Chemistry & Biology, Volume *22*

Supplemental Information

The Tetracycline Destructases: A Novel Family of Tetracycline-Inactivating Enzymes

Kevin J. Forsberg, Sanket Patel, Timothy A. Wencewicz, and Gautam Dantas

Supplemental Data

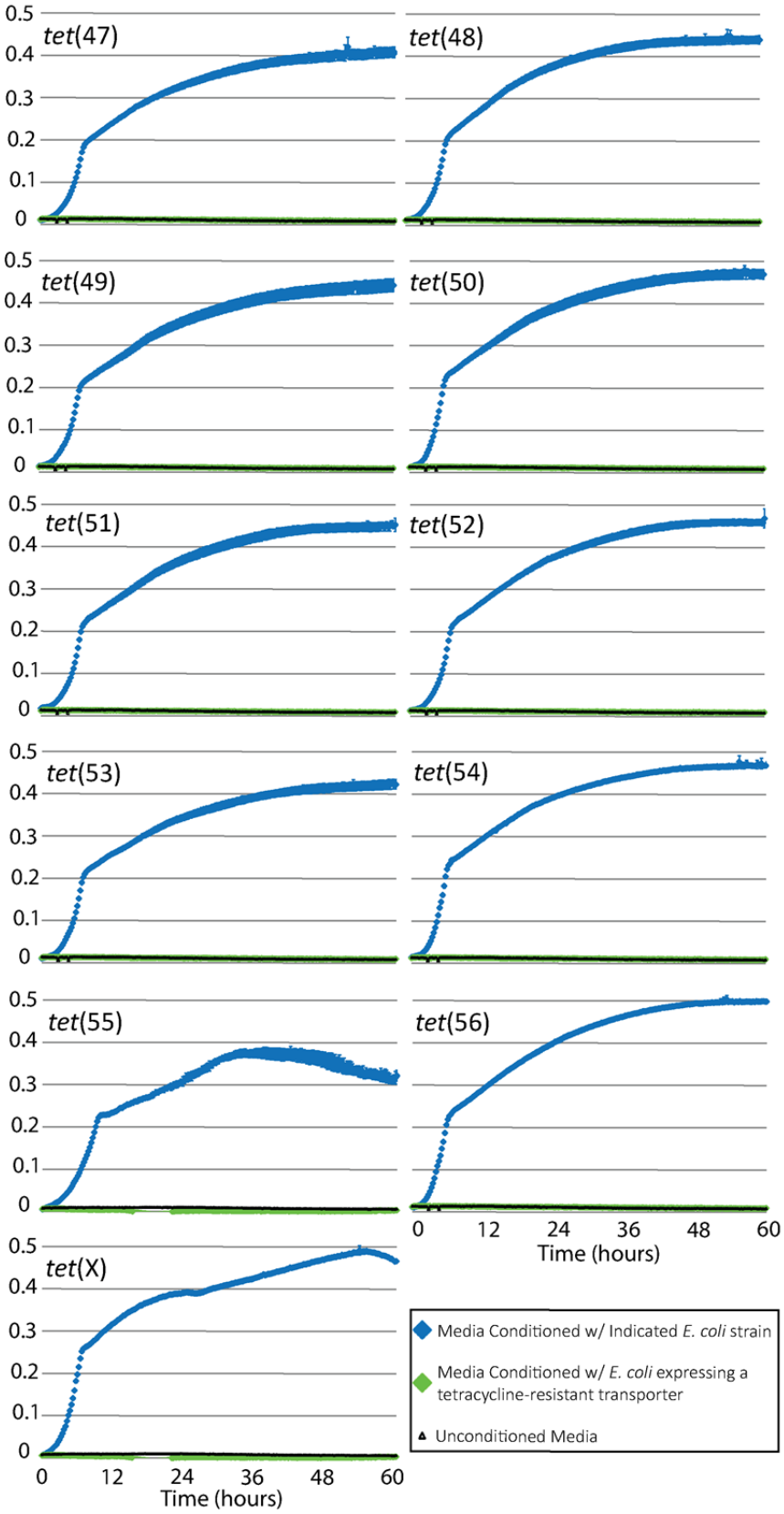


Figure S1, related to figure 1 and main text: Tetracycline-susceptible *E. coli* grown in pre-conditioned, tetracycline-containing media. Y-axes give the absorbance at 600nm, corrected for the background value of a media-only control. Each panel depicts growth of the susceptible strain in media conditioned by *E. coli* expressing (i) the indicated inactivation gene (blue), (ii) a resistant transporter (green), or (iii) unconditioned media (black). The *tet*(X) ORF was used as a positive control for tetracycline inactivation. Values are the average of three trials; error bars depict st. dev.

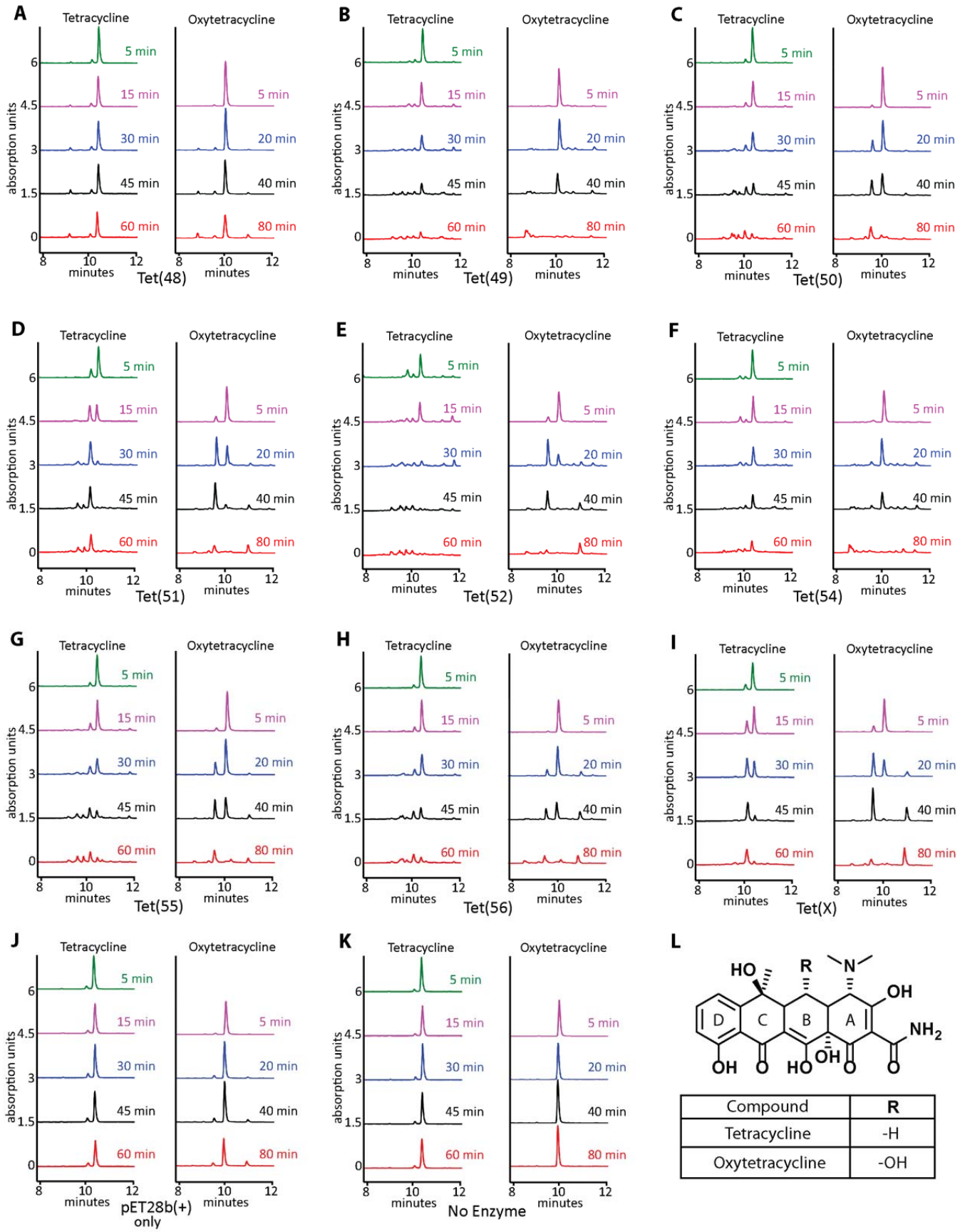


Figure S2, related to figures 3 and 4: Tetracycline and oxytetracycline degradation is catalyzed by diverse flavoenzymes. (A-K) Reverse phase HPLC separation of tetracycline and enzymatically-catalyzed degradation products at 260nm. See figure S3 for absorbance at 363nm. Elimination of antibiotic substrates at both wavelengths indicates that both the β-tricarbonyl (L, ring A) and aryl β-diketone chromophores are disrupted (L, rings B-D).

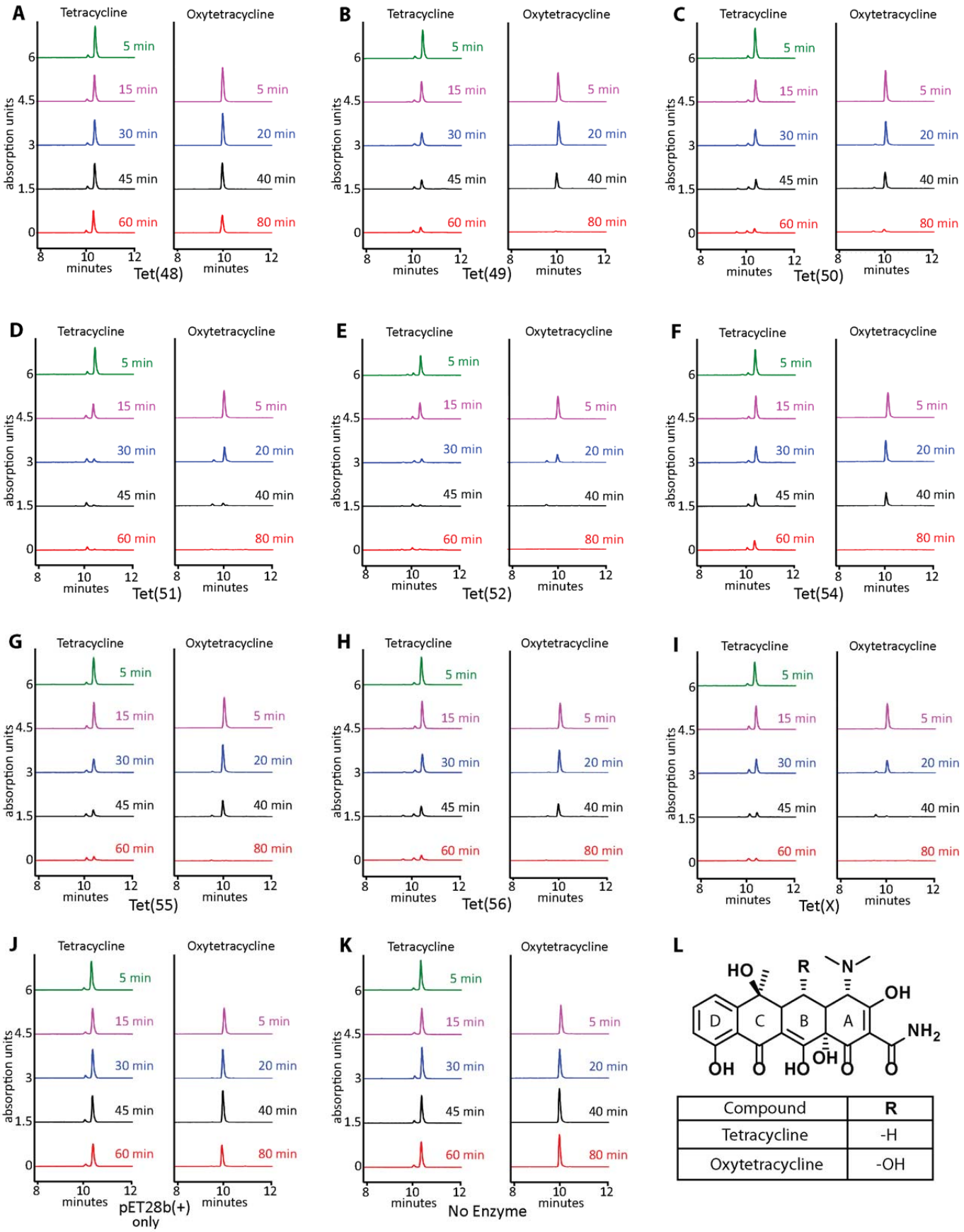
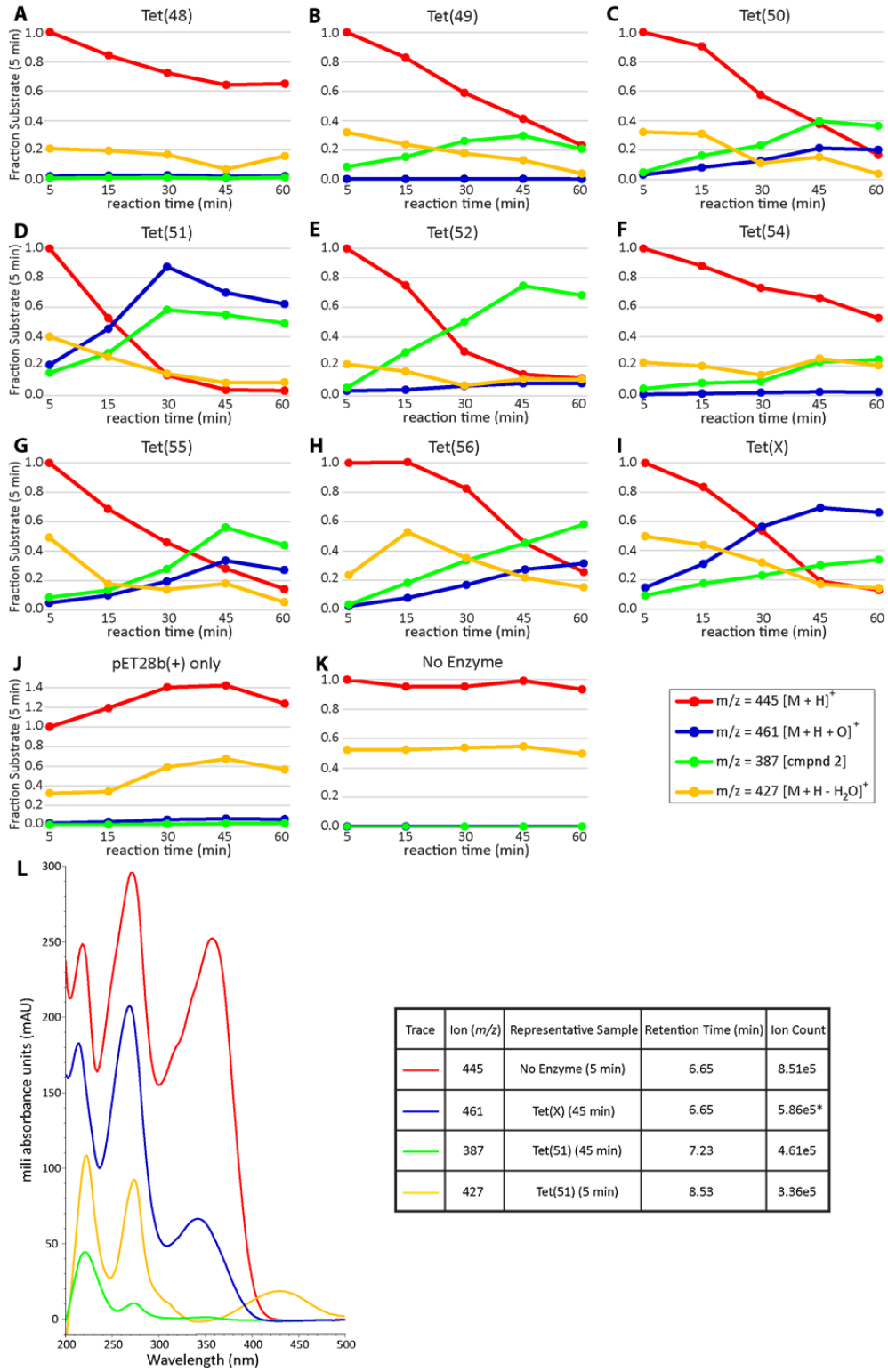


Figure S3, related to figures 3 and 4: Tetracycline and oxytetracycline degradation is catalyzed by diverse flavoenzymes. (A-K) Reverse phase HPLC separation of tetracycline and enzymatically-catalyzed degradation products at 363nm. See figure S2 for absorbance at 260nm. Elimination of antibiotic substrates at both wavelengths indicates that both the β-tricarbonyl (L, ring A) and aryl β-diketone chromophores are disrupted (L, rings B-D).



