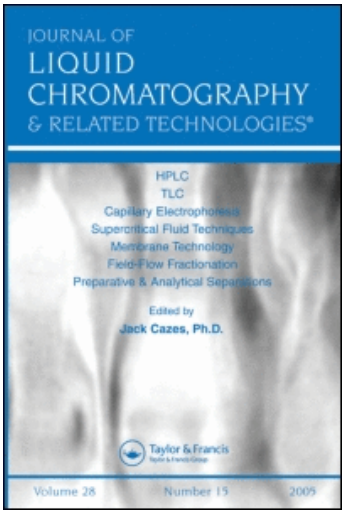

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF HYDROGEN PEROXIDE WITH PEROXYOXALATE CHEMILUMINESCENCE DETECTION

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ABSTRACT

A high performance liquid chromatographic method with peroxyoxalate chemiluminescence (PO-CL) detection was developed for the determination of hydrogen peroxide. Hydrogen peroxide separated by a reversed phase column was detected with the CL produced by the PO-CL reaction using a mixture of 2,4,6,8-tetrathiomorpholinopyrimido[5,4-d]pyrimidine and bis[2-(3,6,9-trioxadecyloxycarbonyl)-4-nitrophenyl]oxalate or bis(2,4,6-trichlorophenyl)oxalate in acetonitrile as a post column reagent. Hydrogen peroxide could be determined over the range of 5-100 pmol on column and the detection limit was 188 fmol at a signal-to-noise ratio of 3. Relative standard deviation of 10-replicate measurements of peak heights for 50 pmol was 2.36%. The detection system applied to the assay of hydrogen peroxide in cola drinks. Trace amounts of hydrogen peroxide could be determined in six cola drinks. The levels were 68.4±2.6-174.2±8.4 nmol/dl. Hydrogen

peroxide contaminated in commercially available hydroperoxides was also determined by the proposed method. The amounts obtained were 0.061% for t-butyl hydroperoxide and 0.003% for cumene hydroperoxide.

INTRODUCTION

It is well known that hydrogen peroxide can be enzymatically generated by the reaction of various oxidases. Therefore, sensitive and selective methods for the determination of hydrogen peroxide have been requisite for the field of biological or biomedical chemistry. For this purpose, a peroxyoxalate chemiluminescence (PO-CL) detection system is very suitable because of its highly selectivity and sensitivity to hydrogen peroxide [1-5]. Recently, we have developed new fluorescent components for PO-CL, i.e., pyrimido-[5,4-d]pyrimidine derivatives, and utilized for the sensitive determination of hydrogen peroxide and glucose by a photographic technique [3]. By this PO-CL system, glucose and uric acid in serum also have been sensitively determined using a flow injection method [4]. On the other hand, to our knowledge, a high performance liquid chromatographic (HPLC) method with CL detection for the assay of hydrogen peroxide has not been developed. In this paper, thus, we tried to develop a HPLC method with PO-CL detection to analyze

hydrogen peroxide using a mixture of 2,4,6,8-tetrathiomorpholinopyrimido[5,4-d]pyrimidine (TMP) and bis-(2,4,6-trichlorophenyl)oxalate (TCPO) or bis[2-(3,6,9-trioxadecyloxycarbonyl)-4-nitrophenyl]oxalate (TDPO) in acetonitrile as a post column CL reagent. Then, we applied the method to the assay of hydrogen peroxide in ascorbic acid, cola drinks and some commercially available peroxides.

MATERIALS

TMP was synthesized as described previously and recrystallized from chloroform-methanol [6]. TDPO, hydrogen peroxide (30%), ascorbic acid, and m-chloroperbenzoic acid were obtained from Wako Pure Chemical Co. (Osaka, Japan). TCPO and imidazole were purchased from Tokyo Kasei Kogyo Ltd. (Tokyo, Japan). t-Butyl hydroperoxide (Katayama Chemical Co., Tokyo, Japan), cumene hydroperoxide (Nacalai tesque, Tokyo, Japan), and catalase (6500 U/ml, Bheringer Manheim Yamanouchi, Tokyo, Japan) were used as obtained. All other reagents used were analytical grade. Acetonitrile used HPLC grade (Wako). Water was deionized and further purified by a WL 21P purifying system (Yamato, Tokyo, Japan).

