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**Research Article**

# Proposal of a Micro Analysis for Singlet Oxygen Absorption Capacity using a Disposable 96-Well Microplate

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**Abstract:** A micro Singlet Oxygen Absorption Capacity (SOAC) method was proposed in this study. Recently, a SOAC assay was developed to assess the total quenching activity of singlet oxygen by carotenoids and phenolic antioxidants in foods and plants. The SOAC value was measured in ethanol-chloroform-$D_2O$ (50:50:1, v/v/v) solution at 35°C using a glass cuvette or a 24-well glass microplate. In order to reduce reagent, sample volume, analysis time and improve efficiency in high-throughput analyses, in the present study, the measurement of SOAC values by using a specially-made disposable glass-bottom 96-well microplate which is resistant to the organic solvent was performed for vegetable extracts (tomato, carrot and red paprika),SOAC values obtained using this protocol was in good accordance with the reported results, suggesting that this disposable 96-well microplate can be used for the measurement of reliable SOAC values, the total antioxidant capacity of general antioxidants and food extracts can be evaluated by measuring the SOAC and Oxygen Radical Absorbance Capacity (ORAC) values by using the same instrument with 96-well microplate system.

**Keywords:** Disposable 96-well glass-bottom microplate, high-throughput analysis, singlet oxygen, SOAC value, vegetable extracts

## INTRODUCTION

Reactive Oxygen Species (ROS) such as singlet oxygen ($^1O_2$), superoxide ($O_2^-$) and hydroxyl radical (OH·) produce oxidative damage to DNA (Devasagayam *et al*., 2004; Valluru *et al*., 2014), proteins and other macromolecules, inducing many multifactorial degenerative diseases such as diabetes (Ha *et al*., 2008), cardiovascular disease (Sugamura and Keaney Jr, 2011; He and Zuo, 2015), cancer (Waris and Ahsan, 2006), neurodegenerative disorders (Uttara *et al*., 2009) and aging (Liochev, 2013). Therefore, the research on antioxidants has raised attention for their prevention from these diseases (Flora *et al*., 2007; Ames *et al*., 1993).

Oxygen radical absorbance capacity (ORAC) using fluorescein as the probe, is an international standard method for measuring antioxidant capacities (Dávalos *et al*., 2004; Ou *et al*., 2013). After being adapted to a high-throughput assay format using a 96-well microplate, ORAC has been widely accepted in the nutraceutical, pharmaceutical and food industries (Huang *et al*., 2002).

Lipophilic antioxidants mainly include carotenoids and vitamin E family which is a group of compounds with neuroprotective properties (Boccardi *et al*., 2016). They play a critical role in biological defense systems against many multifactorial degenerative diseases.

In recent years, a Singlet Oxygen Absorption Capacity (SOAC) assay method has been established to assess the total quenching activity of singlet oxygen ($^1O_2$) based on a kinetic study of a quenching reaction of $^1O_2$. This measurement was performed in ethanol-chloroform-$D_2O$ solution at 35°C by using 4-methyl-1, 4-etheno-2, 3-benzodioxin-1(4H)-propanoic acid (endoperoxide [EP]) as a $^1O_2$ generator, 2, 5-diphenyl-3,4-benzofuran (DPBF) as a $^1O_2$ absorption probe and α-tocopherol (α-Toc) as a standard compound (Ouchi *et al*., 2010; Aizawa *et al*., 2011; Mukai *et al*., 2012; Takahashi *et al*., 2016). However, in this method, a large volume (1.0 mL) of freshly prepared EP solution is required. Due to a high cost of reagents, especially EP and limited numbers of samples in a simultaneous determination, development of a micro SOAC method is necessary in order to improve efficiency in high throughput analyses. Takahashi *et al*. (2016) developed

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Table 1: Employed concentrations, half-lives ($t_{1/2}$) of blank (DPBF Only) and α-Toc of different concentrations

| | Blank | α-Toc 1 | α-Toc 2 | α-Toc 3 |
|---|---|---|---|---|
| Concn/$10^{-4}$ M | 0 | 1.133 | 2.832 | 5.665 |
| $t_{1/2\ (min)}$ | 26.97 | 27.73 | 30.54 | 34.48 |
| | 25.02 | 26.87 | 29.00 | 30.67 |
| | 26.06 | 27.62 | 30.54 | 34.48 |
| | 27.40 | 29.88 | 32.09 | 35.91 |
| $t_{1/2\ min}$ (ave±sd) | 26.36±0.91 | 28.03±1.12 | 30.54±1.09 | 33.89±1.95 |

(n = 4)

a SOAC method using a 24-well glass microplate, volume of reagents (DPBF, EP solution) was cut down to 300 μL, but the cost of a 24-well glass microplate which cannot be disposable is also a constraint for the general application of SOAC assay method. Thus, in the present study, measurement of SOAC values by using a disposable 96-well glass-bottom microplate which is resistant to the organic solvent was proposed.

