SUPPORTING INFORMATION

IDENTIFICATION OF MUTAGENIC AROMATIC AMINES IN RIVER SAMPLES WITH INDUSTRIAL WASTEWATER IMPACT

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Total page number: **21**, including 1 figure and 2 tables

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S1. Materials and Methods

S1.1. Reference Standards and Reagents

Blue Rayon (BR) was purchased from Funakoshi Co. (Tokyo, Japan). Gradient grade methanol (Merck) and 28% ammonium hydroxide (Alfa Aesar) were used for the cleaning and extraction of BR. LC-MS-grade water and methanol used for LC-HRMS analysis were supplied from Sigma-Aldrich (Chromasolv brand). Formic acid (analytical reagent grade, 98%) was purchased from Merck. Reference standards were obtained from Fluka, Alfa Aesar and Sigma-Aldrich, except for 2,8-phenazinediamine which was synthesized by Synchem (Germany), all having more than 97% purity. Dimethylsulfoxide (bioreagent ≥ 99.7%) used for the Ames test was obtained from Sigma-Aldrich.

S1.2 LC-HRMS Analysis

The linear gradient elution was performed with 0.1% formic acid as eluent A and methanol as eluent B as follows: 90:10 at 0 min for 3.2 min, to 5:95 at 21 min, then held for 20 min followed by a 9 min re-equilibration. The injection volume was 5 µl and the column and the autosampler temperatures were kept at 30^{o}C and 8 ^{o}C respectively. The HRMS analysis was performed with a Thermo QExactive Plus instrument with a heated ESI source operated at 300^{o}C. The transfer capillary temperature was set to 300^{o}C. The spray voltage was 3.8 kV, the sheath gas flow rate was 45 a.u. and the auxiliary gas flow rate was 1 a.u. Additional analytical runs were made separately for derivatized and underivatized samples to obtain HRMS/MS data using higher energy collisional dissociation (HCD) with stepped collision energies of 20, 35 and 50% for derivatized samples and 30, 50 and 70% for underivatized samples. All fragment ions generated from the three collision energies were recorded into one spectrum and used for subsequent candidate list generation by MetFrag.

S1.3 MZmine Settings

Masses from individual scans were detected by wavelet transform function with a noise cut-off of 3000. Chromatograms were built with a min time span of 0.1 min and a minimum height of 50,000 a.u, limiting the m/z tolerance to 4.0 ppm. Smoothing was applied on the chromatograms with a filter width of 7 and chromatogram deconvolution was performed with following settings: chromatographic threshold was 80%, search minimum in RT range was 0.1 min, minimum relative height was 30 %, minimum absolute height was 500,000 a.u., minimum ratio of peak

top-to-edge was 2 and peak duration range was 0.1-5.0 min. The peak lists of derivatized and underivatized samples were aligned using the join aligner function setting m/z tolerance of 0.001 m/z with a weight of 70 and a retention time tolerance of 0.2 min with a weight of 30. Among the aligned derivatized and underivatized peak lists, only those peaks with an intensity ratio between derivatized and underivatized samples of <0.1 or > 10 were kept. By these means, also background and blank peaks were removed.

S1.4 MetFrag Settings

ChemSpider was chosen as the database to generate the candidate lists with MetFrag 2.2[1]. For fragment peak match the absolute mass deviation was set to 0.001 m/z and the relative mass deviation was set to 5 ppm. Maximum tree depth was 2 and unconnected compound filter option was chosen for the processing candidate filter.

S1.5 pH-dependent LC Retention Method

Aliquots of underivatized BR samples were separated with a double endcapped C18 column (Zorbax-Extend 100 mm × 2.1 mm, 3.5 µm particle size, Agilent) using the aforementioned gradient elution program in section S1.2 The separation was achieved with three different pH conditions using different mobile phase combinations which are (i) water and methanol both with 0.1% formic acid (pH 2.6) (ii) water and methanol with 2.5 mM ammonium acetate (pH 6.4, adjusted with ammonia) (iii) water and methanol with 2.5 mM ammonium bicarbonate (pH 10.0, adjusted with ammonia). Only full scan chromatograms were acquired with a resolving power of 140,000 (at m/z 200).

S1.6 Synthesis of 2,8-Phenazinediamine

The successful synthesis of 2,8-phenazinediamine was achieved by nitrating the commercially available phenazine-N-oxide and reducing the 3,7-dinitrophenazine-5-oxide to the 2,8-diaminophenazine,based on Otomasu (1958) [2]. For the synthesis, TLC was performed on TLC aluminium sheets—Silica Gel 60 F254 (Merck). Column chromatography was performed on silica gel 40-63 mesh, (Merck). ^{1}H-NMR- and ^{13}C-NMR-spectra were measured on a Bruker Avance in DMSO-D_6 or D_2SO_4 and referenced to TMS. Mass spectra were measured on a Finnigan LCQ^{DECA} (Thermoquest) using APCI (negative mode). Chemicals and solvents were purchased from Sigma-Aldrich, VWR, Alfa Aesar and Fisher Scientific.

As an initial step, the synthesis of 3,7-dinitrophenazine-5-oxide was established by dissolving 3 g (15,3 mmol) of phenazine-n-oxide (Pfaltz& Bauer, USA) in 30 mL concentrated sulfuric acid. The red solution was cooled and 30 mL of nitric acid 68% was slowly added with a dropping funnel. The ice bath was removed and the solution was heated slowly to 100°C in course of 1 hour and held there for 30 min. After cooling down the reaction mixture was poured into ice. The yellow material was filtered off and washed two times with water. After drying 2.8 g of a mixture of dinitrophenazine-N-oxides were obtained. This mixture was purified by crystalizing it from 300 mL of acetone. (Yield: 0.7 g (2,4 mmol); 16%; $C_{12}H_6N_4O_5$; M = 286.20 g/mol; ^{1}H-NMR (D_2SO_4): δ (ppm) = 8,61 [d, 2H]; 9,15 [d, 2H]; 9,84 [s, 2H]; ^{13}C-NMR (D_2SO_4): δ (ppm) = -118.4; 123.9; 133.2; 138.1; 138.2; 148.2).

The second and final step of the synthesis of 2,8-phenazinediamine was achieved as followed: 5 g (17.5 mmol) of 3,7-dinitrophenazine-5-oxide was suspended in 50 mL of methanol. Then a solution of 15 g (79.1 mmol) tin-II-chloride in 30 mL of concentrated hydrochloric acid was added with a dropping funnel. Afterwards the dark suspension was heated in a water bath for 1 h. After cooling down to room temperature, the red-black material was filtered and washed with 200 mL of 2 N hydrochloric acid. The residue was extracted with 300 mL of water and the extract was added slowly to 100 mL of cold concentrated ammonia to obtain the free base from the hydrochloric salt. The solid was separated, washed with water and dried to obtain 1.5 g of a dark red material. This was purified with flash chromatography on silica gel and dichloromethane:methanol 5:1 as liquid phase. The fractions with nearly one spot in TLC were pooled, reduced in volume on a rotary evaporator and stored at –20°C to obtain dark red crystals of the target molecule. (Yield: 0.3 g (1.4 mmol); 8%; C12H10N4; M = 210.23 g/mol; 1H-NMR (DMSO-D6): δ (ppm) = 6,15 [s, 4H; NH2]; 6,92 [s, 2H]; 7,21 [d, 2H]; 7,83 [d, 2H]; 13C-NMR (DMSO-D6): δ (ppm) = -102.1; 122.9; 130.5; 136.2; 145.9; 151.2; MS (ESI-MS): m/z = 211 (100%) [M+H+]).

