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# Contribution of *trans*-aconitic acid to DPPH<sup>•</sup> scavenging ability in different media

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## A B S T R A C T

The antioxidant properties of *trans*-aconitic acid (TAA) alone or in the presence of usual antioxidants were assessed by DPPH<sup>•</sup> assay. The IC<sub>50</sub> 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<sup>•</sup> was located on –CH<sub>2</sub>– methylene bridge because the corresponding radical was more stabilized than COO<sup>•</sup> and C=C<sup>•</sup> 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 μM 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

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juice (de Carvalho-Silva et al., 2014). 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). IC<sub>50</sub> 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, Valentão, & 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 (Célestine-Myrtil & 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) (Malmay, Albet, Putranto, Hanine, & Molinier, 2000; Montoya, Londono, Cortes, & Izquierdo, 2014; Petit et al., 2015; Pislors, Pontalier, & Albet, 2009). The amount available in the molasses is between 7.1 g/kg molasses in Senegal to 23.4 g/kg in Louisiana (Grondin, 2002). Up to now, aconitic acid has been little studied, therefore, its up-grading was investigated as a platform molecule (Piang-Siong, de Caro, Lacaze-Dufaure, Shum Cheong Sing, & Hoareau, 2012), and for its antioxidant properties. Concerning the later, a correlation between antioxidant activity of the leaves extracts and stalks of *Portulaca oleraceae* L. (Purslane) and the concentration of aconitic acid has already been observed (Oliveira et al., 2009). Therefore, the role of aconitic acid in antioxidant activity deserved to be checked.

For this purpose, we sought to evaluate the properties of aconitic acid, as an antioxidant molecule alone or as a promoting agent of an antioxidant formulation containing other known antioxidant molecules.

## 2. Materials and methods

### 2.1. Reagents and solvents

Acetic acid (99.7%), citric acid, *trans*-aconitic acid (TAA, 98%), trolox, gallic acid and 2,2-Diphenyl-1-picrylhydrazyl (DPPH<sup>•</sup>) were purchased from Sigma Aldrich (St Quentin Fallavier, France). Vitamin C and caffeic acid were purchased from Fluka (St Quentin Fallavier, France). Solvents (analytical spectrophotometric grade) were supplied by Carlo Erba (Peypins, France). Antioxidant tests were performed with Biotek Powerwave™ XS spectrophotometer (Winooski, VT), equipped with a thermo regulated cell, to measure the absorbance on a microplate of 96 wells.

### 2.2. Free radical scavenging activity: DPPH assay

The antioxidant capacity was measured using the DPPH test (Brand-Williams, Cuvelier, & Berset, 1995). The DPPH test for

appraising the antioxidant activity of chemicals was widely used in the literature for more than 20 years despite few shortcomings (Apak, Özyürek, Güçlü, & Çapanoğlu, 2016; Foti, 2015; Xie & Schaich, 2014). Brand-Williams et al. measured the antiradical activities of 20 compounds (e.g. vitamin C, caffeic acid, gallic acid). In this spirit, the same DPPH test was applied to investigate antiradical activity of TAA alone and in combination with usual antioxidant (vitamin C, gallic acid, caffeic acid and trolox).

Methanolic solutions of different concentrations of TAA (ranging from 8.62.10<sup>-4</sup> M to 0.1 M) were prepared. 20 µl of the solution to be tested were introduced in each of the 96 wells of the microplate. Then, 280 µl of DPPH<sup>•</sup> at 0.004% (4 mg/100 ml) in methanol were added. Microplate was incubated for 1 h at 30 °C and absorbance was measured at 515 nm. A blank prepared with 20 µl methanol in 280 µl of DPPH<sup>•</sup>, was also taken through the same procedure to determine its antioxidant capacity.

The radical-scavenging activity of the samples expressed as an 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 (IC<sub>50</sub>). 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<sup>•</sup>, at five minutes intervals for one hour.

### 2.3. Computational study

In order to get an insight into the activity of TAA on DPPH<sup>•</sup> 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, & Frisch, 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).

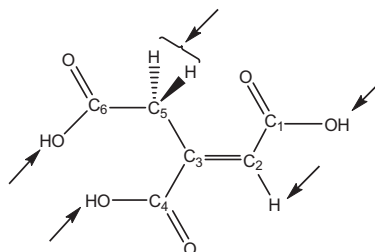


Fig. 1. *Trans*-aconitic acid numbering and studied positions of hydrogen abstraction.

