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Contributed Paper

Development of Sequential Injection Analysis Using Peristaltic Pump and Electrochemical Detection for Antioxidant Capacity Test by ABTS Assay†

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ABSTRACT

Sequential injection analysis (SIA) with electrochemical detection was developed for antioxidant capacity test by 2, 2′-azino-bis(3-ethybenzothiazoline-6-sulfonic acid) (ABTS assay) with collaboration of researchers from chemistry and computing departments. Manifold and sequential steps of the SIA system were designed prior to the computer program was written in Processing Language along with fabrication of electronic circuit using Arduino board for semi-automatic controlling of the bi-directional Masterflex peristaltic pump (model 77521-40). The attaining pump drive controller program provided the following advantages: 1) Connection with the Arduino board so that the voltage in pulse width modulation mode was given to the pump according to the flow rate and time required. 2) User command screens to define flow rate, number of step and time of each step in the SIA system. 3) Pump operation timer with the warning sound and message for change of port position and flow direction at the end of each step. Precision of results and user convenience were improved more than those without using the pump drive controller program. Amperometric detection after chemical reaction was performed by laboratory made electrochemical flow-through cell with three electrode system. The ABTS radical cation left after reaction with antioxidant gave the cathodic current on glassy carbon working electrode at the applied potential of -0.10 V vs. Ag/AgCl. The standard antioxidant, gallic acid showed linear calibration curve in the concentration range of 0-70 ppm (R^2 = 0.9969). Antioxidant capacity tests of various commercial ginger samples were reported in gallic acid equivalent unit with satisfactory results. The sample throughput rate of the SIA system was 9 samples/h at the constant flow rate of 2.0 mL/min.

Keywords: ABTS assay, antioxidant capacity, electrochemical detector, ginger infusion, pump drive controller program, sequential injection analysis

1. INTRODUCTION

Roles and importance of antioxidants in prevention of oxidative stress which causes a wide range of chronic and acute disease processes [1] arouse scientists in searching antioxidants and evaluation of antioxidant capacity in various samples. Several methods for measuring the antioxidant capacity of biological samples, fruits and herbs have been proposed and reviewed [2-5]. One of the most widely used methods for antioxidant capacity assays in vitro is the 2, 2′-azino-bis(3-ethybenzothiazoline-6-sulfonic acid) (ABTS assay). This radical-scavenging assay evaluates the antioxidant activity by the scavenging power on the generated radical and the left reagent is classically monitored by spectrophotometry. Review on flow injection methods for fast screening of antioxidant capacity assays was given by Magalhaes, et al. [6]. Incorporation of the flow techniques with relevant automation to those assays provided the main advantages as enhancement of sample throughput, suitable for routine use and screening purpose.

Sequential injection analysis (SIA) and Flow injection analysis (FIA) base on similar principles in controlled partial dispersion and reproducible sample handling [7] but the non-continuous flow of SIA gives the advantage in reducing sample and reagent consumption. SIA has been applied to ABTS assay using spectrophotometric detection [8-10]. Besides having chromogenic property, the ABTS reagent showed electrochemical behaviour as an effective redox mediator in electro-catalytic reaction with the laccase enzyme [11-12]. Previous works on ABTS assay using flow injection analysis with peristaltic pumps and electrochemical detection (FIA-ECD) [13-14] and SIA-ECD with a syringe pump [15] have been reported. Although, the syringe pumps are more suitable for SIA works as they offer pulse-less and highly precise flow but they require priming before use, have a limited reservoir volume and are relatively expensive, employing peristaltic pumps from the classical FIA are also possible [16]. Reports on the home-made or called in-house written program based on LabVIEW software [17] and Microsoft QuickBasic [10] to control their peristaltic pumps in the SIA systems were published. Both works used the same type of peristaltic pump, Gilson Minipuls 3 (Villier le Bel, France).

The aim of our work was also to develop the in-house computer software to control the Masterflex peristaltic pump (Cole-Parmer Instrument Co., USA) of the SIA-ECD system and apply to the ABTS assay. Application on antioxidant capacity test of the ginger samples was also presented.

2. MATERIALS AND METHODS

2.1 Reagents and Samples

The ABTS reagent as diammonium salt was purchased from Fluka. Standard antioxidant gallic acid (GA) monohydrate, 98% was from Riedel-de-Haen (Germany). Potassium persulfate was from Merck Germany). The other common reagents were of analytical grade from Fluka. Deionized water was used for the preparation of all solutions. The $ABTS^{o+}$ cation radical solution was prepared by oxidation the ABTS reagent with potassium persulfate as previously described [15]. The concentration of $ABTS^{o+}$ solution determined from spectrophotometry was 0.51 mM. The carrier solution, 0.1 mM phosphate buffer pH 7.0 was prepared from disodium hydrogen phosphate and sodium dihydrogen phosphate.

Instant ginger samples of various Thai brands were purchased from the local

supermarkets. For ginger infusion, accurate weight (1.XXXX g) of ginger powder was added to 25 mL deionized water and heated up to 70°C in water bath for 30 min. The residue was filtered off through 11 µm Whatman® filter paper and the volume of the supernatant was made to 25 mL with deionized water.

