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Sugar matters: sugar moieties as reactivity-tuning factors in quercetin O-glycosides

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Abstract

Quercetin, one of the most abundant flavonoids in plant-based foods, commonly occurs in nature in various glycosylated forms. There is still a less explored aspect regarding the cause of its glycosides diversity, depending on the sugars moiety attached. This work focuses on four wide-spread quercetin glycosides—hyperoside, isoquercitrin, quercitrin and rutin—by testing property-tuning capacity of different sugar moieties and thus explain and predict some of their functions in plant-based food. Electron paramagnetic spectra of the semiquinone anion radicals of these glycosides were interpreted in terms of hyperfine coupling constants and linewidths, highlighting a clear link between spin density trends, the identity of the bound sugar, as well as their reactivity corroborated with their modelled structures. Redox potential and lipophilicity were connected to a specific flavonoid-enzyme interaction and correlated with their prooxidant reactivity assessed by oxidation of ferrous hemoglobin. Hyperoside and isoquercitin—galactose and glucose glycosides—exhibit the highest prooxidant reactivity owing to their lowest redox potential and lipophilicity whereas rutin and quercitrin—rutinose and rhamnose glycosides—behave *vice versa*. The ability of the tested glycosides to undergo HAT or SET-type reactions has also been tested using five different analytical assays, including inhibition of cytochrome *c*-triggered liposome peroxidation. In most cases, rutin proved to be the most unreactive of all four tested glycosides considering either steric or redox reasons whereas the reactivity hierarchy of the other three glycosides were rather assay dependent.

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Introduction

It is a fact nowadays that plant secondary metabolites are responsible for plants high adaptation to their environment and stress resistance, as well as for their nutritional properties for humans[1]. One class of such metabolites is that of flavonoids—plant polyphenols and most relevant antioxidants in human diet[2]. Flavonols—most important and widespread class of flavonoids—include, among others, quercetin, kaempferol and myricetin. Following their biosynthesis which occurs via phenylpropanoid pathway from phenylalanine[3], by action of various UDP-glycosyltransferases[4], flavonols aglycone can be converted to their glycosylated form, either O- or C-glycosides.

Flavonols effect on human health has been extensively studied. Their bioavailability is connected to their absorption in the small intestine[5]. Studies have shown that the absorption of glycosides is much higher than that of the corresponding aglycones[6–8]. Thus, the sugar moiety identity is important because it modulates the specificity of β-glycosidase mediated hydrolysis[9]; and hydrophobicity of the molecule itself. In fact, it has been shown that the greater the glycosylation degree, the better and faster the absorption[10]. Some clinical studies have highlighted the fact that the sugar moiety is cleaved in the small intestine before the glycoside is released into the bloodstream[6,11]. Others have proven that glycosides are transported through the brush border membrane by the SGLT1 glucose carrier[12]. Quercetin glycosides have also been detected in blood plasma[13], which indicates that they can be absorbed in the small intestine. A recent study has highlighted the opposite effects of quercetin and its glycosides in interaction with human α7 nicotinic acetylcholine receptor: quercetin enhances the inward current of the receptor, whereas its glycosides inhibit it, due to the presence of the sugar moiety[14]. This study has also emphasized that sugar moiety may confer a degree of specificity with regard to the receptor inhibition.

Health benefits bequeathed by flavonoids are nowadays more and more familiar to consumers[15–17]. In fact, the most accessible and popular sources of quercetin and especially its glycosides are fruits, vegetables and tea, onions, apples, chili pepper and lettuce among[18]. Moreover, a study[19] reports an increase in plant food supplements among various age groups in Europe, noting that the most popular ones are based on *Ginkgo biloba* (ginkgo), *Oenothera biennis* (evening primrose) and *Cynara scolymus* (artichoke)—all three rich in quercetin and its glycosides. Even though quercetin supplement consumption is on the rise, the uptake is twice

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higher when absorbed as isoquercitrin from onions[7]. Moreover, diets rich in fruits and vegetables are regarded as healthy and recommended worldwide[20], small antioxidants such as flavonoids being appreciated lately. Considering this and the high diversity of glycosides from natural sources, a clear methodology is needed in order to determine the molecular properties endowed by the sugar moiety and to predict their impact on health.

Besides being highly appreciated in fruits and vegetables, quercetin glycosides are now considered promising prodrug candidates as well due to their observed activities and hence the legitimate question: does the identity of the sugar moiety play a role or is it mainly about the aglycone? What impact on nutrition and health can these glycosides have based on their structure? It is interesting to add that frequently, a plant species produce various glycosides for the same aglycone[21,22], leading to another justified question: is there a physiological reason why the plant acts in this manner? Given all these important implications, glycosides appear not just merely forms of aglycone transport and storage, but important players in modulating absorption, antioxidant activity and biomolecule interaction, with considerable influences on nutrition and health. Therefore, the identity of the sugar moiety might play a more important role than expected. Regarding all these, the present work aims at comparing one of the most abundant and notable flavonol aglycone—quercetin—to its main glycosides- the 3-O ones (Figure 1). This comprehensive systematic approach explores their intrinsic redox reactivities and hence shed light on how the identity of the sugar moiety might tune the biological activity of the glycosides.

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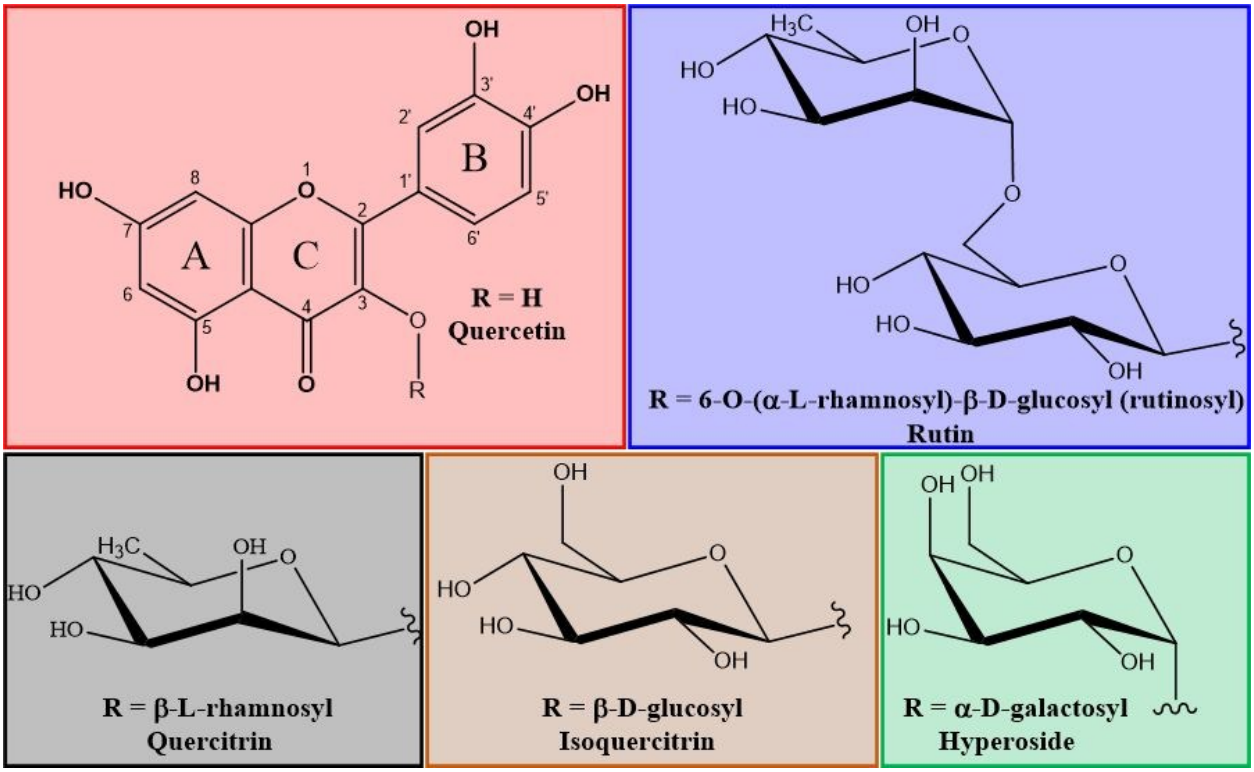

Figure 1. *Chemical structure of the quercetin aglycone and its glycosides studied in this work. For clarity, the color code is kept throughout the text and figures.*

Materials and methods

All compounds used had analytical grade purity and were purchased from Sigma-Aldrich. All stock solutions were prepared in HPLC grade methanol, purchased from Merck.

