Role of Laccase as an Enzymatic Pretreatment Method to Improve Lignocellulosic Saccharification

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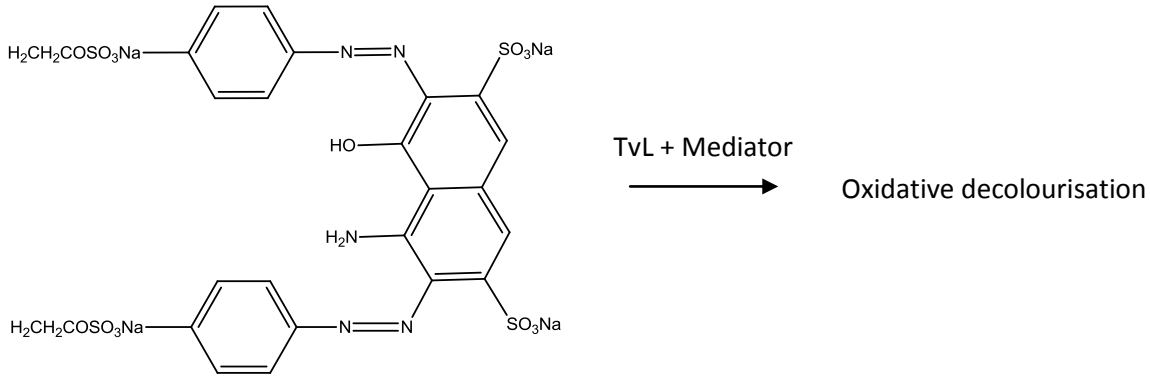
Supplementary Information

Contents

1. Experimental

1.1. Phenolic and synthetic mediator screening using TvL and Reactive Black-5 (RB-5)

Reactions were set up in 96-well plates. 50μM RB-5 was incubated with 100mU *Trametes versicolor* laccase (TvL) in the presence of a panel of mediators at dye:mediator concentrations of 1:1, 1:5 and 1:10. Reactions were covered using aero-seals and were incubated at 25°C and 150rpm. At given timepoints the reactions were analysed by measuring the absorbance at 598nm. No laccase-no mediator (NLNM), and laccase without mediator (TvL NM) were included as negative controls and % decolourisation was calculated based on both controls to produce two separate sets decolourisation data.



1.2. Oxidation of veratryl alcohol (1) by TvL and LMS

Oxidation reactions were performed using 3mM (**1**) in 0.1M sodium acetate buffer pH 5.0. Commercial TvL was used at either high (1.6U/ml) or low (0.4U/ml) concentrations. Synthetic mediators and dyes/indicators were investigated at both 3:1 and 1:1 (substrate: mediator) ratios.

Phenolic mediators were investigated using their optimal concentration established from the RB-5 screen (1:1, 1:5 or 1:10 dye:mediator). Reactions were incubated at 25°C at 200rpm for 24h. Reactions were acidified to pH 2.0 with 1M HCl and extracted twice using 2x volumes of ethyl acetate. Ethyl acetate was evaporated using the Genevac EZ and residues were resuspended in 75% methanol. Veratryl aldehyde (**2**) production was monitored by reversed phase HPLC on an Agilent 1100 series HPLC + LC/MSD SL ESI mass spectrometer. Separation was achieved using an isocratic mobile phase of 60% methanol in super pure water both with 0.1% formic acid at a flow rate of 0.2ml/min for 20 min using a C:18(2) 250 x 2.00mm 5μ column. MS was performed in positive mode using a mass range 100-1500MW (MS spray chamber conditions: Drying gas flow 12.0lmin^{-1}, nebuliser pressure 50psig, drying gas temperature 350°C and capillary voltage 5000V). Authentic standards of (**1**) & (**2**) were used for the identification and quantification of substrate and product. Conversion was calculated based on the difference between the peak areas of the substrate and the product.

1.3. Acid-pretreated wheat straw washing

Wheat straw (WS) slurry was received from NREL, (National Renewable Energy Laboratory, USA) having previously undergone acid pretreatment (dilute sulphuric acid used at 17mg/g dry weight biomass, 158°C, 5min residence time and at 30% total solids concentration). Slurries were washed three times with distilled H_2O at a biomass to water ratio 1:100 to remove products from acid pretreatment such as pentose sugars and their degradation products. Solids were filtered through a buchner funnel under a vacuum pump and collected on filter paper. Dry weight estimation was determined by drying biomass in an oven at 80°C for 24h.

1.4. Laccase addition to acid-pretreated WS: Preliminary and optimised studies

For successive laccase and cellulase treatments, acid-pretreated WS (0.6g d.w WS) both washed and unwashed was treated with 0.8-100U/g (d.w WS) TvL with 0.05M sodium citrate buffer pH5.0 at 5% consistency at incubated at 28°C and 200rpm. Aliquots were taken over 22h and assayed for phenol content. Laccase was denatured by heating samples to 90°C for 15min before samples were hydrolysed in the saccharification assay.

Laccase and mediator optimisation studies were carried out on washed WS using the above treatment conditions except 0.1M sodium acetate pH4.0 was used instead of citrate buffer at pH5.0 because pH4.0 is optimal for laccase activity and pH5.0 is optimal for hydrolysis. 3X wash steps after the laccase/LMS treatment with dH_2O was included to remove the enzyme and associated soluble products to create a step-wise laccase and cellulase treatment. Different concentrations of TvL (50-150U/g and 500-4000U/g d.w WS) and 1-HBT (2.5, 5 and 7.5% w/w WS) were investigated for reaction optimisation.

Optimised laccase and laccase-mediator sytem (LMS) treated WS experiments were set up to typically contain 0.6-1g (d.w WS) at 5% consistency (the d.w was consistent for each individual experiment), 0.1M sodium acetate buffer pH 4.0, 28°C, 200rpm for 40h. An optimal TvL concentration of 150U/g TvL (g/d.w WS) and 5% mediator (w/w WS) was used in all experiments unless otherwise stated. All experimental conditions ran in triplicate and data expressed as an average. Additional studies with corn and sorghum stover were preformed as described under optimised conditions with TvL and were subjected to the APE step as described in 1.6

1.5. Folin-ciocalteu assay for total phenol estimation

Total phenol content was determined by modification of the Folin-Ciocalteau method [1]. 20μl sample was mixed with 80μl water and 50μl Folin's reagent. The reaction was incubated for 3min at room temperature. 250μl 20% sodium carbonate was added and the reactions were incubated for 30min in the dark. Absorbance was measured at 725nm. Total phenol was expressed as g/L based on catechol standards.

1.6. Alkaline-peroxide extractions

An alkaline-peroxide extraction procedure was developed based on previous work [2]. After enzymatic treatments, samples were subjected to an alkaline extraction step reinforced with peroxide using 1% NaOH and 3% H_2O_2 (both w/w d.w WS) at 80°C for 1h. Samples were centrifuged to remove the liquid fractions and the extraction step was repeated 2x. After the last extraction, 3x water washing steps were applied to remove lignin soluble products, H_2O_2 and to neutralise the biomass before saccharification.

1.7. Saccharification experiments

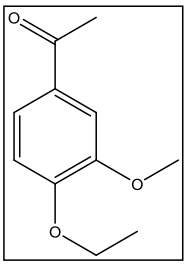
Laccase treated samples were hydrolysed using cellulase GC-220 (69FPU/ml) (Genencor) containing a mix of the three hydrolytic enzymes for cellulose hydrolysis (endo-β-1,4-glucanase, exo-glucanase and cellobiohydrolase). Reactions were run at 5% consistency with 0.5M sodium citrate buffer pH5.0 at 50°C and 200rpm. GC-220 was added in the activity range of 1.4-3.5FPU depending on the dry mass of starting material in each experiment. Reactions were monitored daily up to 3d by sampling the liquid fraction and stopping the reaction by diluting 1:10 10mM H_2SO_4 for immediate analysis or freezing for later analysis. All experimental conditions ran in triplicate and data expressed as an average.

1.8. Glucose quantification with HPLC-RID

Hydrolysis samples quenched in H_2SO_4 were filtered through a 0.2μM membrane (Millipore) before analysis on an Agilent 1200 series HPLC with RID detection using an Aminex HPX 87H column at 55°C. Each run was 15 min with 5mM H_2SO_4 mobile phase and a 0.6ml/min flow rate. D-glucose standards were prepared in the range 0-12g/L and a calibration curve was produced to quantify glucose from hydrolysis samples.

*1.9. Synthesis of 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (dimer **5**)*

