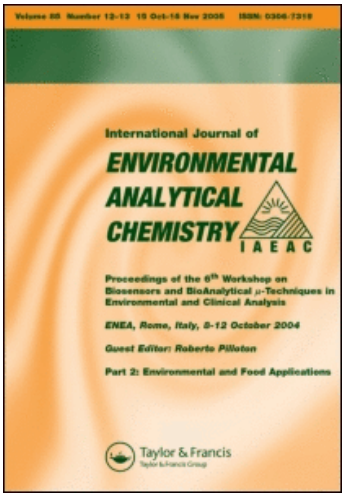


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# SIMULTANEOUS DETERMINATION OF HYDROGEN PEROXIDE AND ORGANIC HYDROPEROXIDES IN WATER

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The kinetics of the reactions of $H_2O_2$ and of methyl, ethyl, *tert*-butyl, and cumene hydroperoxides with $I^-$ were investigated in the presence and absence of molybdate as catalyst. These results were utilized to develop an analytical method for the simultaneous determination of $H_2O_2$ and organic hydroperoxides in aqueous solutions. The total amount of $H_2O_2$ and organic hydroperoxides can be determined by the spectrophotometric measurement of $I_3^-$ formed quantitatively during 30 min of heating at 60°C.

Catalase selectively decomposes $H_2O_2$ in solutions containing organic hydroperoxides. The total amount of the latter can therefore be determined iodometrically after $H_2O_2$ decomposition.

In the oxidation of leuco crystal violet to crystal violet by $H_2O_2$ and organic hydroperoxides, horseradish peroxidase exerts similar activities in the reactions involving methyl and ethyl hydroperoxides and $H_2O_2$, but its activity is much lower with *tert*-butyl and cumene hydroperoxide. It was observed that acetate buffer is unsuitable for pH adjustment in this type of hydroperoxide determination in consequence of the slow oxidation of the dye in the blank solution.

*Keywords:* Hydrogen peroxide determination; Organic hydroperoxide determination; Spectrophotometry; Iodine; Catalase; Horseradish peroxidase

## INTRODUCTION

Hydrogen peroxide ($H_2O_2$) and organic peroxides are generally formed simultaneously in low concentrations in water treatment [1–3] and in free radical-mediated processes in the atmosphere [4]. Their properties lead this type of compound to play important roles in different fields, including laboratory diagnostic tests [5,6].

Appropriate, widely applicable and simple analytical methods for the quantitative determination of $H_2O_2$ and organic hydroperoxides are therefore necessary [7].

The most commonly used methods are based on chemiluminescence [8,9], fluorescence [10,11], electrochemical reactions [12] or the enzymatic oxidation of dyes [13,14].

$H_2O_2$ and organic hydroperoxides are known to display very different reactivities in a reaction such as $I_3^-$ formation from $I^-$. These reactivity differences could possibly allow

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the separate determination of $H_2O_2$ and organic hydroperoxides in the same sample. It seemed interesting to investigate the kinetics of reduction of various organic hydroperoxides and $H_2O_2$ by $I^-$ with a view to the simultaneous determination of these compounds.

The selectivities of catalase and horseradish peroxidase (HRP) in the decompositions of these compounds were also investigated.

## EXPERIMENTAL

### Materials

Cumene hydroperoxide (pract, ~80% in cumene), *tert*-butyl hydroperoxide (purum, ~70% in water), leuco crystal violet (LCV) (Aldrich, Hungary), $H_2O_2$ (Perhydrol, Merc Suprapur, 30% in water), horseradish peroxidase (HRP) (Sigma, EC 1.11.1.7, 220 purpurogallin units/mg solid), catalase (Sigma, C-40, from bovine liver, 14 000 units/mg), sodium iodide, sodium acetate, acetic acid, D-tartaric acid, sodium hydroxide, ammonium molybdate tetrahydrate and phosphate salts, all of analytical grade, were used without further purification. Methyl and ethyl hydroperoxides were synthesized and purified according to the literature [15].

