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Antioxidant Capacity Assays by Sequential Injection Analysis using a Peristaltic Pump and Low-Cost Amperometric Detection with Pencil Lead Electrodes

Pachara Meanha[1], Jakkrapun Kongkedsuk[2], Apisake Hongwitayakorn[2] and Rasamee Chaisuksant[1,*]

ABSTRACT

Miniaturization of electrode and flow cell assemblies was accomplished using low-cost pencil graphite leads and micropipette tips for sequential injection analysis (SIA) with amperometric detection (AD). The Masterflex peristaltic pump of the SIA-AD system was semi-automatically controlled by the in-house electronic circuit controller and pump drive computer software written in the Processing language. The developed SIA-AD was applied to rapid screening tests of antioxidant capacity by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay and reducing capacity measurement. By ABTS assay, the cathodic current signal of the ABTS cation radicals left after the reaction with the antioxidants in the sample was monitored at the applied potential -0.10 V vs. Ag/AgCl whereas by the reducing power measurement, anodic current from oxidation of antioxidants in the sample at +0.50 V vs. Ag/AgCl was detected. The peak current from each sample assay was calibrated with a gallic acid (GA) antioxidant standard conducted using the same procedure and expressed as the total antioxidant capacity of the sample in equivalent units (GAE). The total antioxidant capacities of some herb infusions were determined by ABTS assay and reducing power measurement using the proposed SIA-AD with sample throughputs of 22 and 36 samples/hr, respectively. Classical spectrophotometric ABTS assay was also carried out for comparison. The total antioxidant capacities in GAE found in the herb samples by all assays were in consensus for the series with black tea >> cinnamon > ginger > safflower ≈ chrysanthemum.

Keywords: ABTS assay, antioxidant capacity, pencil electrode, reducing power, SIA-AD

INTRODUCTION

Relationships between electrochemical behavior which is correlated to chemical structure and antioxidant activity of many phenolic compounds have been demonstrated (Gunckel *et al.,*1998; Cheng *et al.,* 2002; Janeiro and Brett 2004; Simic *et al.,* 2007; Aguirre *et al.,* 2010). Recently, a review on electrochemistry as a tool for studying antioxidant properties was published (Sochor *et al.,* 2013). Voltammetry has been used for the characterization and determination of the antioxidant capacity of plant extracts, beverages and blood samples; for example, cyclic voltammetry (Chevion *et al*., 2000; Kilmatin *et al.,* 2003; Sousa *et al*., 2004; Yukovleva *et al.,* 2007; Chaisuksant *et al.,*2012), differential pulse voltammetry (Janeiro and Brett, 2004; Ziyatdinova *et al.,* 2013) and

1 Department of Chemistry, Faculty of Science, Silpakorn University, Nakhon Pathom 73000, Thailand.

2 Department of Computing, Faculty of Science, Silpakorn University, Nakhon Pathom 73000, Thailand.

* Corresponding author, e-mail: CHAISUKSANT_R@silpakorn.edu

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square wave voltammetry (Pohanka *et al.,* 2009; Goiris *et al.*, 2012). Using voltammetry, the redox reaction of an antioxidant on an inert working electrode (mostly, glassy carbon electrodes) is detected during a potential scan. The more powerful the antioxidant as a reducing agent, the less positive the oxidation potential, with the ranking of the oxidation potential established on the inert electrode being a good indication of the antioxidant strength within the specified electrolyte medium. The peak current or peak area obtained from the voltammogram (i-E response) also provides a quantitative measurement for the total antioxidant capacity (TAC) of the sample in terms of the equivalent concentration to a given standard antioxidant. Another prevalent electrochemical method is amperometry, by which the current from an electrode reaction of the target species at a fixed potential is measured and this method is preferable for detection by coupling to the flow techniques. Incorporation of the flow techniques with relevant automation to antioxidant capacity assays offers advantages for routine analysis and screening tests as there is a decreased analysis time and less reagent/sample consumption compared to the conventional batch methods. A rapid test for antioxidant capacity employed flow injection analysis (FIA) with amperometric detection (AD) based on the reducing activity of antioxidants has been presented using a glassy carbon working electrode with an operating potential of +0.40 V vs Ag/AgCl for wines (Mannino *et al.,* 1998) and at +0.50 V vs Ag/AgCl for olive oils (Mannino *et al.,* 1999), lipophilic food extracts (Buratti *et al.,* 2001) and honeybee products (Buratti *et al.,* 2007). The FIA-AD method for electrochemical index measurement of antioxidants was proposed by Blasco *et al.* (2005). The electrochemical index term was defined as the strength of the antioxidant capacity measured at different applied potentials. Antioxidants are classified as "high capacity" when their oxidation on a glassy carbon electrode occurs at a potential of +0.30 V and as "medium capacity" for oxidation at +0.50 V and at +0.80V for total phenolics' oxidation. The oxidation current of each sample was calibrated and expressed as equivalent concentration units of a representative/standard antioxidant in the same way as by voltammetry.

