LIGNIN DEGRADATION USING LIGNOLYTIC ENZYMES

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DECLARATIONS

No portion of work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Ayshemgul Nuramat

DEDICATION

I dedicate this thesis to my husband, ***Ahmed Ibrahim,*** *who has supported me unwaveringly in every step of this unforgettable journey. He has cheered me up when I was stressed and disappointed; He has laughed at me when I was worried for every little thing; He has wiped my tears away with his love; and most importantly, he has believed in me that I can make it. So, thank you, Ahmed, for everything!*

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ABSTRACT

Lignin is the only plant biomass that contains aromatic groups in its structure and can provide a wide range of low molecular weight aromatic chemicals if its depolymerisation can be achieved successfully. Currently, lignin is mainly produced as a waste by-product by the paper and pulp industry and biorefineries. Therefore, the transformation of the phenolic-rich lignin into value added aromatic platform chemicals can be regarded of primary concern to improve the economic profitability of biorefining. Moreover, being a renewable resource, the consumption of fossil fuels will be reduced if lignin can be utilised efficiently. Between chemical degradation and enzymatic degradation, the latter could be a more sustainable method to break down lignin due to its enhanced substrate specificity and ability to preserve the aromatic ring structure compared with chemical processing. Therefore, laccase from *Trametes versicolor* (LTV), lignin peroxidase (LiP) and manganese peroxidase (MnP) were studied to determine the scope to depolymerise both water-soluble and insoluble lignins under mild reaction conditions. The enzymatic activity and stability of all three enzymes was investigated and optimum assay conditions were achieved. LTV was found to be the most stable enzyme as it maintained 55 % of its activity at least for the first 6 h at 30 °C whereas LiP was deactivated after 2 h at 25 °C, and MnP was deactivated after 1 h at 28 °C. However, LTV stability decreased at higher temperatures during the oxidation of 2,2'-azino-bis (3-ethylbenthiazoline-6-sulphonic acid (ABTS). One of the non-phenolic lignin model compounds, veratryl alcohol, was oxidised by LTV in the presence of ABTS, thus confirming the published data. The enzymatic degradation of Organosolv lignin (OSL) by LTV resulted in the formation of 2,6-dimethoxy-1,4-benzoquinone (DBQ). The OSL degradation by LTV was not improved by ethanol addition as a co-solvent although ethanol could stabilise LTV at 40 % (v/v). LTV catalysed the degradation of Kraft lignin although it indicated little effect on lignosulphonates. Lastly, the effect of varying the concentrations of 92 ionic liquids (ILs) and their equivalent metal salts on LTV activity was investigated to find a suitable co-solvent to improve the poor mass transfer in OSL degradation. The study showed that 62 ILs were laccase compatible at an IL concentration of 6 % (w/v) and more than 50 % laccase activity was retained in 18 ionic liquids up to 10 % (w/v), and 80 % (v/v) of dioctyl sulfosuccinate quaternary ammonium salt, $[N_{4,4,4,4}][AOT]$. However, there was a progressive loss of activity when the concentrations of the ILs increased. Further study on the enzymatic degradation of ILs-pre-treated OSL is currently ongoing in our research group so that the decomposition of water-insoluble lignin will be understood more comprehensively.

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ABBREVIATIONS

LTV	Laccase from *Trametes versicolor*
LAB	Laccase from *Agaricus bisporous*
LiP	Lignin Peroxidase
MnP	Manganese Peroxidase
VP	Versatile Peroxidase
OSL	Organosolv lignin
ABTS	2,2'-azino-bis (3-ethylbenthiazoline-6-sulphonic acid
ILs	Ionic liquids
UV-Vis	Ultraviolet–visible spectrophotometry
HPLC	High Performance Liquid Chromatography
GPC	Gel Permeation Chromatography
GC-MS	Gas Chromatography-Mass Spectroscopy
EtOH	Ethanol
EA	Ethyl acetate
DCM	Dichloromethane
µL	Micro liter
µM	Micro molar
mM	Milli molar
mg/mL	Milligram per mille
w / v	Weight by volume
v /v	Volume by volume
MW	Molecular weight
U/ mL	Unit per millilitre
U/ mg	Unit per milligram
g/ L	Gram per liter
s	second
v_o	Initial rate of reaction
K_m	Dissociation constant of the enzyme-substrate complex
V_{max}	Maximum velocity of an enzymatic reaction
nm	Nano meter
T	temperature

RT	Retention time
OD	Optical Density
DBQ	2,6-dimethoxy-1,4-benzoquinone
[IL]	concentration of ionic liquids
C_2min	1-ethyl-3-methylimidazolium
C_4min	1-butyl-3-metylimidazolium
C_6min	1-hexyl-3-metyllimidazolium
C_8min	1-octyl-3-metylimidazolium
C_{10}min	1-decyl-3-metylimidazolium
AOT	Bis(2-ethylhexyl) sulfosuccinate sodium salt
OAc or CH_3CO_2	Acetate
HCO_2	Formaldehyde
Cl	Choloride
NTf_2	Bis(trifluoromethylsulfonyl)amide
BF_4	Tetrafluoroborate
PF_6	Hexafluorophosphate
$N_{4,4,4,4}$	Quaternary or tetrabutyl ammonium
$P_{4,4,4,4}$	Quaternary or tetrabutyl phosphonium
$N_{1,1,2,3}$	Dimethyl-ethylpropylammonium
$N_{1,1,2,4}$	Dimethyl-ethyl-butylammonium
$N_{1,1,4,8}$	Dimethyl-butyl-octylammonium
$N_{1,1,4,10}$	Dimethyl-butyl-decylammonium
$N_{1,8,8,8}$	Methyl-trioctylammonium
$N_{1,1,2,2OH}$	Ethyl(2-hydroxyethyl)dimethylammonium
$N_{1,1,2,3OH}$	Ethyl(3-hydroxyethyl)dimethylammonium
$N_{1,1,4,2OH}$	Butyl(2-hydroxyethyl)dimethylammonium
$N_{1,4\ (C3OAc)2}$	Butyl-(3-diacetylpropyl)methylammonium
$N_{1,4,Pyrr}$	Butyl-(methyl-pyrrolidinium)ammonium
$N_{1,12\ (OH)2}$	1-dodecyl-(dihydroxyl)methylammonium
$N_{1,1,2,2CN}$	Ethyl-(2-cyanamide)dimethylammonium
C_4mpy	1-butyl-3-methylpyridinium
C_6mpy	1-hexyl-3-methylpyridinium
C_8mpy	1-octyl-3-methylpyridinium

C_4mpyr	1-butyl-3-methylpyrrolidinium
$P_{4,4,4,5}$	Tributyl-pentylphosphonium
$P_{4,4,4,6}$	Tributyl-hexylphosphonium
$P_{4,4,4,7}$	Tributyl-heptylphosphonium
$P_{4,4,4,8}$	Tributyl-octylphosphonium
$P_{4,4,4,12}$	Tributyl-dodcylphosphonium
$P_{4,4,4,14}$	Tributyl-tetradecylphosphonium
$P_{5,5,5,5}$	Tetrapentylphosphonium
$P_{6,6,6,3}$	Trihexyl-propylphosphonium
$P_{6,6,6,4}$	Trihexyl-butylphosphonium
$P_{6,6,6,5}$	Trihexyl-pentylphosphonium
$P_{6,6,6,6}$	Tetrahexylphosphonium
$P_{6,6,6,7}$	Trihexyl-heptylphosphonium
$P_{6,6,6,8}$	Trihexyl-octylphosphonium
$P_{6,6,6,14}$	Trihexyl-tetradecylphosphonium
$P_{8,8,8,1}$	Trioctyl-methylphosphonium
$P_{8,8,8,4}$	Trioctyl-butylphosphonium
$P_{8,8,8,5}$	Trioctyl-pentylphosphonium
$P_{8,8,8,6}$	Trioctyl-hexylphosphonium
$P_{8,8,8,7}$	Trioctyl-heptylphosphonium
$P_{8,8,8,8}$	Tetraoctylphosphonium
$P_{8,8,8,10}$	Trioctyl-decylphosphonium
$P_{8,8,8,12}$	Trioctyl-dodecylphosphonium
$P_{8,8,8,14}$	Trioctyl-tetradecylphosphonium

1 BIODEGRADATION OF LIGNIN

1.1 Introduction

As far as sustainable development is concerned, there is a practical need to replace petroleum based production with renewable resources. Furthermore, the constant increase in the price of oil due to fossil fuel depletion tends to give rise to the demand for an alternative and reproducible resource for both energy and chemical production. Therefore, global interests have been increasingly determined to find a better solution to this dilemma, especially focusing on biomass-based renewable substitutes for fossil fuels to reduce the energy crisis and the release of greenhouse gases into the atmosphere (Anastas & Warner, 1998, McKendry, 2002, Fernando *et al.*, 2006, Ragauskas *et al.*, 2006, Ayhan, 2009, Novaes *et al.*, 2010). Moreover, renewable resources can also meet the demands of green chemistry contributing to the reduction or elimination of waste at all stages of manufacturing.

Lignocellulose is a major component of biomass and has been used for polysaccharide and sugar production by high temperature treatment thus far (Pérez *et al.*, 2002, Limayem & Ricke, 2012). In such processes, most attention has been given to converting the cellulose and hemicelluloses into fuels by delignification methods, yet, insufficient effort has been made to make good use of the lignin itself, which is also formed during lignocellulose based wood pulping process (Malherbe & Cloete, 2002). Therefore, better solutions are needed to transform lignin into useful compounds to reduce the waste.

Lignin is the second most abundant natural constituent of the plant biomass after cellulose (McKendry, 2002, Wout Boerjan, 2003, Shleev *et al.*, 2006). It is the only available natural source for the production of aromatic chemicals except for existing traditional routes in the chemical industry (Effendi *et al.*, 2008, Zakzeski *et al.*, 2010). It would be possible to produce different kinds of valuable aromatic and non-aromatic chemicals if the lignin could be broken down efficiently. For this purpose, several degradation methods have been commonly tried, such as pyrolysis, hydrogenation, selective oxidation, as well as decomposition under hydrothermal and supercritical conditions (Zakzeski *et al.*, Kruse & Gawlik, 2002, Sun & Cheng, 2002, Mohan *et al.*, 2006, Fang *et al.*, 2008, Yanik *et al.*, 2008, Hermans *et al.*, 2009, Zakzeski *et al.*, 2010, Pandey & Kim, 2011, Munk *et al.*, 2015). However, most of these pathways are performed at high temperatures using expensive

chemical catalysts containing metals such as molybdenum or palladium, and so additional disposal costs are incurred dealing with the waste products from those processes (Dorrestijn *et al.*, 2000). Such methods do not seem very economic or productive. Moreover, some of the useful linkages in the lignin structure cannot be preserved through these harsh processes. Hence, enzymes seem to be a better option to be used in such processes to resolve those problems mentioned above. This is because enzymes are biological catalysts and they are not only accelerate a reaction in the same way that chemical catalysts do, but they also work under very mild conditions, for example at low pH and operation temperature (Frederick A. Bettelheim, 2010). In addition, they are substrate-specific catalysts and therefore they should be extremely helpful to improve the selectivity of reactions and so the number of by-products which caused by traditional chemical catalysis could be decreased. As a result, selective degradation of lignin-containing biomass using enzymes is a preferred methodology compared to the other methods for producing value added chemicals from lignin due to the mild nature of the technique (Otjen, 1987, Tuomela *et al.*, 2000, Baldrian, 2006, Nyanhongo *et al.*, 2012, Lange *et al.*, 2013).

1.2 Literature survey

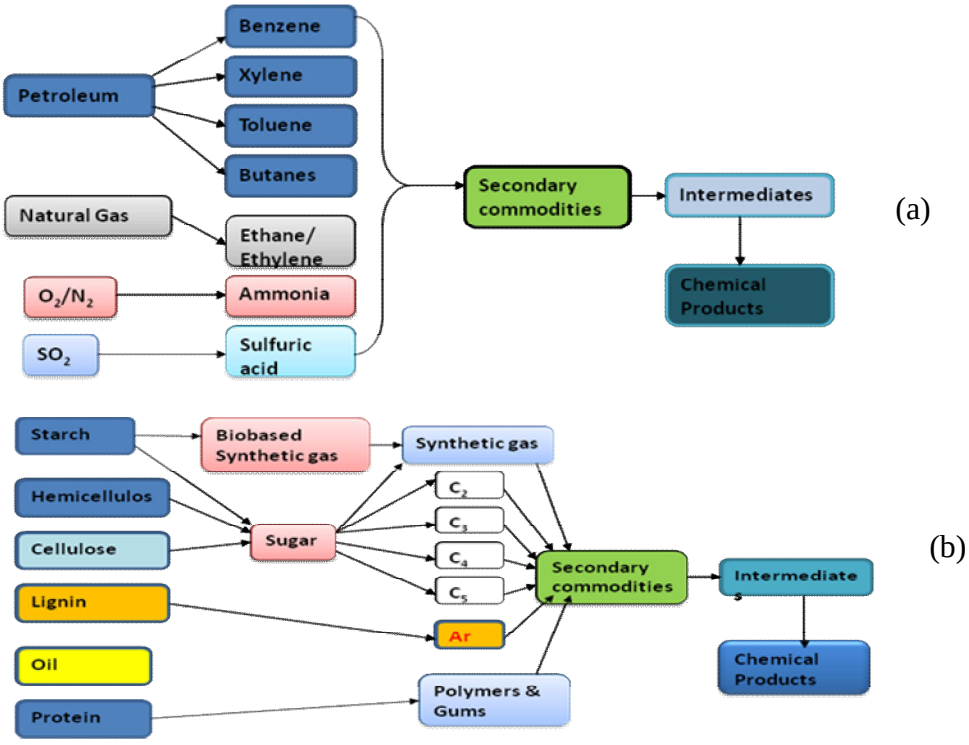
1.2.1 Biomass composition and biorefineries

Biomass is a sustainable resource in nature because its raw materials are cheap and reproducible. For more than a decade, interest has risen in the use of biomass as a renewable source of chemicals, and in some cases this is considered a much better option than the production of biofuels due to the increased value of chemical products over fuel products, and concern over the efficiency of biofuel production (Balandrin *et al.*, 1985, Karagöz *et al.*, 2005, Fernando *et al.*, 2006, Ayhan, 2009).

Plant biomass, crops and trees, have long been recognised as a key supplier of energy as they convert solar energy into chemical energy, and transform carbon dioxide and other nutrients into complicated natural polymers and small molecules (Balandrin *et al.*, 1985, Anastas & Warner, 1998, McKendry, 2002, Anastas, 2003). Plant biomass includes three main natural polymers, cellulose, hemicellulose and lignin, which make up the structures of plant cells, and some low molecular weight organic and inorganic substances (Hofrichter, 2002, Malherbe & Cloete, 2002, Sato *et al.*, 2006). A variety of aliphatic chemicals including sugars, alcohols, aldehydes and acids can be obtained from both cellulose and hemicelluloses

(Sasaki *et al.*, 1998, Kruse & Gawlik, 2003, Werpy & Petersen, 2004). However, only lignin contains a considerable number of aromatic groups in its structure (Faulon & Hatcher, 1994, Dorrestijn *et al.*, 2000, Zakzeski *et al.*, 2010) and so has the possibility to provide a wide range of low molecular weight aromatic chemicals (González *et al.*, 2004, Okuda *et al.*, 2004, Werpy & Petersen, 2004). Therefore, finding a suitable and efficient route to depolymerise lignin would appear to have key importance for lignocellulose based biomass conversion. The biomass chemical flow chart as a proposed replacement for the petroleum flow chart is shown in Scheme 1.1 to illustrate some of the differences between these two processes.

The main technologies reported to produce chemicals from biomass are: (a) biomass refining or pre-treatment, (b) thermo-chemical conversion (gasification, pyrolysis, hydrothermal upgrading), (c) fermentation and bioconversion, and (d) product separation and improvement (Ayhan, 2009). Traditionally, both developed and developing countries all make fuels for transportation using oil refineries. Some of these have recently been partly replaced with ethanol or other liquid chemicals produced from sugars and polysaccharides by using sustainable biorefineries (Clark, 2007, Ayhan, 2009, Fatih Demirbas, 2009, Octave & Thomas, 2009, Cherubini, 2010, FitzPatrick *et al.*, 2010), and the current progress as well as challenges towards producing even more sustainable biorefineries have been discussed (García *et al.*, 2014, Lanzafame *et al.*, 2014, Machani *et al.*, 2014, Sousa-Aguiar *et al.*, 2014).



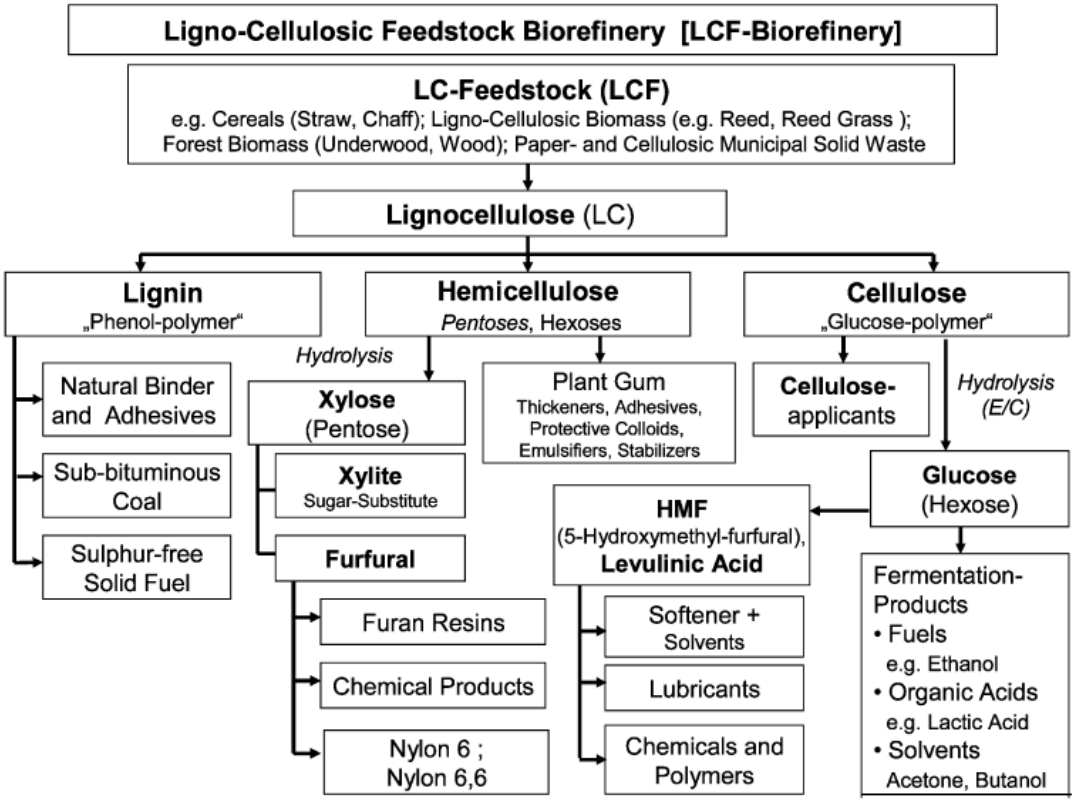
Scheme 1.1: Comparison of (a) petro and (b) biomass based chemical products flow sheet (from Werpy *et al.*) *(Werpy & Petersen, 2004)*. Notes for the abbreviations used in this scheme: C_2 represents the chemicals which are composed of 2 carbon atoms in its molecules, such as ethanol (C_2H_5OH); C_3 stands for propanol (C_3H_7OH); C_4 corresponds to butanol (C_4H_9OH); C_5 as a symbol of pentanol, and the Ar stands for the chemicals that have aromatic or phenolic structures in their molecules.

A biorefinery is a facility for biomass treatment that can combine the bioconversion methods and equipment to produce power (electricity, fuels *etc.*) and variety of possible chemicals from renewable biomass feedstock (Ragauskas *et al.*, 2006, Fatih Demirbas, 2009, Cherubini, 2010). This could replace the use of limited fossil resources to minimise the emission of harmful gases into the atmosphere (McKendry, 2002, Fernando *et al.*, 2006, Ayhan, 2009, Octave & Thomas, 2009).

Tremendous effort is currently being made to find feasible methodologies to produce bio-fuels, such as bio-ethanol from agricultural residues containing cellulose and waste wood, especially in the United States and Europe. If a successful cellulosic ethanol industry is established to replace the fuels that are traditionally produced from petroleum based natural resources, then large amounts of lignin will be produced as a by-product. Another common method used to extract chemicals from biomass makes use of enzymes produced by microbial activity. However, lignocellulosic biomass is more complex and often requires some form of pre-treatment before fermentation can be applied (McKendry, 2002). The pre-treatment step

weakens the lignocellulosic structure to allow fermentation to proceed (Montane *et al.*, 1998). Thus, lignin is produced as a by-product of fermentation. However, it is structurally resistant to biological decomposition (Asada *et al.*, 2005).

Currently, lignin is mainly used for fuels production from biorefineries (e.g. the ethanol production). Although the anaerobic processes (fungi or bacteria) are suitable for lignin modification, they are not able to attack the aromatic rings at all. It requires long incubation times to achieve degradation, and therefore these disadvantageous conditions limit its industrial practicability (Wong, 2009, Tolbert *et al.*, 2014). Therefore, to convert the phenolic-rich waste product, lignin, into higher-values chemicals or materials will be a second main concern to improve the economic feasibility of biorefinering (Lignoworks, 2014). Being an aromatic-rich sustainable natural resource, lignin could provide an amazing opportunity to build a lignocellulose-led biorefinery because it is an extremely abundant raw material, which makes up approximately 30 % of the weight and 40 % of the energy content in lignocellulosic biomass (Kamm & Kamm, 2004, Limayem & Ricke, 2012, Menon & Rao, 2012). Therefore, it plays a significant role in the production of performance materials and value added aromatic platform chemicals (Werpy & Petersen, 2004, Holladay *et al.*, 2007, Cherubini, 2010, Menon & Rao, 2012). Furthermore, lignin can be used as a natural adhesive and binder, while some other useful products could be generated through the hydrolysis of cellulose and hemicelluloses. A schematic representation of a lignocellulosic feedstock biorefinery (LCF-Biorefinery) is given in Scheme 1.2 as an example to show some of the possible chemicals that could be produced using all three sustainable sources ~~of~~ for biorefineries.



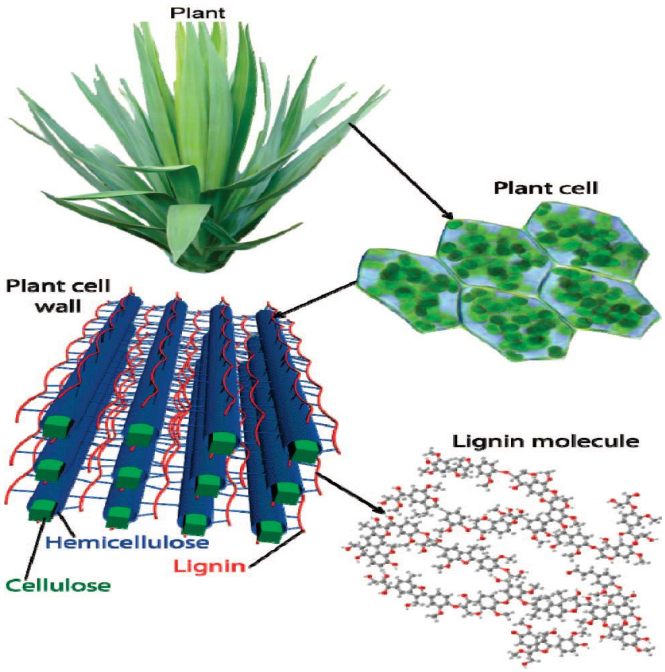
Scheme 1.2: A schematic diagram of lignocellulosic feedstock (LCF) biorefinery, taken from (Kamm & Kamm, 2004).

1.2.2 Lignin and its characteristics

1.2.2.1 Lignin

The name "lignin" comes from the Latin word "lignum", which means wood (Chakar & Ragauskas, 2004, Wong, 2009). It is a three dimensional natural polymer and is found as a cell-wall component in all vascular plants. Lignin coexists with cellulose in hardwoods and softwoods, and its content in wood stems varies between 15 % and 40 % depending on the source of the plant. Lignin acts as a "water channel" in stems and transports water through the cell wall (Kirk & Lynch, 1987, Hofrichter, 2002, Wout Boerjan, 2003). Furthermore, it also acts as a "permanent glue" which holds the whole carbohydrate polymers in the wood stem, which makes the wood stronger. In addition, lignin defends the plant from attackers such as microorganisms and insects (Lewis & Yamamoto, 1990, Collinson & Thielemans, 2010, Zakzeski *et al.*, 2010, Lange *et al.*, 2013). Scheme 1.3 describes a schematic representation of lignin in biomass, emphasising its location and structure. As familiarised earlier, lignin is also a waste product from biorefineries and agriculture in which it could

become a potential source of sustainable aromatic compounds applied for many industrial processes (Novaes *et al.*, Pandey & Kim, Amen-Chen *et al.*, 2001, Grabber, 2005, Novaes *et al.*, 2010, Zakzeski *et al.*, 2010, Pandey & Kim, 2011, Lange *et al.*, 2013).



Scheme 1.3: Schematic representation of the location and structure of lignin in lignocellulosic material, taken from (Zakzeski *et al.*, 2010).

1.2.2.2 Chemistry of lignin

Lignin originates from three major components of phenylpropane units. They are also referred to as monolignols (Wout Boerjan, 2003, Davin & Lewis, 2005). These units are coniferyl, sinapyl, and *p*-coumaryl alcohol. Their structures are shown in Figure1.1.

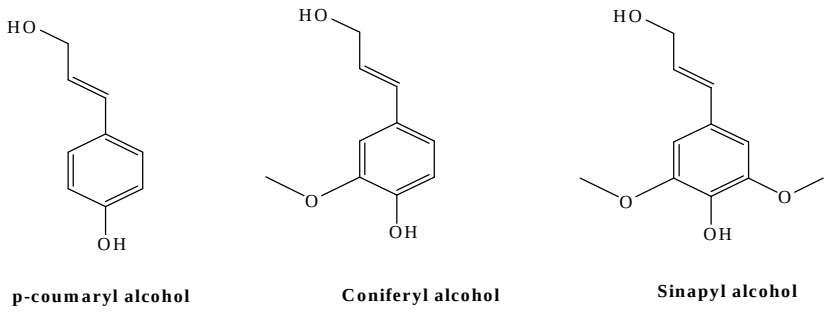


Figure 1.1: The three major components of lignin (Whetten *et al.*, 1998, Wong, 2009)

Lignin is a very complicated and stable polyaromatic compound by nature. The schematic representations of softwood, hardwood lignin and the major linkages maintaining their structures are shown in Figure 1.2 and Figure 1.3 to explain their chemical and structural characteristics.

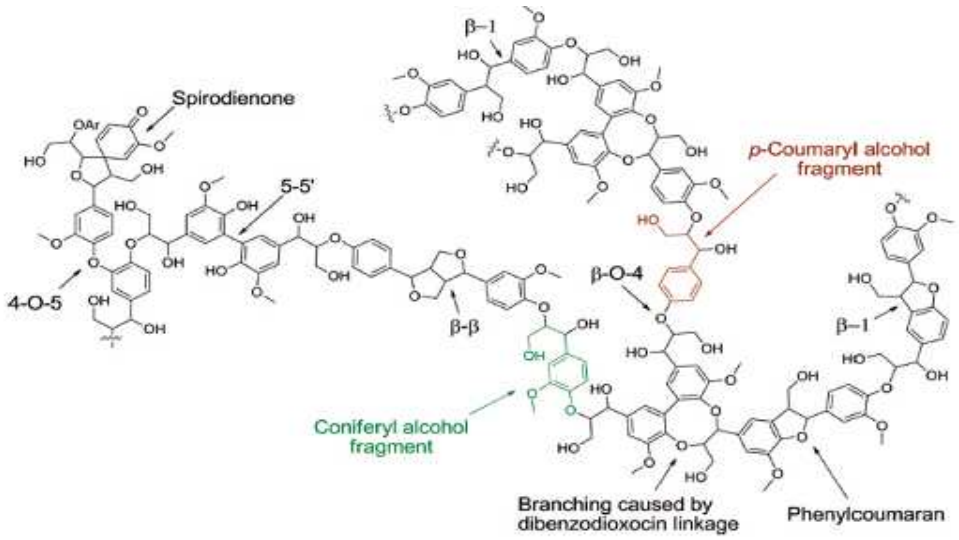


Figure 1.2: Schematic representation of a soft wood lignin (taken from (Zakzeski *et al.*, 2010)

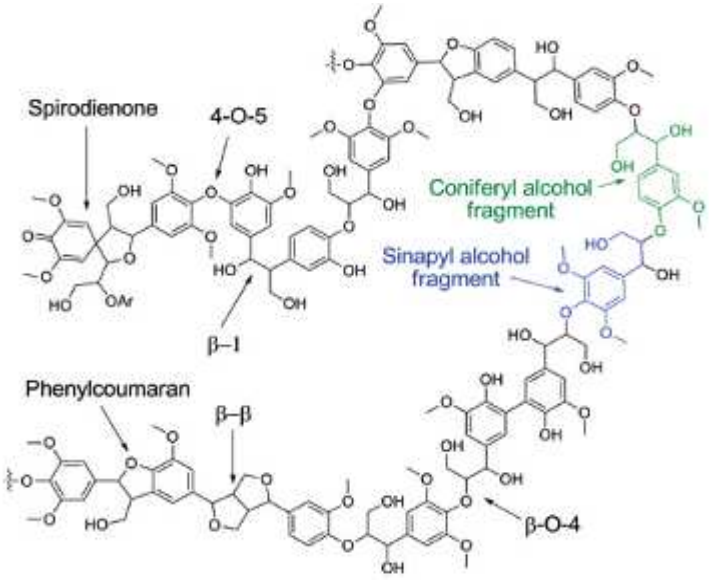


Figure 1.3: Schematic representation of a hard wood lignin (taken from (Zakzeski *et al.*, 2010)

Although lignin is a complex polymer, but there are some common linkages within its structure that allow us to comprehend one of the key features in its formation. These linkages are β-*O*-4 (phenylpropane β-aryl ether), α-*O*-4 (phenylpropane β-aryl ether), 5-5 (biphenyl

and dibenzodioxocin), β-5 (phenylcoumaran), 4-*O*-5 (diaryl ether), β–β (dibenzodioxocin) linkage, and β-1 (1,2- diaryl propane). Among them, the β-*O*-4 linkage is dominant and consists of more than half of the linkages in all types of native lignin (approximately 46 % of softwood and 60 % of the hardwood) structures (Guerra *et al.*, 2006, Lange *et al.*, 2013) (Dorrestijn *et al.*, 2000, Munk *et al.*, 2015). The chemical structures of these main linkages in lignin are shown in Figure 1.4.

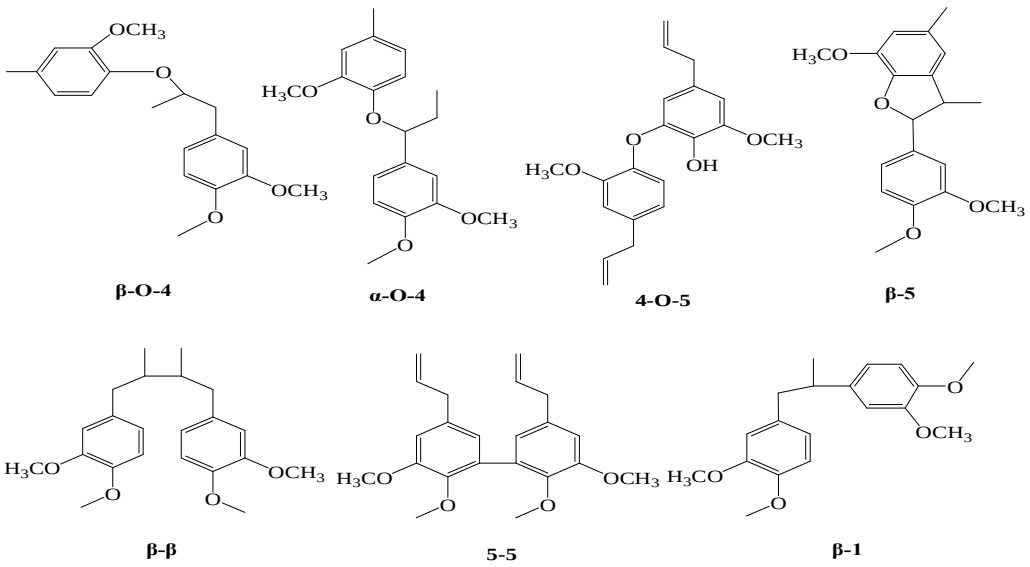


Figure 1.4: Common linkages between phenylpropane units in lignin (Adapted from (Karhunen, 1995, Chakar & Ragauskas, 2004, Lange *et al.*, 2013))

Usually, lignin from hard wood species is more resistant to degradation than soft wood because it contains a higher methoxyl content due to the presence of guaiacyl and syringyl (sinapyl) units. Softwood has a lower methoxyl content as it contains only guaiacyl (coniferyl) units (Ralph *et al.*, 2004, Liu *et al.*, 2008, Wong, 2009).

1.2.2.3 Industrial preparation of different types of lignin

Industrial lignin refers to the lignin that is produced as a by-product from chemical pulping, as well as other forms of biomass-based processes, for example, biorefineries. Chemical pulping is a way of removing lignin from wood (delignification) by chemically modifying it to produce soluble parts of the polymer (Biermann, 1996, Lora & Glasser, 2002).

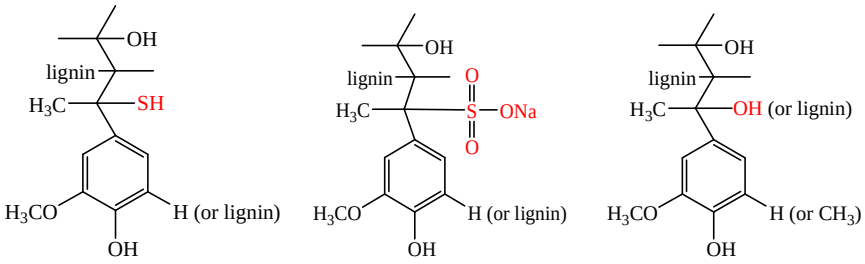
As previously mentioned, lignin is generated from both soft wood and hard wood species.

The sulphur-containing lignin is mainly produced from soft wood. It includes Kraft lignin (lignin alkali with low sulphur content) and lignosulphonates (lignosulphonic acid sodium salt, higher sulphur content), which have been available for many years. Lignosulphonate can be found in large quantities (around 1 million tonnes of solids per year), and Kraft lignin is found in more moderate quantities (around 100,000 tonnes of solids per year) (Boeriu *et al.*, 2004). Lignosulphonate is the sulphonated lignin that is the removed components from wood by sulphite pulping. Hardwood lignosulphonate and softwood lignosulphonate are obtained from waste pulping liquor concentrate by the Howard process (Nunn *et al.*, 1985) after removing and recovering of the sulphur. The molecular weights of their monomers are about 188 Da, and 215–154 Da, respectively. Lignosulphonate can be dissolved using acidic and basic aqueous solutions, as well as some highly polar organic solvents. However, hydrolysis reactions including excessive sulphonations may happen during its production (Lange *et al.*, 2013).

As for Kraft lignin, wood chips are treated with NaOH and Na_2S at high temperatures, and the Kraft lignin is a brown insoluble material that can be collected as a residue from the "black liquor" using a pH-controlled method in the chemical pulping process. It is structurally highly modified, as approximately 70 % to 75 % of the hydroxyl groups become sulphonated during the standard Kraft pulping procedures. Degradation results in an estimated average monomer molecular weight of 180 Da (Lange *et al.*, 2013). Therefore, Kraft lignin can be dissolved in alkali, basic solutions and highly polar organic solvents. It stated that up to 98 % of chemical pulp production in the United States and 92 % in the world actually comes from the Kraft pulping (Ragauskas, Sixta, 2006), which increased its popularity. Kraft lignin and lignosulphonate and their behaviours and characteristics significantly differ from natural lignin because they go through severe modifications during pulping process.

There are some other non-sulfur containing lignins that can be obtained from many different treatments applied in industry. They are soda lignin, Organosolv lignin, steam explosion lignin, hydrolysis lignin and oxygen delignification lignin. These non-sulphur types of lignin are primarily produced by biofuel production, and most of them are not yet commercially available (El Mansouri & Salvadó, 2006). Among these non-sulfer lignins, the organosolv lignin is mainly obtained by treating the wood parts with organic solvents during wood pulping (Johansson *et al.*, 1987, Pye & Lora, 1991). Therefore, it has a less degraded structure than others (e.g. lignosulphonates) and can be easily separated from the pulping

solvents either by solvent elimination or by precipitating organosolv with water, which is then followed by distillation to recycle the organic solvents. The components after solvent removal can be used as a feedstock for further treatment in order to supply many useful intermediates to be used in chemical and pharmaceutical industries (Johansson *et al.*, 1987). Most Organosolv lignin is not soluble in acidic aqueous solutions, but can be dissolved in basic solutions and in many polar organic solvents. Its is estimated that the monomer molecular weight is around 188 Da (Lange *et al.*, 2013). Figure 1.5 shows the basic structural units of commercialised Kraft lignin, lignosulphonate and the Organosolv lignin from Sigma-Aldrich (UK) as examples:



Kraft lignin Lignosulphonate Organosolv lignin

Figure 1.5: Basic structures of Kraft lignin, lignosulphonate and Organosolv lignin (adapted from http://www.sigmaaldrich.com/catalog/product/aldrich)

1.2.3 Common lignin degradation methodologies

It is well known that lignin is a polyphenolic organic complex, and the different chemical bonds in its cross linked network are difficult to break down by natural processes. Several conventional lignin depolymerisation methodologies generalised in the literature are pyrolysis, hydrogenation and the selective oxidation (Dorrestijn *et al.*, 2000, Pandey & Kim, 2011, Lange *et al.*, 2013).

1.2.3.1 Pyrolysis

Pyrolysis uses the effect of heating at high temperature in a controlled atmosphere (Barton, 1979, McKendry, 2002), such as argon gas and, in some cases, under a vacuum (Britt *et al.*, 1999) to break the bonds within the lignin molecules to produce liquid or gas. This produces free radicals, which are very reactive, and thus they will go on to react further with the remaining molecules, breaking the lignin into smaller fragments. For example, the pyrolytic

degradation of lignin occurs at 200 °C to 400 °C, and the ether linkages within the lignin are broken to yield small amounts of guaiacols (Dorrestijn *et al.*, 2000). However, in this process, char is formed when increasing the temperature to improve catechol production. Also, large proportions of hydrogen, carbon monoxide and carbon dioxide are formed as gaseous products (Dorrestijn *et al.*, 2000, Álvarez *et al.*, 2005, Blazsó, 2005). Hence, pyrolysis may bring about some rapid extraction of chemicals from the lignin, but the process is very poor in selectivity and yield, and energy intensive.

1.2.3.2 Catalytic hydrogenation

Catalytic hydrogenation is a method usually applied by dissolving the lignin in an organic solvent under a hydrogen atmosphere. It is reported that the possible products from this method include phenol, alkyl substituted phenols, catechols, guaiacols and aldehydes (Dorrestijn *et al.*, 2000). Char is also produced and at higher temperature the amount of char increases, and benzene, toluene, and xylene (BTX) form as products. The maximum yields of monophenols obtained from such process is around 30 wt% which can only be achieved by using expensive catalysts including metals such as Mo/Pd/Ru/Co/Ni *etc*. in the presence of expensive organic solvents (e.g. dioxane and phenols) at temperatures between 300 °C and 500 °C (Dorrestijn *et al.*, 2000, Pandey & Kim, 2011). Therefore, catalytic hydrogenation is a poor method for obtaining chemicals from lignin as the product distribution is difficult to control and it has poor yields. This method is often not an economic process due to the high expense of the solvents and catalysts.

1.2.3.3 Selective oxidation

Selective oxidation has also been used to obtain useful chemicals from lignin. For example, oxidation using basic conditions produces aromatic aldehydes with yields of up to 50 wt% (Dorrestijn *et al.*, 2000, Martínez *et al.*, 2010). The most important parameter in the oxidation of lignin to produce chemicals is to find a selective oxidant, of which nitrobenzene is the most selective. However, separating it from the products is difficult. So, transition metal oxides such as CuO and Fe_2O_3 are often used instead and show~~n~~ useful properties (Dorrestijn *et al.*, 2000).

1.2.3.4 The oxidative transformation of lignin using enzymes

Enzyme assisted oxidations are highly attractive because they can enhance and expand the range of standard methods used for traditional oxidation processes. Also, they replace the toxic oxidants that have been used to catalyse chemical oxidations, and avoid the formation of by-products that are harmful to the environment. Furthermore, enzymatic reactions play a significant role in organic synthesis as they allow many oxidation and other reactions to be performed in a selective and efficient manner. The enzymes that can participate in oxidation processes are dehydrogenases, oxidases, oxygenases, and peroxidases (Wong, 2009, Rodgers *et al.*, 2010, Hajdok *et al.*, 2011, Kudanga *et al.*, 2011, Lange *et al.*, 2013).

A considerable amount of literature has highlighted the biodegradation of lignin using oxidative lignolytic enzymes (Hofrichter, 2002, Higuchi, 2004, Riva, 2006, Grabber *et al.*, 2008, Hamid, 2009, Wong, 2009, Lange *et al.*, 2013, Singh & Singh, 2014), which have better properties compared to the conventional depolymerisation methods in terms of improving the overall efficiency of chemicals. This is because these enzymes can react selectively at certain linkage positions on the lignin structure and also facilitate the lignin depolymerisation under very mild conditions such as low temperature and pH, and also do not need expensive rare metal catalysts (Dashtban *et al.*, 2010, Pandey & Kim, 2011, Lange *et al.*, 2013, Mogharabi & Faramarzi, 2014). Therefore, lignolytic enzymes can be considered as better biocatalysts to convert lignin based waste materials into valuable phenolic aromatic chemicals compared to other biocatalysts.

1.3 Lignin degrading enzymes

1.3.1 Laccases and common mediators for lignin degradation

Laccase is potentially one of the most useful oxidases among the lignin oxidising enzymes. Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) was first identified in the 1880s as a protein-like substance that catalysed the lacquer curing process (Hatakka, 1994, Mayer & Staples, 2002). The name “laccase” was implemented in the 1890s with one of the defining reactions catalysed using this enzyme, in which it showed the ability to oxidise hydroquinone (Eriksson, 2009). Laccases are widely spread amongst the fungi and other varieties of plant species, in insects, and, also in a bacterium called *Azospirillum lipoferum* (Mayer & Staples, 2002, Riva, 2006, Munk *et al.*, 2015). These laccases participate in lignin biosynthesis, degradation of plant cell walls, plant pathogenicity, and so on (Baldrian, 2006, Rodriguez

Couto & Toca Herrera, 2006, Arora & Sharma, 2010).

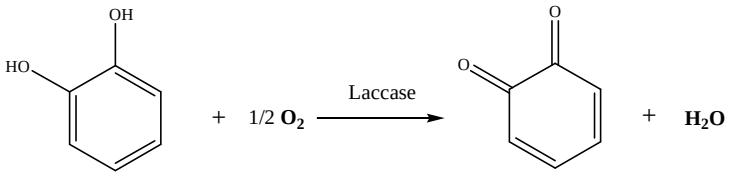


Figure 1.6: Reduction of molecular oxygen (O_2) to water (H_2O) by laccase (Mayer & Staples, 2002, Octavio *et al.*, 2006)

Laccase is one of the polyphenol oxidases that contain copper atoms in the catalytic centre. It catalyses the oxidation of a wide variety of substrates (mainly phenolic substrates) by one-electron oxidation and at the same time reduces the molecular oxygen to water by four-electron reduction (Figure 1.6) (Claus, Messerschmidt, 1998, Baldrian, 2006, Arora & Sharma, 2010, Singh & Singh, 2014). The structure of the laccase active-site and its catalytic cycle are shown in Figure 1.7 (a) and (b). There are four copper ions on the laccase active site: a mononuclear "blue" copper ion (T_1 site) and a tri-nuclear copper cluster (T_2/T_3 site), which includes one T_2 copper ion and two T_3 copper ions. During the oxidation by laccase, the T_1 site accepts electrons from reducing substrates, and these electrons are transferred onto the tri-nuclear T_2/T_3 cluster where the molecular oxygen is activated and reduced to H_2O (Claus, Leonowicz *et al.*, 2001, Riva, 2006, Kudanga *et al.*, 2011). The crystal structure of laccase has been studied by a few researchers (Hakulinen *et al.*, 2002, Piontek *et al.*, 2002, Rodgers *et al.*, 2010). The structure found in the study performed by Rodgers *et al*. is shown in Figure 1.7 (c). Compared to other lignolytic enzymes, laccase can be named as one of the most 'eco-friendly' enzymes because it is directly oxidised by air and it won't produce any toxic peroxide intermediates during its oxidation (Riva, 2006).

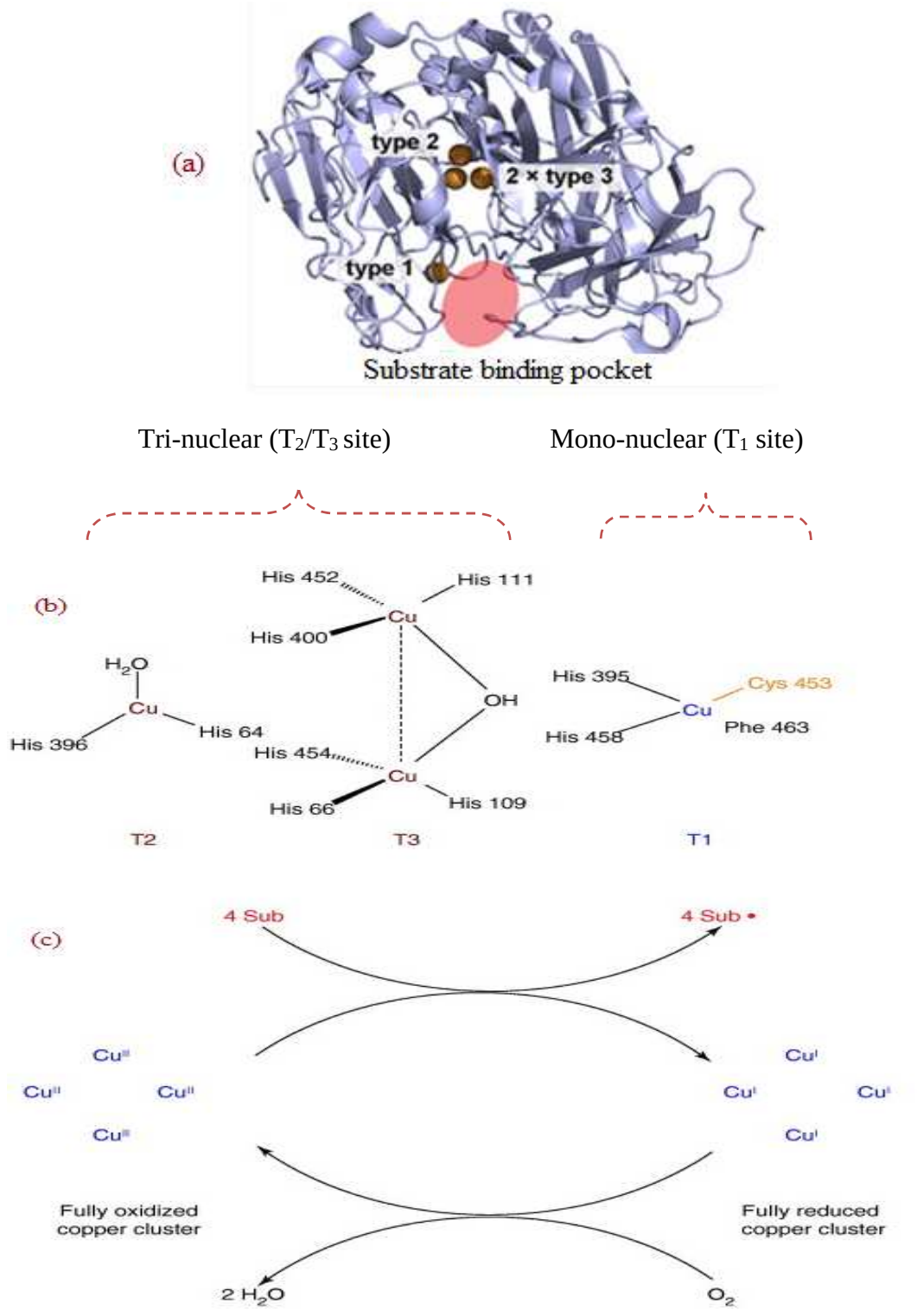


Figure 1.7: The active-site structure of laccase, the catalytic cycle, and the crystal structure: (a) A ribbon model of the X-ray crystal structure from T. *versicolor* Lac1 (PDB 1kya) with the coppers (orange circles) labelled by type and the organic substrate binding pocket highlighted in pink (Rodgers *et al.*, 2010). (b) Model of the catalytic cluster of the laccase from *Trametes versicolor* made of four copper atoms. (c) Schematic representation of a laccase catalytic cycle (Sub: substrate molecule; Sub•: oxidised substrate radicals)(Riva, 2006)

As to lignin, because of the large molecular size of laccase, it cannot enter the fibre secondary wall of plant cells to contact it directly (molecular weight, around 70,000) (Bourbonnais *et al.*, 1997). This is also because of its lowest redox potential (0.5 V to 0.8 V) among lignolytic enzymes (Cañas & Camarero, 2010, Munk *et al.*, 2015). Nevertheless, the presence of a redox mediator is an advantage during the laccase catalysed depolymerisation and mineralisation of lignin (Bourbonnais *et al.*, 1995, Johannes & Majcherczyk, 2000, Elegir *et al.*, 2005, Morozova *et al.*, 2007). In fact, there are around 80 % to 90 % of non-phenolic parts in the lignin structure, and therefore, it seems particularly difficult to break down the lignin using laccase alone (Bao *et al.*, 1994, Bourbonnais *et al.*, 1998, Wong, 2009, Munk *et al.*, 2015). So, by using a suitable mediator it can be possible for laccase to contact with lignin and achieve the successful depolymerisation of lignin. The laccase-catalysed lignin degradation in the presence of ABTS, a most commonly used mediator, is given as an example in Figure 1.8 to demonstrate such a transformation.

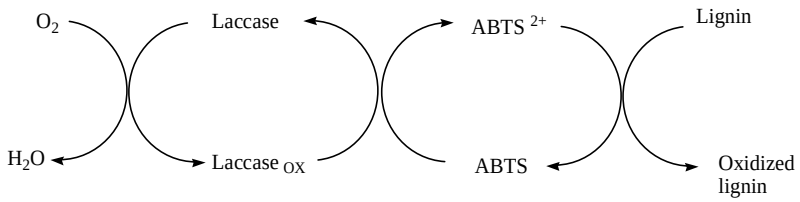
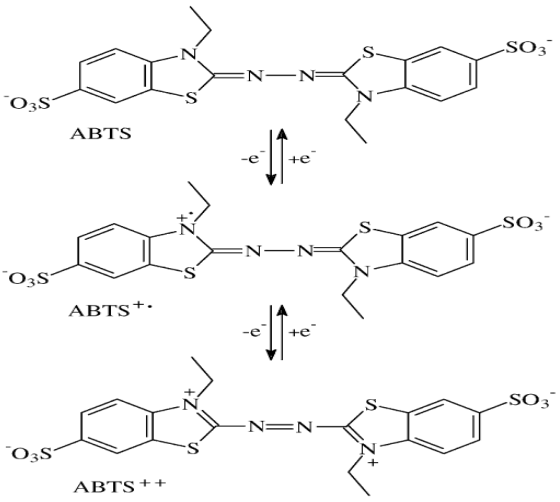


Figure 1.8: Schematic representation of lignin degradation by laccase in the presence of a mediator, ABTS

The oxidative mediation of laccase enzyme mainly takes place as follows. The laccase is oxidised by a dioxygen (O_2) molecule, which is the stoichiometric oxidant of the reaction, and is converted into water. The oxidised laccase then promotes the oxidation of the mediator (ABTS) and is returned to its original form. The oxidised mediator ($ABTS^{2+}$) then oxidises the targeted substrate (lignin) and reduces it to its original form to complete the whole oxidation cycle (Bourbonnais *et al.*, 1998, Riva, 2006).

ABTS was found to be the very first synthetic mediator and the best substrate for laccase (Bourbonnais & Paice, 1992, Cañas & Camarero, 2010). It fits the term of "redox mediator" very well in which ABTS speeds up the reaction rate by shuttling electrons from the substrate (compounds to be oxidised) of the primary electron donors to the electron accepting compounds (Bourbonnais & Paice, 1990). The initial oxidation of ABTS involves two stages (Scheme 1.4). In the first stage, the $ABTS^{+\bullet}$ cation radical is formed by fast oxidation

followed by the formation of the ABTS $^{2+}$ dication in the slow oxidation mode of the cation radical (ABTS $^{+\bullet}$) (Bourbonnais & Paice, 1990, Riva, 2006).



Scheme 1.4: Oxidation of ABTS in the presence of laccase (Majcherczyk *et al.*, 1999, Fabbrini *et al.*, 2002, Morozova *et al.*, 2007)

Most of the laccase mediators are aromatic compounds, which are known to be phenolic fragments of lignin (Bourbonnais *et al.*, 1997). There are more than 100 types of mediators that have been identified so far (Canas & Camarero, 2010). They include some natural mediators, such as acetovanillone, syringaldehyde, vanillic acid, guaiacol, which are phenolic fragments of lignin and therefore can be classified as lignin model compounds, and some more synthetic mediators including 1-hydroxybenzotriazole (HBT), violuric acid (VIO), and N-hydroxyacetanilide (NHA) and so on (Johannes & Majcherczyk, 2000, Camarero *et al.*, 2005, Riva, 2006). The structures of some common laccase mediators and lignin model compounds (natural mediators) are shown in Figure 1.9.

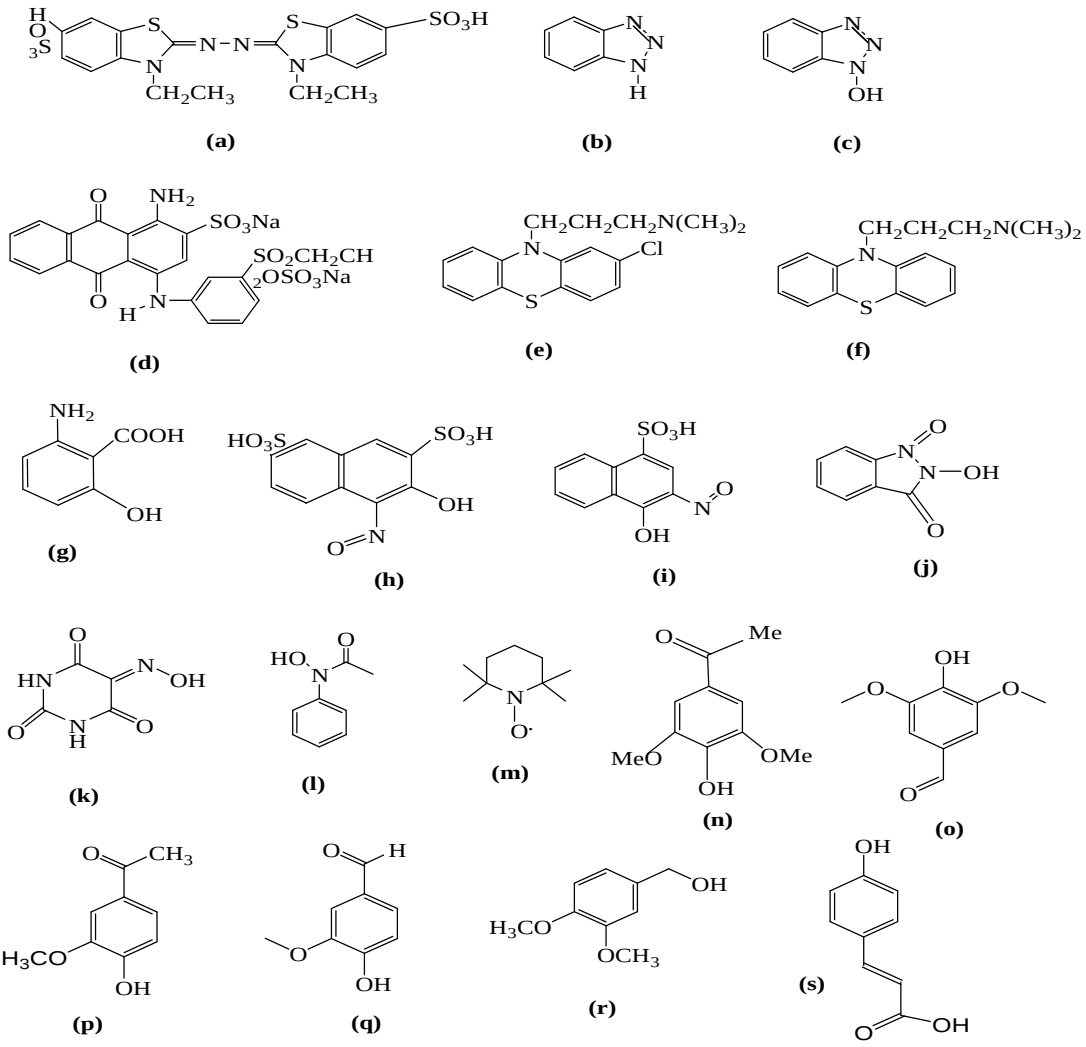


Figure.1.9: The chemical structures of some natural and synthetic mediators and the lignin model compounds which also can be worked as laccase mediators:

(a) 2,2'-azino-bis (3-ethylbenthiazoline-6-sulphonic acid, ABTS); (b) benzotriazole; (c) 1-hydroxybenzotriazole (HBT); (d) remazol brilliant blue; (e) chlorpromazine; (f) promazine; (g) 1-nitroso-2-naphthol-3,6-disulphonic acid; (h) 2-nitroso-1-naphthol-4-sulphonic acid; (i) 3-Hydroxyanthranilic acid (HAA); (j) N-hydroxyphtaimide (HPI); (k) violuric acid (VLA); (l) N-hydroxyacetanilide (NHA); (m) 2,2,6,6-tetramethylpiperidine-1-yloxy (TEMPO); Lignin models: (n) acetosyringone; (o) Syringaldehyde; (p) vanillin; (q) acetovanillone; (r) veratryl alcohol; (s) p-coumaric acid (Bourbonnais *et al.*, 1997, Riva, 2006, Cañas & Camarero, 2010)

1.3.2 Lignin peroxidase

Lignin peroxidase (ligninase or diarylpropane peroxidase, EC 1.11.1.14, LiP) is a lignin-modifying enzyme and belongs to the heme-containing peroxidases. It was first discovered in 1983 in ligninolytic cultures of *Phanaerochete chrysosporium* (Eriksson, 2009, Wong, 2009). It likely has several different forms and they are known to also occur in a number of other white-rot fungi.

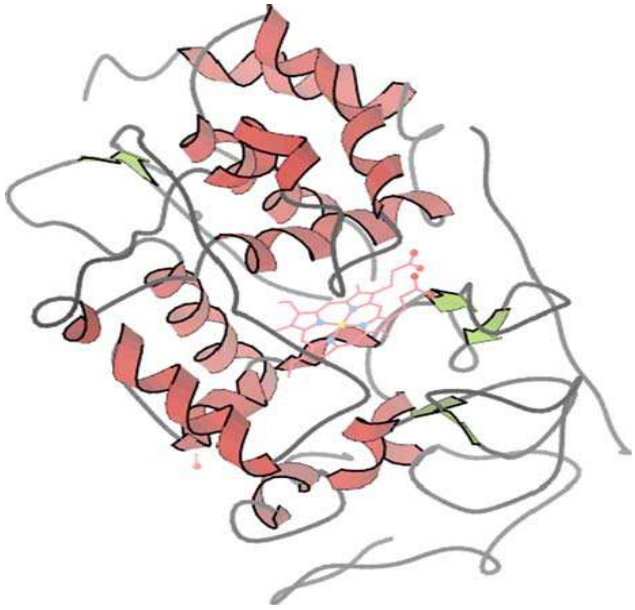


Figure 1.10: The structure of *Phanaerochete chrysosporium* lignin peroxidase (LiP) (Poulos *et al.*, 1993)

The LiP isozymes are glycoproteins that have a molecular weight (MW) of 38 kDa-46 kDa, with an isoelectric point (pI) value of 3.2 to 4.0 (Blodig *et al.*, 2001). LiP works under acidic environment as its pH optimum was reported as 3. The crystal structure of *Phanaerochete chrysosporium* lignin peroxidase (LiP) shown in Figure 1.10 has been determined (Edwards *et al.*, 1993, Choinowski *et al.*, 1999, Blodig *et al.*, 2001); LiP is not a substrate-specific enzyme and can oxidise both phenolic aromatic substrates and a variety of non-phenolic lignin model compounds too. Moreover, it also works on number of organic compounds which have higher redox potential (up to 1.4 V) with the addition of H_2O_2 (Valli *et al.*, 1990).

LiP oxidises the substrates by two continuous one-electron oxidation steps (Scheme 1.5). Firstly, it is oxidised by H_2O_2 to form compound-I (a two electron oxidised intermediate) and water. Then compound-I oxidises a substrate molecule (RH_2) by one electron reduction of compound-I, producing compound-II and oxidised substrate radical (RH•). Compound-II then uses another substrate molecule (RH_2), again by reducing one electron of substrate (RH_2) and returning LiP to its original form (Poulos *et al.*, 1993, Schick Zapanta & Tien, 1997). The oxidation mechanism of a substrate (RH_2) by LiP is shown in Scheme 1.5.

$$\text{Enzyme (LiP)} + H_2O_2 \longrightarrow \text{Compound I} + H_2O$$

$$\text{Compound I} + RH_2 \longrightarrow \text{Compound II} + RH\bullet$$

$$\text{Compound II} + RH_2 \longrightarrow \text{Enzyme (LiP)} + RH\bullet$$

Scheme 1.5: Two step oxidation of LiP (modified from (Wong, 2009))

1.3.3 Manganese peroxidase

Manganese peroxidase (MnP, EC1.11.1.13) is another type of heme-containing extracellular fungal peroxidase, and it was also identified in lignolytic cultures of *Phanerochaete chrysosporium*. MnP catalyses the conversion of Mn^{2+} to Mn^{3+} in the presence of H_2O_2 (Hofrichter, 2002, Wong, 2009). MnP performs slightly worse than LiP in the presence of acidic reaction environment. Its pI value is around 4.5 and the molecular weight is near to 46 KDa. MnP is produced as a series of isozymes often coded and differentially regulated by different genes (Pease & Tien, 1992).

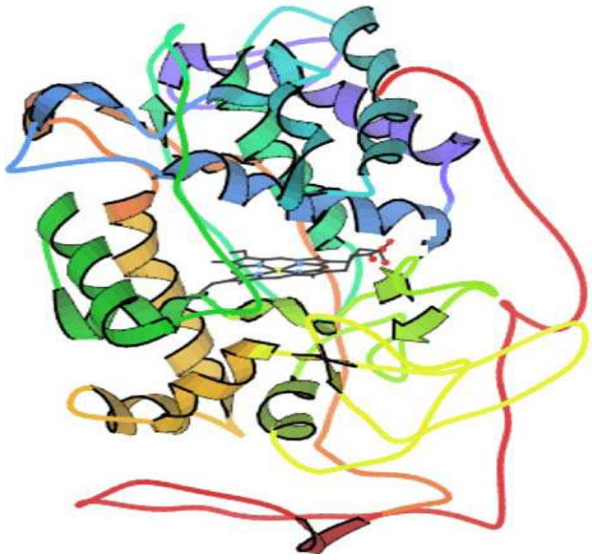


Figure 1.11: Three-dimensional structure of P. Chrysosporium manganese peroxidise (Sundaramoorthy *et al.*, 2005) (PDB1yyd)

The overall structure of MnP from *Phanerochaete chrysosporium* is similar to LiP, which composed of two domains with the heme located between them (Wong, 2009). The 3D structure of MnP is shown in Figure 1.13. MnP and LiP share similar mechanism. The only difference is that MnP uses Mn^{2+} to transform the compound I to compound II without depending on another reducing substrate. Then, returning to its native ferric form, the enzyme

has to oxidise another Mn (II). Therefore, MnP oxidises Mn^{2+} to Mn^{3+} using H_2O_2, which then oxidises phenolic compounds such as lignins, phenols, phenolic lignin model compounds, and high molecular weight chlorolignins (Eriksson, 2009, Lange *et al.*, 2013). H_2O_2 is generated using fungal oxidases such as glucose oxidases, glyoxal oxidases, aryl alcohol oxidases, and methanol oxidase and so on (Eriksson, 2009).

$$MnP + H_2O_2 \longrightarrow MnP\text{-}I + H_2O$$

$$MnP\text{-}I + Mn^{2+} \longrightarrow MnP\text{-}II + Mn^{3+}$$

$$MnP\text{-}II + Mn^{2+} \longrightarrow MnP + Mn^{3+} + H_2O$$

Mn^{3+} in turn mediates the oxidation of organic substrates.

$$Mn^{3+} + RH \longrightarrow Mn^{2+} + R + H^{+}$$

Scheme 1.6: Two-step oxidation of MnP

1.3.4 Versatile Peroxidase

It has been reported that some white rot fungi (*Pleurotus* and *Bjerkandera* species) produce hybrid (versatile) peroxidases (VP) that exhibit the characteristics of both LiP and MnP so that they participate in the oxidation of both phenolic and non-phenolic lignin structures (Camarero *et al.*, 1999, Ruiz-Dueñas *et al.*, 2009). This group of enzymes is not only specific for Mn^{2+} as for MnP, but also oxidises phenolic and non-phenolic substrates as for LiP. For example, veratryl alcohol, methoxybenzenes, and lignin model compounds can be oxidised in the absence of manganese. Moreover, the oxidation mechanism of VP is very like that of LiP and other heme peroxidases. The enzyme VP catalyses electron transfer from an oxidisable substrate and this also includes the formation and reduction of two intermediate compounds (Niladevi, 2009, Wong, 2009, Lange *et al.*, 2013). The molecular structure of VP is closer to LiP than to MnP (Heinfling *et al.*, 1998, Ruiz-Dueñas *et al.*, 1999, Moreira *et al.*, 2005, Ruiz-Dueñas *et al.*, 2006). The three-dimensional structure of P. *eryngii* versatile peroxidase is shown in Figure 1.12 as an example.

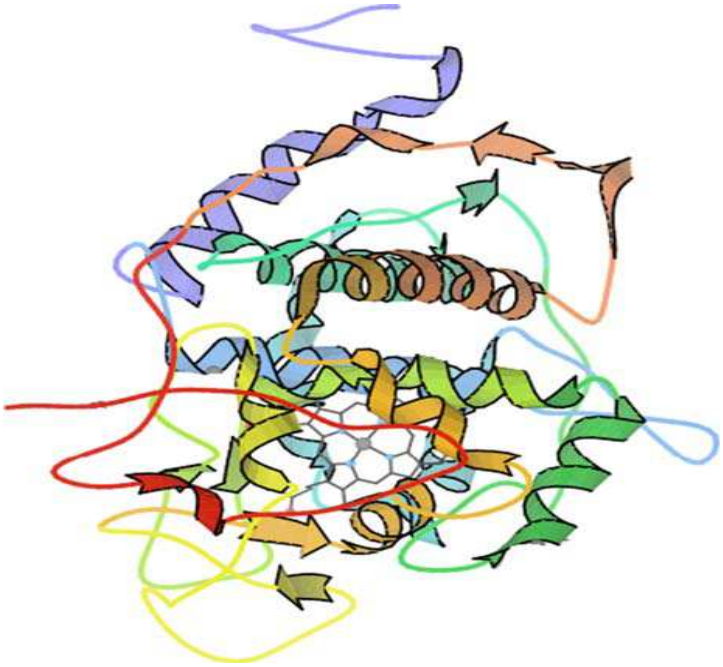


Figure 1.12: Three-dimensional structure of Pleurotus eryngii versatile peroxidase (Wong, 2009)

In summary, these heme-containing peroxidases (LiP, MnP and VP) are able to oxidise a variety of phenolic and non-phenolic lignin model compounds as well as lignin without using mediators because of their relatively high redox potentials (Valli *et al.*, 1990, McEldoon *et al.*, 1995). For example, LiP has the highest redox potential and can oxidise substrates which have similar potential between 0.81 V and 1.49 V, whereas MnP and horseradish peroxidase (HRP) have lower redox potentials and therefore they can oxidise relatively less strong substrates (E'_o= 0.81 V to 1.12 V) (Tuor *et al.*, 1992). Predominantly laccase can oxidise the phenolic substrates as it has the lowest redox potential among them (E'_o= 0.5 V to 0.80 V)(Call & Mücke, 1997). However, it can oxidise non-phenolic substrates too in the presence of a suitable mediator. Therefore, by comparison, the oxidation ability of these enzymes decreases in this order: LiP> MnP> laccase.

However, peroxidases have several disadvantages compared with Cu-containing laccases. First of all, these peroxidases use H_2O_2 to activate the catalytic cycle, whereas laccases only require O_2 in the atmosphere for the same purpose. Thus, the laccase-catalysed oxidation process is more likely a safer proposition compared to H_2O_2-assisted enzyme activation as some of the possible peroxide intermediates may occur when H_2O_2 is used in the reactions. Secondly, all of these lignolytic enzymes are commercially available except versatile peroxidase, but, laccase is the cheapest among them, and a very well-studied enzyme compared to the others. Moreover, enzyme stability and activity of laccases are greater than

those of peroxidases. Therefore, laccase has attracted a higher degree of attention compared to the other alternatives in the relevant research and development work. Lastly, the broad range of available mediators has extended the oxidation potential of laccase, and has enabled laccase to interact with both phenolic and non-phenolic substrates. Thereby, it has wider applications in different kinds of bio-transformations as well as in organic synthesis (Madhavi & Lele, 2009, Witayakran & Ragauskas, 2009, Rodgers *et al.*, 2010, Jeon & Chang, 2013) than peroxidases. In this project, all of these lignolytic enzymes will be investigated over both water-soluble and-soluble lignins in order to identify their role in lignin degradation and the product distribution.

1.3.5 The enzymatic transformation of lignin model compounds

The structural complexity and variability in the source of lignin has led to the use of some simpler, low molecular weight model compounds as a necessary preliminary study to understand ~~the~~ lignin depolymerisation. In fact, such lignin models are comprised of the phenolic or non-phenolic fragments of lignin containing only one or two of the main linkages of lignin. Even though these smaller compounds are not exact copies of the lignin complex network, and also have different levels of solubility from the native and industrial lignin. Understanding their interaction with lignin-degrading enzymes can certainly be helpful to improve and consolidate current knowledge concerning the complex nature of lignin degradation (Crestini & Argyropoulos, 1998, Rochefort *et al.*, 2004, Wong, 2009, Zakzeski *et al.*, 2010, Munk *et al.*, 2015). Also, the development of analytical methods to identify the products from less complicated model compounds will be much simpler and applicable to the analysis of actual lignin degradation products in the end. Therefore, a considerable amount of research has been focused on the study of lignin model compounds with the aim of understanding the formation and break down mechanism of the lignin and its product analysis (Bourbonnais & Paice, 1990, Bourbonnais *et al.*, 1997, Bourbonnais *et al.*, 1998, Crestini *et al.*, 2003, Zakzeski *et al.*, 2010, Pandey & Kim, 2011, Kosa & Ragauskas, 2012, Lange *et al.*, 2013). The following are some relevant examples extracted from the literature.

In 1997, R. Bourbonnais *et al.* studied the reactivities of several mediators and laccases with Kraft pulp and lignin model compounds. They found that the source of laccases affect their reactivity when they were tried to remove the lignin during Kraft pulping. The mediators tested alongside laccase in their study were 2,2’-azinobis (3-ethylbenzthiazoline-6-sulphonate)

(ABTS) and 1-hydroxy- benzotriazole (HBT). Furthermore, they have investigated ~~such~~ laccase and the above mentioned mediators towards dimeric model compounds oxidation. Their results showed that ABTS and HBT do not share the similar mechanisms in the oxidation process. The same research group (Bourbonnais *et al.*, 1998) investigated the laccase reaction with veratryl alcohol as a small lignin model compound using electrochemical analysis. In this experiment, it was indicated that laccase can oxidise the non-phenolic lignin model compound veratryl alcohol in the presence of ABTS as a mediator. This was because the ABTS dication ($ABTS^{+\bullet}/ABTS^{2+}$) was the responsible intermediate during this process to transfer electrons from the laccase to the veratryl alcohol. In 2004, S. Kawai *et al*. conducted a study of the use of laccase towards a non-phenolic lignin model dimer, which called 1-(4-ethoxy-3-methoxyphenyl)-2-(2,6-dimethoxyphenoxy) ethanol. Luckily, a there were great number of depolymerisation products were obtained from that reaction in the presence of HBT as a mrdiator (Kawai *et al.*, 2004). Very recently, a fairly comprehensive review on the topic of most common transformation routes for lignin was published by H. Lange and Crestini *et al.* (2013). They compared the products obtained from the lignin depolymerisation and some phenolic lignin models which are oxidised by laccase and a laccase-HBT mediator system. Initially, they re-confirmed the work done earlier by Fabbrini M *et al*. (2012) and the findings by the R. Bourbonnais group, in which the oxidation with ABTS happens via an electron transfer (ET) process whereas the oxidation by HBT occurs via a hydrogen atom abstraction (HAA) process, and the latter one is possibly easier to control.

Many studies have attempted to explain the use of laccase in lignin degradation in the presence of ABTS (Bourbonnais & Paice, 1992, Bourbonnais *et al.*, 1995, Riva, 2006, Cañas & Camarero, 2010). Bourbonnais *et al*. (1995) conducted a study of the oxidation of softwood Kraft lignin by laccase from *Trametes versicolor* (LTV) during the electro-oxidation of lignin using the electrochemically generated $ABTS^{2+}$ dication. It was shown in their study that the laccase catalytic activity increased by the addition of ABTS as a mediator due to the fact that the degradation of non-phenolic lignin structures requires $ABTS^{2+}$ (Bourbonnais *et al.*, 1995, Bourbonnais *et al.*, 1998, Fabbrini *et al.*, 2002). Another two examples of laccase catalysed oxidation of phenolic and non-phenolic lignin model compounds are shown in Figure 1.13 (a) and (b), respectively.

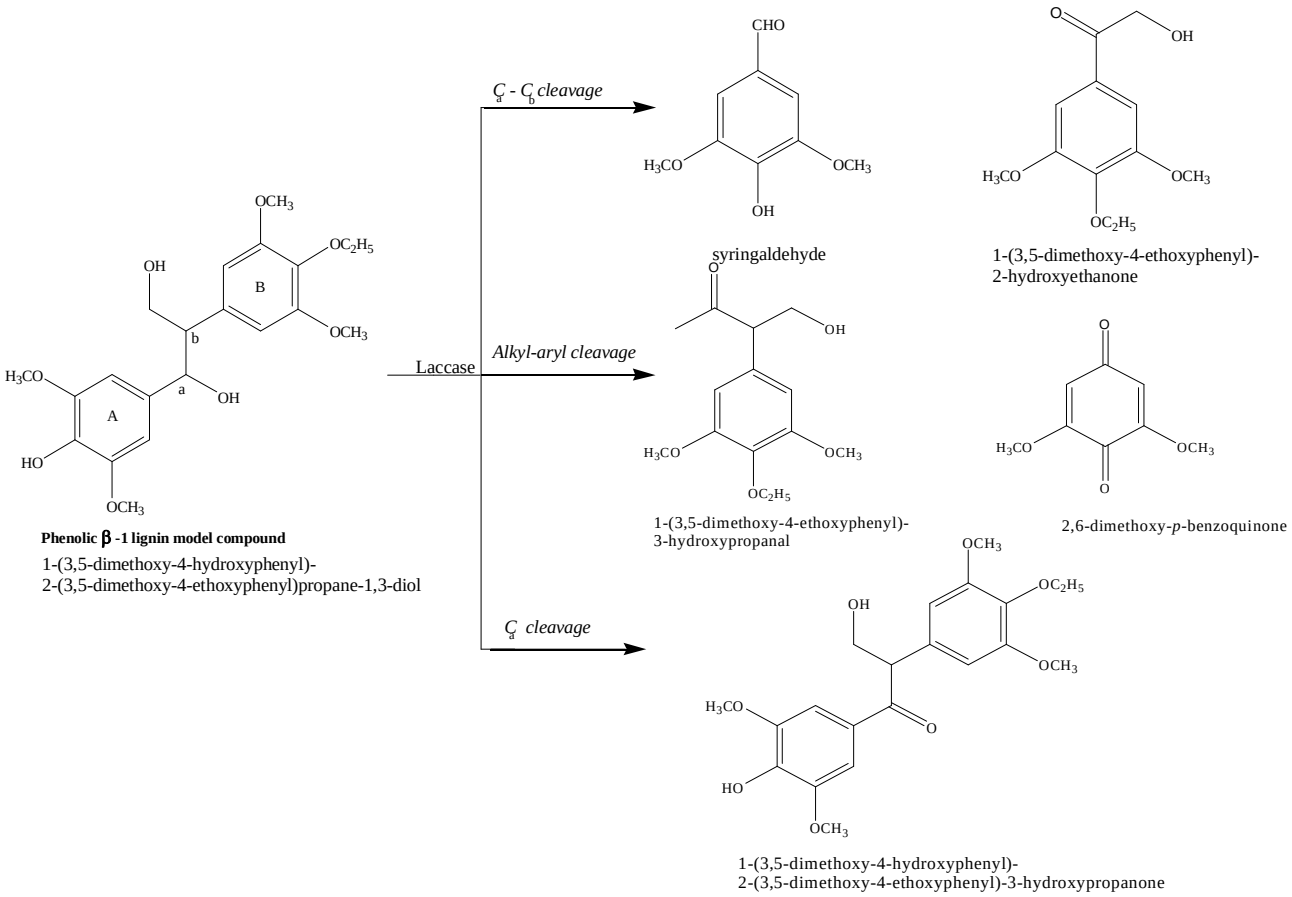


Figure 1.13 (a): Laccase-catalysed oxidation of a phenolic β-1 lignin model compound (Kawai *et al.*, 1988)

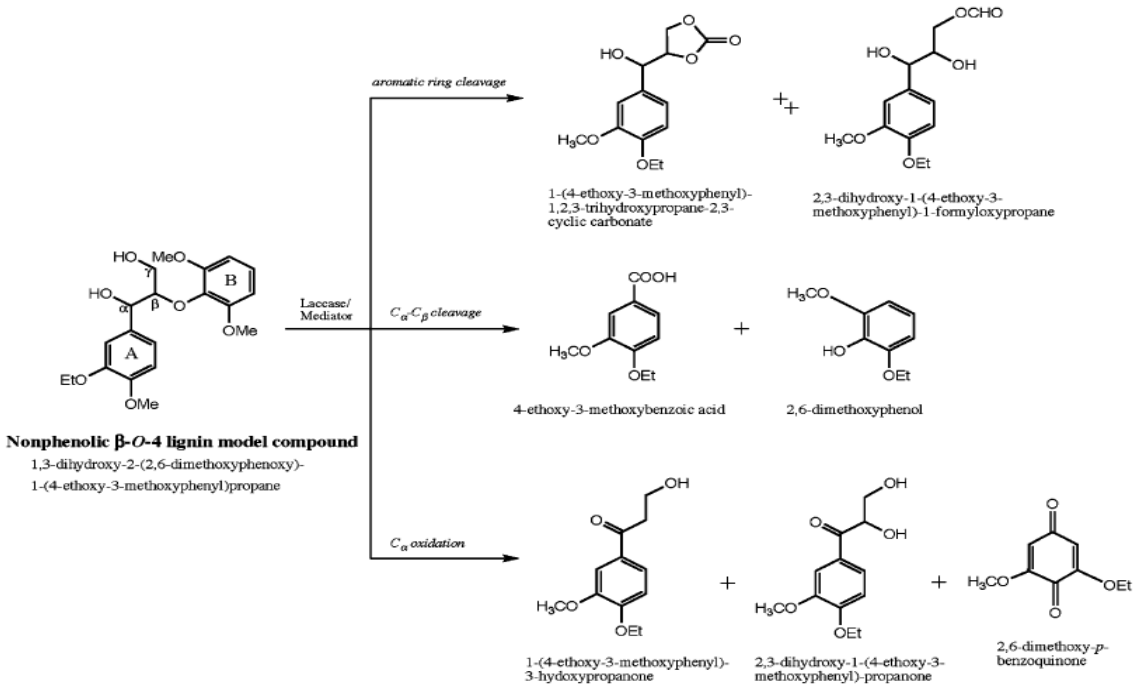


Figure 1.13 (b): Laccase-catalysed oxidation of a non-phenolic β-*O*-4 lignin model compound (Kawai *et al.*, 1999, Kawai *et al.*, 2002)

In addition, laccases can be used in many biotechnological processes with the assistance of those mediators, such as the detoxification of industrial liquid waste, mostly from the paper and pulp, textile, petrochemical industries, and food industries (Arora & Sharma, 2010, Brijwani *et al.*, 2010, Canas & Camarero, 2010, Pradeep & Sridhar, 2013). Also, they can remove xenobiotic substances and produce polymeric products too, and thereby can also be used in bioremediation (Baldrian, 2006, Octavio *et al.*, 2006, Riva, 2006, Rodríguez Couto, 2006, Morozova *et al.*, 2007, Arora & Sharma, 2010, Claus & Strong, 2010, Giardina *et al.*, 2010, Kudanga *et al.*, 2011, Rodriguez-Couto & Toca-Herrera, 2012, Mogharabi & Faramarzi, 2014).

Lignin peroxidase catalyses the H_2O_2-dependent oxidation of 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenyl)-1,3,dihydroxypropane, the non-phenolic lignin model compounds (Figure 1.14) in lignin depolymerisation by oxidative cleavage of *β*-O-4 ether bonds and C_α–C_β linkages (Bourbonnais & Paice, 1990, KERSTEN, 1990); the enzyme also catalyses the oxidation of aromatic C_α alcohols to $C_{\alpha\text{-oxo}}$ compounds, hydroxylation, quinone formation, and aromatic ring cleavage (Eksioglu *et al.*, 2009).

