Application Note

Water Analysis

Identification of Fluoxetine Transformation Products After Photo-and Biodegradation

Using an Agilent 1290 Infinity II LC /6545 Quadrupole Time of Flight (Q-TOF) System

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Abstract

The intake of antidepressants has increased by more than 60% over the past decade. Fluoxetine, developed in the 1980s, quickly became one of the most popular antidepressants for the treatment of various depressive disorders. This study investigates the transformation pathways of fluoxetine by photo- and biodegradation to enable the comparison of abiotic transformation products with biologically formed metabolites. This application note describes a workflow based on high-resolution mass spectrometry with statistical analysis that was used to identify and characterize the formed transformation products. Furthermore, the study highlights the importance of considering fluoxetine's broad range of metabolites both in freshwater systems and ecotoxicity tests.

Introduction

Fluoxetine (FLX; trade name Prozac) is a frequently used antidepressant of the class of selective serotonin reuptake inhibitors (SSRI) and is among the 20 most prescribed pharmaceuticals in the USA. 10% of the consumed drug is excreted unchanged and about 20% as norfluoxetine (NFLX), which is the major human metabolite. FLX and NFLX have been detected in samples from surface water, sediments, and aquatic biota and are described as acutely toxic substances for aquatic organisms.[1] The high toxicity of FLX and the even higher toxicity of its metabolite and transformation product (TP) NFLX highlight the importance of other TPs, which might be formed in water and accumulate in sediment and biota.

Studies on FLX degradation by oxidation processes revealed TPs that were formed by hydroxylation, O-dealkylation (cleavage of the C-O bond), and defluorination of the CF_3 moiety to a carboxyl group (COOH).[2] However, in these studies, TPs containing acidic functional groups, which typically show the highest sensitivity in the negative electrospray ionization mode, were insufficiently considered. Biologically formed metabolites apart from NFLX have been very rarely described in the literature. In the present study, the focus was on the identification of photochemical TPs of FLX. Furthermore, zebrafish (*Danio rerio*) embryos were exposed to FLX and the findings of biologically formed metabolites were compared to abiotic TPs. Identification of TPs/metabolites was performed using ultrahigh performance liquid chromatography (UHPLC) coupled to electrospray ionization (ESI) and accurate-mass quadrupole time-of-flight (Q-TOF) mass spectrometry. UHPLC-ESI/Q-TOF MS provides the appropriate resolution and sensitivity to identify unknown transformation products. Accurate mass and accurate mass fragments have been used to assign chemical formulas and to identify unknowns. The original publication of this study can be found in Environmental Science & Technology.[3]

Materials and methods

Photodegradation experiment

Photochemical experiments were performed in a buffered aqueous solution with simulated sunlight. The experiments were conducted at pH 6, 8, and 10 in ultrapure water and in surface water to cover direct and indirect photochemical processes. The initial FLX concentration was 15 mg/L and the samples were exposed to the simulated sunlight in triplicates for 28 hours, with one control, which was kept in the dark.

Biotransformation of FLX in the zebrafish embryo

Wild-type zebrafish (*Danio rerio*) were used for biotransformation experiments of FLX. After 48 hours, the zebrafish embryos were divided into an exposure group (ultrapure water was replaced by 5 mg/L FLX solution) and a control group. FLX metabolites were identified after 96 hours in triplicates of five pooled embryos. The embryo extraction was conducted by sonication in a mixture of acetonitrile and water (v/v 3:2), spiked with the internal standard FLX-d5. After centrifugation, the supernatant was analyzed directly by LC/Q-TOF.

Analytical methods

The TPs formed by photolysis and biodegradation were analyzed by liquid chromatography (Agilent 1290 Infinity II LC) coupled to quadrupole time-of-flight mass spectrometer (Agilent 6545 LC/Q-TOF). The LC conditions are described in Table 1. The eluent was diverted to waste between minute 4.4 and 4.8 to prevent high FLX concentrations from contaminating the ion source. Additional information on the instrument parameters is given in Table 2. Fragmentation data were obtained by targeted MS/MS mode (precursor isolation width was set to 1.3 Da and the collision energies were set to 10, 20, and 40 eV).

