Scientific paper

Evaluation of Antioxidant Activity Using an Improved DMPD Radical Cation Decolorization Assay

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Received: 27-09-2006

Abstract

An improved decolorization method for measuring the antioxidant activity of food samples using *N,N*-dimethyl-*p*-phenylenediamine (DMPD) is developed. DMPD radical cation ($DMPD^{\bullet+}$) is generated through a reaction between DMPD and potassium persulfate and is subsequently reduced in the presence of hydrogen-donating antioxidants. This assay has a clear edge over the previous DMPD assay (the DMPD/$FeCl_3$ assay) for the determination of antioxidant activity on a number of counts. The chemistry involves the generation of a more stable DMPD radical mono-cation by a reaction with potassium persulfate. The DMPD radical cation generator does not involve Fe(II) ions, which through Fenton's Reaction could cause negative deviation in the antioxidant activity of food extracts. The assay can equally be applied to the determination of antioxidant capacity of both hydrophilic and lipophilic antioxidants. The experimental procedure is rapid, inexpensive and ensures sensitivity along with reproducibility in the measurement of antioxidant activity of hydrophilic and lipophilic compounds and thus has a promising aspect of use in screening large number of fruit samples. The effectiveness of new DMPD decolorization assay was verified by evaluating the antioxidant capacity of different fruit samples.

Keywords: DMPD radical cation, antioxidant activity, hydrophilic, lipophilic, TEAC.

1. Introduction

Several approaches based upon generation of different radical cations through different mechanisms have been introduced for the measurement of total antioxidant activity (TAA) of body fluids, food extracts, and pure compounds [1–16]. Amongst a variety of radical cations available, *N*-alkylated-*p*-phenylenediamines have been found to be fairly persistent in solution after undergoing monoelectronic oxidation e.g., as seen in *N,N*-dimethyl-*p*-phenylenediamine. Since the oxidation potential of radical cation of this amine is compatible with the reduction potential of the alkoxy and peroxy radicals so it can be used for the measurement of oxidative status of different biological samples [17]. Production of the DMPD radical cation forms the basis of one of the spectrophotometric methods that has previously been applied to the measurement of labile sulphide in proteins, water soluble fraction of tomatoes, red wines, green tea infusion, pomegranate juice and antioxidant activity of wines and oxidative status of human plasma [18–24]. The previous $DMPD^{\bullet+}$ assay was based on generation of a violet colored radical cation resulting from the reaction of DMPD with $FeCl_3$ at an acidic pH [25]. This has been criticized on the basis that the metals being an integral part of fruits/vegetable samples could contribute towards the oxidation of DMPD and thus causing a negative deviation from the actual antioxidant values. The presence of Fe(II) and other metals in the fruit and vegetable samples may enhance the production of Reactive Oxygen Species (ROS) through Fenton's Reaction. Thus the recycling of iron from ferric to ferrous form, by a reducing agent can maintain an ongoing Fenton Reaction resulting in the generation of hydroxyl radicals and an ultimate negative deviation to the antioxidant activity (Fig. 1).

Secondly, the stability of the $DMPD^{\bullet+}$ could also be questioned in view of the vulnerability of iron solution being used for the generation of the radical cation due to air oxidation. In the present work, a more appropriate version for the assay has been reported in which the radical is generated in a more stable form through the reaction between DMPD and potassium persulfate. This has absorption maxima at wavelengths 517.4 and 552 nm as calculated by the 1st derivative technique (Fig. 2).

Addition of antioxidants reduces the pre-formed radical cation ($DMPD^{\bullet+}$), to a certain extent and on a time-

