วิธีวิเคราะห์ปริมาณกรดเบนโซอิกและการประยุกต์ใช้
Analytical Methods for Determination of Benzoic Acid and Their Applications

นิพนธ์ต้นฉบับ **Original Article**

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วารสารไทยเภสัชศาสตร์และวิทยาการสุขภาพ2564;16(4):372-379.

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บทคัดย่อ

วัตถุประสงค์: เพื่อพัฒนาและตรวจสอบวิธีวิเคราะห์ปริมาณกรดเบนโซอิกในผลิตภัณฑ์อาหาร **วิธีการศึกษา:** พัฒนาวิธีวิเคราะห์ด้วยการปรับค่าต่าง ๆ และสภาวะของระบบในแต่ละวิธีให้เหมาะสม ตรวจสอบทุกวิธีวิเคราะห์ และทดสอบวิเคราะห์ปริมาณกรดเบนโซอิกในอาหาร **ผลการศึกษา:** การวิเคราะห์ด้วยวิธีโครมาโทกราฟีเหลวสมรรถนะสูงใช้คอลัมน์ C18 เป็นวัฏภาคนิ่ง วัฏภาคเคลื่อนที่ประกอบด้วยแอมโมเนียม อะซิเตตบัฟเฟอร์ และเมทานอล ตรวจวัดที่ความยาวคลื่น 235 นาโนเมตร ช่วงความเป็นเส้นตรงของกราฟมาตรฐานที่ใช้ในการศึกษานี้คือ 7.8 – 500 µg/ml (r^2 > 0.999) ค่าขีดจำกัดการตรวจหา (limit of detection; LOD) และค่าขีดจำกัดการวิเคราะห์เชิงปริมาณ (limit of quantitation; LOQ) เท่ากับ 2.5 และ 3.8 ng/ml ตามลำดับ การวิเคราะห์ด้วยอัตราไวโอเลตและวิสิเบิลสเปกโทรสโกปี วัดค่าการดูดกลืนแสงที่ความยาวคลื่น 240 นาโนเมตร ช่วงความเป็นเส้นตรงของกราฟมาตรฐานคือ 3 – 150 µg/ml (r^2 > 0.999) ค่า LOD และ LOQ เท่ากับ 14.2 และ 43.1 ng/ml ตามลำดับ การวิเคราะห์ด้วยวิธีการไตเตรทใช้สารละลายโซเดียมไฮดรอกไซด์ที่ทราบความเข้มข้นแน่นอนและใช้ฟีนอล์ฟทาลีนเป็นอินดิเคเตอร์ ช่วงความเป็นเส้นตรงของกราฟมาตรฐาน คือ 25 – 500 µg/ml (r^2 > 0.999) ค่า LOD และ LOQ เท่ากับ 6.0 และ 25.0 µg/ml ตามลำดับ ตรวจสอบกรดเบนโซอิกโดยใช้สารละลายเฟอริกคลอไรด์ 2% และคอปเปอร์ซัลเฟต 5% ได้สารประกอบในรูปตะกอนสีส้มและสีฟ้าตามลำดับ น้ำยาทั้งสองชนิดสามารถตรวจสอบกรดเบนโซอิกได้ที่ความเข้มข้นตั้งแต่ 500 µg/ml ขึ้นไป วิธี 4 วิธีนี้สามารถตรวจพบปริมาณกรดเบนโซอิกในตัวอย่างอาหาร เครื่องดื่มผสมถังเช่าและเส้นขนมจีนสดอยู่ในระดับที่ยอมรับได้เมื่ออ้างอิงจากประกาศของสำนักงานคณะกรรมการอาหารและยาของประเทศไทย **สรุปผลการศึกษา:** โครมาโตกราฟีเหลวสมรรถนะสูง อัตราไวโอเลตและวิสิเบิลสเปกโทรสโกปี การไตเตรท การตรวจสอบด้วยปฏิกิริยาการตกตะกอนโดยใช้สารละลายเฟอริกคลอไรด์และคอปเปอร์ซัลเฟต ที่ได้รับการพัฒนาและทดสอบ สามารถนำมาประยุกต์ใช้ในการวิเคราะห์กรดเบนโซอิกในตัวอย่างอาหารและเครื่องดื่มได้

