Contribution of *trans*-aconitic acid to DPPH· scavenging ability in different media

William Piang-Siong [a], Pascale de Caro [b,c], Arnaud Marvilliers [a], Xavier Chasseray [a], Bertrand Payet [a], Alain Shum Cheong Sing [a,*], Bertrand Illien [a]

[a] Université de la Réunion, Faculté des Sciences et Technologies, LCSNSA (Laboratoire de Chimie des Substances Naturelles et des Sciences des Aliments), 15 avenue René Cassin, CS 92003 – RE-97744 Saint Denis Cedex 9, La Réunion, France
[b] Université de Toulouse, INP, LCA (Laboratoire de Chimie Agro-Industrielle), ENSIACET, 4 allée Emile Monso, F-31030 Toulouse, France
[c] INRA, UMR 1010 CAI, F-31030 Toulouse, France

ARTICLE INFO

Chemical compounds studied in this article:
Trans-Aconitic acid (PubChem CID: 444212)
1,1-Diphenyl-2-picrylhydrazyl radical (PubChem CID: 2735032)
Caffeic Acid (PubChem CID: 689043)
Gallic acid (PubChem CID: 370)
Citric acid (PubChem CID: 311)
Acetic acid (PubChem CID: 176)
Ascorbic acid (PubChem CID: 54670067)
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (PubChem CID: 40634)

Keywords:
Trans-aconitic acid
Antioxidant activity
Density functional theory
B3LYP
Vitamin C
Synergy
Additive effect

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 CH_2 methylene bridge because the corresponding radical was more stabilized than COO and C=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 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

* Corresponding author.
E-mail address: alain.shum@univ-reunion.fr (A. Shum Cheong Sing).

http://dx.doi.org/10.1016/j.foodchem.2016.07.083

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_{50} 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) (Malmary, Albet, Putranto, Hanine, & Molinier, 2000; Montoya, Londono, Cortes, & Izquierdo, 2014; Petit et al., 2015; Pislor, 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 ($DPPH^{\cdot}$) 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 $8.62.10^{-4}$ 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 $DPPH^{\cdot}$ 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 $DPPH^{\cdot}$, 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;

$$\%\text{Inhibition} \quad \left(1 \quad \frac{A_{sample}}{A_{blank}}\right) \times 100$$

where A_{blank} is the absorbance of the blank and A_{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_{50}). 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 $DPPH^{\cdot}$, at five minutes intervals for one hour.

2.3. Computational study

In order to get an insight into the activity of TAA on $DPPH^{\cdot}$ 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).

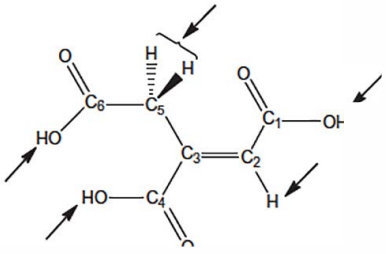


Fig. 1. *Trans*-aconitic acid numbering and studied positions of hydrogen abstraction.

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 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

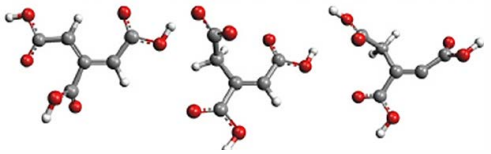
3.1. Experimental and theoretical study of the radical scavenging activity of aconitic acid

The DPPH· test was used to monitor the inhibition percentage versus aconitic acid concentrations. It was then possible to deduce the value of IC_{50}, found to be equal to 70 mM corresponding to the effective TAA concentration for 50% inhibition (Supplementary data Fig. S1). Nevertheless, compared to IC_{50} values of conventional antioxidants, such as vitamin C (24.4 μM) and trolox (16.1 μM) (Payet, 2005), the IC_{50} 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 ($pKa_1 = 2.80$; $pKa_2 = 4.46$; $pKa_3 = 6.30$) are involved in the scavenging process, DPPH· tests were also applied to acetic ($pKa = 4.75$) and citric acids ($pKa_1 = 3.13$; $pKa_2 = 4.76$; $pKa_3 = 6.40$). 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: $C_1OO^{\cdot}$, $C_4OO^{\cdot}$, $C_6OO^{\cdot}$, $C_2^{\cdot}$, $HC_5^{\cdot}$ (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)·) optimization. Thus 35 geometry optimizations of TAA(H)· 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 $COO^{\cdot}$, $HC_5^{\cdot}$ and $C_2^{\cdot}$ radical species were gathered in Table 1. The results for the other 18 radical species can be found in Supplementary material (Table S2). Five $HC_5^{\cdot}$ conformers were optimized. Their relative energies (ΔE = 0 14 kJ/mol) were lower than those of the twelve $COO^{\cdot}$ species (ΔE = 89 100) and of the four $C_2^{\cdot}$ conformers (107 118). In $HC_5^{\cdot}$ radical, C_1, C_2, C_3, C_5 and C_6 atoms were almost in the same plane; C_2C_3 and C_3C_5 bond lengths were nearly equal, respectively 139.6 139.9 and 139.2 139.6 pm. Thus the higher stability of $HC_5^{\cdot}$ radical came from a better delocalization of π electrons over carbon skeleton than in $COO^{\cdot}$ and $C_2^{\cdot}$ radicals. Accurate relative G4(MP2) 6X $\Delta\Delta_f H$ (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· was located on CH_2 methylene bridge because the corresponding radical was more stabilized than $COO^{\cdot}$ and $C_2^{\cdot}$ radicals. Therefore the following reaction mechanism of DPPH· with TAA can be suggested (Fig. 2).