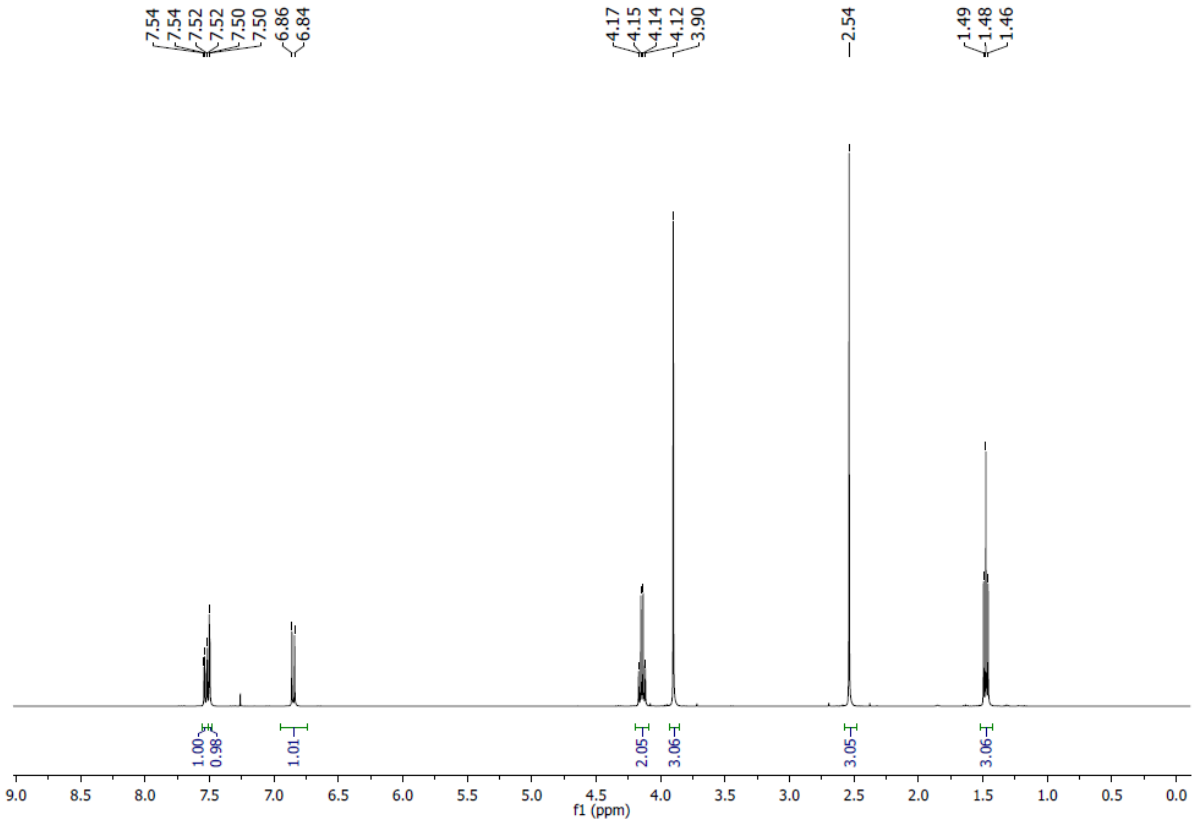
1-(4'-ethoxy-3'-methoxyphenyl)ethan-1-one (**S1**)



To a solution of acetovanillone (3.0 g, 18.1 mmol) in DMF (60 cm^{-3}) was added K_2CO_3 (3.0 g, 21.7 mmol). After 20 mins at room temperature, ethyl iodide (1.75 cm^{-3}, 21.7 mmol) was added and the resulting mixture was heated at 50°C for 20 h. After cooling to room temperature, diethyl ether (250 cm^{-3}) and aqueous NaOH (1 M, 250 cm^{-3}) were added. After separation of the organic phase, the aqueous phase was extracted with diethyl ether (2 x 200 cm^{-3}). The combined organics were washed with water (200 cm^{-3}), dried over $MgSO_4$, filtered and concentrated under

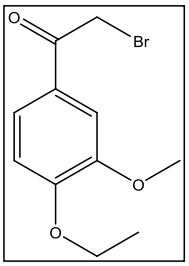
reduced pressure to give the title compound **S1** (2.86 g, 81%) which required no further purification.

δ_H (400MHz, $CDCl_3$) 7.53 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.50 (d, *J* = 2.0 Hz, 1H), 6.85 (d, *J* = 8.4 Hz, 1H), 4.14 (q, *J* = 7.0 Hz, 2H), 3.90 (s, 3H), 2.54 (s, 3H), 1.48 (t, *J* = 7.0 Hz, 3H). δ_C (100MHz, $CDCl_3$) 196.9, 152.8, 149.1, 130.3, 123.3, 110.9, 110.3, 64.5, 56.1, 26.3, 14.7.



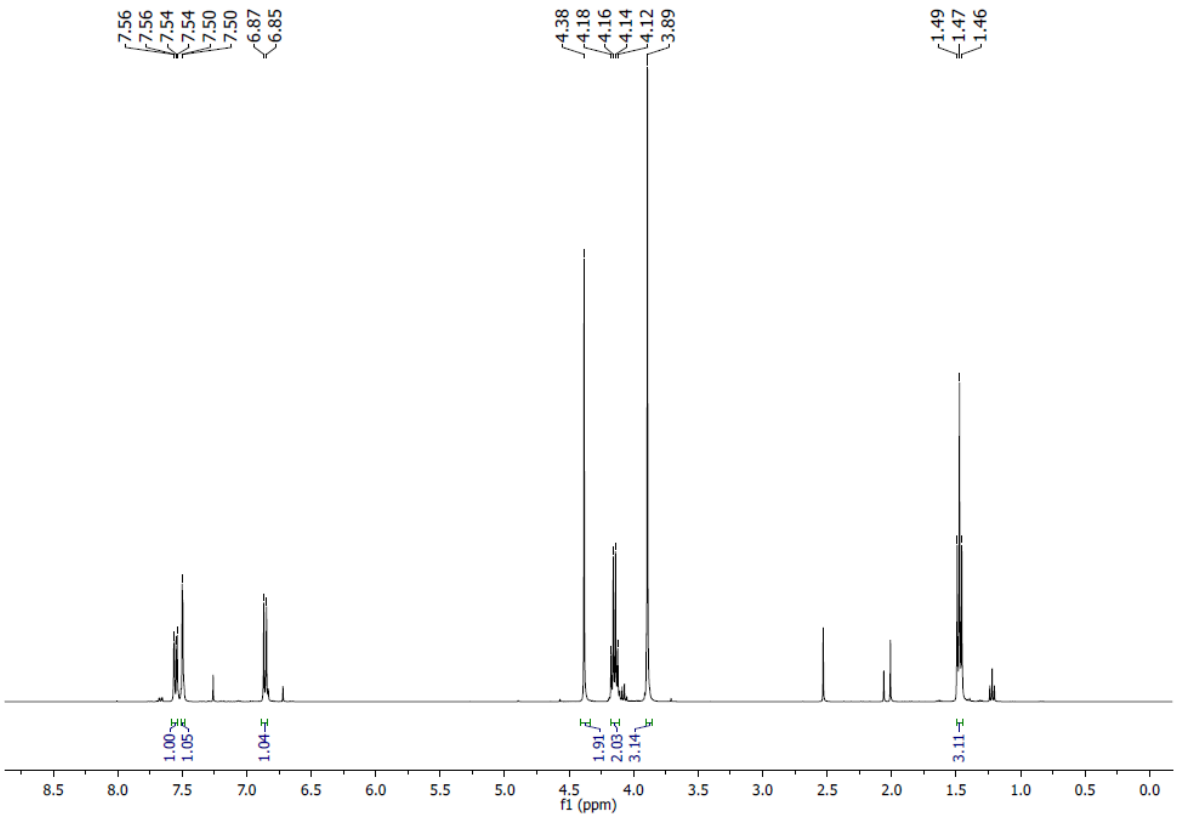
NMR of product from step 1 (**S1**)

2-bromo-1-(4'-ethoxy-3'-methoxyphenyl)ethan-1-one (**S2**)



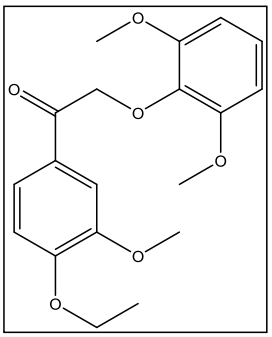
A mixture of ketone **S1** (2.86 g, 14.7 mmol) and $CuBr_2$ (5.25 g, 23.5 mmol) in ethyl actetate (25 cm^{-3}) was heated at reflux for three hours before cooling to room temperature. Filtration with copious ethyl acetate washings and concentration under reduced pressure afforded the crude product **S2** along with starting material **S1** in a 15 : 2 ratio (3.53 g) which was used in the next step without further purification.

δ_H (400MHz, $CDCl_3$) 7.55 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.50 (d, *J* = 2.0 Hz, 1H), 6.86 (d, *J* = 8.4 Hz, 1H), 4.38 (s, 2H), 4.15 (q, *J* = 7.0 Hz, 2H), 3.89 (s, 3H), 1.47 (t, *J* = 7.0 Hz, 3H).



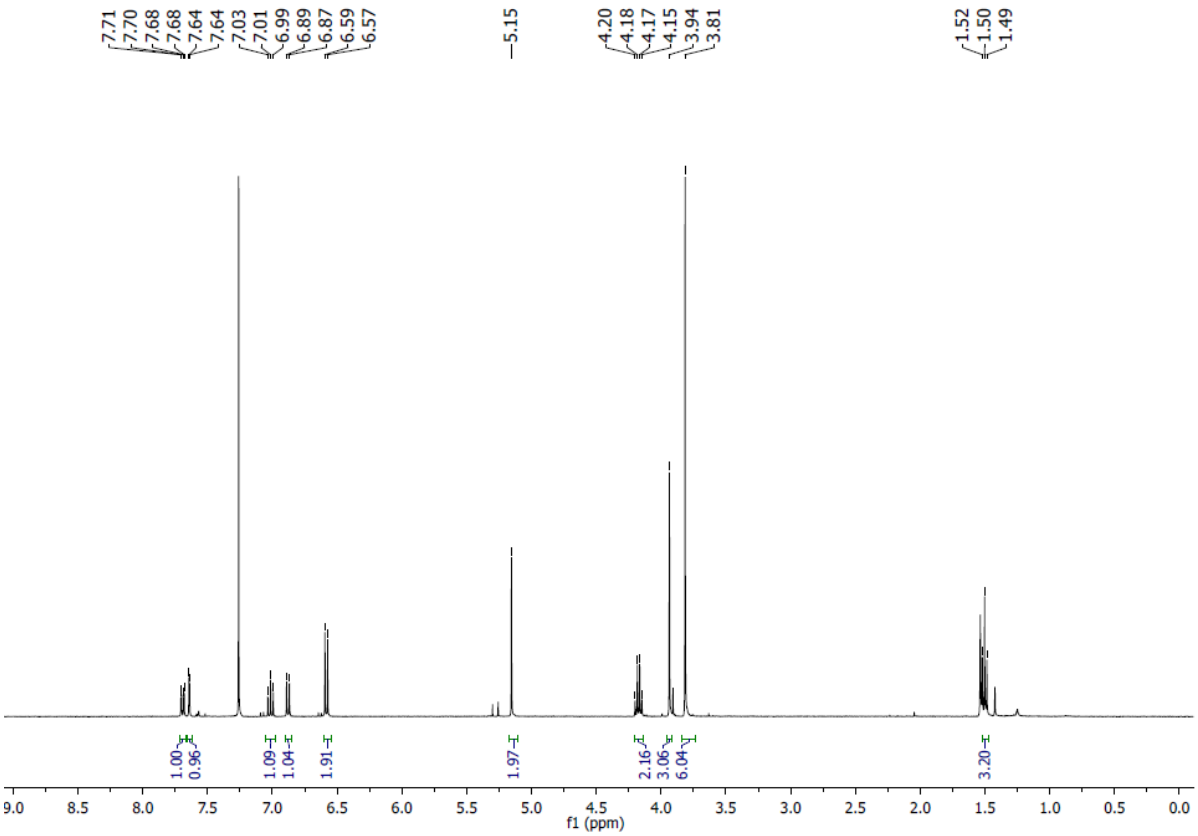
NMR of product from step 2 (**S2**)