Solutions were made with water purified in a MILLI-Q RG (Millipore) system. Standard (stock) $H_2O_2$ solutions were prepared by dilution of 0.01 M solution, which was standardized with permanganate solution [16].

Spectrophotometric measurements were performed with a Hewlett Packard (type HP8452A) diode array spectrophotometer. The stopped-flow measurements were carried out with a Hi-Tech Scientific Ltd (type SF-61) spectrophotometer.



## THE $I_3^-$ METHOD [16]

Into a 10.00 $cm^3$ volumetric flask, 2 $cm^3$ acetate buffer (pH = 4), 1 $cm^3$ 1 M KI solution (prepared freshly each day), 0.1 $cm^3$ saturated ammonium molybdate solution and the predetermined volume of the stock hydroperoxide solution were measured, and the volume was made up to 10.00 $cm^3$. The absorbance was measured at 352 nm. The apparent molar absorbance of $I_3^-$ was determined with $I_3^-$ generated from standard $KBrO_3$ solution in the presence of 0.1 M KI to be $26200 \pm 107\ M^{-1}\ cm^{-1}$. This value is in good agreement with the value of $26\,400\ M^{-1}\ cm^{-1}$ determined by Klassen *et al.* [16].

## ENZYMATIC METHOD BASED ON HRP AND LCV [13,14]

Into a 10.00 $cm^3$ volumetric flask, 3 $cm^3$ pH = 4 buffer, 0.5 $cm^3$ LCV (1 mM solution in 0.06 M HCl), 20 μL HRP solution and the calculated amount of a hydroperoxide were measured and the volume was made up to 10.00 $cm^3$. For pH adjustment, acetate [13], phosphate [14] or tartrate (0.4 M, based on our own results) were used. The absorbance of the solution was measured at 592 nm. The apparent molar absorbance of the crystal violet (CV) formed from the LCV was determined with standard $H_2O_2$ solution in tartrate buffer to be $75\,600 \pm 298\ M^{-1}\ cm^{-1}$.

### Combined Catalase – $I_3^-$ Method

Into a 10.00 $cm^3$ volumetric flask, 0.2 $cm^3$ pH = 7 buffer (0.05 M phosphate) 150 μL catalase solution (2000 SU/$cm^3$) and a mixture of $H_2O_2$ and organic hydroperoxides were measured. The solution was left to stand until the $H_2O_2$ had been decomposed. After 10 min, 2 $cm^3$ pH = 4 (0.1 M) acetate buffer and 1 $cm^3$ 1 M KI solution were added, and the volume was made up to the mark with water. The solution was next heated at 60°C for 30 min in the dark. The absorbance caused only by the organic hydroperoxides was measured at room temperature at 352 nm. Another aliquot of the sample was treated in the same way, but without the addition of catalase, and the total amount of organic hydroperoxides and $H_2O_2$ was determined.

## RESULTS AND DISCUSSION

### Kinetics of Reactions of Hydroperoxides with $I^-$

The reaction of $H_2O_2$ with slightly acidified KI to produce $I_2$ followed by titration with standard sodium thiosulfate solution is the Kingzett method [17,18].

At a large excess of KI, the equilibrium between $I_2$, $I^-$, and $I_3^-$ strongly favors $I_3^-$. Measurement of the absorbance of $I_3^-$ is the basis of the spectrophotometric method. As a consequence of the relatively large molar absorbance of $I_3^-$, this method is suitable for the determination of peroxides at concentrations above 5 μM.

The reaction between $H_2O_2$ and $I^-$ is slow at room temperature, but is accelerated by the catalyst ammonium molybdate [19,20]. Ovenston and Rees [21] showed that a neutral or slightly acidic solution is needed for accurate measurements and the reaction must be carried out in the absence of oxygen (i.e. under nitrogen or carbon dioxide).

The $I_3^-$ method can be used for the determination of organic hydroperoxides as well as peroxide [22,23], but their lower reactivity necessitates the application of special reaction conditions (temperature, solvent, etc.).

