

SUPPLEMENTARY MATERIAL

Ultra-high performance liquid chromatography - high resolution mass spectrometry profiling and hepatoprotective activity of purified saponin and flavonoid fractions from the aerial parts of wild spinach (*Chenopodium bonus-henricus* L.)

Zlatina Kokanova-Nedialkova^{1*}, Paraskev Nedialkov^{1*}, Magdalena Kondeva-Burdina²

¹Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Sofia,

2 Dunav Str., 1000 Sofia, Bulgaria

²Laboratory of drug metabolism and drug toxicity, Department of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Pharmacy, Medical University of Sofia, 2 Dunav Str., 1000 Sofia, Bulgaria

***Corresponding authors: Zlatina Kokanova-Nedialkova, Paraskev Nedialkov,**
Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Sofia, 2 Dunav Str., 1000 Sofia, Bulgaria

E-mails: zlatina.kokanova@pharmfac.mu-sofia.bg, pnedialkov@pharmfac.mu-sofia.bg.

Abstract: A ultra-high performance liquid chromatography - high resolution mass spectrometry profiling method was used for a comprehensive study of flavonoid and saponin-rich fractions from the aerial parts of wild spinach (*Chenopodium bonus-henricus* L.). Thirty-six compounds, respectively **22** saponins of eight sapogenins (phytolaccagenin, bayogenin, medicagenic acid, 2 β -hydroxygypsogenin, 2 β -hydroxyoleanoic acid, 2-hydroxy-30-nor-gypsogenin, 2-hydroxyakebonic acid, and akebonic acid) together with **12** flavonoid glycosides of 6-methoxykaempferol, isorhamnetin, patuletin, spinacetin as well as two ecdysteroids (20-hydroxyecdysone and polypodine B) were detected. The occurrence of sapogenins 2-hydroxy-30-nor-gypsogenin, 2-hydroxyakebonic acid, and akebonic acid in the *Chenopodium* genus is reported here for the first time. The flavonoid and saponin-rich fractions showed *in vitro* hepatoprotective and antioxidant activity comparable to those of flavonoid complex silymarin (60 μ g/mL) in a model of metabolic bioactivation, induced by CCl₄. All tested fractions, compared to silymarin, significantly reduced the cellular damage caused by CCl₄ in rat hepatocytes, preserved cell viability and GSH level, decreased LDH leakage, and reduced lipid damage. The results showed that saponin-rich fractions F3A and F3B possessed better hepatoprotective activity than flavonoid-rich fractions (F2A and F2B). The most active was fraction F3B and this is probably due to the synergism between the saponins and some acylated flavonol glycosides found there.

Keywords: *Chenopodium bonus-henricus*, Amaranthaceae, UHPLC-HRMS, hepatoprotective activity

Table of Contents

Table S1: Characterization of flavonoids of the aerial parts of <i>C. bonus-henricus</i> L. in the negative ion mode	6
Table S2: Effects of co-administration of fractions F2A, F2B, F3A, F3B and silymarin complex (S)(60 µg/mL) with CCl ₄ on the parameters, which characterized functional-metabolic status of isolated rat hepatocytes	7
Figure S1: UHPLC-HRMS based profiling of MeOH extract from the aerial parts of <i>C. bonus-henricus</i> L. in the positive ion mode and detected flavonoids and ecdysteroids	8
Figure S2: UHPLC-HRMS based profiling of MeOH extract from the aerial parts of <i>C. bonus-henricus</i> L. in the negative ion mode and detected saponins	9
Figure S3: MS ² spectrum of Compound 1	10
Figure S4: MS ² spectrum of Compound 2	10
Figure S5: MS ² spectrum of Compound 3	11
Figure S6: Pseudo MS ³ spectrum of precursor at <i>m/z</i> 317.0656 due to aglycone of compound 3	12
Figure S7: MS ² spectrum of Compound 4	13
Figure S8: MS ² spectrum of Compound 5	13
Figure S9: MS ² spectrum of Compound 6	14
Figure S10: MS ² spectrum of Compound 7	14
Figure S11: Pseudo MS ³ spectrum of precursor at <i>m/z</i> 317.0656 due to aglycone of compound 7	15
Figure S12: MS ² spectrum of Compound 8	16
Figure S13: MS ² spectrum of Compound 9	17
Figure S14: MS ² spectrum of Compound 10	18
Figure S15: MS ² spectrum of Compound 11	18
Figure S16: MS ² spectrum of Compound 12	19
Figure S17: Pseudo MS ³ spectrum of precursor at <i>m/z</i> 317.0656 due to aglycone of compound 12	20

Figure S18: MS ² spectrum of Compound 13	21
Figure S19: MS ² spectrum of Compound 14	21
Figure S20: MS ² spectrum (NCE=10) of Compound 15	22
Figure S21: MS ² spectrum (NCE=25) of Compound 15	22
Figure S22: MS ² spectrum (NCE=10) of Compound 16	23
Figure S23: MS ² spectrum (NCE=25) of Compound 16	23
Figure S24: MS ² spectrum (NCE=10) of Compound 17	24
Figure S25: MS ² spectrum (NCE=25) of Compound 17	24
Figure S26: MS ² spectrum of Compound 18	25
Figure S27: MS ² spectrum of Compound 19	25
Figure S28: MS ² spectrum of Compound 20	26
Figure S29: MS ² spectrum (NCE=10) of Compound 21	26
Figure S30: MS ² spectrum (NCE=35) of Compound 21	27
Figure S31: MS ² spectrum of Compound 22	27
Figure S32: MS ² spectrum of Compound 23	28
Figure S33: MS ² spectrum of Compound 24	28
Figure S34: MS ² spectrum of Compound 25	29
Figure S35: MS ² spectrum of Compound 26	29
Figure S36: MS ² spectrum of Compound 27	30
Figure S37: MS ² spectrum of Compound 28	30
Figure S38: MS ² spectrum of Compound 29	31
Figure S39: MS ² spectrum of Compound 30	31
Figure S40: MS ² spectrum of Compound 31	32
Figure S41: MS ² spectrum of Compound 32	32
Figure S42: MS ² spectrum of Compound 33	33
Figure S43: MS ² spectrum of Compound 34	33

Figure S44: MS ² spectrum of Compound 35	34
Figure S45: MS ² spectrum of Compound 36	34
Figure S46: Base peak chromatograms in negative mode of A). the MeOH extract of aerial parts from <i>C. bonus-henricus</i> and B). the mixture of reference compounds. The numbering is according to the Table 1	35
Figure S47: TIC chromatograms in positive mode of A). the MeOH extract of aerial parts from <i>C. bonus-henricus</i> and B). the mixture of reference compounds. The numbering is according to the Table 1	36

Table S1: Characterization of flavonoids of the aerial parts of *C. bonus-henricus* L. in the negative ion mode.

No.	tr ¹ (min)	Frs ²	Identification	Ion mode	Found <i>m/z</i>	Composition (δ ppm)
1 ³	7.57	F2A F2B	Patuletin-3-O- $[\beta$ -Api(1 $\rightarrow$ 2)]- β -Glu(1 $\rightarrow$ 6)- β -Glu	[M-H] ⁻	787.1923	C ₃₃ H ₃₉ O ₂₂ (-0.54)
2 ³	8.49	F2A F2B F3B	Patuletin-3-O- β -Glu(1 $\rightarrow$ 6)- β -Glu	[M-H] ⁻	655.1515	C ₂₈ H ₃₁ O ₁₈ (1.53)
3	8.83	F2A F2B	Isorhamnetin-Hex-Hex-Pent	[M-H] ⁻	771.1984	C ₃₃ H ₃₉ O ₂₁ (0.77)
4 ³	8.94	F2A F2B	6-methoxykaempferol-3-O- $[\beta$ -Api(1 $\rightarrow$ 2)]- β -Glu(1 $\rightarrow$ 6)- β -Glu	[M-H] ⁻	771.1980	C ₃₃ H ₃₉ O ₂₁ (0.21)
5 ³	9.41	F2A F2B	Spinacetin-3-O- $[\beta$ -Api(1 $\rightarrow$ 2)]- β -Glu(1 $\rightarrow$ 6)- β -Glu	[M-H] ⁻	801.2080	C ₃₄ H ₄₁ O ₂₂ (-0.49)
6 ³	10.40	F2A F2B F3B	6-methoxykaempferol-3-O- β -Glu(1 $\rightarrow$ 6)- β -Glu	[M-H] ⁻	639.1567	C ₂₈ H ₃₁ O ₁₇ (1.73)
7	10.50	F2A F2B	Isorhamnetin-Hex-Hex	[M-H] ⁻	639.1570	C ₂₈ H ₃₁ O ₁₇ (2.20)
10 ³	11.09	F2A F2B F3B	Spinacetin-3-O- β -Glu(1 $\rightarrow$ 6)- β -Glu	[M-H] ⁻	669.1671	C ₂₉ H ₃₃ O ₁₈ (1.45)
11 ³	14.28	F2B	Patuletin-3-O-(5'''-O-E-FA)- β -D-Api(1 $\rightarrow$ 2) $[\beta$ -D-Glu(1 $\rightarrow$ 6)]- β -D-Glu	[M-H] ⁻	963.2390	C ₄₃ H ₄₇ O ₂₅ (-1.19)
12	15.74	F2B	Isorhamnetin-Hex-Hex-Pent-FA	[M-H] ⁻	947.2468	C ₄₃ H ₄₇ O ₂₄ (1.68)
13 ³	15.82	F2B F3A ⁴ F3B	Spinacetin-3-O-(5'''-O-E-FA)- β -D-Api(1 $\rightarrow$ 2) $[\beta$ -D-Glu(1 $\rightarrow$ 6)]- β -D-Glu	[M-H] ⁻	977.2520	C ₄₄ H ₄₉ O ₂₅ (-3.82)
14 ³	16.17	F2B F3B	6-methoxykaempferol-3-O-(5'''-O-E-FA)- β -D-Api(1 $\rightarrow$ 2) $[\beta$ -D-Glu(1 $\rightarrow$ 6)]- β -D-Glu	[M-H] ⁻	947.2451	C ₄₃ H ₄₇ O ₂₄ (0.06)

¹ tr - retention time of detected compounds in the MeOH extract.

² Fraction containing the compound.

³ Flavonoids compared with a reference compound.

⁴ The compounds were in trace.

Api, apiose; Glu, glucose; FA, ferulic acid; Pent, pentose; Hex, hexose.

Table S2: Effects of co-administration of fractions F2A, F2B, F3A, F3B and silymarin complex (S)(60 µg/mL) with CCl₄ on the parameters, which characterized functional-metabolic status of isolated rat hepatocytes.

Group	Cell viability (%)		LDH activity (µmol/min/10 ⁶ cells)		GSH level (nmol/10 ⁶ cells)		MDA level (nmol/10 ⁶ cells)	
Control	85 ± 5.2		0.102 ± 0.01		18 ± 2.8		0.052 ± 0.01	
86 µM CCl ₄	32 ± 4.8 ^a	↓62*	0.423 ± 0.02 ^a	↑315*	5 ± 2.3 ^a	↓72*	0.253 ± 0.01 ^a	↑387*
F2A + CCl ₄	55 ± 6.2 ^b	↑72**	0.313 ± 0.02 ^b	↓26**	10 ± 3.2 ^b	↑100**	0.171 ± 0.01 ^b	↓32**
F2B + CCl ₄	52 ± 3.8 ^b	↑63**	0.328 ± 0.01 ^b	↓22**	10 ± 4.2 ^b	↑100**	0.178 ± 0.02 ^b	↓30**
F3A + CCl ₄	58 ± 3.9 ^b	↑81**	0.318 ± 0.01 ^b	↓25**	12 ± 2.1 ^b	↑140**	0.160 ± 0.02 ^b	↓37**
F3B + CCl ₄	66 ± 4.2 ^b	↑106**	0.231 ± 0.01 ^b	↓45**	15 ± 5.2 ^b	↑200**	0.124 ± 0.01 ^b	↓51**
S + CCl ₄	66 ± 3.1 ^b	↑106**	0.225 ± 0.01 ^b	↓47**	15 ± 4.1 ^b	↑200**	0.121 ± 0.01 ^b	↓52**

Significance levels: ^ap < 0.001 vs. control; ^bp < 0.001 vs. CCl₄.

* Effect vs control (%)

** Effect vs CCl₄ (%)

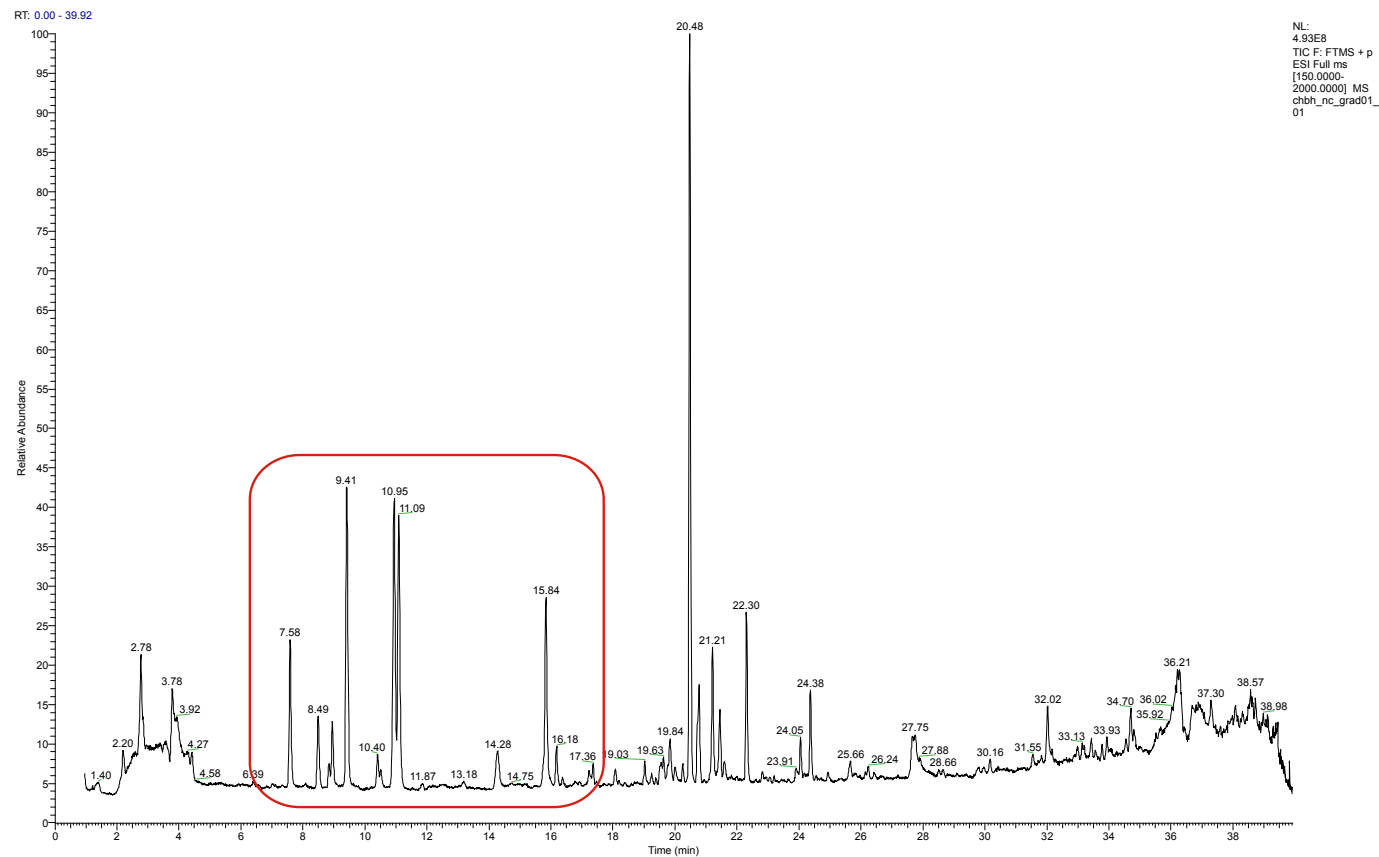


Figure S1: UHPLC-HRMS based profiling of MeOH extract from the aerial parts of *C. bonus-henricus* L. in the positive ion mode and detected flavonoids and ecdysteroids

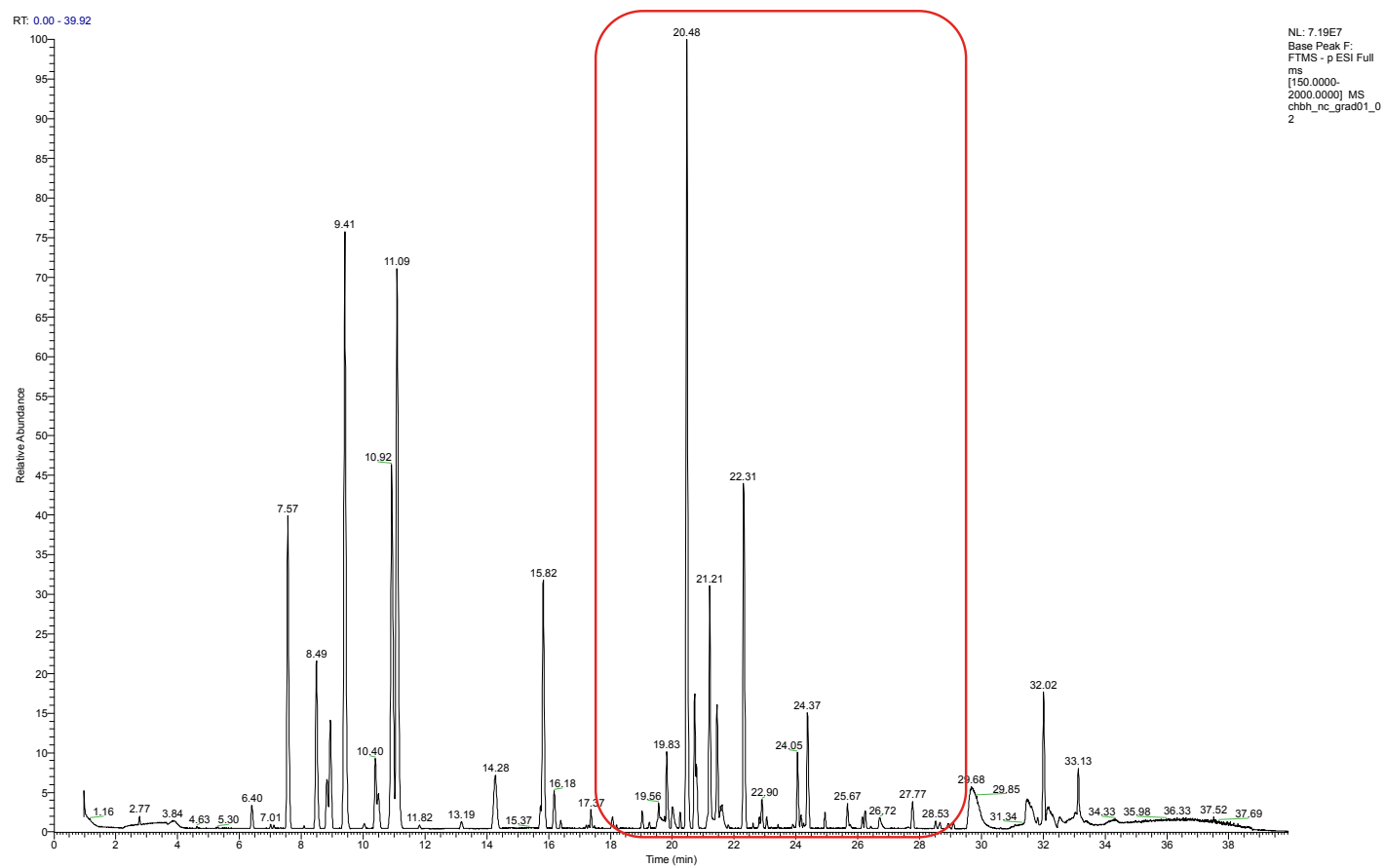


Figure S2: UHPLC-HRMS based profiling of MeOH extract from the aerial parts of *C. bonus-henricus* L. in the negative ion mode and detected saponin

