INVESTIGATION OF ENZYMATIC OLIGOMERIZATION OF RUTIN

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ABSTRACT

The enzymatic polymerization of rutin catalyzed by laccase from Trametes versicolor was investigated under different operating conditions of temperature, pH, solvent, enzyme and substrate concentrations. The highest weight-average molecular mass ($\overline{M_w}$) was about 3900 g/mol. Highest masses were obtained for lowest pH and temperature set point. The production of oligomers was favored when using a cosolvent with a high dielectric constant.

MALDI-TOF analyses showed the presence of rutin oligomers with a polymerization degree of up to 6, resulting from simple bridges between rutin unities. ^{1}H-NMR analyses showed the presence of C-C or C-O linkages in the structure of oligomers, involving both the sugar and phenolic parts of rutin.

Oligomers were characterized by a solubility 4200 times higher than rutin. A molecular modeling study of the hexamer indicated a dense network of H-bonds with water molecules. Fractions enriched with oligomers were obtained by tangential diafiltration. The antioxidant activity of oligomers was shown to decrease with $\overline{M_w}$, while the xanthine oxidase inhibitory activity increased.

***Keywords**: Laccase, rutin, oligomerization, MALDI-TOF, antioxidant activity, molecular modelling.*

INTRODUCTION

Flavonoids are a class of phenolic secondary metabolites of plant that have recently received keen attention due to their antioxidant, antimicrobial and anticarcinogenic properties. Many of these compounds are already used in pharmaceutical, cosmetic and food preparations. Unfortunately, the use of some of them is limited by their low solubility and stability in both lipophilic and aqueous media. Therefore, to improve these properties different techniques of derivatisation are suggested, among them the enzymatic polymerization. This way allows the control of polymer structure due to the regioselectivity of the enzyme and can be conducted under mild operating conditions of temperature, pH and pressure. Different sources of enzyme are used in polymerization processes: transferases, hydrolases, lyases, isomerases, ligases and oxidoreductases (peroxidases and laccases) [1]. The latter is most often used [2-7]. Laccases compared to peroxidases present the advantage of using dioxygen instead of hydrogen peroxide [8] and are able to catalyze a large panel of substrates (ferrocyanidines, aniline, phenol etc.).

The first data obtained in the case of the enzymatic polymerization of simple phenols and some flavonoids (rutin, catechin, quercetin) indicated that the polymerization of these compounds affected their solubility in water [9-11] and their radical scavenging activity [12-15]. The magnitude of these effects depends on the degree of polymerization of the synthesized oligomers [14]. However, for flavonoids, the exact effect of the operating conditions both on the weight-average molecular mass ($\overline{M_w}$) of the oligomers and their biological activities is not yet clear. In the case of simple phenols and aglycone flavonoids, some authors observed that the nature of the solvent affects the molecular mass and the structure of the oligomers. Two dimeric products resulting from either C-C or C-O bonds between monomeric unities were described, depending on the nature and the ratio of the solvent [16, 17]. The

structure of the oligomers was also influenced by the pH [18, 19]. A low pH favored the formation of C-C bridges [20]. The temperature affected only the conversion yield of the reaction [21].

Reported data concerning the antioxidant activity of flavonoid oligomers like rutin and catechin are still subject to controversy. Kurisawa *et al.*[11] observed an increase of the antioxidant power of the oligomers compared to that of rutin, while Desentis-Mendoza *et al.*[15] reported a decrease of the antioxidant activity. The xanthine oxidase inhibitory activity, reported by Kurisawa *et al.* in the case of catechin oligomers [12, 13], increased compared to that of catechin. For rutin oligomers, no data are available concerning this activity. These contradictions could be due to the variability of oligomer structure ($\overline{M_w}$, PDI, C-C or C-O bridges) from one study to another.

The aims of this paper are i) to investigate the effect of temperature, pH, substrate and enzyme concentration, and solvent nature on the $\overline{M_w}$ and the polydispersity (PDI) of rutin oligomers, ii) to identify the type and the localization of the linkage occurring during rutin oligomerization and, iii) to investigate some physical and biological properties of rutin oligomers (solubility, antioxidant and xanthine oxidase inhibitory activities) in function of their $\overline{M_w}$.

Rutin oligomerization was catalyzed by the laccase from *Trametes versicolor*, under different operating conditions of temperature, pH, in several solvents. MALDI-TOF, SEC-UV, FTIR and NMR techniques were used to characterize the mass distribution and the structure of the oligomers. Rutin was chosen in this study for its known vasorelaxation [22], anti-inflammatory [23], and hepatoprotective [24] activities. Moreover, rutin is a glycosylated compound with several reactive hydroxyl groups both in sugar and phenolic parts. However, the implication of the glycosidic hydroxyl groups for oligomers synthesis has never been reported.

EXPERIMENTAL

Materials

Laccase from *Trametes versicolor* (E.C. 1.10.3.2, 21.4 U/mg) and dihydroxybenzoic acid (DHB, matrix for MALDI-MS) were purchased from Fluka. Rutin, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), xanthine and xanthine oxidase (E.C. 1.17.3.2, grade IV from bovine milk, 0.2 U/mg protein) were from Sigma. All chemical solvents were of HPLC grade from VWR.

Oligomerization reactions

Reactions were carried out in a Chemspeed ASW2000 automated synthesizer (Chemspeed Ltd). This platform allows automatic preparation of samples for analyzes: sampling from reaction media, dilution of samples and injection to HPLC system or spotting on MALDI target for MS analysis. Eight reactions were simultaneously performed. Rutin was suspended in 27 mL reactor vessels in 10 mL of methanol/water, solvent/water or methanol/buffer mixture (30:70, v/v) (citrate or phosphate ammonium buffer 0.01 M at different pH ± 0.2). Laccase solution (3 U/mL) was added to the mixture. The reactions media were stirred at 600 rpm for at least 24 h, at different temperatures (± 1 °C).

SEC analysis

Relative masses of oligomers were evaluated by size exclusion chromatography (SEC) (HPLC LaChrom, UV 280 nm LaChrom L-7400, Tosoh TSKgel α 3000 column, 60 °C). Dimethylformamide (DMF) with 1 % LiBr was used as the mobile phase (1 mL/min). Molecular mass calibration was obtained by using standards of polystyrene. The obtained data allowed the determination of number-average molecular mass, weight-average molecular mass, weight-average molecular mass index and polydispersity:

$$\overline{M_n} = \frac{\sum_i n_i M_i}{\sum_i n_i}$$: number-average molecular mass

$$\overline{M_w} = \frac{\sum_i w_i M_i}{\sum_i w_i}$$: weight-average molecular mass

$I_M = \frac{\overline{M_w}}{M_1}$: weight-average molecular mass index

$PDI = \frac{\overline{M_w}}{\overline{M_n}}$: polydispersity (≥ 1)

Where n_i represents the number of molecules of oligomers having a mass of M_i at a polymerization degree of i, and w_i weight fraction of chains at a polymerization degree i. The quantity of oligomers was expressed in arbitrary units of area (a.u.). Error values were determined by three repetitions of the same reaction.

