In search of the optimal macrocyclization sites for neurotensin.

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Supplementary Information

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I- Additional figures

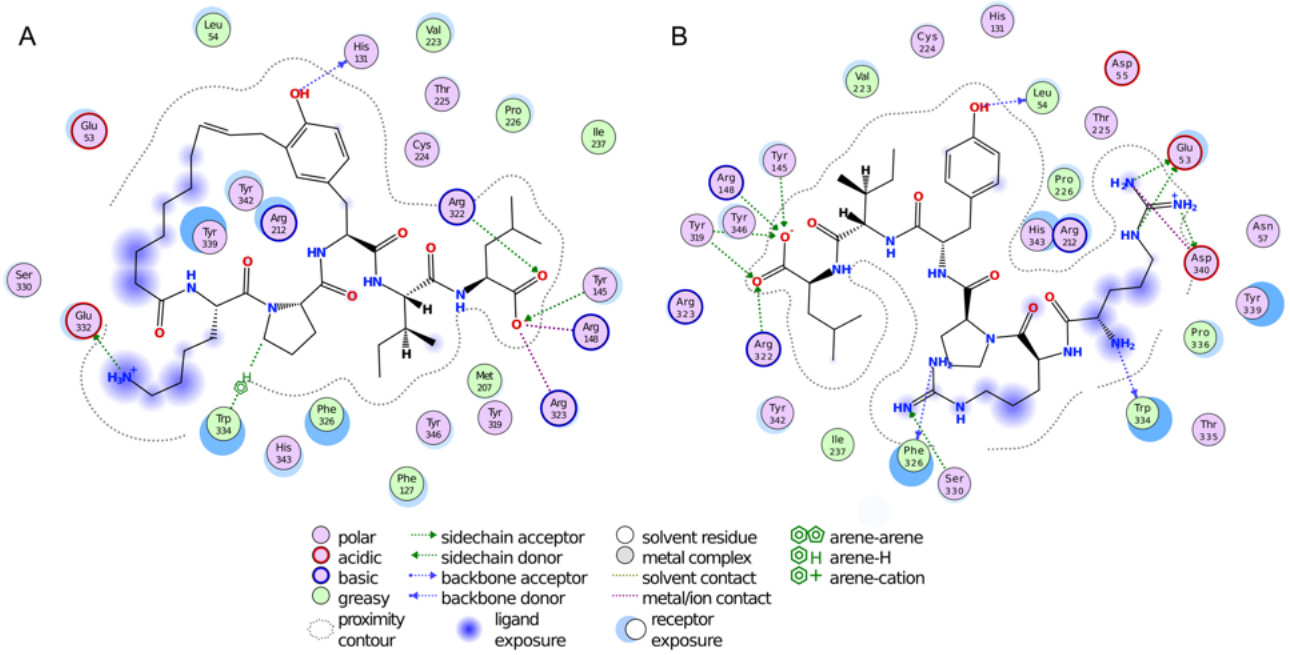

Figure S1. Molecular interactions between hNTS1 and ligands. **A** Compound **10** docked in hNTS1 (homology model based on the rNTS1 crystal 4grv). **B** NT (8-13) in the same homology model. Interactions between the peptide carboxylate and the four receptor residues are conserved, while the Tyr of **10** is shifted and interacts with His^{131} instead of Leu^{54}. The number of *N*-terminal interactions between **10** and the receptor is reduced because of the missing polar groups (namely the *N*-terminal α-amine and the lateral chain of Arg^{8}).

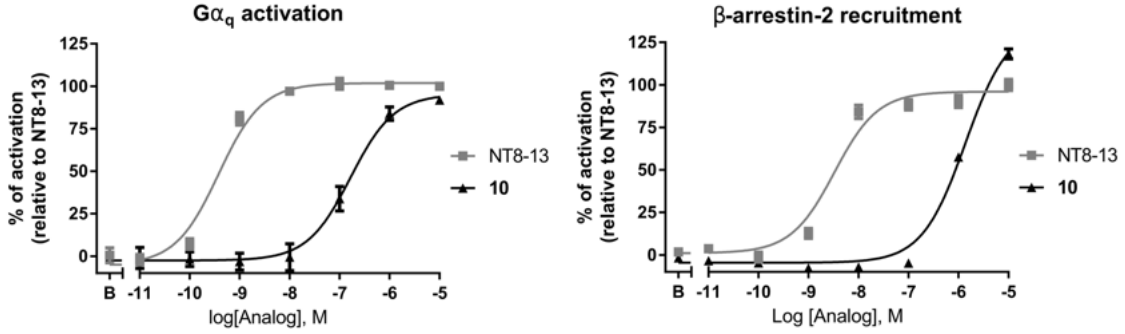

Figure S2. Functional activity of **10** compared to NT (8-13). G protein activation and β-arrestin recruitment were assayed using BRET biosensors.

II- Chemistry

NB : in the following, yields are not optimized.

1. Reagents and solvents

Chemical reagents (except for peptide synthesis reagents, see below) were purchased from Sigma-Aldrich at the highest purity available and used with no further purification. Regular solvents came from Fisher Chemicals and anhydrous solvents from EMD Milipore Corporation.

2. Peptide Synthesis

Peptides were synthesized using standard Fmoc chemistry on 2-chlorotrityl resin (loading: 0.75 mmol/g) obtained from Chem Impex or Matrix Innovation. Fmoc-protected amino-acids and coupling reagents were purchased from these manufacturers as well, in the highest purity available and were used as received. Manual peptide synthesis was performed in 12 mL polypropylene cartridges with 20 µm PE frit from Applied Separations (USA). All quantities given below are for 100 µmol of peptide (130 mg of resin).

Coupling of the first amino acid to the resin

The resin was swelled in DCM for 15 min, then stirred for 30 min with 1.5 mL of a DCM solution containing 5 eq of the Fmoc-protected amino-acid and DIEA (104 µL, 6 eq). The resin was washed with DCM (2 × 5 mL), iPrOH (2 × 5 mL), and DCM (2 × 5 mL). Unreacted sites were capped by stirring 30 min with 1.5 mL of a DCM/MeOH/DIEA (7:2:1) solution and the resin was washed again as described above.

Fmoc Deprotection

Fmoc groups were deprotected by treating the resin with a 20% piperidine/DMF solution (2 × 10 min). The resin was then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), iPrOH (2 × 5 mL), DCM (2 × 5 mL) and DMF (2 × 5 mL).

Coupling of commercial amino acids

The resin was treated with a solution of Fmoc-amino-acid (0.5 mMol, 5 eq), HATU (95 mg, 5 eq) and DIEA (104 µL, 6 eq) in DMF (1.5 mL) for 30 min, then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), iPrOH (2 × 5 mL), DCM (2 × 5 mL) and DMF (2 × 5 mL).

Coupling of non-commercial or costly amino-acids

The resin was treated with a solution of Fmoc-amino acid (0.2 mMol, 2 eq), HATU (38 mg, 2 eq) and DIEA (52 µL, 3 eq) in DMF (1.5 mL) for 2 h, then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), iPrOH (2 × 5 mL), and DCM (2 × 5 mL). The procedure was repeated if the resin beads turned blue when submitted to the Kaiser test.[1]

Acetylation of (*o*-allyl)Tyrosine

Subsequent to coupling of Fmoc-(*o*-allyl)tyrosine, the resin was stirred for one hour in the presence of acetic anhydride (19 µL, 2 eq) and DIEA (35 µL, 2 eq) in DCM (1.5 mL). This step was repeated once or until reaction completion was confirmed by mini-cleavage followed by UPLC-MS analysis. The resin was then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), iPrOH (2 × 5 mL), DCM (2 × 5 mL) and DMF (2 × 5 mL).

Ring-Closing Metathesis

Dry resin, Hoveyda-Grubbs 2nd generation catalyst (12.5 mg, 0.2 eq) and *p*-benzoquinone (11 mg, 1 eq) were placed in a microwave tube which was first purged with anhydrous argon for 10 min. Anhydrous DCE (1.5 mL) was added before submitting the mixture to microwave irradiation (50°C, 60 min) on a Discover SP apparatus from CEM. The resin was then washed with DCM (5 × 5 mL).

De-acetylation of (*o*-allyl)Tyrosine

The resin was treated with 20% Piperidine/DMF (1.5 mL) for 1h. This step was repeated once or until reaction completion was confirmed by mini-cleavage followed by UPLC-MS analysis. The resin was then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), iPrOH (2 × 5 mL), DCM (2 × 5 mL) and DMF (2 × 5 mL).

Simultaneous resin cleavage and side-chain deprotections

The resin was transfered to a 20 mL glass vial and stirred for 2 h with 3 mL of a TFA/DCM/TiS (50:49:1) solution. The peptide was then precipitated in 20 mL of cold tBME, centrifugated (3000 rpm, 15 min, 4°C) and dried *in vacuo*.

Peptide Purification

1 Kaiser, E.; Colescott, R.L.; Bossinger, C.D. and Cook, P.I. *Anal Biochem.* **1970**, 34(2), 595–598.

The crude product was resuspended in water/acetonitrile (7:3) and purified on a preparative HPLC-MS system from Waters (column XSELECT™ CSH™ Prep C18 (19 x 100 mm) packed with 5 µm particles, UV detector 2998, MS SQ Detector 2, Sample manager 2767 and a binary gradient module) using acetonitrile and water + 0.1 % formic acid as eluents. Isolated fractions were then lyophilized and their purity was assessed using a Waters UPLC H-Class with UV detection PDA equipped with an Acquity UPLC® CSH™ C18 (2.1 x 50 mm) column packed with 1.7 µm particles with the following gradient: acetonitrile and water with 0.1% TFA (0→0.2 min: 5% acetonitrile; 0.2→1.5 min: 5%→95%; 1.5→1.8 min: 95%; 1.8→2.0 min: 95%→5%; 2.0→2.5 min: 5%). MS spectra were recorded on a Waters SQD 2 detector (electrospray) instrument. In general, *E* and *Z* isomers around the unsaturation did not separate. High resolution mass spectra (HRMS) of all analogues were obtained using electrospray infusion ESI-Q-Tof from maXis.

3. Synthesis of amino-acid derivatives

Linker B (Fmoc-Tyr-*o*-Allyl-OH)[2]

Fmoc-Tyr(Allyl)-OH (5 g, 11.3 mmol) was suspended into 40 mL anhydrous toluene under argon atmosphere at 0°C. Diethylaluminum chloride (12.7 mL of 1.8 mol/L solution in toluene, 2 eq) was added. The reaction was stirred at room temperature for 4 h then quenched at 0°C by addition of 25 mL 6N HCl. The organic phase was washed with brine, dried over $MgSO_4$, filtered then evaporated. The product and remaining starting material were purified by flash chromatography. The product was then solubilized in water/acetonitrile 50:50 and lyophilized, to give 1.5 g of the desired product (30 % yield). ^{1}H NMR ($CDCl_3$ /CD_3OD 9:1, 400 MHz): 7.7 (d, *J* = 7.5 Hz, 2H), 7.5 (t, *J* = 7.0 Hz, 2H), 7.33 (t, *J* = 7.5 Hz, 2H), 7.24 (t, *J* = 7.4 Hz, 2H), 6.85 (d, *J* = 1.9 Hz, 1H), 6.81 (dd, *J* = 8.1, 1.9 Hz, 1H), 6.65 (d, *J* = 8.1 Hz, 1H), 5.92 (m, 1H), 5.0 (dq, *J* = 1.7, 6.61 Hz, 1H), 4.96 (t, *J* = 1.4 Hz, 1H), 4.49 (t, *J* = 5.9 Hz, 1H), 4.29 (m, 2H), 4.13 (t, *J* = 7.1 Hz, 1H), 3.47 (br, 4H), 3.28 (d, *J* = 6.6 Hz, 2H), 3.03 (dd, *J* = 5.3, 14.1 Hz, 1H), δ 2.94 (dd, *J* = 6.5, 14.1 Hz, 1H). ^{13}C NMR ($CDCl_3$ /CD_3OD 9:1, 100 MHz): δ 173.83, 153.55, 143.80, 141.30, 136.78, 131.23, 128.24, 127.76, 67.07, 127.12, 126.32, 115.36, 115.65, 125.13, 120.0, 54.86, 49.37, 47.16, 37.14, 34.28.

Linker A (Fmoc-Ser(Allyl)-OH)

2 *The Claisen Rearrangement: Methods and Applications*; Hiersemann, M., Nubbemeyer, U., Eds.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, **2007**.

Boc-Ser(Allyl)-OH: Sodium hydride (4.9 g, 122 mmol, 2.5 eq) was suspended into 20 mL DMF in a dry round-bottom flask under argon atmosphere and placed at 0°C. Boc-Ser-OH (10 g, 48.8 mmol) was dissolved into 80 mL DMF and this solution was added slowly to the flask. After stirring for 30 minutes at 0°C, allyl bromide (5.2 mL, 48.8 mmol, 1 eq) was added dropwise. The reaction was stirred at room temperature for 4h, then quenched with 500 mL of NH_4Cl saturated aqueous solution. This solution was washed with ethyl acetate (250 mL) then its pH was adjusted to 1-2 by addition of 6N HCl. The product was extracted with 3x300 mL ethyl acetate. The organic phase was washed with brine, dried over $MgSO_4$, filtered and evaporated in vacuo. The product was purified by flash chromatography and obtained as a yellow oil (60 % yield). NMR caracterisation concurred with what was previously described.[3]

Fmoc-Ser(Allyl)-OH: Boc-Ser(Allyl)-OH (650 mg, 2.7 mmol) was dissolved into 10 mL of DCM/TFA (50:50) and stirred for 2 hours. The solvent was evaporated under reduced p-ressure and the crude was dissolved into 10 mL of water. The pH of this solution was adjusted to 8-9 by addition of $NaHCO_3$. Fmoc-Cl (1049 mg, 4.1 mmol, 1.5 eq) was dissolved in THF and this solution was poured over the aqueous solution. The mixture was stirred overnight and THF until completion of the reaction (TLC) was removed by evaporation. The aqueous phase was washed with diethyl ether (5 mL) and its pH adjusted to 2-3 by addition of 1N HCl. The product was extracted with 3x10 mL ethyl acetate. The combined organic phases were washed with brine (10 mL), dried over $MgSO_4$, filtered and evaporated *in vacuo*. The product was purified by flash chromatography and obtained as a white solid (55 % yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.59 (dd, *J* = 7.2, 3.2 Hz, 2H), 7.38 (t, *J* = 7.3 Hz, 2H), 7.31 (t, *J* = 8.1 Hz, 2H), 5.84 (m, 1H), 5.66 (d, *J* = 8.5 Hz, 1H), 5.26 (d, *J* = 17.0 Hz, 1H), 5.2 (d, *J* = 10.6 Hz, 1H), 4.54 (m, 1H), 4.41 (m, 2H), 4.23 (t, *J* = 7.1 Hz, 1H), 4.02 (d, *J* = 5.7 Hz, 1H), 3.94 (dd, *J* = 9.5, 3.5 Hz, 1H), 3.69 (dd, *J* = 9.5, 3.5 Hz, 1H). ^{13}C NMR ($CDCl_3$ 75.4 MHz): δ 154.90, 136.72, 124.34, 124.21, 121.83, 114.22, 108.27, 107.62, 105.64, 100.52, 98.56, 53.02, 49.92, 47.85, 34.61, 27.65.

III- Biological methods

1. Binding Assays

3 As described in Boal, A. K.; Guryanov, I.; Moretto, A.; Crisma, M.; Lanni, E. L.; Toniolo, C.; Grubbs, R. H.; O'Leary, D. J. *J. Am. Chem. Soc.* **2007**, *129* (22), 6986–6987.

Cell Culture. CHO-K1 cells stably expressing the human NTS1 receptor (hNTS1) (ES-690-C from PerkinElmer, Montréal, QC, Canada) were cultured in Ham's F12 medium at 37°C in a humidified chamber at 5% CO_2. Culture media were supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 20 mM HEPES, and 0.4 mg/mL G418.

Competitive Radioligand Binding Assay on hNTS1. Upon reaching 80% confluence, CHO-K1 cells expressing hNTS1 were washed with PBS and frozen at -80°C until use. Cells were scrapped-off the dish with 10 mM Tris, 1 mM EDTA, pH 7.5 and centrifuged at 3,000 rpm for 15 min at 4°C. The pellet was then resuspended in 50 mM Tris-HCl, pH7.5, containing 0.2% BSA (binding buffer). Competitive radioligand binding experiments were performed by incubating 15 µg of freshly prepared cell membranes expressing hNTS1 with 45 pM of ^{125}I-[Tyr^3]-NT (2200 Ci/mmol) (purchased from PerkinElmer, Billerica, MA) in binding buffer in the presence of increasing concentrations of the analogues ranging from 10^{-11} to 10^{-4} M for 60 min at 25°C. After incubation, the binding reaction mixture was transferred into Polyethyleneimine-coated 96-well filter plates (glass fiber filters GF/B, Millipore, Billerica, MA). Reaction was terminated by filtration, and plates were washed three times with 200 µL of ice-cold binding buffer. Glass filters were then counted in a γ-counter (2470 Wizard2, PerkinElmer, Missisauga, ON, Canada). Non-specific binding was measured in the presence of 10^{-5} M unlabeled NT 8–13 and represented less than 5% of total binding. IC_{50} values were determined from the competition curves as the unlabeled ligand concentration inhibiting half of the ^{125}I-[Tyr^3]-NT-specific binding.

