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# Different values obtained by the FRAP method for the determination of slowly and rapidly reacting phenols

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## ORIGINAL RESEARCH PAPER

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**ABSTRACT**

A number of methods have been applied to measure total antioxidant capacity (TAC), including FRAP, which is based on reducing the amount of iron ions in a complex compound. Researchers often use measurement of absorbance 10 min after mixing a sample with the FRAP reaction solution to calculate TAC. The FRAP solution has been shown to alter absorbance over time by ca 0.0010–0.0020 per hour, under storage conditions. This article intends to show that some substances do not fully or sufficiently react within the common analysis period. It is evident from the results that some substances react more quickly and others very slowly. Absorbance in relation to various phenols was measured. Compared to the levels of absorbance at 10 min, mean absorbance at 48 h was higher by 5,395% for vanillin, 426% for caffeic acid, 170% for sinapinic acid, 67% for gallic acid, 19% for syringic acid, and only by 4% for Trolox. Results for vanillin and caffeic acid indicate potential auto-catalysis.

**KEYWORDS**

FRAP, absorbance, reaction time, phenols, Trolox

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## 1. INTRODUCTION

Compounds that inhibit oxidation processes are called antioxidants. They are added to food to protect against oxidation changes. Oxidative changes cause free radicals that attack cellular components.

There has been considerable interest in adding antioxidants to food as a possible means of preventing cancer (Lobo et al., 2010).

The content of such antioxidants is often denoted as the given Total Antioxidant Capacity (TAC) value, which describes the food (Sies, 2007). Several methods may be employed to determine this antioxidant capacity, including FRAP, DPPH, ORAC, etc. (Prior et al., 2005), each giving different TAC results for the same foodstuff. This is caused by a different reaction mechanism occurring in each method, which is why selection of a specific method impacts the TAC value (Apak et al., 2016a, b, c). Due to the different reaction mechanism, it is recommended to always use more than one method of measuring TAC (Prior et al., 2005). TAC is usually expressed for a specific, pure antioxidant. When Trolox is utilised as standard, the so-called TEAC (Trolox Equivalent Antioxidant Capacity) is calculated, making it possible to apply Trolox as the standard for any method (Paulová et al., 2004).

The Ferric Reducing Antioxidant Power (FRAP) method constitutes an approach based on electron transfer, specifically based on the reducing action of antioxidants. In this method, iron in the oxidised form of $Fe^{III}$ is reduced to $Fe^{II}$ at acidic pH (Benzie and Strain, 1996). In an acidic aqueous environment, the resultant central iron atom complex ($Fe^{III}$) with 2 ligands (2 molecules of 2,4,6-tris(2-pyridyl)-S-triazine, TPTZ) is yellow. When an iron ion ($Fe^{III}$) is reduced to a ferrous ion ($Fe^{II}$) by exposing it to antioxidants, the complex changes colour - from yellow to blue. Antioxidant capacity is determined by the intensity of the blue colour, or, as the case may be, by absorbance at 593 nm, when the frequency of the wavelength absorbs the emerging "blue" complex containing the $Fe^{II}$.

However, not all substances or compounds in food samples react completely or to exactly the same degree within 10 min of the act of mixing (Fraga et al., 2014). In the case of slow-reacting substances, a significant part of the antioxidant remains present in unreacted form (as well as reacted) in the measuring cuvette under examination, even after the 10-min mark. Comparing absorbance measurements taken at 10 min and at later times could highlight the significant presence of the unreacted form of the antioxidant. Based on such a comparison, it is possible to determine which antioxidants react quickly and which react slowly (Chvátalová et al., 2008). From this view, it is expected that time in measurements will be more monitored.

## 2. MATERIALS AND METHODS

Known FRAP method including following chemicals were used: methanol (99.9%, Sigma-Aldrich, Germany), $FeCl_3$ (97%, Sigma-Aldrich, Germany), TPTZ 2,4,6-tri(2-pyridyl-1,3,5-triazine) (99%, Acros Organics, China), 35% HCl (p.a., Ing. Petr Švec - PENTA, Czech Republic), $CH_3COONa$ (p.a., Ing. Petr Švec - PENTA, Czech Republic), acetic acid (99.99%, Sigma-Aldrich, USA), and deionised water. Aqueous solutions were prepared as follows: a $FeCl_3$ solution by weighing 0.081 g of $FeCl_3 \cdot 6H_2O$ into 25 mL; a TPTZ solution by pipetting 88.25 µL of 35% HCl and weighing 0.078 g of TPTZ into 25 mL; and an acetate buffer by pipetting 4 mL of

