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Doctoral Thesis

Laccases and lignin: development of functional polymers and antioxidant activity measuring methods

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CONTENTS

Abstract

Laccase catalysed radical reactions were investigated related functionalisation of lignin and formation of quinone from syringaldazine for measurement of antioxidant activity. Lignin is the second major abundant natural polymer in the world. Industrial lignins are by-products from the pulp and paper industry, as well as from other biomass-based industries. Industrial lignins are classified as non-toxic with a high potentially value, inexpensive and available in huge amounts. Here, the ability of laccases from *Trametes villosa* (TvL), *Myceliophthora thermophila* (MLV), *Trametes hirsuta* (ThL) and *Bacillus* spore laccase (BsL) for the modification of industrial lignin for application as dispersants and adhesives was demonstrated. The modification of lignosulphonate showed that the dispersion of lignosulphonate properties generally increased in all treated samples demonstrating the ability of the laccase-mediator (HBT) system. The Mw for TVL and THL treated samples increased from 28400 to 191100 and 58800, respectively. Use of complementary analytical procedures revealed that the loss of aromatic cross-signals in the HSQC spectra obtained after the enzymatic treatment were a result of deprotonation of the lignin benzenic rings, as revealed by the 1D-NMR spectra while the appearing strong signals of aromatic carbons in ^{13}C NMR spectra after 83 h of incubation in TvL treated samples were a sign of the formation of new ether and C-C aryl-aryl or aryl-alkyl linkages causing strong polymerization as confirmed by SEC (increase in Mw). FTIR, ^{13}C-NMR spectra and Py-GC/MS analysis of treated samples suggested no substantial structural changes in the calcium lignosulphonate aromatic structure, a good indication of the ability of TvL and ThL-HBT systems to limit their effect to the substituent for effective cross-linking without degrading the lignin backbone. Organic-inorganic hybrid polymeric materials derived from silica precursors and lignin were successfully produced using sol gel process. Activation of lignin by laccase increased the interaction between lignin and tetraethylorthosilicate (TEOS) resulting in interpenetrating polymers. The lignin concentration affected the viscosity, curing time and tensile strength. Si-NMR analysis of the hybrids between trimethyl SiO_3 and pure lignin molecules (ferulic and sinapic acid) showed new signals indicating the formation of hybrids. In a second phase, laccase was used for the production of tetramethoxy azobismethylene quinone (TMAMQ) to be used for the measurement of antioxidant activity. Antioxidant activity measuring method using TMAMQ fulfills the criteria of a good antioxidant measuring method as postulated by Prior et al, 2005 such as (1) simplicity, (2) defined endpoint, (3) instrumentation is simple and readily available; (4) has good reproducibility within-run and between-days; (5) is adaptable for assay of both hydrophilic and lipophilic antioxidants; (6) can be adapted to "high-throughput" analysis for routine quality control analyses and a wide of analytical pH range among other advantages.

Zusammenfassung

Laccase-katalsierte Radikalreaktion wurden hinichtlich des Potential zur Funktionalisierung von Lignin und zur Erzeugung von Radikalen für die Messung von Antioxidantien untersucht. Lignin ist nach Cellulose das zweithäufigste natürliche Polymer der Welt. Etwa 18-35 % der Masse verholzter Pflanzen besteht aus Lignin. Industrielle Lignine entstehen als Nebenprodukte in der auf Biomasse basierenden Industrie, zum Beispiel der Zellstoff- und Papierindustrie. Sie sind nicht toxisch, kostengünstig, in großen Mengen verfügbar und weisen zudem ein hohes chemisches Potential auf, wodurch sie ökonomisch einen großen Wert besitzen. Aufgrund ihrer reaktiven funktionellen Gruppen, wie aromatische Hydroxyl-Gruppen, können durch enzymatische Modifikationen neue, umweltfreundliche Produkte erzeugt werden. Das Enzym Laccase ist in der Lage industrielle Lignine zu oxidieren. Die daraus entstehenden Verbindungen finden Anwendung als Dispergiermittel oder Klebstoffe. Die Laccase-mediierte Modifikation von Ligninsulfonat führte zu einer erhöhten Dispergierbarkeit des Sulfonats in allen behandelten Proben, wodurch die Funktionsfähigkeit des Laccase-HBT-Systems demonstriert wurde. Das Molekulargewicht (MW-Wert) der mit Laccasen aus *Trametes villosa* (TVL) und *Trametes hirsuta* (THL) behandelten Proben erhöhte sich von 28400 auf 191100 bzw. 58800. Weiters wurde mittels 1D-NMR Spektroskopie gezeigt, dass der Verlust von aromatischen Kreuzsignalen im HSQC Spektrum, welcher nach enzymatischer Behandlung von Ligninsulfonat beobachtet wurde, auf die Deprotonierung des Benzenrings zurückzuführen ist. Das Aufscheinen starker Signale aromatischer Kohlenstoff-Atome im ^{13}C NMR-Spektrum von Proben, welche 83 Stunden mit TVL inkubiert worden waren, weist auf die Bildung neuer Ether- und C-C-Bindungen (aryl-aryl oder aryl-alkyl) hin. Die Entstehung von Polymeren konnte mittels SEC verifiziert werden (Erhöhung des MW-Werts). FTIR, 13C-NMR-Spektroskopie und Py-GC-MS Analyse der behandelten Proben weisen darauf hin, dass die aromatischen Calzium-Ligninsulfat Struktur nicht beträchtlich verändert wurde. Dieses Ergebnis ist ein Indiz für die Fähigkeit der TVL und des THL-HBT Systems, effektives Vernetzen der Substituenten zu katalysieren ohne dabei das Lignin-Rückgrad zu zerstören. Mit Hilfe des Sol-Gel-Verfahrens was es möglich aus Silica-Vorläufern und Lignin organisch-anorganische Hybrid-Polymer-Materialien zu produzieren. Die Aktivierung von Lignin mittels Laccase führte zu einer erhöhten Interaktion zwischen Lignin und Tetraethylorthosilikat (TEOS) wodurch adhäsive, sich stark durchdringende Polymere generiert werden konnten. Dabei nahm die Konzentration des Lignins Einfluss auf die Viskosität, die Aushärtungszeit und die Bruchfestigkeit des Produkts. Die Bildung von Hybriden zwischen Trimethyl-SiO_3 und Lignin-Monomeren (Ferulsäure und Sinapinsäure) wurde durch zusätzliche Signale in der Si-NMR-Analyse indiziert. Durch Umsetzung von Syringaldazin mit Laccase konnte Tetramethoxy-Azobisbethylen-Quinon (TMAMQ) hergestellt werden, welches erfolgreich zur Messung antioxidativer Aktivität herangezogen wurde. Die Verwendung von TMAMQ erfüllt aufgrund (1) der Einfachheit, (2) des definierten Endpunkts, (3) der einfachen und leicht erhältlichen Geräteausstattung, (4) der guten Reproduzierbarkeit innerhalb einer Serie und

zwischen Serien, (5) der Anwendbarkeit sowohl für hydrophile als auch für lipophile Antioxidantien sowie (6) der Anwendbarkeit für „high-throughput"-Analysen, Routine-Qualitätskontrollen und anderer Vorteile wie die Anwendbarkeit über einen weiten pH-Bereich alle Kriterien einer guten Methode zur Aktivitätsmessung von Antioxidantien wie es Prior et al, 2005 postulierte.

1

General Introduction

One of the major agenda of the 21st century is to protect the world from pollution by means of 3R method (reduce, reuse and recycle) by implementing cleaner industrial practices, or use of more eco-friendly, biodegradable and non-toxic renewable raw materials. In order to achieve these goals improvement in production technologies or maximum exploitation of resources (zero emission principle) has to practice. One of the highly ranked utilized materials is higher plant biomass. Most higher plants are build up of lignin which is one of the most abundant waste material resulting mostly from the pulp and paper industry. Plant biomass is actually largely composed of cellulose, hemicellulose, and lignin. However, maximum socio-economical exploitation of lignin has been minimal up to date.

Although the amount of lignin extracted in pulping operations around the world is estimated to be over 70 million tons per year, less than 2 % is actually recovered for utilization as a chemical product (Lora, 2008). A particularly promising solution for up-grading industrial lignins is through biotechnology, with a possibility to minimize negative environmental impacts as well as increase profitability (Sena-Martins et al, 2008).

Enzyme technology is one of the fields of applied biotechnology that is growing tremendously. In fact, technical enzymes represented the largest part of the market with a value of approximately US$ 1 billion in 1999 with

enzymes for detergent being the largest single market for enzymes valued at around US$ 500 million, and the other dominating markets are baking, beverage and dairy as well as feed and paper and pulp (Schäfer et al., 2002). Enzyme technology has already progressed from the biotransformation of small substrates to biotransformation of synthetic polymers, with an important breakthrough using novel enzymes to enhance the activity of synthetic polymer substrates (Guebitz and Cavaco-Paulo, 2007). Fungal laccases are suitable enzymes for modifying industrial lignin. Laccase (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductase) are a cluster of oxidative glycoprotein enzymes mostly found in higher plants (Riva, 2006) and the white-rot-fungi. Laccase was first reported by Yoshida at the end of the 19th century as a component of the resin ducts of the lacquer tree *Rhus vernicifera* (Yoshida, 1883). Naturally laccases have been known to play different roles among, them in *Bacillus* species laccase are involved in the assembly of UV-resistant spores (Sharma, et al, 2007) and moreover laccase also catalysed formation of cuticle (sclerotization) of insect (Kramer, et al, 2001). In addition, laccase are involved in the formation of cell wall of the plant cells (Sonoki, et al, 2005) and responsible for protection of fungal pathogens (Pezet, et al, 1992). Laccase also collaborate with other oxidoreductase (peroxidase) during delignification by white-rot-fungi (Mayer and Staples, 2002).

1.1. Mechanism of laccase substrate oxidation

Laccases, abstract one electron of particular substrate molecule especially phenolic compounds or aliphatic amine forming reactive radical or hydroquinone derivatives (Dence and Lin, 2002) (Fig. 1.)

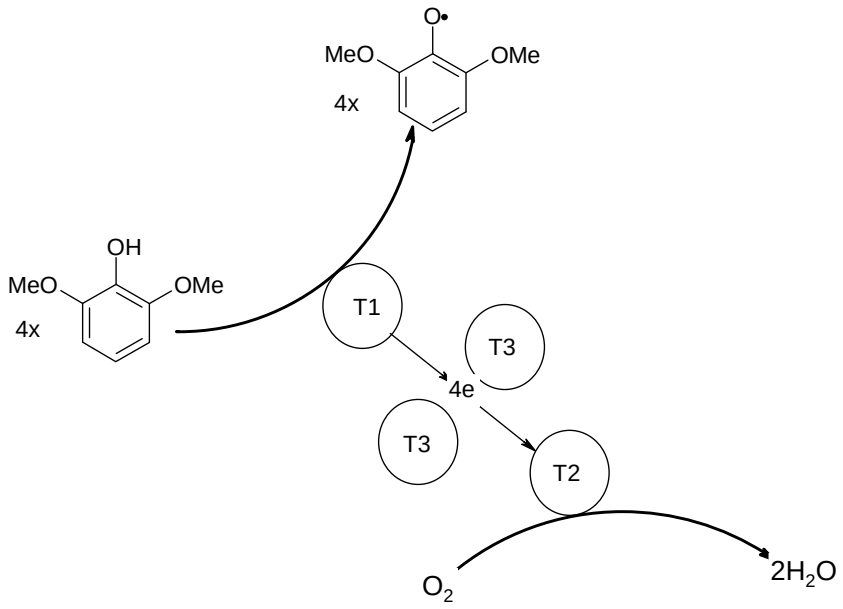

Fig. 1. Laccases catalytic oxidation mechanism of 4 molecules of (2,6-dimethoxyphenol) laccase substrates. Type 1 (T1) is the site where substrate oxidation takes place. Type Type 3 (T3) copper form a trinuclear cluster for electron transfer while (T2) is where reduction of molecular oxygen and release of water takes place.

The complete reaction results in reduction of oxygen to water. That phenomenon has been showed nicely by Nugroho Prasetyo, et al. (2009) that stochiometrically it can be mentioned in order for laccase to reduce one molecule of oxygen to 2 water molecules they need to abstract 4 electrons of the substrate (Fig.1). Finally the produced radicals go under further non enzymatic reactions ultimately producing dimers, oligomers, etc (Riva, 2006).

The substrate of laccase can be broadened to non phenolic compounds like aliphatic alcohol, sugar derivative, and many more (Bourbonnais and Paice, 1990) due to mediators (Fig. 2). ABTS (2,2-azinobis-3 ethylbenzenthiazoline-6- sulfonic acid) was the first identified laccase mediator (Xu, et al, 2000). Implementation of mediator in industrial scale seems to be promising increasing the capability of laccase. Unfortunately, the mediators seem to increase the cost of production process and sometimes they are toxic.

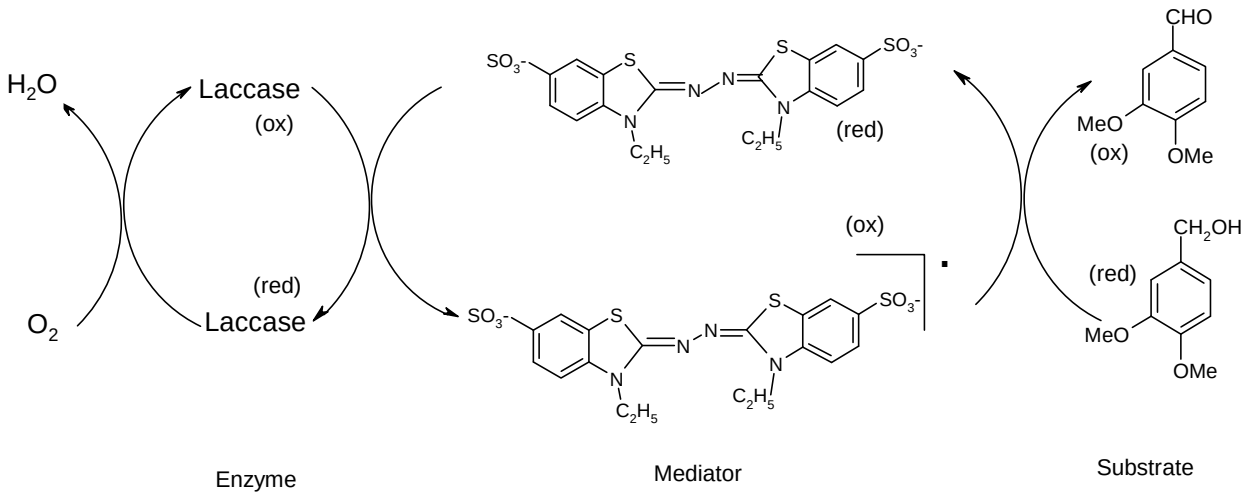

Fig.2. Schematic representation of laccase-catalyzed redox cycles for veratryl alcohol oxidation in the presence of ABTS as mediator.

Nevertheless, conceivably renewable, economic, and environmental friendly natural derived laccase-mediator obtained from plant phenols like *p*-coumaric acid, syringaldehyde, and acetosyringone have been proposed (Camarero, et al, 2007). Although the study of the natural mediator is limited, but this issue is very urgent due to the demand of the laccase application in the industrial processes become higher.

This is because, laccases obtained from fungal sources typically have high redox potential and thus can efficiently oxidise a broad range of aromatic compounds like monophenols, diphenols, polyphenols, methoxy-substituted phenols, aromatic amines and diamines, benzenethiols and even some inorganic compounds such as iodine by using oxygen as an electron acceptor (Xu, et al 1996). Taking advantage of the ability of laccase to oxidize phenolic compound laccases are being explored for a number of applications ranging from food, wastetreatment, devopment of novel functional polymers (Rodríguez Couto and Herrera, 2006). Also emerging literature is suggestive of a major role for laccases in improving lignin properties for enhanced industrial applications in the synthesis of adhesives, epoxy, copolymer, polyefin, chelating agent or coating materials. However, most of the studies have not done a detailed characterization of the

modifications effected by laccase making it impossible to get a real picture of the limitations and opportunities to effectively use lignin. It was also our interest to develop novel functional polymers combining the advantages of silanes and lignins. Additionally, the novel feature of this work was to screen the different laccase generated radicals/quinones for their ability to measure antioxidant activity for application food, health and cosmetic industries.

1.2. Aim of this work

The current work attempts to address the application of industrial lignin to produce much more advantageous and versatile products such dispersion agent and functional polymers like adhesive through laccases oxidation, and analyze the product using a number complementary analytical methods such as the HPLC-MS, FTIR, and NMR. Additionally, the novel feature of this work is to develop laccase-generated reactive species from lignin molecules that can be used as an antioxidant activity measurement.

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Section 1

Development of laccase modified lignin based functional polymers

Industrial lignin

The term "industrial lignin" is usually to lignin by product of the pulp and paper industries. Therefore, industrial lignin properties are influenced by the type of pulping process. For instance, the lignosulphonate are more soluble in water than kraft lignin due to substitution of sulphonate group in their structure. Besides the pulping process industrial lignin also has huge different variety of moieties depending on the origin of the plant.

In fact, low-purity, high heterogeneity, unpleasant smell and dark colour (Gosselink et al., 2004a,b) are mainly causes of problems for the low market value of industrial lignin. However, several techniques to improve quality of industrial has been tried to improve its utilization. For example recently, progress has been made in the use of these industrial lignins as feedstock for novel chemicals (Boudet et al., 2003), and high quality dispersants made from chemically modified kraft lignin (Gosselink et al., 2004a).

Enzymatic approaches for industrial lignins modification

Environmental friendly processes and products are the main issues in 21century world agenda. Technology without any considering of environmental problem is now impossible. Enzyme is the one of the emerging modern technology from that could provide a solution. enzymatic processes require mild conditions such room temperature and pH between 4.5-7 value condition rather than extreme (very acidic or alkaline condition). In addition, another good thing about enzyme, it can be employed through immobilization into such fix material and it can be reused conveniently until several industrial cycling processes (Dur´an et al., 2002). Nevertheless, the undoubting advantages offered by enzymes justify the intensive research that has been done in this field for the last three decades (Sena-Martin, et al, 2008).

Learning from nature, in the case of industrial lignin, the good candidate to modify lignin obvious come from microorganism using different variety of oxidoreductase. Huge variety of microorganism could degrade lignin through different mechanism, but only white-rot basidiomycetes are

known organisms that are able render lignin molecule to carbon dioxide and water. Although, almost all of white-rot fungi are interested to be studied, but the genus *Trametes* is probably the most actively investigated in the phylum of Basidiomycota for lignolytic enzyme formation and application (Nyanhongo et al., 2007) due to they are easy to be cultured, they can grow on cheap media such as corn cobs, straw, peanut shells and even sawdust (Rodríguez Couto, 2004; Rodríguez Couto and Sanromán 2005) and it has a good performance in lignin degradation. The lignolytic enzymes that make T*rametes* very attractive are laccase (Lac, EC 1.10.3.2) and manganese peroxidase (MnP, EC 1.11.1.13), while lignin peroxidase (LiP, EC 1.11.1.14) has only rarely been reported in *Trametes* (Nyanhongo et al., 2007). Further, the other oxidoreductases have also been found in *Trametes*, the not so commonly known cellobiose dehydrogenase (CDH, EC 1.1.99.18) and pyranose 2-oxidase (P2O, EC 1.1.3.10), since they are supposed to be involved in lignin degradation (Zámocký et al, 2006; Henriksson, et al, 2000; Giffhorn, 2000). A large body of evidence shows that *Trametes* is among the most versatile of white-rotters with ongoing intensive research into applications in bioremediation, effluent treatment, the pulp and paper industry, the food industry, synthetic chemistry, biofuels, cosmetics, biosensors and the textile industry, amongst others (Nyanhongo et al., 2007).

From industrial point of view, the most important mechanism through either oxidation by laccase or peroxidase for example are generating radicals and subsequently lead to formation of increasing reactivity from the lignin. Further polymerisations of the lignin can occurr in a random non-enzymatic way to form three-dimensional polymers of higher *M*w and with a variety of new linkages, as a result, a wide variety of new materials with distinct properties can be obtained (Sena-Martins, et al, 2008).

Applications of industrial lignins

Novel, added-value, environmental friendly, and enzyme-based products of modified industrial lignin are becoming more marketable for some recent years, Herein below, some examples of the application of industrial

lignin are described in order to provide a general picture of the variety of new and eco-friendly products.

Dispersant

Lignosulphonates permits a reduction in the amount of water used for forming the board and consequently a decrease of the drying energy), agrochemicals (for instance, to make water dispersible or wettable powders), dye stuffs (where they may serve as grinding aids as well), bitumen (specially when preparing cold-mix bitumen emulsions), pigments (particularly carbon black used in inks) (Lora, 2008).

Water reducers in a concrete

Concrete is the most important material in the world due to it is a backbone of almost all infrastructures. The occurrence of the concrete mainly depend on curing time, therefore reduction of water consumption in the concrete production is crucial rule, it is allow to produce stronger concrete when the water content is very low. Lignosulphonate is the one of good water reducer agent for concrete production. The dispersant capacity of lignosulphonates (mainly calcium and sodium) is the basis for their use as additives that help increase the workability of concrete and allow a reduction in the amount of water required for proper mixing and handling (Lora, 2008). However, lignosulphonates tend to cause some retardation of concrete setting, particularly when their sugar content is high, and they may also cause air entrainment (Lora, 2008).

Wood composites

Wood composite production is predicted to increase from 15.4 to 16.8Mm3 over the period 2002–2006, an 8.3% increase (Stewart, et al, 2008). Production of wood composites, such as fibre or particleboards, follows a basic process: solid wood is fragmented into small strands, chips or fibres; then they are supplemented with a binder and pressed to form a wood-like structure again (Sena-Martins, et al, 2008). The mostly used as adhesive for

wood composites are urea-formaldehyde resins, phenol-formaldehyde resins and melamine-formaldehyde resins. Unfortunately, the formaldehyde-based resins release a harmful amount of formaldehyde during the manufacturing and use of wood composites (Sena-Martins, et al, 2008).

Copolymers

The copolymerization of different lignins (organosolv lignin obtained from delignification of beech and spruce pulp, Indulin AT and a synthetic hydroxypropylated lignin) with vanilic acid, diisocyanate and acrylamide catalysed by laccase (Milstein et al., 1994; Mai et al., 2000; Hüttermann et al., 2001) can be one the breakthrough utilization of the enzymatic modification of industrial lignin become new generation of plastic (Sena-Martins et al, 2008). In addition, lignin from straw pulp reacted with with cresol, using horseradish peroxidase as polymerisation catalytic enzyme was also resulting promising copolymers that can be used as replacement for normal phenolic resins (Liu et al., 1999).

Grafted industrial lignin trough enzyme modification enhanced biodegradability of the new products, significantly higher graft efficiency and a better molecular weight control of the reaction products were obtained.

Coatings

Coating and painting can be used for protecting wood-based products but some time also for asthetic purposes. Coating materials generally can be done by classic chemistry mechanism. However, Bolle and Aehle, (2001) has demonstrated the enzymatic generated industrial lignin (either LS, kraft and organosolv lignins) based coating using laccase employed mediator ABTS (2,2-azino-bis,3-ethylbenzthiazoline-6-sulfonic acid) revealed copolymerization agents with highly improvement of waterproof property.

Chelating agent

There are still no studies in enzymatic modification of industrial lignin resulting chelating agent but a promising study has been done by Gonćalves and Benar (2001) showed that several industrial lignin obtained from sugarcane acetosolv pulping with a polyphenoloxidase after treated by conventional hydroxymethylation and oxidation give result an increase in the number of hydroxyl and carbonyl groups. Hence, the chelating capacity of treated lignin was improved. The increasing capacity of chelating complexes pulp an dpaper industry can be use for wheather removing heavy metals in pulping process or removing metal in its waste water treatment system.

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Polymerization of lignosulfonates by the laccase-HBT (1hydroxybenzotriazole) system improves dispersibility

The ability of laccases from *Trametes villosa* (TvL), *Myceliophthora thermophila* (MtL), *Trametes hirsuta* (ThL) and *Bacillus subtilis* (BsL) to improve the dispersion properties of calcium lignosulfonates 398 in the presence of HBT as a mediator was investigated. Size exclusion chromatography showed an extensive increase in molecular weight of the samples incubated with TvL and ThL by 107% and 572% from 28400 Da after 17 h of incubation, respectively. Interestingly, FTIR spectroscopy, ^{13}C NMR and Py-GC/MS analysis of the treated samples suggested no substantial changes in the aromatic signal of the lignosulfonates, a good indication of the ability of TvL/ThL-HBT systems to limit their effect on functional groups without degrading the lignin backbone. Further, the enzymatic treatments led to a general increase in the dispersion properties, indeed a welcome development for its application in polymer blends.

Keywords: Calcium lignosulfonates, Laccase, Enzymatic modification, Dispersion, Polymerization

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2.1. Introduction

Lignin, the second most abundant polymer on Earth constituting 30% of non-fossil organic carbon, is currently under-utilized (Boerjan et al., 2003). Industrial lignins constitute the main by-products of the pulp and paper industry regarded as waste material, imposing disposal problems and their major applications have been limited to providing fuel for firing the pulping boilers (Mohan and Karthikeyan, 1997). Only approximately 2% of the lignins are used commercially (1 million tons/year of lignosulfonates and less than 100,000 tons/year of kraft lignins) (Gargulak and Lebo, 2000, Gosselink et al., 2004 and Lora and Glasser, 2002). However, over the recent years there has been a renewed interest in using lignin as a renewable raw material. This is partly due to new stringent environmental waste management regulations together with the demand for replacement of oil based products with renewable materials and the new possibilities offered by emerging technologies. Consequently, investigations into increasing the application of lignin in existing and novel polymer blends for mortar, construction materials, adhesives, biodegradable plastics, polyurethane copolymers, paints, dye dispersants, in pesticides and printed circuit boards (Sena-Martins et al., 2008, Stewart, 2008, Lora and Glasser, 2002, Hüttermann et al., 2001 and Kosbar et al., 2001), are increasing.

