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Contribution of trans-aconitic acid to DPPH\(^*\) scavenging ability in different media

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ABSTRACT

The antioxidant properties of trans-aconitic acid (TAA) alone or in the presence of usual antioxidants were assessed by DPPH\(^*\) assay. The IC\(_{50}\) value equal to 70 mM was very high compared to usual antioxidants (vitamin C and trolox). A joint experimental/theoretical study suggested that hydrogen atom abstraction in TAA by DPPH\(^*\) was located on \(-\text{CH}_2–\) methylene bridge because the corresponding radical was more stabilized than COO\(^–\) and C=\(\equiv\)C\(^–\) radicals. In combination with antioxidants (vitamin C, gallic acid, caffeic acid, trolox), synergy or additivity effects were noticed. The magnitude of the synergistic effect varied between 1.06 and 1.24 depending on the type and concentration of antioxidant for a concentration of TAA equal to 22.3 mM. Especially, the addition of TAA at a concentration below 32 mM to a solution containing 20 \(\mu\)M of vitamin C had a synergy effect. Beyond this concentration, TAA showed an additive effect.

1. Introduction

Antioxidants are listed as additives in food (Perrin & Meyer, 2002; Shahidi, 2000), cosmetic (Alander, Andersson, & Lindström, 2006) and pharmaceutical (Celestino et al., 2012) products for the properties they generate in biological and organic media. They are either of synthetic origin (BHT, BHA, propyl gallate) (Freitas & Fatibello-Filho, 2010), or of natural origin (ascorbic acid, vitamin E, carotenoids, polyphenols) (Bruun-Jensen, Skovgaard, Madsen, Skibsted, & Bertelsen, 1996; Martí, Pérez-Vicente, & García-Viguera, 2002). These natural antioxidants present in the plant extracts are increasingly sought to replace the synthetic compounds (Gramza & Korczak, 2005; Wagner, Wotruba, & Elmadfa, 2001). The advantage of natural extracts is to associate one or more antioxidants with other metabolites that often have promoting effects on the antioxidant activity. Thus, the role of certain organic acids, in particular polyacids, was recently highlighted. Methanolic extracts of chamomile with a high content of phenolic acids, flavonoids and organic acids showed a strong antioxidant potential (Guimarães et al., 2013). Phenolic compounds and organic acids may contribute to the biological activity of Brazilian tropical fruit...
The antioxidant activity of turnip extracts was correlated with the total amount of phenolic compounds and organic acids (Fernandes et al., 2007). Specifically, citric acid added to a rosemary extract containing rosmarinic acid (antioxidant) showed a synergistic effect to prevent hydroperoxide formation (Hraš, Hadolin, Knez, & Bauman, 2000). Similarly, the simultaneous presence of oxalic acid, citric acid and malic acid enhanced the antioxidant activity of an extract of the roots of Madagascar periwinkle (Pereira et al., 2010). IC50 value of the extracts of quince (such as pulp, peel and jam) can be correlated with the concentration of vitamin C and citric acid (Silva et al., 2004). A model system was developed to test the influence of a few acids (acetic, malic and citric acids) on ascorbic acid by the DPPH test (Scalzo, 2008). Other polyacids (itaconic acid, cis-and trans-aconitic acid or fumaric acid) are often present in the acid fraction along with acetic, malic or citric acids in the extracts or in certain food or cosmetics preparations (Blass, Pratt, & Cosentino, 2013; Silva, Azevedo, Pereira, Valentai, & Andrade, 2013). However, their contribution to biological activity remains unclear.

