Method for quantification of antioxidant capacity of processed fruit juices exploring the formation of the Fe(II)/3-hydroxy-4-nitroso-2,7-naphthalenedisulfonic complex

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Method for quantification of antioxidant capacity of processed fruit juices
exploring the formation of the Fe(II)/3-hydroxy-4-nitroso-2,7-naphthalenedisulfonic complex

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Abstract

The formation of the Fe(NRS)$_3^{4+}$ complex was used in a modified spectrophotometric method to quantify the total antioxidant capacity (TAC) of processed fruit-juice samples. The procedure is based on the reduction of Fe(III) to Fe(II) in aqueous buffered solution (Tris; pH 8.0) containing the 3-hydroxy-4-nitroso-2,7-naphthalenedisulfonic complex acid (H$_2$NRS). The absorbance values at 730 nm ($A_{730\ nm}$) of the Fe(NRS)$_3^{4+}$ complex obtained with juice samples were compared with $A_{730\ nm}$ values obtained with a standard ascorbic acid solution and then subsequently used to quantify and express the TAC of the samples. Good positive relationship between TAC values in the analysed samples (grape, orange, passion fruit, cashew, peach and strawberry) was found for both Fe(NRS)$_3^{4+}$ complex (proposed method) and ABTS$^{•-}$ radical-free method (used as reference method). The TAC values obtained with Fe(NRS)$_3^{4+}$ and ABTS$^{•-}$ also showed a positive correlation with the Folin-Ciocalteu index values. Fructose, glucose, BHT and BHA did not interfere in the TAC quantification of the processed fruit juice samples with the proposed method. However, ascorbic acid (natural or added as additive, E300) and its derivatives (E301, E302, E303, E315 and E316) will interfere positively. As a 10-fold dilution is required and the absorbance measurements are performed at 730 nm, the proposed method was not subject to color interference from the tested samples. The procedure suggested here is simple, easy to perform, reproducible, does not use organic solvents and does not need expensive equipment.

**Keywords:** processed juice, total antioxidant capacity, 3-hydroxy-4-nitroso-2,7-naphthalenedisulfonic acid, Fe(III).
Introduction

The so-called oxidative stress (which is attributed to the harmful effects caused by the excess of free radicals) can damage proteins and DNA and is also correlated with the generation of a broad spectrum of illnesses related to cardiovascular disease (atherosclerosis), mild cognitive impairment and neurological illness (Alzheimer’s and Parkinson’s diseases) aging disorders and even certain types of cancer\(^1,2\).

Evidence suggests that those diseases can be retarded or even avoided by ingestion of foods products containing antioxidant compounds (particularly vitamins and phenolics), hence a well-balanced human healthy diet appears to be an important factor\(^3\).

In tune with this new healthier lifestyle trend packaged fruit juice has become one of the most consumed beverages mainly because their greater convenience and by the fact of being an important source of vitamins, phenolic compounds, minerals and fibers\(^4,5\). On the label of such products there is sufficient information on the nutritional data (e.g. total fat, total carbohydrate, sugar, sodium, total calories etc.), but none on the total antioxidant capacity (TAC). One of the reasons may be the fact that there is no method globally adopted for quantifying the TAC for this type of beverage yet. Thus, it is highly desirable to count on lower-cost and easier-to-perform methods available to quantify this parameter\(^6\).

The 3-hydroxy-4-nitroso-2,7-naphthalenedisulfonic acid (H\(_2\)NRS, Figure 1) is a water-soluble organic diacid with two protons (sulfonate groups) dissociating at pH<1.0 and the -OH group with a pK 7.46±0.05 (I=0; T=25°C)\(^7\). Its disodium salt (Na\(_2\)NRS) quickly reacts with Fe(III) forming a greenish-yellow complex, which has been employed for total iron quantification in standard samples\(^8\), dietary supplements\(^9\), serum\(^10,11\) and stream water\(^12\).

Fe(II) also reacts with NRS\(^2-\) (1:3 Fe(II):NRS ratio) in a buffered aqueous solution (Tris; pH 8.0) yielding a very stable yellow-green Fe(NRS)\(_3^+\) complex (calculated dissociation constant \(1\times10^{-23}\))\(^13\) which exhibit an absorption peak at 730 nm\(^9,14,15\). In fact, a recent comprehensive study based on the reduction of
Fe(III) to Fe(II) by several antioxidant compounds in aqueous solution (Tris; pH 8.0) containing the NRS$^{2-}$, giving rise to the Fe(NRS)$_3^{4+}$ complex, originated an modified FRAP (Ferric Reducing Antioxidant Power) assay$^{15}$.

