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# *Trtametes versicolor* Laccase Mediated Oxidation of Flavonoids. Influence of the Hydroxylation Pattern of Ring B of Flavonols

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**Abstract:** Several flavonols were chemically transformed upon treatment by *Trametes versicolor* laccase; the majority of the major oxidation products were isolated (HPLC) as pure compounds and their structures unambiguously established through spectroscopic methods (HPLC-MS), when possible. In some cases, compounds were difficult to obtain either completely pure or in sufficient amounts. In these cases, structural hypotheses were formed on the basis of the HPLC-MS fragmentations. Correlation between the structure of the oxidation products and the substitution pattern of the starting materials revealed mechanistic features of the transformation.

**Key words:** *Trametes versicolor*, Laccase, flavanoids, oxidation

## INTRODUCTION

The enzymatic oxidation of polyphenols is involved in numerous biological mechanisms. Since the radicals formed through this oxidation process are relatively stable, they can undergo several coupling reactions, which are of major interest in lignin, tannins and melanin biosynthesis. However, these reactions are also responsible for changes in the polyphenol profile during manufacture of foods and beverages.

The browning of foods associated with polyphenol oxidation is generally considered to be detrimental. Some enzymatic browning reactions are nevertheless beneficial to the overall acceptability of foods. The production of black, oolong and green tea depends on enzymatic browning reactions for colour and flavour development. There is probably a contribution of enzymatic browning to colour development during coffee processing and colour development in cacao is facilitated by polyphenol oxidase during fermentation. Polyphenol oxidases are also responsible for the development of the characteristic golden brown colour in dried fruits such as grape fruits, prunes, dates and figs and blanching is generally required for inactivation of the enzyme after colour development, in order to minimize discolouration.

Flavonoids compose one of the major classes of polyphenolic chemicals involved in enzymatic browning. They are plant secondary metabolites derived from the phenyl propanoid pathway. In seeds, they act in protection against predators and pathogens (Dixon *et al*., 2002), increase seed coat (testa)-imposed dormancy (Debeaujon *et al*., 2000; Pourcel *et al*., 2005) and protect against UV radiation (Winkel-Shirley, 2002). Among the flavanoids, flavonols are yellow pigments (Fig. 1),

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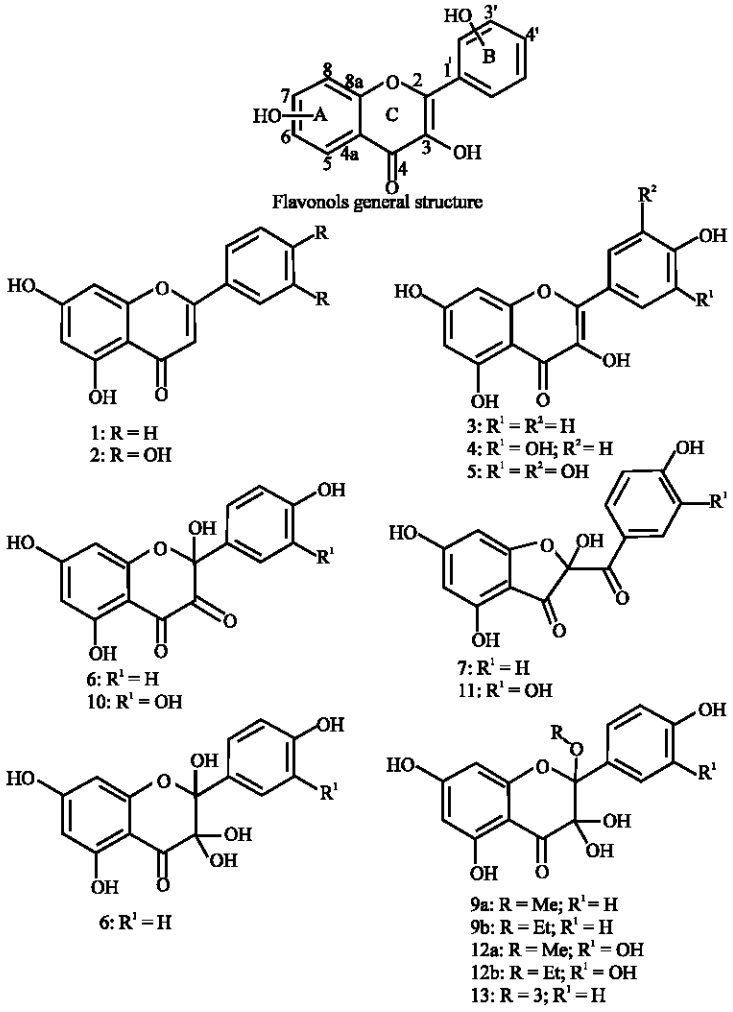