Most of these acids can be obtained on an industrial scale by biotechnological processes or by extraction of industrial by-products (Cao, Du, Gong, & Tsao, 1996; Li & Punt, 2013). Knowledge of their contributions to the antioxidant activity would bring new development prospects for these products. In this perspective, aconitic acid, the major acid representing approximately 60% of organic acids in sugar cane molasses, was studied (Célestin-Mytil & Parfait, 1988). Aconitic acid can also be obtained by dehydration of citric acid (Bruce, 1937; Cranston, 1951, 1955). It is naturally present in sugar cane and can be isolated from by-products of the sugar industry (molasses, vinasse) (Malmary, Albet, Putranto, Hanine, & Molinier, 2000; Montoya, Londono, Cortes, & Izquierdo, 2014; Pettit et al., 2015; Pislor, Pontalier, & Albet, 2009). The amount available in the molasses is between 7.1 g/kg in Senegal to 23.4 g/kg in Louisiana (Grondin, Albet, 2009). Up to now, aconitic acid has been little studied, therefore, IC50 values; inhibition percentage was calculated according to the absorbance values;

\[
\text{%Inhibition} = \left(1 - \frac{A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100
\]

where \(A_{\text{blank}}\) is the absorbance of the blank and \(A_{\text{sample}}\) is the absorbance of the product, at 515 nm, after one hour. Inhibition percentage increases with antioxidative activity.

The concentration for which 50% inhibition is obtained, is called inhibition concentration (IC50). It was calculated from the graph obtained by plotting “inhibition percentages” versus “sample concentrations”.

For the kinetics study of a sample (mixture of antioxidant and TAA), measurements of absorbance at 515 nm were performed, immediately after addition of DPPH, at five minutes intervals for one hour.

### 2.3. Computational study

In order to get an insight into the activity of TAA on DPPH scavenging, radicals of TAA with different positions of hydrogen atom abstraction (Fig. 1) were theoretically studied. Geometries were optimized at the B3LYP/6-31+G(d,p) level using the Gaussian 09 program (Frisch et al., 2013) and tight convergence criteria. An ultrafine grid was used to ensure rotational invariance of the results. All stationary points were confirmed as true minima via vibrational frequency calculations in the harmonic approximation. The popular B3LYP Hybrid density functional (Becke, 1993; Stephens, Devlin, Chabalowski, & Frish, 1994) is widely used to study organic compound geometry (Sousa, Fernandes, & Ramos, 2007). 6-31+G(d,p) Pople style basis set is large enough to describe the structure of organic molecules (even for hydrogen-bonded complexes) at this B3LYP level (Koné, Illien, Graton, & Laurence, 2005).

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**Fig. 1.** Trans-aconitic acid numbering and studied positions of hydrogen abstraction.
Then G4(MP2)-6X composite procedure was applied to improve relative energies of the most stable radical conformers. For 28 hydrogen-abstraction reactions, this procedure achieves 1.7 kJ mol⁻¹ ΔH (0 K) mean absolute deviation to W1 reference values (Chan, Deng, & Radom, 2011).

2.4. Statistical analysis

XLSTAT-Pro Version 2007.4 (Addinsoft, France) was used to calculate Student-Fisher and Friedman tests.

3. Results and discussion

3.1. Experimental and theoretical study of the radical scavenging activity of aconitic acid

The DPPH* test was used to monitor the inhibition percentage versus aconitic acid concentrations. It was then possible to deduce the value of IC₅₀, found to be equal to 70 mM corresponding to the effective TAA concentration for 50% inhibition (Supplementary data – Fig. S1). Nevertheless, compared to IC₅₀ values of conventional antioxidants, such as vitamin C (24.4 μM) and trolox (16.1 μM) (Payet, 2005), the IC₅₀ of the aconitic acid is rather high. We can deduce from these results that aconitic acid has weak radical scavenging properties and cannot therefore be considered as an effective antioxidant.

In order to investigate if acid functions of TAA (pKₐ = 2.80; pKₐ₂ = 4.46; pKₐ₃ = 6.30) are involved in the scavenging process, DPPH* tests were also applied to acetic (pKₐ = 4.75) and citric acids (pKₐ₁ = 3.13; pKₐ₂ = 4.76; pKₐ₃ = 6.40). Inhibition percentages at 0.1 M concentration were found (several different starting points had led to the same radical geometries). Sketched, energies and geometrical parameters for the most stable COO⁻, C₃ and C₂ radical species were gathered in Table 1. The results for the other 18 radical species can be found in Supplementary material (Table S2). Five HC₅ conformers were optimized. Their relative energies (ΔE = 0–14 kJ/mol) were lower than those of the twelve COO⁻ species (ΔE = 89–100) and of the four C₂ conformers (107–118). In HC₅ radical, C₁, C₂, C₃, C₅ and C₆ atoms were almost in the same plane; C₂C₃ and C₂C₅ bond lengths were nearly equal, respectively 139.6–139.9 and 139.2–139.6 pm. Thus the higher stability of HC₅ radical came from a better delocalization of π electrons over carbon skeleton than in COO⁻ and C₂ radicals. Accurate relative G4(MP2)-6X ΔAH (0 K) values for molecules in Table 1 showed the same trend as relative B3LYP energies. In a nutshell, hydrogen atom abstraction in TAA by DPPH* was located on –CH₂– methylene bridge because the corresponding radical was more stabilized than COO⁻ and C₂ radicals. Therefore the following reaction mechanism of DPPH* with TAA can be suggested (Fig. 2).