2-(2'',6''-dimethoxyphenoxy)-1-(4'-ethoxy-3'-methoxyphenyl)ethan-1-one (**S3**)



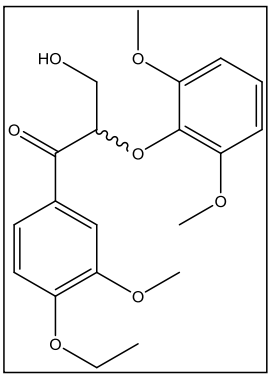
A mixture of syringol (2.6 g, 16.9 mmol) and K_2CO_3 (2.3 g, 16.9 mmol) in acetone (35 cm^{-3}) was stirred at room temperature for 30 mins before the addition of crude bromide **S2** (3.53 g) in acetone (10 cm^{-3}). After 3 h at room temperature, ethyl acetate (200 cm^{-3}) and aqueous NaOH (1 M, 200 cm^{-3}) were added. After separation of the organic phase, the aqueous phase was extracted with ethyl acetate (2 x 100 cm^{-3}). The combined organics were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Flash chromatography (5 : 1 then 7 : 2 cyclohexane : ethyl acetate) afforded the title compound **S3** (2.16 g, 42% over 2 steps).

δ_H (400MHz, $CDCl_3$) 7.69 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.64 (d, *J* = 2.0 Hz, 1H), 7.01 (t, *J* = 8.4 Hz, 1H), 6.88 (d, *J* = 8.4 Hz, 1H), 6.58 (d, *J* = 8.4 Hz, 2H), 5.15 (s, 2H), 4.17 (q, *J* = 7.0 Hz, 2H), 3.94 (s, 3H), 3.81 (s, 6H), 1.50 (t, *J* = 7.0 Hz, 3H). δ_C (100MHz, $CDCl_3$) 193.7, 153.3, 152.8, 149.1, 136.7, 128.2, 124.1, 123.0, 111.0, 110.8, 105.3, 75.3, 64.4, 56.1, 56.1, 14.6.



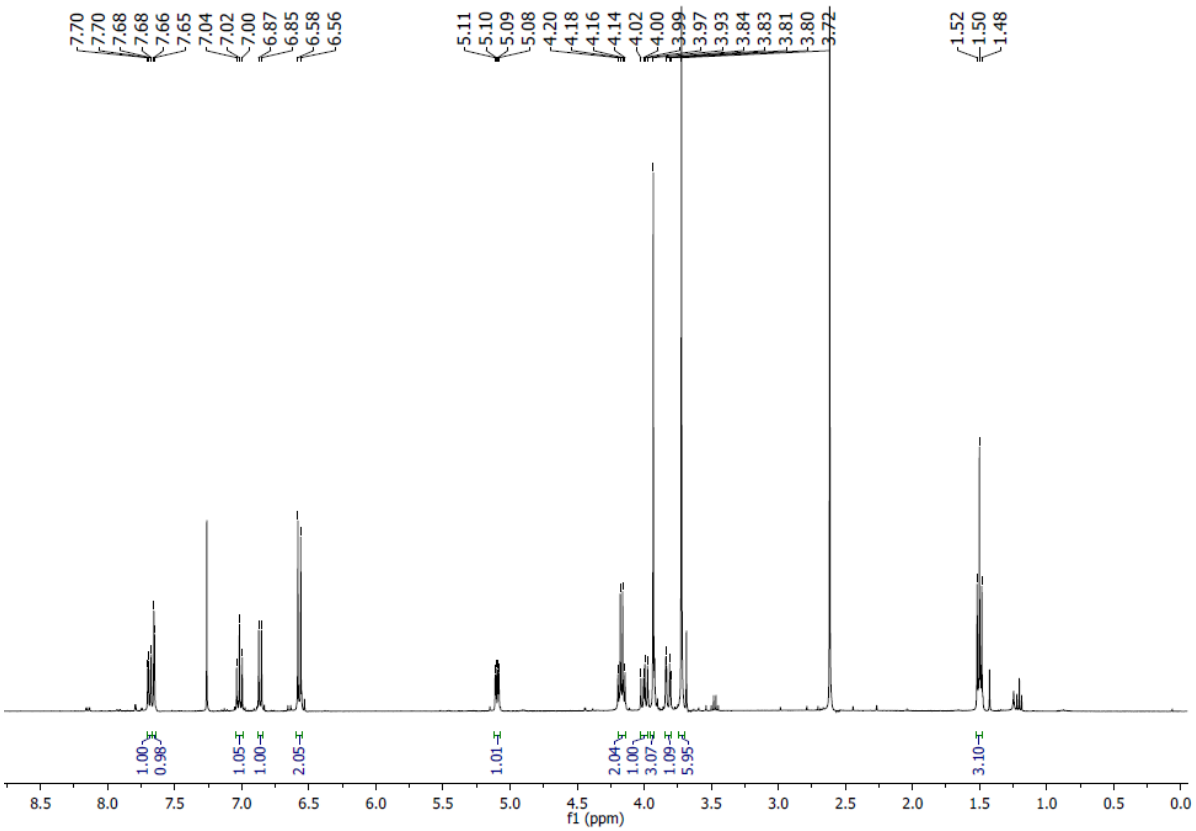
NMR of product from Step 3 (**S3**)

2-(2'',6''-dimethoxyphenoxy)-1-(4'-ethoxy-3'-methoxyphenyl)-3-hydroxypropan-1-one (**7**)



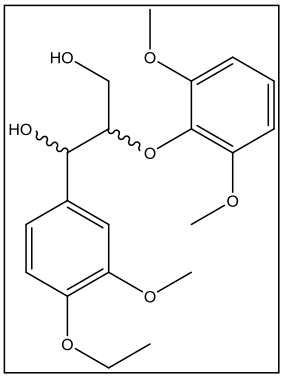
To a mixture of ketone **S3** (500 mg, 1.44 mmol) and paraformaldehyde (63 mg, 2.16 mmol) in DMSO (9.3 cm^{-3}) was added K_2CO_3 and the resulting solution stirred at room temperature for 20 h before the addition of diethyl ether (200 cm^{-3}) and water (200 cm^{-3}). After separation of the organic phase, the aqueous phase was extracted with ethyl acetate (2 x 100 cm^{-3}). The combined organics were dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the title compound **S4** (400 mg, 74%).

δ_H (400MHz, $CDCl_3$) 7.69 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.65 (d, *J* = 2.0 Hz, 1H), 7.02 (t, *J* = 8.4 Hz, 1H), 6.86 (d, *J* = 8.4 Hz, 1H), 6.57 (d, *J* = 8.4 Hz, 2H), 5.10 (dd, *J* = 7.9, 3.0 Hz, 1H), 4.17 (q, *J* = 7.2 Hz, 2H), 4.00 (dd, *J* = 12.2, 7.9 Hz, 1H), 3.93 (s, 3H), 3.82 (dd, *J* = 12.2, 3.0 Hz, 1H), 3.72 (s, 6H), 1.50 (t, *J* = 7.2 Hz, 3H).



NMR of product from step 4 (ketone **7**)

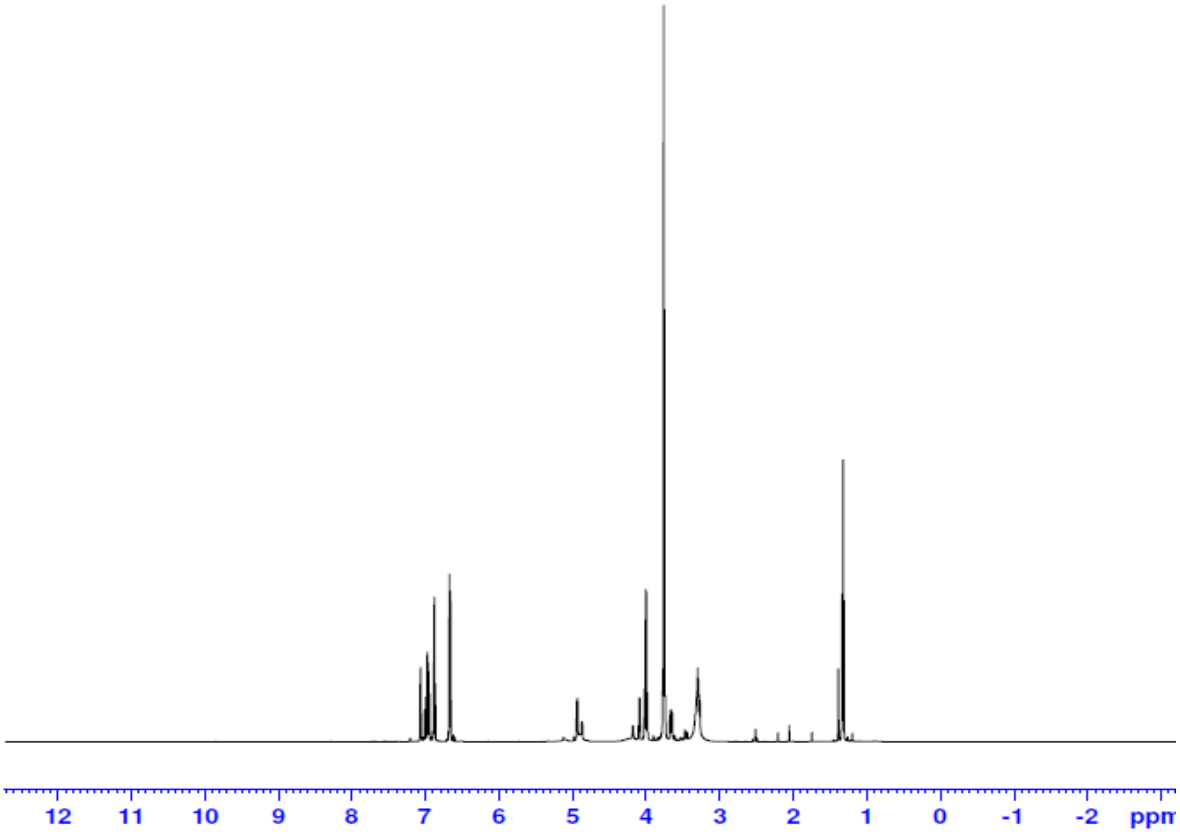
2-(2'',6''-dimethoxyphenoxy)-1-(4'-ethoxy-3'-methoxyphenyl)propane-1,3-diol (dimer **5**)



