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Chemo-enzymatic preparation and characterization of renewable oligomers with bisguaiacol moieties: promising sustainable antiradical/antioxidant additives†

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The synthesis, structural characterization and properties of new bio-based oligomers with bisguaiacol-type moieties esterified by diverse aliphatic linkers are described. These oligomers, produced by oxidative oligomerization of renewable dihydroferulic acid-based bisphenols with commercially available *Trametes versicolor* laccase, are proposed as promising sustainable antiradical/antioxidant additives for polymers. This environmentally friendly biocatalyzed process is performed under very mild conditions in open vessels and aqueous solution at room temperature. Modifications of the reaction conditions (use of an organic co-solvent up to 80% v/v and increase of the reaction temperature up to 60 °C) revealed that the polymerization degree of the oligomers may be controlled by adjusting the nature and the ratio of the co-solvent, the reaction time and the reaction temperature. Thermal analyses (TGA and DSC) demonstrated that these phenolic oligomers exhibit high thermal stability and that their T_d 5% and T_g can be easily tailored by playing with both the structure of the bisphenol and the degree of polymerization. Similarly, these phenolic oligomers exhibit tunable potent antiradical/antioxidant activity as shown by DPPH analyses. These aliphatic–aromatic oligomers with bisguaiacol-type moieties are thus promising as easily accessible, eco-friendly antiradical/antioxidant additives for the stabilization of polymers in packaging and other applications.

Introduction

In the last decades, there has been an increasing demand on the part of both consumers and industries for non-toxic and renewable synthons that could be used to replace fossil-based building blocks for the preparation of additives, polymers and resins.[1–6] In this way, there have been many reports on the use of biobased phenolic functional polymers acting as flame retardants or antioxidants in polymer matrices.[7–10]

The addition of phenolic antioxidants is the most convenient and effective way to keep polymers, such as polyolefins and polyesters, from thermo- and photo-oxodegradation. It is well known that hindered phenolics can be used as antioxidant additives for packaging polymers.[11–13] Moreover, they can act to prevent oxidative deterioration of packaged food products and thus may induce major economic and health benefits. To prevent oxidation, antioxidant additives, such as butylated hydroxyanisole (BHA), 3,5-di-*t*-butyl-4-hydroxytoluene (BHT), *t*-butylhydroquinone (TBHQ), and propyl gallate, have been used in food packaging polymer formulations. However, issues regarding their potential toxicity due to mutagenic, endocrine disruption and carcinogenic activities have been raised as such small phenolic antioxidants may be released from the polymer matrices when in contact with aqueous media.[14] Therefore, in order to advantageously replace these hazardous additives, non-toxic natural small phenolics (*e.g.*, caffeic acid, natural rosemary extracts, ascorbic acid, α-tocopherol, curcumin, and flavonoids) have been incorporated into various packaging materials.[15–23]

A new trend for antioxidant development is to prepare high molecular weight antioxidant additives through the co- or

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homopolymerization of a functional monomer bearing hindered phenolic antioxidants.[24,25] The expected advantages of such phenolic polymers or oligomers include lower volatility, greater chemical and thermal stability, and a lower tendency to be released from the polymer matrix into the contact medium (*e.g.*, food, liquid). Monomeric antioxidants reported in the literature are based on acrylate derivatives[25] and maleimide derivatives[26] of BHT, caffeic acid,[27] or *p*-hydroxyphenylacetic acid.[28]

A greener approach based on the use of lignins derivatives as sustainable and biocompatible antioxidants for polymer stabilization, and more particularly in packaging, is also becoming increasingly popular.[29] Found in all vascular plants, lignins are natural crosslinked polyphenolic materials and account for approximately 30% of the organic carbon in the biosphere. Obtained from the oxidase-catalyzed polymerization of three *p*-hydroxycinnamic monomeric alcohols (aka monolignols) exhibiting various substitutions (*p*-coumaric, coniferylic and sinapylic alcohols),[30] lignins thereby exhibit promising concentrations of hindered free phenols conferring them with potent antioxidant properties, due to their radical scavenging capacity.[31–35] Nevertheless, although interesting antioxidant activities have been reported for a wide range of lignins,[36–39] they are negatively impacted by lignins' heterogeneity and by the presence of byproducts (*e.g.*, carbohydrates), these structural factors being linked not only to their botanical origin, but also to the industrial processes used for the fractionation of the lignocellulosic biomass.[31,33,34] In view of these drawbacks, the use of industrial lignins as antioxidant additives, although being of economical interest, remains hazardous. Therefore, the use of low molecular weight phenolics derived from lignocellulosic biomass for the preparation of synthetic pure oligomers with a high content of free phenols appears to be a very promising sustainable approach to bio-based antioxidants.

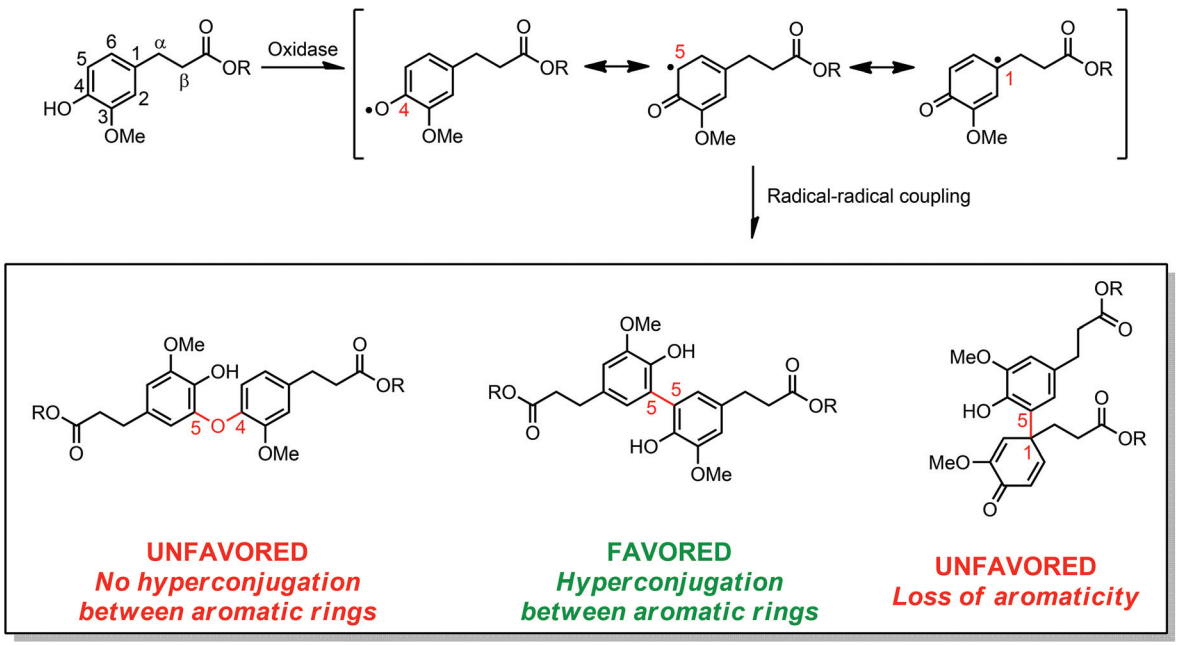
While linear polyphenolic structures can be efficiently synthesized through classical polymerization techniques,[24–26] enzymatic-catalysis with peroxidases[27,28,40–42] and laccases[43–49] has also been successfully used for the polymerization of phenolic monomers. For instance, Ambrogi *et al.*[27] efficiently homopolymerized methyl caffeate in presence of horseradish peroxidase. However, only one of the two free phenol groups of the monomer was preserved in the resulting linear polyphenolic polymer, lowering the antioxidant potential of the material. As a matter of fact, enzyme-mediated oxidation of phenolics promotes the formation of the corresponding phenoxy radicals that can lead to various regioisomeric radicals, depending on conjugation features, recombination of which may result in cross-linked networks with different coupling patterns such as C–C and C–O bonds, as observed in native and industrial lignins.[50] The preparation of well-structured linear polyphenols *via* oxidase-catalyzed polymerization remains therefore a synthetic challenge.