S1.7 Ames Fluctuation Assay and Mutagenicity Evaluation

The Ames test was conducted exactly as described in Hug et al [3]. 2-aminoanthracene was used as a positive control and spontaneous reversions were evaluated with DMSO as the negative control. The mutagenic activity of the positive control and samples were determined in triplicates and fitted to the exponential equation S1. *Y* represents the number of positive wells and *x*

represents the concentration in mg BR equivalent. The constant *a* was set to 48 as the maximum revertant number and the slope of the equation, represented by a*b, was used to express the mutagenic activity in revertants / mg BR eq.

$$y = a * (1 - e^{-bx}) \qquad \textbf{Eq. S1}$$

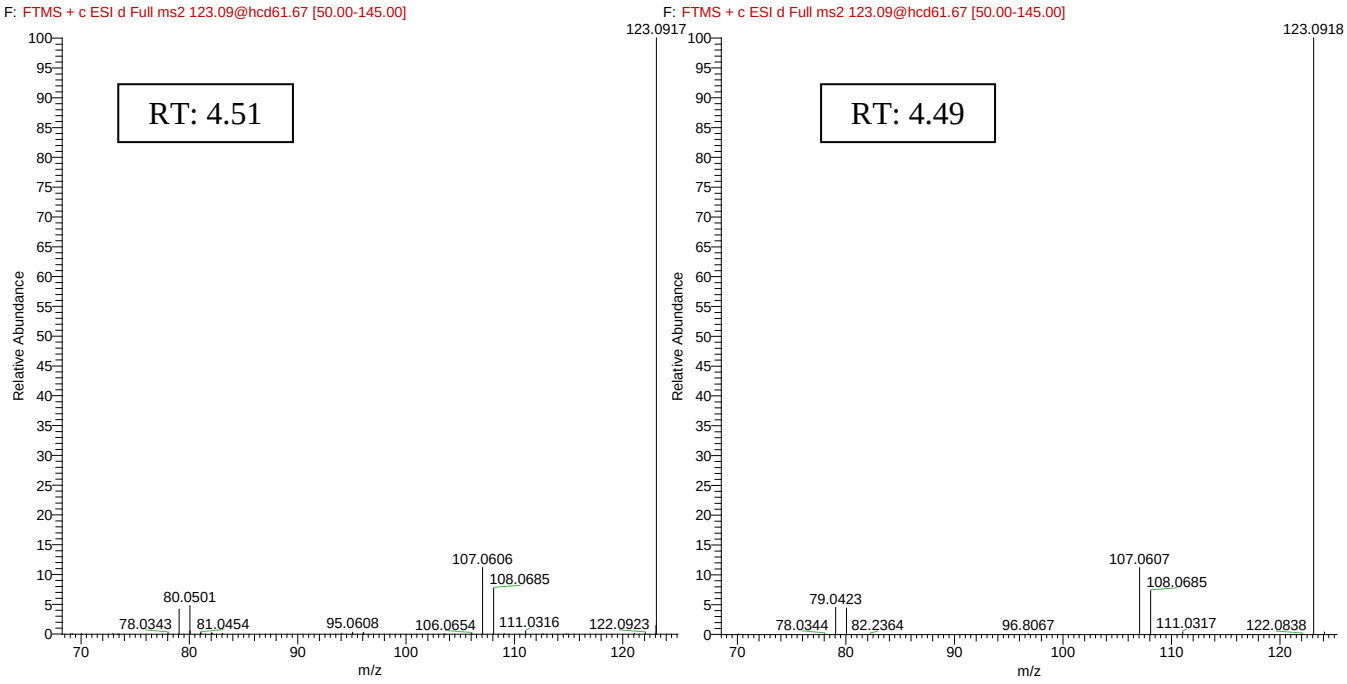
S2. Results and Discussion

S2.1 MS/MS Spectra of Identified Compounds and Respective Reference Standards

The MS/MS spectra of identified compounds and reference standards were acquired with the stepped collision energies as given in section S1.2. In addition to the analyte spectra of all identified compounds, the derivative spectra of 2,8-phenazinediamine and the derivative of the respective reference standard are also given below as an example. The MS/MS spectrum of doxazosin was acquired with HCD 45 of normalized collision energy to have a direct comparison with the existing MassBank spectrum record number: EQ329303 (*SPLASH* code: splash10-0006-0079100000-06d36a9b7afe6cf0c6e9 [4])

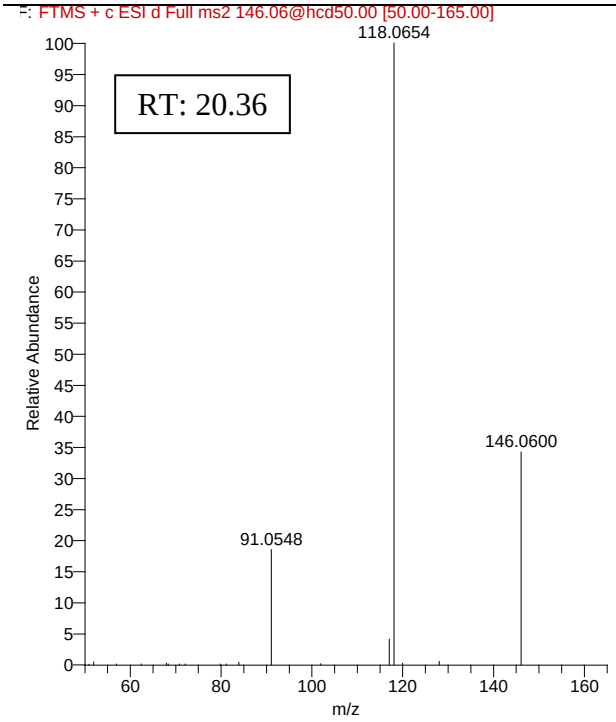
4-Dimethylaminopyridine Analyte [M+H]+: 123.0915

Analyte | Reference Standard



3-Formylindole Analyte [M+H]+: 146.0599

Analyte



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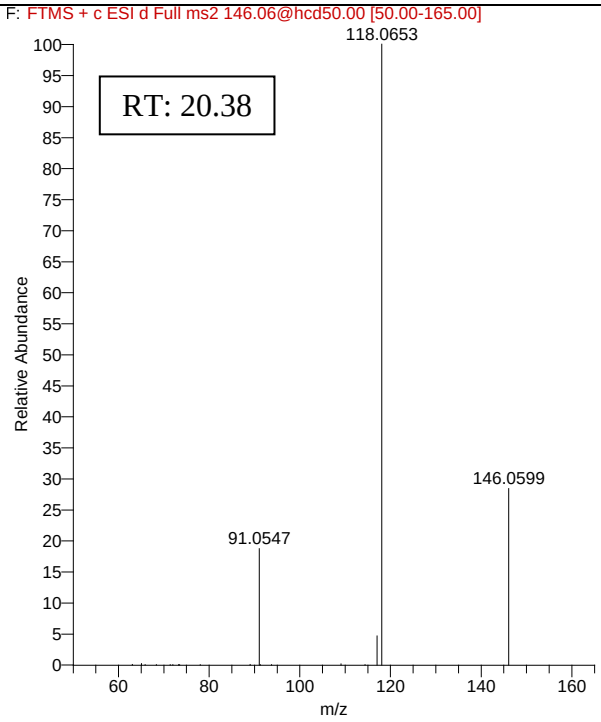
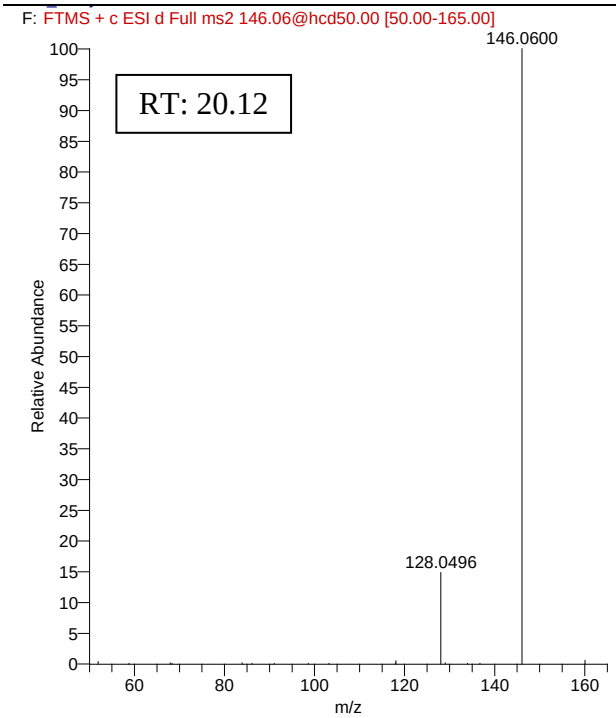


