Note

Inter-laboratory Validation Study of a Singlet Oxygen Absorption Capacity Assay Method for Determining the Antioxidant Capacities of Antioxidant Solutions and Food Extracts

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Singlet oxygen absorption capacity (SOAC) is an assay method used to evaluate the singlet oxygen quenching capacity. We evaluated the precision of the SOAC assay method in an inter-laboratory validation study using 3 antioxidant solutions and 3 food extracts. This study involved 14 participating laboratories, with data sets from 8 successfully participating laboratories subjected to statistical analysis. In accordance with the harmonized protocol, this study showed that the intermediate precision relative standard deviation (RSDint) and the reproducibility relative standard deviation (RSDR) ranged from 3.4 to 9.9%, and from 6.2 to 14.0%, respectively, and the HorRat values ranged from 0.87 to 1.97. Therefore, the SOAC assay method was validated by this inter-laboratory study in accordance with the internationally harmonized protocol.

Keywords: singlet oxygen, antioxidant, singlet oxygen absorption capacity, carotenoids, inter-laboratory validation study

Introduction

Carotenoids are natural pigments found mainly in fruits and vegetables. Mammals cannot synthesize carotenoids de novo, and therefore carotenoids must be obtained from the daily diet as vitamins. About 40 carotenoids are regularly identified in human serum and milk (Khachik et al., 1997). Carotenoids can quench singlet oxygen due to their chemical structure and this characteristic contributes to their beneficial effects. The dietary intake of carotenoid-rich fruits and vegetables has been associated with reduced risk of common diseases, including cancer, cardiovascular disease, age-related macular degeneration, and cataract formation (Rock et al., 1998). Hama et al. (2012) reported that astaxanthin prevented UV-induced skin damage by scavenging singlet oxygen production, and Li et al. (2010) showed that zeaxanthin and lutein prevented the light-induced production of singlet oxygen in human retinal pigment epithelium/choroid.
method allows evaluation of the singlet oxygen absorption capacity of both hydrophilic and lipophilic antioxidants in a variety of food extract such as from vegetables, fruits, and edible oils (Aizawa et al., 2011; Mukai et al., 2012; Iwasaki et al., 2015; Mukai et al., 2015). Moreover, the SOAC values of food extracts are nearly equal to theoretical values calculated from each SOAC value of antioxidants contained in these food extracts (Aizawa et al., 2011). These earlier reports suggest that the SOAC assay is a useful method for evaluating the singlet oxygen absorption capacity of food extracts without requiring analysis of the extract components.

Takahashi et al. (2016) modified the original SOAC assay method to allow the use of a 24-well glass microplate and a microplate reader because only a very limited number of laboratories have instruments comparable to a UV-Vis spectrophotometer equipped with a six-channel cell positioner. Moreover, the modified method allows higher throughput by measuring three samples simultaneously. However, the method’s precision has not been evaluated in an inter-laboratory validation study. The precision of the method is needed when the SOAC values of antioxidants/food extracts refer to the reported papers and create an antioxidant database. Therefore, in this study, we conducted inter-laboratory tests in accordance with the internationally harmonized protocol to validate the modified SOAC assay method.

Materials and Methods

Reagents and chemicals EP and (±)-α-Toc (CAS number: 10191-41-0) were purchased from Wako Pure Chemicals Industries (Tokyo, Japan). DPBF was purchased from Tokyo Kasei Chemicals (Tokyo, Japan). Capsanthin, astaxanthin, and zeaxanthin were purchased from Extrasynthese (Genay, France). All other chemicals were reagent grade.

Instruments A 24-well glass microplate (FU-24, NGS Precision, Tokyo, Japan) was initially used (laboratories 1-3); however, because the manufacture of this glass microplate has been discontinued, a 24-well microplate made of polytetrafluoroethylene (PTFE; iTEC SCIENCE Co, Ltd., Ibaraki, Japan) was developed and also used (laboratories 4-14). A custom-made quartz glass plate (128 × 85 × 2 mm) was used to cover the 24-well glass microplate.