Fig 1: Structure of flavanoids

characterized by a fully conjugated flavan backbone through a 3-hydroxy-pyranone C ring. They are often present in glycosidic forms and compose one of the main flavanoid sub-classes. These phytochemicals are often referred as antioxidants on account of their ability to protect against damage caused by Reactive Oxygen Species (ROS). By their ability to react with and damage many structures, ROS are implicated in decreases such as cancers and artherosclerosis. Both natural and synthetic antioxidants act against ROS and the use of antioxidants in food technology enhances product stability, quality and shelf life. Recent research has revealed the disadvantages of synthetic antioxidants and their possible injurious properties for human health, compared to natural antioxidants.

In a program dedicated to the study of the total synthesis (Es-Safi *et al*., 2006a; Beauhaire *et al*., 2005; Boyer *et al*., 2005a) and the chemical (Es-Safi and Ducrot, 2006b; Boyer *et al*., 2005b, 2006) or enzymatic oxidation of polyphenols, we examine the products of flavonol oxidation mediated by *Trametes versicolor* laccases. Indeed, the main transformations undergone by polyphenols in fruits and plant during food processing are caused by oxidative reactions catalysed by polyphenoloxidases, such as laccases, catechol oxidases and tyrosinases and by peroxidases. Oxidation proceeds in the

presence of molecular oxygen for most polyphenol oxidases and with hydrogen peroxide for peroxidases (Dehon *et al*., 2002; Lopez Serrano and Barcelo, 2003). Autoxidation and chemical oxidation processes may also occur (Guyot *et al*., 1996).

Laccases are part of a large group of enzymzes, called the blue copper oxidases and they are present in plant and in a large variety of fungi (Claus, 2004; Riva, 2006). Assuming that the course of the enzymatic oxidation of polyphenols would be more influenced by the chemical structure of the starting polyphenols than by the nature of the enzyme used, we turned to the use of *Trametes versicolor* laccase, a reliable, commercially available fungus enzyme with a low substrate specificity (Bertrand *et al*., 2002; Kollmann *et al*., 2005) in order to study flavonol oxidation. Indeed, preliminarily experiments (Ghidouche and Ducrot, unpublished results) with model compounds showed that this enzyme produced the same oxidation profiles as laccases and peroxidases from vegetal sources, which are more difficult to obtain from sustainable sources and harder to handle.

Other reports have described the enzymatic oxidation of flavonols, but most of them concentrated on the kinetic features of the reaction, via changes in UV absorption by the reaction mixture, due to the formation of oxidation products (Fenoll *et al*., 2003). Only a few authors have tried to determine the precise structure of the oxidized products and the mechanistic pathway of their formation. Moreover, most efforts have focused on the enzymatic oxidation of quercetin and quercetin glycosides. In order to allow chemical mechanism determination for this type of reactions, we decided to examine the influence of B and C ring substitution in our enzymatic system, using several commercially available flavonols. Previous authors have pointed out the importance of B ring oxidation, since laccases are reported to have a phenol or catechol oxidase activity, giving rise in most cases either to oxidized monomeric species or oligomeric compounds, obtained through radical-radical couplings. Our starting compounds were two compounds with no hydroxy group at C-3, namely chrysin 1 and luteolin 2 and three flavonols with a hydroxy group at C-3, exhibiting various hydroxylation patterns on their B ring, namely kaempferol 3, quercetin 4 and myricetin 5.