2-Hydroxyquinoline Analyte [M+H]+: 146.0599

Analyte



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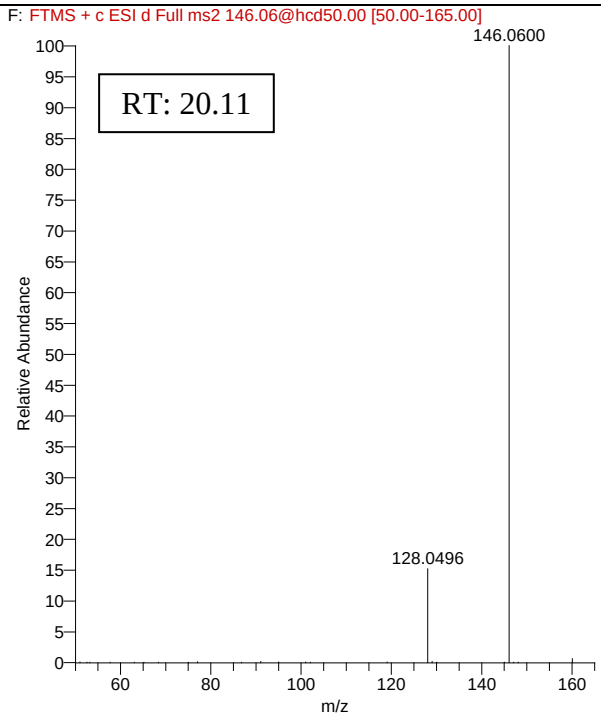
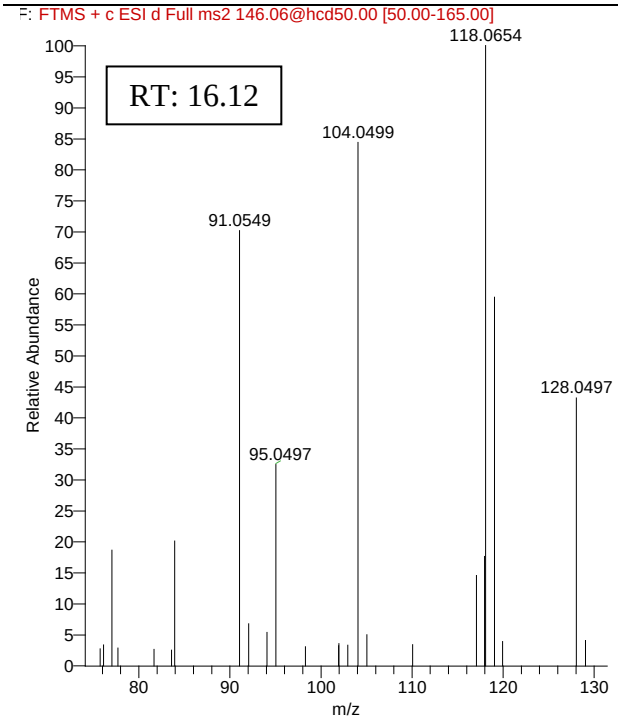


4-Hydroxyquinoline Analyte [M+H]+: 146.0599

Analyte



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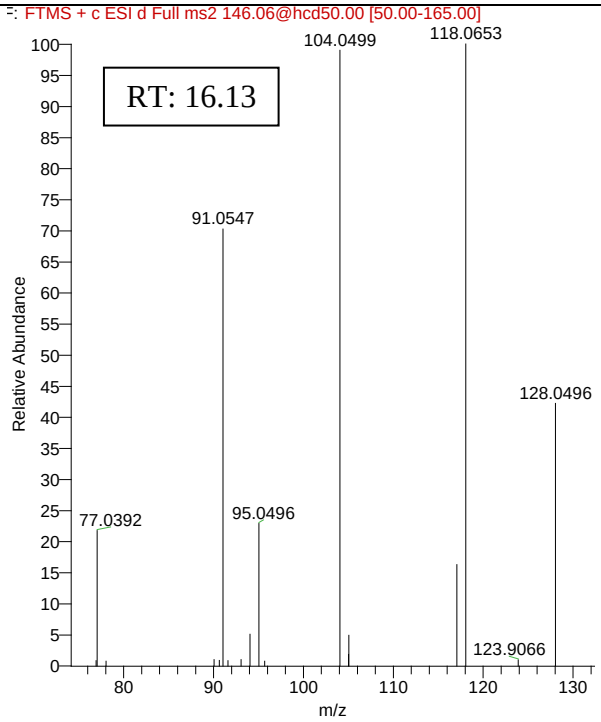
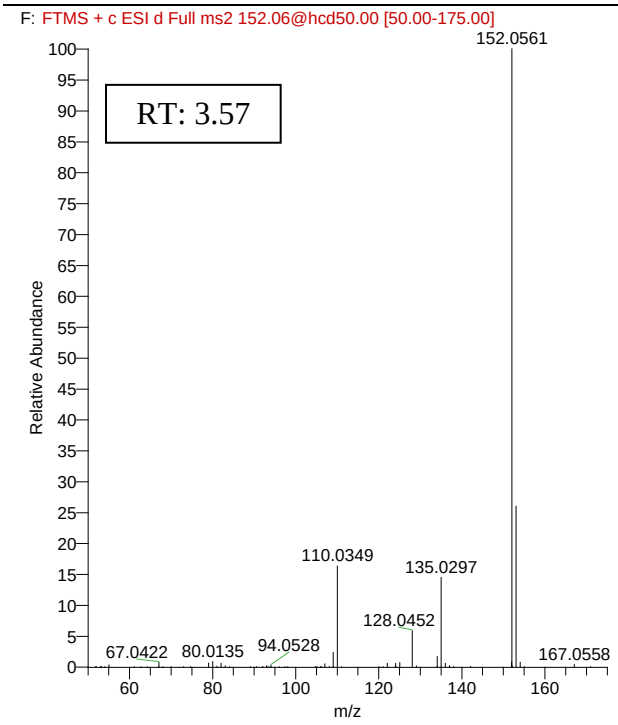


Guanine Analyte [M+H]+: 152.0565

Analyte



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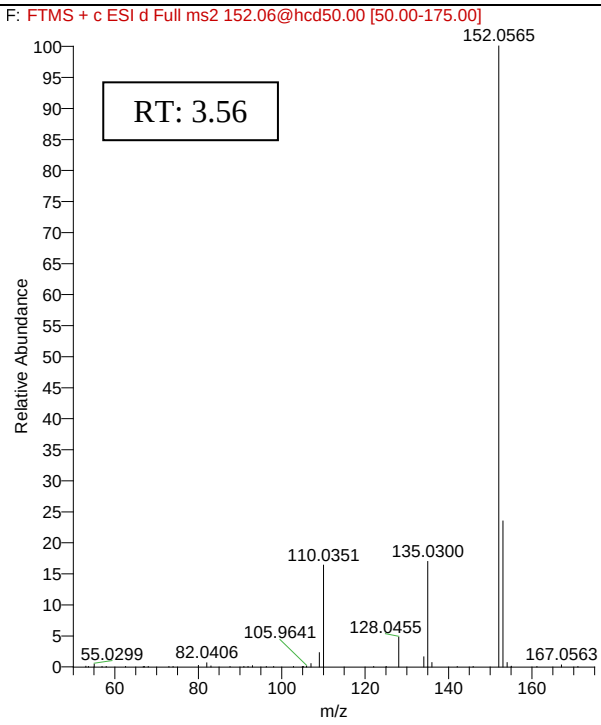
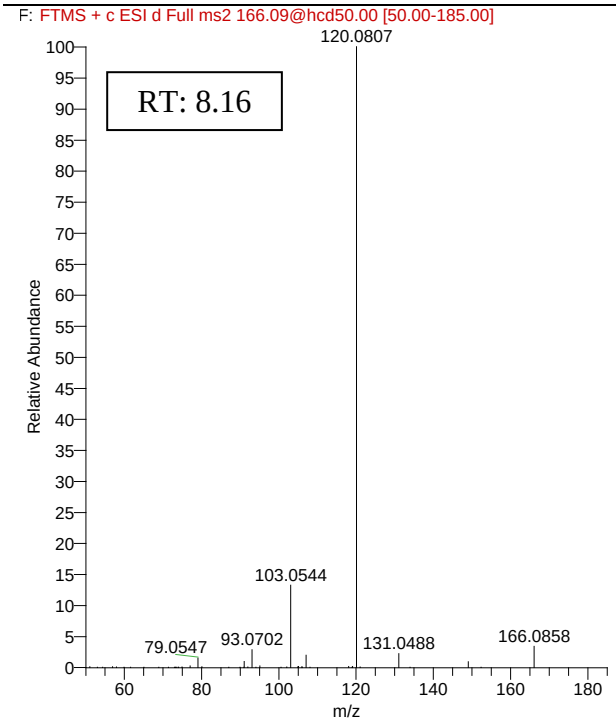