Table 1. LC-Parameters for Q-TOF.

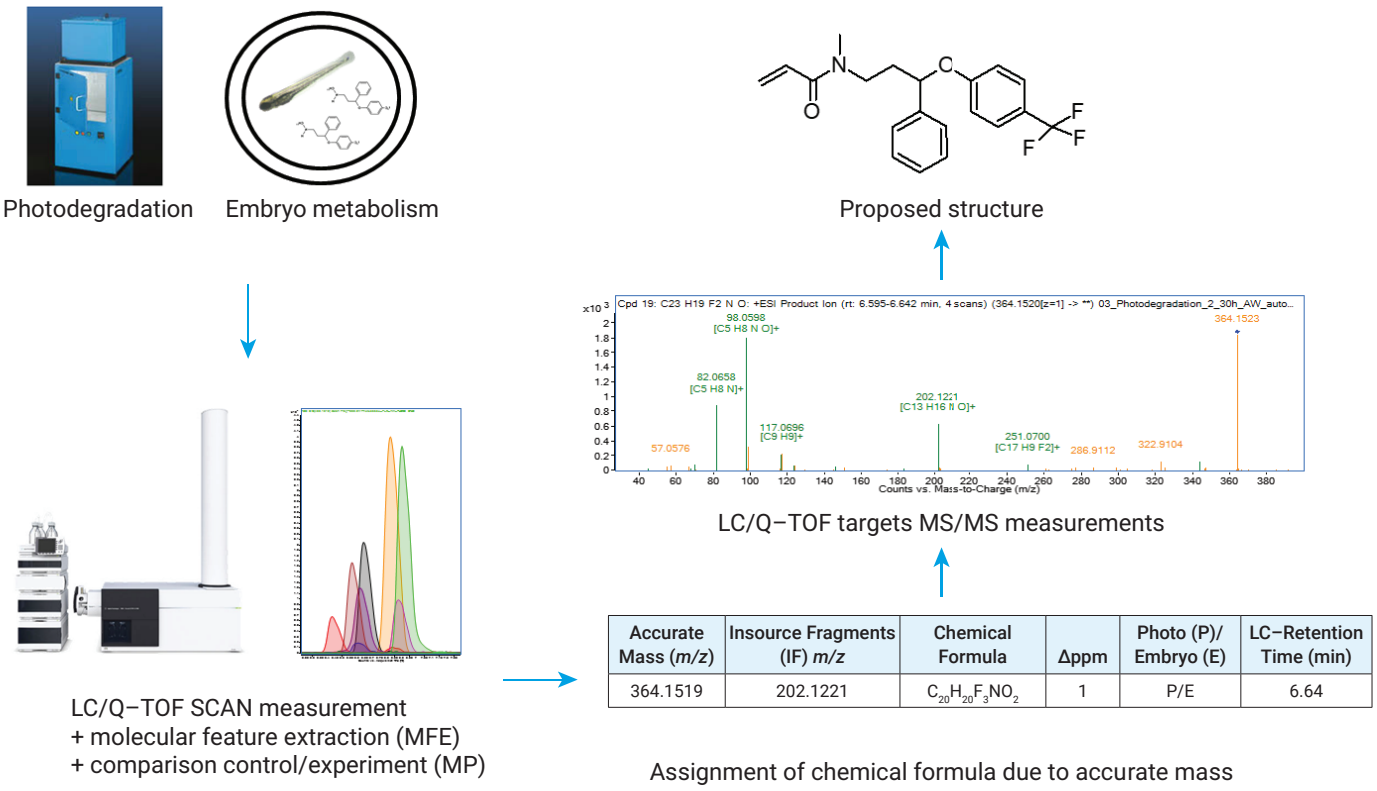
Parameter	Value	
Instrument	1290 Infinity I LC binary pump	
Mobile Phase	Positive: A) 0.1% formic acid, in water B) 0.1% formic acid, in acetonitrile	Negative: A) 0.1 % acetic acid and 0.1 mM ammonium acetate, in water B) 0.1% acetic acid, in acetonitrile
Gradient	**Time (min)** / **%B** 0 / 5 1 / 5 8 / 100 15 / 100 15.1 / 5	
Flow rate	0.40 mL/min	
Column	Agilent InfinityLab Poroshell 120 EC-C18 (2.7 µm, 2.1 × 100 mm)	
Temperature	40 °C	
Injection Volume	5 µL	