Data Analysis. Competitive radioligand binding data were plotted using Prism 7 (GraphPad, La Jolla, CA) using the One-site-Fit Log(IC_{50}) and represented the mean ± SEM of at least three separate experiments each done in triplicate.

2. BRET Assays[4]

CHO-K1 cells (ATCC, CCL-61) were cultured in DMEM-F12 supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 20 mM HEPES at 37°C in a humidified chamber at 5% CO_2. Cells were seeded into 10-cm petri dishes at a density of 1.5 × 10^6 cells; 24 h after seeding, cells were transfected with plasmids coding for hNTS1-GFP10/RlucII-β-arrestin2, or with hNTS1/Gαq-

4 Galés, C.; Rebois, R. V.; Hogue, M.; Trieu, P.; Breit, A.; Hébert, T. E.; Bouvier, M. *Nature Methods* **2005,** 2(3), 177–144.

RlucII/Gβ_1/GFP10-Gγ_1 using PEI. Cells were transferred into 96-well plates at a concentration of 50 000 cells/well 24 h after transfection and incubated at 37°C overnight. Then, cells were washed with PBS and 90 µL of HBSS was added in each well; cells were then stimulated with increasing concentrations of each analog ranging from 10^{-11} to 10^{-5} M for 20 min (β-arrestin2 recruitment) or 5 min (Gα_q activation) at 37°C. After stimulation, coelenterazine 400A was added in each well to a final concentration of 5 µM and the plate was read using filter selected for BRET2 measurement on a Mithras 2 plate reader (Berthold Technologies, Tennessee, USA). BRET2 ratio was determined as $GFP10_{em}$ /$RlucII_{em}$. Data were analyzed using GraphPad Prism 7, normalization was done by using the BRET2 ratio of nonstimulated cells as 0% and the ratio of cells stimulated with 10^{-6} M NT 8–13 as 100% of activation. EC_{50} values were determined using the dose response-stimulation log(agonist) vs response (three parameters) and represent the mean ± SEM of at least three separate experiments, each performed in triplicate.

3. Plasma Stability Assay

Rat plasma was obtained by centrifugation of rat blood (13000 rpm, 5 min, 4°C). 6 µL of a 1 mM aqueous solution (10% DMSO) of peptide were incubated with 27 µL of rat plasma at 37°C for 1, 2, 4 and 8 hours (1, 3 and 5 min for NT 8–13). At these times, proteolytic degradation was halted by adding 70 µL acetonitrile/ethanol (1:1), 0.5 µM nicotinamide solution and vortexing. Samples were kept on ice until centrifugated (13000 rpm, 5 min, 4°C) and the supernatant was filtered on a 4 mm nylon 0.2 µm syringe filter and analyzed by UPLC-MS. Each experiment was repeated three times. Data were analyzed using GraphPad Prism 7's one phase decay equation.

IV- Modeling procedures

All modelling was done using MOE.[5]

Superimposition to NT 8-13: Macrocycles (**1**, **2** and **6**) were built using MOE's builder then minimized on all atom using Amber 10: EHT forcefield and 0.1 kcal/mol/A^2 RMS as a gradient. The X-ray structure of rNTS1-bound NT 8-13 (pdb ID 4grv)[6] was loaded and both structures were

5 *Molecular Operating Environment (MOE)*, 2016.08; Chemical Computing Group ULC, 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, **2017**

6 White, J. F.; Noinaj, N.; Shibata, Y.; Love, J.; Kloss, B.; Xu, F.; Gvozdenovic-Jeremic, J.; Shah, P.; Shiloach, J.; Tate, C. G.; Grisshammer, R. *Nature* **2012**, *490* (7421), 508–513.

protonated and their partial charges were calculated. NT 8-13 was set as "fixed" and the structures were superimposed using the Flexible Alignment module. The iteration limit was fixed to 200, the failure limit to 20 and the energy cutoff to 15 Kcal/mol.

Docking: Macrocycles 7-10 were built using MOE's buider, minimized, protonated and partial charges were calculated. Conformational search with the "Conformatial Search" module were carried out on the Z and E isomers independently using Amber10:EHT as forcefield and the LowModeMD as a method, performing molecular dynamics perturbations along low frequency vibrational mode. Rejection limit was fixed to 100, iteration limit to 10 000, RMS gradient to 0.005 and MM iteration limit to 500. Two conformations were judged equal if the optimal heavy atom RMS superposition distance was less than 0.25A. The conformations obtained were then docked into a hNTS1 homology model (built from rNTS1 X-ray structure 4grv using MOE's tools).

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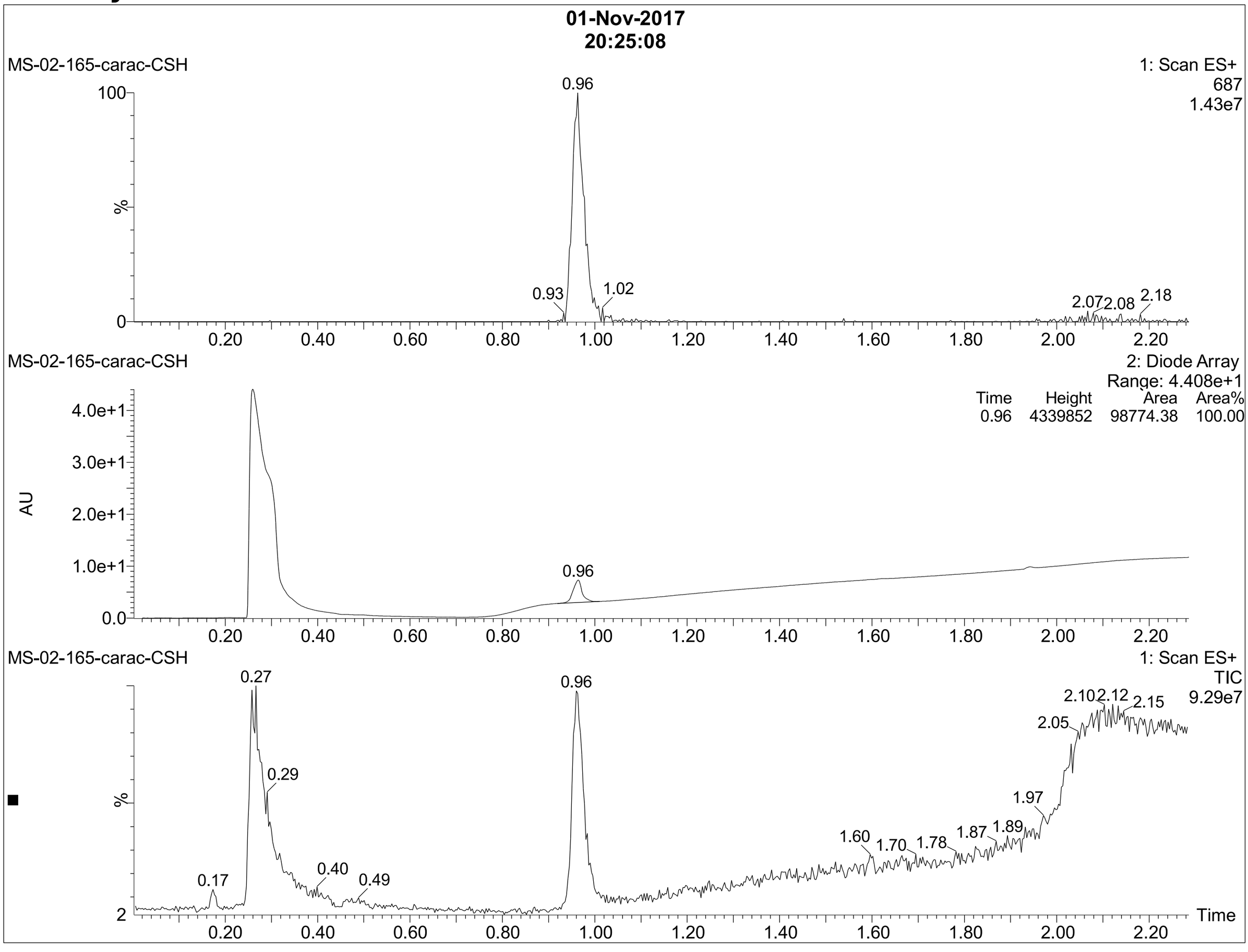
Supplementary Information

Compounds caracterisation

Compound	Obtained (mg)	Molecular Formula	Molecular Weight (g/mol)	Purity (%)	Exact Mass (calculated)	Exact Mass (found)	Ion
1	7.7	$C_{34}H_{51}N_7O_8$	685.8	100	686.3872	686.3853	$[M+H]^+$
1-L	24	$C_{36}H_{55}N_7O_8$	713.9	99.5	714.4185	714.4182	$[M+H]^+$
2	6.5	$C_{34}H_{51}N_7O_8$	685.8	100	686.3872	686.3880	$[M+H]^+$
3	7.1	$C_{34}H_{51}N_7O_8$	685.8	100	686.3872	686.3869	$[M+H]^+$
4	5.8	$C_{34}H_{51}N_7O_8$	685.8	100	686.3872	686.3883	$[M+H]^+$
5	14.8	$C_{34}H_{51}N_7O_8$	685.8	96.9	686.3872	686.3873	$[M+H]^+$
6	20.6	$C_{36}H_{55}N_7O_{10}$	745.9	100	746.4083	746.4089	$[M+H]^+$
7	8.4	$C_{33}H_{50}N_6O_8$	658.8	90.7	659.3763	659.3757	$[M+H]^+$
8	8.4	$C_{38}H_{59}N_7O_8$	741.9	100	742.4498	742.4475	$[M+H]^+$
9	2.8	$C_{39}H_{61}N_7O_9$	771.9	100	772.4604	772.4622	$[M+H]^+$
10	3.3	$C_{42}H_{66}N_6O_8$	783.0	100	783.5015	783.5023	$[M+H]^+$
10-L	4.4	$C_{44}H_{70}N_6O_8$	811.1	100	811.5328	811.5329	$[M+H]^+$
Linker A	-	$C_{21}H_{21}NO_5$	367.4	97	390.1312	390.1323	$[M+Na]^+$
Linker B	-	$C_{27}H_{25}NO_5$	443.5	99	466.1625	466.1636	$[M+Na]^+$

Macrocycle 1



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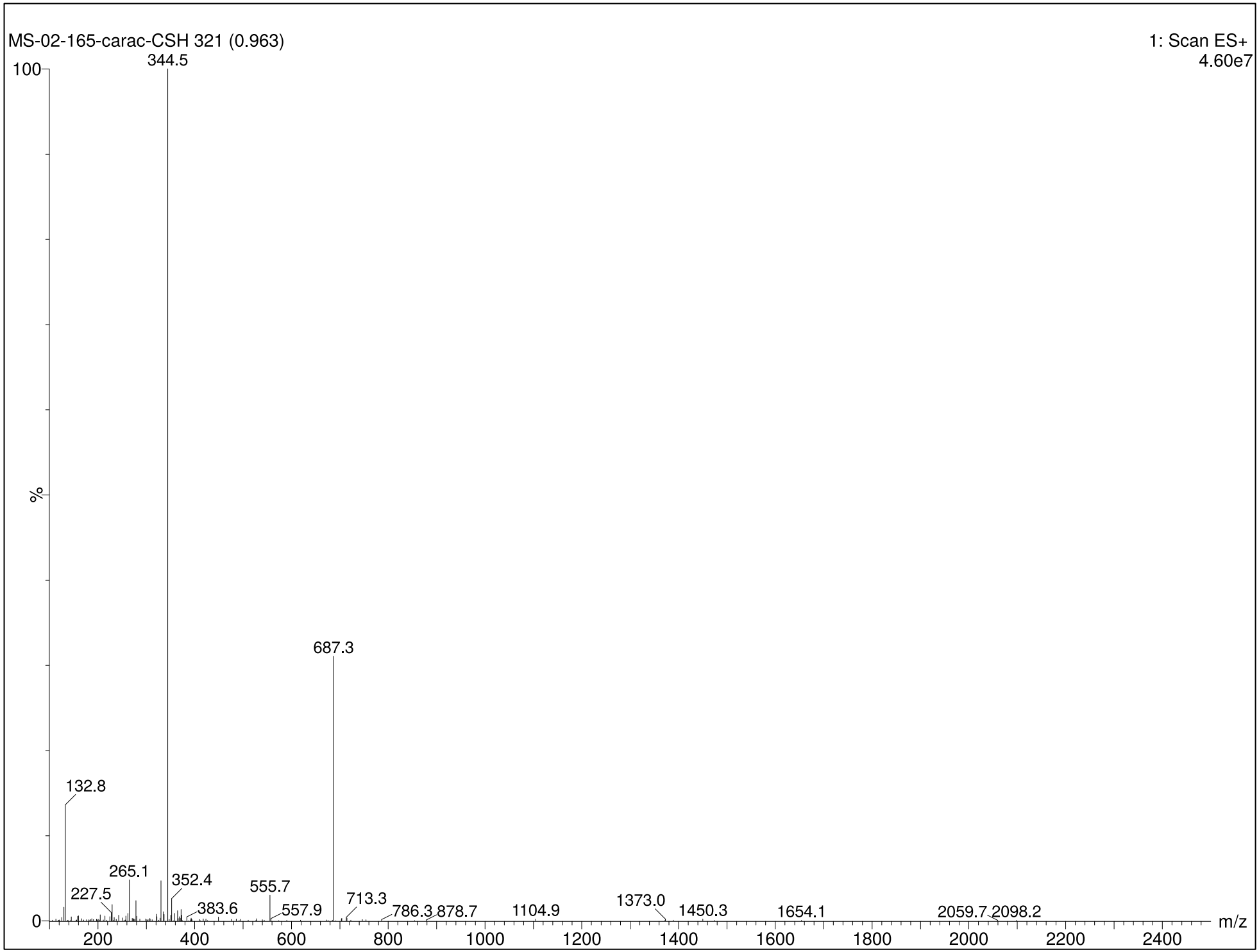

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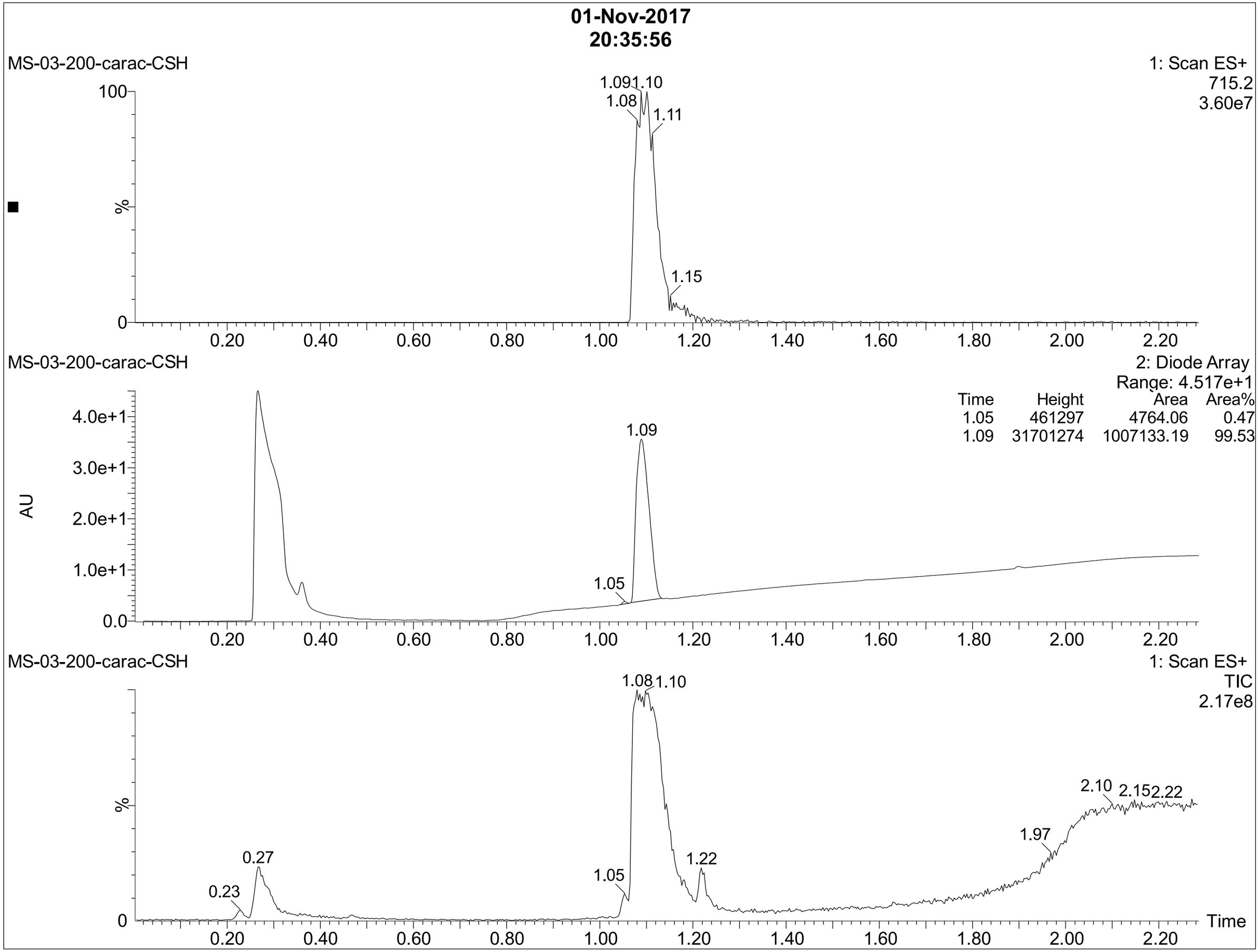