This modified FRAP assay mentioned above was employed to quantify the TAC of several processed fruit juice samples in the present paper. Since these beverages contain natural (or added) antioxidants the addition of aliquots of these samples in an aqueous solution containing Fe(III) also promoted the generation of Fe(II) which in the presence of NRS$^{2-}$ (pH 8.0) formed the same the Fe(NRS)$_3^{4+}$ complex. The absorbance values recorded at 730 nm ($A_{730 \text{ nm}}$) of the bright green water-soluble Fe(NRS)$_3^{4+}$ complex obtained with fruit juice samples were compared with $A_{730 \text{ nm}}$ values of the same complex formed after the addition of an ascorbic acid standard solution and used to quantify and express the TAC of each juice.

From the environmental perspective, the suggested procedure may also be an interesting alternative since it does not use organic solvents, such as the methods based on the extinction of the DPPH$^{16}$ and ABTS$^{17}$ radical-free methods.

For comparison purposes, the TAC values obtained with the Fe(NRS)$_3^{4+}$ complex were correlated with values obtained with the ABTS$^{••}$ radical-free method$^{2,17}$ and with the Folin-Ciocalteu index (FC index)$^{14}$.

**Experimental**

**Apparatus**

All absorbance measurements were recorded in a HPUV 8453 (Agilent) spectrophotometer using a 1.0 cm optical path length using glass cell.

**Reagents**
Reverse osmosis water (Quimis Q842-210, Diadema, Brazil) was used to prepare all solutions except when another solvent is indicated otherwise.

All reagents used were of analytical-grade.

A 1.0×10⁻² mol/L disodium salt of 3-hydroxy-4-nitroso-2,7-naphthalenedisulfonic acid solution, Na₂NRS, (C₁₀H₅NO₈S₂Na₂, 99.7%, FW 377.26 g/mol, J.T. Baker, Center Valley, USA) was prepared by dissolving 0.9432 g in 250 mL of water. A 2.5×10⁻³ mol/L solution was obtained by appropriate dilution.

A 1.8×10⁻³ mol/L iron(III) sulfate (Fe₂(SO₄)₃.5H₂O, 99.7%, FW 489.9 g/mol, Merck, Darmstadt, Germany) solution was prepared by dissolving 0.0441 g in 100.0 mL of water adding 4 drops of concentrated sulfuric acid (H₂SO₄, FW 98.08 g/mol, J.T. Baker, Center Valley, USA).

A 1.0×10⁻¹ mol/L 2-amino-2-hydroxymethyl-propane-1,3-diol, Tris, (C₄H₁₁NO₃, 99.7 %, FW 121.1 g/mol, Merck, Darmstadt, Germany) solution (pH 8.0) was prepared by dissolution of 3.029 g in 250.0 mL of water.

A 1.0×10⁻² mol/L ascorbic acid, AA, (C₆H₈O₆, 99.7 %, FW 176.1 g/mol, Merck, Darmstadt, Germany) standard solution was prepared by dissolving 0.176 g in a 100.0 mL volumetric flask containing water. A 1.0×10⁻⁴ mol/L solution was obtained by accurate dilution.

The Folin-Ciocalteu reagent (FCR) was prepared as recommended by Brazilian Pharmacopeia and it is described elsewhere.

A 10% (m/v) sodium carbonate (Na₂CO₃, 99 %, FW 105.99 g/mol, Vetec, Rio de Janeiro, Brazil) solution was prepared in water.

A 5.0×10⁻³ mol/L gallic acid, GA, (C₇H₆O₅, 99%, FW 170.12 g/mol, Sigma-Aldrich, St. Louis, USA) standard solution was prepared by dissolving 0.0941 g in a 100.0 mL volumetric flask containing water. A 5.0×10⁻⁴ mol/L solution was obtained by accurate dilution.

A 7.0×10⁻³ mol/L 2,2’-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt, ABTS, (C₁₈H₁₆N₄O₆S₄, FW 548.68 g/mol, Sigma-Aldrich, St. Louis, USA) solution was prepared by dissolving 0.192 g in ethyl alcohol in a 50.0 mL volumetric flask.
A $1.4 \times 10^{-1}$ mol/L potassium persulfate ($K_2S_2O_8$, FW 270.33 g/mol, Neon Comercial Ltda., São Paulo, Brazil) solution was prepared by dissolving 0.3784 g in 10.0 mL of water just before use.