From the review of Megalhaes *et al.* (2009), the automatic flow methods applied to radical scavenging assays as 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) assay and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay have been popular since the radical reagents of these two assays are supposed to mimic the reactive nitrogen/oxygen species found *in vivo* and their optical properties facilitate spectrophotometric detection. Milardovic *et al.* (2007a,b) reported the FIA system with biamperometric detection of the $ABTS^{o+}$/ABTS redox couple for determination of the TAC in herbs, juices and alcoholic beverages. However, a continuous flow system consumes large amounts of reagent and generates waste. As far as is known, the current author group was the first to develop sequential injection analysis (SIA) using AD for analysis of TAC by ABTS assay (Chan-Eam *et al.,* 2011). The SIA system operated with a syringe pump controlled by the in-house software program written in LabVIEW 8.0TM provided sample throughput of 40 samples/hr. Later the same group developed the SIA-AD system using a bi-directional peristaltic pump (Kongkedsuk *et al.,* 2013). The system was semi-automatically controlled by the in-house pump drive controller (PDC) program version 1.0 written in the Processing language and applied to ABTS assay. The sample throughput rate was 9 samples per hour at a constant solution flow rate of 2.0 mL/min.

Pencil lead has been used as the carbon material of electrodes in electrochemical analysis due to its low cost and having the most characteristics compatible with the more expensive glassy carbon electrode (Bond *et al.,* 1997; Demetriades *et al.,* 2004; King *et al.,* 2010; Wang and Kawde, 2011). The use of a pencil lead electrode to evaluate the antioxidant capacity and total phenolic content of tea infusions was

presented by Buratti *et al.* (2008) with FIA-AD at the applied potential of +0.50 V and +0.80 V, respectively.

This work continued to use the SIA-AD system with a peristaltic pump and the PDC program version 1.1 was further developed from the previous version (Kongkedsuk *et al.,* 2013). The flow rate of each step in the SIA protocol can be adjusted. Thus, the sample throughput rate increased. In addition, the low-cost, low-tech detector cell for the SIA-AD system was fabricated from pencil lead (graphite) fixed to small micropipette tips as the electrodes and cell body. The same SIA-AD manifold was applied to two types of TAC assay using ABTS reagent and reducing power measurement. The results of the assays with the SIA-AD system were compared to those obtained by the classical spectrophotometric ABTS assay.

MATERIALS AND METHODS

Reagents and samples

All reagents were of analytical grade. ABTS reagent as diammonium salt was sourced from Fluka (Buchs, Switzerland) and potassium persulfate from Merck (Darmstadt, Germany). The $ABTS^{o+}$ radical was prepared by oxidation of the ABTS reagent with potassium persulfate as previously described (Chan-Eam *et al.,* 2011). A concentration of the $ABTS^{o+}$ radical solution of 0.5 mM was used in the SIA-AD while by classical spectrophotometry, the radical solution was diluted with deionized water to give 0.7 absorbance at 734 nm before assay. The antioxidant standard was 98% gallic acid monohydrate from Riedel-de-Haen (Seelze, Germany). A 50 mM phosphate buffer, pH 7.0, prepared from disodium hydrogen phosphate and sodium dihydrogen phosphate was used as the supporting electrolyte and carrier in all electrochemical measurements. Herb samples were black tea, ginger, cinnamon, safflower and chrysanthemum in dried form packages, locally produced in Thailand. Sample preparation was performed by infusion of an accurate weight (1.0000 g) of dried powder in 25 mL deionized water at 70°C for 30 min. The residue was filtered off through 11µm WhatmanTM filter paper and the volume of the supernatant was made to 25 mL again with deionized water. Further dilution was made to be in the proper concentration according to the working range of each method.