Buffer solutions were prepared as follows: (1) 10 (or 100) mM imidazole buffer (pH 7.0); imidazole (0.68

g for 10 mM, 6.80 g for 100 mM) was dissolved in 800 ml H_2O, adjusted the pH at 7.0 with nitric acid, and diluted to 1000 ml with water, (2) 0.5 M phosphate buffer (pH 7.0); 0.5 M KH_2PO_4 was mixed with 0.5 M Na_2HPO_4 to adjust the pH at 7.0.

METHODS

HPLC Apparatus

The flow system used is shown in Fig. 1. The HPLC system consisted of two LC 6A HPLC pumps (Shimadzu, Kyoto, Japan), a 7125 injector (Rheodyne, Cotati, CA, USA) with 20 µl sample loop, a separation column [STR ODS-II (150 x 4.6 mm, I.D., 5 µm, Shimadzu), CHEMCOSORB 5-ODS-UH (150 x 4.6 mm, I.D., 5 µm, Chemco, Tokyo, Japan) and TSK ODS-80TM (250 x 4.6 mm, I.D., 5 µm, Tosoh, Tokyo, Japan), a detector [825-CL and UVI-DEC-100-1V, Jasco, Tokyo, Japan], a SC-77 signal cleaner (Sic, Tokyo, Japan), and a FBR-1 recorder (Tosoh). For the measurement of three dimensional chromatograms, a Shimadzu LC-9A HPLC system equipped with SPD-M6A photodiode array UV-VIS detector was used.

HPLC conditions

The PO-CL reaction conditions for the determination of hydrogen peroxide were examined by a STR ODS-II

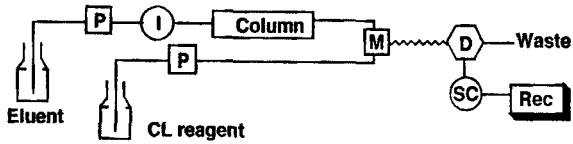

FIGURE 1. Flow system for HPLC-CL detection.

P: pump; I: injector; M: mixing tee; D: detector; SC: signal cleaner; Rec: recorder.

reversed phase column with an eluent of 10 mM imidazole buffer(pH 7.0)-acetonitrile (90/10, v/v) at a flow rate of 1 ml/min. A mixture of TMP and TDPO in acetonitrile was used as a post column reagent at a flow rate of 1 ml/min. Hydrogen peroxide in cola drinks and ascorbic acid was separated with TSKgel ODS-80TM with an eluent of 10 mM imidazole buffer (pH 7.0)-acetonitrile (60/40, v/v) at a flow rate of 1 ml/min. A post column reagent used was a mixture of TMP and TDPO in acetonitrile at a flow rate of 1 ml/min. For the separation of hydrogen peroxide from other peroxides, CHEMCOSORB 5-ODS-UH was used with an eluent of 10 mM imidazole buffer (pH 7.0)-methanol (30/70, v/v) at a flow rate of 0.8 ml/min. A mixture of TMP and TCPO in acetonitrile was used as a post column reagent at a flow rate of 1 ml/min.

Measurement of Time Course for the Consumption of Hydrogen Peroxide with Catalase

To a sample solution (700 µl) were added 300 µl 0.5 M phosphate buffer (pH 7.0) and 10 µl catalase

(6500 U/ml), and incubated at 37 °C for 90 min. At every 30 min, a portion (200 µl) of the mixture was pipetted, passed through a membrane filter (0.45 µm), and the filtrate was injected onto HPLC.

Measurement of the Stability of the Post Column Reagent

TCPO-TMP: a mixture of TCPO (0.3 mM) and TMP ($5x10^{-6}$ M) in acetonitrile was kept in an amber bottle at room temperature. A 20 µl portions of the mixture was injected onto HPLC at a definite interval. TCPO, TMP and a decomposed product of TCPO were separated on STR-ODS-II column using a mobile phase of acetonitrile-water (95/15, v/v) at a flow rate of 1.0 ml/ml, and monitored at 280 nm.

TDPO-TMP: a mixture of TDPO (0.3 mM) and TMP ($5x10^{-6}$ M) in acetonitrile was kept in an amber bottle at room temperature. A 20 µl portions of the mixture was injected onto HPLC at a definite interval. TDPO, TMP and a decomposed product of TDPO were separated on STR-ODS-II column with a mobile phase of acetonitrile-water (99/1, v/v) at a flow rate of 1.0 ml/min, and monitored at 310 nm.