Table 2. Q-TOF operating parameters.

Parameter	Value
Instrument	Agilent 6545 LC/Q-TOF
MS1 Mass Range	50 to 1,000 *m/z*
MS2 Mass Range	50 to 1,000 *m/z*
MS1 Acquisition Rate	3 spectra/s
MS2 Acquisition Rate	5 spectra/s
Collision Energy	10, 20, and 40 eV
Dry Gas Temperature	225 °C
Drying Gas Flow Rate	6 L/min
Sheath Gas Temperature	375 °C
Sheath Gas Flow Rate	12 L/min
Nebulizer Gas	40 psi
Nozzle Voltage	± 300 V
Capillary Voltage	± 3,200 V
Fragmentor Voltage	160 V

Workflow for the identification of TPs by nontarget screening

The detection of possible TPs was conducted by molecular feature extraction of the scan data (*m/z* 50 to 500) using the Agilent MassHunter Qualitative Analysis software (version B.07.00). The extraction and comparison with the control revealed the features present in the samples, which were defined by the parameters *m/z*, retention time, and signal intensity. Chemical structures were proposed based on MS/MS data, in-source fragmentation, retention time behavior, and literature information. The overall workflow is depicted in Figure 1. In detail, the data sets from controls versus treatments were evaluated with the Agilent Mass Profiler software (version B.08.00). An upregulated fold-change filter of five (log_2(experiment/control)>2.32) was used to select TPs formed during the experiments. Chemical formulas were generated for the relevant accurate masses based on constraints on the elemental composition derived from the main compound FLX. The metabolite identification approach with Mass Profiler is shown for TP 338 in zebrafish embryo extracts in Figure 2. Based on the accurate mass measurements chemical formulas were assigned for a mass error below 10 ppm.



Accurate Mass (*m/z*)	Insource Fragments (IF) *m/z*	Chemical Formula	Δppm	Photo (P)/ Embryo (E)	LC–Retention Time (min)
364.1519	202.1221	$C_{20}H_{20}F_3NO_2$	1	P/E	6.64

Figure 1. Workflow for the identification of TPs. Untreated and treated samples of irradiation and metabolism experiments were analyzed and data extracted using the Agilent Mass Profiler software (MP). Target MS/MS was conducted to acquire mass spectra for features with assigned chemical formula, which were significantly higher in samples with longer exposure time.

