Supporting Information for:

Profiling of Diferulates (Plant Cell Wall Cross-Linkers) Using Ultrahigh-performance Liquid Chromatography-Tandem Mass Spectrometry

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Supplemental Information: Methods

Separation and analysis of diferulates using LC/MS/MS

The LC/MS system consisted of two Prominence LC-20AD (Shimadzu Corp.) pumps connected to a QTRAP 3200 hybrid-linear ion trap mass analyzer (Applied Biosystems) equipped with an electrospray ionization source (ESI). Diferulic acid standards, synthesized Ac-Am and Di-Am diferulates, and NaOH-hydrolyzed and AFEX^{TM}-treated corn stover extracts were analyzed directly using a fused core Ascentis Express C18 (50 × 2.1 mm; 2.7 µm particles) column (Supelco, USA). Binary gradient elution was used with 0.15% aqueous formic acid (A) and methanol (B) under the following conditions and linear gradients: 0-1 min 5% B, increase to 30% B at 20 min; 40% B at 25 min; sudden increase to 99% B at 25.01 min and held until 27 min; returned to initial conditions at 27.01 min and held until 30 min. Injection volume, flow rate and column temperature were 5.0 µL, 0.4 mL/min and 50 °C.

Fragment ions were generated in both positive and negative ion modes for $[M+H]^+$ and $[M-H]^-$ ions using collision induced dissociation (CID) with nitrogen as collision gas, using enhanced product ion scanning. Major product ions generated at collision energy of 20 eV were subjected to a second CID stage in MS^3 scans. Accurate mass measurements were performed using a Waters LCT Premier orthogonal acceleration time-of-flight (TOF) mass spectrometer with the same chromatography system and multiplexed non-selective collision induced dissociation through three collision potentials (15, 45, and 70 V) with spectrum acquisition of 0.2 s/function.[42]

Profiling of process byproducts following AFEX^{TM} pretreatment of corn stover

AFEX^{TM} treated corn stover was extracted with hot water (20 mL/g at 60 °C) for 2 h. The mixture was cooled and filtered through Whatman filter paper (grade 40, pore size 8 µm).

Filtrates were centrifuged for 10 min at 10,000*g,* and supernatant was used directly for LC/MS analysis via injection with no further processing. Electrospray ionization in negative mode was employed for analysis of process byproducts. Nontargeted peak detection, integration, and retention time alignment was performed using Waters MarkerLynx software.

Table S1

Table S1. Fragment ions from CID MS/MS spectra of $[M+H]^+$ from diferulic acid standards using 20 eV collision energy, with suggested consecutive losses of side-chains and relative abundance for each product ion. MS^3 performed on major product ions to support these suggested pathways.

Type of ion	Neutral Loss	*m/z*	8-O-4	8-8 NC	8-8 C	8-5 C	8-5 NC	5-5
$[M+H^+-H_2O]^+$	18	369	10	100			100	
$[M+H^+-2H_2O]^+$	36	351	100	48	24	35		100
$[M+H^+-CO_2]^+$	44	343				40		
$[M+H^+-H_2O-CO]^+$	46	341		43	95		90	
$[M+H^+-CO-CH_3^{\cdot}]^+$	61	326	37					
$[M+H^+-H_2O-CO_2]^+$	62	325	32			100	18	
$[M+H^+-2H_2O-CO]^+$	64	323		40	55	20		10
$[M+H^+-2H_2O-CH_3OH]^+$	68	319						18
$[M+H^+-H_2O-CO-CH_3OH]^+$	78	309					55	
$[M+H^+-2H_2O-CO_2]^+$	80	307				50		
$[M+H^+-H_2O-CO-CO_2]^+$	90	297		49	100			
$[M+H^+-H_2O-CO_2-CH_3OH]^+$	94	293				35		
$[M+H^+-2H_2O-2CH_3OH]^+$	100	287						4
$[M+H^+-H_2O-CO-CO_2-CH_3OH]^+$	122	265		30	54	26		
$[M+H^+-H_2O-C_7H_8O_2]^+$	142	245		71				
$[M+H^+-H_2O-2CO-CO_2-CH_3OH]^+$	150	237			12			
$[263-CO_2]^+$	168	219	33					
$[219-H_2O]^+$	186	201	15					
$[M+H^+-C_{10}H_{10}O_4]^+$	194	193	70					
$[219-CH_3OH]^+$	210	177	15					
$[M+H^+-C_{10}H_{10}O_4-CO_2]^+$	238	149	15					

