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Diphenylbenzidine as a Probe for Determining Antioxidant Capacity of Plant Extracts

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ABSTRACT

A new method for measuring antioxidant capacity using diphenylbenzidine (DPB) as a probe was developed. The aim of the study is to invent a moderately stable, intense color probe that can be linearly decolorized by the increasing amount of antioxidant compounds. DPB was prepared by oxidizing diphenylamine with either ceric ammonium nitrate or ferric chloride in formic acid solution. DPB probe is deep purple and decolorization of the probe is linearly proportional to the quantity of antioxidant compounds. The DPB batch assay was applied to assess the antioxidant capacity of ascorbic acid. IC_{50} value of ascorbic acid measured by DPB assay (1.2 ppm) was compared with those determined by widely accepted methods such as DPPH and ABTS methods (1.3 and 1.6 ppm, respectively). Antioxidant capacity of plant extracts assessed by DPB assay also satisfyingly correlated with the lipid peroxidation inhibition capacity. Two main advantageous features of DPB probe that make it applicable to the flow injection analysis (FIA) are the fast preparation step that allows an on-line synthesis of the probe and the brief incubation period enabling an inexpensive, fast, semi-automatic analysis, especially when a large number of samples are involved.

Keywords: Antioxidant capacity; Colorimetric method; Diphenylbenzidine; FIA

1. Introduction

Due to the health danger of free radicals, foods that contain high level of antioxidant compounds are of high interest. As a consequence, many assays have been developed as a tool for evaluating the antioxidant capacity in diets. There are a variety of analytical techniques used to detect the quantity of the radical probe such as a cyclic voltammetry (CV) [1], electron spin resonance (ESR) [2], high-performance liquid chromatography (HPLC) [3], UV-visible spectroscopy [4] and Flow Injection Analysis (FIA) [5]. In colorimetric method, the antioxidant capacity will be proportional to the decreasing amount of the radical

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probe as evidenced by the fading of its color. Radical probes that are widely used are DPPH [6-7] and ABTS radical cation [8-9]. Nevertheless, the oxidation of DPPH to DPPH radical and the oxidation of ABTS to ABTS radical cation are rather time-consuming. This drawback makes these probes less attractive for the application in flow injection analysis.

Diphenylbenzidine (DPB) [10-11] the oxidation product of diphenylamine (DPA), is deep purple in color and is oxidized readily by the polyphenolic compounds making it suitable to be applied as a probe for measuring the antioxidant capacity of polyphenolic antioxidants.

FIA markedly provides several favorable features such as simplicity, versatility, cost effectiveness, and high sample throughput that are advantageous for rapid and reliable determination of antioxidant capacity in both pure compounds and complex matrices. Therefore, in this work a novel, expedient assay utilizing purple DPB as a probe for determining antioxidant capacity of plant extracts was proposed. DPB probe will be applied to both batch and FI methods.

Bamboo leaves and tea leaves are known to be rich in bioactive polyphenol constituents. Therefore, their antioxidant capacities are of interest. In this study, antioxidant capacities of these plants will be evaluated using the newly developed DPB method.

2. Materials and Methods

2.1 Chemicals

Diphenylamine (DPA), Ascorbic acid, Ceric ammonium nitrate (CAN), Butylated hydroxytoluene (BHT), Ferrous sulfate, and Titron X 100 were purchased from Sigma. Ammonium thiocyanate was obtained from Fluka. Ferric chloride, and Formic acid were purchased from Merck. Deionized-distilled water was used throughout.

2.2 Instrumentation

UV-visible spectrometer CECIL 1010, Peristaltic pump ISMATEC A unit of IDEX Corporation, Tube C-Flex Tubing, Cole-Parmer Instrument Company, 0.1 cm diameter, Six-port valve injector, Visible spectrometer JENWAY 6061 Colori Meter, Recorder by Cole Parmer model-201 b-1830 Chart Recorder.

2.3 Batch assay

2.3.1 Optimization for the preparation of DPB

Four conditions for the preparation of DPB were investigated.