## MATERIALS AND METHODS

Methanol, ethanol and acetonitrile (Carlo Erba, Italy) were of analytical grade. Myricetin, luteolin and chrysin were purchased from Extrasynthese (Genay, France), quercetin and kaempferol from Sigma (St Louis, MO, USA).

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) was obtained from *Trametes versicolor* cultures (Bertrand *et al*., 2002 and references cited therein).

Assays for laccase activity were performed by measuring the enzymatic oxidation of ABTS at 420 nm ($\epsilon = 3.6*10^4$ $cm^{-1}$ $M^{-1}$ for the oxidation product). The reaction mixture contained 1 mM ABTS in a 0.1 M $Na_2HPO_4$/ citric acid buffer (pH 3) in a volume of 1 mL. The buffer solution was saturated with air by bubbling prior to the experiment. After adding the enzymatic solution, the increase in absorbance was followed during the first two minutes at 30°C.

### Oxidation Procedure

### Analytical Scale

Analytical scale incubation of kaempferol or myricetin was carried out in glass vials containing 100 µL of the flavonol methanolic solution (10 mg $mL^{-1}$), 300 µL laccase and 600 µL of a citrate/phosphate buffer, 0.1 M, pH4 (total volume of 1 mL). For quercetin, chrysin and luteolin, the incubation media contained 200 µL of the flavonol methanolic solution (5 mg $mL^{-1}$), 300 µL laccase and 500 µL citrate/phosphate buffer, 0.1 M, pH 4.

The reactions were started by the addition of the enzyme. The incubation was conducted under air agitation at 30°C for 3 h.

The same procedure was repeated for each flavonol changing the solvent (methanol, ethanol and acetonitrile).

A 10 μL volume of the reaction mixture was injected every 1 h in a Varian HPLC system, including a Varian pump 9012 and a Varian 9065 diode array detector set at 254 and 282 nm. The column was a C18 TSK gel ODS-88 (packing 5 μm) (4.6*250 nm). The elution conditions were the following: flow rate 1 mL $min^{-1}$, solvent A 0.5% acetic acid in water; solvent B 0.5% acetic acid in acetonitrile. Linear gradient from 15-80% B in 20 min was used, followed by washing and reconditioning of the column.

The chromatographic analysis of myricetin oxidation was carried out using a chromatographic system consisting in a Waters 2695 separation module equipped with an autosampler and a Waters 2487 detector set at 254 and 282 nm. The column was a 150*2.0 nm i.d. Interchrom UP5ODB# 15E (Uptishere 5 μm ODB), used under the following elution conditions: Flow rate 0.1 mL $min^{-1}$, solvent A acetonitrile/water/acetic acid 95/5/0.5, solvent B: Water/acetonitrile/acetic acid 95/5/0.5, linear gradient from 10-80% A in 30 min, followed by washing and reconditioning of the column.

**Preparative Scale**

The incubation medium (100 mL) contained 10 mL of a flavonol solution in methanol (10 mg $mL^{-1}$), 30 mL of laccase 2 U $mL^{-1}$ and 60 mL of a citrate/phosphate buffer 0.1 M, pH 4. The reaction was started by the addition of the enzyme extract suspended in the same buffer solution. The incubation was conducted for 2 h at 30°C.

The suspension was centrifuged at 6000 rpm for 10 min; the supernatant was then filtered under vacuum through an ultra filtration, Ø 44.5 mm (Millipore). The 100 mL filtrate was then concentrated to a final volume of 15 mL by evaporation.

**Purification Procedure**

The myricetin oxidation products were separated on a high semi-preparative HPLC system including a Waters 600 pump, a manual U6K injector, a Uvicord SII UV detector (Pharmacia LKB) set at 254 nm and a REC 102 recorder (Pharmacia Biotech). The column was a C18 Kromasil 250*20 mm (10 μm packing). Serial injections (1 mL) were carried out under the following elution conditions: flow rate 18 mL $min^{-1}$, solvent A (0.5% acetic acid in water), solvent B (0.5% acetic acid in acetonitrile). Linear gradient was used from 15-50% B in 25 min, followed by washing and reconditioning the column. The fractions corresponding to the major HPLC peaks were collected and dried for further LC/MS and NMR analysis.

