Reactive oxygen species, including free radicals, are formed by exogenous chemicals and endogenous metabolic processes in the human body. The body’s antioxidant defense system is comprised of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase, transition metal binding proteins such as ferritin and ceruloplasmin, and hormones such as estrogen and melatonin. Other antioxidants of dietary origin include vitamins, polyphenols, and carotenoids. Oxidative stress occurs when the body’s antioxidant defenses are overwhelmed. Cellular and extracellular macromolecules (ie, proteins, lipids, and nucleic acids) can suffer oxidative damage, which may initiate or promote the development of atherosclerosis, inflammation, neoplasia, and possibly the aging process itself.1-6

Blood contains many antioxidants that inhibit or limit oxidative damage.7 The concentrations of these antioxidants can be measured individually; however, this approach is time consuming and expensive. Also, the results may not reflect the total antioxidant capacity (TAC),8 which is of greater practical use given the considerable interest in antioxidants as nutritional supplements to maintain health and prevent disease. Several methods have been developed to measure the TAC and the most common of these methods are the oxygen radical absorbance capacity (ORAC)9, ferric reducing antioxidant power (FRAP),10 the total radical trapping antioxidant potential (TRAP),11 and the trolox equivalent antioxidant capacity (TEAC) assays.12 In this study, we compared the performance of the ferric reducing capacity (FRC) assay with the FRAP assay, carried out according to the methods reported by Benzie and Strain.10

**ABSTRACT**

**Objective:** To compare the performance of the ferric reducing capacity (FRC) assay with the ferric reducing antioxidant power (FRAP) assay to measure the total antioxidant capacity (TAC) of serum samples.

**Methods:** Serum from 10 healthy ethnic Chinese adult volunteers was assayed for TAC levels. A Sunrise absorbance reader was used for measurements of absorbance changes. The kinetic force of the reaction was monitored at 2-minute intervals for as long as 10 minutes at room temperature. The difference in absorbance between the blank and sample specimens was calculated.

**Results:** No significant increase in signal was observed at the 2-minute mark for the vitamin C reaction kinetic manifestations of FRC or FRAP. However, over time an increase was observed in absorbance levels in readings with serum. The dose-response lines for both assays showed strong linearity up to the 2000 μmol/L concentration of vitamin C tested at the 4-minute mark. Serum TAC ranged from 264 to 610 μmol/L of vitamin C equivalents for FRC and from 172 to 418 for FRAP. A strong positive correlation was observed between the TAC of the 10 serum samples measured by both assay methods.

**Conclusions:** The FRC assay is a useful alternative to the FRAP assay for measuring the TAC of serum due to its simplicity and speed of use and because it does not require specialized equipment. However, normal ranges should be established in the population intended before our results are put to clinical use.

**Keywords:** clinical chemistry, antioxidant assay, free radicals

**Abbreviations**

TAC, total antioxidant capacity; ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant power; TRAP, total radical trapping antioxidant potential; TEAC, trolox equivalent antioxidant capacity; FRC, ferric reducing capacity; TPTZ, 2,4,6-tri-pyridyl-s-triazine; Fe⁶-TPTZ, ferric tripyridyltriazine; Fe⁶-TPTZ, ferrous tripyridyltriazine; FeCl₃, 6H₂O, ferric chloride hexahydrate

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Materials and Methods

Reagents and Equipment

TPTZ (2,4,6-tri-pyridyl-s-triazine), ferric chloride hexahydrate (FeCl₃·6H₂O), sodium acetate trihydrate, and vitamin C (L-ascorbic acid) were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO). Glacial acetic acid and hydrochloric acid were purchased from Merck and Co., Inc. (Whitehouse Station, NJ). The FRC assay kit was a gift from K B Lim Skin Clinic Pte Ltd, Singapore. Vitamin C solutions of 62.5, 125.0, 250.0, 500.0, 1000.0 and 2000.0 μmol/l were prepared for use as standards in both assays.

Serum from 10 healthy ethnic Chinese adult volunteers was assayed for TAC levels after obtaining their informed consent. This study was conducted according to the principles of the Declaration of Helsinki.

A Sunrise absorbance reader (Tecan Group Ltd, Männedorf, Switzerland) was used for absorbance measurements. The reaction rate was determined by absorbance measurements taken at 2-minute intervals for as long as 10 minutes at room temperature. The difference in absorbance between the blank and sample specimens was calculated. Absorbance readings were made in duplicate and their means were used for analysis.

FRAP Assay

The FRAP assay was carried out as described by Benzie and Strain, with minor modifications for assay on a 96-well microplate. The FRAP reagent was prepared by mixing 25 ml of 300.0 mmol/L acetate buffer, 2.5 ml of 10 mmol/L TPTZ solution, and 2.5 ml of 20 mmol/L FeCl₃ solution in a 10:1:1 ratio. 10 μL of sample was mixed with 200 μL of FRAP reagent; the contents were mixed vigorously. Ferric tripyridyltriazine (FeIII-TPTZ) complex is reduced to ferrous tripyridyltriazine (FeII-TPTZ) form in the presence of antioxidants and develops an intense blue color, with maximum absorption at 593 nm.