Condition 1. DPA (0.1 mmol, 0.0169 g) was dissolved in 5 mL of formic acid. Separately, CAN (0.2 mmol, 0.1096 g) was dissolved in 45 mL of formic acid. The two solutions were then mixed together to produce a solution of intense purple color. One mL of the resulting solution was diluted 60-fold with formic acid and the absorbance at 580 nm of the solution was monitored every minute up to 10 min and every 10 min after that until 1 h.

Condition 2. DPA (0.1 mmol, 0.0169 g) was dissolved in 5 mL of formic acid. Similarly, $FeCl_3$ (0.3 mmol, 0.0487 g) was dissolved in another 5 mL of formic acid. Blending of both solutions provided a deep purple color solution. One mL of the resulting solution was diluted with formic acid to 20 mL and the absorbance at 580 nm of the solution was measured every minute up to 15 min and every 15 min after that until 45 min.

Condition 3. DPA (0.0169 g) was dissolved in 5 mL of EtOH. In another beaker, CAN (0.1096 g) was also dissolved in EtOH. The two solutions were then combined

Condition 4. DPA (0.0169 g) was dissolved in 5 mL of EtOH. Likewise, $FeCl_3$ (0.0487 g) was dissolved in another 5 mL of EtOH. The two solutions were then mixed together.

2.3.2 Determination of antioxidant capacity of ascorbic acid solution by the DPB assay

A stock solution of 10 ppm ascorbic acid was diluted to various concentrations including 0.4, 0.8, 1.2 and 1.6 ppm. Subsequently, 1 mL of sample solution was mixed with 2 mL of the solution of DPB prepared in condition 1. The mixture was incubated for 5 min at room temperature. Absorbance at 580 nm of samples were measured.

2.3.3 Determination of antioxidant capacity of ascorbic acid solution by the DPPH assay

An aliquot of each dilution (0.3-1.4 ppm) of ascorbic acid (1 mL) was added to 3 mL of the DPPH solution (0.25 mM DPPH in ethanol, prepared daily). The mixture was incubated for 30 min at room temperature. The absorbance was measured at 520 nm with the UV 1700-Shimadzu spectrometer.

2.3.4 Determination of antioxidant capacity of ascorbic acid solution by the ABTS assay

The working stock solution was prepared by mixing a 7.4 mM ABTS solution with a 2.6 mM potassium persulfate solution in an equal quantity and allowing them to react for 12-16 h at room temperature in the dark. One mL of the resulting $ABTS^{\bullet+}$ solution was diluted with 60 mL of distilled water. Fresh $ABTS^{\bullet+}$ solution was prepared for each experiment.

An aliquot of each dilution (0.3-2.0 ppm) of ascorbic acid (1 mL) was mixed with 3 mL of $ABTS^{\bullet+}$ solution and incubated in a dark condition for 5 min. Then the absorbance was recorded at 734 nm using the UV 1700-Shimadzu spectrometer.

2.3.5 Determination of antioxidant capacity of bamboo leaf extracts by the DPB assay

Bamboo leaves were washed, oven dried at 80 ºC, and extracted in 4 solvents of different polarities (EtOH, EtOAc, CH_2Cl_2, and hexane). A sample of 6 g of chopped bamboo leaves was soaked in 100 mL of the extracting solvent for 3 h at room temperature. After the removal of solvent, crude extracts were weighed and re-dissolved in EtOH (5 mL) to make a stock solution. Different concentrations of samples (0.03, 0.05, 0.10, 0.15, and 0.20 mg mL^{-1}) were prepared. A one mL of sample solution was combined with 2 mL of DPB solution *(condition 1).* The mixture was incubated for 5 min at room temperature. Absorbance at 580 nm of samples were recorded. The % inhibition was calculated according to the following equation

$$\%\text{inhibition} = \left(1 - \frac{A_{sample}}{A_{cpntrol}}\right) \times 100,$$

where A_{sample} is absorbance of sample at 580 nm and $A_{control}$ is absorbance of control at 580 nm. The antioxidant capacity is then compared in terms of IC_{50}.

