JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 8477–8480 8477
DOI:10.1021/jf9044292

New Analytical Method for Investigating the Antioxidant Power of Food Extracts on the Basis of Their Electron-Donating Ability: Comparison to the Ferric Reducing/Antioxidant Power (FRAP) Assay

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Several analytical approaches are available for investigating the antioxidant power for antioxidants, and they are based on a variety of chemical principles, such as oxygen radical absorbance capacity (ORAC), Trolox equivalent antioxidant capacity (TEAC), and ferric reducing/antioxidant power (FRAP). This paper reports a new rapid method for investigating antioxidant power on the basis of the electron-donating ability. This method is called chemiluminescence analysis of antioxidant power (CAAP). The electrons donated from antioxidants are capable of inducing chemiluminescence in the presence of lucigenin and a base. Thus, the intensity of chemiluminescence induced by antioxidants is proportional to their electron-donating ability (antioxidant power). It was found that the correlation between CAAP and FRAP was positive (r = 0.959) and statistically significant ($p < 0.05$). In addition to the FRAP assay, the rapid CAAP assay is convenient for investigating the antioxidant power of herbal extracts.

KEYWORDS: Antioxidant power; CAAP; FRAP; chemiluminescence; ROS

INTRODUCTION

Reactive oxygen species (ROS) are thought to be involved in many human disorders, including inflammation (*1*, *2*), neurodegeneration (*3*, *4*), cardiodysfunction (*5*, *6*), and cancer (*7*, *8*). Therefore, removal of excess ROS should reduce the complications associated with ROS. Many strategies have been applied to reduce the content of internal ROS and, among these, supplementation of antioxidants is the most widely accepted because of its convenience. Antioxidants are capable of scavenging ROS because of their chemical properties, serving as (a) free radical terminators (antioxidants provide hydrogen for terminating chain reactions induced by free radicals), (b) oxygen scavengers (antioxidants donate electrons to neutralize reactive oxygen species), and (c) metal chelators (*9*−*11*).

Before supplementation, the antioxidant power of an antioxidant has to be investigated because it is a marker for its ROS scavenging ability. Several assays have been used to investigate the antioxidant power of antioxidants, such as oxygen radical absorbance capacity (ORAC)(*12*), ferric reducing/antioxidant power (FRAP) (*13*), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent antioxidant capacity (TEAC)(*14*). The ORAC assay is used to investigate the hydrogen-providing ability of antioxidants, and the FRAP and TEAC assays are used to investigate the electron-donating ability of antioxidants (*9*).

Here, we describe a new method for investigating the antioxidant power of food extracts by chemiluminescence analysis. This method is called the chemiluminescence analysis of antioxidant power (CAAP), and it is designed to investigate the antioxidant power of food extracts on the basis of their electron-donating ability. The electrons donated from antioxidants are capable of inducing chemiluminescence in the presence of high pH (>7). Hence, the intensity of chemiluminescence induced by antioxidants is proportional to their electron-donating ability (antioxidant power). In this experiment, the correlation between CAAP and FRAP is examined. Epigallocatechin gallate (EGCG) and arginine (**Figure 1**) serve as examples to illustrate why food extracts are capable of inducing chemiluminescence on the basis of their electron-donating ability.

MATERIALS AND METHODS

Food Extracts. All of the food extracts were kindly donated by Numen Biotech Co. Ltd. (Taipei, Taiwan). The batch numbers for these extracts were 200509GTE (for green tea extract), 200601GS (for grape seed extract), 200402RR (for *Rhodiola rosea* extract), 200706AMLA (for *Emblica officinalis* extract), 200410Elu (for Siberian ginseng extract), and 200811AE (for apple extract).

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Published on Web 07/08/2010

Chemicals and Reagents. In addition to GT-EGCG (EGCG obtained from Numen Biotech. Co. Ltd.), other chemicals and reagents used in this study were from Sigma-Aldrich Inc. (St. Louis, MO).

