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Isolation of quercetin's salts and studies of their physicochemical properties and antioxidant relationships

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Abstract—Hydroxyflavones in alkaline solutions show high free radical scavenging activities. Quercetin, one of these hydroxyflavones may be submitted to chemical reactions yielding a mixture of mono-, di- and tri-sodium salts. These salts were recovered after solubilization and stepwise precipitation in methylalcohol/ethylacetate solvents. The different salts were analyzed using sodium emission spectrophotometry and nuclear magnetic resonance to determine the number of acid hydrogens at pH 10 and the position of these acid hydrogens. Our study demonstrates that among the three salts of quercetin, the di-sodium compound is endowed with the more efficient scavenging properties in a phosphate buffer at physiological pH 7.4. Physicochemical parameters and free hydroxyl radical scavenging activity relationships were also determined, allowing to explain the mechanisms whereby hydroxyl groups exert their radical scavenging activities.

1. Introduction

As early as in 1966, the antioxidant properties of flavonoid molecules were investigated.[1] Quercetin is one of the most studied flavonoids and is characterized by the presence of five hydroxyl groups (Fig. 1). The superoxide antioxidant activities of quercetin have been documented primarily in several experimental models,[2–4] notably in lipid peroxidation systems[5,6] and are likely due to the beneficial effects generated the 3′ and 4′ hydroxyl groups participating to the catechol ring (B). Subsequent analysis has shown that quercetin had some anticarcinogenic properties.[7–9]

In contrast, some flavonoid molecules may display prooxidant activities that are, in the case of quercetin, attributed to the three hydroxyl groups (Fig. 1) situated at positions 3, 5 and 7 of the A and C rings.[10] In presence of Fe^{3+}-EDTA at pH 7.4, quercetin enhances hydroxyl radical formation from H_2O_2 in the deoxyribose assay. Bleomycin-dependent DNA damage was increased by quercetin in the presence of Fe^{3+}.[11] In addition, toxicity studies have been performed with quercetin reporting its mutagenicity in the Ames' test model and even its carcinogenic potential.[7] Previous investigations[12–14] have shown that the three acidic hydrogens of quercetin have the following pK_a and may be involved in reactions.

$$pK_1 = 7.7 \text{ (position 3)} > pK_2 = 8.77 \text{ (position 4}') > pK_3 = 9.81 \text{ (position 7)}.$$

These opposite data about quercetin activities led us to change the 3, 4′ and 7 acid forms of quercetin into salt

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Abbreviations: MDA: malondialdehyde; TBA: thiobarbituric acid; [D]: concentration of the deoxyribose; [S]: concentration of the scavenger substance; $A°$: absorbance at 532 nm after reaction of malondialdehyde with thiobarbituric acid in absence of scavenger substance; A: absorbance at 532 nm after reaction of malondialdehyde with thiobarbituric acid in presence of scavenger substance; QH_3: Quercetin; $(QH_2)^-$: Quercetin mono-anionic; $(QH)^{2-}$: Quercetin di-anionic; Q^{3-}: Quercetin tri-anionic

Keywords: Isolation; Quercetin's salts; Antioxidant activity; Physicochemical properties.

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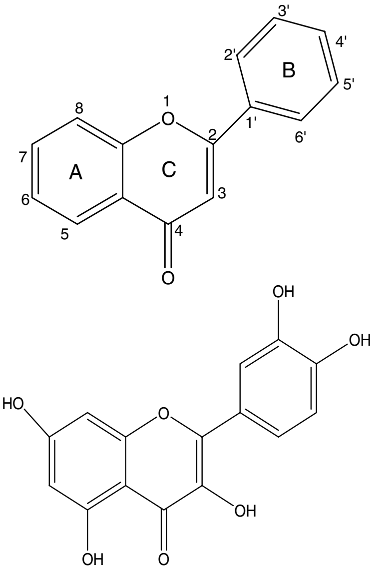

Figure 1. Quercetin structure and hydrogen positions.

derivatives in order to neutralize the harmful activities of this molecule and to obtain more stable and more active compound. The aim of our study was thus to save the protective properties of the quercetin molecule while discarding its undesirable pro-oxidant effects.

In the present study, numerous physicochemical and antioxidant properties of these salts were evaluated after isolation of each salt from the mixture. Sodium (Na) spectrophotometry emission and nuclear magnetic resonance (NMR) studies were undertaken to determine its physicochemical properties. Absorption in UV–vis was used to determine its antioxidant properties.