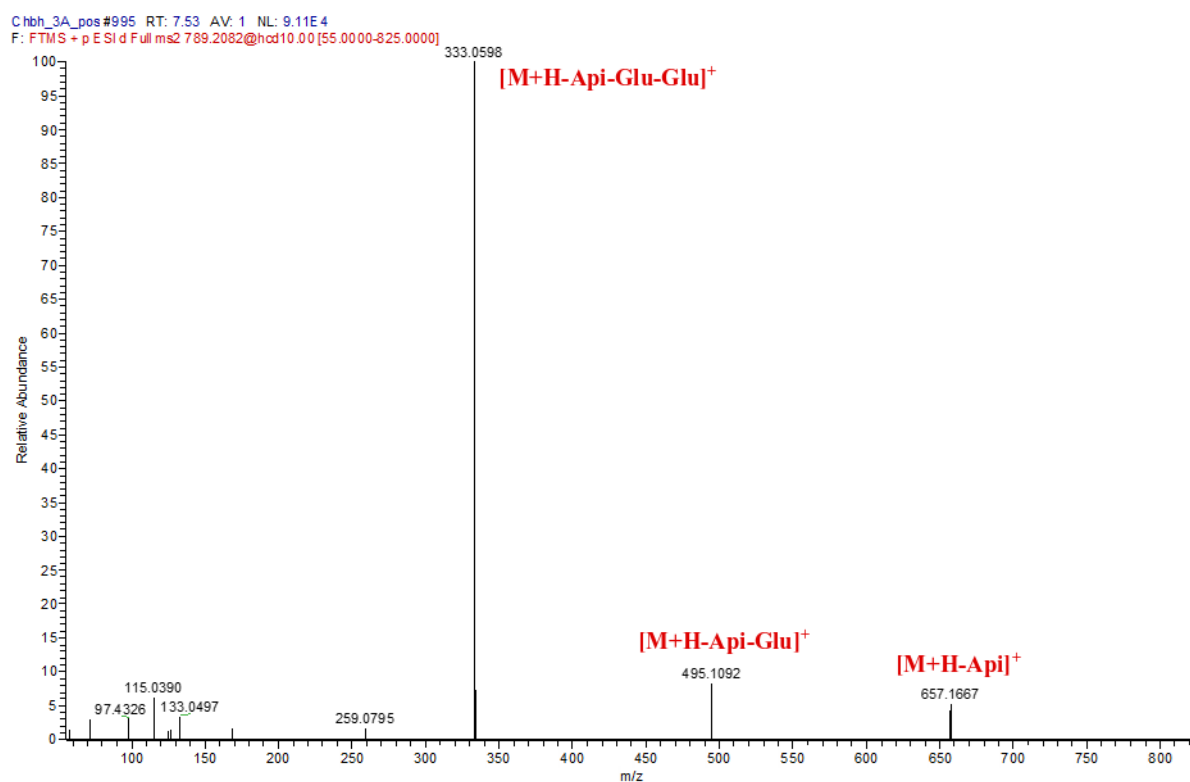


Figure S3: MS² spectrum of Compound 1

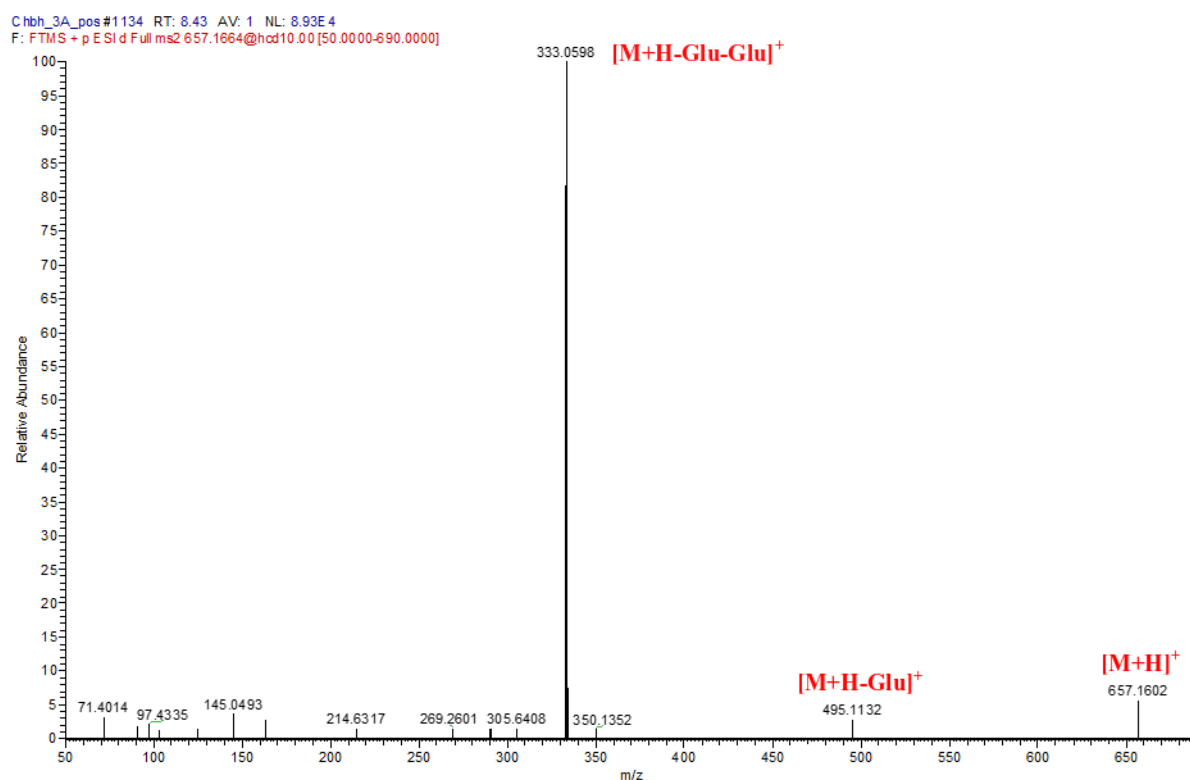


Figure S4: MS² spectrum of Compound 2

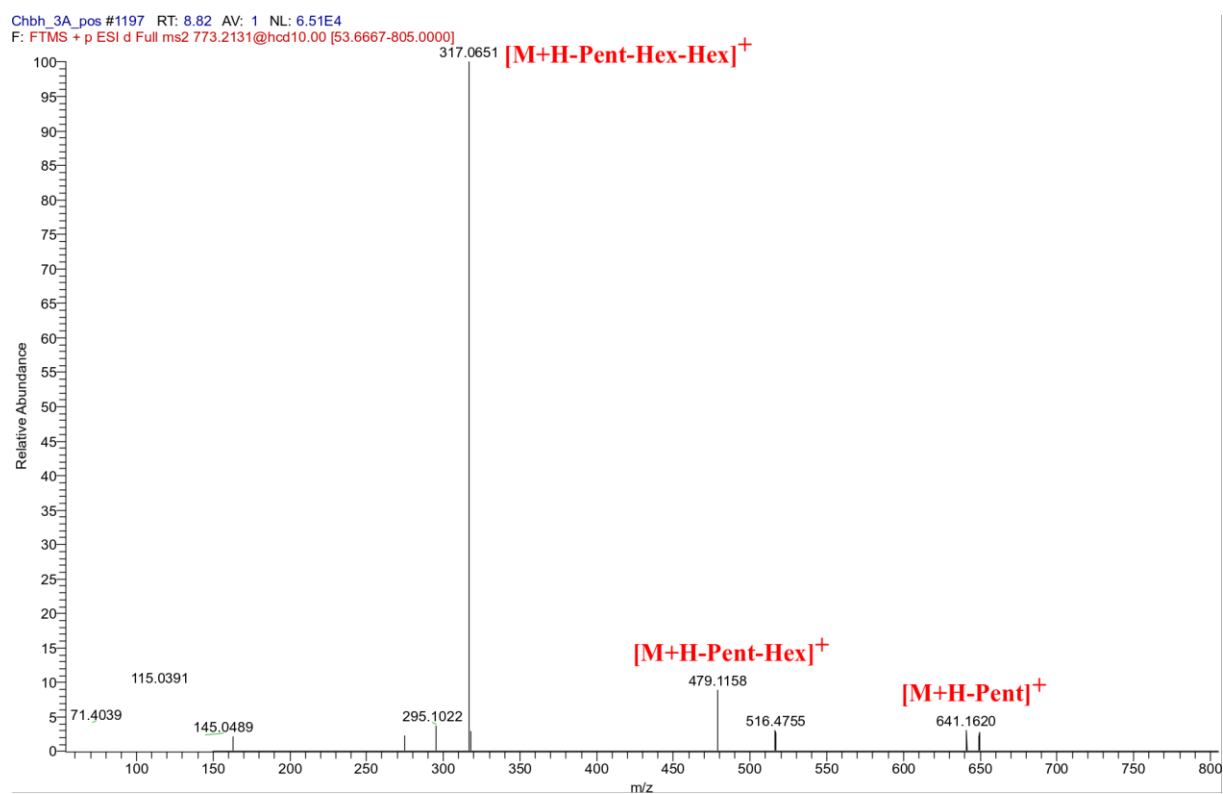


Figure S5: MS² spectrum of Compound **3**

Chbh_NC_flav_PRM03 #742 RT: 8.88 AV: 1 NL: 2.42E5
 F: FTMS + p ESI Full ms2 317.0656@hcd60.00 [50.0000-340.0000]

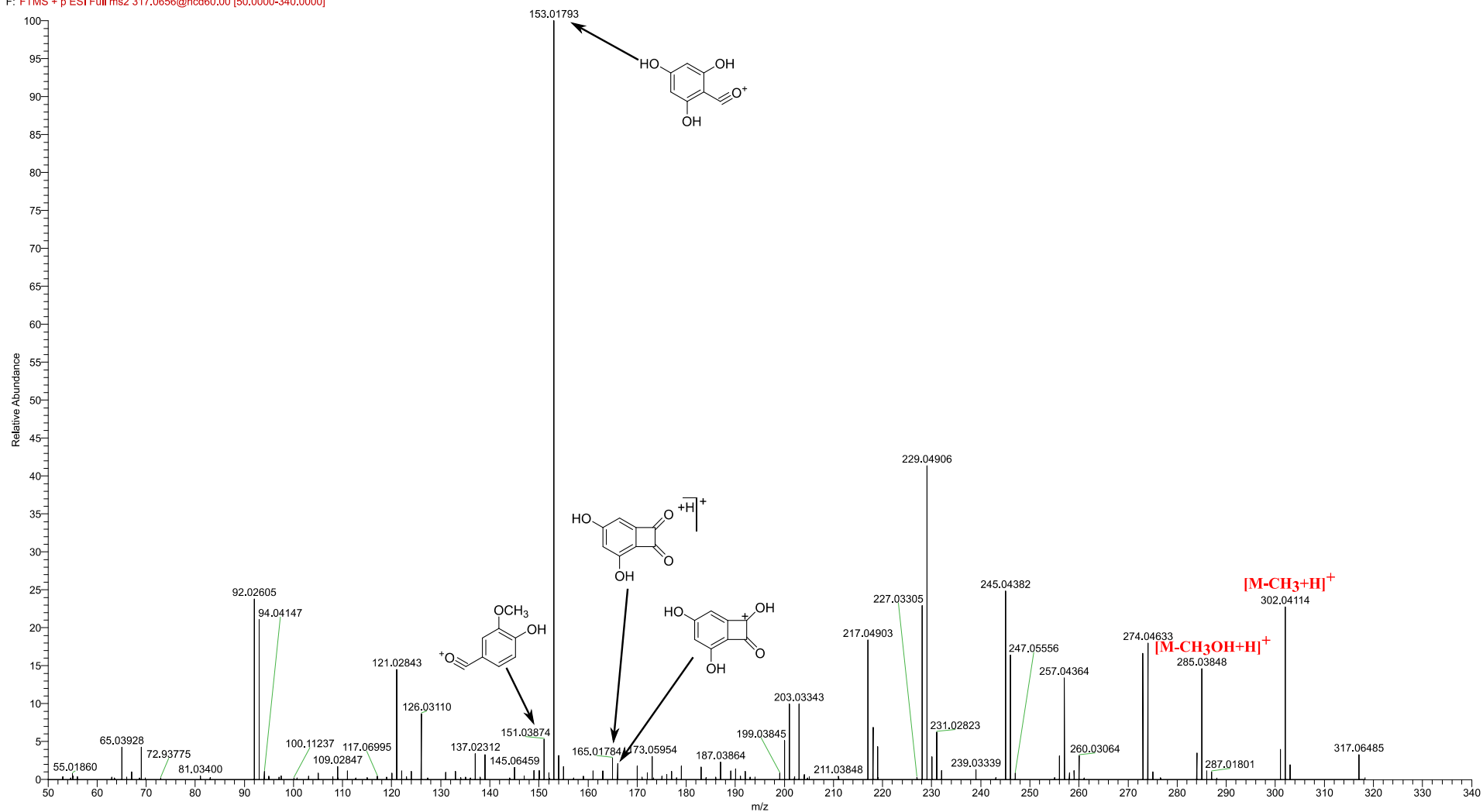


Figure S6: Pseudo MS³ spectrum of precursor at *m/z* 317.0656 due to aglycone of compound 3