Figure S1

Figure S1. Enhanced product ion (MS/MS) spectra of $[M+H]^+$ from synthetic diferulate Ac-Am and Di-Am

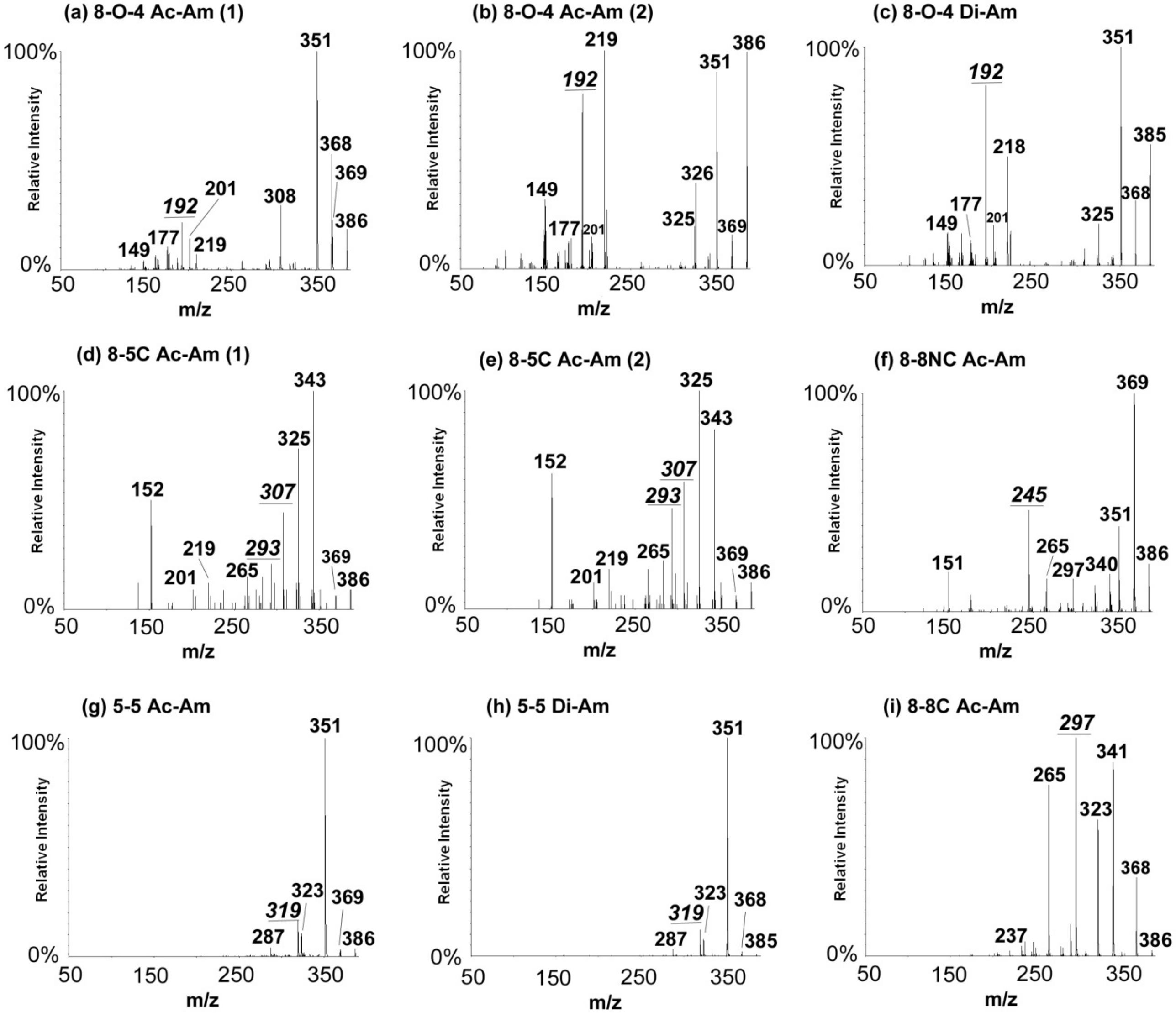
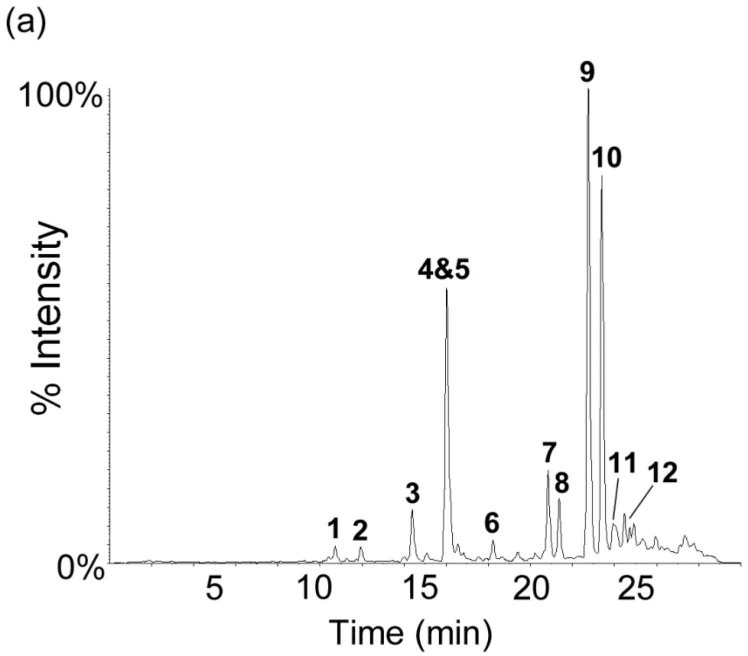


