

Human Metabolite Lamotrigine-N2-glucuronide Is the Principal Source of Lamotrigine-Derived Compounds in Wastewater Treatment Plants and Surface Water

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SUPPORTING INFORMATION

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WWTPs

The five WWTPs (WWTP 1-5) are situated in the metropolitan area of the city of Barcelona. They are conventional WWTPs equipped with mechanical treatment (pre-treatment and primary clarifier), followed by activated sludge treatment with denitrification and nitrification, and phosphate removal. The parameters given correspond to hydraulic retention time (HRT), average daily flow and population equivalents (PE).

They were as follows: WWTP - 1: HRT – 0.5 d, flow - 40,000 m³d⁻¹, 165, 000 PE; WWTP - 2: HRT – 0.3 d, flow – 240,800 m³d⁻¹, 1,142,000 PE; WWTP - 3: HRT – 0.3 d, flow – 50 000 m³d⁻¹, 229,000 PE; WWTP - 4: HRT – 0.6 d, flow – 33,000 m³d⁻¹, 220,000 PE; WWTP - 5: HRT – 0.32 d, flow -45,900 m³d⁻¹, 198,000 PE.

Sampling of wastewater

Wastewater samples were provided by the staff of WWTPs involved in the study. All samples were 24-hour composite samples taken in period from November 2013 to January 2014. All samples were collected using automatic sampler but the sampling mode was different between the WWTPs sampled.

WWTP - 1 and WWTP - 4 use a time-proportional sampling mode. In the case of WWTP - 1 250 mL samples are collected every 15 minutes in 24 1-liter bottles. These are later combined and integrated to a single sample. WWTP - 4 uses likewise a time-proportional mode but with less frequent sample uptake – 250 ml are collected every hour over the course of 24 hours. Like in the case of WWTP - 1, samples are integrated after all the 24-h samples are collected.

WWTP - 2 uses a flow-proportional sampling mode based on the official Catalan regulations.¹

A total volume of 8 L is collected over the course of 24 hours with the following average distribution; 72 % from 11 a.m. to 22 p.m., 10 % from 11 p.m. to 2 a.m., 6 % from 3 a.m. to 6

a.m. and 12 % from 7 a.m. to 10 a.m. WWTP - 3 uses a volume-proportional sampling mode. In this case, a sample of approximately 100 mL is taken every 1000 m³ of volume to reach 5 L daily collected wastewater.

Surface water samples

Surface water samples were taken as grab samples. They were located along the two rivers located near metropolitan area of the city of Barcelona. 7 samples were taken from Llobregat river and 6 samples from Besos river (**Figure SI-1**). Llobregat samples were marked LL-1-7 and Besos samples BS-1-6. Both Llobregat and Besos river receive discharge from the WWTP 1-4 analyzed.

These surface water samples have been used previously for detection based detection of iodinated contrast media photodegradates and in this work were re-analyzed for suspect screening and quantification of lamotrigine and its derived compounds.

Table SI-1, which shows physico-chemical properties and location of the surface water samples, is reproduced from previous published work by Zonja et al.²

Figure SI-1. 13 sampling sites of Besos and Llobregat rivers.

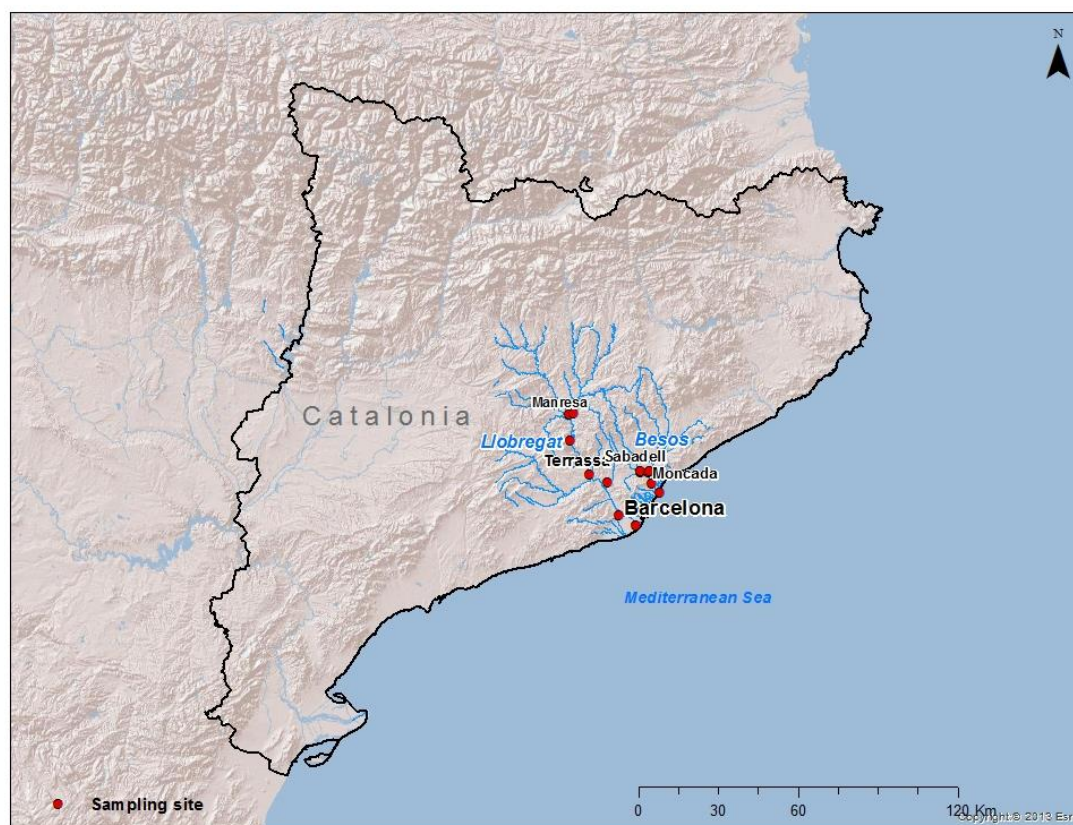


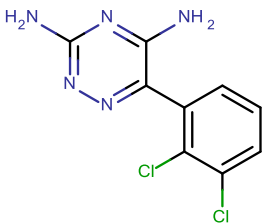
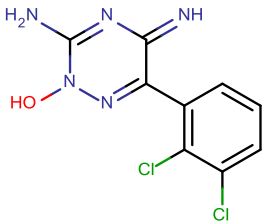
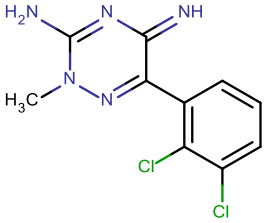
Table SI-1. Physico-chemical properties and location of the samples

SL	pH	T	O ₂		σ	UTM coordinates		
		[°C]	[mg/L]	[%]		Zone	X	Y
BS-1	8.3	16.1	8.30	86.2	1484	31	429192	4593550
BS-2	8.0	14.0	8.71	91.4	1165	31	429307	4593848
BS-3	8.3	13.5	9.25	96.2	838	31	432000	4593213
BS-4	8.1	15.3	9.10	95.4	1447	31	432651	4593766
BS-5	7.9	17.2	7.04	86.2	1388	31	433154	4589203
BS-6	7.8	16.8	6.33	80.5	35009	31	435804	4585677
LL-1	7.9	8.2	8.99	91.6	757	31	403881	4616871
LL-2	7.9	9.2	9.30	96.2	1087	31	405907	4617415
LL-3	7.8	7.9	9.04	92.8	1139	31	403792	4607459
LL-4	8.1	8.9	8.96	92.4	1220	31	410078	4594291
LL-5	8.1	14.2	8.72	90.4	1407	31	416614	4590452
LL-6	8.0	10.3	9.09	94.7	1395	31	420247	4577928
LL-7	7.3	12.1	*	*	6050	31	426109	4573894

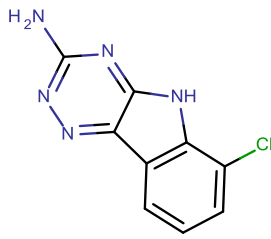
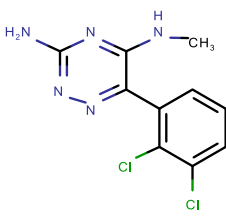
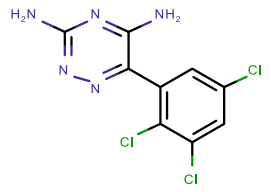
BS - Besos river, LL – Llobregat river, SL - sampling location, T – temperature, σ – conductivity; * probe malfunctioned

Suspect list

Table SI-2³. Suspect list comprised of lamotrigine and derived compounds

Name	Structure	SMILES	Type	Exact mass ³⁵ Cl [M+H] ⁺	Exact mass ³⁷ Cl [M+H] ⁺	Elemental composition	Log P	IUPAC name	Ref.
Lamotrigine (LMG)		<chem>NC1=NN=C(C(N)=N1)C1=C(Cl)C(Cl)=CC=C1</chem>	Parent compound	256.0151	258.0122	C ₉ H ₇ Cl ₂ N ₅	1.93	6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine	
Lamotrigine N2-oxide (LMG-N2-oxide)		<chem>NC1=NC(=N)C(=NN1O)C1=C(Cl)C(Cl)=CC=C1</chem>	Minor human metabolite	272.0100	274.0071	C ₉ H ₇ Cl ₂ N ₅ O	1.76	3-amino-6-(2,3-dichlorophenyl)-5-imino-2,5-dihydro-1,2,4-triazin-2-ol	4, 5
N2-methyl-lamotrigine (N2-Me-LMG)		<chem>CN1N=C(C(=N)N=C1N)C1=C(Cl)C(Cl)=CC=C1</chem>	Minor human metabolite	270.0308	272.0278	C ₁₀ H ₉ Cl ₂ N ₅	1.92	6-(2,3-dichlorophenyl)-5-imino-2-methyl-2,5-dihydro-1,2,4-triazin-3-amine	4, 5

Lamotrigine-N2-glucuronide (LMG-N2-G)		<chem>NC1=C(N=[N+](C2OC(C(O)C(O)C2O)C(O)=O)C(N)=N1)C1=C(Cl)C(Cl)=CC=C1</chem>	Major human metabolite	432.0472	434.0443	$C_{15}H_{16}Cl_2N_5O_6$	-3.01	3,5-diamino-2-(6-carboxy-3,4,5-trihydroxyoxan-2-yl)-6-(2,3-dichlorophenyl)-1,2λ ⁵ ,4-triazin-2-ylum	4, 5
Lamotrigine-N5-glucuronide (LMG-N5-G)		<chem>NC1=NC(NC2OC(C(O)C(O)C2O)C(O)=O)C(N=N1)C1=C(Cl)C(Cl)=CC=C1</chem>	Minor human metabolite	432.0472	434.0443	$C_{15}H_{16}Cl_2N_5O_6$	-1.94	6-1-triazin-5-yl]amino-3,4,5-trihydroxyoxane-2-carboxylic acid	4, 5
Lamotrigine-impurity A (OXO-LMG)		<chem>NC1=NC(=O)C(N=N1)C1=C(Cl)C(Cl)=CC=C1</chem>	USP Lamotrigine Related Compound C (impurity)	256.9991	258.9962	$C_9H_6Cl_2N_4O$	1.69	3-amino-6-(2,3-dichlorophenyl)-5,6-dihydro-1,2,4-triazin-5-one	6
Lamotrigine-impurity B (diOXO-LMG)		<chem>ClC1=CC=CC(C2N=NC(=O)NC2=O)=C1Cl</chem>	Lamotrigine Impurity D	257.9832	259.9802	$C_9H_5Cl_2N_3O_2$	2.01	6-(2,3-dichlorophenyl)-3,4,5,6-tetrahydro-1,2,4-triazine-3,5-dione	*7
PhotoTP-1	Not identified	Not identified	PhotoTP@ pH7	259.0148	261.0118	$C_9H_9Cl_2N_4O$	-	-	8

PhotoTP-2		<chem>NC1=NN=C2C(NC3=C2C=CC=C3Cl)=N1</chem>	Photo-degradation product @pH7	219.0312	221.0282	$C_9H_6ClN_5$	1.39	6-chloro-5H-[1,2,4]triazino[5,6-b]indol-3-amine	8
PhotoTP-3	Not identified	Not identified	PhotoTP@pH7	256.0151	258.0122	$C_9H_7Cl_2N_5$	-	-	8
N5-methyl-lamotrigine (N5-Me-LMG)		<chem>CNC1=NC(N)=NN=C1C1=C(Cl)C(Cl)=CC=C1</chem>	Lamotrigine impurity	270.0308	272.0278	$C_{10}H_9Cl_2N_5$	2.23	6-(2,3-dichlorophenyl)-N5-methyl-1,2,4-triazine-3,5-diamine	*
Chlorinated lamotrigine (Cl-LMG)		<chem>NC1=NN=C(C(N)=N1)C1=C(Cl)C(Cl)=CC(Cl)=C1</chem>	Pharmacopeia reported impurity	289.9762	291.9732	$C_9H_6Cl_3N_5$	2.53	6-(2,3,5-trichlorophenyl)-1,2,4-triazine-3,5-diamine	*

* Toronto Research Chemicals, <http://www.trc-canada.com> (accessed June 7th 2015)

Screening method for wastewater samples and re-analyzed surface water samples

Chromatographic conditions for the suspect screening were as follows:

The mobile phases used were: (A) acetonitrile with 0.1 % formic acid and (B) water (0.1 % formic acid). Elution was accomplished with the following solvent gradient: 0 min (3 % A) - 1 min (3 % A) – 17 min (95 % A), - 21 min (95 % A) – 21.5 min (3% A) and stabilized until 25 min. The flow rate was $300 \mu\text{L min}^{-1}$, the column temperature was held at 40 °C and the auto sampler was set to the temperature of 8 °C.

For the MS screening method, Q-Orbitrap-MS was equipped with Electrospray ionization interface (ESI) which operated in the positive ion mode with the following settings: spray voltage +3.0 kV, heater temperature 250 °C, and capillary temperature 350 °C.

For the detection method, a data dependent scan was applied with the following parameters; Full scan and data dependant-MS² were both set to resolution of 35 000 [full width at half maximum (FWHM)], with the full scan range of m/z 100-1000. Quadrupole isolation window was set to m/z 2 and the normalized collision energy (NCE) to 35.

Suspect screening of WWTPs and surface water - preliminary results

Preliminary wastewater analysis

Results of wastewater which are shown in **Table SI-3** show that while N2-Me-LMG and LMG-N2-Oxide were only sporadically detected in effluents samples, LMG-N2-G was the most abundant LMG-related compound in influent samples followed by LMG. The predominance of the glucuronide with respect to the parent compound is in agreement with the reported excretion pattern in humans as summarized in the introduction.^{9, 10} Comparison of the levels of LMG and its related compounds in influent and effluent samples shows a substantial decrease in the concentration of the glucuronide, while LMG was measured at higher levels in the treated sewage. This observation would match the expectation of extensive deconjugation during biological WW treatment unmasking LMG. However, the two metabolites N2-Me-LMG and LMG-N2-Oxide (quantified using LMG as a standard), found at low concentrations in influent WWTP 1 and 4 seem to be formed in those WWTPs while there was no apparent change in concentration between influent and effluent of WWTPs 2 and 3. Also, high concentrations of the reported LMG impurity, OXO-LMG, were found in effluent samples of WWTP 1 and 2 at higher concentrations than in influent samples suggesting the possibility of its formation during biodegradation process in the secondary treatment of the WWTP.

Table SI-3. Screening for lamotrigine and related compounds in WWTPs and surface water.