Nevertheless, massive exploitation of lignin is hampered by its huge physico-chemical heterogeneity owing to its inherent variety of aromatic units, inter-unit linkages, functional groups, and molecular size (Vázquez et al., 1999, Glasser and Sarkanen, 1989 and Li et al., 1997). This is a result of both the heterogeneity of this plant polymer and its degradation during the pulping process resulting in a polydisperse material (Dence and Lin, 1992) which has high interfacial tension and lacks interfacial adhesion properties, making it difficult to achieve desired degree of dispersion in polymer blends (Cazacu et al., 2004). Most lignin polymer blends have been reported to be immiscible due to low entropy (Flory, 1953) indicating that the ability of lignin to mix strongly depends on its active properties (Ekeberg et al., 2006). For example, increasing lignin concentration in thermoplastics and rubber blends

negatively affects the tensile force and melt flow index of the product (Alexy et al., 2000), while its polydispersity limits its addition between 5% and 10% the level of the resin weight in adhesive synthesis (Turunen et al., 2003). In short, the deterioration of the mechanical properties of all polymer blends where lignin has been used, is attributed to the poor adhesion and dispersion of the lignin particles which create defects that act as stress concentrators (El Mansouri and Salvado, 2006). Therefore, industrial applications of lignins require modifications to improve its dispersion properties among other physico-chemical characteristics.

In line with this demand, in this study, the effect of laccase-mediator treatment on dispersion properties of lignosulfonates was investigated and correlated to chemical changes of the polymer. Lignosulfonates are highly cross-linked anionic polymers in which the essentially hydrophobic backbone is rendered hydrophilic by substitution with sulfonate groups (Askvik et al., 2001). In the presence of mediators, laccases (benzenediol: oxygen oxidoreductases, EC.1.10.3.2) can also oxidize the non-phenolic moieties in lignin (Call and Mücke, 1997 and Leonowicz et al., 2001) and this has been widely studied in the pulp and paper industry in relation to delignification (Chandra and Ragauskas, 2005, Balakshin et al., 2001, Elegir et al., 2005 and Rochefort et al., 2004). Among the known mediators, 2,2'-azinobis-(3-ethylbenzthiazoline)-6-sulfonate (ABTS) and 1-hydroxybenzotriazole (HBT) are the most commonly used (Ibarra et al., 2006, Leonowicz et al., 2001, Baiocco et al., 2003 and Call and Mücke, 1997) and effective. Although, a number of studies have demonstrated the ability of laccase to modify technical lignins (Sena-Martins et al., 2008, Milstein et al., 1990, Milstein et al., 1994, Hernández Fernaud et al., 2006, Lund and Ragauskas, 2001 and Popp et al., 1991), detailed characterization of the resulting chemical changes is still lacking. Here, we relate for the first time the enzymatic improvement of the dispersion properties of lignosulfonates to the chemical changes of the polymer. Detailed analysis was carried out by employing a number of different complementary techniques among them fluorescence monitoring, different

nuclear magnetic resonance (NMR) techniques, Fourier transform infrared (FTIR) spectroscopy, size exclusion chromatography and chemical analysis.

2.2. Materials and methods

2.2.1. Materials

Calcium lignosulfonate samples were provided by Borregaard (Sarpsborg, Norway). Among the enzymes used, NS51002 – *Trametes villosa* laccase (TvL) and NS 51003 – *Myceliophthora thermophila* laccase (MtL) were supplied by Novozymes (Bagsvaerd, Denmark). *Trametes hirsuta* laccase (ThL) and *Bacillus* spore laccase (BsL) were produced as previously described ([Almansa et al., 2004] and [Held et al., 2005]). All the other reagents used were of analytical grade purchased either from Sigma–Aldrich or Merck.

2.2.2. Laccase activity assay

The activity of laccase was determined spectrophotometrically by monitoring the oxidation of 2,2′-azinobis-(3-ethylbenzthiazoline)-6-sulfonate (ABTS) to its cation radical (ε_{436} = 29,300 M^{-1} cm^{-1}) as substrate at 436 nm in 50 mM sodium succinate buffer at pH 4.5 and 30 °C using quartz cuvette of path length 10 mm (Nugroho Prasetyo et al., 2009) and activity expressed in nano katals (nkat) corresponding to 1 nmol of substrate converted per second.

2.2.3. Polymerization of calcium lignosulfonates

Calcium lignosulfonate samples were incubated with each of the above laccases separately. Briefly, 1 g of the calcium ligninosulfonate sample was dissolved in 50 ml double distilled water in 250 ml Erlenmeyer flasks and 1-hydroxybenzotriazole (HBT) or ABTS (1 mM final concentration) added to the reaction mixture. The reaction was started by adding a 30 nkat ml^{-1} laccase activity as determined at the optimum pH value of the individual experiments. Samples were then incubated at 30 °C while shaking at 150 rpm. Samples were withdrawn at regular intervals and fluorescence intensity measured

instantly while the other part of the sample was immediately frozen by immersing in liquid nitrogen. The frozen samples were lyophilized using the Labconco Freeze Dry System/FreeZone© 4.5 Liter Benchtop Model 77500 (Vienna, Austria). The freeze drier was operated at a temperature of −48 °C and at a vacuum pressure of 3×10^{-4} mbar. These freeze dried samples were stored in the dark in sealed tubes at 4 °C until further analysis.

2.2.4. Fluorescence intensity measurements

During enzymatic polymerization fluorescence intensity was monitored (Ex 355 nm/Em 400 nm) at defined time intervals using TECAN Infinite M200 plate reader (Tecan Austria GmbH, Grödig, Austria). A lignin sample of 100 µl was added to a solution of 2-methoxyethanol (Thomson et al., 2005) and water (2:1 v/v) and then thoroughly mixed before measuring.

2.2.5. FTIR analysis

FTIR spectra were obtained on a Perkin–Elmer Spectrum 2000 instrument by the attenuated total reflectance (ATR) technique. Spectra were recorded in the 4000–600 cm^{-1} range with 16 scans at a resolution of 4.0 cm^{-1} and an interval of 1.0 cm^{-1}. Sulfonate groups were also detected by FTIR at 1145 and 647 cm^{-1}.

2.2.6. Size exclusion chromatography

All lignosulfonates, being highly soluble in water, were analyzed using three TSK-gel columns (3000 PW, 4000 PW, 3000 PW) coupled in series with 0.1 M sodium hydroxide as the eluant. Flow rate was 1 mL/min and detection was done by UV at 280 nm.

2.2.7. NMR analysis

Solution NMR spectra, including ^{1}H NMR, ^{13}C NMR and heteronuclear single quantum correlation (HSQC) 2D-NMR spectra were recorded on 40 mg of lignosulfonate dissolved in 0.75 mL of DMSO-d_6 using a Bruker AVANCE

500 MHz as previously described (Ibarra et al., 2006). A semiquantitative analysis of the HSQC cross-signal intensities was performed (Heikkinen et al., 2003 and Zhang and Gellerstedt, 2007) including separate volume integrations and comparison in each of the regions of the spectrum, which contain cross-signals of chemically analogous carbon–proton pairs. Cross-polarization magic-angle spinning (CPMAS) ^{13}C NMR spectra of solid lignosulfonate samples were recorded for 9 h on a Bruker AVANCE DSX 300 using the standard pulse sequence, a time domain of 4 K, a spectral width of 41,666 Hz, a contact time of 2 ms, and an interpulse delay of 4 s. Signals were assigned by comparison with the literature (Bardet et al., 2006, Capanema et al., 2004, Lebo et al., 2008, Liitiä et al., 2003, Lundquist, 1981, Lutnaes et al., 2008, Martínez et al., 1999, Ralph et al., 1999, Ralph et al., 2004 and Robert, 1992).

2.2.8. Photon-correlation spectroscopy (PCS) and Zeta-potential measurements

The surface charge of the oxidized solutions was measured in terms of Zeta-potential in a Zetasizer Nano Series (Malvern Instruments Inc., Worcester, UK). This method measures how fast a particle moves in a liquid when an electrical field is applied i.e. its velocity. The aggregation behaviour of treated lignosulfonate particles in solution was therefore performed by determining its electrophoretic mobility. The size distribution of the oxidized samples was also measured by photon-correlation spectroscopy.

2.2.9. Dispersion properties

The Turbiscan MA 2000 from Sci-Tec Inc (Sandy Hook, USA) was used to assess the stability of suspensions (Mengual et al., 1999). Different enzyme-modified lignosulfonates are rated after their ability to stabilise a standard suspension. A similar procedure has been used to follow the sedimentation of suspensions (Balastre et al., 2002) and creaming of emulsions (Roland et al., 2003). The Turbiscan technology consists in measuring backscattering and transmission intensities versus the sample height in order to detect particle size

change (coalescence, flocculation) and phase separation (sedimentation, creaming).

2.2.10. Py-GC/MS

The pyrolysis of the lignosulfonates (approximately 100 µg) was performed in duplicate with a model 2020 micro-furnace pyrolyzer (Frontier Laboratories Ltd., Yoriyama, Japan) directly connected to an Agilent 6890 GC/MS system equipped with a 30 m × 0.25 mm i.d., 0.25 µm HP 5MS fused silica capillary column. The detector consisted of an Agilent 5973 mass selective detector (EI at 70 eV). The pyrolysis was performed at 500 °C. The GC/MS conditions were as follows: the oven temperature was held at 50 °C for 1 min and then increased up to 100 °C at 30 °C/min, from 100 to 300 °C at 10 °C/min and isothermal at 300 °C for 10 min. The carrier gas used was helium with a controlled flow of 1 ml/min. The compounds were identified by comparing the mass spectra obtained with those of the Wiley and NIST computer libraries and that reported in the literature (Faix et al., 1990 and Ralph and Hatfield, 1991). Sulfonate groups were also detected by Py-GC/MS**.**

2.3. Results and discussion

2.3.1. Fluorescence intensity

Fluorescence spectroscopy was used as a sensitive and simple analytical tool to optimize modification of calcium lignosulfonates with different laccases at different pHs. A similar trend was observed in fluorescence changes (decrease in fluorescence) when lignosulfonates were treated with laccases in the presence of either HBT or ABTS (Fig. 1a and b). TvL and ThL were effective in reducing fluorescence under acidic conditions (pH 4.0 and 4.5) while the BsL and the MTL were more effective at pH above 6 (Fig. 1a and b). The MTL performed slightly better than the BsL in the presence of both ABTS or HBT as mediators (Fig. 1a and b). Although the decrease in fluorescence measured for TvL and ThL treated lignin at pH 4.0 and 4.5 was almost similar in the presence of ABTS or HBT, samples incubated with the

latter performed slightly better. Similarly, at the same pH (4.5), the ThL reduced fluorescence was 2058 AU in ABTS supplemented samples and 2828 AU in HBT incubated samples. Fluorescence is an intrinsic property of lignin attributed to conjugated carbonyl, biphenyl, phenylcoumarins and stilbene groups (Albinsson et al., 1999 and Lundquist et al., 1978). Therefore, the observed decrease in fluorescence intensity in this study upon incubation with laccases indicated modification of these functional groups present in lignin. The destruction or modification of biphenyl groups, for example, has been shown to affect fluorescence intensity (Castellan et al., 1992). Here, the decrease in fluorescence was used as an indication of the extent of modification of the calcium lignosulfonates. The observed different modifications by the different enzymes maybe attributed to the different redox potential of the laccases. For example TvL and ThL are high redox potential laccases with redox potentials of approximately +790 mV (Rebrikov et al., 2006 and Tadesse et al., 2008) while MTL and BsL are low redox potential laccases (460 mV and 455 mV, respectively) (Tadesse et al., 2008 and Melo et al., 2007).

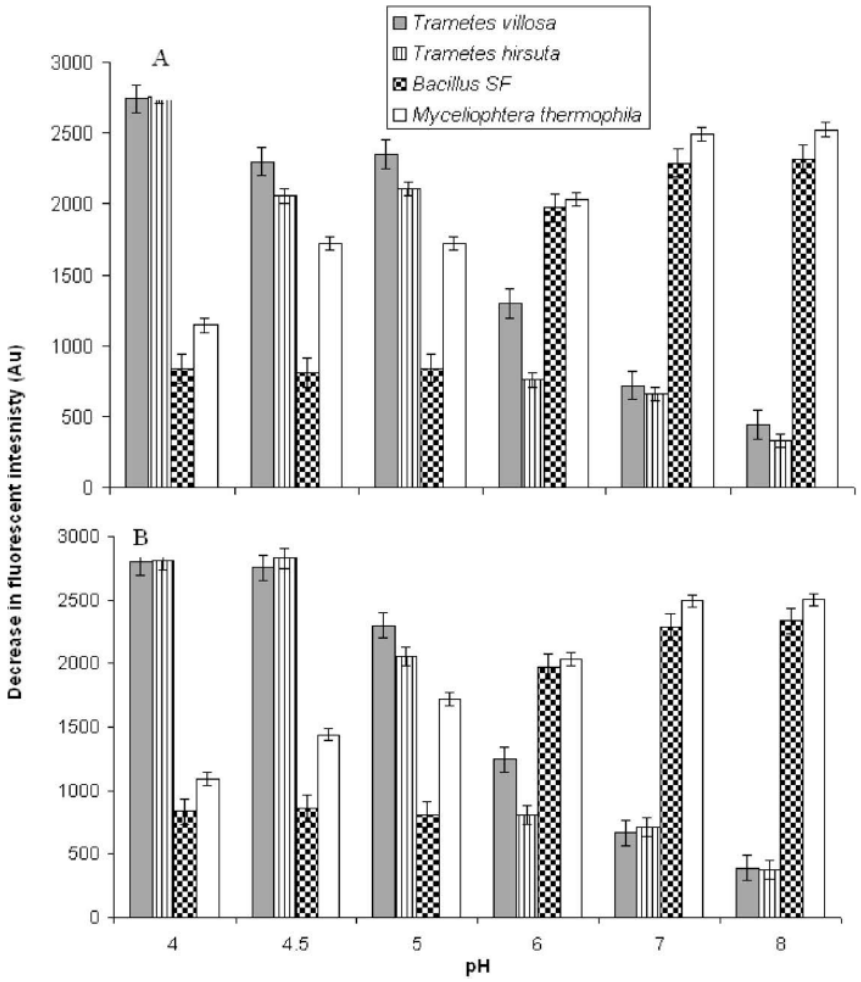

Fig.1. Fluorescence decrease of lignosulfonate after incubation with different laccases in the presence of HBT (A) and ABTS (B) at different pHs after 83 h of incubation. Data is an average of 3 independent replicates ± standard deviation.

2.3.2. Gel permeation chromatography

The calcium lignosulfonate samples incubated with TvL and ThL underwent extensive polymerization. The Mw increased by 74% after 17 h of incubation with ThL and by 370% in TvL-treated samples (Table 1) supplemented with 0.5 mM HBT. Polymerization as a central feature during laccase oxidation of lignin moieties has also been reported by previous authors (Ishihara and Miyazaki, 1972; Hüttermann et al., 1980; Elegir et al., 2007). As indicated earlier by Karhunen et al., (1990a) and Karhunen et al., (1990b), the radicals generated by laccases underwent resonance

stabilisation forming different mesomeric forms that coupled in many possibilities forming inter-unit linkages which include β-O-4, β-5, 5-5, β-β, 5-O-4 resulting in polymers of different sizes. In this study, the increase in Mw was accompanied by a decrease in phenolic groups and carboxylic groups. Several authors have observed a similar decrease in phenolic groups (Shleev et al., 2006, Grönqvist et al., 2005, Rittstieg et al., 2002 and Buchert et al., 2002). This indicates that the laccase-HBT oxidized phenolic substituents and generated phenoxy radicals which underwent coupling reactions leading to the observed polymerization. The content of carboxylic groups in lignin decreased by 2% for ThL and by 2.4% for TvL after 17 h of incubation, respectively (Table 1.)

Table 1. Changes in molecular weight (Mw) and functional group content after treatment of lignosulfonates with different laccases in the presence of HBT.

Laccase	Time (h)	Mw	Mn	Pdi	Ar–OH (%)	COOH (%)
Trametes hirsuta	0	30800	1900	16.21	1.9	7.5
	0.5	29700	1900	15.63	–	–
	17	53800	4300	12.51	1.4	5.6
Trametes villosa	0	29400	1800	16.33	1.9	7.5
	0.5	40400	2950	13.69	1.5	5.6
	17	140100	8250	16.98	1.0	5.1

Mn-number average molecular weight; Pdi-polydispersity.

The effect of increasing incubation time and doubling HBT concentration was investigated in subsequent experiments (Table 2). Increasing incubation time to 83 h and HBT concentration to 1 mM (final concentration) resulted in 107% and 572% increase in Mw of ThL and TvL incubated calcium lignosulfonate samples, respectively. The changes in Mw are clearly visible in size exclusion chromatography (Fig. 2 ThL, TvL). The Mw of TvL incubated samples clearly changed resulting in a narrower Mw band, comparing chromatograms of samples incubated for 0 h and 83 h. Although there was a clear modification of lignosulfonates in ThL samples, the

modifications are different from those obtained in TvL samples. Previous researchers have also reported polymerization of lignosulfonates by laccases (Leonowicz et al., 1985, Hatakka et al., 1996 and Bae and Kim, 1996), although the use of ABTS as a laccase mediator was repeatedly resulted in depolymerizaion of lignin (Hernández Fernaud et al., 2006 and Bourbonnais et al., 1995) and was even shown to be incorporated in polymerization products (Rittstieg et al., 2002).

Table 2. Changes during incubation of *Trametes hirsuta* and *Trametes villosa* laccase-HBT systems with lignosulfonates.

Laccase	Time (h)	Mw	Mn	Inorganic S (%)	Organic S (%)	Ar-OH (%)	COOH (%)
T. hirsuta							
	0	28400	2650	0.8	5.4	1.8	7.4
	17	43100	3800	0.8	5.0	1.4	6.1
	83	58800	5250	1.0	5.1	1.1	5.4
T. villosa							
	0	28400	2650	0.9	5.2	1.9	7.4
	17	142400	9200	1.0	4.9	1.1	5.2
	83	191100	10500	1.0	5.1	1.0	5.0

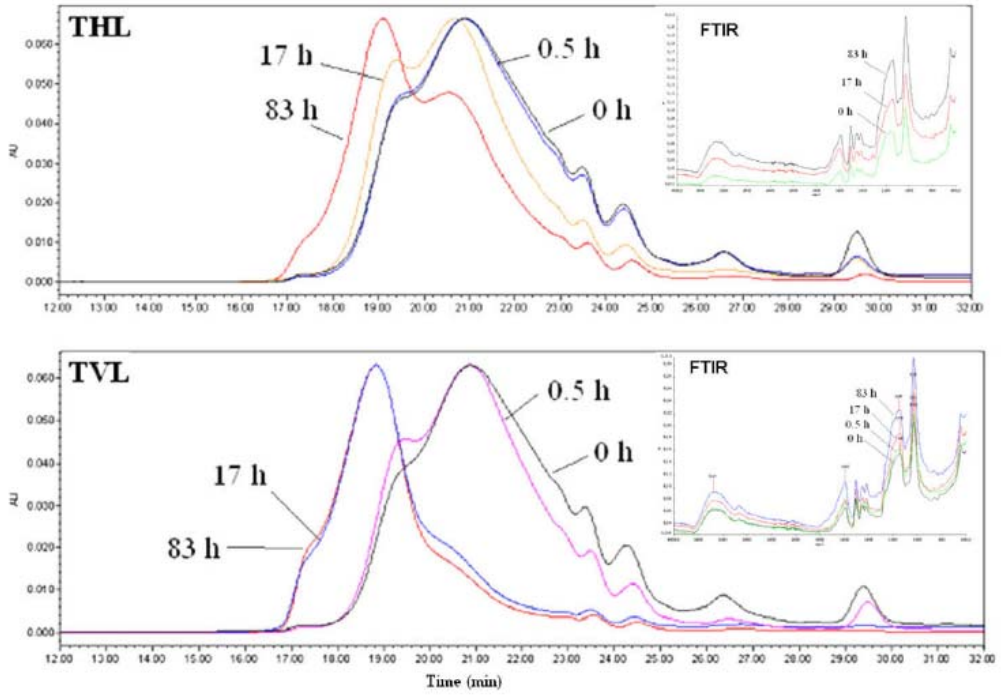

Fig. 2. Polymerization of lignosulfonates by *Trametes hirsuta* laccase (THL) and *Trametes villosa* laccase (TVL) in the presence of HBT after 0, 0.5, 17 and 83 h of incubation as monitored by size exclusion chromatography. Inserts show results of FTIR analysis of the lignosulfonates.

An increase in incubation time and HBT concentration lead only to a marginal further decrease in phenolic and carboxylic groups. The phenolic content decreased from 1.4 mmol g^{-1} in the untreated lignosulfonate sample to 0.85 mmol g^{-1} after 83 h incubation with ThL and to 0.45 mmol g^{-1} after incubation with TvL (Table 2). There was a small decrease in the organic sulfur content and a slight increase in inorganic sulfur content in both samples upon enzyme treatment (Table 2). This loss of sulfonic acid groups might be responsible for the in the Zeta-potential. This parameter increased from 0.65 to 2.4 mV in ThL-treated samples and from 0.6 to 2.2 mV in TvL-treated samples. The particle size [Z-average (d.nm)] increased from 369.4 to 942.8 in ThL and 311.0 to 421.2 in TvL samples indicating aggregation of particles. Unlike TvL-treated samples for 83 h, ThL samples became partly insoluble.

2.3.3. FTIR analysis

FTIR spectroscopy at mid-infrared region (4000–600 cm^{-1}) was applied to monitor structural changes occurring during incubation of the calcium lignosulfonate with the ThL and TvL (Fig. 2 ThL and TvL). In the FTIR spectra bands at 1595 cm^{-1} and 1520 cm^{-1} suggest aromatic ring vibrations and at 1033 cm^{-1}, aromatic C–H in-plane deformation (Fig. 2 ThL and TvL). Sulfonate groups are shown by bands at around 1145 cm^{-1} (asymmetric and symmetric $–SO_2–$ vibrations) and one band at around 647 cm^{-1} (from S–O structure). Further the stretching vibrations of alcoholic and phenolic OH groups involved in hydrogen bonds were detected between 3500–3200 cm^{-1}. Further analysis of the region between 1645 and 1760 cm^{-1}, reveals no noticeable changes in carbonyl or carboxylic acid group present or generated by the treatments in the structures. As a general conclusion, according to the FTIR data, no substantial changes were introduced in the calcium lignosulfonates samples during incubation with both TvL and ThL.

2.3.4. Dispersion properties

Indeed, the dispersant properties of the enzyme treated lignosulfonate were significantly improved as shown in Fig. 3. A low value of delta

backscattering indicates a more stable suspension. The increased Mw and reactivity of the enzyme treated lignosulfonates could have enhanced its miscibility. Previously, prepared lignosulfonates by phenolation were shown to increase dispersibility by over 30% for gypsum paste than the commercial lignosulfonate (Matsushita and Yasuda, 2005). They attributed the improvement in dispersion properties to increased Mw and sulfur contents of the preparations. This is inline with the observation in this study where an increase in Mw and reactivity were noted and a very marginal loss of sulfur. The fact that laccase did not remove sulfur is very encouraging because sulfonate groups are important for imparting solubility properties to lignins.

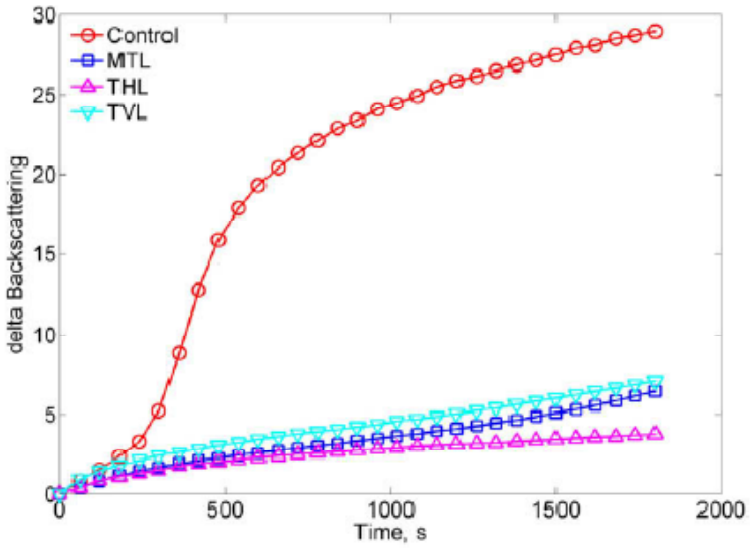

Fig.3. Dispersibility of lignosulfonates polymerized with laccases from *Myceliopthera thermophilia*, *Trametes hirsuta* and *Trametes villosa* (MTL, THL and TVL) in the presence of HBT, based on multiple light scattering (BS - Backscattered and T - transmitted light compared to a control and to chemically modified lignosulfonate after 17 h incubation.