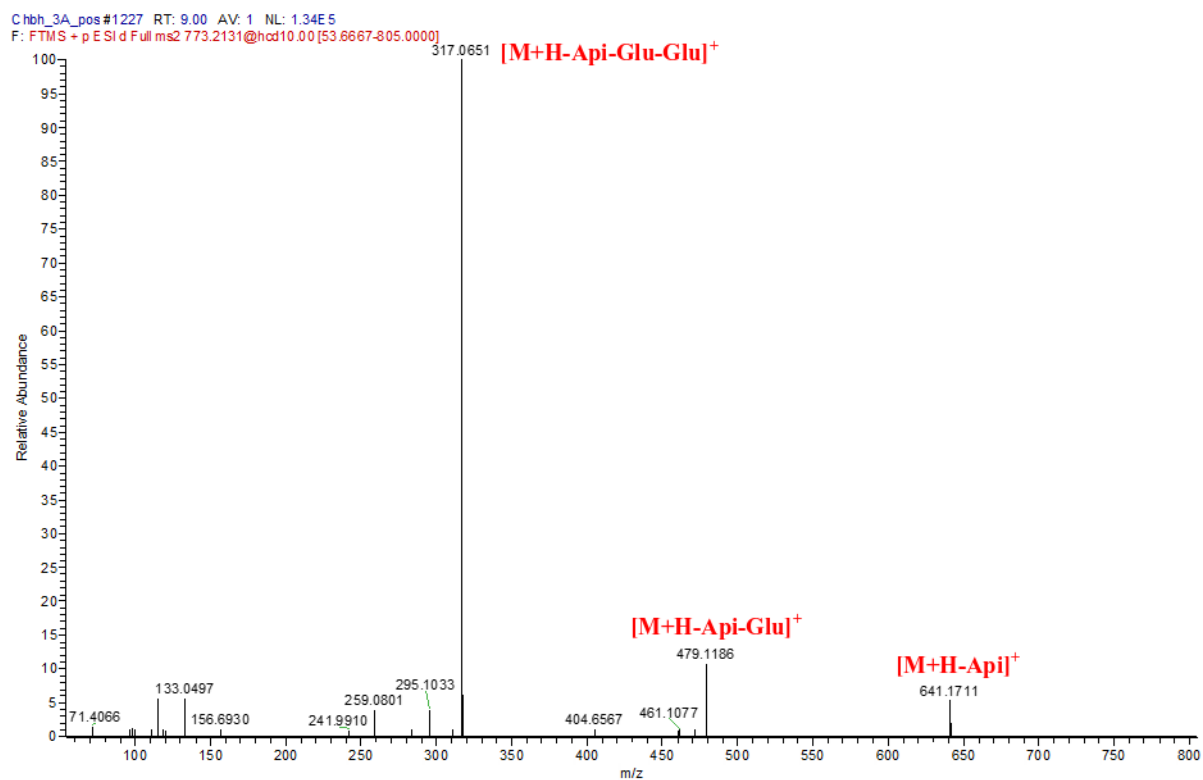


Figure S7: MS² spectrum of Compound 4

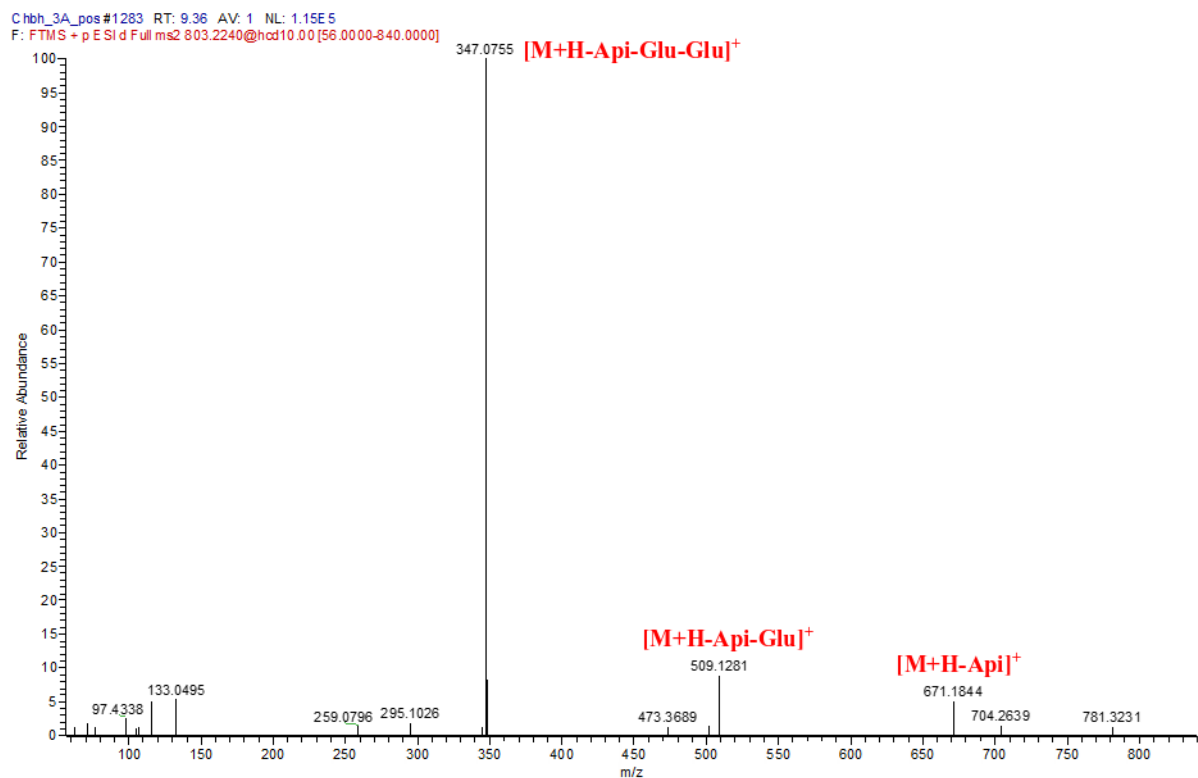


Figure S8: MS² spectrum of Compound 5

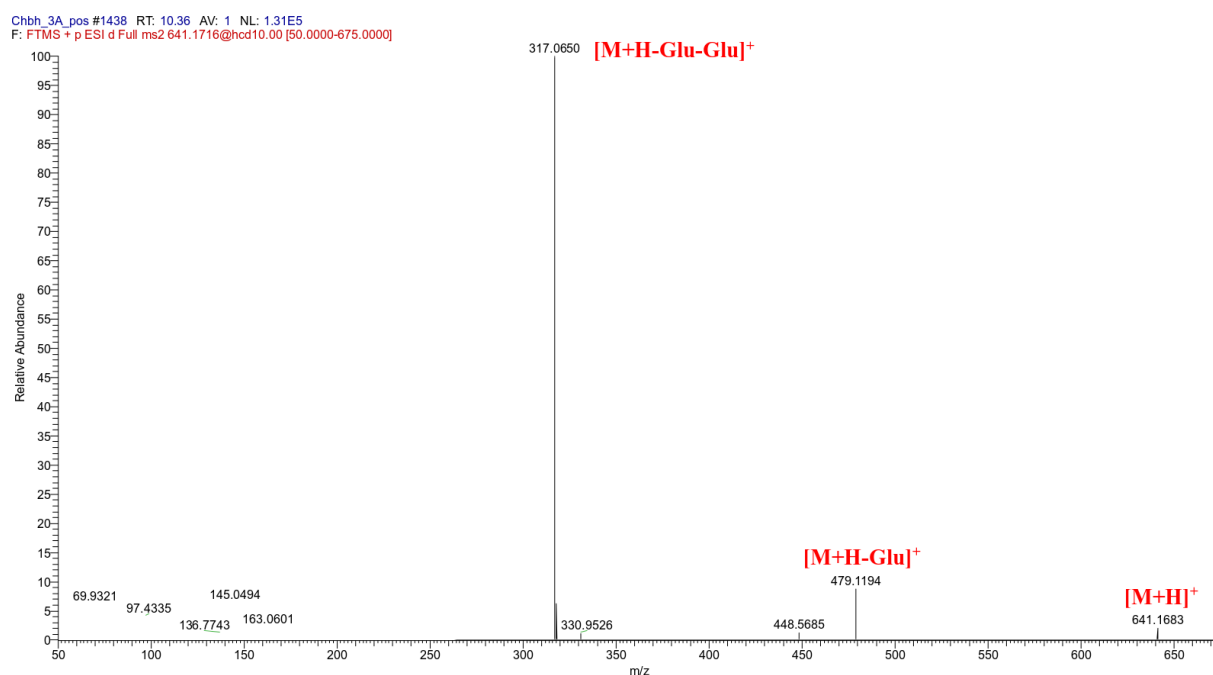


Figure S9: MS² spectrum of Compound 6

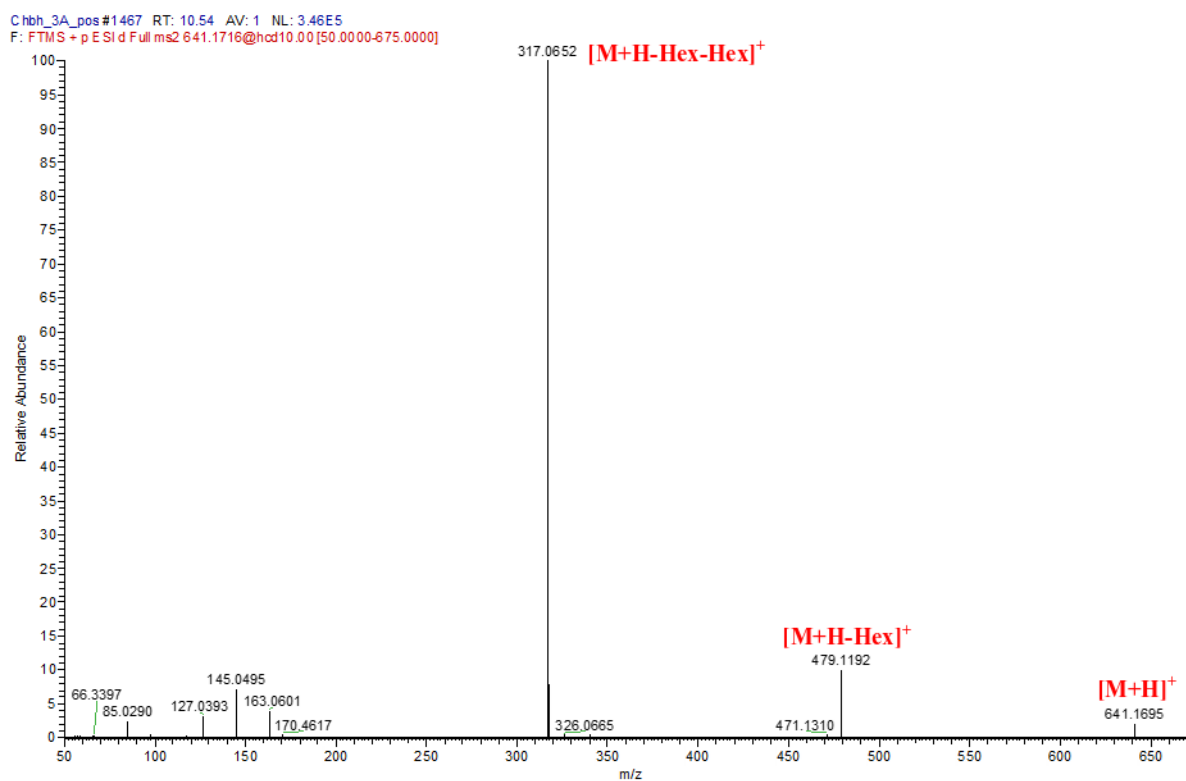


Figure S10: MS² spectrum of Compound 7

Chbh_NC_flav_PRM03 #1390 RT: 10.55 AV: 1 NL: 1.15E5
 F: FTMS + p ESI Full ms2 317.0656@hcd60.00 [50.0000-340.0000]

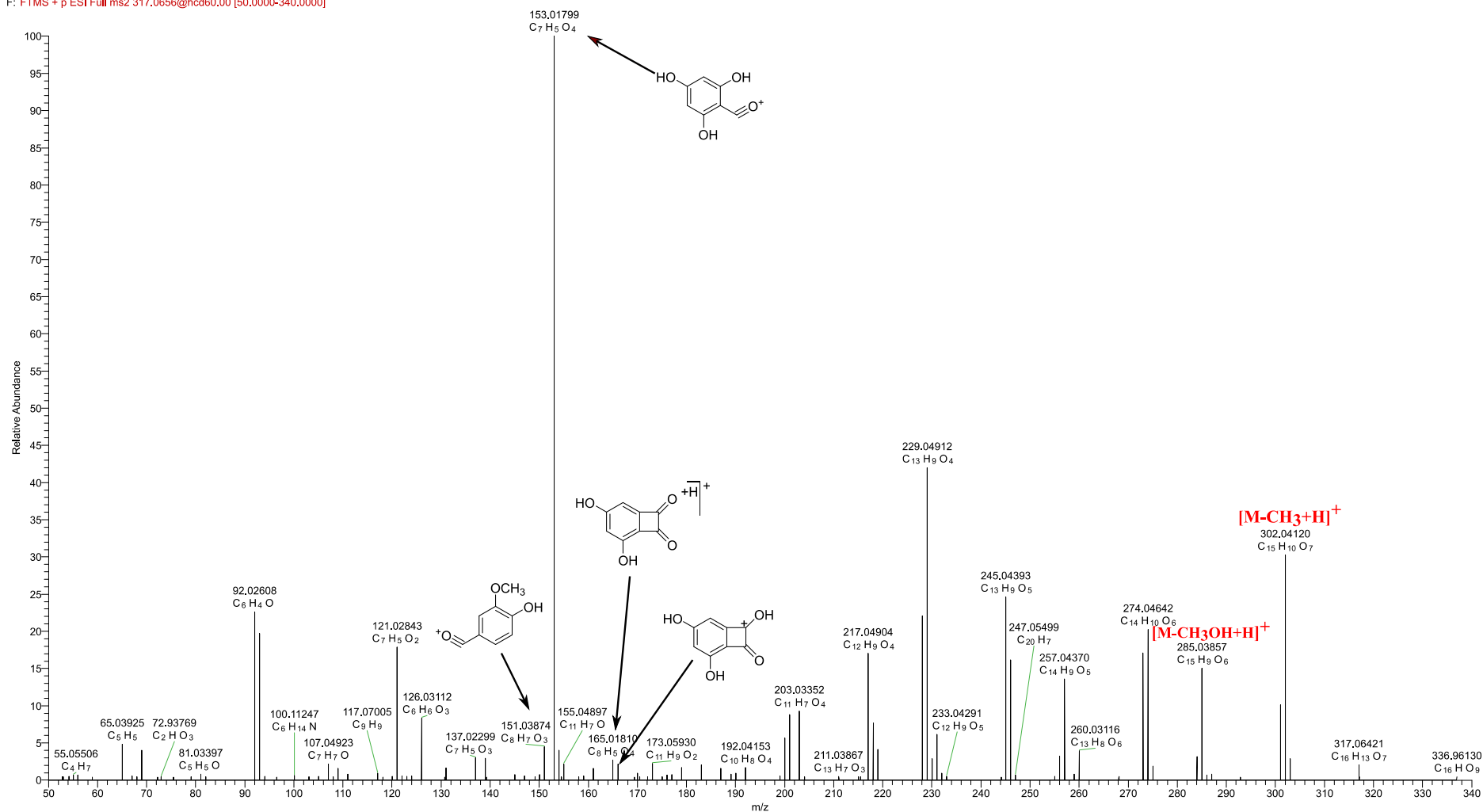


Figure S11: Pseudo MS³ spectrum of precursor at *m/z* 317.0656 due to aglycone of compound 7

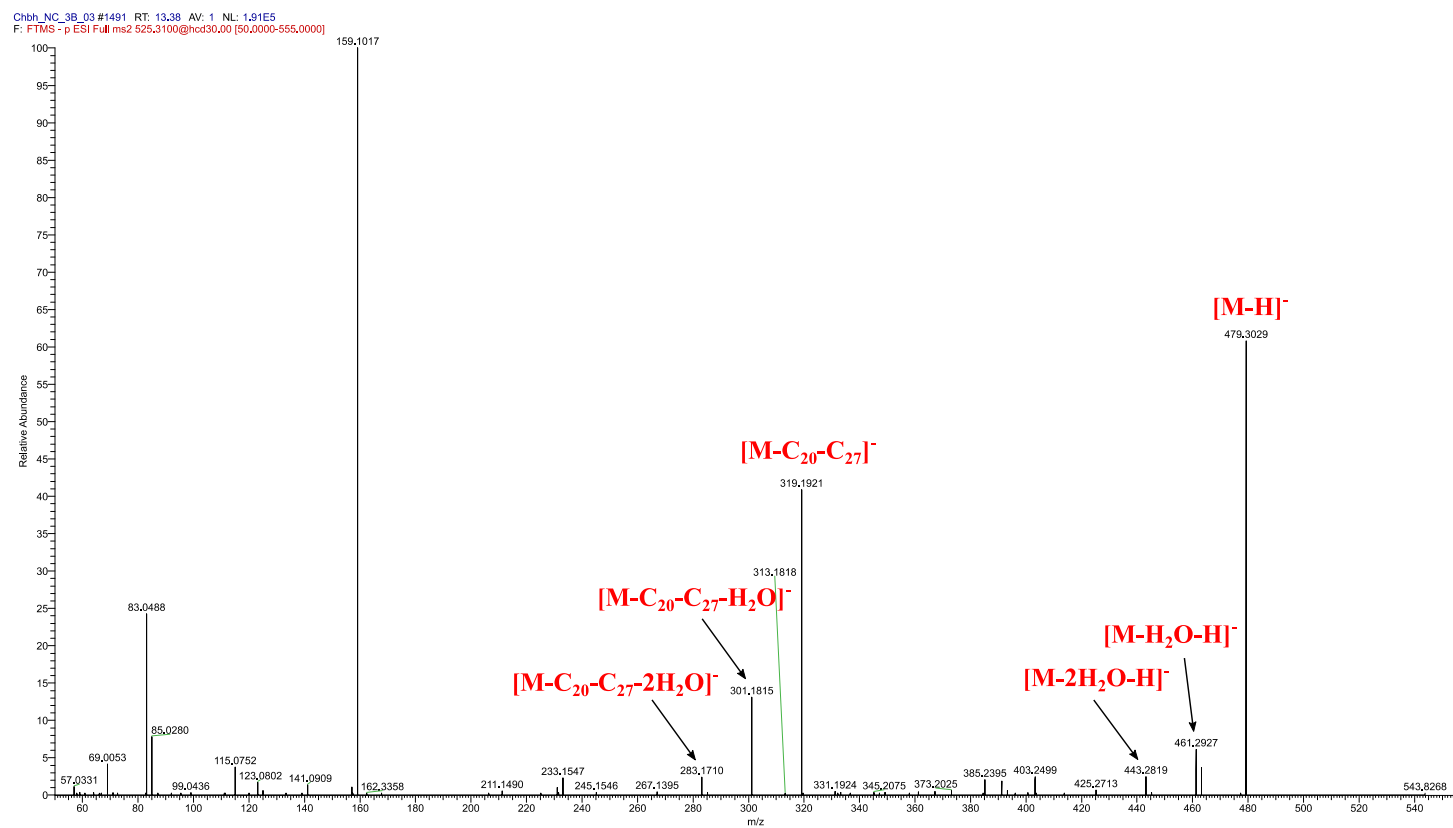


Figure S12: MS² spectrum of Compound 8

Chbh_NC_3B_04 #1319 RT: 12.64 AV: 1 NL: 1.82E5
F: FTMS - p ESI Full ms2 541.3000@hcd35.00 [50.0000-570.0000]