Test samples Capsanthin, astaxanthin, and zeaxanthin were dissolved at 5 mg/L in ethanol/chloroform/D$_2$O (50:50:1, v/v/v) solvent to provide the antioxidant solutions. Carrot, tomato, and red paprika (bell pepper) were purchased from retail stores in Ibaraki, Japan, in December 2014, and were cut into small pieces, snap-frozen in liquid nitrogen, and lyophilized; samples were then pulverized using a grinder mill (GM-200; Retsch, Haan, Germany). Carrot powder (9.9 g dry weight (DW)), tomato powder (8 gDW), and red paprika powder (7 gDW) were divided into several batches (about 1 gDW each) and extracted with the ethanol/chloroform/D$_2$O solvent three times using an accelerated solvent extractor (ASE-350; Dionex, San Jose, CA, USA). The extracts from each vegetable were pooled and diluted to 1 L with ethanol/chloroform/D$_2$O solvent. Aliquots (about 20 mL) of each antioxidant solution or extract were portioned into 50 glass vials; 5 vials were used for the homogeneity test, and the remainder were stored at −20°C until transport to the participants.

Homogeneity of the test samples Five vials (units) were chosen at random for each test material and each unit was split into 2 equal parts (unit subsamples). The SOAC values of each unit subsample were measured as described below under repeatability conditions, i.e., the same method on identical test items in the same laboratory by the same operator using the same equipment within a short time scale.

The within- and between-unit standard deviations for the SOAC values were calculated by one-way analysis of variance (ANOVA) and by applying the F-test at the 95% confidence level. All p values of the test materials for the validation study were greater than 0.05, indicating that the samples were homogeneous (data not shown).

Stability of the test samples The stabilities of the antioxidant solutions and food extracts were verified prior to the inter-laboratory study. Antioxidant solutions and food extracts were prepared in a manner similar to that described above, and about 20-mL aliquots of each antioxidant solution or food extract were placed in each of 16 vials. No significant changes in the SOAC values were observed by t-tests in the test materials during storage at −20°C for 7 weeks (data not shown).

Consequently, after preparation, all test samples were stored at −20°C until distribution. All test samples were packed in insulated boxes along with a cooling bag and sent to participants in chilled containers. Upon receipt of the test samples, participants were asked to immediately store the test samples in a freezer (−20°C) until use. In addition, samples were to be analyzed within 7 weeks, ensuring adequate stability.

SOAC measurement using a microplate reader The SOAC values of the test samples were measured according to the method described by Takahashi et al. (2016). Briefly, 6-mL, 3-mL, and 2-mL aliquots of each test sample were transferred into 10-mL brown volumetric flasks and diluted with ethanol/chloroform/D$_2$O solvent. The undiluted and three diluted test samples, 1.5 mM α-Toc solution, and ethanol/chloroform/D$_2$O solvent (300 μL each) were added to the wells of a 24-well microplate using a digital analytical syringe (eVol XR; SGE Analytical Science, Victoria, Australia) on ice. DPBF solution (0.19 mM, 300 μL) and EP solution (1.2 mM, 300 μL) were added next to the wells of the same 24-well microplate using a digital analytical syringe, but were not added to the baseline wells. The final volume of each well was adjusted to 900 μL with ethanol/chloroform/D$_2$O solvent. Immediately after completion of these additions, the 24-well microplate was tightly sealed with a quartz glass cover and transferred to the microplate reader, which had been pre-incubated to 35°C. The absorbance at 413 nm of each well was measured.
inter-laboratory Validation of SOAC assay method

Table 1. Inter-laboratory study results for the determination of the average relative SOAC values of the test samples using the SOAC assay method