Trace	Ion (*m/z*)	Representative Sample	Retention Time (min)	Ion Count
	445	No Enzyme (5 min)	6.65	8.51e5
	461	Tet(X) (45 min)	6.65	5.86e5*
	387	Tet(51) (45 min)	7.23	4.61e5
	427	Tet(51) (5 min)	8.53	3.36e5

Figure S4, related to figure 4: Tetracyline degradation yields multiple products. (A-K) The relative proportions of ion counts attributable to tetracycline (*m/z* for $[M+H]^+$ equals 445 in positive ion mode) and associated reaction products (see legend). The data is generated from the reactions using tetracycline depicted in figures S2-S3. Putative structures for the indicated products are depicted in figures 6 and S6. (L) Representative spectra for ions discussed in this manuscript. The asterisk (*) denotes a sample where multiple ions (*m/z* 445 and 461) were not sufficiently resolved by retention time; 78% of the represented sample (based on raw ion counts) can be attributed to the ion with mass 461. Observed retention times are consistent with the expected polarity of the structures depicted in figure S6. The ion with an *m/z* value of 387 does not absorb beyond 280nm, consistent with the loss of conjugation in figure S6.

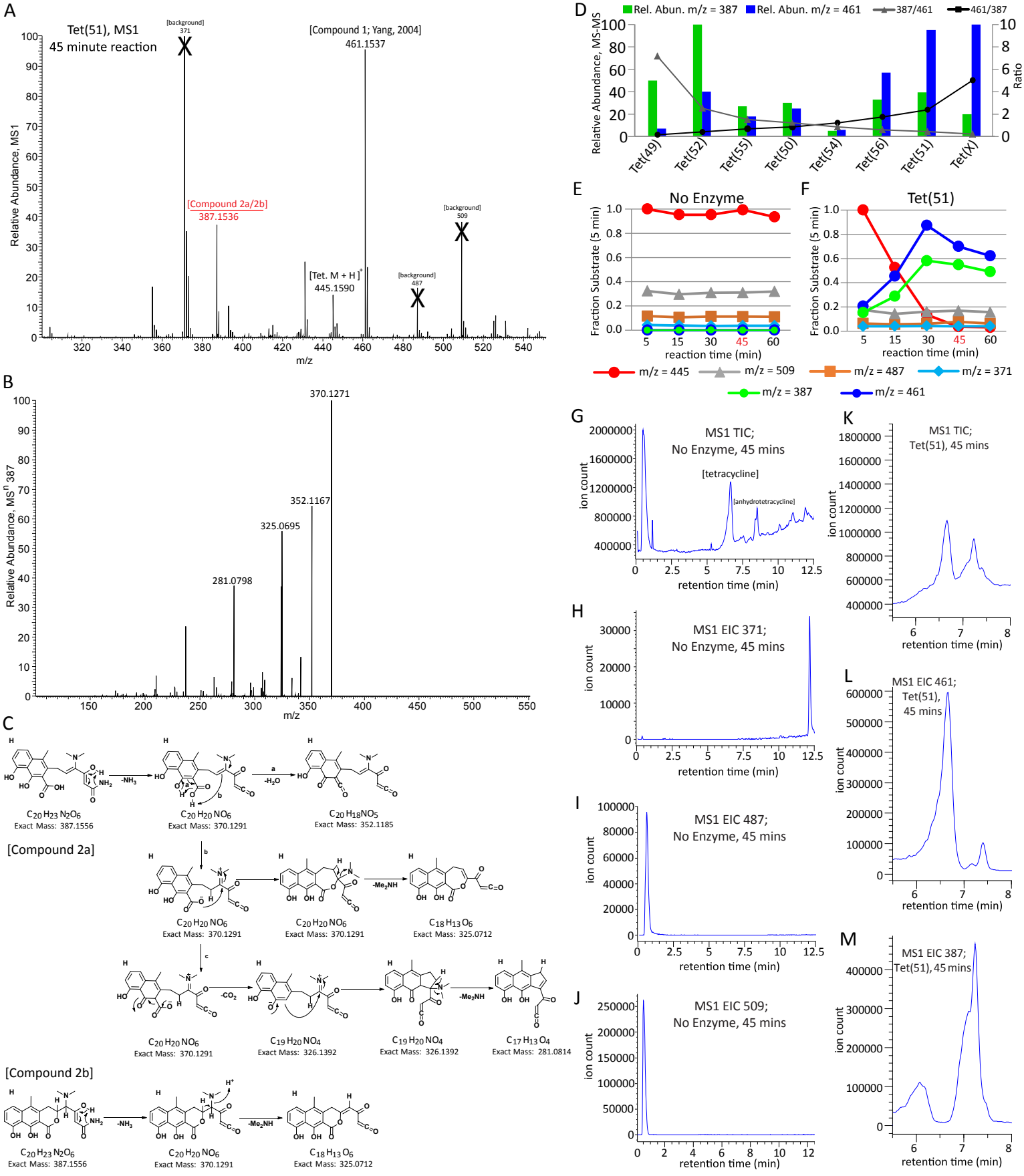

A
Tet(51), MS1
45 minute reaction
[background]
371
[Compound 1; Yang, 2004]
461.1537
[Compound 2a/2b]
387.1536
[Tet. M + H]+
445.1590
[background]
487
[background]
509
Relative Abundance, MS1
m/z
B
370.1271
352.1167
325.0695
281.0798
Relative Abundance, MSn 387
m/z
C
C20 H23 N2O6
Exact Mass: 387.1556
C20 H20 NO6
Exact Mass: 370.1291
C20 H18NO5
Exact Mass: 352.1185
[Compound 2a]
C20 H20 NO6
Exact Mass: 370.1291
C20 H20 NO6
Exact Mass: 370.1291
C18 H13 O6
Exact Mass: 325.0712
C20 H20 NO6
Exact Mass: 370.1291
C19 H20 NO4
Exact Mass: 326.1392
C19 H20 NO4
Exact Mass: 326.1392
C17 H13 O4
Exact Mass: 281.0814
[Compound 2b]
C20 H23 N2O6
Exact Mass: 387.1556
C20 H20 NO6
Exact Mass: 370.1291
C18 H13 O6
Exact Mass: 325.0712
D
Rel. Abun. m/z = 387
Rel. Abun. m/z = 461
387/461
461/387
Relative Abundance, MS-MS
Ratio
Tet(49)
Tet(52)
Tet(55)
Tet(50)
Tet(54)
Tet(56)
Tet(51)
Tet(X)
E
No Enzyme
Fraction Substrate (5 min)
reaction time (min)
F
Tet(51)
Fraction Substrate (5 min)
reaction time (min)
m/z = 445
m/z = 509
m/z = 487
m/z = 371
m/z = 387
m/z = 461
G
MS1 TIC;
No Enzyme, 45 mins
[tetracycline]
[anhydrotetracycline]
ion count
retention time (min)
H
MS1 EIC 371;
No Enzyme, 45 mins
ion count
retention time (min)
I
MS1 EIC 487;
No Enzyme, 45 mins
ion count
retention time (min)
J
MS1 EIC 509;
No Enzyme, 45 mins
ion count
retention time (min)
K
MS1 TIC;
Tet(51), 45 mins
ion count
retention time (min)
L
MS1 EIC 461;
Tet(51),
45 mins
ion count
retention time (min)
M
MS1 EIC 387;
Tet(51), 45 mins
ion count
retention time (min)

Figure S5, related to figures 4 and 5: Flavoenzymes oxidize tetracycline in multiple fashions. (A) Representative MS1 spectra from direct-injection, high resolution tandem MS. All spectra are available in supplementary dataset 1. Peaks marked with an 'X' indicate background ions associated with the reaction, as shown in (E) and (G-J). The product labeled in red was used to generate the fragmentation profile pictured in (B). (B) The MS^n fragmentation profile for the compound with an *m/z* of 387 is consistent with the proposed structure of compounds **2a**/**2b** in figures 6 and S6, as schematized in (C). (D) The relative abundance values and associated ratios for the two major reaction products (*m/z* = 387 and *m/z* = 461), as quantified by high-resolution tandem MS using all tetracycline-active enzymes. The distinct product profiles indicate differential oxidative preferences for the various flavoenzymes. (E-F) Ion counts for background ions and reaction products in tetracycline degradation reactions, from the reactions depicted in figures S2-S4. Background ions are present in all reactions and do not change over time; representative LC-MS traces from the 45 minute timepoint in (E) are depicted in (G-J). Reaction products increase in abundance as reactions progress at the expense of substrate; representative LC-MS traces from the 45 minute timepoint in (F) are depicted in (K-M). The two product peaks in (M) are consistent with the multiple reaction products proposed in figures 6 and S6. TIC; total ion count. EIC; extracted ion count.

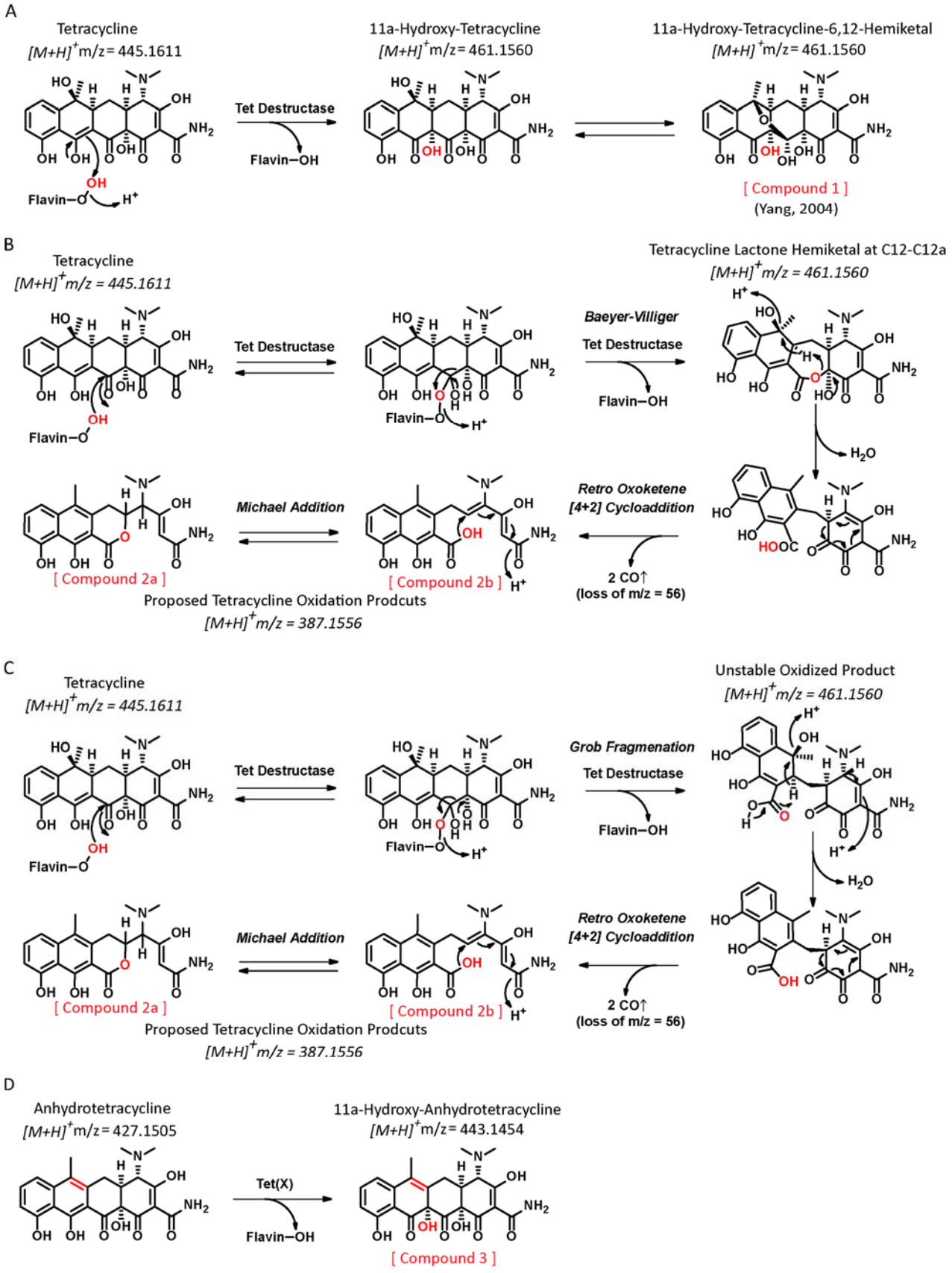


Figure S6, related to figure 6: Reactions schemes for the enzymatic degradation of tetracycline and anhydrotetracycline. (A) Monooxygenation of tetracycline as described in (Yang, 2004). (B) Production of compounds **2a**/**2b** by a Baeyer-Villiger reaction. (C) Production of compounds **2a**/**2b** by a Grob fragmentation. (D) Monooxygenation of anhydrotetracycline, depicted per the activity described in (Yang, 2004).

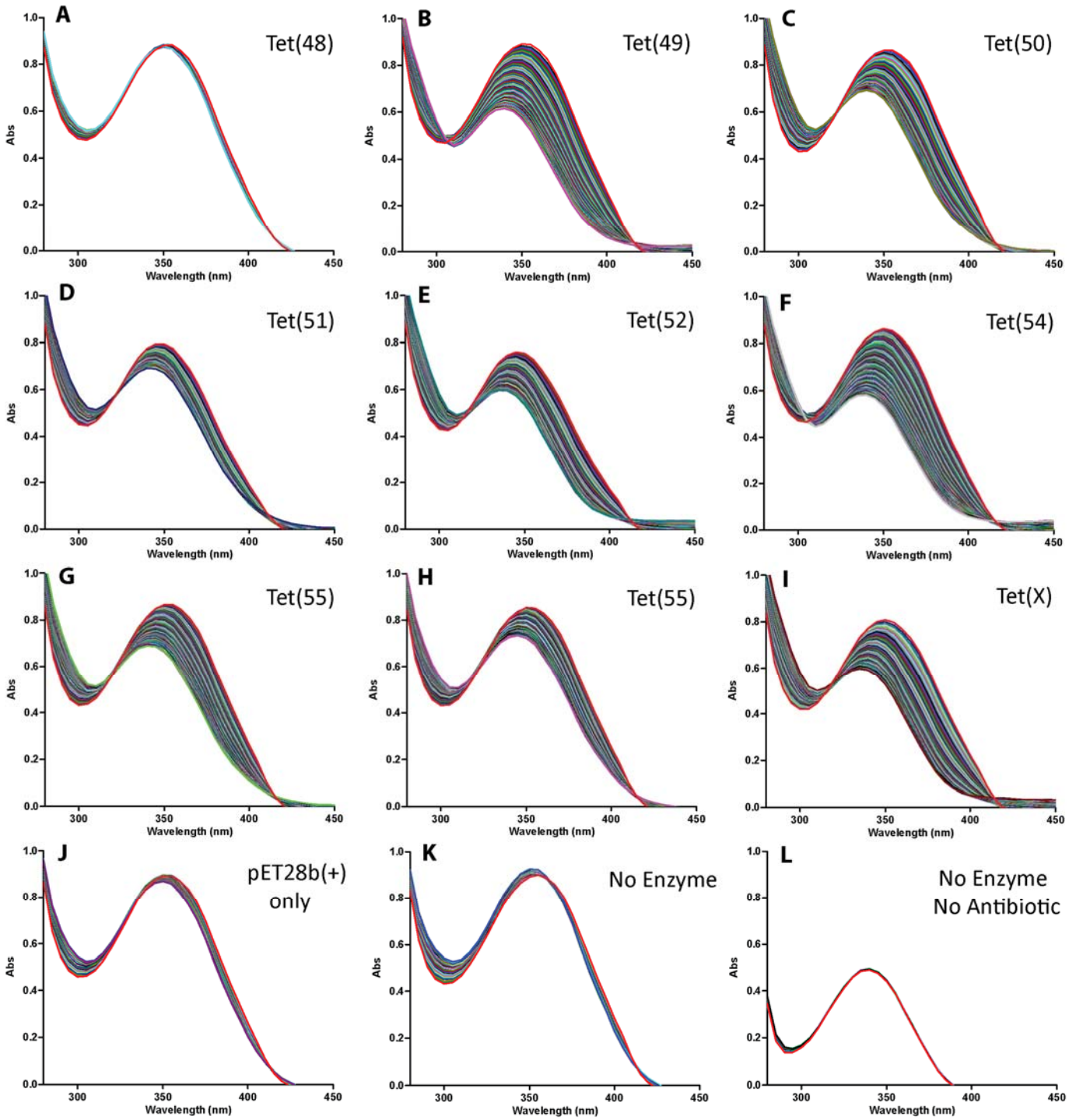


Figure S7, related to figure 3: UV-visible spectrum of enzymatic oxytetracycline degradation. (A-L) Each panel shows the elimination of oxytetracycline over the course of three hours, in a reaction containing the indicated purified enzyme (or control), oxytetracycline, and an NADPH regeneration system. Absorbance scans were taken at one minute intervals. The rainbow pattern depicts a spectral change over time; absorbance at 360nm or 400nm always decreased with time.

Table S1, related to table 1: Metagenomic contigs containing tetracycline-inactivating enzymes, as reported in Forsberg *et. al. Nature*, 2014. For predicted functions depicted below, both Pfam numbers and nucleotide coordinates are given. Genbank accession numbers are given.

Genotype	Soil_Contig	Full Contig Accession Number	Syntenic Antibiotic Resistance Genes	Other Notable Functions Found on Contig
tet(47)	S08_6	KJ693557	none	PF00118: cpn60/GroEL chaperonin (2..928)
tet(48)	S08_12	KJ693683	PF04655: aminoglycoside phosphotransferase (3..344)	PF13310: RhuM family virulence protein (1650..1814)
tet(49)	S11_5	KJ694493	none	
tet(50)	S11_7	KJ694463	none	
tet(51)	S14_2	KJ695235	none	
tet(52)	S14_3	KJ695236	none	PF02517: Putative bacteriocin immunity factor (1..831)
tet(53)	S15_14	KJ695302	none	
tet(54)	S19_4	KJ696048	PF04655: aminoglycoside phosphotransferase (2..430)	PF00239: Resolvase, N terminal domain (2009..2602)
tet(55)	S20_12	KJ696343	none	
tet(56)-2	S17_9	KJ695851	none	

Table S2, related to main text and experimental procedures: Genbank accession numbers for all described tetracycline resistance genes and reaction-specific conditions for their amplification from metagenomic selections via PCR.