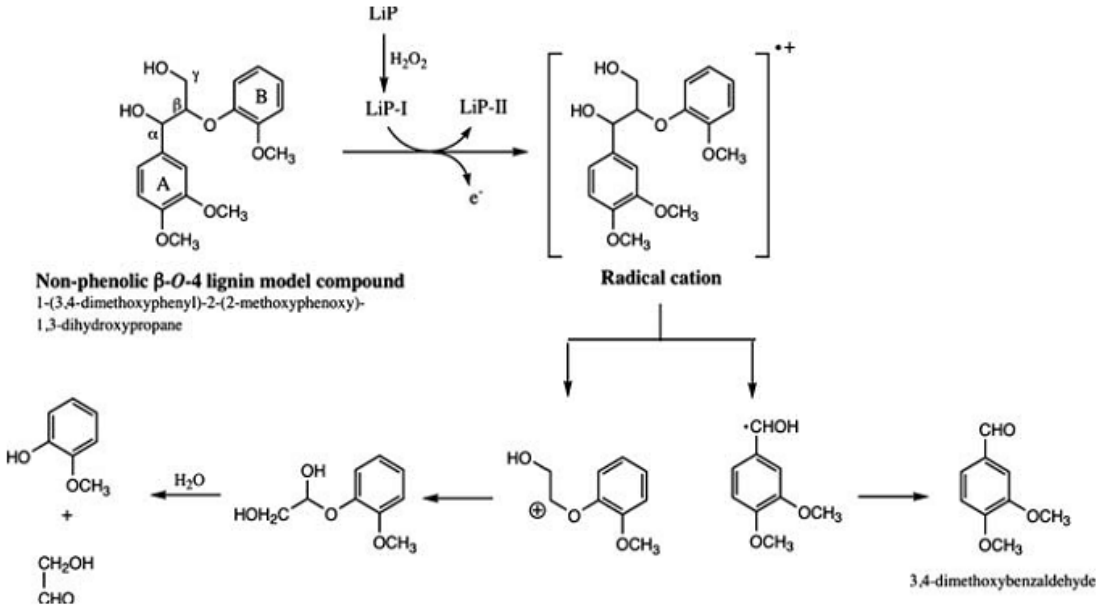


Figure 1.14: LiP-catalysed oxidation of non-phenolic β-O-4 lignin model compound (Wong, 2009)

Examples of oxidation the phenolic and non-phenolic lignin model compounds by MnP are given in Figure 1.15 (a) and Figure 1.15(b).

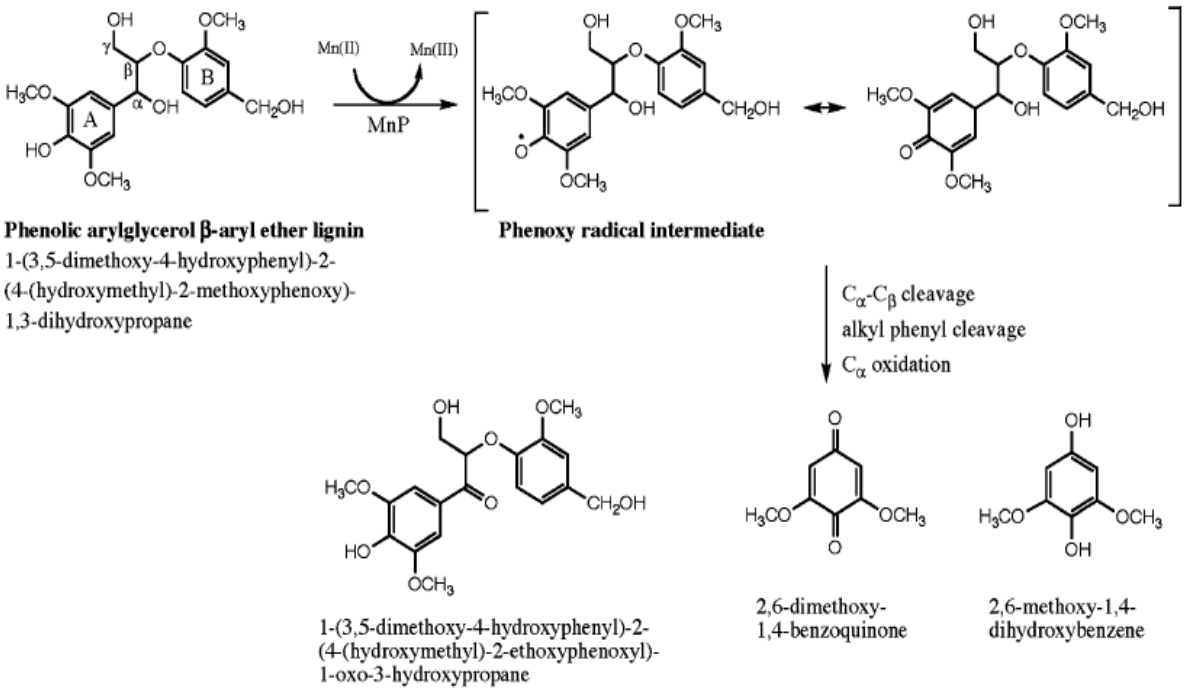


Figure 1.15(a): MnP-catalysed oxidation of phenolic arylglycerol β–aryl ether lignin model compound (taken from (Tuor *et al.*, 1992))

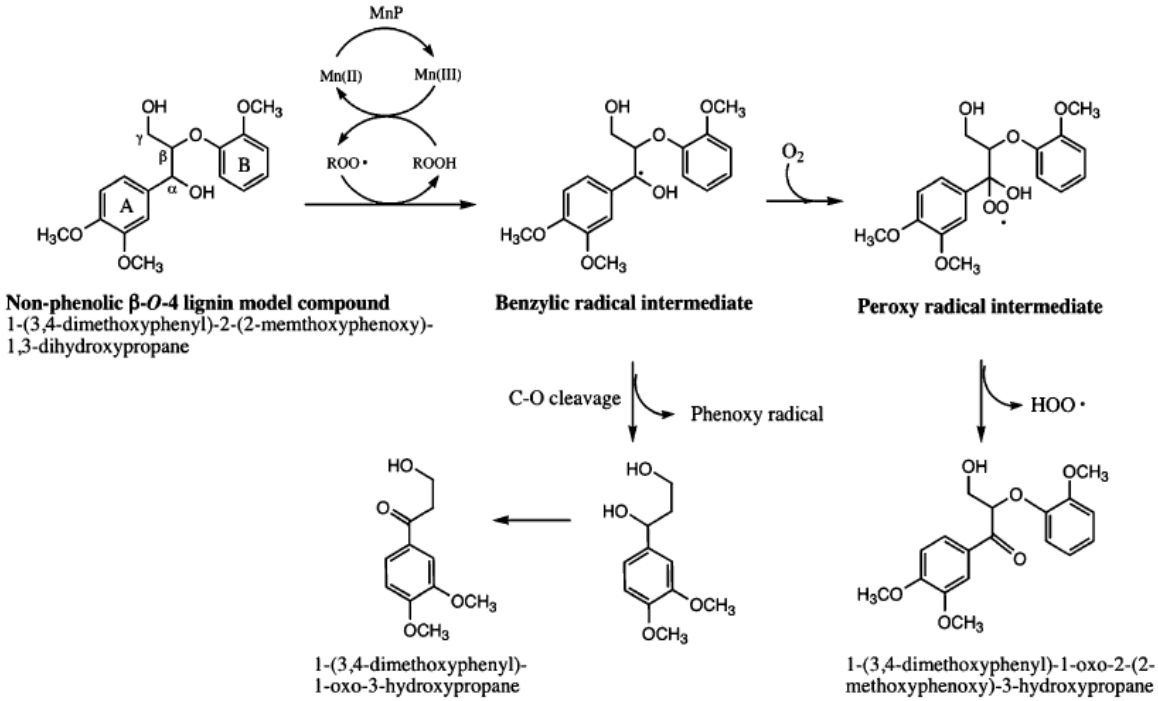


Figure 1.15 (b) MnP-catalysed oxidation of non-phenolic β-O-4 ether lignin model compound (taken from (Bao *et al.*, 1994, Kapich *et al.*, 2005))

Versatile peroxidase (VP) which was originated from *Pleurotus* bjerkandera able to oxidise Mn^{2+} and both phenolic and non-phenolic aromatic compounds (Heinfling *et al.*, 1998). For instance, VP oxidises Mn^{2+} to Mn^{3+}; degrades the non-phenolic lignin model veratryl glycerol-β-guaiacyl ether yielding veratryl aldehyde; and oxidises veratryl alcohol and p-dimethoxybenzene to veratryl aldehyde and *p*-dimethoxybenzoquinone, respectively in the same way as LiP oxidise them (Higuchi, 2004).

1.3.6 The enzymatic degradation of lignin

Native and industrially processed lignin has a much more complex structure, for instance, a higher molecular weight and lower solubility than the comparative lignin model compounds. Therefore, they may act differently during their reaction with enzymes. Enzymatic transformation of lignosulphonate could be performed in the presence of ligninolytic enzymes (Hatakka, 1994, Leonowicz *et al.*, 1999, Crestini *et al.*, 2003). As to the oxidative reactions of lignin model compounds using other members of lignolytic enzymes, numbers of studies were carried out. For example, lignin peroxidise (LiP) was used as catalysts to transform some phenolic model compounds (e.g. guaiacol, vanillyl alcohol, catechol, syringic acid, acetosyringone, *etc*.), lignin oligomers and some of the non-phenolic lignin model compounds (such as veratryl alcohol, diarylpropane lignin model dimmers *etc*.) (Banci *et al.*, 1999, Hofrichter, 2002, Wong, 2009).

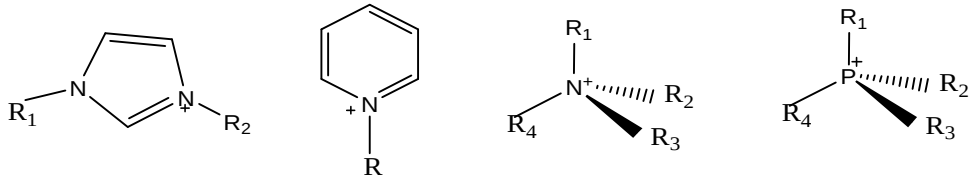
As more comprehensive example, Martinez *et al*. performed a study using all 3 types of lignolytic enzymes (fungal lignin peroxidase, manganese peroxidase and laccase) towards the oxidative degradation of alkali wheat straw lignin (Martinez-Inigo & Kurek, 1997). Furthermore, M. Hofrichter *et al.* demonstrated that milled pine wood was disassembled in the presence of MnP from *Phlebia radiate* (Hofrichter, 2002). Using a surfactant (Tween 80) in their reaction has greatly improved the pine wood depolymerisation process. Another piece of evidence that MnP can degrade lignin was when Forester *et al.* (1988) treated spruce ball-milled lignin with Mn^{3+}-pyrophosphate plus glutathione (GSH), and observed that the HPLC elution profile of the treated lignin was altered significantly towards more hydrophilic products that eluted earlier than the bulk of the lignin (Hofrichter, 2002). The results from Forester *et al* suggest that an increase in the phenolic structure of lignin may lead an increase the reactivity of MnP-catalysed reactions (Kantelinen *et al.*, 1993, Hofrichter, 2002).

1.3.7 Using Ionic liquids as solvents for lignin degradation

1.3.7.1 Ionic liquids and their properties

Although laccase catalysed lignin degradation can be enhanced by the addition of a suitable mediator, however, the heterogeneity of the water-insoluble Organosolv lignin reaction is still a major disadvantage which needs improvement. It is therefore desirable to find a solvent system which does not cause deactivation of laccase and also increases the solubility of Organosolv lignin in order to overcome the poor mass transfer between laccase and the water-insoluble Organosolv lignin. Being one of the major merits of green chemistry, Ionic liquids (ILs) possess some promising competence in contrast to the conventional organic solvents (Holbrey & Seddon, 1999, Welton, 2004, Hobbs & Thomas, 2007, Poliakoff & Licence, 2007).

Ionic liquids are salts composed of a large organic cation and an anion that can be either organic or inorganic. They are mostly liquid at room temperature (Holbrey & Seddon, 1999**,** Welton, 1999, Sheldon, 2001, Hallett & Welton, 2011). The structure of some most commonly used anions and commonly used cations is shown in Figure 1.16:



1 3- Dialkylimidazolium Alkylpyridinium Tetraalkylammonium Tetraalkylphosphonium

Figure 1.16 Examples of most common cation and anions in ionic liquids (Welton, 1999)

(Anoins: BF_4^-, PF_6^-, SbF_6^-, NO_3^-, $CF_3SO_3^-$, $(CF_3SO_3)_2N^-$, $ArSO_3^-$, $CF_3CO_2^-$, $CH_3CO_2^-$ and $Al_2Cl_7^-$)

Ethylammonium nitrate ($[EtNH_3][NO_3]$) was the first example of an ionic liquid which was first found by P. Walden and co-workers in 1914. It is liquid at room temperature (Earle Martyn & Seddon Kenneth, 2002). Room temperature ionic liquids (RTILs) exhibit certain properties which make them attractive media for performing green catalytic reactions. They are non-flammable, highly thermo stable, non-toxic and non-volatile (Welton, 1999, Chiappe & Pieraccini, 2005, Hallett & Welton, 2011), so they

can be used as green alternatives to traditional volatile organic compounds (VOCs). Ionic liquids have been employed in the place of organic solvents in many chemical and bio-chemical transformations (Gordon, 2001, Sheldon, 2001, Park & Kazlauskas, 2003, Zhao *et al.*, 2005, van Rantwijk & Sheldon, 2007), as they allow the processes to be performed under fairly mild conditions. This is due to their adjustable physical and chemical properties as compared to conventional organic solvents (Holbrey & Seddon, 1999, Blanchard & Brennecke, 2000, Angell *et al.*, 2007, Clark & Tavener, 2007, Fraser & MacFarlane, 2009). Therefore, ionic liquids (IL) with the above mentioned properties have attracted much attention in academia and in industry with the hope of eliminating or reducing hazards and waste materials, and in order to simplify or optimise the chemical and engineering processes (Anastas & Kirchhoff, 2002, Sheldon, 2005, El Seoud *et al.*, 2007, Plechkova & Seddon, 2008). Moreover, some task specific ILs have also displayed excellent physical characteristics including the ability to dissolve polar and non-polar organic, inorganic, and polymeric compounds (Lee, 2006). Such ILs have been honoured as 'designers solvents' due to their ion flexibility in structure which enables them to be tailored according to the needs of the researchers (Anastas & Kirchhoff, 2002, Pietro Tundo, 2007, Plechkova & Seddon, 2008). Interest in ionic liquids has grown steadily because of their potential applications as "green solvents" by providing the possibility of using them for clean manufacturing in the chemical industry (Gordon, 2001, van Rantwijk *et al.*, 2003, Sheldon, 2005, Zhao, 2006, Zhu, 2008, Hallett & Welton, 2011, Chatel & Rogers, 2014). Much effort has been extended to the design of ILs suitable for reactions that use enzymes as the catalyst, with particular attention to enzyme and substrate solubility, and enzyme activity and selectivity (Sheldon *et al.*, 2002, Park & Kazlauskas, 2003, Zhao, 2010, Naushad *et al.*, 2012, Kelley & Rogers, 2015). However, the selection of ionic liquids to be used in biotransformations, thanks to their catalytic activity, still needs experimental screening in the proposed reaction media, especially due to the fact that interactions between enzymes and ionic liquids have yet to be fully understood (Zhao, 2005, Shipovskov *et al.*, 2008, Tavares *et al.*, 2008, Wood & Stephens, 2010, Lars Rehmann, 2012, Hamidi, 2013, de Diego *et al.*, 2014).

On the other hand, it should be noted that not all ILs are actually 'green' or non-toxic (Stephens & Licence, 2011, Bubalo *et al.*, 2014). Number of studies have shown that ILs which contain anions such as Cl^-, HCO_2^-, $CH_3CO_2^-$, PF_6^-, BF_4^- are generally classed as not enzyme friendly as they generate toxic gases when reacted with water and these should be

taken into account when designing and performing biotransformation in ILs. The effect of cations is not as significant as anions when it comes to the toxicity of ILs towards enzymes (Zhao, 2005, Zhao, 2006, Zhao *et al.*, 2006, Zhao *et al.*, 2006, Arning *et al.*, 2008, Yang, 2009).

1.3.7.2 Ionic liquids as potential solvents for lignocellulosic biomass conversion

Rogers and co-workers discovered that several imidazolium based ILs can dissolve a large amount of cellulose (Moens & Khan, 2002, Swatloski *et al.*, 2002). This encouraged several other groups to test a variety of other ILs for a similar purpose (Barthel & Heinze, 2006, Zhao, 2008, Wang *et al.*, 2012). ILs have also been found able to dissolve lignin and lignocelluloses (Zhu, 2006, Fort *et al.*, 2007, Kilpelainen *et al.*, 2007, Mikkola *et al.*, 2007, Pinkert *et al.*, 2009, Zavrel *et al.*, 2009, Liu *et al.*, 2010, Mäki-Arvela *et al.*, 2010, Mora‐Pale *et al.*, 2011, Moniruzzaman & Ono, 2012, Chatel & Rogers, 2014). Simmons *et al.* (Trevor J. Simmons, 2009) reported that the ionic liquid (1-ethyl-3-methylimidazolium acetate, [Emim][CH_3COO]) -mediated the selective extraction of lignin from wood leading to enhanced enzymatic cellulose hydrolysis. Moniruzzaman and Ono proposed an enzymatic delignification of wood biomass in an aqueous system using RTILs as a co-solvent (Moniruzzaman & Ono, 2012).

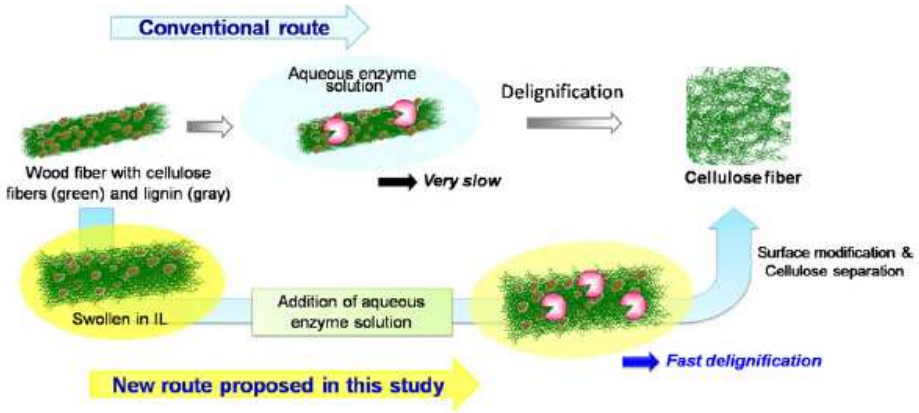


Figure 1.17: Comparison of enzymatic delignification versus conventional delignification of wood (taken from (Moniruzzaman & Ono, 2012)).

They proved that the substrate and product solubility increased in the presence of ILs and therefore the enzyme has potentially easier access to IL swollen wood cells (Figure 1.17).

However, most of these researchers focused on the delignification of lignocellulosic biomass in order to achieve high purity cellulose fibres from wood and therefore lignin was not their main interest. More recently, because of the ability of dissolving lignin and extracting it from lignocellulosic biomass, the popularity of ILs has increased further (Pu *et al.*, 2007, Lee *et al.*, 2009, Tan *et al.*, 2009, Mora‐Pale *et al.*, 2011, Brandt *et al.*, 2013, Prado *et al.*, 2013). Very recently, Gregory Chatel and Robin D. Rogers reported that their group have been exploring a more sustainable method of using ILs to recover all three parts of lignocellulosic biomass, which is particularly useful as it described the opportunities to obtain lower molecular weight compounds from lignin by summarising the previous studies of the oxidation of both lignin model compounds and native lignins (Chatel & Rogers, 2014). It is worth mentioning that only one example, among their list of enzymatic oxidation reactions of lignin and its model compounds, has described the enzymatic oxidation of lignin model compound in ILs, in which *Melanocarpus albomyces* laccase catalysed the oxidation of 2,6-dimethoxyphenol, coniferyl alcohol in the presence of 0-40 % of 1-allyl-3-methylimmidazolium chloride ([Amim]Cl) and water mixture at room temperature (Lahtinen *et al.*, 2013). To date, no evidence has been published regarding the enzymatic degradation of native lignin by use of ILs as yet. The reason behind this is that lignin has a highly stable and heterogeneous structure and changes take place to its composition and structure because of the types of biomass, types of wood as well as the difference in harvesting seasons *etc*. Enzymatic degradation of lignin has become attractive because of its advantages over chemical catalysis. However, poor mass transfer delays its accessibility to lignin, hence the yield of lignin degradation is still very low at 9.8 % (Hamidi, 2013). Therefore, exploring a suitable solvent/co-solvent to address the mass transfer issue has led the research direction of this study towards finding both enzyme and lignin friendly ILs.

Ionic liquids have been studied as solvents for laccase-catalysed reactions (Hinckley *et al.*, 2002, Turner *et al.*, 2005) in an aqueous system in order to avoid deactivation. Enhanced activity can be obtained with water-insoluble substrates (Hinckley *et al.*, 2002), and laccase activity can also be stimulated by mixing ionic liquids with water (Shipovskov *et al.*, 2008, Lars Rehmann, 2012, Hamidi, 2013). These studies have provided guidance with regard to the selection of ILs for both water-miscible and -immiscible substrates. On the subject of the effect of ILs with respect to the activity of laccase, Hani has screened 106 different ILs with the aim of finding laccase from *Agaricus bisporus* (LAB) compatible ILs (Hamidi, 2013), and Lars (Rehmann *et al.*, 2012) has screened 63 ILs to identify their effect on LTV.

However, limitations concerning how to differentiate the degree of compatibility of ILs with the proposed enzymes, in particular LTV, have to be taken into account as both groups of authors performed their screening experiments only at 0.6 % (w/v) of ILs concentration. Therefore, in order to expand the understanding in relation to the effect of ILs (92 in total) of laccase activity at various concentrations has become another desirable part of this project in order to improve the laccase catalysed degradation of water-insoluble lignin.

1.4 Objectives of current study

This project firstly investigates the enzymatic oxidation of veratryl alcohol, a non-phenolic lignin model compound, using a commercially available enzyme, laccase from *Trametes versicolor* (LTV), and a well-studied laccase mediator, 2,2'-azino-bis (3-ethylbenthiazoline-6-sulphonic acid (ABTS). This is used as a bench mark reaction to identify the effect of LTV with regard to simple model compounds, which in turn will be used for understanding the complex nature of the oxidative depolymerisation of lignin. Furthermore, the project seeks to identify the effect of LTV on different types of lignin, and to develop suitable depolymerisation methodology for each type of lignin in order to determine the differences between their possible degradation products according to the source of the lignin, respectively. It then moves onto other lignin degrading enzymes, such as lignin peroxidase (LiP) and manganese peroxidase (MnP) with the aim of establishing their enzymatic assay conditions, and carries out a preliminary study regarding their capabilities of depolymerising two types of lignins (Organosolv and Kraft). Finally, the catalytic activity of LTV in the presence of different kinds of ILs using ABTS as a substrate will be studied systematically to identify how these ILs affect the LTV activity according to their structural properties, and their concentrations used. As a result, some laccase compatible ILs will be identified from the above screening study in order to choose a suitable solvent system to be used for degrading the water-insoluble lignin. Overall, this project aims to provide valuable experimental data through a comprehensive study of the enzymatic degradation of various types of lignins in aqueous reaction media using a combination of many useful analytical techniques which could be applied to similar processes in future research.

2 MATERIALS AND METHODS

2.1 Materials

Laccase from *Trametes versicolor* (LTV), lignin peroxidase (LiP), manganese peroxidase from *Nematoloma frowardii* (MnP), lignosulphonate (lignosulphonic acid sodium salt, higher sulphur content), Kraft lignin (lignin alkali with low sulphur content) and other chemicals used for this study were obtained from Sigma Aldrich, Scientific Laboratory Supplies Ltd. (SLS), and Fisher Scientific Ltd. in the UK. Organosolv lignin was supplied by Lignol Innovations Ltd. (Canada). Another one batch of lignosulphonates was provided by Borregaard UK Ltd. All chemicals were used as received except that vacuum distillation was applied to veratryl alcohol. The ionic liquids for the laccase activity screening study were kindly provided by Queens University Ionic Liquids Lab (QUILL) and the group led by Prof Peter Licence at the School of Chemistry, University of Nottingham, UK. The bibenzyl and hexameric lignin model compounds used for the GPC calibration were obtained from Dr Gary Sheldrake at the School of Chemistry and Chemical Engineering of Queen's University (Belfast, UK).

2.2 Methods for LTV activity measurement and for the lignin degradation reactions catalysed by LTV

2.2.1 Preparation of LTV stock solution

LTV solution was prepared using laccase from *Trametes versicolor* (22.4 U/mg). Laccase powder (10 mg) was added to the stock solution of citric buffer (pH 4.5, 10 ml) to produce a 1 mg/mL of LTV stock solution. It was then pipetted into 20 Eppendorf tubes where each tube contained 500 µl of laccase and stored in the freezer below -18 °C for future use. Each tube was used once and then discarded.

2.2.2 Preparation of buffers for LTV related reactions

2.2.2.1 Ammonium citric buffer

Stock solutions of ammonium acetate and citric acid solution were prepared (100 mM, 1L each). The pH of 0.1 M ammonium acetate ($C_2H_5CO_2NH_4$, MW=77.08 g.mol^{-1}) solution was measured using a pH meter, and then mixed with 0.1 M citric acid monohydrate ($C_6H_8O_7.H_2O$, MW = 210.14 g.mol^{-1}) solution until the buffer reached 4.5.

2.2.2.2 Sodium acetate buffer

The sodium acetate buffer (50 mM, pH 3.5) was prepared by dissolving 1.0255 g sodium acetate (CH_3CO_2Na, MW= 82.0340 g.mol^{-1}) in water (250 mL), and its pH was adjusted by adding acetic acid (glacial). The final pH 3.5 of this buffer was checked by using a pH meter after calibration (pH range between 4.0 and 7.0).

2.2.2.3 Sodium citric buffer

Stock solutions of trisodium citric and citric acid solution were prepared (100 mM, 1L each). The pH of 0.1 M trisodium citrate dihydrate ($C_6H_5O_7Na_3.2H_2O$, MW=294.12 g.mol^{-1}) was measured using a pH meter, and then mixed with 0.1 M citric acid monohydrate ($C_6H_8O_7.H_2O$, MW = 210.14 g.mol^{-1}) until the buffer reached 4.5.

2.2.3 Preparation of ABTS

The required solution of 2, 2'-azino-bis-3-ethylbenthiazoline-6-sulphonic acid, ABTS ($C_{18}H_{24}N_6O_6S_4$, MW = 548.68 g.mol^{-1}) was prepared freshly for each experiment to prevent its self-oxidation by air.

2.2.4 Measuring the activity of LTV

The catalytic activity of LTV was measured using ABTS as a redox substrate. The rate of ABTS oxidation was determined spectrophotometrically using the absorbance data of ABTS measured at 420 nm.

LTV (0.025 mg/ml) was diluted from LTV stock solution (1 mg.ml^{-1}) in sodium citric buffer (pH 4.5) and ABTS (10 mg) was dissolved into water (5 ml) to make its 4 mM solution. Firstly, the absorbance of LTV solution in buffer without ABTS was measured at 420 nm:

895 µl of citric buffer (0.1 M, pH 4.5) was mixed with LTV (5 µl, 0.025 $mg.ml^{-1}$) in buffer. The ABTS (100 µl, 4 mM) was then added to the above mixture to start the reaction and its absorbance was measured at 420 nm for 1h using an Agilent 8453 UV-Vis spectrophotometer (Agilent Technologies Ltd., UK) at 30 °C. The temperature was maintained by using a temperature controlled water-bath (Grant Instruments (Cambridge) Ltd.) with a rotary pump (Gilson Minipuls 3) attached to the spectrophotometer. The reaction rate of ABTS oxidation was obtained by using the molar extinction coefficient of 36000 $M^{-1}.cm^{-1}$ (Shipovskov *et al.*, 2008).

2.2.5 Study of the effect of ionic liquids on laccase activity with regard to ABTS oxidation

The catalytic actvity of laccase from *Trametes versicolor* **(**LTV) was evaluated in the presence of ionic liquids (ILs) when using ABTS as a substrate. ILs were tested using several different concentrations.

2.2.5.1 UV-Vis spectrophotometric assay in the presence of ILs

LTV activity was measured in 96 well quartz plates for testing the effect of different ionic liquids using a FLUOstar Optima Microplate Reader (BMG Labtech Ltd., UK). A quartz plate was used in this assay as some of ionic liquids can dissolve polypropylene plate. The total assay volume for each well was 300 µl. Each well contained of citric buffer (260 µl of 100 mM, pH 4.5), ILs (9 µl of 3 %, v/v), LTV (2 µl of 0.025 mg/ml) and ABTS (29 µl of 4 mM) respectively. A picture of microplates and assay are shown in Figure 2.1(a,b). Each assay was done in triplicate and the mean reaction rates are reported. Control experiments without adding ILs were also measured.

Figure.2.1 (a) Visual image of a FLUOstar Mircoplatereader (http://www.bmglabtech.com)

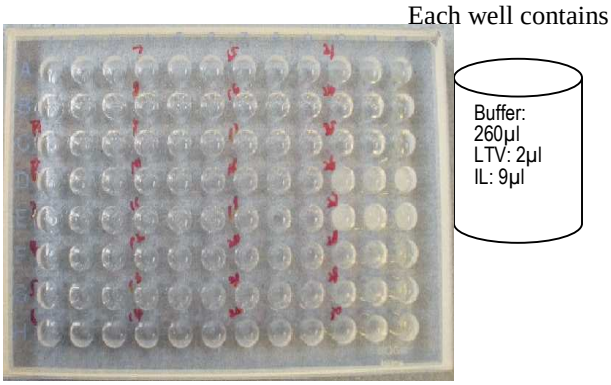


Figure.2.1 (b) a visual image of 96 well quartz microplate assays

2.2.5.2 High throughput screening of ILs at lower concentrations

UV-Vis spectrophotometric assay was replaced by Micro plate reader assay in for the high throughput screening of 92 different ILs. LTV activity was measured over a range of IL concentrations from 0.6 % to 10.0 % (w/v) using a FLUOstar Mircoplatereader. These assays (total volume of 300 µL) contained 100 mM sodium citrate buffer pH 4.5 (269-239 µL) pH 4.5), 0.025 $mg.ml^{-1}$ laccase solution (2 µl) in sodium citrate buffer and ionic liquid (0-30 µl). Each solution of water immiscible ionic liquids was vortexed vigorously before addition to obtain a relatively homogeneous solution and also to minimise the error caused by water immiscibility. The reaction mixture was equilibrated for 60 seconds. The reaction was then started by adding ABTS solution (29 µl, 4 mM in distilled water), resulting in an initial substrate concentration of 0.4 mM and an initial total assay volume of 300 µl. The formation of the $ABTS^{2+}$ radical cations was monitored at 420 nm (Szutowicz *et al.*, 1984) every 65 s at 37 °C for 5 h. The initial reaction rates were calculated monitoring over the first 715 s after substrate addition.

2.2.5.3 High throughput screening of ILs at higher concentrations

Higher concentrations of ILs were then screened to determine their effect on LTV activity once the most laccase compatible ionic liquids were determined from 2.2.5.3. The only difference being that each IL solution was prepared by weighing the targetted pure ionic liquid then adding it into water to prepare the needed concentrations, namely 20 %, 40 %,

60 %, 80 % and 100% (w/v), into the laccase activity assays. Most of the ILs tested in high concentration range was formed biphasic system, so they were vortexed and centrifuged, and then the upper layer was mixed with LTV prior to measurement. Appropriate control reactions (without the addition of ILs/ABTS/LTV) were also run.

The initial rate of reaction (v_0) for ABTS oxidation reactions in presence of IL was calculated by measuring the absorbance of $ABTS^{2+}$ at 420 nm in triplicate by time. The LTV activity was then determined by comparing the measured v_0 with the v_0 of the control experiment in the absence of ILs. Errors were calculated as standard deviations throughout all measurements. The assay contents of with and without IL are shown in in Table 2.1.

Table 2.1 The LTV activity assay contents of with and without ILs.

[IL] (W/V, %)	Volume of buffer (µl)	Volume of IL (µl)	Volume of LTV (µl)	Volume of ABTS (µl)
Control	269	0	2	29
0.6	267.2	1.8	2	29
2	263	6	2	29
4	257	12	2	29
10	239	30	2	29
20	209	60	2	29
40	149	120	2	29
60	129	180	2	29
80	29	240	2	29
100	0	269	2	29

Note: Last assay was prepared to examine the absorbance of pure IL with LTV and ABTS in the absence of buffer and therefore it was not included in the discussion.

2.2.6 LTV catalysed veratryl alcohol oxidation

2.2.6.1 Preparation of laccase solution in buffer

This solution was prepared from the 0.22 mL of LTV stock solution (1 $mg.ml^{-1}$or 22.4 U/mL) in a sodium acetate buffer (2.2.1) to obtain a final concentration of LTV (0.5 U/mL) in the 10 mL of total assay.

2.2.6.2 Preparation of veratryl alcohol solution

Veratryl alcohol was prepared freshly for each use by dissolving 0.0084g of veratryl alcohol

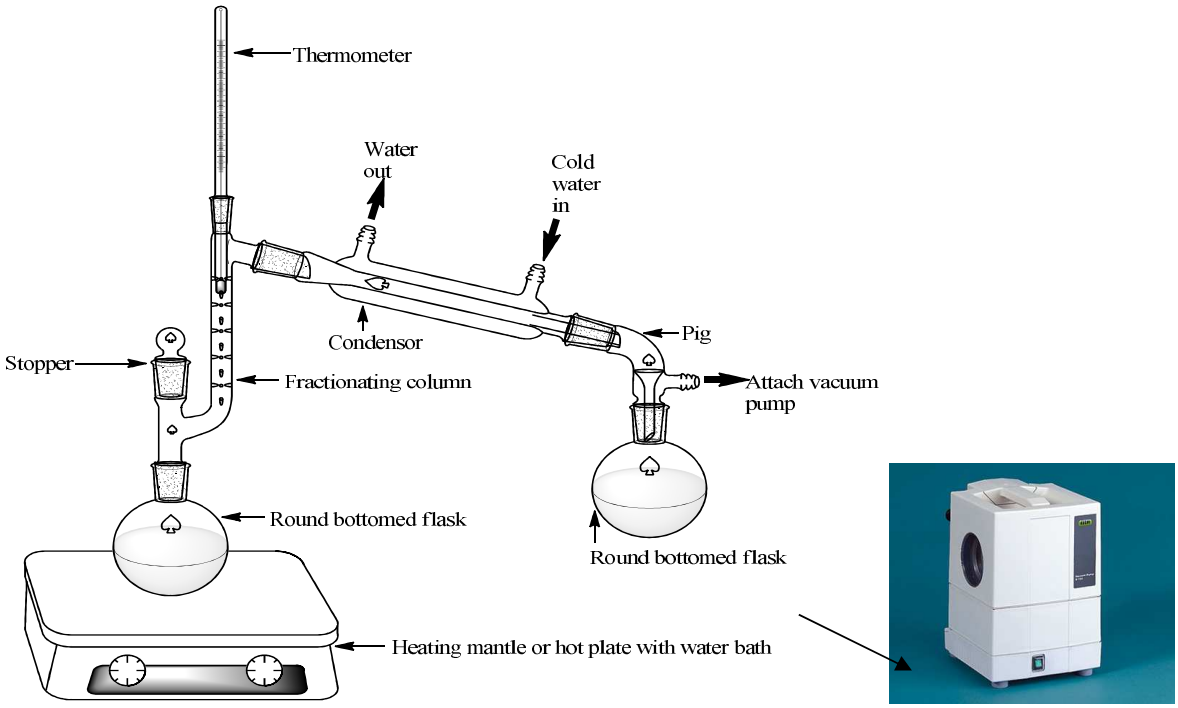


Figure 2.2: Schematic illustration for vacuum distillation of veratryl alcohol

Obtained veratryl alcohol after distillation was compared with the original product before distillation using Gas chromatography and mass spectroscopy (GC-MS) to confirm the veratryl alcohol was purified. The distilled product was confirmed by GC-MS analysis (Appendix A.1) using veratryl alcohol and veratryl aldehyde as standards.

2.2.6.3 Veratryl alcohol oxidation with LTV in the presence of ABTS

The oxidation of veratryl alcohol was performed by the adding 0.5 U/ml LTV to the solution of 3 mM veratryl alcohol and 1 mM ABTS in a 50 mM sodium acetate buffer (pH 3.5), and incubated at 30 °C for 48 h at a shaking speed of 200 rpm. The final reaction volume was 10 mL. Control reactions were also undertaken under the same conditions without laccase or ABTS. Samples were taken from both of the reaction and control experiments at intervals, and were analysed and quantified by HPLC analysis. Calibrations were made using authentic standards of veratryl aldehyde and veratryl alcohol both before and after the reaction took place. Experiments were carried out in triplicate, and their mean values were reported.

2.2.7 Organosolv oxidative degradation by LTV

A mixture of Oraganosolv lignin (29 g/L), 16 mL of 100 mM ammonium acetate buffer (pH 4.5), 250 µL of 0.025 mg/mL LTV solution in the same buffer, and 11 mL of 4 mM ABTS was incubated at 30 °C for 24h. All reaction vessels were capped with foil and incubated at 30 °C in a Sartorrius Certomat® BS-1shaker with a shaking speed of 200 rpm. Reaction was terminated by adding 10 µL concentrated sulphuric acid to each 2.5 mL of aqueous mixture and centrifuged at 20 °C at 1000 rpm in order to separate the unreacted solid lignin materials from the rest of the aqueous fraction. A portion from the aqueous fraction (1 mL) was mixed with 333 µL methanol, and its pH was adjusted to 12 by adding 1M NaOH solution prior to GPC analysis. Another 1 mL sample from the same aqueous fraction after phase separation was extracted using 4 mL ethyl acetate (EA) twice (2 mL each) and the EA layer of 2 times extraction was combined together, and evaporated under a fume hood overnight until its dryness. It then resuspended with acetone and analysed by GC-MS. Control reactions, without adding LTV, ABTS, and in the absence of both LTV and ABTS, was also performed. The component missed out from any control reaction mixtures was replaced by equal amount of buffer. All reactions were done in duplicate. Mean are reported with error bars. This procedure was applied in all Organosolv lignin degradation reactions with LTV between 30 - 60 °C unless otherwise specified.

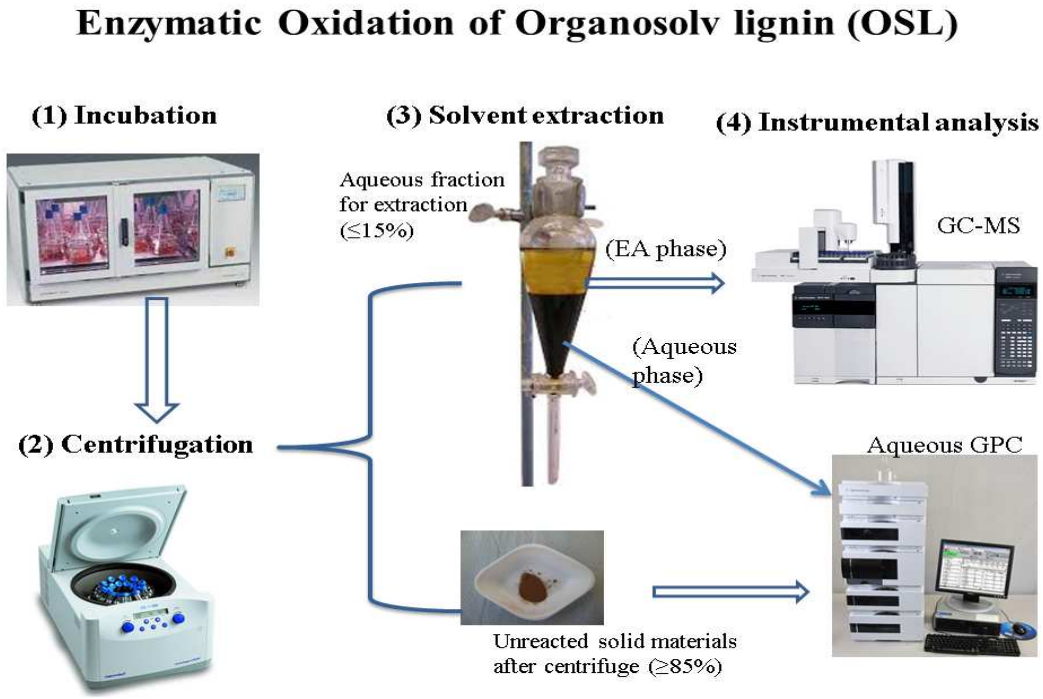


Figure 2.3: Schematic representation of OSL degradation by laccase and product analysis

2.2.8 The OSL degradation by LTV in the presence of ethanol

2.2.8.1 The effect of ethanol addition on LTV activity during its oxidation of ABTS

The oxidation of ABTS by LTV in the presence of ethanol was monitored at 420 nm using an Agilent 8453 UV-visible spectrophotometer (Agilent Tech UK Ltd). The aim of this study is to find out the best ethanol concentration in the assay mixture, and hence to identify the effect of ethanol on the activity of LTV. A mixture of 0.025 $mg.ml^{-1}$ of LTV (250 µL), 0.1 M ammonium acetate buffer pH 4.5 (24 mL) and ethanol (0-40 %, v/v) was incubated at 30 °C in a flat bottom flask (250 mL) and sampled (900 µL) at intervals. Reactions were started by adding 4 mM of ABTS (100 µL) into the samples. Reaction was performed in triplicates.

2.2.8.2 The LTV-catalysed depolymerisation of OSL in the presence of ethanol

The OSL degradation reaction was investigated in the presence of 10 and 20% of ethanol. All the reaction conditions were as same as that of the OSL degradation at 30 °C which previously described except the required amount of ethanol was added by reducing the amount of buffer in the reaction mixture. To prepare the complete reaction mixture (a dark brown suspension, total volume 35 mL) in a flat-bottom flask (250 mL), Organosolv lignin (2 g) was dissolved in EtOH (10 % and 20 % respectively, v/v) and mixed with 100 mM of sodium citrate buffer pH 4.5, 4 mM of ABTS (11 ml) and 0.025 mg/ml of LTV solution in a buffer (250 µL). The final concentration of Organosolv lignin was 57 g/L. Control reactions without adding LTV, ABTS and ethanol were also performed. The method was the same as in the complete reaction mixture but the components omitted were replaced with equivalent volumes of buffer. All reaction vessels were incubated at 30 °C at a shaking speed of 200 rpm. Samples (8.7 mL) were taken at regular intervals during the 24 hour incubation. Aqueous samples were acidified by adding concentrated H_2SO_4 (50 µL). The samples were centrifuged at 20 °C, 10,000 rpm for 10 min to separate the solid residues and the aqueous phase. Aqueous samples were extracted with EA and analysed with GC-MS.

2.2.9 Lignosulphonate oxidative degradation by LTV

The reaction procedure was the same as described in 2.2.6 except a 2-folded lignin increased concentration (57 g. L^{-1}) was used and the reaction was buffered by 100 mM sodium acetate

buffer pH3. As lignosulphonate sodium salt is water soluble, so there was no need to centrifuge to remove the unreacted solid materials. Reactions completed in duplicates and mean of duplicate measurements were reported. A simplified reaction process image of the enzymatic digestion of the lignosulphonates is shown in Figure 2.4.

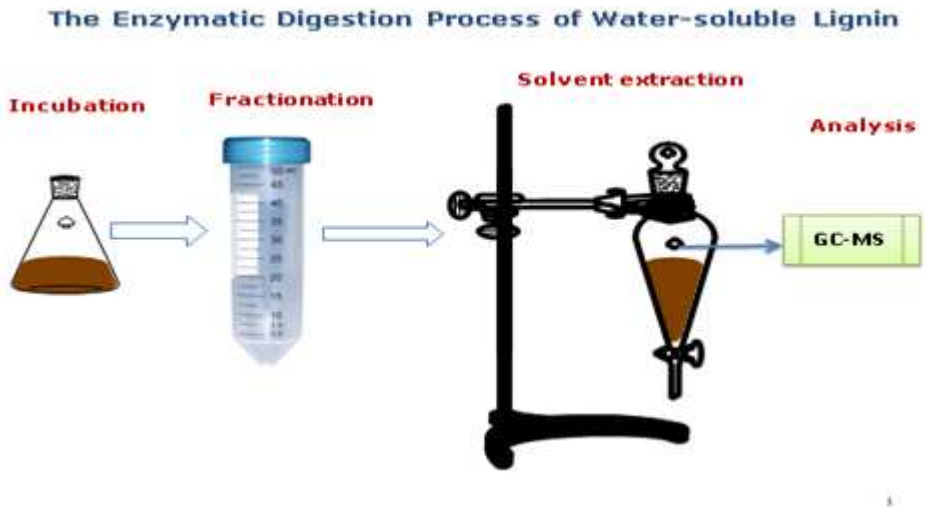


Figure 2.4: Schematic representation of water soluble lignosulphonate degradation

2.2.10 Kraft lignin oxidative degradation by LTV

The same method for lignosulphonate degradation reaction was applied to Kraft lignin degradation with LTV except the reaction mixture was adjusted to pH 4.5 by adding acetic acid (glacial) before adding the LTV.

2.3 Methods for LiP activity determination and for the lignin degradation reactions catalysed by LiP

2.3.1 Preparation of sodium tartrate buffer

Sodium tartrate buffer was prepared by dissolving 3.75 g of L-tartaric acid (MW=150.09 $g.mol^{-1}$) in 500 mL deionised water and 1 g of sodium hydroxide (MW=40.00 $g.mol^{-1}$) in 500 mL deionised water to produce their 100 mM solutions. The acid solution was added into the base solution at room temperature (18 °C) until the pH was 3.0 with thorough mixing using a magnetic stirrer.

2.3.2 Preparation of lignin peroxidase (LiP)

The solution of LiP was prepared by dissolving 10 mg of LiP (powder light brown, specified activity > 0.1 U/mg) in 1 mL of sodium tartrate buffer (pH 3.0). LiP was freshly prepared for each experiment.

2.3.3 Preparation of veratryl alcohol solution

Veratryl alcohol was prepared freshly for each use by dissolving 0.0084g of veratryl alcohol (MW=168.19 $g.mol^{-1}$) in 5 mL of sodium tartrate buffer (pH 3.0).

2.3.4 Preparation of H_2O_2 solution

Hydrogen peroxide (H_2O_2) was prepared by diluting concentrated H_2O_2 solution (≥ 30%) in deionised water to 0.4 mM. The hydrogen peroxide solution was prepared daily in order to avoid any possible thermal decomposition.

2.3.5 LiP activity assay

The activity of LiP was determined spectrophotometrically by observing the rate of veratryl aldehyde formation from veratryl alcohol oxidation by measuring the absorbance increase at 310 nm (Figure 2.5). The measurement was performed at 28 °C. The assay (total volume, 1.0 ml) contained 50 mM sodium tartrate buffer pH 3.0 (650 µL), 10 mM veratryl alcohol (50 µL), and 0.4 mM H_2O_2 in water (250 µL). The reaction was started by the addition of 10 mg/ml LiP solution in the sodium tartrate buffer (50 µL). The hydrogen peroxide solution was prepared freshly for each use, and its concentration was determined at 240 nm using an extinction coefficient of 39.4 $M^{-1}.cm^{-1}$ (Nelson & Kiesow, 1972). The concentration of lignin peroxidase was determined by measuring its absorbance at 408 nm using an extinction coefficient of 169 mM^{-1} (Kirk *et al.*, 1986).

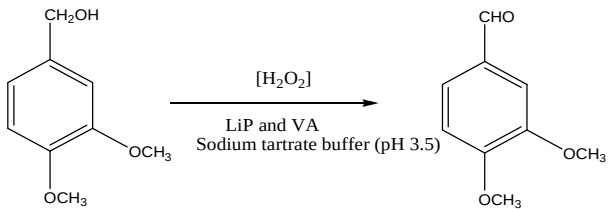


Figure 2.5: The oxidation of veratryl alcohol by LiP

2.3.6 LiP catalysed Organosolv lignin degradation

To prepare the complete reaction mixture (heterogeneous solution, total volume 12 mL) in a flat-bottomed flask (250 mL), 0.685 g lignin was mixed with 100 mM sodium tartrate buffer pH 3 (7.8 ml), 10 mM veratryl alcohol (0.6 ml), 10 $mg.ml^{-1}$ LiP (0.6 ml) and 0.4 mM H_2O_2 solution (3.0 ml). The final concentration of lignin was 57 g. L^{-1}. Control reactions without individually adding LiP and H_2O_2 solution, veratryl alcohol, and without all three of these items were also performed. The method in control reactions was the same as in the complete reaction mixture but the components missed out were replaced by equivalent volumes of buffer. All reaction vessels were capped with foil and incubated at 28 °C in a shaker at 200 rpm. Samples (4 mL) were taken at regular intervals during the 24 h of incubation.

2.3.7 LiP catalysed Kraft lignin degradation

Kraft lignin degradataion with LiP was performed as same method as it described in 2.3.6 except reaction was shortened for 5h.

2.4 Methods for MnP activity determination and for the lignin degradation reactions catalysed by MnP

2.4.1 Preparation of sodium malonate buffer

The sodium malonate buffer was prepared by dissolving 5.203 g of malonic acid (MW=104.06 $g.mol^{-1}$) and 2.0 g of sodium hydroxide (MW= 40.00 $g.mol^{-1}$) in 500 mL of deionised water, respectively. Each of these solutions was prepared with a final concentration of 100 mM. The acid solution was added into the base solution to adjust the pH of the final solution until 4.5 was obtained.

2.4.2 Preparation of $MnSO_4$ solution

$MnSO_4$ solution was prepared by dissolving 0.006 g of $MnSO_4$ (MW=246.48 $g.mol^{-1}$) in deionised water (100 mL) to produce a final concentration of 0.25 mM of solution. It was then diluted to 35 µM to be used for actual reactions.

2.4.3 Preparation of manganese peroxidase (MnP)

Mnganese peroxidase was prepared by dissolving 5 mg of MnP from *Nematoloma frowardii* (powder light brown, activity ≥20 U/g or 0.02 U/ mg) in 1 mL of sodium malonate buffer (pH 4.5) with a final concentration of 1 mg/ml. It was then diluted to the concentration of 0.05 $mg.ml^{-1}$ and used in the actual reactions. MnP was freshly prepared for every experiment to prevent from activity loss.

2.4.4 Preparation of H_2O_2 solution

Hydrogen peroxide (H_2O_2) was prepared by diluting concentrated H_2O_2 solution (≥ 30%) in deionised water to 0.2 mM.

2.4.5 MnP activity assay

The MnP activity was determined by monitoring the oxidation of Mn^{2+} to Mn^{3+} (Figure 2.6). The initial rate of Mn (III) malonate formation was determined spectrophotometrically in a quartz cuvette by following the initial increase in absorbance at 270 nm using the extinction coefficient of 11,590 $M^{-1}.cm^{-1}$. The assay contained 100 mM sodium malonate buffer pH 4.5 (600 µL), 35 µM $MnSO_4$ (50 µL), and 0.2 mM H_2O_2 in water (250 µL) and 3 mM ABTS (50 µL). The reaction was initiated at 25 °C by the addition of 0.05 $mg.ml^{-1}$MnP solution in a buffer (50 µL) to the above assay mixture. The total volume of the assay was 1 ml.

$$2\ Mn^{2+} + H_2O_2 + 2H^+ \longrightarrow 2\ Mn^{3} + 2H_2O$$

Figure 2.6: The oxidation of Mn (II) (Muzzarelli, 1990)

2.4.6 MnP catalysed Organosolv lignin degradation

The reaction mixtures included Organosolv lignin (57.14 g/L), sodium malonate buffer (100 mM, pH4.5), $MnSO_4$ (35 µM), ABTS (3 mM), MnP (0.05 mg. mL^{-1}), and 0.2 mM H_2O_2 (3 mL). The total reaction volume was 12 mL. Control reactions without adding MnP and H_2O_2, Mn^{2+} and ABTS and without all of the above in a malonate buffer were also performed. The

method for the controls was the same as in the complete reaction mixture but the components missed were replaced with equivalent volumes of malonate buffer. All reaction vessels were incubated at 25 °C at a shaking speed of 200 rpm for 24 h. Reactions were observed at a time interval of 24 h and terminated by adding concentrated sulphuric acid into the cooled mixtures. The samples were centrifuged at 20 °C, 10,000 rpm for 5 min to separate the solid residues from the aqueous phase. Part of aqueous phase was extracted with ethyl acetate and analysed with GC-MS to identify the possible depolymerisation products released from OSL.

2.4.7 MnP catalysed Kraft lignin degradation

The complete reaction mixture contained 2g Kraft lignin, 0.1 mg/ mL MnP, 0.20 mM H_2O_2, 35 µM $MnSO_4$ solution, 3 mM ABTS and 100 mM sodium malonate buffer (pH 4.5). Control reactions without adding MnP and H_2O_2, $MnSO_4$ and ABTS, and without all of the above 4 components were also performed. The method in the control reactions was the same as in the complete reaction mixture but the components missed out were replaced by equivalent volumes of above buffer. All reaction vessels were capped with foil and incubated at 25°C in a shaker at 200 rpm for 5 h.

2.5 Analytical techniques

Various analytical methods were used in this study to monitor and identify the reaction products from lignin degradation, as well as to evaluate the function of enzymes.

2.5.1 High Performance Liquid Chromatography (HPLC)

An Agilent 1200 model ~~of~~ HPLC with an integrated data system was used for the identification of oxidation products from veratryl alcohol. The column was a reverse phase Waters X Terra ® RP 18 column (3.0×250mm) fitted with a Guard column which prevents column damage. An ultraviolet (UV) single wavelength detector (λ= 230 nm) was used for quantitative analysis. For analysing the veratryl alcohol oxidation product, the separation was obtained using linear gradients ranging from 5 % MeOH to 100 % MeOH at flow rate of 1 mL/min at 37 °C. Compounds were detected at 280 nm using a UV detector. The veratryl alcohol oxidation product was identified and quantified by comparing the chromatogram obtained and retention time with the calibrated authentic standards of veratryl alcohol and

veratryl aldehyde. The calibrations for this reaction were performed with veratryl alcohol and veratryl aldehyde using commercially available compounds (Sigma-Aldrich) within the appropriate concentration range as described below.

2.5.1.1 Preparation of the HPLC standards

Veratryl alcohol ($C_9H_{12}O_3$, MW=168.19 g.mol^{-1}, 0.0084 g) and veratryl aldehyde ($C_9H_{10}O_3$, MW=166.17 g.mol^{-1}, 0.0083 g) were dissolved in MQ-water (5 mL) respectively to make 10 mM solutions. Each sample was run under the HPLC condition described in Section 2.3.3, and a cocktail or mixed sample of these two compounds at same concentration was analysed to make sure the minimum error could occur by the retention time difference between the individual run and the mixed sample.

2.5.1.2 The calibration procedure of HPLC standards

The calibration of the standards was performed using the five-point calibration method. Sample concentrations prepared by diluting the stock solution (10 mM) of standards veratryl alcohol and veratryl aldehyde. The calibration curve obtained is shown in Figure 2.7.

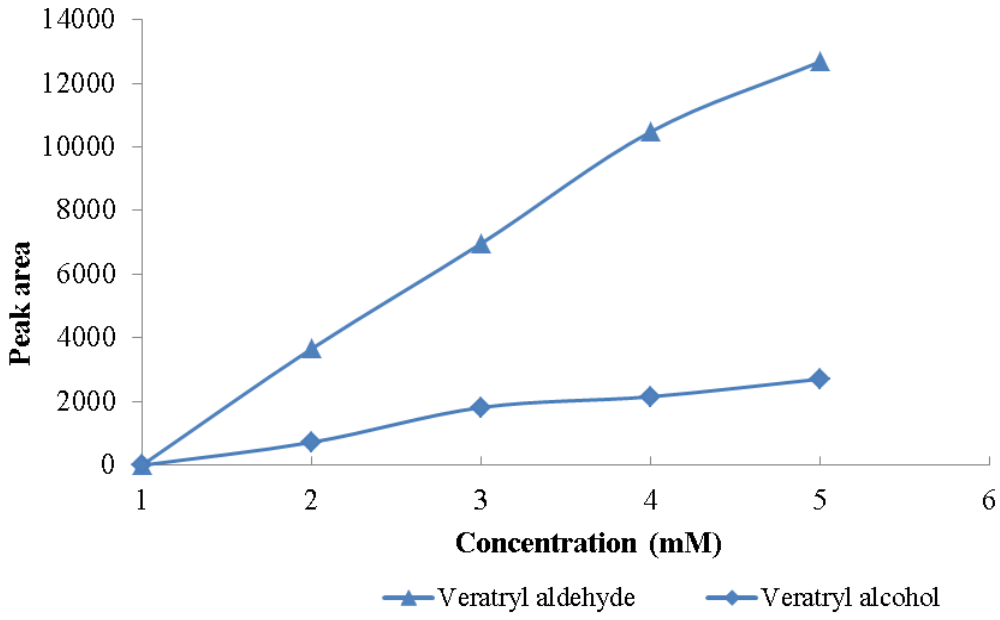


Figure 2.7: The HPLC calibration curve for veratryl alcohol and veratryl aldehyde

2.5.2 Gas Chromatography Mass Spectroscopy (GC-MS)

2.5.2.1 Sample preparation for the oxidative degradation of Organosolv lignin by LTV

Aqueous samples (each 2.5 mL) were cooled to room temperature and acidified by adding concentrated sulphuric acid, H_2SO_4 (10 µL). The samples were centrifuged at 20 °C, 6000 rpm for 10 min using an Eppendorf centrifuge (5810R) to separate the solid residues from the aqueous phase. A portion (1 mL) was extracted with ethyl acetate (EA) twice with with the total volume of 4 mL (1:4, v/v). The EA extracts were dried under a fume hood and re-dissolved in acetone (1 mL) for GC-MS analysis. The samples were vortexed in 1.5 mL of polypropylene tubes using an Epondorf Minispin®/Minispin Plus centrifuge for 3 mins at 6000 rpm to separate any undissolved solid parts in the samples. Authentic standards of lignin monomers (5 mM) were dissolved in methanol and analysed by GC-MS to be used as external standards.

2.5.2.2 Sample preparation for the oxidative degradation of lignosulphonates and Kraft lignin by LTV

All aqueous samples (8.7 mL) were cooled to room temperature and acidified by adding concentrated sulphuric acid H_2SO_4 (50 µL). Both lignin types were water-soluble and therefore there was no need to centrifuge the solutions before ethyl acetate extraction. A portion (4 mL) was extracted with ethyl acetate (16 mL). The extracts were left to evaporate under a fume hood and re-suspended in DCM (1 mL) for GC-MS analysis. The rest were as same as described in 2.5.2.1.

2.5.2.3 Sample preparation for the oxidative degradation of Organosolv lignin by LiP and MnP

A portion of acidified samples (2 mL) were extracted by 8 mL ethyl acetate (1:4, v/v) and dried under a fume hood before re-concentrated with 1 mL dichloromethane (DCM). Rest of the procedure were as same as described in 2.5.2.1.

2.5.2.4 Product identification by GC-MS for all types of lignins

The samples from the ethyl acetate extracts were analysed by a GC-MS from Agilent Technologies (7890A) gas chromatography (GC) system with a MS-5975C triple-axis detector mass spectroscopy (MS) and integrated autosampler (model number 7693). The column used was a HP-5MS (Agilent technologies) with 30 m x 0.25 mm (internal diameter) non-polar bonded phase capillary column with a phase thickness of 0.25 µm. The carrier gas was high purity helium at 0.55 ml/min (30 cm/s) with a split ratio of 65:1. The temperature program started at 70 °C for 2 min and then increased to 230 °C at a rate of 7 °C/min. The column was held at this temperature for 5 min. The injector and the GCMS detector were kept at 250 °C and 280 °C, respectively. The solution containing the sample was injected (1 µl) from the autosampler. Each sample was run for 35 mins. Fragmentation was achieved by electron ionisation (EI) (positive ion ionisation) at 70 eV, the source temperature was 180 °C, the interface temperature was 240 °C and the mass resolution was 300 units. Identification of the compounds was carried out by comparing the mass spectra with those contained in NIST08 MS library and calibrated authentic standards. For the identification of samples from MnP and LiP catalysed reactions, methods were all same except the injection method was modified to splitless.

2.5.3 Gel Permeation Chromatography (GPC)

In this project, a Jordi Gel Sulfonated Plus 10,000A column (250×10 mm) fitted with a guard column was used for aqueous samples analysis. The aqueous samples (25 µL) from lignin degradation reactions and the standard chemicals for GPC calibration were adjusted to pH 12 using sodium hydroxide (1M), and analyzed using this Jordi column with a solvent mixture of water (90%) and methanol (10%, v/v) as mobile phase with the flow rate of 1mL/min. Solid samples were dissolved in the above mixture first, and their pH were adjusted for 12 before injection. These were monitored by a refractive index (RI) detector at 35°C. Obtained results were reviewed and compared with the calibration curve of those standards to determine the molecular weight of samples.

2.5.3.1 The development of GPC calibration using authentic standards

A set of authentic pullulan standards were obtained from Sigma-Aldrich (UK) with molecular weights of 342, 1080, 5900, 9600, 21100, 47100 and 107000 (Sigma-Aldrich, 2013) and

were used for the determination of molecular weights of the lignin degradation products in this study. Samples were prepared for GPC analysis according to the suggested methods in the information sheet provided. Each standard (0.005 g) was dissolved in the same mobile phase solvent (2 mL) as used for the GPC analysis of the lignin degradation reaction samples, wherein methanol and water (9:1, v/v) was used and the pH of this mixed mobile phase was adjusted to 12 with sodium hydroxide (1 M).

2.5.3.2 Calibration of pullulan standards to determine the molecular weights changes in lignin degradation

The GPC results analysed from each type of lignin degradation by enzymes were compared with the calibrated authentic pullulan standards. The calibration curves of the standards were made according to a relative and narrow standard calibration method (Waters, 2013). After running the series of pullulan standards, a polynomial fit was then performed, and the resulting log M versus retention time calibration curve was plotted (Waters, 2013). The molecular weights of Organosolv, Kraft lignin and lignosulphonates, before and after the enzymatic depolymerisations, were then determined using this calibration curve (Figure 2.8). Data represents of 3 replicates with the standard deviation of no larger than 0.01.

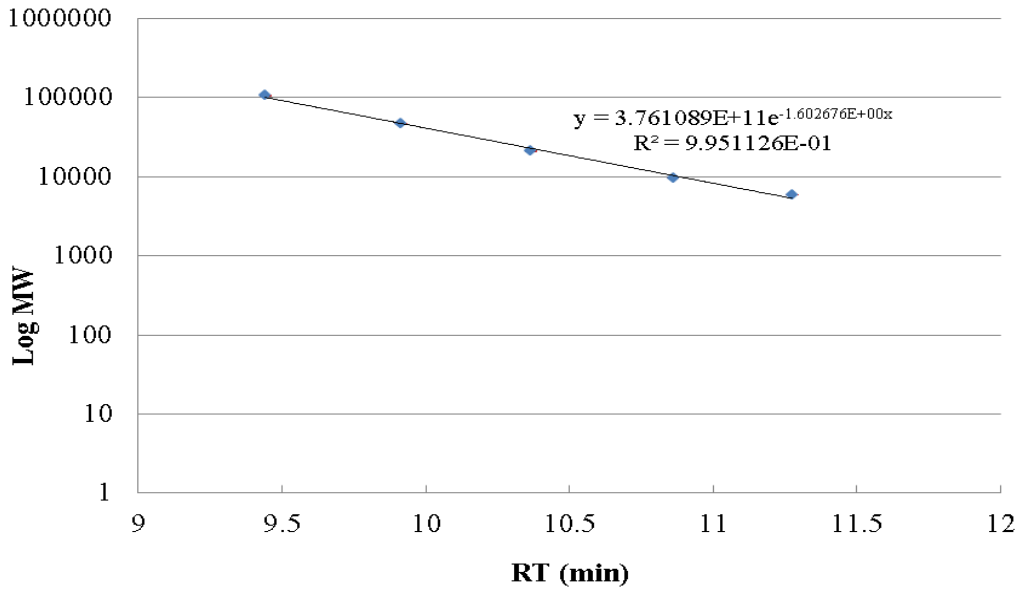


Figure 2.8: GPC calibration curve using pullulan standards

3 OXIDATIVE DEGRADATION OF ORGANOSOLV LIGNIN WITH LIGNOLYTIC ENZYMES

3.1 INTRODUCTION

Organosolv lignin is not water-soluble, and this may limit its wider usage as compared to other types of lignin (lignosulphonate and Kraft lignin). However, it possesses some advantages over the others such as higher purity and lower molecular weight. Also, the Organosolv process is a technique to solubilise lignin and hemicellulose using organic solvents during the pulping process and therefore, it does not require extreme conditions to prepare lignin and most importantly it is free from sulphides. Hence, the Organosolv process can be considered as relatively environmentally friendly method to obtain more purified lignin which in turn can be transformed into value added aromatic products from its depolymerisation (Zakzeski *et al.*, 2010, Lange *et al.*, 2013).

In this study, Organosolv lignin was chosen as a substrate for depolymerisation by laccase from *Trametes versicolor* (LTV), using ABTS as a mediator. Firstly, the activity and thermo-stability of LTV were studied because suitable operating conditions are crucial in enzymatic reactions. Laccase was applied into the oxidation of a non-phenolic lignin model compound, veratryl alcohol, and thus the catalytic ability of LTV was verified. Oxidation of Organosolv lignin was then investigated under aqueous reaction conditions using LTV in the presence of ABTS as a mediator. Other influential factors that may affect lignin depolymerisation such as incubation time and temperature, an extra supply of enzyme and mediator were also studied. Furthermore, using an organic solvent (e.g. ethanol) mixture with water was performed as a parallel study according to a hypothesis that this could improve the mass transfer between the laccase and lignin by increasing the homogeneity of this reaction system. To meet this end, the percentage of ethanol which could be used in this aqueous reaction was also investigated through a set of laccase activity tests so that it did not cause laccase deactivation. Finally, other lignolytic enzymes such as lignin and manganese peroxidases were also investigated to test their ability to catalyse Organosolv lignin depolymerisation.

3.2 RESULTS AND DISCUSSION

3.2.1.1 Laccase activity measurement using ABTS as a substrate

This experiment was performed to determine the rate of ABTS oxidation by LTV. The rate of ABTS oxidation by LTV is affected by the ABTS concentration (or [ABTS]), the reaction temperature and also the pH of the working buffer. To this end, a spectrophotometric assay of ABTS oxidation by LTV was performed under various reaction conditions. The reaction mixture contained sodium citrate buffer, ABTS and LTV.

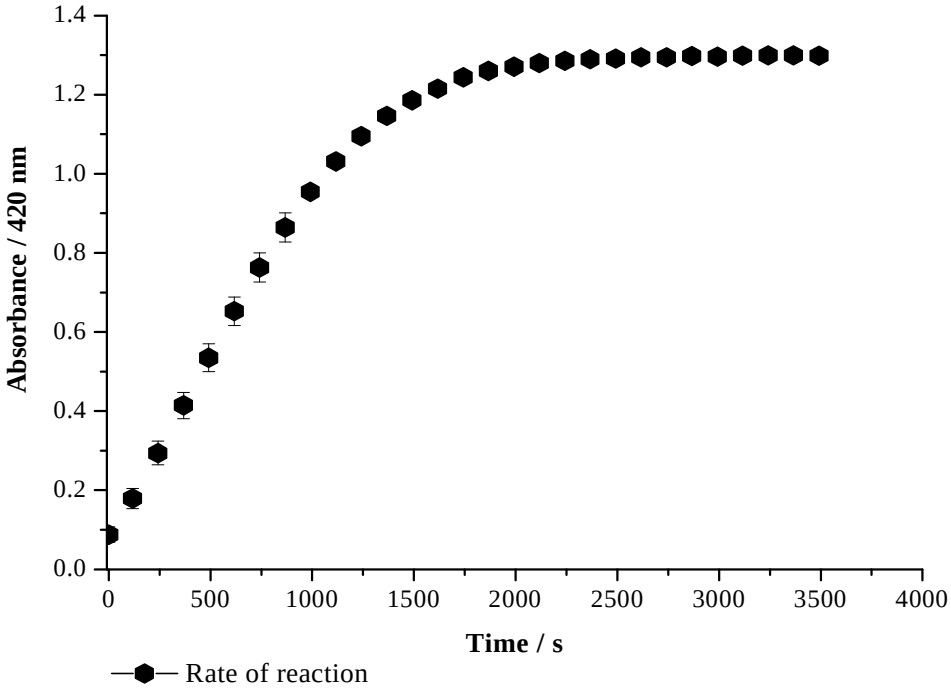


Figure 3.1: The time course of ABTS oxidation by LTV.

The absorbance of ABTS was recorded for 1h at 420 nm at 22 °C. The total volume of this assay was 1 mL and contained 895 µL citric buffer (100 mM, pH 4.5), 5 µL of LTV (0.025 mg/ml) and 100 µL of ABTS (4mM)).

The absorption values (OD) of $ABTS^{2+}$ increased at a linear rate until about 2000 s and then gradually slowed down. The data represent the mean of three replicates with an error of less than 2% (Figure 3.1). The reaction rate (v) was calculated from the linear region (first 800 s) of the curve. The rate of ABTS oxidation was $0.93 \pm 0.01\ \mu M^{-1}.\ s^{-1}$.

3.2.1.2 The effect of ABTS concentration on LTV activity

This study was designed to measure the effect of ABTS concentration (or [ABTS]) on LTV activity towards oxidation of ABTS. To this end, a spectrophotometric assay of ABTS

oxidation by LTV was measured under various [ABTS]. The reaction mixture contained sodium citrate buffer, ABTS and LTV.

The rate of reaction at each [ABTS] over time was recorded and was then replotted against reaction time to show the effect of [ABTS] towards the rate of ABTS oxidation. The measurement was conducted the same as stated in 3.1.1.1.The rate of reaction increased when the [ABTS] increased due to the fact that the rate of product formation depends on the substrate concentration in an enzymatic reaction (Marangoni, 2003). The rate of reaction increased with the increase in [ABTS] and then it started to level off (Figure 3.2). The maximum oxidation rate was observed at 4 mM of [ABTS].

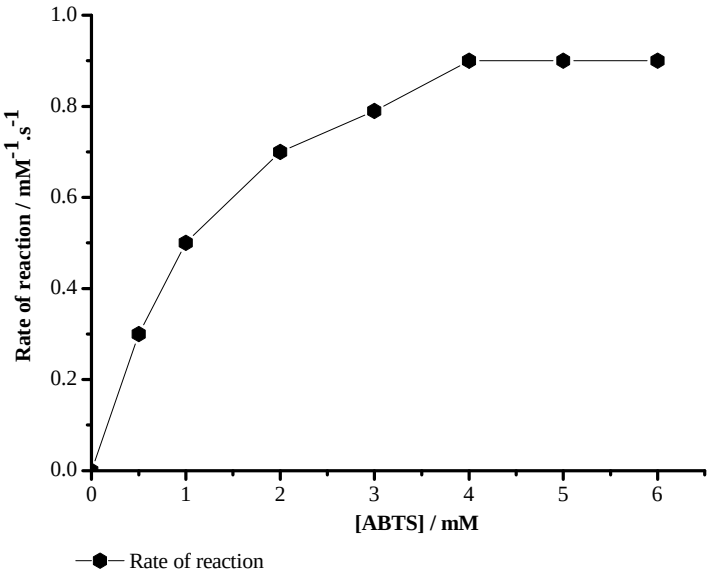


Figure 3.2: The Effect of [ABTS] on the rate of LTV catalysed oxidation.

The total volume of this assay was 1 mL and contained 895 µL citric buffer (100 mM, pH 4.5), 5 µL of LTV (0.025 mg/ml) and 100 µL of various concentration of ABTS. The data represent the mean of three replicates with a standard deviation of less than 1%.

This is because of that at the given constant pH, LTV concentration and temperature conditions of the current measurement, the active sites of LTV became saturated with the high ABTS concentration, and the enzyme-substrate complex (LTV-ABTS couple) must disassociate from each-other to make more room in the active sites of LTV so that it can accommodate more substrate to react with (Cornish-Bowden, 1995). Therefore any further rise in the [ABTS] after 4 mM did not affect the rate of the reaction.

3.3 Laccase thermo-stability study

Laccases were reported as a stable enzyme at higher operating temperature and so it has been applied as catalyst in many types of bio-transformations and in organic synthesis. However, the differences in sources of laccases and other reaction conditions during their catalysis may cause variations in their activity as well as their stability performances. To the best of the author's knowledge, the optimum temperature of laccase from various sources below was found to be between 30-60 °C. As for the activity of LTV only was also significantly vary between 45-80 °C (Rancano *et al.*, 2003, Baldrian, 2004, Koschorreck *et al.*, 2008, Hilden *et al.*, 2009, Patrick *et al.*, 2009, Reiss *et al.*, 2011, Zhu *et al.*, 2011, Lu *et al.*, 2012). Therefore, the thermal stability of LTV was re-examined in the current study and determined by incubating the LTV in the ammonium acetate buffer (pH 4.5) for 24h at the temperature range between 30 and 60 °C in order to identify the effect of reaction temperature on LTV activity.

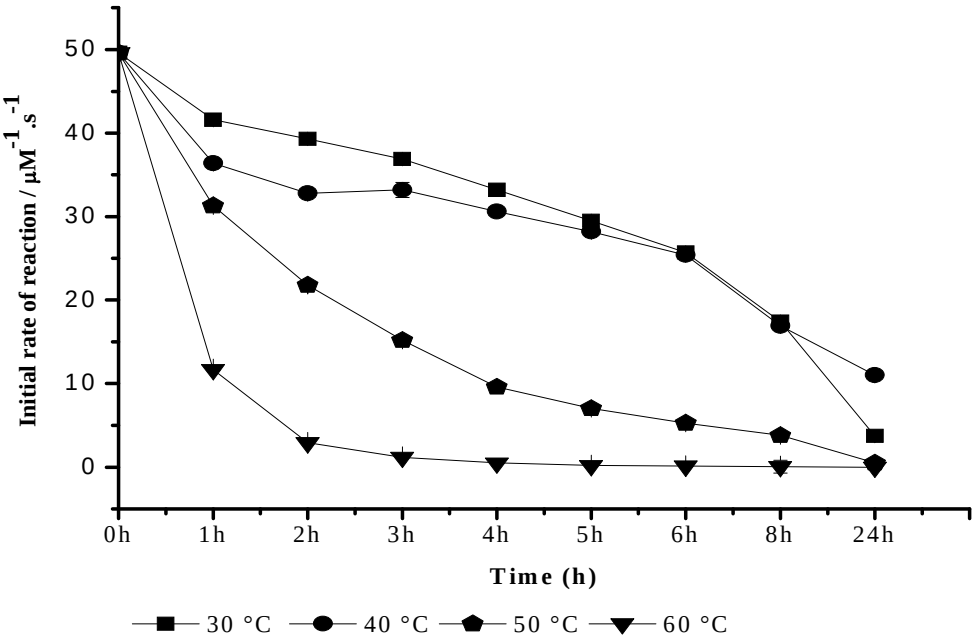


Figure 3.3: Thermo-stability test of LTV between 30 and 60 °C.

The reaction mixture contained LTV (0.25 mg.ml^{-1}, 250 µL), ammonium acetate buffer pH 4.5 (0.1 M, 16 mL) and deionised water (8 mL), and incubated at four fixed temperatures respectively, and sampled (900 µL) at intervals. The reaction was started by adding 100 µL of ABTS (4 mM) into the above mixture 900 µL respectively. The oxidation of ABTS by LTV was monitored at 420 nm at the designated reaction temperatures over the 24 h incubation. The data represents the mean of triplicate experiments, and the errors were not larger than 1% with standard deviation.

The initial reaction rate of ABTS oxidation at each observation temperature was determined by measuring the absorbance (OD) increase of ABTS $^{2+}$ at 420 mm in triplicate at room temperature (21°C), and then plotting the mean OD of triplicate samples against incubation time. The residual activity of LTV was reported through the changes in rate of ABTS oxidation at designated temperature and time. It was then compared with the rate of control reaction which was measured by sampling from the buffered enzyme solution just before the incubations started. It shows that LTV activity decreased significantly with the incubation time and increasing temperature (Figure 3.3). LTV lost more than 85 % of its activity after 1h of incubation at 60 °C while it still maintained 55 % of its activity at least for the first 6 hr between 30 and 40 °C. However, the decrease in LTV activity between 30 °C and 40 °C was not significant except the loss of LTV activity was slower at 30 °C than 40 °C. Therefore, the most suitable operating temperature for the LTV-catalysed reaction was chosen between 30-40 °C.

3.4 Veratryl alcohol oxidation using LTV in the presence of ABTS as a mediator

Prior to perform the LTV catalysed actual lignin reactions, it is necessary to evaluate the laccase ability towards the oxidation of simpler model compounds which resembles lignin. To this end, veratryl alcohol (3,4-Dimethoxybenzyl alcohol) was chosen as a non-phenolic lignin model compound and its reaction with LTV was examined in the presence of ABTS. It was reported that veratryl alcohol cannot be oxidised by laccase alone as the known redox potential of laccase is not higher than 0.8V whereas the redox potential of veratryl alcohol is around 1.2 (Dolphin, 1991). However, this reaction can be preceded with the assistance of a mediator or co-oxidant, e.g. ABTS (Muheim *et al.*, 1992, Smith *et al.*, 2009). Therefore, veratryl alcohol oxidation by LTV in the presence of ABTS (Figure 3.4) was re-examined in this project as a benchmark reaction to understand the laccase catalysed depolymerisation of lignin.

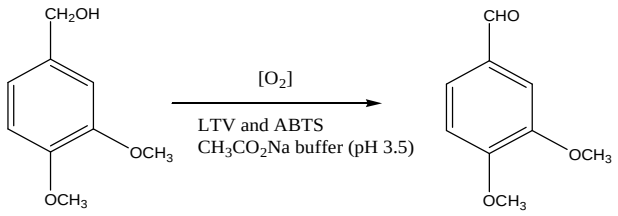


Figure 3.4: Laccase catalysed oxidation of veratryl alcohol to veratryl aldehyde

A number of laccase mediators have been used to perform veratryl alcohol oxidation in the literature (Bourbonnais & Paice, 1987, Schmidt *et al.*, 1989, Bourbonnais & Paice, 1990, Muheim *et al.*, 1992, Bourbonnais *et al.*, 1997, Fritz-Langhals & Kunath, 1998, Majcherczyk *et al.*, 1999, Balakshin *et al.*, 2000, Fabbrini *et al.*, 2002, Morozova *et al.*, 2007, Díaz-González *et al.*, 2011, Larson *et al.*, 2013), such as 2,2,6,6-tetramethylpiperidine-1-yloxy (TEMPO), 1-hydroxybenzotriazole (HBT) and ABTS. In this study, ABTS was chosen as a favoured mediator after a comprehensive literature survey (Appendix A.2). The prior studies in the literature indicated that the reaction with ABTS proved to be at least 85 times more efficient than HBT, although both of them can accelerate the laccase catalysed oxidation of veratryl alcohol by yielding a similar amount of aldehyde, 93 % with HBT and 95 % with ABTS, respectively (Bourbonnais *et al.*, 1997). In the case of TEMPO, the conversion of the above reaction was reported as high as 99 % in the presence of tert-butanol as a co-solvent (Larson *et al.*, 2013). However, insufficient details were given in this literature regarding how the assay was performed. In addition, the concentration of the mediator used was nearly equal to the veratryl alcohol concentration which was not suggested for this reaction by other researchers. Furthermore, the veratryl alcohol is a water-miscible substrate and so there is no need for a co-solvent for its reaction with water-miscible ABTS and laccase. Last but not the least, the concentration of ABTS should not be too high in the reaction because the ABTS itself is also a good substrate for laccase, and it may compete with veratryl alcohol for the active sites of laccase (Balakshin *et al.*, 2000). Therefore, taking all of the above factors and previous studies into consideration, the veratryl alcohol oxidation was carefully designed as follows.

The oxidation of veratryl alcohol reaction mixture contained LTV (0.5 U/ml), veratryl alcohol (3 mM) and ABTS (1 mM) in a sodium acetate buffer (pH 3.5), and incubated at 30 °C for 48 h at a shaking speed of 200 rpm. Control reactions without laccase or ABTS were also undertaken under the same conditions. The components missed out from the control reaction mixtures were replaced with equal amount of sodium acetate buffer. The reactions were sampled at intervals and monitored using High Performance Liquid Chromatography (HPLC). The amount of veratryl aldehyde produced was quantified and reconfirmed using a single point external standard method (academy, 2014) using the authentic standards of veratryl alcohol and aldehyde. The data presented are the mean of three replicates.

There was no oxidation product from veratryl alcohol formed in the control reactions where there was no LTV or ABTS present in the reaction (Appendix 3-1). The amount of veratryl aldehyde was significantly increased with increasing incubation time and with the consumption of veratryl alcohol, but 43.94 % of veratryl alcohol (1.27 mM) still remained unconverted after 48 hrs of incubation (Figure 3.5). Therefore, the results obtained here show agreement with the literature in which veratryl alcohol oxidation is considered as a fairly slow reaction process (Majcherczyk *et al.*, 1999, Shleev *et al.*, 2006). However, the reaction yield in this particular study was lower than the findings of Balakshin *et al*. (2000) as they have achieved more than 90% conversion with the same type of laccase, but used some different reaction conditions (Balakshin *et al.*, 2000). For instance, they have applied nearly 3 times higher concentration of veratryl alcohol and 17 times more ABTS in their oxidation reaction, and also flushed with oxygen to achieve better conversion.