คำสำคัญ: กรดเบนโซอิก, โครมาโทกราฟีเหลวสมรรถนะสูง, อัตราไวโอเลตและวิสิเบิลสเปกโทรสโกปี, ไตเตรท, เฟอริกคลอไรด์, คอปเปอร์ซัลเฟต

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Abstract

Objectiv: To develop and validate the analytical methods for the determination of benzoic acid in foodstuffs. **Methods:** The system configurations and conditions of the methods were optimized. Method validations were performed for all analytical methods. The methods were then applied for the quantitative analyses of benzoic acid in foodstuffs. **Results:** HPLC system was developed using C18 column as a stationary phase with ammonium acetate buffer and methanol as a mobile phase. Detection wavelength of 235 nm was used. The linear range was 7.8 - 500 µg/mL (r^2 > 0.999). Limit of detection (LOD) and limit of quantitation (LOQ) were 2.5 and 3.8 ng/mL, respectively. For UV-visible spectrophotometric method, the optimum wavelength was observed at 240 nm, the linear range was 3 - 150 µg/mL (r^2 > 0.999). LOD and LOQ were 14.2 and 43.1 ng/mL, respectively. Acid-base titration technique was performed using an exact concentration of standard sodium hydroxide solution as a titrant and phenolphthalein T.S. as an indicator. The linear range of the method was 25 - 500 µg/mL (r^2 > 0.999). LOD and LOQ were 6.0 and 25.0 µg/mL, respectively. Precipitation methods using 2% ferric chloride and 5% copper (II) sulfate solution were developed and yielded beige-tan and blue precipitate of metal-aromatic acid complex, respectively. Both methods could detect benzoic acid at the concentration of higher than 500 µg/mL. These methods were applied to determine benzoic acid in foodstuffs (cordyceps herbal drink and fresh rice noodle). Acceptable ranges of benzoic acid according to Thai FDA regulation were found. **Conclusion:** HPLC, UV-visible spectrophotometry, titration, ferric chloride test and copper sulfate test were developed and validated. All methods were successfully applied to determine benzoic acid in food and beverage.

Keywords benzoic acid, HPLC, UV-vis spectrophotometry, titration, ferric chloride, copper sulfate

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Introduction

Benzoic acid is a preservative that is widely used as a food additive to preserve and extend shelf life in various foods. It is usually in form of salt, such as sodium salt, calcium salt, etc. In Thailand, benzoic acid and its derivatives are allowed as food preservatives to prevent the growth of microorganisms such as fungi, yeast and bacteria.[1] Even though no accumulation is not observed after receiving small amount of benzoic acid and benzoate salts, the exposure at high dose can lead to abnormalities, e.g., nausea, vomiting, fatigue, headache, allergic reactions and urticaria. Nonetheless, some

people may experience severe symptoms and unconsciousness. In animal studies, there were reports of hepatotoxicity, nephrotoxicity, neurotoxicity and teratogenicity of prolonged exposure to large amount of these substances.[2]

In 2013, there was a report of benzoic acid exposure in Thai population by consumption of processed food. It reported that the amount of exposure was lower than that of the acceptable daily intake (ADI)[3] which is not more than 5 mg/kg body weight.[4] However, in the report, it was interesting that in children aged 3 - 5.9 years receiving benzoic acid at a higher risk level (33 per cent of ADI) compared to other age groups. Exposure to the high risk-products combined with other products at the average level was assessed and it was found that these children probably received benzoic acid up to 92 per cent of ADI. Each day, children also received benzoic acid from other foods which this substance and its salts are allowed as food additive, so there was a chance of over intake of benzoic acid. In addition, recent reports showed that the quantity of benzoic acid in foods was higher than those prescribed by the Food and Drug Administration.[5,6] Therefore, this raised awareness about the safety of consumption of foods and beverages available in the market.

