Identification of new transformation products during enzymatic treatment of tetracycline and erythromycin antibiotics at laboratory scale by an on-line turbulent flow liquid-chromatography coupled to a high resolution mass spectrometer LTQ-Orbitrap

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Abstract

This work describes the formation of transformation products (TPs) by the enzymatic degradation at laboratory scale of two highly consumed antibiotics: tetracycline (Tc) and erythromycin (ERY). The analysis of the samples was carried out by a fast and simple method based on the novel configuration of the on-line turbulent flow system coupled to a hybrid linear ion trap - high resolution mass spectrometer. The method was optimized and validated for the complete analysis of ERY, Tc and their transformation products within 10 min without any other sample manipulation. Furthermore, the applicability of the on-line procedure was evaluated for 25 additional antibiotics, covering a wide range of chemical classes in different environmental waters with satisfactory quality parameters.

Degradation rates obtained for Tc by laccase enzyme and ERY by EreB esterase enzyme without the presence of mediators were ~78% and ~50%, respectively. Concerning the identification of TPs, three suspected compounds for Tc and five of ERY have been proposed. In the case of Tc, the tentative molecular formulas with errors mass within 2 ppm have been based on the hypothesis of dehydroxylation, (bi)demethylation and oxidation of the rings A and C as major reactions. In contrast, the major TP detected for ERY has been identified as the "dehydration ERY-A", with the same molecular formula of its parent compound. In addition, the evaluation of the antibiotic activity of the samples along the enzymatic treatments showed a decrease around 100% in both cases.

1. Introduction

During last decades, antibiotic compounds have been considered “emerging contaminants” due to their high consumption and pseudo-persistence in the environment (Fatta-Kasinos D., 2011; Richardson *et al.*, 2011). The main input into the environment for pharmaceuticals is through waste water treatment plants (WWTPs) (Richardson *et al.*, 2011). This mild elimination represents an environmental problem since antibiotics can find their way to natural environment trough the effluent wastewaters by their direct discharged into the river. Due to the low degradability of some of the antibiotics by conventional wastewater treatment, different research groups have been focusing the study on alternative degradation processes in order to increase the removal efficiency (Konstantinou *et al.*, 2010). However, these alternative treatments can also lead to the formation of transformation products (TPs) of the pollutants that might be either more persistent or toxic than the parent compound (Watkinson *et al.*, 2007; Farré *et al.*, 2008; Onesios *et al.*, 2009; Michael *et al.*, 2013). In this context, in order to better assess the removal efficiency of such alternative treatment methods, the detection of known and unknown compounds (including TPs) is of high importance as well as to assess their toxicity.

Nowadays, different methods are available which allow the detection and quantification of a multiple class of known antibiotics in contaminated environmental compartments, most of them based on liquid chromatography coupled to mass spectrometry in tandem (LC-MS/MS) (Richardson, 2012; Gros *et al.*, 2013). During recent years, the development of on-line methodologies has increased due to their advantages such as the reduction of sample manipulation, the decrease of the analysis time and the increase in robustness of the analytical method. Nevertheless, and in spite of their excellent analytical performance, these approaches are slowly implemented for routine analysis. In this work the use of a turbulent flow chromatography as a sample pre-treatment step before a screening analysis will be discussed. The main difference between the turbulent flow chromatography with the normal on-line solid phase extraction (SPE) is that, in the latest, laminar flow is used. The turbulence inside the extraction column (flow ≥ 1.5 ml/min) allows the good removal of high molecular weight compounds, whereas small molecules are trapped by the column pores, being a mixed mechanism of sorption and size exclusion chromatography (Thermo Scientific; Llorca, 2012). This technique has been used for the analysis of pharmaceuticals, their metabolites and known transformation products in environmental water samples (López-Serna *et al.*, 2012) or for the analysis of more complicated matrices such as food (Mottier *et al.*, 2007; Stolker *et al.*, 2010). In addition to the common techniques based on the detection of known antibiotics in the environment, different analytical methodologies are being developed in order to detect and identify “new” contaminants and unknown substances that reach WWTPs through the so-called non-target analysis (Krauss *et al.*, 2010). Regarding the identification, the most widely used methodology for organic unknown compounds is based on MS analysis and, in particular, on high resolution mass spectrometry (HRMS) (Hernández *et al.*, 2012).