Table 1
Most stable radical conformers for $COO^{\cdot}$, $HC_5^{\cdot}$ and $C_2^{\cdot}$ radical TAA(−H)· species.

Name	$HC_5^{\cdot}$_1	$C_6OO^{\cdot}$_1	$C_2^{\cdot}$_1
Sketch			
Energy (ua)	−683.023701	−682.989864	−682.982877
Dipole (D)	1.85	4.85	0.96
ΔE B3LYP*	0.0	88.8	107.2
d[C_1C_2] (pm)	147.1	148.4	144.9
d[C_2C_3] (pm)	139.6	134.7	131.9
d[C_3C_4] (pm)	151.7	150.1	149.9
d[C_3C_5] (pm)	139.4	150.9	151.5
d[C_5C_6] (pm)	147.0	150.9	152.0
dih[$C_2C_3C_5C_6$] (°)	174.8	-81.8	−108.3
$\Delta(\Delta_f H_0)$ G4(MP2)-6X*	0.0	87.9	100.6

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

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).

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

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

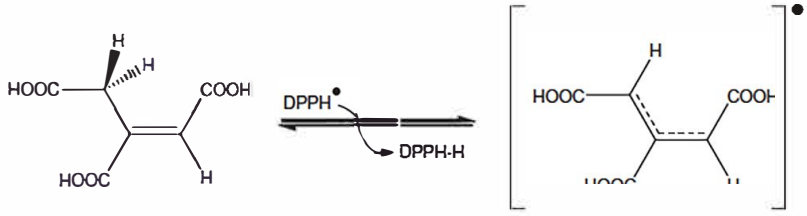


Fig. 2. Possible reaction mechanism of TAA with DPPH·.

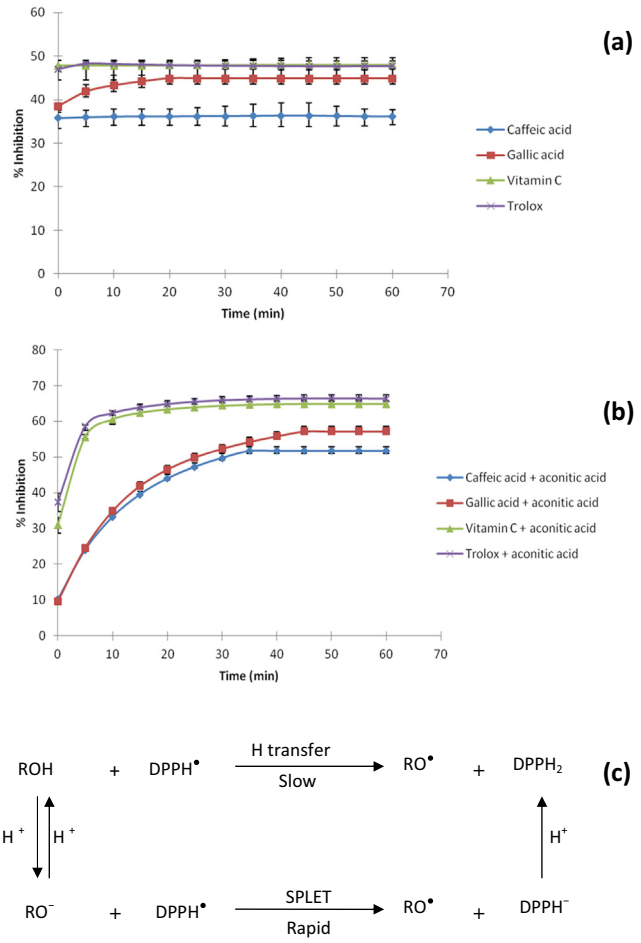


Fig. 3. Kinetic studies of aconitic acid solution mixed with conventional antioxidants (15 μM caffeic acid, 5 μM gallic acid, 22.5 μM vitamin C and trolox). (a) Inhibition percentages of standard antioxidants versus time. (b) Inhibition percentages of standard antioxidants mixed with aconitic acid (22.3 mM) versus time. (c) Reaction between DPPH· 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·, 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 p*Ka* of aconitic acid (p*Ka* = 2.80), pH of the solution is low and ROH form predominates. 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 respec tively) 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 per centages 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· 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 rela tive 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 2
Inhibition percentages of different antioxidants in solution, in association with an organic acid at 0.067 N in methanolic solution.

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

* 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.

Antioxidant	Concentration (μM)	$\%I_{mixture}$	$\%I_{theoretical}$	SE
Vitamin C	15	46.1 ± 1.6	40.6 ± 1.6	1.14*
	22.5	64.8 ± 0.7	56.4 ± 1.3	1.15*
Caffeic acid	5	37.8 ± 1.0	31.1 ± 2.0	1.22*
	15	51.7 ± 0.6	46.4 ± 1.4	1.11*
Gallic acid	2	30.6 ± 0.9	24.7 ± 0.8	1.24*
	5	57.1 ± 0.6	53.8 ± 0.9	1.06*
Trolox	15	46.5 ± 0.5	39.7 ± 0.6	1.18*
	22.5	66.3 ± 0.6	56.1 ± 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 (% $I_{mixture}$) and the theoretical value (% $I_{theoretical}$) (Liu, Shi, Ibarra, Kakuda, & Xue, 2008; Zanfini, Corbini, La Rosa, & Dreassi, 2010).

$$SE \quad \frac{\%I_{mixture}}{\%I_{theoretical}} \tag{1}$$

with

$$\%I_{theoretical} \quad \%I_{antioxidant} + \%I_{acid} \quad \frac{\%I_{antioxidant} \times \%I_{acid}}{100} \tag{2}$$

% $I_{antioxidant}$ and % I_{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 % $I_{mixture}$ 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 antiox idant 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 con centration (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 antioxi dants, 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 μM and two ranges of concentrations in aconitic acid were selected according to potential food additive applications or antiparasitic formulations: between 10 and 800 μM (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:

gain $\%I_{mixture}$ $\%I_{vitamin\ C}$

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 μM) or that of trolox (IC_{50} = 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 CH_2 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.