Gene Name	Gene Length (bp)	Accession No.	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	Annealing Temp.	Extension Time (min)
tet(47)	1248	KR857681	ATGCGCTGTCATTTCCGATG	TCATTCATAATCCGGTAACGAAAT	56C	1.5
tet(48)	1200	KR857682	ATGACATTCCTATTTAAGGAATTTAAAGGT	TTATTTATAGGCTGAATACTCAGGTAATTT	57C	1.5
tet(49)	1164	KR857683	ATGTCTGCAATAAATAAAATTCTCGTAATA	TTATTCATAATTAGGTAACGAAATCATAT	55C	1.5
tet(50)	1167	KR857684	ATGACTAAACATATAAAAATTCTTGTAATAGGG	TTATTCGTATTGTGGCAAGTTTATTGAATTAG	57C	1.5
tet(51)	1164	KR857685	ATGCCTATCATAAATAAAATTCTGGTAATA	TCATTCATAATTCGGCAACG	55C	1.5
tet(52)	1173	KR857686	ATGTCTAATGTGAATAAAATTCTCG	TCAGTGCGGATTGGCGTA	55C	1.5
tet(53)	1164	KR857687	ATGTCTACTATAAATAAAATTCTCGTGATCGG	TCATTCATACTCTGGCAACGAAAT	59C	1.5
tet(54)	1167	KR857688	ATGTCTACTATAAAAAAAATTCTCGTG	TCATTCATACTCAGGCAGCTT	55C	1.5
tet(55)	1158	KR857689	ATGCCCCACACCAAAAAAAT	TTAATACTCCGGCAACTCGAT	57C	1.5
tet(56)	1170	YP_003456135	ATGTCTAAAAATATCAAAATTCTCGTC	CTATGATGATTCATATTGAGGTAAGG	57C	1.5
tet(X)	1167	JQ990987	ATGACAATGCGAATAGATACAGA	TTATACATTTAACAATTGCTGAAACG	55.4C	1.5
Tetracycline-Resistant Transporter	1506	KF630260	ATGGAGAACACTCGTATGTCTT	TCAGGCCGCTGTTCG	57C	2

Table S3, related to experimental procedures: Experimental details for detecting tetracycline inactivation via growth assays in conditioned-media. N.G. indicates no growth was observed.

Tetracycline Resistance Gene	Batch	OD600 of starter culture	Volume of starter culture in 10ml	Time Media Conditioned (hrs)	OD600 at filtration
tet(47)	1	0.41	10µl	24	0.72
tet(48)	1	0.4	10µl	24	0.56
tet(49)	1	0.37	10µl	24	0.72
tet(50)	1	0.54	10µl	24	0.97
tet(51)	1	0.43	10µl	24	0.64
tet(52)	1	0.36	10µl	24	0.7
tet(53)	1	0.35	10µl	24	0.75
tet(54)	1	0.41	10µl	24	0.66
tet(55)	2	0.45	10µl	42	0.59
tet(56)	1	0.17	20µl	24	0.77
tet(X)	2	0.43	10µl	42	0.61
Ctrl: Tetracycline resistant transporter (batch #1)	1	0.51	10µl	24	0.49
Ctrl: Tetracycline resistant transporter (batch #2)	2	0.46	10µl	42	0.53
Ctrl: None (unconditioned media, batch #1)	1	0.32	10µl	24	N.G.
Ctrl: None (unconditioned media, batch #2)	2	0.47	10µl	42	N.G.

Table S4, related to experimental procedures: Experimental details for quantifying tetracycline levels in media supporting the growth of various tetracycline resistance genes. N.G. indicates no growth was observed.

Tetracycline Resistance Gene	OD600 of starter culture	Volume of starter culture in 10ml	Time Media Conditioned (hrs)	OD600 at filtration
tet(47)	0.97	14.17µl	48	0.45
tet(48)	0.99	13.89µl	48	0.45
tet(49)	0.91	15.11µl	48	0.35
tet(50)	1.38	10µl	48	0.43
tet(51)	0.97	14.17µl	48	0.50
tet(52)	0.74	18.58µl	48	0.55
tet(53)	0.94	14.63µl	48	0.41
tet(54)	0.88	15.63µl	48	0.28
tet(55)	0.87	15.81µl	48	0.61
tet(56)	0.92	14.95µl	48	0.43
Ctrl: Tetracycline resistant transporter	0.95	14.47µl	48	0.39
Ctrl: None (unconditioned media)	1.0	13.75µl	48	N.G.

Table S5, related to experimental procedures: Optimized parameters for MRM transitions used to detect both tetracycline and the internal standard PIPES from minimal growth media.

Q1 MS	Q3 MS	Time (msec)	ID	Declustering Potential (Volts)	Collision Energy (Volts)
445.3	410.2	150	Tetracycline	100	28
445.3	154.1	150	Tetracycline	60	37
445.3	98.1	150	Tetracycline	65	60
303	195.2	150	PIPES	100	33
303	152.1	150	PIPES	100	33
303	70.1	150	PIPES	100	33

Table S6, related to experimental procedures: Reaction-specific conditions for the addition of BamHI and NdeI cut sites to tetracycline resistance genes via PCR amplification.

Gene Name	Gene Length (bp)	Accession No.	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	Annealing Temp.	Extension Time (min)
tet(47)	1248	KR857681	TAATACATATGATGCGCTGTCATTTCCGATG	TAAATGGATCCTCATTCATAATCCGGTAACGAAAT	56C	1.5
tet(48)	1200	KR857682	TAATACATATGATGACATTCCTATTTAAGGAATTTAAAGGT	TAATTGGATCCTTATTTATAGGCTGAATACTCAGGTAATTT	57C	1.5
tet(49)	1164	KR857683	TAATACATATGATGTCTGCAATAAATAAAATTCTCGTAATA	TAATTGGATCCTTATTCATAATTAGGTAACGAAATCATAT	55C	1.5
tet(50)	1167	KR857684	TAATACATATGATGACTAAACATATAAAAATTCTTGTAATAGGG	TAATTGGATCCTTATTCGTATTGTGGCAAGTTTATTGAATTAG	57C	1.5
tet(51)	1164	KR857685	ATAATCATATGATGCCTATCATAAATAAAATTCTGGTAATA	TAATTGGATCCTCATTCATAATTCGGCAACG	55C	1.5
tet(52)	1173	KR857686	ATTATCATATGATGTCTAATGTGAATAAAATTCTCG	TTTATGGATCCTCAGTGCGGATTGGCGTA	55C	1.5
tet(53)	1164	KR857687	TAATACATATGATGTCTACTATAAATAAAATTCTCGTGATCGG	TAATTGGATCCTCATTCATACTCTGGCAACGAAAT	59C	1.5
tet(54)	1167	KR857688	TAATACATATGATGTCTACTATAAAAAAAATTCTCGTG	TAATTGGATCCTCATTCATACTCAGGCAGCTT	55C	1.5
tet(55)	1158	KR857689	TAATACATATGATGCCCCACACCAAAAAAAT	TAATTGGATCCTTAATACTCCGGCAACTCGAT	57C	1.5
tet(56)	1170	YP_003456135	ATTAACATATGATGTCTAAAAATATCAAAATTCTCGTC	TAATTGGATCCCTATGATGATTCATATTGAGGTAAGG	57C	1.5
tet(X)	1167	JQ990987	TAATACATATGATGACAATGCGAATAGATACAGA	TAATTGGATCCTTATACATTTAACAATTGCTGAAACG	55C	1.5

Table S7, related to experimental procedures: Details of enzyme purification. For the empty-vector control, a percent extinction coefficient of 10 was used to estimate total background protein. OD600 indicates optical density, i.e. the absorbance of the culture at 600nm light. NA; not applicable.

Genotype	OD600 at induction	OD600 at harvesting	Enzyme Mol Wt (KDa)	A280 Ext. Coefficient	A280	Concentration (mg/ml)	Volume Recovered (ml)	Amt. Recovered (mg)
Tet(47)	0.25	0.73	43.95	40340	Purification Unsuccessful			
Tet(48)	0.23	0.46	45.00	44350	2.149	2.18	1.25	2.73
Tet(49)	0.23	0.60	43.79	42860	5.538	5.66	1	5.66
Tet(50)	0.24	0.87	43.63	42860	28.276	28.78	0.75	21.59
Tet(51)	0.22	0.37	43.97	41370	3.733	3.97	1.4	5.55
Tet(52)	0.29	0.66	44.10	34380	6.865	8.81	1.5	13.21
Tet(53)	0.33	0.58	43.62	42860	Purification Unsuccessful			
Tet(54)	0.35	0.57	44.07	49850	4.636	4.1	1.1	4.51
Tet(55)	0.31	1.02	42.92	45840	39.474	37	0.75	27.75
Tet(56)	0.47	0.77	44.02	42860	19.561	20.1	1.7	34.17
Tet(X)	0.43	0.70	43.71	36440	25.768	30.9	1.2	37.08
empty pET28(b)+	0.37	0.71	NA	ε1% = 10.0	8.839	9	1.1	9.9

Supplemental Experimental Procedures

Sub-Cloning of Tetracycline Resistance Genes into pZE21

For each soil-derived, putative FAD-dependent oxidoreductase, the full-length ORF was subcloned from the purified PCR-product that resulted from amplifying the resistance-conferring metagenomic DNA fragments in original the tetracycline selections (Forsberg et al., 2014). PCRs were performed in a total volume of 25µl using the following reagents: 1µl of template (amplified PCR product), 1µl of 10mM dNTPs (NEB, Ipswich, MA), 2.5µl of 10x ThermoPol buffer (NEB), 0.5µl of Taq polymerase (NEB, 5U/µl), and 1µl each of forward and reverse primer, at 10µM. All reactions were incubated at 94ºC for 10 minutes, subjected to 35 cycles of: (i) 94ºC for 45 seconds, (ii) reaction-specific annealing temperature for 45 seconds, and (iii) 72ºC for a reaction-specific extension time, and finally incubated at 72ºC for 10 minutes before being held at 4ºC. Target-specific accession numbers and reaction-specific primer sequences/cycling conditions are indicated in table S2.

The *Legionella* homolog was cloned from a genomic DNA preparation from *L. longbeachae* (ATCC 33462). Lawns of *L. longbeachae* grown on charcoal yeast extract agar plates were given generously by Dr. Joseph Vogel (Washington University in St. Louis) and cells collected by scraping and combining growth from three agar plates. Cells were pelleted and genomic DNA extracted via bead-beating and phenol-chloroform extraction. Cell pellets were re-suspended in 500µl "Buffer A" (200mM NaCl, 200mM Tris, 20mM EDTA), and 210µl of 20% SDS and 500µl of phenol:chloroform:IAA (25:24:1, pH7.9) were added to the mixture. The solution was then added to 250µl of 0.1mm zirconium beads (BioSpec Products, Bartlesville, OK, cat#1107910) before cooling on ice for five minutes. Cells were lysed by bead-beating with the mini bead-beater 24 (BioSpec Products, cat#112011) at maximum intensity for two minutes, cooled on ice for two minutes, and beat again for two minutes. Lysed cells were spun at 6800 rcf at 4ºC for three minutes and the aqueous phase transferred to a pre-spun phase-lock gel tube (5Prime, Gaithersburg, MD, cat#2302820). One volume of phenol:chloroform:IAA (25:24:1), pH7.9 was added to the tube and mixed by inversion. Tubes were then spun at 20800 rcf at room temperature for five minutes. The aqueous phase was transferred to a new microcentrifuge tube and one volume of -20ºC isopropanol and 1/10th volume 3M NaOAc (Ambion, Austin, TX, cat#AM9740, pH 5.5) added. After overnight storage at -20ºC, the tube was spun in a pre-chilled (4ºC)

tabletop centrifuge before a wash with 500µl of room-temperature ethanol was performed. After washing, the tube was spun down a final time, residual ethanol removed by evaporation, and the DNA pellet re-suspended in 50µl of warm, nuclease-free H_2O. An aliquot of this genomic DNA preparation (2µl, 340ng) was used for PCR-amplification of the target homolog with the conditions outlined in the preceding paragraph, and according to the reaction-specific parameters outlined in table S2.

Both control genotypes (*tet(X)* and the tetracycline-resistant transporter) were amplified via PCR using template extracted from functional selections with fecal metagenomic libraries. Templates were extracted exactly as outlined previously for selections with soil libraries (Forsberg et al., 2014). The transporter was amplified from accession #KF630260, as described previously and with reaction-specific and gene-specific information provided in table S2. We PCR-amplified *tet(X)* (sequence identical to accession #JQ990987) using 2.5µl of template and the PFX DNA polymerase, per suggested methods (http://tools.invitrogen.com/content/sfs/manuals/platinumpfx_pps.pdf), and according to the details provided in table S2.

All purified PCR products were cloned 9bp downstream of the ribosome binding site within the pZE21 expression vector(Lutz and Bujard, 1997) and transformed into *E. coli* MegaX cells (Invitrogen, Carlsbad, CA). To facilitate cloning, Taq-amplified PCR products were blunted using the END-IT enzyme mix (Epicentre, Madison, WI cat#ER0720) and ligated to the expression vector using the Fast-Link DNA ligation kit (Epicentre, cat#LK11025). PCRs performed with PFX (a blunt-end polymerase) were phosphorylated (rather than blunted) prior to ligation using T4 PNK (Epicentre, cat#P0505H) per manufacturer's recommendations.

All sub-cloned genes were verified to confer tetracycline resistance when expressed in *E. coli* MegaX at a minimum of 16µg/ml and subjected to Sanger sequencing to confirm appropriate sequence and orientation. In some instances, small discrepancies between the confirmatory Sanger sequence and the original Illumina-generated, assembled sequence were observed (Forsberg et al., 2014), none of which impacted resistance-conferring activity when expressed in *E. coli*. In two constructs (*tet*(47) and *tet*(48)), multiple in-frame start codons were present in the assembled metagenomic contigs which resulted in several potential open reading frames for which *a priori* predictions of optimality were not obvious. In both cases, the longest potential open

reading frame was cloned from its metagenomic source (1248bp and 1200bp for *tet*(47) and *tet*(48), respectively); accession numbers corresponding to the exact sequences used in this study are reported in table S2. Accession numbers for the original metagenomic contigs are reported in table S1.

Determination of Tetracycline Minimum Inhibitory Concentrations

Determination of all minimum inhibitory concentrations was performed in liquid media using Mueller-Hinton broth (2g Beef Infusion Solids, 1.5g Starch, 17.5g Casein hydrolysate, pH 7.4, in a final volume of 1L) supplemented with 50µg/ml kanamycin (the resistance marker on the pZE21 expression vector) and tetracycline at the reported concentrations. All minimum inhibitory concentrations were performed in a final volume of 200µl, with a 2.5% initial cellular inoculum. Growth of *E. coli* transformants was profiled via absorbance measurements at 600nm (OD600) using the Synergy H1 microplate reader (Biotek Instruments, Inc, Winooski, VT) for a minimum of 48 hours at 37ºC. Cultures were protected from light. MIC determinations were performed using *E. coli* strains transformed with all soil-derived tetracycline inactivation genes and the homolog from *L. longbeachae*.

Tetracycline Media-Darkening Growth Assays

E. coli transformants expressing either the tetracycline-resistant transporter (control) or a putative tetracycline-inactivating protein were grown in 8ml of Luria-Bertani (LB) broth (5g Yeast Extract, 5g NaCl, 10g of Tryptone in 1L H2O) at 37ºC for 4 days, protected from light, and shaking at 225 rpm. Cultures were inoculated with 10µl of starter culture grown to an optical density that ranged from 0.35 to 0.54 (excepting the transformant expressing the ORF from *L. longbeachae*, for which 20µl of starter culture with an optical density of 0.17 was used). To the cultures indicated in figure 2, tetracycline was added at a concentration of 100µg/ml, excepting the *tet*(55) construct, as this drug concentration inhibited the growth of this transformant. For this culture only, 32µg/ml tetracycline was used. After 96 hours, images were taken using a standard digital camera.

Detecting Tetracycline Inactivation with Conditioned-Media Growth Assays

To test for the ability of the *E. coli* transformants expressing the various tetracycline resistance proteins to inactivate the antibiotic, we grew each transformant in tetracycline-containing media, filtered out cells, and compared the cytotoxicity of the spent supernatant to either (i) unconditioned media, or (ii) media conditioned

with an *E. coli* transformant encoding a tetracycline-resistant transport protein. To condition media, an inoculum of log phase *E. coli* transformants was introduced to 10ml of Mueller-Hinton broth containing 50μg/ml kanamycin (the resistance marker on the pZE21 expression vector) and 8μg/ml tetracycline. The inoculated cells were determined to be in log phase via absorbance measurements at OD600 using the Synergy H1 microplate reader (Biotek Instruments, Inc) every 15 minutes and were introduced into fresh media at the densities and volumes outlined in table S3. Cultures were grown until reaching an OD600 measurement of ~0.5-1.0, at which point cells were pelleted and the supernatant passed through a 0.2μM filter. Due to variable growth rates, cultures used to condition media were grown for different periods of time to reach the appropriate cellular density: one batch was grown for 24 hours while a second was allowed to progress 42 hours. As detailed in table S3, the appropriate negative controls (unconditioned media and transporter-conditioned media) were repeated for both intervals. To verify that the initial concentration of tetracycline (8μg/ml) entirely prevented the growth of our tetracycline-susceptible *E. coli* strain (MegaX + an empty pZE21 vector), the control strain was inoculated into Mueller-Hinton broth containing 50μg/ml kanamycin and 8μg/ml tetracycline, per table S3. No growth was observed (table S3), confirming the potency of the tetracycline antibiotic.

