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Sequential injection analysis with electrochemical detection as a tool for economic and rapid evaluation of total antioxidant capacity

S. Chan-Eam[a,b], S. Teerasong[a,c], K. Damwan[d], D. Nacapricha[a,b], R. Chaisuksant[d,*]

[a] Flow Innovation-Research for Science and Technology Laboratories (First Labs), Thailand
[b] Department of Chemistry, Center of Excellence for Innovation in Chemistry Faculty of Science, Mahidol University, Bangkok 10400, Thailand
[c] Department of Chemistry, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand
[d] Department of Chemistry, Faculty of Science, Silpakorn University, Nakorn Pathom 73000, Thailand

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ABSTRACT

This work presents a new flow-based coupled electrochemical technique for evaluation of "total antioxidant capacity (TAC)". A sequential injection (SI) with amperometric detection was applied to the TAC analysis of commercial instant ginger infusion beverages using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. Besides having chromogenic properties, the ABTS reagent behaves as an electroactive species at the glassy carbon electrode in phosphate buffer pH 7.0, the decrease of the cathodic current signal of the $ABTS^{\bullet+}$ radical after reaction with antioxidants can be monitored. The SI system, furnished with an in-house electrochemical detection cell (ECD), was optimized with respect to the applied potential, sample and reagent volume, and flow rate to the detector. Gallic acid was used as the standard antioxidant and the capacity was reported as gallic acid equivalent (GAE) unit. TAC measurements of ginger infusions at the optimum condition were performed using the proposed technique and also with the classical batch spectrophotometric ABTS assay. TAC values obtained from our method and the standard method are in good agreement ($r^2 = 0.956$). The SI-amperometric technique provided satisfactory precision (4.11% RSD) with rapid sample throughput (40 samples h^{-1}). Also using this method, the consumption of the expensive ABTS reagent was greatly reduced.

1. Introduction

Presently, people have increasing interest in healthy living. There are various food products available for promoting health care. Many kinds of herbs are processed as herbal drinks, such as tea, chamomile, mint, basil, cinnamon, catnip, aloe vera, ginseng and ginger. Ginger (*Zingiber offcinale* Rosc.), which belongs to the family Zingiberaceae, has a long history of cultivation and its rhizome part has been used as a spice and for medicinal purposes for over 2000 years [1]. Ginger contains pungent phenolic substances (e.g., gingerols, shogaols, paradols and zingerone) with antioxidative, anti-inflammatory, anti-tumor, anti-carcinogenic and anti-mutagenic activities [1–4]. Petroleum ether extracts of the ginger rhizome exhibited high antioxidant activity comparable to those of commercial antioxidants such as Sustane 20 and Sustane HW-4 [5]. Moreover, cinnamic acid and gallic acid were also reported as the major antioxidant phenolic compounds in ginger [6]. Natural ginger has been processed into instant ginger tea or ginger infusion powder and is one of the popular beverages in Asian countries like China, India, Indonesia, Vietnam and Thailand [7,8]. However, as far as we know, no antioxidant capacity level for the processed ginger products of Thailand has been given on any package labels. Information of the antioxidant capacity can be used to guarantee the quality of the products to the customers as well as for the process monitoring. Analytical technique that can be employed in the manufacture industry should be simple, convenient, automatic and economic.

A number of methods have been presented to evaluate the total antioxidant capacity (TAC) [9–17]. The most common in vitro methods are based on radical scavenging, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [13] and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay [14,15], or reducing capacity, such as ferric reducing antioxidant power (FRAP) [16]. ABTS is the most widely used reagent because it is a simple and convenient assay. The method is a decolorization reaction. Mixing of the oxidizing agent (peroxide or persulfate) and ABTS in aqueous solution generates the radical cation ($ABTS^{\bullet+}$), which is blue-green in color. After the addition of an antioxidant, $ABTS^{\bullet+}$ is reduced back to ABTS and the blue-green color disappears. The remaining $ABTS^{\bullet+}$ can be measured via absorption at wavelength 734 nm. Although the classical ABTS assay is uncomplicated, it is time consuming in order to reach the equi-

* Corresponding author. Tel.: +66 34 255797; fax: +66 34 271356.
E-mail address: rasameec@su.ac.th (R. Chaisuksant).