Ferulic acid, one of the three *p*-hydroxycinnamic acids found in lignocellulosic biomass is a sustainable and valuable chemical feedstock. Present in relatively large quantities in wheat and rice brans as well as in sugarbeet pulp or sugarcane bagasse, its production at the industrial scale from these biorefineries and food industries byproducts is currently being investigated, particularly in our team. According to the best estimates, prices could be as low as $1–3 per kg, a reasonable target price for a commercial monomer. Ferulic acid can also be readily synthesized from vanillin, a phenolic compound industrially produced from lignins (sale price *ca.* $6–15 per kg); however this synthetic pathway requires extra synthetic and purification steps leading to a higher production cost. In order to understand the mechanisms of biocatalytic oxidation of natural phenolics, we have previously developed an expertise in lignin related phenol chemistry,[51–53] that allowed us to design a new methodology for the production of renewable bisphenols through a lipase-mediated process to efficiently esterify dihydroferulic acid — a derivative of ferulic acid — with various bio-based aliphatic polyols[54] such as isosorbide, 1,3-propanediol, 1,4-butanediol and glycerol, four commercially available compounds with prices in the range of $1–5 per kg. After having demonstrated that these bisphenols can be used as monomers for the synthesis of high thermal stability polymers, whose T_g could be easily tuned by adjusting the nature of the polyol,[55–58] we investigated their potential as antiradical/antioxidant additives. Structure–activity relationship studies of a library of bis- and trisphenols based on ferulic acid and sinapic acid showed that such compounds possess antiradical/antioxidant activities similar to that of Irganox® 1010, a widely used commercially available antioxidant additive in polypropylene.[59]

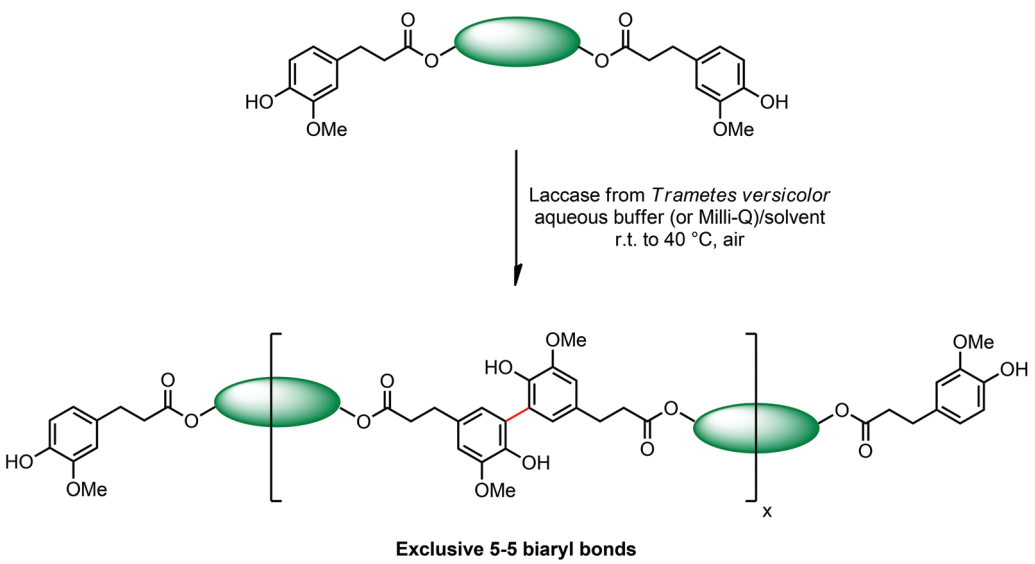
We then turned to the use of these bisphenols as phenolic monomers for the preparation of renewable linear polyphenolic oligomers through oxidative oligomerization (oxidation of the phenol to the phenoxy radical and subsequent radical–radical coupling). Indeed, because of their specific structural design (*i.e.*, no benzylic unsaturation and only one methoxy group in the *ortho*-position to the phenol), these bisphenols were expected to preferentially oligomerize *via* a regioselective 5–5′ biaryl bond as described in Scheme 1. The resulting phenolic oligomers with bisguaiacol moieties should exhibit not only high thermostability (highly conjugated bisguaiacol moieties) and biodegradation ability (internal ester bonds of the starting bisphenols), and lower diffusion capability, but also high antioxidant properties due to the presence of one phenol on each aromatic ring along the oligomeric chain.

As we dedicate ourselves to the production of renewable materials through sustainable synthetic processes, an oxidase-catalyzed oligomerization was envisaged. Peroxidases[27,28,40–42] and laccases,[43–49,60,61] which are involved in lignin biosynthesis and oxidative degradation, are known to initiate such cascade reactions. Compared to peroxidases that require a harsh co-oxidant (H_2O_2), laccases prove more suitable catalysts at an industrial scale since they use oxygen from the air as co-oxidant, allowing the reactions to be performed in open reactors.

In this paper we report a facile biocatalytic access to structured linear polyphenolic polymers with bisguaiacol-type



Scheme 1 Anticipated regioselectivity of the oxidative radical–radical coupling of dihydroferulates.



Scheme 2 Laccase-catalyzed oligomerization of dihydroferulic acid-based bisphenols through exclusive 5–5 biaryl bonds.

moieties *via* a convenient laccase-catalyzed oligomerization of renewable dihydroferulic acid-based bisphenols (Scheme 2).[62] The thermal properties and antioxidant activities of the resulting phenolic oligomers are also presented.