It is found that the determination of benzoic acid content in foodstuffs still requires laboratory equipment and method that is time-consuming and costly; while detection methods of steroids, borax, salicylic acid or formalin are rapid, easy-to-use test kits. Currently, the method for analyzing benzoic acid in food and beverage are both qualitative and quantitative analyses. In qualitative analytical methods, ferric chloride test and Mohler's test are easy to use, convenient and less time-consuming to determine whether benzoic acid has been added to foodstuffs.[7,8] However, these methods cannot be used to quantitate the amount of benzoic acid in food and beverages, while titration, spectrophotometry and chromatography is available for quantification. Nevertheless, the Association of Official Analytical Chemistry (AOAC) has established liquid chromatography and gas chromatography as standard methods for the determination of benzoic acid.[9] Both chromatographic techniques provide accurate and precise results. However, these methods are complicated, time-consuming and expensive. In Thailand, the analysis of benzoic acid in food is not routinely practiced. Benzoic acid test kit that is available in the market can be only applied to rice noodle products and it provides positive result at a limit of detection of more than 1,000 mg/kg.[10] Thus, its sensitivity is inadequate for other products with lower permitted level of benzoic acid.[1] Less time-consuming, less costly and/or more environment-friendly techniques will provide more cpractical methods for the determination of benzoic acid in foodstuffs in the market. In this study, we aimed to develop and validate the analytical methods for the determination of benzoic acid, i.e., HPLC, UV-visible spectrophotometry, titration, ferric chloride test and copper sulfate test and applied to food and beverage.

Methods

Chemicals and reagents

Benzoic acid was purchased from Sigma-Aldrich, St. Louis, Missouri, United States (> 99.5% purity by HPLC). Hydroquinone (internal standard; IS), sodium hydroxide, copper (II) sulfate, ferric chloride, hydrochloric acid, sodium chloride, acetic acid, ammonium acetate, hydrochloric acid, ammonium hydroxide and phenolphthalein TS were purchased from Sigma-Aldrich, St. Louis, Missouri, United States. Methanol, ethanol and chloroform were purchased from RCI Labscan (Bangkok, Thailand). All reagents were of analytical grade or HPLC grade. Cordyceps herbal drink was purchased from a supermarket. Fresh rice noodle was obtained from a local market near the University of Phayao.

Preparation of stock solutions and calibration standards[11]

Stock solution of benzoic acid and hydroquinone were prepared in methanol at 2,000 and 200 µg/mL, respectively. Both stock solutions were kept at 4 °C until use. For the standard curve of HPLC analysis, the working solution were prepared as 2-fold serial dilutions with methanol to make final concentrations of 7.81, 15.62, 31.25, 62.5, 125, 250, 500 µg/mL with 50 µg/mL of IS in all samples. The same dilution method was used to yield final concentrations of 3, 5, 10, 20, 50, 100, 150 µg/mL for UV-visible spectrophotometry and 25, 50, 100, 150, 200, 250, 500 µg/mL for titration technique. QC samples were prepared at 25, 200, 400 µg/mL; 9, 75, 120 µg/mL; and 80, 220, 400 µg/mL for HPLC, UV-visible spectrophotometry and titration method, respectively. For ferric chloride and copper sulfate tests, working solutions were prepared to yield final concentrations of 200, 250, 500, 750 and 1,000 µg/mL.

Method developments and validations

1. High-performance liquid chromatography (HPLC)[11-13]

All analytical procedures were performed on a Shimadzu UFLC model composed of SPD20A UV/VIS detector and LC-20AD liquid chromatograph (Shimadzu Corporation, Singapore). The chromatographic condition was optimized. The separation was carried out on a C18 column (Inertsil, 150 mm x 4.6 mm, 5 µm with 5 µm guard cartridge, GL Sciences Inc. Japan) at ambient temperature. Mobile phase consisted of 0.01M ammonium acetate buffer: methanol (40:60 v/v), pH 4.5. The flow rate was 0.7 mL/min with the total run time of 5.5 min. The injection volume was 20 µL. The detection wavelength was 235 nm. LC solutions software (Shimadzu Corporation, Singapore) was used for HPLC operation. All data were analyzed using PostRun Analysis software (Shimadzu Corporation, Singapore).

2. UV-visible spectrophotometry[12,14]

All UV absorbance were acquired using V-630 UV-VIS spectrophotometer (JASCO Deutschland GmbH, Germany), a double-beam spectrophotometer with single monochromator and silicon photodiode detectors. The optimized wavelength of benzoic acid solution was 240 nm. The operation procedures were performed on JASCO V-630 UV-VIS software (JASCO Deutschland GmbH, Germany).