Phenylalanine Analyte [M+H]+: 166.0862

Analyte



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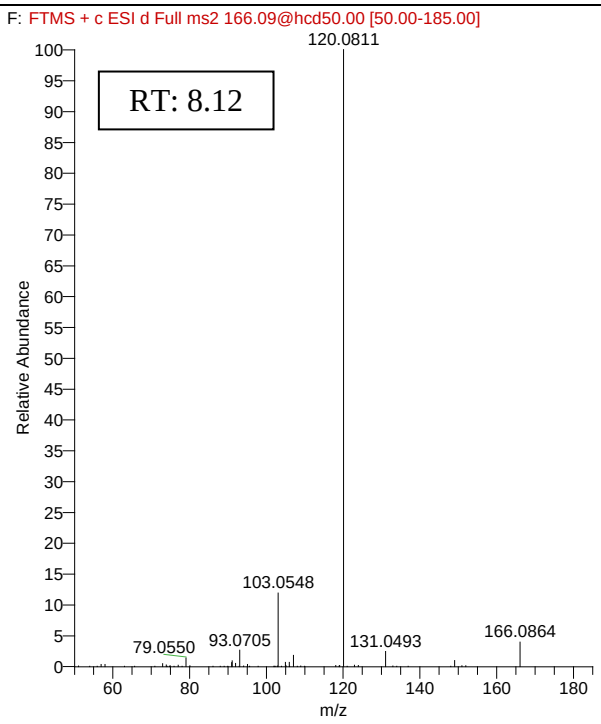
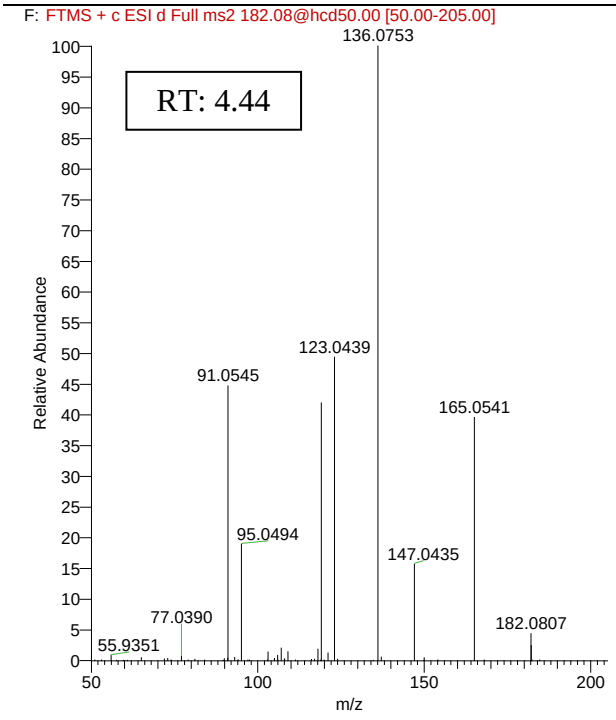


Tyrosine Analyte [M+H]+: 182.0811

Analyte



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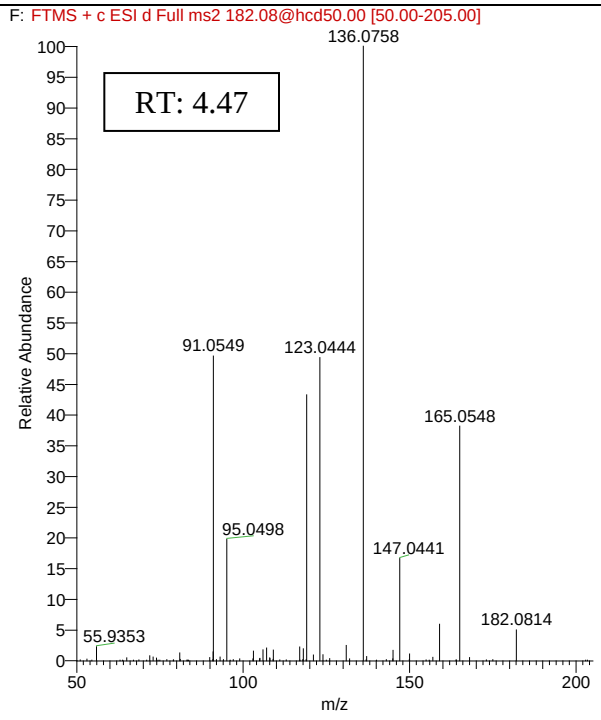
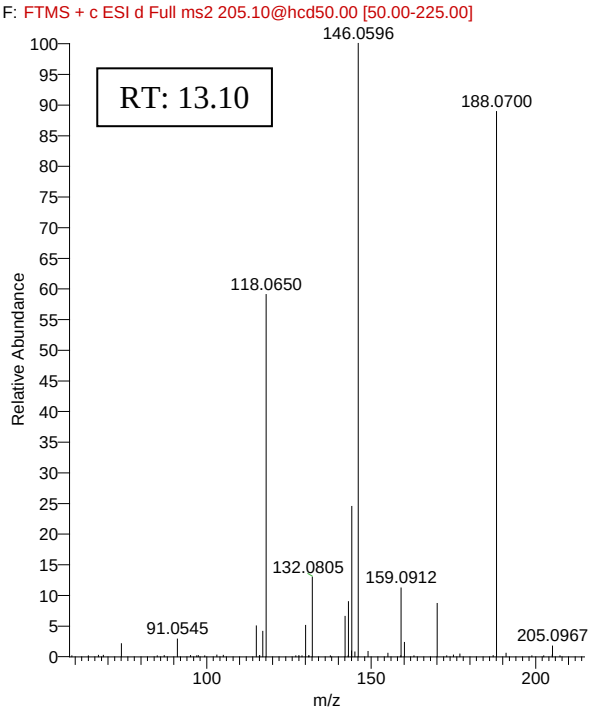


Tryptophan Analyte [M+H]+: 205.1970

Analyte



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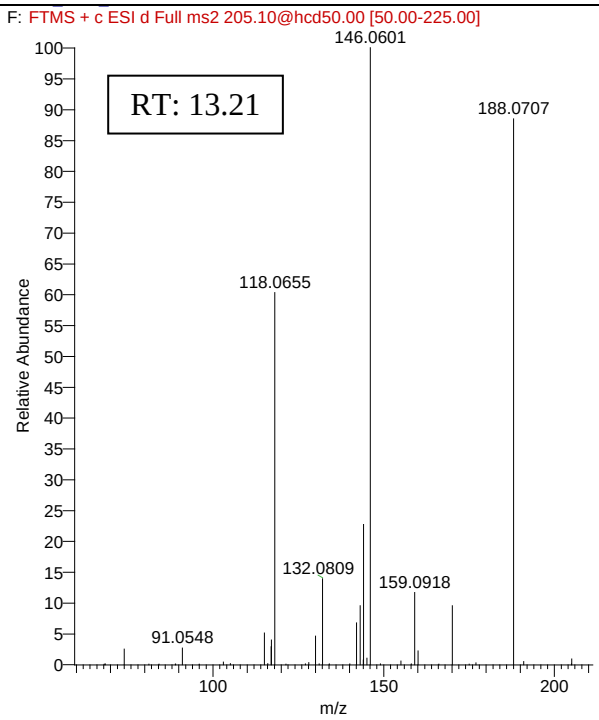
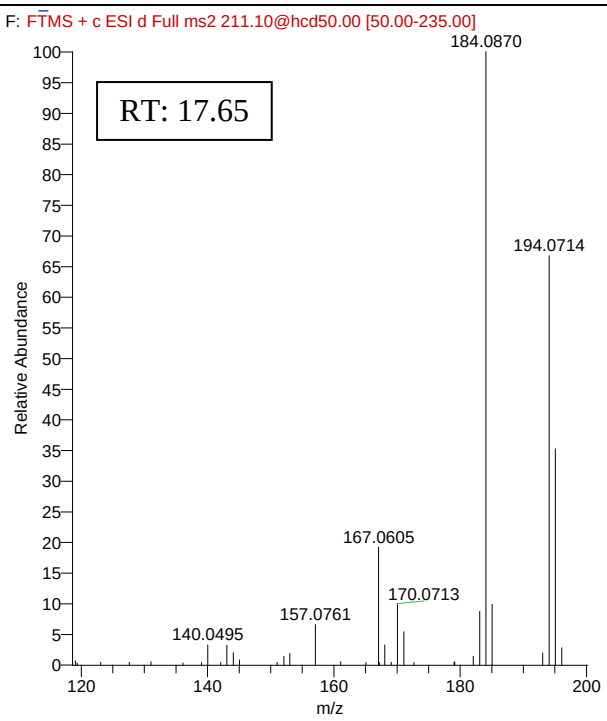