A $2.0 \times 10^{-3}$ mol/L 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Trolox®, ($C_{14}H_{18}O_4$, FW 250.29 g/mol, Sigma-Aldrich, St. Louis, USA) solution was prepared by dissolving 0.0250 g in ethyl alcohol in a 50.0 mL volumetric flask.

**Sample preparation**

The fruit juice samples (all in carton packaging) were purchased in local supermarkets and kept at room temperature in the laboratory. All samples were within the expiration date established by the supplier.

For the TAC quantification (with Fe(NRS)$_3^{4-}$ complex and ABTS$^{•+}$ radical-free method) and the FC index (with FCR), 1.0 mL of each sample was weighed and transferred to a 10.0 mL volumetric flask and the volume completed with water. All analyses were performed in replicas (n 3).

**Quantification of FC index in fruit juices with the FCR**

A calibration graph was obtained by mixing aliquots (100-400 µL) of a $5.0 \times 10^{-4}$ mol/L GA standard solution with 200 µL of FCR in a 5.0 mL volumetric flask, which was completed with a 10% Na$_2$CO$_3$ solution.

The multiple standard addition method$^{16,19}$ was used in all sample analyses as follows: 500 µL of diluted fruit juice samples were transferred to five volumetric flasks (5.0 mL) followed by the addition of 200 µL of FCR. In four out of five volumetric flasks, aliquots (100-400 µL) of $5.0 \times 10^{-4}$ mol/L GA solution were added and the volume completed with the same 10% Na$_2$CO$_3$ solution.

In both graphs (calibration and multiple standard additions with the samples) the absorbance was measured at 715 nm after 30 minutes using water as reference solution. The FC index was expressed as mg GA per g fruit juices (FJ).
Quantification of TAC using the ABTS$^{*+}$ radical-free method

The ABTS$^{*+}$ radical-free method was based on the procedure previously described\textsuperscript{2,17} with slight modifications.

Briefly, a 5.0 mL of $7.0 \times 10^{-3}$ mol/L ABTS solution was mixed with 88 µL of $1.4 \times 10^{-1}$ mol/L $\text{K}_2\text{S}_2\text{O}_8$ solution for conversion of ABTS into ABTS$^{*+}$ radical cation. This ABTS$^{*+}$ solution, which was kept in a stoppered dark flask at room temperature for 16 hours and was diluted with ethyl alcohol just before use until the absorbance value recorded at 734 nm, $A_{734\text{nm}}$, was (0.70±0.05).

Initially, five diluted solutions were prepared transferring 0.5; 2.5; 5.0; 7.5 and 10 mL of a $2.0 \times 10^{-3}$ mol/L Trolox® to five 10.0 mL volumetric flasks and completed with ethyl alcohol (except for the last solution). A five-point calibration graph was obtained transferring 50 µL of these diluted solutions to a 5.0 mL volumetric flasks and completed with the ABTS$^{*+}$ solution above prepared.

Then, a calibration graph with FJ (10-fold v/v diluted) was obtained. Aliquots of 100-800 µL of these diluted juice solutions were transferred to a 5.0 mL volumetric flask, which was completed with the same ABTS$^{*+}$ solution.

All the $A_{734\text{nm}}$ values were recorded after 6 minutes using ethyl alcohol as reference solution. The TAC values were as expressed as µg Trolox® per g FJ.

Quantification of TAC in with the proposed FRAP assay (Fe(NRS)$_3^{3+}$ complex)

Calibration with AA solution

In seven 5.0 mL volumetric flasks the following reactants were added (the order of addition is mandatory): 500 µL $2.5 \times 10^{-3}$ mol/L $\text{Na}_2\text{NRS}$, 100 µL $1.8 \times 10^{-3}$ mol/L Fe(III), 100-700 µL $1.0 \times 10^{-4}$ mol/L AA standard solution, 300 µL $1.0 \times 10^{-4}$ mol/L Tris and the volume completed with water. The AA final concentration ranged from ($0.352-2.47$)$\times 10^{-3}$ mg/mL.
Absorbance measurements were recorded at 730 nm ($A_{730\text{nm}}$) after 30 minutes. A freshly prepared solution containing $250 \times 10^{-6}$ mol/L Na$_2$NRS, $36 \times 10^{-6}$ mol/L Fe(III) and $6.0 \times 10^{-3}$ mol/L Tris was used as reference solution. A calibration graph ($A_{730\text{nm}}$ vs. $C_{\text{AA}}$, in mg/mL) obtained is described by the equation $A_{730\text{nm}} = a + b \times C_{\text{AA}}$.