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librium state. The electrochemical (EC) behavior of ABTS has been reported [18] and ABTS has been used as the redox mediator for catalytic reaction of laccase enzyme [19–21]. Later, ABTS assay was applied to flow injection techniques in order to reduce the analysis time [22–24]. In 2007, Milardovic et al. reported the use of a flow injection analysis (FIA) system with biamperometric detection for determination of TAC in alcoholic beverages [25] and another system for tea, wine and juices [26]. Both systems employed online enzymatic generation of $ABTS^{\bullet+}$ radical. Analysis rates were 42 and 37 samples h^{-1}, respectively. However, continuous flow system requires large reagent consumption and waste.

Recently, various flow based techniques were reviewed for fast screening of antioxidant capacity [27]. Sequential injection analysis (SIA), the second generation of flow based method, was first reported for ABTS assay by Pinto et al. in 2005 [28]. The technique was improved and published a few years later [29,30]. Clearly, SIA has advantages over FIA in reducing reagent and sample consumption because SIA is based on non-continuous flow [31]. The syringe pump in SIA is under computer control, thus an automated system can be developed for monitoring the TAC in large number of samples. As yet, no report has proposed SIA with an electrochemical detection for assessing TAC.

This work presents a new economic system for evaluation of TAC based on $ABTS^{\bullet+}$ radical scavenging. The SIA was employed for microliter handling in order to reduce the ABTS reagent, which is an expensive chemical. Detection of the remaining $ABTS^{\bullet+}$ radical, after reaction with antioxidant, was achieved via an amperometric method using an in-house electrochemical detection cell (ECD). The developed procedure was applied to monitor the TAC in commercial ginger drinks in term of gallic acid equivalent, GAE (mg g^{-1} sample), which then represents the total amount of antioxidant capacity in each sample.

2. Experimental

2.1. Reagents and solutions

All the reagents used were of analytical grade. 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was from Fluka (Germany) and potassium persulfate (di-potassium peroxodisulfate) was from Merck (Germany). The antioxidant standard was gallic acid (GA) monohydrate, 98% from Riedel-de Haën (Germany). A 0.10 mM phosphate buffer pH 7.0, prepared from Na_2HPO_4, 98% and NaH_2PO_4, 99% from Fluka (Germany), was used as supporting electrolyte and carrier in the flow system. Aqueous solutions of GA standard were prepared daily using ultrapure water (Branstead EASYpure II, USA). The stock $ABTS^{\bullet+}$ solution was prepared by mixing 0.0548 g of ABTS and 0.0094 g of $K_2S_2O_8$ in 100 mL ultrapure water and stored in dark for 12 h before analysis. The concentration of $ABTS^{\bullet+}$ solution was determined from spectrophotometric method using molar extinction coefficient 1.5×10^4 L mol^{-1} cm^{-1} at 734 nm [14]. For the SIA experiment, this $ABTS^{\bullet+}$ solution was directly used. For the classical experiment, the $ABTS^{\bullet+}$ solution was diluted to give 0.7 absorbance at 734 nm.

2.2. Sample

Instant ginger powder sample of various Thai brands were purchased from the local supermarkets. Sample solutions were freshly prepared by dissolving an accurate weight of sample powder (in the range of 1–9 g as the recommended amounts on the package labels) in 50 mL boiling deionized water. After stirring, the infusions were left to reach room temperature (about 10 min). The sample was then filtered through the 11 μm Whatman® filter paper if necessary. These solutions were analyzed using the developed SIA method and the classical spectrophotometric method.