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  $1.7 \text{ kJ mol}^{-1}$   $\Delta_r H$  (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 $\cdot$  test was used to monitor the inhibition percentage versus aconitic acid concentrations. It was then possible to deduce the value of IC<sub>50</sub>, found to be equal to 70 mM corresponding to the effective TAA concentration for 50% inhibition (Supplementary data – Fig. S1). Nevertheless, compared to IC<sub>50</sub> values of conventional antioxidants, such as vitamin C (24.4  $\mu\text{M}$ ) and trolox (16.1  $\mu\text{M}$ ) (Payet, 2005), the IC<sub>50</sub> 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 ( $\text{pK}_{\text{a}1} = 2.80$ ;  $\text{pK}_{\text{a}2} = 4.46$ ;  $\text{pK}_{\text{a}3} = 6.30$ ) are involved in the scavenging process, DPPH $\cdot$  tests were also applied to acetic ( $\text{pK}_{\text{a}} = 4.75$ ) and citric acids ( $\text{pK}_{\text{a}1} = 3.13$ ;  $\text{pK}_{\text{a}2} = 4.76$ ;  $\text{pK}_{\text{a}3} = 6.40$ ). Inhibition percentages at 0.1 M concentration are 67.6%, 1.9% and 2.4% for TAA, acetic acid and citric acid respectively (Supplementary data – Table S1). As inhibition percentages of citric and acetic acids are nearly equal

and lower than TAA's, it is suggested that acid functions might not play an important role in the scavenging process of TAA, whereas the double bond might be decisive in this process.

Theoretical study was focused on the relative stabilities of TAA radicals. H atom abstraction can occur in five different positions on the TAA skeleton: C<sub>1</sub>OO $\cdot$ , C<sub>4</sub>OO $\cdot$ , C<sub>6</sub>OO $\cdot$ , C<sub>2</sub>, HC<sub>5</sub> (see Fig. 1 for carbon atom numbering system). In order to search for the global minimum of each radical species, geometries of the seven lowest-energy conformers of TAA were used as starting point for each radical (TAA(-H) $\cdot$ ) optimization. Thus 35 geometry optimizations of TAA(-H) $\cdot$  were performed and 21 different geometries were found (several different starting points had led to the same radical geometries). Sketches, energies and geometrical parameters for the most stable COO $\cdot$ , HC<sub>5</sub> and C<sub>2</sub> radical species were gathered in Table 1. The results for the other 18 radical species can be found in Supplementary material (Table S2). Five HC<sub>5</sub> conformers were optimized. Their relative energies ( $\Delta E = 0\text{--}14 \text{ kJ/mol}$ ) were lower than those of the twelve COO $\cdot$  species ( $\Delta E = 89\text{--}100$ ) and of the four C<sub>2</sub> conformers (107–118). In HC<sub>5</sub> radical, C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>, C<sub>5</sub> and C<sub>6</sub> atoms were almost in the same plane; C<sub>2</sub>C<sub>3</sub> and C<sub>3</sub>C<sub>5</sub> bond lengths were nearly equal, respectively 139.6–139.9 and 139.2–139.6 pm. Thus the higher stability of HC<sub>5</sub> radical came from a better delocalization of  $\pi$  electrons over carbon skeleton than in COO $\cdot$  and C<sub>2</sub> radicals. Accurate relative G4(MP2)-6X  $\Delta_r H$  (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 $\cdot$  was located on -CH<sub>2</sub>- methylene bridge because the corresponding radical was more stabilized than COO $\cdot$  and C<sub>2</sub> radicals. Therefore the following reaction mechanism of DPPH $\cdot$  with TAA can be suggested (Fig. 2).

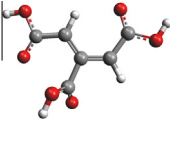
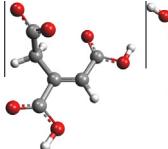
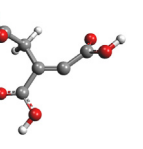
#### 3.2. Kinetic study of aconitic acid solution mixed with conventional antioxidants

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).

