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Determination of organic peroxides by liquid chromatography with on-line post-column ultraviolet irradiation and peroxyoxalate chemiluminescence detection

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Abstract

A HPLC method was developed for the simultaneous determination of organic peroxides and hydrogen peroxide with peroxyoxalate chemiluminescence (PO-CL) detection following on-line UV irradiation. Organic peroxides [i.e., benzoyl peroxide (BP), *tert*.-butyl hydroperoxide (BHP), *tert*.-butyl perbenzoate (BPB), cumene hydroperoxide (CHP)] were UV irradiated (254 nm, 15 W) to generate hydrogen peroxide, which was determined by PO-CL detection. The conditions for UV irradiation and PO-CL detection were optimized by a flow injection analysis (FIA) system. Generation of hydrogen peroxide from peroxides with on-line UV irradiation also was confirmed by the FIA system by incorporating an enzyme column reactor immobilized with catalase. The separation of four organic peroxides and hydrogen peroxide by HPLC was accomplished isocratically on an ODS column within 30 min. The detection limits (signal-to-noise ratio=3) were 1.1 μ*M* for hydrogen peroxide, 6.8 μ*M* for BP, 31.3 μ*M* for BHP, 7.5 μ*M* for BPB and 1.3 μ*M* for CHP. The proposed method was applied to the determination of BP in wheat flour.

Keywords: Peroxyoxalate chemiluminescence detection; Organic peroxides; Peroxides

1. Introduction

Peroxides are widely used in the food industry. For example, hydrogen peroxide is legally allowed as a food additive in Japan because of its bleaching and sanitizing properties and benzoyl peroxide is used as a flour bleacher in many countries. In foodstuffs, increased amounts of lipid hydroperoxides contribute to increased toxicity and loss of taste and flavor [1,2]. Recently, lipid hydroperoxides have become the focus of attention as a causative factor in some diseases, such as heart disease and aging [3,4]. In non-food uses, peroxides are employed as initiators in polymer production and are often found in air: volatile organic peroxides, such as *tert*.-butyl perbenzoate and di-*tert*.-butyl peroxide are, identified in indoor air [5] as well as gaseous hydrogen peroxide in the atmosphere [6,7]. Because peroxides are hazardous and may have adverse biological effects, sensitive and selective methods for the determination of organic peroxides and hydrogen peroxide are required in the fields of food, clinical and environmental chemistries.

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For the simultaneous determination of peroxides, several high-performance liquid chromatographic (HPLC) methods have been reported, which include UV [8,9], electrochemical [10], fluorescence [11,12] and chemiluminescence (CL) detection [13,14]. The UV detection method uses simple apparatus but it is less sensitive. Fluorescence method involves the reaction of the peroxide and *p*-hydroxyphenylacetic acid with horseradish peroxidase, which forms the fluorescent *p*-hydroxyphenylacetic acid dimmer. A limitation of the method is that it is analyte specific because of the selectivity of the enzyme [11]. Although fluorescent labeling with diphenyl-1-pyrenylphosphine is highly sensitive and successfully applied to lipid hydroperoxide in human plasma [12], it is a relatively time-consuming procedure. CL methods using luminol [13] and isoluminol [14] are extremely sensitive, however, they are pH dependent catalytic reactions with cytochrome *c* and peroxidase and thus the choice of mobile phase solvent is limited.

We previously reported a HPLC method with peroxyoxalate chemiluminescence (PO-CL) detection for determining hydrogen peroxide, which was successfully applied to hydrogen peroxide assay in cola drinks [15]. Sufficient CL intensity, however, was only observed for hydrogen peroxide and the sensitivity of the method was not sufficient for the trace analysis of organic peroxides. In the present study, we investigated the HPLC–PO-CL detection system for the determination of organic peroxides based on the fact that organic peroxides can be converted to hydrogen peroxide by on-line UV irradiation [16]. Organic peroxides [i.e., benzoyl peroxide (BP), *tert*.-butyl perbenzoate (BPB), cumene hydroperoxide (CHP), *tert*.-butyl hydroperoxide (BHP)], were the analytes, which generate hydrogen peroxide to be detected by PO-CL detection after UV irradiation, as well as hydrogen peroxide. The conditions for UV irradiation and PO-CL detection were optimized by a flow injection analysis (FIA) system. Generation of hydrogen peroxide from organic peroxide by UV irradiation also was confirmed by the FIA system using an enzyme column reactor in which catalase was immobilized. To evaluate the applicability of the proposed method, a determination of BP spiked into wheat flour was examined.