**Calibration with FJ solution**

The following reactants were added in four 5.0 mL volumetric flasks: 500 µL $2.5 \times 10^{-3}$ mol/L Na$_2$NRS, 100 µL $1.8 \times 10^{-3}$ mol/L Fe(III), 100-800 µL of diluted (1:10 v/v) FJ, 300 µL $1.0 \times 10^{-1}$ mol/L Tris and the volume completed with water.

$A_{730\text{nm}}$ values were obtained after 30 minutes using the same reference solution as described above for the calibration graph with AA solution ($250 \times 10^{-6}$ mol/L Na$_2$NRS, $36 \times 10^{-6}$ mol/L Fe(III) and $6.0 \times 10^{-3}$ mol/L Tris). A graph ($A_{730\text{nm}}$ vs. $C_{\text{FJ}}$, FJ concentration in mg/mL) obtained is described by the equation $A_{730\text{nm}} = a + b \times C_{\text{FJ}}$.

**Calculation method for quantification of the TAC of FJ**

With the equation $A_{730\text{nm}} = a + b \times C_{\text{AA}}$ is calculated the $A_{730\text{nm}}$ value corresponding to a 1.0 mg/mL AA standard solution. This $A_{730\text{nm}}$ value is placed into the equation $A_{730\text{nm}} = a + b \times C_{\text{FJ}}$, giving the concentration of FJ (in mg/mL), which corresponds to the antioxidant capacity of a 1.0 mg/mL AA standard solution. Correcting the sample dilution (10-fold) and knowing the juice sample mass transferred to a 10.0 mL volumetric flask the TAC values were calculated and expressed as g FJ per g AA. The TAC values can be more easily obtained with the equation below:

$$TAC \ (g \ FJ/g \ AA) = \left\{ \frac{b' \times m_{FJ} \times 1000}{[(a + b) - a']} \right\}$$  \hspace{1cm} (1)
In which \( a \) and \( b \) are the linear and angular coefficients of the calibration curve with AA, respectively. \( a' \) and \( b' \) are the linear and angular coefficients of the curve with diluted juice, respectively. \( m_{FJ} \) is the mass (in g) of juice.
Results and Discussion

General features of the chemical reaction used in the proposed method

The chemical aspects of the reduction reaction of Fe(III) to Fe(II) in aqueous solution containing NRS\(^2\), used in the present study for the quantification of the TAC of FJ, were detailed in a previously published study\(^{15}\).

Basically, Fe(II) formed by the reduction of Fe(III) by a suitable reducing agent reacts with 3-fold excess of NRS\(^2\) at 6.8 < pH < 8.5\(^{15}\) forming a greenish-yellow water-soluble Fe(NRS)\(_3\)\(^{4+}\) complex\(^{10,11,20-22}\), which increase in absorbance values at 730 nm are related to the concentration of this reducing agent\(^{14,15}\).

Figure 2 shows the absorption spectra (330 nm – 990 nm) of aqueous solution containing Fe(III) \(36 \times 10^{-6}\) mol/L; Na\(_2\)NRS \(250 \times 10^{-6}\) mol/L, Tris \(6.0 \times 10^{-3}\) mol/L and AA (0.354; 0.705; 1.06; 1.41; 1.76; 2.11 e 2.47) \(\times 10^{-3}\) mg/mL, with formation of the Fe(NRS)\(_3\)\(^{4+}\) complex according to equation (2). The Fe(NRS)\(_3\)\(^{4+}\) complex formed has three peaks of maximum absorption: 360, 422 and 730 nm. According to figure 2, it is noted that at 730 nm there is an apparent sensitivity about 3-fold lower than 360 nm or 422 nm. However, the blank reagent (Fe(III) \(36 \times 10^{-6}\) mol/L; Na\(_2\)NRS \(250 \times 10^{-6}\) mol/L and Tris \(6.0 \times 10^{-3}\) mol/L) absorbs more strongly in these last two wavelengths. Therefore, all absorbance measurements recorded and used in this proposed method were carried out at 730 nm (\(A_{730\text{nm}}\)) where the absorbance value of the blank reagent is smaller (Figure 2). This may be an advantage in the proposed method since spectrophotometers used for measurements in the visible spectrum are less expensive.

\[
2 \text{Fe(NRS)}_3^{3+} + \text{AA} + \text{H}_2\text{O} \rightarrow 2 \text{Fe(NRS)}_3^{4+} + \text{DehydroAA} + 2 \text{H}^+ \quad (2)
\]
Antioxidant standard chosen

