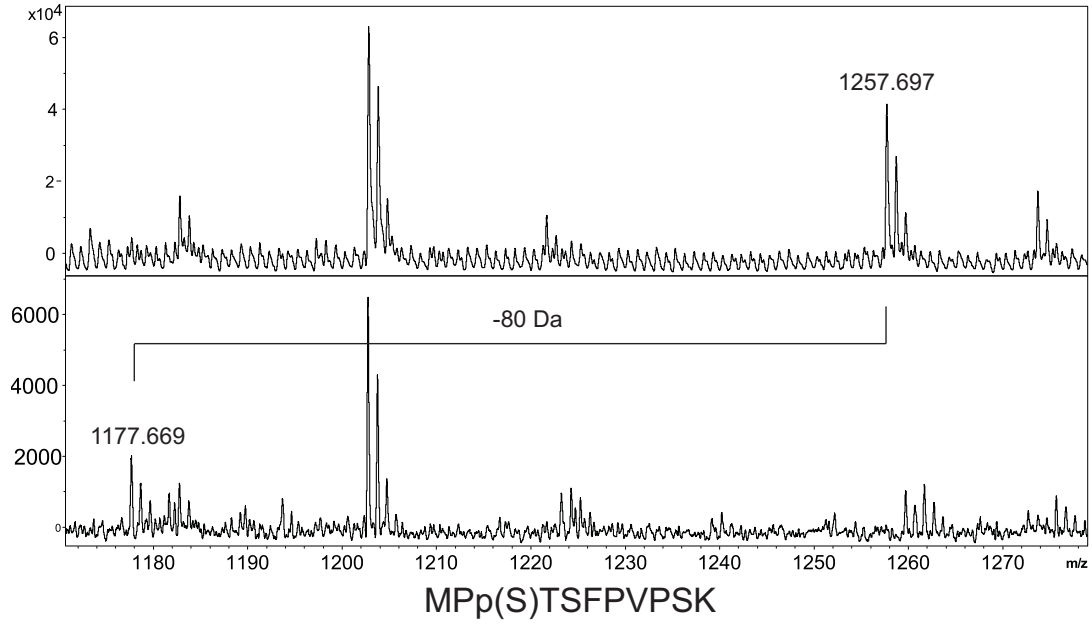
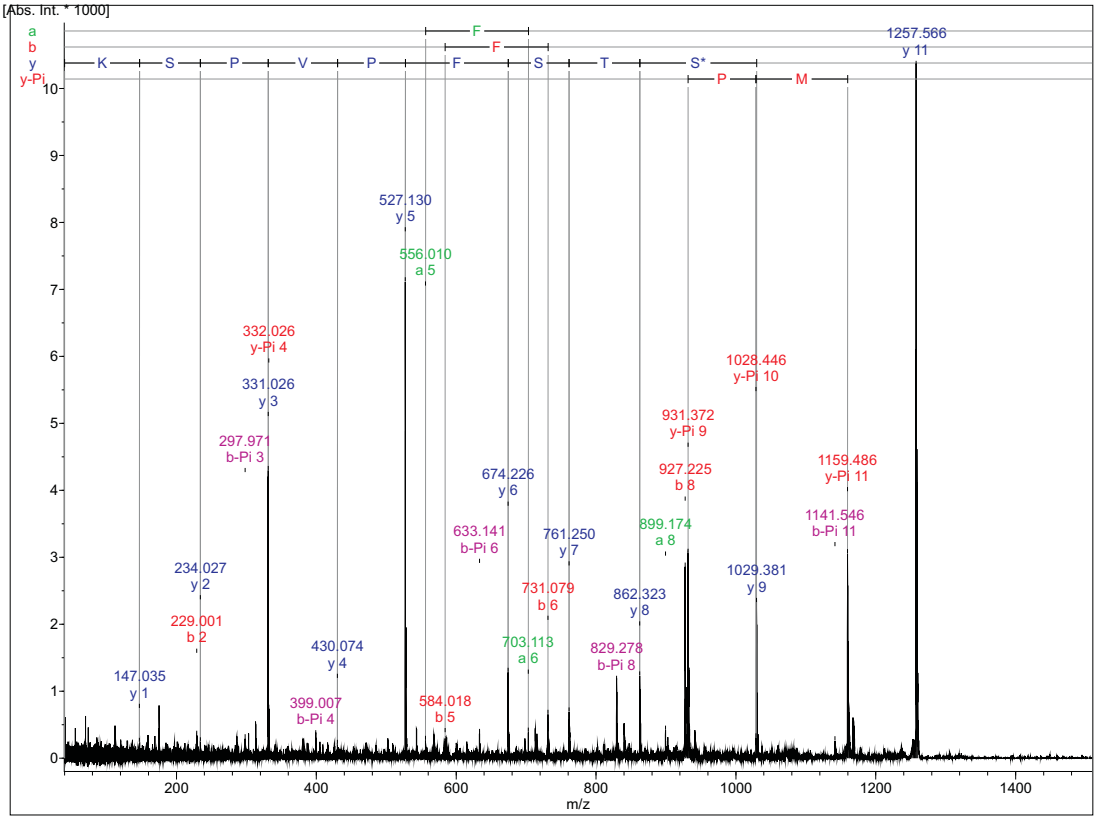
Supplementary image 1.1: PKA phosphorylated NFAT *m/z* 1257.55