Apparatus and instrumentation

The SIA system was designed as shown in Figure 1. The bi-directional peristaltic pump was sourced from Masterflex (Cole-Parmer Instrument Co.; Vernon Hills, IL, USA) with the pump drive model 77521-40, pump head 07519-10 and cartridges 7519-85. The cartridge tubing was two-stop Tygon tubing of 1.02 mm internal diameter (i.d.) connected to the PTFE holding/mixing coil of 1.07 mm i.d., 200 cm length polytetrafluoroethylene (PTFE) tubing. The in-house pump drive controller (PDC) program written in Processing language monitored the pump drive through an Arduino microcontroller electronic circuit for switching on/off and the voltage was applied in pulse width modulation mode to drive the eight rollers of the pump head at the corresponding speed with the speed calibrated to a flow rate of the solution in milliters per minute. The PDC program version 1.1 was further developed from the previous work (Kongkedsuk *et al.,* 2013) so that variation in the flow rate in each step of the SIA protocol was possible in order to decrease the analysis time.

An electrochemical detector cell was made of a 20–200 µL micropipette tip with its tip cut off and sealed with Epoxy glue. The upper side was covered with a polyvinyl chloride (PVC) cap with three holes to fix the three electrodes. The inlet and outlet of 1.02 mm i.d. PTFE tubes were fixed on the cell body. The pencil electrode (PE) was made from 35 mm length graphite pencil lead (2B, STEADLER; Nuremberg, Germany), wiring to a copper rod (0.5 mm outside diameter, o.d.; 45 mm length) with a thin copper wire (0.13 mm o.d.,

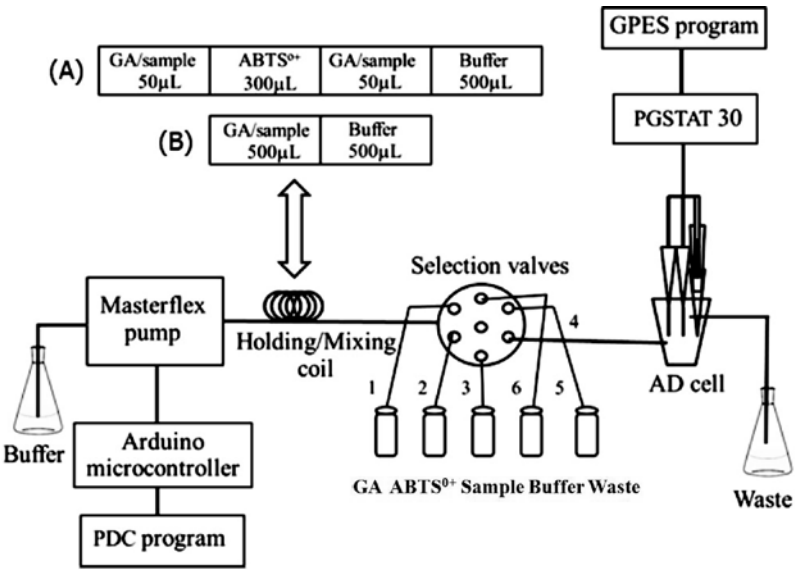

Figure 1 Schematic diagram of the sequential injection analysis with amperometric detection (SIA-AD) manifold with protocols for: (A) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay; (B) reducing power measurement

10 cm) and applied silver paint (SPI supplies) for electrical connection. A micropipette tip was used as the electrode body to cover the fragile pencil rod, sealed with PVC glue and the exposed pencil rod was 10 mm in length. The PE was pretreated by soaking for 12 hr in 50% HNO_3 before a further 2 hr in ethanol followed by rinsing thoroughly with deionized water. Two PEs being working and counter electrodes were then fixed on the PVC cap of the detector cell together with a laboratory made Ag/AgCl (saturated KCl) reference electrode fabricated with the micropipette tip body. The three electrodes were connected to the Autolab PGSTAT30-potentiostat controlled by the GPES software version 4.9.007 (Eco Chemie; Utrecht, the Netherlands) and data were recorded in the chrono-amperometric mode**.**

ABTS assay by SIA-AD

The SIA-AD procedure is shown in Table 1 for the ABTS assay, the sequential injection procedure composed of six steps in one cycle. The aspirated steps into the holding/mixing coil are as follows: antioxidant standard/sample was aspirated in two segments (step 1 and 3) and the ABTS^{o+} solution was aspirated as the middle segment between the standard/sample segments (step 2). The phosphate buffer, carrier solution was aspirated to finish the segment sequence (step 4). Then, the direction of the flow was changed forward and backward for mixing the solutions (step 5) before the solution was propelled to the AD cell (step 6). The analytical signal of AD was the cathodic current from reduction of the ABTS^{o+} radical at the applied potential of -0.10 V vs Ag/AgCl on the PE working electrode. The peak currents from the antioxidant samples were calibrated with the calibration curve of gallic acid (GA) in the concentration range 0–40 ppm. The antioxidant capacity results were expressed in GAE units.