Figure S2

Figure S2. Retention time and high resolution mass measurements of diferulic acids identified in products from NaOH-hydrolyzed corn stover.



Peak #	Retention time	*m/z*	Theoretical Mass	Δm (ppm)^{a}	Compound
1	10.8	387.1062	387.1080	-4.6	8-5C Di-Ac (*cis*)^{b}
2	12.0	387.1069	387.1080	-2.8	Unknown Diferulate^{c}
3	14.5	387.1074	387.1080	-1.5	8-8C Di-Ac
4	16.2	387.1063	387.1080	-4.4	8-8NC Di-Ac
5	16.1	387.1069	387.1080	-2.8	8-5NC Di-Ac
6	18.4	387.1067	387.1080	-3.4	Unknown Diferulate^{c}
7	20.9	387.1068	387.1080	-3.0	5-5 Di-Ac
8	21.6	387.1072	387.1080	-2.1	8-5NC Di-Ac
9	22.9	387.1078	387.1080	-0.5	8-O-4 Di-Ac (*trans*)
10	23.4	387.1075	387.1080	-1.3	8–5C Di-Ac^{b}
11	23.8	387.1062	387.1080	-4.7	8–O–4 Di-Ac (*cis*)^{d}
12	24.8	387.1065	387.1080	-3.9	4–O–5 Di-Ac

a) Δm (ppm)=(measured mass-theoretical mass/measured mass) × 10^{6}
b) The huge retention time difference between the two 8–5C isomers is attributed to differences in intramolecular hydrogen bonds
c) These two diferulates have fragmentation patterns similar to 8–8C
d) Annotated as a *cis*-isomer (one or both double bonds) as abundance increases upon photoirradiation.

Figure S3

Figure S3. CID MS/MS spectra of diferulic acids from NaOH-hydrolyzed corn stover. Labels 1-12 correspond to the peaks in Table S-2