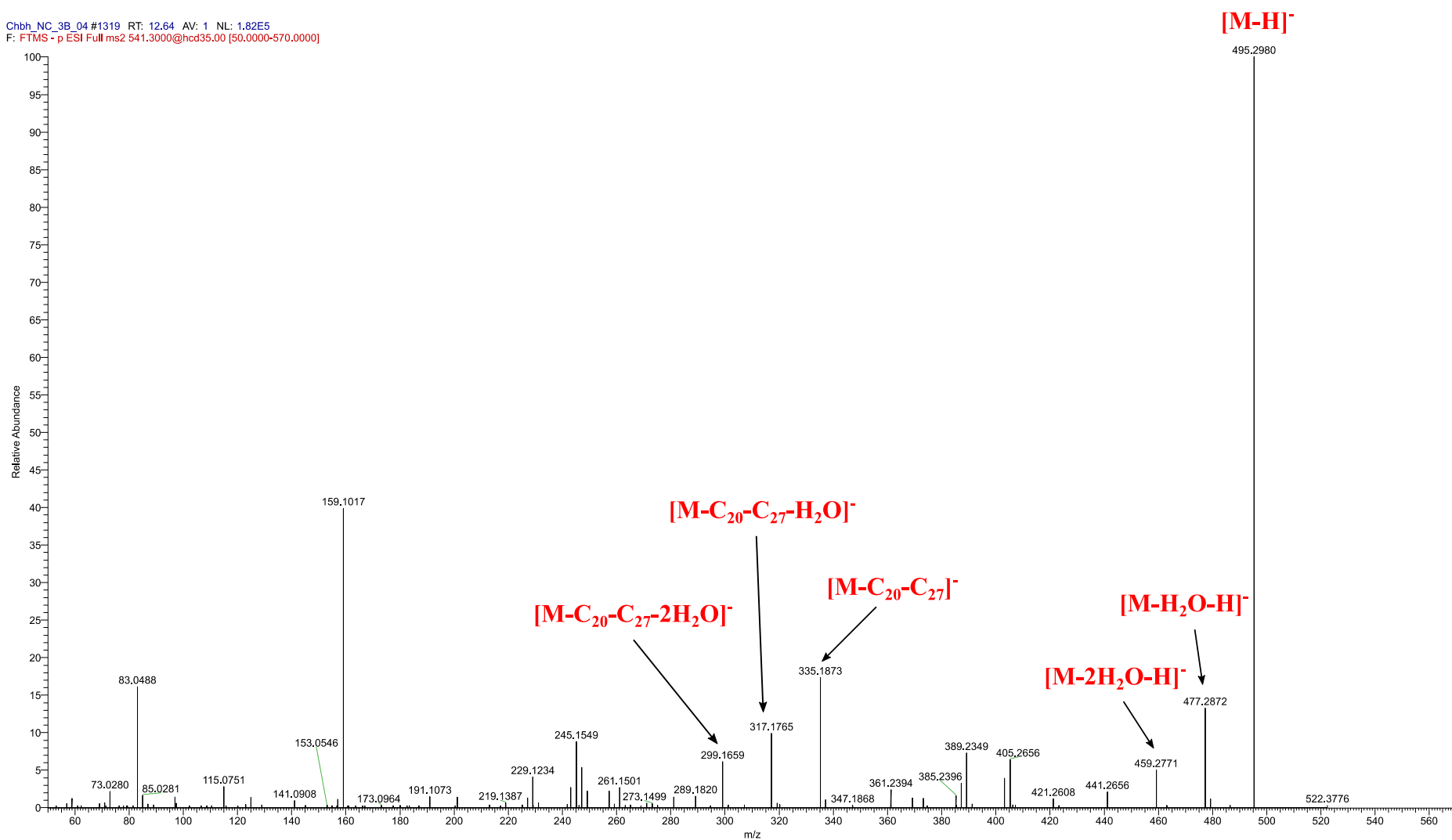


Figure S13: MS² spectrum of Compound 9

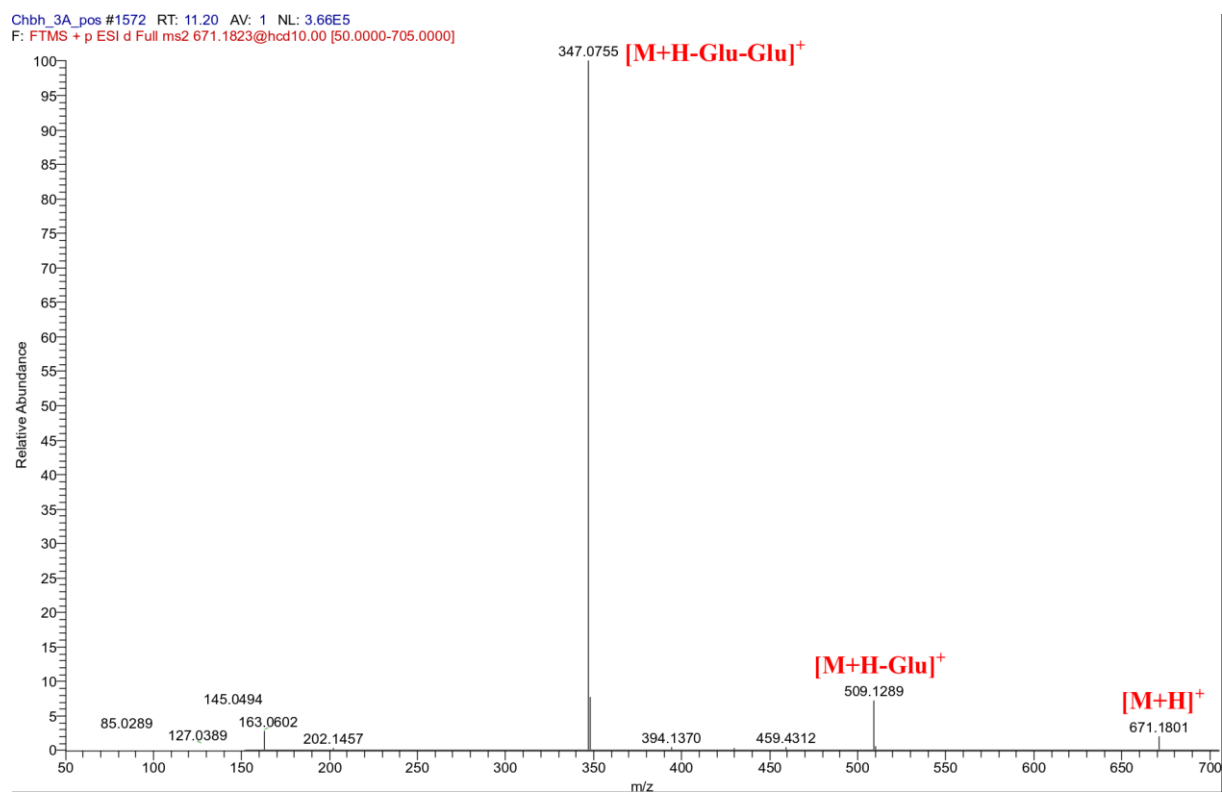


Figure S14: MS² spectrum of Compound 10

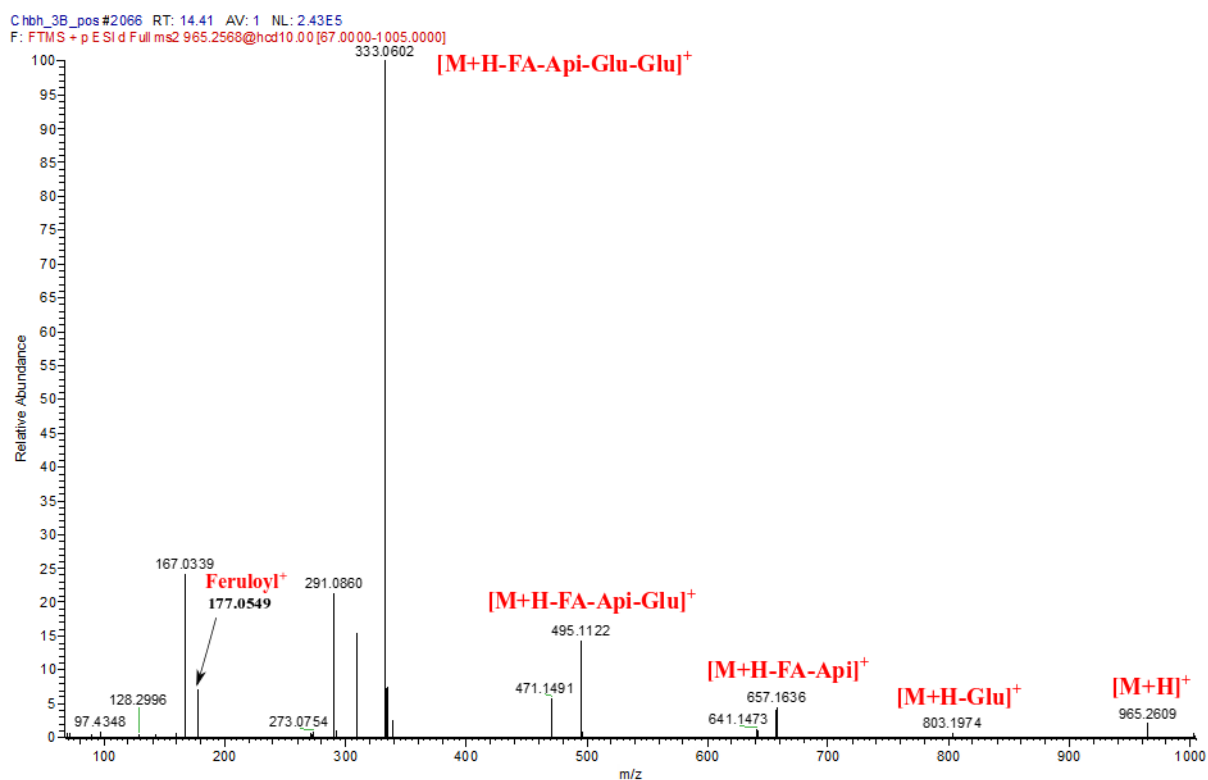


Figure S15: MS² spectrum of Compound 11

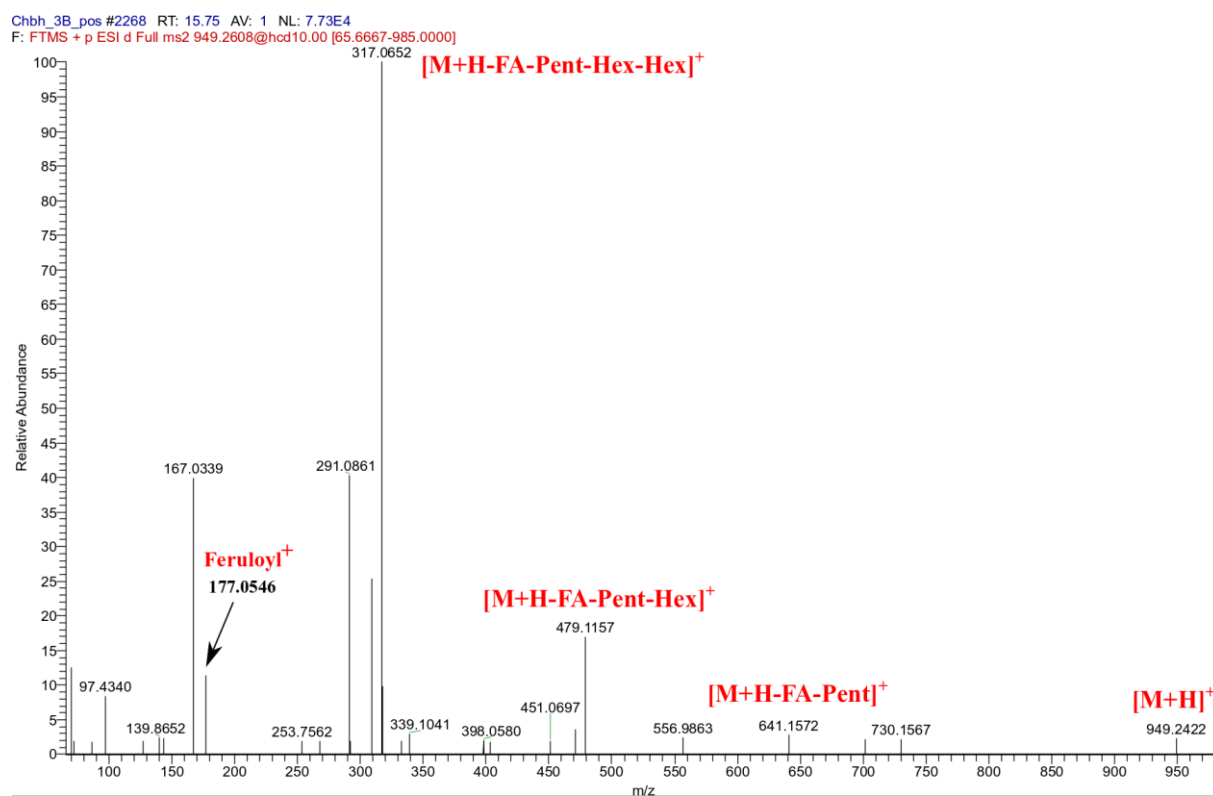


Figure S16: MS² spectrum of Compound 12

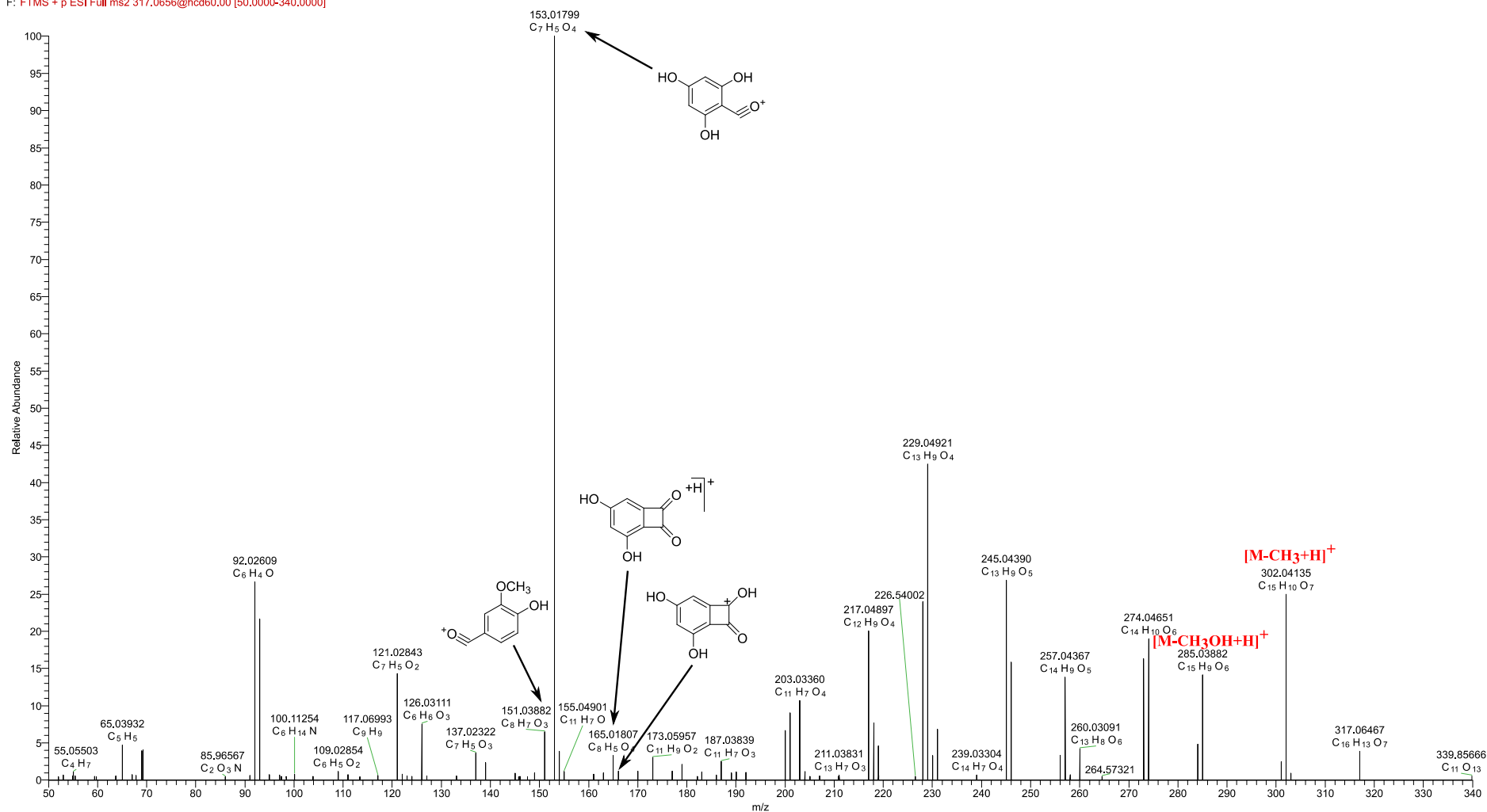


Figure S17: Pseudo MS³ spectrum of precursor at m/z 317.0656 due to aglycone of compound 12

