Simvastatin: *In-Vitro* Metabolic Profiling of a Potent Competitive HMG-CoA Reductase Inhibitor

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Table S1: Concentration of Protein (Calibration curve of BSA)

Standard	Concentration µg/ml	Readings
1	20	0.0175
2	40	0.0365
3	60	0.0550
4	80	0.0725
5	100	0.1030
Microsomes suspension (1µl)	34.2	0.029

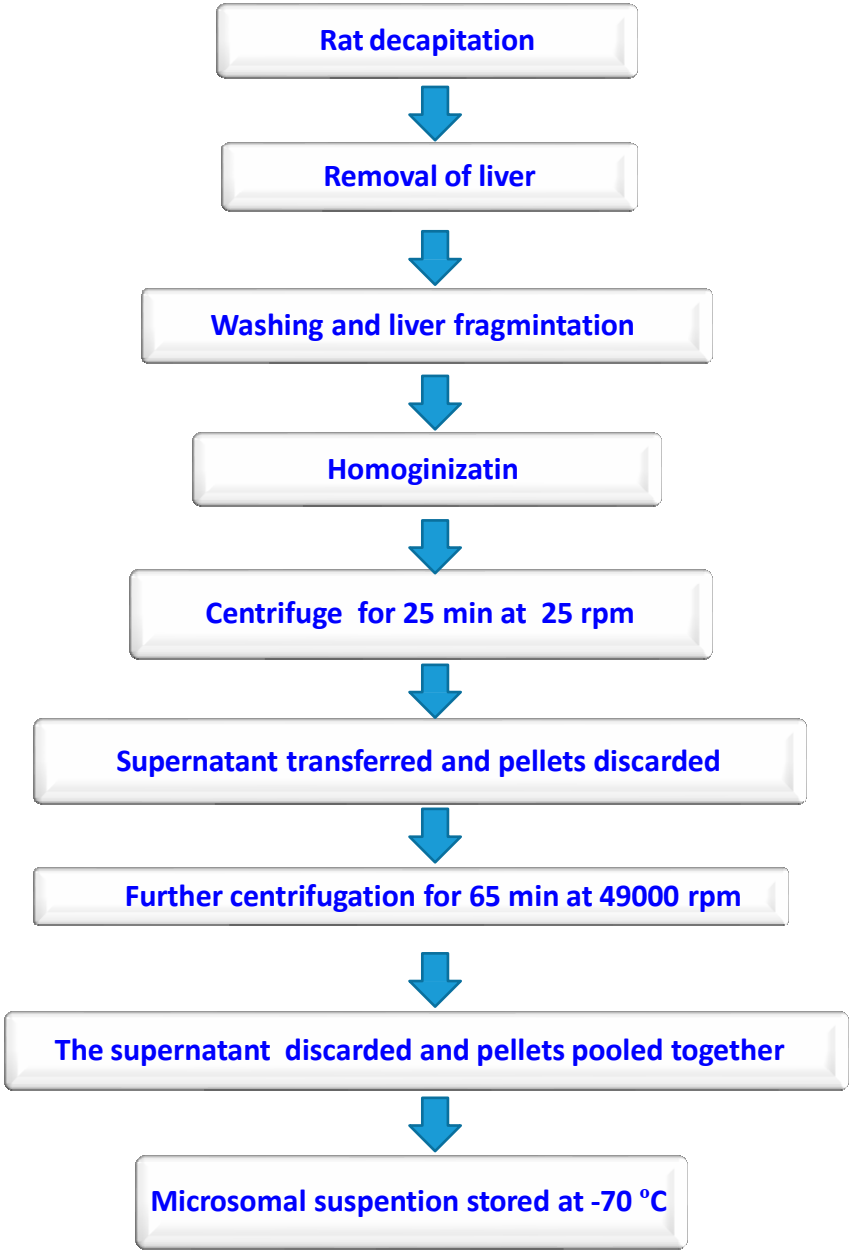


Figure S1. Schematic representation of rat liver microsomes preparation step

Table S2: RLMs Incubations

Incubation	Microsomes	Simvastatin	NADPH	MgCl2	Buffer
Test1	80µl	1µl	100µl	100µl	719µl
Test2	80µl	1µl	100µl	100µl	719µl
Control 1	80µl	0µl	100µl	100µl	720µl
Control 2	80µl	1µl	0µl	100µl	819µl
Control 3	0µl	1µl	100µl	100µl	799µl

RLMs Incubation procedure

The various components of the incubation reaction were added following a specific order as the following:

1. Appropriate volume of ice-cold phosphate buffer solution (0.08M KH_2PO_4/NaH_2PO_4, pH 7.4) was prepared.
2. Appropriate volume of an ice cold was freshly prepared $MgCl_2$ solution (20.33 mg/ml).
3. 4µl (20µm) of a 100mM stock solution of the Simvastatin was added to incubation tubes.
4. An appropriate volume of the RLMs suspension was added (50µl of microsome suspension to give the required concentration in the incubation mixture which is 2 mg/ml).
5. Test tubes containing the incubation mixtures were transferred to a shaking water bath and allowed to stand at a temperature of 37 °C in the water bath for 5 minutes (pre incubation).
6. 1mM of NADPH (8.33 mg/ml) was added after the incubation mixtures have reached 37 °C.
7. The incubation mixture was allowed to run for 90 minutes.
8. The reaction was terminated by the addition of 2 ml of ice cold acetonitrile.
9. The mixture was centrifuged for 10 minutes at 14000 rpm and the supernatant was transferred to a fresh container and was subjected to evaporation under a stream of nitrogen.
10. The residue will be reconstituted in of the mobile phase and transferred to HPLC vials for analysis.

Table S3: Hepatocytes incubations

Incubation type	Hepatocytes suspension	SV	ACN used for solubilization of Simvastatin
Test	2ml	2µl	0µl
Control 1	2ml	0µl	2 µl
Control 2	0ml	2µl	2ml

The components of the incubation reaction were added on a specific order as the following:

a- Two ml of the preincubated hepatocytes suspension was add to the appropriate container at room temperature except for control 2.

b- Two µl of a 100mM stock solution of SV was added to tubes except that of control.

c- To tubes containing control 1, add 2 µl of ACN solvent.

4- The vials were incubated at 37°C for 2 hr in a shaking water bath.

5- By adding 2 ml of ice-cold acetonitrile, the reaction was stopped.

6- Vials were centrifuged at 14,000 rpm for 10 min. then the supernatant was discarded.

7- The supernatant was evaporated under stream of Nitrogen (N_2).

8- The residue was reconstituted in 1ml H_2O/ACN (50:50) of the mobile phase and transferred to HPLC vials for analysis.

Isolated Perfused Rat Liver Hepatocytes

For the preparation of the isolated perfused rat hepatocytes, enzymatic method was used, in which collagenase perfusion is applied as the principle of disintegration of the liver [1]. The collagenase treatment digests the architecture of the liver and allows the hepatocytes to be released. A buffer medium containing collagenase enzyme was circulated continuously through the liver via the portal vein in order to achieve maximum exposure of intracellular spaces to collagenase. A reservoir containing the perfusion medium was held at 37 °C in a water bath cabinet. The perfusion medium comprised calcium free Hank's solution[72] and gassed with a mixture comprising 95% O_2 and 5% CO_2. The solutions were added in the order above into 2 liter brown bottle and bubbled with 5% CO_2/ 95% O_2 for 10 minutes. Meanwhile 9.71g $NaHCO_3$ was taken, dissolved in 1000 ml distilled H_2O and bubbled with 5% CO_2/ 95% O_2 for 10 minutes. Then the $NaHCO_3$ solution was added to the salt solution. The solutions for perfusion of the rat liver and preparation of the cell suspension were stored at 4 °C after sterile filtration with a filter/storage system.

[1] Berry, M. N.; Friend, D. S. High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *J Cell Biol.* **1969**, *43*, 506-520

Perfusion of Rat Liver Hepatocytes Protocol

Rat (Sprague dawley) was anaesthetized with Urethane at dose (1g/Kg) [2]. The abdomen was opened by a long midline incision and two perpendicular cuts at the level of the kidneys. The guts are moved to the left, out of the abdominal cavity, giving free access to the portal vein and the inferior vena cava (IVC). Prior to cannulation, the thread was placed loosely around the v. portal, and around the v. cava inferior above the right kidney.

Then the perfusion is started with Hank 1 buffer, and the IVC was opened below the right kidney to allow drainage of perfusion. The chest cavity was cut widely open, the v. cava superior. Cannulated through the left atrium, and the thread around v. cava (inf). In the abdominal cavity is tightened. Heparin 0.1 ml (1000 units /ml) was injected into the inferior vena cava to prevent blood clotting inside blood vessels. All solutions were bubbled with 5% CO_2/95% O_2 and heated to 37 °C during the perfusion period. The perfusion pump containing plain was turned on with a flow rate 10-15ml/min so that the tubing or cannula can be inserted into the portal vein. The perfusate was switched to Hank I buffer for 10 min. without recirculation. The total perfusate volume at this time amounts to approximately 150 ml. The liver was then perfusate with Hank II buffer for 15-30 min. that contained 2 mM Ca^{2+} and 0.02% (w/v) collagenase type 1V from Sigma equivalent to 92 units/ml of collagen digestion activity.

[2]. Fish, R. E. M. R. E. *Anesthesia and Analgesia in Laboratory Animals*. Academic Press, London: **1997**

Perfusion of Rat Liver Hepatocytes Protocol

The collagenase perfusion usually continued for 15-25 min depending upon the appearance of the liver. When the liver was fully digested, and showed signs of disintegration when touched with forceps, the perfusion was halted immediately by stopping the pump then the liver was cut out and placed in the 150ml petri dish and the perfusion tube was detached. The liver was spread using two forceps into 100 ml Krebs-Albumin Buffer that contained 1% (w/v) BSA., followed by filtering of the dispersed liver through a sterile cotton gauze mesh to remove remaining connective tissue and clumps of cells then the filtrate was collected in a 50 ml conical centrifuge tube and the cells allowed settling down by gravity.

The final volume was 15 ml (2ml will be used in the next step for each incubated sample). Finally, the cells were counted and viability of cells was determined by using haemocytometer [3].

[3]. Strober, W. Trypan blue exclusion test of cell viability. *Curr. Protoc. Immunol.* **2001**, *3,* A.3B.1-A.3B.2

Table S4: Gradient solvent system used in HPLC separation of microsomal incubations

Time in min.	0	5	10	15	20	25	30
% Solvent A	70	70	50	30	50	70	70
% Solvent B	30	30	50	70	50	30	30

Table S5: Optimized MS parameters for SV

Agilent Ion Trap 6320	**Drying Gas (N2) Temp. & Flow**	**Mode**	**Nebulizer Pressure**	**Scan range (Da)**	**Smart target**	**Max Accumulation time**
	325 °C 10 Liter/min	Positive/ Negative	50 psi	100-1000	10,000.	300000 µs
Agilent Triple Quad 6410	Gas Temp. & Flow	Mode	Nebulizer Pressure	Scan range	Frag (V)	Capillary Volt
	350 °C 12 Liter/min	Positive/ Negative	55	20-1000	135	4000

Buffers used for the preparation of RLMs

Homogenization buffer	**0.1M phosphate buffer pH~7.4 15% KCl (10mM EDTA optional)**
Wash buffer	0.1M phosphate buffer pH~7.4 15% KCl (1mM EDTA optional)
Storage buffer	0.1M phosphate buffer pH~7.4 20% glycerol (0.1mM EDTA optional)

Solutions of Lowry method

Analytical reagents	Description
Reagent A	1 g of Cupric Sulphate ($CuSO4.H_2O$) was dissolved in 100 ml distilled water and stored at 4ºC.
Reagent B	4 g of Potassium Sodium Tartarate ($KNaC_4H_4O_6.4H_2O$) were dissolved in 100 ml of distilled water and stored at 4ºC.
Reagent C	4 g Sodium Hydroxide (NaOH) and 30g anhydrous Sodium Carbonate (Na_2CO_3) were dissolved in 1 L of distilled water and stored at 4ºC.

Lowry assay protocol was done according to the following procedures [4]

1. Bovine serum albumin (BSA) was dissolved to obtain a concentration of 5 mg/10 ml in distilled water. From this stock solution 0, 200, 400, 600, 800, and 1000 µl of the BSA standard were added to clean test tubes.
2. Completed to 1ml with distilled water to get a 0, 100, 200, 300, 400, and 500 µg/ml of the standard curve.
3. Two hundred fifty µl from each tube were transferred into a clean test tube, in duplicates.
4. Ten, five and one µl from microsomal suspension were transferred into clean test tubes and completed with distilled water to 250 µl.
5. The standards and samples were made up to a final volume of 0.25 ml with distilled water.
6. One fourth ml of freshly prepared dye reagent (1 ml A + 1 ml B + 20 ml C) was added to each of the samples and the standard tubes vortex gently.
7. Samples and the standard tubes were incubated for 10 min. at room temperature.
8. Three-quarters of ml of freshly diluted 1:10 Folin-phenol reagent was added to each sample and vortex gently during adding.
9. Samples were allowed to stand for 10 min at 50 °C
10. The absorbance of each sample was determined at 660 nm against a reagent blank containing all of the assay components except protein (i.e. Identical to 0 µg standard).

[4]. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J Biol. Chem.* **1951**, *193*, 265-275.

10 mM Tris-HCl buffer pH 7.4:

1. For a 1 M solution, 12.1 g of Tris base was dissolved in 80 mL of deionized water.
2. The pH was adjusted to 7.4 value by slowly adding approximately 6-7 mL concentrated HCl [Adding concentrated HCl to the Tris buffer will increase the temperature of the solution, which affects the pH. Allow the solution to cool to room temperature before making final adjustments to the pH (using more HCl if necessary)].
3. The volume of the solution was adjusted to 100 mL with deionized water.
4. 0.22 μm sterile filter was used to filter Tris-HCl.
5. To obtain a 10 mM Tris-HCl pH 7.4 solution, 1M Tris-HCl pH 7.4 was diluted in 1:100 with deionized water [for example, add 1mL of 1M Tris-HCl pH 7.4 to 99 mL of deionized water].

Preparation of RLMs:

Differential Centrifugation Method

The Rat Liver Microsome (RLMs) were prepared following a previously reported work [5]. In brief, homogenizing the tissue, mostly liver, in a more or less defined and reproducible manner. Buffers, tools and centrifuge rotors were kept cold for the time of the preparation procedure, in order to preserve enzymes activity. Harvested liver was removed and weighed, then tissue was rinsed thoroughly with ice cold buffer at pH 7.4. The liver was cut into small pieces and then homogenized with mechanical homogenizer. The homogenate tissue was centrifuged at differential centrifugation forces, started with a low centrifugal force of about 9000×g in order to remove the undisrupted cells, nuclei, and mitochondrial. The resulting supernatant is then centrifuged at a higher force to precipitate the microsomes. The microsomal pellet is resuspended in a final suspension buffer and is then ready for use in incubation studies. CYP450 activity was verified via testing the ability of the prepared microsomes to transform phenytoin to *p*-hydroxyphenytoin. In order to increase the purity or enzyme activity of the final preparation, there is a number of additions to the preparation buffers were used. For example, the addition of EDTA in decreasing concentration to the preparation buffers (10 mM in homogenization buffer, 1mM in wash buffer and 0.1mM in final buffer) was thought to stabilize flavin monooxygenase (FMO) activity.[64] The addition of potassium chloride (1.15 % w/v) to the homogenization buffer further purifies the preparation by the removal of blood and cytoplasmic contaminants. Glycerol was added to the final storage buffer to preserve enzyme activity in storage Sprague Dawley rats (weight: 400 gm) were obtained from the animal facility in the College of Pharmacy. Table 16 shows the composition of buffers used for the preparation of liver RLMs.