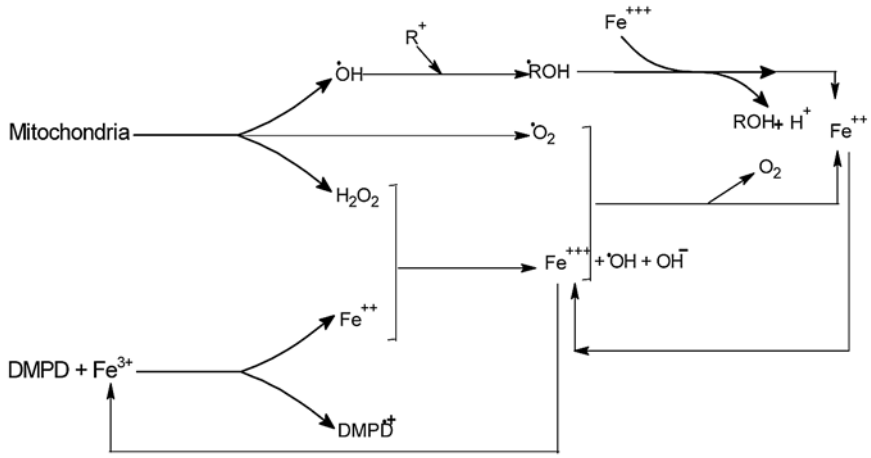

Fig. 1: The negative deviation caused by recycling of the ferrous to the ferric form by Fenton Reaction inside the biological systems

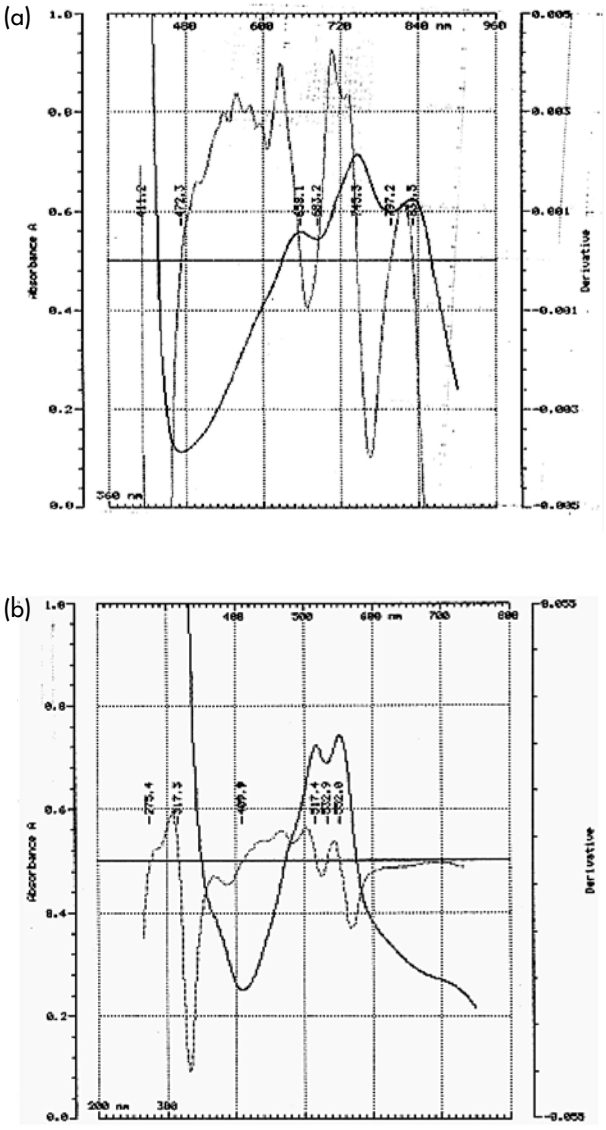

Fig. 2: Normal spectrum (solid line) and First derivative (dotted line) of a) ABTS radical cation in methanol and b) of DMPD radical cation in water

scale depending upon the antioxidant activity, the concentration of the antioxidant and the duration of the reaction. The extent of decolorization on addition of standard/sample solutions to $DMPD^{\bullet+}$ was determined in terms of percentage inhibition, which was a function of concentration and time. The Trolox Equivalent Antioxidant Capacity (TEAC) was then calculated by comparing with the percent inhibition caused by Trolox as a standard under the same conditions. The method is applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts.

2. Materials and Methods

Trolox (Hoffman-La Roche) (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid; Aldrich Chemical Co., Gillingham, Dorset, UK) was used as an antioxidant standard. Trolox (2.5 mM) was prepared in ethanol or 5 mM phosphate buffered saline (PBS), pH 7.4, to be used as a stock standard, as described previously [1]. Fresh working standards were prepared daily on dilution with ethanol. ABTS, 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, potassium persulfate (di-potassium peroxdisulfate) and ascorbic acid (95% pure) were obtained from Sigma-Aldrich (Poole, Dorset, UK) and HPLC grade ethanol from Rathburn Chemicals Ltd. (Walkerburn, Peebleshire, Scotland). DMPD was purchased from Fluka (Schweiz, Switzerland) and ascorbic acid from Sigma-Aldrich (95% pure). The antioxidant activity was assessed as described below. Experiments were performed on the spectrophotometer model Cecil CE 7200.