After conditioning and filtration, supernatants were used as growth media for the tetracycline-susceptible *E. coli* strain. In all cases, growth assays were performed in a final volume of 225μl supernatant inoculated with 5μl of tetracycline-susceptible *E. coli* at an OD600 value of 0.6. Growth was profiled via absorbance measurements at 600nm using the Synergy H1 microplate reader (Biotek Instruments, Inc) every 15 minutes for a minimum of 60 hours at 37ºC. Cultures were protected from light. Absorbance values were corrected for the background absorbance of the filtered supernatant by subtracting measurements of un-inoculated controls from those receiving inoculum.

Quantifying Tetracycline Levels via Liquid Chromatography-Tandem Mass Spectrometry

To confirm *E. coli* transformants expressing the various tetracycline resistance proteins inactivate the antibiotic, levels of the drug were quantified in growth media by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Strains were grown in 10ml of M9 minimal media (1X M9 minimal salts [Sigma, St. Louis, MO, cat #M6030], 2mM $MgSO_4$, 100μM $CaCl_2$) supplemented with 9g/L glucose, 100mg/L thiamine

(Sigma #T4625), 100mg/L leucine (Sigma #L8912), 8μg/ml tetracycline (Sigma #268054), and 15.1 mg/L PIPES (piperazine-N,N′-bis(2)-ethanesulfonic acid; Sigma #P6757, used as an internal control for quantification via mass spectrometry). Cultures were inoculated with transformants previously grown for 13 hours at 37ºC and normalized to the optical density of the transformant, according to table S4. Strains were allowed to grow for 48 hours, shaking continuously at 225rpm and protected from light, to OD600 values given in table S4. Subsequently, cells were pelleted and supernatants passed through a 0.2μM filter before tetracycline levels were quantified via LC-MS/MS by Dr. Sophie Alvarez at the Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center (St. Louis, MO USA).

To quantify absolute tetracycline levels, LC-MS/MS was performed using filtered supernatants diluted three-fold and transferred to HPLC vials. The LC–MS/MS system used is composed of a Shimadzu (Kyoto, Japan) LC system with two Shimadzu solvent delivery pumps (model LC10AD), a Shimadzu integrated controller (SCL10Avp), a Valco (Houston, TX) two-position diverter valve, and a LEAP CTC PAL autosampler with a 50 μl sample loop. This LC system is interfaced with an AB Sciex (Framingham, MA) 4000 QTRAP mass spectrometer equipped with a TurboIonSpray (TIS) electrospray ion source. Source parameters were set as follows: curtain gas, 20 arbitrary units (a.u.); source gas 1, 50 a.u.; source gas 2, 50 a.u.; collision activated dissociation, high; interface heater, on; temperature, 500 ºC; ion spray voltage, +5500. Both quadruples (Q1 and Q3) were set to unit resolution. Analyst software (version 1.4.2) was used to control sample acquisition and data analysis. The 4000 QTRAP mass spectrometer was tuned and calibrated according to the manufacturer's recommendations. Tetracycline and the internal standard used (PIPES) were detected using 3 MRM transitions previously optimized using each standard (tetracycline, Sigma #268054; PIPES, Sigma #P6757) as detailed in table S5.

For LC separation, a ZORBAX Eclipse XDB-C18 column (4.6 mm x 150 mm, Agilent, Santa Clara, CA) was used flowing at 0.5 ml/min. The gradient was from 95% solvent A (0.1% [v/v] formic acid in Milli-Q water), held for 2 min, to 80% solvent B (100% Methanol) in 8 min. The LC was then ramped to 95% B and held for 2.5 min, then ramped back to initial conditions (95% solvent A) in 1 min and re-equilibrated for an additional 2 min. For quantification, a series of standard samples containing different concentrations of

tetracycline and a constant concentration of PIPES was prepared. The peak area in samples was first normalized in the same manner used for optimization of the standards and then quantified according to the standard curve. The linear range of the standard curve for tetracycline quantification was between 0.008 and 10 µM, with an r^2 value of 0.999.

Sub-Cloning of Tetracycline Resistance Genes into pET28b(+)

All genes encoding putative tetracycline-inactivating enzymes were cloned into the pET28b(+) vector (Novagen, Darmstadt, Germany) digested using BamHI and NdeI restriction enzymes. Digestion of the pET28b(+) vector was performed in a total volume of 50µl using the following reagents mixed well and incubated at 37ºC overnight: (i) 2.5µg of purified vector DNA, isolated via miniprep with a Qiagen purification kit (Venlo, Netherlands, cat#27104) per manufacturer's recommendations, (ii) 5µl NEB cutsmart buffer, (iii) 0.67µl BamHI-HF (100U/µl, NEB), and (iv) 3µl NdeI (20U/µl, NEB). Subsequently, digested vector was dephosphorylated using 1/10th volume calf intestinal phosphatase (CIP, 10U/µl, NEB) with 1/10th volume NEB buffer #3 followed by incubation at 37ºC for three hours. BamHI and NdeI cut sites were added to each cloned putative tetracycline-inactivating gene using ~1-2ng of template (subcloned genes in pZE21) and the PFX DNA polymerase, per suggested protocols (http://tools.invitrogen.com/content/sfs/manuals/platinumpfx_pps.pdf). All reactions were incubated at 94ºC for 10 minutes, subjected to 35 cycles of: (i) 94ºC for 45 seconds, (ii) reaction-specific annealing temperature for 45 seconds, and (iii) 72ºC for a reaction-specific extension time, and finally incubated at 72ºC for 10 minutes before being held at 4ºC. Reaction-specific primer sequences/cycling conditions are indicated in table S6.

Purified PCR product was digested in a 50µl reaction incubated at 37ºC overnight and containing (i) 30µl of pure amplicon (2 - 4.8µg DNA), ii) 5µl NEB cutsmart buffer, (iii) 0.67µl BamHI-HF (100U/µl, NEB), (iv) 3ul NdeI (20U/µl, NEB), and (v) 1µl of DpnI (20U/µl, NEB, to linearize residual pZE21 vector containing sub-cloned tetracycline resistant genes). To facilitate cloning, each digested amplicon was ligated to digested pET28b(+) using the Fast-Link DNA ligation kit (Epicentre, cat#LK11025) per manufacturer's recommendations. All cloned genes were subjected to Sanger sequencing to confirm appropriate sequence and orientation.

Enzyme Purification

After ligation into pET28b(+), all putative tetracycline-inactivating genotypes were purified by miniprep and transformed into BL21-Star(DE3) *E. coli* cells (Life Technologies, Carlsbad, CA) via heat-shock. Enzymes were purified using similar protocols but in two separate groups of six; each group was purified on a separate day. Group #1 consisted of genotypes Tet(47), Tet(48), Tet(49), Tet(50), Tet(51), and Tet(52). Group #2 consisted of genotypes: Tet(53), Tet(54), Tet(55), Tet(56), Tet(X), and an empty pET28b(+) vector. Overnight cultures of each strain were prepared and 500µl used to inoculate 1L LB broth containing 50µg/ml kanamycin and grown to the optical densities detailed in table S7, at which time samples were cooled to 15ºC and 500µl of 1M Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce expression. Cells were then allowed to grow at 15ºC overnight and harvested the following day, at optical densities given in table S7. For each sample, 1ml of both uninduced and induced cells were frozen at -20ºC for use in SDS protein gels.

The following buffers were used in purification: *Lysis buffer* (50mM K_2HPO_4, 500mM NaCl, 5mM Beta-Mercaptoethanol, 20mM imidazole, 10% glycerol), *Elution buffer* (50mM K_2HPO_4, 500mM NaCl, 5mM Beta-Mercaptoethanol, 300mM imidazole, 10% glycerol), and *Dialysis buffer* (50mM K_2HPO_4, 150mM NaCl, 1mM DTT, 5% Glycerol). Samples were kept in a cold room at 4ºC or on ice throughout the purification process. After overnight induction with IPTG, each sample was pelleted by centrifugation at 10,000g for 10 minutes at 4ºC, supernatants discarded, and cells resuspended in 40ml of lysis buffer containing SIGMA*FAST* protease inhibitor (Sigma cat#S8820) at a concentration of one tablet in 100ml buffer. Resuspended cells were subsequently flash-frozen in liquid nitrogen and thawed in a room-temperature water bath. Cells were then lysed using an EmulsiFlex-C5 homogenizer (Avestin, Ottawa, Canada) at a minimum psi of 15,000, lysates collected, and clarified at 50,000 rpm for 35 minutes at 4ºC (samples in group #2 were clarified at 39,000 rpm). From each sample, approximately 1ml of both lysate and clarified supernatant was stored for use in SDS protein gels.

Proteins were purified from supernatant by elution through Ni-NTA agarose beads (Invitrogen cat#R901), using 8ml of beads for samples in group #1 and 15ml for group #2 samples. After beads were washed twice with 50ml diH_2O, clarified supernatant was mixed with the beads while gently rocking at 4ºC for

30 to 60 minutes to facilitate binding of the His_6-tagged enzymes to the Ni-NTA beads. Bound beads were allowed to settle and the supernatant permitted to flow through purification columns and the drippings collected. Beads were subsequently washed with 40ml of lysis buffer, with wash also collected by drip. Proteins were then eluted from Ni-NTA beads by flowing elution buffer over beads three separate times, each elution was collected separately, and mixed before dialysis. Samples in group #1 were eluted with three volumes of 20ml elution buffer while group #2 samples used elution volumes of 10ml. All group #1 samples were dialyzed using 10 kDa molecular weight cut-off SnakeSkin Pleated Dialysis tubing from Thermo Scientific (Waltham, MA) in 2L dialysis buffer overnight before transfer into a fresh 4L of dialysis buffer the following day, and allowed to dialyze for additional 5 hours, reaching a final 1:80 dilution. Each sample from group #2 was dialyzed in 1L of dialysis buffer overnight using the same tubing; for a final 1:34 dilution. For each sample, aliquots of supernatant flow-through, washes, and each elution were maintained for use in SDS protein gels.

Running SDS Protein Gels

For each sample, an SDS protein gel was run using uninduced cells, induced cells, lysate, clarified supernatant, flowthrough, wash, elution #1, elution #2, and elution #3. The volume of induced cells used was normalized to the number of uninduced cells collected, per the OD600 values reported in table S7 such that band intensities were comparable within samples and facilitated inference of proper expression induction. Cell aliquots were spun down at 9,000 rpm at 4ºC and supernatants discarded before pellets were resuspended in diH_2O. One part resuspended pellet was mixed with two parts SDS gel loading dye (containing 10% w/v SDS, 50% v/v glycerol, 0.25M Tris-HCl pH 6.8, 0.5M dithiothreitol, 0.25% w/v bromophenol blue), heated at 90ºC for ten minutes, centrifuged at 13,000 rpm for 20 minutes at room temperature, and 10µl of sample loaded into gel wells. For lysates, clarified supernatants, flowthrough, washes, and all elutions, three parts sample was mixed with one part loading dye and heated at 90ºC for ten minutes. For lysates and clarified supernatants, 10µl of sample was loaded on SDS gels whereas 15µl was used for flowthrough, washes, and all elutions. As a size marker, 12µl of BioRad (Hercules, CA) Precision Plus Dual Xtra Standard (cat#161-0377) was included. The loaded SDS gels (BioRad Any kD Mini-PROTEAN TGX Gel; cat#456-9033) ran for 10 minutes at 150 volts and then 35 minutes at 185 volts. Gels were subsequently stained with coomassie blue dye and then destained

by gentle rocking overnight using a 10/10/80 ratio of acetic acid to methanol to water. Gels were imaged the subsequent day; the expected size of all tetracycline-inactivating enzymes is approximately 44KDa. For samples Tet(47) and Tet(53), no band of the desired size was visible in any elution, indicating purification was unsuccessful. These samples were not carried through to further *in vitro* experimentation.

Concentrating, Quantifying, and Storing Enzymes

To concentrate samples, dialyzed eluate was loaded into a 10kDa centrifugal filtration unit (Millipore, Darmstadt, Germany, cat#ACK5010PG), removing proteins smaller than 10KDa. Before sample was loaded, concentration columns were conditioned with 15ml of dialysis buffer and centrifuged at 4,500 rpm for 15 minutes at 4ºC. Samples were then concentrated in 15ml aliquots and precipitate avoided by periodic gentle pipetting. All samples were concentrated to volumes below 1.7ml and concentration quantified using A280 measurements taken with a NanoDrop Spectrophotometer (Thermo Scientific). For each enzyme, extinction coefficients were determined using ExPASy's ProtParam tool (http://web.expasy.org/protparam) assuming reduced cysteines; all parameters, concentrations, and yields are reported in table S7. Notably, solutions of purified protein showed a yellow hue in a concentration-dependent fashion, indicating likely flavin content (Yang et al., 2004).

Preparing *In vitro* Enzymatic Reactions

All reactions were performed using 100mM TAPS buffer (Sigma #T5130), pH 8.5, unless otherwise noted. Inactivation reactions contained 1.4mM antibiotic (either tetracycline or oxytetracycline), 350µg enzyme, and an NADPH regenerating system in a total volume of 564µl. For reactions using anhydrotetracycline, 1mM substrate was used with 200ug enzyme in a 475ul reaction. The NADPH regenerating system consisted of the following components (final concentrations): glucose-6-phosphate (40mM, Sigma #G7879), $NADP^+$ (4mM, Sigma #N5755), $MgCl_2$ (1mM, Sigma #M9272), and glucose-6-phosphate dehydrogenase (4U/ml, Sigma #G2921). Before addition to the reaction, the regenerating system was prepared as a 4X stock as follows. The glucose-6-phosphate and $NADP^+$ were dissolved in TAPS and the mixture pH'ed to 8.4 before the addition of $MgCl_2$ and glucose-6-phosphate dehydrogenase. The solution was then incubated at 37ºC for 30 minutes to generate NADPH before the regeneration solution was added to each reaction. The reaction was started by the

addition of a putative tetracycline-inactivating enzyme. Before enzyme addition, the pH of each reaction was measured and verified to be between 8.1 and 8.3. Enzymes were thawed on ice with FAD added to a final concentration of 5μM. FAD was also added to 'no-enzyme' controls, matched to the highest concentration in any other experiment. The same reactions were used for UV-visible spectroscopy (following dilution), reverse phase HPLC, and (where applicable) LC-MS and high-resolution tandem MS.

UV-visible Spectroscopy

After enzyme addition, reactions were immediately mixed and 25μl added to 975μl TAPS (40-fold dilution) such that absorbance readings would fall within the dynamic range of the spectrometer used (a Cary 60 UV/Vis system, Agilent). For reactions using anhydrotetracycline, a 50-fold dilution factor was used. Absorbance then was measured from 280nm to 450nm or 550nm light, at 1-5nm intervals, for up to 3.5 hours. Time course spectra can be seen in figures 3, 6, and S7.

Sampling Reactions

From each reaction, samples were taken at indicated timepoints by transferring a 50μl volume into a 200μl quencher solution comprised of equal parts acetonitrile (Sigma #34851) and 0.25M HCl. Reactions were quenched under acidic conditions because prior work with Tet(X) demonstrated that enzymatic degradation products of tetracyclines are unstable at neutral pH, but stable at low pH (Moore et al., 2005; Yang et al., 2004). In many cases, soil-derived enzymes showed similar initial products as Tet(X) by reverse phase HPLC. Reactions that appeared to progress slowly accumulated less degradation product, consistent its lower stability at neutral pH. Slower reactions expose products to unfavorable pH levels for longer periods of time and would therefore be expected to result in diminished levels of any compound unstable at the reaction's pH. After an aliquot of reaction was quenched, the sample was centrifuged at 10,000 rpm for 10 minutes at 4°C and 200μl transferred to a suitable vial for reverse phase HPLC.

High Performance Liquid Chromatography to Separate Tetracycline Degradation Products

Products generated from enzymatic inactivation of both tetracycline and oxytetracycline were separated by reverse phase HPLC using a Phenomenex (Torrance, CA) Luna C18 column (5μm, 100 Å, 4.6 x 250mm, part #00G-4252-E0). The column was first equilibrated using H_2O containing 0.1% trifluoroacetic acid (solvent

A), before ramping to 25% solvent B (acetonitrile containing 0.1% trifluoroacetic acid). Products of enzymatic activity were then eluted using 25µl of sample along a linear gradient from 25% to 75% solvent B over 14 minutes at a flow rate of 1ml/min. HPLC was performed using a Beckman Coulter (Pasadena, CA) system (gold 126 solvent module + gold 168 detector module) attached to an autosampler such that samples were allowed to run in continuous succession overnight.