[5]. Mountney, A.; Boutte, A. M.; Gilsdorf, J.; Lu, X. C.; Tortella, F. C.; Shear, D. A. Intravenous Administration of Simvastatin Improves Cognitive Outcome following Severe Traumatic Brain Injury in Rats. *J Neurotrauma.* **2016**, *33*, 1492-1500

Preparation of RLMs:

Calcium Aggregation Method [6]:

The method of Schenkman and Cinti with minor modifications, was used for isolation of microsomes. Briefly, excised livers (20 g) were thawed, finely chopped with a pair of scissors, and homogenized with four volumes (80 ml) of ice cold 10 mM Tris-HCl buffer containing 0.25 M sucrose, pH 7.4, in a Potter glass homogenizer equipped with a Teflon pestle. The homogenate was centrifuged at 13,000 × g for 10 min at 4°C in a refrigerated centrifuge (Biofuge Stratos), and the precipitate was discarded. To the supernatant, calcium chloride was added to yield a final concentration of 10 mM. The solution was stirred for 15-20 min, and then centrifuged at 25,000 × g for 10 min at 4°C. The firmly packed pellets of microsomes were re-suspended by homogenization in 100 mM Tris-HCl buffer containing 20% w/v glycerol and 10 mM EDTA, pH 7.4. The microsomes were stored at –70°C until use.

[6]. Lyer, P. S. W. P. S. S. K. Isolation and catalytic competence of different animal liver microsomal fractions prepared by calcium-aggregation method. *Indian J Pharm. Sci.* **2006**, *2*, 262-265

Preparation of Perfusion Solution.

The composition of Hanks Buffer (10 X)

A. **Hanks Buffer** (10 X).
The salts listed in Table S-7 were dissolved in 1000 ml distilled H_2O and stored at 4 °C.

NaCl	80.0 g
KCl	4.0 g
$MgSO_4.7H_2O$	2.0 g
$Na_2HPO_4.2H_2O$	0.6 g
KH_2PO_4	0.6 g

The composition of Krebs-Henseleit Buffer (2 X)

B. **Krebs-Henseleit Buffer** (2 X)
The following stocks stated in Table 20 were all stored at 4°C.

Solutions	VOLUME
Distilled H_2O	785 ml
16.09% NaCl	200 ml
1.10% KCl	150 ml
0.22 M KH_2PO_4	25 ml
2.74% $MgSO_4.7H_2O$	50 ml
0.09 M $CaCl_2.2H_2O$	100 ml

The composition of Hank1 buffer

C. **Hank I (500 ml).**

Compound	QUANTITY
$NaHCO_3$	1.05 g
Hepes	1.50 g
BSA (fraction V Cat.No.9418, Sigma Chemical Co.)	3.33 g
EGTA	114 mg
Distilled H_2O	450 ml
Hank's Buffer (10 X)	50 ml

The composition of Hank II buffer

D. **Hank II** (500 ml).
The pH was adjusted with 5M NaOH to 7.4.

$NaHCO_3$	1.05 g
Hepes	1.50 g
$CaCl_2.2H_2O$	147 mg
Distilled H_2O	450 ml
Hank's Buffer (10 X)	50 ml

The composition of Krebs-Hepes buffer

E. **Krebs-Hepes Buffer** (500 ml).
The pH was adjusted with 5M NaOH to 7.4.

Hepes	1.5 g
Distilled H_2O	250 ml
Krebs-Henseleit Buffer (2 X)	250 ml

The composition of Krebs -Albumin Buffer

F. **Krebs -Albumin Buffer** (500ml).
The pH was adjusted with 5M NaOH to 7.4.

Hepes	**1.5 g**
BSA	5 g
Distilled H_2O	250 ml
Krebs-Henseleit Buffer (2 X)	250 ml

Cell Counting Protocol

1. An aliquot of cell suspension was centrifuged 5 min at 100*g* and discard supernatant.

2. The cell pellet was resuspend in 1 ml phosphate buffer saline (PBS)

3. 1 part of 0.4% trypan blue and 1part cell suspension (dilution of cells) was mixed. Then the mixture was allowed to incubate ~3 min at room temperature. Cells counted within 3 to 5 min of mixing with trypan blue, as longer incubation periods will lead to cell death and reduced viability counts. Mixing can be performed in a well of a microtiter plate or a small plastic tube using 10 to 20 µl each of cell suspension and trypan blue.

4. A drop of the trypan blue/cell mixture was applied to a haemocytometer. Haemocytometer was placed on the stage of a binocular microscope and focus on the cells.

5. The unstained (viable) and stained (nonviable) cells was counted separately in the hemacytometer.

6. Calculate the percentage of viable cells as follows:

Viability (%) = (no. of viable cells × 100)/[(no. of viable cells) + (no. of dead cells)].

Averaging of two readings of the microsomal preparations showed the protein concentration is 34.2 mg/ml.

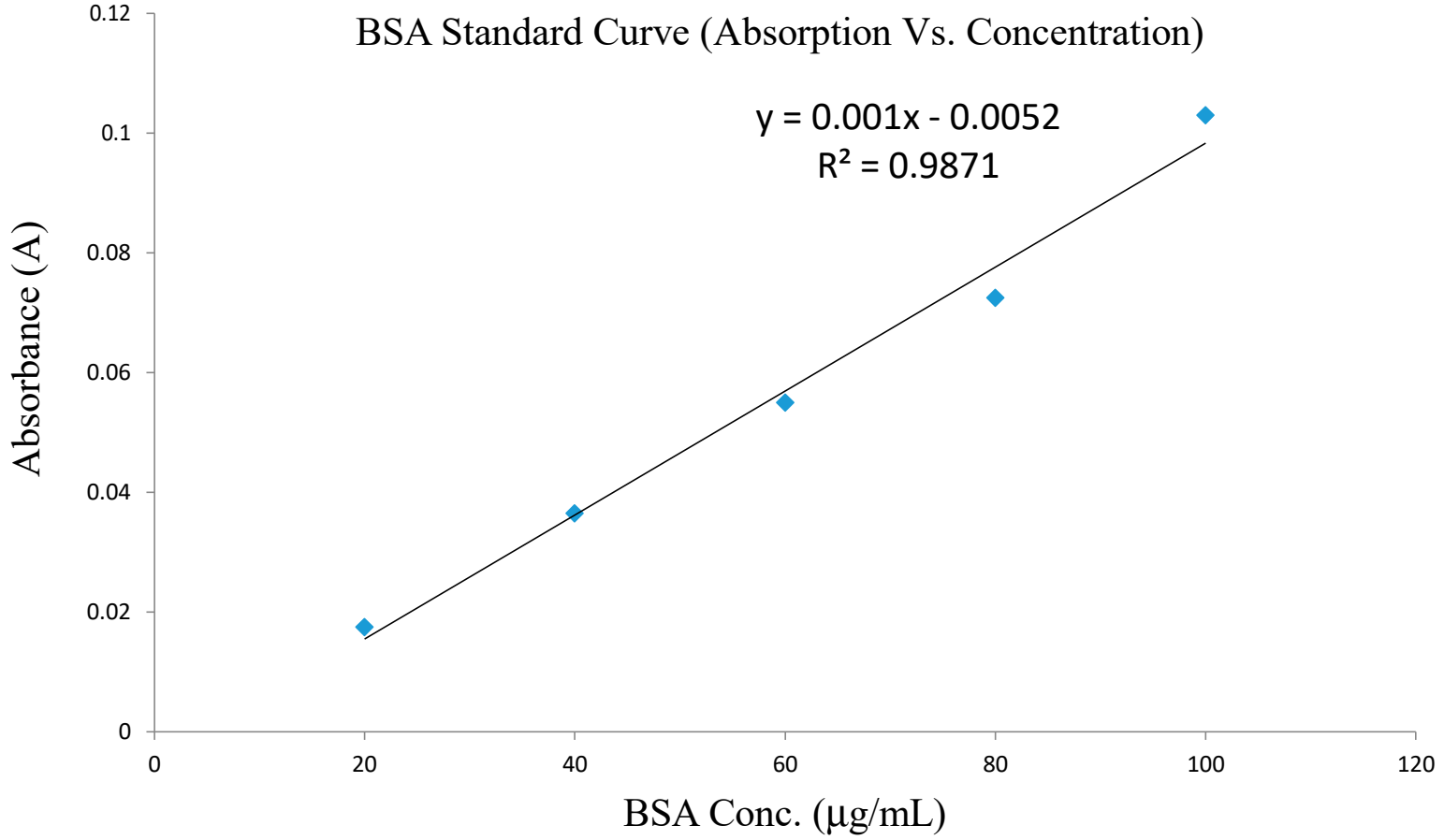


Figure S2: Calibration curve of different standard of protein concentration

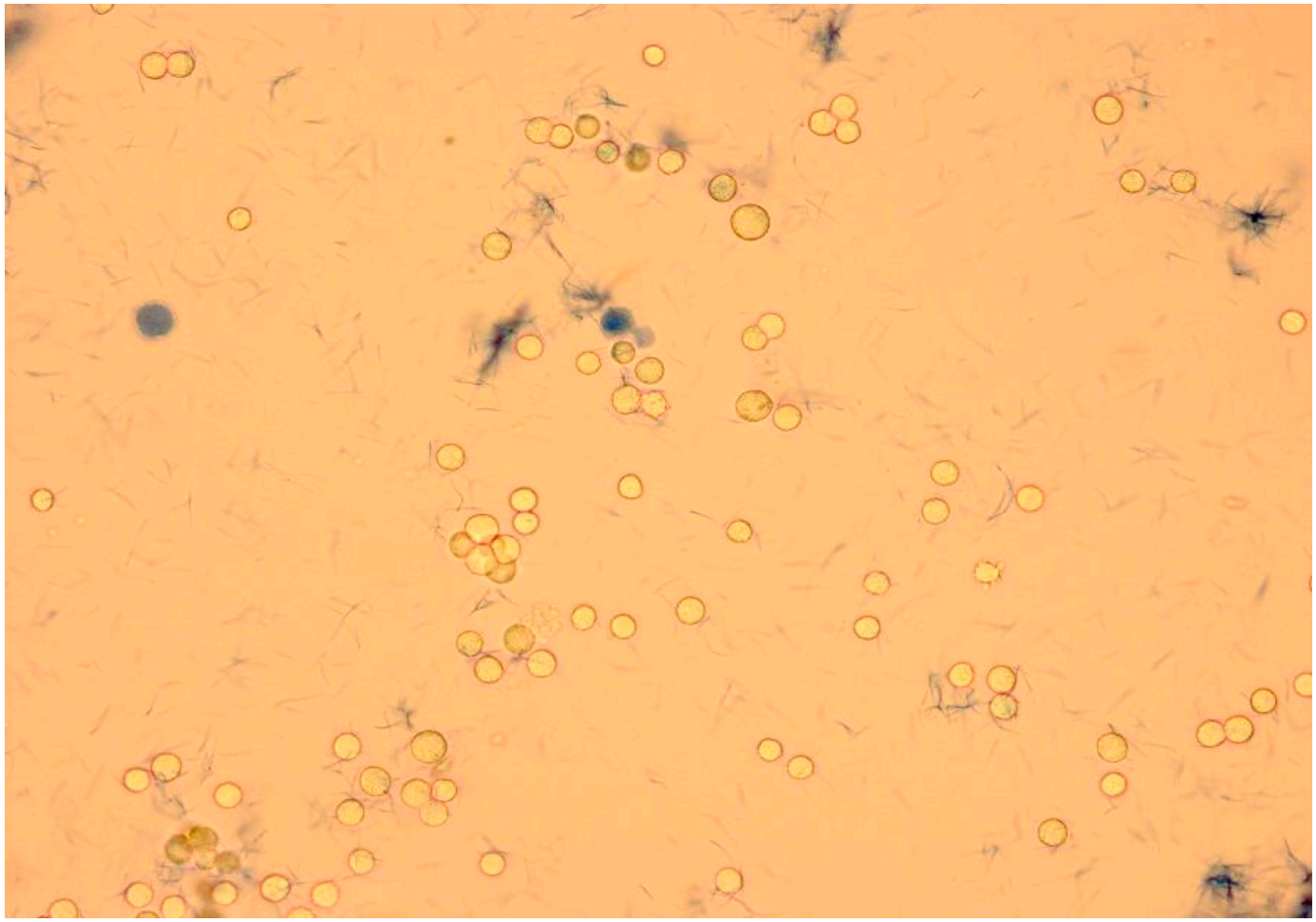


Figure S3**.** Number of cell unstained (viable) and stained (nonviable) cells

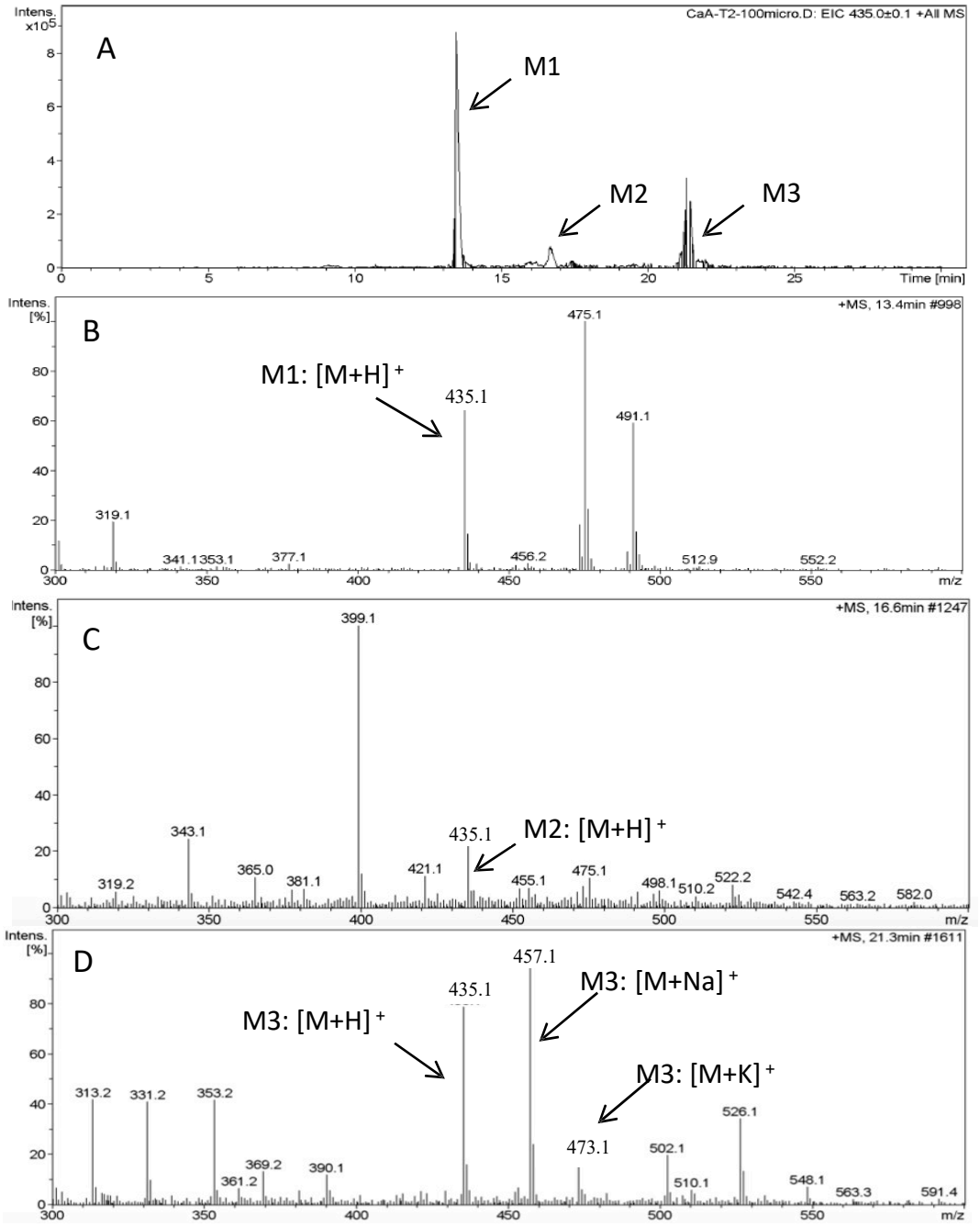


Figure S4. Extracted ion chromatogram (EIC) and mass spectra of possible mono-hydroxylated metabolites in Ion-Trap LC-MS in positive mode:

A) EIC at *m/z* 435;

B) Possible mono-hydroxylated metabolite **M1** at Rt = 13.4 min.

