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Homogeneous and heterogeneous methods for laccase-mediated functionalization of chitosan by tannic acid and quercetin

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ABSTRACT

Homogeneous and heterogeneous methods for functionalization of chitosan with quercetin or tannic acid using laccase from *Trametes versicolor* is presented, yielding a bio-based product with synergistic antioxidant and antimicrobial properties. HPLC–SEC analysis and cyclic voltammetry kinetic studies showed that laccase catalyzes the oxidation of quercetin to electrophilic *o*-quinones, which undergo to an oligomer/polymer-forming structures. On the other hand, tannic acid was converted into gallic acid, its dimers, partially gallic acid esterified glucose and glucose, when exposed to laccases. ATR-FTIR spectroscopy provided evidence that quercetin *o*-quinones undergo coupling reactions with amino groups of chitosan via Schiff-base and Michael addition mechanisms under heterogeneous method, while oxidized tannic acid cross-linked with chitosan by hydrogen and electrostatic interactions under both methods. All polyphenols functionalized chitosans showed greatly improved ABTS$^{\bullet+}$ cation radicals scavenging capacity, compared with the untreated chitosan, while antimicrobial activities significantly depended on the mode of functionalization and type of microorganism.

1. Introduction

There is a growing interest in exploiting biological reactions using biological polymers for producing effective antioxidant/antimicrobial-based materials used in food processing, packaging, textile and pharmaceutical/medical applications (Muzzarelli et al., 2012). Among the polysaccharides, chitosan is currently the most promising product with many biological activities (Muzzarelli & Muzzarelli, 2009). It may be expected that the functionalization of chitosan with phenolic quinones through coupling with amines to yield either Schiff-bases or Michael-type adducts, may enhance its antioxidant activity, antimicrobial and antifungal properties against a wide range of microorganisms (Aljawish et al., 2012). Ranging from simple phenolic molecules to highly polymerized compounds with molecular weights greater than 30 kDa, the occurrence of this complex group of substances in plant foods is extremely variable as well as their biological activities (Hirvonen et al., 2001; Ovaskainen et al., 2008).

Recently, several reports involving numbers of assays for the determination of the antioxidant capacity of a variety of molecules have been introduced (de Queiroz Ferreira & Avaca, 2008). Among the tasted polyphenols the well-known polyphenolic compounds tannic acid and quercetin have been documented to possessing the highest antioxidant capacity (Pulido, Bravo, & Saura-Calixto, 2000) as well as spectrum of various pharmacological activities (Jung et al., 2010). On the basis of these reports, we hypothesized that tannic acid and quercetin are the most efficient choice for inhibiting the ABTS$^{\bullet+}$ cation radicals and investigated the antioxidant effect of these compounds, incorporated in/on chitosan films.

Tannins are natural polyphenols widely distributed in woody and herbaceous plants which can be divided into two distinct groups: condensed tannins based on polymeric flavonoids (proanthocyanidins) such as catechin and gallocatechin, and hydrolyzable tannins which comprise mainly gallotannins and ellagitannins (Widsten et al., 2010). Tannic acid (1,2,3,4,6-pentagalloyl-O-glucose) is tannin, which can be hydrolyzed under mild acidic or alkaline conditions to yield carbohydrate (glucose) and phenolic acids (mainly gallic acid). Hot water or enzymes (such as tannase) also produce tannic acid hydrolysis (Aelenei, Popa, Novac, Lisa, & Balaita, 2009).

Quercetin (3,3′,4′5,7-pentahydroxyflavone) is a flavonoid of widespread occurrence in nature whose medicinal properties have been extensively demonstrated in the literature, especially the antioxidant capacity (Timbola, de Souza, Giacomelli, & Spinelli, 2006). The antioxidant properties of quercetin have been attributed to its capacity to scavenge free radicals generated in aqueous phase, increasing the resistance of lipids against peroxidation (Pietta, 2000). Despite this, the electrochemical behavior of quercetin has

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been often studied in organic solvents due to constraint imposed by its solubility characteristics. Evidently, the stability of intermediate species originated by oxidation reactions is substantially different depending on the surroundings, i.e. organic or aqueous media. The knowledge of its electrochemical behavior in aqueous media is relevant to get insight into the quercetin action as an antioxidant.

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) mainly catalyzes the oxidation of *p*-hydroxyphenols and can act on a great variety of substrates. There are several advantages in the laccase polymerization of phenolic compounds: (i) the reaction takes place in the absence of toxic reagents such as formaldehyde, (ii) laccase catalyzes the polymerization of a great variety of phenol monomers, (iii) the phenolic compounds with more than two reactive groups can be polymerized selectively, and (iv) the structure and solubility of the polymer can be controlled by changing the conditions of the reactions (Desentis-Mendoza et al., 2006).

The main purpose of this research was thus to develop novel, stable and bio-based formulations, prepared from chitosan and natural antioxidants, catalyzed by laccase, in consideration of attaining of synergistic antimicrobial and antioxidative activities.

2. Materials and methods

2.1. Materials

Medium molecular weight chitosan (M_w of 421.5 kDa and polydispersity index (PDI) of 3.82 determined by HPLC/SEC) with a degree of deacetylation ~90%, was purchased from Mahtani Chitosan Pvt. Ltd., India. Tannic acid, quercetin, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and laccase from *Trametes versicolor* were obtained from Sigma–Aldrich and used without further purification. All reactions were conducted in deionized water. Other reagents used were of analytical grade.

2.2. Laccase activity

The activity of the laccase was determined by monitoring the oxidation of ABTS at 436 nm ($\varepsilon_{436} = 2.9 \times 10^4\ M^{-1}\ cm^{-1}$) as reported by Niku-Paavola, Karhunen, Salola, and Raunio (1988). The assay reaction mixture contained a 200 μL enzyme solution, 100 μL of freshly prepared 1 mM ABTS, and a 700 μL 100 mM citrate buffer of pH 4.5. Enzyme activity was expressed in units, U, defined as μmol of ABTS oxidized per min.

The pH and temperature activity profile of laccase were determined between pH 4.5 and 7.5 at 30 °C, and at pH 4.5 between 20 and 70 °C.

2.3. Oxidation of tannic acid and quercetin by laccase

Tannic acid and quercetin were dissolved and dispersed, respectively, at 10 mM concentration in a 100 mL phosphate buffer (100 mM) of pH 6.5, and then reacted with 153 U of laccase for 24 h at 30 °C under constant stirring. Final reaction products were collected, centrifuged for 2 h at 11,000 rpm and washed off three times with water and in the case of quercetin additional with ethanol to eliminate non-reacted compounds. The pellet containing the laccase produced compounds was frozen at −20 °C and then lyophilized for 24 h using Mini-lyotrap LTE equipment.