2.3.6 Lipid peroxidation inhibition capacity (LPIC) of bamboo leaf extracts

A modified ferric thiocyanate method [12] was used to determine the inhibition of lipid peroxidation based on the extract's ability to protect oil from heat-induced oxidation. Briefly, in a 50-mL beaker, 1 mL of 1.1 mg mL^{-1} ascorbic acid solution, 1 mL of soybean oil, and 10 mL EtOH were mixed and stirred vigorously at 50 ºC for 15 min. Then 0.1 mL of the mixture, 0.1 mL ammonium thiocyanate, and 0.1 mL ferrous sulfate were placed into 4.7 mL of EtOH and stirred for 3 min. The absorbance of the resulting red mixture was determined.

The procedure was repeated by replacing ascorbic acid solution with 1.1 mg mL^{-1} bamboo leaf extract solutions.

2.4 DPB flow injection assay

2.4.1 Solvent system for the flow based DPB assay

Five solvent systems have been examined for the application in the FI system. In general, DPA (0.1692 g) was dissolved in 25 mL of the solvent and $FeCl_3$ (0.5406 g) was dissolved in another 25 mL of the solvent. Two solutions were then combined.

Solvent system: (1) formic acid, (2) EtOH, (3) 1:1 EtOH:H_2O, (4) 1:1 HNO_3:H_2O, (5) 5% formic acid.

2.4.2 Reagents for the flow based DPB assay

Ascorbic acid (0.0200 g) was dissolved in 20 mL H_2O to make a stock solution of 1,000 ppm. Subsequently, the stock solution was diluted into samples of different concentrations including 20, 50, 100, and 150 ppm.

BHT (0.0200 g) was dissolved in 200 mL of 3:7 H_2O:acetone to make a stock solution of 100 ppm BHT. The stock solution was further diluted into samples of different concentrations including 10, 20, 30, and 40 ppm.

A sample of 0.25 g ground tea leaves was extracted in 20 mL of 1:1 acetone:H_2O and centrifuged at 300 rpm for 1 h. The supernatant was firstly diluted with 20 mL of 1:1 acetone:H_2O, and diluted again with DI water to 800 mL. 1, 2, 3, and 4 mL of the stock solution were pipetted into volumetric flasks and the volume was adjusted to 10 mL with DI water. Concentrations of sample solutions are 31, 63, 94, and 125 ppm, respectively.

2.4.3 FI analyzer

The laboratory-made FI analyzer depicted in Fig. 1 consists of an Ismatec peristaltic pump, a visible spectrometer JENWAY 6061 Colori Meter, Recorder by Cole Parmer model-201 b-1830 Chart Recorder, equipped with a low pressure six-port valve injection. C-Flex Tubing, 0.10 cm i.d., was used for the construction of mixing and reaction coils. The length of the first coil is 150 cm and the length of the second coil is 130 cm. Solutions of DPA in 5% formic acid, $FeCl_3$ in 5% formic acid, and H_2O (carrier) were pumped into the system. The reaction of starting materials was carried out in the first mixing coil. The sample was injected and reacted with the probe at the second reaction coil. The peak responses were detected and plotted on a chart recorder. A 300 µL volume of sample and a 3.15 mL min^{-1} flow rate were applied. A certain amount of DPA may precipitate. Cleaning the tubing after the experiment with Titron X 100 is recommended.

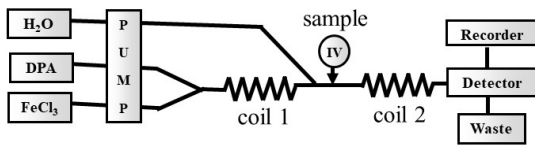

Fig. 1. The laboratory-made FI analyzer.