C) Possible mono-hydroxylated metabolite **M2** at Rt = 16.6 min.

D) Possible mono-hydroxylated metabolite **M3** at Rt = 21.3 min.

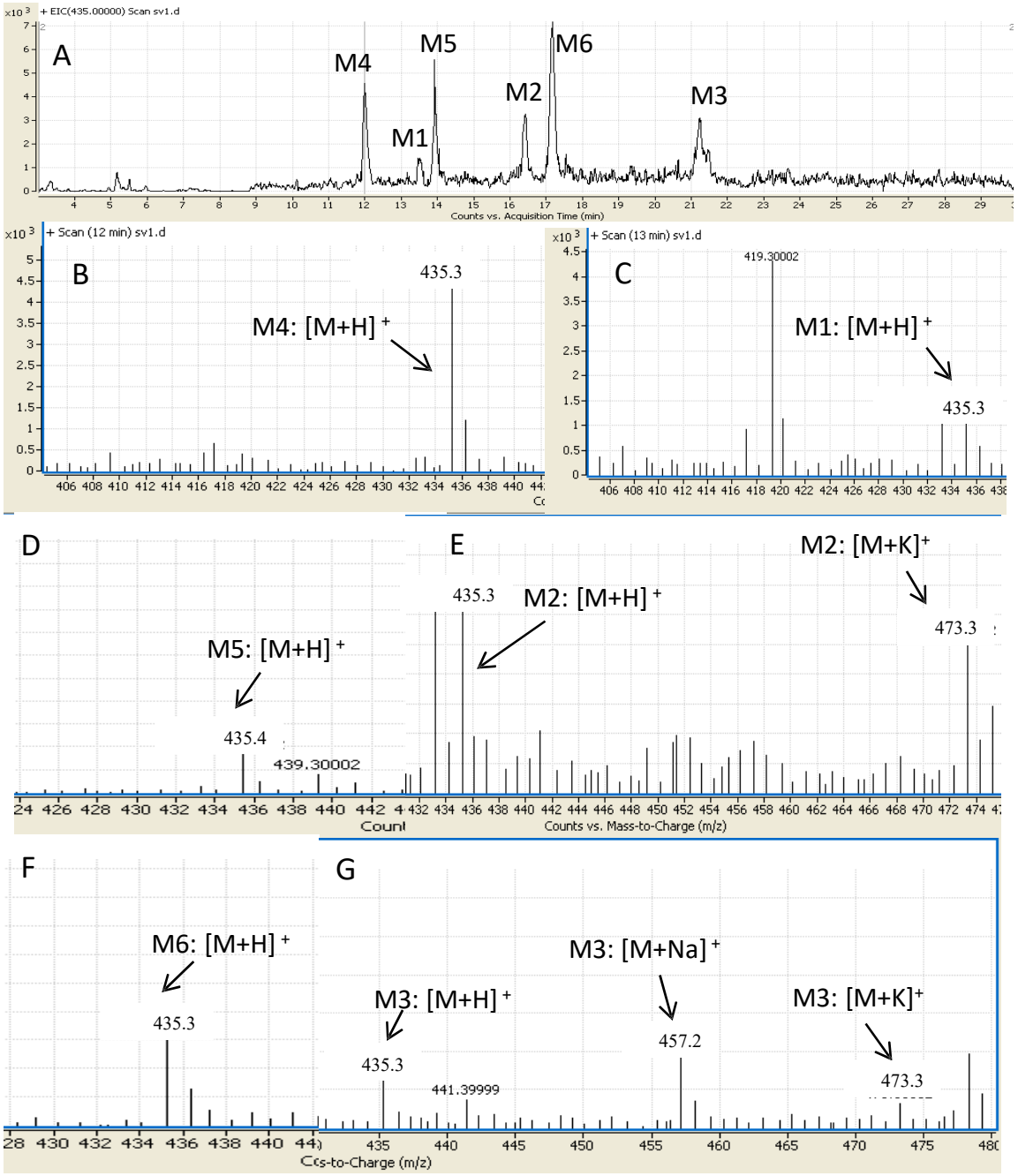


Figure S5. Extracted ion chromatogram (EIC) and mass spectra of possible monohydroxylated metabolites in QqQ LC-MS in positive mode:

A) EIC at *m/z* 435;
B) Mass spectrum of **M4**;
C) Mass spectrum of **M1**;
D) Mass spectrum of **M5**;
E) Mass spectrum of **M2**;
F) Mass spectrum of **M6**;
G) Mass spectrum of **M3**.

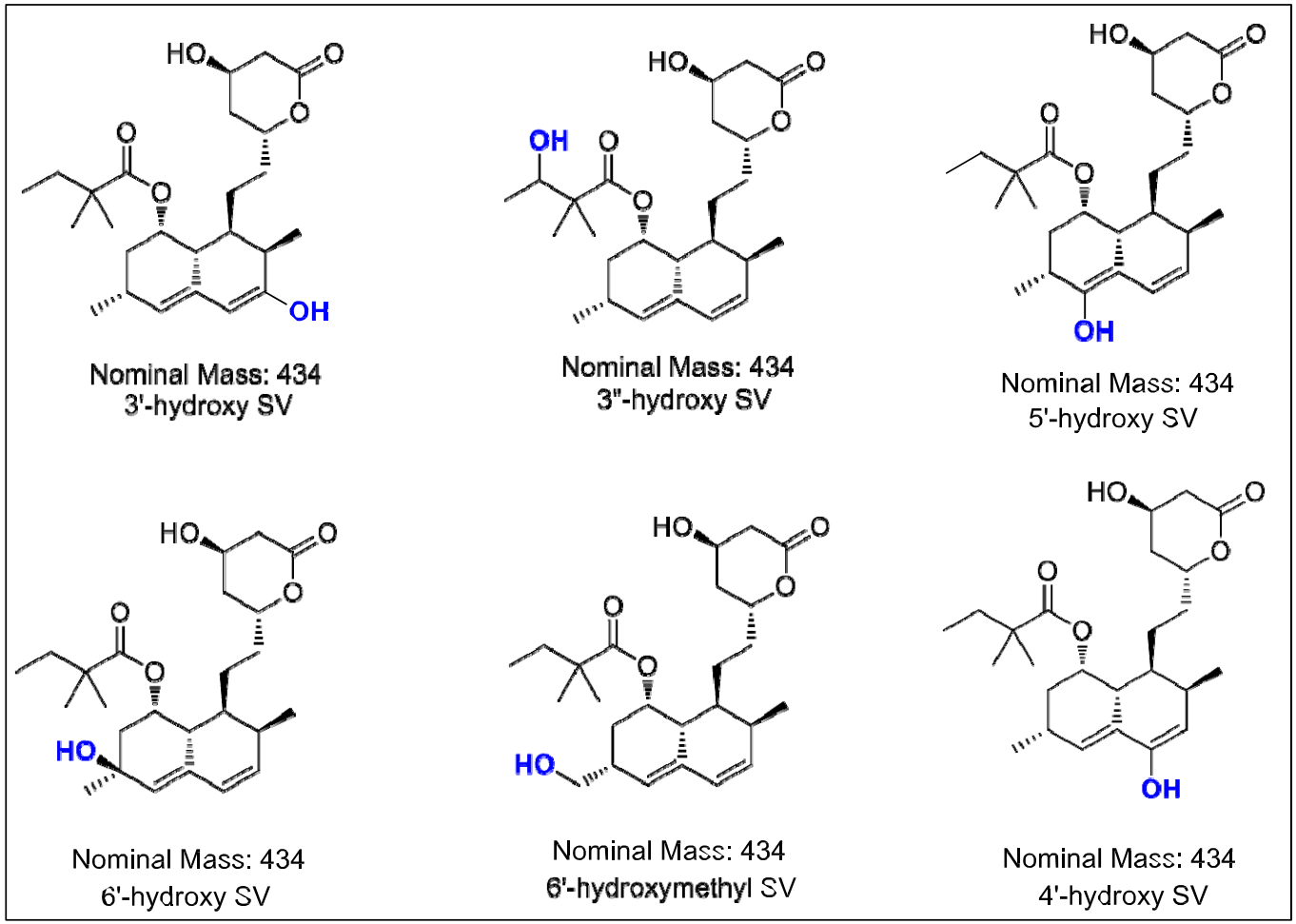


Figure S6. Structures of possible six mono-hydroxylated metabolites (**M1-M6**)

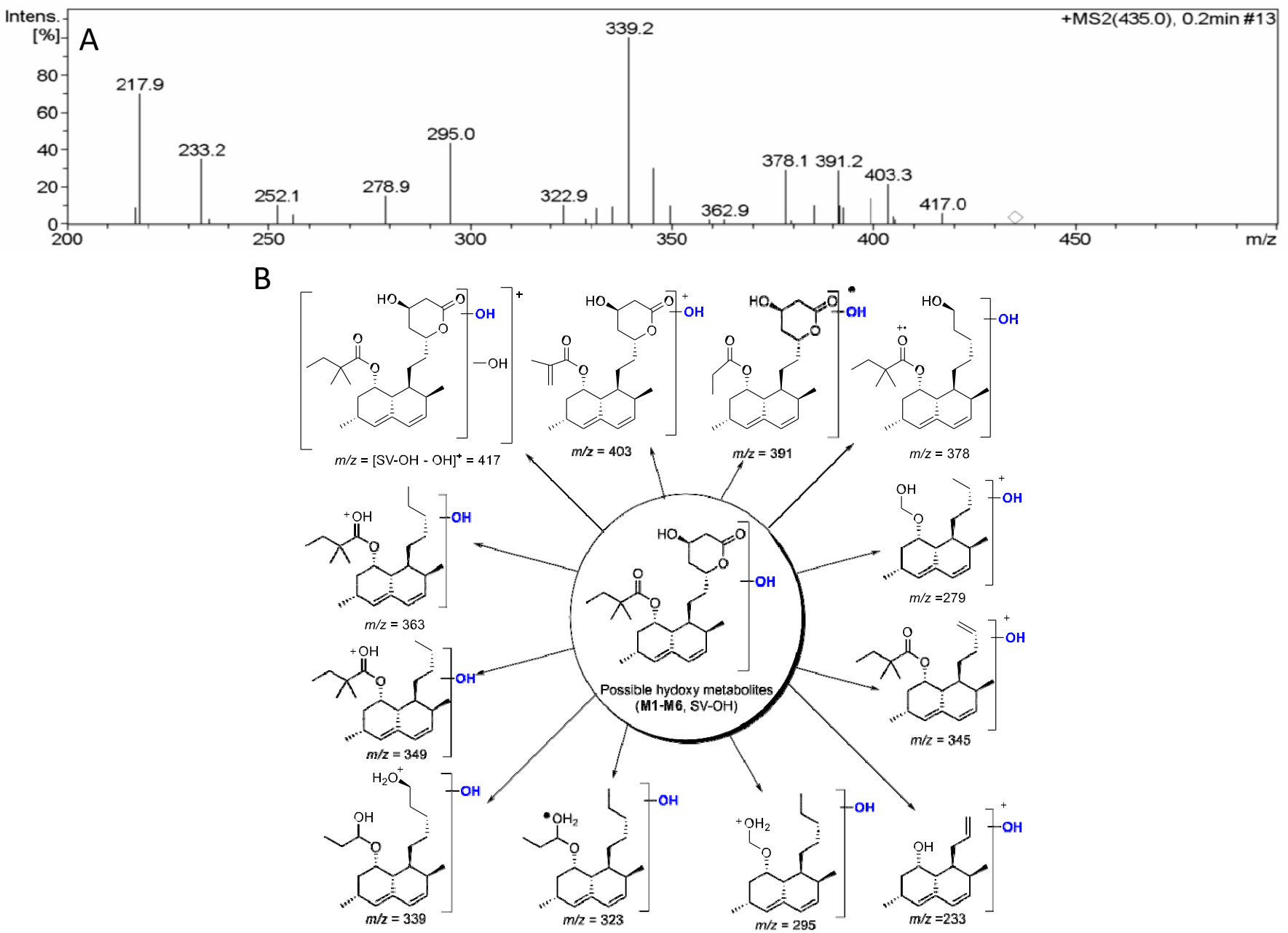


Figure S7. MS^2 spectra of **M1/M2/M3/M4/M5/M6**: (A) Fragmentation spectra of possible SV-OH at *m/z* = 435 in positive mode; and (B) Possible fragmentation pattern of possible SV-OH

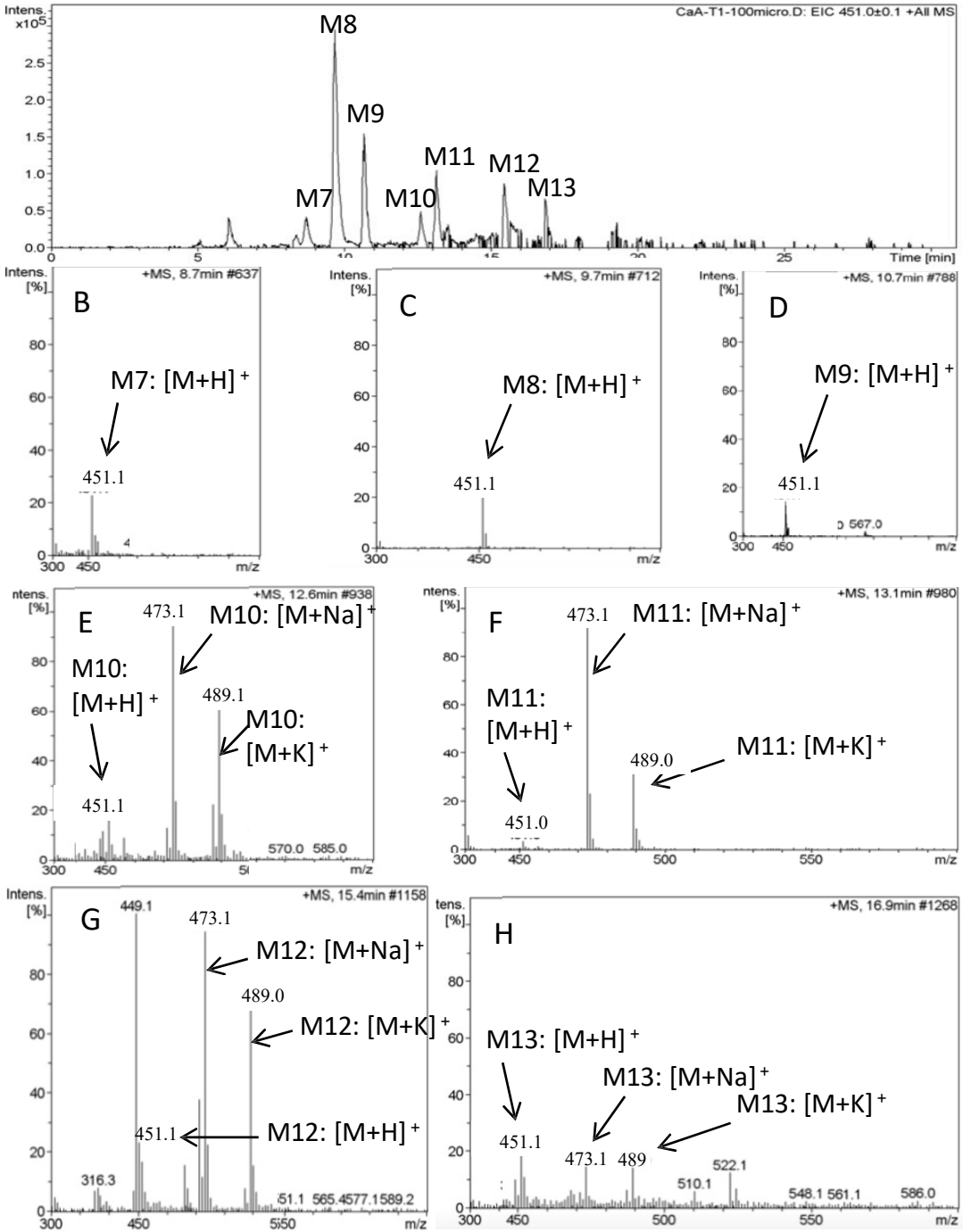


Figure S8. Extracted ion chromatogram (EIC) and mass spectra of possible dihydroxylated metabolites in Ion-Trap LC-MS in positive mode:

A) EIC at *m/z* = 451;
B) Mass spectrum of **M7** at 8.7 min.;
C) Mass spectrum of **M8** at 9.7 min.;
D) Mass spectrum of **M9** at 10.7 min.;
E) Mass spectrum of **M10** at 12.6 min.;
F) Mass spectrum of **M11** at 13.1 min.;
G) Mass spectrum of **M12** at 15.4 min.;
H) Mass spectrum of **M13** at 16.9 min.

