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Original Article

Fast and simple method for semiquantitative determination of calcium propionate in bread samples

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ABSTRACT

Calcium propionate has been widely used as a preservative in bakery and in bread. It is sometimes not carefully used, or a high concentration is added to preserve products. High consumption of calcium propionate can lead to several health problems. This study aims to develop a fast and simple semiquantitative method based on color complex formation for the determination of calcium propionate in a bread sample. A red–brown complex was obtained from the reaction of ferric ammonium sulfate and propionate anion. The product was rapidly formed and easily observed with the concentration of propionate anion >0.4 mg/mL. A high-performance liquid chromatography (HPLC) method was also developed and validated for comparison. Twenty-two bread samples from three markets near Bangkok were randomly selected and assayed for calcium propionate using the above two developed methods. The results showed that 19/22 samples contained calcium propionate >2000 mg/kg. The results of the complex formation method agreed with the HPLC method.

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1. Introduction

In the countryside areas of Thailand, some health problems come from dietary foods. Local food manufacturers do not usually know about good manufacturing practices, as well as country food laws. Calcium propionate has been widely used as a preservative in bakery and in bread. It has a

potency for preventive bacteria and fungi. Calcium propionate is listed as E number 282 in the Codex Alimentarius and is allocated an acceptable daily intake as “not specified” or “not limited” [1,2]. However, the fact that acceptable daily intake is not specified does not mean that unlimited intake is acceptable. It could, in principle, be allowed for use in foods in general with no limitation other than being in accordance with good manufacturing practices. The United

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States Food and Drug Administration recommended that the daily intake of calcium propionate for adults is 1 mg/kg/d [3]. If 1 kg of food was taken per day for a man of 50 kg body weight, the amount of calcium propionate in the food should not exceed 2000 mg/kg.

In Thailand, there were notifications of the ministry of Public Health of Thailand announced in 1984 and 1989. The allowed concentration of calcium propionate in food according to those announcements was 0.2%. Although oral calcium propionate might not cause severe toxicity, long term health effects have been reported. Propionate preservatives can contribute to or cause hyperactivity [4], visual hallucinations [5], irritability, restlessness, inattention, and sleep disturbance in some children [6–8]. Some researchers found that brief infusions of propionic acid in rats produced short bouts of behavioral, i.e., hyperactivity, object fixation, social impairments and other effects such as seizures, similar to those seen in autistic spectrum disorders [9,10].

Food safety is a key factor of good health. Unfortunately, some preservatives are illegally added or exceed regulation limits in foodstuffs. Several analytical methods have been developed for determination of these illegally used preservatives including food contaminants [11–13]. For propionic acid, the reported quantitative determination methods were gas chromatography [14] and gas chromatography/mass spectrometry [15,16]. Although these methods have been accepted for their accurate and precise results, but they still have some drawbacks such as requiring expensive instruments and well-trained analysts. Moreover, toxic organic solvent waste was also produced. In this study, a simple and cheap method of calcium propionate investigation in bread products based on the colorimetric method was developed. The developed colorimetric method was successfully applied to determine calcium propionate in bread products and the results were in agreement with high-performance liquid chromatography (HPLC). This indicated the accuracy of the colorimetric method.

2. Materials and methods

2.1. Preparation of standard solution of calcium propionate

About 200 mg of calcium propionate was accurately weighted into a 100-mL volumetric flask, then dissolved and diluted with deionized water. Further dilution of this stock solution was employed with deionized water to obtain a standard solution of 0.6 mg/mL and 0.4 mg/mL.

2.2. Preparation of ferric ammonium sulfate test solution

About 8.0 g of ferric ammonium sulfate was dissolved and made up to 100 mL with deionized water.

2.3. Bread sample preparation

A sample was cut into small pieces and 10 g weighed accurately into a 250-mL beaker. Some 100 mL of deionized water

was added, and the mixture was left standing for 2 hours before filtering. The filtrate was used to test for calcium propionate in the colorimetric method. For the HPLC assay, the filtrate was further diluted 10 times with a mobile phase before injection.