Electron paramagnetic spectroscopy measurements. Each compound (quercetin – Quer, rutin – Rut, quercitrin – Qrt, hyperoside – Hyp and isoquercitrin – Isq) was dissolved in methanol at 3.1 mM. From this stock solution, an aliquot of 16.2 µL was mixed with 28.8 µL of methanol-water solvent. The semiquinone anion radical formation was then triggered by addition of 5 µL of 0.5 M NaOH methanol solution upon vigorous mixing[23], aerobically, and the solution was rapidly transferred to a glass capillary and placed in the iris of the Bruker EPR spectrometer. The final content of methanol varied from 100% to 40%. Anaerobic trials were also tested—all reagent and vials were purged with argon prior mixing. The measurements were performed on an X-band EPR spectrometer with parameters as follows: field center of 3514 G, 40 G sweep width, modulation amplitude of 0.5 G and 40 ms conversion time. All spectra were recorded in 2024 points. Each signal was measured six times, in triplicates. Spectra simulation was performed

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using WinSim software developed by Duling[24]. Before simulation, all spectra were resampled to 1024 points by applying the interpolation function from XEPR software.

Spectrophotometric pKa determination[25]. Britton-Robinson universal buffer was used for these UV-Vis titration experiments. The universal buffer consists of 0.04 M H_3BO_3, 0.04 M acetic acid, 0.04 M H_3PO_4 and was prepared and adjusted to corresponding pHs, varying from 2 to 13 with a step of 0.5 units. A volume of 1000 µL of each buffer had been purged for 3 minutes with argon then an aliquot of 15 µL of each compound from 2 mg/mL stock solution was added. The UV-Vis molecular absorption spectra were recorded in 190 to 800 nm region. The pKas were determined at different wavelength values and reported for each compound.

Molecular modelling. The equilibrium geometries, normal mode vibrational frequencies and thermodynamic potentials for neutral and deprotonated species were computed using the M06-2X[26] exchange-correlation DFT functional considering the Def2-TZVP[27] basis set as is implemented in the GAUSSIAN09[28] program package. The theoretical pKa values were obtained considering the Born-Haber thermodynamic cycle based on the hydronium ion[29] and the solvation effects were taken into account using the CPCM[30] **C**ontinuum **P**olarizable **C**onductor **M**odel. The theoretical g-tensors and hyperfine couplings were calculated using the EPR/NMR module implemented in the ORCA[31,32] program package considering the B3LYP[33] hybrid DFT functional and the Def2-TZVPP[27] as well as the auxiliary Def2/JK[34] basis sets.

Lipophilicity determination via HPLC. Methanol and all buffering reagents (glacial acetic acid, 25% ammonia solution, ammonium acetate) of HPLC grade were used throughout chromatographic measurements of lipophilic properties. Uracil was used as an unretained compound to determine HPLC column dead time. For each flavonol, the test solution was prepared by dilution of stock solution to obtain a concentration of 1 mM. For HPLC mobile phases, water and methanol both with the addition of buffer to obtain suitable pH have been used. Buffer solutions of pH 7.4 and higher were made by dissolving 0.05 mol of ammonium acetate in 1 L of phase adjusted to a given pH using ammonia solution, while buffer with a pH 4.5 were prepared by acidifying 50 mM ammonium acetate of mobile phase using acetic acid. Chromatographic analyses were carried out on an Agilent 1100 Series HPLC-DAD system under conditions previously described[35]. The chromatograms were recorded at 280 and 360 nm. The retention times of each compound were measured under isocratic elution mode using a mixture of methanol and aqueous buffer in different proportions as a mobile phase. The HPLC runs for

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minimum five different mobile phase compositions with 5% increment of methanol were performed in duplicate. These measurements were carried out at 37°C under different pH conditions (4.5, 7.4, 8.5 and 10.0). The obtained retention data were used to calculate parameters of linear solvent strength model in which the intercept denoted as $\log k_w$ is the main lipophilicity descriptor corresponding to 0% organic modifier. Finally, the $\log k_w$ value was converted to distribution coefficient ($\log D$) taking into account the column phase ratio ($\varnothing$) as has been reported previously[35]. The phase ratio of HPLC column was determined for each pH according to Moldoveanu et al.[36] and the obtained values were: $\log\varnothing_{4.5}$ = -0.2964, $\log\varnothing_{7.4}$ = -0.3093, $\log\varnothing_{8.5}$ = -0.2958, $\log\varnothing_{10.0}$ = -0.2651.

Complexation with $AlCl_3$[37]. For this, an aliquot of 435 µL deionized water was mixed thoroughly with 15 µL 310 µM solution of each compound, 150 µL of a 2% $AlCl_3$ and 150 µL of 1M solution of sodium acetate. This mixture was incubated for 10 minutes at room temperature. Finally, a 150 µL aliquot of 0.1 M NaOH solution was added upon vigorous mixing, followed by 40 minutes incubation at room temperature. Afterwards, the spectra were recorded for each compound between 300 and 700 nm. All measurements were performed in duplicate.

Prooxidant and laccase enzymatic activity. Redox potential determination. The protocol for prooxidant activity and mechanism was previously described in detail[38]. Briefly, a solution of 15 µM freshly-prepared oxy-hemoglobin via dithionite reduction followed by desalting-column treatment was prepared in a sodium acetate buffer with a pH of 5.5. An aliquot of 965 µL from this solution was mixed with 20 µL of each compound from a 310 µM stock solution. The process started upon addition of 15 µL of *S. sclerotiorum* laccase from a 3 µM stock. The reaction has been monitored kinetically at 575 nm for 20 min. All measurements were performed in duplicate. The turnover of laccase with all studied compounds was also determined as previously described. The redox potentials of the tested compounds were determine via cyclic voltammetry, in PBS buffer using Ag/AgCl, KCl(1M) as reference electrode, according to our previous studies[38].

Antioxidant capacity determination assays. Five distinct assays—based on different mechanisms, in different conditions—were used in order to evaluate the antioxidant capacity of the studied compounds. ***1. Inhibition of induced peroxidation of liposomes assay***[39] – Liposomes were obtained by 20 minutes sonication of 0.2 mg/mL soy lecithin in PBS buffer (pH of 7.4). An aliquot of 300 µL of such homogenous liposome solution was mixed thoroughly with specific