The concentrations here presented are semi-quantitative and are based on using lamotrigine as a standard. This table was used solely; (i) to check whether the suspect compounds are present in the samples and (ii) to see, in very relative terms, if there is an increase or a decrease of these compounds between the influent and the effluent of WWTPs.

ngL ⁻¹		LMG-N2-G	LMG	N2-Me-LMG	N2-LMG-Oxide	OXO-LMG
WWTP 1	<i>influent</i>	476	520	< 5	< 5	18
	<i>effluent</i>	137	1254	23	13	96
WWTP 2	<i>influent</i>	33	21	< 5	< 5	< 5
	<i>effluent</i>	6	97	< 5	< 5	8
WWTP 3	<i>influent</i>	6	13	< 5	< 5	< 5
	<i>effluent</i>	< 5	18	< 5	< 5	< 5
WWTP 4	<i>influent</i>	88	45	< 5	< 5	< 5
	<i>effluent</i>	37	325	7	< 5	15
Surface water**	<i>Ll- 1</i>	< 5	41	< 5	< 5	< 5
	<i>Ll- 2</i>	< 5	41	< 5	< 5	< 5
	<i>Ll- 3</i>	< 5	37	< 5	< 5	< 5
	<i>Ll- 4</i>	6	64	< 5	< 5	7
	<i>Ll- 5</i>	54	1011	18	< 5	77
	<i>Ll- 6</i>	14	139	< 5	< 5	13
	<i>Ll- 7</i>	8	111	< 5	< 5	9
	<i>Bs- 1</i>	< 5	237	6	< 5	18
	<i>Bs- 2</i>	< 5	162	< 5	< 5	15
	<i>Bs- 3</i>	6	234	8	< 5	18
	<i>Bs- 4</i>	< 5	172	< 5	< 5	29
	<i>Bs- 5</i>	< 5	141	< 5	< 5	23
	<i>Bs- 6</i>	< 5	64	< 5	< 5	7

*Concentration limit of quantification was set arbitrarily to 5 ngL⁻¹ since at this point standards of other compounds were not available

**BS - Besos river, LL – Llobregat river

Activated sludge experiments

Before the biodegradation experiments, 50 mL of a mixture of WWTP-1 influent and effluent were extracted using the 4 cartridges. This was done in order to test whether it would be possible only to use one cartridge for biodegradation experiment. 50 mL was tested since this was the volume intended to be taken after each time-dependent sampling point in the batch reactors. For this purpose, the 50 mL were passed through the four cartridges which were connected in series. Each cartridge was eluted separately as in the analysis of wastewater; however, the extracts were not joined together. They were pre-concentrated to 200 μ L and injected separately to LC-QExactive-Orbitrap-MS.

The results are given in the **Table SI-4**. As can be seen, all of the compounds tested were retained in the HLB cartridge and thus only HLB cartridge was used for all of the biodegradation batch experiments. The numbers in the **Table SI-4** do not represent real recovery since at this point only LMG was available. What the numbers represent is the percentage of area found in the extract of the four cartridges.

Table SI-4. Relative recovery of lamotrigine and derived compounds in activated sludge.

Matrix (volume)	Cartridge	LMG	LMG-N2-G	OXO-LMG	N2-Me-LMG	LMG-N2- oxide
Batch experiments (50 mL)	HLB [%]	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0
	PPL [%]	0	0	0	0	0
	MAX [%]	0	0	0	0	0
	MCX [%]	0	0	0	0	0

Biodegradation of lamotrigine, $^{13}\text{C}_3$ -lamotrigine and lamotrigine N2-glucuronide - 6 days

Lamotrigine in Activated Sludge

Figure SI-2. Degradation of lamotrigine over the course of 6 days in activated sludge bioreactor at concentration $5\ \mu\text{gL}^{-1}$

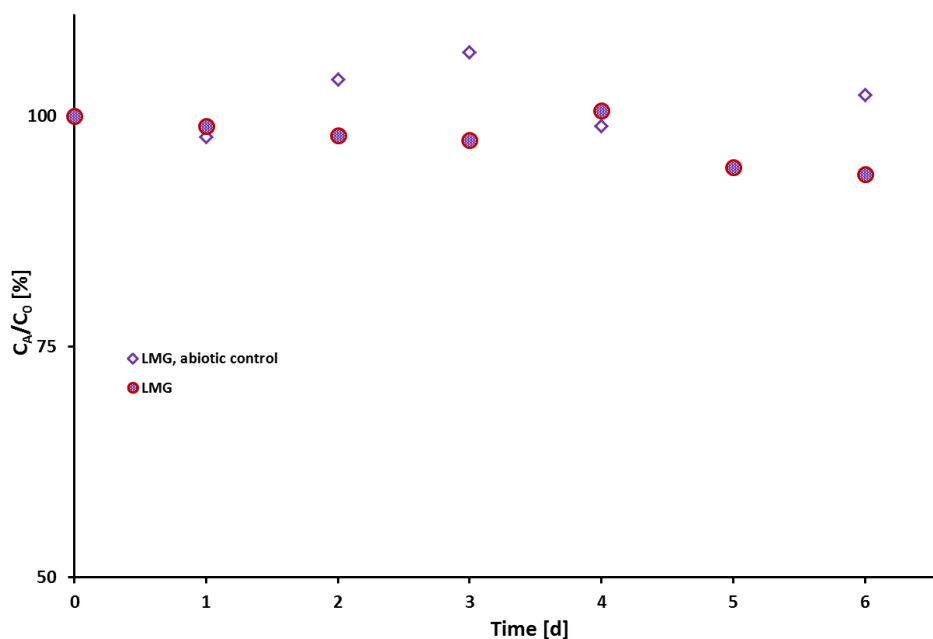
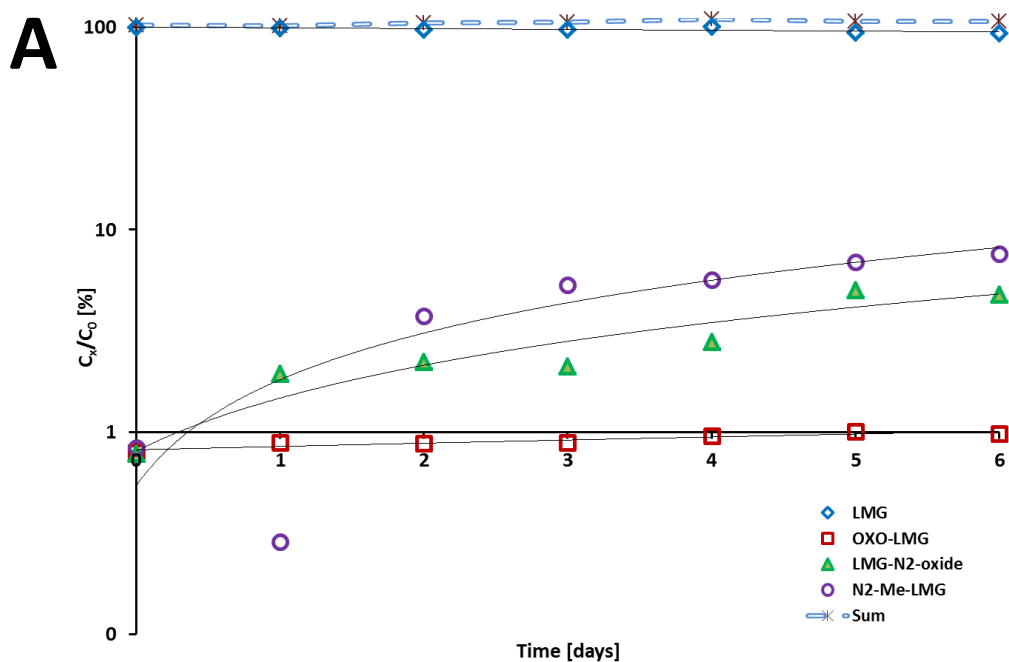
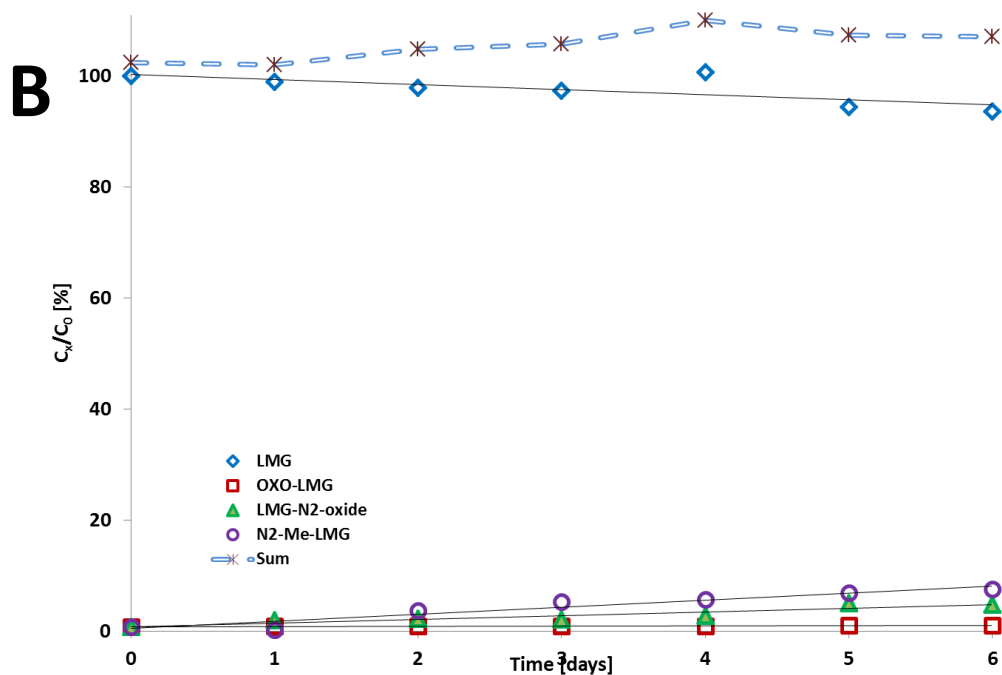


Figure SI-3. Mass balance for lamotrigine over the course of 6 days in activated sludge bioreactor at concentration of $5\ \mu\text{gL}^{-1}$ showed as (A) logarithmic scale and (B) linear scale. Calculations were performed based on molar concentrations.





Lamotrigine- $^{13}\text{C}_3$ in Activated Sludge – TP identification

Lamotrigine – $^{13}\text{C}_3$, a labelled internal standard of lamotrigine was also degraded in activated sludge, where the concentration of $50\ \mu\text{gL}^{-1}$ was used for identification purposes. As can be seen from the **Figures SI-4. and SI-5**, both $^{13}\text{C}_3$ -LMG-N2-oxide- (SI-4) and N2-Me- $^{13}\text{C}_3$ -LMG (SI-5) were formed. The chromatographic peaks of isotopically labeled compounds had a negative RT defect of 0.02 min while the $[\text{M}+\text{H}]^+$ of the parent ions deferred by 3.0101 of the exact mass. The same mass defect is seen in the high mass fragments before the phenyltriazine ring breaks (Figures SI-4 and 5; A' and B').

Figure SI-4. Detection of LMG-N2-oxide and $^{13}\text{C}_3$ -LMG-N2-oxide in activated sludge degradation of LMG and $^{13}\text{C}_3$ -LMG, respectively. A shows the chromatogram and A' MS² spectrum at NCE 35 of LMG-N2-oxide. B and B' show the same for $^{13}\text{C}_3$ -LMG-N2-oxide.