2.1. Sample Preparations

The juices of citrus fruits, which were purchased from local fruit market, Lahore, Pakistan, were extracted, filtered and diluted with deionized water. The samples were stored in refrigerator at 4 °C.

2.2. Imporoved DMPD$^{\bullet+}$ Assay Protocol – Decolorization Assay

0.209 g of DMPD was dissolved in 10 ml deionized water to form 100 mM solution. DMPD radical cation was produced by reacting 100 µL of DMPD stock solution with 50 µL of potassium persulfate solution (0.4 mM) and making the final volume 10 mL with acetate buffer (pH 5.6). The solution was incubated in the dark at 25 °C for 3–4 hours before use. The radical was stable in this form for several hours when stored in the dark at 4 °C. For the study of phenolic compounds and food extracts, the DMPD$^{\bullet+}$ solution was diluted with acetate buffer (pH 5.6), to an absorbance of 0.70 to 0.80 at 517.4 nm and equilibrated at 25 °C (Fig. 3).

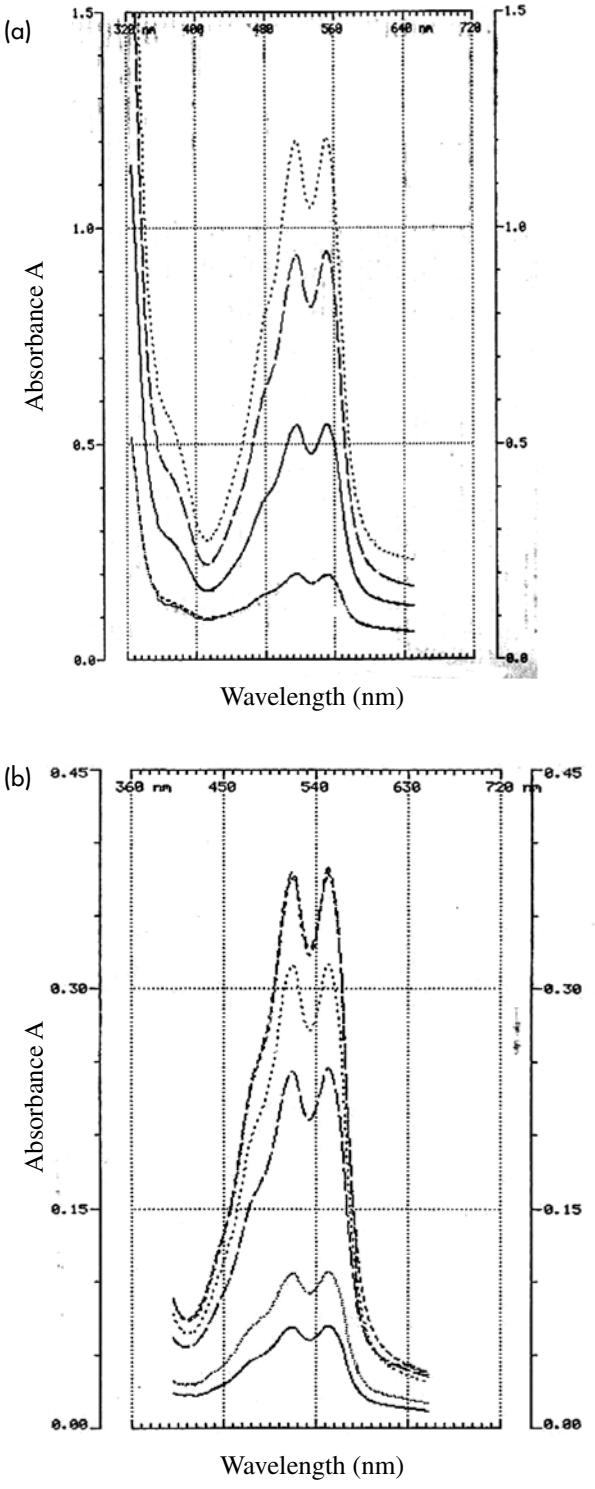

Fig. 3: Visible spectrum of DMPD radical cation produced by reacting DMPD with (A) $FeCl_3$ and (B) $K_2S_2O_8$.