2. Experimental

2.1. Materials and reagents

CHP and hydrogen peroxide (30%) were obtained from Wako (Osaka, Japan). BP and BPB were from Nacalai Tesque (Tokyo, Japan), and BHP was from Katayama (Tokyo, Japan). Solutions of hydrogen peroxide, CHP, BPB and BHP (0.1 *M*), and solution of BP (0.01 *M*) were prepared in acetonitrile. These solutions were diluted appropriately with the carrier solution (for FIA) or mobile phase (for HPLC) to prepare the working solutions. Bis(2,4,6-trichlorophenyl)oxalate (TCPO) and imidazole were obtained from Tokyo Chemical Industry (Tokyo, Japan); imidazole was recrystallized from acetonitrile before use. Catalase from bovine liver (Boehringer Mannheim-Yamanouchi, Tokyo, Japan) was used to prepare the immobilized enzyme column reactor (IMER). 2,4,6,8-Tetrathiomorpholinopyrimido[5,4-*d*]pyrimidine (TMP) prepared by our previous method [17] was dissolved in acetonitrile, adjusted to 0.1 m*M*, and stored at 4 °C until use. Distilled water was passed through a Pure Line WL21P system (Yamato Scientific, Tokyo, Japan). Other chemicals were of extra pure grade.

2.2. HPLC–PO-CL system

The HPLC system (Fig. 1) consisted of two LC-9A liquid chromatographic pumps (Shimadzu, Kyoto, Japan), a Rheodyne 7125 injector (Cotati, CA, USA) with a 20-μl sample loop, a low-pressure mercury lamp (15 W, 254 nm, Sigemi Standard, Tokyo, Japan), a Chemcosorb 5-ODS-UH column

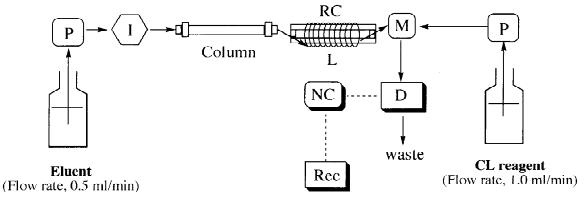

Fig. 1. HPLC system for the determination of peroxides. P, Pump; I, injector; L, low-pressure mercury lamp; RC, reaction coil; M, mixing tee; D, chemiluminescence detector; NC, noise filter; Rec, recorder.

(150×4.6 mm I.D., Chemco, Tokyo, Japan), a CLD-10A chemiluminescence detector (Shimadzu), a UNI-1 noise filter (Union, Gunma, Japan), and an FBR-1 recorder (Tosoh, Tokyo, Japan). PTFE tubing (4.0 m×0.5 mm I.D., GL Sciences, Tokyo, Japan) coiled around the low-pressure mercury lamp was used as the on-line for UV radiation reactor. Imidazole–HNO_3 buffer (20 m*M*, pH 7.5) containing 60% acetonitrile as a mobile phase and a mixture of 0.5 m*M* TCPO and 1.5 μ*M* TMP in acetonitrile (50:50, v/v) were used as the post column CL reagent. The flow-rates of the mobile phase and the CL reagent were set at 0.5 and 1.0 ml/min, respectively.

The tubing coil length for UV irradiation and the conditions for PO-CL detection were optimized with an FIA system in which the separation column was removed from the HPLC system. Imidazole–HNO_3 buffer (20 m*M*, pH 7.5) was used as the carrier solution.

The catalase IMER used to confirm the generation of hydrogen peroxide was prepared as described in our previous report [18]. The IMER was connected between the on-line reactor and the mixing tee in the FIA system.

2.3. Assay procedure for the determination of BP in wheat flour

Wheat flours used were obtained at local markets in Japan. A 1-ml volume of ethanol was added to the wheat flour (0.5 g) or spiked wheat flour with BP standard solution. The suspension was sonicated for 3 min, centrifuged at 650 *g* for 5 min, and the supernatant collected, filtered through a disk filter (polytetrafluoroethylene, 0.45 μm), and a 20-μl aliquot injected into the HPLC system.