Synergetic 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 mM. Beyond this value, the additive effect replaces the synergistic effect. This result shows that the measure ment of anti oxidant properties of natural extracts should take into account both the contents of antioxidative molecules and organic acids.

Acknowledgments

This work was performed in the framework of the V2ARUN pro ject, supported by FUI France (Fonds unique interministériel, France) and labelled by Qualitropic (pôle de compétitivité).

We are grateful to eRcane for its collaboration and Regional council of l'île de la Réunion for their support. BI and AM wish to express their gratefulness to the "Centre de Calcul de l'Université de La Réunion" (CCUR) for computer time.

Table S1. Inhibition percentage of three organic acids at a concentration of 0.1 mol.L^{-1}

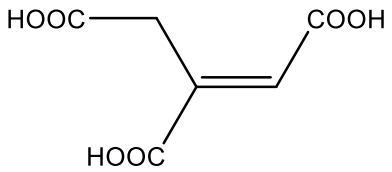
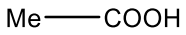
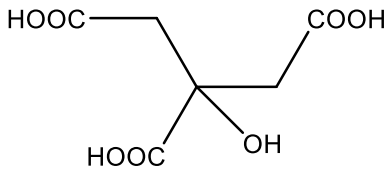
	TAA	Acetic acid	Citric acid
			
Inhibition %	67.6 ± 0.6	1.9 ± 0.2	2.4 ± 0.2

Table S2. Sketch, energy, dipole moment, relative energy, selected bond lengths and dihedral angles for each TAA(−H)• radical optimized at the B3LYP/6-31+G(d,p) level.

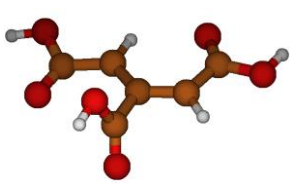
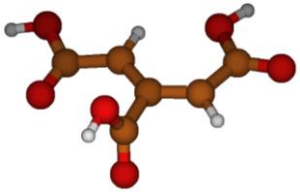
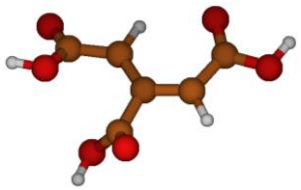
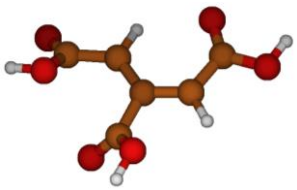
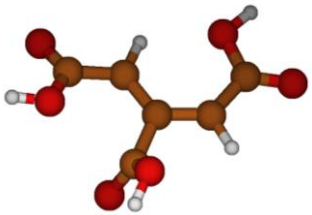
Name	**$HC_5^{\bullet}$_1**	**$HC_5^{\bullet}$_2**	**$HC_5^{\bullet}$_3**	**$HC_5^{\bullet}$_4**	**$HC_5^{\bullet}$_5**
Sketch					
Energy (ua)	-683.023701	-683.021239	-683.021215	-683.020751	-683.018531
Dipole (D)	1.85	3.09	1.98	3.82	2.02
ΔE ($kJ.mol^{-1}$)	0	6.46	6.53	7.74	13.6
d[C_1C_2] (pm)	147.1	147.0	147.0	147.0	147.0
d[C_2C_3] (pm)	139.6	139.9	139.8	139.8	139.9
d[C_3C_4] (pm)	151.7	151.9	151.3	151.1	151.6
d[C_3C_5] (pm)	139.4	139.2	139.6	139.6	139.5
d[C_5C_6] (pm)	147.0	147.0	147.3	147.6	147.2
dih[$C_1C_2C_3C_4$] (°)	-179.7	-179.6	178.3	174.8	174.9
dih[$C_1C_2C_3C_5$] (°)	1.3	1.6	-0.8	-0.3	-0.9
dih[$C_2C_3C_5C_6$] (°)	174.8	-179.6	-172.8	-174.8	-175.5

Table S2. (Continuation)

