Colorimetric Assay Based on Green Analytical Method for Determination of Acetic Acid using Red Cabbage (*Brassica oleraceae L. var.*) as a Natural Reagent

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Abstract

A green portable micro-scale colorimetric technique was developed for acetic acid determination. This system is controlled via an application on mobile phone. Furthermore, red cabbage (*Brassica oleraceae L. var.*) extracts were used as an alternative natural reagent for the quantification of acetic acid. The purple color of cyanidin contained in the red cabbage extracts reacted with acetic acid to produce a magenta color substance and showed maximum absorption wavelength at 525 nm. The various parameters for optimum condition were investigated. Under the suitable condition, a linear calibration graph in the range of 1.00-5.00%w/v acetic acid ($y = 0.063x + 0.142$, $R^2 = 0.9777$) was obtained with LOD and LOQ of 0.03 and 0.20%w/v, respectively. The tests were produced 6 times ($n = 6$) to ensure the precision of the proposed technique, and showed 3.4, 2.2, and 1.0%RSD for 1.00, 3.00, and 5.00%w/v of acetic acid, respectively. The proposed method was successfully applied to determine acetic acid in vinegar samples. The results agreed well with the standard method with a non-significant difference at 95% confidence interval. The proposed method presented many advantages such as using a cost-effective and environmental friendly analysis system, less toxic and producing less waste.

Keywords: Colorimetric assay, Green analytical method, Acetic acid, Red cabbage, Natural reagent

Introduction

Acetic acid (CH_3COOH) or ethanoic acid is known as a popular food additive to maintain the pH value and also to flavor the taste of food. Moreover, acetic acid is also used as raw materials for dyes, plastic, pesticides, adhesive stuff, and synthetic polymers industry. Because of the corrosive property of acetic acid, it can cause a gastrointestinal disease in case of an excessive dose. The concentration of acetic acid deriving from natural fermentation is

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suggested at 4%w/v, while artificial vinegar in the range of 4–7%w/v. This percentage has been set by the Ministry of Public Health in Thailand (Ministry of Public Health, 2000).

The conventional method for acetic acid determination is titration (AOAC method). This method is based on acid placed inside an Erlenmeyer flask to react with a base or standard solution inside a burette and using phenolphthalein as indicator. The color change of the mixture solution inside the Erlenmeyer flask indicated the end point of titration. Titration provides a basic step and reliable data. However, it generates large amount of waste, uses chemical indicators and requires a tedious work. Recently, the concept of green chemistry proposed to reduce the amount of waste, encourage the use of safer solvent or reagent and also avoid environmental contamination (Anastas, 1999). Since 1990, the use of micro total analysis (μTAS) has been growing rapidly. This method combines analysis process in a single device and also produces micro-liters of waste (Manz et al., 1990). The replacement of toxic/harmful chemical reagent with natural reagent and also the reduction of the amount of waste produced are significant advantages. Some techniques such as flow-based techniques coupled with natural reagent were published and described as greener methods. For example, the researches using spectrophotometric method coupled with flow injection/sequential injection such as exploiting guava leaf extracts (Settheeworrarit et al., 2005), green tea extracts for iron determination (Pinyou et al., 2010) and approaches using heartwood of *Ceasalpinia* sappan linn. for aluminium assay were published (Siriangkhawut et al., 2016). Supharoek et al. (2018) reported employing turmeric and lime as natural reagents for acetic acid determination by using a sequential injection system. Another technique combined with flow based analysis has been reported by using titration method to determine acetic acid with various mode detection such as conductometry (Tavares Araújo et al., 2005), gas chromatography (Wittmann et al., 2000; Xie et al., 2005; Lin et al., 2014) and spectrophotometry (Gonzalez-Rodriguez et al., 2001).

Red cabbage (*Brassica oleraceae L. var.*) contains a chemical called mono and di-acylated cyadinin (Cy) anthocyanin that changes color depending on pH value (Ahmadiani et al., 2016). Red cabbages juice appears reddish-pink in acidic condition, purple in neural condition, and bluish-green or even intense yellow in alkaline/basic condition. Red cabbage is known as a natural pH indicator and also used as food/pharmaceutical dye (Chigurupati et al., 2002). Prietto et al. (2017) developed a pH sensitive film containing anthocyanin from black-bean seed coat and red cabbage extracts. It was found that pH sensitive film from red cabbage

extracts gave higher color stability than black-bean seed coat extracts. A visual indicator for fish deterioration by using chitosan/corn starch blend film with extracts from red cabbage has been invented by Silva-Pereira et al.. They reported that this system is very sensitive to pH and very useful for quality control of food storage and conditioning. Red cabbage extracts can chelate with some transition metals (Silva-Pereira et al., 2015). Khaodee, et al. (2014) demonstrated the effectiveness of the simultaneous naked-eye detection for 4 metal ions by using cyanidin extracted from red cabbage as a chelating agent. Therefore, considering all previous informative data, it was relevant to use a red cabbage (*Brassica oleraceae L. var.*) as an alternative natural reagent for acetic acid quantification in this research.

