), designing and fabricating for programmed liquid flow (Komatsu et al., 2021), manipulating the liquid flow by printing dissolvable compounds

on paper to delay to pass through the detection zone (Lutz et al., 2013), and using nanoparticles for optical enhancement (Huang et al., 2016; Morbioli et al., 2017)

Generally, PADs are comprised of the paper as a substrate, molecular probe or recognition element, and analytes. The strategies for detection depend on the reaction on each platform. The first attempt for this study is the development of the distance-based paper device (dPAD) for the detection of lead (Pb) in food matrices. The dPAD is an approach relying on a color distance generated by the reaction of analytes traveling along the microfluidic paper channel. The length is usually proportional to the analyte's concentration. Therefore, semi-quantification and quantification analysis can be straightforward achieving without the need for additional sophisticated readout equipment.

The dPADs can integrate into broad applications such as environmental detection, molecular diagnostic, and food safety management. In this study, the implementation of the dPAD into metal ion detection in food matrices is proposed. The dPAD was designed to have a detection channel connected with the sample loading area. The detection channel was pre-impregnated with polyethyleneimine (PEI). The sample analyte was mixed with CA color in the microcentrifuge tube for the complex formation of CA-Pb. This CA-Pb complex will be absorbed in the sample loading area, while the remaining of the CA color traveled to form a complex with PEI on the detection channel. Therefore, reducing the color distance at the detection channel was proportional to the Pb concentration in the sample. As a proof of concept, the dPAD could be applied to determine Pb in century eggs.

The sensitivity of the developed dPAD was relatively high. In addition, interference by the sample matrices affected the distance measurement.



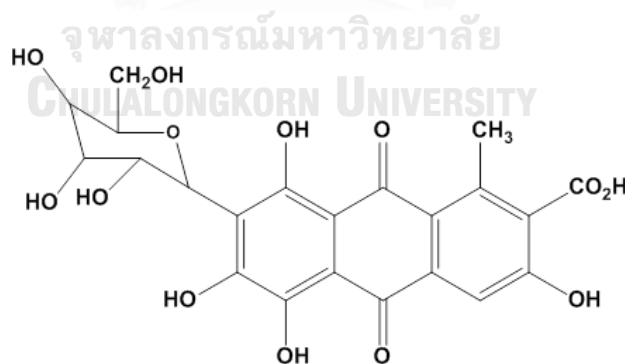
Therefore, the standard addition technique was used to minimize the interference in the food matrices. Standard solution of Pb (8.0, 9.0, and 10.0  $\mu\text{g}\cdot\text{mL}^{-1}$ ) was added to the samples before sample analysis. The drawing PAD was combined with distance readout for Pb sample at lower or higher concentrations than the cut-off (2.0  $\mu\text{g}\cdot\text{mL}^{-1}$ ). The results obtained by the developed dPAD were in good agreement with those obtained by atomic absorption spectroscopy (AAS). The dPAD is thus promising in terms of its rapidity and inexpensive cost of production. This article has been published in Biosensors, DOI: 10.3390/bios11030090. The details of the technology and novelty of the developed dPAD were described in Part 2.

Alongside physical, chemical, and biological hazards in food safety management, hazards exist regarding religious or consumer faith, such as halal and kosher status. Halal describes non-permissible substances to be used or consumed by Muslims. In Arabic, the word 'halal' means permissible, while 'haram' means non-permissible. Haram items include porcine and porcine derivatives, prohibited food ingredients of animal origin, alcohol, and products that have not been prepared with clean equipment (Hanzaee & Ramezani, 2011). Because food is a fundamental principle of life, Muslim awareness regarding the consumption of food contaminated by non-permissible substances, especially ingredients that originate from unlawful animals, has been increasing. Moreover, the complexity of food science and technology is well-known to Muslim consumers. Therefore, information on food sources and their processing is gaining greater awareness. This fact has led to the introduction of science-based analytical approaches to halal safety management.

The detection of porcine and its by-products has received the most attention from scientists in this area (Hassan et al., 2018). The detection of porcine substances relies on porcine protein (Hassan et al., 2018)50,51),

volatile compounds (Nurjuliana et al., 2011), and nucleotides (Nikzad et al., 2017). Methods such as LC-MS/TOF, FTIR, or real-time-PCR provide sensitivity and high throughput. However, specialized instruments and well-trained personnel are required. The implementation of analytical methods could provide sufficient information regarding risk contamination and authentication of unclearly labelled products, and is not limited to the information of the actual sources of the samples e.g. gelatin. Hence, implementing analytical methods into halal safety assessment could facilitate and enhance the quality and safety of halal products.

E120, or carminic acid, has been widely used as a color additive in food, cosmetics, and pharmaceutical products. The chemical structure of carminic acid is presented in Figure 1. This color is derived from an insect origin. Therefore, it is not permitted in halal products. However, no analytical method has been used before to detect CA to support halal safety assessment and certification. Thus, the development of an analytical technique for the determination of CA was proposed in this thesis.



*Figure 1 structure of the carminic acid (CA)*

The sensitive analytical technique for the detection of CA proposed in this study was based on the ability of CA to form a redox reaction. The differential pulse voltammetry (DPV) of CA was found at a carbon-screen

printed electrode (SPE). CA gives a single well-defined oxidation peak at ca. +0.207 V and a single reduction peak at -0.791 V, vs. Ag/AgCl in 100 mM citrate buffer solution at pH 3.0. A linear response of CA was obtained in the range of 0.01 to 0.40 mM for the bare electrode. The limit of detection (LOD) was 33.68  $\mu$ M. Furthermore, the proposed method was validated and successfully applied to the determination of CA in spiked samples of lipstick and food beverages, with satisfactory recoveries in the range of 80.1–123.6%. The effect of potentially interfering compounds on the peak current response of CA was also studied. The presence of an alternative synthetic red color, carmoisine (CM), did not interfere with the characteristic potential of CA. Therefore, the simultaneous detection of CA in the presence of carmoisine was achieved. Compared with the bare electrode, a modified AuNP/PEI SPE that offered good charge-transport characteristics significantly enhanced the sensitivity. This method was the first application of an electrochemical biosensor with a good performance for the rapid and specific detection of CA, that can be utilized in halal assurance safety management. The details of the technology and novelty of this proposed electrochemical sensor were described in Part 2. The manuscript was planned to submit to Food Technology and Biotechnology (FTB).

The information of this dissertation was submitted in partial fulfillment towards one of the requirements for a Ph.D. degree in Biomedical Science, Chulalongkorn University.

## **2. Background and significance of the study**

### *2.1 Hazardous substances in food*

In the context of food safety management, there are three types of hazards: biological hazards, chemical hazards, and physical hazards. The presence of physical hazards in food can be eliminated through appropriate hygienic practices (GHP). Moreover, sanitary conditions can reduce and

prevent foreign materials from unintentionally being introduced to food. Chemical hazards include chemical pollutants, metal ions, pesticides, insecticides, etc. Exposure can occur by absorption from soil and water, residues of veterinary drugs and supplements, and heavy metals used in pesticides and insecticides, which can cause harm to humans (Sonone et al., 2020). Biological hazards, such as mycotoxins, natural toxins, and environmental toxins may also be introduced to food and cause foodborne illness and other adverse health effects. A systematic preventive approach has been introduced to reduce and eliminate hazards in food, including hazard analysis and critical control points (HACCP) to control risks and improve food safety and quality.

## 2.2 *Food safety assessment*

There are four steps of risk assessment: i) hazard identification, ii) hazard characterization, iii) exposure assessment, and iv) risk characterization. Assessment helps estimate the feasibility and the severity of an adverse health effect that could occur due to exposure to hazards. Also, it can be used to evaluate substances that have been added intentionally to food, such as food additives, processing aids, and chemicals from agricultural or veterinary sources. In addition, the substances that occur unintentionally include chemical contaminants, biological contaminants, and environmental contaminants were included to those risk assessments. The assessment supports decisions around legal measures that should be taken to prevent harm by a food.

Safety during the food supply chain aims to preserved food safety and quality. The production process must be safe from microbial, chemical, personally related incidents and environmental hygiene (Fung et al., 2018). It was reported that food could be contaminated during harvest, slaughtering, preparation, production, storage, and distribution. Food safety and quality can

be conducted from the initial stage to the final stage of food production. Therefore, several activities have been involved such as implementing the good agricultural practice (GAP), hazard analysis and critical control point (HACCP), and good manufacturing practice (GMP) to minimize the contamination in finished products.

### 2.3 *Lead contamination in food*

Regarding the analysis of hazards, the detection of heavy metals in food and food-related products is crucial because it can cause several adverse effects on health. Heavy metals have a bioaccumulative nature; they can accumulate in living organisms (Crosby, 1977; Hao et al., 2019; Järup, 2003). The main threats of heavy metals for humans are associated with exposure to arsenic (As), copper (Cu), and lead (Pb). Pb is currently used in various products and industries, including the agricultural, electricity, battery, paint, and food wares industries (Kumar et al., 2020). Pb can be deposited in organisms from natural sources and anthropogenic sources. For example, Pb in pesticides used during crop production can be absorbed in the soil and released into the water. Pb can be absorbed by plants and cannot be entirely removed by washing. Contamination of Pb in food can also occur intentionally by illegal methods in some types of food products. One of the products that may contain a high level of Pb is century eggs, because of the addition of lead oxide (PbO) to facilitate the penetration of sodium hydroxide (NaOH). The procedure involves the reaction of sodium carbonate ( $\text{Na}_2\text{CO}_3$ ), water ( $\text{H}_2\text{O}$ ), and calcium oxide (CaO), of the piking coating mud into the eggs (Wang & Fung, 1996). As a result, misuse of lead oxide can be found during century eggs production to shorten formation time. Therefore, Pb can be found at a higher concentration than those was allowed by the Food and Drugs Administration (FDA) in century eggs (Xu et al., 2014).

#### 2.4 *Analytical method for detection of lead*

Most available detection tools for Pb depend on sophisticated and large instruments, such as inductively coupled plasma mass spectroscopy (ICP-MS) (Bonney et al., 2002; Ndung'u et al., 2004) and atomic absorption spectroscopy (AAS) (Rada-Mendoza et al., 2018; Wongkalasin, 2017). Colorimetric-based detection using spectrophotometry techniques has also been demonstrated, using the complexation properties of Pb with dyes (Sakamaki et al., 2017). The sensitivity of colorimetric-based methods has been improved by using nanomaterials' enzyme-mimic activities (nanozyme) to provide an abundance of active edges and a large specific surface area for promoting the enzyme-catalyzed reaction (Tang et al., 2020). However, the color is interfered with by the sample matrices, which either disrupt the reaction of the reagent and the analyte or interfere with the spectrometric light measurement. The detection of Pb with fluorescence techniques improves the limit of detection (Liang et al., 2017). The combination of color measurement with smartphone detection facilitates the implementation of portable strips for visualization and quantification analysis (Wang et al., 2019).

#### 2.5 *Distance-based measurement*

Recognizing the sensitivity and significant challenges of colorimetric-based detection methods for PAD, the distance-based paper device (dPAD) was introduced. The dPAD can be applied for semi- and quantitative analysis, by measuring the color distance traveling along the hydrophilic channel. Therefore, the dPAD is an instrument-free method (Chutvirasakul et al., 2020; Hongwarittorn et al., 2017; Zhang & Yang, 2021).

#### 2.6 *Religious-based component in food and cosmetic products*

Some components or ingredients of food and other related products are forbidden by religious obligations (Neio Demirci et al., 2016). The issue of consumer faith and perceptions can challenge food producers to produce

food that complied with food safety assurances and specific religious regulations. Regarding Islamic obligations, the diet consumed by Muslims must be considered halal. To this end, the halal certification system was established for determining whether a product is halal or not (Ratanamaneichat & Rakkarn, 2013). The concept of food safety regarding halal certification emphasizes haram in the quality assurance control. Halal quality assurance includes preparation, processing, handling, and storage. Halal applications should be implemented in all processing stages, “from farm to table”, to produce safe, hygienic, and healthy products (Mohd, 2004).

The criteria of halal refer to the nature, origin, and processing of items and ingredients. However, the source of the ingredients is becoming more complex. In addition, composites of products may be mixed, or contaminated with haram. Like emulsions or aromas, numerous ingredients are unclear in terms of origin, and they are not obligated to declare this on the food label. Haram ingredients could be unintentional contamination due to a lack of education around the processing of halal products. Contamination with porcine gelatine, enzymes, or glycerine used as an emulsifier during production is an example of haram products (Ameer & Othman, 2012). Therefore, laboratory analysis of specific ingredients of halal output could help to identify non-compliant and compliant ingredients.

Islamic law allows all products except alcohol, porcine products, blood, meat from cadavers, and meat from animals that were not slaughtered according to Islamic principles. Most insects are also forbidden, including Cochineals, which are used as a natural red colorant; however, honey and shellac are permissible (Additives, 2015; Dahlan et al., 2016; Regenstein et al., 2003). Besides haram food products, there are also suspected (mashbooh) products, which refer to products with unknown origins or those for which

uncertainty exists regarding permission or prohibition. Practically, Muslim are recommended to avoid consuming those food products.

### 2.7 *The use of carminic acid in food and cosmetic products*

The products derived from cochineal were named by the Codex Alimentarius, using an international numbering system (INS), as No. 120, and the Colour Index (C.I.), with the generic name C.I. Natural Red 4, and the constitution number C.I. No. 75470 (Organization, 2011). This color is authorized as a food additive in the European Union (EU), as per Annex II to Regulation (EC) No 1333/2008. According to the evaluation by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2000 and the Scientific Committee for Food (SCF), a daily intake (ADI) of 5.0 mg/kg/body weight (bw)/day was reported (*Evaluations of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)*, 2020). The proteinaceous content in the extract can cause allergies in some individuals. Therefore, appropriate purification steps are needed to remove allergic proteins as much as possible (Additives, 2015). This color is widely used in numerous foods and beverages, because it has high chemical and biological stability (Chung et al., 2001). It is used at a concentration up to the maximum permitted level (MPL) regulated by the Codex and the General Standard of Food Additives (GSFA) (Greig, 2012).

One of the potential artificial substitutes for carminic acid is carmoisine (INS 124). Although artificial colors possess superior technological stability at a lower price, consumer demand for natural colors often prevents their use. Therefore, other pigments of plant origin, such as carotenoids (INS 160), anthocyanins (INS 163), and betalains (INS 162), are permissible as food additives in the European Union (Müller-Maatsch & Gras, 2016) and International Numbering System (INS).



The use of synthetic dyes and carminic acid to enhance the appearance of products and improve the coloring strength is often reported, since they share the same color properties (Moradi-Khatoonabadi et al., 2015; Morbioli et al., 2017). Chromatographic separation of exogenous colorants after pre-concentration on a polyamide column for quantification of acid-water soluble artificial dyes has previously been reported (Ordoudi et al., 2018; Rongso et al., 2020) however, the detection of a low concentration of carminic acid may still not be achieved.

## 2.8 *Analytical method for detection of carminic acid*

There are several analytical detection methods that have been used for the determination of carminic acid. A satisfactory and straightforward method for the determination of carminic acid in complex food matrices is spectrophotometry (Heydari et al., 2015). The official method established by FAO/WHO is spectrophotometry in acidic conditions, with diluted hydrochloric acid (Additives, 2015; Rudometova et al., 2020). Detection by high-performance liquid chromatography (HPLC) could be performed with diode array detection, and using fluorescent detection (Carvalho & Collins, 1997; Tran et al., 2014). The use of HPLC usually provides simultaneous detection of several analytes within a single run. LC-MS/MS is used for the detection of the unauthorized addition of dyes in herbs and spices; this was developed under compromised conditions to achieve a detection limit of 0.1–2.5 mg/kg, as regulated by the European Rapid Alert System for Food and Feed (RASFF) (Bessaire et al., 2019; Sabatino et al., 2012). Although HPLC and LC-MS/MS offer many advantages, the main drawback is their environmental impact, as many toxic chemicals are used and the running cost is high. Additionally, the scope of carminic acid detection has been recently expanded into medicine and pharmaceutical samples (Masár et al., 2020).

### 2.9 *Electrochemical detection method*

The most common methods for determining color additives are HPLC, spectrophotometry, and capillary electrophoresis. According to analytical protocols, electrochemical-based determination has several advantages, such as decreasing the analytical time because no pre-treatment steps are required. Moreover, this approach is suitable for detection in diluted samples to remove the effects of matrices from the samples. Square wave voltammetry (SWV) has been proposed to identify carminic acid in foodstuffs, in a hanging mercury drop electrode (HMDE). The application of an HMDE for differential pulse voltammetry (DPV) has achieved a detection limit of 0.16  $\mu\text{M}$  (Yilmaz et al., 2014). The detection of carminic acid using a modified graphite electrode (GE) with a conductive polymer (polypropylene) and bimetallic nanoparticles were previously reported. The detection limit was at the nanomolar level (Arslan & Çakır, 2016) with modification of the carbon electrode with reduced graphene oxide and methionine (Akkapinyo et al., 2021). Therefore, the scope of the determination of carminic acid has applicability for the analysis of carminic acid in medicine and pharmaceutical samples.

### 3. **Research problems**

- i) Most of the paper-based analytical devices for detection of lead are demonstrated in water samples, which usually not be implemented for food samples. Food samples are known for their complex matrices and high interference background. Century eggs, which are likely to be found Pb at high concentration, are among the food matrices that generally affect assay detection.
- ii) The safety assessment in halal products is a very crucial procedure in halal certification, especially for consumer protection of Muslims living in non-Muslim society. However, only a few

detection techniques have been involved in the halal certification system. Furthermore, most of the detection techniques have been focused on the detection of porcine and its derivatives. The detection techniques for another relevant non-permissible compound such as food additive are thus a challenge.

#### 4. Research questions

- i) Is a portable distance-based paper analytical device applicable for the detection of metal ion in food matrices?
- ii) Is the portable sensitive DPV technique at the SPE capable of detecting carminic acid in food and cosmetic products?
- iii) Is the developed analytical detection technique potential to be applicable at the site of need?

#### 5. Scope

This study aimed to develop analytical detection techniques with the potential to assess hazards that might be contaminated in food, cosmetics, or other related products.

A distance-based paper device (dPAD) developed with a paper substrate was created in our laboratory using a wax printing technique. A complex of immobilized PEI and Pb generated a distance proportional to the concentration of Pb in the sample. The standard addition technique was used to provide a sufficient concentration of Pb for measurement. The concept of the drawing paper-based device (drawing-PAD) was introduced to differentiate samples with higher or lower concentrations than the cut-off concentration regulated by the FDA.