Liquid Chromatography Mass Spectrometry of Tetracycline Degradation Products

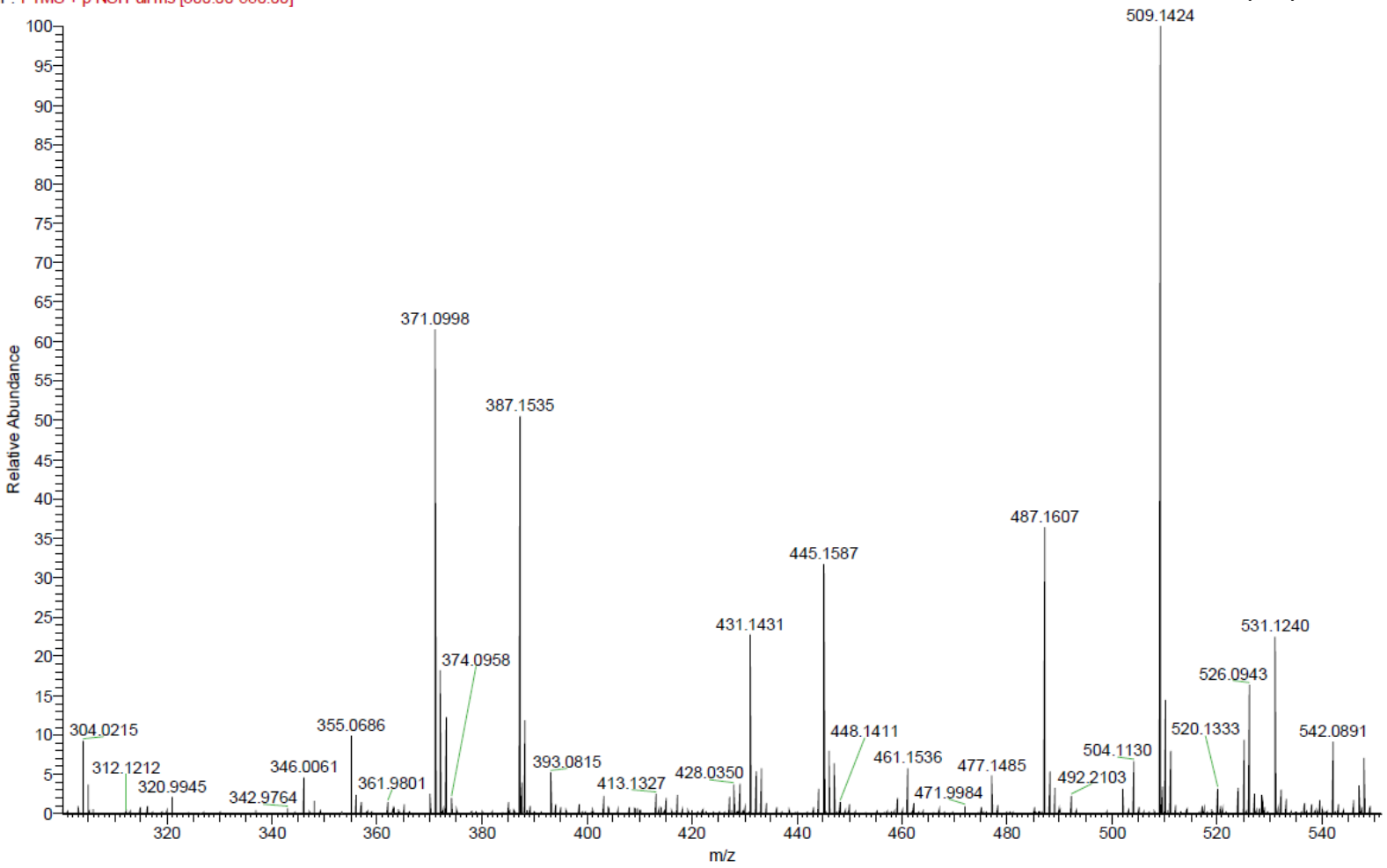
After reverse phase HPLC as described above, samples were analyzed by LC-MS using an Agilent single quadrupole LC/MS 6130. Reaction products were first separated by reverse phase HPLC using a Phenomenex Gemini C18 column (5µm, 110 Å, 2 x 50mm, part #00B-4435-B0). The column was equilibrated using H_2O plus 0.1% formic acid and reaction products eluted using 30µl of sample along a linear gradient to 95% acetonitrile plus 0.1% formic acid over 14 minutes at a flow rate of 0.5ml/min. Electrospray ionization was used for analysis of reaction products by mass spectrometry and ion counts for a particular *m/z* peak determined by peak height. Panels in figures 4, 5, S4, and S5 depict ion counts of various analytes from each enzymatic (or control) reaction, normalized to the counts observed for peak associated with the tetracycline substrate (*m/z* of 445) at the first timepoint taken (5 minutes post reaction initiation).

High Resolution Tandem Mass Spectrometry of Tetracycline Degradation Products

Reactions were performed as described above and 45 minute timepoints were quenched and frozen before analysis by high-resolution tandem mass spectrometry. Samples were diluted 6x with 50% MeOH/0.1% formic acid and run on LTQ-Orbitrap Velos by direct infusion using the Advion Triversa nanomate. The samples were acquired using a high resolution (60,000) mass spectrometer. The MS scan was acquired from 300-550 m/z. The 387 m/z compound was fragmented by MS2 and MS3-CID with collision energies of 15 and 25 eV respectively. Detailed parameters associated with any given spectra are given in supplemental dataset 1.

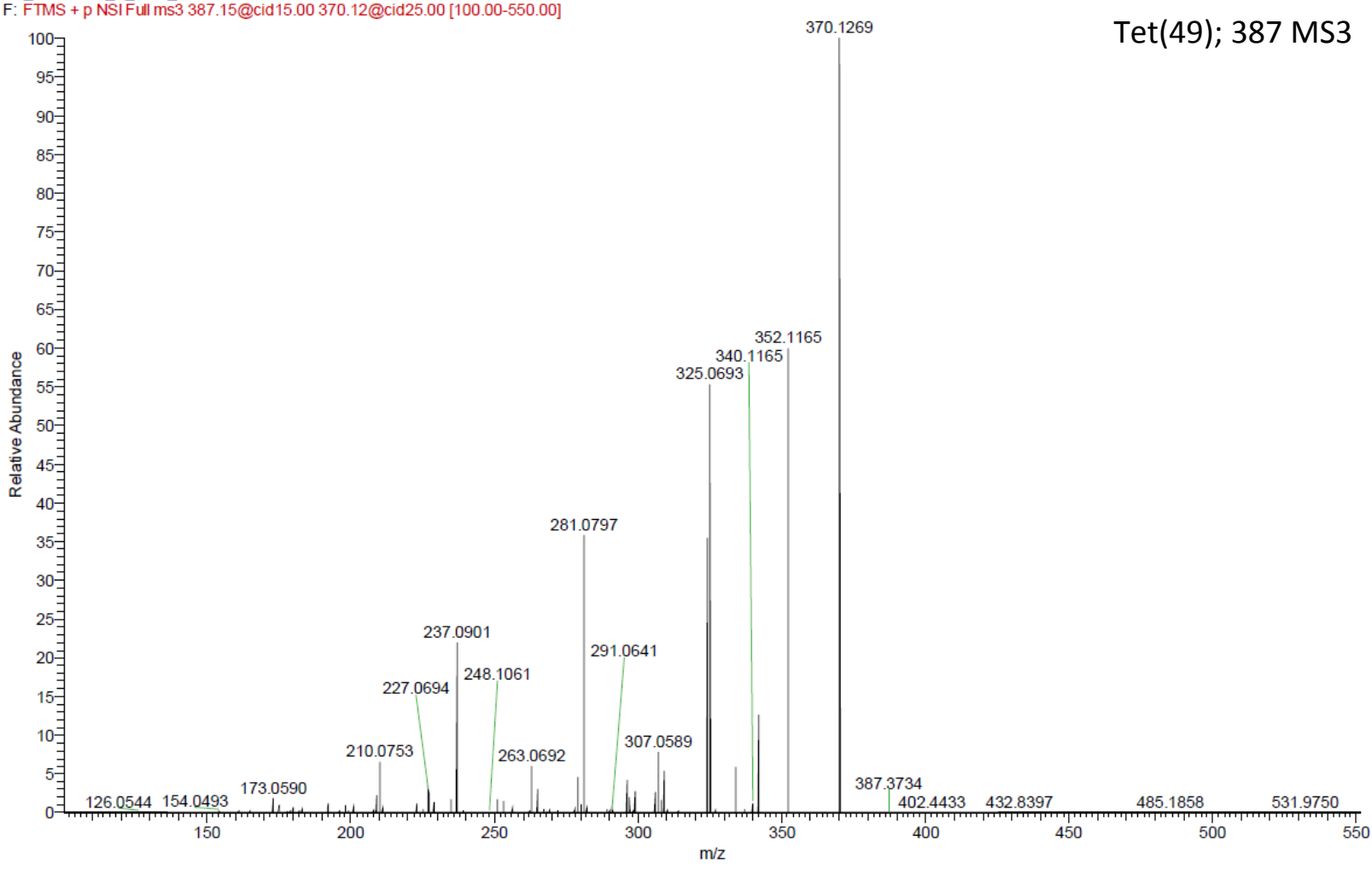
KF_20150422_A_MS #2-13 RT: 0.03-0.33 AV: 12 NL: 6.67E6
F: FTMS + p NSI Full ms [300.00-550.00]

Tet(49) MS1



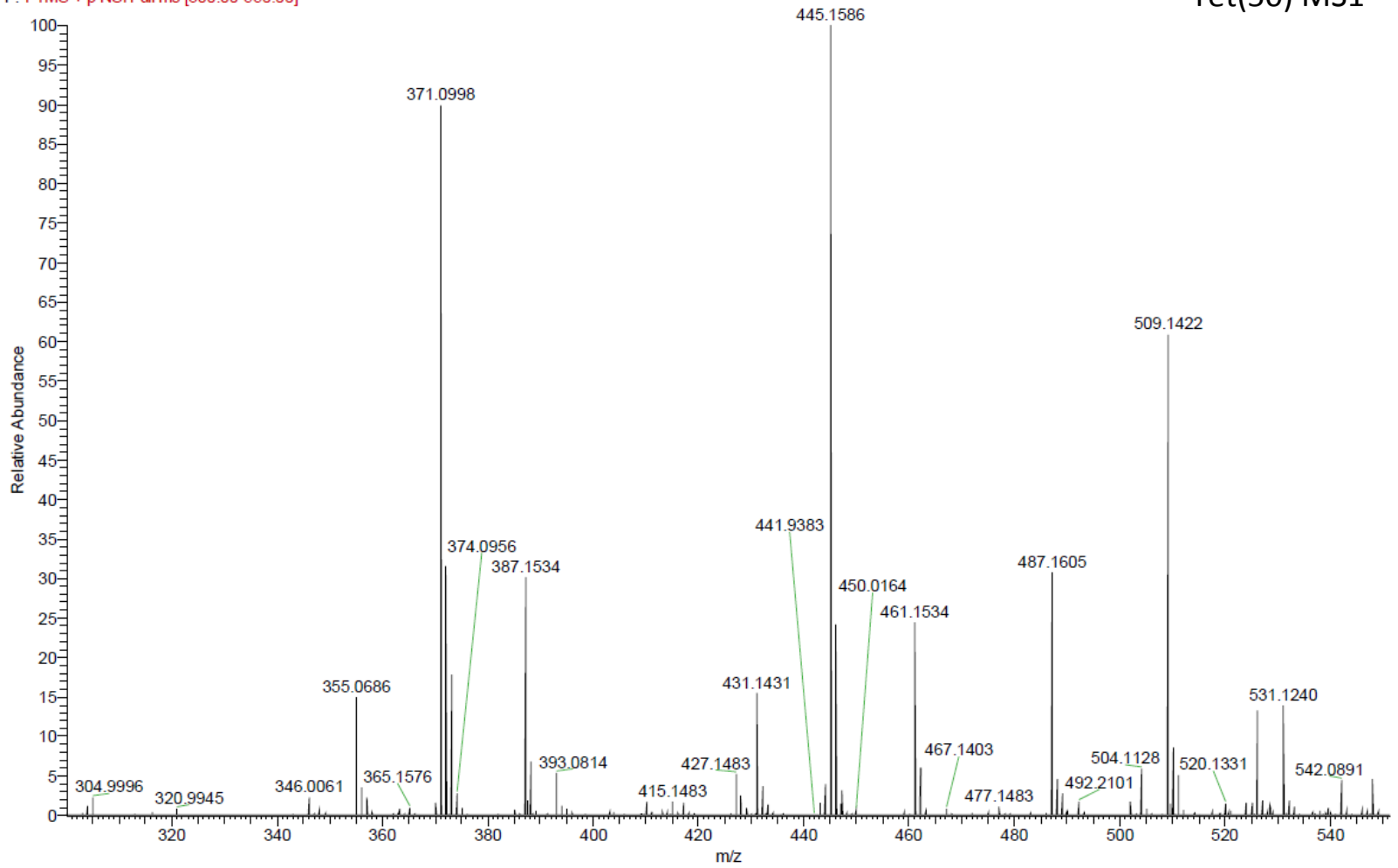
KF_20150422_A_MS3_387 #2-13 RT: 0.03-0.22 AV: 12 NL: 9.68E4
F: FTMS + p NSI Full ms3 387.15@cid15.00 370.12@cid25.00 [100.00-550.00]

Tet(49); 387 MS3



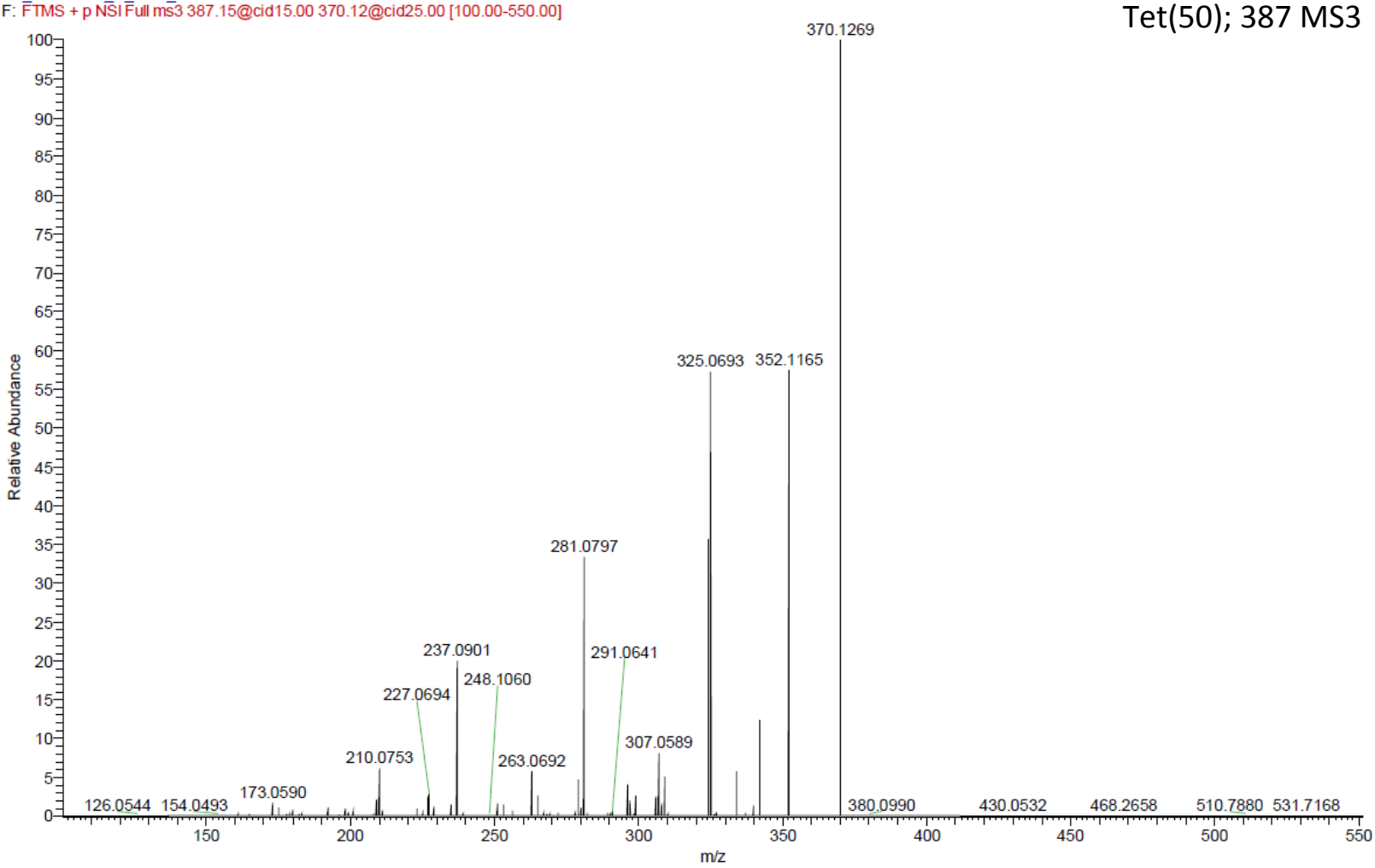
KF_20150422_B_MS #2-13 RT: 0.03-0.34 AV: 12 NL: 7.78E6
F: FTMS + p NSI Full ms [300.00-550.00]