MALDI analysis

Absolute masses were determined by MALDI-TOF technology using DHB matrix (5 mg/mL in acetonitrile/water (30:70, v/v) with 0.1 % TFA). After 24 h of reaction, samples were automatically withdrawn from reaction media and diluted two fold (acetonitrile/water (30:70, v/v) with 0.1 % TFA). Solutions (3 µL) were spotted online on MALDI target with DHB matrix (1 µL).

Samples analyzes were realized on MALDI-TOF Ultraflex (Bruker Daltonik) using the reflectron operating mode. All spectra were obtained in the positive ion mode and ionization was performed with a 337 nm pulsed nitrogen laser. Spectra were obtained by accumulation of at least 600 laser shots and calibrated using the statistical method of FlexAnalysis software (Bruker). All samples were spotted in triplicate.

UV analysis

The UV spectra of rutin solutions and of reaction media containing oligomers were determinated on a UV6000LP spectrometer (Spectra System, Thermofinnigan).

FTIR analysiss

IR analyzes were achieved by ATR-FT-IR spectroscopy using FT-IR spectrometer Tensor 27 (Bruker). The solutions containing oligomers were diluted with water/methanol (70:30, v/v) then deposited on the ATR unit. Solvents were evaporated under vacuum at 50 °C to give a film which was analyzed. Spectra were obtained by the accumulation of 1024 scans, with a 2 cm^{-1} resolution. Spectra treatment included H_2O/CO_2 correction, smoothing by Savitzky-Golay relation (9 points) and normalization.

NMR analysis

^{1}H-NMR analyzes of rutin and 3 KDa permeat powders was performed on a Bruker DRX 300 MHz spectrometer, at 300 K, after dissolution in DMSO-d_6. ^{1}H-NMR spectra of the 4 powders of the aliquots obtained at 30 min, 1 h, 2 h and 3 h of reaction (0.2 g/L of rutin and 0.3 U/mL of laccase in methanol/water, 30:70, v/v) were recorded on a Bruker 600 MHz spectrometer with a cryoprobe at 293 K in CD_3OD/D_2O (30:70, v/v) at a concentration of 3 g/L.

A diffusion-ordered spectroscopy (DOSY) NMR technique was used to aid the deconvolution of the complex system. A diffusion delay time of 100 ms was applied, in CD_3OD/D_2O (30/70, v:v). The self–diffusion coefficient is affected by the viscosity of the solvent. The mobility of the molecules can be converted into their hydrodynamic radius (R), which is more informative to determine the size of the particles and to follow the formation of aggregates through the Stokes-Einstein law expression:

$R = \frac{k_B T}{D f \pi \eta}$ where η is the viscosity of the solvent ($\eta_{methanol}$= 0.156 .10^{-3} kg.m^{-1}.s^{-1} and η_{water}= 1.000 kg.m^{-1}.s^{-1}), k_B the Boltzmann constant (1.38.10^{-23} kg.m^2.s^{-2}.K^{-1}), T the temperature (293 K) and R the hydrodynamic radius of the soluble molecule, f a constant between 4 (slip) and 6 (stick) depending on the boundary conditions. Here the stick value is used.

Peaks related to rutin:

^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) = 0.99-5.36 (H of sugars), 6.20 (6-H), 6.39 (8-H), 6.85 ($J_{H5'/H6'}$ = 0.2 Hz, 5'-H), 7.54 (2'-H), 7.55 ($J_{H5'/H6'}$ = 0.2 Hz, 6'-H), 9.17 (3'-OH), 9.65 (4'-OH), 10.85 (7-OH), 12.60 (5-OH).

Peaks related to rutin oligomers:

^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) = 0.99-6.17 (H of sugars), 6.18 (6-H), 6.40 (8-H), 6.86 ($J_{H5'/H6'}$ = 0.2 Hz, 5'-H), 7.97 ($J_{H5'/H6'}$ = 0.2 Hz, 6'-H).

Antioxidant activities determination

Scavenging activity and xanthine oxidase inhibitory activity of different pools of oligomers were analyzed. Samples were prepared from reaction media after 24 h of synthesis (methanol/water, 30:70, v/v, 20 °C), by successive filtration processes: on a 50 KDa membrane to remove the enzyme, then on 8, 5 and 3 KDa membranes by a tangential diafiltration process (V/V). Afterwards, the fractions were lyophilized. Scavenging free radical power was evaluated by the DPPH test as described by Burda et Oleszek [25]. A solution of rutin or oligomers (1 mL), at different concentrations (from 10^{-6} to 10^{-4} M, concentrations were calculated from $\overline{M_w}$) in methanol, was added to 2 mL of a DPPH solution (10 mg/L in methanol/water, 80:20, v/v). A reference sample was prepared by adding 1 ml of methanol in 2 mL of DPPH solution. Rutin and oligomers absorbance for each concentration was evaluated at 527 nm, after 15 min, at 23 °C. The antiradical activity was calculated as a percentage of DPPH discoloration using the following equation:

$$antiradical\ activity = 100 \times \left(1 - \frac{absorbance\ of\ sample - absorbance\ of\ oligomers}{absorbance\ of\ reference}\right).$$

The antiradical activity of flavonoids is expressed as the final concentration that results in half-maximal DPPH discoloration (IC50) and calculated by exponential regression analysis. Each absorbance was evaluated in triplicate.

To determine the inhibition of xanthine oxidase activity the oligomeric powders were solubilized in phosphate buffer (pH 7.5, 50 mM). The rutin was dissolved in a minimum of DMSO and then in buffer. Tests solutions were prepared by adding 1600 µL of buffer, 300 µL of flavonoids solution (10^{-5}– 4.10^{-4} M), 1000 µL of a solution of xanthine (0.15 mM, initially dissolved in a minimum of NaOH 2 % (w/v), then diluted in buffer) and 100 µL of a solution of xanthine oxidase (0.2 U/mL). The reaction was monitored for 6 min at 295 nm. Two samples were prepared, the first without flavonoids to determine the total uric acid production, and the second without enzyme to measure the absorbance of flavonoids at 295 nm for the range of concentrations. The inhibition of xanthine oxidase by flavonoids is expressed as the final concentration that results in half-maximal enzyme velocity (IC50) and calculated by standard curve regression analysis. All measurements were realized in triplicate.