2.4. Size exclusion chromatography

Size-exclusion chromatography (SEC) was used to evaluate the molecular weights (M_w) of the chitosan and synthesized phenolic compounds. These analyses were performed using a High-Performance Liquid Chromatography Series 1200 (Agilent Technologies), using a Plaquagel-OH column. Retention was detected on the Refractive Index Detector (RID) and the Variable Wavelength Detector (VWD), with a detection interval of 190–400 nm equipped with a standard cell. A chitosan solution (addition of HCl) was diluted to a concentration of 1 g/L, filtered, and injected within the system having a 0.5 M $NaNO_3$ and 0.01 M NaH_2PO_4 mobile phase, with a flow-rate of 1 mL/min. Linear polyethylene standards with molecular weights between 6000 and 463,000 g/mol were used to record the calibration curve when determining chitosan M_w. The phenolic products were dissolved in tetrahydrofuran (1 g/L), filtrated with 0.22 μm filter and 10 μL was injected and subsequently eluted through a Zorbax PSM bimodal column. DMSO (100%) was used as a mobile phase, with a flow-rate of 1 mL/min. Polystyrene standards with molecular weights between 1000 and 4000 g/mol were used for column calibration/estimation of the samples molecular weights. All HPLC/SEC data were evaluated using Chem Station computer software (Agilent Technologies).

2.5. Electrochemical experiments

Voltammetric experiments were performed using a PGSTAT101 Autolab controlled by Autolab NOVA software version 1.5. All the experiments were carried out in a 100 mL Metrohm cell with a triple-electrode configuration. The working electrode was a glassy carbon (GCE) with a surface diameter of 3 mm (Metrohm). The counter and reference electrodes were platinum (Metrohm) and Ag/AgCl (Metrohm) electrodes, respectively. The renewal of the glassy carbon surface was achieved by polishing with 1.0 and 0.3 μm alpha-alumina (Micropolish, Buehler) on a micro cloth-polishing pad (Buehler), followed by washing in an ultrasonic Selecta bath for 2 min. The tannic acid and quercetin (0.1 mM) oxidation by laccase was studied using 35 U of laccase in 100 mM phosphate buffer (pH 6.5) at a 50 mV/s scan rate, within a thermostated-cell (100 mL) at 30 °C, and the voltammetric curves were recorded every hour for 5 h, and then after 24 h.

2.6. Preparation of chitosan films

Chitosan film forming solution was prepared by dissolving 1 g chitosan powder in total volume of 50 mL of water with HCl (pH near 2). Undissolved material was centrifuged out. To determine the material weight, the pellet was frozen at −20 °C and then lyophilized for 24 h. After the known undissolved material weight, the chitosan solution was diluted to 1% (w/v), and the pH was adjusted to 5.8–6.0 using NaOH. Chitosan films were prepared by adding 5 mL of a 1% (w/v) chitosan solution to 7 cm-diameter Petri dishes, and then oven-dried overnight at 45 °C. The dried films were neutralized by immersion in 1 M NaOH for 3 h, thoroughly washed with water and phosphate buffer (100 mM, pH 6.5), and stored in phosphate buffer at 4 °C.

2.7. Enzymatic functionalization of chitosan

Heterogeneous grafting method was conducted by incubating the chitosan films for 24 h in 100 mL buffer (100 mM phosphate buffer, pH 6.5), containing polyphenols (10 mM) and laccase (153 U), under constant shaking at 30 °C. The control samples were treated in the same way but without adding the enzyme. After the reaction, the films were rinsed out extensively with ethanol and water.

Homogeneous method was performed by first preparing separately chitosan (1%, w/v), polyphenols (10 mM), and laccase (153 U) solutions, and the pH of each solution was adjusted to a target value of pH 4.5. The chitosan solution was heated to 30 °C and an individual polyphenol solution/dispersion was added under constant stirring. After homogeneous distribution laccase solution was

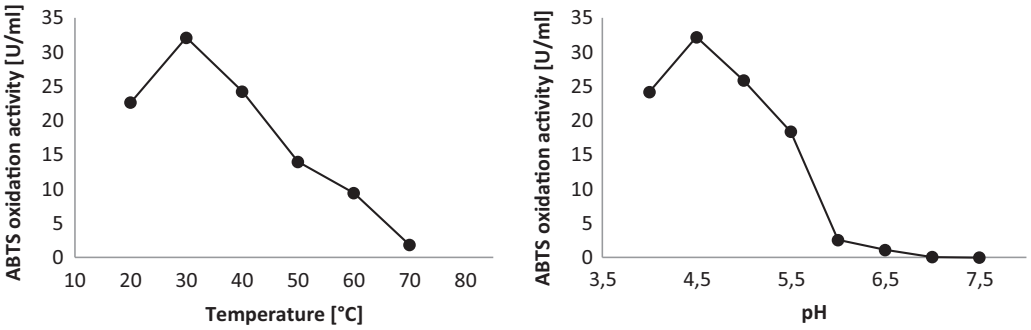

Fig. 1. Laccase-ABTS oxidation activity (a) at pH 4.5 versus different temperatures and (b) at 30 °C in different pH mediums.

slowly added while stirring, and allowed to react for 24 h at 30 °C. The laccase–chitosan conjugates, i.e. pellet was removed from the solution by centrifugation at 5000 rpm for 5 min. The supernatant solution was then cast onto 7 cm-diameter Petri dishes and oven-dried overnight at 45 °C. The dry films obtained were peeled off, thoroughly washed with ethanol and water.

2.8. ATR-FTIR analysis

The spectra of chitosan films, functionalized chitosan with laccase-oxidized tannic acid and quercetin, their initial structure and laccase-synthesized compounds were recorded using a Perkin-Elmer Spectrum One FTIR spectrometer with a Golden Gate ATR attachment and a diamond crystal. The absorbance measurements were carried out within the 650–4000 cm^{-1}, with 16 scans and a resolution of 4 cm^{-1}.

2.9. Antioxidant activity

The antioxidant activity was determined by mixing 1 mg of the test samples and a 300 μL $ABTS^{\bullet+}$ free radical solution prepared by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Before usage, the $ABTS^{\bullet+}$ solution was diluted with phosphate buffer saline (PBS) in order to reach an absorbance of 0.700 ± 0.025 at 734 nm. The inhibition of the $ABTS^{\bullet+}$ radical was monitored at 734 nm and 25 °C, and the percentage of inhibition of this radical was calculated at the end of 10 and 30 min using Eq. (1), where $A_{control}$ is the initial concentration of the $ABTS^{\bullet+}$ and A_{sample} is the absorbance of the remaining concentration of $ABTS^{\bullet+}$ in the presence of the sample. All data are the averages of triplicate experiments.

$$\text{Inhibition or scavenging effect } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (1)$$

2.10. Antimicrobial activity

The antimicrobial properties of the treated samples were evaluated by the American Standard Test Method (ASTM) E2149-01 (standard test method for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions). *Escherichia coli* (ATCC 25992), *Salmonella enterica* (ATCC 13076) a Gram-negative bacterium, *Listeria monocytogenes* (ATCC 7644) a Gram-positive bacterium and *Candida albicans* (ATCC 90028) fungus, were selected due to their resistance to common antimicrobial agents. The incubated test culture in a nutrient broth was diluted using a sterilized 0.3 mM phosphate buffer (KH_2PO_4; pH 6.8) to give a final concentration of $1.5–3.0 \times 10^5$ colony-forming units (CFU)/mL. This solution was used as a working microorganism suspension. The sterilized chitosans' films were cut into small pieces (1 cm × 1 cm) and transferred to a 250 mL Erlenmeyer flask containing 50 mL of the working suspension. All flasks were loosely capped, placed in the incubator, and shaken for 1 h at 37 °C and 120 rpm using a Wrist Action incubator shaker. The initial level of micro-organisms was determined in the following way: a sample (1 mL) was serially diluted and plated (inoculated) in duplicate in nutrient agar. The inoculated plates were incubated at 37 °C for 24 h and the surviving cells were counted. The average values of the duplicates were converted to CFU/mL in the flasks by multiplying by the dilution factor. The antimicrobial activity was expressed in terms of *R*% reduction of the organism after contact with the test specimen compared to the number of bacterial cells of the inoculum before the addition of the test specimen. The percentage reduction was calculated using the following equation (Lim & Hudson, 2004a, 2004b):

$$\text{Reduction rate : } \quad R \ (\%) = \frac{B - A}{B} \times 100 \quad (2)$$

where *A* is CFU/mL for the flask containing test chitosan film samples, and *B* is CFU/mL for the flask used to determine "*A*" before the addition of the test samples.

