Iron-citrate complexes and free radicals generation: Is citric acid an innocent additive in foods and drinks?

Isabelle Gautier-luneau[1,2,*], Perrine Bertet[1], André Jeunet[1], Guy Serratrice[1] & Jean-Louis Pierre[1]

[1]*Laboratoire de Chimie Biomimétique, LEDSS, UMR CNRS 5616, Université Joseph Fourier, BP 53, 38041, Grenoble Cedex 9, France;* [2]*Laboratoire de Cristallographie, CNRS, 25 avenue des Martyrs, BP 166, 38042, Grenoble Cedex 9, France; *Author for correspondence (E-mail: Isabelle.Gautier-Luneau@grenoble.cnrs.fr)*

Abstract

The generation of free radicals (Fenton chemistry) from various iron citrate complexes has been studied. Spin trapping methods have been used. The results can question concerning the innocence of added citric acid in foods and cold drinks. We concluded that in absence of pathological situation citric acid is probably not dangerous but it may become dangerous in situation of oxidative stress and/or iron overload.

Introduction

Citric acid which plays numerous physiological roles (Glusker 1980) is ubiquitous in nature (it accounts for 5% by weight of lemon juice and 0.3% by weight of teeth and bone). The citrate concentration controls several other metabolite pathways. Citric acid is also the allosteric inhibitor of phosphofructokinase, the basic enzyme in the glycolysis pathway. In higher organisms it regulates the utilization of calcium contained in foods.

Ferric citrate plays a paramount role in iron metabolism in living systems. Citrate is present in blood plasma in submillimolar concentration and promotes the bioavailability of dietary non-heme iron (it is believed to be one of the major components of a pool of Fe^{III} that is not bound to transferrin) (Parkes *et al.* 1991). Citrate has also been proposed to be a constituent of the low molecular weight cytosolic iron pool (Bakkeren *et al.* 1985). The presence of Fe^{II} and a reducing agent is required for activation of the Krebs cycle enzyme aconitase. The iron A ternary enzyme-

Fe^{II}-citrate complex has been detected at the active site of aconitase (Villafranca & Mildvan 1972): one iron of the iron-sulfur cluster expands its coordination number from four to six through the formation of a chelate ring with the citrate.

A survey of the literature reveals that the coordination chemistry of iron citrate was long time remained fuzzy (Pierre & Gautier-Luneau 2000). The different experimental conditions used for the preparation of the so-called ferric citrates (stoechiometry, pH and temperature) lead to different species, which, moreover, can be in equilibrium in solution (Gautier-Luneau *et al.* 2005). We have unambiguously characterized theses species.

Citric acid and its salts have many applications in everyday life. Among others, it is used as an additive in foods and in the production of cold drinks and effervescent salts (Milewska 1988).

Owing to the fact that the mobilization of iron may, under certain conditions, lead to the formation of highly toxic hydrogen radicals (Pierre & Fontecave 1999), one can question concerning the innocence of adding citric acid in large amounts in foods or in drinks. In this paper we have studied

the catalytic role of iron-citrate complexes in the formation of hydroxyl radicals. We have also shown the formation of hydroxyl radical from commercial orange juices in presence of Fe^{III}.

Material and methods

All chemical reagents were from commercial sources and of the purest grade. All the drinks (orange soda, orange juice, lemon soda, ice-tea) are from self-service stores.

The reaction mixture were prepared from the following solutions: pure DMPO; H_2O_2 solution 0.1 M or 2 M; $Fe(NO_3)_3 \cdot 9H_2O$ solution 0.1 M or $Fe(ClO_4)_3 \cdot 9H_2O$ solution 0.1 M or 0.2 mM; iron-citrate solution with $[Fe^{III}] = 0.1$ M or 0.2 mM prepared from $Fe(ClO_4)_3 \cdot 9H_2O$ and citric acid with molar ratio Fe:cit equal to 1:2, 1:10 and 1:100; sodium ascorbate solution 20 mM; tris(hydroxymethyl)aminomethane (Tris) buffer 0.05 M, pH 7.0. 100 μL of the reaction mixtures were prepared by adding 5 μL of DMPO, 10 μL of H_2O_2, 10 μL of Fe^{III} or Fe:cit solution, 5 μL of sodium ascorbate and 70 μL of Tris buffer to complete the solution or 75 μL of Tris buffer if sodium ascorbate is not added (final pH was equal to 7.0; pH was measured from 1 mL of a solution with the same composition). The concentrations in the reaction mixture were 0.02 mM or 10 mM in Fe^{III} and 10 mM or 0.2 M in H_2O_2. The reactions (room temperature, aerobic conditions) have been monitored by ESR spectroscopy.