Materials and methods

Materials

All reagents, HCCA (α-cyano-4-hydroxycinnamic acid), *Trametes versicolor* laccase (ref: 51639-5G, lot result 13.6 U g^{-1}) were purchased from Aldrich Chemical Co. and were used as received. Compounds were purified on a Puriflash 430 purchased from Interchim, using Si–OH phase columns.

Laccase activity assay with ABTS

Laccase activity was determined by the ABTS method.[63] The nonphenolic dye ABTS is readily oxidized by the enzyme into the cation radical at acidic pH. The concentration of the cation radical responsible for the intense blue-green color is given by the UV absorption at 420 nm and can be correlated to enzyme activity. Oxidation of ABTS was monitored by measuring the

absorption at 420 nm (A_{420}) with a molar extinction coefficient of 3.6×10^4 M^{-1} cm^{-1} (ε_{420}). The reaction mixture contained 0.9 mL of 1.0 mM substrate (ABTS), 2.0 mL of 0.1 M sodium acetate buffer (pH 4.5), and 0.1 mL of a 50 mg L^{-1} laccase solution. Absorbance was read at 420 nm in a spectrophotometer against a suitable blank. One unit (U) was defined as the amount of the laccase that oxidized 1 μmol of ABTS per min. The ABTS assay on the laccase used in this work revealed an activity of 13.6 U g^{-1}.

Characterization

^1H, ^{13}C and HSQC NMR spectra were recorded at 20 °C in d_6-acetone on a Bruker Advance at 400 MHz and 100 MHz respectively; reference was given by the residual undeuterated acetone peak at 2.05 ppm in the case of ^1H, and d_6-acetone peaks at 205.87 and 30.6 ppm in ^{13}C; δ are given in ppm. TGA and DSC were recorded under an inert atmosphere at 10 °C min^{-1} on a Q500 and a Q100 from TA Instrument respectively.

GPC analysis

Gel Permeation Chromatography (GPC) was performed at 25 °C on a GPC system equipped with a Gilson 305 pump, an Ultimate 3000 ACC Dionex© injector, an Ultimate 3000 PDA Dionex© detector at 254 nm and PLgel columns purchased from Agilent Technology (300 × 7.5 mm, 5 μm, 300 Å) in THF (stabilized with BHT) (flow rate 1 mL min^{-1}) using Igepal® standard (441, 735 and 1983) purchased from Aldrich Chemical Co.

MALDI-TOF analysis

Absolute masses were determined by MALDI-TOF technology using an HCCA matrix (10 mg mL^{-1} in acetonitrile/water (80 : 20, v/v) with 0.1% TFA). Dried samples were diluted in acetonitrile/water (80 : 20, v/v) with 0.1% TFA to obtain a 10 mg mL^{-1} solution. 1 : 1 sample : matrix solutions (1 μL) were spotted online on MALDI target.

Samples analyses were realized on a MALDI-TOF Reflex™ III (Bruker Daltonik) using the reflectron operating mode. All spectra were obtained in the positive ion mode at 20 kV and ionization was performed with a 337 nm pulsed nitrogen laser (8 Hz). Spectra were obtained by the accumulation of at least 600 laser shots and calibrated using the statistical method of FlexAnalysis software (Bruker). All samples were spotted in triplicate.

General procedure for the laccase-catalyzed oligomerization of bisphenols

Bisphenol (6, 25, 30 or 40 g L^{-1}) was dissolved in a suitable co-solvent (among 21 different organic solvents have been tested, see Fig. 4) before adding phosphate/citrate buffer (pH 2.3, 2.9, 3.4, 3.7, 4.2, 5.6, 7.7, 8.8) or Milli-Q water (pH 5.2). A solution of *Trametes versicolor* laccase in phosphate citrate buffer (or Milli-Q water) at different loading (2, 10, 100, 1000 U per $mmol_{substrate}$) was then added to the reaction mixture, to finally reach volumetric ratios of co-solvent/aqueous phase of 1/4, 2/3, 3/2, and 4/1. The reaction mixture was stirred for a given time (8, 24, 48, 96 or 120 hours) at a given temperature (20, 40, 60 or 80 °C). The reaction was quenched by adding dichloromethane (50 mL). The organic layer was then washed with water (2 × 100 mL) to remove buffer salts, dried over anhydrous magnesium sulfate, filtered and concentrated under vacuum to recover oligomers and residual bisphenol. The crude mixtures were directly analyzed by GPC without further purification to determine the bisphenol conversion and degree of polymerization.

Antioxidant assay using DPPH analysis

Determination of antiradical activity was based on the procedure used by Brand-Williams *et al.*[64] with some modifications. Analyses were performed in a microwell plate at 25 °C under constant agitation and in acetonitrile.

For each compound to be tested, 6 solutions in acetonitrile with different molar concentrations were prepared. DPPH solution in acetonitrile (2105 μmol L^{-1}) was also prepared and stored at 5 °C.

10 μL of each compound solution was added in different wells, and the latter were completed with 190 μL of DPPH solution being added. In order to subtract the absorbance of acetonitrile and the natural reduction of DPPH, a well with 200 μL of acetonitrile and a well with 190 μL of DPPH with 10 μL of acetonitrile were also prepared. The decrease in absorbance was monitored by UV-visible spectroscopy at 520 nm with a microwell plate reader, automatically recording one point every 5 minutes during 7.5 hours. At the end of the UV analysis, each sample of tested compound reached a stable absorbance value, corresponding to a percentage of remaining DPPH at equilibrium. Thanks to this % of remaining DPPH at a steady state we were able to determine the EC_{50} value, corresponding to the amount of tested compound needed to reduce 50% of the initial population of DPPH. EC_{50} values will then allow us to compare the antioxidant capacity of the bisphenols and their oligomers knowing that the lower the value, the higher the antiradical activity.

Results and discussion

Laccase-catalyzed oligomerization of biobased bisphenols

According to Pion *et al.*,[54] bisphenols were successfully obtained under mild conditions with good to excellent yields with regards to the polyol (**1**: 50%, **2**: 92%, **3**: 95%, **4**: 92%). Reaction times were adapted according to the polyol. For clarity, **1**, **2**, **3** and **4**, have been named GDF, PDF, BDF and IDF, respectively (where P = 1,3-propanediol, B = 1,4-butanediol, I = isosorbide, G = glycerol, and DF = dihydroferulate) (Fig. 1).