The conventional spectrophotometer or colorimeter of a common laboratory involves a large amount of reagent and sample in milliliters such as UV–Vis spectrophotometer. Purchase and maintenance cost are expensive and it requests to use a computer program. Therefore, it is rarely used for on-site analysis. In recent years, the mini-spectrophotometer has been developed. In 2017, USHIO Inc. created PiCOEXPLORER, a device aimed to provide a better signal and precision. PiCOEXPLORER is a compact system and a portable device of a new generation as small size colorimeter. It is based on RGB analysis system and allows displaying RGB intensity and absorbance for each Red (R), Green (G), and Blue (B) mode. The device is controlled by a program using an application on mobile phone with either iOS or Android operating systems. This portable device is convenient for on-site micro-scale analysis. PiCOEXPLORER is known as a new colorimeter device giving more advantages than the conventional method of spectrophotometer. This device was used in this research as a compact and modern detector. In addition, this method was demonstrated effectiveness of natural reagent (Red cabbage) for acidity determination. (USHIO Inc., 2017).

Objective

This research focused on developing a simple, cost-effective, environmentally friendly, compact and modern method by using PiCOEXPLORER as a colorimeter device and red cabbage extracts as an alternative natural reagent for acetic acid determination in vinegar samples instead of using acid-base titration. The performance of the proposed method was demonstrated with vinegar samples.

Materials and methods

Reagents and chemicals

A standard solution was prepared by diluting glacial acetic acid (≥ 99.8%, Analytical reagent grade, RCI-Labscan) in deionized water to obtain 50% (w/v). The stock solution of acetic acid was standardized by titration with sodium hydroxide and using phenolphthalein as indicator. Working standard (1.00–5.00%w/v) acetic acid was daily prepared by diluting the stock solution with deionized water.

Red cabbage extracts

Red cabbage extracts were simply extracted by using deionized water as a solvent. A 100 g chopped red cabbage was pasted inside a mortar then 80 mL of water was added and extracted for 30 min, then filtrated through a white fabric sheet. The filtrated solution was rinsed and made up a volume of 100 mL in a volumetric flask with deionized water. The extracts solution was filtrated again with a Whatman no. 1 filter paper. The extracts were prepared daily.

Instrumentation

The signals were detected by using a green micro-scale colorimeter via PiCOEXPLORER™ Model PAS-110, purchased from Ushio Inc.. Thermo Scientific Genesys 840-208100 UV–Vis spectrophotometer with matched quartz cell was used for scanning the spectra of proposed compound.

Operating steps

Firstly, PiCOEXPLORER was connected to PAS-110 mobile phone application. Then, the “standard curve button” was pressed for setting the concentration unit and standard concentration. Next, 125 µL of 100%w/v red cabbages extract were pipetted and mixed with 75 µL of acetic acid standard/sample solution and placed inside the measurement chamber and covered with chamber lid (Figure 1(a) and 1(b)) using deionized water mixed with red cabbage extracts as blank. Then, the signal of blue light absorbance (blue color sensor) was used for linear calibration graph as shown in Figure 1(c) and Figure 1(d), respectively.

