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# Oxygen electrode as a new tool to evaluate hydroxyl radical-scavenging ability

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ABSTRACT

An oxygen electrode was applied to determine hydroxyl radical (•OH) levels for the first time. The method is based on the determination of •OH generated by the Fenton reaction using the reaction of •OH with a scavenger and the resulting radical consuming an oxygen molecule stoichiometrically. Thus, the •OH-scavenging abilities of antioxidant reagents, as well as the concentration of •OH, can be determined by the measurement of consumption of dissolved oxygen using an oxygen electrode. A good correlation between the present method and conventional colorimetry was obtained for the estimation of the •OH-scavenging activities of antioxidants. Furthermore, the results correlated with the •OH-scavenging rate constants of the reagents evaluated by a "cupric ion reducing antioxidant capacity (CUPRAC)" assay. We applied the present method to estimate the •OH-scavenging abilities of commercially available alcoholic drinks.



## 1. Introduction

Reactive oxygen species, including hydroxyl radicals (•OH), are generated in various biological systems and often cause oxidative stress, which is related to aging and diseases such as cancer, cardiovascular disease, Alzheimer's disease, and Parkinson's disease [1]. A number of methods have been developed for the determination of •OH levels. These methods include electron spin resonance (ESR) with spin trapping agents [2,3], high-performance liquid chromatography (HPLC) analysis of products formed by a reaction with •OH [1,4], luminol chemiluminescence [5–8], and colorimetry using suitable color-producing reagents [9,10].

In the present study, we developed a new method for the detection of •OH using an oxygen electrode. Although various kinds of substances are readily determined using an oxygen electrode coupled to enzymes (so-called enzyme electrodes) [11], we were particularly interested in the application of an oxygen electrode to determine biologically important short-lived species such as •OH. We have previously applied an oxygen electrode to analyze lipid peroxidation mechanisms by the measurement of the consumption of dissolved oxygen [12], and the present investigation is an extension of the use of the electrode to determine •OH levels, which also involves oxygen consumption. The present method is based on the determination of •OH generated by the Fenton reaction using a scavenging reaction expressed by the following reaction sequences:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \quad (1)$$

$$RH + {}^{\bullet}OH \rightarrow R^{\bullet} + H_2O \quad (2)$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (3)$$

where RH shows an •OH-scavenger. Eq. (1) shows the Fenton reaction. The radical species (R•) produced by the reaction of •OH and its scavenger (Eq. (2)) further reacts with an oxygen molecule to produce a peroxy radical (ROO•) as shown in Eq. (3) [13–15]. In most antioxidant reagents tested in the present study, ROO• was expected to decompose or disproportionate, leading to the oxidation products of the reagents, such as the corresponding carboxylic acids or aldehydes, and not to cause chain reactions accompanied by further utilization of oxygen molecules [13,15]. Hence, we can determine the concentration of •OH through the consumption of dissolved oxygen using an oxygen electrode. The •OH-scavenging abilities of various antioxidants determined by the present method compared favorably with those determined by conventional colorimetry, monitoring of the bleaching of *p*-nitrosodimethylaniline [9], and the •OH-scavenging rate constants evaluated from a "cupric ion reducing antioxidant capacity (CUPRAC)" assay [10].

## 2. Experimental

### 2.1. Reagents

The reagents were obtained from the following sources: iron (II) sulfate heptahydrate, dimethyl sulfoxide (DMSO), D-mannitol, D-

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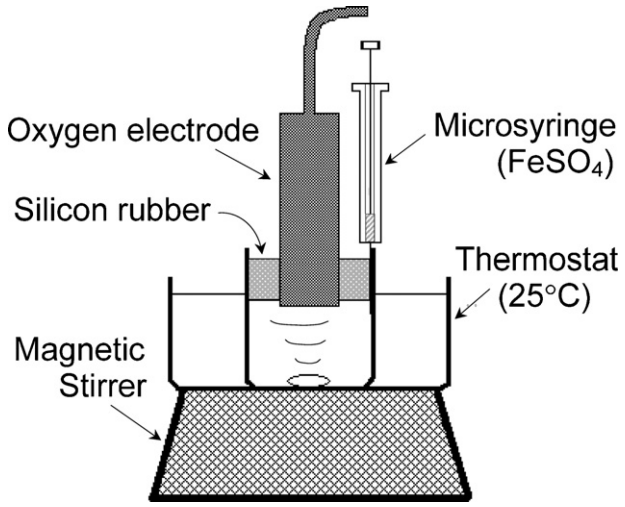


**Fig. 1.** Experimental setup for monitoring •OH-scavenging reaction using an oxygen electrode.

glucose, L-lysine, and trisodium citrate dihydrate were from Wako (Osaka, Japan); $H_2O_2$ (35 wt.% in water), sodium acetate trihydrate, methanol, ethanol, and 2-propanol were from Ishizu Seiyaku (Osaka, Japan); *N*,*N*-dimethyl-4-nitrosoaniline was from Aldrich (Milwaukee, WI, USA); sodium formate was from Kanto Kagaku (Tokyo, Japan). All other reagents were of analytical reagent grade.

### 2.2. Alcoholic drinks

Three different kinds of alcoholic drinks were used for the application of the method: shochu (Japanese distilled liquor) (Mugi-koji; Takara-Shuzo, Kyoto, Japan), sake (Gekkeikan; Gekkeikan, Kyoto, Japan), and whiskey (Suntory Old; Suntory, Osaka, Japan).