Availability of the Post Column Reagent

Twenty µl of H_2O_2 (containing 50 or 100 pmol) was injected on to HPLC at a definite interval, separated

on STR-ODS-II column with a mobile phase of 100 mM imidazole buffer (pH 7.0)-acetonitrile (60/40, v/v) at a flow rate of 1 ml/min, reacted with a post column reagent of TCPO (0.3 mM) and TMP ($5x10^{-7}$ M) in acetonitrile or TDPO (0.3 mM) and TMP ($2.5x10^{-7}$ M) in acetonitrile, and the resulted CL was monitored.

RESULTS AND DISCUSSION

Post Column PO-CL Reagent

In the previous study, we used a mixture of TCPO and TMP in acetonitrile as the post column reagent for the flow injection analysis of hydrogen peroxide derived from glucose or uric acid by the enzymatic reaction with immobilized enzyme reactor [3]. In this case, three delivery pumps were used for a carrier solvent, TCPO and TMP solutions. Some research works on PO-CL have revealed that a mixture of hydrogen peroxide and TCPO (or TDPO) could be used as a PO-CL reagent for the determination of fluorophores, and consequently, one of the two delivery pumps for reagents could be reduced [5-7]. On the other hand, as the post column reagent, a mixture of fluorescent compound and aryloxalate in acetonitrile seemed to be applicable for the determination of hydrogen peroxide. Thus we studied first the

use a mixture of fluorescent compound (TMP) and TCPO (or TDPO) in acetonitrile as a post column PO-CL reagent. First, the stability of the reagent mixture was examined. A HPLC method with UV detection was used for the measurement of time course of the relative peak heights for TCPO (or TDPO) and TMP in acetonitrile. The chromatograms obtained were shown in Fig. 2. As shown in Fig. 3, the relative peak heights for TCPO (or TDPO) alone were stable at least for 24 h. TCPO in the mixture with TMP was also stable, but TDPO was less stable compared to TCPO and decomposed gradually. TMP itself in the mixture with TCPO or TDPO was less stable than oxalates coexisted.

The availability of a mixture of TMP and TCPO or TDPO in acetonitrile as the post column reagent was measured as a function of relative CL intensity produced from the reaction with a known concentration of hydrogen peroxide. The time course of CL intensity for hydrogen peroxide (100 pmol/injection) was shown in Fig. 4. The relative CL intensity obtained with TMP-TDPO reagent was ca. 1.5 times larger than that with TMP-TCPO reagent and both CL intensities were almost constant at least for 8h. These results show that a mixture of TMP and TCPO or TDPO in acetonitrile could be used as a post column reagent within 8h under the conditions used.

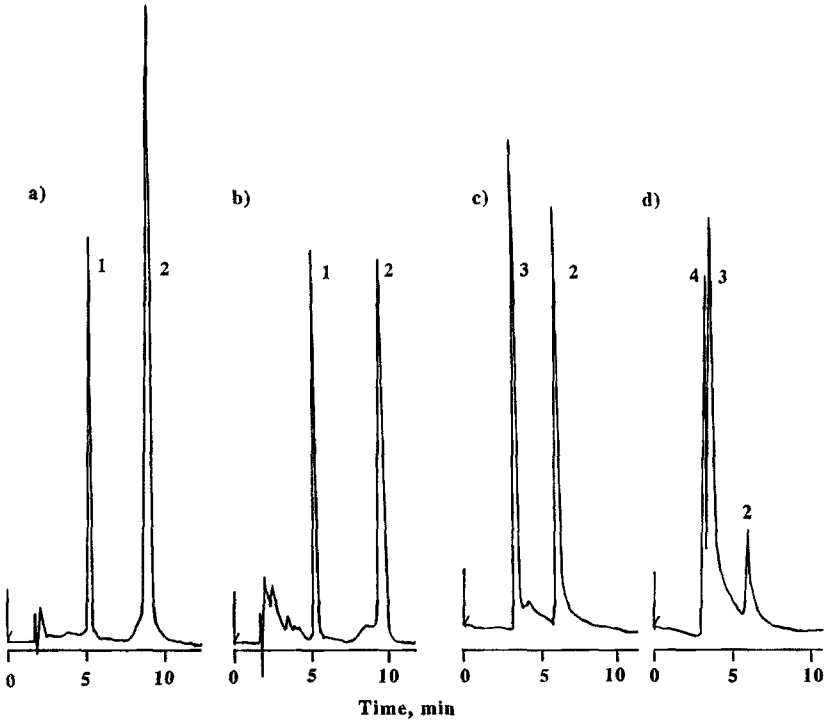

FIGURE 2. Chromatograms for TCPO-TMP and TDPO-TMP solutions in acetonitrile.

a); TCPO-TMP at 0h, b); TCPO-TMP at 24h after; c); TDPO-TMP at 0 h, d); TDPO-TMP at 24 h after; Peaks: 1; TCPO, 2; TMP, 3; TDPO, 4; Decomposed product. Other experimental conditions are described in the text.

