Original Article

Employing natural reagents from turmeric and lime for acetic acid determination in vinegar sample

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A B S T R A C T

A simple, rapid and environmentally friendly sequential injection analysis system employing natural extract reagents was developed for the determination of acetic acid following an acid–base reaction in the presence of an indicator. Powdered lime and turmeric were utilized as the natural base and indicator, respectively. Mixing lime and turmeric produced an orange to reddish-brown color solution which absorbed the maximum wavelength at 455 nm, with absorbance decreasing with increasing acetic acid concentration. Influential parameters including lime and turmeric concentrations, reagent and sample aspirated volumes, mixing coil length and dispensing flow rate were investigated and optimized. A standard calibration graph was plotted for 0–5.0 mmol/L acetic acid with \( r^2 = 0.9925 \). Relative standard deviations (RSD) at 2.0 and 4.0 mmol/L acetic acid were less than 3% (n = 7), with limit of detection (LOD) and limit of quantification (LOQ) at 0.12 and 0.24 mmol/L, respectively. The method was successfully applied to assay acetic acid concentration in cooking vinegar samples. Results achieved were not significantly different from those obtained following a batchwise standard AOAC titration method.

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1. Introduction

Ethanoic acid (CH₃COOH) is also known as acetic acid. When undiluted, glacial acetic acid is a clear, colorless liquid organic compound with a strong odor of vinegar which can be used to corrode sediments in water pipes, especially sanitary sewer pipes. In the food industry, acetic acid is utilized as an additive to control pH levels and also to improve the taste. Furthermore, acetic acid is widely used to produce plastics, dyes, pesticides, synthetic polymers and glue. Due to its corrosive properties, acetic acid can cause stomach diseases. In Thailand, the Ministry of Public Health has set the concentration of acetic acid in fermented and distilled vinegar at not less than 4% w/v, while artificial vinegar varies from 4% to 7% w/v [1].
Batchwise titration is a standard AOAC method for the determination of acetic acid [2], based on an acid–base reaction between acetic acid and standard sodium hydroxide using phenolphthalein as an indicator. This standard procedure is simple with rapid and reliable results. However, disadvantages include the generation of excessive waste, use of extensive glassware, tedious operation steps, and the need for synthetic reagents.

Recently, developments in green analytical techniques, as procedures which provide economical and environmentally friendly advantages, have become important aspects in sustainable analytical chemistry [3–5]. Substituting toxic chemicals with alternative less harmful reagent and minimizing waste products using flow-based analytical techniques can achieve greener results [6,7]. Acid–base titrations with flow-based analysis using various detection systems such as spectrophotometry [8], conductometry [9], amperometry [10], multi-commutated segmented flow systems [11], gas chromatography [12–14] and proton nuclear magnetic resonance (1H NMR) spectroscopy [15] have all been utilized for acetic acid quantitative assay. The use of chemical reagents extracted from plants including butterfly pea flower, orchid flower, and beet root with a simple lab-on-chip approach was reported as a green analytical method for acidity assay in vinegar samples [16].

Turmeric (Curcuma domestica) is widely employed as a medicinal plant [17] for antiseptic, antibacterial [18], anti-inflammatory/arthritis [19,20] and cancer treatment [21,22], with uses also as a dye in the food and cosmetic industries [23].

Curcumin is the main component of turmeric extracts which expresses a yellow color in acid and/or neutral medium while presenting as orange or reddish-brown in the base condition [24–26] as a natural pH indicator. Traditionally, lime (hydrated lime) was generated by calcining shells at a boiling temperature. After boiling, the remaining calcined shells were added to the water and calcium hydroxide (Ca(OH)₂) was produced [27,28]. Lime solution was used to soak fruits and/or vegetables such as banana and pumpkin before food preparation. Lime was also typically mixed with betel leaves and chewed with Areca nut as a mild stimulant [28,29].

