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Ferricyanide chronoamperometric total antioxidant capacity assay for green tea

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ABSTRACT

In our previous study, we established a ferricyanide chronoamperometric total antioxidant capacity (FC-TAC) assay using a carbon screen-printed disposable microchip, and then the TAC value of vegetable extraction was estimated. In the present study, we further conducted the FC-TAC assay for green tea (Japanese tea), and the TAC value of the green tea was estimated using several extracting conditions for drinking and compared with the value obtained from an optical method using 2,2-diphenyl-1-picrylhydrazyl (DPPH). First, as a typical antioxidant contained in the green tea, the response to trolox, ascorbic acid, or catechin was investigated by the FC-TAC assay, and the results corresponded well with the results obtained using the conventional DPPH assay ($r > 0.973$). Next, real sample applications were examined in the FC-TAC assay using a total of 20 samples from four types of tea. The results of the present FC-TAC assay showed a relatively sensitive response to the antioxidants contained in the green tea as compared with the DPPH assay, and an acceptable correlation ($r = 0.804$) was obtained with a good slope of regression curve ($y = 1.01x$).

KEYWORDS: green tea, total antioxidant capacity, ferricyanide, chronoamperometry, catechin

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1. INTRODUCTION

Increasing healthy life expectancy is one of the most significant criteria in reducing healthcare costs in Japan [1], and it is achieved by continuous control of physical conditions, e.g., eating and drinking habits [2]. Antioxidants act as a defense mechanism of an organism against pathologies associated with the attack of free radicals [3]. Thus, the antioxidant properties of food and drinks have recently been a focus of attention; especially green tea (*Camellia sinensis*) has been a focus of attention as an appealing drink having excellent antioxidant properties [4]. The tea made by extracting *C. sinensis* leaves is known as a traditional health drink containing ascorbic acid (As; vitamin C), catechins (a kind of polyphenol), flavonoids, and α-tocopherol (vitamin E) as typical antioxidants and is mainly categorized based on the manufacturing process into three types, namely green tea, oolong tea, and red tea. The green tea (Japanese tea) is made by heat treatment without a fermentation process and is commonly categorized into four types, namely normal green tea (green tea of middle grade; Sencha), coarse tea (Bancha), roasted tea (Hōjicha), and powdered green tea (Matcha). Due to non-fermentation, green tea is intrinsically rich in catechins.

For measuring the total antioxidant capacity (TAC) of food and drinks, many estimation methods have been developed, and optical methods have most commonly been employed [5-7]. An optical method using 2,2-diphenyl-1-picrylhydrazyl (DPPH) is conventionally employed as a spectrophotometric

method for the TAC assay [8, 9]. However, this method involves a complex procedure, requires expensive equipments, is time consuming for color development, and is affected by light absorption and scattering substances in the tea sample.

To overcome such negative aspects of the optical method, we tried the possibility of using an electrochemical method for the TAC assay. In our previous study, we established a ferricyanide chronoamperometric total antioxidant capacity (FC-TAC) assay using a carbon screen-printed disposable microchip (CSPD microchip), and the TAC value of vegetable extraction was estimated [10]. In fact, we have developed several CSPD microchips for use in clinical [11-13], environmental [14, 15], and food analyses [16, 17]. In the present study, the CSPD microchips were employed in the FC-TAC assay for green tea [10]. In the FC-TAC assay, the green tea sample was prepared in the usual manner.

2. MATERIALS AND METHODS

2.1. Materials

Potassium hexacyanoferrate(II) (potassium ferricyanide), methanol (MeOH), l-ascorbic acid (As), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Wako Pure Chemicals (Osaka, Japan). Catechin (Ct) hydrate was purchased from Sigma-Aldrich Co. LLC (Missouri, USA). Trolox (Tx; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Merck Millipore Co. (Darmstadt, Germany). The other chemicals used in this study were of reagent grade. Ultrapure water was used in the experiments for the preparation of potassium ferricyanide solution (290 mM) and 80% (v/v) MeOH solution.