Since laccases use the oxygen from the air as co-oxidant, the four bisphenols were oligomerized in the presence of a commercially available laccase from *Trametes versicolor* in open vessels under different operating conditions of reagent concentrations, temperature, pH, in several solvent mixtures. MALDI-TOF, GPC and NMR techniques were used to

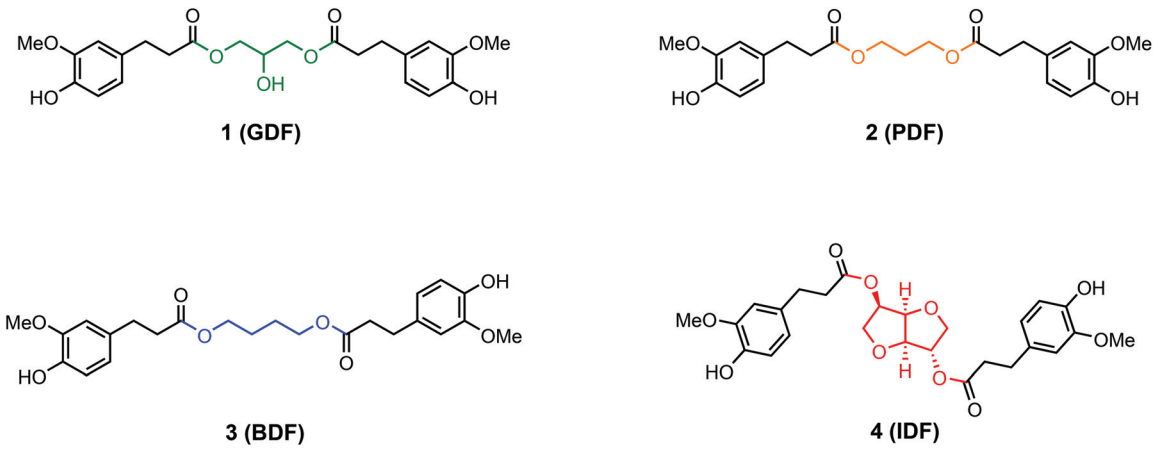


Fig. 1 Biobased bisphenols to be oligomerized through laccase-mediated oxidation.

characterize the mass distribution and the structure of the oligomers. Bisphenol conversion, DP_n and DP_w were measured through GPC analysis to determine the influence of the reaction conditions.

Oligomers of IDF, PDF, BDF, and GDF — named PIDF PPDF, PBDF and PGDF, respectively — were analyzed by NMR spectroscopy in deuterated acetone. The ^{1}H NMR spectra of the oligomers exhibit a significant complexification of the aromatic area (6–8 ppm) compared to the spectra of the corresponding bisphenols. However, at this stage, there is no sufficient proof of exclusive biaryl bonding between monomers. Fortunately, the formation of aryl–aryl bonds was confirmed using HSQC data (Fig. 2). Indeed, the ^{13}C NMR spectra of the four oligomers show a new signal at 126 ppm corresponding to the quaternary carbons involved in the biaryl coupling (5o) (Fig. 3). HSQC analysis confirmed the presence of biaryl bonds as it shows, in the aromatic area, only the signals for the C–H of the terminal phenols (in green) and the signals for the C–H of the internal phenols (in red). Unambiguous attribution of the signals was possible thanks to the presence of the characteristic HMBC correlations between the aromatic protons and the benzylic methylene carbon of the dihydroferulic moiety. Indeed, from the five proton signals attributed to aromatic protons, only one doesn't exhibit such a correlation with a benzylic methylene and this absence allowed its attribution to 5t. Further HMBC correlations between the aromatic protons and the quaternary aromatic carbons thereafter allowed the separation of the two sets of aromatic signals corresponding to the internal and terminal phenols.

MALDI-TOF analysis of PBDF (Fig. 4) showed well-detectable peaks in the mass range up to 3600 *m*/*z*, suggesting the presence of significant amounts of oligomers up to a DP_n of 8. Analysis of the clusters of the pseudomolecular ion peaks revealed a repetitive unit of 444 Da corresponding to the molecular weight of BDF minus two Hs [M − 2H]. The *m*/*z* value of the different fragmentation peaks showed that the fragmentation may occur through cleavage of the biaryl bond, probably favored by the presence of the free phenol in the *ortho* position.

All the data above confirm that, as expected, laccase-mediated oligomerization of this type of bisphenol proceeds exclusively through a 5–5 biaryl coupling pattern.

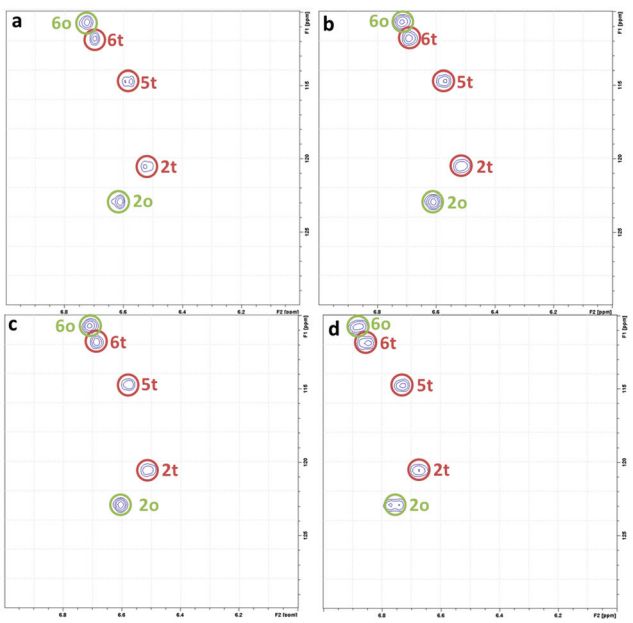


Fig. 2 HSQC in $(CD_3)CO$ of (a) PPDF, (b) PGDF (c) PBDF, (d) PIDF.

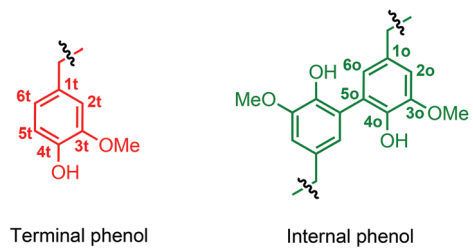


Fig. 3 Representation of terminal phenols (t) and internal phenols (o).

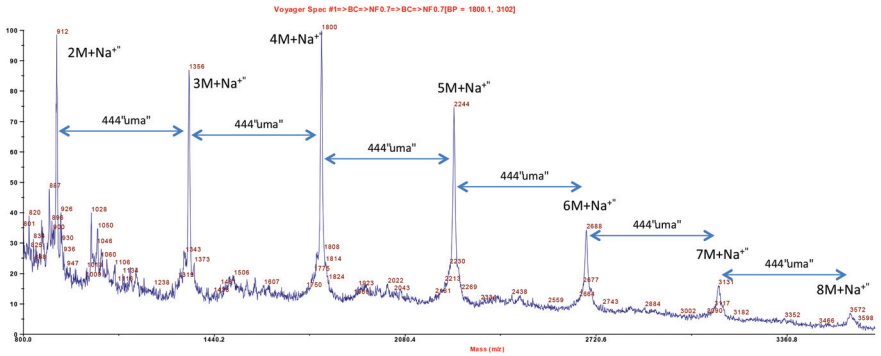


Fig. 4 MALDI-TOF for PBDF oligomer.