The sample solutions of citrus juices were prepared through extraction and dilution with de-ionized water in such a manner that a 10µl aliquot of each dilution into the assay produced 20%–50% inhibition of the blank absorbance.

After addition of 3.49 ml of DMPD$^{\bullet+}$ solution ($A_{517.4nm}$ = 0.750 + 0.020) stabilized in ethanol or acetate buffer, to 10 µl of diluted sample solutions or Trolox standard solutions (final concentration 0–15 µM), the absorbance was noted up to 6 minutes at 30 °C. Appropriate solvent blanks were run in each assay. All determinations were carried out at least three times, and in triplicate, for each concentration of the standards and samples. The percentage inhibition of absorbance at 517.4 nm is calculated and plotted as a function of concentration of antioxidants and Trolox for the standard reference data.

2.3. ABTS$^{\bullet+}$ Assay protocol

ABTS$^{\bullet+}$ Assay protocol as developed by Re *et al*. [27] was followed. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation (ABTS$^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. For the study of antioxidant activity of citrus juices, the ABTS stock solution was diluted with methanol to an absorbance of 0.70 (+0.02) at 745 nm and equilibrated at 30 °C. After addition of 3.4 ml of diluted ABTS$^{\bullet+}$ solution (A_{734nm} = 0.700 + 0.020) to 100 µl of diluted sample solutions, the absorbance reading was taken at 25 °C exactly 1 min after initial mixing up to 6 minutes. Appropriate solvent blanks were run in each assay. All determinations were carried out at least three times, and in triplicate, on each occasion and at each separate concentration of the standards and samples. The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of concentration of antioxidants and of Trolox for the standard reference data.

2.4. DMPD/$FeCl_3$ Assay Protocol

The standard protocol as developed by Fogliano *et al*. [25] was followed. 100 mM DMPD solution was prepared by dissolving 209 mg of DMPD in 10 mL of deionized water; 1mL of this solution was added to 100 mL of

0.1 M acetate buffer, pH 5.25, and the colored radical cation ($DMPD^{\bullet+}$) was obtained by adding 0.2 mL of solution of 0.05 M ferric chloride (final concentration 0.1 mM). An absorbance of 0.900 + 0.100 units which represents the uninhibited signal was obtained. Standard solutions of Trolox and ascorbic acid were prepared in deionized water and methanol respectively. 100 µL of diluted samples were introduced into the sample and %age inhibition of the radical cation was calculated for the standards and sample solutions.

3. Results and Discussion

3.1. Standardization of the Improved Assay

The basic principle underlying the DMPD decolorization assay is that DMPD, at an acidic pH, forms a purple-colored radical cation by reacting with a suitable oxidizing agent. The UV-Visible spectra of the DMPD radical cation generated by using $K_2S_2O_8$ and $FeCl_3$ as oxidizing agents show a maximum absorbance at 517.4nm and 552nm (Fig. 2). The 1st derivative graphs of the radical cations $ABTS^+$ and $DMPD^{\bullet+}$ in methanol and deionized water respectively were obtained by using CECIL CE 7200 UV-Visible Spectrophotometer with band-width 2nm and path length 10mm.

Standard and sample antioxidants that are able to transfer a hydrogen atom to DMPD radical cation scavenge the color of the solution proportionate to their amount. The reduction of DMPD radical cation is rapid and shows a stable end point. The optimization of different parameters i.e. concentration of DMPD and oxidizing agent, pH etc. for the formation of a stable DMPD radical cation with considerable sensitivity was carried out (Fig. 4). It was found that both the pH and the concentration ratio between DMPD and $K_2S_2O_8$ were central to the effectiveness of the assay.

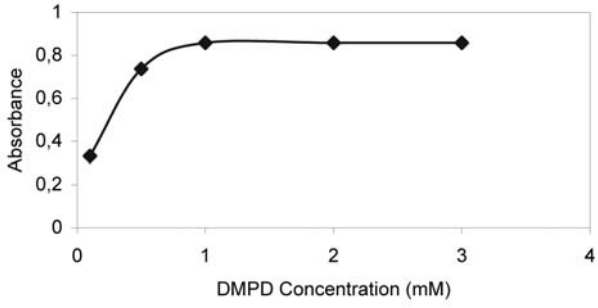

Fig. 4: Dependence of color formation on DMPD concentration. Absorbance at 517.4 nm was recorded at different time intervals after potassium persulfate addition.