2. Materials and methods

2.1. Reagents and chemistry

Ascorbic acid, 2-deoxy-D-ribose, thiobarbituric, trichloroacetic, 5-chlorosalicylaldehyde, 3,5-dichloro-2-hydroxybenzaldehyde, 2,3,5-trichlorophenol, 2,3-dichloro-4-hydroxybenzaldehyde, 2,3,5-trichloro-4-hydroxybenzaldehyde and quercetin were purchased from Aldrich (St Quentin Fallavier, France). Hydrogen peroxide, iron (II) ammonium sulfate, methyl alcohol, ethyl acetate, methyl alcohol-d_6 and dehydrated ethylenediamine tetraacetic acid di-sodium salt were obtained from Fluka (St Quentin Fallavier, France). Catechol, 3,4-dihydroxybenzaldehyde, cyclohexanol, phenol, 3-hydroxy-benzaldehyde, 2-hydroxybenzaldehyde, 4-hydroxy-benzaldehyde, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid and 3,4-dihydroxyphenyl acetic acid were obtained from Acros (Noisy Le Grand, France). All other chemicals were of analytical grade.

2.2. Apparatus

The microcomputer pH vision 6072N model from Electronics LTD was used for the pH determination of the reaction mixture. The absorbance measurements for the free radical assays were performed on a BECKMAN UV–Visible detector (Model 65), which was also used for the kinetic study of the precipitation. The ^{1}H NMR spectra were measured on a BRUKER 200 MHz spectrometer. All the Na atomic emission spectra were performed on a PERKIN ELMER OPTIMA 3300 DV. The pK_a values were determined by titration on a METROHM ion analyses, with combined pH glass electrode (Herisau, Switzerland).

2.3. Preparation and isolation of the three salts of quercetin

Quercetin (0.1 g) was dissolved in 40 mL of ethyl acetate. Then, a solution of sodium hydroxide dissolved in methyl alcohol was slowly added until the required pH < pH of quercetin degradation 10.2:[15] pH 7.7 for the formation of the mono-salt, pH 8.8 for the di-salt and pH 9.8 for the tri-salt. At the same time, the methyl alcohol was added to prevent the precipitation of the salt before reaching the required form (7.3 mL for the mono-salt, 19.6 mL for the di-salt and 28 mL for the tri-salt). After precipitation and filtration, the recovered compounds were dried and then analyzed using Na emission spectrophotometry to confirm the number of Na corresponding to each molecular species and by NMR to determine the position of this Na and the corresponding pK_a.

2.4. Sodium emission spectrophotometer

The number of Na atoms corresponding to each form of salt was determined as follows: taking into account the molecular mass (MM) of each quercetin derivative (324.57 g, 347.87 g, 371.17 g, respectively). Then, 29.41 mg of quercetin mono-sodium salt was added to 1 L of HNO_3 at (2%) in order to precipitate the quercetin and isolate the Na in the solution obtained after filtration on 0.45 µm filters. This solution was calculated to contain 2.08 mg of Na per litre. Then, 16.03 mg of quercetin di-sodium salts was added to 1 L of HNO_3 at (2%) and finally 11.67 mg of quercetin tri-sodium salts was added to 1 L of HNO_3 at (2%) and in the same way as the quercetin mono-sodium salt, the Na was isolated and was calculated to contain, respectively, 2.15 and 2.17 mg/L.

2.5. Deprotonation sites of the three salts by ^{1}H NMR

The compounds obtained after precipitation as described above and corresponding to the three quercetin salts, were used to investigate the deprotonation sites by ^{1}H NMR spectral measurements. All ^{1}H NMR spectra were measured on a Bruker 200 MHz spectrometer and the tetramethylsilane (TMS) was used as standard compound to obtain hydrogen atom chemical shifts. The compounds were dissolved in methyl alcohol-d_6.

The method consisted in measuring the ^1H NMR spectra of the quercetin and each one of its salts (mono-, di- and tri-sodium salts) in order to determine chemical shift variations and deprotonation site relationships. This relationship was evaluated by comparing ^1H NMR spectra and by determining the position of the shielding effect triggered by 1, 2 or 3 deprotonation procedures. The more is the chemical shift of a hydrogen atom decreased, the less is the distance recorded between this hydrogen and the site of deprotonation.

2.6. Fenton test

This test is used by different authors to evaluate the hydroxyl radical scavenging activity of a compound.[16] The samples were incubated at 37 °C for 15 min in a phosphate buffer, at pH 7.4 (24 mM NaH_2PO_4–Na_2HPO_4 in 15 mM NaCl) with deoxyribose (0.6 mM), ascorbic acid (0.6 mM), hydrogen peroxide (0.855 mM), the substances tested (0–0.5 mM) and the EDTA (0.02 mM) with $(NH_4)_2Fe(SO_4)_2$ (0.02 mM) in order to initiate the reaction. EDTA and $(NH_4)_2Fe(SO_4)_2$ were premixed just before the addition to the reaction mixture. A 1.5 mL amount of 2.8% (w/v) of cold trichloroacetic acid were then added, and an aliquot of 1 mL of the incubation was mixed with 1 mL of thiobarbituric acid reagent (1% w/v, in 0.05 M NaOH), followed by a heating at 100 °C for 15 min and a cooling at room temperature. The absorbance was determined at 532 nm against appropriate blanks.

The method is based on the specific reaction of deoxyribose with hydroxyl radicals generated from ascorbic acid/Fe^{2+}/EDTA/H_2O_2, which produce some malondialdehyde (MDA), which can be evaluated by the reaction with the thiobarbituric acid (TBA).