2.3. Apparatus and instrumentation

The SIA system was constructed as shown in Fig. 1. The SIA module was assembled from a syringe pump equipped with an eight-port selection valve (Kloehn Versa Pump 6, USA). The 10-mL zero dead volume syringe was fitted with a 1.48 mL holding coil (1.59 mm i.d. × 745 mm length). The pump and valve were computer controlled by means of the in-house software written with LabVIEW 8.0™. Electrochemical measurements were carried out with a potentiostat, microAutolabIII (Metrohm, The Netherlands) with GPES software (version 4.9.005) for data setting and analysis. For the flow amperometric detection, the SIA system was furnished with an in-house electrochemical flow-cell (made of acrylic resin with cell volume of 350 μL) and three-electrode system composed of an Ag/AgCl reference electrode (RE), 3 mm diameter glassy carbon (GCE) working electrode (WE) and a second GCE as counter electrode (CE) (dotted area of Fig. 1). Before measurement, the GCE was polished with alumina using a damp polishing cloth (Metrohm, The Netherlands). In the study of the electrochemical behavior of ABTS reagent, cyclic voltammograms were measured in a standard electrochemical cell with the same three-electrode system used in the SIA. A pH-meter (Thermo Orion model 420, USA) with combination glass electrode (Orion 9156BNWP, USA) was employed for pH adjustment of the buffer solution.

The classical batch method was carried out using diluted $ABTS^{\bullet+}$ reagent with GA as standard (final concentrations 0.00–0.80 ppm). After 30 min reaction time, the absorbance was measured at 734 nm with a single-beam Jenway 6405 UV/Vis Spectrophotometer (UK), equipped with a cuvette of 10 mm light path (Agilent Technologies 1000-0544, Germany). TAC values were calculated via EC_{50}, 50% effective concentration, and expressed in GAE unit (mg g^{-1} sample) as described by Boonyuen et al. [32].

2.4. Sequential injection procedure

As shown in Table 1, the sequential injection procedure has four main steps for one cycle. Firstly, 4500 μL of phosphate buffer was aspirated into the system at a flow rate of 18 mL min^{-1}. Next, two 37.5 μL segments of standard or sample (port 1–4 for GA standards and port 5 for sample, respectively) partition with a 375 μL segment of $ABTS^{\bullet+}$ solution (port 6) were sequentially aspirated into a holding coil at the flow rate of 18 mL min^{-1}. Then, syringe was moved forward and backward three times to induce zone mixing. Finally, the sample solution was propelled to the detection cell through port 7 at the flow rate of 12 mL min^{-1}. A decreased signal (compared with blank sample) is observed because $ABTS^{\bullet+}$ is reduced back to ABTS by antioxidant compounds in the sample.

3. Results and discussion

3.1. Electrochemical behavior of $ABTS^{\bullet+}$ and GA

Fig. 2a shows the electrochemical behavior of $ABTS^{\bullet+}$ on a glassy carbon working electrode by cyclic voltammetry (CV). The cyclic voltammograms were obtained in phosphate buffer solution pH 7.0 (curve 0) containing $ABTS^{\bullet+}$ (curves 1–5). An applied potential between −0.50 and +1.20 V vs. Ag/AgCl at the scan rate of 0.10 V s^{-1} was used. Curves 1–5 represent the signal corresponding to final concentrations of 0.05, 0.15, 0.26, 0.40 and 0.51 mM $ABTS^{\bullet+}$, respectively. Similar to previous reports in acetate buffer pH 5 [19,22], two oxidation peaks were observed for the oxidation of ABTS to its cation radical ($ABTS^{\bullet+}$) and dication ($ABTS^{2+}$). These two anodic peaks have shown E_{pa} at 0.55 and 0.96 V (vs. Ag/AgCl).