3. Results and discussion

3.1. Determination of laccase pH and temperature activity optimum

Initially, the optimal conditions for laccase oxidation were investigated with ABTS as a substrate. As shown in Fig. 1 it exhibits high enzyme activity over broad pH and temperature ranges with optimum activity at pH 4.5 and at temperature of 30 °C. The activity decreased with increasing pH and temperature, and according to the optimal activity only 0.015% and 5.6% activity was remained at pH 6.5 and at temperature of 70 °C, respectively.

3.2. Cyclic voltammetry measurements during enzymatic oxidation of tannic acid and quercetin

Tannic acid and quercetin redox properties and the availability of examined compounds to transfer electrons to the electrode during the laccase oxidation were investigated by cyclic voltammetry (CV). CV measurements leading to the determination of physicochemical parameters for antioxidants (e.g. redox potential, number of electrons transferred, etc.) are relevant not only for evaluating the antioxidative abilities but also for understanding their reaction mechanisms.

During an enzymatic oxidation of tannic acid and quercetin, depolymerization and polymerization, respectively, processes occurred, which resulted in a decrease of anodic peak current and new peaks detection after 24 h (Figs. 2 and 3). To confirm that the electrodic processes were associated with tannic acid or quercetin oxidation, experiments were performed with only those two polyphenols, only laccases, and combination of polyphenols

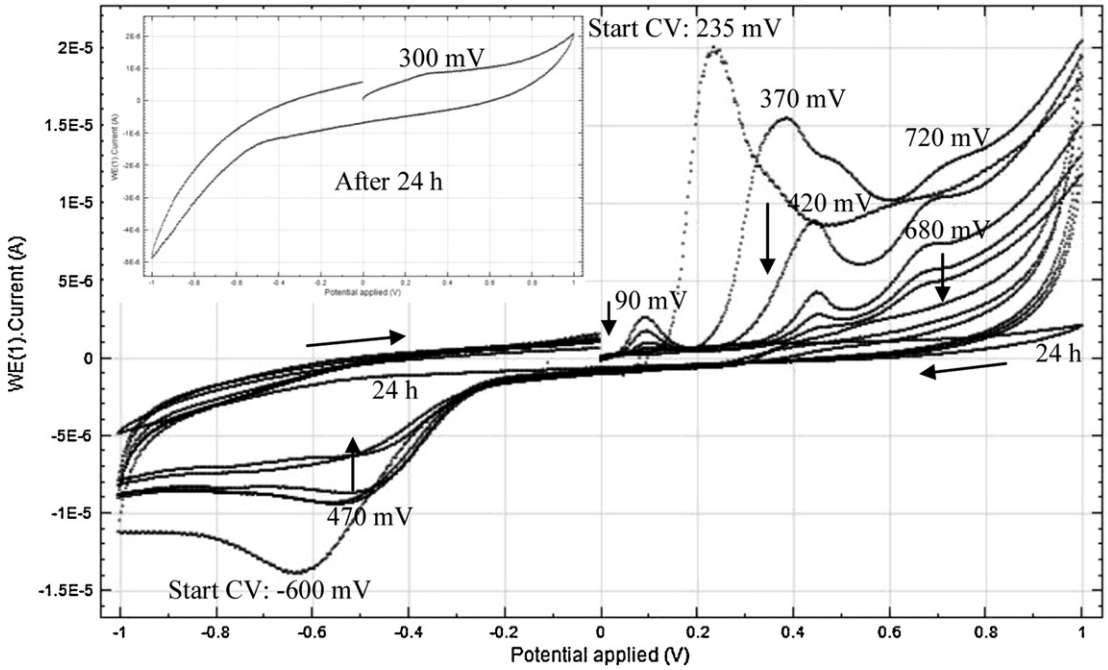

Fig. 2. Time decreasing cyclic voltammograms of 0.1 mM tannic acid in the presence of laccase (35 U) in the 100 mM phosphate buffer, pH 6.5 at 30 °C. Scan rate: 50 mV/s.

and laccases. A characteristic increase in current was observed only in the presence of polyphenols.

0.1 mM tannic acid at pH 6.5, without the presence of laccases, generated a well-defined anodic peak at 253 mV. After laccase addition, cyclic voltammogram profile changed; peak moved to the around 420 mV and new one appeared at around 720 mV (Fig. 2). Newly formed cyclic voltammogram profile corresponds to the gallic acid profile at pH 6.5, reported by our previous work (Božič, Gorgieva, & Kokol, 2012).

Free hydroxyl groups on the glucose or partially gallic acid esterified glucose were oxidized and detected with anodic peak at 90 mV after 2 h with laccase incubation. Conformation of glucose peak was performed with separate 0.1 mM glucose solution at pH 6.5 (data not shown). All new formed peaks, indicating converted molecules, decreased with time and after 24 h only small irreversible anodic peak at 300 mV was detected. During enzymatic oxidation tannic acid undergoes aggregation processes, by which electrode surface was blocked or limited, but remained dimers of gallic acid were still able to be detected at 300 mV. No new polymeric products were detected with CV, which is in accordance with the SEC and ATR-FTIR results. Proposed tannic acid oxidation mechanism, catalyzed by laccases is shown in Fig. 4.