2.2. Sample preparation and extraction of antioxidants

In the present study, we examined four types of tea: normal green tea (green tea of middle grade), coarse tea (containing roasted tea), and powdered green tea. For the first two types of tea, each sample solution was prepared in the usual manner by using hot water (90 ml pure water at 80 °C) and the constituents were extracted from 3 g of dried tea leaves with an extraction time of 1 min. A sample solution of powdered green tea was prepared in the same manner using 40 ml of hot water. The sample solution was moved to another cup and centrifuged to remove any leaves, then diluted 100 times using 80% (v/v) MeOH. Standard solutions of Tx were prepared using 80% (v/v) MeOH to obtain the concentrations of 0, 25, 50, 75, and 100 μM. Standard solutions of As or Ct were also prepared in the same manner.

2.3. FC measurement

The FC measurements were performed using an electrochemical analyzer (Model: CHI-1202, BAS, Inc., Tokyo, Japan) according to the previous study [10]. The sample solution (27 μL) containing tea extracts diluted in 80% (v/v) MeOH was dropped onto the electrode surface of the microchip, and the FC measurement was started immediately after dropping 3 μL of 290 mM potassium ferricyanide solution into the sample solution. A potential was applied to a working electrode (WE) at 0.8 V for 10 s after a 20 s rest. Finally, the current output obtained after 10 s application was used as the measured value.

2.4. DPPH test

A 200 μM DPPH solution (1.5 mL) dissolved in the MeOH solution was mixed with the same volume of trolox primary standard solution in a 96-well microplate. The mixture was incubated for 60 min for color development, and the absorbance of the mixture at 517 nm was measured using a microplate reader (Ultra Evolution, TECAN Group Ltd., Switzerland). On the basis of a calibration curve for Tx, the trolox equivalent antioxidant capacity (TEAC) value was calculated and used for estimation of the real tea sample.

2.5. Trolox equivalent value of green tea antioxidant

In the FC-TAC assay for green tea, the TAC value of each antioxidant contained in the green tea sample was obtained by substitution of the sensor value (A) to the slope ($A \cdot L \cdot mol^{-1}$) of the calibration curve, and then the value of the antioxidant concentration ($mol \cdot L^{-1}$) was calculated. In the case of Tx, the value of the TEAC (mgTxEQ/g) in the sample was calculated from its molar concentration. In the case of the DPPH assay, the TEAC value was calculated by the same manner as in the FC-TAC assay. The TEAC was calculated in the same manner in the cases of As and Ct as well.

3. RESULTS AND DISCUSSION

3.1. Calibration curves and correlation

The electrochemical measurement for the TAC assay of green tea was performed according to the method used in the previous study and was accomplished by a mediator-detecting system using potassium ferricyanide (FC) as an electron acceptor [10]. The principle of the FC-TAC assay was to apply an oxidative potential to ferrocyanide, which is produced by the reduction of ferricyanide with antioxidants. The output current was observed through the electrochemical analyzer when ferrocyanide was electrochemically oxidized on the electrode surface. The amount of ferrocyanide was proportional to the concentration of the antioxidant.

Tx was used as an antioxidant analog for the estimation of the TEAC value and as the standard for the present FC-TAC assay. In the calibration curve for Tx, excellent correlation was obtained in the present FC-TAC assay with a determination coefficient (r^2) of 0.999 and an averaged relative standard deviation (RSD_{av}) of 8.1% (n = 5). Then a calibration curve for Tx was made using the conventional DPPH assay, and the results correlated well with the results obtained using the present FC-TAC assay (r = 0.996). In the same manner, a calibration curve for As or Ct was obtained with both assays. The results are summarized in table 1. Good correlations were obtained for both assays (r > 0.973), although slight influences of the colors originating from Ct were observed in the

Table 1. Comparison between the present FC-TAC assay and the conventional DPPH assay.

Standard	Number of plots (n)	Linear regression curve		Correlation coefficient (r)
		slope (μA/mM)	y-intercept (μA)	
Antioxidant(s)				
Tx	5	5.45	0.9	0.996
As	5	5.95	0.7	0.995
Ct	5	4.34	0.7	0.973

Table 2. Green tea categories used in the present study.