Here, a simple, green analytical approach following acid-base titration using a sequential injection (SI) system was proposed. Natural pH indicators from turmeric extracts and a natural base from hydrated lime were titrated with acetic acid in vinegar samples. The SI system used spectrophotometric detection as an automatic function to reduce reagent consumption and waste generation. Maximum wavelength absorption of the reddish-brown hydrated lime and turmeric mixture was at 455 nm, and absorbance decreased proportionally to the concentration of acetic acid. Both physical and chemical parameters were studied and results obtained using the proposed method were compared with AOAC standards.

2. Materials and methods

2.1. Chemicals and reagents

Standard analytical reagent grade acetic acid at 99.8% was obtained from QRéC, New Zealand. The working solution of acetic acid was prepared daily by dilution with deionized (DI) water (Milli-Q, Millipore, Sweden).

The natural base was obtained from lime. Hydrated lime was purchased from a local market in Maha Sarakham Province in March 2016. Wet lime was desiccated by heating at 100 °C in an oven (Binder, Germany) and then crushed to powder using an agate mortar. Powder lime was stored in a high density plastic lab bottle (HDPE) and kept at desiccator. A stock solution of lime was prepared by vortexing 2.0 g of powdered lime with 10 mL DI water for 1 min and then centrifuging at 4032 × g (6000 rpm) for 5 min. The supernatant was filtered using Whatman filter paper No. 1 and stored in a HDPE. The extraction procedure was repeated three times. The working solution of lime was freshly made by pipetting 5.0 mL of stock lime solution and diluting to 50 mL with DI water. Reagent bottles were sealed with air inlet clean-up devices consisting of a 50 mL syringe filled with soda lime/sodium hydroxide in a ratio of 1:1 (w/w) to prevent atmospheric carbon dioxide (CO₂) gas reacting with hydroxide in solution to produce a gradual lime concentration change and affect the precision of the analysis [30–32].

The natural indicator was obtained from turmeric powdered form (Chiang Mai, Thailand). Turmeric natural indicator raw material was kept at 4 °C in refrigerator. A stock turmeric solution was generated by extracting 0.4 g of turmeric powder in 10 mL of 96% ethanol (Merck, Darmstadt, Germany) by vortex mixer for 1 min. The solution was then centrifuged at 6000 rpm for 5 min, passed through filter paper Whatman No. 1 and stored in an ambient bottle for light protection. The extraction method was operated twice. Working solutions of turmeric were prepared daily by pipetting 5.0 mL of turmeric stock solution into a 50 mL volumetric flask.

2.2. Apparatus and operations

The sequential injection (SI) system with a spectrophotometer for quantification of acetic acid is illustrated in Fig. 1a. A bidirectional syringe pump (5000 μL, CAVRO, San Jose, CA) and a 10-port selection valve (Valco Instruments, Houston, TX) were employed to aspirate and dispense all reagent and carrier solutions. A holding coil (250 cm PTFE tubing; OD 1/16”, ID 0.03”) was used to protect the solution from flowing into the syringe barrel. A detection unit, including a tungsten halogen lamp light source, a spectrophotometer (AvaSpec-3648 Star-Line, Avantes, Netherlands) and optic fiber cables (ID 400 nm, 2 mm) with a flow-through cell (Quartz 10 mm path length, 80 μL internal volume) were utilized to monitor the absorbance at 455 nm. A mixing coil (PTFE tubing, ID 0.5 mm, 100 cm long) and PTFE tubing (ID 0.5 mm) were connected to the port of the selection valve. AvaSoft 8.0 for Avantes fiber optic spectrometer was used for data acquisition. In-house created software based on Visual Basic 6.0 was used to automatically operate the sequential injection system [33].

Operational steps for acetic acid determination are presented in Table 1. The holding coil, the flow-through cell and the PTFE tubing were connected to the port of the rotary selection valve and initially filled with DI water as the carrier solution. All tubing connected with the reagents was filled with their respective solutions and 4 mL of carrier solution was aspirated into the syringe barrel. Natural reagents (lime
and turmeric solution) and standard acetic acid or samples were then sequentially aspirated into a holding coil in accordance with the solution sequence (Fig. 1b). Finally, the aspirated zones at the holding coil were dispensed to the spectrophotometric flow cell to measure the absorbance at 455 nm.