The detection of carminic acid in food and cosmetic products has also been demonstrated in this study. Carminic acid is considered a religious hazard that should not be used in halal food and cosmetic products. A

sensitive electrochemical-based detection method was studied, involving a carbon screen-printed electrode and using the DPV approach. This technique can detect carminic acid even when carmoisine (CM) is also present in the products. It was found that the Interference by other common compounds usually existing in food and cosmetic products was also ruled out to ensure that the technique will be applicable for real sample matrices. The assay sensitivity was found to be much improved by modification of the carbon electrode with a composition of gold nanoparticles (AuNP) and PEI.

## 6. Outcomes

- a) Two analytical techniques were found to have the potential for utilizing in safety assessment, including i) dPAD, for the detection of lead in food samples, and ii) an electrochemistry-based technique for detecting carminic acid in food and cosmetics.
  - i. dPADs combined standard addition technique and straight-line drawing interpretation were feasible for detecting Pb in food matrices.
  - ii. The sensitive electrochemistry-based detection technique was feasible for the detection of carminic acid in food and cosmetics.
- b) The cost for detecting both targets analyzes in food and cosmetic safety assessments are much lower than the available conventional methods.

## Part 2

### A Simple Distance Paper-Based Analytical Device for the Screening of Lead in Food Matrices

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**Abstract:** A simple and rapid distance paper-based analytical device (dPAD) for the detection of lead (Pb) in foods is proposed herein. The assay principle is based on competitive binding between carminic acid (CA) and polyethyleneimine (PEI) to Pb in a food sample. The paper channels were pre-immobilized with PEI, before reacting with a mixture of the sample and CA. Pb can strongly bind to the CA; hence, the length of the red color deposition on the flow channel decreased as a lower amount of free CA bound to PEI. The dPAD exhibited good linear correlation, with ranges of 5–100  $\mu\text{g}\cdot\text{mL}^{-1}$  ( $R^2 = 0.974$ ) of Pb. Although, the limit of detection (LOD) of this platform was rather high, at 12.3  $\mu\text{g}\cdot\text{mL}^{-1}$ , a series of standard additions (8.0, 9.0, and 10.0  $\mu\text{g}\cdot\text{mL}^{-1}$ ) can be used to interpret the cutoff of Pb concentrations at higher or lower than 2  $\mu\text{g}\cdot\text{mL}^{-1}$ . The presence of common metal ions such as calcium, magnesium, nickel, and zinc did not interfere with the color distance readout. The validity of the developed dPAD was demonstrated by its applicability to screen the contamination of Pb in century egg samples. The results obtained from the dPAD are in accordance with the concentration measured by atomic absorption spectroscopy (AAS) ( $n = 9$ ). In conclusion, this proposed dPAD, combined with the standard addition method, could be applied for screening Pb contamination in food matrices. This platform is, therefore, potentially applicable for field measurements of Pb in developing countries, because it is cheap and rapid, and it requires no significant laborious instruments.

**Keywords:** distance paper-based device; carminic acid; polyethyleneimine; lead detection; standard addition; food matrices

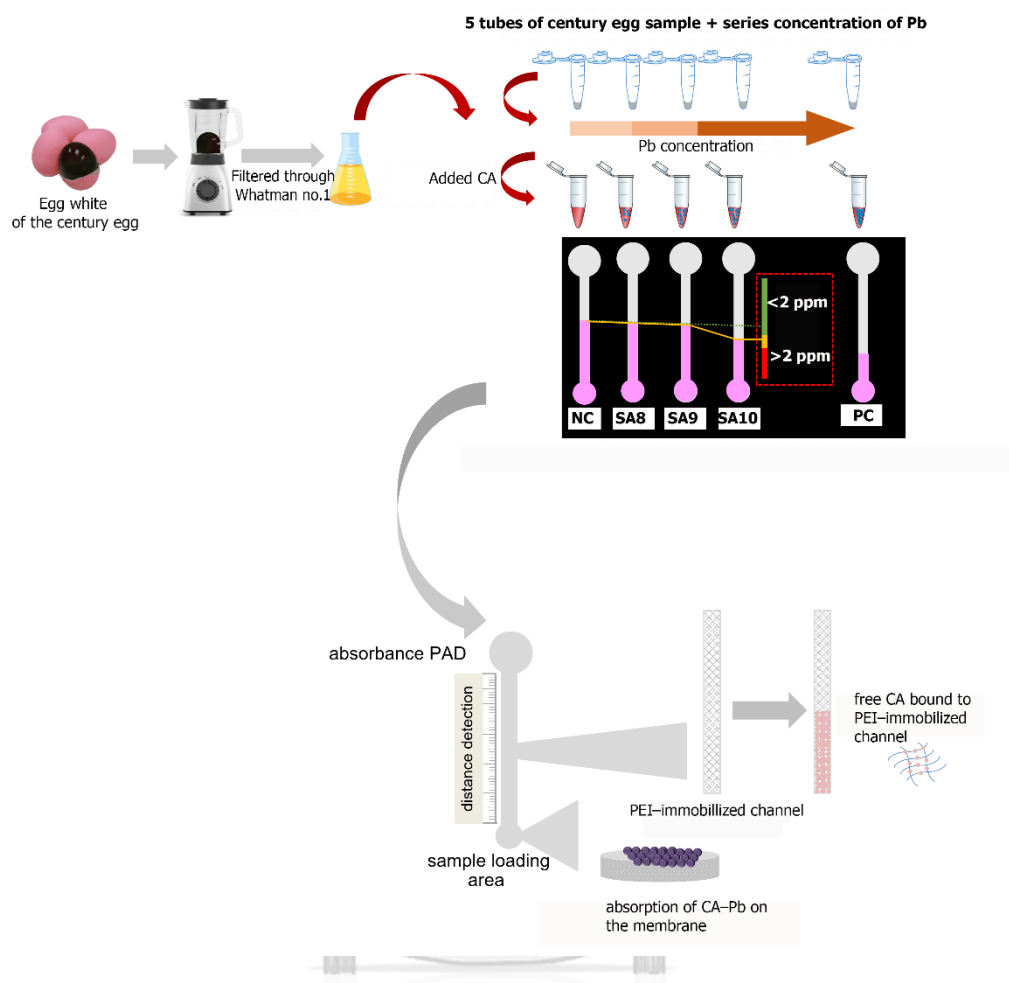


Figure 2 Graphical abstract

## 1. Introduction

Century egg, otherwise known as preserved egg or Pidán, is one of the famous Chinese and Thai cuisine ingredients because of its unique taste, high nutrition, and affordability. In the manufacturing process, there is likely the misuse of lead oxide, with it being added into the pickling mixture in order to reduce the ripening time and also to help improve the texture of the century eggs. However, the lead (Pb) content in the pickling mixture could also permeate through the eggshells and egg membrane into all parts of the eggs, thereby accumulating in the egg whites and yolks (Tu & Zhao, 2017; Zhao et al., 2014). Hence, the Pb content in century eggs has become a major safety

concern because food consumption is a major pathway of toxic compounds for human beings (Xu et al., 2014). Pb is indicated as a neurotoxic element, which could be the cause of long-term serious health problems. Accumulation of Pb in the human body could damage not only the nervous system, but also the immune system (Mishra, 2009). The permissible content of Pb in century eggs regulated by China's food safety standards is less than  $2 \mu\text{g}\cdot\text{mL}^{-1}$  [3] which corresponds to the value permissible by the Food and Drug Administration (FDA) of Thailand (*Notification of the Ministry of Public Health (No. 236) B.E. 2544 (2001)*, 2001).

The existing reliable methods that can be used for the quantitation of Pb are atomic absorption spectroscopy (AAS), inductively coupled plasma optical emission spectrometry (ICP/AES), and inductively coupled plasma mass spectroscopy (ICPM) (Tokalioğlu, 2012). The major advantage of these methods is their high sensitivity, as they can detect analytes at very low concentrations with exceptional reproducibility. However, those methods are expensive and time-consuming, and they require well-trained personnel. Therefore, chemical and biological sensing technologies have been considered as alternatives for the rapid and low-cost detection of Pb (Mazumdar et al., 2010; Wang et al., 2015). To achieve sensitivity and to improve selectivity for the detection of Pb and other heavy metals simultaneously, several strategies have been proposed. These methods are based on  $\text{K}^+$ -induced specific DNA G-quadruplexes (Zhu et al., 2018), gold nanoparticles (AuNPs) (Hung et al., 2010), and DNAzymes (Memon et al., 2019). However, these methods are based on the assistance of instruments. Nevertheless, for methods of on-site determination, users are attracted not only because of their reliable platforms, but also because they are lightweight and cheap, and they offer rapid detection. The paper-based assay method is, thus, attracting considerable attention as a matrix for fabricating a



point-of-care device that is lightweight and highly abundant. Moreover, paper is made from cellulose fibers with a high volume-to-surface ratio and permits the transport of fluids via capillary forces without requiring external pumping equipment.

The colorimetric measurement of Pb using paper-based devices has already been established, in which the method is based on the reduction of silver nitrate ( $\text{AgNO}_3$ ) using sodium borohydride ( $\text{NaBH}_4$ ) as a reducing agent and polyvinyl alcohol (PVA) as a capping agent. In the presence of Pb, the aggregation of  $\text{AgNO}_3$  results in a reddish-yellow color that can be observed and quantitated using a smartphone and ImageJ software (Shrivastava et al., 2019). Generally, the quantitation of the color intensity on the paper can be complicated by some factors, including different color perceptions and lighting effects. These factors reduce the sensitivity and reproducibility of an assay. Therefore, this assay method usually requires an external paper scanner and specific programs for image analysis.

The distance paper-based analytical device (dPAD) has advantages over a colorimetric assay because it does not require any extra instruments for measurement and quantitation. The measurement is simple, requiring only a readout of the color distance from the nearby ruler. Distance-based detection is one of the promising semi-quantitative analysis methods with superiority to the colorimetric detection-based method (Cai et al., 2020; Cate, Adkins, et al., 2015; Cate et al., 2013; Gerold et al., 2018; Kalish et al., 2019; Yamada et al., 2018). Several approaches have been proposed for improving the performance of the dPADs, such as using organic solvent to enforce the opening of wax valves, which facilitates reagent mixing and incubation in distance paper-based analytical devices (Chen et al., 2019). The wax printing of a microchannel of a dPAD platform on a plastic substrate could accomplish a reduction of the volume of an analyte (Qamar et al., 2019). The

distance-based approach can also be performed on a cotton thread substrate, which is an alternative material to paper for the fabrication of the fluidic sensor for the analysis of environmental contaminants (Nilghaz et al., 2014; Xu et al., 2018). In addition, by combination with a pre-concentration approach, such as headspace microextraction (Phoonsawat & Dungchai, 2021), solid-phase extraction (Quinn et al., 2018), and a portable heater (Ninwong, Sangkaew, et al., 2020), the sensitivity of measurements can be improved. Furthermore, there are several reports of using distance-based applications for clinical diagnosis such as chloride test (Taghizadeh-Behbahani et al., 2019), potassium (Soda et al., 2019), alkaline phosphatase, and cardiac troponin I (Khachornsakkul & Dungchai, 2021; Zheng et al., 2020). For the detection of metal ions, the dPADs have also been previously reported using the principle based on the deposition of chemical chelating compounds, such as dithizone (Cai et al., 2017) and a synthesized porphyrin (Pratiwi et al., 2017). Interestingly, the growing interest in dPAD application is reflected by the increasing number of publications during recent years, by using several strategies such as G-quadruplex-DNAzyme (Wu et al., 2020), starch hydrolysis (Chen et al., 2020), ionophore-doped ion-selective nanospheres (Du et al., 2020; Shibata et al., 2019), and synthesized fluorescent ligands (Nguyen et al., 2020; Ninwong, Sangkaew, et al., 2020).

In this research, we propose a distance-based detection method for Pb-contaminated food matrices using century eggs as an example. The potential chelating agent used was carminic acid (CA). It is generally known that CA is red in color, which is produced by insects, and it has been used historically as a natural food dye because it is a less toxic substance. Moreover, there are previous reports of a complex formation between CA and Pb in buffer solutions (Inoue et al., 2018; Sakamaki et al., 2017). The color of the solution changes from red to purple when Pb is present, as can be detected by a

spectrophotometer. However, the color development when CA is deposited directly along a hydrophilic channel on paper cannot be observed because of color fading due to the exposure of CA to light and oxygen (Berrie & Strumfels, 2017). Moreover, the negative functional group of CA molecules does not promote chemical interaction between CA and cellulose paper during the reaction. The dispersion of dye in porous paper leads to a faint and milder color on white paper. To overcome such a problem, herein, the paper surface was modified with positively charged molecules, i.e., polyethyleneimine (PEI), to entrap the dyes to enhance the color intensity on paper, as this approach has been successfully applied previously for entrapping unbound hydroxynaphthol blue as a loop-mediated isothermal amplification (LAMP) solution proposed by our group (Hongwarittorn et al., 2017). PEI is composed of repeating units of amine groups and a  $\text{CH}_2\text{CH}_2$  spacer, and it possesses strong binding capacity to negatively charged compounds. PEI has been successfully used to modify the paper surface to quantify the initial concentration of genomic DNA (Hongwarittorn et al., 2017).

In this article, we propose an indirect approach for the screening of low concentrations of Pb in food matrices using a dPAD. PEI is an imperative immobilizing agent in the flow channels of porous paper, allowing to trap free negatively charged dyes and CA, thereby forming a color distance readout. In this study, the measurement was based on the distance given by free CA bound to immobilized PEI on paper after forming a complex with Pb in the solution. As such, a shorter distance implies a higher Pb concentration. To improve the sensitivity of our proposed method to ensure its efficiency for the detection of Pb contamination in food samples, a series of Pb concentrations were added to a sample and performed in parallel on the same device. A straight line was obtained by connecting the top of the color distance of the serial concentrations of Pb to see the trend of the curve, in

which the Pb concentration could be determined through the specified color index, as this approach was inspired by the method for screening the albumin-to-creatinine ratio described by Hiraoka et al. (Hiraoka et al., 2020). By using this approach, the dPAD reported herein can be used to determine Pb contamination at the cutoff level for food safety control for century eggs. Furthermore, it is economically and environmentally friendly, and it is superior to other conventional methods because it is easy to operate, it is portable, and it does not require any external instruments. Our proposed method provides great promise for quick decision-making regarding food safety concerns because of a standalone and read-by-eye detection method.

## **2. Materials and Methods**

### *2.1 Chemicals and Reagents*

AAS standard grades of Pb, Ca, Mg, Cu, Ni, and Zn at concentrations of 1000 mg·L<sup>-1</sup> in 3% nitric solution were purchased from Merck KGaA (Darmstadt, Germany). Hydroxyethyl piperazineethanesulfonic acid (HEPES) of Vetec<sup>TM</sup> reagent grade, branched PEI with an average molecular weight of 25 kDa, and CA were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). A buffer composition, including sodium dihydrogen phosphate and Tris(hydroxymethyl)aminomethane, was obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Analytical reagent grade sodium hydrogen diphosphate, potassium cyanide, and sodium acetate were purchased from Ajax Finehem (Sydney, Australia). American Chemical Society (ACS) grade acetic acid (glacial) and hydrochloric acid fuming 37% were purchased from Merck KGaA (Darmstadt, Germany). All buffer solutions were prepared in ultrapure (UP) water with a resistivity of 18.2 MΩ·cm (Thermo Fisher Scientific, Abingdon, UK). Whatman<sup>TM</sup> filter paper, Grade 1004 (No. 4), was purchased from GE Healthcare Life Sciences (Hatfield, UK). A wax printer was employed for paper fabrication (Fuji Xerox, Tokyo, Japan, ColorQube8870). The

Multiskan GO<sup>TM</sup> microplate reader (Thermo Fisher Scientific, UK) was used for spectrophotometric studies. Fourier-transform infrared (FT-IR) analysis was archived by FT-IR Tensor II of Bruker (Karlsruhe, Germany). SEM images were collected using SEM Quanta 250 (FEI<sup>TM</sup>, Hillsboro, OR, USA). AAS was analyzed by AGILENT 280 FS at the Scientific and Technological Research Equipment Centre (STREC), Chulalongkorn University, Thailand.

## 2.2 *Design and Fabrication of the dPAD*

The dPAD was designed using the Microsoft Office PowerPoint 2010 program. The designed pattern was printed onto filter paper using the wax printing technique (Lu et al., 2010) to create hydrophilic and hydrophobic barriers. In our design, as depicted in Figure 3, a sheet of paper composed of five channels was used for one sample measurement. With each channel, a circular shape with a diameter of 7.0 mm was created for the sample loading area. The circular shape was connected to a straight hydrophilic channel with a width of 2.5 mm and a length of 40 mm and was used as the detection area. A larger circular shape (diameter of 9.0 mm) was designed on top of the detection channel to assist fluid flow by capillary action along the straight hydrophilic channel. After wax printing, the designed dPAD was heated to 150 °C for 2 min to melt the wax and to allow it to penetrate through the thickness of the membrane paper, as well as to create hydrophobic barriers to manipulate the fluid flow. After that, the wax-printed paper was attached by double-sided adhesive tape to a plastic transparent sheet in order to strengthen the device and to control the flow during the analysis.