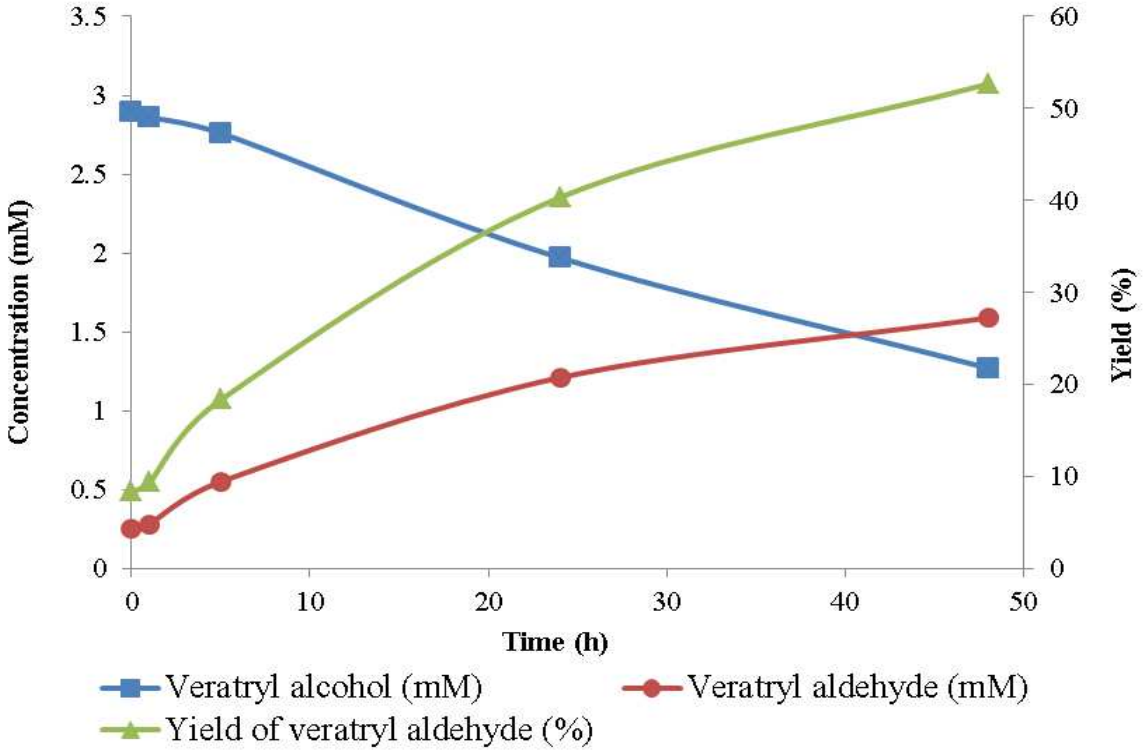


Figure 3.5: Veratryl alcohol oxidation to veratryl aldehyde.

Reaction mixtures contained 3 mM of veratryl alcohol (VA), 50 mM of sodium acetate buffer (pH 3.5), 1 mM of ABTS and 0.5 U/ml of LTV. Reactions were performed in triplicate. Mean of triplicate analysis are reported with the error not higher than 1%. The amount of produced veratryl aldehyde and the consumed veratryl alcohol were determined by HPLC.

The results shown above confirm that LTV can oxidise veratryl alcohol into veratryl aldehyde in the presence of ABTS as a mediator. Furthermore, the enzymatic oxidation of veratryl alcohol is found to be a very slow reaction. This finding implies that extending the incubation time for similar processes may be helpful, for example for the depolymerisation of lignin using laccase, as it is much more complicated than veratryl alcohol. In fact, this reaction could be extended in order to obtain the highest conversion of veratryl alcohol to veratryl aldehyde. However, the main objective of the current study was to verify the ability of laccase with respect to the oxidation of non-phenolic lignin model compounds, and therefore this particular study was not focused to achieve the optimum yielding condition in this case.

3.5 Organosolv lignin degradation with LTV using ABTS as a mediator

This study was aimed to investigate the oxidation ability of laccase towards water-insoluble Organosolv lignin. A mixture of oraganosolv lignin, LTV, ABTS and ammonium citric buffer was incubated at 4 different temperatures, namely at 30 °C, 40 °C, 50 °C and 60 °C respectively, for 24h. Control reactions, without adding LTV, ABTS, and in the absence of both LTV and ABTS, were also performed. The component missed out from any control reaction mixtures was replaced by equal amount of buffer. The reaction was terminated by adding sulphuric acid at room temperature and fractionated using centrifuge. Once the liquid fraction was separated from the solid fraction, a portion of the aqueous fraction and the unreacted solid material were analysed by Gel Permeation Chromatography (GPC), and another portion of aqueous fraction was extracted with ethyl acetate (EA) and analysed by Gas Chromatography-Mass Spectroscopy (GC-MS).

3.5.1 The effect of temperature on Organosolv lignin degradation by laccase

To prepare the complete reaction mixture (total volume 35 mL) in a flat-bottomed flask (250 mL), Organosolv lignin (1 g) was suspended in deionised water (7 mL) and mixed with 100 mM an sodium citric buffer pH 4.5 (16 ml), 4 mM ABTS (11 ml) and 0.25 $mg.ml^{-1}$LTV solution in a buffer (250 µL). The final concentration of Organosolv lignin was 28.5 g. L^{-1}. Reactions (with all components) and control reactions (without LTV/ABTS/ both ABTS and LTV) were performed in duplicate. The method used was the same as in the complete reaction mixture but by replacing the omitted items with equivalent volumes of buffer.

Samples (2.5 mL) were taken for analysis at regular intervals during the 24 hours of incubation. Both the reactions and analyses were performed in duplicate and the mean data were reported.

3.5.1.1 Laccase catalysed Organosolv lignin degradation at 30 °C

(1) GPC analysis:

GPC has been applied to lignin related research for more than a decade as it is a particularly suitable method for studying the size/molecular weight of difference of compounds in a mixed sample, especially for polymer analysis (Pellinen & Salkinoja-Salonen, 1985, Bourbonnais *et al.*, **1995,** Wong & de Jong, 1996, Majcherczyk & Hüttermann, 1997, Fredheim *et al.*, 2002, Cathala *et al.*, 2003, Ringena *et al.*, 2006).The calculated molecular weight (MW) from the GPC analysis of each reaction mixture by time course using the calibrated pullulan standard (Appendix A.3) is shown in Table 3.1.

Table 3.1 MW changes between complete reaction and control experiments from Organosolv lignin oxidation by laccase at 30°C.

Reactions	Mean RT (min)	Estimated mean MW of products (t=0 h)	Estimated mean MW of products (t=1 h)	Estimated mean MW of products (t=5 h)	Estimated mean MW of products (t=24 h)
Complete reaction with LTV & ABTS	11.94	1850 ± 0.05	2320 ± 0.03	1940 ± 0.07	1940 ± 0.00
	12.22	1160 ± 0.10	1240 ± 0.04	1220 ± 0.09	1230 ± 0.00
Control without LTV, but with ABTS	11.93	1880 ± 0.11	1990 ± 0.90	2090 ± 0.01	1800 ± 0.01
	12.23	1170 ± 0.15	1170 ± 0.18	1300 ± 0.30	1530 ± 0.05
Control without ABTS but with LTV	11.77	2400 ± 0.11	2110 ± 0.09	1980 ± 0.01	1890 ± 0.01
	12.17	1260 ± 0.04	1170 ± 0.17	1220 ± 0.01	1180 ± 0.05
Control without LTV & ABTS	11.84	2150 ± 0.05	2050 ± 0.00	2050 ± 0.04	1980 ± 0.07
	12.17	1260 ± 0.04	1220 ± 0.00	1280 ± 0.03	1180 ± 0.00

The results from GPC analysis showed that OSL could be affected by laccase as some differences occurred to the MWs of the reaction samples. In the 2 group of enzymatic reactions, experiments which contained laccase enzymatic reactions, the most distinctive change was happened to the control reaction sample where there was no ABTS but LTV was in the reaction, in which the estimated MW at the retention time (RT) at 11.77 and 12.17 were reduced progressively by the incubation time whereas there was a MW increase was occurred to the complete reaction sample at 1h (RT=11.94 min) and it dropped at 5h but has not changed much even after 24h of incubation. This indicates that laccase has an effect on MW reduction of the OSL when it applied as catalyst for this reaction, and lignin might be go through polymerisation in the presence of ABTS. In the non-enzymatic control reactions, there was not any significant difference occurred. However, a slightly decrease in MW can also be seen in the control sample with the absence of both LTV and ABTS. Therefore, these findings imply that the OSL reaction is a quite complicated process as there was both an increase (polymerisation) and a decrease in molecular weight (depolymerisation) as found in the enzymatic and non-enzymatic reactions at the early stage of incubation. These basic findings in GPC increased the author's interest in the product analysis of ethyl acetate (EA) extracts of the same reaction samples. Therefore, GC-MS analysis was applied to the products in the organic phase after separating the above aqueous samples using EA as an extraction solvent.

(2) GC-MS analysis

The products extracted into the EA phase from the same reaction as described above were identified by GC-MS and listed in Table 3.3. The chemical structures of products formed from Organosolv lignin degradation by LTV are shown in Figure 3.6.

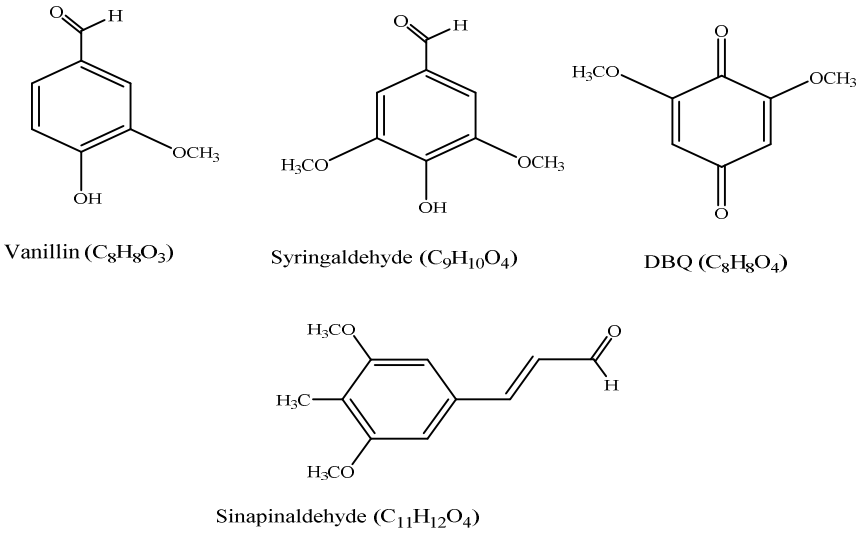


Figure 3.6: Chemical structures of Organosolv lignin depolymerisation products

The product identities were confirmed based on a comparison of mass spectra of authentic standards and the NIST library data (Appendix A.4.1). The data represents the mean of two replicates and standard deviation. Control samples (Organosolv lignin without LTV/ABTS or without both of them) contained the same amount of lignin (29 g/L) as the complete reaction mixtures with LTV and ABTS. The concentration of the released compound was calculated according to the peak area of the products compared to the peak area of the authentic standard (5 mM).

The method applied for reactions from OSL degradation at 30-60 °C in Figure 3.7, Figure 3.8, Figure 3.9 and Figure 3.10 was as follows: A mixture of Organosolv lignin (29 g/L), 16 mL of 100 mM ammonium acetate buffer (pH 4.5), 250 µL of 0.025 mg/mL LTV solution in the same buffer, and 11 mL of 4 mM ABTS was incubated at the temperatures of 30 °C or 40 °C or 50 °C, and or 60°C for 24h with a shaking speed of 200 rpm. Reaction was terminated by adding 10 µL concentrated sulphuric acid to each 2.5 mL of aqueous mixture and centrifuged at 20 °C at 1000 rpm to separate the unreacted solid lignin materials from the rest of the aqueous fraction. A portion from the aqueous fraction (1 mL) sample from the same aqueous fraction after phase separation was extracted using 4 mL ethyl acetate (EA) twice (2 mL each) and the EA layer of 2 times extraction was combined together, and evaporated under a fume hood overnight until its dryness. It then resuspended with acetone and analysed by GC-MS. Complete reaction done in the presence of both LTV and ABTS as Figure 3.7 (a). Control reactions, with ABTS but without adding LTV shown as Figure 3.7 (b); with LTV but without ABTS as Figure 3.7 (c); and in the absence of both LTV and ABTS shown as Figure 3.7 (d) were also performed. The component missed out from any control reaction mixtures was

replaced by equal amount of buffer. All reactions were done in duplicate. Mean are reported with error bars.

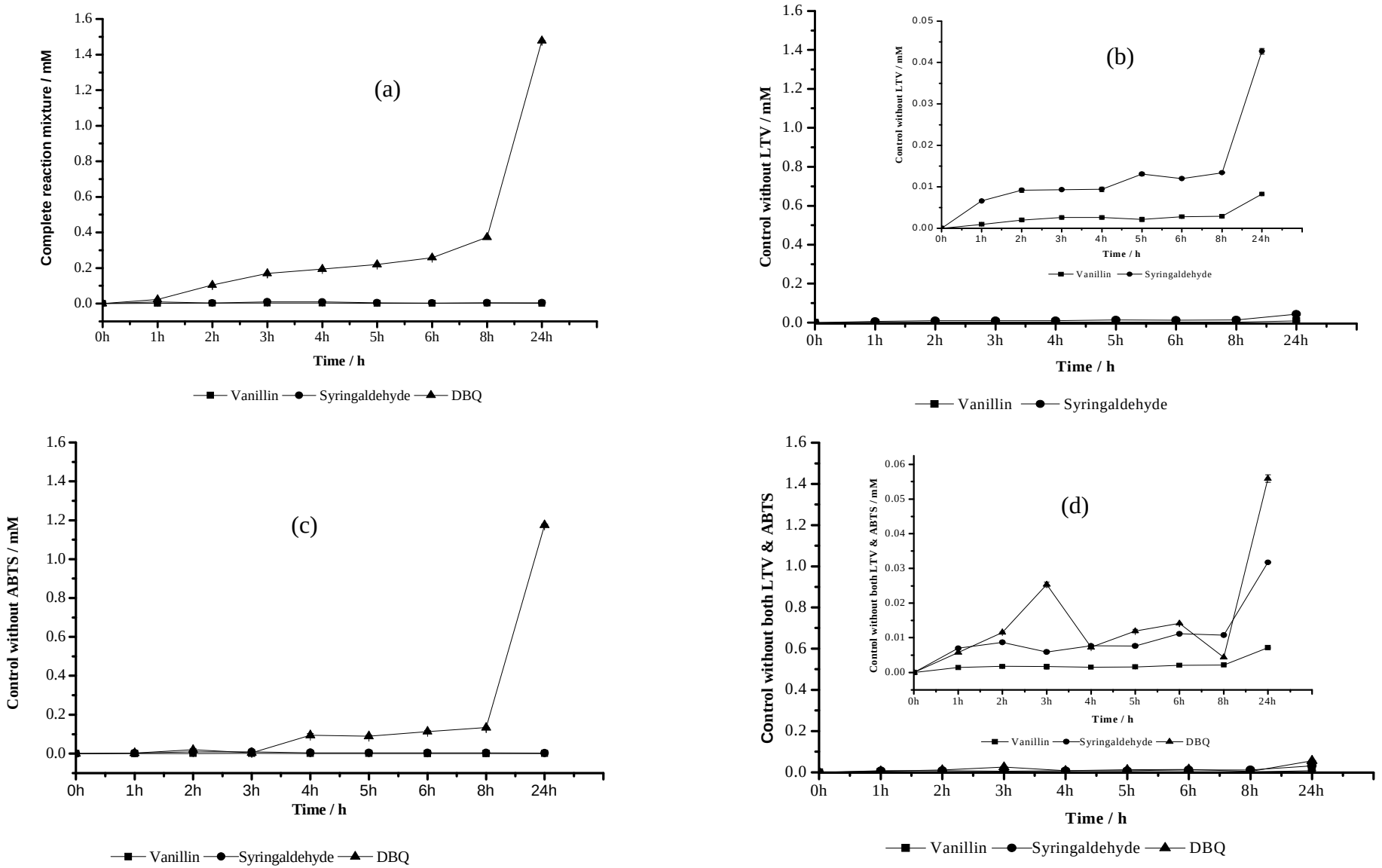


Figure 3.7: The product concentration *versus* reaction time graph for Organosolv lignin degradation products at 30 °C for 24h.

As shown in Figure 3.7, the main compound formed from LTV assisted Organosolv lignin depolymerisation at 30 °C for 24 h was DBQ and its concentration increased as the incubation time increased. Although it was also formed in the control reaction in the absence of ABTS but with LTV (Figure 3.7 (c), but its highest concentration (1.4 mM) was found in the complete reaction sample (Figure 3.7 (a) at 24 h. However, this confirms further that DBQ was only produced where there was laccase present in the reaction. Vanillin and syringaldehyde appeared in all reaction conditions but there was hardly any significant difference has happened to them compared to DBQ at all-time intervals. These results are consistent with previous results which were obtained using HPLC analysis. It implies that LTV contributes to Organosolv lignin oxidative degradation. The observed GC-MS chromatograms (24h as shown an an example) for all samples from this reaction and controls are given in Appendix A.4.1. Other minor peaks that appeared on the chromatogram were considerably smaller, and contamination mainly came from the plastic syringe and filters used in sample preparation, which contained plasticisers such as tributyl phosphate (TBP).

3.5.1.2 Laccase catalysed Organosolv lignin degradation at higher temperatures

The Organosolv lignin reaction with LTV was also investigated at temperatures of 40 °C, 50 °C and 60 °C respectively in order to determine the effect of reaction temperature on the enzymatic depolymerisation products from Organosolv lignin. The method except for the incubation temperature was as same as those of performed 30 °C. The degradation products formed at higher temperatures from Organosolv depolymerisation were analysed by GC-MS after EA extractions. Reactions were performed in duplicate, and the mean was reported.

The GC-MS identified that the major products from Organosolv degradation at 40 °C were as same as those ~~were~~ formed at 30 °C. The product distribution is observed as a form of changes in product concentration versus incubation time (Figure 3.8). However, these compounds also appeared in control reactions without LTV or ABTS at 6 h and 24 h (Figure 3.8 b and Figure 3.8 c). The only difference was that the concentration of vanillin and syringaldehyde after enzymatic treatments were significantly smaller than in the original Organosolv in which there was no LTV and ABTS present in the reaction mixture (Figure 3.8 d). This indicates that some of these compounds might have converted into 2, 6-dihymethoxy-1, 4-benzoquinone (Bozell *et al.*, 1995). The GC-MS chromatograms are shown (Appendix A.4.2).

The time course profile of the samples from the complete reaction mixture and all 3 controls sample with the presence of both LTV and ABTS) are shown in Figure 3.8 based on their concentration variations by time.

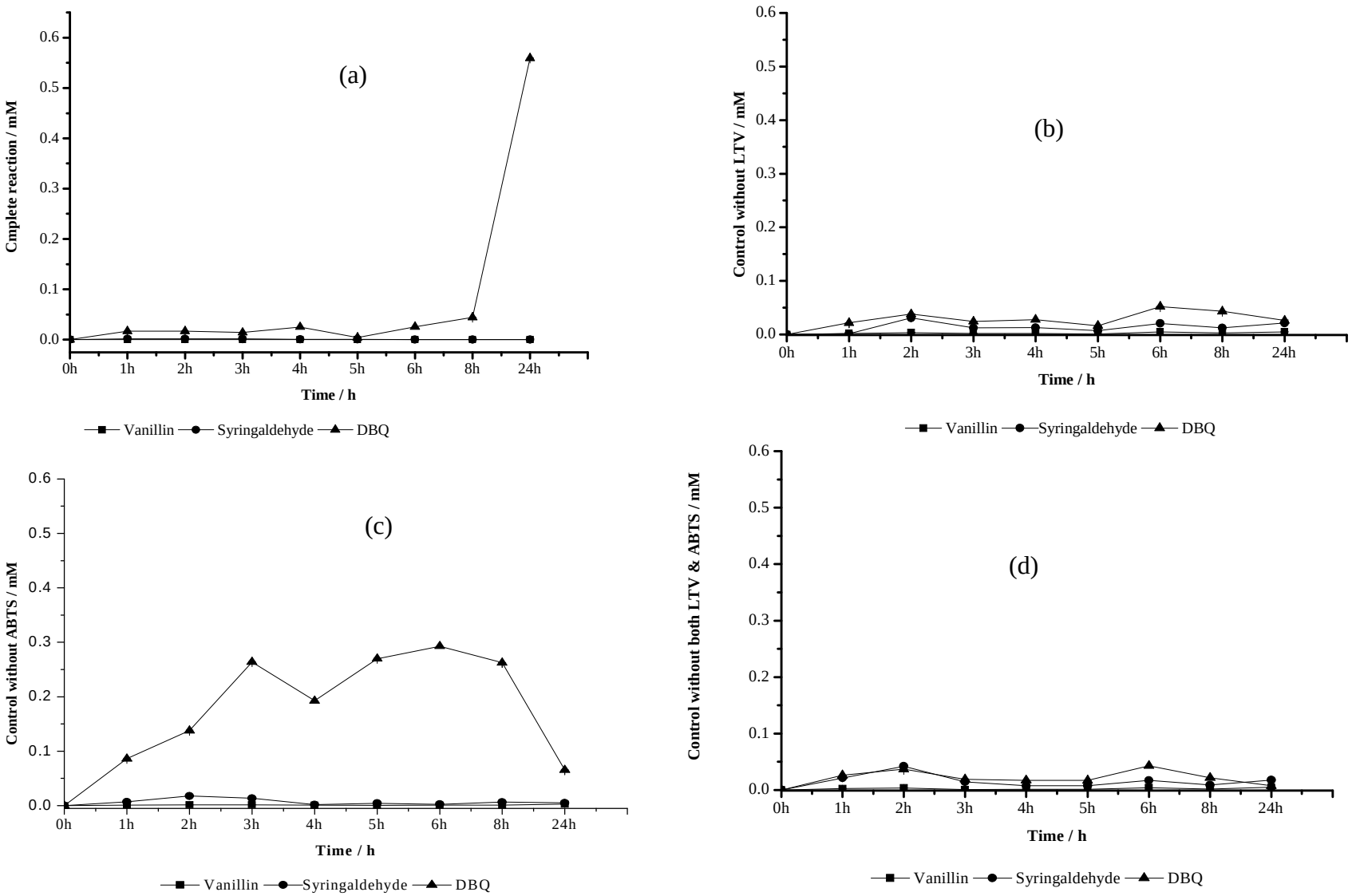


Figure 3.8: The product concentration *versus* reaction time graph for Organosolv lignin degradation products at 40 °C for 24 h.

The main products from the analysis at 40 °C are similar to the findings at 30 °C. In contrast, the concentration of the 2, 6-dihydroxy-1, 4-benzoquinone (DBQ) steadily increased by time and only increased significantly in the presence of laccase as the incubation time increased. However, the amount of DBQ formed in the reaction performed at 40 °C was less than half of that produced at 30 °C. No significant changes occurred in control in which LTV was absent from the reaction. These results indicate that LTV has contributed to Organosolv lignin oxidative degradation, but the reaction temperature has considerable impact on the distribution of products formed.

The main products found from the reactions performed at 50 °C were noticeably different from the observation of degradation at 40 °C (Figure 3. 9). At 50 °C Concentration of products in the each sample were increased compared to product concentrations formed at the lower temperatures. This is because the elevated temperature has increased the solubility of OSL and so more materials were released accordingly. However, the degree of increase is very much similar in both complete and control reactions. Some decline took place to all samples before 3 h. However, a constant increase occurred in the main product (DBQ) after 3 h of incubation until 8 h and then remained almost the same until 24 h. The time course profile of the above reaction samples based on their concentration changes are given in Figure 3.9. It confirms further that the amount of product (DBQ) is related to the incubation temperature and the incubation time. Meanwhile, there was a possibility of lignin repolymerisation with laccase in the reaction at a higher temperature. The GC-MS chromatograms are shown (Appendix A.4.3).

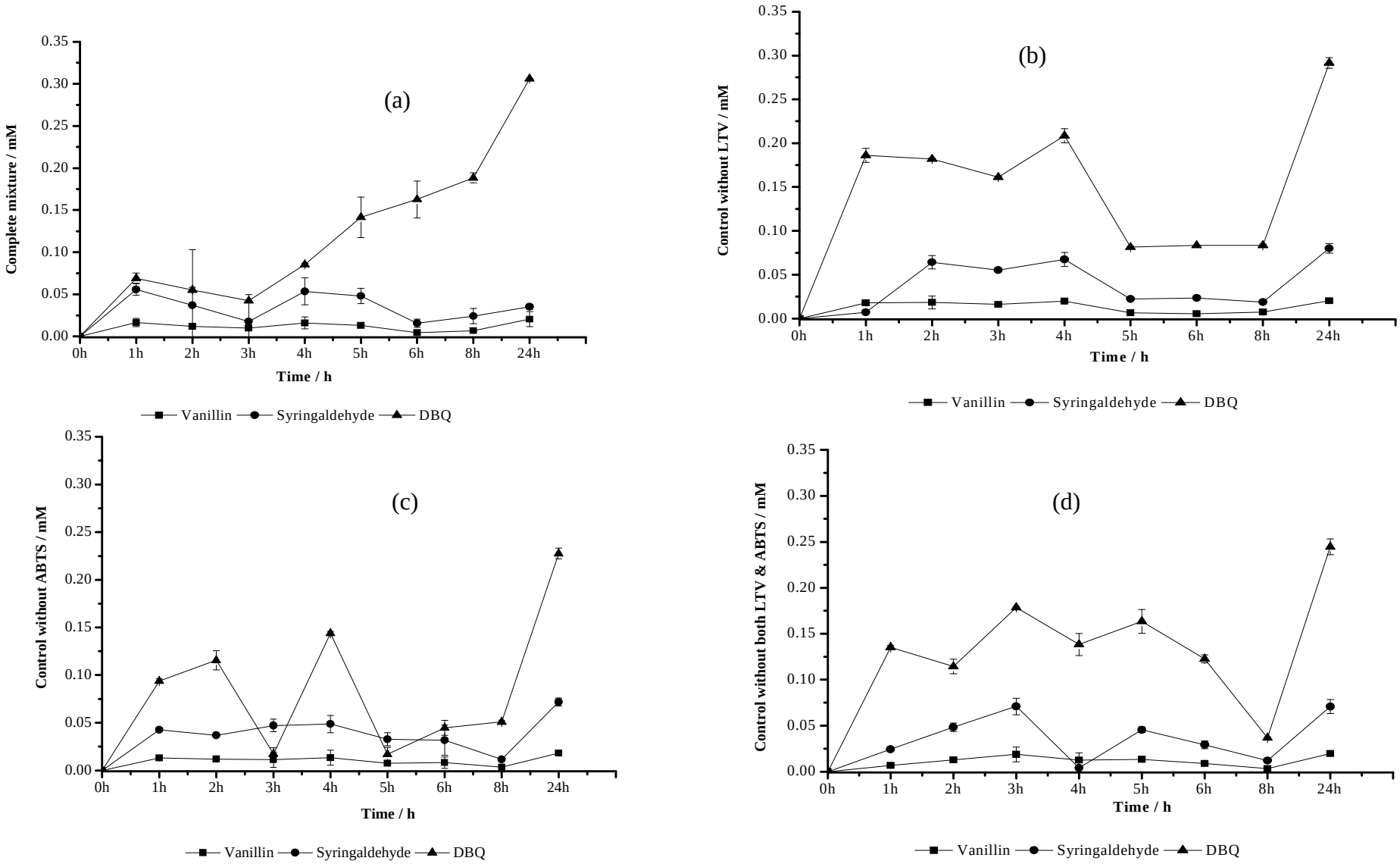


Figure 3.9: The product concentration *versus* reaction time graph for Organosolv lignin degradation products at 50 °C for 24 h.

A further experiment was planned to observe the product distribution for Organosolv degradation at a slightly higher temperature in the presence of LTV.

The major products identified by GC-MS from Organosolv degradation at 60 °C were vanillin, 2, 6-dihydroxy-1, 4-benzoquinone and syringaldehyde again. Nevertheless, these compounds also appeared in the control reactions with and without enzymatic treatment. The concentration of DBQ in the complete reaction had a tendency of increase with the reaction time and the rise happened more distinctively at 24h. However, the level of increase was not too significant and its concentration much lower compared to the reaction done at 30 °C (Figure 3.7). In addition, this trend was almost the same in all reaction conditions tested. The full chromatograms of controls are shown in Appendix. No other new products were identified from the enzymatic treatment of Organosolv lignin at 60 °C. So, this is apparently not a suitable reaction temperature. The time course profile of the above reaction samples based on their concentration changes are given in Figure 3.10. The GC-MS chromatograms are shown (Appendix A.4.4).

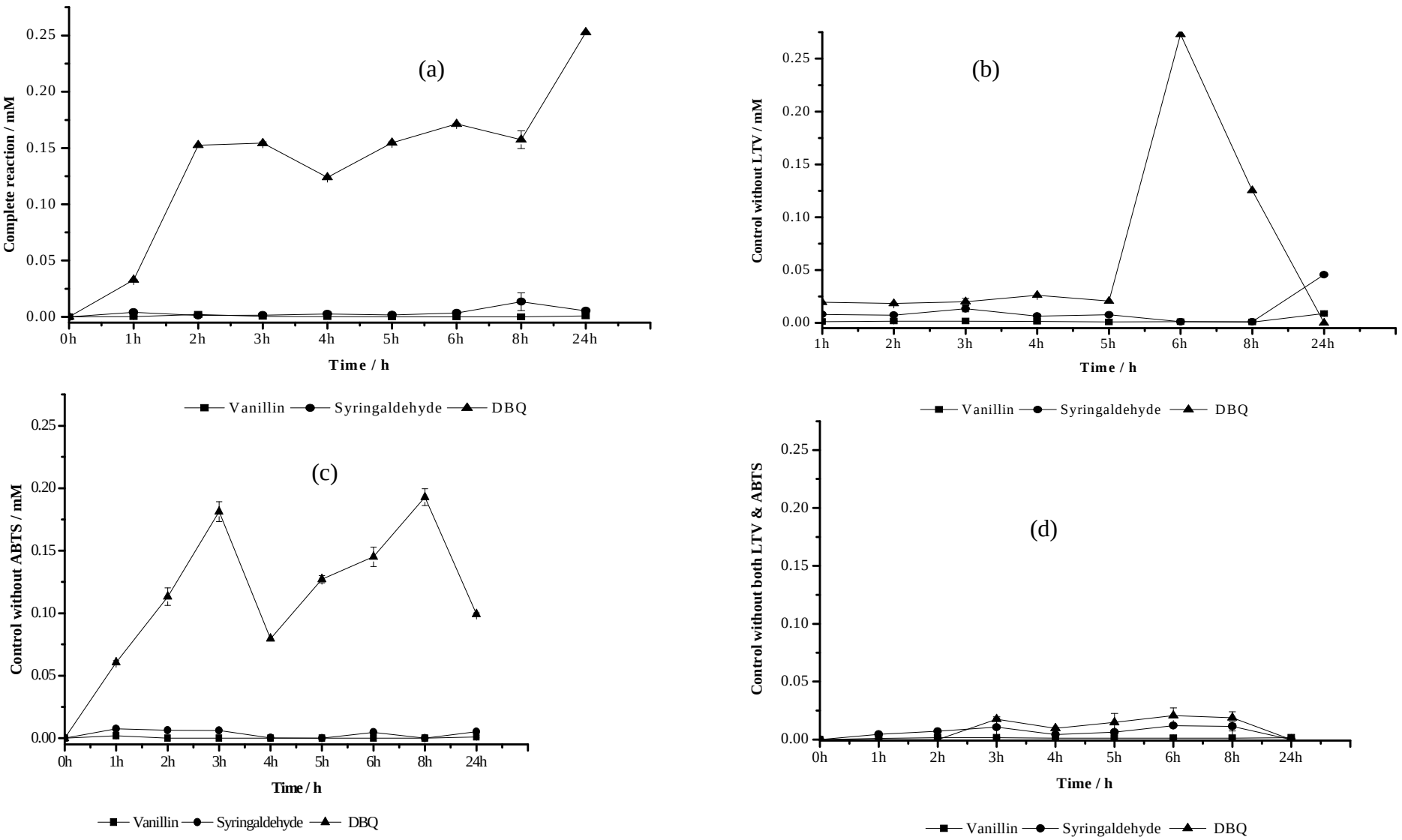


Figure 3.10: The product concentration *versus* reaction time graph for Organosolv lignin degradation products at 60 °C for 24 h.

To conclude, the temperature effect on OSL degradation by LTV could be described as below (Figure 3.11). It indicates that the concentration of DBQ decreased with the incubation temperature increased and it shown a good agreement of the LTV stability experiment in which LTV lost its activity when temperature increased (Figure 3.3). Control reactions were not compared here as the concentrations of these products in them were considerably low.

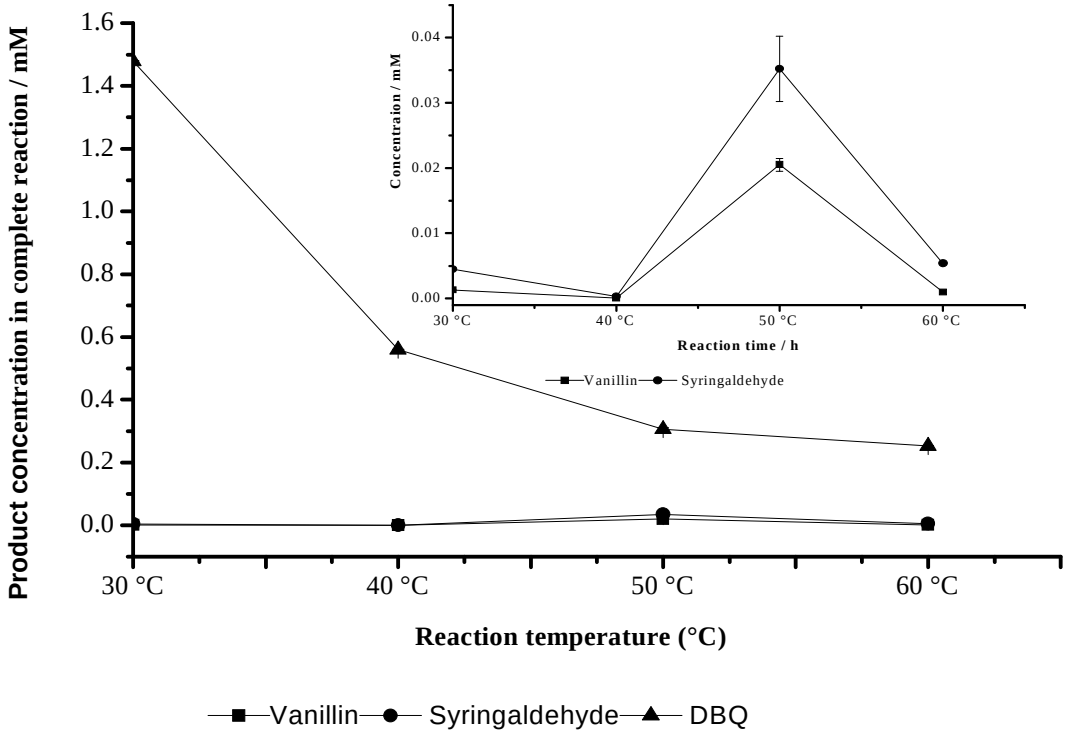


Figure 3.11: The comparison of product concentration versus incubation temperature for the complete reaction from the GC-MS identified from OSL degradation by LTV concentration

The following conclusions can be drawn from the above study regarding the temperature effect on enzymatic treatment of OSL. Laccase has an effect on OSL depolymerisation. The only main product from enzymatic depolymerisation of Organosolv lignin is 2,6- DBQ and its concentration decreases when the reaction temperature increases (Figure 3.11). This result is in agreement with the findings from LTV thermal stability study in which because the LTV almost maintains its activity at the temperatures between 30-40 °C (Figure 3.3). However, 30 °C is the best temperature which suits most to the OSL depolymerisation reactions as DBQ has achieved its highest amount of 1.46 mM (Figure 3.7) at 30 °C. Also 5-6 h of reaction time is more applicable, but the concentration of products increases when the incubation time is extended at a lower temperature. The main products from each incubation

temperature are listed in Table 3.2 so that our conclusion and product identification regarding OSL degradation by LTV will be easier to comprehend.

Table 3.2: The list of main compounds from laccase assisted OSL degradation

Incubation temperature	**GC-MS identified compounds**			
30°C	2,6-dimethoxy benzoquinone	Syringaldehyde		Vanillin
40°C	2,6-dimethoxy benzoquinone	Syringaldehyde	Vanillin	3,5-dimethoxy -4-hydroxy Cinnamaldehyde
50°C	Syringaldehyde	Vanillin	2,6-dimethoxy Benzoquinone	3,5-dimethoxy -4-hydroxy Cinnamaldehyde
60°C	Syringaldehyde	2,6-dimethoxy benzoquinone		Vanillin

3.5.2 The effect of extended incubation time on Organosolv lignin degradation

The previous studies suggested that the optimum reaction temperature for the enzymatic depolymerisation of the Organosolv lignin should be at 30 °C. In addition, it can be found the product concentration versus incubation temperature and time analysis that there was a tendency of continuous rise for the complete reaction sample in all range of temperature investigated. Therefore, the following experiments were set up at 30 °C to understand how the extended incubation time will affect the OSL depolymerisation by LTV among other factors. All reaction conditions were the same as described earlier (3.5.1.1) except the reaction time was extended to 6 days (120 h). Samples were taken at intervals both from the complete reaction mixture and the relevant controls, and then analysed by GC-MS as before. Reactions were performed in duplicate, and the mean are reported with standard deviations. The findings from GC-MS are mainly discussed here to explain the effect of the long reaction hours on the OSL depolymerisation (Figure 3.12). The GC-MS chromatograms are shown (Appendix A.5).

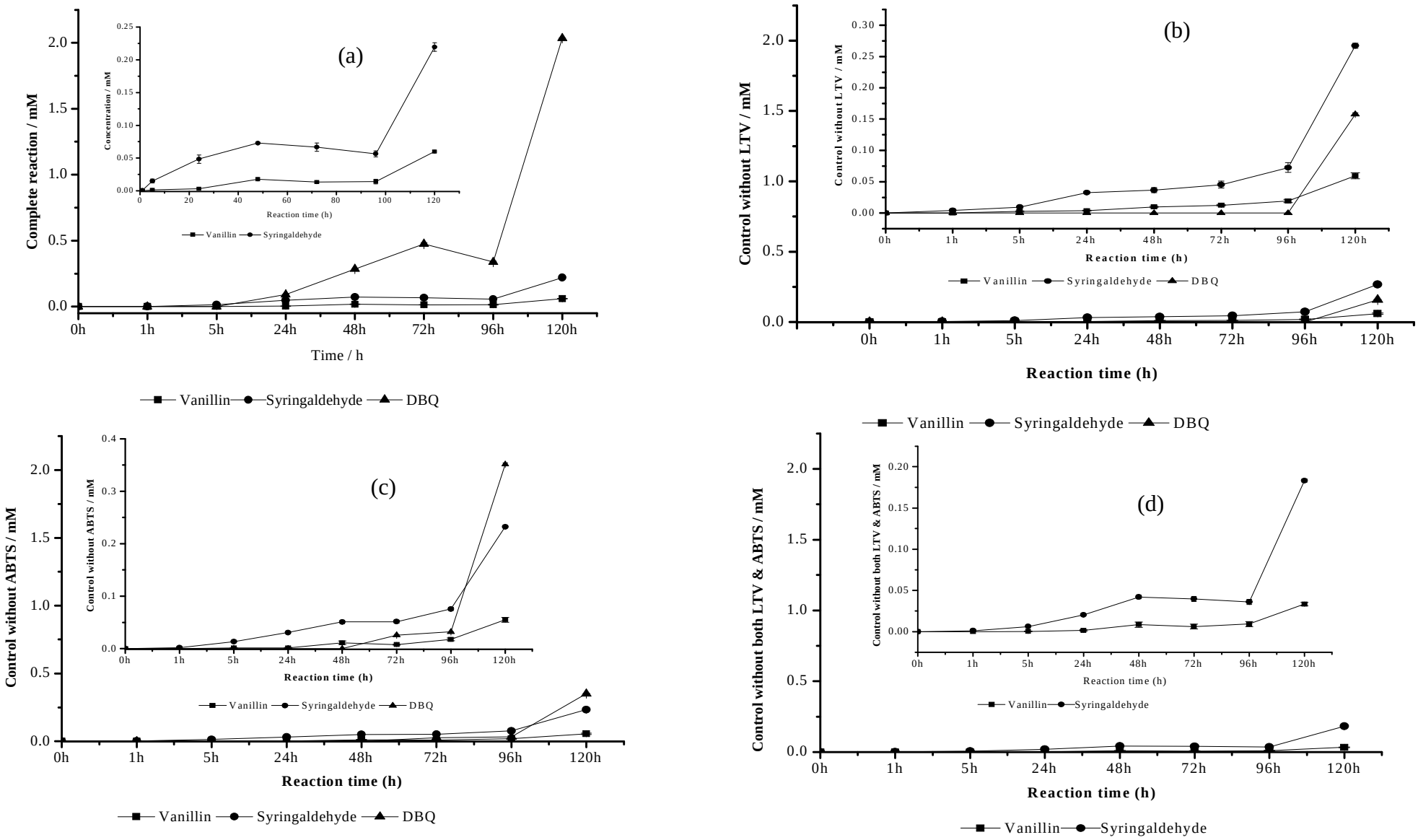


Figure 3.12: The time course profile of Organosolv treatment with LTV at 30 °C for 6 days.

Still, DBQ was found to be a product which formed by the enzymatic degradation of OSL by LTV and this was confirmed the earlier experiment (Figure 3.7). It was obvious that there was no DBQ was found in the control without LTV but ABTS (Figure 3.12 b) and the control without both LTV and ABTS (Figure 3.12 d). The highest concentration (0.09 mM) of DBQ was found in the complete reaction where both LTV and ABTS were present in the reaction (Figure 3.12 a). After 24 h, some changes took place in which the peak areas of main compounds were increased, but there was no formation of new degradation product from OSL. DBQ was also appeared in the control reaction where LTV was absent (Figure 3.12 b) at 120 h and in the control which there was no ABTS but LTV was present, after 72h (Figure 3.12 c) respectively. Though, the amount of DBQ in both cases was only 0.0024 mM and 0.0016 mM, respectively. Therefore, it could be confirmed that 2,6-dimethoxy benzoquinone was the product of LTV catalysed OSL degradation. On the other hand, no remarkable changes occurred to vanillin whereas the concentration of syringaldehyde was gradually increased with time, yet 10-fold smaller than DBQ. In addition, the unusual increase of the amount of DBQ has happened during the analysis, in which OSL was reacted for 120 h (Figure 3.12 a) compared to the amount of DBQ formed at 24h. So, more in depth research has to be done to reconfirm this result. It is not considered as an enzymatic process after LTV deactivated, however, it is possible that thermos-hydrolysis has happened to the OSL with the prolonged reaction time and thus it lead the unusual increase in the concentration of DBQ.

3.5.3 The effect of laccase concentration on Organosolv lignin degradation

The aim of this particular study was to observe whether a change in enzyme concentration had an effect on the Organosolv degradation reaction. The results were confirmed through GC-MS analysis. Reactions were performed in duplicate, and means are reported (Figure 3.13).

As shown in Figure 3.13, Organosolv oxidation with LTV resulted in formation of 2,6-dimethoxy-benzoquinone (DBQ) and its highest concentration (1.46 mM) was obtained when [LTV] at 0.25 mg/ml in the reaction. In addition, when the [LTV] was increased further, the concentration of products from OSL degradation was decreased (Figure 3.15). This might be because the LTV was saturated with degradable fractions of OSL when applied at 1 mg/mL in the designated conditions, and this situation did not change after this point, or a further rise in the concentration may drive the lignin to polymerise back (Bollag *et al.*, 1988, Youn *et al.*,

1995, Mattinen *et al.*, 2008). Therefore, the most suitable enzyme concentration for the OSL degradation by LTV was found at 0.25 mg/ml.

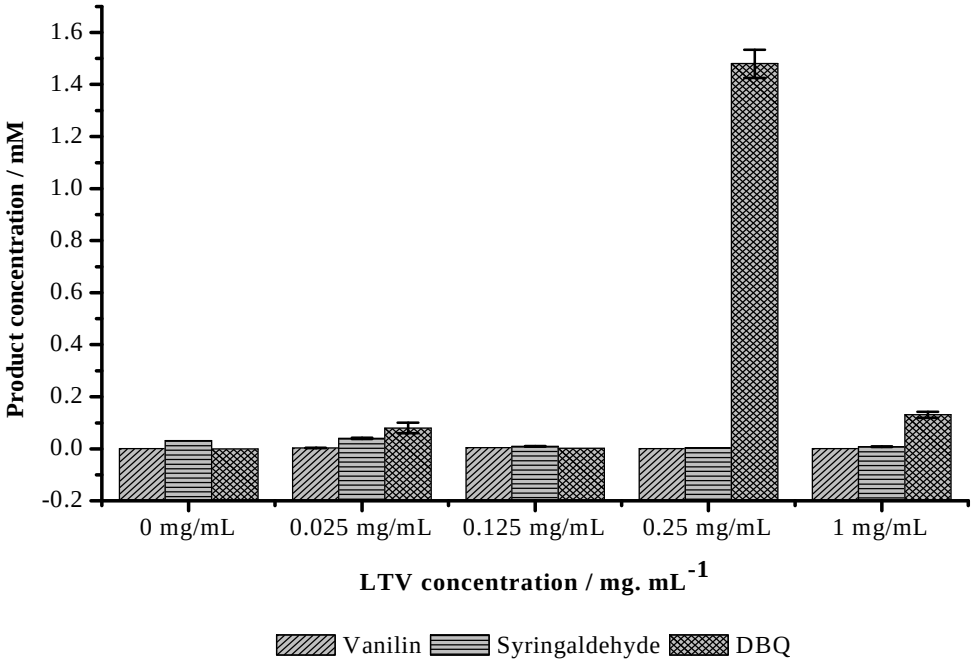


Figure 3.13: The effect of laccase concentration on the degradation products of OSL.

Reaction mixture included 1 g OSL (29 g/L), 4 mM ABTS (11 mL), 100 mM ammonium acetate buffer (23 mL) and 4 different concentration of LTV and incubated at 30 °C for 24 h. Both reaction and analysis were performed in duplicate and mean are reported with a standard error not larger than 5%. The GC-MS chromatograms are shown (Appendix A.6).

3.5.4 The effect of additional LTV and ABTS addition on Organosolv lignin degradation

The LTV catalysed OSL reaction was not improved by increasing the LTV concentration. Therefore, the following experiments were designed to determine the effect of adding LTV or ABTS for a second time during the 24 h of the reaction cycle. LTV and ABTS were added after 5 h of reaction in order to prevent the laccase from deactivation. Control reactions were also performed without the second addition of LTV, ABTS, and without adding both of LTV plus ABTS. The results obtained were identified and confirmed by GC-MS analysis. The reactions were done in duplicate, and the mean are reported with the error bar using the standard deviations between parallel experiments in the same conditions (Figure 3.14).

The GC-MS chromatograms are shown (Appendix A.7).

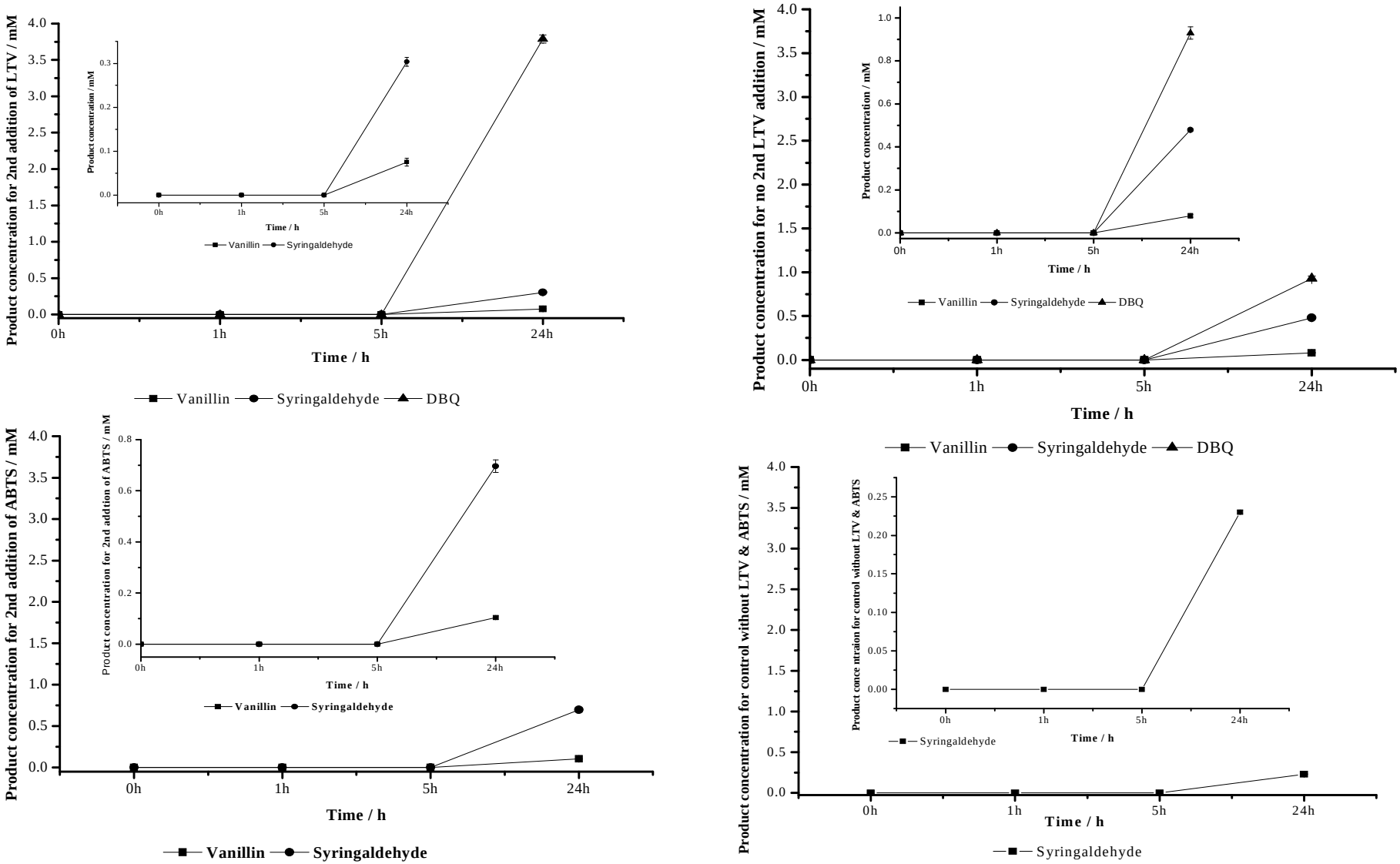


Figure 3.14: The effect of extra LTV & ABTS addition over 24 h of incubation at 30 °C. Original reaction mixture included 1 g OSL (29 g/L), 4 mM ABTS (11 mL), 250 µL LTV (0.25 mg/ mL) and 100 mM ammonium acetate buffer (23 mL). LTV and ABTS were added for second time after 5 h of reaction with the same concentration and amount as they were added at the start of the reaction.

Vanillin, DBQ and syringaldehyde still were the three main products identified from the OSL digestion with extra supply of LTV as expected (Figure 3.16). Luckily, a good improvement of lignin digestion was found when the LTV was supplied for the second time to sustain its activity as the concentration of DBQ significantly increased by 4 times compared to the control reaction without second addition of LTV (0.93 mM). Vanillin was barely affected by ABTS or LTV addition, but the concentration of syringaldehyde decreased with the extra addition of LTV, and increased with the extra ABTS addition. This indicates that actually the increased amount of DBQ may be formed by syringaldehyde oxidation with the laccase. This finding is in agreement with earlier research (Rittstieg *et al.*, 2002). Therefore, it is beneficial to add both laccase and ABTS from time to time into the depolymerisation process to prevent enzyme deactivation and therefore to achieve a better digestion of OSL.

3.5.5 The effect of a co-solvent addition on Organosolv lignin degradation

This study was aimed at improving laccase accessibility to lignin by adding a co-solvent to the lignin degradation reaction so that its enzymatic depolymerisation process could be facilitated. The reason for choosing ethanol was that it is a less expensive and harmful solvent than those organic solvents e.g. methanol, acetonitrile, acetone, isopropanol and DMSO *etc.*) which were screened as laccase benign solvents were tested by some other researchers for same enzyme but from different origin (Uzan *et al.*, 2010, Yan *et al.*, 2015); also it is very convenient to recover ethanol from the chemical reaction by simple distillation and can be reused. In addition, ethanol was also used in the typical Organosolv pulping as a lignin extraction solvent because it is relatively cheap and easy to recover from the process (Johansson *et al.*, 1987). In this study, firstly, the effect of ethanol on LTV was tested at different concentrations of ethanol. The water insoluble OSL degradation by LTV was then performed in the presence of chosen 10 % and 20 % ethanol (v/v) in an aqueous reaction mixture using ABTS as a mediator. Therefore, the current study provides a comparison between the different chemicals that could be released from the enzymatic digestion of lignin with the addition of ethanol and the process where there is no ethanol included. LTV was incubated for 24 h at 30 °C in the presence of ethanol (0-40 %, v/v) and the activity of the samples taken from this mixture was measured using ABTS as a substrate. The initial reaction rate was determined by plotting the OD against the incubation time (Figure 3.15)

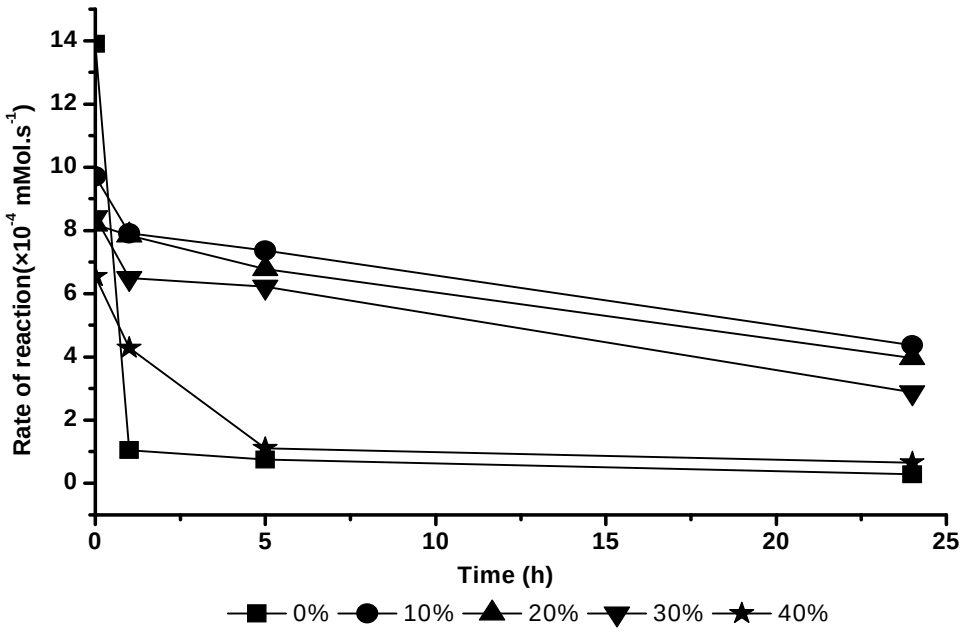


Figure 3.15: The effect of ethanol on LTV activity.

The mixture of 0.025 mg.ml^{-1} of LTV (250 µL), 0.1 M ammonium acetate buffer pH 4.5 (24 mL) and ethanol (0-40 %, v/v) was incubated at 30 °C. The data represent the mean values of three replicate assays; errors were less than 2 %, so the error bars are not shown.

Ethanol inhibited LTV activity since the activity dropped as soon as the first sample was taken immediately after ethanol was added by 70 %, 61 %, 59 % and 47 % respectively compared with the control. LTV lost more than 90 % of the activity after 1 h in the absence of ethanol. Surprisingly, ethanol stabilised LTV, especially in the range 10-30 % (v/v) since only about 43 % (10-20 %) and 46.5 % (30 %, v/v) of activity had been lost after 1 h. When the ethanol concentration was increased up to 40 % (v/v), the enzyme became less stable but still maintained 31% of activity over the first 1 h whereas only 7.5 % of activity was left in the control reaction (Figure 3.15). This result indicates that the ethanol addition enhances LTV stability. Therefore, 10-20 % (v/v) of ethanol was chosen as a reasonable range of co-solvent concentration to be used for the Organosolv degradation process.

Laccase assisted OSL degradation was studied in an aqueous reaction system at 30 °C in the presence of ethanol. The samples from the complete reaction mixture and controls were separated from the solid residue by a centrifuge and extracted by EA prior to GC-MS identification. The aqueous samples were directly analysed by GPC whereas products in the EA extracts were monitored by GC-MS. Both the reaction and analysis were all carried out in

duplicates. Samples from the EA extract were analysed by GC-MS and compared with the NIST library matches for every individual chemical extracted. Product identities were confirmed by comparing the retention time and mass spectra with those of the authentic standards.

The identified major products from the OSL degradation in the presence of 10 % ethanol (v/v) mixture at 30 °C were vanillin, syringaldehyde and syringol (Table 3.3). The chemical structures of products formed from Organosolv lignin degradation by LTV are shown in Figure 3.16.

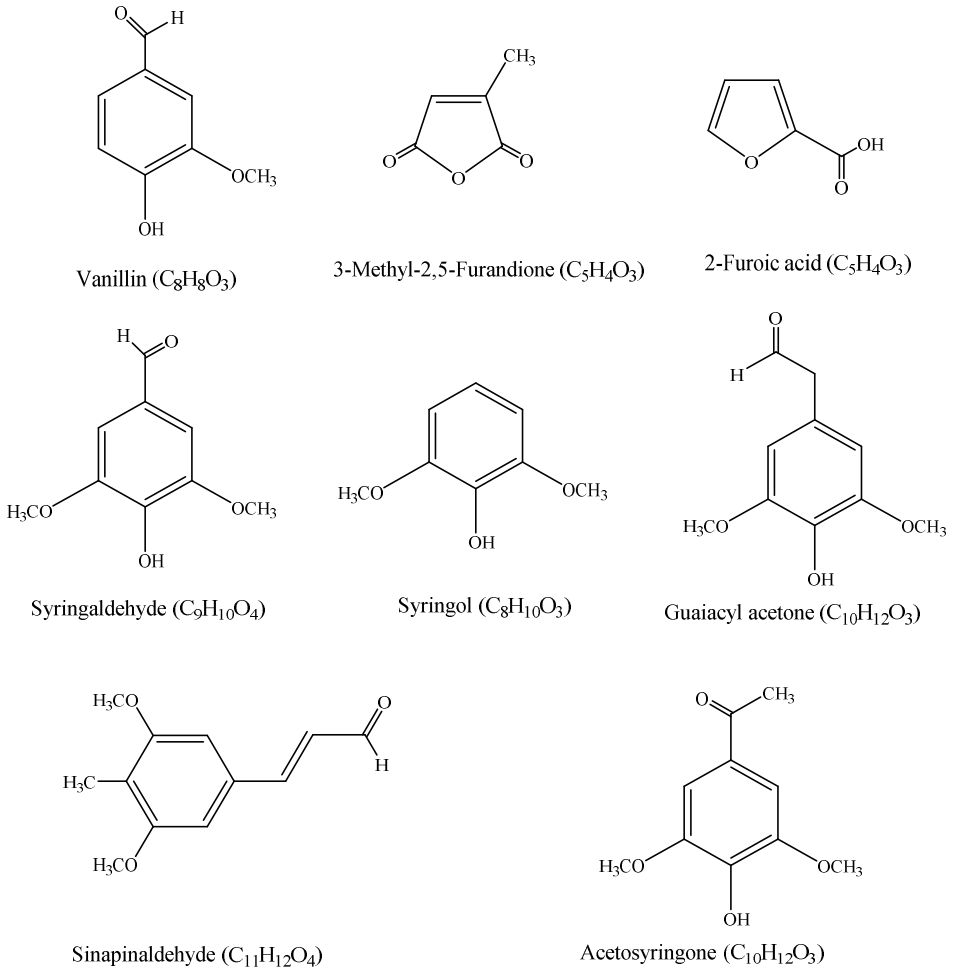


Figure 3.16: The structure of identified chemicals from OSL degradation by LTV in the presence of 10% ethanol.

In addition, guaiacylacetone and acetosyringone were formed as minor products in the control sample where there was no ABTS. However, vanillin and syringaldehyde and all other monomers, identified from the complete reaction sample, also appeared in all three control reactions undertaken (Figure 3.16). Therefore, there is no significant difference

between the reaction sample and controls. As for the 3-methylenedihydro-2,5-furandione which was tentatively identified from the NIST library (itaconic anhydride, RT~ 6 min) and 3,5-dimethoxy-4-hydroxycinnamaldehyde was identified by comparing with the standard (Sinapaldehyde, RT~18 min), and both of them were appeared in every cases regardless of with and without the presence of LTV or ABTS. The GC-MS chromatograms are shown (Appendix A.8.1).

Table 3.3: The compounds identified by GC-MS in the EA extracts from OLS degradation by LTV in the presence of 10 % ethanol (v/v) at 24 h of incubation.

No.	Compounds	Retention time (min)	
		Product	External standard
1	Vanillin	11.24	11.52
2	2,5-Furandione, 3-methyl-	4.46	n.a
3	Syringaldehyde	14.43	14.69
4	Syringol	10.52	10.54
5	Guaiacyl acetone	15.58	n.a
6	Acetosyringone	15.20	n.a

n.a: not available (authentic standard is not available)

They may formed because of the solubilities of some lower molecular weight compounds were increased with the addition of ethanol into the reaction mixture, and were released from OSL material more easily than in the absence of ethanol. The data for the distribution of monomers detected from each case at 24 h was arranged separately in Figure 3.16 according to the peak area changes of each compound. The graph showed that the most significant changes that took place in the compounds after 24 h of incubation were 3-methyl, 2,5-Furandione and syringaldehyde (Figure 3.17).

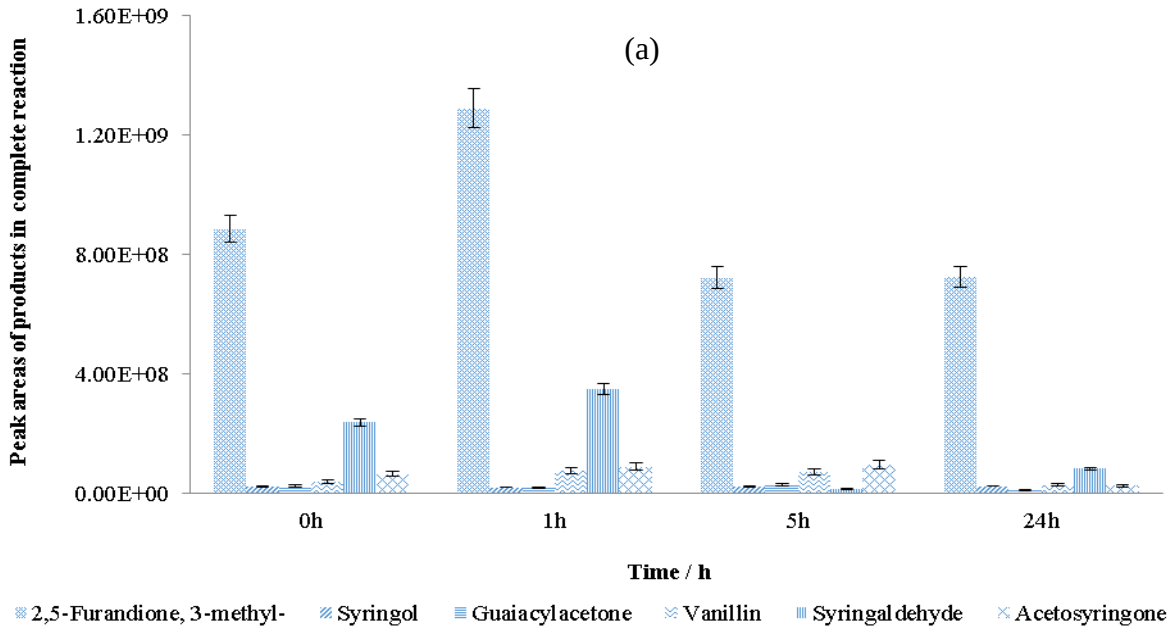


Figure 3.17(a): The time course profile of complete reaction (in the presence of both LTV&ABTS) for the product distribution in OSL degradation by LTV in the presence of 10% ethanol. Data represent the mean of two replicates with the error less than 15%.

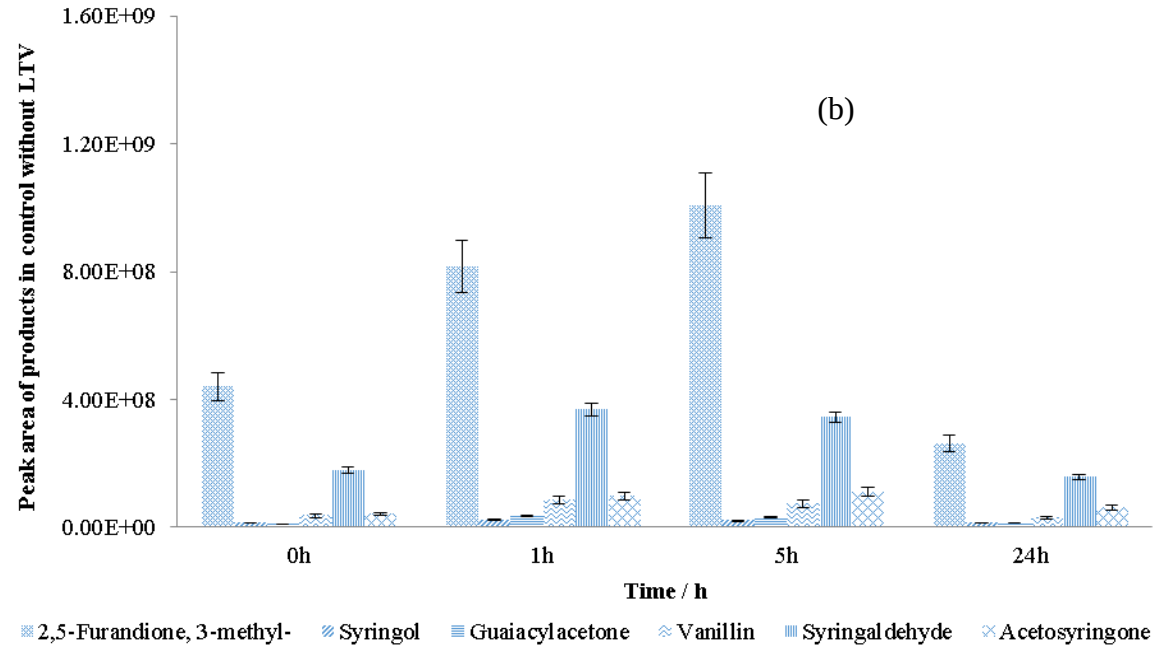


Figure 3.17 (b): The time course profile for control reaction (without LTV but with ABTS) of the product distribution in OSL degradation by LTV in the presence of 10% ethanol. Data represent the mean of two replicates with the error less than 15%.

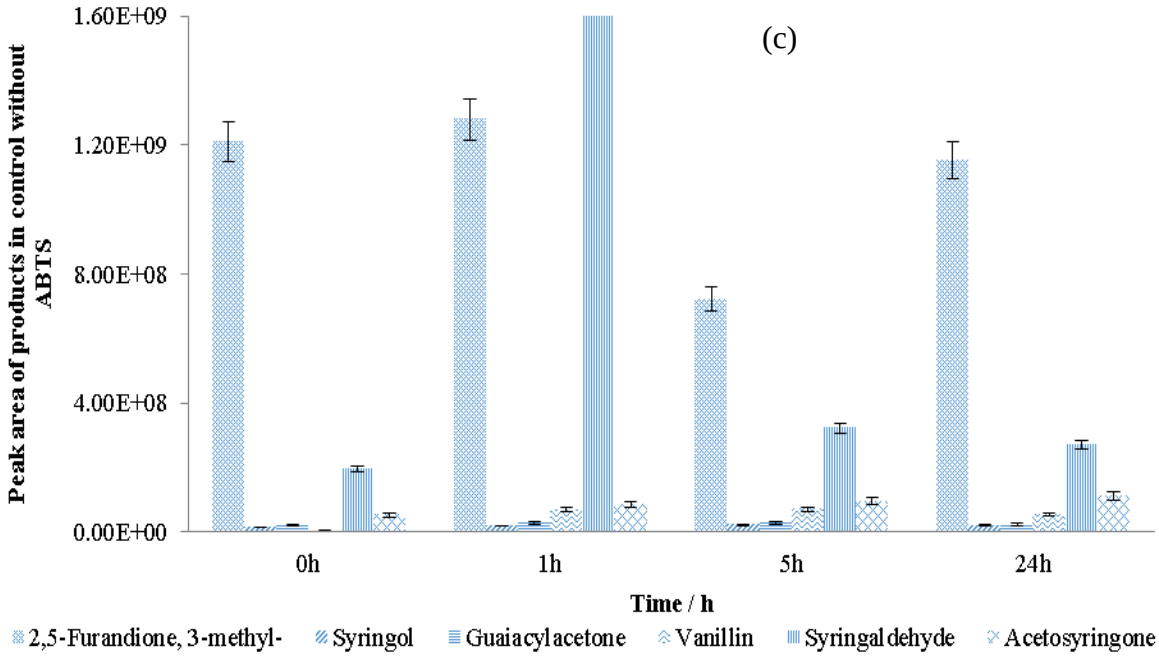


Figure 3.17 (c): The time course profile for control reaction (without ABTS but with LTV) of the product distribution in OSL degradation by LTV in the presence of 10% ethanol. Data represent the mean of two replicates with the error less than 15%.

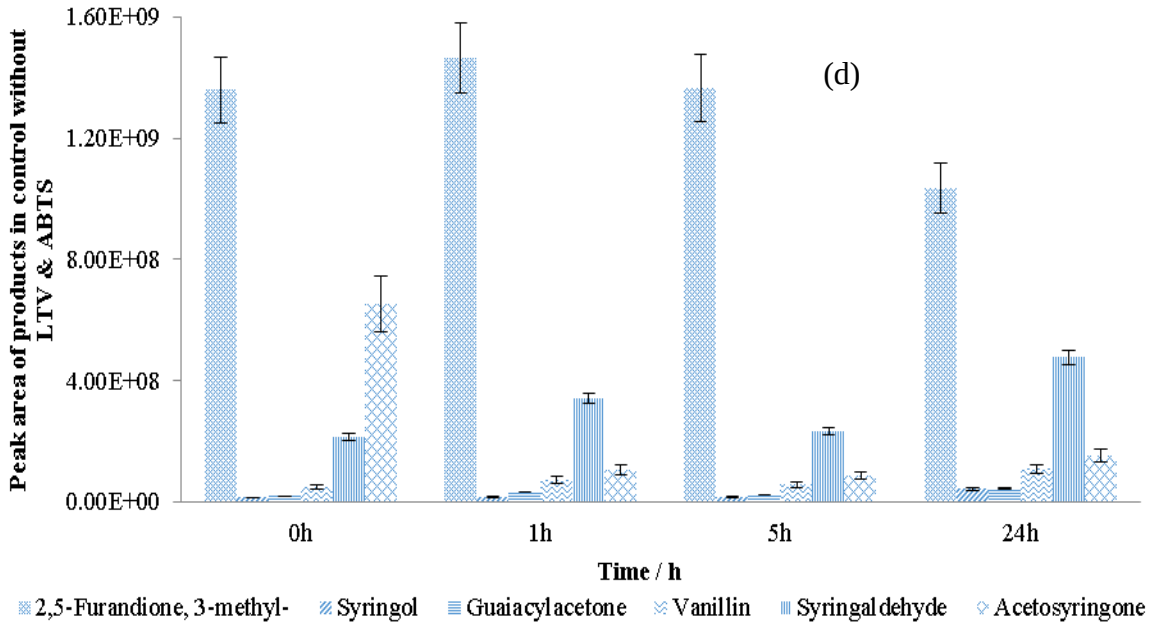


Figure 3.17: The time course profile for the product distribution in OSL degradation by LTV in the presence of 10% ethanol, without the addition of both LTV and ABTS. Data represent the mean of two replicates with the error less than 15%.

All compounds increased during the first 1 h of incubation, and then dropped at 5 h except for 3-methyl- 2, 5-furandione which increased in the absence of ABTS. This compound may come from the OSL material itself and dissolved in the ethanol to be released into the reaction mixtures. For syringaldehyde, there was no considerable effect of adding LTV or ABTS as its peak area increased with increasing incubation time regardless of enzyme or mediator addition (Figure 3.17). One surprising finding was that DBQ was not detected from the samples with ethanol added. Therefore, it is not clear whether the laccase actually oxidised the lignin in the presence of ethanol or some new compounds were released from lignin itself because of their solubility in ethanol.

The OSL degradation using LTV experiment was investigated further in the presence of ethanol (20 %, v/v) with all corresponding control experiments at 30 °C. All other relevant details for performing the reaction, sample preparation and the analysis were as same as the study of degradation of OLS by LTV in the presence of 10% ethanol, which was discussed earlier. Product identities which confirmed by GC-MS analysis of the OSL degradation by LTV in the presence of 20% ethanol are shown in Table 3.4.

In addition to the major products identified by GC-MS, there was a small amount of a few other compounds such as dihydromethylene 2,5-Furandione (RT=5.81 min), butanedioic monoester (RT=7.76 min), 3-methyl-2,4,6-trimetheylphenol (RT=12.33 min), vanillic acid (RT=13.14 min) and 3,5-dimethoxycinnamaldehyde (RT=17.75 min,) which were released from the controls when there was no ABTS or LTV. However, 3-methyl-2, 5-furandione, vanillin and syringaldehyde as well as all other monomers identified from the complete reaction sample also appeared in all three other control reactions undertaken (Table 3.4). The GC-MS chromatograms are shown (Appendix A.8.2).

Table 3.4: Compounds detected from OSL degradation by LTV in the presence of 20% ethanol and their peak areas detected by GC-MS

No.	Compounds	Retention time (min)	
		Product	External standard
1	Vanillin	11.24	11.52
2	2,5-Furandione, 3-methyl-	4.46	n.a
3	Syringaldehyde	14.43	14.69
4	Syringol	10.52	10.54
5	Guaiacylacetone	12.88	n.a
6	Acetosyringone	15.20	n.a

n.a: not available (authentic standard is not available)

Therefore, no significant difference could be seen between the reaction sample and controls for the major products identified by GC-MS analysis. Moreover, a few monomers disappeared in the total reaction sample and control without LTV, but all the main monomers identified from the enzymatic process were formed in the controls without ABTS or both LTV and ABTS. These findings suggest the variation between each case analysed was mainly caused by the ethanol addition since a greater amount of lignin material was dissolved and subsequently released into the solution. Meanwhile, it is hard to identify why the monomers in the complete reaction mixture or control without LTV disappeared due to the enzymatic interaction or perhaps this was caused by some other reason.

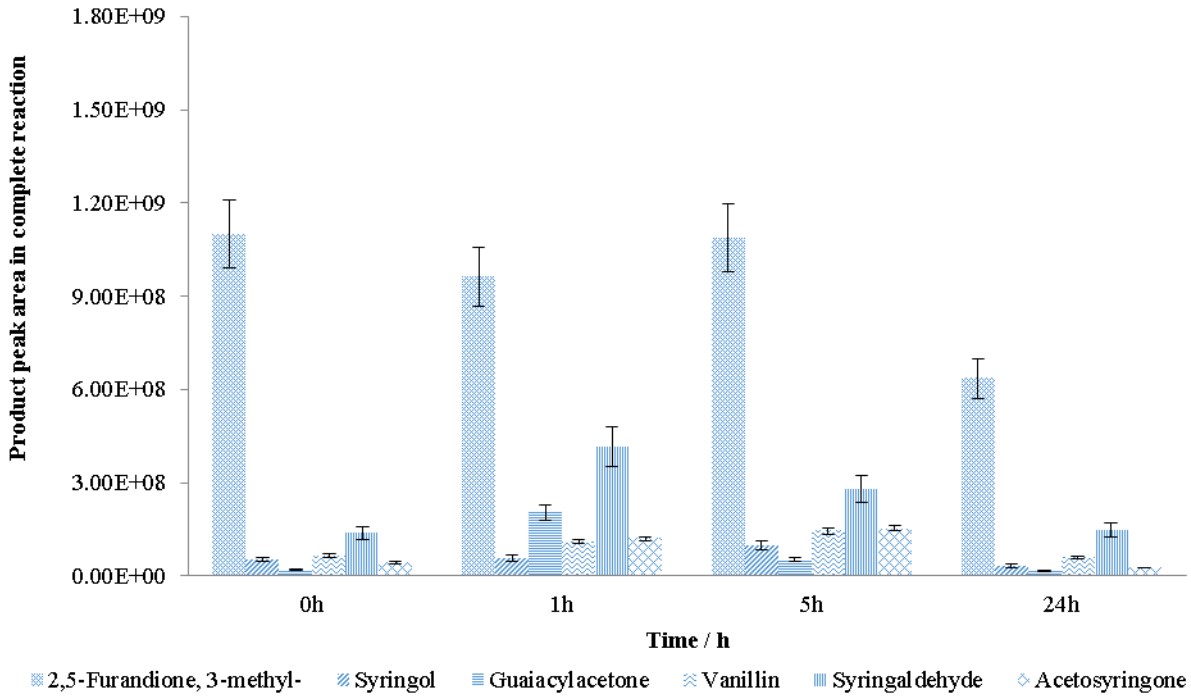


Figure 3.18 (a): The time course profile for the product distribution in OSL degradation by LTV in the presence of 20% ethanol (Complete reaction). Data represent the mean of two replicates with the error less than 15%.

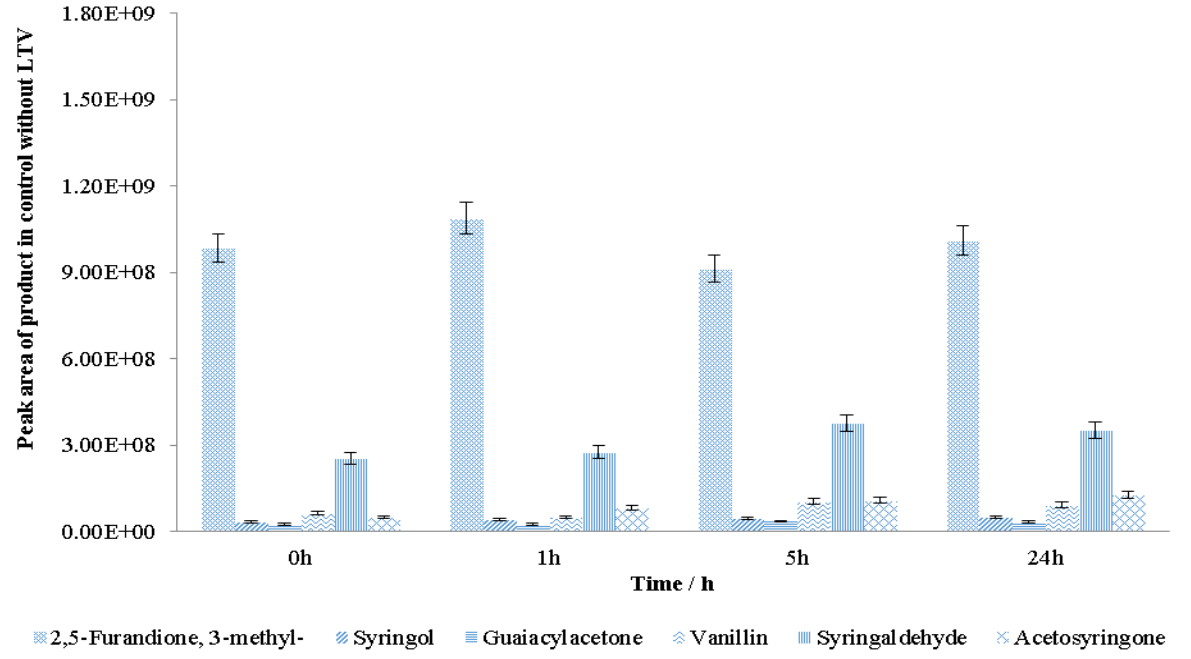


Figure 3.18 (b): The time course profile for the product distribution in OSL degradation without LTV in the presence of 20% ethanol. Data represent the mean of two replicates with the error less than 15%.

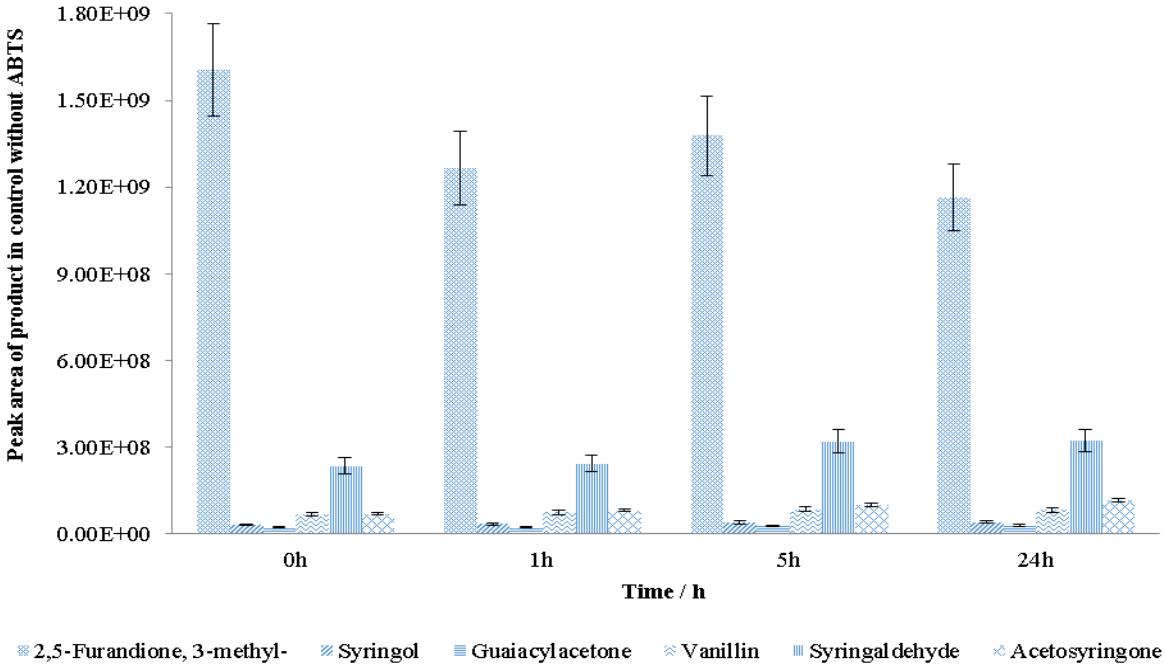


Figure 3.18 (c): The time course profile for the product distribution in OSL degradation without ABTS in the presence of 20% ethanol. Data represent the mean of two replicates with the error less than 15%.