For example, sulfonation leads to water-soluble anionic polymers and high-dispersibility gypsum paste (Matsushita et al., 2008 and Li et al., 2009). The dispersing efficiency increased as the surface tension decreased, suggesting that the fluidity of the gypsum paste increased with the polymer adsorption on the gypsum particle surface (Matsushita et al., 2008). This phenomenon

may also be attributed to the observed increase in dispersion properties in this study.

2.3.5. NMR analysis of enzymatically-modified lignosulfonates

In contrast to FTIR data, the HSQC NMR analysis (Fig. 4) showed decreases in the intensities of cross-signals in the three main regions of the lignosulfonate spectrum, corresponding to aromatic (δ_H/δ_C 5.5–8/105–140 ppm), aliphatic oxygenated (δ_H/δ_C 2.5–5.5/50–105 ppm) and aliphatic non-oxygenated (δ_H/δ_C 0–3/0–50 ppm) ^1H–^{13}C correlations, albeit with very different decrease intensities in each of them. The HSQC spectra were obtained from the same amount of sample (40 mg in 0.75 mL of DMSOd_6) and normalized to the residual DMSO cross-signal (δ_H/δ_C 2.5/40 ppm). The above decreases were observed in both the samples treated with TvL (Fig. 4a and b) and ThL (Fig. 4c and d).

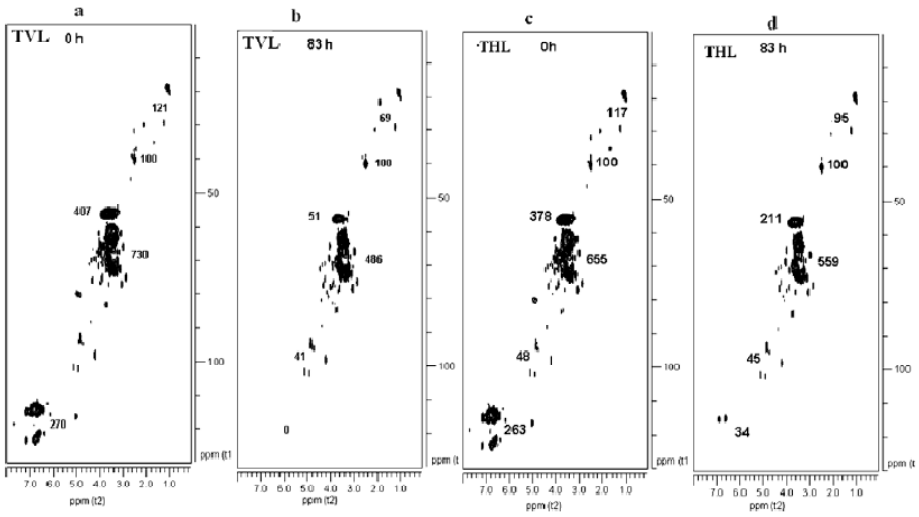

Fig. 4. HSQC 2D-NMR analysis of lignosulfonate modification by *Trametes hirsuta* laccase (THL) (right) and *Trametes villosa* laccase (TVL) (left) in the presence of HBT after 0 h (a and c, respectively) and 83 h (b and d, respectively) of incubation.

The integrals of the main groups of ^1H–^{13}C correlation signals (from bottom to top: aromatic signals, anomeric polysaccharide signals, different oxygenated aliphatic signals, methoxyl signal, and non-oxygenated aliphatic signals) are indicated, referred to the residual DMSO signal (as 100%).

After 83 h incubation the aromatic cross-signals found in the HSQC spectrum of the control (0-h) lignosulfonate, which corresponded to H_2–C_2, H_5–C_5 and H_6–C_6 correlations (with δ_H/δ_C 6.91/114.7, 6.65/114.9 and 6.75/122.9 ppm, respectively) completely disappeared in the TvL-treated samples. On the other hand, they were still partially visible in the ThL-treated samples although with very strongly reduced intensities. In addition, the methoxyl cross-signal (with δ_H/δ_C 3.72/56.2 ppm) significantly decreased in both the TvL and ThL-treated samples, together with those of the most abundant β-O-4′ linked α-sulfonated side-chains including H_β–C_β correlation (with δ_H/δ_C 4.93/80.1 ppm), while polysaccharide and other oxygenated aliphatic cross-signals remained practically unaffected by the laccase-mediator treatment. Finally, only a few and small cross-signals of non-oxygenated aliphatic correlations were observed in the lignosulfonate spectra including that from the methyl of the acetate buffer used for the enzymatic treatment (with δ_H/δ_C 1.1/19 ppm).

The disappearance of the aromatic ^1H–^{13}C correlation signals in the TvL treated lignosulfonate after 83 h of incubation, as shown by HSQC 2D-NMR, was initially unexpected. Therefore this sample and its (0-time) control were further analyzed by ^1H NMR, and by both liquid and solid state ^{13}C NMR (Fig. 5). The latter was used to solve eventual solubility problems due to enzymatic polymerization, although no DMSO insoluble material was observed in the NMR tubes.

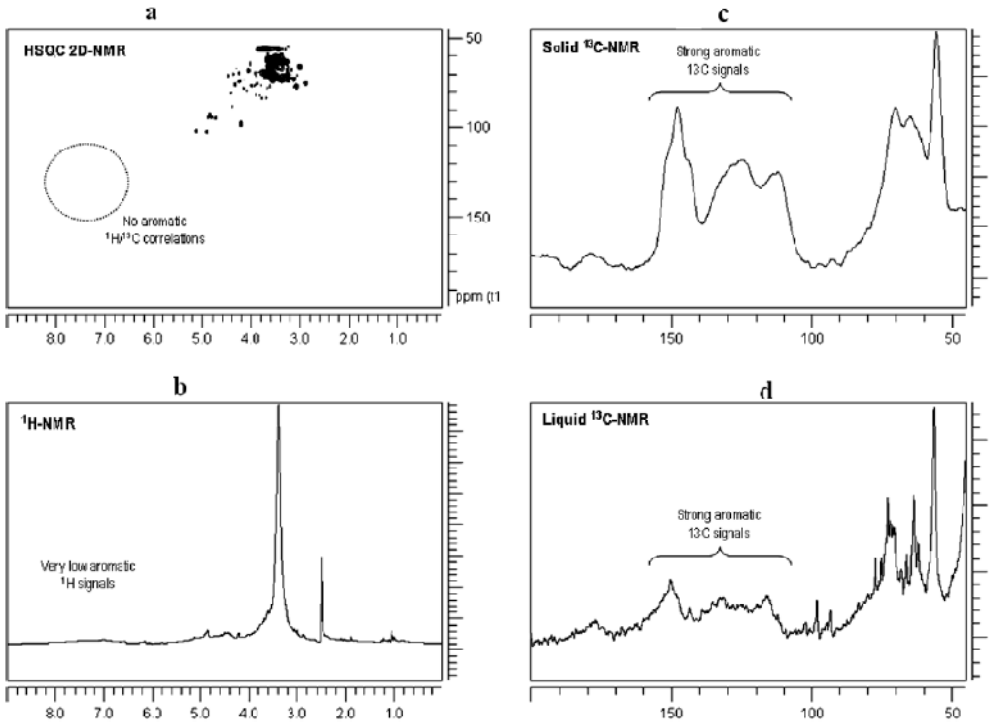

Fig. 5. Comparison of aromatic signals in (a) HSQC 2D-NMR spectrum (^{1}H–^{13}C correlation), (b) ^{1}H NMR spectrum, (c) ^{13}C NMR spectrum, and (d) CPMAS ^{13}C NMR spectrum of spruce lignosulfonate after 83 h incubation with *Trametes villosa* laccase (TVL)-HBT system.

The loss of aromatic cross-signals in the HSQC spectra obtained after the enzymatic treatment (Fig. 5a) was due to deprotonation of the lignin benzenic rings, as revealed by the 1D-NMR spectra. In this way, no aromatic proton signals were found in the 1H NMR spectrum of the lignosulfonate treated with TvL for 83 h (Fig. 5b) while strong signals of aromatic carbons appeared in the ^{13}C NMR spectra obtained either in solution (Fig. 5c) or in the solid state using the CPMAS technique (Fig. 5d). This suggests formation of new ether and C–C aryl–aryl or aryl–alkyl linkages as a result of the enzymatic attack on the lignosulfonate aromatic nuclei causing the strong polymerization observed by SEC. After initial condensation reactions between the phenoxy radicals formed by the action of the enzyme on the phenolic units present in the initial lignosulfonate sample, the high redox-potential laccase-HBT system most probably cause additional oxidative attack on the

non-phenolic lignin nuclei resulting in additional deprotonation and condensation reactions.

2.3.6. Py-GC/MS of enzymatically-modified lignosulfonates

The chemical composition of the lignosulfonates was analyzed by Py-GC/MS (Fig. 6). The compounds released arise mainly from lignin moieties with minor amounts of carbohydrates and sulfur compounds being present. Among the lignin derived compounds, only guaiacyl derivatives were detected, as corresponds to a lignosulfonate from softwood. The most interesting observation obtained from Py-GC/MS pyrograms was the decrease in the intensity of the lignin peaks (4-methylguaiacol, 4-ethylguaiacol, guaiacylacetone, 4-vinylguaiacol, homovanillylalcohol, eugenol, *cis*- and *trans*-isoeugenol, dihyroconiferyl alcohol and *trans*-coniferaldehyde), in both TvL and ThL-treated samples (Fig. 6). The decrease in the different lignin moieties was accompanied by a concomitant increase in sulfur dioxide and dimethyldisulfide. Lignin markers were very much present as detected by Py-GC/MS in all the samples during the whole incubation period despite a slight decrease at longer incubation periods (Fig. 6). The FTIR and ^{13}C NMR spectra together with Py-GC/MS chromatograms suggesting no substantial structural changes in the calcium lignosulfonate aromatic structure are a good indication of the ability of TvL and ThL to limit their effect to effective cross-linking, without degrading the lignin backbone. These data are in line with reports by Martinnen et al. (2008) who also did not observe substantial differences in the aromatic signals after laccase treatment of lignin.

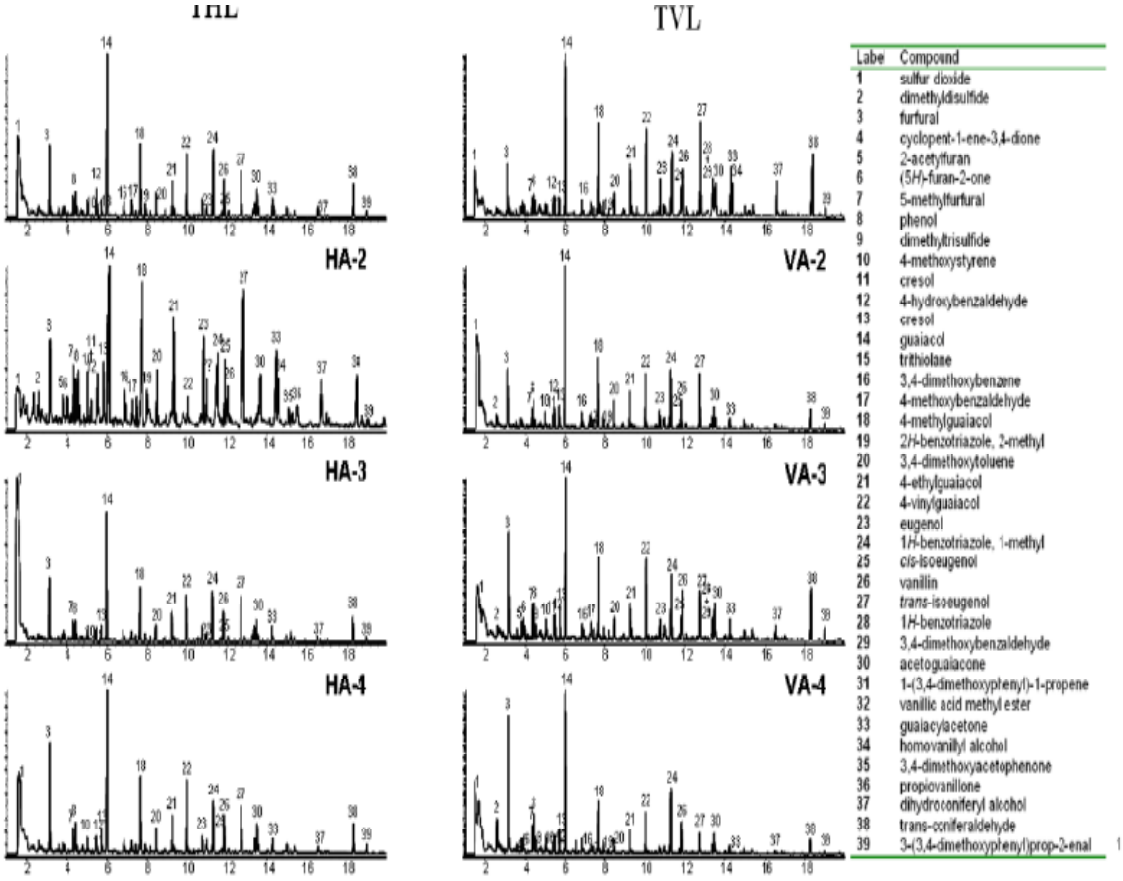

Fig. 6. Py-GC/MS of calcium lignosulfonate during incubation with *Trametes hirsuta* laccase (THL) and *Trametes villosa* laccase (TVL) in the presence of HBT.

The seemingly contradictory aromatic data by HSQC 2D-NMR, showing the disappearance of the aromatic cross-signals, may be due to the strong polymerization produced by the laccase-HBT treatment. This resulted in new carbon–carbon and carbon–oxygen linkages leading to condensation and/or modification reactions in such a way that most lignin aromatic carbons were unprotonated, the remaining ones being below the HSQC detection level. Some problems associated with 2D-NMR spectroscopy are related to the short T_1 and T_2 relaxation times (Garver et al., 1996 and Zhang and Gellerstedt, 2007 suggested the degree of polymerization as one of the factors affecting short T_2 values in HSQC NMR. Our observation seems to also vindicate earlier comments by Capanema et al. (2004) who emphasized a need for caution when analyzing 2D-NMR spectra of lignin data that must be complemented with 1D (^{1}H and ^{13}C NMR) spectra.

2.4. Conclusions

Size exclusion chromatography analysis of ThL-HBT and TvL-HBT treated lignosulfonate resulted in extensive polymerization leading to 107% and 572% increase in Mw from 28 400 Da, respectively. New ether and C–C aryl–aryl or aryl–alkyl linkages were detected as causing the strong polymerization as confirmed by FTIR, ^{13}C NMR spectra and Py-GC/MS chromatograms. Nevertheless, the treatment did not affect the lignin backbone, a good indication of the ability of TvL and ThL-HBT systems to limit their effect to the functional groups only. As a result, the dispersant properties of the enzyme treated lignosulfonate increased significantly.

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3

Functional polymers derived from enzyme mediated coupling of lignin onto silanes

Organic-inorganic hybrid polymeric materiales derived from silica precursors and lignin were sucessfully produced using sol gel process. Activation of lignin by laccase increased the interaction between lignin and tetraethylorthosilicate (TEOS) resulting in interepenetrating polymers. The lignin concentration affected the viscosity, curing time and tensile strength. Si-NMR analysis of the hybrids between trimethylSiO3 and pure lignin molecules (ferulic and sinapic acid) showed new signals indicating the formation of hybrids.

Key Words: Tetraethylorthosilicate (TEOS), lignin,laccase, adhesive

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3.1. Introduction

The use of wood and wood-based products is an integral part of human life. Among the major wood product categories used for different purposes are sawn timber, wood-based panels, woodchips, paper and paper products, poles and railway sleepers etc. Despite its attractive properties (low thermal extension, low density and high mechanical strength), wood materials are highly hygroscopic and biodegradable. Traditionally two approaches namely; chemical and thermomechanical wood processing technologies have been employed to improve wood properties (Sander and Koch, 2001; Rowell,2004; Tjeerdsma and Militz, 2005). Regrettably, in recent years most of these methods are highly discouraged due to their negative effect on human health and environmental pollution problems. It is this sudden shift in world policy that has seen the emergency of biological based mechanisms (enzymatic processes) gaining favor as the most suitable processes. One such promising option is the use of oxidative enzymes like laccases in improving wood properties. Laccase (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductase) catalyse the monoeletronic oxidation of phenols and aromatic or aliphatic amines to reactive radicals in a redox reaction in which molecular oxygen is simultaneously reduced to water. Laccases therefore can be used to create radicals on wood surface at ambient temperatures when they react with lignin moieties to which functional molecules of interest can be coupled (Kudanga et al., 2008). A summary of the applications of laccase in grafting of low molecular weight molecules onto lignocellulose material is provided in a recent review article (Widsten and Kandelbauer, 2008).

However, although silanes are among the most versatile widely used chemicals as coupling agents and for solid surface property modification (Donath et al 2006a, Donath et al 2006b) the use of laccases as coupling agent has not been studied**.** Silane coupling chemicals bear many functional groups capable of reacting with OH-rich surface and these functional groups can also be tailored depending on application. Several chemical processes using silane as coupling agents have been developed to transform hydrophilic biopolymers into hydrophobic polymers (Saka et al 1999, Saka and

Ueno, 1997; Saka and Yakake, 1993). For instance, textile manufacturers have been using a thin physically adhering coating of silicone for many years, to impart water repellence to fabric (Sodano 1979). In wood, the utilization of silane coupling agents in chemical processes has been reported to increase the compatibility between the wood fibres and as well as increasing hydrophobicity by converting the polar hydroxyl groups of wood into hydrophobic moieties (Kokta et al. 1990; Ulrich 1991; Raj and Kokta 1992). Similarly silanes have been used since the 1940s as adhesion promoters between polymers and oxidized surfaces.

To this effect, this study first of its kind, explores the possibility of developing an enzyme based technologie using laccases to produce lignin reactive species which then can be used to couple to silanes. Enzymatic coupling of lignin to silane molecules can greatly benefit the production of modified woodfiber thermoplastics composites; lignin based organic-inorganic hybrid adhesives, hydrophobic wood material as well as other wood with other interesting improved surface properties (antimicrobial, flame retardants).

3.2. Material and methods

3.2.1. Materials

Phenolic compounds (caffeic acid, 4-hydroxybenzoic acid, sinapic acid, pyrogallol, gallic acid, syringic acid, guaiacol, catechol, syringaldazine; 2,6-dimethoxyphenol and eugenol) and silane precursors (Ethoxhytrimethylsilane and 1,3-Bis(3-aminopropyl)-1,1,3,3-tetramethyldisiloxane) were obtained from Sigma.

Lignins from different sources were kindly provided by industrial partners in the frame of the European Union Biorenew project supported by the European Community. Four types of lignosulphonate shown in Table 1 were supplied by Borregard Ligno-Tech, Norway.

Table 1. Lignosulphonate sample

Name	Raw material	Cation	Treated
Ca –lingo	Softwood, Norway spruce (*Picea abies*)	Ca	Filtered
Na-ligno	Softwood, Norway spruce (*Picea abies*)	Na	Ultrafiltered Ionexchanged
Mod-Na ligno	Softwood, Norway spruce (*Picea abies*)	Na	Ultrafiltered Desulfonated Oxidized
Hardwood	Hardwood (*Eucalytus grandis*)	Ca	Filtered Heat treated

Sulphur-free lignin [a mixture of wheat straw (*Triticum* sp) and sarkanda grass (*Saccharum munja*)] was supplied was supplied by Granit RD, Switzerland. Tetraethylorthosilicate (TEOS) was obtained from Sigma-Aldrich Chemie GmbH, Germany and ethanol absolute (96%) was purchased from Austr Alco Österr Alkoholhandels GmbH, Austria. The rest of the chemical used were purchased either from Merck or Sigma-Aldrich.

3.2.2. Methods

3.2.2.1. Laccase activity assay

The activity of laccase was determined spectrophotometrically by monitoring the oxidation of 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonate (ABTS) (ε_{436} = 29,300 $M^{-1}cm^{-1}$) as substrate at 436 nm in 50 mM sodium succinate buffer at pH 4.5 and 37 °C. The spectrophotometric measurements were done by recording the absorbance in the time scan mode for 2 min.

3.2.2.1. FTIR analysis

IR spectroscopy studies were performed for each group experiment of the lignin. IR transmission spectra were recorded directly to sample on a A Genesis Series FTIR Spectrometer of Analytical Technology Inc. (ATI Mattson, USA) equipped with a single reflection diamond attenuated total reflection probe was used.

3.2.2.1. NMR analysis

^{29}Si (59.3 MHz) NMR spectra were recorded on a Varian MercuryPlus 300 spectrometer. Samples for ^{29}Si spectra were either dissolved in deuterated solvents or in cases of reaction samples and DME solutions measured with a D_2O capillary in order to provide a lock frequency signal. NMR shifts were referenced to solvent residual peaks. To eliminate the temperature dependence of chemical shifts, spectra were recorded at 25^{O}C and samples were allowed to equilibrate thermally for 10 min.

3.2.2.2. Preparation of lignin-silica hybrid adhesive

Lignin-silica hybrid was performed by oxidation of each lignin using laccase and following by sol-gel method proposed by by Stöber (Matsoukas and Gulari, 1988). Each type of lignin with different weight (0.1;0.25;1.0;2.0 g) was diluted in stirred beaker glass using sodium succinate buffer 0.05M pH 4.5. Oxidation reaction was started by adding 5 ml of diluted crude laccase at 40^{O}C for 20 minutes. The stirring rate was 150 revolutions per minute. Reaction was continued by adding of TEOS and leaves it for 1 h to make TEOS become hydrolyzed. Then for the final reaction (condensation) was added sodium hydroxide and ethanol until giving a solution with concentration in the range TEOS (8mM), sodium hydroxide (15mM) and ethanol (40mM) for 3 h. The adhesive product in the bottom of the beaker glass then ready for the following measurement.

3.2.2.3. Pull-out test

Pull-out tests were carried out in adhesive joints produced between two similarly treated strips (10x20 mm) of lignin contain paper about 0.2 mm thick. Immediately after surface treatment was carried out, adhesive solution (1 mg) was applied to each strip to be joined, and left to dry for 10 min. The strips were immediately placed in contact and pressed in the clamp subsequently leave it for one night in the oven at 30 ^{O}C to achieve a suitable joint. Force strength determination was measured using pull out equipment (Zwick/Materialprüfung 1435) at Institute of Paper, Pulp and Fiber Technology TU Graz, Austria.

3.2.2.4. Viscosity determination

The rheological properties of the adhesive was investigated using a Rheometric Scientific rheometer (RM 115- Rheomat) equipped with a rotational concentric cylinder (DIN 114). The gap between the inner and the outer cylinder is 1 mm. The instrument applied a time-varying deformation on the sample and measured the transmitted torque. Controlled shear rate (CSR) experiments were carried out as follows: shear rate was increased to 1^{-1} in 10s and maintained for 300 s to provide a uniform and standardized state in all the solutions.

3.2.2.5. Curing time

Curing time of the lignin-silica hybrid adhesive was measured by drying time of adhesive on the glass plate in room temperature. One drop of adhesive put on the glass plate and the time of the adhesive become dry was determined.

3.3. Results and Discussion

Fluorescence spectroscopy monitoring was used as a sensitive and simple analytical tool to monitor or follow the modifications during incubation of laccase with lignin samples. The fluorescence intensity rapidly decreased during the first 60 min of incubation in all samples. After 180 min the

fluorescence intensity was below 500 AU representing over 81 % reduction in fluorescence intesnity (Fig 1). Fuorescence is an intrinsic property of lignin attributed to aromatic structures such as conjugated carbonyl, biphenyl, phenylcoumarins and stilbene groups [13]. The destruction or modification of these chromophores lead to the decrease in the fluorescence. Previous studies have shown that modification of biphenyl groups resulted in decrease in fluoresecne intesntity [14].

In this study monitoring the decrease in fluorescence was used to decide the incubation time needed before adding TEOS. From the Fig 1 it is clear that after the first hour of incubation of lignin with laccase significant decrease in fluorescence was achieved. Therefore TEOS was introduced into the reaction mixture after 1 hr pre-incubation of lignin with laccase in order to facilitate the reaction of oxidized lignin and hydrolyzed TEOS. Sol–gel process was chosen as the method of choice for lignin-silica hybrid synthesis because it is a versatile method used to obtain homogeneous interpenetrating organic–inorganic hybrid composites polymers [15]. The application of adhesives to join different materials is mainly inflenced by the rheological properties of these solutions. For this reason viscosity studies were carried out. Ca-Ls samples showed the highest viscosity although generally the viscosity increased in all samples with increasing lignin concentration up to a certain point and then decreased (Fig 2). Rheological properties of an adhesive are extremely important in determining their spreading and penetration over the surface of application. The solution without lignin (control) exhibited a Newtonian behaviour, i.e. there was no variation of viscosity by increasing the shear rate. The increase in the apparent viscosity after adding lignin maybe ascribed to the creation of bonds between the silanol groups and reactive lignin units leading to the formation of a tridimensional physical structure. It is quite interesting to note that the tensile strength was increased during the presence of lignin in all sample. This is not surprising since inorganic-organic hybrid polymer have been shown to posses the good properties of organic polymers such as flexibility, low density, toughness and formability while the inorganic part contributes with surface hardness, modulus strength [16].