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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</thead>
<tbody>
<tr>
<td>Capsanthin (mol α-Toc/mol)</td>
<td>121.3</td>
<td>129.3</td>
<td>139.7</td>
<td>133.6</td>
<td>118.9</td>
<td>129.9</td>
<td>136.6</td>
<td>131.7</td>
<td>128.7</td>
<td>104.2</td>
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<tr>
<td>Zeaxanthin (mol α-Toc/mol)</td>
<td>126.8</td>
<td>144.6</td>
<td>114.6</td>
<td>123.2</td>
<td>138.5</td>
<td>120.1</td>
<td>134.2</td>
<td>130.5</td>
<td>177.7</td>
<td>153.2</td>
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<tr>
<td>Astaxanthin (mol α-Toc/mol)</td>
<td>124.7</td>
<td>161.3</td>
<td>119.3</td>
<td>138.7</td>
<td>131.3</td>
<td>142.0</td>
<td>109.3</td>
<td>132.7</td>
<td>129.3</td>
<td>176.0</td>
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<tr>
<td>Tomato (μmol α-Toc/gDW)</td>
<td>106.0</td>
<td>136.7</td>
<td>118.0</td>
<td>130.7</td>
<td>133.3</td>
<td>130.0</td>
<td>148.7</td>
<td>133.3</td>
<td>168.7</td>
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<td>Red paprika (μmol α-Toc/gDW)</td>
<td>139.6</td>
<td>146.2</td>
<td>144.0</td>
<td>170.3</td>
<td>141.1</td>
<td>161.5</td>
<td>125.7</td>
<td>134.5</td>
<td>141.8</td>
<td>193.7</td>
</tr>
<tr>
<td>Carrot (μmol α-Toc/gDW)</td>
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<td>144.0</td>
<td>141.8</td>
<td>158.6</td>
<td>145.5</td>
<td>149.1</td>
<td>133.0</td>
<td>152.0</td>
<td>173.2</td>
<td>195.2</td>
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<tr>
<td>Astaxanthin (mol α-Toc/mol)</td>
<td>150.5</td>
<td>133.8</td>
<td>135.6</td>
<td>149.4</td>
<td>133.4</td>
<td>156.3</td>
<td>161.9</td>
<td>146.3</td>
<td>178.1</td>
<td>150.6</td>
</tr>
<tr>
<td>Astaxanthin (mol α-Toc/mol)</td>
<td>265.0</td>
<td>236.4</td>
<td>234.3</td>
<td>267.1</td>
<td>257.1</td>
<td>274.3</td>
<td>265.0</td>
<td>245.7</td>
<td>265.0</td>
<td>294.3</td>
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<tr>
<td>Astaxanthin (mol α-Toc/mol)</td>
<td>259.3</td>
<td>263.6</td>
<td>260.7</td>
<td>284.3</td>
<td>262.9</td>
<td>258.6</td>
<td>302.1</td>
<td>258.6</td>
<td>302.1</td>
<td>259.3</td>
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<tr>
<td>Astaxanthin (mol α-Toc/mol)</td>
<td>123.7</td>
<td>123.7</td>
<td>116.2</td>
<td>118.2</td>
<td>124.2</td>
<td>132.3</td>
<td>126.3</td>
<td>122.7</td>
<td>121.7</td>
<td>152.5</td>
</tr>
<tr>
<td>Astaxanthin (mol α-Toc/mol)</td>
<td>107.6</td>
<td>114.1</td>
<td>113.1</td>
<td>118.2</td>
<td>118.7</td>
<td>128.3</td>
<td>117.2</td>
<td>157.6</td>
<td>137.9</td>
<td>137.9</td>
</tr>
</tbody>
</table>

a. Results of laboratories 1-8 were used for the statistical analyses. Results of laboratories 9 and 10 were excluded, because laboratory 9 did not follow the protocol, and absorbance of DPBF solution of laboratory 10 was beyond the tolerable ranges. Laboratories 11-14 failed to measure SOAC values of test samples.

every 30 s for 120 min at 35°C.

Calculation of the average relative SOAC value As described in previous reports (Ouchi et al., 2010; Aizawa et al., 2011; Mukai et al., 2012; Iwasaki et al., 2015), the relative SOAC value is defined as:

\[
\text{Relative SOAC value} = \left( \frac{(t_{1/2}^{\text{Sample}} - t_{1/2}^{\text{blank}})}{(t_{1/2}^{\alpha\text{-Toc}} - t_{1/2}^{\text{blank}})} \right) \times \left( \frac{[\alpha\text{-Toc}]}{[\text{Sample}]} \right)
\]

where \( t_{1/2} \) is the half-life of DPBF, and [α-Toc] and [Sample] are the concentration of the α-Toc and test sample (mol/L; antioxidant solutions, or gDW/L; food extracts), respectively. A pseudo-first-order condition is satisfied in the time range 40 < t < 70 min in this method (Takahashi, et al., 2016). Therefore, \( t_{1/2} \) was calculated using Equation 2:

\[
t_{1/2} = \ln 2/k,
\]

where \( k \) is the slope of the ln (absorbance) versus t plot of the disappearance of DPBF in the time range 40 < t < 70 min. Therefore, the relative SOAC values were calculated from each \( t_{1/2} \) value of the diluted test samples, and these values were averaged. If the difference between the \( t_{1/2} \) values of the test sample and the blank \( (t_{1/2}^{\text{Sample}} - t_{1/2}^{\text{blank}}) \) was smaller than 5 min, this value was excluded from the average relative SOAC value.