3. Results

3.1 The batch DPB assay

3.1.1 Condition for the preparation of DPB

When DPA is oxidized, it will dimerize to provide the colorless DPB (Fig. 2). The second oxidation will turn the colorless DPB into DPB semiquinone, the intermediate oxidation product which is green in color. Further oxidation will lead to the purple DPB which is the final oxidation product. The purple DPB is moderately stable, while the green DPB semiquinone is rather unstable as evidenced by the gradual disappearing of the green color. Therefore, particular oxidizing agents and solvents will be investigated in order to achieve the purple DPB.

Experimental outcomes clearly exhibited that formic acid is an appropriate solvent for the preparation of the purple DPB. The oxidation of DPA with either CAN or $FeCl_3$ performed in formic acid

persistently gave a deep purple solution of DPB (molar absorptivity = 32,500 $M^{-1}cm^{-1}$). When the oxidations were carried out in EtOH *(conditions 3 and 4)*, the reactions failed to give to purple DPB probe and the resulting green solution faded over time.

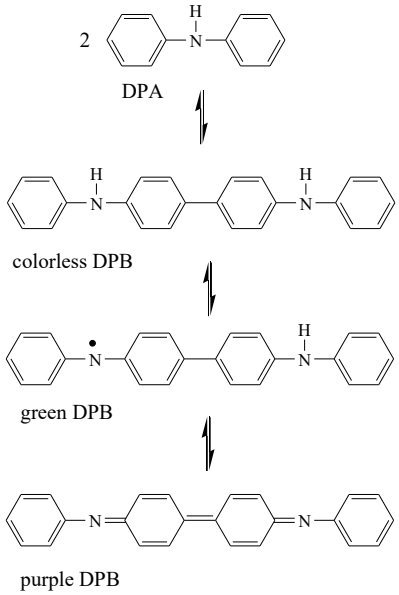

Fig. 2. Oxidation of DPA.

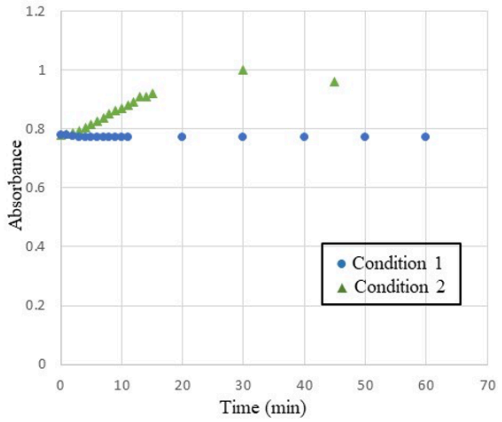

Fig. 3. The time-absorbance curve of DPB obtained from conditions 1 and 2.

For the oxidizing agent, both CAN and $FeCl_3$ were apparently able to produce the purple DPB product. However, as evidenced in the time-absorbance curve in Fig. 3, the absorbance of the purple solution obtained from condition 1, which used CAN as the oxidant, remained constant at all times during the 1-h period of absorbance monitoring. In condition 2, which used CAN as the oxidant, the absorbance fluctuated more during the monitoring. Therefore, condition 1 was the optimal condition and was utilized in the batch DPB assay.

3.1.2 Determination of antioxidant capacity of ascorbic acid solution by the DPB assay

Ascorbic acid was utilized as the standard antioxidant compound. Upon increasing the amount of ascorbic acid, the linear decolorization of DPB probe was observed with the R^2 value of 0.9997. Accordingly, the IC_{50} of ascorbic acid is 1.2 ppm. For method validation, the IC_{50} value of ascorbic acid obtained by the DPB assay was compared to IC_{50} values determined by the widely accepted standard assays such as DPPH and ABTS methods. Experimental results revealed that IC_{50} values of ascorbic acid evaluated by DPPH and ABTS methods are 1.3 and 1.6 ppm, respectively. Data from 3 assays agrees nicely and therefore ensure the validity of the new DPB probe.