Influence of co-solvent nature and volume ratio

It has been reported that the use of laccases can be done in the presence of several organic co-solvents[46,65,66] in order to increase the solubility of the substrate and oligomers formed. These studies indicated that the nature and the proportion of the solvent impact the conversion, the polydispersity and the structure of the polymers. We therefore investigated the influence of organic co-solvents on the laccase-mediated oligomerization of bisphenol (Fig. 5).

Solvents with different polarities were tested: alcohols, halogenated solvents, ketones, esters, as well as cyclic/heterocyclic/aliphatic solvents; leading to mono or biphasic systems. The influence of each solvent was evaluated at r.t. after 24 h of reaction.

As previously reported in the literature,[67,68] Fig. 4 shows a very high tolerance of *Trametes versicolor* laccase towards organic solvents. Even though the use of some co-solvents, especially halogenated ones, resulted in rather low conversions after 24 hours (*ca*. 5%), higher conversions can be reached after longer reaction times (72, 96 hours). Among all the twenty-one solvents tested, ethanol, isopropanol, acetonitrile, acetone, dimethoxyethane and dioxane were those that allowed >90% conversion in 24 hours. Ethanol was preferred as it is the most environmentally friendly and the cheaper solvent among the five above.

To determine the impact of the co-solvent ratio on the oligomerizations, reactions have been performed in aqueous buffer : ethanol mixtures with an ethanol ratio of up to 80% (Fig. 6).

The data in Fig. 6 show a tolerance of laccase to more than a 60% volume ratio of ethanol. Increasing the volume ratio co-solvent/buffer is therefore possible to a certain extent if needed for solubility reasons with respect to the starting bisphenol.

In summary, this laccase-mediated oligomerization process can be efficiently performed with a wide range of co-solvents, and thus can be accommodated to substrates with different solubilities by varying the nature and the % v/v ratio of the co-solvent. These data also showed that the dielectric constant of

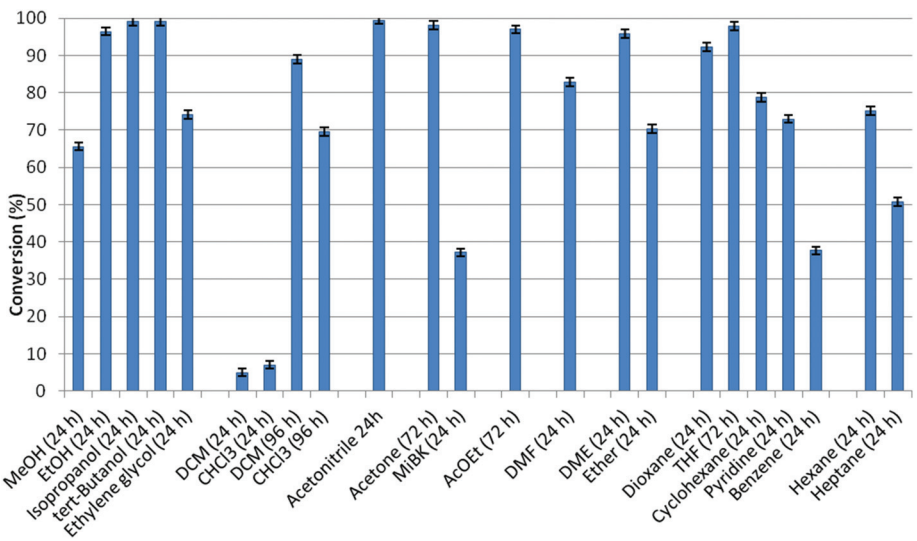


Fig. 5 Study of laccase-catalyzed oligomerization of PDF using different co-solvents, 30 g L^{-1}, 4.2 phosphate/citrate buffer : co-solvent (70 : 30), 50 U per $mmol_{substrate}$, r.t., after 24, 72 or 96 hours.

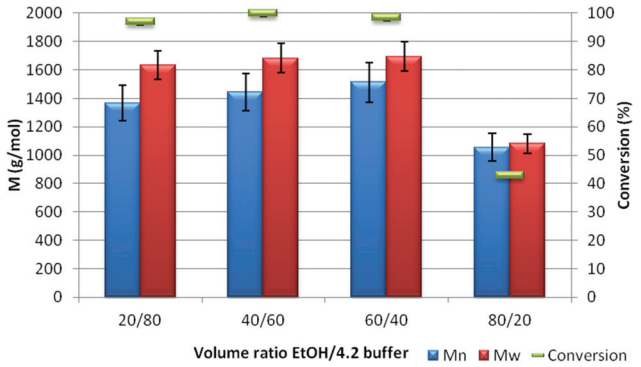


Fig. 6 Study of laccase-catalyzed oligomerization of PDF using different volume ratios (ethanol/4.2 buffer), 10 U per $mmol_{substrate}$, at r.t., after 120 hours, and at 25 g L^{-1}.

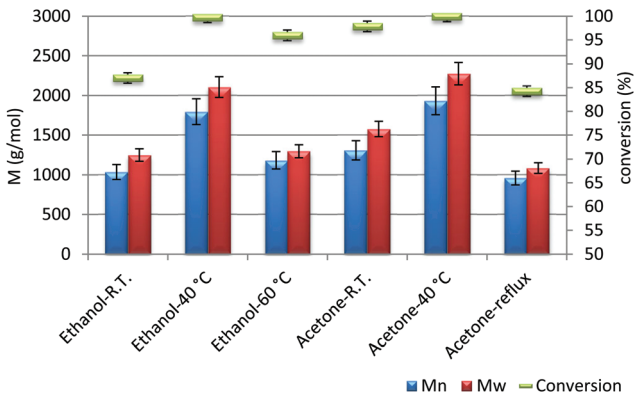


Fig. 7 Study of laccase-catalyzed oligomerization of GDF using different temperature and co-solvents (30% volume ratio), 50 U per $mmol_{substrate}$, at pH 4.2, after 120 hours, and at 25 g L^{-1}.

the co-solvent (ε) is an important factor in enzymatic oligomerization. Indeed, the constant ε is implicated in dissociation phenomena and is likely to decrease the chelation effect of bisphenols and their oligomers, which are supposed to decrease the activity of the enzyme. Enzyme inhibition by chelation phenomena has already been demonstrated by Kim *et al.*,[68] who studied the effect of catechin-aldehyde polycondensates on xanthine oxidase activity. Another effect of the solvent could be the alteration of the enzyme stability.