Inter-laboratory studies of SOAC measurement The inter-laboratory study was performed in accordance with an internationally harmonized protocol (Horwiz, 1995) as recommended by CAC/GL 28-1995 and involved 14 participating laboratories analyzing 3 antioxidant solutions and 3 food extracts. Participants received a shipment containing 12 containers of test samples comprising two sets of test samples and each set consisted of 6 different solutions. Each sample was provided in duplicate and was randomly labeled. Participants were also provided with a method protocol and an electronic evaluation and reporting sheet (MS Excel format). Participants were requested to follow the method protocol exactly.

Statistical analyses of the inter-laboratory study data Data could not be used from laboratories in which i) the SOAC measurements were unsuccessful due to leakage of solution from the 24-well glass or PTFE microplates, ii) the protocol was not followed, or iii) an extremely low absorbance of the DPBF solution was measured. All other data were subjected to the statistical tests described in the internationally harmonized protocol (Horwiz, 1995) recommended by CAC/GL 28-1995. The Cochran test and single and double Grubbs tests were conducted to detect outlying variances and outlying data set averages, respectively. The one-tail test at a probability value of 2.5% was applied to the Cochran test, and a probability value of 1.25% to the single and double Grubbs tests.

Results and Discussion Measurements were unsuccessful at four of the laboratories due to instrumentation problems. Laboratories 11-13 reported that the solvent leaked from the bottom of the 24-well PTFE microplate, and laboratory 14 reported that the solvent spilled between the glass plate lid and 24-well microplate due to the swinging motion of the microplate reader. Therefore, further development of the 24-well microplate is required for the SOAC assay method. The values obtained for the various samples by the remaining 10 participating laboratories are given in Table 1. Laboratory 9 did not follow the method protocol and used too high a concentration of α-Toc. The data from laboratory 10 showed low absorption at 413 nm of DPBF at time zero, suggesting that the DPBF may have degraded prior to
the measurement. Thus, the results from these laboratories were also excluded from the statistical analysis. In this study, differences in the microplates did not produce an observable effect.

The data sets of the 8 remaining participating laboratories were subjected to the statistical tests described in the Protocol for the Design, Conduct and Interpretation of Method Performance Studies (Horwitz, 1995), using the Cochran test to identify outlying variances and the single and double Grubbs tests to detect outlying data set averages. No data sets were removed as Cochran outliers or single Grubbs outliers (Table 1). The intermediate precision and reproducibility were calculated as defined by the protocol (Horwitz, 1995). The RSD$_{int}$ and RSD$_b$ of the test samples ranged from 4.3 to 9.9%, and from 7.5 to 14.0%, respectively (Table 2).

The precision data obtained in this inter-laboratory study were compared with the predicted levels of precision obtained from the Horwitz equation,

\[
\text{HorRat} = \frac{\text{RSD}_b}{\text{RSD}_a} = \frac{\text{RSD}_b}{2C^{0.5}}.
\]

where \(C\) is the commonly measured concentration of the analyte in the sample, expressed as a mass fraction. In the case of oxygen radical absorbance capacity methods, the predicted RSD$_b$ of each sample was calculated relative to the concentration of Trolox, the standard antioxidant (Watanabe et al., 2012 and 2016). In this method, \(\alpha\)-Toc is used as a standard antioxidant, and the antioxidant capacity of a sample is expressed as the \(\alpha\)-Toc equivalent. Therefore, the predicted RSD$_b$ of each sample was calculated from the final concentration of \(\alpha\)-Toc. A mass fraction of 0.5 mM for \(\alpha\)-Toc was calculated by multiplying the volume concentration by the molecular weight of \(\alpha\)-Toc, and the predicted RSD$_b$ was calculated to be 7.13% according to the Horwitz equation. The HorRat value, the ratio of RSD$_b$ (measured) to the predicted RSD$_b$ (Horwitz), gives a comparison between the actual precision and the precision predicted by the Horwitz equation. The calculated HorRat values can be used as a performance parameter, indicating the acceptability of the precision of the method. A HorRat value of <2 usually indicates satisfactory reproducibility, whereas a value of >2 usually indicates unsatisfactory performance of the method. The HorRat values of the test samples ranged from 0.96 to 1.97 (Table 2). All HorRat values of the test samples were less than 2, indicating that the SOAC measurement was validated by an inter-laboratory test in accordance with the internationally harmonized protocol (Horwitz, 1995).

In this study, we demonstrated the precision of the SOAC assay method by inter-laboratory validation involving 14 participating laboratories using 3 antioxidant solutions and 3 food extracts. However, the measurements were unsuccessful at four of the laboratories due to instrumentation problems and at two laboratories due to technical issues. Therefore, the development of a 24-well microplate is required for the SOAC assay method and protocol method.

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References