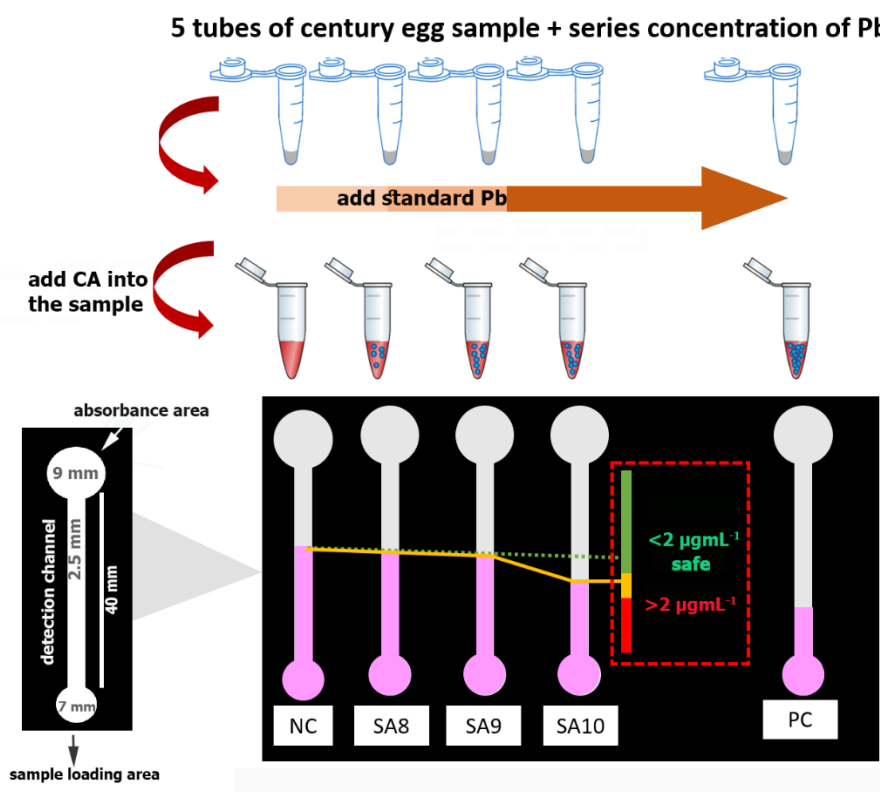


Figure 3 Demonstration of the screening analysis of lead (Pb) in century egg samples using the developed distance paper-based analytical device (dPAD) combined with the standard addition technique for evaluation at the cutoff concentration of Pb. CA, carminic acid. NC, negative control; SA8, SA9, and SA10, sample with standard Pb addition at the concentration of 8, 9, and 10  $\mu\text{g mL}^{-1}$ , respectively; PC, positive control.

### 2.3 Modification of the Membrane Surface by PEI

The hydrophilic paper channels were modified by immobilizing the PEI to improve the absorption efficiency of CA on the dPAD. In this study, the effect of PEI on the color distance of CA was studied at PEI 0.02% and 0.05%. The aqueous solution of PEI was prepared in UP water. Then, the aqueous solution of PEI (7.5  $\mu\text{L}$ ) was immobilized along the paper channels using the manual pipetting technique. After that, the channels were allowed to dry at

room temperature. The PEI-immobilized dPAD was kept under dry conditions using a desiccator.

To find a suitable concentration of PEI, the CA solution was dissolved in 0.1 M HEPES buffer (pH 7.0) to gain a final concentration of 0.0 – 2.0 mmol·L<sup>-1</sup>. A solution of 20 µL of CA solution was pipetted into the sample loading area of the dPAD and left to wick along the detection channel until the solution reached the top of the dPAD. The concentration of PEI able to entrap CA, but not too high to block the wicking solution, was selected as an appropriate concentration. In addition, the interaction of PEI and CA on the membrane was further characterized with SEM analysis.

#### 2.4 The Assay Principle

The assay principle refers to integrating the binding property of CA to Pb under appropriate pH conditions. The CA–Pb complex could be trapped in the sample loading area, allowing only free CA to wick through the hydrophilic detection channel, entrapped by the pre-impregnated PEI. In brief, the reagent buffer (0.4 mmol·L<sup>-1</sup> CA solution) was mixed with the analyte sample at a 1:1 (v/v) ratio and incubated for 5 min at room temperature. After mixing, the solution mixture (20 µL) was loaded into the sample loading area of the dPAD. The color distance was read when the solution reached the top of the platform. Therefore, the color distance of CA visualized from the detection channel was reduced when presented with high concentrations of Pb. The formation of the color distance was easily observed on the immobilized detection channel of the dPAD. The color distance could be measured with a Vernier scale meter to obtain two-digit accuracy. Additionally, the screening of Pb in the sample could be estimated using the naked eye after drawing a line and interpretation together with the standard addition method described in Section 2.5.

## 2.5 Real Sample Analysis

To demonstrate the applicability of our proposed dPAD for the screening of Pb-contaminated food matrices, century eggs spiked with Pb were used as tested samples. UP water was added to the whites (50 g) of the century eggs to gain a 1:1 (w/v) ratio and it was homogenized using a blender. Then, the supernatant was filtered through a Whatman No. 1 membrane filter with the assistance of a peristaltic pump. The filtrated supernatant of the sample was divided into nine portions and spiked with Pb to obtain a final concentration ranging from 0  $\mu\text{g}\cdot\text{mL}^{-1}$  to 3  $\mu\text{g}\cdot\text{mL}^{-1}$ , which were used as blind samples ( $n = 9$ ). Then, the Pb level in the blind samples was determined with the proposed dPAD using the protocol of the standard addition technique. In brief, the food samples (50  $\mu\text{L}$ ) were aliquoted into five microcentrifuge tubes, to which the standard Pb was added to gain final concentrations of 0.0, 8.0, 9.0, 10.0, and 40.0  $\mu\text{g}\cdot\text{mL}^{-1}$ . Herein, samples with 0 and 40  $\mu\text{g}\cdot\text{mL}^{-1}$  Pb were used as a negative and positive control, respectively. The reagent buffer (0.4  $\text{mmol}\cdot\text{L}^{-1}$  CA solution in 0.1 M HEPES buffer at pH 7.0) was added to each microcentrifuge tube at the ratio of 1:1 (v/v) and mixed thoroughly, before incubating for 5 min at room temperature to allow the binding of CA and Pb. Then, 20  $\mu\text{L}$  of the solution mixture was loaded into each channel of the PEI-immobilized dPAD. The red color distances from each channel were observed, and a line was drawn from low to high concentrations. The level of Pb in the food samples was determined by simply observing the straight line connecting the top of a series concentrations through the color identification chart, as demonstrated in Figure 3. The validity of the dPAD for Pb detection was confirmed by the AAS standard method.



### 3. Results

#### 3.1 Distance-Based Detection Method

The potential of using CA for the rapid screening of Pb with the approach of distance-based color formation by the CA–Pb complex was investigated. A preliminary study was performed using CA immobilized directly onto the designed detection channels of the dPAD. However, red color formation along the channels was not observed as expected. The color fading might be due to the penetration of CA color through the porous membrane. Moreover, it was found that CA could be moved to the top of the dPAD when the solution was applied, which implies that the CA did not strongly adsorb into the paper channels. Therefore, it was assumed that it is not possible to directly apply CA to the dPAD to perform color distance detection of Pb. Therefore, the hydrophilic channel needed additional modification by a cationic polymer to enhance the binding with free CA and generating a color distance. In our study, the strong positively charged polymer PEI was used for modification of the paper surface.

The suitable concentration of PEI for immobilizing the detection channel of the dPAD was investigated. The PEI solution was prepared by dissolving it in ultrapure water. The results demonstrate that PEI at the concentration of 0.1% could block the membrane pores, leading to CA not being able to move along the detection channel. Therefore, in this study, PEI was then diluted to gain final concentrations of 0.02% and 0.05% (w/v). Various concentrations of CA were loaded into the sample loading area and allowed to travel along the PEI-immobilized detection channels. It was found that the color distance increased as the CA concentration increased. A saturation curve was found when the concentration of CA reached 2.0 mmol·L<sup>-1</sup>. Linearity was observed in a range of 0.2–1.0 mmol·L<sup>-1</sup> in both PEI concentrations, as shown in Figure S1 of the Supplementary Materials. It was

found that PEI at the concentration of 0.02% afforded a 1.6-fold higher sensitivity than that obtained from 0.05% PEI. In addition, using PEI at 0.02% immobilization, the color distance observed on the PEI-immobilized channel exhibited color stability, as the color distance precipitated on the channels did not fade with time. Thus, it could be suggested that the CA color stabilized by forming a complex with PEI on the dPAD. Unless otherwise stated, our dPAD was prepared using Whatman membrane No. 4 and the channels were pre-immobilized with 0.02% PEI before analysis.

### 3.2 *Effect of pH*

pH affects the net charge of PEI, thereby influencing the binding of PEI to CA. Therefore, various buffer and pH values, including sodium acetate buffer (pH 6.0), HEPES buffer (pH 6.0 and 7.0), sodium phosphate buffer (pH 6.0, 7.0, and 8.0), and Tris-HCl (pH 8.0 and 9.0), were investigated. It was found that the acidic buffer gave the shortest color distance, relying on the stronger interaction between PEI and CA (see Figure S2, Supplementary Materials). The color distances measured from CA dissolved in alkaline buffer were longer than those observed for the acidic buffer. Interestingly, no binding interaction was observed between PEI and CA when using phosphate buffer, whether under acidic or basic pH. Using phosphate buffer, the CA color moved through the PEI-immobilized detection channel without absorption or precipitation. The precipitation of a color distance measured when using CA prepared in phosphate buffer was, thus, not detectable. Thus, HEPES buffer, which is an ampholyte from a zwitterion structure, provided a broad range of buffering capacity of an environmentally relevant pH region, in addition to biological medium compatibility. A further advantage of HEPES is that it is a compound, whereby the binding constant to metal ions is negligible. Therefore, HEPES buffer (pH 7.0) was selected as an appropriate buffer solution for this method.

### 3.3 SEM Analysis

The complex formation between PEI and CA on the membrane of dPAD was investigated with SEM. Various conditions were modified on the bare cellulose-based membrane to see surface morphology changes, as shown in Figure 4. Compared to the unmodified cellulose membrane in Figure 4a, dense surface morphologies were found when a surface membrane was modified by PEI (Figure 4b), suggesting that the PEI was successfully coated onto the membrane surface. However, the membrane pores were still noticeable. On the contrary, the surface of the unmodified membrane with deposition of the CA solution was no different to the bare membrane (Figure 4c). This means that the CA did not interact with the paper cellulose fiber. Interestingly, particles with spherical morphology were clearly observed when the CA solution was added to the PEI-modified surface membrane (Figure 4 d,e). This suggests that these distinctive circular structures are indicative of the condensation of highly positively charged PEI with negatively charged CA, forming stable spherical shape particles with sizes ranging from 1 to 5 microns. The condensation of PEI with CA resulted in visual detection on the paper membrane. This condensed spherical structure is rather similar to that of PEI complexation with DNA, which is very useful for gene delivery systems, as well as for the protection of DNA from enzyme degradation (Baker et al., 1997).

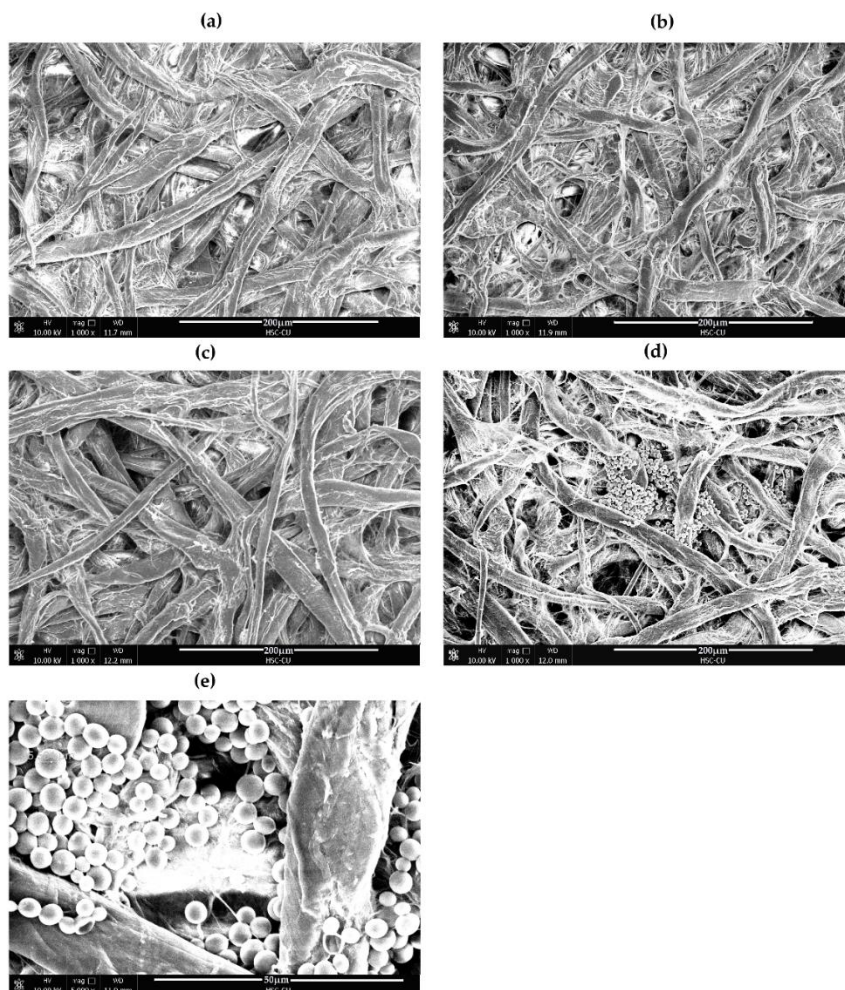


Figure 4 (a) Bare Whatman grade 1004; (b) polyethyleneimine (PEI) immobilized on Whatman filter; (c) CA immobilized on Whatman filter; (d) PEI-CA complex on Whatman filter (1000x); (e) PEI-CA complex on Whatman filter (5000x).

### 3.4 Measurement of Pb on the dPAD

The reaction of the CA and Pb complex was prepared using CA at a concentration of  $0.8 \text{ mmol} \cdot \text{L}^{-1}$  in  $0.1 \text{ M}$  HEPES buffer (pH 7.0). The reduction in color distance when the Pb concentration was increased is demonstrated in Figure 5. Then, a suitable concentration of CA was determined by testing the concentrations of  $0.8$  and  $0.4 \text{ mmol} \cdot \text{L}^{-1}$ . It was found that, when using CA at the concentration of  $0.4 \text{ mmol} \cdot \text{L}^{-1}$ , a higher sensitivity was obtained when

comparing the slope of the linear regression with that that observed when using CA at the concentration of  $0.8 \text{ mmol}\cdot\text{L}^{-1}$  (Figure 6). In addition, using  $0.4 \text{ mmol}\cdot\text{L}^{-1}$  CA, the analytical range of the measurement was found to be broader when the Pb concentration ranged from  $5 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$  to  $100 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ . Therefore, CA at the concentration of  $0.4 \text{ mmol}\cdot\text{L}^{-1}$  was selected for further study. Using  $0.8 \text{ mmol}\cdot\text{L}^{-1}$  of CA, the excess dye might interfere with the distance formation, as a faint red color was observed within the channel, especially at low Pb concentrations. Therefore, the color distance measured at  $20 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$  was not different from that at  $0 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ . According to the condition of using  $0.4 \text{ mmol}\cdot\text{L}^{-1}$  of CA, the limit of detection (LOD) ( $3 \text{ SD/slope}$ ) was calculated as  $12.28 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ . Although the LOD of this method is higher than the permissible level of lead in food, using standard addition methods can report the existing lead contamination in real samples.

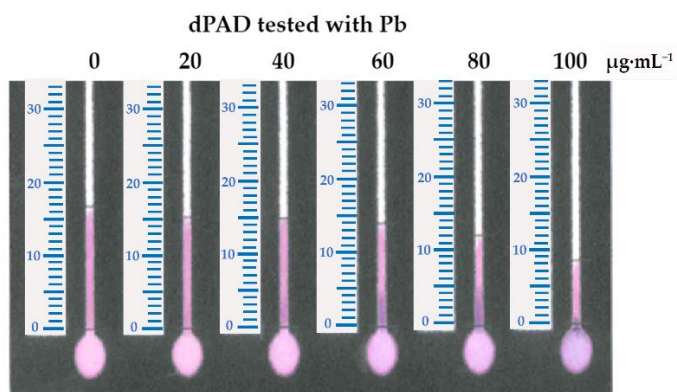


Figure 5 the decrease in the color distance on the PEI-immobilized dPAD observed when introducing Pb at concentrations ranging from 0 to  $100 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$  with  $0.8 \text{ mmol}\cdot\text{L}^{-1}$  CA in  $0.1 \text{ M}$  hydroxyethyl piperazineethanesulfonic acid (HEPES) buffer (pH 7.0). The reaction was performed at room temperature for 5 min of incubation.

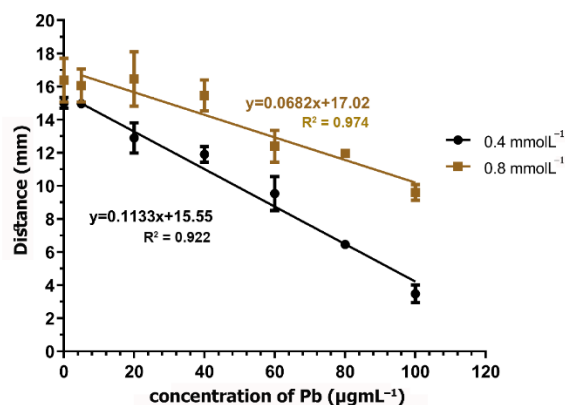


Figure 6 Color distance reduction when increasing the concentration of Pb using 0.4 and 0.8 mmol·L<sup>-1</sup> CA, with the Pb concentration ranging from 0 to 100  $\mu\text{g}\cdot\text{mL}^{-1}$  in 0.1 M HEPES buffer (pH 7.0). Error bars represent triplicate measurements.

### 3.5 Interferences with Other Metal Ions

The selectivity of CA toward Pb was evaluated in the presence of other potential metal ions. The interference study was conducted using 20  $\mu\text{g}\cdot\text{mL}^{-1}$  Pb. Interfering ions, including Ca, Cu, Ni, Zn, and Mg, were prepared at a concentration ranging from 0 to 1000  $\mu\text{g}\cdot\text{mL}^{-1}$ . The effect of these interfering ions was studied by introducing each of them to the Pb solution separately. The interference ratio was defined as the concentration ratio of the interfering ions that produced a  $\pm 5\%$  signal change when presented with 20  $\mu\text{g}\cdot\text{mL}^{-1}$  Pb.