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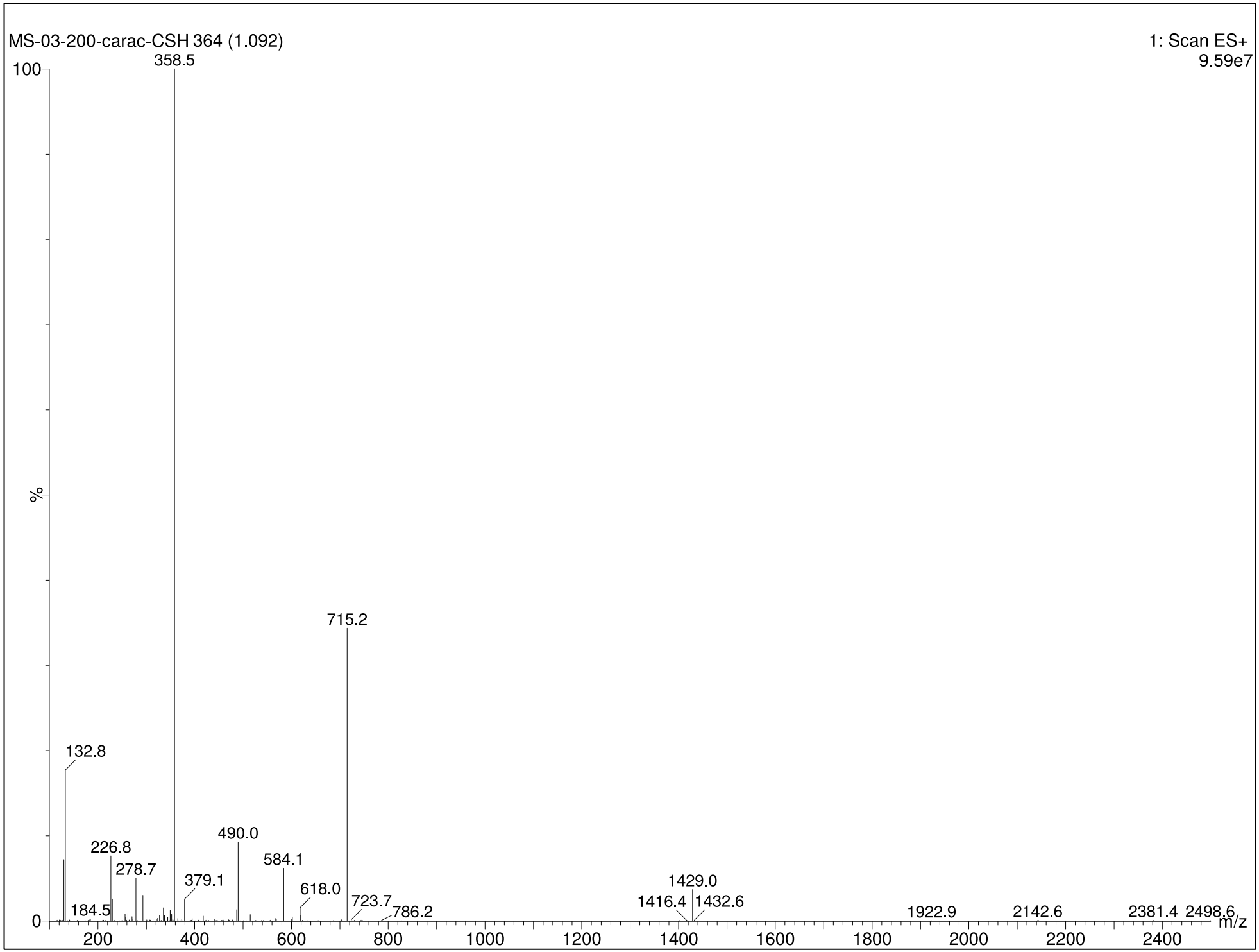

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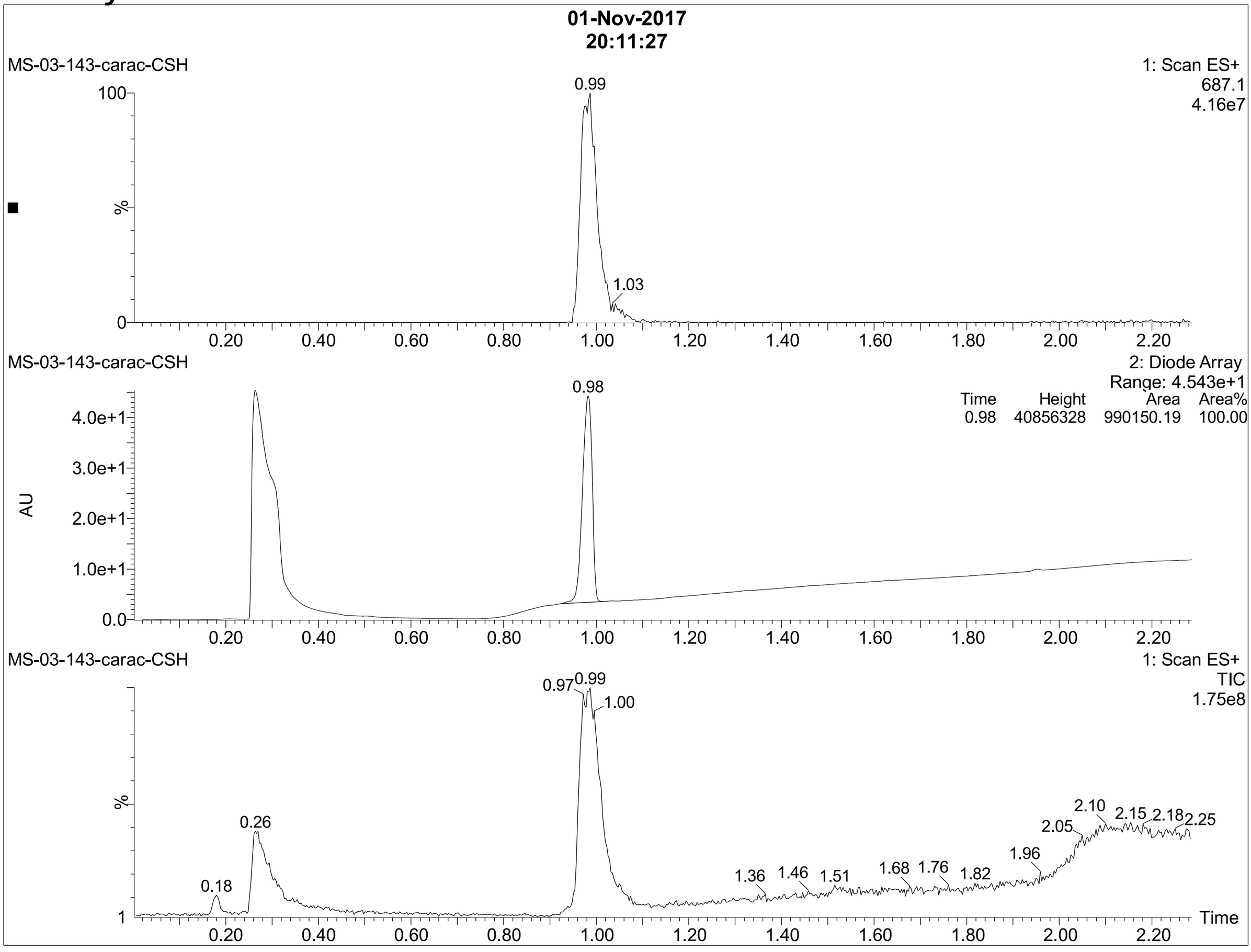

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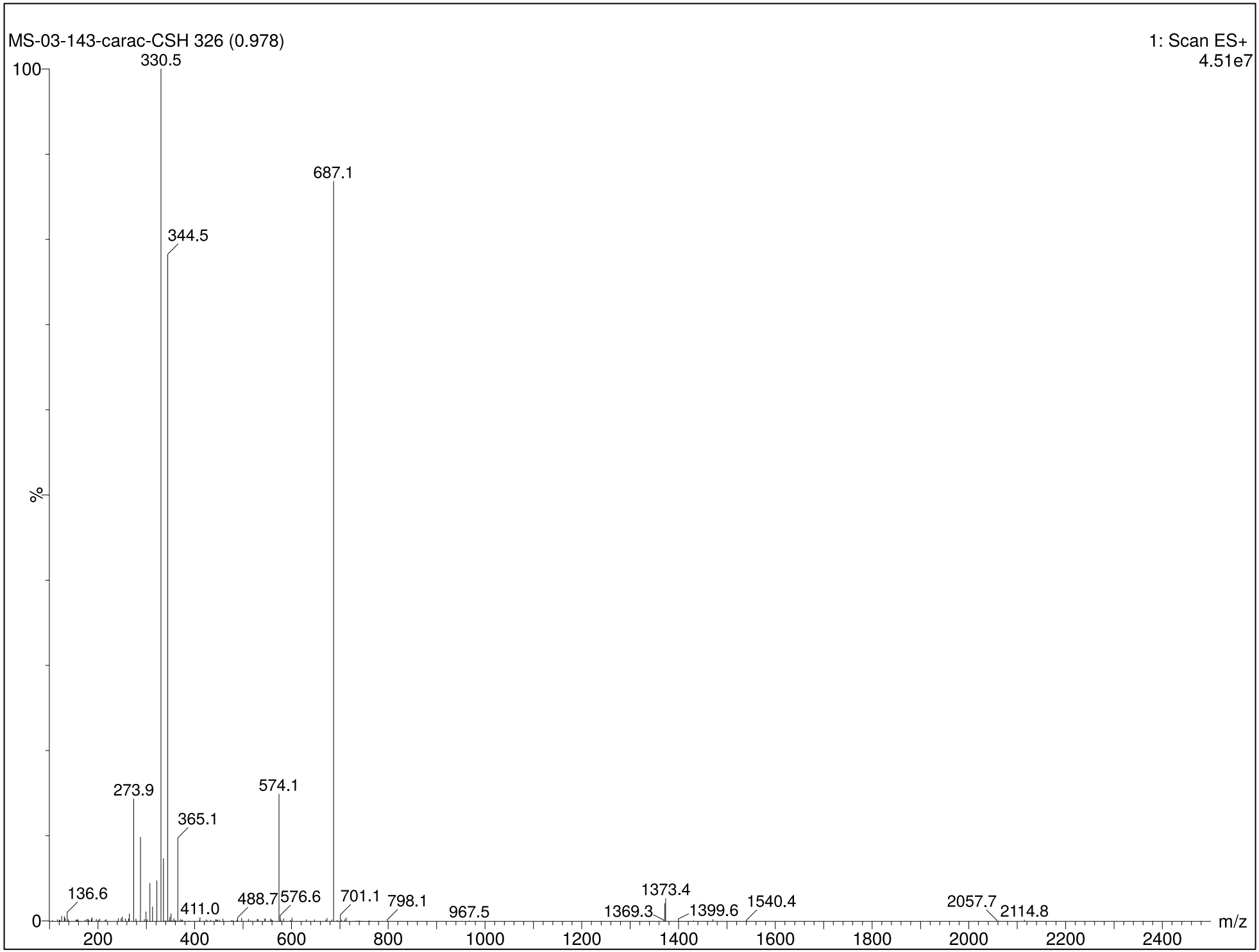

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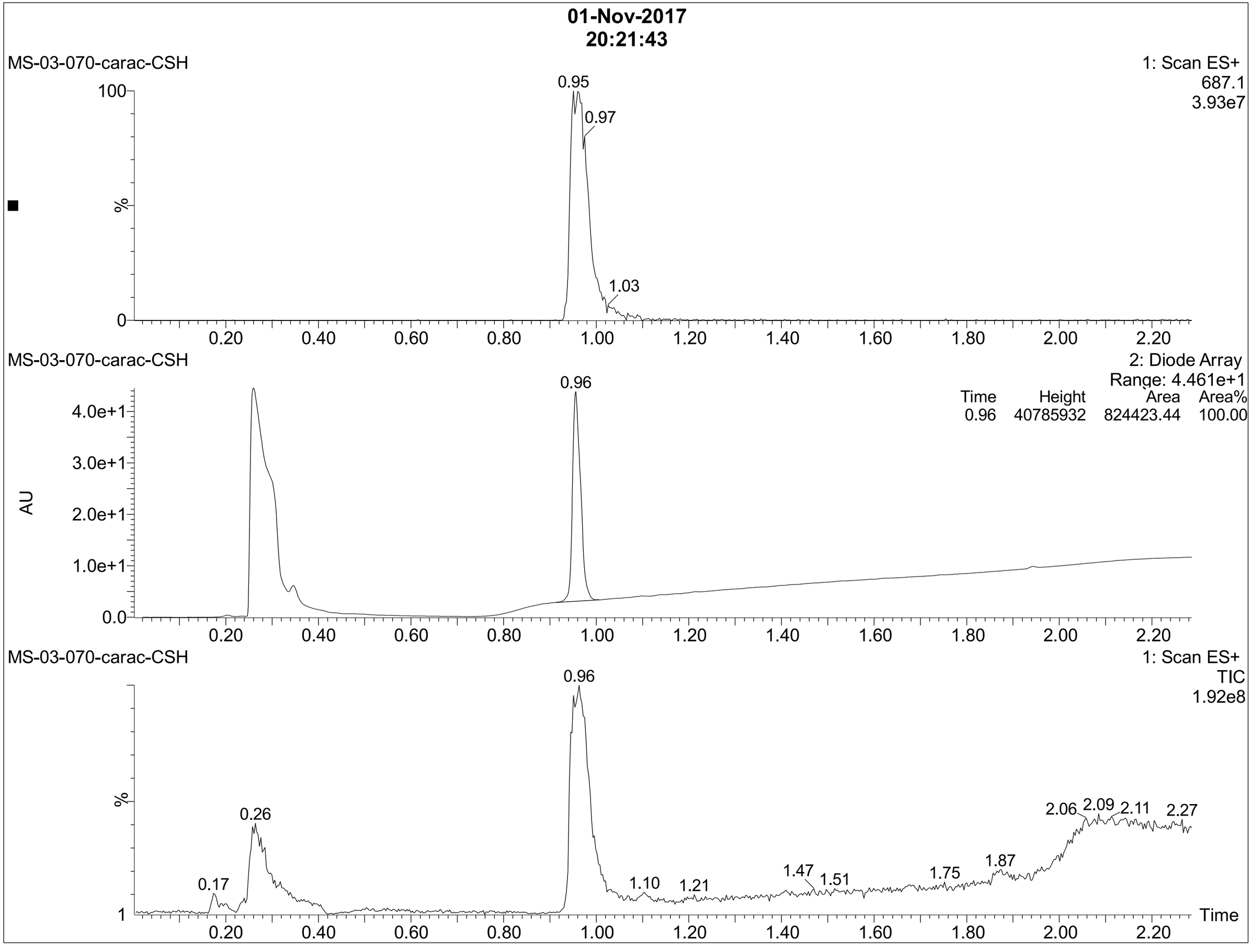