The AA was used as standard compound to calculate and express the TAC of processed FJ because it reduces rapidly Fe(III) in aqueous solution (pH 8.0) containing NRS\textsuperscript{2-} equation (2). Additionally, it is biologically active compound and is cheaper than other standard antioxidants (e.g. Trolox\textsuperscript{®}). Another reason for its use is due to the excellent reproducibility obtained with its calibration curves. A typical calibration curve for AA is linear from (0.20-1.4)\times10^{-5} mol/L, meaning (0.35-2.5)\times10^{-3} mg/mL, and is described by the equation $A_{730\text{nm}} = -0.01527 + 30860\times[\text{AA}]$ ($r^2 = 0.996$; n = 7) where [AA] is the concentration of AA in mol/L (Figure 2, inset). The AA average apparent molar absorptivity value (at 730 nm) obtained with the Fe(NRS)\textsubscript{34-} complex is $(3.2\pm0.2)\times10^4$ L/ cm mol for 23 calibration curves with a relative standard deviation as 6.9%.

Time required for $A_{730\text{nm}}$ and order of reagent addition

As previously mentioned, a longer time is recommended to allow the complete reaction of Fe(III)/complexes with antioxidants\textsuperscript{23}, so a 30 minute-time was chosen to perform all $A_{730\text{nm}}$ measurements in the proposed method.

Regarding the AA quantification in pharmaceutical formulations it was pointed out that the colour of the Fe(NRS)\textsubscript{34+} complex remained stable for until 30 minutes when protected from light exposure. To avoid the gradual decrease of the absorbance value over time it was necessary to wrap the flask containing the complex with carbon paper\textsuperscript{24}.

However, no decrease in $A_{730\text{nm}}$ values was observed in the proposed procedure during the 30 minutes period measurement (either in the samples or in the standard AA solution) even when the flask was exposed to daylight inside the laboratory.

The reactants addition order is quite important and must be obeyed as described in the general procedure. Otherwise the reaction does not occur and lack of reproducibility is observed.
Possible interfering compounds in the proposed method

It was pointed out in a previous study that reducing sugars did not interfere even when 100-fold more concentrated than AA $1.0 \times 10^{-6}$ mol/L\textsuperscript{15}.

BHT (2,6-bis(1,1-dimethylethyl)-4-methylphenol) and BHA (a mixture of 2-\textit{tert}-Butyl-4-hydroxyanisole and 3-\textit{tert}-butyl-4-hydroxyanisole) it can be tolerated up to 10-fold higher AA $1.0 \times 10^{-6}$ mol/L\textsuperscript{15}. However, as the solutions of these two antioxidants had to be prepared in 50:50 v/v water:ethanol mixture they are not likely to be in large amounts in the aqueous juice samples.

These observations leads to the conclusion that there should be no interference of D-(-)-Fructose, D-(+)-Glucose, BHT and BHA in the TAC quantification of processed FJ with the proposed method.

On the other hand, sodium ascorbate (E301), calcium ascorbate (E302), potassium ascorbate (E303), erythorbic acid (E315) and sodium erythorbate (E316), widely used as food preservative will positively interfere. Moreover, the fact that the proposed method cannot differentiate the AA commonly added as a vitamin supplement or antioxidant (E300) from the AA originating from the fruit itself must be borne in mind. Regarding the analyzed samples, it is necessary to mention that all the information listed (three tables) is provided by the suppliers who do not specify the amount of these additives in the package label.

Results with samples

In the present modified FRAP assay Fe(II) was generated from the reduction of Fe(III) by the antioxidants present in the samples of processed FJ in solution containing NRS\textsuperscript{2-} (Tris buffer; pH 8.0). The varieties of juice analyzed were chosen based on their convenience on supermarket and that is the reason for having more samples of grape and orange juices (respectively 10 and 7 samples). The other samples (specifically 1 passion fruit, 1 cashew, 2 peaches and 2 strawberry) were classified into a single group named \textit{miscellaneous}.  

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For comparison of the TAC values the same samples were analyzed with the Fe(NRS)$_3^{3+}$ complex (proposed method) and with the method based on the scavenging of ABTS$^{••}$. In addition, the same samples were reacted with the FCR to quantify FC index values.

Tables 1, 2 and 3 show the results obtained with these three methods.

Considering only the grape juice samples (Table 1) a good positive relationship, represented by adjusted $r^2$ 0.703 ($P<0.002$), between the TAC values obtained with Fe(NRS)$_3^{3+}$ complex and with ABTS$^{••}$ radical-free method was found. Both TAC values (Fe(NRS)$_3^{3+}$ and ABTS$^{••}$) showed a good positive correlation with the FC index values (respectively adj. $r^2$ 0.837 ($P<0.0002$) and 0.800 ($P<0.0005$)).