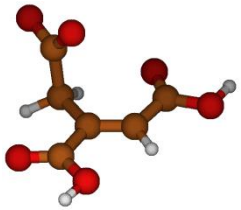
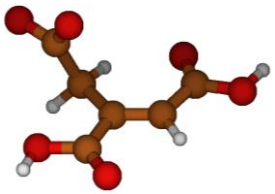
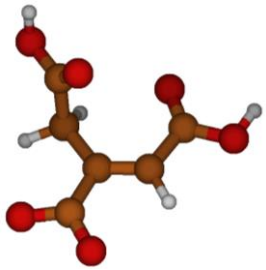
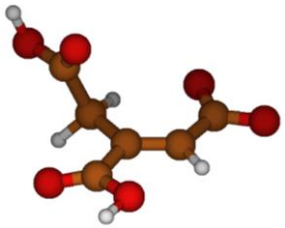
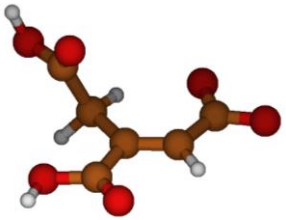
Name	**$C_6OO^\bullet$_1**	**$C_6OO^\bullet$_2**	**$C_4OO^\bullet$_1**	**$C_1OO^\bullet$_1**	**$C_1OO^\bullet$_2**
Sketch					
Energy (ua)	-682.989864	-682.989353	-682.989032	-682.988551	-682.987790
Dipole (D)	4.85	3.14	3.25	2.59	4.29
ΔE (kJ.mol^{-1})	88.8	90.2	91.0	92.3	94.3
d[C_1C_2] (pm)	148.4	148.6	148.3	147.2	147.0
d[C_2C_3] (pm)	134.7	134.6	134.5	134.6	134.5
d[C_3C_4] (pm)	150.1	150.6	148.8	150.2	150.7
d[C_3C_5] (pm)	150.9	151.0	150.4	150.5	150.7
d[C_5C_6] (pm)	150.9	150.7	152.3	152.1	152.0
dih[$C_1C_2C_3C_4$] (°)	-179.8	-179.9	-179.9	178.9	179.9
dih[$C_1C_2C_3C_5$] (°)	-0.9	-0.1	-0.7	0.1	0.3
dih[$C_2C_3C_5C_6$] (°)	-81.8	-106.5	-77.2	-107.6	-110.2

Table S2. (Continuation)

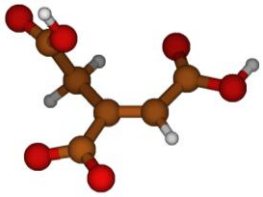
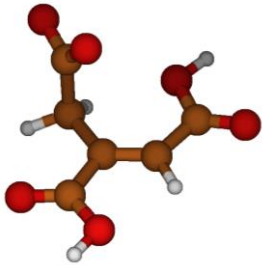
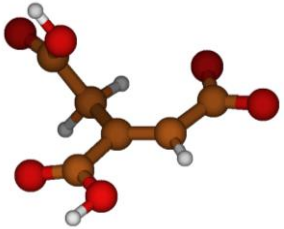
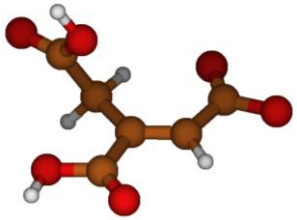
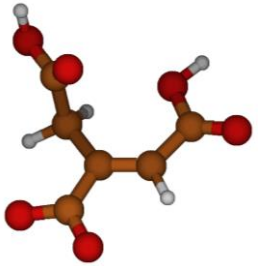
Name	**$C_4OO^\bullet$_2**	**$C_6OO^\bullet$_3**	**$C_1OO^\bullet$_3**	**$C_1OO^\bullet$_4**	**$C_4OO^\bullet$_3**
Sketch					
Energy (ua)	-682.987661	-682.987514	-682.987065	-682.986652	-682.986454
Dipole (D)	3.41	3.15	3.76	3.28	4.58
ΔE (kJ.mol^{-1})	94.6	95.0	96.2	97.3	97.8
d[C_1C_2] (pm)	148.4	148.3	147.1	147.0	148.4
d[C_2C_3] (pm)	134.6	134.8	134.7	134.6	134.6
d[C_3C_4] (pm)	149.0	150.3	150.3	150.7	148.9
d[C_3C_5] (pm)	150.5	151.1	150.6	150.8	150.5
d[C_5C_6] (pm)	152.5	150.8	152.4	152.4	152.2
dih[$C_1C_2C_3C_4$] (°)	179.8	-178.9	179.5	179.8	-179.0
dih[$C_1C_2C_3C_5$] (°)	0.9	0.4	0.7	0.7	0.4
dih[$C_2C_3C_5C_6$] (°)	-105.8	-75.6	130.1	-112.8	-79

Table S2. (Continuation)

