Supplemental Information

Chemical Standardization of Milk Thistle (*Silybum marianum* L.) Extract Using UHPLC-MS/MS and the Method of Standard Addition

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Analysis of milk thistle (*Silybum marianum*) extract using high-resolution UHPLC-MS

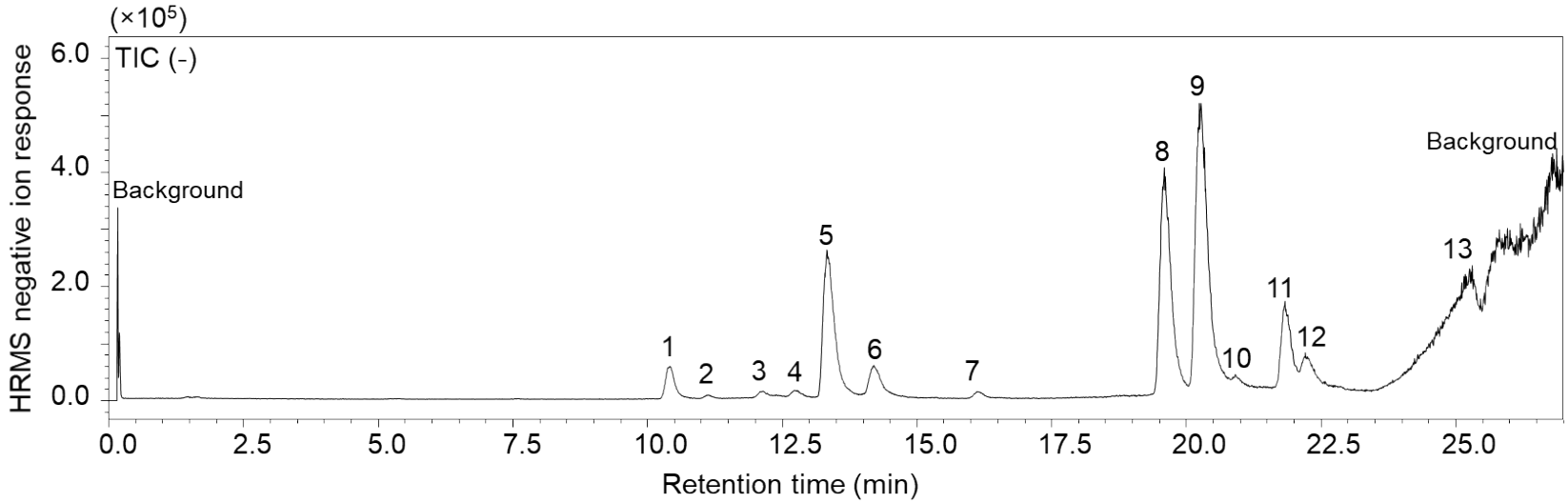


Figure 1. Total ion chromatogram of milk thistle extract (10 µg/mL) analyzed on a Shimadzu 9030 QToF mass spectrometer with negative ion electrospray. As described in the Methods section, reversed phase UHPLC gradient elution was used from water to methanol (both solvents containing 0.1% formic acid). Peak assignments are shown in **Supplemental Table 1**.

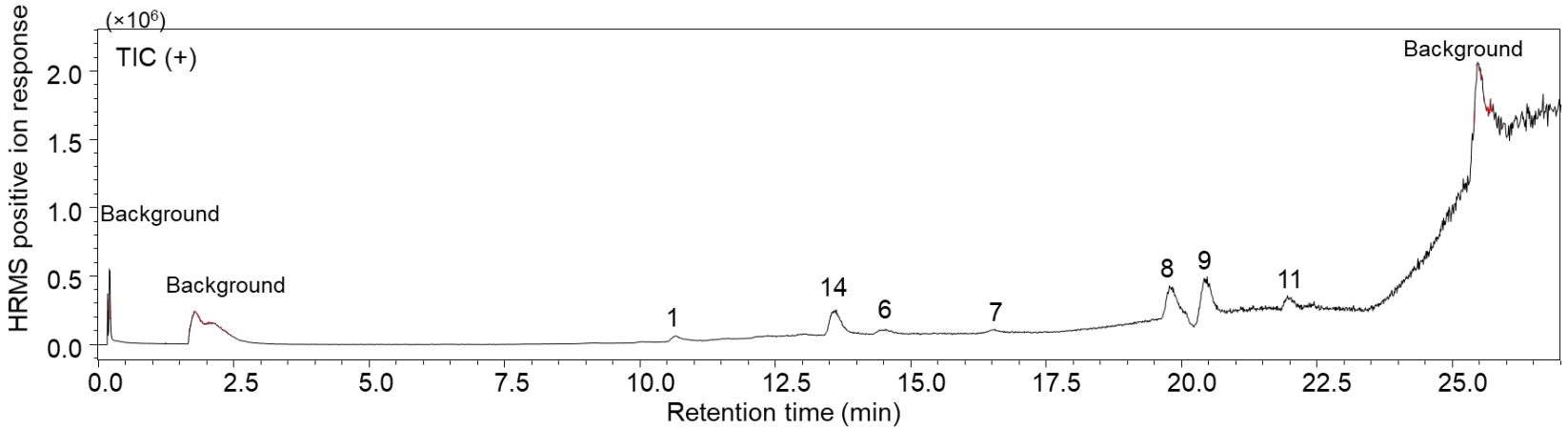
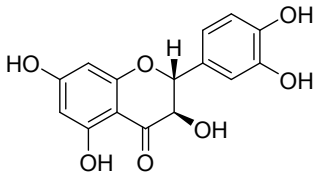
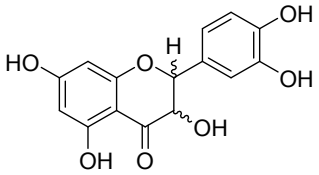
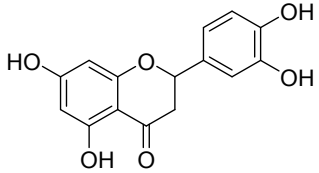
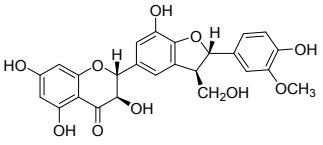
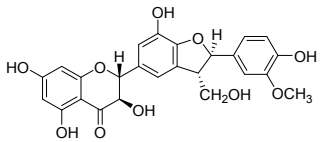
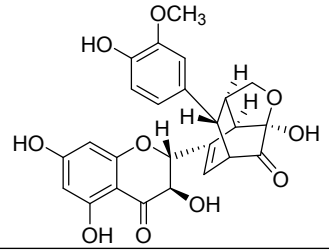
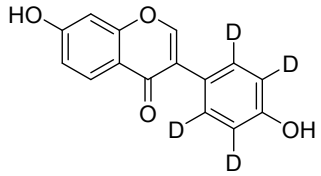
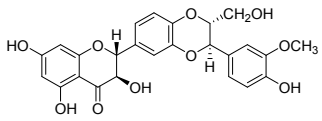
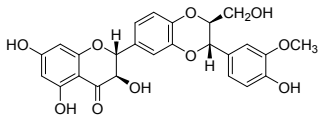
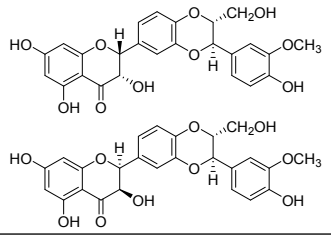
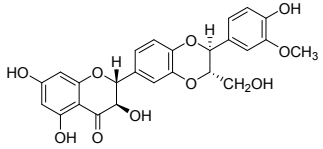
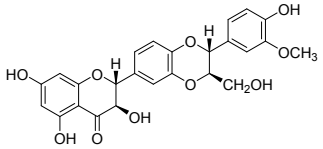
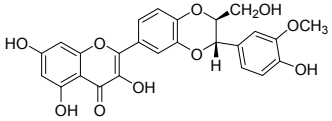
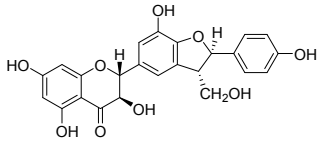


Figure 2. Total ion chromatogram of milk thistle extract (10 µg/mL) analyzed on a Shimadzu 9030 UHPLC-QToF mass spectrometer with positive ion electrospray. Reversed phase UHPLC gradient elution was used as in **Figure 1** above. Peak assignments are shown in **Supplemental Table 1**. Note that the retention times of the analytes in this positive ion electrospray chromatogram are shifted by 0.24 min compared with the corresponding negative ion chromatogram in **Figure 1** due to variations in chromatographic conditions. The d_4-daidzein internal standard was used to verify the shift in the retention time. (See Table 1, Peak 7).

Table 1. Peak assignments for the high-resolution UHPLC-MS analyses of milk thistle extract (*Silybum marianum*) shown in **Supplemental Figures 1** and **2.**

Peak number	Retention time (min)	Measured mass (*m/z*) (-) top (+) bottom	ΔM (ppm) (-) top (+) bottom	Compound Molecular Formula Confidence	Chemical Structure	Comments
1	10.42	303.0485 305.0554	8.2 1.3	Taxifolin $C_{15}H_{12}O_7$ Reference standard		HRMS supports molecular formula. MS/MS supports structure via reference standard. Retention time matches that of reference standard.
2	11.14	303.0485 -	8.2 -	Taxifolin isomer $C_{15}H_{12}O_7$ Tentative		HRMS supports molecular formula. MS/MS supports structure through comparison of taxifolin major fragment ion of *m*/*z* 125. The stereochemical configuration is unknown.
3	12.13	287.0538 -	8.0 -	Eriodictyol $C_{15}H_{12}O_6$ Tentative		HRMS supports molecular formula of taxifolin minus a hydroxyl group.(eriodictyol) MS/MS supports structure through comparison of taxifolin major fragment ion of *m*/*z* 125
4	12.76	481.1101 -	8.1 -	Neusilychristin $C_{25}H_{22}O_{10}$ Tentative		Eluting shorly before silychristin A, neusilychristin A, the MS/MS data matches that of silychristin [1].