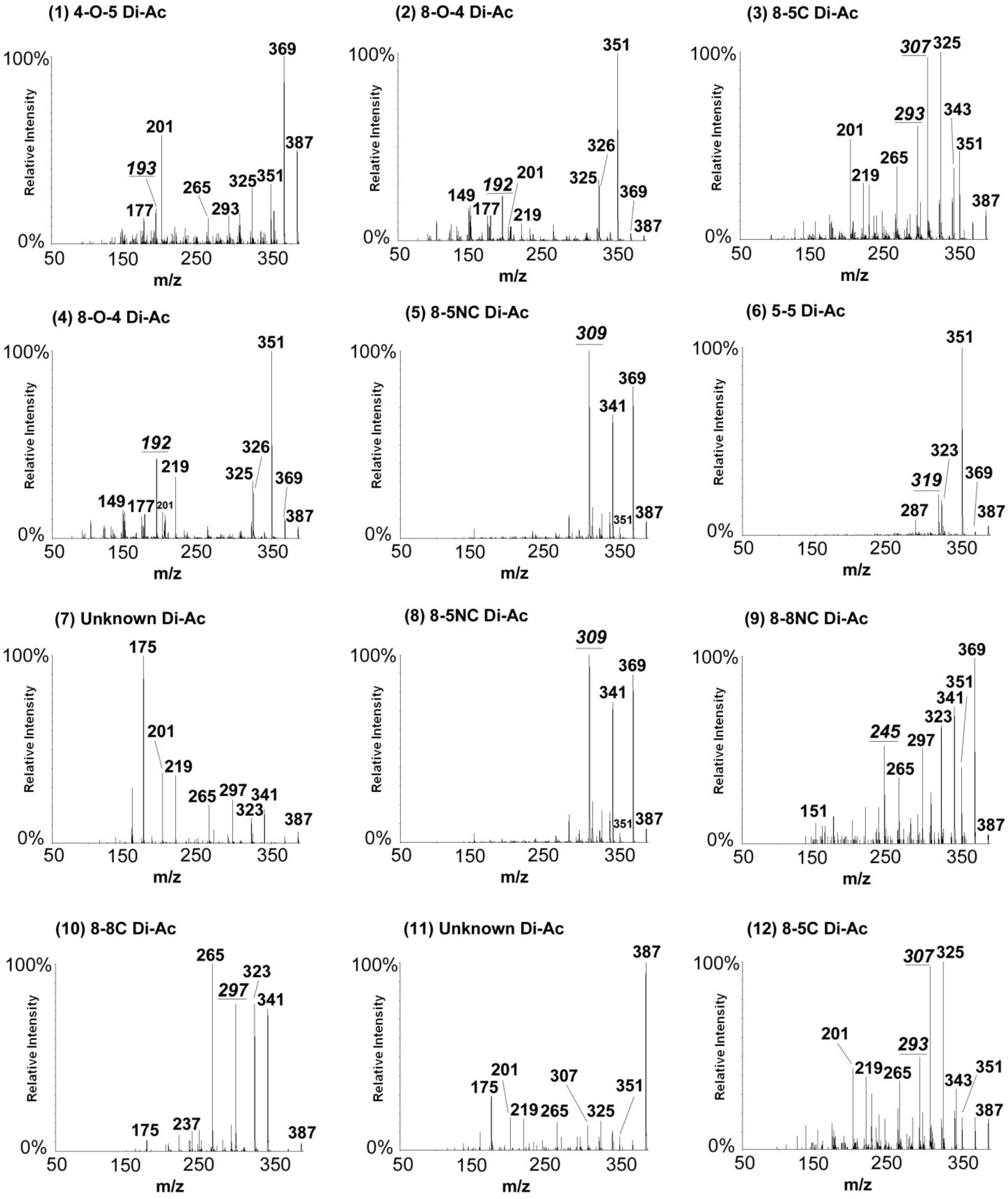
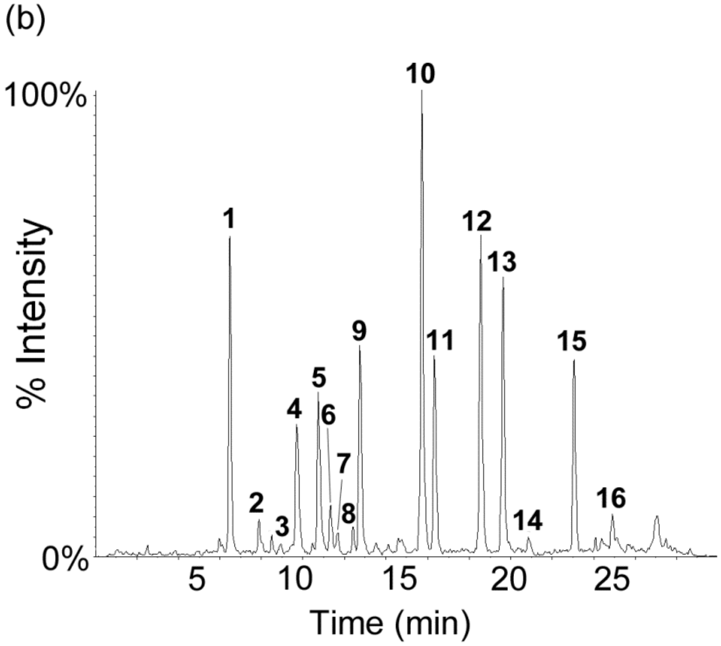


Fig S4

Table S4. Retention time and high resolution mass measurements of Di-Ac, Ac-Am and Di-Am diferulates identified in products from AFEX-treated corn stover.

