

Determination of benzoyl peroxide and benzoic acid in wheat flour by high-performance liquid chromatography and its identification by high-performance liquid chromatography–mass spectrometry

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Abstract

An HPLC method on C₁₈ column using a gradient mobile phase is proposed for the separate determination of residual benzoyl peroxide (BP) and benzoic acid (BA) in flour and wheat products. The recoveries obtained were quite excellent, from 96.0 to 99.3% for BP added to the flour, and 91.3% for BA added to the flour. Analysis of 10 samples of commercial foods such as flour and wheat products, detected 0.7 µg/g of BP in imported noodles. Furthermore, we successfully verified the existence of BP by LC–MS. These methods are simple and reliable for determination and verifying the amount of BP and BA in foods since now the use of BP as a food additive is permitted in many countries.

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1. Introduction

Freshly milled wheat flour is not suitable for producing high quality wheat products as it contains protein splitting enzyme and pigments such as carotenoids. During flour storage for several weeks, known as aging, reactions take place between flour components and oxygen. Oxidizing agents are often added to flour in order to accelerate its natural maturing, and benzoyl peroxide (BP) is among the most common oxidizing agents used for wheat flour. In Japan, the United States, Canada, China, and Taiwan for example, the use of BP is permitted to shorten the aging period. It is also known that adding BP stabilizes the oxidation of the ingredients, bleaching and sterilizing the flour. On the other hand, BP also has an explosive nature. When dry, it may explode when impacted or heated and may catch fire or explode when it contacts a reducing substance, so that in many countries BP itself is standardized [1,2]. In Japan, to prevent the explode,

diluted BP is listed as food an additive. BP is diluted to contain 19.0–22.0% with one or more of aluminum potassium sulfate, calcium salts of phosphate, calcium sulphate, calcium carbonate, magnesium carbonate and starch [3]. The Japanese regulations permit the use of diluted BP in flour below 0.30 g/kg.

Normally, about 3 g of BP are added to 20 kg of wheat flour. Added BP is decomposed easily to benzoic acid (BA) and other substances, like biphenyl and phenylbenzoate [4]. With the current method for analysis of food additives in Japan, BP is converted to benzoic acid using potassium iodide and this is measured by gas chromatography [5]. In December 1999, the Chigasaki Health Center in Kanagawa prefecture detected BA in instant noodles imported from Hong Kong [6]. In this case, a relatively complex test was employed in order to determine whether the detected BA had been added or was derived from BP.

There are several approaches for analyzing BP in flour or wheat products. With the AOAC method, BP is degraded to BA in the presence of powdered Fe and hydrochloric acid, and determined by colorimetry [7]. Also in many other reports BP was detected after decomposition to BA, using

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ion chromatography [8] or GC [9]. With these methods, however, it is impossible to clarify whether the BA was generated during the test or by the natural decomposition of added BP while the food was processed or preserved. Using HPLC chromatography [10,11], BP and BA levels can be determined separately under different isocratic conditions. This approach was applied for flour and wheat products, and in addition a confirmation test with LC–MS was performed.

2. Experimental

2.1. Materials

Domestic flour, instant noodles and Japanese noodles were purchased from local markets in Tokyo or Kanagawa.

2.2. Chemicals

In Japan, diluted BP is permitted as food additives, but is not now produced due to self-restraint of the flour industry. Therefore, from the viewpoint of the safety, hydrous BP was used for the experiments. Diluted BP of USP grade was obtained from Aldrich, and hydrous BP from Sigma–Aldrich Japan (SAJ 1st grade). The content of BP in the hydrous BP was determined by the method of JECFA [1] and FCC [2]. About 250 mg of sample, accurately weighed was dissolved in 15 ml of acetone in a 100-ml glass-stoppered bottle. Add 3 ml of 50% potassium iodide solution and swirl for 1 min. Titrate immediately with 0.1 mol/l sodium thiosulfate (without addition of starch as an indicator). Each ml of 0.1 mol/l sodium thiosulfate is equivalent to 12.112 mg of BP. Perform a blank test in the same manner and make any necessary correction. All the other chemicals were of the highest grade available. Millipore filters used were Millipore Millex R-LH SLLH HI3 NL (0.5 μm).