![Fig. 2. Possible reaction mechanism of TAA with DPPH.](image)

Four conventional antioxidants were selected to determine the behaviour of aconitic acid when mixed with each of them. The concentration of aconitic solution was 22.3 mM (Scalzo, 2008). The results of this kinetic study were reported in Fig. 3 (part a, b, c).

First, kinetics of the reaction between each of the four standard antioxidants and DPPH* were monitored by measuring inhibition percentages (part a). We note that the inhibition percentages are nearly constant over time. The percentage of inhibition has levelled off at 36.1 ± 1.3, 44.9 ± 0.8, 47.7 ± 0.7, 48.0 ± 1.7 for caffeic acid, gallic acid, vitamin C and trolox respectively.

In part b, kinetic profiles from mixtures between a standard antioxidant and aconitic acid are presented. In these cases, the time required to reach the maximum value for inhibition percentage depends on the nature of the antioxidant: the first part of Fig. 3b indicates that the reaction kinetics between the antioxidant and radical DPPH* is slowed for several tens of minutes. However, the second part shows a levelling off of the percentage of inhibition, indicating the end of the reaction between the antioxidant and radical DPPH*. Slower kinetics of reaction between an antioxidant (phenols, curcumin or vitamin E) and DPPH* radical in the presence of acetic acid was reported by several authors.
mechanism and hydrogen transfer.

Inhibition percentages of standard antioxidants mixed with aconitic acid (22.3 mM) versus time. (b) Inhibition percentages of different antioxidants in solution, in association with an organic acid at 0.067 N in methanolic solution.

The samples with methanol, without any addition of acid, represent the blank samples. A Friedman test was performed (threshold risk \( \alpha = 5\% \)) to observe the differences in activity caused by the addition of an acid, compared to the acid free sample. The addition of acetic acid or citric acid does not generate significant differences compared to the blank except for the mixture, trolox + citric acid, which has a relatively small difference (4.9%). Then, for a given antioxidant, firstly, the inhibition percentages increase with the antioxidant concentrations, secondly, the inhibition percentages obtained with the addition of acetic acid and citric acid are quite similar. Lo Scalzo (Scalzo, 2008) found the same trends for vitamin C in combination with acetic and citric acid in ethanol for the same concentrations, but inhibition percentages were different. It is well known that DPPH test is sensitive to solvent (Sharma & Bhat, 2009). Moreover, Lo Scalzo used a lower initial concentration (19.2 µM) than in our study (95 µM).

On the other hand, mixtures with aconitic acid generate a significant increase in the inhibition percentage, statistically validated by Friedman test. Thus, for a given antioxidant, the relative gain in antioxidant activity in combination with TAA is higher for the lowest antioxidant concentration (+58% vitamin C, +111% caffeic acid, +200% gallic acid, +65% trolox) whereas it becomes weak or null for the highest antioxidant concentrations.

3.3. Comparison of threshold values of inhibition percentage of mixtures

Table 2 contains the maximum values of inhibition percentages obtained for mixtures containing an organic acid (acetic acid, citric acid and aconitic acid) and usual antioxidants (vitamin C, caffeic acid, gallic acid and trolox).

3.4. Demonstration of the synergistic or additive effect

From percentages of inhibition obtained for the mixtures with aconitic acid, synergistic effects can be calculated (Table 3).

