Electronic Supplementary Material (ESI) for ChemComm.

Supporting information

A study on the effect of synthetic α-to-β^3-amino acid mutations on the binding of phosphopeptides to 14-3-3 proteins

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I. Supplementary methods

Peptide synthesis

All peptides were synthesized by standard Fmoc SPPS using an automated Intavis Multipep RSi peptide synthesizer. Peptides containing a C-terminal amide were synthesized using a Rink Amide resin, whereas a preloaded 2-Chlorotrityl resin was used for peptides with a C-terminal carboxylate. Fmoc protected β-amino acids were used in the same manner as Fmoc protected α-amino acids. All reactions were performed N-methyl-pyrrolidone (NMP). Fmoc deprotection was performed in 20% (v/v) piperidine and acid activation in a solution of 1.2 M diisopropyl ethylamine (DIPEA) and 0.4 M (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 8-fold excess). Each coupling reaction was subsequently performed twice for one hour in 0.5 M amino acid solution (10-fold excess) and followed by capping through acetylation (pyridine/acetic anhydride/DMF, 1/1/1, v/v/v). The last N-terminal amino acid was either capped through acetylation or left as the free amine. The final peptides were cleaved off the resin in trifluoracetic acid (TFA), H_2O, triisopropylsilane (TIS) and ethanedithiol (EDT) (92.5/2.5/2.5/2.5, v/v/v/v) for 5 hours at room temperature and precipitated in 20 mL diethyl ether (Et_2O) at -30 °C. The precipitated peptide suspension was stored at -30 °C for ten minutes, centrifuged at 2000 rpm and decanted. Twenty milliliters of fresh Et_2O at -30 °C were added, the suspension stored at -30 °C for another ten minutes and centrifuged again. The solvent was decanted and the remaining pellet dried in the open air. Peptides were then purified by mass triggered reverse phase HPLC on an appropriate gradient of H_2O/acetonitrile + 0.1% TFA. The resulting peptide solutions were lyophilized and stored at -30 °C until further use.

Protein expression

Proteins 14-3-3σΔC and full length 14-3-3σ & 14-3-3ζ were expressed according to the following general protocol. Plasmids were transformed into NiCo21(DE3) cells (New England Biolabs) according to the manufacturers protocol. Colonies were grown on LB agar plates + 100 µg/mL ampicillin overnight at 37 °C. One colony was picked and precultured in 25 mL sterile LB medium + 100 µg/mL ampicillin overnight at 37 °C. 2 TB medium + 100 µg/mL ampicillin was then inoculated with the preculture and incubated at 37 °C until an OD_{600} of ~1.0 was reached. Protein expression was then induced with 0.4 mM IPTG and the cells incubated overnight at 18 °C. Cells were then harvested by ultracentrifugation at 20000 rpm at 4°C, flash frozen in liquid nitrogen and stored at -80°C until further purification.

Prior to purification, the cell pellets were thawed and resuspended in 10 mL/g pellet lysis buffer (25 mM Tris, pH = 8.0, 150 mM NaCl, 5% v/v glycerol, 10 mM imidazole, 4 mM BME and 1 mM PMSF). The cells were then lysed twice by homogenization using an EmulsiFlex-C3 homogenizer. The lysate was incubated with benzonase (Merck Millipore) for 15 minutes and then centrifuged at 20000 g for 15 minutes. The supernatant was applied in overnight circulation at 4 °C to a 5 mL HisTrap column pre-equilibrated with 20 column volumes (CV) lysis buffer. The column was then washed with 20 CV wash buffer (25 mM Tris, pH = 8.0, 300 mM NaCl, 5% v/v glycerol, 25 mM imidazole and 4 mM BME) and the protein eluted with 40 mL elution buffer (20 mM HEPES, pH 8.0, 100 mM NaCl, 5% v/v glycerol, 250 mM imidazole and 4 mM BME). The protein was then pipetted into a SpectrumLabs Spectra/Por 10000 Da MWCO dialysis bag and

dialysed overnight at 4 °C against dialysis buffer (25 mM HEPES, pH = 8.0, 100 mM NaCl, 4 mM BME, 2 mM MgCl2). For the σΔC protein, 1:500 mg/mg TEV protease was added to the dialysis bag. The full length proteins were then concentrated to ~50 mg/mL using 10000 Da MWCO Amicon spinfilters, aliquoted, flash frozen in liquid nitrogen and stored at -80 °C until further usage. The σΔC protein was instead applied to a 5 mL HisTrap column pre-equilibrated with 20 CV dialysis buffer. The flowtrough was captured and concentrated to ~50 mg/mL using 10000 Da MWCO Amicon spinfilters. The concentrated σΔC protein was then applied to a HiLoad superdex 75 16/60 SEC column using an Äkta FPLC apparatus. The fractions containing protein were then pooled, concentrated to ~50 mg/mL protein, flash frozen in liquid nitrogen and stored at -80°C until further use.

Fluorescence Polarization

The YAP peptides were dissolved to a concentration of 5 mM in pure MilliQ water. A mastermix containing the labeled reporter peptide (FITC-ERα) at 100 nM and 14-3-3 protein (1 µM in case of 14-3-3ζ & 5 µM in case of 14-3-3σ and 5 µM when comparing the various isoforms) was made in FP buffer (10 mM HEPES, pH = 7.50, 150 mM NaCl, 0,1% (v/v) Tween 20). Then a 2-fold dilution series starting from 250 uM (in case of 14-3-3ζ) or 500 uM (in case of 14-3-3σ) of the unlabeled YAP peptides was made in the mastermix in black Corning low volume, round bottom 384-well assay plates (Corning #4514). Fluorescence polarization was then measured in a Tecan Safire II at room temperature (excitation wavelength 485 (20) nm and emission wavelength 535 (25) nm). All data points are measured in triplicate. The IC_{50}-values were then determined by plotting the data in Origin scientific graphing software and fitting the curves with a 3-parameter sigmoidal dose response curve.

Isothermal titration calorimetry

All ITC measurements were performed on a Microcal™ iTC200 calorimeter. The device cell was filled with a solution of 80 µM of the 14-3-3 protein in ITC buffer (100 mM NaCl, 25 mM HEPES, 10 mM $MgCl_2$, 0.5 mM TCEP and PH 7.40). The syringe was filled with a 1 mM solution of the peptide in ITC buffer. Reference cell was filled with MilliQ water. The peptide was then titrated to the protein at 37 °C though 19 injections of 2 ❍l with 180 seconds spacing between each injection. Additional settings: Reference power: 5 µCal/s; initial delay: 60 s and stirring speed: 750 rpm. Data was processed using Origin 7 (Originlab) software containing an automated module for calculation and fitting of ITC data.

Crystallography

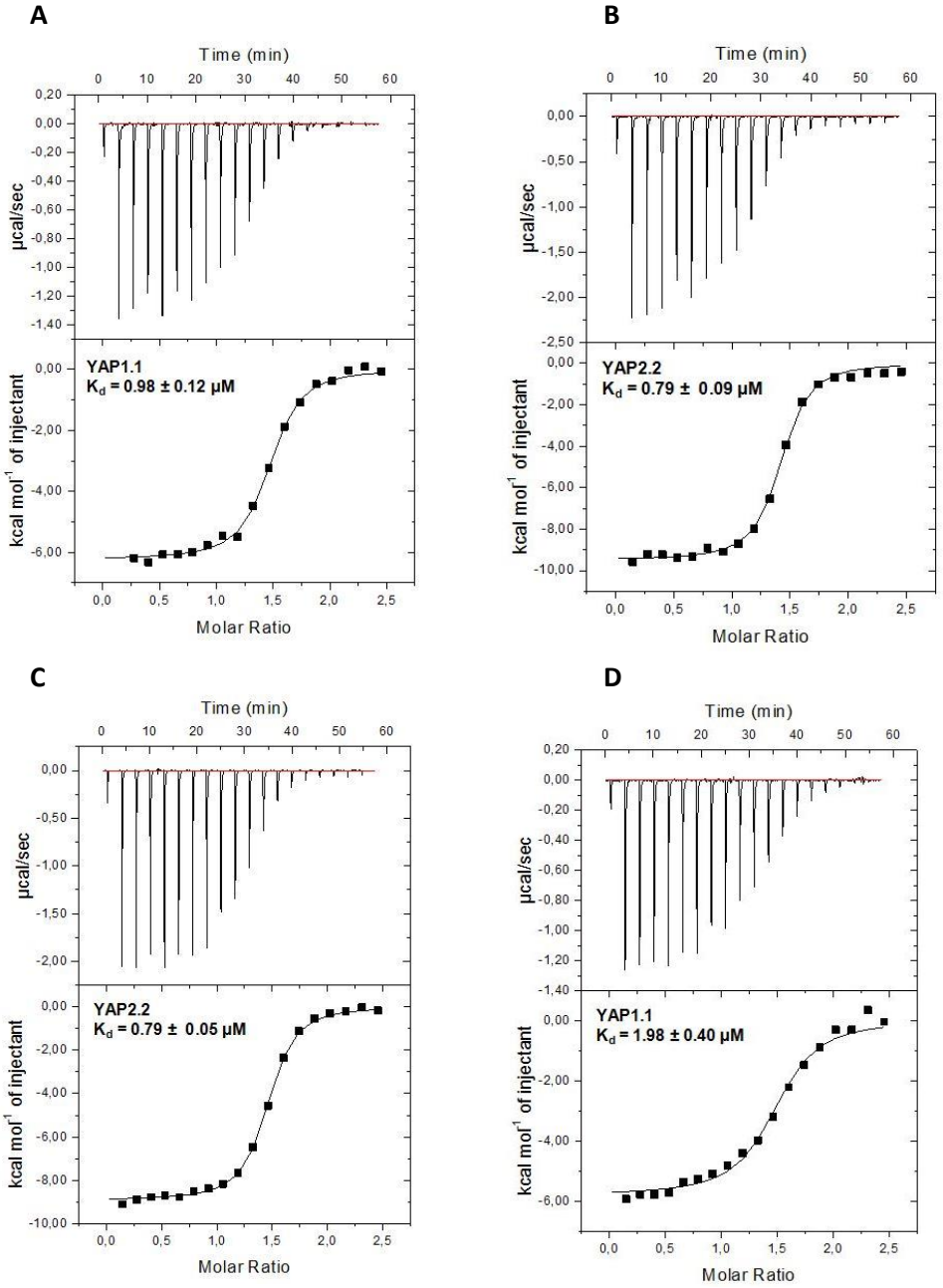
Crystals of the binary complex of the YAP peptides and 14-3-3σΔC were grown by mixing 10 mg/mL 14-3-3σΔC with the appropriate YAP peptides in a molar ratio of 1 : 2 in 10 mM HEPES pH 7.4, 150 mM NaCl and 2 mM BME and incubating overnight at 277K. The formed complex was then set up for crystallization by mixing 1 : 1 (v/v) with 0.095 M HEPES pH 7.5, 26% PEG400, 0.19 M CaCl2, 5% glycerol and incubating in a sitting drop at 277 K. Crystals grew within a week. X-ray diffraction data was collected at 100 K at the p11 beamline of the PETRA-III synchrotron of the DESY facility in Hamburg, Germany using a Pilatus 6M-F detector.[1,2] The data was indexed and integrated using iMosflm and scaled and merged using Aimless.[3] Phasing was done by molecular replacement using Phaser[4,5] and 3MHR as a starting model and was

followed by iterative rounds of refinement and manual model building using Phenix.Refine[6] and Coot[7] respectively. Model validation was performed using MolProbity.[8] Figures were created using PyMol.

Table S1: Crystallography data collection and refinement statistics of βYAP3, βYAP7 and βYAP3.2 in complex with 14-3-3σΔC.