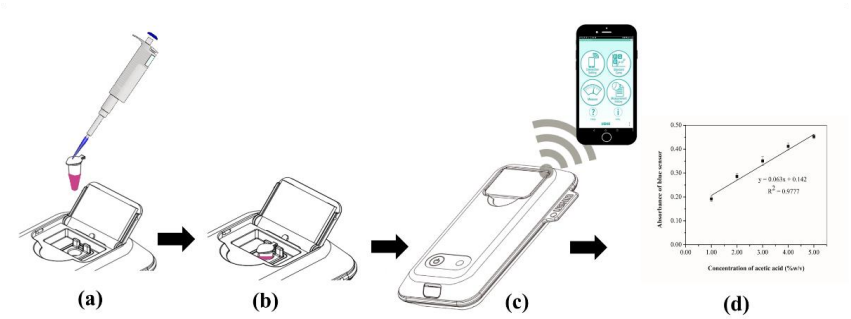
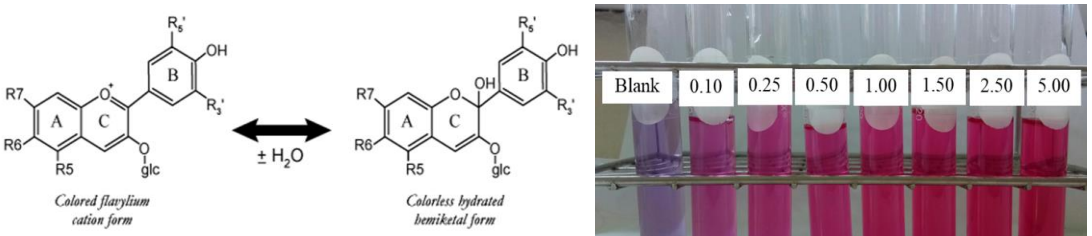


Figure 1 Operating steps: (a) pipetting 125 µL of red cabbage extracts with 75 µL of acetic acid/sample in a PCR tube (b) placing the mixture inside PiCOEXPLORER chamber (c) measuring the absorbance via standard curve mode with the PAS-110 application (d) constructing of linear calibration graph.

Results

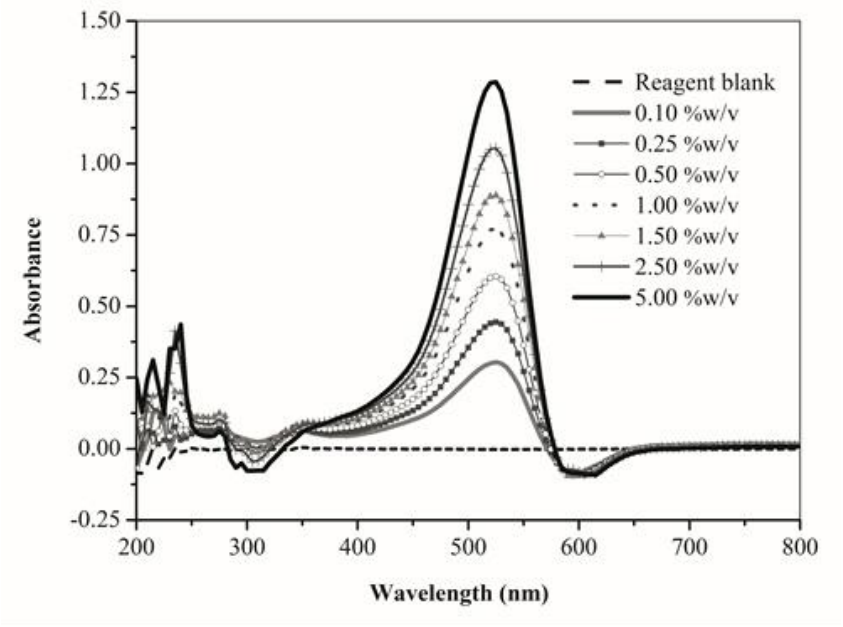
Spectra characteristics of interaction between red cabbage extracts and acetic acid

Red cabbages extract is known as an acid-base natural indicator by color changes depending on pH value. It contains anthocyanin which changes color in an hydrabase anion form (pH 7–8, blue) to flavylium cation (pH > 3, red) in acidic solution (Khaodee et al., 2014; Galán-Vidalv et al., 2014). Acidic acid is a weak acid with a pH in range of 3–4. Therefore, it's protonate at hydroxyl group in hydrated bemiketal form to flavylium cation form as shown in Figure 2(a). This explains the interest for using it in this research as natural reagent for acid determination. In a preliminary study, absorption spectra of the proposed compound (Magenta color as shown in Figure 2(b)) from the reaction between various acetic acid concentrations (0.10–5.00 % w/v) with red cabbage extracts (as shown as Figure 2(c)) were investigated by using red cabbages extracts (Purple color) as blank. The proposed compound spectra were observed at 525 nm as the maximum absorption which is in the absorption of blue color sensor wavelength (400–540 nm) in PiCOEXPLORER device. The absorbance of compounds increased proportionally according to acetic acid concentration. The results showed that red cabbage extracts were possible to use as alternative natural reagent for acetic acid determination in the pH range of 3–4.