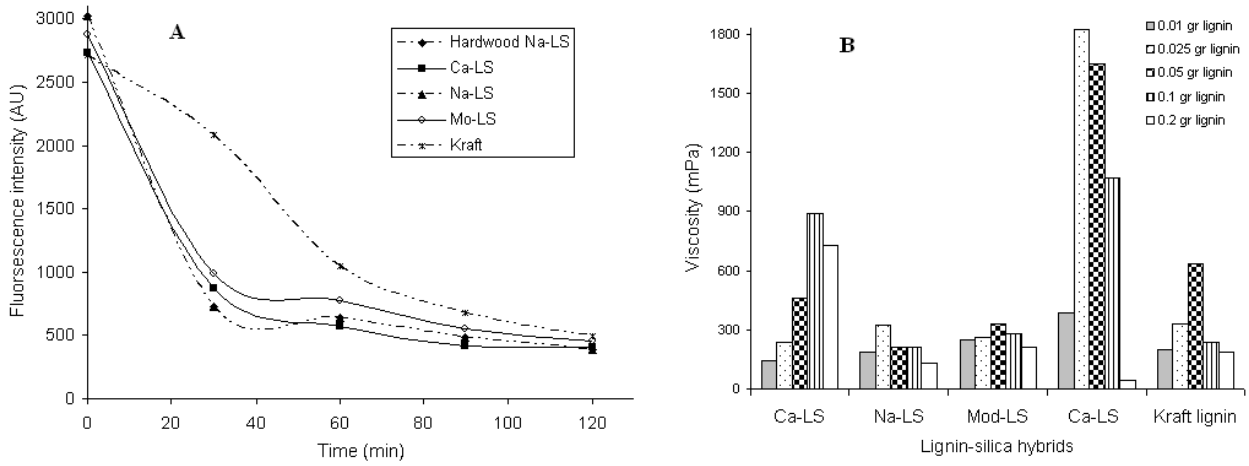

Fig 1. Effect of laccase on the fluorescence intensity (**A**) and effect of lignin concentration on viscosity lignin-silica hybrids (**B**).

The presence of lignin also influenced the curing time. At lower concentrations of lignin the curing time was higher than at higher lignin concentrations (Fig 3). For example with Ca-LS, the curing time was 50 % less at lignin concentrations above 0.1 g. Surprisingly the curing time of samples with CA-Ls Hard wood was short and almost similar at all different concentrations. Therefore different lignins had different influence on the viscosity, curing time and tensile strength. For example the pull test showed different polymers showed different tensile strength with Ca-LS polymers supplemented with 0.5 g lignin showing the highest force (135 N). Except for kraft lignin, increase in lignin concentration positively affected the tensile strength in the other samples**.** Various approaches of wood treatment with inorganic silicon compounds [16] have been widely reported. Wood treated with silanes showed enhanced dimensional stability especially when the hydrolysis and the condensation of the silanes was steered to proceed within the cell wall.

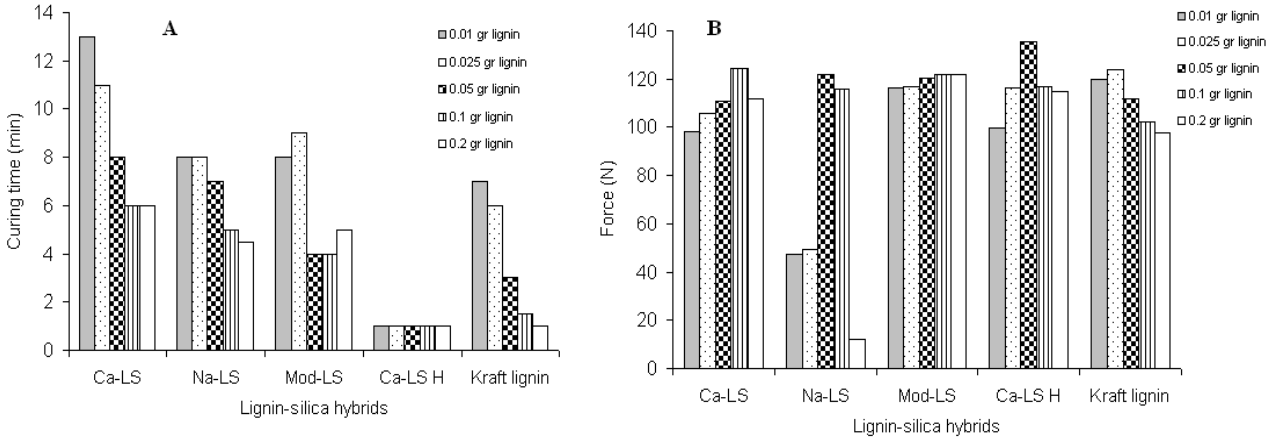

Fig 2. Effect of lignin concentration on viscosity and curing time

As shown in Fig 3 new signals were obtained when ferulic or sinapic acid was incuabted with trimethylSiO3 in the presence of laccase. No signal was detected when silane precursor was incubated with either feulic acid or sinapic acid in the absence of laccase (Fig 3). This shows the ability to produce interpenetrating hybrid polymers between lignin and sillane precursors.

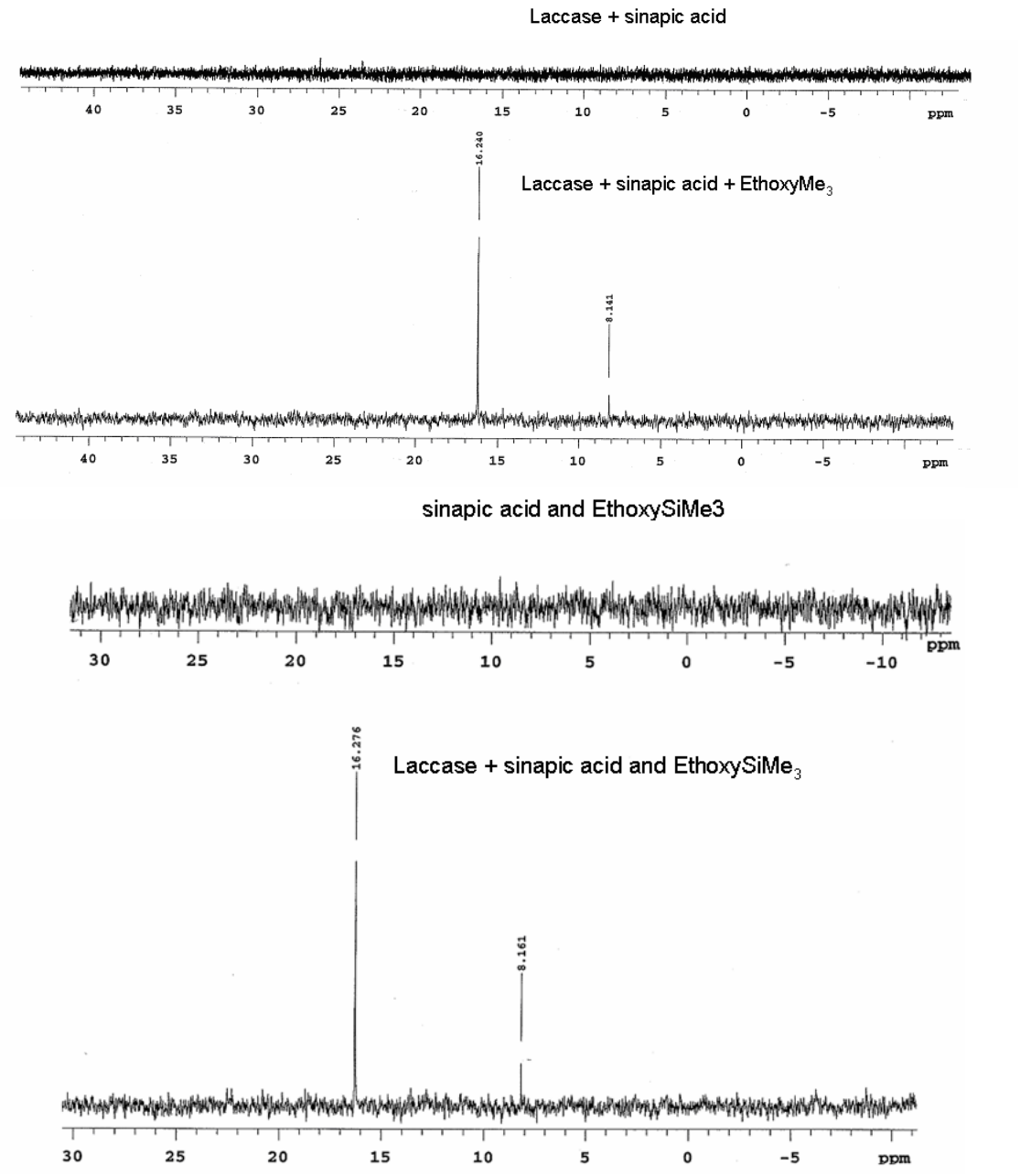

Fig 3. Si-NMR studies of TEOS/ sinapic acid and TEOS/ferulic acid hybrids

3.5. Conclusions

In summary, lacases plays an major role in activating lignin thereby increase the interaction between lignin and TEOS resulting in interepenetrating polymers. The concentration of lignin has a direct bearing on the viscosity, curing time and tensile strength. However, the laccase mediated lignin-silica hybrid requires further optimization since the resulting

polymer absorbs water. Nevertheless this study shows the great potential of developing lignin-silica adhesives and provides the base for producing materiales which have a wide potenial application in functionalization of lignocellulosic materials and other synthetic polymers. This is because silica is such a versatile molecule which enables the attachment of several functional molecules.

3.5. References

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Section 2

Developing antioxidant activity measuring methods

Introduction of the antioxidant science

The popularity of oxidative stress term has been increasing since end of 20th century (Kohen and Nyska, 2002; Ames, et. al., 1993) concomitant with the developing of the health consciousness. Most well known diseases like cancer, cardiovascular, immune-system decline, brain dysfunction, and cataracts are mainly attributed to oxidative stress. The occurrence of oxidative stress in the body is started from excessive generation of radical species i.e., nitric oxide radical (NO•), superoxide ion radical ($O•_2^-$), hydroxyl radical (OH•), peroxyl (ROO•) and alkoxyl radicals (RO•), singlet oxygen ($^1O_2$) towards mopped up agent like glutathione peroxidase, catalase, superoxide dismutases, and albumin reveal cells damage afterwards health problems. Antioxidants become much more famous not only because of their ability to quench the radical and prolong the self life of the food stuffs (Giasson, et.al., 2002), because they readily give an electron to radical species

Laccase generated oxidized lignin as a tool for measuring antioxidant activity

The radical produced by laccase oxidation can be used as radical model for antioxidant quenching ability. To date, several attempts have been made to develop antioxidant-measuring methods (Magalhaes et al, 2008; Perez-Jimenez, et al, 2008; Prior, et al, 2005; Sanchez-Moreno, 2002; Prior and Cao, 1999), nevertheless, an internationally standardized method does not exist (Huang, et al, 2005; Prior, et al, 2005). The available methods developed to date are generally classified into two categories: inhibition methods (in which the inhibition of oxidative damage of the target molecule is measured in the presence of antioxidants) and methods based on direct measurement of radical scavenging activity by antioxidants present in the sample. The inhibition methods are generally expensive and time-consuming (Fogliano, et al, 1999). The radical scavenging methods employing 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2,9-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals, although widely used (Perez-Jimenez, et al, 2008), are difficult to compare among different laboratories. This makes it difficult to perform strict quality control for antioxidant products in the food and

nutraceutical industry (Huang, et al, 2005). Recent international conferences on antioxidants have acknowledged the urgent need for the development of appropriate standardized methods which can reliably be used for estimating antioxidant activity given their importance in the food and health industries (Finley, 2005; Schaich, 2005). Laccase-generated ABTS radicals have been used to measure antioxidant activity (Kulys and Bratkovskaja, 2007). However, laccase activity was not inhibited and therefore the possibility of laccase to reoxidize reduced ABTS radicals was not considered. Laccases (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductase) belong to the so-called blue-copper family of oxidases. These enzymes perform monoelectronic oxidation of phenols and aromatic or aliphatic amines to corresponding reactive radicals (Liers, et al, 2007; Riva, 2006). The produced free radical is further transformed into a quinone in a second enzymatic oxidation step or by spontaneous disproportionation (Harkin et al, 1974; Johannes and Majcherczyk, 2000; Leonowicz, et al, 1984). The resulting quinone can react with similar molecules or others in its surrounding milieu to produce polymeric products (Minussi, et al, 2002). The reactivity of these generated species together with the observation by Isaac and Van Eldik (1997) that ascorbic acid (an antioxidant) can reduce quinones and phenolic radicals is what motivated us to investigate the ability of laccase oxidized phenols to oxidize antioxidant molecules and in the process use them to quantify antioxidant activity of a given sample. The ability of laccase to form reactive species has attracted a lot of scientific and industrial interest in a number of areas including wood, textile, food, fuel cell, biosensor, and paper industries (Camarero, et al, 2007; Kim, et al, 2007), as well as application in free radical chemistry (Rodríguez Couto and Herrera, 2006). Generation of such reactive species by laccases is easy and does not require special handling conditions.

This study aims to provide a deep picture of the variety of new products developed through laccase oxidation using several type of lignosulphonate as raw materials. In addition, it introduces fundamental aspects related to the laccase used to modify the lignin structure to make more versatile like substitute material (dispersion agent) and functional polymer such an

adhesive. Additionally, the novel feature of this work is to develop laccase-generated reactive species from lignin molecules that can be used as an antioxidant activity measurement.

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4

Antioxidant activity assay based on laccase-generated radicals

A novel antioxidant activity assay was developed using laccase-oxidized phenolics. In a three-step approach, phenolic compounds were first oxidized by laccase. Laccase was then inhibited using 80% (v/v) methanol which also stabilized the oxidized phenolics which were then used to measure antioxidant activities of ascorbic acid and Trolox. From a number of laccase-oxidized phenolics screened for potential use in the measurement of antioxidant activities, syringaldazine emerged the best, giving results comparable to the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, which is currently used in conventional methods. Like DPPH radicals, two moles of stoichiometric oxidized syringaldazine were reduced by one mole of either ascorbic acid or Trolox. For the first time we show that antioxidant activity can be correlated to oxygen consumption by laccase. Reduction of one molecule of oxygen corresponded to oxidation of four molecules of syringaldazine which in turn is reduced by two molecules of Trolox or ascorbic acid. This study therefore demonstrates the great potential of using laccase-oxidized syringaldazine for the measurement of antioxidant activity.

Keywords: Laccase-oxidized phenolics; Antioxidant assay; Syringaldazine.

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4.1. Introduction

Nutritional and nutraceutical (health-promoting factors) aspects of foodstuffs are gaining increasing importance worldwide. Among the nutraceutical compounds, the demand for antioxidants has greatly increased. Antioxidants are not only known to inhibit oxidation in foods thereby increasing shelf life [1], but they also quench free radicals in vivo thereby preventing the development of many pathophysiological conditions such as atherosclerosis, cancer, and Alzheimer's [2, 3]. The antioxidants achieve this by reacting with free radicals, chelating catalytic metals, and also scavenging reactive oxygen species [4]. In food, antioxidants prevent oxidation of lipids and minimize rancidity thereby increasing shelf life [5]. The oxidation of lipids in foods is responsible for the formation of off-flavors and undesirable chemical compounds which may be detrimental to health. Free radical oxidation of the lipids in foods has historically been a major problem for food processing industries to the extent that early attempts to measure antioxidant activities were mainly focused on lipid protection [6].

Although several attempts have been made to develop antioxidant-measuring methods [7–11], an internationally standardized method does not exist [9, 12]. The available methods developed to date are generally classified into two categories: inhibition methods (in which the inhibition of oxidative damage of the target molecule is measured in the presence of antioxidants) and methods based on direct measurement of radical scavenging activity by antioxidants present in the sample. The inhibition methods are generally expensive and time-consuming [6]. The radical scavenging methods employing 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2,9-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals, although widely used [8], are difficult to compare among different laboratories. This makes it difficult to perform strict quality control for antioxidant products in the food and nutraceutical industry [12]. Recent international conferences on antioxidants have acknowledged the urgent need for the development of appropriate standardized methods which can reliably be used for estimating antioxidant activity given their importance in the food and health industries [13, 14].

Laccase-generated ABTS radicals have been used to measure antioxidant activity [15]. However, laccase activity was not inhibited and therefore the possibility of laccase to reoxidize reduced ABTS radicals was not considered. Laccases (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductase) belong to the so-called blue-copper family of oxidases. These enzymes perform monoelectronic oxidation of phenols and aromatic or aliphatic amines to corresponding reactive radicals [16, 17]. The produced free radical is further transformed into a quinone in a second enzymatic oxidation step or by spontaneous disproportionation [18–20]. The resulting quinone can react with similar molecules or others in its surrounding milieu to produce polymeric products [21]. The reactivity of these generated species together with the observation by Isaac and Van Eldik [22] that ascorbic acid
(an antioxidant) can reduce quinones and phenolic radicals is what motivated us to investigate the ability of laccaseoxidized phenols to oxidize antioxidant molecules and in the process use them to quantify antioxidant activity of a given sample. The ability of laccase to form reactive species has attracted a lot of scientific and industrial interest in a number of areas including wood, textile, food, fuel cell, biosensor, and paper industries [23, 24], as well as application in free radical chemistry [25]. Generation of such reactive species by laccases is easy and does not require special handling conditions.

This study therefore illustrates a three-step approach in which laccases are used to oxidize different phenolic molecules in the first step, followed by inhibition of laccase in order to avoid interference with the antioxidant-measuring step. The third step is the use of the laccase-oxidized phenolics to estimate the antioxidant activity of a given sample. The antioxidants (ascorbic acid and vitamin E) were chosen since ascorbic acid (vitamin C) is considered the most important water-soluble antioxidant in extracellular fluids capable of neutralizing reactive oxygen species in the aqueous phase [26], whereas vitamin E is the major lipid-soluble antioxidant effective in chain-breaking within the cell membrane thereby protecting membrane fatty acids from oxidants [3]. Furthermore, the study demonstrates the stoichiometric

relationship between laccase-oxidized syringaldazine, the consumed oxygen, and the amount of antioxidant required to regenerate the substrate.

4.2. Materials and method

4.2.1. Chemicals and enzyme

Phenolic compounds (caffeic acid, 4-hydroxybenzoic acid, sinapic acid, pyrogallol, gallic acid, syringic acid, guaiacol, catechol, syringaldazine, 2,6-dimethoxyphenol, and eugenol) were obtained from Sigma. The antioxidants ascorbic acid and Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a derivative of vitamin E, were purchased from Merck. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and 2,2′-azinobis-(3-ethylbenzothiazoline)-6-sulphonate (ABTS) were also purchased from Sigma. All other chemicals used in this study were either purchased from Merck or Sigma. The Trametes hirsuta laccase was produced and purified as previously described by Almansa et al. [27].

4.2.2. Laccase activity assay

The activity of laccase was determined spectrophotometrically by monitoring the oxidation of 2,2′-azinobis-(3-ethylbenzothiazoline)-6-sulphonate (ABTS) (ε436=29,300 M−1 cm−1) as substrate at 436 nm in 50 mM sodium succinate buffer at pH 4.5 and 37 °C. The spectrophotometric measurements were done by recording the absorbance in the time scan mode for 2 min.

4.2.3. Generation of phenolic radicals for measurement of antioxidant activity

Stock solutions (10 mM) of each of the abovementioned phenolic compounds dissolved in methanol were prepared and kept in the dark until further use. The phenolic compounds were used in reaction mixtures at final concentrations of 30–140 μM in 50 mM sodium succinate buffer at pH 4.5. The reaction was started by adding 50 μl of laccase (20 nkat ml−1) to make a final volume of 1 ml. The reaction mixture was incubated at 37 °C, while monitoring the oxidation process in a 1-cm-pathway, disposable, UV transparent cuvette using an Hitachi U-2001 UV–Vis spectrophotometer. During the screening phase laccase oxidation of phenolic compounds was monitored by means of wavelength scans in the range 900–200 nm for 9 min (every cycle, 3 min) in

order to identify the absorbance maxima of transformation products. Thereafter, ascorbic acid was added in twofold molar excess to the calculated concentration of generated radicals and wavelength scans continued for another 9 min.

4.2.4. Laccase inhibition

The IC50 values were determined by incubating laccase with the following commonly used and previously studied laccase inhibitors [27]: sodium azide, sodium fluoride, and potassium cyanide. Different concentrations of these inhibitors in the range 0–90 μM were incubated with 50 μl laccase (20 nkat ml−1) and ABTS as substrate in 50 mM sodium succinate buffer at pH 4.5 and 37 °C. The ability of methanol and ethanol to inhibit laccase activity was assessed in the concentration range 0–90% (v/v) using ABTS as the substrate as described above.

4.2.5. Stability of laccase oxidized phenolics in the presence of different inhibitors

The maximum concentrations of inhibitors required to completely repress laccase activity as determined above were added to laccase-oxidized phenolics. The effect of inhibitor on the stability of the oxidized phenolics was then monitored spectrophotometrically at regular intervals at the respective absorption maxima of each radical, while incubating the radicals in the dark for 30 min.

4.2.6. Antioxidant activity test with ascorbic acid and Trolox

Oxidized phenolics for use in the antioxidant activity test were prepared by incubating respective phenolic molecules with 50 μM laccase (20 nkat ml−1) as described above, while monitoring formation of product at the respective absorption maxima until complete conversion. Methanol was added to this stock solution of laccase-oxidized phenolics to a final concentration of 80% (v/v) to stop laccase activity and stabilize the oxidized phenolics. Different concentrations of ascorbic acid and Trolox dissolved in methanol (0–20 μM) were then added to 800 μl of the laccase-generated radicals dissolved in 80% methanol at concentrations to give an initial absorbance of 0.8. The mixture was thoroughly mixed and incubated at 25

°C, while shaking at 140 rpm until full completion of the reaction as evidenced by no further decrease in absorbance. The stoichiometric reduction of laccase-generated radicals by ascorbic acid and Trolox was compared to that of the well-known DPPH radical under similar conditions as described above.

4.2.7. Measurement of oxygen and antioxidant consumption

Laccase (20 nkat ml−1) was incubated with syringaldazine (0.48 mM) in doubly distilled water and the oxygen consumption was monitored using a Rand Brothers Digital Model 20 Oxygen sensor (Cambridge, UK). After complete oxidation of syringaldazine by laccase, varying amounts of Trolox and ascorbic acid (60, 80, 100 μM) were added and the oxygen consumption was followed at each concentration level. At each stage the reaction was monitored until a plateau was reached. This enabled one to relate the amount of oxygen consumed to the amount of syringaldazine in the initial reaction and the amount of laccase-oxidized syringaldazine reduced by antioxidants. The actual oxygen concentration in the reaction mixture used for the calculation of consumed oxygen was determined using the method originally described by Winkler [28].

4.3. Results and discussion

4.3.1. Screening various phenolic compounds for use in the measurement of antioxidant activity.

Various phenolic compounds were oxidized by laccase and used to estimate the antioxidant activity of ascorbic acid and Trolox. Laccase oxidation of all phenolic molecules produced colored oxidized products, and those compounds giving sharp peaks in Vis spectra after oxidation were selected. Consequently catechol, 2,6-dimethoxyphenol, guaiacol, sinapic acid, and syringaldazine were chosen (Fig. 1). Although caffeic acid, pyrogallol, gallic acid, syringic acid, and eugenol were oxidized by laccase, the spectra of the resulting products showed multiple peaks and/or peaks that were too broad. Nevertheless, although ABTS showed a broad peak it was also retained for further studies to allow comparison with data from the

literature (Fig. 1). Of all the phenolic molecules oxidized by laccase, catechol required prolonged incubation periods to achieve complete oxidation.

Upon addition of ascorbic acid after 9 min to the laccasegenerated radicals, the peaks and the colour of catechol, 2,6-dimethoxyphenol, guaiacol, and oxidized syringaldazine disappeared within 3 min (Fig. 1). In the case of sinapic acid, an additional product peak was detected between 400 and 450 nm after incubation of the formed radicals with ascorbic acid (Fig. 1). Guaiacol and ABTS radicals required a longer reaction time (6 min) with ascorbic acid for their complete disappearance.

