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Simple flow injection for screening of total antioxidant capacity by amperometric detection of DPPH radical on carbon nanotube modified-glassy carbon electrode

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ABSTRACT

An amperometric flow injection (FI) method suitable for evaluation of 'total antioxidant capacity' (TAC) is presented. In this method, a carrier stream of a solution of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) continuously flows through an electrochemical cell, furnished with a carbon nanotube modified-glassy carbon electrode (CNT/GC) as the working electrode. At the applied voltage of 0.05 V (vs. Ag/AgCl), DPPH• is reduced resulting in a constant electric current. For measurement of the TAC, a sample zone containing antioxidant(s) is injected into the carrier stream therein reduction reaction of DPPH• occurring within the sample zone. The decreased amount of the radical in the sample zone leads to a drop of the amperometric signal at the CNT/GC electrode. We have also compared the performance of the CNT/GC electrode to the unmodified GC electrode using cyclic voltammetry. The sensitivity of the CNT/GC electrode was more than twenty five times greater than the bare GC electrode. The study of the sweep rate dependence showed that the cathodic and anodic current of 0.1 mM DPPH solution varied linearly (r^2=0.998) with the square root of the scan rate, from 0.02 to 0.12 Vs^{-1}. These results demonstrated that the CNT/GC electrode is appropriate for the quantitation of antioxidants via amperometric detection of the residual concentration of non-reacted DPPH•. We obtained linear calibrations for all the antioxidants tested including gallic acid, catechin, quercetin, caffeic acid and Trolox. The system offers rapid sample throughput (45 samples h^{-1}) and good precision of 3.2% R.S.D., for 20 μL-injection of 2.5 μM Trolox (n=30). This method was applied to evaluate the TAC of extracts of some Thai indigenous vegetables.

1. Introduction

The protective effect against 'oxidative stress' [1,2] by dietary intake of antioxidant compounds, either as food additives or as pharmaceutical supplements, has been widely recognized [2,3]. Antioxidant compounds from natural sources like phenolic acids, polyphenols and flavonoids can scavenge free radicals and reactive oxygen species (ROS) such as H_2O_2 and $O^{2-\bullet}$ that are present in biological fluids, and thereby inhibit the 'oxidative stress' [2]. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Antioxidants from fruits and vegetables, such as vitamin C, vitamin E, carotenes, and phenolic acids have also been shown to scavenge ROS, that can damage lipid and protein [3]. Thus, it is believed that consumption of food containing antioxidants can reduce the oxidative stress condition in our body as well as delay aging [4,5].

There has been an increasing number of assessments of 'total antioxidant capacity' (TAC) in medicinal plants and biological fluids. A variety of assays have been developed, including colorimetric [6–9], fluorimetric [10,11], and chemiluminescence [12–15] methods. Colorimetric method is based on reaction between a chromogenic radical and the antioxidant. These, chromogenic compounds include 2,2′-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid ($ABTS^{\bullet+}$) [16,17] and 2,2-diphenyl-1-picrylhydrazyl (DPPH•) [18,19], which react directly and rapidly with most antioxidant compounds.

DPPH• free radical reacts with an antioxidant (AH) or a radical species (R•) according to reactions (1) and (2).

$$DPPH^{\bullet} + A-H \rightarrow DPPH-H + A^{\bullet} \quad (1)$$

$$DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH-R \quad (2)$$

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Antioxidant can donate a hydrogen atom to DPPH$^{\bullet}$ and thus decrease the concentration of the stable free radical (DPPH$^{\bullet}$), resulting in the color change from violet to pale yellow. Non-reacted DPPH$^{\bullet}$ can be monitored by spectrometric or electrochemical method [20].

In the spectrometric method, the absorbance of DPPH$^{\bullet}$ is measured at 518 nm. This method was first reported in 1958 by Blois [18] and further modified by Brand-Williams et al. [21]. It is the commonly accepted assay for evaluating the antioxidant content in various types of samples including, wine [22], fruit [23], medicinal plant extracts [24], synthesized compounds [25,26] and purified natural compounds [27,28]. Recently an automatic procedure for DPPH$^{\bullet}$ assay was developed based on multi-syringe flow injection analysis (MSFIA) [29] for screening and evaluation of antioxidant scavenging capacity. Although these methods are simple, colorimetric detection of DPPH$^{\bullet}$ is prone to interference from color and turbidity of the samples. The methods usually require a pretreatment of samples. Separation of color or matrix interferences by HPLC with post-column detection using DPPH$^{\bullet}$ is one strategy for colored samples with complex matrices [30,31].