Determination of Hydrogen Peroxide

A HPLC-CL determination of hydrogen peroxide was examined using STR-ODS-II and a mixture of TDPO and TMP in acetonitrile as an analytical column and a post column CL reagent, respectively. Hydrogen peroxide was separated at a retention time of ca. 2 min with a

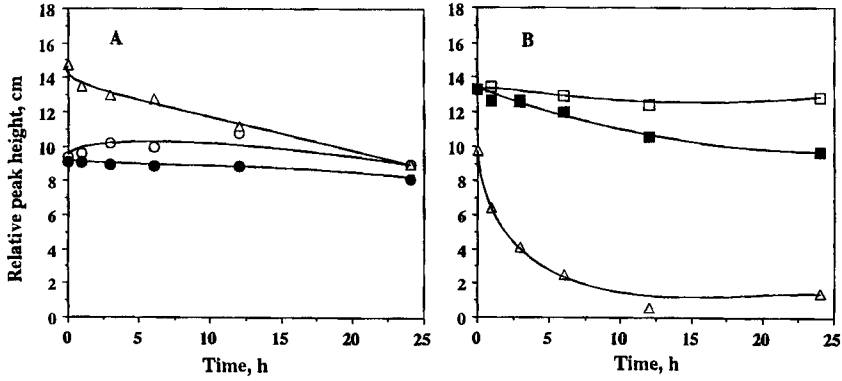

FIGURE 3. Stabilities of the post column CL reagents.

A: (○)TCPO, (●)TCPO in TCPO-TMP, (△) TMP in TCPO-TMP; B: (□)TDPO, (■)TDPO in TDPO-TMP, (△)TMP in TDPO-TMP. Other experimental conditions are described in the text.

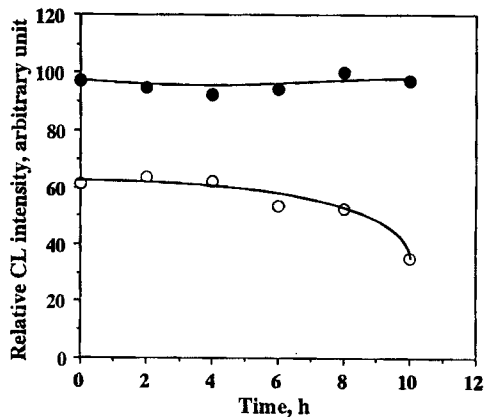

FIGURE 4. Availabilities of the post column CL reagents.

Sample: H_2O_2, 100 pmol/injection); TDPO-TMP (●); TCPO-TMP (○). Other experimental conditions are described in the text.

mobile phase of 10 mM imidazole buffer (pH 7.0)-CH_3CN (90/10, v/v) at a flow rate of 1 ml/min. Optimization of the CL reagent concentration was examined with hydrogen peroxide (50 pmol/injection). Though the relative CL intensity was increased with an increase of the concentration TDPO and TMP, the back-ground level was also increased; a 1:1 mixture of 0.6 mM TDPO in acetonitrile and $5x10^{-7}$ M TMP in acetonitrile gave the largest S/N ratio, which was used as a post column reagent. In this work, we studied the effect of a signal cleaner (SC77, SIC) on chromatograms and found that it increased the S/N ratio more than 5 times. The calibration curve for H_2O_2 was linear over the range from 5 to 100 pmol (r=0.999), and the detection limit was 188 fmol ($9.9x10^{-8}$ M, S/N=3). The relative standard deviation of peak heights for 10-replicate measurements of 50 pmol H_2O_2 was 2.36%. The sensitivity of this method is higher than that of our previous flow-injection analysis (FIA) with CL detection method ($5x10^{-8}$ M)[4] and comparable to that of PO-CL method using immobilized fluorophores ($1x10^{-8}$ M)[1], but slightly lower than those of PO-CL detection with fiber-optic sensor ($2.5x10^{-9}$ M)[2] and FIA-PO-CL method using sulforhodamine 101 as a fluorescent component ($3x10^{-9}$ M, S/N=2)[5].