**LC-MS**

LC-MS analyses were performed with a chromatographic system (Alliance) consisting of a Waters 2695 separation module equipped with an autosampler and a Waters 2487 detector set at 254 and 282 nm. The column was a 150*20 mm i.d Inteechrom UP5ODB# 15 E (Uptishere 5 μm ODB). The elution conditions were the same as for analytical analyses; the injected volumes were 5 μL each at a flow rate of 0.1 mL $min^{-1}$. The fractions were previously dissolved in a acetonitrile/water solution (½ v/v). The HPLC system was coupled to a Quattro LC/MS/MS triple quadrupole mass spectrometer (Micromass, Manchester, UK), equipped with a pneumatically assisted Electrospray Ionisation Source (ESI). All the fractions were analysed in both negative and positive mode. Data acquisition and processing were carried out with a Mass-Lynx NT 4.0 data system.

**NMR Spectroscopy**

NMR data ($^{1}H$: 300 MHZ; $^{13}C$: 75.5 MHZ) were recorded on a VARIAN Mercury Plus 300 instrument or a Bruker Avance 600 (for HMBC and HSQC experiments). All NMR spectra were recorded in deuteriated dimethyl sulfoxide in 5 mm tubes.

## RESULTS AND DISCUSSION

The choice of the starting materials (Fig. 1) was influenced by already published results on the structure-antioxidant activity relationships of flavonoids. It was indeed clearly established that the B ring is the most important site for H transfer and consequently antioxidant capacity. In contrast, the A-ring seems to be less important. On the other hand, the $\Delta^{2,3}$ double bond should also contribute to the antioxidant activity, since it ensures π-electron delocalization between the B- and the C- ring, which contributes to the stabilization of the RO· radicals formed in the oxidation process, after H-abstraction (Saskia *et al*., 1996). The role of the 3-OH group is still under debate. *In vitro* studies demonstrated that this hydroxy group contributes to the antioxidant potential. Indeed, blocking the 3-OH group (e.g., rutin) or removing it (e.g., luteolin) significantly decreases the activity (Rice-Evans *et al*., 1996). The influence of the 3-OH group on the metabolism of quercetin has also been demonstrated (Steiner *et al*., 2002), since quercetin is believed to coordinate to the copper containing active site of the 2, 3-dioxygenase issued from human intestinal bacteria through the 3-OH group and the carbonyl at C-4, allowing H-abstraction and oxidation at C-2 (Balogh-Hergovich *et al*., 1997).

A novelty of our study is the use of a laccase as the enzymatic oxidant. Other studies most often reported the oxidation of flavonoids either with chemical radical initiators, like DPPH, or enzymatic system, like PPO (polyphenol oxidase; in this case, the flavonoids are not the primary substrate, the oxidation being mediated by the o-quinone from caffeoyl quinic acid). Therefore, in these two cases, oxidation of the flavonols may occur through either a mono- or a di-electronic transfer (Fig. 2). The possibility of a di-electronic transfer may indeed favor the oxidation of diphenol through formation of semiquinones and quinones and the thermodynamics of the reaction would thereby be directed by the stability of the potentially formed products, rather than by the stability of the primary radicals formed through H-abstraction.