## MATERIALS AND METHODS

**Materials:** DPBF, a-Toc, EP and sea sand (30~50 mesh) were obtained from Wako Chemicals, Japan. Tomato, carrot and paprika extracts were prepared according to a procedure similar to that used by Wu *et al*. (2004) and Ouchi *et al*. (2010). The method is as follows: fresh vegetables (carrot, tomato and paprika) were cut and freeze-dried. 1.00 g of freeze-dried powder sample was mixed with 5 g of sea sand. Sample and sea sand were transferred to in a screw mouth test tube (50 mL) and were extracted with 10 mL of ethanol/chloroform/$D_2O$ (50 : 50 : 1, v/v/v) for 1 h, three or more times of extraction was repeated, until there was no change of color of solvent. The extracts were combined in a volumetric flask, the concentration (g/L) of samples were calculated and the extract was used to measure the SOAC value. a-Toc was used as a standard reference and applied concentrations were shown in Table 1, applied concentrations of three vegetable extracts were shown in Table 2.

**Measurements of Half-Life ($t_{1/2}$) of DPBF by a microplate reader:** Measurements of $t_{1/2}$ of DPBF were performed in ethanol/chloroform/$D_2O$ (50:50:1, v/v/v) solution by using a microplate reader (Infinite M200, TECAN) equipped with a temperature control system. A specially-made 96 Well Glass Bottom Plate (GP960SA, MATSUNAMI GLASS IND., LTD, Japan) was used. After addition of 100 μL samples and 100 μL DPBF ($7.95\times10^{-5}$ M), 100μL of EP solution ($4.68\times10^{-4}$ M) was added to each well by a multiple pipette. Room temperature was less than 20°C, as the production of $^1O_2$ due to the thermal decomposition of EP occurs at 25°C. The microplate was covered tightly with a transparent plastic seal (RAPID CRS, BioChromatom, Japan) which is resistant to the organic solvent to avoid loss of solvent before moving to the microplate reader which has been preheated to 35.0±0.5°C. Thirty seconds was taken from the addition of EP to the beginning of measurements of UV-vis absorption spectra, which were performed at 413 nm at one-minute intervals for 90 min.

**Analyses of SOAC values based on the half-Life ($t_{1/2}$) of DPBF:** The analysis of the decay curve was performed at $0 < t < 60$ min when a microplate was applied, while the analysis of decay curve was performed at $5 < t < 60$ min in the original large volume method (Ouchi *et al*., 2010), and according to their study, there exists the following relationship between S and half-life ($t_{1/2}$) for the analyzed period:

$$t_{1/2} = \ln 2/S \quad (1)$$

where, S are slopes of the first-order plots (that is, ln(absorbance) vs t) of degradation of DPBF:

$$t_{1/2}^{\ sample}/\ t_{1/2}^{\ Blank} = 1 + (k_Q^{\ sample}/\ k_d)\times[sample] \quad (2)$$

$k_d$ is the rate of natural deactivation of $^1O_2$ in the solvent, the value of $k_d$ in ethanol/chloroform/$D_2O$ (50:50:1, v/v/v) is $3.03\times10^5$ $s^{-1}$(Di Mascio *et al*., 1989; Beutner *et al*., 2001):

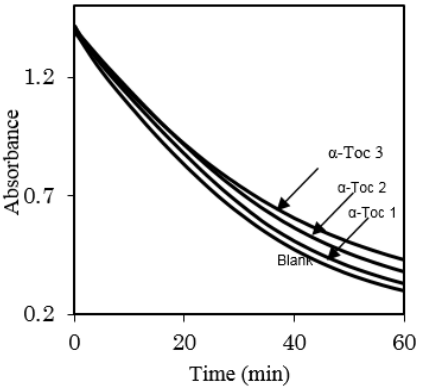
$$\text{Relative SOAC value} = \{(\ t_{1/2}^{sample} - t_{1/2}^{blank})/\ (\ t_{1/2}^{\alpha\text{-}Toc} - t_{1/2}^{blank})\}\times\{[\alpha\text{-Toc}]\ /[sample]\} \quad (3)$$

where, [α-Toc] and [sample] are concentrations of α-Toc and sample, given on a weight basis (g/L), respectively.