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small volumes of each tested compound so that the final concentration of each compound varied from 5 to 120 nM. The reaction was triggered by addition of cytochrome *c* solution to a final concentration of 2 μM and recorded on kinetic mode overnight at 235 nm using a microplate reader (Tecan spectrophotometer). All measurements were performed in triplicates. ***2. DPPH bleaching assay***[40] – a stock of DPPH was prepared in methanol with a concentration of 1 mM. In order to perform the assay, 45 μL of this DPPH solution were pipetted in a cuvette along with a mixture of methanol-water and 15 μM of each tested compound to a final volume of 1000 μL. The mixtures of methanol-water had a percentage of methanol varying from 40 to 100% from ten to ten units. After the addition of the compound, the reaction mixture was vigorously shaken and incubated for 20 minutes. Afterwards, the absorbance was measured at 517 nm. All measurements have been performed in duplicates. The final results were given as bleaching percentages and were calculated as follows: $(A_0-A_1)*100/A_0$, where A_0 is the initial absorbance and A_1 is the final value, respectively. ***3. ABTS radical bleaching assay***[40] – an ABTS radical stock solution was prepared by the enzymatic way, in the presence of zucchini peroxidase and hydrogen peroxide in a pH 5.5 acetate buffering system. A volume of 80 μL of this solution was mixed with a corresponding volume of a mixture of methanol-water with percentages of methanol equal to 92 and varying from 40 to 90% from ten to ten units. Tested compounds were added to a final concentration of 5 μM and the mixture was incubated at room temperature for 20 minutes. The absorbance was then measured at 730 nm. All measurements have been performed in duplicates. The final results were given as bleaching percentages and were calculated as follows: $(A_0-A_1)*100/A_0$, where A_0 is the initial absorbance and A_1 is the final value, respectively. ***4. Cupric ion reducing antioxidant capacity (CUPRAC) method***[41] – a solution of bis(neocuproine)Cu(II) was prepared by mixing 2 mM neocuproine with 1 mM pentahydrated $CuSO_4$ in 1M ammonium acetate buffer of pH 7 and incubating the reagent for 10 minutes. 600 μL of this solution were mixed with 300 μL deionized water in a cuvette along with volumes of each compound. The final concentrations of each compound varied from 1.5 to 30 μM. The absorbance was measured at 450 nm and all measurements have been performed in duplicates. ***5. Oxygen radical absorption capacity (ORAC) method***[40] – a 21.6 nM solution of fluorescein was prepared in PBS buffer, pH of 7.4. A second solution was made by dissolving AAPH (short for 2,2'-azino-bis-(2-amidino-propane)) in PBS buffer, pH of 7.4, to a final concentration of 530 mM. In a 3 mL cuvette 2.7 mL of fluorescein solution were added along with a volume of

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compound originally in a methanol solution. This mixture was incubated at 37°C for 3 minutes, followed by the addition of 300 µL AAPH solution upon vigorous mixing. The final concentration of tested compound was 1 µM for each compound tested. The reaction progress was observed by means of fluorescence spectroscopy on kinetic mode at the constant temperature of 37°C. The excitation wavelength used was 485 nm, while the emission was recorded at 315 nm. All measurements have been performed in duplicates.

Results and discussion

EPR spectroscopy assessment and pKa determination

Polyphenolics free radicals, generated in various ways, have been extensively studied—including EPR spectroscopy[42–46]. Theoretical calculations have been previously employed on quercetin and related flavonols, revealing that the hydroxyl groups of ring B and C are the sites involved in the process[47], their interplay being important in determining the radical reactivity. Herein, semiquinone anion radicals of quercetin and its glycosides have been produced in alkaline medium in the presence of molecular oxygen, generating superoxide anion radical as well[42,43,46]. All free radicals generated reach the highest spin concentration in 10 minutes, after that, their signal starts to decay in either one hour (for quercetin), or longer (for all glycosides). Representative EPR spectra of all five studied compounds—measured in various methanol/water ratios—are shown in Figure 2 and in greater details in Figures S1 and S2. The higher the methanol content, the higher the radical stability for quercetin, as its semiquinone is barely detectable at 40% methanol and completely undetectable bellow. The glycosides radical stability is influenced *vice versa* by methanol content. It does not come as a surprise that quercetin spectrum is different from its glycosides, showing that the attached sugar moiety strongly influences the structure and thus the reactivity of the radical. Quercetin EPR profile is characterized by six lines with intensities highly dependent upon the methanol content. The number of arising EPR lines is given by the three protons in the B ring that can be magnetically coupled with the free electron. Quercetin owns five hydroxyl groups, even six if the keto tautomerism is considered[48].

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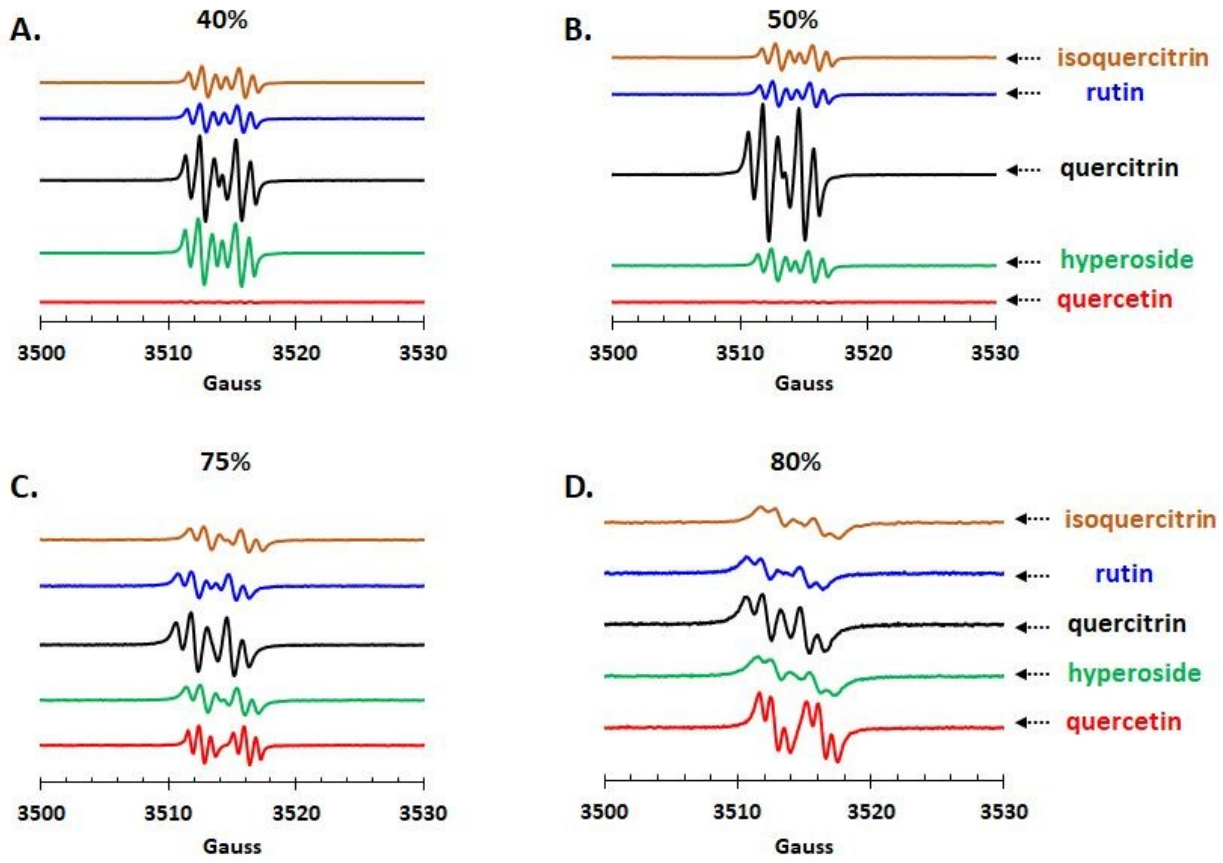

Figure 2. *X-band EPR spectra of the semiquinone anion radicals of the quercetin and its studied glycosides (1 mM) in water-methanol solution, recorded at several methanol percentages:* ***A****. 40%,* ***B****. 50%,* ***C****. 75% and* ***D****. 80%, at room temperature, 10 mW power.*

Previous studies have agreed that the most stable radical would be the one formed at 4' position, the loss of an electron occurring after a deprotonation step[47,49]. A preceding theoretical study has explored quercetin radicals from the perspective of a double deprotonation process[47] with the result of an anionic radical, whereas another study has concluded that quercetin can form a double-anionic radical, with two deprotonated hydroxyl groups[46]. Since our working conditions are strongly alkaline (pH 12), in order to know what species dominates the process, pKa values have to be precisely determined. For this purpose, by using an anaerobic titration setup and by monitoring absorption molecular spectra, three well-defined pKas were determined—hence, four distinct species—for quercetin, whereas all glycosides displayed only two values, as shown in Figures S3 and S4.