The color distance observed from the solution of Pb together with Ca, Ni, and Mg did not decrease significantly, even when their concentrations were increased to 20 times higher than that of Pb. The interference by Zn ions was observed as a color change from violet to red when its concentration was increased to 10 times higher than that of the 20  $\mu\text{g}\cdot\text{mL}^{-1}$  Pb signal. Therefore, the tolerance ratio of Pb to Zn calculated from the color distance of the dPAD was 10. However, interference by Cu was observed on

the dPAD, in which it produced >5% change of the color distance when an equivalent concentration of Cu was added to the Pb test sample. In addition, the color intensity of CA significantly decreased in the test solution consisting of Cu. This suggests that Cu is able to bind with CA, similarly to Pb. In addition, the precipitated compound trapped upon the sample loading area was distinguished. Accordingly, there was no free CA wicking through the detection channel to perform a color distance when the Cu concentration was increased to higher than  $120 \mu\text{g}\cdot\text{mL}^{-1}$ . Hence, a significant reduction in free CA molecules was also noticed from the significant reduction in the color distance on the dPAD (Table 1). The decrease in the Pb color distance was not only due to the binding property of both Cu and Pb toward CA, but also because of the oxidation of CA in the presence of Cu (Tütem et al., 1996). This can lead to the loss of color of CA, and it was, thus, not surprising that a decrease in Pb signal distance was dramatically observed. To demonstrate the reduction in the effect of Cu using the dPAD, potassium cyanide (KCN) was used as a masking agent. In this study, KCN was prepared at the concentration of 5% and was added to the testing solution comprising Pb and various concentrations of Cu. The percentage reduction was calculated by comparison with the color distance of Pb alone. It was found that, in the presence of KCN, the color distance reduction was deemed not to have decreased dramatically, as the same reduction was found previously when KCN was not introduced. The tolerance ratio for Pb to Cu was, thus, found to be better, as the concentration of Cu could be increased to  $40 \mu\text{g}\cdot\text{mL}^{-1}$  (see Figure S3, Supplementary Materials). Therefore, it could be concluded that the interfering effect of Cu can be eliminated by the addition of KCN as a masking reagent.

Table 1 Interference ratio of foreign ions.

Sample	dPAD
Tested foreign ions	Interference ratio of other metal ions to Pb
Cu	1
Ca	>20
Ni	>20
Zn	10
Mg	>20

### 3.6 Proof of Concept for Analysis of Pb on the dPAD

To confirm that the developed dPAD was capable of detecting Pb, it was tested with drinking water obtained from a household water dispenser. However, Pb was not detectable because the sensitivity of the dPAD was not high enough. The drinking water was then spiked with standard Pb to gain final concentrations of 5.0, 20.0, 50.0, and 100.0  $\mu\text{g}\cdot\text{mL}^{-1}$ . As shown in Figure 7, the reduction in color distance was related to the increasing concentration of Pb. In addition, precipitation of the CA-Pb complex could clearly be observed on the dPAD when testing with Pb at a concentration higher than 50.0  $\mu\text{g}\cdot\text{mL}^{-1}$  (Figure 7 insert). Therefore, it is believed that our proposed dPAD can be applied for the determination of Pb in real samples. In addition, colorimetric-based test strips commercially available on the market were also used for the determination of Pb in parallel with our dPAD. According to the package insert, the strips can provide a detection range of 0–500  $\mu\text{g}\cdot\text{mL}^{-1}$ . The color changed from yellow to orange when the concentration of Pb was in



a range of  $0\text{--}100\ \mu\text{g}\cdot\text{mL}^{-1}$ , while it turned to red-violet when the concentration of Pb increased to  $500\ \mu\text{g}\cdot\text{mL}^{-1}$ . The color changes of the commercial test strips could be observed rapidly within a minute. However, it was rather difficult to distinguish the yellow-brown color against a paper background color, particularly at low Pb concentrations. Therefore, there could be a source of error in the variation of individual color perceptions. In conclusion, as a proof of concept, dPADs fabricated using PEI-immobilized detection channels have the ability to screen for Pb on the basis of color distance visualization.

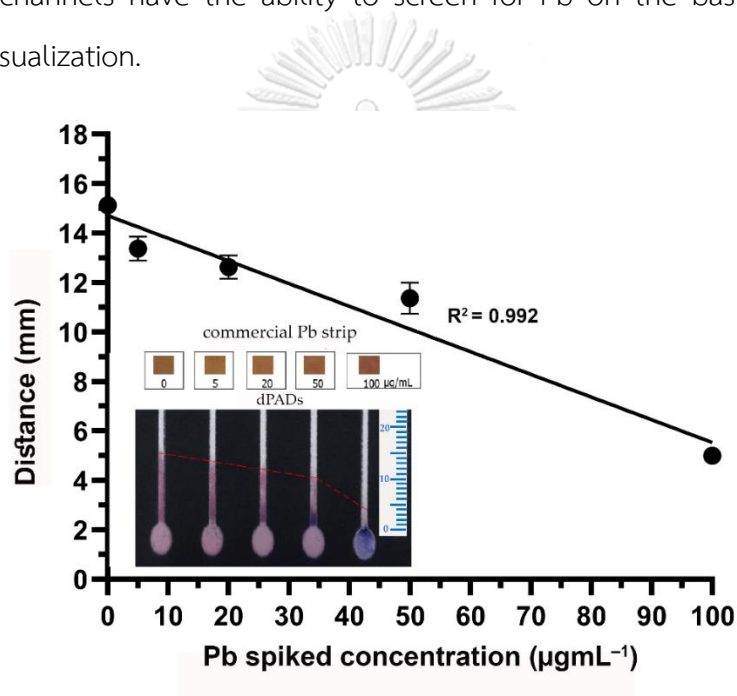


Figure 7 Drinking water with Pb ( $0\text{--}100\ \mu\text{g}\cdot\text{mL}^{-1}$ ) tested with commercial strips and the dPAD using  $0.4\ \text{mmol}\cdot\text{L}^{-1}$  CA ( $n = 3$ ).

### 3.7 Real Food Sample Analysis

To implement the dPAD for the screening of Pb in food matrices, century eggs were used as a representative food sample because they are one of the most relevant pickling products associated with the high consumption of Pb. The supernatant obtained from the homogenized egg whites from the century egg samples was tested using the dPAD. However,

the supernatant was more viscous than drinking water, because it was composed of abundant ovalbumin, other proteins, and lipids. In our study, we found that the viscosity of the supernatant can influence the color distance formation, as per the color distance reduction of ~14%–20% compared to that from drinking water. The precision of the developed method was determined at four Pb concentrations (0.0, 5.0, 20.0, and 40.0  $\mu\text{g}\cdot\text{mL}^{-1}$ ) spiked in the egg white supernatant.

To study intra-day and inter-day reproducibility, 153 pieces of dPADs were fabricated in the same batch and assayed with different spiked Pb concentrations. For the intra-day precision assay, each concentration was performed in 18 replicates. Color distances of  $10.91 \pm 0.68$ ,  $10.50 \pm 0.54$ ,  $9.73 \pm 0.49$ , and  $6.25 \pm 0.35$  mm were observed. The intra-day precision was expressed as % Coefficient of Variation (CVs) of 6.28%, 5.16%, 2.67%, and 5.66%, when tested with spiked Pb at concentrations of 0.0, 5.0, 20.0, and 40.0  $\mu\text{g}\cdot\text{mL}^{-1}$ , respectively ( $n = 72$ ). Inter-day reproducibility was performed on three different days and each concentration was conducted in nine replicates. The color distances of the century egg spiked with 0.0, 10.0, and 40.0  $\mu\text{g}\cdot\text{mL}^{-1}$  were obtained at  $10.84 \pm 0.76$ ,  $9.96 \pm 0.36$ , and  $6.41 \pm 0.55$  mm, which could be expressed as %CVs of 7.05%, 3.66%, and 8.67%, respectively ( $n = 81$ ). As the %CVs of both intra-day and inter-day reproducibility were less than 10%, the precision and reproducibility of our proposed dPADs were acceptable.

The standard addition technique is a well-known technique for minimizing the effect of sample matrices that interfere with the measurement of an analyte signal. To overcome the variation of the viscosity from different sample matrices, the standard addition method was exploited. Furthermore, the standard addition technique was also used to assist the dPAD's performance for the screening of low concentrations of the target analytes. Different concentrations of Pb were added to the supernatant of the egg

whites of the century egg samples; then, they were tested in parallel, together with negative and positive controls, using the same dPAD, as described in Section 2.4.

Blinded samples with Pb levels ranging from  $0 \mu\text{g}\cdot\text{mL}^{-1}$  to  $3 \mu\text{g}\cdot\text{mL}^{-1}$  ( $n = 9$ ) were used to evaluate the developed dPAD. To discriminate samples with Pb levels over and below the cutoff permissible levels ( $2 \mu\text{g}\cdot\text{mL}^{-1}$ ) according to the national standard, the concept of a drawing line interpretation was combined in our device. A straight line was drawn by connecting the top of the color distance of each channel. A reduction trend line could be distinguished on the basis of whether a sample contained Pb at higher or lower levels than the cutoff concentration. Therefore, the concentration of Pb measured under our proposed device could be expressed as  $\text{Pb} \leq 2 \mu\text{g}\cdot\text{mL}^{-1}$  and  $>2 \mu\text{g}\cdot\text{mL}^{-1}$ . These samples were also tested with the AAS standard technique to confirm the actual concentrations. The Pb levels determined with this proposed platform were in accordance with the concentrations measured by AAS. Only one sample (No. 3) was found to have ambiguous results. The dPAD expressed the concentration as  $\geq 2 \mu\text{g}\cdot\text{mL}^{-1}$ , while, the actual concentration of Pb by AAS was  $1.9568 \mu\text{g}\cdot\text{mL}^{-1}$  (Table 2). However, the overestimation of  $0.0432 \mu\text{g}\cdot\text{mL}^{-1}$  could be due to the variations in color distance on the dPAD affecting the straight trend line, which is a possible source of error. Nevertheless, our purpose was to design a dPAD to use for screening purposes; thus, this was acceptable, since Pb contamination was not ruled out. Interestingly, all of the samples were also analyzed using the same commercial rapid test strips mentioned in Section 3.6. The color changes observed on the commercial strips were interpreted as “zero”, suggesting that all samples had Pb at lower levels than the kit’s detection limit, despite the detection range of  $0\text{--}500 \text{ mg}\cdot\text{L}^{-1}$  claimed by in package insert.

*Table 2 Determination of Pb in blinded spiked century eggs on the dPAD compared to the concentrations measured by atomic absorption spectroscopy (AAS).*

Sample	dPAD ( $\mu\text{g}\cdot\text{mL}^{-1}$ )	AAS ( $\mu\text{g}\cdot\text{mL}^{-1}$ )
Sample 1	$\geq 2$	2.8916
Sample 2	$\geq 2$	2.8643
Sample 3	$\geq 2$	1.9568
Sample 4	$\geq 2$	2.8728
Sample 5	$\geq 2$	2.0063
Sample 6	$\geq 2$	2.0012
Sample 7	$< 2$	0.0803
Sample 8	$< 2$	0.0923
Sample 9	$\geq 2$	2.8950

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#### 4. Discussion

##### 4.1 CA-PEI Complexation Using the dPAD Approach

The color fading limited the use of CA directly as a chemosensing agent for Pb under the distance-based strategy. The fading was caused by photobleaching of anthraquinone, which occurs due to the accumulation of tautomeric photoreactive triplet state exposed with oxygen (Stapelfeldt et al., 1993). Moreover, the color fading of anthraquinone dye could be attributed to emissions from tautomeric states following excited-state internal electron transfer and from the population of a dark, nonradiative state that irreversibly

leads to a colorless molecule (Comini et al., 2017; Tan et al., 2017) [46,47]. Therefore, the membrane surface of the dPAD needed further modification before interaction with the CA. Herein, the strategy of opposite charge interaction between negatively charged CA and strong positively charged PEI was utilized.

PEI is a polymer with a high concentration of amine groups in its structure. The amine groups provided a good environment for the formation of a CA-PEI complex, as reported in the literature (Bayraktutan, 2019). The redshift spectrum of approximately 52 nm observed in our experiment could be described by the complex formation between PEI and CA (see Figure S4, Supplementary Materials). Additionally, the binding between PEI and CA could be described as an anthraquinone moiety of the CA-bearing anionic group interacting with the cationic hyperbranched structure of PEI (Böhm et al., 2011). According to our approach, the interaction between PEI and CA was reserved on the hydrophilic detection channel of the dPAD. A surface membrane pretreated with PEI for absorption of other dyes has previously been reported to improve the absorption efficiency (Wang et al., 2017). The absorption performance in the hydrophilic channel of our dPAD could be explained in a similar manner. In comparison to these membrane modifications, bare membrane substrates could be used for the absorption of CA. However, it was observed that, after dropping the solution into the sample loading area, the impregnated CA was easily desorbed from the paper channel and moved toward the upper part of the channel. On the contrary, surprisingly, the PEI-modified membrane substrate was found to be complexed with CA with clear color formation along the paper channels. This was confirmed by the presence of spherical structures of PEI condensation on the paper surface when observed under SEM. This could be explained in a similar manner to that of PEI-DNA complexes (Baker et al., 1997;

Choosakoonkriang et al., 2003; Hildebrandt et al., 2003; Hou et al., 2011). Therefore, we assumed that the binding interaction of PEI and the anionic dye CA on the membrane substrate could take place in a comparable manner. In addition, the hydrophilicity property of the membrane after treatment with PEI remained. The binding interaction between PEI and CA on the membrane surface is, thus, promising for further application for the measurement of particular compounds by using the distance-based indirect strategy.

There are many factors that contributed to the color distance of our proposed dPAD. First, an excess concentration of PEI could have been trapped in the membrane of porous fibers (Sinclair et al., 2018), resulting in obstruction of the flow of CA. Therefore, using high concentrations of PEI for immobilization on the membrane can result in a shorter color distance formation. Second, the pH of the system contributed to the variation of the color distance measured on the PEI-immobilized channel, because it plays a major role in its electrostatic interaction with CA on the hydrophilic detection channel. A short color distance was observed from the acidic solution in comparison to the color distance under basic conditions. This can be explained because, under acidic conditions, the PEI chain is strongly positively charged due to the protonation of the secondary amine along the backbone chain of PEI (Curtis et al., 2016). In addition to the positive charge of PEI, the deprotonated forms of CA were also pH-dependent ( $pK_{a1} = 3.39$ ;  $pK_{a2} = 5.78$ ;  $pK_{a3} = 8.35$  (Atabey et al., 2012), which could suggest that the deprotonated state of CA promotes the binding interaction of CA to PEI. Third, the detection channel's width refers to the area available for the complex formation of PEI immobilized on the membrane and free CA (Guan & Sun, 2020). This factor affects the slope of the linear regression line. In our experiment, a hydrophilic channel width of 2.5 mm was defined before wax printing. This width is workable, as suggested in the literature, as the channel width for the

distance-based approach should not exceed 4.7 mm because the detection range will be unacceptably low (Cate, Noblitt, et al., 2015).

#### 4.2 *Detection of Pb in Food Samples*

The detection of Pb in food samples using the paper-based device is a challenge because it can be significantly influenced by the sample matrix. A bismuth-modified electrode fabricated by screen-printing on paper can increase the sensitivity of Pb detection for food and environmental analysis (Chaiyo et al., 2016). The paper-based assay was successfully implemented for selective detection of Pb in the environmental water using AgNPs/PVA as a plasmonic colorimetric probe, in which the color development relied on the aggregation of specific synthesized silver nanoparticles (Shrivastava et al., 2019). In addition, paper-based colorimetric detection has been proposed for Pb detection in a water sample, by using sodium rhodizonate as a colorimetric reagent for direct Pb detection on a PAD (Satarpai et al., 2016). Nevertheless, most of the colorimetric detection on paper-based devices described previously relied on image analysis. However, simple and instrument-free detection by color visualization has not yet been reported for the detection of Pb in food matrices.

In this study, the developed dPAD proved promising for the screening of Pb in a complex food sample matrix. However, Cu showed partial interference with the color distance reduction. The interference by Cu is due to the complex formation between CA and Cu (Atabey et al., 2012); hence, false-positive results could have occurred. However, the interference by Cu can be diminished by adding KCN. It has been reported that KCN reacts with Cu to form  $[\text{Cu}(\text{CN})_3]^{2-}$ , which usually occurs more easily than with Pb (Chooto et al., 2010). Although KCN is an effective agent to mask and diminish the effect of Cu, it is not recommended for use in food analysis because of its high toxicity. Alternative masking reagents for the Cu should be further

investigated such as thiosemicarbazide and its derivatives (Ibrahim et al., 2018; Youssef et al., 2012) and potassium iodine (Beltrán et al., 2016). In addition to Cu, other ions such as  $\text{Al}^{3+}$ ,  $\text{Cr}^{3+}$ , or  $\text{Fe}^{3+}$  could interfere with the assay as those ions can potentially bind to the CA (Sakamaki et al., 2017). Among those ions, only  $\text{Fe}^{3+}$  is possibly found in a very small amount in egg white (Sunwoo & Gujral, 2015). Nevertheless, common iron masking agents such as NaF (Ninwong, Ratnarathorn, et al., 2020; Smaniotto et al., 2009; Zhang et al., 2015) can be used to reduce the interference effect by  $\text{Fe}^{3+}$  and achieve better specificity.

The applicability of the proposed dPAD for testing Pb in drinking water is very promising, because the color distance reduced proportionally to the increase in Pb concentrations. However, the sensitivity of the dPAD was still not sufficient for implementation in real drinking water samples, as it needs a detection limit as low as  $0.01 \mu\text{g}\cdot\text{mL}^{-1}$  and  $0.5 \mu\text{g}\cdot\text{mL}^{-1}$  for beverages in a sealed container (*Notification of Ministry of Public Health (No. 356) B.E. 2556 (2013)*, 2013). There is a report of using polyvinyl alcohol-capped silver nanoparticles to enhance the sensitivity of paper-based assays of Pb in water. This colorimetric detection accomplished the detection range of  $0.02\text{--}1.0 \mu\text{g}\cdot\text{mL}^{-1}$ , albeit with the assistance of Image J analysis (Shrivastava et al., 2019). For real food sample matrices, the color reduction from urease inhibition by Pb was demonstrated in water and milk. The sensitivity of the method was approximately  $8.0 \mu\text{g}\cdot\text{mL}^{-1}$ , which is a similar level to our proposed method, and it still did not meet the FDA regulation (*Notification of Ministry of Public Health (No. 356) B.E. 2556 (2013)*, 2013; *Notification of the Ministry of Public Health (No. 236) B.E. 2544 (2001)*, 2001).