To a solution of ketone **7** (400 mg, 1.06 mmol) in methanol (8 cm^{-3}) at 0°C was added $NaBH_4$ (84 mg, 2.2 mmol). After 2 h at 0°C, the reaction mixture was partitioned between diethyl ether (100 cm^{-3}) and water (100 cm^{-3}). After separation of the organic phase, the aqueous phase was extracted with diethyl ether (2 x 100 cm^{-3}). The combined organics were dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the title compounds dimer **5** (230 mg, 57%) as a 5 : 2 ratio of *threo* : *erythro* isomers. The major and minor isomers were assigned based on comparison of the ^{13}C chemical shifts with those of a closely related structure (2-(2'',6''-dimethoxyphenoxy)-1-(4'-methoxy-3'-methoxyphenyl)propane-1,3-diol)[3].

δ_H (500MHz, DMSO, 50°C) 7.06 (d, *J* = 1.8 Hz, 0.71H), 7.00 (br. s, 0.29H), 6.97 (t, *J* = 8.4 Hz, 1H), 6.94 (dd, *J* = 8.3, 1.8 Hz, 0.71H), 6.88-6.85 (m, 1.29H), 6.66 (d, *J* = 8.4 Hz, 0.58H), 6.66 (d, *J* = 8.4 Hz, 1.42H), 4.93 (d, *J* = 4.8 Hz, 0.71H), 4.86 (d, *J* = 4.8 Hz, 0.29H), 4.17 (q, *J* = 4.7 Hz, 0.29H), 4.08 (q, *J* = 4.8 Hz, 0.71H), 3.99 (q, *J* = 7.0 Hz, 2H), 3.77-3.72 (m, 9.29H), 3.65 (dd, *J* = 11.5, 5.0 Hz, 0.71H), 3.45 (dd, *J* = 11.8, 4.1 Hz, 0.29H), 3.27 (dd, *J* = 11.5, 4.6 Hz, 0.71H), 1.31 (t, *J* = 7.0 Hz, 3H). δ_C (125MHz, DMSO, 50°C) 152.9 (minor), 152.8 (major), 148.6 (minor), 148.4 (major), 146.9 (minor), 146.9 (major), 136.3 (major), 135.8 (minor), 135.0 (minor), 134.8 (major), 123.3 (major), 123.2 (minor), 119.1 (minor), 118.9 (major), 112.8 (minor), 112.8 (major), 111.1 (minor), 111.1 (major), 105.9 (minor), 105.8 (major), 86.8 (major), 85.9 (minor), 72.0 (minor), 71.4 (major), 63.8 (minor), 63.8

(major), 60.2 (major), 59.7 (minor), 55.9 (minor), 55.9 (major), 55.4 (major + minor), 14.7 (major + minor).



NMR of product from step 5 (dimer **5**)

1.10. β-O-4 dimer oxidation studies

1-(4-Hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol **3** and 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol **4** were prepared as 10mM stock solutions in dimethylforamide (DMF). Oxidation reactions were performed as described by Li *et al.*,[4] with some modifications. Each reaction contained 1mM dimer **3** or **4**, 1mM 1-HBT, and 2U/mL commercial *T. versicolor* laccase in 50mM sodium acetate buffer pH 4.5. Reactions were carried out at 25°C and 150rpm agitation. 500µl aliquots were taken after 24h and acidified with 1M HCl. The extraction and analysis procedure was the same as described for the veratryl alcohol oxidation reaction. Oxidation studies of 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (dimer **5**) with TvL and 1-HBT was carried out according to Kawai *et al.*, [5]. Reactions were analysed and monitored by reversed phase HPLC on an Agilent 1100 series HPLC + LC/MSD SL ESI mass spectrometer. Separation was achieved using an isocratic mobile phase of 60% methanol in super pure water both with 0.1% formic acid at a flow rate of 0.2ml/min for 30 min using a C:18(2) 250 x 2.00mm 5µ column. MS was performed in positive mode using a mass range 100-1500MW (MS spray chamber conditions: Drying gas flow 12.0ml/min^{-1}, nebuliser pressure 50psi, drying gas temperature 350°C and capillary voltage 5000V).

*1.11 Characterisation of ketone **6***

Due the lack of an authentic standard for the ketone product **6**, scale-up followed by preparative HPLC and NMR analysis was required. The biotransformation as previously described for dimer **4** was scaled up using 1mM dimer **4**, 10U TvL and 1mM 1-HBT. The reaction ran for 72h and product formation was monitored by LC-MS as described in the procedure above. The reaction was acidified to pH 2.0 and extracted with 2x volumes ethyl acetate. The combined organics were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Following resuspension in 65% methanol, preparative HPLC was used to isolate the ketone product **6** for NMR analysis. Preparative reverse phase HPLC was performed on an Agilent 1200 series HPLC with UV/Vis detection at 280nm using a C:18 250 x 21.20mm Hyperclone 5μ ODS column with separation achieved using an isocratic mobile phase of 60% methanol in super pure water at a flow rate 3.0ml/min for 30min. Fractions were collected coinciding with the elution of ketone **6** (21.5-23.2min) and pooled before evaporating under reduced pressure.

δ_H (400MHz, $CDCl_3$) 7.75 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.62 (d, *J* = 2.0 Hz, 1H), 7.00 (ddd, *J* = 8.0, 7.2, 1.6 Hz, 1H), 6.92 (dd, *J* = 8.0, 1.6 Hz, 1H), 6.89 (d, *J* = 8.4 Hz, 1H), 6.89 (dd, *J* = 8.0, 1.6 Hz, 1H), 6.83 (ddd, *J* = 8.0, 7.2, 1.6 Hz, 1H), 5.41 (t, *J* = 5.3 Hz, 1H), 4.07 (d, *J* = 5.3 Hz, 2H), 3.95 (s, 3H), 3.92 (s, 3H), 3.86 (s, 3H). δ_C (100MHz, $CDCl_3$) 195.0, 154.0, 150.5, 149.2, 147.0, 128.1, 123.7, 121.2, 118.5, 112.3, 111.0, 110.1, 84.6, 63.8, 56.1, 56.0, 55.8.

MS of **6** was generated by liquid chromatography followed by electrospray ionization (ESI). The *m/z* of **6** is 332.13. ESI generated the molecular ion $[MH^+]$ = 333.0 (332.12 + 1), [M+Na] = 355.0 (332.12 + 22.9) and [2M+Na] = 687.3 (332.12 (x2) +22.9)

*1.12 Characterisation of ketone **7***

Ketone **7** was produced via step 4 in the synthesis of dimer **5** therefore was used as an authentic standard in the LC-MS analysis of the biotransformation of dimer 5. MS of **7** was generated by liquid chromatography followed by electrospray ionization (ESI). The *m/z* of **7** is 376.15. ESI generated the molecular ion $[MH^+]$ = 377.0 (376.15 + 1), [M+Na] = 399.0 (376.15 + 22.9) and [2M+Na] = 775.3 (376.15 (x2) +22.9)

1.13. Lignin extraction by organosolv

Lignin was extracted from the solid WS substrate fraction following enzymatic treatments by organosolv delignification. The substrate was mixed with 60:40% w/w ethanol: H_2O at a solid/liquid-ratio of 1:9 (g d.w WS) for 2h at 126°C. After 2h the solid and liquid fraction was separated by centrifugation and lignin was precipitated from the liquid fraction by dilution with acidified refridgerated water (3:1 water:liquid fraction) as described by [6].