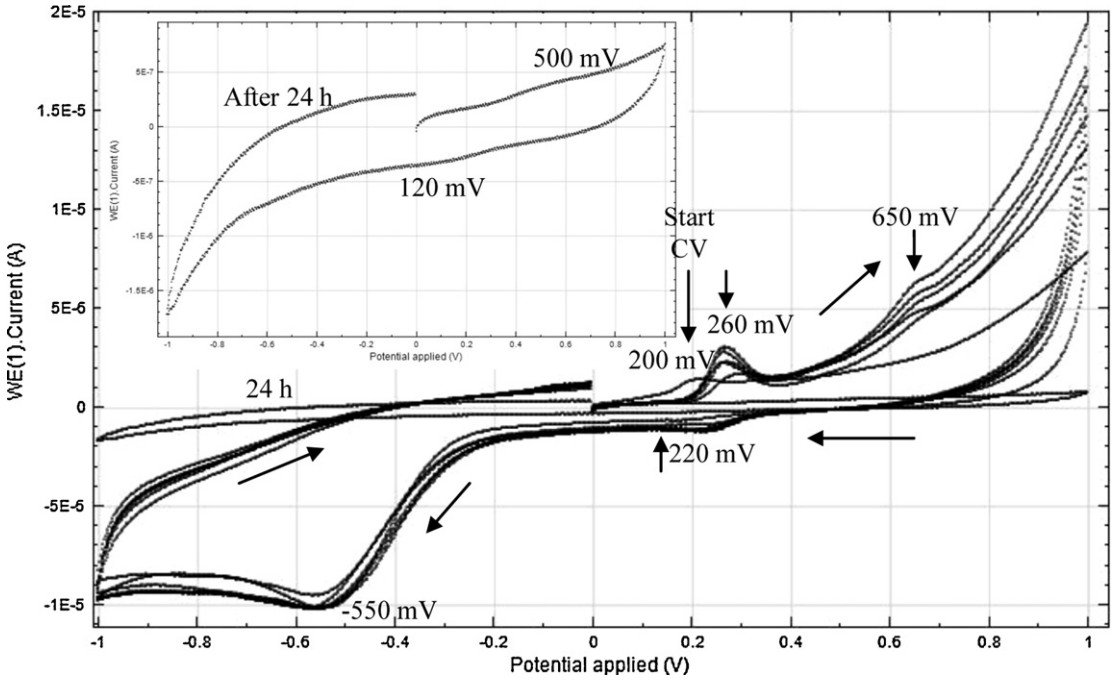

Fig. 3. Time decreasing cyclic voltammograms of 0.1 mM quercetin in the presence of laccase (35 U) in the 100 mM phosphate buffer, pH 6.5 at 30 °C. Scan rate: 50 mV/s.

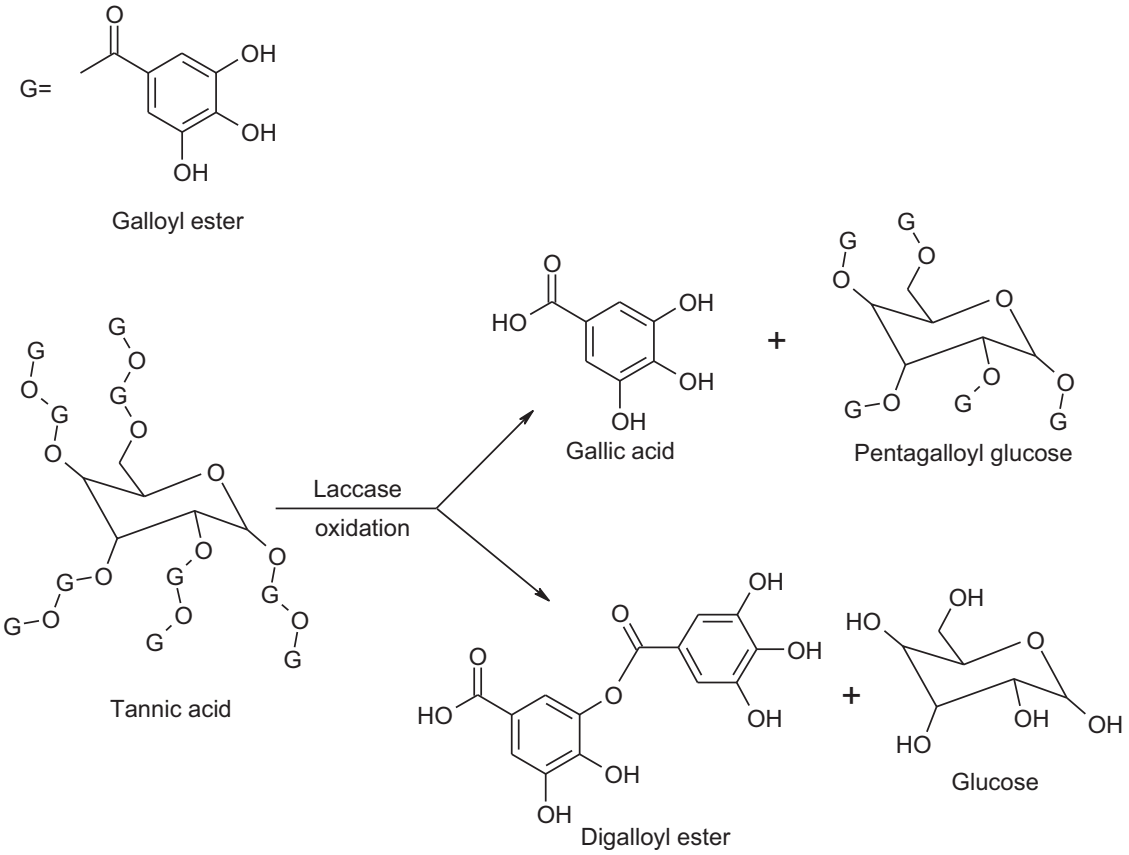

Fig. 4. Proposed tannic acid oxidation mechanism, catalyzed by laccases.

The cyclic voltammograms of 0.1 mM quercetin alone and in the presence of laccase in 100 mM phosphate buffer pH 6.5 are shown in Fig. 3. Oxidation can occur either in a single one-electron, single two-electron or in two one-electron steps. Under these experimental conditions, one reversible single two-electron two-proton process at 200 mV (versus Ag/AgCl) was observed for quercetin without the presence of laccases, which corresponds to the two electron oxidation of the *ortho* 3′,4′-dihydroxy substitute on B-ring of quercetin leading to the formation of the unstable *ortho*-quinone structure (Simić, Manlović, Šegan, & Tedorović, 2007). Because of instability of the active *ortho*-quinone only its partial reduction is observed at 220 mV reverse cathodic reduction peak (Timbola et al., 2006). In the presence of laccase, a significant change in the shape of the corresponding signal was observed. Peak at 200 mV increased and moved to 260 mV and new one appeared at 650 mV. A plausible interpretation for the peak at 260 and 650 mV is that laccase forms an electrochemically active and unstable product, i.e. laccase catalyzes the removal of hydrogen atoms from hydroxyl groups of the *ortho* 3′,4′-dihydroxy substituent on the B ring and *meta* 5,7-dihydroxy substituent on the A ring, respectively, and therefore it improves solubility and readiness for electrochemical reactions taking place at the electrode/solution interface. In addition, the formation of a visible brown compound during the laccase oxidation, indicated the formation of *ortho*-quinone or its tautomers from quercetin, which can undergo various polymerization processes between themselves and thus producing oligomeric/polymeric compounds with complicated structures (Timbola et al., 2006). With time cyclic voltammograms decreased and after 24 h peak at 260 mV disappeared, suggesting consumption of quercetin in solution, and hardly detectable peak at 500 mV appeared, indicating new electro-active polymerized compound generated by the enzymatic reaction.

3.3. SEC analysis of laccase catalyzed products at pH 6.5

Laccase activity at pH 6.5 was remarkably decreased (Fig. 1), therefore, enzymatic coupling ability of phenolic units under mild reaction conditions, giving opportunity for environmentally benign system, has been investigated. Molecular weights of the quercetin oligomers/polymers obtained by laccase oxidation/polymerization at pH 6.5 were estimated by SEC (Table 1).