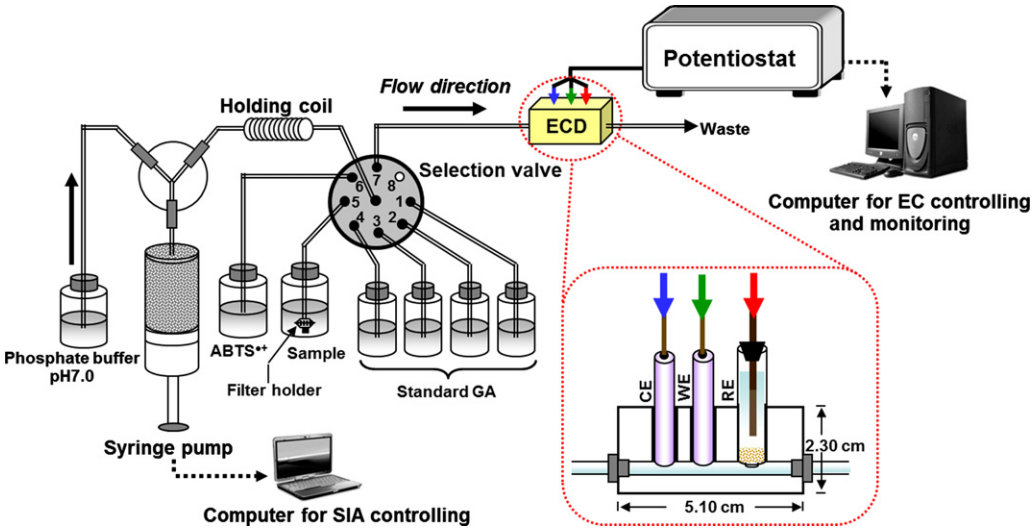

Fig. 1. Schematic diagram of the SIA system for the evaluation of TAC with an in-house flow-through ECD (1.9 cm width × 5.1 cm length × 2.3 cm height). CE, counter electrode; WE, working electrode; RE, reference electrode.

In this phosphate buffer pH 7.0, only the first oxidation step gave a quasi-reversible reaction (ΔE_p = 110 mV) since on the reverse scan only one equal reduction peak current at E_{pc} at 0.44 V (vs. Ag/AgCl) was observed. This first step gave $E_{1/2}$ = 0.495 V (estimated from $E_{1/2} = (E_{pa} + E_{pc})/2$), corresponding to the ABTS/ABTS$^{\bullet+}$ couple. On increasing the concentration of ABTS, the response of the first reaction step increased in both anodic and cathodic directions. The cyclic voltammogram of standard GA was also investigated under the same condition (curve 6). The GA gave a broad irreversible oxidation starting from 0.20 V. Accordingly, an applied potential of −0.10 V was selected for the amperometric evaluation of TAC in the flow system in order to avoid interferences from electrode reaction of other species besides the ABTS reagent in the system. Using EC flow-through detection cell, linearity between current responses and ABTS$^{\bullet+}$ concentrations at potential of −0.10 V is shown in Fig. 2b.

3.2. Effect of applied potential

The effect of applied potential on the sensitivity for the SI system was studied for voltages −0.20 and 0.20 V. From the electrochemical properties of ABTS$^{\bullet+}$ and GA observed in the batch system (Fig. 2a), only cathodic current from the ABTS$^{\bullet+}$ radical reduction process was needed at the applied potential. In the negative potential range, oxidation of the antioxidants in the sample can be avoided. However, the more negative applied potential resulted in higher background current due to the voltage limit of the system. Experimental results revealed that sensitivity reached a maximum at −0.10 V and decreased when the applied potential was more positive than 0.00 V. Thus, −0.10 V was selected.

3.3. Selection of reagent and sample volume

The most important factor in the development of this system is to reduce analysis cost per sample, especially the amount of ABTS reagent. The volume of reagent and sample were optimized using 0, 10 and 60 ppm standard GA solutions. It was observed that the solution volume needed for the proposed SIA method can be reduced to microliter level. We found that 375 μL of ABTS$^{\bullet+}$ was sufficient to cover the analysis range (0–100 ppm GA) with satisfactory signal. Thus, ABTS$^{\bullet+}$ solution at 375 μL was selected for all further experiments. A volume of 37.5 μL of sample segment was finally selected. With these two optimum values, the consumption of ABTS reagent was much less than that in previous FIA methods around 4-fold by volume or 1000-fold by mole [25,26].

3.4. Effect of flow rate on EC detector

In order to lower the analysis time, flow rate of the reaction zone to the detection cell was explored. Experiments were carried out at flow rate of 6, 9 and 12 mL min^{-1} with GA in concentration range as in the former experiment. The sensitivity of the three rates was founded to be similar. Thus, a rapid system could be operated at flow rate of 12 mL min^{-1}, with analysis time of 88 s sample^{-1}. At higher flow rate, there is risk of air bubble appearing in the flow line.