2.4. Testing for calcium propionate

Some 1.0 mL of 0.6 mg/mL calcium propionate standard solution was added to a 1.0-mL bread sample preparation. The color of the reaction was monitored after a few drops of ferric ammonium sulfate test solution (TS) was added. The color of the reaction tube was compared with the standard preparation tube and blank tube. Standard preparation was performed by adding a few drops of ferric ammonium sulfate TS to 2.0 mL of 0.4 mg/mL of calcium propionate. The blank tube was performed by adding a few drops of ferric ammonium sulfate TS to 2.0 mL of deionized water. A red–brown color complex was formed indicating the presence of calcium propionate. For the sample containing calcium propionate < 2000 mg/kg, the color was weaker than that of the standard tube, but stronger than that of the blank tube.

2.5. HPLC condition

Quantitative determination of calcium propionate in bread samples was employed using Shimadzu model LC-10 AD (Shimadzu Corporation, Kyoto, Japan) equipped with an SPD-10A UV detector. A symmetry C18 HPLC column (4.6 mm × 150 mm, 5 µm) was utilized. A mixture of 0.05% phosphoric acid and methanol with the ratio of 90 and 10 by volume was the successively mobile phase. The flow rate was 1.0 mL/min and the presence of calcium propionate was monitored at 210 nm.

2.6. HPLC method validation

Method validation parameters, i.e., linearity, accuracy precision, and specificity were evaluated. Linearity was performed by using a series concentration of standard solutions in the range of 25.0–250.0 µg/mL. The concentrations (X-axis) were then plotted with their corresponding peak areas (Y-axis). Linear least square regression was used to calculate the correlation coefficient (*r*).

Accuracy of the HPLC method was studied with standard addition approach. Three concentrations, low, middle, and high, of standard calcium propionate covering the linearity range were spiked into equal amounts of bread samples. Three replicates were performed for each concentration level. Accuracy of the method was expressed as recovery percent of standard found from the sample.

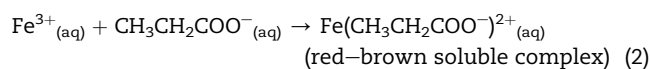
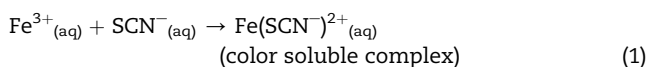
Precision was evaluated with three concentrations of the desired compound covering linearity range and three replicate samples for each concentration. Precision was expressed as relative standard deviation percent (% RSD) of replicate samples of each concentration.

Specificity of the HPLC method was obtained by comparing the chromatograms of the sample with the standard and standard spiked-sample. Retention times of calcium propionate in the sample and standard spiked-sample might equal that of the calcium propionate standard.

3. Results and discussion

3.1. Semiquantitative determination of calcium propionate

From the results, it could be seen that the red–brown complex can be formed from the reaction of ferric ions and propionate ions with the complex ratio of 1:1. The ratio of complex formation was already proved by the mole-ratio method which was described by Yoe and Jones [17]. The chemical reaction used in this study was similar to that which occurs between ferric ions and thiocyanate ions in Volhard's method as displayed in Eq. (1) [18]. Therefore, the chemical reaction between ferric and propionate ions was proposed as in Eq. (2).



The reaction was clearly observed for calcium propionate equal to or higher than 0.4 mg/mL. To confirm the limit of detection (LOD) of the reaction, a graph of probability of identification (POI) (Y-axis) and calcium propionate concentration (X-axis) was constructed [19]. Series concentrations of calcium propionate, 0.0–1.0 mg/mL, 10 replicates for each concentration, were prepared and tested with ferric ammonium sulfate TS. Then, the POI of each concentration was calculated using the following equation: POI = number of positive results/10.

The concentration corresponding to POI = 0.95 was set as the LOD [19]. As shown in Figure 1, POI equal to 0.95 or 95% positive results was observed when the concentration of calcium propionate in the solution was up to 0.4 mg/mL.