Chemiluminescence Analysis. The chemiluminescence induced by food extracts was measured by mixing arginine (Arg) and lucigenin in distilled deionized water (DDW). In this study, 0.1 mL of food extract solution (0.1 mg/mL), 0.2 mL of lucigenin solution (1 mg/mL), and 0.75 mL of DDW were added to a chemiluminescence analyzer (CLA-FS1, Tohoku Electronic Industrial Co. Ltd., Rifu Town, Miyagi, Japan). After 20 s of mixing, 0.2 mL of arginine solution (1 mg/mL) was added by injection, and the resulting intensive chemiluminescence was measured for 60 s. The chemiluminescence data for the various food extracts are expressed as EGCG equivalents (μM).

Polyphenol Analysis. The polyphenol content of the food extracts was measured with Folin−Ciocalteu reagent (*15*). In this analysis, 15 mL of the food extract solution (0.1 mg/mL) was diluted with 80% (v/v) ethanol to give concentrations of 0.094, 0.188, and 0.375 mg/mL. Then, 0.5 mL of the diluted solution, 6.5 mL of DDW, 0.5 mL of Folin−Ciocalteu reagent (2 N), and 5 mL of Na_2CO_3 buffer (7%, w/v) were placed into test tubes and, after incubation at room temperature for 90 min, the absorbance at 620 nm was measured for all samples. In this experiment, gallic acid served as a standard and the polyphenol content of the food extracts is expressed as a percentage (%).

Ferric Reducing/Antioxidant Power Assay. The FRAP assay in this experiment was modified from that described by Othman et al. (*16*). The FRAP reagent was prepared by mixing acetate buffer (246.1 mg of sodium acetate in 10 mL of 10% (v/v) acetic acid), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) solution (31.23 mg of TPTZ and 0.044 mL of 37% HCl in 10 mL of distilled water), and $FeCl_3$ solution (54.06 mg of $FeCl_3 \cdot 6H_2O$ in 10 mL of distilled water) in a ratio of 10:1:1 (by volume). The FRAP reagent (0.5 mL), DDW (0.05 mL), and 0.016 mL of food extract solution were transferred into vials and incubated at room temperature for 4 min, and then the absorbance at 593 nm was measured. During this experiment, DDW and $FeSO_4$ were used as the blank and standard, respectively. The FRAP data for food extracts are expressed as $FeSO_4$ equivalents (μg/mL).

Statistical Analysis. All experimental data are expressed as mean ± standard deviation (SD). ANOVA and Pearson's correlation coefficient were used for data analysis. Statistical significance was set at $p < 0.05$.

RESULTS

The principle of chemiluminescence analysis is that light-emitting materials, such as luminol or lucigenin, are oxidized by ROS and release chemiluminescence that is detected by a chemiluminescence analyzer. **Figure 2** shows that the chemiluminescence induced by pure EGCG and arginine (EGCG+Arg) was detected by a chemiluminescence analyzer (**Figure 2a**) and that the chemiluminescence was suppressed in a dose-dependent manner by the addition of superoxide dismutase (SOD) (**Figure 2b**).

Table 1 summarizes the chemiluminescence induced by various food extracts in the presence of arginine. The chemiluminescence intensity (expressed by EGCG equivalents, μM) of the extracts with added arginine was ranked in the order GT-EGCG (green tea EGCG, 218.15 ± 9.93 μM) > GTE (green tea extract, 191.90 ± 8.56 μM) > RR (*R. rosea* extract, 33.66 ± 2.65 μM) > GS (grape seed extract, 18.08 ± 0.57 μM) > AMLA (*E. officinalis* extract, 10.33 ± 0.57 μM) > Elu (Siberian ginseng extract, 4.24 ± 0.24 μM) > AE (apple extract, 2.41 ± 0.12 μM).