To follow the FDA regulation, we implemented a standard addition technique to facilitate the usage of the dPAD for the screening of Pb in real food samples. Nevertheless, our device can detect Pb at the  $2.0 \mu\text{g}\cdot\text{mL}^{-1}$



level; therefore, the dPAD combined with standard addition is very promising as an alternative tool for the low-cost and simple detection of Pb in food matrices, particularly in developing countries.

The standard addition technique helps the interpretation of results, especially when there is existing interference from complex sample matrices. In addition, it facilitates the measurement when the concentration of analytes is likely to be low. In this study, a series of concentrations of standard Pb were used for each measurement. Because various concentrations of Pb were spiked in the tested sample, different color distances were expected to be observed on the dPAD. The amount of Pb existing in food samples can be estimated by drawing a straight line connecting each detection channel, a concept that is similar to that previously reported for albumin index evaluation (Hiraoka et al., 2020). When applying it to food matrices, it seems that the viscosity effects of food fluids influence the flow on a dPAD, in which a higher viscosity results in a slower rate (Lee et al., 2017), consequently affecting the color distance (Noiphung et al., 2018). In our study, the supernatant obtained from the century egg extractions was rather viscous, which might have affected the color distance formation. However, because of the use of the standard addition method, the variation in the sample viscosity could be diminished. In this study, using the standard addition method combined with drawing a straight line was effective for the screening of Pb at a cutoff level of  $2.0 \mu\text{g}\cdot\text{mL}^{-1}$ . Our proposed method was superior to that of commercial rapid test strips for Pb screening. The color change observed on commercial strips was rather difficult to interpret because of the interference of the color background of the paper. Moreover, the color change of different concentrations was not obvious. Generally, distance-based detection is advantageous over the colorimetric method, because the bias from individual color perceptions can be diminished.

## 5. Conclusions

The developed dPAD proposed in this study provides superior advantages in terms of simplicity, rapidity, and affordability for screening the contamination of Pb in century eggs. Thanks to the strong binding between PEI and CA, the length of the red color deposition on the flow channel could be clearly observed, and it referred to the Pb content in the tested samples. Additionally, the proposed device is very promising for food safety screening, particularly in developing countries. In addition to its affordability and portability, the method also meets the requirements of using fewer chemical reagents, and it is more environmentally friendly in comparison to the use of other chelating compounds. Because of the visual detection of the color distance color formation and the simplicity of drawing a curve to estimate the lead content, extra instruments for color intensity analysis are no longer required. Distance-based visualization combined with the standard addition technique was shown to be a simple and effective method for the screening of Pb in food matrices because it can accurately determine Pb at the  $2.0 \mu\text{g}\cdot\text{mL}^{-1}$  cutoff level, which is the safety-regulated limit in many countries. Moreover, the dPAD can be performed at neutral pH levels; therefore, it has the potential for broad applications in different fields, such as environmental and biological samples. Nevertheless, the sensitivity of this platform needs to be further improved for widespread implementation in other food matrices or biological samples. Multifactorial optimization and appropriate pre-concentration of the samples could also probably solve this limitation.

## 6. Supplementary Materials:

### 6.1 *PEI concentration optimization for immobilization on the dPAD*

To determine the appropriate concentration of PEI for the distance measurement on the dPAD, 7.5  $\mu\text{L}$  of PEI at the concentrations of 0.02% and 0.05% was deposited in the hydrophilic channel of the dPAD. Various CA

concentrations ( $0.0\text{--}2.0\text{ mmolL}^{-1}$ ) were tested. The results demonstrate that the color distance on the dPAD increased when the CA concentrations increased (Figure S1).

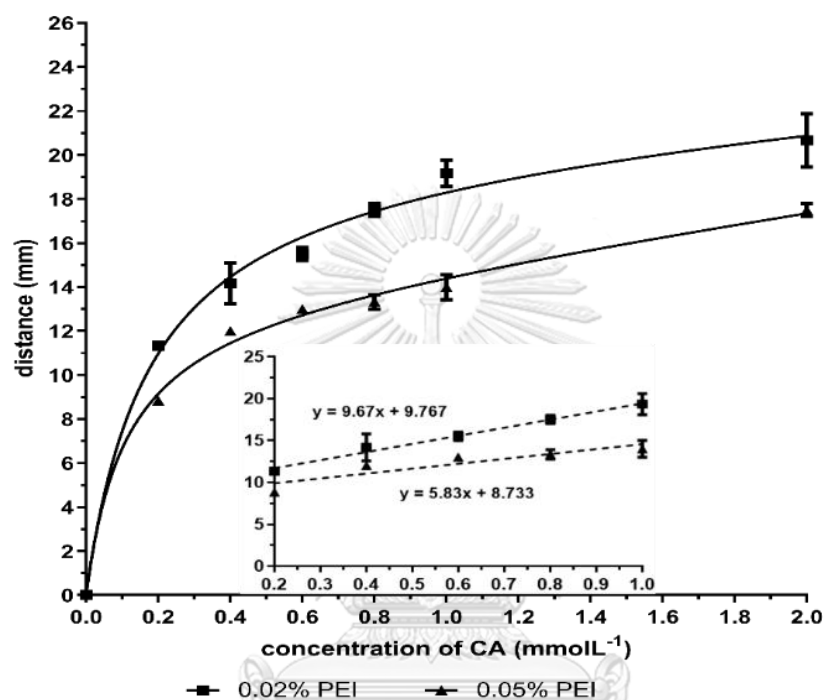


Figure S1 The proportional relationship between the color distance with the carminic acid (CA) concentration on the polyethyleneimine (PEI)-immobilized distance paper-based analytical device (dPAD) using PEI at the concentrations of 0.02% and 0.05% ( $n = 3$ ). Inset: Linear regression analysis and the best-fit line of CA between the ranges of  $0.2$  and  $1.0\text{ mmolL}^{-1}$ .

## 6.2 Effect of pH on the color distance of CA on the PEI-immobilized dPAD

The effect of pH on the adsorption behavior of CA on the PEI-immobilized membrane was investigated using various types of buffers and pH levels. The buffer solution, including sodium acetate buffer (pH 6.0),

HEPES buffer (pH 6.0 and 7.0), sodium phosphate buffer (pH 6.0, 7.0, and 8.0), and tris-HCl (pH 8.0 and 9.0), was tested with  $0.8 \text{ mmolL}^{-1}$  of CA (Figure S2).

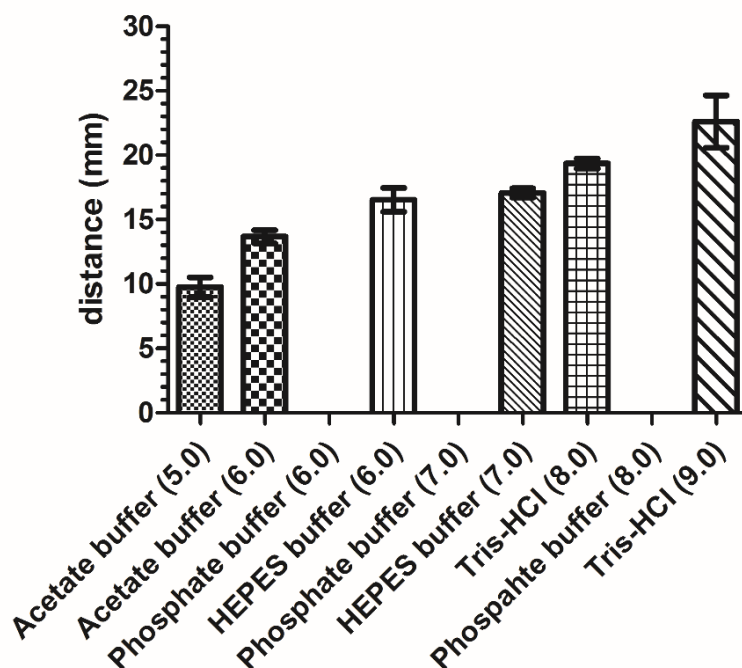


Figure S2 The effect of pH on the color distance observed on the PEI-immobilized dPAD with 0.02% PEI. All buffers were prepared at the concentration of 0.1 M. CA was prepared at the concentration of  $0.8 \text{ mmolL}^{-1}$  ( $n = 3$ ).

### 6.3 Effect of copper on the distance observed with the dPAD

The detection of Pb on the dPAD was affected by copper (Cu). In this study, the solution mixture was prepared with CA and a mixture of  $20 \mu\text{g mL}^{-1}$  of Pb and  $20 \mu\text{g mL}^{-1}$  of Cu. The distance was measured, and it was found to be affected by Cu by a color distance reduction of >5%. To demonstrate that the effect of Cu could be eliminated by KCN, KCN was added to the solution of CA and, the solution mixture of Pb and Cu was then added into the CA solution. The reduction of the distance is shown in Figure S3.

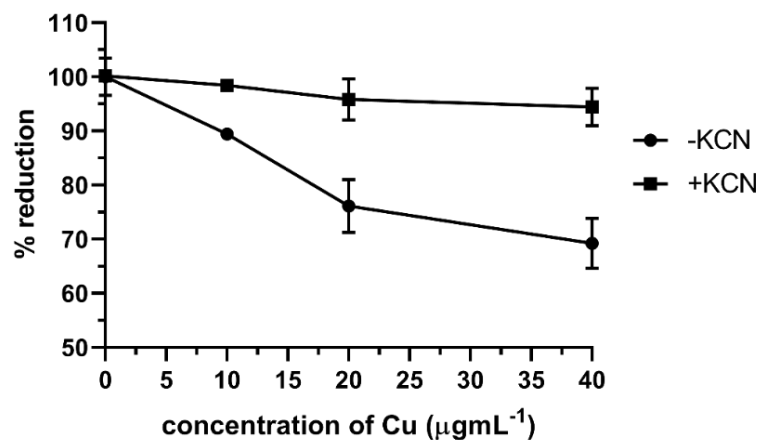


Figure S3 The effect of Cu on the measurement of Pb in the presence and absence of 5% potassium cyanide (KCN) ( $n = 3$ ).

#### 6.4 CA-PEI complexation

The complex formation between CA and PEI was confirmed in batch solution by determination of the absorption spectrum shift in 0.1 M HEPES buffer (pH 7.0). The maximum absorption spectrum shift from 514 to 566 nm of CA exhibited an interaction of CA and PEI, as seen in Figure S4.

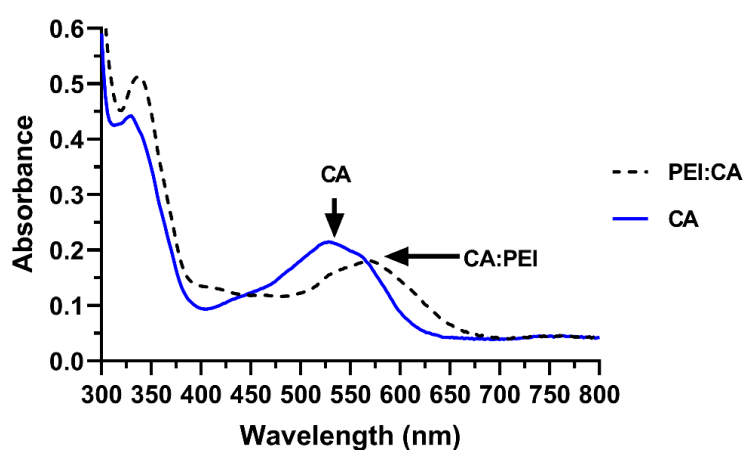


Figure S4 The absorption spectrum shift of CA by means of the addition of PEI in 0.1 M of HEPES buffer (pH 7.0).

## An Electrochemistry-Based Technique for the Detection of Carminic Acid to Assure the Safety of Halal Products

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### SUMMARY

*Research background:* Carminic acid (CA) is a red dye which originates from the Cochineal insect. This dye cannot be used in halal products. Therefore, a detection method for carminic acid is very important for consumer protection. For halal certification, integration of the information at the site is necessary to confirm that the product does not contain and is not contaminated with CA.

*Experimental approach:* The electrochemical detection of CA in the presence of carmoisine (CM) was performed at the AuNP/PEI electrode vs. Ag/AgCl under acidic conditions (pH 3.0). The characteristic potential of CA was observed at the reduction of  $-0.791$  V. To improve the sensitivity, modification of the surface electrode using composited AuNP/PEI was demonstrated.

*Results and conclusions:* The sensitive DPV technique with an unmodified electrode with the potential scan from  $-1.5$  to  $+1.5$  V exhibited the characteristic signal of CA ( $-0.791$  V). This potential was not interfered with by CM, and other co-existing compounds usually found in food and cosmetics. A detection range of  $10\text{--}400$   $\mu\text{M}$  ( $R^2 = 0.99$ ,  $y = 38.08x + 1.636$ ) was achieved with an LOD of  $33.68$   $\mu\text{M}$ . The precision of this technique was demonstrated by a %CV of  $2.78\%$  to  $9.52\%$ . The recovery determined by spiked CA in the food matrices, as well as cosmetics, ranged from  $84.1\%$  to  $123.6\%$ . The results obtained from the DPV analysis were comparable to those obtained by ultra-HPLC. Furthermore, improvement of the sensitivity of the DPV was achieved by modification of the surface of the electrode with composited AuNP/PEI. The sensitivity obtained at the modified electrode was 24 times higher than the unmodified electrode. In addition, sample preparation, such as filtration and separation, was not needed for the proposed method. Therefore, the method is promising in terms of simplicity and rapidity, for integration into on-site halal assurance, certification, and authentication.

Key words: Halal, Food and cosmetic safety assessment, Food authentication, Food additive monitoring.

## 1. Introduction

‘Halal’ is an Arabic word which means ‘permissible for Muslims to consume or use’ (Al-Teinaz et al., 2020). On the contrary, ‘haram’ means ‘non-permissible for Muslims to consume or use’. According to Islamic law, haram substances, such as dog, pig, blood (Rohman & Windarsih, 2020), and animals slaughtered in an inappropriate manner (Mohd Yusoff et al., 2021; Sohaib et al., 2020), cannot be used in halal products. Therefore, the halal certification system has an important role in providing assurance to the Muslim consumer, especially for Muslims living in non-Muslim societies, in order to preserve their identity and fulfil Muslim religious obligations. Halal-certified products are products handled according to Islamic law, with a high standard of hygiene, and which consistently meet a certain standard of cleanliness and safety (Azmi et al., 2018).

The global halal market has experienced increasing demand for halal-certified products, which has contributed to the positive performance of halal businesses (Ab Talib et al., 2017). In terms of product types, the halal food market has shown remarkable growth, and the industry’s success is due largely to building consumer trust via compliance with international halal standards and requirements. Additionally, halal-certified products are guaranteed in terms of their cleanliness and safety for consumption and are made in a hygienic manner. The biggest non-food halal market is cosmetics, in which demand is growing because consumers tend to have a positive view of the quality of halal cosmetics (Divianjella et al., 2020; "Halal-certified cosmetics and personal care products are gaining popularity," 2013; "Halal cosmetics: a market that cannot be ignored," 2014; Khalid et al., 2021). Despite the rapid growth of the global halal food industry, there remain few halal cosmetics currently available on the market. This is because the production of halal cosmetics faces many challenges. This fact is because



many chemicals and ingredients used in cosmetics need to be clarified regarding their halal status before introducing them into the production process (Hajipour et al., 2015). Chemicals and ingredients derived from unlawful animals or obtained in an inappropriate manner cannot be used in halal food and cosmetics (Hussain-Gambles, 2020; Karahalil, 2020; Nordin et al., 2021).

Food additives that have been approved by the FDA can often be used in halal production, except those that might have originated or become contaminated with haram substances. Therefore, detection of ingredients is required for halal authentication (Hashim & Hashim, 2013; Soon et al., 2017). Alongside porcine derivative ingredients, a group of colors is widely used in many kinds of foods and cosmetics. Any color additive to be used in food and other FDA-related products has to be approved by the FDA in order to ensure that they are safe for consumption (Harp & Barrows, 2015). These colors can be derived either by chemical synthesis, or by extraction from microbials, animals, and plants. Most natural colors are derived from plants, such as carotenoid, melanins, and quinins, and are frequently used as colorings in food (Dufossé, 2006) because of their availability, cost efficiency, and stability. Color produced by microorganisms through the fermentation process is another promising avenue for production of food and cosmetic color additives (Tuli et al., 2015), and currently include cycloprodigiosin (Nawaz et al., 2021) and lycopene (Chen et al., 2016; Ma et al., 2015). However, there is a color additive that is obtained through extraction from the dried body of a small insect. The insect-derived color is known as carminic acid (CA), or C.I. 75470, and is extracted from female cochineal insects, which are parasites of cacti. This color has been used as a colorant in many industries, such as the food and beverage, cosmetic, pharmaceutical, and textile industries (Al-Teinaz, 2020; Othman et al., 2021). It is considered

non-halal because of the insect origin, and the extraction and purification process (Fatih Gültekin 2020; Pöhl, 2016). Muslim consumers, therefore, should reject products associated with CA. Moreover, the potential impact of natural products on human health is of increasing concern among consumers, because natural products can contain allergen proteins (Takeo et al., 2018). CA can be used in a mixture with other substances to obtain different shades, which can also improve the color stability compared to single-color additions. Furthermore, mixtures of colors with safe diluents has facilitated the use of these colors in products (Chung et al., 2016).

The techniques used for detection of CA currently rely on highly sensitive instruments. The use of high-performance liquid chromatography (HPLC) is promising for quantification of CA in food matrices, such as yogurt (Carvalho & Collins, 1997). The Raman and surface-enhanced Raman scattering approaches have been used for the quantification of CA in solid candies (Garrido et al., 2019). Differential pulse polarography (DPV) in the hanging mercury drop electrode (DME) method improves sensitivity, and reduced the time required for measurement (Yilmaz et al., 2014). Even though these techniques are promising in terms of their high sensitivity and accuracy, they require specialised instruments, and well-trained personnel to operate them and interpret the results. Therefore, they do not allow for portable analysis at the point-of-need.

Until now, there has been no validated method or laboratory registered with the purpose of halal certification or authentication of CA. In this study, a new and robust technique for determination of CA was developed using disposable screen-printed carbon electrodes to provide a simpler method to detect CA for halal product assurance, with high sensitivity and rapid determination, and with simple sample manipulation. This method represents the first attempt to facilitate halal authentication and inspection of halal

color additives, specifically CA in the presence of CM, without the need for additional separation procedures.