Reducing power measurement by SIA-AD

The same SIA-AD manifold was employed for the reducing power measurement with only three aspirated steps. The 500 µL of antioxidant standard or sample solution was first aspirated at flow rate 3.0 mL/min for 10 s, followed

Table 1 SIA-AD protocol for ABTS assay

Step	Valve position	Volume (μL)	Time (s)	Flow rate (mL/min)	Flow direction	Event
1	1/3	50	3	1	Reverse	Standard/sample zone 1 aspirated
2	2	300	6	3	Reverse	Reagent zone aspirated
3	1/3	50	3	1	Reverse	Standard/sample zone 2 aspirated
4	6	500	10	3	Reverse	Carrier aspirated
5	6	-	6	2	Forward	Zones mixing
		-	6	2	Reverse	
		-	6	2	Forward	
		-	6	2	Reverse	
		-	6	2	Forward	
		-	6	2	Reverse	
6	4	-	100	3	Forward	Zones sent to AD

by an equal volume of 50 mM phosphate buffer to finish the antioxidant zone and the solution was propelled to the detector cell for amperometric detection at the applied potential + 0.50 V vs Ag/AgCl at the same flow rate for 80 s. The oxidation peak current of the sample was calibrated with GA which was carried out in the concentration range 0–80 ppm and the result was expressed also in GAE units.

ABTS assay by spectrophotometry

The radical $ABTS^{o+}$ was generated by the reaction of 7 mM ABTS solution with 2.45 mM potassium persulfate (final concentration) and allowed to stand for 12–16 hr in the dark at room temperature. The resulting solution was diluted with water to give an absorbance of 0.70 (± 0.05) at 734 nm before use. Two mL of this solution was mixed with several volumes of each sample and the total volume was adjusted to 4.0 mL with water. The absorbance at 734 nm was measured by a Lambda 35 UV/VIS spectrophotometer after standing for 30 min. The antioxidant capacity of the sample in GAE units was obtained by calibration with the calibration curve of 0.20–1.0 ppm of GA.

RESULTS AND DISCUSSION

Analytical performance

Electrochemical methods for determination of TAC require definite redox activity of electroactive species which can be any reagent of the assay or the antioxidants themselves in the sample. Cyclic voltammograms of the ABTS reagent and standard GA were examined in 50 mM phosphate buffer, pH 7.0 medium within the fabricated detector cell. Their electrochemical behaviors on the pencil lead electrode were not different from the previous work on a glassy carbon working electrode (Chan-Eam *et al.,* 2011). The quasi-reversible couple ($ABTS/ABTS^{o+}$) showed $E_{1/2}$ = 0.50 V vs Ag/AgCl (estimated from $(E_{pa}+ E_{pc})/2$) with a peak separation of 80 mV, whereas GA showed irreversible oxidation at the onset potential 0.13 V vs Ag/AgCl. The applied potential at -0.10 V vs Ag/AgCl was used for amperometric detection of $ABTS^{o+}$ as for the work on the glassy carbon working electrode.

The performance of the two types of TAC tests with the proposed SIA-AD system were examined. From the ABTS assay, the scavenging power of the antioxidant was monitored from

the cathodic current of $ABTS^{o+}$ left while by the reagentless method, the reducing power measurement, the anodic current from the oxidation of antioxidants present in the sample at the fixed applied potential 0.50 V vs Ag/AgCl was measured for calibration. Optimization of the SIA-AD was investigated using the following: AD cell feature, reagent/antioxidant volume ratio, zone sequence, flow rate and number of zone mixing cycles. Then, the optimized parameters were chosen according to sensitivity and the analysis time. The inlet and outlet tubes in a perpendicular direction fixed to the flow cell with the inlet point being 10 mm lower than the outlet point provided a good peak shape and a higher peak current signal than the other features. The cell volume was found to be 260 µL (calculated from aspiration of the solution at a fixed flow rate). The reagent/antioxidant volume ratio at 3:1 was the optimum, at which the $ABTS^{o+}$ volume of 300 µL could cover the GA concentration up to 40 ppm. The $ABTS^{o+}$ zone was sandwiched between two 50 µL of sample to ensure sufficient mixing of the reaction solution. The $ABTS^{o+}$ volume at the same concentration was half-reduced with the micropipette tip flow cell when compared to the previous work using a 350 µL acrylic flow cell (Kongkedsuk *et al.,* 2013) and this reagent consumption was even less than the 375 µL of $ABTS^{o+}$ used in the SIA-AD system with the syringe pump (Chan-Eam *et al.,* 2011). However, the shortened linear length of the calibration curve from 0–70 ppm of GA was compromised to be 0–40 ppm with this decreasing reagent volume. The flow rate of each step in the SIA-AD protocol could be adjusted between 1–3 mL/min with the command from the in-house-developed PDC program version 1.1 and the analysis time was158 s/sample so that the sample throughput was 22 samples per hour.