Electron Spin Resonance (ESR) measurements were recorded on a Bruker ESP 300 E spectrometer operating at 9.4 GHz (X band, 100 KHz modulation). 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) has been used as a spin-trap for hydroxyl radical. The corresponding signal is a quadruplet with 1:2:2:1 intensities (Buettner 1987). In all the cases, these relative intensities were observed. The amount of $OH^\bullet$ was calculated in arbitrary units (A.U.) from the intensity of the second signal and by taking account the gain of the receiver.

Results and discussion

Spin trapping experiments with DMPO as a spin-trap were carried out to study the ability of various buffered solutions containing iron (III) salt and citric acid to promote the formation of hydroxyl radicals in the presence of hydrogen peroxide and in the presence or not of a reducing agent (ascorbic acid). The classical signal of the DMPO-OH radical, issued from the coupling between DMPO and hydroxyl radicals was observed for about an half hour, reflecting the continuous formation of hydroxyl radicals.

The curves presented in Figure 1 exhibit the formation of hydroxyl radical as a function of time in solution containing equimolar concentrations of Fe^{III} and H_2O_2 (10 mM). A very low amount of $OH^\bullet$ was observed for solutions without citric acid whatever the ferric salt (nitrate or perchlorate) and in the presence or not of ascorbic acid. On the contrary, high amount of $OH^\bullet$ was detected for the solution containing Fe:cit (1:2) in the presence or not of ascorbic acid. In this case, after ten minutes a marked decrease of $OH^\bullet$ was observed owing to the default of H_2O_2. Consequently, further experiments were realized by using a large excess of H_2O_2 with respect to Fe^{III}. In these conditions, Figure 2 shows that the production of $OH^\bullet$ remains constant during at less 30 min. The two sets of experiments realized at concentrations in Fe^{III} of 0.02 mM and 10 mM and for various molar ratio Fe:cit showed a marked increase of the production of $OH^\bullet$ with an increase of $[Fe^{III}]$ (about 20 AU and 300 AU for 0.02 mM and 10 mM in $[Fe^{III}]$ respectively). Furthermore, the increase of the concentration of citric acid (Fe:cit
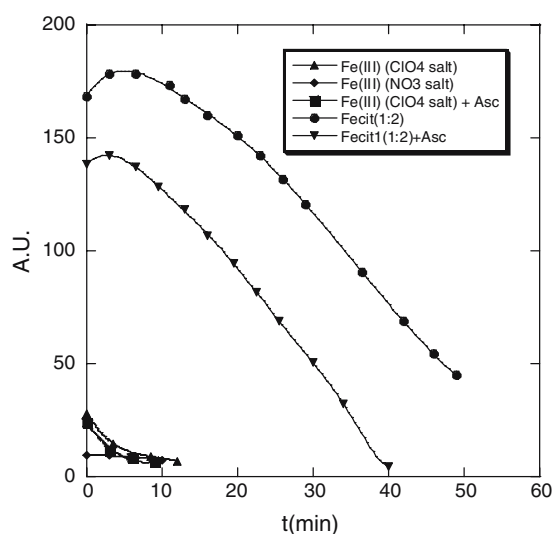

Figure 1. Formation of hydroxyl radical as a function of time in solution containing $[Fe^{III}] = [H_2O_2] = 10$ mM in the presence or not of citric acid and ascorbic acid. Final pH = 7.0.
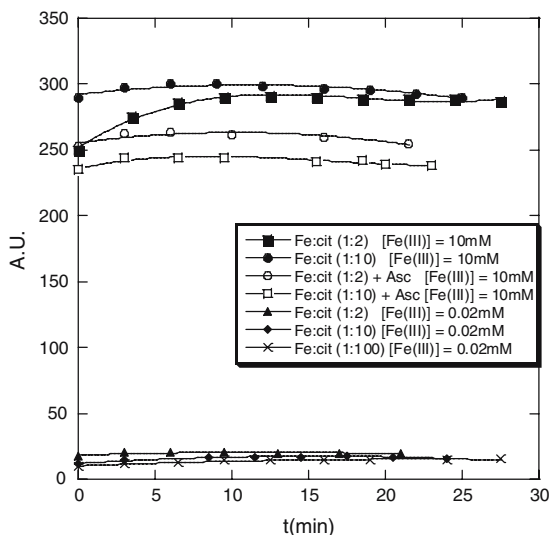
Figure 2. Formation of hydroxyl radical as a function of time in solution of ferric citrate in molar ratio Fe:cit equal to 1:2, 1:10; 1:100, with $[Fe^{III}] = 10$ mM or 0.02 mM, $[H_2O_2] = 200$ mM in the presence or not of ascorbic acid. Final pH = 7.0.

ratios 1:2, 1:10, 1:100) does not influence the amount of OH$^\bullet$. It should be noted that the experiments performed in the presence of ascorbic acid gave similar results to those performed in its absence indicating that a reducing agent is not necessary to promote the Fenton reaction.