2.3. Vinegar samples and sample preparation

Vinegar samples used in this work were 2 fermented, 6 distilled and 2 artificial vinegars. They were purchased from local markets in Maha Sarakham Province, Thailand. The essential component in vinegar is acetic acid by using water as its constituent. Moreover, food additives following sulfur dioxide and L-ascorbic acid were not allowed more than 70 mg and 400 mg per 1 kg of vinegar. All samples were kept at 4 °C until prepared for quantitative analysis. Before quantification of acetic acid by developed method, vinegar samples were diluted with DI water to achieve final concentrations of standard acetic acid in the calibration curve.

2.4. Method validation and statistical analysis

The method validations such as linearity of calibration graph, LOD and LOQ of proposed method and also precision were performed. The linear range of acetic acid calibration was investigated between 0 and 5.0 mmol/L of acetic acid. LOD and LOQ were studied by determination the absorbance of blank solution (containing turmeric and lime solution without acetic acid) in 7 times. Then, LOD and LOQ was calculated from $X + 3 \text{SD}$ and $X + 10 \text{SD}$, respectively, where $X$ and SD is the average concentration of acetic acid and standard deviation of acetic acid in the blank. % RSD value at 2.0 and 4.0 mmol/L of acetic acid was utilized to evaluate precision of developed method using 7 injections. Student’s $t$-test was used for comparison between the results obtained by developed method with standard AOAC method.

2.5. Standard method [2]

Conventional batchwise titration based on the reaction of acetic acid with sodium hydroxide (Univar, Australia) in the presence

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![Fig. 1 - Sequential injection system for determination of acetic acid using natural reagent; (a) Schematic diagram of the proposed method; SP, syringe pump; HC, holding coil; MC, mixing coil; LS, lime solution; TS, turmeric solution; AA, standard or sample acetic acid; (b) aspiration sequence of the solution volume in µL.](image)

**Table 1 - Operational steps for acetic acid determination.**

| Step | Syringe pump valve | Description | Volume (µL) | Flow rate (µL s⁻¹) | Selection valve |
|------|-------------------|-------------|-------------|-------------------|----------------|
| 1    | [↑] In            | Aspirate DI water to syringe | 4000 | 200 | – |
| 2    | [↑] Out           | Aspirate lime to holding coil | 50 | 50 | 9 |
| 3    | [↑] Out           | Aspirate turmeric to holding coil | 50 | 50 | 8 |
| 4    | [↑] Out           | Aspirate acetic acid standard or sample to holding coil | 50 | 50 | 7 |
| 5    | [↑] Out           | Aspirate lime to holding coil | 50 | 50 | 9 |
| 6    | [↑] Out           | Aspirate turmeric to holding coil | 50 | 50 | 8 |
| 7    | [↑] Out           | Aspirate acetic acid standard or sample to holding coil | 50 | 50 | 7 |
| 8    | [↑] Out           | Dispense solution to detector | Empty | 120 | 2 |

* [↑] downward direction, [↑] upward direction.
of phenolphthalein indicator was employed as the standard to compare results against the proposed method. Sodium hydroxide solution (as a secondary standard) was standardized with potassium hydrogen phthalate (KHP, QR&Co, New Zealand).