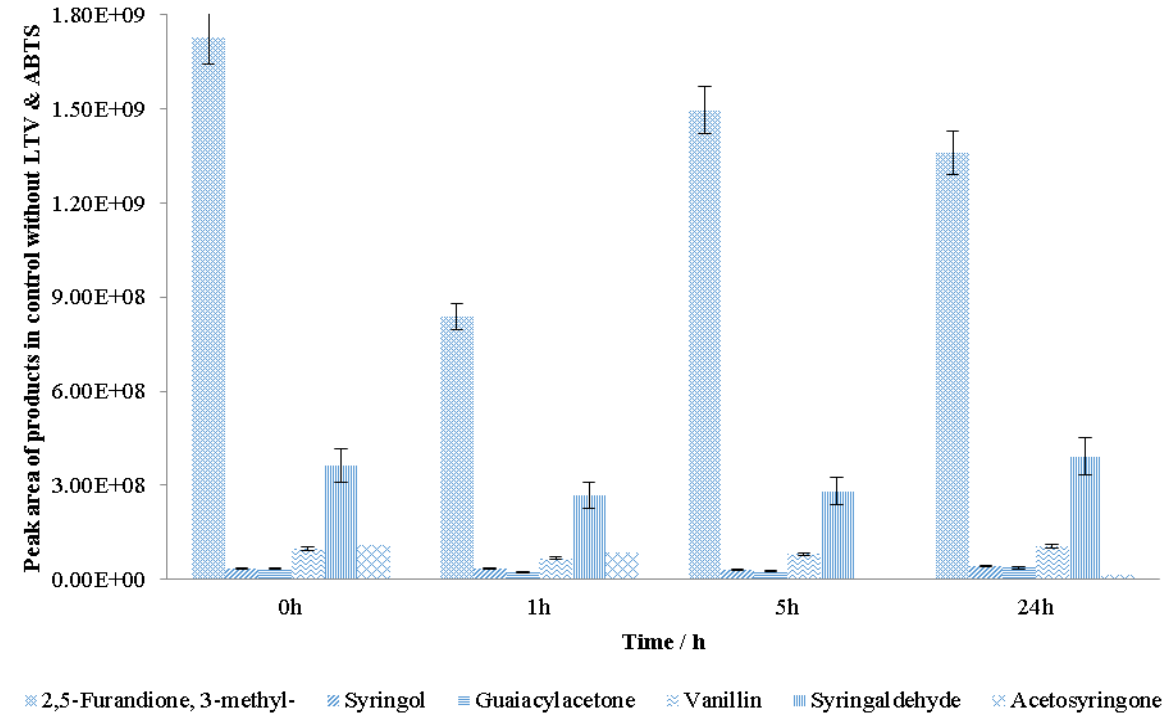


Figure 3.18 (d): The time course profile for the product distribution in OSL degradation in the control without both LTV and ABTS. Data represent the mean of two replicates with the error less than 15%.

Figure 3.18 showed that the most significant changes which formed with the 20 % ethanol addition were 3-methyl, 2,5-Furandione and syringaldehyde. This finding was identical to the results where 10 % (v/v) of ethanol was used in OSL degradation. Other monomers were scarcely affected by the enzymatic or non-enzymatic depolymerisation of Organosolv lignin in all conditions studied. Overall, the 3-methyl- 2,5-furandione decreased after 5 h of incubation in the complete reaction, controls without ABTS and in the absence of both LTV and ABTS except in the control where no LTV was present. This reducing trend became more obvious in the complete reaction where there was LTV and ABTS present (Figure 3.18a). With regard to syringaldehyde, the overall amount increased with increasing incubation time (Figure 3.18 (a, b)). This outcome was similar for vanillin (Figure 3.18 (a, b)). However, a decreasing trend can be seen in the complete reaction sample after 1 h of reaction (Figure 3.18 (a, b)). All other compounds were not affected that much with and without the laccase present.

Conclusions from the optimisation study with respect to the OSL degradation by LTV are as follows:

A considerable amount of effort has been made to depolymerise OSL and to identify the possible factors which might influence the enzymatic reaction. The enzyme activity and the basic depolymerisation reaction conditions have also been studied systematically. One of the major findings from this study was that incubation temperature had a significant effect on laccase performance in terms of OSL depolymerisation, by which a lower reaction temperature (30-40 °C) is more suitable than a higher temperature (50-60 °C) (Figure 3.7, Figure 3.8, Figure 3.9 and Figure 3.10). This is consistent with the thermal stability of LTV (Figure 3.4) although higher incubation temperatures improve the mass transfer between the reaction materials and should enable more efficient lignin digestion; this is not applicable to the enzymatic process, as the lower temperature is crucial to maintain enzyme activity (Figure 3.3). The results obtained from the OSL degradation from 30 °C to 60 °C showed that that Therefore, a further extension of the incubation time was suggested at a lower operating temperature (Figure 3.7). However, there was no increase in degradation products compared to the control and no new products appeared even after a six days long incubation (Figure 3.11).

LTV concentration has an important influence on OSL degradation as the highest concentration of DBQ, the main product from OSL oxidation, was formed with the laccase at

0.25 mg. ml^{-1} (Figure 3.13). Similarly, its activity is a key point to stress as the amount of DBQ increased by 39 % compared with the reaction when there was no extra supply of LTV into this reaction system (Figure 3.14). Another constructive result achieved by this study was that LTV can be stabilised in the presence of ethanol up to 40 % (v/v) (Figure 3.15). Therefore, this result might be useful to run an enzymatic reaction in a co-solvent to improve substrate binding to LTV and also for water-insoluble compounds (for example, lower molecular weight compounds such as veratryl alcohol or some other dimers etc. However, using ethanol as co-solvent did not improve the OSL oxidative degradation by LTV.

3.6 Peroxidase catalysed oxidative degradation of Organosolv lignin

Lignin peroxidase (LiP) and manganese peroxidases (MnP) were reported to have higher redox potential and were able to oxidise both phenolic and non-phenolic structures of lignin, and therefore had wider substrate ranges compared to laccases (Valli *et al.*, 1990, Tuor *et al.*, 1992, Call & Mücke, 1997, Hofrichter, 2002, Kapich *et al.*, 2005, Wong, 2009). However, peroxidase-assisted lignin oxidation is a very challenging task due to the complex nature of the lignin polymer itself and also based on the fact that peroxidases are not very stable during oxidation reactions (Aitken & Irvine, 1989, Qiu *et al.*, 2009, Asgher *et al.*, 2012, Yadav *et al.*, 2012). In this project, both enzymes were investigated to understand their properties by developing their assays and then used for degrading OSL to identify their oxidation ability with regard to lignin related complex polymers.

3.6.1 Enzymatic degradation of OSL using lignin peroxidase (LiP) as catalyst

3.6.1.1 The enzymatic activity assay of LiP

This study sets out to confirm and optimise the activity of LiP using veratryl alcohol as a substrate. It is influenced by the concentrations of LiP, VA and H_2O_2, the operating temperature, incubation time and the pH value of the buffer.

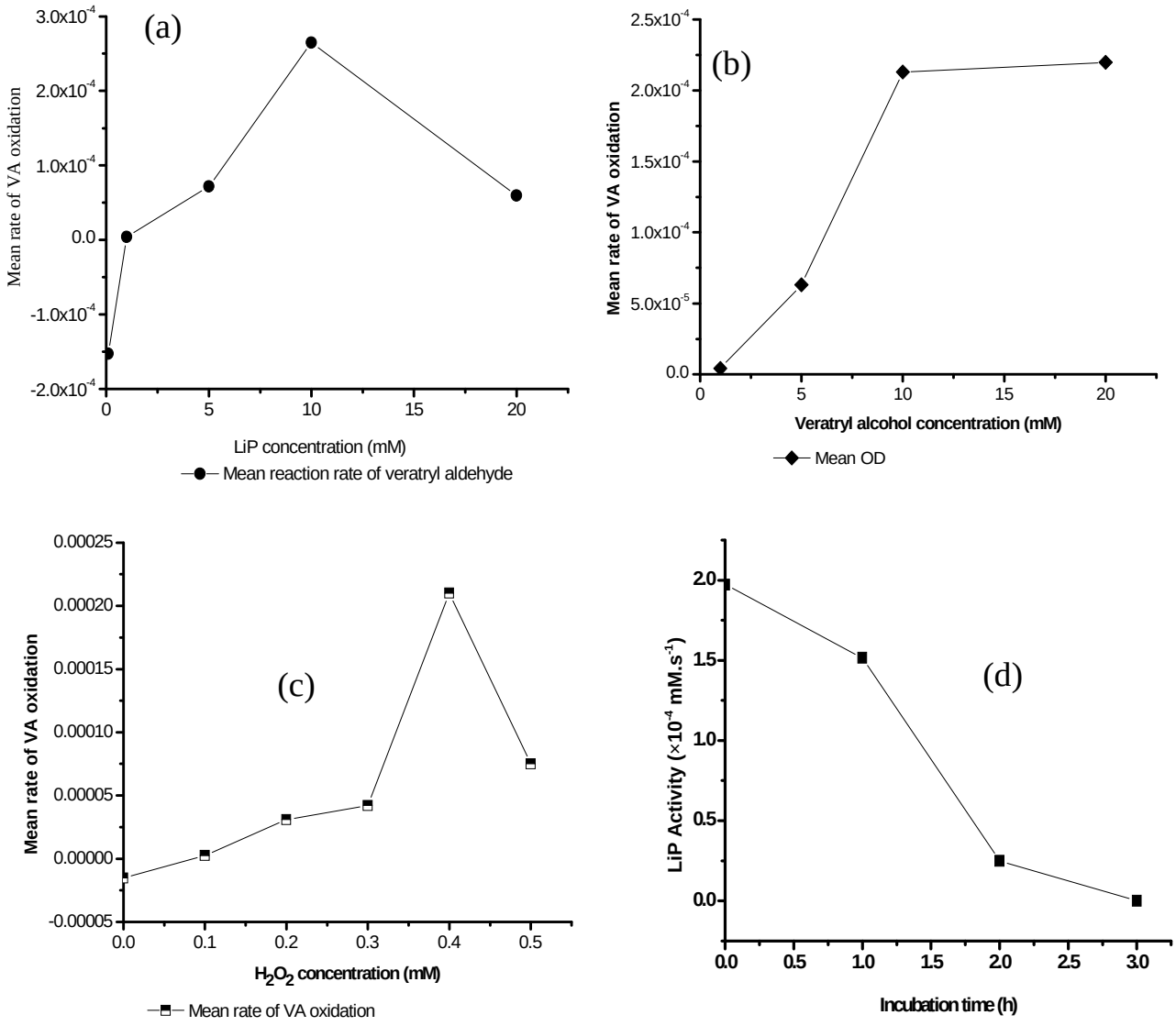


Figure 3.19: The oxidation of veratryl alcohol by LiP. (a) The effect of VA concentration; (b) The effect of LiP concentration; (c) The effect of H_2O_2 concentration; (d) The stability of LiP versus incubation time. Date represents of triplicated measurements with the standard deviation of less than 0.05. Therefore, they were not shown in the graph.

To this end, a spectrophotometric assay of veratryl alcohol oxidation using LiP was examined under various reaction conditions. However, factors such as pH and temperature were chosen according to a brief literature survey (Table 2 in the Appendix A.9) and used as stated in the literature (Koduri & Tien, 1994, Tien & Ma, 1997). The reaction mixture contained sodium tartrate buffer, VA, LiP and H_2O_2. The reaction was initiated with the addition of LiP.

As shown in Figure 3.18, the reaction rate of veratryl alcohol oxidation to veratryl aldehyde is affected by many factors. At the beginning of the reaction, the reaction rate increased

smoothly with the lower concentrations of substrate (VA) or enzyme (LiP) or its co-substrate (H_2O_2). However, this rate did not increase notably by adding more substrate (VA) into the assay mixture when all the other conditions remained the same. This result indicated that the LiP was already saturated with 10 mM of VA and the extra addition of veratryl alcohol did not cause the reaction to perform any faster (Figure 3.19 a). The same took place with the LiP concentration, however, the reaction slowed down with an increase in the amount of LiP as its oxidation rate decreased from 0.26 $\mu M^{-1}.s^{-1}$ to 0.06 $\mu M^{-1}.s^{-1}$ when the concentration of LiP changed from 10 $mg.ml^{-1}$ to 20 $mg.ml^{-1}$ (Figure 3.19 b). The most suitable concentration was found at 0.4 mM for the H_2O_2 as the rate dropped once its concentration rose (Figure 3.19 c). Finally, the activity of LiP was measured against its incubation time at 28 °C (Koduri & Tien, 1994, Chung & Aust, 1995). It turned out that LiP is stable during the first 1 h although it lost activity by 13 % whereas LTV was still maintained 55 % of its activity at least for the first 6 hr at 30°C. LiP activity almost vanished after 2 h and was fully deactivated after 3 h of incubation. Therefore, the recommended time for LiP catalysed oxidation assays is not more than 3 h (Figure 3.19 d). The veratryl alcohol oxidation by LiP was appeared to follow Mechaelis-Menten kinetics; however, there were not enough data points from this experiment to determine the kinetic parameters (K_m and V_{max}) of the reaction yet.

3.6.1.2 Attempt to degrade OSL using lignin peroxidase

The oxidative depolymerisation of OSL was investigated using LiP as a catalyst. The reaction was performed under the most suitable conditions for LiP activity as previously determined in section 3.6.1.1. The reaction mixture included OSL (57.14 g/L), sodium tartrate buffer (50 mM, pH 3), LiP (10 mg/ mL), veratryl alcohol (10 mM), and H_2O_2 (0.4 mM). Total volume was 12 mL. Control reactions without adding LiP and H_2O_2, VA, and without all of the above were also performed. The method was as same as in the complete reaction mixture but the components omitted were replaced with equivalent volumes of tartrate buffer. All reaction vessels were incubated at 28 °C at a shaking speed of 200 rpm. Reactions were observed for a 24 h time interval and terminated by adding concentrated sulphuric acid into the cooled mixtures. The samples were centrifuged at 20 °C, 10,000 rpm for 5 min to separate the solid residues and the aqueous phase. Part of the aqueous phase was extracted with ethyl acetate and analysed with GC-MS to identify the possible depolymerisation products released from the OSL. However, none of the samples extracted with ethyl acetate contained any

products as there was nothing that could be observed from the GC chromatograms after repeated analysis. It might be caused by column contamination or other unknown reasons too.

3.6.2 Enzymatic degradation of OSL using manganese peroxidase (MnP) as catalyst

3.6.2.1 The enzymatic activity assay of MnP

The enzymatic activity of MnP was investigated as a parallel study to the previously optimised LiP assay and to test its oxidation ability on the oxidative degradation of OSL.

The initial rate of Mn (III) malonate formation was measured spectrophotometrically at 270 nm using $MnSO_4$ as a substrate to determine the activity of LiP. This oxidation took place in the presence of H_2O_2 and the addition of ABTS as an assisting agent. The assay mixture (1 mL) contained a sodium malonate buffer (100 mM, pH 4.5), $MnSO_4$ (0.25 mM), and H_2O_2 (0.4 mM) and ABTS (4 mM). The reaction was initiated at 25 °C by the addition of MnP solution in a buffer (5 mg. ml^{-1}). The potential factors that influenced this oxidation were carefully investigated. They were the concentrations of MnP, $MnSO_4$, ABTS, H_2O_2 as well as the operating temperature and the pH value of the working buffer. The factors such as pH and temperature were chosen according to a brief literature survey (Table 3 in the Appendix A.9) and used as stated as in the literature.

The results showed that the rate of Mn^{3+} formation is affected by several factors. First of all, the concentration of the enzyme was varied to identify the most suitable amount of MnP. The reaction rate increased steadily to a maximum at a MnP concentration of 0.05 mg/ mL (Figure 3.30 a). In the case of observing the substrate concentration, the reaction rate reached a maximum rate of 2.65 $\mu M^{-1}.s^{-1}$ when the concentration of Mn^{2+} was 35 µM (Figure 3.20 b). The oxidation reaction by MnP also followed a Mechaelis-Menten kinetics, but too few data points were available to measure K_m and V_{max} from this experiment. The optimum concentration of H_2O_2 was at 0.2 mM and the reaction was slowed down when the concentration increased (Figure 3.20 c). This is because a high concentration of H_2O_2 might prevent Mn^{2+} from binding or it could possibly destabilise the MnP. On the other hand, in the presence of ABTS during Mn^{2+} oxidation, H_2O_2 has been shown to enhance and extend the MnP enzyme performance (Collins *et al.*, 1998). Therefore, the optimum concentration of ABTS for MnP oxidation of Mn^{2+} was also tested and its most suitable amount was found at 3 mM (Figure 3.20 d). Last, but not least, the stability of MnP is a very important issue to be considered. Therefore, it was incubated up to 6 h to identify its activity changes (Figure 3.20

e). Overall, its activity decreased slowly but surely as the incubation time increased and it was totally deactivated after 6 h. This suggests that enzymatic transformation using MnP could be a preferred choice to consider, yet, its stability is a key factor to bear in mind.

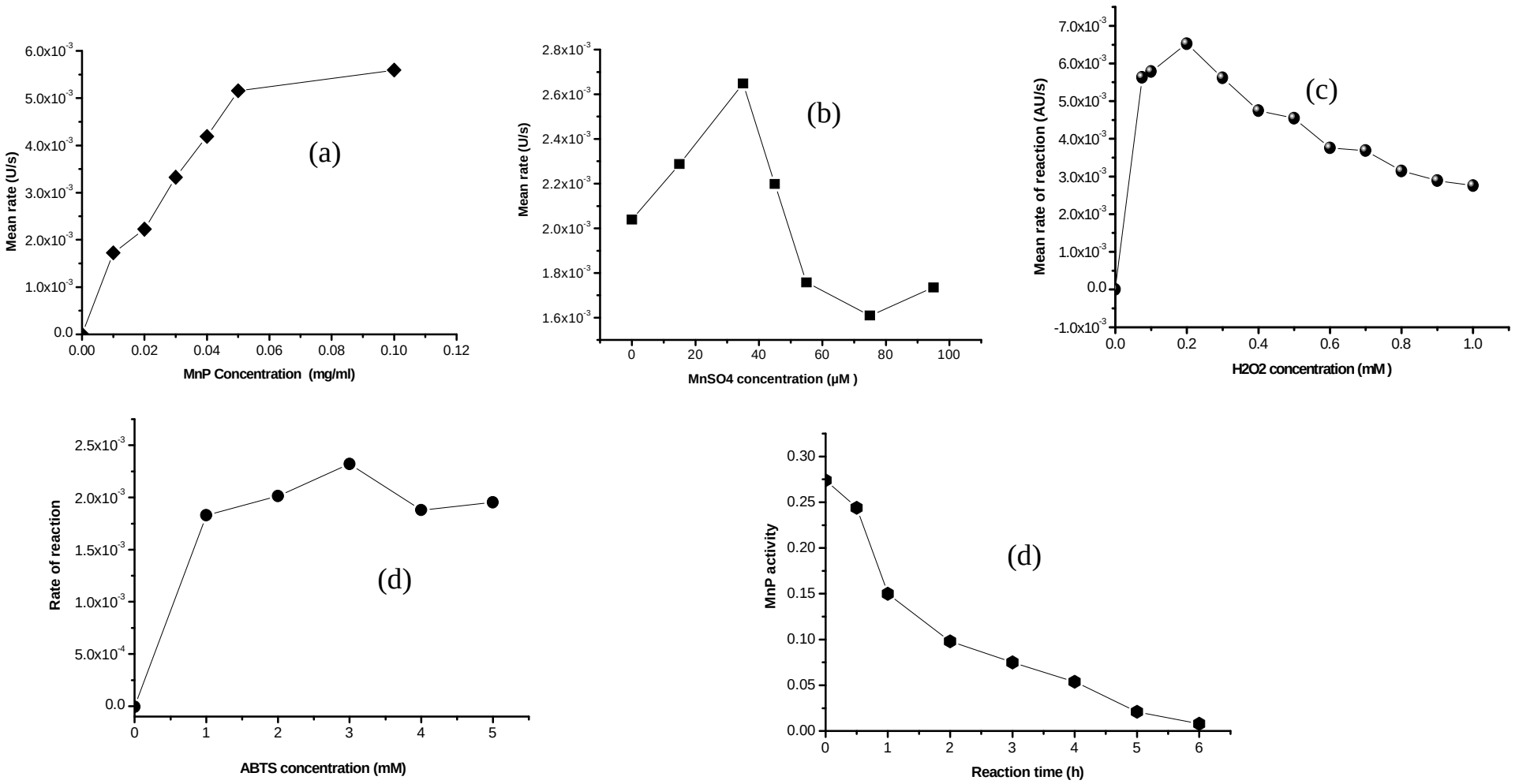


Figure 3.20: The MnP catalysed oxidation of Mn (II) to Mn (III) using as catalyst.The measurement was conducted to establish the MnP catalysed Mn^{3+}reaction conditions by identifying the optimum reaction condition for the following influential factors:
(a) The effect of MnP concentration; (b) The effect of $MnSO_4$ concentration; (c) The effect of H_2O_2 concentration; (d) The effect of ABTS concentration; (e) The stability of MnP versus incubation time. Date represents of triplicated measurements with the standard deviation of less than 0.1. Therefore, they were not shown in the graph.

3.6.2.2 Attempt to degrade Organosolv lignin using manganese peroxidase (MnP)

A manganese peroxidase catalysed Organosolv lignin oxidation experiment was performed in order to examine the catalytic ability of MnP to depolymerise lignin. The results are shown in Figure 3.21. GCMS chromatograms are shown in Appendix A.10.

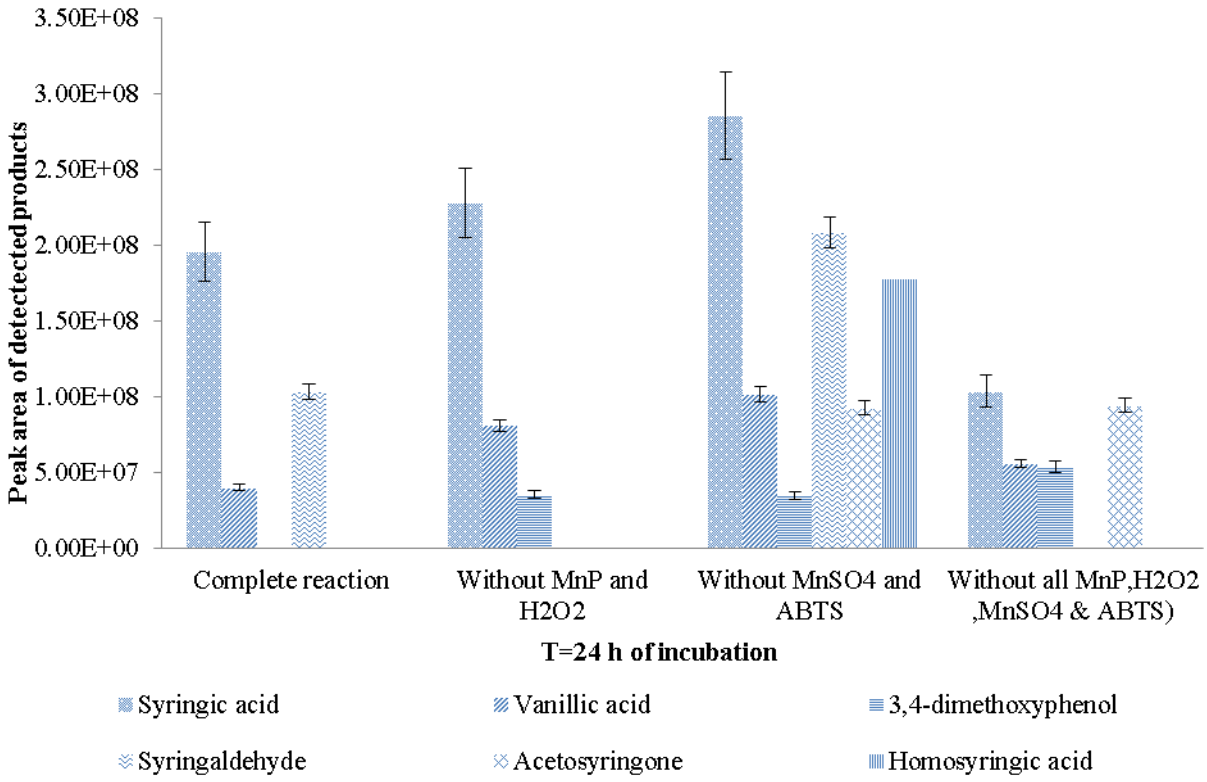


Figure 3.21: The product distribution in ethyl acetate extracts from Organosolv degradation using MnP as a catalyst. Data represents the mean value of two replicates.

The chemicals identified from OSL depolymerisation by MnP are shown in Fig. 3. 22.

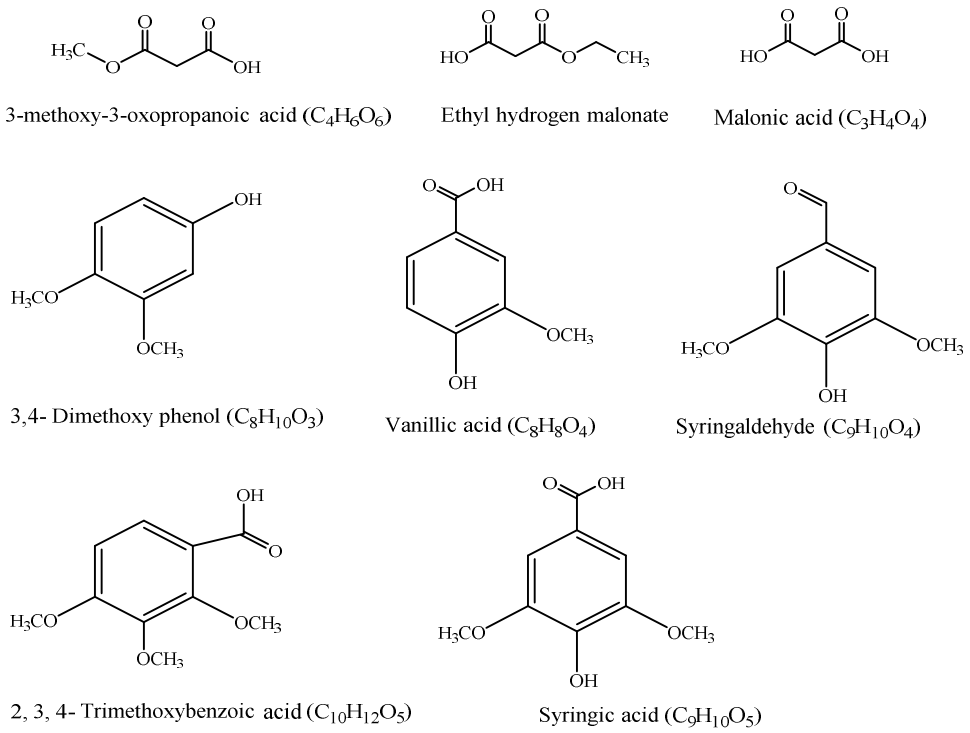


Fig. 3.22: The structure of chemicals identified from the OSL degradation by MnP

The main products that appeared from the Organosolv reaction with MnP were syringic acid (RT=15.97 min), vanillic acid (RT=13.15 min) and syringaldehyde (RT=14.36 min). Some tiny quantities of minor compounds such as 1,2-dimethoxy-4-methylbenzene (RT=8.94 min), 3,4-dimethoxyphenol (RT=10.53 min), acetosyringone (RT=15.17 min) and homosyringic acid (RT=15.72 min) were also formed (Figure 3.21). However, they could also be found in the control reactions as well. In the case of the complete mixture with enzyme, mediator and ABTS, the main product was found to be syringic acid and it presumably appeared from the oxidation of syringaldehyde (Figure 3.21). It was present in the control sample without enzyme as well, but all potential compounds (syringic acid, acetosyringone and homosyringic acid) which could be produced by syringaladehyde related oxidation were found in the control reaction where there were no enzyme and mediators. However, the amount of these compounds was higher than in the complete reaction sample (Figure 3.21). Likewise, vanillic acid was found with the highest level in the control reaction where there was no mediator present, although it could also be seen in all cases studied. Therefore, enzyme may have stimulated the reaction to degrade lignin mildly, though with very low product concentrations according to their peak area level as seen in Figure 3.21. The syringic acid, acetosyringone and vanillic acid were also found in the last control in which there was no enzyme and

mediators added into the reaction. Hence, the enzymatic depolymerisation of OSL is a really complicated process and the reaction conditions should be studied further in order to learn the real effect of the enzyme MnP with respect to product variation.

4 OXIDATIVE DEGRADATION OF SULPHONATED LIGNIN BY LIGNOLYTIC ENZYMES

4.1 Introduction

This study is aimed at understanding the interaction between lignolytic enzymes with sulphonated lignins using laccase from *Trametes versicolor* (LTV), lignin peroxidase (LiP) and manganese peroxidase (MnP) and the product distributions originating from the enzymatic treatment of each sulphonated lignin. At first, the catalytic ability of laccase was tested on the biodegradation of water soluble lignins provided by four different sources. They were lignosulphonate and Kraft lignin purchased from Sigma-Aldrich (UK), and another one type of lignosulphonate donated by Borregard Ligno Tech (England), respectively. Reactions were performed under aqueous conditions using a laccase mediator system (LTV-ABTS couple). The study then moved onto the observation of depolymerisation of Kraft lignin using MnP and LiP.

The reaction mixture contained lignin (2 g), ammonium acetate buffer (100 mM, pH 4.5), ABTS (11 ml of 4 mM), and the LTV enzyme solution in a buffer (250 µL of 0.25 $mg.ml^{-1}$). The final concentration of lignin in this reaction was 57.14 g/L. This gave a homogeneous solution as this type of lignin (the lignosulphonate from Sigma-Aldrich) was completely water-soluble. Control reactions without adding LTV or ABTS and without both of the above were also performed. The method in the controls was exactly as same as in the complete reaction mixture but the components missed were replaced by equivalent volumes of buffer.

The laccase-catalysed reactions of all these lignins were performed at 30 °C hereafter as it was the most suitable reaction temperature as found in a previous part of this project (Chapter 3).

4.2 Results and Discussion

4.2.1 The LTV catalysed depolymerisation of lignosulphonate (Sigma-Aldrich)

In total, five compounds were identified from the GC-MS analysis of lignosulphonate oxidative degradation by using a laccase mediator system (Figure 4.1). It confirmed that the products formed from water soluble lignosulphonate were significantly different from those identified from the Organosolv lignin treatment under the same reaction conditions. The chemical structures of the products formed from lignosulphonate depolymerisation are shown in Figure 4.1.

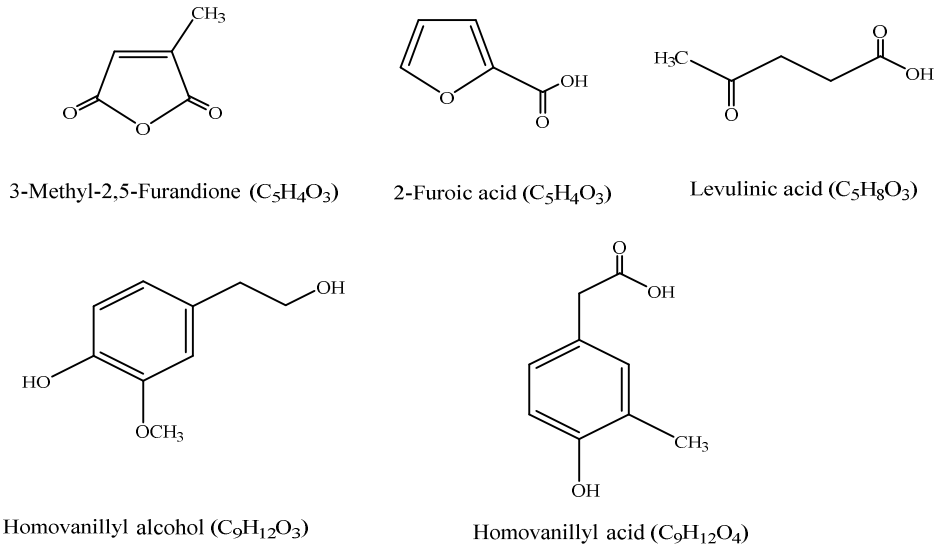


Figure 4.1: Chemical structures of lignosulphonate depolymerisation products identified by the GC-MS.

The major products identified from the lignosulphonate degradation by laccase are shown in Table 4.1 with their retention time in GC-MS analysis. Product identification was tended to confirm by NIST Library identification.

Table 4.1: Identified products by GC-MS from lignosulphonate degradation by LTV.

No.	Compounds	Retention time of Product	Retention time of external standard
1	Homovanillyl alcohol	12.88	13.00
2	Homovanillic acid	14.24	n.a
3	2,5-Furandione, 3-methyl-	4.49	n.a
4	Levulinic acid	6.01	n.a
5	2-Furoic acid	6.19	n.a

(n.a: These products were unavailable.)

However, these products identified by GC-MS in the complete reaction sample, also appeared in all the other three control reactions undertaken, regardless of laccase and ABTS additions into the reaction mixture (Figure 4.2a) and without laccase (Figure 4.2b) or ABTS (Figure 4.2c), and with both of laccase and ABTS absent from the reaction (Figure 4.2d).

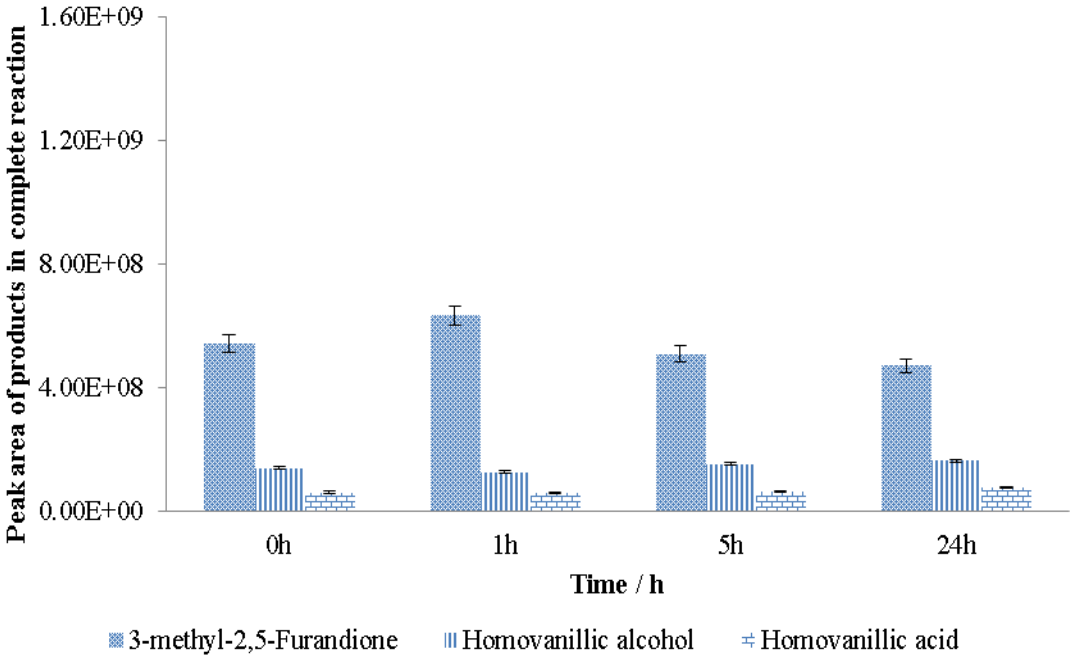


Figure 4.2 (a): The time course profile of lignosulphonate digestion by laccase in the presence of ABTS (complete reaction)

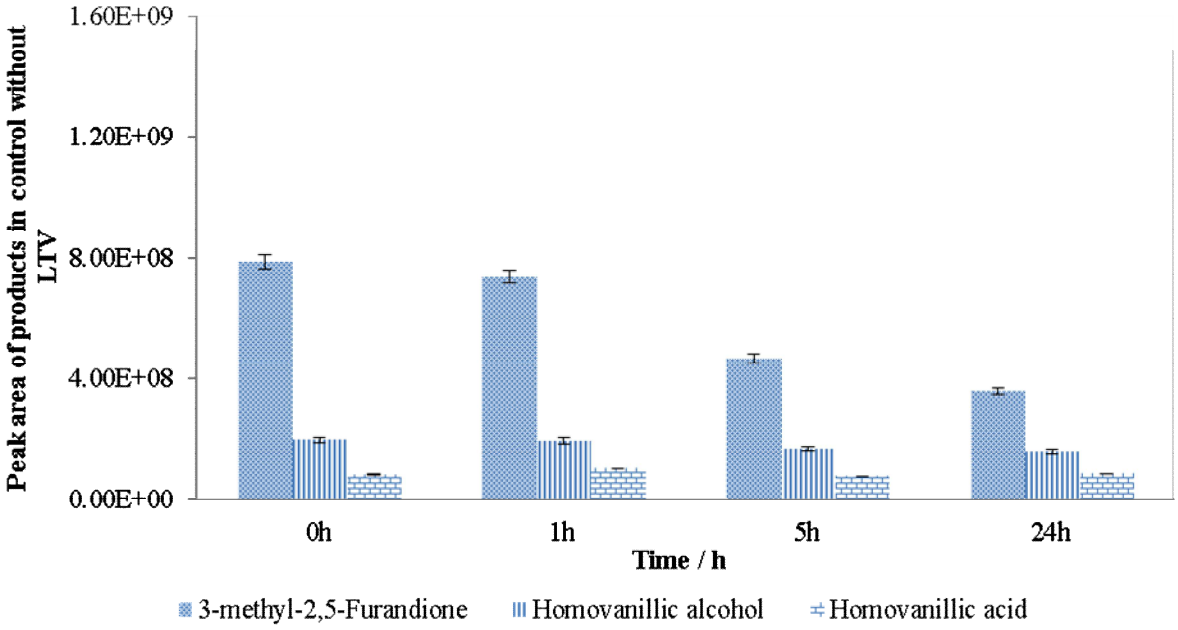


Figure 4.2 (b): The time course profile of lignosulphonate treatment with ABTS in the absence of laccase.

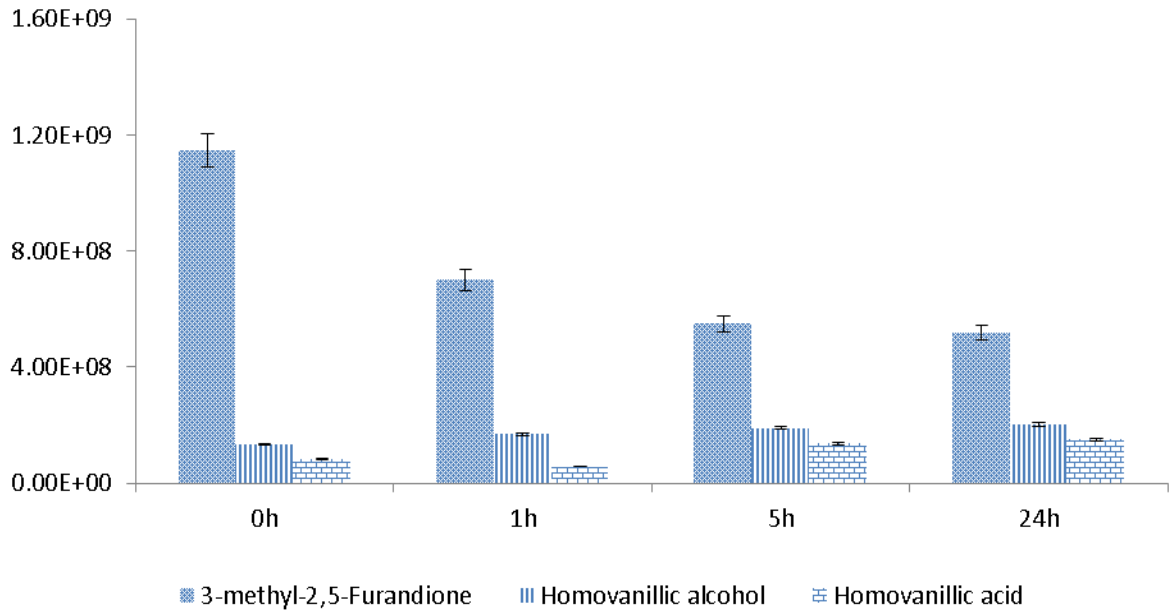


Figure 4.2 (c): The time course profile of lignosulphonate digestion by laccase in the absence of ABTS.

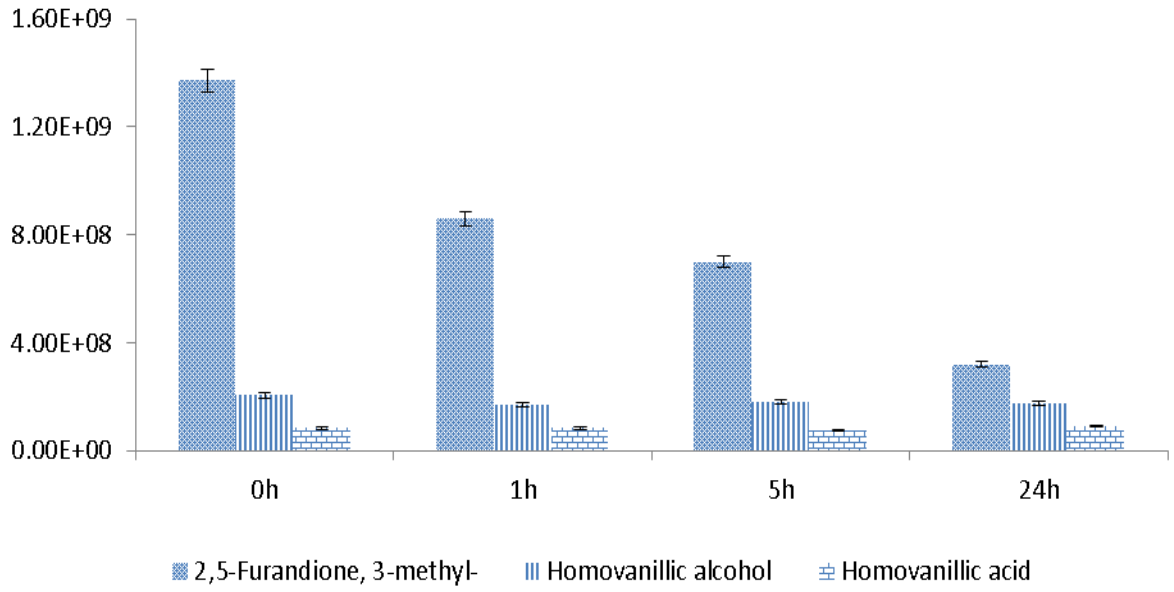


Figure 4.2 (d): The time course profile of lignosulphonate in buffer in the absence of laccase and ABTS.

The reaction mixture contained lignin (2 g), ammonium acetate buffer (100 mM, pH 4.5), ABTS (11 ml of 4 mM), and the LTV in a buffer (250 µL of 0.25 $mg.ml^{-1}$). The final concentration of lignin was 57.14 g/L. The reaction was terminated by adding concentrated sulphuric acid H_2SO_4 and samples were taken at regular intervals during the incubation. All aqueous mixtures were centrifuged for 1 min at 10,000 rpm at 20 °C before the extraction was applied. A portion (4 mL) was extracted with ethyl acetate two times (total volume of 16 mL). The extracts were left to evaporate under a fume hood and re-dissolved in acetone (1 mL) and analysed by Gas Chromatography–Mass Spectrometry (GC-MS).

The GC chromatograms obtained for these compounds are given in GCMS chromatograms are shown in Appendix A.11.1 as a reference for comparing the product distribution in each case (chromatograms are given for samples at 24 h only). The most distinctive change that occurred among these products was 3-methyl-2,5-Furandione, for which its peak area reduced with increasing incubation time in all cases studied (Figure 4.2 a, b, c, d). However, such difference was too small in the complete reaction sample compared with the significant changes in all three control reactions (Figure 4.2a). The peak area of all monomers identified remained almost identical from the time when the reaction started (0 h) until the end of reaction (24 h) (Table 4.1 and Figure 4.2a). As a result, there was no substantial difference between the reaction samples and the relevant controls performed above. Thus, it can be concluded that this batch of lignosulphonate lignin, which was supplied by Sigma-Aldrich, was barely affected by the laccase assisted treatment.

4.2.2 The LTV catalysed depolymerisation of lignosulphonate (Borregaard)

This experiment was set to ascertain if any further variations could be found using another source of lignosulphonate labelled as DP590 from Borregaard Lignin Tech (UK). This lignin was treated and analysed in the same way as the lignosulphonate samples previously studied, with the only exception of shortening the reaction time. As the extension of the incubation time was not found to be helpful for lignosulphonate reactions with a laccase mediator system, this lignin was reacted for only 6 h to see the difference caused by both enzymatic and non-enzymatic processes. Product identification was completed by GC-MS analysis and the results shown here were collected from both complete and control reactions at the beginning of the reactions (0 h) and at the end of the reactions (6 h). The chromatograms obtained at these sampling times are overlaid in Appendix A.11.2.

There were seven different compounds identified from this type of lignosulphonate (Figure 4.3).

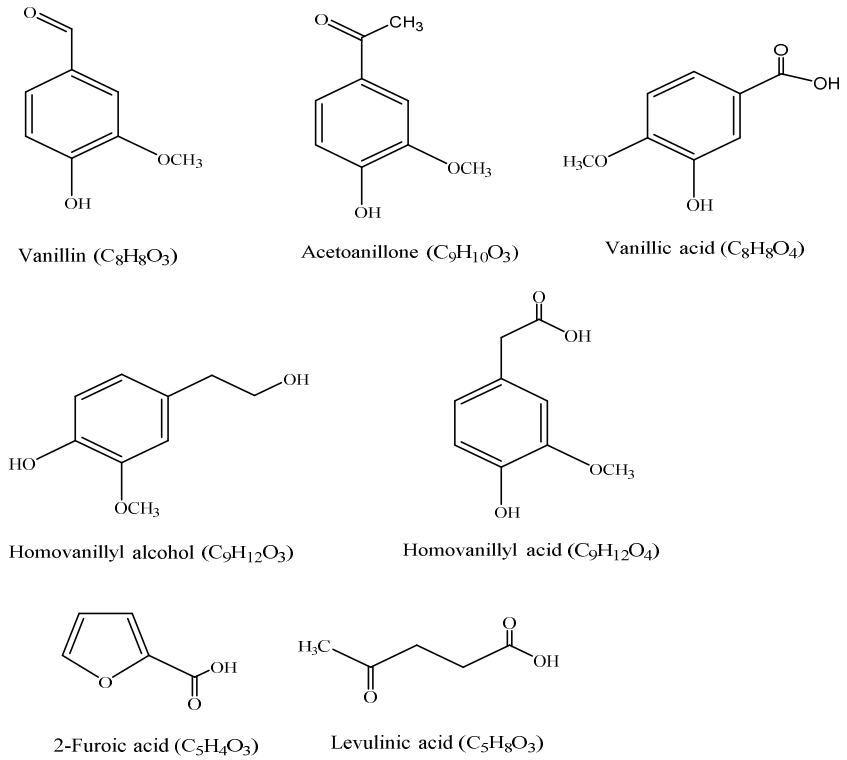


Figure 4.3: Chemical structures of lignosulphonate (DP590) depolymerisation products by laccase at 30 °C.

The time course profile for all cases investigated are shown in Figure 4.4 a, b, c to show the changes of products from both the complete reaction and the controls due to changes in the incubation time. The comparison shown below is based on samples analysed after only 1 h of incubation as that was the time when the more noticeable changes occurred to those products (Figure 4.4).

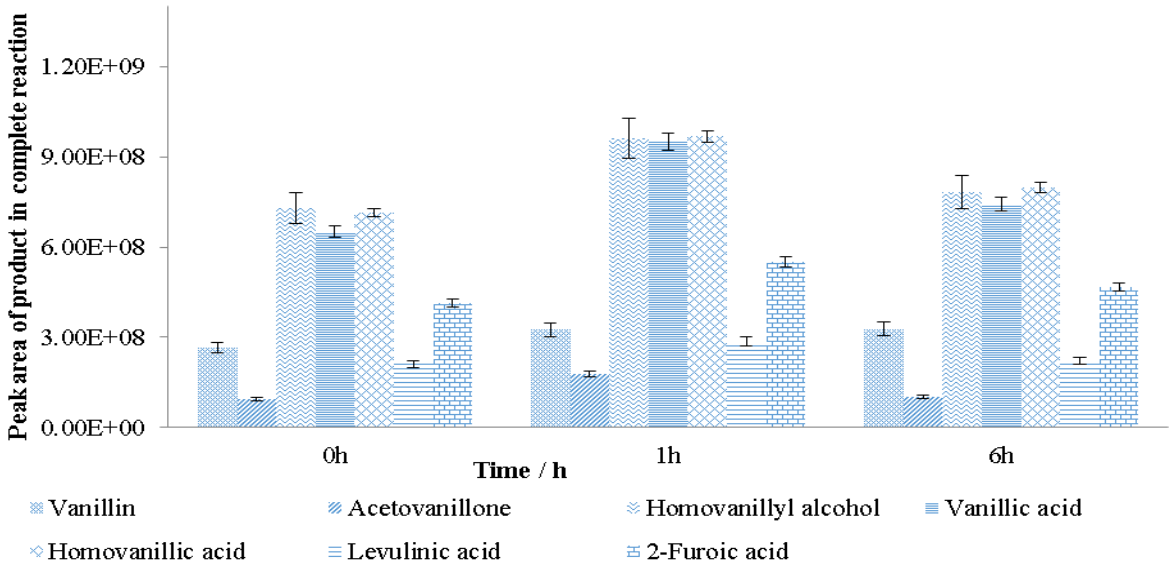


Figure 4.4 (a): The time course profile of lignosulphonate (DP590) in the presence of laccase and ABTS (complete reaction).

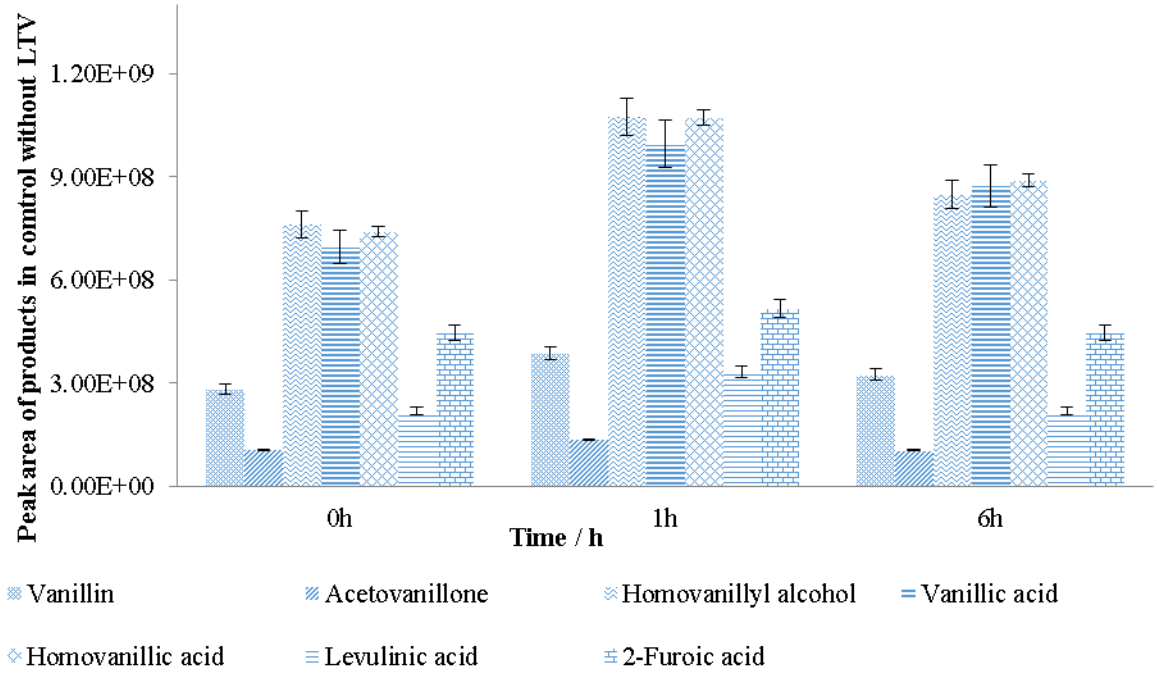


Figure 4.4 (b): The time course profile of lignosulphonate (DP590) in the absence of LTV.

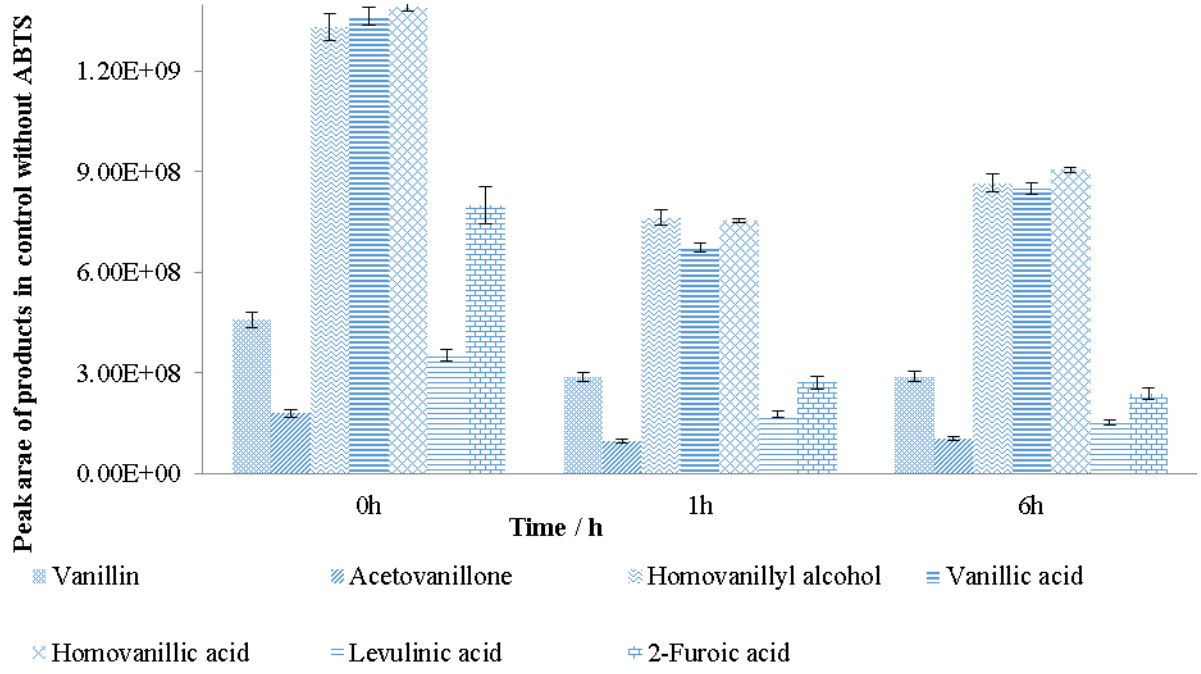


Figure 4.4 (c): The time course profile of lignosulphonate (DP590) in the absence of ABTS.

There were five main products produced from lignin (DP590) after enzymatic digestion with LTV, and two more new compounds, levulinic acid and 2-furoic acid (Figure 4.3 and Figure 4.4). Among them, homovanillic alcohol, vanillic acid and homovanillic acid were the most abundant chemicals that could be seen with the highest peak areas obtained from GC-MS analysis. At the beginning of the reactions, all of these three main products were found in both the complete reaction and the controls (Figure 4.4 a, b, c). Interestingly, the highest amount of the above products was found in the control reaction where there was LTV but no ABTS added into the reaction mixture at the beginning of reaction (Figure 4.4c), and that remained as the highest amongst all cases examined regardless of the incubation time changes and enzymatic or non-enzymatic reactions that were performed (Figure 4.4). Other compounds, such as levulinic acid and 2-furoic acid decreased over time when no ABTS was in the reaction (Figure 4.4c) whereas these products were influenced only slightly by changes in the reaction time both in the complete reaction (Figure 4.4a) and the control reaction without LTV (Figure 4.4b). Likewise, the vanillin and acetovanillone were barely affected by the incubation time or type of the reaction. Moreover, an extended reaction time did not increase the amount of any of these compounds in general. To conclude, these products identified from lignosulphonate (DP590) may be mainly formed by the addition of ABTS rather than LTV into the reaction. Or perhaps there are other factors that should be taken into

account concerning lignosulphonate degradation as it is a very complicated reaction system which may be affected by more variables than discussed above.

With regard to the preliminary studies of lignosulphonates so far, laccase has demonstrated a small effect on lignosulphonates but it has very little impact on their degradation (Chapter 4.1.1 and 4.1.2) compared to the earlier study of the enzymatic depolymerisation of Organosolv lignin (Chapter 3.2). The compounds formed from each lignin are different in two types of lignosulphonates because of the lignin source. However, it is still not clear that why those compounds are affected more by the presence of a mediator (ABTS) than LTV (Section 4.1.2). Therefore, other lignolytic enzymes were not examined for their effect on lignosulphonates as yet, as the main focus at this stage was to find out the initial difference(s) that might be occurring in the product distribution between water-soluble (lignosulphonates) and –insoluble lignins (Organosolv).

4.3 The Kraft lignin depolymerisation with lignolytic enzymes

In this particular study the oxidative degradation of Kraft lignin was investigated using LTV, LiP and MnP in order to understand the effects of these enzymes on the product distribution upon degradation.

4.3.1 Kraft lignin depolymerisation with LTV

4.3.1.1 Observation of Kraft lignin oxidation by laccase for 24h

Kraft lignin was depolymerised by LTV and its products were identified after fractionation of the samples. As this type of lignin has been previously prepared under very high pH conditions, it is required to adjust its pH to the pH optimum for LTV (4.5) before the reaction starts. Kraft lignin (2 g) was mixed with sodium acetate buffer (16 mL), and distilled water (7 mL), and the pH of this mixture was adjusted by adding acetic acid to obtain pH 4.5 before adding the LTV into this mixture to start the reaction. The reactions were stopped by acidifying the reaction mixtures by adding concentrated sulphuric acid, and resultant liquid was fractionated to separate the solid materials from aqueous phase. Subsequently, the aqueous phase was extracted with EA. Samples from the EA extract were analysed by GC-

MS. The chemical structures of GC-MS identified compounds from Kraft lignin degradation by LTV at 30 °C are shown in Figure 4.5.

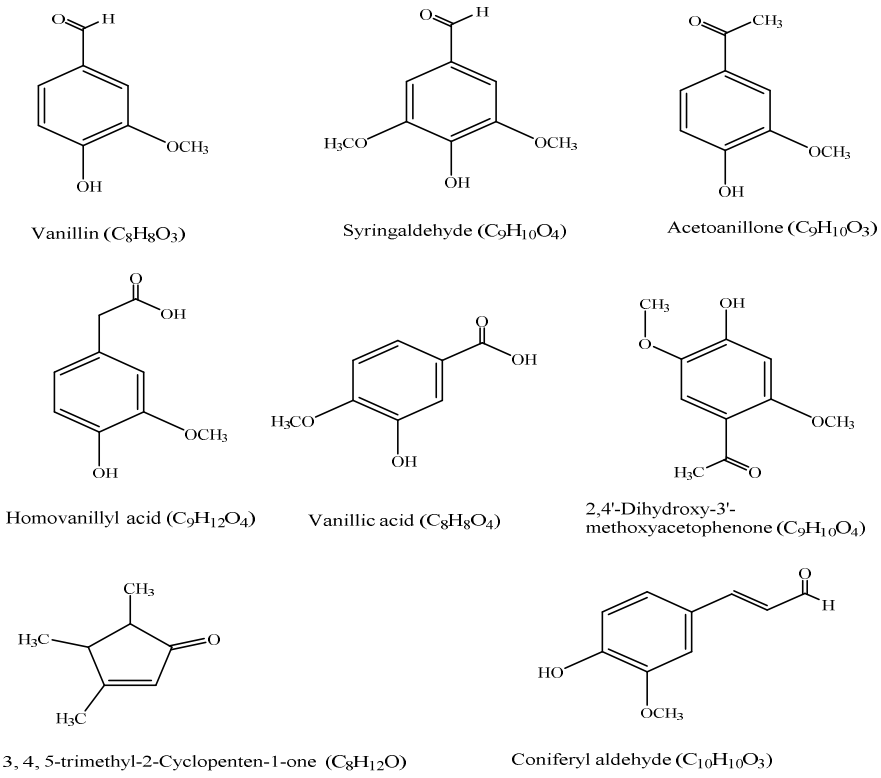


Figure 4.5: Chemical structures of identified compounds from Kraft lignin (from Sigma-Aldrich) depolymerisation by laccase

The major products formed from Kraft lignin depolymerisation by laccase at 30 °C are listed in Table 4.4 with the retention times of these products are separated on the GC column. Their related GC chromatograms are shown in Appendix A as references. Vanillin, acetovanillone and homovanillic acids were the main products from this lignin whereas 3,4,5-trimethyl-2-Cyclopenten-1-one, vanillic acid, syringaldehyde, 2,4'-Dihydroxy-3'-methoxyacetophenone and the 4-Hydroxy-2-methoxycinnamaldehyde were identified as minor products.

Table 4.2: The GC-MS identified products from Kraft lignin depolymerisation by LTV

No.	Compounds	Retention time (min)	
		Product	External standard
1	3,4,5-trimethyl-2-Cyclopenten-1-one	6.71	n.a
2	Vanillin	11.22	11.52
3	Acetovanillone	12.32	12.65
4	Vanillic acid	13.21	13.54
5	Homovanillic acid	14.23	n.a
6	2,4'-Dihydroxy-3'-methoxyacetophenone	14.59	n.a
7	4-Hydroxy-2-methoxycinnamaldehyde	15.20	n.a

The time course profile for the distribution of the major products in both the complete reaction and the controls are compared in Figure 4.6.

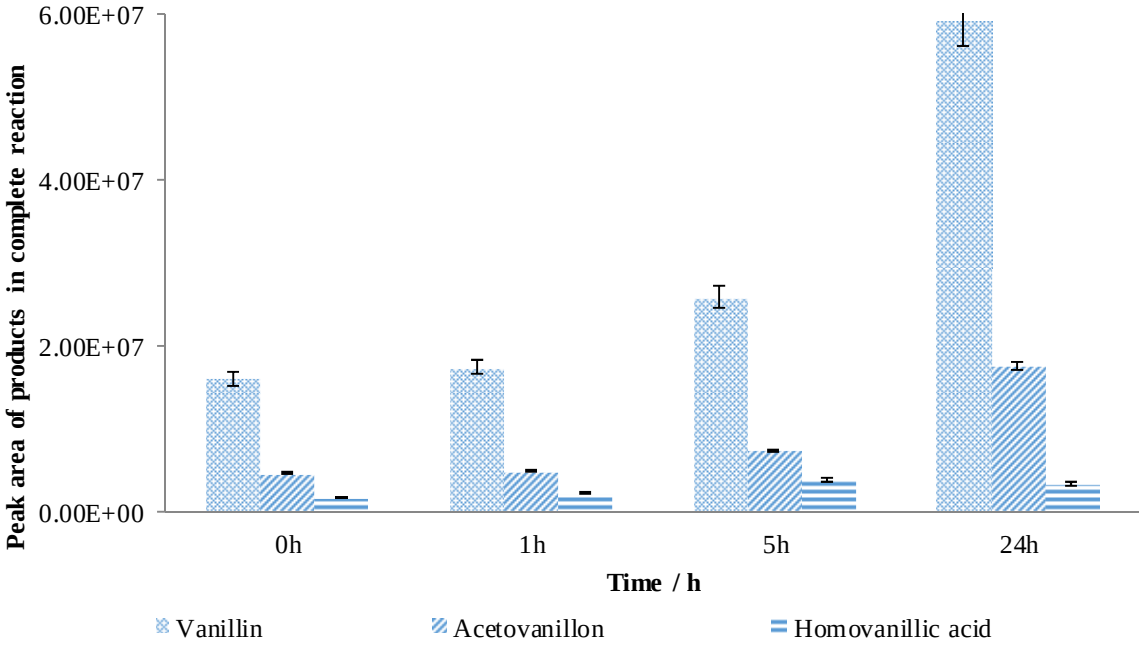


Figure 4.16 (a): The time course profile of Kraft lignin digestion by LTV in the presence of laccase (complete reaction)

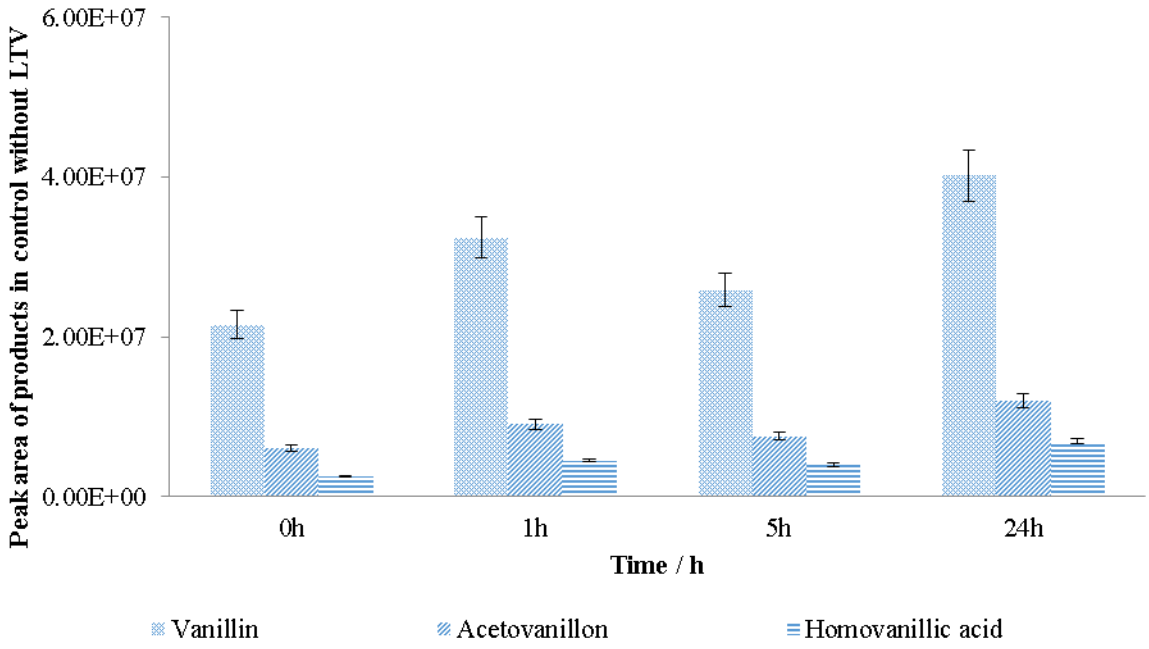


Figure 4.6 (b): The time course profile of Kraft lignin digestion without LTV but in the presence of ABTS

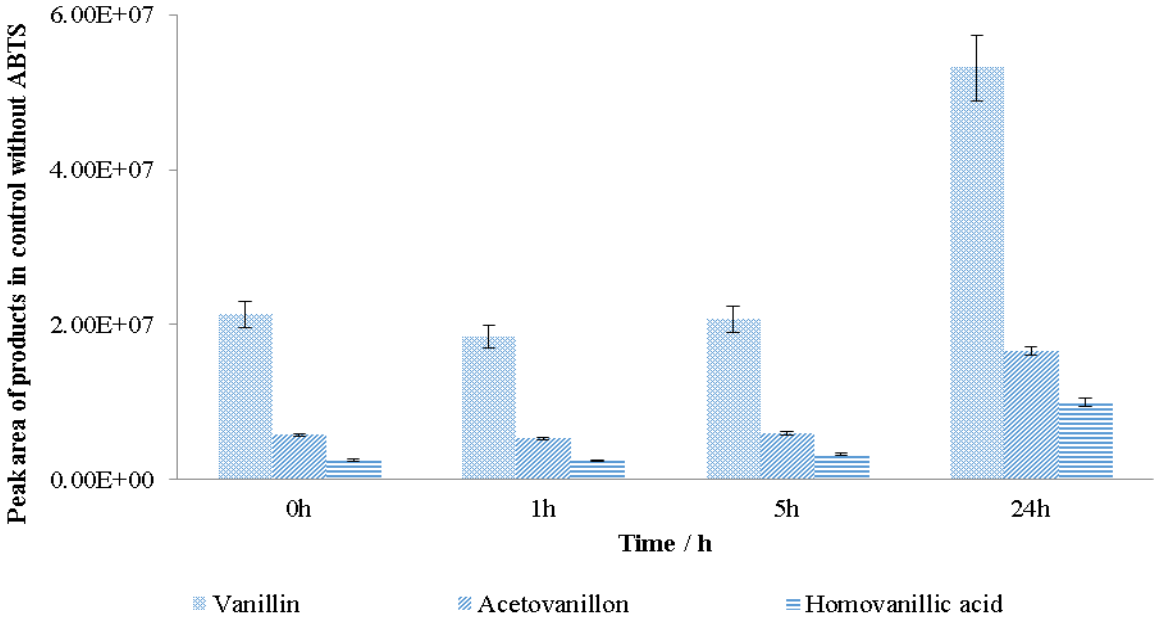


Figure 4.6 (c): The time course profile of Kraft lignin digestion without ABTS but in the presence of LTV

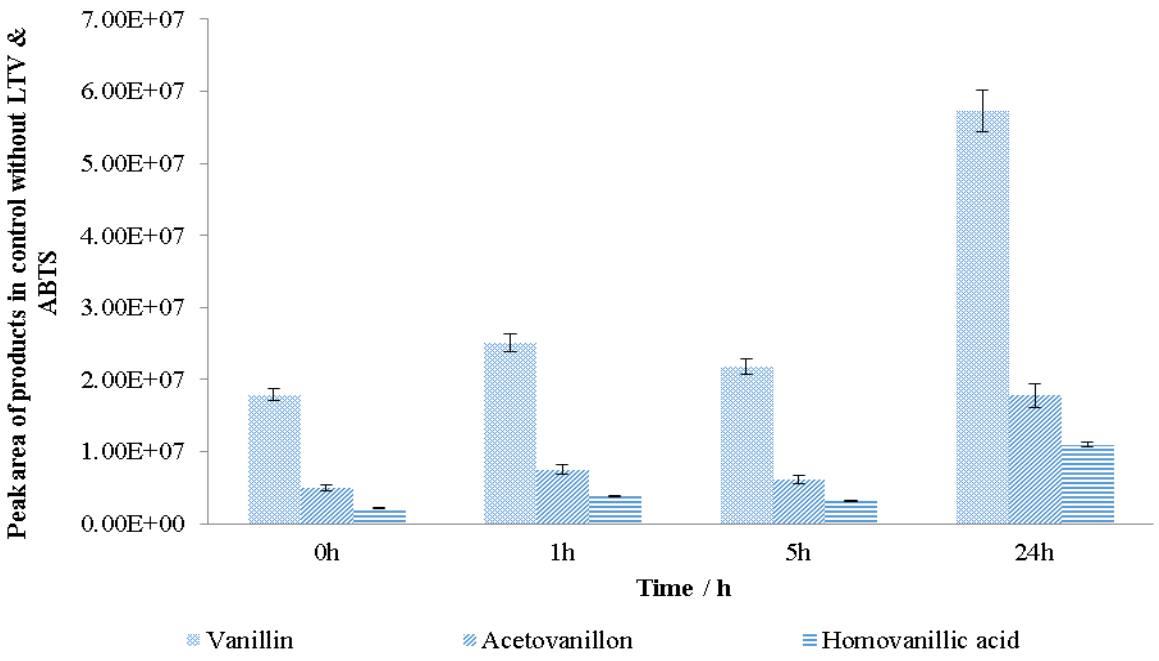


Figure 4.6 (d): The time course profile of Kraft lignin digestion without both LTV and ABTS

As shown in Figure 4.6, the main finding was that the peak area of vanillin increased as the incubation time increased. The highest peak area for vanillin can be seen at 24 h of incubation, but its peak area in the enzymatic reaction and non-enzymatic reactions were almost identical. The lowest peak area of vanillin was in the control where no LTV was present (Figure 4.6 b). However, there was no significant difference observed in the results for both the complete reaction and all other three controls with and without adding the LTV as shown in Figure 4.12 (a, b, c, d). The figure indicates that extending the reaction time is advantageous for Kraft lignin to release monomers, but that there is very little benefit in running this reaction under enzymatic conditions or this reaction needs improvement on reaction conditions.

Samples from both of the complete reaction and the controls were taken at intervals and analysed by GPC, and compared with the pullulan standard curve in order to estimate the MW of products. The calculated MWs are listed in Table 4.5 to show the relative changes in MW between complete reaction and controls.

Table 4.3 The estimated MWs of both complete and control experiments from Kraft lignin oxidation by LTV at 30°C for 24h. Data represent the mean of duplicate sample with the standard deviation less than 5%.

No.	Reactions	Mean RT for each peak (min)	Estimated mean MW of products (t=0 h)	Estimated mean MW of products (t=1 h)	Estimated mean MW of products (t=5 h)	Estimated mean MW of products (t=24 h)
1	Kraft lignin incubation in buffer in the presence of both LTV & ABTS	9.56	83550 ± 0.01	83280 ± 0.01	83145 ± 0.01	83280 ± 0.01
		10.33	24440 ± 0	23800 ±0	23860 ± 0.31	24280 ± 0.01
		11.80	2320 ± 0	2840 ± 0	2840 ± 0.23	2630 ± 0.01
		12.51	740 ± 0	745 ± 0	760 ± 0.01	735 ± 0.01
2	Kraft lignin incubation in buffer without LTV but with ABTS	9.56	83550 ± 0.01	83815 ± 0	83410 ± 0.35	82880 ± 0
		10.34	24090 ± 0.21	24795 ± 0.3	24280 ± 0.11	23820 ± 0.01
		11.69	2730 ± 0.43	2780 ±0.01	2360 ± 0	2670 ± 0.07
		12.50	745 ± 0.01	660 ± 0.01	755 ± 0.01	710 ± 0
3	Kraft lignin incubation in buffer without ABTS but with LTV	9.56	83680 ± 131	8420 ± 0.04	84490 ± 0.51	83280 ± 0.01
		10.32	24675 ± 0.07	24655 ± 0.21	26200 ± 0.01	243210 ± 0.12
		11.66	2885 ± 0.47	2820 ± 0	2925 ± 0.01	3125 ± 0.01
		12.52	732 ± 1	715 ± 0	1000 ± 0.01	680 ± 0
4	Kraft lignin incubation in buffer in the absence of both LTV & ABTS	9.55	84490 ± 0.71	86610 ± 0.12	8445 ± 0	83680 ± 0.01
		10.32	24675 ± 0.15	24810 ± 0	24970 ± 0.01	24550 ± 0.01
		11.61	3110 ± 0.1	2970 ± 0	3200 ± 0	3520 ± 0.11
		12.51	732 ± 0.11	740 ± 0	750 ± 0	720 ± 0

In general, the estimated MWs of products in each case decreased as the incubation time increased (Table 4.3). This means that the lignin was depolymerised by laccase. For example, after 24h of incubation, at RT=9.56 min, the MW of both the complete reaction and all three controls decreased by 270, 670, 400 and 810 respectively in the order of complete reaction, control without LTV, control without ABTS and control without both LTV and ABTS in the reaction. Therefore, this indicates that the Kraft lignin released some monomers by the time

of extended incubation. However, the possibility of depolymerisation in non-enzymatic reactions (no.2 and no.4) was higher than in the enzymatic reaction (no.1 and no.3). These findings were consistent with the findings from the GC-MS analysis discussed previously. Therefore, laccase has shown no effect on Kraft lignin depolymerisation under the designated reaction conditions.

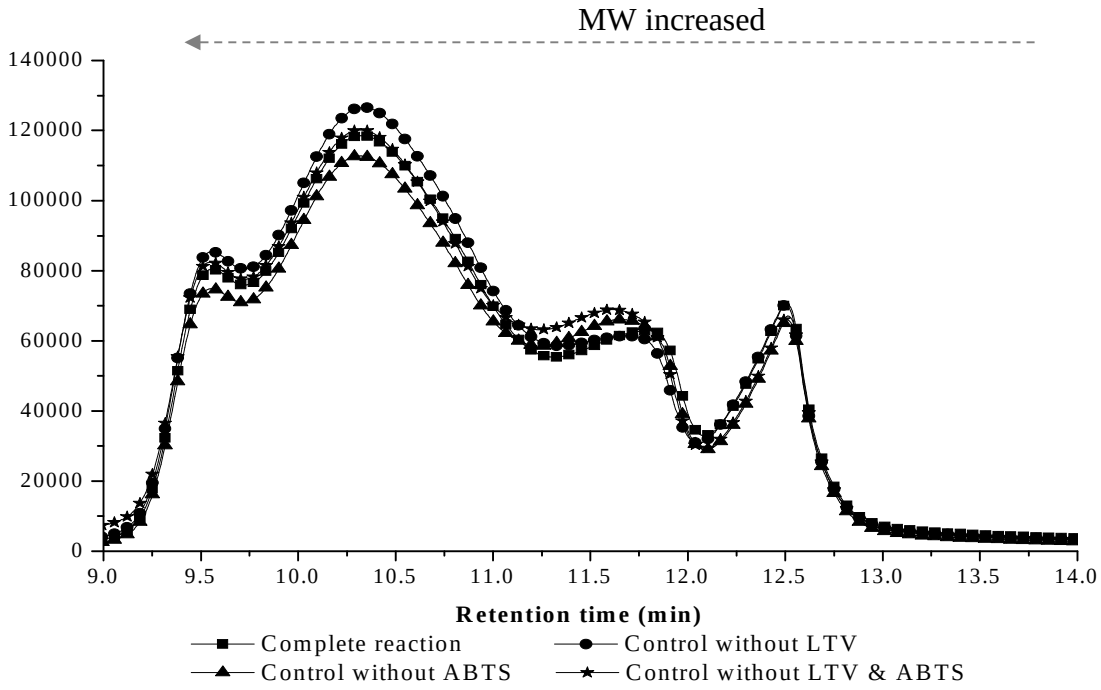


Figure 4.7(a): The overlaid GPC chromatograms of Kraft lignin depolymerisation by laccase and mediator at 30 °C at the beginning of the reaction (t=0 h)

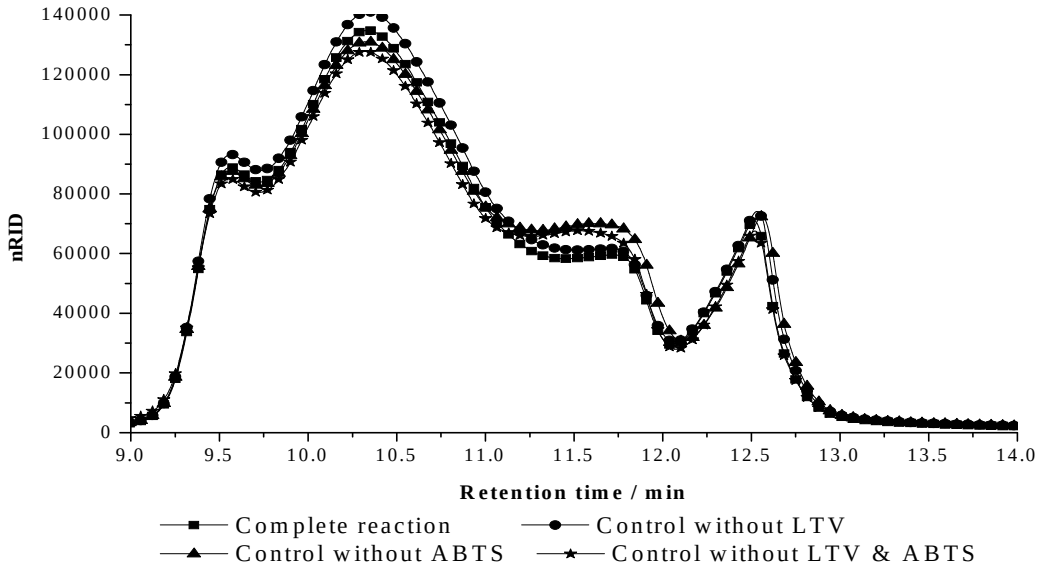


Figure 4.7(b): The overlaid GPC chromatograms of Kraft lignin depolymerisation by laccase and mediator at 30 °C at the end of the reaction (t=24 h)

4.3.1.2 Re-examining the Kraft lignin oxidation by laccase for 6 days

The reaction of Kraft lignin oxidation by laccase in the presence of ABTS was investigated again using a slightly different method as reported by R. Bourbonnais and co-workers (Bourbonnais *et al.*, **1995**) to determine the MW changes of products from both the complete reaction and the controls after three and six days of incubation using GPC. The estimated MW of samples from each reaction are given in Table 4.6 to show the relative changes in MW over the incubation time as well as the effect of ABTS addition for 3 h into the ongoing reaction after three days.

Table 4.4: MW changes between the complete reaction and the control experiments from Kraft lignin oxidation by laccase over six days of incubation.

No.	Reactions	Mean RT for each peak (min)	Estimated mean MW of products (t=0 h)	Estimated mean MW of products (t=24 h)	Estimated mean MW of products (t=3 days)	Estimated mean MW of products (t=6 days)
1	Lignin incubation in buffer without the addition of both LTV & ABTS	9.57	82285 ± 0.10	80780 ± 0.32	80070 ± 0.00	87730 ± 0.01
		10.35	23480 ± 0.23	23950 ± 0.45	24400 ± 0.00	25700 ± 0.05
		11.46	3960 ± 0.11	4000 ± 0.61	3950 ± 0.12	4015 ± 0.03
		12.27	1085 ± 0.14	1050 ± 0.01	1055 ± 0.00	1000 ± 0.08
2	Lignin reaction with LTV but without ABTS for three days and six days	9.56	83210 ± 0.00	82090 ± 0.08	81105 ± 0.32	80650 ± 0.13
		10.34	23760 ± 0.46	24420 ± 0.00	24930 ± 0.00	23400 ± 0.46
		11.44	4130 ± 0.21	4125 ± 0.71	4070 ± 0.11	3780 ± 0.07
		12.26	1100 ± 0.00	1065 ± 0.12	1075 ± 0.00	765 ± 0.07
3	Lignin incubation with both LTV and ABTS for three days, and further extended until six days	9.57	82150 ± 0.14	82285 ± 0.70	80520 ± 0.01	84830 ± 0.41
		10.34	23880 ± 0.71	24535 ± 0.56	24815 ± 0.41	24440 ± 0.60
		11.43	4165 ± 0.31	4105 ± 0.09	4080 ± 0.01	3845 ± 0.00
		12.26	1100 ± 0.23	1090 ± 0.00	1080 ± 0.01	950 ± 0.00
4	Pre-incubation of lignin and laccase for three days and then addition of ABTS for 3 h, and then extended up to six days	9.57	81500 ± 0.00	80650 ± 0.01	79625 ± 0.00	76930 ± 0.01
		10.38	22430 ± 0.33	23270 ± 0.87	23420 ± 0.50	26180 ± 0.76
		11.49	3790 ± 0.10	3800 ± 0.11	3740 ± 0.43	3905 ± 0.00
		12.29	1050 ± 0.33	1040 ± 0.27	1000 ± 0.60	925 ± 0.45

Reaction conditions: The complete reaction mixture contained Kraft lignin (4 g), LTV (0.1 U/mL), ABTS (1 mM) and sodium acetate buffer (0.05 M, pH 5) such that the reaction volume was 200 mL. After incubation at 50 °C with a shaking speed of 200 rpm for the designated time period the reaction was terminated by adding 0.5 mL of 5N NaOH and 1.25 mL of ethanol to the 5 mL of aqueous reaction mixture. It was then fractionated by centrifuge at 8000 rpm for 10 min at 20 °C. The aliquots were filtered using a 0.45 µm filter prior to the GPC analysis. Both reactions and analysis were done in two replicates, and their mean data was reported. In the complete reaction, lignin was incubated with both LTV and ABTS for three days and further extended until six days. The other three control reactions were (1) lignin incubation in buffer without the addition of both LTV & ABTS; (2) lignin incubation with LTV but without ABTS for three days and further extended up to six days and (3) pre-incubation of lignin and laccase for three days and then the addition of ABTS for 3 h, respectively.

