Comparison of Spectrophotometric Assays Determining Total Antioxidant Capacity

Toplam Antioksidan Kapasiteyi Belirleyen Spektrofotometrik Yöntemlerin Karşılaştırılması

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ÖZET

Amaç: Çalışmanın amacı, doğrudan antioksidan kapasiteyi ölçen dört tam otomatik fotometrik yöntem arasında yer alan ferrik iyon indirgeyici güç, total antioksidan durum, total antioksidan yanıt ve total tiyol yöntemlerinin kapsamlı olarak değerlendirilmesi ile belirtilen yöntemlerin güçlü ve zayıf yönlerini açığa çıkarmaktı.

Gereç ve Yöntem: Kan örnekleri 125 sağlıklı bireyden alındı. Analitik tayinlerde serum örnekleri ile çeşitli endojen ve eksojen antioksidan moleküller kullanıldı. Yöntemlerin analitik performans özellikleri analiz edildi ve bu yöntemlerin sonuçları arasındaki ilişkiler incelendi.

Bulgular: Bu çalışmadan elde edilen verilere göre, ferrik iyon indirgeyici güç yöntemi tiyol bileşiklerini, karotenoid ve ürik asidi ölçmek için yetersizdi. Total antioksidan yanıt yöntemi için tekrarlanabilirlik skorları diğer üç yöntemden daha düşüktü ve ürik asidi ölçmek için total antioksidan yanıt yöntemi zayıftı; bununla birlikte total antioksidan yanıt, diğer yöntemlerden daha yüksek doğrusallığa sahipti. Total antioksidan durum yöntemi, hemoliz ve lipemi etkileşiminden en az etkilenen yöntemdi. Total tiyol yöntemi saptama sınırı, hassasiyet ve tekrarlanabilirlik açısından etkili bulundu.

Sonuç: Halen, bir bileşiğin antioksidan aktivitesini belirlemek için kullanılan standart bir yöntem bulunmamakla birlikte, farklı yöntemlerin kullanılması ve farklı yöntemlerden elde edilen sonuçların değerlendirilmesi, antioksidan kapasitenin değerlendirilmesi için uygun bir yoldur.

Anahtar kelimeler: antioksidan kapasite; FRAP; TAS; TAR; TTL

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ABSTRACT

Aim: The aim of this study was to extensively evaluate four fully-automated photometric methods directly measuring antioxidant capacity; ferric ion reducing power, total antioxidant status, total antioxidant response, total thiol and to reveal the weak and strong sides of the assays.

Material and methods: Blood samples were obtained from 125 healthy subjects. Serum samples and several endogenous and exogenous antioxidant molecules were used for analytical determinations. The performance characteristics of the methods were compared and the relationships between these methods' results were investigated.

Results: According to the data obtained from this study, the ferric ion reducing power method was inadequate for measuring thiol compounds, carotenoid and uric acid. Repeatability scores for the total antioxidant response method were lower than the other three methods, and the total antioxidant response method was found to be weak to measure uric acid. However, total antioxidant response method had a higher linearity than the other methods. The total antioxidant status method was the least affected by hemolysis and lipemia interference. Total thiol method—with the lowest limit of detection, high sensitivity, and repeatability—was found to be efficient.

Conclusion: Though there is currently no standard method used to determine the antioxidant activity of a compound, the use of different methods and assessing the results gathered from the different methods is a convenient way to evaluate antioxidant capacity.

Key words: antioxidant capacity; FRAP; TAS; TAR; TTL

INTRODUCTION

Over the past several years, oxidative stress has been described as the impairment of the equilibrium between oxidants and antioxidants [1]. Free radical generation appears sustainably during normal cellular function [2]. When overproduction of a reactive oxygen species (ROS) occurs, it leads to modifications in several cell organelles [3]. As a result, oxidative stress is suspected to be involved in various disease processes [2, 4].