For analysis of antioxidant in colored or complex samples, electrochemical methods are more advantageous than spectrometric. Cyclic voltammetry with glassy carbon (GC) electrode has been used for evaluation of antioxidant capacity of blood plasma, tissue homogenates and some plant extracts [32]. Voltammetric behavior of some antioxidants was investigated by using differential pulse voltammetry on GC electrode [33]. Amperometric analysis of antioxidant on GC electrode in flow injection (FI) format was also reported for determination of total phenolic compounds in foods [34] and of antioxidant capacity in honey, propolis and royal jelly [35]. An automated ABTS$^{\bullet+}$ assay by sequential injection analysis (SIA) was developed by Chan-Eam et al. [36] using amperometric detection on GC electrode for analysis of TAC in instant ginger infusion beverages.

Carbon nanotubes (CNT) form an increasingly important group of nanomaterials with unique geometrical, mechanical, electronic and chemical properties [37,38]. Such properties of CNT make them extremely attractive for use in electrochemical detection. Recent studies [39,40] demonstrated that CNT can impart high electrocatalytic activity and decreased surface fouling to electrochemical devices. In view of these remarkable properties of the CNT, a number of applications, employing this relatively new material for electrochemical quantitation, have been reported. CNT graphite-based electrode was employed and its performance was investigated using caffeic acid as a model compound [41]. A modified GC electrode with CNT and Nafion was used to demonstrate its selectivity towards quercetin, which has anti-inflammatory and antioxidant properties [42]. However, in evaluation of the total antioxidant capacity (or TAC) in food and plant extracts, the measurement of the capacity arising from the presence of all compounds that can inhibit the oxidative reactions is desired. Thus, selectivity towards a single compound is therefore not necessary. Although CNT-modified carbon electrodes have been used for electrochemical detections of some compounds with antioxidant activity [41,42], none of these studies are suitable for rapid screening of TAC. In drug discovery, where there is large number of samples for TAC screening per day, a flow technique is more suitable.

This work describes the development of an electrochemical method based on the use of stable radical DPPH$^{\bullet}$ as an alternative to the spectrometric DPPH$^{\bullet}$ methods, which are not suitable for colored plant extracts. In this system, we monitored amperometric signal from reduction reaction of DPPH$^{\bullet}$ at a CNT modified glassy carbon electrode, as the radical is continuously flowed through the electrochemical flow-cell. Injection of a plug of a solution containing antioxidant will decrease the amount of the DPPH$^{\bullet}$ from the reaction between antioxidant and the radical. The drop of the DPPH$^{\bullet}$ signal from baseline level is related to the amount of the reacted DPPH$^{\bullet}$ in the plug. It has been demonstrated that at the optimized voltage, the system could detect five major antioxidant compounds such as caffeic acid, quercetin, gallic acid, catechin and Trolox. In this work, we focused on the analysis of some ethanol-soluble and water-soluble antioxidants typically present in selected food and plants. The proposed method was applied to determine TAC in extracts of various Thai indigenous vegetables.

2. Experimental

2.1. Chemical and reagent

All chemicals used were of analytical reagent grade. Deionized-distilled water was employed for standard and reagent preparations.

DPPH (2,2-diphenyl-1-picrylhydrazyl), quercetin, gallic acid and caffeic acid were purchased from Sigma-Aldrich (St. Louis, USA). Catechin and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Acros Organic (Geel, Belgium). A stock solution (5 mM) of DPPH was prepared by dissolving the appropriate amount in absolute ethanol (Carlo Erba; Milan, Italy). The DPPH solution was ultra-sonicated (Crest 575HT, NJ, USA) for about 2 h and kept in the dark to minimize decomposition by light. Ten millimolar standard solutions of water-soluble antioxidants (Trolox and gallic acid) were prepared using deionized-distilled water. Other antioxidants were prepared in absolute ethanol. Phosphate buffer (0.03 M, pH 7.0) containing 0.03 M KCl and absolute ethanol (60:40, v/v) was used as the buffer electrolyte in all electrochemical measurements.