It was observed that a stable $DMPD^{\bullet+}$ was obtained by mixing 100 µl of DMPD (100 mM) solution with 50 µl of $K_2S_2O_8$ (0.4 mM) in 10 mL acetic acid buffer (5.6 pH) and incubating the solution in dark for 3.5–4 hours at 25 °C. The solution was then diluted with acetate buffer (pH 5.6) to an absorbance unit 0.7 to 0.8, which is of crucial importance for better sensitivity and a sufficient inhibition range. Although the kinetics of color scavenging of radical cation on addition of antioxidant may be helpful in determining the potency of antioxidants, yet the method can be used more conveniently by measuring the color reduction at a fixed time point. The range of linearity as found by using Trolox as the standard antioxidant falls between 0.1 and 15 µg that is quite comparable with the range obtained in ABTS radical cation assay. The assay is quite reproducible with sufficient sensitivity. Moreover, DMPD is almost hundred times cost effective than ABTS.

A dose-response curve using Trolox from seven different sets of experiments (Fig. 5) is highly reproducible with low standard deviation.

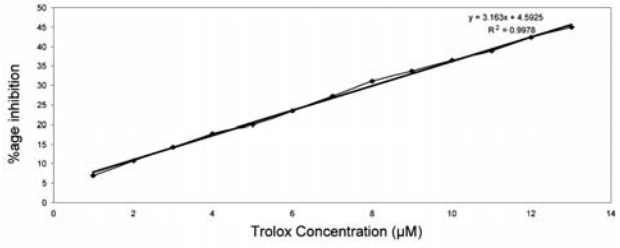

Fig. 5: Concentration-response curve (straight line represents trend line) for the absorbance at 517.4 nm for $DMPD^{\bullet+}$ as a function of concentration of standard Trolox solution.

Inhibition of the absorbance at 517.4 nm is linear between 0.3 to 13 mM of Trolox. The linear equation calculated is:

$$A_{517}\ (\text{inhibition}) = 3.163\ (\mu\text{M of Trolox}) + 4.5925$$
$$R^2 = 0.9978$$

This method gives measurement of the antioxidant activity of a range of hydrophilic and lipophilic samples by calculating the percentage inhibition of DMPD radical cation at 517.4 nm on the addition of antioxidant samples. Figure 6 illustrates the effects of time duration on the in-

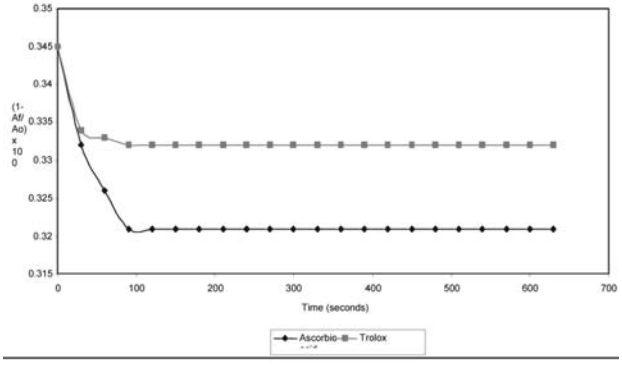

Fig. 6: Effect of the duration of interaction of specific antioxidants on the suppression of the absorbance of the $DMPD^{\bullet+}$

Table 1: Trolox Equivalent Antioxidant Activity (mmol/liter) of citrus fruit extracts