Substances neutralizing the potentially harmful effects of free radicals are grouped together in what is referred to as the antioxidant defense system [5]. The antioxidant system is responsible for cellular protection against oxidative stress [3]. In vitro and in vivo antioxidant measurement methods have been developed and implemented by several different techniques so far [6]. Various terms, such as "capacity," "activity," "power," "parameter," "potential", "potency", and "activity" are used to define antioxidant capacity [7]. According to the reaction mechanisms, the determination of antioxidant capacity can be divided into two main groups: the reactions based on hydrogen transfer (HAT) and the reactions based on single electron transfer (ET) [8, 9].

Various tests for the assessment of antioxidant capacity are available, but no standardized methods exist that measure antioxidant capacity [6-8]. The aim of this study was to comprehensively evaluate the analytical performance characteristics of four different automated methods: the ferric reducing ability of plasma (FRAP) [10], total antioxidant status (TAS) [11], total antioxidant response (TAR) [12], and total thiol (TTL) [13, 14]. FRAP method is based on the principle of the reduction of the ferric-tripyridyltriazine complex (Fe^{+3} -TPTZ) by the antioxidants at low pH to the blue ferrous form (Fe^{+2} -TPTZ). The change of the absorbance is measured [10]. TAS method is based on the reduction of colored 2, 2'-azino-bis (3-ethylbenzotiazoline-6-sulphonic acid) (ABTS) radical to a colorless reduced form by the antioxidants that are present in the sample [11]. In TAR method Fe^{+2} - O-dianizidine complex reacts with hydrogen peroxide and hydroxyl radical occurs. This radical oxidizes o-dianizidine molecules to yellow-brown dianizidyl radicals. Color formation increases with forward oxidation reactions. Antioxidants in the sample suppress oxidation reactions and the color formation. This reaction is monitored spectrophotometrically [12]. TTL method is based on the reduction of 5, 5'-dithiobis- (2-nitrobenzoic) acid (DTNB) molecule by thiols

to disulfide form and a molecule of 5-thionitrobenzoic acid. This reaction is observed on the spectrophotometry [13, 14].

In this study these methods were compared and their advantages and disadvantages were discussed.

MATERIALS AND METHODS

Study protocol

Blood samples were obtained from 125 healthy subjects. All participants had a normal medical history, and normal laboratory and clinical findings. Subjects with any acute or chronic diseases were excluded. None of the participants were currently using any medication, nor were they cigarette smokers or alcohol abusers. The study was approved by the local ethics committee and written informed consents were received from all participants. After an overnight fasting period, blood samples were taken into tubes, and the serum samples were separated by centrifugation at 1800 g for 10 min and were stored at -80 ℃ until the analysis was performed. A Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan), which had a temperature-controlled cuvette holder, and a c501 automated analyzer (Roche Hitachi, Mannheim, Germany) were used in this study.

Analytical determinations

FRAP assay

FRAP measurement was performed using the method originally developed by Benzie and Strain [10].The method was applied to the automated analyzer. The sample volume was 20 μl (obtained from the mixture of the 10 μl sample with 30 μl distilled water), so the working reagent volume was 150 μl. The absorbance at 593 nm was read, and a 0–10 min reaction time window was used.

TAS assay

The TAS assay was performed according to Erel's method [11]. When the method was performed on the automated analyzer, the sample volume was 6 μl; the volume of reagent 1 was 100 μl, and the volume of reagent 2 was 10 μl. The absorbance was taken at 660 nm. Originally, absorbance was read before the mixing of reagent 1 and 2. Then, the last absorbance was taken at the end of the incubation period (10 min).

TAR assay

The TAR assay was performed according to Erel's method [12]. When the method was applied to the automated analyzer, the sample volume was 4.5 μl; the volume of reagent 1 was 180 μl, and the volume of reagent 2 was 10 μl. The absorbance was taken at 444 nm. Absorbance was first read prior to the mixing of reagents 1 and 2. The last absorbance was read when the reaction chart reached a plateau line (10 min).