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These findings did not come as a surprise, considering the fact that one hydroxyl group is blocked by glycosylation. The pKa1 and pKa3 values significantly decreases after glycosylation, highlighting that the effects of the sugar moiety upon acidity of the hydroxyl groups and important correlation with lipophilicity and redox potential (*vide infra*). The 10.41 pKa2 value (measurable only for quercetin), can be attributed to the 3-OH group, the group that is no longer present in glycosides. On the same note the pKa1 can be attributed to the 4'-OH whereas pKa3 for 3'-OH. Similar analysis of two quercetin homologous compounds, kaempferol, which lacks the 3'-OH group and luteolin, which lacks 3-OH group confirmed the correct assignment of the pKas (Figure S3). In addition, the 4' – 3 – 3' deprotonation order has been confirmed by theoretical calculations as well. Our approach was performed in two steps.

First, we calculate the deprotonation Gibbs free energies for each of the 3 positions, using the Born-Haber thermodynamic cycle based on the hydronium ion[29]. The obtained values were 26.44 kcal/mol for 4', 28.46 kcal/mol for 3 and 28.71 for 3' kcal/mol, respectively. The corresponding pKa values are: 5.40 for 4', 7.02 for 3 and 7.22 for 3', respectively. The smallest value was assigned to the lower pKa: pKa1 = 5.40.

In the second step, with quercetin deprotonated in the 4' position, the deprotonation Gibbs free energies for the other 2 position were calculated. The obtained values were 33.75 kcal/mol for 3 and 40.27 kcal/mol for 3', respectively. The corresponding pKa values are: 11.25 for 3 and 16.46 for 3', respectively. The smallest value was assigned to pKa2 = 11.25. Following the same procedure, the highest pKa value was to be calculated with the quercetin doubly deprotonated, from the deprotonation Gibbs free energies for the 3'position. Unfortunately, the lack of convergence in the geometry optimization with solvent model prevented us from obtaining credible numerical results for pKa3.

When it comes to the pKa values of hydroxyls in position 5 and 7, previous studies highlighted the difficulty of determining their characteristic; in most of the cases, their values are close to those of other hydroxyl groups on quercetin, thus they cannot be properly determined[49]. However, the protons in these groups are placed too far from the radical center as to influence the EPR signal and thus they are not of interest in this discussion. Therefore, considering that the working pH is well above the third pKa value of quercetin and its glycosides, the protons that can interact with the unpaired electron in in the generated radical are 6'-H_1, 5'-H_2 and 2'-H_3, all located on the B ring, as shown in Figure 3[44]. Simulations of all EPR signals have been

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performed by 3 inequivalent protons in order to determine the g value, the linewidth and the values for all three hyperfine coupling constants for the measured spectra. All simulated parameters are grouped in Table S1, in the supplementary material.

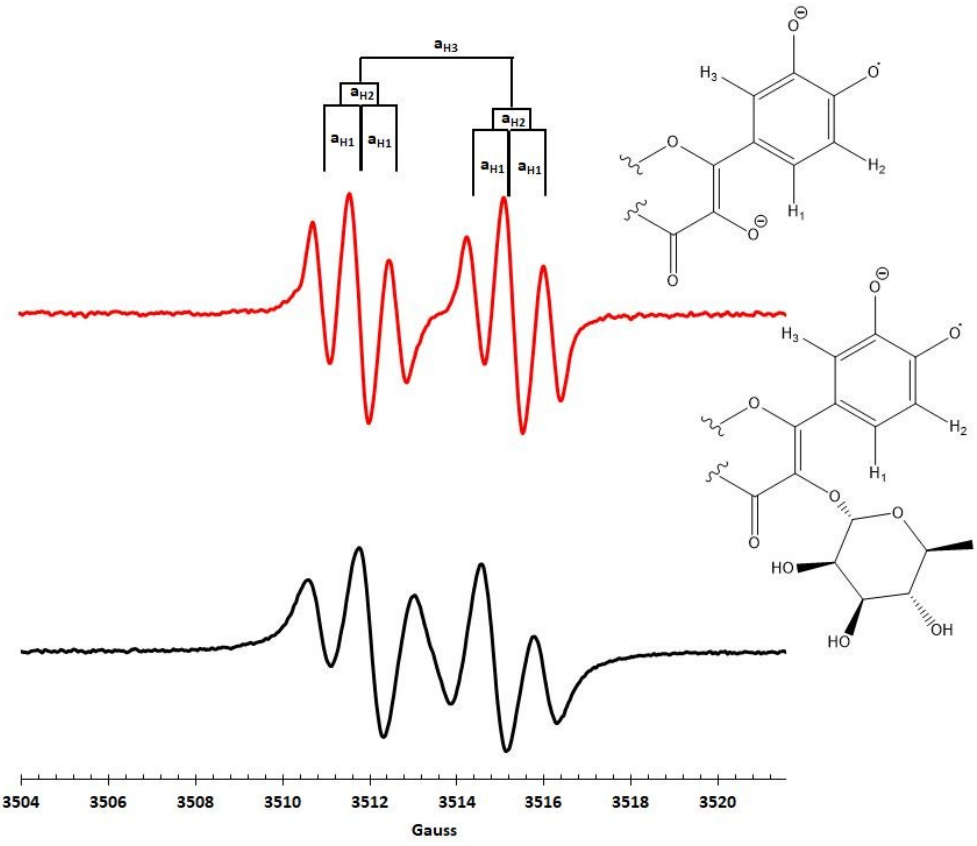

Figure 3. *Hyperfine splitting of two representative EPR spectra (quercetin – top and quercitrin – bottom) and their schematic structures with labelled hydrogen atoms involved in the splitting.*

The linewidth of an EPR spectrum is related to spin relaxation when the Boltzmann magnetization is regained. Generally speaking, this parameter is mainly influenced by the spin-spin relaxation at room temperature, since at this temperature dipole-dipole interaction is less important due to high translational movements. A previous study has highlighted that the linewidth is dependent on solvent viscosity due to modulation of diffusion rate: a less viscous environment gives rise to higher linewidths[50]. In the present study, the trends of linewidth evolution as function of methanol content is presented in Figure 4. There is a general trend among all generated radicals to exhibit higher values of linewidth as the percentage of methanol increases and the viscosity decreases[51]. Still, there are slight differences in the absolute values of

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the linewidths for the studied compounds, which seem to be correlated to the degree of lipophilicity (*vide infra*). Hyperoside and isoquercitrin, whose sugar moieties are enantiomers, have almost identical linewidth, whereas this parameter decreases from rutin to quercitrin, both of them having a deoxy moiety directly attached to the quercetin skeleton. In this sense, hydrogen bonding might also play its role in modulating relaxation times.

The simulated values for all three hyperfine coupling constants are given in Table S1, of the supplementary material. Generally speaking, it is known that the hyperfine coupling constants of protons are highly correlated to the spin density at the adjacent atom[52], in this case carbon atoms. Intramolecular hydrogen bonding[53,54] as well as local geometries are also determining the magnitude of this interaction. The evolution of hyperfine coupling constants is related to that of linewidth in a proportional manner if other factors do not come in play[50]. The variation of hyperfine coupling constants with the methanol content is presented in Figure 4. The second constant in terms of magnitude is the only one that varies according to the linewidth, whereas the other two show a reverse pattern.