Peak number	Retention time (min)	Measured mass (*m/z*) (-) top (+) bottom	ΔM (ppm) (-) top (+) bottom	Compound Molecular Formula Confidence	Chemical Structure	Comments
5	13.35	481.1102 -	7.9 -	Silychristin $C_{25}H_{22}O_{10}$ Reference standard		HRMS supports molecular formula. MS/MS supports structure via reference standard. Retention time matches that of reference standard
6	14.20	481.1101 -	8.1 -	Silydianin $C_{25}H_{22}O_{10}$ Reference standard		HRMS supports molecular formula. MS/MS supports structure via reference standard. Retention time matches that of reference standard
7	16.15 16.40	257.0735 259.0899	8.5 1.2	d_4-Daidzein internal standard $C_{15}H_6D_4O_4$ Reference standard		HRMS supports molecular formula. MS/MS supports structure via reference standard. Retention time matches that of reference standard
8	19.60	481.1102 483.1283	7.9 0.41	Silybin A $C_{25}H_{22}O_{10}$ Reference standard		HRMS supports molecular formula. MS/MS supports structure via reference standard. Retention time matches that of reference standard
9	20.27	481.1103 483.1284	7.7 0.2	Silybin B $C_{25}H_{22}O_{10}$ Reference standard		HRMS supports molecular formula. MS/MS supports structure via reference standard. Retention time matches that of reference standard

Peak number	Retention time (min)	Measured mass (*m/z*) (-) top (+) bottom	ΔM (ppm) (-) top (+) bottom	Compound Molecular Formula Confidence	Chemical Structure	Comments
10	20.93	481.1102 -	7.9 -	A silymarin isomer, likely 2,3-*cis*-silybin A $C_{25}H_{22}O_{10}$ Tentative		Based on structural similarity, the suggested silybin isomer is 2,3-cis-silybin A, which has been reported [1]. The HRMS data supports a silybin isomer and the MS/MS matches that of other silybins.
11	21.85	481.1103 483.1254	7.7 6.4	Isosilybin A $C_{25}H_{22}O_{10}$ Reference standard		HRMS supports molecular formula. MS/MS supports structure via reference standard. Retention time matches that of reference standard
12	22.26	481.1102 -	7.9 -	Isosilybin B $C_{25}H_{22}O_{10}$ Reference standard		HRMS supports molecular formula. MS/MS supports structure via reference standard. Retention time matches that of reference standard
13	25.38	479.0948 -	7.5	2,3-Dehydrosilybin B $C_{25}H_{20}O_{10}$ Reference standard		HRMS supports molecular formula. MS/MS supports structure via reference standard. Retention time matches that of reference standard
14	13.59	- 453.1176	- 0.88	In source fragment of silychristin $C_{24}H_{20}O_9$ Tentative		Common fragment ion for silybins and isosilybins. The retention time suggests an in source fragment ion of silychristin. Fragmentation data of silychristin match literature data [2]

HRMS and MS/MS of peaks in the milk thistle extract chromatograms in Figures 1 and 2

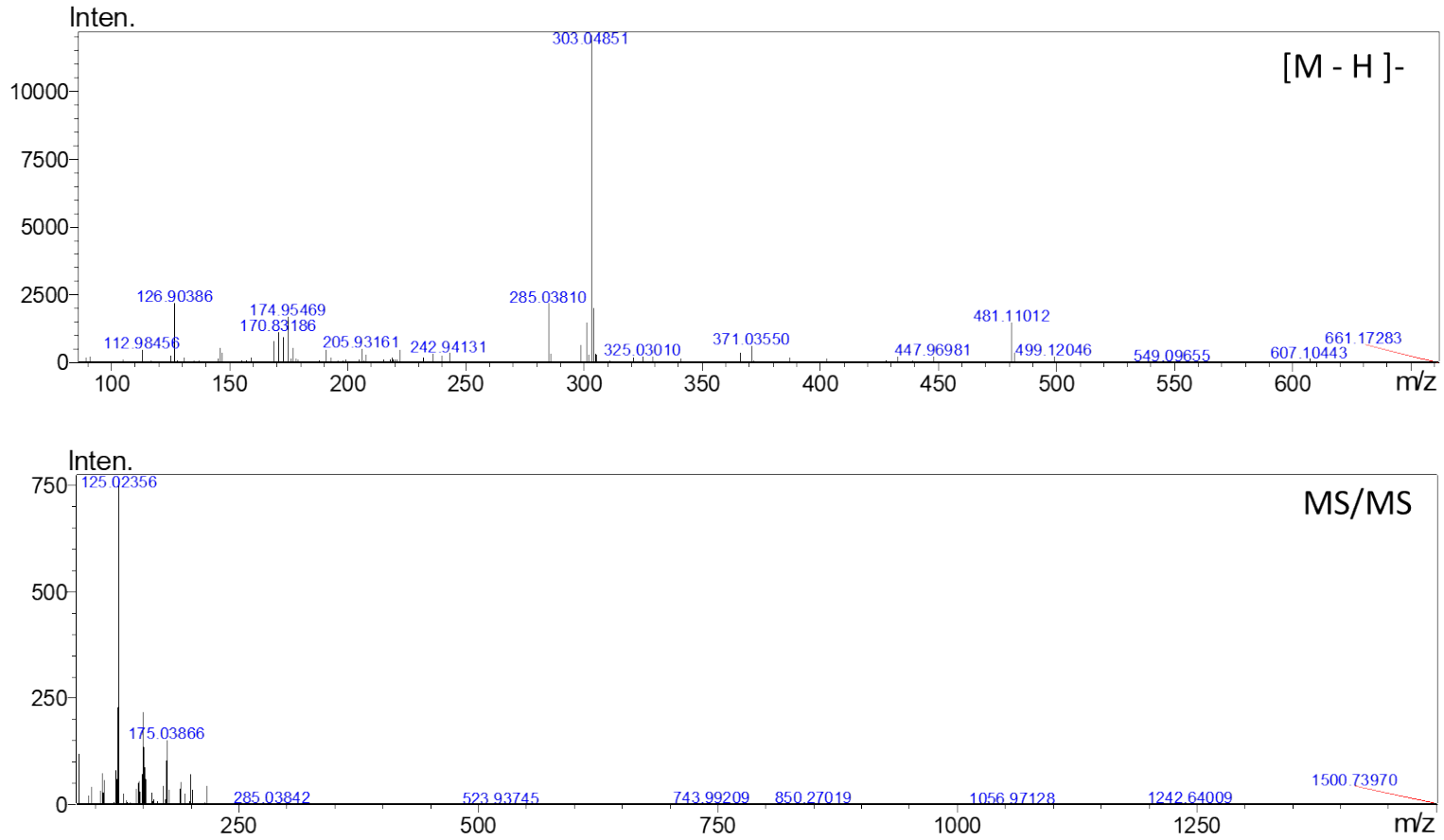


Figure 3. Negative ion electrospray HRMS (top panel) and product ion tandem mass spectrum (bottom panel) of peak 1 (taxifolin; $[M-H]^-$) of *m*/*z* 303.04854 eluting at 10.42 min

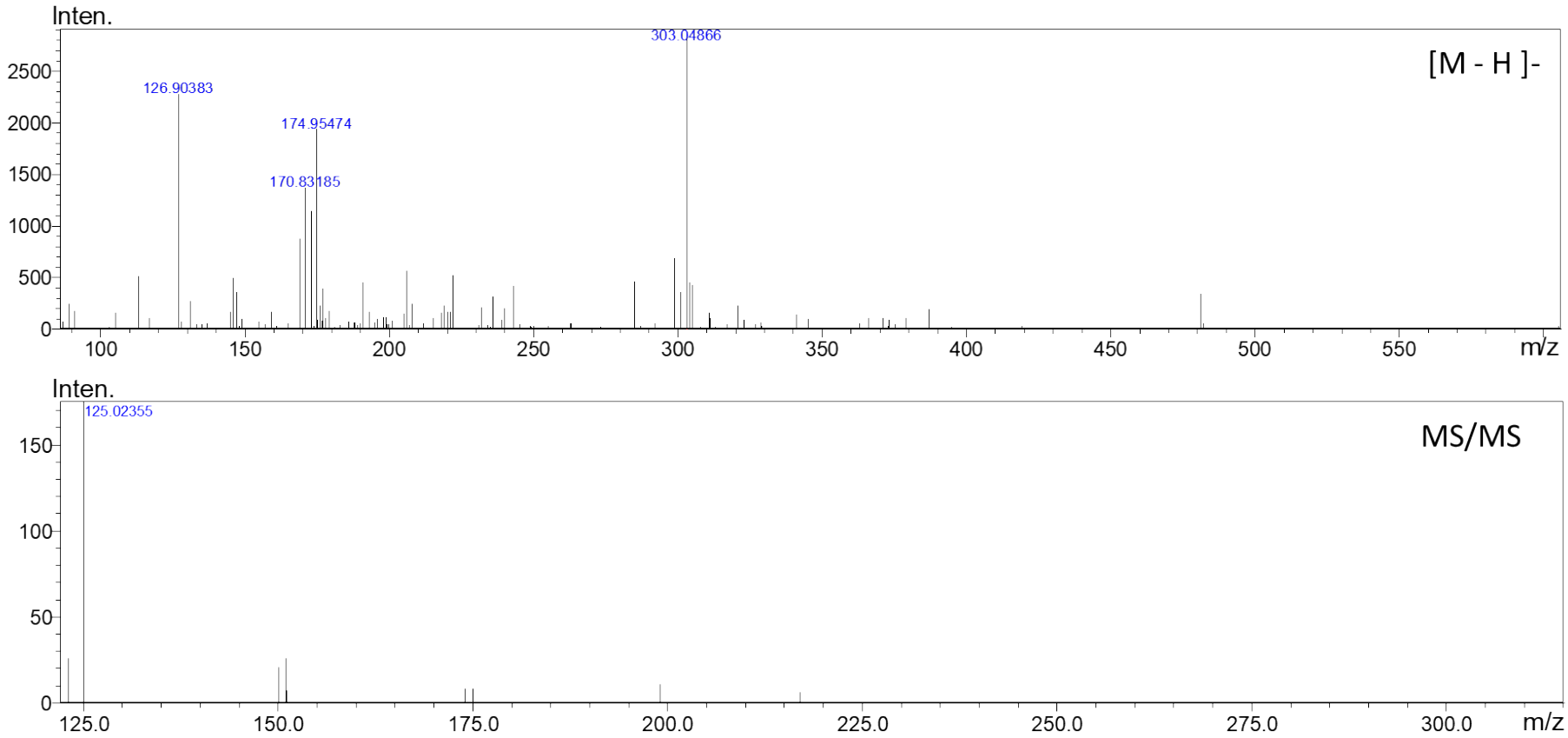


Figure 4. Negative ion electrospray HRMS (top panel) and product ion tandem mass spectrum (bottom panel) of peak 2 (taxifolin isomer; $[M-H]^-$) of *m*/*z* 303.04854 eluting at 11.14 min