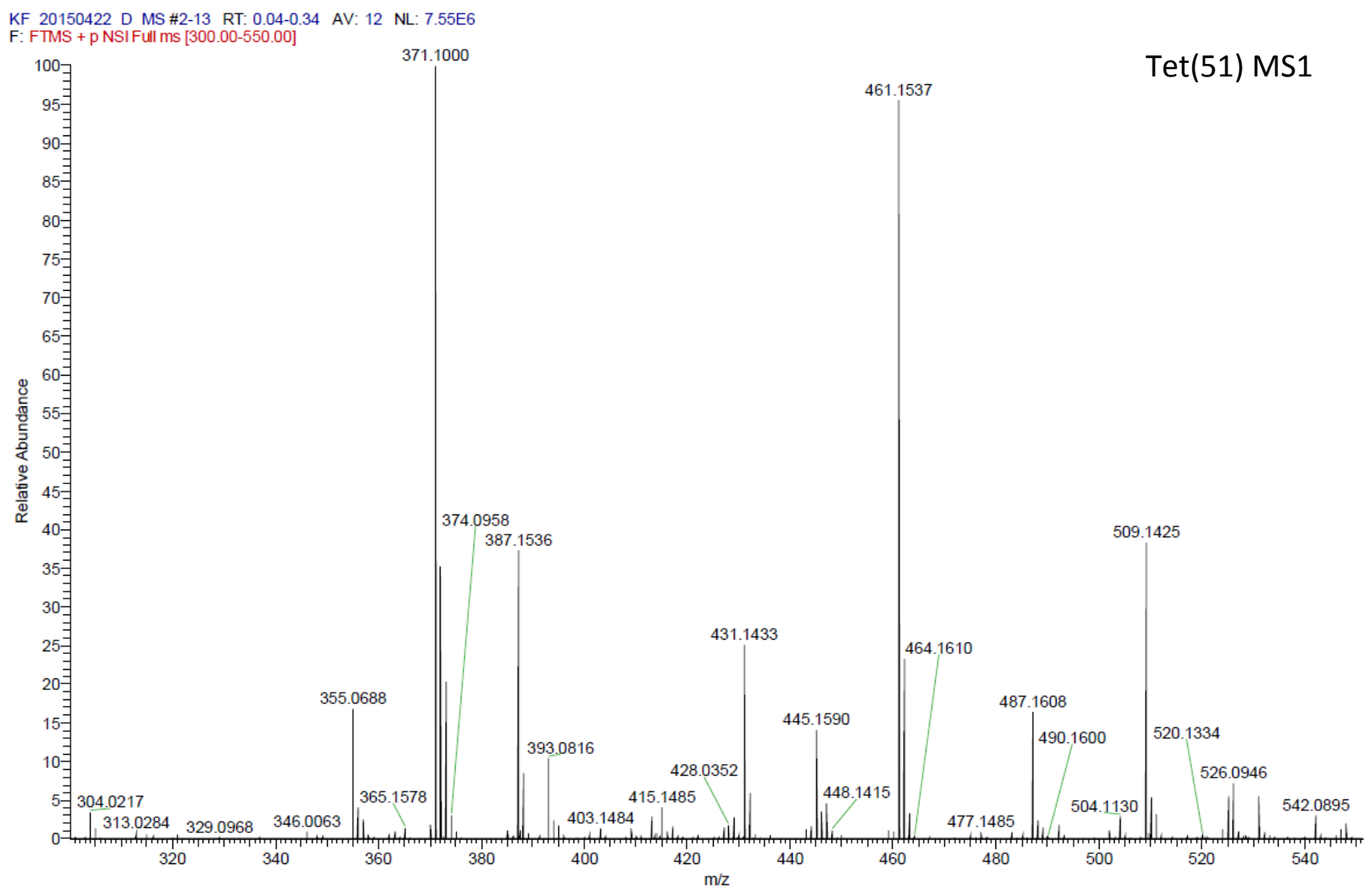
Tet(50) MS1

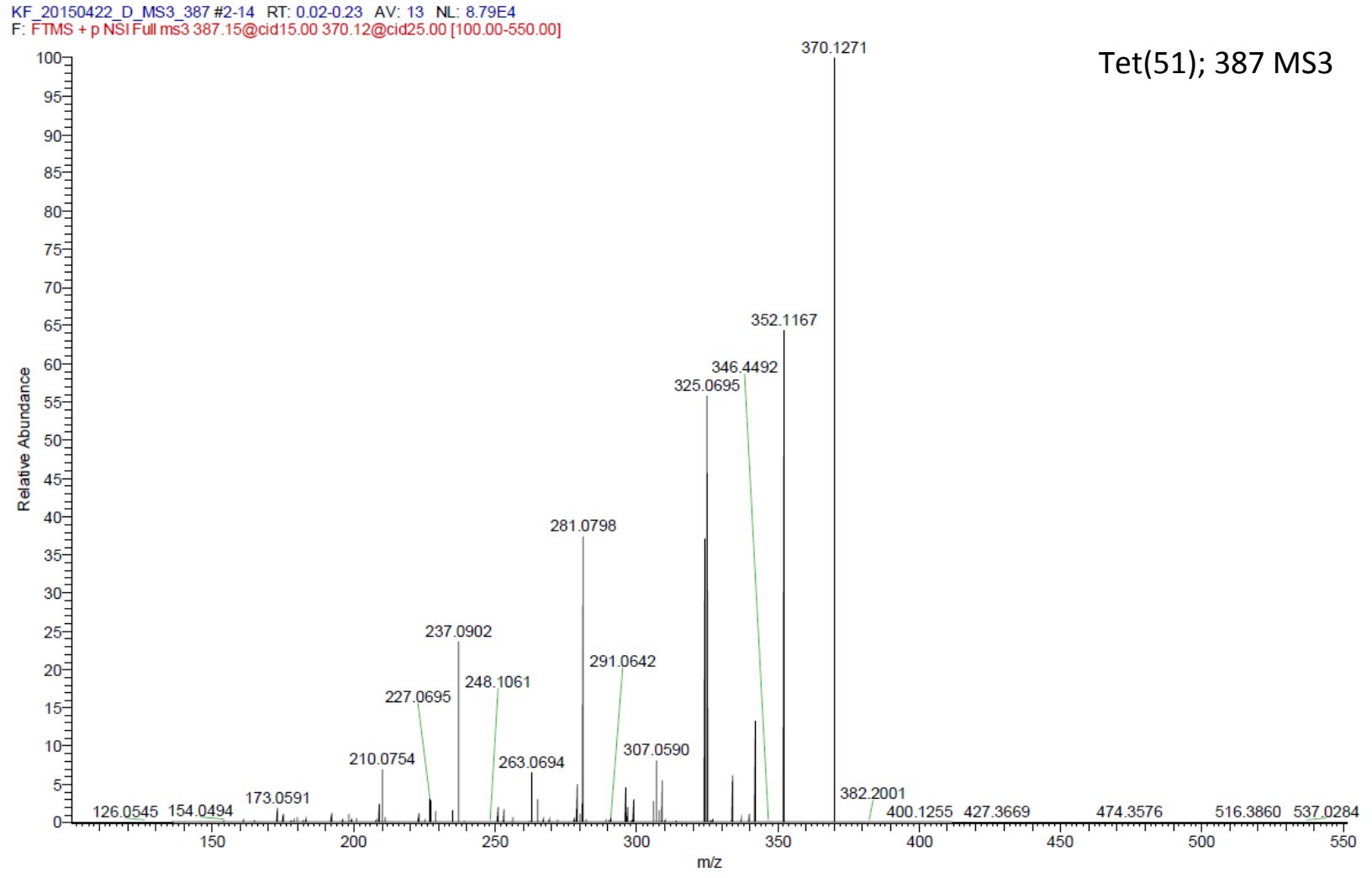


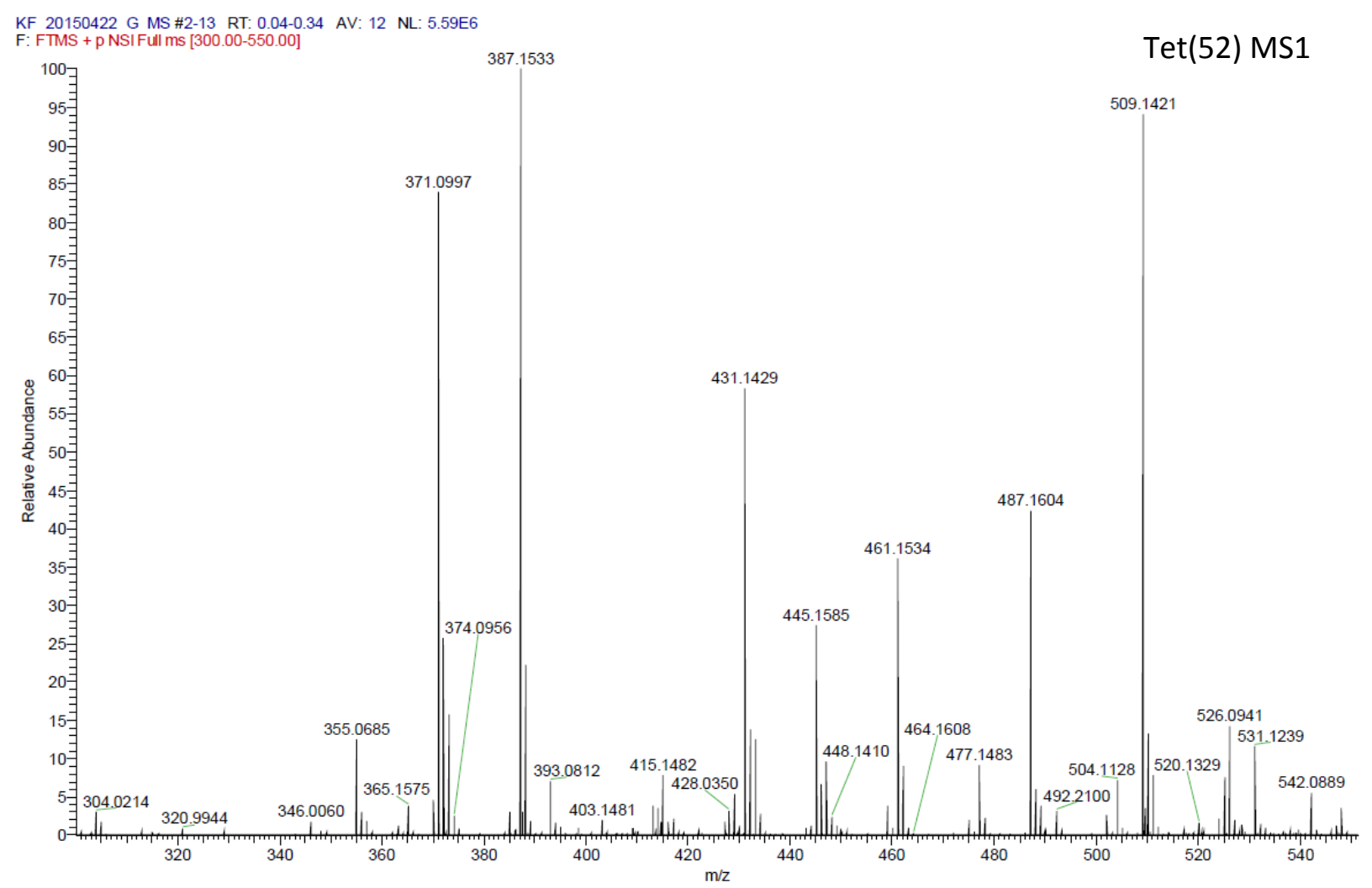
KF_20150422_B_MS3_387 #2-14 RT: 0.02-0.24 AV: 13 NL: 7.98E4
F: FTMS + p NSI Full ms3 387.15@cid15.00 370.12@cid25.00 [100.00-550.00]