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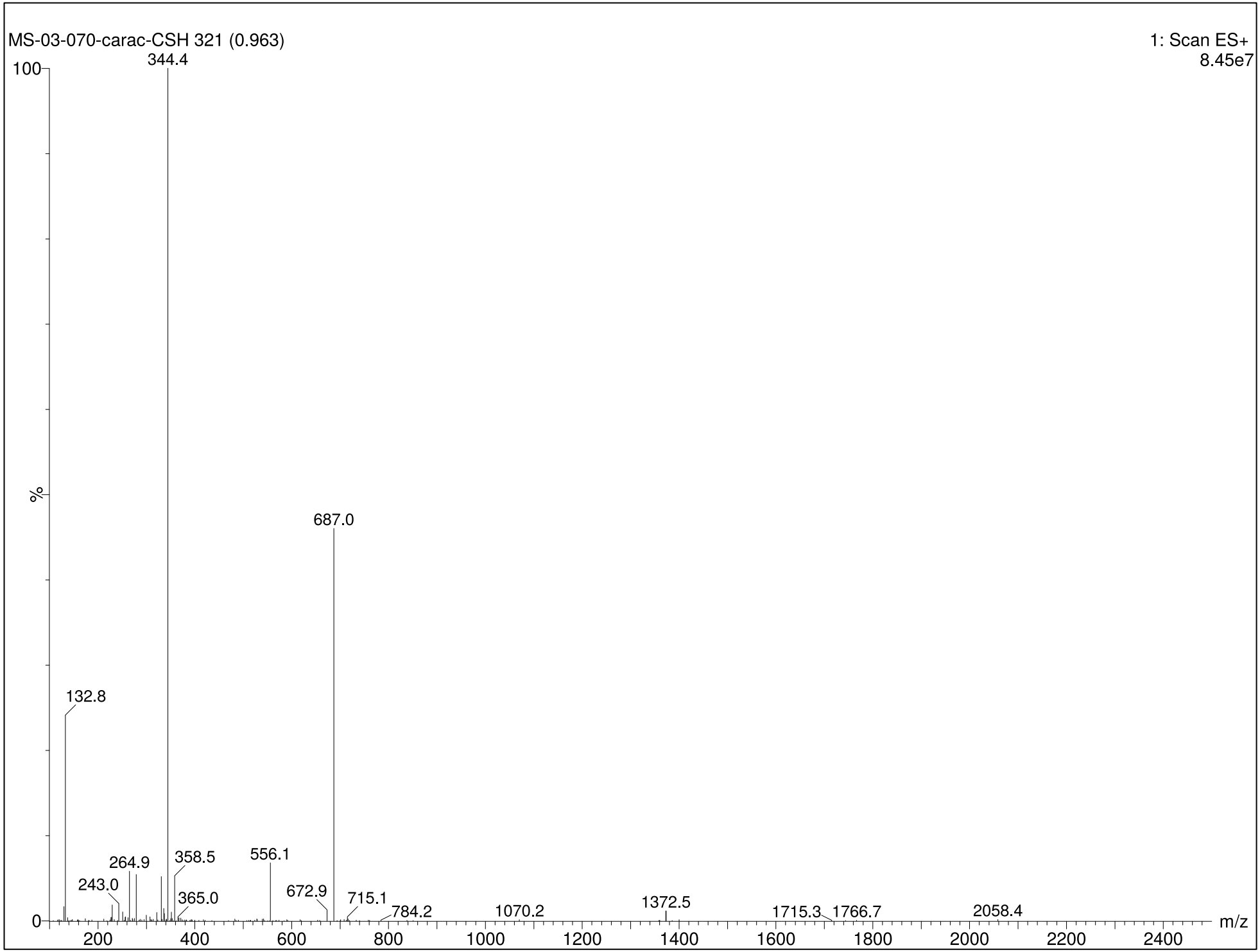

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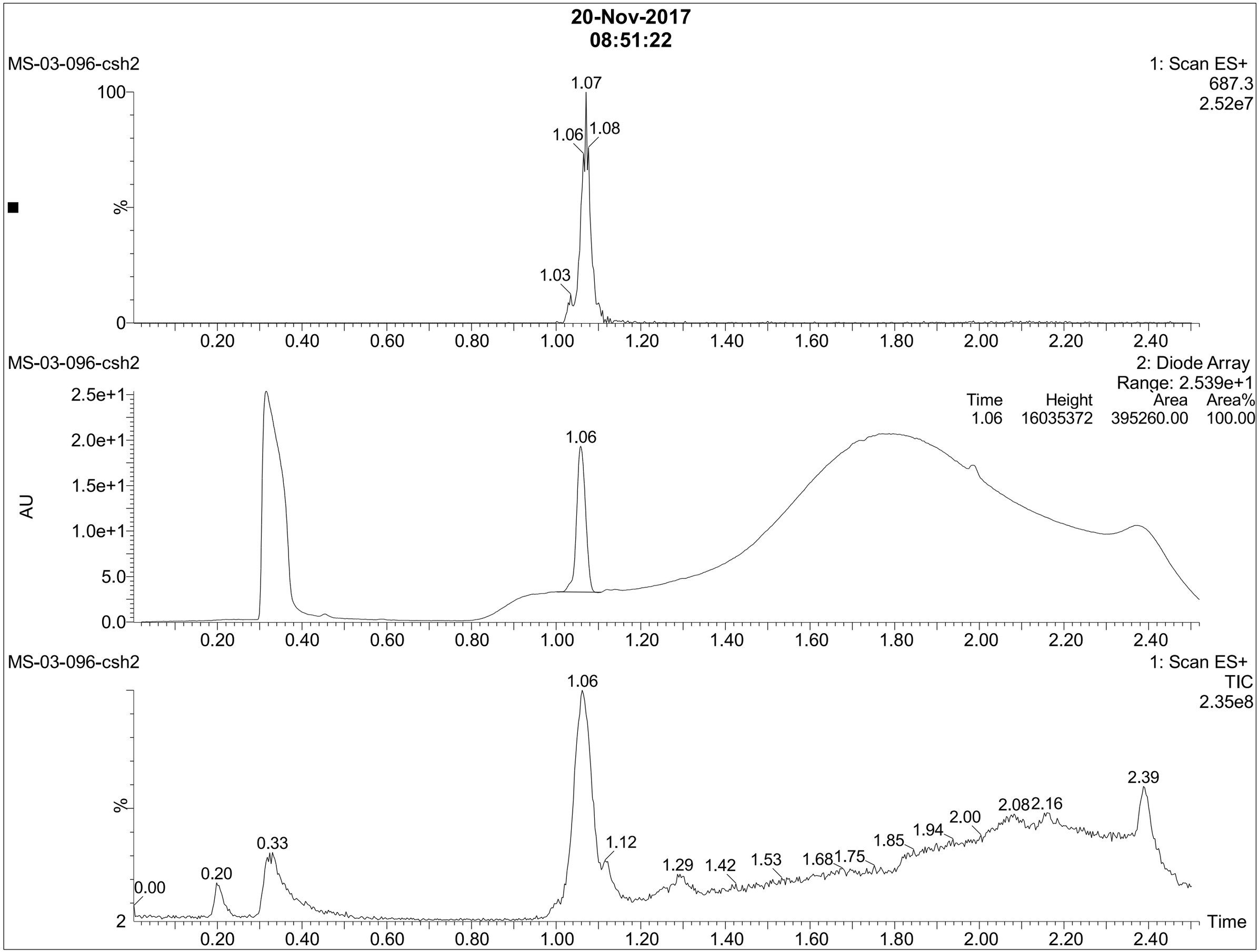

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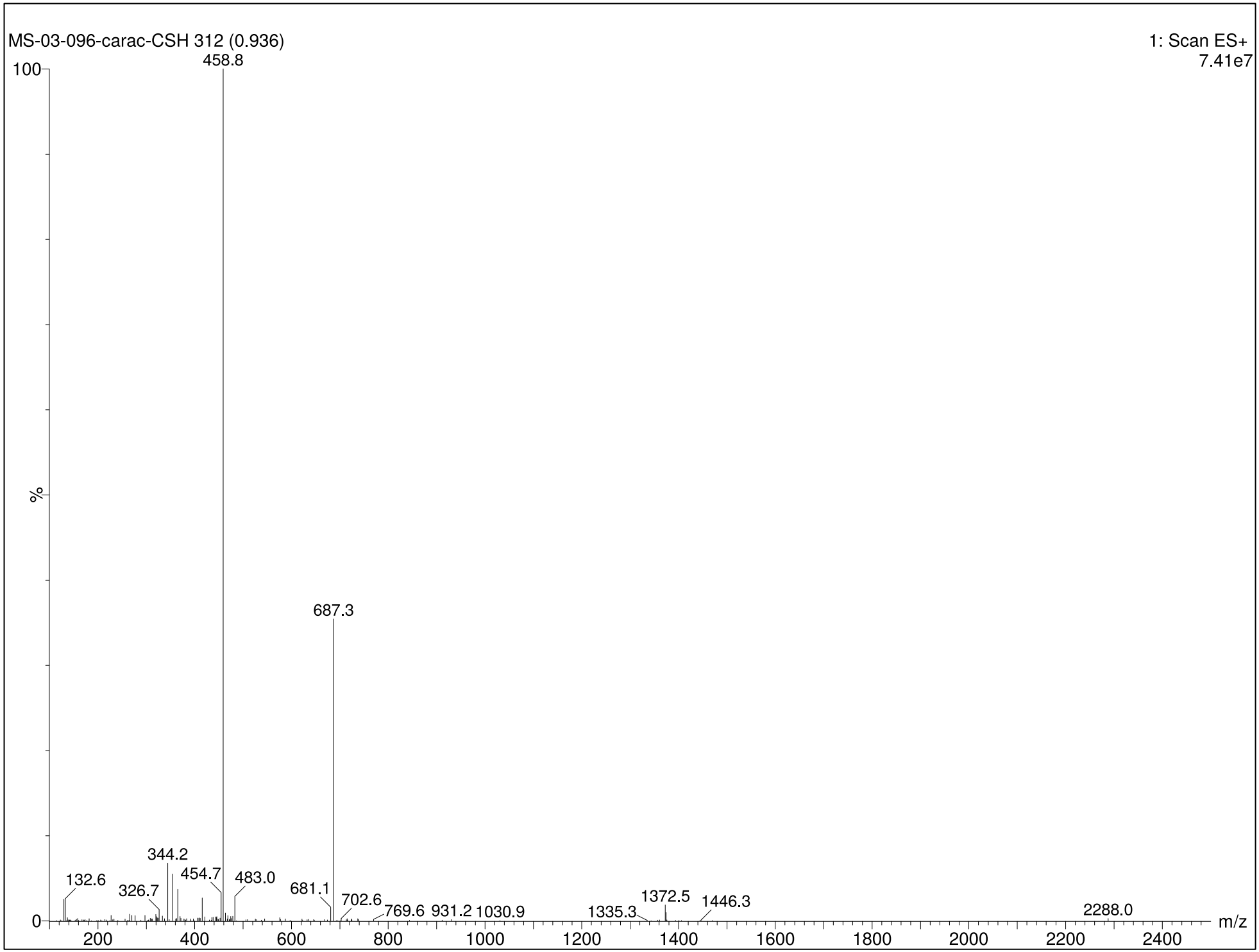

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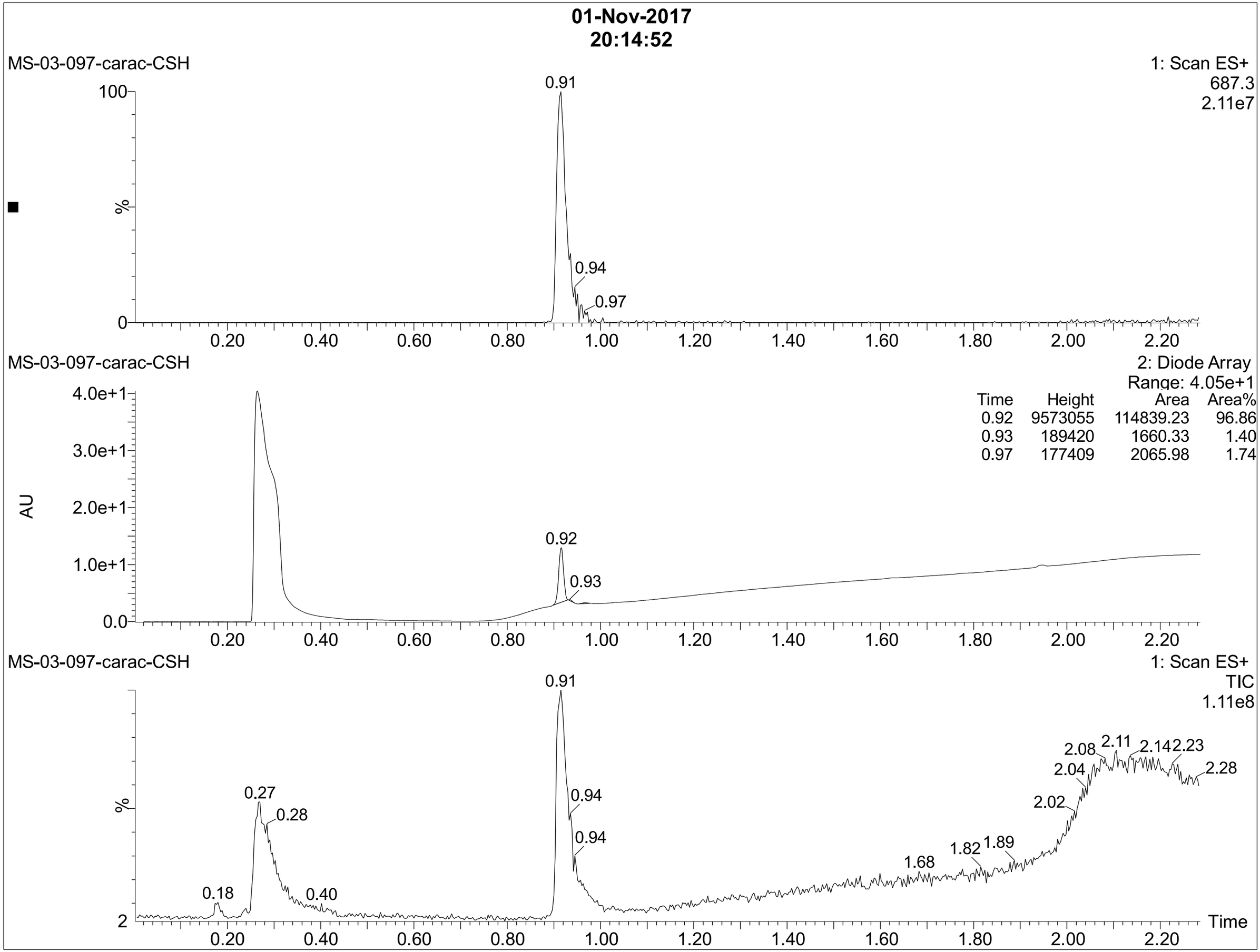

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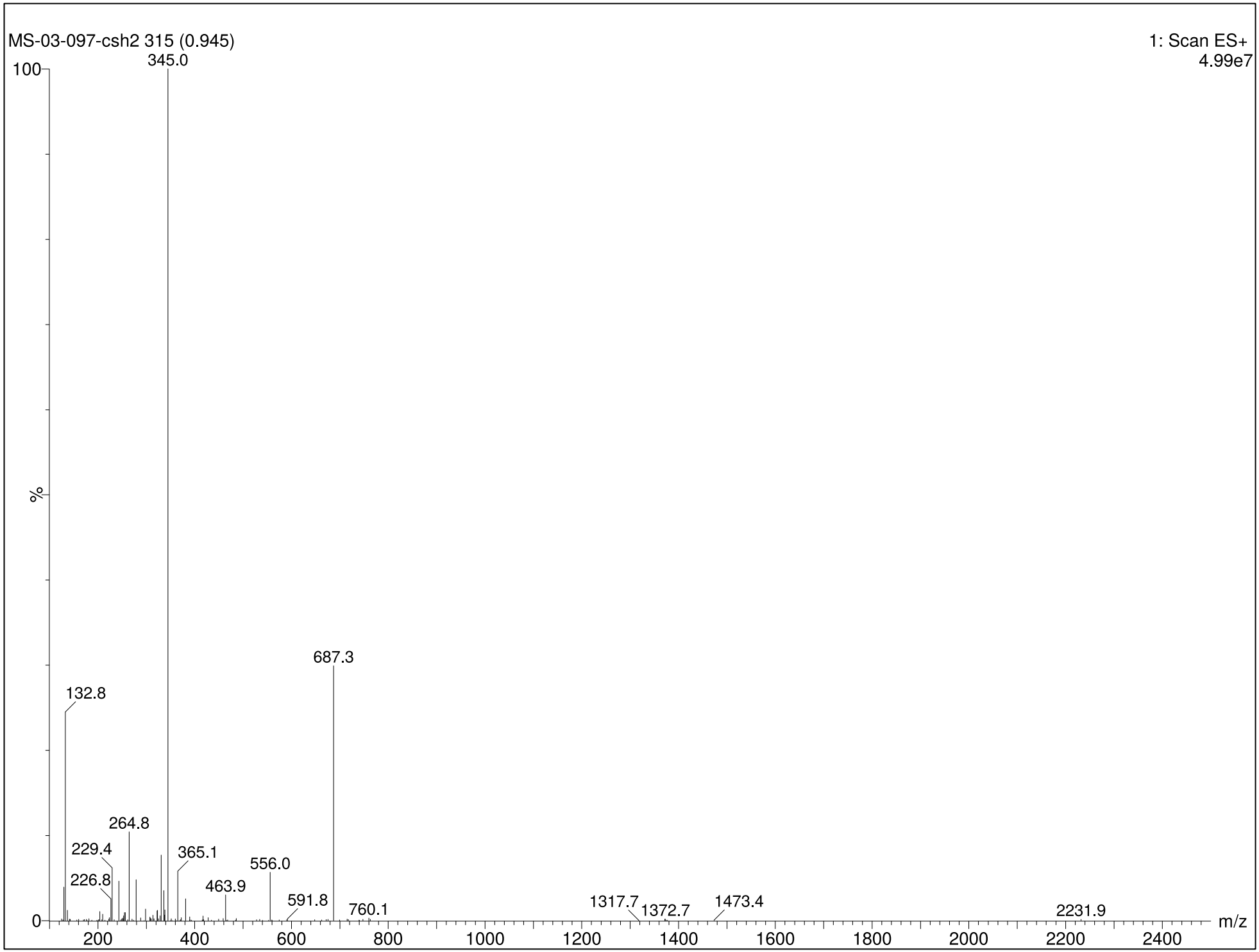

