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Evaluation of Antioxidant Activity by Flow Injection Analysis with Electrochemical Detection

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The activities of various natural antioxidants, such as certain vitamins, enzymes, and natural polyphenols, have attracted attention. Several methods for evaluating antioxidant activity are known. In this review, we focus on the electrochemical detection of radical compounds in studies that evaluate antioxidant activity using flow injection analysis (FIA) with electrochemical detection.

Keywords Antioxidant, flow injection analysis, electrochemical detection, DPPH, ABTS, Trolox

1. Introduction

Antioxidants protect us against various oxidative injuries caused by active oxygens. In particular, antioxidant vitamins such as vitamin C (ascorbic acid) and E (α-tocopherol), antioxidant enzymes such as superoxide dismutase (SOD), and many other plant-derived polyphenols are known as radical scavengers, which reduce the radical species to harmless forms. There have been numerous studies on evaluation methods of the antioxidant activity as a radical scavenger [1,2]. In order to evaluate the antioxidant activity, an observation of electron transfer between an antioxidant and a synthetic radical, as well as active oxygens or lipid peroxy radical produced in vitro (these are also generated in living systems), has been extensively carried out. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical [3] and ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation [4,5] are often used as synthetic radicals. From the measurements of these compounds, information regarding the electron transfer rate and the stoichiometry of the radical scavenging reaction between the antioxidant and the radical species can be obtained. The evaluation of such reduction/oxidation activity by electrochemical measurements has also been extensively studied [6-11]. In electrochemical measurements, the equilibrium of the redox reaction is controlled by electrode potential, and the concentration or the number of electrons that participate in the reaction is evaluated from the current.

Flow injection analysis (FIA) is a technique that enables stable mixing of samples, control of reaction time, and prompt detection with good repeatability. In this mini review, recently published articles in which the antioxidant activity of various natural antioxidants was evaluated by FIA with electrochemical techniques are introduced. Particularly, articles on electrochemical detection of radical compounds are summarized herein.

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2. DPPH radical scavenging activity of antioxidant

The synthetic organic radicals DPPH• and $ABTS^{•+}$ are often used as oxidants. These radical species have strong absorption in the visible light region and their concentration changes can be easily detected from the decrease of their absorption. Therefore, in general, an absorption decrease due to the addition of an antioxidant is measured by spectrophotometry in order to evaluate the antioxidant activity. However, for the application of this method to a colored sample, or to achieve higher sensitivity, the electrochemical detection technique is often preferred.

DPPH• can be obtained as a solid reagent in the radical state, which can be dissolved in alcohol and used directly in experiments. DPPH• is known to exhibit a reversible redox reaction from cyclic voltammogram [12,13]. In the article[12], an antioxidant solution was injected into a continuous flow carrier solution (pH 7.0 phosphate buffer) containing 0.25 mM DPPH• and 0.03 M KCl. DPPH• was reduced by the antioxidant to the DPPH-H neutral form, which is less colored. Amperometric detection of the unreacted DPPH• radical was carried out using multi-walled carbon nanotube (MWCNT) modified glassy carbon (GC) electrode fixed at 0.05 V *vs*. Ag/AgCl (KCl sat.) reference electrode. A decrease in the cathodic (reduction) current was observed when DPPH• was reduced by the antioxidant. By immobilizing MWCNT on GC, an increase in current due to an increase in the electrode surface area was observed, and thus improved sensitivity was achieved. Linearity was observed between the current decrease and added antioxidant concentration. The limits of detection (LOD) obtained for gallic acid, catechin, quercetin, caffeic acid, and Trolox were 0.04, 0.02, 0.03, 0.08, and 0.04 μM, respectively. In

addition, the total antioxidant capacity (TAC) for extracts of Thai indigenous vegetables obtained by this method (electrochemical detection of DPPH•) was comparable with that obtained by a commonly used method based on the change in absorbance of DPPH•.

Similarly, the correlation between the TAC obtained by the amperometric detection at −0.1 V *vs*. Ag/AgCl on the screen-printed gold electrode and that by spectrophotometric detection was revealed for DPPH• or $ABTS^{\bullet+}$ [13]. The system was applied to the monitoring the antioxidant capacity of wines during wine-making.

3. $ABTS^{\bullet+}$ scavenging activity of antioxidant

Chemical oxidation by adding an oxidizing agent or electrolytic oxidation is required for $ABTS^{\bullet+}$ generation from commercially available neutral ABTS. While DPPH• is not soluble in water, $ABTS^{\bullet+}$ is water soluble and, thus, radical scavenging reaction can be observed in 100% aqueous solution. It is known that ABTS also exhibits a reversible electrochemical response similar to DPPH [4]. In an FIA system [14], $ABTS^{\bullet+}$ was generated by an enzymatic reaction. That is, ABTS was oxidized (using horseradish peroxidase, HRP) by H_2O_2, which was generated by a glucose oxidation reactor in a glucose oxidase (GOD) immobilized column. After the redox reaction with an antioxidant in a mixing coil, unreacted $ABTS^{\bullet+}$ was detected as a cathodic current by interdigitated electrodes (IDE) installed downstream. A linear calibration curve was obtained in the range 20 μM – 1 mM of Trolox, which is a synthetic water-soluble antioxidant (called water soluble vitamin E). Good agreement was found between the result obtained by the FIA method and that by the spectrophotometric detection of $ABTS^{\bullet+}$ for the antioxidant capacity of 14 alcoholic beverages (wines and spirits).