Solubility and rheological characterization

The solubility of rutin oligomers was evaluated at 30 °C in water while the solubility of rutin was determined for all operating conditions of the reaction.

To obtain a rheological characterization of the oligomers, a reaction medium (methanol/water, 30:70, v/v, 24 h of reaction) was lyophilized and analyzed. Several samples of oligomers were prepared at different concentrations in water and analyzed at 20 °C on a StressTech rheometer coupled with a RMS Lauda temperature controller. All tests were conducted with 40 mm diameter parallel-plate geometry at 20 °C. A solvent trap was used during measurement to limit evaporation. Viscosity was then measured at shear stress ranging from 0.05 to 10 Pa, according to the concentrations. The overlap concentration was obtained by the representation of the viscosity at the plateau for a shear rate of 300 s^{-1} depending on oligomer concentration. All measures were realized in triplicate.

Molecular modeling analysis

The molecular dynamic (MD) simulations were performed with Insight II (msi) programs, on an O2 SGI workstation, employing the force-field cff, on Discover 3. The first step was a minimization in vacuum by using a steepest descent, a conjugate gradient descent and a Newton-Raphson algorithm until the energy gradient was smaller than 0.1 kcal/(mol.Å). A dielectric constant ε = 2.0 F/m was used in all minimizations. The second step is a molecular dynamic simulation to explore the conformational space of rutin and its oligomers. The dynamics run for 10 ps, at 600 K. The lowest-energy conformation was minimized with a convergence of 0.1 kcal/(mol. Å). This conformation was selected for molecular modeling in solvent and for an enthalpic study.

The enthalpic study was carried out using the semiemperical AM1 method, with HyperChem software. Optimization was performed with conjugated gradient (algorithm Polak-Ribiere) with a self consistent field (SCF) of 0.001 kcal/mol in vacuum. The bond dissociation energy [26] was calculated as the energy of the radical resulting from the abstraction of one hydrogen minus the energy of the neutral molecule. The most stable radicals localized either on oxygen or a carbon atom were obtained by the abstraction of the hydrogen atom of the 4'-OH, and the hydrogen atom of the 6'-CH.
Molecular modeling in water was performed in a cubic cell with periodic boundary conditions. NVT molecular dynamic was carried out in a box of 40.0 Å side length containing 1294 water molecules plus one molecule of rutin with a cutoff of 14.0 Å and a dielectric constant of 2.0, at 300 K. The MD simulations run for 200 ps. For the simulation of the hexamer of rutin, the same conditions were used, with a cubic cell of 50.0 Å side length containing 1679 water molecules. Successive and short minimization/dynamic procedures were applied with a convergence of 0.001 kcal/(mol. Å) and a run duration of 8 ps.

RESULTS AND DISCUSSION

To investigate the kinetic of the enzymatic polymerization of rutin catalyzed by the laccase, two reactions, with and without addition of the enzyme, were carried out in a methanol/water medium with an initial pH of 7.0, at a constant temperature of 20 °C. The rutin consumption, the weight-average mass index (I_M) and the polydispersity (PDI) of synthesized oligomers were followed during 55 hours of incubation. The results indicated that in the absence of the enzyme, the concentration of rutin remained constant without any change of the medium coloration. In the presence of the enzyme (Figure 1), the rutin was completely depleted from the medium during the four first hours of the reaction. Both I_M and PDI increased respectively up to 3.44 and 1.24 during the first 24 hours of incubation and then decreased slightly. The increase of I_M, even after the total consumption of the monomer, indicated the presence of an enzyme reactivity towards oligomeric species, which leads to the elongation of the chain length.

1. Influence of pH and temperature on polydispersity, weight-average molecular mass and production of oligomers

The oligomerization of rutin, in the presence of the laccase from *Trametes versicolor* was studied at different pH and temperatures. The results, after 24 h of incubation, are summarized in Figure 2. For the given temperature of 20 °C, the increase of pH led to a slight decrease of the PDI and a variable I_M. The highest I_M was reached at a pH of 5.0, when the enzyme activity is optimal [27].
In methanol/water medium, a higher I_M and heterogeneity in oligomers size were observed at 10 °C compared to 20 and 50 °C. These results could be explained by a thermic deactivation of the laccase, which leads to a lower concentration of radical species. In such conditions, the elongation of the chains will be favored. The diminution of I_M at 50 °C could be attributed to a larger concentration of radical species which favor coupling reactions and so, the termination of the synthesis. The comparison of these data to those reported by Kurisawa *et al.* [11] with the laccase from *Myceliophtora*, indicating that the effect of pH and temperature is independent to the origin of laccase.
The effects of pH and temperature were also quantified on the oligomers production. The results are summarized in Figure 2. The pH has no significant influence on the oligomers production, while an increase of temperature favors their synthesis. The highest production of oligomers was reached at 50 °C, when the solubility of rutin and the laccase activity [27] are favored.

2. Effect of enzyme and rutin concentrations

The enzyme is responsible for the generation of radical species in the medium. The concentration of these radical species can affect the chain length of the oligomers. The elongation of the oligomeric chains can also be affected by the initial concentration of rutin. So, both enzyme and rutin concentrations were varied. The results (Table 1) showed that, the initial concentration of enzyme and substrate has no significant influence neither on the PDI nor on the I_M.
Rutin oligomers were obtained by polymerization of rutin catalyzed by laccase from *Trametes versicolor* in methanol/water (30:70, v/v), at 20 °C, for 24 h. Weight-average molecular mass index (I_M) and polydispersity (PDI) were determined by SEC-UV analyzes.
This observation is in agreement with those reported by Kurisawa *et al.* [11]. The concentration of enzyme influences the kinetic of the reaction but not the chain length of the oligomers. For a high

concentration of rutin (9 g/L), the I_M remained constant (3.44) although the rutin was not totally consumed. This behavior could be attributed to the enzyme deactivation or to a sequestering effect of the oligomers toward the enzyme, leading to its inactivation.
To verify this assumption, either 30 U of enzyme or 30 U of enzyme and 30 mg of rutin were added after 24 hours of reaction (standard reaction, with 3 g/L of rutin). In spite of the feeding with fresh enzyme with or without additional rutin, no change was observed. These data suggested an inactivation of the laccase which could be due to a sequestering effect of rutin or/and its oligomers toward the copper of the active site of the enzyme, as it is mentioned by Desentis-Mendoza *et al.* [15].