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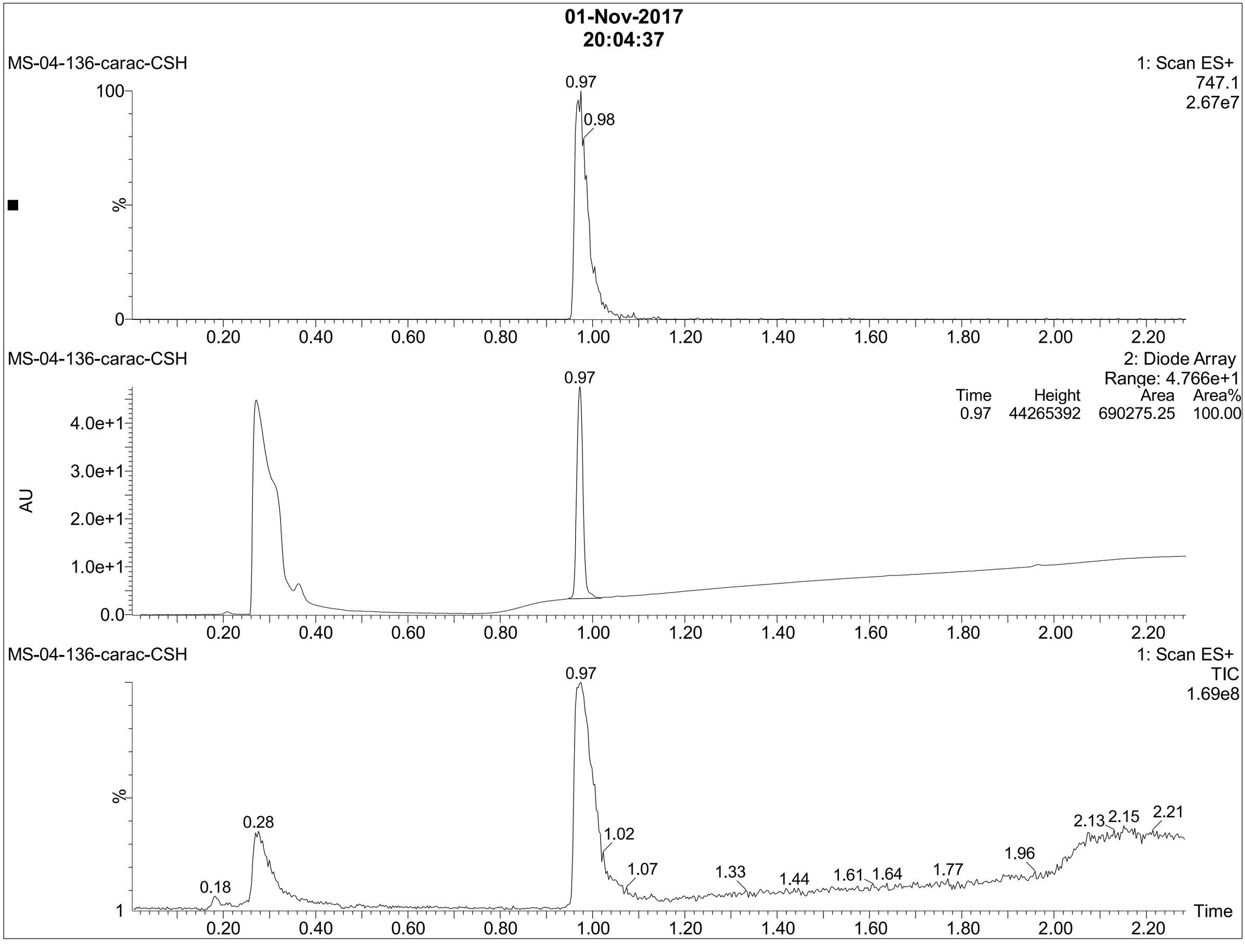

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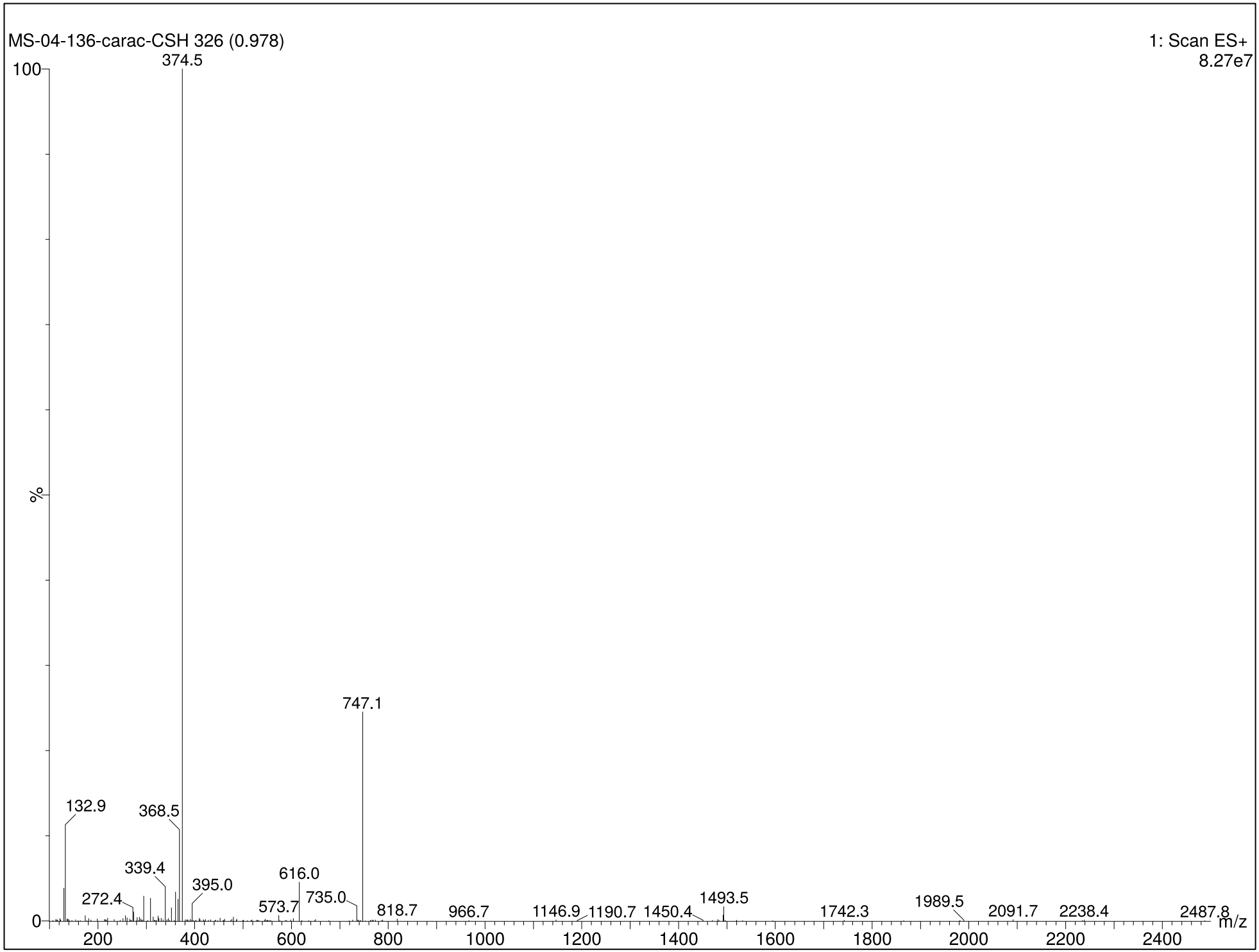
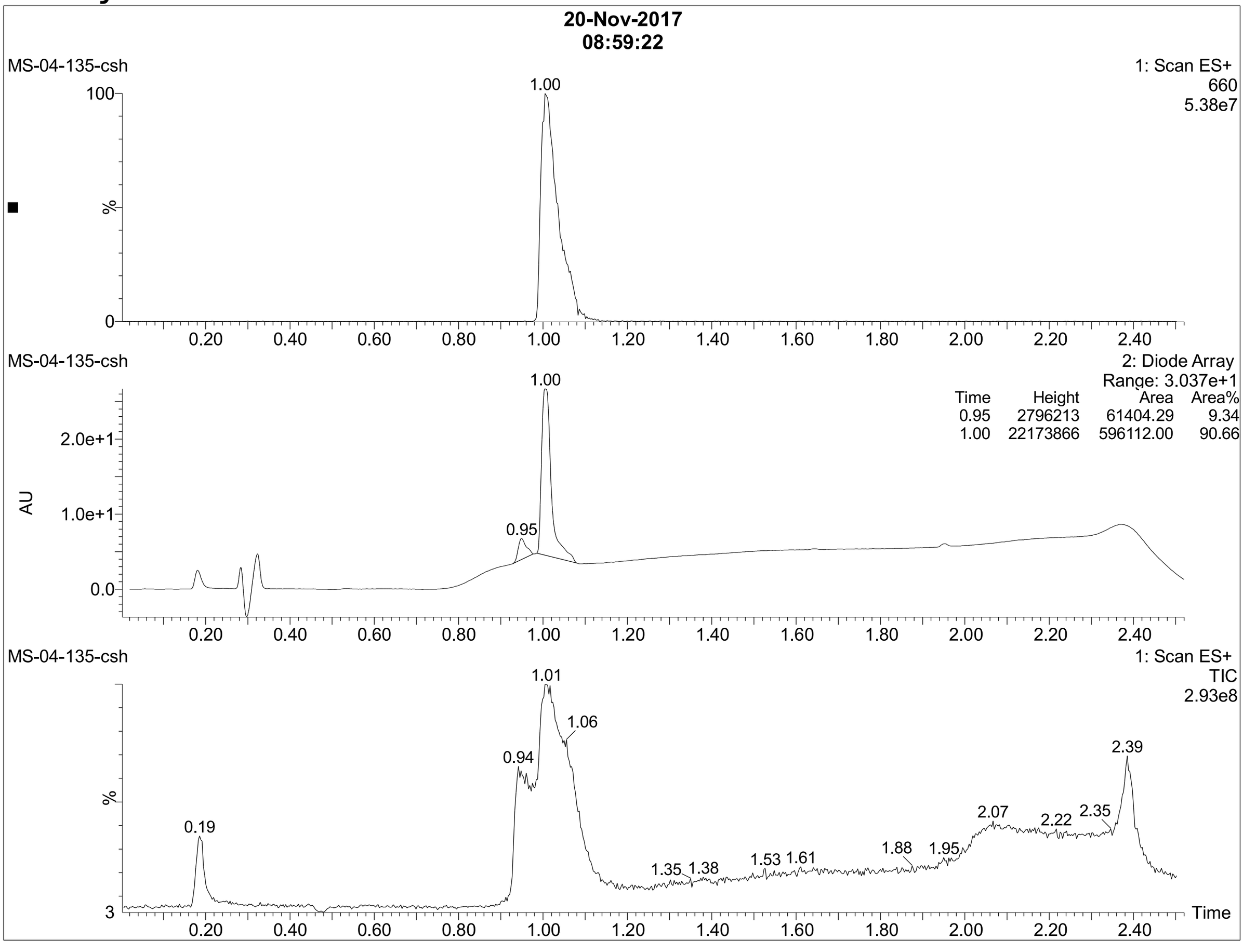
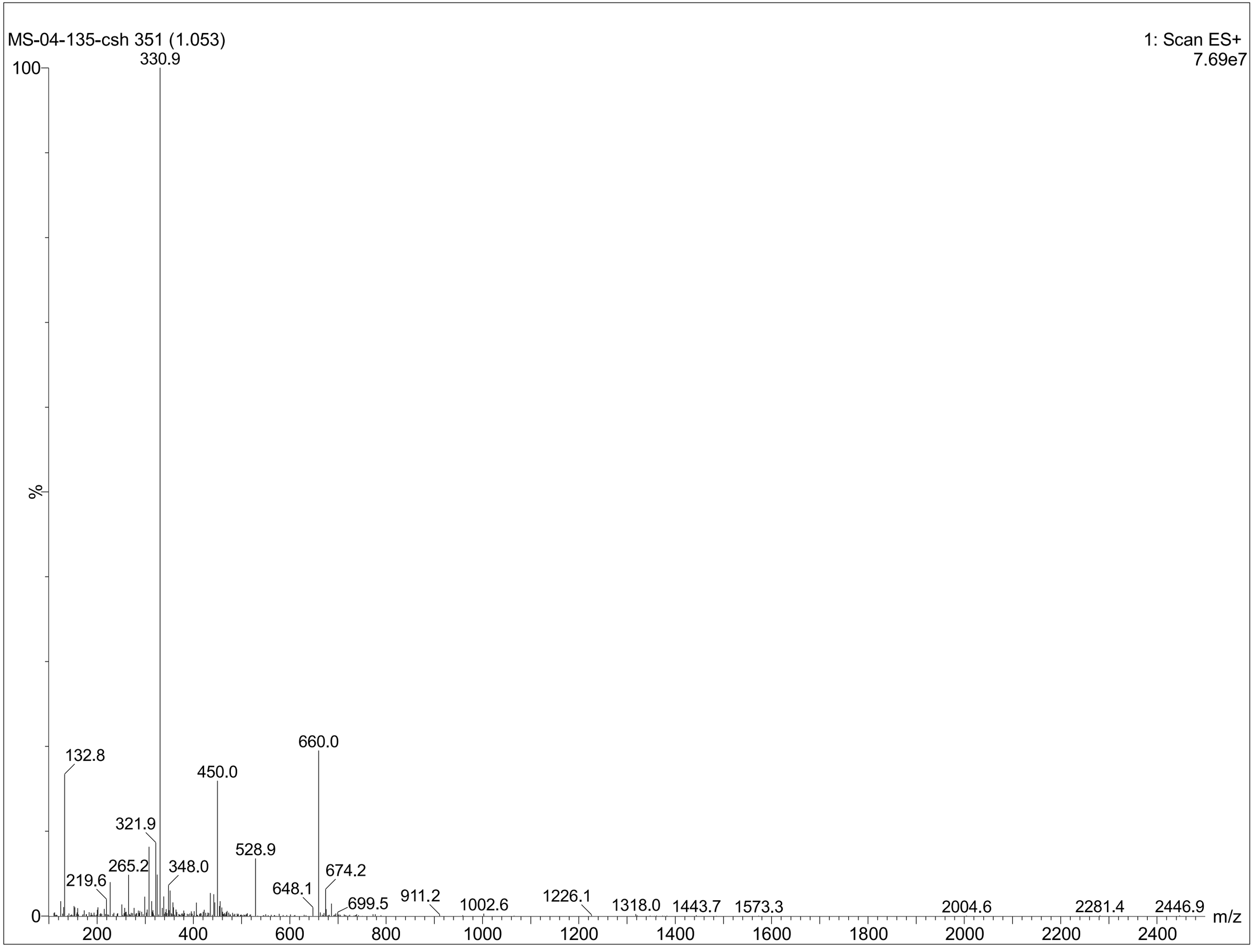