In another article [15], ABTS was oxidized by $K_2S_2O_8$ to generate $ABTS^{\bullet+}$; and the antioxidant capacity measurement of ginger powder using the $ABTS^{\bullet+}$ was measured by sequential injection analysis (SIA) with ECD, where ABTS was oxidized off-line. The unreacted $ABTS^{\bullet+}$ after the mixing with antioxidant was detected at GC electrode with applied potential of 0.1 V *vs*. Ag/AgCl. It also showed good correlation between gallic acid equivalent (GAE) antioxidant capacity by the electrochemical and that by a spectrophotometric detection of $ABTS^{\bullet+}$.

Sander *et al.* [16] reported the FIA system with ECD for quantification of phenolic antioxidant in natural dissolved organic matter (DOM). The $ABTS^{\bullet+}$ generated off-line by enzymatic oxidation was used as an oxidant, and its reduced form (ABTS) produced upon reduction by antioxidants was detected amperometrically (anodic current) at ECD with GC electrode. The limit of quantitation (LOQ) for Trolox was 22 pmol by electrochemical detection, while it was 980 pmol by spectrophotometric detection monitored at 728 nm (the absorbance maximum of $ABTS^{\bullet+}$). The LOQ obtained by the electrochemical detection system is lower because the base signal is much smaller than that in spectrophotometric measurement. This FIA system was applied to the quantification of electron-donating phenolic moieties in natural DOM by determining the number of electrons transferred from these moieties to $ABTS^{\bullet+}$.

It has also been reported that $ABTS^{\bullet+}$ was generated by electrochemical oxidation of ABTS in pH 7.4 buffered solution at 0.70 V *vs*. Ag/AgCl [17]. When the applied potential was too high, ABTS was oxidized to the divalent cation. The conversion efficiency decreased as the flow rate increased. The authors reported that sufficient efficiency (over 60%) was obtained by flow-through electrochemical cell when its flow rate was less than 0.2 mL min^{-1}. Thus, ABTS radicals can be generated more conveniently without an additional oxidizing agent, enabling the construction of a simpler analysis system. The LOD of 1.6 μM for Trolox was obtained by spectroscopic detection at 734 nm of $ABTS^{\bullet+}$. For 19 antioxidants and 6 tea samples, a good agreement between Trolox equivalent antioxidant capacity obtained by the FIA method and that obtained by the general batch method.

4. Potentiometric detection of antioxidant activity using ferricyanide/ferrocyanide redox couple

Shpigun *et al.* reported that antioxidant activity was evaluated using a Fe complex, which is not an organic radical [18]. When an antioxidant was injected into the ferricyanide/ferrocyanide mixed carrier solution, reduction of ferricyanide to ferrocyanide proceeds in accordance with the antioxidant activity of the sample. Here, the negative shift of the equilibrium potential of the solution with the change in ferricyanide/ferrocyanide ratio was observed by flow injection potentiometric measurements (FIP). A wide antioxidant concentration range from 1 μM to 10 mM was determined by optimizing the concentrations of ferricyanide and ferrocyanide. This technique enables a wide dynamic range measurement using simple apparatus.

5. Summary

The methods for evaluation of antioxidant activity by FIA with electrochemical detection techniques were introduced. The combination of electrochemistry and FIA is very effective for the analysis of antioxidants. These systems were also used for

activity measurement of real samples. Since various antioxidants are present as a mixture in real samples, antioxidant capacity is mostly expressed as a relative value to a certain stable antioxidant. Trolox is often used as a standard. Trolox undergoes a relatively stable two-electron oxidation [19,20]. Other than Trolox, gallic acid [15] and ascorbic acid [18] are also used as standards. Validation of these FIA systems using ECD was carried out by comparison with spectrophotometric detection of DPPH• or ABTS•$^{+}$. Since it is simply a difference of the detection method, electrochemical or spectroscopic, it seemed natural to obtain a linear relationship. However, there are several reports on their antioxidant activity by evaluating the electrochemical properties of the antioxidant itself [10,21-24]. Some papers indicated that the data obtained by the electrochemical measurements have a good correlation with the DPPH scavenging activity [10,21,23]. This means that antioxidant activity can be evaluated only by electrochemical measurement without using radical species. These results show important physicochemical properties of antioxidants related to radical scavenging activity. Additionally, in order to comprehensively understand the antioxidant reactions, it is necessary to evaluate the entire reaction, including the reaction products of antioxidants. It is considered desirable that the product obtained after the electron transfer is safely inactivated. From this point of view, there is a need to analyze the reaction products [25]. It is expected that further research in this field will continue to develop in the future.

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