A)



B)



C)

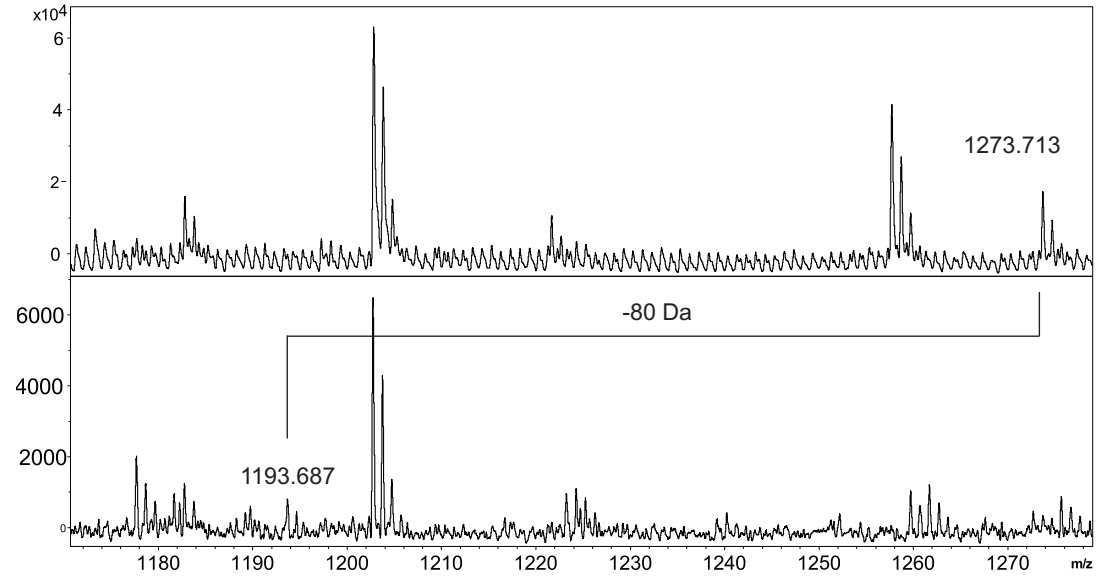
Monoisotopic mass of neutral peptide Mr(calc): 1256.5512
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
S3 : Phospho (ST), with neutral losses 97.9769(shown in table), 0.0000
Ions Score: 77 Expect: 2.2e-09
Matches (highlighted): 15/204 fragment ions using 21 most intense peaks

#	b	b^0	Seq.	y	y*	y^0	#
1	132.0478		M				11
2	**229.1005**		P	1028.5411	1011.5146	1010.5306	10
3	298.122	280.1114	S	**931.4884**	914.4618	913.4778	9
4	**399.1697**	381.1591	T	**862.4669**	845.4403	844.4563	8
5	486.2017	468.1911	S	**761.4192**	744.3927	743.4087	7
6	633.2701	615.2595	F	**674.3872**	657.3606	656.3766	6
7	730.3229	712.3123	P	**527.3188**	510.2922	509.3082	5
8	**829.3913**	811.3807	V	**430.266**	413.2395	412.2554	4
9	926.444	908.4335	P	**331.1976**	314.171	**313.187**	3
10	1013.4761	995.4655	S	234.1448	217.1183	216.1343	2
11			K	**147.1128**	130.0863		1

Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation from Biotools. Part C: Mascot search results and ion table.