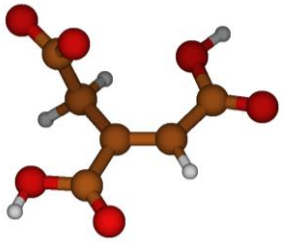
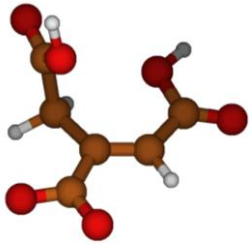
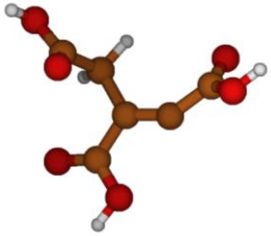
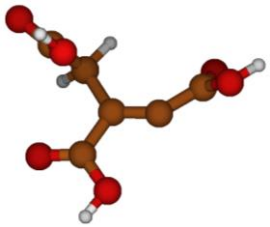
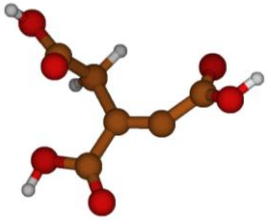
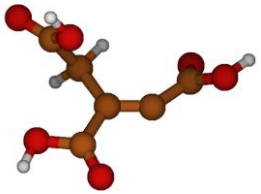
Name	**$C_6OO^{\bullet}$_4**	**$C_4OO^{\bullet}$_4**	**$C_2^{\bullet}$_1**	**$C_2^{\bullet}$_2**	**$C_2^{\bullet}$_3**
Sketch					
Energy (ua)	-682.986258	-682.985664	-682.982877	-682.981861	-682.979595
Dipole (D)	3.15	2.26	0.96	3.21	2.34
ΔE (kJ.mol^{-1})	98.3	99.9	107.2	109.9	115.8
d[C_1C_2] (pm)	148.3	148.3	144.9	144.4	145.2
d[C_2C_3] (pm)	134.7	134.7	131.9	131.7	132.2
d[C_3C_4] (pm)	150.7	149.1	149.9	149.8	150.2
d[C_3C_5] (pm)	151.1	150.8	151.5	151.8	151.8
d[C_5C_6] (pm)	150.9	152.4	152.0	152.2	151.9
dih[$C_1C_2C_3C_4$] (°)	-178.7	-179.4	174.6	173.7	175.9
dih[$C_1C_2C_3C_5$] (°)	0.5	1.6	-4.6	-5.0	-3.5
dih[$C_2C_3C_5C_6$] (°)	-95.4	-83.2	-108.3	-114.0	-110.4

Table S2. (Continuation)

Name	**$C_2^{\bullet}$_4**
Sketch	
Energy (ua)	-682.9788612
Dipole (D)	1.30
ΔE (kJ.mol^{-1})	117.7
d[C_1C_2] (pm)	145.0
d[C_2C_3] (pm)	132.2
d[C_3C_4] (pm)	150.1
d[C_3C_5] (pm)	152.1
d[C_5C_6] (pm)	152.1
dih[$C_1C_2C_3C_4$] (°)	175.7
dih[$C_1C_2C_3C_5$] (°)	-3.2
dih[$C_2C_3C_5C_6$] (°)	-114.5

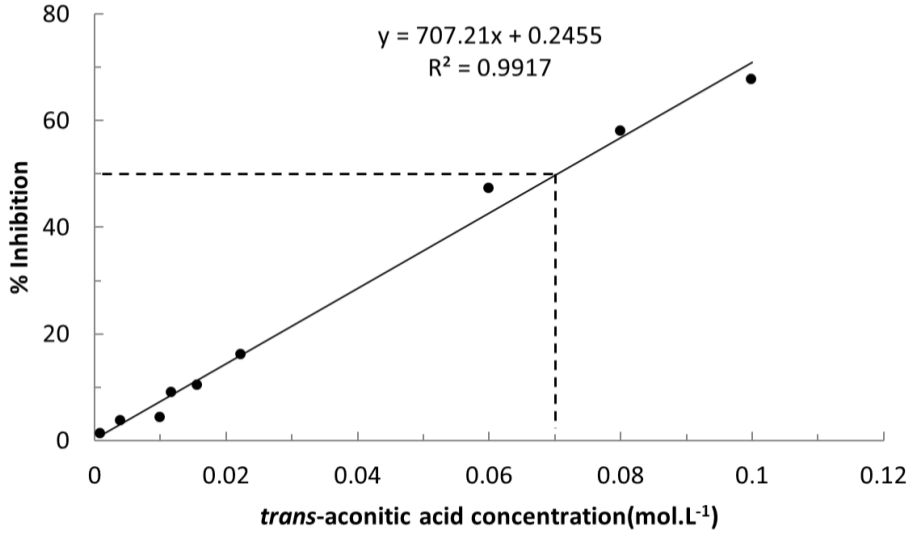


Figure S1. Determination of IC_{50} for *trans*-aconitic acid

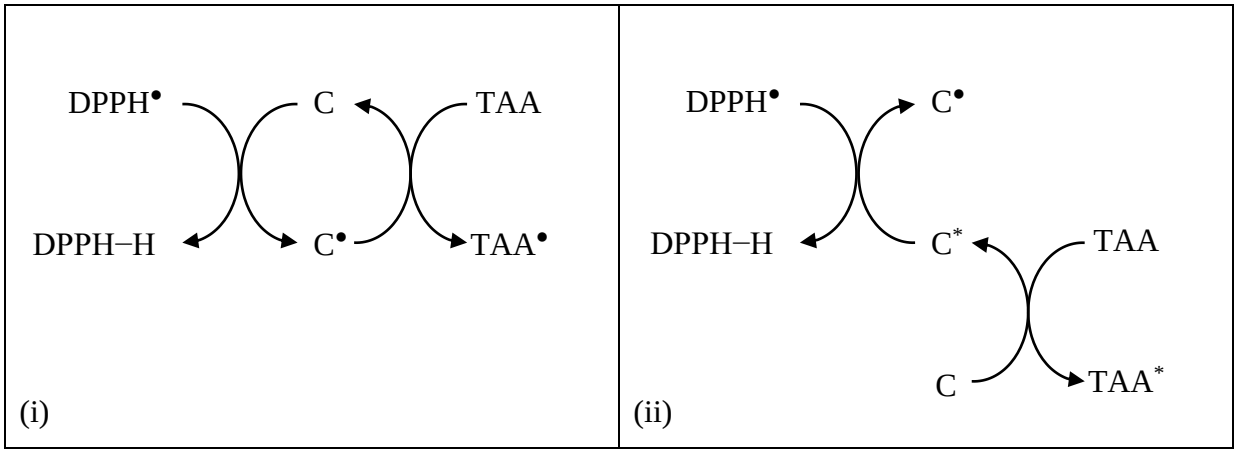


Figure S2. Mechanism suggested for i) regeneration of vitamin C (C) by aconitic acid (TAA), (ii) for activation of Vitamin C (C*) by aconitic acid (TAA).