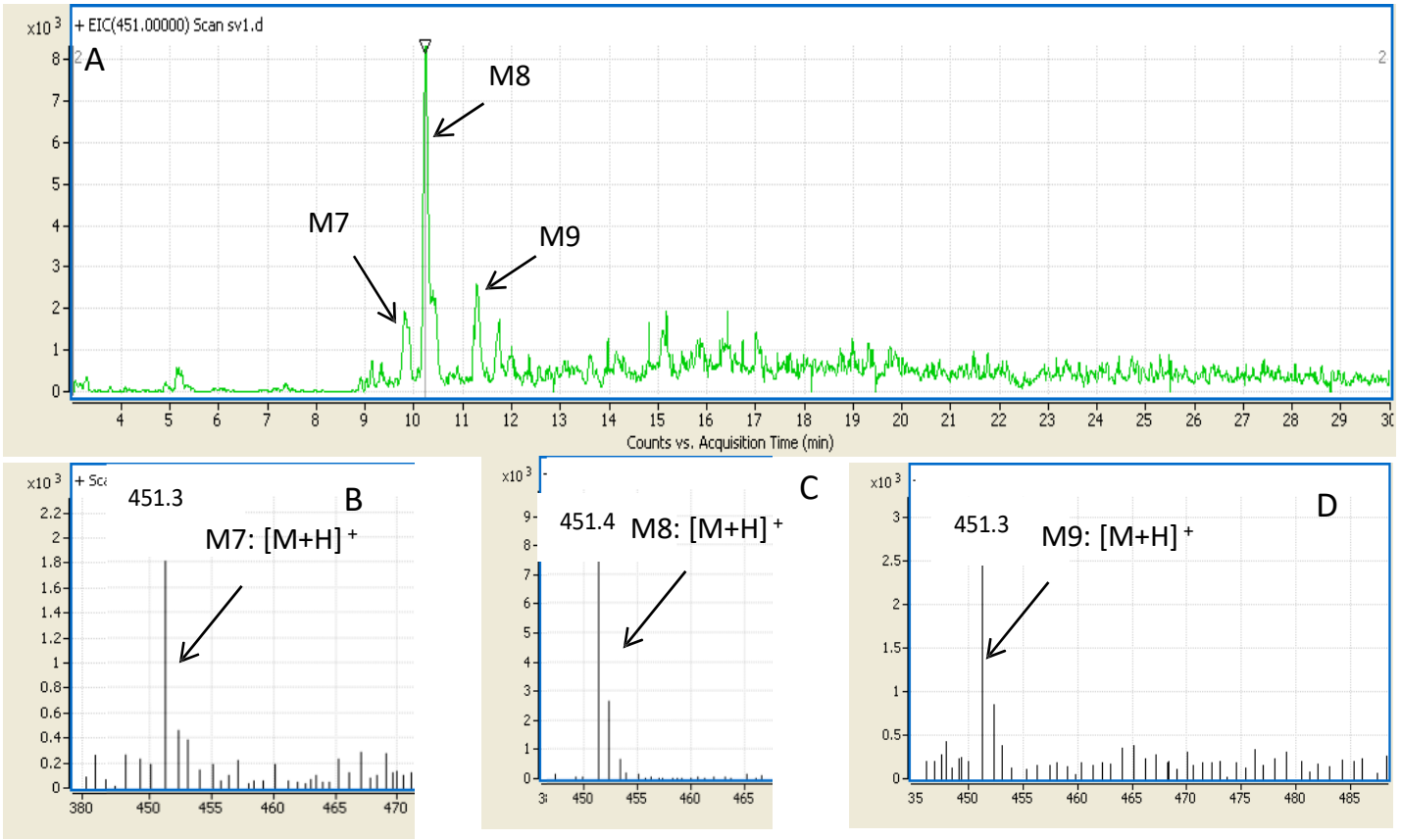


Figure S9. Extracted ion chromatogram (EIC) and mass spectra of possible dihydroxylated metabolites in QqQ LC-MS in positive mode: A) EIC at *m/z* 451; B) Mass spectrum of possible **M7** at 9.9 min.; C) Mass spectrum of possible **M8** at 10.2 min.; D) Mass spectrum of possible **M9** at 11.2 min.

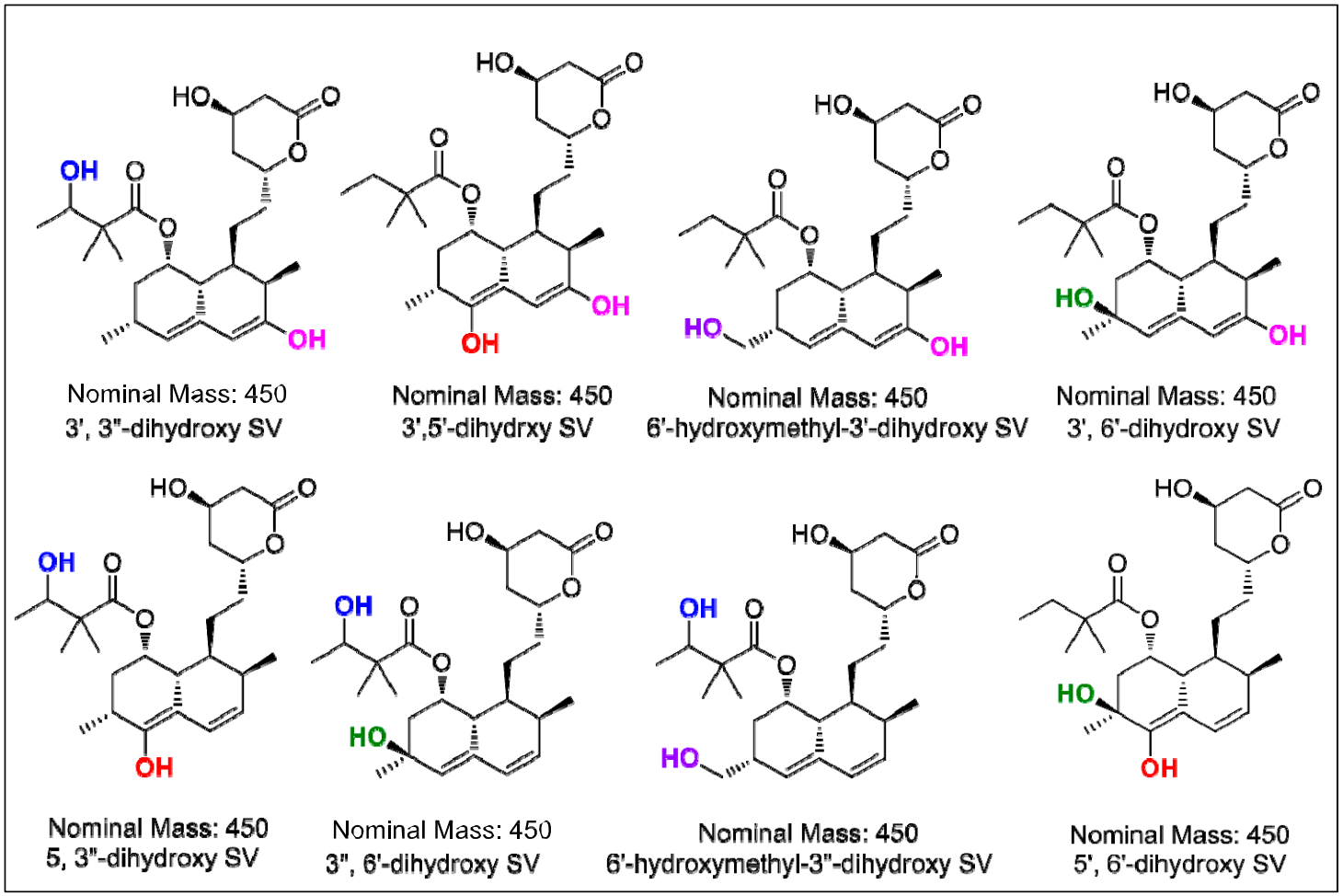


Figure S10. Chemical structure of possible dihydroxylated metabolites **(M7-M13)**

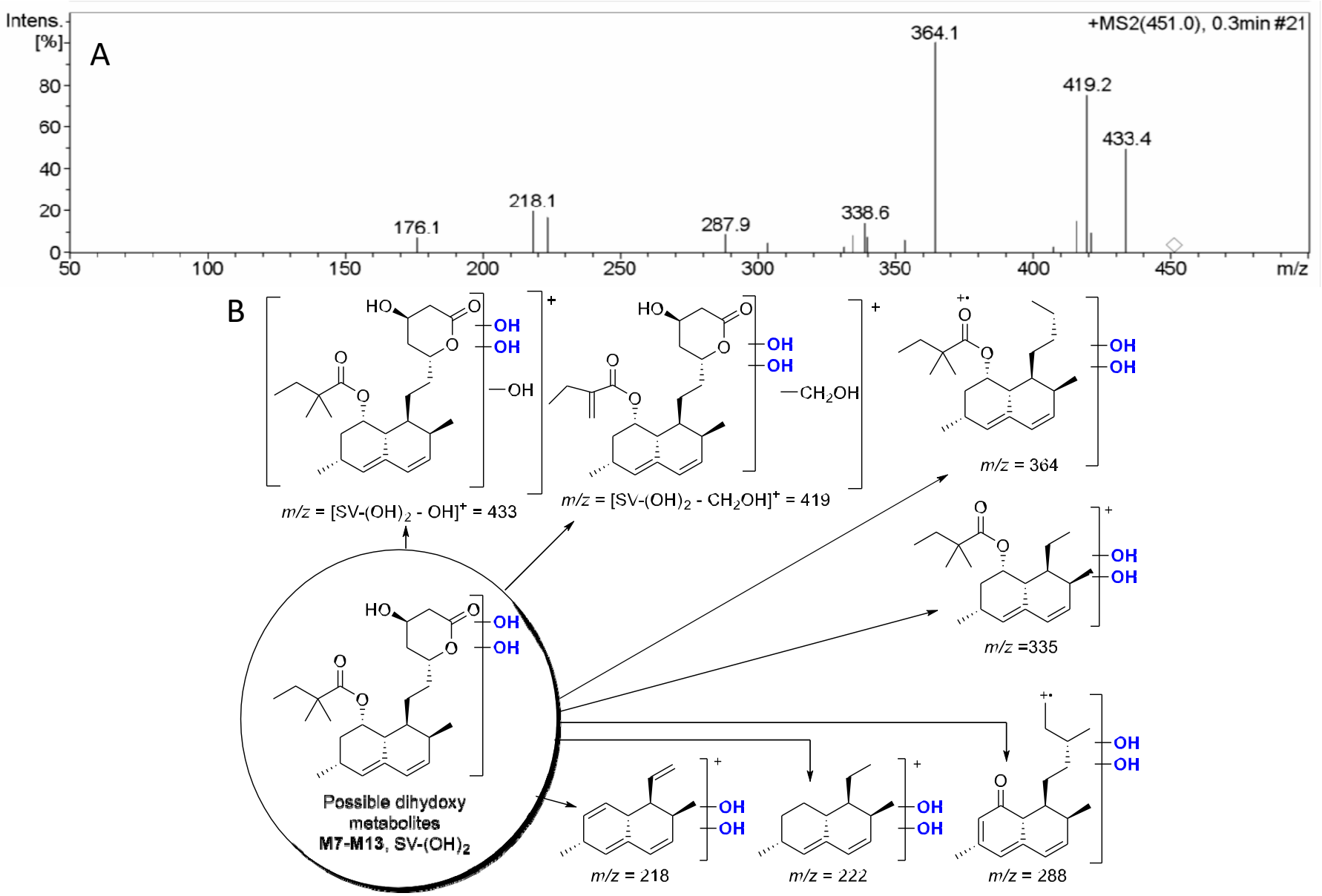


Figure S11. MS^2 spectra of **M7/M82/M9/M10/M11/M12/M13**: (A) Fragmentation spectra of possible SV-$(OH)_2$ at *m/z* = 451 in positive mode; and (B) Possible fragmentation pattern of possible SV-$(OH)_2$

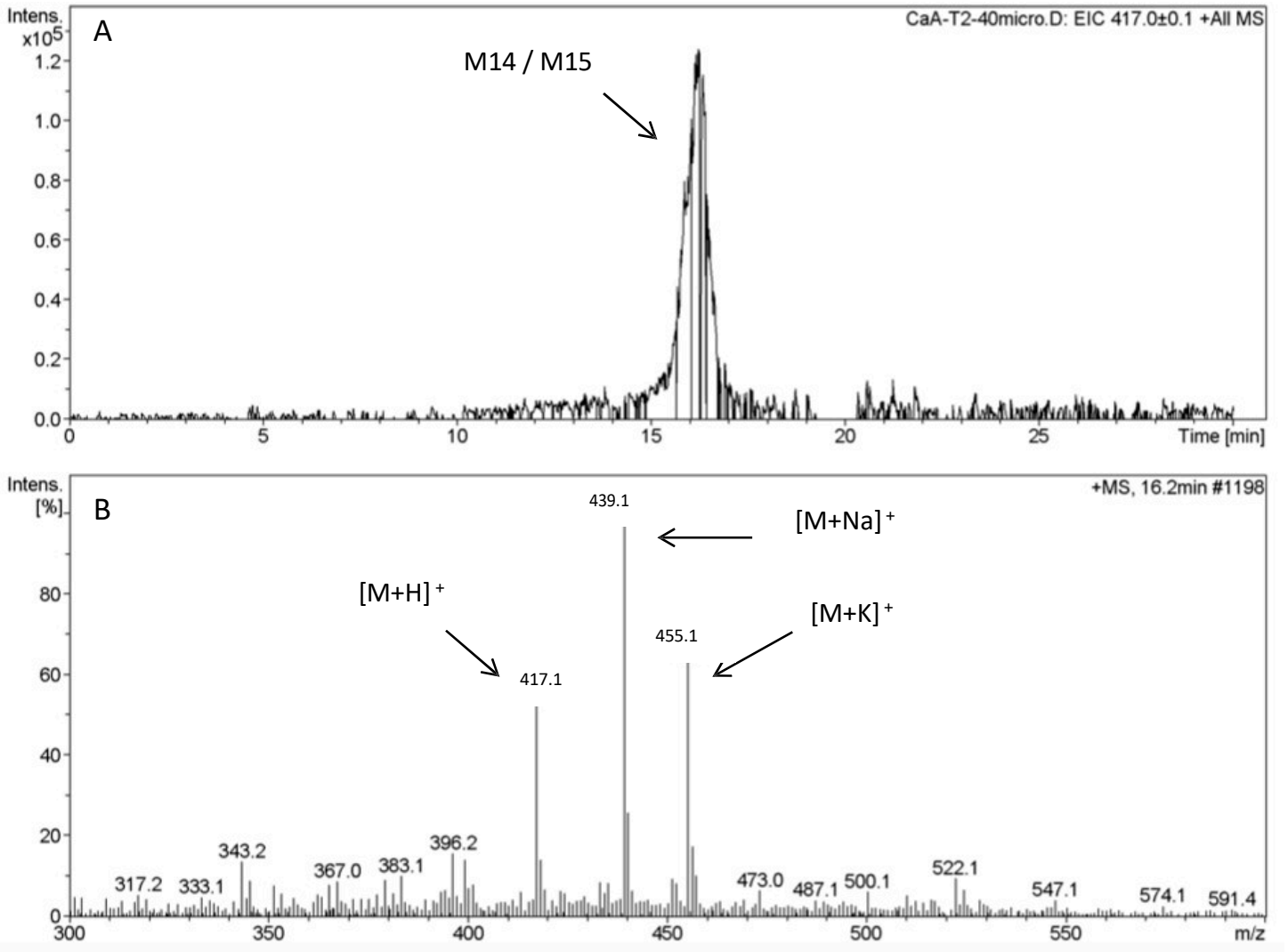


Figure S12. Extracted ion chromatogram (EIC) and mass spectrum of possible exomethylene metabolites in Ion-Trap LC-MS in positive mode: A) EIC at *m/z* 417; B) mass spectrum of possible **M1**4 / **M1**5 at 16.2 min.