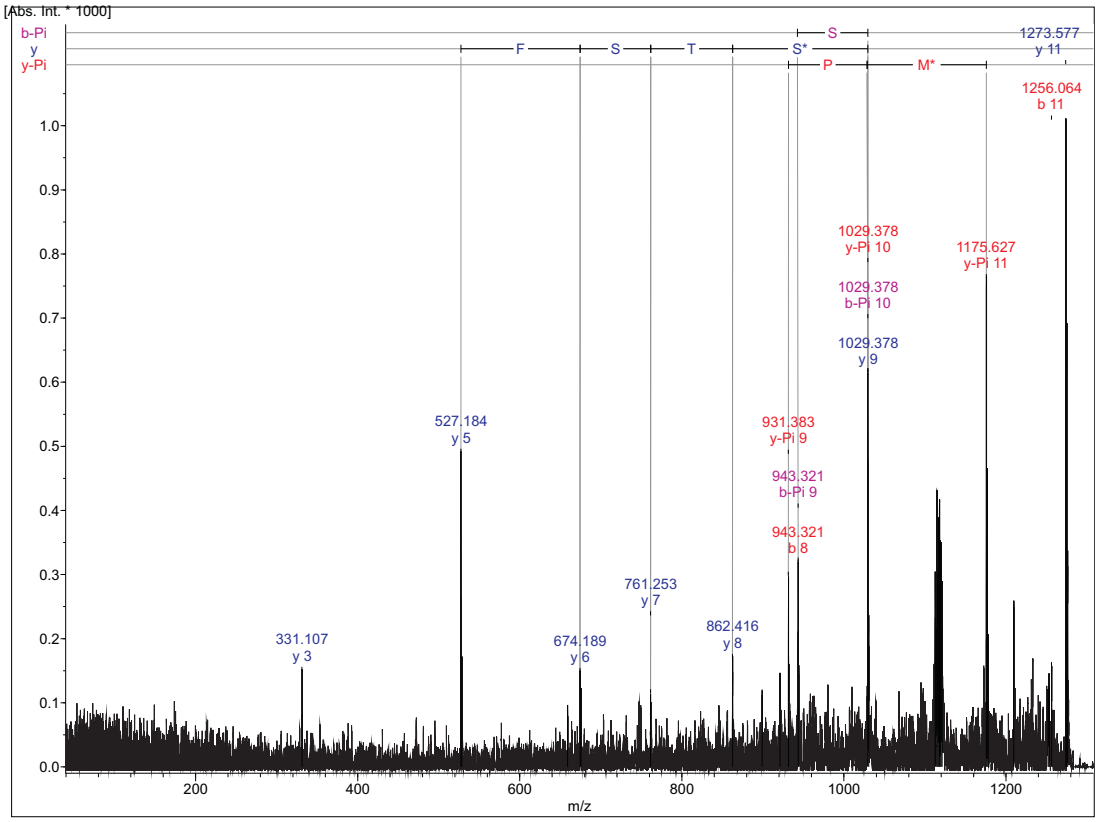
Supplementary image 1.2: PKA phosphorylated NFAT *m/z* 1273.54

A)



ox(M)Pp(S)TSFPVPSK

B)



C)

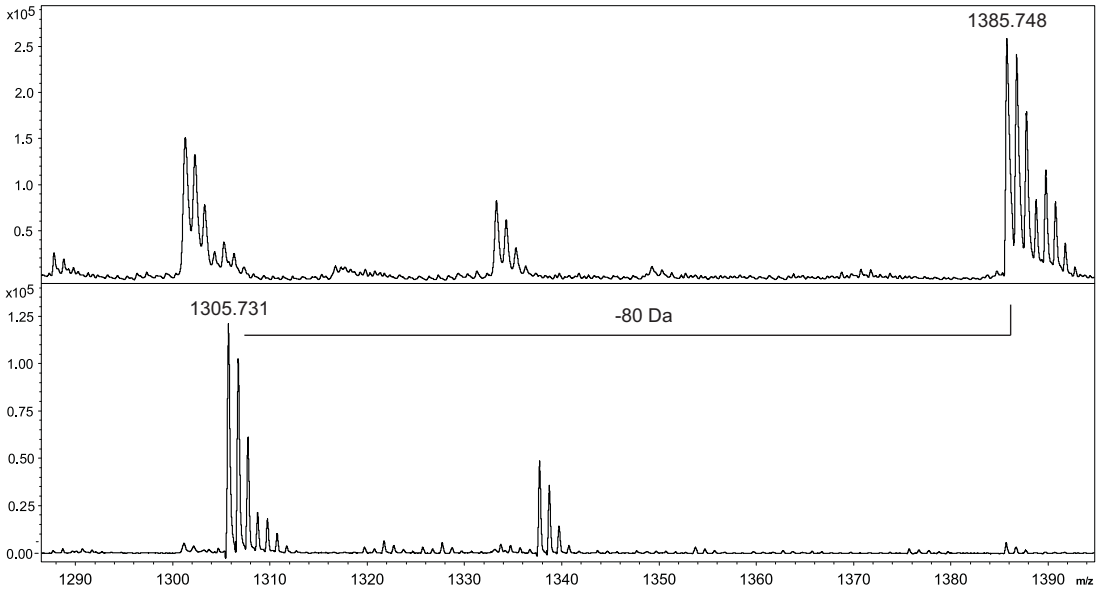
Monoisotopic mass of neutral peptide Mr(calc): 1272.5461
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
M1 : Oxidation (M), with neutral losses 63.9983(shown in table), 0.0000
S3 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
Ions Score: 42 Expect: 6e-06
Matches (highlighted): 11/265 fragment ions using 16 most intense peaks