The rate constant for the reaction of a given scavenger with hydroxyl radicals, can be determined by the competition between the tested compounds and the deoxyribose for hydroxyl radical. The rate constant is given by Eq. 1:

$$\frac{1}{A} = \frac{1}{A^\circ}\left(1 + \frac{K_s[S]}{K_D[D] + K_x}\right) \tag{1}$$

in which A is the absorbance value recorded at 532 nm for a given concentration of the scavenger [S], A° the absorbance without the scavenger, [D] the concentration of deoxyribose (0.6 mM in our experiments), K_D the rate constant of deoxyribose and K_s the one of the studied compound; K_x is a constant depending on the experimental conditions and representing the part of hydroxyl radicals, which react with all other reagents (for instance with Fe^{2+}-EDTA, H_2O_2...) in the assay mixture, with the exception of deoxyribose. As a result, the rate constant could be expressed as

$$\frac{A^\circ}{A} = \left(1 + \frac{K_s S}{K_D[D] + K_x}\right) \tag{2}$$

in which the plot of A°/A against [S] allowed to determine the slope "a" and therefore

$$K_s = a(K_D \times [D] + K_x) \tag{3}$$

2.7. Hydroxyl radical scavenging as function of pH

The solutions of quercetin 0.15 mM were prepared at different pH going from 8 to 10.4. These solutions were diluted in order to have a concentration of 0.5 mM in a phosphate buffer at pH 7.4. Then, the Fenton test was carried out for this series as described above. As a result, the pH values of the prepared solutions reflect the relative abundance of each molecular species according to the following equations:

$$QH_3 \overset{K_1}{\rightleftharpoons} QH_2^- + H^+ \quad pK_1 = 7.7$$

$$QH_2^- \overset{K_2}{\rightleftharpoons} QH^{2-} + H^+ \quad pK_2 = 8.77$$

$$QH^{2-} \overset{K_3}{\rightleftharpoons} QH^{3-} + H^+ \quad pK_3 = 9.81$$

with

$$K_1 = \frac{[QH_2^-][H^+]}{[QH_3]}(1) \quad K_2 = \frac{[QH^{2-}][H^+]}{[QH_2^-]}(2)$$

$$K_3 = \frac{[Q^{3-}][H^+]}{[QH^{2-}]}(3)$$

knowing that

$$\begin{aligned}[QH_3]_t &= [QH_3] + [QH_2^-] + [QH^{2-}] + [Q^{3-}] \\ &= [QH_3]\left(1 + \frac{[QH_2^-]}{[QH_3]} + \frac{[QH^{2-}]}{[QH_3]} + \frac{[Q^{3-}]}{[QH_3]}\right)\end{aligned}$$

so

$$[QH_3] = \frac{[QH_3]_t}{1 + \frac{K_1}{[H^+]} + \frac{K_1K_2}{[H^+]^2} + \frac{K_1K_2K_3}{[H^+]^3}}$$

$$[QH_2^-] = \frac{[QH_3]K_1}{[H^+]}, \quad [QH^{2-}] = \frac{[QH_2^-]K_2}{[H^+]}$$

$$\text{and} \quad [Q^{3-}] = \frac{[QH^{2-}]K_3}{[H^+]}$$

2.8. Hydroxyl radical scavenging activity of quercetin di-salt of sodium

Quercetin di-salts ranging from 0.15 to 0.5 mM were prepared by dissolution of the di-salt of quercetin isolated previously and tested for hydroxyl radical scavenging activity in Fenton reaction system as above described.

2.9. Physicochemical parameters and hydroxyl radical scavenging activity relationships

Eighteen compounds were chosen in order to study the pK_a-hydroxyl radical scavenging activity relationships.

These compounds have the same phenol or catechol ring, and different substitutions were undertaken making them responsible for the changes of the acidity of the hydroxyl group of the phenol ring. The pK_a of the most acidic hydroxyl of this cycle were determined using a Metrohm apparatus with a combined pH glass electrode and NaOH 0.1 M as reagent. Then, these compounds were tested by Fenton reaction to evaluate the hydroxyl radical scavenging velocity values. The same tests were also performed for the salt form of these molecules.

3. Results and discussion

3.1. Sodium emission spectrophotometry

The Na content of each salt of quercetin was expressed in mg of Na per litre. The theoretical and practical results were reported in Table 1. These results demonstrated clearly that the compound recovered at pH 7.7 corresponded to the quercetin mono-sodium salt and the compounds obtained at pH 8.8 and 9.8 corresponded to the quercetin di-sodium and tri-sodium salts, respectively.

3.2. Acid hydrogen sites of the three salts

The ^{1}H NMR spectra obtained for quercetin and the different salts let us to determine the chemical shift variations of each of five hydrogen atoms of quercetin. The chemical shift variation of each hydrogen atom as function of the deprotonation number is plotted in Figure 2.