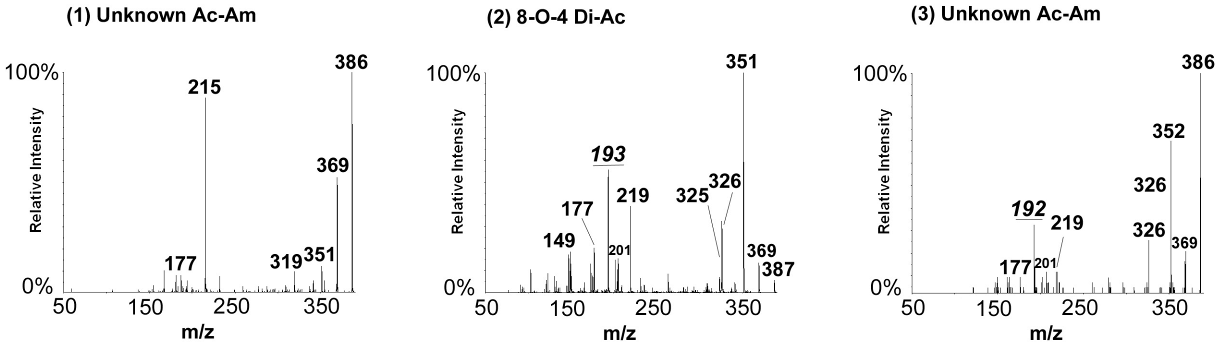


Peak #	Retention time	*m/z*	Theoretical Mass	Δm (ppm)	Compound
1	6.4	387.1547	387.1556[a]	-2.3	Feruloyl amide
2	7.8	385.1390	385.1400	-2.6	8-8C Di-Am
3	8.5	385.1387	385.1400	-3.4	8-5NC Di-Am
4	9.7	386.1230	386.1240	-2.6	8-8C Ac-Am (2)
5	10.7	386.1235	386.1240	-1.3	8-8C Ac-Am (1)
6	11.3	386.1226	386.1240	-3.6	Unknown Diferulate Ac-Am
7	11.6	386.1242	386.1240	-0.5	8-5NC Ac-Am
8	12.4	385.1387	385.1400	-3.3	5-5 Di-Am
9	12.7	386.1231	386.1240	-2.3	8-8NC Ac-Am
10	15.7	385.1392	385.1400	-2.1	8-O-4 Di-Am
11	16.3	386.1233	386.1240	-1.8	5-5 Ac-Am
12	18.5	386.1237	386.1240	-0.8	8-O-4 Ac-Am (2)
13	19.6	386.1235	386.1240	-1.3	8-O-4 Ac-Am (1)
14	20.8	386.1225	386.1240	-3.9	Unknown Diferulate Ac-Am
15	23.0	387.1069	387.1080	-2.8	8-O-4 Di-Ac
16	24.9	386.1222	386.1240	-4.7	Unknown Diferulate Ac-Am

a) This is the mass of a non-covalent dimer ($[2M+H]^+$) of feruloyl amide.
The 387 > 193 MRM transition which was used for 8–O–4-diferulic acid generates signal for this ion.

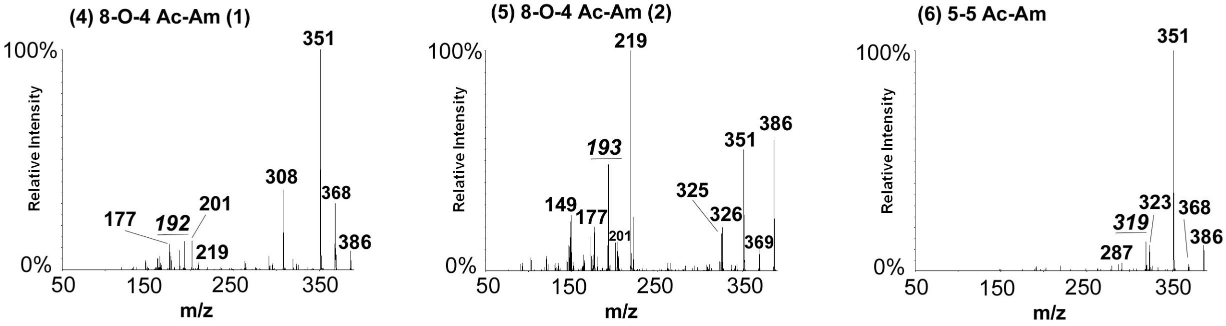
Figure S5

Figure S5. CID MS/MS spectra of Di-Ac, Ac-Am and Di-Am diferulates identified in products from AFEX^{TM}-treated corn stover. Labels 1-16 correspond to the peaks in Table S3.