(a)

(b)



(c)

Figure 2 (a) The reaction of cyanidin transform between flavylium cation form and hydrated bemiketal form in acid–base condition. (b) The color of red cabbage extracts (blank) and red cabbage extracts with 0.10–5.00 %w/v of acetic acid (c) Absorbance spectra of the proposed compound from the reaction between various acetic acid concentrations with red cabbage extracts by using red cabbage extracts as blank which is measuring with UV–Vis spectrophotometer.

Parameters optimization

The various parameters of the proposed method such as concentration of red cabbage extracts, extraction time, reaction time and volume of red cabbage extracts to obtain the best sensitivity and precision for optimum condition were investigated.

Effect of the concentration of red cabbage extracts

The first parameter having the most effect on sensitivity is the reagent concentration. Therefore, the concentration of red cabbage extracts (10, 20, 30, 40, 50, 60, 80 and 100%w/v) was investigated by studying the effect of the absorbance of red (R), green (G) and blue (B) light of 1.00%w/v acetic acid after it reacts with red cabbage extracts. The color of red cabbage extracts varied depending on pH. It was extracted by using deionized water as solvent because the color pigments can be dissolved in water and to follow the green chemistry concept. The results showed that the absorbance of red (R) decreased, the absorbance of blue and green light enhanced to 80%w/v of red cabbage extracts then the absorbance of green light was constant while blue absorbance slightly still enhanced until 100%w/v. This is due to the amount of cyanidin from red cabbages extracts and also because the proposed compound has a magenta color therefore absorbance B and G increased with the concentration of cyanidin in red cabbages extracts as shown in Figure 3. Thus, it can be concluded that 100%w/v of red cabbage extracts was chosen as the optimum concentration for further studies because it gave the highest sensitivity and precision. In addition, the absorbance of blue light (blue color sensor) was chosen as a mastered signal for other parameters.

Effect of extraction time

Cyanidin pigment contained in red cabbages is a water soluble compound. Therefore, water was selected as a solvent for extraction. The effect of extraction time was also evaluated since a longer extraction time should allow more proposed pigments dissolved in extracts but will also lead to the decomposition or oxidation of the natural reagent. Extraction time varied from 10 to 60 min. The results showed that the absorbance of blue (B) increased with the extraction time from 10 to 20 min then slightly increased until 30 min and slightly decreased from 30 to 60 min. An extraction time of 30 min was chosen because it gave the highest absorbance and precision as shown in Figure 4.

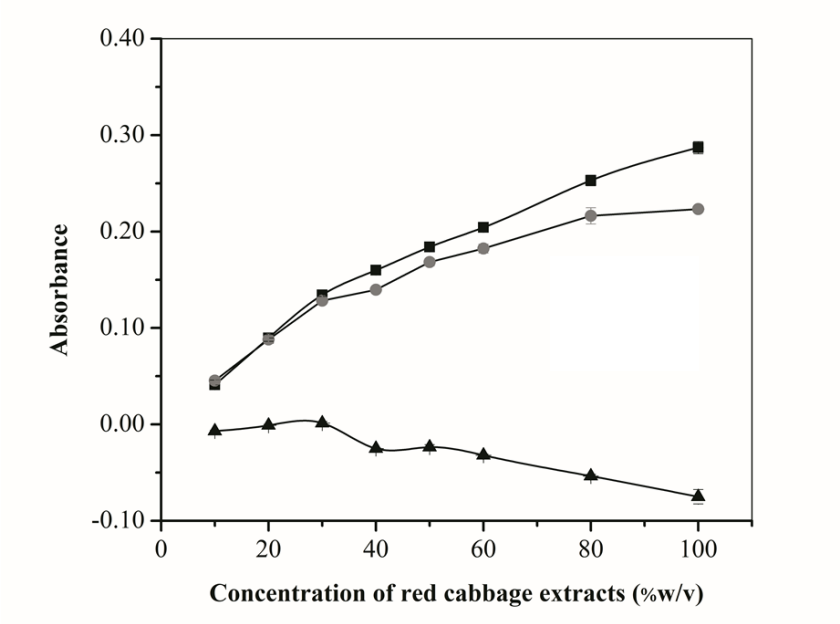


Figure 3 Absorbance of red (▲), green (●) and blue (■) light color sensor recorded by PiCOEXPLORER device for the study of red cabbages extracts concentration effect in range of 10 to 100%w/v on 1.00%w/v of acetic acid.