The objective of this work was to apply the on-line turbulent flow technology as sample pre-treatment method coupled to a hybrid linear ion trap – Orbitrap mass spectrometer for the detection of unknown compounds after enzymatic treatments with the aim to remove the complex matrix of the samples. Although other on-line pre-treatments coupled to a HRMS have been developed for similar purposes (Kovalova *et al.*, 2012; Gros *et al.*, 2014), this is the first time that turbulent flow in combination with HRMS as a pre-treatment step has been used for screening analysis. The developed methodology was applied to the study of unknown degradation products of two antibiotics (Tc and ERY) generated through a novel enzymatic decontamination treatment for waters (ENDETECH; de Gunzburg *et al.*, 2012). Both type of antibiotics are hardly metabolized and further excreted from the body arriving unaltered to WWTPs. For example, Tc arrives almost unaltered to WWTPs, where it is eliminated/redistributed between 60 and 80% (Jia *et al.*, 2009; Pailler *et al.*, 2009; Watkinson *et al.*, 2009; Gros *et al.*, 2010) using conventional activated sludge treatment (CAS) and then discharged into the media through the effluent. Similar pattern is observed for ERY, where the problematic associated to the discharged amounts into the environment from WWTPs is still present at ng/L (Gros *et al.*, 2012). Another problem is associated to the presence of these compounds into the media since it can induce the development of the so called "environmental antibiotic-resistant pathogens" (Hirsch *et al.*, 1999; Bautitz and Nogueira, 2007; Alighardashi *et al.*, 2009; Khan *et al.*, 2010; Huerta *et al.*, 2013). The development of this resistance to antibiotics could cause problems at environmental levels (maybe affecting biota and, finally, human health) although this information is scarce and contradictory (Rizzo *et al.*, 2013). Nonetheless, and as an example, Szczepanowski *et al.* (2009) investigated this effect in WWTPs. The authors detected relevant antibiotic-resistant genes to Tc and macrolides among other antibiotics in WWTP bacteria from Germany. Similar pattern is observed for ERY (macrolide), which also has been described to affect the resistance of bacterial communities in activated sludge (Li and Zhang, 2010) but, in contrast, not in biofilms at environmental relevant concentrations (Wunder *et al.*, 2013). Because of these problems, different authors have studied an alternative degradation processes for both compounds as well as the generation of any transformation products (Tc (Halling-Sørensen *et al.*, 2002; Parka and Chounga, 2007; Chen and Huang, 2010; Khan *et al.*, 2010) and ERY (Kim *et al.*, 2004a; 2004b; Luiz *et al.*, 2010; Pengelly, 2010)).

The studies described in the present work include the biodegradation efficiency of i) Tc at laboratory scale by *laccase* enzyme and (ii) ERY by EreB esterase, both treatments without mediators. The study of TPs generated has been studied by means of a screening method based on on-line turbulent flow chromatography (TFC) coupled to LC–(ESI)–LTQ Orbitrap.

2. Experimental section

2.1 Materials

Chemicals. Pure standards of target compounds (Table S1) were of high purity grade (>90%). All analytes were purchased from Sigma-Aldrich. The calibration mixture used for high resolution mass spectrometry purposes was supplied by Thermo Fisher Scientific (LTQ ESI Positive Ion Calibration Solution and ESI Negative Ion Calibration Solution). Tetracycline hydrochloride (97.5% purity grade) for biodegradation experiments was purchased from Molekula (Dorset, United Kingdom).

Solvents. All the solvents were of high purity. HPLC grade methanol, acetonitrile and water were supplied by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) solution was from Panreac and formic acid 98% was from Merck (Darmstadt, Germany). For high-performance-liquid chromatography-high resolution mass spectrometry HPLC grade methanol and water (Lichrosolv) were supplied by Thermo Fisher Scientific.

Biodegradation experiments. Laccase from *Trametes versicolor* (Sigma 51639 product) for the degradation of Tc was supplied by Sigma-Aldrich. EreB esterase from *Escherichia coli* for the degradation of ERY was produced by c-LEcta.

Extraction cartridges. Solid phase extraction cartridges for off-line sample treatment Oasis HLB (60 mg 3 ml) were from Waters Corporation (Miltford, MA, U.S.A).

Water samples. Different waters were collected during 2013 including effluent and influent water from La Roca WWTP (Catalonia), surface river water from La Muga River (Catalonia) and tap water from Girona (Catalonia). Influent, effluent and river waters were previously filtered through 0.45 µm nylon filter (Millipore, Billerica, MA, USA).

2.2 Biodegradation experiments

The biodegradation experiments for Tc and ERY were carried out at laboratory scale for the degradation of 100 µg/ml by laccase enzyme and EreB esterase, respectively. More detailed information about the experimental procedure can be seen in section 1 from the Supporting Information.

2.3 Sample pre-treatment

Depending on the experiment type the samples were treated by different procedures before the analytical process. More detailed information can be seen in section 2 from the Supporting Information.

2.4 Instrumental analysis by liquid chromatography coupled to mass spectrometry

The quantification of the selected antibiotics in the samples from the degradation processes was carried out in a Waters Acquity Ultra-Performance™ liquid chromatograph system coupled to a hybrid quadrupole - linear ion trap QqLIT (5500 QTRAP from Applied Biosystems, Foster City, CA, USA).

In contrast, the evaluation of the samples for the presence of any transformation product was performed in an Aria TLX-1 turbulent flow chromatograph coupled to a HRMS LTQ-Orbitrap (Thermo Fisher Scientific).