The results show that Kraft lignin was depolymerised as the incubation time increased for both the reaction of lignin incubated with LTV without ABTS for three days and when the lignin was pre-incubated with LTV for three days and then reacted with ABTS for 3 h. In the case of the lignin reaction with LTV for three days without ABTS (reaction no. 2 in Table 4.4), the average MW calculated for the largest peak appeared at RT=9.56 min on the GPC chromatograms with 83210 at the beginning of the reaction (t=0 h), and it progressively decreased by 2105 after three days and 2560 after six days of reaction respectively. A more significant descending trend was found in the lignin sample pre-incubated with LTV for three days and then reacted with ABTS for 3 h, in which the average MW of Kraft lignin steadily decreased by 1870 after three days and 4570 after six days respectively. This is compared to the average MW data of 81500 at t=0 h (reaction no. 4 in Table 4.4) and indicates that laccase is able to depolymerise the Kraft lignin without the addition of ABTS (reaction no. 2 in Table 4.4), but the depolymerisation can take place more effectively with the addition of ABTS after pre-incubation of lignin with LTV for three days (reaction no. 4 in Table 4.4). This finding is slightly different from the finding of Bourbonnais and co-workers (Bourbonnais *et al.*, 1995) who discovered that laccase has a polymerisation effect on lignin and that depolymerisation can only be achieved by the addition of ABTS into the enzymatic reaction of Kraft lignin with laccase. However, the present results are consistent with the work of the above researchers in that LTV can depolymerise lignin more effectively with the support of ABTS. As for the lignin reaction in the presence of both LTV and ABTS, the lignin was inclined to polymerisation in this case after six days of incubation with an average MW rise of 2675 compared to the 82155 at t=0 h (reaction no. 3 in Table 4.4). However, an exception was found under the same reaction conditions in which the MW of lignin dropped to 80520 after three days of incubation (reaction no. 3 in Table 4.4). Therefore, the results obtained from the lignin oxidation in the presence of both LTV and ABTS show that LTV has both a depolymerisation effect (after three days) and a polymerisation effect (after 6 days) on the Kraft lignin used in this study. Regarding the control experiment where lignin was incubated without the addition of LTV or ABTS, there was a decrease in the average MW by 1500, 2210 which represent a thermo- degradation of lignin by itself over the incubation time of three days. However, there was an increase after six days by 5450 compared to the data at the beginning of this reaction (reaction no. 1 in Table 4.4). In short, laccase has mainly shown a depolymerisation effect on Kraft lignin (reactions no. 2 and no. 4 in Table 4.4), but it also plays a repolymerisation role on this lignin in the case of incubation when both LTV and ABTS were present (reaction no. 3 in Table 4.4). Moreover, the Kraft lignin itself can be

thermally polymerised on its own as the reaction temperature and time increase (reaction no. 1 in Table 4.4).

4.3.2 The attempts to degrade Kraft lignin by peroxidases

As reviewed earlier, both lignin peroxidase and manganese peroxidase haven been used for the oxidative degradation of certain lignin model compounds including dimeric and oligomeric lignin derivatives. In this study, manganese and lignin peroxidases (LiP and MnP) were used to attempt to degrade Kraft lignin in order to understand their role in native lignin degradation so that their oxidation ability with regard to the lignin polymer could be evaluated.

4.3.2.1 Kraft lignin degradation using lignin peroxidase (LiP)

The compounds identified from GC-MS analysis for this reaction are shown in Figure 4.8.

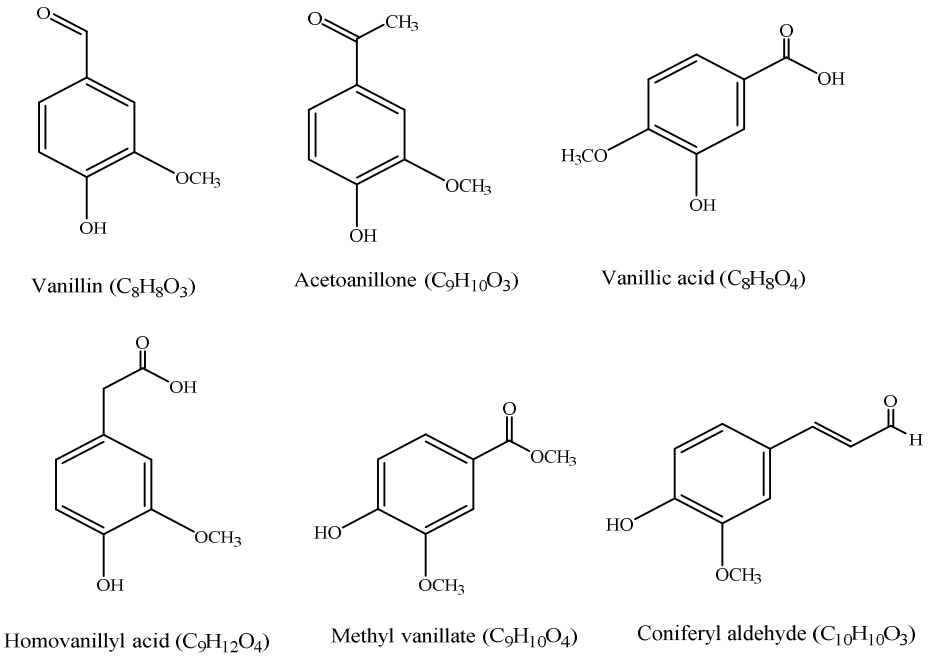


Figure 4.8: Chemical structures of identified compounds from Kraft lignin (from Sigma-Aldrich) depolymerisation by lignin peroxidase

The retention times of the main products detected from the Kraft lignin reaction with LiP are listed in Table 4.7 to show the difference between the complete reaction and all three controls. The chromatograms obtained at these sampling times are overlaid in Appendix A.11.4.

Table 4.5: Identification of products formed from Kraft lignin depolymerisation by LiP at 5h.

No.	Compounds	Retention time of product	External standard
1	Vanillin	11.22	11.52
2	Acetovanillone	12.32	12.65
3	Methyl vallinate	12.44	n. a
4	Vanillic acid	13.21	13.54
5	Homovanillic acid	14.23	n. a
6	Coniferylaldehyde	15.18	n. a

The time course profiles of the six major compounds from this reaction and their retention times are given in Figure 4.9 with all the relevant controls.

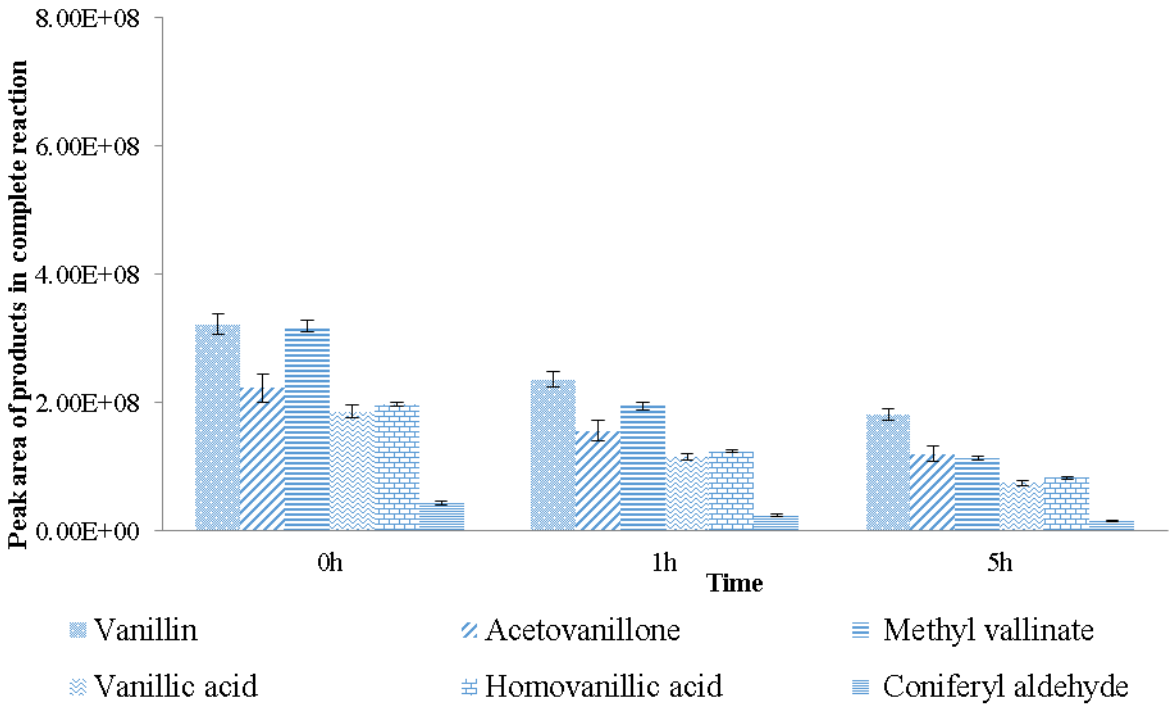


Figure 4.9 (a): The time course profile of Kraft lignin degradation by LiP in the presence of veratryl alcohol (complete reaction).

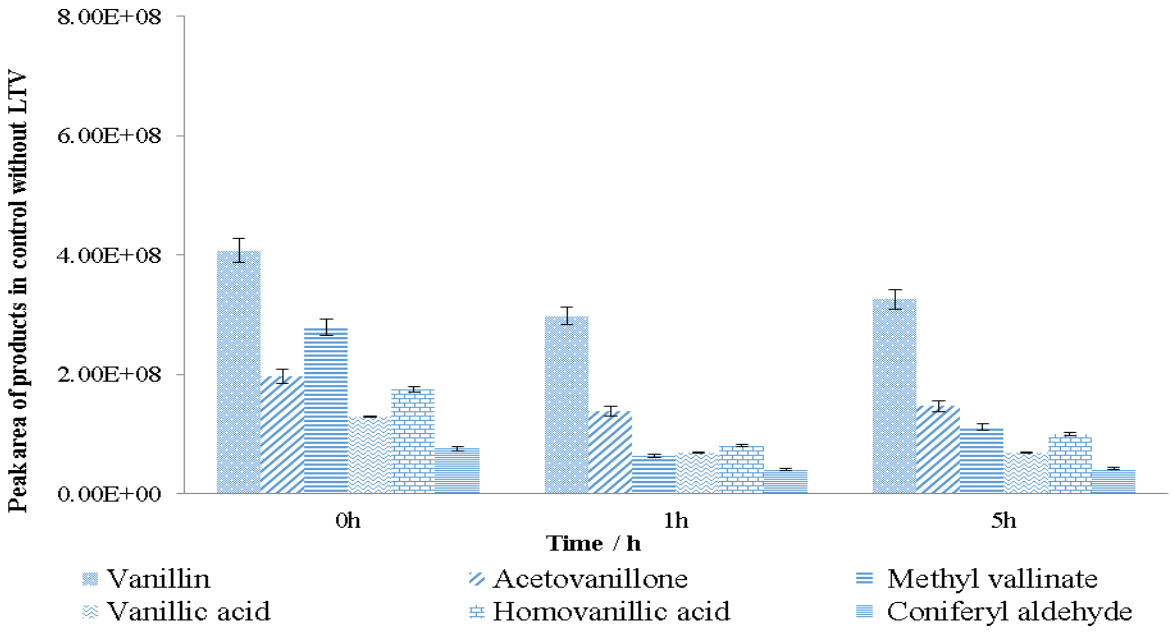


Figure 4.9 (b): The time course profile of Kraft lignin degradation (Control without LiP).

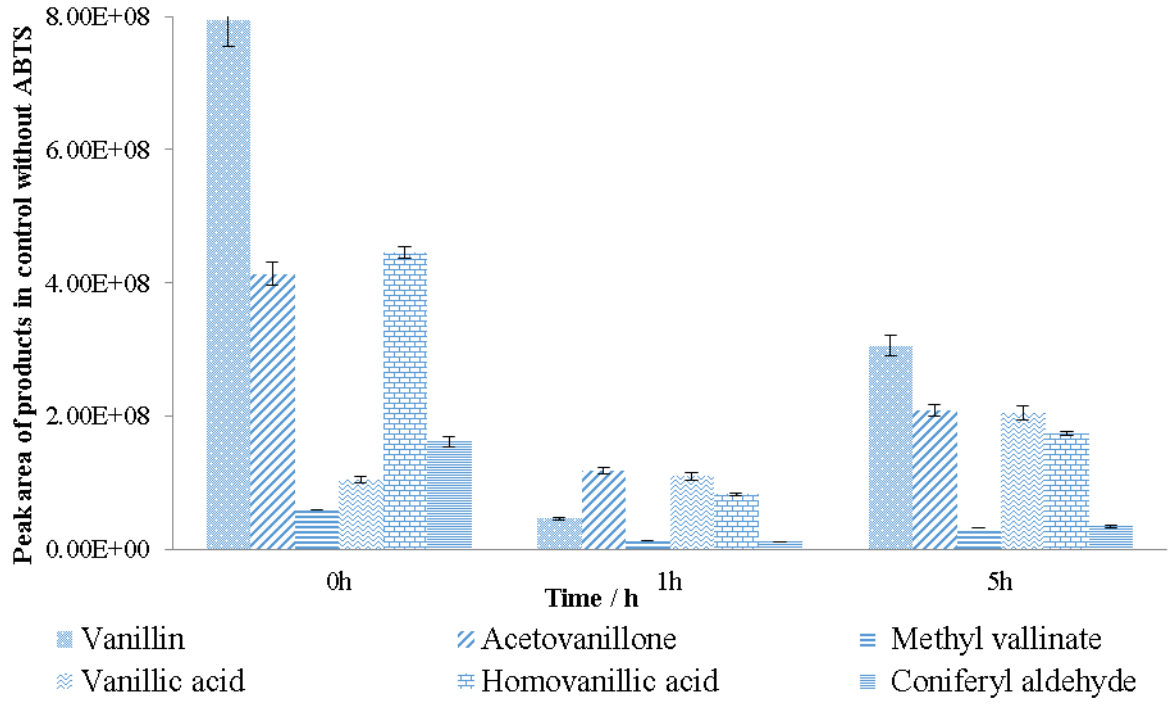


Figure 4.9 (c): The time course profile of Kraft lignin degradation (Control without veratryl alcohol).

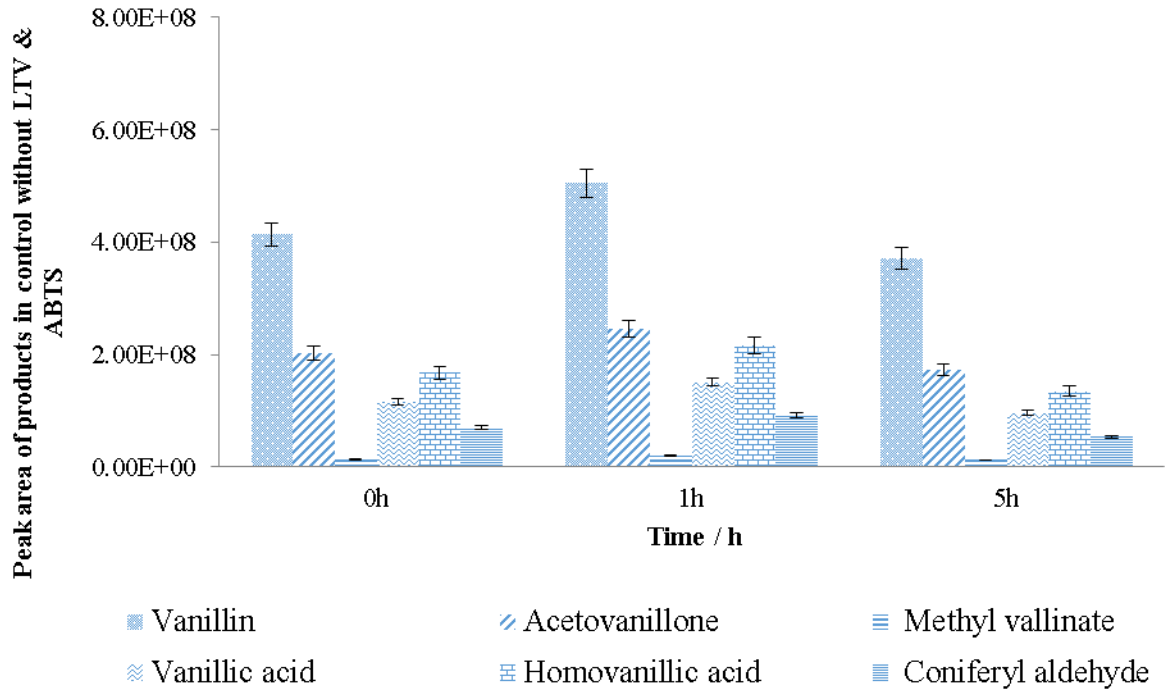


Figure 4.9 (d): The time course profile of Kraft lignin degradation (Control without both LiP and veratryl alcohol).

The reaction mixture contained 57 g/L Kraft lignin, 10 mg/mL LiP, 0.4 mM H_2O_2, and 10 mM veratryl alcohol and 50 mM sodium tartrate buffer (pH3). Control reactions without adding LiP and H_2O_2, veratryl alcohol, and without all of the above three components were also performed. The method in the control reactions was the same as in the complete reaction mixture but the components missed out were replaced by equivalent volumes of buffer. All reaction vessels were capped with foil and incubated at 28 °C in a shaker at 200 rpm for 5 h. Samples (11.6 mL) were taken at regular intervals and centrifuged at 1000 rpm for 5 mins at 20 °C prior to EA extraction. The aqueous sample and EA ratio used was 1:4 (v/v). EA extracts were dried under fume hood, re-suspended in dichloromethane (DCM) and analysed by GC-MS using splitless injection method. Both this experiment and the analysis were undertaken in two replicates. Mean are reported with errors smaller than 10%.

It can be seen that the peak areas of the compounds formed from the complete reaction tend to decrease as the reaction time is extended (Figure 4.9 a). Among the controls, not many important changes take place with incubation time for the products identified from the control reaction in which there was no enzyme (Figure 4.9 b). The most distinctive changes occurred in the sample which had no veratryl alcohol but contained LiP as the peak areas of almost all compounds were apparently higher than in the complete reaction when the reaction started

(0 h). The peaks then declined to the lowest level at 1h of reaction time compared with every other result obtained, and then increased again at 5 h (Figure 4.9 c). As for the control reaction which did not include any enzyme or mediator, the peak areas of the compounds rose at 1 h of incubation, but dropped slightly when the incubation time was extended to 5 h (Figure 4.9 d). In summary, no new compound was formed because of the enzymatic reaction. The control without adding veratryl aldehyde seemed more productive than the complete reaction and the other controls tested. Yet, this was not expected. It is an agreed fact by many researchers in this field that the degradation of lignin polymer is not a simple process from which a clear point can be concluded to tell what is really happening with the enzymatic reaction, because the possible products from lignin are so much closer in terms of molecular weight and other properties.

4.3.2.2 Kraft lignin degradation using manganese peroxidase (MnP)

There were six compounds detected from the Kraft lignin treatment with MnP (Figure 4.10).

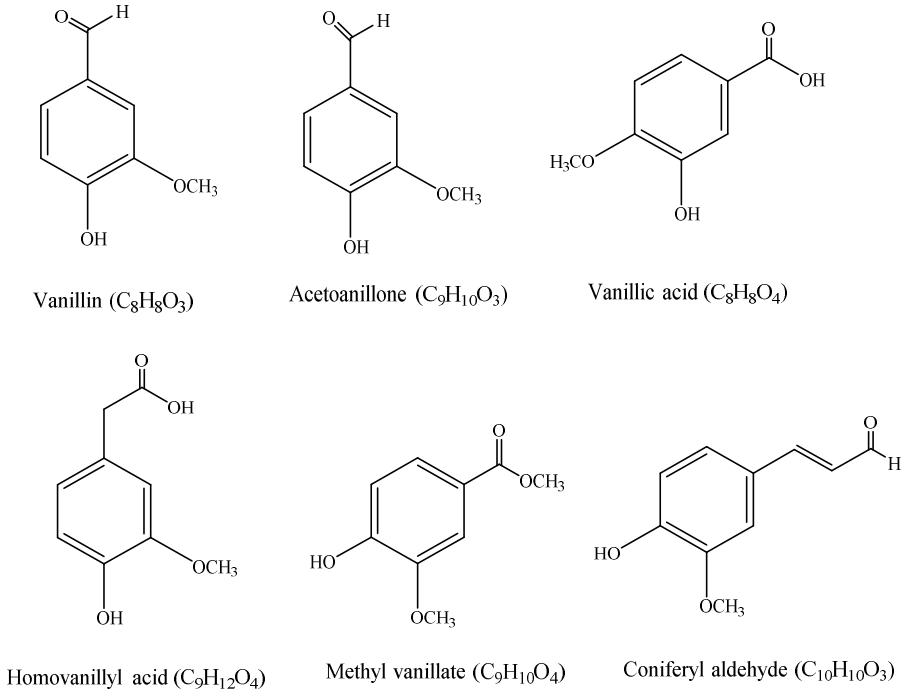


Figure 4.10: Chemical structures of identified compounds from Kraft lignin by MnP (from Sigma-Aldrich) depolymerisation by manganese peroxidase

It appeared that the products formed by both MnP and LiP catalysis are the same. The retention times detected by GC-MS of the main products during incubation are listed in Table 4.6. The chromatograms obtained at 5h are overlaid in Appendix A.11.5.

Table 4.6: Identification of products formed from Kraft lignin depolymerisation by MnP.

No.	Compounds	Retention time (min)	
		Product	External standard
1	Vanillin	11.22	11.52
2	Acetovanillone	12.32	12.65
3	Methyl vallinate	12.44	n. a
4	Vanillic acid	13.21	13.54
5	Homovanillic acid	14.23	n. a
6	Coniferylaldehyde	15.18	n. a

(n.a: a standard to the product was not available)

The time course profiles of the six major compounds from this reaction and their retention times are given in Figure 4.11 with all the relevant controls.

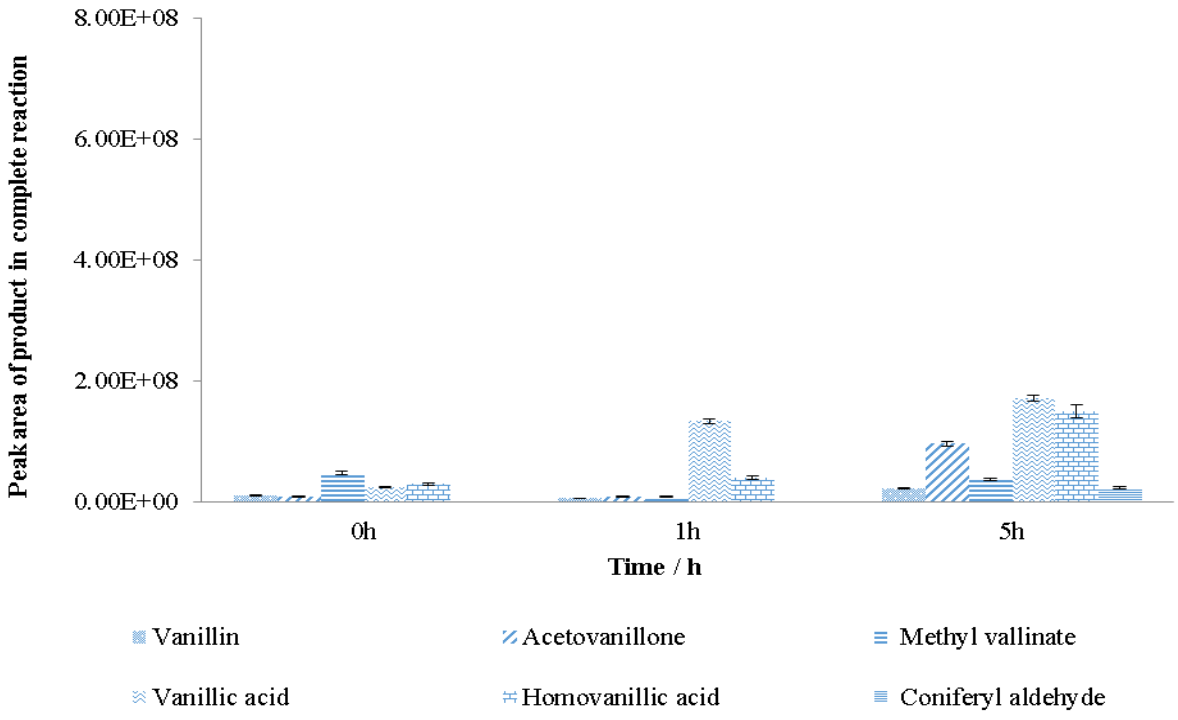


Figure 4.11 (a): The time course profile of Kraft lignin degradation by MnP in the presence of $MnSO_4$ (complete reaction)

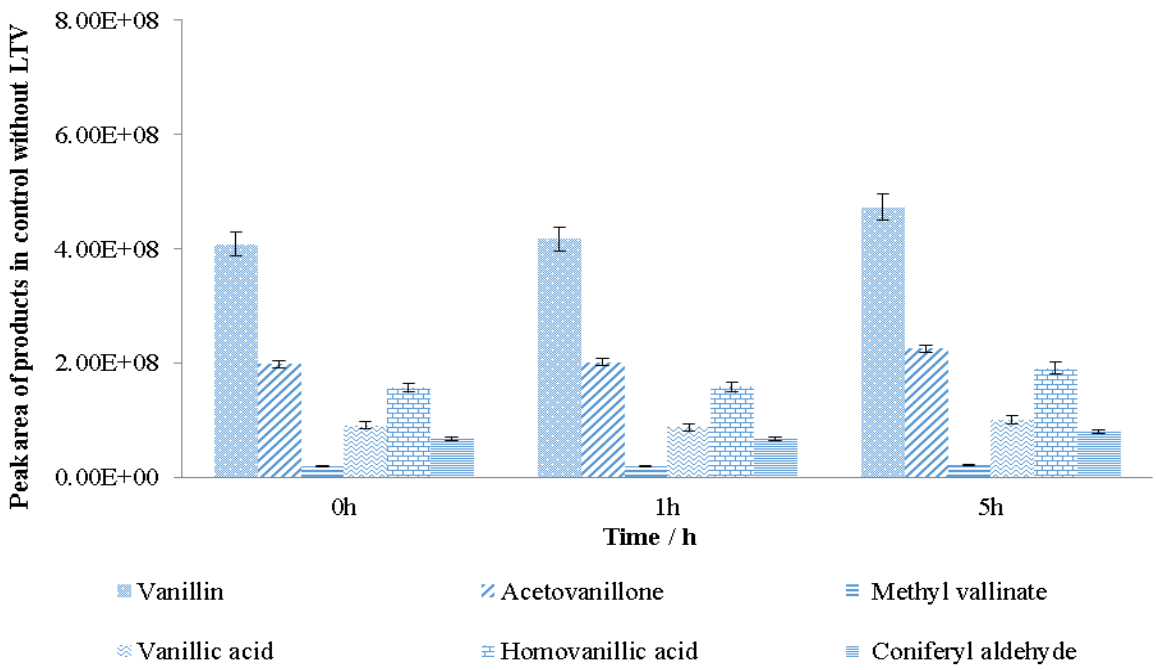


Figure 4.11 (b): The time course profile of Kraft lignin degradation in the absence of MnP

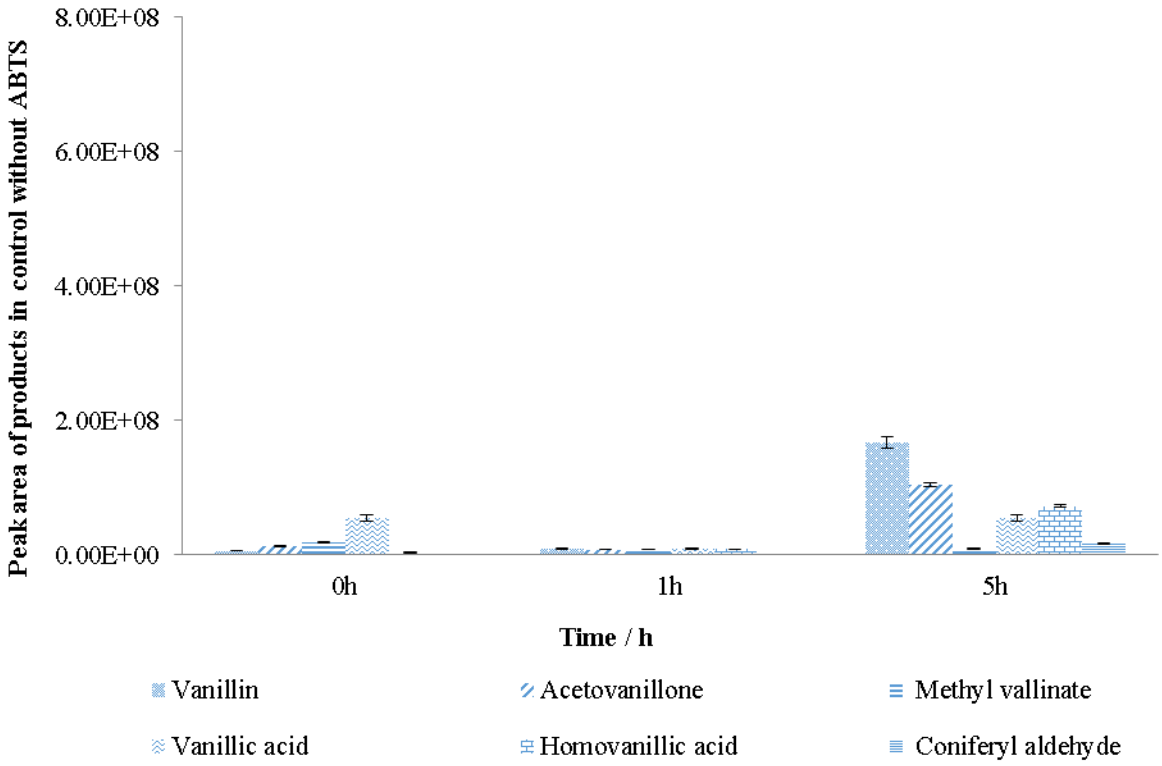


Figure 4.11 (c): The time course profile of Kraft lignin degradation in the absence of $MnSO_4$

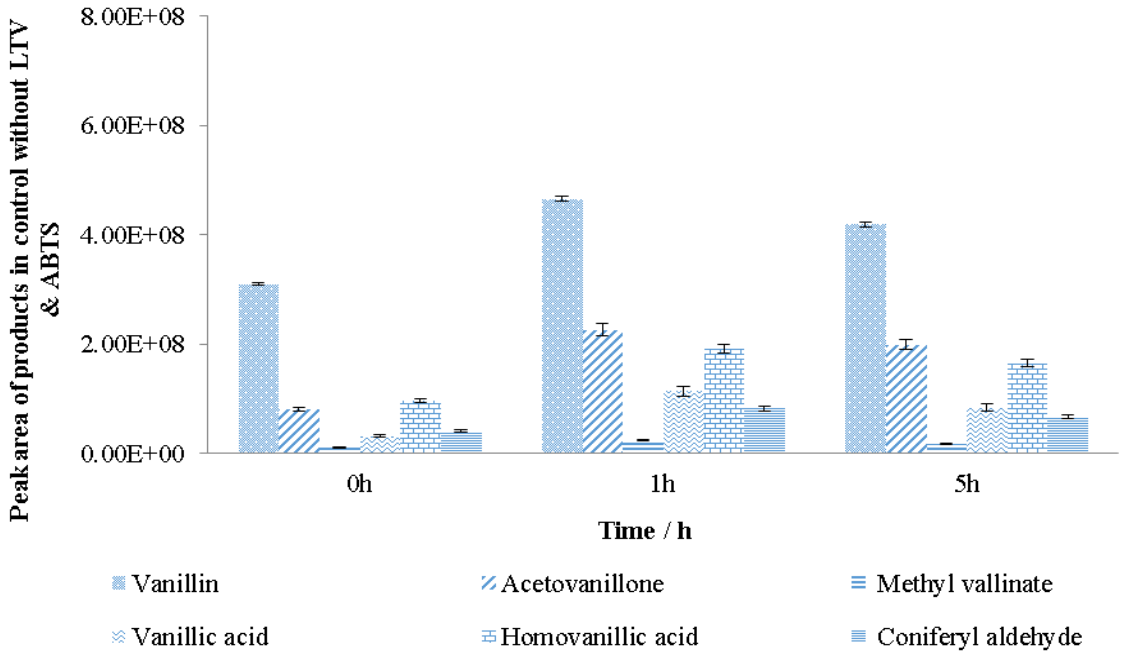


Figure 4.11 (d): The time course profile of Kraft lignin degradation in the absence of both MnP and $MnSO_4$

The complete reaction mixture contained 2g Kraft lignin, 0.1 mg/ mL MnP, 0.20 mM H_2O_2, 35 µM $MnSO_4$ solution, 3 mM ABTS and 100 mM sodium malonate buffer (pH 4.5). Control reactions without adding MnP and H_2O_2, $MnSO_4$ and ABTS, and without all of the above 4 components were also performed. The method in the control reactions was the same as in the complete reaction mixture but the components missed out were replaced by equivalent volumes of above buffer. All reaction vessels were capped with foil and incubated at 25°C in a shaker at 200 rpm for 5 h. Samples (11.6 mL) were taken at regular intervals and centrifuged at 1000 rpm for 5 mins at 20 °C prior to EA extraction. The aqueous sample and EA ratio used was 1:4 (v/v). EA extracts were dried under fume hood, re-suspended in dichloromethane (DCM) and analysed by GC-MS using splitless injection method. Both this experiment and the analysis were undertaken in two replicates. Mean are reported with errors smaller than 10%.

In the case of the complete reactions (Figure 4.11a), the peak area of the main products increased over time. A similar trend was found in the control where there was no LTV in the reaction (Figure 4.11b). For the control which did not contain ABTS but did include LTV in the reaction mixture, the peak areas of the products decreased after 1 h of incubation, but increased again at 5 h (Figure 4.11c), which shows both the depolymerisation and re-polymerisation effects of MnP with regard to this lignin. However, the situation completely reversed when it came to the control in which no MnP or $MnSO_4$ were present in the reaction mixture (Figure 4.11d). This indicates that the Kraft lignin can be depolymerised on its own over the reaction time. A comparison was made to show the product distribution changes between the complete reaction and the controls at 5 h, and this indicated that the amounts of the compounds were higher in the complete reaction rather than the other three controls as

expected. The results for the control without the addition of MnP or $MnSO_4$ were identical at 5 h.

4.4 Conclusions

4.4.1 Lignosulphonate reactions with laccase

Lignins from four different sources were processed by LTV under the same conditions. Although some difference could be seen in the products formed from each lignin provided by different sources, however, the LTV indicated too little a role in the depolymerisation of lignosulphonates as there was no significant difference between the enzymatic reactions and non-enzymatic reactions according to the results obtained so far, regardless of lignin solubility in an aqueous reaction system.

4.4.2 Kraft lignin reactions with laccase and both peroxidases

With respect to the Kraft lignin oxidation by laccase for 24 h and acidic treatment to terminate the reaction (Section 4.3.1.1), there was an increasing trend in general in the amount of products produced over the incubation time regardless of all situations as observed by GC-MS analysis. This indicated that Kraft lignin can be depolymerised on its own over time and the addition of an enzyme or mediator has little effect on its alteration. However, in a slightly different study (Section 4.3.1.2) which was conducted using the adopted method which referred to Bourbannais *et al.* (1995), laccase has mainly shown a depolymerisation role with regard to Kraft lignin which was enhanced by the addition of ABTS after the pre-incubation of this lignin with LTV as a permitted step.

5 The Effect of Ionic liquids on the Oxidation of ABTS by Laccase

The main aim to carry on this study is to find LTV compatible ionic liquids to be used as a co-solvent in the water-insoluble Organosolv degradation by LTV/ABTS system. Therefore, 92 ILs were screened to identify their effects on laccase catalysed ABTS oxidation at various concentrations.

5.1 Results and Discussion

5.1.1 Spectrophptometric assay for LTV activity on ABTS in presence of [C2mim][Lactate]

The activity of LTV was measured using ABTS as substrate in the presence of the ionic liquid [C2mim][Lactate]. The experiments were performed as regular spectrophotometric assay first (without adding IL to reaction mixture) as a control and then with [C2mim] [Lactate] (60%, v/v). Reactions were started by adding ABTS. The obtained spectra are shown in Figure 5.1.

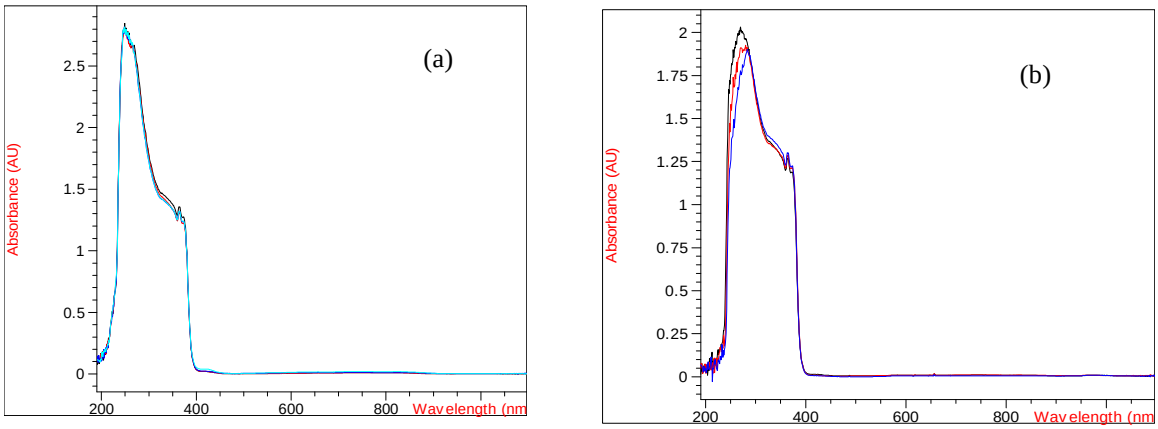


Figure 5.1: (a) Spectrum of ABTS after oxidation by LTV as a catalyst in (a); (b) presence of [C2mim] [Lactate]

The ionic liquid has no much effect on the spectrum of oxidized ABTS. However, the detected absorbance of ABTS is decreased as compare to the control experiment. This shows that [C_2mim] [Lactate] inhibited LTV activity because the rate of reaction is decreased drastically when C_2mim [lactate] (60%, v/v) was added to the reaction mixture (Figure 5.2).

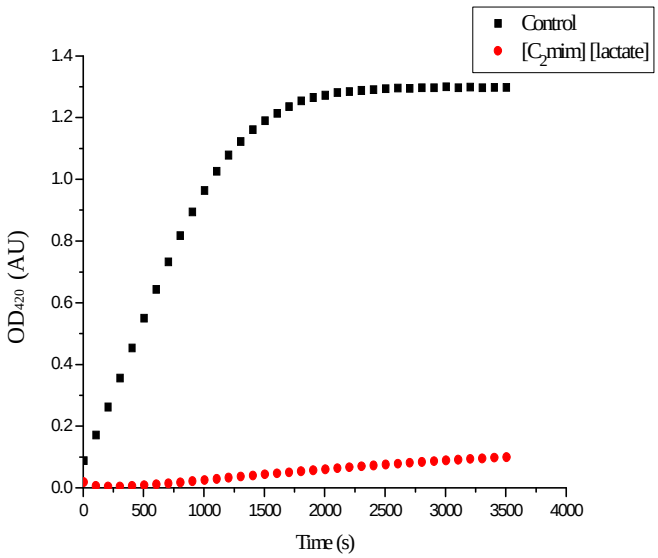
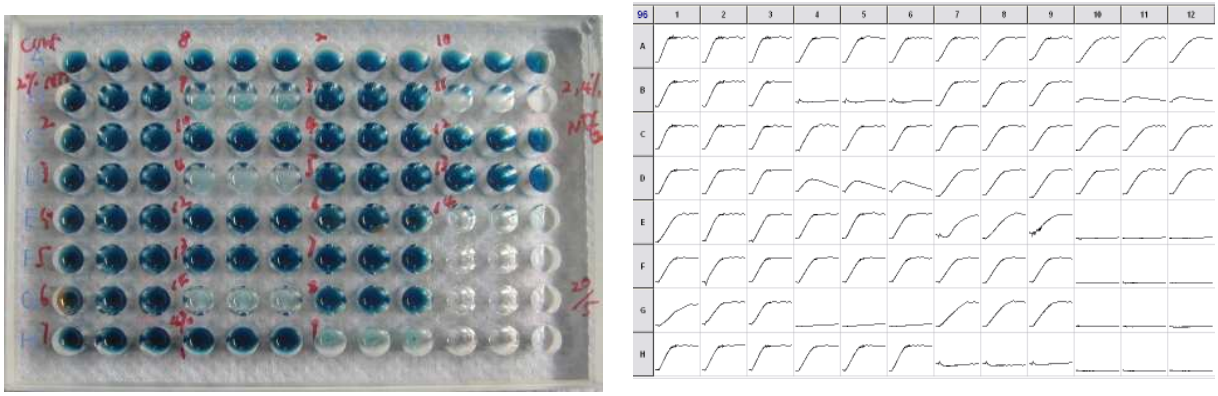


Figure 5.2: LTV activity in presence and absence of [C_2mim] [Lactate]

5.1.2 Microscale laccase assay

LTV activity towards the oxidation of ABTS was measured in parallel using ABTS as the substrate in 96-well plates in a total volume of 300 µl to minimise the usage of ionic liquids and maximise experimental throughput (Figure 5.3).



(a) (b)

Figure 5.3: (a) An image of ABTS colour change in the presence of different ILs in a 96- well plate assay; (b) Comparison of OD_{420} for ILs which contained NTf_2 as anion using 2 % to 4 % [IL] to show the difference of the measured activity curve versus control.

The formation of the $ABTS^{2+}$ radical cations was monitored at 420 nm. Assay (300 µL) contained 100 mM sodium citrate buffer pH 4.5 (263-257µL), 0.025 $mg.ml^{-1}$ laccase solution (2 µl) in sodium citrate buffer and 2-4 % (v/v) ionic liquids (0-12µl). Each solution of water immiscible ionic liquids was vortexed vigorously before addition to obtain a relatively homogeneous solution and also to minimise the error caused by water immiscibility. The reaction mixture was equilibrated for 60 seconds. The reaction was then started by adding 4 Mm ABTS solution (29 µl). Absorbance recorded at every 65 s at 37 °C for 5 h. The initial reaction rates were calculated monitoring over the first 715 s after substrate addition.

Initially, the assay system was validated by measuring the activity in the absence of ILs, as in a conventional spectrophotometric assay. The reaction mixture was equilibrated and the reaction was initiated by adding ABTS. The initial reaction rate (v_0) was 0.10 ± 0.04 $\mu M^{-1}.s^{-1}$. The initial reaction rate (v_0) was confirmed in a 96-well microplate reader and there was no difference in the measured reaction rate was found as the v_0 was measured as 0.10 ± 0.01 $\mu M^{-1}.s^{-1}$. Control experiments without adding ILs/LTV/ABTS were also performed because of a few factors affecting the accuracy of the measured data. Some of the ionic liquids were not water-soluble and so formed a biphasic system in water, such as most of the phosphonium (e.g. $[P_{6,6,6,14}]$ $[NTf_2]$, $[P_{8,8,8,4}]$ [Cl] and $[P_{8,8,8,12}]$ $[CH_3CO_2]$) and ammonium based ionic liquids (e.g. $[N_{1,1,4,8}]$[AOT], $[N_{1,1,2,4}]$[AOT] and $[N_{1,1,2,3OH}]$[AOT]) and and some of the imidazolium based ionic liquids (e.g. $[C_{10}mim][Cl]$, $[C_2mim][PF_6]$, $[C_4mim][NTf_2]$). Most of the sodium bis(2-ethylhexyl) sulphosuccinate (AOT) containing ionic liquids formed highly viscous phases when dissolved or mixed with water prior to measurement, and this made the measurement more difficult. A few apparently water miscible and/or immiscible ionic liquids formed an emulsion or droplets when mixed with water (e.g. AOT, OAc, Cl or HCO_2 containing ionic liquids). The phase behaviours and properties of the above discussed ILs are listed in Table1, Table 3 and Table 4 of Appendix A.13, respectively, with water miscibility and other behaviours observed during the actual experiments. All such factors may affect the light transmittance by scattering the light during measurement and so affecting the accuracy of the collected data. Hence, further investigations were also made before and after the OD measurement by observing the phase behaviour, such as colour changes, turbidity and the soft or hard gel crystals that were formed.

The effect of each IL was determined by measuring the absorbance of ABTS oxidation by LTV in the presence of such IL in the mixture, and then the initial rate of this reaction was compared to the initial rate of control reaction where IL was absent from the measurement. The primary aim was to determine how compatible these ionic liquids were towards laccase. It would have been useful to report the apparent change in the K_m and V_{max} values as compared to those found in control experiments in the absence of ionic liquids. However, it was not possible to measure such kinetic parameters accurately for most of the quaternary phosphonium ionic liquids due to the light scattering of such ILs during measurement. Therefore, the effect of water- miscible and -immiscible ionic liquids was measured by determining the initial rate of reaction to calculate the residual

activity of LTV instead of determining the corresponding kinetic parameters.

5.1.2.1 The effect of imidazolium based ionic liquids on LTV activity.

Imidazolium based ionic liquids (22 in total) were screened to test their effect on LTV activity with regard to ABTS oxidation. Control experiments without adding ionic liquids were conducted beforehand. The aim of this study was to test the effect on laccase activity of the length of the alkyl side chain, and of the anions and cations of the imidazolium based ionic liquids. Some imidazolium chlorides were also included due to their capability to dissolve cellulose and similar carbohydrates and with the intention of observing their effects on LTV activity (Zavrel *et al.*, 2009, Fendt *et al.*, 2010, Mäki-Arvela *et al.*, 2010). Water solubility and the measured raw laccase activity data of these ionic liquids are shown in Table 1 in the Appendix A.13. The results obtained are given in Figure 5.4.

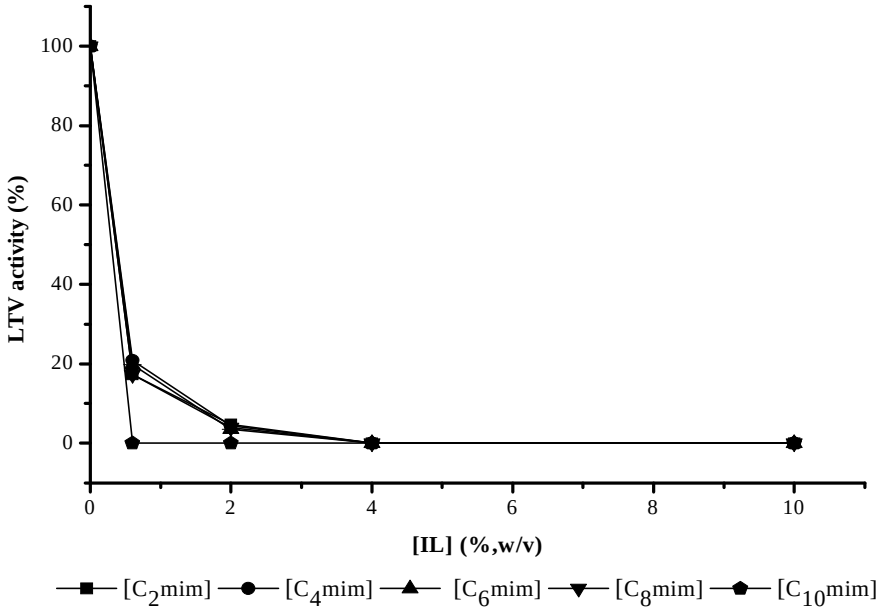


Figure 5.4 (a): Effect of IL concentration of different alkyl side chain length of imidazolium chlorides ([$C_x$mim][Cl]) on LTV activity. Data represent triplicate analyses with a standard deviation less than 0.1. Therefore, the error bar is not shown in the graph.

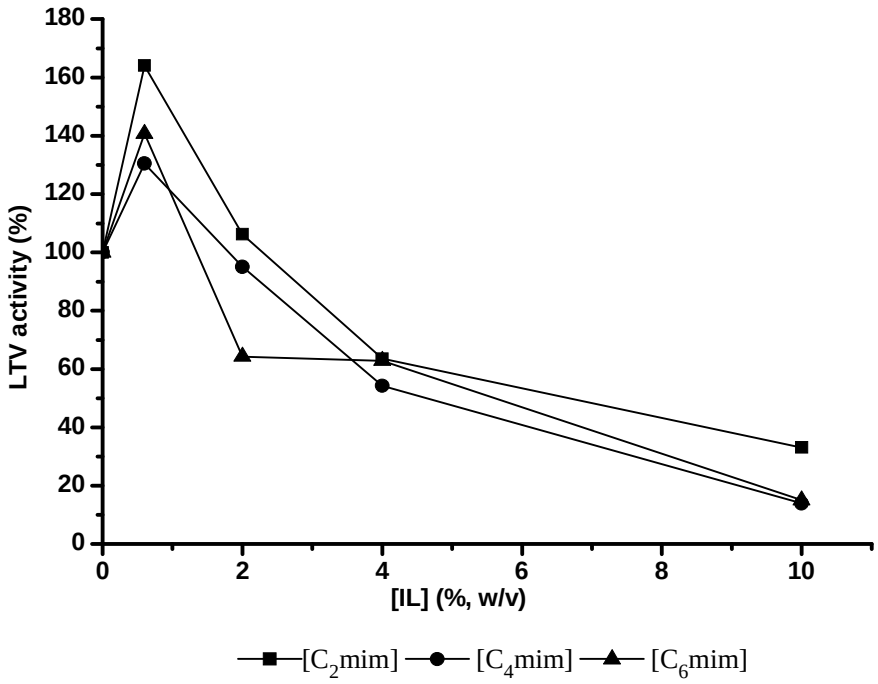


Figure 5.4 (b): Effect of IL concentration of different alkyl side chain length of imidazolium salts which contained [AOT] as anion and with different imidazolium cation on LTV activity.

LTV activity was measured over a range of IL concentrations from 0.6 % to 10.0 % (w/v). These assays (total volume of 300 µL) contained 100 mM sodium citrate buffer pH 4.5 (269-239 µL) pH 4.5), 0.025 $mg.ml^{-1}$ laccase solution (2 µl) in sodium citrate buffer and ionic liquid (0-30 µl). The reaction mixture was equilibrated for 60 seconds. The reaction was then started by adding 4 mM ABTS solution (29 µl). The formation of the $ABTS^{2+}$ radical cations was monitored at 420 nm and recorded at every 65 s at 37 °C for 5 h. The initial reaction rates were calculated monitoring over the first 715 s after substrate addition. The measurements were done in triplicates. The mean rate of reaction reported with the error less than 1%.

Imidazolium chlorides were found to decrease laccase activity when 0.6 % (w/v) was used, (Figure 5.4a). LTV was inhibited by these ILs at concentrations higher than 2 % (w/v). The length of the alkyl side chain had little effect except that $[C_{10}mim]Cl$ was inhibitory at all concentrations tested (Table 1 in the Appendix A.13). AOT-containing imidazolium based ionic liquids (Figure 5.4b), unlike those containing Cl, were found to stimulate laccase activity when less than 2 % (w/v) was used and maintained 60 % of laccase activity until a concentration of 4 % (w/v). Among the AOT-containing imidazolium ILs, lthough $[C_2mim][AOT]$ caused the highest stimulation and least inhibition (Figure 5.4b), however, the difference between them is not significant (Table 1 in the Appendix A.13). These results are not in agreement with the findings of by Zhao *et al*. who found that an increase in alkyl side chain has an obvious inhibition effect on enzyme activity (Zhao, **2006**). However, there was no data given for the effect of AOT in their work.

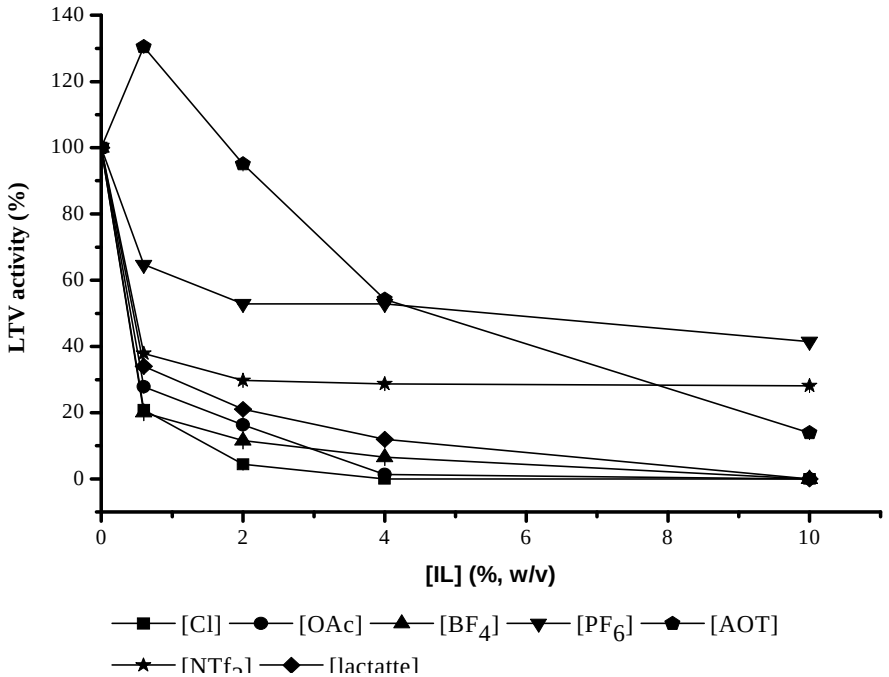


Figure 5.5 (a): The effect of anions on LTV activity when Im = C_4mim.

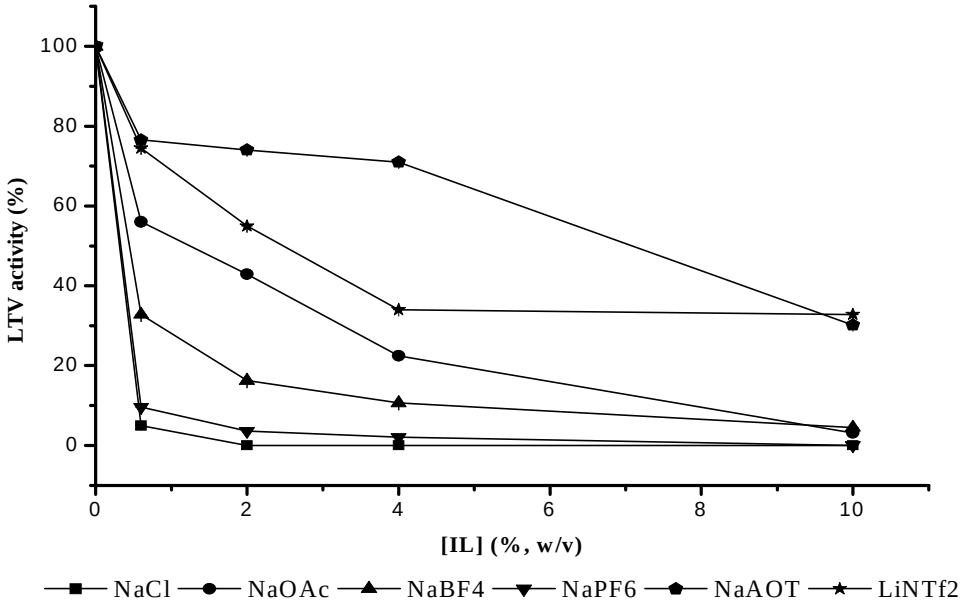


Figure 5.5 (b): The effects of anions on LTV activity when equivalent sodium or lithium salts as control.

LTV activity was measured over a range of IL concentrations from 0.6 % to 10.0 % (w/v). These assays (total volume of 300 µL) contained 100 mM sodium citrate buffer pH 4.5 (269-239 µL) pH 4.5), 0.025 mg.ml^{-1} laccase solution (2 µl) in sodium citrate buffer and ionic liquid (0-30 µl). The reaction mixture was equilibrated for 60 seconds. The reaction was then started by adding 4 mM ABTS solution (29 µl). The formation of the $ABTS^{2+}$ radical cations was monitored at 420 nm and recorded at every 65 s at 37 °C for 5 h. The initial reaction rates were calculated monitoring over the first 715 s after substrate addition. The measurements were done in triplicates. The mean rate of reaction reported with the error less than 1%.

All of the C_4mim^+ salts inhibited LTV activity, except for [C_4mim][AOT] which stimulated LTV activity when supplied at 0.6 % (w/v). Half of LTV activity remained until the concentration of [C_4mim][AOT] was up to 4% (w/v), however, it inhibited LTV nearly by 85% at the concentration of 10% (w/v). When it comes to the comparison of the effect of anions that were contained in sodium and lithium salts, the residual LTV activity was retained even in the presence of 10 % (w/v) of the anions AOT^-, NTf_2^- and PF_6^- (Figure 5.5a). LTV maintained its activity only in 0.6 % (w/v) NaCl, but caused complete inhibition at 2% (Figure 5.5b).

When the effects of NTf_2^--containing lithium salt and C_4mim based ILs were compared, the retained LTV activity was not too different until a concentration of 10 % (w/v), as it supports about 33% in the former while it was around 35 % in the latter. The $NaPF_6$ was a strong inhibitory to the LTV and only 10 % of its activity was observed at 0.6 % (w/v), and it was deactivated totally at 10 % (w/v). In contrast, nearly 50 % of LTV activity was retained when C_4mim was used as the cation. This is mainly because C_4mim PF_6 is a water-immiscible IL and forms a biphasic system when it is mixed with water whereas $NaPF_6$ is a completely water soluble salt. This may suggest that the ionic interaction between laccase and C_4mim PF_6 is far lower than the normal salt $NaPF_6$. In a biphasic system, laccase may be surrounded by the essential aqueous nano-environment to maintain its activity whereas it is exposed to the salt solution formed by water-miscible $NaPF_6$. As for chlorides, both sodium and C_4mim salts were found to be inhibitory above 2 % (w/v). BF_4^- inhibited LTV activity more than PF_6^- since the former soluble in water. In general, the anions affected LTV activity more than the cations. In the case of the C_4mim salts tested, the effect of anions on the LTV activity decreased in the order of [Cl] > [BF_4] > [CH_3CO_2] > [NTf_2] > [PF_6] > [AOT] whereas the order for the equivalent sodium or lithium salts was [Cl] > [PF_6] > [BF_4] > [CH_3CO_2] > [NTf_2] > [AOT], in general. Some of the findings agreed with Zhao, who found [Cl] to cause more inhibition than [CH_3CO_2], but slightly disagreed with the result for the effect of [PF_6] and [BF_4] anions due to the water solubility difference of these normal salts and their corresponding ILs (Zhao, 2006, Zhao *et al.*, 2006). Therefore, it should be noted, however, that such differences may depend on the enzyme.

5.1.2.2 LTV activities in the presence of organic acid-containing imidazolium ILs and sodium salts

Imidazolium based ILs and sodium salts of various organic acids (12 in total) were tested to determine their compatibility with LTV. All of the liquids and salts were water miscible. The structural details and the resultant raw activity data of these salts are shown in Table.2 of Appendix A.12. Except for [formate], the C_2mim^+ and sodium salts containing organic acid anions stimulated LTV activity at 0.6 % (w/v), but were inhibitory at higher concentrations (Figure 5.6a and b). The highest activity was found to be induced by [glycolate], whereas [formate] caused total deactivation of LTV even at the lowest concentration. The kosmotropic anion, formate (HCO_2^-), may have stronger interactions with water molecules and behaved as a kosmotropic anion and thereby it caused a higher inhibition to LTV (Zhao *et al.*, 2008**,** Yang, 2009). LTV activity was decreased by the investigated anions, at a concentration of salt above 0.6 % (w/v), in the following order: [glycolate] < [lactate] < [octanoate] < [decanoate] < [furoate] < [formate] (Figures 5.6a and 5.6b).

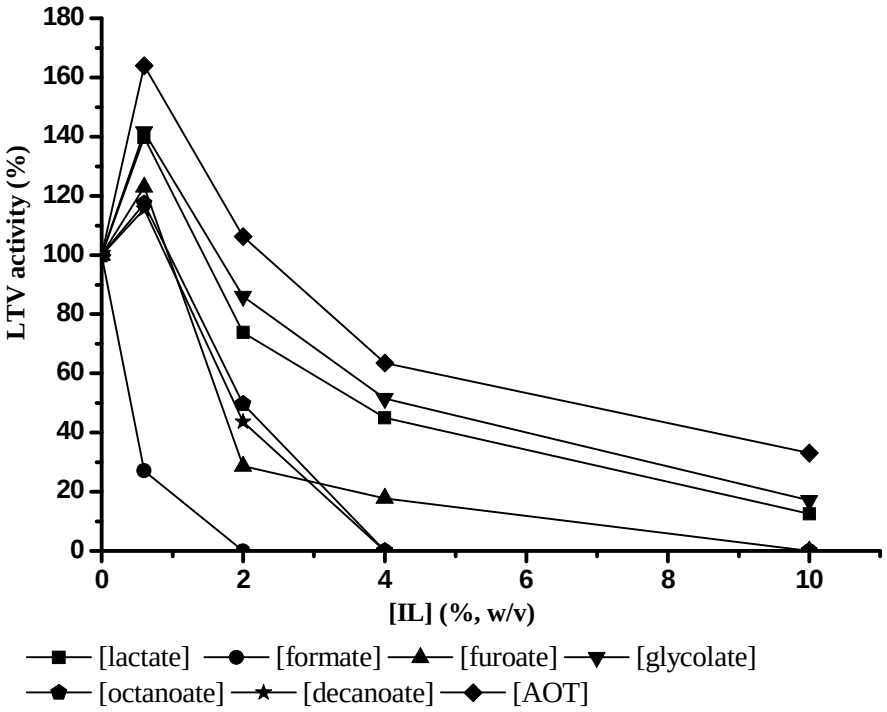


Figure 5.6(a): The effects organic acid containing imidazolium ILs on LTV activity when the cation was C_2mim^+.

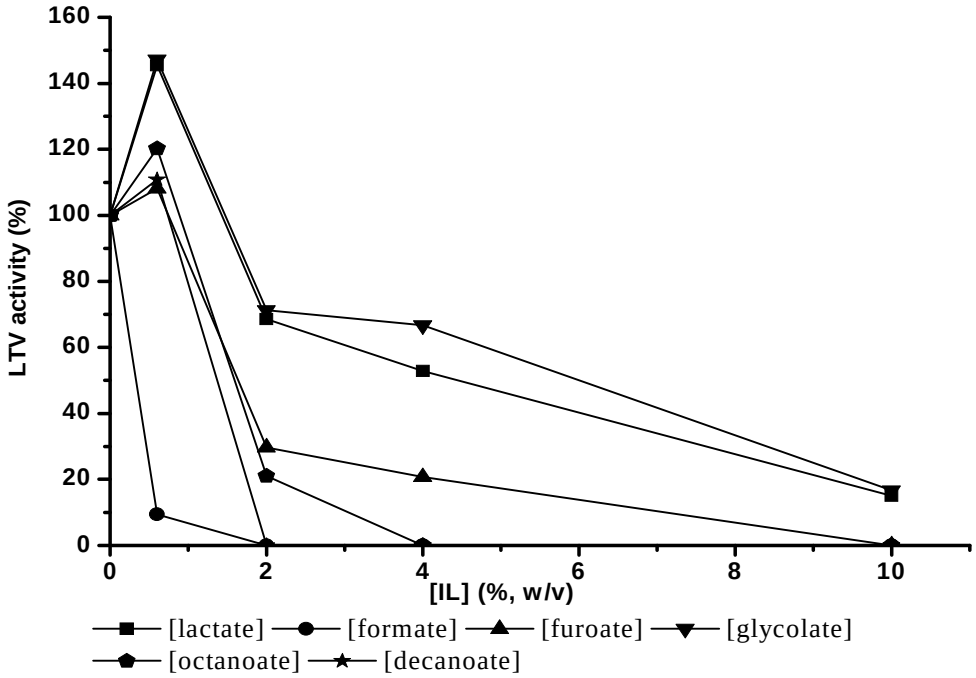


Figure 5.6 (b): The effects of equivalent sodium salts

LTV activity was measured over a range of IL concentrations from 0.6 % to 10.0 % (w/v). These assays (total volume of 300 µL) contained 100 mM sodium citrate buffer pH 4.5 (269-239 µL) pH 4.5), 0.025 $mg.ml^{-1}$ laccase solution (2 µl) in sodium citrate buffer and ionic liquid (0-30 µl). The reaction mixture was equilibrated for 60 seconds. The reaction was then started by adding 4 mM ABTS solution (29 µl). The formation of the $ABTS^{2+}$ radical cations was monitored at 420 nm and recorded at every 65 s at 37 °C for 5 h. The initial reaction rates were calculated monitoring over the first 715 s after substrate addition. The measurements were done in triplicates. The mean rate of reaction reported with the error less than 1%.

It is worth mentioning that, to date, no relevant literature can be found to illustrate the effect of such organic anions in the reported Hofmeister series yet (Kunz *et al.*, 2004, Hua, 2005, Zhang & Cremer, 2006**,** Zhao, 2006, Zhao *et al.*, 2006**,** Zhao *et al.*, 2006). Additionally, LTV activity was increased when a less chaotropic cation, Sr^{2+}, was added. Furthermore, additional tests have been conducted on [lactate] salts containing [Na], [Sr^{2+}] and [C_2mim], for which it was found that LTV activity was enhanced at a concentration of 0.6 % (w/v); also, 50-60 % of LTV activity was maintained at 4 % (w/v) of [C_2mim][AOT], sodium lactate and sodium glycolate (Figure 5.6 and Table1 and 2 in the Appendix A.13). At higher concentrations of ILs and the equivalent salts, for example at 10 % (w/v) of both cases, the LTV activity was gradually reduced and reached zero for the above mentioned 3 lactate salts (Figures 5.6a and 5.6b). The influence of such salts has been found in the following order: $[Sr]^{2+} > [Na]^{+} > [C_2mim]^{+}$. This is because of the kosmotropicity which increases while the

ion charge increased. These findings are consistent with those of Zhao and his co-workers (Zhao *et al.*, 2006).

5.1.2.3 The effect of [AOT] and [NTf_2] -containing quaternary ammonium ILs towards LTV activity

Previous results suggested that better retention of LTV activity is obtained in the presence of [AOT] and [NTf_2] containing ILs. Therefore, the influence on the LTV activity of quaternary ammonium based salts containing [AOT] and [NTf_2] anions were investigated. Control experiments in the absence of an ionic liquid were carried out. [Li][NTf_2] was also tested as a control. Structural details and raw activity data of these salts are shown in Table 3 in the Appendix A.13. A comparison of the results is given in Figure 5.7.

All the tested ammonium salts were water-immiscible. The general trend was the same as that illustrated for the ILs already discussed, since LTV activity decreased when the ionic liquid concentration increased. Interestingly, the reduction of LTV activity found to occur for this class of ILs was not such a drastic manner as for the other ILs.

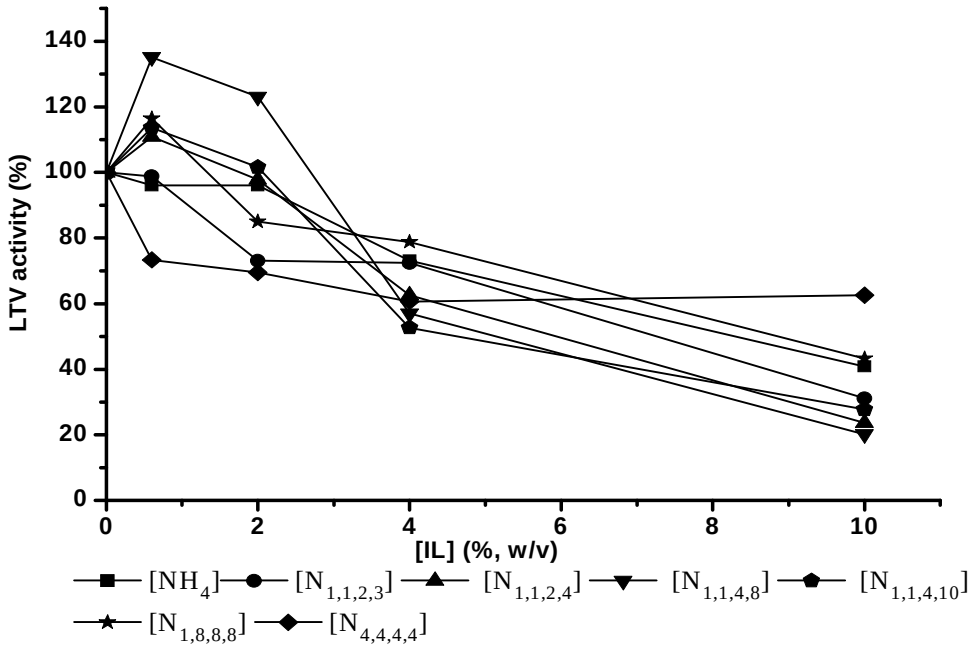


Figure 5.7(a): The effect of alkyl chain length on LTV activity when AOT is the anion for quaternary ammonium based ILs.

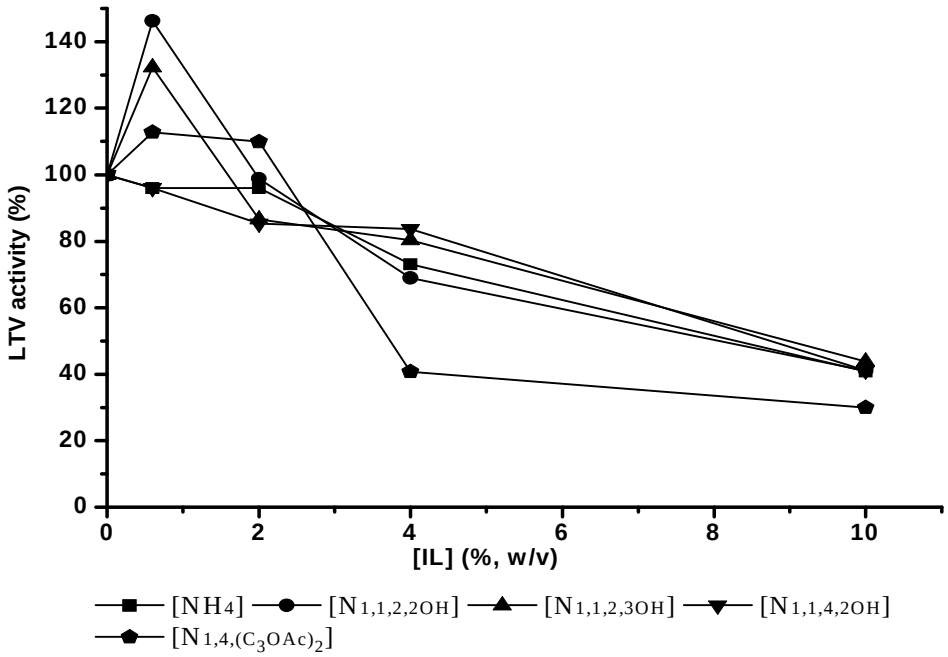


Figure 5.7(b): The effect of substituents on the quaternary ammonium side chain towards LTV activity when AOT is an anion.

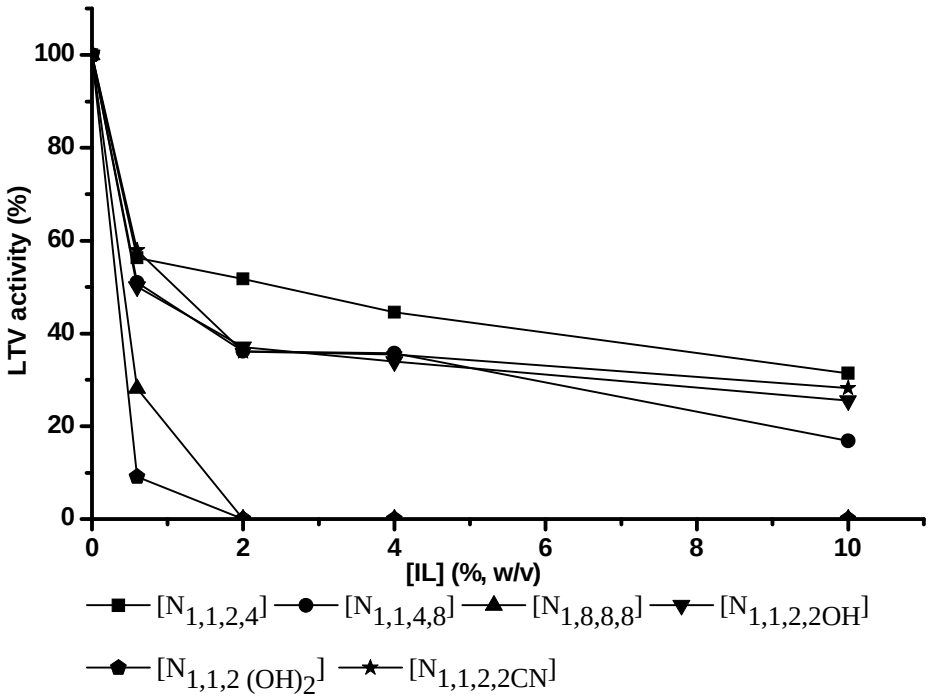


Figure 5.7(c): The effect of quaternary ammonium salts on LTV activity when NTf_2 is an anion.

Reaction conditions of Figure 5.7 were: LTV activity was measured over a range of IL concentrations from 0.6 % to 10.0 % (w/v). These assays (total volume of 300 µL) contained 100 mM sodium citrate buffer pH 4.5 (269-239 µL) pH 4.5), 0.025 $mg.ml^{-1}$ laccase solution (2 µl) in sodium citrate buffer and ionic liquid (0-30 µl). The reaction mixture was equilibrated for 60 seconds. The reaction was then started by adding 4 mM ABTS solution (29 µl). The formation of the $ABTS^{2+}$ radical cations was

monitored at 420 nm and recorded at every 65 s at 37 °C for 5 h. The initial reaction rates were calculated monitoring over the first 715 s after substrate addition. The measurements were done in triplicates. The mean rate of reaction reported with the error less than 1%.

$[N_{1,1,4,8}][AOT]$ stimulated LTV activity over the range 0-2 % (w/v). These salts, $[N_{1,1,2,4}][AOT]$, $[N_{1,1,4,10}][AOT]$ and $[N_{1,8,8,8}][AOT]$, stimulated LTV activity at 0.6 % (w/v) and all three caused inhibition at higher concentrations. The $[NH_4][AOT]$ salt was tested as a control and it was an inhibitory along with $[N_{1,1,2,3}][AOT]$ and $[N_{4,4,4,4}][AOT]$ at all concentrations tested. Nevertheless, some activity was still retained in $[N_{4,4,4,4}][AOT]$, $[NH_4][AOT]$ and $[N_{1,8,8,8}][AOT]$ whilst $[N_{1,1,2,3}][AOT]$ and $[N_{1,1,4,10}][AOT]$ inhibited LTV almost completely at 10 % (w/v). Similar constant activity was maintained in the presence of $[N_{4,4,4,4}][AOT]$ salt at almost all concentrations and higher (e.g. 62%) than the residual LTV activity data of the control (e.g. 41% in $[NH_4]$ [AOT]).

When [ILs] concentrations were in the range 0.6-10 %, the LTV activity changed only a little by adding the different tetra alkyl ammonium ILs. Only changes of 27-37 % were observed (*versus* control). Most significantly, more than 60 % of the LTV activity was maintained for most of the salts in this group. This evidence suggests that there is a remarkable compatibility towards laccase for ILs having [AOT] as the anion rather than containing $[NTf_2]$.

Hydroxyl-containing quaternary ammonium based ILs affected the LTV activity in the following order: $[N_{1,1,1,2OH}] < [N_{1,1,1,3OH}] < [N_{1,1,4,2OH}]$. LTV activity decreased when the alkyl side chain length of some quaternary ammonium cation increased, e.g. $[N_{1,4,\ (C3OAc)2}]$ (Figure 5.7 b). Similar results were found for $[N_{1,\ 1,\ 4,\ 8}]$ and $[N_{1,\ 1,\ 4,\ 10}]$, whose effect on LTV activity resulted in the order $[N_{1,\ 1,\ 4,\ 8}] > [N_{1,\ 1,\ 4,\ 10}]$ (Figure 5.7a). This also occurred with the NTf_2 containing ILs in Figure 5.7 (c) where the LTV activity decreased as the alkyl side chain length of the quaternary ammonium cation increased, such as $[N_{1,\ 1,\ 2,\ 4}]$ and $[N_{1,\ 1,\ 4,\ 8}]$. Conversely, $[N_{1,8,8,8}]$ that has a carbon side chain length longer than $[N_{4,4,4,4}]$, was found to promote LTV activity by 16 % while $[N_{4,4,4,4}]$ decreased it by 27 %. Such evidence may be explained by the fact that $[N_{4,4,4,4}]$ bears four identical substituents on the ammonium cation and so all may contribute equally to such an effect, thus resulting in very little noticeable difference in the LTV activity. Another interesting finding revealed by investigating ILs containing a quaternary ammonium based cation was that just a single hydroxyl substituent on the ammonium cation caused a difference when the similar ILs were compared. For example, $[N_{1,1,2,3OH}]$ promoted LTV activity by 32 % (*versus* control) which was more than $[N_{1,1,2,3}]$ perhaps due to the electron donating –OH substitute. $[N_{1,4,(C_3OAc)_2}][AOT]$ also

improved LTV activity by 13 % (*versus* control) when used at 0.6 % (w/v), but decreased its activity by 70 % when used at 10 % (w/v).

The substituent on the structure of the ILs appears to be relevant. [Li][NTf_2] has been tested as a control for [NTf_2] containing salts, and showed good compatibility with LTV at 0.6 % (w/v). At 0.6 % (w/v), laccase retained its activity at 74 % versus the control, but caused inhibition after 4 % (w/v) with a residual activity of 34 % (Table 3 in AppendixA.13).

It is worth noting that some mismatch phenomena (e.g. compared to the known Hofmeister series) were found for this group of ILs as most of these were not water-miscible, and formed a gel or emulsion when mixed with water. This is also another factor to take into consideration when screening the effects of ILs on LTV activity.

5.1.2.4 The Effect of the [AOT] containing Pyridinium and Pyrrolidinium Ionic Liquids on LTV activity

One pyrrolidinium and three pyridinium based ILs were tested in order to evaluate their compatibility with laccase. All of the liquids had [AOT] as the anion and all formed highly viscous and gel-like mixtures in water, with the only exception being [C_4mpy] which is water-miscible. The relevant structure and measured activity details are given in Table 4 in Appendix A.13.

In the concentration range 0.6 to 2.0 % (w/v), all of the liquids stimulated LTV activity and decreased it gradually at higher concentrations (Figure 5.8). For the three pyridinium ILs, the LTV activity increased as the alkyl side chain length on the pyridinium cation increased at all concentrations except 2 % (Figure 5.8). For instance, the highest LTV activity was found to be 139 % using [C_8,mpy][AOT] while the lowest at 114 %, resulted from [C_4mpy] [AOT]. This was contrary to the findings from imidazolium salts or other ionic liquid types where an increase of alkyl side chain length resulted in a decrease of enzyme activity. When different cations with the same alkyl side chain were considered, [C_4,mpyr] increased LTV activity more than [C_4mpy] for all given concentrations.

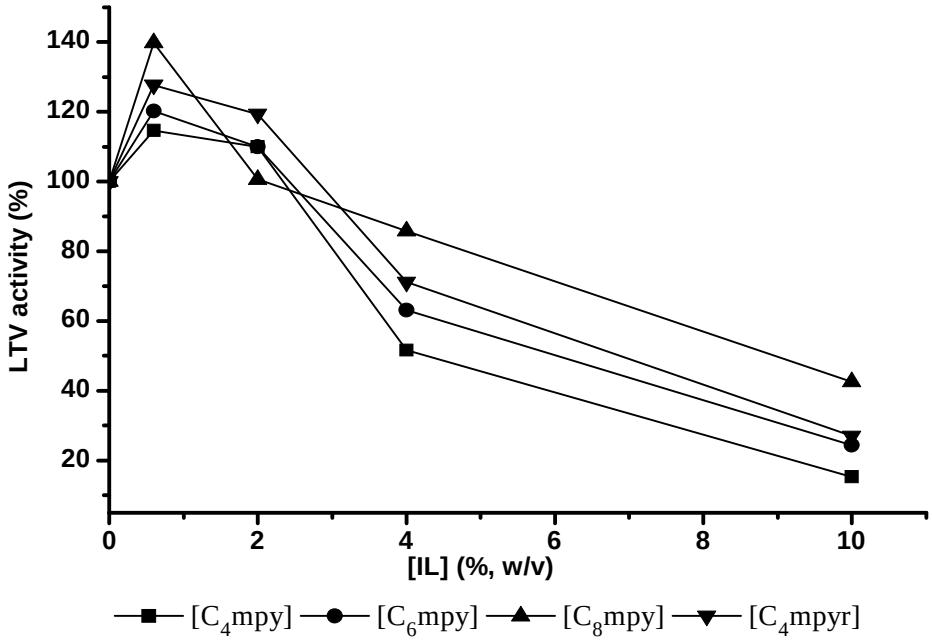


Figure 5.8: LTV activity in the presence of pyridinium and pyrrolidinium based ILs

LTV activity was measured over a range of IL concentrations from 0.6 % to 10.0 % (w/v). These assays (total volume of 300 µL) contained 100 mM sodium citrate buffer pH 4.5 (269-239 µL), 0.025 $mg.ml^{-1}$ laccase solution (2 µl) in sodium citrate buffer and ionic liquid (0-30 µl). The reaction mixture was equilibrated for 60 seconds. The reaction was then started by adding 4 mM ABTS solution (29 µl). The formation of the $ABTS^{2+}$ radical cations was monitored at 420 nm and recorded at every 65 s at 37 °C for 5 h. The initial reaction rates were calculated monitoring over the first 715 s after substrate addition. The measurements were done in triplicates. The mean rate of reaction reported with the error less than 1%.