2.2 Instrumentation

The SIA system as in Figure 1 was designed to enable the ABTS assay. The bi-directional peristaltic pump of Masterflex (Cole-Parmer Instrument Co. USA) comprised of the pump drive model 77521-40, pump head 07519-10 and cartridges 07519-85. The cartridge tubing was the

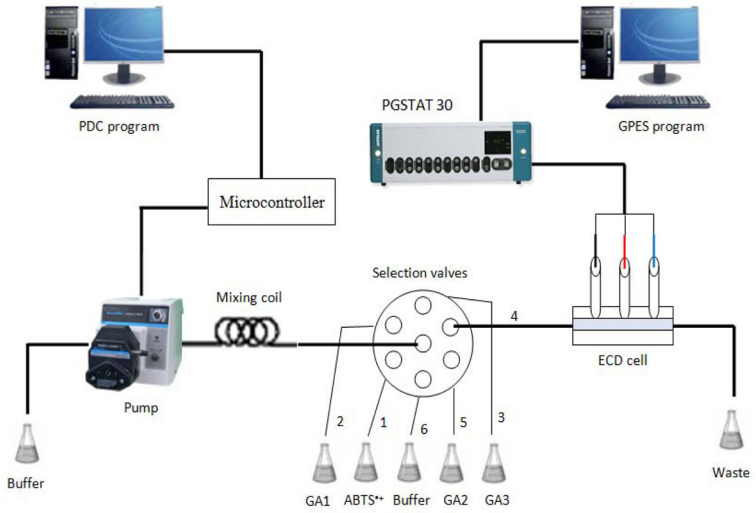

Figure 1. Schematic diagram of our SIA-ECD system.

two-stop Tygon tubing of 1.02 mm i.d. which connected to the holding/mixing coil of 1.07 mm i.d., 200 cm length PTFE tubing.

The Pump Drive Controller (PDC) program was developed on a MacBook featuring Intel Core 2 Duo processor with 2GB memory. The interface electronic circuit was the voltage extender of 0-5 V from Arduino microcontroller to 0-10 V as required to the pump drive. The PDC software was written in Processing Language to monitor the pump drive for switching on/off and the voltage was applied in Pulse Width Modulation (PWM) mode to drive the eight rollers of the pump head at the corresponding speed. The speed of the pump was then, calibrated to flow rate of solution in mL/min.

A six-port valve (V-341, Upchurch Scientific, USA) served as the selection valve connected to mixing coil, various solutions and electrochemical detector (ECD) cell via PTFE tubing. The valve was manually turned to each position at the specified time with assistance of the warning message on the monitoring screen and voice from the PDC program.

The flow-through cell body was constructed in-house from transparent acrylic resin with cell volume of 350 µL. The working electrode was a glassy carbon electrode (GCE) (3 mm-diameter disk), the reference electrode was a Ag/AgCl (sat. KCl) and another GCE served as a counter electrode. The three electrodes were connected to the potentiostat- Autolab PGSTAT30 and controlled by the GPES software version 4.9.007 (Eco Chemie, The Netherlands) for applying potential and data processing in the chrono-amperometry

mode. Pretreatment of the GCEs was done by polishing with alumina on a damp polishing cloth (Metrohm, The Netherlands) before fixing to the flow cell.

2.3 SIA-ECD Procedure

Our accomplished PDC programs can be installed on Intel Mac (Intel Core 2 Duo machine with at least 2GB memory and OS × 10.4 or later version) or Window PC (Intel Core 2 Duo or later version with at least 1.06 GHz speed and at least 2 GB memory). The first PDC program version 1.0 can control pump to drive solution at a fix flow rate. Figure 2 illustrated the user command screens of the PDC program while working. The main program window was for description of the experiment.

Figure 2. Computer screens of the PDC program: (a) Main window for project description, (b) Calibration Process window for flow rate calibration and (c) Definition of Step window.

Other command windows were opened from the menu of this main window.

Experimental work started with the Calibration Process Window to calibrate the potential signal-PWM and the flow rate. The relationship between PWM and flow rate was reported in regression equation before a fix flow rate was chosen for solution aspiration. The step sequence of SIA was created in the Definition of Steps Window as the protocol sequence in Table 1. The flow rate of 2 mL/ min. was used throughout. The aspirated sequence into the holding/mixing coil are as follows: antioxidant standard/sample was aspirated in two segments (step 1 and 3) and the $ABTS^{o+}$ radical 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 three times for mixing the solutions before the solution was propelled to ECD cell. The analytical signal of amperometric detection was the cathodic current from reduction at the applied potential -0.10 V vs Ag/AgCl on GCE working electrode of the $ABTS^{o+}$ radical left after reaction with the antioxidant. The peak current from antioxidant sample was calibrated with the calibration curve of standard gallic acid. The antioxidant capacity results were expressed as the gallic acid equivalent (GAE) unit in mg gallic acid/g ginger powder.

Table 1. SIA protocol sequence for the ABTS assay.