Several commercial drinks (orange soda or non gaseous orange juice, lemon soda, natural lemon juice and ice tea) were studied in order to test their
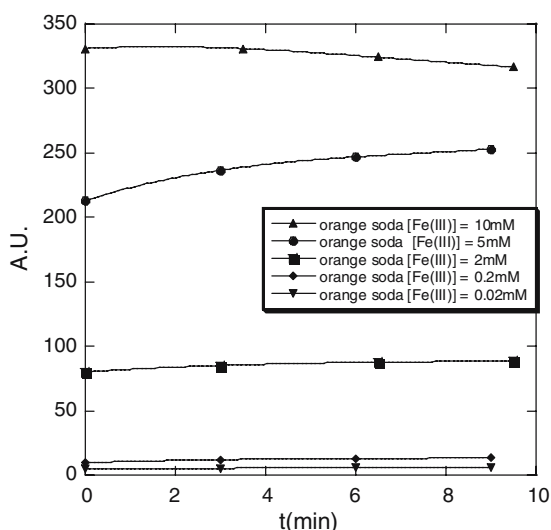

Figure 3. Formation of hydroxyl radical as a function of time in solution of orange soda (commercial drink) in presence of H_2O_2 (200 mM) and for different concentrations of Fe^{III}.
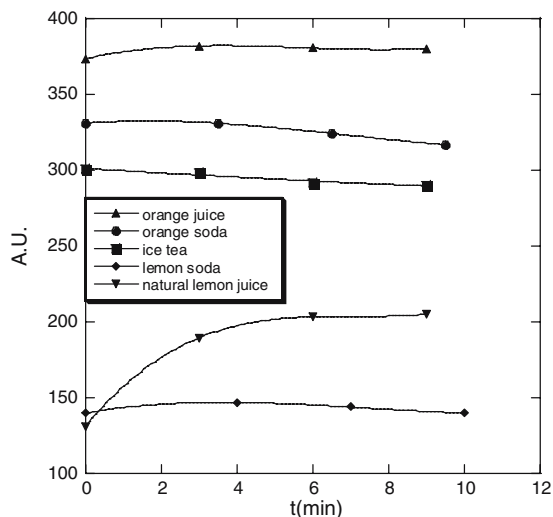

Figure 4. Formation of hydroxyl radical as a function of time in solution of commercial drinks in presence of H_2O_2 (200 mM) and Fe^{III} (10 mM).

ability to the formation of hydroxyl radical. All of the experiments were carried out in the presence of added H_2O_2 (200 mM) in large excess with respect to Fe^{III} (10 mM). The results are depicted in the Figures 3 and 4, respectively. At first, the influence of the concentration in Fe^{III} was studied on orange soda. The curve of the Figure 3 clearly shows that the production of OH$^\bullet$ largely increase with $[Fe^{III}]$. The curves of the Figure 4 evidence the production of OH$^\bullet$ in several commercial drinks. This production was in the range 150–380 A.U. depending on the type of drink, that is similar to the range value (250–300 A.U.) observed for the previously examined solutions containing citric acid (Figure 2). All the drinks are labeled to contain citric acid. Our results suggest that the presence of citric acid in the drinks favors the Fenton reaction and so, the formation of hydroxyl radical. The large range of OH$^\bullet$ production is not easy to interpret since the concentration of the added components in the drinks are not indicated. The presence of anti-radical components in some drinks could explain that a smaller amount of OH$^\bullet$ was observed.

Conclusion

It is well known that Fe^{III} is poorly soluble and the primary role of citric acid is to maintain soluble Fe^{III}. We have shown that in some conditions,

hydroxyl radicals are formed. Surprisingly, the potential toxicity of citric acid seems to be occulted, despite the publication of papers open to alert the opinion concerning this toxicity.

It has been shown that mitochondrial lipid peroxidation could be induced by Fe^{II}-citrate (Castilho et al. 1999; Santos et al. 2001). Hydroxyl radical formation from the auto-reduction of a ferric citrate complex has been evidenced (Gutteridge 1991).

In humans "free" iron is almost non existent and iron potentially chelatable by citric acid is probably scarce, owing to the fact that the complexing ability of citric acid is lower than that of the natural iron chelators (transferrin, ferritin, apo ironproteins...). Of course, the concentration of Fe^{III} and even H_2O_2 used in our experiments are higher than those of normal biological situation and in the absence of a pathological situation, one can hope that citric acid is not dangerous. But in situation of oxidative stress (excess of hydrogen peroxide production) and/or iron overload (hemochromatosis...) it may be postulated that citric acid could become dangerous, amplifying the formation of hydroxyl radicals.

Acknowledgments

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