Table 2 gives the polyphenol content of several food extracts, which was ranked in the order GTE (90.5 ± 3.2%) ≅ GT-EGCG (90.2 ± 1.8%) > GS (55.7 ± 1.3%) > RR (41.2 ± 2.2%) > AE (28.0 ± 2.3%) > AMLA (12.2 ± 0.2%) > Elu (4.1 ± 0.2%).

Table 3 summarizes the results of the FRAP assay for various food extracts. High electron-donating ability is associated with a high FRAP value, and the value is proportional to the antioxidant power of an analyte. The FRAP value was ranked in the order GT-EGCG (3104 ± 16 μg/mL) > GTE (2548 ± 21 μg/mL) > GS (1090 ± 10 μg/mL) > RR (782 ± 5 μg/mL) > AMLA (577 ± 5 μg/mL) > Elu (163 ± 2 μg/mL) > AE (24 ± 1 μg/mL).

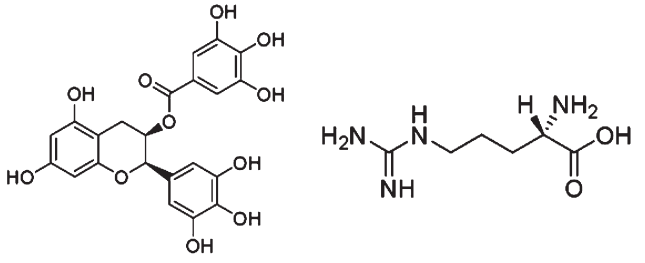

Figure 1. Chemical structures of green tea EGCG and arginine.

Table 1. Chemiluminescence (CL) Induced by Arginine and Food Extracts[a]

sample	EGCG equivalent (μM)
RR	33.66 ± 2.65
GTE	191.90 ± 8.56
GT-EGCG	218.15 ± 9.93
AMLA	10.33 ± 0.57
Elu	4.24 ± 0.24
AE	2.41 ± 0.12
GS	18.08 ± 0.57

[a] Data are expressed as mean (n = 6) ± SD. GT-EGCG represents EGCG obtained from Numen Biotech. Ltd.

Table 2. Total Polyphenol Content Analysis of Food Extracts[a]

sample	polyphenol (%)
RR	41.2 ± 2.2
GTE	90.5 ± 3.2
GT-EGCG	90.2 ± 1.8
AMLA	12.2 ± 0.2
Elu	4.1 ± 0.2
AE	28.0 ± 2.3
GS	55.7 ± 1.3

[a] Data are expressed as mean (n = 3) ± SD. GT-EGCG represents EGCG obtained from Numen Biotech. Ltd.

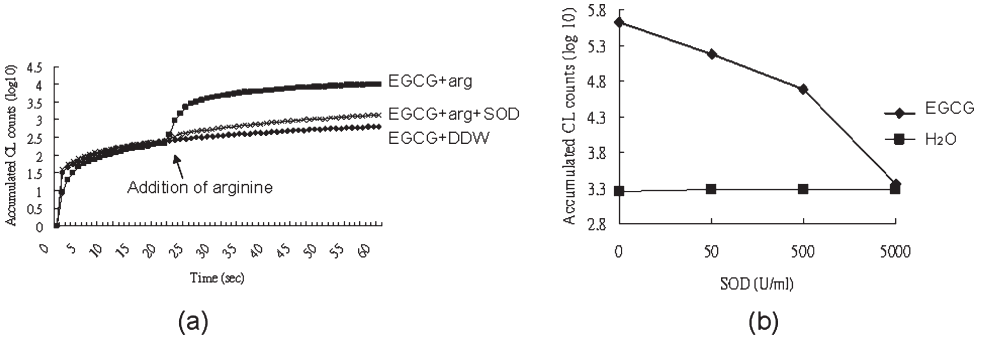

Figure 2. Chemiluminescence induced by EGCG+Arg (**a**) and suppressed by SOD treatment in a dose-dependent manner (**b**).