3. Titration[12,14,15]

Working solutions of benzoic acid in methanol were titrated using 0.005N sodium hydroxide. The exact concentration of the titrant had been determined using potassium hydrogen phthalate solution. Phenolphthalein TS was used as an indicator.

4. Detection of benzoic acid using precipitation methods

A. Precipitation method using ferric chloride[8]

Analytical concentrations of benzoic acid solution were pre-treated by neutralization using 30% ammonium hydroxide in water and then heating to eliminate ammonia. The beige-tan precipitate of ferric benzoate complex was formed after adding 100 µL of 2% ferric chloride. The intensities of the precipitate color were visually determined by performing the reaction for 5 replications. Color intensities forming by the same concentration of benzoic acid were compared between replications to see whether they were similar. Color intensities forming by the different concentrations of benzoic acid were compared to determine whether they could be distinguished from others.

B. Precipitation method using copper (II) sulfate

The procedure of this method was similar to the precipitation method using ferric chloride. The 5% copper (II) sulfate were used instead of ferric chloride solution. The formation of copper benzoate was observed as blue precipitate.

5. Method validation

Method validations followed the AOAC Guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals.[12] The measurements in the interferences of benzoic acid in food and beverage were determined by analyzing the specificity of the methods using substances commonly used in foods. Linear regressions were determined as coefficient of variations (r^2). Intra-day precisions and accuracies were determined with the QC samples at three concentrations on three consecutive days. The accuracy and precision of the method was expressed by per cent recovery (% recovery) and per cent relative standard deviation (% RSD), respectively.

Sample preparations

1. Sample preparation for HPLC, UV-vis spectro-photometry and titration techniques

One gram of pulverized fresh rice noodle or cordyceps herbal drink was weighted into centrifuge tube before adding 2 mL of warm water. The sample was mixed thoroughly and centrifuged at 4,000 rpm for 10 min. One milliliter of supernatant was collected for further partition technique using 1 mL of chloroform. After partition, 0.5 mL of organic layer was collected and chloroform was removed by evaporation at 60 °C. The residue was reconstituted using 0.5 mL of methanol before the analysis. Sample preparation could be scaled up for acquired volume of sample for each analytical technique.

2. Sample preparation for precipitation methods

Fresh rice noodle was pulverized and weighed for 30 g, then 30 mL of acidified methanol (pH 4.0) was added. The sample was mixed thoroughly and set aside for 10 min before filtered to obtain clear solution for the analysis. In case of cordyceps herbal drink, the analysis by precipitation methods was not applicable since the endpoint colors were interfered by the color from sample obtained after sample preparation.

Data and statistical analysis

The study results are described by mean and standard deviation (SD).

Results

High performance liquid chromatography (HPLC)

The optimized condition of HPLC was sensitive enough for the determination of benzoic acid in foodstuffs according to the permitted levels by Thai FDA. The retention of IS and benzoic acid was 2.9 and 4.6 minutes (Figure 1), respectively. The LOD and LOQ were observed by signal to noise ratio of 3:1 at 2.5 ng/mL and 10:1 at 3.8 ng/mL, respectively. The standard curve was constructed at 7.8 – 500 µg/mL. Specificity of the method was tested using methyl paraben and propyl paraben solutions. Both substances did not interfere the analyses of benzoic acid and IS. The intra-day and inter-day accuracies at low, medium and high concentrations (25, 200, 400 µg/mL) were in the range of 99.3 - 100.9%. The intra-day and inter-day precisions were 0.2 - 1.9% (Table 1).

UV-visible spectrophotometry

The optimal wavelength of the analysis was 240 nm. The LOD and LOQ of the method were determined by 3.3 SD/slope and 10 SD/slope, respectively.[12] When SDs were obtained from ten times measurement of blank methanol and slopes were obtained from standard curve, the LOD and LOQ were 14.2 and 43.1 ng/mL, respectively. Glucose and sucrose were used to determine specificity of the method. The highest absorbances of both substances were observed at 260 - 270 nm. The standard curve was construct at 3 - 150 µg/mL. The intra-day and inter-day accuracies were in the range of 99.3 - 101.4%. The intra-day and inter-day precisions were 0.5 - 0.7% (Table 1).