3. Effect of solvent

Different works reported the effect of the solvent on the enzymatic oligomerization of simple phenols [3, 16, 28, 29]. These studies indicated that the nature and the proportion of the solvent modify the yield, the I_M and the structure of the polymers. For more complex phenols like flavonoids, there are no data available concerning the effect of the solvent on their oligomerization.
To investigate the effect of the solvent on the enzymatic oligomerization of rutin, seven protic polar, aprotic polar and aprotic apolar solvents were tested (Figure 3a) at a solvent/water ratio equal to 30:70 (v/v). In the case of methanol, three percentages (70, 50 and 30 %) were used. The influence of each solvent was evaluated at 20 °C after 24 h of reaction. The results indicated that the nature of the solvent affects mainly the production of oligomers. For a given ratio (30:70 v/v), no significant change was observed for I_M and PDI. The highest and the lowest production of oligomers were obtained in water (8.11 a.u. area) and THF/water medium (0.28 a.u. area) respectively. These results can not be attributed to the solubility of rutin in such media which is 0.14 g/L in water versus 9.25 g/L in THF/water. However, a correlation was observed between the dielectric constant (ε) of the cosolvent and the production of oligomers (Figure 3b).
The increase of methanol/water ratio, led to a decrease of the I_M, PDI and the oligomers production, as it was reported by Kurisawa *et al.* [11] in the presence of the laccase from *Myceliophtora.* For methanol/water ratio superior to 70 % no activity was observed.
In conclusion, our data showed that the dielectric constant (ε) is an important factor to explain the effect of the solvent on the enzymatic oligomerization of rutin. The constant ε is implicated in dissociation phenomena and is likely to diminish the chelation effect of rutin and its oligomers, which is supposed to decrease the activity of the enzyme. In fact, the enzymatic inhibition by a chelation phenomenon was already demonstrated by Kim *et al.* [30], that studied the effect of catechin-aldehyde polycondensates on xanthine oxidase activity. Another effect of the solvent could be the alteration of the enzyme stability.

4. Structural investigation

To characterize the structure of the oligomers, MALDI-TOF, UV, FTIR and NMR analyzes were realized after 24 h of incubation in a methanol/water medium (70:30, v/v).
MALDI-TOF analysis allowed the determination of the absolute masses of rutin oligomers. These were identified as di-, tri-, tetra-, penta- and hexamers of rutin (Figure 4). No higher masses were observed whatever the operating conditions tested. The relative percentage of each oligomer depends on the operating conditions (pH, temperature, concentration of enzyme, etc.).
The gap of mass due to the addition of one rutin shows the abstraction of two hydrogen atoms. This result indicates that the connection mode between rutin units is a simple bridge. According to the bibliographic data this linkage could occur between two B rings of rutin [31]. Depending on pH [18, 32] and solvent [29], it could be either a C-O or a C-C linkage. Rutin presents several reactive hydroxyl groups both on the phenolic rings and the sugar part. So, this linkage could be also localized on the sugar moiety. To elucidate the type and the localization of the linkage between two units of rutin, further investigations using UV, FTIR, ^{1}H-NMR at 300 MHz, diffusion ordered spectroscopy NMR (DOSY) and ^{1}H-NMR at 600 MHz with cryoprobe analyzes were realized.
The UV-visible spectrum of rutin, in methanol, presented two maxima of absorption at 282 and 359 nm due to the π-π* transition of the aromatic electrons. For rutin oligomers the 359 nm band was much larger and presented a hypsochromic shift of 11 nm. This behaviour suggests an implication of the B ring of rutin in the formation of oligomers. In fact, Marckam [33] observed that the presence of a substitution on the 5, 7 and 4' positions of phenolic rings led all time to an hypsochromic shift.

In comparison to rutin spectrum, the IR analysis of oligomers showed a broadening of the absorption bands and a new peak at 1130 cm^{-1} (Figure 5). This peak indicated the formation of a new ether bond (C-O). Similar results were reported by Mejias *et al.* [31] in the presence of peroxidase.
The ^{1}H-NMR analysis of rutin oligomers was already reported by Kurisawa *et al.* [11]. These authors observed 3 broad peaks at δ 0.8-1.3, 3.0-4.0, 4.4-5.0 ppm but these data didn't allow to identify the type and the localisation of the linkage established during the oligomers formation. In order to complete these results, different complementary techniques were used: DOSY and ^{1}H-NMR with cryoprobe.
The DOSY analysis was expected to separate the oligomers in function of their hydrodynamic radius and then improve the spectrum resolution. Unfortunately, this analysis didn't permit to separate the oligomers and the spectrum was similar to that reported by Kurisawa *et al.* [11]. However, this technique gave the global self-diffusion coefficients ($8.4.10^{-11}$ $m^2.s^{-1}$) and the hydrodynamic radius (29.8 Å) of the oligomers.
To overcome the heterogeneity of the reaction medium and then to increase the resolution of ^{1}H-NMR analysis, four aliquots were taken from the reaction medium after 30 minutes, 1 h, 2 h and 3 h of synthesis. These samples were heated at 95 °C for 5 minutes to inactivate the enzyme, then lyophilized and analyzed by ^{1}H-NMR, at 600 MHz, with cryoprobe, at a concentration of 3 mg/mL (D_2O:CD_3OD, 70:30, v/v). For the first sample (30 min of reaction), the ^{1}H-NMR spectrum revealed four new signals with a multiplicity of 2 at 7.17, 7.26, 7.31, 7.52 ppm (Figure 6).
These peaks could result from the upfield shift of the 6'H signal and the downfield shift of the 5'H signal of rutins linked together, by a C-O bridge (C2'-O4'). The evolution with time of NMR spectra revealed a progressive broadening of all signals both in aromatic and sugar zones. This profile suggests that several types of bridges should occur during the oligomerization of rutin, involving both the aromatic and the sugar parts of the flavonoid.
The data obtained by different techniques confirm the presence of a C-O linkage and suggest that this linkage occurres on the phenolic and sugar parts of rutin. However these data don't allow to conclude about the presence or the lack of the C-C linkage. For this reason, the reaction medium was filtrated on a 3000 Da membrane by tangential flow filtration to obtain a fraction enriched with oligomers with low molecular weight. As expected, the permeate contained mainly residual rutin and dirutin. This was lyophilized then dissolved in DMSO-d_6 and analyzed by ^{1}H-NMR spectroscopy. The analysis of the spectrum showed a limited number of signals in the aromatic zone, a downfield shift of the 6'H signal and the absence of the 2'H peak. This behaviour suggests a symmetrical structure which results from a C2'-C2' bridge between two rutin units (Figure 7). So, it is clear that both C-C and C-O linkages were established during rutin oligomerization.