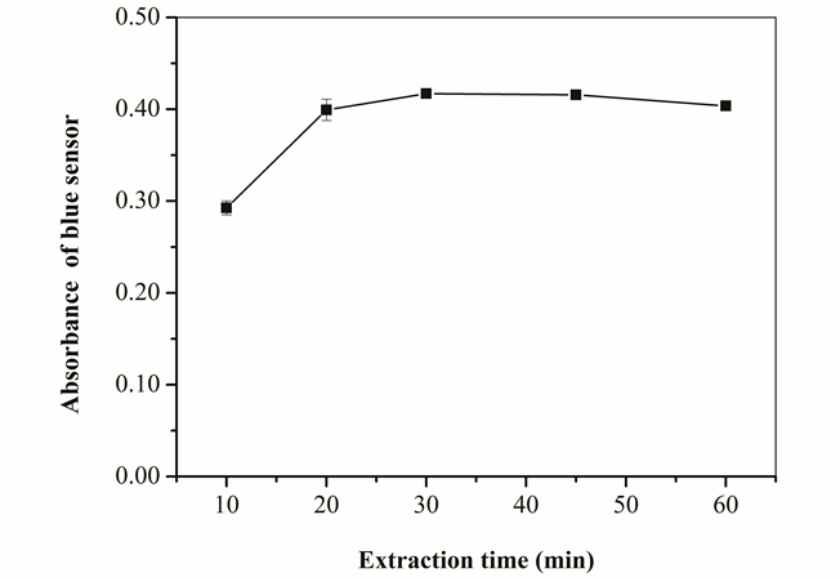


Figure 4 The effect of extraction time of red cabbage extracts on sensitivity and precision of 1.00%w/v acetic acid in range 10 to 60 min.

Effect of reaction time

Various reaction times (0, 5, 10, 15, 20, 25, and 30 min) were investigated in order to obtain the highest sensitivity and precision even though usually the rate of acid–base reaction is fast. The variation of reaction time indicated that the absorbance of blue light was very slightly increasing with reaction time from 0 to 15 min but after 20 min the signal was slightly decreasing until 30 min as shown in Figure 5. It due to stability of this proposed compound and their pigment in red cabbage was decomposed (Chigurupati et al., 2002). Thus, in this case the reaction time of 0 min (measurement immediately after mixed) was chosen to save analysis time.

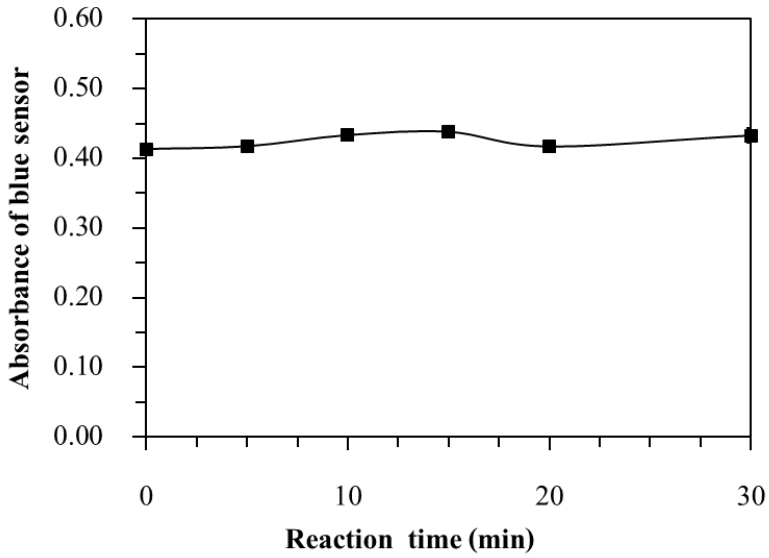


Figure 5 The effect of reaction time of red cabbage extracts on sensitivity and precision of 1.00%w/v acetic acid in range 0 to 30 min.

Effect of red cabbage extracts volume

According to the limit of PCR tube size, the total maximum volume could not exceed 200 µL. So, in this research, the total volume of mixture between reagent and sample solution was 200 µL. Volume of reagent was studied in ranges of 25 to 175 µL by monitoring the absorbance of blue light after mixing with a volume of sample solution to obtain 200 µL. The results indicated that the absorbance was increasing with the volume of red cabbage extract until 125 µL and then the absorbance was decreasing as shown in Figure 6. It due to

the amount of red cabbage extract have to many volumes (>150 µL) when comparation with amount of acetic acid. Therefore, the optimum volume of this study was 125 µL of red cabbage extracts and 75 µL of sample solution.