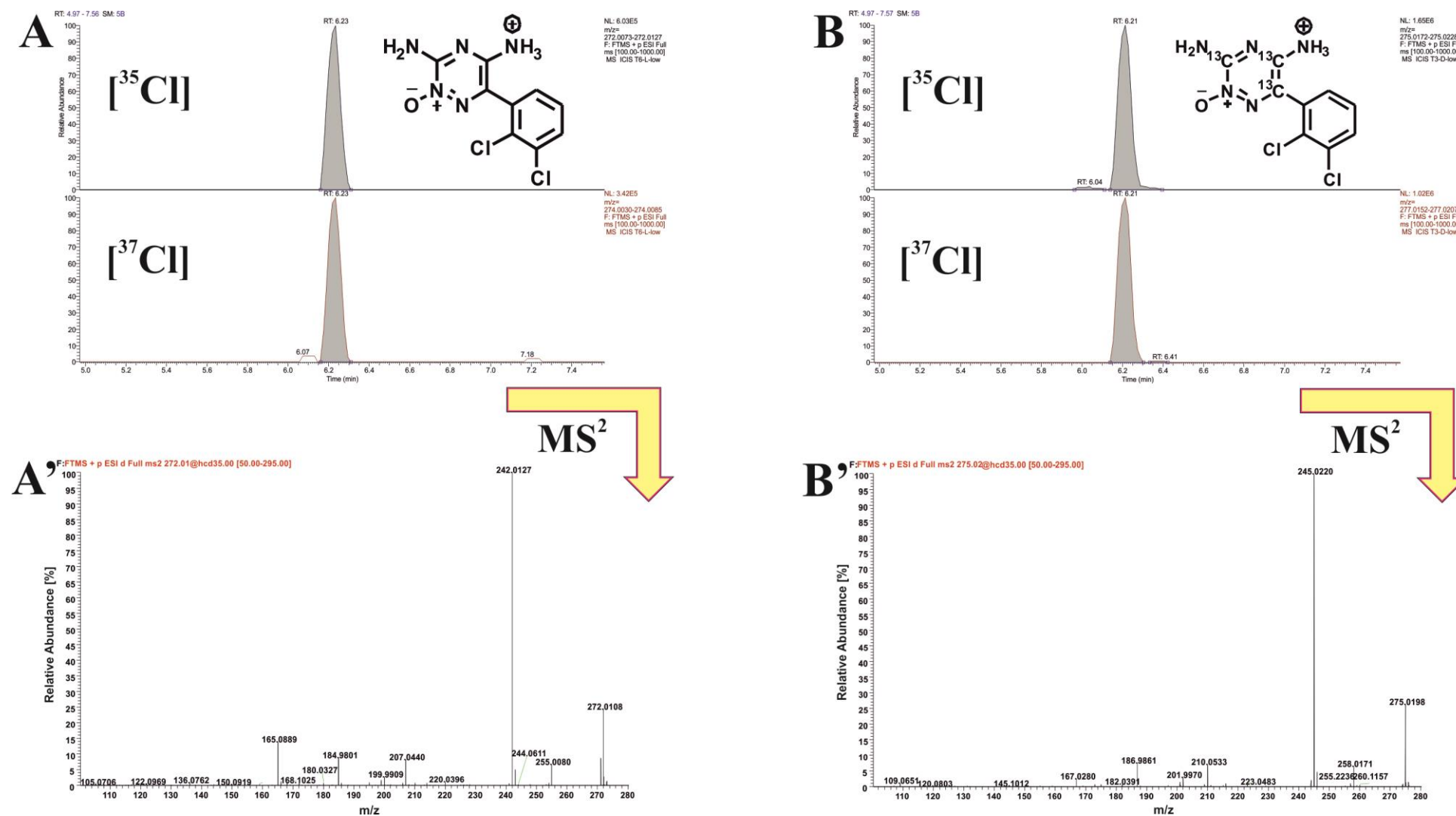
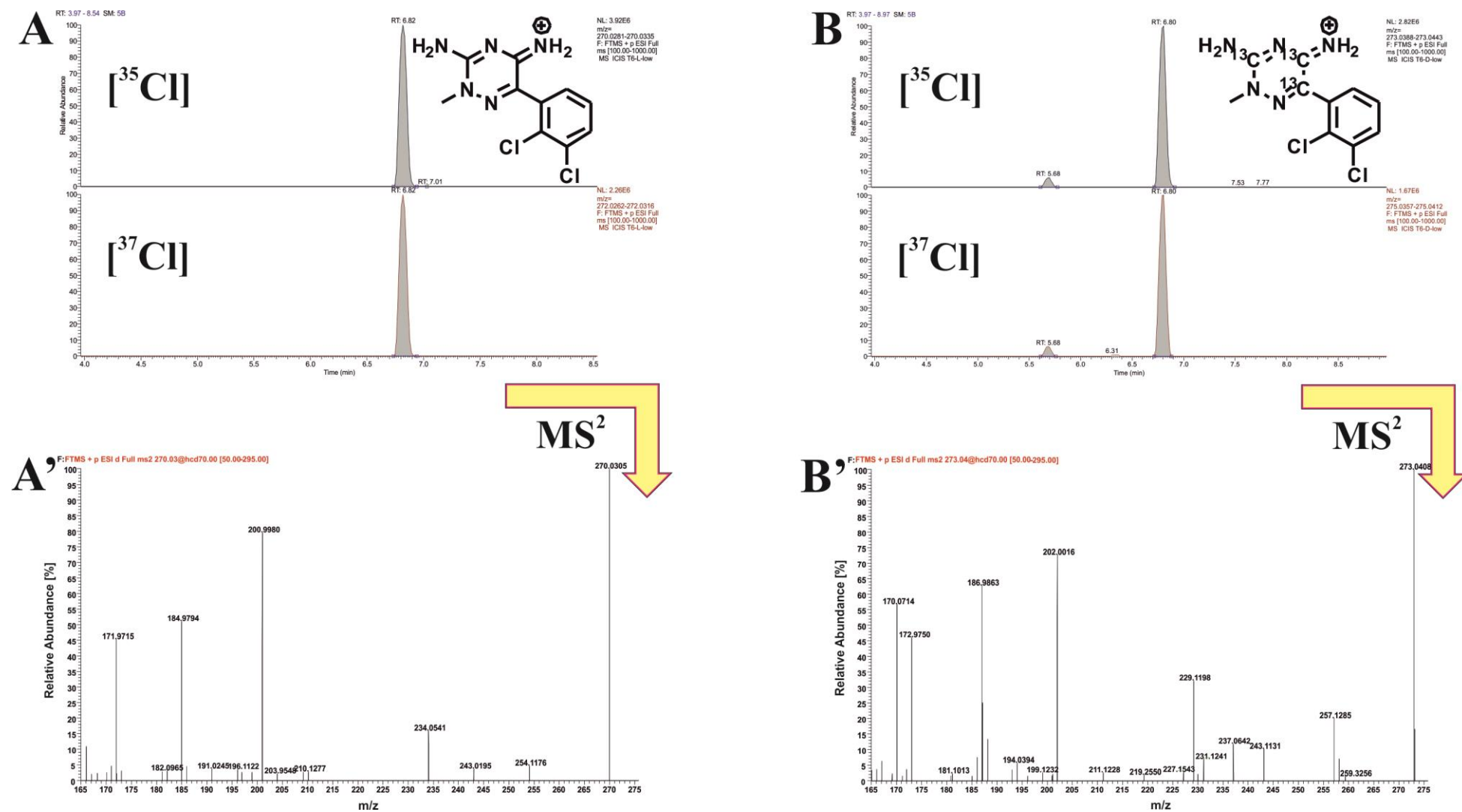
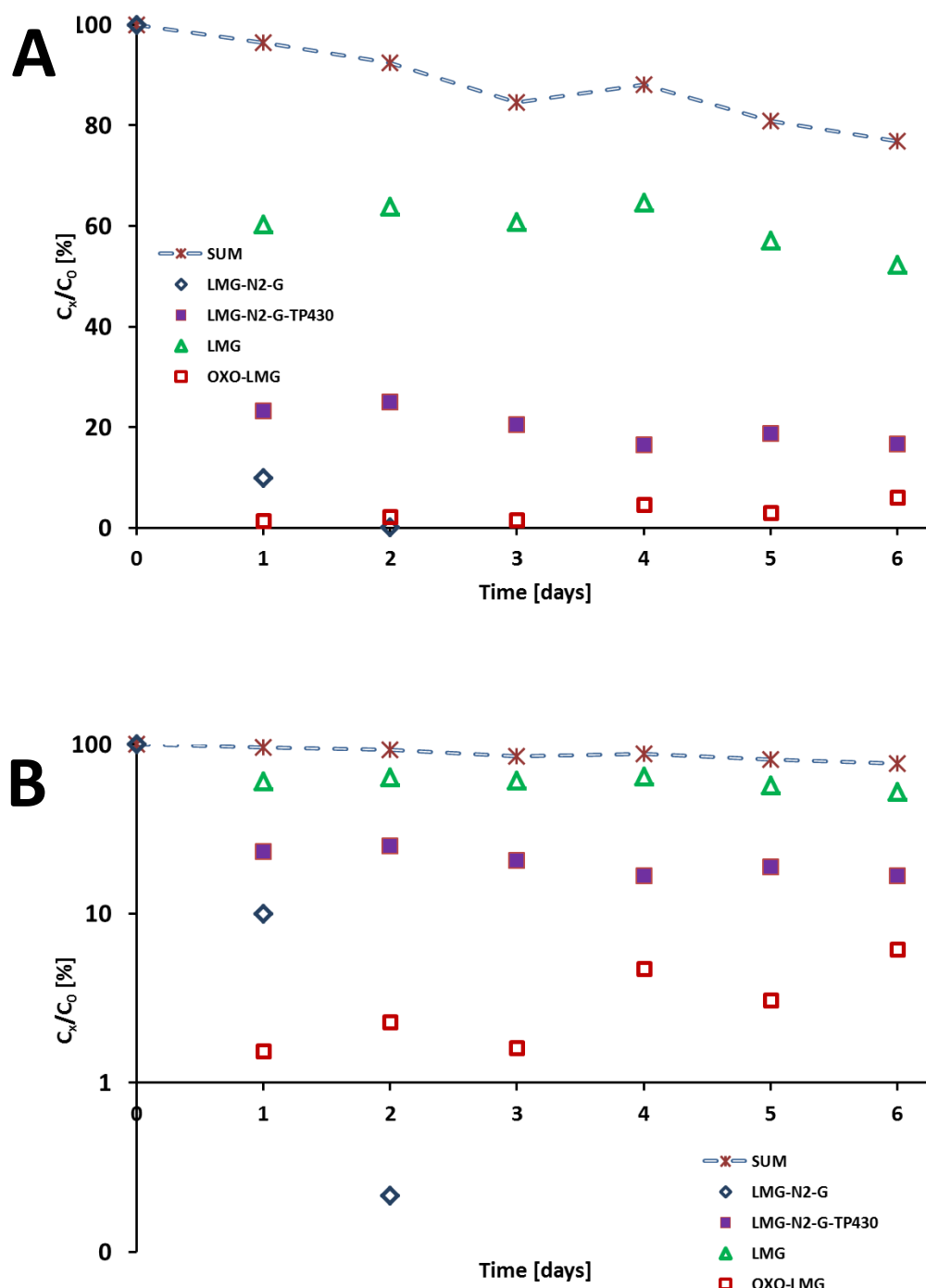


Figure SI-5. Detection of N2-Me-LMG and N2-Me-¹³C₃-LMG in activated sludge degradation of LMG and ¹³C₃-LMG, respectively. A shows the chromatogram and A' MS² spectrum at NCE 35 of N2-Me-LMG. B and B' show the same for N2-Me-¹³C₃-LMG.



Lamotrigine N2-glucuronide in Activated Sludge

Figure SI-6. Mass balance for LMG-N2-G over the course of 6 days in activated sludge bioreactor at concentration of $5 \mu\text{gL}^{-1}$ showed as (A) linear scale and (B) logarithmic scale. Calculations were performed based on molar concentrations.



Biodegradation of lamotrigine N2-glucuronide - 8 hours

Background concentration in activated sludge for biodegradation experiments

Table SI-5. Background concentration of activated sludge.

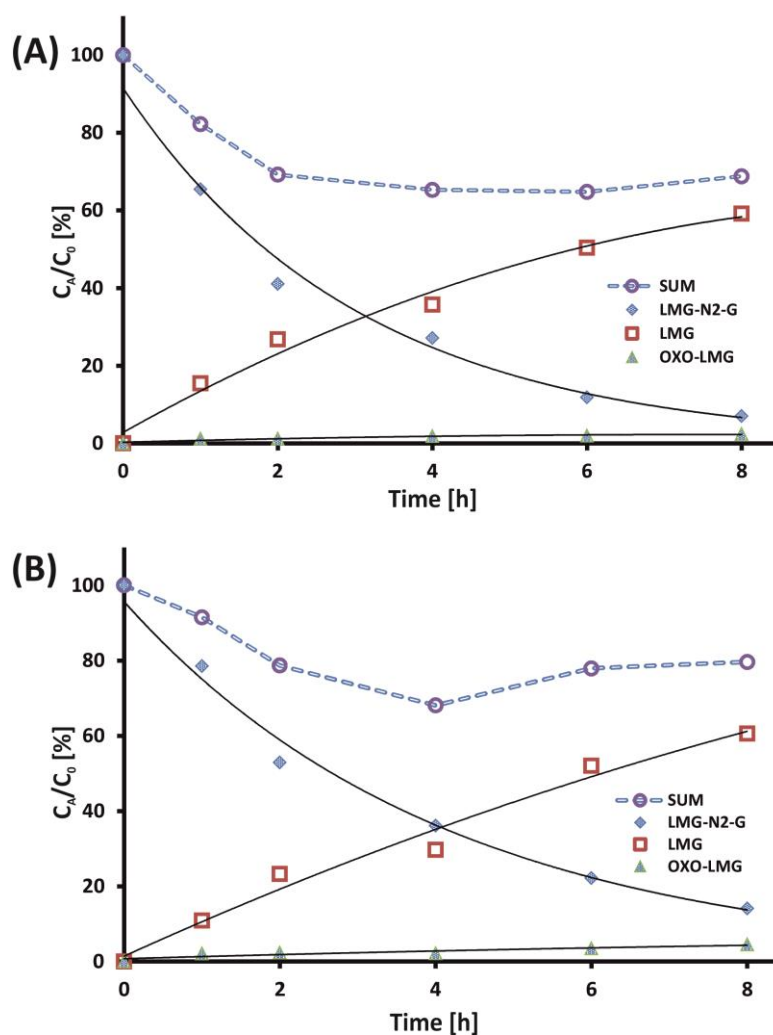
Concentration ngL ⁻¹	LMG	LMG-N2-G	OXO-LMG	N2-Me-LMG	LMG-N2- oxide	LMG-N2-G- TP430
May 2014	402	n.d.	33	15	7.3	7.0
August 2014 (I)	284	n.d.	100	9.6	n.d.	47
November 2014	120	n.d.	21	n.d.	n.d.	n.d.

n.d.- not detected

Mass balance of LMG-N2-G with its TPs LMG and OXO-LMG

Figure SI-7. Degradation of (A) 0.5 µgL⁻¹ and (B) 5 µgL⁻¹ of lamotrigine N2-glucuronide in activated sludge reactor over the course of 8 hours.

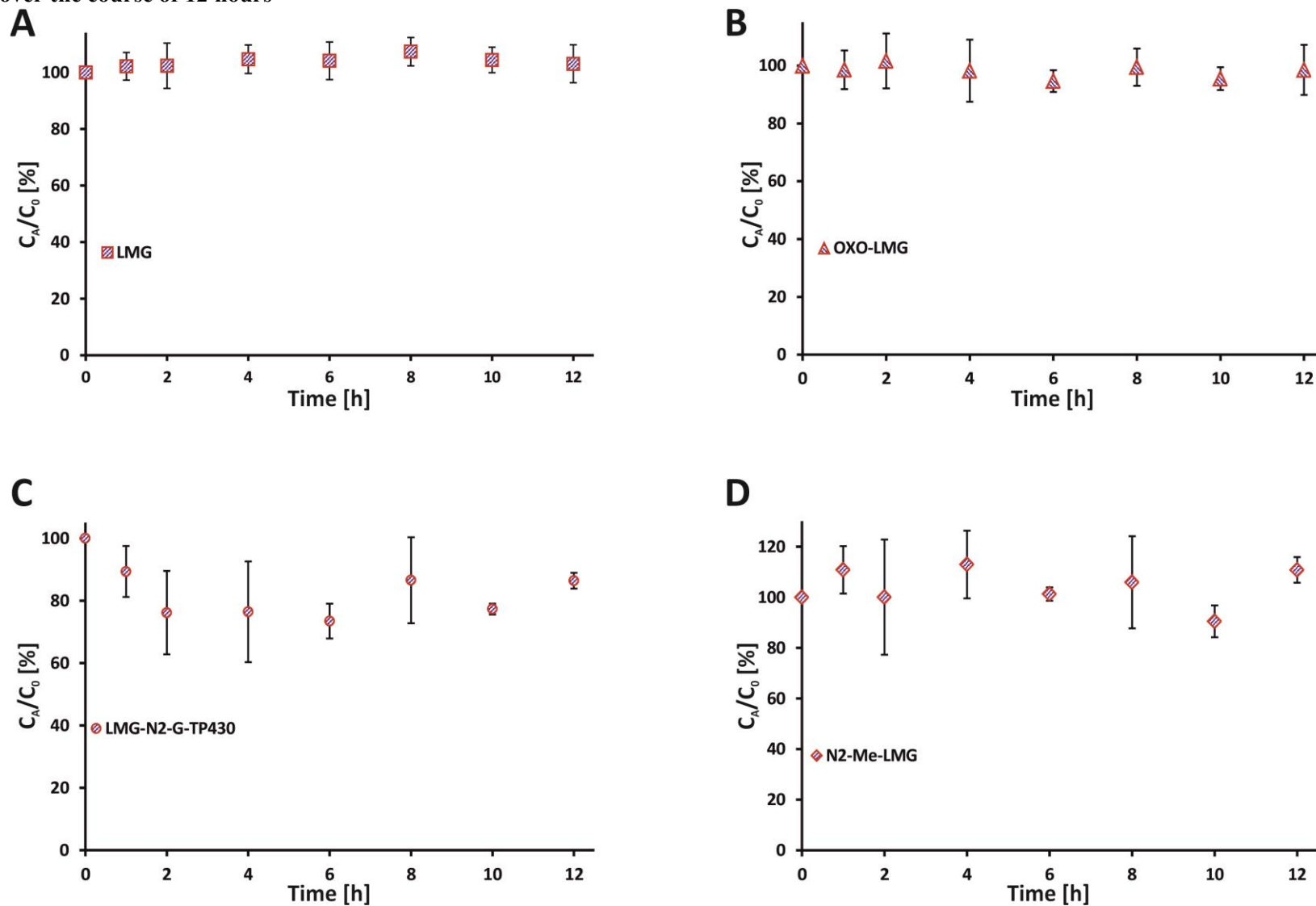
The graph shows the two LMG-N2-G TPs; LMG and OXO-LMG. Dashed line shows the mass balance as sum of the two TPs and LMG-N2-G. Points are given as average of 4 reactors (2 in Aug.(I) and 2 in Nov. 2014.) for the biotic degradation.