More detailed information about the methodology can be found in section 3 from the Supporting Information as well as the data processing procedure for each case.

3. Results

3.1 Optimization of on-line turbulent flow chromatography

The optimization of the method for the analysis of ERY, Tc and their transformation products was based on different steps that can be seen in section S4 of the Supporting Information. The optimum methodology for the on-line TFC-LC-(ESI)-LTQ Orbitrap was validated according to Commission Decision 2002/657/EC (European_Commission, 2002) in buffered water for a whole set of antibiotics (see Table S1) including ERY and Tc. The analytical quality parameters are summarized in Table S3 and an example of chromatogram can be seen in Figure S1. In addition, the method was tested for different water types including tap water, surface river water as well as effluent and influent waters from WWTP. The results are summarized in Tables S4 and S5.

The MLOD working in full scan mode with mass accuracy of 60,000 were, in general, between 0.01 and 0.07 ng/ml and MLOQ in the range of 0.02 – 0.70 ng/ml. Although these limits were much higher than those reported for the same analytes in more sensitive instruments working in selected reaction monitoring (i.e., QqLIT (Gros *et al.*, 2013)), the still good sensitivity working in full scan acquisition mode should be considered as one advantage of LTQ-Orbitrap mass spectrometer. The combination of the good sensitivity in full scan and the high resolution offers better capabilities for the detection and identification of unknown compounds based on mass accuracy (< 5 ppm) (Gros *et al.*, 2014). Additionally, the performance of MS^n experiments for structural elucidation of TPs should be taken as an advantage (Llorca, 2012). The percentage of recovery of the on-line extraction method was tested at three different spiking levels. The values were ranging between 51 to 115% for all the antibiotics and between 69 – 84% for Tc and 103-115% for ERY in buffered water. As regards to method precision, it was evaluated by the interday and intraday percentage of relative standard deviation (%RSDs) with values below 20%. The applicability of the method to other water matrices showed recoveries between 50 and 120% in almost all waters tested. In addition, no significant deviation of the retention times was observed between real and buffered waters remarking the efficiency of the clean-up process. Finally, the precision showed values below 30% in all the cases (Tables S4 and S5).

The validation of the method proved its applicability for the study of different type of antibiotics in water samples when a pre-cleaning step is necessary. This high-throughput methodology significantly reduces sample manipulation (the analysis is completed in 10 min)

preventing sample contamination and, consequently, increasing its robustness. In addition, this process helps to maintain clean the ultra-high sensitive LTQ-Orbitrap and thus increases its lifetime. Finally, the method was applied to the analysis of samples from enzymatic degradation experiments where a clean-up was necessary

3.2 Degradation of tetracycline

3.2.1 Tetracycline degradation rates

The results of the study based on degradation of 100 µg/ml of Tc at lab-scale with laccase, without the presence of mediators, reached the 78% of removal after 18 hours of exposure (Figure 1 A) and the decrease of the inhibitory activity was estimated to 87% ± 10%, according to the bioassay. These elimination rates can be improved by the use of mediators as shown by Suda *et al.* (2012). The authors showed that tetracycline antibiotics were completely removed after 1h of exposure in the presence of 1-hydroxybenzotriazole (a redox mediator). The same effect was observed by Wen *et al.* (2009) in another study using a crude lignin peroxidase from *Phanerochaete chrysosporium* (white rot fungus) with degradation rates up to 99% after 30 min of exposure in the presence of veratryl alcohol as a mediator.

3.3.2 Identification of transformation products of tetracycline

The chromatograms generated working in full scan mode from the sample at t=0 and after the enzymatic treatment were compared using SIEVE software. Different peaks were found in the chromatogram from the treated sample and not in the samples at time 0, and can thus be considered as potential TPs of Tc degradation (see an example in Figure S2). In order to confirm their identity and to provide a plausible structure, structural elucidation studies based on MS^2 parallel experiments were performed as it has been explained before. These spectra allowed the elucidation of the fragmentation of the molecule based on the proposed structure. This fragmentation was also compared with the fragmentation pattern of Tc in order to look for common fragments of the potential TPs. For this reason, previous experiments achieving the MS^6 fragmentation of this compound were carried out and the elucidation postulated in Figure S3. Finally, the molecular structure for every TP was tentatively proposed by the confirmation of exact mass and ring-double bound equivalent number (RDB).