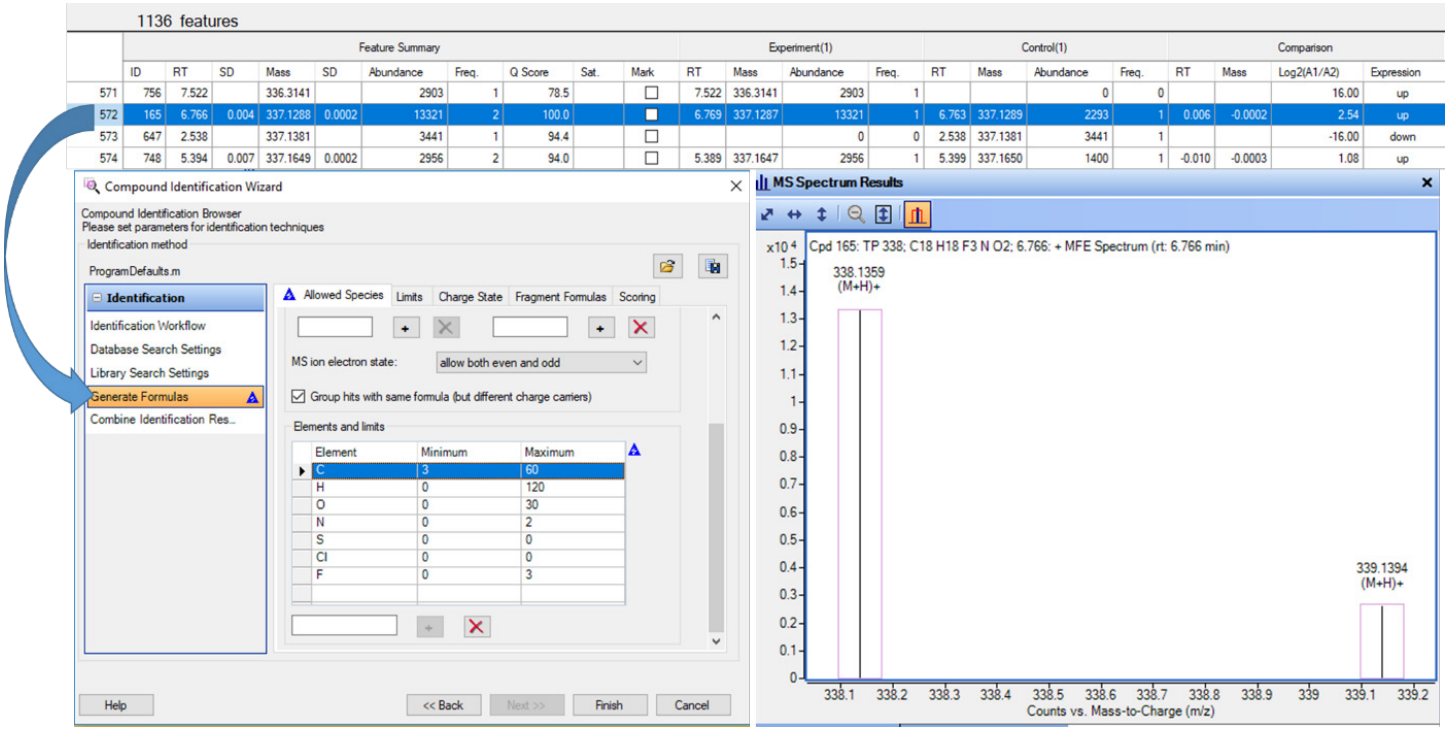


Figure 2. Selection of an upregulated feature from experiment versus control comparison, assignment of a chemical formula, and matching of the isotope pattern. The fold change filter was set to (log_2(experiment/control)>2.32).

Results and discussion

FLX degradation and NFLX formation by photolysis

After 28 hours in surface water, 0.5% of the degraded molar FLX concentration was detected as the TP NFLX, which is the main metabolite found in humans. The small amount of NFLX detected in our study implies that further TPs of FLX were most likely present. Control experiments in the dark showed negligible (<4%) losses of FLX in all matrices and no formation of TPs. Therefore, sorption, biodegradation, and nonphotolytic abiotic degradation of FLX could be excluded.

Identified photolysis TPs of FLX

After direct and indirect photolysis with simulated sunlight, the C-O bond cleavage of FLX primarily produced 3-(methylamino)-1-phenyl-1-propanol (TP 166) and 4-(trifluoromethyl)phenol (TFMP) (Figure 3). The formation of TFA during direct photolysis was also observed.