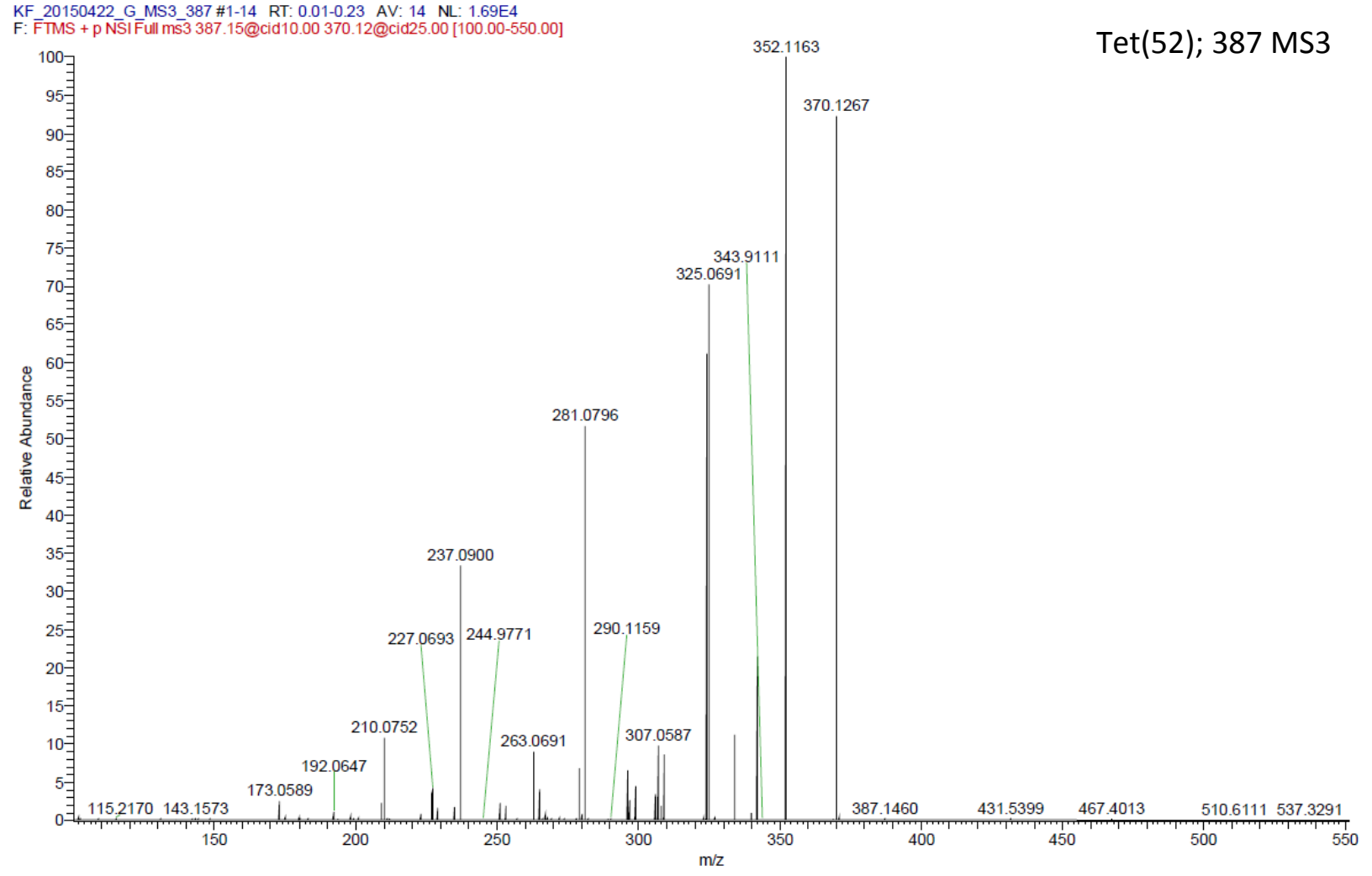
Tet(50); 387 MS3

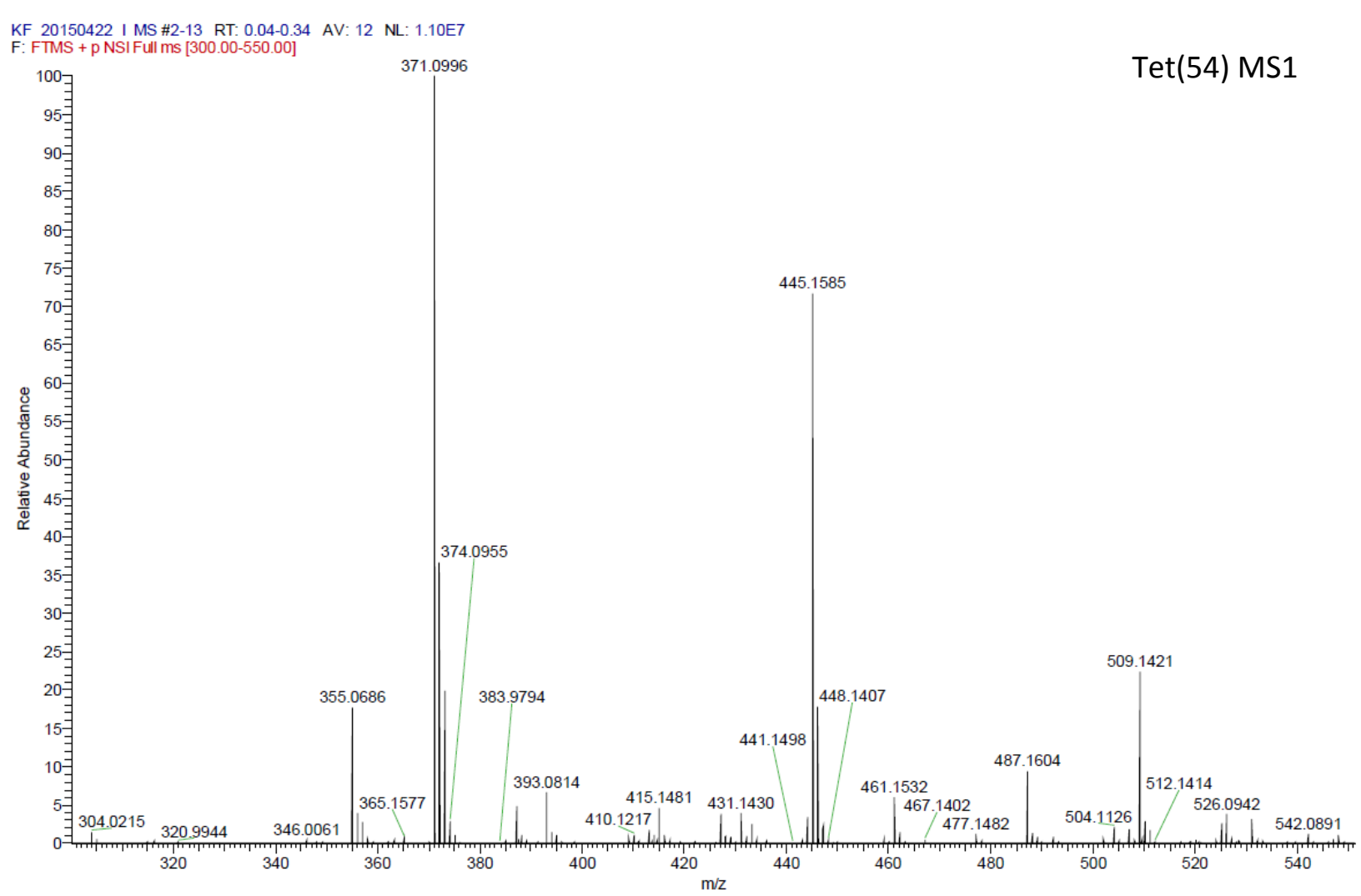


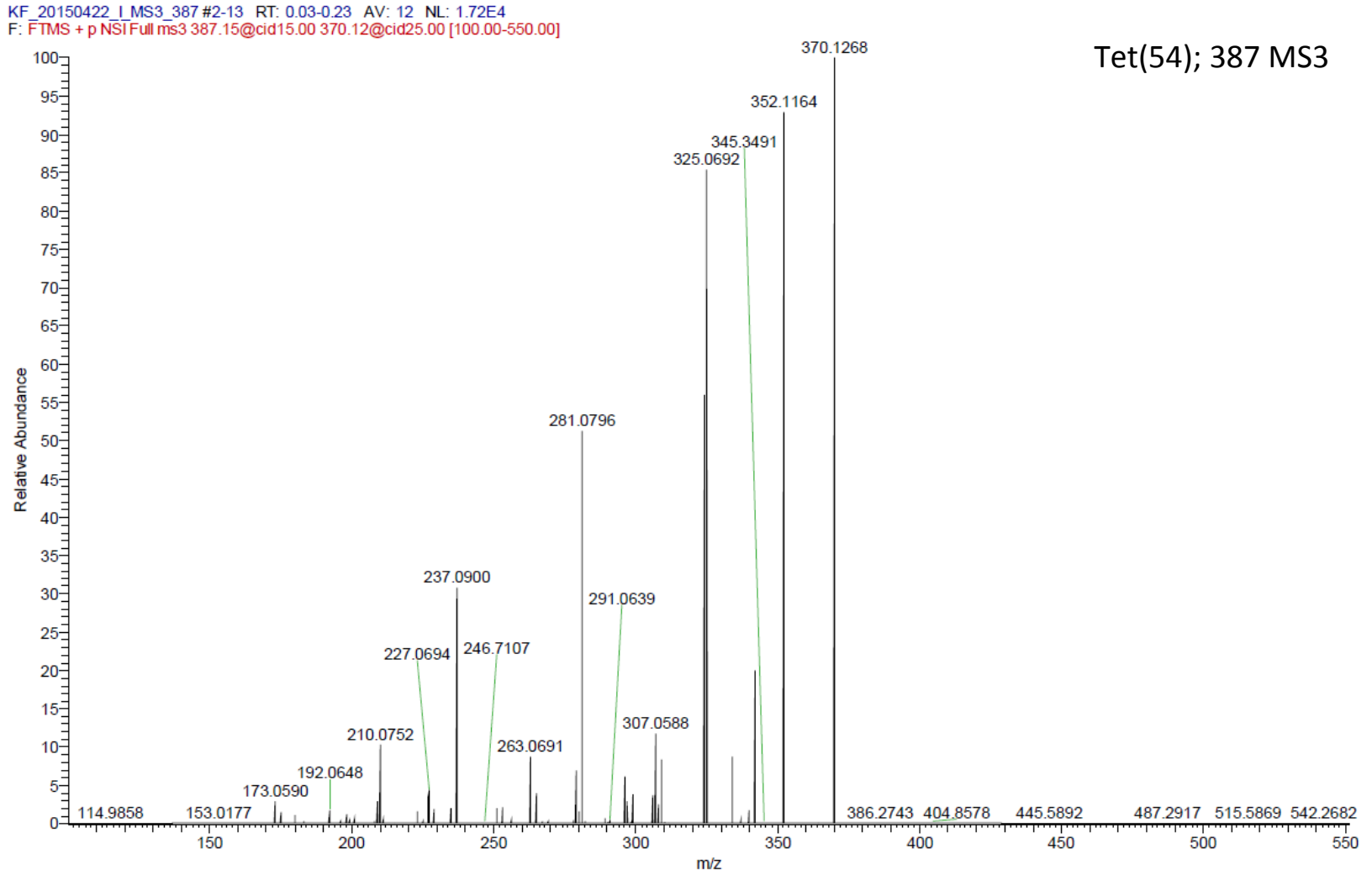

KF_20150422_D_MS #2-13 RT: 0.04-0.34 AV: 12 NL: 7.55E6
F: FTMS + p NSI Full ms [300.00-550.00]
Tet(51) MS1
Relative Abundance
m/z
371.1000
461.1537
374.0958
387.1536
509.1425
431.1433
464.1610
355.0688
487.1608
445.1590
490.1600
520.1334
393.0816
428.0352
526.0946
448.1415
415.1485
365.1578
504.1130
542.0895
304.0217
313.0284
329.0968
346.0063
403.1484
477.1485

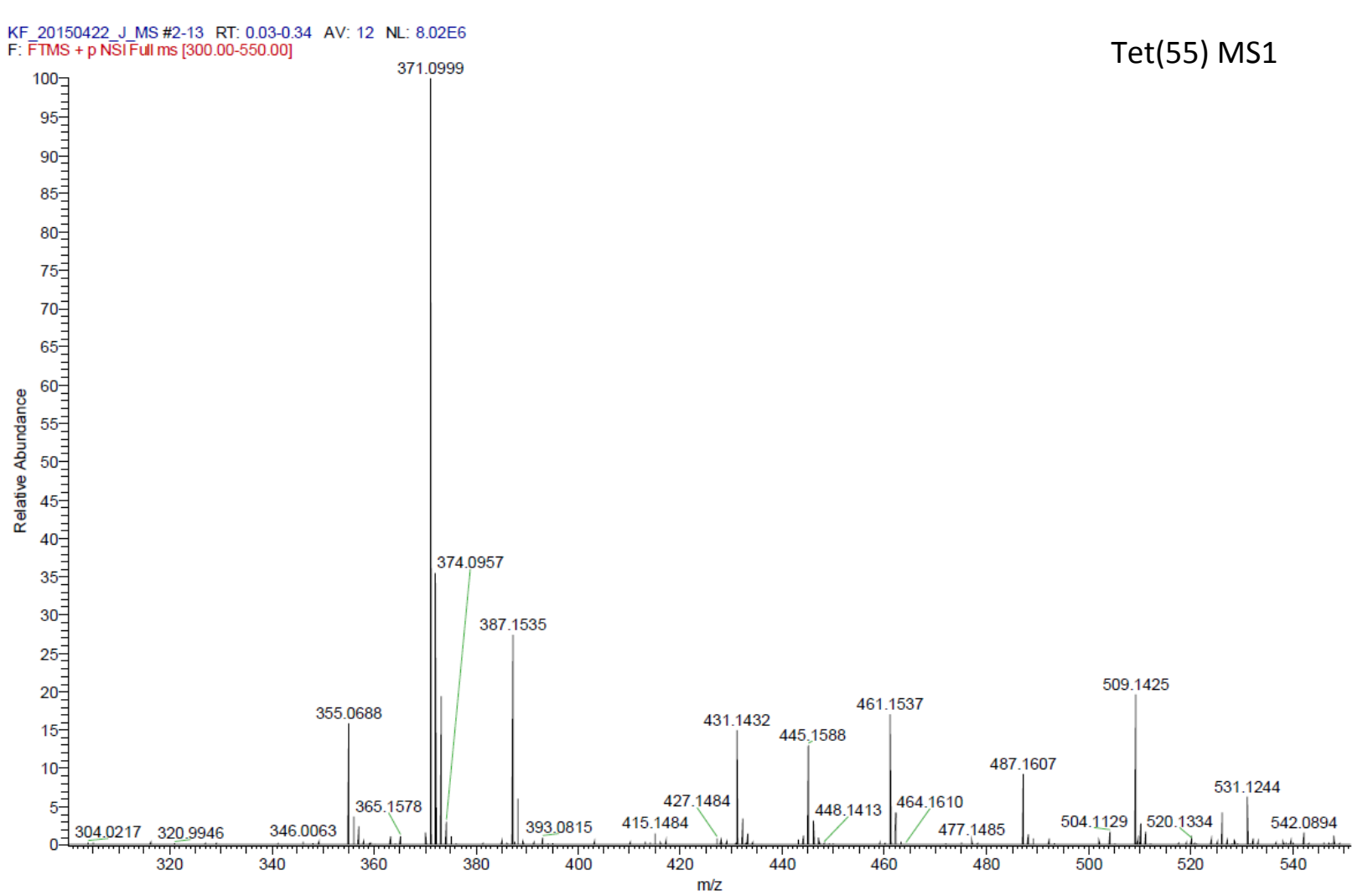

KF_20150422_D_MS3_387 #2-14 RT: 0.02-0.23 AV: 13 NL: 8.79E4
F: FTMS + p NSI Full ms3 387.15@cid15.00 370.12@cid25.00 [100.00-550.00]
Tet(51); 387 MS3
Relative Abundance
m/z
370.1271
352.1167
346.4492
325.0695
281.0798
237.0902
291.0642
248.1061
227.0695
307.0590
210.0754
263.0694
382.2001
173.0591
126.0545
154.0494
400.1255
427.3669
474.3576
516.3860
537.0284

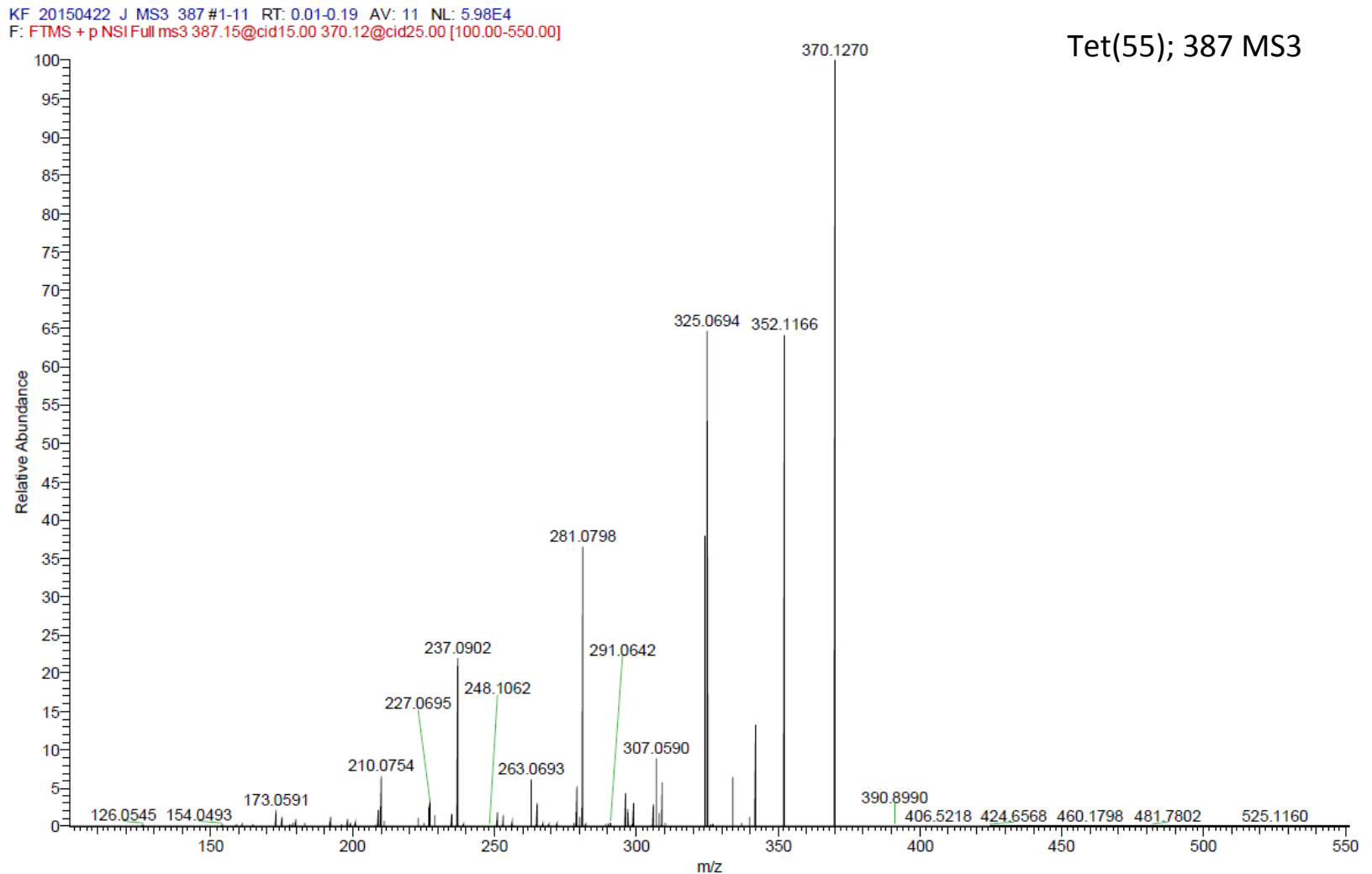

KF_20150422_G_MS #2-13 RT: 0.04-0.34 AV: 12 NL: 5.59E6
F: FTMS + p NSI Full ms [300.00-550.00]
Tet(52) MS1
Relative Abundance
m/z
387.1533
509.1421
371.0997
431.1429
487.1604
461.1534
445.1585
374.0956
355.0685
464.1608
526.0941
531.1239
448.1410
477.1483
393.0812
415.1482
428.0350
504.1128
520.1329
542.0889
365.1575
492.2100
304.0214
320.9944
346.0060
403.1481


KF_20150422_G_MS3_387 #1-14 RT: 0.01-0.23 AV: 14 NL: 1.69E4
F: FTMS + p NSI Full ms3 387.15@cid10.00 370.12@cid25.00 [100.00-550.00]
Tet(52); 387 MS3
Relative Abundance
m/z
352.1163
370.1267
343.9111
325.0691
281.0796
237.0900
227.0693
244.9771
290.1159
210.0752
263.0691
307.0587
192.0647
173.0589
115.2170
143.1573
387.1460
431.5399
467.4013
510.6111
537.3291


KF_20150422_I MS #2-13 RT: 0.04-0.34 AV: 12 NL: 1.10E7
F: FTMS + p NSI Full ms [300.00-550.00]
Tet(54) MS1
Relative Abundance
m/z
371.0996
445.1585
374.0955
355.0686
383.9794
448.1407
509.1421
441.1498
487.1604
393.0814
461.1532
512.1414
415.1481
467.1402
526.0942
365.1577
431.1430
477.1482
504.1126
542.0891
304.0215
320.9944
346.0061
410.1217