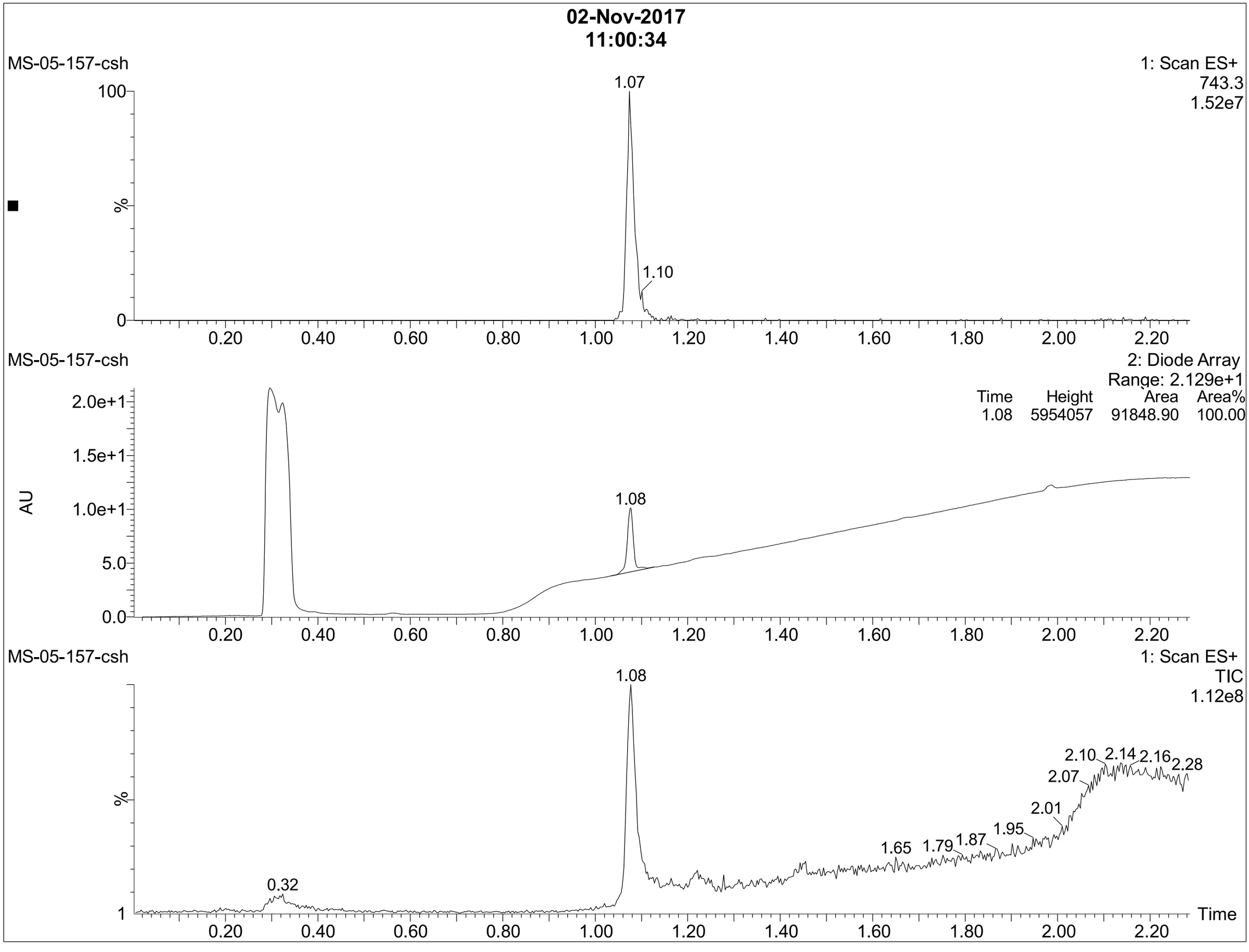
Macrocycle 7



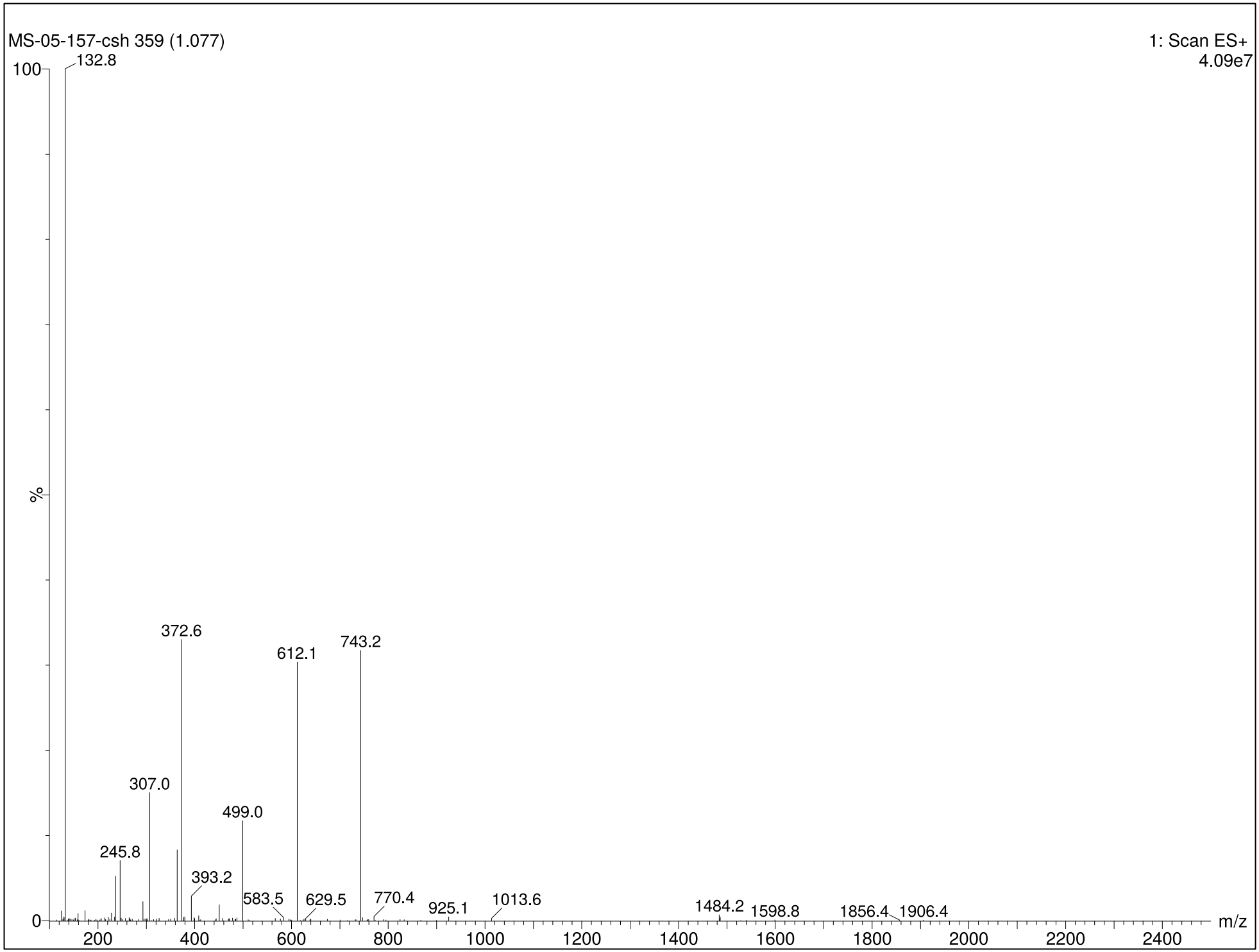
Macrocyle 7


MS-04-135-csh 351 (1.053)
1: Scan ES+
7.69e7
100
%
0
330.9
132.8
450.0
660.0
321.9
528.9
265.2
348.0
674.2
219.6
648.1
699.5
911.2
1002.6
1226.1
1318.0
1443.7
1573.3
2004.6
2281.4
2446.9
m/z
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400