TTL assay

The assay was performed using the method that was developed by Ellman and modified by Hu [13, 14]. The procedure for the automated analyzer was as follows: the sample volume was 10 μl; the volume of reagent 1 was 110 μl, and the volume of reagent 2 was 10 μl. The absorbance was taken at 412 nm. First, absorbance was read before the mixture of reagent 1 and 2; the last absorbance was taken when the reaction graph reached a plateau line (10 min).

Statistical analyses

As the data distributed normally, independent sample t-tests were used for comparison. Pearson's correlation coefficients and linear regression analyses were performed to observe the relationships between assays. In all analyses, a p value of less than 0.05 was considered statistically significant. SPSS software version 22.0 was used for statistical calculations (SPSS Inc., Chicago, IL, USA).

RESULTS

Reaction kinetics of pure antioxidants

The reaction kinetics of pure antioxidants are shown in Figures 1–4. As shown in Figure 1, the FRAP assay revealed that the antioxidant effects of vitamin C, (±)-catechin, Trolox, and α-tocopherol were fast and were completed at the beginning of the reaction. In addition,

the antioxidant effect on DTT was lower than on the other compounds and was completed later in the reaction. The effect of uric acid proved to consist of little or no reaction. Although the antioxidant effects of β-carotene and GSH were close to each other, they were weaker than the reactions of other stronger antioxidants. The antioxidant effects of gallic acid and bilirubin were found to be much more than other antioxidants, such as vitamin C and Trolox.

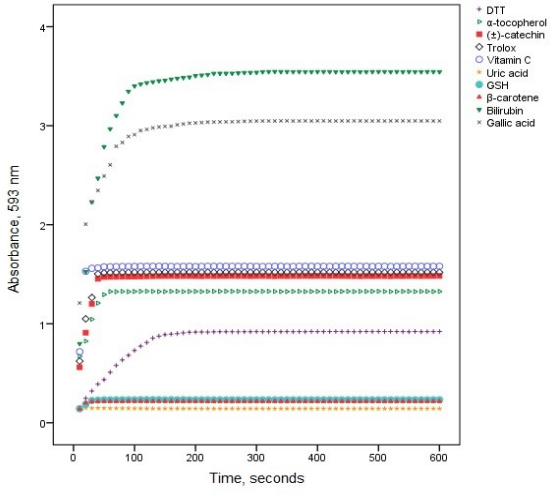

Figure 1. Reaction kinetics of some antioxidants in the FRAP assay

In the TAS assay, the antioxidant effects of vitamin C, Trolox, bilirubin, and DTT were rapid and took place at the start of the reactions. The reactions of uric acid, α-tocopherol, and GSH occurred later than other antioxidants. In particular, the latest effect was observed with uric acid. The effects of gallic acid and (±)-catechin were expressed at the beginning of reactions within seconds and were more powerful than other antioxidants (Figure 2).

As shown in Figure 3 within the TAR assay, the antioxidant effect of vitamin C was slower than that of DTT, uric acid, and GSH. The effect of β-carotene was faster than that of Trolox, α-tocopherol, gallic acid, and (±)-catechin. Of the all antioxidants assessed, gallic acid had the latest reaction. Bilirubin was found to have the fastest and highest reaction. In the TTL assay, DTT had a higher antioxidant effect than GSH (Figure 4).