3. Results and discussion

3.1. Optimization of UV irradiation

As the peroxides generated hydrogen peroxide while passing through the on-line UV irradiation reactor, the length of the reaction coil was considered to affect the sensitivity. The effect of coil length, ranging from 2.0 to 6.0 m, on CL intensity was examined (Fig. 2). CL intensities for organic peroxides were largest at a coil length of 3.0 or 4.0 m except for hydrogen peroxide whose intensity decreased with an increase in coil length. The result regarding hydrogen peroxide is due to the decomposition of hydrogen peroxide by UV irradiation. For the organic peroxides, the generation of hydrogen peroxide and decomposition of hydrogen peroxide are considered to occur simultaneously. From this experiment, 4.0 m of coil length, corresponding to about 1.6 min of irradiation time, was selected as optimal.

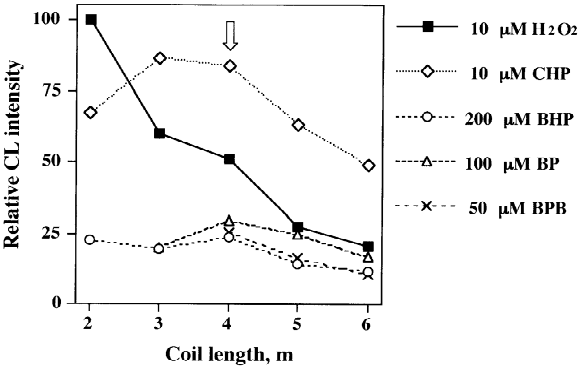

Fig. 2. Effect of coil length on CL intensity. Carrier solution, 20 m*M* imidazole–HNO_3 buffer (pH 7.5); CL reagent, a mixture of 0.5 m*M* TCPO and 1.5 μ*M* TMP in acetonitrile (50:50, v/v).

3.2. Optimization of CL conditions

An imidazole–HNO_3 buffer as a carrier solution and a mixture of TCPO and TMP as post column CL reagent were used for CL detection [18]. To obtain the highest hydrogen peroxide sensitivity, the concentration and pH of the imidazole–HNO_3 buffer were optimized. The concentration of imidazole–HNO_3 buffer (pH 7.5) was examined over the range of 10 to 30 m*M*; the maximum CL intensity was observed at 20 m*M*. The CL intensities of peroxides increased with an increase in pH from 7.25 to 8.00. Adaptation of this buffer as a mobile phase to HPLC separation of the organic peroxides with an ODS column, pH 7.50 was tentatively selected.

We previously reported that a combination of TCPO and TMP as the PO-CL reagents was most

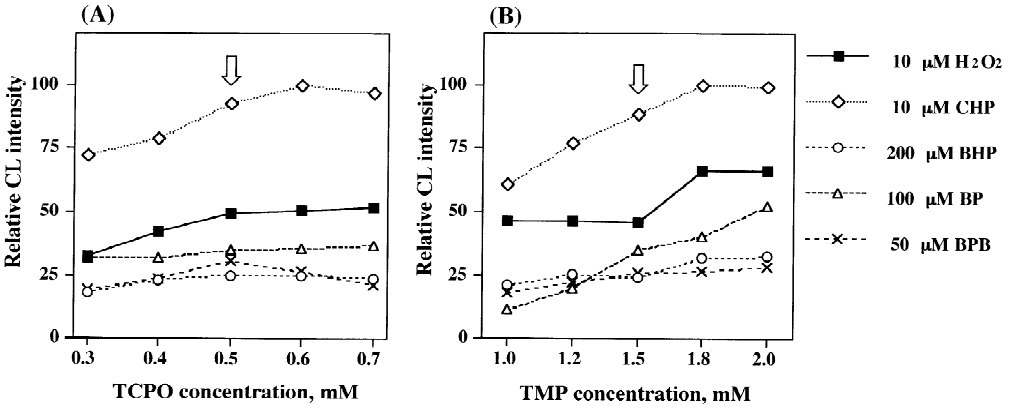

Fig. 3. Effect of concentration of (A) TCPO and (B) TMP on CL intensity. Carrier solution, 20 m*M* imidazole–HNO_3 buffer (pH 7.5); CL reagent, (A) a mixture of TCPO and 1.5 μ*M* TMP in acetonitrile (50:50, v/v), (B) a mixture of 0.5 m*M* TCPO and TMP in acetonitrile (50:50, v/v).

suitable for determining hydrogen peroxide [19]. The effects of the concentrations of CL reagents on CL intensity thus were investigated. As shown in Fig. 3, the CL intensities increased when both reagent concentrations were increased. The background noise level, however, increased simultaneously, and signal-to-noise (*S*/*N*) ratio was not improved: 0.5 m*M* TCPO and 1.5 μ*M* TMP were chosen for further experiments as described in the previous report [19].