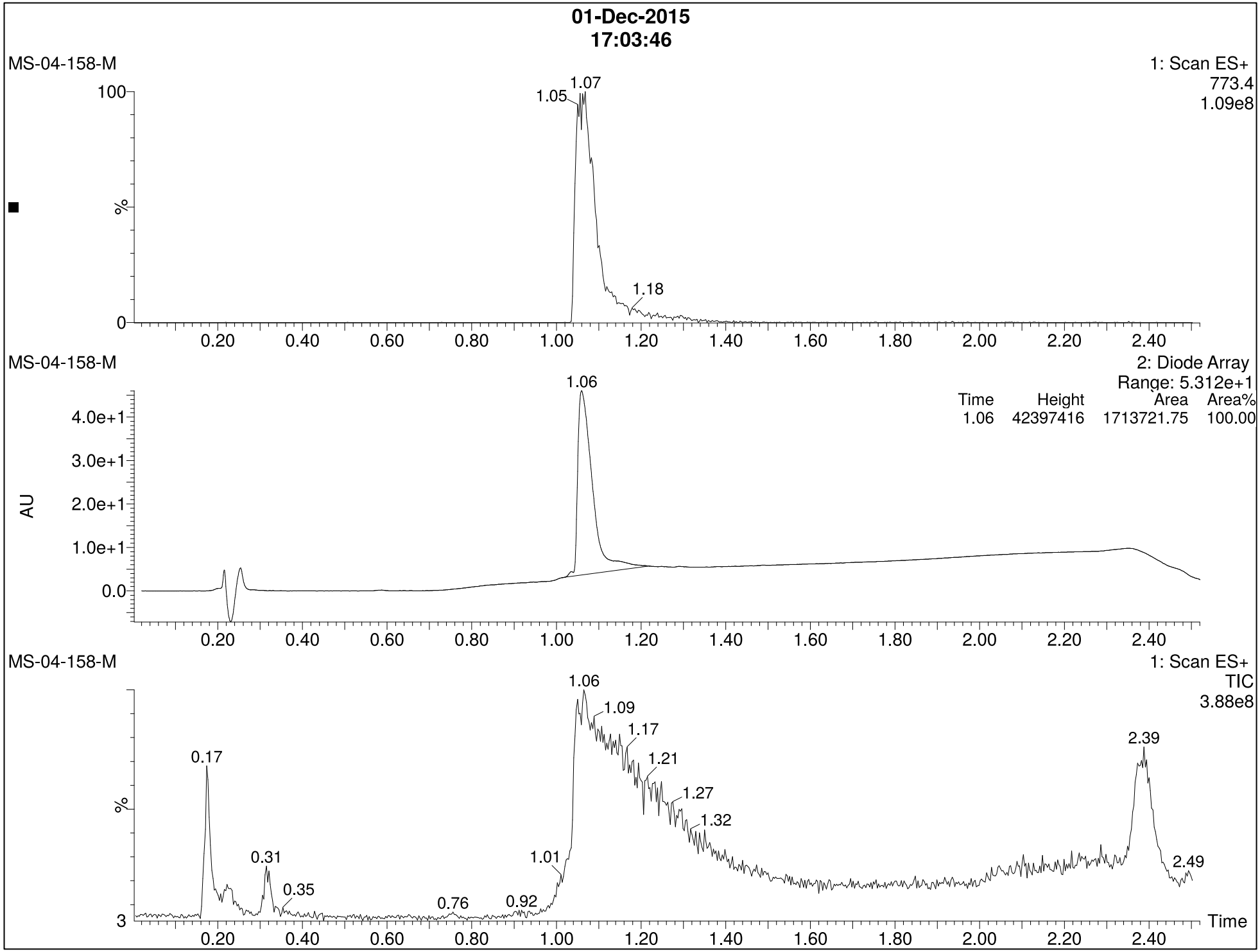
Macrocycle 8



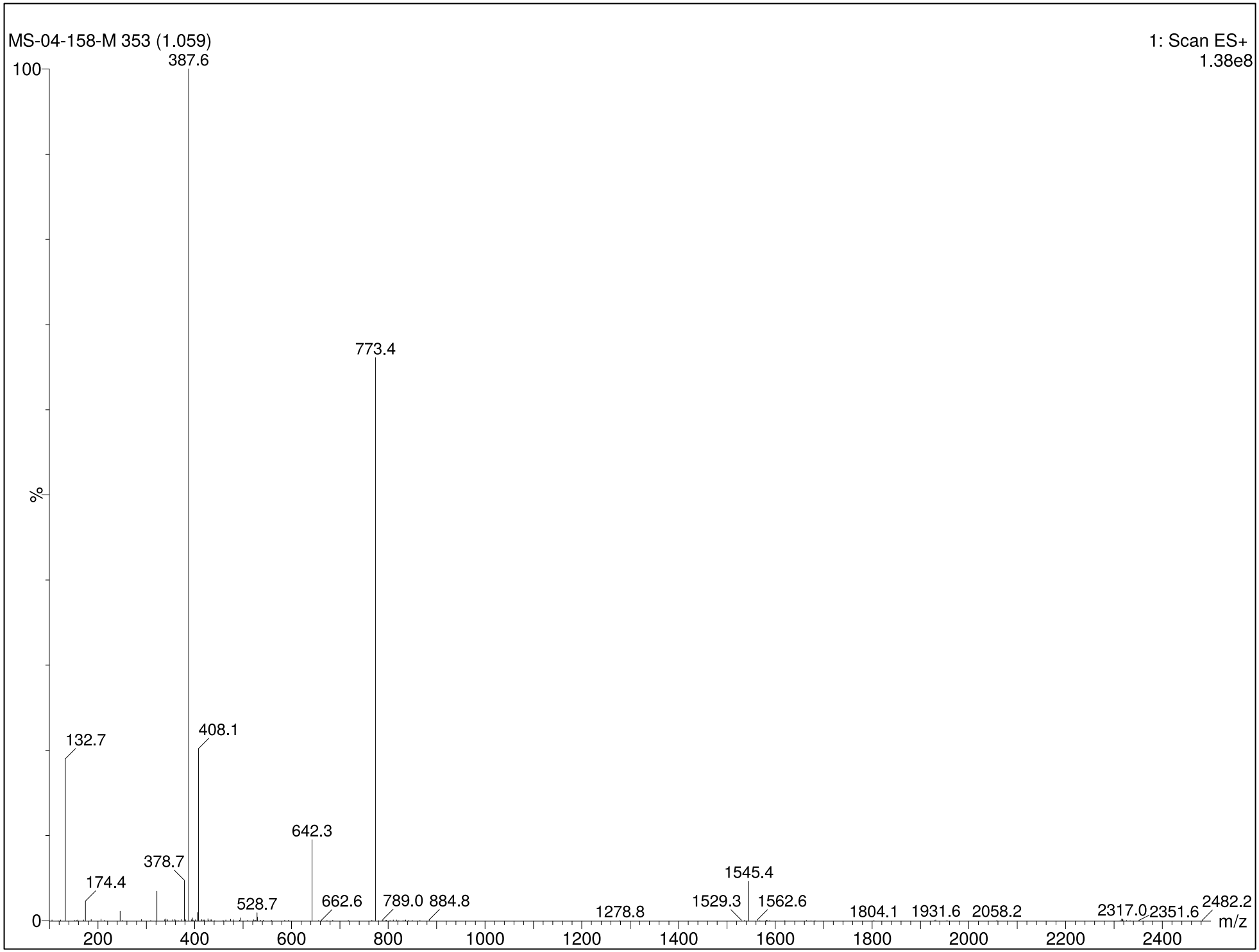
Macrocycle 8



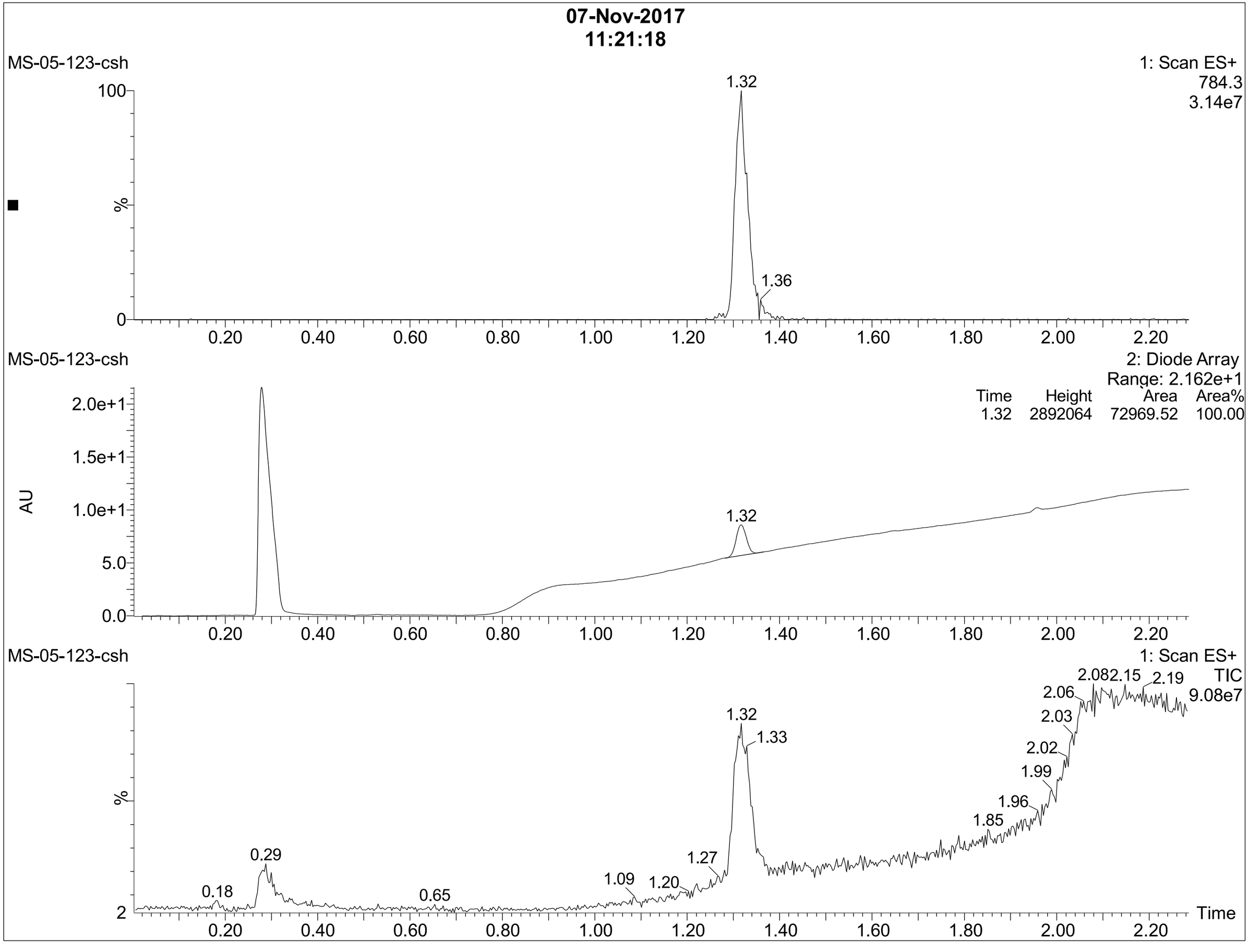
Macrocycle 9



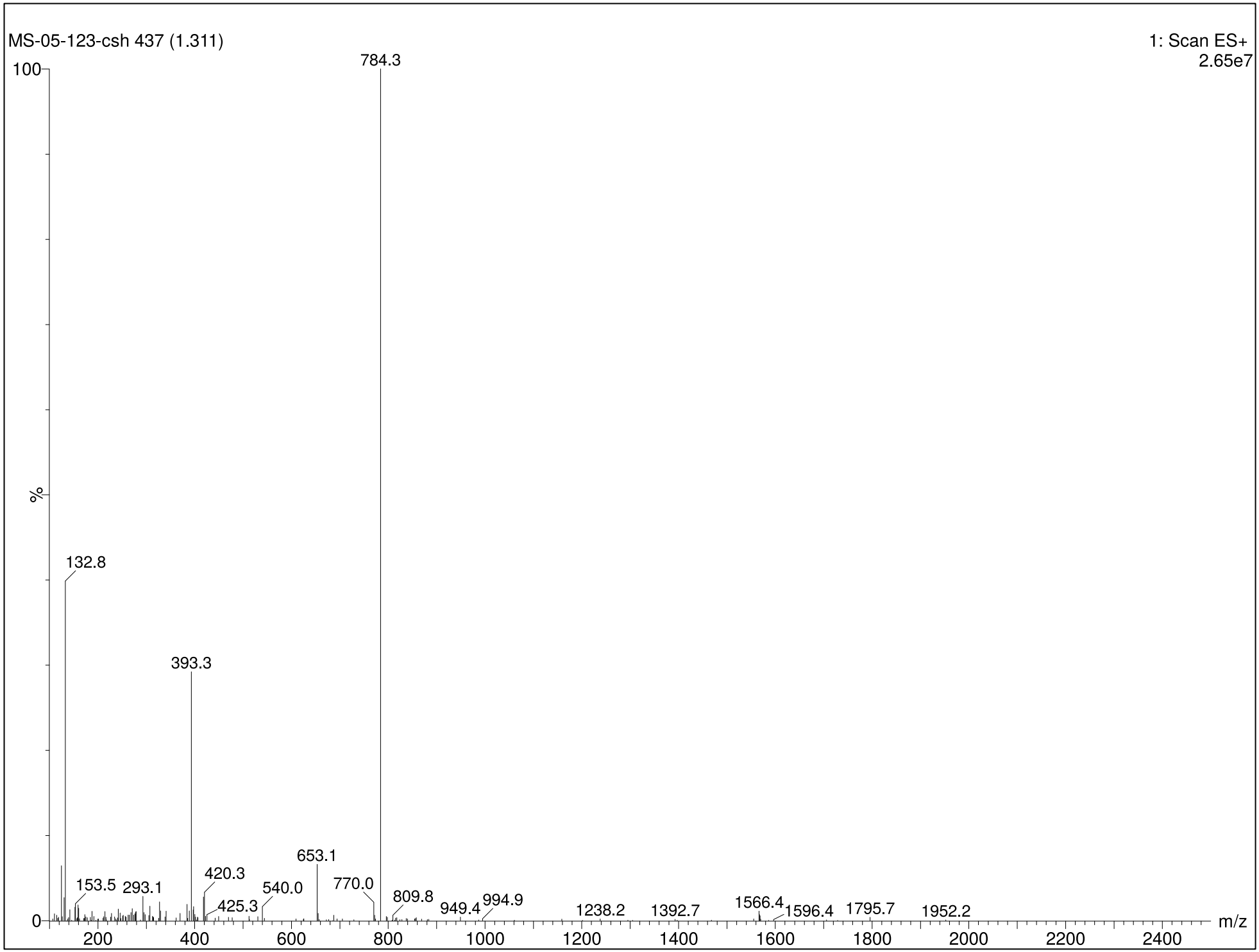
Macrocycle 9



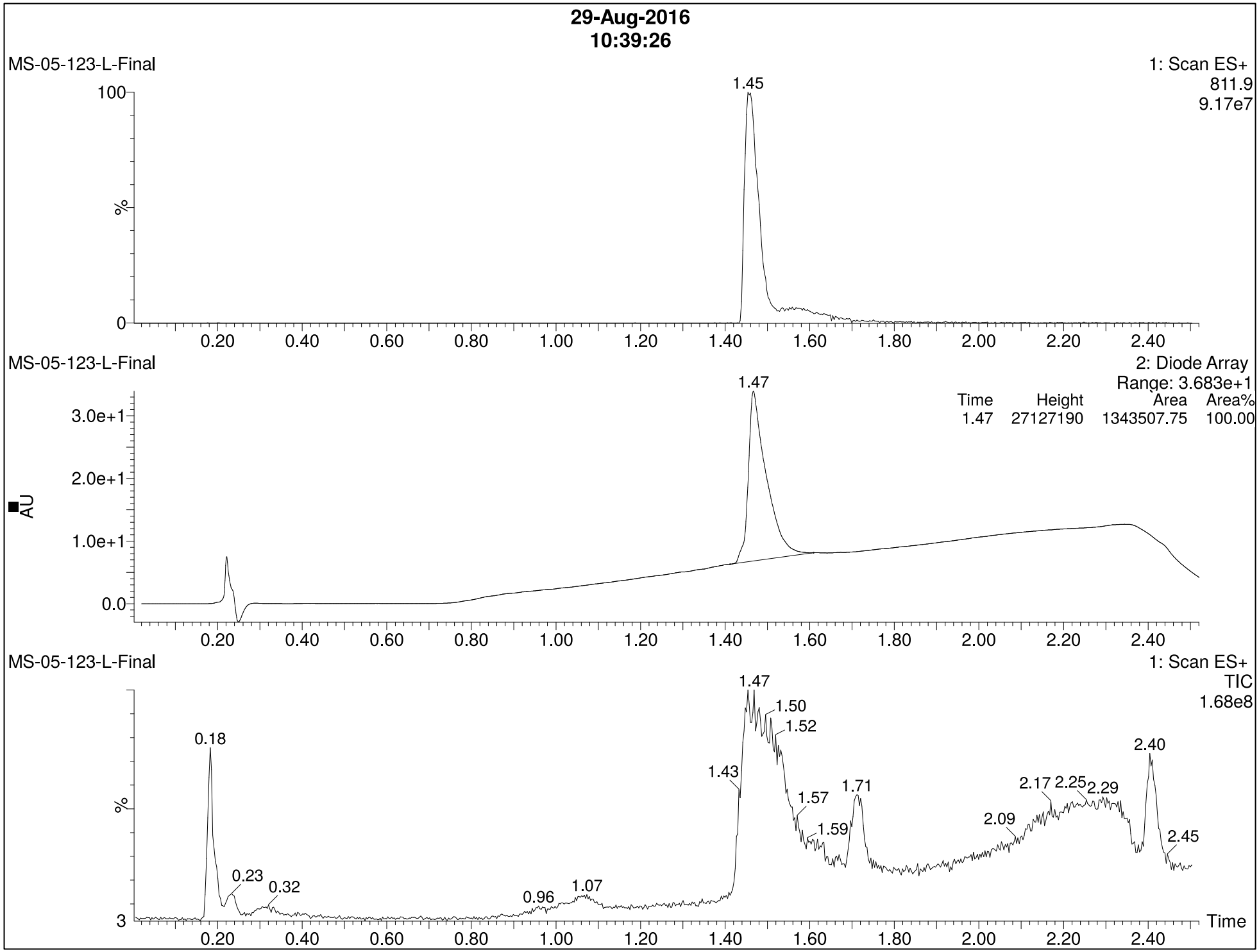
Macrocycle 10



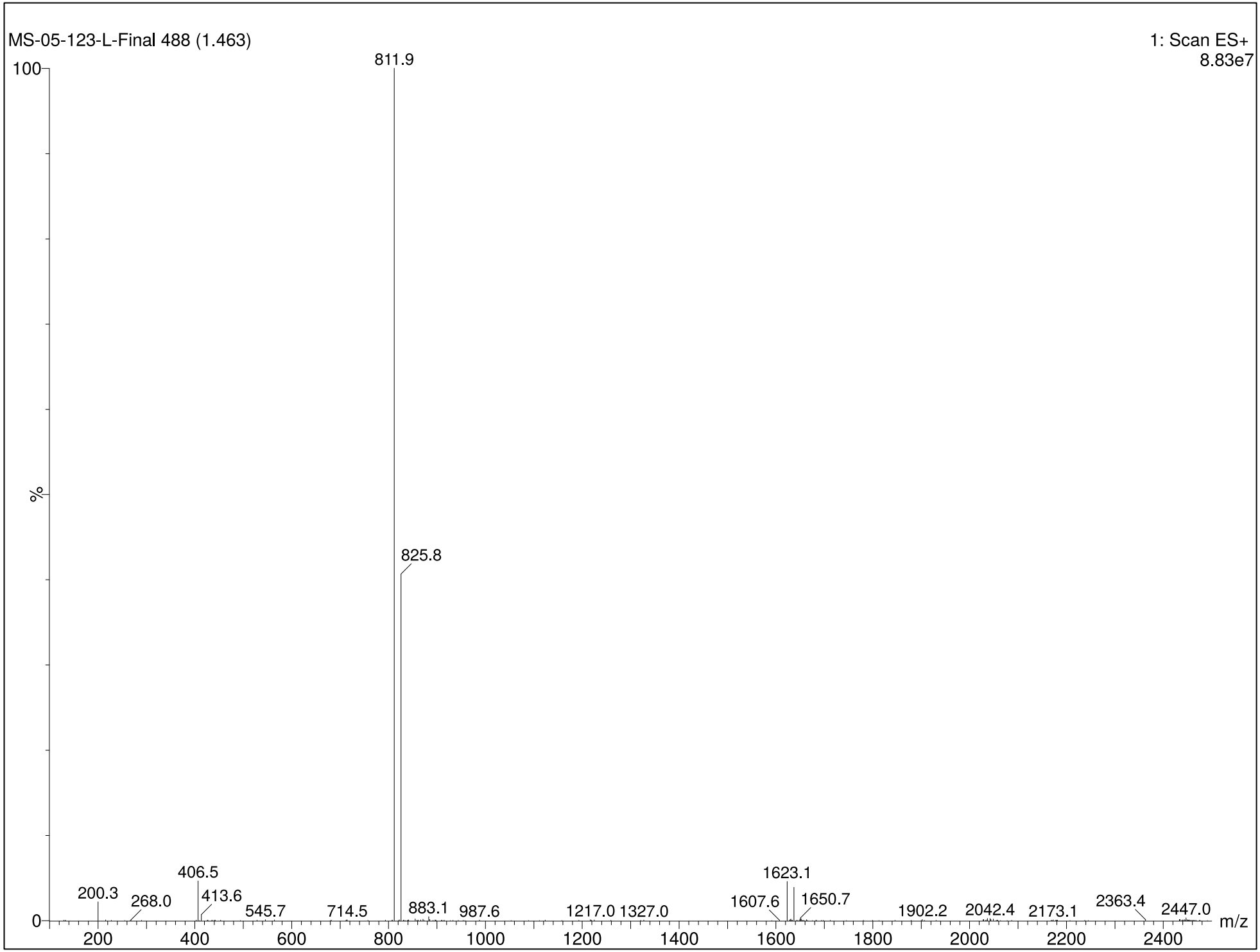
Macrocycle 10



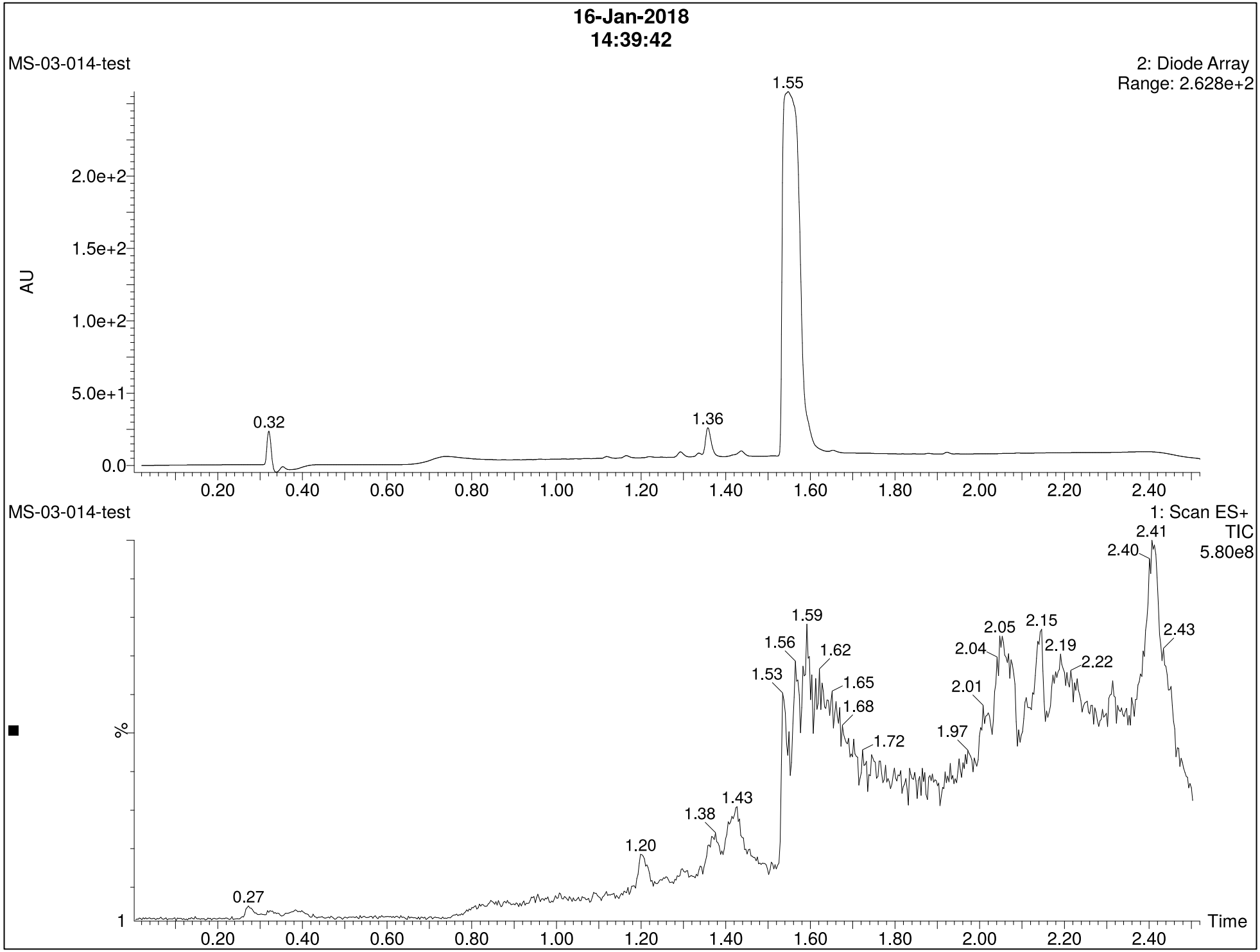
10-L (precursor of 10)