The DPB probe has many advantageous features, for instance, the preparation was simple and rapid, by simply mixing the solution of DPA with the solution of the oxidant, the purple DPB probe is formed instantaneously, compared to 12-16 h for the preparation of the ABTS probe. Furthermore, the reaction between the DPB probe and the antioxidant compound is fast, requiring only 5 min of incubation, compared to 30 min for the DPPH assay. These advantages of the DPB assay make it a beneficial alternative to the existing conventional methods. While DPPH assay is typically conducted in EtOH or EtOH/H_2O media, DPB and ABTS assays are carried out primarily in aqueous media. As a result, DPB assay is fit mainly for

polar antioxidants such as polyphenolic compounds.

3.1.3 Determination of antioxidant capacity of bamboo leaf extracts by the DPB assay

Antioxidant capacities of bamboo leaves extracted by 4 solvents of different polarities (EtOH, EtOAc, CH_2Cl_2, and hexane) were investigated. The probes were apparently quenched in a dose-dependent manner. Percentage inhibition was calculated and plotted against the concentration of the extract to determine the IC_{50} value as illustrated in Fig. 4. The major antioxidant compounds found in plant extracts are the polyphenolic compounds which are polar organic compounds. Therefore, polar organic solvents are anticipated to be a more efficient extracting solvent than non-polar solvents.

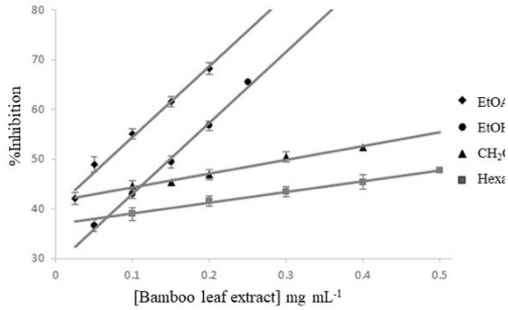

Fig. 4. Percentage inhibition of bamboo leaf extracts (n=5).

As expected, EtOAc-soluble extract exhibited the highest antioxidant capacity with the lowest IC_{50} value of 0.069±0.005 mg mL^{-1} (R^2 = 0.9852), followed by EtOH-soluble extract with IC_{50} value of 0.150± 0.006 mg mL^{-1} (R^2 = 0.9955). CH_2Cl_2 and hexane were far less effective extracting solvents with the IC_{50} values of 0.306± 0.024 (R^2 = 0.9955) and 0.608±0.018 mg mL^{-1} (R^2 = 0.9813), respectively.

3.1.4 Lipid peroxidation inhibition capacity (LPIC) of bamboo leaf extracts

A large number of assays have been developed to assess the *in vitro* activity of antioxidants. The ferric thiocyanate method [13] is one of the practical techniques for assessing the degree of lipid peroxidation based on the intensity of the color of ferric thiocyanate complex. Once the lipid has been oxidized by a radical to form lipid peroxide (LOOH) it will be readily reduced by Fe^{2+} to yield lipid alkoxyl radical ($LO^{\cdot}$) and Fe^{3+} (Fig. 5). $LO^{\cdot}$ will be further reduced with Fe^{2+} to provide LOH and more Fe^{3+}. Ferric ions generated will react with thiocyanate ions (SCN^-) to form the red ferric thiocyanate complex with the maximum absorption at 460 nm.

$$LOOH + Fe^{2+} \rightarrow LO^{\cdot} + {}^{-}OH + Fe^{3+}$$
$$LO^{\cdot} + Fe^{2+} + H^{+} \rightarrow LOH + Fe^{3+}$$
$$Fe^{3+} + SCN^{-} \rightarrow [Fe(SCN)]^{2+} \text{ (red)}$$

Fig. 5. Reactions between LOOH and Fe^{2+} that leads to the formation of ferric thiocyanate complex.