#	b	b^0	Seq.	y	y*	y^0	#
1	84.0444		M				11
2	181.0972		P	1126.518	1109.4915	1108.5074	10
3	348.0955	330.0849	S	**1029.4652**	1012.4387	1011.4547	9
4	449.1432	431.1326	T	**862.4669**	845.4403	844.4563	8
5	536.1752	518.1647	S	**761.4192**	744.3927	743.4087	7
6	683.2436	665.2331	F	**674.3872**	657.3606	656.3766	6
7	780.2964	762.2858	P	**527.3188**	510.2922	509.3082	5
8	879.3648	861.3542	V	430.266	413.2395	412.2554	4
9	976.4176	958.407	P	**331.1976**	314.171	313.187	3
10	1063.4496	1045.439	S	234.1448	217.1183	216.1343	2
11			K	147.1128	130.0863		1

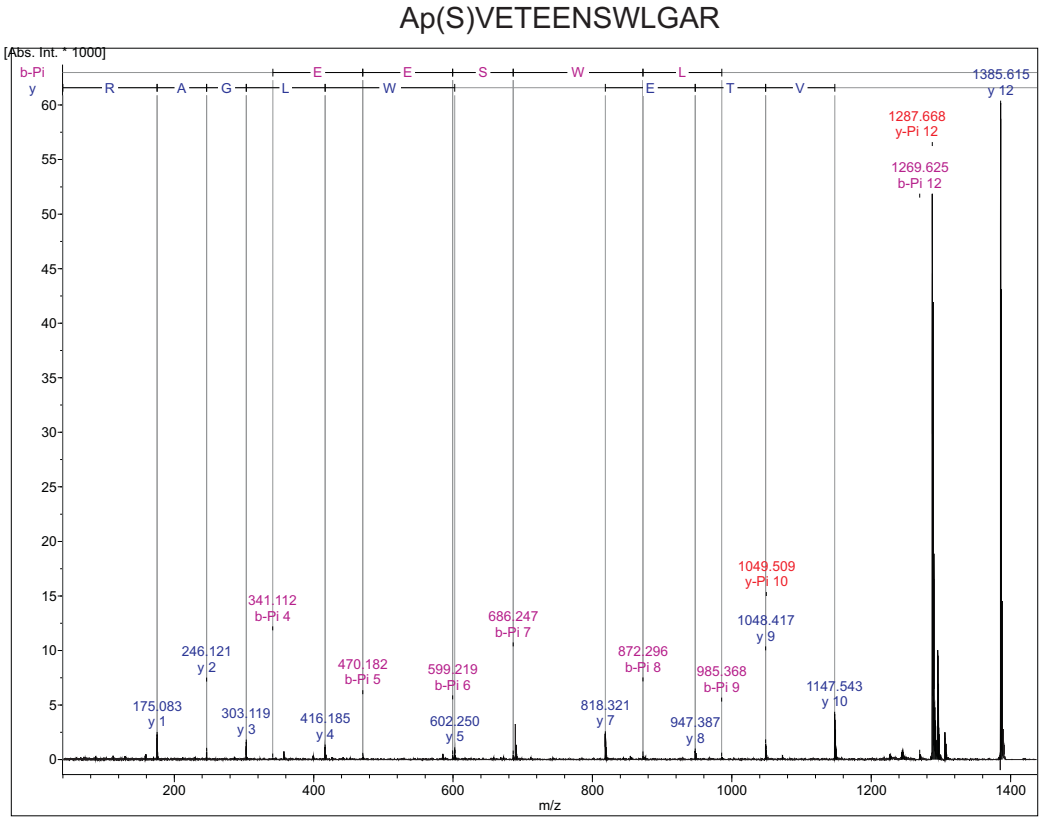
Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation from Biotools. Part C: Mascot search results and ion table.