As the attack of the DPPH\(^+\) on the antioxidant acid is limited, the mechanism of proton transfer preferentially takes place. This later occurs at a slow rate, which explains the kinetic profile observed in the presence of aconitic acid. The results are then consistent with the SPLET mechanism.

The threshold values obtained (51.7 ± 0.6, 57.1 ± 0.6, 64.8 ± 0.7, 66.3 ± 0.6 for caffeic acid, gallic acid, vitamin C and trolox respectively) are higher than the inhibition percentages observed for the standard antioxidants (part a). It means that mixtures with aconitic acid lead to higher inhibition percentages after a latency period (20–50 min depending on the antioxidant).

3.3. Comparison of threshold values of inhibition percentage of mixtures

Table 2 contains the maximum values of inhibition percentages obtained for mixtures containing an organic acid (acetic acid, citric acid and aconitic acid) and usual antioxidants (vitamin C, caffeic acid, gallic acid and trolox).

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Concentration (µM)</th>
<th>Blank</th>
<th>Acetic acid (67 mM)</th>
<th>Citric acid (22.3 mM)</th>
<th>TAA (22.3 mM)</th>
<th>Relative gain in antioxidative activity with TAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>15</td>
<td>29.1 ± 1.7</td>
<td>32.6 ± 0.5</td>
<td>33.8 ± 1.1</td>
<td>46.1 ± 1.6</td>
<td>58%</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>48.0 ± 1.7</td>
<td>52.2 ± 0.7</td>
<td>51.6 ± 1.3</td>
<td>64.8 ± 1.7</td>
<td>35%</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>91.7 ± 0.2</td>
<td>92.0 ± 0.1</td>
<td>90.9 ± 1.3</td>
<td>91.8 ± 0.2</td>
<td>0%</td>
</tr>
<tr>
<td>Caffeic Acid</td>
<td>5</td>
<td>17.9 ± 1.8</td>
<td>16.9 ± 0.8</td>
<td>19.4 ± 0.6</td>
<td>37.8 ± 1.0</td>
<td>111%</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>36.1 ± 1.3</td>
<td>34.9 ± 0.4</td>
<td>38.7 ± 1.4</td>
<td>51.7 ± 0.6</td>
<td>43%</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>56.6 ± 1.6</td>
<td>55.8 ± 1.4</td>
<td>52.4 ± 1.6</td>
<td>68.9 ± 1.0</td>
<td>22%</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>2</td>
<td>10.2 ± 1.1</td>
<td>12.0 ± 1.1</td>
<td>14.3 ± 0.5</td>
<td>30.6 ± 0.9</td>
<td>200%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>44.9 ± 0.8</td>
<td>46.2 ± 0.6</td>
<td>46.2 ± 1.2</td>
<td>57.1 ± 1.0</td>
<td>27%</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>61.7 ± 1.1</td>
<td>61.5 ± 1.1</td>
<td>56.2 ± 1.4</td>
<td>67.9 ± 1.1</td>
<td>10%</td>
</tr>
<tr>
<td>Trolox</td>
<td>22.5</td>
<td>28.1 ± 1.8</td>
<td>30.4 ± 0.7</td>
<td>33.0 ± 1.3</td>
<td>46.5 ± 0.5</td>
<td>65%</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>47.7 ± 0.7</td>
<td>48.5 ± 0.6</td>
<td>53.7 ± 0.5</td>
<td>66.3 ± 0.6</td>
<td>13%</td>
</tr>
</tbody>
</table>

* Significant difference according to Friedman test (\( P = 0.05 \) et n = 6).
Table 3

Synergistic Effect (SE) and inhibition percentages for antioxidant/organic acid mixtures.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Concentration (μM)</th>
<th>%I_mixture</th>
<th>%I_theoretical</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>15</td>
<td>46.1 ± 1.6</td>
<td>40.6 ± 1.6</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>64.8 ± 0.7</td>
<td>56.4 ± 1.3</td>
<td>1.15</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>5</td>
<td>37.8 ± 1.0</td>
<td>31.1 ± 2.0</td>
<td>1.22</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>51.7 ± 0.6</td>
<td>46.4 ± 1.4</td>
<td>1.11</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>2</td>
<td>30.6 ± 0.9</td>
<td>24.7 ± 0.8</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>57.1 ± 0.6</td>
<td>53.8 ± 0.9</td>
<td>1.06</td>
</tr>
<tr>
<td>Trolox</td>
<td>15</td>
<td>46.5 ± 0.5</td>
<td>39.7 ± 0.6</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>66.3 ± 0.6</td>
<td>56.1 ± 0.7</td>
<td>1.17</td>
</tr>
</tbody>
</table>