By the reducing power measurement, the fixed applied potential at +0.50 V vs Ag/AgCl was chosen as oxidation of most antioxidant samples had occurred. This reagentless method needs no chemical reaction but only an electrode reaction of the antioxidants present in the sample. A wide linear range of the calibration curve up to 80 ppm GA was possible. The sample throughput was up to 36 samples/hr at the constant flow rate of 3 mL/min by the same SIA-AD manifold with fewer sequence steps.

The response signals from the ABTS assay and reducing power measurement including the calibration curves are shown in Figures 2 and 3 and the analytical performance of both methods is summarized in Table 2. The two methods are based on different principles, so the current signal changes with the antioxidant concentration appear in opposite ways. Pretreatment of PE by soaking in 50% HNO_3 and ethanol before use could reduce the background signal from inorganic/organic impurities in the pencil lead which also contained clay and polymers (Bond *et al.,* 1997). When passivation of the PE occurred, as determined from the significant decrease in the peak current at the same concentration of the GA aspirated, electrochemical surface cleaning (Chaisuksant *et al.,* 2012) was easily performed without dislodging the PEs. The 0.10 mM $NaHCO_3$ solution was aspirated to be retained in the AD cell followed by cycling the applied potential between -1.30 to +1.60 V vs. Ag/AgCl at a scan rate of 200 mV/s for 3–5 cycles before flushing with the carrier stream.

Sample analysis

The proposed SIA-AD system was used to determine the TAC of the herbal infusions and the results are shown in Table 3. The antioxidant capacity in the GAE values of the herb infusion samples from the ABTS assay by SIA-AD, the reducing power measurement by SIA-AD and the classical ABTS assay all produced the same series: black tea >> cinnamon > ginger > safflower ≈ chrysanthemum. Statistical analysis of the results using one-way analysis of variance and Tukey's test revealed no significant difference ($P < 0.05$) between SIA-AD and spectrophotometric detection of the ABTS assay but there was a significant

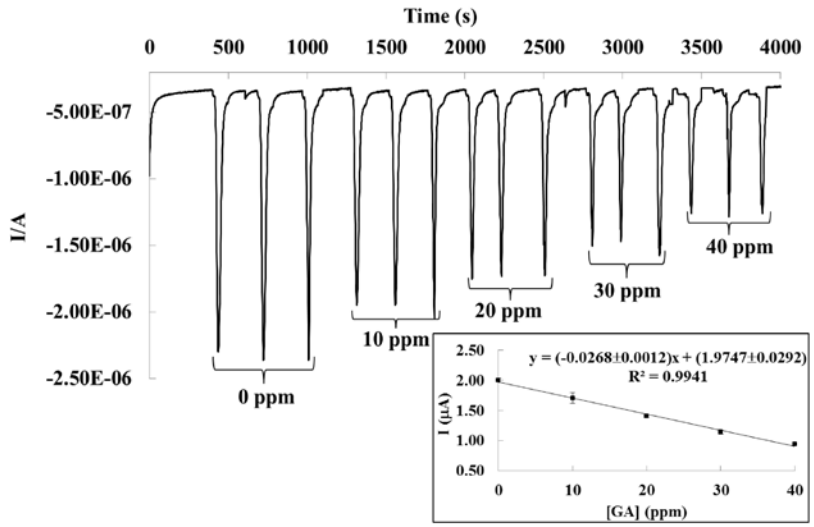

Figure 2 Amperometric response signal and calibration curve from the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay using sequential injection analysis with amperometric detection (SIA-AD)