1.14. Py-GCMS with TMAH

1mg of extracted lignin from laccase treated samples was placed into a quartz pyrolysis sampling tube with quartz wool capped at the end. 7μl tetramethylammoniumhydroxide (TMAH) was added to the lignin before loading onto the Chemical Data System (CDS) 5200 series pyroprobe pyrolysis unit. The probe was heated to 600°C for 10s. Pyrolysed organic lignin fragments were analysed using an Agilent 7890A GC fitted with a HP-5 fused capillary column (J+W Scientific; 5%

diphenyldimethypolysiloxane; 30m length, 0.32m internal diameter, 0.25μm film thickness) coupled to an Agilent 5975 MSD single quadrupole mass spectrometer operating in electron ionisation (EI) mode (scanning a range of m/z 50 to 600 at 2.7 scans s^{-1}, ionisation energy 70eV). The pyrolysis transfer line and injector temperatures were set at 350 ˚C, the heated interface at 300 ˚C, the EI source at 230˚C and the MS quadrupole at 150˚C. Helium was used as the carrier gas and the compounds were introduced in split mode (split ratio 50:1) with a split flow rate of 50ml/min. The oven temperature started at 40°C and was held for 2 minutes and increased to 220˚C at 2.5˚C min^{-1}, then held at this temperature for 1 min before being heated to 300˚C at 20˚C min^{-1} whereby it was held at this temperature for 11 min. The total run time was 90min. Products were identified and named by comparison to previously published data [7, 8]

2. Supplementary Results

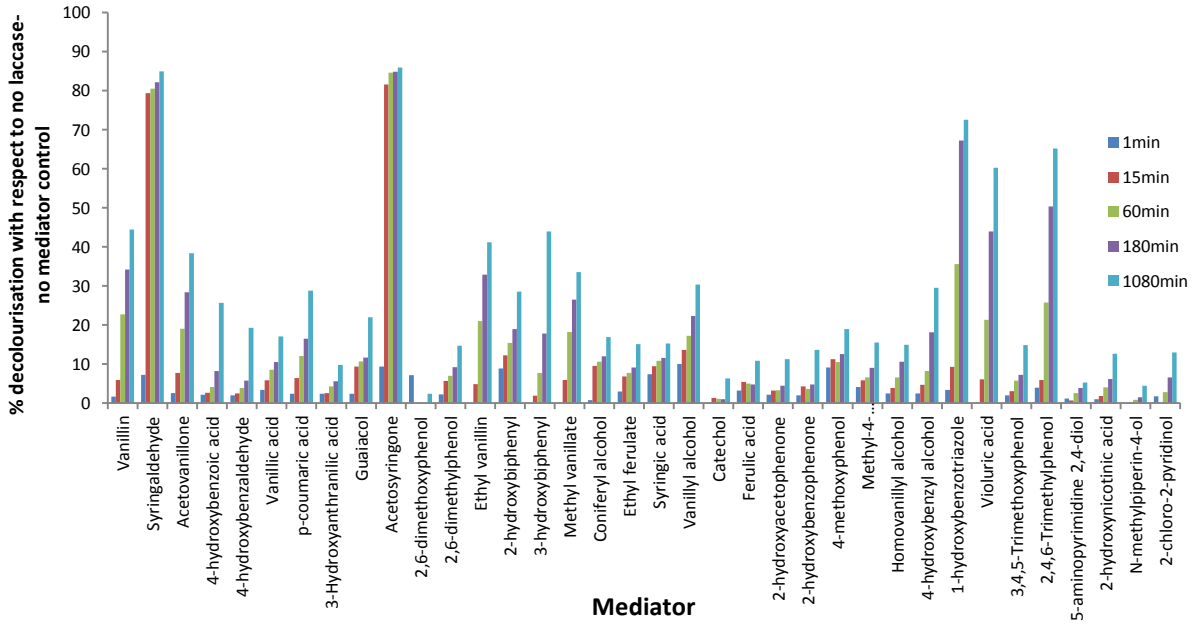
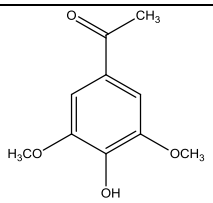
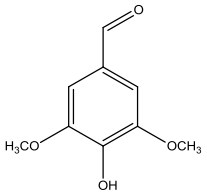
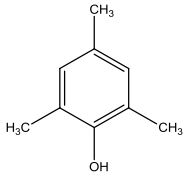
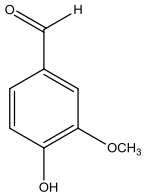
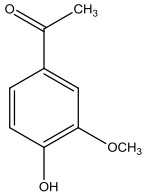
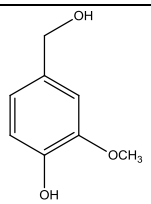
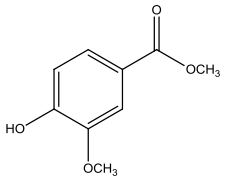
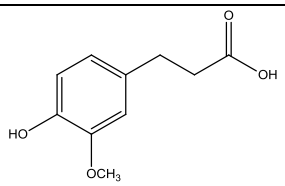
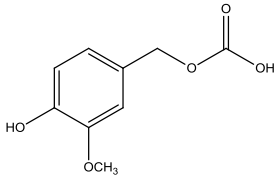
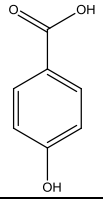
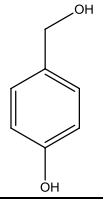
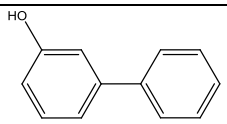
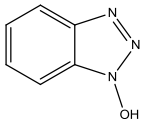
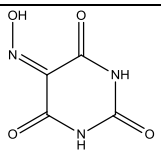


Figure 1. % decolourisation at of RB-5 in the presence of a range of phenolic and synthetic mediators using a 1:1 dye:mediator-ratio.

Table 1: The 12 phenolic mediators that induced the greatest % decolourisation of Reactive Black-5 with *Trametes versicolor* laccase. Data is presented at all dye:mediator-ratios examined and at two timpoints (3h and 18h). Structures are grouped according to structural similarities. 1-HBT and violuric acid are included as synthetic mediator controls.

Mediator	Structure	Ratio dye:med	Decolourisation	
			3 h	18 h
1. Acetosyringone		1:1 1:5 1:10	**83.6 (0.1)** 74.0 (0.1) 69.0 (0.6)	**83.9 (0.2)** 71.8 (0.1) 67.7 (0.7)
2. Syringaldehyde		1:1 1:5 1:10	**81.0 (0.7)** 76.1 (0.9) 71.0 (0.8)	**83.2 (0.3)** 75.2 (0.8) 71.4 (0.6)
3. 2,4,6-Trimethylphenol		1:1 1:5 1:10	46.2 (1.4) **78.1 (0.8)** 76.9 (0.8)	61.3 (1.3) **81.7 (0.1)** 78.4 (0.2)
4. Vanillin		1:1 1:5 1:10	29.6 (1.2) **41.6 (1.0)** **39.1 (1.0)**	36.9 (1.0) **52.5 (0.8)** **56.0 (1.0)**
5. Acetovanillone		1:1 1:5 1:10	23.4 (0.6) **31.9 (0.4)** **26.4 (1.66)**	31.4 (1.3) **39.2 (0.7)** **40.2 (1.23**)
6. Vanillyl alcohol		1:1 1:5 1:10	17.5 (1.0) **19.9 (0.7)** 12.1 (0.5)	23.8 (1.0) **34.3 (0.4)** 26.9 (0.3)
7. Methyl Vanillate		1:1 1:5 1:10	25.5 (1.4) 31.4 (0.4) **34.9 (0.6)**	30.7 (1.1) 36.7 (0.4) **42.4 (0.61)**
8. p-coumaric acid		1:1 1:5 1:10	9.25 (0.5) **24.7 (0.2)** 19.7 (0.5)	18.4 (1.3) **40.2 (0.4)** 40.8 (1.4)

9. Ethyl vanillin		1:1 1:5 1:10	32.0 (0.8) 39.5 (1.5) **39.3 (0.33)**	38.7 (0.5) 50.0 (1.4) **51.6 (0.3)**
10. 4-Hydroxybenzoic acid		1:1 1:5 1:10	1.2 (0.4) **12.9 (1.4)** 5.2 (1.45)	17.2 (0.5) **46.8 (2.1)** 44.2 (1.06)
11. 4-hydroxybenzyl alcohol		1:1 1:5 1:10	14.3 (1.3) **20.2 (0.9)** **14.7 (1.1)**	22.8 (1.5) **24.2 (0.2)** **25.9 (1.3)**
12. 3-hydroxybiphenyl		1:1 1:5 1:10	16.7 (0.6) 20.2 (1.3) **21.6 (0.96)**	41.6 (0.5) 52.6 (1.0) **57.6 (0.9)**
13. 1-Hydroxybenzotriazole		1:1 1:5 1:10	**66.1 (1.0)** 65.1 (0.7) 62.8 (0.3)	**69.5 (0.6)** 68.0 (0.4) 68.8 (0.9)
14. Violuric acid		1:1 1:5 1:10	42.0 (2.0) **80.5 (0.2)** 79.3 (0.7)	55.9 (1.2) **81.9 (0.2)** 80.4 (0.7)

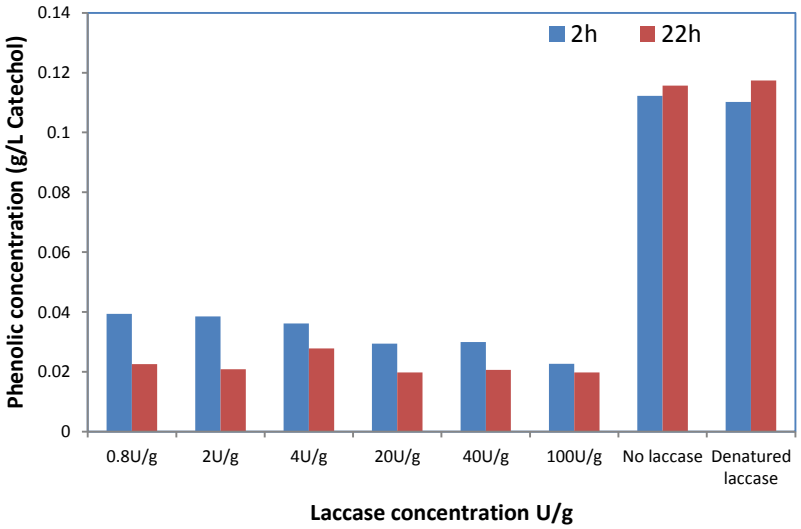


Figure 2. Phenol content from acid-treated wheat straw slurries treated with different concentrations of TvL. No laccase and denatured laccase samples were used as negative controls.