## **2. Materials and methods**

### *2.1 Chemicals and instruments*

The CA and other chemicals were purchased from Sigma Aldrich (Sigma-Aldrich, St. Louis, MO, USA). (Darmstadt, Germany). Milli Q water (Millipore, Billerica, MA, USA) was used to prepare the samples. Citric acid buffer pH 2.5–3.0 was prepared using citric acid from Ajax Finehem (Australia). Sodium dihydrogen phosphate was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Analytical reagent grade sodium hydrogen diphosphate and sodium acetate were purchased from Ajax Finehem (Australia). Acetic acid (glacial) and hydrochloric acid (HCl) 37% were purchased from Merck KGaA (Darmstadt, Germany). Glycine was purchased from Sigma Aldrich (Sigma-Aldrich, St. Louis, MO, USA). All buffer solutions were prepared in ultrapure water, with a resistivity of 18.2 MΩ.cm (Thermo Fisher Scientific, U.K.). Gold nanoparticles (AuNP) were purchased from Kestral Bioscience (Bangkok, Thailand). For ultra-HPLC analysis, the mobile phase solution for ultra-HPLC was filtered through a 0.22 µm nylon filter (Merck Millipore, Billerica, MA, USA) before use. All buffer solutions were prepared at a concentration of 100 mM to obtain appropriate pHs. All pH measurements were performed using a pH meter Metrohm 780, with a standard uncertainty of 0.1 mV (Metrohm, Herisau, Switzerland). The disposable strips of a silver pseudoreference electrode and a graphite counter electrode (ItalSens IS-C) were purchased from PalmSens® (PalmSens, Utrecht, The Netherlands). All viscous samples were pipetted through a Pos-D™ positive-displacement pipette (Mettler-Toledo International Inc, Columbus OH, USA).

## 2.2 *Detection of CA on a screen-printed carbon electrode*

The characteristics and electrochemical conditions for the detection of CA were studied using various buffer types and pH values. The acidic buffer solution, including Glycine-HCl buffer pH 2.5–3.0, sodium citrate buffer pH 3.0–6.0, and sodium acetate buffer pH 4.0–5.0, were prepared at a concentration of 0.1 M. CA, and CM (0.1 mM) was prepared at the above-mentioned pH values to study the electrochemical behaviour with DPV using a screen-printed carbon electrode at a scan rate of 0.1 Vs<sup>-1</sup>, in the potential window of –1.5 V to 1.5 V, keeping the pulse amplitude and width of 0.1 V and 0.05 seconds, respectively. The equilibration time for the electrode was 3 seconds. The operation program was PStTrace 5.8, which was installed on an Android mobile system. The effects of possible interfering agents were defined as the ratio of interference to CA concentration, which gave an error of the current intensity of less than 10.0% in the determination of CA by the recovery of CA calculated from the standard curve, using the DPV method.

## 2.3 *Surface modification of the electrode*

The surface of the carbon screen printed electrode was modified using several strategies. The first was using the solution PEI (3.0  $\mu$ L) for 5 minutes at room temperature to allow the physical absorption of PEI onto the surface of the electrode. The excess PEI was removed by rinsing with DI several times. The PEI-modified electrode was dried before use. The second strategy used gold nanoparticles (AuNPs). The concentrated gold nanoparticles were prepared by centrifugation of 40 nm AuNP at 10,000 rpm for 10 minutes. The pelleted AuNPs were deposited onto the electrode surface. The AuNP modified surface was attached with PEI for 5 minutes, then rinsed several times with excess DI and left until it dried. Thirdly, composites of AuNPs with PEI were obtained by mixing the solution of PEI and 80 nM AuNPs (1:1 v/v). The mixture solution was sonicated for 10 minutes at room temperature. The

colloid solution of the composite AuNPs/PEI was immediately deposited onto the electrode surface, and left until it dried. All modified electrodes were tested with the standard CA at a concentration of 0.2 mM, using DPV. The appropriate modification conditions were selected for further study.

#### 2.4 *Ultra-HPLC*

Ultra-HPLC separations were performed using a Supelco analytical Titan C18 column (5.0 cm × 2.1 mm, 1.9 μm) (Sigma Aldrich, USA). The analytical Ultra-HPLC apparatus consisted of an LC30 AD pump (Shimadzu, Japan) and SPD20A detector (Shimadzu, Japan). The gradient mobile phase system was used for the analysis of CA and CM at 40 °C, by using a photodiode array (PDA) detector at a wavelength of 526 nm. The mobile phase consisted of solvent A (0.1 M sodium phosphate buffer pH 5.5) and solvent B (methanol) with a flow rate of 0.5 mLmin<sup>-1</sup>. The initial mobile phase composition was a gradient from 0 to 45% of solvent B within 2.30 minutes, and was maintained at 45% solvent B for 1.40 minutes, changed linearly to 10% (3.70–4.20 minutes), and then returned to the initial conditions and kept for chromatograph column equilibrium for 6.0 minutes. The mobile phase was filtered through a 0.22 μm nylon membrane filter, and ultra-sounded for degasification before use. The injection volume was 20 μL. For analysis, the dye color (CA and CM) was dissolved in 0.1 M citric acid buffer pH 7.0 at a concentration of 0.5 mM.

#### 2.5 *Real sample analysis*

As a proof-of-concept demonstration for the detection of CA in cosmetic samples, lipstick tint was prepared by melting 3.0 g of aloe vera balm with varying concentrations of a color mixture (CA and CM) to obtain a final concentration of 0.1 % w/w. The solution was then placed at room temperature to settle. Color analysis was performed by sample dilution with a citric acid buffer, pH 3.0, at 1: 1 (w/v), and was vortexed. The solution was

tested with DPV and ultra-HPLC. Additionally, commercially available foods and lipstick tints with red coloring were purchased from a supermarket in Bangkok, Thailand, for method validation. The samples were stored at 4 °C until analysis.

### 3. Result and discussion

#### 3.1 *Detection of CA on a screen-printed carbon electrode*

The polarographic behaviour of CA has been studied previously on a DME electrode using Britton–Robinson buffer pH 2.0 (Yilmaz et al., 2014). However, the width of the potential window of the mercury electrode limited its selectivity, therefore, combining it with preliminary separation techniques such as liquid-liquid or solid-phase extraction, or column, paper, or thin layer chromatography, is required (Vyskočil & Barek, 2009). On the other hand, disposable carbon screen-printed electrodes exhibit a broad potential window and low background current (Couto et al., 2016; Wang et al., 1998). Therefore, modified SPE could be the suitable platform for sensitive detection of CA in the presence of co-existing dye. Furthermore, carbon-based SPEs have shown inferior behaviour due to its good cathodic potential as well as the less of toxicity from mercury oxidation (Barón-Jaimez et al., 2014; Xu et al., 2021). In addition, they allow portable analysis, and enable the use of minimal infrastructure with low cost. Therefore, a carbon screen-printed electrode was employed for the detection of CA. The structure of CA comprised of the anthraquinone moiety linked with a monosaccharide, methyl and 4 of hydroxyl groups. It is a tetraprotic acid with confirmed by 4 different of pKa values 2.81–3.42, 5.43–6.20, and 8.10–8.94 (Atabey et al., 2012).

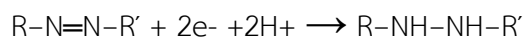
An alternative of CA is CM. CM belongs to the group of azo dyes which are used as food additives (E-122). E-122 is considered as halal color which can be used not only in food and beverage but also cosmetic and drugs.

However, the health effect from the azo dye was also reported. Therefore, it must be used with the level does not exceed than  $100 \text{ mg}\cdot\text{Kg}^{-1}$  in chewy gum and soft candy and  $150 \text{ mg}\cdot\text{Kg}^{-1}$  in beverages (*Food additive details (Updated up to the 42nd Session of the Codex Alimentarius Commission (2019))*). In some food products, the mixture of CA and CM has been reported in which to improve the appearance of food products (Bessaire et al., 2019; Müller-Maatsch & Gras, 2016). Another azo dye such as amaranth (E-123) usually used in medicines instead of using in food and cosmetic because of low of ADI ( $0.05 \text{ mg}\cdot\text{Kg}^{-1}$ ). Therefore, CM was selected for demonstration of determination of CA in the presence of synthesis azo dyes.

In this study, the electrochemical behaviour of CA and CM on the carbon screen-printed electrode was studied with a DPV scan potential range of  $-1.5 \text{ V}$  to  $1.5 \text{ V}$ , at a scan rate of  $0.1 \text{ Vs}^{-1}$ . The effect of the supporting electrolyte was examined over the pH range of 2.5 – 6.0 in different buffers. The well-shape of oxidation and reduction responses were obtained for all tested electrolytes. CA exhibited electrochemical properties because of the phenolic and o-quinone in its structure (Yilmaz et al., 2014; Ziyatdinova et al., 2020). The electrooxidation of CA resulted in color reduction (Tütem et al., 1996). The DPV response of CA at the reductive region was shifted to more negative (from  $-0.581 \text{ V}$  to  $-0.940 \text{ V}$ ) with an increasing pH, while the oxidative current of CA was observed within the potential range of  $+0.397 \text{ V}$  to  $+0.017 \text{ V}$ . For glycine-HCl buffer at pH 2.5 and 3.0, the response currents were similar, however the shift of the signal ( $\sim 60 \text{ mV}$ ) was observed at both the oxidation and reduction potentials. The response signal was higher for CA prepared in citric acid buffer than that prepared in sodium acetate and glycine-HCl buffer (See Figure. S5, supplementary materials). The maximal electrolytic reduction (negative potential) was found when using  $0.1 \text{ M}$  citric acid buffer pH 6.0, while the oxidation of CA (positive potential) was higher

when using 0.1 M citric acid buffer pH 3.0 (Figure 8). Therefore, the oxidation and reduction of CA at the carbon electrode was pH dependent, similar to that observed using a DME electrode (Yilmaz et al., 2014). The oxidation of CM at potential 0.0 V and +0.186 V were also observed in 0.1 M citric acid buffer pH 6.0. The effect of pH on the electroactivity of a group of azo dye is regardless to their substitutes (Pliuta et al., 2021). These peaks were slightly different from that of the oxidation peak of CA. Therefore, to eliminate this source of error, this pH is not suitable for the detection of CA if CM is also included in the color mixture.

The electrochemical behaviour of CM was also investigated under the same buffers and pH conditions of CA. It was found that CM could be oxidized at the positive potential. A significant oxidation peak of CM was observed around +0.62V and + 0.60 V for CM prepared in glycine-HCl pH 2.5 and 3.0, respectively. A similar pattern was observed for citric acid (+0.30 V to +0.46V) and acetate buffer (+0.36 to +0.43 V), in which the peak was shifted to a lower potential with increasing pH. It has been reported that the reduction of azo dyes takes two stages in pH below 3. In addition, for azo dyes with the pH close to the neutral condition, the reduction in the stage 2 does not occurred. The reductions of CM are as following reaction:



The oxidation of CM was thus pH dependent (Chebotarev et al., 2020; Lipskikh et al., 2017; Nezhad et al., 2018). The electrolytic reduction of CM exhibited reduction peaks around -0.129V and -0.501V for CM prepared in citric acid buffer at all pH values tested in this study. In addition, the reduction potential of -0.791V was not observed for CM prepared in strong acidic buffer conditions (pH 2.5–4.0). The small response in the reduction region was observed when CM was prepared in



sodium acetate and citrate buffer at pH 5.0 and 6.0. Meanwhile, at the oxidative potential, the responsive potential for CM was near that found for CA (see Figure. S6, supplementary materials). Therefore, the responsive potential of the oxidative state might not be suitable as a marker for detection of CA where CM is included in the system. Hence, 0.1 M citric acid buffer at pH 3.0 was selected as the most appropriate pH conditions for electrochemical detection of CA in the presence of CM. The specific potential for oxidation-reduction of CA and CM are promising for both qualitative and quantitative analysis. The voltametric separation of CA and CM can be achieved on the carbon electrode without additional processes, such as microwave-assisted hydrothermal elimination (Chang et al., 2010).

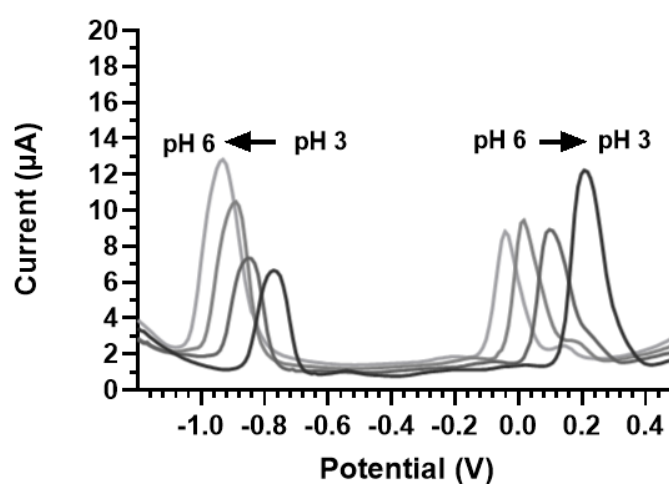


Figure 8 Electrochemical responses of 0.1 mM CA prepared in citric acid buffer pH 3.0–6.0.

### 3.2 Simultaneous determination of CA in the presence of CM

After ensuring the position of the reduction signals of the individual CA and CM, the detection of CA in the presence of CM was carried out in the selected supporting electrolyte. As can be seen from Figure 9, the potential marker of CA could be clearly seen when the addition of 0.1 mM CA into the system included 0.5 mM of CM. In addition, increasing the CA concentration from 0.1 to 0.5 mM led to good linearity of the current response. This implies

that the DPV technique is promising for the detection of CA, even when the sample is mixed or contaminated with CM. In addition, under the selected conditions, the detection of CA did not require an additional separation protocol. This technique is also faster than chromatographic paper, because it does not require any incubation time (~40 minutes). Although this technique is more expensive than chromatographic paper, it can be used without a toxic solvent system

Furthermore, the effect of CM on the detection of CA was studied by increasing the concentration of CM, while keeping the concentration of CA constant at 0.1 mM. Two significant oxidation responses of CM were particularly emphasized at the potential of +0.007 V and +0.526 V upon increasing the CM concentration (see Figure. S7, supplementary materials). Moreover, the individual signal of CA at the potential of +0.207 V was interfered with by CM when CM was present at concentrations exceeding 8-fold that of CA. The oxidation peak of CM at high concentrations could be ascribed to the oxidation of the hydrolysis product of CM. However, the signal at the potential of -0.791 V of CA could still be observed, even when the concentration of CM was more than 10 times higher than that of CA. Therefore, this technique could provide spontaneous detection of CA in the presence of chemically synthesized CM.

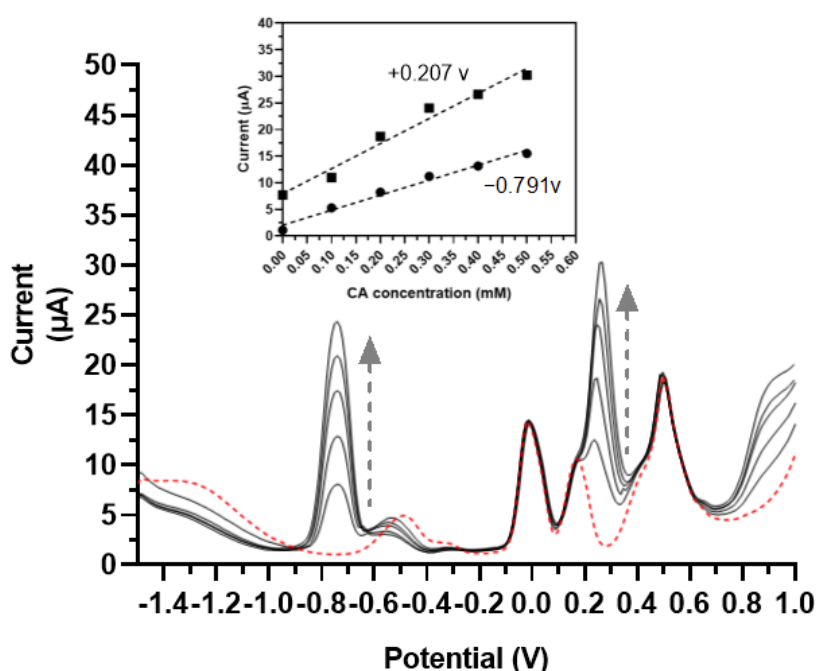


Figure 9 DPV voltammograms of 0.5 mM CM in the presence of a CA concentration ranging from 0–0.5 mM. The voltammogram was carried in 0.1 M citric acid buffer pH 3.0. Insert: increase of the current signal at the oxidation and reduction peak of  $-0.791$  and  $+0.207$  V. The red line is the chromatogram of 0.5 mM CM without CA.

### 3.3 Limit of detection of CA

The DPV method has higher current sensitivity compared to CV in electrochemical reactions. The reduction peak current and concentration exhibit a linear correlation in the range of 0.01–0.40 mM (Figure 10), with a corresponding equation of  $y = 38.08x + 1.636$  and  $y = 63.62x + 3.031$  obtained at a potential of  $-0.791$  V and  $+0.207$  V, respectively ( $R^2$  of 0.99). The limit of detection (LOD), calculated by the  $3 \times \text{SD}/\text{slope}$  of the signal potential of  $-0.791$  V and  $+0.207$  V, were  $33.68 \mu\text{M}$  and  $46.1 \mu\text{M}$ , respectively. The sensitivity obtained using the carbon screen printed electrode was higher than those observed by DME, which was reported on previously with an LOD of  $0.16 \mu\text{M}$  (Yilmaz et al., 2014). However, due to the high selectivity at the

reductive potential of CA, it provided simultaneous detection without using additional separation instrumentation.

As demonstrated in the above session, CM did not have an adverse effect on the detection of CA (Figure 11). Different types of substances that commonly co-exist food and cosmetics samples, which could be potent interfering agents, were also studied. The effects of the interference compounds were calculated from the peak current, in which changes to the current of more than 10% of the current of 0.1 mM CA were noted. A group of cosmetic-grade humectants, including monopropyleneglycol, was prepared in DI water. The interferences were prepared at concentrations exceeding 100-fold (w/w) that of CA. The effect of ascorbic acid, which is a substance used as an antioxidant in many foods and cosmetics, was also studied. For ascorbic acid, 0.1 mM CA was spiked into the 100 mM stock solution of ascorbic acid. The final concentration of ascorbic acid was calculated as 100 times higher than that of CA. It was found that the oxidation of ascorbic acid at the potential of +0.117 V did not significantly alter the reduction current of CA (-0.791 V). Other types of substances, including glucose, the amino acid L-cysteine, and sodium benzoate, were also studied. We found that at a concentration 100 times higher than that of CA, there was no interfering effect in the detection of CA. Therefore, it could be concluded that the substances did not have a significant effect on the voltammogram of CA, and no interference was observed. Therefore, electrochemical analysis based on DPV on a disposable screen-printed electrode enabled sensitive and specific detection of CA. These results are presented in Table 3.