Under the above conditions, the effects of flow-rates of the carrier solution and CL reagent on CL intensity were examined. The flow-rate of the carrier solution was examined over the range of 0.3 to 0.7 ml/min at the fixed flow-rate of CL reagent (1.0 ml/min); the maximum peak heights were observed at 0.5 ml/min for each peroxide. The flow-rate of the CL reagent provided the largest peak height at 1.0 ml/min (examined range: 0.8–1.2 ml/min) for each peroxide when the flow-rate of the carrier solution was set at 0.5 ml/min.

For the optimization of CL conditions by the FIA system, imidazole–HNO_3 buffer was used as a carrier solution. The buffer solution, however, is mixed with organic solvent for the use as a mobile phase on HPLC separation of the peroxides. In this study, we selected a combination of imidazole–HNO_3 buffer and acetonitrile, according to the previous report [15]. The effect of acetonitrile content in the carrier solution was thus examined over the range 0–60% (v/v) (Fig. 4). This content influences PO-CL reaction and the decomposition yield of peroxide. A correlation between CL intensity for each peroxide and acetonitrile content could not be observed under the experimental conditions used. The results, however, indicated that 60% of acetonitrile in the carrier solution gave preferable CL and could be applied to HPLC separation of organic peroxides with PO-CL detection after UV irradiation.

We confirmed that the CL signals obtained with the FIA system were based on the hydrogen peroxide generated from organic peroxides after UV irradiation. To confirm this, the catalase IMER was inserted into the FIA system. The peaks of each peroxide

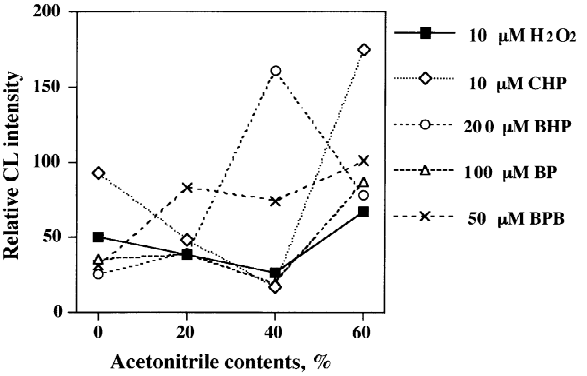

Fig. 4. Effect of acetonitrile contents in the carrier solution on CL intensity. Carrier solution, a mixture of acetonitrile and 20 m*M* imidazole–HNO_3 buffer (pH 7.5); CL reagent, a mixture of 0.5 m*M* TCPO and 1.5 μ*M* TMP in acetonitrile (50:50, v/v).

completely disappeared after passing through the IMER, as well as that of hydrogen peroxide. Fig. 5 shows representative recorder responses for hydrogen peroxide and CHP.

3.3. Separation of peroxides

For the separation of organic peroxides and hydrogen peroxide a HPLC system equipped with an ODS column was used. Using an isocratic mobile phase composed of 20 m*M* imidazole–HNO_3 buffer (pH 7.5) containing 60% acetonitrile, separation was achieved within 30 min. The retention times of peroxides were as follows: 5.0 min for hydrogen peroxide, 7.0 min for BHP, 9.5 min for CHP, 19.0 min for BPB and 29.5 min for BP. A typical chromatogram of standard peroxides is shown in Fig. 6.

3.4. Calibration curves and detection limits

The results obtained with the proposed method are summarized in Table 1. Calibration curves obtained with the standard peroxides showed good linear relationships ($r=0.998$) between concentration and CL intensity. The detection limits for hydrogen peroxide, BP, BHP, BPB and CHP at an S/N ratio of 3 were 1.1 μ*M* (22.0 pmol/injection), 6.8 μ*M* (136.0 pmol/injection), 31.3 μ*M* (626.0 pmol/injection), 7.5 μ*M* (150.0 pmol/injection) and 1.3 μ*M* (26.0 pmol/injection), respectively.

The detection limits were compared with those obtained by the same HPLC system in which the low-pressure mercury lamp was turned off. The results can be summarized as follows. (1) The sensitivity for hydrogen peroxide with UV radiation decreased 40-fold because of decomposition. (2) The sensitivities obtained with UV irradiation were ca. 10^5 and 100-times higher for CHP and BHP, respectively, than those without UV irradiation. (3) Although no CL intensities for BP and BPB were observed even at 0.1 *M* without UV irradiation, these compounds could be sensitively determined with UV irradiation.