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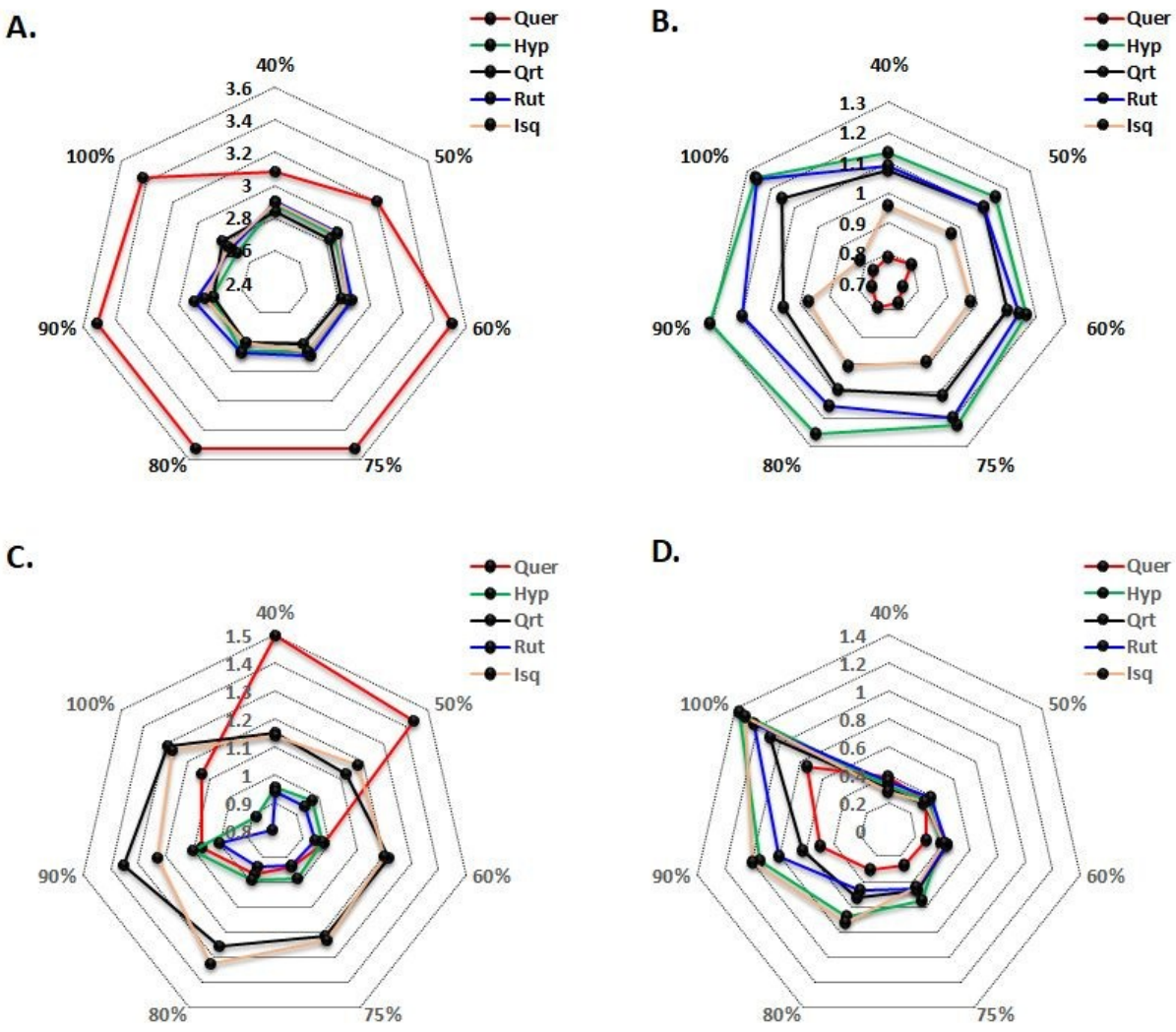

Figure 4. *Hyperfine coupling constants of the proton H1 (A), H2 (B), H3 (C) and linewidth (D) for quercetin and its glycosides as function of methanol content. The values are determined from the experimental spectra applying WinSim software. The detailed values are presented in Table S1.*

The assignment of hyperfine coupling constants to their corresponding proton on the catechol moiety was approached by molecular modelling. Considering the high pH required for the EPR assay, all calculated radicals were completely deprotonated (Figure S5). It is noteworthy that, at least to the authors' knowledge, no other previous study has explored the parameters of completely deprotonated flavonoids. As for quercetin semiquinone radical, the simulated hyperfine coupling constants are in good to very good correlation to the experimental ones. The

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highest coupling constant corresponds to proton H1 (Figure 3), in accordance with the fact that hydrogen bonding tends to increase spin density on protons. Another possible explanation consists of changes in spin density due to loss of planarity. The smallest constant is attributed to the proton H2, because it is the farthest from the coumarin unit and lacks the possibility of establishing hydrogen bonds. Moving to quercetin glycosides (structures in Figure S5), the presence of sugars clearly affects the structure, as the catechol ring will be twisted according to saccharide identity (Table S2). As a result of this torsion, all coupling constants are modified. The observed trend is most probably due to catechol protons interaction with the hydroxyl moieties in the sugar. A clear decrease can be seen in constant corresponding to H1, the decrease being directly attributable to the torsion angle in monosaccharides: the more tilted the catechol ring, the higher the coupling constant. Rutin, comprising a disaccharide unit, does not follow this trend.

When it comes to comparing the hyperfine constants of glycosides with each other, one can notice that the magnitude of those corresponding to H_2 and H_3 are not greatly affected by the sugar. On the other hand, the case of H_1 is slightly different with respect to each glycoside, especially to quercitrin: the hyperfine values determined are higher in this case, whereas the others display similar values. The part of the B ring containing H_1 comes closer to the sugar upon glycosylation, thus the polarity and the number of feasible hydrogen bonds is higher.

Redox potential, lipophilicity, enzyme interaction and prooxidant reactivity

As an intrinsic property of redox active compounds such as quercetin and its glycosides, the reduction potential plays an important role not only in modulation of antioxidant ability and cellular redox homeostasis, but it also dictates the interaction of the molecules with oxido-reductase enzymes. As known, the value of the reduction potential is strongly dependent upon pH in the case of flavonols, as deprotonation favors the reduction capacity[48]: the higher the pH, the better the electron donating abilities due to the lower value of the redox potential. Quercetin has been thoroughly studied electrochemically in order to determine the mechanism of its oxidation in various conditions[55,56]. It was established that its very good reduction ability is due to the structural elements in both B and C rings, namely the hydroxyl groups at positions 3-C, 3'-C and 4'-C and the double bond between 2-C and 3-C. It was highlighted that the B ring is the most active, being the first oxidized, followed by the 3-OH group. Previous studies have

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concluded that the glycosylation of 3-OH renders a greater potential value, such as for rutin and quercitrin[57,58]. As expected, quercetin is assigned the lowest potential value, rendering the best antioxidant abilities. What is remarkable is that there are significant differences in the potential value between the glycosides depending on the identity of the sugar. Clearly, there are two groups distinguishable by the redox potential: hyperoside and isoquercitrin have the lowest values, whereas quercitrin, followed closely by rutin, display the highest ones (Figure 5). There seems to be another intrinsic property that may come into play with the observed hierarchy: the degree of hydrophobicity or lipophilicity. At a molecular level, lipophilicity is an important property correlated with the number of interactions between the biologically active molecule and its target/receptor, ranging from hydrogen bonds to hydrophobic forces. Still, this property highly impacts the degree of membrane-passing capacity: if it is too elevated, the molecule will remain bound to the membrane, whereas a low lipophilicity would prevent the molecule from entering the cell. Lipophilicity is vital when discussing biologically active compounds because it can modulate absorption, distribution, metabolism and excretion (ADME) , as well as toxicity and efficiency of the molecules[59]. In the present work, lipophilicity has been determined for all five compounds at four different pHs. The complete profile of the analysis is presented in supplementary material. As can be observed in Figure 5 and Figure S6, there is a clear trend in lipophilicity variation for the studied compounds, except for the highest pH, where the change in hierarchy may be attributed to secondary processes due to quercetin increased reactivity. The values corresponding to each compound do not necessarily come as a surprise if the structure is considered: the presence of the sugar, no matter its nature, should render a higher number of sites able to establish hydrogen bonds and thus would increase the hydrophilicity of the entire glycoside. This property is clearly evidenced by quercetin which, at pHs lower than 10, has the highest determined lipophilicity. When it comes to comparing the derivatives property, the structure of sugar should be analyzed more closely: both isoquercitrin and hyperoside contain very similar sugar moieties, one of them being glucose and the other, its enantiomer, galactose. Both of them display a supplementary hydroxyl group compared to rhamnose, the sugar contained in quercitrin. With this in mind, the lipophilicity of quercitrin should be higher, due to its lower hydrogen bonding capacity. Rutin, on the other side, displays a rutinose moiety, formed by condensation of glucose and rhamnose. Even though the number of hydroxyl moieties is greater than in any other glycoside, its dimension might not be promoting a hydrophilic

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character. Indeed, quercitrin is described by the highest degree of lipophilicity, followed by rutin and then by both isoquercitrin and hyperoside.