3. Results and discussion

3.1. Variation and stability of natural reagents

Lime consisting of Ca(OH)$_2$ is a strong base, but only slightly soluble in water. Thus, to obtain sufficient concentration and volume of base solution, the extraction procedure was repeated three times and precipitation of Ca(OH)$_2$ was observed when the lime extract solution was stored in a refrigerator at 4°C. Therefore, solutions of natural reagents were prepared daily and reproducibility of the extraction was studied by remaking the natural reagent solutions seven times daily. Freshly prepared natural reagents were reacted with acetic acid at 1.0, 3.0 and 5.0 mmol/L, giving RSD at 7.88%, 7.95% and 7.45%, respectively and indicating that this method was robust. Moreover, the stability of natural reagent extracted was studied by measurement the absorbance of blank at 455 nm for at least 8 h. It was observed that the absorbance decreased approximately 5.08%. This indicated that there was no significant change of absorbance with time. So, the natural reagent extracts was stabled at least 8 h, which adequate for this automatic Si-spectrophotometric method. In addition, the stability of raw material was determined by comparison between the two slopes of calibration at first day and 2 months later. It was found that the sensitivity was decreased about 8.78%. Hence, we recommended that the calibration graph should be prepared in daily to prevent the variability inherent of experiment.

3.2. Characterization of lime and turmeric

Lime powder was analyzed by X-ray diffraction (XRD, Bruker model D8 Advance, Germany) to identify its components. The XRD spectrum of lime powder was compared with XRD spectra in the library (Fig. 2) and corresponded to Portlandite mineral (pattern COD 1008780), indicating that the major component of lime powder was calcium hydroxide (Ca(OH)$_2$) as a strong base. After dissolution, the pH of the solution was 13.444. Concentration of Ca(OH)$_2$ was also standardized with KHP at 35.7 ± 0.5 mmol/L (n = 3).

Curcumin is an active pigment in turmeric and extraction by ethanol produced a yellow solution. The pKa values of curcumin were 7.7–8.5 for pKa$_1$, 8.5–10.4 for pKa$_2$ and 9.5–10.7 for pKa$_3$ to dissociate three hydrogen atoms in the enol form of curcumin [25,34]. Curcumin solution color changed from yellow to orange or reddish-brown in the pH range 7.5–8.5 [24,25].

3.3. Absorption spectra of natural reagents

The active ingredient present in turmeric is curcumin as an acid-base indicator that is yellow in acidic and neutral solutions and orange or reddish-brown in basic solutions [24–26]. Extracted turmeric solution absorbed visible light at 420 nm (Fig. 3a). An orange and/or reddish-brown color was obtained (Fig. 3b) after mixing turmeric solution (indicator) with lime solution (base), which provided maximum wavelength at 455 nm. Absorbance at 455 nm decreased with increasing acetic acid concentration because acetic acid reacted with hydroxide in the solution resulting in a pH change, with the color of the mixed solution altering from orange and/or reddish-brown to yellow. The molecular configuration of curcumin in acid/neutral solution and alkaline solution involved the detection principal of the proposed method is demonstrated in Scheme 1. In acid/neutral and alkaline conditions, the curcumin are presented in diketone and enolate form, respectively.

3.4. Optimization of sequential injection-spectrophotometer for automatic determination of acetic acid employing natural reagent

To obtain an automatic system with low chemical consumption and rapid analysis as the aim of green analytical chemistry, sequential injection analysis with spectrophotometric detection utilizing natural reagent was developed to assay acetic acid in a cooking vinegar sample.

3.4.1. Effect of aspirated sequence profiles

The effect of aspirated sequential profiles of lime solution, turmeric solution and standard acetic acid or samples were created and investigated using 300 µL total volume. Different segment profiles are demonstrated in Table 2. Highest absorbance was achieved using sequential order number 1. Therefore, this segment was utilized throughout the experiments.

3.4.2. Dilution of turmeric solution and aspirated turmeric volume

To ensure that the absorbance signals did not exceed the measurement limit of the spectrophotometer, turmeric extract solutions were diluted with DI water by pipetting 3.0, 4.0, 5.0 and 6.0 mL amounts into a 50 mL volumetric flask. Diluted turmeric solutions were used for acetic acid detection following sequential injection. Absorbance signal increased with increasing turmeric solution extracts. However, 5.0 mL of turmeric was selected for further experiments because it showed sufficient sensitivity to assay acetic acid.