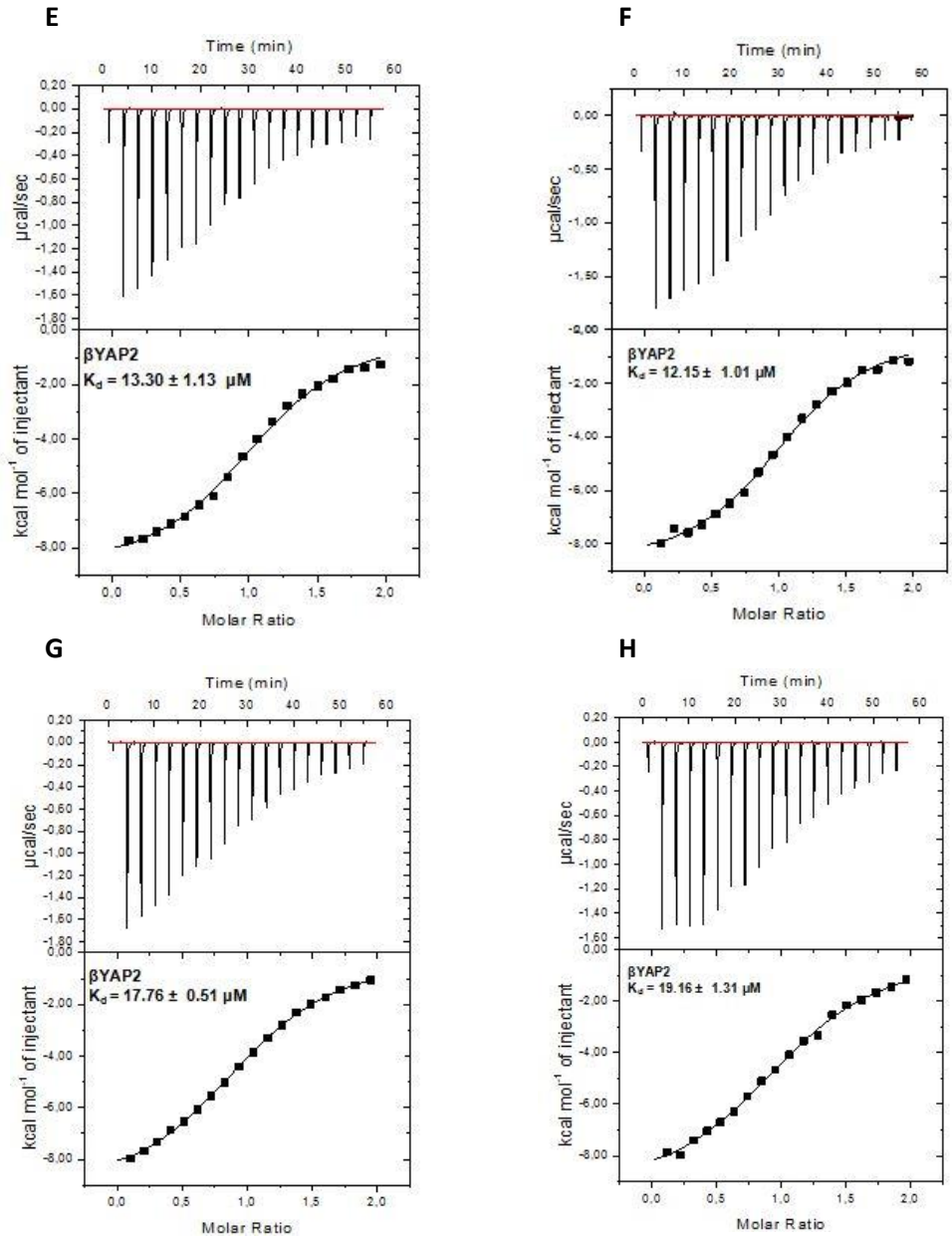
	βYAP2	**βYAP2.2**	**βYAP3**	**βYAP3.2**	**βYAP4**	**βYAP5**	**βYAP7**
PDB code	6G6X	6G8P	6G8J	6G8Q	6G8K	6G8L	6G8I
Data collection							
Resolution (Å)[a]	1.13 (1.13-1.15)	1.90 (1.94-1.90)	1.47 (1.50-1.47)	1.85 (1.89-1.85)	1.25 (1.27-1.25)	1.37 (1.39-1.37)	1.60 (1.63-1.60)
Space group	$C222_1$	$C222_1$	$C222_1$	$C222_1$	$C222_1$	$C222_1$	$C222_1$
Cell parameters (Å)	82.118, 111.849, 62.630 α=β=γ= 90°	82.037, 111.499, 62.460 α=β=γ= 90°	82.149, 112.019, 62.680 α=β=γ= 90°	82.108, 111.900, 62.620 α=β=γ= 90°	82.260, 11.730, 62.510 α=β=γ= 90°	82.258, 111.969, 62.650 α=β=γ= 90°	82.118, 112.170, 62.550 α=β=γ= 90°
R_{merge}[a,b]	0.082 (1.36)	0.085 (0.18)	0.099 (0.97)	0.126 (0.665)	0.101 (0.853)	0.133 (0.640)	0.161 (1.53)
Average $I/\sigma_{(I)}$[a,b]	12.1 (1.6)	18.6 (9.7)	12.1 (2.2)	11.4 (2.6)	10.8 (1.8)	10.0 (2.0)	10.2 (2.0)
$CC_{1/2}$ (%)[a,b,c]	0.998 (0.767)	0.992 (0.983)	0.994 (0.627)	0.995 (0.770)	0.995 (0.669)	0.992 (0.708)	0.979 (0.708)
Completeness (%)[a,b]	100 (99.9)	100 (96)	97.8 (94.4)	99.3 (90.8)	99.9 (98.8)	99.8 (96.0)	100 (100)
Redundancy[a,b]	11.2 (8.1)	11.2 (8.2)	12.3 (11.1)	11.1 (7.3)	11.7 (7.6)	9.9 (4.4)	11.5 (11.5)
Refinement							
Number of protein/solvent/ligand atoms	2309/399/-	2122/349/-	2150/375/-	2071/357/-	2181/387/-	2203/426/-	2103/383/-
R_{work}/R_{free} (%)	14.96/16.78	13.3/15.9	15.89/17.26	14.40/17.54	14.10/15.97	13.57/15.72	14.96/17.34
Unique reflections used in refinement	107651	22861	48296	24785	79638	60814	38473
R.m.s. deviations from ideal values bond lengths (Å) / bond angles (°)	0.008/0.95	0.004/0.67	0.004/0.63	0.009/0.89	0.009/1.0	0.008/0.98	0.010/0.89
Average protein/solvent/ligand B-factor ($Å^2$)	111.9/-/-	98.6/-/-	78.6/-/-	85.4/-/-	112.6/-/-	XX	65.4/-/-
Ramachandran favored (%)	98.3	98.3	98.3	98.3	98.3	98.3	98.3
Ramachandran allowed (%)	1.7	1.7	1.7	1.7	1.7	1.7	1.7
Ramachandran outliers (%)	0	0	0	0	0	0	0

II. Supplementary Figures



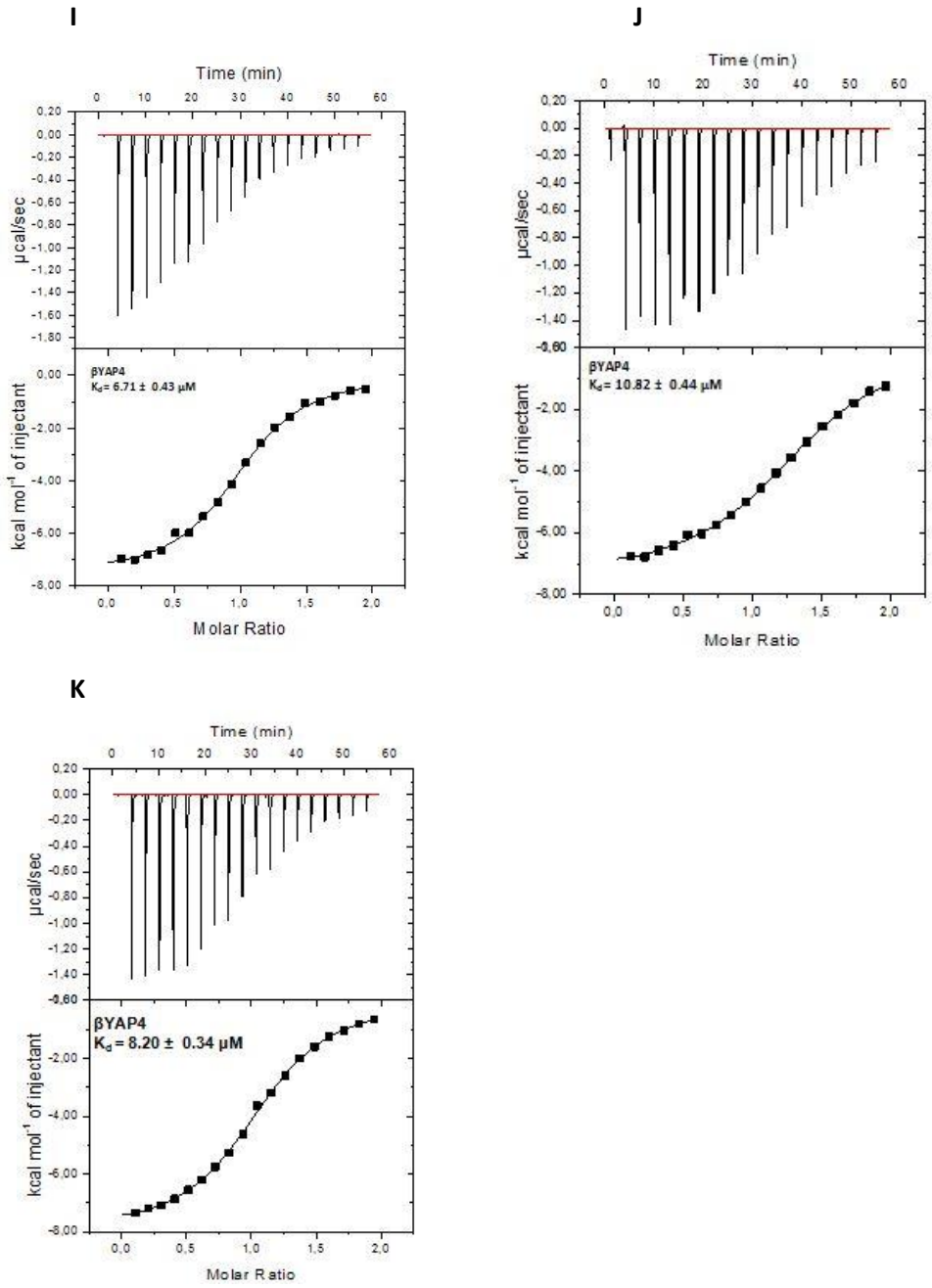
	Peptide	Protein	N (sites)	K_a (M^{-1})	ΔH (cal/mol)	ΔS (cal/mol/deg)
A	**YAP1.1**	*14-3-3ζ*	1.42 ± 0.00884	1.02E6 ± 1.23E5	-6229 ± 62.80	7.40
B	**YAP2.2**	*14-3-3ζ*	1.37 ± 0.00741	1.26E6 ± 1.44E5	-9484 ± 79.56	-2.67
C	**YAP1.1**	*14-3-3σ*	1.45 ± 0.0204	5.05E5 ± 1.02E5	-5791 ± 115.2	7.42
D	**YAP2.2**	*14-3-3σ*	1.41 ± 0.00453	1.26E6 ± 8.61E4	-8918 ± 44.54	-0.851

Figure S1: ITC data of YAP peptides **A:** YAP1.1 against 14-3-3ζ **B:** YAP2.2 against 14-3-3ζ **C:** YAP1.1 against 14-3-3σ **D:** YAP2.2 against 14-3-3σ



	Peptide	Protein	N (sites)	K_a (M^{-1})	ΔH (cal/mol)	ΔS (cal/mol/deg)
E	**βYAP2**	*14-3-3ζ*	1.11 ± 0.0139	7.52E4 ± 6.37E3	-8955 ± 169.8	-6.57
F	**βYAP2**	*14-3-3 ζ*	1.09 ± 0.0133	8.23E4 ± 6.87E3	-8946 ± 162.0	-6.36
G	**βYAP2**	*14-3-3σ*	1.04 ± 0.00517	5.63E4 ± 1.61E3	-9404 ± 72.19	-8.59
H	**βYAP2**	*14-3-3 σ*	1.11 ± 0.0127	5.22E4 ± 3.56E3	-9557 ± 175.6	-9.23

Figure S2: ITC data of βYAP peptides **E:** βYAP2 against 14-3-3ζ **F:** βYAP2 against 14-3-3ζ **G:** βYAP2 against 14-3-3σ **H:** βYAP2 against 14-3-3σ.