concentrated $CH_3COOH$ and weighing 0.775 g $CH_3COONa$ into 250 mL, the subsequent pH of the buffer equalled 3.6. The Ferric Reducing Antioxidant Power (FRAP) solution was prepared by mixing all 3 sub-solutions at a ratio of 1:1:10. For absorbance measurements, 2000 µL of the reaction mixture (described above) and 25 µL of the prepared sample (phenol acid or Trolox or deionised water) were pipetted into a cuvette, which was then mixed for 10 s by a cuvette shaker at laboratory temperature, 1,000 r.p.m. (IKA MS 3 Digital, Staufen, Germany). The absorbance of samples was gauged on a spectrometer (Specord 50 PLUS Analytic Jena, Jena, Germany) at a wavelength of 593 nm, at various intervals after mixing, at 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 60, 120, 180, 240, 360, 480, 600, 1,170, 1,440, and 2,880 min.

The method used to calculate the resulting absorbance of the tested phenolic substances: The background of the pure FRAP solution ($A_{FRAP}$) was subtracted from the measured absorbance of the phenolic substance at a given time; the difference was then divided by the concentration (in mM) and multiplied by a hypothetical concentration of 0.1 mM. The absorbance values obtained in this manner for the various phenolic substances ($A_P$) were then compared in relation to different intervals of measurement.

## 3. RESULTS AND DISCUSSION

The measurement time is not strict, for example, Guo et al. (2003) measured absorbance at 10 min, Jakovetić Tanasković et al. (2018) at 5 min, and Thaipong et al. (2006) at 30 min, after mixing a sample containing the antioxidants with the FRAP solution (an acidified TPTZ complex solution with $Fe^{III}$). Using a calibration line, TAC can be calculated from the absorbance measured in this manner, e.g. according to the Trolox standard. Original works (Benzie and Strain 1996) measured absorbance at 8 min. Detailed measurement of absorbance over time showed that some reactions still continue.

### 3.1. The absorbance background of a pure FRAP solution

Under laboratory conditions, a separate FRAP (2000 µL) solution with clean water (25 µL) acting as a blank sample demonstrated the absorbance of ($A_{FRAP}$), which rose over time (Fig. 1). Mean absorbance equalled 0.1633 at 10 min and 0.2200 at 48 h. After 6 h (i.e. a working day), the samples would therefore show increased values for absorbance by 0.0109, i.e. an increase of

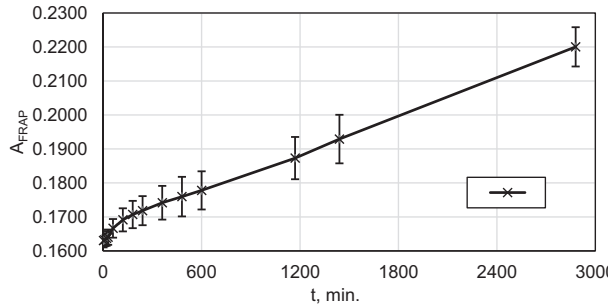


*Fig. 1.* The absorbance of the pure FRAP solution over time (FRAP Background)

6.67%. The value of absorbance of the FRAP solution stored in the laboratory at the 2-day interval could potentially reach 0.0567, i.e. an increase of 34.72%. Conversely, at intervals prior to 10 min, there was very little difference visible in the absorbance of the pure FRAP solution, i.e. just 0.18%, at the 10-min interval.

The average rate of the increase in absorbance over time of $dA_{FRAP}/dt$ was determined on the basis of the difference between absorbance values measured at least 8 min apart. The absorbance of the pure FRAP solution did not rise linearly. The increase in $dA_{FRAP}/dt$ equalled approximately 0.0020 per hour until 3 h had elapsed (except for a single peak); after 4 h it equated to approximately 0.0010 per hour (Fig. 2).

## 3.2. Absorbance of phenolic acids and Trolox

Due to the marked difference in absorbance of various phenolic substances at 10 min, it was necessary to prepared solutions: 0.0676 mM gallic acid; 0.0969 mM syringic acid; 0.1071 mM caffeic acid; 0.0910 mM sinapinic acid; 0.7361 mM vanillin, and 0.4994 mM Trolox. For comparison purposes, absorbance values were recalculated for 0.1 mM (Fig. 3).