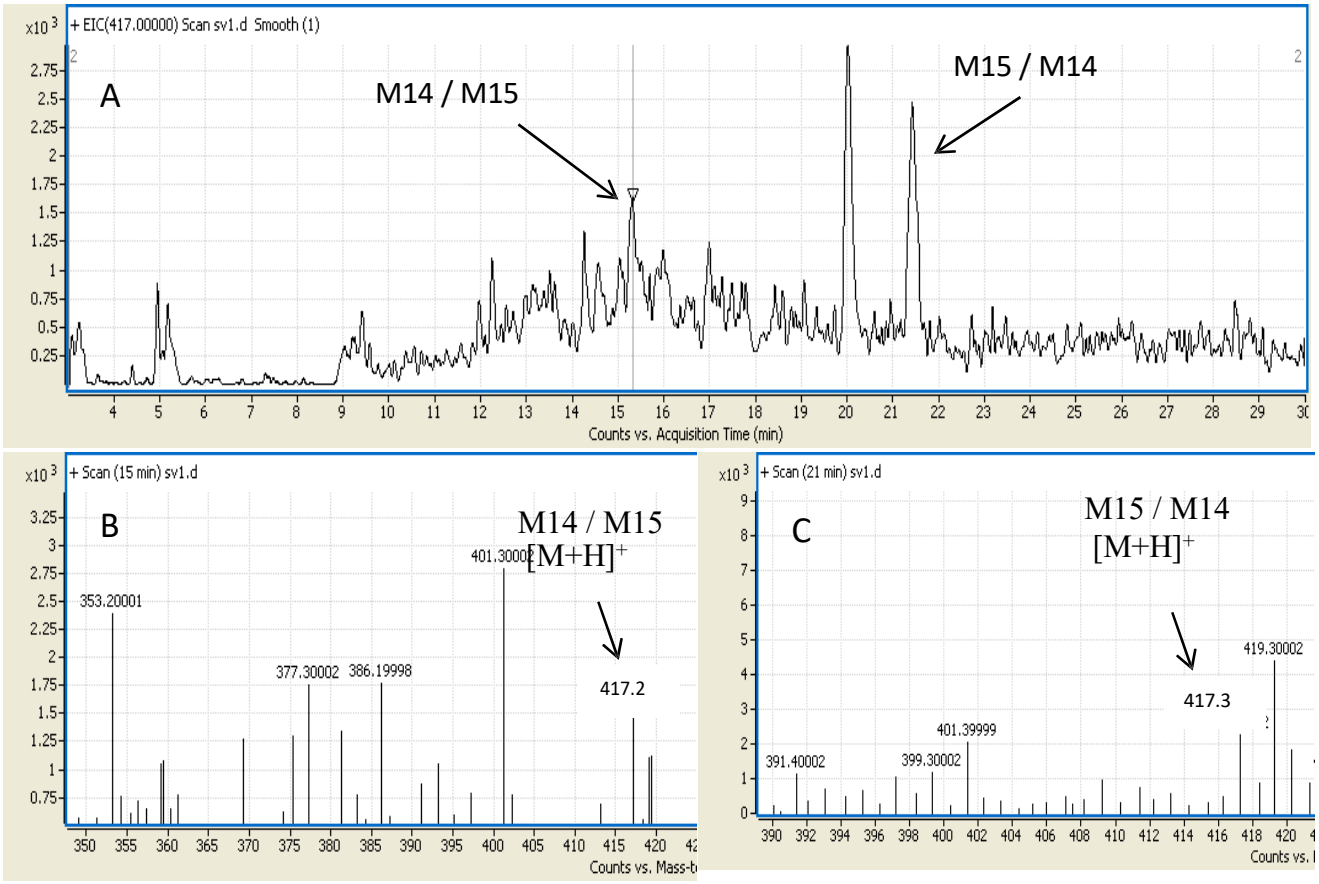


Figure S13. Extracted ion chromatogram (EIC) and mass spectra of possible exomethylene metabolites in QqQ LC-MS in positive mode: A) EIC at *m/z* 417; B) Mass spectrum of **M14** at 15.3 min.; C) Mass spectrum of **M15** at 21.3 min.

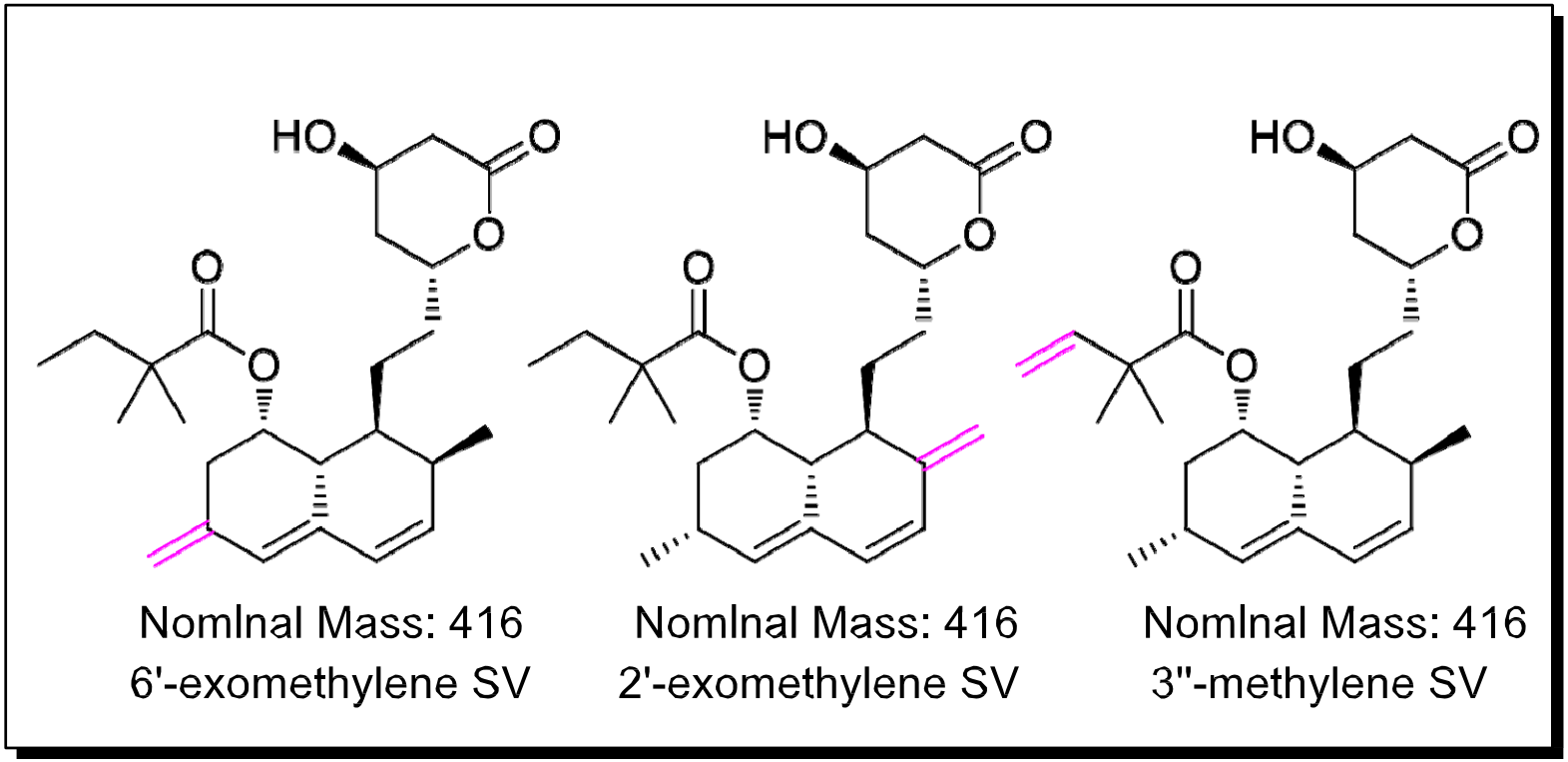


Figure S14. Chemical structures of possible (exo)methylene-SV metabolites **(M14-M15)**

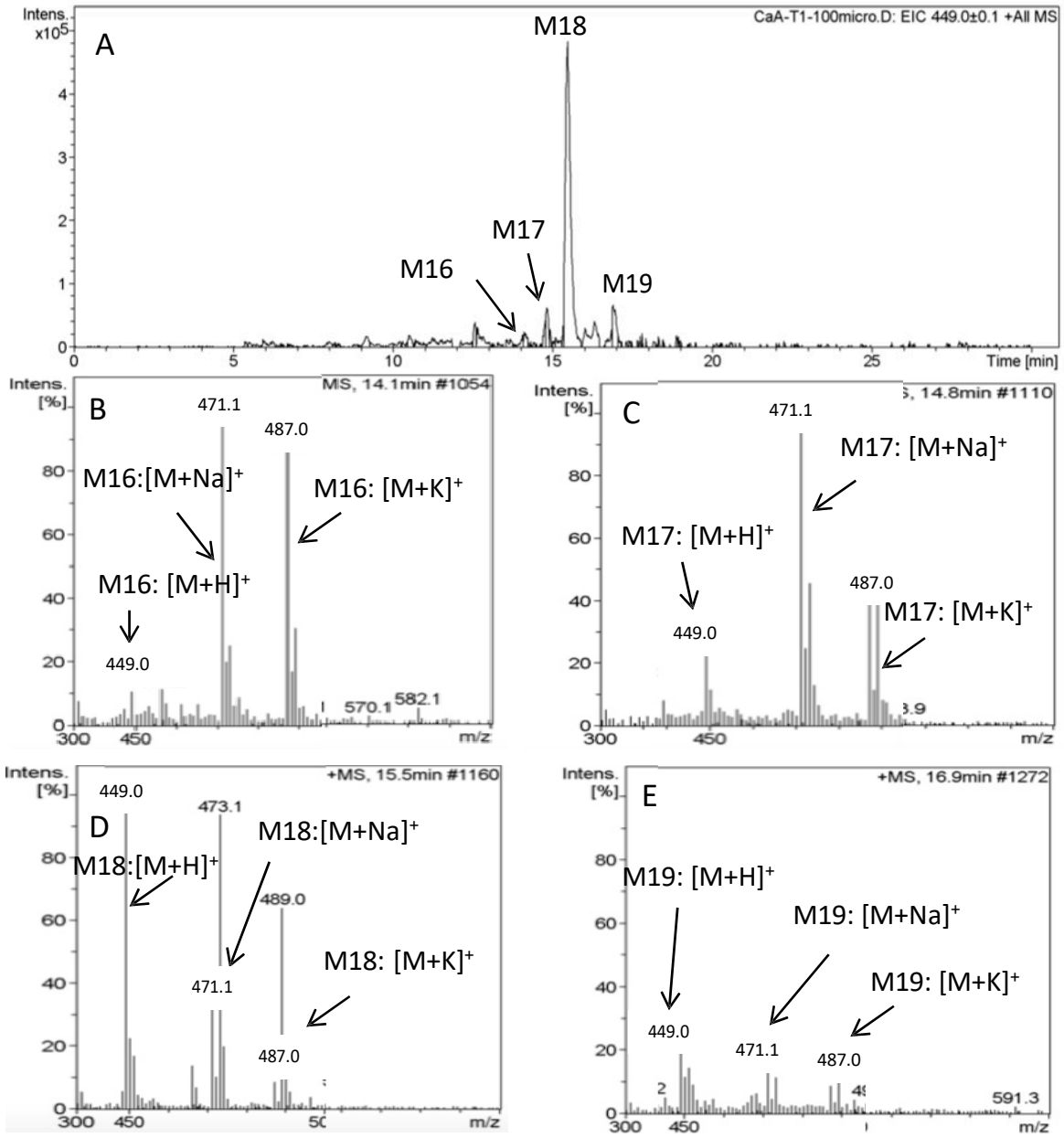


Figure S15. Extracted ion chromatogram (EIC) and mass spectra of possible oxidized metabolites (carboxyl-SV) in Ion-Trap LC-MS in positive mode;
A) EIC at *m/z* 449;
B) Mass spectrum of **M16** at 14.1 min.;
C) Mass spectrum of **M17** at 14.8 min.;
D) Mass spectrum of **M18** at 15.5 min.;
E) Mass spectrum of **M19** at 16.9 min.