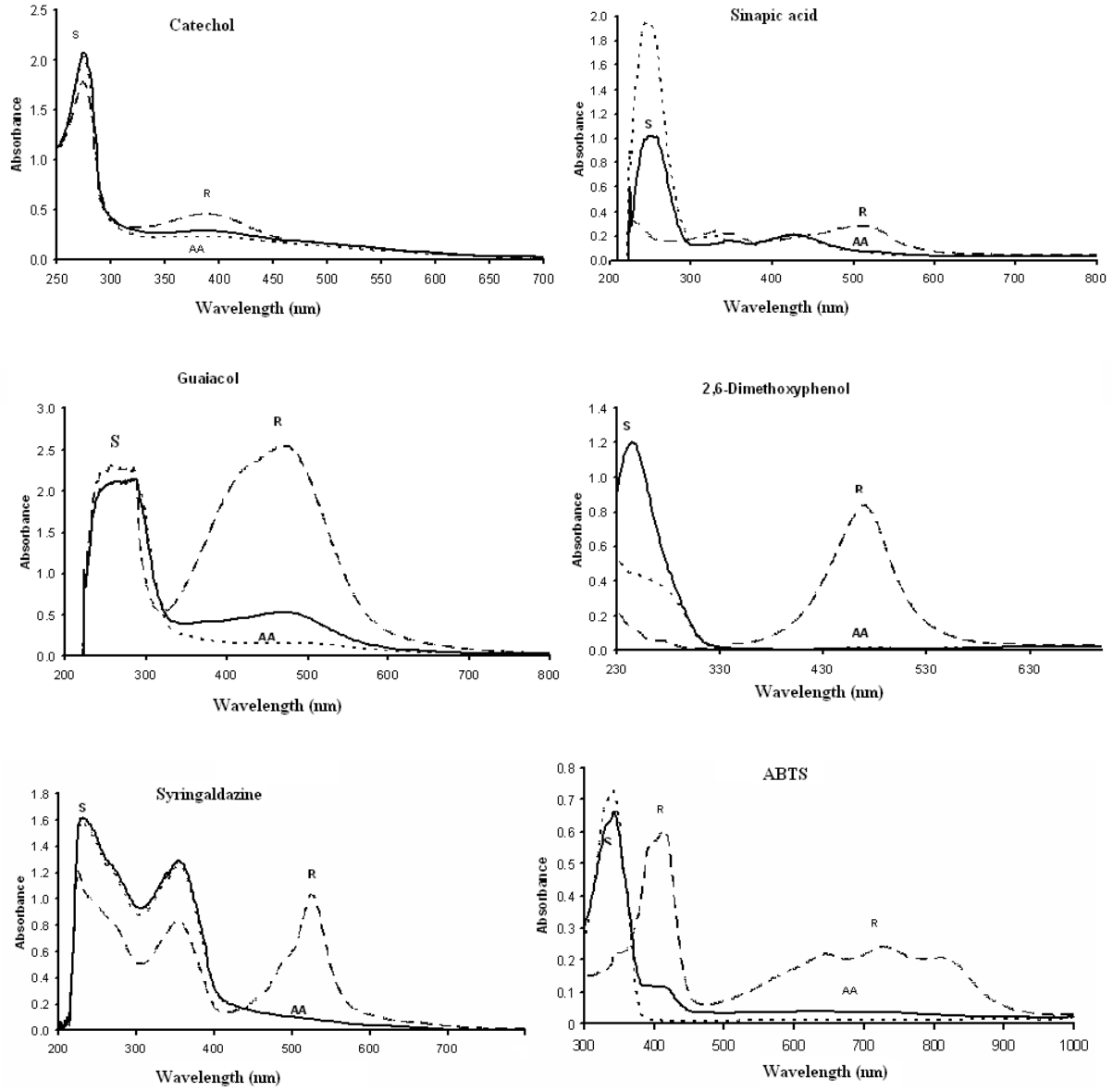


Fig.1 Chromatograms of UV–Vis absorption spectra of various laccase substrates (S, solid lines) at time zero, oxidized phenolics (R, dashed lines) after 9 min of incubation with laccase, and the effect ofascorbic acids as antioxidant (AA, dotted lines) on the laccase oxidized phenolics after 18 min of incubation.

4.3.2. Inhibition of laccase and effect of inhibitor on the stability of laccase oxidized phenolics

In order to avoid laccase from reoxidizing the reduced oxidized phenolics and thereby interfering with the antioxidant activity measurement step, screening for efficient laccase inhibitors was carried out. Table 1 shows the concentrations of the tested inhibitors needed to inhibit T. hirsuta laccase activity by 50% (IC50). The most potent inorganic inhibitor of laccase activity was potassium cyanide, followed by sodium azide, and finally sodium fluoride (Table 1). The organic solvents (methanol and ethanol) were also effective in inhibiting T. hirsuta laccase with IC50 values of 40% and 45% v/v, respectively. The laccase inhibition experiments were consistent with the data reported for Trametes sp. laccases [27, 29, 30].

Table 1. Concentration of inhibitors required to inhibit *T. hirsuta* laccase activity (20 nkat ml^{-1}) by 50 % in 5 min.

Inhibitor	**I_{50}**
Potassium cynaide	30 µM
Sodium azide	40 µM
Sodium fluoride	50 µM
Organic solvents	
Methanol	40 % v/v
Ethanol	45 % v/v

Figure 2 shows the effect of different laccase inhibitors on enzymatically formed oxidized products. Sodium fluoride and sodium azide interfered with the sinapic acid oxidation products leading to a continued decrease in the absorbance. However, the oxidized sinapic acid was very stable during the first 5 min in the absence of the inhibitors. Oxidized syringaldazine product concentration continued to decrease in the control as well as in the presence of inhibitors (Fig. 2b). The presence of all the inorganic inhibitors in oxidized syringaldazine seemed to destabilize the products. The oxidized 2,6-

dimethoxyphenol was relatively stable in buffer, sodium azide, and potassium cyanide during the whole incubation period lasting 30 min (Fig. 2c).

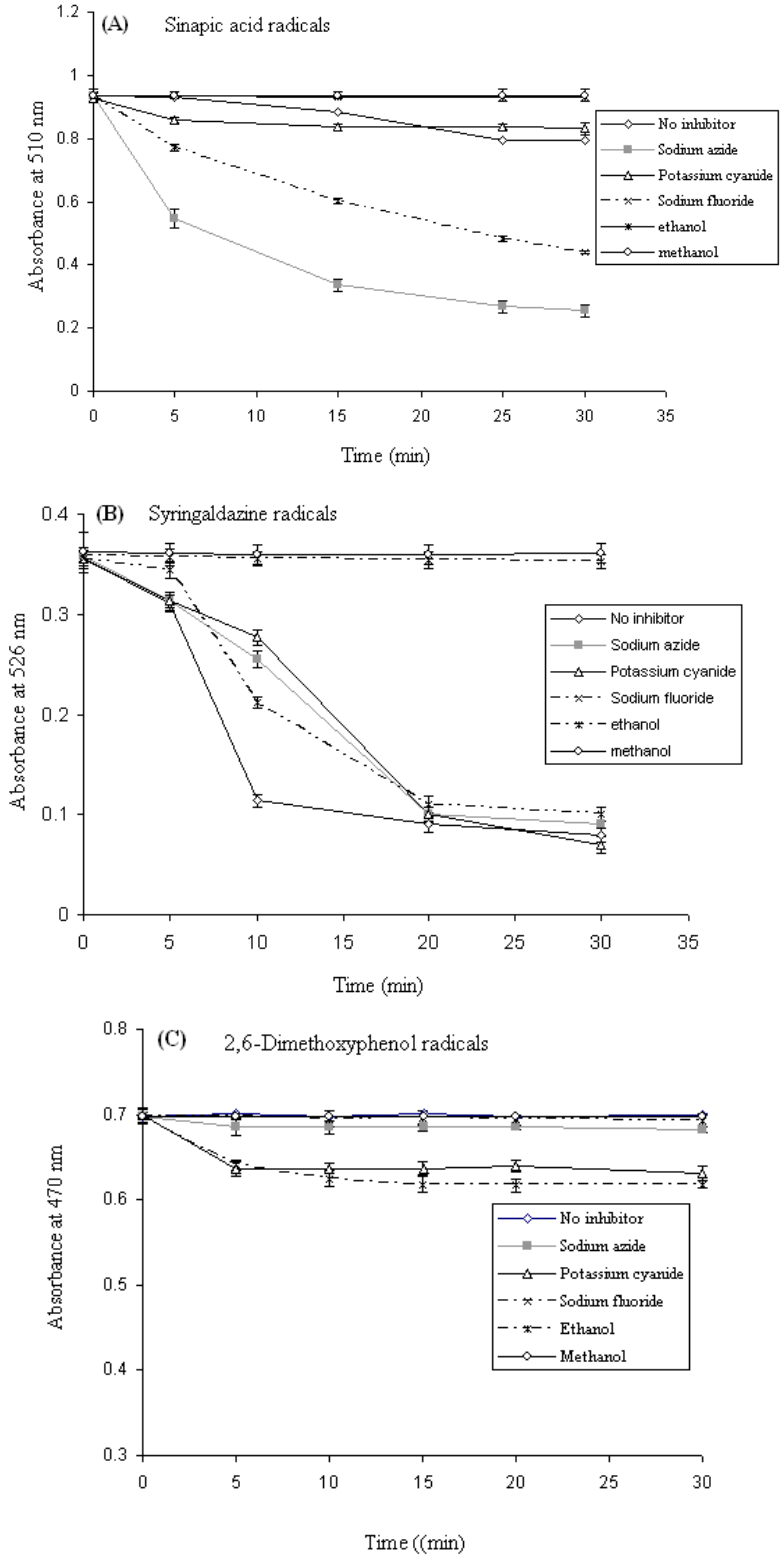


Fig.2. Effect of inhibitors and organic solvents (ethanol and methanol) on the stability of radicals. The laccase oxidized phenolics were incubated with inhibitors and organic solvents in the dark for 30 min, while monitoring changes in absorption.

Interestingly, apart from inhibiting laccase activity, the organic solvents had a stabilizing effect on laccase-oxidized phenolics (Fig. 2). Methanol (80% v/v) was very effective in inhibiting laccase as well as imparting a stabilizing effect on laccase-oxidized 2,6-dimethoxyphenol, syringaldazine, and sinapic acid during the 30-min incubation period (Fig. 2). Laccase-oxidized syringaldazine and sinapic acid were equally stable in 90% ethanol, although the concentration of oxidized 2,6-dimethoxyphenol slightly decreased. Methanol was therefore chosen as the appropriate laccase inhibitor as well as the stabilizing solvent of laccaseoxidized phenolics.

4.3.3. Antioxidant measurement with ascorbic acid

The relationship between the concentration of the antioxidant (ascorbic acid) and reduction of the laccase-oxidized phenolics was linear (Fig. 3).

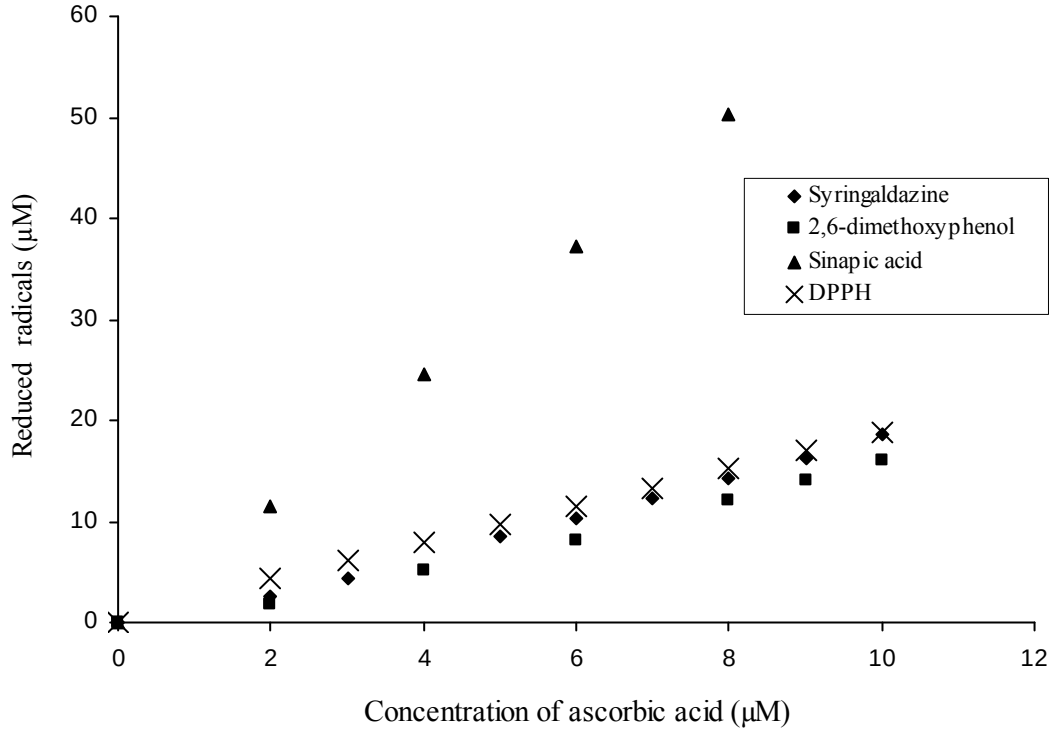


Fig. 3. Measurement of antioxidant activity of ascorbic acid using laccase-generated radicals and DPPH radicals. The laccase oxidized phenolics and the DPPH radicals were incubated with different concentrations of ascorbic acid for 5 min at 25 °C, while shaking at 150 rpm.

It is well known that antioxidant compounds effect electron or hydrogen atom transfers to quench the radicals leading to discoloration proportional to their amount [6]. Accordingly, 1.8 µM DPPH radicals, 1.9 µM oxidized syringaldazine, and 2 µM oxidized 2,6-dimethoxyphenol were reduced by 1 µM ascorbic acid (Table 2) which is close to the calculated theoretical value of 2 µM oxidized phenolic reduced by 1 µM ascorbic acid.

Table 2. Amount of syringaldazine radicals reduced by either ascorbic acid or Trolox. The values of the reduced radicals are an average of three independent experiments while incubating reaction mixtures at 25 °C and shaking at 150 rpm for 6 min.

Radical	Absorption maxima (nm)	Extinction coefficient (M^{-1} cm^{-1})	Ascorbic acid (1 µM)		Trolox (1 µM)	
			Reduced radicals (µM)	Correlation coefficient (r)	Reduced radicals (µM)	Correlation coefficient (r)
DPPH	515	1.21×10^3	1.8 ± 0.2	0.996	1.9 ± 0.2	0.996
Syringaldazine	526	3.13×10^4	1.9 ± 0.2	0.991	2.3 ± 0.3	0.999
Sinapic acid	510	4.82×10^3	6.1 ± 0.1	0.993	-	-
2,6-DMP	470	3.34×10^4	2 ± 0.2	0.994	-	-
ABTS	415	5.48×10^3	6.3 ± 0.4	0.999	10.1 ± 0.2	0.997
	436	1.47×10^3	7 ± 0.3	0.998	16.2 ± 0.3	0.989
	734	1.49×10^3	8.8 ± 0.5	0.996	18.9 ± 0.5	0.991

(-) no reaction

This effectively means that after laccase oxidation of syringaldazine and 2,6-dimethoxphenol the product is deficient of one electron. This observation is in contrast to data previously reported [31] which suggest that laccase performs a two-electron abstraction from one molecule of syringaldazine. This, however, maybe attributed to the redox potential of the used laccase and reaction conditions. The best correlation between antioxidant and laccase-oxidized phenolic concentration was observed with syringaldazine and 2,6-dimethoxyphenol with a correlation coefficient (r) of 0.99 (Fig. 3; Table 2). Like the DPPH radical which is characterized as a stable free radical that does not dimerize [32], it is also speculated that oxidized syringaldazine and 2,6-dimethoxyphenol do not form dimers when dissolved in 80% (v/v) methanol as

confirmed by no change in absorbance measurements. Although it has been reported that 2,6-dimethoxyphenol tends to form dimers after prolonged incubation in buffer solutions [33], it seems the organic solvents (methanol and ethanol) used in this study tend to stabilize these products as evidenced by no change in absorbance value after 30 min of incubation and disappearance of radical once antioxidant was added.

However, oxidized sinapic acid showed a complex behavior, with 6.1 µM of the oxidized product being quenched by 1 µM ascorbic acid (Table 2). The only explanation for this strange behavior can be attributed to the new product peak emerging between 400 and 450 nm observed in wavelength scans (Fig. 1). This may suggest the possibility of coupling reactions between oxidized sinapic acid with ascorbic acid. Similarly, analysis of antioxidant activity of ascorbic acid with laccase-generated ABTS radicals showed a more complex behavior which is in agreement with previous studies on ABTS cation radicals [15, 34]. Further, Osman et al. [35] and our group (Rittstieg et al. [36]) provided evidence for the formation of covalent adducts between ABTS radical cations and polyphenols. Indeed it is these adducts which make it difficult to analyze the reaction stoichiometry with ABTS, and a similar explanation can be attributed to the results obtained with oxidized sinapic acid and ABTS radicals in this study.

4.3.4. Antioxidant activity test with Trolox

Oxidized syringaldazine was completely quenched in less than 2 min upon addition of Trolox and revealed a correlation coefficient of 0.99. The calculated consumption of laccase-oxidized syringaldazine and DPPH radicals corresponded to 2 µM per 1 µM of Trolox (Table 2). This stoichiometric relationship is consistent with the above results obtained for ascorbic acid as summarized in Table 2. Similarly, Friaa and Brault [37] observed the reduction of DPPH radical to its hydrazine analog (DPPH-H) with a measured stoichiometry of 2. In contrast, incubation of laccase oxidation products of 2,6-dimethoxyphenol with Trolox did not show a significant decrease of the oxidized 2,6-dimethoxyphenol concentration even in the presence of a tenfold molar excess concentration of Trolox to calculated laccase-oxidized

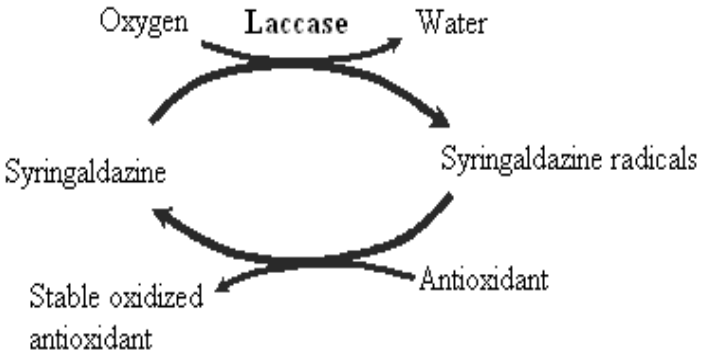
products. The behavior of oxidized 2,6-dimethoxyphenol in the presence of Trolox was surprising given its good performance with ascorbic acid. The reaction conditions such as the solvent and pH have been shown to have an important effect on the reducing capacity of antioxidants [12]. Under acidic conditions the reducing capacity may be suppressed due to protonation on antioxidant compounds, whereas under basic conditions proton dissociation of phenolic compounds would enhance reducing capacity [12]. Guo et al. [38] got different values when water, acetone, ethanol, and methanol were used as solvents. Further, hydrogen atom transfer reactions are solvent and pH dependent [12]. It is therefore interesting to investigate the effects of these factors with regard to the behavior of oxidized 2,6-dimethoxyphenol in the presence of Trolox.

As summarized in Table 2, only syringaldazine was consistent and behaved like DPPH radicals in the presence of ascorbic acid and Trolox as antioxidants. The general explanation for the unexpected behavior of some of the laccase-oxidized phenolics when incubated with either ascorbic acid or Trolox as antioxidants may be found in the reaction conditions as discussed above and the presence of coupling reactions as reported for ABTS. Re et al. [39] and Berg et al. [40] observed substantial differences associated with Trolox and quercetin as antioxidant. Supporting the same argument Fogliano et al. [6] also observed different kinetic data between Trolox and ascorbic acid when applied to the scavenging of *N,N*-dimethyl-p-phenylenediamine radicals.

4.3.5. Oxygen consumption measurements

Oxidation of phenolic molecule by laccase results in oneelectron abstraction thereby forming a radical and simultaneously reducing molecular oxygen to water [31]. As illustrated in Scheme 1 laccase oxidation of syringaldazine results in one-electron abstraction and simultaneously reduces molecular oxygen to water. Since it is also known that antioxidants reduce radicals back to their parent compounds it is also possible that laccase-oxidized syringalazine is reduced back to syringaldazine (Scheme 1).

Scheme 1. Laccases oxidize syringaldazine resulting in the formation water and syringaldazine radicals, addition of the antioxidants reduce the syringaldazine radicals back to their parent coumpound (syringaldazine).



Consequently it was observed that laccase oxidation of 485.4 µM syringaldazine resulted in consumption (reduction) of 123.8 µM oxygen molecules representing a 3.9:1 molar ratio, respectively (Figs. 4 and 5).

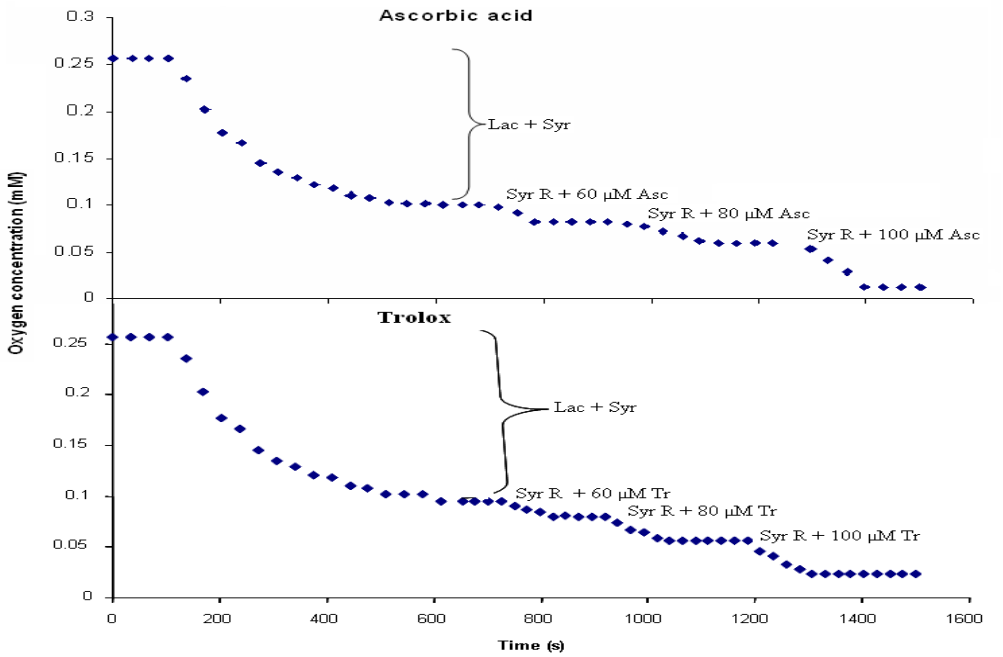


Fig.4. Oxygen consumption during incubation of laccase (20 nkat ml−1) with syringaldazine (Lac + syr) before and after addition of varying amounts of ascorbic acid (Asc) and Trolox (Tr).

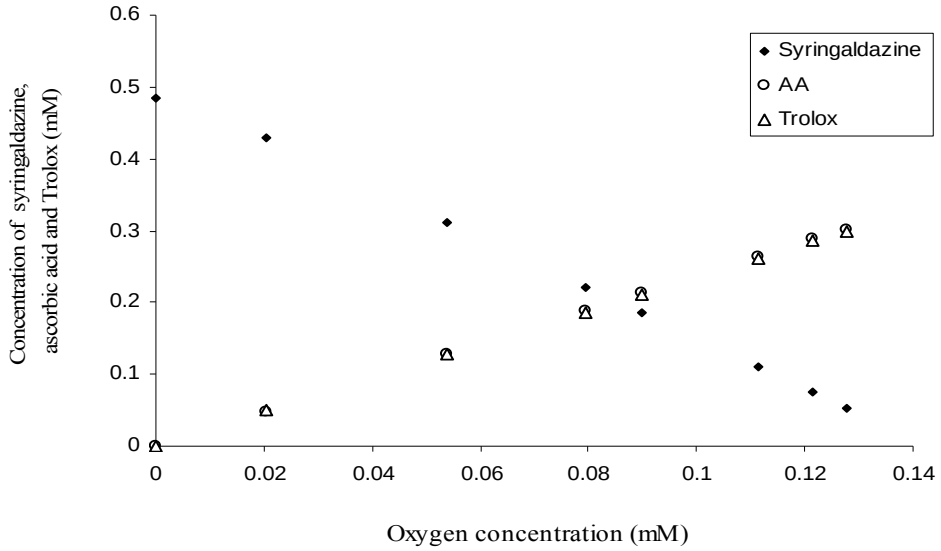


Fig.5. Relationship between laccase-oxidized syringaldazine and consumed oxygen, and syringaldazine radicals reduced by varying amounts of antioxidants (ascorbic acid and Trolox) and the oxygen consumed when reduced syringaldazine is reoxidized by laccase.

This value is close to the calculated theoretical values of 4:1 molar ratio (syringaldazine abstracted electrons/reduced oxygen) as expected and therefore the variation was due to experimental conditions. Stoichiometrically this means that in order for laccases to reduce one molecule of oxygen to two water molecules they need to abstract four electrons from four molecules of syringaldazine. Thus, the oxygen consumption experiments confirmed that laccase abstracts one electron from a syringaldazine molecule. However, other authors suggested that this oneelectron abstraction might ultimately lead to coupling and disproportionation reactions. Nevertheless, even potentially resulting quinones have been described to oxidize ascorbic acid [22]. The consumption of oxygen occurred without any delay after introducing either Trolox or ascorbic acid (Fig. 4). In the first phase, the amount of oxygen correlated to the amount of syringaldazine oxidized by the laccase (Fig. 4). Subsequently, after addition of varying amounts of antioxidants, a fraction of oxidized syringaldazine was reduced back to the parent compound. From Fig. 4 it can be observed that by increasing the antioxidant concentration (Trolox and ascorbic acid) from 60 to 100 μM the oxygen

consumption also increased as shown by the increase in slope. As shown in Fig. 5 oxygen consumption increases with increase in addition of Trolox and ascorbic acid to oxidized syringaldazine. The reduced syringaldaizine was in turn reoxidized by laccase as illustrated in Scheme 1, while simultaneously reducing oxygen to water. Thus, the amount of oxygen consumed correlated to the amount of antioxidant added. In both cases (ascorbic acid and Trolox) approximate stoichiometric ratios of two moles of antioxidants reduced per four moles of oxidized syringaldazine which in the presence of laccase reduced one molecule of oxygen with standard deviations of ± 0.4 (n=3). Therefore by monitoring the oxygen consumption it was possible to calculate the amount of antioxidants required to reduce a certain amount of oxidized syringaldazine (Fig. 5). Laccases have previously been used in biosensors for quantification of phenolic compounds [41, 42]; therefore application of the same principle which uses quantification of oxygen consumption means that an automated method for antioxidant activity becomes feasible.