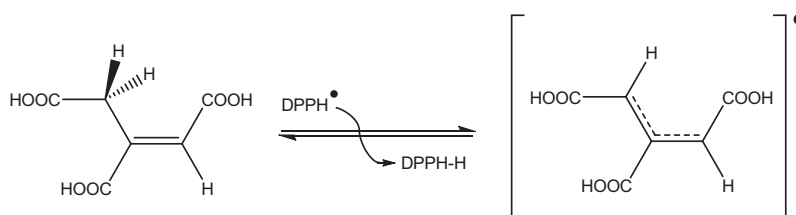
First, kinetics of the reaction between each of the four standard antioxidants and DPPH $\cdot$  were monitored by measuring inhibition percentages (part a). We note that the inhibition percentages are nearly constant over time. The percentage of inhibition has levelled off at  $36.1 \pm 1.3$ ,  $44.9 \pm 0.8$ ,  $47.7 \pm 0.7$ ,  $48.0 \pm 1.7$  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 $\cdot$  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 $\cdot$ . Slower kinetics of reaction between an antioxidant (phenols, curcumin or vitamin E) and DPPH $\cdot$  radical in the presence of acetic acid was reported by several authors

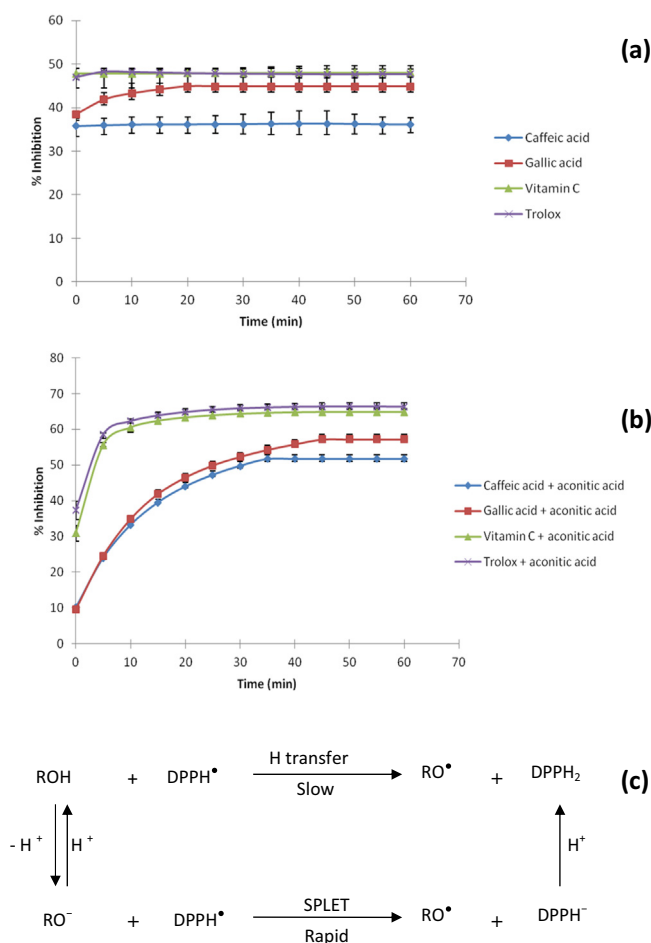
**Table 1**  
Most stable radical conformers for COO $\cdot$ , HC<sub>5</sub> and C<sub>2</sub> radical TAA(-H) $\cdot$  species.

Name	HC <sub>5</sub> _1	C <sub>6</sub> OO_1	C <sub>2</sub> _1
<b>Sketch</b>			
<b>Energy (ua)</b>	-683.023701	-682.989864	-682.982877
<b>Dipole (D)</b>	1.85	4.85	0.96
<b><math>\Delta E</math> B3LYP*</b>	0.0	88.8	107.2
<b>d[C<sub>1</sub>C<sub>2</sub>] (pm)</b>	147.1	148.4	144.9
<b>d[C<sub>2</sub>C<sub>3</sub>] (pm)</b>	139.6	134.7	131.9
<b>d[C<sub>3</sub>C<sub>4</sub>] (pm)</b>	151.7	150.1	149.9
<b>d[C<sub>3</sub>C<sub>5</sub>] (pm)</b>	139.4	150.9	151.5
<b>d[C<sub>5</sub>C<sub>6</sub>] (pm)</b>	147.0	150.9	152.0
<b>dih[C<sub>2</sub>C<sub>3</sub>C<sub>5</sub>C<sub>6</sub>] (°)</b>	174.8	-81.8	-108.3
<b><math>\Delta(\Delta_r H_0)</math></b>	0.0	87.9	100.6
<b>G4(MP2)-6X*</b>			

\*  $\Delta E$  and  $\Delta(\Delta_r H_0)$  given in  $\text{kJ mol}^{-1}$ . The most stable radical is taken as reference.