Titration

The LOD and LOQ of the method were determined by 3.3 SD/slope and 10 SD/slope, respectively.12 When SDs were obtained from ten times measurement of blank methanol and slopes were obtained from standard curve, LOD and LOQ were 6 and 25 µg/mL, respectively. In the validation, the standard curve of benzoic acid was constructed at 25 – 500 µg/mL. The intra-day and inter-day accuracies and precisions of the method were in the range of 99.4 - 100.5% and 0.2 - 0.7%, respectively (Table 1).

Detection of benzoic acid using precipitation methods

The specificities of the methods were investigate using organic acid, i.e., ascorbic acid and citric acid. Both acids did not interact with ferric chloride and copper (II) sulfate (Figure 2A). The precipitate color visually obtained from 5 replications of the analysis using the same concentration of benzoic acid resulted in the same intensities and could be distinguished from those obtained from the different concentrations. The LOD of both analytical methods was 500 µg/mL (Figure 2B). The color intensities of the precipitate were developed to standard color bands in the concentration range of 500 - 1000 µg/mL (Figure 2C).

5. Determination of benzoic acid in fresh rice noodle and cordyceps herbal drink

The recoveries of benzoic acid in fresh rice noodle and cordyceps herbal drink analyzed by HPLC, UV-visible spectrophotometry and titration were in the range of 32.9 - 37.8% and 71.5 - 81.5%, respectively (Table 2). The quantities of benzoic acid in fresh rice noodle and cordyceps herbal drink after calculation by weight were in the range of 70.5 - 94.8 mg/kg and 146.2 - 172.9 mg/kg, respectively. To apply ferric chloride and copper (II) sulfate tests to the determination of benzoic acid content in fresh rice noodle, UV-visible spectrophotometry was used as a standard method. The recovery of benzoic acid from fresh rice noodle was 39.8%. The color intensities of the precipitates were equivalent to the concentration of benzoic acid between 500 - 750 and 750 – 1,000 µg/mL for sample spiked with benzoic acid and solvent spiked with benzoic acid, respectively (Figure 2D). These results corresponded to the values obtained from UV-visible spectrophotometry which were 586.4 and 857.3 µg/mL, respectively (Table 2). Benzoic acid was not detected in un-spiked samples using precipitation methods because the concentration of benzoic acid in the samples (188 µg/mL) was lower than LOD. These results were also confirmed by UV-visible spectrophotometry. Benzoic acid content (188 µg/mL) in fresh rice noodle could be calculated to 62.7 mg/kg of the sample.

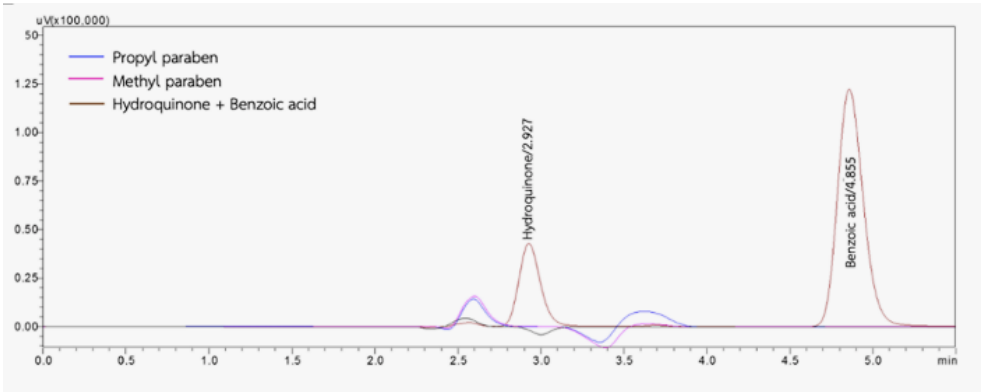

Figure 1 HPLC chromatograms of benzoic acid, hydroquinone (IS), methyl paraben and propyl paraben. The retention time of benzoic acid, IS 4.86 are and 2.93 min, respectively. Methyl paraben and propyl paraben were not interfered the analyses.