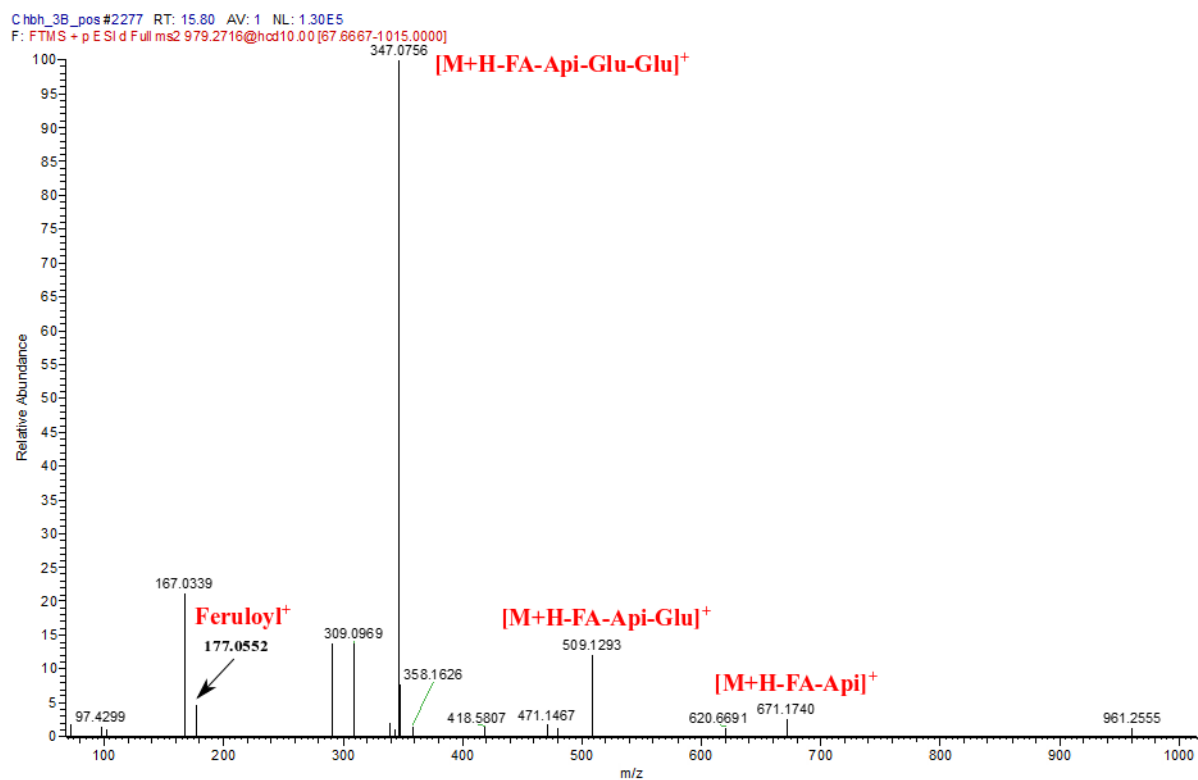


Figure S18: MS² spectrum of Compound 13

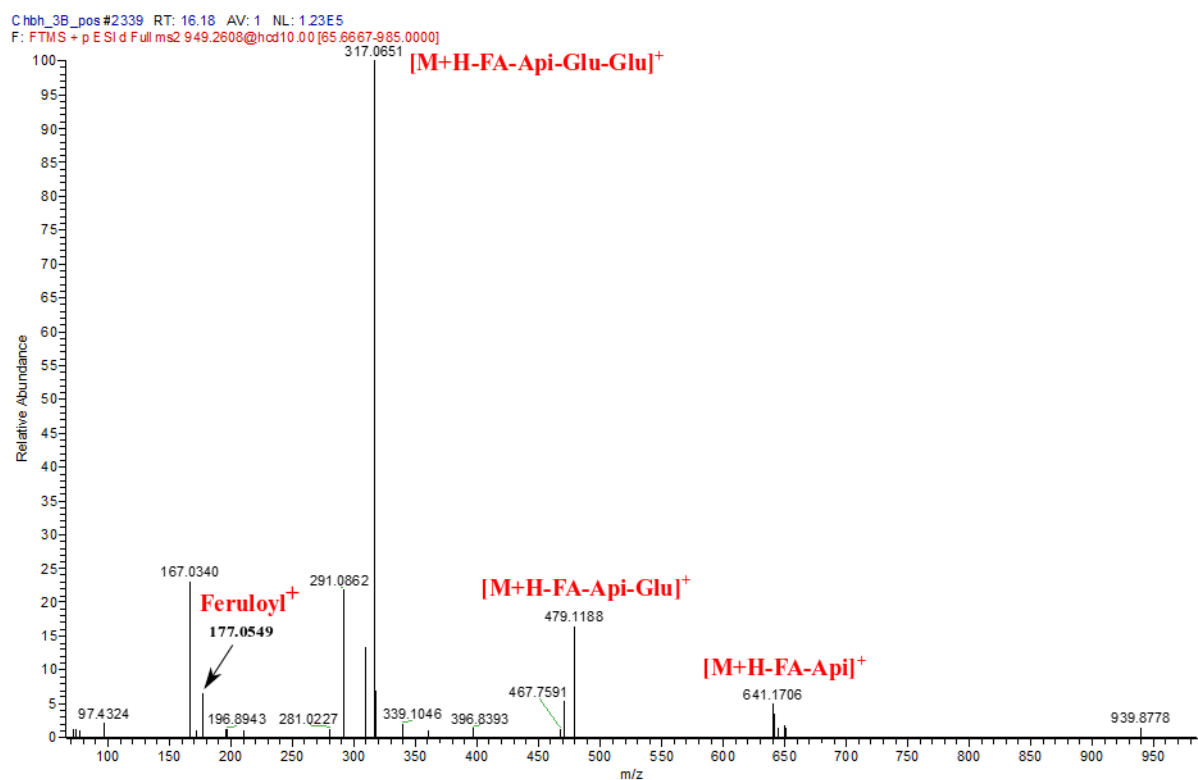


Figure S19: MS² spectrum of Compound 14

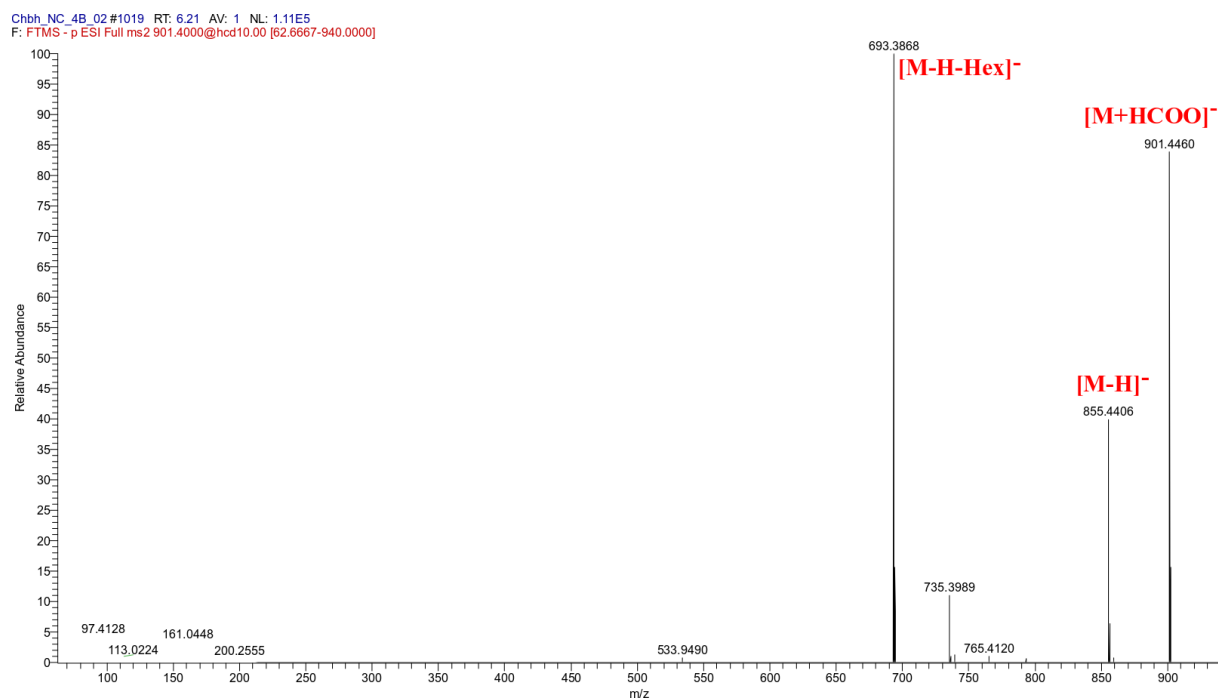


Figure S20: MS² spectrum (NCE=10) of Compound **15**

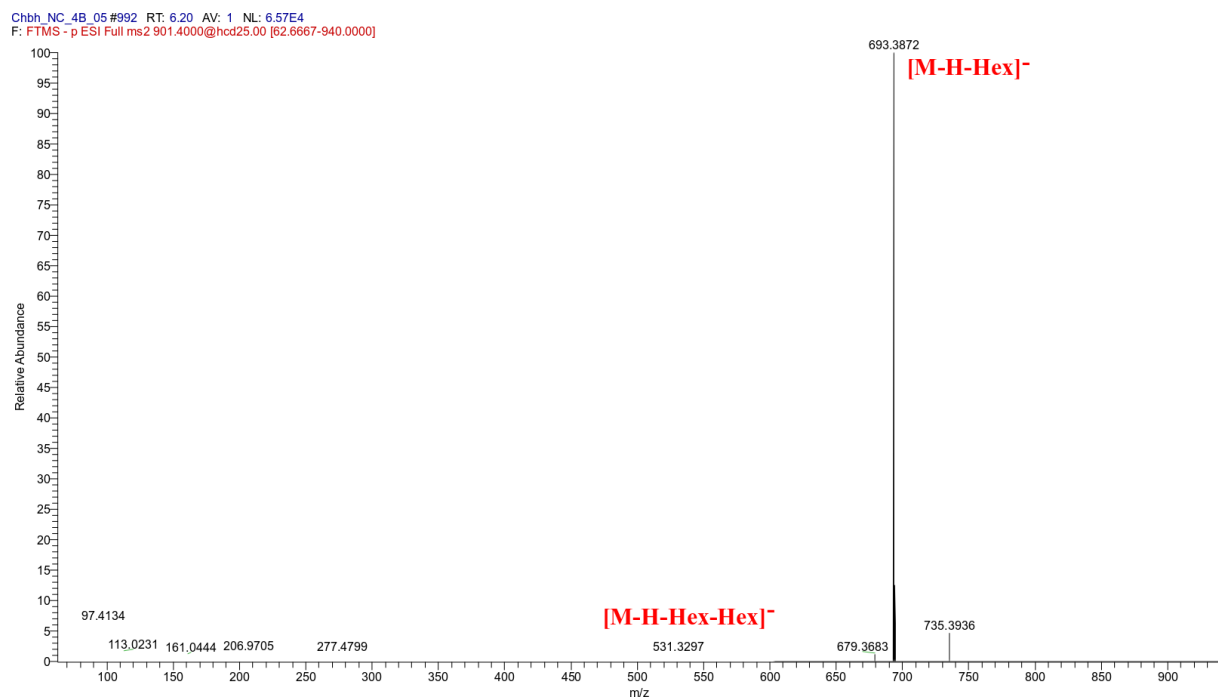


Figure S21: MS² spectrum (NCE=25) of Compound **15**

Chbh_NC_4B_02 #1129 RT: 6.45 AV: 1 NL: 3.33E4
F: FTMS - p ESI Full ms2 841.4000@hcd10.00 [58.3333-875.0000]

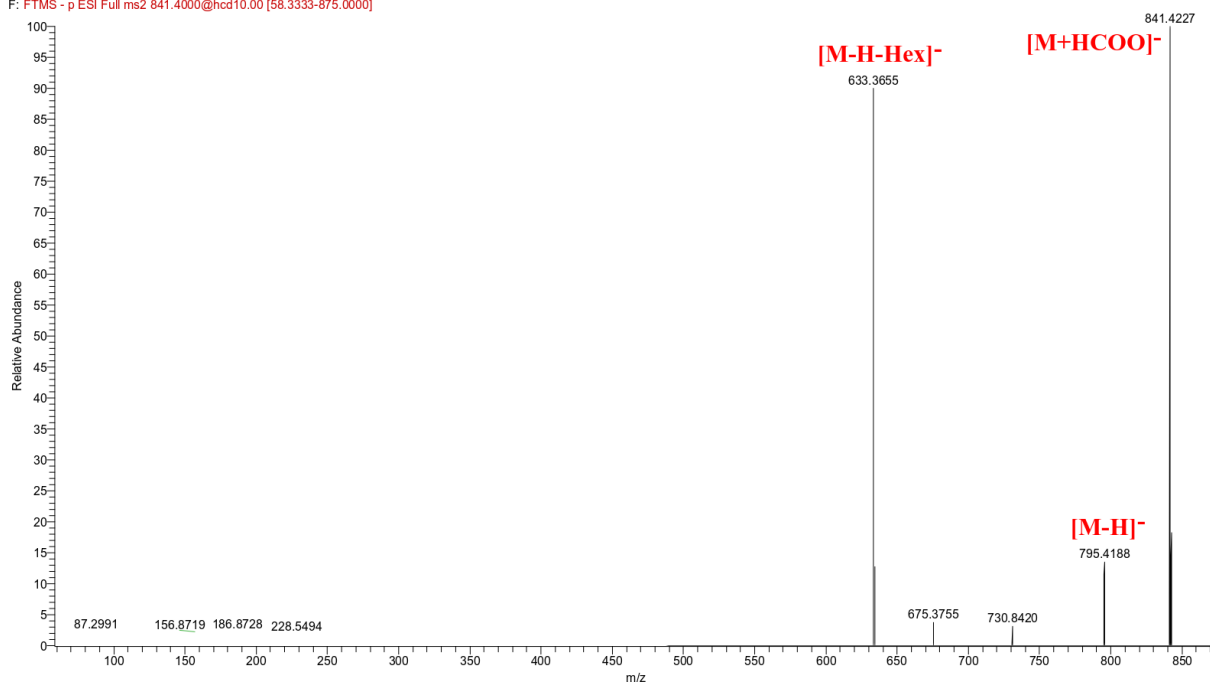


Figure S22: MS² spectrum (NCE=10) of Compound **16**

Chbh_NC_4B_05 #1109 RT: 6.46 AV: 1 NL: 3.77E4
F: FTMS - p ESI Full ms2 841.4000@hcd25.00 [58.3333-875.0000]

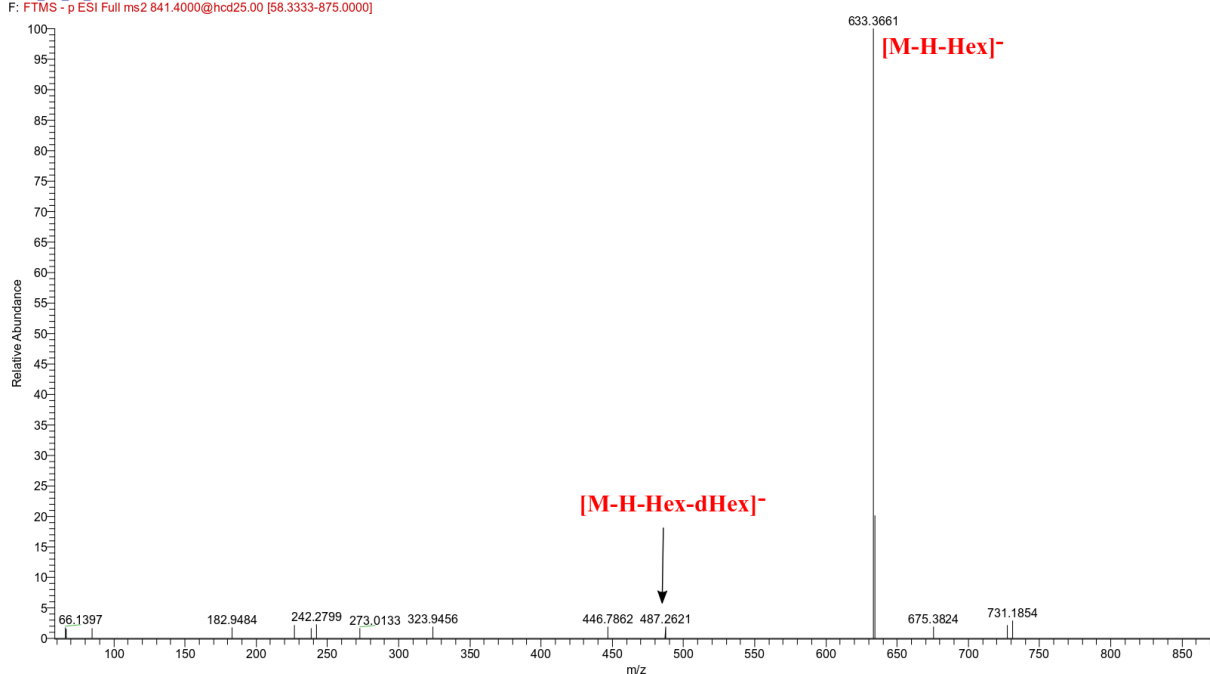


Figure S23: MS² spectrum (NCE=25) of Compound **16**

Chbh_NC_4B_Q2#1258 RT: 6.73 AV: 1 NL: 2.05E5
F: FTMS -p ESI Full ms2 1033.5000@hcd10.00 [71.6667-1075.0000]

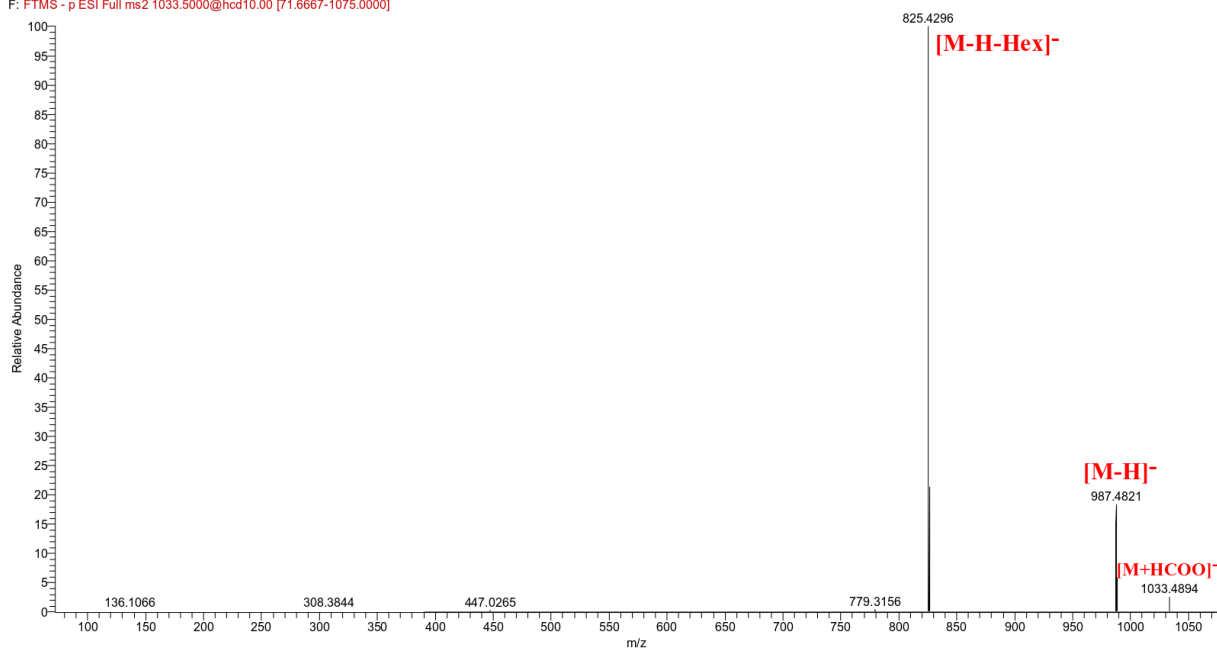


Figure S24: MS² spectrum (NCE=10) of Compound 17

Chbh_NC_4B_Q5#1231 RT: 6.73 AV: 1 NL: 1.16E5
F: FTMS -p ESI Full ms2 1033.5000@hcd25.00 [71.6667-1075.0000]

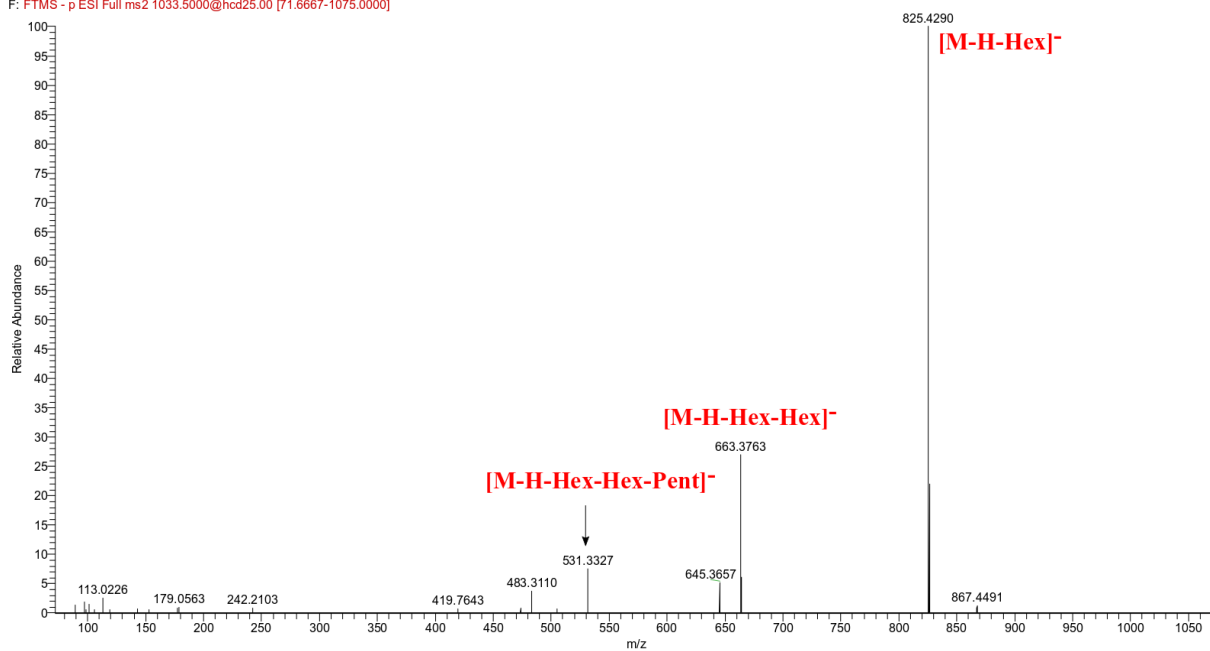


Figure S25: MS² spectrum (NCE=25) of Compound 17

Chbh_NC_4B_04 #1546 RT: 7.41 AV: 1 NL: 1.06E5
 F: FTMS - p ESI Full ms2 871.4000@hcd20.00 [60.3333-905.0000]

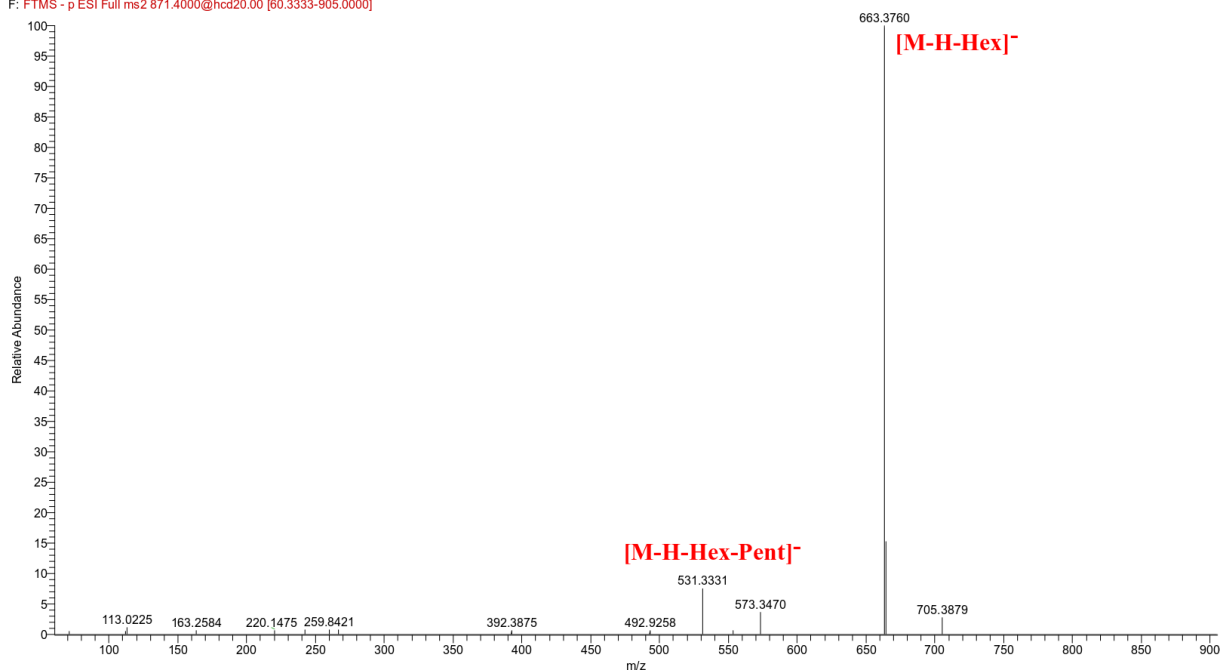


Figure S26: MS² spectrum of Compound 18

Chbh_NC_4B_04 #1633 RT: 7.61 AV: 1 NL: 1.43E6
 F: FTMS - p ESI Full ms2 871.4000@hcd20.00 [60.3333-905.0000]