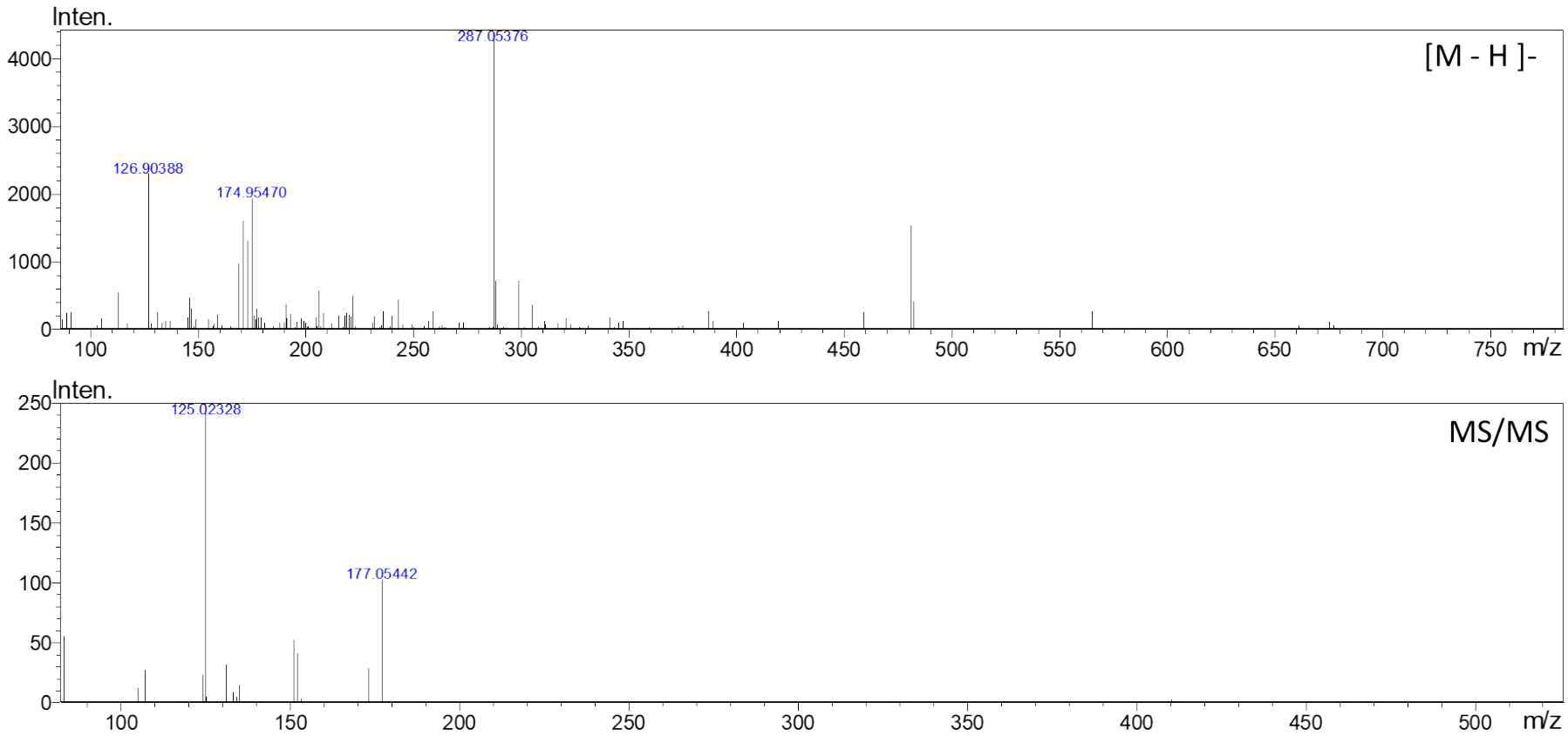


Figure 5. Negative ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 3 (probably eriodictyol; $[M-H]^-$) of *m*/*z* 287.0538 eluting at 12.13 min

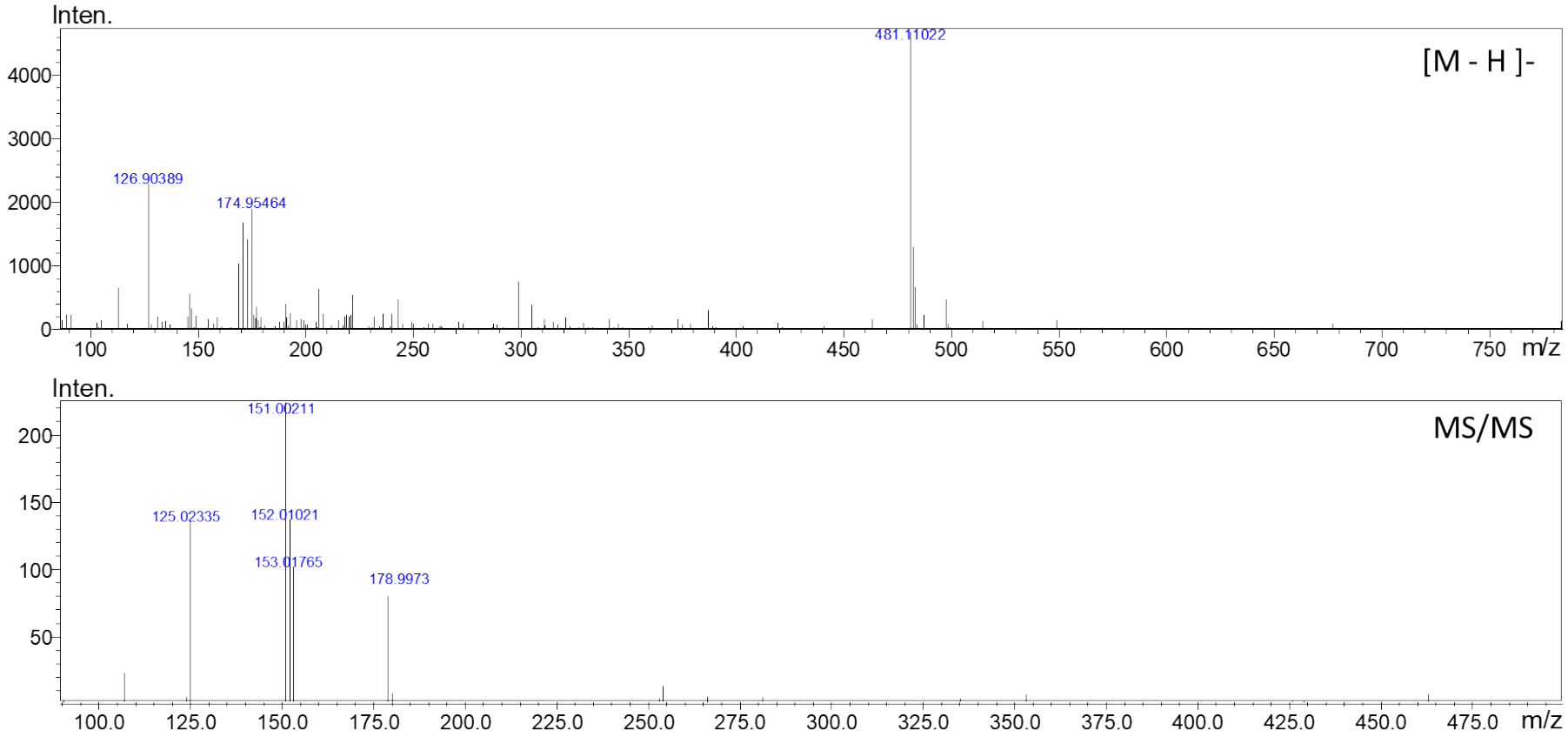


Figure 6. Negative ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 4 (neusilychristin; $[M-H]^-$) of *m*/*z* 481.1101 eluting at 12.76 min

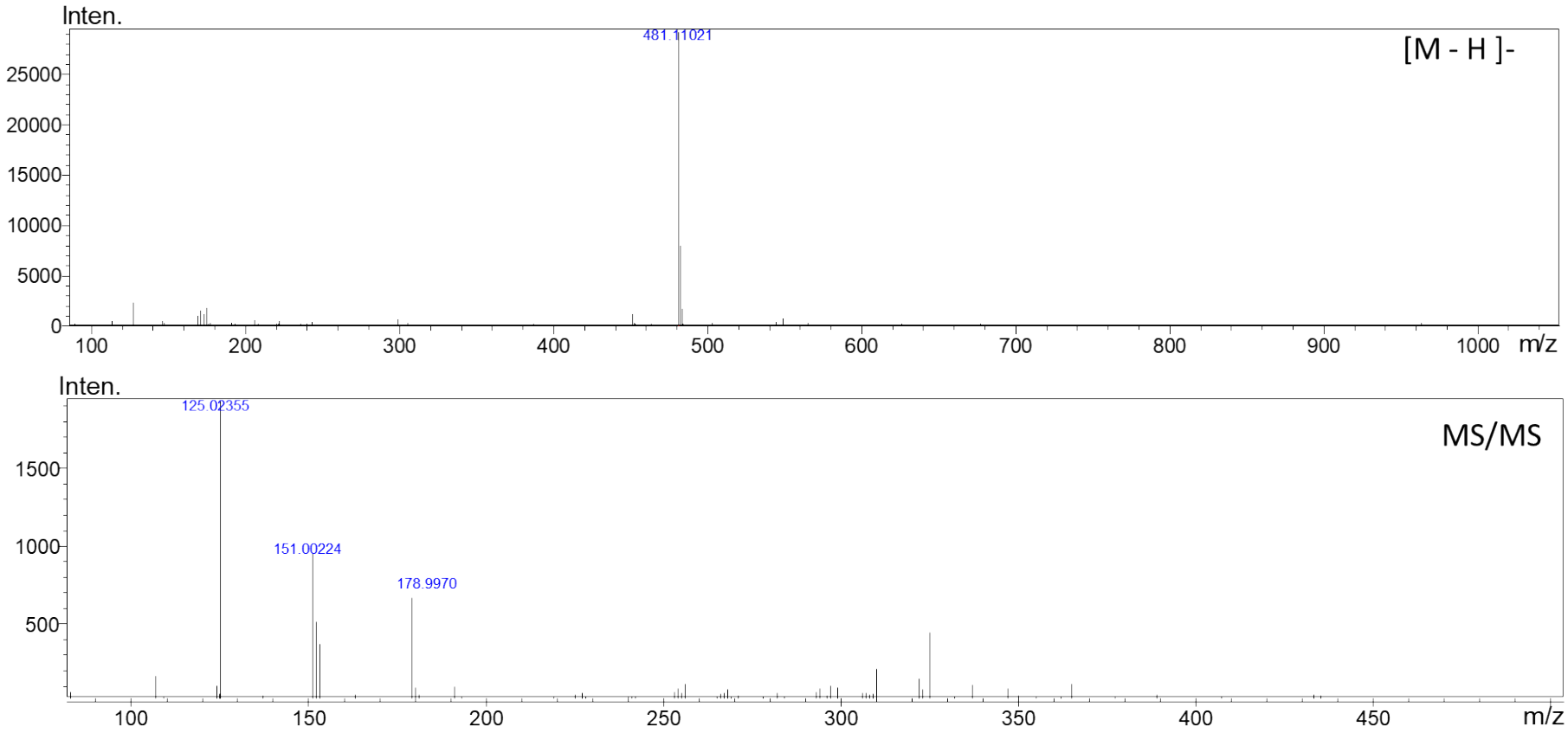


Figure 7. Negative ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 5 (silychristin; $[M-H]^-$) of *m*/*z* 481.1102 eluting at 13.35 min