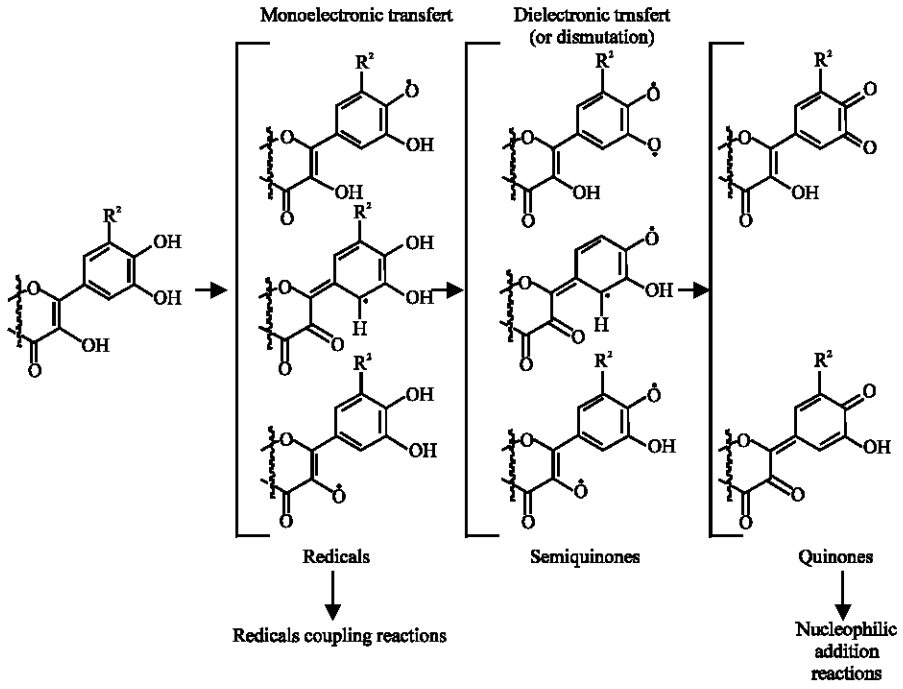



Fig. 2: General oxidation pathways for flavonols

In the case of a laccase, the complete mechanism of the reaction including the pathway leading to oxygen reduction, is not clearly determined (Enguita *et al*., 2004 and references cited therein). However, mono-electronic transfer is privileged, which allows investigation of the stability of the various RO radicals potentially formed as primary products of the oxidation. Indeed, in this case, quinones may only be the result of radical dismutation, the kinetics of which would probably not be competitive with those of a radical coupling reaction. Indeed, among the four copper atoms in the structure of the laccase, only one is involved in the oxidation of the substrate. The three other copper atoms are responsible for the oxygen reduction and oxidation of the Cu(I) atom of the active site of the enzyme.

The first feature arising from our study was the non oxidation of both 1 and 2, thereby confirming the crucial role of the 3-OH group in the oxidation process. This also showed that the presence of a highly reactive catechol type B-ring is not sufficient to allow a one-electron oxidative process to occur, also reflecting the postulated role (DFT studies by Trouillas *et al*., 2004, 2006) played by the hydroxy group at C-3 in the first steps of flavonol oxidation.

Turning to the oxidation of flavonols 3 and 4, we were not surprised to observe that these two compounds, although not exhibiting the same hydroxylation pattern on ring B, nevertheless presented similar behavior in our oxidation conditions. Indeed, the reaction mixture reflected in both cases the presence of only few compounds, MS analysis of which showed that the major ones were isomeric products, corresponding to the addition of one oxygen atom (M + 16). Depending on the solvent used, the other reaction products were either dimeric compounds in aprotic conditions (acetonitrile) or products resulting from solvent addition in protic media (MeOH or EtOH). In this later case, changing from methanol to ethanol was the key to unambiguously characterizing this solvent addition through MS analysis. Later, NMR analysis confirmed the structure of compounds 6-9 and 10-12, as oxidation products of kaempferol and quercetin, respectively. Structures of these products have already been described in the literature (Krishnamachari *et al*., 2002; Hvattum *et al*., 2004 and references cited therein).