TPs 326a and 326b are hydroxyl products of FLX and were measured in ESI (+) mode. Both TPs showed the characteristic MS fragment *m/z* 44.05 (C_2H_6N) of the unchanged alkylamine side chain. TP 326a was also detected in ESI (−) and showed the original TFMP moiety (*m/z* 161.0217) as a fragment. The observed fragments suggest that hydroxylation occurred on the benzyl moiety of FLX. TPs 182a and 182b may be formed by hydroxylation of TP 166 and TP 148 by loss of water from TP 166. TP 148 was only detectable as the in-source fragments *m/z* 117.07 (C_9H_9) and *m/z* 91.0548 (C_7H_7). A decrease of sheath gas temperature in the Agilent Jet Stream (AJS) source from 400 to 300 °C revealed the peak of the precursor TP 148.

Furthermore, seven TPs were derived from the substitution of the -CF_3 group and further hydroxylation and O-dealkylation. The initial transformation reaction was the nucleophilic substitution of -CF_3 with –COOH, which formed TP 286a (Figure 3). Further O-dealkylation and hydroxylation formed six TPs.

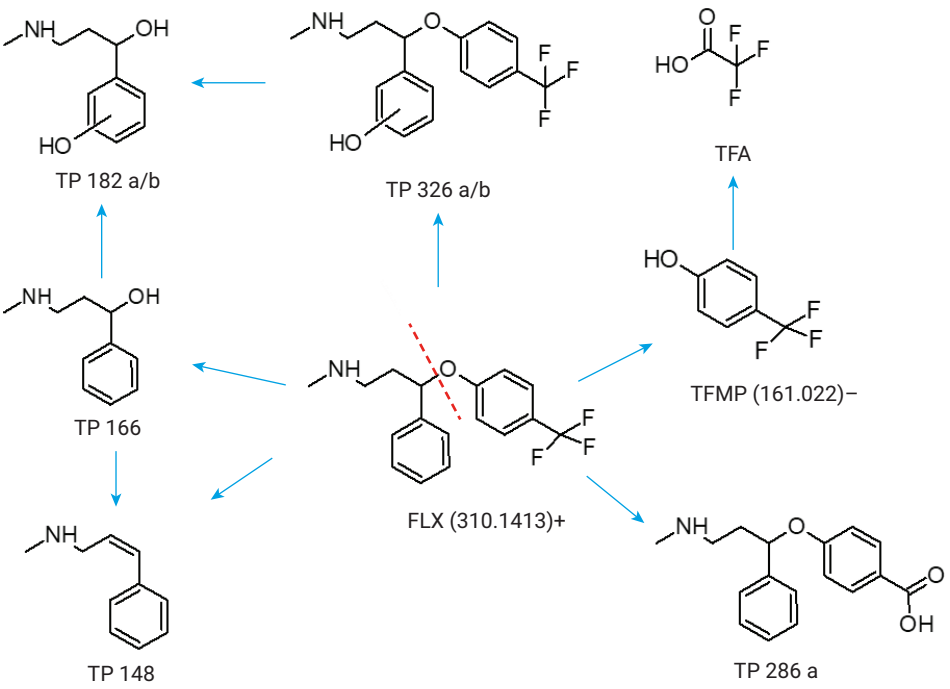


Figure 3. Scheme for O-dealkylated, oxidated, and hydroxylated TPs by direct and indirect FLX-photolysis. TFMP and TFA were measured by ESI (–), all other TPs by ESI (+). TP 326a and TP 286a were measured by both ESI (–) and ESI (+).

Various N-acylation reactions are known for primary and secondary amines in biotic systems. This study observed photo-induced N-acylation which, it is believed, has never been observed before for amines in general. Most of the TPs showed a characteristic in-source fragment (IF) formed by cleavage of the TFMP moiety. Five TPs were detected after N-acylation with aldehydes and a further five TPs that were associated with reactions with carboxylic acids.