	Peptide	Protein	N (sites)	K_a (M^{-1})	ΔH (cal/mol)	ΔS (cal/mol/deg)
I	**βYAP4**	*14-3-3ζ*	1.01 ± 0.00812	1.49E5 ± 9.59E3	-7569 ± 83.55	-0.741
J	**βYAP4**	14-3-3σ	1.34 ± 0.00606	9.24E4 ± 3.77E3	-7397 ± 51.51	-1.13
K	**βYAP4**	*14-3-3σ*	1.07 ± 0.00567	1.22E5 ± 5.06E3	-7978 ± 59.19	-2.45

Figure S3: ITC data of βYAP peptides **I:** βYAP4 against 14-3-3ζ **J:** βYAP4 against 14-3-3σ **K:** βYAP4 against 14-3-3σ.

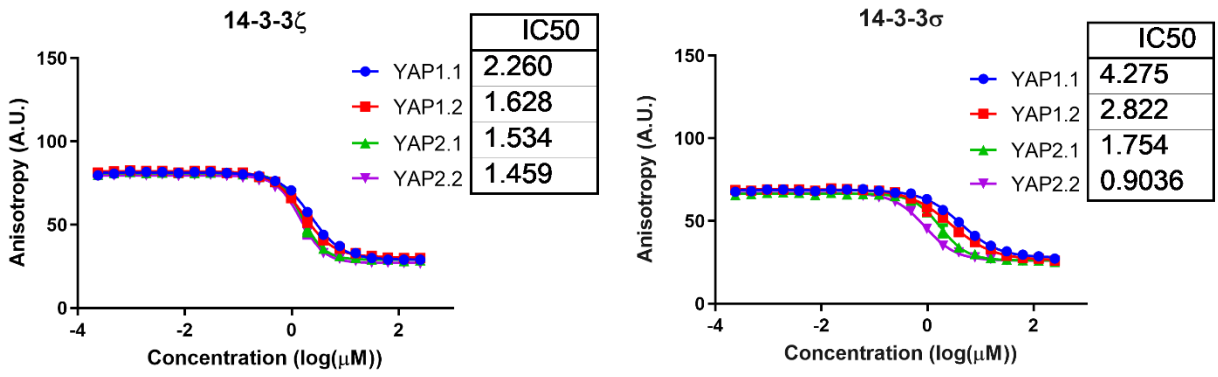


Figure S4 Competitive FP results of YAP peptides against 14-3-3ζ and 14-3-3σ. (1 μM 14-3-3ζ and 100 nM FITC-ERα).

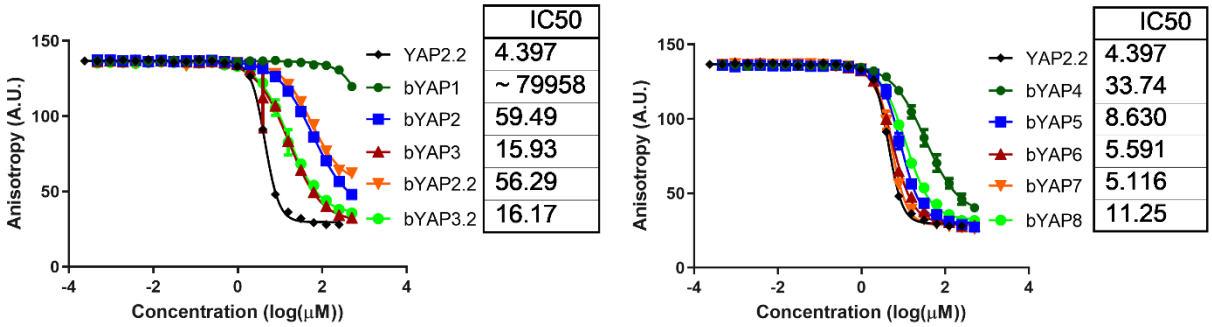


Figure S5 Competitive FP results of βYAP peptides against 14-3-3ζ. (5 μM 14-3-3ζ and 100 nM FITC-ERα).

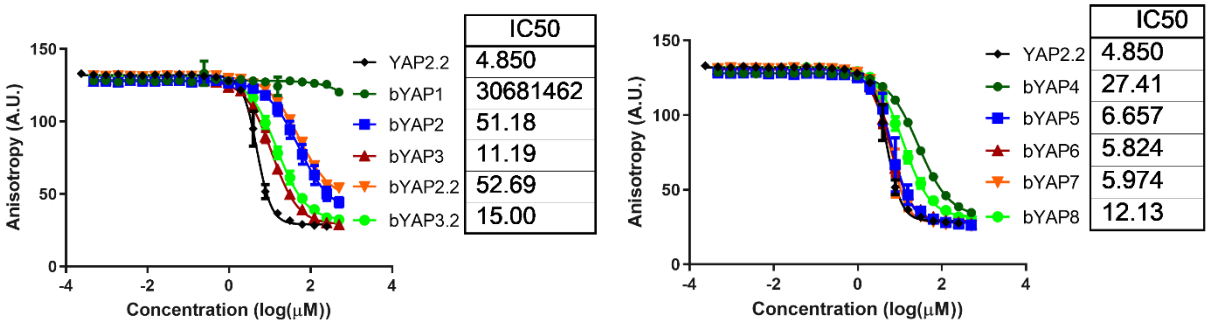


Figure S6 Competitive FP results of βYAP peptides against 14-3-3σ. (5 μM 14-3-3σ and 100 nM FITC-ERα).

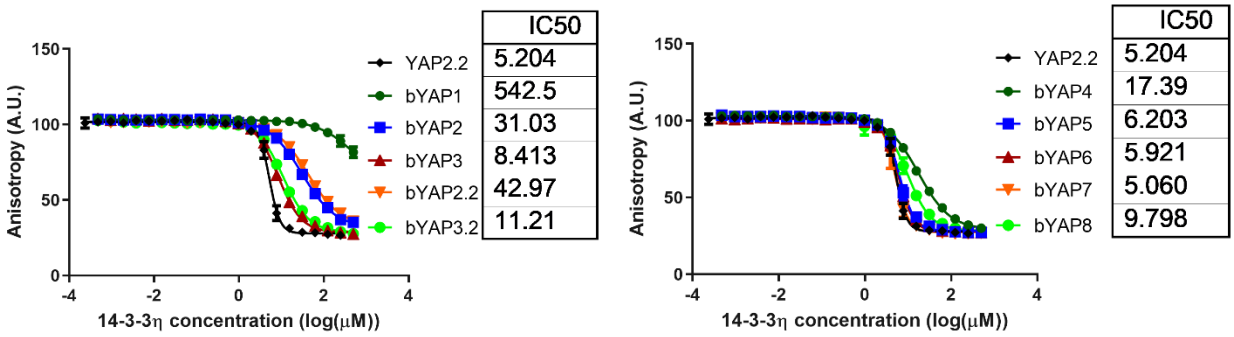


Figure S7 Competitive FP results of βYAP peptides against 14-3-3η. (5 μM 14-3-3η and 100 nM FITC-ERα).

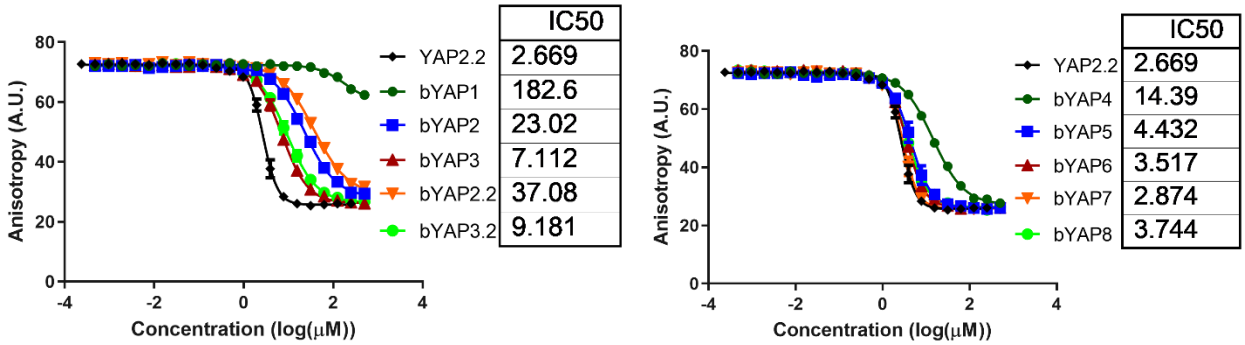


Figure S8 Competitive FP results of βYAP peptides against 14-3-3ε. (5 μM 14-3-3ε and 100 nM FITC-ERα).

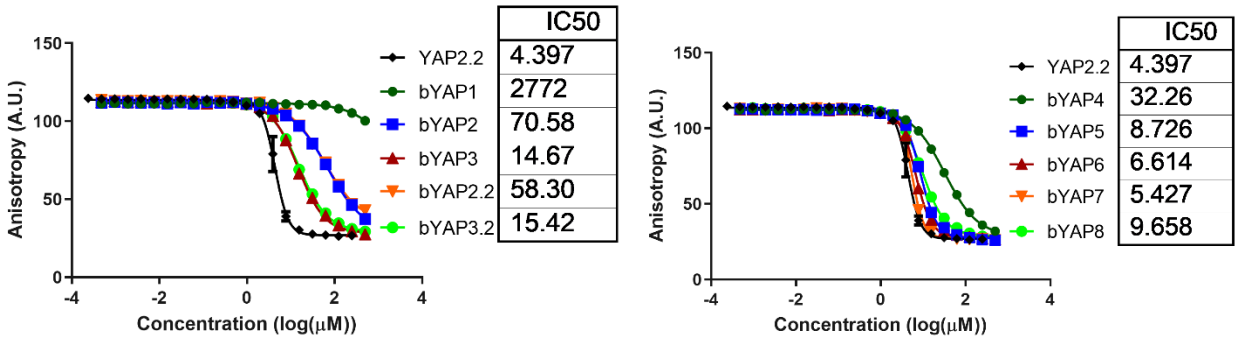


Figure S9 Competitive FP results of βYAP peptides against 14-3-3τ. (5 μM 14-3-3τ and 100 nM FITC-ERα).