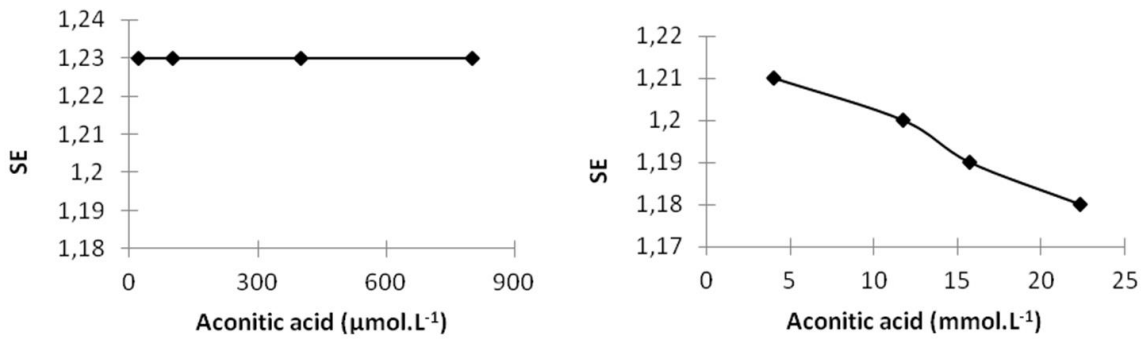


Figure S3. Synergistic effect versus aconitic acid content coupled to vitamin C over two concentration ranges

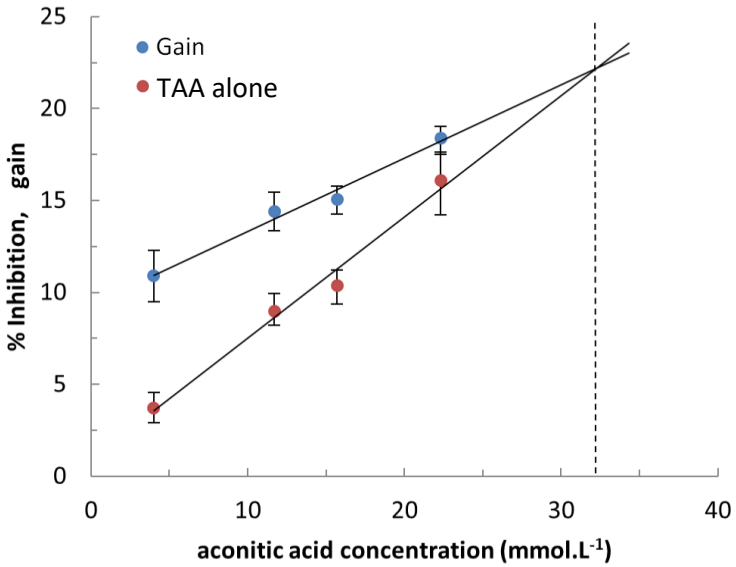


Figure S4. Intersection between percentage inhibition of aconitic acid and gain in oxidative activity of Vitamin C

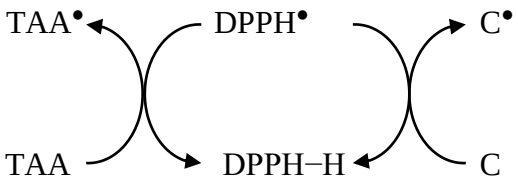


Figure S5. Representation of the effect of vitamin C (C) and aconitic acid (> 32 mM) on the DPPH•

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.

References

Alander, J., Andersson, A.-C., & Lindström, C. (2006). Cosmetic emollients with high stability against photo-oxidation. *Lipid Technology, 18*(10), 226–230.

Apak, R., Özyürek, M., Güçlü, K., & Çapanoğlu, E. (2016). Antioxidant activity/capacity measurement. 1. Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays. *Journal of Agricultural and Food Chemistry, 64*(5), 997–1027.

Becke, A. D. (1993). Density-functional thermochemistry. III. The role of exact exchange. *Journal of Chemical Physics, 98*(7), 5648–5652.

Blass, J. P., Pratt, L., & Cosentino, S. (2013). Cream for stimulating mitochondrial activity in the skin. US Patent 8,435,547 B2.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT Food Science and Technology, 28*(1), 25–30.

Bruce, W. F. (1937). Aconitic acid. *Organic Syntheses, 17*, 1.

Bruun-Jensen, L., Skovgaard, I. M., Madsen, E. A., Skibsted, L. H., & Bertelsen, G. (1996). The combined effect of tocopherols, L-ascorbyl palmitate and L-ascorbic acid on the development of warmed-over flavour in cooked, minced turkey. *Food Chemistry, 55*(1), 41–47.

Cao, N., Du, J., Gong, C., & Tsao, G. (1996). Simultaneous production and recovery of fumaric acid from immobilized Rhizopus oryzae with a rotary biofilm contactor and an adsorption column. *Applied and Environmental Microbiology, 62*(8), 2926–2931.

Célestine-Myrtil, D. A., & Parfait, A. (1988). HPLC determination of organic acids in sugar cane and its industrial by-products. *International Sugar Journal, 90*, 28–32.