Regarding the orange juice (Table 2) an excellent positive correlation between TAC values obtained with Fe(NRS)$_3^{3+}$ complex and the ABTS$^{••}$ radical-free method was verified (adj. $r^2$ 0.937; $P<0.0004$). Here again both TAC values (Fe(NRS)$_3^{3+}$ and ABTS$^{••}$) showed a good positive relationship with the FC index values (respectively adj. $r^2$ 0.840; $P<0.004$, and 0.763 $P<0.001$).

Also, for miscellaneous group FJ (Table 3) a good positive correlation between the TAC values with the Fe(NRS)$_3^{3+}$ complex and ABTS$^{••}$ radical-free method (adj. $r^2$ 0.762, $P<0.02$) was found. Probably due to the heterogeneity of these samples, the correlation of the FC index values with TAC values measured with the Fe(NRS)$_3^{3+}$ complex (adj. $r^2$ 0.775; $P<0.02$) and with ABTS$^{••}$ radical-free method (adj. $r^2$ 0.601; $P<0.07$) showed a somewhat lower agreement.

Taking into account the above results, it seems correct to state that the proposed method can be used as an alternative procedure to quantify the TAC of these processed FJ samples.

The proposed method has the advantage of being more attractive environmentally speaking, since the reaction is not conducted in organic solvents (e.g. methanol or acetone) and the ligand can be recovered as described in previous work$^{14,15}$. The preparation of the samples in the proposed method was done only by dilution (no extraction or centrifugation steps was used), which makes the suggested procedure less tedious. No turbidity was observed in any analysis, probably due to 10-fold dilution required, which supports the use of the proposed method for a sample containing a certain amount of pulp (dietary fibre).
The concordance between both methods used for TAC quantification (Fe(NRS)$_3^{4-}$ and ABTS$^{•+}$) and FC index values suggests that compounds oxidized by the FCR are responsible for the antioxidant capacity found in these samples (Figure 3). It is well known that FCR is not specific for polyphenols and that another group of antioxidants may react with the FCR and overestimate the polyphenolic content$^{25}$. On the other hand, the FC index results obtained in this study (standard addition method with GA solution) do not invalidate the assumption that polyphenols are responsible for TAC values in these samples. It is well known that grape juices generally have greater polyphenolic content than FJ like orange, passion fruit, cashew, peach and strawberries, evaluated in this study. In fact, for the samples tested the higher the FC index (grape juices $1.02±0.47 >$ orange juices $0.275±0.070 >$ miscellaneous $0.258±0.037$), the higher the antioxidant capacity (either obtained with Fe(NRS)$_3^{4-}$ complex or with the ABTS$^{•+}$ radical-free method).

Other analytical potentialities

The results obtained in this study lead us to believe that the proposed reaction can also be used to measure the TAC of different samples of plant origin (other than medicinal plant extracts$^{15}$ and processed FJ) such as wines, beers, teas and fruits.

In addition, since Fe(III) is reduced by tyrosine to Fe(II) under the same experimental conditions of this proposed method$^{15}$ this reaction can be used to quantify the antioxidant capacity of biological samples such as serum, follicular fluid or tears.

In fact, a recent study$^{26}$ has shown that TAC of blood serum sample can be achieved using cysteine (which strongly reduces Fe(III) in solution containing NRS$^{-2}$) as antioxidant standard. This allows us to infer that the reaction of the proposed method can also be used in complex biological samples.

Conclusion
The formation of the aqueous Fe(NRS)_3^{4-} complex (Tris buffer; pH 8.0) was suitably utilized in a modified FRAP assay to quantify the TAC of processed FJ. Fructose, glucose, BHT and BHA did not interfere but AA (natural or added) and its derivatives interfere positively.

A known limitation of the spectrophotometric methods is the possible interference of coloured samples but as a 10-fold dilution is required and the absorbance measurements are performed at 730 nm, the proposed method is not subject to colour interference of samples of grape, orange, passion fruit, cashew, peaches and strawberries juice samples.

The procedure suggested here is simple, easy to perform, reproducible, does not use organic solvents and can be used in routine analysis since reagents are not expensive and the equipment is commonly available in most laboratories.

As the proposed procedure was designed to quantify the TAC, it is not possible to define the contribution of each antioxidant individually. However, as expected, the sample group with highest FC index (probably polyphenols and vitamins) presented higher antioxidant capacity.