Three different TPs were detected during degradation experiments of Tc: TP 618, TP 396 and TP 431. The proposed structures are summarized in Table 1 and an approximation of their formation ratio based on chromatographic peak areas are shown in Figure 1 B. As it can be seen, the degradation rate of Tc is near to 70% while TPs generated are estimated to reach the 2.3% for TP 431 and 8% for TP 618 and TP 396. This difference in mass balance could indicate the final mineralization of Tc. However, more studies would be necessary in order to confirm this hypothesis. As regards to molecular structure for the compounds, TP 431 could be generated in a metabolic pathway where Tc is oxidized in the position 5 generating the corresponding ketone (oxytetracycline) and the amino group is bi-demethylated in the position 4

(Table 1). This last bi-demethylation step was described by Halling-Sorensen *et al.* (2003) during the abiotic degradation of oxytetracycline in soil interstitial water. Although, based on this theory the formation of oxytetracycline is a necessary pre-step for the formation of TP 431, its presence was not confirmed in our experiments. Nonetheless, the molecular formula of this compound has been confirmed by the loss of the amino group during MS^2 experiments and RDB equivalents. However, more identification points would be necessary in order to assure the position of the oxidation process. In contrast, the formation of TP 396 (suggested structure in Table 1) can be explained by dehydrogenation in position 12 generating the 12-dehydrotetracycline, the subsequent water elimination in the position 6 and the oxidation in the position 4. The oxidation of the position 4 has been reported in a previous work by Kahn *et al.* (2010) during ozonation of Tc. Finally, the molecular formula postulated for TP 618 (TP 618 A and B in Table 1) remains unclear after structural elucidation studies and further efforts should be done in order to know the position of the R group and its correct structural formula. To the authors' knowledge, this is the first time that these TPs have been detected during the enzymatic degradation of Tc with laccase.

3.4 Degradation of erythromycin

3.4.1 Erythromycin degradation rates

Degradation rate of ERY by EreB esterase achieved 52% after 16 h of treatment (Figure 1 A). The qualitative assay of antibiotic activity also showed a decrease after the treatment. The efficiency of different organisms in the degradation of ERY has been tested before by, for example, Alighardashi *et al.* (2009). The authors observed the high efficiency in the inactivation of this compound by sludge bacteria. Another example was given by Kim *et al.* (2004b) with the study of mineralization of ERY by sediment microbial. The results showed the partial or complete mineralization probably due to the increase in the microbial population density that metabolizes this compound. In addition, the degradation in aqueous solution at different pH was studied by the same authors and different mechanistic models were proposed (Kim *et al.*, 2004a).

3.4.2 Identification of transformation products of erythromycin

Regarding the studies based on TPs for ERY, a longer analytical chromatographic column was used instead of the Hypersil GOLD: Acquity UPLC® HSS T3 (2.1 x 100 mm; 1.8 µm), in order to obtain a better chromatographic separation between the TPs. The chromatographic deconvolution (by SIEVE software) of the samples showed the presence of 5 suspected TPs in the treated sample: TP 734, TP 752, TP 750, TP 748 (A) and TP 748 (B). The summary of the main proposed structures can be seen in Table 1 and the tentatively degradation pathways are shown in Figure 2. The hypothetic structures were elucidated through exact mass, MS^2 fragmentation and RDB equivalents of molecular ion and the fragments. With the aim of identifying the most common fragments of ERY, as in the case of Tc, MS^n

experiments were previously performed. The proposed pathway (Figure S4) is confirmed by a previous study carried out by Haghedooren *et al.* (2006).

During data analysis, TP 734 was identified as the major degradation product. The ratio formation of this compound is estimated to reach the 100% whereas only 50% of degradation of ERY was observed (Figure 1 C). This can be explained by de different ionization efficiency in ESI. Concerning to the hypothetic pathway formation for this TP (TP 734, "dehydration erythromycin A"), it was also identified by Pengley (Pengelly, 2010) as the major product of ERY treated with EreB esterase by the formation of the intermediate TP 752 as it has been tentatively proposed in Figure 2. This last TP was also detected in other enzymatic study (with ERY esterase) carried out by Kim *et al.* (2004b) in aquaculture sediment containing ERY-resistant Pseudomonas species. Moreover, other biodegradation studies have reported this compound (Feldman *et al.*, 1963; Flickinger and Perlman, 1975; Kim *et al.*, 2002).

Additionally, other minor TPs were detected. For example, TP 748 (A) (known as erythromycin E) could be generated during the biodegradation of ERY (Vara *et al.*, 1989; Gerhard, 1999). In this case, the estimation of the calculated formation ratio was around 7%. The TP 750 could be generated by the oxidation of the methyl group in the position C2 (~ 1%) and its consequent dehydrogenation could lead to TP 748 (B) (~ 5%). In addition, a structural isomer compound of TPs 748 (A) and (B), namely TP 748 (C) (t_r = 8.09 min, see Table 1), was observed. This compound was proposed to be generated through the methylation of the hydroxyl group in the position C6 but with a minimum ration formation (0.5%). As regards to TP 720 (~2.8%), this one could be generated by the L-cladinose demethylation of ERY. To conclude with minor TPs, the dehydroxylation of the C11 (the most likely position) was proposed for the generation of TP 718 (also named erythromycin D) with a ratio formation of 5%. The consequent L-cladinose demethylation of this TP could lead the TP 704 (ratio of 0.4%). However, the generation of TP 720 (erythromycin C) and TP 718 (erythromycin D) is quite peculiar since they have been described to be formed by different biosynthesis pathways to generate ERY by an irreversible reaction (Vara *et al.*, 1989; Gerhard, 1999).