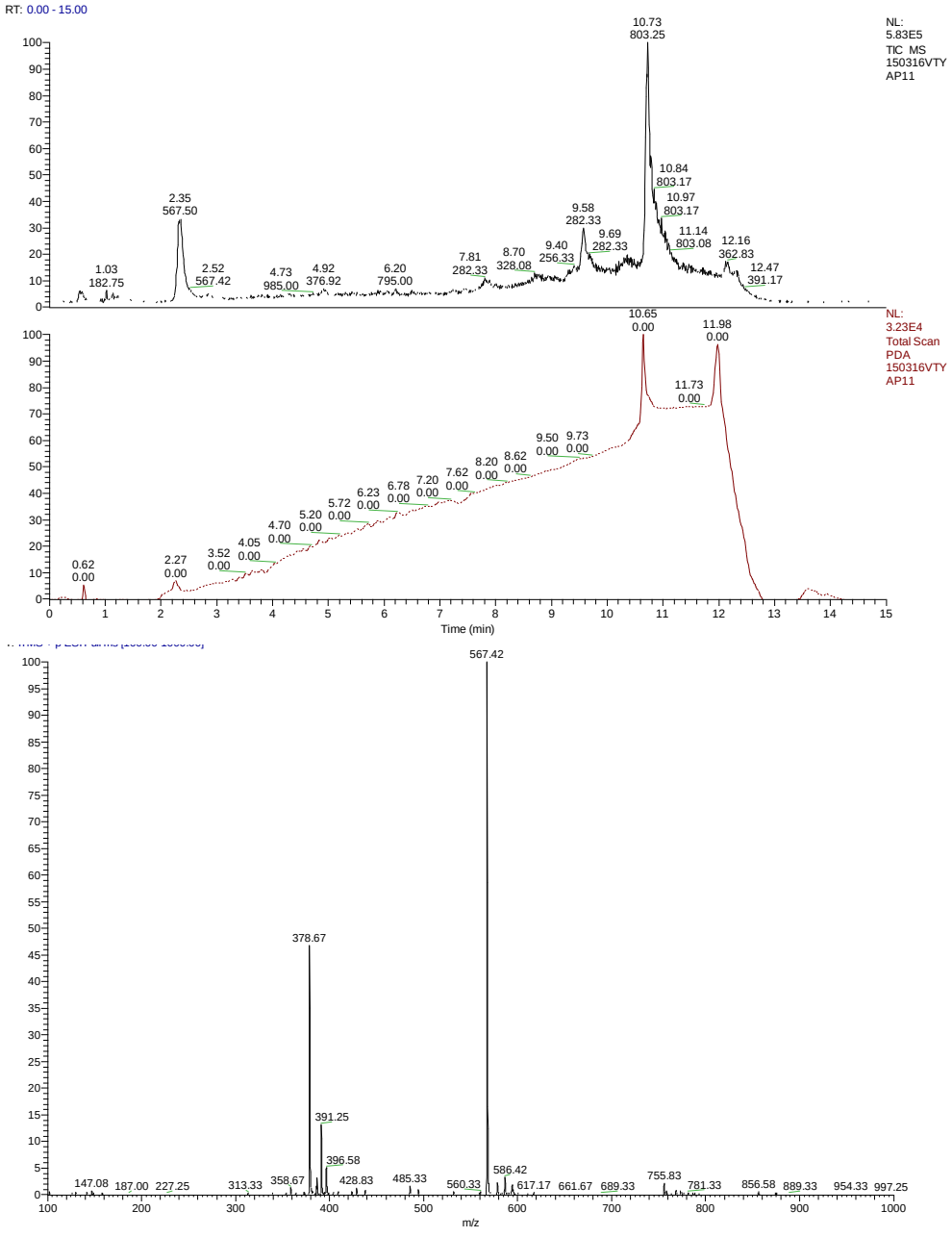
YAP 1.1 **H_2N-R A H pS S P A S L Q-COOH**

Expected mass:

$[M+H]^+$ =1133.5

$[M+2H]^{2+}$= 567.3

$[M+3H]^{3+}$ = 378.5



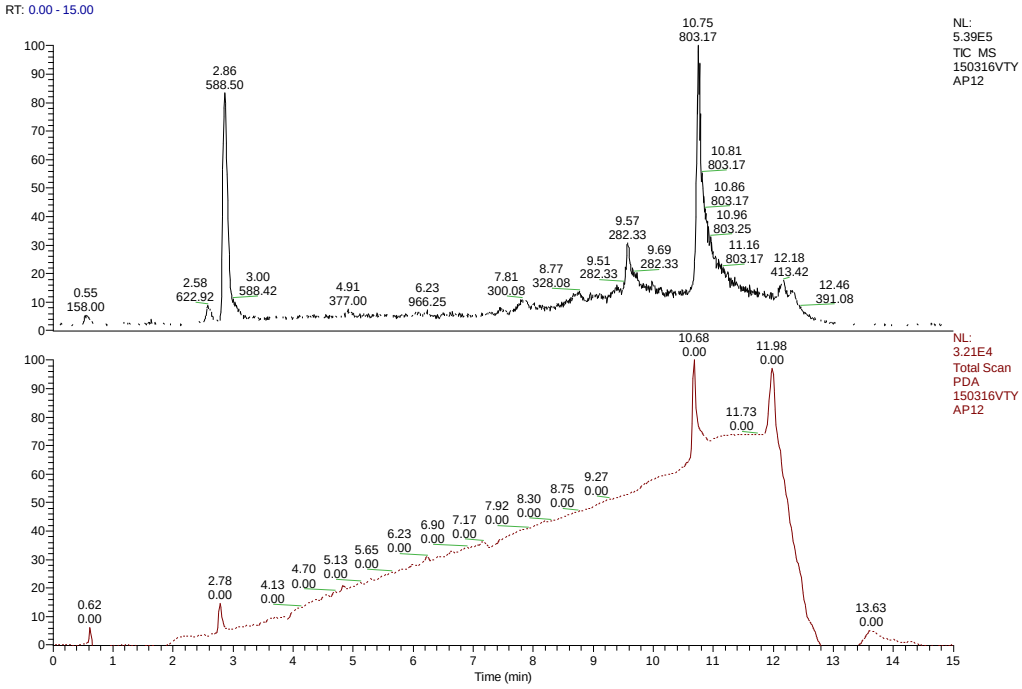
YAP 1.2 **Ac-R A H pS S P A S L Q-COOH**

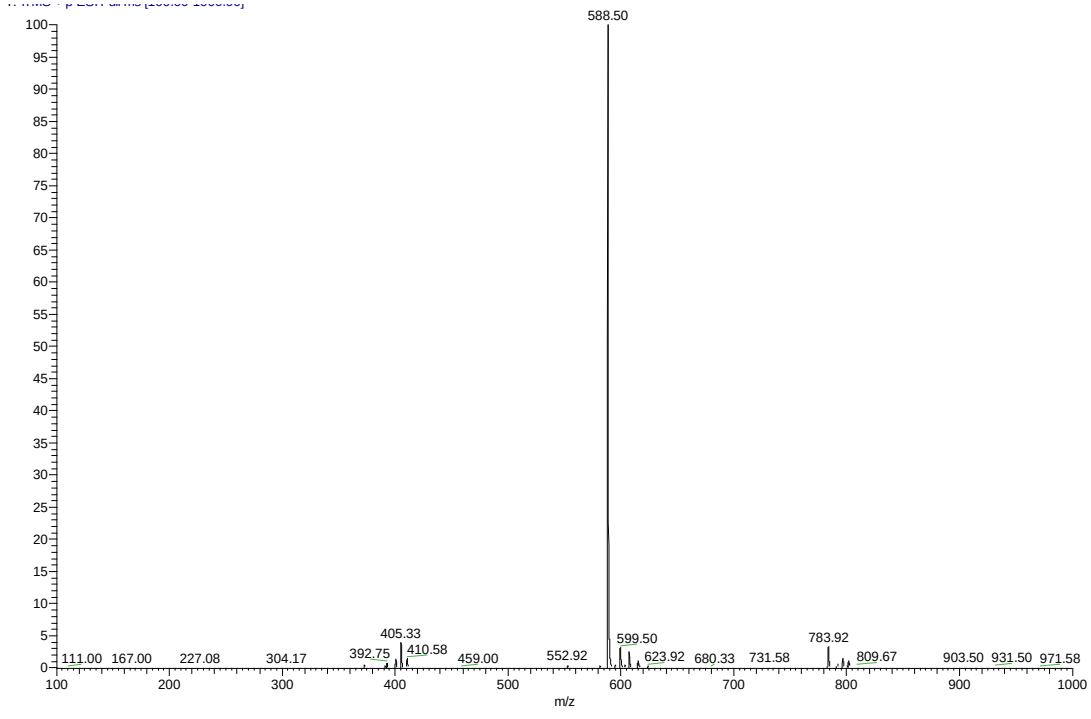
Expected mass:

$[M+H]^{+}$ = 1175.5

$[M+2H]^{2+}$ = 588.3

$[M+3H]^{3+}$ = 392.5





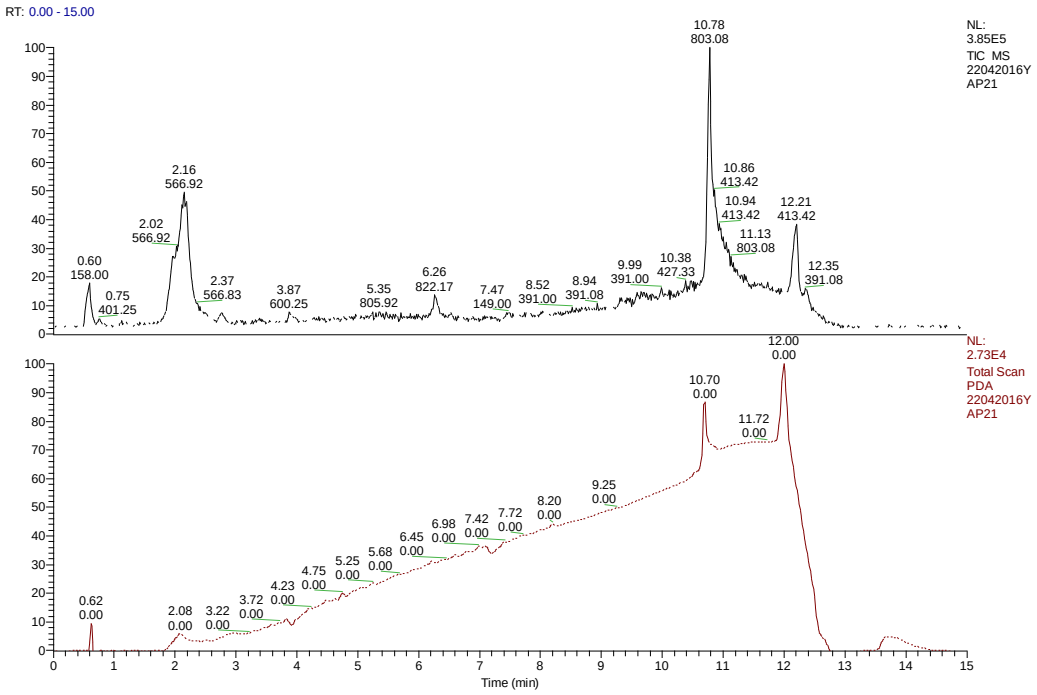
YAP 2.1 **H_2N-R A H pS S P A S L Q-$CONH_2$**

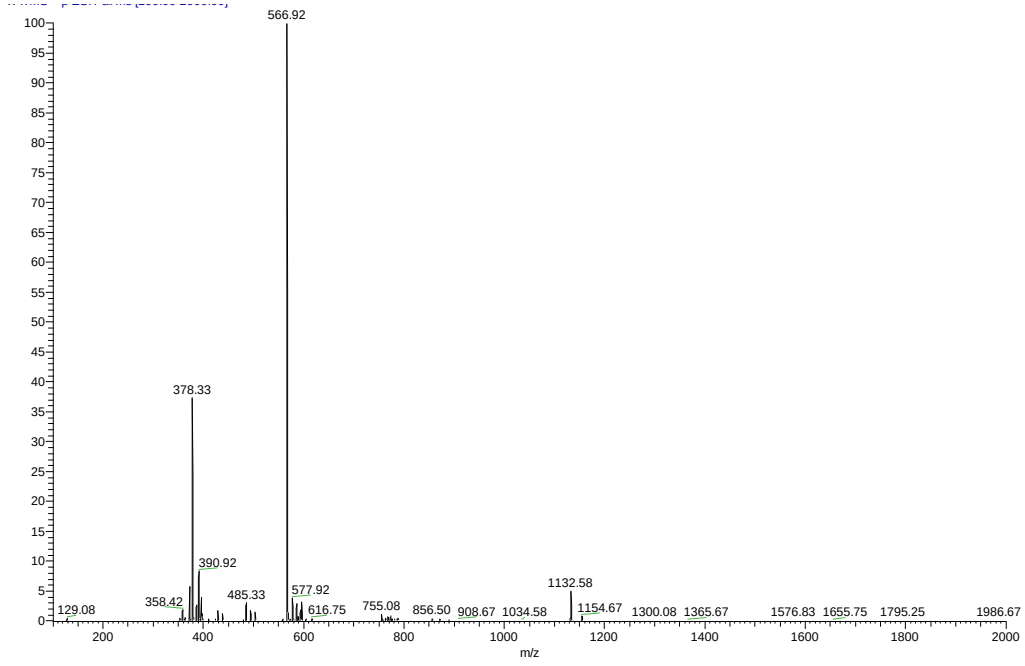
Expected mass:

$[M+H]^+$ = 1132.5

$[M+2H]^{2+}$ = 566.8

$[M+3H]^{3+}$ = 378.2





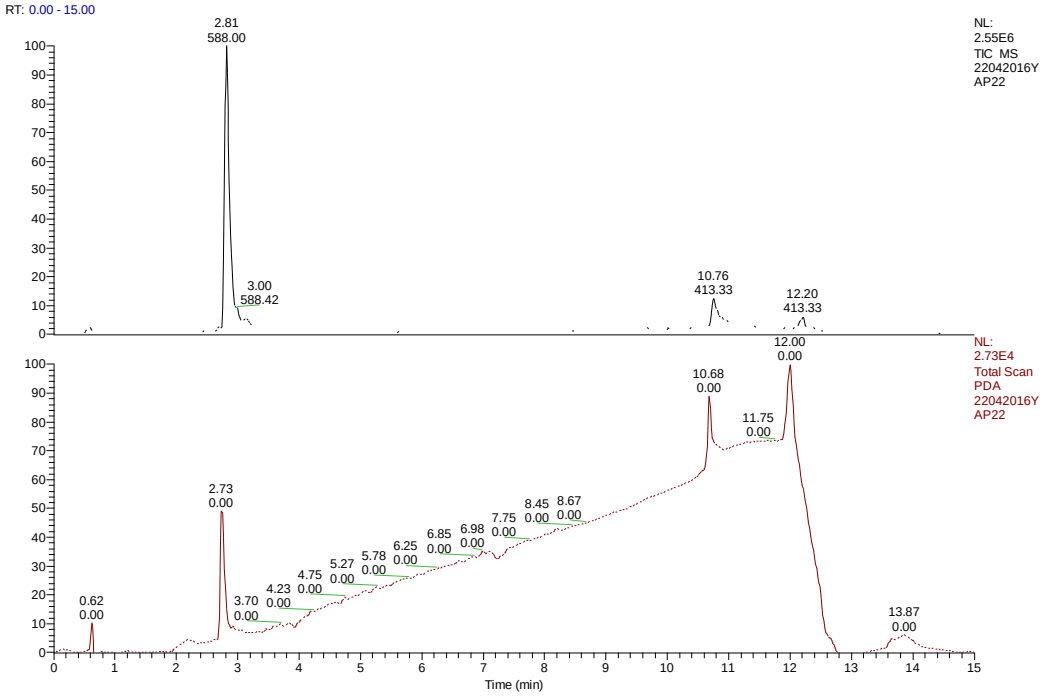
YAP 2.2 **Ac-R A H pS S P A S L Q-CONH$_2$**

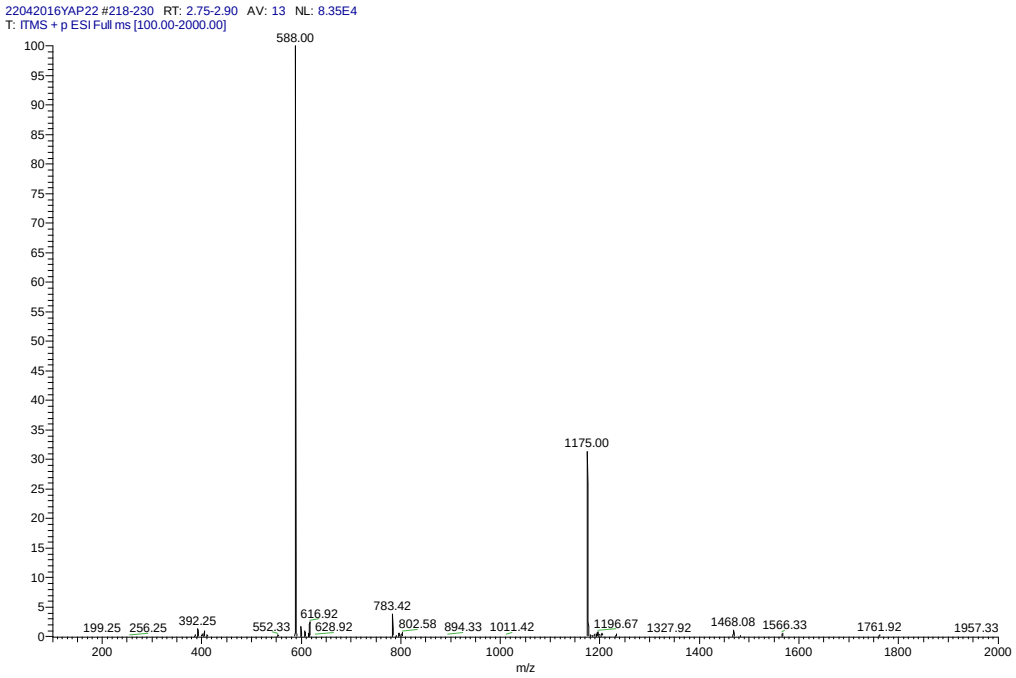
Expected mass:

$[M+H]^{+}$ = 1174.5

$[M+2H]^{2+}$ = 587.8

$[M+3H]^{3+}$ = 392.5





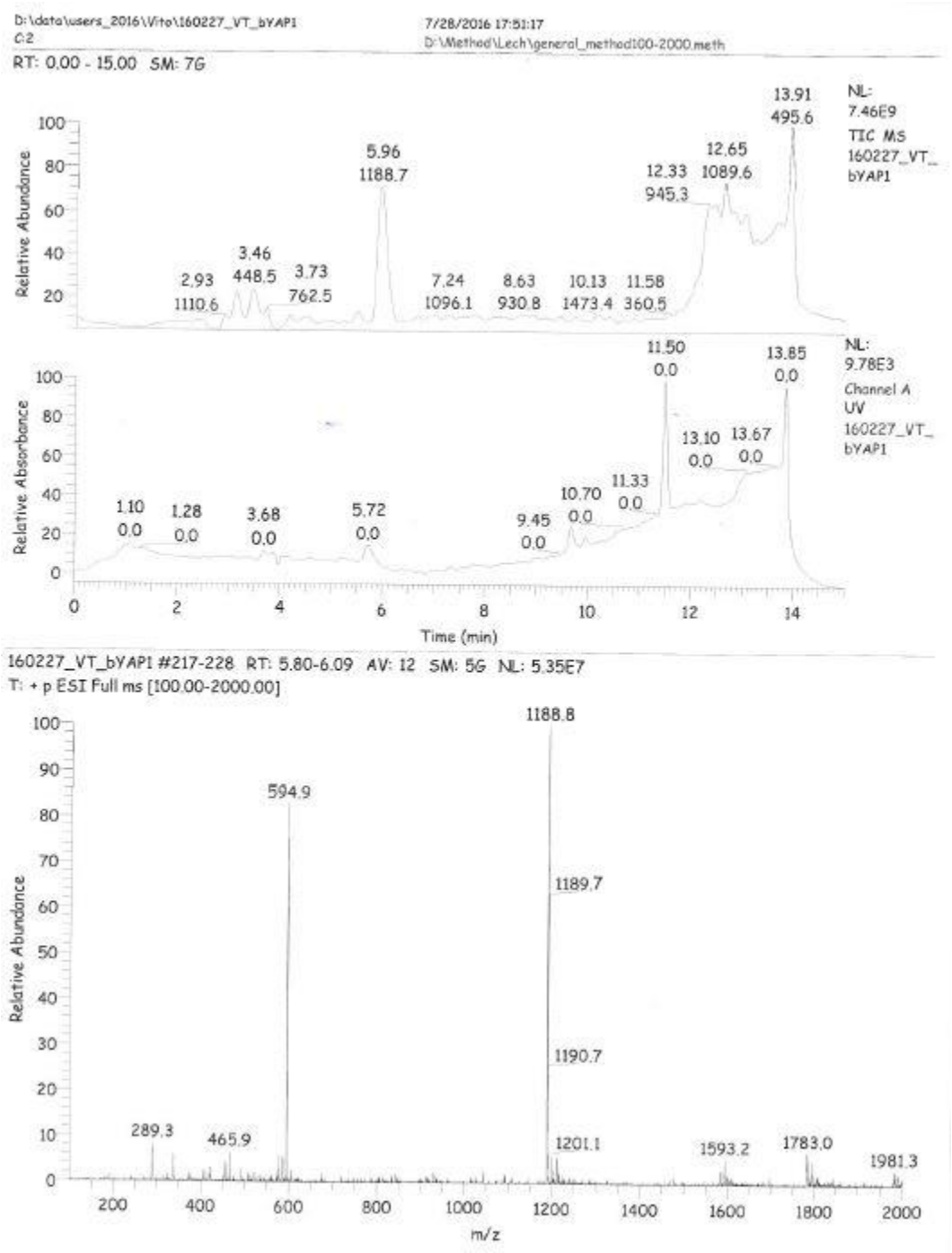
βYAP 1 **Ac-R A H pS βS P A S L Q-CONH$_2$**

Expected mass:

$[M+H]^+$ = 1188.5

$[M+2H]^{2+}$ = 594.8

$[M+3H]^{3+}$ = 396.8



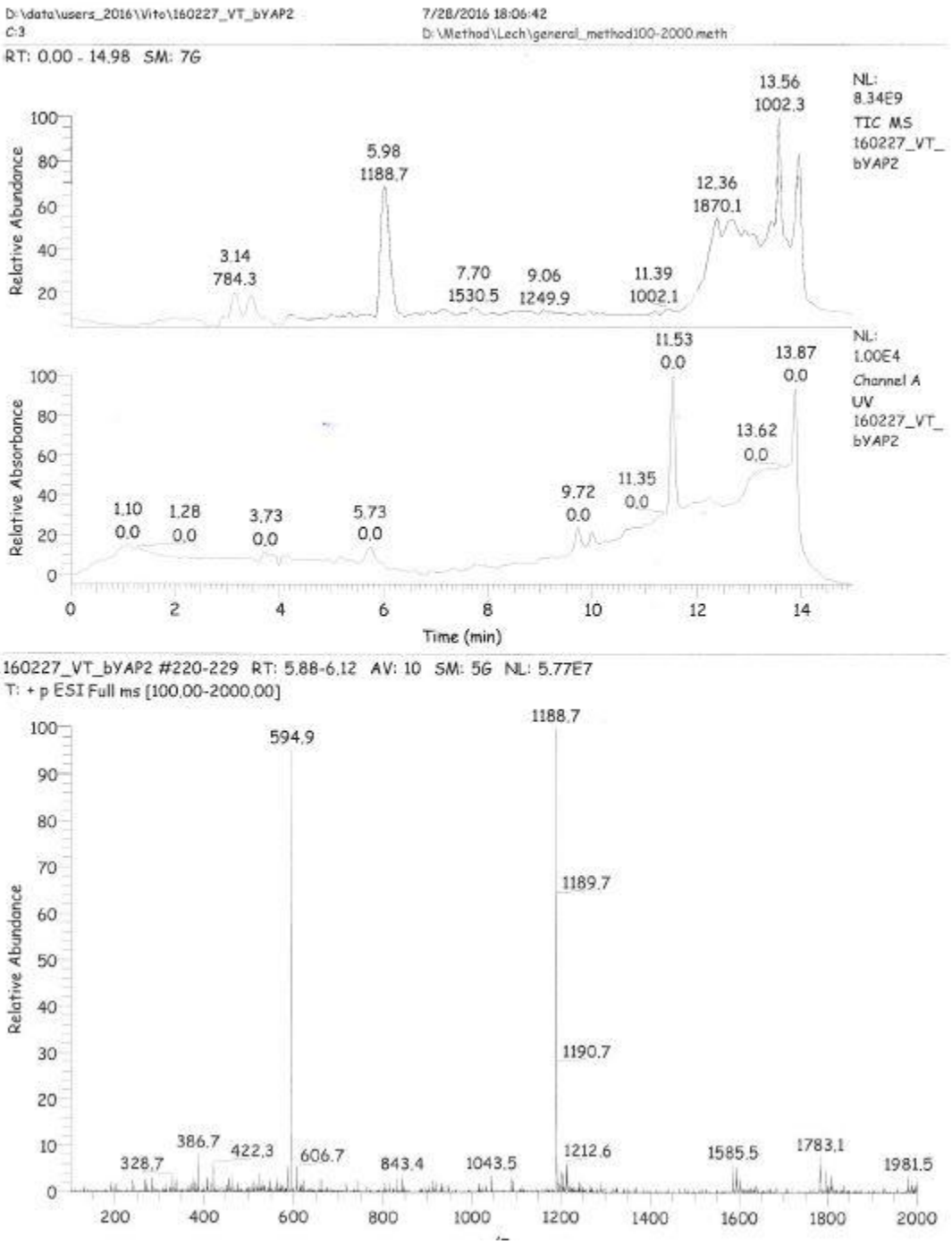
βYAP 2 **Ac-R A H pS S βP A S L Q-CONH$_2$**