The hydroxyl groups of B and A-ring in quercetin, possessing electron donating properties and being a radical target, formed phenolic radicals by laccase catalysis. The phenolic radicals react with a second radical to form *o*-quinones, which are highly reactive electrophilic molecules and spontaneously polymerize in a non-enzymatic pathway. In spite of very low activity, laccase induced quercetin polymerization in 100 mM phosphate buffer (pH 6.5) at 30 °C and in 24 h yielded the polymers of broad distribution with

Table 1
SEC analysis (average molecular weights (M_w), retention time (Rt) peak areas and degree of polymerization (DP)) of quercetin oligomers/polymers obtained by laccase oxidation at pH 6.5.

	M_w (g/mol)/Rt (min)/area (%)/DP	
M_w (tannic acid) = 1701.20 g/mol	No new higher M_w compound detected	
	900	2300
	5.421	5.134
M_w (quercetin) = 302.24 g/mol	54.34	45.66
	3	8

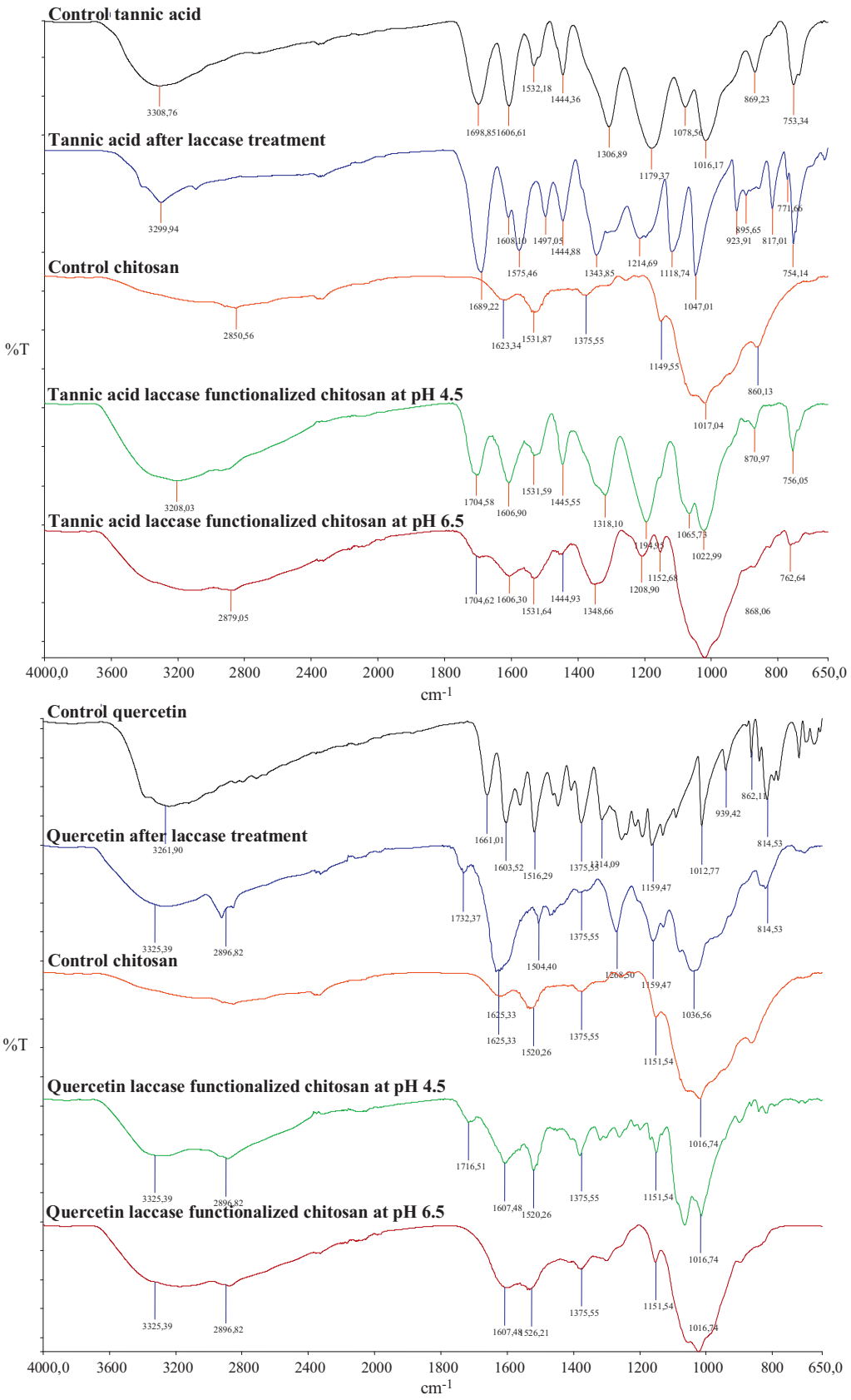

Fig. 5. ATR-FTIR spectra of chitosan, quercetin and tannic acid control compound, quercetin oligomers/polymers, tannic acid aggregates; and chitosan functionalized with quercetin and tannic acid at pH 4.5 and 6.5 at 30 °C for 24 h.

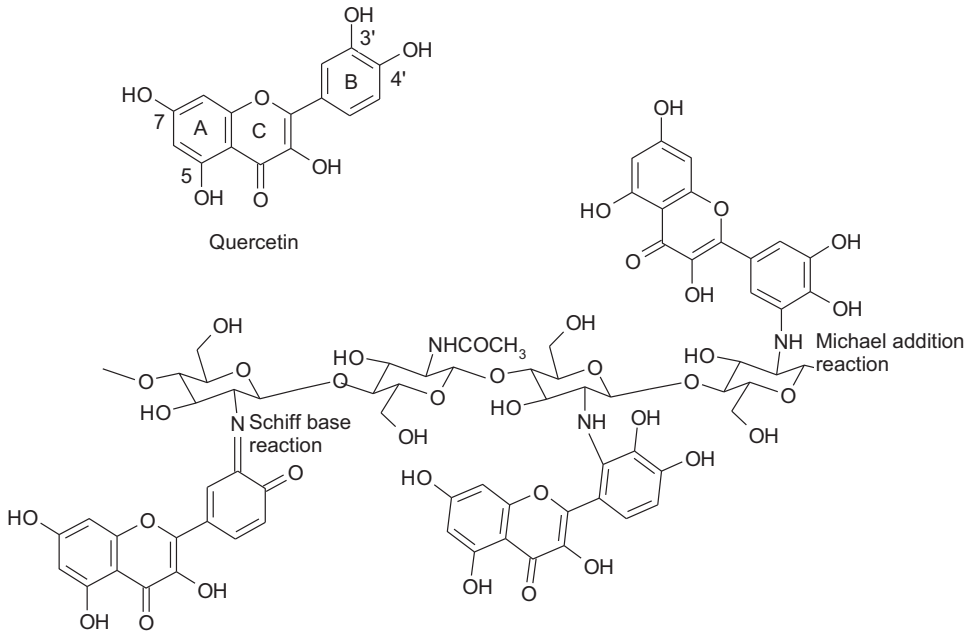

Fig. 6. Proposed structures of the final quercetin functionalized chitosan polymeric products obtained by laccase catalyzed reactions.