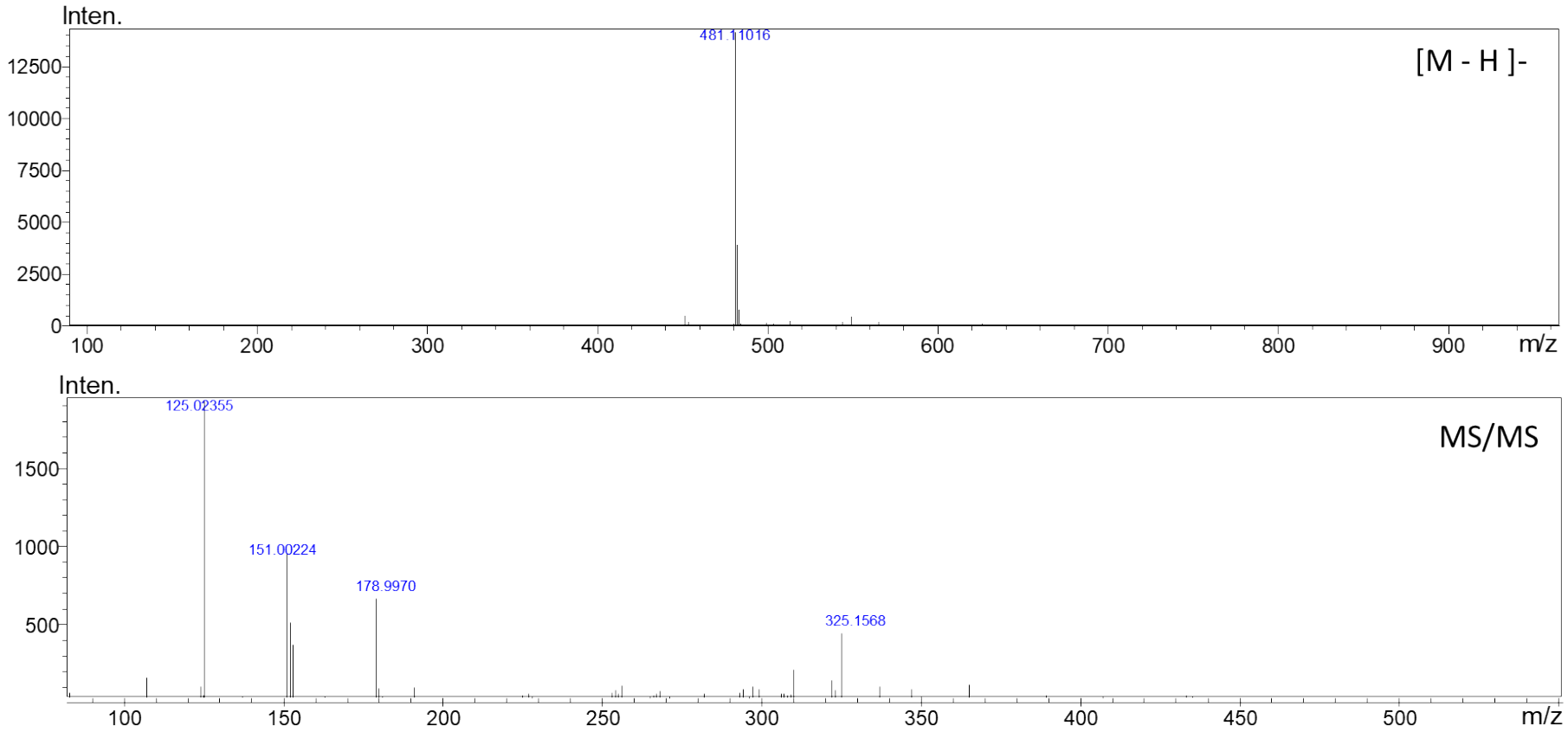


Figure 8.Negative ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 6 (silydianin; $[M-H]^-$) of *m*/*z* 481.1101 eluting at 14.20 min

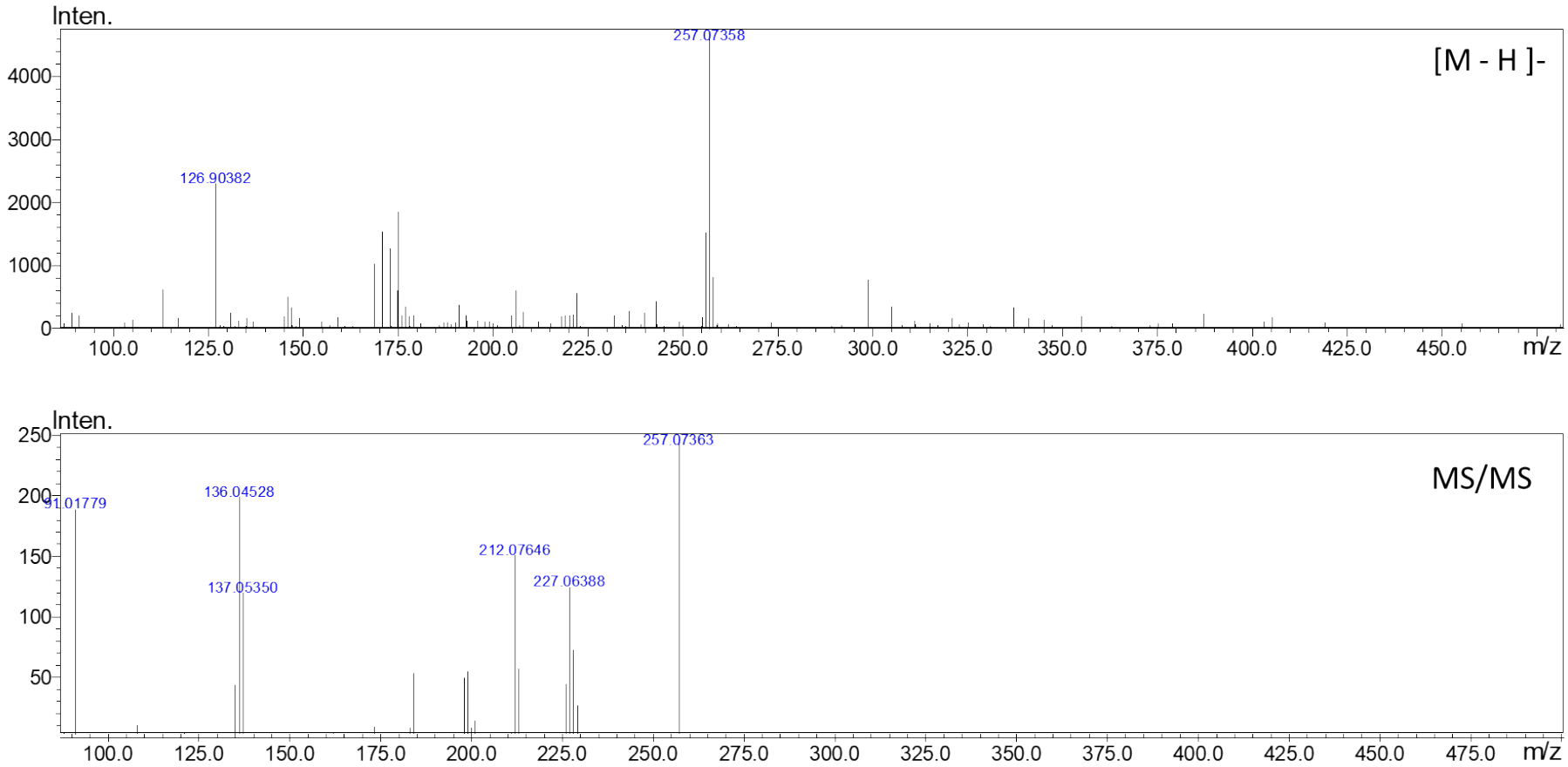


Figure 9. Negative ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 7 (internal standard, d_4-daidzein; $[M-H]^-$) of *m/z* 257.0735 eluting at 16.15 min

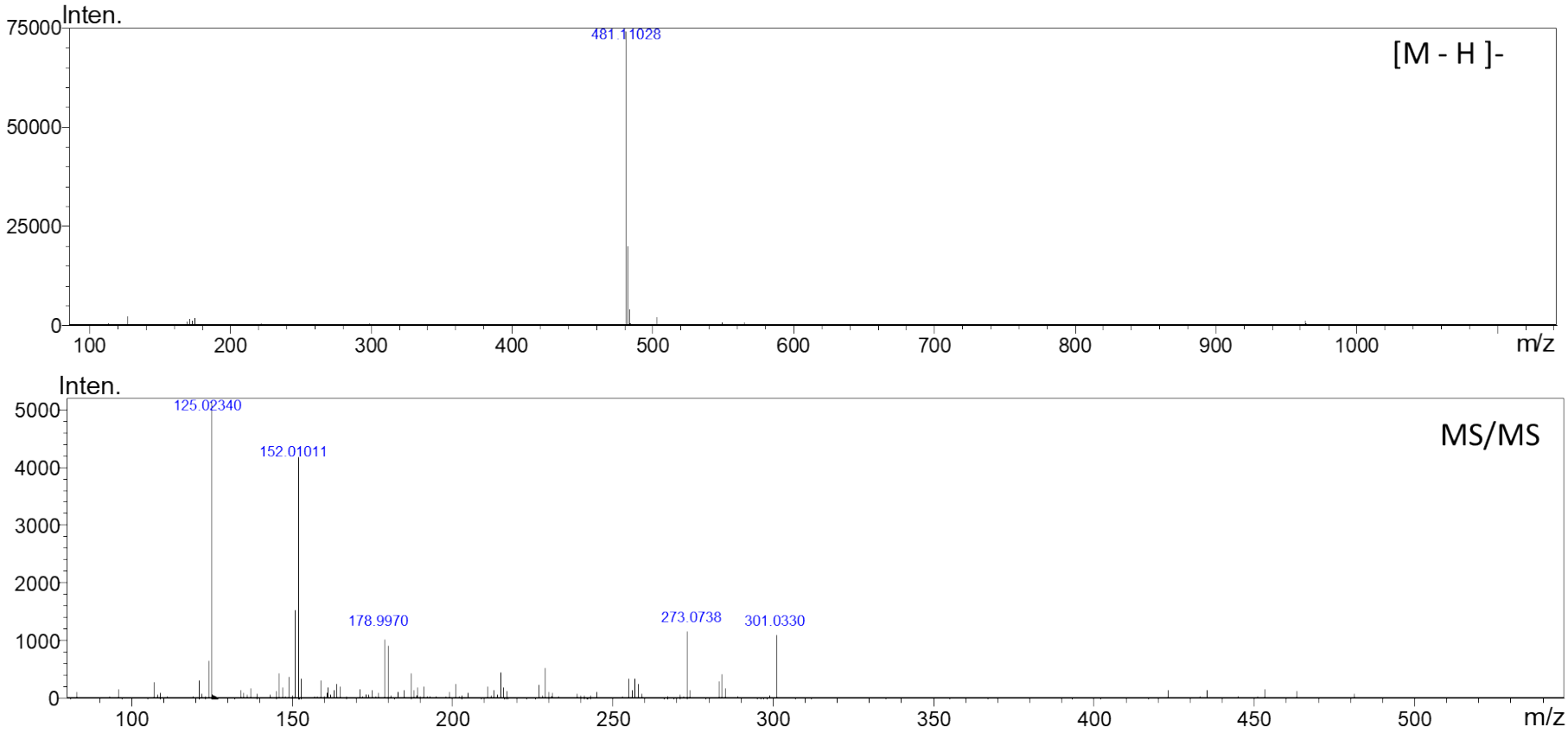


Figure 10. Negative ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 8 (silybin A; $[M-H]^-$) of *m*/*z* 481.1102 eluting at 19.60 min