Stability of LMG-N2-G transformation products

For stability of transformation products of LMG-N2-G, activated sludge was taken in August 2014. (Aug.2014 (II)) from the same WWTP-5 plant and diluted to 0.9 g_{ss} with WW effluent. The reactors were not spiked and pH was adjusted to 7.0 ± 0.4 . The degradation of diluted activated sludge was performed in four batch reactors (**Table 1**) and background concentration levels measured at T0 were considered as starting concentration. Four compounds were found to be present with the following concentrations: LMG ($194 \pm 13 \text{ ngL}^{-1}$), OXO – LMG ($55 \pm 4 \text{ ngL}^{-1}$), LMG-N2-G-TP430 ($17 \pm 1 \text{ ngL}^{-1}$) and N2-Me-LMG ($4.1 \pm 0.3 \text{ ngL}^{-1}$). LMG-N2-G and LMG-N2-oxide were not present in this activated sludge samples. Results of the TP stability are shown in **Figure SI-8**.

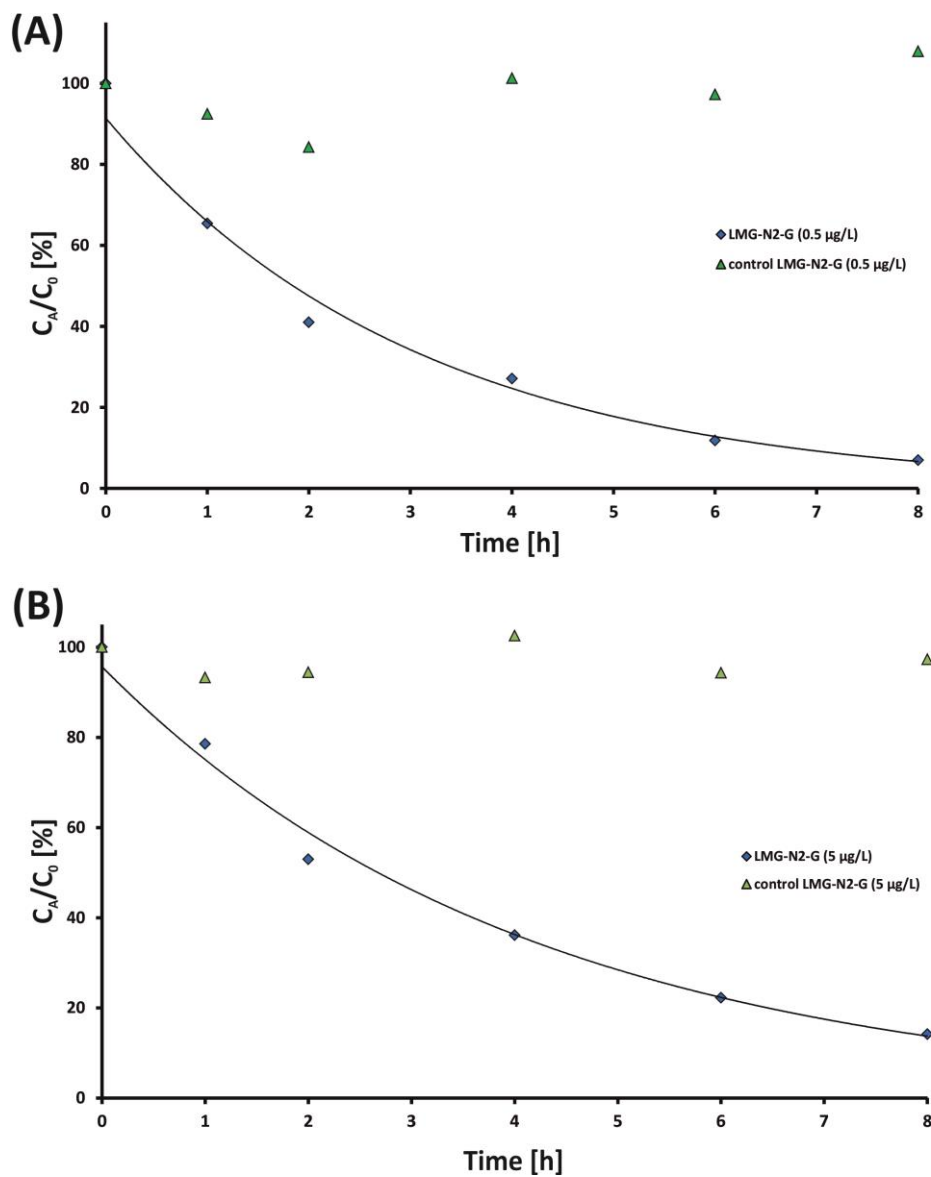
Figure SI-8. Stability of LMG-N2-G TPs: (A) 194 ngL⁻¹ LMG; (B) 55 ngL⁻¹ OXO-LMG; (C) 17 ngL⁻¹ LMG-N2-G-TP430 and LMG TP (D) 4.1 ngL⁻¹ N2-Me-LMG over the course of 12 hours



Abiotic experiments

Figure SI-9. Degradation of (A) $0.5 \mu\text{gL}^{-1}$ and (B) $5 \mu\text{gL}^{-1}$ lamotrigine N2-glucuronide in comparison with control reactors (activated sludge control).

Points are given as average of 4 reactors (Aug.(I) and Nov. 2014.) for the biotic degradation and as average of 3 reactors for the control reactors (Nov. 2014.).



Identification of TPs using HRMS

The LC method used for identification was shortened to 10 minutes. The mobile phases employed were the same as before: (A) acetonitrile with 0.1 % formic acid and (B) water (0.1 % formic acid). Elution now had the following solvent gradient: 0 min (10 % A) - 1 min (10 % A) – 6 min (95 % A) - 7 min (95 % A) – 8 min (10 % A) and stabilized until 10 min. The flow rate was $300 \mu\text{L min}^{-1}$, the column temperature was held at 40 °C and auto sampler temperature was set to 8 °C.

Electrospray ionization interface (ESI), as before, operated in the positive ion mode with the following settings: spray voltage +3.0 kV, heater temperature 250 °C, and capillary temperature 350 °C.

The Q-Exactive-MS method was set at a resolution of 140,000 for both scan mode and MS/MS experiments. The full scan was set to range of m/z 100 – 500. Isolation window was m/z 2 and inclusion list turned on and filled with the list of investigated masses. For pseudo- MS^3 (pMS^3) experiments *In-source ionization* was applied in order to produce the given m/z before breaking the ion in the HCD.

Figures of fragmentation pattern and MS spectra have been arranged in order that the fragmentation pattern and spectra have the same number i.e. **Figure SI-10** shows the fragmentation pattern (named: Figure SI-10.X-A., X = 1 - 4), MS^2 and pMS^3 spectra (named: Figure SI-10.X-B., X = 1 - 4) of a compound described.

Fragmentation patterns in **Figures SI-10.X-A, X= 1-4** are drawn with **black** and **red** arrows. **Black arrows** were drawn in cases where a given fragment has been seen in a pMS^3 spectrum of its direct predecessor. **Red arrows** were drawn all other cases and they represent the fragments which have been seen in the spectrum of its indirect predecessor.

Red arrows were used only in cases when it was not possible to do a pMS³ experiment on a given mass and thus establish its direct predecessor.

Details on a specific MS² and pMS³ performed can be found in the header of each spectrum.

Example below is given for LMG for MS² and LMG-229 for pMS³.

	Analyzer	Ionization	In-source	Experiment type	Selected mass	NCE	Mass range
LMG-MS ²	FTMS	p ESI	-	Full ms2	256.01	@hcd 60	50-259
LMG-229 pMS ³	FTMS	p ESI	sid=35	Full ms2	229.01	@hcd 15	50-231

NCEs (normalized collision energy) used throughout the experiments were 10, 15, 20, 35 and 60. Unless otherwise stated, the NCEs 15 and 20 were used with the ramp of 30%.

Accurate mass measurements of protonated LMG, LMG-N2-G and LMG-N2-G-TP430 as determined by LC-QExactive-MS in MS/MS or pseudo MS³ modes can be found in **Table SI-6**.

LAMOTRIGINE (LMG)

Figure SI-10.1-A. Fragmentation pattern of Lamotrigine (LMG)

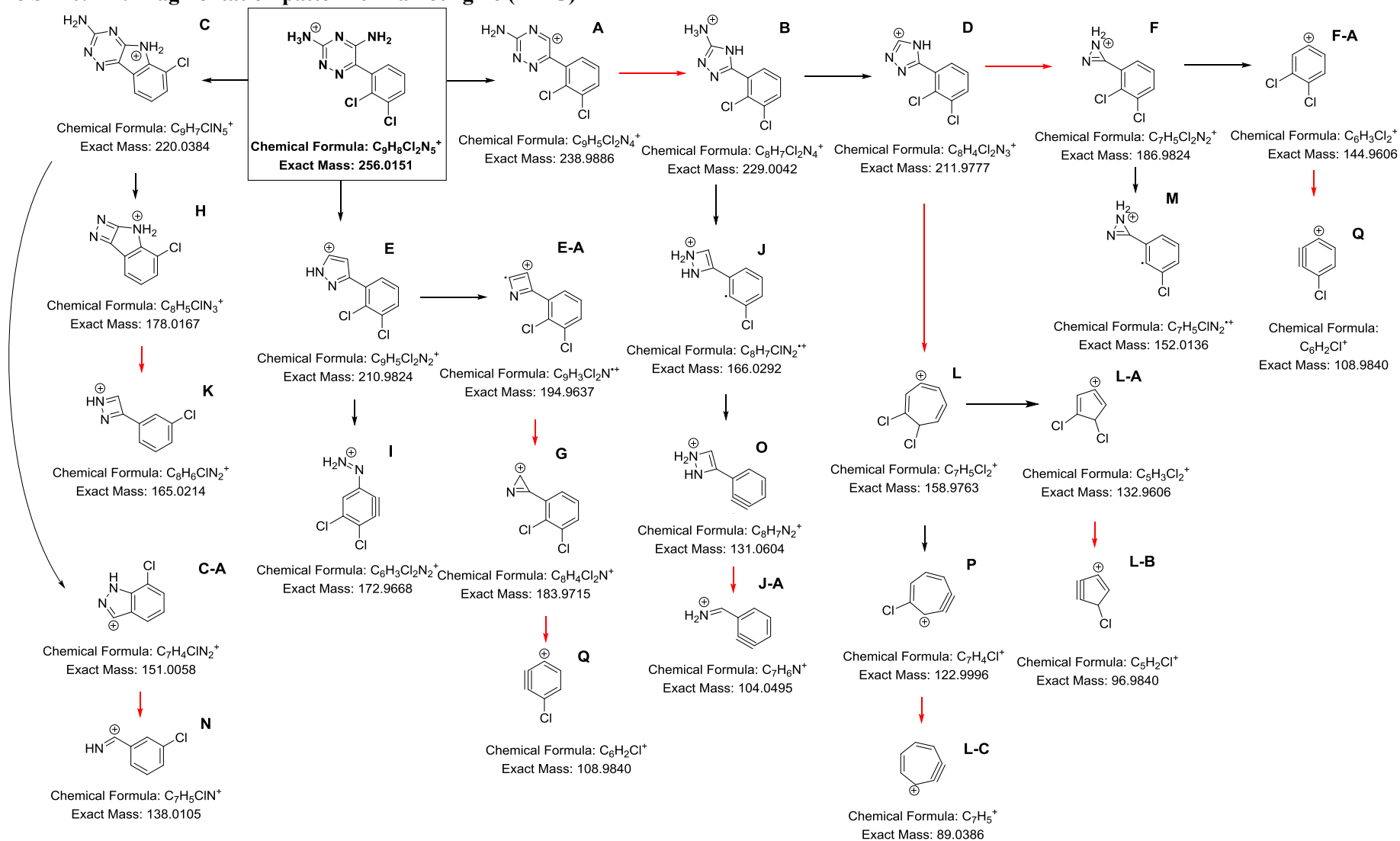


Figure SI-10.1-B. MS² and pMS³ spectra of Lamotrigine (LMG)

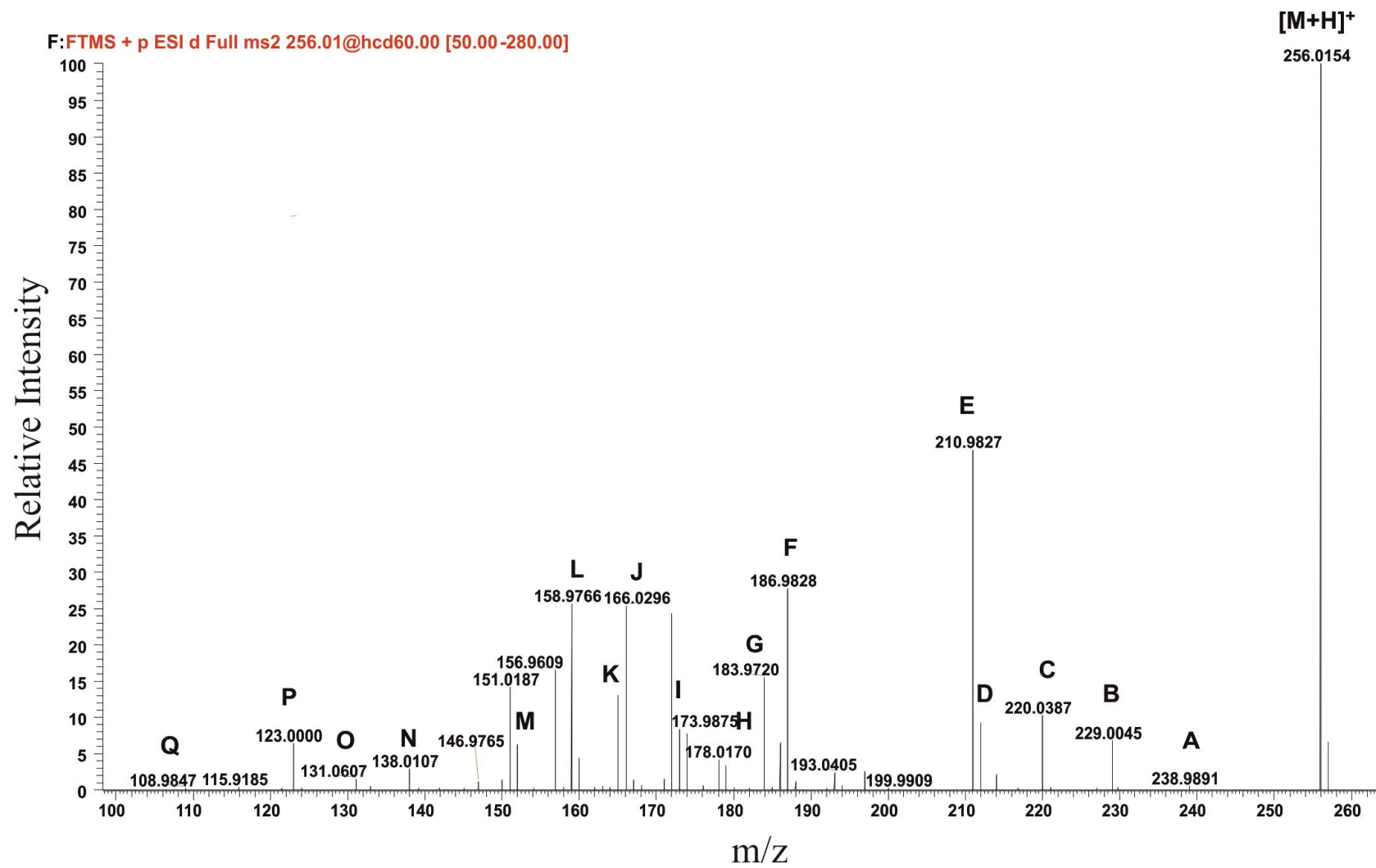
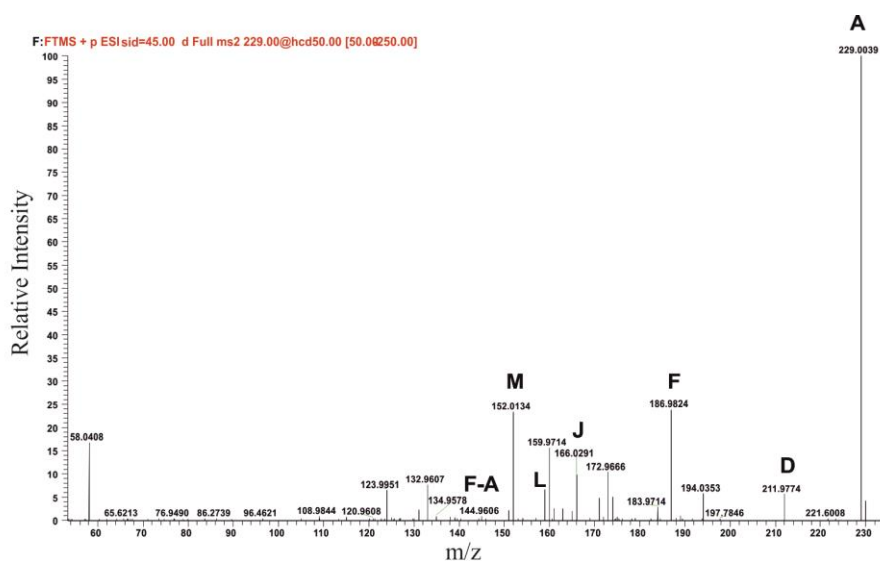
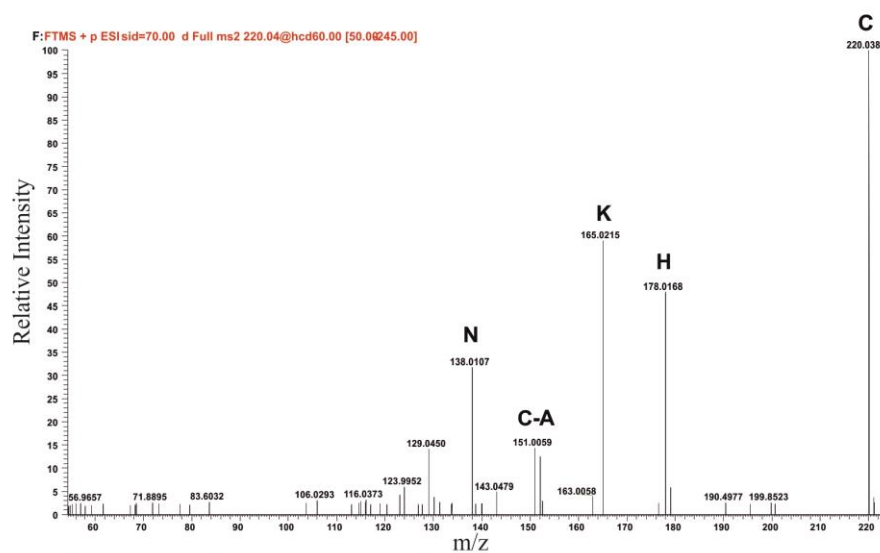