5.1.2.5 Effect of quaternary phosphonium ionic liquids on LTV activity

The effect on LTV activity of ILs composed of quaternary phosphonium cations and Cl, HCO_2, CH_3CO_2, AOT and NTf_2 as the anions was investigated. Solubility and other applicable details are shown in Table.5 in Appendix A.13.

The majority of such ILs inhibited LTV when used at a low ionic liquid concentration, whereas the quaternary ammonium based ILs were mostly found to be highly compatible with LTV at lower concentrations (0.6-2%, w/v) in Figure 5.7. The degree of inhibition once again was found to depend on the anion and the length of the alkyl side chain attached to the phosphonium cation. Different to the findings in the cases of imadazolium ILs, the shorter carbon side chains inhibited more than the longer side chain for quaternary phosphonium ILs. However, in some cases, this effect could not be generalised as some specific differences were also exhibited by this group. For example, the LTV activity obtained for P_4 cations was

inhibited by 19-28 %, while the P_8 cations showed an inhibition varying in the range of 55-83.50 % (Table.5 in Appendix A.13). All P_4 ILs tested had Cl as the anion and all of them were water miscible. The LTV activity decreased when their side chain length was increased. One P_5 ionic liquid was tested, showing complete inhibition, due to the presence of the HCO_2^- anion which easily formed a strong H-bond in water, so deactivating the LTV. All the P_6 ILs tested inhibited LTV activity except $[P_{6,6,6,14}][AOT]$ and $[P_{6,6,6,14}][NTf_2]$, in which the former showed the best compatibility with LTV amongst the tested phosphonium ILs. Furthermore, all the P_6 ILs included in this study were found to be water immiscible, with the only exception being $[P_{6,6,6,3}][Cl]$. In the P_8 ILs group, $[P_{8,8,8,10}][CH_3CO_2]$ showed the highest activity (83.50 % *versus* control) while $[P_{8,8,8,6}][Cl]$ displayed the lowest activity (40 % versus control). All of the tetra alkyl phosphonium based ILs proved to be LTV inhibitors. In fact, the anions (Cl^-, $CH_3CO_2^-$ and HCO_2^-) in these ILs were categorised as strong kosmotropes in the Hofmeister classification (Yang, 2009). Still, with regard to the P_8 group ILs, by maintaining the same cation $[P_{8,8,8,12}]$, the LTV activity was decreased in the following order: $CH_3CO_2^- > HCO_2^- > Cl^-$ at a concentration of 0.6 % (w/v). The higher activity shown by $[P_{8,8,8,12}][CH_3CO_2]$ as compared to $[P_{8,8,8,12}][Cl]$ was surprising as $CH_3CO_2^-$ is more kosmotropic than Cl^- (Zhao, 2006, Zhao *et al.*, 2006, Yang, 2009) and so it should decrease the activity of LTV. However, instead, the $[P_{8,8,8,4}][Cl]$ and $[P_{8,8,8,4}][CH_3CO_2]$ behaved as expected (Zhao *et al.*, 2006). The effect on LTV activity of the different side chain lengths of the P_8 cations ($[P_{8,8,8,1}]$ to $[P_{8,\ 8,\ 8,\ 10}]$) of the ILs bearing the same anion Cl was also investigated. The shorter side chain length supported LTV activity at low ionic liquid concentrations, as expected. However, all the quaternary phosphonium ILs inhibited LTV activity at [IL] higher than 0.6 % (Table. 5 in the Appendix A.13). Clearly, they should not be suggested to support laccase catalysed reactions.

5.2 Screening of LTV-compatible ILs at higher concentrations

With the hope to use the most laccase-compatible ILs in future work, some of the most enzyme friendly ILs were screened at higher concentrations. One quaternary ammonium ($[N_{4,4,4,4}][AOT]$) and four imidazolium-based salts ($[C_2mim][PF_6]$, $[C_4mim][PF_6]$, $[C_2mim][NTf_2]$ and $[C_4mim][NTf_2]$), were tested. All of the ILs were water immiscible. They were tested at concentrations varying in the range 20-100 % (w/v). The previous screening results, obtained for them when the [IL] was between 0.6 and 10 % (w/v), were also included

in the further investigation in order to compare the overall trend (Figure 5.9). Solubility and other applicable details of these ionic liquids are shown in Table.6 in Appendix A.13.

There was a progressive loss of activity which continued when the concentrations of [C_2mim][PF_6], [C_4mim][PF_6], [C_2mim][NTf_2] and [C_4mim][NTf_2] were increased beyond 10 % (w/v) except for [$N_{4,4,4,4}$][AOT], where activity was retained even up to 80 % (w/v). [$N_{4,4,4,4}$][AOT] was found to be the most LTV compatible ionic liquid (Figure 5.9). An amount of 50 % of LTV activity was still retained when used at a concentration of 20-60 % (w/v). The highest compatibility was found at 10 % (w/v). However, LTV activity was completely inhibited in the pure [$N_{4,4,4,4}$][AOT]. Both the [NTf_2] containing ILs supported LTV activity at concentrations of less than 10 % (w/v). [C_4mim][NTf_2] inhibited LTV activity drastically at a concentration of 20 % (w/v), whereas [C_4min][NTf_2] still supported LTV activity until 60 % (w/v).

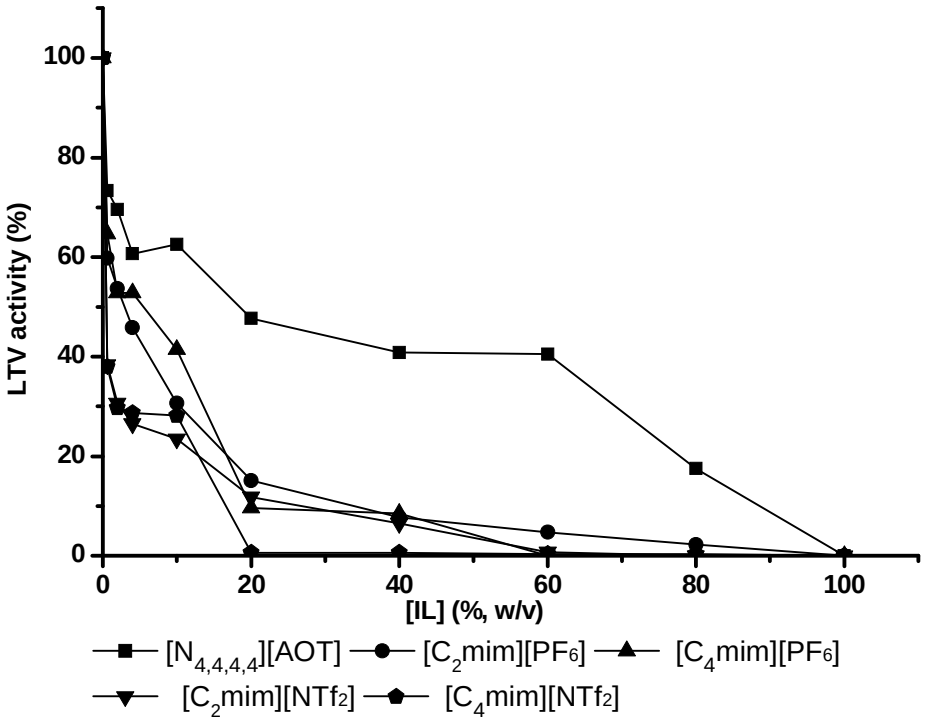


Figure 5.9: Screening of LTV compatible ILs with higher concentration

LTV activity was measured over a range of IL concentrations from 20 % to 100 % (w/v). These assays (total volume of 300 µL) contained 100 mM sodium citrate buffer pH 4.5 (209-0 µL), 0.025 $mg.ml^{-1}$ laccase solution (2 µl) in sodium citrate buffer and ionic liquid (60-300 µl). The reaction mixture was equilibrated for 60 seconds. The reaction was then started by adding 4 mM ABTS solution (29 µl). The formation of the $ABTS^{2+}$ radical cations was monitored at 420 nm and recorded at every 65 s at 37 °C for 5 h. The initial reaction rates were calculated monitoring over the first 715 s after substrate addition. The measurements were done in triplicates. The mean rate of reaction reported with the error less than 1%.

No significant difference was observed between [C_2mim][PF_6] and [C_4mim][PF_6] regarding their effects on the LTV activity, up to a concentration of 40 % (w/v). [C_4min][PF_6] inhibited laccase totally at 60 % (w/v), while [C_2min] [PF_6] still kept a little LTV activity (2 %) up to 80 % (w/v). Such difference in behaviour was mainly caused by the different alkyl side chains (Figure 5.9).

5.3 Conclusions

A total of 92 different ILs or salts were tested. To the best of the author's knowledge, this represents the largest number of ILs tested for their compatibility with an enzyme under a whole range of ionic liquid concentrations. Obviously, the developed methodology can be transferred to a large number of different enzymes.

Notable LTV activity was observed in 62 ILs to catalyse the ABTS oxidation reaction in the case of a 0.6 % (w/v) concentration of ILs; nearly 44 of the ILs were compatible with LTVuntil 4 % (w/v) and more than 50 % LTV activity was maintained in 18 ILs until10 % (w/v). Almost half of the phosphonium salts tested, except $P_{6,6,6,14}$ [AOT], were proven to be LTV inhibitors even if used at 0.6 % (w/v). However, the P_8 group of quaternary phosphonium ILs did not inhibit the enzyme at 0.6 % (w/v) even though they contained strongly kosmotropic anions (Cl^-, OAc^- or HCO_2^-). The rest of the mostly LTV compatible salts are shown in Figure 5.9 with a higher concentration. As for most of the quaternary phosphonium and some of the [AOT] containing ammonium salts, the measured LTV activity was either too low to estimate kinetic parameters or the ILs caused too much light scattering to allow accurate determination of the reaction rate (Table 3 and Table 5 in the Appendix A.13). In general, biphasic ionic liquid-water systems proved to be more suitable for enzymes than solutions of water-miscible ILs, particularly in the case of laccase. This is, perhaps, not unanticipated, because the interaction between ions and enzyme does affect enzyme activity.

This study confirmed further that the enzyme performance depended strongly on the nature of the anion. [NTf_2] and [AOT] were found to be the most suitable anions to prepare laccase-compatible water- immiscible ILs. The most LTV friendly salts in the water-miscible group were lactate or glycolate containing imidazolium or sodium salts with short alkyl chains (Figure 5.5). The effect of the component ions on enzyme activity in aqueous media can be illustrated with their kosmotropicity according to the Hofmeister series (Zhang & Cremer, 2006). Kosmotropic anions and chaotropic cations stabilise

enzymes in most cases, while chaotropic anions and kosmotropic cations have a destabilising effect (Hua, 2005, Constantinescu *et al.*, 2007). In a review published by Jenkins and Marcus (1995) stated that "effects in dilute homogeneous salt solutions do not bear directly on effects observed near surfaces, including those of macromolecules, such as proteins". Therefore, the Hofmeister series is best used for qualitative analysis of the effect of ILs on enzymes. However, there is still a need for a more comprehensive study, for example, concerning the effects of organic anions and the greater complexity of inorganic anions except for those previously examined (Zhao, 2010).

In conclusion, LTV activity decreased when the ionic liquid concentration increased in all cases included in this comprehensive screening. The effect of imidazolium chlorides, alkanoates, lactate, glycolate and furoate salts were similar to the equivalent sodium salts, indicating that the anion is more important than the cation. All of these salts were water miscible. LTV was more active in the presence of water-immiscible ILs, especially with [AOT] contained ILs. Furthermore, the ILs may be most useful where the enzymatic process needs to be operated in a biphasic system (e.g. for the delivery of water-insoluble substrates or extraction of inhibitory products).

6 Discussion and conclusions

6.1 Summary of results

The aim of this project was to study the enzymatic depolymerisation of both water-soluble and –insoluble lignins to obtain lower molecular weight aromatic chemicals. As the second most important component of plant biomass, only lignin contains aromatic groups within its structure. Currently, lignin is mainly produced as a waste by-product from the paper and pulp industry and from biorefineries. Therefore, to explore a suitable methodology to convert the phenolic-rich lignin into performance materials and value added aromatic platform chemicals is a key issue to improve the economic profitability of biorefining. Secondly, the consumption of fossil fuels could possibly be reduced if lignin can be utilised efficiently. To date, the most promising routes to degrade lignin are chemical degradation and enzymatic degradation. Enzymatic degradation has drawn much attention because of the mild nature and enhanced substrate specificity of the enzymes as well as the ability to preserve the aromatic nuclei in the lignin structure compared with chemical oxidation.

Huge efforts have been made to depolymerise water-soluble lignosulphonate over the water-insoluble Organosolv lignin (Hamidi, 2013). Also, the enzyme is favoured to be operated within an aqueous reaction environment and thereby there is less need to consider the hindrance of poor mass transfer between the enzyme and its substrate (lignin) which occurs if there is insoluble, as long as the other reaction conditions can be established. The sulphite-free Organosolv and lower sulphite containing Kraft lignins have more native lignin- like structures that are preserved throughout their preparation methods in industry (Martínez *et al.*, 2010, Zakzeski *et al.*, 2010, Munk *et al.*, 2015), which although they are not aqueous soluble makes them as attractive substrates. In fact, 92 % of world pulp production comes from Kraft pulping (Ragauskas) Therefore, the study of the degradation of Organosolv and Kraft lignin has significance to expand current knowledge regarding the product availability and distribution in various lignin.

The main findings of this thesis are:

- LTV oxidised one of the non-phenolic lignin model compounds, veratryl alcohol in the presence of ABTS (Figure 3.6), but it lost activity significantly at higher reaction temperatures (Figure 3.4).
- LTV oxidised the formation of 2,6-dimethoxy-1,4-benzoquinone (DBQ) from Organosolv lignin depolymerisation (Table 3.2) and the concentration of DBQ was decreased with increasing reaction temperature (Figure 3.13), which agreed with the findings of the LTV stability study (Figure 3.4).
- An extra supply of LTV improved the reaction (Figure 3.16). When the reaction was extended to 120 h, there were neither new products formed nor any significant increase in the DBQ concentration (Figure 3.14). At higher temperatures, DBQ was also formed by thermal decomposition of the lignin (Figure 3.11 and Figure 3.15)
- The addition of ethanol as a co-solvent did not improve the Organosolv lignin degradation although it did show a stabilisation effect on the LTV (Figure 3.17). This indicated that it is possible to run an enzymatic reaction with laccase in a co-solvent where the substrate is relatively easier to bind with LTV, and also for water-insoluble compounds, but perhaps not for a substance which is as complicated as lignin.
- LTV was found to be the most stable enzyme among the three lignolytic enzymes as it maintained 55 % of its activity for at least the first 6 hr at 30 °C (Figure 3.3) whereas LiP was deactivated after 2 h at 28 °C (Figure 3.24d), and MnP was deactivated after 3 h at 25 °C (Figure 3.25e).
- Neither MnP nor LiP indicated any effect on the degradation of Organosolv lignin (Figure 3. 26).
- LTV catalysed the depolymerisation of water soluble lignosulphonates from three different sources were studied under the same conditions. However, LTV showed too little effect in their depolymerisation (Figure 4.2.1, Figure 4.2.2 and Figure 4.2.3 of Chapter 4) compared to the enzymatic depolymerisation of Organosolv lignin (Chapter 3.2).
- LTV catalysed the decomposition of Kraft lignin after 6 days (Table 4.6) while no improvement was found by the reactions of MnP (Figure 4.17) or LiP (Figure 4.20).
- A number of ILs such as $[C_2mim][PF_6]$**,** $[C_4mim][PF_6]$**,** $[C_2mim][NTf_2]$ and $[C_4mim][NTf_2]$ were discovered to be LTV compatible at 10 % (w/v) (Figure 5.4) whereas dioctyl sulfosuccinate quaternary ammonium salt, $[N_{4,4,4,4}][AOT]$, retained its

activity until 80 % (w/v) (Figure 5.9) and therefore was found to be the most LTV compatible ionic liquid.

6.2 Discussion and Future Recommendations

The optimisation of the reaction conditions of LTV catalysed veratryl alcohol oxidation was developed in this project and it was discovered that one of the major problems with the LTV catalysed reaction was its deactivation at higher temperatures although it sustained its activity for 6 h at a lower incubation temperature. A number of ILs that were identified, for example, $[C_2mim][PF_6]$**,** $[C_4mim][PF_6]$**,** $[C_2mim][NTf_2]$ and $[N_{4,4,4,4}][AOT]$, were discovered to be compatible with LTV at concentrations of 10 % and 80 % (w/v) respectively for the first time in this project. These can be applied for the oxidation of LTV catalysed reactions of water-insoluble substrates to overcome the poor mass transfer between the enzyme and the substrates.

Although several researchers have demonstrated that laccase catalyses the decrease in MW of lignin, there is only one example where the formation of low MW products was demonstrated from the depolymerisation of lignosulphonate catalysed by LTV and laccase from *Agaricus bisporus* (LAB) comprehensively, namely the work of Hamidi (Hamidi, 2013). This current study demonstrated that LTV can catalyse the depolymerisation of water-insoluble Organosolv lignin to 2,6-dimethoxy-1,4-benzoquinone (DBQ). However, at higher temperatures, the reaction would occur purely by the thermal decomposition of lignin itself, which suggests that LTV barely accelerated this natural chemical reaction. In addition, the research in this thesis has demonstrated that LTV catalyses the decomposition of Kraft lignin although low MW products have not been identified as yet. It can be understood from this highly challenging project that it is extraordinary difficult to catalyse the depolymerisation of lignin using enzymes for a number of reasons. Firstly, the structure of lignin is incredibly complicated and there are also problems such as the mass transfer in this reaction regardless of the heterogeneity or homogeneity of the reaction system. There is also the major issue of the stability of the enzyme. The evidence of prolonged incubation of this reaction also showed little improvement in the catalytic ability of the enzyme to degrade lignin. The addition of ethanol improved the solubility of the lignin, but it deactivated the enzyme when used with lignin.

Further studies are needed to understand the effect of LiP and MnP on Organosolv and other degradations of lignin as the relevant literature suggests the dimeric and oligomeric lignin model compounds can be oxidised by these lignolytic enzymes because of their higher redox potential (Tuor *et al.*, 1992, Kapich *et al.*, 2005, Wong, 2009). Therefore, to perform the oxidation of some simpler lignin model compounds using these enzymes may increase understanding with regard to their role in lignin degradation. However, their lower stability and the peroxide intermediates which may occur in the presence of H_2O_2 during such oxidations may increase the challenges of lignin degradation further.

The enzyme tends to lose activity all the time during these reactions which indicates that there is less possibility of obtaining a decent depolymerisation of the lignin using an enzymatic process. Therefore, it is really difficult to perform and optimise such reactions. However, the extended work performed with ILs shows that there is potential scope in which ILs can be used to solubilise the lignin. Reiman and co-workers (Shleev *et al.*, 2006) have shown that using ILs allows one to partition the mediator with the ILs and keep it separate from the enzyme which prevents the enzyme from suffering inactivation. Therefore, further work is needed to develop an IL to keep the mediator away from the enzyme and allow the lignin to be degraded. Potentially, the immobilisation of enzymes might be useful although it has been mentioned in the literature that it significantly improves the stability of the enzyme such as when using an emulsion type system which could improve the activity of the enzyme (Jolivalt *et al.*, 2000, Durán *et al.*, 2002, Zhu *et al.*, 2007, Kudanga *et al.*, 2011, Jesionowski *et al.*, 2014). However, it is not easy to implement that concept practically into a complex lignin reaction system. Thus more work is needed to determine if immobilisation could improve the laccase stability. The ILs may be helpful but they have a limitation as far as enzyme deactivation is concerned. The pre-treatment of lignin before the depolymerisation with laccase may be another improvement as it allows the separation of lower MW compounds in the lignin material before the enzymatic reaction takes place (Milstein *et al.*, 1993, Zhao *et al.*, 2009). This then allows the comprehension of the real effect of laccase on the transformation of the higher MW fractions of lignin into smaller MW chemicals. To conclude, although lignin has great diversity of monomers in its structure, however, because of its unusual complexity this hinders the gaining of a real insight into the reaction mechanism of the lignin degradation in order to carry out any further optimisation of the reaction.

However, recently lignin model compounds have been studied by Shoulder's group (Forsythe *et al.*, 2013) and Westwood's group (Lancefield & Westwood, 2015). Both sets of authors have published work on the preparation of hardwood and softwood lignin model polymers, and the structures of these compounds would appear to be much less complex than the native lignin when attempting to comprehend their mechanism. The related research to the above work concerning the enzymatic degradation of these polymeric model compounds is currently ongoing in our laboratory to optimise the laccase reaction system further.

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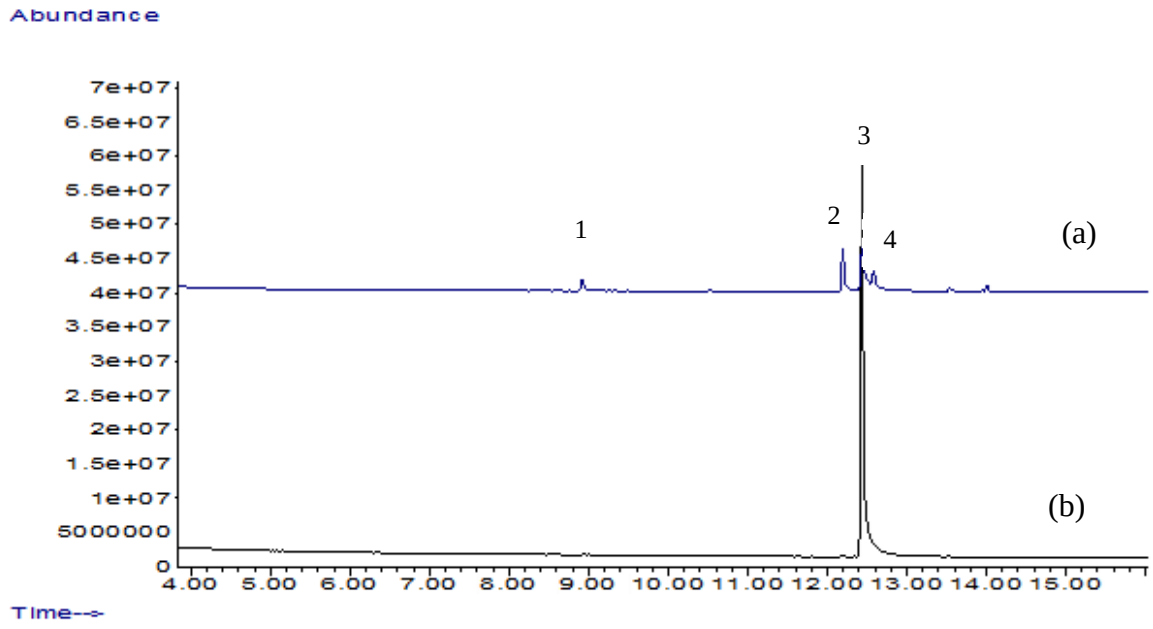
APPENDICES

Appendix A.1 GC-MS Confirmation of Veratryl Alcohol Before and After Distillation

The veratryl alcohol was vacuum distilled using an oil bath at 170 ^{0}C with the pressure of P = 1021 mbar. Upon distillation, the collected fraction of veratryl alcohol and the purchased veratryl alcohol before distillation were analysed by GC-MS to confirm its purification from the trace contaminant chemical, contaminant methyl-3-methoxy-4- hydroxybenzoate. The samples were injected using split mood (65:1).

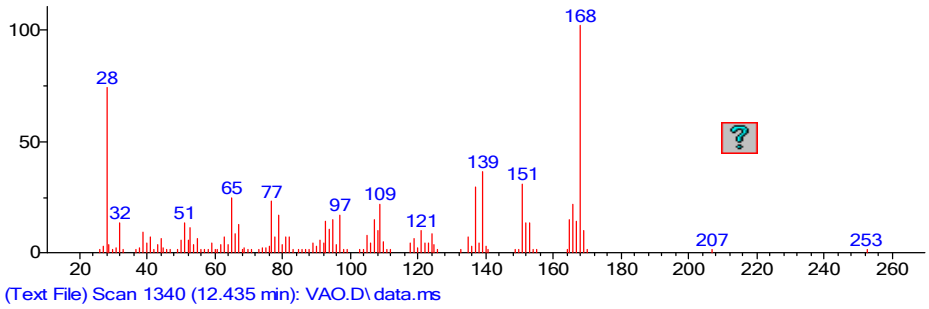
A.1.1: Chromatogram of veratryl alcohol before (a) and after distillation (b).



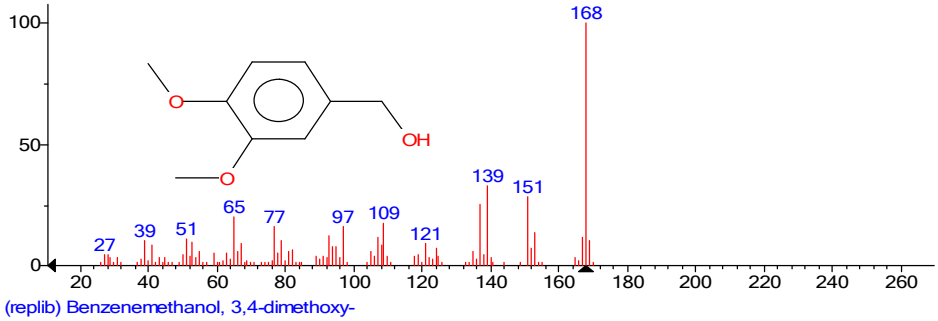
The veratryl alcohol before distillation was appeared at RT = 12.43 min (peak 3) and it was mainly contaminated by 3, 4-dimethoxytoluene (peak 1, RT = 8.92 min), veratraldehyde (peak 2, RT = 12.20 min) and homoveratric acid (peak 4, RT= 12.58 min).

Mass Spectrum of veratryl alcohol and its main contaminant (e.g. veratryl aldehyde) and their matches with National Institute of Standard and Technology (NIST) Library data search.

(1) Veratryl alcohol:

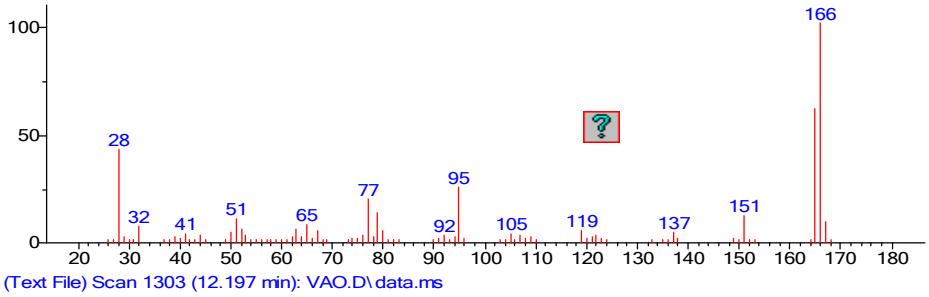


NIST library match: Veratryl alcohol (or Benzenemethanol, 3,4-dimethoxy-) ; Formula: $C_9H_{12}O_3$; MW: 168 ; CAS#: 93-03-8; NIST#: 6495



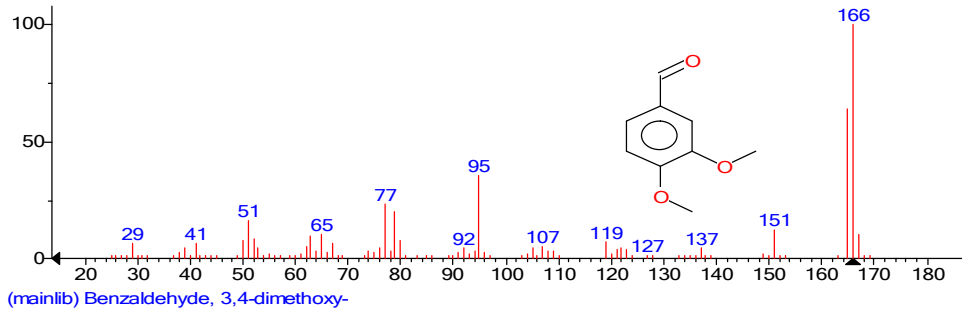
The retention time of the veratryl alcohol identified matched with its standard and the NIST library match probability for veratryl alcohol after the distillation reached to 89.3% whereas it was 81.2% before distillation. However, this was not a good enough match due to the purity of veratryl alcohol was still not very high. It indicates that it is too difficult to purify veratryl alcohol completely from the trace contaminants of veratryl aldehyde although the traces of GC chromatogram (b) shown there was too little possibility of impurity after distillation.

(2) Veratryl aldehyde:



NIST library match: Veratryl aldehyde (or Benzaldehyde, 3,4-dimethoxy-); Formula: $C_9H_{10}O_3$; MW: 166; CAS#: 120-14-9; NIST#: 352844;

The probability of veratryl aldehyde in the NIST library match was 86.7%, which indicates that it has contaminated with other impurities, e.g. veratryl alcohol as their peaks was not clearly separate from each other.



(mainlib) Benzaldehyde, 3,4-dimethoxy-

Appendix A.2: Brief Summary of Literature about Veratryl Alcohol Oxidation LTV using different mediators

No.	[Subs] (mM)	[Mediator]/ (mM)	Total volume (mL)	laccase source & conc. (U/mL)	Buffer (pH & conc.)	Reaction temp. (^{0}C) & time (h)	Co-solvent (%)	Flush with O_2	Conversion/Yield (%)	HPLC /GC conditions	References
1	[VA]= 5mM	[TEMPO]= 4.5 mM	*0.5 mL*	*Trametes versicolor* (got from Fluka) [LTV]=36.8U/mL, but the one available now is 0.5 U/mL only	pH 4.4 McIlvaine buffer, but its conc. was not given	T=30^{0}C for 24h	20% (v/v) *tert*-butanol	none	99-100	HPLC: mobile phase was 10 - 75 % (v/v) aqueous ACN with 0.025 % trifluoroacetic acid. Flow rate was 1 ml/min. UV detector at λ=278 nm	Troy M. Larson and Amber M. Anderson *et al*. Combinatorial evaluation of laccase-mediator system in the oxidation of veratryl alcohol. *Biotechnol Lett* .2013, 35:225–231
2	[VA]= 15 mM	[Methyl syringate (Msy) or acetosyringone (Asy)]= 0.25 to 15 mM [Sub]/[M]=1:1	not given	*Trametes villosa* from Novozymes. [Lac]=1 U/ml	Tartrate buffer (50 mM pH 4)	T=50^{0}C, 15 h	2% (v/v) EtOH	none	not specified	HPLC: with a linear gradient of methanol (5–100% v/v) in 0.086% (v/v) H_3PO_4 at 1 ml/min flow rate. λ=280 nm	María Díaz-González and Teresa Vida. Phenolic compounds as enhancers in enzymatic and electrochemical oxidation of veratryl alcohol and lignins. *Appl Microbiol Biotechnol*. 2011, 89:1693–1700
4	Initial [VA] = 8.57 mM	[ABTS] =17.14 mM	not given	*Coriolus versicolor*, [Lac] =0.434 U/mL	Sodium acetate buffer (50 mM, pH 4.5)	T= 30^{0}C for 24-48h. GC method given in the paper.	None	Yes	>90%	**GC:** Internal standard solution (3, 4-dimethoxyacetophe none)	Balakshin, Mikhail Yu.; Chen, Chen-Loung; Gratzl, Josef S. *et al* .Kinetic studies on oxidation of veratryl alcohol by laccase-mediator system: Part 1. Effects of mediator concentration. *Holzforschung*. 2000, 54(2): 165-170
5	[VA] was not given	HOBT (1-hydroxy-1H-benzotriazole). Concentrations was not given	not given	*Trametes versicolor*. Concentrations was not given	buffer (pH 4.5)	45^{0}C	None	none	not specified		Elke Fritz-Langhals and Brigitte Kunath. Synthesis of aromatic aldehydes by laccase -mediator assisted oxidation. *Tetrahedron*

											Letters. 1998, 39: 5955-5956.
6	[VA] = 20 mM	[TEMPO] = 6mM	3 ml	*Trametes villosa*, [lac] =3 U/ml,	Citric buffer (pH = 3.5, 0.1M)	24 h at r.t	None	yes	99%	**GC:** using Acetophnone as internal standard	Maura Fabbrini, Carlo Galli et al.Comparing the catalytic efficiency of some mediators of laccase. *Journal of Molecular Catalysis B: Enzymatic*. 2002, 16, 231–240.
7	[VA] = 3 mM	[ABTS]= 0.8 mM		Laccase was not specified. [lac] =0.1U/ml	Sodium acetate buffer (0.1M, pH = 3.0)	24 h at r.t.	None	yes	not specified	**HPLC:** 50% can or 40% methanol with flow rate of 1ml/min	Muheim, Andreas; Fiechter, Armin. On the mechanism of oxidation of non-phenolic lignin model compounds by the laccase-ABTS couple. *Holzforschung*, 1992, 46(2): 121-6.
8	[VA] = 2 mM	[ABTS]= 1 mM	*10 mL*	*Trametes versicolor*, [lac]= 0.5 U/mL	sodium acetate buffer (0.05 M, pH 5)	22^0C, 24h	None	none	not specified	HPLC analysis	R. BOURBONNAIS and M. G. PAICE *et al.* Reactivities of Various Mediators and Laccases with Kraft Pulp and Lignin Model Compounds. *Appl. and Environ. Microbio*.Dec. 1997: 4627–4632.
											R. BOURBONNAIS and M. G. PAICE *et al.* Oxidation of non-phenolic substrates. *FEBS letters*. 1990, 267 (1): 99-102.

Appendix A.3: GPC calibration using authentic pullulan standards

The calculation of the MW of products in each sample was confirmed *via* the retention times of the authentic pullulan standards (Table 3.2). The retention time of each standard is proportional to its MW. Calibration curves were generated according to a relative standard method (Waters, 2013). After running the series of pullulan standards, a polynomial fit was performed and the resulting log M versus retention time calibration curve was plotted. The MW changes of Organosolv, Kraft lignins after enzymatic decomposition were then determined using this pullulan standard's calibration curve.

Table 3.2: Retention times of a series of pullulan standards based on their MW. Data represent mean of triplicate measurement.

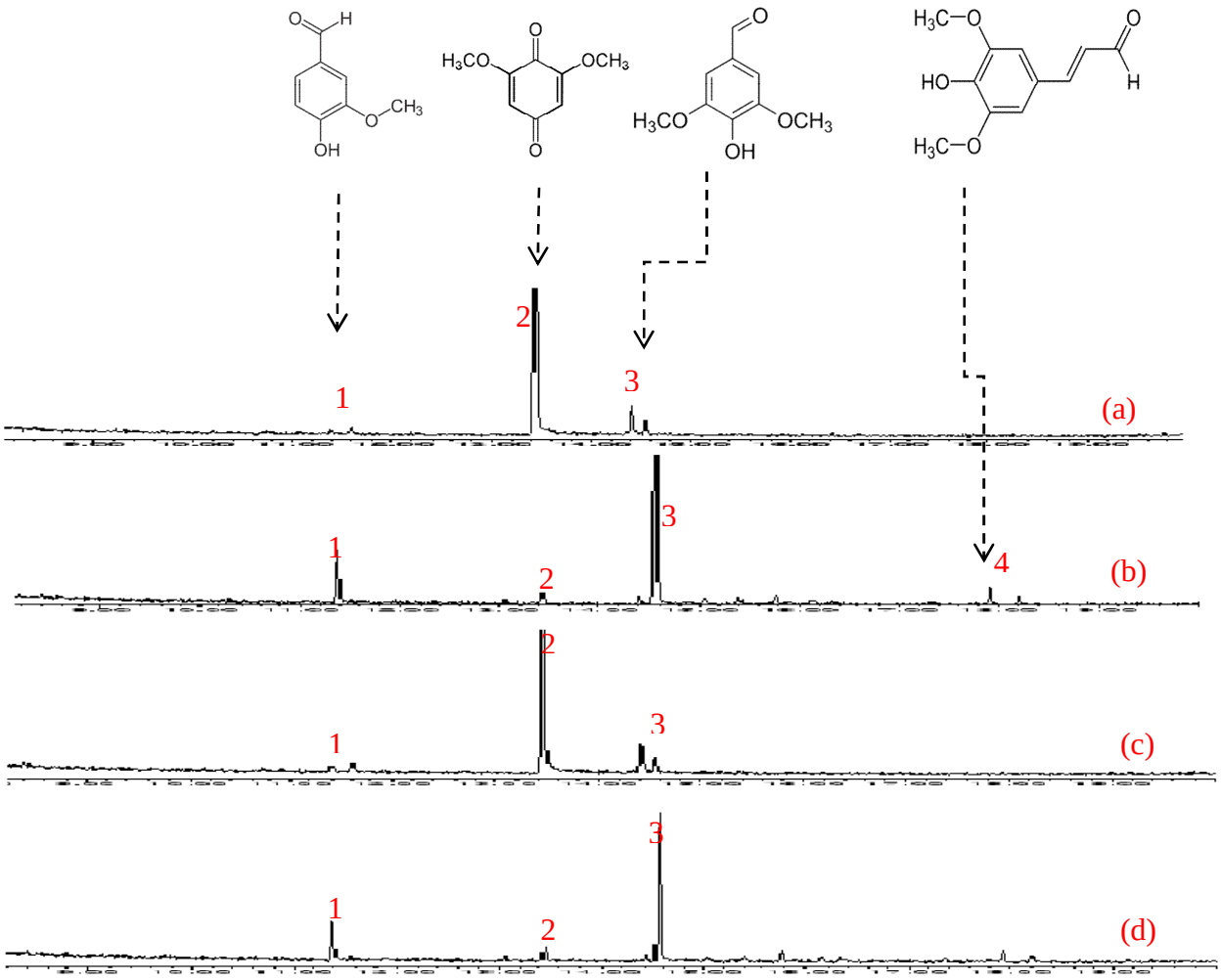
Name of standard	MW of standard	Mean RT of standards
Pullulan	342	13.97
	1080	13.46
	5900	11.28
	9600	10.86
	21100	10.37
	47100	9.91
	107000	9.44

Appendix A.4: GC-MS identification of Organosolv lignin degradation by LTV

The products formed after enzymatic treatment of Organosolv lignin by LTV in the presence of ABTS as mediator. Sample was incubated at 30-60 °C for 24 h at a shaking speed of 200 rpm. The sample was evaporated to dryness and the dried sample of ethyl acetate extract fraction was re-dissolved in acetone. The chromatogram represents the duplicate analysis.

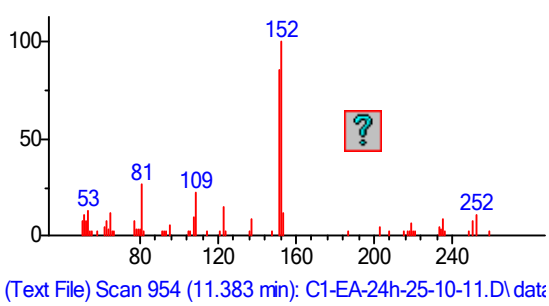
A4.1: GC chromatograms of OSL degradation by LTV in the presence of ABTS at 30 ^{0}C

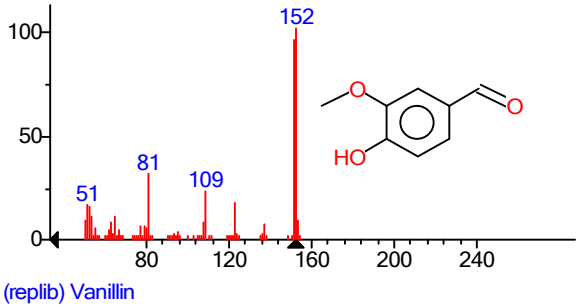
There was no significant difference shown for the degradation products formed at different incubation times except the peak areas increase of the detected compounds. Therefore, the GC chromatogram and mass spectra are given for the samples of 24h reactions for reference only. The symbols used in these chromatograms are: (a) complete reaction; (b) control without LTV; (c) control without ABTS; (d) without LTV & ABTS.



The main products formed from Organosolv degradation by LTV at 30 ^{0}C were vanillin (peak 1), DBQ (peak 2), syringaldehyde (peak 3) and trace amount of 3,5-Dimethoxy-4-hydroxycinnamaldehyde (sinnapaldehyde, peak 4).

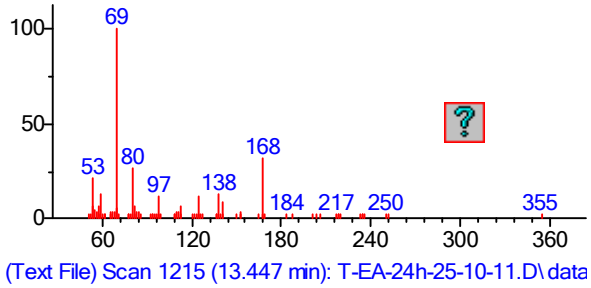
(1) RT=13.38 min----Vanillin

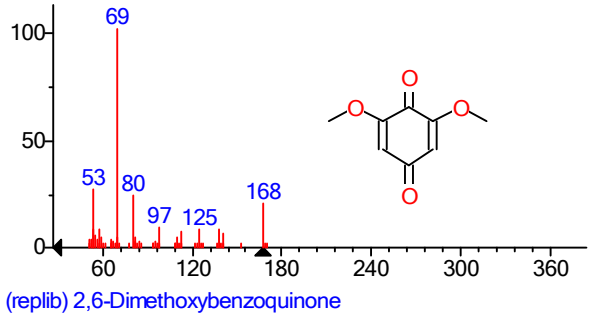




NIST library confirmation: Vanillin; Formula: $C_8H_8O_3$; MW: 152; CAS#: 121-33-5; NIST#: 227894
The probability match with NIST library was only 85% versus the probability of authentic standard was 87.8%. So, it could be considered as a close match with its standard, however, cannot be accepted as a perfect match.

(2) RT=13.45 min----2,6-Dimethoxybenzoquinone (DBQ)

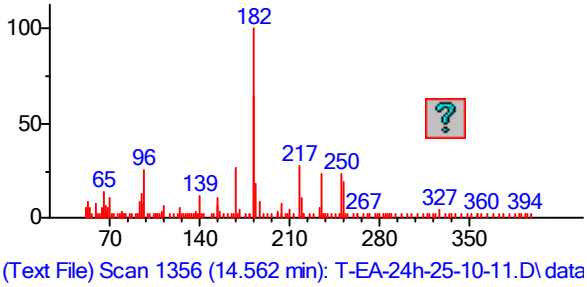


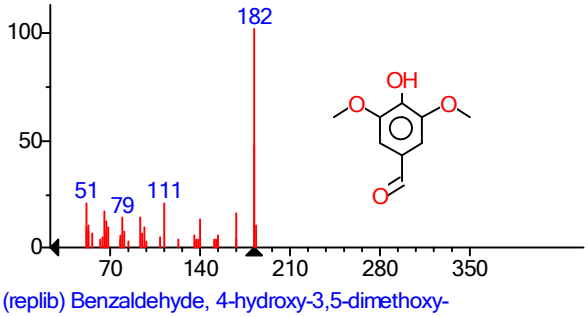


NIST library confirmation: 2, 6-Dimethoxybenzoquinone; Formula: $C_8H_8O_4$; MW: 168; CAS#: 530-55-2; NIST#: 136693

The probability match of DBQ with NIST library was only larger than 95 % versus the probability of authentic standard was 91.2%. So, it could be considered as a very close match with its standard. However, there were contaminants shown in the mass spectrum which might be caused by the mixture of dimeric compounds or other unknown materials from the reaction.

(3) RT=14.56 min---- Syringaldehyde



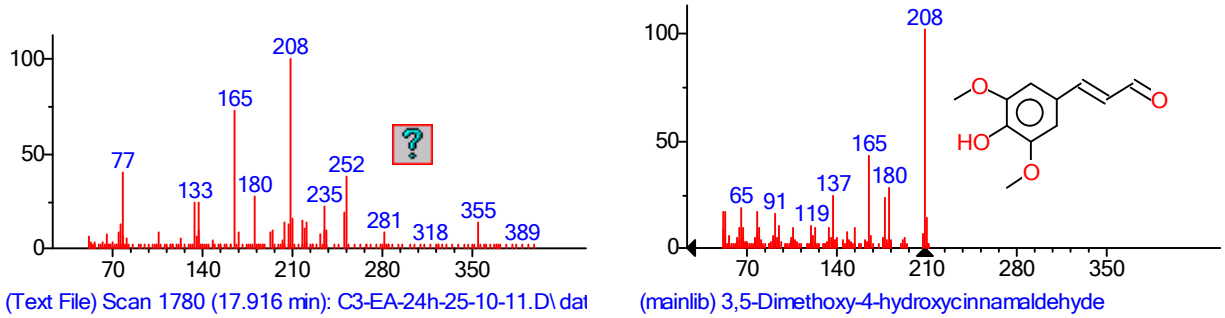


NIST library confirmation: 4-hydroxy-3, 5-dimethoxy- Benzaldehyde; Formula: $C_9H_{10}O_4$; MW: 182; CAS#: 134-96-3; NIST#: 22235

The probability match of syringaldehyde with NIST library was only larger than 75 % versus the probability of authentic standard was 76.4%. So, it could be considered as a very close match with its standard. However, there

were contaminants shown in the mass spectrum which might be caused by the mixture of dimeric compounds or other unknown materials from the reaction.

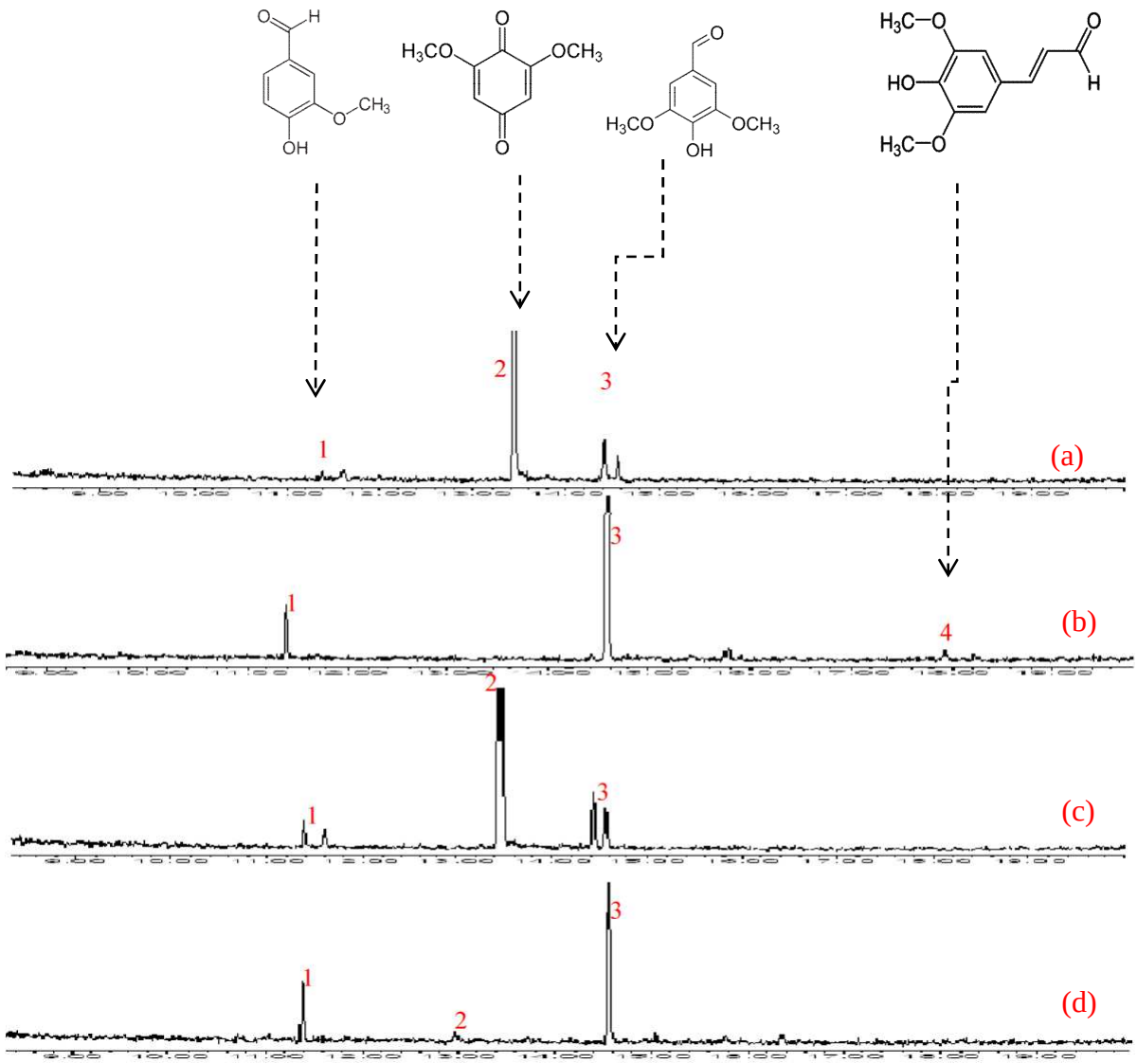
(4) RT=17.92 min---3,5-Dimethoxy-4-hydroxycinnamaldehyde (Sinapaldehyde)



NIST library confirmation: 3,5-Dimethoxy-4-hydroxycinnamaldehyde; Formula: $C_{11}H_{12}O_4$; MW: 208; CAS#: 87345-53-7; NIST#: 250264

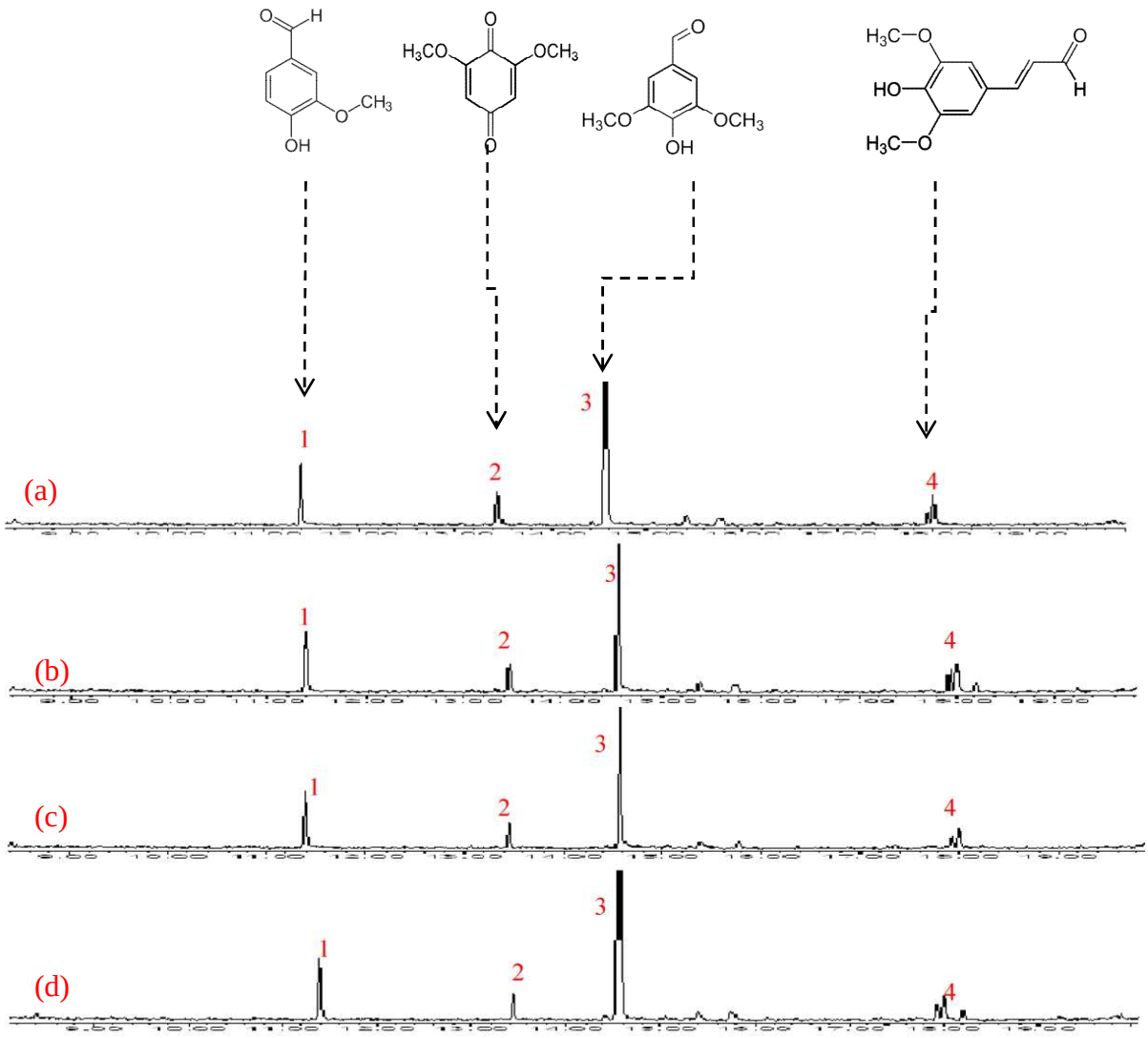
The NIST library match for this compound was only about 35% and there was no standard available. Therefore, it is a very poor match and might be mixed with other compound from the reaction.

A4.2: GC-MS identification of OSL degradation by LTV in the presence of ABTS at 40 ^{0}C



The GC-MS identified major products from organosolv degradation at 40 °C were vanillin (peak 1), syringaldehyde (peak2), DBQ (peak3) and sinapaldehyde (peak4). Other small peaks are contaminants. The symbols used in these chromatograms are: (a) complete reaction; (b) control without LTV; (c) control without ABTS; (d) without LTV & ABTS. The organosolv degradation products from the reaction at 40°C are similar with those formed at 30 °C. Only difference was that trace amount of sinapaldehyde was formed in the sample of control without LTV only. MS data is shown below. Other mass spectra data for the same products are not repeated.

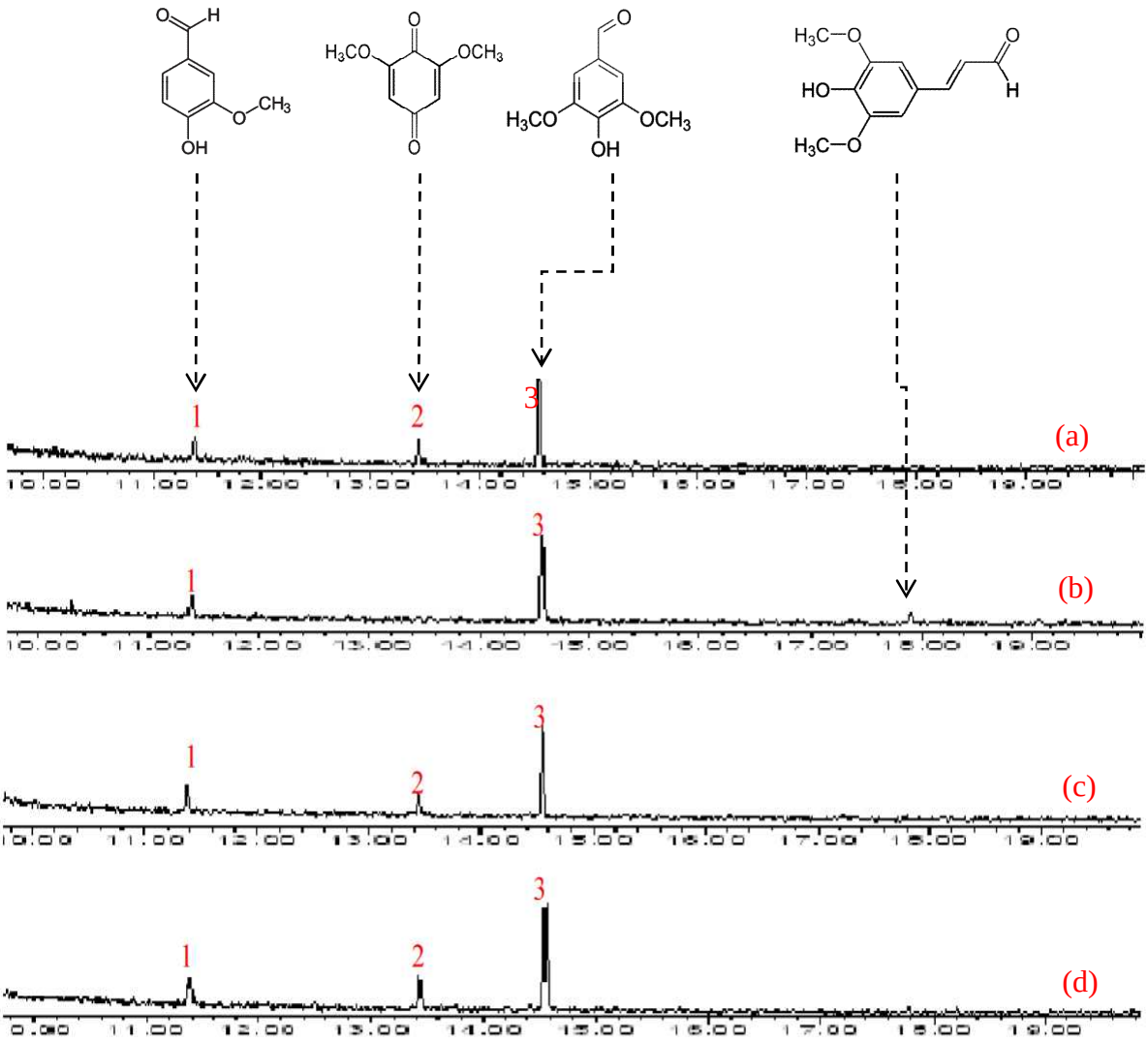
A4.3: GC-MS identification of OSL degradation by LTV in the presence of ABTS at 50 ^{0}C



The GC-MS identified major products from organosolv degradation at 50 °C were vanillin (peak 1), DBQ (peak2), syringaldehyde (peak3) and sinapaldehyde (peak4). The symbols used in these chromatograms are: (a) complete reaction; (b) control without LTV; (c) control without ABTS; (d) without LTV & ABTS. The organosolv degradation products from the reaction at 50°C are similar with those formed at 40 °C except the peak area of sinapaldehyde was increased. Therefore, the mass spectra data for the same products are not repeated again.

A4.3: GC-MS identification of OSL degradation by LTV in the presence of ABTS at 60 ^{0}C

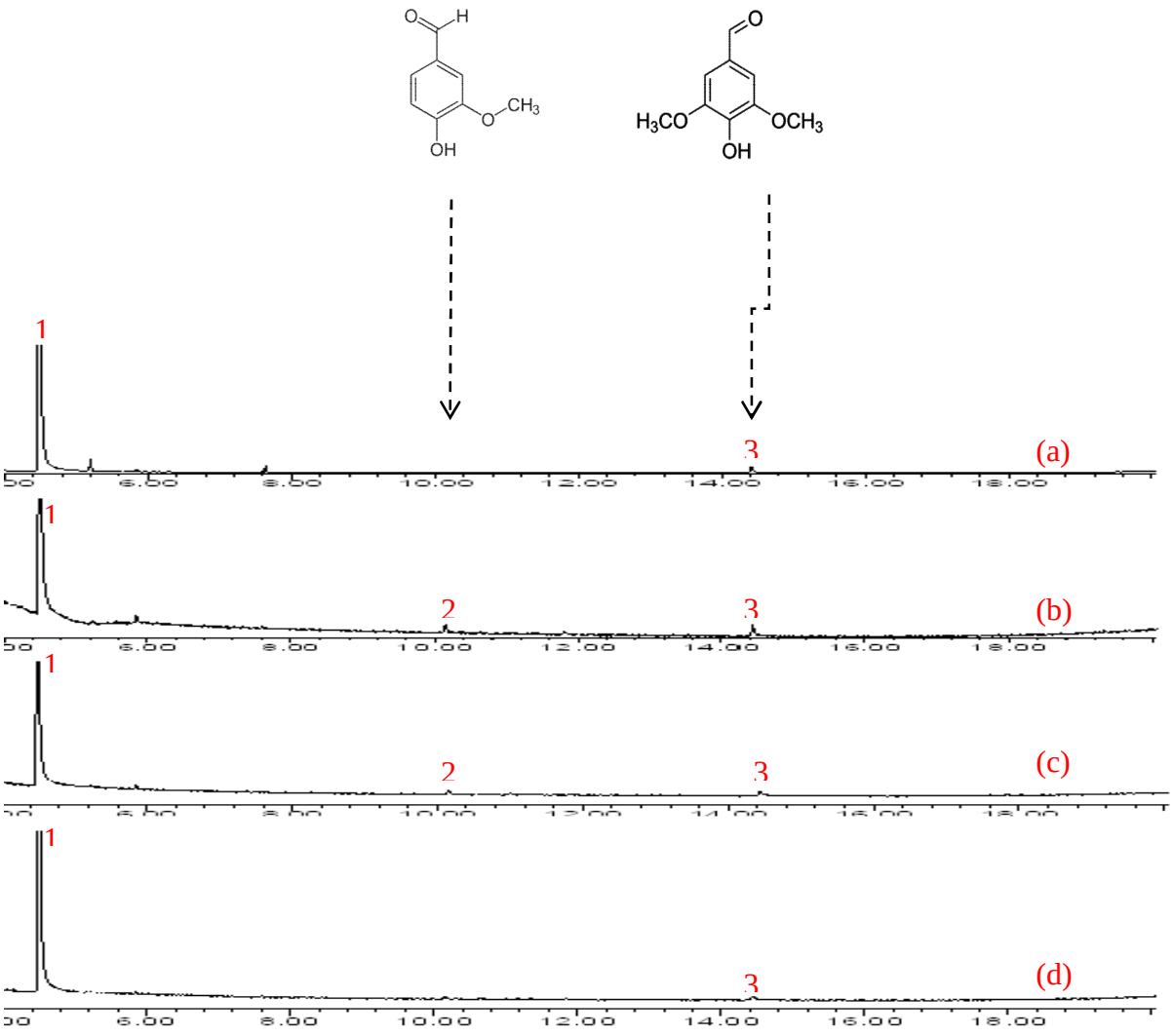
As LTV was proven to be losing its activity very quickly at higher temperature, therefore, the OSL reaction at 60 ^{0}C was only incubated for 6h. The symbols used in these chromatograms are: (a) complete reaction; (b) control without LTV; (c) control without ABTS; (d) without LTV & ABTS.



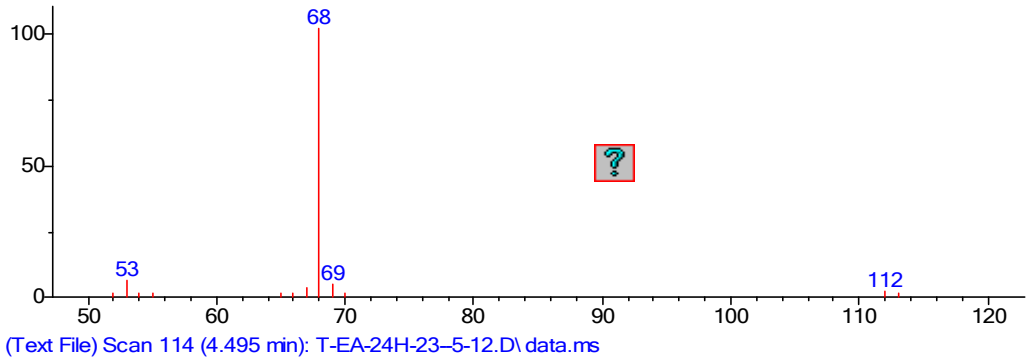
The GC-MS identified major products from organosolv degradation at 60 °C were vanillin (peak 1), DBQ (peak2), syringaldehyde (peak3) and sinapaldehyde (peak4). The organosolv degradation products from the reaction at 60°C are similar with those formed at 40 °C and 50 °C except the peak areas of all products were apparently decreased. It indicates that 60 ^{0}C is not an ideal reaction temperature for LTV catalysed OSL degradation.

Appendix A.5 GC-MS identification of OSL degradation by LTV at 30 ^{0}C for 6 days extended reaction

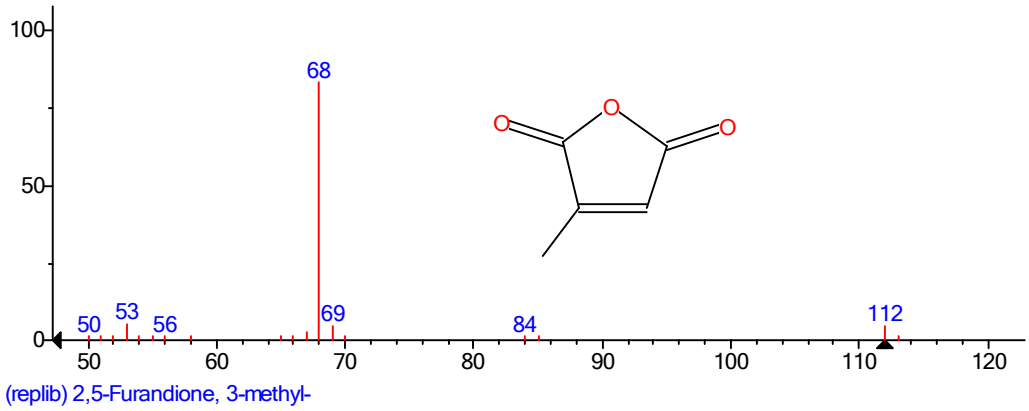
A5.1: GC-MS identification of OSL treatment by LTV - ABTS system at 30 ^{0}C for 1h



The compounds identified at 1h of incubation from OSL tretment with LTV were 3-methyl-2,5-Furandione (peak 1 at RT=4.49 min), vanillin (peak 2) and syringaldehyde (peak 3).
The mass spectra of 3-methyl-2,5-Furandione was confirmed by NIST library as follows.



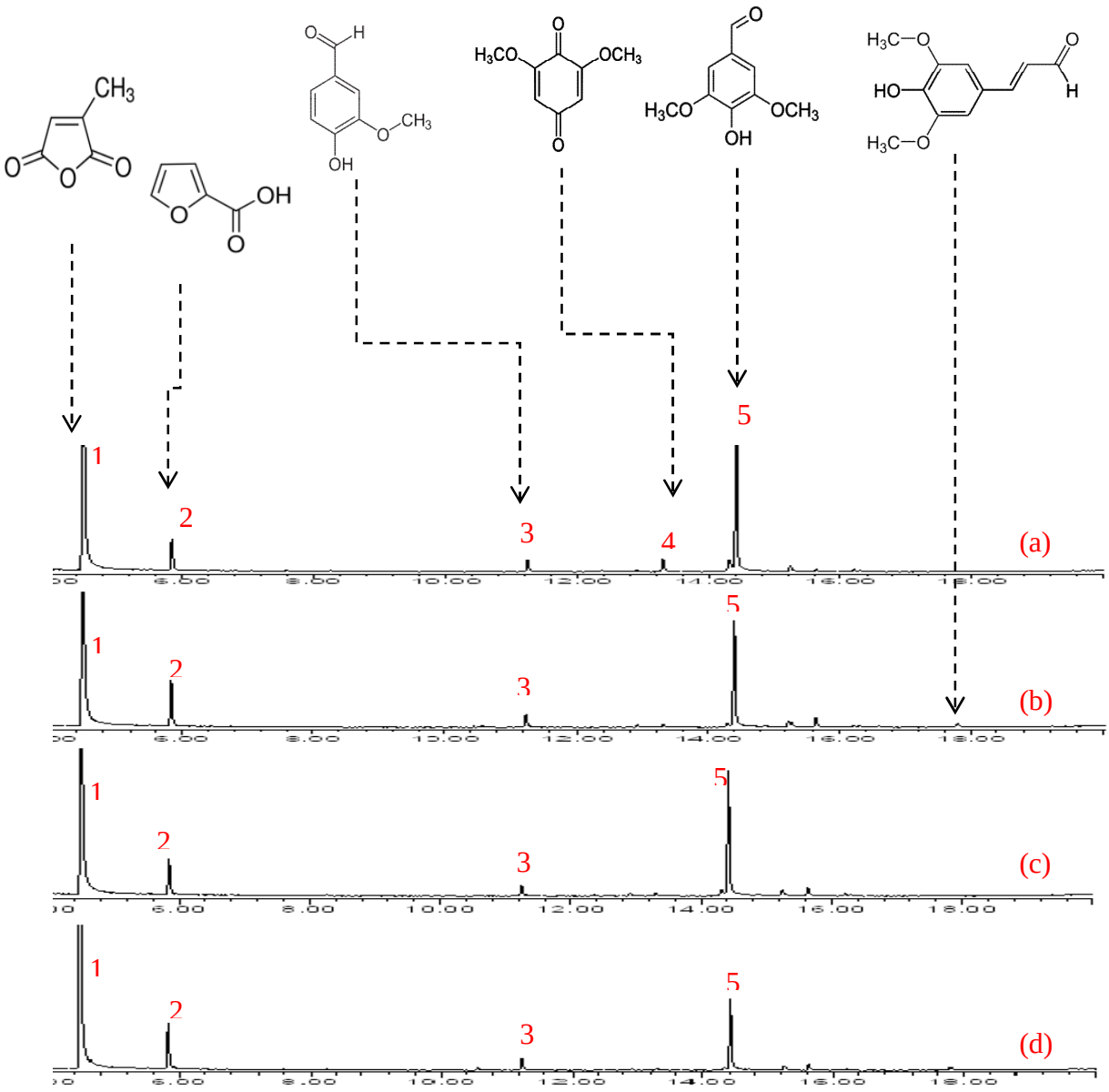
NIST library confirmation: 2,5-Furandione, 3-methyl-; Formula: $C_5H_4O_3$; MW: 112; CAS#: 616-02-4; NIST#: 227741



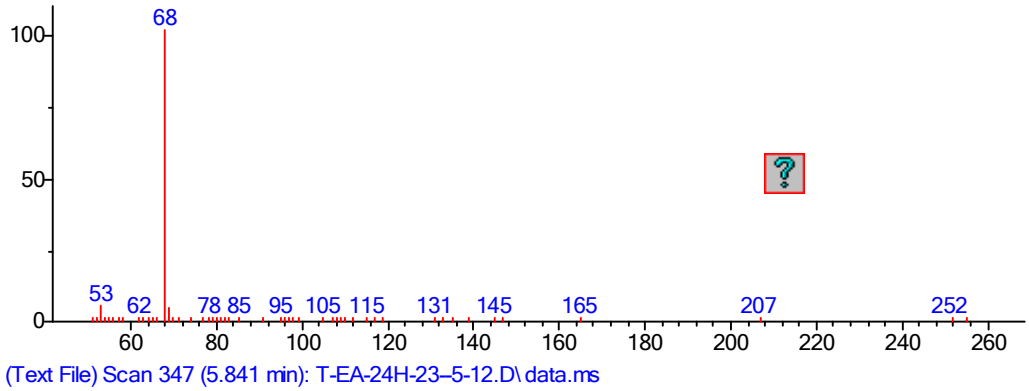
The probability of 3-methyl- 2,5-Furandione in the NIST library match was only 58 % and there was no standard available to confirm this compound. So, the identification was only resulted from the NIST library match as a preliminary match.

The rest of the compounds were not newly formed. So, their mass spectra identification are not repeated.

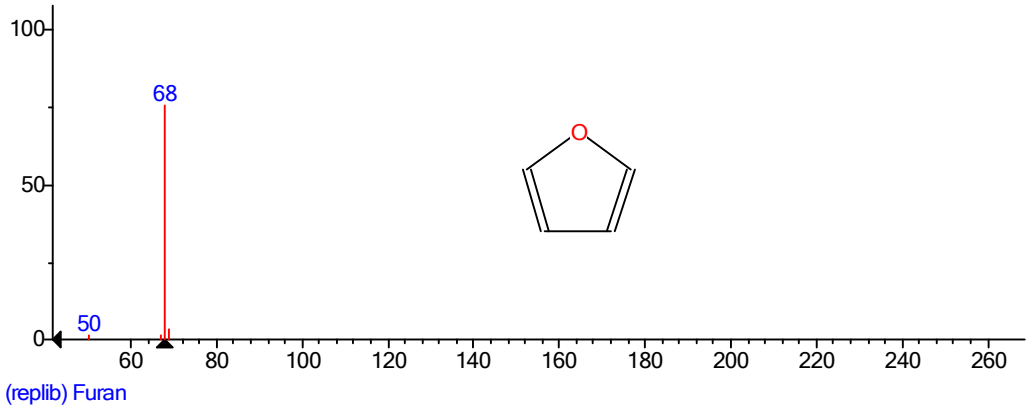
A5.2: GC-MS identification of OSL treatment by LTV-ABTS at 30 ^{0}C for 24h



Except the furan (RT=5.84 min, peak 2), all other chemicals were formed from earlier reactions too. Therefore, their GC-MS identifications are not repeated. The mass spectrum of furan was as follows.



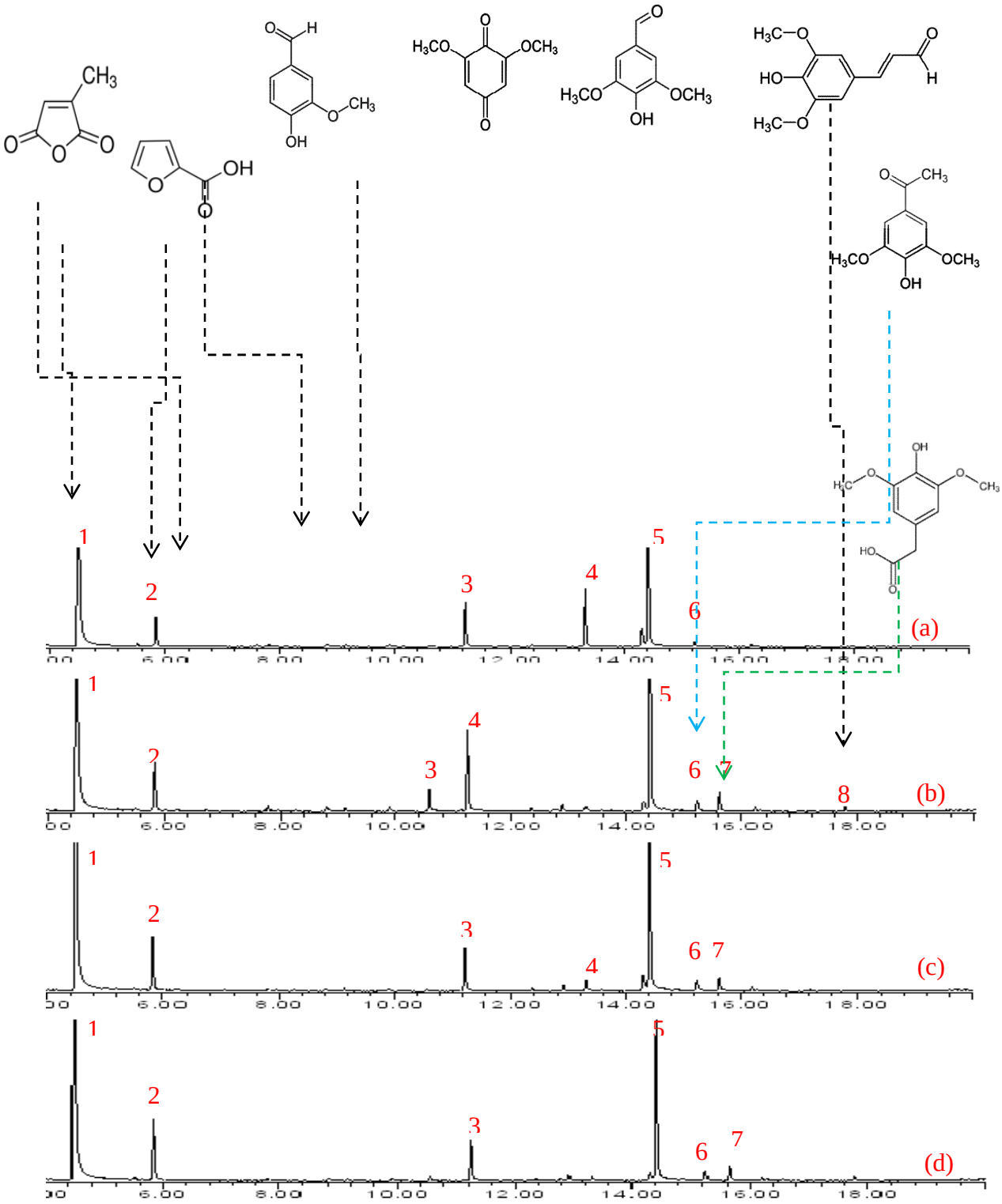
NIST library confirmation: Furan; Formula: C_4H_4O; MW: 68; CAS#: 110-00-9; NIST#: 19050



The probability of Furan in the NIST library match was only 36 % and there was no standard available to confirm this compound. Therefore, the identification was only resulted from the NIST library match as a preliminary identification.

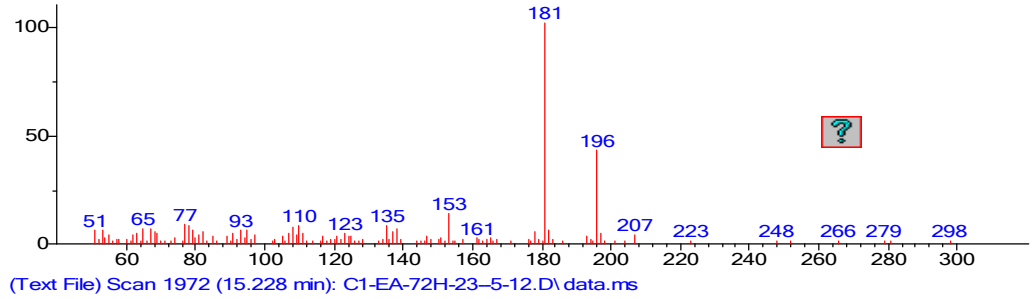
The rest of the compounds were not newly formed. So, their mass spectra identification are not repeated.

A5.3: GC-MS identification of OSL treatment by LTV-ABTS at 30 ^{0}C for 72h

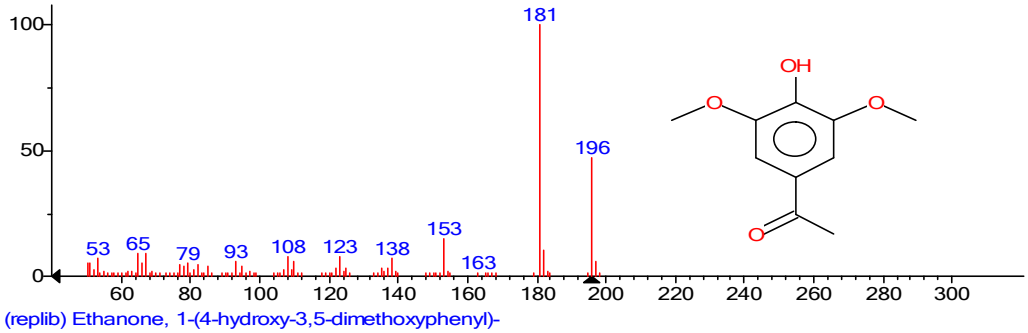


Except acetosyringone (peak 6 at RT=15.23 min) and homosyringic acid (peak 7 at RT=15.63 min), all other chemicals were formed in previous reactions. So, their mass spectra data are not shown. The observed mass spectra of the above 2 new compounds which mainly idenfied from the control reactions of OSL degradation by LTV at 30 ^{0}C for 72 h were as follows:

(1) Acetosyringone

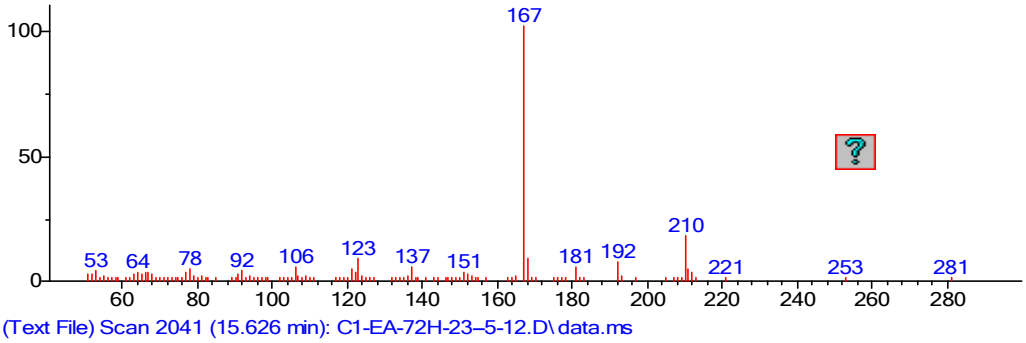


NIST library confirmation: Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-; Formula: $C_{10}H_{12}O_4$; MW: 196; CAS#: 2478-38-8; NIST#: 291615

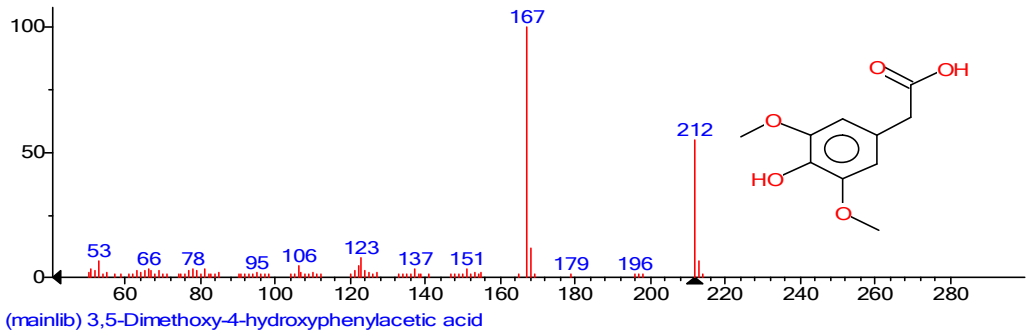


The probability of acetosyringone in the NIST library match was only 62.4 %, but there was no standard available to confirm it. Also, there was possibility mixing with higher MW compounds as well from the mass spectrum. So, this identification by the NIST library match was only a preliminary match.