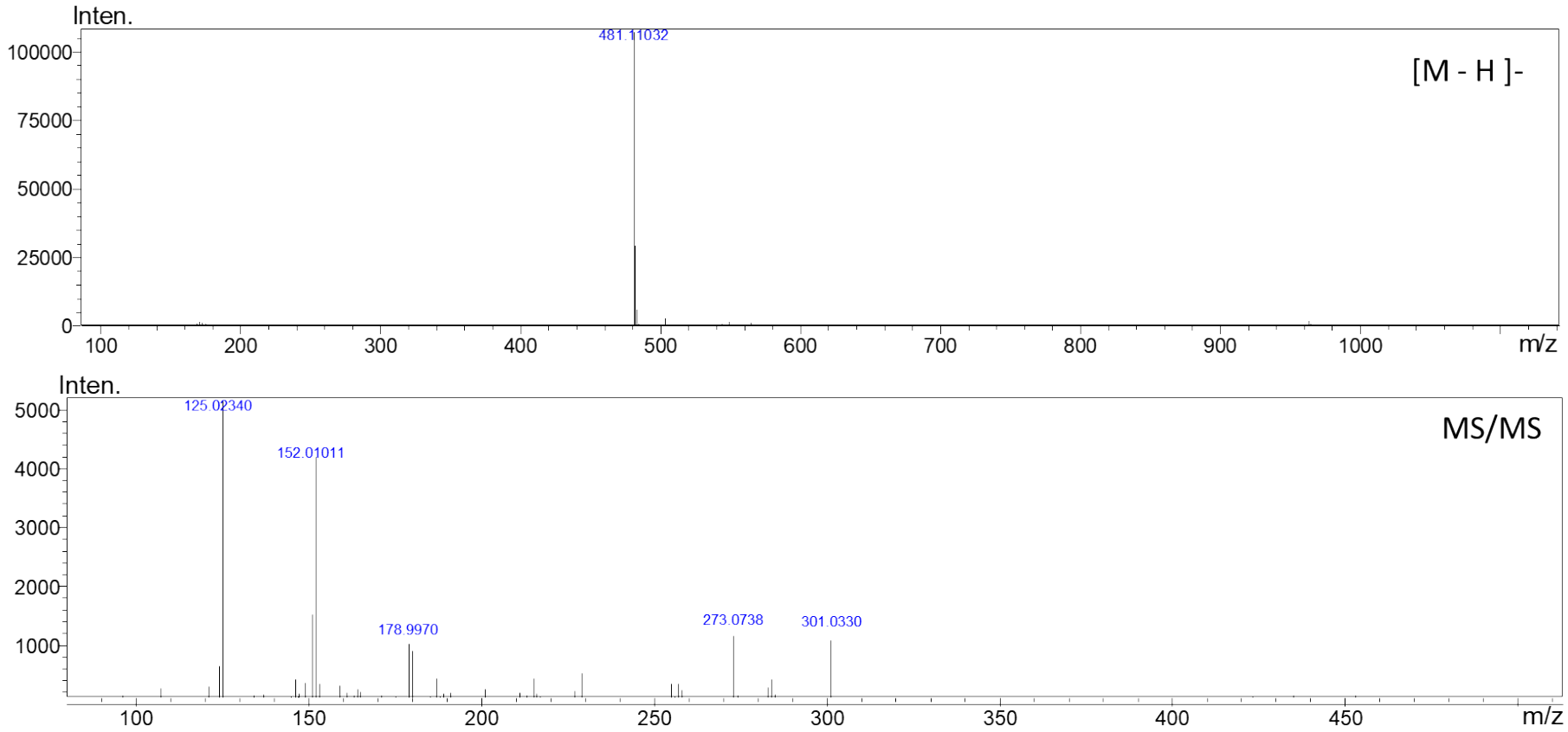


Figure 11. Negative ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 9 (silybin B; $[M-H]^-$) of *m*/*z* 481.1103 eluting at 20.27 min

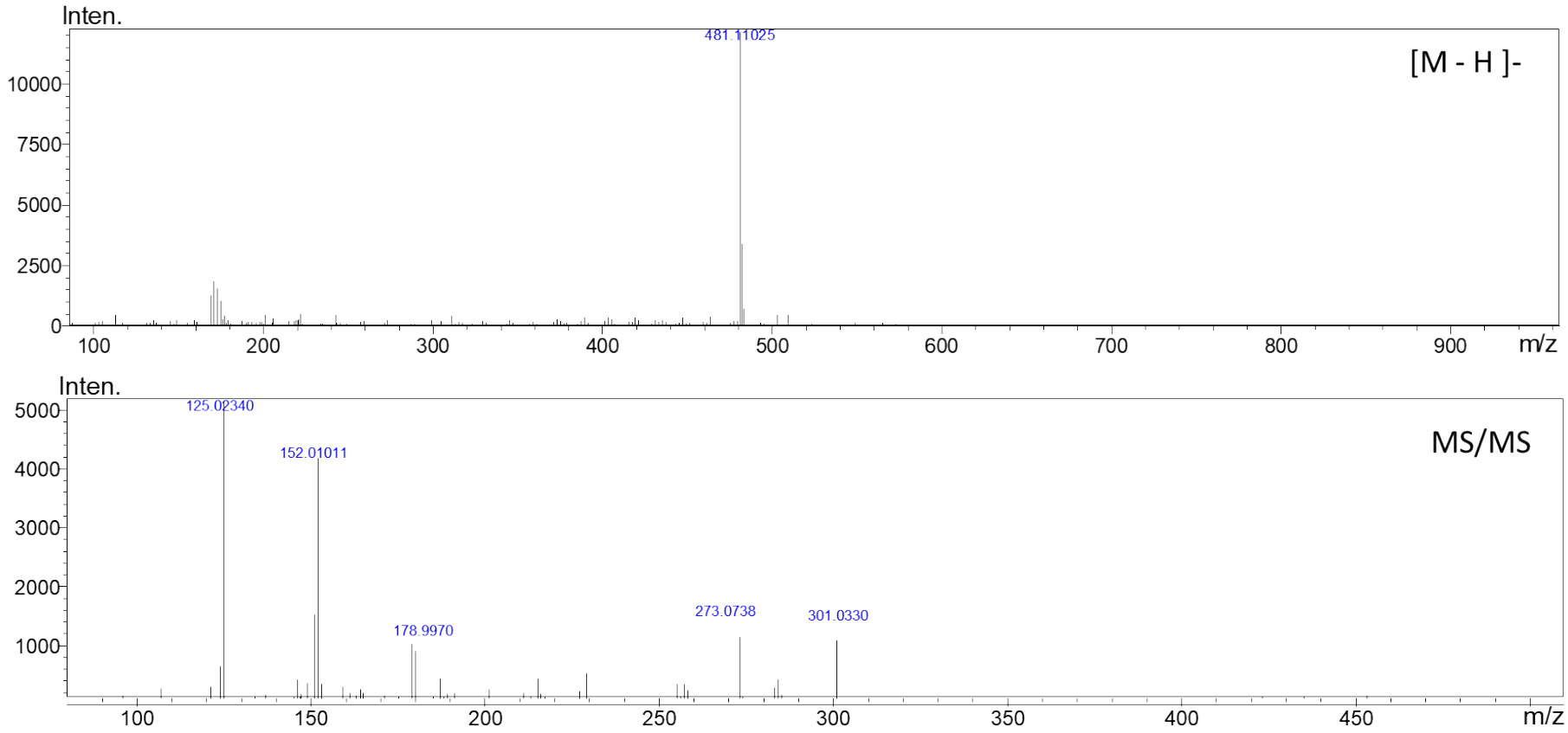


Figure 12. Negative ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 10 (silymarin isomer, likely 2,3-cis-silybin A; $[M-H]^-$) of *m/z* 481.1102 eluting at 20.93 min

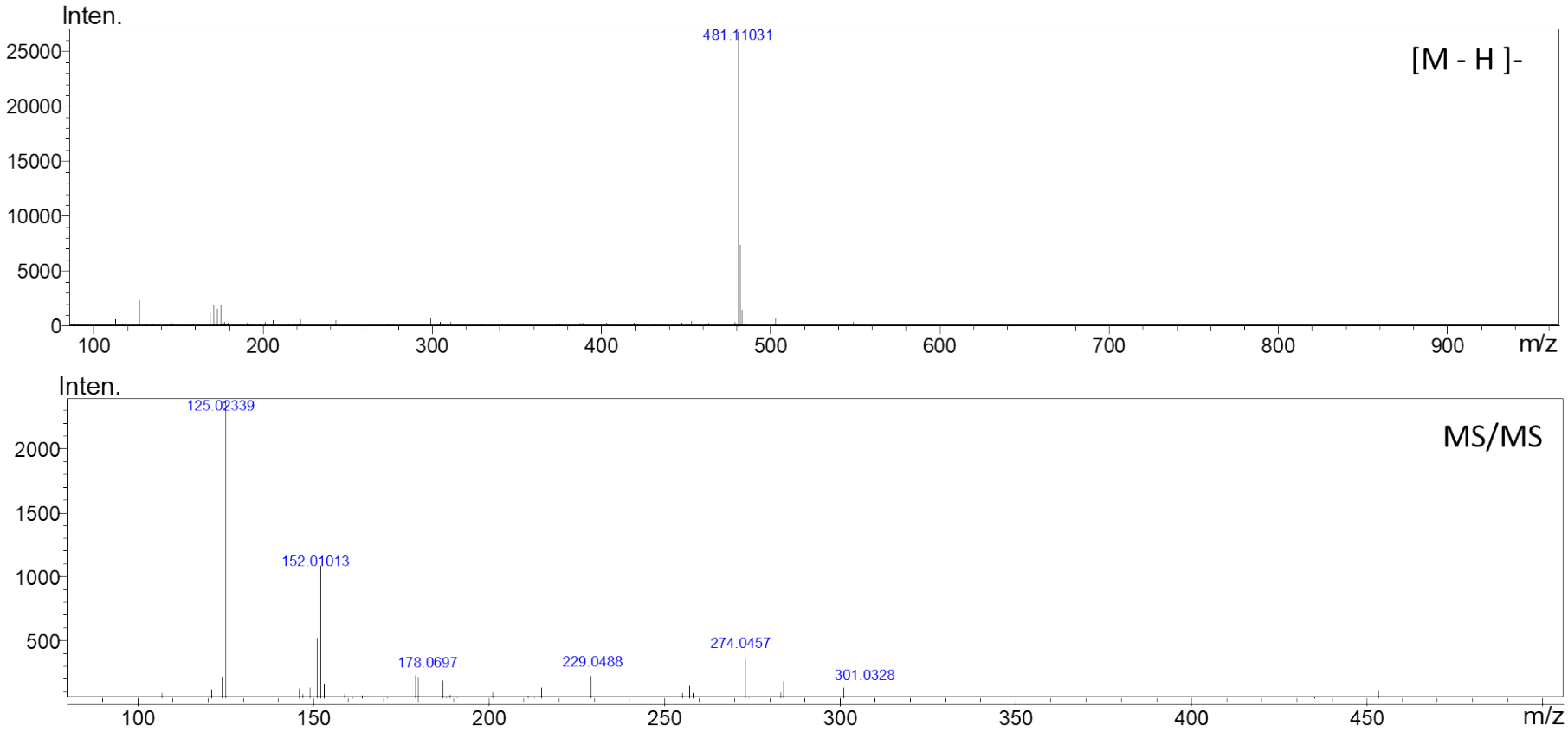


Figure 13. Negative ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 11 (isosilybin A; $[M-H]^-$) of *m*/*z* 481.1103 eluting at 21.85 min