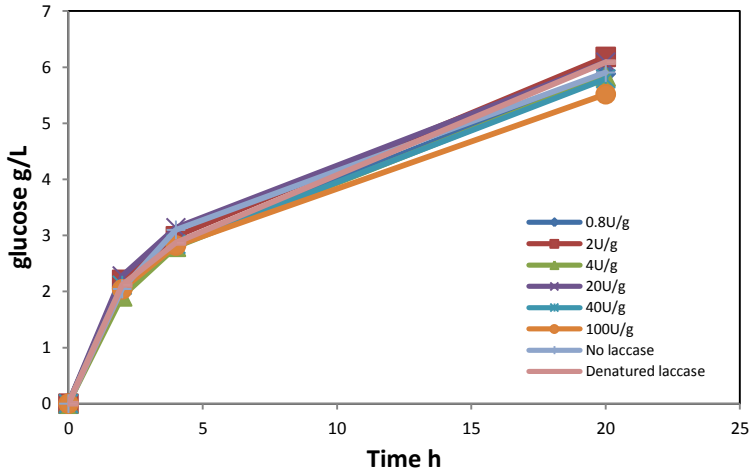


Figure 3. Glucose release from acid-pretreated wheat straw slurries treated with different concentrations of TvL with no alkaline peroxide extraction. No laccase and denatured laccase samples were used as negative controls.

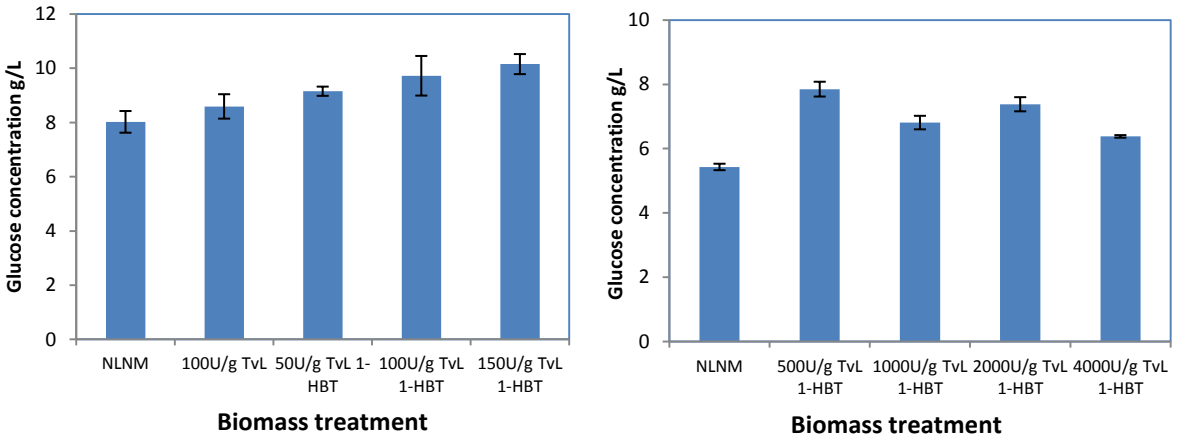


Figure 4. Glucose release from acid-pretreated wheat straw subjected to treatment with different concentrations of TvL and 5% 1-HBT. Concentration was investigated at both a low ranges (50-150U/g, left) and high ranges (500-4000U/g, right) before alkaline-peroxide extraction.

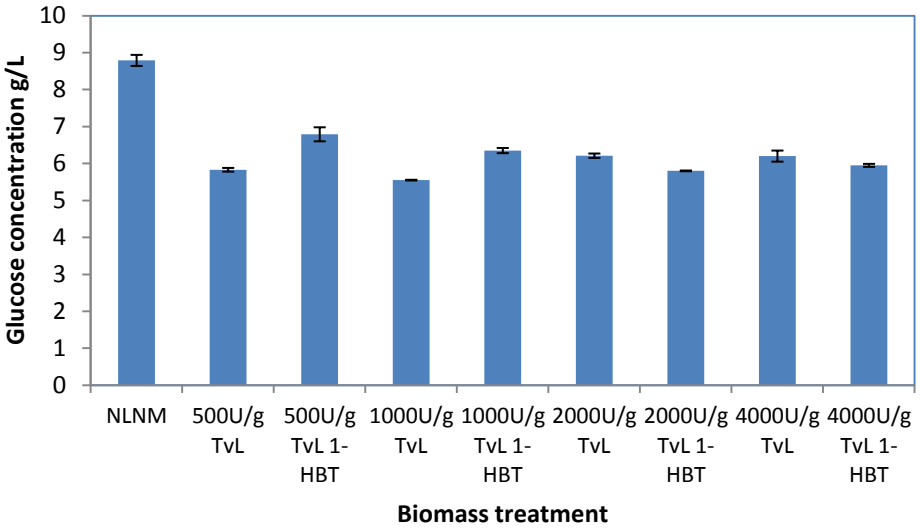


Figure 5. Glucose release from acid-pretreated wheat straw subjected to TvL at 500-4000U/g with and without 1-HBT and without alkaline-peroxide extraction.

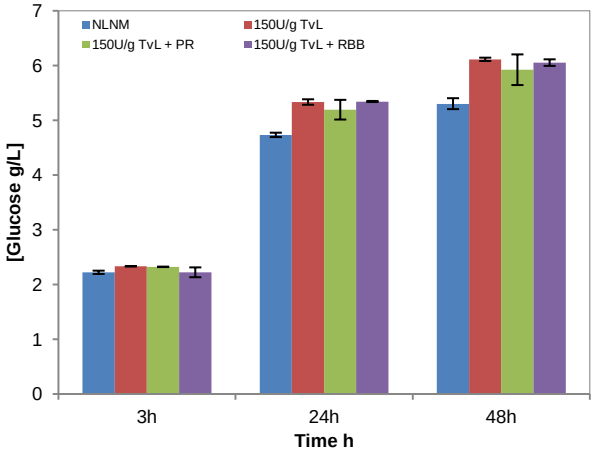


Figure 6: Glucose release following hydrolysis of acid pretreated wheat straw incubated with and without TvL and the mediators PR and RBB

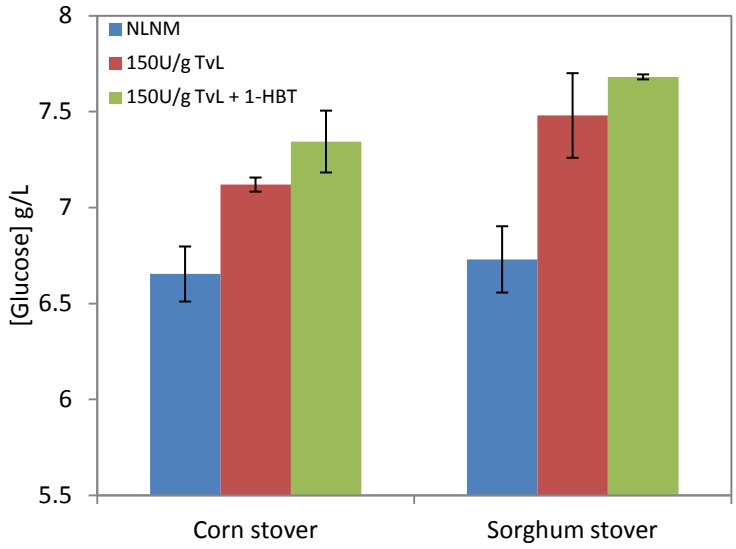


Figure 7. Glucose release from corn stover and sorghum stover hydrolysis following treatment with TvL and 1-HBT and APE. Increases in glucose release were lower than those observed previouslywith wheat straw. This is most likely due to the finer milling/smaller particle size of the wheat straw, owing to the larger surface area of this substrate.

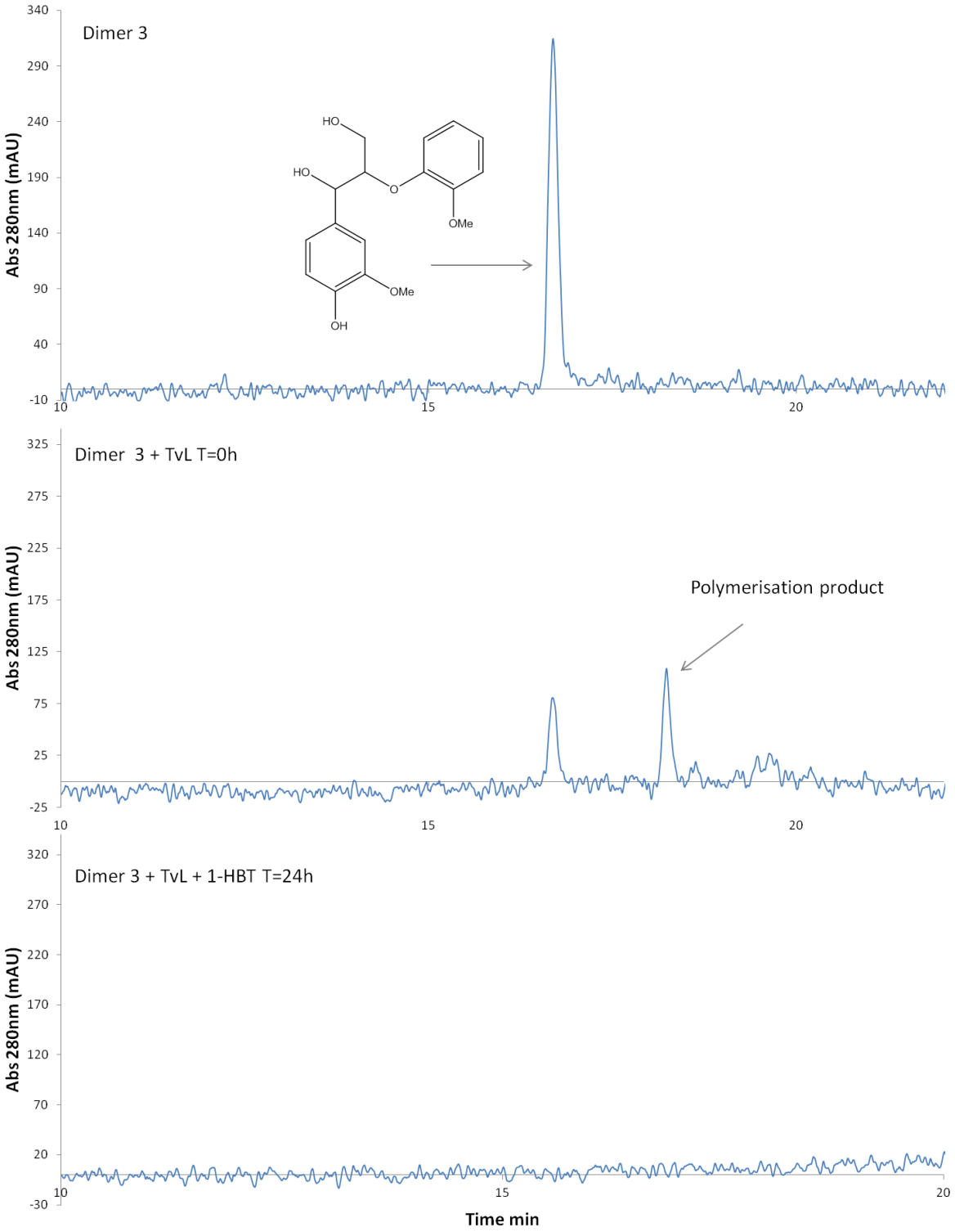


Figure 8: UV traces following the oxidation of dimer **3**. Top: The retention time of the standard compound of **3**. Middle: UV trace of **3** upon addition of TvL whereby the disappearance of **3** is noticeable and a dimerisation product appears. Bottom: The complete disappearance of **3** after 24h incubation with TvL and no single products are detected.

