Untangling Lignin Degradation: An In Silico Approach for Identifying **Unknown Phenolic Compounds and Lignin Oligomers** Woo-Young Song¹, Youri Yang² and Tae-Young Kim^{1,*}

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Overview

> This study presents a comprehensive LC-MS/MS strategy combining suspect screening with *in silico* MS/MS for identifying lignin degradation products. > This strategy enabled the detection of 28 lignin degradation products that exhibited significant increases following CtLac enzyme treatment.

Introduction

- Lignin, one of the most abundant natural aromatic polymers, represents a promising renewable feedstock for producing value-added aromatic compounds.
- > However, unlocking its full potential through enzymatic conversion of lignocellulosic biomass hinges on deciphering the complex mixture of phenolic compounds (PCs) and lignin oligomers (LOs) formed during degradation.
- > This study presents a comprehensive strategy for identifying unknown PCs and LOs in lignin degradation products by combining suspect screening and *in silico* MS/MS identification.

Results and Discussion



- > The strategy was applied to analyze PCs and LOs derived from various wood types treated with CtLac, an oxidative enzyme.
- > A key feature of this approach is the combination of accurate mass defect filtering specific to lignin with structural elucidation of lignin-derived fragment ions, which allows for the isolation and characterization of chemical features containing phenylpropanoid moieties, the building blocks of lignin..

Materials and Methods



Figure 1. MS/MS-based structural characterization of 12 PCs detected in six types of CtLac-treated woods. Upper and lower spectra are MS and MS/MS spectra of each PC, respectively. Asterisk represents the precursor ion peak. **Red dotted line indicates a bond fragmentation corresponding to each product ion.**

- > The suspect screening analysis of six intact woody biomass treated with CtLac identified 14 tentative PCs out of 24 from the suspect list, including 12 PCs confirmed with MS/MS-based structural identification.
- \succ The neutral loss of 15 Da (CH₃, methyl) was detected from G- and S-type PCs containing a methoxy group. Other neutral losses corresponding to different functional groups on the propanoyl position were observed: 18 Da (H_2O) , $30 \text{ Da} (\text{CH}_2\text{O}), 42 \text{ Da} (\text{C}_2\text{H}_4\text{O}) \text{ and } 44 \text{ Da} (\text{CO}_2).$

Suspect screening of PCs in six wood enzymatic degradation products

 Table 2. Peak area ratios of PCs between CtLac-treated six wood biomass and enzyme blank

Label	Compound	Retention time	m/z	Cypress	Ginkgo	Pine	Maple	Oak	Poplar
	H-type								
А	Catechol	9.58	109.0295	$0.99^{a} \pm 0.06^{b}$	0.83±0.09*	0.98 ± 0.07	1.02 ± 0.07	$0.89 \pm 0.03*$	0.86±0.11
В	<i>p</i> -Coumaryl alcohol	12.22	149.0608	0.99 ± 0.09	0.61±0.20	1.01±0.10	1.07 ± 0.22	1.02 ± 0.26	1.13±0.51
С	<i>p</i> -Hydroxybenzaldehyde	11.48	121.0294	1.04 ± 0.05	1.17±0.13	1.14±0.09*	1.22±0.03*	1.07 ± 0.11	1.06 ± 0.17
D	p-Hydroxbenzoic acid	10.08	137.0244	0.91±0.09	$0.49 \pm 0.06*$	$0.64 \pm 0.04*$	0.71±0.11*	$0.50 \pm 0.03*$	$0.73 \pm 0.12*$
Е	Cinnamic acid	14.82	147.0451	0.99 ± 0.04	1.07 ± 0.08	1.09 ± 0.07	1.13±0.05*	0.96 ± 0.04	0.92 ± 0.10
F	<i>p</i> -Coumaric acid	9.82	163.0400	1.01±0.09	0.94±0.11	1.00 ± 0.05	1.10±0.05*	$0.88 \pm 0.02*$	0.92±0.12
G	Caffeic acid	12.63	179.0349	1.23±0.14*	1.27±0.13*	1.29±0.08*	1.27±0.06*	1.13±0.06*	1.14±0.19
	<i>G-type</i>								
Н	Guaiacol	10.89	123.0451	1.01±0.09	0.98±0.11	1.01±0.06	1.12±0.06*	$0.85 \pm 0.03*$	$0.79 \pm 0.09 *$
Ι	Coniferyl alcohol	12.16	179.0714	0.97 ± 0.06	$0.39 \pm 0.08*$	0.78 ± 0.06 *	$0.87 \pm 0.07*$	$0.68 \pm 0.03^*$	$0.65 \pm 0.07*$
J	Vanillin	12.10	151.0400	1.07 ± 0.05	1.17±0.12	1.21±0.03*	1.13±0.04*	$1.10 \pm 0.04*$	1.02 ± 0.10
K	Vanillic acid	10.89	167.0349	0.91±0.10	$0.67 \pm 0.10^{*}$	$0.83 \pm 0.07*$	$0.85 \pm 0.01*$	$0.43 \pm 0.02*$	$0.40 \pm 0.05*$
L	Ferulic acid	12.22	193.0506	$0.85 \pm 0.07*$	$0.76 \pm 0.07 *$	$0.88 \pm 0.07*$	1.04 ± 0.05	$0.88 \pm 0.01*$	0.83 ± 0.08 *
	S-type								
М	Sinapyl alcohol	12.79	209.0814	ND ^c	ND	ND	0.95 ± 0.05	0.75±0.02*	0.79±0.09*
Ν	Syringaldehyde	12.29	181.0506	ND	ND	ND	1.12±0.04*	1.07±0.03*	0.96 ± 0.08