4. Conclusions

The novel combination of TFC coupled to LTQ-Orbitrap has been proved to be a useful methodology for the analysis of 27 antibiotics in water samples as well as for the investigation of transformation products of target antibiotics after an enzymatic treatment. The method allows low sample manipulation and time consumption as well as high robustness and precision. Another advantage is the structural elucidation of the compounds by means of accurate mass of the molecular ion as well as the fragment ions from MS^2 experiments performed in parallel. The analytical approach has been used for the clean-up of complex samples from the enzymatic treatment of Tc and ERY and to identify transformation products. The promising results (quantitative removal of antibiotic concentration and activity) situate the enzymatic decontamination as a reliable complementary water treatment for elimination of antibiotics. In

addition, the study of transformation products denoted the generation of three tentatively TPs for Tc and five for ERY. Further studies are needed in order to decrease the degradation time and to enlarge the applicability of the enzymatic treatment.

Acknowledgments

This work has been founded by the European Community's Seventh Framework Program ENDETECH research project [FP7/2007-2013] under grant agreement n°282818. ML wants to acknowledge the collaborative work of all the partners from the ENDETECH project. This study has been co-financed by the European Union through the European Regional Development Fund (FEDER). This work was partly supported by the Generalitat de Catalunya (Consolidated Research Group: Catalan Institute for water Research 2014 SGR 291). Thermo Scientific is acknowledged for the columns.

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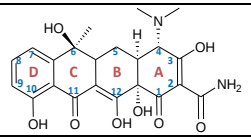
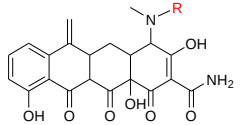
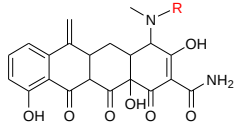
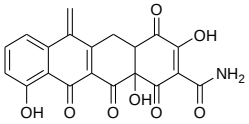
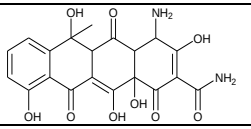
467

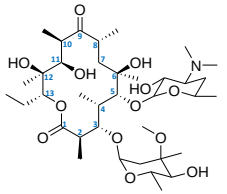
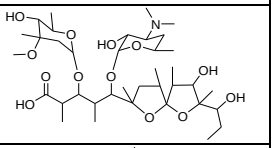
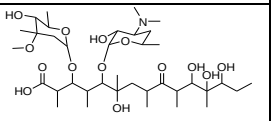
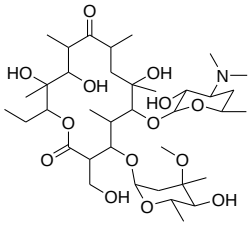
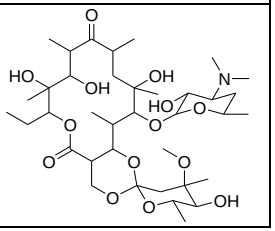
Figure captions

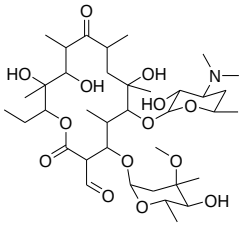
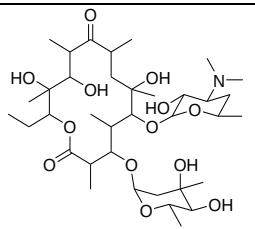
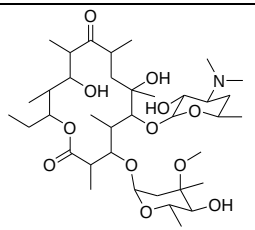
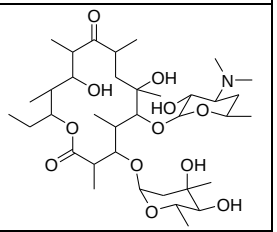
Figure 1: **A)** Biodegradation rates of 100 µg/ml of Tc by *Laccase* from *Trametes versicolor* (17.5 µg/ml) after 18 h and 100 µg/ml of ERY by EreB esterase (1.8 µg/ml) after 16 h; **B)** ratio formation of detected TPs during Tc degradation studies; **C)** ratio formation of detected TPs during ERY degradation studies by EreB. A: Area of the corresponding chromatographic peak, Ao: Peak area of Tc at time 0 and A: Area at time 18 h or 16 h

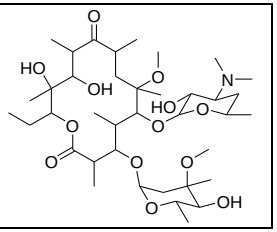
Figure 2: proposed degradation pathways of ERY by EreB esterase