2.3. Instruments

2.3.1. HPLC apparatus and conditions

HPLC analyses were performed on a LC-10 series (Shimadzu, Kyoto, Japan) liquid chromatograph system equipped with two LC-10ADvp pump and an SPD-10AVvp spectrophotometric detector, and an SPD-M10Avp photodiode array detector, and a SIL-10ADvp autosampler. Data processing was carried out with a Class-VP LC workstation. The HPLC system consisted of an Inertsil ODS-80A column (250 mm \times 4.6 mm i.d.) (GL Science, Tokyo, Japan) and an Inertsil ODS-3 guard column (10 mm \times 4 mm i.d.) of the same material. The detection wavelength was set at 235 nm and the Column oven at 40 °C. For isocratic separation, the mobile phase was a mixture of acetonitrile–water (55:45) and the flow rate was 1 ml/min. The conditions for the gradient were as follows: mobile phase A consisted of water–glacial acetic acid (1000:1) and mobile phase B of

acetonitrile–glacial acetic acid (1000:1). After 10 min, 18% B was increased to 60% and held there for another 13 min with a flow rate of 1.2 ml/min.

2.3.2. LC–MS

LC–MS measurements were carried out with a Waters 2690 separation module, coupled to a Waters ZQ2000 LC–MS with an electrospray ionization source (Waters, Milford, MA, USA).

For the LC-ESI⁺-MS a split system 1/4 was used to introduce the effluent into the ES. The capillary voltage was held at +3.2 kV and the cone voltage was set to 10 V. The source temperature was 120 °C and desolvation temperature was 450 °C. Mass spectra were acquired by scanning from m/z 50 to 300 and data were processed using Mass Lynx software. The separation was performed in an TSK-Gel ODS-80Ts column (150 mm \times 4.6 mm i.d.) (Toso, Tokyo, Japan) under chromatographic conditions of: flow-rate of 1.0 ml/min, sample injection volume of 20 μl and 75% methanol as the mobile phases.

2.3.3. Procedure

An approximately 10.0 g of flour sample was weighted accurately and 50 ml of acetonitrile was added and the mixture shaken with a magnetic stirrer for 15 min. The sample solution was filtrated through a 0.5 μm filter and injected into the HPLC system.

For LC–MS analysis, approximately 10.0 g of flour sample was weighted accurately and 10 ml of acetonitrile was added.

A standard stock solution was prepared by dissolving an accurately weighted quantity of hydrous BP, equivalent to about 100 mg of BP, in 100 ml acetonitrile. The concentration of the standard solution was determined by the compendial iodometric procedure described in Section 2.2.

3. Results and discussion

3.1. Measurement of the content of BP in hydrous BP

Following the assay by titration adopted in JECFA [1] and FCC [2], we measured the content of BP in hydrous BP to use as a standard. The reagent from Aldrich (USP grade) contained $74.8 \pm 0.4\%$ ($n = 5$) of BP and that from Sigma–Aldrich Japan (SAJ 1st grade) $72.5 \pm 0.7\%$ ($n = 5$). The USP hydrous BP is regulated to contain not less than 65.0% and not more than 82.0%.

For this report, acetonitrile was used as a solvent to prepare the BP solutions to match the mobile phase for HPLC. Therefore, to confirm the lack of influence of the solvent in the titration, we used acetonitrile instead of acetone and the content obtained was $74.3 \pm 0.3\%$ ($n = 5$) almost the same as that with acetone under identical conditions. We decided to use acetonitrile for preparation of the standard solution.

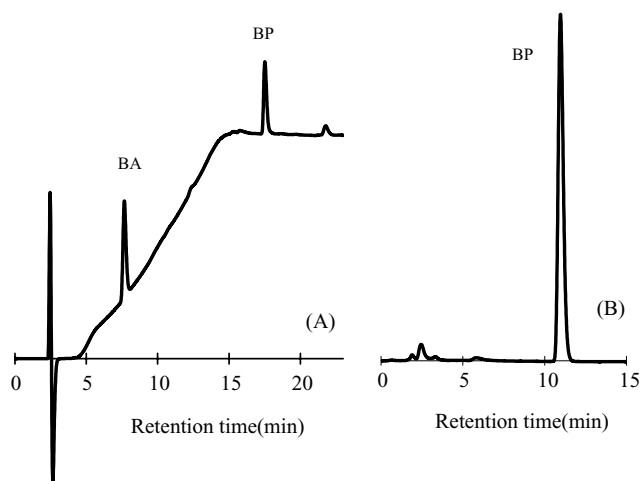


Fig. 1. HPLC chromatograms for the elution of standard preparations of benzoic acid (BA) and benzoyl peroxide (BP) in the gradient condition (A) and in the isocratic condition (B).