(2) Homosyringic acid



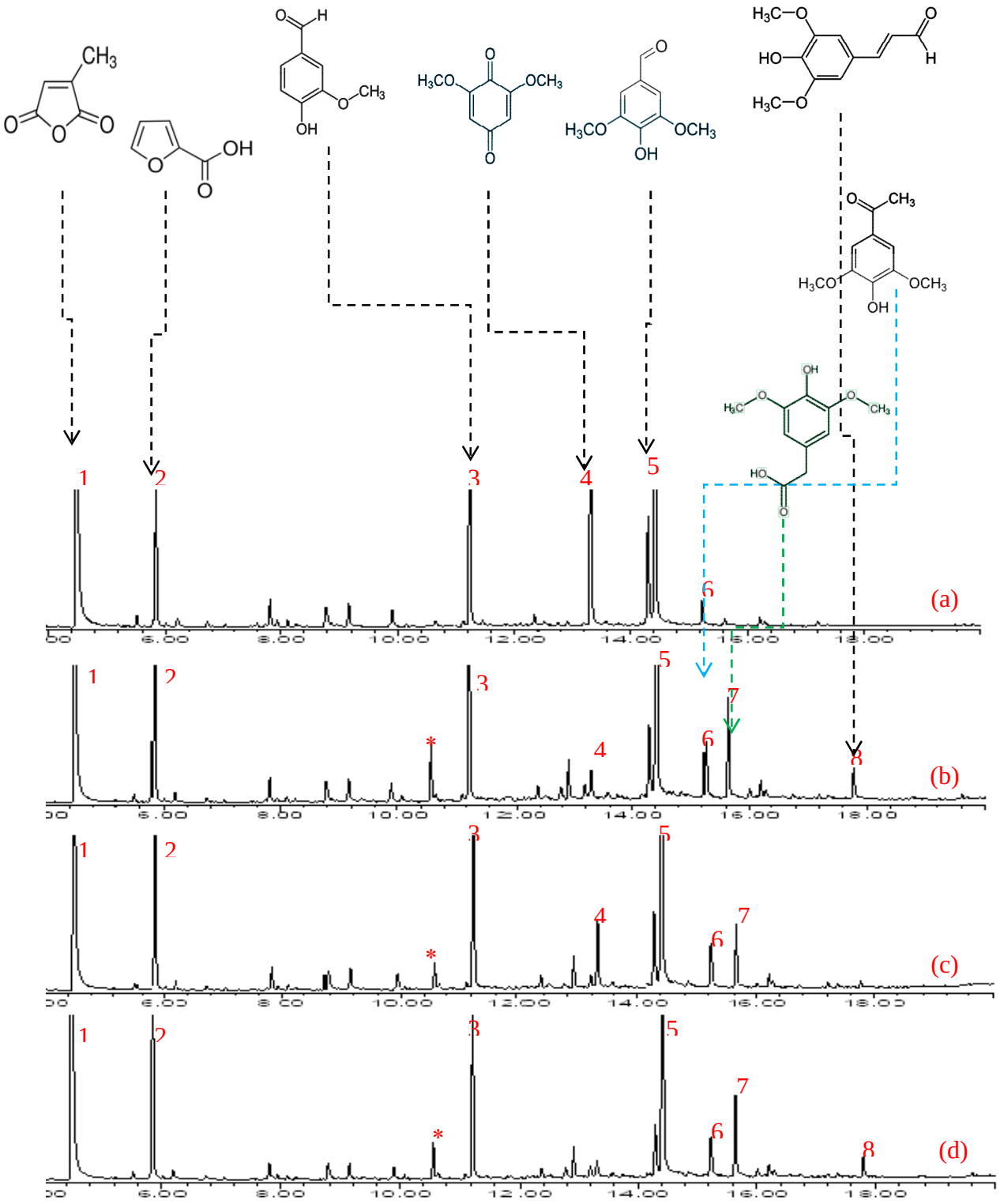
NIST library match: Name: 3,5-Dimethoxy-4-hydroxyphenylacetic acid; Formula: $C_{10}H_{12}O_5$; MW: 212; CAS#: 4385-56-2; NIST#: 134852



The probability of homosyringic acid in the NIST library match was only below 30%, and there was no standard available to confirm it. So, this identification by the NIST library match was only a preliminary match.

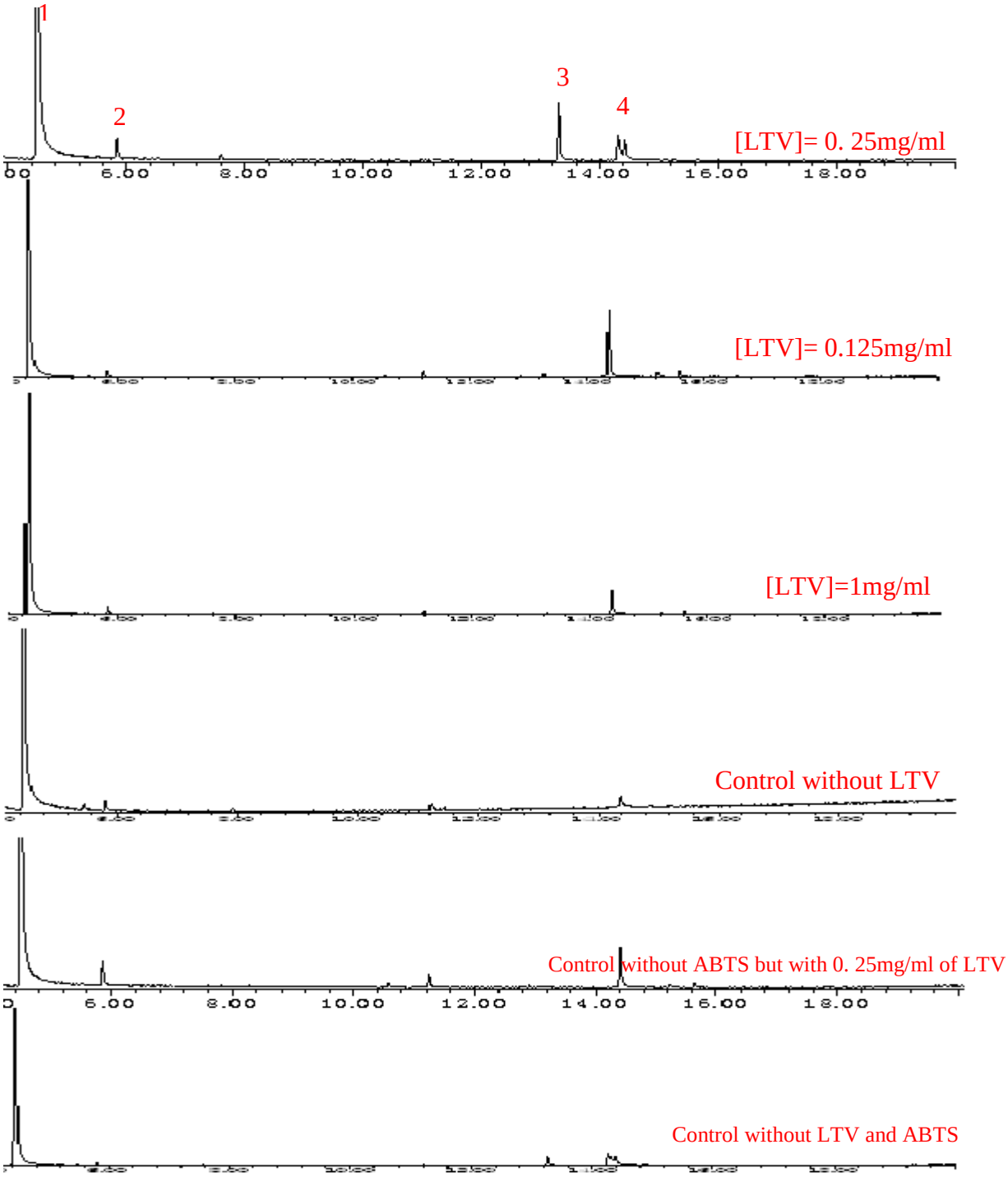
A5.4: GC-MS identification of OSL treatment by LTV-ABTS at 30 ^{0}C for 120 h

The main compounds formed from OSL degradation by LTV after 120 h are identical to the compounds previously identified at 72h of incubation apart from the peak area increase for all of them with the increasing reaction time. Therefore, the mass spectra details are not repeated.



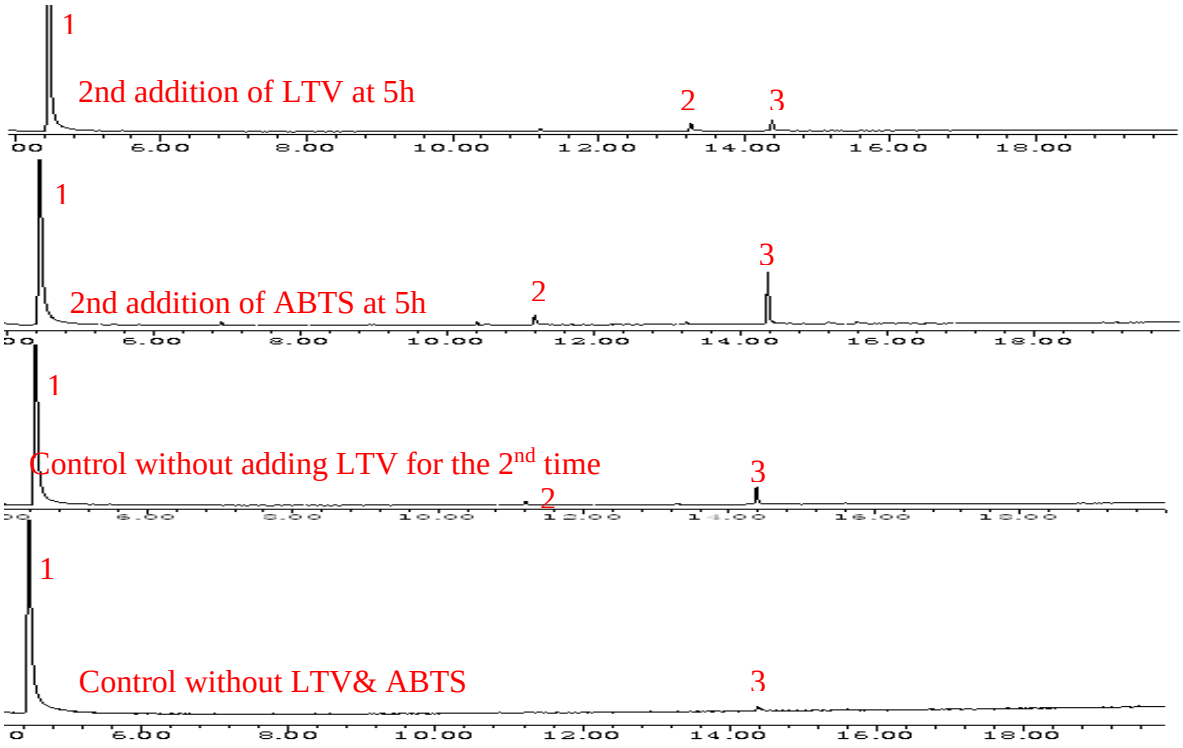
Appendix A.6: Chromatograms to verify the effect of LTV concentration on OSL degradation by LTV at 30 ^{0}C

The comparison of the observed GC-MS chromatogram from the samples of EA extracts after OSL degradation by LTV. The reaction mixtures contained OSL (57 g/L) mixed with LTV (at 4 different concentrations), 4 mM ABTS and 100 mM ammonium citrate buffer pH 4.5. All reactions and corresponding controls were incubated at 30 ^{0}C with the shaking speed of 200 rpm for 24h.



Appendix A.7: Chromatograms to verify the effect of extra addition of LTV & ABTS on OSL degradation at 30 ^{0}C

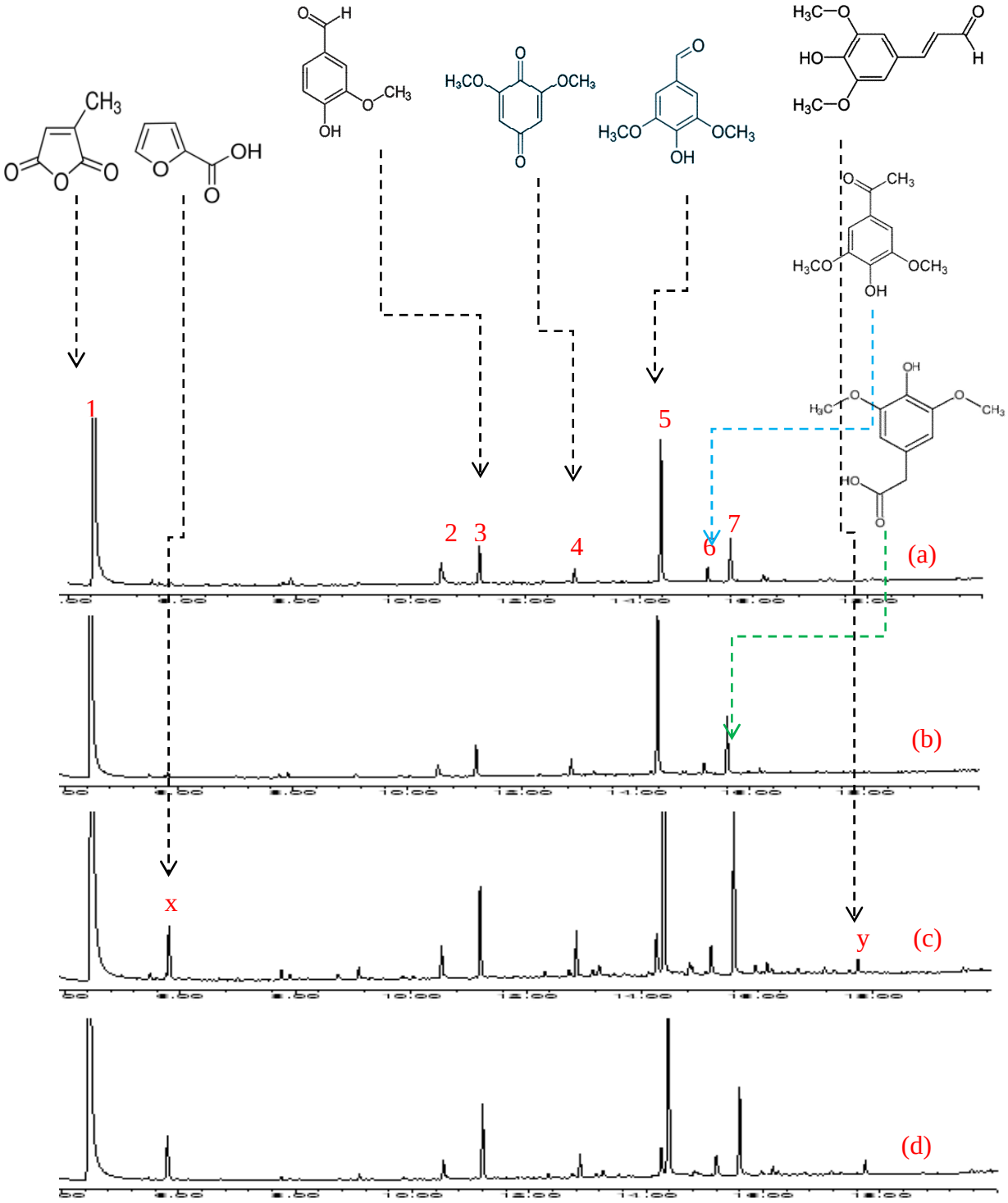
The comparison of the observed GC-MS chromatogram from the samples of EA extracts after second addition of 0.25mg/ml LTV and ABTS (4mM) after 5h of ongoing reaction. All reactions and controls were incubated at 30 ^{0}C for 24h at a shaking speed of 200 rpm.



Appendix A.8: Chromatograms FOR OSL degradation by ltv at 30 ^{0}C in the presence of ethanol

A8.1: GC-MS identification of OSL treatment by LTV-ABTS at 30 ^{0}C with 10% ethanol

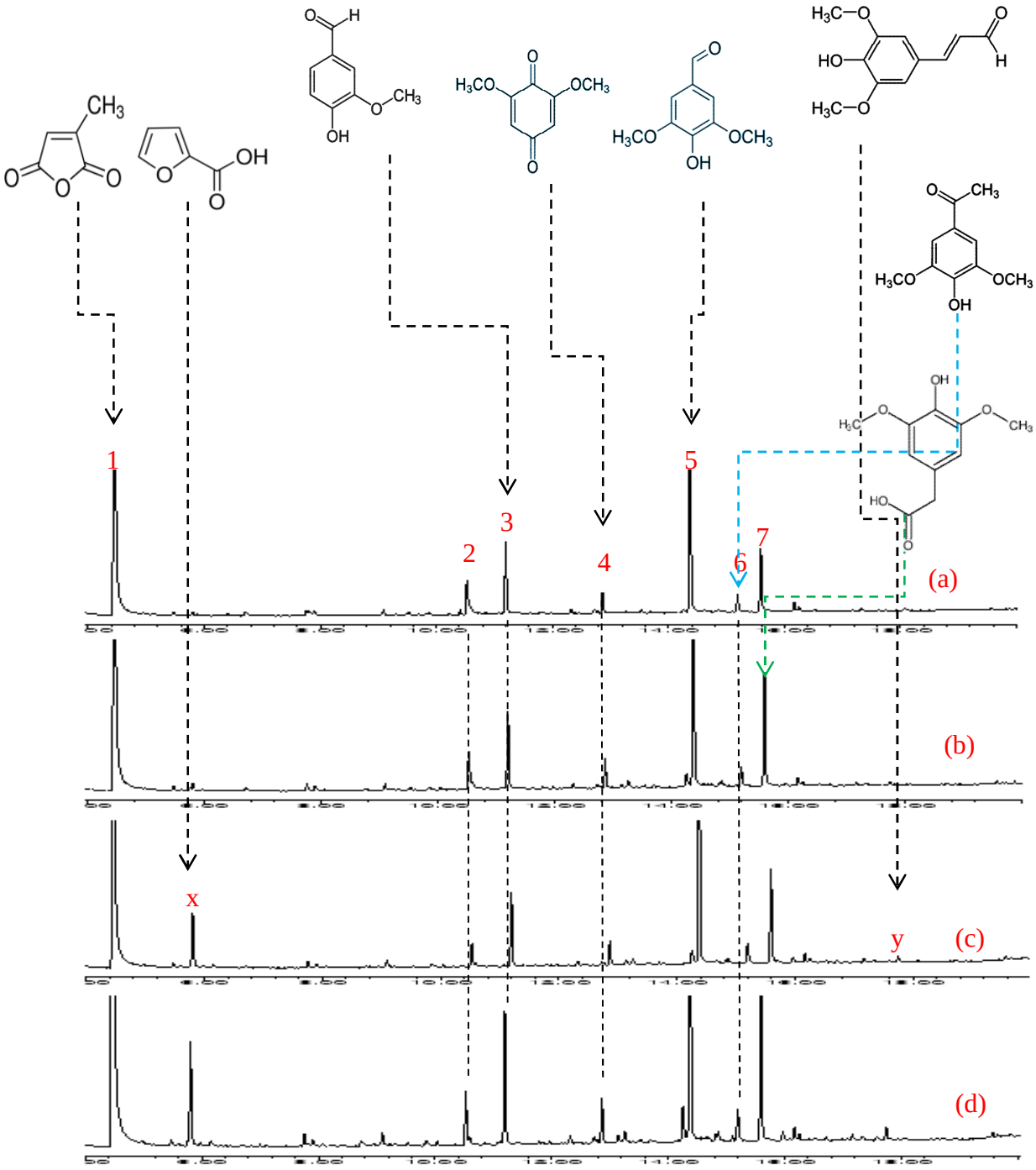
OSL (57g/L) was reacted with 0.25 mg/mL LTV and 4 mM ABTS for 24h at 30 ^{0}C in the presence of 10% ethanol. The symbols used in the chromatograms are: (a) complete reaction; (b) control without LTV; (c) control without ABTS; (d) control without LTV & ABTS.



Observed mass spectra of GC-MS identified compounds produced from OSL degradation by LTV in the presence of 10% of ethanol were similar to the compounds which were formed from 72h of extended reaction products of OSL. So, there is no need for repeated explaination.

A8.2: GC-MS identification of OSL treatment by LTV-ABTS at 30 ^{0}C with 20% ethanol

OSL (57g/L) was reacted with 0.25 mg/mL LTV and 4 mM ABTS for 24h at 30 ^{0}C in the presence of 20% ethanol. Symbols are used in the chromatograms: (a) complete reaction; (b) control without LTV; (c) control without ABTS; (d) control without LTV & ABTS.



Appendix A.9: A brief literature survey for developing LiP and MnP activity assays

Table 2: Lignin peroxidase (LiP) Assays and the relevant reactions catalysed by LiP

No.	Substrate	Buffer	pH	Assay conditions (Λ, ε, T) and time etc.	References
1	Veratryl alcohol (VA)	Sodium titrate (50 mM)	3.5	V_{final}= ?; [VA]= (2-4) mM, [H_2O_2]= 0.4 Mm [LiP]= 0.05µM ; Λ= 310 nm; T= 28°C (additional: Km= 172 µM; ε = 9300 M^{-1} cm^{-1})	Ming Tien and Dengbo Ma. Oxidation of 4-Methoxymandelic Acid by Lignin Peroxidase Mediation by Veratryl Alcohol. *The Journal of Biological Chemistry*, 1997, 272, 8912-8917
2	VA	Sodium titrate (0.33M)	3.0	V_{final}=? ; [VA] = 3 mM/0.33 M buffer (1ml); [LiP] = 0.25-0.30 unit/2ml (in water). To start with [H_2O_2] =54mM (15.4µL); T= 25°C, Λ=310 nm, ε = 9300 M^{-1} cm^{-1}	Simo Sarkanen R A Razal. Lignin peroxidase: toward a clarification of its role in vivo. *The Journal of Biological Chemistry*, 1991, 266, 3636-3643.
	Guaiacol (GA)		4.0	V_{final}=? [GA]= 1.32 mM/ 0.33 M buffer , [H_2O_2] =300 µM; [LiP] =? T= 30°C, Λ=436 nm, ε= 6390 M^{-1} cm^{-1}	
3	VA	Sodium Titrate (25 mM)	3.5	V_{final}=? ; [VA]= ?; [LiP]= 0.1 µM ;To start with [H_2O_2]=0.4 mM, T= 28°C, Λ=310 nm, ε= 9300 M^{-1} cm^{-1}	Rao S. Koduri and Ming Tien. Kinetic Analysis of Lignin Peroxidase: Explanation for the Mediation Phenomenon by Veratryl Alcohol. *Biochemistry*, 1994, *33* (14), 4225–4230
	Anisyl alcohol (AA)			V_{final}=? ; [AA]= 4 mM, [LiP]= 0.5 µM ,To start with [H_2O_2] =0.3 mM; T= 28°C, Λ=280 nm (oxidation product of AA was quantified by HPLC)	
4	Guaiacol	Sodium Titrate buffer (25 mM)	3.5	V_{final}=? ; [GA]= 2 mM, [LiP]= 0.1 µM, To start with [H_2O_2]=300 µM; Λ= 470 nm, ε = 26,600 M^{-1} cm^{-1}, T= 28°C for 180 or 240s	Rao S. Koduri and Ming Tien. Oxidation of Guaiacol by Lignin Peroxidase. *The Journal of Biological Chemistry*, 1995, 270, 22254-22258.
	VA		3.5	V_{final}=? ; [VA]= 2 mM, [LiP]= 0.1 µM, To start with [H_2O_2] =300 µM; Λ= 310 nm, ε= 9300 M^{-1} cm^{-1}, T= 28°C for 180 or 240s	
5	Veratryl Alcohol (4 mM)	Sodium Titrate buffer (0.1M)	3.5	V_{final}=? ; [VA]= 2 mM, [LiP]= 0.1 µM, To start with [H_2O_2] =300 µM; Λ= 310 nm, ε = 9300 M^{-1} cm^{-1}, T= 28°C	Namhyun Chung and Steven D. Veratryl Alcohol-Mediated Indirect Oxidation of Phenol by Lignin Peroxidase. *Archives of Biochemistry and Biophysics*, 1995, Vol 316 (2), 733–737
	Phenol			V_{final}=?; [Phe]= 100 µM, [LiP]= 0.4 µM, To start with [H_2O_2]=3 mM; T= 28°C, Λ= 398 nm, ε = 6400 M^{-1} cm^{-1}	

6	Guaicol and 3 of β–O-4- phenolic lignin model compounds (dimer, trimer and tetramers)	Sodium tartrate (35 mM)	3.5	V_{final}=?, [H_2O_2] =?, [Subs]=0.5-1 mM, [LiP] =1.5µM ; Λ= 417 nm (guaiacol), T=28°C	Lucia Banci and Simone Ciofi-Baffoni *et al*. Lignin and Mn Peroxidase-Catalyzed Oxidation of Phenolic Lignin Oligomers. *Biochemistry*, 1999, *38* (10), 3205–3210
7	VA	Sodium tartarate buffer (1 ml of 125 mM)		[VA]= 10 mM (500 µl), [H_2O_2] = 2mM (500 µL), [LiP] = 0.1 µM ; T=25°C, Λ=310 nm, ε = 9300 M^{-1} cm^{-1}	Daljit S. Arora and Paramjit K. Gill. Comparison of two assay procedures for lignin peroxidase. Enzyme and Microbial Technology, 2001 (28), 602–605
	Dye Azure B		3.0	[Dye Azure B]= 0.160 mM, [H_2O_2] = 2mM (500 µL); [LiP] = 0.1 µM; Λ=561 nm, ε=? T=25°C	
8	2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)	Sodium tartarate buffer (50 mM)	3.5	[tetramer]= 50 µM, [H_2O_2] $_{final}$= 0.2 mM (10 µL). $V_{reaction}$=15 ml and initially contained [tetramer] = 110 mM and [LP H8 isozyme] = 1 µM. After initiation of the reaction, an additional aliquot of enzyme (0.5 nmol) and H_2O_2 (150 nmol) were added in every 2 min to yield a final conc. of 1.5 mM and 0.14 mM, respectively. Aliquots (1 ml) were removed at different time points from the reaction mixture & added to 2 ml of ACN and analyzed by HPLC. T=28°C	Tunde Mester and Katia Ambert-Balay. Oxidation of a tetrameric nonphenolic lignin model compound by lignin peroxidase. *The Journal of Biological Chemistry*, 2001, 276, 22985-22990.
9	VA (400 µL 10 mM) as mediator for 17ß-Estradiol	tartarate buffer (400 µL)	3.0	[VA]= 10 mM (200 µL), [H_2O_2]= 2 mM (200 µL), [LiP]= 0.02U/ml, $V_{(Enz)}$ =400 µL, Assay runs at rt. for 1 min; Λ=310 nm	Liang Mao and Junhe Lu *et al*. Transformation of 17ß-Estradiol Mediated by Lignin Peroxidase: The Role of Veratryl Alcohol. *Archives of Environmental Contamination and Toxicology*, 2010, Vol 59 (1) , 13-19
10	Nonphenolic trimeric lignin model compounds 1 and 2	Sodium tartarate buffer (50 mM)	3.5	V_{final}=1 ml; V_{buffer}= 800 µL, [VA] = (0.1-0.5) mM, [LiP] = 4.6mM (50 µL, prepared in pH 4 same buffer), [H_2O_2] =?; Λ= 310 nm, T= 25°C	Enrico Baciocchi and Claudia Fabbri et al. Lignin Peroxidase-Catalyzed Oxidation of Nonphenolic Trimeric Lignin Model Compounds: Fragmentation Reactions in the Intermediate Radical Cations. The Journal of Organic Chemistry 2003 68 (23), 9061-9069
11	VA	Sodium tartarate buffer (0.1M)	3	V_{final}=? ; [VA] = 0.4 mM, [LiP] = (1-5) µg protein /ml; 0.1% Tween 80; [H_2O_2] = 0.54mM; Λ= 310 nm, T= 37°C, ε = 9300 M^{-1} cm^{-1} (Addition of H_2O_2 started the reaction.)	MING TIEN AND T. KENT KIRK. Lignin-degrading enzyme from Phanerochaete chrysosporium: Purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. Natl. Acad. Sci. USA 1984 Biochemistry Vol. 81, 2280-2284
12	VA	Not specified	2.5	V_{final}=? [VA]= 2 mM, [H_2O_2] = 0.4 mM, [LiP] = ;(OD=0.2/min), [tartaric acid] = 50 m *M*; T=?; Λ=310 nm, ε= 9300 M^{-1} cm^{-1}	Tien, M., and Kirk, T. K. Lignin Peroxidase of *Phanerochaete chrysosporium. Methods in Enzymology.*, 1988,161, 238–249

(Note: question mark (?) is used for the unspecified reaction conditions in the literature)

Table 3: Manganese peroxidase (MnP) Assays and the relevant reactions catalysed by MnP

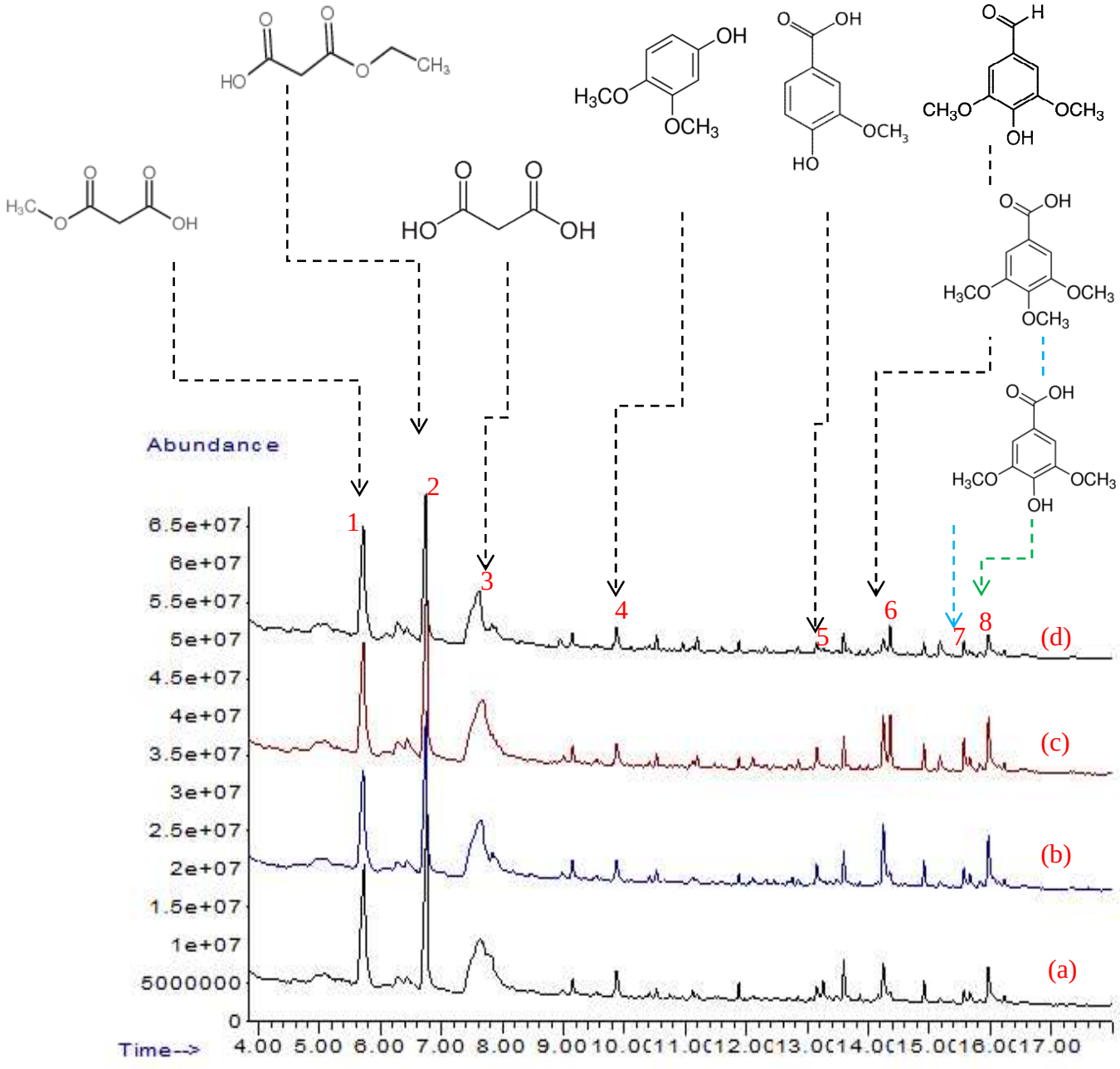
No.	Substrate	Buffer	pH	Assay conditions (Λ, ε, T) and time etc.	Reference
1	Phenol red	Sodium malonate (50 mM)	4.5	V_{final}= 1.0 ml; [$MnSO_4$] = 0.2 mM, [H_2O_2] = 0.1 mM, [phenol red] =0.0025%, [buffer] = 50 mM (pH 4.5); Λ= 431 nm; T= 25°C	FREDERICK S. ARCHIBALD. A new assay for lignin-type peroxidases employing the dye azure B. *Appl. Environ. Microbiol.* ,1992, 3110-3116.
2	2,6-dimethoxyphenol (10 mM)	sodium lactate/ malonate/ oxalate/ tartrate/ malate/ succinate (50 mM)	4.5	V_{final}= 3.0 ml; [MnP] =1 µg/ml; [H_2O_2] =0.01-0.1 mM, [$MnSO_4$]=10 ×[H_2O_2]=0.1-1 mM; ; T= 25°C; Λ= 330 nm; ε_{270}=11.59 (malonate buffer)	Hiroyuki Wariishi *et al.* Manganese (II) oxidation by manganese peroxidase from the basidiomycete Phanerochaete chrysosporium. Kinetic mechanism and role of chelators. *The Journal of Biological Chemistry*, 1992, 267, No. 33, 23689-23695.
3	$MnSO_4$	Sodium malonate (50 mM)	4.5	V_{final}= 3.0 ml; [$MnSO_4$] = 0.2 mM, [H_2O_2] = 0.1 mM; Λ= 270 nm; T= 25°C	M. G. PAICE and I. D. Reid. Manganese Peroxidase, Produced by *Trametes versicolor* during Pulp Bleaching, Demethylates and Delignifies Kraft Pulp. *Appl. Environ. Microbiol*., 1993, 59 (1): 260.
4	MBTH and DMAB (Dye)	Sodium succinic-lactic acid (100 mM)	4.5	V_{final}= 2ml; [3-methyl-2-benzothiazolinone hydrazone (MBTH)] =0.07 mM, [3-(dimethylamino) benzoic acid (DMAB)] = 0.99 mM, [$MnSO_4$] = 0.3 mM, [MnP] =17 nM, [H_2O_2] =0.05 Mm. The reaction was initiated by addition of H_2O_2. Λ= 590 nm; T= 37°C	Maria del Pilar Castillo and John Stenstrom et al. Determination of Manganese Peroxidase Activity with 3-Methyl-2-benzothiazolinone Hydrazone and 3-(Dimethylamino) benzoic Acid. *Analytical Biochemistry*, 1994, 218, 399-404.
5	Phenol red	sodium succinate (20 mM)	4.5	V_{final}= 1.0 ml; [$MnSO_4$] = 0.1 mM, [H_2O_2] = 0.1 mM, [phenol red] =0.0025%, [buffer] = 50 mM (pH 4.5); 1 mg of bovine serum albumin (BSA, Sigma) ml^{-1}, of [phenol red] = 0.1 $mg.ml^{-1}$. The reaction was started by the addition of H_2O_2 to final concentration of 0.1 mM and was stopped after 1 min with 50 ml of 10% NaOH; Λ= 610 nm; T= 30°C	T Vares and M Kalsi et al. Lignin Peroxidases, Manganese Peroxidases, and Other Ligninolytic Enzymes Produced by Phlebia radiata during Solid-State Fermentation of Wheat Straw. *Appl. Environ. Microbiol*. 1995, 61, 10, 3515-3520.

6	Phenol red	sodium lactate (25 mM)	4.5	V_{final}=? [phenol red]= 0.01%, [$MnSO_4$] = 100µM, egg albumin (0.1%), [H_2O_2] =100 µM in 1.0 ml of 20 mM Na-succinate buffer. Λ= 610 nm; T= 30°C (5 min) and terminated with the addition of 2 N NaOH (40µL).	Masaaki Kuwahara, Jeffrey K. Glenn *et al*. Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. *FEBS LETTERS*, 1984, 169 (2), 247-250.
7	$MnSO_4$	Sodium malonate (50 mM)	4.5	V_{final}=? [$MnSO_4$] = 0.2 mM, [H_2O_2] = 0.1 mM; Λ= 270 nm; T=?	FREDERIC H. PERIE and MICHAEL H. GOLD. Manganese regulation of manganese peroxidase expression and lignin degradation by the white rot fungus Dichomitus squalens. *Applied and Environmental Microbiology*, 1991, 2240-2245
8	Aromatic substrate	Sodium malonate (50 mM)	4.5	V_{final}=1 ml, [$MnSO_4$] = 0.5mM, MnP=5pg, [glutathione (GSH)] =5mM, [H_2O_2] =100 µM under anaerobic condition; Reactions were initiated by adding H_2O_2 (0.2 mM). Λ= 270nm; T= 37°C.	Thiol-mediated oxidation of nonphenolic lignin model compounds by manganese peroxidase of Phanerochaete chrysosporium. *Journal of Biochemistry*, 1989, 264 (24), 14185-14191.
9	$MnSO_4$	Sodium lactate buffer (100 mM)	4.5	V_{final}=? [$MnSO_4$] = 100 µM, [H_2O_2] = 100 µM; MnP=100 ng, V_{MnP} =1-15 µL; Λ= 240 nm; T=?	Tien, M., and Kirk, T. K. (1988) *Methods Enzymol.* 161, 238–249
10	Guaicol and 3 of β–O-4-phenolic lignin model compounds (dimer, trimer and tetramers)	Sodium tartrate (20 mM)	4.5	V_{final}=? ; [H_2O_2] =? , [Subs]=0.5-1 mM; [MnP]=1.5µM for 1.5s; Λ= 417 nm, T=28°C	Lucia Banci and Simone Ciofi-Baffoni *et al*. Lignin and Mn Peroxidase-Catalyzed Oxidation of Phenolic Lignin Oligomers. *Biochemistry*, 1999, *38* (10), 3205–3210.

(Note: question mark (?) is used for the unspecified reaction conditions in the literature)

Appendix A10: GC-MS identification of OSL degradation by MnP

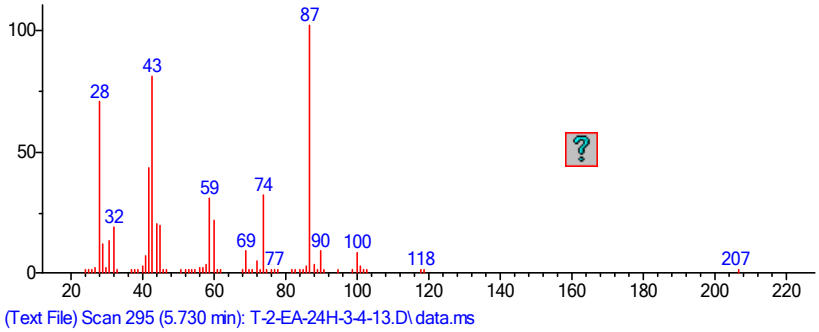
The overlay of total ion chromatograms for MnP catalysed OSL degradation reaction products. Symbols are used in the chromatograms: (a) complete reaction; (b) control without MnP; (c) control without $MnSO_4$; (d) control without both MnP & $MnSO_4$.



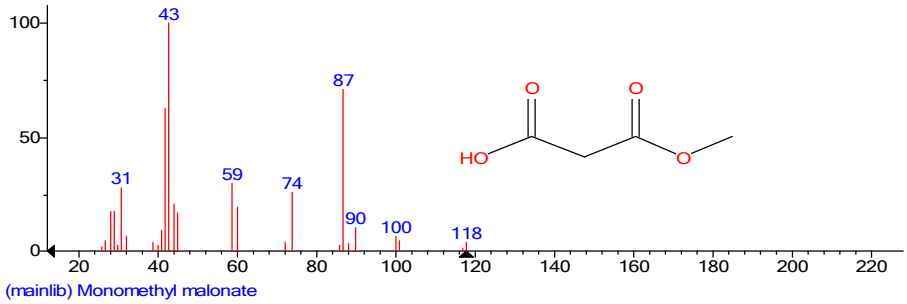
Among the identified compounds formed from both cmplete reaction and controls, the 3-methoxy-3-oxopropanoic acid (peak 1, RT = 5.69 min), ethyl hydrogen malonate (peak2, RT = 6.73 min), malonic acid (peak3, RT = 7.60 min) were unlikely related to the enzymatic degradation reaction of and they might came from the reaction components. The 3,4- dimethoxyphenol (peak4, RT = 10.53

min), vanillic acid (peak5, RT = 13.15min), syringaldehyde (peak6, RT = 14.36 min), 2,3,4-Trimethoxybenzoic acid (peak7, RT = 15.57 min) and syringic acid (peak8, RT = 15.97 min) were more likely produced from OSL reaction with MnP and the co-oxidants. The mass spectrum identification of these compounds are as follows.

(1) 3-Methoxy-3-oxopropanoic acid (RT = 5.69 min)

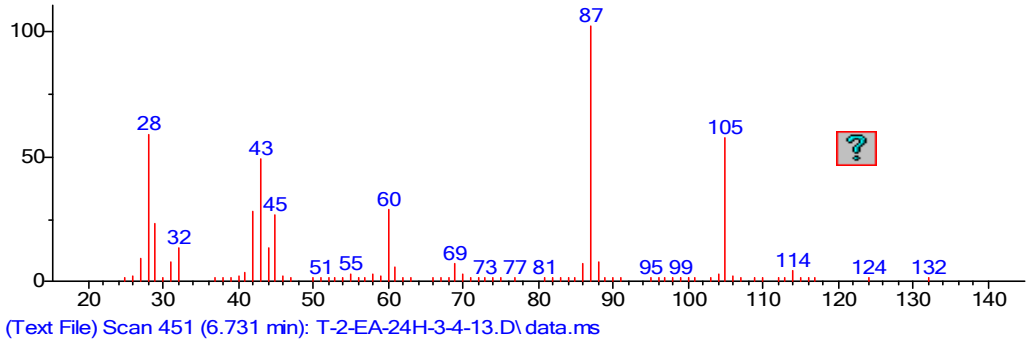


NIST library matches: Monomethyl malonate; Formula: $C_4H_6O_4$; MW: 118; CAS#: 16695-14-0; NIST#: 105250

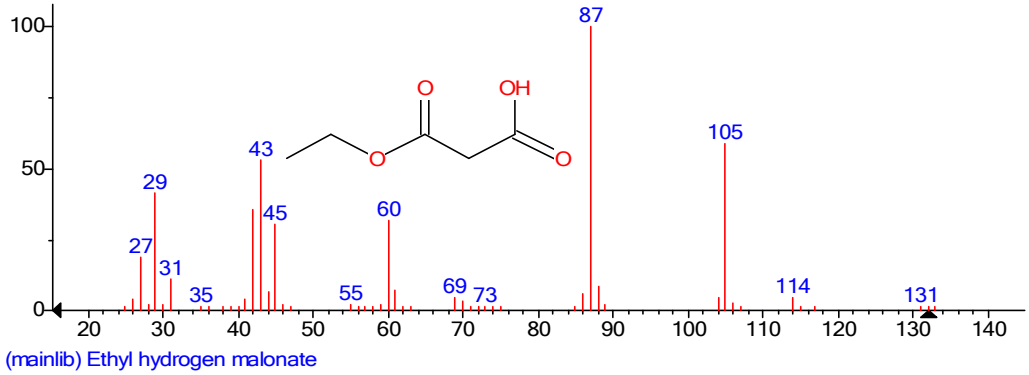


The probability of 3-Methoxy-3-oxopropanoic acid in NIST library identification was higher than 95%, however, there was no authentic standard available to confirm this compound further. So, this was a preliminary result.

(2) Ethyl hydrogen malonate (RT = 6.73 min)

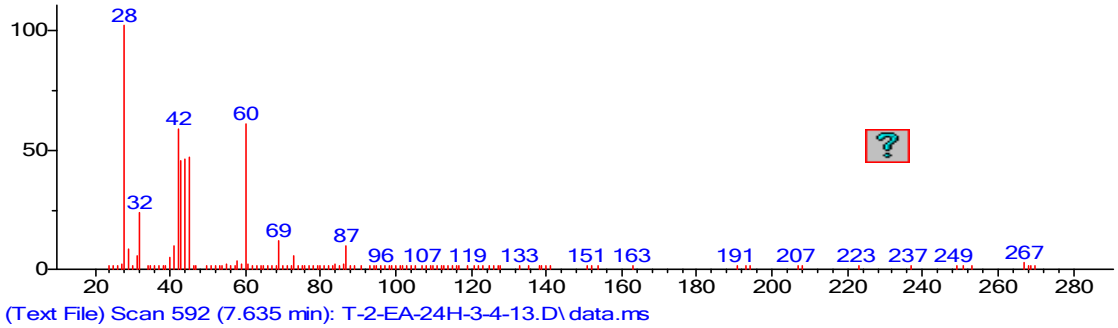


NIST library matches: Ethyl hydrogen malonate; Formula: $C_5H_8O_4$; MW: 132; CAS#: 1071-46-1; NIST#: 343651



The probability of ethyl hydrogen malonate in NIST library identification was nearly 95%, however, there was no authentic standard available to confirm this compound further. So, this was a preliminary result.

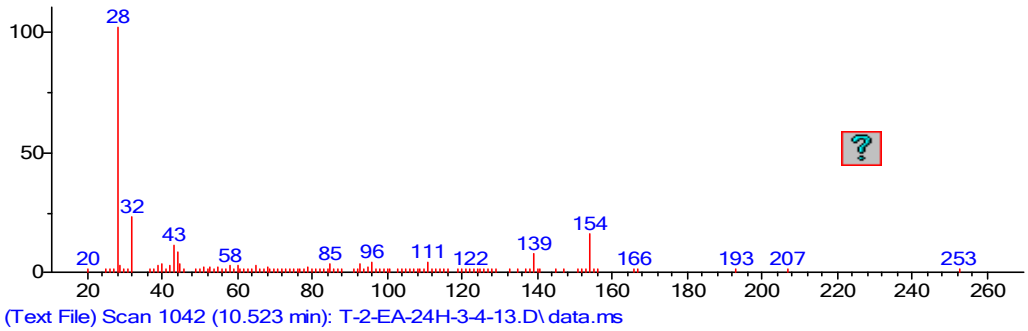
(3) Malonic acid (RT = 7.63 min)



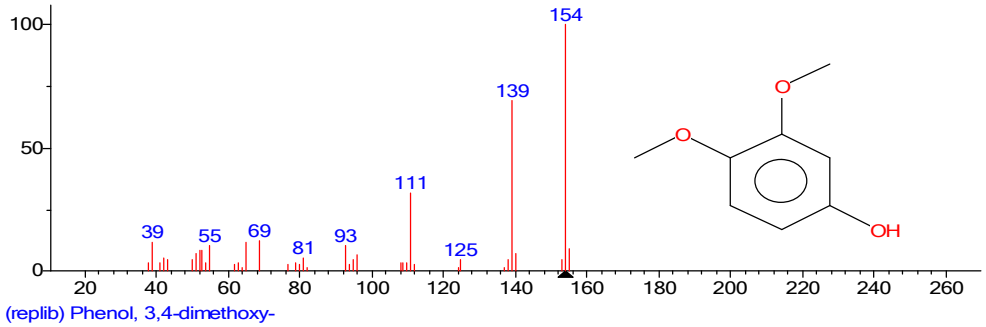
NIST Library matches: Propanedioic acid; Formula: $C_3H_4O_4$; MW: 104; CAS#: 141-82-2; NIST#: 20032

The probability of propanedioic acid in NIST library identification was about 65% only. So, this was a very poor match and needs to be repeated in the future investigation.

(4) 3,4-Dimethoxy-Phenol (RT = 10.53 min)

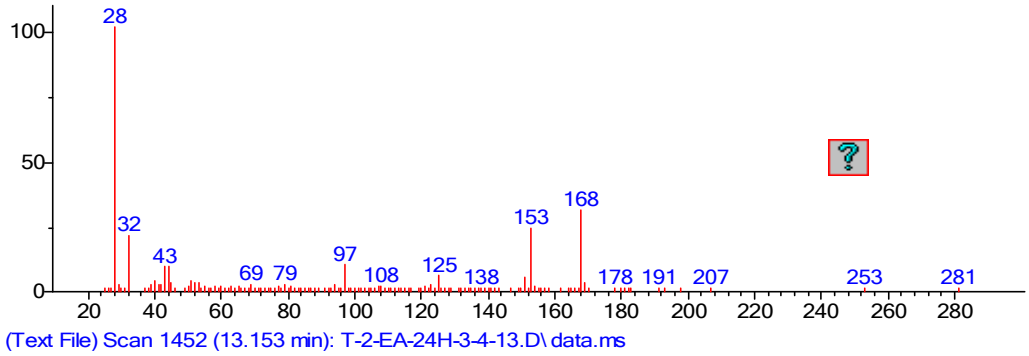


NIST Library confirmation: Phenol, 3,4-dimethoxy-, Formula: $C_8H_{10}O_3$; MW: 154; CAS#: 2033-89-8; NIST#: 33989

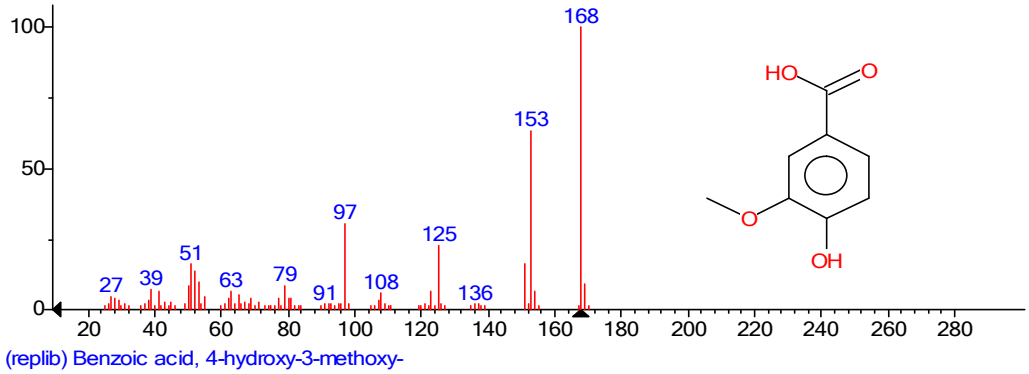


The probability of 3,4-dimethoxy phenol in NIST library identification was about 35% only. So, this was a very poor match and needs to be repeated in the future investigation.

(5) Vanillic acid (RT = 13.15 min)

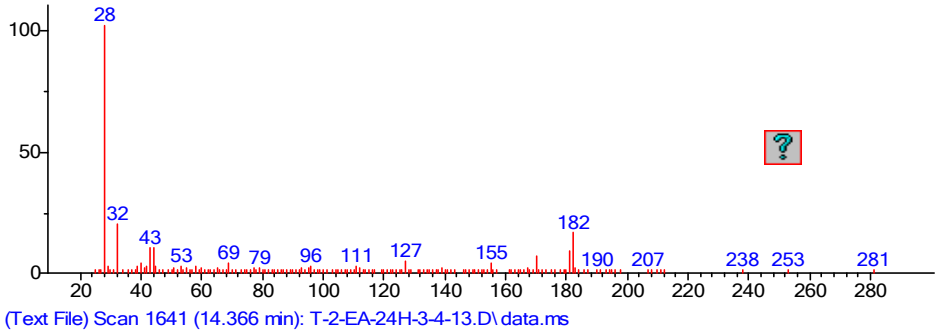


NIST Library confirmation: 4-hydroxy-3-methoxy- Benzoic acid; Formula: $C_8H_8O_4$; MW: 168; CAS#: 121-34-6; NIST#: 6514

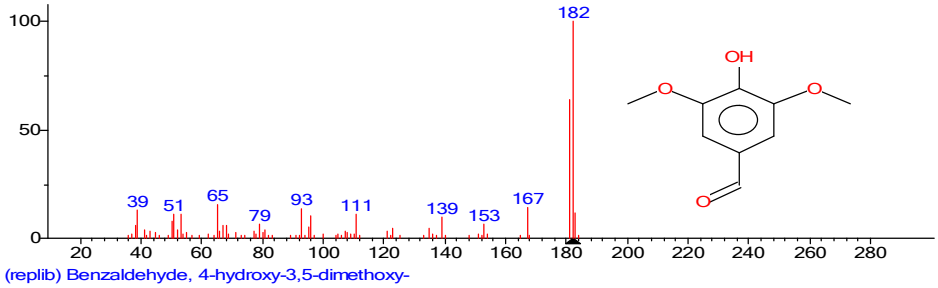


The probability of vanillic acid in NIST library identification was about 60% only. So, this was a very poor match and needs to be repeated in the future investigation.

(6) Syringaldehyde (RT = 14.37 min)

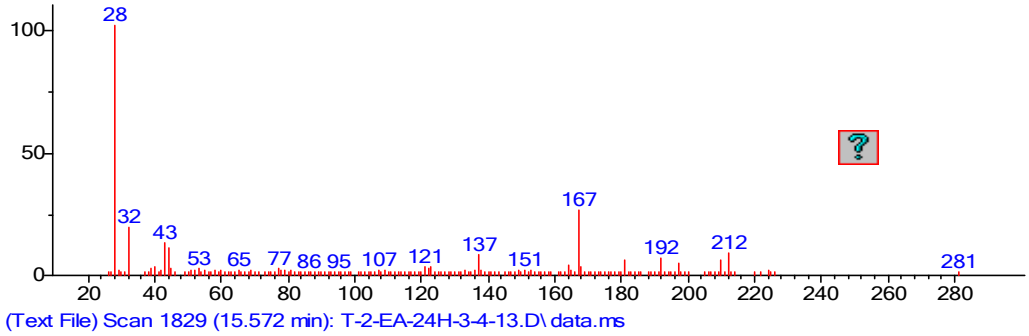


NIST Library confirmation: Name: 4-hydroxy-3,5-dimethoxy-Benzaldehyde; Formula: $C_9H_{10}O_4$; MW: 182; CAS#: 134-96-3; NIST#: 250256

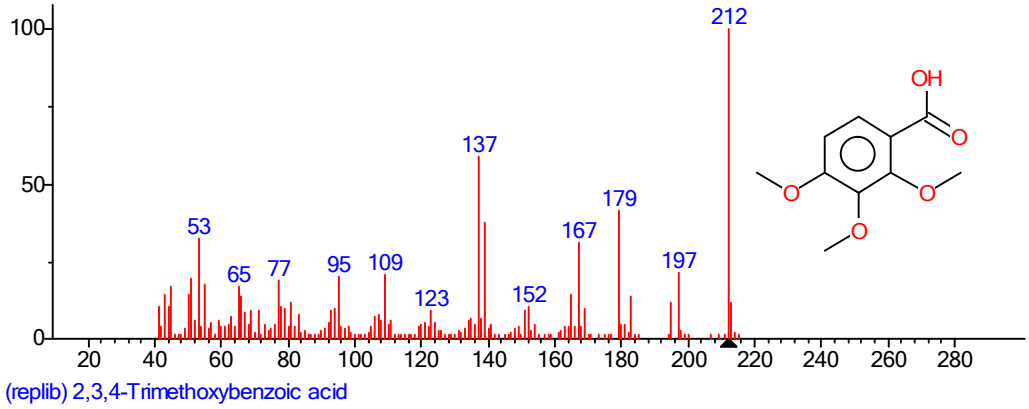


The probability of syringaldehyde in NIST library identification was about 20% only. So, this was an extremely poor match.

(7) 2,3,4-Trimethoxybenzoic acid (RT = 15.57 min)

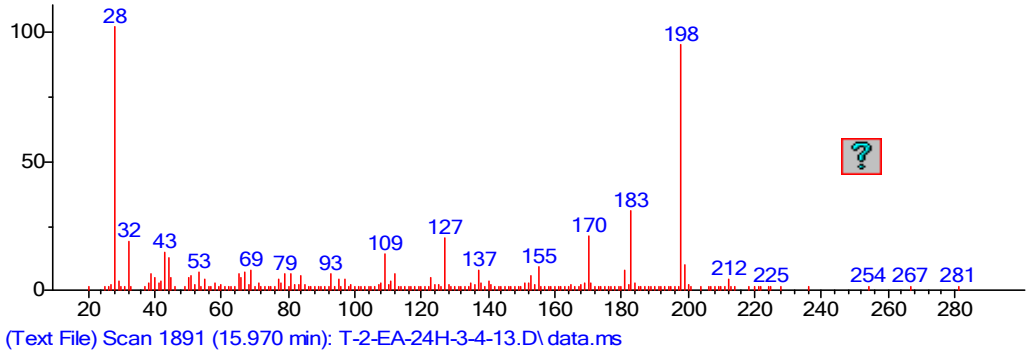


NIST Library confirmation: Name: 2,3,4-Trimethoxybenzoic acid; Formula: $C_{10}H_{12}O_5$; MW: 212; CAS#: 573-11-5; NIST#: 116043

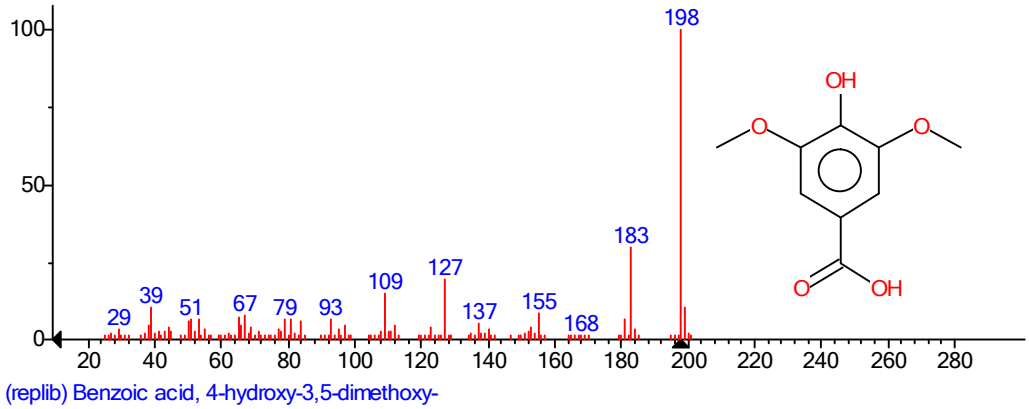


The probability of 2,3,4-trimethoxybenzoic acid in NIST library identification was about 40% only. So, this was a very poor match and needs to be repeated in the future investigation.

(8) Syringic acid (RT = 15.97 min)



NIST Library confirmation: 4-hydroxy-3, 5-dimethoxy- Benzoic acid; Formula: $C_9H_{10}O_5$; MW: 198; CAS#: 530-57-4; NIST#: 234147

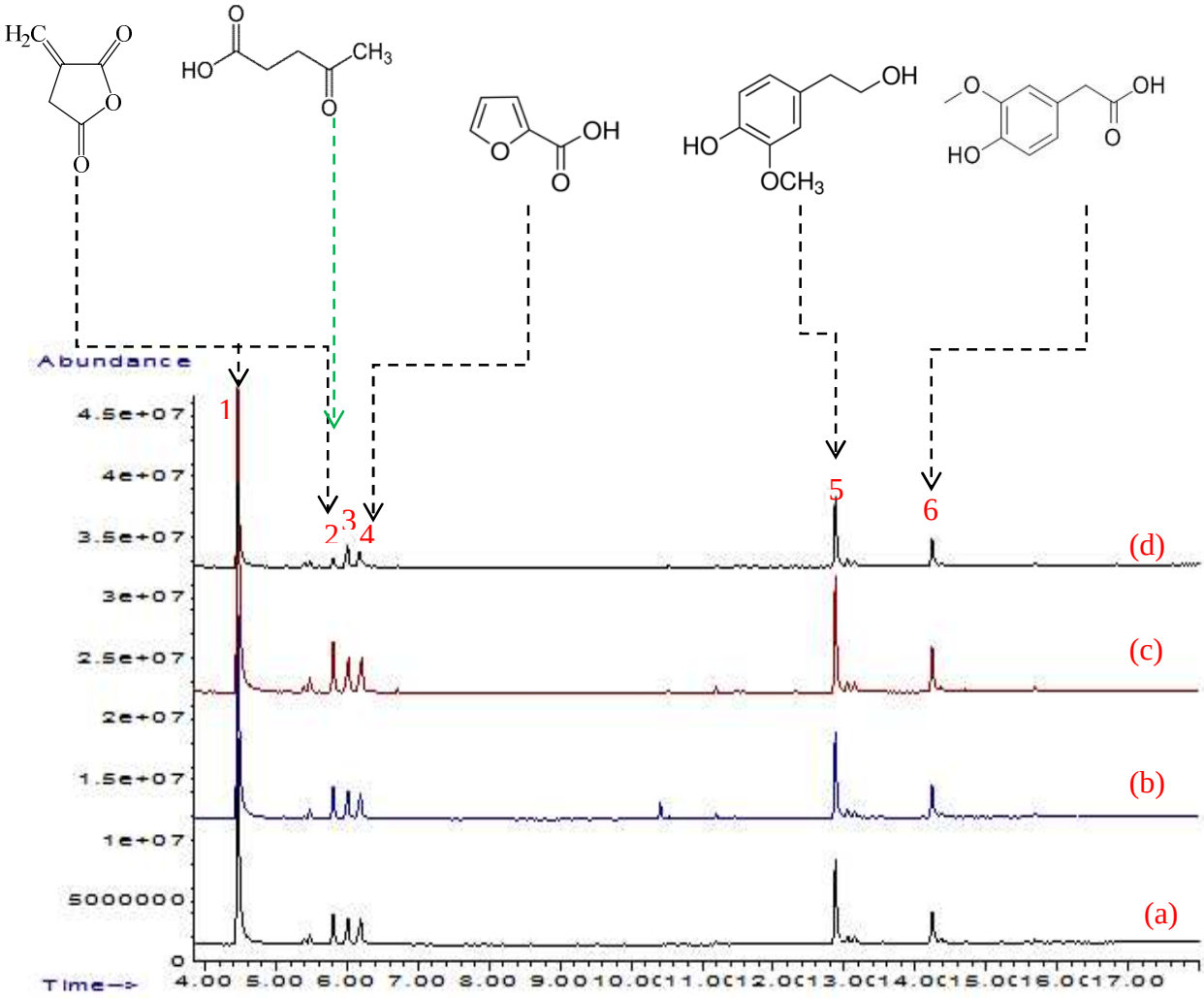


The probability of syringic acid in NIST library identification was about 80% versus its standard was 78% by NIST library match. So, it can be a close match, yet with low purity.

Appendix A11: GC-MS identification of Lignosulfonates degradation by LTV

A11.1: Chromatograms of lignosulphonate from Sigma-Aldrich:

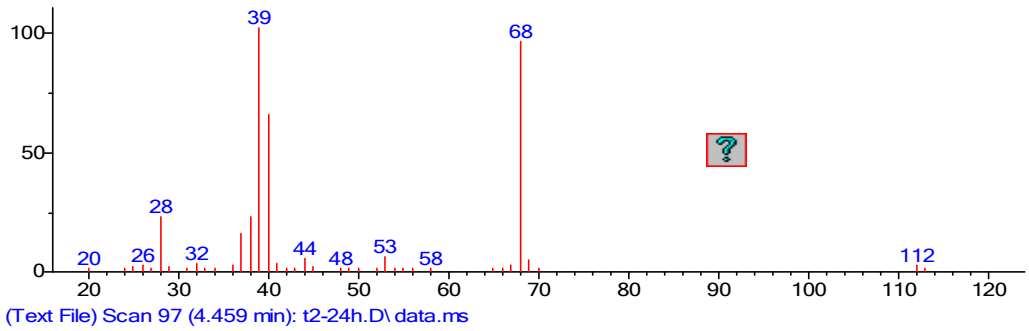
Lignosulphonate from Sigma-Aldrich was treated by LTV in the presence of ABTS at 30C for 24h with the shaking speed of 200 rpm. Samples from reaction and controls were acidified, extracted with EA and the dried extracts were suspended with acetone before GC-MS analysis.



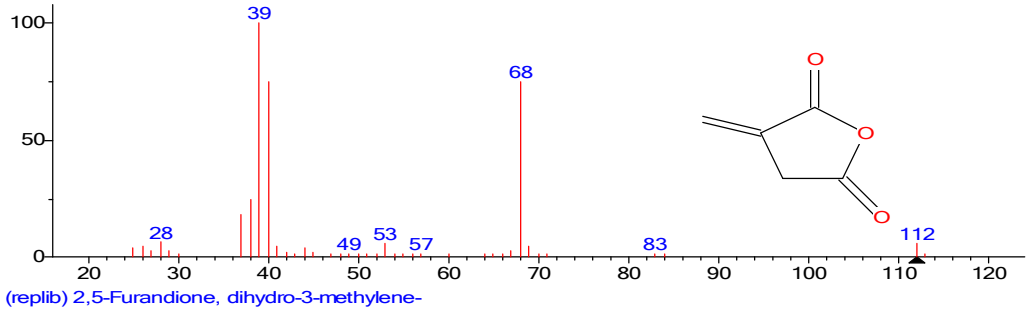
Mass spectrums of identified chemicals are as follows:

(1) 3-Methylenedihydro-2,5-furandione (peak 1 at RT=4.46 min and peak 2 at 5.81 min)

(a) RT=4.46 min

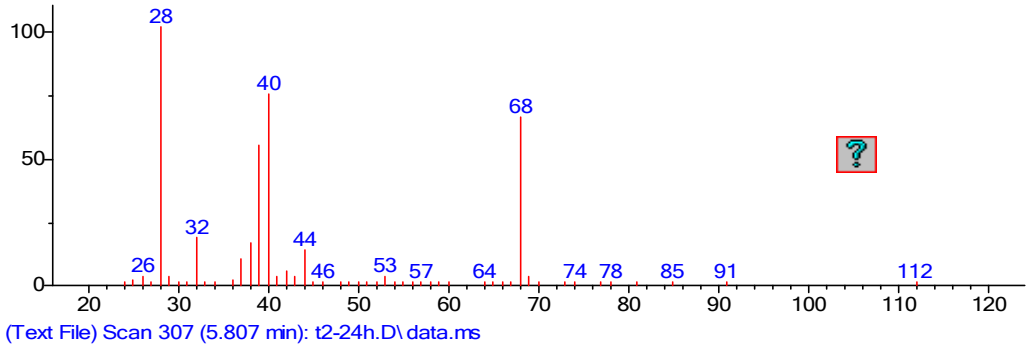


NIST library match: Name: 2,5-Furandione, dihydro-3-methylene-, Formula: $C_5H_4O_3$; MW: 112; CAS#: 2170-03-8 NIST#: 197008

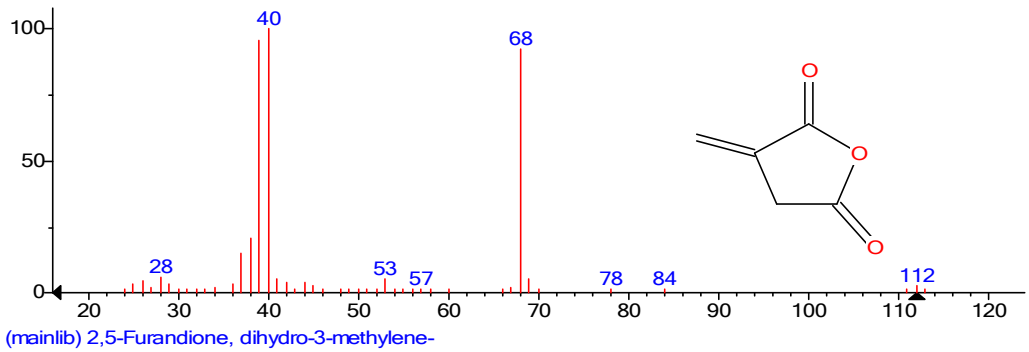


The probability of dihydro-3-methylene-2,5-Furandione in NIST library identification was about 55%. However, there was no standard available to confirm this result. So, it should be reconsidered in the future investigation.

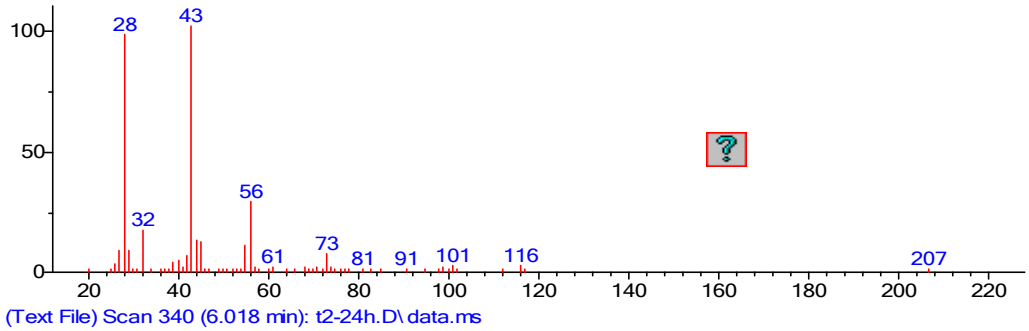
(b) RT=5.81 min



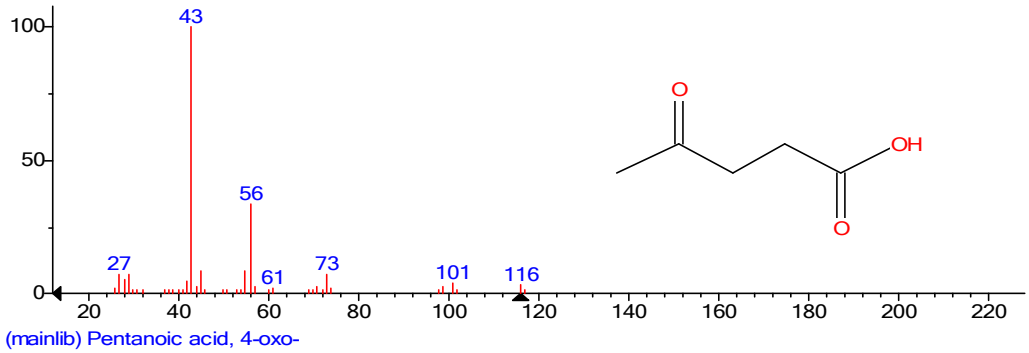
NIST library match: Name: 2,5-Furandione, dihydro-3-methylene-; Formula: $C_5H_4O_3$; MW: 112 CAS#: 2170-03-8 NIST#: 229857



(2) Levulinic acid (peak 3, RT=6.02 min)

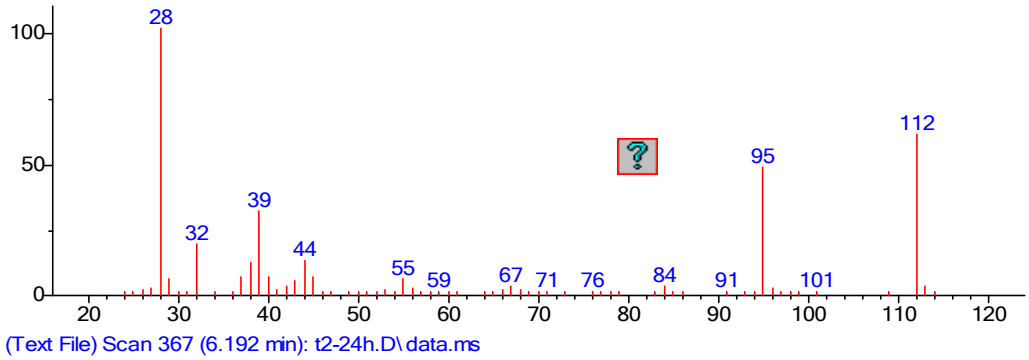


NIST library match: Name: Pentanoic acid, 4-oxo-; Formula: $C_5H_8O_3$; MW: 116; CAS#: 123-76-2; NIST#: 1979

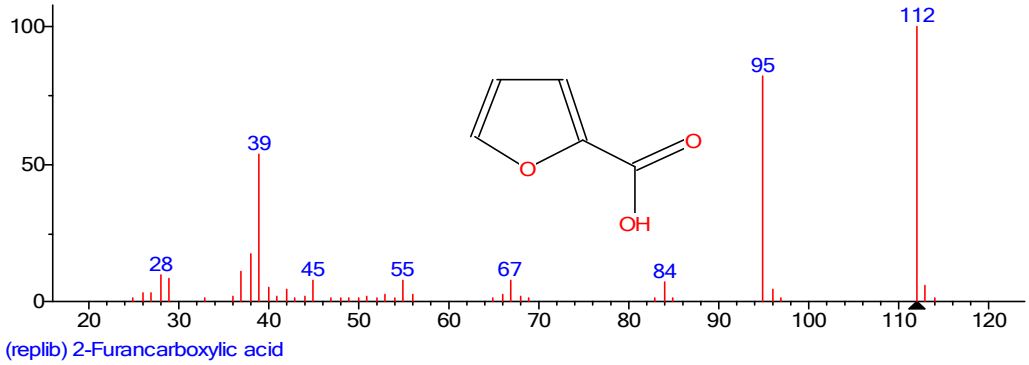


The probability of levulinic acid in NIST library identification was about 90%. It could be a close match, yet, not with very high purity.

(3) 2-Furoic acid (peak 4, RT=6.192 min)

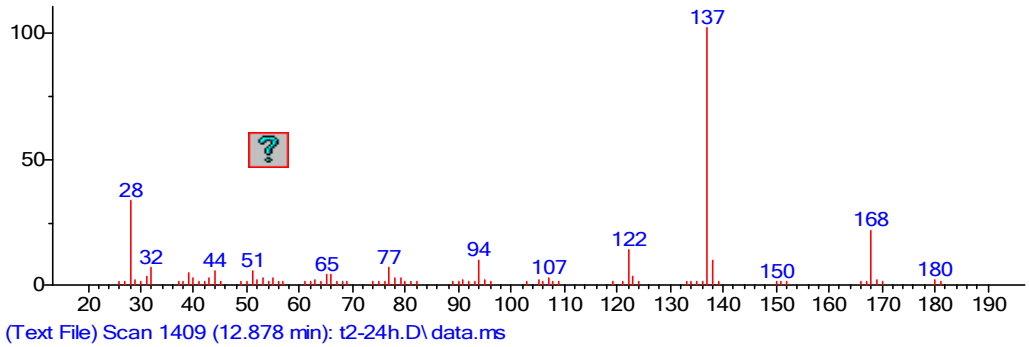


NIST library match: Name: 2-Furancarboxylic acid; Formula: $C_5H_4O_3$; MW: 112 CAS#: 88-14-2 NIST#: 221171

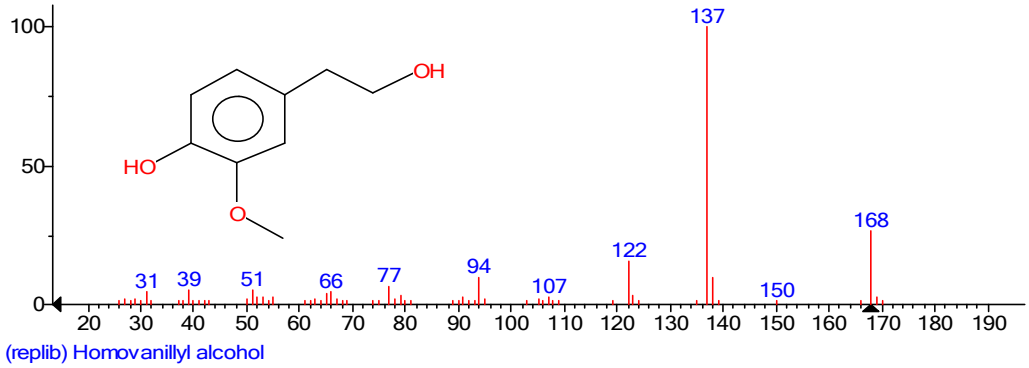


The probability of 2-Furancarboxylic acid in NIST library identification was about 80%. So, it could be a close match, yet, not with very high purity.

(4) Homovanillyl alcohol (peak 5, RT=12.88 min)

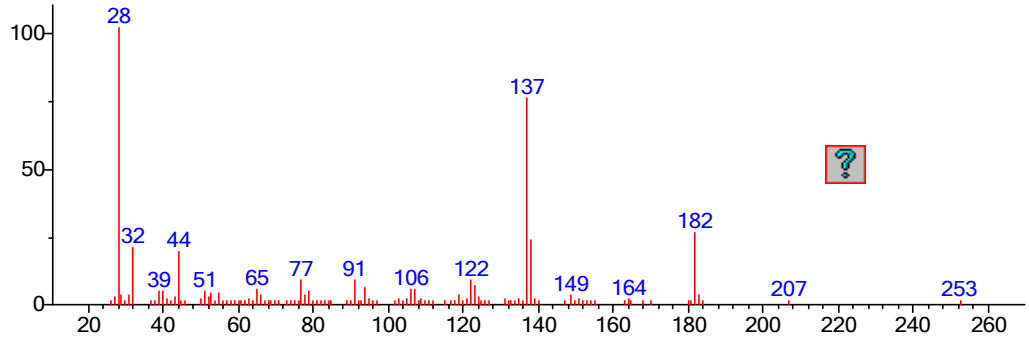


NIST library match: Name: Homovanillyl alcohol; Formula: $C_9H_{12}O_3$; MW: 168; CAS#: 2380-78-1; NIST#: 133524



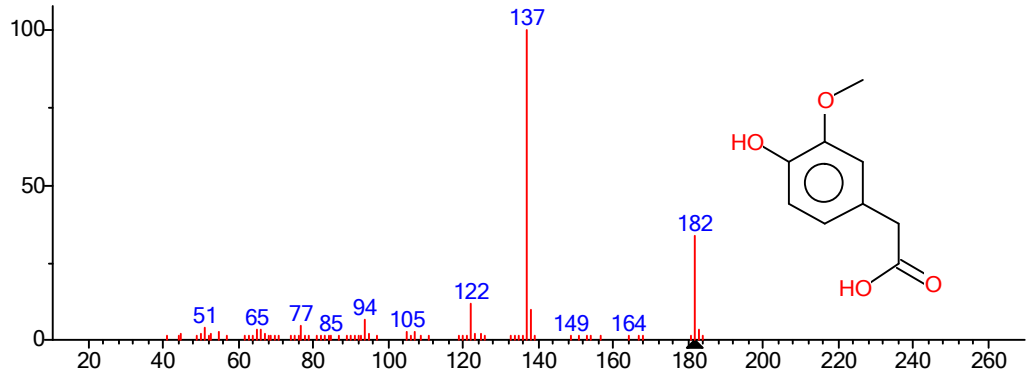
The probability of homovanillyl alcohol in NIST library identification was higher than 90% where as its standard was 94.8%. So, it could be a close match, yet, not with very high purity.

(5) Homovanillic acid (peak 6, RT=14.28 min)



(Text File) Scan 1621 (14.238 min): t2-24h.D\data.ms

NIST libraryName: Benzeneacetic acid, 4-hydroxy-3-methoxy-; Formula: $C_9H_{10}O_4$; MW: 182; CAS#: 306-08-1; NIST#: 248367

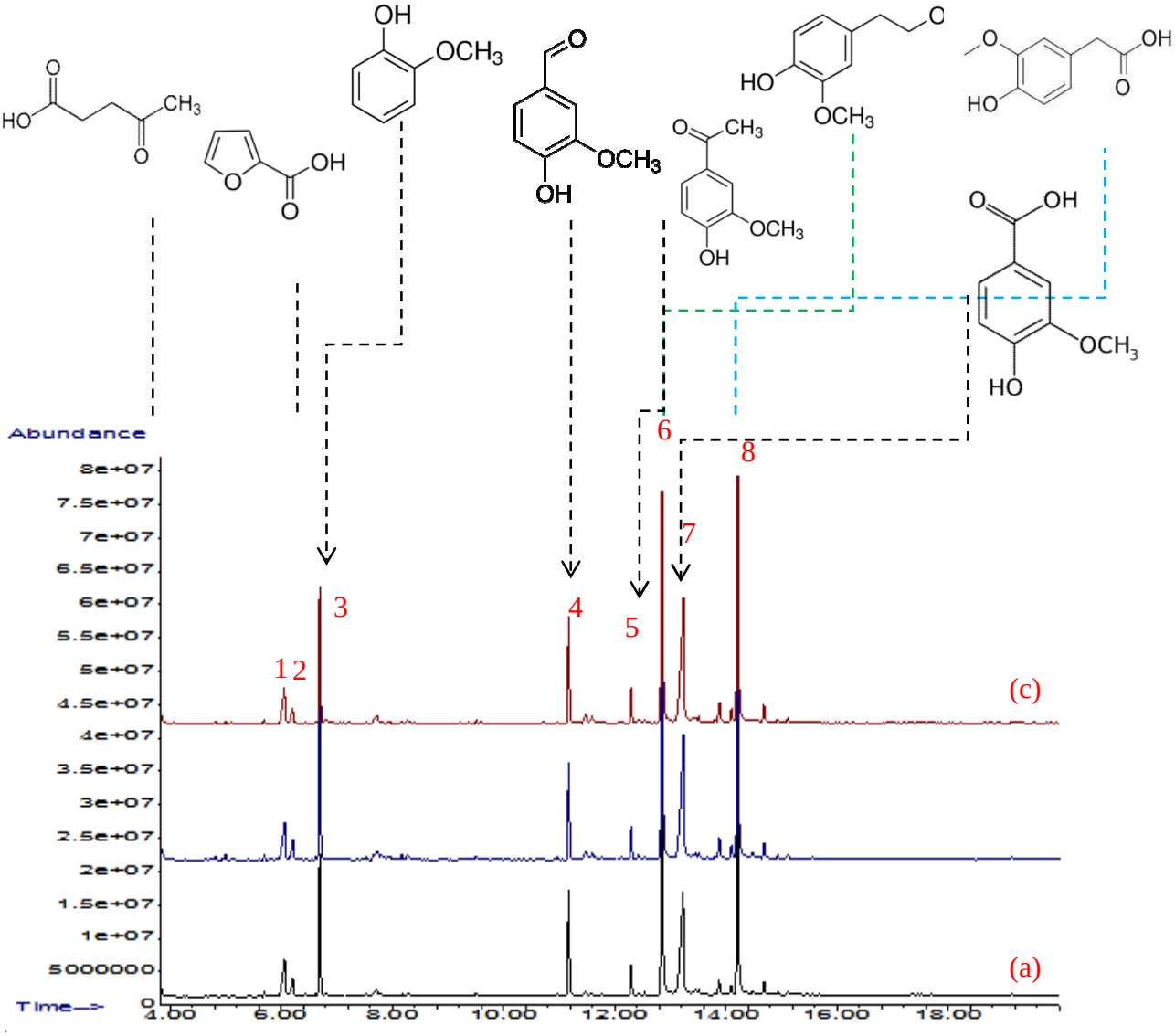


(replib) Benzeneacetic acid, 4-hydroxy-3-methoxy-

The probability of homovanillic acid in NIST library identification was less than 60%, but there was no standard available to confirm this result.