The sensitivity for BP indicates that the proposed method is 6–410-times more sensitive than the methods using UV detection [8,9], and as sensitive as

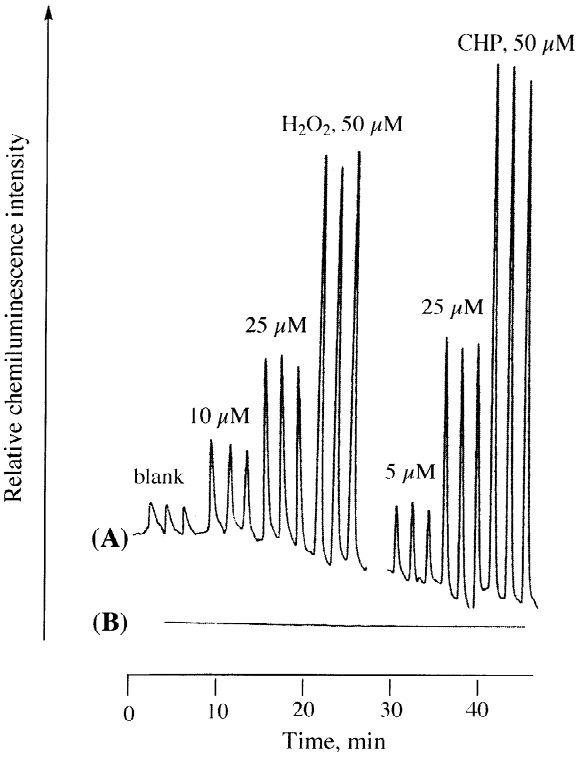

Fig. 5. Recorder responses for (A) hydrogen peroxide and CHP, and (B) those obtained with catalase treatment.

those with fluorescence detection [11,16]. Among the peroxides tested, BHP had a relatively low sensitivity. The reason for this phenomenon could be elucidated that BHP does not have an aromatic ring in its structure, which may limit its rate of hydrogen peroxide generation.

3.5. Determination of BP in wheat flour

For determination of organic peroxides in foodstuffs, to evaluate the applicability of the proposed method, a determination of BP spiked into wheat flour was examined. Ethanol was used for extraction of BP [20]. The effect of sonication time on recovery was studied by adding 2 ml of ethanol to wheat flour spiked with 50 ppm of BP. A constant and maximum recovery of BP was made by sonication for >2 min: the sonication time chosen was 3 min.

A calibration curve of recovered BP from wheat

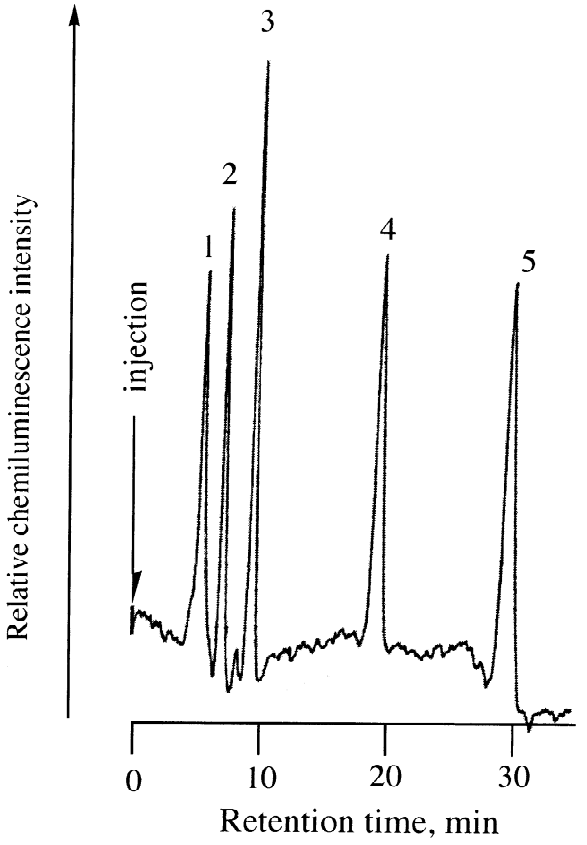

Fig. 6. Chromatogram of a standard mixture of peroxides. Peaks: 1=hydrogen peroxide (15 μ*M*); 2=BHP (400 μ*M*); 3=CHP (20 μ*M*); 4=BPB (100 μ*M*); 5=BP (100 μ*M*).

flour (12.5–75.0 ppm) showed good linearity (r= 0.992). The proposed method could detect as low as 4.1 ppm of BP at an S/N ratio of 3. Within- and between-day precisions of the method were evaluated by using wheat flour samples spiked with BP standard (50 ppm). Table 2 lists the mean±standard deviation (SD) of recovered BP and relative standard deviation (RSD). Within- and between-day precisions were 2.6% (n=5) and 4.1% (n=5), respectively, and recoveries were 93.8±2.4 and 94.0±4.2%, respectively. A typical chromatogram of wheat flour spiked with standard BP is shown in Fig. 7.