Determination of Hydrogen Peroxide in Ascorbic Acid and Cola Drinks

Trace amounts of hydrogen peroxide have been found in drinks containing ascorbic acid or caramel. Therefore, a quantitative determination of H_2O_2 contaminated in commercially available ascorbic acid and cola drinks was studied using ODS-80TM and a mixture of TDPO and TMP in acetonitrile as an analytical column and a post column CL reagent, respectively. The eluent used was 10 mM imidazole buffer/CH_3CN(60/40, v/v). Retention times for ascorbic acid and H_2O_2 were confirmed by a three dimensional UV detection. Separation of H_2O_2 spiked in cola drink was also achieved by the same conditions (Fig. 5). The peak corresponded to H_2O_2 in ascorbic acid and cola was disappeared with treatment of catalase (Fig. 6). Figure 7 shows typical chromatograms for CL detection of H_2O_2 in ascorbic acid and cola drink. Contents of hydrogen peroxide determined in two commercially available ascorbic acids were $1.49 \pm 0.01\%$(n=4) and $1.30 \pm 0.01\%$(n=4). Several kinds of cola drinks were assayed and 68.4 - 174.2 nmol/dl of H_2O_2 could be determined. These results are briefly summarized in Table 1.

Determination of Other Peroxides

As peroxides have been known to cause serious damages for living cells, it is biologically important

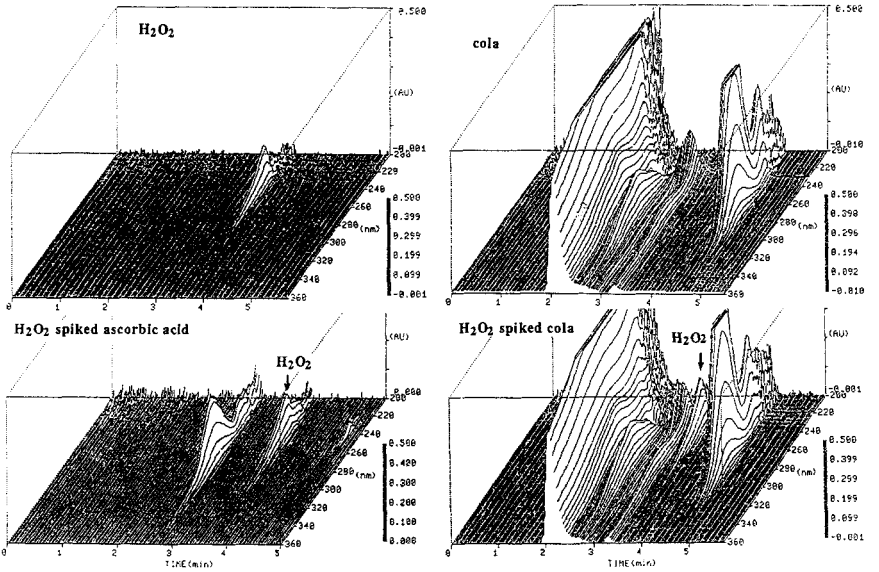

FIGURE 5. Chromatograms with three dimensional UV detection for H_2O_2, H_2O_2 spiked ascorbic acid and H_2O_2 spiked cola.

Sample: H_2O_2; $1x10^{-2}$ M.

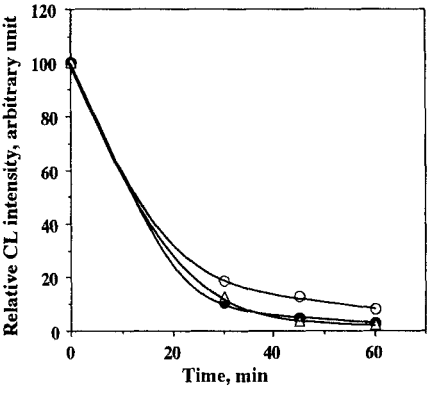

FIGURE 6. Time course for catalase reaction with H_2O_2.

Sample: ascorbic acid; $1x10^{-4}$ M, H_2O_2; $5x10^{-7}$ M, cola; undiluted.