Supplementary image 1.3: PKA phosphorylated NFAT *m/z* 1385.61

A)



B)



C)

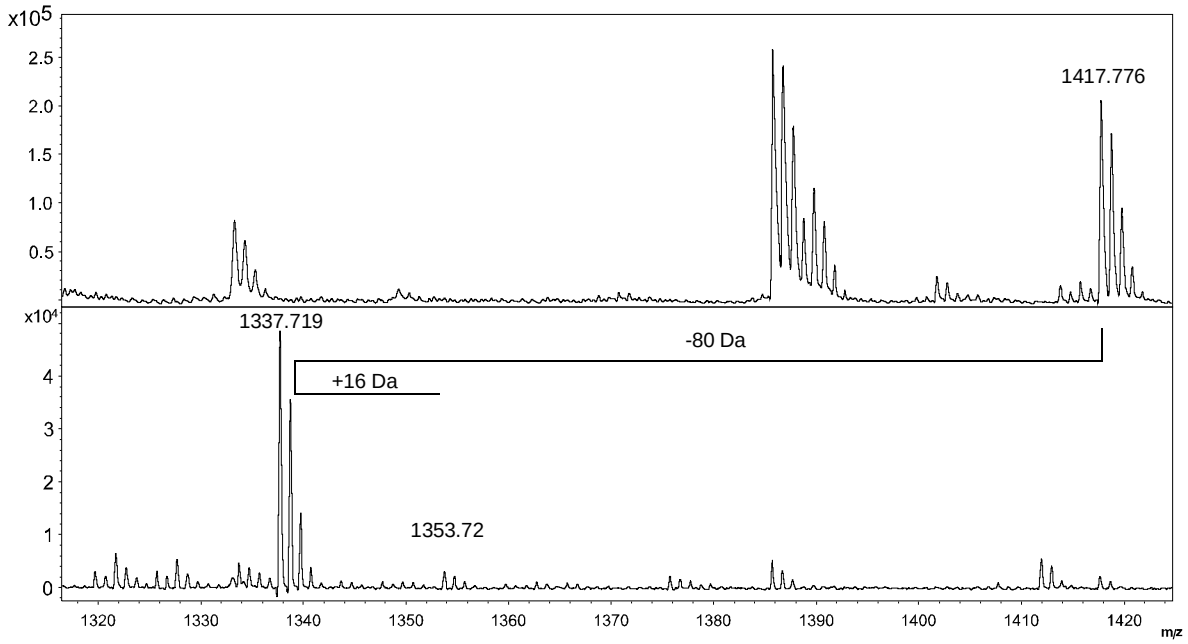
Monoisotopic mass of neutral peptide Mr(calc): 1384.6024
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
S2 : Phospho (ST), with neutral losses 97.9769(shown in table), 0.0000
Ions Score: 106 Expect: 2.3e-12
Matches (highlighted): 13/220 fragment ions using 16 most intense peaks

#	b	b⁰	Seq.	y	y*	y⁰	#
1	72.0444		A				12
2	141.0658	123.0553	S	1216.5957	1199.5691	1198.5851	11
3	240.1343	222.1237	V	**1147.5742**	1130.5477	1129.5636	10
4	341.1819	323.1714	T	**1048.5058**	1031.4793	1030.4952	9
5	470.2245	452.214	E	**947.4581**	930.4316	929.4476	8
6	599.2671	581.2566	E	**818.4155**	801.389	800.405	7
7	686.2992	668.2886	S	**689.3729**	672.3464	671.3624	6
8	872.3785	854.3679	W	**602.3409**	**585.3144**		5
9	985.4625	967.452	L	**416.2616**	399.235		4
10	1042.484	1024.4734	G	**303.1775**	286.151		3
11	1113.5211	1095.5105	A	**246.1561**	229.1295		2
12			R	**175.119**	158.0924		1