(1) Unknown Ac-Am

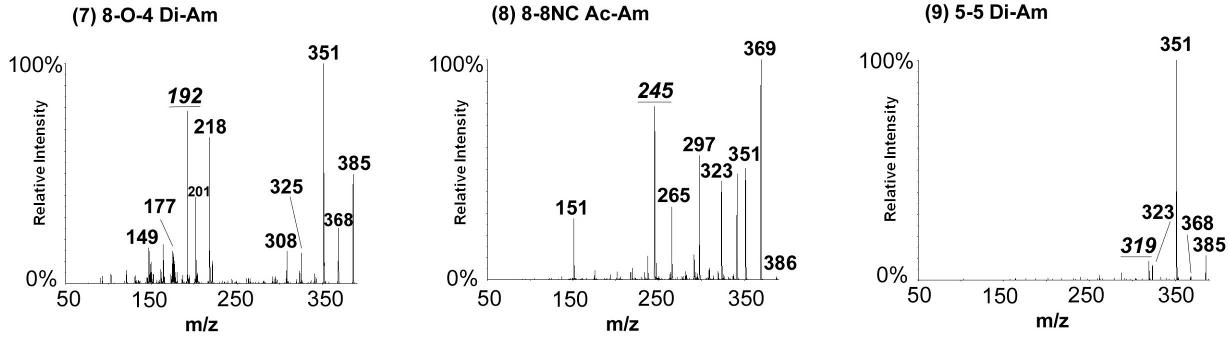
(2) 8-O-4 Di-Ac

(3) Unknown Ac-Am


(4) 8-O-4 Ac-Am (1)

(5) 8-O-4 Ac-Am (2)

(6) 5-5 Ac-Am

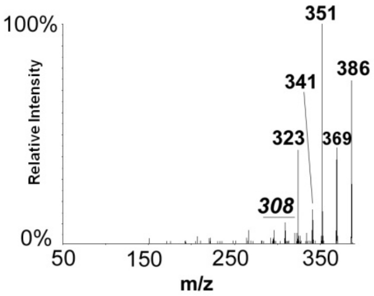

(7) 8-O-4 Di-Am

(8) 8-8NC Ac-Am

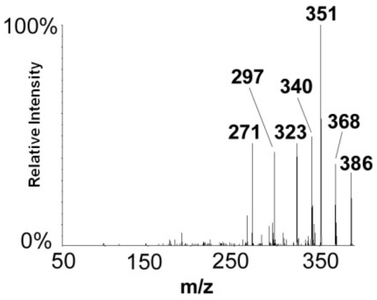
(9) 5-5 Di-Am

Figure S5 (Continued)

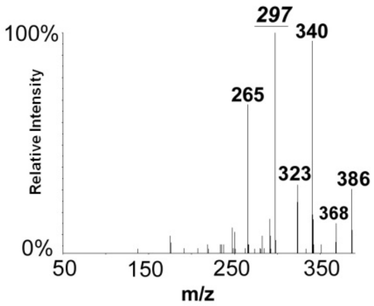
(10) 8-5NC Ac-Am



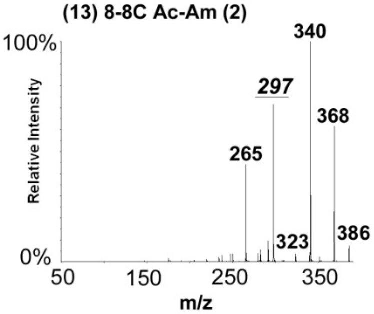
(11) Unknown Ac-Am



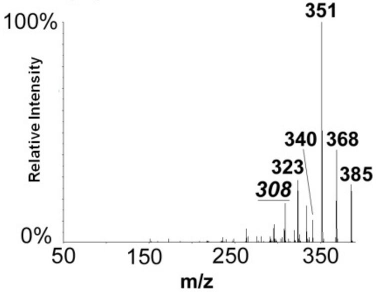
(12) 8-8C Ac-Am (1)



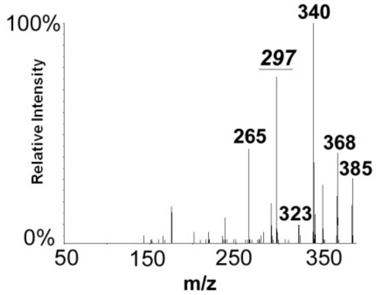
(13) 8-8C Ac-Am (2)