5. Solubility, antioxidant activity and inhibitory effect on xanthine oxidase of rutin oligomers

To study the solubility, reaction media containing rutin oligomers were lyophilized. The powder was dissolved in water, at 30 °C. The solubility was about 600 g/L. This solubility is 4200 fold higher than rutin solubility value. The solubility of polymers could also be described by a critical overlap concentration (C*). This parameter characterizes the beginning of the contact between macromolecular coils [34]. In the case of oligomers with an I_M of 3.44, the rheological investigation showed that this factor was around 20 wt %.
To explain the high solubility of rutin oligomers, the interactions and especially the H-bonds between theses molecules and water were evaluate by a molecular modeling study. The lowest energy conformation of rutin monomer and rutin hexamer furnished by molecular dynamic were introduced into two distinct water cells. The hexamer was built on the basis of O4'-C6' bridges which implicate the most stable radicals ($O^{\bullet}$, $C^{\bullet}$). In water, rutin showed a folded structure where the rhamnose and the ring A became closer together. Six intermolecular H-bonds were established with the water molecules. The hexamer revealed an unfolded structure where sugars offer a large contact with the surrounding solvent. The number of intermolecular H-bonds between the hexamer and water molecules was evaluated to 94. This dense network of H-bonds could explain the high solubility of the hexamer compared to rutin.

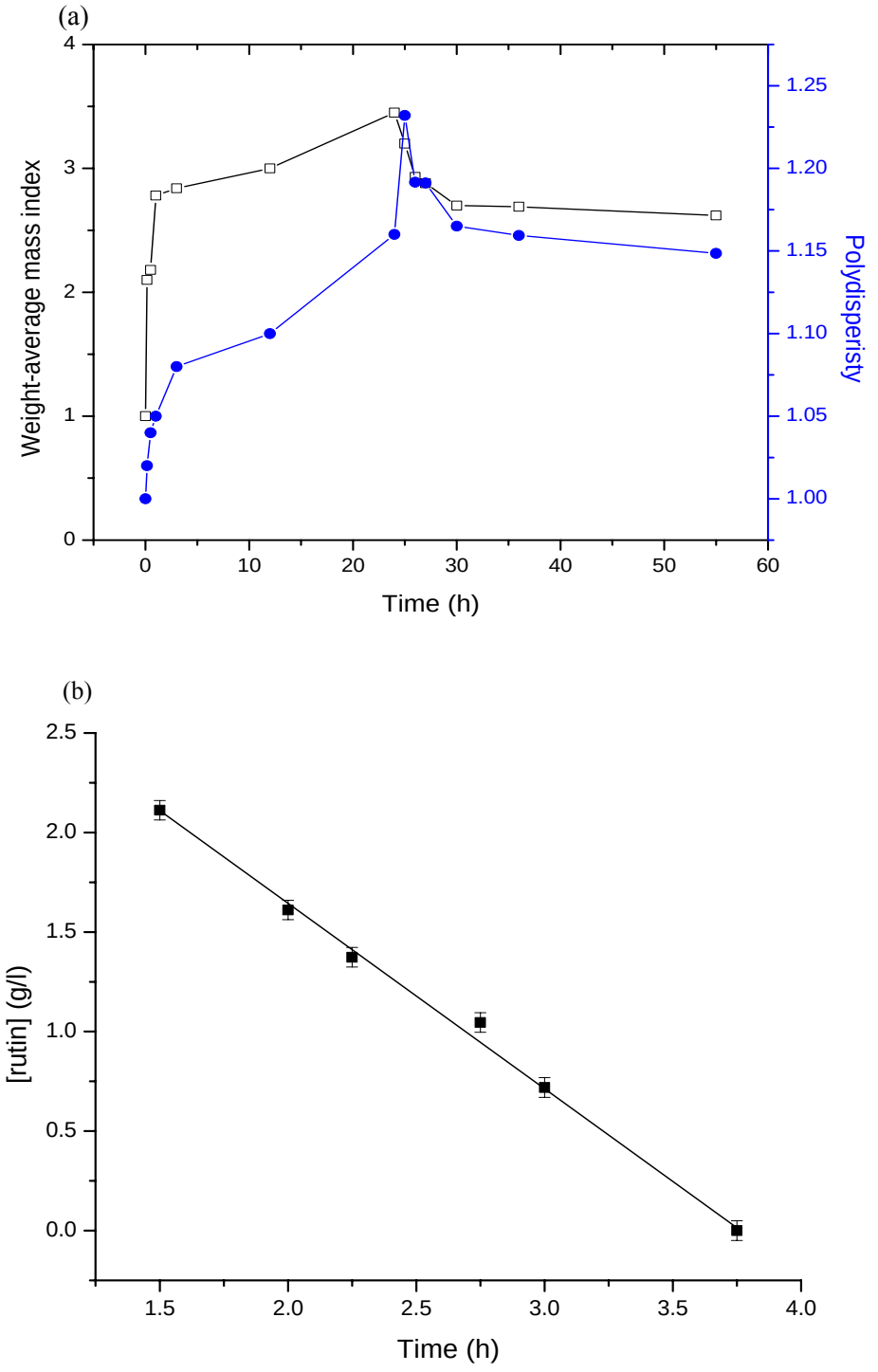


Figure-1: Kinetics of rutin (3 g/L) oligomerization by the laccase from *Trametes versicolor* (3 U/mL) in methanol/water (30:70, v/v) at 20 °C: -□-: weight-average molecular mass index ± 10 % and -●- : polydispersity ± 2 %(a), and kinetic of consumption of rutin (b).