Figure SI-10.1-B. MS² and pMS³ spectra of Lamotrigine (LMG) – *continued*

LMG – 229 pMS³



LMG – 220 pMS³



LMG – 210 pMS³

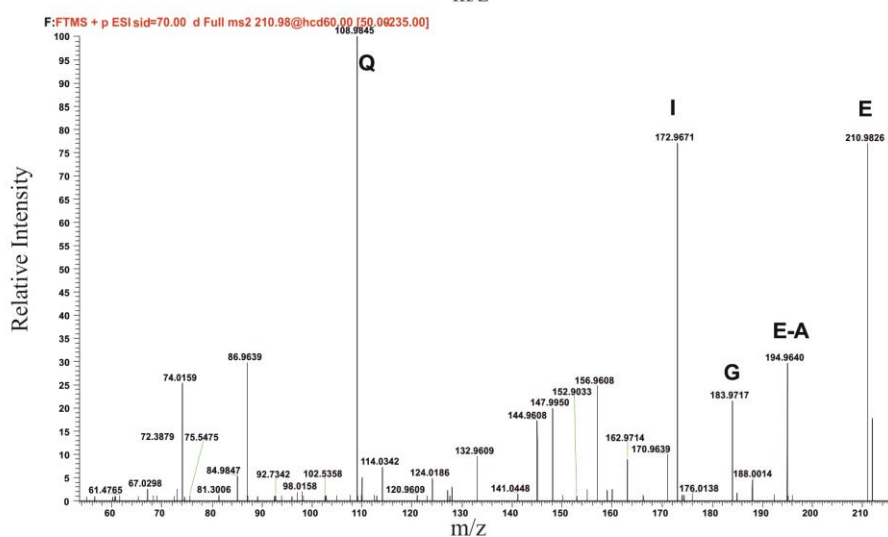
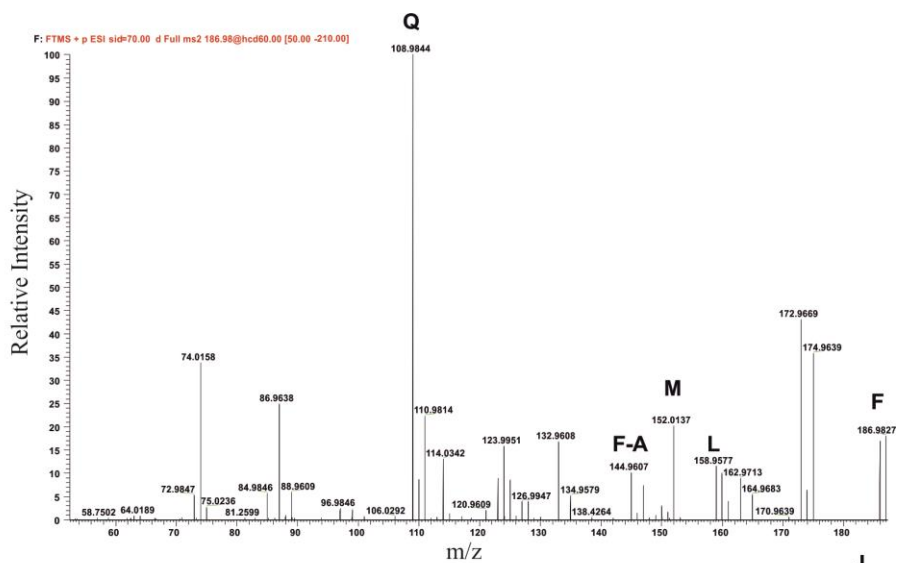
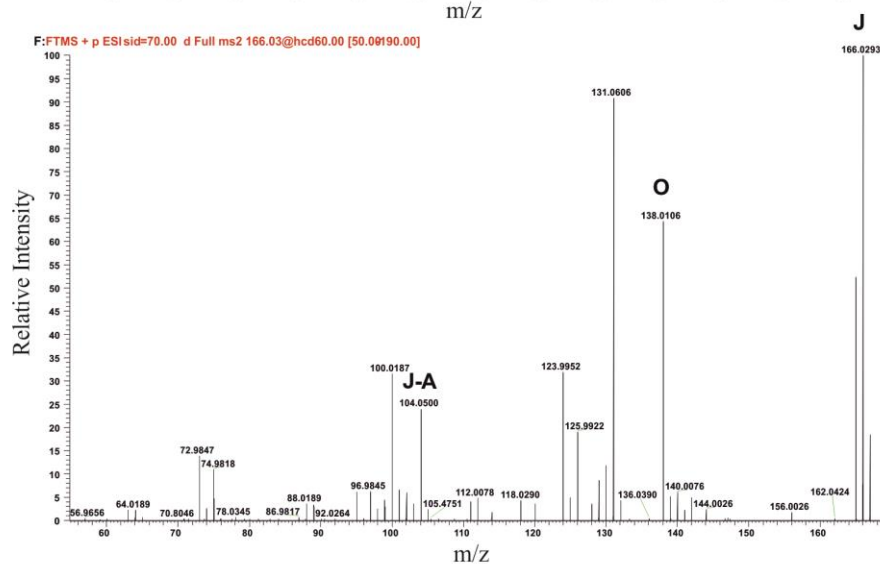


Figure SI-10.1-B. MS² and pMS³ spectra of Lamotrigine (LMG) - *continued*

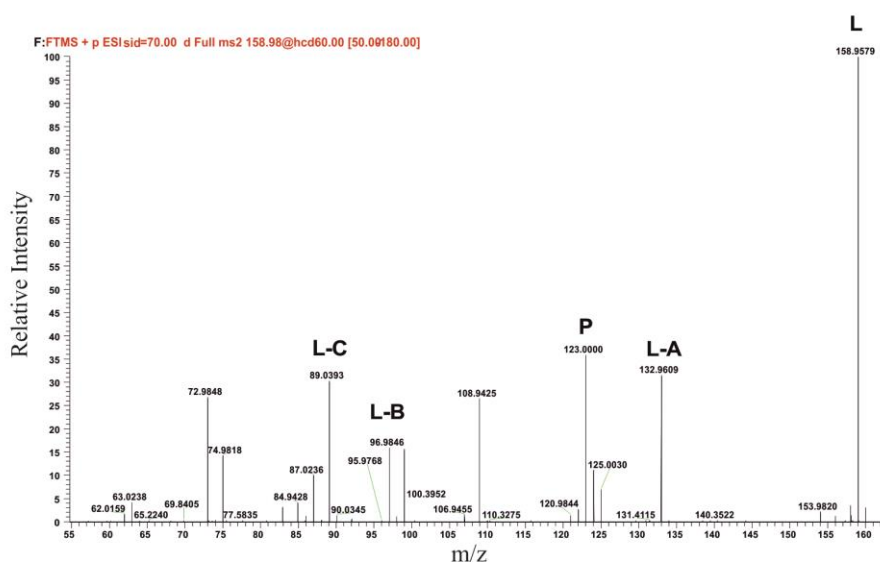
LMG – 186 pMS³



LMG – 166 pMS³



LMG – 158 pMS³



LAMOTRIGINE-N2-GLUCURONIDE (LMG-N2-G)

Figure SI-10.2-A. Fragmentation pattern of lamotrigine-N2-glucuronide (LMG-N2-G)

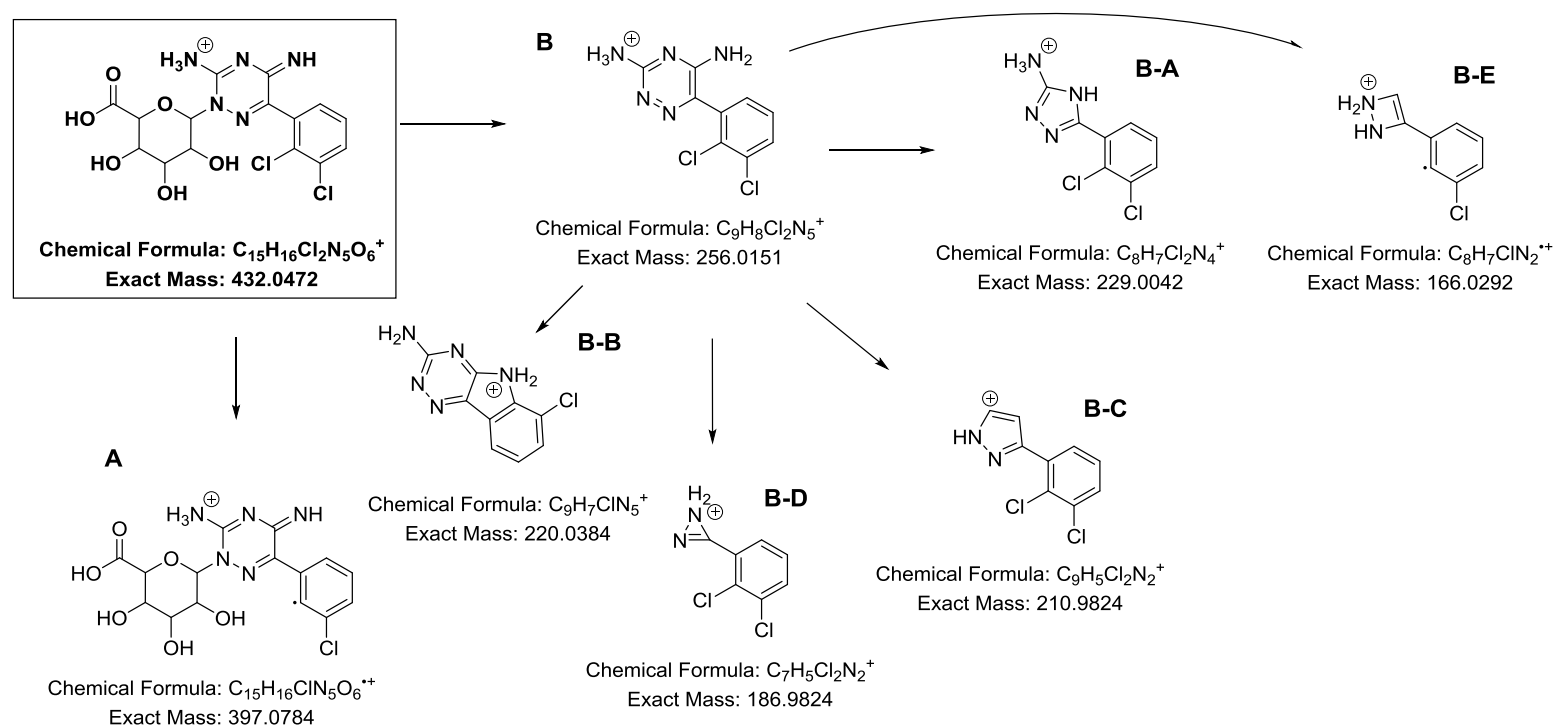
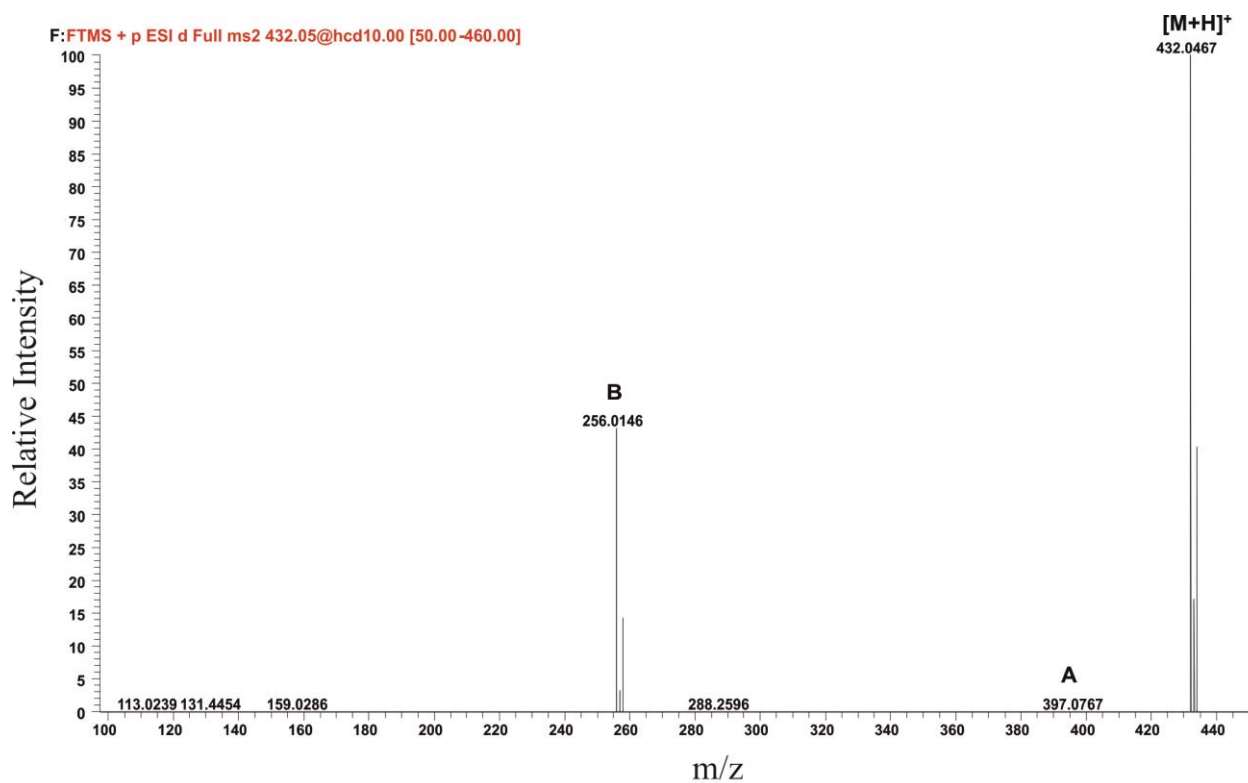
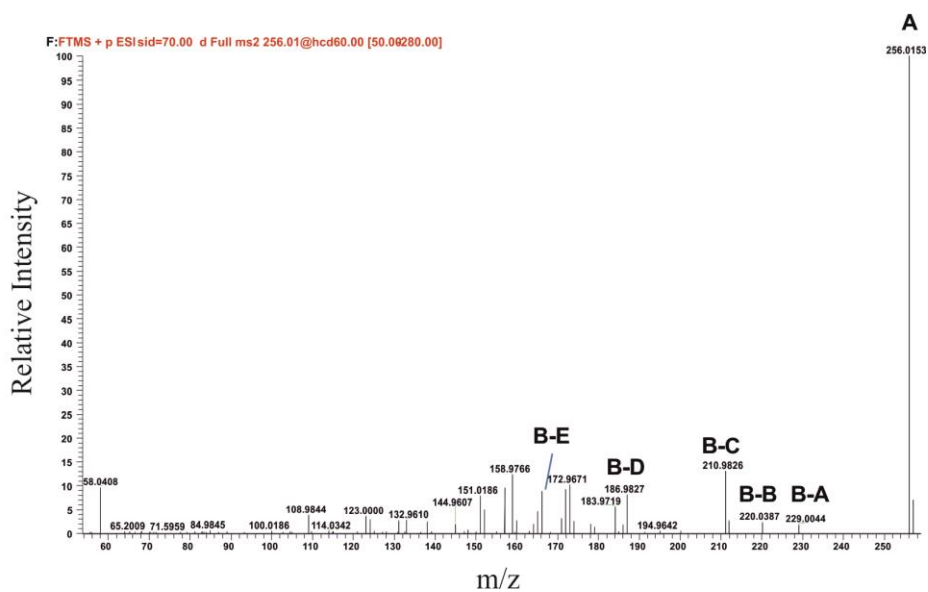