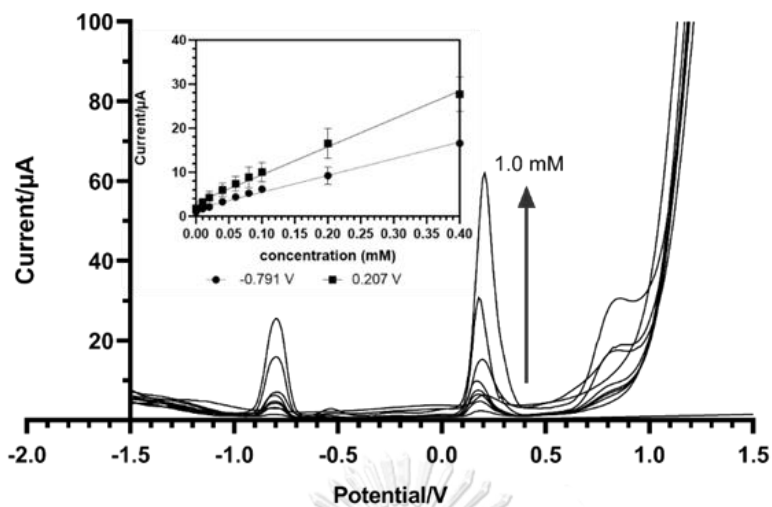


Figure 10 Standard curve of carminic acid in 0.1 M citric acid buffer pH 3.0. The concentration of carminic acid ranged from 0–1.0 mM. Insert; the linearity obtained for signal potentials of  $-0.791$  V and  $+0.206$  V

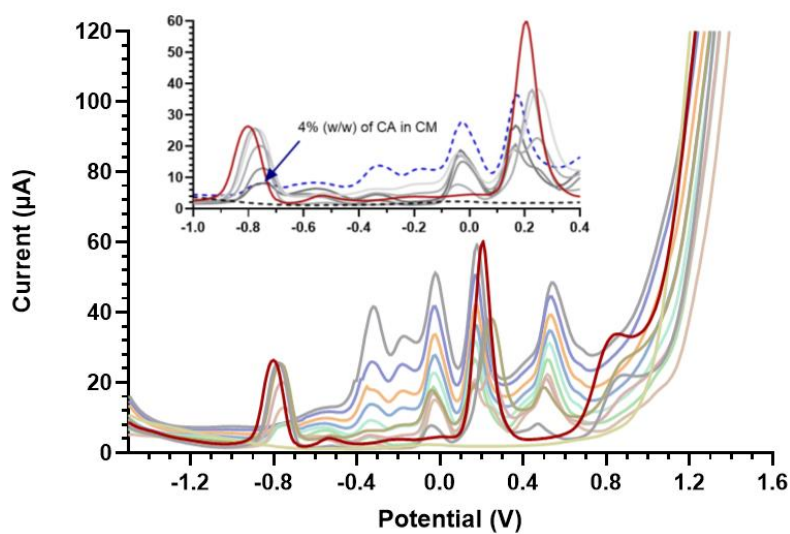


Figure 11 Simultaneous detection of CA in the presence of CM. The red line represents the DPV spectrum of CA in citric acid buffer pH 3.0. The marker signal of CA was found when CA was mixed with CM down to 4.0 % (w/w).

*Table 3 Interfering effects for the determination of 0.1 mM CA by DPV*

Interfering ion	Tolerance ratio
Glycerine	100
Polyethylene glycol	100
Ascorbic acid	100
Glucose	100
L-Cysteine	100
Sodium benzoate	200

#### 3.4 Proof-of-concept for detection of CA in water-based lipstick

The tint lipstick formulation was prepared in our laboratory, using aloe vera as the main ingredient. Mixtures of CA and CM were prepared at different ratios, with the total dye at the final concentration of 0.1% (w/w). Tint-1 contained neither CA nor CM. Tint-2 was formulated with 0.33 mM of CA and 1.67 mM of CM. Tint-3 was formulated with 0.67 mM of CA and 1.33 mM of CM. The prepared liquid lipsticks were diluted in the 0.1 M citric acid buffer pH 3.0, before being subjected to analysis with DPV. The lipstick with aloe vera alone (tint-1) did not exhibit any electrochemical current at the carbon electrode. The recovery of the CA content in tint-1 and tint were 84.5 and 84.1%, respectively.

For ultra-HPLC, the sample was passed through a 0.22  $\mu\text{M}$  filter before analysis. A fast linear gradient, at a flow rate of 0.5 mL min<sup>-1</sup>, of a binary mobile system of methanol and 0.1 M phosphate buffer pH 5.5, was used to separate the compounds of interest with a reasonably short run time of 6.0

min. The separation chromatograms of CA and CM were exhibited at the rt of 2.55 and 3.55 minutes (See Figure S8, supplementary materials). The concentrations of CA calculated by the calibration curves of DPV and the ultra-HPLC technique are compared in Table 4.

The precision was studied at 0.33 mM and 0.67 mM of CA. The CA was added to the lab-made water-based lipstick ( $n=20$ ). The prepared lipstick was tested by 1:1 (v/v) dilution with 0.1 M citric acid buffer pH 3.0. The current at the marker position ( $-0.791$  V) were  $10.19 \pm 0.97$   $\mu$ A and  $14.08 \pm 0.39$   $\mu$ A and the coefficient variation (%) were 9.52% and 2.78%, respectively. Inter-day precision ( $n=10$ ) exhibited  $1.047 \pm 0.087$  and  $1.412 \pm 0.083$   $\mu$ A with the CV of 8.27% and 5.88%, respectively. The lower of the CV attributed to the precision of the proposed technique.

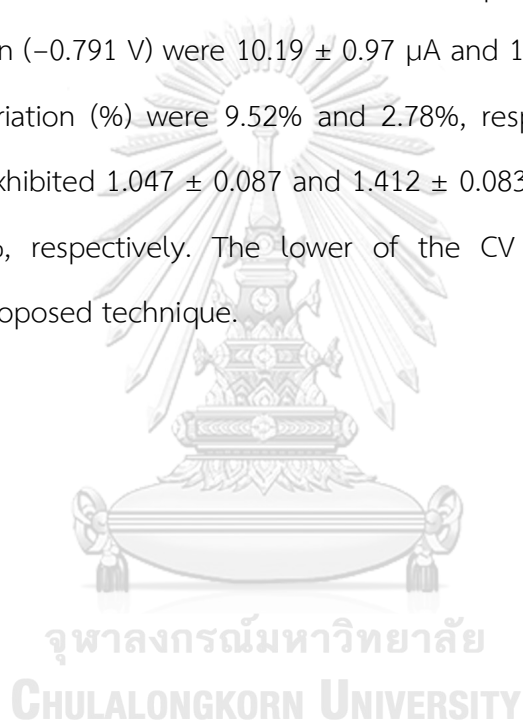


Table 4 Comparative studied for the determination of CA in water-based lipstick using DPV and ultra-HPLC ( $n = 3$ )

Formula	CA concentration (mM)	
	Ultra-HPLC (%recovery)	DPV at $-0.791$ V (%recovery)
Tint-1 (without CA)	ND	ND
Tint-2 (0.33 mM CA)	$0.33 \pm 0.064$ (101.0)	$0.28 \pm 0.026^*$ (84.5)
Tint-3 (0.67 mM CA)	$0.49 \pm 0.048$ (73.4)	$0.55 \pm 0.044^*$ (84.1)
ND : Not detectable		

### 3.5 Detection of CA in the commercial lipstick tint and raw material color additives

The commercial food red color was diluted 200 times with buffer solution, before it was subjected to analysis by DPV and ultra-HPLC. The CM potential of the commercial food red coloring could be confirmed with the  $rt$  at 3.5 minutes by the ultra-HPLC chromatogram. Three of beverages with red coloring were collected from a supermarket in Bangkok for CA analysis. Beverage C exhibited a signal of  $-0.791$  V, in which the concentration calculated from the calibration curve was  $0.014 \pm 0.001$  mM by ultra-HPLC and  $0.012 \pm 0.002$  mM by DPV. This concentration is lower than the LOD of the developed technique; therefore, the standard solution of CA was spiked



to gain the final concentrations of 0.05, 0.10, and 0.25 mM. Recovery of 85.30 to 95.83% was obtained by ultra-HPLC, and 101.9 to 123.9% by DPV. Furthermore, no CA was found in the other beverages (A and B), as demonstrated in Table 5.

The cosmetic-grade raw materials were supplied from Sensient Technologies (Thailand) Co., Ltd. Blind testing was performed for these raw materials, which are used as color additives in cosmetic products. All sample was prepared at the concentration of  $0.25 \text{ mg mL}^{-1}$  in citric acid buffer pH 3.0. The samples investigated with DPV exhibited a significant current of CA at the reductive  $-0.791 \text{ V}$  for raw materials A and B, which was in accordance with the ultra-HPLC chromatogram of CA, as demonstrated with an  $r_t$  of 2.55 minutes. The concentrations of CA analyzed by ultra-HPLC for raw materials A and B were  $0.222 \pm 0.011$  and  $0.416 \pm 0.0101 \text{ mM}$ , respectively. The concentrations of CA calculated by DPV were  $0.209 \pm 0.002$  and  $0.490 \pm 0.004 \text{ mM}$  for raw materials A and B, respectively. Furthermore, raw material C exhibited the characteristic voltammogram of CM, which is in accordance with the spectrum peak at an  $r_t$  of 3.5 minutes on ultra-HPLC. The results indicated the potential of the DPV technique with disposable carbon screen-printed electrodes.

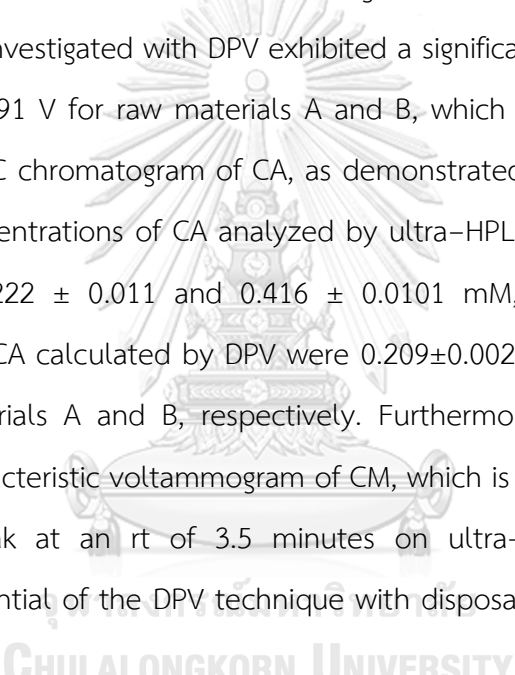


Table 5 Surveillance study of the commercial water-based lipstick available in the market in Bangkok, Thailand and raw materials used as color additive in cosmetic products

Sample name	added conc. (mM)		ultra-HPLC		DPV		CA detectable
	found conc. (mM)	recovery %	found conc. (mM)	recovery %	found conc. (mM)	recovery %	
Food colorant	CM*		CM*		CM*		No
Beverage A	ND		ND		ND		No
Beverage B	ND		ND		ND		No
Beverage C	0.0	0.014±0.001			0.012±0.002		Yes

Sample name	added conc. (mM)	ultra-HPLC		DPV		CA detectable
		found conc. (mM)	recovery %	found conc. (mM)	recovery %	
Raw-material-A  (0.25 mgmL <sup>-1</sup> )	0.10	0.097±0.023	85.30±20.3	0.138±0.003	123.6±2.34	Yes
	0.25	0.262±0.022	95.83±6.30	0.26±0.009	101.9±3.38	
Raw-material-B  (0.25 mgmL <sup>-1</sup> )		0.222±0.011		0.209±0.002	93.17±6.8	Yes
				0.490±0.004	109.02±4.7	

ND: not detectable, CM\*: CM detected, n=3

### 3.6 *The enhancement of the sensitivity of detection by surface modification of the composite AuNP with PEI for modification*

Generally, electrode modification with nanomaterial enhances sensitivity of detection. The nanostructure provided specific surface area for promoting reaction at the electrode surface. In this study, the modification of the surface electrode was performed with different strategies, including using AuNPs, electrolytic polymer, and AuNP composited polymer (PEI). Branched-PEI (MW of 25 k) has high density of cation of imine on its structure. Functionalized AuNPs' surface with PEI was shown to be effective procedure for improving the surface property of AuNPs to be accumulated with nitrite ( $\text{NO}_2^-$ ) as a result of increasing its detection performance (Talbi et al., 2019). Figure 12 shows the improvement of the current signal using the electrode modified with 80 nM AuNPs alone, and electrode modified by the layer-by-layer deposition of AuNPs with various concentrations of PEI, ranging from 0.0 to 0.5%. Enhancement of the detection sensitivity was observed in all conditions of electrode modifications. The optimum current for CA was obtained using AuNPs with a PEI of 0.2%. However, the large error bar ( $n=4$ ) implied variation of the layer-by-layer deposition method which could be due heterogeneous electron transfer at interface of modified electrode (Mirčeski et al., 2007).

The homogeneity on the electrode surface was improved by single step casting modification. The nanocomposites of AuNPs functionalized with PEI was prepared by ultrasonic-assisted fabrication (Arrigo et al., 2018; Zhang et al., 2009). It has been previously reported that PEI has the ability to act as a reducing agent and stabilizing agent for gold nanoparticles (AuNPs) (Sun et al., 2005; Wang et al., 2005). Therefore, increased colloidal stability of the ultrasonicate-induced fabrication was observed by the red color of the solution (Belhout et al., 2019). The colloidal of AuNPs was obtained by mixing

(1:1, v/v) at room temperature, and ultrasonication for 10 minutes. The solution of AuNP/PEI was immediately deposited on the carbon screen printed electrode for 6 minutes at 60°C. The modified electrode was cleaned with citric acid buffer pH 3.0, and dried at room temperature before use. It was found that modification of the surface electrode with the nanocomposite AuNPs/PEI was promising for improvement of the sensitivity of the CA sensors. The highest performance of the AuNP/PEI modified electrode was obtained when using 0.05% PEI. Reduction of the current was obtained when the PEI concentration was increased by more than 0.1% (Figure 13). Therefore, modification of the solid carbon electrode with nanocomposites AuNP/PEI at the concentration of 0.05% PEI was selected for studying the analytical performance of CA detection. Furthermore, at a very low concentration of CA, the reduction peak of CA was overlayed by the background signal of the modified electrode. Although, the signal potential of CM was dominant at the potential of 0.0 V instead that was observed at +0.207V when the nanocomposites AuNP/PEI modified electrode was tested (See Figure S9, supplementary materials). This was suggested by the electrostatic binding of CM at the PEI-modified electrode. Owing to enhance specific signal of the CM current at 0.0 V, the signal current of the CA detection could be selected at the oxidation potential (+0.297 V). As reported in Table 6, the LOD for the detection of CA was 1.42  $\mu\text{M}$ , however, with a lowering of the detection range from 10–400  $\mu\text{M}$  to 10–100  $\mu\text{M}$  ( $R^2 = 0.986$ ). In conclusion, the sensitivity of the detection was greater by approximately 24 times, compared to the unmodified electrode.

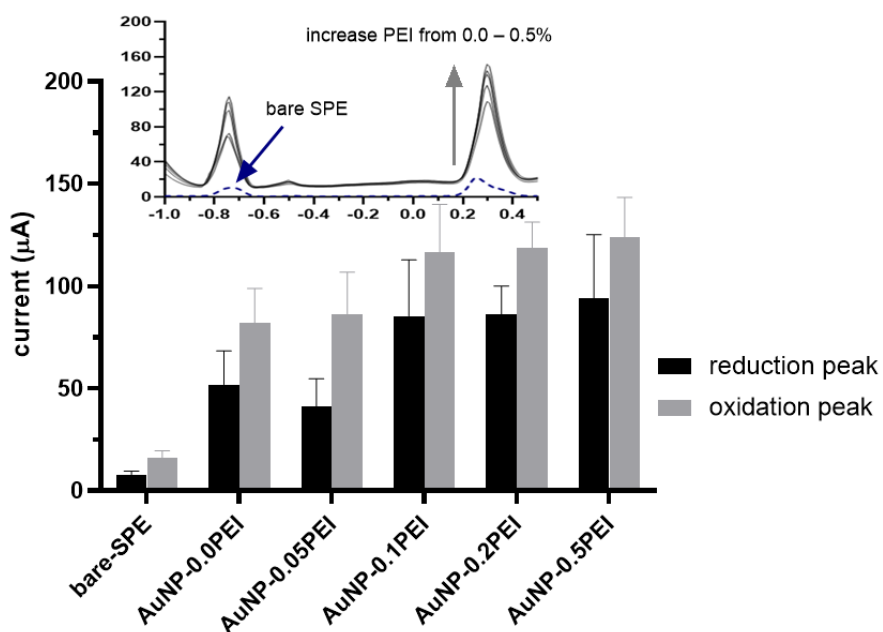


Figure 12 The sensitivity of the CA detection at the modified electrode.

The DPV was scanned in the potential range of  $-1.5$  to  $1.5$  as the modified electrode vs Ag/AgCl. Insert: the DPV behaviour of modified electrode compared with the unmodified SPE (blue).