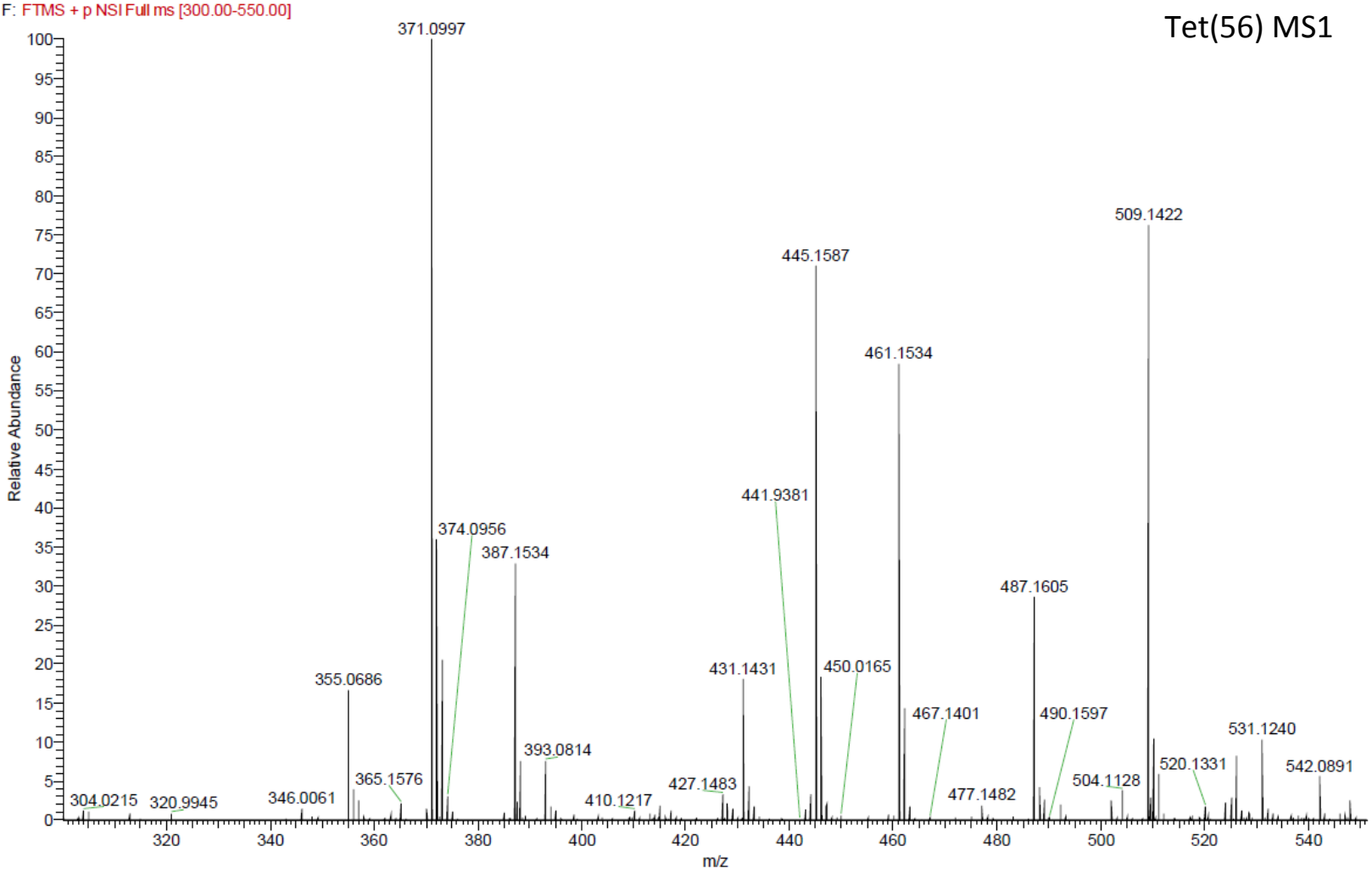

KF_20150422_I_MS3_387 #2-13 RT: 0.03-0.23 AV: 12 NL: 1.72E4
F: FTMS + p NSI Full ms3 387.15@cid15.00 370.12@cid25.00 [100.00-550.00]
Tet(54); 387 MS3
Relative Abundance
m/z
370.1268
352.1164
345.3491
325.0692
281.0796
237.0900
291.0639
227.0694
246.7107
307.0588
210.0752
263.0691
192.0648
173.0590
114.9858
153.0177
386.2743
404.8578
445.5892
487.2917
515.5869
542.2682


KF_20150422_J_MS #2-13 RT: 0.03-0.34 AV: 12 NL: 8.02E6
F: FTMS + p NSI Full ms [300.00-550.00]
Tet(55) MS1
Relative Abundance
m/z
371.0999
374.0957
387.1535
355.0688
431.1432
445.1588
461.1537
509.1425
487.1607
531.1244
427.1484
448.1413
464.1610
365.1578
393.0815
415.1484
477.1485
504.1129
520.1334
542.0894
304.0217
320.9946
346.0063


KF_20150422_J_MS3 387#1-11 RT: 0.01-0.19 AV: 11 NL: 5.98E4
F: FTMS + p NSI Full ms3 387.15@cid15.00 370.12@cid25.00 [100.00-550.00]
Tet(55); 387 MS3
Relative Abundance
m/z
370.1270
325.0694
352.1166
281.0798
237.0902
291.0642
248.1062
227.0695
307.0590
210.0754
263.0693
173.0591
390.8990
126.0545
154.0493
406.5218
424.6568
460.1798
481.7802
525.1160

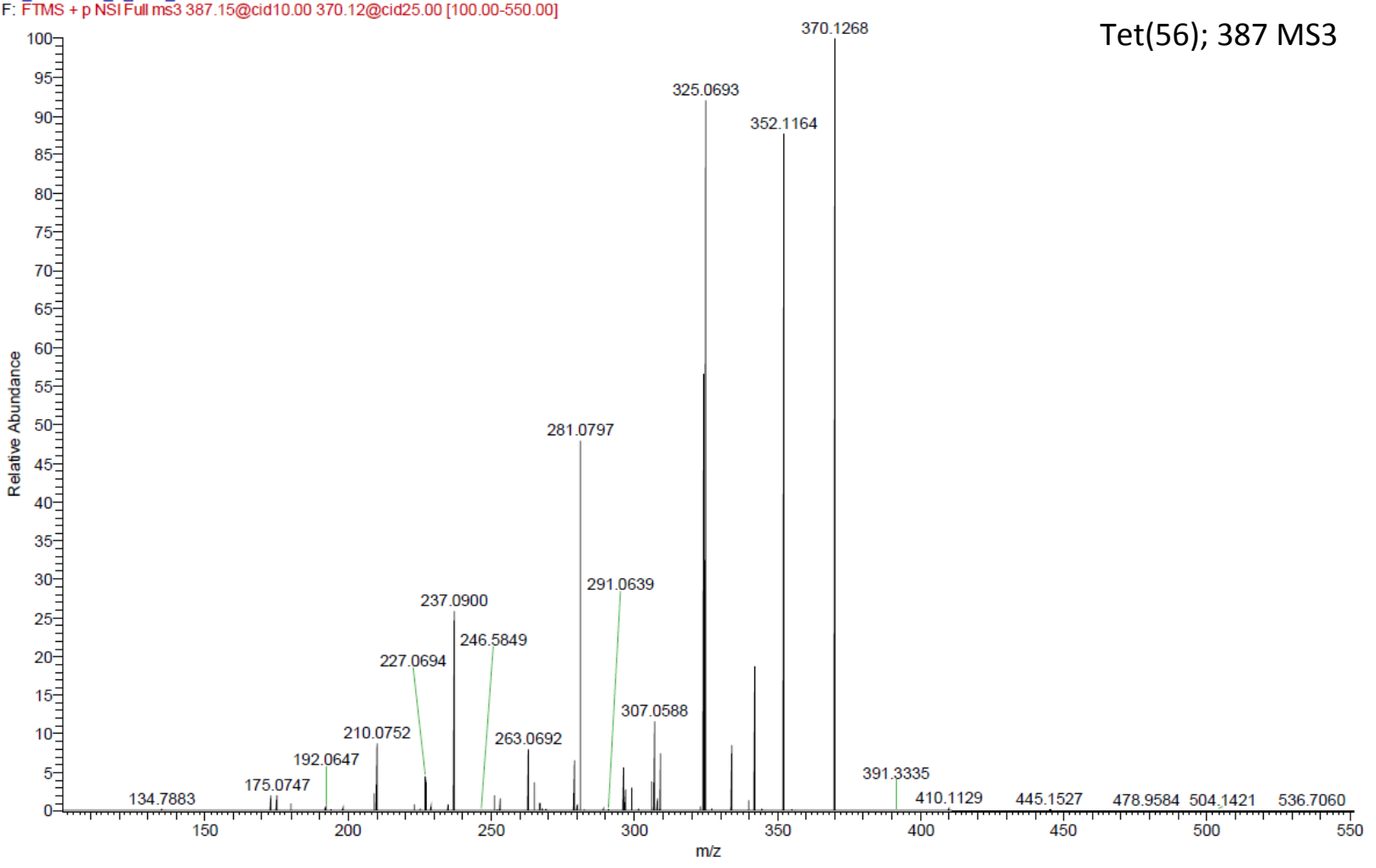
KF_20150422_K_MS #2-13 RT: 0.03-0.34 AV: 12 NL: 7.48E6
F: FTMS + p NSI Full ms [300.00-550.00]

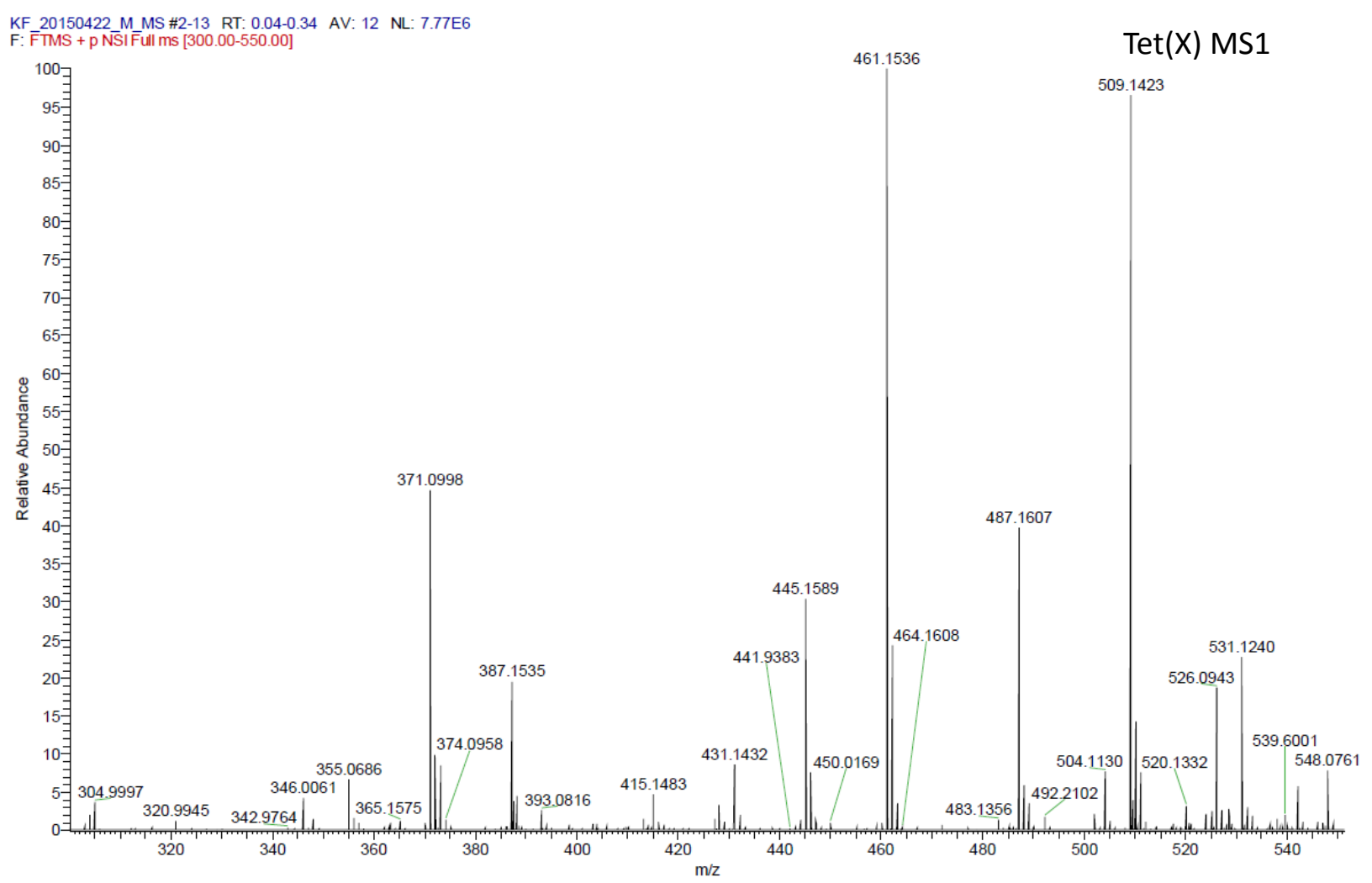
Tet(56) MS1

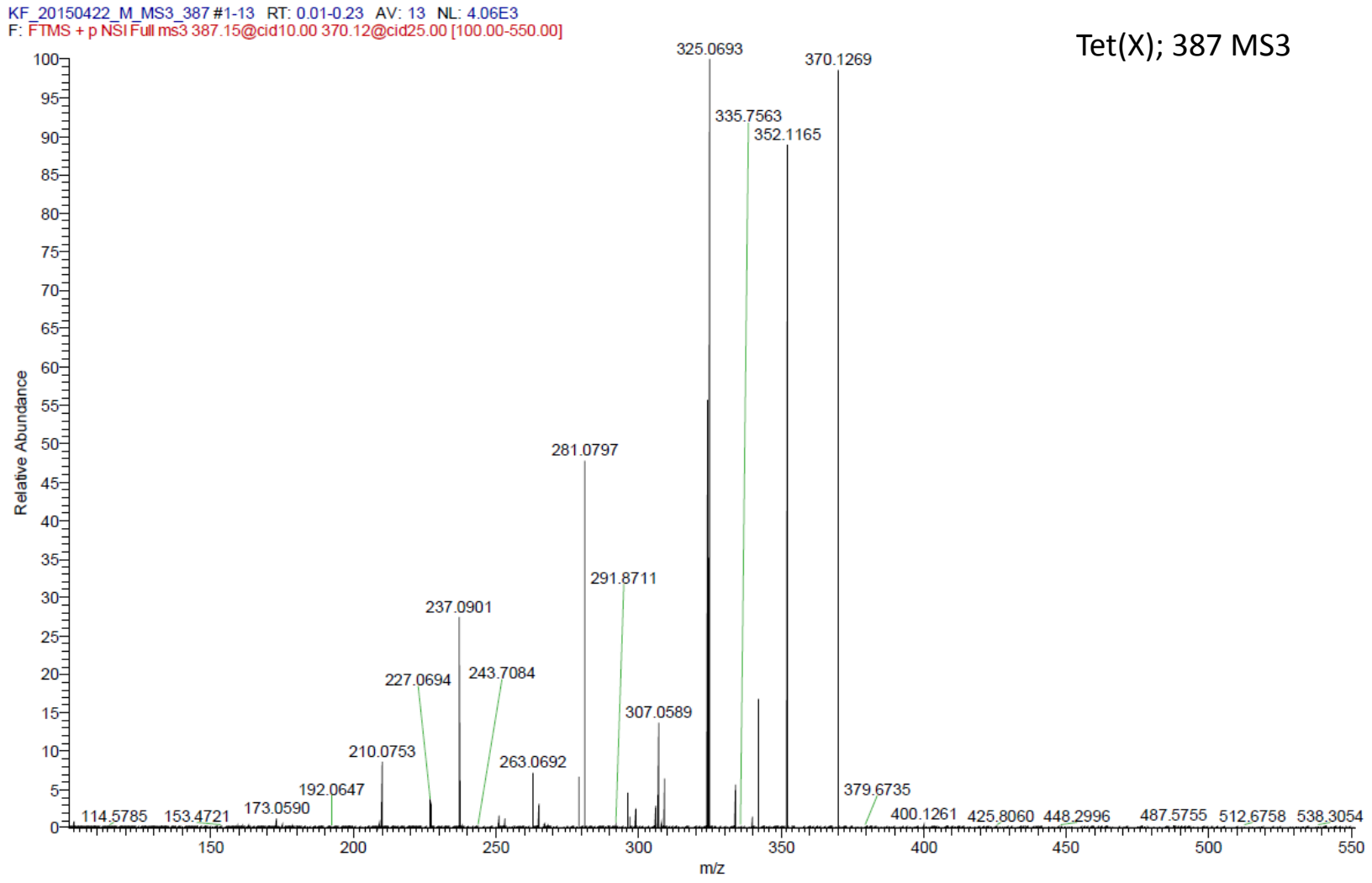


KF_20150422_K_MS3_387 #1-14 RT: 0.01-0.24 AV: 14 NL: 1.45E4
F: FTMS + p NSI Full ms3 387.15@cid10.00 370.12@cid25.00 [100.00-550.00]

Tet(56); 387 MS3




KF_20150422_M_MS #2-13 RT: 0.04-0.34 AV: 12 NL: 7.77E6
F: FTMS + p NSI Full ms [300.00-550.00]
Tet(X) MS1
Relative Abundance
m/z
461.1536
509.1423
371.0998
487.1607
445.1589
464.1608
441.9383
387.1535
531.1240
526.0943
374.0958
539.6001
431.1432
450.0169
504.1130
520.1332
548.0761
355.0686
346.0061
415.1483
492.2102
304.9997
320.9945
342.9764
365.1575
393.0816
483.1356


KF_20150422_M_MS3_387 #1-13 RT: 0.01-0.23 AV: 13 NL: 4.06E3
F: FTMS + p NSI Full ms3 387.15@cid10.00 370.12@cid25.00 [100.00-550.00]
Tet(X); 387 MS3
Relative Abundance
m/z
325.0693
370.1269
335.7563
352.1165
281.0797
291.8711
237.0901
227.0694
243.7084
307.0589
210.0753
263.0692
192.0647
379.6735
173.0590
114.5785
153.4721
400.1261
425.8060
448.2996
487.5755
512.6758
538.3054