Table 3. FRAP Assay of Food Extracts[a]

sample (0.1 mg/mL)	FRAP (μg/mL)
RR	782 ± 5
GTE	2548 ± 21
GT-EGCG	3104 ± 16
AMLA	577 ± 5
Elu	163 ± 2
AE	24 ± 1
GS	1090 ± 10

[a] Data are expressed as mean (n = 3) ± SD. GT-EGCG represents EGCG obtained from Numen Biotech. Ltd.

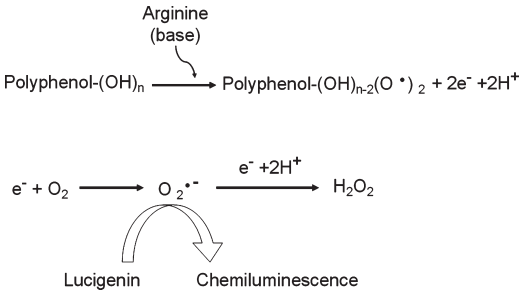

Figure 3. Proposed mechanism of EGCG-induced reactive oxygen species (modified from ref *17*).

DISCUSSION

This study showed that EGCG+Arg was capable of inducing chemiluminescence (**Figure 2a**), which was suppressed by the addition of SOD (**Figure 2b**). These findings indicate that EGCG induced ROS in the presence of arginine and that ROS might be superoxide anions. This is because the chemiluminescence induced by EGCG+Arg was totally suppressed by the addition of SOD (5000 U/mL), which is specific for neutralizing superoxide anions. Arakawa et al. (*17*) reported that polyphenols release 2 equiv of electron at basic pH, and these electrons are accepted by oxygen due to the high electron affinity for oxygen. **Figure 3** shows the proposed mechanism whereby polyphenols are capable of initiating chemiluminescence. In the figure, arginine served as a base due to its basic amino derivative (**Figure 1**). Pure EGCG served as a polyphenol (**Figure 1**) and released electrons in the presence of arginine, and the electrons were immediately accepted by oxygen. The oxygen increased its reactivity due to the acceptance of electrons, and then the reactive oxygen (superoxide anion) oxidized lucigenin (the light-emitting material), which released chemiluminescence that was detected by a chemiluminescence analyzer. This might explain why EGCG induced chemiluminescence in the presence of arginine. In addition to arginine, EGCG induced chemiluminescence in the presence of other bases, such as NaOH (please refer to Figure A in the Supporting Information). Furthermore, we found that antioxidants without a phenol group (such as β-carotene) did not induce chemiluminescence in the presence of arginine (please refer to Figure B in the Supporting Information). This may be due to the relatively weak electron-donating ability in the absence of the phenol group.

The above findings show that the chemiluminescence induced by EGCG is associated with its electron-donating ability, which is associated with its antioxidant power. The FRAP assay is often used to determine the antioxidant power of food extracts on the basis of their electron-donating ability. The principle of the FRAP assay is that the electrons donated from a herbal extract are capable of reducing ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). Therefore, the concentration of ferrous ions is proportional to the electron-donating ability of the herbal extract. As a result, the FRAP value (antioxidant power) of food extracts is correlated positively with their electron-donating ability.

Table 4. Correlation between FRAP, Chemiluminescence, and Polyphenol Content of Food Extracts

comparison	Pearson's correlation coefficient	p
FRAP versus CL counts	0.959	<0.05
FRAP versus polyphenol content	0.921	<0.05
polyphenol content versus CL counts	0.839	0.83

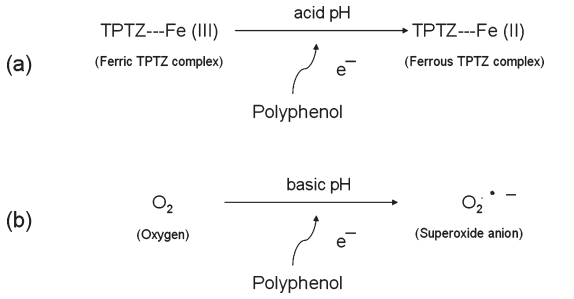

Figure 4. Principle of the FRAP assay. (**a**) The electron donated from the polyphenol reduces ferric ions to ferrous ions in acid pH. (**b**) The electron donated from the polyphenol reduces oxygen to superoxide anion in basic pH.