A11.2: Chromatograms of lignosulphonate from Borregaard (DP590) was treated with LTV at 30C for 6h.

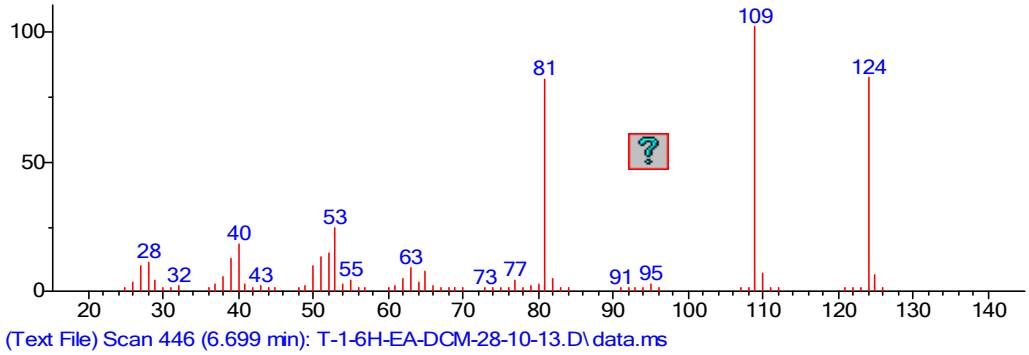
Lignosulphonate from Sigma-Aldrich was treated by LTV in the presence of ABTS at 30C for 24h with the shaking speed of 200 rpm. Samples from reaction and controls were acidified, extracted with EA and the dried extracts were suspended with acetone before GC-MS analysis. The symbols used are: (a) complete reaction; (b) control without LTV; (c) control without ABTS.



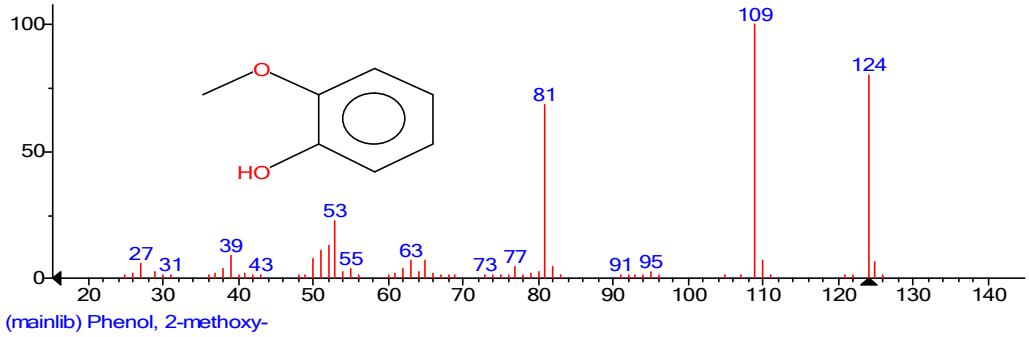
The compounds identified from lignosulphonate degradation by LTV were: Levulinic acid (peak 1, RT=6.03 min), 2-Furoic acid (peak2, RT=6.18 min), guaiacol (peak 3, RT=6.67 min), vanillin (peak 4, RT=11.20 min), acetovanillone (peak 5, RT=12.30 min), homovanillyl alcohol (peak 6, RT=12.86 min), vanillic acid (peak 7, RT=13.23 min) and homovanillic acid (peak8, RT=14.21 min). Except guaiacol and vanillic acid, all others

were identified in previous sample analysis, so the following mass spectra are shown for those 2 compounds only.

(1) Guaiacol

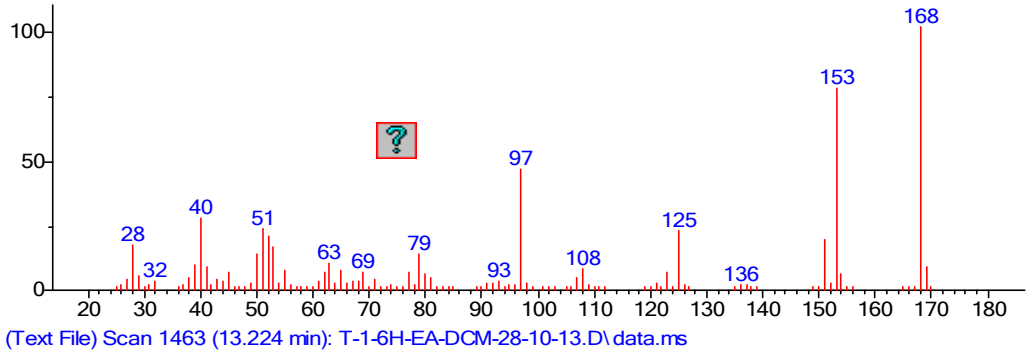


NIST library match: Name: Phenol, 2-methoxy-; Formula: $C_7H_8O_2$; MW: 124; CAS#: 90-05-1; NIST#: 291437

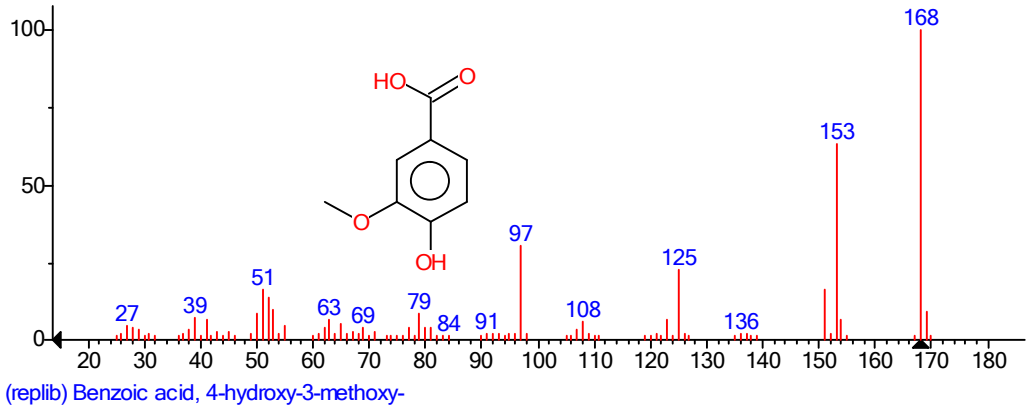


The probability of guaiacol in NIST library identification was nearly 90% whereas its standard was 77.5% only. So, it can be considered as a good match, yet with lower purity.

(2) Vanillic acid

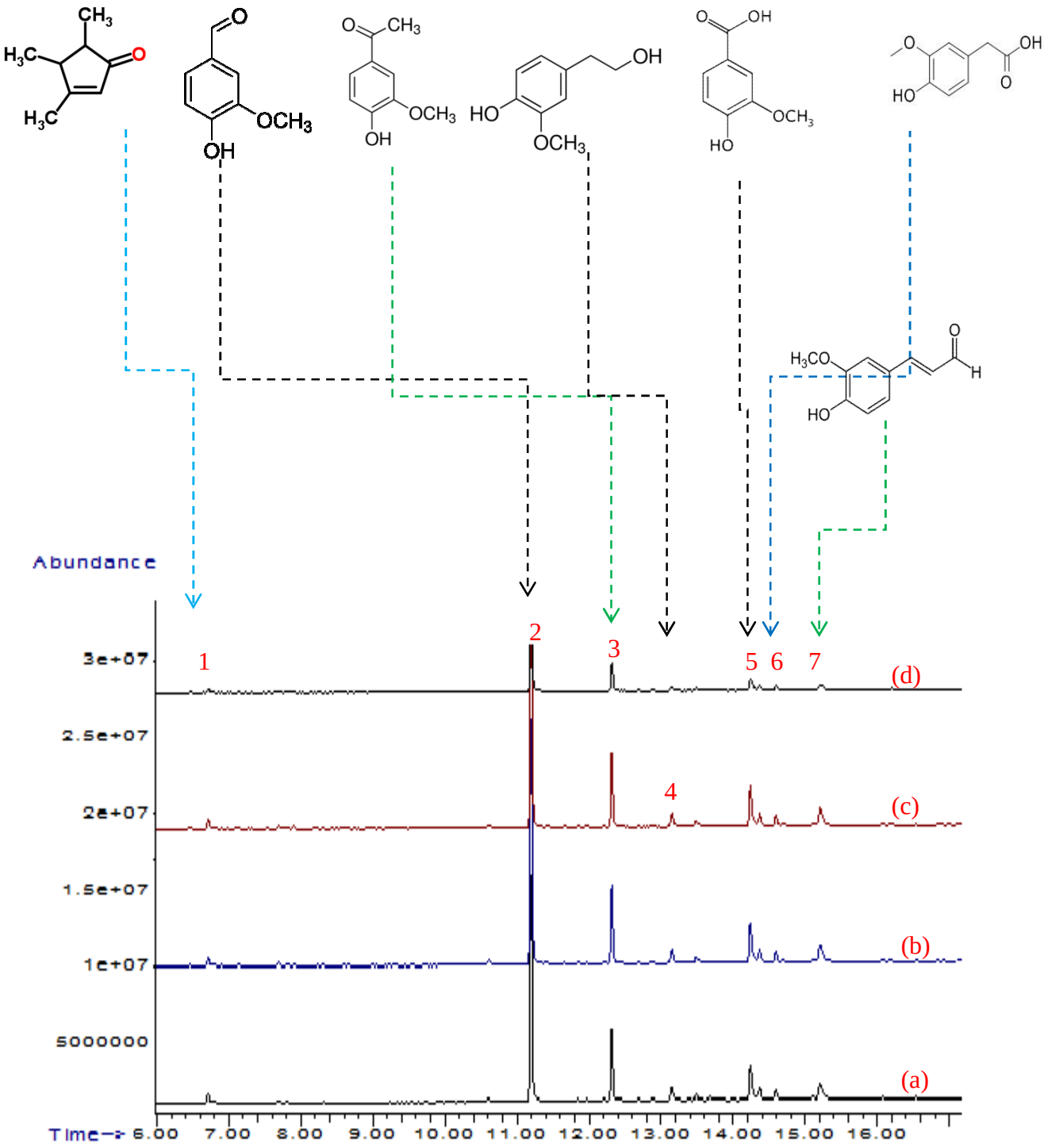


NIST library match: Name: Benzoic acid, 4-hydroxy-3-methoxy-; Formula: $C_8H_8O_4$; MW: 168; CAS#: 121-34-6; NIST#: 6514



The probability of vanillic acid in NIST library identification was less than 70% whereas its standard was 54% only. So, it can be considered as a good match, but with low purity.

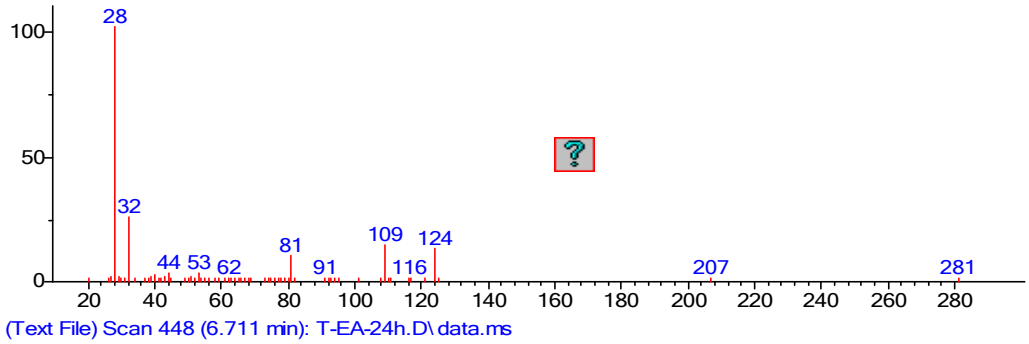
Appendix A11.3: GC-MS identification of Kraft lignin degradation by LTV



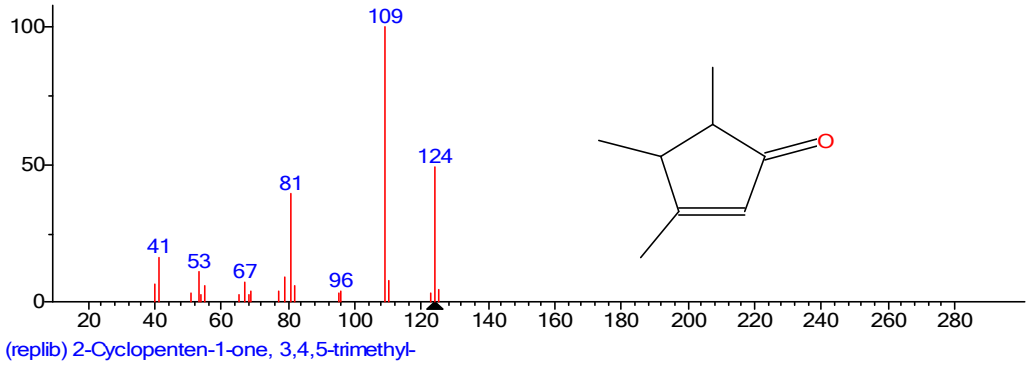
The compounds identified from Kraft lignin degradation by LTV were: 3,4,5-Trimethyl-2-cyclopenten-1-one (peak 1, RT=6.71 min), vanillin (peak 2, RT=11.20 min), acetovanillone (peak 3, RT=12.30 min), homovanillyl alcohol (peak 4, RT=12.86 min), vanillic acid (peak 5, RT=13.17 min), homovanillic acid (peak6, RT=14.21 min) and the 4-Hydroxy-2-methoxycinnamaldehyde (Coniferyl aldehyde, peak 7, RT=15.50 min). Except

guaiacol, vanillic acid and the coniferyl aldehyde, all others were identified in previous sample analysis, so the following mass spectra are shown for those 3 compounds only.

(1) 3,4,5-Trimethyl-2-cyclopenten-1-one

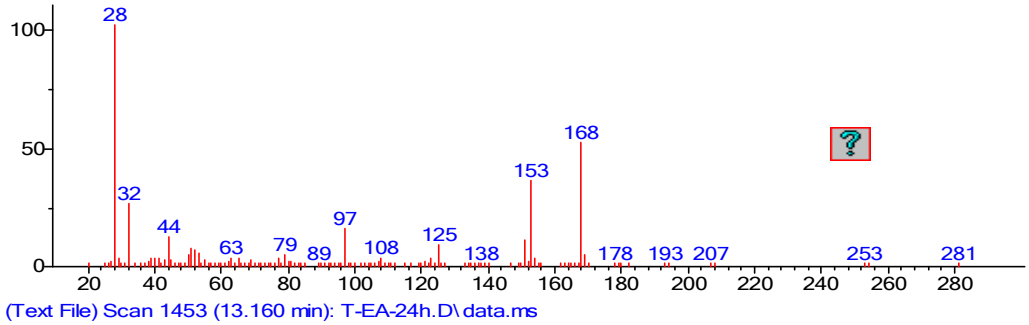


NIST library match: Name: 2-Cyclopenten-1-one, 3,4,5-trimethyl-; Formula: $C_8H_{12}O$; MW: 124 CAS#: 55683-21-1; NIST#: 27161

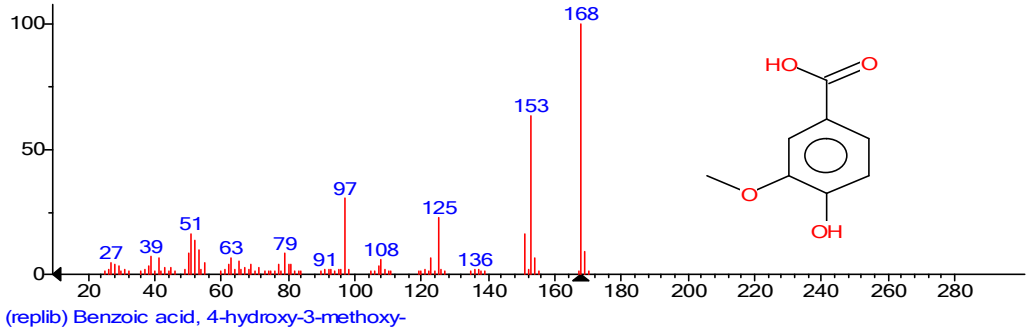


The probability of 3,4,5-trimethyl-2-Cyclopenten-1-one in NIST library identification was less than 40% and there was no standard available to confirm this match. So, it can be considered as a very poor match.

(2) Vanillic acid

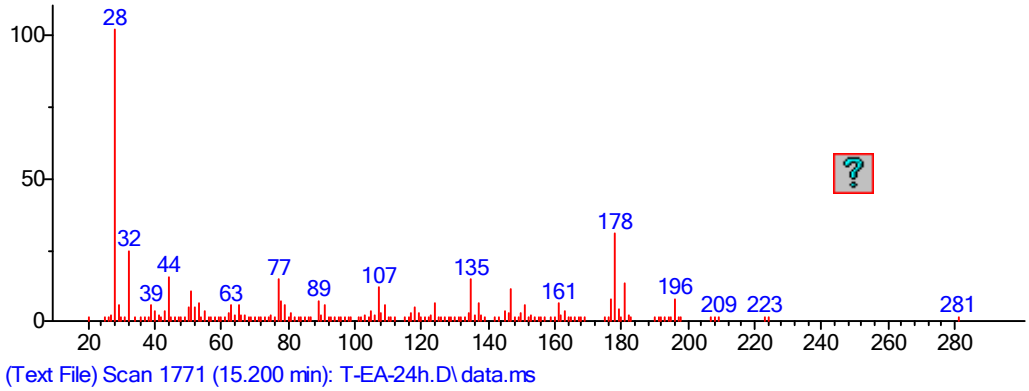


NIST library match: Name: Benzoic acid, 4-hydroxy-3-methoxy-; Formula: $C_8H_8O_4$; MW: 168; CAS#: 121-34-6; NIST#: 6514

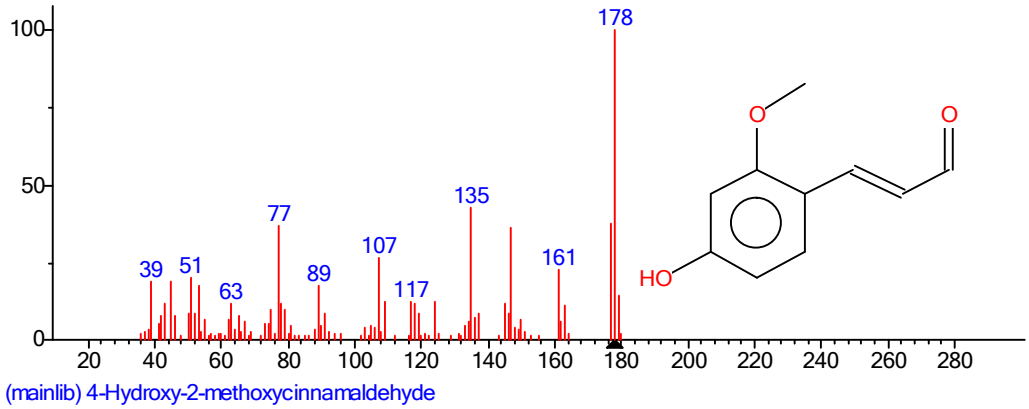


The probability of vanillic acid in NIST library identification was 65% whereas its standard was only 54% match in NIST. So, it can be considered as a good match, yet with low purity.

(3) Conifereyl aldehyde

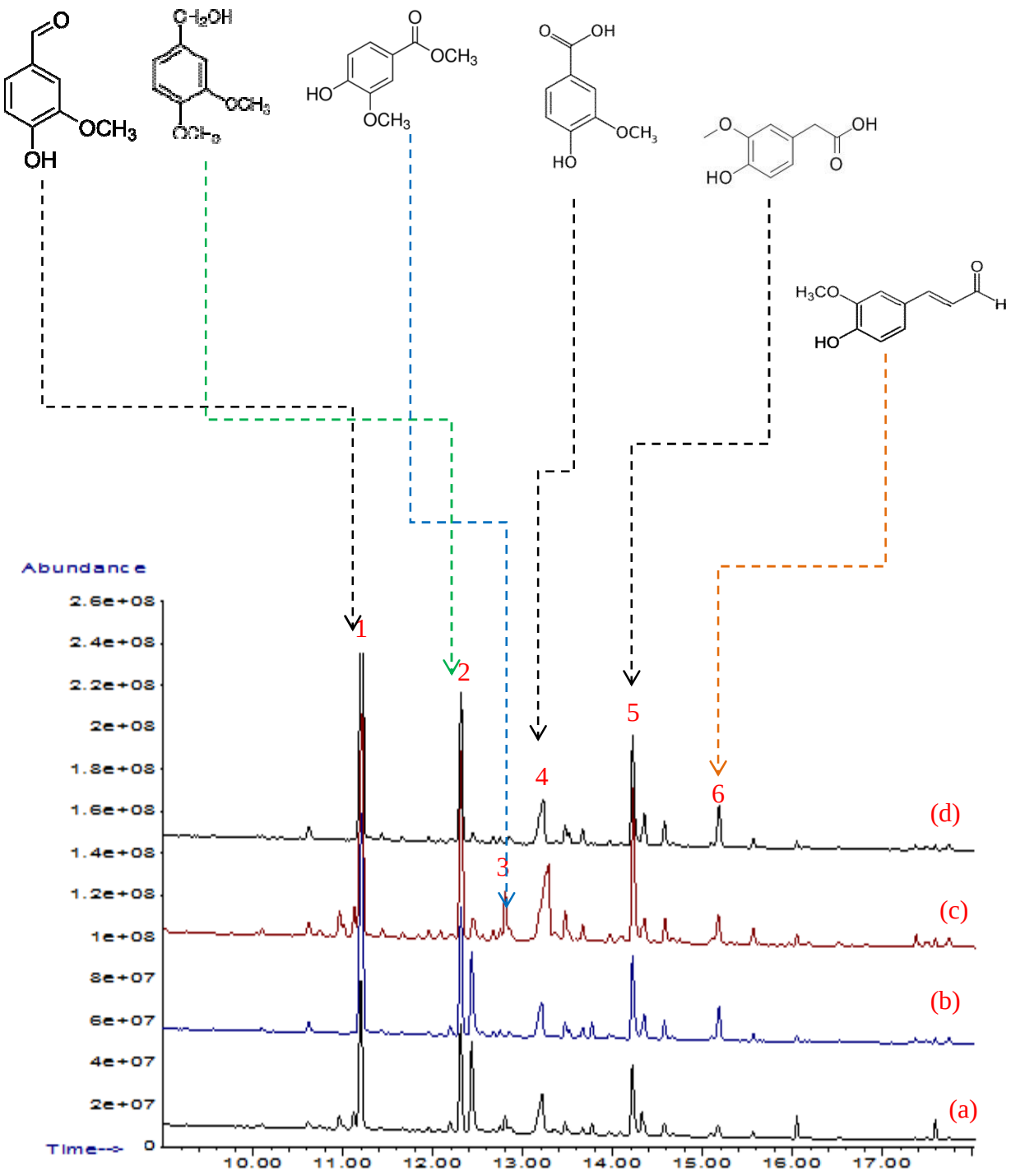


NIST library match: Name: 4-Hydroxy-2-methoxycinnamaldehyde; Formula: $C_{10}H_{10}O_3$; MW: 178; CAS#: 127321-19-1; NIST#: 250265

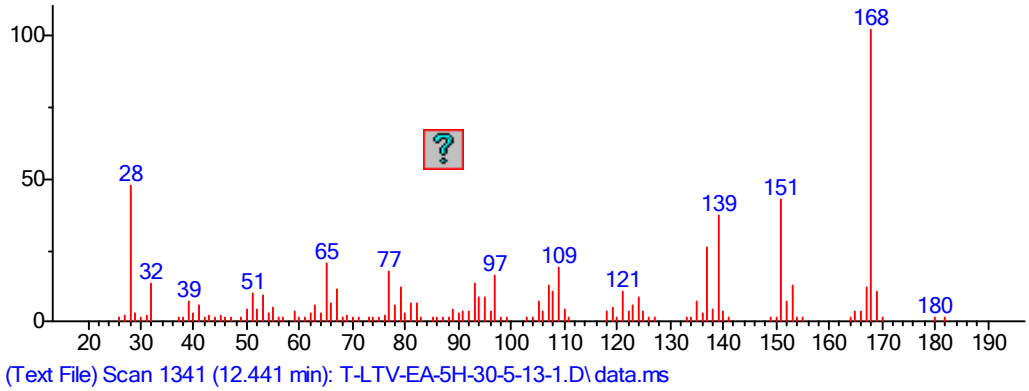


The probability of coniferyl aocohol in NIST library identification was 70 %, but there was no standard chemical available to confirm this preliminary result.

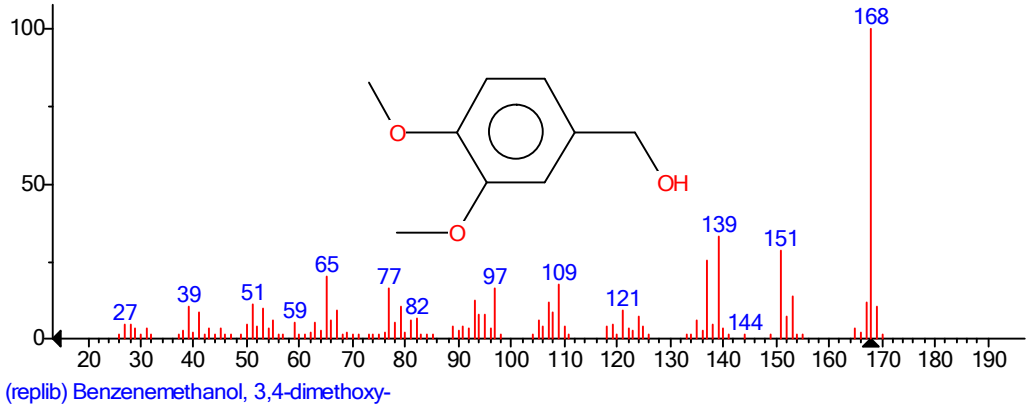
Appendix A11.4: GC-MS identification of Kraft lignin degradation by LiP



The compounds identified from Kraft lignin degradation by LiP were: vanillin (peak 1, RT=11.20 min), veratryl alcohol (peak 2, RT=12.44 min), homovanillyl alcohol (peak 3, RT=12.86 min), vanillic acid (peak 4, RT=13.17 min), homovanillic acid (peak5, RT=14.21 min) and coniferyl aldehyde (peak 6, RT=15.50 min). Except veratryl alcohol, all other compounds were identified in the previous analysis, so the following mass spectra are shown for veratryl alcohol only. This compound was originated from reaction material.

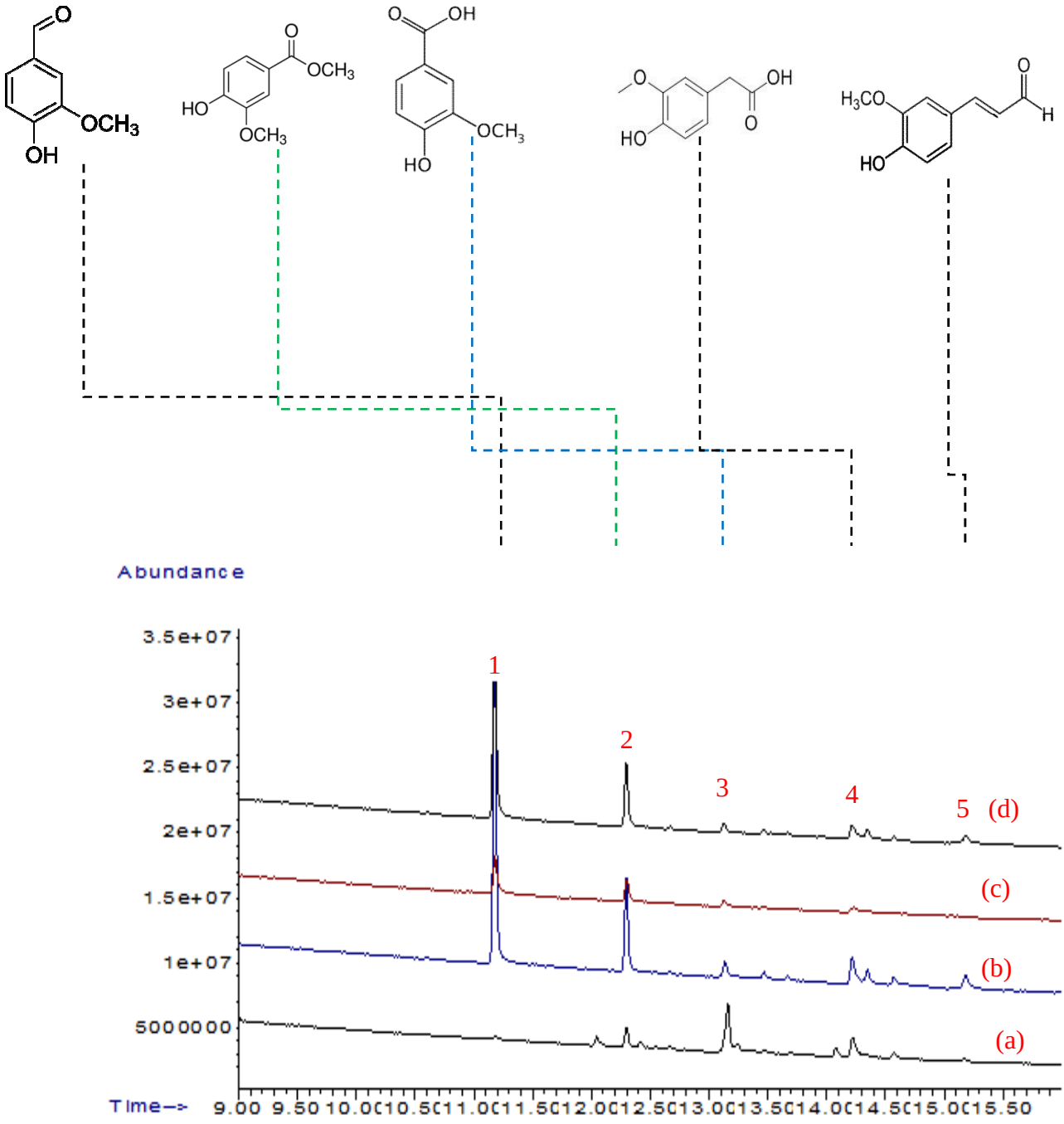


NIST library match: Name: Benzenemethanol, 3,4-dimethoxy-; Formula: $C_9H_{12}O_3$; MW: 168; CAS#: 93-03-8; NIST#: 6495



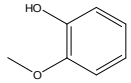
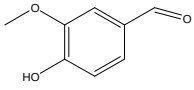
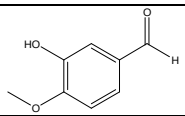
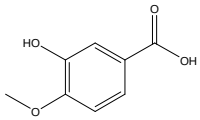
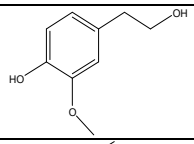
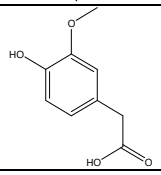
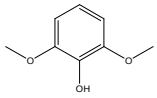
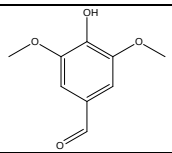
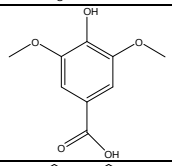
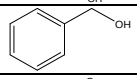
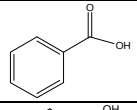
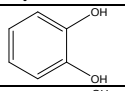
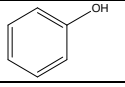
The probability of 3,4-dimethoxy- Benzenemethanol (veratryl alcohol) in NIST library identification was 93 % whereas its standard was 81.2% match in the NIST library. Therefore, this can be considered as a good match, however, its purity is still lower for a perfect match.

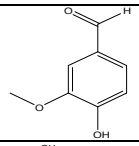
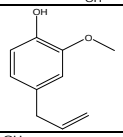
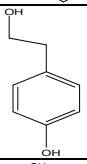
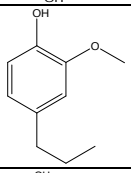
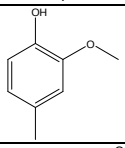
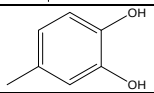
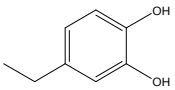
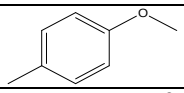
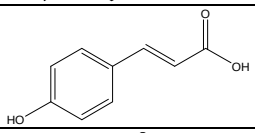
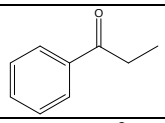
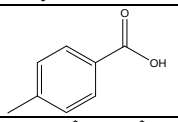
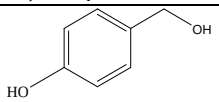
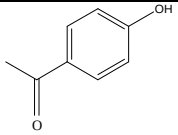
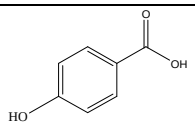
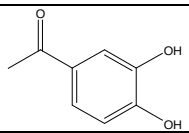
Appendix A11.4: GC-MS identification of Kraft lignin degradation by MnP

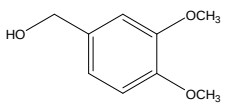
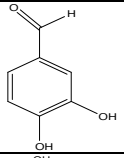
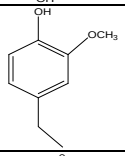
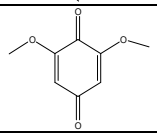
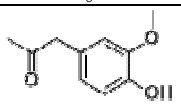
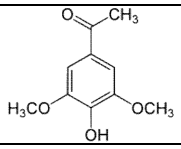
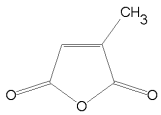
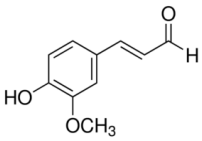
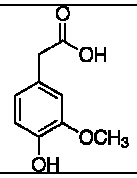
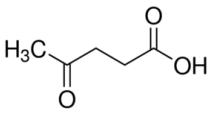
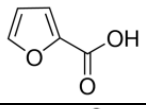
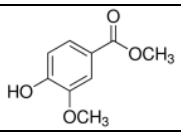


The compounds identified from Kraft lignin degradation by MnP were: vanillin (peak 1, RT=11.20 min), homovanillyl alcohol (peak 2, RT=12.86 min), vanillic acid (peak 3, RT=13.17 min), homovanillic acid (peak4, RT=14.21 min) and coniferyl aldehyde (peak 5, RT=15.50 min). All compounds were identified in the previous analysis, so mass spectra are shown.

Appendix A.12: The authentic standard chemicals to be used for GC-MS identification of the depolymerisation products from various types of lignin (concentration was 5mM for each available standard shown in this list)

Name & Formula	Structure	MW	RT (min)	GC-MS detected possibility (%)
Guaiacol $C_7H_8O_2$		124.14	7	77.5
Vanillin $C_8H_8O_3$		152	11.52	71.8
Isovanillin $C_8H_8O_3$		152.14	12.26	52.3
Isovanillic acid $C_8H_8O_4$		168.15	14.23	63.2
Homovanillyl alcohol $C_9H_{12}O_3$		168	13.00	94.8
Vanillic acid $C_9H_{10}O_4$		182	13.54	54
Syringol $C_8H_{10}O_3$		154.16	10.84	82.3
Syringaldehyde $C_9H_{10}O_4$		182	14.69	76.4
Syringic acid $C_9H_{10}O_5$		198.17	16.34	78
Benzyl alcohol C_7H_8O		108	6.11	55.1
Benzoic acid $C_7H_6O_2$		122.12	8.18	56.6
Pyrocatechol $C_6H_6O_2$		110.11	8.55	81.6
Phenol C_6H_6O		94.11	5.20	73.5

Acetovanillone $C_8H_8O_4$		168	12.65	72.8
Eugenol $C_{10}H_{12}O_2$		164.21	10.95	34.9
4-hydroxy benzoethanol $C_8H_{10}O_2$		138	11.78	55
2-methoxy-4-propylphenol $C_{10}H_{14}O_2$		166.22	11.07	87.6
2-hydroxy-5-methylanisole $C_8H_{10}O_2$		138	8.59	65.2
4-methylcatechol $C_7H_8O_2$		124	9.89	73.2
4-ethylcatechol $C_8H_{10}O_2$		138	11.20	80
4-methylanisole $C_8H_{10}O$		122	5.92	50.3
P-coumaric acid $C_9H_8O_3$		164	15.87	57.2
Propiophenone $C_9H_{10}O$		134	8.87	40.7
P-toluic acid $C_8H_8O_2$		136	9.73	70.3
4-hydroxybenzyl alcohol $C_7H_8O_2$		138.12	10.65	55.1
4-hydroxybenzoic aldehyde $C_7H_6O_2$		122.12	10.90	75.8
4-hydroxybenzoic acid (PHBA) $C_7H_6O_3$		138.12	12.71	66
3,4-dihydroxy-Benzaldehyde $C_7H_6O_3$		138.12	13.68	71.6

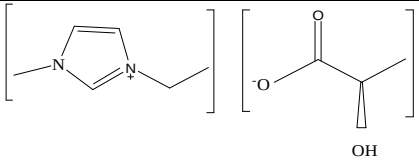
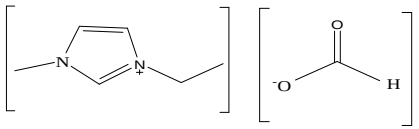
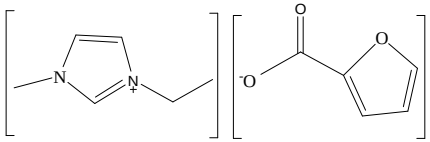
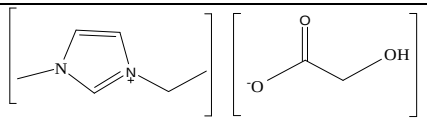
Veratryl alcohol (3,4-dimethoxy- Benzyl alcohol) $C_9H_{12}O_3$		168.19	12.75	81.2
3,4-dihydroxy- Benzoic acid $C_7H_6O_4$		154	15.27	73.5
4-ethyl-2-methoxy- Phenol (4-Ethylguaiacol) $C_9H_{12}O_2$		152.19	9.85	79
2,6-Dimethoxy-1,4-benzoquinone $C_8H_8O_4$		168	13.69	91.2
Guaiacyl acetone $C_{10}H_{12}O_3$		180.20	none	None
Acetosyringone $C_{10}H_{12}O_4$		196.19	none	None
2,5-Furandione, 3-methyl- $C_5H_4O_3$		112.08	none	None
4-Hydroxy-2-methoxycinnamaldehyde or coniferylaldehyde $C_{10}H_{10}O_3$		178.18	none	None
Homovanillic acid $C_9H_{10}O_4$		182.17	none	None
Levulinic acid $C_5H_8O_3$		116.11	none	None
2-Furoic acid $C_5H_4O_3$		112.08	none	None
Methyl vallinate $C_9H_{10}O_4$		182.17	none	None

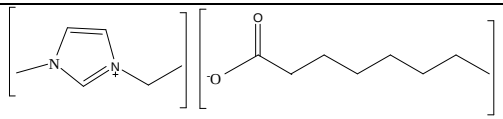
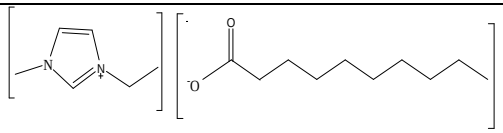
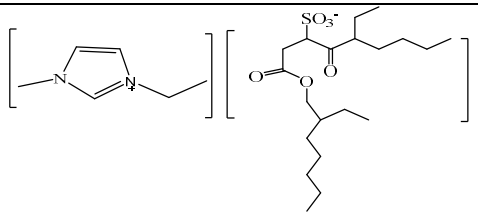
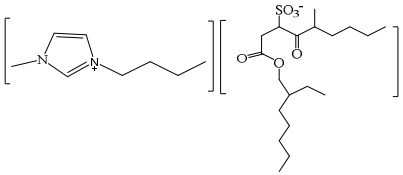
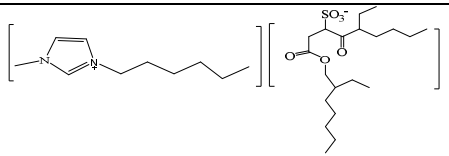
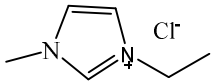
Some of the standard chemicals became available when the author has completed this study. Therefore, the data shown as none were the results at the time of this piece of work has been done.

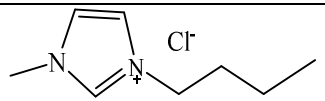
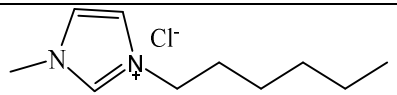
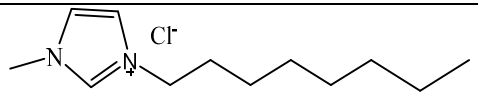
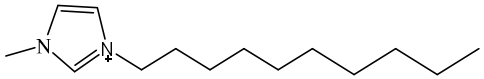
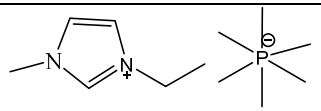
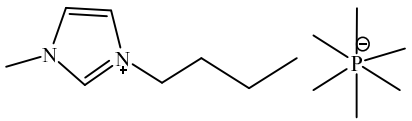
Note: Appendix A.12 indicates that most of the possible lignin depolymerisation products are very close in their molecular weight and other physical and chemical properties. Therefore, these facts could increase the challenges of the downstream processes of breaking down the lignin and the purification of main products and by-products from each other. Also, the NIST library matches are concentration-dependant, so even though the NIST % matches of most of the products were low, however, the products which have their retention times matched with the available authentic standards, could be reliable. Therefore, all these factors should be taken into account to explain the poor matched results of the reaction products with the identification by NIST library, and the standards.

Appendix A13: List of Chemical Structures and water miscibility properties of ILs which were studied in this thesis.

Table1. Activity of LTV in the presence of imidazolium based ionic liquids

Ionic Liquid	**Structure**	**[IL] (%, w/v)**	**V_0[mM.s^{-1}] ×10^{-4}**	**LTV activity = ($V_{o\ (IL)}$/ $V_{o\ (control)}$)× 100%**	**Water miscibility**
Control		0	10.73 ± 0.04	100	
[C_2mim][lactate]		0.6	15.0 ± 0.07	140	miscible
		2	7.92 ± 0.05	74	
		4	4.83 ± 0.08	45	
		10	1.35 ± 0.03	12.5	
[C_2mim][formate]		0.6	2.90 ± 0.04	27	miscible
		2	0	0	
		4	0	0	
		10	0	0	
[C_2mim][furoate]		0.6	13.2 ± 0.05	123	miscible
		2	3.08 ± 0.04	29	
		4	1.91 ± 0.05	18	
		10	0	0	
[C_2mim][glycolate]		0.6	15.2 ± 0.07	142	Miscible
		2	9.23 ± 0.05	86	
		4	5.53 ± 0.06	51.5	

		10	1.83 ± 0.01	17	
[C_2mim][octanoate]		0.6	12.6 ± 0.07	117	Miscible
		2	5.33 ± 0.02	50	
		4	0	0	
		10	0	0	
[C_2mim][decanoate]		0.6	12.4 ± 0.06	116	miscible
		2	4.68 ± 0.02	44	
		4	0	0	
		10	0	0	
[C_2mim][AOT]		0.6	17.6 ± 0.03	164	gelish mixture
		2	11.4 ± 0.04	106	
		4	6.82 ± 0.08	64	
		10	3.55 ± 0.02	33	
[C_4mim][AOT]		0.6	14.0 ± 0.08	130	immiscible,gelish mixture
		2	10.2 ± 0.08	95	
		4	5.85 ± 0.04	54	
		10	1.49 ± 0.05	14	
[C_6mim][AOT]		0.6	14.3 ± 0.08	140	immiscible, emulsion
		2	9.71 ± 0.08	64	
		4	7.31 ± 0.06	63	
		10	3.31 ± 0.06	15	
[C_2mim][Cl]		0.6	1.85 ± 0.01	17	miscible, colorless crystal
		2	0	5	
		4	0	0	

		10	0	0	
[C_4mim][Cl]		0.6	2.24 ± 0.01	21	miscible, colorless crystal
		2	0.47 ± 0.01	4	
		4	0	0	
		10	0	0	
[C_6mim][Cl]		0.6	2.12 ± 0.006	20	miscible, light yellow liquid
		2	0.38 ± 0.01	3.5	
		4	0	0	
		10	0	0	
[C_8mim][Cl]		0.6	1.85 ± 0.01	17	miscible, transparant liquid
		2	0.42 ± 0.01	4	
		4	0	0	
		10	0	0	
[C_{10}mim][Cl]		0.6	0	0	difficult to solve, bubbles appreadlight yellow gel
		2	0	0	
		4	0	0	
		10	0	0	
[C_2mim][PF_6]		0.6	6.42 ± 0.01	60	immiscible, crystal formed
		2	5.76 ± 0.01	54	
		4	4.92 ± 0.02	46	
		10	3.29 ± 0.02	31	
[C_4mim][PF_6]		0.6	6.94 ± 0.02	65	immiscible, yellow liquid
		2	6.32 ± 0.01	53	
		4	5.67 ± 0.05	53	
		10	4.45 ± 0.01	41.5	

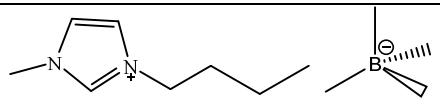
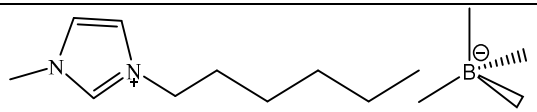
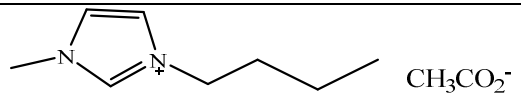
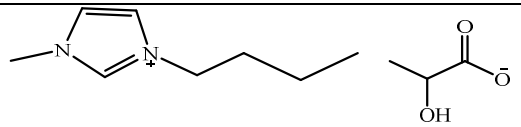
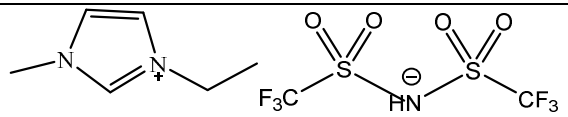
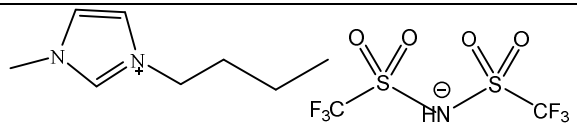
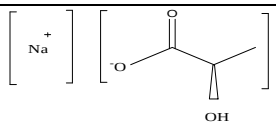
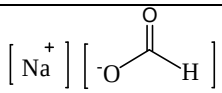
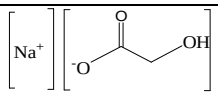
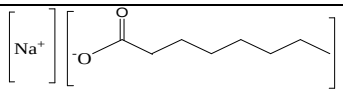
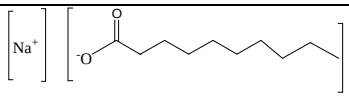
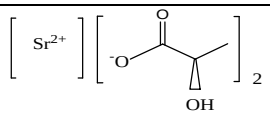
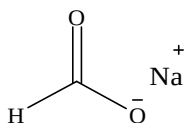
$[C_4mim][BF_4]$		0.6	2.15 ± 0.02	20	miscible, turbid liquid
		2	1.24 ± 0.01	11.5	
		4	0.70 ± 0.01	6.5	
		10	0	0	
$[C_6mim][BF_4]$		0.6	1.66 ± 0.01	15.5	immiscible, light yellow liquid
		2	0.77 ± 0.01	11.5	
		4	0	0	
		10	0	0	
$[C_4mim][OAc]$		0.6	2.98 ± 0.01	28	miscible, light colourless liquid
		2	1.75 ± 0.01	16	
		4	0.14 ± 0.01	1.3	
		10	0	0	
$[C_4mim][lactate]$		0.6	3.67 ± 0.02	34	miscible, gold yellow liquid
		2	2.25 ± 0.02	21	
		4	1.29 ± 0.01	12	
		10	0	0	
$[C_2mim][NTf_2]$		0.6	4.12 ± 0.03	38	immiscible, light yellow liquid
		2	3.29 ± 0.01	31	
		4	2.85 ± 0.01	26.5	
		10	2.34 ± 0.02	23.5	
$[C_4mim][NTf_2]$		0.6	4.07 ± 0.01	38	immiscible, light yellow liquid
		2	3.19 ± 0.01	30	
		4	3.08 ± 0.01	29	
		10	3.02 ± 0.01	28	
St.Dev: less than 0.08					

Table2. LTV activity on the presence of equivalent sodium salt

Equivalent salt	Structure	[IL] (%, w/v)	U_0[mM.s^{-1}] ×10^{-4}	LTV activity = ($U_{0\,(IL)}$/ $U_{0\,(control)}$)× 100%	Water miscibility
Control		0	10.73 ± 0.04	100	All are miscible and forms transparent solution except sodium chloride
[Na][lactate]		0.6	15.6 ± 0.06	145	
		2	7.36 ± 0.04	68.5	
		4	5.67 ± 0.03	53	
		10	1.61 ± 0.01	15	
[Na][formate]		0.6	1.01 ± 0.01	9.4	
		2	0	0	
		4	0	0	
		10	0	0	
[Na][glycolate]		0.6	15.8± 0.10	147	
		2	7.64 ± 0.09	71	
		4	7.15 ± 0.05	67	
		10	1.79 ± 0.02	17	
[Na][octanoate]		0.6	12.9 ± 0.06	120	
		2	2.25 ± 0.02	21	
		4	0	0	
		10	0	0	
[Na][decanoate]		0.6	11.9 ± 0.10	111	
		2	0	0	
		4	0	0	
		10	0	0	
[Sr^{2+}][lactate]		0.6	19.4 ± 0.04	181	
		2	9.73 ± 0.09	91	
		4	6.00 ± 0.06	56	
		10	0	0	
NaCl	Na^+Cl^-	0.6	0.54 ± 0.01	5	
		2	0	0	
		4	0	0	
		10	0	0	
NaOAc		0.6	6.01 ± 0.02	56	
		2	4.60 ± 0.01	43	
		4	2.41 ± 0.06	22.5	
		10	0.34 ± 0.01	3	

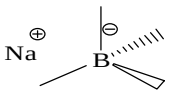
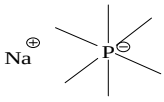
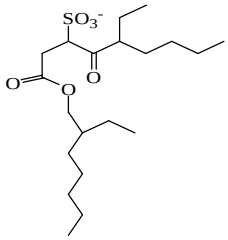
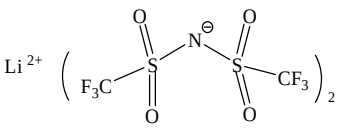
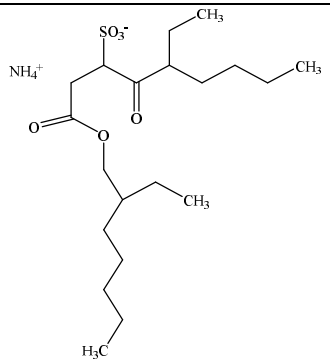
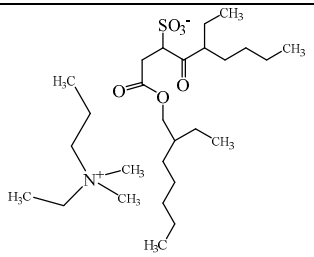
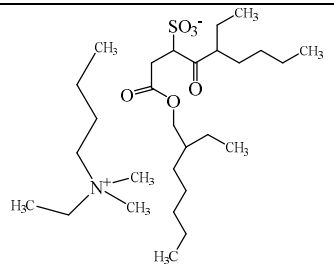
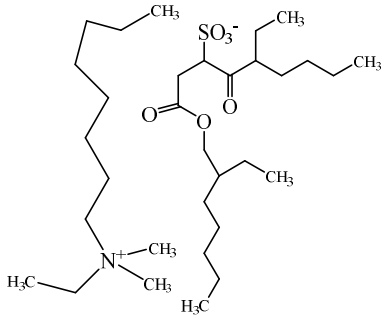
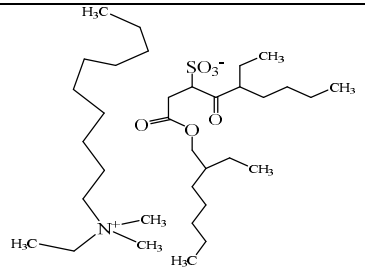
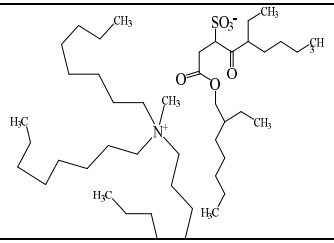
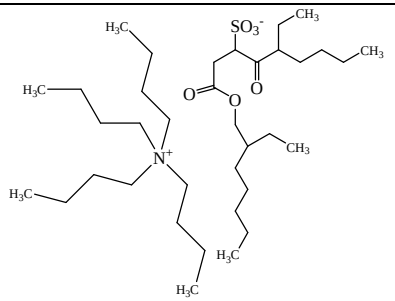
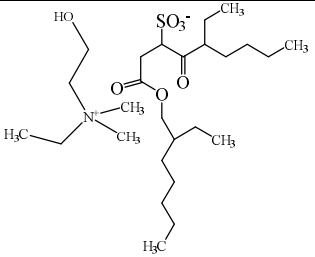
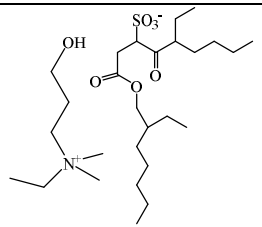
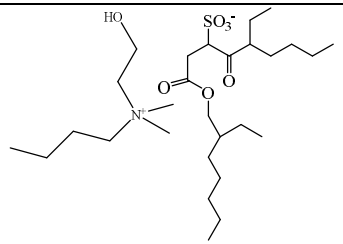
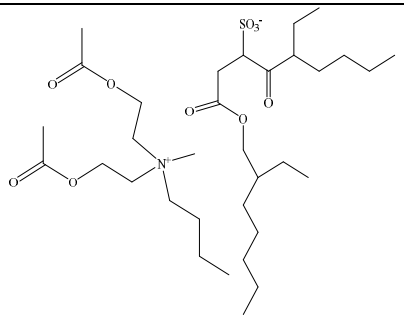
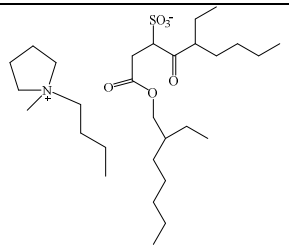
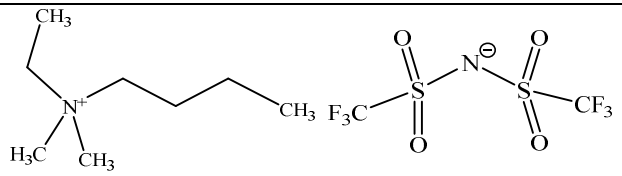
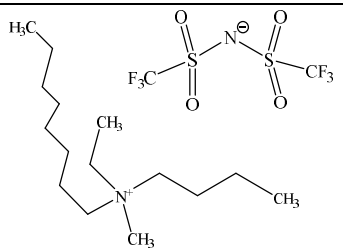
$NaBF_4$		0.6	1.88 ± 0.01	33	
		2	0.93 ± 0.02	16	
		4	0.61 ± 0.01	11	
		10	0.26 ± 0.01	4.5	
$NaPF_6$		0.6	1.03 ± 0.01	10	
		2	0.38 ± 0.01	4	
		4	0.22 ± 0.02	2	
		10	0	0	
AOT		0.6	8.21 ± 0.03	76.5	
		2	7.94 ± 0.01	74	
		4	7.62 ± 0.01	71	
		10	3.24 ± 0.01	30	
$LiNTf_2$		0.6	7.99 ± 0.02	74.5	
		2	5.89 ± 0.01	55	
		4	3.65 ± 0.01	34	
		10	3.52 ± 0.01	33	
St.Dev: less than 0.1					

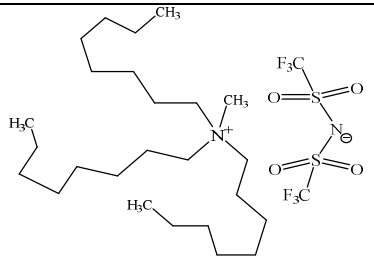
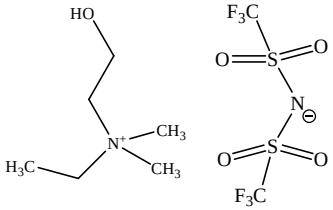
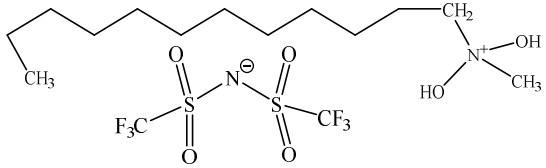
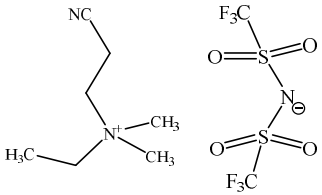
Table3. Activity of LTV in the presence of quaternary ammonium based ionic liquids

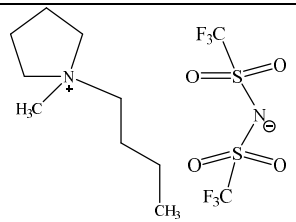
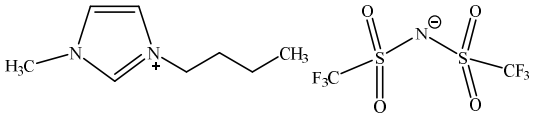
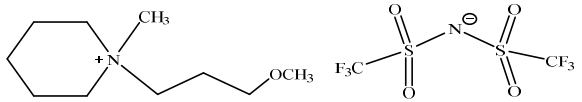
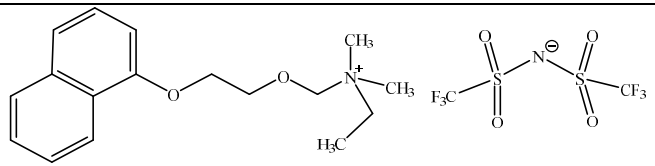
Ionic Liquid	**Structure**	**[IL] (%, w/v)**	**V_o[mM.s^{-1}] ×10^{-4}**	**LTV activity = ($V_{o\ (IL)}$/ $V_{o\ (control)}$) × 100%**	**Water miscibility**
Control		0	10.73 ± 0.04	100	
[NH_4][AOT]		0.6	10.3 ± 0.05	96	immiscible, gelish mixture
		2	10.3 ± 0.05	96	
		4	9.77 ± 0.04	73	
		10	4.38 ± 0.03	41	
[$N_{1,1,2,3}$][AOT]		0.6	10.6 ± 0.04	99	immiscible, emulsion
		2	7.84 ± 0.01	73	
		4	7.77 ± 0.07	72	
		10	3.37 ± 0.06	31	
[$N_{1,1,2,4}$][AOT]		0.6	11.9 ± 0.07	111	immiscible,

		2	10.5 ± 0.08	98	gelish mixture
		4	6.71 ± 0.08	62.5	
		10	2.54 ± 0.15	24	
$[N_{1,1,4,8}][AOT]$		0.6	14.5 ± 0.05	135	immiscible, emulsion
		2	13.2 ± 0.05	123	
		4	6.12 ± 0.09	57	
		10	2.16 ± 0.08	20	
$[N_{1,1,4,10}][AOT]$		0.6	12.2 ± 0.02	114	immiscible, highly viscous, emulsion
		2	10.9 ± 0.05	101.5	
		4	5.65 ± 0.02	53	
		10	2.97 ± 0.06	28	
$[N_{1,8,8,8}][AOT]$		0.6	12.5 ± 0.06	116.5	immiscible, emulsion
		2	9.12 ± 0.03	85	
		4	8.46 ± 0.03	79	

		10	4.64 ± 0.08	43	
$[N_{4,4,4,4}][AOT]$		0.6	7.87 ± 0.05	73	immiscible, emulsion
		2	7.46 ± 0.02	69.5	
		4	6.51 ± 0.03	61	
		10	6.71 ± 0.01	62.5	
$[N_{1,1,2,2OH}][AOT]$		0.6	15.7 ± 0.09	146	immiscible, emulsion
		2	10.6 ± 0.02	99	
		4	7.40 ± 0.05	69	
		10	4.41 ± 0.01	41	
$[N_{1,1,2,3OH}][AOT]$		0.6	14.2 ± 0.02	132	immiscible, highly viscous and gelish mixture
		2	9.29 ± 0.04	87	
		4	8.62 ± 0.03	80	
		10	4.71 ± 0.01	44	
$[N_{1,1,4,2OH}][AOT]$		0.6	10.3 ± 0.07	96	immiscible, viscous mixture
		2	9.16 ± 0.03	85	
		4	8.98 ± 0.04	84	
		10	4.42 ± 0.01	41	

$[N_{1,4\ (C3oAc)2}][AOT]$		0.6	12.1 ± 0.07	113	immiscible,highly viscous, emulsion
		2	11.8 ± 0.05	110	
		4	4.37 ± 0.04	41	
		10	3.22 ± 0.02	30	
$[N_{1,4,Pyrr}][AOT]$		0.6	5.63 ± 0.14	52.5	immiscible, forms soft gel
		2	4.56 ± 0.07	43	
		4	3.51 ± 0.07	33	
		10	2.22 ± 0.05	21	
$[N_{1,1,2,4}][NTf_2]$		0.6	6.04 ± 0.10	56	immiscible
		2	5.55 ± 0.02	52	
		4	4.78 ± 0.02	44.5	
		10	3.37 ± 0.03	31	
$[N_{1,1,4,8}][NTf_2]$		0.6	5.47 ± 0.07	51	immiscible, forms emulsion
		2	3.88 ± 0.06	36	
		4	3.84 ± 0.12	36	
		10	1.81 ± 0.02	17	

$[N_{1,8,8,8}][NTf_2]$		0.6	3.02 ± 0.11	28	immiscible
		2	0	0	
		4	0	0	
		10	0	0	
$[N_{1,1,2,2OH}][NTf_2]$		0.6	5.37 ± 0.08	50	immiscible
		2	3.98 ± 0.02	37	
		4	3.64 ± 0.04	34	
		10	2.74 ± 0.02	25.5	
$[N_{1,12,(OH)2}][NTf_2]$		0.6	0.98 ± 0.01	9	immiscible
		2	0	0	
		4	0	0	
		10	0	0	
$[N_{1,1,2,2CN}][NTf_2]$		0.6	6.21 ± 0.12	58	immiscible
		2	3.87 ± 0.06	36	
		4	3.8 ± 0.08	35	
		10	3.03 ± 0.04	28	

$[N_{1,4,Pyrr}][NTf_2]$		0.6	5.71 ± 0.07	53	immiscible
		2	4.91 ± 0.06	46	
		4	3.68 ± 0.02	34	
		10	3.06 ± 0.01	28.5	
$[C_4mim][NTf_2]$		0.6	6.61 ± 0.04	62	immiscible, forms emulsion
		2	3.88 ± 0.03	36	
		4	3.51 ± 0.01	33	
		10	2.60 ± 0.006	24	
$[N_{1,4,C3OH,Py}]$ $][NTf_2]$		0.6	5.64 ± 0.09	52.5	Immiscible
		2	4.98 ± 0.03	46	
		4	4.10 ± 0.04	38	
		10	2.30 ± 0.13	21	
$[N_{1,1,1,C1OC2OC10H8}]$ $[NTf_2]$		0.6	3.41 ± 0.03	32	immiscible
		2	2.57 ± 0.08	24	
		4	1.20 ± 0.01	11	
		10	0	0	

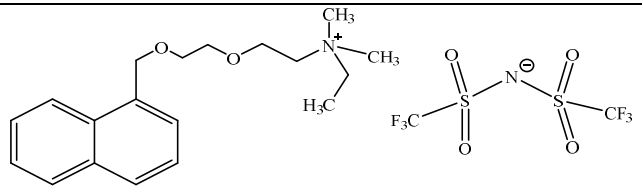
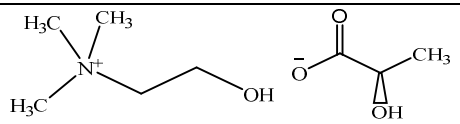
$[N_{1,1,1,C2OC2OC10H8}]$ $[NTf_2]$		0.6	6.06 ± 0.04	56.5	Immiscible
		2	4.29 ± 0.07	40	
		4	3.92 ± 0.08	36.5	
		10	2.82 ± 0.03	26	
$[N_{1,1,1,2OH}]$[lactate]		0.6	15.1 ± 0.06	141	miscible
		2	6.90 ± 0.04	64	
		4	6.74 ± 0.03	63	
		10	1.61 ± 0.01	15	

Table4. Activity of LTV in the presence of pyridinium and pyrrolidinium based ionic liquids

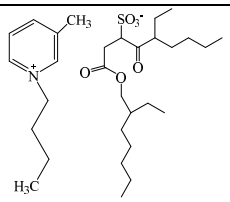
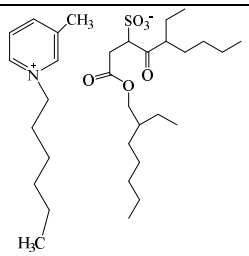
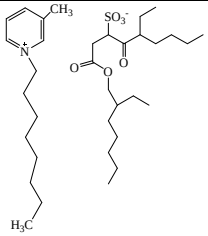
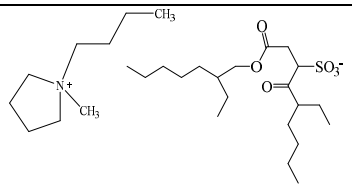
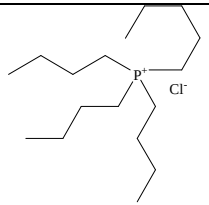
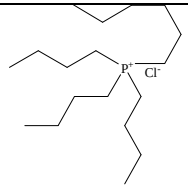
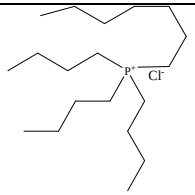
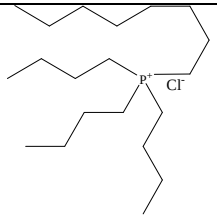
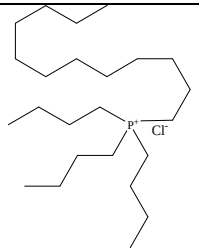
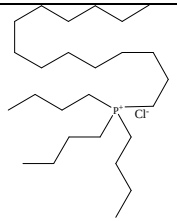
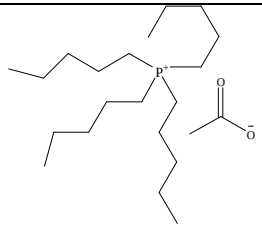
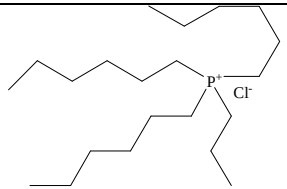
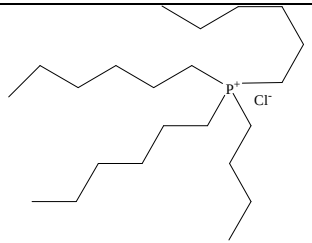
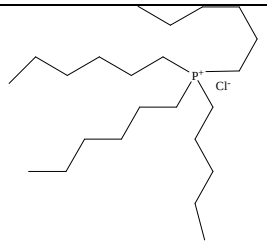
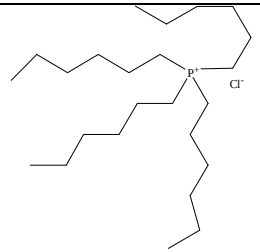
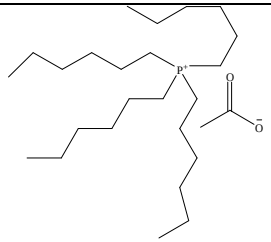
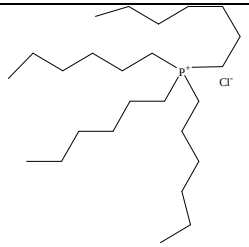
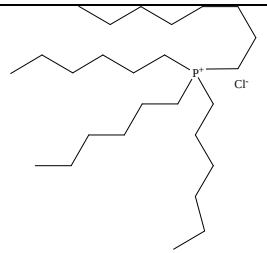
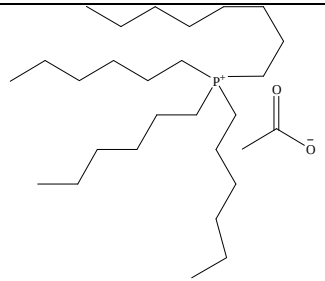
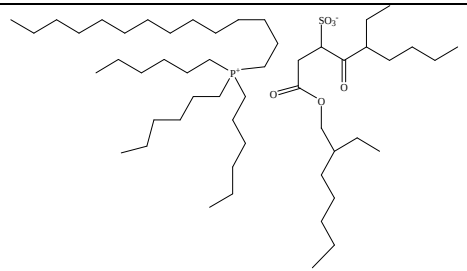
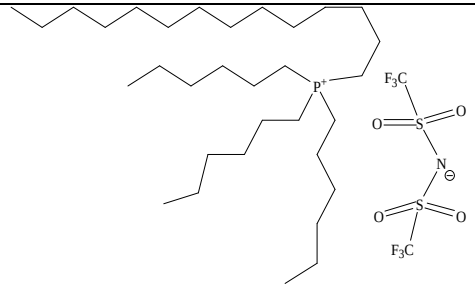
Ionic Liquid	Structure of ILs	[IL] (%, w/v)	V_0[mM.s^{-1}] ×10^{-4}	LTV activity = ($V_{0\,(IL)}$/ $V_{0\,(control)}$)× 100%	Water miscibility
Control		0	10.73 ± 0.04	100	
[C_4mpy]][AOT]		0.6	12.3 ± 0.06	115	viscous, miscible
		2	11.8 ± 0.04	110	
		4	5.54 ± 0.01	52	
		10	1.64 ± 0.03	15	
[C_6mpy]][AOT]		0.6	12.9 ± 0.09	120	viscous, immiscible
		2	11.8 ± 0.03	110	
		4	6.77 ± 0.01	63	
		10	2.61 ± 0.09	24	
[C_8mpy]][AOT]		0.6	15.0 ± 0.05	140	immiscible ,highly viscous, gelish mixture
		2	10.8 ± 0.06	100	
		4	9.21 ± 0.05	86	
		10	4.57 ± 0.02	42.5	
[C_4mpyr][AOT]		0.6	13.7 ± 0.06	128	immiscible ,highly viscous, gelish mixture
		2	12.8 ± 0.04	119	
		4	7.64 ± 0.03	71	
		10	2.90 ± 0.01	27	
St.Dev: less than 0.1					

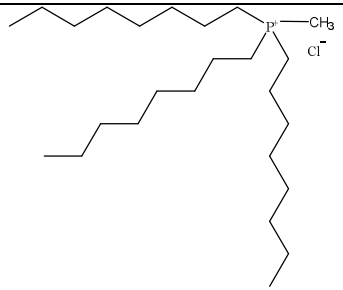
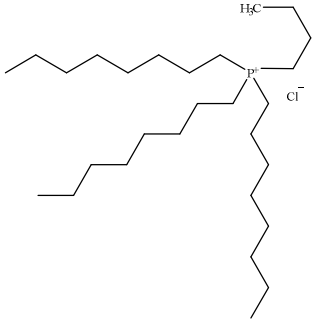
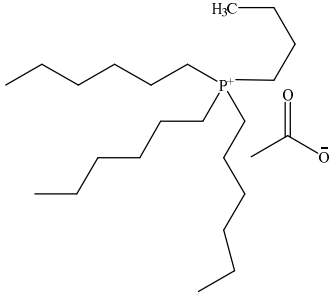
Table5. Activity of LTV in the presence of phosphonium based ionic liquids

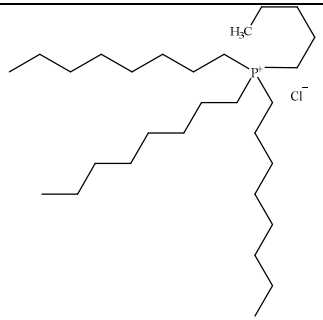
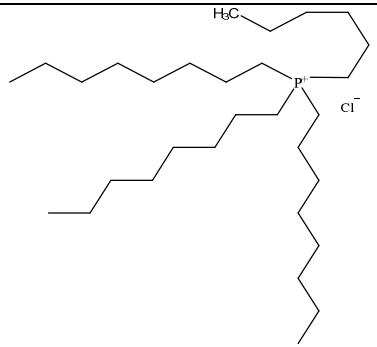
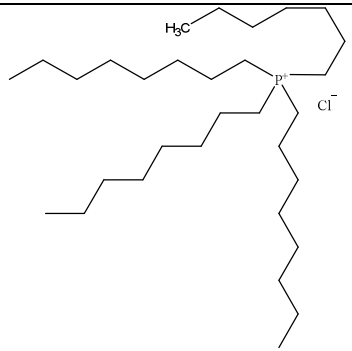
Ionic Liquid		Structure of ionic liquids	[IL] (%, w/v)	V_0[mM.s^{-1}] ×10 $^{-4}$	LTV activity = ($V_{0\ (IL)}$/ $V_{0\ (control)}$)× 100%	Water miscibility
Control			0	10.73 ± 0.04	100	
P_4 group	$[P_{4,4,4,5}][C1]$		0.6	3.09 ± 0.02	29	miscible
	$[P_{4,4,4,6}][C1]$		0.6	2.14 ± 0.01	20	miscible
	$[P_{4,4,4,7}][C1]$		0.6	0	0.00	miscible
	$[P_{4,4,4,8}][C1]$		0.6	0	0.00	miscible

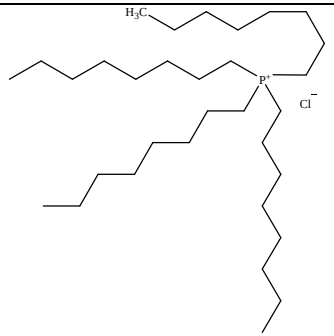
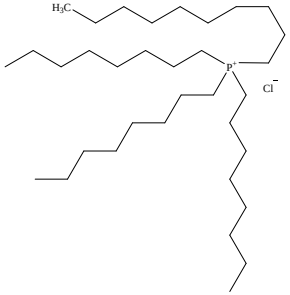
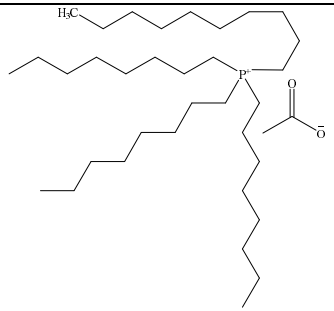
	$[P_{4,4,4,12}][C1]$		0.6	0	0.00	miscible
	$[P_{4,4,4,14}][C1]$		0.6	0	0.00	miscible
P_5 group	$[P_{5,5,5,5}][OAc]$		0.6	0	0.00	miscible
P_6 group	$[P_{6,6,6,3}][C1]$		0.6	0	0.00	miscible
	$[P_{6,6,6,4}][C1]$		0.6	0	0.00	immiscible

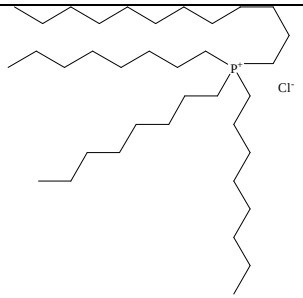
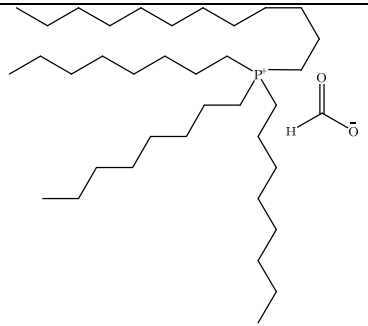
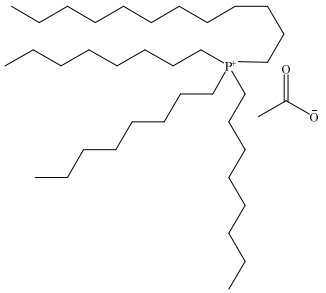
	$[P_{6,6,6,5}][C1]$		0.6	0	0.00	immiscible
	$[P_{6,6,6,6}][C1]$		0.6	0	0.00	immiscible
	$[P_{6,6,6,6}][HCO_2]$		0.6	0	0.00	immiscible
	$[P_{6,6,6,7}][C1]$		0.6	0	0.00	immiscible

	$[P_{6,6,6,8}][C1]$		0.6	0	0.00	immiscible
	$[P_{6,6,6,8}][HCO_2]$		0.6	0	0.00	immiscible
	$[P_{6,6,6,14}][AOT]$		0.6	10.6 ± 0.06	99	immiscible
	$[P_{6,6,6,14}]$ $[NTf_2]$		0.6	5.10 ± 0.04	47.5	immiscible
			2	4.62 ± 0.03	43	
			4	3.70 ± 0.04	34.5	
			10	2.86 ± 0.03	27	
			0.6	0.54 ± 0.004	5	

P_8 group	$[P_{8,8,8,1}][C1]$		0.6	8.48 ± 0.02	79	immiscible
	$[P_{8,8,8,4}][C1]$		0.6	8.22 ± 0.05	77	immiscible
	$[P_{8,8,8,4}][OAc]$		0.6	7.56 ± 0.04	70.5	miscible

	$[P_{8,8,8,5}][C1]$		0.6	7.04 ± 0.09	66	immiscible
	$[P_{8,8,8,6}][C1]$		0.6	4.26 ± 0.02	40	immiscible
	$[P_{8,8,8,7}][C1]$		0.6	4.78 ± 0.03	44.5	miscible

	$[P_{8,8,8,8}][C1]$		0.6	5.83 ± 0.09	54	immiscible
	$[P_{8,8,8,10}][C1]$		0.6	5.54 ± 0.09	52	immiscible
	$[P_{8,8,8,10}][OAc]$		0.6	8.96 ± 0.07	83.5	immiscible

	$[P_{8,8,8,12}][C1]$		0.6	6.72 ± 0.06	63	immiscible
	$[P_{8,8,8,12}][HCO_2]$		0.6	7.72 ± 0.04	72	immiscible
	$[P_{8,8,8,12}][OAc]$		0.6	8.21 ± 0.07	76.5	immiscible

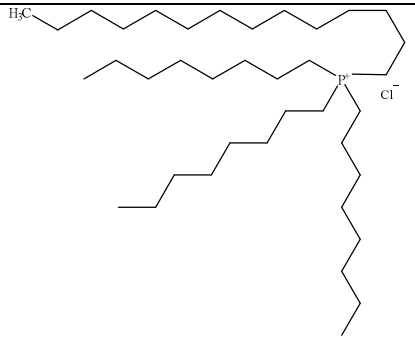
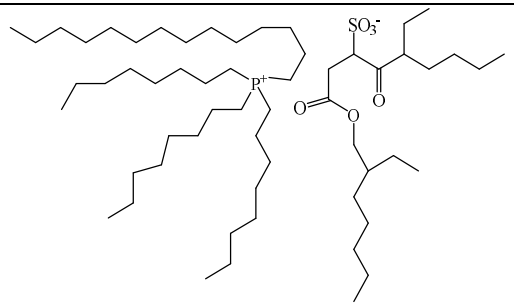
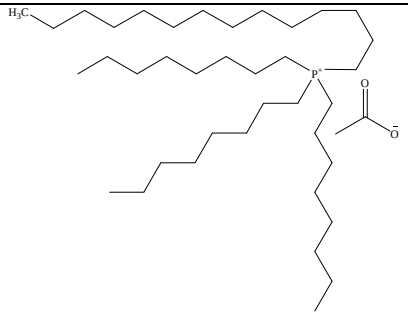
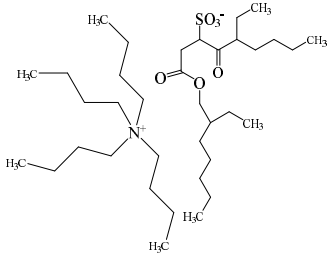
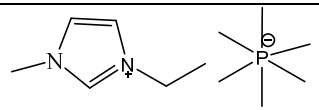
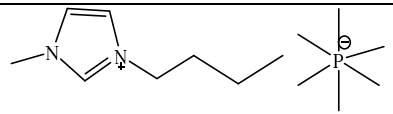
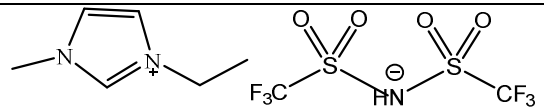
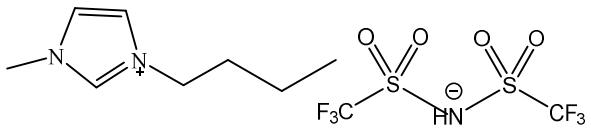
	$[P_{8,8,8,14}][C1]$		0.6	5.93 ± 0.03	55	immiscible
	$[P_{8,8,8,14}][AOT]$		0.6	0	0.00	immiscible
	$[P_{8,8,8,14}][OAc]$		0.6	7.92 ± 0.04	74	immiscible
St.Dev: less than 0.1						

Table6. Activity of LTV in the presence higher concentrated LTV compatible ionic liquids

Ionic Liquid	Structure	[IL] (%, w/v)	V_o[mM.s^{-1}] ×10^{-4}	LTV activity = ($V_{o\ (IL)}$/ $V_{o\ (control)}$)× 100%	Water miscibility
Control		0	10.73 ± 0.04	100	
$[N_{4,4,4,4}][AOT]$		20	5.12 ± 0.07	48	immiscible, but mixed with water as emulsion
		40	4.38 ± 0.07	41	
		60	4.35 ± 0.03	40.5	
		80	1.88 ± 0.09	17.5	
		100	0	0	
$[C_2mim][PF_6]$		20	1.62 ± 0.01	15	immiscible, clearly separate from water as solid particle
		40	0.83 ± 0.03	8	
		60	0.51 ± 0.01	5	
		80	0.24 ± 0.01	2	
		100	0	0	
$[C_4mim][PF_6]$		20	1.03 ± 0.02	10	immiscible, clearly separate from water as light yellow liquid
		40	0.91 ± 0.02	8.5	
		60	0	0	
		80	0	0	
		100	0	0	
$[C_2mim][NTf_2]$		20	1.27 ± 0.03	12	immiscible, clearly separate from water as light yellow liquid
		40	0.70 ± 0.01	6.52	
		60	0.08 ± 0.01	0.74	
		80	0	0	
		100	0	0	

$[C_4mim][NTf_2]$		20	0.07 ± 0.002	0.65	immiscible, clearly separate from water as light yellow liquid
		40	0.07 ± 0.002	0.65	
		60	0.04 ± 0.002	0.37	
		80	0.04 ± 0.002	0.37	
		100	0	0	
St. Dev was smaller than 0.1					