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Original Article

A rapid and sensitive spectrophotometric method for the determination of benzoyl peroxide in wheat flour samples

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ABSTRACT

A simple, rapid, and sensitive spectrophotometric method for the determination of benzoyl peroxide (BPO) in wheat flour samples was developed. The detection principle is based on BPO reacted with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to obtain a blue-green colored product that was detected at 415 nm by spectrophotometry. The effect of factors influencing the color reaction was investigated. Under the selected conditions, the linear range for quantification of BPO was observed between 0.2–1.0 mg L^{-1} with $r^2 = 0.998$. The limit of detection (LOD) was 0.025 mg L^{-1}. The developed method obtained superior precision (relative standard deviation < 2%) using 11 repeatability at 0.2 mg L^{-1}, 0.6 mg L^{-1}, and 0.8 mg L^{-1}. The proposed methodology was successfully applied to determine BPO in wheat flour samples.

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1. Introduction

Benzoyl peroxide (BPO) is widely used as an initiator and catalyst for polymerization processes [1–4]. Moreover, it is commonly used as a food additive in flour, resulting in a bright white flour color, because of its bleaching property [5]. BPO has been used as an acne treatment because it works as a peeling agent. It increases skin turnover, clears pores, and reduces bacterial count (specifically *Propionibacterium acnes*) as well as acts directly as an antimicrobial [6,7].

An excessive BPO can not only annihilate nutrients in flour, but it has effects on human health especially when BPO is in flour. It can decompose into benzoic acid and other

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deleterious substances, such as biphenyl and phenylbenzoate, that cause tissue damage and diseases [8,9].

The specific maximum concentration of BPO in food is 0.05 g kg^{-1} in the USA and UK [10]. In 2009, the Codex Alimentarius Commission determined that the amount of BPO in wheat flour was < 75 mg kg^{-1} [9]. Recently, BPO has been strictly forbidden as an additive to flour in the European Union and China (since May 1, 2011) [8]. Hence, a rapid, sensitive, and selective methodology for quantification of BPO in food samples needs to be developed.

There are many analytical methods for determining BPO. The Association of Official Analytical Chemists method [11] is the standard method for quantification of BPO in wheat flour. This method is based on dissociation of BPO to benzoic acid in the presence of iron and hydrochloric acid as the catalyst. The benzoic acid product is detected by colorimetric reaction. Even though this method is simple, it has low sensitivity for measuring the analyte. Chromatographic techniques such as liquid chromatography [12,13], high-performance liquid chromatography (HPLC) [14–19], gas chromatography [20], capillary electrophoresis [21], and ion chromatography [22] have been developed and used for the detection of BPO in flour samples. Therefore, chromatographic method can determine many analytes simultaneously [23]. Moreover, these methods are provided high accuracy and high precision. Flow injection analysis (FIA) is an alternative method for the determination of BPO, and it is rapid, automatic, and has a high sample throughput [24–26]. Generally in FIA, the reagents continuously flow in narrow tubing aided by the operation of a peristaltic pump in order to obtain a continuously generated baseline, which results in a lot of wasted generations. Chemiluminescence [10,27–29], fluorescence [8,30], differential pulse voltammetry [31], and amperometry [6,32] have been reported as useable methods and highly sensitive for determining BPO. Colorimetric reactions based on chromogenic reagents such as *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPDA) [24,33], *N*-ethyl-2-naphthylamine (NENA) [34], *N,N*-dimethyl-*p*-phenylenediamine [35], iodine in acidic medium [26], ferric thiocyanate [36], *β*-cyclodextrin [37], and 3,3′,5,5′-tetramethylbenzidine (TMB) [9] have been developed for the determination of BPO. These methods provide high sensitivity and selectivity, low detection limit, and high precision and accuracy. However, to the best of our knowledge, the use of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a chromogenic reagent has not been presented for BPO detection.