2,3-Phenazinediamine Analyte [M+H]+: 211.0977

Analyte



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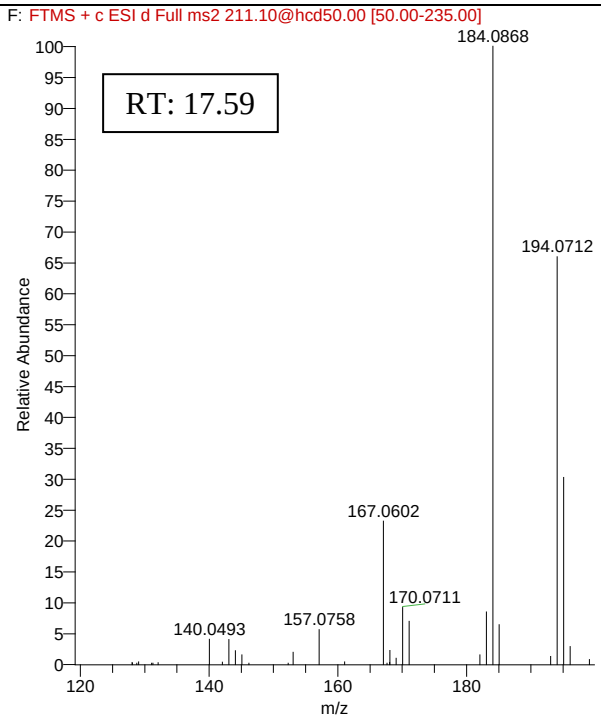
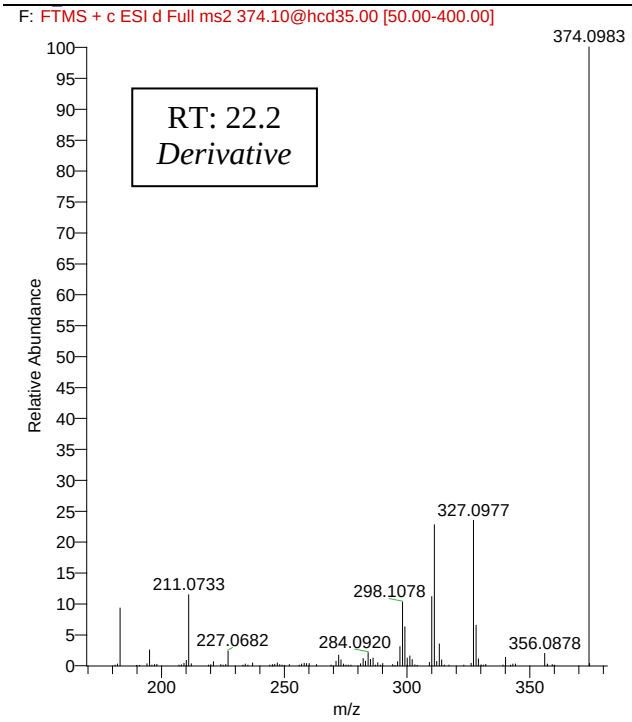
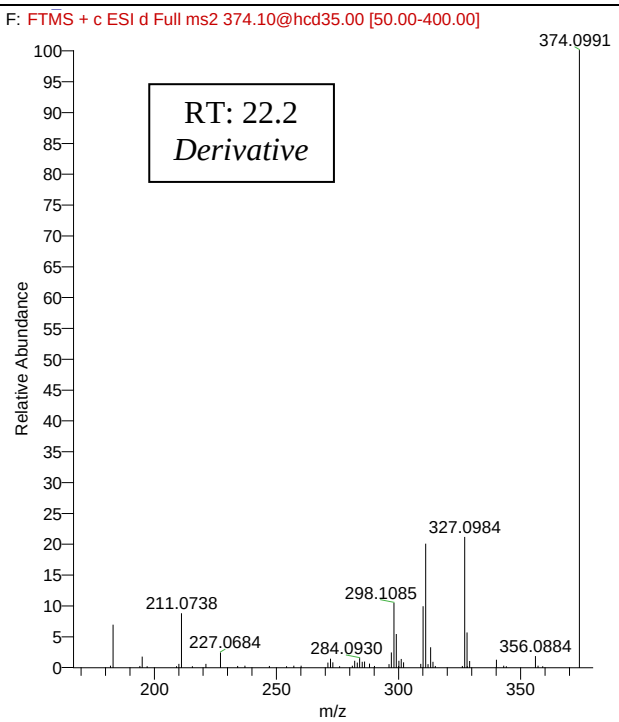


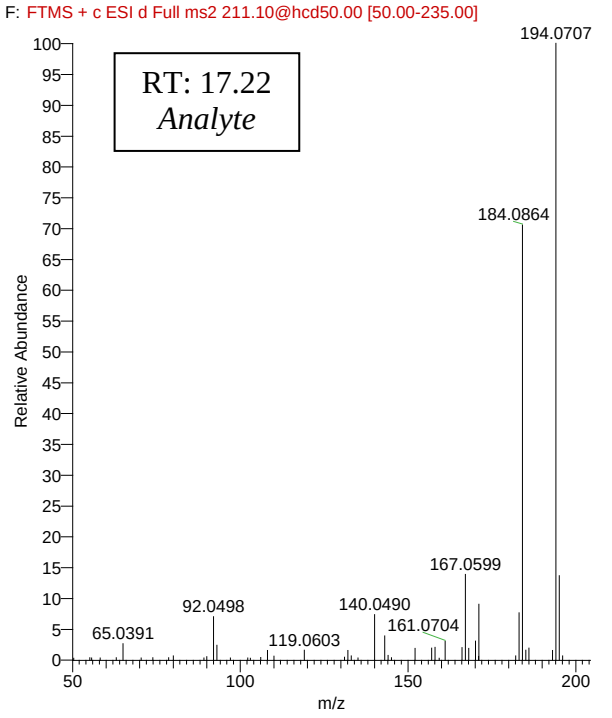
2,8-Phenazinediamine Analyte [M+H]+: 211.0977 Derivative [M+H]+: 374.0995