* Significant difference according to Student-Fischer test (P = 0.05. for n = 6) to compare mixture percentages and theoretical percentages.

The synergistic effect (SE) of a mixture is defined by the ratio of the experimental value of the inhibition percentage of the antioxidant/organic acid mixture (%I_mixture) and the theoretical value (%I_theoretical) ([Liu, Shi, Ibarra, Kakuda, & Xue, 2008; Zanfini, Corbini, La Rosa, & Dreassi, 2010]).

\[
SE = \frac{\%{I}_{mixture}}{\%{I}_{theoretical}}
\]  

with

\[
\%{I}_{theoretical} = \%{I}_{antioxidant} + \%{I}_{acid} - \frac{\%{I}_{antioxidant} \times \%{I}_{acid}}{100}
\]

\%I_{antioxidant} and \%I_{acid} respectively represent the percentage of the antioxidant and of the organic acid used alone.

A Student-Fischer test was performed with a risk threshold of \( \alpha = 5\% \), in order to identify significant differences between %I_mixture and %I_theoretical. This test is an essential preliminary step before calculating SE.

A synergistic effect is found when the SE is greater than 1. In the cases where significant differences (obtained by Friedman test) were observed between the blank (antioxidant without acid) and the sample (with acid), SE values were calculated. The results are shown in Table 3. The SE values calculated for the highest antioxidant concentrations (45 μM for vitamin C and trolox, 22.5 μM for caffeic acid and 10 μM for gallic acid) are very closed to one and were not shown in Table 3 (there is no more SE effect). The highest synergistic effect (1.24) was found for the lowest gallic acid concentration (2 μM). Then for higher gallic acid concentration (5 μM and 10 μM) SE values were decreased (SE = 1.06 and 1.00 respectively). The same trend was observed for the other antioxidants, the synergistic effect was also decreased with the increase of the antioxidant concentration. This synergistic effect at low concentrations can be explained either by a regeneration of the antioxidant concentration. This synergistic effect at low concentrations should not be too high to allow synergistic effect.

4. Conclusion

Already known for its acidifying properties, trans-aconitic acid had never been studied for its antioxidant properties. The method of DPPH test helped to highlight a too low radical scavenging activity to be exploited; an IC50 value of 70.4 μM was found for TAA, less than that of vitamin C (IC50 = 24.4 μM) or that of trolox (IC50 = 16.1 μM), an analogue of vitamin E. The theoretical study showed that carboxylic acid moiety is probably not involved in the reaction mechanism with DPPH. In fact, hydrogen atom abstraction in TAA by DPPH was located on –CH2– methylene bridge because the corresponding radical was more stabilized than COO– and C=C radicals.

A synergistic effect resulted from the combination of trans-aconitic acid and an antioxidant, such as vitamin C, gallic acid, caffeic acid, while the combination with citric acid and acetic acid showed no effect under the same conditions. Note that the antioxidant concentration should not be too high to allow synergistic effect.

This property has the advantage of enhancing the effectiveness of the tested conventional antioxidants, which could be used at lower concentrations. Kinetics of reactions of DPPH with the antioxidants, in the presence of aconitic acid, have shown a latency period before reaching a stable inhibition percentage, which is in agreement with a HAT mechanism.

Synergistic effect has been studied particularly for vitamin C at a concentration of 20 μM. For this antioxidant commonly used in food industry, the synergistic effect of aconitic acid is active below a concentration of 32 μM. Beyond this value, the additive effect replaces the synergistic effect. This result shows that the measurement of anti-oxidant properties of natural extracts should take into account both the contents of antioxidative molecules and organic acids.

Acknowledgments

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