2.2. Preparation of plant extract

Thai indigenous plants were purchased from the local supermarkets in Ubon Ratchathani Province, Thailand. Six vegetables (tummy wood, red-bead tree, rice paddy herb, metchun, smartweed and teaw) were selected as samples for TAC assay. Botanical names of these vegetables are *Careya sphaerica* Roxb. (tummy wood), *Adenanthera pavonina* Linn. (red-bead tree), *Limnophila aromatica* Merr. (rice paddy herb), *Syzygium gratum* (Wright) S.N. (metchun), *Polygonum odoratum* Lour. (smartweed), and *Cratoxylum formosum* Dyer (teaw).

The extraction scheme previously described by Silva et al., for fruits and leaves [43] was adopted. Fresh vegetable leaves were dried in an oven at 40 °C for 48 h to constant weight, followed by powdering in mortar. Three consecutive extractions with hexane (3 mL × 25 mL) were performed on 2.5 g portions of plant powder to remove both pigments and lipids. Centrifugation (ALC Centrifuge 4218, Milano, Italy) at 3000 rpm was carried out for 5 min and the organic solvent discarded. A 10 mL aliquot of 2% (w/v) of aqueous sodium metabisulphite was then added to the solid residue (to stabilize the antioxidant), followed by 25 mL methanol:water (80:20, v/v). The supernatant was separated out and the solid further extracted two times with the methanol:water solution. The 3 methanolic fractions were pooled together before filtering through a 0.25 μm cellulose membrane prior to the determination of TAC.

2.3. Preparation of the CNT-modified GC electrode

The multi-wall carbon nanotubes (ca. 95% purity) were purchased from NanoLab Inc. (MA, USA). The CNT were further

purified by stirring in a 2 M nitric acid solution for 20 h at room temperature, followed by repeated washing with deionized-distilled water before drying in an oven at 120 °C. Purified CNT were immobilized onto a polished GC electrode (CH Instrument, USA) using dimethylformamide (DMF) as the dispersing agent. A 2 mg portion of CNT was added to 1 mL of DMF, followed by sonication for 3 h. A suitable volume of CNT mixture in DMF was casted on the surface of GC electrode. The electrode was allowed to dry at room temperature for 1 h in a fume hood. Finally, the electrode was rinsed with deionized-distilled water before use. In this work, cyclic voltametric study was carried out to find the optimal volume of CNT mixture required for the casting process.

2.4. Cyclic voltammetry

An Autolab potentiostat PGSTAT 12 (Eco-chemie, Netherlands), equipped with the GPES 4.9 software, was used for all the cyclic voltammetric studies. The active surface area of the GC electrode was approximately 0.07 cm^2. A three-electrode system, consisting of the working electrode (GC or CNT/GC electrode), a reference electrode (Ag/AgCl/KCl (sat.)) and a counter electrode (platinum wire) was employed. Measurements were carried out in the phosphate-ethanol buffer solution as supporting-electrolyte.

2.5. Amperometric detection of DPPH• on CNT/GC electrode by flow injection

Fig. 1 shows schematic diagram of the FI system for amperometric detection of DPPH• at CNT/GC electrode. A Shimadzu pump (model LC-10AD, Japan) was used for the liquid flow. A Rheodyne injector (model 7725, USA), fitted with 20 μL sample loop was used for injecting antioxidant standards and samples. Electrochemical measurements were carried out with a Shimadzu electrochemical flow-through detector (model L-ECD-6A, Japan). Chromatographic software Class VP 6.0 was used for data collection and processing. The area of working electrode was 0.36 cm^2. Amperometric detection was monitored for a flow of 0.25 mM DPPH• in the phosphate-ethanol buffer solution. This DPPH• solution was used as the carrier stream in the FI experiments. Trolox was utilized as the reference antioxidant. A Change in amperometric signal was plotted against the concentration of Trolox to give the calibration curve.