Table 1. LC-MS/MS conditions for analysis of lignin degradation products

LC Parameters		Conditions							MS Parameters	Value
0	Zorbax C18 (3.0 x 100 mm, 1.7 μm)							Ion Polarity	Negative	
Mobile Phase		A: Water with 0.1% acetic acid,							Mass Range	30 - 1,500 m/z
		B: ACN with 0.1% acetic acid							Mass Resolution	30,000
Flow Rate		0.4 IIIL/min						-	Data Dependent Mode	Top 5
Gradient	Time (min)	0	12	15	20	20.1	28	-		15 + 0.05 × (/-) V
	Solvent B (%)	1	45	80	80	1	1		Collision energy	$13 + 0.03 \times (m/z) V$



^aEIC peak area ratio of the PC standard between the two different additives. ^bStandard deviation of peak area ratio (n=3). *Significantly changed compound abundance (P<0.05). Bold indicates compounds significantly increased after CtLac treatment.

- \succ <u>S-unit PCs</u> were detected in <u>hardwoods</u> but <u>not in softwoods</u>, consistent with previous reports on the differential monomer type ratio (H/G/S) of hardwoods and softwoods: hardwood lignin (H/G/S = trace:50:50, GS-lignin) and softwood lignin (H/G/S = 4:96:trace, G-lignin) [1].
- > The CtLac treatment had <u>varying effect</u> on the production of PCs in <u>different types of woody biomass</u>. Notably, cypress showed <u>no significant increase of benzaldehyde chemicals</u> (*p*-hydroxybenzaldehyde and vanillin), which were significantly increased by CtLac treatment in a previous work with grass lignin [2].

In silico MS/MS-based characterization of PCs and LOs in six wood enzymatic degradation products

Table 2. Peak area ratios of *in silico* identified 14 PCs and LOs between CtLac-treated six wood biomass and enzyme blank.

Label	Compound	Retention time	m/z	Peak area ratio	
	Cyprus*				
А	2-Hydroxy-3-phenyl-2-propenal	15.61	147.0447	$25.5^{a}\pm1.1^{b}$	
В	Gingerol	17.36	293.1751	7.00 ± 0.01	
С	Komodoquinone B	14.67	355.0816	42.0 ± 1.4	
D	Glycosmisic acid	15.48	371.1133	37.9±1.1	
	Maple				
Е	4-Hydroxybenzyl alcohol	8.28	123.0449	15.6±1.4	
F	Homovanillic acid	8.29	181.0503	17.1±1.3	
G	C.I. Natural Red 20	12.93	287.092	90.0±5.0	
Н	Sesamolinol	14.01	371.1127	6.99±0.26	
	Pine				
T	Cinnamul alashal	14.02	133 0657	5 50+0.03	

- ► <u>Different types of woods</u> produced <u>unique</u> oligomers with different linkage types by CtLac treatment.
- For cyprus and pine, an increase of (8-5)-type dimer (glycosmisic acid, m/z = 371.1131, RT =



1. By exploiting accurate mass defect filtering specific to lignin, followed by structural elucidation of lignin-derived fragment ions, we effectively isolated and characterized lignin degradation products from <u>complex matrices</u> of biomass degradation product containing lipids and carbohydrates.

2. The changes of degradation products released from wood biomass after CtLac treatment exhibited unique patterns depending on wood divisions (hardwoods and softwoods) and species.

References

1. Yang, Y., Song, W. Y., Hur, H. G., Kim, T. Y., & Ghatge, S. (2019). Thermoalkaliphilic laccase treatment for enhanced production of high-value benzaldehyde chemicals from lignin. International journal of biological macromolecules, 124, 200-208. 2. Hon, D. N., & Shiraishi, N. (2000). Wood and cellulosic chemistry, revised, and expanded. CRC press.