ABTS is a chromogenic substrate for enzyme peroxidase activity measurement similar to TMB which can be oxidized by hydrogen peroxide in the presence of peroxidase enzyme as a catalyst. The color of the solution changes from light yellow-green to a blue-green color. The maximum absorption wavelength (λ_{max}) was observed at 415 nm. Therefore, a novel method of utilizing ABTS as a reagent detectable by spectrophotometry for BPO in food samples was investigated.

2. Materials and methods

2.1. *Reagents and chemicals*

The chemical reagents used throughout this study were analytical grade and utilized without any further purification. The deionized water was purified by Milli-Q, Millipore apparatus. ABTS was obtained from Sigma-Aldrich (Sigma-Aldrich,

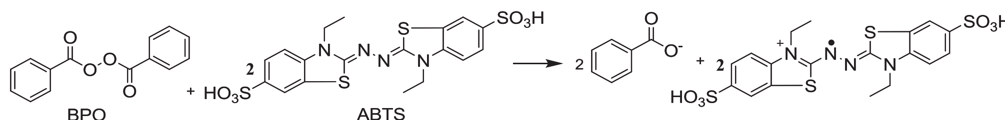

Fig. 1 – The possibility reaction of the proposed system.

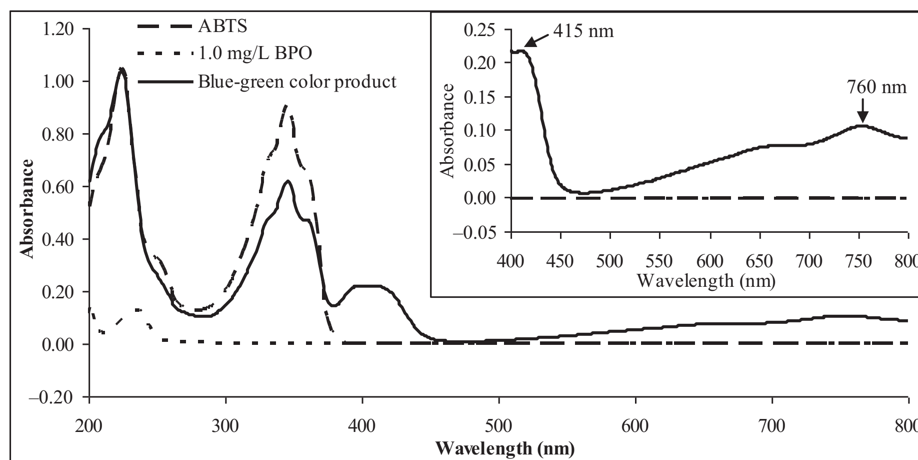

Fig. 2 – Absorption spectra of 1.0 mg L^{-1} BPO (dotted line), ABTS 10 mg L^{-1} (broken line) and the blue-green color solution after mixed 1.0 mg L^{-1} BPO with 10 mg L^{-1} ABTS (unbroken line). ABTS = 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); BPO = benzoyl peroxide.

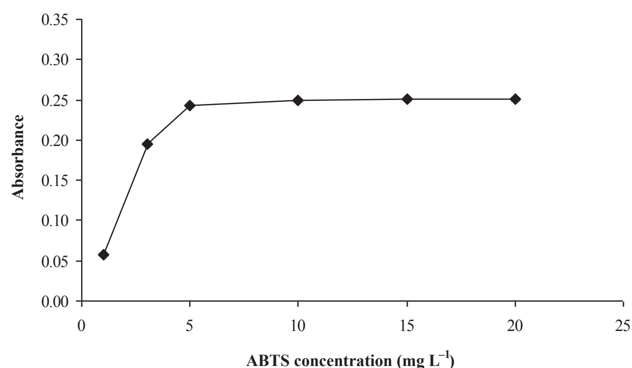

Fig. 3 – Effects of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) concentration.