2.6. The colorimetric method

A colorimetric method, based on measurement of the percentage of scavenging activity of DPPH• as a function of the concentration of Trolox, was employed for validation. This method was adopted from the method of Boateng et al. [44]. Plant extract (0.1 mL) was added to 2.9 mL of methanolic solution of DPPH• (6×10^{-5} M). The solution was shaken, stored in the dark for 1 h and the absorbance was measured at 517 nm. All the plant extracts had no absorbance at 517 nm. For control, 0.1 mL methanol was used. The percentage DPPH• scavenging activity of the sample was calculated using Eq. (3), where $A_{control}$ is the absorbance of the control (DPPH• solution without test sample) and $A_{std/sample}$ is the absorbance after addition of standard or test sample.

$$\%\ \text{DPPH}^{\bullet}\ \text{radical scavenging activity} = \left[\frac{A_{control} - A_{std/sample}}{A_{control}}\right] \times 100 \quad (3)$$

Radical scavenging activity of extracts was expressed in terms of milligrams of Trolox equivalent per gram of dry plant (mg of Trolox g^{-1} of sample) using the calibration curve of Trolox.

2.7. Method validation

Samples of Thai vegetable extracts were evaluated for TAC equivalent to the Trolox radical scavenging activity using the developed amperometric flow system in Fig. 1. Sample solutions were diluted (5 or 10 times) before injection. The amperometric results are compared with the results obtained from the colorimetric method [44].

3. Results and discussion

3.1. Cyclic voltammetry of DPPH• on GC and CNT/GC electrodes

Fig. 2(a) shows the cyclic voltammograms (CVs) for DPPH• solution, together with corresponding background from GC and CNT/GC electrodes. The CNT/GC exhibited well defined reversible peaks at 0.327 and 0.305 V, whereas the GC electrode exhibited the oxidation and reduction peaks at 0.362 and at 0.310 V, respectively. The potential differences between the oxidation and reduction peaks were 22 mV (CNT/GC) and 52 mV (GC). The reduction peak current, after background subtracted, was 0.41 μA for GC electrode and was 10.40 μA for CNT/GC electrode. The peak current obtained from the CNT/GC was approximately 25 times greater than that obtained from GC electrode. Therefore, it is expected, that CNT/GC electrode should provide a better sensitivity than bare GC electrode.

The electrochemical mechanism of DPPH• on CNT/GC electrode has not been investigated. However, there have been some reports of cyclic voltamograms of DPPH• on GC electrodes [45,46] and on a paraffin-impregnated graphite electrode (PIGE) [47]. In these works, the authors observed the oxidation of DPPH•, similarly to what is observed in Fig. 2(a). These authors suggested that the anodic and cathodic peaks belong to B/B′ redox couple as shown in Scheme 1. Therefore, it is likely that the oxidation behavior of DPPH• at CNT/GC and GC are the same as that reported in the system of Milardovic et al. [45,46] and Zhuang et al. [47].

Fig. 2(b) shows the cyclic voltammograms of a DPPH• solution on CNT/GC at various scan rates. As shown in the inset of Fig. 2(b), cathodic and anodic peak currents (μA) vary linearly with the square root of scan rate ($V^{1/2}s^{-1/2}$) within the scan range of 0.02–0.12 V s^{-1}. Linear regression analysis gave r^2 value of 0.998. These

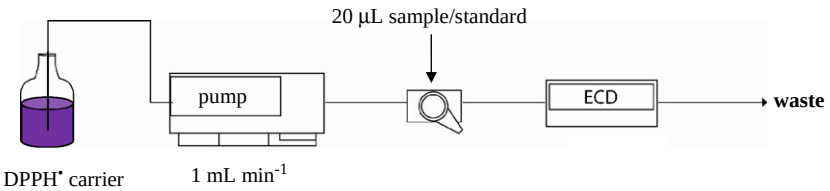


Fig. 1. FI manifold for evaluation of TAC with amperometric detection using carbon-nanotubes modified glassy-carbon (CNT/GC) as a working electrode (WE). Optimal conditions: potential, 0.05 V (vs. Ag/AgCl); carrier, 0.25 mM DPPH in phosphate buffer solution (0.03 M, pH = 7.0) containing 0.03 M KCl and 40% (v/v) ethanol; flow rate, 1 mL min^{-1}.