Expected mass:

$[M+H]^+$ = 1188.5

$[M+2H]^{2+}$ = 594.8

$[M+3H]^{3+}$ = 396.8



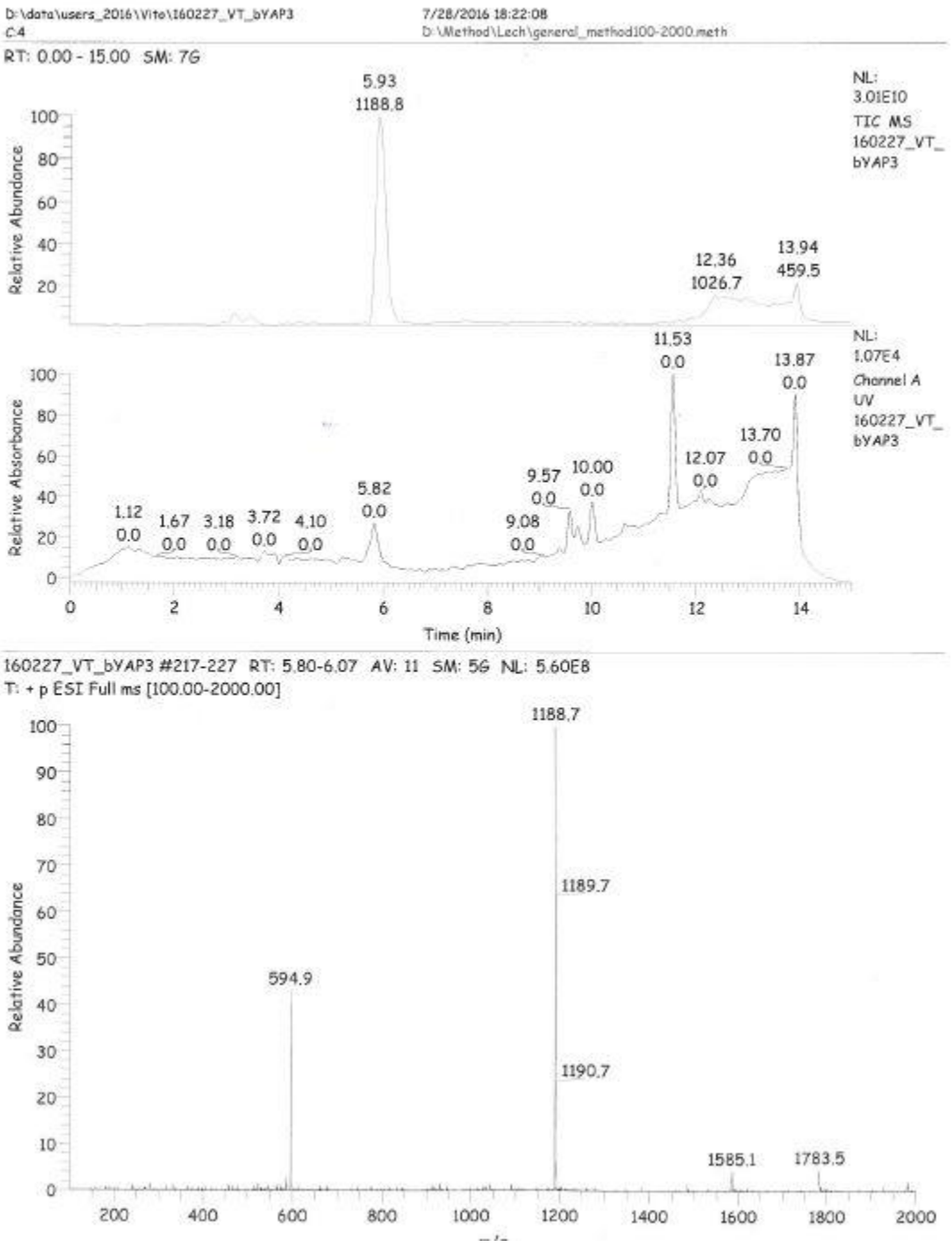
βYAP 3 **Ac-R A H pS S P βA S L Q-CONH$_2$**

Expected mass:

$[M+H]^+$ = 1188.5

$[M+2H]^{2+}$ = 594.8

$[M+3H]^{3+}$ = 396.8



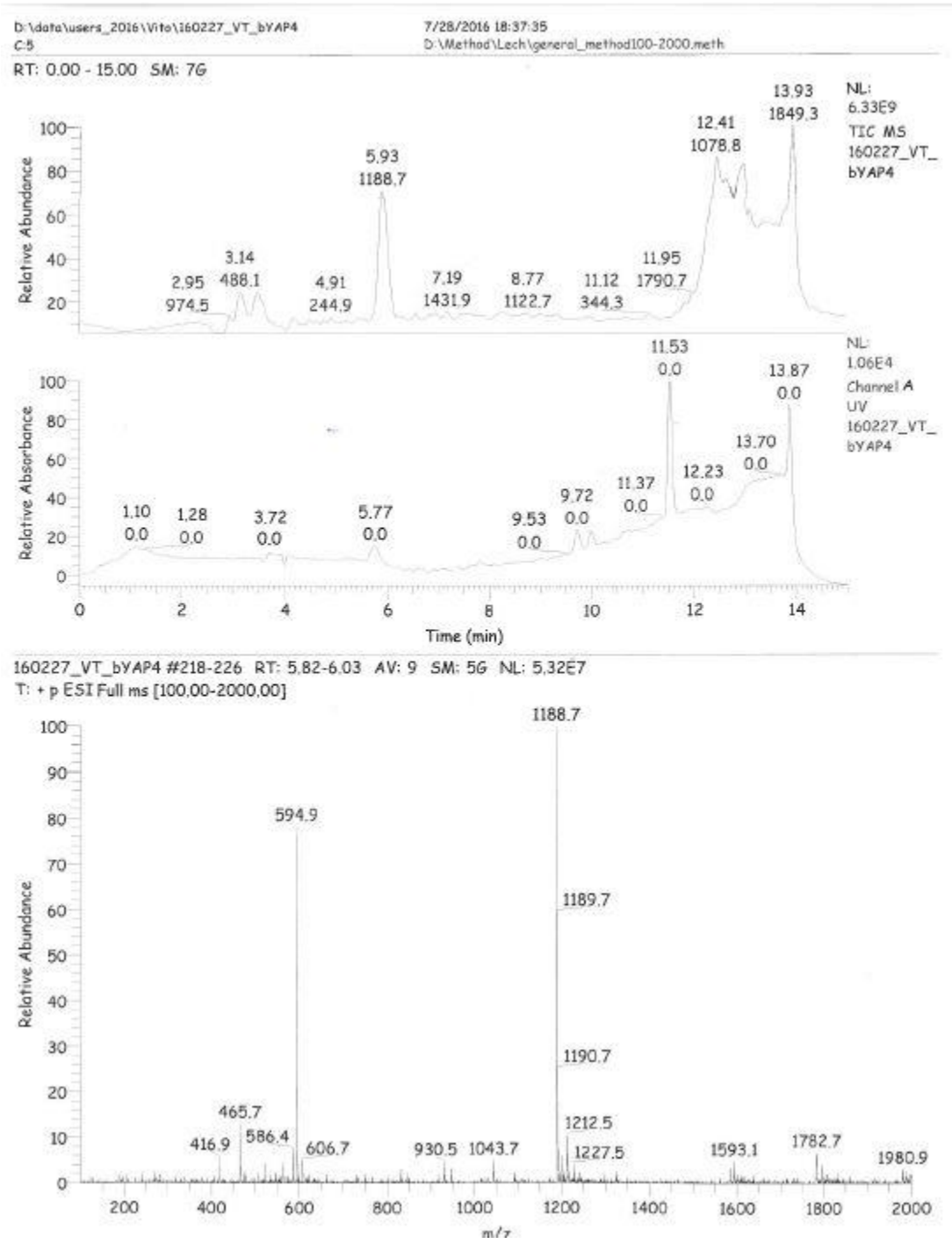
βYAP 4 **Ac-R A H pS S P A βS L Q-CONH$_2$**

Expected mass:

$[M+H]^{+}$ = 1188.5

$[M+2H]^{2+}$ = 594.8

$[M+3H]^{3+}$ = 396.8



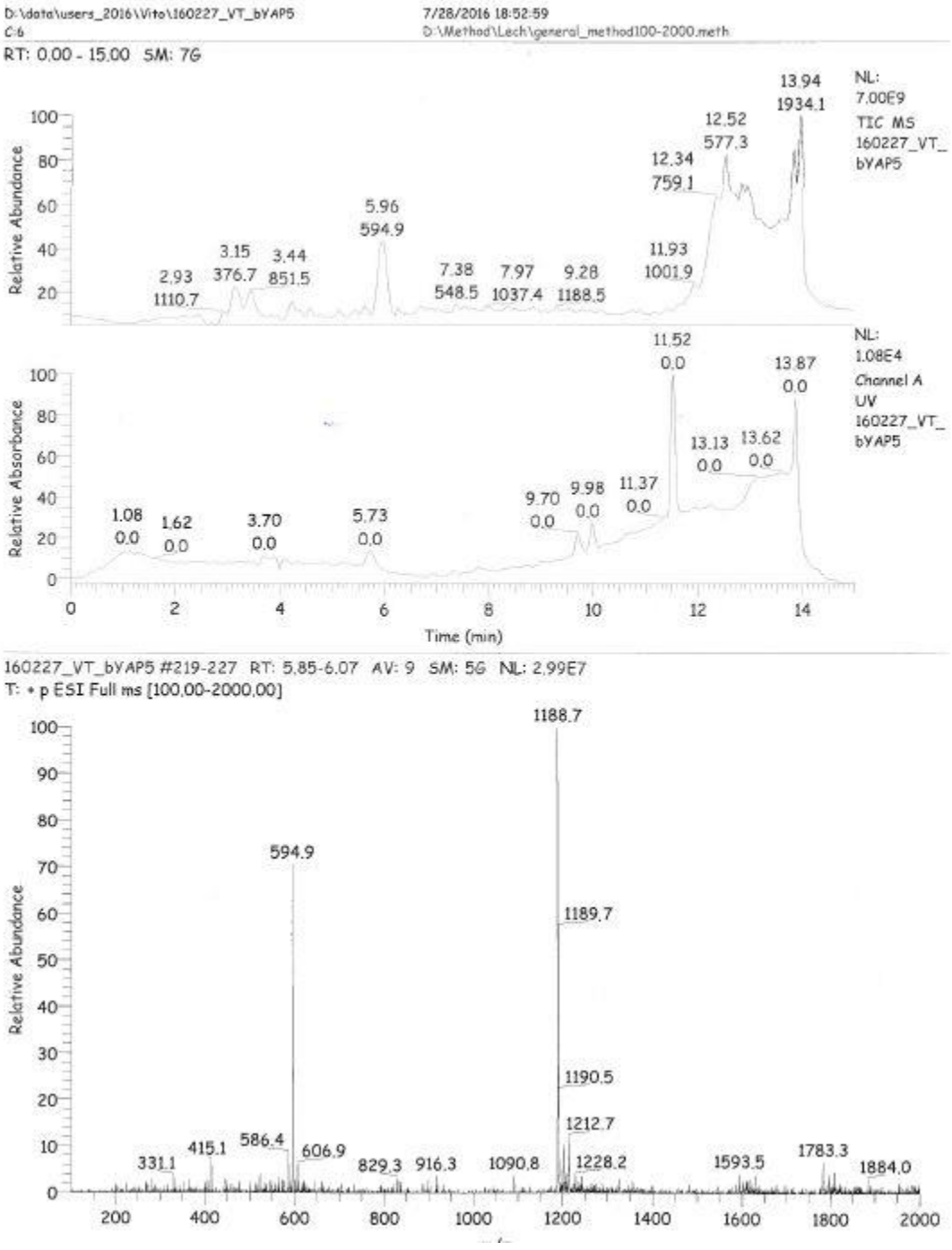
βYAP 5 **Ac-R A H pS S P A S βL Q-CONH$_2$**