The results showed that the first deprotonation induced a shielding effect on the H_6, H_8, $H_{2'}$ and $H_{6'}$. Therefore, the first deprotonation was carried out in the ring between the two couples of hydrogen, namely in the ring C of the molecule (Fig. 1). The ring C offered only one single deprotonation possibility, occurring in position 3, where the first acid hydrogen was therefore situated. In the case of dual deprotonation procedure, a larger shielding effect was recorded confirming that the ring containing hydrogens 6 and 8 was preferentially affected. In this ring, there exist two deprotonation possibilities involving hydroxyl groups situated at 5- and 7-positions. It was however, easier to deprotonate the hydroxyl group on the position 7 than that of position 5 where the hydrogen can make bond with the oxygen of the carbonyl group. We concluded from these considerations that the second deprotonation was preferentially carried out at position 7.

Both shielding and deshielding effects were noted along the three deprotonating steps. The $H_{6'}$, $H_{2'}$ and $H_{5'}$ in the ring B showed a shielding effect but the H_6 and H_8 showed also a deshielding effect in the ring A. The former effect was attributed to the deprotonation of hydrogen in 4′-position in the ring B, because of its conjugation with the ring C, which explain its better acidity by comparison with the hydroxyl group on the 3′-position. The deshielding effect could be due to the breaking of the hydrogen bond between the hydroxyl in 5-position and the carbonyl group.

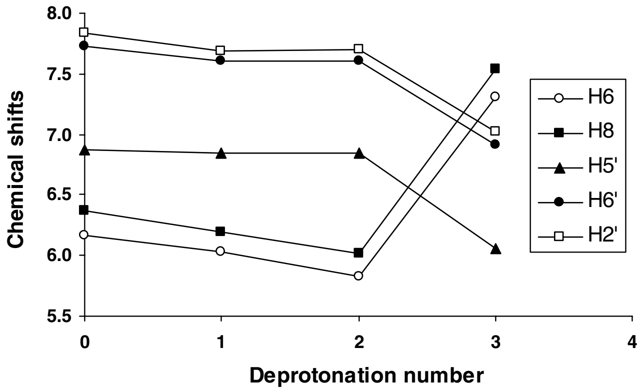

Figure 2. Chemical shifts as function of deprotonation number. Chemical shifts of each of the hydrogens of the quercetin as function of the deprotonation number.

This study concludes that the $pK_{a1} = 7.7$ could be attributed to the hydroxyl on the 3-position, the $pK_{a2} = 8.77$ to the hydroxyl on the 7-position instead of the 4′-position as described previously.[12–14] Finally, the $pK_{a3} = 9.8$ corresponded to the hydroxyl in 4′-position instead of the 7-position.

3.3. Hydroxyl radical scavenging as a function of pH

The hydroxyl radical scavenging activities of the different quercetin salts obtained at different pH, were proportional of the $A°/A$ as described in the above-mentioned Eq. 2. The variation of the $A°/A$ as function of the pH of salt preparation was shown in the Figure 3. We noted that between pH 9.0 and 9.6 the hydroxyl radical scavenging activity increased.

Then, at $pH > 10.0$, the hydroxyl radical scavenging activity did not show variation. It was very important to note that the buffer decreased rapidly the pH to 7.4 but maintained the salt forms unmodified during the reaction time. Evidence that the physicochemical structure of salt forms was kept unaltered was provided by the variation of the hydroxyl radical scavenging activity at this critical pH.

Table 1. Sodium content of each salt of quercetin

Compounds	Compounds concentration (mg/L)	Sodium concentration calculated(mg/L)	Sodium concentration measured (mg/L)
Mono-salt (pH 7.7)	29.40 ± 0.20	2.08 ± 0.01	2.07 ± 0.02
Di-salt (pH 8.8)	16.03 ± 0.10	2.15 ± 0.01	2.16 ± 0.01
Tri-salt (pH 9.8)	11.17 ± 0.13	2.17 ± 0.02	2.25 ± 0.10

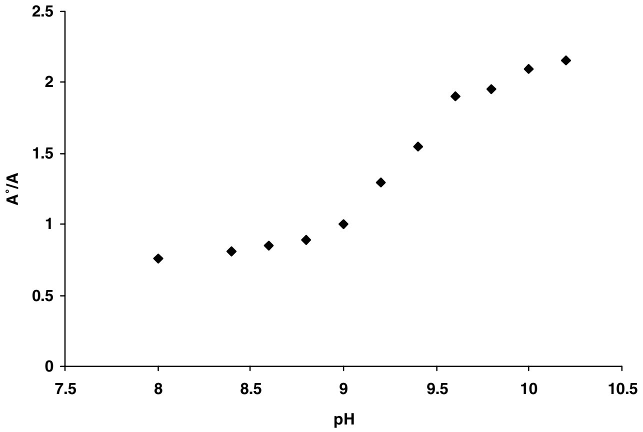

Figure 3. Hydroxyl radical scavenging as function of pH. Hydroxyl radical scavenging activity, represented by the ratio of the absorbances in absence and presence of the scavenger substance, as function of the pH of the salt solution and not the pH of the mixture Fenton reaction, which is fixed at 7.4.