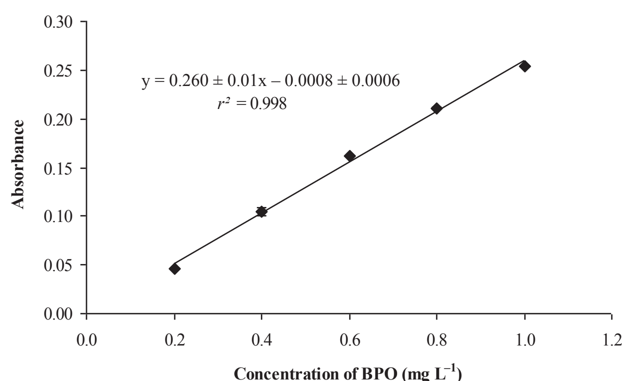

Fig. 4 – Calibration curve graph of the developed method.

Oakville, Canada). BPO was purchased from Panreac (Panreac, Barcelona, Spain). Ethanol 96% came from Merck (Merck, Darmstadt, Germany).

The stock solution of chromogenic reagent as ABTS 1000 mg L^{-1} was prepared by dissolving 0.1 g of ABTS in 100 mL of deionized water.

1000 mg L^{-1} BPO stock solution was generated by dissolving BPO 0.1 g in 100 mL of 96% ethanol. The working solution of BPO was prepared fresh daily by diluting appropriate volume of stock BPO in 96% ethanol.

2.2. *Apparatus*

UV-Vis spectrophotometer model Lambda 25 from Perkin-Elmer was used to measure absorbance (Perkin-Elmer, USA).

2.3. *Sample preparation for BPO assay*

Nonadditive wheat flour (flour blank) and flour samples were purchased from local markets in Maha Sarakham Province, Thailand. All samples were stored at 4°C until prepared for quantitative assay. Flour sample (0.5 g) and spiked sample were transferred to a centrifuge tube and 5 mL of ethanol was added. Then, sonication of the sample for 5 minutes was completed followed by shaking of the solution for 5 minutes with a vortex mixer. The supernatant was collected after being centrifuged at 4070*g* for 10 minutes.

2.4. *Analytical method for assay of BPO*

The analytical procedures for the quantification of BPO were started by pipette 1000 μL of extracted solution to 10 mL volumetric flask. Then, 1000 μL of 100 mg L^{-1} ABTS was added. Finally, the solution was made up to 10 mL with 96% ethanol, and the solution was reacted for 1 minute. The solution developed from light-green to a green color immediately without any catalysts, which provided maximum absorbance at 415 nm. The content of BPO in the real sample was calculated using the linear regression equation of standard curve.

2.5. *Calibration graph*

The calibration graph for the determination of BPO was investigated in the range 0.2–1.0 mg L^{-1} by dilution with appropriate volume of 100 mg L^{-1} standard BPO between 20 μL and 100 μL followed by adding 1000 μL of 100 mg L^{-1} ABTS. Afterward, the 96% ethanol was utilized to adjust volume to 10 mL. The calibration graph was created by plotting absorbance (y-axis) with concentration of BPO in mg L^{-1} (x-axis).

2.6. *Reference method*

In order to confirm the content of BPO in the real sample, HPLC was used as reference method. Here, briefly the operational conditions of HPLC (Agilent 1100 series HPLC, Germany) adapted from Saiz et al [17] were as follows: isocratic separation on a ACE® C-18-AR column (5 μm, 250 mm × 4.6 mm i.d.; Advanced Chromatography Technologies Ltd., United Kingdom); methanol/water (80:20) mobile phase at 1.0 mL/min; 20 μL of sample loop. The eluted compound was detected at 227 nm.

Table 1 – Analytical table of merits.

Linear range (mg L^{-1})	Linear equation	Correlation coefficient (r^2)	LOD (mg L^{-1})	%RSD
0.2–1.0	$Abs = (0.260 \pm 0.010)C_{BPO} - (0.0008 \pm 0.0006)$	0.998	0.025[a]	< 2%[b]

Abs = absorbance; C_{BPO} = concentration of BPO; LOD = limit of detection; RSD = relative standard deviation.
[a] LOD was calculated by 3σ of blank/slope (σ is standard deviation of blank).
[b] Studied at 0.2 mg L^{-1}, 0.4 mg L^{-1}, and 0.8 mg L^{-1} of BPO with 11 repeats.