**Fig. 2.** Possible reaction mechanism of TAA with DPPH $\cdot$ .



**Fig. 3.** Kinetic studies of aconitic acid solution mixed with conventional antioxidants (15  $\mu\text{M}$  caffeic acid, 5  $\mu\text{M}$  gallic acid, 22.5  $\mu\text{M}$  vitamin C and trolox). (a) Inhibition percentages of standard antioxidants versus time. (b) Inhibition percentages of standard antioxidants mixed with aconitic acid (22.3 mM) versus time. (c) Reaction between DPPH $\cdot$  and an antioxidant (ROH) according to SPLET mechanism and hydrogen transfer.

(Litwinienko & Ingold, 2003, 2004; Musialik & Litwinienko, 2005; Scalzo, 2008). Based on the reaction mechanisms involving the DPPH $\cdot$ ; this result can be explained with the SPLET mechanisms coupled with the hydrogen transfer (part c). The SPLET mechanism is initiated by the formation of an alkoxide or a phenoxide, and has a high constant reaction rate. Given the pKa of aconitic acid (pKa = 2.80), pH of the solution is low and ROH form predominates.

**Table 2**  
Inhibition percentages of different antioxidants in solution, in association with an organic acid at 0.067 N in methanolic solution.

Antioxidant	Concentration ( $\mu\text{M}$ )	Blank	Acetic acid (67 mM)	% Inhibition Citric acid (22.3 mM)	TAA (22.3 mM)	Relative gain in antioxidative activity with TAA
Vitamin C	15	29.1 $\pm$ 1.7	32.6 $\pm$ 0.5	33.8 $\pm$ 1.1	46.1 $\pm$ 1.6*	58%
	22.5	48.0 $\pm$ 1.7	52.2 $\pm$ 0.7	51.6 $\pm$ 1.3	64.8 $\pm$ 0.7*	35%
	45	91.7 $\pm$ 0.2	92.0 $\pm$ 0.1	90.9 $\pm$ 1.3	91.8 $\pm$ 0.2	0%
Caffeic Acid	5	17.9 $\pm$ 1.8	16.9 $\pm$ 0.8	19.4 $\pm$ 0.6	37.8 $\pm$ 1.0*	111%
	15	36.1 $\pm$ 1.3	34.9 $\pm$ 0.4	38.7 $\pm$ 1.4	51.7 $\pm$ 0.6*	43%
	22.5	56.6 $\pm$ 1.6	55.8 $\pm$ 1.4	52.4 $\pm$ 1.6	68.9 $\pm$ 1.0	22%
Gallic acid	2	10.2 $\pm$ 1.1	12.0 $\pm$ 1.1	14.3 $\pm$ 0.5	30.6 $\pm$ 0.9*	200%
	5	44.9 $\pm$ 0.8	46.2 $\pm$ 0.6	46.2 $\pm$ 1.2	57.1 $\pm$ 0.6*	27%
	10	61.7 $\pm$ 1.1	61.5 $\pm$ 1.1	56.2 $\pm$ 1.4	67.9 $\pm$ 1.1	10%
Trolox	15	28.1 $\pm$ 1.8	30.4 $\pm$ 0.7	33.0 $\pm$ 1.3	46.5 $\pm$ 0.5*	65%
	22.5	47.7 $\pm$ 0.7	48.5 $\pm$ 0.6	53.7 $\pm$ 0.5	66.3 $\pm$ 0.6*	13%
	45	91.6 $\pm$ 0.3	91.7 $\pm$ 0.1	90.8 $\pm$ 0.6	91.7 $\pm$ 0.1	0%

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

As the attack of the DPPH $\cdot$  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  $\pm$  0.6, 57.1  $\pm$  0.6, 64.8  $\pm$  0.7, 66.3  $\pm$  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).

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 DPPH $\cdot$  concentration (19.2  $\mu\text{M}$ ) than in our study (95  $\mu\text{M}$ ).

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.