### 2.3. Preparation of solutions

The solutions of $FeSO_4$ and $H_2O_2$ were made up fresh each time using Milli-Q water. The stock solutions of scavengers were prepared in 10 mM $Na_2HPO_4/NaH_2PO_4$ (pH 6.0).

### 2.4. Measurement of oxygen consumption

The assay mixture consisting of the required concentrations of $H_2O_2$ and scavenger dissolved in 10 mM $Na_2HPO_4/NaH_2PO_4$ (pH 6.0) was put in a cylindrical glass vessel (inner diameter 21 mm, height 40 mm) and then the oxygen electrode (P-type; Biott., Tokyo, Japan) was set and covered with silicon rubber (Fig. 1). The reaction was started by injecting $FeSO_4$ with a microsyringe into the mixture. The total volume of the reaction mixture was 3.5 ml. Oxygen consumption was measured with stirring for about 2 min. The measurements were performed in a thermostat adjusted to 25 °C.

### 2.5. Bleaching of p-nitrosodimethylaniline

Colorimetry using *p*-nitrosodimethylaniline [9] was performed under the same conditions as the oxygen electrode method. The assay mixture consisted of 25 μM *p*-nitrosodimethylaniline, 0.5 mM $H_2O_2$, and the required concentration of scavenger dissolved in 10 mM $Na_2HPO_4/NaH_2PO_4$ (pH 6.0). The mixture was put in a conventional quartz cell (light path length 1 cm) for measuring the absorbance and set to a cuvette holder of an Ocean Optics USB2000 miniature fiber-optic spectrometer (Dunedin, FL, USA). A fiber-optic cable was connected to conduct light, and Ocean Optics OOIBase32 software was used to process the data. The reaction was started by adding $FeSO_4$ to a final concentration of 0.3 mM. Measurements were performed at room temperature (about 25 °C) with stirring and a decrease in the absorbance at 440 nm of *p*-nitrosodimethylaniline was monitored for 2 min.

## 3. Results and discussion

### 3.1. Detection of hydroxyl radicals by oxygen consumption

Generation of •OH by the Fenton reaction is known to increase in acidic solutions [16]. To avoid measurements in highly acidic media, assays were performed in nearly neutral media by selecting 10 mM $Na_2HPO_4/NaH_2PO_4$ (pH 6.0) as the reaction buffer, in which the rate of oxygen consumption decreased down to 85% of those in pH <5 but was still very effective. The use of other buffers, such as Hepes–NaOH, Mes–NaOH, and Tris–$H_2SO_4$, was less effective than that of the phosphate buffer because of smaller rates of oxygen consumption. Typical traces of oxygen consumption are shown in Fig. 2. In this experiment, we used mannitol, a highly selective •OH scavenger, which is known not to react with reactive oxygen species except for •OH [17]. After the reaction was started with the injection of $FeSO_4$, rapid oxygen consumption was observed within 20 s. It should be pointed out that the response time (90% of final signal) of the oxygen electrode was rather long (less than 20 s) (specifications for oxygen electrode; Biott., Tokyo, Japan), and thus monitoring the •OH-scavenging reaction through the measurement of oxygen consumption required a certain amount of time. The decrease in oxygen concentration correlated with the reaction sequences shown in the Introduction, indicating that R• (mannitol radical), formed by the reaction of mannitol with •OH, further reacted with an oxygen molecule to produce ROO• (the peroxy radical of mannitol). This result was in good accordance with a previous ESR study [13], showing that the mannitol radical reacted with an oxygen molecule stoichiometrically and the resulting peroxyl radical did not participate in further reactions with oxygen molecules. The fact that the level of oxygen consumption became constant after a sufficient reaction time also supported the view that the peroxy radical of mannitol did not take part in further consumption of oxygen molecules. The rates of oxygen consumption, estimated from the reaction traces shown in Fig. 2, as well as the total amounts of oxygen consumed, were enhanced with the increasing concentration of mannitol. Similar experiments were performed by changing the

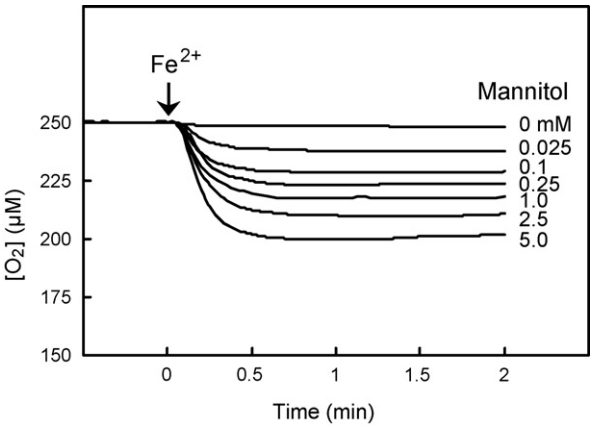


**Fig. 2.** Dynamic response of the oxygen electrode caused by the reaction of •OH with mannitol. The concentration of mannitol was changed between 0 and 5 mM with the same concentrations of $H_2O_2$ (0.5 mM) and $FeSO_4$ (0.3 mM) in 10 mM $Na_2HPO_4/NaH_2PO_4$ (pH 6.0). The reaction was started by the addition of $FeSO_4$. The total volume of the reaction mixture was 3.5 ml.