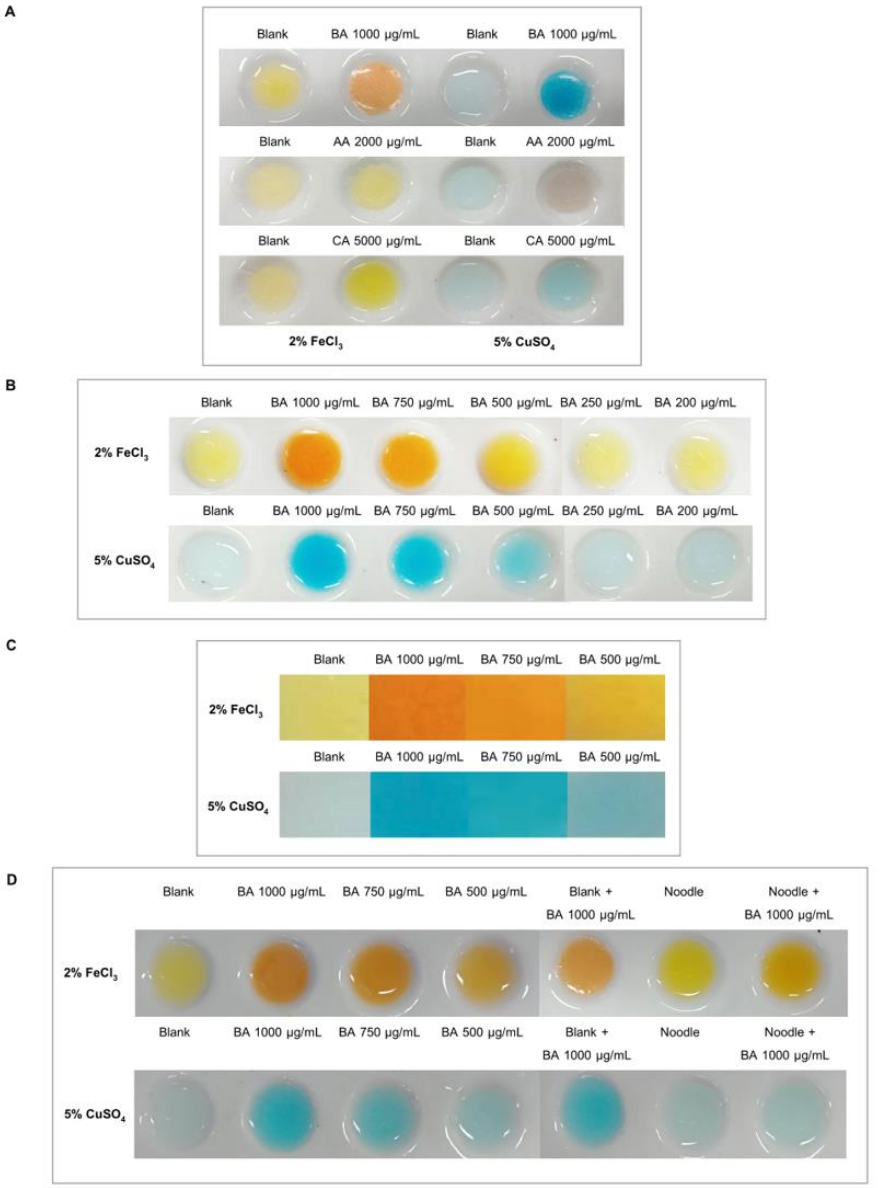

Figure 2 The results from the detection of benzoic acid (BA) by precipitation methods using ferric chloride and copper (II) sulfate. (A) Determination of specificity using ascorbic acid (AA) and citric acid (CA) (B) Determination of LODs (C) Standard color bands obtained from the developed methods (D) Analysis of benzoic acid content in fresh rice noodle.

Table 1 Results from method validations of HPLC, UV-visible spectrophotometry, and titration.