The method was applied to the analyses of BP in five kinds of wheat flour made in Japan but it was not be detected in any of the samples tested. BP however is used as a flour bleacher in many countries and this method may be useful in identifying BP in wheat flour imported from other countries.

4. Conclusions

Using on-line post-column UV irradiation, organic peroxides and hydrogen peroxide could be determined simultaneously with HPLC–PO-CL detection, and the five peroxides tested were separated within 30 min. Among them (except for hydrogen peroxide), aromatic peroxides might be more readily determined than alkyl peroxides. The proposed method does not require a time-consuming labeling procedure and has satisfactory reproducibility and sensitivity, which is comparable to sensitive fluorescence methods. The proposed method should be useful for the determination of organic peroxides in the fields of food, clinical and environmental chemistries. The applicability of the method to lipid hydroperoxide analysis is currently being investigated.

Table 1
Calibration curves and detection limits of the HPLC–PO-CL method

Peroxide	Calibration curve[a]					Detection limit[c] (μ*M*)	
	Range (μ*M*)	Slope[b]	Intercept[b]	r	n	UV irradiation	Without UV irradiation
H_2O_2	6–60	0.50±0.02	−0.0077±0.16	0.998	5	1.1	$2.5\cdot10^{-2}$
CHP	8–80	0.54±0.01	0.12±0.34	1.000	5	1.3	$1.0\cdot10^{5}$
BHP	160–1600	0.018±0.0002	−0.23±0.18	1.000	5	31.3	$3.1\cdot10^{3}$
BP	40–400	0.073±0.003	0.78±0.29	0.998	5	6.8	n.d.[d]
BPB	40–400	0.070±0.003	0.66±0.25	0.998	5	7.5	n.d.[d]

[a] CL intensity (arbitrary unit) versus concentration (μ*M*).
[b] Data presented as mean±SD of three experiments.
[c] Detection limit at a S/N ratio of 3.
[d] n.d.=Not detected at 0.1 *M*.

Table 2
Precision and recovery of the HPLC–PO-CL method for BP in wheat flour

Spiked concentration (ppm)	Within-day (n=5)			Between-day (n=5)		
	Found, ppm (mean±SD)	RSD (%)	Recovery, % (mean±SD)	Found, ppm (Mean±SD)	RSD, %	Recovery, % (Mean±SD)
50	46.9±1.2	2.6	93.8±2.4	47.0±2.1	4.1	94.0±4.2

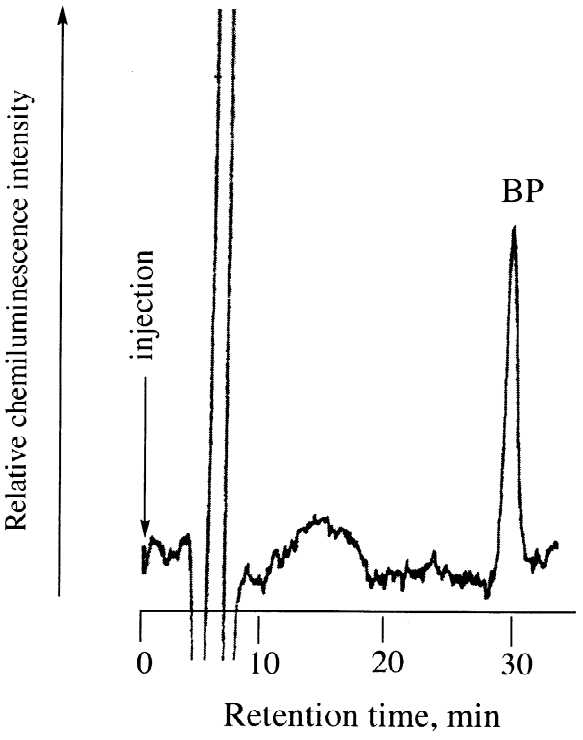

Fig. 7. Chromatogram of wheat flour spiked with standard BP (50 ppm).

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