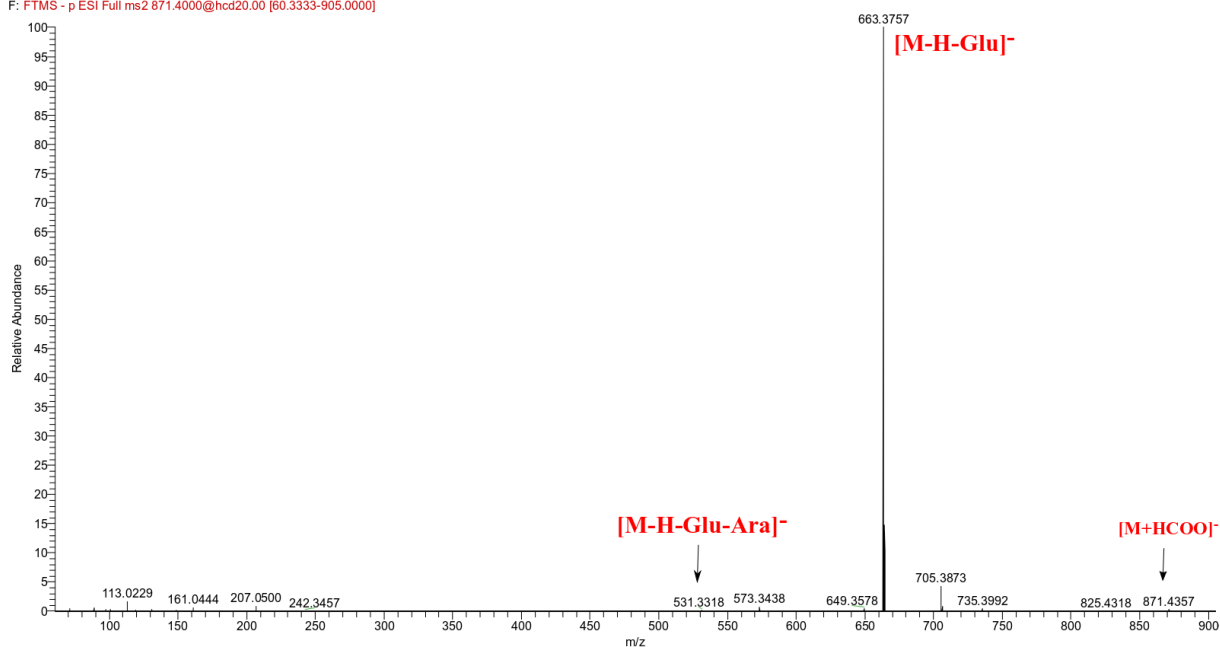


Figure S27: MS² spectrum of Compound 19

Chbh_NC_4B_04 #1779 RT: 7.94 AV: 1 NL: 3.35E5
F: FTMS - p ESI Full ms2 807.4000@hcd20.00 [56.0000-840.0000]

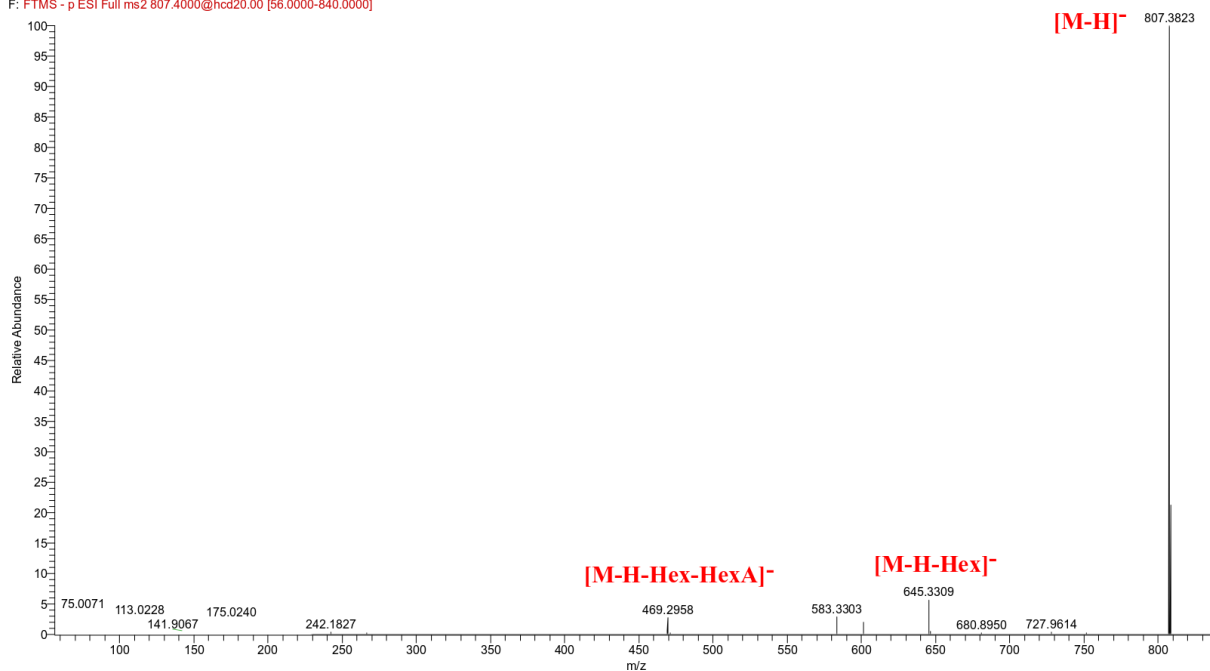


Figure S28: MS² spectrum of Compound 20

Chbh_NC_4B_02 #1807 RT: 7.94 AV: 1 NL: 9.56E5
F: FTMS - p ESI Full ms2 1017.5000@hcd10.00 [70.3333-1055.0000]

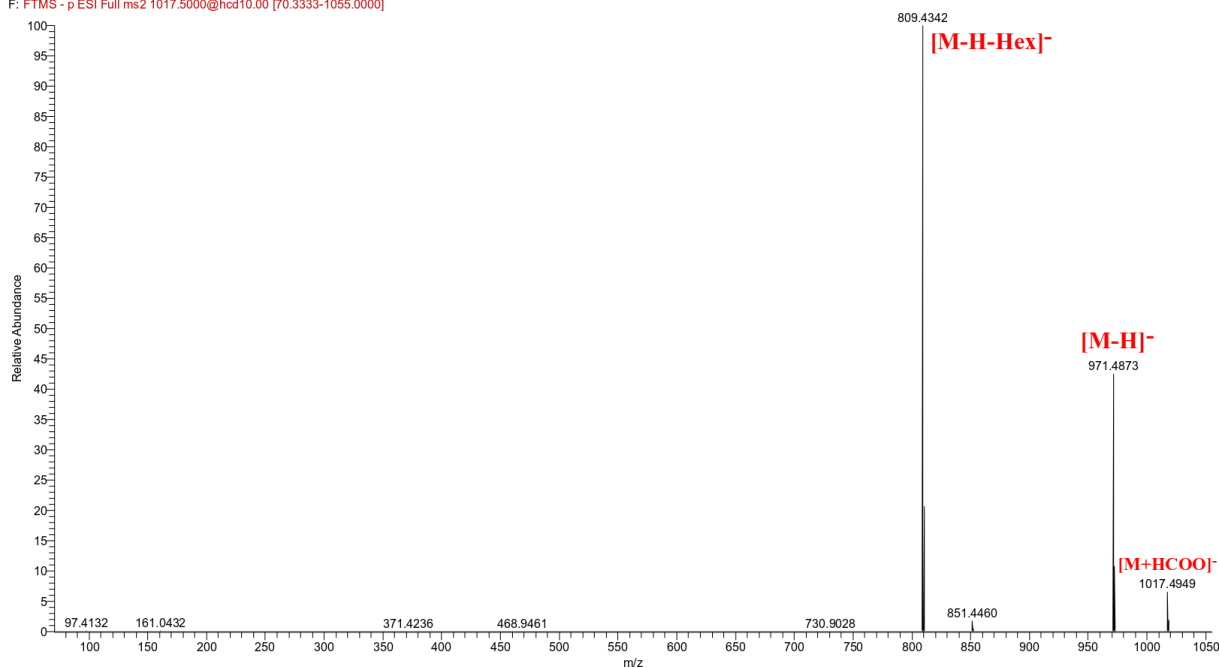


Figure S29: MS² spectrum (NCE=10) of Compound 21

Chbh_NC_4B_07#1757 RT: 7.97 AV: 1 NL: 1.74E5
F: FTMS -p ESI Full ms2 1017.5000@hcd35.00 [70.3333-1055.0000]

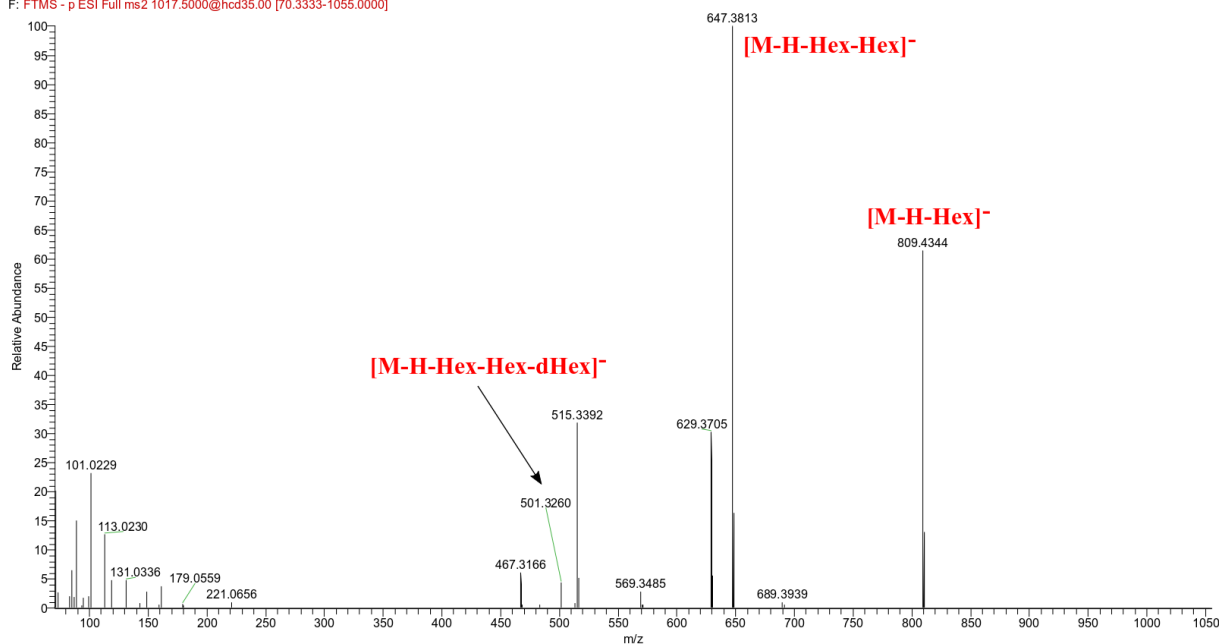


Figure S30: MS² spectrum (NCE=35) of Compound 21

Chbh_NC_4B_04#2014 RT: 8.47 AV: 1 NL: 5.94E5
F: FTMS -p ESI Full ms2 825.4000@hcd20.00 [57.3333-860.0000]

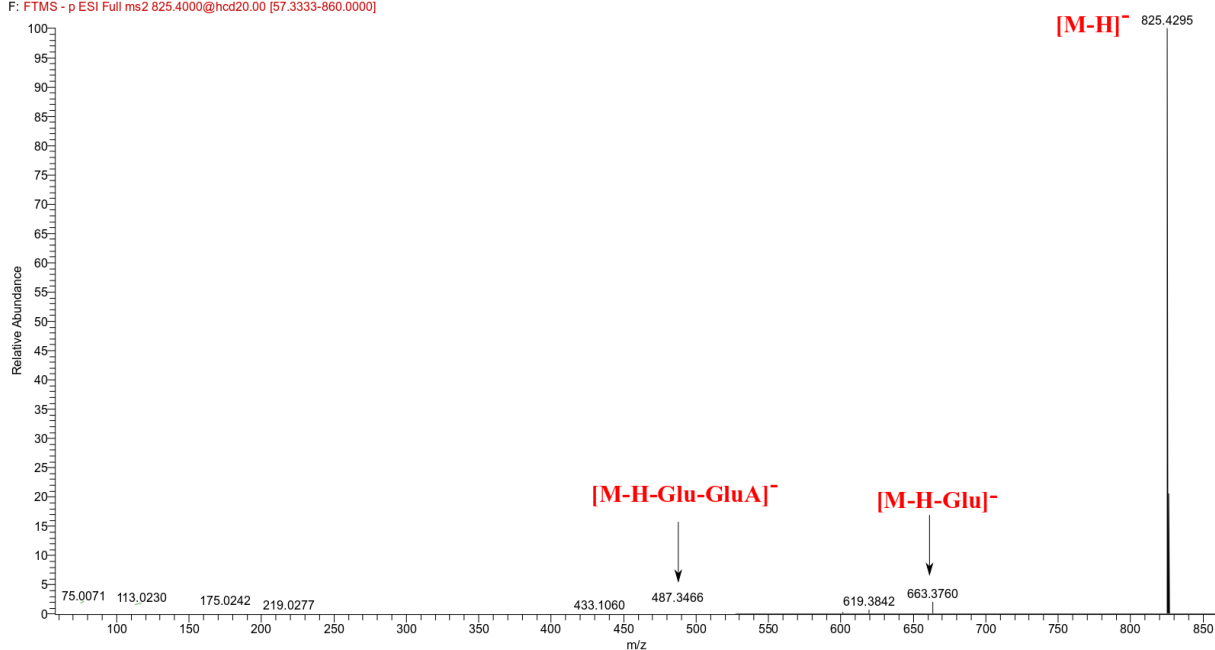


Figure S31: MS² spectrum of Compound 22

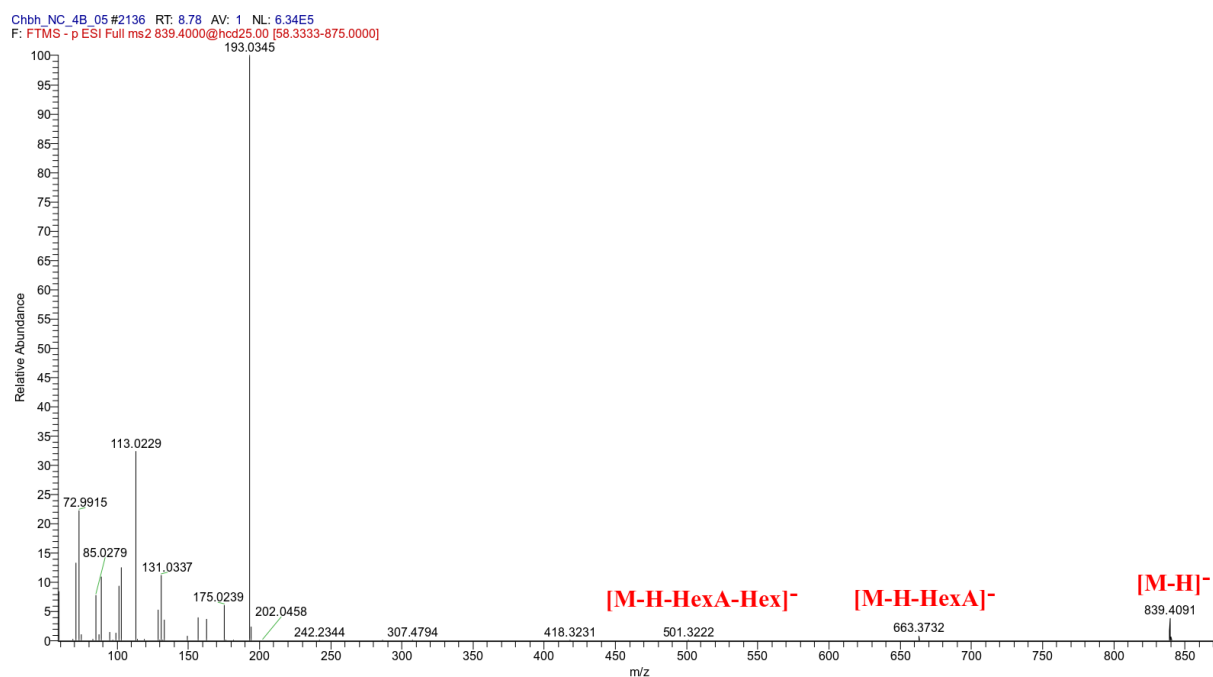


Figure S32: MS² spectrum of Compound 23

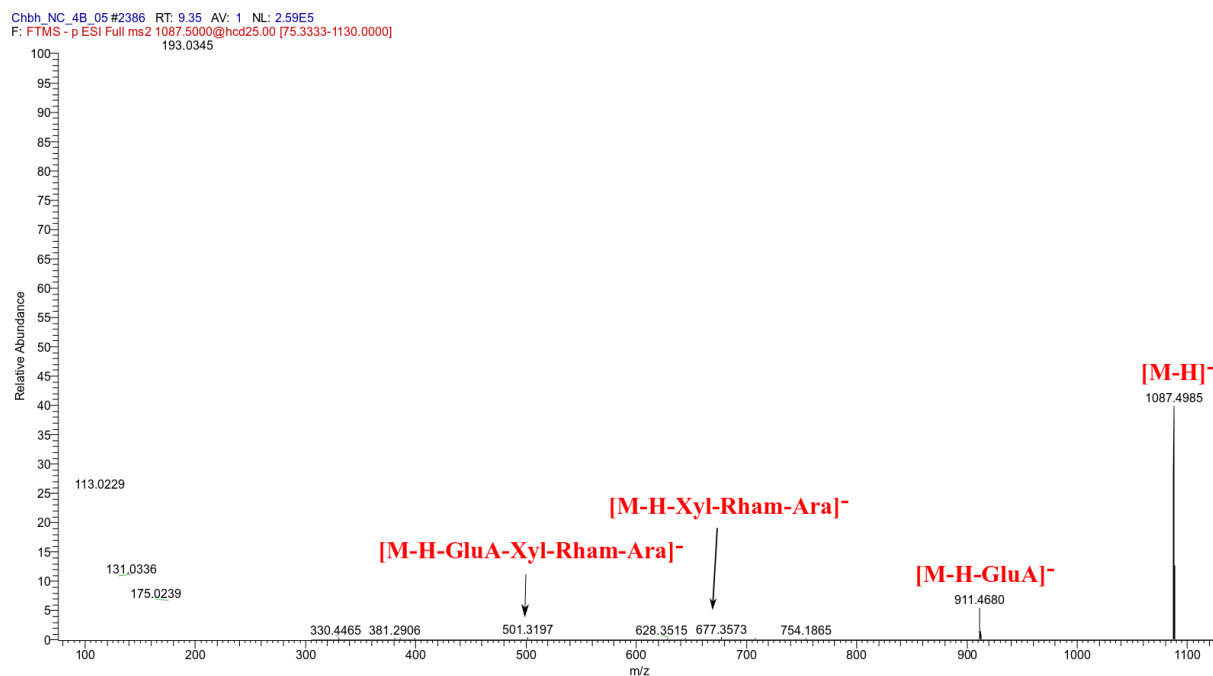


Figure S33: MS² spectrum of Compound 24

Chbh_NC_4B_02 #2526 RT: 9.55 AV: 1 NL: 5.35E4
 F: FTMS -p ESI Full ms2 739.4000@hcd10.00 [51.6667-775.0000]