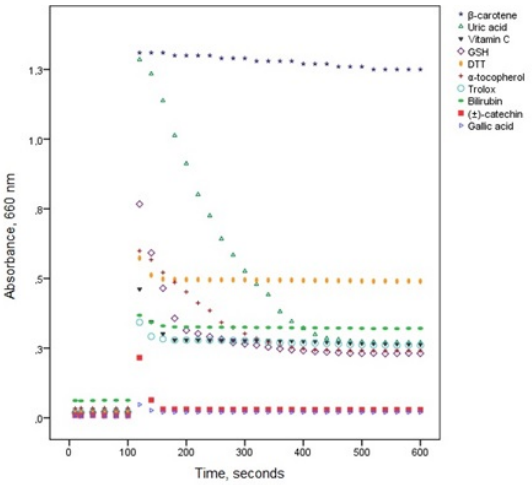

Figure 2. Reaction diagrams of antioxidants solutions in the TAS assay

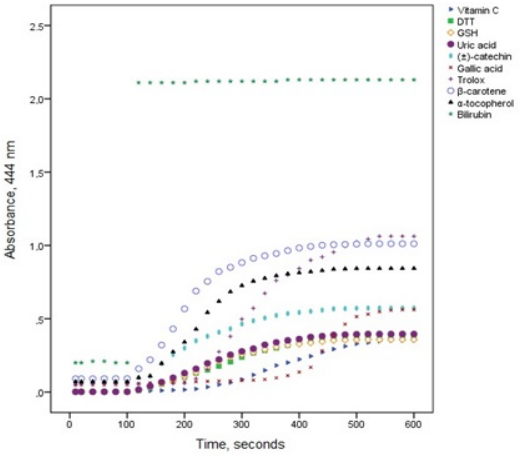

Figure 3. Reactions graphs of pure antioxidants in the TAR assay

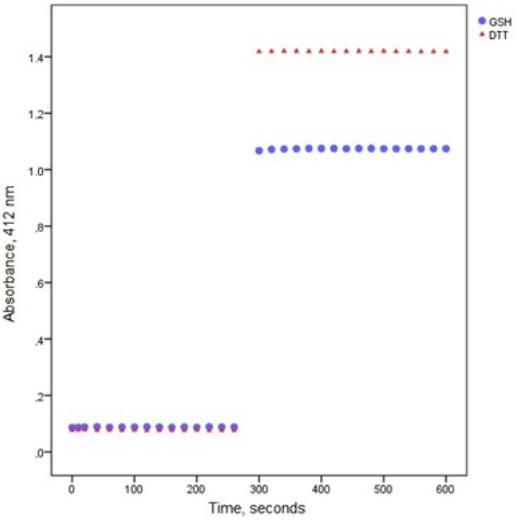

Figure 4. Reaction charts of individual antioxidants in the TTL assay

Linearity of the assays

The linearity of the FRAP, TAS, and TAR assays were performed using serial dilutions of the Trolox solution. The upper limit of the linearity in the FRAP assay was 6.0 mmol Trolox equivalent/l. In the regression analyses, the r value was 0.99 ($p < 0.001$, Sy /x = 0.147), while the slope was 0.94 ($p < 0.001$, Sy/x = 0.037), and the intercept was 0.07 ($p = 0.334$, Sy/x = 0.064). In the TAS assay, the upper limit of the linearity in the assay was 620 mmol Trolox equivalent/l. In the regression analyses, the r value was 0.99 ($p < 0.001$, Sy/x = 0.028), the slope was 0.97 ($p < 0.001$, Sy/x = 0.036), and the intercept was -0.004 ($p = 0.863$, Sy/x = 0.023). The upper limit of the linearity in the TAR assay was 5.0 mmol Trolox equivalent/l. In the regression analyses, the *r* value was 0.99 ($p < 0.001$, Sy/x = 0.136), the slope was 1.248 ($p < 0.001$, Sy/x = 0.025), and the intercept was -0.401 ($p < 0.001$, Sy/x = 0.055). The linearity of the TTL assay was performed by serial dilutions of the DTT solution. The upper limit of the linearity in the assay was 4.0 mmol/l. In the regression analyses, the *r* value was 0.99 ($p < 0.001$, Sy/x = 79.5), the slope was 1203 ($p < 0.001$, Sy/x = 20.12), and the intercept was 75.21 ($p = 0.057$, Sy/x = 34.52).

Analytical sensitivity

Analytical sensitivity, which is the slope of the calibration line, for the FRAP, TAS, and TAR assays were 8.7 × 10-5, 1.05 ×10-5, and 6.67 × 10-4 [Absorbance/(Amount)], [A X (mmol/l)-1], respectively. The analytical sensitivity of the TTL assay was 1.96 × 10-4 [Absorbance/(Amount)], [A X (μmol/l)-1].