Sample		Material			Manufacturing process	Remarks
Category	Feature	Picking season	Picking site	Growth condition		
Sencha	Decocted tea	Spring	First- or second-flushed fresh leaves	Exposed directly to sunlight	Steaming, kneading and drying	It is the most common green tea in Japan.
Matcha	Powdered ceremonial tea	Spring	First-picked fresh leaves after removing veins	Shading from direct sunlight as is the case with Gyokuro*	Steaming, milling and drying	It is made from Tencha**
Bancha	Decocted coarse tea	Summer or autumn	Third- or fourth-flushed coarse leaves	Exposed directly to sunlight	Steaming, kneading and drying	It consists of well-grown leaves.
Hōjicha	Roasted Sencha or Bancha				Roasting of Sencha or Bancha	It is categorized into roasted Sencha.

*Gyokuro is the finest green tea. **Tencha is made by steaming and drying leaves (not kneading).

conventional DPPH assay (r = 0.973). Thus, these results clearly showed the potentiality of the present FC-TAC assay as an estimation method for evaluating the antioxidant capacity of green tea.

3.2. Real sample application and comparison with conventional DPPH assay

Table 2 summarizes the categories and features of the green tea, and table 3 shows 20 kinds of commercially available green tea that were used in the present study. In table 4, the results of real sample application are summarized.

In the present FC-TAC assay, the TEAC values obtained were different depending on the variety of green tea, and relatively high TEAC values were obtained for Sencha. The reason for this result could be attributed to the fact that the leaves used to make Sencha were grown under strong sunlight in spring and steamed to retain the maximum amount of antioxidant substances. Compared with

Table 3. Commercially available green tea sample used in the present study.

Category		Planting area		Manufacturer*	Remarks
Code	**Trade name**	**District**	**Prefecture**		
Sencha					
S_1	Hien_no_Tsuyu	Chubu	Shizuoka	1	Deep steamed
S_2	Yūki_Sencha	Kyushu	Kumamoto	1	Middle steamed
S_3	Miyako_no_Homare	Chubu	Shizuoka	1	Deep steamed and second flushed
S_4	Reihō	Chubu	Shizuoka	1	Deep steamed and first flushed
S_5	Awase_Midori	Kyushu	Kagoshima	2	Contains rare-breed leaves
S_6	Sayama_Sencha	Kanto	Saitama	2	Strong taste
Matcha					
M_1	Samidori	Kinki	Kyoto	3	
M_2	Senjin_no_Mukashi	Kinki	Kyoto	3	
M_3	Ogurayama	Kinki	Kyoto	3	
M_4	Shikibu_no_Mukashi	Kinki	Kyoto	3	
M_5	Yomo_no_Kaori	Kinki	Kyoto	3	
M_6	Yūki_Matcha	Kinki	Kyoto	1	
M_7	Tokusen_Yūki_Matcha	Kinki	Kyoto	1	
M_8	Rakuraku_Matcha	Kinki	Kyoto	1	
Bancha					
B_1	Shizuoka_Bancha	Chubu	Shizuoka	1	
B_2	Sarani_Oishii_Shizuoka_Bancha	Chubu	Shizuoka	1	
B_3	Bancha	Chubu	Shizuoka	4	
B_4	Uji_Bancha (Nagomi)	Kinki	Kyoto	4	
Hōjicha					
H_1	Sannen_Bancha	Kinki	Kyoto	5	Aged for three years and roasted
H_2	Kyō_Bancha	Kinki	Kyoto	6	

*1) Kanekoen Co. Ltd., 2) Ito En Ltd., 3) Yamamasa Koyamaen Co. Ltd., 4) Maruyamaen Co. Ltd., 5) Harimaen Seicha Co. Ltd., 6) Lupicia Co. Ltd.

Table 4. Correlation between the present FC-TAC assay and the conventional DPPH assay.