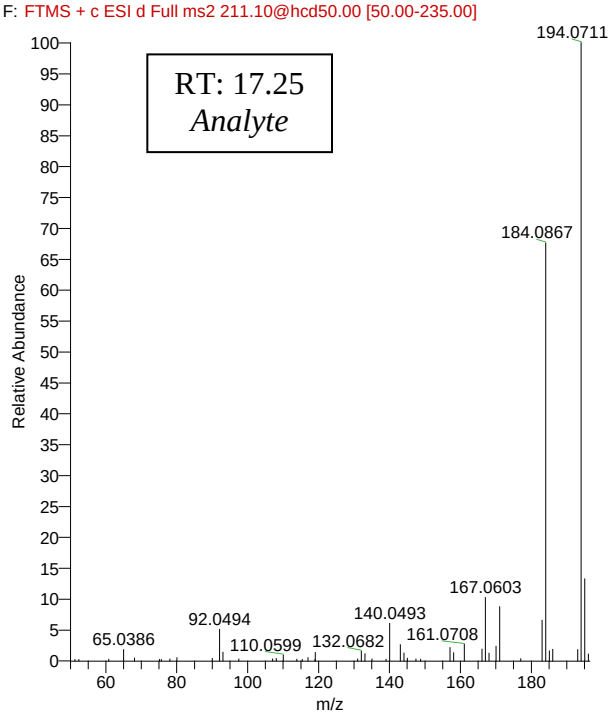
Derivative / Analyte



Derivative / Reference Standard

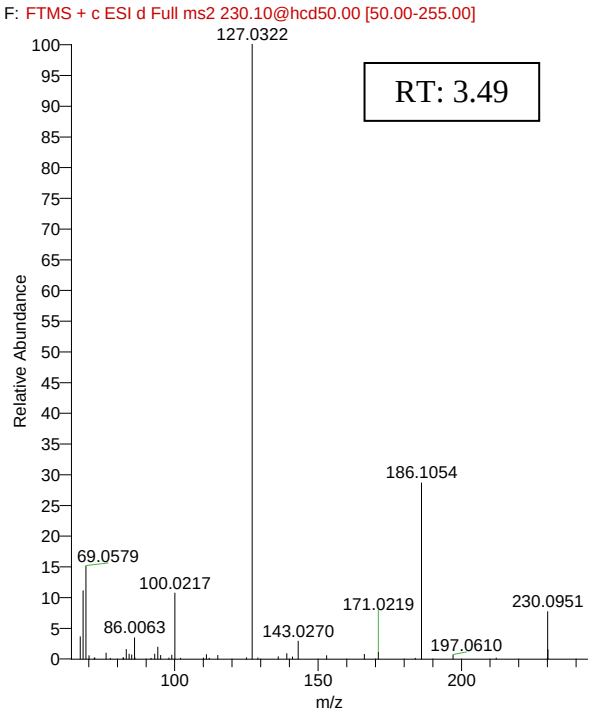






L-(+)-Ergothioneine Analyte [M+H]+: 230.0956

Analyte



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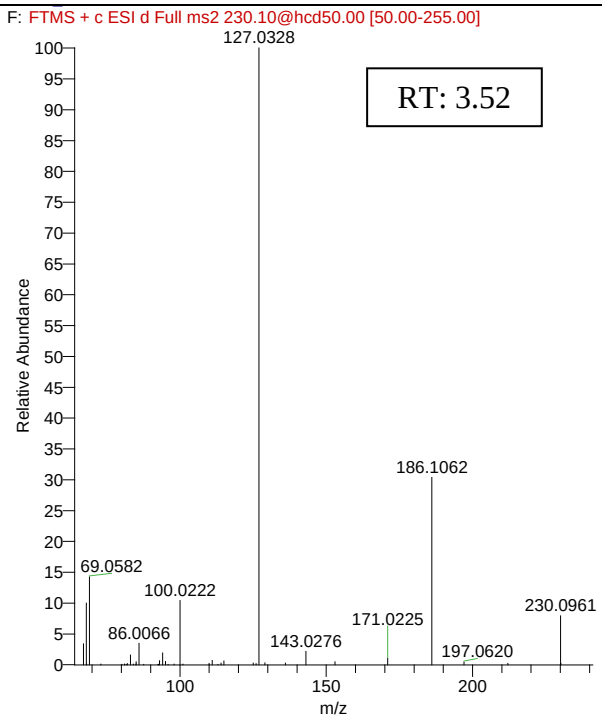
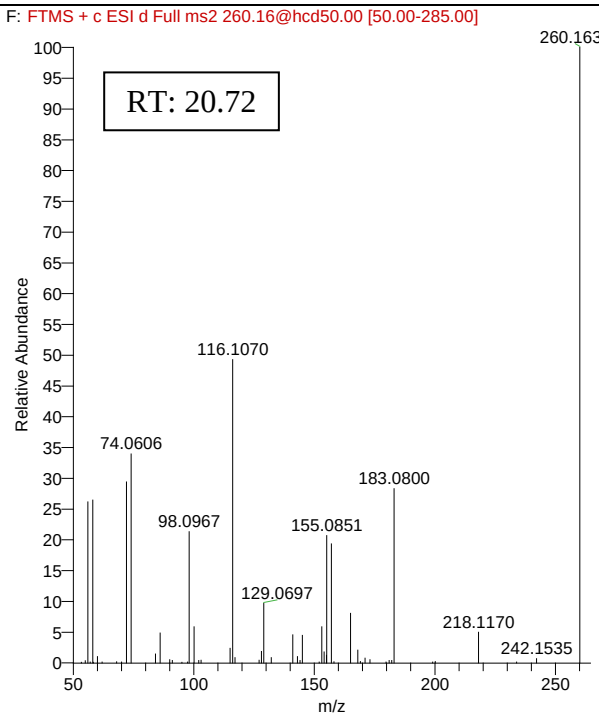


Propranolol Analyte [M+H]+: 260.1643

Analyte



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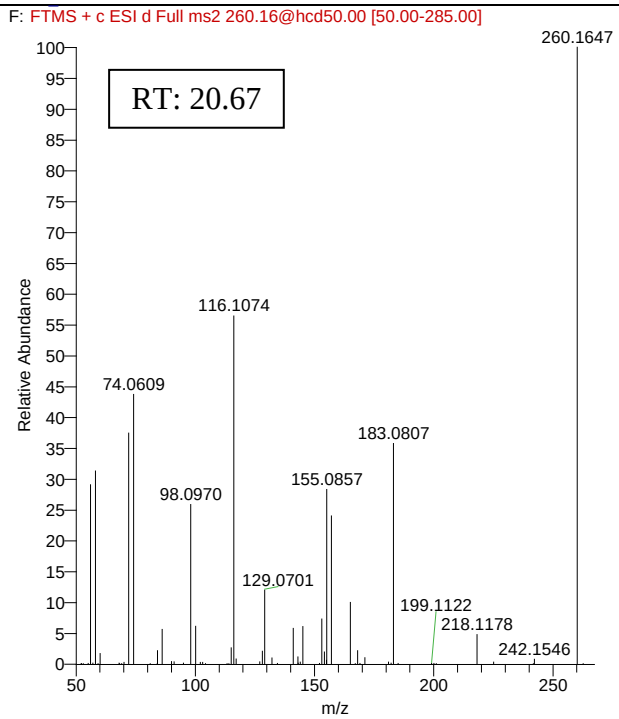
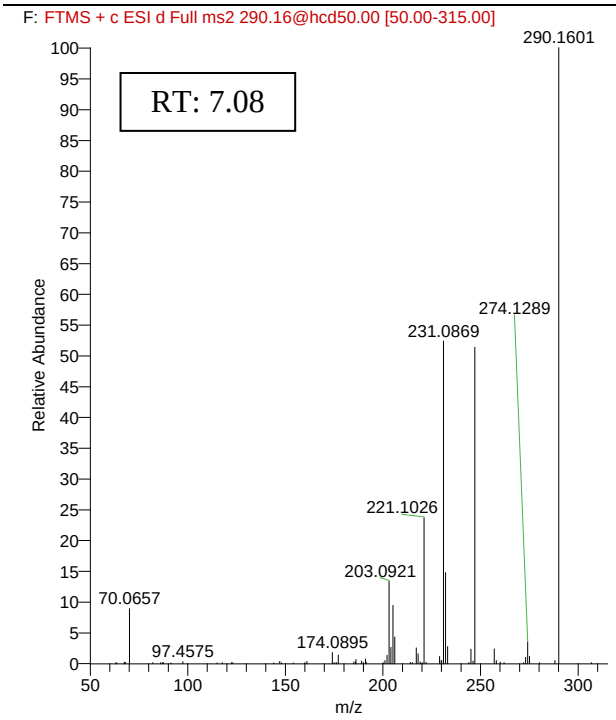