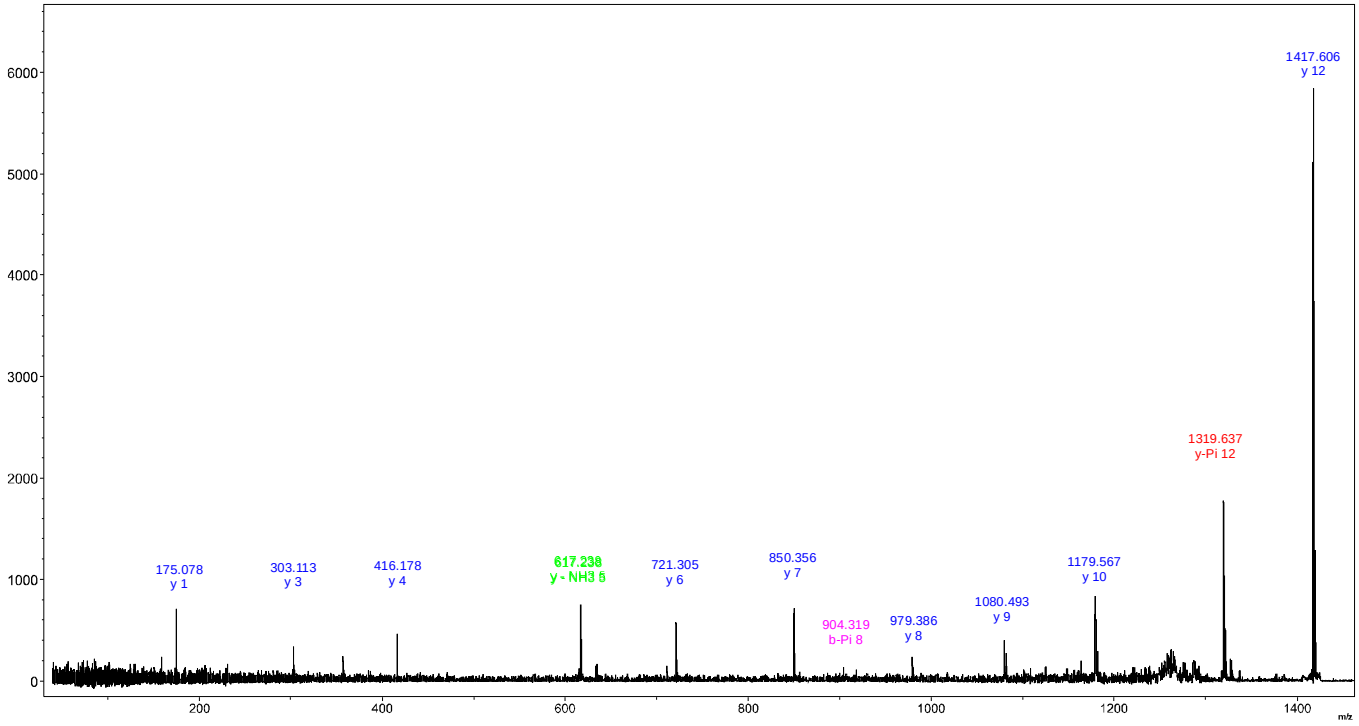
Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation from Biotools. Part C: Mascot search results and ion table.

Supplementary image 1.4: PKA phosphorylated NFAT *m/z* 1417.59

A)



B)