4.4. Conclusion

In summary, this study has revealed the great potential of using lacasse-oxidized phenolics for measurement of antioxidant activity. Laccase-oxidized syringaldazine performed best in the assay and was comparable to DPPH radicals in estimating antioxidant activity of the model antioxidant compounds (ascorbic acid and Trolox). Nevertheless, the other screened phenolic molecules may also be useful provided more intensive studies are undertaken to fully understand the electron/hydrogen transfer mechanisms and to find appropriate reaction conditions.

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5

Laccase-generated tetramethoxy azobismethylene quinone (TMAMQ) as a tool for antioxidant activity measurement

The potential of laccase-generated tetramethoxy azobismethylene quinone (TMAMQ) for measuring antioxidant activity of a wide range of structurally diverse molecules present in food and humans was investigated for the first time. All the tested antioxidants including simple phenolics, polyphenols and vitamins quenched TMAMQ. The antioxidant activity of phenolics and polyphenolics depended on the position and number of hydroxyl groups on the benzene ring. Equally interesting was the ability of amino acids like cysteine, tryptophan and methionine as well as peptides (glutathione) and proteins (albumin) to quench TMAMQ, demonstrating the great potential of TMAMQ for analysis of antioxidant activity of serum samples. Further, TMAMQ is promising is a more reliable tool for measuring antioxidant activity of amino acids when considering conflicting reports on antioxidant activity of some of the amino acids. The extracts from various food samples showed varying antioxidant activity with highest for spinach (4.36 mg methanol extract/mmol TMAMQ) followed by kiwi (13.95 mg methanol extract/mmol TMAMQ) and lettuce (40 mg methanol extract/mmol TMAMQ). The use of the laccase generated TMAMQ can be exploited for the development of laccase based biosensors for complex and coloured samples thereby facilitating online monitoring of antioxidants in food, cosmetic and health industries.

Keywords: Antioxidant activity assa; Antioxidants; Tetramethoxy azobismethylene quinone; Laccase

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5.1. Introduction

The human body is exposed to a large variety of reactive species (free radicals) from both endogenous and exogenous sources. Endogenous free radical species (superoxide, nitric oxide and hydrogen peroxide) are products of normal cellular function. These cellular functions include mitochondrial respiration (Serrano, Goni, & Saura-Calixto, 2007), activated phagocytes, arachidonic acid metabolism, ovulation and fertilization (Magalhaes, Segundo, Reis, & Lima, 2008; Singh, Sharad, & Kapur, 2004). Exogenous sources of free radicals include pollutants such as car exhaust, industrial contaminants encompassing many types of nitrogen reactive species, drugs and xenobiotics (toxins, pesticides, herbicides etc.) (Kohen & Nyska, 2002; Valko et al., 2007). Cell damage caused by free radicals has been implicated in the pathogenesis of at least 50 diseases conditions (Dalle-Donne, Rossi, Colombo, Giustarini, & Milzani, 2006; Halliwell, 1994). Similarly, in food, for example the oxidation of lipids by free radicals has historically been a major problem for food processing industries responsible for the formation of off-flavours and undesirable chemical compounds which may be detrimental to health (Jadhav, Nimbalkar, Kulkarni, & Madhavi, 1996).

To protect the cells and organs against free radicals, biological systems have evolved a highly sophisticated and complex antioxidant protection system. These antioxidants therefore constitute the body's first line of defence against free radical damage. The antioxidants include biologically built-in mechanism of neutralizing free radicals for example glutathione peroxidase, catalase, and superoxide dismutases, glutathione and albumin (Singh et al., 2004; Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006). The exogenous sources of antioxidants are mainly of dietary origin including vitamin C, tocopherols, carotenoids, flavonoids (Singh et al., 2004; Valko et al., 2006). Endogenous and exogenous antioxidants function interactively and synergistically to neutralize free radicals. When the availability of antioxidants is limited, cell damage and food oxidation occurs. Strangely, despite the well recognized importance of antioxidants for human health and food preservation, currently there is no nutritional standard index available related to antioxidants for food

labelling because of the lack of standardised methods (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002). However, recently determining antioxidant capacity has become a very active research topic as recently demonstrated by international efforts to standardise assay methods (Prior, Wu, & Schaich, 2005).

Recently, we have discovered that laccase oxidised syringaldazine was a reliable tool for measuring antioxidant activity of vitamin C and vitamin E (Nugroho Prasetyo et al., 2009). The performance of laccase oxidised syringaldazine was comparable to the commercially available 2,2-diphenyl-l-picrylhydrazyl (DPPH) radical. Syringaldazine is a yellowish compound which is converted to tetramethoxy azobismethylene quinone (TMAMQ) with maximum absorbance at 530 nm (deep purple colour) upon oxidation by laccases (Harkin, Larsen, & Obstharkin, 1974; Holm, Nielsen, & Eriksen, 1998). It is a well known laccase substrate which is also used to detect peroxidase activities. The reaction of laccase with syringaldazine first generates a free radical and loss of the second electron can either proceed enzymatically or by disproportionation forming a deep purple coloured quinone (TMAMQ – Fig. 1) which is not prone to polymerisation under appropriate conditions (Kuznetsova & Romakh, 1996; Thurston, 1994). Further, electrochemical and pulse radiolysis studies of the oxidation of syringaldazine confirmed a reversible two-electron-two-proton transfer leading to the formation of a deep purple compound (Hapiot, Pinson, Neta, & Rolando, 1993).

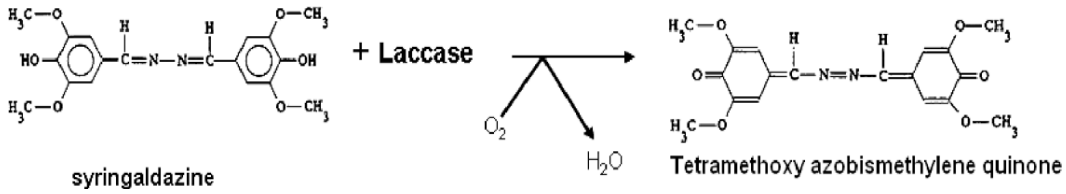

Fig.1. Laccase oxidation of syringaldazine results in the formation of a water molecule and tetramethoxy azobismethylene quinone (TMAMQ).

Syringaldazine can also be oxidised by chlorine and is currently used for the colorimetric determination of chlorine in water (Bauer & Rupe, 1971;

Cooper, Roscher, & Slifker, 1982). This work, is the first to explore the ability of laccase generated TMAMQ to measure the antioxidant activity of a wide variety of structurally different antioxidants relevant to both the food industry and human health. The study is also extended to investigate the ability of laccase generated TMAMQ to measure antioxidant activity of crude extracts of known important dietary sources. This knowledge will allow the development of laccase based biosensors for complex thereby facilitating online monitoring of antioxidants in food, cosmetic and health industries.

5.2. Materials and methods

5.2.1. Chemicals and enzyme

All the used antioxidants molecules were of analytical grade. The phenolics were purchased from Sigma–Aldrich, Steinheim, Germany while the flavonoids were purchased from Carl Roth GmbH, Karlsruhe Germany. All the other chemicals were purchased from Merck, Darmstadt, Germany. The Trametes hirsuta laccase was produced and purified as previously described by Almansa, Kandelbauer, Pereira, Cavaco, and Guebitz (2004). Food samples of onion (*Allium cepa*), green and fermented tea (*Camellia sinensis*), kiwi (*Actinidia arguta*), apple *(Malus domestica*), carrot (*Daucus carota* subsp. sativus), roasted coffee (*Coffea robusta*), lettuce (*Lactuca sativa*), spinach (*Spinacea oleracea*), pumpkin seed (*Curcubita maxima*), tomato (*Solanum lycopersicum*) and garlic (*Allium sativum*) were purchased from local markets in Graz, Austria.

5.2.2. Laccase activity assay

The activity of laccase was determined spectrophotometrically by monitoring the oxidation of 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (ε 436 = 29,300 M^{-1} cm^{-1}) as a substrate at 436 nm in 50 mM sodium succinate buffer at pH 4.5 and 30 ^{O}C (Nugroho Prasetyo et al., 2009). The spectrophotometric measurements were done by recording the absorbance in the time scan mode for 2 min using a Hitachi U-2001 UV–Vis spectrophotometer.

5.2.3. Generation of TMAMQ

TMAMQ stock solutions were prepared by incubating syringaldazine (0.17 mM) with 50 µl of laccase (20 nkat ml^{-1}) in 50 mM sodium succinate buffer at pH 4.5. The reaction mixture (1 ml) was incubated at 30 ^{O}C for 10 min while shaking at 140 rpm in a thermomixer (Eppendorf AG, Germany). The oxidation process was monitored at 530 nm using a Hitachi U-2001 UV–vis spectrophotometer in disposable cuvettes of 1 cm pathway. To this stock solution of laccase generated TMAMQ, methanol was added to a final concentration of 80% (v/v) to stop laccase activity and to stabilize the TMAMQ (Nugroho Prasetyo et al., 2009).

5.2.4. Determination of antioxidant activity of pure molecules

Different concentrations of pure antioxidant molecules dissolved in methanol (0–20 lM) were added to 800 µl of TMAMQ dissolved in 80% methanol (final absorbance of 0.8) in order to obtain a dose response curve. The mixture was thoroughly mixed and incubated at 30 ^{O}C while shaking in a thermomixer (Eppendorf AG, Germany) at 140 rpm until full completion of the reaction as evidenced by no further decrease in absorbance. The degree of decoloration of the solution indicates the scavenging efficiency of the added antioxidant sample. The stoichiometrical reduction of TMAMQ (1 µM of TMAMQ reduced by 1 µM antioxidant) was then calculated from a dose response curve of added antioxidant.

5.2.5. Preparation of food samples and extraction of antioxidants

5.2.5.1. Recovery of simple phenolic compounds

Food samples were purchased fresh from local markets, and the edible parts blended using a blender GT800 (Uetendorf, Switzerland) and then freeze-dried. The procedure followed for extraction of antioxidants was as previously described by Saura-Calixto, Serrano, and Goni (2007) with slight modifications. The first extraction procedure involved suspending 0.5 g of freeze dried food sample in 20 ml of 98% methanol/water (50:50 v/v) in a 250 ml Erlenmeyer

flask and incubating at 25 _C while mixing at 150 rpm for 1 h. The mixture was then centrifuged at 2500 g for 20 min and the supernatant recovered. The residue was then washed with 20 ml of 98% acetone/water (70:30 v/v) and centrifugation repeated as described above to maximally recover remaining antioxidants as recommended by previous researchers (Perez-Jimenez et al., 2008). The antioxidant activity of the different extracts was determined separately after adjusting pH to 7 using NaOH to stabilize the extracts (Perez-Jimenez et al., 2008).

5.2.5.2. Recovery of hydrolysed tannins

The residues arising from methanol extracts were mixed with 20 ml of methanol and 2 ml of concentrated sulphuric acid. Samples were then incubated at 85 ^{O}C for 20 h in a shaking water bath. The samples were then centrifuged at 2500 g for 20 min and supernatants recovered; subsequently the pH of supernatant was adjusted to 7 using NaOH. This fraction contained hydrolysable tannins and other phenolics linked to carbohydrates and proteins (Hartzfeld, Forkner, Hunter, & Hagerman, 2002).

5.2.5.3. Recovery of condensed tannins

The remaining residues from the two extraction processes were further treated with 37% HCl/98% butanol/FeCl3 (5:95, v/v) at 100 ^{O}C for 3 h. Samples were centrifuged as described above and the supernatants recovered. The precipitates were further washed twice with 37% HCl/98% butanol (5:95 v/v) (Porter, 1989). The samples were then centrifuged at 2500 g for 20 min and supernatants recovered. The pH of the antioxidant fractions from this extraction step corresponding to condensed tannins were adjusted to 7 as described above.

5.2.6. Determination of antioxidant capacity of food samples

The different extracts obtained above were incubated separately ith the TMAMQ as described above for pure antioxidants. The extracts were mixed in varying amounts to obtain a dose response curve. The mixture was thoroughly mixed and incubated at 30 ^{O}C while shaking at 140 rpm until full completion of the reaction as evidenced by no further decrease in

absorbance. The stochiometrical reduction of TMAMQ was then calculated from the decrease in absorbance as described above.

5.2.7. Antioxidant activity of oils

Extra-virgin olive oil and maize oil produced by Warenhandels-AG, Salzburg, Austria and Unilever, Wien, Austria respectively were used directly without any modification. Total antioxidant capacity was determined directly after diluting aliquots in ethyl acetate (Espin, Soler-Rivas, & Wichers, 2000). The mixture was thoroughly mixed and incubated at 30 ^{O}C while shaking at 140 rpm until full completion of the reaction as evidenced by no further decrease in absorbance. The stoichiometrical reduction of TMAMQ was then calculated from the dose response curve as described above.

5.2.8. Measurement of total phenols

The phenol content of the extracts was determined according to the Folin–Ciocalteu procedure (Singleton & Rossi, 1965). Briefly, test sample (100 µl) was mixed with 100 µl of Folin–Ciocalteau reagent and swirled. Subsequently, 500 µl of nanopure water, 50–100 µl of sample, and 100 µl of Folin–Ciocalteu reagent were added to a 2 ml eppendorf reaction tube. The contents were mixed and allowed to stand for 5–8 min at 25 ^{O}C. Thereafter, 1 ml of a 7% sodium carbonate solution was added, followed by the addition of nanopure water. Solutions were mixed and allowed to stand at 30 ^{O}C again for 2 h. Total phenolic content was determined using TECAN Infinite M2000 plate reader (Tecan Austria GmbH, Grödig, Austria) at 650 nm wavelength. Total phenolic content was determined as gallic acid equivalents (GAE) expressed as milligrams per litre of gallic acid per milligram of extract (Peschel et al., 2006).

5.3. Results and discussion

In a first stage, the antioxidant scavenging activity of different pure molecules (phenolics, amino acids, peptides, proteins and vitamins) was investigated followed by the antioxidant activity of food extracts.

5.3.1. Antioxidant activity of phenolics

Among phenolic compounds, phenolic acids, flavonoids and tannins are regarded as the main dietary antioxidants (Pellegrini et al., 2007; Sikora, Cieslik, & Topolska, 2008) and were therefore selected. The antioxidant activity of simple phenolics increased with increasing number of hydroxyl groups in the order (guaiacol [0.01 lM] < pyrogallol [5.79 lM]) (Table 1). A similar trend was also observed with hydroxybenzoic acids in the order 4-hydroxybenzoic acid [no activity] < 2,5-hydroxybenzoic [0.63 lM] < gallic acid [3.91 lM] (Table 1). Substitution of the 3- and 5-hydroxyl group in gallic acid with methoxy groups as in syringic acid reduced the antioxidant activity from 3.91 lM to 1.03 lM, respectively. However the presence of the two methoxy groups in syringic acid adjacent to the OH group enhanced antioxidant activity as compared with vanillic acid (Table 1).

Table 1. Amount of TMAMQ quenched by 1µM of the phenolics tested.

Phenolics	**Number of OH**	**Amount of TMAMQ (µM) reduced by 1 µM of phenolics**
Simple phenolics		
Guaiacol	1	0.01 ± 0.004
Catechol	2	2.57 ± 0.30
Pyrogallol	3	5.78 ± 0.14
Hydroxybenzoic acids		
4-hydroxybenzoic acid	1	-
2,5-hydroxybenzoic acid	2	0.63 ± 0.02
Gallic acid	3	3.91 ± 0.02
Vannilic acid	1	0.67 ± 0.03
Syringic acid	1	1.03 ± 0.04
Hydroxycinnamic acids		
ρ-coumaric	1	-
Cafeic acid	2	2.47 ± 0.31
Eugenol	1	0.2 4 ± 0.15
Ferulic acid	1	1.23 ± 0.12
Sinapic acid	1	3.58 ± 0.22

(-) no antioxidant activity detected

The antioxidant activity of hydroxycinnamic acids also increased with increasing number of hydroxyl groups (Table 1). Like the monohydroxy benzoic acid (4-hydroxybenzoic acid), p-coumaric acid showed no antioxidant activity in terms of hydrogen donating capacity towards TMAMQ. However, the presence of another hydroxyl group for example in caffeic acid as compared with p-coumaric showed an antioxidant activity of 2.47 lM (Table 1). The antioxidant behaviour of hydroxybenzoic acids and hydroxycinnamic acids in the presence of TMAMQ is consistent with previous observation (Rice-Evans, Miller, & Paganga, 1996; Villano, Fernandez-Pachon, Moya, Troncoso, & Garcia-Parrilla, 2007) when these respective antioxidants were incubated with ABTS and N,N-diphenyl-picryl hydrazyl (DPPH) radicals. The introduction of methoxyl groups at meta and para positions in hydroxybenzoic acids increased the antioxidant activity when comparing vannilic acid (0.67 lM) and syringic acid (1.03 lM). A similar trend was also observed for hydroxycinnamates when comparing caffeic acid (2.47 lM) to sinapic acid (3.58 lM). However, substitution of the 3-hydroxyl group only of caffeic acid by a methoxy group (ferulic acid) considerably decreased the antioxidant activity of hydroxycinnamates (Table 1). The antioxidant activity of the tested hydroxycinnamic acids was in the order sinapic acid > cafeic acid > ferulic acid > p-coumaric acid (Table 1). In agreement with previous studies using other assay procedures, the antioxidant activity of phenolic antioxidants depended on the number and positions of the hydroxyl groups in relation to the carboxyl functional group and presence of methoxyl groups (Balasundram, Sundram, & Samman, 2006; Monach, Williamson, Morand, Scalbert, & Rémésy, 2005; Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999) For example, the increase in antioxidant activity of phenolics with increasing degree of hydroxylation and the decrease in antioxidant activity with substitution of the hydroxyl groups at the 3- and 5-position with methoxyl groups as in syringic acid has also been reported by Rice-Evans and her coworkers (Rice-Evans et al., 1996). Further, in this study just as in previous studies (Andreasen, Landbo, Christensen, Hansen, & Meyer, 2001), generally the hydroxycinnamic acids exhibited higher antioxidant activity compared to

the corresponding hydroxybenzoic acids. The high antioxidant activity of hydroxycinnamic acids has been attributed to the presence of CH=CH–COOH group, which ensures greater H-donating ability and radical stabilization than the –COOH group in the hydroxybenzoic acids (Rice-Evans, Miller, & Paganga, 1997). However, comparing ferulic acid and eugenol which basically share similar functional groups, the presence of $CH_2CH=CH_2$ negatively influenced the antioxidant activity of the later (Table 1).

5.3.2. Antioxidant activity of polyphenols

The antioxidant activity of anthocyanins, flavonols, flavones and flavan-3-ols was investigated (Table 2). Among the anthocyanins tested, delphinidin chloride reduced 5.40 µM TMAMQ, pelargonidin chloride reduced 3.68 µM TMAMQ and paeonidin chloride reduced 2.89 µM of TMAMQ. Cyanin chloride and oenin chloride each reduced 0.88 µM and 0.94, respectively. malvidin chloride (1 µM) showed the highest antioxidant activity reducing 13.01 µM of TMAMQ although in previous studies its antioxidant activity was similar to that of peonidin.

Table 2. Amount of TMAMQ quenched by 1µM of the polyphenolics tested.

Antioxidants	Position and number of free -OH substituents	Amount of TMAMQ (µM) reduced by 1 µmol antioxidant
Anthocyanidins		
Malvin chloride	7, 4´	0.17 ± 0.03
Oenin chloride	5, 7, 4´	0.94 ± 0.20
Malvidin chloride	3, 5, 7, 4´	13.01 ± 0.51
Pelargonidin chloride	3, 5, 7, 4´	3.68 ± 0.32
Paeonidin chloride	3, 5, 7, 4´	2.89 ± 0.44
Cyanin chloride	3, 5, 7, 3´, 4´	0.88 ± 0.21
Delphinidin chloride	3, 5, 7, 3´, 4´, 5´	5.40 ± 0.52
Flavan-3-ols		
Epigallocatechin gallate	3,5,7,3´,4´,3´´,4´´, 5´´	14.33 ± 2.00
catechin	3, 5, 7, 3´, 4´	5.98 ± 0.41
Flavonols		
Quercetin	3, 5, 7, 3´, 4´	4.80 ± 0.31
Flavones		
Rutin hydrate	5, 7, 3´, 4´	2.01 ± 0.21

This result is consistent with results obtained with hydroxbenzoic and hydroxycinnamic acids when comparing antioxidants with one methoxyl group and two methoxyl groups demonstrating the advantage of this method over the DPPH and ABTS methods. Increase in methoxyl groups enhances the electron donating ability of the antioxidant. The B-ring hydroxyl configuration has been reported to be the most significant determinant for radical scavenging ability of flavonoids (Balasundram et al., 2006; Burda & Oleszek, 2001; Cao, Sofic, & Prior, 1996). The antioxidant activity of quercetin (4.80 lM), a flanovol was more than twice that of rutin hydrate (2.01 lM) a flavone, similar to that reported by (Lien, Ren, Bui, & Wang, 1999; Silva et al., 2002). This also adds evidence to the premise that flavonols are more effective free radical scavengers than flavones (Lien et al., 1999; Rice-Evans et al., 1996) and that glycosylation of flavonoids reduces their antioxidant activity when compared to the corresponding aglycones. Although quercetin has an identical number of hydroxyl groups in the same positions as catechin, the 2,3-double bond in the C ring and the 4-oxo group maybe responsible for enhancing the antioxidant activity of the later. Epigallocatechin gallate had an antioxidant activity three times that of catechin (Table 2) which shows the contribution of gallic units on the former. Generally the antioxidant activity responses of polyphenols and phenolics were in good agreement with the proposed structureactivity relationship as summarised by previous reports (Balasundram et al., 2006; Heim, Tagliaferro, & Bobilya, 2002; Rice-Evans et al., 1997).

5.3.3. Antioxidant activity of endogenous antioxidants and free amino acids

Of the 21 free amino acids tested, only cysteine, tryptophan, serine, arginine, methionine and glycine quenched TMAMQ as shown in Table 3. A higher antioxidant activity (0.47) was obtained with cysteine (containing a free –SH group) followed by tryptophan (0.16), an aromatic containing amino acid. Many contradictory results are found in the literature when comparing the antioxidant activity of amino acids. For example, Trianitis and co-workers (Triantis, Yannakopoulou, Nikokavoura, Dimotikali, & Papadopoulos, 2007) using the luminal-sodium hypochlorite chemiluminescence observed

antioxidant activity with 13 of the amino acids listed in Table 3 while another previous report (Perez-Jimenez & Saura-Calixto, 2006) using ORAC and ABTS recorded antioxidant activity for tyrosine, trypotophan and arginine. Interestingly, Meucci and Mele (1997) using ABTS and Triantis et al. (2007) using DPPH reported antioxidant activity only for a few amino acids.

Table 3. Antioxidant activity of free amino acids and other serum antioxidants as measured with laccase generated TMAMQ.

Antioxidant	**µmol TMAMQ reduced per 1 µmol antioxidant**
Amino acids	
Cysteine	4.7×10^{-1}
Tryptophan	1.6×10^{-1}
Serine	4.0×10^{-4}
Arginine	4.7×10^{-3}
Methionine	9.0×10^{-4}
Aspartic acid	-
Lysine	-
Proline	-
Histidine	-
Alanine	-
Tyrosine	-
Valine	-
Isoleucine	-
Threonine	-
Phenylalanine	-
Glutamic acid	-
Asparagine	-
Leucine	-
Gylcine	3.0×10^{-5}
Other serum antioxidants	
Uric acid	6.6×10^{-1}
Glutathione	3.0×10^{-1}
Albumin	1.8×10^{-1}
Vitamins	
L-ascorbic acid 6-palmitate	0.86 ± 0.03
Vitamin E	1.26 ± 0.12
β-carotene (Vitamin A precursor)	0.05 ± 0.02
Oleuropein	0.82 ± 0.21

In another report, Ahmad, Al-Hakim, Adel, and Shehata (1983) found that lysine, arginine, glutamic acid, methionine, and hydroxyproline had high antioxidant activity. From structural point of view lysine and glutamic acid may not be antioxidants and this was confirmed by TMAMQ. This may indicate the superiority of TMAMQ as a more reliable tool to measure antioxidant activity of serum amino acids and proteins. Nevertheless, despite this controversy, the antioxidant activities of cysteine, tryptophan, methionine just as observed with TMAMQ are well established (Hernandez-Ledesma, Davalos, Bartolome, & Amigo, 2005; Levine, Mosoni, Berlett, & Satdtman, 1996; Masella, Di Benedetto, Vari, Filesi, & Giovannini, 2005; Pastore, Federici, Bertini, & Piemonte, 2003). The antioxidant activities of cysteine, methionine and glycine when discussed in connection with the observed antioxidant activity of glutathione and serum albumin justify its already known role as a major antioxidant source in humans. Further, the observed antioxidant activity of cysteine, glutathione and albumin strengthens the crucial role of sulphydryl groups as source of antioxidants in proteins or peptides. For example glutathione is a tripeptide composed of cysteine, glutamic acid and glycine which is viewed as the principal non-protein thiol involved in the antioxidant cellular defence (Masella et al., 2005; Pastore et al., 2003). Albumin which also showed antioxidant activity with TMAMQ is believed to contribute 70% of the free radical-trapping activity of serum (Bourdon & Blache, 2001) and its antioxidant activity is attributed to cysteine residues (Oettl & Stauber, 2007). Another widely reported human endogenous antioxidant is uric acid which also quenched TMAMQ (Table 3). Uric acid is reported as a selective antioxidant responsible for quenching hydroxyl radicals and hypochlorous acid (Genestra, 2007; Kohen & Nyska, 2002).