Our results showed that the quercetin di-salt manifested the most efficient hydroxyl radical scavenging properties at pH 9.6 giving to this derivative the most interesting biological potential. It was also concluded that the quercetin di-salt was characterized by the higher solubility in the reaction mixture.

In alkaline solution, the quercetin reached its higher free hydroxyl radical scavenging activity after two deprotonation steps in 3- and 7-positions, as demonstrated previously. The isolated quercetin sodium di-salt has been shown stabile during 1 h at pH 2.3 and six weeks for pHs situated in-between 6 and 8 (*data in process*). For a future oral application of this molecule, it will be recommended to protect it by coating the final dosage forms (tablets, capsules) from the gastric juice using gastro-resistant coating, allowing the content to reach unaltered the less aggressive intestinal compartment. For topical applications, there is no problem to formulate it into cream or gel since skin pH does not fall below pH 5.

In the quercetin di-salt, the catechol ring is kept unmodified where the most adsorbing radical site is located. This is supported by Movileanu et al. studies that highlighted the importance of this group.[17] Indeed, the quercetin in alkaline solution (pH 8) showed an interaction with planar lipid bilayers.[17] In our investigation, we demonstrated that the most polar sites of the quercetin at pH 8 are in 3- and 7-positions. Moreover, the deprotonation of the hydroxyl in 4′-position requires a pH > 9.8 as demonstrated in our study, explaining why the most antioxidant active site is not modified. For a topical application, this interaction will be suitable because the drug efficiency does not require blood crossing. In the case of oral administration, we have shown that the intestinal absorption of the quercetin di-salt is achieved through the mediation of specific active transport systems (*data in process*).

3.4. Hydroxyl radical scavenging activity of quercetin di-salt of sodium

The high hydroxyl radical scavenging activity of the quercetin di-salt was substantiated by the high constant value obtained in the Fenton reaction system: $K_s = (1.4 \pm 0.1) \times 10^{10}\,M^{-1}\,s^{-1}$ (Figs. 4–6). K_x the constant dependent on the experimental conditions was evaluated in our case at $4.5 \times 10^9\,M^{-1}\,s^{-1}$. This constant value could be considered to be very high when compared to that recorded for troxerutin reaching the highest level in the same Fenton reaction system.[18]

3.5. Physicochemical parameters and hydroxyl radical scavenging activity relationships

The first studied parameters were the pK_a and the salt formation effect on the hydroxyl radical scavenging activity. For this purpose, experiments were carried out

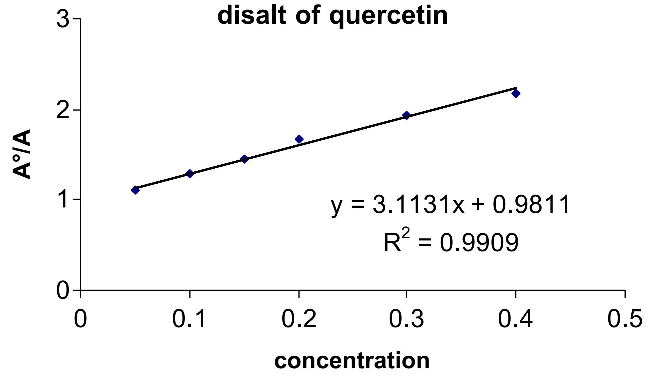

Figure 4. Experimentation of hydroxyl radical scavenging activity of the quercetin di-salt. Hydroxyl radical scavenging constant ($K_s = 1.4 \times 10^{10}$). This constant was obtained by Eq. 3: $K_s = a(K_D \times [D] + K_x)$. '*a*' is the slope obtained by the plot of the ratio of the absorbances in absence and presence of the di-salt of quercetin as function of the concentrations of this salt.

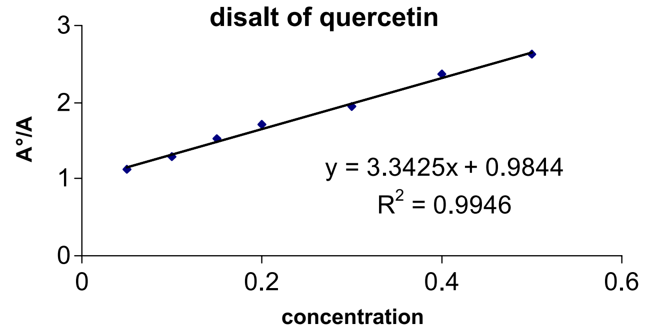

Figure 5. Experimentation of hydroxyl radical scavenging activity of the quercetin di-salt. Hydroxyl radical scavenging constant ($K_s = 1.5 \times 10^{10}$). This constant was obtained by Eq. 3: $K_s = a(K_D \times [D] + K_x)$. '*a*' is the slope obtained by the plot of the ratio of the absorbances in absence and presence of the di-salt of quercetin as function of the concentrations of this salt.