Figure 4 shows the fragment spectrum of TP 408, an N-acylation TP of FLX with succinic acid. The IF (*m/z* 246.1116) is the second most abundant fragment at 10 eV. Furthermore, the FLX characteristic fragment 44.0489 is detected, as well as *m/z* 117.0699, which was a common fragment of all TPs.

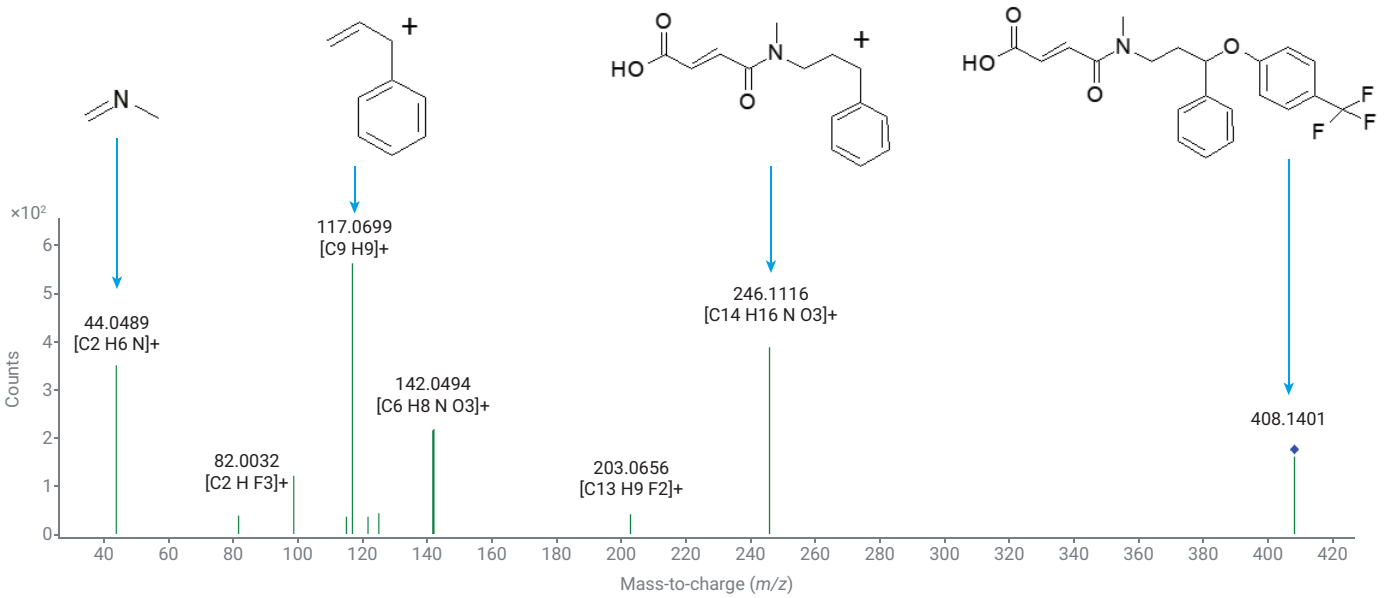


Figure 4. Fragmentation pattern at 10 eV collision energy of the TP 408.

Uptake and transformation of FLX in zebrafish embryos

In zebrafish embryos, FLX, NFLX, and TFMP accumulated and the already identified TPs 338, 364, 352, 410, and 326a/b (after photolysis) could be confirmed.[4] Three new metabolites were also identified: 1) TP 326 c was tentatively identified as hydroxylamine of FLX, based on fragments and increased retention time in comparison to TPs 326a and 326b. 2) A methylated FLX derivative was proposed for TP 324. 3) TP 409 the only chemical formula that could be proposed so far is, $C_{22}H_{27}F_3N_2O_2$.