3.2. Examination of HPLC conditions

Although various conditions are used for reversed-phase HPLC for BP, we have analyzed BP and BA simultaneously using gradient analysis, referring to the USP method [10]. Fig. 1 shows a chromatogram of BP and BA. The retention time of BP was 17.5 min and that of BA was 7.6 min. Since the maximum absorption of BP was at 195 and 235 nm, a wavelength of 235 nm was used for measurement taking into account interference by food ingredients. The calibration curve based on the peak area showed an excellent linearity in the range of 0.07–15 $\mu\text{g/g}$ as BP and in the range of 0.25 to 15 $\mu\text{g/g}$ as BA.

When measuring BP in a lower concentration, we performed analyses under isocratic conditions with 55% acetonitrile (Fig. 1). The calibration curve showed excellent linearity in the range of 0.02–15 $\mu\text{g/g}$. However, under these conditions, BA could not be detected simultaneously since it was retained only for 2.9 min and was contained in the solvent peak.

3.3. Recovery test

We conducted a recovery test on one product each for flour, instant noodles, and Japanese wheat noodles confirmed with the method not to produce BP and BA.

When 7, 30, or 60 $\mu\text{g/g}$ of BP were added to flour, under conditions of gradient elution, the recoveries were in the range of 96.0–99.3%. With BA, 5 or 10 $\mu\text{g/g}$ was added, and recovery was about 91.3%, as shown in Table 1. On isocratic analysis, the recovery of BP was as good as 99.4–99.6%.

Next, when 0.7 or 7 $\mu\text{g/g}$ of BP was added to instant noodles and udon noodles, the recoveries were 97.1–99.4%, and the standard deviation ranged from 0.4 to 3.2% (Table 2). Fig. 2 shows an HPLC chromatogram with udon noodle no.

Table 1
Recoveries of benzoyl peroxide and benzoic acid from flour

	Added amount ($\mu\text{g/g}$)	Recovery (%) ^a	
		Gradient	Isocratic
Benzoyl peroxide	7	98.1 \pm 2.6	99.4 \pm 1.2
	30	99.3 \pm 0.5	99.6 \pm 0.6
	60	96.0 \pm 1.9	99.4 \pm 0.5
Benzoic acid	5	91.3 \pm 0.7	–
	10	91.3 \pm 1.7	–

^a Values are averages of five determinations \pm standard deviations.

Table 2
Recoveries of benzoyl peroxide from wheat products

Sample	Added amount ($\mu\text{g/g}$)	Recovery (%) ^a
Instant noodle no. 1	0.7	98.1 \pm 1.8
	7	97.3 \pm 1.5
Noodle no. 1	0.7	97.1 \pm 3.2
	7	99.4 \pm 0.4

^a Values are averages of five determinations \pm standard deviations.

1 used as the sample, either without addition (A) or with 7 $\mu\text{g/g}$ of BP (B).

3.4. Effect of the diluent on the recovery test

Since standard diluted BP was unavailable, in this study hydrous BP was used. To confirm the lack of any influence of the diluent, we conducted a recovery test with gradient analysis using calcium carbonate or starch to dilute BP to 20% (Table 3). The recoveries were 99.5 \pm 0.6% when 30 $\mu\text{g/g}$ of BP with calcium carbonate was added to flour, and 99.4 \pm 0.8% when starch was used ($n = 5$). In both cases good results were obtained, so that we can conclude that diluted BP can be determined by the same procedure as hydrous BP.

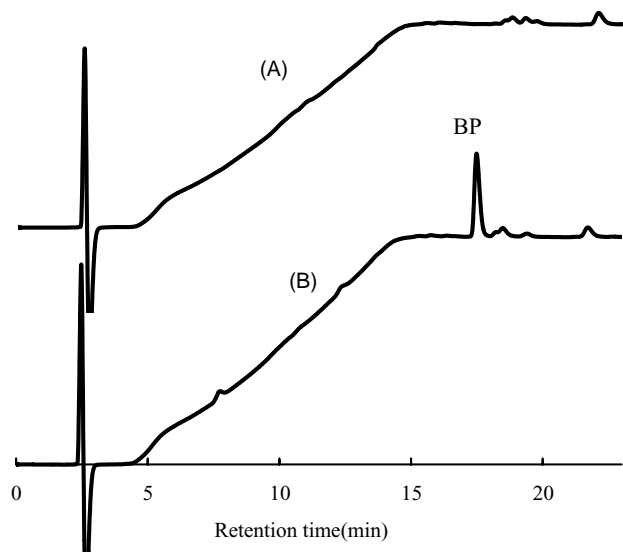


Fig. 2. HPLC for benzoyl peroxide-spiked and unspiked noodle of no. 1. (A) Unspiked sample; (B) spiked sample (7 $\mu\text{g/g}$).