6,7-Dimethoxy-2-(1-piperazinyl)-4-quinazolinamine Analyte [M+H]+: 290.1607

Analyte



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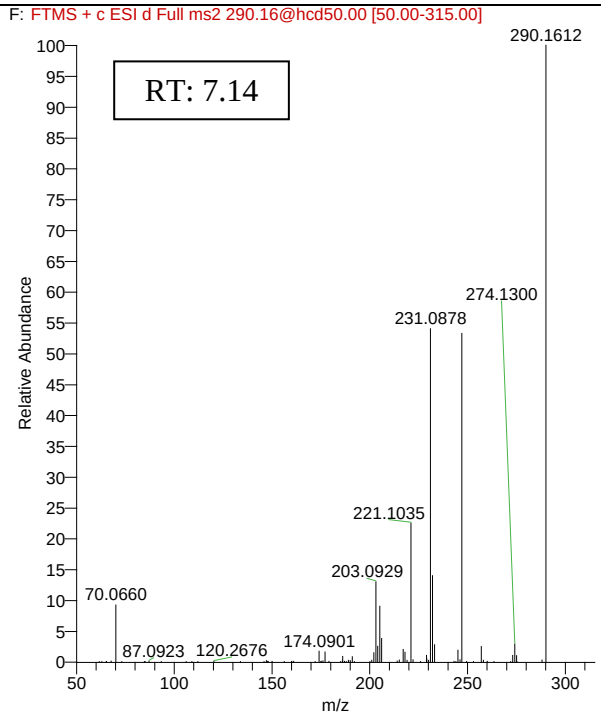
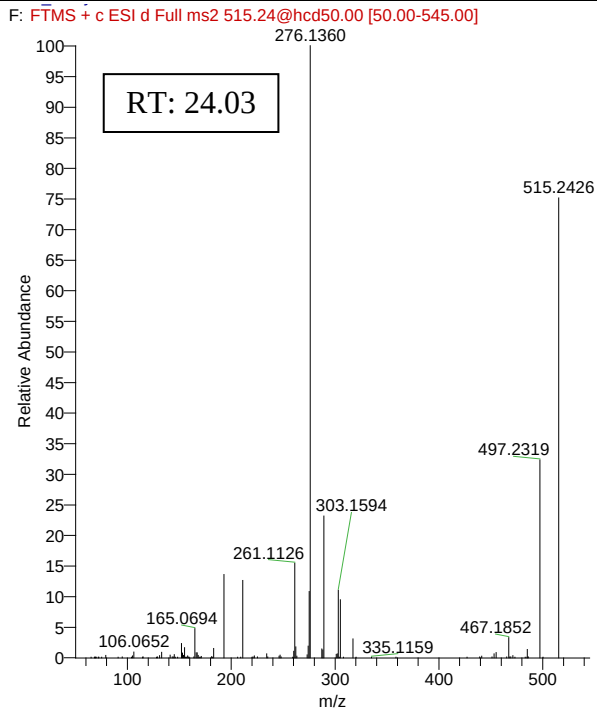


Telmisartan Analyte [M+H]+: 515.2444

Analyte



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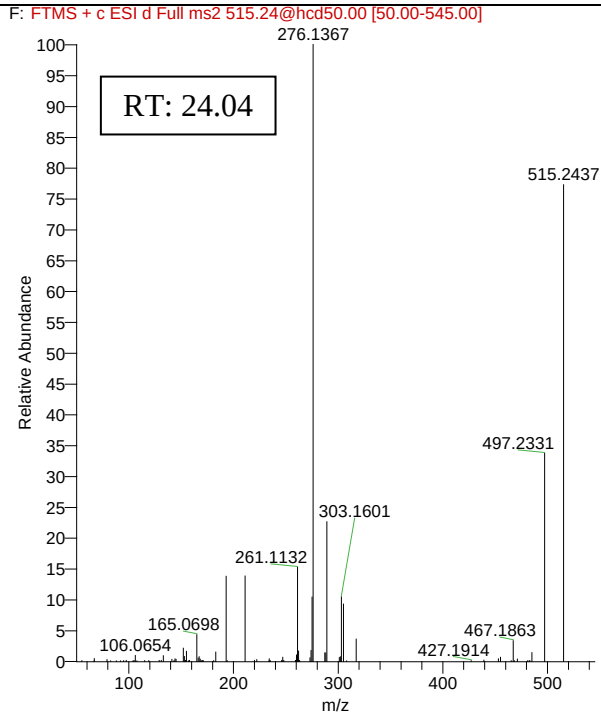
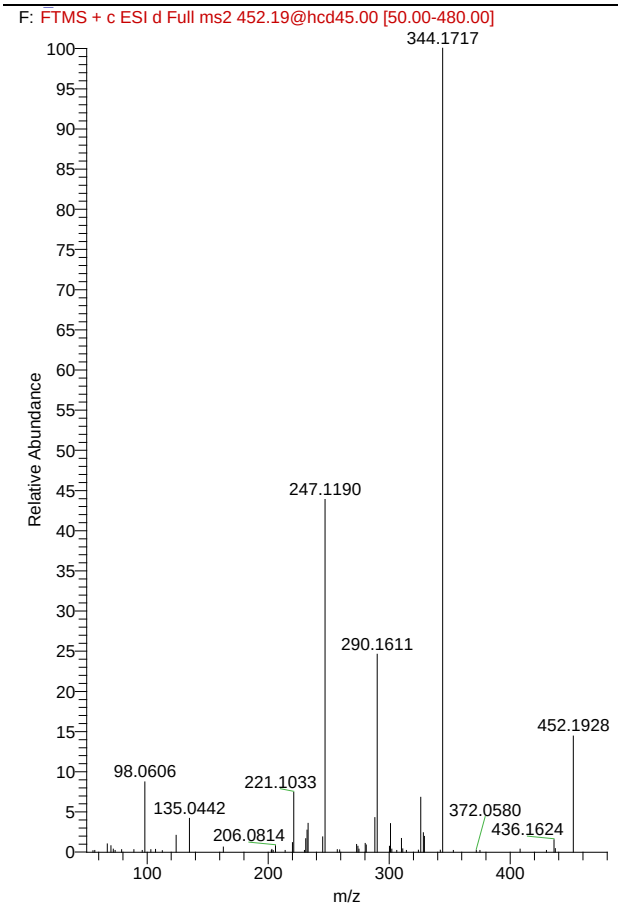


Doxazosine Analyte [M+H]+: 452.1927

Analyte



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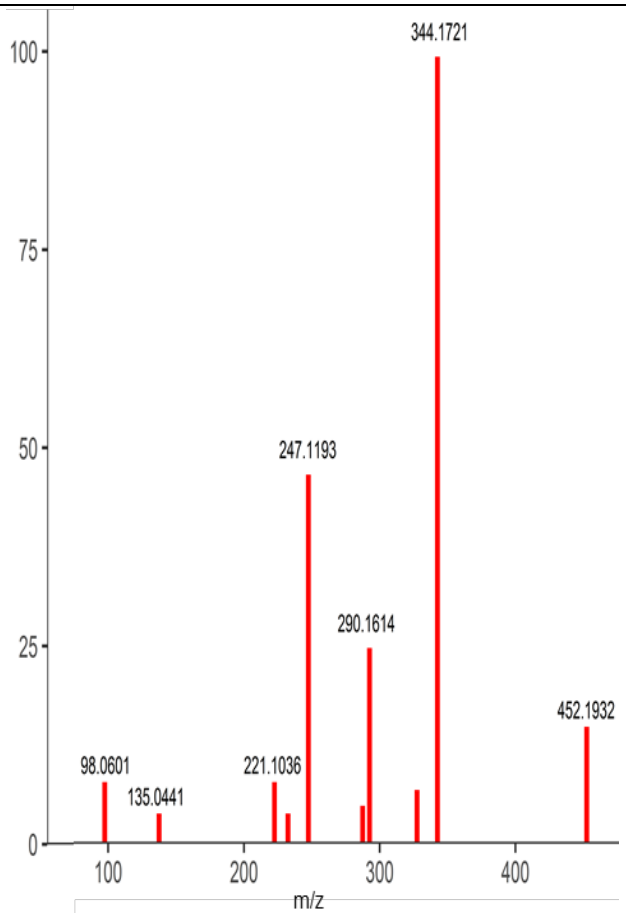

Table S1. Detailed summary of unidentified compounds

Detected Protonated mass [M+H]+ (*mass accuracy ppm*)	**Neutral Formula**	**Detection Frequency (# of samples)**	**Candidates with a MetFrag score > 0.7 (ref#>10)**	**# of Candidates complying with pH-dependent LC retention (Ref >10)**	**# of Candidates complying with HDX (Ref >10)**	**# of Candidates complying with both methods (Ref >10)**	**Confidence Level**	**Rank / Score (Ref#) of tested reference standard**	**Notes on the tested reference standard**
109.0763 (+2.9)	$C_6H_8N_2$	6	104 (29)	43 (17)	26 (14)	19 (13)	Level 4	3 / 0.96 (207)	compound known to be produced / occurred at the site
124.0870 (+0.9)	$C_6H_9N_3$	6	165 (65)	53 (48)	31 (24)	19 (14)	Level 4	2 / 0.76 (118)	compound known to be produced / occurred at the site
133.0760 (-0.0)	$C_8H_8N_2$	3	100 (81)	58 (45)	35 (33)	20 (19)	Level 4	1 / 0.92 (42)	compound known to be produced / occurred at the site
136.0756 (-0.4)	$C_8H_9N_O$	5	67 (15)	27 (5)	53 (15)	24 (5)	Level 4	1 / 0.84 (153)	highest ranked candidate fitting the experimental data
140.1181 (-1.2)	$C_7H_{13}N_3$	5	244 (54)	70 (51)	61 (44)	56(41)	Level 4	1 / 0.70 (134)	compound known to be produced / occurred at the site
151.0324 (-0.6)	$C_7H_6N_2S$	6	78 (27)	44 (18)	41 (17)	30 (15)	Level 4	-	none of the remaining candidate structures were aromatic amines
160.0869 (-0.5)	$C_9H_9N_3$	6	224 (55)	102 (37)	103 (24)	60 (18)	Level 4	1 / 0.78 (136)	compound known to be produced / occurred at the site