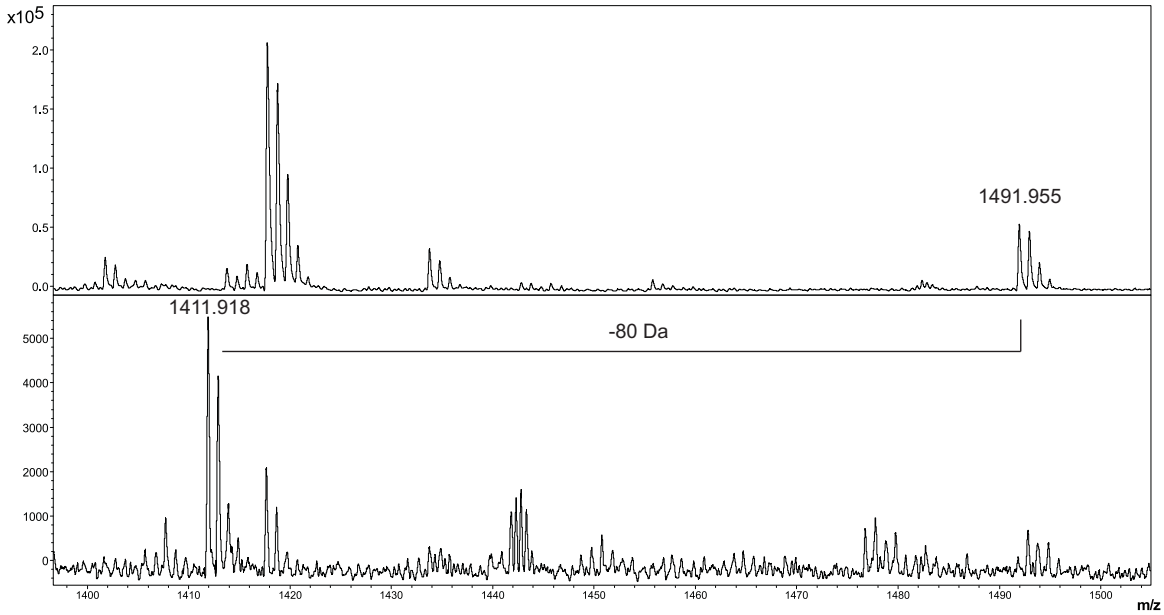
#	b	Seq.	y	y*	#
1	72.0444	A			12
2	141.0658	S	1248.586	1231.559	11
3	240.1343	V	1179.564	1162.538	10
4	341.1819	T	1080.496	1063.469	9
5	470.2245	E	979.4479	962.4214	8
6	599.2671	E	850.4054	833.3788	7
7	686.2992	S	721.3628	704.3362	6
8	904.3683	W	634.3307	617.3042	5
9	1017.452	L	416.2616	399.235	4
10	1074.474	G	303.1775	286.151	3
11	1145.511	A	246.1561	229.1295	2
12		R	175.119	158.0924	1

Monoisotopic mass of neutral peptide Mr(calc): 1416.5922
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
S2 : Phospho (ST), with neutral losses 97.9769(shown in table), 0.0000
W8 : Dioxidation (W)
Ions Score: 79 Expect: 1.2e-06
Matches (Highlited): 12/219 fragment ions using 18 most intense peaks

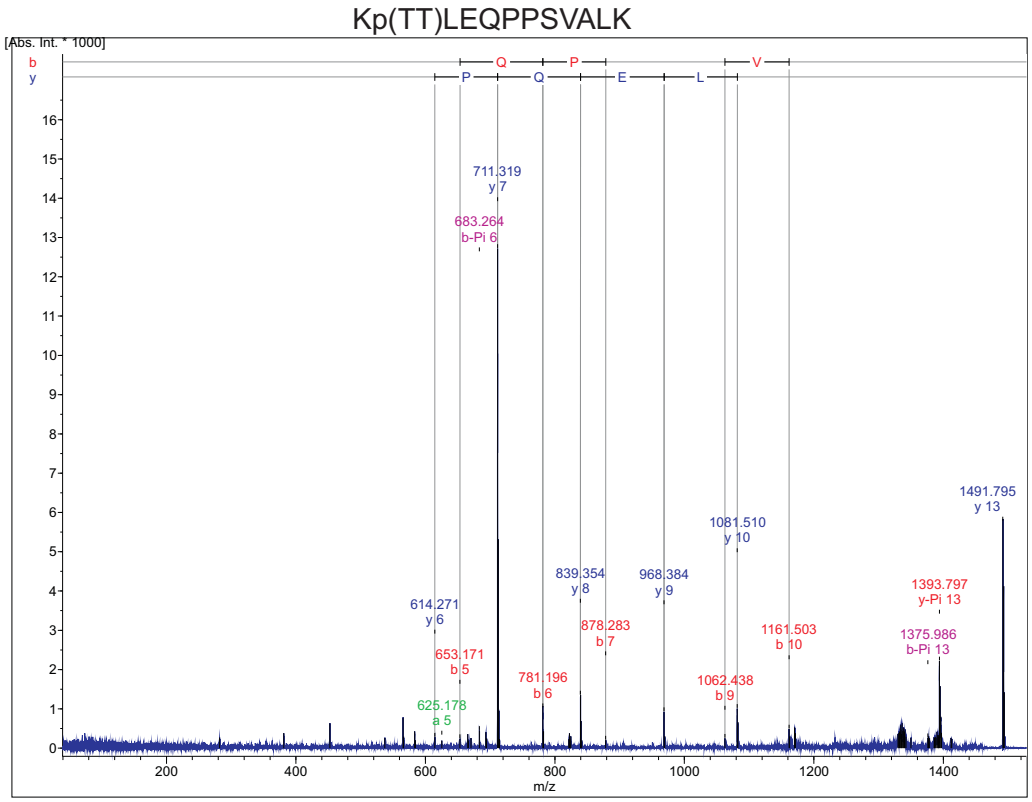
Part A: mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation. Mascot search results and ion table.