As mentioned above, we hypothesized that the intensity of the chemiluminescence induced by herbal extracts is correlated with their FRAP values and polyphenol contents. To investigate this hypothesis, we determined the arginine-induced chemiluminescence (**Table 1**), polyphenol content (**Table 2**), and FRAP value (**Table 3**) for various food extracts. **Table 4** summarizes the relationships between FRAP, chemiluminescence, and polyphenol content for food extracts. A positive correlation was observed for FRAP versus chemiluminescence (Pearson's correlation coefficient = 0.959, $p < 0.05$) and for FRAP versus polyphenol content (Pearson's correlation coefficient = 0.921, $p < 0.05$). The correlation between polyphenol content and chemiluminescence was positive (Pearson's correlation coefficient = 0.839) but not statistically significant ($p > 0.05$). This may be due to the small number of samples analyzed.

Some studies (*16*, *18*) have shown that the antioxidant power (FRAP value) of herbal extracts is correlated with their polyphenol content, and our findings are in agreement (**Table 4**, FRAP versus polyphenol content). Furthermore, our results demonstrate the positive correlation between FRAP and chemiluminescence on the basis of the electron-donating ability of the food extracts. **Figure 4** illustrates why the positive correlation between FRAP and chemiluminescence was observed.

In **Figure 4a**, the electrons released from the polyphenol are capable of reducing ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), which react with TPTZ to produce a ferrous−TPTZ complex. The complex exhibits great absorbance at a wavelength of 593 nm. Therefore, the absorbance at 593 nm for an antioxidant is proportional to its electron-donating ability as well as its FRAP value (antioxidant power). **Figure 4b** shows that the electrons released from the polyphenol are capable of reducing oxygen to yield superoxide anion. Afterward, the superoxide anion reacted with the light-emitting material (lucigenin) to release intensive chemiluminescence, which was detected by a chemiluminescence analyzer. Therefore, the intensity of the chemiluminescence for an antioxidant is proportional to its electron-donating ability. The link between FRAP and arginine-induced chemiluminescence is the electron-donating ability, which explains why a positive correlation was observed between the FRAP and chemiluminescence assays.

The electron-donating ability is an important parameter for investigating the antioxidant power of food extracts. Here, we observed that the chemiluminescence induced by food extracts in the presence of arginine (basic amino acid) was correlated significantly with their FRAP (antioxidant) values. We demonstrated that the positive correlation between chemiluminescence and FRAP for food extracts was due to their electron-donating ability. This is the first study to investigate the antioxidant power of food extracts via chemiluminescence analysis based on their electron-donating ability. We suggest that the chemiluminescence method for investigating the antioxidant power of food extracts is a rapid and convenient alternative to the FRAP assay.

ABBREVIATIONS USED

AE, apple extract; AMLA, emblica officinalis extract; Arg, arginine; CAAP, chemiluminescence analysis of antioxidant power; DDW, distilled deionized water; EGCG, epigallocatechin gallate; Elu, Siberian ginseng extract; FRAP, ferric reducing/antioxidant power; GS, grape seed extract; GTE, green tea extract; ORAC, oxygen radical absorbance capacity; ROS, reactive oxygen species; RR, rhodiola rosea extract; TEAC, Trolox equivalent antioxidant capacity; TPTZ, 2,4,6-tris(2-pyridyl)-1,3,5-triazine; Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

Supporting Information Available: EGCG and β-cartoene activity in chemiluminescence (Figures A and B). This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review December 16, 2009. Revised manuscript received June 22, 2010. Accepted June 23, 2010. This study was supported in part by the Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH99-TD-B-111-004) and in part by the Taiwan Department of Health Cancer Research Center of Excellence (DOH99-TD-C-111-005).