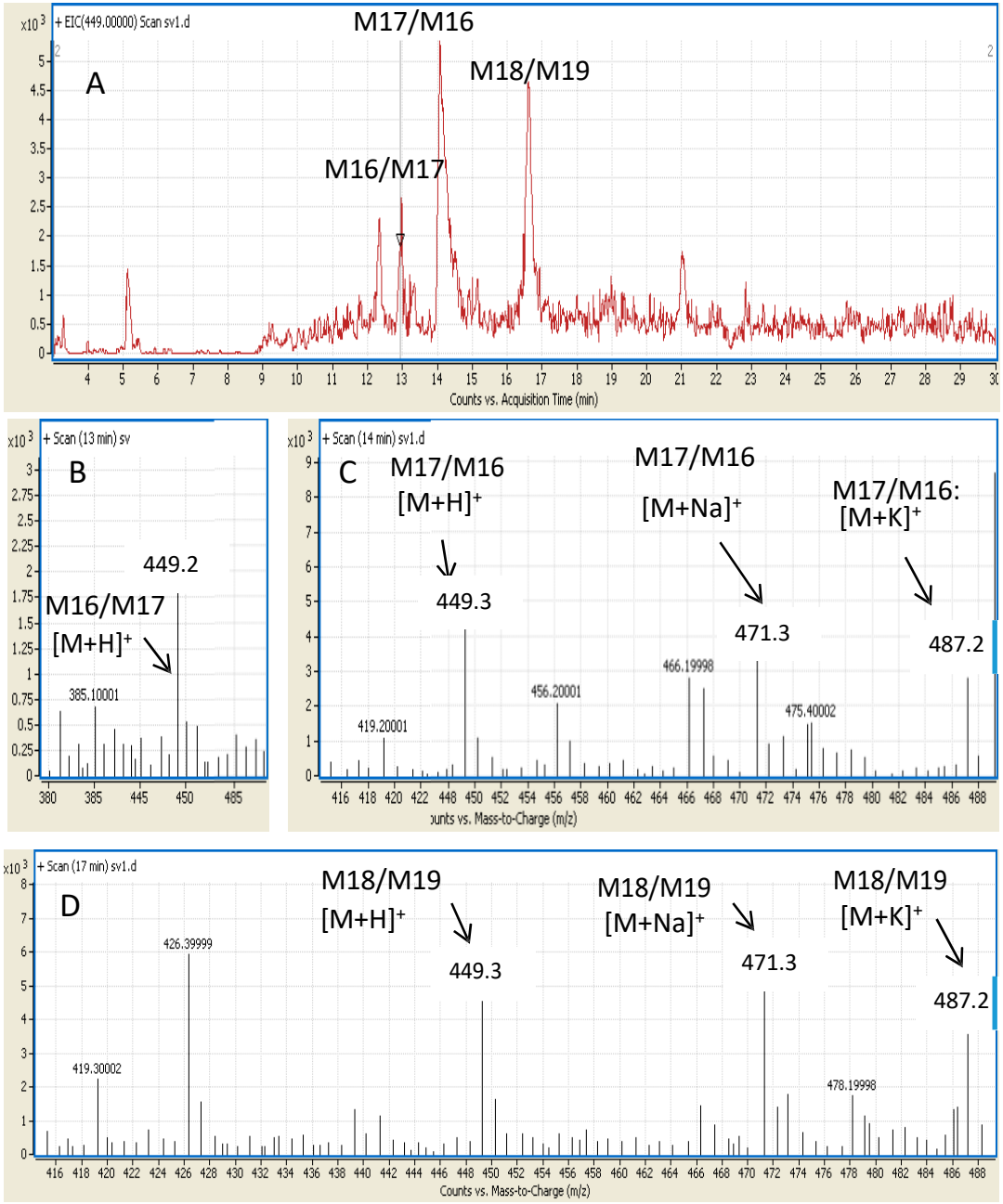


Figure S16. Extracted ion chromatogram (EIC) and mass spectra of possible oxidized metabolites (carboxyl-SV) in QqQ LC-MS in positive mode:
A) EIC at *m/z* 449;
B) MS of **M16**/**M17** at 13.0 min.;
C) MS of **M17**/**M16** at 14.1 min.;
D) MS of **M18**/**M19** at 16.9 min.

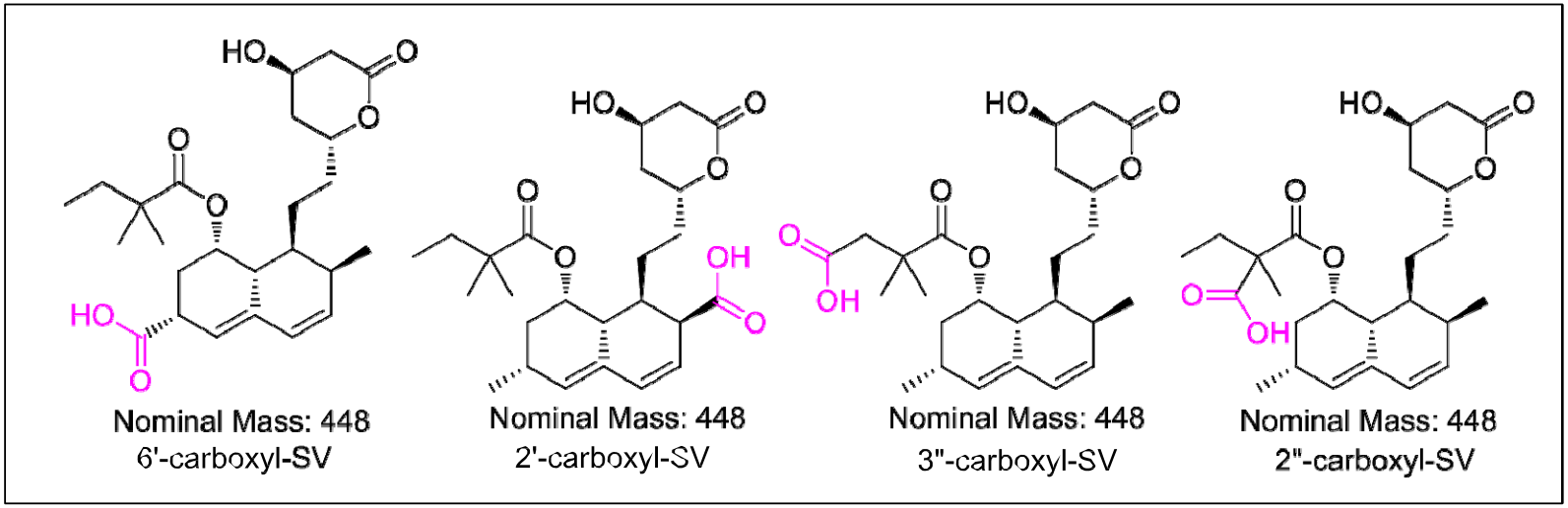


Figure S17. Chemical structures of possible carboxyl-SV metabolites **(M16-19)**

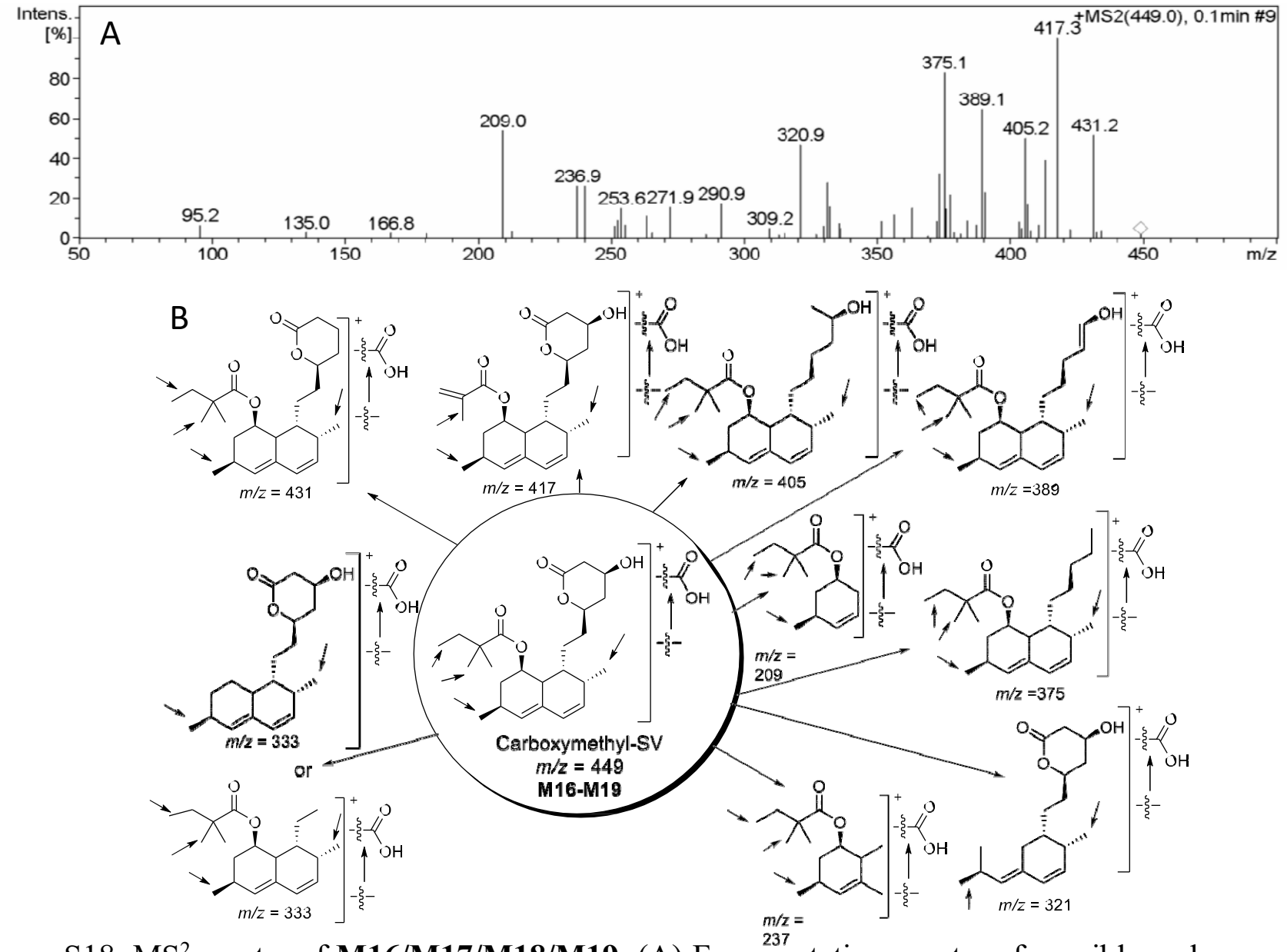


Figure S18. MS^2 spectra of **M16/M17/M18/M19**: (A) Fragmentation spectra of possible carboxymethyl-SV at *m/z* = 449 in positive mode; and (B) Possible fragmentation pattern of possible carboxymethyl-SV

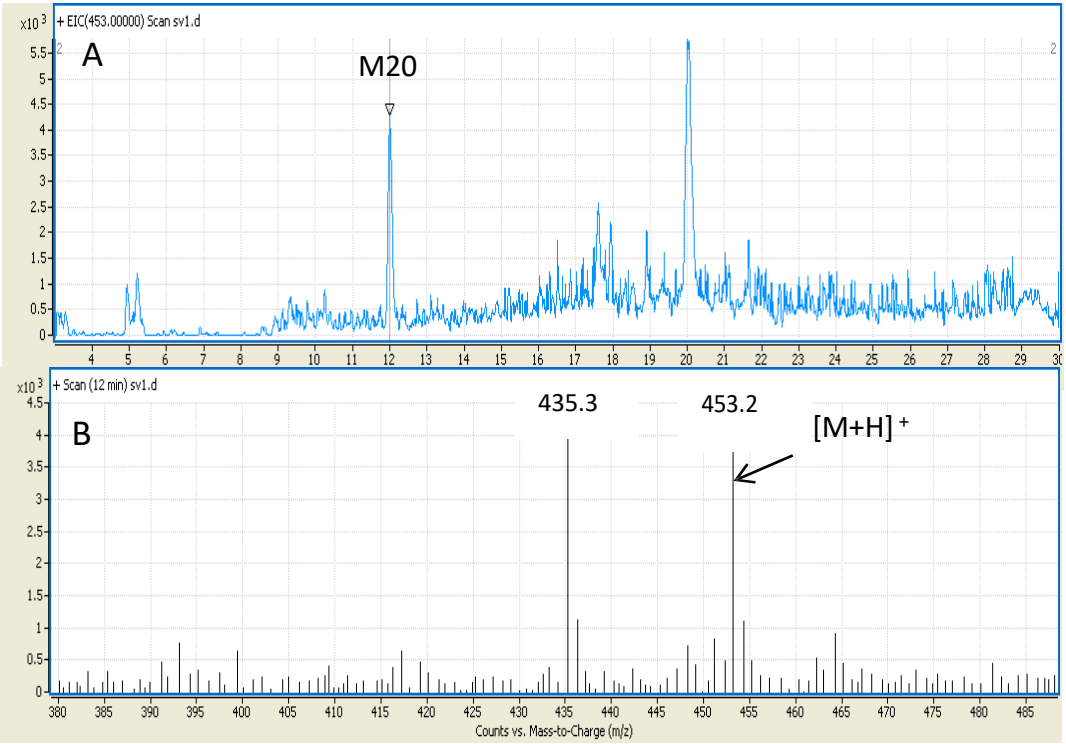


Figure S19. Extracted ion chromatogram (EIC) and mass spectrum of possible dihydrodiol-SV in LC- QqQ MS in positive mode: A) EIC at *m/z* 453; B) Mass spectrum of **M20** at 12.0 min.

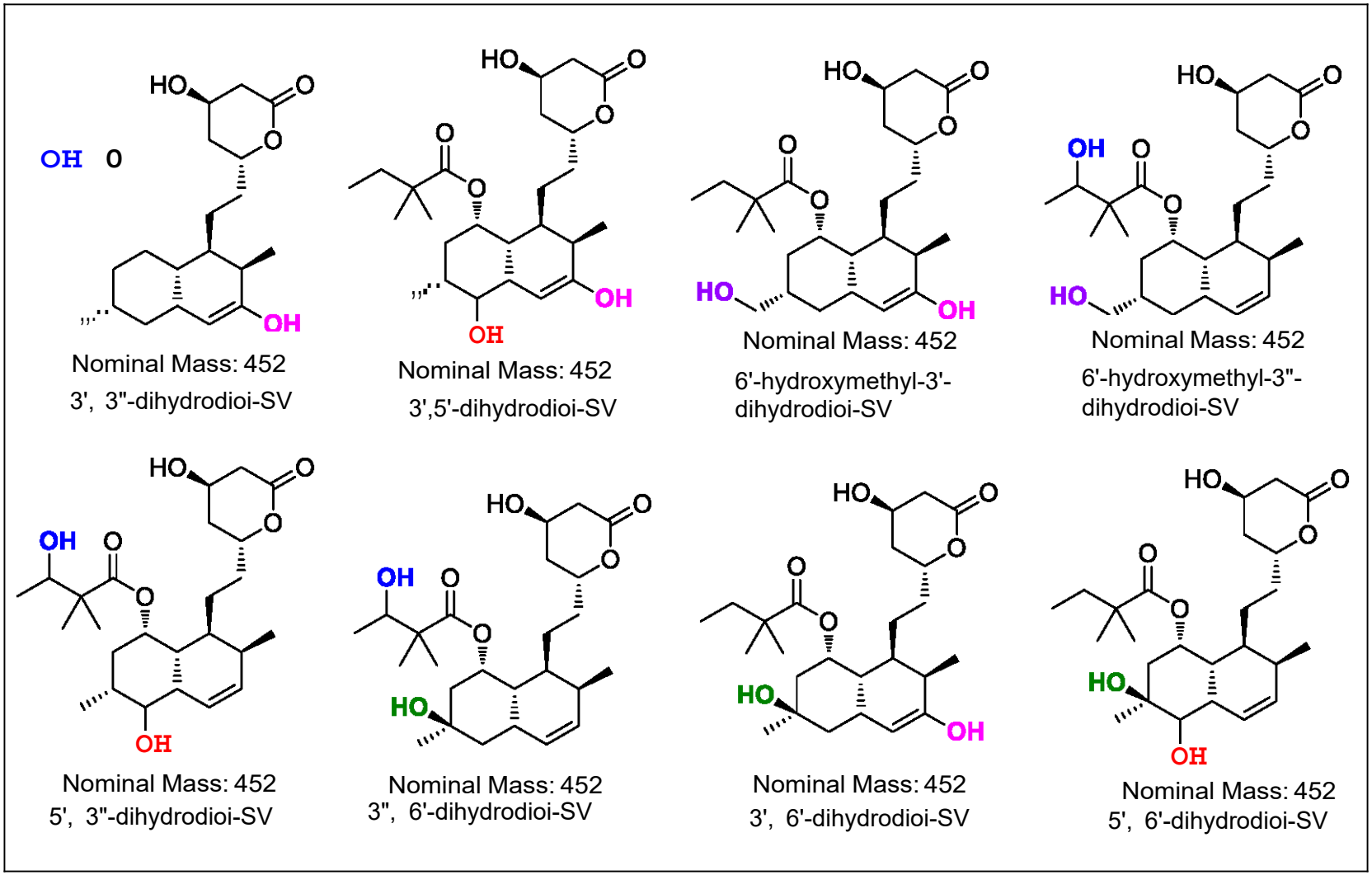


Figure S20. Chemical structures of possible dihydrodiol metabolites (M20)