5.3.4. Antioxidant activity of vitamins and other compounds

The antioxidant activity of vitamin E was almost similar to that of ascorbic acid (Table 3) as determined in a previous study (Nugroho Prasetyo et al., 2009). The antioxidant activity of b-carotene was weak compared to Vitamin C and E (Table 3). Similarly low antioxidant activity of b-carotene was also reported

using DPPH radical (Jimenez-Escrig, Jimenez-Jimenez, Sanchez-Moreno, & Saura-Calixto, 2000; Lee, Ozcelik, & Min, 2006). This may be attributed to antioxidant mechanisms of carotenoids which have been known to quench singlet oxygen radicals primarily by physical mechanism in which the excess energy of the singlet oxygen is transferred to the carotenoid's electron rich structure. The excited carotenoid then enters into its ground state by losing the extra energy as heat (Sies & Wilhelm, 2004). This means that the electron/hydrogen transfer mechanism is not the major route justifying the low antioxidant values. Equally interestingly was oleuropein which gives extra-virgin olive oil its bitter, pungent taste, which was also able to quench 0.82 lM TMAMQ confirming earlier reports that it is a powerful antioxidant (Tripoli et al., 2005).

5.3.5. Total phenolic content and antioxidant activity of extracts of food samples

The antioxidant activity of common fruits and vegetables found in Austrian food markets (Graz) was investigated. The different antioxidants in the food samples were extracted sequentially using different solvents (methanol, acetone, acidified methanol and butanol). The antioxidant activity as well as the total phenols of each solvent extract was analysed separately as summarised in Table 4 and Fig. 2, respectively. The different solvents extracted different antioxidants as reflected by the different antioxidants activities obtained with the different fractions (Table 4). The antioxidant activity of the methanol extract of spinach was the highest (4.36 mg extract/mmol TMAMQ) followed by kiwi (13.95 mg extract/mmol TMAMQ) and lettuce (40 mg extract/mmol TMAMQ), respectively. The rank order of the antioxidant activity of the methanol extracts of spinach, kiwi and lettuce reflected the total phenols content of the respective fractions in that order (Fig. 2). This antioxidant activity rank order agrees well with the Oxygen Radical Absorbance Capacity (ORAC) method which ranked spinach > white onion > tomato > carrot (Ou et al., 2002). Other previous reports (Cao et al., 1996; Kaur & Kapoor, 2001; Prior & Cao, 2000) also consistently reported high antioxidant activity of the same food samples using the ORAC assay. In other

similar studies, (Perez-Jimenez et al., 2008) also observed that efficient extraction of antioxidants required the use solvents with different polarities. The different solvents extracted different antioxidants as reflected by the different antioxidants activities obtained with the different fractions. The antioxidant capacities of kiwi have been previously attributed to high levels of vitamin C present in the fruit (Chun et al., 2005; Halvorsen et al., 2002; Wang, Cao, & Prior, 1996) and could explain the antioxidant activity of the methanol extract. Although methanol extracts exhibited the highest concentration of phenolics in the other samples (Fig. 2), in pumpkin seeds the total phenolics were highest in hydrolysed tannins fractions. Surprisingly the acetone fraction of the pumpkin seeds showed the highest antioxidant activity of 13.44 mg extract per 1 mmol TMAMQ (Table 4). The data also show that different solvents are required for the extraction of different antioxidants contained in various food samples (Table 4).

Table 4. Antioxidant activity of different food extracts (mg dry weight) as measured with laccase generated TMAMQ.

Food sample	Methanol/ water (50:50 v/v)	Acetone/water 70:30 v/v	Acidified methanol	Acidified butanol/$FeCl_3$
	mg dry weight of the extract required to reduce 1 mmol TMAMQ			
Fruits and vegetables				
Apple	555.56 ±1	149.25 ± 3	555.56 ± 2	188.68 ± 2
Carrots	98.04 ± 2	270.27 ± 1	476.19 ± 4	185.19 ± 4
Garlic	588.24 ± 2	135.14 ± 3	53.19 ± 3	36.63 ± 3
Kiwi	13.95 ± 1	44.25 ± 1	68.03 ± 3	140.85 ± 2
Lettuce	40.98 ± 3	181.82 ± 3	270.27 ± 1	263.16 ± 2
Onion	344.83 ± 2	39.37 ± 4	370.37 ± 3	46.08 ± 3
Pumpkin seed	158.73 ± 3	13.44 ± 2	153.85 ± 3	79.37 ± 3
Spinach	4.36 ± 0.5	30.40 ± 2	100.00 ± 2	476.19 ± 1
Tomato	61.73 ± 3	38.31 ± 3	49.75 ± 4	82.64 ± 2
Beverages		***Water extract***		
Fermented tea (*Camellia sinensis)*		1.15 ± 0.02		
Green Tea (*Camellia sinensis)*		0.59 ± 0.01		
Coffee		0.65 ± 0.03		
Oils		***Oil extract***		
Maize corn oil		26.75 ± 0.01		
Extra-virgin olive oil		20.32 ± 0.03		

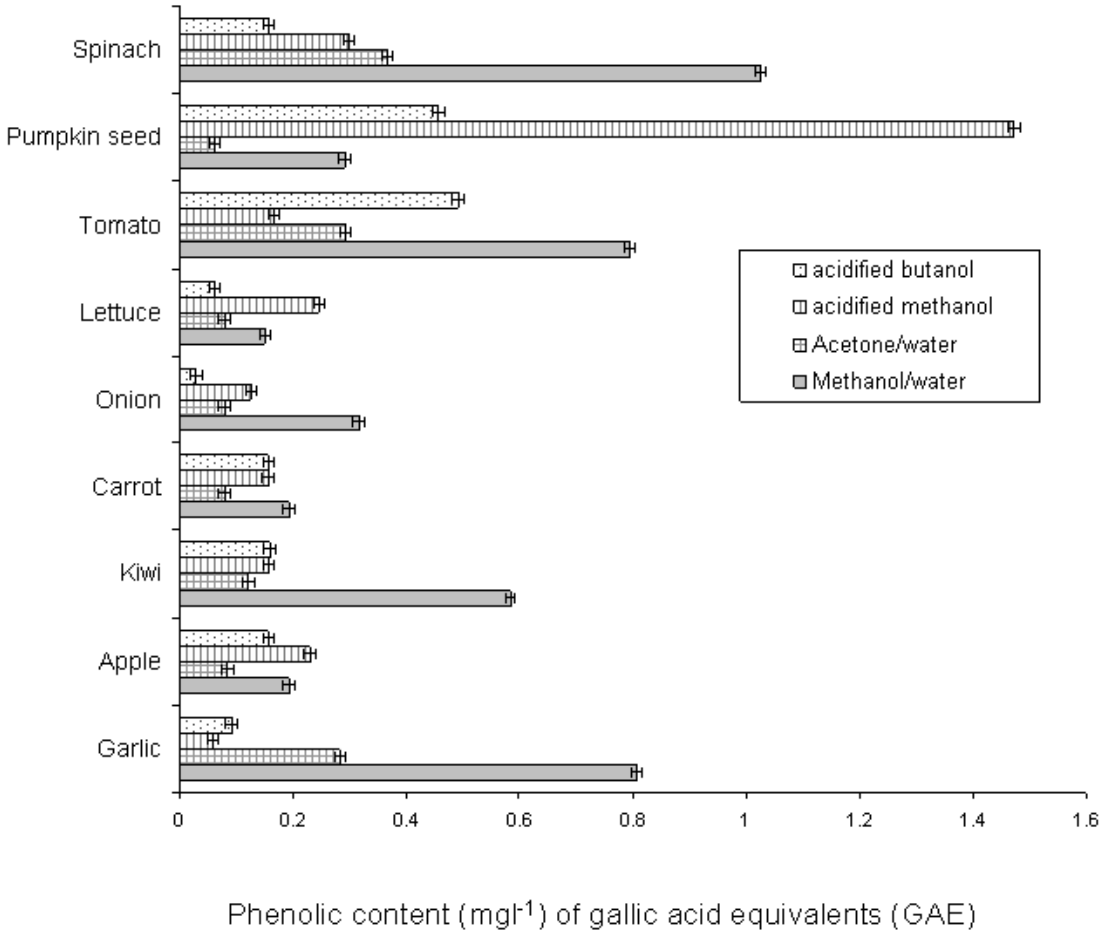

Fig. 2. Total phenol content in the different food extracts.

The hydrolysed tannins and condensed tannins fractions of garlic acid also showed a high antioxidant activity when compared to the antioxidant activity of other fractions (Table 4). The antioxidant activity of tomato fractions and kiwi were generally high for all the fractions (Table 4). High levels of carotenoids especially lycopene in tomato and tocopherols in all chlorophyll containing tissues for example lettuce and spinach have been shown to contribute significantly to their antioxidant activity (Piironen, Syvaeoja, Salminen, & Koivistoinen, 1986). The antioxidant activity of all extracts from apples was generally low (Table 4) which probably can be justified by earlier reports that the major source of antioxidants in apples are found in the peel which is believed to contain about 7-times higher antioxidants than the pulp (Sikora et al., 2008).

5.3.6. Antioxidant activity of beverages and oils

The water extracts obtained from green, fermented teas and coffee were very active in quenching TMAMQ (Table 4) as compared to food samples in Table 4. The antioxidant activity of teas is mainly attributed to catechins namely; epigallocatechin gallate, epigallocatechin, gallic acid, epicatechin and catechin (Balasundram et al., 2006; Sikora et al., 2008). The antioxidant activity of green tea (0.59 g/mmol) was twice as effective as that of fermented tea (1.15 mg/mmol (Table 4). The decrease in antioxidant activity in fermented tea is attributed to changes occurring during the fermentation process where the flavanols in green tea leaves (mainly catechins and their gallic esters) undergo an oxidative polymerization by polyphenol oxidases, turning the leaves black (Daglia, Papetti, Gregotti, Berte, & Gazzani, 2000; Richelle, Tavazzi, & Offord, 2001; Wei, Zhou, Cai, Yang, & Liu, 2006). The antioxidant activity of coffee was lower as compared to that of green tea (Table 4). Coffee processing like in teas leads to the modification and loss of some polyphenolic antioxidants during roasting (Daglia et al., 2000; Tubaro, Micossi, & Ursini, 1996). Extra virgin olive oil quenched TMAMQ and its activity of 20.32 ml may partly be attributed to the presence oleuropein already shown above to quench TMAMQ as well as other phenolics described by earlier researchers as a powerful antioxidants both in vivo and in vitro (Tripoli et al., 2005). Maize oil also showed antioxidant activity (Table 4). Different oils have different antioxidants-contributing compounds as described by Pennington (2002).

Although generally the rank order of antioxidant activity of most of the known pure antioxidants and food samples investigated in this work agree with previous studies using different approaches (ORAC, DPPH, ABTS), the actual values obtained vary. There may be several underlying factors among which are effects of solvents. Solvents have been shown to influence the reaction of antioxidants (Dangles, Dufoura, & Fargeixa, 2000) as well as the reaction mechanism favouring hydrogen atom transfer in apolar solvents and electron transfer mechanisms in polar solvents (Perez-Jimenez & Saura-Calixto, 2006; Saito & Kawabata, 2004). There is therefore a need to investigate the

effects of solvents as well as other reaction conditions which might influence accurate measurement of antioxidant activity of given samples using TMAMQ.

5.4. Conclusions

All known different classes of antioxidants molecules tested (dietary and endogenous) were able to quench TMAMQ. This observation demonstrates the great potential application of TMAMQ for antioxidant activity measurement in both food, cosmetic and health industries. The TMAMQ seem to be more reliable than both DPPH and ABTS method when considering antioxidant activity of amino acids and this may well extend to antioxidant activity of serum proteins. Adding to this interesting observation, the extensive information accrued in the development of laccase based biosensors for phenolics detection in wine, beer, tea and vegetable extracts (Franzoi, Dupont, Spinelli, & Vieira, 2009; Gamella, Campuzano, Reviejo, & Pingarron, 2008; Jarosz-Wilkolazka, Ruzgas, & Gorton, 2004, 2005; Merle et al., 2008; Portaccio et al., 2006; Roy, Abraham, Abhijith, Kumar, & Thakur, 2005; Shleev et al., 2006)

makes the development of an automated method for antioxidant activity determination feasible. In this case the immobilized laccase on the biosensor can be designed in such a way that laccase oxidises immobilized syringaldazine to TMAMQ which is further reduced to syringaldazine upon addition of a sample containing antioxidants molecules. The resulting oxygen consumption can be easily monitored (Nugroho Prasetyo et al., 2009) and converted into electrochemical signals.

5.5. References

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6

Cellular and plasma antioxidant activity assay using Tetramethoxy Azobismethylene Quinone (TMAMQ)

The rates of the reduction of Tetramethoxy Azobismethylene Quinone (TMAMQ) by cellular and plasma antioxidants were investigated. The highest reduction rate (*k*) was obtained with ascorbic acid (1.11 x 10^{-2} $\mu M^{-1}s^{-1}$) while glutathione showed the lowest 2.94 x 10^{-5} $\mu M^{-1}s^{-1}$. Comparing TMAMQ and the commercially available antioxidant method (TAC), clearly shows a similar trend although the values differ. Nevertheless, TMAMQ has many attractive properties, among them its high sensitivity (only a minute plasma sample is required) and the reaction proceeds until steady state (all antioxidants have reacted) without the need to stop the reaction. TMAMQ is very stable in acetonitrile (1.5 months) and this has direct implications on reducing cost and experimental steps. Unlike many other commercially available methods antioxidant activity with TMAMQ is possible over a wider pH range (4 – 7.5). TMAMQ is therefore a highly promising antioxidant activity assay method.

Key words: cellular antioxidants; plasma antioxidants; kinetics

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6.1. Introduction

The human body is constantly exposed to a large variety of reactive species (free radicals) which under certain conditions exceed the antioxidant capacity of the body resulting in oxidative stress. Oxidative stress has been implicated in various pathological conditions involving cardiovascular disease, atherosclerosis, cancer, neurological disorders, diabetes, ischemia/reperfusion, ageing etc. (Dalle-Donne et al., 2005; Dhalla, et al., 2000; Jenner, 2003; Sayre, et al., 2001). However, to protect the cells and organs against free radicals, biological systems have evolved a highly sophisticated and complex antioxidant system (Nugroho Prasetyo et al., 2009). These antioxidants constitute the body's first line of defence against free radical damage. The antioxidants include biologically in-built mechanism of neutralizing free radicals comprising endogenous enzyme systems (superoxide dismutase, glutathione peroxidase, catalase) and non-enzymatic antioxidants [glutathione (GSH), ascorbic acid (Vitamin C), uric acid, albumin, gamma and a-tocopherol (Vitamin E), carotenoids, flavonoids etc].

Among the non–enzymatic antioxidants, glutathione, Vitamic C and E play a central and complementary role in quenching free radicals in and around the cell. For example glutathione and vitamin C are complementary water-soluble antioxidants which scavenge reactive oxygen species (ROS) in the fluid outside and within the cell. Vitamin C is considered the most important water-soluble antioxidant in extra-cellular fluids capable of neutralizing ROS in aqueous phase to prevent their entry into cells (Bagchi and Puri,1998). Glutathione is the major soluble antioxidant in the cell compartments comprising [cytosol (1–11 mM), nuclei (3–15 mM), and mitochondria (5–11 mM)] (Masella, et al., 2005). Unlike glutathione and vitamin C, vitamin E is the most effective lipid-soluble antioxidant present in human cells (Burton et al.,1983). Vitamin E as an antioxidant is involved in protecting membrane fatty acids from oxidants. Ascorbic acid and a-tocopherol are believed to be regenerated by reduction with glutathione (Jacob, 1995).

In blood, albumin is code named "sponge or tramp streamer" of the circulation due to its ability to mop a wide range of antioxidants and bind many foreign molecules, metals or organic molecules alike. Its antioxidant activity accounts for more than 70 % in serum (Roche et al., 2008). Another important serum antioxidant is uric acid. Given the importance of the antioxidants described above, any method that can accurately monitor the levels of these cellular antioxidants will be a welcome development to fight against oxidative stress related diseases. Measurement of the antioxidant status of biological fluids could be used as an early warning sign of possible disease. However, although several methods have been developed and are commercially available, questionable sensitivity, unreliability, non-reproducible results and procedural difficulties hamper all making it difficult to have a standardized internally accepted method (Prior, et al., 2005; Perez-Jimenez et al., 2008, Nugroho Prasetyo et al., 2010). For example, the total peroxyl radical trapping assay developed by Wayner et al., 1985 (the most widely used method two decades ago) had a serious problem in that the oxygen eletrode used does not maintain its stability during the whole incubation time. While comparison of the oxygen radical absorbance capacity assay (ORAC) with the Randox Trolox equivalent (Randox- TEAC) and the Ferric reducing ability (FRAP) assays showed no correlation among them rendering the results meaningless for practical applications.

In continued efforts to develop an effective total antioxidant biomarker, Nugroho Prasetyo et al (2009) recently developed a highly promising antioxidant activity method based on laccase generated Tetramethoxy Azobismethylene Quinone (TMAMQ). In the subsequent screening studies, it was shown that TMAMQ was reduced by all known and tested antioxidants including glutathione, uric acid, albumin, Vitamin C and E (Nugroho Prasetyo et al 2010). In this study the kinetic reduction of TMAMQ by cellular antioxidants (glutathione, uric acid, Vitamin C and E) is investigated by plotting the initial velocities (V_0) of the reaction as a function of concentration of reactants. The initial velocity was defined as the initial slope of a graph of the concentrations of reactants as a function of time measured over a range

of times such that only a small fraction of TMAMQ has been reduced (Arnaut et al 2007). Preceding the kinetic study, the influence of pH and solvent on the TMAMQ stability was investigated in order to define its ph-range. Finally, the ability of TMAMQ to measure the antioxidant activity of clinical samples was compared with a commercially available method (based on chromogenic substrate tetramethlybenzidine monitored at 450 nm) used for measuring the total antioxidant activity of serum and EDTA plasma.

6.2. Materials and methods

6.2.1. Enzyme and chemicals

All antioxidant molecules were of analytical grade. The cellular and plasma antioxidants were purchased from Sigma-Aldrich, Steinheim, Germany. All the other chemicals were purchased from Merck, Darmstadt, Germany. *Trametes modesta* laccase was produced and purified as previously described by Nyanhongo et al. (2002).

6.2.2. Production of TMAMQ

TMAMQ was produced as previously described (Nugroho Prasetyo et al., 2009; 2010). Briefly, syringaldazine (0.17mM) was incubated with 50µl of laccase (20 nkat ml^{-1} determined as previously described by Nugroho Prasetyo, et al., 2009) in 50mM sodium citrate buffer at pH 4.5 for 10 min at 30 °C while shaking at 140 rpm using in a Themomixer (Eppendorf AG, Germany). The reaction was then immediately frozen in liquid notrigen and then lyophilized using a Labconco Freeze Dry System / FreeZone© 4.5 Liter Benchtop Model 77500 (Vienna, Austria). The freeze drier was operated at a temperature of -48 °C and at a vacuum pressure of 3 x 10^{-4} mbar. The freeze dried TMAMQ was then dissolved in ice cold methanol and centrifuged at 13 000 rpm to precipitate the enzyme. The resulting supernatant containing TMAMQ was used for antioxidant activity assay.

6.2.3. Effect of pH and solvent on TMAMQ

The effect of pH on TMAMQ stability was studied by incubating freeze dried TMAMQ at different pH ranging from 4-14 and monitoring changes using

wavelength scan in the range 200 – 900 nm. The effect of solvents was investigated by dissolving and incubating lyophilized TMAMQ in the respective solvent (methanol, ethanol, ethylacetate, acetonitrile) at 4 °C, -20 °C and room temperature. The residual TMAMQ was measured at defined incubation times during the incubation period.

6.2.4. Monitoring kinetics reduction of TMAMQ

Spectrophotometric data were acquired by incubating different TMAMQ concentrations (in excess with respect to antioxidants) with the different concentrations of the individual antioxidants. The rate of reduction of TMAMQ was monitored at 530 nm using either a stop flow spectrometer PBP Spectra Kinetic 05-109 Monochromator from Applied Photophysics, UK and the above mentioned UV-Vis spectrometer. The temperature of all the reagents was first adjusted to 25 °C and the temperature in the cell kept constant at the similar temperature by means of a thermoset bath. Typical procedure consisted of adding a freshly prepared solution of the antioxidant to 2 ml of a freshly prepared solution of TMAMQ in methanol, placed in the spectrometer cell. Spectra were recorded every 0.5 s until the reaction reached plateau.

6.2.5. Data analysis

The exact concentration of TMAMQ was calculated from a calibration curve. For each antioxidant concentration tested, data were fitted by using a software programme Origin Pro 7.5 programme to obtain the kinetic parameters and confirmed by mathematical derivation. The kinetic parameters were estimated using curve fittings achieved through least-square regression analysis and yielded optimized values for the parameters.

6.2.6. Antioxidant activity measurement of plasma samples

A commercial antioxidant activity assay "Total Antioxidant Capacity (TAC)" (LDN, Nordhorn, Germany) was used. The handling and measurements were conducted as described in the accompanying manual. For TMAMQ, standards, controls and serum samples were first diluted by transferring 5 µl into 200 µl double distilled water and then 60 µl of the diluted samples were transferred into 340µl TMAMQ, total volume 400 µl. Samples were incubated

until the reaction reached a plateau (approximately 20 min) before reading absorbance at 530 nm using a TECAN Infinite M200 plate reader (Tecan Austria GmbH, Grödig, Austria).

6.3. Results and discussion

6.3.1. The effect of pH on TMAMQ

The effect of pH on electron transfer reactions in measuring antioxidant activity has already been established (Sang, et al, 2002; Arts, et al, 2004, Huang, et al, 2005). Therefore the study of the effect of pH on TMAMQ is extremely important in order to define its effective pH range. TMAMQ was stable in a broad pH range spanning from pH 4 – 7.5 (Fig 1). Comparing TMAMQ with the well known current antioxidant activity measuring methods shows that it has a broader pH range than the ORAC (Cao, et al., 1993), TRAP (Ghiselli, et al., 1995), TEAC (Miller, et al., 1993), TOSC (Winston, et al., 1998), PSC (Adom, et al., 2005), and FRAP (Benzie, et al., 1996) .

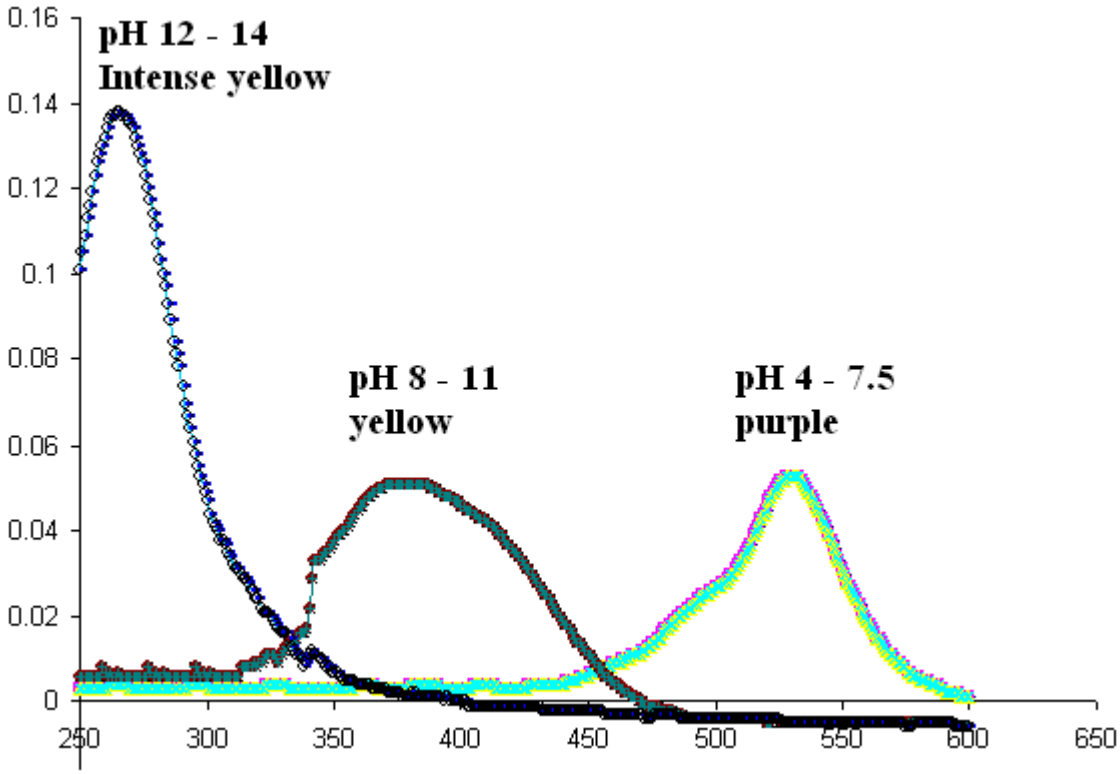

Fig 1. Effect of pH on TMAMQ

TMAMQ is purple within the pH range 4- 7.5 and shifts to yellow in the pH range 8-11, intense yellow (pH 12-14). However, TMAMQ is stable over the human physiological pH 7.5. This observation is consistent with several studies, which reported that most antioxidants are effective in the pH range 4.6-7.4 (Rajendiran and Balasubramanian, 2007; Labrinea and Georgiou, 2004; Schlesier, et al, 2002). For, example, the cellular non-enzymatic antioxidants operate within the pH range 7.3 - 7.5 (Roos and Boron, 1981; Luby-Phelps, 2000).