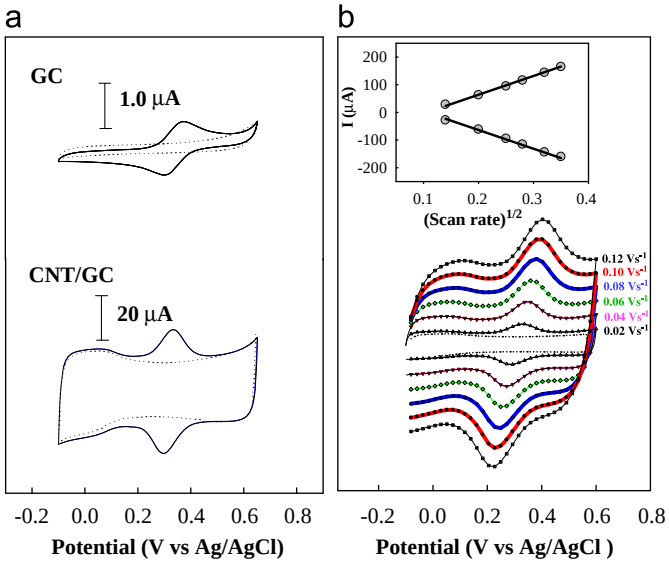
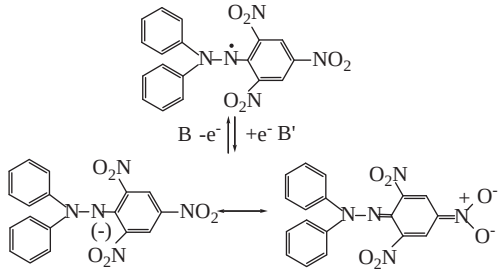


Fig. 2. Cyclic voltametric results of 0.1 mM of DPPH in 0.03 M phosphate buffer (pH 7.0) containing 0.03 KCl in 40% (v/v) ethanol showing (a) CVs obtained from GC and CNT/GC electrodes using scan rate of 0.01 Vs^{-1} (solid line); background voltammograms are shown as dotted lines (b) CVs obtained at various scan rates on CNT/GC electrode with the inset showing the linear relationship between the reduction currents and the square root of the scan rate, slope of 683.52 μA $(sV^{-1})^{1/2}$and r^2=0.998.



Scheme 1. Mechanism of oxidation/reduction of DPPH•.

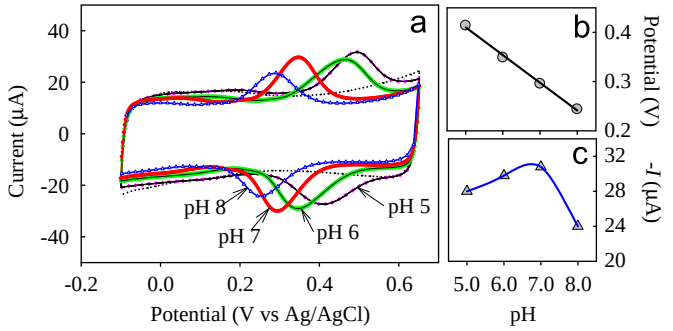


Fig. 3. Results from cyclic voltammetry at various pHs for 0.1 mM of DPPH• in 0.03 M phosphate buffer containing 0.03 KCl and 40 % (v/v) ethanol showing (a) CVs on CNT/GC electrode, (b) peak potential and (c) peak current for various pH.