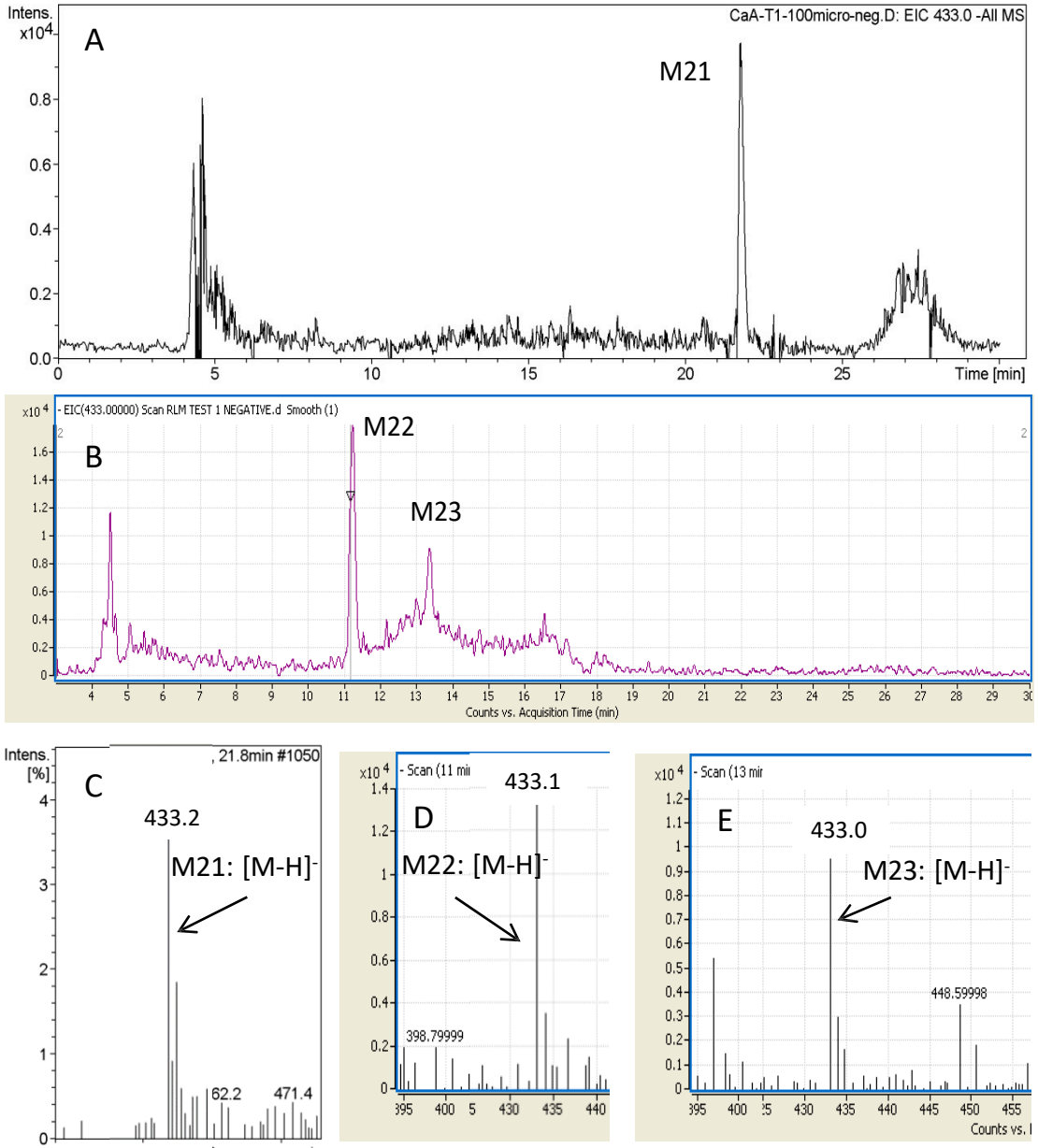


Figure S21. Extracted ion chromatogram (EIC) and mass spectra of possible exomethylene SVA metabolites in Ion-Trap / QqQ LC-MS in negative mode:
A) EIC at *m/z* 433 in Ion-Trap;
B) EIC at *m/z* 433 in QqQ-MS;
C) MS of **M21** at 21.8 min in Ion trap - MS.;
D) MS of **M22** at 11 min in QqQ MS.;
E) MS of **M23** at 13.3 min in QqQ MS.

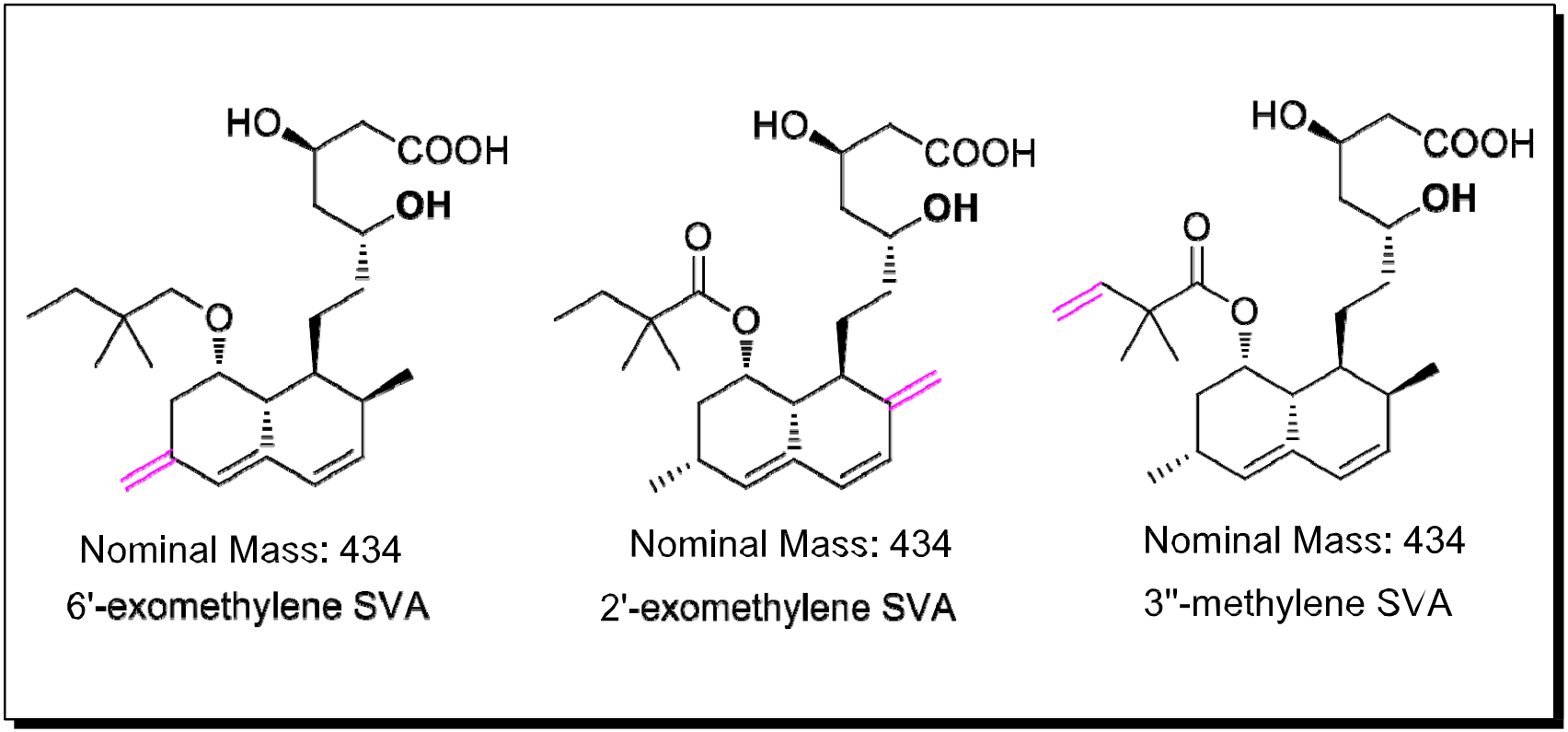


Figure S22. Chemical structures of possible (exo)methylene-SVA metabolites **(M21-M23)**

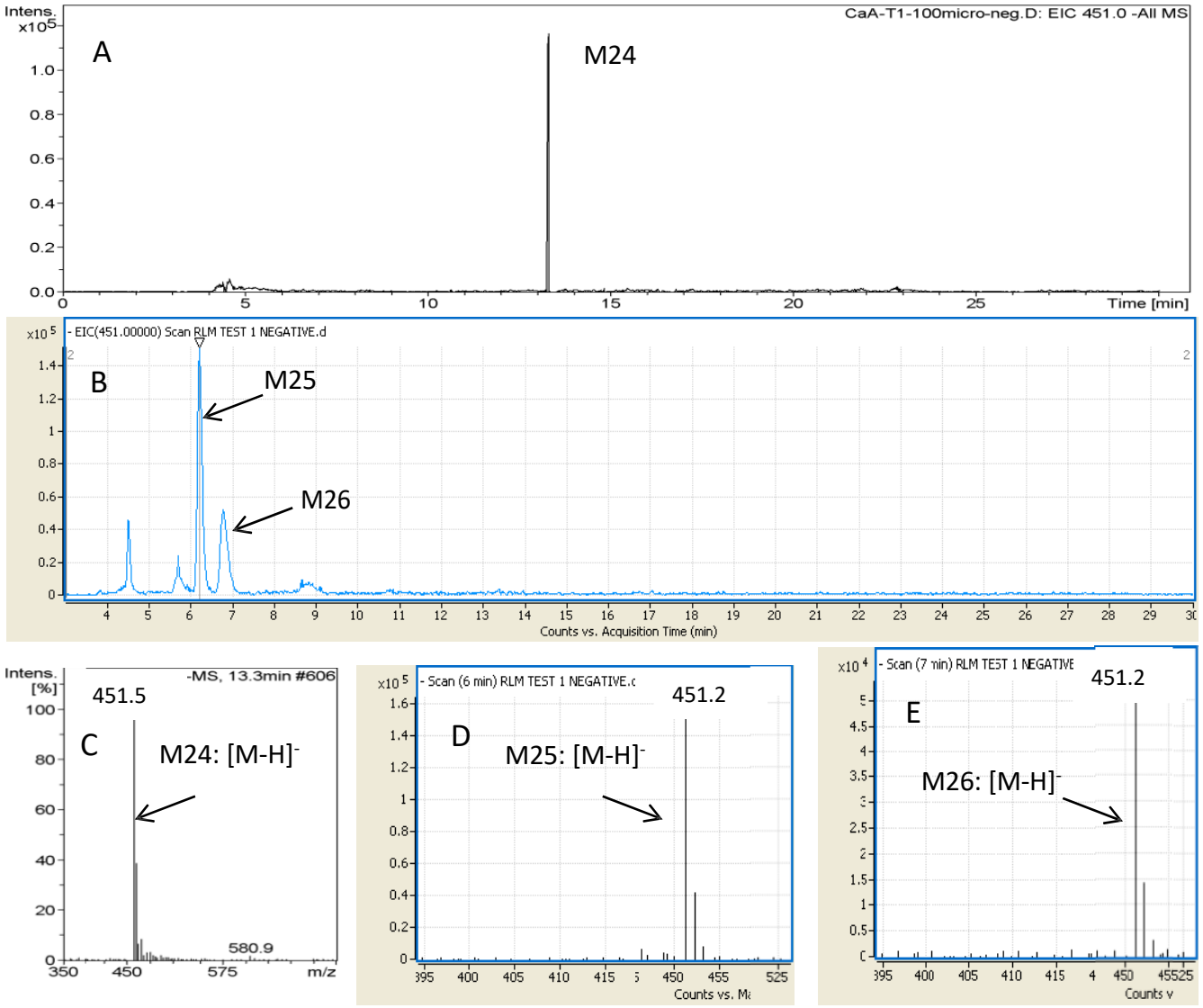


Figure S23. Extracted ion chromatogram (EIC) and mass spectra of possible hydroxyl-SVA metabolites in Ion-Trap / QqQ-LC-MS in negative mode: A) EIC at *m/z* 451 in Ion-Trap; B) EIC at *m/z* 451 in in QqQ MS; C) Mass spectrum of **M24** at 13.3 min in Ion trap -MS.; D) Mass spectrum of

M25 at 6.2 min. in QqQ MS; E) Mass spectrum of **M26** at 6.9 min. in QqQ MS.

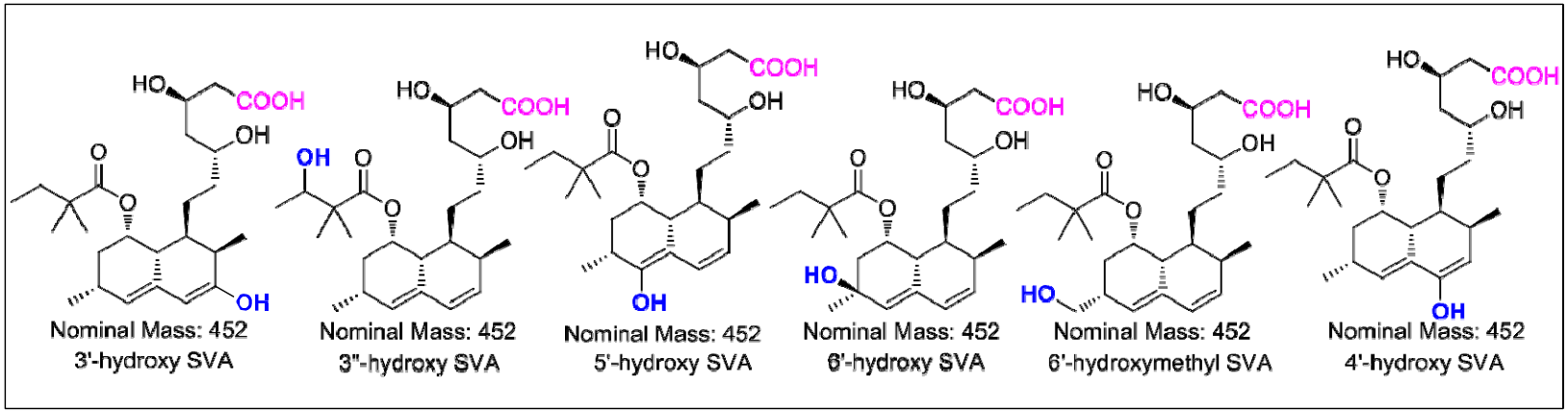


Figure S24. Chemical structures of possible hydroxyl-SVA metabolites **(M24-M26)**