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References


Captions of Tables

Table 1. The antioxidant capacity (with the Fe(NRS)$_{3}^{4+}$ complex and ABTS$^{•+}$) and the FC index values of the grape juices analysed.

Table 2. The antioxidant capacity (with the Fe(NRS)$_{3}^{4+}$ complex and ABTS$^{•+}$) and the FC index values of the orange juices analysed.

Table 3. The antioxidant capacity (with the Fe(NRS)$_{3}^{4+}$ complex and ABTS$^{•+}$) and the FC index values of miscellaneous juices analysed.

Captions of Figures

Figure 1. Structure of the anion of the 3-hydroxy-4-nitroso-2,7-naphthalenedisulfonic acid.

Figure 2. Absorption spectra with formation of the Fe(NRS)$_{3}^{4+}$ complex before and after addition of a standard ascorbic acid solution, where (a) Fe(III) $36 \times 10^{-6}$ mol/L + NRS $250 \times 10^{-6}$ mol/L; Tris $6.0 \times 10^{-3}$ mol/L; (b) to (h) = (a) + ascorbic acid (0.200; 0.400; 0.600; 0.800; 1.00; 1.20 e 1.40)$\times 10^{-5}$ mol/L, respectively. Absorbance measurements after 30 minutes using water as reference solution.

Figure 3. 3-D graph comparing the antioxidant capacity (Fe(NRS)$_{3}^{4+}$ complex and ABTS$^{•+}$ method) and FC index for all analysed samples. The FC index is expressed as mg gallic acid per g juice fruit; ABTS$^{•+}$ is the antioxidant capacity expressed as μg trolox per g juice fruit and Fe(NRS)$_{3}^{4+}$ complex (proposed method) is the antioxidant capacity expressed as g fruit juice per g ascorbic acid.
**Table 1.** The antioxidant capacity (with the Fe(NRS)$_3^{+}$ complex and ABTS$^{**}$) and the FC index values of the grape juices analysed.

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<th>Additives</th>
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<td></td>
<td>Fe(NRS)$_3^{+}$ complex</td>
<td>ABTS$^{**}$</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.324 ± 0.010</td>
<td>411 ± 17</td>
<td>0.928 ± 0.004</td>
</tr>
<tr>
<td>2</td>
<td>0.395 ± 0.010</td>
<td>673 ± 41</td>
<td>1.64 ± 0.090</td>
</tr>
<tr>
<td>3</td>
<td>0.215 ± 0.020</td>
<td>408 ± 17</td>
<td>0.820 ± 0.003</td>
</tr>
<tr>
<td>4</td>
<td>0.373 ± 0.030</td>
<td>326 ± 7</td>
<td>0.993 ± 0.020</td>
</tr>
<tr>
<td>5</td>
<td>0.213 ± 0.010</td>
<td>324 ± 15</td>
<td>0.693 ± 0.040</td>
</tr>
<tr>
<td>6</td>
<td>0.147 ± 0.020</td>
<td>176 ± 5</td>
<td>0.306 ± 0.017</td>
</tr>
<tr>
<td>7</td>
<td>0.418 ± 0.030</td>
<td>822 ± 53</td>
<td>1.44 ± 0.32</td>
</tr>
<tr>
<td>8</td>
<td>0.271 ± 0.020</td>
<td>462 ± 19</td>
<td>0.601 ± 0.006</td>
</tr>
<tr>
<td>9</td>
<td>0.253 ± 0.010</td>
<td>282 ± 13</td>
<td>0.841 ± 0.083</td>
</tr>
<tr>
<td>10</td>
<td>0.664 ± 0.070</td>
<td>839 ± 66</td>
<td>1.90 ± 0.12</td>
</tr>
</tbody>
</table>

FC index = expressed as mg gallic acid/g juice fruit; ABTS$^{**}$ = expressed as μg trolox/g juice fruit; Fe(NRS)$_3^{+}$ complex (proposed method) = expressed as g fruit juice/g ascorbic acid; Additives as reported in the package: E300 = ascorbic acid and E316 = sodium erythorbate; NI = no information.
Table 2. The antioxidant capacity (with the Fe(NRS)$_{3}^{4-}$ complex and ABTS$^{••}$) and the FC index values of the orange juices analysed.