6.3.2. Effect of solvents on TMAMQ stability

The best solvent for TMAMQ was acetonitrile for each temperature during the whole incubation period. The concentration of TMAMQ remained almost constant during the whole incubation period (Fig 2) loosing <1 % TMAMQ. However, TMAMQ was not stable in methanol and ethanol (Fig 2) resulting in 96 % and 71 % of TMAMQ after 45 days of incubation. This maybe attributed to the hydrogen-bond-accepting properties of protic solvents (ethanol and methanol) which negatively affect stability of radicals (Litiwinienko and Ingold, 2007) leading to the reduction of TMAMQ. On the contrary, acetonitrile as an aprotic solvent lacking the hydrogen bonding properties stabilized TMAMQ. Therefore, acetonitrile can be considered the best solvent for storing TMAMQ. This overcomes problems of wasting reagents as well as cutting on steps and time, all having direct implications on reducing the cost of the assay.

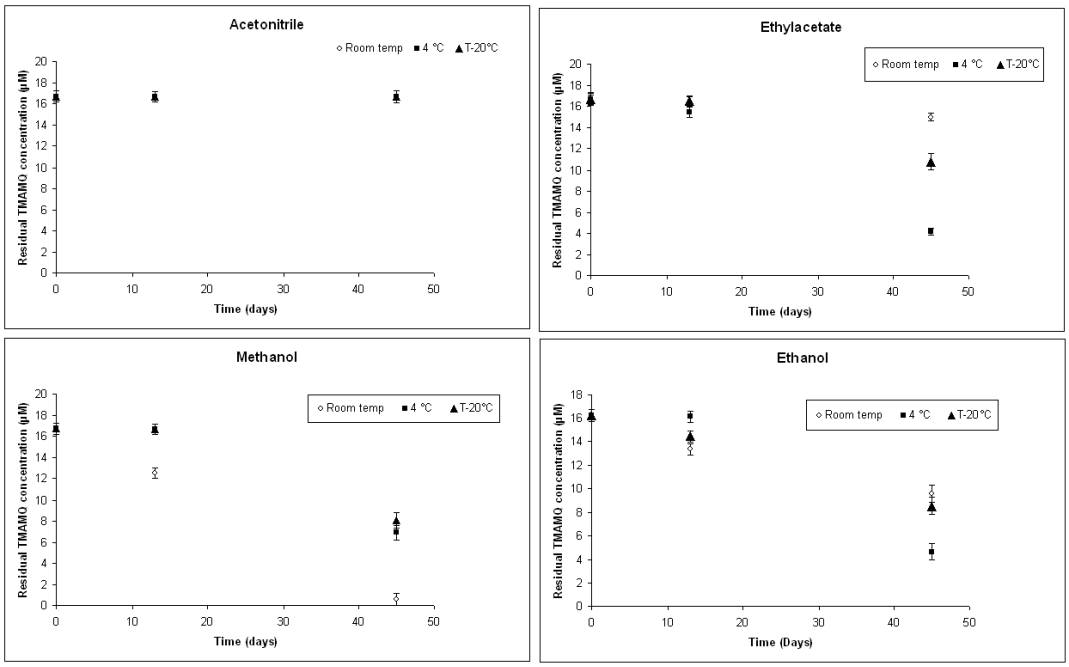

Fig 2. Effect of solvent on the stability of TMAMQ. Results are an average of three independents experiments with a standard deviation of ± 2.

6.3.3. Kinetic reduction of TMAMQ by the cellular and plasma antioxidants

The kinetic reduction of TMAMQ by the different cellular antioxidants was determined based on the initial rates. TMAMQ was in excess under all experimental conditions. Based on this (Eq. 1) was proposed for the overall reaction.

$$\mathrm{TMAMQ} + \mathrm{AOXH} \xrightarrow{k} \mathrm{Syringaldazine} + \mathrm{AOX}\bullet \qquad (1)$$

Where AOXH is the antioxidant, *k*- is the rate reaction constant, syringaldazine is a product of the reduction of TMAMQ and AOX $^{\bullet}$ is the oxidized antioxidant (AOXH). Since our interest was aimed at determining the rates of reduction for TMAMQ, the first part of the equation was considered. Therefore according to Eq. (1) the rate reaction constant of the reduction of TMAMQ can be expressed as in equation (2).

$$-\frac{d[TMAMQ]}{dt} = -\frac{d[AOXH]}{dt} = k[TMAMQ]^m[AOXH]^n \qquad (2)$$

where $-\frac{d[TMAMQ]}{dt} = -\frac{d[AOXH]}{dt}$ represents the rate of the reaction, *k* rate constant, *m* and *n* represent orders of the reduction of TMAMQ, respectively. Accordingly, a direct plot (time vs concentration of reduced TMAMQ) was plotted in order to determine the initial rates calculated from the time of introduction of the respective antioxidant (as indicated by arrow in Fig 3). As clearly shown in Fig 3 the evolution of the different reaction kinetics depended on the nature of the antioxidant. Ascorbic acid, Trolox, a-tocopherol, gamma tocopherol rapidly reacted with TMAMQ as evidenced by its rapid disappearance. Doubling the concentration of TMAMQ or increasing the concentration of either trolox or a-tocopherol increased the steepness of the slope (Fig 3).

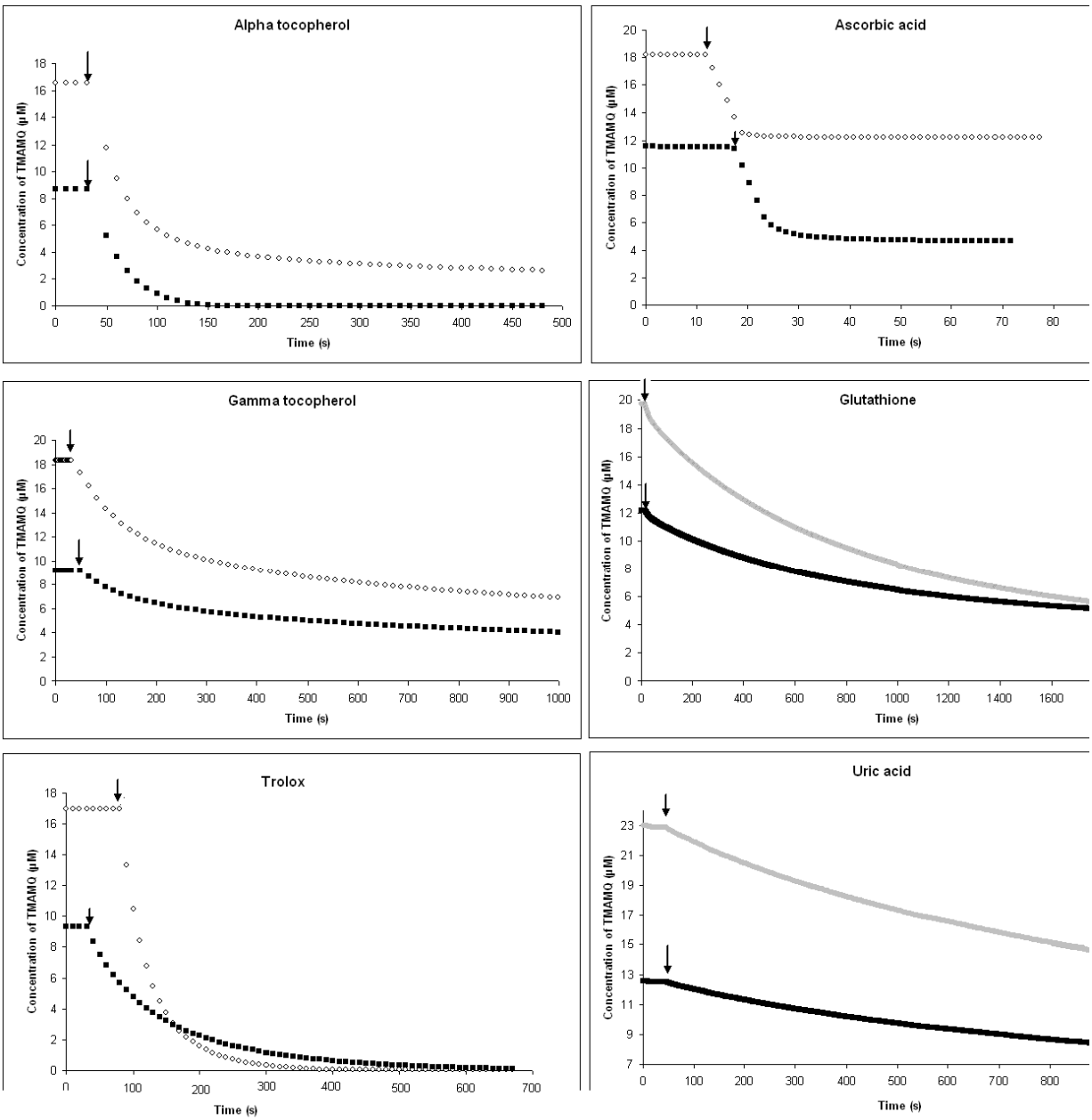

Fig. 3. Reduction profile of TMAMQ in the presence of different antioxidants. Reduction of TMAMQ was measured at half and approximately double its concentration while fixing the concentration of the respective antioxidant.

Ascorbic acid was the fastest in reducing TMAMQ with a rate of 1.11 x 10^{-2} $\mu M^{-1}s^{-1}$ requiring between <6 s to reach plateau while the rest required more than 100 s (Fig 3). Brand-Williams et al. (1995) using DPPH radical observed a similar trend comparing ascorbic acid (required <1 min to reach steady state) while a-tocopherol required 30 min to reach steady state. Uric acid and glutathione reacted slowly with TMAMQ (Table 1) and this is also confirmed by the length

of incubation time of >800 s required to reach steady state (Fig 3). Interestingly in the tocopherol family, trolox showed comparatively higher reactions rates with TMAMQ than a-tocopherol and gamma tocopherol (Table 1). This maybe attributed to their difference in chemical structure. Previous studies calculating theoretical bond dissociation enthalpy and ionisation potentials as a measure of hydrogen or electron transfer capability of various antioxidants placed a-tocopherol as the best hydrogen or electron donor while in trolox the carboxylic group was reported to influence its antioxidants activity depending on solvent (Friaa and Brault, 2006). However, consistent with findings in this study previous studies have also demonstrated higher reaction rates of ascorbic acid than a-tocopherol in its ability to donate electrons (Castro et al., 2006). Nevertheless, the reactions rates of ascorbic acid, trolox, gamma tocopherol and a-tocopherol with TMAMQ were magnitudes higher than the rest. Re, et al. (1999) also observed fast reduction kinetics with ascorbic acid, trolox and a-tocopherol. Although the more rapid the absorbance decreases, the more potent the antioxidant activity of the compound in terms of hydrogen-donating ability (Suja et al., 2004), these orders of the reaction rates do not correspond to their physiological functions. For example, this does not mean that ascorbic acid is more physiologically potent than glutathione.

Table 1. Rates of reduction of TMAMQ by individual cellular and plasma antioxidants

Antioxidant	**1 mol TMAMQ reduced per 1 lmol antioxidant**	***k* ($\mu M^{-1}s^{-1}$)**
a -tocopherol	1. 98 ± 0.2	1.98×10^{-3}
Gamma-tocopherol	1.97 ± 0.2	1.80×10^{-3}
Uric acid	0.66 ± 0.03^	1.12×10^{-5}
Trolox	1.9 0 ± 0.2*	4.47×10^{-3}
Ascorbic acid	2.3 ± 0.3*	1.11×10^{-2}
Glutathione	0.3 ± 0.03^	2.94×10^{-5}

* Nugroho Prasetyo et al (2009); ^ Nugroho Prasetyo et al (2010).

The slow reaction rate of glutathione as evidenced in this study was also observed using the FRAP assay (Janaszewska and Bartosz, 2002) and ABTS radical (Re, et al., 1999). The ineffectiveness of glutathione to reduce DPPH was attributed to the relative oxidation-reduction potentials of the alkienyl groups of thiosulfinates compared with a-tocopherol and ascorbic acid (Larson, 1997; Xiao and Parkin, 2002; Janaszewska and Bartosz, 2002), and this might also be true for the reduction of TMAMQ in this study. The rate of reduction of TMAMQ by albumin was 4.13 x 10^{-3} $\mu g^{-1}s^{-1}$. The slow reaction rates with TMAMQ have direct implication on overall incubation time. Careful consideration has to be made for such slow reactors as uric acid and glutathione when measuring the total antioxidant activity of cellular and plasma samples. In a similar study incubating antioxidant samples for 4 - 6 min using the TEAC method showed that the reactions had not completely reacted (Perez-Jimenez et al 2008).

6.3.4. Reduction of TMAMQ by clinical serum samples

The reduction of TMAMQ by the serum samples was compared with a commercially available TAC method 20 subjects at baseline and after 3 weeks i.e. a total of 40 serum samples. The reduction of TMAMQ required approximately 16 min to reach steady state. From Fig 4, it is clear that the reduction proceeded through two distinct phases (initial phase very fast, followed by a slow). Considering the reaction rates described elsewhere in this study, it can be speculated that antioxidants like ascorbic acid, tocopherol were responsible for the fast reaction phase while glutathione, uric acid and albumin were predominant in the second phase. The reaction proceeded until all the antioxidants were exhausted as shown in Fig 4.This is a quite an interesting property of TMAMQ as it guarantees that all antioxidants in the system have reacted.

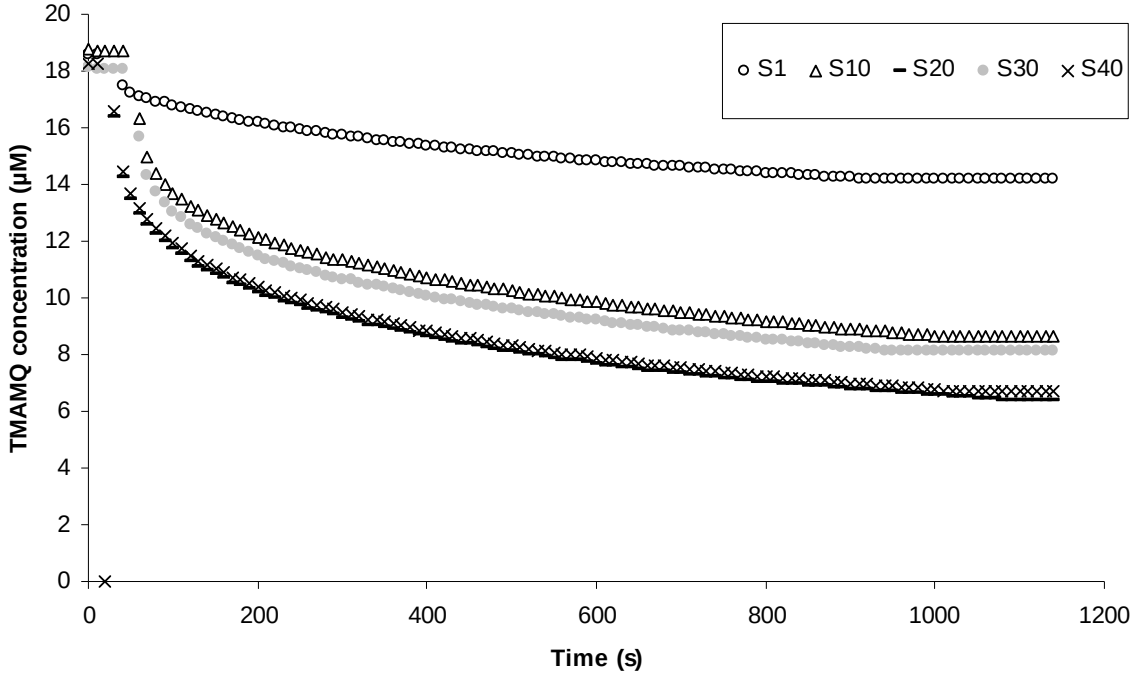

Fig. 4. Reduction profile of TMAMQ by antioxidants of selective serum samples

The general trend of the reduction of TMAMQ was similar to TAC assay (Fig 5). Nevertheless, the fact that incubation of antioxidants with TMAMQ is done until all the antioxidants have completely reacted as evidenced by no change in absorbance (until reaching a plateau) is an attractive property. The absence of correlation between ORAC, FRAP and TEAC was blamed on the end–points while the FRAP suffered from the disadvantage that it did not measure glutathione (Cao and Prior, 1998). In the case of TEAC, antioxidants quench ABTS radical produced by first oxidizing the substrate with H_20_2 in the presence of a peroxidase and metmyoglobin, respectively, conditions which make it difficult to exclude the direct interaction of an added antioxidant at subsequent stage with the residual substrates leading to reduced or increased production of radical species as noted by Cao and Prior, (1998). Another interesting advantage of TMAMQ is that its maximum absorbance at 530 nm compared to TAC measured at 450 nm is far from interference by many biological molecules absorbing in the UV range.

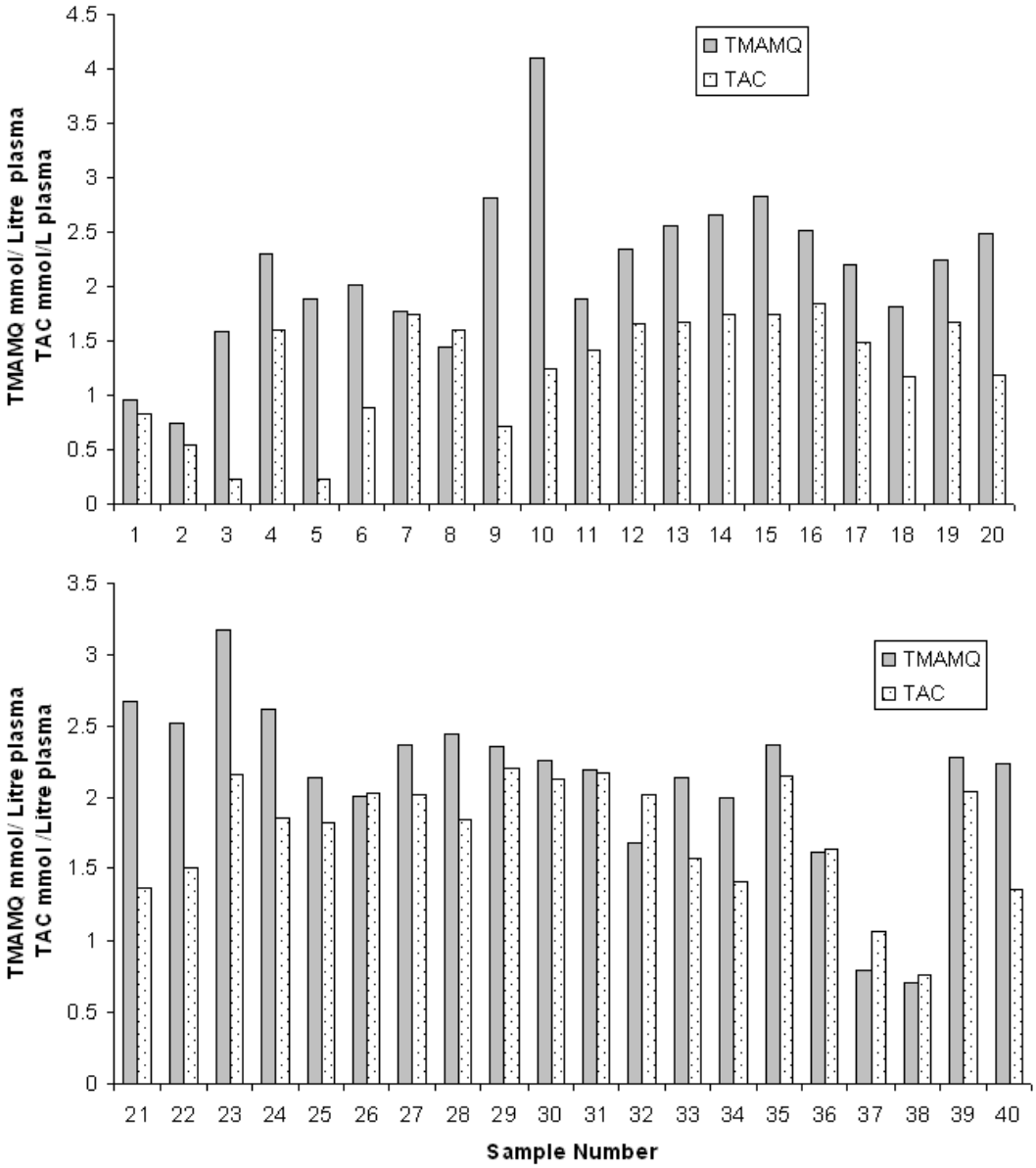

Fig. 5. Concentration of TMAMQ reduced by the different plasma samples

This may explain the general lower values obtained as compared to TAC (Fig 5). Arnao et al (2000) also suggested that the difference between TEAC (436 nm) and DPPH radical (515 nm) method were due to the fact that the later is measured at a wavelength closer to the visible spectrum than the former. TMAMQ is therefore a very attractive molecule for antioxidant activity assay worth pursuing.

Considering the results of this study and the previous studies (Nugroho Prasetyo, 2009; 2010), TMAMQ as an antioxidant activity measuring method

fulfills the criteria of a good antioxidant measuring method as postulated by Prior et al, 2005. It is simple and easy since TMAMQ can be provided readymade and the stock solutions made in acetonitrile are stable over 2 month. Since it is possible to store TMAMQ this means that the assay can be adapted for "high-throughput" analysis for routine quality control analysis or for just a single sample. The reactions are incubated until all antioxidants have reacted (until plateau) which guarantees accurate total antioxidant activity estimation. The assay has a high reproducibility within-run and between-days and can be used for both hydrophilic and lipophilic samples. Another very attractive feature is the wide pH range in which the antioxidant can be investigated. The assay is also highly sensitive as it requires a very small amount of sample (instead of the recommended 25 µl serum sample for the TAC, even 5 µl of the serum sample was diluted 40 times).

6.4. Conclusion

From this study it is clear that TMAMQ can be perfectly applied for cellular as well as plasma and serum antioxidant activity assay at physiological pH. The fact that stock solutions prepared in acetonitrile are stable makes the assay flexible in terms of number of samples that can be assayed per given time and hence cost-effective. The rate of reduction of TMAMQ was highest with ascorbic acid followed by the tocopherol family. The kinetic reduction of TMAMQ by the different individual cellular antioxidants highlight the need for careful consideration of slow reactors like glutathione, uric acid and albumin in order to obtain accurate information regarding the antioxidant activity of plasma samples. This was also confirmed by the reduction profiles of clinical samples tested which showed a two phase reaction (first fast phase) and a slow phase. Although TMAMQ and TAC showed similar reduction trends the values were different demonstrating different sensitivities of the methods. In conclusion, TMAMQ is an ideal molecule for total antioxidant activity assay.

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7

General conclusion and future perspectives

The potential of laccase to improve industrial lignin properties has been demonstrated in this study. *Trametes villosa* laccase (TvL) and *Trametes hirsuta* laccase (ThL) -HBT systems were more effective in in modifying lignin based on decreasing the fluorescence intensity than *Myceleoptera thermophila* laccase (MLV) and *Bacillus* SF (BsL) treated samples. This maybe attributed to the different redox potential of these laccases. ThL and TvL treated calcium lignosulfonate samples underwent extensive polymerization leading to 107 % and 572 increase in Mw, respectively. The loss of aromatic cross-signals in the HSQC spectra obtained after the enzymatic treatment was due to deprotonation of the lignin benzenic rings, as revealed by the 1D-NMR spectra while the appearing strong signals of aromatic carbons in ^{13}C NMR spectra after 83 h of incubation in TvL treated samples were a sign of the formation of new ether and C-C aryl-aryl or aryl-alkyl linkages causing strong polymerization observed by SEC. The FTIR and ^{13}C-NMR spectra and Py-GC/MS chromatograms suggesting no substantial structural changes in the calcium lignosulphonate aromatic structure are a good indication of the ability of TvL and ThL-HBT systems to limit their effect to the substituents for effective cross-linking without degrading the lignin backbone. Generally the dispersant properties of the enzyme treated lignosulfonate increased

significantly.

Lacases plays a major role in activating lignin thereby increase the interaction between lignin and tetraethylorthosilicate (TEOS) resulting in interpenetrating polymers for applications. The concentration of lignin has a direct bearing on the viscosity, curing time and tensile strength. However, the laccase mediated lignin-silica hybrid requires further optimization since the resulting polymer absorbs water. Nevertheless this study shows the great potential of developing lignin-silica adhesives and provides the base for producing materiales which have a wide potential application in functionalization of lignocellulosic materials and other synthetic polymers. This is because silica is such a versatile molecule which enables the attachment of several functional molecules.

One of the greatest achievements on this study was the development of novel antioxidant activity assays based on lacasse-oxidized phenolics. Laccase oxidized syringaldazine generates tetramethoxy azobismethylene quinone (TMAMQ), which emerged as the best, comparable to conventional DPPH radical in estimating antioxidant activity of the model antioxidant compounds (ascorbic acid and Trolox). Moreover, all known different classes of antioxidants molecules tested (dietary and endogenous) were able to quench TMAMQ. In addition, TMAMQ can be used effectively for the measurement of the plasma and cellular non enzymatic antioxidants under physiological conditions. Furthermore, from kinetic study of antioxidant reacting with TMAMQ showed that there is no adduct generated and interefered in the reaction, this led to the future perpective of this method can be challenged to other method available in the market. Adding to this interesting observation, also the extensive information accrued to development of laccase based biosensors for phenolics detection in wine, beer, tea and vegetable extracts.

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