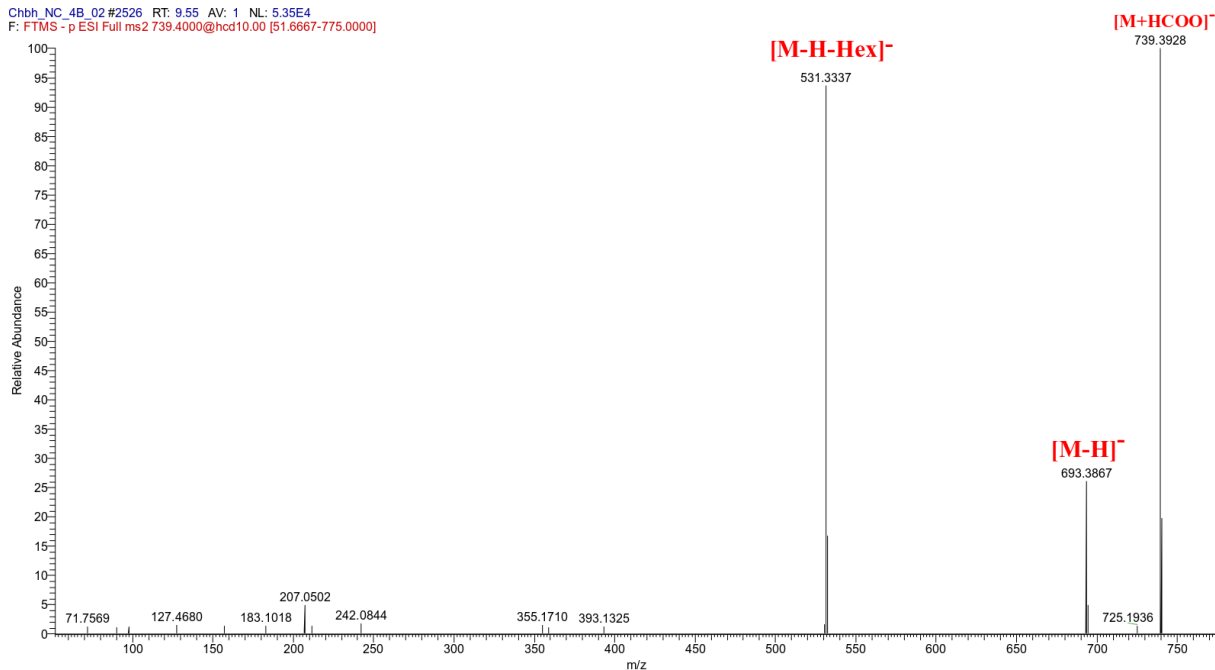


Figure S34: MS² spectrum of Compound 25

Chbh_NC_4B_03 #2605 RT: 9.77 AV: 1 NL: 1.27E6
 F: FTMS -p ESI Full ms2 823.4000@hcd15.00 [57.3333-860.0000]

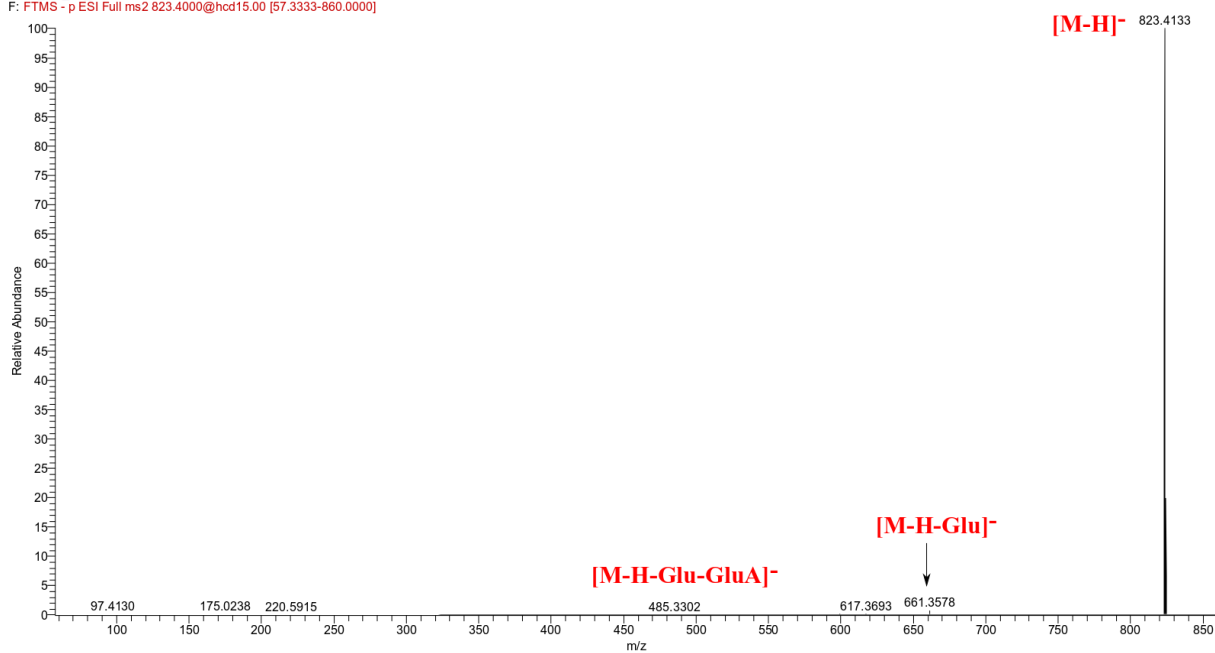


Figure S35: MS² spectrum of Compound 26

Chbh_NC_4B_05 #2763 RT: 10.20 AV: 1 NL: 7.09E3
 F: FTMS -p ESI Full ms2 793.4000@hcd25.00 [55.3333-830.0000]

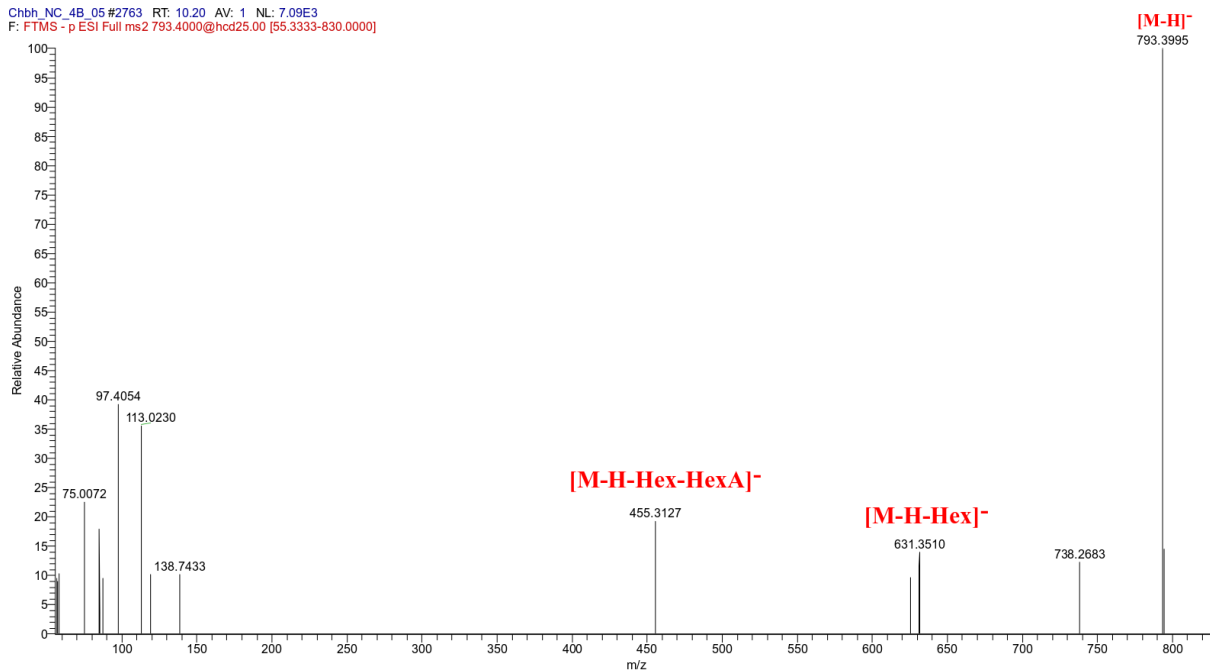


Figure S36: MS² spectrum of Compound 27

Chbh_NC_4B_04 #2886 RT: 10.44 AV: 1 NL: 9.04E4
 F: FTMS -p ESI Full ms2 827.4000@hcd20.00 [57.3333-860.0000]

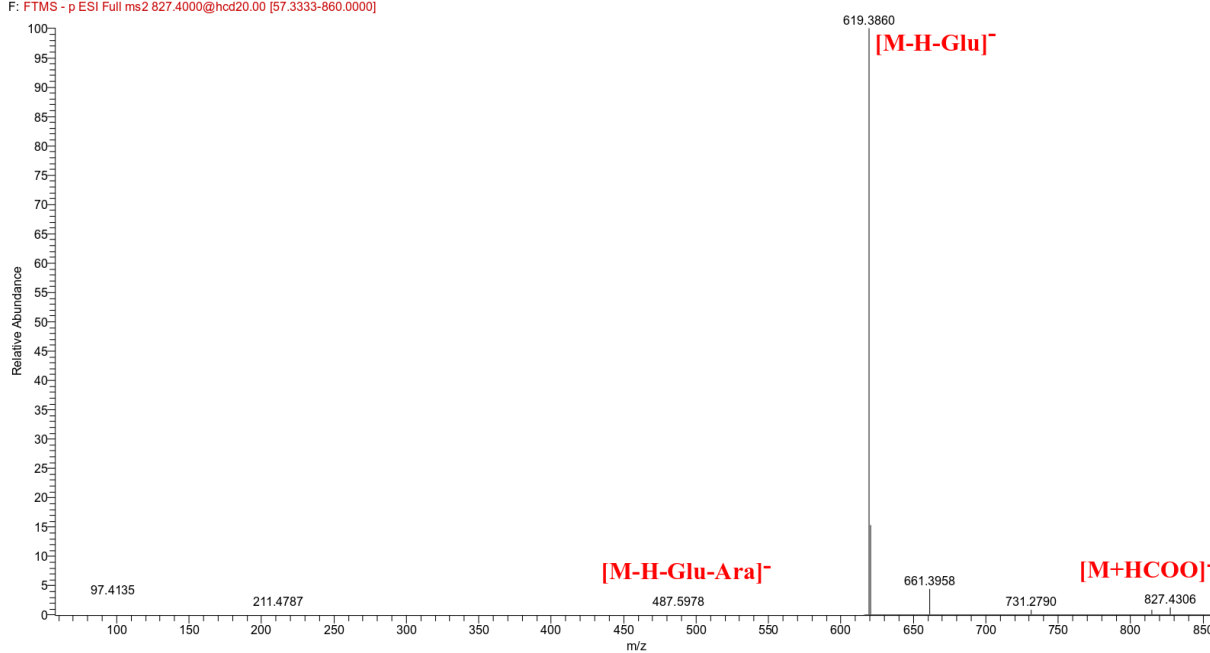


Figure S37: MS² spectrum of Compound 28

Chbh_NC_4B_06 #3188 RT: 11.19 AV: 1 NL: 1.69E4
 F: FTMS - p ESI Full ms2 777.4000@hcd30.00 [54.0000-810.0000]

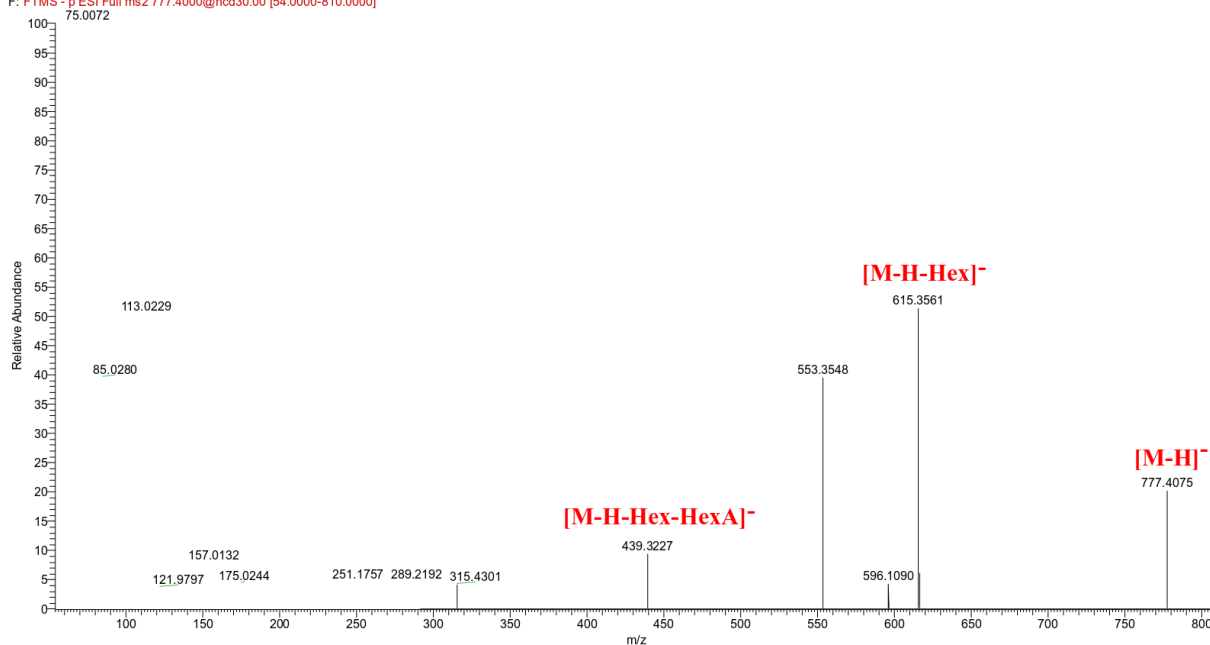


Figure S38: MS² spectrum of Compound 29

Chbh_NC_4B_04 #3597 RT: 12.00 AV: 1 NL: 2.54E5
 F: FTMS - p ESI Full ms2 809.4000@hcd20.00 [56.3333-845.0000]



Figure S39: MS² spectrum of Compound 30

Chbh_NC_4B_03 #3699 RT: 12.17 AV: 1 NL: 1.25E5
F: FTMS - p ESI Full ms2 663.4000@hcd15.00 [50.0000-695.0000]

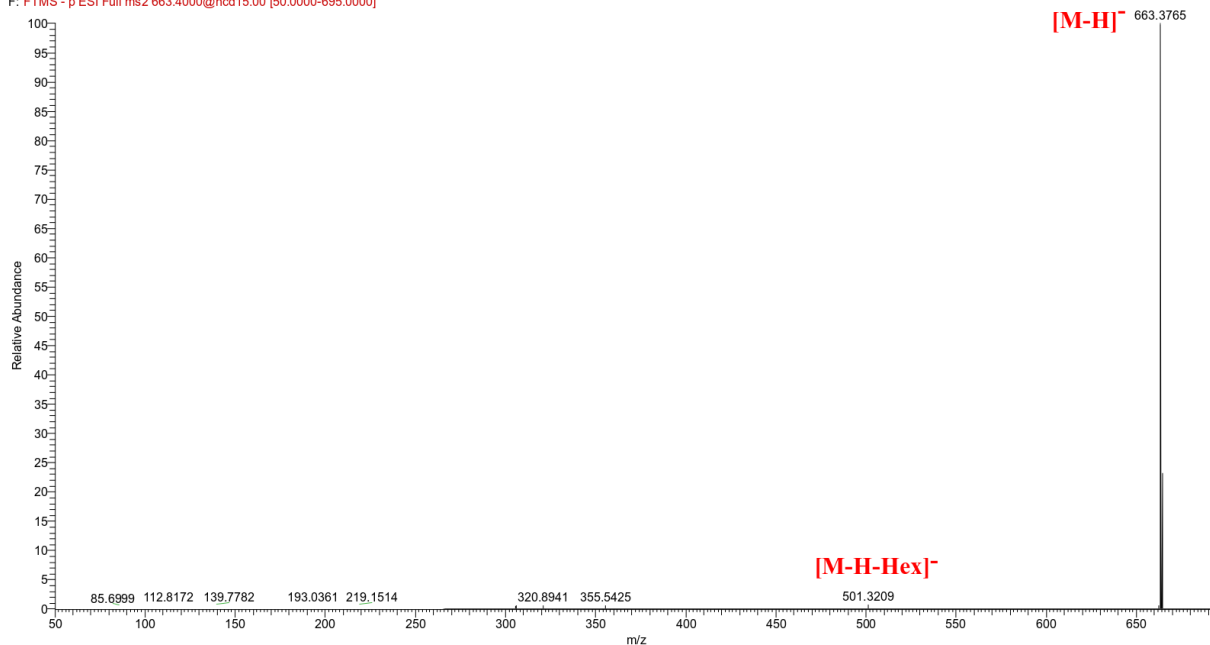


Figure S40: MS² spectrum of Compound 31

Chbh_NC_4B_03 #3756 RT: 12.30 AV: 1 NL: 1.12E6
F: FTMS - p ESI Full ms2 709.4000@hcd15.00 [50.0000-740.0000]

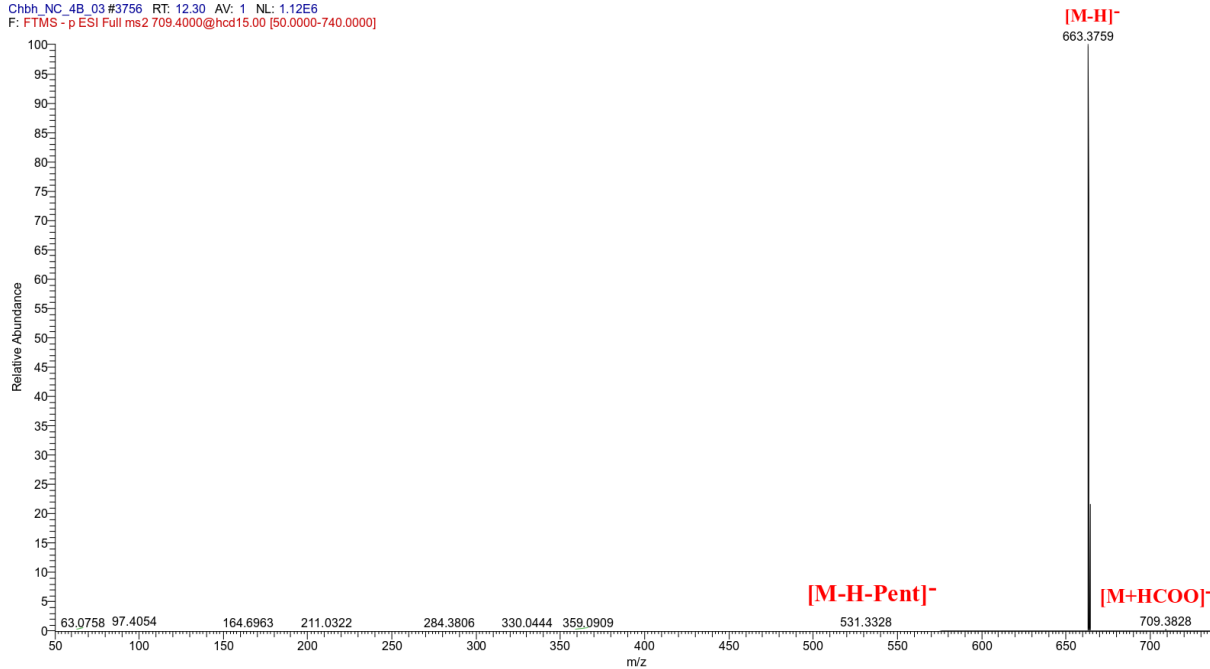


Figure S41: MS² spectrum of Compound 32

Chbh_NC_neg_4B #6267 RT: 14.02 AV: 1 NL: 1.72E4
F: FTMS -p ESI d Full ms2 645.3296@hcd25.00 [50.0000-675.0000]