In the first series of experiments, the kinetics of the reactions of $H_2O_2$ and organic hydroperoxides (methyl, ethyl, *tert*-butyl, and cumene hydroperoxides) with $I^-$ were studied at 25°C in solution of pH = 4, in the presence or absence of ammonium molybdate.

The rate equation for the formation of $I_3^-$ is

$$-\frac{d[\mathrm{PO}]}{dt} = \frac{d[I_3^-]}{dt} = k[I^-]^2[\mathrm{PO}] \tag{1}$$

where [PO] and [$I^-$] are the concentrations of peroxide and $I^-$, respectively, at reaction time $t$, and $k$ is the rate coefficient. At a very large excess (~100 times) of $I^-$, $I_3^-$ formation can be described by a pseudo-first-order rate equation. Substitution and integration result in the equation:

$$\ln\frac{[\mathrm{PO}]_o}{[\mathrm{PO}]_t} = k't \tag{2}$$

where $k' = k[I^-]^2$. The kinetic data calculated from the spectrophotometric measurements (without the addition of molybdate) are presented in Fig. 1.

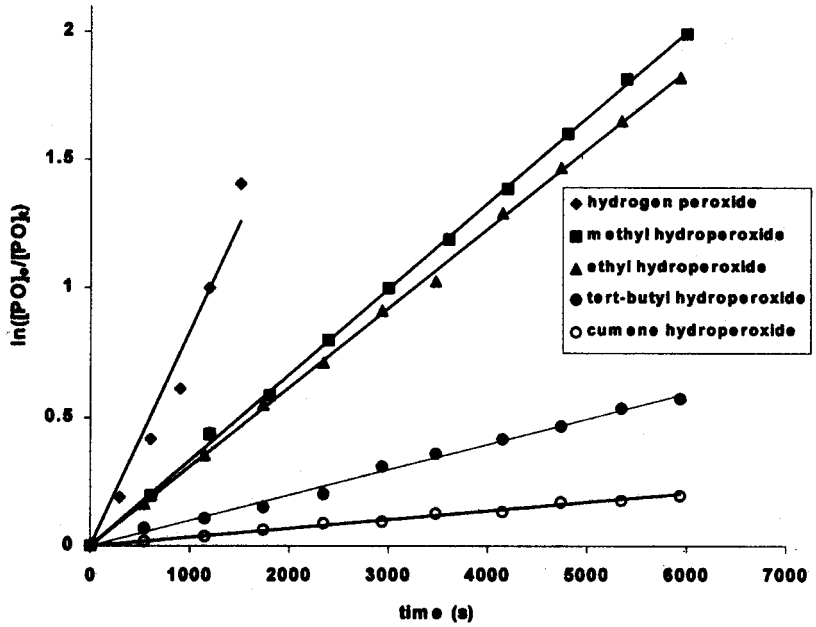


FIGURE 1 First-order kinetic representation of the reactions hydroperoxides with $I^-$ without molybdate catalyst.

TABLE I First-order rate constants of the reactions of the different hydroperoxides with $I^-$

| *Compound* | *Rate coefficients* ($s^{-1}$) | |
|---|---|---|
| | *Without molybdate* | *With molybdate* |
| Hydrogen peroxide | $8.4 \times 10^{-4}$ | $3.3 \times 10^{-1}$ |
| Methyl hydroperoxide | $3.3 \times 10^{-4}$ | $6.7 \times 10^{-4}$ |
| Ethyl hydroperoxide | $3.1 \times 10^{-4}$ | $6.2 \times 10^{-4}$ |
| *tert*-Butyl hydroperoxide | $9.7 \times 10^{-5}$ | $2.1 \times 10^{-4}$ |
| Cumene hydroperoxide | $3.3 \times 10^{-5}$ | $1.4 \times 10^{-4}$ |

It can be seen that the kinetic data can be described by a pseudo-first-order rate equation. The reaction kinetic measurements were also carried out with the addition of molybdate as catalyst. The reduction of $H_2O_2$ was very rapid, and was therefore measured by a stopped-flow spectrophotometric method. This reaction too was of pseudo-first order. The first-order rate constants (taking into consideration the $I^-$ concentration) are given in Table I.