Analytical recovery

The recovery of the FRAP, TAS, and TAR assays were determined by adding 2 mmol/l vitamin C to serum samples. The mean percentage recovery of the FRAP, TAS, and TAR assays were 136–141%, 110–114%, and 128–132%, respectively. The percentage recovery of the TTL assay was determined by adding 200 μmol/l GSH to serum samples. The mean percentage recovery of the TTL assay was 97–102%.

Limit of detection

The detection limit of the methods was determined by performing the zero calibrator 10 times. The detection limit was calculated as the mean of the zero calibrator + 3 SD. The detection limits of the FRAP, TAS, and TAR assays were 0.070, 0.091, and 0.184 mmol Trolox equivalent/l, respectively. The detection limit of the TTL assay was 3.92 μmol/l.

Precision

The precision of the methods was determined by evaluating the three levels within the serum pool. Within run variability was determined by analyzing duplicates of 20 serum samples. Within day, (between run) variability was calculated on the basis of two runs and 20 determinations with duplicates of serum. Between days, variability was calculated by analyzing frozen aliquots of serum pools on 20 working days. The mean ± SD values of serum for each method at high, medium, and low levels and the coefficient variation percentage (CV %) of each assay are given in Table 1.

Table 1. The precision of the assays determining antioxidant capacity

A) The precision of the FRAP assay

	Mean ± SD	CV %
Within run		
Low	0.395 ± 0.02	2.74
Medium	0.550 ± 0.03	2.71
High	0.920 ± 0.03	1.33
Within day		
Low	0.405 ± 0.02	3.53
Medium	0.562 ± 0.02	3.14
High	0.942 ± 0.04	3.57
Between day		
Low	0.396 ± 0.01	2.16
Medium	0.550 ± 0.02	3.21
High	0.920 ± 0.03	2.05

B) The precision of the TAS assay

	Mean ± SD	CV %
Within run		
Low	0.807 ± 0.13	20.55
Medium	0.826 ± 0.12	15.68
High	0.883 ± 0.13	16.15
Within day		
Low	0.785 ± 0.12	8.22
Medium	0.803 ± 0.13	7.18
High	0.900 ± 0.13	8.67
Between day		
Low	0.816 ± 0.14	15.63
Medium	0.803 ± 0.13	13.92
High	0.908 ± 0.14	12.19

C) The precision of the TAR assay

	Mean ± SD	CV %
Within run		
Low	0.807 ± 0.13	20.55
Medium	0.826 ± 0.12	15.68
High	0.883 ± 0.13	16.15
Within day		
Low	0.785 ± 0.12	8.22
Medium	0.803 ± 0.13	7.18
High	0.900 ± 0.13	8.67
Between day		
Low	0.816 ± 0.14	15.63
Medium	0.803 ± 0.13	13.92
High	0.908 ± 0.14	12.19

D) The precision of the TTL assay

	Mean ± SD	CV %
Within run		
Low	296.8 ± 3.99	0.91
Medium	367.8 ± 4.26	0.85
High	374.7 ± 3.83	0.80
Within day		
Low	297.3 ± 4.08	0.15
Medium	368.4 ± 4.29	0.24
High	375.1 ± 4.14	0.48
Between day		
Low	296.7 ± 4.23	1.35
Medium	368.2 ± 4.30	1.10
High	374.8 ± 4.34	1.03

Interference

A hemoglobin standard was used for experiments of hemolysis interference. Serum samples with 20 mg/dl, 40 mg/dl, 60 mg/dl, 80 mg/dl, 100 mg/dl, 200 mg/dl, 400 mg/dl, 600 mg/dl, 800 mg/dl, and 1000 mg/dl of hemoglobin concentration were obtained by adding appropriate amounts of hemoglobin standard to the serum. In the FRAP, TAS, TAR, and TTL methods, more than 10% interferences were observed over 400 mg/dl, 800 mg/dl, 200 mg/dl, and 200 mg/dl of hemoglobin concentrations, respectively.