DP 3 and 8, indicating similar M_w of newly formed molecules with different arrangements.

In the case of tannic acid the same chromatogram profiles were obtained before and after laccase treatment, indicating an absence of higher M_w polymerization products, also confirmed by CV results. On the other hand, tannic acid-depolymerization products, as a consequence of their low M_w and therefore out-of-range for controlled elution through used column, were not observed.

In addition, it should be noted that both washed and dried polyphenols modified chitosan films, prepared by the heterogeneous and homogeneous method were exposed to the dissolving process in order to determine the molecular weight. However, we were unable to dissolve any of those films in the tetrahydrofuran as organic solvent or water with HCl (pH 3). Laccase oxidized a phenolic compound via a single electron oxidation to form phenolic free radicals in the chitosan solution. The phenolic free radicals coupled with each other to form dimer and polymeric substances. We believe that these hydrophobic phenolic moieties form gel network junctions, contributing to the increase in phenolic hydroxyl groups, which can form stronger hydrogen bonds than a single phenolic hydroxyl group, which could be one of the explanations for the insolubility of the homogeneous method functionalized chitosan-phenolic systems.

3.4. ATR-FTIR analysis of laccase oxidized tannic acid and quercetin and their functionalized chitosans

The ATR-FTIR spectra of the tannic acid and quercetin functionalized chitosans, tannic acid and quercetin control compounds and their laccase oxidized products are shown in Fig. 5.

The quercetin oligomers/polymers spectra exhibit a different profile compared to the monomers. It has typical polyphenol characteristics, showing a broad peaks centered at 3330 and 1375 cm^{-1} due to the vibration of O—H linkage of phenolic and hydroxyl groups, at 1450–1600 cm^{-1} due to the aromatic ring C=C stretching, and C=O stretching vibration at 1200–1300 cm^{-1}, respectively. The peak at 1000–1150 cm^{-1}, corresponding mainly to ethers (C—O—C), increased compared to phenolics monomers spectra, indicating extended polymerization (Yamada, Abe, & Tanizawa, 2007). Furthermore, the vibration band of carbonyl stretching appeared in the region 1730–1750 cm^{-1} for the quercetin oligomers/polymers spectra, while in the monomer spectra they are missing. This can be attributed to the intermediate step involving the formation of a quinone, i.e. *o*-quinone in C3′ and C4′ or in C5 and C7. New strong peaks at 1625 and 1730 cm^{-1} are probably due to the quinone moiety absorption, detectable also when oxidation reaction was performed in chitosan solution at pH 4.5 (Fig. 5) (Desentis-Mendoza et al., 2006). Besides, the disappearance of the characteristic fingerprint region for both monomers in polymer spectra can be observed.

The ATR-FTIR spectrum of the tannic acid soluble aggregates, formed by the reaction of laccase with a 10 mM tannic acid solution at pH 6.5 is similar to the tannic acid control showing a broad absorption band between 3600 and 2500 cm^{-1}, which is due to the presence of numerous hydroxyl groups. There are some strong bands typical of poly(hydroxyl) compounds assigned to OH bending and C—OH stretching vibrations, and several other peaks characteristics of skeletal vibrations, aromatic compounds and substituted benzene rings. However, some distinctive features have also been observed as possible consequence of the tannic acid depolymerization. Several peaks are not only shifted slightly, but also reduced or increased in intensity. The C=O stretching band of the carbonyl group at around 1700 cm^{-1} (tannic acid control) increased and shift to lower wave numbers at around 1690 cm^{-1} for tannic acid aggregates. Some bands related to the hydroxyl groups have been transformed drastically. The strong OH bending at 1606 cm^{-1} become very broad and split into two new, small one at 1608 cm^{-1} and larger one at 1575 cm^{-1} due to the stronger amorphous nature of the aggregates (Iglesias, Garcia de Saldana, & Jaen, 2001). Unfortunately these spectral changes cannot be definitively interpreted because these signals overlap with the NH stretching peaks and amide peaks associated with proteins, which could be present as tannic acid phenolic groups are an excellent hydrogen donor that forms strong hydrogen bonds with the protein's carboxyl group. Furthermore, peaks located in the region of 900–650 cm^{-1} are appreciably changed, which signifies that the substituents on benzene ring are changed, i.e. release of bonded gallic acid dimers (Chen, Li, Lian, & Jiang, 2009). No new gallic acid polymerization products were detected by ATR-FTIR or SEC, which is probably due to the limited accessibility of laccase to gallic acid residues that

are subjected to steric hindrance of the still present bulky dimer of gallic acid esterified to glucose.

Untreated chitosan spectrum (control) show strong absorption band in the 3200–3500 cm^{-1}, attributed to —OH and —NH stretching and signals at about 1633, 1532 and 1380 cm^{-1} attributed to the amide I, II, and III modes of the residual N-acetyl groups, respectively. The absorption band at 1150 cm^{-1} (anti-symmetric stretching of C—O—C bridge) and at 1080 cm^{-1} (skeletal vibration involving the C—O stretching) is characteristic of chitosan saccharide structure (Muzzarelli, Tanfani, Scarpini, & Laterza, 1980; Sousa, Guebitz, & Kokol, 2009). The ATR-FTIR spectrum of quercetin grafted chitosans at pH 6.5 showed changes involving shoulder of the 1650 cm^{-1} band, attributed to the C=N stretching mode of imines, supporting the occurrence of a Schiff-base reaction. Analogously, the Michael addition-type reaction can be supported by the trend of the conversion of some primary amines into secondary amines, NH stretching at 3300–3500 cm^{-1} and NH bending around 1530 cm^{-1}, which became broader due to an increase in the aromatic and non-aromatic stretching hydroxyl groups of quercetin polyphenol, providing direct information on its structural changes occurring during the reaction (Muzzarelli & Muzzarelli, 2002). The expected schematic coupling reaction between reactive *o*-quinones enzymatically produced from quercetin and reactive amine groups in chitosan structures is shown in Fig. 6.

In the case of quercetin loaded in chitosan solution at pH 4.5, characteristic bands of free quercetin monomer, associated with aromatic bending and stretching (around 1100 and 1600 cm^{-1}), —OH phenolic bending (1200–1400 cm^{-1}) disappeared, while the absorption peaks assigned to —OH stretching (3200–3550 cm^{-1} and 1365–1375 cm^{-1}), C=O stretching (1625 and 1730 cm^{-1}), aromatic ring C=C stretching (1450–1600 cm^{-1}), C—O stretching vibration (1200–1300 cm^{-1}) and C—O—C stretching (1000–1150 cm^{-1}) of quercetin oligomers/polymers appeared which confirms the undisturbed quercetin polymerization induced by laccases in the 1% chitosan solution at pH 4.5. Furthermore, spectra of chitosan with incorporated quercetin in the presence of laccases show covered pattern on their informative peaks as the spectra of the control chitosan and quercetin after laccase treatment. This indicates that there is no interaction between active groups of oxidized quercetin with functional groups of chitosan at pH 4.5.