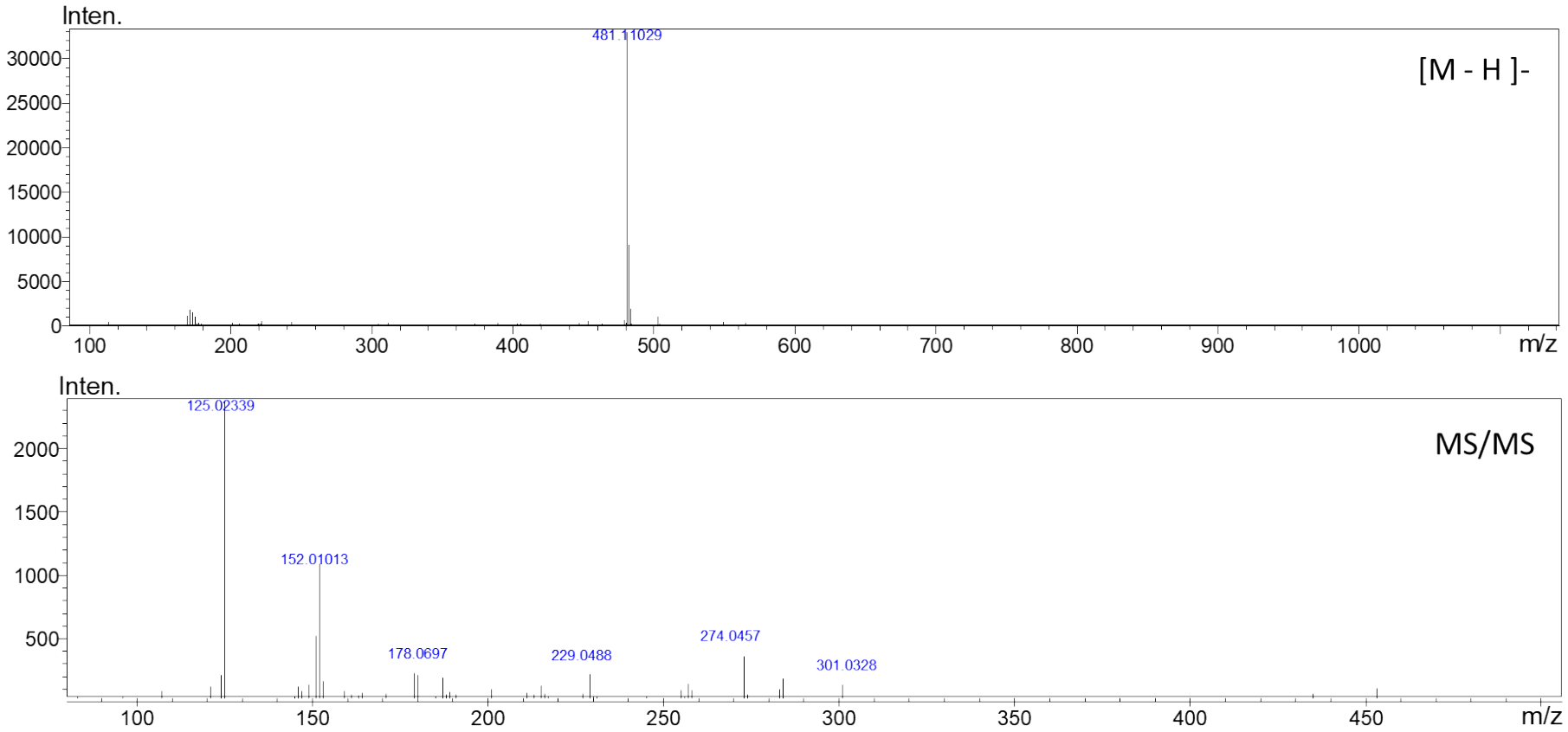


Figure 14. Negative ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 12 (isosilybin B; $[M-H]^-$) of *m*/*z* 481.1102 eluting at 22.26 min

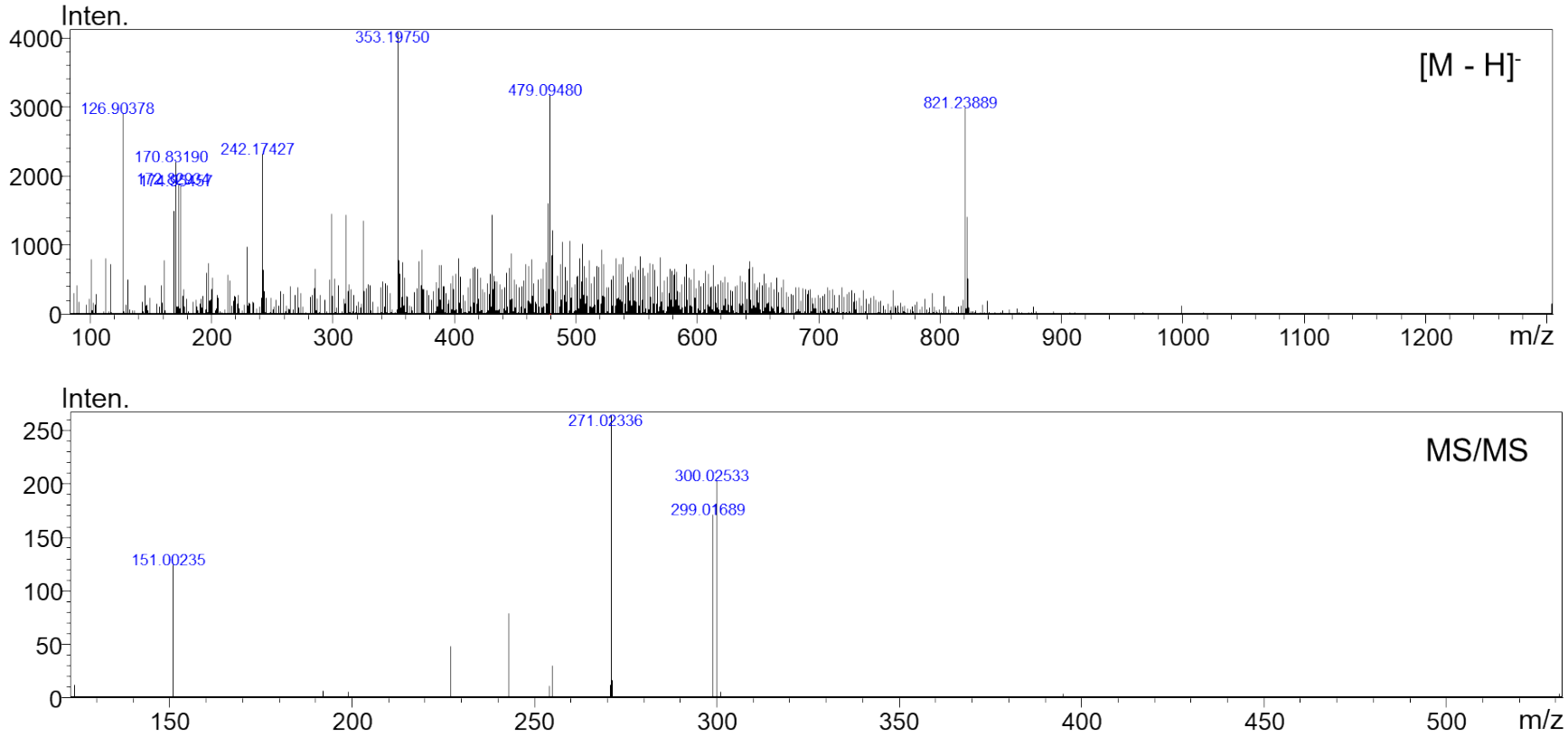


Figure 15. Negative ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 13 (2,3-dehydrosilybin B; $[M-H]^-$) of *m*/*z* 479.0948 eluting at 25.34 min