Bilirubin interference experiments were performed by using the bilirubin standard. Samples with 2.5 mg/dl, 5 mg/dl, 7.5 mg/dl, 10 mg/dl, 20 mg/dl, 30 mg/dl, 40 mg/dl, 50 mg/dl, and 60 mg/dl of bilirubin concentrations were achieved by adding various amounts of the bilirubin standard to the serum pools. More than 10% interferences were obtained over 2.5 mg/dl, 5 mg/dl, 7.5 mg/dl, and 20 mg/dl of bilirubin concentrations in the FRAP, TAS, TAR, and TTL assays, respectively.

Clinoleic emulsion (20%) was used for the experiments of lipemia interference, and 20% of lipid emulsion was added to the serum pool in different proportions to simulate lipemia-induced turbidity. Triglyceride concentrations within the serum pool containing the highest lipid emulsion was 2000 g/l. This concentration was recognized as the 2000 lipemia index. In the FRAP, TAS, TAR, and TTL methods, the test results were not affected until reaching the 500, 1000, 500, and 500 lipemia indexes for each method, respectively.

Dilution

The sensitivity of the methods were assessed under different serum dilutions. With different serum dilutions, the resulting values were evaluated against expected values. The dilution of the serum did not affect the results of the assays.

The relationship between methods determining antioxidant capacity and antioxidant components of serum

As seen in Table 2, both the TAS and FRAP methods were significantly correlated with serum uric acid, total protein, and total bilirubin levels. While the TAR assay had a positive significant correlation with total

bilirubin and total protein, the TAR assay did not achieve a statistically significant relationship with uric acid. The TTL assay correlated positively at high levels with total protein concentrations, but it did not have a significant relationship with uric acid or total bilirubin levels. The association of the TAS assay results with both the FRAP and TAR assays were statistically significant. There was no significant relationship between the TAS and the TTL methods. In addition, there was no significant relationship between the FRAP assay and either the TAR or TTL assays. A statistically positive correlation was also found between the TAR and the TTL methods.

DISCUSSION

Measurement of the total antioxidant capacity of a tissue or biological fluid shows its oxidant buffering potential [15]. Numerous methods have been developed to measure total antioxidant capacity for various compounds [6-8]. Methodological aspects, such as the chemistry of the method, the target molecules, the differences of measurement conditions, pH, wavelength, and reaction time all play important roles in assessing the results of antioxidant capacity measurement methods [9, 16]. The four different photometric methods evaluated in this study are based on different fundamental principles [10-14].

Cao et al. found that within runs and between runs, the CV of the FRAP assay were 3.5% and 5.6%, respectively [17]. In another study, Jansen et al. obtained a FRAP method CV as 11.4% [18]. In this study, the CVs of all levels of the FRAP method were lower than those obtained in the literature (Table 1). In the TAS method, the within and between run CVs obtained were lower in this study, although the CV of the TAS method was under 4% in both studies [11]. Jansen et al. [18] found the CV of the TAS method was higher compared to both Erel's study [11] and this research. Though the CVs of the TAR assay were lower than 3% across all three levels in Erel's work [12], the CV obtained was over 10% in this study. The low reproducibility rate of the TAR method may be due to the increased number of steps in the method's procedure when compared with the other methods.

The TTL method was affected by hemolysis interference, perhaps due to the spectral absorbance of hemoglobin because the strongest characteristic wavelength of light absorbed by hemoglobin was 415 nm. The TTL method was monitored spectrophotometrically at 412 nm. Why the TAR method was affected at lower concentrations of hemoglobin than the other evaluated methods may be the yellow-brown color that was formed in the principle method. At high hemoglobin concentrations, hemoglobin may have led to the increase in absorbance by causing darkening of this color.