S. Citrus Fruit	TEAC (ABTS Method)			TEAC (DMPD/$FeCl_3$ Method)			TEAC (DMPD/$K_2S_2O_8$ Method)		
	Direct HAA†	HAA‡	LAA§	Direct HAA†	HAA‡	LAA§	Direct HAA†	HAA‡	LAA§
1. Citrus aurantium dulcis	16.19 ± 0.11	17.28 ± 0.17	0.74 ± 0.14	16.00 ± 0.17	16.08 ± 0.20	0.75 ± 0.24	0.75 ± 0.24	14.88 ± 0.09	0.53 ± 0.05
2. Citrum aurantium var	3.00 ± 0.08	3.4 ± 0.24	0.48 ± 0.16	8.20 ± 0.06	8.80 ± 0.17	0.31 ± 0.16	6.00 ± 0.07	6.04 ± 0.17	0.21 ± 0.09
3. Citrum medica	19.40 ± 0.13	19.3 ± 0.19	3.80 ± 0.07	38.04 ± 0.11	40.20 ± 0.09	1.60 ± 0.14	19.29 ± 0.13	21.90 ± 0.11	2.01 ± 0.06
4. Citrum aurantium var	7.10 ± 0.18	7.20 ± 0.14	0.62 ± 0.21	7.50 ± 0.20	8.10 ± 0.09	0.33 ± 0.11	3.98 ± 0.10	4.69 ± 0.08	0.31 ± 0.10
5. Citrus paradisi	4.56 ± 0.07	4.64 ± 0.15	0.44 ± 0.09	6.92 ± 0.18	6.96 ± 0.13	0.42 ± 0.19	4.28 ± 0.12	4.44 ± 0.12	0.34 ± 0.07
6. Citrum sinensis	7.21 ± 0.14	7.24 ± 0.04	0.42 ± 0.15	8.30 ± 0.13	8.88 ± 0.17	0.22 ± 0.10	4.90 ± 0.05	5.62 ± 0.12	0.20 ± 0.09
7. Citrum aurantium	1.28 ± 0.12	1.372 ± 0.11	0.30 ± 0.21	7.50 ± 0.08	8.01 ± 0.11	0.34 ± 0.24	2.21 ± 0.08	2.23 ± 0.04	0.29 ± 0.14

† = Direct Hydrophilic Antioxidant Activity, ‡ = Hydrophilic Antioxidant Activity, § = Lipophilic Antioxidant Activity n + SD = > each performed in triplicate at three separate concentrations.

teraction of standard antioxidants with DMPD radical cation generated through an improved DMPD Assay.

The results demonstrate that the reaction of antioxidants with $DMPD^{\bullet+}$ is completed by 100 seconds. The standard dose-response curve was obtained by plotting a range of concentrations of standard antioxidants against their corresponding % inhibition. The values of TEAC were determined by comparing the percentage inhibition of the given sample with the percentage inhibition obtained by using standard antioxidant i.e. Trolox (Table 1).

3.2. Fruit Samples

Direct Hydrophilic Antioxidant Activity (direct HAA), Hydrophilic Antioxidant Activity (HAA) and Lipophilic Antioxidant Activity (LAA) of seven members of citrus family were evaluated by using the above three methods (Fig. 7 and Table 1).

In order to relate the contributions of hydrophilic and lipophilic components of the samples with the direct hydrophilic antioxidant activity (without separating the sample into hydrophilic and lipophilic components) of citrus members, these components were extracted separately in aqueous and organic phases. For this purpose the fruit extracts and solvents (ethyl acetate, n-hexane and dichloromethane) were mixed in a 1:1 (v/v), and after 1h of gentle shaking the samples were centrifuged at 1800 x g for 10 minutes. The two phases formed were separated. Subsequently, the hydrophilic antioxidant activity (HAA) and the lipophilic antioxidant activity (LAA) were determined.

Table 1 shows the values of direct HAA, HAA and LAA of the seven citrus members, expressed as Trolox equivalents. It is evident from the Figure that, in all cases the Total Antioxidant Activity (TAA = HAA + LAA) was