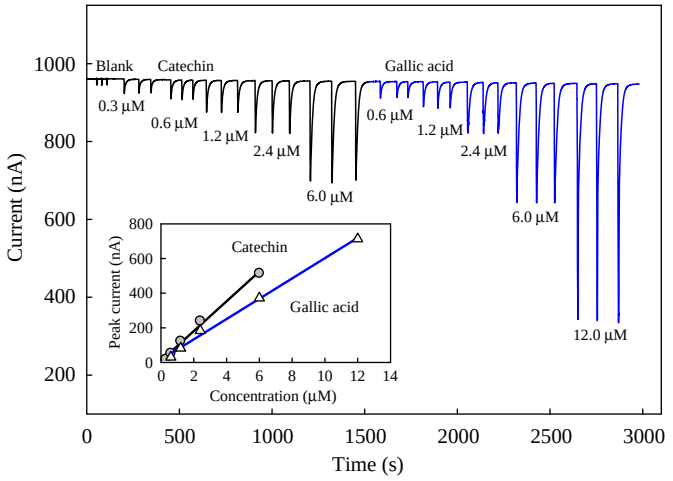


Fig. 4. Examples of signal profiles of the FI system obtained for injections of catechin and gallic acid standards. Operating potential for CNT/GC: 0.05 V (vs. Ag/AgCl); other conditions as in Fig. 1. The inset shows the linear relationship between the signal of antioxidant and the concentration.

results indicated that the current is limited by semifinite linear diffusion of DPPH• to the CNT/GC electrode.

The effect of buffer pH on reduction peak current and peak potential was investigated for pH 5–8 using the 0.03 M phosphate buffer containing 0.03 M KCl and 40% (v/v) ethanol as supporting electrolyte (Fig. 3a). It was observed that the values of peak potential shifted slightly towards less positive values when the pH increased. Fig. 3(b) shows the linear dependency (r^2=0.997) observed in the pH range of 5–8 with a slope of −56.3 mV/pH. This value of the slope indicates the exchange of one electron in the electrochemical reduction of B/B′ redox couple at CNT/GC. Fig. 3(c) shows that the maximum peak current was observed at pH 7.0. Thus, pH 7.0 was selected as the optimum pH for amperometric detection of DPPH•.

3.2. Optimization of the CNT loading

Optimization of the amount of CNT was first investigated using cyclic voltammetry to find the most sensitive condition to detect DPPH•. The experiment was carried out by casting the mixture of CNT in DMF (2 mg mL^{-1}) with 10, 20, 30 and 40 μL, onto the bare GC electrode. Every electrode compositions yielded linear calibration plots over the tested concentration range from 0.025 to 0.100 mM DPPH• (data not shown). The sensitivity (slope) increased with increasing CNT loading, from 10 to 30 μL. However, the noise level dramatically increased at 40-μL loading leading to the drop in sensitivity as compared to the 30-μL loading. We therefore chose 30 μL as the optimal loading. In the preparation of the CNT/GC electrode for use in flow injection system, the volume of CNT mixture 155 μL added had the same optimal volume/area ratio as employed in the cyclic voltammetric experiment.

3.3. Analytical performance

Using the optimum potential for the CNT/GC electrode (0.05 V vs. Ag/Ag/Cl), the flow system in Fig. 1 was employed to study the analytical performance for gallic acid, catechin, quercetin, caffeic acid and Trolox. We observed well-defined negative peaks from all the antioxidants. Representative signal profiles from multiple injections of catechin and gallic acid are shown in Fig. 4. The inset is the linear calibration graphs of these two antioxidants.

Table 1 summarizes the analytical performance of our flow system including the linear calibration equations of all five antioxidants (gallic acid, catechin, quercetin, caffeic acid Trolox). The limits of detection (3σ) are at the low μM levels. The system provides good precision (% R.S.D.=3.2) for 20 μL injections (n=30) of 2.5 μM Trolox. Sample throughput of this system is 45 samples h^{-1}.

Table 1
Analytical performance of the proposed CNT/GC-FI method investigated on five antioxidant standards.

Antioxidant	Linear range (μM)	Calibration parameter			Precision (RSD)[a]	Limit of detection[b] (μM)
		Intercept (nA)	Slope (nAμM^{-1})	r^2		
Gallic acid	0.6–12	15.9	58.6	0.996	1.3	0.04
Catechin	0.3–6	9.10	85.9	0.993	1.5	0.02
Quercetin	0.3–6	7.50	111	0.995	1.5	0.03
Caffeic acid	0.6–12	19.8	760	0.996	2.0	0.08
Trolox	0.3–8	14.9	80.1	0.996	1.2	0.04

[a] Calculated from the signal of 0.8 μM of each antioxidant standard ($n=3$).
[b] Calculated from 3σ ($n=10$) of the signals from the lowest concentration of each working range.