Table 2. The relationship between methods determining antioxidant capacity and antioxidant components of serum

n = 125	TAS assay	TAR assay	TTL assay	Total protein	Uric acid	Total Bilirubin
FRAP assay	r = 0.934 p < 0.001	r = 0.129 p = 0.154	r = -0.063 p = 0.485	r = 0.22 p = 0.014	r = 0.952 p < 0.001	r = 0.265 p = 0.003
TAS assay		r = 0.18 p = 0.16	r = -0.022 p = 0.810	r = 0.22 p = 0.014	r = 0.924 p < 0.001	r = 0.282 p = 0.001
TAR assay			r = 0.332 p < 0.001	r = 0.182 p = 0.044	r = 0.125 p = 0.169	r = 0.307 p = 0.001
TTL assay				r = 0.405 p < 0.001	r = -0.053 p = 0.562	r = 0.074 p = 0.416

FRAP, ferric ion reducing power; TAS, total antioxidant status; TAR, total antioxidant response; TTL, total thiol.
The r value is the Pearson correlation coefficient.
The p value is the value of significance.

Bilirubin absorbed light strongly between 340–500 nm [19]. In a strongly acidic medium, the absorption of bilirubin transposes to the UV wavelengths. Under alkaline conditions, bilirubin loses a few of its absorption properties [19]. Due to the reductant substance prospect of bilirubin, the FRAP, TAS, and TAR methods may have experienced interference by bilirubin. Because bilirubin can react with H_2O_2 [19], bilirubin may have caused interference in both the TAS and TAR assays' results. The TTL method may have not been affected by the bilirubin's reductant character because the principle of the method is not based on electron transfer.

The FRAP method measures the ferric reducing ability, and it is different from other ET-based methods, such as TAS and TAR, because there are no free radicals or oxidants in the FRAP method. The antioxidant capacity of an antioxidant against any oxidant may not operate in line with the reduction ability of a ferric ion to a ferrous ion [21]. These difference led to lower results in the FRAP method than those obtained in the TAS and the TAR methods.

Ruskovska et al. [24] and Jansen et al. [18] found quite strong correlations between uric acid levels and the FRAP and TAS assays, in a similar manner to this study. Likewise, Jansen et al. found no statistically significant correlations between uric acid values, and either the TAR or the TTL methods [18]. Similarly to this study, both Erel [11] and Jansen et al. [18] found strong relationships between the TAS and FRAP methods. Because acidic pH forms a principle of the TAR method, the uric acid could not have been determined or may have been determined to be very weak in the TAR method. The TTL method, using a different mechanism than the other three assessed methods, comprised an antioxidant method that reflected the presence of free thiol groups. The absence of correlation between the TTL method and uric acid may explain the lack of relationships between both the TAS and FRAP methods. Due to the insufficient measurement of molecules that contain sulfhydryl groups by the FRAP method, the lack of correlation between the FRAP and the TTL methods was not unexpected.

In brief, The FRAP assay was based on only iron ions; so this reaction was not specific, and it was insufficient to measure thiol compounds, such as glutathione. Despite these results, the FRAP method was simple, cheap, and did not require special equipment. The TAR method was inadequate for measuring uric acid, an important antioxidant component of serum, due to the pH of the assay principle. In addition, the reproducibility of the TAR method was lower when compared to the other three methods, but the TAR method had a higher linearity than the others. Due to its simple and easy application, the TAS method could be used in many laboratories for antioxidant capacity research studies. Not to be affected by lipemia and hemolysis interferences were advantages of this method. The TTL assay was a very good method, providing the lowest limit of detection, high sensitivity, and high reproducibility.

This study evaluated and reported on the limitations and strengths of several antioxidant capacity methods. Currently, there is no standard method to determine the antioxidant activity of a compound. Methods of determining antioxidant activity depend on various parameters, such as substrate, reaction conditions, the nature of the compound to be analyzed, and the speed and sensitivity of the analysis. Therefore, using different methods for evaluating antioxidant capacity and assessing the results through multiple methods provided more accurate and complete results.

Conflict of interest

There is no conflict of interest.

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