If the lipid molecule was protected by antioxidant compounds, a small amount of lipid peroxide would be produced. A lesser quantity of Fe^{2+} would be oxidized to Fe^{3+} resulting in the slight amount of ferric thiocyanate complex being formed, thereby, producing a lighter red solution. In particular, the LPIC value of an extract can reflect its own antioxidant capacity. The ferric thiocyanate method is therefore investigated in conjunction with the DPB assay to find the correlation between methods. LPICs of ascorbic acid and bamboo leaf extracts are presented in Table 1.

Table 1. LPIC of ascorbic acid and bamboo leaf extracts.

Entries	Sample (1.1 mg mL^{-1})	Absorbance at 460 nm
1	Control	>2
2	Ascorbic acid	0.810
3	EtOAc-soluble extract	0.960
4	EtOH-soluble extract	1.006
5	CH_2Cl_2–soluble extract	1.052
6	Hexane-soluble extract	1.117

Obviously, ascorbic acid had the highest LPIC. The second highest was EtOAc-soluble extract, followed by EtOH, CH_2Cl_2, and hexane-soluble extracts, respectively. The results of LPIC of bamboo leaf extracts agreed nicely with the results of antioxidant activity. Noticeably, absorbances of extract samples (n=4) had no significant difference at 95% confidence level (Q = 0.827).

3.2 DPB flow injection assay

The DPB probe served the batch assay effectually. Yet, to apply the probe to the flow-based assay, some aspects had to be fine-tuned.

3.2.1 Oxidant and Solvent system for the flow based DPB assay

Both CAN and $FeCl_3$ can effectively oxidize DPA to DPB. However, CAN is so reactive that once it is dissolved in the solvent, it should be mixed with the solution of DPA immediately. Unfortunately, for the flow-based assay, DPA and the oxidant are separately dissolved in the solvent and are gradually pumped to mix in the system. Therefore, $FeCl_3$ is favored as the oxidant for the FI assay.

For the solvent system, the reaction of DPA and $FeCl_3$ in formic acid generated purple color of DPB straightaway. On the other hand, other solvent systems such as EtOH, EtOH:H_2O, and HNO_3:H_2O failed to give the purple color. Only deep green solutions were observed in these solvent systems. Nevertheless, concentrated acid is not suitable for the flow-based system. Therefore, 5% formic acid solution was utilized instead as the solvent and purple color of DPB was observed instantly upon mixing the solutions of two starting materials.

3.2.2 DPB FI assay for determination of antioxidant capacity of samples

The assessment of antioxidant capacity of the sample by the DPB FI assay has been explored. Three samples (ascorbic acid, BHT, and tea leaf extract) were utilized. Apparently, each sample could linearly decolorize the purple DPB probe. The R^2 values were in the range of 0.9634-0.9931. As summarized in Table 2, BHT exhibited the highest antioxidant capacity with the IC_{50} value of 27 ppm (RSD = 2.08, n = 3), followed by ascorbic acid with the IC_{50} value of 72 ppm (RSD = 4.38, n = 3). Tea leaf extract demonstrated the lowest antioxidant capacity with the IC_{50} value of 106 ppm (RSD = 1.87, n = 3). The concentration ranges for samples used in this study are reported in Table 2.

Table 2. Antioxidant capacity of samples determined by the DPB FI assay.

Sample	IC_{50} (ppm)	% RSD (n=3)	[ ] range (ppm)
BHT	27	2.08	10-40
Ascorbic acid	72	4.38	20-150
Tea leaf extract	106	1.87	30-125

4. Conclusions

The DPB assay definitely offers various advantages. Primarily, the preparation procedure is really simple and fast. Upon mixing DPA with the oxidizing agent, the DPB probe is instantly generated compared to 12 h for the preparation of the ABTS probe. This feature allows the probe to be synthesized on-line in the FIA system. Furthermore, the DPB probe is relatively stable and can be decolorized rapidly in a dose-dependent manner (5 min incubation, compared to 30 min for DPPH method). The starting materials are also inexpensive. In addition, the DPB flow-based assay is semi-automatic allowing a faster screening of the antioxidant capacity of samples. Besides, a lesser amount of chemicals is required and less waste is produced.

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