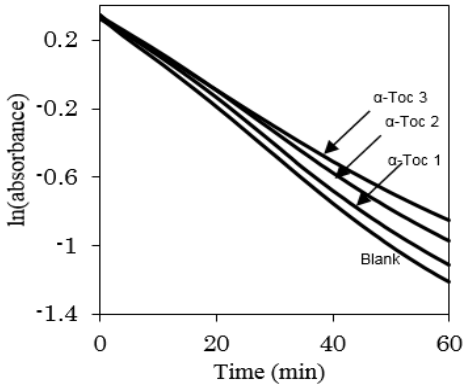
**Data analysis:** All experiments were carried out at least in triplicates. The data were subjected to multifactor Analysis of Variance (ANOVA), followed by the Least Significant Difference (LSD) test to determine the significant difference between samples at $p<0.05$ level using the software SPSS V.16.

## RESULTS AND DISCUSSION

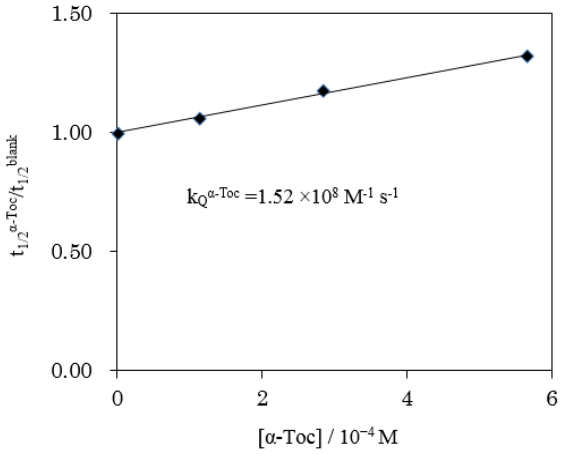
**Measurements of half-Life ($t_{1/2}$) of DPBF by a micro SOAC method for α-Toc:** Figure 1a shows an example of measurement of the reaction between DPBF and EP in the absence (blank) and presence of α-Toc. The values of first-order decay rate constant ($S_{blank}$, $S_{\alpha\text{-}Toc}$) were estimated by analyzing the decay curve of DPBF, as shown in Fig. 1b. Half-life of DPBF calculated using




(a)




(b)




(c)

Fig. 1a: Change in absorbance of DPBF at 413nm during the reaction of DPBF with $^1O_2$ in the absence (blank) and presence of α-Toc in ethanol/chloroform/$D_2O$, $[DPBF]_{t=0} = 7.95\times10^{-5}$ M and $[EP]_{t=0} = 4.68\times10^{-4}$ M. [α-Toc] refers to Table 1;
Fig. 1b: The plot of ln (absorbance) versus time
Fig. 1c: The plot of $t_{1/2}^{\alpha\text{-Toc}}/t_{1/2}^{blank}$ versus [α-Toc]

Eq. (1) was listed in Table 1. Different with procedure described by Aizawa *et al*. (2011), the analysis of the decay curve for this micro-method was performed at 0 < t < 60 min, as for a small assay volume (300 μL) in each well of a 96-well microplate, 5 min of lag time in the original method was not necessary for the solution temperature increasing from <25°C to 35°C. As shown in Fig. 1a and 1b, the DPBF decay curve started decreasing linearly from the beginning of measurement (t = 0). The values of $t_{1/2}^{\alpha\text{-Toc}}$ and $t_{1/2}^{blank}$ were calculated, as shown in Table 1, at a low concentration of α-Toc, the values of $t_{1/2}$ are all less than 60 min, which is in accordance with that reported by Ouchi *et al*. (2010).

To ascertain the reliability of analysis, a plot of $t_{1/2}^{\alpha\text{-Toc}}/t_{1/2}^{blank}$ versus [α-Toc] is shown in Fig. 1c. A linear relation between $t_{1/2}^{\alpha\text{-Toc}}$ and [α-Toc] should be expected, when a steady-state analysis would be fulfilled. In the current study, the linear correlation coefficient (R) observed in Fig. 1c was 0.999, suggesting this method is stable and reliable. The $k_Q^{\alpha\text{-Toc}}$ value obtained is $1.52\times10^8$ $M^{-1}$ $s^{-1}$, similar to the result reported by Aizawa ($1.24\times10^8$ $M^{-1}$ $s^{-1}$) (Aizawa *et al*., 2011).