It was found that the $H_2O_2$ reacts much faster than the organic peroxides. The reactivities of methyl and ethyl hydroperoxides proved to be very similar to one another, whereas *tert*-butyl and cumene hydroperoxides reacted more slowly with $I^-$. The reaction of $H_2O_2$ was accelerated almost $10^3$-fold by the catalyst, but those of the organic hydroperoxides were accelerated only moderately (2–3-fold) by the addition of the catalyst.

The determined rate coefficients permit an estimation of the effects of the organic hydroperoxides on the $H_2O_2$ concentration determination under the usual

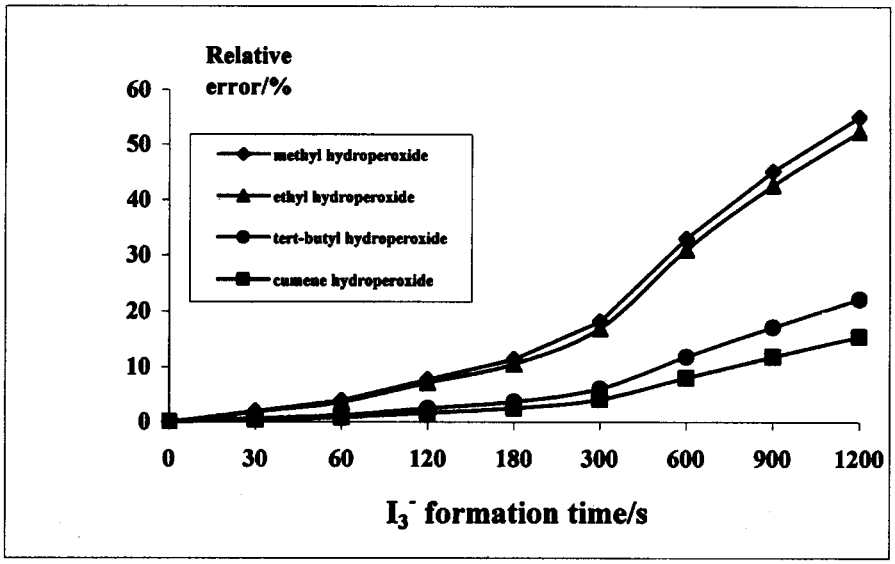


FIGURE 2 Calculated relative errors caused by the presence of organic hydroperoxides on $H_2O_2$ determination as a function of the $I_3^-$ formation time.



spectrophotometric measurement conditions. The results of error calculations are presented in Fig. 2.

It was presumed here that $H_2O_2$ and organic peroxides were present at the same concentration. It can be seen that $H_2O_2$ determination is strongly influenced by the presence of organic hydroperoxides, and it is also evident that the organic hydroperoxides do not react quantitatively with $I^-$ at room temperature. The rates of reduction of peroxides by $I^-$ are known to increase with increase of temperature [22,23]. The reaction must be performed in the dark.

In the next series of experiments, the effects of temperature on the reactions of the hydroperoxides with $I^-$ were investigated. It was established that all of the investigated hydroperoxides react quantitatively with $I^-$ within 30 min at 60°C. During this time, no thermal decomposition of the peroxides or $H_2O_2$ was observed. This means that the total amount of $H_2O_2$ and hydroperoxides can be determined by measurement of the absorbance of the $I_3^-$ formed within a reliable analysis time (30 min) if the reactions are performed at 60°C.

## Selectivity of HRP

$H_2O_2$ decomposition by HRP followed by stoichiometric formation of the highly colored CV by oxidation of LCV, is a frequently-used specific method for the determination of $H_2O_2$ [13,14]. It seemed plausible to apply this method of $H_2O_2$ determination in samples containing other organic hydroperoxides, although the selectivity of this enzymatic method has not been clearly demonstrated [4,13,24,25].