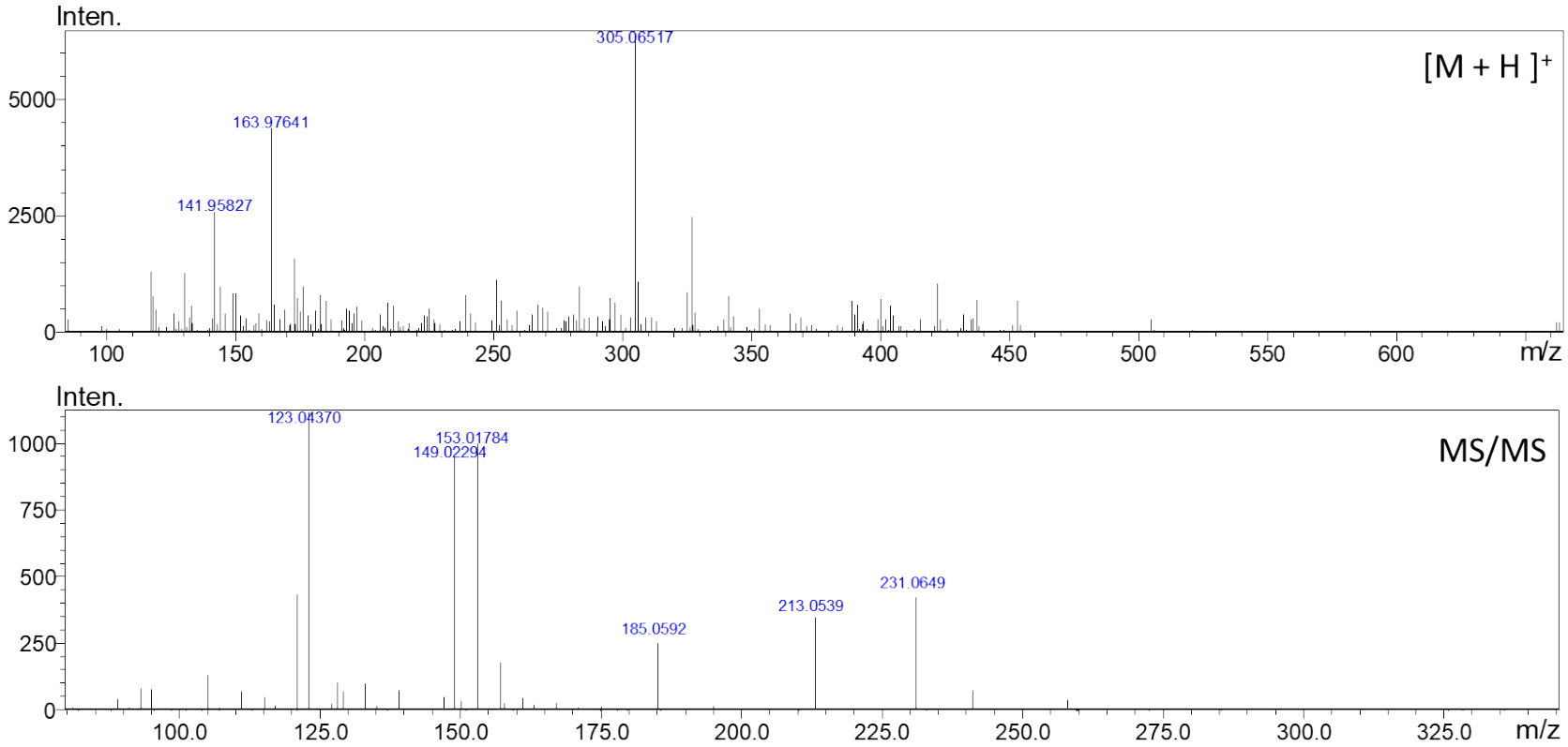


Figure 16. Positive ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 1 (taxifolin; $[M+H]^+$) of *m*/*z* 305.0651 eluting at 10.64 min

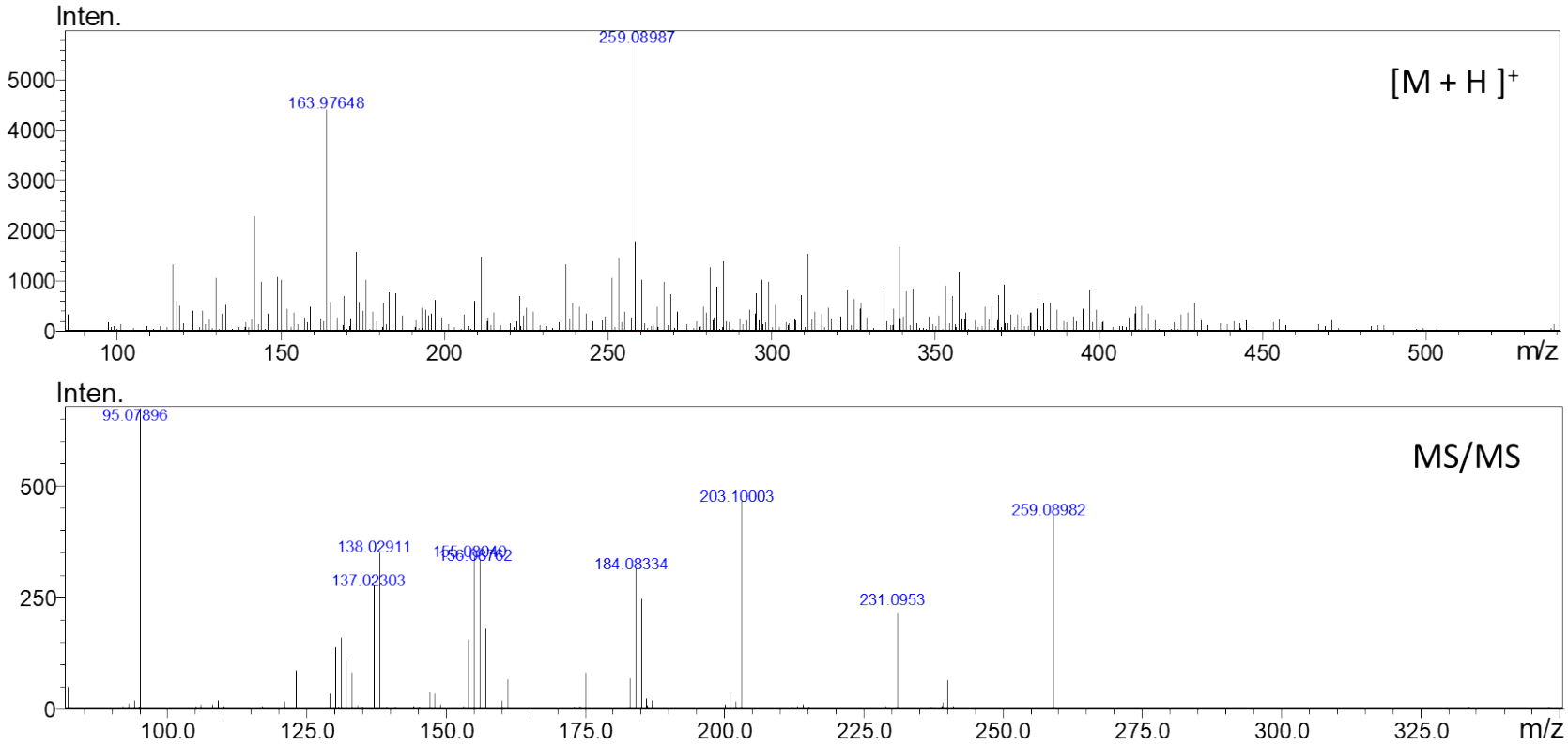


Figure 17. Positive ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 7 (internal standard, d_4-daidzein; $[M+H]^+$) of *m*/*z* 259.0899 eluting at 16.51 min

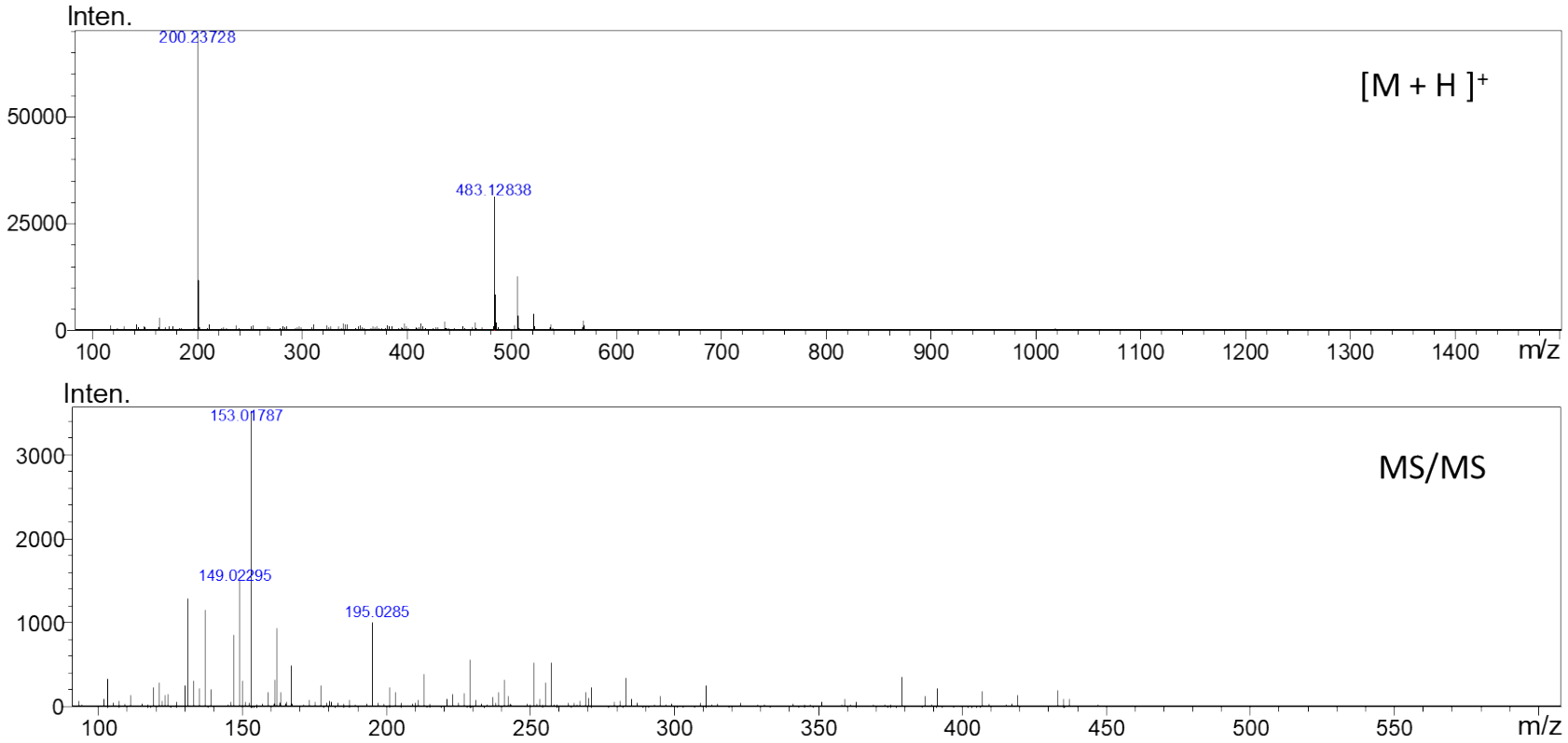


Figure 18. Positive ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 8 (silybin A; $[M+H]^+$) of *m*/*z* 483.1283 eluting at 19.80 min