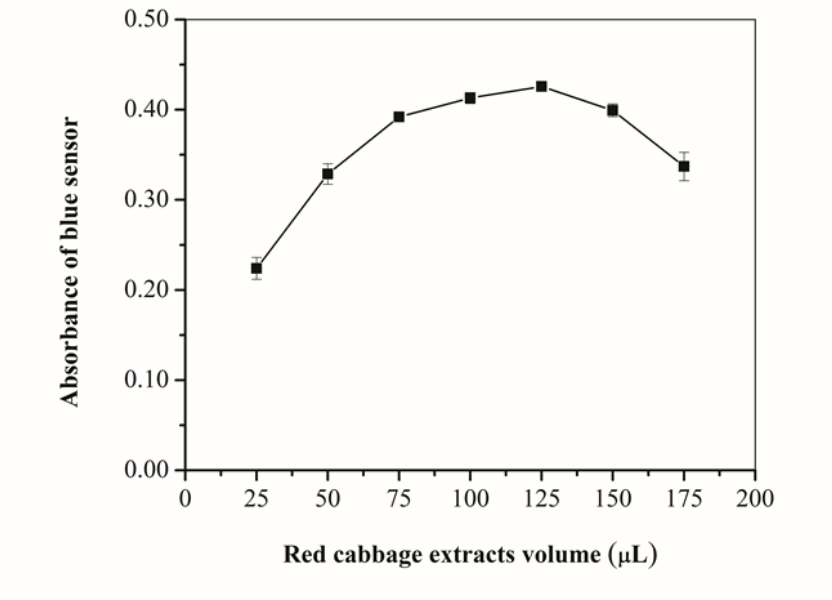


Figure 6 The effect of volume of red cabbage extracts to sensitivity and precision of 1.00%w/v acetic acid in total volume 200 µL.

The optimum conditions described before (shown below in Table 1) were applied for studying analytical characteristics. These included the linearity range, the limit of detection (LOD), the limit of quantitation (LOQ) and also the precision of the proposed method. It was found that the linearity range of acetic acid was between 1.00–5.00%w/v (Figure 7), 0.03%w/v of LOD and 0.20%w/v of LOQ, respectively. The precision of the proposed method was studied in acetic acid concentration of 1.00, 3.00 and 5.00%w/v. The results found that %RSD of studied concentration were 3.4, 2.2, and 1.0%, respectively. In this case, the percentage of recovery by spiking acetic acid concentration in vinegar samples for sample preparation step was also studied. The percent recoveries were found between 87–96 %.

Table 1 Analytical characteristics of proposed method under optimum conditions

Parameters studied	Acetic acid standard solution
Linear regression equation [a]	y = 0.063x + 0.142 (R^2 = 0.9777)
Linear range (%w/v)	1.00–5.00
LOD (3σ of blank) (%w/v)	0.03
LOQ (10σ of blank) (%w/v)	0.20
%RSD (*n=6*) over the linear range	1.0–3.4

a. Y is signal in Absorbance (blue color sensor), and X is acetic acid concentration

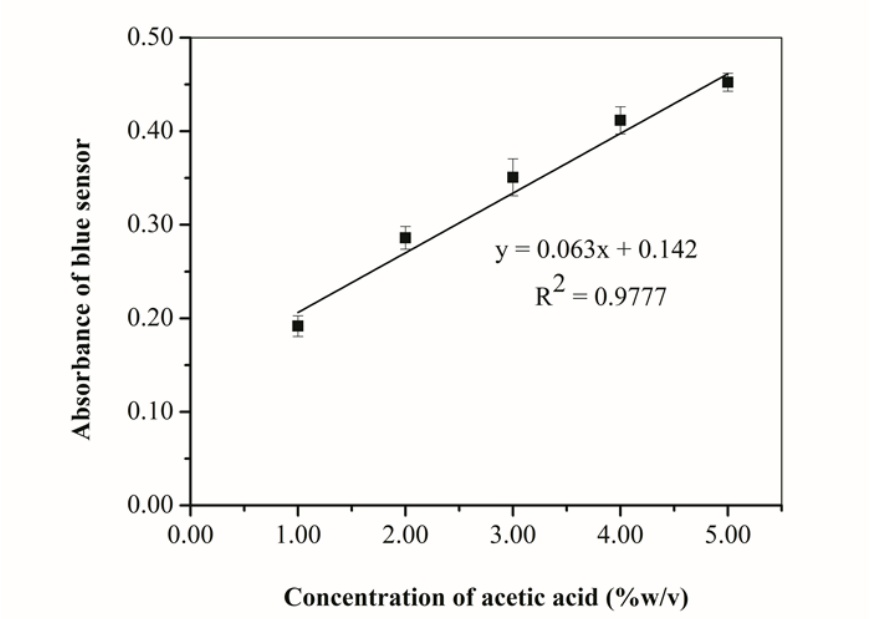


Figure 7 The linearity range of acetic acid concentration under optimum condition by using red cabbage extract as a natural reagent monitored by PiCOEXPLORER device.