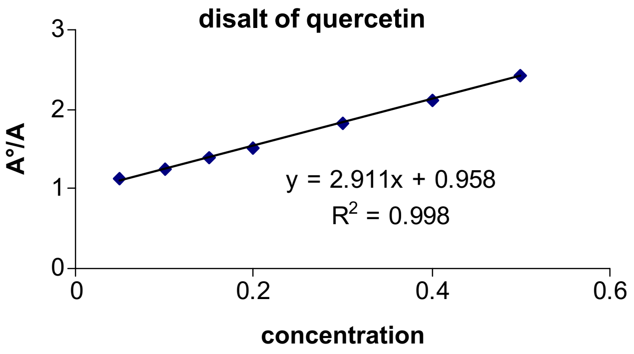

Figure 6. Experimentation of hydroxyl radical scavenging activity of the quercetin di-salt. Hydroxyl radical scavenging constant ($K_s = 1.3 \times 10^{10}$). This constant was obtained by Eq. 3: $K_s = a(K_D \times [D] + K_x)$. '$a$' is the slope obtained by the plot of the ratio of the absorbances in absence and presence of the di-salt of quercetin as function of the concentrations of this salt.

using a series of mono-hydroxylated molecules characterized by different pK_a values. Results reported in Figure 7 showed that there is a negative correlation between scavenging activity and pK_a values situated above 7.6 but that both parameters exhibit parallel declines below this breaking point.

Indeed, in Figure 8, we noted that for all the mono-hydroxylated molecules studied, the salt formation did not have any effect on the scavenging activity except in the case of 5-chlorosalicylaldehyde (pK_a = 7.6) whose salt manifested an activity twofold more important than that of the acid form. We could therefore conclude that the formation of the salt did not reduce the hydroxyl radical scavenging activity.

A second study was performed to evaluate the effect of the presence and the position of another hydroxyl group in the phenol ring.

In Figure 9, compounds with similar pK_a and with two hydroxyl groups on the phenol ring in the *ortho*-, *meta*- and *para*-positions were reported. The results demonstrated that on the one hand, the presence of the second hydroxyl in the *ortho*-position increased the scavenging activity comparison to the molecule with only one hydroxyl group. On the other hand, the presence of the hydroxyl groups in *para*- or *meta*-positions decreased the scavenging activity at half.

The third study of the physicochemical parameters and hydroxyl radical scavenging activity relationships was focused on the effect of the acid function. Figure 10 showed that this function had a negative effect on the scavenging activity in the presence of one or two hydroxyl groups.

Figure 11 showed that whatever the substitution (except for the acid substitution) responsible of the pK_a of the hydroxyl in the catechol function, the scavenging activity kept the same value for the same pK_a and it increased threefold if the pK_a decreased from 9.68 to 7.5.

In conclusion, the parameters shown to be considered in the molecules selection for the best hydroxyl radical scavenging activity are as followed: the pK_a of the hydroxyl group, the number and the position of the other hydroxyl groups and finally the presence of the acid function.

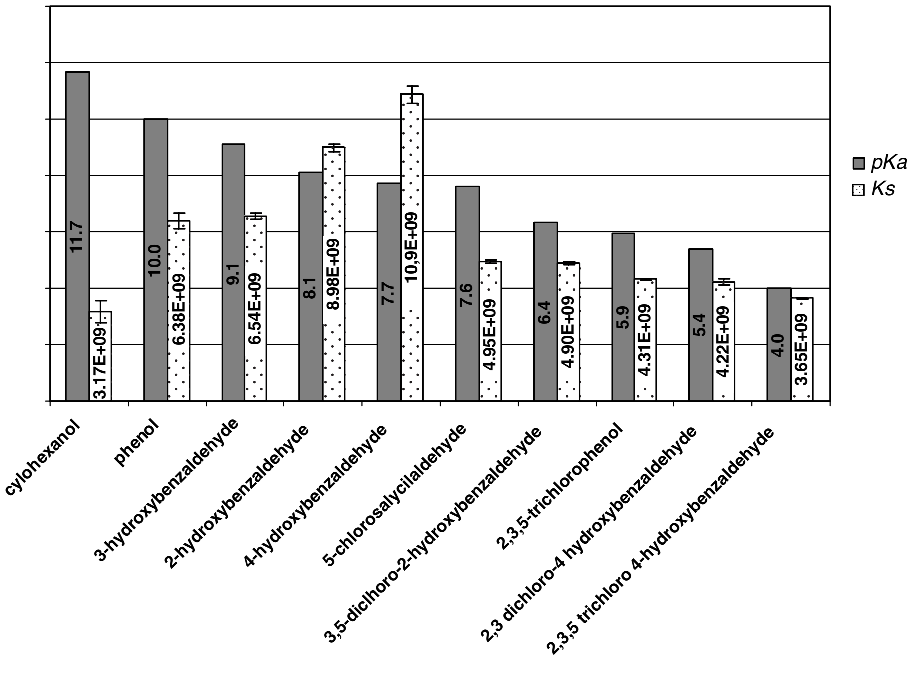

Figure 7. pK_a and K_s average relationship of different mono-hydroxylated molecules. Comparison of the pK_a (acid constant) and the K_s (hydroxyl radical scavenging constant) of different substances in order to evaluate the effect of the pK_a on the scavenging activities.