The $H_2O_2$ concentration was first determined using the method described by Mottola *et al.* [13]. It was important to recognize that the different protonated forms of CV differ considerably in absorbance and it is therefore crucial to fix the pH of the solution

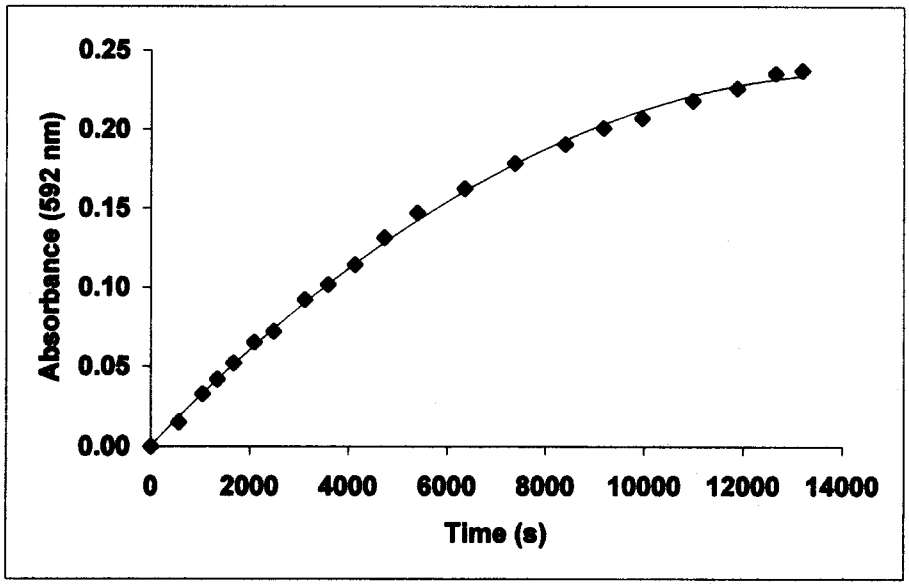


FIGURE 3 Change in time of the absorbance of a solution containing 2 M acetate buffer of pH = 4, LCV and HRP.



during the analysis. According to the method described by Zhang and Wong [14], the pH optimum is at approximately 4 and Mottola *et al.* [13] used acetate buffer for pH adjustment. It was very surprising that in the blank solution (containing only acetate buffer, LCV, enzyme and probably dissolved oxygen) a continuous increase in absorptivity was observed, as shown in Fig. 3. We have no any interpretation for this experience.

It was concluded that acetate buffer cannot be used for the analysis under the applied conditions. Various buffers were examined and finally 0.4 M tartrate ($pK_{s2} = 4.36$) was selected as an appropriate medium. The apparent molar absorbance of CV in tartrate buffer at 592 nm ($\varepsilon = 75\,600 \pm 298\,M^{-1}\,cm^{-1}$) was determined. The $H_2O_2$ gives an excellent calibration plot in the absence of organic hydroperoxides, under the conditions applied in the Experimental part.

In the next series of experiments, the selectivity of $H_2O_2$ determination by the HRP LCV method was investigated in the presence of methyl, ethyl, *tert*-butyl, and cumene hydroperoxides. The measurements were performed with standard organic hydroperoxide and $H_2O_2$ ($5 \times 10^{-6}$ M) solutions. The results showed that methyl and ethyl hydroperoxides and $H_2O_2$ reacted very rapidly, whereas *tert*-butyl and cumene hydroperoxides formed CV only very slowly. The conversion of *tert*-butyl hydroperoxide during 24 h was only 3.5% and that of cumene hydroperoxide was 18.5%. On the basis of these results, it was concluded that the method based on peroxidase and LCV cannot be used for the specific determination of $H_2O_2$ in the presence of methyl or ethyl hydroperoxide, but it is suitable for determination of their total amount. *tert*-Butyl and cumene hydroperoxides do not have considerable influences on the determination when the time between the CV formation and its absorbance measurement is not more than about 1 h. These compounds cause negligible interference in $H_2O_2$ determination.