Application of proposed method for acetic acid determination in vinegar samples

Various brands of vinegar samples available in department stores and markets were purchased. The sample preparation procedure was 2-flod diluted before analysis as described previously in the procedure of the experimental part. A graph of the proposed method was constructed by plotting between absorbance of blue light and concentration of acetic acid. The amounts of acetic acid in samples were summarized as shown below in Table 2. The result was validated with titration method as a standard method. It was found that the results

from the proposed method were in good correlation with the standard method, considering the slope of the correlation graph (0.9946) which is close to 1 and R^2 value of 0.9377. Both methods were also compared using the paired *t*-test. It was not found any significant difference between the two methods at 95% confidence interval for all the samples.

Table 2 Determination of acetic acid in vinegar samples by the proposed method and standard method based on acid–base titration

Sample	CH_3COOH in vinegar samples (%w/v) (Mean±SD)	
	Proposed method	standard method
1	5.00±0.09	4.98±0.02
2	4.86±0.13	4.94±0.03
3	4.90±0.05	5.07±0.02
4	4.73±0.08	4.72±0.01
5	5.03±0.11	5.08±0.04
6	4.23±0.02	4.22±0.01
7	4.99±0.07	5.06±0.02
8	5.27±0.09	5.20±0.06
9	4.81±0.08	4.77±0.06
10	5.06±0.08	5.06±0.02

Discussion and suggestions

This research developed a green colorimetric method based on a micro-volume scale with PiCOEXPLORER device. This new device was successfully used with red cabbage extract as an alternative natural reagent for determination of acetic acid in vinegar samples. Red cabbage extract provides an alternative for inexpensive and easily available reagent for assay amount of acetic acid without the need of purification prior to use. In addition, red cabbage extracts should be daily prepared before use.

The proposed method provides an alternative to other methods which is simple, low cost, environmental friendly, has high precision and reduces the sample and reagent consumption in microliter (Ueda et al., 2010; Wongwilai et al., 2010). Moreover, acetic acid contents obtained by the proposed method and the reference method were in good agreement as compared by the paired *t*-test at 95% confidence level and by a correlation graph.

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References

Ahmadiani, N., Robbins, R. J., Collins, T. M., & Monica Giusti, M. (2016). Molar absorptivity (ε) and spectral characteristics of cyanidin-based anthocyanins from red cabbage. *Food Chemistry*, *197*, 900–906. https://doi.org/10.1016/j.foodchem.2015.11.032

Anastas, P. T. (1999). Green chemistry and the role of analytical methodology development. *Critical Reviews in Analytical Chemistry*, *29*(3), 167–175. https://doi.org/10.1080/10408349891199356

Chigurupati, N., Saiki, L., Gayser, C., & Dash, A. K. (2002). Evaluation of red cabbage dye as a potential natural color for pharmaceutical use. *International Journal of Pharmaceutics*, *241*, 293–299. https://doi.org/10.1016/S0378-5173(02)00246-6

Galán-Vidal, C. A., Castaήeda-Ovando, A. Páez-Hernández, M. E., & Contreras-Lopez, E. (2014). Determination of nitrites in commercial sausages by anthocyanins degradation. Experimental design and optimization. *Journal of the Mexican Chemical Society*, *58*(2), 180–184. https://doi.org/10.29356/jmcs.v58i2.175

González-Rodríguez, J., Pérez-Juan, P., & Luque de Castro, M. D. (2001). Two-parameter determination in vinegar by a flow injection–pervaporation system. *Analyst*, *126*, 1177–1181. https://doi.org/10.1039/b102185k

Khaodee, W., Aeungmaitrepirom, W., & Tuntulani, T. (2014). Effectively simultaneous naked-eye detection of Cu(II), Pb(II), Al(III) and Fe(III) using cyanidin extracted from red cabbage as chelating agent. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, *126*, 98–104. https://doi.org/10.1016/j.saa.2014.01.125

Lin, S., Zhang, J., Gao, Y., Zhang, X., Song, S., & Long, Z. (2014). Rapid and sensitive gas chromatography-triple quadrupole mass spectrometry method for the determination of organic acids in tobacco leaves. *Analytical Methods*, *6*, 5227–5235. https://doi.org/10.1039/c4ay00688g

Manz, A., Graber, N., & Widmer, H. M. (1990). Miniaturized total chemical analysis systems: a novel concept for chemical sensing. *Sensors and Actuators, B1*, 244–248. https://doi.org/10.1016/0925-4005(90)80209-I

Ministry of Public Health. (2000). Notification of the Ministry of public Health (No. 204): vinegar., Bangkok: Prachachon.