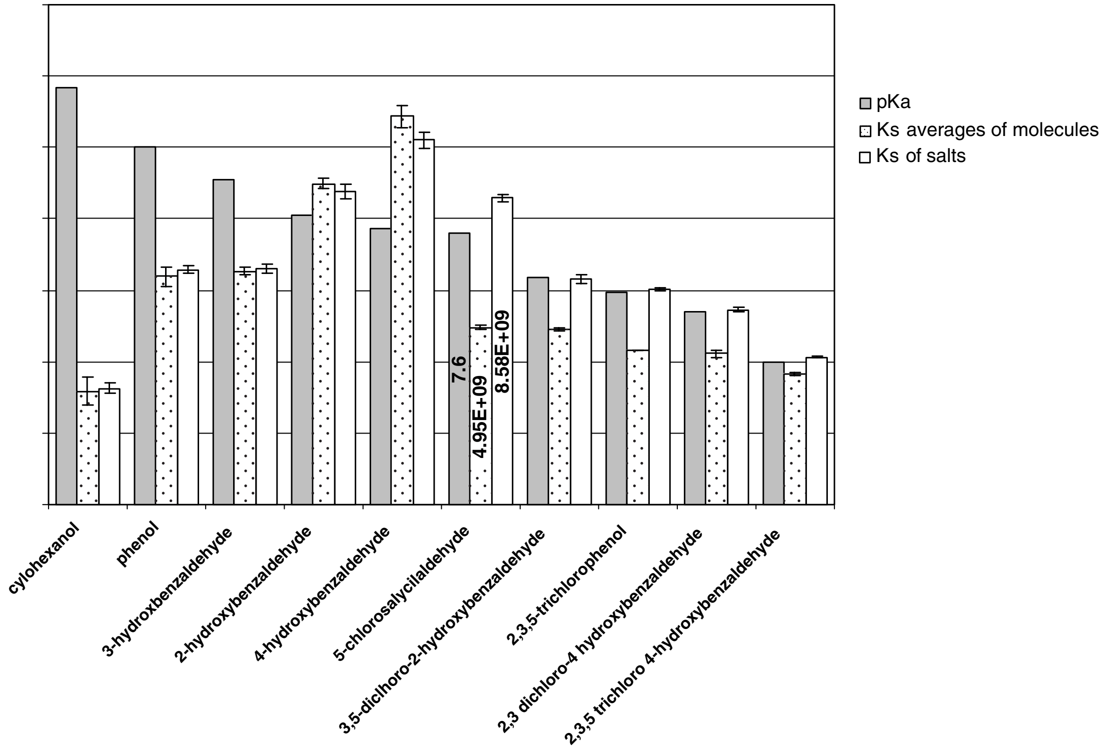

Figure 8. Effect of deprotonation on the scavenging activity as function of pK_a. Comparison of the K_s (hydroxyl radical scavenging constant) of different substances and the K_s of their corresponding salts in order to evaluate the effect of the salification on the scavenging activities.

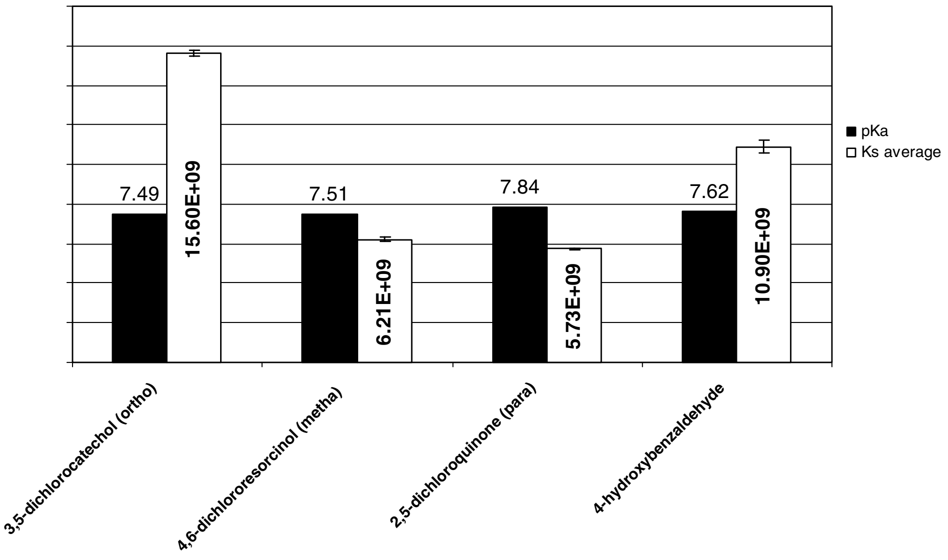

Figure 9. Effect of the hydroxide presence and position on the scavenging activity. Comparison of the K_s (hydroxyl radical scavenging constant) of a phenol substance with one hydroxide and the K_s of four others with two hydroxides in order to evaluate the effect of the position of the second hydroxide on the scavenging activity.