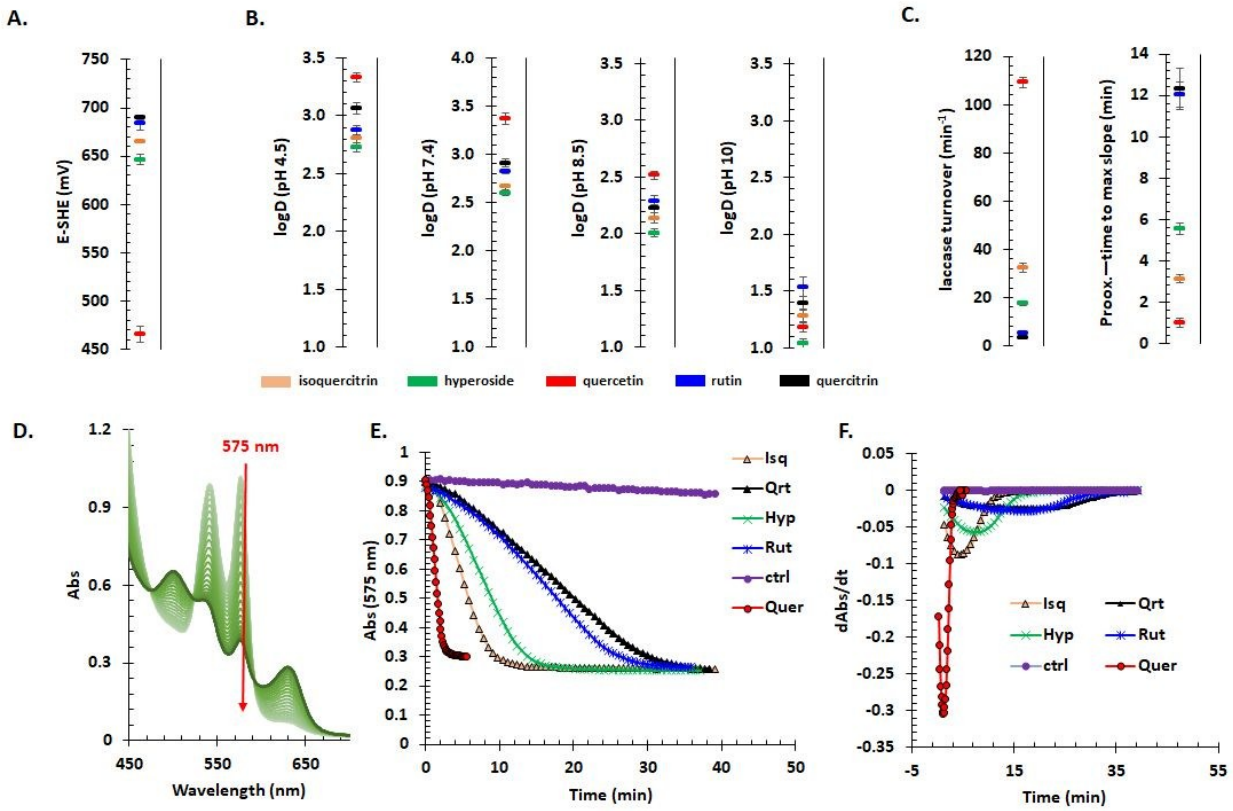

Figure 5. ***A.*** *Redox potential of the studied compounds, measured in PBS using cyclic voltammetry.* ***B****. Lipophilicity expressed as logD determined using reversed phase HPLC at different pH values.* ***C****. Laccase assay turnover and prooxidant parameter (time to maximum slope) as determined from the hemoglobin oxidation kinetic curves.* ***D****. Spectrophotometric hemoglobin oxidation (from ferrous to ferric form) in presence of laccase and quercetin glycosides.* ***E****. Kinetic profiles of hemoglobin oxidation in the presence of laccase and quercetin and its glycosides at 575 nm.* ***F****. Time derivative profiles of the 575 nm kinetic curves.*

There is a very good positive correlation between the value of the redox potential and that of the lipophilicity, as well as a negative correlation between these two properties and the EPR spectrum linewidth (*vide supra*). Structurally speaking, the fact that the B ring of the quercetin moiety is tilted with 38° off the rest of the molecule[60] may promote interactions between its features and the sugar contained in the glycoside, thus modifying the electron density distribution. The resulting properties changes, though not necessarily dramatic, can prove of high

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importance when it comes to interaction between these compounds and biological entities, such as enzymes.

Laccase is a copper containing oxido-reductase that is known for its high promiscuity towards numerous classes of plant secondary metabolites, including flavonoids. Its mechanism of action involves one-electron oxidation which can be followed by other reactions[38]. It has been previously shown that flavonoids are very good laccase substrates due to the high number of hydroxyl groups which allows a high binding affinity at the active site[61]. The binding is mediated mainly through hydrogen bonds with histidine, glutamate and aspartate[62]. In the present study, the turnover number of laccase for all five compounds has been determined and summarized in Figure 5. When it comes to glycosides, the turnover number decreases and it seems to be correlated with the redox potential. Isoquercitrin and hyperoside are poorer laccase substrates compared to quercetin. Still, some steric factors might come into play as their sugars have different hydroxyls orientations. When it comes to rutin and quercitrin, their efficiency as substrates is closely linked to their potential, the characteristic laccase turnover number being two orders of magnitude smaller. Herein steric factors may also play an important role: quercitrin can form fewer hydrogen bonds and rutin being the structurally bulkiest glycoside, has a reduced ability to properly interact with active site residues.

Overall, the sugar moiety is not only tuning the molecule properties (reduction potential and lipophilicity), it also plays a role in modulating affinity for the studied laccase. Quercetin and its glycosides not only interact with an enzyme as substrates, they can also play the role of inhibitors. Quercetin is known for its competitive inhibiting activity in some kinases involved in cancer[63], but its glycosides are also important players in deactivation of some enzymes. Generally speaking, quercetin glycosides are reported to interact with enzymes by hydrophobic (π-π stacking with aromatic amino acids) and van der Waals interactions (reported for Val, Leu and Ile) through the aglycone moiety, as well as hydrogen bonds through hydroxyl groups in the sugar, sometimes inducing conformational changes in the protein. Even though they are considered inhibitors with medium affinity and selectivity, there are examples of glycosides that act efficiently, mainly due to hydroxyl groups in key positions or due to size and conformation of the sugar—in fact, the efficiency can be reduced to the number of interactions that ensure the attachment in the binding pocket of the protein. Their structure is similar to that of ATP, making them good competitors for ATP-dependent enzymes binding sites. Common examples of

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competitive inhibition include quercetin 3-O-L-rhamnoside (quercitrin) for p90 S6 ribosomal kinase[64], quercetin-3-O-galactoside (hyperoside) for some isoforms of cytochrome P450[65] and quercetin 3-O-glucoside (isoquercitrin) for dipeptidyl peptidase-4 in type 2 diabete[66]. However, other types of inhibition can be seen, such as non-competitive type of quercitrin to sortase A[67] or uncompetitive of quercetin 3-O-rutinose (rutin) to DNA isomerase IV in some bacteria[68].,

Another interesting aspect of flavonoids redox behaviour is related to their ability to act as prooxidants in certain conditions. This course of action is of interest due to the fact that this capacity is involved in certain physiological processes. Herein, a prooxidant assay involving laccase and oxy-hemoglobin was performed[38]. Basically, laccase converts quercetin or its glycosides into free radicals by one-electron oxidation. The produced radical and/or the oxidized product will oxidize ferrous hemoglobin to its met form until all flavonoid is consumed. The faster the hemoglobin oxidation, the better the prooxidant activity. A kinetic profile corresponding to this process is presented in Figure 5. Taking into account this mechanism of prooxidant activity exploitation, it is important to note that the time required for oxy-hemoglobin transformation is linked to laccase turnover on that specific compound, as the generation of the free radical might be a rate limiting step. Quercetin exhibited the highest prooxidant activity, completely oxidizing hemoglobin in less than 2 minutes. When it comes to glycosides, their time for reaction completion was in good agreement with laccase turnover, quercitrin being the least prooxidant of them all.