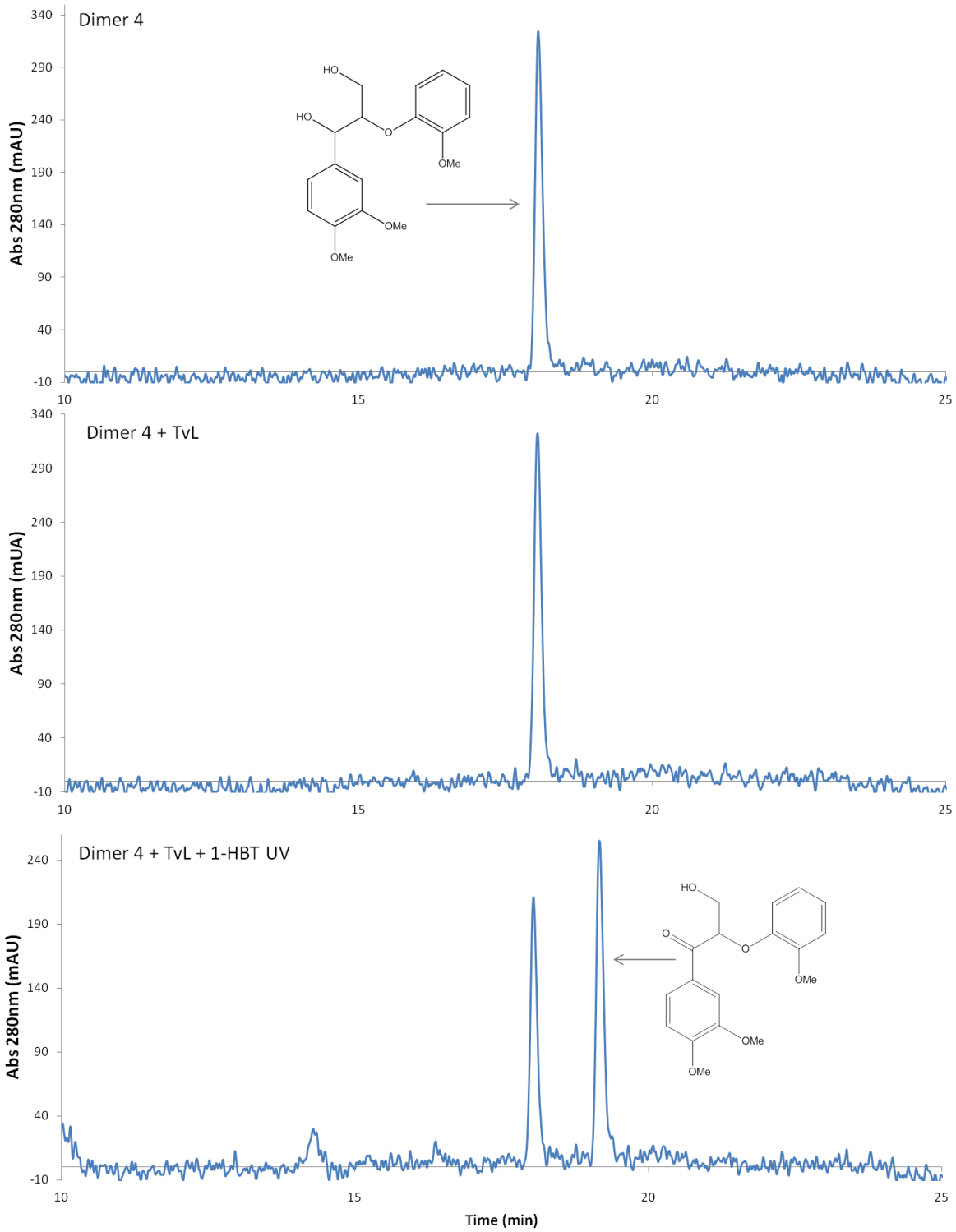


Figure 9: UV traces following the oxidation of dimer **4**. Top: The retention time of the standard compound of **4**. Middle: UV trace of **4** upon addition of TvL whereby no products are detected. Bottom: The reaction of **4** with TvL and 1-HBT whereby the Cα oxidation product **6** is identified

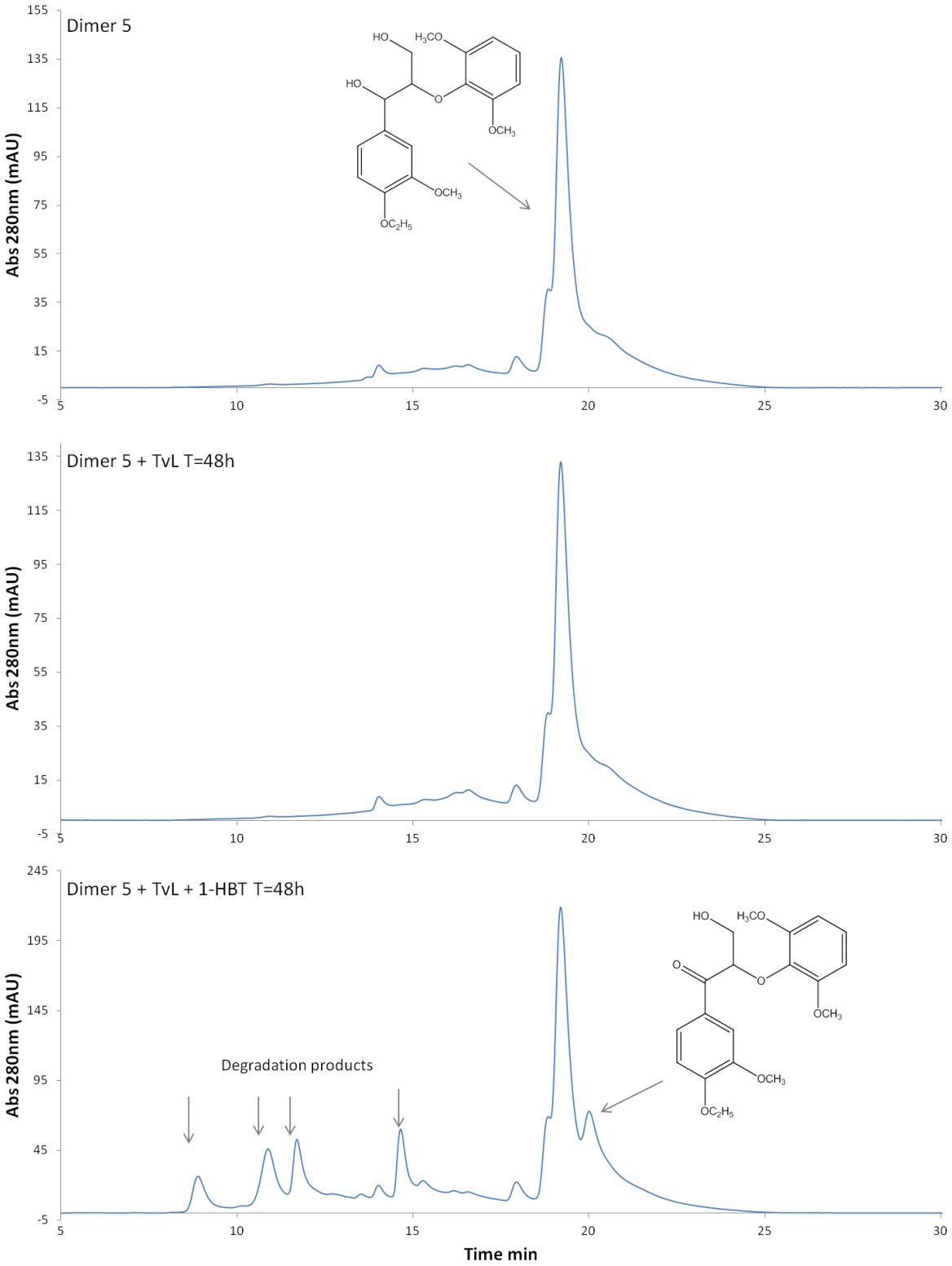


Figure 10: UV traces following the oxidation of dimer **5**. Top: The retention time of the standard compound of **5**. Middle: UV trace of **5** upon addition of TvL whereby no oxidation products are detected. Bottom: The reaction of **5** with TvL and 1-HBT whereby the production of Cα oxidation product **7** was confirmed. Additional products that were confirmed by LC-MS and comparison to published literature were also identified.