Table 3
Effect of diluents on the recoveries from flour

Diluent	Added amount ($\mu\text{g/g}$)	Recovery (%) ^a
Calcium carbonate	30	99.5 \pm 0.6
Starch	30	99.4 \pm 0.8

^a Values are averages of five determinations \pm standard deviations.

3.5. Detection limit

With HPLC, the detection limits were calculated using the JIS high-performance liquid chromatographic method [12] on the basis of S/N ratios. The detection limits under gradient conditions were 30 ng/ml for BP and 20 ng/ml for BA (S/N = 3, $n = 5$). With isocratic analysis the detection limit for BP was estimated to be 2 ng/ml (S/N = 3, $n = 5$), the approach being 10 times more sensitive than gradient analysis.

3.6. Change of amount of BP and BA in the flour bleaching process

It is well known that BP is a free radical initiator, causing carotenoid oxidation by a typical free radical mechanism. And in those reactions, benzoic acid is identified as one of the main final products. We added 30 $\mu\text{g/g}$ of BP to flour, an amount in the range normally added to flour, in order to follow change in BP and BA at various time points. As shown in Fig. 3, the content of BP was 29.5 $\mu\text{g/g}$ and the recovery was 99.3% immediately after addition. After 3 h of bleaching, the content of BP was significantly reduced at 4.0 $\mu\text{g/g}$, and after 8 h the value was almost zero. The content of BA was 2.8 $\mu\text{g/g}$ immediately after the B addition,

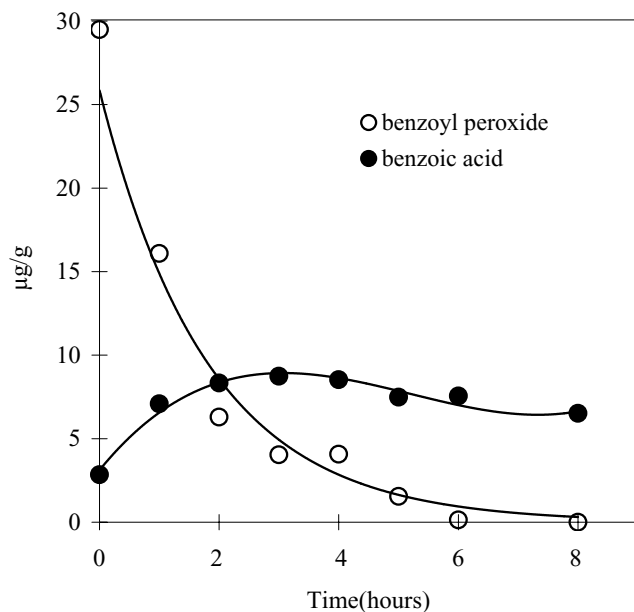


Fig. 3. Amount of benzoic acid and benzoyl peroxide in flour during bleaching.

Table 4
Analytical results for benzoil peroxide in flours and wheat products

Sample	Country	Contents ($\mu\text{g/g}$) ^a	
		Benzoyl peroxide	Benzoic acid
Flour no. 1	Japan	N.D.	N.D. ^b
Flour no. 2	Japan	N.D.	N.D.
Flour no. 3	China	N.D.	N.D.
Instant noodle no. 1	Hong Kong	N.D.	N.D.
Instant noodle no. 2	Hong Kong	0.7 \pm 0.1	N.D.
Instant noodle no. 3	Hong Kong	N.D.	N.D.
Instant noodle no. 4	China	N.D.	N.D.
Noodle no. 1	China	N.D.	N.D.
Noodle no. 2	China	N.D.	N.D.
Chinese noodles	Thailand	N.D.	N.D.

^a Values are averages of three determinations \pm standard deviations.