**Measurements of Half-Life ($t_{1/2}$) of DPBF with a micro SOAC method for three vegetables and analysis of relative SOAC values:** In order to simplify the procedure, for each measurement, which means only one concentration of each sample was performed in one microplate, for example, data of samples (tomato -1, carrot-1, paprika -1) was obtained at the same time, by using the same blank and α-Toc data. Measurement of the time dependence of the absorbance of each sample solution (carrot, tomato and paprika) at 413 nm (data were not shown) was used for the correction of the baseline for each decay curve since the absorptions of β-carotene and lycopene overlap with that of DPBF at 413 nm. The values of $S_{tomato}$, $S_{carrot}$, $S_{paprika}$ and for $t_{1/2}^{tomato}$, $t_{1/2}^{carrot}$ and $t_{1/2}^{paprika}$ obtained are listed in Table 2. As for each time of test, UV-Vis absorption spectra of new blank and standard compound (α-Toc) were measured, so we didn't list any of the $S_{blank}$, $S_{a\text{-}toc}$ and $t_{1/2}^{blank}$, $t_{1/2}^{a\text{-}toc}$ obtained in four times. As shown in Table 2, all the values of $t_{1/2}$ were less than 60 min, indicating that the determination of $t_{1/2}$ from the decay curve of DPBF is appropriate.

According to Eq. (2), $t_{1/2}$ increases linearly with increasing concentration of antioxidants. In fact, in the current study, as for different concentration of samples, a blank was measured every time, in this case, $t_{1/2}^{sample}$ corrected by each $t_{1/2}^{blank}$ is needed. In the case of tomato and carrot extract, the measurements were repeated two times for the same extracts (tomato-2 and -3) and (carrot-2 and -3), the $t_{1/2}$ obtained for tomato-2 and -3, carrot-2 and -3 are similar to each other, which suggests the reliability of this micro SOAC method. Furthermore, as listed in Table 2, the relative SOAC values for vegetable extracts are as follows: paprika

Table 2: Employed concentrations, first-order decay rates (S), Half-Lives ($t_{1/2}$) and relative SOAC values of samples (Carrot, Tomato and Paprika) obtained by using a 96-well microplate

| | Carrot-1 | Carrot-2 | Carrot -3 | Carrot -4 |
|---|---|---|---|---|
| Concn(g/L) | 2 | 12.5 | 12.5 | 20 |
| S | 0.0245 | 0.0145 | 0.0150 | 0.0141 |
| $t_{1/2\ (min)}$ | 28.29 | 47.80 | 47.48 | 49.16 |
| Relative SOAC value | 0.0524 | 0.0500 | 0.0388 | 0.0441 |
| Relative SOAC value (ave±sd): 0.046±0.006 (n = 4) Reported (ave±sd): 0.051± 0.001 * | | | | |
| | Tomato-1 | Tomato-2 | Tomato-3 | Tomato -4 |
| Concn (g/L) | 2 | 12.5 | 12.5 | 20 |
| S | 0.0125 | 0.0127 | 0.013 | 0.0126 |
| $t_{1/2\ (min)}$ | 28.96 | 54.58 | 53.73 | 55.01 |
| Relative SOAC value | 0.0631 | 0.0660 | 0.0509 | 0.0557 |
| Relative SOAC value (ave±sd): 0.059±0.007 (n = 4) Reported (ave±sd): 0.060±0.003* | | | | |
| | Paprika-1 | Paprika-2 | Paprika-3 | Paprika-4 |
| Concn(g/L) | 1 | 2 | 4 | 6 |
| S | 0.0221 | 0.0201 | 0.0146 | 0.0124 |
| $t_{1/2\ (min)}$ | 31.36 | 34.48 | 44.43 | 52.12 |
| Relative SOAC value | 0.1376 | 0.1394 | 0.1445 | 0.1427 |
| Relative SOAC value(ave±sd): 0.141±0.003 (n = 4) Reported (ave±sd): 0.124±0.008* | | | | |

***** Relative SOAC values reported by Aizawa *et al*. (2011)

(0.141) > tomato (0.059)> carrot (0.046), which is also in accordance with results reported by other researchers (Aizawa *et al*., 2011).

## CONCLUSION

A micro SOAC method was proposed in this study, in which the required volume of reagent and sample were cut down to about 1/10 compared with the original method using a glass cuvette. And the use of a disposable 96-well microplate is also easy to operate in high throughput analysis. This micro SOAC method combined with micro ORAC method is supposed to be useful to estimate both lipophilic and hydrophilic antioxidative extracts, its application would be presumably in nutrition and healthy food to measure the antioxidant capacity of diets. Measurement of the SOAC values, as well as the ORAC values for antioxidants in eggs from hens fed with different feed, are now in progress in our laboratory.

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