475 **Table 1**: Accurate mass measurements of Tc and ERY after enzymatic degradations and proposed molecular structures for the TPs

Retention time (min)	Compound	Ion	Measured mass [m/z]	Elemental composition	Calculated mass [m/z]	Relative error [ppm]	Double-bond equivalents (RDB)	Proposed structure
Tc								
4.68 (on-line) 3.08 (off-line)	Tc	$[M+H]^+$ $[M+H-H_2O]^+$ ***** $[M+H-H_2O\ -NH_3]^+$ *****	445.1608 427.1505 410.1239	$C_{22}H_{25}N_2O_8$ $C_{22}H_{23}N_2O_7$ $C_{22}H_{20}NO_7$	445.1605 427.1500 410.1234	0.579 1.223 1.150	11.5 12.5 13.5	
Tc degradation by lab-scale experiments (results obtained by on-line TFC experiments)								
5.19	TP 618 (A)	$[M+H]^+$ $[M+H-R]^+$ ***** $[M+H-R-NH_3]^+$ *****	618.1894 412.1267 395.1005	$C_{24}H_{28}N_9O_{11}$ $C_{21}H_{20}N_2O_7$ $C_{21}H_{17}NO_7$	618.1903 412.1265 395.1000	1.456 0.479 1.384	15.5 13.0 14.0	 R: $C_3H_8O_4N_7$
5.19	TP 618 (B)	$[M+H]^+$ $[M+H-R]^+$ ***** $[M+H-R-NH_3]^+$ *****	618.1894 412.1267 395.1005	$C_{26}H_{30}N_6O_{12}$ $C_{21}H_{20}N_2O_7$ $C_{21}H_{17}NO_7$	618.1916 412.1265 395.1000	-3.594 0.479 1.384	15.0 13.0 14.0	 R: $C_5H_{10}O_5N_4$
5.66	TP 396	$[M+H]^+$ $[M+H-\ NH_3]^+$ ***** $[M+H-NH_3-CO]^+$ *****	396.0714 379.0449 351.0500	$C_{20}H_{14}NO_8$ $C_{20}H_{11}O_8$ $C_{19}H_{11}O_7$	396.0713 379.0448 351.0499	0.018 0.148 0.202	14.5 15.5 14.5	
5.95	TP 431	$[M+H]^+$ $[M+H-\ NH_3]^+$ *****	431.1087 396.0717	$C_{20}H_{19}N_2O_9$	431.1085	0.449	12.5	
ERY								

7.48 (on-line) 4.49 (off-line)	ERY	[M+H]$^+$ [M+H- (L-cladinose)]$^+$ *	734.4693 576.3748	$C_{37}H_{68}NO_{13}$ $C_{29}H_{54}NO_{10}$	735.4763 576.3742	1.065 1.001	4.5 3.5	
ERY degradation by lab-scale experiments (results obtained by on-line TFC experiments)								
7.96 †	TP 734 (dehydration ERY)	[M+H]$^+$ [M+H- (L-cladinose)]$^+$ *	734.4688 576.3745	$C_{37}H_{68}NO_{13}$ $C_{29}H_{54}NO_{10}$	735.4763 576.3742	0.317 0.480	4.5 3.5	
7.46 †	TP 752	[M+H]$^+$ [M+H- OH]$^+$ * [M+H- (L-cladinose)]$^+$ *	752.4768 735.4733 594.3876	$C_{37}H_{70}NO_{14}$ $C_{37}H_{69}NO_{13}$ $C_{29}H_{56}NO_{11}$	752.4791 735.4763 594.3847	-2.966 -4.191 4.731	3.5 4.0 2.5	
7.56 †	TP 750	[M+H]$^+$ [M+H- (L-cladinose)]$^+$ * [M+H- [O-(L-cladinose)]]$^+$ *	750.4643 592.3701 576.3752	$C_{37}H_{68}N\ O_{14}$ $C_{29}H_{54}N\ O_{11}$ $C_{29}H_{54}N\ O_{10}$	750.4634 592.3691 576.3742	1.143 1.557 1.764	4.5 3.5 3.5	
7.69 †	TP 748 (A) (ERY-E)	[M+H]$^+$ [M+H-[O- (L-cladinose)]]$^+$ *	748.4482 576.3748	$C_{37}H_{66}NO_{14}$ $C_{29}H_{54}NO_{10}$	748.4479 576.3742	0.612 0.983	5.5 3.5	