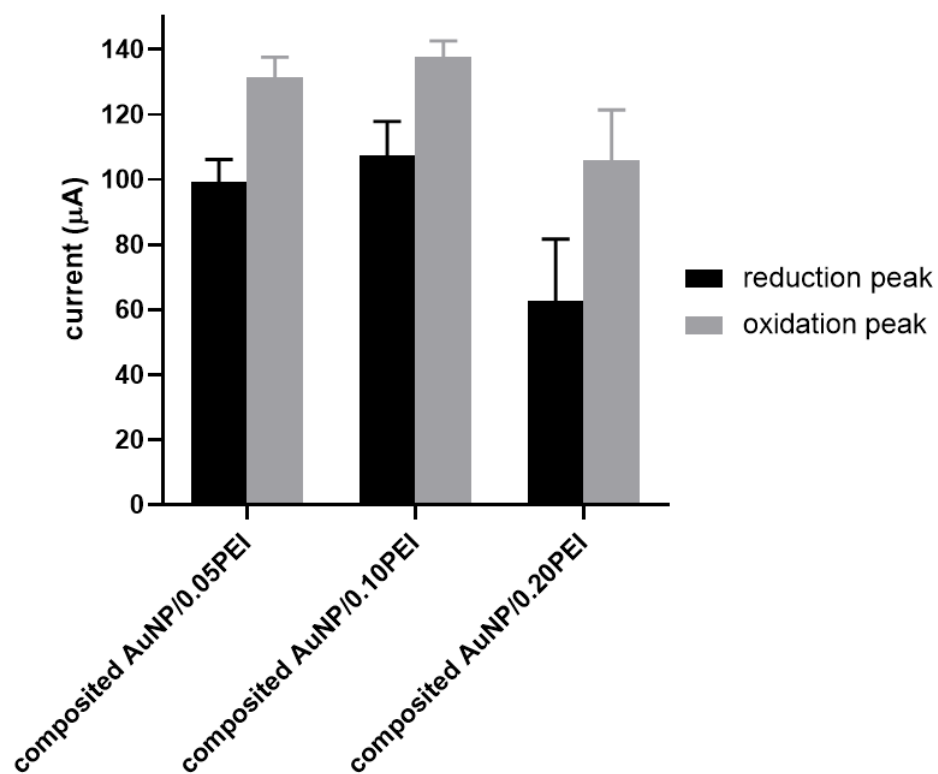


Figure 13 Detection of CA at the modified electrode with the composited AuNP/PEI. All parameters used for CA analysis were similar to those used previously.

*Table 6 The comparison of the performance of unmodified electrode and composited AuNP/PEI electrode vs Ag/AgCl*

Modification SPE	Detection range ( $\mu\text{M}$ )	Calibration curve	LOD ( $\mu\text{M}$ )
Bare-SPE	10–400	$y = 38.08x + 1.636$	33.68
Modified-AuNP/PEI	10–100	$y = 991.32x - 5.451$	1.42

#### 4. Conclusion

In this work, we demonstrated the feasibility of using disposable screen-printed electrodes for the monitoring of unlawful substances that could be found in food and cosmetic products. The unmodified electrode with DPV achieved simultaneous detection, even in high-complexity sample matrices and with combinations of permissible and non-permissible colors. This technique provided comparable sensitivity, without the need for separation by chromatographic methods. Therefore, this technique is simpler and faster than paper and liquid chromatography. In addition, the device is portable, and a smart phone can be used for interpretation of the results. Therefore, it could be applied at the site of need. It could therefore be a useful tool and a novel method for supporting halal product assignment, accreditation, and authentication. Additionally, as the DPV method did not require any extraction method, it is defined as direct analysis. The simple and rapid sample preparation phase avoids time loss in the analyzes and allows multiple analyzes to take place within a reasonable amount of time.

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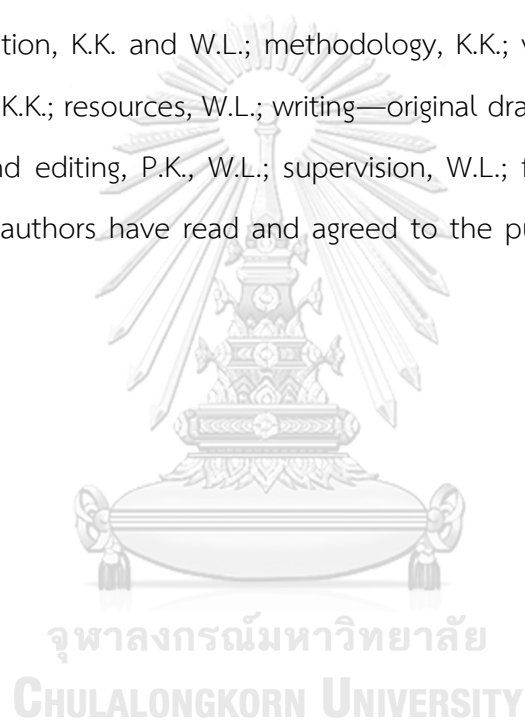
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**Conflict of interest**

The authors declare no conflict of interest.

**Authors' contribution**

Conceptualization, K.K. and W.L.; methodology, K.K.; validation, K.K. and W.L.; investigation, K.K.; resources, W.L.; writing—original draft preparation, K.K.; writing—review and editing, P.K., W.L.; supervision, W.L.; funding acquisition, W.D. and V.N. All authors have read and agreed to the published version of the manuscript.



## 5. Supplementary materials

All supplementary materials are available at: [www.ftb.com.hr](http://www.ftb.com.hr) and as following:

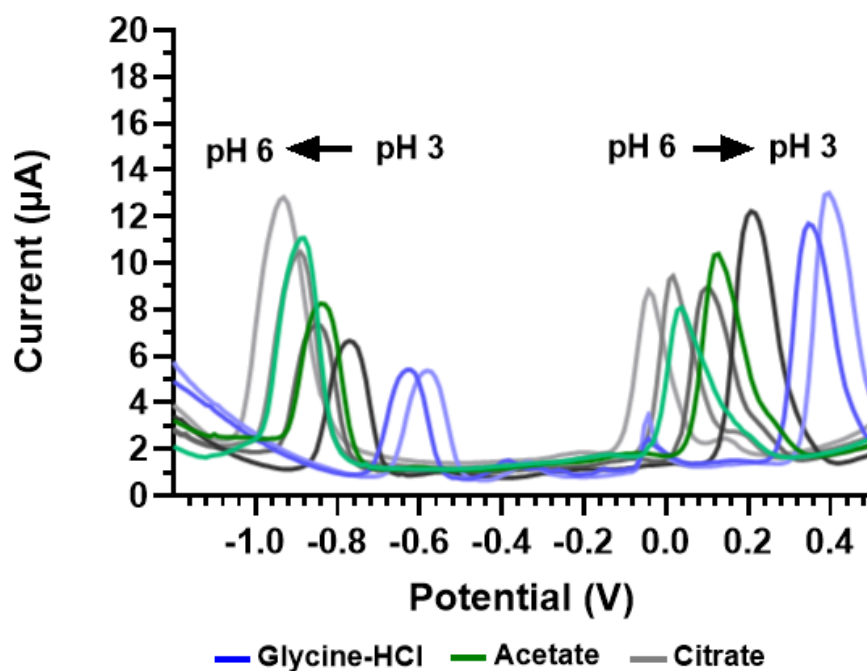


Figure S5 The reduction behaviour of CA in various buffers and pHs. The highest DPV responses was observed when CA was prepared in citric acid buffer

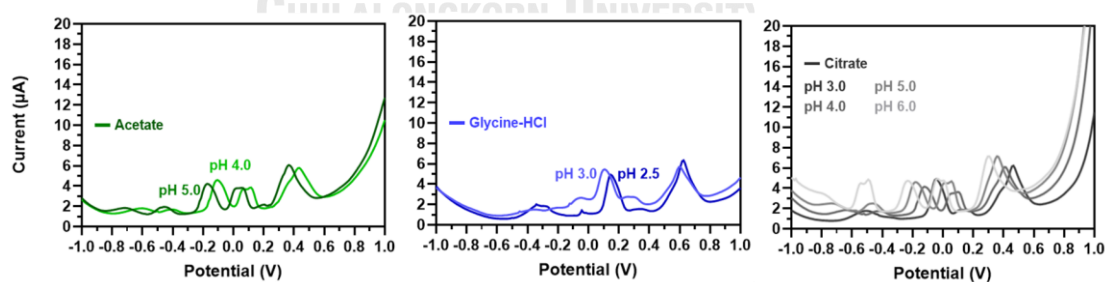


Figure S6 The DPV chromatogram of 0.1 M CM in various buffers and pHs condition. The characteristic signal of CA at the potential of  $-0.79$  volt did not observe at the strong pH conditions (pH 2.5 – 4.0). The small signal response was observed at pH 5.0 and 6.0 of acetate and citrate buffer.

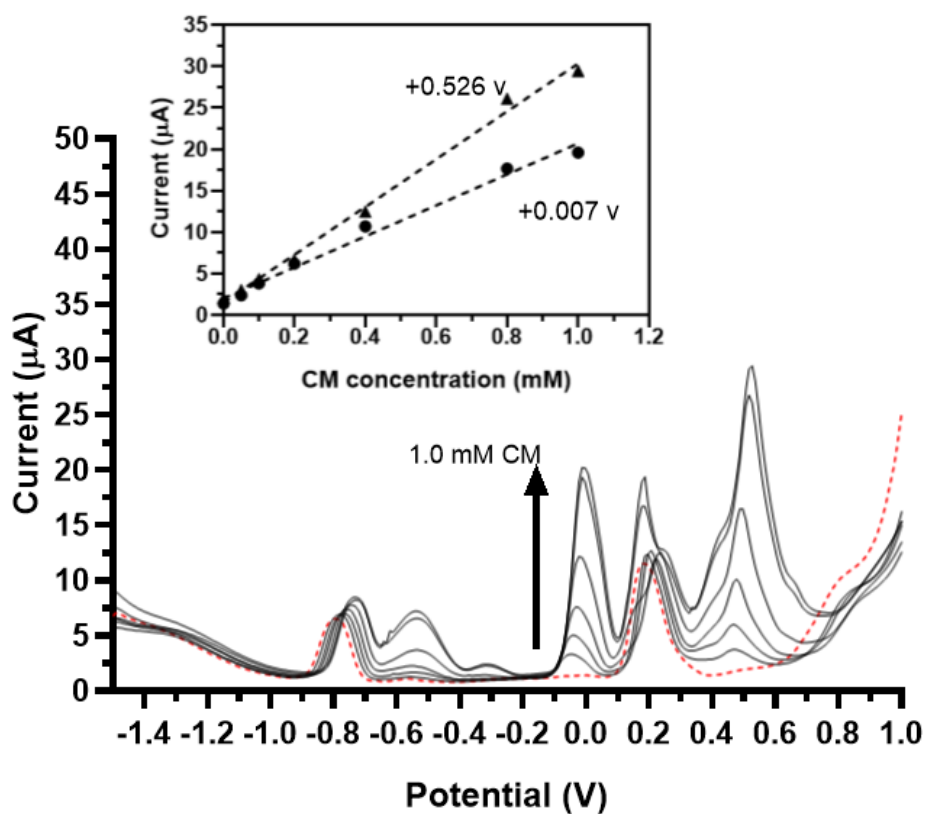


Figure S7 The effect of CM on the detection of CA in 0.1 M citric acid pH 3.0. The characteristic potential response of CA at  $-0.791$  volts still detectable and did not interfered by the CM. Insert; the linearity increases of the current signal of CM at the potential of  $+0.007$  volts when increase CM concentration.

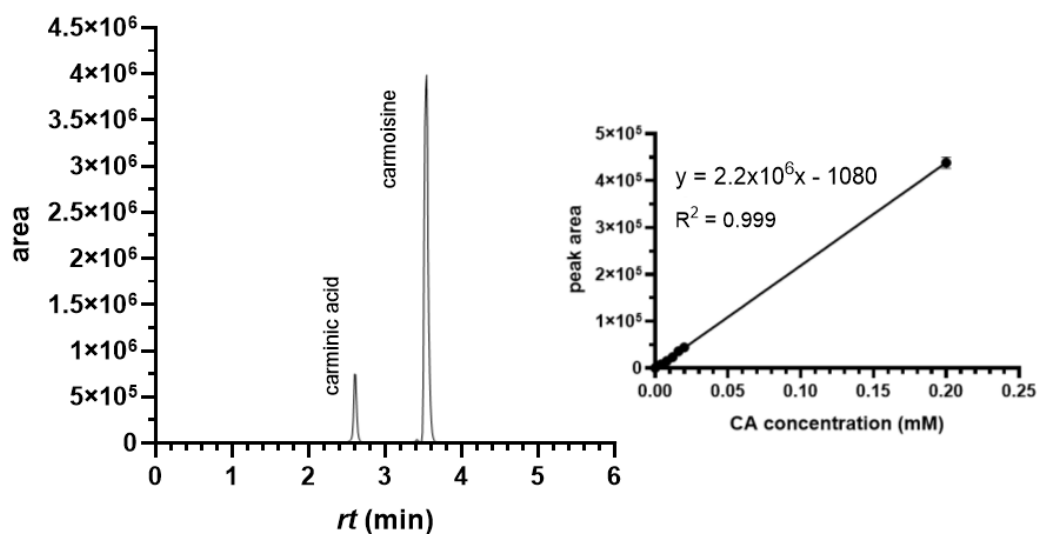


Figure S8 The chromatogram of a mixture of CA and CM in the 0.1 M citric acid buffer. The solution mixture was run through C18 column using gradient solvent system consisted of methanol and 0.1 M sodium phosphate buffer pH 5.5 as a mobile phase with a flow rate of 0.5 mLmin<sup>-1</sup>. The CA and CM could be observed at 2.55 minutes and 3.55 minutes, respectively. Insert: the calibration curve of CA in citric acid pH 3.0.

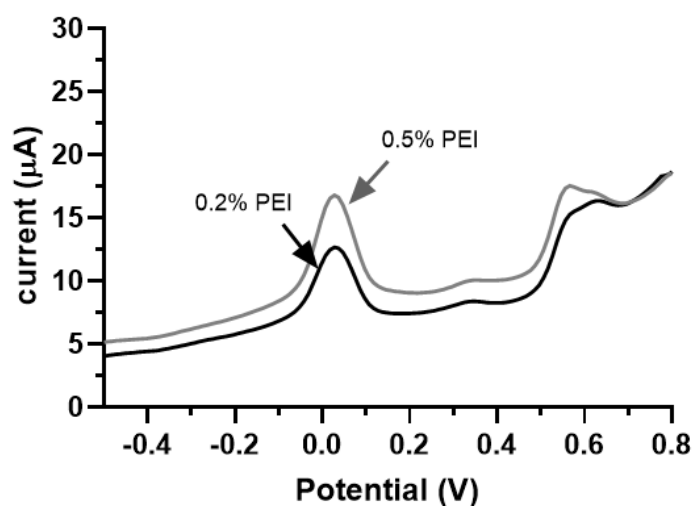


Figure S9 The DPV responses for CM detected with the AuNP/PEI modified electrode. The peak at the potential of  $\sim 0.20$  V could not be observed under the detection condition.

## Part 3

### 1. Conclusion

Two analytical approaches have been proposed as following:

- i) a simple distance paper-based analytical device for the screening of lead in food matrices
- ii) an electrochemistry-based technique for the detection of carminic acid to assure the safety of halal products

These analytical approaches can assist the safety assessment in terms of chemical and haram hazards analysis.

The distance-based paper device was fabricated by immobilizing the detection channel with PEI. The color of CA precipitated on the immobilized detection channel after forming a complex with Pb in solution was proportional to the Pb content in the sample. The contaminated Pb in century eggs samples were detectable using a standard addition technique combined with a drawing-PAD. The potential of the developed dPAD for screening of Pb from blinded spiked century egg samples and reported as lower or higher  $2 \text{ mg mL}^{-1}$ , agreed with the actual Pb concentration found in the same samples as determined with AAS.

The analysis of hazard that was related to the consumer obligation was also proposed herein. Because CA was recognized as one of the non-permissible ingredients for Muslim consumers, the method for determination of CA was in high demand to assist halal product assessment. In this study, an electrochemistry-based method was proposed. The DPV technique was employed to analyze CA in the solution mixtures because the specific potential of oxidation and reduction of CA under acidic solution at the carbon-screen printed electrode can be obtained. Interestingly, the

modification of the electrode's surface with PEI and gold nanoparticle composite can dramatically improve the sensitivity of the method. Its sensitivity is sufficient to detect CA in raw materials and available products such as lipsticks and beverages.

## **2. Limitations and suggestions for further works**

The limitation of this study depends on each approach as following:

- i) A simple distance paper-based analytical device for the screening of lead in food matrices
  - a. According to our preliminary investigation, the stability of the developed dPADs is acceptable, as the devices can be stored in a desiccator for at least one month. Further long-term stability should be investigated.
  - b. The detection mechanism relies on the capillary action of a paper substrate, which could be affected by the humidity and temperature of the environment.
  - c. The application of the developed dPADs was demonstrated in spiked century eggs samples. However, analyzing other food matrices with different viscosity and ion interferences may not be applicable.
  - d. The limit of detection for the developed dPAD was due to the color formation along the immobilized detection channel. Therefore, the homogeneous immobilizing of the vicious polymer (PEI) by other techniques such as screening or printing can be maximized the reaction uniformly and enhance the sensitivity.

- e. To enhance the sensitivity, pre-treatment of the sample is promising by designing the sample loading with filtering out interferences.
  - f. The sample pre-concentration on the dPADs before analysis by simple heating and evaporation on the membrane paper is possible. Alternatively, using water-absorbent materials to generate continuous flow and received high solution volume can be specially designed.
  - g. Although KCN is an effective agent to mask and diminish the effect of Cu, it is not recommended for use in food analysis because of its high toxicity. Alternative masking reagents for the Cu should be further investigated such as thiosemicarbazide and its derivatives
  - h. In this study, Fe<sup>3+</sup> didn't study for interference. However, Fe<sup>3+</sup> is possibly found in a very small amount in egg white. Nevertheless, common iron masking agents such as NaF can be used to reduce the interference effect by Fe<sup>3+</sup> and achieve better specificity.
- ii) An electrochemistry-based technique for the detection of carminic acid to assure the safety of halal products
- a. In this study, the specificity of the method for analysis of CA under real sample matrix was investigated only in the presence of the other color additive, CM. Therefore, the method needs further investigation when analyzing the actual samples existing with several food additive compounds.

- b. The unknown shelf-life stability of the modified electrode limited the applications for analyzing outside laboratories. Thus, it implies further study in terms of storage stability, ruggedness, and robustness.
- c. After modification by a composited of AuNP/PEI, the homogeneity of electrode surface should be confirmed by conductive atomic force microscopy (AFM) measurement.
- d. In this study, the commercial electrodes were entirely used. However, the in-house fabrication of carbon screen-printed electrodes is a choice in the future to reduce the assay cost.





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