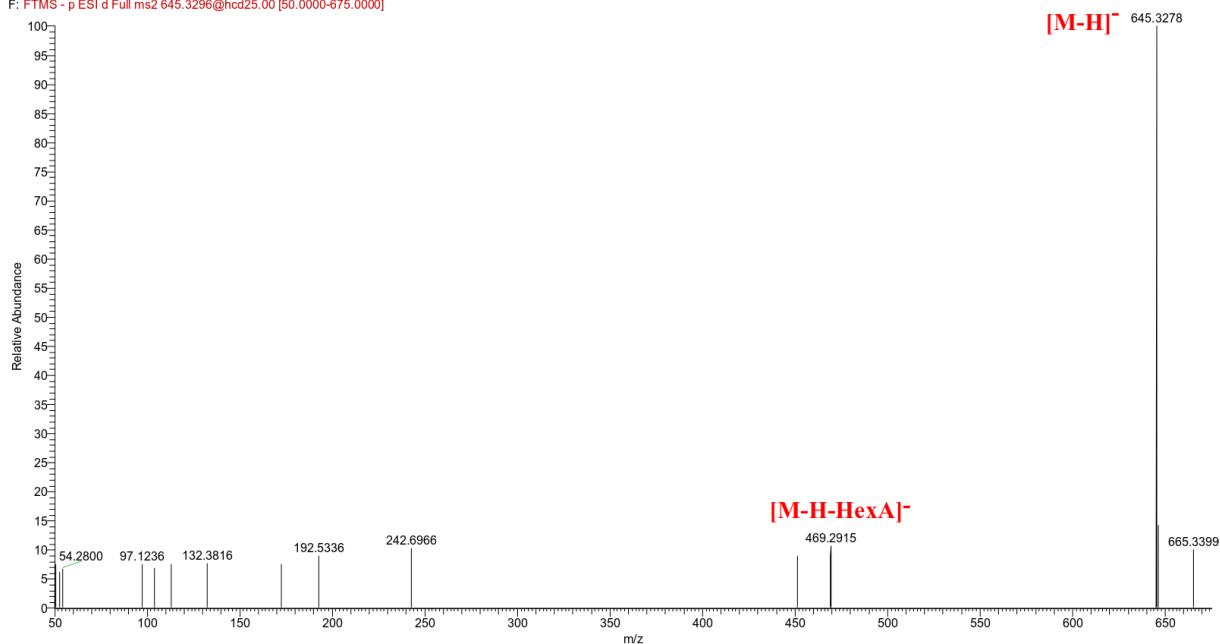


Figure S42: MS² spectrum of Compound 33

Chbh_NC_4B_04 #2207 RT: 14.52 AV: 1 NL: 4.10E4
F: FTMS -p ESI d Full ms2 677.3562@hcd35.00 [50.0000-710.0000]

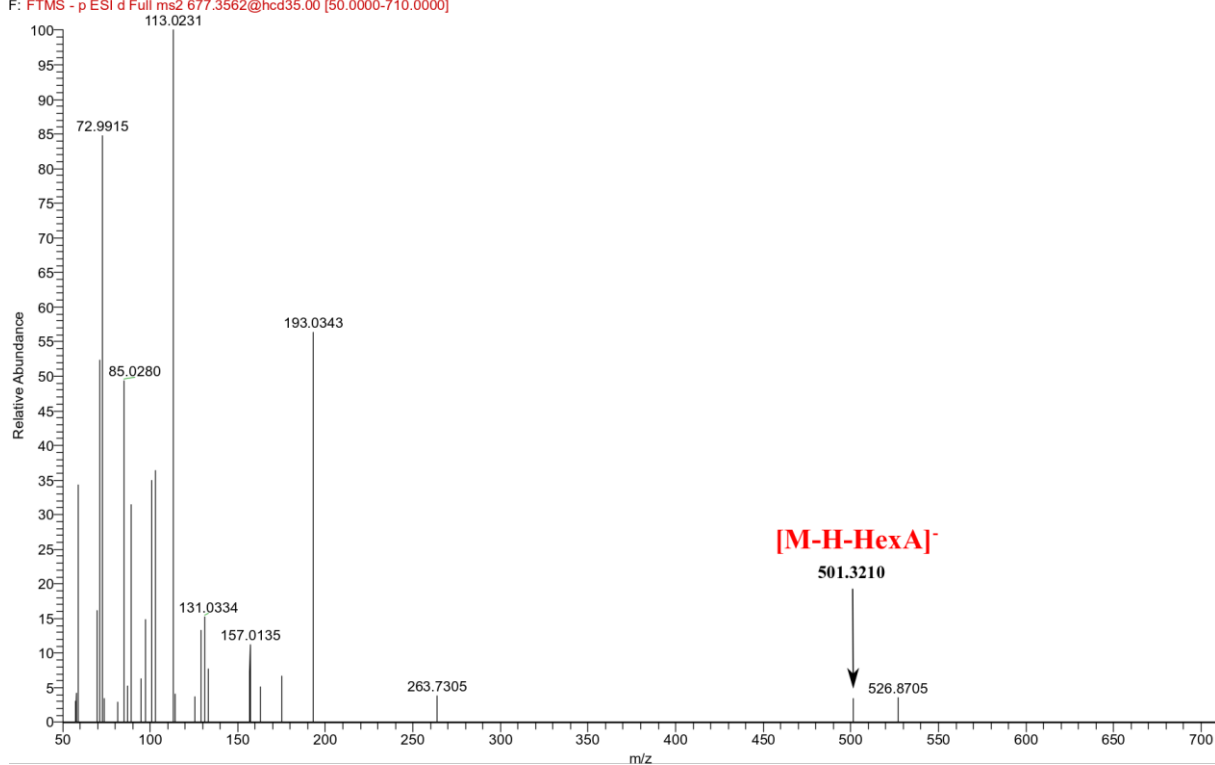


Figure S43: MS² spectrum of Compound 34

Chbh_NC_4B_04 #4824 RT: 14.67 AV: 1 NL: 6.04E5
F: FTMS - p ESI Full ms2 663.4000@hcd20.00 [50.0000-695.0000]

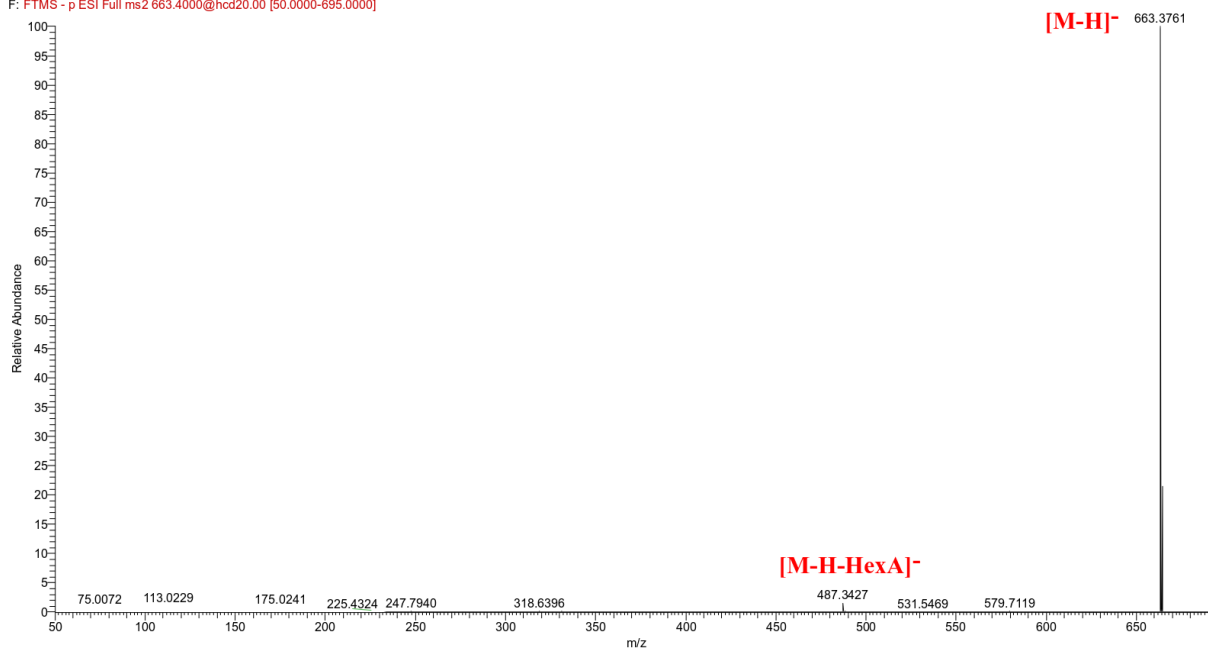


Figure S44: MS² spectrum of Compound 35

Chbh_NC_4B_02 #5691 RT: 16.43 AV: 1 NL: 1.29E6
F: FTMS - p ESI Full ms2 661.3000@hcd10.00 [50.0000-695.0000]

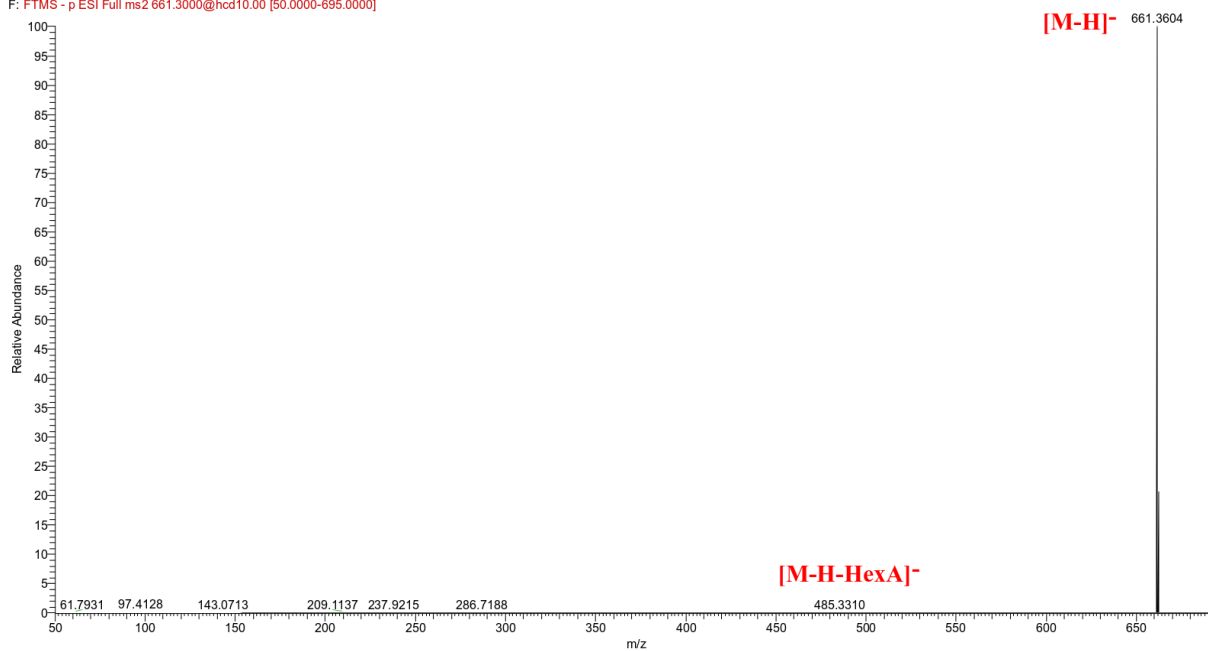


Figure S45: MS² spectrum of Compound 36

RT: 0.36 - 27.62

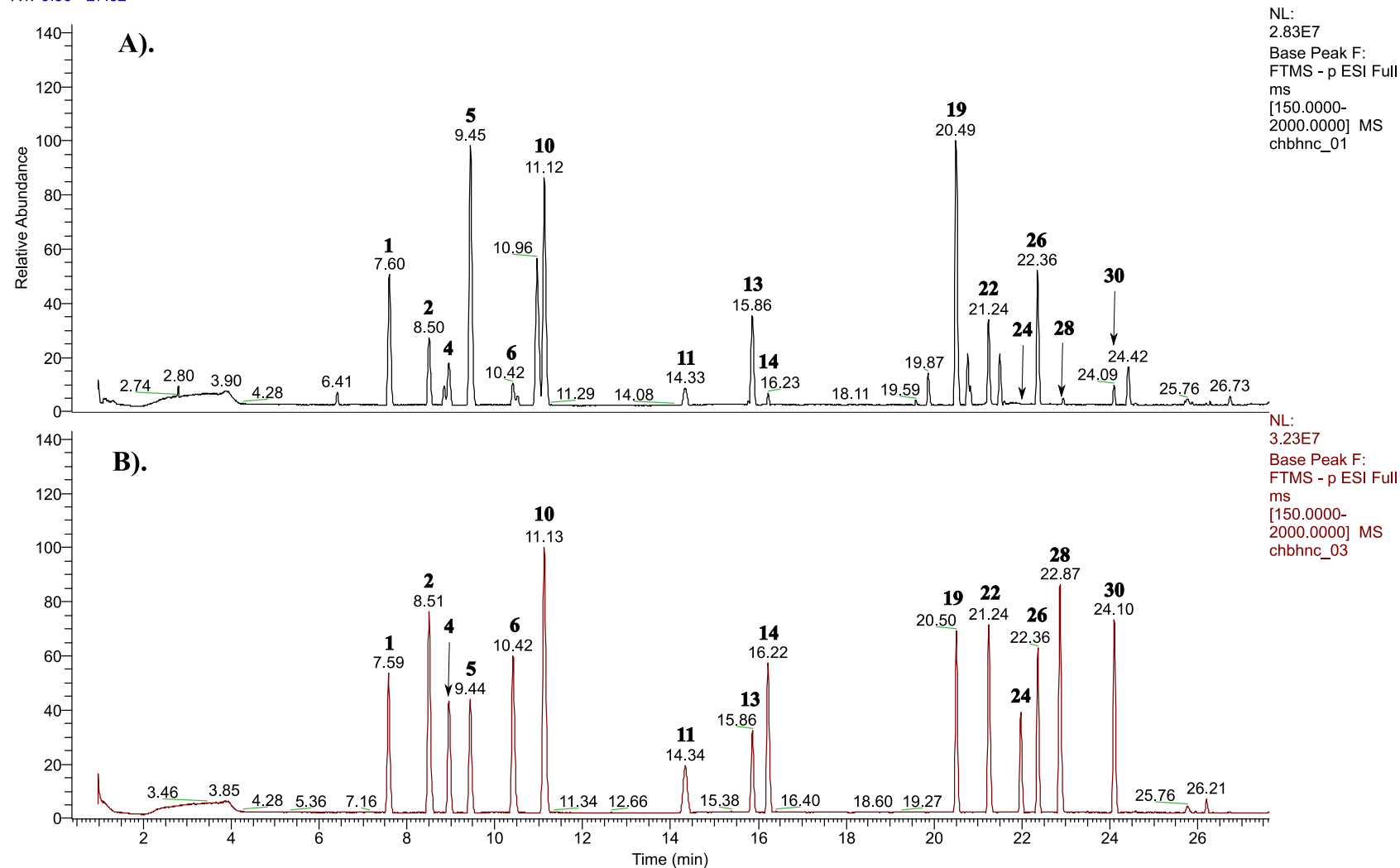


Figure S46: Base peak chromatograms in negative mode of A). the MeOH extract of aerial parts from *C. bonus-henricus* and B). the mixture of reference compounds. The numbering is according to the Table 1.

RT: 0.53 - 27.49

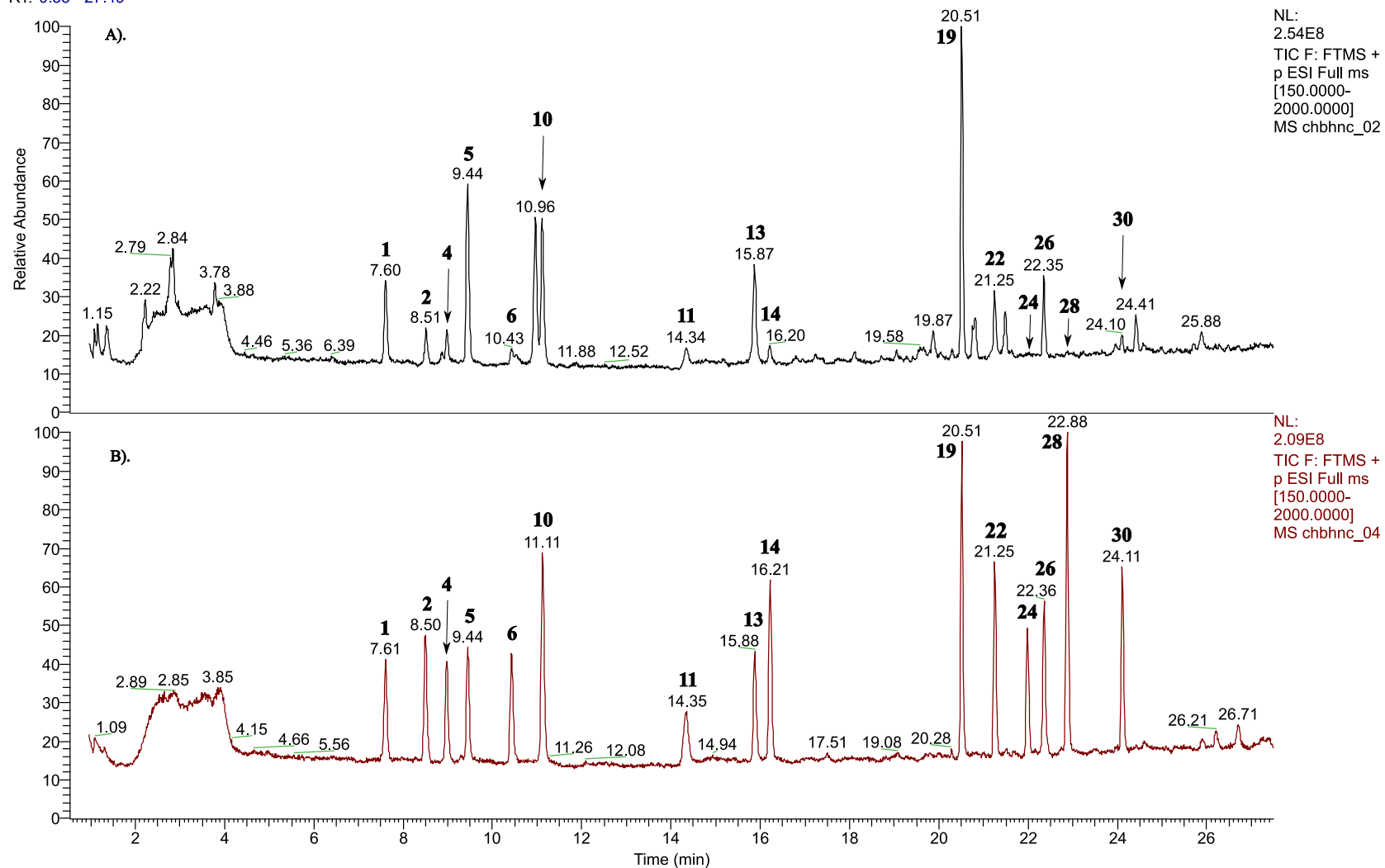


Figure S47: TIC chromatograms in positive mode of A). the MeOH extract of aerial parts from *C. bonus-henricus* and B). the mixture of reference compounds. The numbering is according to the Table 1.