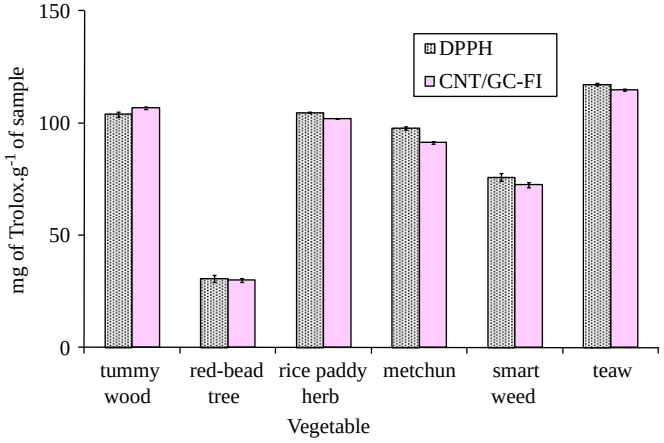


Fig. 5. TAC values for extracts of Thai indigenous vegetables obtained from our method compared with the values obtained from a reference method (DPPH• colorimetric method, [44]).

3.4. Application to Thai indigenous vegetable extracts

The proposed CNT/GC-FI method was applied to assess the 'total antioxidant capacity' (TAC) of six Thai vegetables/herbs (tummy wood, red-bead tree, rice paddy herb, metchun, smart-weed and teaw). Determination was carried out in triplicate for a sample. The data obtained by the proposed method were compared with the values obtained from DPPH• colorimetric methods and are shown in Fig. 5. Antioxidant contents were expressed as mg Trolox equivalent per gram of dry plant (mg of Trolox g^{-1} of sample). Using the paired t-test [48], the results obtained from CNT/GC-FI are not significantly different from the results of the spectrometric DPPH method [$t_{observed}=1.6990$, where $t_{critical}=2.5706$, $P=0.05$]. Among these Thai vegetables, tummy wood and teaw exhibited the highest TAC values.

4. Conclusions

This work presents a method development that utilizing amperometric detection of DPPH• to measure the 'total antioxidant capacity' or TAC of vegetable extracts. In the flow system, the level of DPPH• monitored continuously at the amperometric cell is reduced on injection of a plug of antioxidants. There is a reaction between DPPH• and the antioxidant which lowers the amount of the DPPH• in the plug leading to a decrease in the DPPH• signal. This decrease has a linear correlation with the concentration of the antioxidant in the injected plug. It was found that 0.05 V (vs. Ag/AgCl) was the optimal voltage for monitoring DPPH•. Trolox was used as the reference antioxidant for calibration. The TAC value in the sample is reported as mg equivalent to Trolox g^{-1} sample.

The developed method is very simple since the FI system can be constructed easily using common laboratory equipment. In this work, a HPLC system, i.e., pump, injection valve and the electrochemical detector was set up for the single-line FI system (Fig. 1). In order to improve the sensitivity, carbon nanotubes was casted onto the glassy carbon working electrode of the HPLC electrochemical detector.

The final FI method is rapid (sample throughput of 45 samples h^{-1}) and is suitable for analysis of plant extracts, which often are colored. The pigments can interfere with any conventional colorimetric method for determination of TAC. Thus an electrochemical DPPH• method is applicable to all types of plant extracts.

Novelty statement

This work presents a new flow injection (FI) method for measuring the so-called 'total antioxidant capacity' or TAC of vegetable extracts. The method utilizes amperometric detection of DPPH•. We employed CNT to improve the sensitivity of detection on glassy carbon electrode. Trolox was used as the reference antioxidant for calibration. Unlike colorimetric methods, pigments do not interfere our method in the TAC measurement. This electrochemical DPPH• method is applicable to all types of plant extracts.

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