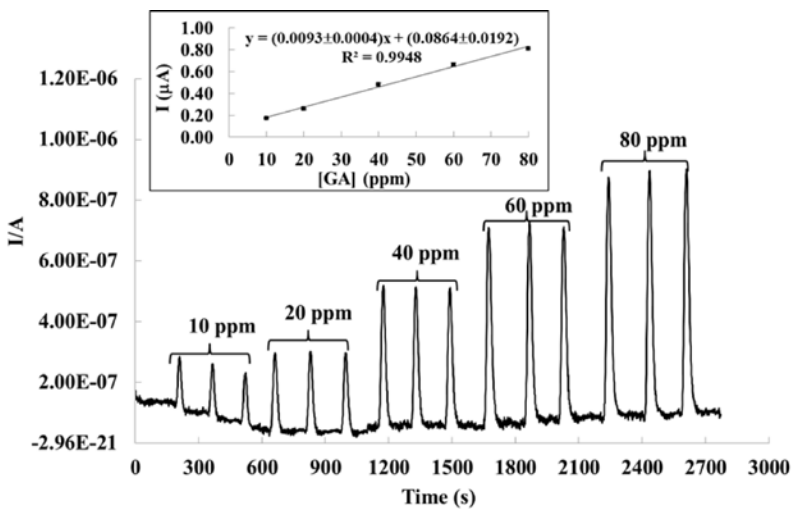

Figure 3 Amperometric response signal and calibration curve from the reducing power measurement using sequential injection analysis with amperometric detection (SIA-AD).

difference between the ABTS assay and reducing power measurement for all samples except the tea sample. This might have been due to the different mechanisms of the methods and the tea sample needed much more dilution than the other samples, especially, for the spectrophotometric detection which provided a narrower working concentration range, compared to the electrochemical detection. Since no single assay can accurately reflect all antioxidants in a sample that contains several substances (Prior *et al.,* 2005), more than one assay based on different reactions should be used. The proposed SIA-AD can possibly apply to other widely used radical scavenging methods

Table 2 Analytical characteristics of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay and reducing power measurement by the proposed sequential injection analysis with amperometric detection

Parameter	ABTS assay	Reducing power measurement
E_{appl}, (V vs Ag/AgCl)	-0.10	+0.50
Reagent conc., volume (mM, μL)	0.50, 300	0
Sample volume (μL)	100	500
Linear working range (ppm GA)	0–40	10–80
Regression coefficient	0.9941	0.9948
Sensitivity (μA/ppm GA)	-0.0268	0.0093
% RSD of GA detection (conc.), n =10	6.4 (20 ppm)	2.4 (40 ppm)
Detection limit (ppm GA)	4.2	7.2
Sample throughput per hour	22	36

GA = gallic acid

Table 3 Total antioxidant capacity of sample analysis obtained from two methods using the proposed sequential injection analysis with amperometric detection (SIA-AD) and the classical spectrophotometric 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

Sample	GAE (mg/g sample)*		
	ABTS assay by SIA-AD	Reducing power by SIA-AD	ABTS assay by spectrophotometry
Tea	$36.46 \pm 2.25_4$	$37.70 \pm 3.84_4$	$31.93 \pm 0.99_5$
Ginger	$0.90 \pm 0.12_7$	$0.26 \pm 0.03_1$	$0.76 \pm 0.01_6$
Cinnamon	$1.56 \pm 0.15_1$	$0.59 \pm 0.06_5$	$1.57 \pm 0.09_1$
Safflower	$0.15 \pm 0.02_1$	$0.08 \pm 0.00_6$	$0.14 \pm 0.02_0$
Chrysanthemum	$0.17 \pm 0.01_2$	$0.05 \pm 0.00_2$	$0.15 \pm 0.03_0$

*(expressed as gallic acid equivalent (GAE) as mean ± SD, n=3)

for TAC determination such as DPPH assay as the DPPH reagent is also an electroactive species and it was successfully applied with FIA-AD (Amatatongchai *et al.,* 2012). In addition, other standard antioxidants can be used for capacity calibration whenever there is an electrode reaction from any electroactive species being monitored.

CONCLUSION

SIA using a peristaltic pump and amperometric detection with pencil lead electrodes was developed which can be applied to two different mechanisms of antioxidant capacity assays—radical scavenging ABTS assay and reducing power measurement. The low-cost pencil lead could substitute for expensive glassy carbon material in the electrode fabrication and offered the opportunity to scale down the electrode and cell bodies while still retaining good analytical performance. The purpose was to reduce the reagent consumption and hence, lower the disposed waste, which correspond to a reduced cost of analysis also being achieved.

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