Sample	DPPH (n = 3)			Sens (n = 5)		
	TxEQ (mgTxEQ/g)	SD (±)	RSD (%)	TxEQ (mgTxEQ/g)	SD (±)	RSD (%)
Sencha (decocted tea)						
S_1	62.05	0.15	0.24	83.73	5.93	7.1
S_2	61.86	0.19	0.30	65.50	3.48	5.3
S_3	65.16	0.32	0.50	53.99	2.24	4.1
S_4	65.28	0.11	0.17	60.52	8.93	14.8
S_5	61.86	0.25	0.40	46.16	7.80	16.9
S_6	61.58	0.04	0.07	64.71	2.96	4.6
Matcha (powdered ceremonial tea)						
M_1	67.65	0.49	0.73	44.21	2.36	5.3
M_2	68.85	0.35	0.51	50.28	1.76	3.5
M_3	66.43	0.16	0.25	57.05	3.59	6.3
M_4	64.83	0.16	0.25	52.51	2.09	4.0
M_5	64.83	0.39	0.60	51.55	1.97	3.8
M_6	64.85	0.41	0.64	76.85	1.62	2.1
M_7	66.17	0.39	0.59	78.37	2.87	3.7
M_8	51.58	0.64	1.24	30.91	2.60	8.4
Bancha (decocted coarse tea)						
B_1	61.96	0.15	0.24	54.62	4.03	7.4
B_2	62.10	0.31	0.50	44.27	3.30	7.5
B_3	62.88	0.55	0.88	63.77	3.25	5.1
B_4	62.95	0.49	0.79	57.02	2.45	4.3
Hōjicha (roasted Bancha)						
H_1	14.68	0.36	2.47	5.67	2.90	51.2
H_2	10.64	0.14	1.33	7.48	2.83	37.8
	RSD_{av} (%)		0.63	RSD_{av} (%)		10.2

Sencha, the amount of antioxidant substances in the Matcha leaf was relatively low which might be due to the fact that the leaves were grown under a shaded condition and oxidized by powdering during production. As is the case with the Matcha leaf, the amount of antioxidant substances in the Bancha leaf was also low which might be due to the fact that the leaves were harvested before growing enough. In the case of Hōjicha, low TEAC values were observed due to the fact that antioxidant substances contained in the leaf were mostly oxidized and decomposed by roasting during production. The RSD_{av} value of 20 samples obtained by the present assay was 10.2% (n = 5). On the other hand, the TEAC values obtained by the conventional DPPH assay were close to 70 mgTxEQ/g with the exception of roasted green tea (Hōjicha). The RSD_{av} value of 20 samples obtained by this assay was 0.63% (n = 3).

Figure 1 shows a correlation diagram obtained by real sample application. A good slope of $y = 1.01x$ with acceptable correlation (r = 0.804) was observed between the present FC-TAC assay and the conventional DPPH assay. These differences might be due to the differences in the reactive properties between FC and DPPH. This shows that the present FC-TAC assay has the potential as a superior assay for the estimation of the antioxidant

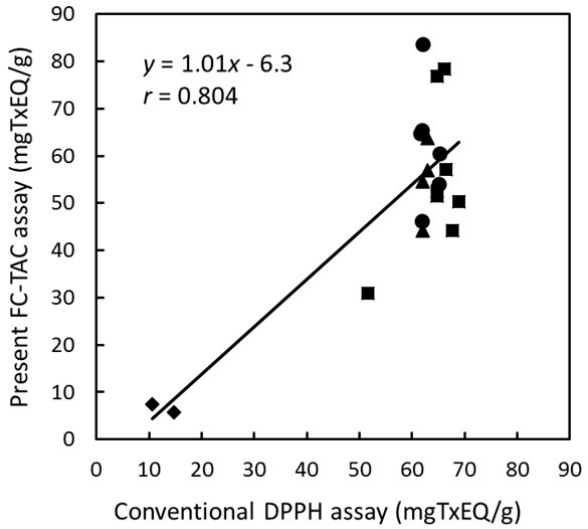

Figure 1. Comparison between the present FC-TAC assay and the conventional DPPH assay. (●) Sencha (decocted tea), (■) Matcha (powdered ceremonial tea), (▲) Bancha (decocted coarse tea), and (♦) Hōjicha (roasted Bancha).

capacity of green tea as compared with the conventional DPPH assay.

4. CONCLUSION

As a subsequent study to the previous FC-TAC assay for vegetables, we studied the FC-TAC assay for green tea. An acceptable correlation with the conventional assay was obtained, and the present FC-TAC assay successfully showed a superior feature in green tea estimation compared with the conventional DPPH assay.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest directly relevant to the content of the present article.

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