3.2. Bread sample preparation

Ten grams of a bread sample was cut into small pieces and soaked in 100 mL of deionized water. Because of the bulk of the bread, < 100 mL deionized water was not enough to soak the sample very well. If 10 g of the sample containing 0.2% of calcium propionate was dissolved in 100 mL deionized water, the resulted solution would contained 0.2 mg/mL of calcium propionate which was lower than LOD of the method (0.4 mg/mL). Therefore, a standard solution of calcium propionate at a concentration of 0.2 mg/mL was added to the sample solution to increase the concentration up to the LOD level.

3.3. Determination of calcium propionate in bread sample by colorimetric method

The colorimetric method described in this study is a semi-quantitative approach. In general, qualitative analysis

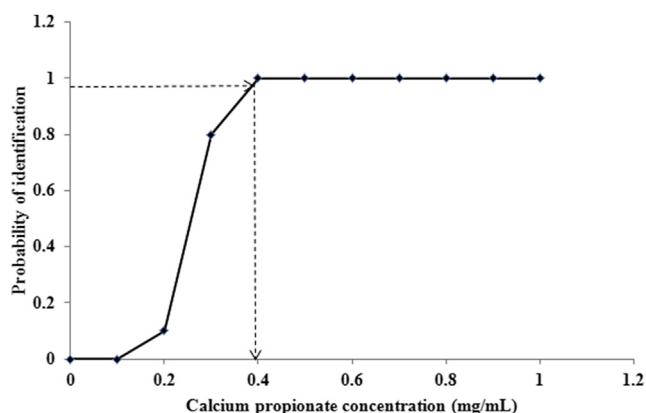


Figure 1 – Graph of probability of identification and calcium propionate concentration indicating the limit of detection of calcium propionate by colorimetric method.

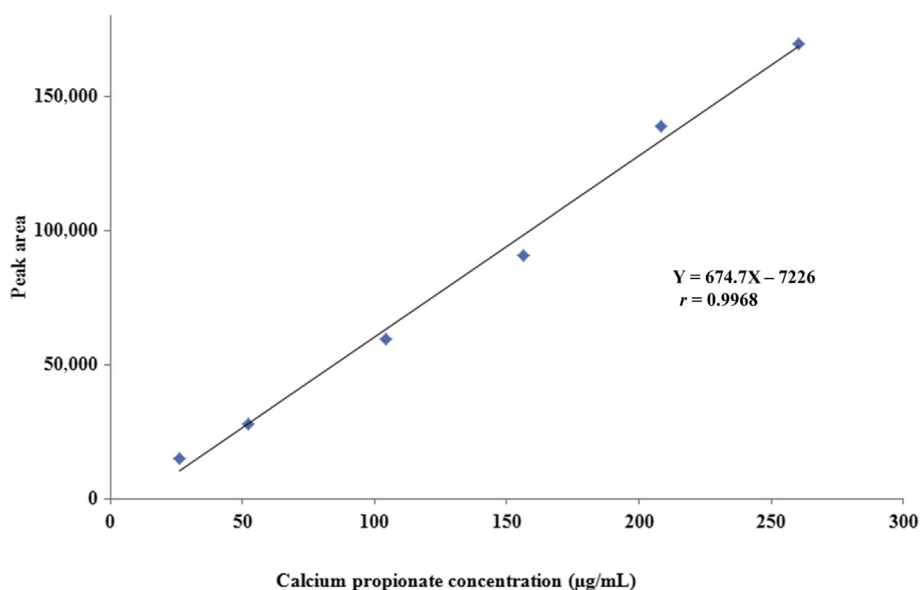


Figure 2 – Linearity graph of the developed high-performance liquid chromatography (HPLC) method for calcium propionate.

establishes the chemical identity of the species in the sample, while quantitative analysis determines the relative amounts of these species in numerical results. Semiquantitative analysis is between qualitative and quantitative methods. It can provide the detection of an analyte presence including the approximate quantity by comparing with a standard at allowance concentration.