Step	Valve position	Volume (μL)	Time (s)	Flow direction	Event
1	2	67	2	Reverse	Std./sample zone 1 aspirated
2	1	600	18	Reverse	Reagent zone aspirated
3	2	67	2	Reverse	Std./sample zone 2 aspirated
4	6	500	15	Reverse	Carrier aspirated
		-	8	Forward	
		-	8	Reverse	
5	6	-	8	Forward	Zones mixing
		-	8	Reverse	
		-	8	Forward	
		-	8	Reverse	
6	4	-	220	Forward	Zones sent to ECD

3. RESULTS AND DISCUSSION

3.1 Semi-automatic Controlling of SIA-ECD

The developed PDC program Version 1.0 can be installed and run on Intel Mac or Window PC as stated before. However, the program will run properly on 32-bit Windows XP. Windows 7 is not recommended for running this program. Although, the direction of flow at the pump drive and valve position of the multi-selection valve were manually drive, the precision of peak current from SIA-ECD with the PDC program was improved when compared to SIA-ECD without the PDC program. It was found that the %RSD of the peak current obtained from three replicate measurements of the pure $ABTS^{o+}$ solution reduced from 4.70 to 4.14% and after reaction with 10 ppm gallic acid, %RSD reduced from 9.39 to 2.75%. Not only the precise volume of reagent and sample were aspirated at the precise time, the PDC program facilitated the user to work step by step with the warning sound and messages for changing the valve position and flow direction. Beginning each SIA work with flow rate calibration process was to ensure the performance of the pump drive and Tygon tubing which is normally less stable and shorter durable than the syringe pump [18].

3.2 Analytical Performance

Optimization of the reagent/sample volume ratio in the sequenced segments was done by varying the aspiration time of each segment with the maximum concentration at 100 ppm of standard GA. The volume ratio at $ABTS^{o+}$ reagent : GA = 4.5:1 was the optimum. At the lower ratio, the signal was at the noise level and at the higher ratio meant to the higher expense of reagent. The volume of $ABTS^{o+}$ at 600 μL was selected to cover the GA concentration range 0-100 ppm for the least volume of a sample segment at 67μL (2 s of aspiration time with the constant flow rate 2.0 mL/min). This consumption of ABTS reagent was about 1.6-fold more than the previous SIA-ECD using syringe pump [15] and about 6.5 fold less than the previous FIA-ECD with the same peristaltic pump and same flow rate [14]. Response signals of the standard GA from our system at concentration range 0-70 ppm is shown in Figure 3 with a calibration curve of linear equation $Y = (-6.208 \pm 0.202)$

X + (609.85 ±8.30) with R^2 = 0.9969; where Y = peak current in nA, X = GA concentration in ppm. The higher concentration of GA resulted in decreasing of the peak current of $ABTS^{0+}$. Precision of peak current measurement from the reaction with 30 ppm GA gave relative standard deviation (RSD) of 2.28% (n = 10). Detection limit calculated from the GA concentration giving a signal equal to the blank signal plus three standard deviation of the blank was 5.60 ppm. Sample analysis time was 305 s, the throughput of 11 samples/h was possible but in practical, 9 samples/h. The new PDC program (Version 1.1) provided variation of the flow rate in each step

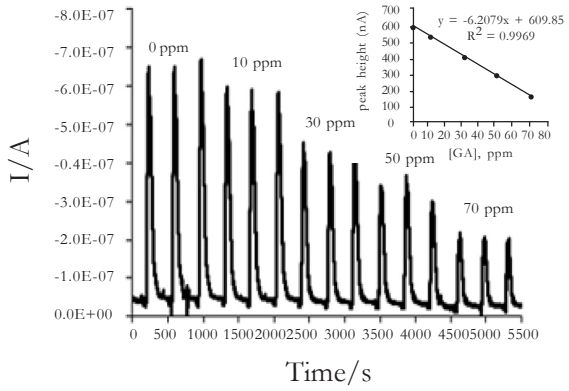

Figure 3. SIA-ECD signals of three replicate measurements of each concentration of GA and calibration curve.

was under investigated to give the higher sample throughput.

3.3 Antioxidant Capacity Test of Instant Ginger Samples

The proposed SIA-ECD for ABTS assay was then applied to determine antioxidant capacity of instant ginger powder from three Thai brands. The results are in Table 2. The GAE values seem correspond to the ginger contents as labeled on their packages.

4. CONCLUSIONS

The SIA-ECD using peristaltic pump was developed for antioxidant capacity test by ABTS assay with collaboration of researchers from chemistry and computer science. The in-house written software and the fabricated electronic interface for the specified pump drive provided ability of using the less expensive instrument and achieved the main advantage of SIA in significant reduction of reagent and sample consumption.

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Table 2. Antioxidant capacity test of instant ginger samples determined from the proposed SIA-ECD for ABTS assay.

Sample	% Ginger labeled	GAE*(mg/g sample)	%RSD
D	85	0.80 ± 0.06	7.8
H	4.46	0.63 ± 0.01	4.0
G	100	1.16 ± 0.04	2.0

*Expressed as mean ± s.d. from triplicate determination.

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