^b Not detected (BA < 0.2 $\mu\text{g/g}$; BP < 0.3 $\mu\text{g/g}$).

and after 3 h of bleaching, had increased to a maximum of 8.7 $\mu\text{g/g}$. Then the content gradually decreased to 6.5 $\mu\text{g/g}$ after 8 h. We also measured the content of BA at the time when it would be expected to peak, 3 h after addition of the maximum acceptable quantity in Japan (60 $\mu\text{g/g}$ as BP), and detected 7.1 \pm 0.3 $\mu\text{g/g}$ of BA ($n = 3$).

In December 1999, the Chigasaki Health Center in Kanagawa Prefecture in Japan detected 60 to 100 $\mu\text{g/g}$ of BA in instant noodles imported from Hong Kong, and these was concern about possible violation of the article 7 of the food sanitation law. The conclusion was that BA had been produced by decomposition of BP since the latter was present [6]. In the present study, as mentioned above, we detected only 7.1 $\mu\text{g/g}$ of BA even when the maximum quantity of BP was added to flour, implying that BA in the Hong Kong instant noodles could not all have been derived from BP. Saiz et al. reported that BP added to flour was not

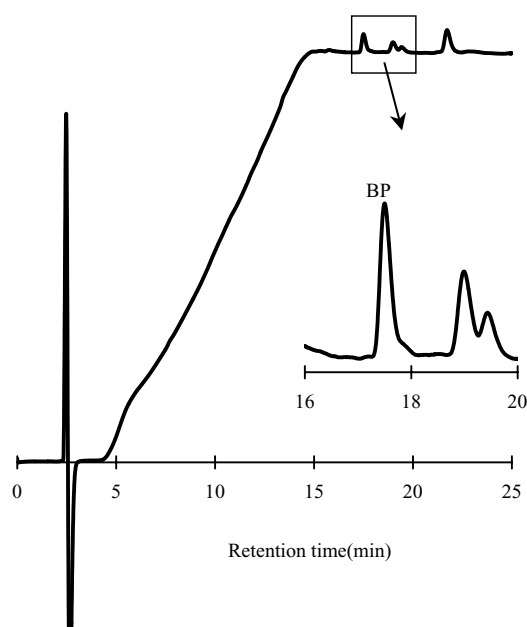


Fig. 4. HPLC of instant noodle of no. 2.