TABLE II Reproducibility and selectivity of iodometric determination of hydroperoxides combined with enzymatic $H_2O_2$ decomposition

| *Hydroperoxide* | *Measured concentrations* ($\times 10^{-6}$ M) *and their standard deviations* | | | |
|---|---|---|---|---|
| | $H_2O_2 + PO^{\#}$ *no catalase* | $H_2O_2 + PO^{\#} + catalase$ | $PO^{\#}$ *no catalase* | $PO^{\#} + catalase$ |
| $H_2O_2$ | $5.01 \pm 0.12$ | $5.03 \pm 0.09$ | 0 | 0 |
| $CH_3OOH$ | $9.91 \pm 0.22$ | $5.04 \pm 0.11$ | $4.94 \pm 0.08$ | $5.07 \pm 0.13$ |
| $C_2H_5OOH$ | $10.01 \pm 0.27$ | $4.96 \pm 0.13$ | $5.10 \pm 0.12$ | $4.93 \pm 0.07$ |
| $(CH_3)_3COOH$ | $10.01 \pm 0.19$ | $5.01 \pm 0.08$ | $5.05 \pm 0.10$ | $4.97 \pm 0.11$ |
| $C_6H_5(CH_3)_2COOH$ | $9.98 \pm 0.22$ | $4.98 \pm 0.12$ | $4.93 \pm 0.13$ | $5.03 \pm 0.09$ |

[#] The PO notation denotes the organic hydroperoxides.



### Selectivity of Catalase

The last series of experiments focused on another means of simultaneous determination of the investigated hydroperoxides. It is known that catalase very efficiently catalyses the decomposition of $H_2O_2$ to oxygen and water [26]. The selectivity of catalase was also investigated for methyl, ethyl, *tert*-butyl, and cumene hydroperoxides. It was observed that catalase selectively decomposed the $H_2O_2$ in the mixtures, leaving the other hydroperoxides in the solution intact. Accordingly, the above described iodometric method was used for the determination of the total amount of $H_2O_2$ and the organic hydroperoxides. In a parallel sample, the amount of organic hydroperoxides was determined in the same way after decomposition of the $H_2O_2$ with catalase. Table II gives the results of ten parallel determinations, in which standard solutions ($c = 5 \times 10^{-6}$ M of each compound) containing $H_2O_2$ and hydroperoxides together and separately were taken for the measurements. The Table also gives the results of the influence of catalase addition on the organic hydroperoxide determinations. It is seen that catalase separately decomposes the $H_2O_2$ and has no influence on the iodometric hydroperoxide determination.

## CONCLUSIONS

The pseudo-first-order rate constants for the reactions of various organic hydroperoxides and $H_2O_2$ with $I^-$ in the presence and absence of ammonium molybdate as catalyst have been determined at room temperature. The catalyst considerably ($\sim$400-fold) increases the rate of reaction of $H_2O_2$ with $I^-$, whereas for the organic hydroperoxides this acceleration effect is much lower (2–3-fold). The rate coefficients are of the same order of magnitude, and the $I_3^-$ formation reaction can therefore not be used for the simultaneous determination of hydroperoxides. The reduction of hydroperoxides by $I^-$ is fast enough and quantitative if the solutions are heated at 60°C for 30 min, and it can be used for determination of the total amount of $H_2O_2$ and organic hydroperoxides. The relative error in the determination is below 3% at a concentration of $5 \times 10^{-6}$ M. Catalase was shown to selectively decompose $H_2O_2$ and not react with the organic hydroperoxides. Consequently, the total amount of organic hydroperoxides can be determined following the decomposition of $H_2O_2$ with catalase. In solution of pH $\approx 7$, the reaction of catalase with $H_2O_2$ is complete within 10 min at 25°C.

The HRP-catalyzed oxidation of LCV to CV is rapid and highly selective for methyl and ethyl hydroperoxides and $H_2O_2$, in contrast with *tert*-butyl and cumene hydroperoxides. LCV is slowly oxidized in air-equilibrated acetate buffer solution in the absence of any peroxide compounds, and its use is therefore not recommended in this type of hydroperoxide determination.

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