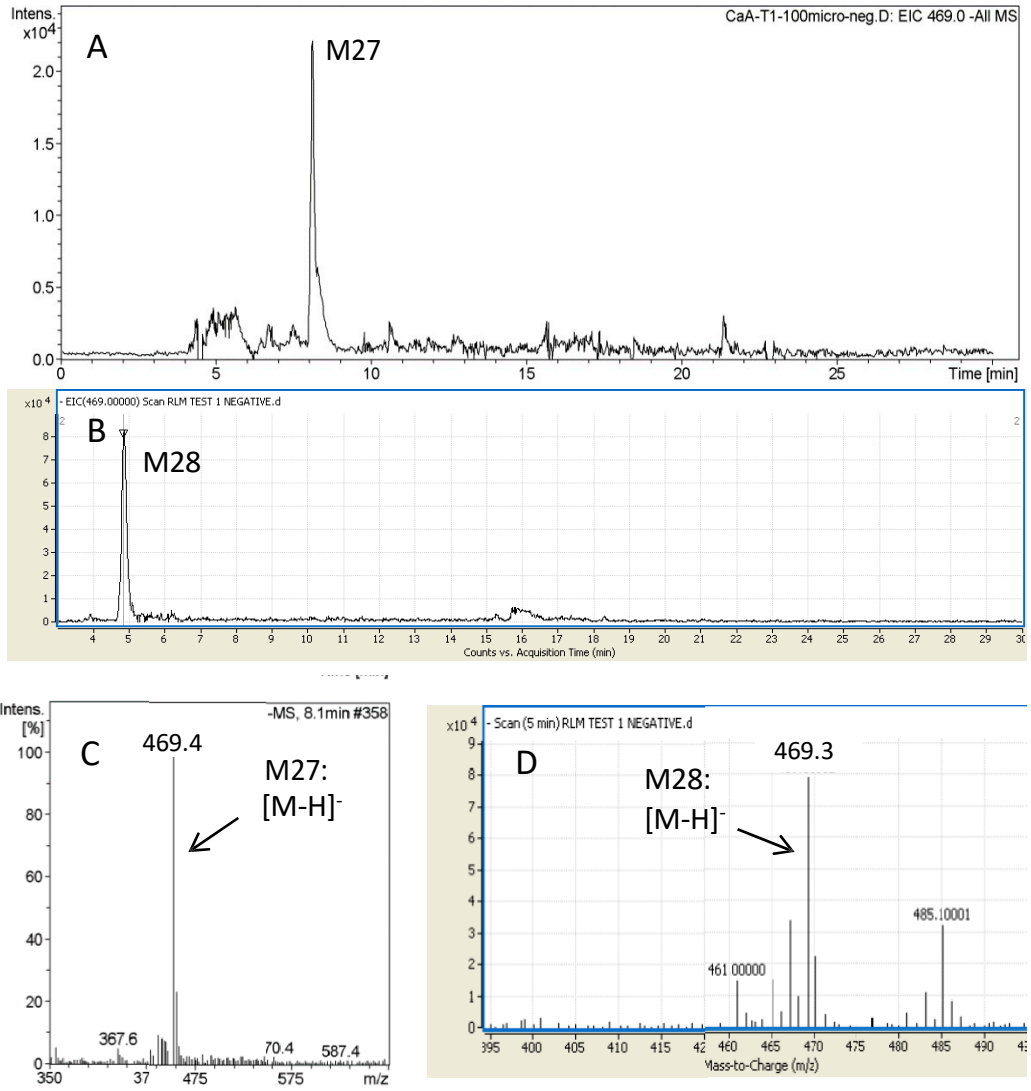


Figure S25. Extracted ion chromatogram (EIC) and mass spectra of possible dihydrodiol SVA metabolites in Ion-Trap / QqQ LC-MS in negative mode:
A) EIC at *m/z* 469 in Ion-Trap;
B) EIC at *m/z* 469 in QqQ;
C) MS of **M27** at 8.1 min. in Ion trap -MS;
D) MS of **M28** at 4.9 min. in QqQ-MS

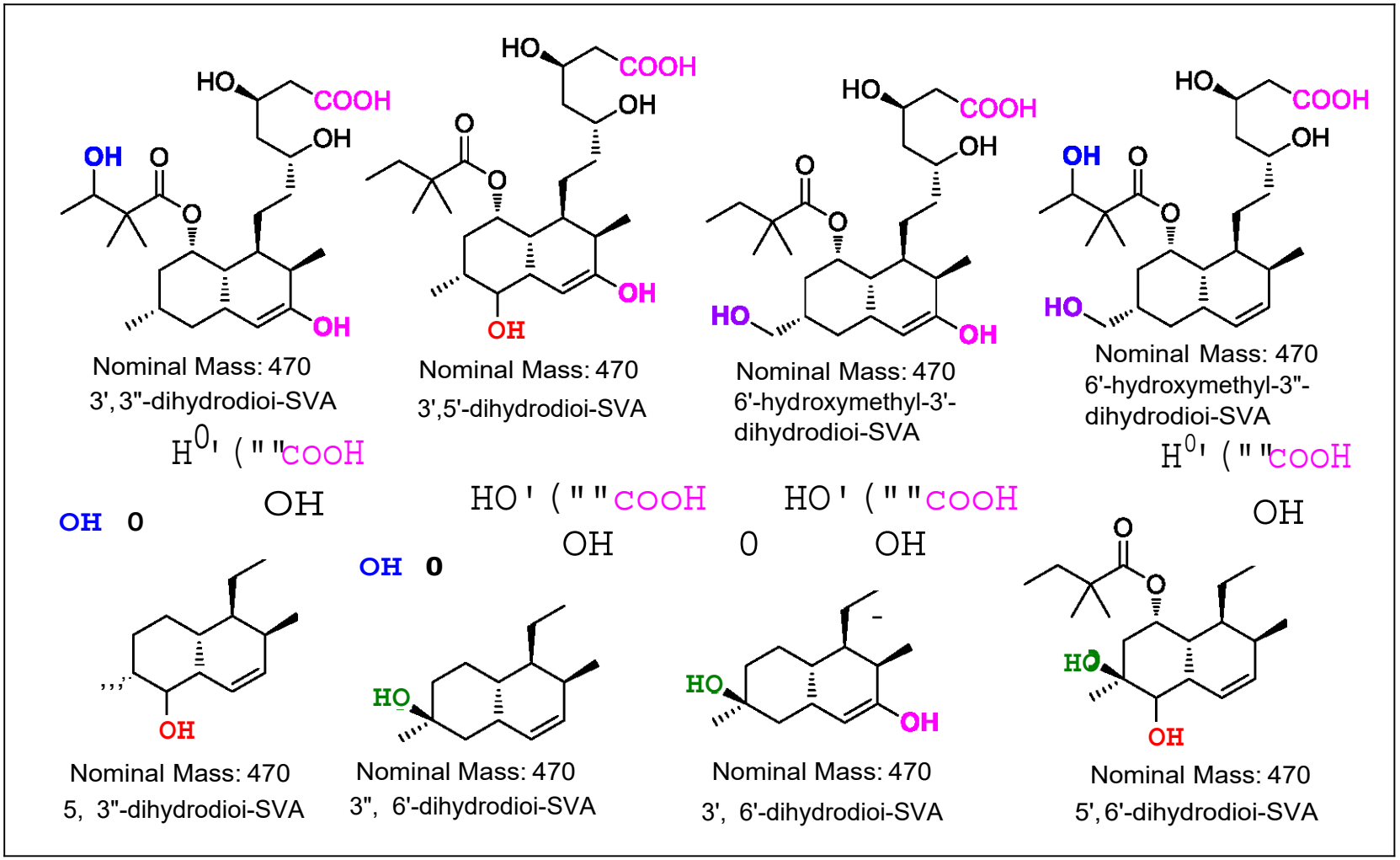


Figure S26. Chemical structures of possible dihydrodiol SVA metabolites (M27-M28)

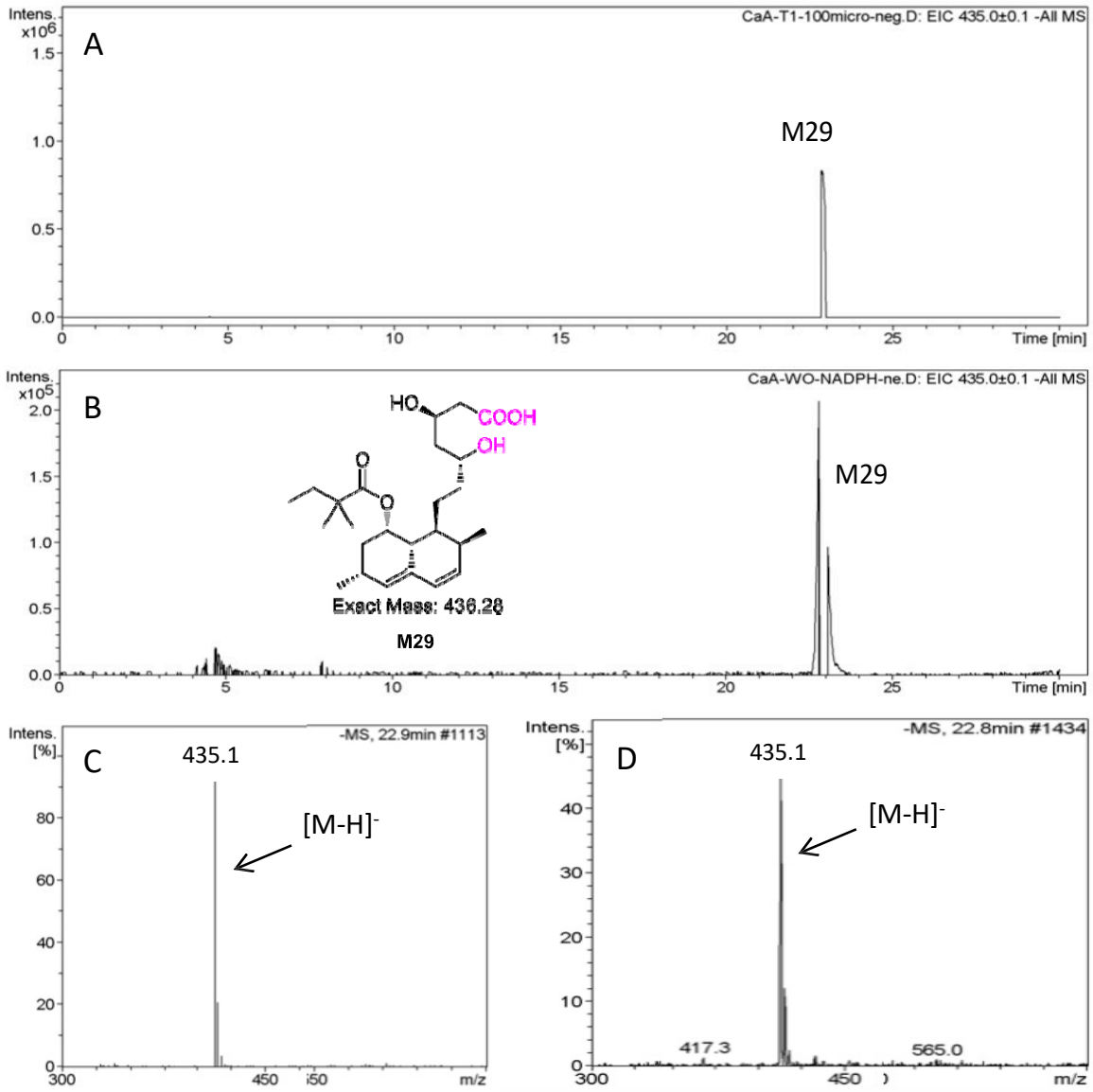


Figure S27. Extracted ion chromatogram (EIC) and mass spectra of possible SVA metabolites in Ion-Trap LC-MS in negative mode:
A) EIC at *m/z* 435 in Ion-Trap;
B) EIC at *m/z* 435 in Ion-Trap without NADPH;
C) MS of **M29** at 22.8 min.;
D) MS of **M29** at 22.8 min. without NADPH.

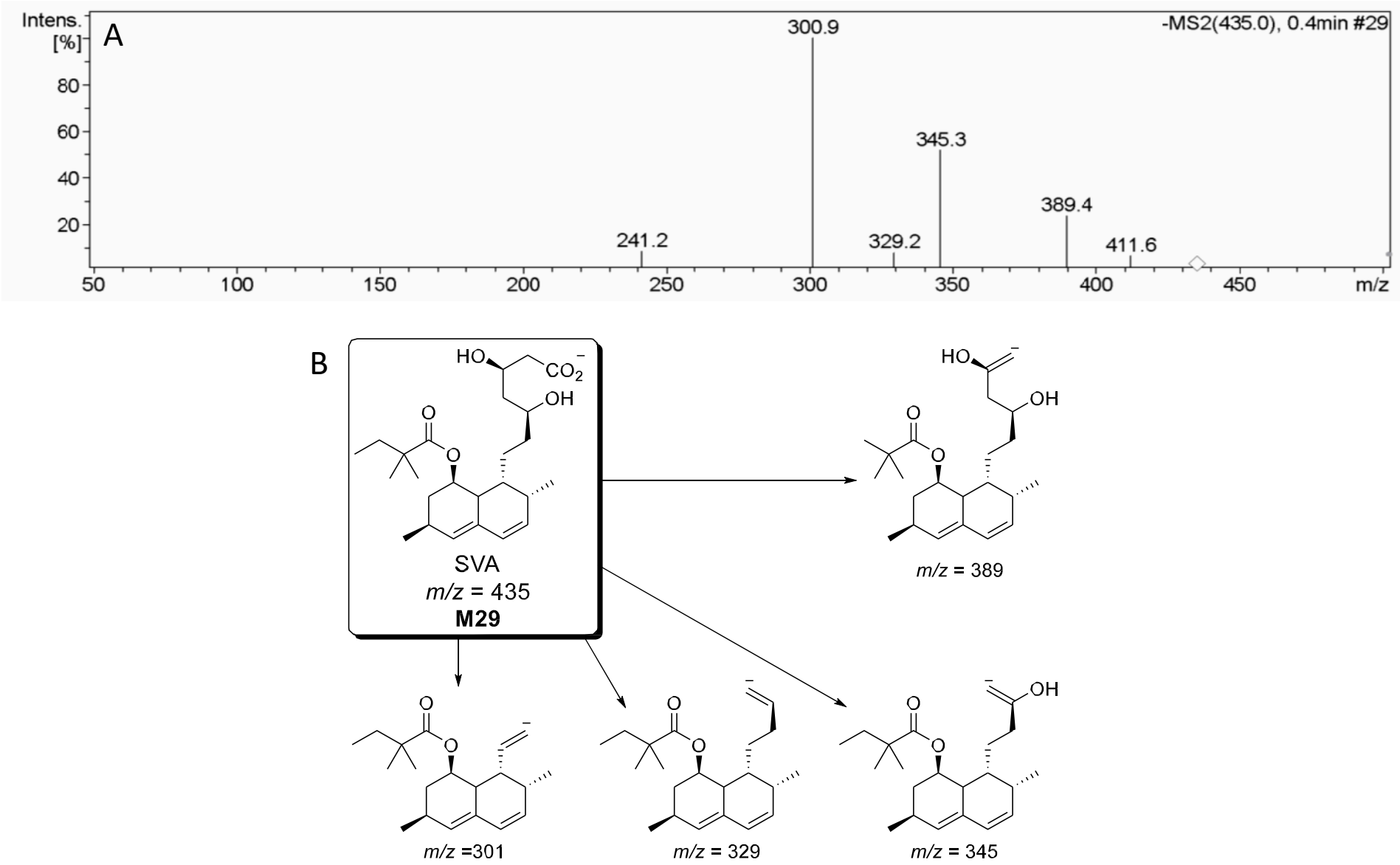


Figure S28. MS^2 spectra of **M29**: (A) Fragmentation spectra of possible SVA at *m/z* = 435 in negative mode; and (B) Possible fragmentation pattern of possible SVA