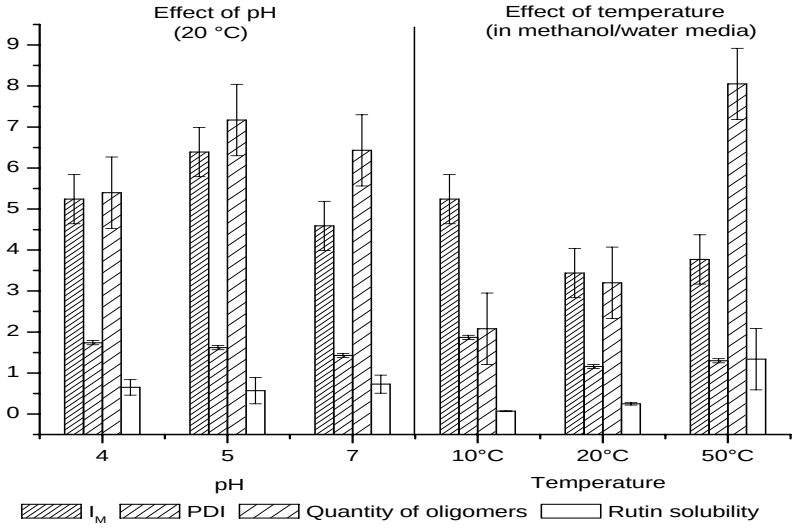
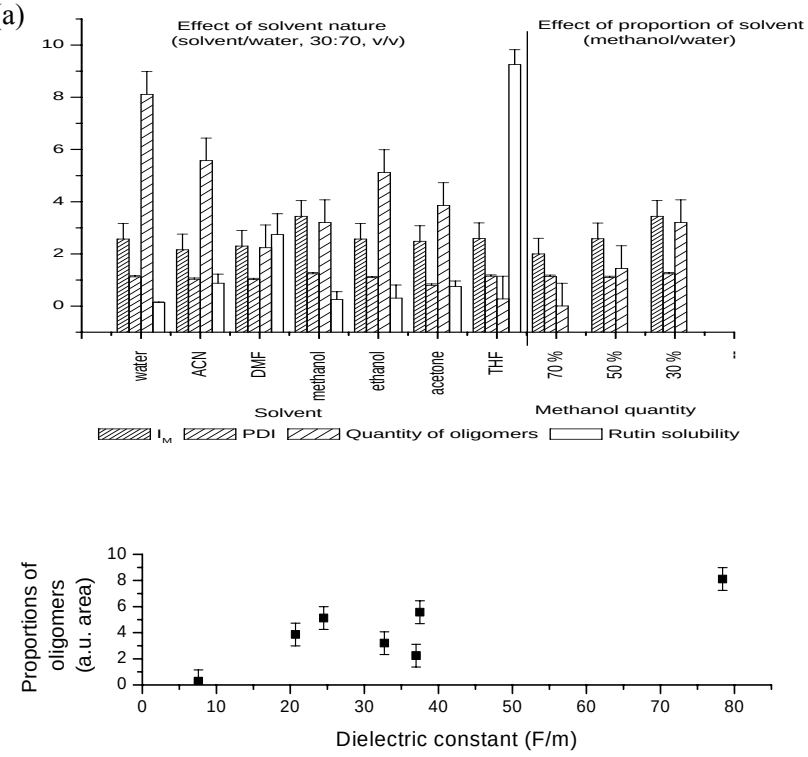


Figure-2: Influence of pH and temperature on weight-average molecular mass index (I_M), polydispersity (PDI), production of oligomers and solubility of rutin. Rutin (3 g/L) oligomerization was catalyzed by the laccase from *Trametes versicolor* (3 U/mL), for 24 h, in methanol/water media at different temperatures (10, 20 or 50 °C) or methanol/ buffer media at pH 4, 5 or 7.

(a)



(b)

Figure-3: Effect of nature and ratio of solvent on weight-average molecular mass index (I_M), polydispersity (PDI), production of oligomers and solubility of rutin (a), and influence of the dielectric constant of the cosolvent on the proportion of oligomers (b). Rutin (3 g/L) oligomerization was catalyzed by the laccase from *Trametes versicolor* (3 U/ml), for 24 h, in solvent/water media (30:70, v/v) at 20 °C.

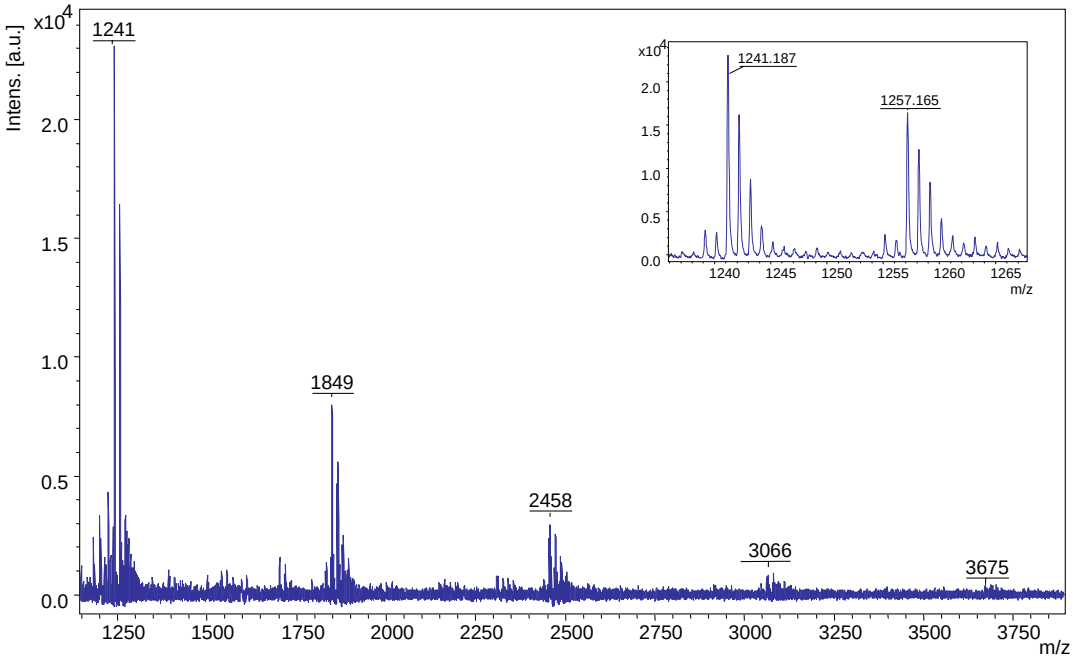


Figure-4: MALDI-TOF positive ion spectrum ($[M - H + Na \text{ or } K]^+$) in the reflectron mode of rutin oligomerization media and isotopic distribution of dimer. Dihydroxybenzoic acid was used as matrix.