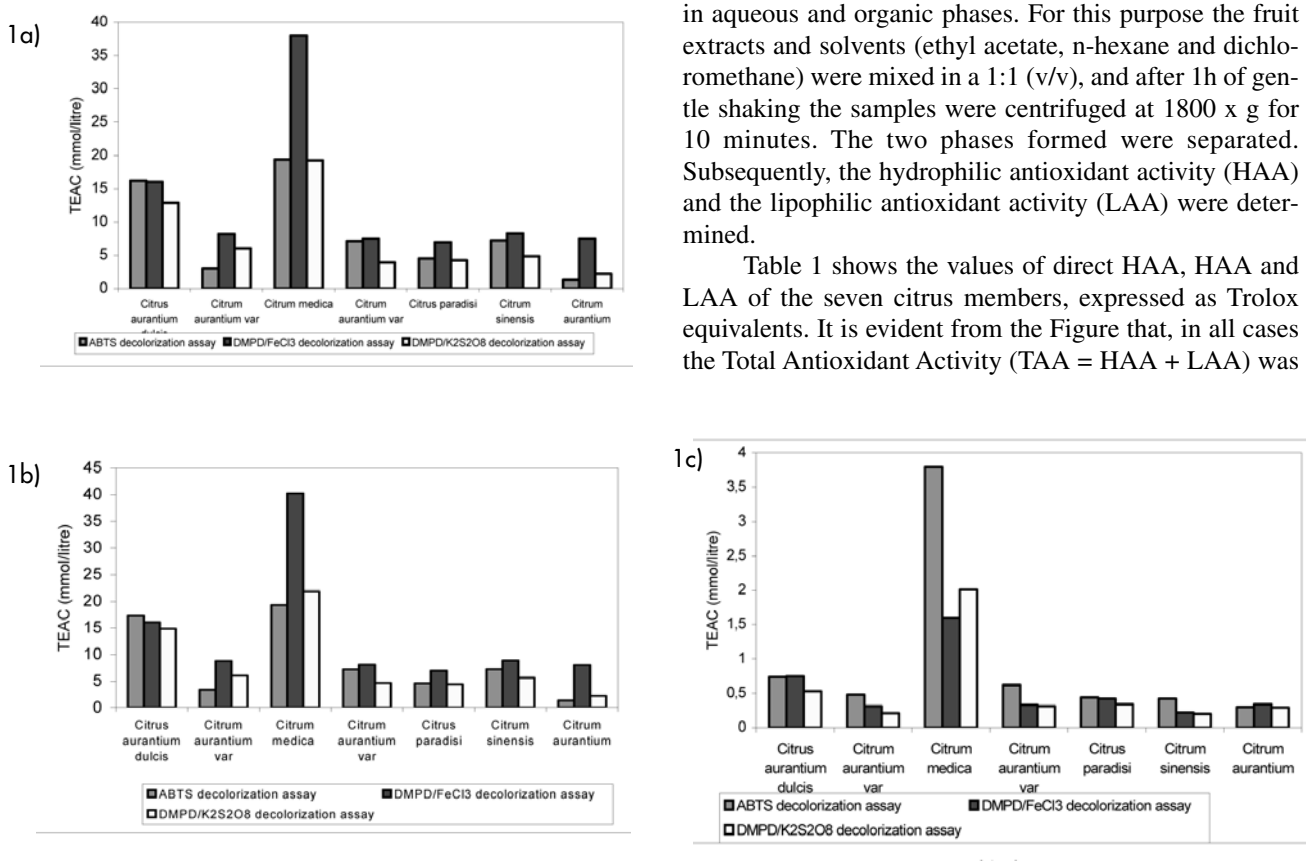

Fig. 7: Graphical comparison of TEAC values (1a) direct hydrophilic antioxidant activity (1b) hydrophilic antioxidant activity (1c) lipophilic antioxidant activity of different citrus fruits using three different methods.

higher than the direct HAA of the concerned citrus member. This clearly shows that the hydrophilic and lipophilic antioxidant compounds present in these samples express their antioxidant activity only in the adequate medium i.e. the antioxidant activity also depends upon the nature of the medium. The conclusion drawn may be useful in explaining the nature of antioxidant compounds and their medium-dependant activity. Furthermore, a general trend that more than 90% of the antioxidants of citrus members are hydrophilic in nature can be seen and it is also in conformation with the already reported data [26]. The TEAC values obtained depict that *Citrum medica* (lemon) has the highest direct HAA, HAA and LAA values for all the three methods followed by *Citrus aurantium dulcis*, which has the second highest TEAC values.

4. Acknowledgement:

We acknowledge the HEC financial support for the research work under HEC Research Scholar Grant No. 042-121235-Ps2-286.

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Povzetek

Razvili smo izboljšano spektrofotometrično metodo za določanje antioksidativne učinkovitosti vzorcev hrane z uporabo *N*,*N*-dimetil-*p*-fenilendiamina (DPMD). Z reakcijo med DPMD in kalijevim persulfatom tvorimo $DPMD^{\bullet+}$, ki ga nato reduciramo v prisotnosti antioksidantov, ki lahko donirajo vodik. Predlagani test ima vrsto prednosti pred obstoječim, saj nastaja stabilnejši $DPMD^{\bullet+}$ v odsotnosti Fe(II), ki bi vsled Fentonove reakcije lahko imeli negativen vpliv na zanesljivost testa. Uporabimo ga lahko za hidrofilne in lipofilne antioksidante, postopek pa je enostaven in poceni ter zagotavlja primerno ponovljivost in občutljivost. Uporabili smo ga za oceno antioksidativne učinkovitosti različnega sadja.