Pinyou, P., Hartwell, S.K., Jakmunee, J., Lapanantnoppakhun, S., & Grudpan, K. (2010). Flow injection determination of iron ions with green tea extracts as a natural chromogenic reagent. *Analytical Sciences*, *26*, 619–623. https://doi.org/10.2116/analsci.26.619

Prietto, L., Mirapalhete, T. C., Pinto, V. Z., Hoffmann, J. F., Vanier, N. L., Lim, L-T., Guerra Dias, A. R., & da R. Zavareze, E. (2017). pH-sensitive films containing anthocyanins extracted from black bean seed coat and red cabbage. *LWT-Food Science and Technology*, *80*, 492–500. https://doi.org/10.1016/j.lwt.2017.03.006

Settheeworrarit, T., Hartwell, S. K., Lapanatnoppakun, S., Jakmunee, J., Christan, G. D., & Grudpan, K. (2005). Exploiting guava leaf extract as an alternative natural reagent for flow injection determination of iron. *Talanta*, *68*, 262–267. https://doi.org/10.1016/j.talanta.2005.07.039

Silva-Pereira, M. C., Teixeira, J. A., Pereira-J únior, V. A., & Stefani, R. (2015). Chitosan/corn starch blend films with extract from Brassica oleraceae (red cabbage) as a visual indicator of fish deterioration. LWT-Food Science and Technology, *61*, 258–262. http://doi.org/10.1016/j.lwt.2014.11.041

Siriangkhawut, W., Khanhuathon, Y., Chantiratikul, P., Ponhong, K. & Grudpan, K. (2016). A green sequential injection spectrophotometric approach using natural reagent extracts from heartwood of *Ceasalpinia sappan* Linn. for determination of aluminium. *Analytical Sciences*, *32*, 329–336. https://doi.org/10.2116/analsci.32.329

Supharoek, S., Ponhong, K., Siriangkhawut, W., & Grudpan, K. (2018). Employing natural reagents from turmeric and lime for acetic acid determination in vinegar sample. *Journal of Food and Drug* Analysis, *26*(2), 583–590. https://doi.org/10.1016/j.jfda.2017.06.007

Tavares Araújo, C.S., Lira de Carvalho, J., Ribeiro Mota, D., de Araújo, C. L., & Coelho, N. M. M. (2005). Determination of sulphite and acetic acid in foods by gas permeation flow injection analysis. *Food Chemistry*, *92*, 765– 770. https://doi.org/10.1016/j.foodchem.2004.10.032

Ueda, M., Lapanantnoppakhun, S., Wongwilai, W., Teshima, N., Sakai, T., & Grudpan, K. (2010). Exploiting a simple water extract of a flower as a natural reagent for acidity assay using a lab-on-chip. *Journal of Flow Injection Analysis*, *27*(1), 57– 60. https://doi.org/10.24688/jfia.27.1_57

USHIO Inc., (2017, June 12). PiCOEXPLORER™ PAS-110 photo absorbance sensor. https://www.ushio.com/product/picoexplorer-pas110/

Wittmann, Gy., Van Langenhove, H., & Dewulf, J. (2000). Determination of acetic acid in aqueous samples, by water-phase derivatisation, solid-phase microextraction and gas chromatography. *Journal of Chromatography A*, *874*, 225–234. https://doi.org/10.1016/s0021-9673(00)00114-x

Wongwilaia, W., Lapanantnoppakhuna, S., Grudpan, S., & Grudpana, K. (2010). Webcam camera as a detector for a simple lab-on-chip time based approach. *Talanta*, *81*, 1137–1141. https://doi.org/10.1016/j.talanta.2010.01.058

Xie, W.Q., & Chai, X. S. (2017). Determination of total acid content in vinegars by reaction-based headspace gas chromatography. *Food Analytical Methods*, *10*(2), 419–423. https://doi.org/10.1007/s12161-016-0595-2