When chitosan films were exposed to tannic acid in the presence of laccases at pH 6.5 or in the chitosan solution at pH 4.5, tannic acid characteristic bands are present without new ones, representing proof that the tannic acid was included (adsorbed) into (onto) chitosan polymer without covalent bonding. Increased free —OH groups of gallic acid compounds resulted into broadened band in the region of 3200–3500 cm^{-1}. Additionally, tannic acid is able to crosslink chitosan by forming multiple hydrogen bonds. Formation of H bonding resulted in a proton donor group to lower frequency values, i.e. 1623 cm^{-1} amide I of chitosan to 1607 cm^{-1} for treated chitosan at both pHs.

3.5. Antioxidant activity of tannic acid and quercetin functionalized chitosan

Antioxidant capacity of products obtained by laccase oxidation of polyphenols and of chitosan films functionalized by polyphenols was studied following $ABTS^{\bullet+}$ cation decolorization assay method. Fig. 7 shows the reducing power of the control tannic acid and quercetin compounds, their laccase reaction products and by them functionalized chitosans through the time.

All tested samples showed strong inhibitory capacity to the $ABTS^{\bullet+}$ cation radicals; however the highest was for quercetin control, its oligomers/polymers and its functionalized chitosans. In general, the activities of flavonoids are known to be limited for only few hours in vivo, although the reaction mechanisms have

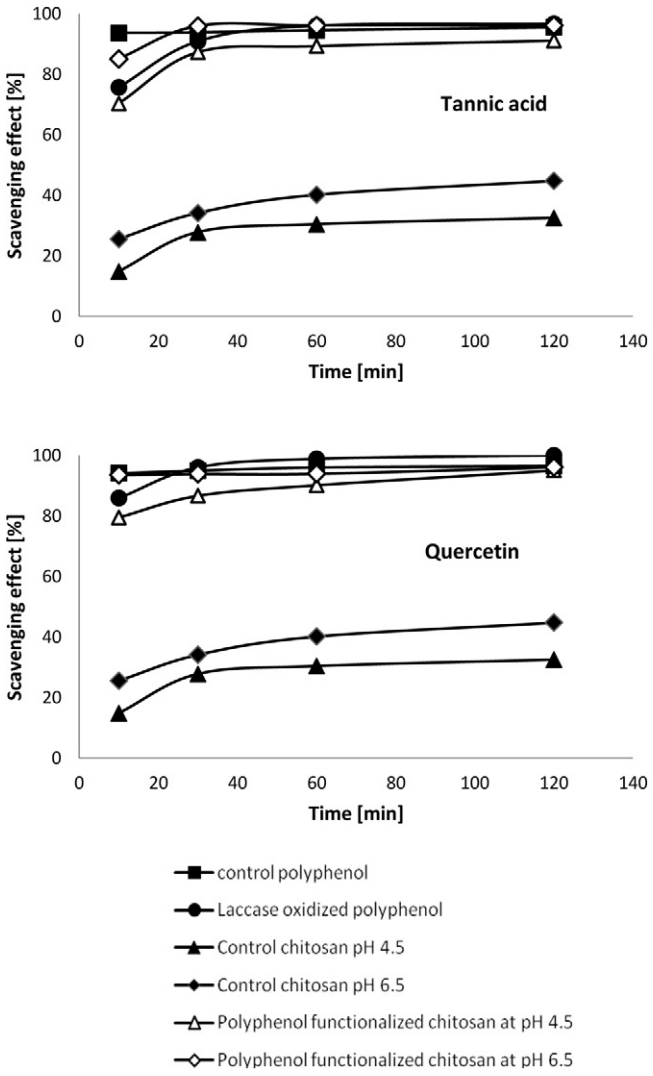

Fig. 7. Time dependent radical inhibition effects of control polyphenolic compounds and their laccase reaction products (reaction conducted at 10 mM polyphenols concentration in 100 ml phosphate buffer (100 mM) pH 6.5 with 153 U of laccase for 24 h at 30 °C under constant stirring), and by the functionalized chitosans at pH 4.5 and 6.5.

not been established yet. In contrast, a relatively high molecular fraction of polymerized flavonoids has been reported to exhibit enhanced physiological properties, such as antioxidant capacity, and a relatively longer circulation time (Kurisawa, Chung, Uyama, & Kobayashi, 2003). An increase in antioxidant capacity can be observed for both, quercetin monomeric control and its oligomeric/polymeric compounds during the time. Despite the lower quercetin oligomeric/polymeric antioxidant capacity at the beginning, only after 30 min, the capacity was higher compared to their monomeric derivates. High insolubility and steric hindrance of the poly(quercetin) might be the reasons that quercetin monomer is more potent free radical scavenger at the beginning of measurements than its polymer. For monomeric flavonoids, the ability to act as antioxidants is dependent on extended conjugation, and molecular weight. The higher scavenging capacity of the poly(quercetin) against free radicals could be attributed to creation of high concentration of phenolic moieties in the molecules. Moreover the reducing power of laccase oxidized tannic acid was lower at the beginning of measurement, but reached higher value after 2 h than tannic acid control. In general, the reducing properties

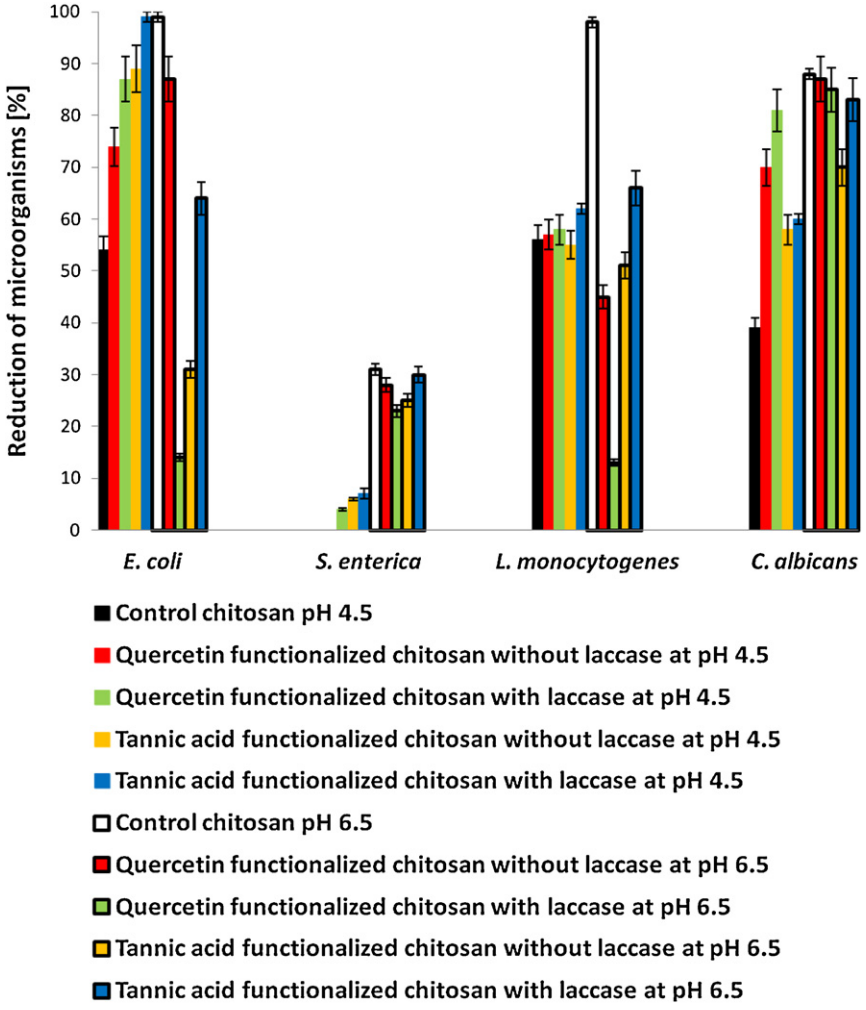

Fig. 8. Antimicrobial/antifungal activity of quercetin and tannic acid functionalized chitosan at pH 4.5 and 6.5 with and without laccase.