Celestino, M. T., de Magalhaes, U. O., Fraga, A. G. M., Carmo, F. A., do Lione, V., Castro, H. C., ... Cabral, L. M. (2012). Rational use of antioxidants in solid oral pharmaceutical preparations. *Brazilian Journal of Pharmaceutical Sciences, 48*, 405–415.

Chan, B., Deng, J., & Radom, L. (2011). G4(MP2)-6X: A cost-effective improvement to G4(MP2). *Journal of Chemical Theory and Computation, 7*(1), 112–120.

Chubb, N. A. L., de Rose, G. F., & Narishetty, S. T. K. (2012). Topical antiparasitic formulations. US Patent 0,316,210 A1.

Cranston, H. A. (1951). Manufacture of aconitic acid. US Patent 2,566,172.

Cranston, H. A. (1955). Manufacture of aconitic acid. US Patent 2,727,066.

de Carvalho-Silva, L. B., Dionísio, A. P., da Pereira, A. C. S., Wurlitzer, N. J., Brito, E. S., de Bataglion, G. A., ... Liu, R. H. (2014). Antiproliferative, antimutagenic and antioxidant activities of a Brazilian tropical fruit juice. *LWT Food Science and Technology, 59*(2), 1319–1324.

Fernandes, F., Valentão, P., Sousa, C., Pereira, J. A., Seabra, R. M., & Andrade, P. B. (2007). Chemical and antioxidative assessment of dietary turnip (Brassica rapa var. rapa L.). *Food Chemistry, 105*(3), 1003–1010.

Foti, M. C. (2015). Use and Abuse of the DPPH· Radical. *Journal of Agricultural and Food Chemistry, 63*(40), 8765–8776.

Freitas, K. H. G., & Fatibello-Filho, O. (2010). Simultaneous determination of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in food samples using a carbon composite electrode modified with Cu3(PO4)2 immobilized in polyester resin. *Talanta, 81*(3), 1102–1108.

Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., ... Bakken, V. (2013). *Gaussian 09 (Version Rev. D.01)*. Wallingford: Gaussian Inc..

Gramza, A., & Korczak, J. (2005). Tea constituents (Camellia sinensis L.) as antioxidants in lipid systems. *Trends in Food Science & Technology, 16*(8), 351–358.

Grondin, T. (2002). *Étude de la composition aromatique de mélasse de canne de la réunion: Mise en évidence de précurseurs d'arôme de nature glycosidique (doctoral dissertation)*. Saint Denis: Université de La Réunion.

Guimarães, R., Barros, L., Dueñas, M., Calhelha, R. C., Carvalho, A. M., Santos-Buelga, C., ... Ferreira, I. C. F. R. (2013). Infusion and decoction of wild German chamomile: Bioactivity and characterization of organic acids and phenolic compounds. *Food Chemistry, 136*(2), 947–954.

Hraš, A. R., Hadolin, M., Knez, Ž., & Bauman, D. (2000). Comparison of antioxidative and synergistic effects of rosemary extract with α-tocopherol, ascorbyl palmitate and citric acid in sunflower oil. *Food Chemistry, 71*(2), 229–233.

Koné, M., Illien, B., Graton, J., & Laurence, C. (2005). B3LYP and MP2 calculations of the enthalpies of hydrogen-bonded complexes of methanol with neutral bases and anions: Comparison with experimental data. *The Journal of Physical Chemistry A, 109*(51), 11907–11913.

Li, A., & Punt, P. (2013). Industrial production of organic acids by fungi. In Maria G. Tuohy (Ed.), *Applications of microbial engineering* (pp. 52–74). CRC Press.

Litwinienko, G., & Ingold, K. (2003). Abnormal solvent effects on hydrogen atom abstractions. 1. The reactions of phenols with 2,2-diphenyl-1-picrylhydrazyl (dpph) in alcohols. *Journal of Organic Chemistry, 68*(9), 3433–3438.

Litwinienko, G., & Ingold, K. (2004). Abnormal solvent effects on hydrogen atom abstraction. 2. Resolution of the curcumin antioxidant controversy. The role of sequential proton loss electron transfer. *Journal of Organic Chemistry, 69*(18), 5888–5896.

Liu, D., Shi, J., Ibarra, A. C., Kakuda, Y., & Xue, S. J. (2008). The scavenging capacity and synergistic effects of lycopene, vitamin E, vitamin C, and beta-carotene mixtures on the DPPH free radical. *LWT Food Science and Technology, 41*(7), 1344–1349.

Malmary, G., Albet, J., Putranto, A., Hanine, H., & Molinier, J. (2000). Recovery of aconitic and lactic acids from simulated aqueous effluents of the sugar cane industry through liquid-liquid extraction. *Journal of Chemical Technology and Biotechnology, 75*(12), 1169–1173.

Martí, N., Pérez-Vicente, A., & García-Viguera, C. (2002). Influence of storage temperature and ascorbic acid addition on pomegranate juice. *Journal of the Science of Food and Agriculture, 82*(2), 217–221.

Montoya, G., Londono, J., Cortes, P., & Izquierdo, O. (2014). Quantitation of trans-aconitic acid in different stages of the sugar manufacturing process. *Journal of Agricultural and Food Chemistry, 62*(33), 8314–8318.

Moriwaki, J., Shimizu, M., Nishide, T., & Koike, S. (2009). Fat or oil composition. US Patent 7,494,680 B2.