Figure SI-10.2-B. MS² and pMS³ spectra of lamotrigine-N2-glucuronide (LMG-N2-G)



LMG-N2-G – LMG pMS³



LAMOTRIGINE-N2-GLUCURONIDE TP430 (LMG-N2-G TP430)

Figure SI-10.3-A. Fragmentation pattern of lamotrigine-N2-glucuronide TP430 (LMG-N2-G-TP430)

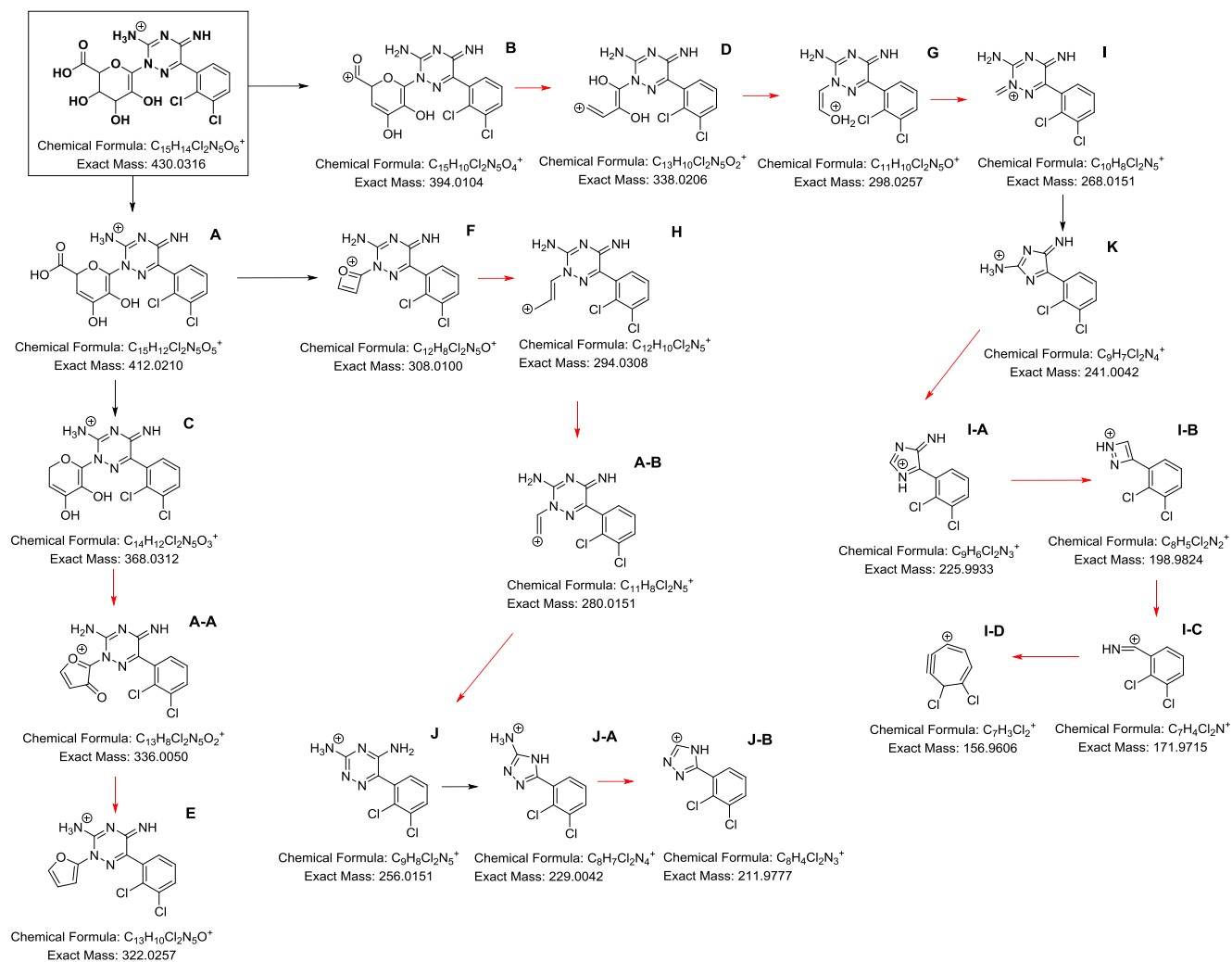


Figure SI-10.3-B. MS² and pMS³ spectra of lamotrigine-N2-glucuronide TP430 (LMG-N2-G-TP430)

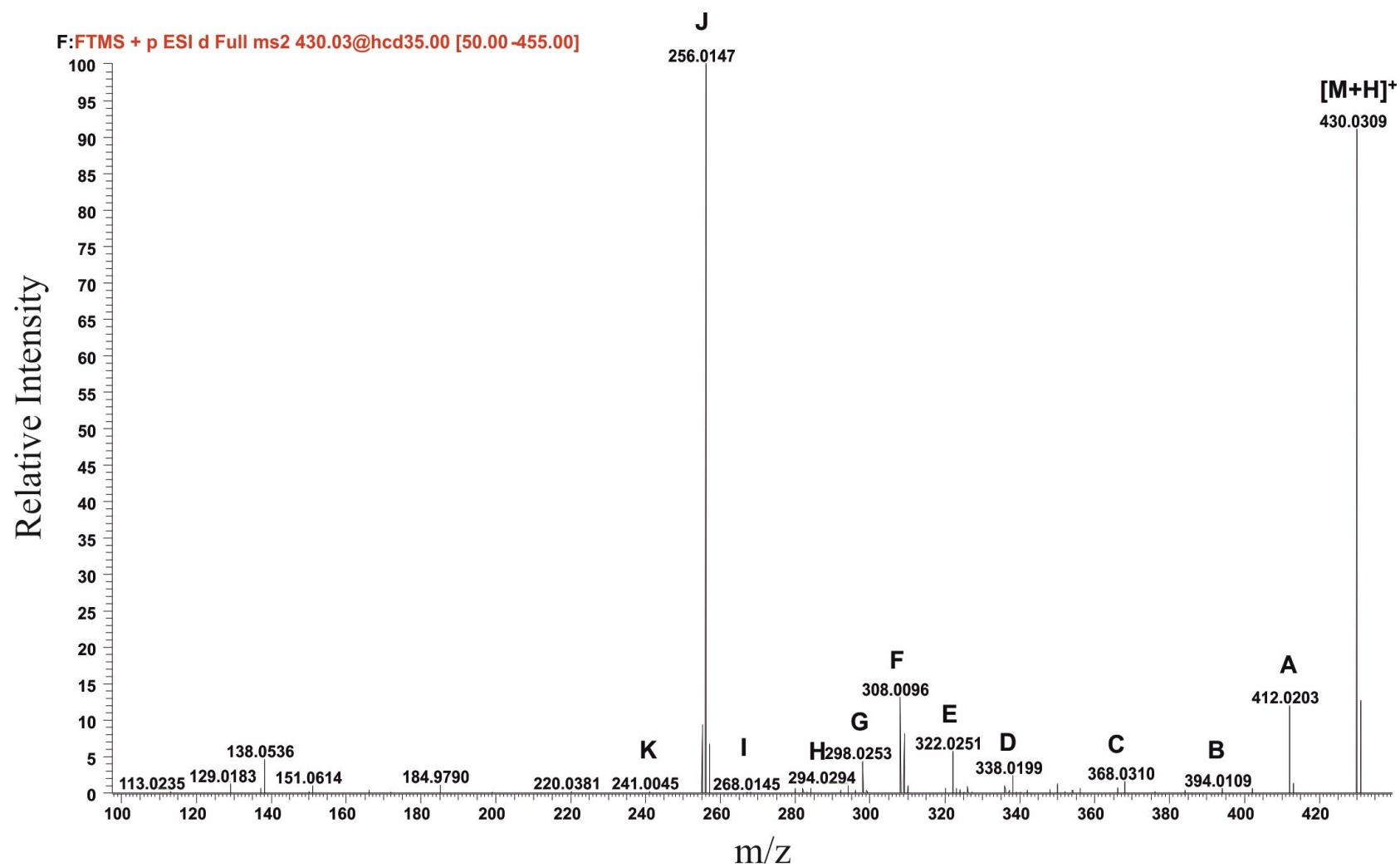
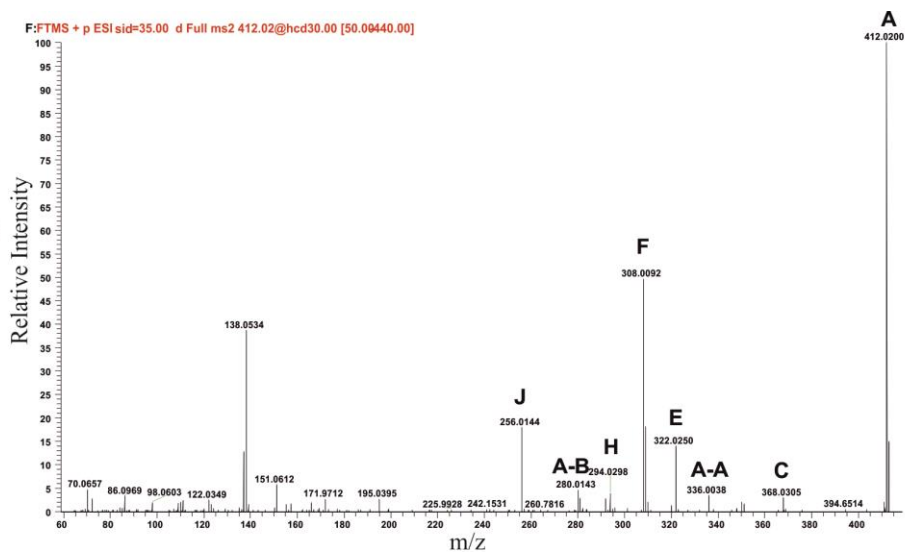
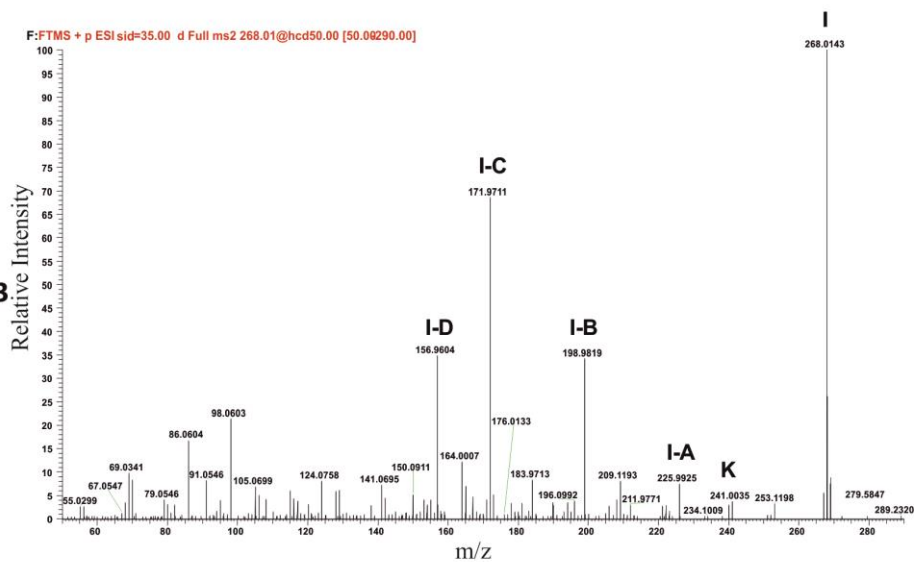


Figure SI-10.3-B. MS² and pMS³ spectra of lamotrigine-N2-glucuronide TP430 (LMG-N2-G-TP430) – *continued*