4. Conclusion

Our physicochemical studies have demonstrated that the two hydroxyl groups in 3′- and 4′-positions participating to the catechol group of quercetin are essential to ensure high radical scavenging activities.

The Na emission spectrophotometry and the H^1 NMR studies of the different quercetin salts point to a mixture of mono-, di- and tri-salts of quercetin. The quercetin 3,7-di-salt was the most active derivative as showed in Figure 3 studying the correlation between the pH of quercetin solution and the hydroxyl radical scavenging activity. The quercetin 3,7-di-salt appeared as a good hydroxyl radical scavenger because of its high solubility in aqueous solution and also of its capacity to neutralize the pro-oxidant activities of quercetin while keeping unmodified the antioxidant activities due to the catechol ring.

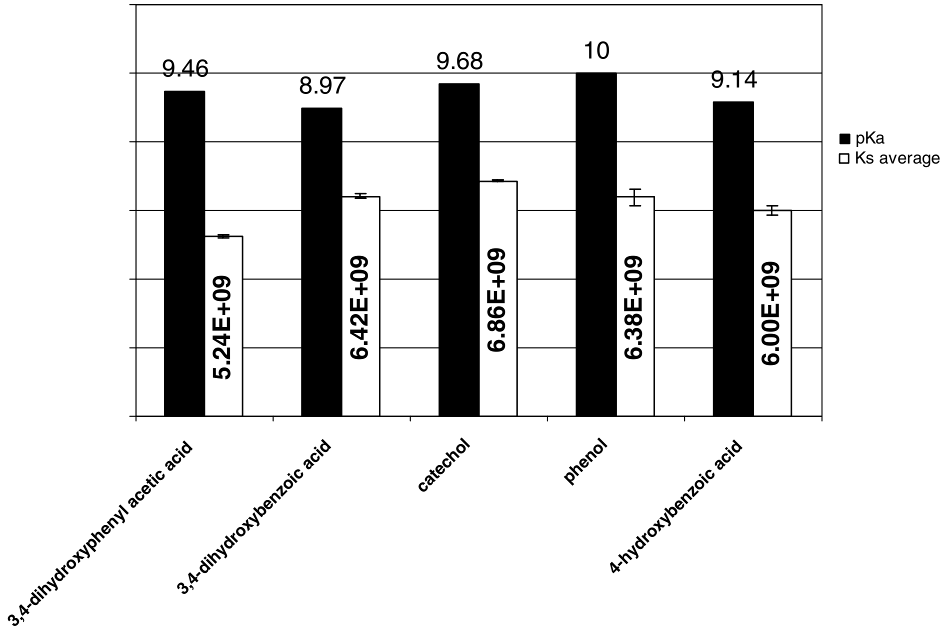

Figure 10. Effect of the acidic function on the scavenging activity. Comparison of the K_s (hydroxyl radical scavenging constant) of a phenol and catechol substances and the K_s of the same groups with an acid group in order to evaluate the effect of the acid group.

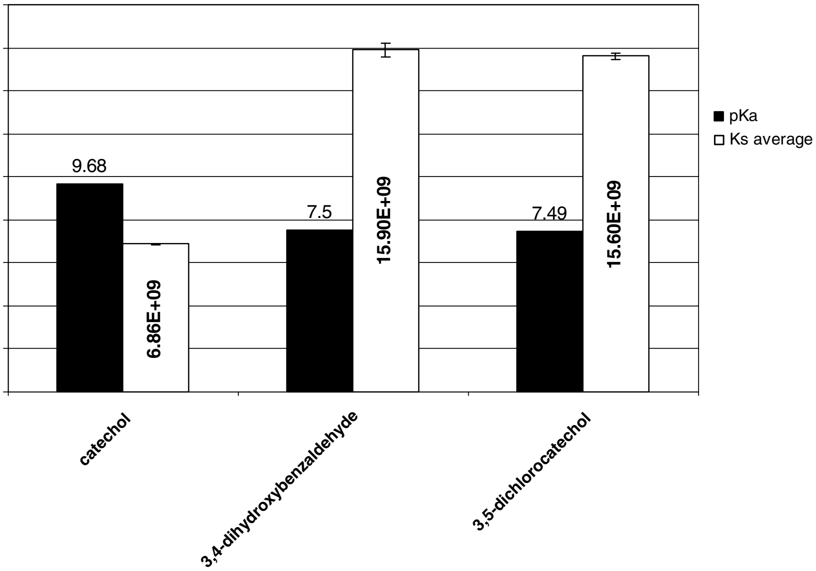

Figure 11. Effect of the pK_a on the catechol function in the scavenging activity. Comparison of the K_s (hydroxyl radical scavenging constant) of the catechol substances and the pK_a in order to evaluate the effect of the pK_a on the scavenging activity in presence of the catechol.

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