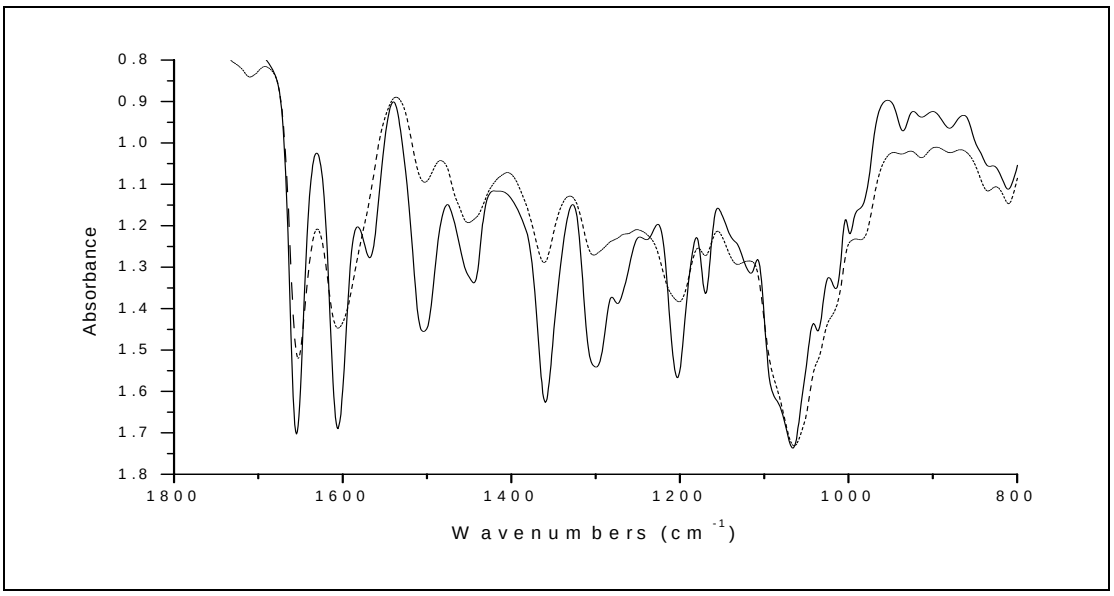


Figure-5: FTIR (diamond ATR) spectra of rutin (─) and reaction media containing rutin and oligomers (---). A film of reaction media has been realized on the diamond ATR for this analysis.

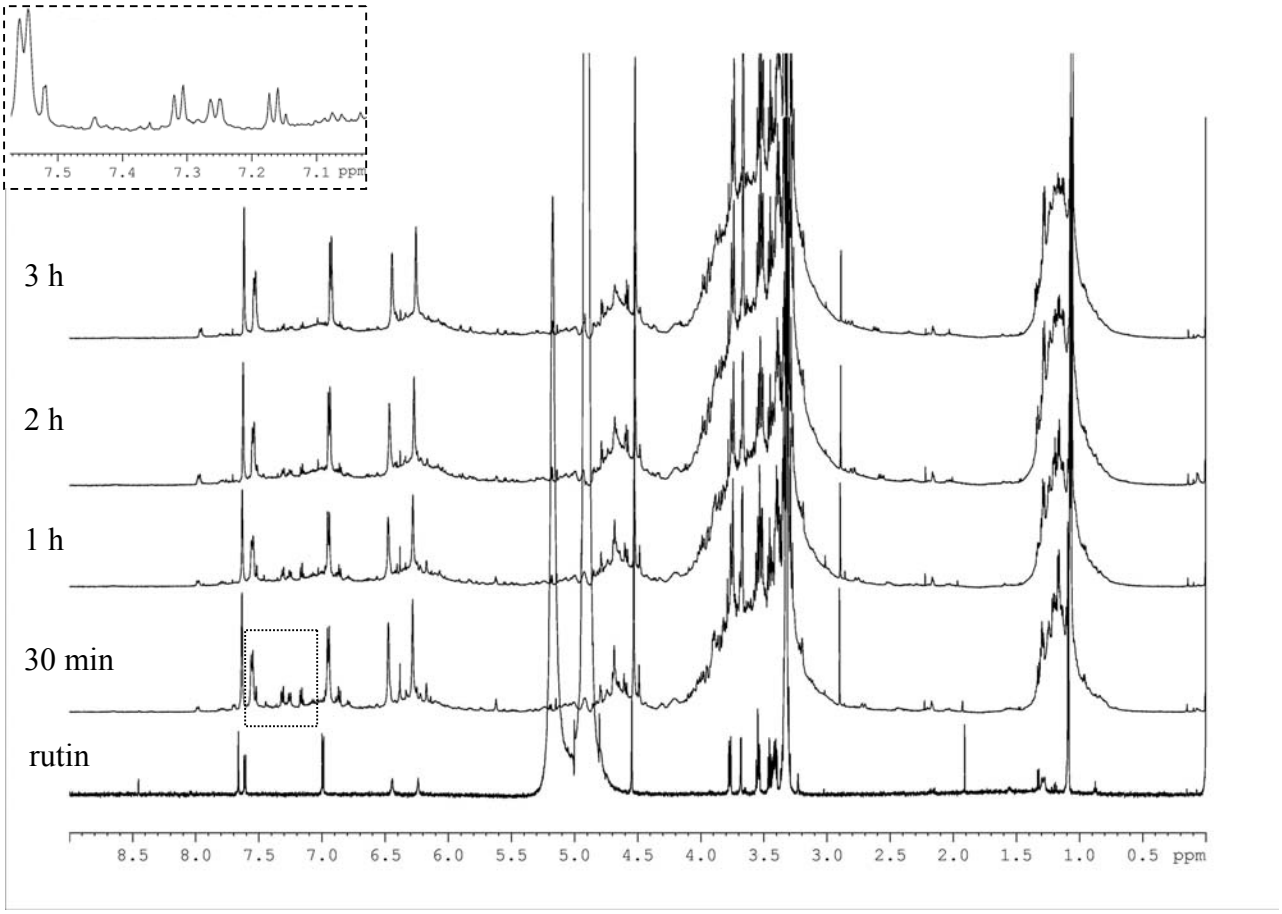


Figure-6: ^{1}H-NMR (600 MHz by cryoprobe) spectra of media containing rutin and oligomers of rutin at four times (30 min, 1 h, 2 h, 3 h) of reaction. Rutin (0.2 g/L) oligomerization was catalyzed by the laccase from *Trametes versicolor* (0.3 U/mL) in CD_3OD/D_2O (30:70, v/v), at 20 °C.

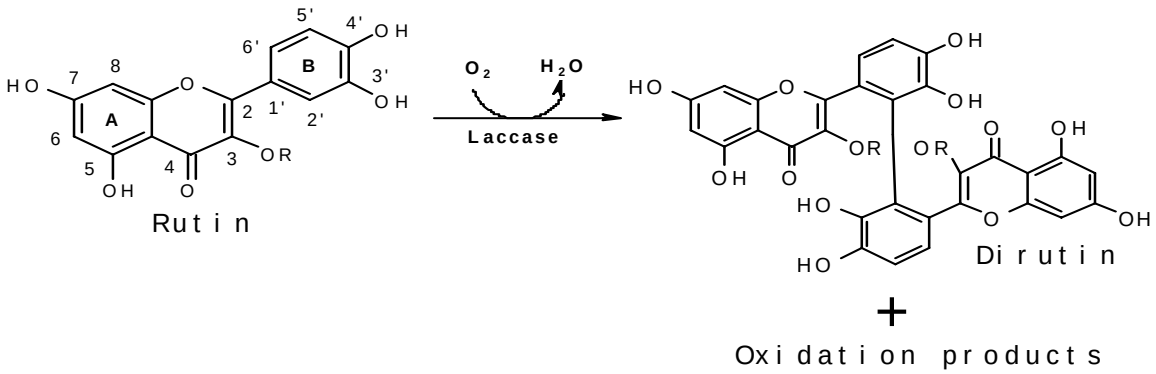


Figure-7: Polymerization reaction of rutin (R: 6-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl) catalyzed by laccase.