Table 2 – Analytical characteristics of some methods for determination of benzoyl peroxide in wheat flour samples.

Detection methods	Sample	Working range	LOD (mg L^{-1})	LOQ (mg L^{-1})	%RSD	Recovery (%)	Advantage	Disadvantage	Refs
AOAC official method (benzoyl peroxide bleach (benzoic acid) in flour)	Wheat flour	0–1.2 mg L^{-1}	—	—	—	—	Simple	Low sensitivity and time-consuming	[11]
TMB-based colorimetric method	Wheat flour	0.67–16 mg L^{-1}	0.45	—	2.68	97–118	Sensitive, convenient and rapid	Batch-wise method and high amount of waste	[9]
Fluorescence based on *N*-methoxy rhodamine-6G spirolactam	Wheat flour	0.2–3.2 mg L^{-1}	0.06	—	—	—	Simple and sensitive	Long time analysis (30 min)	[8]
HPLC	Wheat flour	0.07–0.15 μg g^{-1}	0.03	—	0.4-3.2	96.0–99.3	Simple and reliable	Long time analysis (up to 25 min)	[14]
Differential pulse voltammetry	Wheat flour	0.61–24.2 mg L^{-1}	0.061	—	—	94.8–106	Selective	Using organic solvent	[31]
Ion chromatography with pre-column derivative	Wheat flour	0.12–20 mg L^{-1}	0.019	—	—	81–92	Sensitive and selective	Long time analysis	[22]
Chemiluminescence microfluidic chip	Flour	0.8–100 mg L^{-1}	0.4	—	2.41–3.06	—	Less reagent consumption	Complication device and tedious step of operation	[10]
ABTS-based colorimetric method	Wheat flour	0.2–1.0 mg L^{-1}	0.025	0.087	0.2–1.7	87–104	Rapid, sensitive and selective	Batch-wise method and high waste generation	This study

ABTS = 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); AOAC = Association of Official Analytical Chemists; HPLC = high performance liquid chromatography; LOD = limit of detection; LOQ = limit of quantitation; RSD = relative standard deviation; TMB = tetramethylbenzidine.

3. Results and discussion

3.1. *Possibility of color reaction*

The detectable reaction of the proposed method for examination of BPO in flour samples is based on redox reaction between ABTS and BPO in an ethanol medium. After BPO was reduced by a chromogenic agent such as TMB [9] or ABTS, it became benzoate anion. Therefore, the possibility of this color reaction was shown in Fig. 1. Fortunately, this colorimetric reaction can occur without any catalysts. This reaction is rapid and simple. ABTS was oxidized by BPO as a strong oxidizing agent to provide ABTS radical cation as a blue-green color product. From the previous reports [38–40], the $ABTS^{\bullet+}$ chromophore consists of conjugated π-double bonds system in molecule. Therefore, it absorbs certain wavelengths at visible light. The characteristic strong absorption peak at 380–435 nm (yellow-green color) and board absorption peak from 650 nm to 780 nm (blue-green color) was observed as presented in Fig. 2. In order to achieve high sensitivity, the concentration of BPO in the sample can be quantified by measuring the absorbance at 415 nm. Because the highest molar extinction coefficient ($\varepsilon = 3.6 \times 10^4$ L mol^{-1} cm^{-1}) for ABTS radical cation at 415 nm [38–40] and less background absorption at this wavelength.

3.2. *Effect of ABTS concentration*

ABTS can be oxidized by BPO without any catalyst. Therefore, the effect of ABTS concentration on sensitivity was investigated between 1 mg L^{-1} and 20 mg L^{-1}. The result is illustrated

Table 3 – Recovery and the results for the determination of benzoyl peroxide in wheat flour samples.