7.26 †	TP 748 (B) (from ERY-E)	[[M+H]$^+$ [M+H- CO]$^+$ ***** [M+H-CO-(L-cladinose)]$^+$ *****	748.4482 720.4539 576.3745	$C_{37}H_{66}NO_{14}$ $C_{36}H_{66}NO_{13}$ $C_{29}H_{54}NO_{10}$	748.4479 720.4528 576.3742	0.612 1.433 0.446	5.5 4.5 3.5	
7.66 †	TP 720 (ERY-C)	[M+H]$^+$ [M+H- (L-desmethyl-cladinose)]$^+$ *****	720.4542 576.3749	$C_{36}H_{66}NO_{13}$ $C_{29}H_{54}NO_{10}$	720.4528 576.3742	1.794 1.244	4.5 3.5	
8.05 †	TP 718	[M+H]$^+$ [M+H- [O-(L-cladinose)]]$^+$ *****	718.4746 560.3801	$C_{37}H_{68}NO_{12}$ $C_{29}H_{54}NO_9$	718.4736 560.3793	1.318 1.359	4.5 3.5	
7.74 †	TP 704	[M+H]$^+$ [M+H- (L-desmethyl-cladinose)]$^+$ *****	704.4599 560.3805	$C_{36}H_{66}NO_{12}$ $C_{29}H_{54}NO_9$	704.4579 560.3793	2.807 2.055	4.5 3.5	

8.09 †	TP 748 (C)	$[M+H]^+$ $[M+H-CH_2O]^+$ * $[M+H-CH_3$-[O-(L-cladinose)]]$^+$ ****	748.4851 718.4750 560.3804	$C_{38}H_{70}NO_{13}$ $C_{37}H_{68}NO_{12}$ $C_{29}H_{54}NO_9$	748.4842 718.4736 560.3793	1.232 1.986 2.001	4.5 4.5 3.5	

476 ***** Fragments confirmed by full scan at mass accuracy of 60,000 and by MS^2 experiments

477 † The analytical column was an Acquity UPLC® HSS T3 (2.1 x 100 mm; 1.8 µm), longer than the normally used in order to increase the chromatographic
478 separation between TPs