Table 1
SIA protocol sequence for the experiment.

Step	Flow rate (mL min^{-1})	Volume (μL)	Flow direction	Event
1	18	4500	Reverse	Carrier aspirated
	18	37.5	Reverse	Standard/sample zone segment 1 aspirated
2	18	375	Reverse	Reagent zone aspirated
	18	37.5	Reverse	Standard/sample zone segment 2 aspirated
3	6	–	Reverse	
	6	–	Forward	
	6	–	Reverse	Mixing of zones
	6	–	Forward	
	6	–	Reverse	
	6	–	Forward	
4	12	4950	Forward	Zones sent to EC detector
End of cycle				

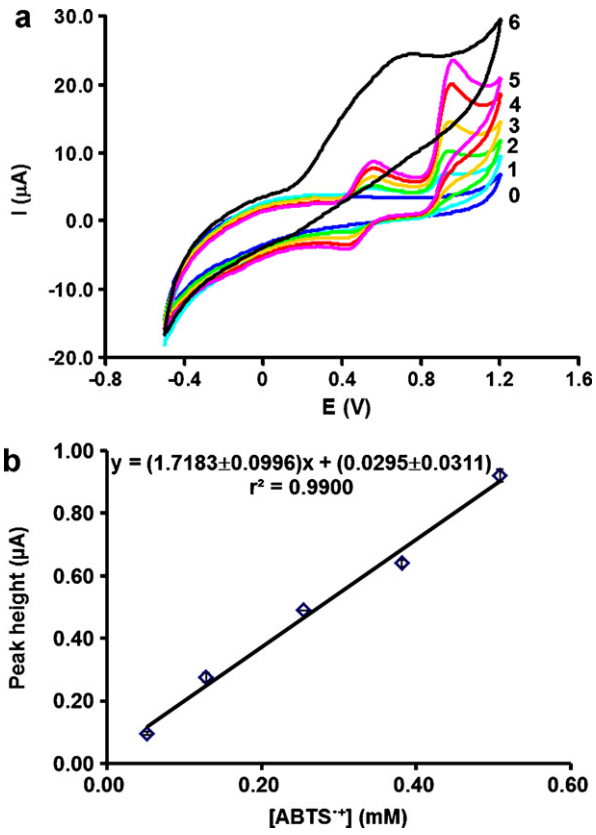

Fig. 2. (a) Cyclic voltammograms of $ABTS^{\bullet+}$ in phosphate buffer pH 7.0 at various concentrations (0.00–0.51 mM) (curves 0–5) and of 200 ppm GA standard (curve 6), obtained at scan rate 0.10 V s^{-1} under batch operation. (b) Linear plot of the current response with $ABTS^{\bullet+}$ concentration at the applied potential of −0.10 V obtained under the SIA operation.

3.5. Analytical performance

The response of standard GA was measured under the optimum condition at room temperature (25 °C). Fig. 3 shows (negative) signals with the GA concentrations (1–100 ppm). The inset shows a calibration graph of a plot of the GA concentration against the peak height. The linear equation was peak height, μA = (−0.0300 ± 0.0014) GA concentration, ppm + (3.1547 ± 0.0846) with r^2 = 0.9937, and %RSD = 4.11 (n = 12). Limit of detection was 7.50 ppm, calculated as a three times standard deviation of blank signal. Sample analysis time was 88 s, so that the throughput of 40 samples h^{-1} was achieved.