The aspirated volume of turmeric solution was investigated between 60 and 120 µL. Sensitivity was enhanced when the aspirated volume of turmeric increased. An aspirated volume of 100 µL was chosen for the proposed method.

3.4.3. Dilution of lime solution and aspirated lime volume

Stock extracts of lime solution were not suitable for acetic acid measurement since they had high concentrations (approximately 35.7 ± 0.5 mmol/L). Dilution of lime was, therefore, studied by pipetting lime solution at 3.0, 4.0 and 5.0 mL into a 50 mL volumetric flask and the final volume was adjusted with DI water. The signal increased with increasing volume of lime because Ca(OH)$_2$ concentration was also increased. However, utilizing lime at 5.0 mL was provided sufficient sensitivity for acetic acid assay by our method. Therefore, 5.0 mL of lime solution was employed throughout the experiment and used to optimize the other parameters.
Moreover, the aspirated volume of lime solution was explored in the range 60–120 μL. Highest sensitivity was achieved at 100 μL (Fig. 4a). Using aspirated volume over 100 μL, the sensitivity was decreased because high concentration of base was negligibly effect to change of pH after react with acetic acid. Moreover, a lot of waste was generated. Hence, an aspirated volume of lime at 100 μL was chosen as the optimum condition for this parameter.

3.4.4. Aspirated volume of acetic acid standard or sample
The aspirated volume of acetic acid standard or sample was examined to attain the best sensitivity for the quantification of acetic acid. A volume of acetic acid between 40 and 130 μL was investigated. Experimental results are presented in Fig. 4b. Net signal at 455 nm increased when the aspirated volume was 100 μL and then remained constant. Moreover, this volume provided a smooth baseline and yielded high reproducibility. Thus, the aspirated volume was optimized at 100 μL.

3.4.5. Mixing coil length
To ensure through mixing of the natural reagents with acetic acid, the length of the mixing coil was investigated in the range 30–150 cm. Fig. 4c shows that the signal decreased as the length of the mixing coil increased because increasing mixing coil length yield to more dispersion in the system.

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Table 2 – Aspiration order of natural reagents and acetic acid.

| Sequence No. | Sequence order          | Volume (µL)       | Net absorbance at 455 nm (n = 3, ±SD) |
|--------------|-------------------------|-------------------|---------------------------------------|
| 1            | LS/TS/AA/LS/TS/AA       | 50/50/50/50/50    | 0.710 ± 0.010                         |
| 2            | LS/TS/AA/LS/AA/TS/AA    | 50/50/30/50/40/30 | 0.482 ± 0.031                         |
| 3            | TS/AA/LS/TS/AA/LS/TS    | 30/50/50/50/50/50 | 0.592 ± 0.009                         |

LS, lime solution; TS, turmeric solution; AA, acetic acid standard.

* Net absorbance = absorbance of blank – absorbance of 5 mmol/L acetic acid.

Fig. 4 – Optimized conditions of sequential injection for green analysis of acetic acid; (a) effect of aspirate volume of lime; (b) effect of sample volume; (c) effect of mixing coil length; (d) effect of dispensing flow rate.

Fig. 5 – SI graph of the proposed method and standard calibration graph for determination of acetic acid in vinegar.
Concentration of acetic acid comparing the proposed method with standard AOAC titration.

| Sample | Koncentration of acetic acid (% w/v) | Proposed method (±SD, n = 3) | AOAC titration (±SD, n = 3) |
|--------|---------------------------------|-----------------------------|-----------------------------|
| 1      | 5.61 ± 0.39                     | 5.89 ± 0.00                 |                             |
| 2      | 5.25 ± 0.35                     | 5.54 ± 0.04                 |                             |
| 3      | 5.60 ± 0.31                     | 5.48 ± 0.04                 |                             |
| 4      | 5.23 ± 0.21                     | 5.36 ± 0.04                 |                             |
| 5      | 4.85 ± 0.34                     | 4.83 ± 0.04                 |                             |
| 6      | 5.03 ± 0.23                     | 5.08 ± 0.04                 |                             |
| 7      | 4.63 ± 0.36                     | 4.69 ± 0.14                 |                             |
| 8      | 5.20 ± 0.34                     | 5.00 ± 0.04                 |                             |
| 9      | 5.09 ± 0.39                     | 5.02 ± 0.04                 |                             |
| 10     | 4.85 ± 0.28                     | 4.73 ± 0.06                 |                             |

n = number of experiments.
SD = standard deviation.
Sample 1–2 = fermented vinegar.
Sample 3–8 = distilled vinegar.
Sample 9–10 = artificial vinegar.