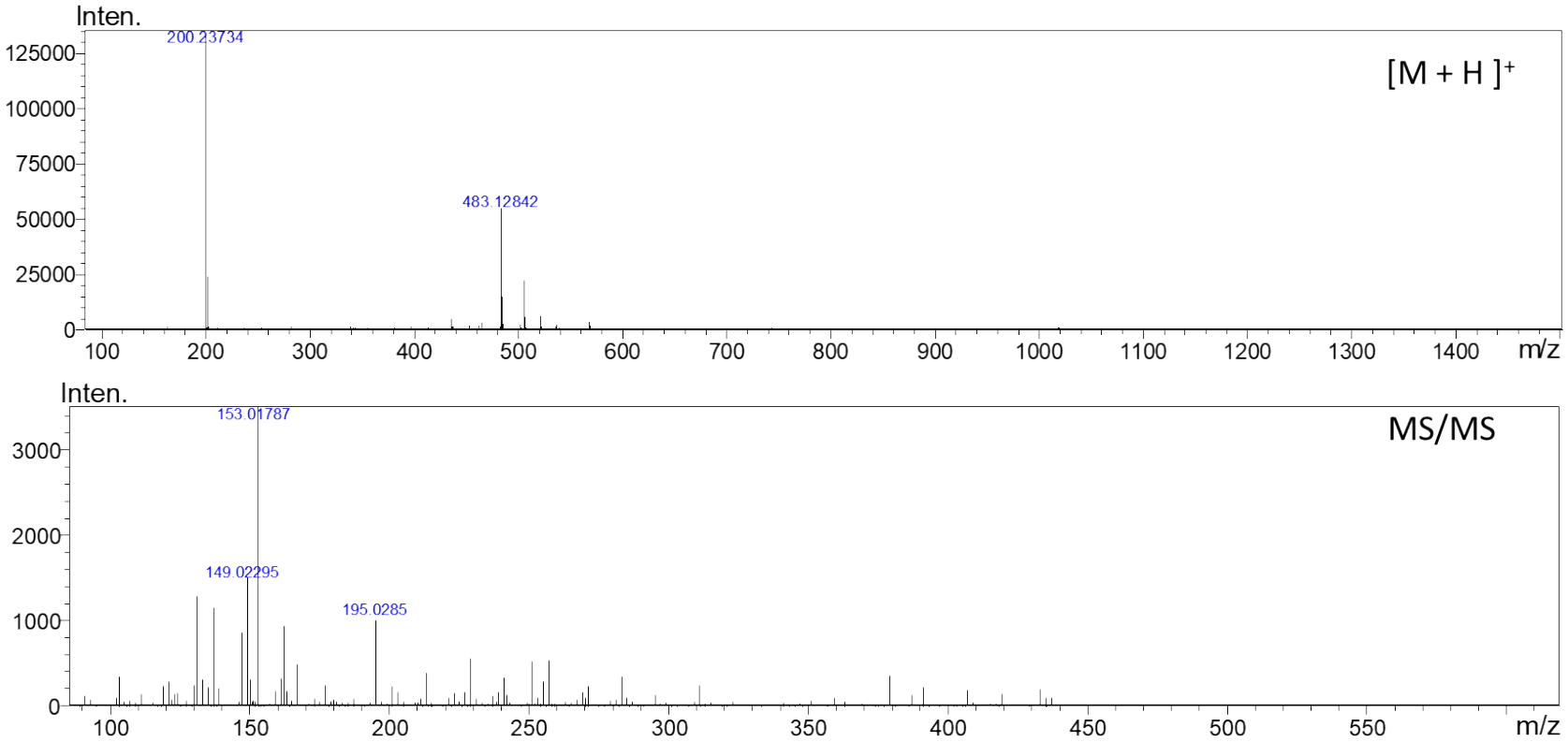


Figure 19. Positive ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 9 (silybin B; $[M+H]^+$) of *m*/*z* 483.1284 eluting at 20.43 min

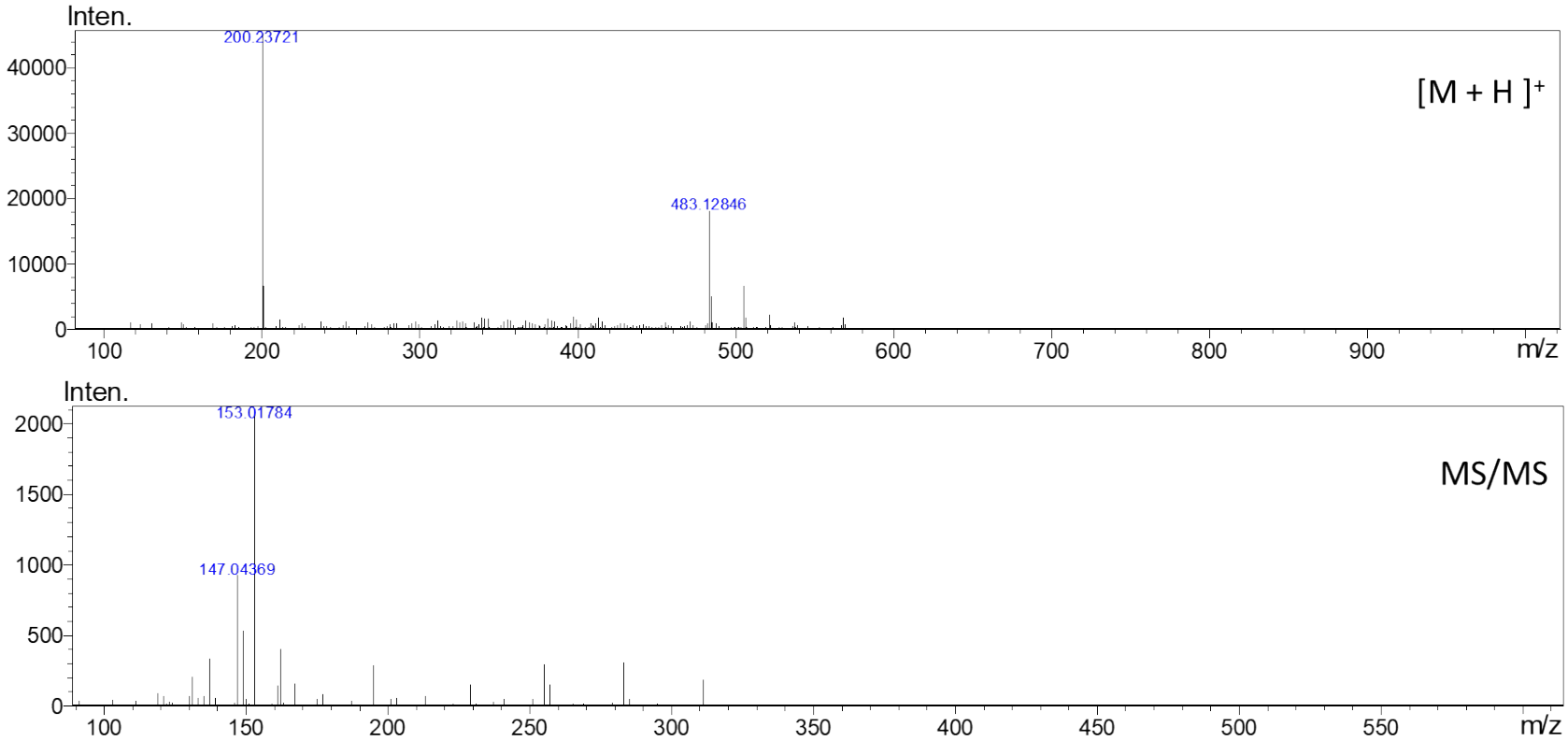


Figure 20. Positive ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 11 (isosilybin A; $[M+H]^+$) of *m*/*z* 483.1254 eluting at 21.99 min

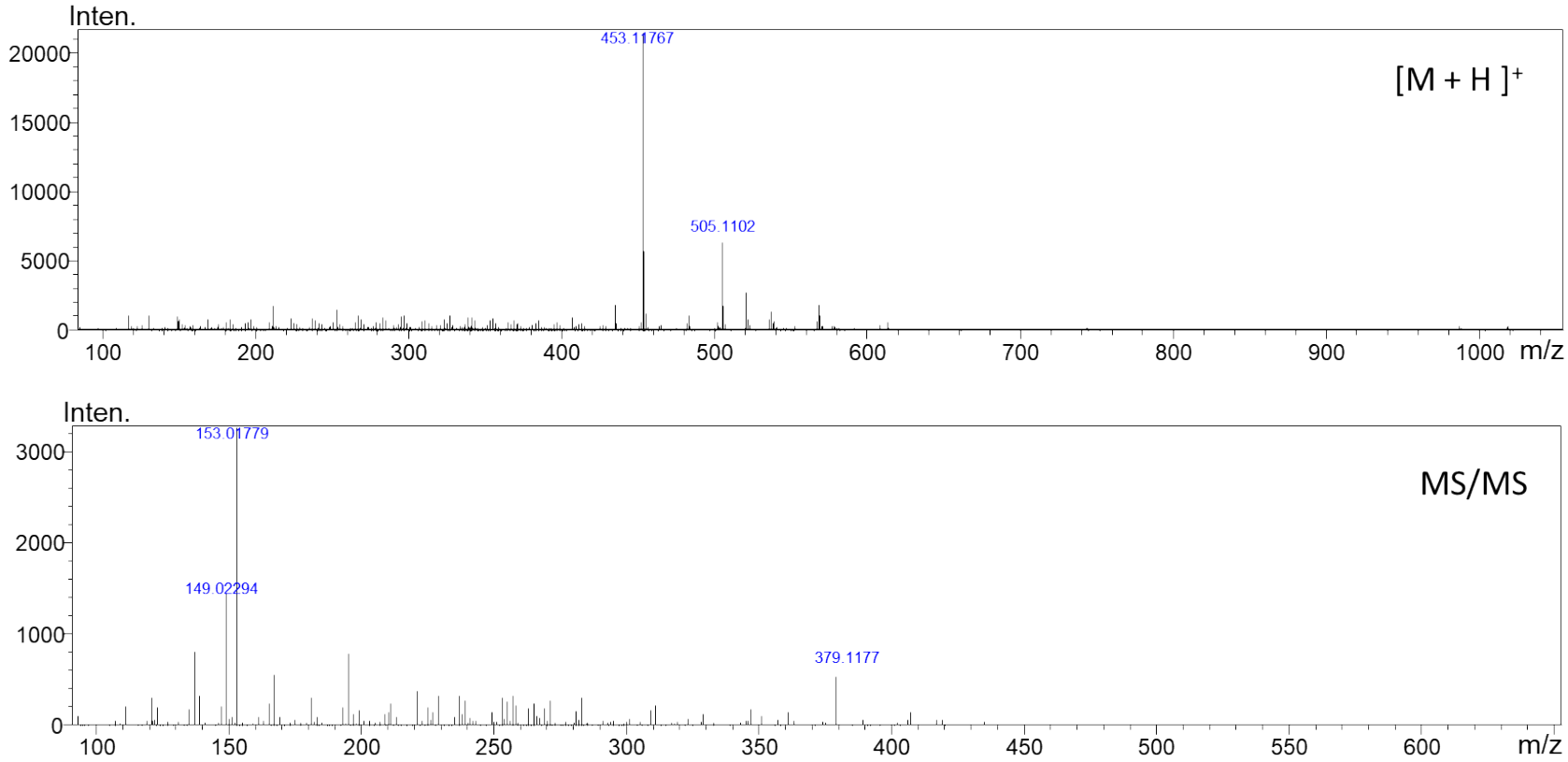


Figure 21. Positive ion electrospray HRMS (top panel) and product ion tandem mass spectrum of peak 14 (in-source fragment of silychristin; $[M+H]^+$) of *m/z* 453.1176 eluting at 13.59 min

References

[1] Csupor, D.; Csorba, A.; Hohmann, J. Recent Advances in the Analysis of Flavonolignans of *Silybum marianum*. *J. Pharm. Biomedical Anal,* **2016**, *130*, 301.

[2] Lee, J. I.; Hsu, B. H.; Wu, D.; Barrett, J. S. Separation and Characterization of Silybin, Isosilybin, Silydianin and Silychristin in Milk Thistle Extract by Liquid Chromatography–Electrospray Tandem Mass Spectrometry. *J. Chromatogr. A* **2006**, *1116*, 57.