Conclusion

The presented workflow demonstrates the identification of abiotic and biotic transformation products using a streamlined workflow based on liquid chromatography high-resolution mass spectrometry (UHPLC/Q-TOF MS) and data evaluation and reduction by a software-based statistical approach. The identification of 26 different photo induced transformation products, along with low concentrations of norfluoxetine, highlights the importance of considering a broad range of transformation products in abiotic fluoxetine degradation processes. The study also indicated similarities between the abiotic and biotic degradation of fluoxetine. Table 3 shows a summary of the identified TPs.

Table 3. Identified TPs from photodegradation (P) and metabolites from zebrafish embryos (E).

Name (pos(+) or neg (-) Ionization)	Chemical Formula	Accurate Mass (*m/z*)	Insource Fragments (IF) *m/z*	Δppm	Photo(P)/ Embryo (E)	LC-Retention Time (min)
NFLX (+)	$C_{16}H_{16}F_3NO$	296.1367	134.0966	0	P	4.42
TFMP (-)	$C_7H_5F_3O$	161.022		0	P/E	5.02
TP 139 (-)	$C_7H_6O_3$	137.024		2	P	2.79
TP 146 (+)	C_9H_7NO	146.06		4	P	1.46
TP 148 (+)	$C_{10}H_{13}N$	148.1121	117.07, 91.09	3	P	3.1
TP 150 (+)	$C_9H_{11}NO$	150.0913		7	P	2.37
TP 152 (+)	$C_9H_{13}NO$	152.107		4	P	1.71
TP 166 (+)	$C_{10}H_{15}NO$	166.1226		3	P	2.21
TP 182a (+)	$C_{10}H_{15}NO_2$	182.1176		5	P	1.25
TP 182b (+)	$C_{10}H_{15}NO_2$	182.1176		5	P	1.62
TP 272 (+)	$C_{16}H_{17}NO_3$	272.1281		1	P	3.44
TP 286a (+/-)	$C_{17}H_{19}NO_3$	286.1438		3	P	2.93
		284.1292		0		
TP 286b (+)	$C_{17}H_{19}NO_3$	286.1438		2	P	3.72
TP 286c (-)	$C_{17}H_{19}NO_3$	284.1292		0	P	4.33
TP 316a (-)	$C_{17}H_{19}NO_5$	316.119		0	P	3.19
TP 316b (-)	$C_{17}H_{19}NO_5$	316.119		0	P	3.43
TP 324 (+)	$C_{18}H_{20}F_3NO$	324.157	162.1263	2	E	4.59
TP 326a (+)	$C_{17}H_{18}F_3NO_2$	326.1367		4	P/E	4.69
TP 326b (+)	$C_{17}H_{18}F_3NO_2$	326.1367		0	P/E	4.90
TP 326c (+)	$C_{17}H_{18}F_3NO_2$	326.1367		0	E	5.51
TP 338 (+)	$C_{18}H_{18}F_3NO_2$	338.1362	176.1074	0	P/E	6.82
TP 352 (+)	$C_{19}H_{20}F_3NO_2$	352.152	268.1339	2	P/E	6.87
TP 364 (+)	$C_{20}H_{20}F_3NO_2$	364.1519	202.1221	1	P/E	6.64
TP 366 (+)	$C_{20}H_{22}F_3NO_2$	366.1672	204.1383	4	P	5.05
TP 378a (+)	$C_{21}H_{22}F_3NO_2$	378.1675	216.1385	1	P	6.42
TP 378b (+)	$C_{21}H_{22}F_3NO_2$	378.1675		1	P	6.85
TP 382 (-)	$C_{19}H_{18}F_3NO_4$	380.1115		2	P	6.18
TP 396 (+)	$C_{20}H_{21}F_3NO_4$	396.1419	234.1125	1	P	6.63
TP 408 (+)	$C_{21}H_{20}F_3NO_4$	408.1417	246.1116	0	P	6.62
TP 409 (+)	$C_{22}H_{27}F_3N_2O_2$	409.2071	247.1799		E	5.68
TP 410 (+)	$C_{21}H_{22}F_3NO_4$	410.1547	248.1285	0	P/E	6.56

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