Sample no.	Original amount[a]	Spiked (μg/g)	ABTS method Found value[b] (μg/g) / Recovery[b] (%)	HPLC Found value[c] (μg/g) / Recovery[c] (%)
1	—	50	46.1 ± 0.1	44.1 ± 4.2
			92 ± 0.2	88 ± 8.4
		75[d]	72.3 ± 0.2	74.9 ± 4.7
			96 ± 0.2	100 ± 6.3
		90	80 ± 0.2	81.6 ± 4.6
			89 ± 0.2	91 ± 5.2
2	—	50	50.9 ± 0.3	46.7 ± 4.8
			102 ± 0.5	93 ± 9.7
		75[d]	74.8 ± 0.2	71.0 ± 3.9
			100 ± 0.3	95 ± 5.2
		90	89.9 ± 0.2	88.5 ± 4.0
			100 ± 0.3	98 ± 4.4
3	—	50	46.5 ± 0.1	49.9 ± 3.8
			93 ± 0.3	99 ± 7.6
		75[d]	69.5 ± 0.2	67.3 ± 5.1
			93 ± 0.3	90 ± 6.8
		90	83.7 ± 0.3	85.5 ± 4.2
			93 ± 0.3	95 ± 4.7
4	—	50	45.4 ± 0.1	46.6 ± 4.2
			91 ± 0.2	93 ± 8.5
		75[d]	64.9 ± 0.2	76.0 ± 4.5
			87 ± 0.3	101 ± 6.0
		90	80.9 ± 0.0	82.0 ± 4.4
			90 ± 0.0	91 ± 4.8
5	—	50	51.8 ± 0.2	50.6 ± 4.2
			104 ± 0.3	101 ± 8.0
		75[d]	72.2 ± 0.2	74.9 ± 4.4
			96 ± 0.3	99 ± 5.9
		90	87.5 ± 0.1	87.8 ± 4.6
			97 ± 0.1	98 ± 5.1
6	—	50	51.1 ± 0.2	54.8 ± 3.9
			102 ± 0.3	109 ± 7.7
		75[d]	68.4 ± 0.0	75.3 ± 4.2
			91 ± 0.1	100 ± 5.7
		90	85.6 ± 0.4	94.0 ± 4.9
			95 ± 0.4	104 ± 5.4

ABTS = 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); HPLC = high performance liquid chromatography.
[a] Not detectable.
[b] Values found are the average of three determinations ($n = 3$) ± the corresponding standard deviation.
[c] Values found are the average of three determinations ($n = 3$) ± the corresponding standard deviation.
[d] Limit value for benzoyl peroxide residues in wheat flour stipulated in Codex Alimentarius Commission.

in Fig. 3. It was found that the absorbance vividly increased by increasing the concentration of ABTS. However, the analytical signal was practically constant in the range of 5–20 mg L^{-1}. So, in the developed method, a concentration of ABTS at 10 mg L^{-1} was used for subsequent procedures.

3.3. *Effect of reaction time*

The reaction time of 1–10 minutes was investigated for improvement of absorbance intensity. It was found that the absorbance values did not increase with increasing reaction time. However, in order to obtain excellence of precision, the reaction time of 1 minute was thoroughly utilized for the experiments.

3.4. *Analytical figure of merits*

Under the selected conditions, the method validations of linearity, limit of detection (LOD), and precision were performed. Linear calibration curve is demonstrated in Fig. 4. Analytical figures of merit are presented in Table 1. The results implied that the linear ranges of the calibration graph are found in ranges of 0.2–1.0 mg L^{-1} with the linear equation:

$$Abs = (0.260 \pm 0.010)C_{BPO} - (0.0008 \pm 0.0006) \quad (1)$$

and correlation coefficient (r^2) of 0.998, where *Abs* is absorbance and C_{BPO} is concentration of BPO. The detection limit provided 0.025 mg L^{-1} at 3σ of blank/slope (σ is standard deviation of blank) corresponding to 0.25 mg BPO per kg wheat flour. Limit of quantitation (LOQ) was 0.087 mg L^{-1} (10σ of blank/slope). Moreover, the relative standard deviation (RSD, $n = 11$) intraday was 1.7%, 0.2%, and 0.4 % at 0.2 mg L^{-1}, 0.6 mg L^{-1}, and 0.8 mg L^{-1} of BPO, respectively. The reproducibility of interday experiments were observed between 3.5% and 4.7% ($n = 11$) at 0.2 mg L^{-1}, 0.6 mg L^{-1}, and 0.8 mg L^{-1} of BPO. These findings indicate that the developed method has excellent precision. Table 2 summarizes the analytical characteristics of the proposed method in comparison to other methods reported in the literatures. Obviously, the proposed method has achieved one of the most sensitive approaches for BPO detection when compared with other previously reported methods. This approached method was detected at 415 nm, which has high molar extinction coefficient (ε) as 3.6×10^4 L mol^{-1} cm^{-1}. Furthermore, fewer background interferences and no effect of sample matric were obtained because of the use of ethanol as extraction solvent and detection media. In addition, this method is suitable for rapid quantification without complicated expensive instruments.

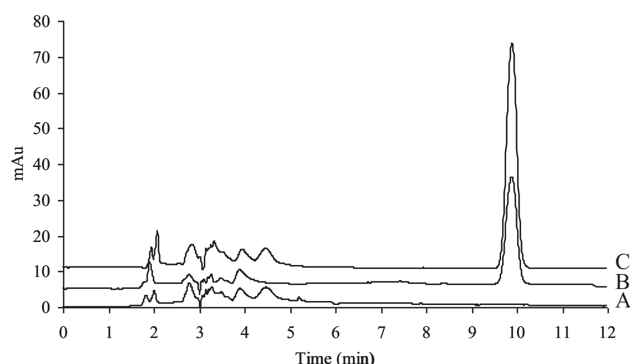

Fig. 5 – Chromatograms of (A) sample No. 6 without spiked benzoyl peroxide (BPO) standard, (B) BPO standard 50 μg g^{-1} and (C) sample No. 6 with spiked BPO 90 μg g^{-1}.

3.5. *Analytical application and recovery*

The proposed method has been used for quantification of BPO in flour samples. The sample preparation procedures are described in the *Materials and methods* section. In order to eliminate the matric interference, ethanol was utilized as extraction solvent for the real sample. Because of inorganic salts, starch and fat are poorly dissolved in ethanol that is used as the extraction solvent and detection media [8,9]. The samples were spiked with BPO standard at different concentrations (50 μg g^{-1}, 75 μg g^{-1}, and 90 μg g^{-1}). The results are summarized in Table 3. The quantification of BPO content depends on the formation of ABTS cation radical. However, there are non-oxidative agents or other bleaching agents such as ammonium persulfate, calcium phosphate, nitrogen dioxide, chlorine dioxide, nitrogen dichloride, and calcium peroxide [41] that can be affected by the determination of BPO by the approach method. Because the solubility of inorganic salts were poor in ethanol extract solutions other peroxides cannot be reacted with ABTS without adding other enzyme peroxidases. Therefore, the high percentage recoveries between 87–104% and 88–109% were obtained for the proposed method and HPLC method [17], respectively. The chromatograms of standard BPO 50 μg g^{-1}, sample No. 6 spiked with standard BPO 90 μg g^{-1} and without spiked BPO are illustrated in Fig. 5. It was found that the retention time of benzoyl peroxide was 9.750 ± 0.016 ($n = 3$). The other compounds and/or other flour-bleaching agent were not coeluted with BPO. Wheat flour samples were analyzed by the HPLC method for comparison. As presented in Table 3. The obtained results from both methods were in good agreement, which evaluated by *t*-test at the 95% confidence limit ($t_{calculate} = 1.77$, $t_{critical} = 2.11$), indicating that there is no significant difference between the two methods.

4. Conclusion

The colorimetric reaction for the determination of BPO using ABTS as the chromogenic reagent was successfully developed. This procedure provided a simple, rapid, and sensitive method for the determination of BPO in wheat flour samples. The results were satisfactory when compared with the HPLC method. Therefore, the proposed method is an alternative procedure for application to determine BPO in real flour samples.

Conflicts of interest

All authors declare no conflicts of interest.

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