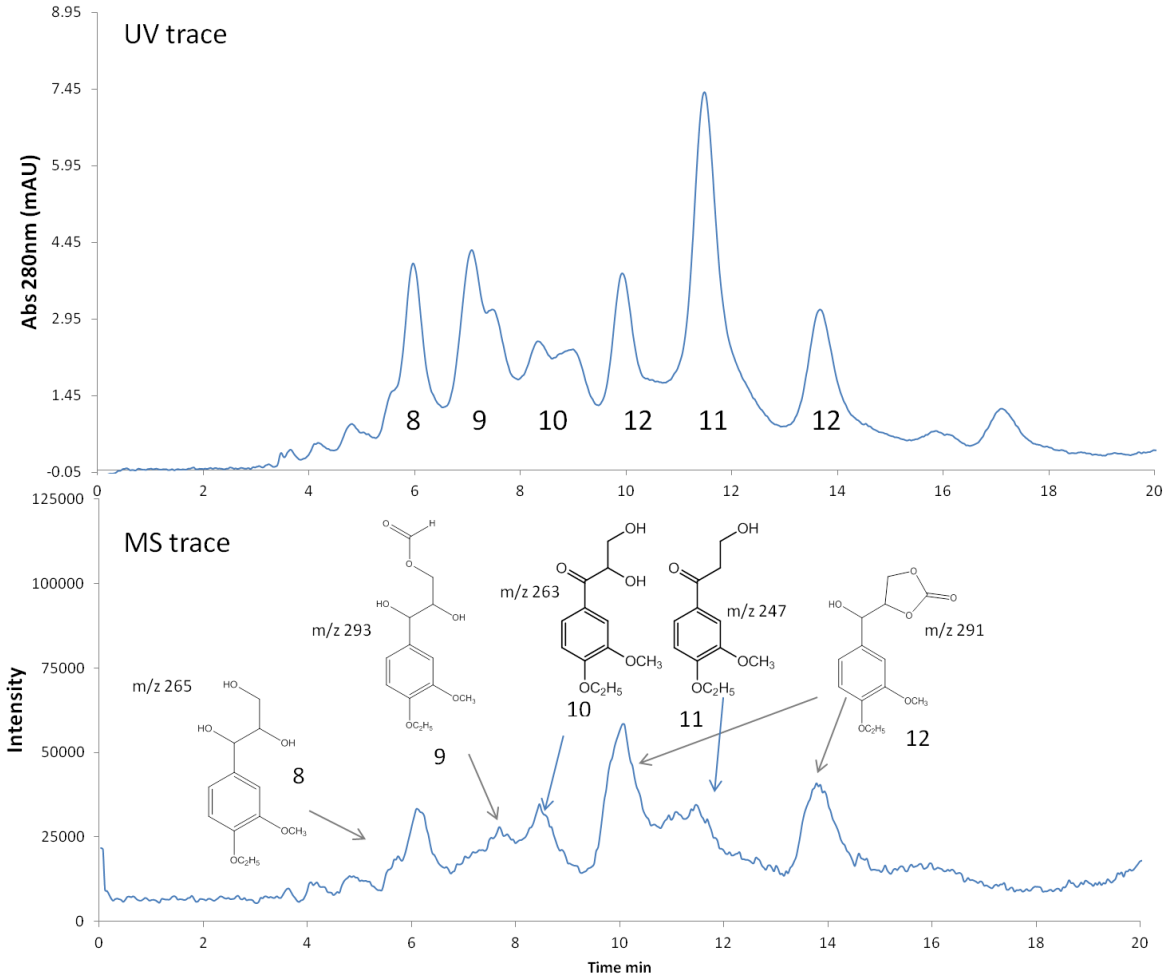


Figure 11. LC-MS traces from the oxidation of dimer **5**. Top: UV Chromatogram shows the region whereby the additional unconfirmed degradation products are eluted. Bottom: The corresponding MS trace to the UV chromatogram. Regions whereby particular masses corresponding to previously referenced degradation products were picked up are annotated by the possible structures.

Table 2: List of identifiable TMAH thermochemolysis products adapted from Vane *et al*., [7]

Label	Molecular weight	Assignment	Characteristic ions
G4	166	3,4-Dimethoxybenzaldehyde	151, 165, 166
G5	180	3,4-Dimethoxyacetophenone	137, 165, 180
G6	196	Methyl, 3,4-dimethoxybenzoate	165, 181, 196
S4	196	3,4,5-Trimethoxybenzaldehyde	125, 181, 196
G7	194	cis-2-(3,4-Dimethoxyphenyl)-1-methoxyethylene	151, 179, 194
G8	194	trans-2-(3,4-Dimethoxyphenyl)-1-methoxyethylene	151, 179, 194
G10	208	*cis*-1-(3,4-Dimethoxyphenyl)-1-methoxy-1-propene	165, 193, 208
P18	161	*trans*-3-(4-Methoxyphenyl)-3-propenoate	161, 192, 133
S5	195	3,4,5-Trimethoxybenzoate	195, 210, 139
S6	226	Methyl, 3,4,5-trimethoxybenzoate	226, 211, 195
G13	208	trans-1-(3,4-Dimethoxyphenyl)-3-methoxy-1-propene	91, 177, 208
S7	209	*cis*-1-(3,4,5-Trimethoxyphenyl)-2-Methoxyethylene	209, 224, 181
S8	209	*trans*-1-(3,4,5-Trimethoxyphenyl)-2-methoxyethylene	209, 224, 181
G14	181	threo/erythro-1-(3,4-Dimethoxy phenyl)-1,2,3-trimethoxypropane	166, 181, 270
G15	181	threo/erythro-1-(3,4-Dimethoxy phenyl)-1,2,3-trimethoxypropane	166, 181, 270
S10	223	*cis*-1-(3,4,5-Trimethoxyphenyl)-methoxyprop-1-ene	223, 238, 195
G18	222	*trans*-3-(3,4-Dimethoxyphenyl)-3-Propenoate	222, 207, 191
S14	211	threo/erythro-1-(3,4,5-Trimethoxy-phenyl)-1,2,3-trimethoxypropane	211, 181, 300
S15	211	threo/erythro-1-(3,4,5-Trimethoxy-propenoate	211, 181, 300

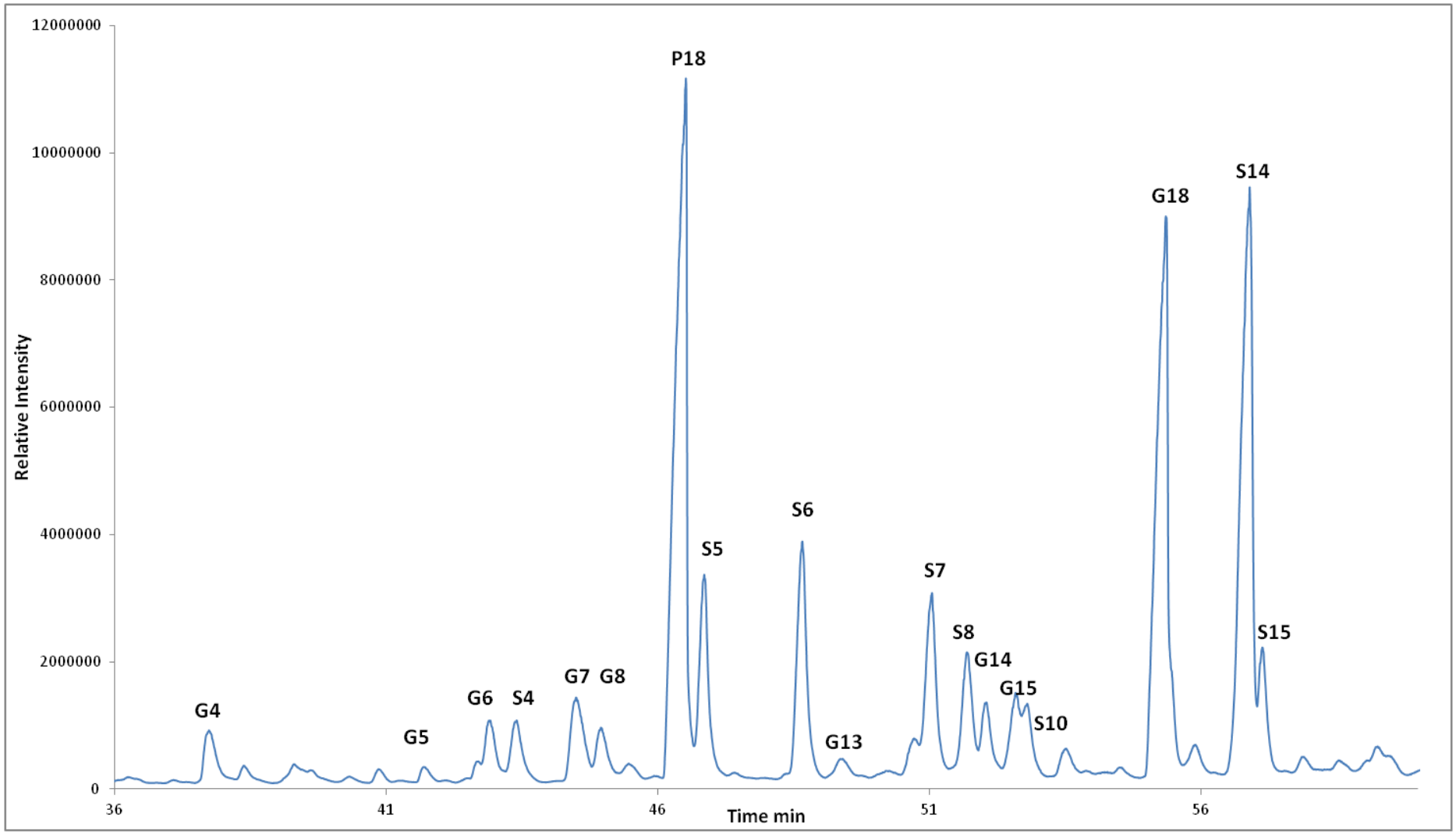


Figure 12. Partial chromatogram of the total ion current (TIC) for the TMAH thermochemolysis products from organosolv extracted wheat straw lignin with no laccase or mediator treatment (NLNM). Products were identified from the information in Table X and by library searches (G:Guaiacyl, S:Syringyl and P:*p*-hydroxyphenyl).

3. References

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