FRC Assay

The FRC assay is similar to the FRAP assay except that it uses 1, 10-Phenanthroline instead of TPTZ. Phenanthroline forms a FeIII-(Phen) complex that is reduced to ferrous tripyridyltriazine (FeII-TPTZ) form in the presence of antioxidants and develops an intense blue color, with maximum absorption at 593 nm.

Statistical Analysis

Statistical analysis was performed with SPSS 16.0 for Windows (SPSS Inc, Chicago, IL). Simple regression analysis was performed to calculate the dose-response relationship of the standard vitamin C solutions. The correlation between the assay methods was assessed using Spearman rank order correlation; P < .05 was considered to be statistically significant.

Results

Figure 1 shows the vitamin C reaction profile of FRC (part A) and FRAP (part B). The curves were flat; also, no significant increase in signal was observed after the first reading at the 2-minute mark. However, over time an increase was observed in absorbance levels compared to initial and blank measurements (Figure 2).

The linearity of the methods was tested using standards prepared with various concentrations of vitamin C. Figure 3, parts A and B, show the dose-response P of both assays relative to vitamin C concentration. Both assays produced a linear response of as high as 2000 μmol/L concentration of vitamin C measured at the 4-minute mark, with a correlation coefficient R² of 0.998 for the FRC assay (Figure 3, part A) and 0.997 for the FRAP assay (Figure 3, part B).

Serum TAC was expressed in terms of μmol/L of vitamin C equivalents. The TAC ranged from 264 through 610 for FRC and from 172 through 418 for FRAP. The mean (SD) and median were 353 (31) and 338 for FRC and 262 (64) and 253 for FRAP. A strong positive correlation was observed between the TAC of the 10 serum samples measured by both assay methods (Figure 4; Spearman rank order correlation rₛ = 0.75, P = .01).

Discussion

Many assays are available to measure antioxidant capacity, but no single method can truly measure the total antioxidant
Figure 1
Kinetic reaction to vitamin C. A, Ferric reducing capacity. B, Ferric reducing antioxidant power.

Figure 2
Serum kinetic reaction. A, Ferric reducing capacity. B, Ferric reducing antioxidant power.
An approximation to the true TAC can be obtained by combining the results of different assay methods; however, this process would be difficult, time consuming, and impractical. The assay that most closely approximates the true TAC is probably the ORAC assay, which measures the reaction until its completion and detects fast- and slow-reacting antioxidants. It has been modified to measure lipophilic and hydrophilic antioxidants. However, temperature control is critical to the validity of the results.

With optimal test conditions, the ORAC assay is considered to be the criterion standard for TAC measurement. However, the long analysis time (approximately 1 hour) and the need for expensive equipment are factors that make the ORAC assay more suitable for research environments than for routine clinical use. The FRAP assay is a simple, rapid, and relatively inexpensive assay developed by Benzie and Strain to determine the TAC of plasma. Antioxidants present in plasma reduce FeIII-TPTZ complex to FeII-TPTZ, a blue-colored complex; the change in absorbance directly reflects the TAC of plasma. The FRC assay is similar to FRAP except that it uses 1, 10-Phenanthroline instead of TPTZ. Phenanthroline forms a FeIII-(Phen)3 complex that is reduced to an orange-red-colored FeII-(Phen)3 complex.

We adapted the FRAP assay for use in a 96-well microplate and compared its performance with that of FRC. TAC was expressed as μmol/L of vitamin C equivalents. The linearity of both tests was assessed using standard concentrations of vitamin C solution from 62.5 through 2000.0 μmol/L; both produced linear calibrations.

We observed an increase in absorbance over time in the 10

**Figure 3**

**Figure 4**
Correlation between ferric reducing capacity (FRC) and ferric reducing antioxidant power (FRAP).
serum samples tested. This increase likely occurs due to proteins such as albumin, and to a much smaller extent from small amounts of slow-reacting polyphenols present in the serum. However, the role of protein in antioxidant defense is unclear; oxidative changes in proteins may be a specific marker of oxidative damage rather than a sign of antioxidant defense. Hence, in the original FRAP assay, Benzie and Strain recommended a 4-minute reaction time so that TAC can be measured without risk of the protein-associated changes in absorbance masking smaller, perhaps more important, changes that occur due to antioxidant activity in the sample. In 9 of the 10 serum samples tested (Figure 2, parts A and B), the increase in signal was minimal. Hence, we recommend the 4-minute reading of TAC, as has been suggested by Benzie and Strain.

The 4-minute TAC of serum ranged from 264 to 610 for FRC and 172 to 418 μmol/L of Vitamin C equivalents for FRAP. Thus both methods appear to be adequate for the testing the TAC of serum. Strong positive correlation was observed between the 2 assay methods (Spearman rank order correlation $r = 0.75, P = .01$). This is not surprising because both measure the reduction of ferric ions. Like FRAP, FRC will also not measure thiol groups.

Although its sample size is small, this study suggests that the FRC assay is a useful alternative to the FRAP assay for measuring the TAC of serum. Like FRAP, the FRC assay is simple, rapid, and does not require specialized equipment other than a UV spectrophotometer. This study was not designed to establish a reference range. Hence, reference ranges should be established by each laboratory if these methods are intended for clinical use. Like FRAP, FRC may also be useful for measuring the antioxidant activity of different foods.

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