Table S1*cont`d*

170.0711 (-0.8)	$C_{10}H_7N_3$	6	41 (10)	4 (3)	13 (5)	3 (3)	Level 4	-	standards not available
174.1025(-0.4)	$C_{10}H_{11}N_3$	6	653 (90)	257 (49)	242 (49)	104 (30)	Level 4	1 / 0.82 (162)	compound known to be produced / occurred at the site
180.1018 (-0.5)	$C_{10}H_{13}NO_2$	5	261 (195)	73 (52)	72 (63)	10 (6)	Level 4	1/ 0.78 (90)	highest ranked candidate fitting the experimental data
195.0586 (-0.3)	$C_9H_{11}N_2OS$	5	71 (48)	35 (24)	22 (14)	15 (10)	Level 4	-	none of the remaining candidate structures were aromatic amines
200.0810 (-0.5)	$C_{11}H_9N_3O$	6	159 (22)	47 (15)	63 (10)	16 (1)	Level 3	-	only one candidate fitting the experimental data (***1-Methoxy-9H-2,4,9-triaza-fluorene***) rank: 1 / 0.71(17) but standard not available
200.1070 (-0.2)	$C_{13}H_{13}NO$	5	188 (44)	-	25 (22)	-	Level 4	1 / 0.95 (148)	highest ranked candidate fitting the experimental data
225.0690 (-1.1)	$C_{10}H_{12}N_2O_2S$	6	487 (35)	108(0)	71(14)	7 (0)	Level 4	-	no candidate fitting the experimental data with a reference number > 10

Table S1*cont`d*

225.1132 (-1.1)	$C_{13}H_{12}N_4$	6	435 (16)	292 (11)	6 (1)	3 (1)	Level 3	-	only one candidate fitting the experimental data (***8-Methylbenzo[f]quinazoline-1,3-diamine***) rank: 1/ 0.76 (12) but standard not available
240.1129 (-0.9)	$C_{14}H_{13}N_3O$	6	349 (22)	117 (7)	61 (3)	49 (1)	Level 3	-	only one candidate fitting the experimental data (***2-(6-Methyl-2-pyridinyl)-2,3-dihydro-4(1H)-quinazolinone***) rank: 1/ 0.73 (16) but standard not available
252.1088 (-1.4)	$C_{10}H_{13}N_5O_3$	6	98 (14)	88 (14)	64 (12)	56 (12)	Level 4	-	-
300.1009 (-0.6)	$C_{15}H_{14}N_5Cl$	6	449 (4)	188 (3)	21 (1)	2 (0)*	Level 4	-	standards not available
334.0617 (-0.9)	$C_{15}H_{13}N_5Cl_2$	6	37 (3)	18 (2)	1 (0)	1 (0)*	Level 3	-	only one candidate fitting the experimental data(***ChemSpider ID: 415984***) rank: 1/ 0.66 (2) but standard not available

Table S1*cont`d*

335.2298 (-1.2)	$C_{15}H_{26}N_8O$	5	10 (2)	10 (2)	3 (1)	3 (1)	Level 3	-	only one candidate fitting the experimental data (***ChemSpider ID: 2350570***) rank: 1 / 1.00 (17) but standard not available
336.2137 (-1.6)	$C_{15}H_{25}N_7O_2$	6	40 (10)	36 (10)	11 (1)	10 (1)	Level 3	-	only one candidate fitting the experimental data (***ChemSpider ID: 466615***) rank: 1 / 0.92 (29) but standard not available
363.2246 (-1.4)	$C_{16}H_{26}N_8O_2$	6	15 (0)	12 (0)	4 (0)	3(0)	Level 4	-	standards not available

*no candidates fitting the experimental data with number of references higher than 10

S2.2 Mutagenicity Evaluation

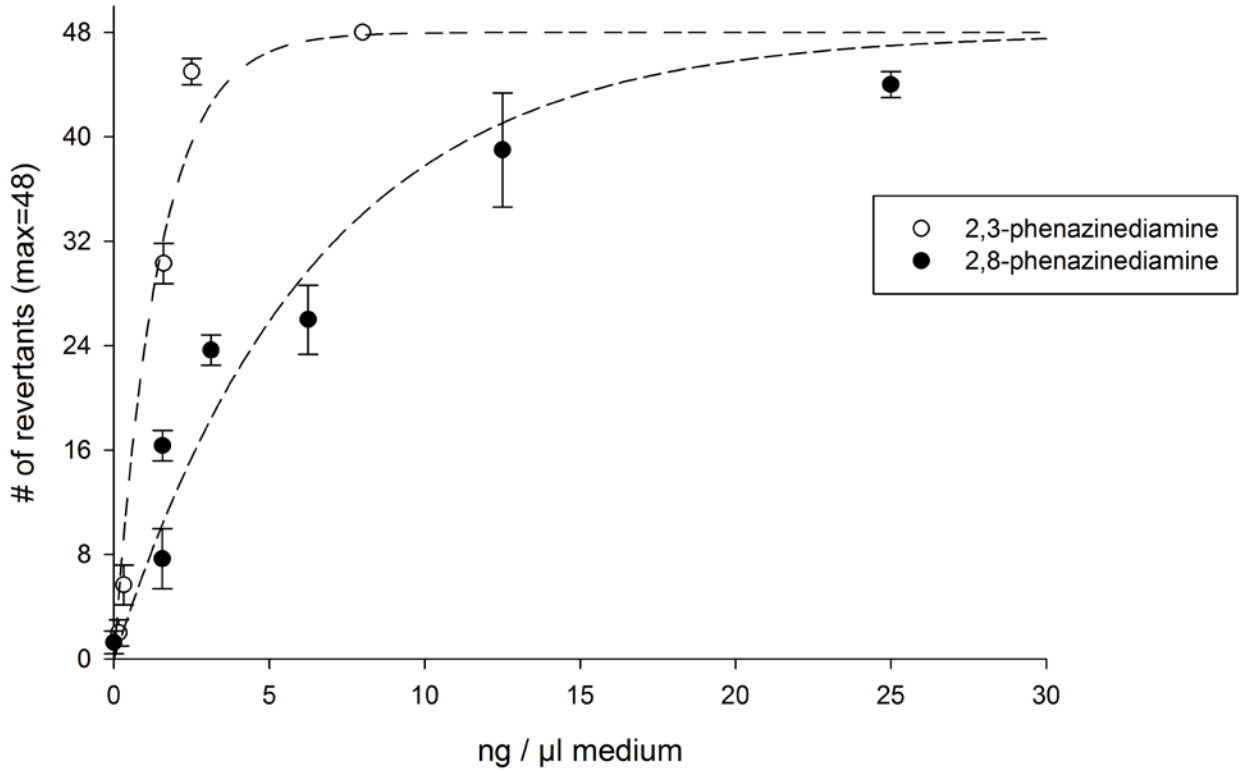

Figure S1. Concentration-response plots of diaminophenazines with fitted curves of the exponential equation

Table S2. Mutagenic activity of BR samples and diaminophenazines isomers detected in each BR sample calculated by equation S1 and expressed by the calculated slopes (a*b) as revertants / mg BR eq.

Sample	Mutagenic activity of BR samples # of rev. / mg BR eq.	Mutagenic activity of 2,3-phenazinediamine # of rev. / mg BR eq.	Mutagenic activity of 2,8-phenazinediamine # of rev. / mg BR eq.
BR1	23.8 (±2.0)	-	20.4 (±2.9)
BR2	47.4 (±1.8)	-	13.3 (±1.9)
BR3	9.4 (±1.4)	35.6 (±4.5)	2.4 (±0.3)
BR4	53.2 (±4.0)	6.7 (±0.8)	18.6 (±0.8)
BR5	116.8 (±6.2)	3.3 (±0.4)	2.4 (±0.3)
BR6	68.4 (±9.6)	1.7 (±0.2)	1.2 (±0.2)

S3. REFERENCES

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