Reducing power and free radical scavenging ability

There is strong evidence that another *in vivo* role of quercetin derivatives is to act as protective agents against UV-B light[69]. Experiments proved that this protecting role is not due to prevention of ROS formation, but to ROS inactivation through two main mechanisms: hydrogen atom transfer (HAT) or single electron transfer (SET)[70]. There are also hybrid mechanisms between these two: single electron transfer followed by proton transfer (SET-PT) and sequential proton loss electron transfer (SPLET). Upon UV-B stress, flavonols accumulate into cells vacuoles especially in leaves which contain high levels of quercetin glycosides. This location seems to be associated with chloroplast protection. Moreover, quercetin and its derivatives are capable of Fe^{2+} and Cu^{2+} complexation, thus preventing the formation of ROS through Fenton-like reactions[71].

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It has already been discussed that quercetin displays very good reductive properties due to its structural features: a planar molecule with extended π-delocalization. In a comparative theoretical study, quercetin antioxidant ability was attributed to some specific structural elements, especially the catechol moiety that is the B ring[47,48,72]. Although 3-OH is of importance in increasing the redox potency of quercetin, it needs to be turned on by the presence of the double bond. Upon 3-OH glycosylation, the resulting quercetin derivative loses the extended planarity between the catechol moiety and the rest of the molecule, planarity which ensures a higher degree of delocalization [48]. Furthermore, the fact that the 3-OH is not free anymore will decrease the antioxidant potency. A recent study has highlighted into more detail the effect of 3-O-glycosylation of quercetin: in both HAT and SET type reactions. 3-O-glycosides exhibit lower antioxidant abilities due to their higher O-H bond dissociation enthalpies and ionization potential compared to quercetin[73]. Moreover, from all possible quercetin-O-glycosides, 3-O-type exhibited the lowest energy of the highest occupied molecular orbital (HOMO), thus the weakest reactivity in oxidation reactions.

In general, a *plethora* of studies have been undergone to assess antioxidant activities of flavonols. Thus, *in vitro* assays can be easily performed in order to test the presumed ROS quenching activities of this class of compounds by various mechanisms[74]. Still, some voices claim that their *in vivo* antioxidant effect in humans is not so relevant, due to their low concentration in blood compared to other endogenous antioxidant molecules such as ascorbic acid. This skepticism has led to interesting discoveries that highlighted the involvement of various forms of quercetin derivatives in inhibiting key enzyme, for example various kinases[75]. However, these assays can offer clues related to the mechanistic behavior of the studied compounds, not necessarily in a physiological relevant reaction, but mainly for other applications. Structurally speaking, there is a clear structure-activity relationship of flavonoids that renders them prone to one oxidation mechanism or another: the presence of the catechol moiety, known as the B ring in flavonols, leads to an inclination towards HAT mechanism, whereas an extended electron delocalization driven by planarity is crucial for SET-type reactions[70]. In the case of the present study, it is obvious by all means and information presented that quercetin is expected to have the highest antioxidant activity with respect to its 3-O-glycosides. Due to the fact that all four glycosides have lost their planarity and share similar electron delocalization on the B ring, it is not to be expected that a pure SET-based assay would

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result in significant differentiation in antioxidant ability. However, due to previous results presented in this study, that the electron distribution on the B ring seems to be affected by the proximity of the sugar, HAT characterized methods should point out clear different H atom donating properties among the selected quercetin derivatives. Steric hindrance impose by the presence of sugar should also be taken into consideration[76].

The two most common antioxidant assays are DPPH (2,2'-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonis acid)) bleaching assays (a TEAC variant), both of them underlined by a mixed mechanism[76]. Both assays make use of bulky artificial stable free radicals that can interact with flavonols. All four glycosides have been tested with respect to these two classic methods, as can be observed in Figure 6 and Figure S7. Surprisingly, the final results do not show any kind of correlation. The DPPH bleaching assay was performed at various mixtures of water-methanol, highlighting that the measured antioxidant activity is modulated by solvent dielectric constant.

One of the most representative pure SET-based assays is the so-called CUPRAC method, short for cupric ion reducing antioxidant capacity[76]. The undelying principle is that upon reduction of Cu(II) to Cu (I), a complex is formed with bis-neocuproine leading to a characteristic yellow colour[41]. In the present study, at lower compound concentration there is no significant difference in antioxidant capacity between quercetin and its glycosides, as can be seen in Figure 6. However, as the concentration level of tested compounds increases, the difference in observed effect becomes clear. Because it is characterized by the most extensive conjugation and degree of planarity, quercetin performs the best with respect to SET oxidation. All glycosides containing one sugar unit behave very similarly, in terms of absolute value and slope of the concentration dependence. However, rutin, which contains a disaccharide, deems a lower antioxidant activity, most probably due to steric hindrance.

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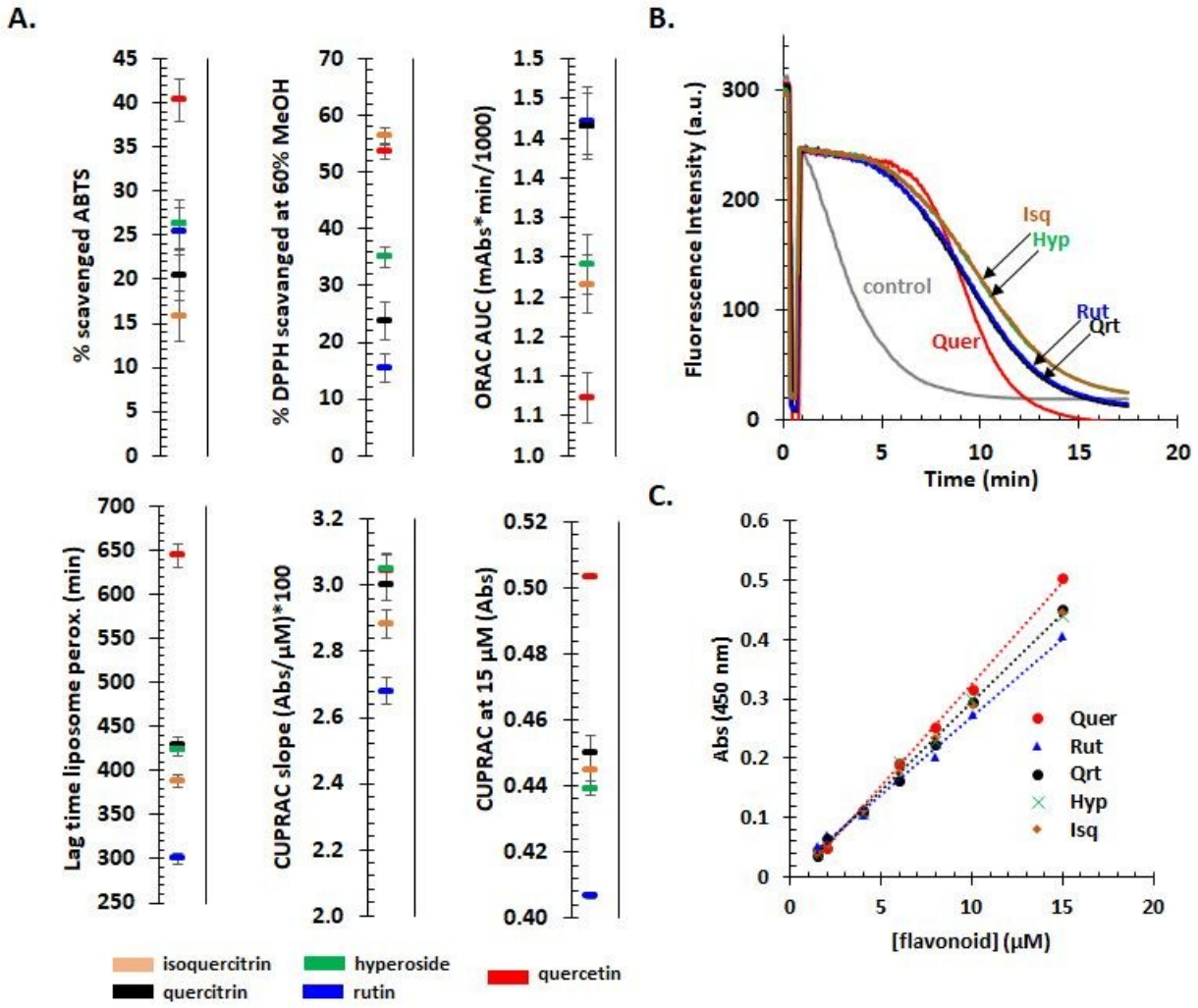