At the 10-min mark of the reaction test, the means of absorbance of the phenolic substances ($A_P$) were as follows: vanillin 0.0015; Trolox 0.0325; caffeic acid 0.0544; sinapinic acid 0.0791; syringic acid 0.0849, and gallic acid 0.1118. Similar or identical values for means of absorbance $A_P$ were achieved as early as the 2-min interval of the reaction time, as follows: for Trolox, caffeic acid, sinapinic acid and syringic acid. Differences (above 10%) were evident in the absorbance of vanillin (0.0004; 73% lower) and gallic acid (0.0947; 15% lower). At the 30-min interval of the reaction period, higher absorbances were recorded for sinapinic acid than syringic acid, although it was the latter that had shown higher values for the same at the prior interval. Additional instances of one substance "overtaking" another in terms of absorbance were observed at subsequent reaction times, with caffeic acid outperforming syringic acid at 180 min, and sinapinic acid at 360 min. The absorbance of vanillin also exceeded that of Trolox at 360 min, while the absorbance levels of caffeic acid were higher than those seen for gallic acid at 480 min. One such instance described above was recorded at 48 h, when the absorbance of sinapinic acid was higher than that of gallic acid.

At the last interval of measurement, i.e. at the 48-h mark of the reaction test, the means of absorbance of phenolic substances ($A_P$) were as follows: vanillin 0.0836; Trolox 0.0338; caffeic

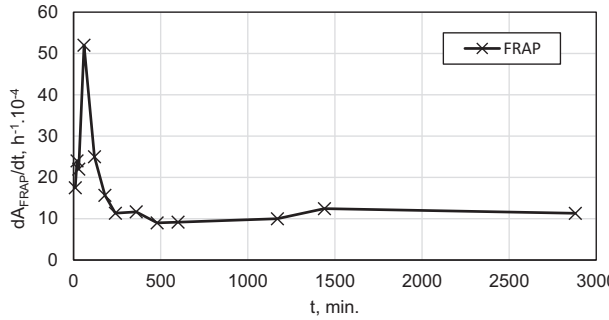


*Fig. 2.* Rate of increase of absorbance over time for the pure FRAP solution

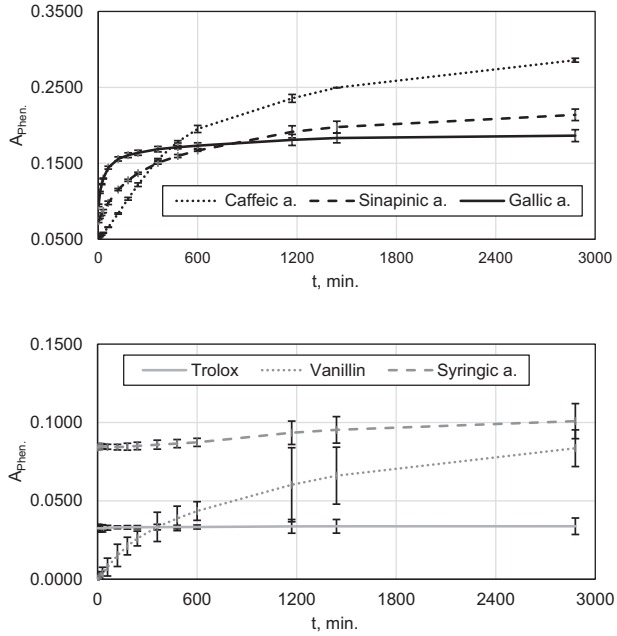


*Fig. 3.* Absorbance of 6 different phenols with the FRAP solution over time

acid 0.2858; sinapinic acid 0.2139; syringic acid 0.1009; and gallic acid 0.1865; this represents a rise in per cent compared to the 10-min mark: 5,473% for vanillin; 4% for Trolox; 425% for caffeic acid; 170% for sinapinic acid; 19% for syringic acid; and 67% for gallic acid.

## 3.3. The rate of increase in phenol absorbance demonstrated for the FRAP method

The average increase in the level of absorbance over time ($dA_P/dt$) was determined on the basis of differences in absorbance values measured at least 8 min apart.

Fast-reacting phenols – i.e. substances with virtually no change in absorbance over time – were Trolox and syringic acid (Fig. 4). In both cases, $dA_P/dt$ was below 0.0010 per hour at an early stage, once 10 min had passed. This means that virtually the whole weighed amount of phenol (Trolox/syringic acid) reacted during the first couple of minutes or even sooner. Syringic acid demonstrated a rise in absorbance of 0.0064 per hour between the 2$^{nd}$ and 10$^{th}$ minutes, whereas subsequent intervals showed values of below 0.0010 per hour. The increase in absorbance for Trolox was less than 0.0008 per hour throughout the observed period – from the 2$^{nd}$ minute to the 48-h mark.