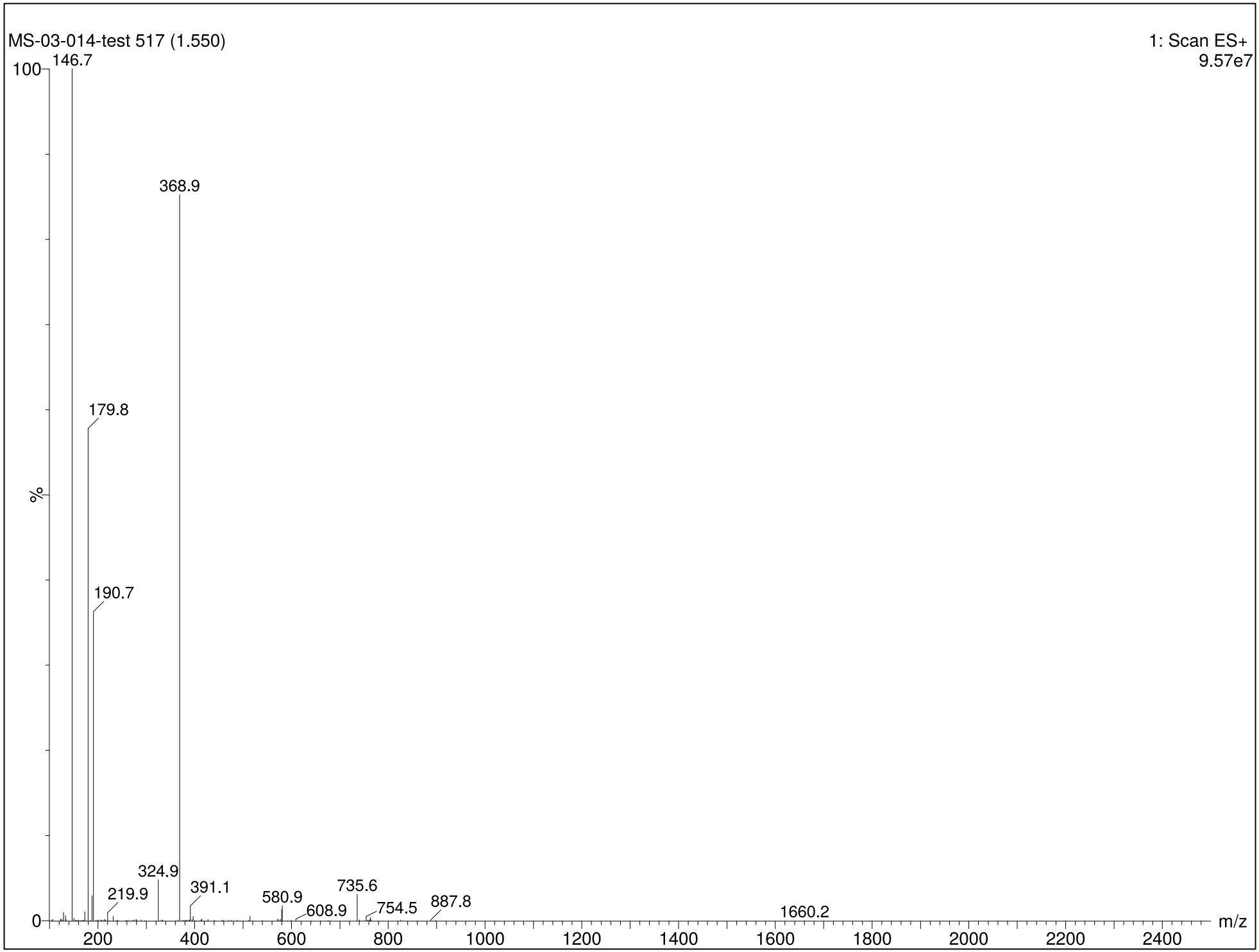
10-L (precursor of 10)



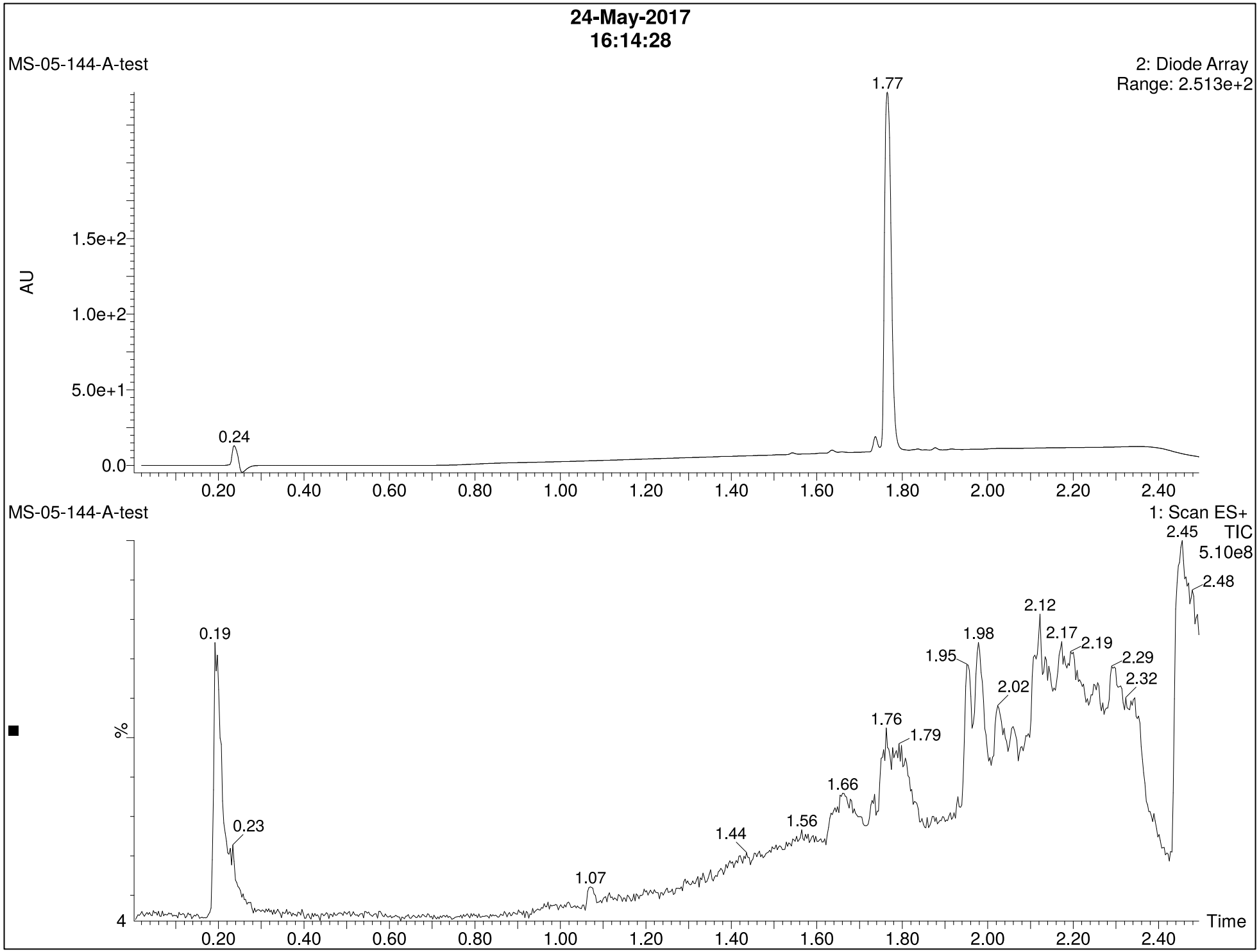
Linker A

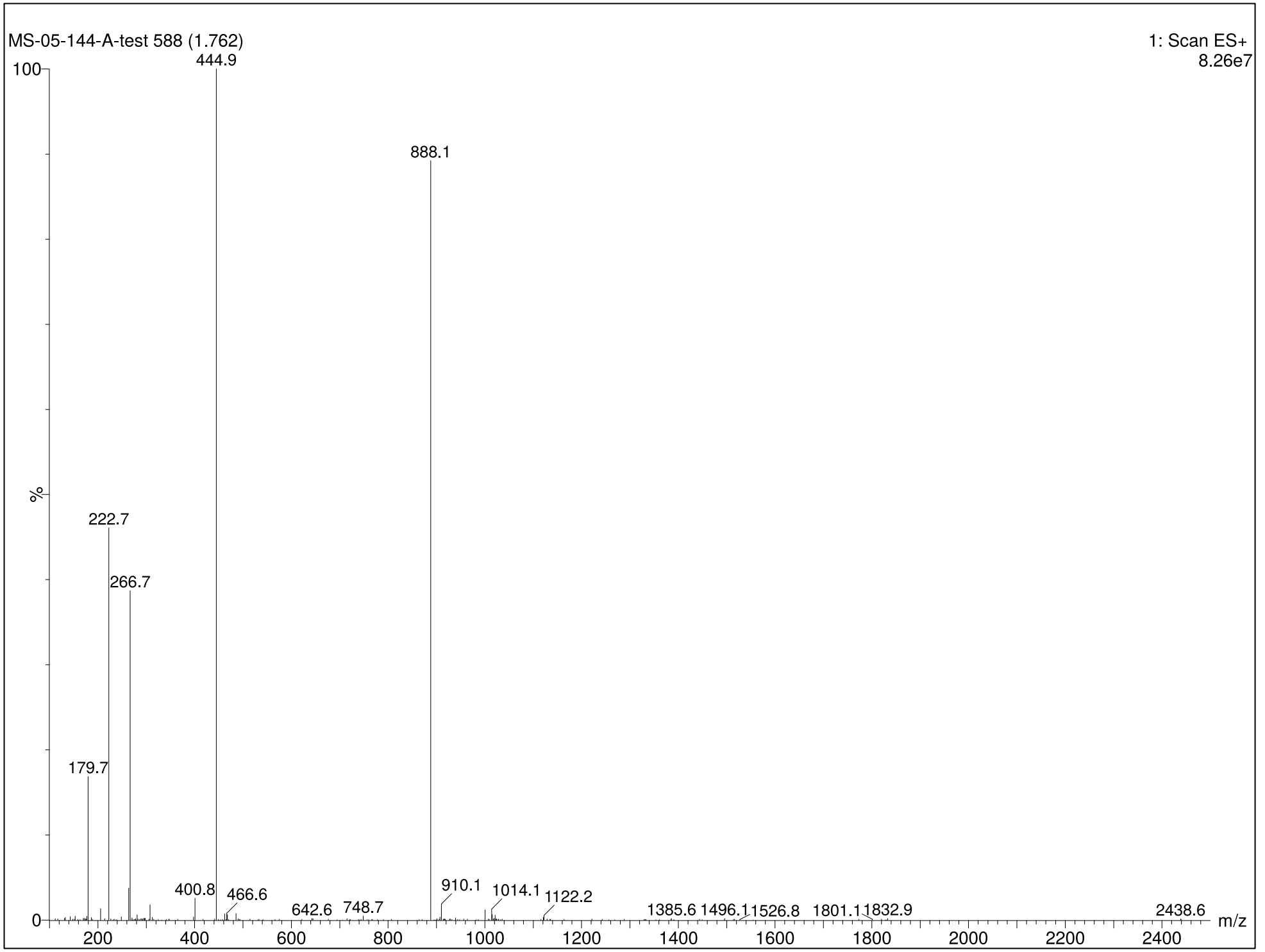

16-Jan-2018
14:39:42
MS-03-014-test
2: Diode Array
Range: 2.628e+2
AU
2.0e+2
1.5e+2
1.0e+2
5.0e+1
0.0
0.32
1.36
1.55
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
2.00
2.20
2.40
MS-03-014-test
1: Scan ES+
TIC
5.80e8
%
1
0.27
1.20
1.38
1.43
1.53
1.56
1.59
1.62
1.65
1.68
1.72
1.97
2.01
2.04
2.05
2.15
2.19
2.22
2.40
2.41
2.43
Time

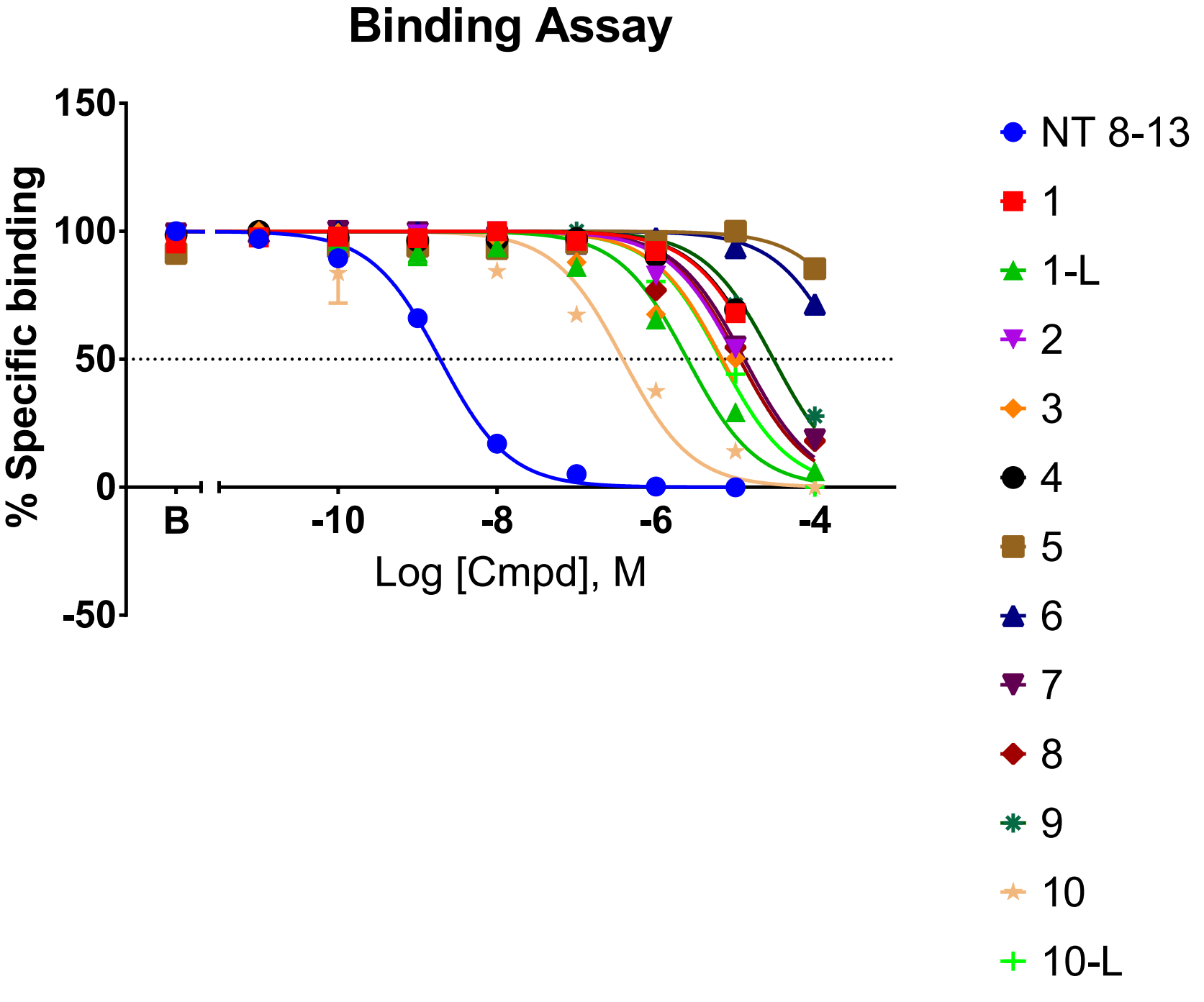
Linker A


MS-03-014-test 517 (1.550)
1: Scan ES+
9.57e7
100
%
0
146.7
368.9
179.8
190.7
324.9
219.9
391.1
580.9
608.9
735.6
754.5
887.8
1660.2
m/z
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400

Linker B


24-May-2017
16:14:28
MS-05-144-A-test
2: Diode Array
Range: 2.513e+2
AU
1.5e+2
1.0e+2
5.0e+1
0.0
1.77
0.24
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
2.00
2.20
2.40
MS-05-144-A-test
1: Scan ES+
TIC
5.10e8
%
4
0.19
0.23
1.07
1.44
1.56
1.66
1.76
1.79
1.95
1.98
2.02
2.12
2.17
2.19
2.29
2.32
2.45
2.48
Time


MS-05-144-A-test 588 (1.762)
1: Scan ES+
8.26e7
100
%
0
444.9
888.1
222.7
266.7
179.7
400.8
466.6
642.6
748.7
910.1
1014.1
1122.2
1385.6
1496.1
1526.8
1801.1
1832.9
2438.6
m/z
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400


Binding Assay
% Specific binding
150
100
50
0
-50
B
-10
-8
-6
-4
Log [Cmpd], M
NT 8-13
1
1-L
2
3
4
5
6
7
8
9
10
10-L