Analytical method	Calibration range (µg/mL)	Linearity (r^2)	LOD (µg/mL)	LOQ (µg/mL)	Concentration of QC samples (µg/mL)	Intra-day analysis*		Inter-day analysis†	
						Accuracy (% recovery)‡	Precision (% RSD)	Accuracy (% recovery)‡	Precision (% RSD)
HPLC	7.8 - 500	> 0.999	0.0025	0.0038	25	100.9 ± 0.2	0.7	99.4 ± 0.5	1.9
					200	100.1 ± 0.9	0.4	100.2 ± 2.3	1.1
					400	100.1 ± 1.0	0.2	99.3 ± 1.9	0.5
UV-visible spectrophotometry	3 - 150	> 0.999	0.0142	0.0431	9	101.4 ± 0.1	0.6	101.2 ± 0.1	0.6
					75	101.1 ± 0.4	0.5	101.2 ± 2.3	0.7
					120	99.3 ± 0.9	0.5	100.0 ± 1.9	0.7
Titration	25 - 500	> 0.999	6.0	25.0	80	100.0 ± 0.5	0.6	99.4 ± 0.8	0.7
					220	99.7 ± 0.2	0.4	100.2 ± 0.6	0.5
					400	100.1 ± 0.2	0.2	100.5 ± 0.4	0.3

* Values from the analyses within one day, † Values from the analyses for 3 consecutive days, ‡ Mean ± SD.

Table 2 Results from determinations of benzoic acid content in food and beverage samples. All analyses were based on the principle of standard-spiked sample preparation.

Analytical method		Samples#					
		Fresh rice noodle			Cordyceps herbal drink		
		Sample (A)	Sample + Benzoic acid (B)	Solvent + Benzoic acid (S)	Sample (A)	Sample + Benzoic acid (B)	Solvent + Benzoic acid (S)
HPLC	Benzoic acid added (µg/mL)	-	31.2	31.2	-	31.2	31.2
	Benzoic acid detected (µg/mL)	23.7 ± 0.0	35.5 ± 0.1	30.4 ± 1.6	43.2 ± 0.0	68.7 ± 0.4	30.9 ± 0.2
	Extraction recovery of benzoic acid (%)	-	37.8 ± 0.5*	97.3 ± 1.3†	-	81.5 ± 1.2*	99.0 ± 0.4†
	Benzoic acid content (mg/kg)‡		94.8			172.9	
UV-visible spectrophotometry	Benzoic acid added (µg/mL)	-	15.6	15.6	-	15.6	15.6
	Benzoic acid detected (µg/mL)	2.9 ± 0.1	9.1 ± 0.1	14.9 ± 0.3	43.2 ± 0.9	55.8 ± 0.5	15.2 ± 0.2
	Extraction recovery of benzoic acid (%)	-	37.2 ± 1.6*	96.1 ± 0.6†	-	81.5 ± 2.9*	98.2 ± 1.7†
	Benzoic acid content (mg/kg)‡		94.2 ± 2.3			172.8 ± 3.6	
Titration	Benzoic acid added (µg/mL)	-	100.0	100.0	-	50.0	50.0
	Benzoic acid detected (µg/mL)	55.2 ± 1.01	88.1 ± 1.5	112.1 ± 1.49	110.6 ± 1.0	147.8 ± 1.0	84.3 ± 0.8
	Extraction recovery of benzoic acid (%)	-	32.9 ± 1.5*	93.6 ± 1.49†	-	71.5 ± 2.0*	98.2 ± 1.5†
	Benzoic acid content (mg/kg)‡		70.5 ± 2.0			146.2 ± 2.0	
Precipitation methods using ferric chloride and copper (II) sulfate	Benzoic acid added (µg/mL)	-	1000.0	1000.0	Not analyzed		
	Benzoic acid detected (µg/mL)	188.0 ± 2.14	586.4 ± 52.9	857.3 ± 32.9			
	Extraction recovery of benzoic acid (%)	-	39.8 ± 5.5*	85.7 ± 3.3†			
	Benzoic acid content (mg/kg)‡		62.7 ± 0.6§				

Values were obtained from 3 replications and are expressed as mean ± SD.

* Extraction recovery = (Benzoic acid detected in sample spiked with benzoic acid (B) - Benzoic acid detected in sample (A) x 100) / Benzoic acid added into sample (B).

† Extraction recovery = (Benzoic acid detected in solvent spiked with benzoic acid (S) x 100) / Benzoic acid added into solvent (S).

‡ Values were obtained from benzoic acid detected in sample and calculated back to 1 kg of sample.

§ Results from precipitation reactions shown in Figure 2D. The exact value obtained from standard method, UV-visible spectrophotometry.