Influence of temperature

Being a critical factor in both substrate solubility and laccase activity, the influence of reaction temperature was evaluated on different substrate/co-solvent couples: GDF, BDF and IDF, in acetone or ethanol (30% volume ratio), pH 4.2, 50 U per $mmol_{substrate}$, after 120 hours and at a substrate concentration where all substrates were totally dissolved (Fig. 7–9).

In the case of GDF (Fig. 6), whatever the co-solvent, 40 °C proves to be an optimum temperature since conversion is almost total and the degree of polymerization is at its maximum.

The results shown in Fig. 7 revealed that, among the solvents tested, oligomerization of IDF is more efficient at r.t. and with acetone as co-solvent. Futhermore, results confirm that heating above 60 °C is unfavorable as it leads to a diminution of laccase activity (decrease of conversion). Further study at r.t. revealed that acetonitrile provided M_n and M_w up to *ca.* 3500 and *ca.* 4100, respectively (Fig. 8).

This study shows different outcomes with BDF with regards to IDF, *i.e.* superior conversion and higher degree of polymerization at r.t. with acetone (Fig. 10). Moreover data confirm that temperatures above 40 °C are unfavorable toward laccase activity.

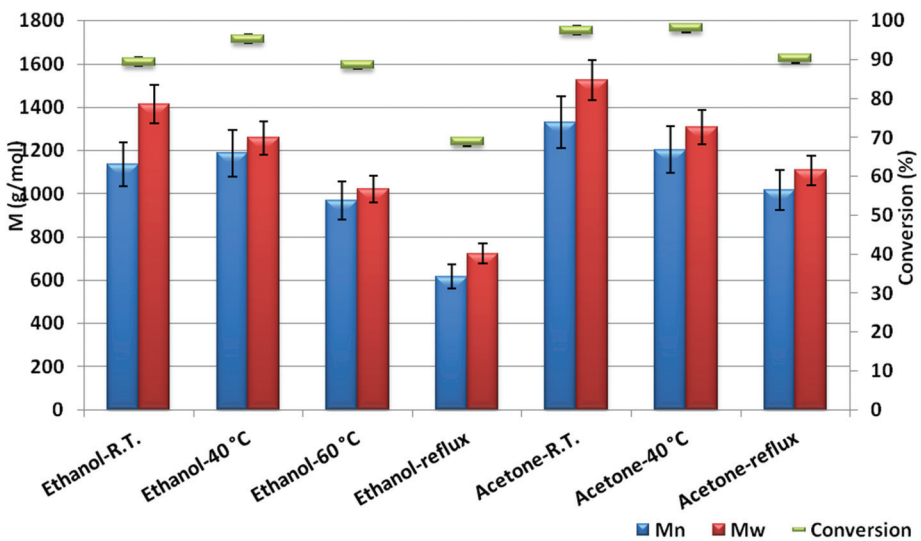


Fig. 8 Study of laccase-catalyzed oligomerization of IDF using different temperature and co-solvents (30% volume ratio), 50 U per $mmol_{substrate}$, at pH 4.2, after 120 hours, and at 40 g L^{-1}.

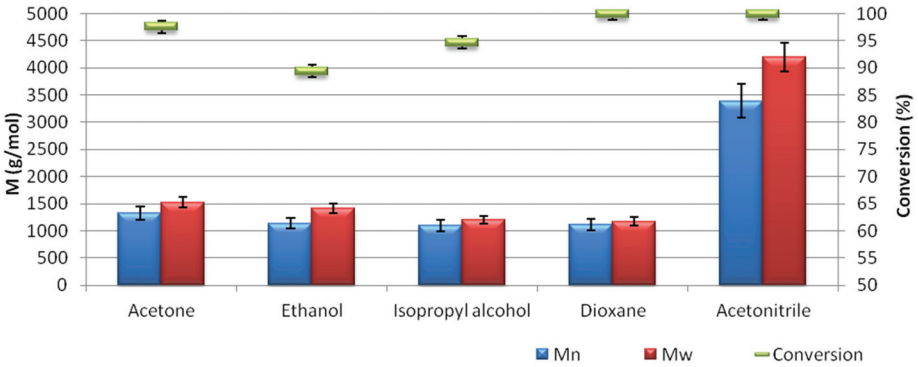


Fig. 9 Study of laccase-catalyzed oligomerization of IDF using different co-solvents (30% v/v ratio), 50 U per $mmol_{substrate}$, at pH 4.2, r.t, after 120 hours, and at 40 g L^{-1}.

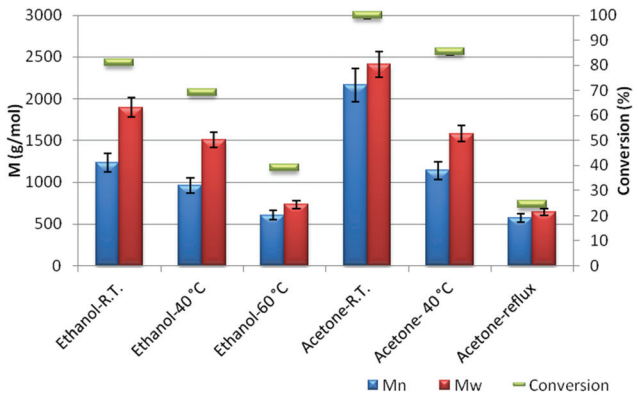


Fig. 10 Study of laccase-catalyzed oligomerization of BDF using different temperature and co-solvent (30% volume ratio, 4.2 buffer), 50 U per $mmol_{substrate}$, after 120 hours, and at 6 g L^{-1}.

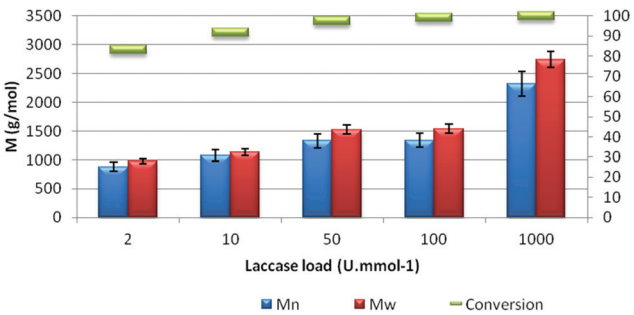


Fig. 11 Study of laccase-catalyzed oligomerization of IDF using different laccase loading (U per $mmol_{substrate}$), 4.2 phosphate/citrate buffer : acetone (70 : 30), r.t., after 120 hours.

These studies revealed that laccase from *Trametes versicolor* allowed the use of a wide range of temperatures (r.t. to 40 °C)[68] and co-solvents. Above 40 °C, laccase activity decreases significantly (Fig. 7, 8 and 10), probably because of the deactivation of the enzyme.[70] Temperature and co-solvent nature and % v/v can be finely adjusted and optimized according to the substrate nature and solubility.