Expected mass:

$[M+H]^+$ = 1188.5

$[M+2H]^{2+}$ = 594.8

$[M+3H]^{3+}$ = 396.8



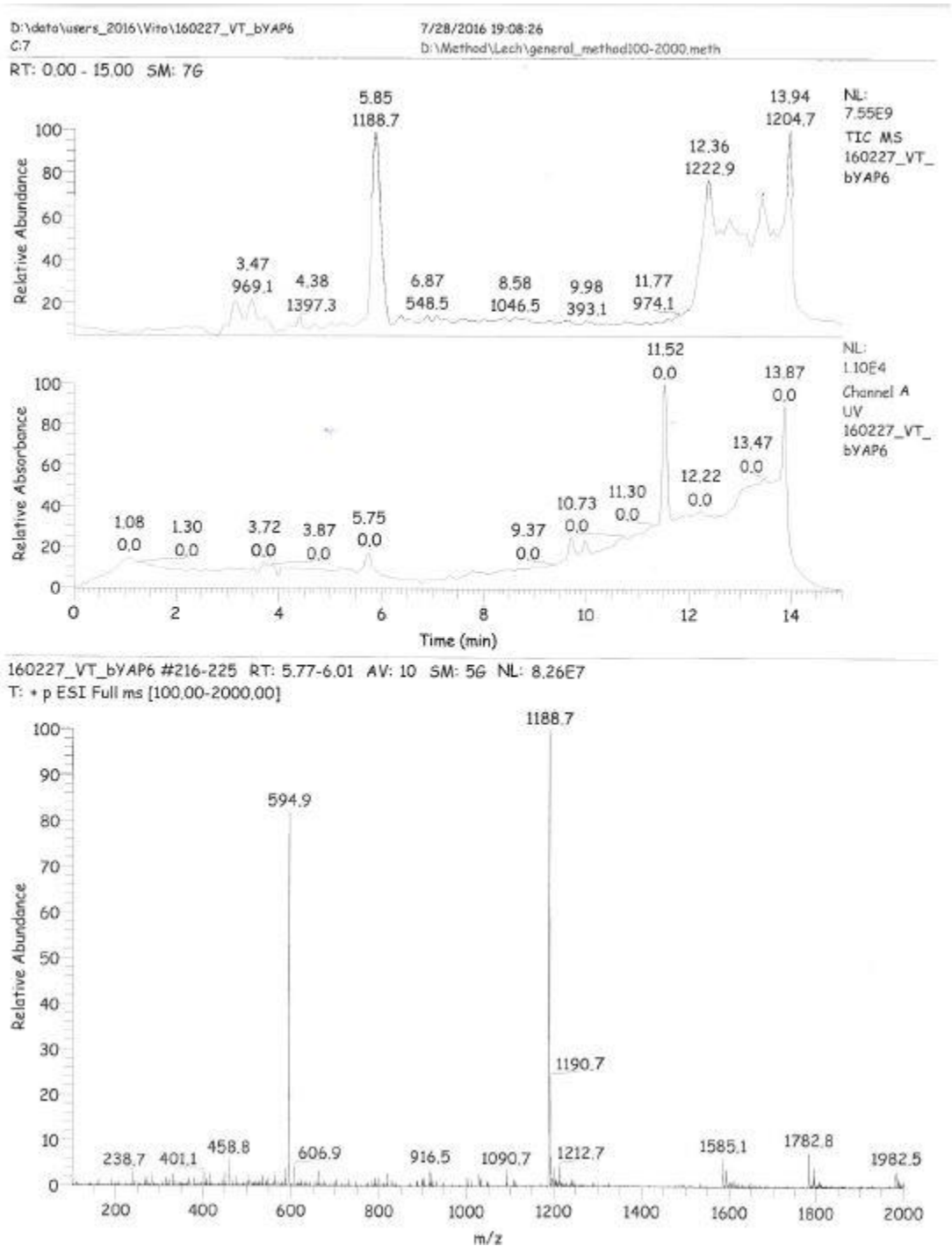
βYAP 6 **Ac-R A H pS S P A S L βQ-CONH$_2$**

Expected mass:

$[M+H]^+$ = 1188.5

$[M+2H]^{2+}$ = 594.8

$[M+3H]^{3+}$ = 396.8



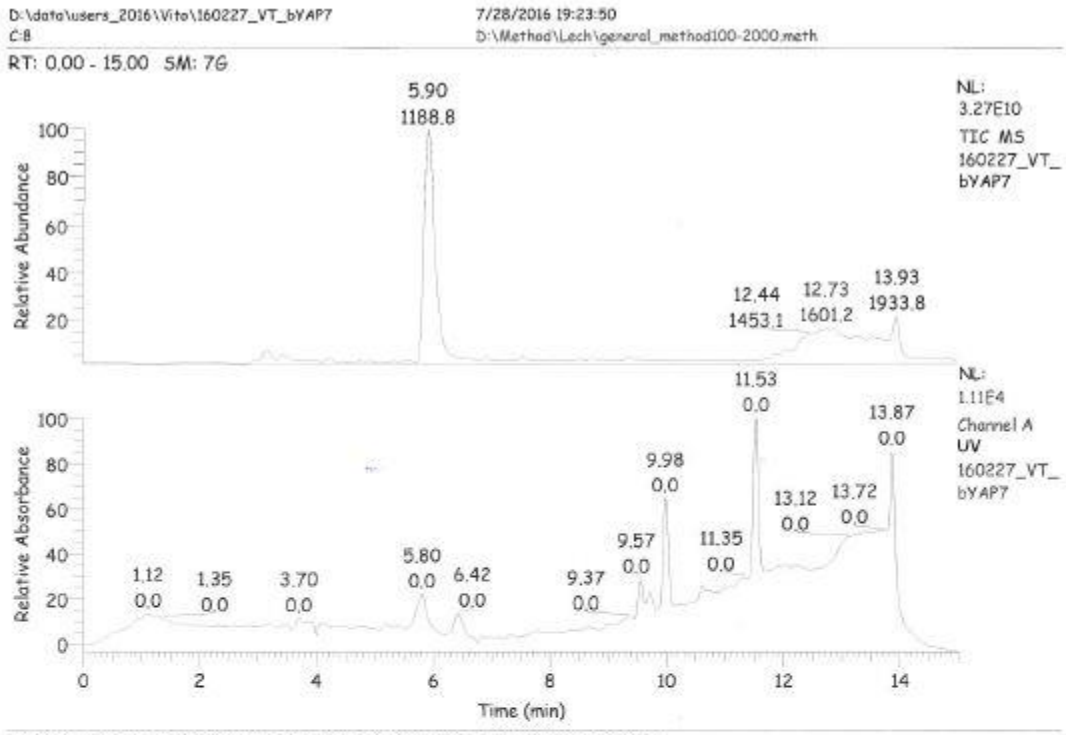
βYAP 7 **Ac-βR A H pS S P A S L Q-CONH$_2$**

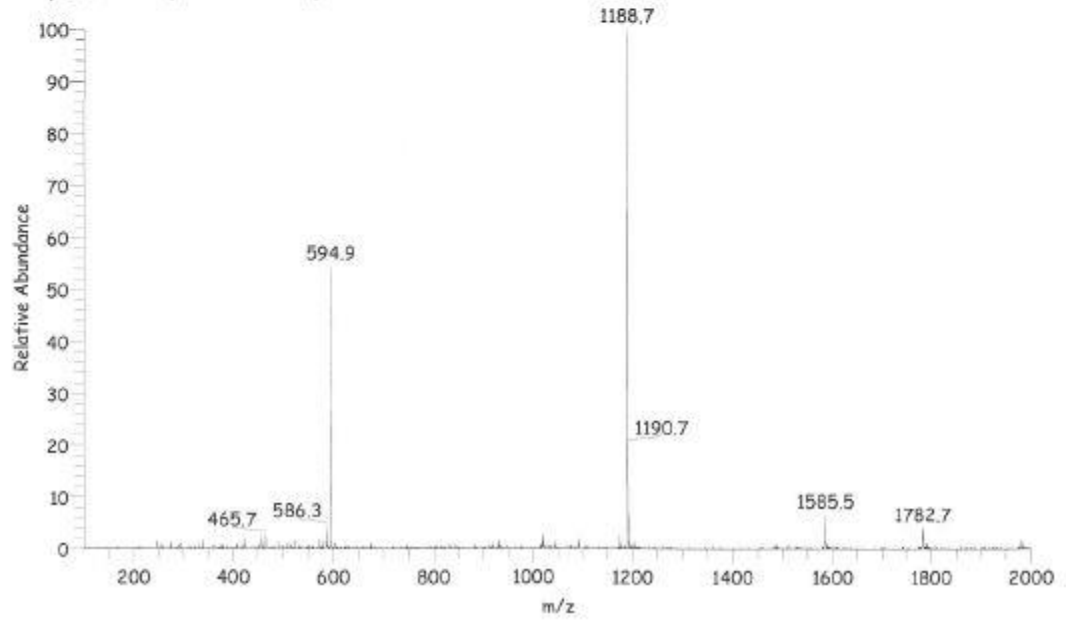
Expected mass:

$[M+H]^+$ = 1188.5

$[M+2H]^{2+}$ = 594.8

$[M+3H]^{3+}$ = 396.8





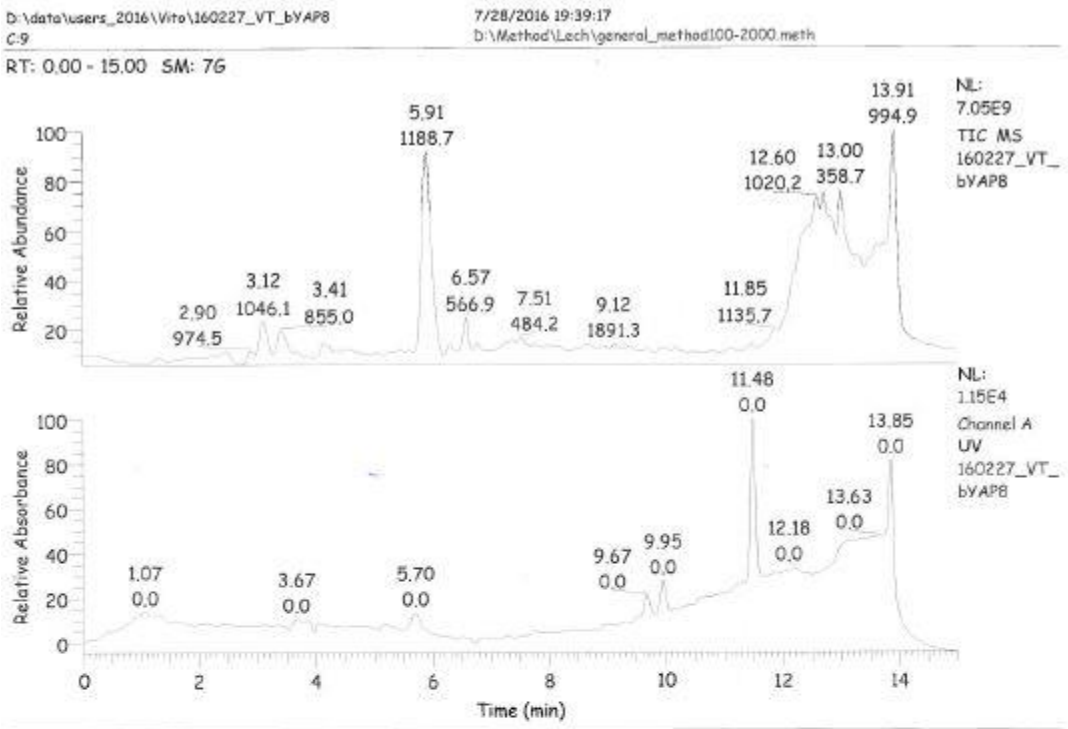
βYAP 8 **Ac-R βA H pS S P A S L Q-CONH$_2$**

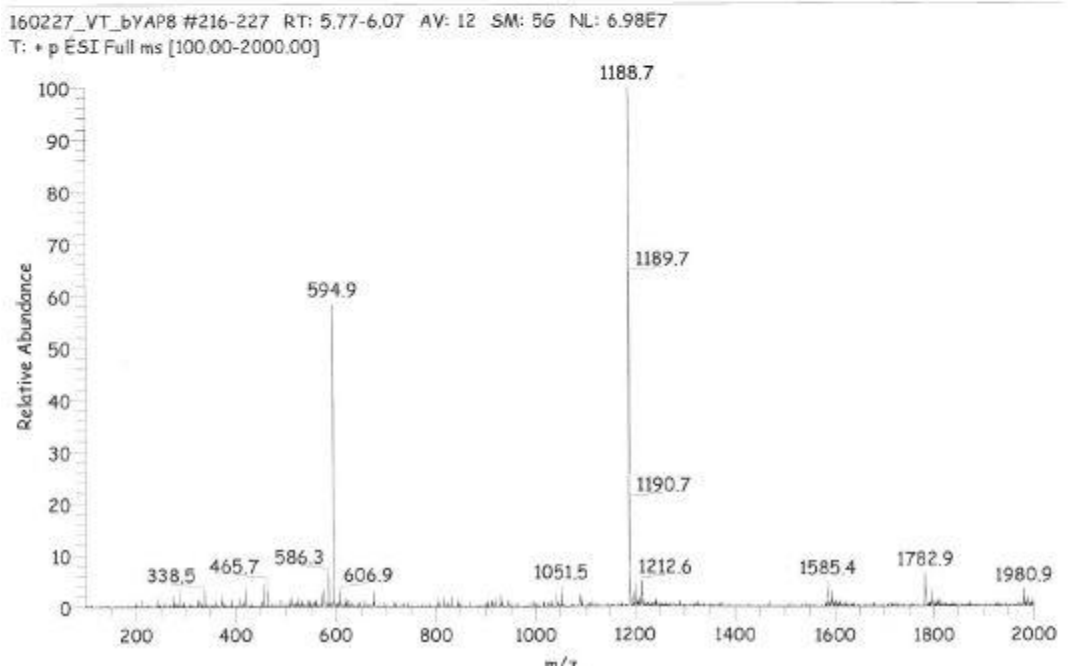
Expected mass:

$[M+H]^+$ = 1188.5

$[M+2H]^{2+}$ = 594.8

$[M+3H]^{3+}$ = 396.8





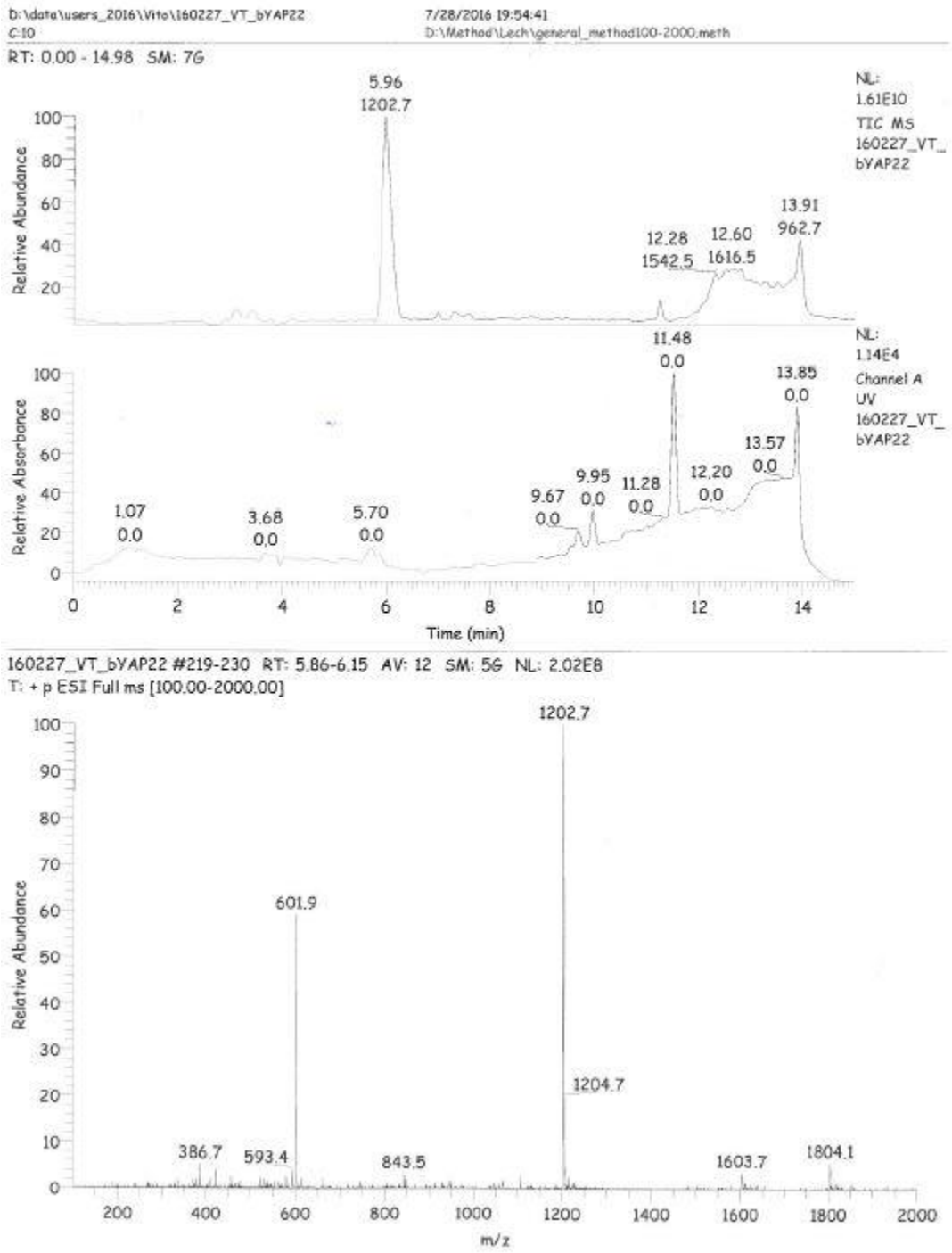
βYAP 2.2 **Ac-R A H pS S βP A S βL Q-CONH$_2$**

Expected mass:

$[M+H]^+$ = 1202.5

$[M+2H]^{2+}$ = 601.8

$[M+3H]^{3+}$ = 401.5



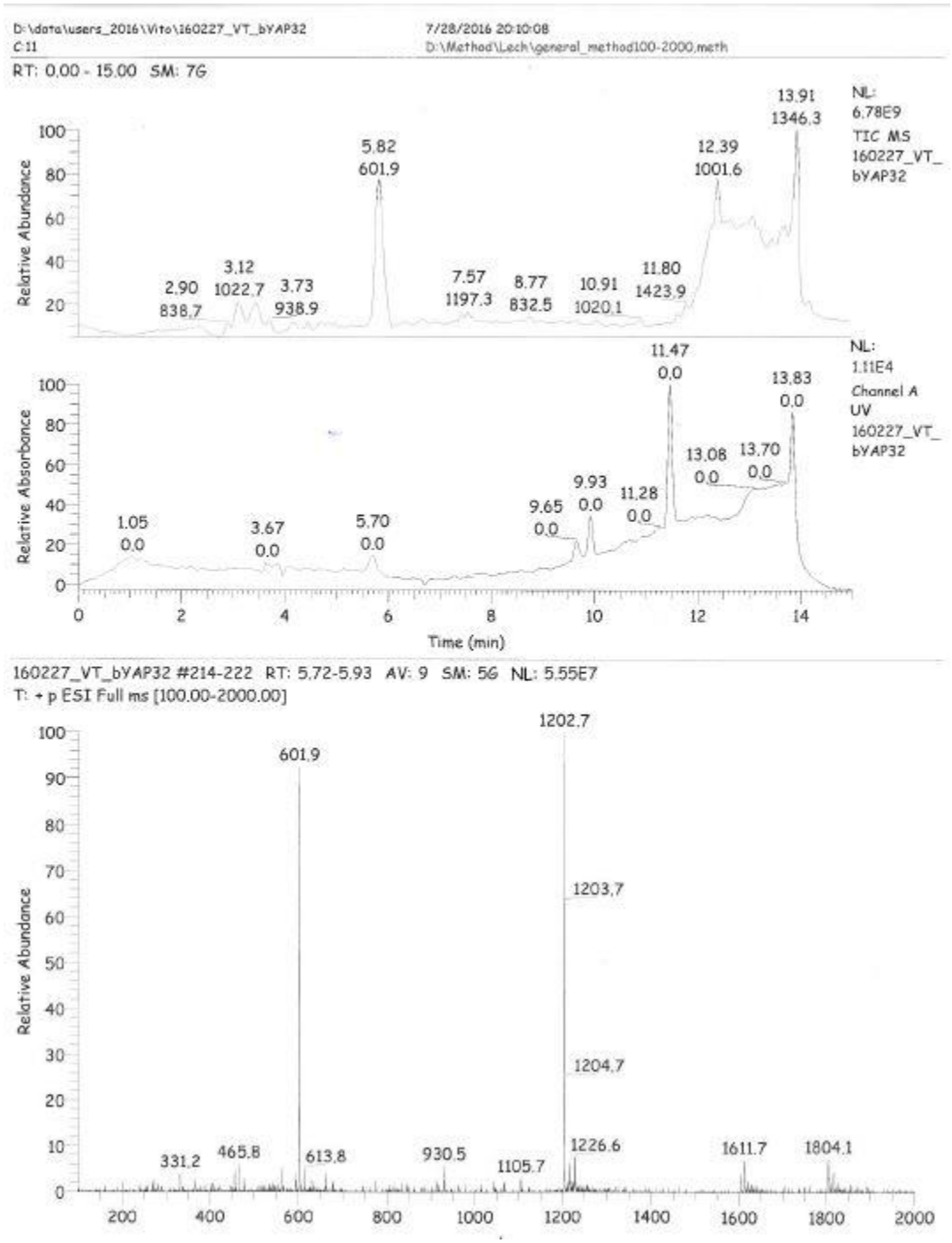
βYAP 3.2 **Ac-R A H pS S P βA S L βQ-CONH$_2$**

Expected mass:

$[M+H]^+$ = 1202.5

$[M+2H]^{2+}$ = 601.8

$[M+3H]^{3+}$ = 401.5



III. References

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