Musialik, M., & Litwinienko, G. (2005). Scavenging of dpph radicals by vitamin E is accelerated by its partial ionization: The role of sequential proton loss electron transfer. *Organic Letters, 7*(22), 4951–4954.

Nagaoka, S., Kakiuchi, T., Ohara, K., & Mukai, K. (2007). Kinetics of the reaction by vitamin E is regenerated which natural by vitamin C. *Chemistry and Physics of Lipids, 146*(1), 26–32.

Niki, E., Noguchi, N., Tsuchihashi, H., & Gotoh, N. (1995). Interaction among vitamin C, vitamin E, and beta-carotene. *The American Journal of Clinical Nutrition, 62*(6 Suppl), 1322S–1326S.

Oliveira, I., Valentao, P., Lopes, R., Andrade, P. B., Bento, A., & Pereira, J. A. (2009). Phytochemical characterization and radical scavenging activity of *Portulaca oleraceae* L. leaves and stems. *Microchemical Journal, 92*(2), 129–134.

Payet, B. (2005). *Valorisation des produits sucriers de la Réunion : Recherche de compléments alimentaires à activité antioxydante* (Doctoral dissertation). Saint Denis: Université de La Réunion.

Pereira, D. M., Faria, J., Gaspar, L., Ferreres, F., Valentão, P., Sottomayor, M., & Andrade, P. B. (2010). Exploiting Catharanthus roseus roots: Source of antioxidants. *Food Chemistry, 121*(1), 56–61.

Perrin, C., & Meyer, L. (2002). Quantification of synthetic phenolic antioxidants in dry foods by reversed-phase HPLC with photodiode array detection. *Food Chemistry, 77*(1), 93–100.

Petit, A., Wu-Tiu-Yen, J., Pislor, E., Pontalier, P.-Y., Albet, J., Hoareau, W., & Corcodel, L. (2015). Method for extracting aconitic acid from products of the sugar cane industry. FR patent EP2921471 (A1).

Piang-Siong, W., de Caro, P., Lacaze-Dufaure, C., Shum Cheong Sing, A., & Hoareau, W. (2012). Effect of catalytic conditions on the synthesis of new aconitate esters. *Industrial Crops and Products, 35*(1), 203–210.

Pislor, E., Pontalier, P.-Y., & Albet, J. (2009). Process for recovering carboxylic acids from sugar cane industry by-products. *Proceedings of the second International Congress on Green Process Engineering*. . Venice, Italy.

Romano, C. S., Abadi, K., Repetto, V., Vojnov, A. A., & Moreno, S. (2009). Synergistic antioxidant and antibacterial activity of rosemary plus butylated derivatives. *Food Chemistry, 115*(2), 456–461.

Roy, G., Letourneau, S., Culver, C., & Behrens, J. (2012). Unsaturated acids for fading protection of colors derived from natural sources used in beverage products. US Patent 8,158,183.

Roy, G., Berardi, R., Chan, W., & Lee, T. (2013). Prevention of synthetic color fading in beverages using botanically derived color stabilizers. US Patent 8,420,141.

Scalzo, R. L. (2008). Organic acids influence on DPPH scavenging by ascorbic acid. *Food Chemistry, 107*(1), 40–43.

Shahidi, F. (2000). Antioxidants in food and food antioxidants. *Food/Nahrung, 44*(3), 158–163.

Sharma, O. P., & Bhat, T. K. (2009). DPPH antioxidant assay revisited. *Food Chemistry, 113*(4), 1202–1205.

Silva, B. M., Andrade, P. B., Valentão, P., Ferreres, F., Seabra, R. M., & Ferreira, M. A. (2004). Quince (Cydonia oblonga Miller) Fruit (Pulp, Peel, and Seed) and Jam: Antioxidant Activity. *Journal of Agricultural and Food Chemistry, 52*(15), 4705–4712.

Silva, L. R., Azevedo, J., Pereira, M. J., Valentão, P., & Andrade, P. B. (2013). Chemical assessment and antioxidant capacity of pepper (Capsicum annuum L.) seeds. *Food and Chemical Toxicology, 53*, 240–248.

Sousa, S. F., Fernandes, P. A., & Ramos, M. J. (2007). General performance of density functionals. *Journal of Physical Chemistry A, 111*(42), 10439–10452.

Stephens, P. J., Devlin, F. J., Chabalowski, C. F., & Frisch, M. J. (1994). Ab initio calculation of vibrational absorption and circular dichroism spectra using density functional force fields. *Journal of Physical Chemistry, 98*(45), 11623–11627.

Takase, H., Koike, S., Sakai, H., Nishide, T., Hase, T., & Murase, T. (2007). Oil composition. US Patent 7,182,971.

Wagner, K.-H., Wotruba, F., & Elmadfa, I. (2001). Antioxidative potential of tocotrienols and tocopherols in coconut fat at different oxidation temperatures. *European Journal of Lipid Science and Technology, 103*(11), 746–751.

Xie, J., & Schaich, K. M. (2014). Re-evaluation of the 2,2-Diphenyl-1-picrylhydrazyl Free Radical (DPPH) Assay for Antioxidant Activity. *Journal of Agricultural and Food Chemistry, 62*(19), 4251–4260.

Zanfini, A., Corbini, G., La Rosa, C., & Dreassi, E. (2010). Antioxidant activity of tomato lipophilic extracts and interactions between carotenoids and alpha-tocopherol in synthetic mixtures. *LWT Food Science and Technology, 43*(1), 67–72.