Influence of laccase loading

The enzyme being responsible for the generation of radicals, the enzyme activity therefore impacts their concentration and affects the chain length of the oligomers. Initial experiments were thus carried out over a range of laccase loading (2–1000 U per $mmol_{substrate}$) with IDF as substrate in a 4.2 phosphate/citrate buffer : acetone (70 : 30) mixture for 120 hours at r.t. (Fig. 10).

As expected, for a given reaction time, increasing laccase load leads to an increase in conversion and also in degree of polymerization (Fig. 11). Even if a 2 U per $mmol_{substrate}$ already yields an 83% conversion, the higher laccase loads allowed a higher degree of polymerization for the same reaction time.

The elongation of the oligomeric chains can also be affected by the concentration of bisphenol. Laccase-mediated oligomerization of BDF was therefore performed at different concentrations in phosphate/citrate buffer (pH 4.2) : co-solvent (40 : 60), at r.t. for 120 hours in the presence of 50 U per $mmol_{substrate}$ (see ESI, Fig. S1†).

Under identical conditions, the three oligomers obtained with different substrate concentrations gave quite similar GPC spectra. We can therefore conclude that the concentration of the substrate (*i.e.*, bisphenol) does not impact the oligomerization process in terms of degree of polymerization.

The observations above are in agreement with those reported by Kurisawa *et al.*[43] Indeed, the concentration of enzyme has a greater influence on the kinetics of the reaction than on the chain length of the oligomers. This behavior could be attributed to a sequestering effect of the oligomers toward the copper of the active site of the enzyme, leading to its inactivation as it was mentioned by Desentis-Mendoza *et al.*[45] Aggregation phenomena of the oligomers could also be advocated to explain these results since further addition of fresh enzyme solution with or without additional bisphenol, did not allow further evolution of the system, indicating that a maximum DP was reached that it was not influenced by the enzyme concentration.

Influence of reaction time

Having shown that 50 U per $mmol_{substrate}$ is an effective laccase load, we then investigated the influence of reaction time in the oligomerization of BDF in a 4.2 phosphate/citrate buffer : acetone (70 : 30) mixture (6 g L^{-1}) in presence of 50 U per $mmol_{substrate}$ at r.t.

Fig. 12 shows that maximum conversion is quickly reached (95% after 8 hours) whereas the degree of polymerization exhibits a steady increase. This proves that the dimerization of BDF is favored when further oligomerization appears to be the limiting step. This could be due either to a decrease of solubility and mobility of the higher oligomers or steric effects preventing them from accessing the enzyme catalytic site.[71] It is also noteworthy to mention that the degree of polymerization reaches, as already demonstrated previously, a maximum and remains relatively constant even after longer reaction time.

Influence of pH

The optimum pH for oligomerization was found to be around 4.5 which is in accordance with the reported data in the literature[69,71] but the activity of laccase remained >90% in a 3.5–6 pH range without significant change of the degree of polymerization. In view of these results, we decided to explore the possibility to replace the aqueous buffer with pure water (Milli-Q quality) and thus simplify and lower the cost of the synthetic procedure. PDF laccase-catalyzed oligomerization was studied in ethyl acetate (30% volume ratio), using 50 U per $mmol_{substrate}$ at different temperatures using buffer phosphate/citrate (pH 4.2) or pure Milli-Q water (pH 5.2) for 24 hours (Fig. 13).

Unexpectedly, equivalent or higher conversions and degrees of polymerization were obtained when the oligomerization was performed without buffered aqueous solution. No matter the temperature, and even at a non optimum pH value, the use of Milli-Q water provided oligomers with longer chain length and in higher yields. These results may be related to the ionic strength of the reaction medium that may promote a decrease of reactivity (or disponibility) of the oligomers *versus* the enzyme through chelation of the phenols, promoting aggregation phenomena, when lowering the pH value also increases the phenate/phenol concentration ratio enhancing the kinetics of the reaction.

As a summary to the study of parameters impacting the laccase-catalyzed oligomerization, Table S1 in the ESI† shows the degree of polymerization obtained for bisphenols oligomerized under a large variety of conditions.

Thermal properties

Thermal properties of PPDF, PIDF, PGDF, and PBDF oligomers were evaluated by thermogravimetry (TGA) and differential scanning calorimetry (DSC). Properties were determined with regards to bisphenol structure and chain length (DP_n) (see ESI, Fig. S1 and S2†).

Whatever the nature of the diol, all oligomers exhibit rather high thermostabilities from 280 °C up to 340 °C. As expected, oligomer chain length has a positive effect on thermal stability. It is also noteworthy to mention that the more flexible the diol, the higher the T_d 5%.

Similarly, DSC analyses revealed that T_g increases with increasing DP_n (Fig. 16†). Also, depending on the bisphenol structure and the degree of polymerization, one can access a wide range of T_g (from 0 °C for a DP_n = 3 PBDF oligomer to 90 °C for a DP_n = 5 PIDF oligomer). As expected and previously observed,[55–58] the use of a rigid bicyclic diol (*e.g.*, isosorbide) provides high T_g while the use of more flexible diol (*e.g.*, 1,4-butanediol) reduces it.

Antioxidant activities

The antioxidant properties of the oligomers were investigated using a modified DPPH assay which determines the H-donor capacity of the antioxidant as a quencher of the stable DPPH free radical.[64] Oligomer samples with very narrow dispersity

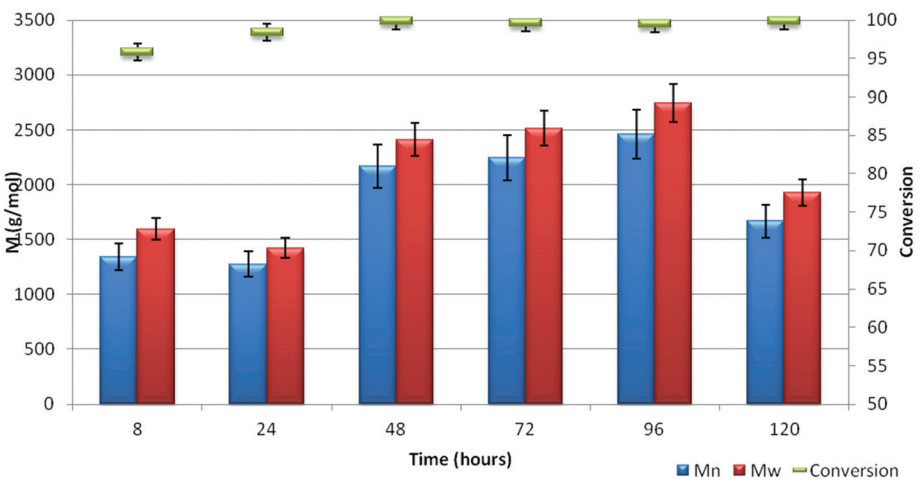


Fig. 12 Study of laccase-catalyzed oligomerization of BDF using 50 U per $mmol_{substrate}$, 4.2 phosphate/citrate buffer : acetone (70 : 30) (6 g L^{-1}), r.t., after 120 hours.