As an example, structure of 12a was determined through its NMR spectra as follows. $^{1}H$ spectrum of 12a exhibited the same resonance pattern for the aromatic protons as did quercetin, but also showed a supplementary resonance at 3.05 ppm (integrating for 3 protons, in agreement with the presence of a methyl group attached to an oxygen atom) (Fig. 3). The $^{13}C$ spectrum was also quite similar to that of quercetin, with two less quaternary $sp^2$ carbon resonances, which were replaced by two resonances at 91.7 and 100.9 ppm. HSQC nad HMBC experiments were performed and allowed the complete assignment of all carbon resonances. Indeed, HMBC correlations were observed for the resonance at 100.9 ppm, with protons of the methyl group as well as with protons H2', H5' and H6'. At the same time, no HMBC correlations were observed for carbon resonance at 91.7, which thereby was attributed to C3, the resonance at 100.9 beeing assigned to C2. These results are only

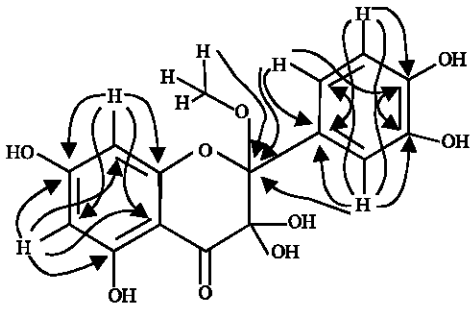
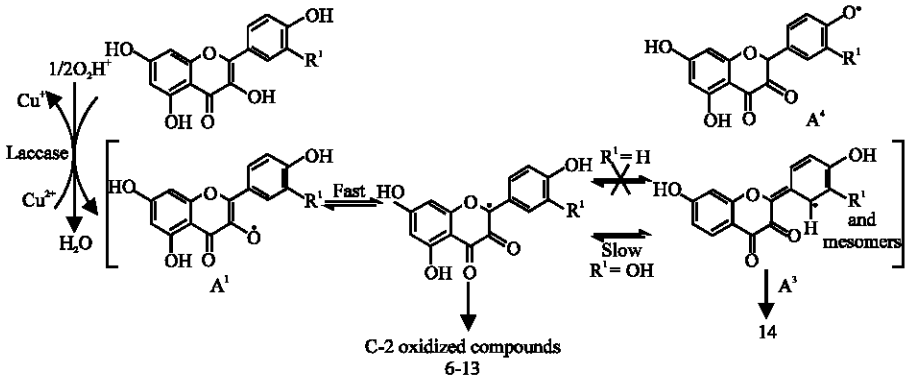



Fig. 3: HMBC correlations observed for 12a

consistent with the introduction of a methoxy group at C2 through trapping of the C2 radical and therefore confirming the possibility of oxidation at C2 through activation of the 3-OH group in a mono-electronic process.

The structures of the dimeric compounds obtained through oxidation of these flavonols in acetonitrile were more difficult to establish, because of their low abundance in the reaction mixture, which did not allow us to obtain them as pure compounds for NMR spectroscopy. However, kaempferol oxidation led to the formation of only one dimeric compound (m/zs 604) and quercetin allowed the formation of two pseudo dimeric compounds at m/z 498 and 618. Examination of the MS spectra of compounds at m/z 604 (13) and 618 (14) obtained from 3 and 4, respectively revealed a behavioural discrepancy between these two flavonols. Indeed, the formation of 14 is compatible with the formation of either a carbon-carbon bond or a carbon-oxygen bond between the two monomeric subunits. At the same time and even if we were not able to determine the atoms involved in this bond, the MS spectra of 13 is only compatible with the presence of a carbon-oxygen single bond between the flavonol subunits. 13 should therefore be the result of the trapping of the C2 radical formed in the first step of the reaction by an hydroxy group of another flavanol molecule in a similar way as this observed for the formation of oxidized monomers 9a, b. This assumption is also supported by the observation in the MS spectrum of 13 of a major fragment at m/z 302, also observed in compounds 9a,b, as the result of the easy cleavage of the OR bond. Unfortunately, we did not get sufficient data to determine which oxygen atom of kaempferol is involved in this interflavanyl linkage. On the other hand, the MS spectrum of 14 did not reveal similar fragmentation (no corresponding fragment at m/z 318), but rather those compatible withe classical fragmentations of the flavan skeleton. This may indicate a carbon-carbon single bond between both quercetin subunits, although we were unable to get sufficient data for a complete structure assignment. The compound at m/z 498 (the structure of which has also not been completely established, since it remains a very minor product of the reaction) should arise from a degradation of the pseudo dimer 14.