<table>
<thead>
<tr>
<th>Orange juices</th>
<th>Antioxidant Capacity</th>
<th>FC index</th>
<th>Additives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe(NRS)$_{3}^{4-}$</td>
<td>ABTS$^{••}$</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.055 ± 0.001</td>
<td>67 ± 0.6</td>
<td>0.224 ± 0.009 E300; E316; Dietary fiber</td>
</tr>
<tr>
<td>2</td>
<td>0.102 ± 0.005</td>
<td>113 ± 2</td>
<td>0.233 ± 0.019 E316</td>
</tr>
<tr>
<td>3</td>
<td>0.217 ± 0.004</td>
<td>179 ± 3</td>
<td>0.390 ± 0.020 E300; E316</td>
</tr>
<tr>
<td>4</td>
<td>0.180 ± 0.006</td>
<td>175 ± 15</td>
<td>0.319 ± 0.006 E316</td>
</tr>
<tr>
<td>5</td>
<td>0.050 ± 0.006</td>
<td>87 ± 2</td>
<td>0.262 ± 0.016 E300; Dietary fiber</td>
</tr>
<tr>
<td>6</td>
<td>0.037 ± 0.002</td>
<td>63 ± 5</td>
<td>0.168 ± 0.001 E300; E316</td>
</tr>
<tr>
<td>7</td>
<td>0.194 ± 0.003</td>
<td>23 ± 2</td>
<td>0.331 ± 0.018 E300</td>
</tr>
</tbody>
</table>

FC index = expressed as mg gallic acid/g juice fruit; ABTS$^{••}$ = expressed as μg trolox/g juice fruit; Fe(NRS)$_{3}^{4-}$ complex (proposed method) = expressed as g fruit juice/g ascorbic acid; Additives as reported in the package: E300 = ascorbic acid and E316 = sodium erythorbate.
Table 3. The antioxidant capacity (with the Fe(NRS)$_3^{4+}$ complex and ABTS$^{•+}$) and the FC index values of miscellaneous juices analysed.

<table>
<thead>
<tr>
<th>Miscellaneous</th>
<th>Antioxidant Capacity</th>
<th>FC index</th>
<th>Additives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe(NRS)$_3^{4+}$ complex</td>
<td>ABTS$^{•+}$</td>
<td></td>
</tr>
<tr>
<td>Cashew</td>
<td>0.338 ± 0.003</td>
<td>342 ± 39</td>
<td>0.326 ± 0.025</td>
</tr>
<tr>
<td>Passion fruit</td>
<td>0.214 ± 0.018</td>
<td>164 ± 1</td>
<td>0.262 ± 0.024</td>
</tr>
<tr>
<td>Peach 1</td>
<td>0.165 ± 0.010</td>
<td>152 ± 6</td>
<td>0.211 ± 0.032</td>
</tr>
<tr>
<td>Peach 2</td>
<td>0.228 ± 0.035</td>
<td>290 ± 13</td>
<td>0.267 ± 0.039</td>
</tr>
<tr>
<td>Strawberry 1</td>
<td>0.202 ± 0.016</td>
<td>211 ± 1</td>
<td>0.261 ± 0.008</td>
</tr>
<tr>
<td>Strawberry 2</td>
<td>0.223 ± 0.007</td>
<td>226 ± 12</td>
<td>0.220 ± 0.048</td>
</tr>
</tbody>
</table>

FC index = expressed as mg gallic acid/g juice fruit; ABTS$^{•+}$ = expressed as μg trolox/g juice fruit; Fe(NRS)$_3^{4+}$ complex (proposed method) = expressed as g fruit juice/g ascorbic acid; Additives as reported in the package: E300 = ascorbic acid and E316 = sodium erythorbate; NI = no information.
Structure of the anion of the 3-hydroxy-4-nitroso-2,7-naphthalenedisulfonic acid.

149x84mm (300 x 300 DPI)
Absorption spectra with formation of the Fe(NRS)₃⁻ complex before and after addition of a standard ascorbic acid solution, where (a) Fe(III) 36×10⁻⁶ mol/L + NRS 250×10⁻⁶ mol/L; Tris 6.0×10⁻³ mol/L; (b) to (h) = (a) + ascorbic acid (0.200; 0.400; 0.600; 0.800; 1.00; 1.20 e 1.40)×10⁻⁵ mol/L, respectively. Absorbance measurements after 30 minutes using water as reference solution.
3-D graph comparing the antioxidant capacity (Fe(NRS)$_3$- complex and ABTS$^{••}$ method) and FC index for all analysed samples. The FC index is expressed as mg gallic acid/g juice fruit; ABTS$^{••}$ is the antioxidant capacity expressed as μg trolox/g juice fruit and Fe(NRS)$_3$- complex (proposed method) is the antioxidant capacity expressed as g fruit juice/g ascorbic acid.