### 3.4. 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).



**Table 3**

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

Antioxidant	Concentration ( $\mu\text{M}$ )	% $I_{\text{mixture}}$	% $I_{\text{theoretical}}$	SE
Vitamin C	15	46.1 $\pm$ 1.6	40.6 $\pm$ 1.6	1.14*
	22.5	64.8 $\pm$ 0.7	56.4 $\pm$ 1.3	1.15*
Caffeic acid	5	37.8 $\pm$ 1.0	31.1 $\pm$ 2.0	1.22*
	15	51.7 $\pm$ 0.6	46.4 $\pm$ 1.4	1.11*
Gallic acid	2	30.6 $\pm$ 0.9	24.7 $\pm$ 0.8	1.24*
	5	57.1 $\pm$ 0.6	53.8 $\pm$ 0.9	1.06*
Trolox	15	46.5 $\pm$ 0.5	39.7 $\pm$ 0.6	1.18*
	22.5	66.3 $\pm$ 0.6	56.1 $\pm$ 0.7	1.17

\* 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 mixture (%  $I_{\text{mixture}}$ ) and the theoretical value (%  $I_{\text{theoretical}}$ ) (Liu, Shi, Ibarra, Kakuda, & Xue, 2008; Zanfini, Corbini, La Rosa, & Dreassi, 2010).

$$SE = \frac{\%I_{\text{mixture}}}{\%I_{\text{theoretical}}} \quad (1)$$

with

$$\%I_{\text{theoretical}} = \%I_{\text{antioxidant}} + \%I_{\text{acid}} - \frac{\%I_{\text{antioxidant}} \times \%I_{\text{acid}}}{100} \quad (2)$$

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

A Student-Fischer test was performed with a risk threshold of  $\alpha = 5\%$ , in order to identify significant differences between %  $I_{\text{mixture}}$  and %  $I_{\text{theoretical}}$ . 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  $\mu\text{M}$  for vitamin C and trolox, 22.5  $\mu\text{M}$  for caffeic acid and 10  $\mu\text{M}$  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  $\mu\text{M}$ ). Then for higher gallic acid concentration (5  $\mu\text{M}$  and 10  $\mu\text{M}$ ) 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 (Nagaoka, Kakiuchi, Ohara, & Mukai, 2007; Niki, Noguchi, Tsuchihashi, & Gotoh, 1995) or by its activation (Romano, Abadi, Repetto, Vojnov, & Moreno, 2009) as it was proposed for vitamin C (Supplementary data – Fig. S2).

### 3.5. Determination of an effective maximum concentration of aconitic acid

Vitamin C was chosen as a standard antioxidant to study the influence of the concentration of aconitic acid on the synergistic effect of the mixture. The concentration of vitamin C was set at 20  $\mu\text{M}$  and two ranges of concentrations in aconitic acid were selected according to potential food additive applications or antiparasitic formulations: between 10 and 800  $\mu\text{M}$  (Chubb, de Rose, & Narishetty, 2012; Roy, Berardi, Chan, & Lee, 2013; Roy,

Letourneau, Culver, & Behrens, 2012; Takase et al., 2007) and between 6 and 60 mM (Moriwaki, Shimizu, Nishide, & Koike, 2009) (Supplementary data – Fig. S3). In the food range, the SE remains constant (between 1.19 and 1.23) despite the increase in aconitic acid. But, a slight decrease of the SE is observed on the second range.

To determine the maximum effective concentration in aconitic acid, a gain in antioxidant activity due to the contribution of aconitic acid is calculated using the following formula:

$$\text{gain} = \%I_{\text{mixture}} - \%I_{\text{vitamin C}}$$

By comparing the gain and the inhibition percentage of aconitic acid, it was possible to determine by extrapolation the maximum useful concentration, equal to 32 mM. Beyond this concentration, the synergistic effect disappears in favor of a simple additive effect (Supplementary data – Fig. S4)

The action of aconitic acid as a co-oxidant may be described according to a joint reaction between DPPH radical and TAA on one hand and between DPPH radical and vitamin C on another hand (Supplementary data – Fig. S5).

## 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  $IC_{50}$  value of 70.4 mM was found for TAA, less than that of vitamin C ( $IC_{50} = 24.4 \mu\text{M}$ ) or that of trolox ( $IC_{50} = 16.1 \mu\text{M}$ ), 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 $\cdot$  was located on  $-\text{CH}_2-$  methylene bridge because the corresponding radical was more stabilized than  $\text{COO}\cdot$  and  $\text{C}=\text{C}\cdot$  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 $\cdot$  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.

Synergetic effect has been studied particularly for vitamin C at a concentration of 20  $\mu\text{M}$ . For this antioxidant commonly used in food industry, the synergistic effect of aconitic acid is active below a concentration of 32 mM. 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.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.07.083>.

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