We thereafter turned to the investigation of the possible oxidation of myricetin 5 by our enzymatic system. Although already assumed to be a substrate for oxidative enzymes (Jimenez, 1999), oxidation products of myricetin have, to our knowledge, never been investigated. As for 3 and 4 (Scheme 1), three solvents (methanol, ethanol and acetonitrile were used in order to see the influence of the solvent in this reaction and potentially to trap the radical at C2 in protic media. However, in this case, although not exhibiting exactly the same chromatographic profile, the reaction mixtures in these three solvents essentially showed the presence of dimers and oxidized dimers at m/z 634 (2M-2H), 648, 650, 664, 666 and 684. Beside these compounds, oxidation reaction, when conducted in protic media, also afforded some degraded dimers at m/z 484 and 620. Focusing our interest on the dimer at




Scheme 1: Postulated oxidation mechanism for 3 and 4

m/z 634, we were able to isolate it as a pure compound and its simple $^1$H NMR spectrum, due to symmetry, (Fig. 4) allowed us to assign it structure 15, resulting from the coupling of two $B^3$ radicals (Scheme 2).

The most important feature arising from this reaction is the fact that no other type of linkage than that occurring in 15 was observed in all the other dimeric oxidation product. The chromatographic profile of the enzymatic oxidation (Fig. 5b) performed with 15 as starting material (although not identical from a quantitative point of view to that observed in the oxidation of 5, Fig. 5a), qualitatively showed the presence of the same oxidized dimers. Moreover, the evolution of the chromatographic profile of the enzymatic oxidation of 5 clearly showed that 15 is first accumulated in the reaction mixture and then transformed into the other compounds and mainly into the compound exhibiting a molecular weight of 650 amu, corresponding to a further oxidation with incorporation of an oxygen atom. According to the structure of the oxidation products of quercetin, structure 16 could be putatively assigned to this compound.

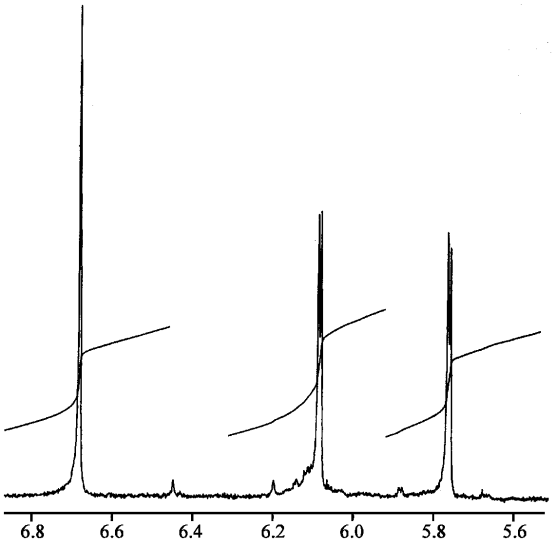
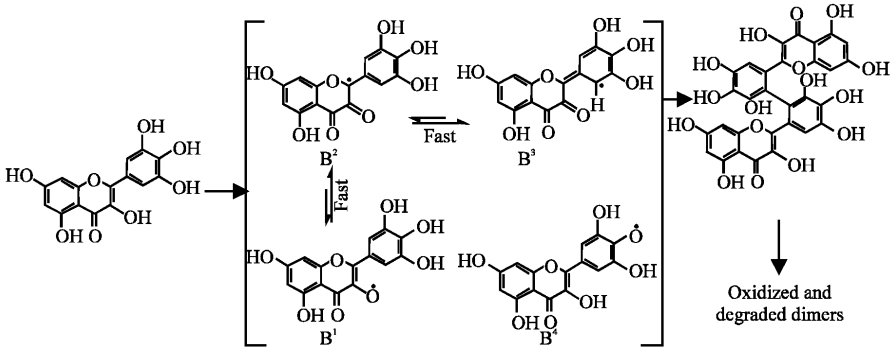