(14) 8-5NC Di-Am



(15) 8-8C Di-Am



(16) Feruloyl amide non-covalent dimer

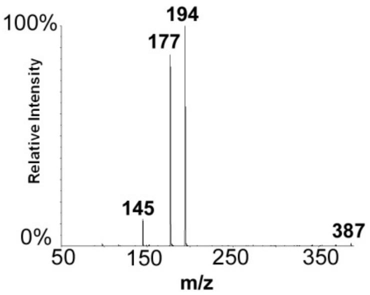
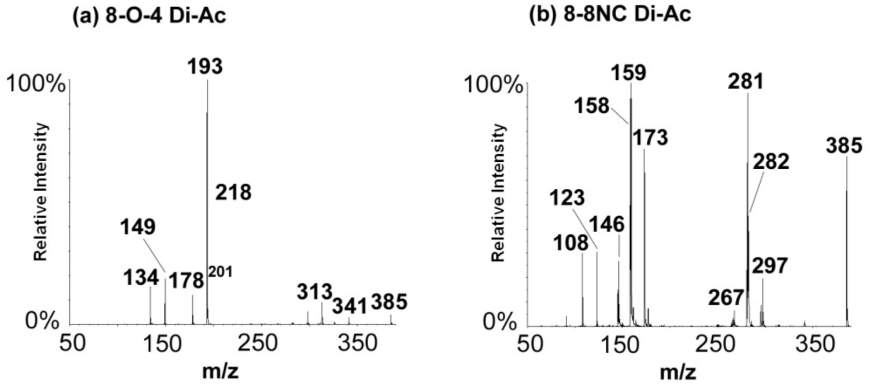
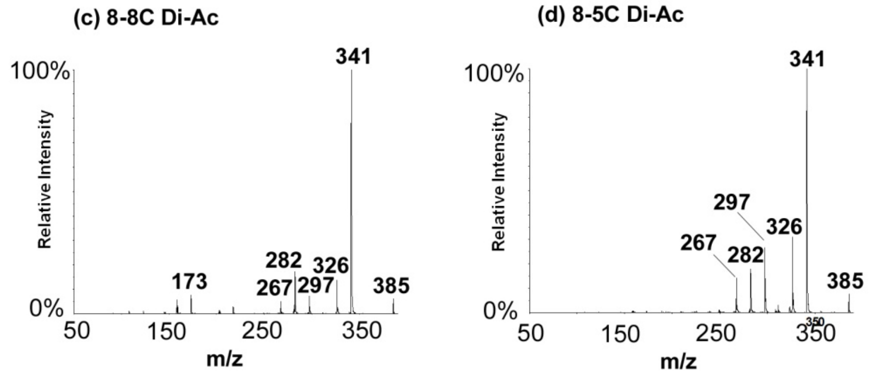


Figure S6

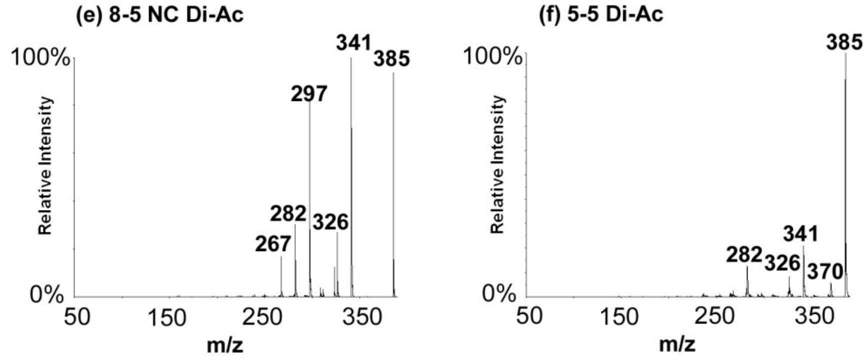
Figure S6. CID MS/MS spectra from [M-H]$^-$ of diferulic acid authentic standards using 20 eV collision energy


(a) 8-O-4 Di-Ac

(b) 8-8NC Di-Ac


(c) 8-8C Di-Ac

(d) 8-5C Di-Ac


(e) 8-5 NC Di-Ac

(f) 5-5 Di-Ac

Figure S7

Figure S7. CID spectra of diferulic acid authentic standards generated using (a) a hybrid linear ion trap (QTRAP 3200), (b) an orthogonal TOF instrument (LCT Premier) with nonselective CID, (c) a triple quadrupole (Quattro Premier XE) and (d) quadrupole/time-of-flight hybrid (QTOF Ultima API). MS/MS spectra are products of $[M+H]^+$.