**LMG-N2-G
TP430 – 412 pMS³**



**LMG-N2-G
TP430 – 268 pMS³**



**LMG-N2-G
TP430 – 256 pMS³**

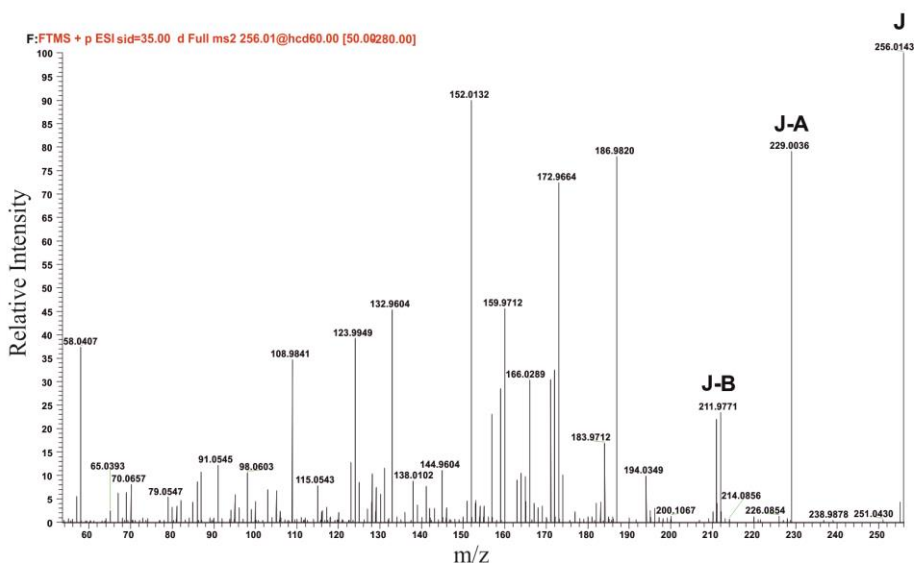


Table SI-6. Accurate mass measurements of protonated LMG, LMG-N2-G and LMG-N2-G-TP430 as determined by LC-QExactive-MS in MS/MS or pseudo MS³ modes

Nominal ion mass	Measured mass [m/z]	Mass error [ppm]	Elemental composition	Calculated mass [m/z]	DBE ^a
Lamotrigine (LMG)					
[M+H] ⁺	256.0154	1.2	C ₉ H ₈ Cl ₂ N ₅	256.0151	7.5
A 238	238.9891	2.1	C ₉ H ₅ Cl ₂ N ₄	238.9886	8.5
B 229	229.0045	1.3	C ₈ H ₇ Cl ₂ N ₄	229.0042	6.5
D 211	211.9774	-1.4	C ₈ H ₄ Cl ₂ N ₃	211.9777	7.5
F 186	186.9824	0.0	C ₇ H ₅ Cl ₂ N ₂	186.9824	5.5
J 166	166.0291	-0.6	C ₈ H ₇ ClN ₂	166.0292	6.0
L 158	158.9760	-1.9	C ₇ H ₅ Cl ₂	158.9763	4.5
M 152	152.0134	-1.3	C ₇ H ₅ ClN ₂	152.0136	6.0
F-A 144	144.9606	0.0	C ₆ H ₃ Cl ₂	144.9606	4.5
C 220	220.0387	1.4	C ₉ H ₇ ClN ₅	220.0384	8.5
H 178	178.0168	0.6	C ₈ H ₅ ClN ₃	178.0167	7.5
K 165	165.0215	0.6	C ₈ H ₆ ClN ₂	165.0214	6.5
C-A 151	151.0059	1.0	C ₇ H ₄ ClN ₂	150.0058	6.5
N 138	138.0107	1.4	C ₇ H ₅ ClN	138.0105	5.5
D 211	211.9775	-0.9	C ₈ H ₄ Cl ₂ N ₃	211.9777	7.5
E 210	210.9827	1.4	C ₉ H ₅ Cl ₂ N ₂	210.9824	7.5
E-A 194	194.9640	1.5	C ₉ H ₃ Cl ₂ N	194.9637	8.0
G 183	183.9717	1.1	C ₈ H ₄ Cl ₂ N	183.9715	6.5
I 172	172.9671	1.7	C ₆ H ₃ Cl ₂ N ₂	172.9668	5.5
Q 108	108.9845	4.6	C ₆ H ₂ Cl	108.9840	5.5
F 186	186.9828	2.1	C ₇ H ₅ Cl ₂ N ₂	186.9824	5.5
M 152	152.0137	0.7	C ₇ H ₅ ClN ₂	152.0136	6.0
F-A 144	144.9607	0.7	C ₆ H ₃ Cl ₂	144.9606	4.5
L-A 132	132.9608	1.5	C ₅ H ₃ Cl ₂	132.9606	3.5
Q 108	108.9844	3.7	C ₆ H ₂ Cl	108.9840	5.5
G 183	183.9720	2.7	C ₈ H ₄ Cl ₂ N	183.9715	6.5
H 178	178.0170	1.7	C ₈ H ₅ ClN ₃	178.0167	7.5

I 172	172.9665	-1.7	C ₆ H ₃ Cl ₂ N ₂	172.9668	5.5
J 166	166.0296	2.4	C ₈ H ₇ ClN ₂	166.0292	6.0
O 131	131.0606	1.5	C ₈ H ₇ N ₂	131.0604	6.5
J-A 104	104.0500	4.8	C ₇ H ₆ N	104.0495	5.5
K 165	165.0211	-1.8	C ₈ H ₆ ClN ₂	165.0214	6.5
L 158	158.9766	1.9	C ₇ H ₅ Cl ₂	158.9763	4.5
L-A 132	132.9609	2.3	C ₅ H ₃ Cl ₂	132.9606	3.5
P 122	123.0000	3.3	C ₇ H ₄ Cl	122.9996	5.5
L-B 96	96.9846	6.2	C ₅ H ₂ Cl	96.9840	4.5
L-C 89	89.0393	7.9	C ₇ H ₅	89.0386	5.5
M 152	152.0132	-2.6	C ₇ H ₅ ClN ₂	152.0136	6.0
N 138	138.0107	1.4	C ₇ H ₅ ClN	138.0105	5.5
O 131	131.0607	2.3	C ₈ H ₇ N ₂	131.0604	6.5
P 123	123.0000	3.3	C ₇ H ₄ Cl	122.9996	5.5
Q 108	108.9847	6.4	C ₆ H ₂ Cl	108.9840	5.5
Lamotrigine-N2-glucuronide (LMG-N2-G)					
[M+H] ⁺	432.0467	-1.2	C ₁₅ H ₁₆ Cl ₂ N ₅ O ₆	432.0472	9.5
A 397	397.0767	-4.3	C ₁₅ H ₁₆ ClN ₅ O ₆	397.0784	10.0
B 256	256.0146	-2.0	C ₉ H ₈ Cl ₂ N ₅	256.0151	7.5
B-A 229	229.0044	0.9	C ₈ H ₇ Cl ₂ N ₄	229.0042	6.5
B-B 220	220.0387	1.4	C ₉ H ₇ ClN ₅	220.0384	8.5
B-C 210	210.9826	0.9	C ₉ H ₅ Cl ₂ N ₂	210.9824	7.5
B-D 186	186.9827	1.6	C ₇ H ₅ Cl ₂ N ₂	186.9824	5.5
B-E 166	166.0288	-2.4	C ₈ H ₇ ClN ₂	166.0292	6.0
Lamotrigine-N2-glucuronide TP430 (LMG-N2-G-TP430)					
[M+H] ⁺	430.0318	0.5	C ₁₅ H ₁₄ Cl ₂ N ₅ O ₆	430.0316	10.5
A 412	412.0203	-1.7	C ₁₅ H ₁₂ Cl ₂ N ₅ O ₅	412.0210	11.5
C 368	368.0305	-1.9	C ₁₄ H ₁₂ Cl ₂ N ₅ O ₃	368.0312	10.5
A-A 336	336.0038	-3.6	C ₁₃ H ₈ Cl ₂ N ₅ O ₂	336.0050	11.5
E 322	322.0250	-2.2	C ₁₃ H ₁₀ Cl ₂ N ₅ O	322.0257	10.5

F 308	308.0092	-2.6	C ₁₂ H ₈ Cl ₂ N ₅ O	308.0100	10.5
H 294	294.0298	-3.4	C ₁₂ H ₁₀ Cl ₂ N ₅	294.0308	9.5
A-B 280	280.0143	-2.9	C ₁₁ H ₈ Cl ₂ N ₅	280.0151	9.5
J 256	256.0144	-2.7	C ₉ H ₈ Cl ₂ N ₅	256.0151	7.5
B 394	394.0109	1.3	C ₁₅ H ₁₀ Cl ₂ N ₅ O ₄	394.0104	12.5
C 368	368.0310	-0.5	C ₁₄ H ₁₂ Cl ₂ N ₅ O ₃	368.0312	10.5
D 338	338.0199	-2.1	C ₁₃ H ₁₀ Cl ₂ N ₅ O ₂	338.0206	10.5
E 322	322.0251	-1.9	C ₁₃ H ₁₀ Cl ₂ N ₅ O	322.0257	10.5
F 308	308.0096	-1.3	C ₁₂ H ₈ Cl ₂ N ₅ O	308.0100	10.5
G 298	298.0253	-1.3	C ₁₁ H ₁₀ Cl ₂ N ₅ O	298.0257	8.5
H 294	294.0294	-4.8	C ₁₂ H ₁₀ Cl ₂ N ₅	294.0308	9.5
I 268	268.0145	-2.2	C ₁₀ H ₈ Cl ₂ N ₅	268.0151	8.5
K 241	241.0035	-2.9	C ₉ H ₇ Cl ₂ N ₄	241.0042	7.5
I-A 225	225.9925	-3.5	C ₉ H ₆ Cl ₂ N ₃	225.9933	7.5
I-B 198	198.9819	-2.5	C ₈ H ₅ Cl ₂ N ₂	198.9824	6.5
I-C 171	171.9711	-2.3	C ₇ H ₄ Cl ₂ N	171.9715	5.5
I-D 156	156.9604	-1.3	C ₇ H ₃ Cl ₂	156.9606	5.5
J 256	256.0147	-1.6	C ₉ H ₈ Cl ₂ N ₅	256.0151	7.5
J-A 229	229.0036	-2.7	C ₈ H ₇ Cl ₂ N ₄	229.0042	6.5
J-B 211	211.9771	-2.7	C ₈ H ₄ Cl ₂ N ₃	211.9777	7.5
K 241	241.0045	1.2	C ₉ H ₇ Cl ₂ N ₄	241.0042	7.5

^a Double-bond equivalents;

Transformation of LMG-N2-G-TP430 to LMG-N2-G-TP431-Amd

LMG-N2-G-TP431-Amd is a transformation product of LMG-N2-G-TP430 which is formed as a result of the amidine hydrolysis (purple transformation in the manuscript) of the LMG-N2-G-TP430. This TP was found at barely detectable concentrations in the experiments over the course of 8 hours of degradation of LMG-N2-G. This was the reason why it was not included in the mass balance of the LMG-N2-G. However, in the initial experiments where LMG-N2-G was degraded over the course of 6 days at concentrations of 50 and 5 μgL^{-1} (concentrations for identifications of TPs and kinetics, respectively), this compound was detected at higher concentrations. In the degradation experiments for identification purposes (50 μgL^{-1} concentration) its abundance was sufficient enough to perform the MS² experiments and to characterize the compound (**Figure SI-12**). In the **Figure SI-12** can be seen the fragmentation spectrum of the LMG-N2-G-TP431-Amd in comparison with the fragmentation spectrum of LMG-N2-G-TP430 (**Figure SI-11**). From the blue box spectra in the **Figure SI-11** it can be seen that while the LMG-N2-G-TP430 under NCE 35 fragments to its most abundant ion with m/z 256.0156 which is LMG. On the other hand, LMG-N2-G-TP431-Amd under the same NCE fragments to m/z 256.9991 which is the exact mass of OXO-LMG (**Figure SI-12**). In the amplified spectra of both compounds it can be seen that the majority of other fragments forming (although with different intensities), are the same with the mass defect of 0.984.

As to reaction pattern, for LMG-N2-G, amidine hydrolysis TP was only detected as an abiotic transformation product which depended on the pH and was not detected in the biodegradation experiments. Furthermore, it was suggested that the reaction of amidine hydrolysis and decojugation happen simultaneously and that this could be the reason why LMG-N2-G-TP433-Amd was not detected in the batch experiments. Since the

LMG-N2-G-TP431-Amd was formed in the same batch reactors (over the course of 6 days) where the pH was carefully monitored to be around 7, the source of this TP could be enzymatic transformation. Unlike in the case of LMG-N2-G-TP433-Amd, the deconjugation of LMG-N2-G-TP431-Amd to form OXO-LMG is far more difficult because of the higher stability of the corresponding glycal (similar to its parent compound LMG-N2-G-TP430).

Figure SI-11. MS² fragmentation of LMG-N2-G-TP430.

The difference with the spectrum in the blue box and the large spectra are the m/z range. The blue box spectrum has the m/z range from 100 – 435 and the enlarged spectrum has the m/z range from 260 - 435.

Letters in the lower spectra correspond to the fragmentation pattern of LMG-N2-G-TP430 as depicted in the Figure SI-9.4.-A.

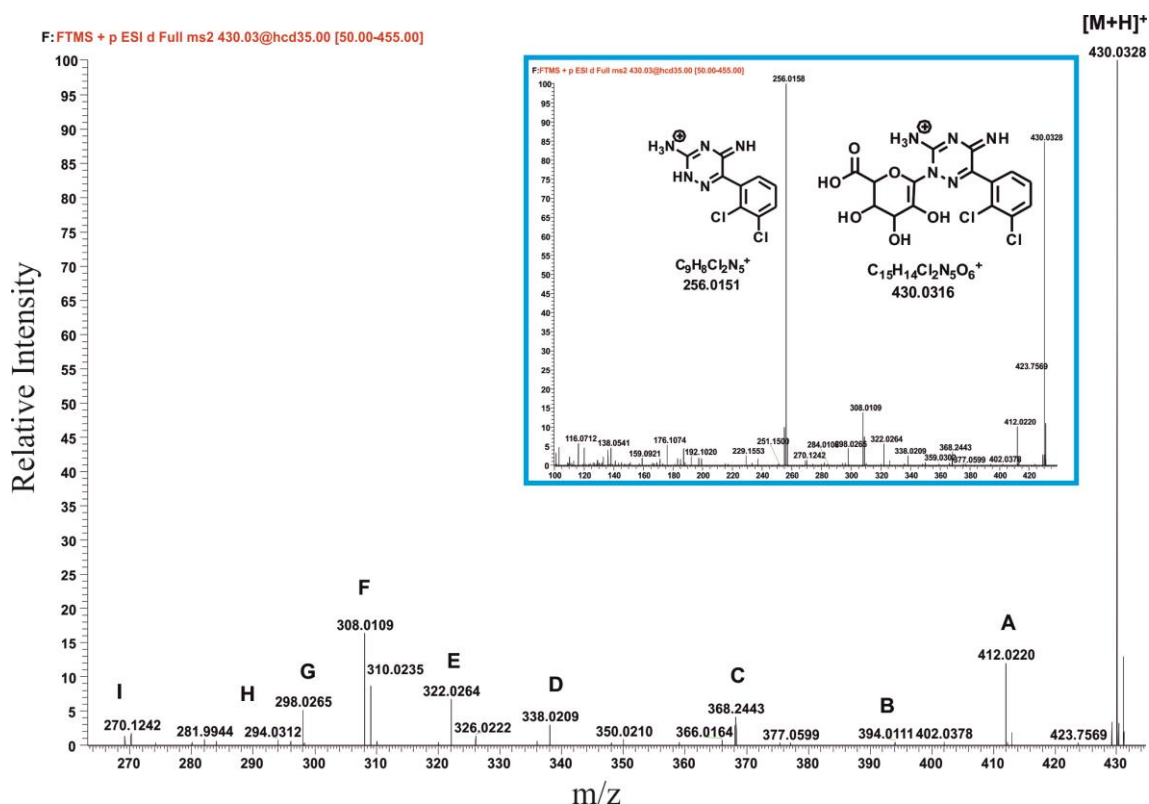
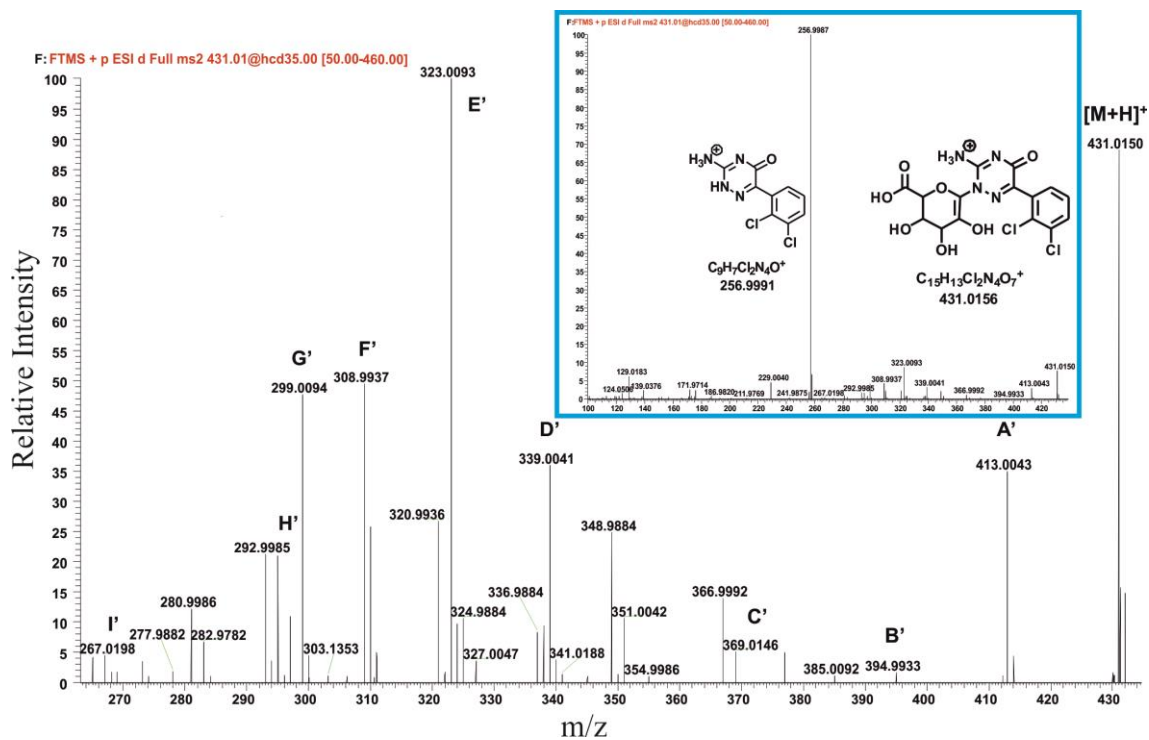