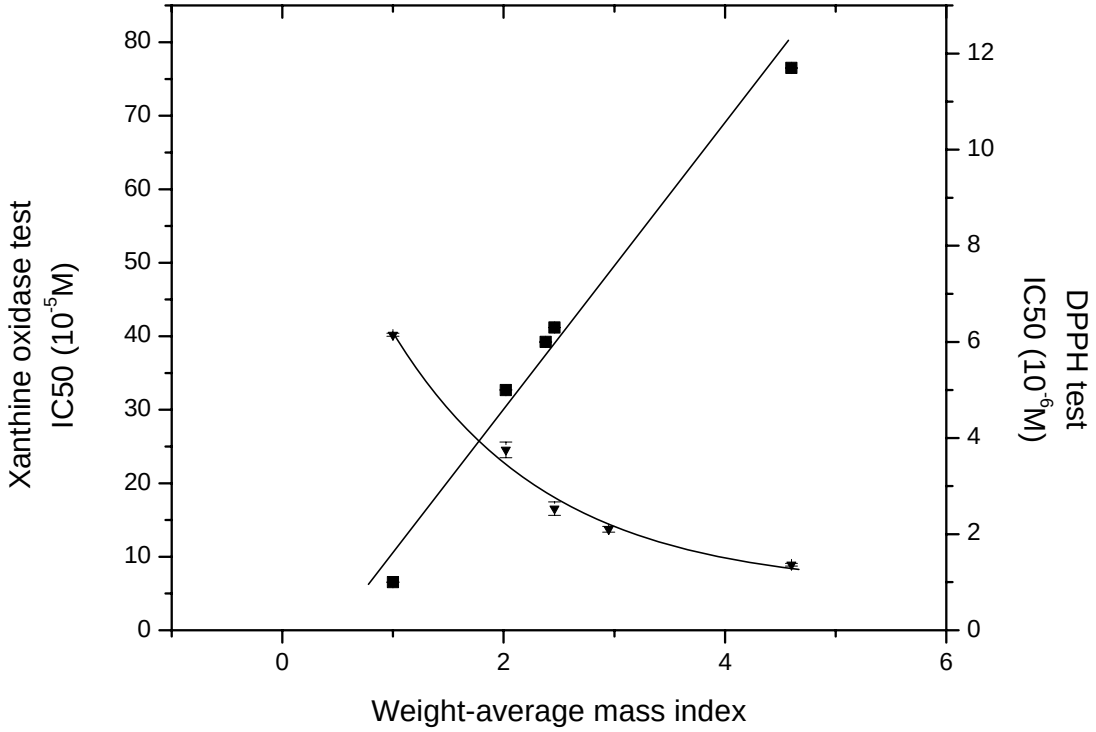


Figure-8: Influence of weight-average molecular mass index of rutin and rutin oligomers on antiradical activity determined by DPPH test (■) and on xanthine oxidase inhibitory activity (▼). The antiradical activity of flavonoids is expressed as the final concentration that results in half-maximal DPPH discoloration (IC50). The inhibition of xanthine oxidase by flavonoids is expressed as the final concentration that results in half-maximal enzyme activity (IC50).

Table-1: Influence of enzyme and substrate concentration on oligomerization of rutin catalyzed by laccase from *Trametes versicolor*.

Enzyme (U/mL)	**Rutin (g/L)**	**I_M**	**PDI**	**Quantity of oligomers (a.u. area)**
3.0	3.0	3.44 ± 0.6	1.24 ± 0.05	3.2 ± 0.87
3.0	9.0	3.44 ± 0.6	1.16 ± 0.05	4.6 ± 0.87
0.1	3.0	2.79 ± 0.6	1.13 ± 0.05	1.0 ± 0.87

The radical scavenging activity of rutin and rutin oligomers was evaluated for each fraction obtained by tangential flow diafiltration with a cut-off of 50, 8, 5, 3 KDa respectively. These fractions, corresponding to retentates and permeate, were characterized by I_M of 4.59, 2.95, 2.46, 2.38 and 2.02. As indicated in Figure 8, the IC50 increased progressively versus I_M, which means a decrease of the radical scavenging activity. The retentate fraction obtained with a membrane of 50 KDa (I_M of 4.59) led to a high IC50 (11.7µM) compared to the IC50 value obtained for rutin (1 µM). This diminution of the antioxidant activity could be attributed to the loss of the free hydroxyl group on C-4' and/or C-3'. In fact Burda and Oleszek [25] reported that these groups play a major role in the antioxidant power. The diminution of the antioxidant activity was also observed by Desentis-Mendoza *et al.* [15], using also the DPPH test.

The xanthine oxidase inhibitory activity of rutin and oligomers was evaluated for each fraction of tangential flow filtration. As indicated in Figure 8, the IC50 decreased progressively versus I_M. The

oligomers of high $\overline{M_w}$ showed a greater inhibitory activity than rutin. The inhibition of the xanthine oxidase activity was followed by Kurisawa *et al.* [12] during the oligomerization of catechin by peroxidase but these authors didn't analyze the effect of $\overline{M_w}$. This behaviour suggests that the hydroxyl groups on C-5 and C-7, the carbonyl group on C-4 and the double bond between C-2 and C-3 are not implicated in the reaction of polymerization. In fact, several authors [35-38] reported that the presence of these groups are responsible for the inhibition of the xanthine oxidase activity.
Moreover, Kim *et al.* [30] observed the chelation of the catechin-aldehyde polycondensates onto the Fe/S and/or the flavin adenine dinucleotide (FAD) center of the xanthine oxidase. So, the high inhibitory activity of the oligomers reported in our study could also be linked to their high chelating capacity.
This work showed that the oligomerization of rutin catalyzed by the laccase from *Trametes versicolor* led to a complex mixture of oligomers containing up to 6 units of rutin linked together by with C-C and C-O bridges involving both the sugar part and the phenolic part of rutin. The $\overline{M_w}$, the amount of oligomers and the PDI depended on pH and temperature conditions. High temperatures led to low $\overline{M_w}$ and high production of oligomers whereas high $\overline{M_w}$ were obtained at low temperature or a pH of 5. The lowest PDI (1.16) was obtained at 20 °C. The solvent nature influenced also the production of oligomers. The increase of dielectric constant medium increases these concentrations.

Rutin oligomers exhibited a solubility 4200 fold higher than that of rutin. A decrease of the antioxidant power and an increase of the xanthine oxidase inhibitory activity were observed when increasing $\overline{M_w}$.

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