For the semiquantitation method of calcium propionate used in this study, the reaction can precisely detect the amount of calcium propionate equal to or greater than 0.4 mg/mL. However, the allowance limit of calcium propionate in bread is 0.2% or 2000 mg/kg. If a 10-g of sample was used in the assay, at least 100 mL of deionized water was needed to soak the sample and dissolve the calcium propionate inside. It was impossible to enrich the concentration by using deionized water < 100 mL because of the bulky bread sample. If calcium propionate was used in bread up to the limit of 2000 mg/kg and

dissolved completely in deionized water 100 mL, the concentration would be 0.2 mg/mL. Unfortunately, this concentration was lower than the LOD of the developed colorimetric method that was 0.4 mg/mL. To reach detection ability, standard calcium propionate was added to the sample solution to increase the final concentration up to 0.4 mg/mL.

In the testing procedure, 1.0 mL of sample solution (the expected concentration was 0.2 mg/mL) was added with 1.0 mL of standard solution 0.6 mg/mL. Therefore, the final concentration of this solution was about 0.4 mg/mL. A few drops of ferric ammonium sulfate reagent was added and the formation of a red–brown complex was observed. The reaction of 2.0 mL of standard solution 0.4 mg/mL was performed for comparison. Some 2.0 mL deionized water was dropped with a few drops of ferric ammonium sulfate reagent and used as a negative control. For interpretation, if the color of the red–brown complex of the sample was stronger than that of the standard, it indicated that the amount of calcium propionate in the sample was > 0.2% or 2000 mg/kg and vice versa.

Table 1 – Accuracy and precision of the developed high-performance liquid chromatography (HPLC) method.

Concentration levels ($\mu\text{g/mL}$)	% Recovery ($\pm$ % RSD, $n = 3$)
50	102.3 $\pm$ 3.3
100	102.4 $\pm$ 3.4
200	106.5 $\pm$ 6.0

RSD = relative standard deviation.

3.4. HPLC method development and validation

Calcium propionate in bread could be separated from the sample matrix by using successive HPLC conditions as described above with a retention time of 5.4 minutes. The developed method was then validated for the intended purpose. Linear equation was $Y = 674.7X - 7226$ with correlation