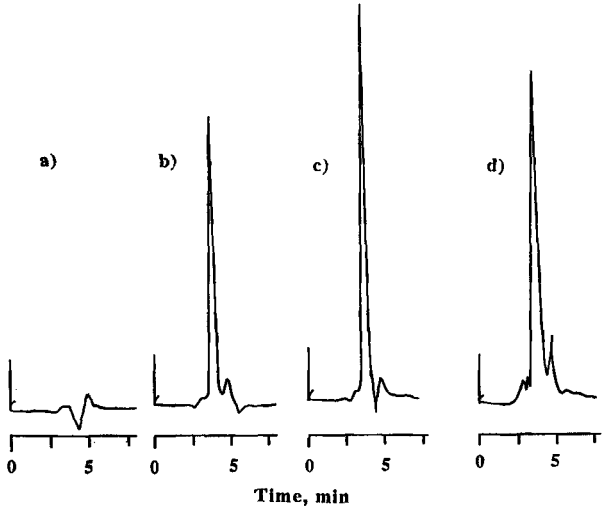

FIGURE 7. Chromatograms for H_2O_2 and H_2O_2 in ascorbic acid and cola drink.

a); blank (H_2O), b); H_2O_2 ($1x10^{-6}$ M), c); ascorbic acid ($2.5x10^{-5}$ M), d); cola (undiluted). Other experimental conditions are described in the text.

TABLE 1. Amounts of Hydrogen Peroxide in Cola Drinks

Sample	nmol/dl	RSD%, (n)
cola A	174.2 ± 8.4	5.54 (10)
cola B	92.5 ± 7.1	7.54 (10)
cola C	114.5 ± 3.8	3.72 (10)
light cola A	85.4 ± 1.9	2.22 (5)
diet cola A	68.4 ± 2.6	3.80 (5)
diet cola B	74.2 ± 2.9	3.84 (5)

to develop sensitive methods to determine a trace amount of them. It is obvious that peroxides can be detected by the PO-CL detection method. Thus we studied the determination of some commercially available peroxides, i.e., t-butyl hydroperoxide, cumene hydroperoxide, and m-chloroperbenzoic acid. For HPLC conditions, a CHEMCOSORB 5-ODS-UH column and a mixture of TCPO and TMP in acetonitrile were used as an analytical column and a CL reagent, respectively. Separation was done with an eluent of 10 mM imidazole buffer (pH7)/MeOH(30/70, v/v). Retention time for H_2O_2, t-butyl hydroperoxide, cumene hydroperoxide and m-chloroperbenzoic acid were 1.9, 3.1, 5.0 and 5.0 min, respectively. In the course of the experiment, we found that H_2O_2 was contaminated in hydroperoxides. Hydrogen peroxide concentrations contaminated in t-butyl hydroperoxide and cumene hydroperoxide were 0.061% and 0.003%, respectively, but could not be detected in m-chloroperbenzoic acid. The relative CL intensities for t-butyl hydroperoxide and cumene hydroperoxide were 0.47 and 0.26 when that of H_2O_2 was taken as 100. This result means that H_2O_2 gave very strong CL compared to other peroxides and, therefore, suitable for oxidation agent in PO-CL system.

In conclusion, HPLC-PO-CL determination method of H_2O_2 was developed for the first time and successfully applied to the assay of H_2O_2 in commercially available

ascorbic acid and cola drinks. Furthermore, the system was utilized for the determination of three commercially available peroxides (t-butyl hydroperoxide, cumene hydroperoxide, m-chloroperbenzoic acid) and H_2O_2 contaminated in them. The proposed H_2O_2 determination method is very sensitive and can detect as low as 188 fmol of H_2O_2. The method might be applied to assay of trace amounts of H_2O_2 generated in biological materials or environmental samples.

REFERENCES

1. G. Gubitz, P. Zoonen, C. Gooijer, N. H. Velthorst, R. W. Frei, Anal. Chem., 57: 2071-2074 (1985).

2. M. S. A.-Latif, G. G. Guilbault, Anal. Chem., 60: 2671-2674 (1988).

3. K. Nakashima, S. Kawaguchi, R. S. Givens, S. Akiyama, Anal. Sci., 6: 833-836 (1990).

4. K. Nakashima, N. Hayashida, S. Kawaguchi, S. Akiyama, Y. Tsukamoto, K. Imai, Anal. Sci., 7: 715-718 (1991).

5. M. Katayama, H. Takeuchi, H. Taniguchi, Anal. Lett., 24: 1005-1015 (1991).

6. K. Nakashima, S. Akiyama, Y. Tsukamoto, K. imai, Dyes Pigm., 12: 21-26 (1990).

7. K. Imai, Y. Matsunaga, Y. Tsukamoto, A. Nishitani, J. Chromatogr., 400: 169-176 (1987).

8. N. Imaizumi, K. Hayakawa, M. Miyazaki, K. Imai, Analyst, 114: 161-164 (1989).

9. N. Hanaoka, J. Chromatogr., 503: 155-165 (1990).

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