are associated with the presence and accessibility of reductones which exhibit antioxidant action by breaking the free radical chain by donating a hydrogen atom. The stronger amorphous nature of the tannic acid aggregates after laccase oxidation limits the reductones accessibility, but reproduced hydroxyl groups after converted tannic acid indicates higher antioxidant capacity.

In the case of the native chitosans, low reduction of $ABTS^{\bullet+}$ (~40%) was observed after 2 h and this may be partly due to the inhibition of radical scavenging because of strong inter- and intra-molecular hydrogen links. The antioxidant ability in scavenging the $ABTS^{\bullet+}$ of quercetin and tannic acid functionalized chitosans at pH 4.5 were 95% and 91%, respectively, and functionalized at pH 6.5 was for both 96%. The scavenging activity of all polyphenols functionalized chitosan derivatives was more than two times higher than native chitosan. Thus, an enhancement of the antioxidant activity in the chitosan derivatives with the products of laccase-catalyzed oxidation of quercetin or tannic acid was demonstrated.

3.6. Antimicrobial activity of tannic acid and quercetin functionalized chitosan

Fig. 8 presents the antimicrobial/antifungal properties of control chitosan and polyphenols functionalized chitosan samples with and without laccase addition.

Antimicrobial reduction of the control chitosan pH 4.5, quercetin and tannic acid functionalized chitosan with and without laccase by homogeneous method at pH 4.5, are similar (i.e. for *L. monocytogenes*) or improved (i.e. for *E. coli* and *C. albicans*), indicating that the incorporation of quercetin and tannic acid into chitosan did not result in loss of antimicrobial efficiency. When using homogeneous functionalization method, availability of positively charged amino groups in the chitosan former, which makes it a bioadhesive for negatively charged microbial surfaces, is not disturbed by covalent coupling and the presence of quercetin and tannic acid made chitosan even more antimicrobial effectively due to the their ability to diminish bacterial cell regeneration (Cushnie & Lamb, 2005). As discussed above, when functionalization proceeded by heterogeneous method at pH 6.5, functional group in chitosan was covalently cross-linked with *o*-quinones (Muzzarelli, Ilari, Xia, Pinotti, & Tomasetti, 1994; Muzzarelli, Littarru, Muzzarelli, & Tosi, 2003) thus, the antimicrobial properties of the chitosan after quercetin grafting and even in the case of tannic acid surface adsorption were comprised. However, quercetin and tannic acid alone also have antifungal and antimicrobial properties (Cushnie & Lamb, 2005; Fras-Zemljič, Kokol, & Čakara, 2011; Widsten et al., 2010). The hydrophobicity of quercetin enables them to interact with the bacterial cell membrane. This structural distortion causes deterioration of the membrane and increases membrane permeability. In addition, it has been postulated that

phenolic components suppress the calcium and potassium transport by partitioning in the lipid phase of the membrane and subsequently altering the local environment of calcium channels (Altiok, Altiok, & Tihminlioglu, 2010). The free hydroxyl group on the benzene ring is deemed essential for the antifungal and antimicrobial activity of both quercetin and tannic acid, and since this group is not affected by the functionalization process, the phenol group should partially compensate for the "lost" antifungal/antimicrobial properties of the amine group on native chitosan. However, the functionalization at pH 6.5, regardless of the polyphenol used, caused a reduction of the antimicrobial effect compared to control chitosan. It should be noted that amount of grafted polyphenols is not controlled and additional work is required to determine the precise effect of polyphenol quantum on antimicrobial activity. The most resistant tested microorganism or insufficient reduction in all tested functionalized chitosan films was with *Salmonella enterica*. These findings match with our previous work (Božič et al., 2012).

However, the highest antimicrobial effect, i.e. against *E. coli* was achieved when by laccase oxidized tannic acid at pH 4.5 was used in the functionalization process. According to Chung, Stevens, Lin, and Wei, (1993), the ester linkage between gallic acid and glucose is important for the antibacterial properties of gallotannins; they found tannic acid to strongly inhibit Gram-positive and Gram-negative bacteria while gallic acid or ellagic acid showed reduced effect. In fact, tannins are often far more toxic to microorganisms than monomeric compounds such as gallic acid or catechin related to them but this cannot be generalized for all microorganisms.

4. Conclusions

In this work, a laccase from *T. versicolor* was used to initiate the oxidation of two polyphenols, tannic acid and quercetin. Laccase efficiently induced the polymerization of quercetin, yielding new oligomeric/polymeric products, while tannic acid underwent depolymerization process with converted tannic acid molecules, i.e. gallic acid, gallic acid dimers, partially gallic acid esterified glucose and glucose. This enzymatic reaction was carried out under mild operational conditions (at 30 °C and pH 6.5), with the quercetin and tannic acid dispersed and dissolved, respectively, in the reaction medium, without harsh acidic solubilization and without organic solvent. Both laccase induced quercetin and tannic acid products, compared to their controls, showed increased antioxidant capacity against $ABTS^{\bullet+}$ cation radicals.

Furthermore, chitosan was functionalized (using homogeneous method at pH 4.5 and heterogeneous method at pH 6.5) with the oxidative products of quercetin or tannic acid catalyzed by the laccase.

In summary, the laccase oxidized quercetin to reactive radicals and under heterogeneously functionalization conditions, radicals covalently bound onto the chitosan free amine groups by Schiff-base and Michael addition reactions. The presence of chitosan in the homogeneously reaction medium had no influence on the oxidative rate of quercetin, but it did hinder covalent coupling due to the protonation state of the chitosan amines at pH 4.5. On the other hand, oxidized tannic acid strongly cross-linked with chitosan only by hydrogen and electrostatic interactions under both used functionalization conditions.

All quercetin or tannic acid functionalized chitosans exhibited greatly amplified $ABTS^{\bullet+}$ cation radicals scavenging capacity compared to untreated chitosan. Furthermore, the effectiveness of the quercetin and tannic acid functionalized by both procedures was tested against *E. coli*, *S. enterica*, *L. monocytogenes* and *C. Albicans*. It has been shown that in general by homogeneous method functionalized chitosans were more effective against *E. coli* and *C. albicans*, while heterogeneous method of chitosan functionalization induced the reduction in antimicrobial activity against all tested microorganisms.

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