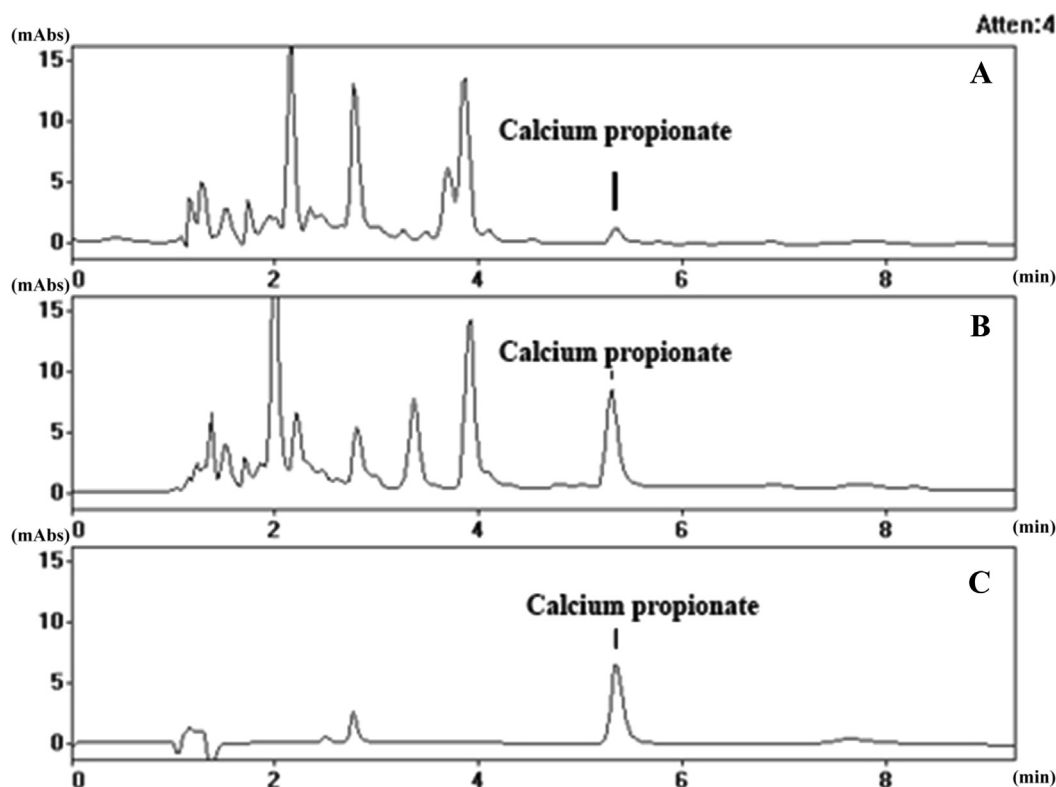


Figure 3 – Chromatograms showing the specificity of the high-performance liquid chromatography (HPLC) method for calcium propionate. Chromatograms of calcium propionate (A) in a bread sample, (B) standard calcium propionate spiked sample, and (C) standard calcium propionate. Chromatographic conditions: the column was a Symmetry C18 HPLC column (4.6 mm $\times$ 150 mm., i.d., 5 μm); the flow rate was 1.0 mL/min; the mobile phase was methanol and 0.05% phosphoric acid (10:90, v/v); the injection volume was 20 μL ; UV detection occurred at 210 nm; and the run time was 9 minutes.

coefficient (r) >0.99 (Figure 2). Recovery percent values of the standard found from standard spiked-samples ranging from 102.3% to 106.5% indicated the accuracy of the developed HPLC method. Precision of the method was acceptable when the % RSD values of replicate samples were < 6.0%. Method validation results are summarized in Table 1. From linearity, accuracy, and precision results, the range of the developed HPLC method could be also claimed at 50.0–200.0 $\mu\text{g/mL}$. In addition, the developed HPLC method had specificity for monitoring of calcium propionate in bread samples. As seen from Figure 3, retention times of calcium propionate in samples and standard spiked-samples were the same as the retention time of the authentic compound. Moreover, the peak area of the standard spiked-sample was in agreement with the summation of calcium propionate peaks in standard and sample chromatograms.

3.5. Sample determination

Twenty-two bread products were randomly sampled from three areas outside Bangkok. Manufacturing information such as mailing address, manufacturing date, expiration date, and food compositions of several samples were not presented. Amounts of calcium propionate in these samples were determined by the developed colorimetry and HPLC methods.

Table 2 – Assay results of calcium propionate in bread samples obtained from the developed colorimetric method and high-performance liquid chromatography (HPLC) method.

Sample No.	Source No.	Calcium propionate from colorimetric method		Calcium propionate from HPLC method (mg/kg)
		< 2000 mg/kg	> 2000 mg/kg	
1	1		✓	8454
2	1	✓		1804
3	1		✓	10,683
4	1		✓	5315
5	1		✓	8717
6	2		✓	7487
7	2		✓	21,327
8	2		✓	10,462
9	2		✓	8966
10	2		✓	16,472
11	2		✓	5778
12	2		✓	16,677
13	2		✓	6846
14	2		✓	44,646
15	2		✓	22,396
16	2		✓	12,731
17	2		✓	12,704
18	3		✓	7903
19	3		✓	7310
20	3	✓		1566
21	3		✓	4855
22	3	✓		1939
Total of 22 samples		3	19	—

HPLC = high-performance liquid chromatography.

From the results shown in Table 2, it could be seen that the results obtained from colorimetry were in agreement with the HPLC method. It was also noticed that 19/22 samples were found to have calcium propionate higher than the recommended concentration of 2000 mg/kg.

In summary, a simple, fast, and cheap colorimetry method was successfully developed and can be used as a screening method for calcium propionate in bread samples. A simple HPLC method was also developed and validated. Twenty-two bread samples were sampled from three areas near Bangkok, and the amounts of calcium propionate in these samples were determined using the developed colorimetry and HPLC methods. Determination results obtained from both methods were similar. From these results, it was seen that the developed colorimetry method was applicable as a screening method for calcium propionate in bread products.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

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