Calibration curves were done three-times to check linearity (as validation parameter) in the common analysis time of 10 min. All three linear regressions had a coefficient of determination $R^2 \geq 0.9989$ (Fig. 5).

The opposite was recorded for gallic acid, sinapinic acid, caffeic acid, and vanillin, which turned out to be slow-reacting phenols. Their absorbance values continued to increase even after 10 min had elapsed, reaching values exceeding 0.0010 per hour (Fig. 3). Between 10 and 20 min

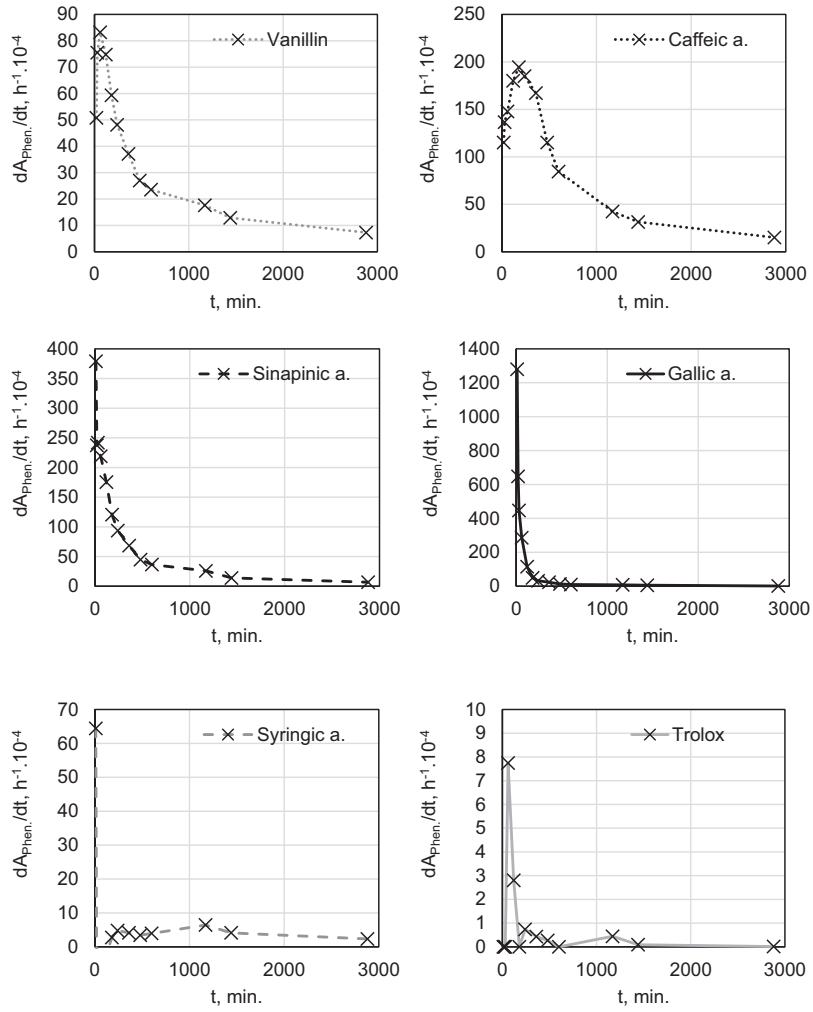


*Fig. 4.* The rate of increase in absorbance over time for 6 different phenols

the $dA_P/dt$ values gauged were 0.0648 per hour for gallic acid, 0.0237 per hour for sinapinic acid, 0.0115 per hour for caffeic acid, and 0.0051 per hour for vanillin. Towards the end of the period of measurement, between the 24- and 48-h marks, increases in absorbance equalled less than 0.0010 per hour for gallic acid, sinapinic acid, and vanillin, and 0.0015 per hour for caffeic acid. It was the latter two substances for which the local maximum was recorded, i.e. the highest $dA_P/dt$ was seen later in the experiment, not early on. Vanillin reached maximum levels between minutes 30 and 60, at 0.0083 per hour, while the same situation was observed for caffeic acid between 120 and 180 min, at 0.0194 per hour.