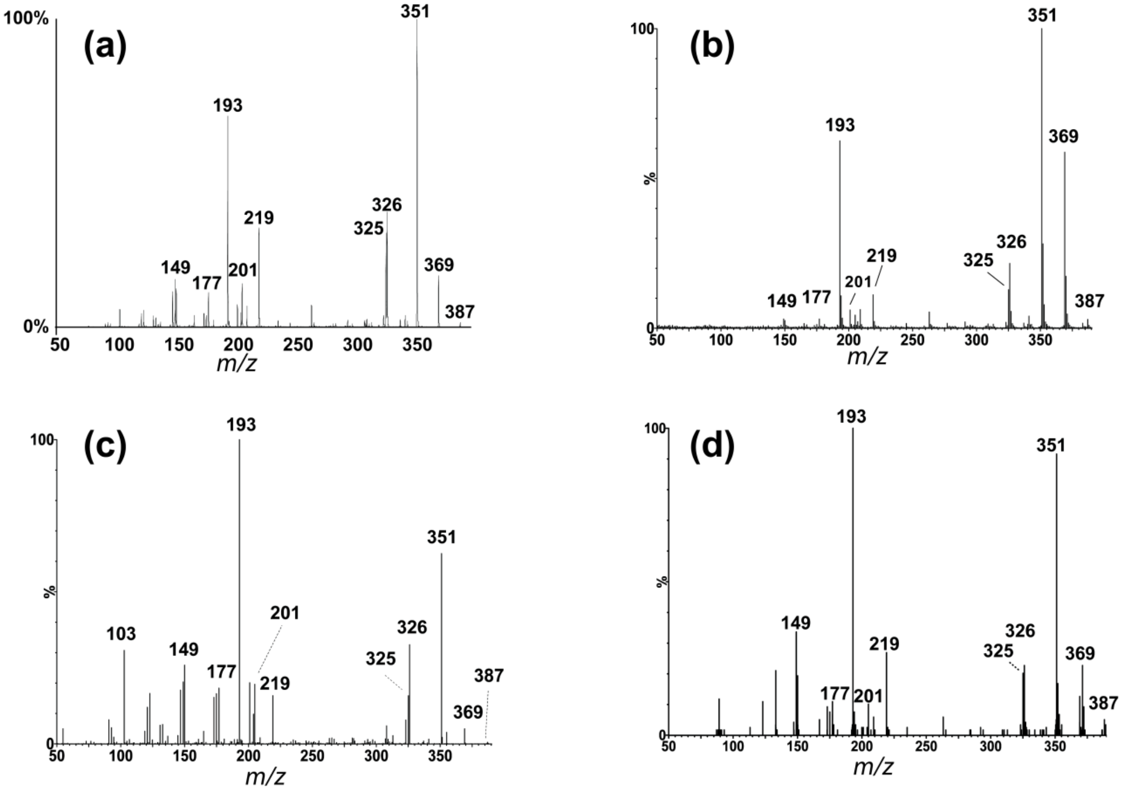
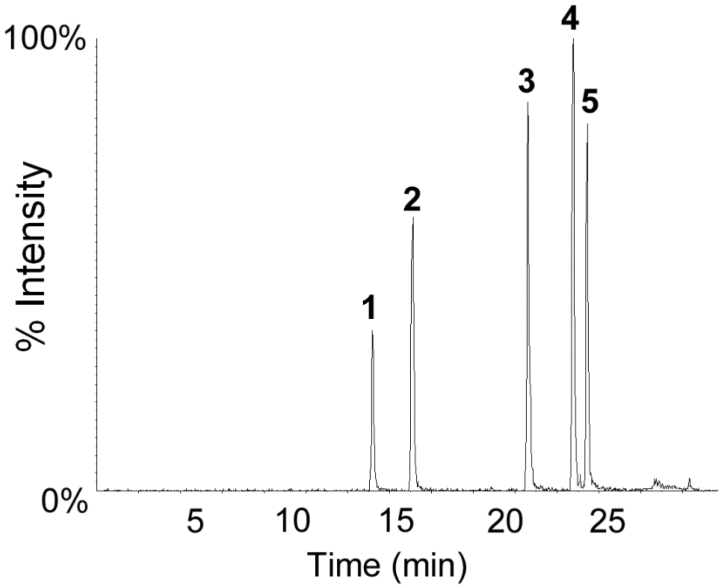


Figure S8

Fig. S8. Extracted ion chromatograms for 5 MRM transitions from five authentic diferulic acid standards at equal concentrations (5 µM; 5 µL injection).



Peak #	Diferulic acid isomer	MRM transition in ESI positive mode	Relative response factor (relative to peak #1)
1	8–8C	387 > 297	1.00
2	8–8NC	387 > 245	1.76
3	5–5	387 > 319	2.37
4	8–O–4	387 > 193	2.47
5	8–5C	387 > 293	1.70