Fig. 4: $_1$H NMR spectrum of 15 (exchangeable protons not shown)




Scheme 2: Postulated oxidation mechanism for 5

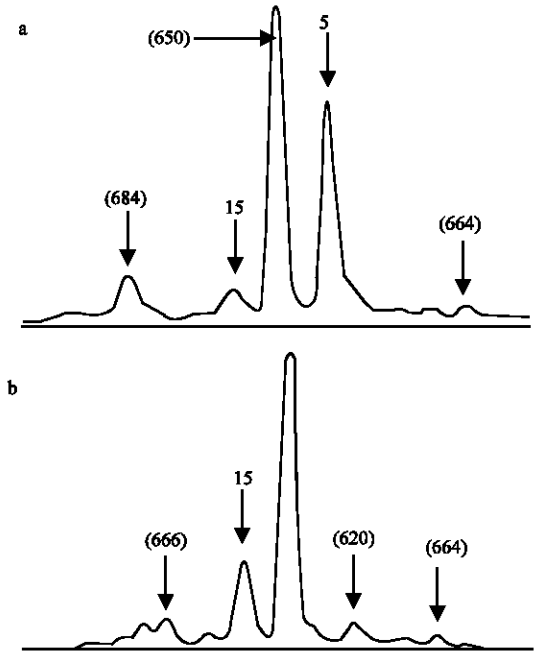
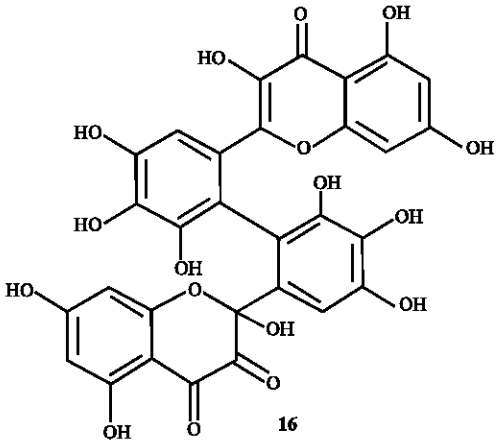



Fig. 5: Chromatographic profiles of the oxidation reaction mixture at 3 h of 5 (a) and 15 (b)




From a mechanistic point of view, our results show that the oxidation of flavonols first occurs at C2 through abstraction of the enolic proton at 3-OH and that, thereafter, the equilibrium between the possible isomeric radicals is directed by the substitution pattern of ring B. In the case of 3 and 4, radicals $A^3$ and $A^4$ are not sufficiently stabilized to allow the significative formation of dimeric compounds linked through a carbon-carbon biphenyl bond. On the other hand, in the case of myricetin 5, although the first formation of the $B^4$ radical could not be excluded, the most probable pathway remains the first formation of the $B^1$ radical, which rapidly isomerizes into the B 2one, which is stabilized by the presence of the three phenolic groups (mainly by this in para position), leading to the formation of 15. However, the presence of compounds resulting from further oxidation of this first

formed dimer, as well as the lack of higher order oligomers, indicates that in dimeric compounds, oxidation thereafter occurs mainly at C2. Moreover, the observation of compounds at m/z 664, 666 and 684 clearly demonstrates that compound 15 may undergo at least three further consecutive oxidations, thereby underlining the high antioxidant activity of 5.

## CONCLUSIONS

Through the observation of the oxidation products of three flavonols (3, 4 and 5), we showed that the first oxidation to occur, takes places not on the phenolic functions, but on the hydroxy group at C3, resulting in the formation of C2 oxidized products. However, the presence of phenolic groups on the B ring is an important feature in the stabilizing of phenyl radicals, which can undergo coupling reactions. Moreover, when observed, these phenyl or phenoxy radicals did not undergo dismutation, since it would results in the formation of quinones (Fig. 2), which would undergo nucleophilic addition reaction with other flavanol monomers, resulting in the formation of C2' (or C3')-C8 (or C6) dimers as already reported in the literature for flavan-3-ols (Guyot *et al*., 1996).

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