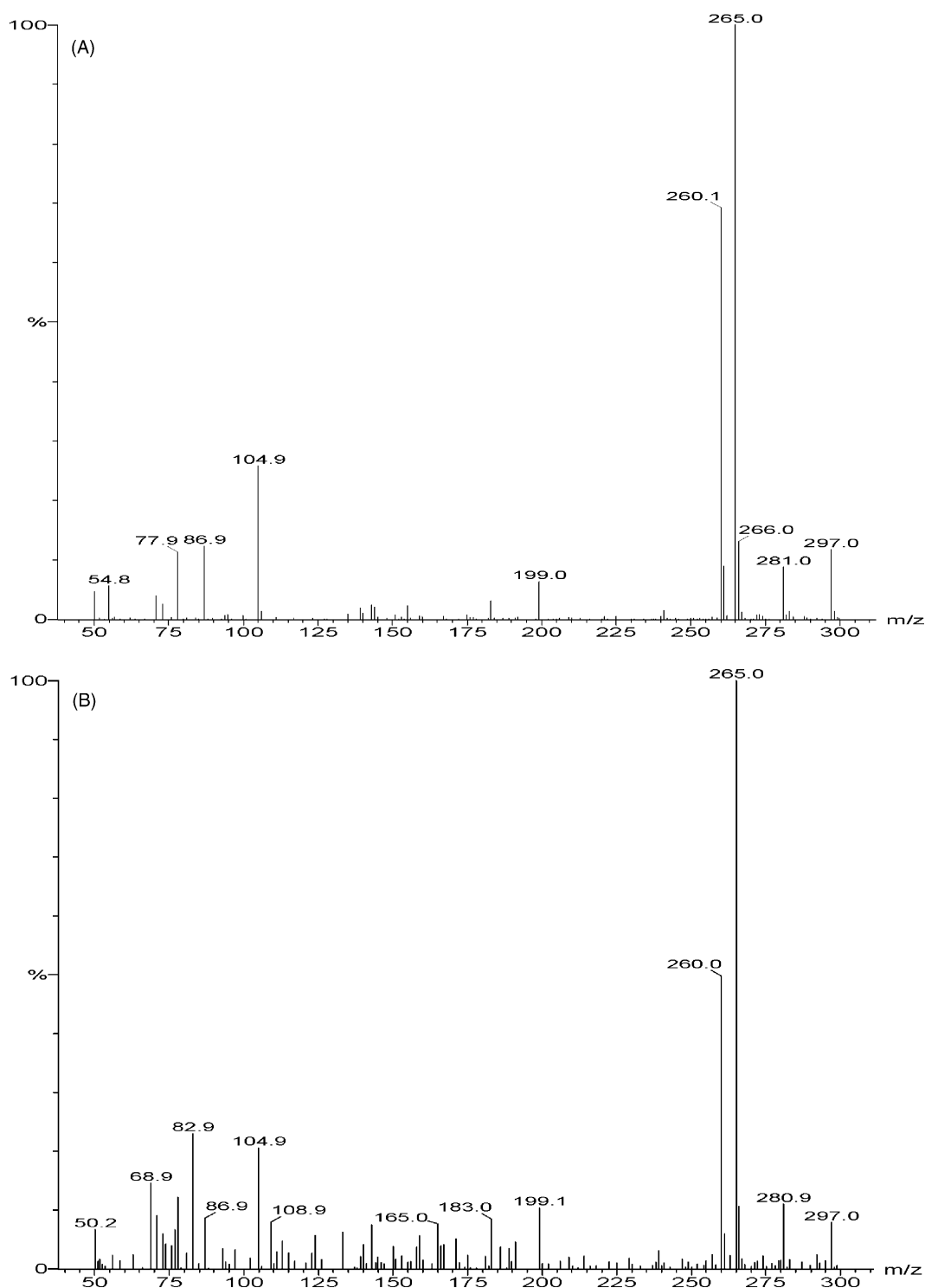


Fig. 5. LC-MS spectrum of a standard solution of benzoyl peroxide (A) and the sample solution of instant noodle no. 2 (B).

decomposed completely and a trace of BP remained [10], although they added more than twice the amount of BP permitted in Japan. In the Chigasaki case we suspect that not only BP but also a fairly high level of BA was introduced during the preparation.

Regarding of decomposition products of BP, and BA retention time. GDDIPATI et al. [4] quantified the amount of

BP decomposition products in the sample. But they did not match the decomposition products we detected.

3.7. Determination of BP in food products

We have analyzed 10 commercial products made of flour, including instant noodles, and Japanese noodles as listed in

Table 4, and detected 0.7 $\mu\text{g/g}$ of BP in one instant noodle product (no. 2) made in Hong Kong. This product was similar to the one seized by the Chigasaki Health Center in Kanagawa prefecture. In the other nine products, no peak detected around the BP retention time and BA was not identified in any of the products. The HPLC chromatogram of noodle no. 2 is shown in Fig. 4.

3.8. Confirmation test with LC–MS

There are two reports on BP analysis with LC–MS in the literature. In one electron impact ionization (EI) MS was employed to verify BA m/z 122 and full-deuteride ($\text{C}_6\text{D}_5\text{CO}_2\text{D}$) m/z 128 produced by the thermal decomposition of BP [13]. In the other, supercritical fluid chromatography (SFC)–MS was used to verify ammonia-added ion m/z 260 [14]. However, we could find no MS analysis report of food extracts. Here, we conducted a confirmation test with LC–MS on the instant noodle no. 2 product in which BP had been detected. Although a mixture of methanol and 1% formic acid was also used for measurement to accelerate ionization, a higher sensitivity was achieved with the mixture of methanol–water (75:25). Ionization was performed in the positive mode with electrospray (ESI) and ions were detected in the scan mode. We used flow injection equipment to automatically optimize capillary voltage, cone voltage, source temperature, and deliquoring temperature to obtain the conditions under which the maximum sensitivity could be attained.

The mass spectrum of both standard and sample solutions showed an m/z 265 sodium-loaded ion and an m/z 105 fragment ion (Fig. 5) with OH removed from the BA produced by the decomposition of BP, verifying the existence of BP.

4. Conclusion

We established a simple and reliable HPLC method for quantifying BP and BA separately in flour and wheat prod-

ucts. The recoveries with this method were quite excellent. And analysis of 10 commercial flour and wheat products in Japan allowed detection of 0.7 $\mu\text{g/g}$ of BP, whose existence could be verified by LC–MS. Although the use of BP has not been reported recently in Japan, this method should be useful for analyzing imported foods since the employment of BP as a food additive is permitted in foreign countries.

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