Figure 6. ***A****. Reducing power and radical scavenging ability of the studied compounds determined using various assays.* ***B.*** *Kinetic profile of ORAC assay for each flavonoid.* ***C.*** *Concentration dependence by CUPRAC method.*

When it comes to hydrogen atom transfer mechanisms, one of the most widely used method of antioxidant potency is oxygen radical absorption capacity (ORAC)[76]. The principle of the method is simple: a compound with antioxidant properties protects fluorescein from oxidation induced by AAPH (2,2'-azino-bis-(2-amidino-propane)) radical, avoinding the decrease in fluorescence. Considering the fact that the B ring in quercetin is neither sterically hindered, nor influence in terms of electron density distribution, it does not come as a surprise that this compounds shows the highest antioxidant activity as shown in Figure 6. The trend established for glycosides is slightly surprinsing: there is a very good correlation between the

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values determined for them and their redox potential value. Still, this observation may be explained by careful examination of HAT mechanism basics. Marcus theory is one of the most important principles in redox reactions. The core relationship states that the reaction barrier or activation energy is directly proportional with the reaction driving force, namely the free enthalpy, and with a reorganization energy[77]. The free enthalpy is as directly connected to another intrinsic property of a compound: its redox potential. Taking into account that the reorganization energy for the studied system should not be high (the reaction does not need a dramatic rearrangement), it can be concluded that the lower the potential, the lower the activitation energy and thus the faster the reaction. This may account for the observed glycoside values with respect to HAT-type reactions.

Another antioxidant activity assay that has been performed in the current study is the inhibition of cytochrome c induced oxidation of liposomes[39]. The interaction of cytochrome c with liposomes is of high physiological impact. When apoptosis is triggered, cytochrome c induces the peroxidation of membranes and of liposomes by acting as a peroxidase[78]. After the reaction is triggered, the propagation of lipid peroxidation is relatively fast. When a compound with antioxidant abilities is added, the peroxidation is delayed. Quercetin and its selected 3-O-glycosides have been assayed at various concentrations. Their sigmoidal kinetic profiles are presented in Figure S8, whereas the concentration dependence curves are shown in Figure S9. The most antioxidant candidate was quercetin, followed by quercitrin, hyperoside, isoquercitrin and, finally, rutin. Considering that this assay is a HAT-based one, it is expected to see a clear link between the determined reductive properties and the potential or lipophilicity of the glycosides. Still, this assay concerns more than meets the eye: because liposomes are involved, not only antioxidant capacity is at stake, but also the capacity to interact with them. As stated, before in this study, interactions between flavonoids and membrane lipids are well known. Small planar flavonols can penetrate the lipidic layer, thus interacting profoundly with them. As the size increases, the compound will stay at the surface of the membrane, interacting poorly. Even though rutin is not the least lipophilic studied glycoside, it is still the structurally the bulkiest, thus its superficial interaction with the liposomes might prevent it from greater scavenging action.

A secondary antioxidant mechanism comprises the chelation of free metal-ions that, otherwise, can induce radical generation by Fenton reactions[76]. This prevention can be achieved

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by a compound rich in chelating functional groups, such as amines or hydroxyls. Flavonols are rich in hydroxyl groups, thus they can participate in metal-chelation. An assay concerning evaluation of this activity involves chelation of Al^{3+} by flavonoids (Figure S10) with the formation of a colored complex[37]: the greater the number of hydroxyl moieties involved in chelation, the more prominent the bathochromic effect of the recorded spectrum. Blockage at any position or steric hindrance results in preventing Al^{3+} chelation; hydroxyl groups from sugar unit are not involved in this coordination. Thus, it is expected that quercetin, with the highest number of free hydroxyl group exhibits the highest bathochromic effect (430 nm). With respect to the number of free hydroxyls, all four glycosides should absorb at exactly the same wavelength (416 nm). Still, quercitrin is different, absorbing at 410 nm. The fact that its sugar is a deoxy type might further prevent chelation compared to the other glycosides.

Conclusions

Quercetin has a fundamentally different behavior compared to its glycosides, being by far more reactive. Still, distinctive features have been observed for glycosides, based on the nature of the sugar they contained. EPR and molecular modeling data support the finding that the higher the torsion angle, the higher the corresponding hyperfine coupling constant. This torsion angle is influenced by the size of the sugar moiety and this explains the observed link between spin densities and steric hindrances through torsion angle. On its part, spin density on free radicals dictates their reactivity, thus a connection between steric hindrance and reactivity can be established. Rutin showed a more equally-distributed spin density, partly explaining it low reactivity in anion radical form, whereas quercetin, without any steric constraints, displayed an uneven spin density and, in consequence, the highest reactivity. Interaction with laccase was clearly linked to redox potential, as this enzyme is an oxidoreductase. Glycosides with lowest redox potential and highest lipophilicity are the best substrates for laccase; rutin and quercitrin were the least reactive substrates whereas isoquercitrin and hyperoside displayed relatively higher reactivity. Phenoxyl radical interaction with oxyhemoglobin recommended both isoquercitrin and hyperoside as being slightly inferior to quercetin in reactivity, whereas rutin and quercitrin displayed an even higher lack of sensitivity. As for reducing power and free radical scavenging abilities, redox potential is the main driving force, but structural elements that promote HAT or SET based mechanism are also relevant. Loss of radical planarity and a more

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balanced spin density distribution render quercetin glycosides as more stable towards both HAT-driven reactions compared to their parent aglycone. Lack of structure-reactivity relationship is obvious in both DPPH and ABTS bleaching assays. Still, when it comes to more sensitive reactions, CUPRAC method, based on a SET mechanism, there was a slightly difference between quercetin and its glycosides; all monosaccharide-containing glycosides behave identically. ORAC assay, an epitome of HAT mechanism, was directly connected to the redox potential of each compound, underlining clear reactivity differences. Inhibition of liposome peroxidation has also been evaluated, connecting the protection role of flavonoids to their lipophilicity: a higher lipophilicity allows a compound to interact with the lipid layer in liposomes in depth, rendering a protection against free radicals. Rutin was the only exception to the trend, aspect that might be explained by steric hindrance.

Our findings point out that the properties of quercetin glycosides are finely tuned by the saccharide moiety, mainly due to steric hindrance, drawing a simple conclusion: sugar really matters. The presented results may come into hand in the future, being new pieces of the puzzle of flavonoid properties. Considering the increasing awareness of consumers towards nutraceuticals, these results may help in understanding the link between food components and their function, opening new perspectives for functional foods design. As more roles for these molecules are discovered and their use as lead compounds in drug synthesis is increasing, the *in vitro* (bio)chemical results presented herein might offer important clues in structure-steric-redox-reactivity modulation.

Conflict of interest

There are no conflicts to declare.

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