Figure SI-12. MS² fragmentation of LMG-N2-G-TP431-Amd.

The difference with the spectra in the blue box and the large spectra are the m/z range. The blue box spectrum has the m/z range from 100 – 435 and the enlarged spectrum has the m/z range from 260 - 435.

Apostrophized letters in the lower spectra are put for comparison between the LMG-N2-G-TP430 and LMG-N2-G-TP431-Amd.



Determination of lamotrigine and derived compounds in wastewater and surface water

The wastewater and surface water samples already run were reanalyzed using Xcalibur from Thermo Scientific since only scan filter was used for quantitation and determination (see LOQ section below).

As mentioned in the manuscript, for WWTP 4 (**Table SI-7**), a procedural error occurred and the internal standard was spiked approximately 1000 lower than the other wastewater samples which caused that in some cases it was below LOQ. Most likely due to this, the total LMG-N2-G mass balance was overestimated by 54 %.

Table SI-7. Concentration of LMG-N2-G and related compounds in the WWTP 4.

nmolL ⁻¹	WWTP 4	
	influent	effluent
LMG-N2-G (1) [³⁷ Cl]/[³⁵ Cl] [%]	1.159 ± 0.066 64	0.498 ± 0.002 62
LMG (2) [³⁷ Cl]/[³⁵ Cl] [%]	0.174 ± 0.006 60	1.270 ± 0.121 64
<i>SUM (1+2)</i>	<i>1.333</i>	<i>(+32 %) 1.768</i>
N2-MeLMG (3) [³⁷ Cl]/[³⁵ Cl] [%]	< LOQ -	0.024 ± 0.003 62
LMG-N2-Oxide (4) [³⁷ Cl]/[³⁵ Cl] [%]	< LOQ -	0.009 ± 0.000 61
<i>SUM (1-4)</i>	<i>1.333</i>	<i>(+34%) 1.792</i>
OXO-LMG (5) [³⁷ Cl]/[³⁵ Cl] [%]	0.048 ± 0.002 62	0.180 ± 0.010 62
LMG-N2-G-TP433-Amd (6) [³⁷ Cl]/[³⁵ Cl] [%]	0.012 ± 0.001 54	< LOQ -
<i>SUM (1-6)</i>	<i>1.393</i>	<i>(+42%) 1.972</i>
LMG-N2-G-TP430 (7) [³⁷ Cl]/[³⁵ Cl] [%]	n.d. -	0.170 ± 0.008 60
<i>SUM all (1-7)</i>	<i>1.393</i>	<i>(+54 %) 2.142</i>

Recovery measurements

For recovery calculations each matrix (WW influent, WW effluent and surface water) a mixture of samples was joined together. As in the screening method, 400 mL of WW influent, 600 mL of WW effluent and 800 mL of surface water were used for pre-concentration. The pH of the mixture was adjusted to 6.5 and then spiked with 200 ng of standards available (including $^{13}\text{C}_3$ -LMG). All experiments were performed in triplicate. The extracts of different cartridges were eluted separately and analyzed separately.

Recovery was calculated as the difference between the sum of concentrations from the four cartridges after the SPE and the blank sample divided with spiked concentration.

The results of the recovery experiments are shown in **Table SI-8**.

Since the four cartridges were analyzed separately, the relative distribution of analytes between them is also reported. The arrows in the table report the order how the cartridges were connected.

As can be seen from the **Table SI-8**, recoveries in all matrices ranged from 68 % (for OXO-LMG in WW influent) to 134 % (for LMG-N2-oxide in WW effluent). What can also be noted is that almost all of the analytes were retained in the first cartridge to be connected in the series – HLB. The only exception to this was the extraction efficiency for LMG-N2-G in surface water samples where LMG-N2-G was distributed between the HLB and BondElute PPL with 72 – 28 % ratio. Since LMG-N2-G was completely extracted when less volume was preconcentrated (400 mL for WW influent and 600 mL for WW effluent), it is likely that the 800 mL of surface water caused saturation of the HLB cartridge. LMG-N2-G-TP433-Amd recovery was determined only in WW influent since this compound was not detected in any of the effluent or surface water samples.

LOQ measurements

Limit of quantification (LOQ) is also shown in the **Table SI-8**. For a compound to be identified a set of parameters must have been fulfilled and match the parent compound. The compound was determined if it matched retention time (± 0.2 min), exact mass accuracy (<5 ppm) and relative chlorine isotope pattern which was calculated for every compound and sample. This value is given below each reported concentration. LOQ values were calculated as signal-to-noise ratio (S/N) higher than 10. $<LOQ$ was reported for compounds where the identification rules applied but the S/N was lower than 10. LOQs determined for all compounds were in the low ngL^{-1} ranges (**Table SI-8**) which afforded the quantification of all of the compounds in the majority of the samples investigated. The highest LOQ determined in all matrices was that of LMG. This was due to a high background noise detected of the m/z 256.0151. Similar to the recovery measurements, LOQ of LMG-N2-G-TP-433-Amd was only determined in the WW influent since this was the only matrix where this compound was detected. As for LMG-N2-G-TP430, LOQ used was the one of LMG-N2-G since it was not possible to obtain this compound as an authentic standard.

Table SI-8. Recoveries and LOQ of LMG and derived compounds in WW influent, WW effluent and surface water.

Arrows denote which was the order of SPE cartridges which were connected in series.

Matrix (volume)	Cartridge	LMG	LMG-N2-G	OXO-LMG	N2-Me-LMG	LMG-N2-oxide	LMG-N2-G TP430	LMG-N2-G TP433-Amd
WWTP influent (400 mL)^a	Rec tot ^b [%]	104 ± 10 ^c	100 ± 8	65 ± 8	85 ± 14	114 ± 9	N/A ^e	104 ± 7
	HLB ^d [%]	99	100	100	100	100	100	95
	PPL ^d [%]	0	0	0	0	0	0	5
	MAX ^d [%]	1	0	0	0	0	0	0
	↓ MCX ^d [%]	0	0	0	0	0	0	0
LOQ [ngL⁻¹]		7.3	0.5	1.1	6.5	4.9	LMG-N2-G	1.0
WWTP effluent (600 mL)	Rec tot [%]	79 ± 10	96 ± 4	99 ± 1	105 ± 2	134 ± 2	N/A	N/A
	HLB [%]	100	100	100	100	100	100	N/A
	PPL [%]	0	0	0	0	0	0	N/A
	MAX [%]	0	0	0	0	0	0	N/A
	↓ MCX [%]	0	0	0	0	0	0	N/A
LOQ [ngL⁻¹]		2.2	0.3	0.5	0.6	2.1	LMG-N2-G	N/A
Surface water (800 mL)	Rec tot [%]	100 ± 13	103 ± 3	118 ± 8	81 ± 5	78 ± 4	N/A	N/A
	HLB [%]	100	72	100	100	100	100	N/A
	PPL [%]	0	28	0	0	0	0	N/A
	MAX [%]	0	0	0	0	0	0	N/A
	↓ MCX [%]	0	0	0	0	0	0	N/A
LOQ [ngL⁻¹]		1.5	0.2	0.2	0.3	1.2	LMG-N2-G	N/A
RT [min]		6.42	4.68	6.87	6.73	6.36	5.72	5.47

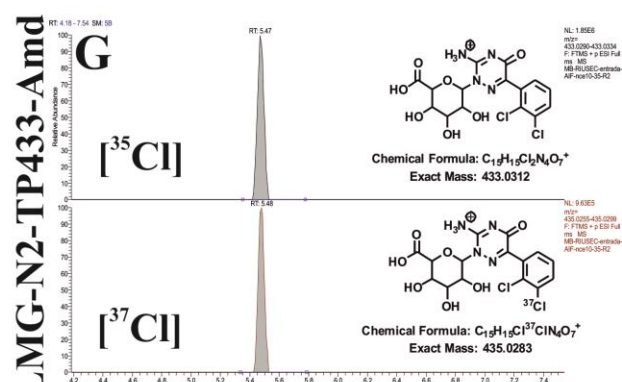
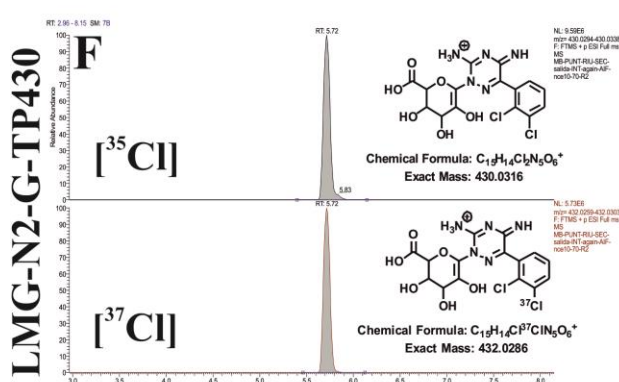
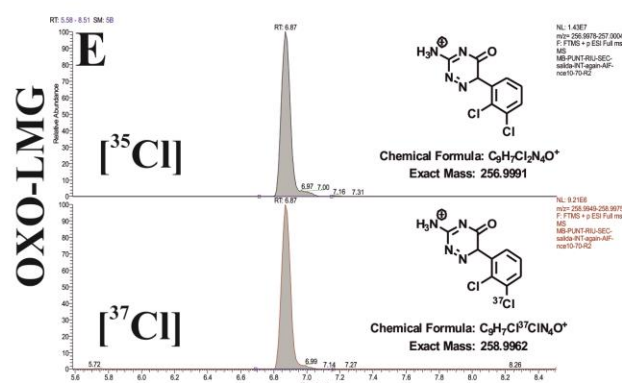
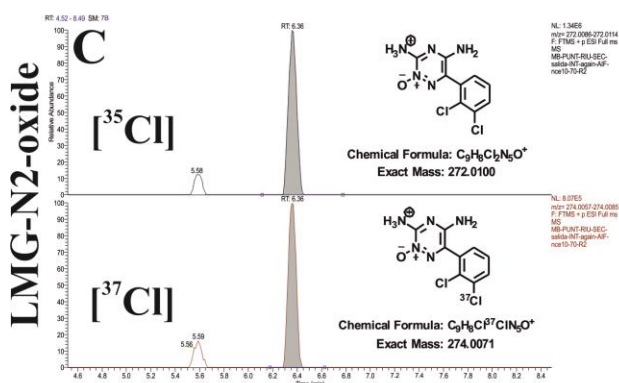
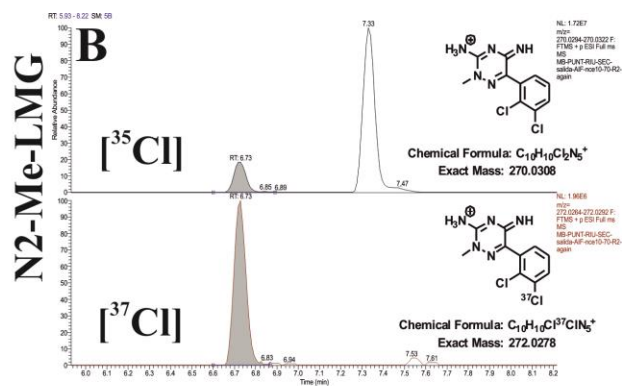
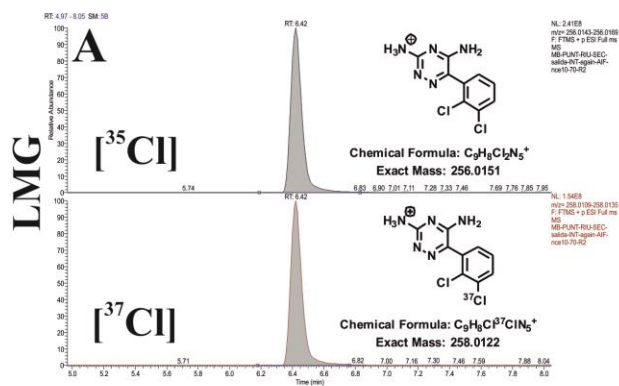
^a preconcentration volume; ^b total recovery of a compound; ^c average of three measurements and standard deviation; ^d percentage of total recovery that was retained in the cartridge; ^e not analyzed

Figure SI-13. Representative chromatograms of detected TPs in influent (LMG-N2-TP433-Amd) and effluent samples (the rest)

Figure SI-13 shows the chromatograms of the (A) LMG, (B) N2-Me-LMG, (C) LMG-N2-oxide, (D) LMG-N2-G, (E) OXO-LMG, (F) LMG-N2-G-TP430 and (G) LMG-N2-G-TP433-Amd together with their corresponding ³⁷Cl isotope peak.

For (B) N2-Me-LMG another peak that eluted later was detected with the same isobaric mass as LMG metabolite but without the corresponding ³⁷Cl isotope and was discarded from further analysis

For (C) LMG-N2-oxide again another peak was detected with the ³⁵Cl and ³⁷Cl isotope ration which indicated that the compound had two chlorine atoms. However, since this compound was detected in only one sample (one WW effluent sample) it was further used as a positive finding of a suspect analyte.



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