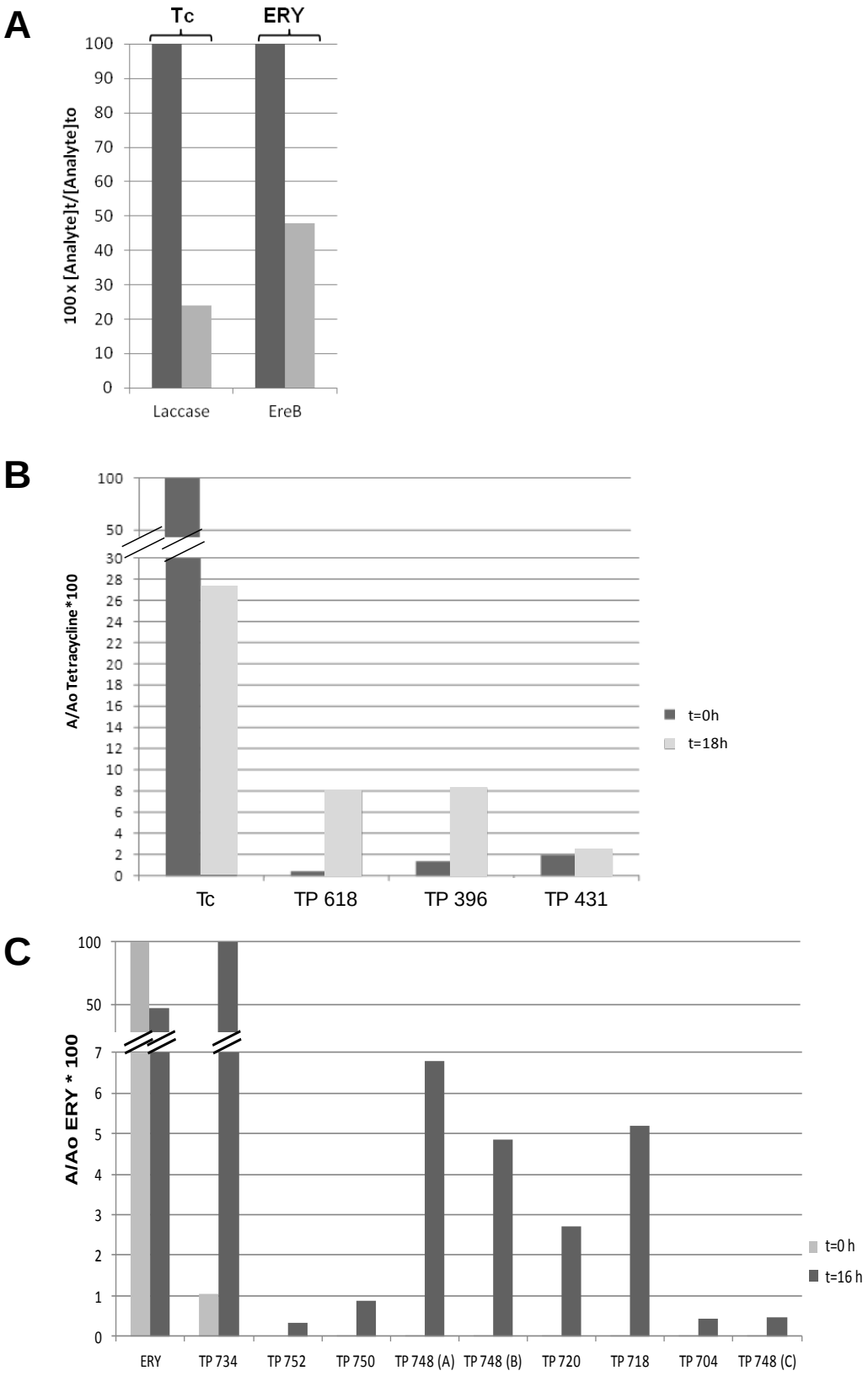
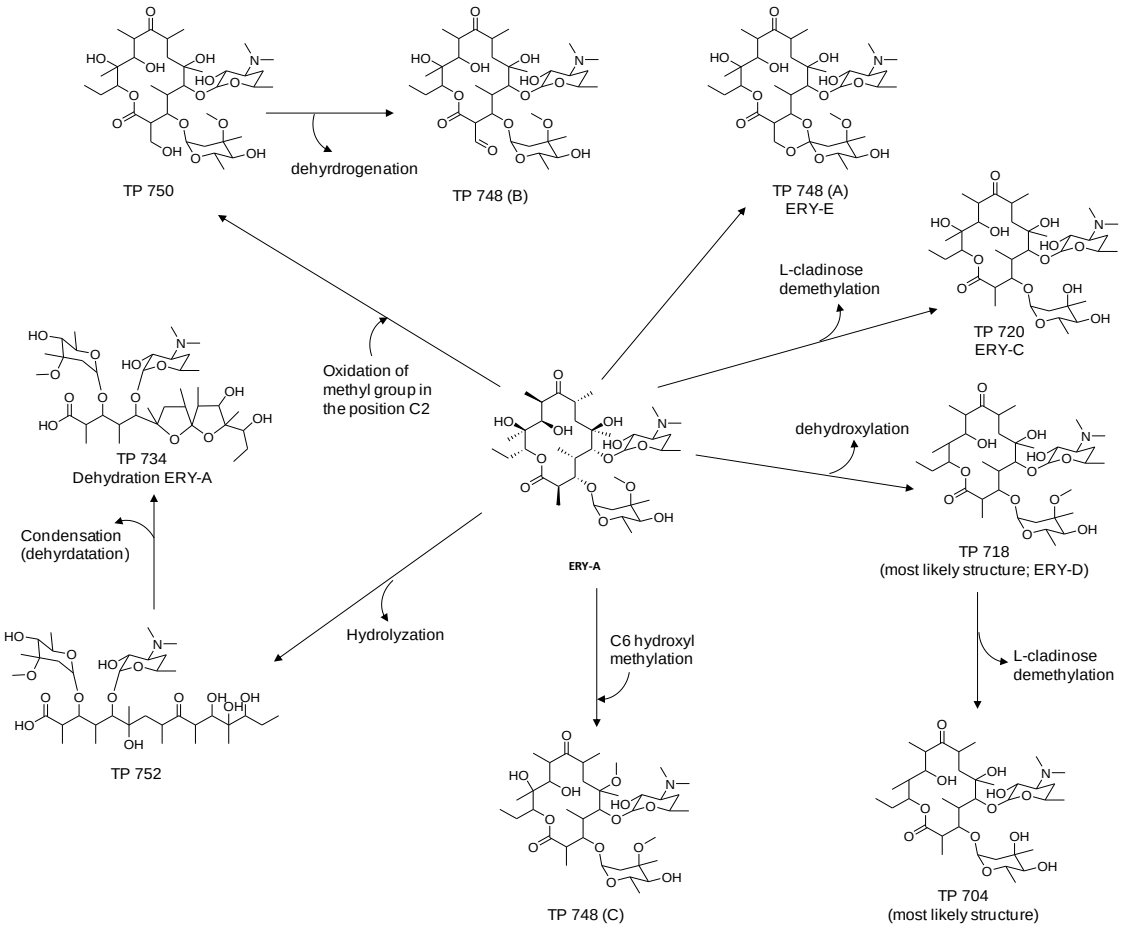

A
Tc
ERY
100 x [Analyte]t/[Analyte]to
Laccase
EreB
B
A/Ao Tetracycline*100
t=0h
t=18h
Tc
TP 618
TP 396
TP 431
C
A/Ao ERY * 100
t=0 h
t=16 h
ERY
TP 734
TP 752
TP 750
TP 748 (A)
TP 748 (B)
TP 720
TP 718
TP 704
TP 748 (C)

Figure 1



481

482 **Figure 2**

Supplementary Material

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