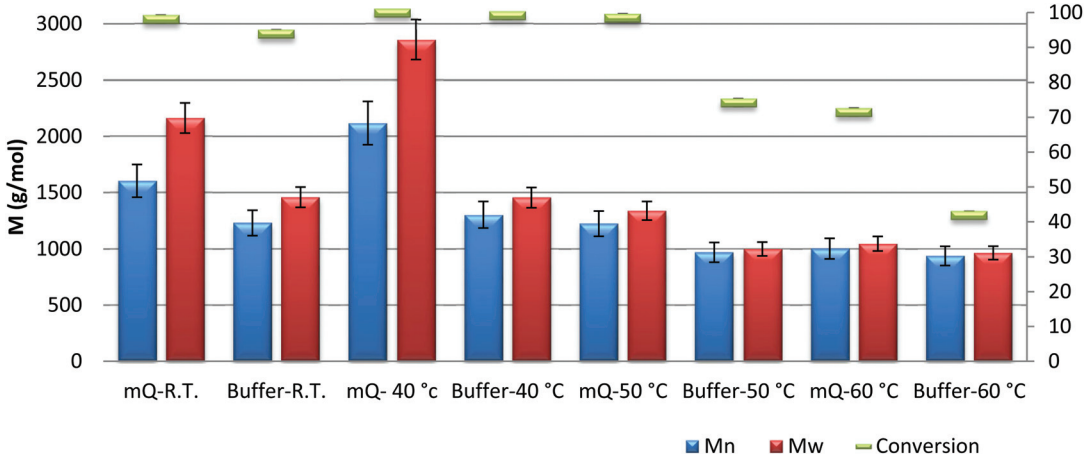


Fig. 13 Study of laccase-catalyzed oligomerization of PDF using different temperatures and aqueous media, ethanol 30% volume ratio, 4.2 buffer, 100 U per $mmol_{substrate}$, after 120 hours, and at 20 g L^{-1}.

(PDI < 1.3) were used to study the effect of the degree of polymerization on their antioxidant capacity. In some cases, samples were purified by flash chromatography on silica gel eluted with cyclohexane : AcOEt from 5 : 5 to 0 : 10 and then AcOEt : EtOH 9 : 1 until low molecular mass oligomers were afforded.

Compared to ferulic acid, BHT and Irganox® 1010 that have an EC_{50} of 9.6, 21.5 and 2.48 nmol respectively, all bisphenols and their corresponding oligomers have EC_{50} values between 3.7 and 0.6 nmol with regards to the chain length and the internal diol (Fig. 14). Similarly to what was observed for the T_g, the structure of the internal diol impacts the antioxidant properties. As a matter of fact, and as previously observed,[59] rigid bicyclic isosorbide provides higher EC_{50} values compared to flexible aliphatic diols (*i.e.*, 1,3-propanediol, 1,4-butanediol and glycerol). In addition, the data show a decrease of the EC_{50} value when the chain length of the oligomers increases which is consistent with the fact that the number of free phenol moieties increases with the DP_n. Furthermore, it is noteworthy to mention that the EC_{50} value seems to reach a plateau despite the further increase of phenolic groups. This behavior can be due to the steric hindrance of internal phenols that are less accessible to reduce the DPPH radical.

From the above set of data, it could be concluded that these ferulic-acid based linear aliphatic-aromatic oligomers with bis-guaiacol-type moieties are highly effective free radical scavengers by H-atom transfer toward DPPH, and even more effective than the corresponding bisphenols, and commonly used antioxidants such as ferulic acid (EC_{50} = 9.6 nmol), BHT (EC_{50} = 21.5 nmol) and Irganox® 1010 (EC_{50} = 2.4 nmol).

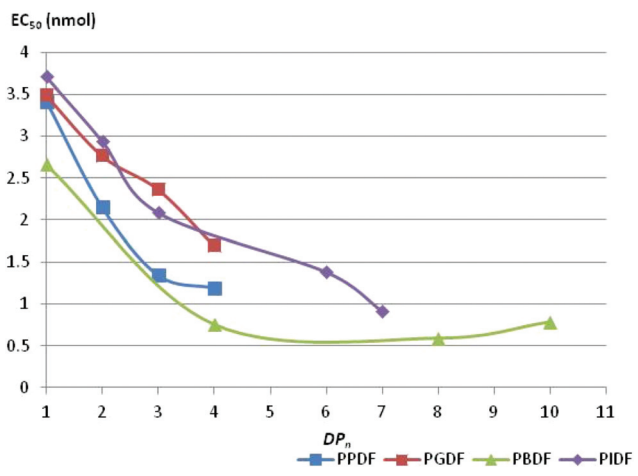


Fig. 14 Evolution of EC_{50} with increasing chain length.

Conclusion

Novel biobased linear aliphatic-aromatic oligomers with bis-guaiacol-type moieties have been successfully synthesized from ferulic acid based bisphenols by a mild and environnementally friendly biocatalytic procedure using laccase from *Trametes versicolor*. It is noteworthy to mention that these bisphenols are 100% biobased and are prepared by an eco-friendly chemo-enzymatic pathway. Based on relatively cheap starting materials ($1–6 per kg) and commercially available enzymes (lipase, laccase), and being performed under mild conditions (*e.g.*, atmospheric pressure, relatively low temperature, open vessel), this synthetic process proves economically viable. We have also demonstrated that depending on the oligomerization conditions (temperature, reaction time, laccase concentration, substrate concentration, co-solvent nature and volume ratio), the degree of polymerization can be controlled. Moreover, considering that the longer the oligomer chain length is, the less soluble it becomes, by playing with the temperature, and the nature and the ratio of co-solvent, one can make oligomers precipitate and stop their growth. DPPH and thermal properties studies revealed that both the T_g and the antioxidant capacity of these highly thermostable linear phenolic oligomers can be tailored by judiciously adjusting

the nature of the bisphenol core (*i.e.*, diol) and the degree of polymerization. On the basis of these results, these renewable linear phenolic oligomers are promising new efficient biocompatible antioxidant additives for a sustainable approach to polymer stabilization with considerable potential advantages over commonly used low molecular weight phenolic antioxidants such as reduced tendency of leaching, high stability to thermal and oxidative injury, lower toxicity, and biodegradation ability due to the ester linkages.

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