An increase in reaction rates or in absorbance over time could indicate auto-catalysis has taken place, when emerging reaction products catalyse the reaction. As a consequence, relatively

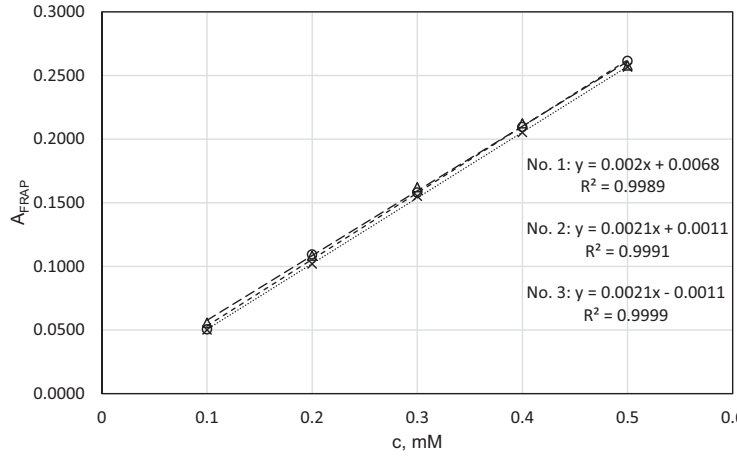


*Fig. 5.* Linearity of calibration with $R^2$ and equations in reaction time 10 min for Trolox (three measurements)

low $dA_P/dt$ values were observed here at early intervals of measurement, which gradually increased to their maximum over time. These values then started to decline, as they did for the other substances, as the rate of reaction dropped due to the constant decrease in the concentration of the reactant.

## 3.4. The effect of increasing absorbance in phenols on TAC during application of the FRAP method

Gauging the absorbance of several phenolic substances at different intervals within 48 h reveals that the measurements taken at the common reaction time of 10 min for the FRAP method could affect the calculation of TAC. The reason is that the absorbance of some substances increases over time, even after dozens of hours, therefore, the aforementioned interval is too short for these substances to react fully, compared with the Trolox standard. This means that the resulting TAC, to which absorbance at 10 min is usually applied, may not fully correspond to the true 'antioxidant capacity' of a specific substance. This is particularly the case for the slow-reacting phenols, i.e. vanillin, caffeic, sinapinic, and gallic acids, for which TAC increases (Trolox equivalent) by approximately 5,300%, 400%, 160%, and 60% (Table 1) between 10 min and 48 h.

*Table 1.* Total antioxidant capacity (TAC) in mM Trolox of various phenols at 10 min and 48 h

| Phenols | TAC, mM at 10 min | TAC, mM at 48 h |
|---|---|---|
| Vanillin (0.7361 mM) | 0.034 ± 0.009 | 1.819 ± 0.042 |
| Caffeic acid (0.1071 mM) | 0.179 ± 0.004 | 0.903 ± 0.011 |
| Sinapinic acid (0.0910 mM) | 0.221 ± 0.006 | 0.575 ± 0.028 |
| Gallic acid (0.0676 mM) | 0.233 ± 0.005 | 0.373 ± 0.028 |
| Syringic acid (0.0969 mM) | 0.253 ± 0.005 | 0.289 ± 0.040 |

## 4. CONCLUSIONS

Detailed measurement of absorbance over time in the case of the FRAP method showed that some reactions or processes continue to go on. The absorbance of the FRAP solution itself changes over time and gradually increases. The absorbance of the pure FRAP solution increased by approximately 0.0020 per hour during storage at laboratory temperature; after the 4-h interval, the value equalled around 0.0010 per hour. This 'FRAP Background' would have affected the absorbance measurements of each phenol if water, not the FRAP solution, had been present in the reference sample (blank), especially at later measuring intervals (following preparation of the FRAP solution).

Another process is the increase in absorbance over time of the measured substances (in addition to the effect of the FRAP solution itself). In other words, some phenolic substances did not react to a sufficient degree within the short assessment interval and consequently showed a significant rise in absorbance at later intervals of analysis. For the first hour from the start of the reaction, the increase in absorbance remained above 0.0200 $hour^{-1}$ for some phenols. For gallic acid an increase from 0.1118 to 0.1226 (i.e. 0.648 $hour^{-1}$) was observed between the $10^{th}$ and $20^{th}$ minute, such a difference in absorbance leads to different TAC values (expressed in Trolox) determined at the $10^{th}$ (0.233 mM) or $20^{th}$ (0.255 mM) min.

In most cases, the highest rate of increase in absorbance (dA/dt) was seen soon after mixing, although the peak values of $dA_{Max.}/dt$ for vanillin and caffeic acid were recorded at later intervals. This could have been an instance of auto-catalysis, whereas emerging products catalysed the reaction between the initial form of the phenol and the FRAP solution.

Measurement of the phenolic substances showed that some substances were not capable of reacting fully (even to a major extent) within 10 min, at which time they were evaluated using the FRAP method to gauge TAC.

## ACKNOWLEDGEMENTS

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