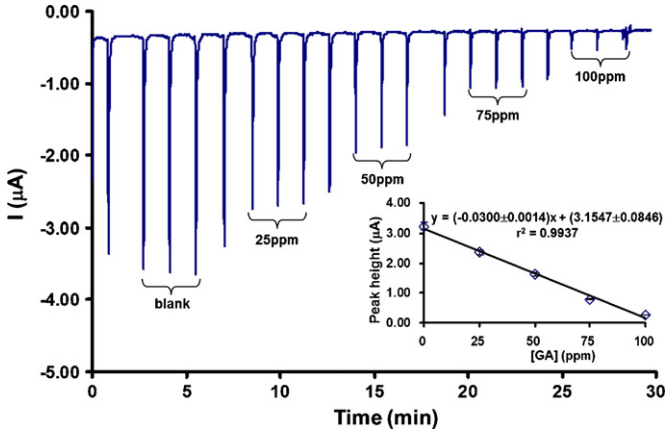

Fig. 3. Signal profile obtained from the proposed SIA system. Three consecutive signals of each GA concentration were used to construct the calibration graph (inset). *Note*: when changing from one standard/sample to another, the first readings were negligible. To ensure the complete flushing of the system with new sample, the readings from next second injections are usable as analysis signals.

Table 2
Comparison between the total antioxidant capacity as GAE from the proposed SIA and the classical method.

Sample	GAE[a] (mg g^{-1} sample)	
	Proposed SIA method	Classical method [32]
A	1.201 ± 0.023	1.195 ± 0.017
B	1.160 ± 0.044	1.029 ± 0.038
C	0.771 ± 0.024	0.855 ± 0.003
D	0.616 ± 0.044	0.558 ± 0.009
E	0.436 ± 0.030	0.372 ± 0.014
F	0.326 ± 0.025	0.273 ± 0.005
G	0.375 ± 0.021	0.199 ± 0.005

[a] Expressed as a mean ± standard deviation obtained from triplicate determinations.

3.6. Evaluation of total antioxidant capacity of ginger infusion

The proposed method was applied to evaluate TAC of local instant ginger powder samples. Seven commercial samples were analyzed by using the SIA method compared with a classical spectrophotometric method [32]. For the SIA method, TAC could be determined using the calibration graph of GA (Fig. 3) and expressed in GAE unit (mg g^{-1} sample). GAE values are summarized in Table 2. The correlation between the SIA and classical methods is good (slope = 0.9096 ± 0.087, intercept = 0.1155 ± 0.064 and r^2 = 0.956). The results from both methods are in good agreement with t_{stat} = 1.83 and t_{crit} = 2.57 at 95% level of confidence [33].

For turbid or cloudy samples, an on-line filtration unit (Millipore-Swinnex, USA) was tested. The filter holder was fitted at the end of the sample port (port 5) and a glass fiber filter (13 mm diameter) was placed inside the holder. Sample particles were thus filtered out to prevent clogging of the flow system. Life time of the filter depends upon the sample matrices. In this work, the on-line filtration unit was evolved for samples of instant ginger infusion. We found that the current signal of the sample, with and without the filtration unit, were comparable with 3.21% relative error. Thus, connecting the holder into the system could be an alternative for direct analysis without any prior sample preparation. The effect of particle or colloidal on amperometric detection may result in the noisy signal but this is not the problem as long as the flow is constant. In this work, the adsorption on the electrode surface was negligible.

4. Conclusions

Many consumers know of the antioxidant property of beverages. Nevertheless, most manufactures are unaware that antioxidant capacity of their product can be measured with approximate quantity of the antioxidants (as TAC). The purpose of our work is to propose the applicability of SIA with electrochemical detection for evaluation of antioxidant capacity, using ginger infusion drinks as an example. Application of this method to other antioxidant samples and standards could also be accomplished as all reducing antioxidants which react with the $ABTS^{\bullet+}$ are expressed as the standard used.

The results demonstrated that SIA together with amperometric detection can be a useful tool in evaluation of TAC by ABTS assay. An advantage of the electrochemical detection for this measurement is that a colored sample has no effect on the detector. For turbid sample, a filter-holder was attached at the end of the sample port for on-line filtration. Moreover, implementation of the

SIA system to the amperometric ABTS assay has significant advantage in reduced consumption of the costly reagent. Since no single antioxidant capacity assay can really reflect the "total antioxidant capacity", our developed technique can be a convenient method for rapid and automatic screening providing data of antioxidant capacity that is comparable with other commonly used assays.

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