Discussions and Conclusion

In this study, four analytic methods were developed and validated. They were HPLC, UV-visible spectrophotometry, titration and precipitation methods using metal-complex forming. It was found that the HPLC developed in this study was specific to benzoic acid and IS. It provided better sensitivity with LOQ of 3.8 ng/mL and requires less analyzing time compared to the HPLC developed in previous studies (60 ng/mL).[16] The standard curve used in this study was constructed in the suitable range for quantitative analysis of benzoic acid in food (7.81 – 500 µg/mL) according to Thai FDA regulation. Moreover, in this study, the standard curves of the wilder range, i.e., 0.0038 – 500 µg/mL, were also performed and the results showed the great linearities over the validation criteria (r^2>0.997).[12] The UV-visible spectrophotometry, like the HPLC, was specific to benzoic acid. The method provided better sensitivity with the LOQ of 4.3 ng/mL compared to the results reported in previous studies (1000 ng/mL).[14] To be compared to HPLC, the UV-visible spectrophotometry offered a narrower range of standard curve due to disobeying of Beer Lambert's law at above 500 µg/mL.

However, the concentration range of benzoic acid in the standard curve that was developed in this study was sufficient for the analysis of food samples in the market. The developed titration method was found to be less sensitive than HPLC and UV-visible spectrophotometry. Nevertheless, the standard curve of this method was adequate for the determination of benzoic acid in food samples. In addition, the lowest concentration of the standard curve used in this study was lower than LOD of the previous study ($\geq$ 50 µg/mL).[14] From the development of titration method, it has been noticed that the concentration of titrant was an essential factor that affected the results. Titration is the least costly quantitative method but there is still a problem of specificity. The acid-base titration might give false positive results from other acids content in food. However, the method can be used as a screening method for the detection of the over-permitted level of benzoic acid.

The qualitative or semi-quantitative analytical methods used ferric chloride and copper (II) sulfate on the principle of metal complex forming precipitation, i.e. ferric benzoate and copper benzoate. It was found that both developed methods were suitable for the determination of benzoic acid at 500 µg/mL and higher concentration. Standard color bands were developed as indicators of benzoic acid in concentrations of 500, 750 and 1,000 µg/mL. The superior advantage over test kit available in the market in Thailand is that the test kit can be used to analyze only rice noodle that contains a concentration of benzoic acid higher than 1000 mg/kg.[10] Both tests do not require complicated analysis tools compared to other colorimetric reactions that require special programs in computer or mobile phone[17] or measurement using spectrophotometer.[18]

The limitations of these analytical methods include non-specificity of the titration method. Since it was acid-base titration, therefore, if tested samples contain other acids, the analyzed concentrations may not be the exact values. While the precipitation methods may not be suitable for colored samples obtained after sample preparation. Because the color from samples may interfere the color resulting from the reaction. However, it is still possible to know whether benzoic acid is present in the sample by observing the forming of precipitate.

This study succeeds in applying the above 4 methods of analysis to food and beverage samples. They were fresh rice noodle and cordyceps herbal drink that the content of benzoic acid as a preservative are regulated by the Food and Drug Administration.[1] It was found that the results from quantitative analyses obtained from HPLC, UV-visible spectrophotometry and titration were comparable. The results of precipitation methods using ferric chloride and copper (II) sulfate were confirmed by UV-visible spectrophotometry. However, the extraction recoveries of benzoic acid from fresh rice noodle reported herein was considerably low. To tackle this problem, other techniques and factors might be considered e.g. polarity and acidity of organic solvents which can be affected the solubility of free form and salt form of benzoic acid, time of extraction, use of auxiliary solvent, salting out technique and other types of extraction such as solid-phase extraction. The appropriate sample preparation methods should also be developed in order to get rid of the matrix effect, especially samples that contain potential analytical interferences. The suitable methods are in our development processes.

In conclusion, this study developed and validated the HPLC, UV-visible spectrophotometry, titration and precipitation methods using ferric chloride and copper (II) sulfate for the detection and measurement of benzoic acid content in two kinds of foods. We expected high specificity, high sensitivity, decrement of analysis time, no requirement of expensive and/or complicated tools, and the application to food and beverage in the market. Furthermore, we aimed that the precipitation methods can be developed as an available test kit shortly. Nevertheless, selection of using method depends on expenditure and the amount of benzoic acid contained in samples. The developed methods can also be further applied to either food or cosmetic products which will be useful for consumer protection.

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