However, employing a mixing coil at 30 cm provided the highest sensitivity but the value of %RSD also increased. Thus, a mixing coil at 100 cm was selected for the proposed method to optimize analytical precision.

3.4.6. Dispensing flow rate
The effect of dispensing flow rate to deliver the aspirated zones and measure absorbance at 455 nm with the spectrophotometer was investigated to optimize sensitivity and high sample throughput. Dispensing flow rate for acetic acid determination was tested from 75 to 150 μL s⁻¹. Analytical net signals are shown in Fig. 4d. Low dispensing flow rates resulted in low sensitivity. Therefore, a dispensing flow rate of 120 μL s⁻¹ was adopted for the proposed method.

3.5. Analytical figure of merits
Under the selected conditions, the linear standard calibration graph and working length were determined in the range 0–5.0 mmol/L of acetic acid with the linear equation as

\[ Abs = -0.2199([\text{acetic acid}] + 1.4388); r^2 = 0.9925 \]

by plotting peak height (absorbance) versus acetic acid concentration. A typical calibration SI graph and calibration curve are presented in Fig. 5. LOD and LOQ were 0.12 and 0.24 mmol/L of acetic acid, respectively. The relative standard deviations (%RSD) for 2.0 and 4.0 mmol/L of acetic acid (n = 7) were 2.50% and 2.06%, respectively. The throughput of the proposed method was 45 samples per hour.

3.6. Application of the proposed sequential injection employing natural reagents for acetic acid assay
Sequential injection utilized natural reagents to measure the acetic acid content in cooking vinegar samples obtained from local stores in Maha Sarakham Province. The concentration of acetic acid obtained by the proposed method was compared with results using the standard AOAC method [2]. Concentrations of acetic acid obtained from both sequential injection and standard titration AOAC methods were not significantly different with t-test at a 95% confidence level (t_calculated = 0.61, t_critical = 2.26 at df = 9) (Table 3). Furthermore, the correlation coefficient (r) between results from the two methods was 0.9125 indicating no evidence of systematic difference between them. To perform the study, all vinegar samples were spiked with acetic acid at 1.0 and 3.0 mmol/L. Satisfactory results were obtained for concentration levels studied with percentage recoveries of 80–104%. However, the other vinegars as strong color could be interfered this analytical method. The working range of calibration utilizing for determination of acetic acid was found at low concentration in the range 0–5 mmol/L. So, the sample was diluted with DI water before analysis by developed method in order to obtain the final amount of acetic acid in the calibration graph. Fortunately, the dilution of sample could be eliminated the color. Therefore, color of vinegar samples could not be interfered.

4. Conclusions
A green sequential injection spectrophotometric method employing natural reagent extracts was successfully developed for analysis of acetic acid. Crude turmeric and lime extracts can replace toxic chemical reagents for the quantification of acetic acid content in cooking vinegar samples. The proposed system significantly increased the performance of acid-base titration for acetic acid assay as a simple, cost-effective method with a high degree of automation and low chemical consumption. The proposed system was reproducible, accurate, and rapid with a sample throughput rate of 45 injections h⁻¹. Satisfactory recovery and high sample measurement frequency proved that the proposed system has high potential as an alternative method for quality control of acetic acid content in vinegar products.

Conflicts of interest
Sam-ang Supharoek, Kraingkrai Ponhong, Watsaka Siriangkhawut and Kate Grudpan declare that they have no conflict of interest.

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