Supplementary image 1.5: PKA phosphorylated NFAT *m/z* 1491.54

A)



B)



C)

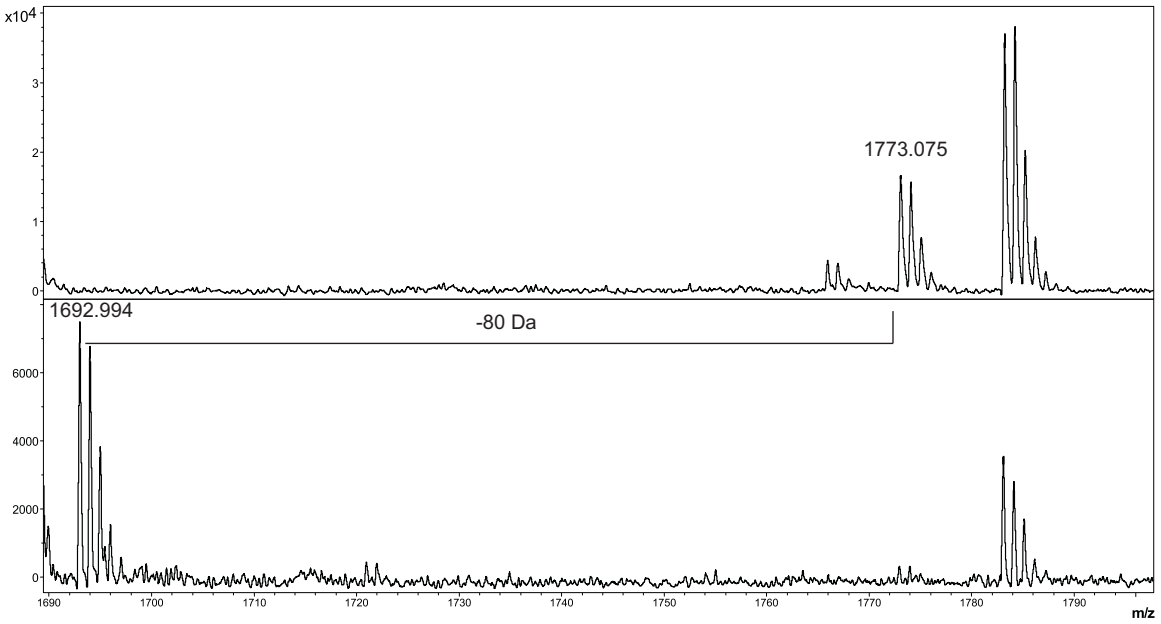
Monoisotopic mass of neutral peptide Mr(calc): 1490.7745
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
T3 : Phospho (ST), with neutral losses 97.9769(shown in table), 0.0000
Ions Score: 24 Expect: 0.00035
Matches (highlighted): 11/285 fragment ions using 13 most intense peaks

#	b	b*	b^0	Seq.	y	y*	y^0	#
1	129.1022	112.0757		K				13
2	230.1499	213.1234	212.1394	T	1265.71	1248.6834	1247.6994	12
3	313.187	296.1605	295.1765	T	1164.6623	1147.6357	1146.6517	11
4	426.2711	409.2445	408.2605	L	**1081.6252**	1064.5986	1063.6146	10
5	555.3137	538.2871	537.3031	E	**968.5411**	951.5146	950.5306	9
6	**683.3723**	666.3457	665.3617	Q	**839.4985**	822.472	821.488	8
7	780.425	763.3985	762.4145	P	**711.44**	694.4134	693.4294	7
8	877.4778	860.4512	859.4672	P	614.3872	597.3606	596.3766	6
9	964.5098	947.4833	946.4993	S	517.3344	500.3079	499.3239	5
10	1063.5782	1046.5517	1045.5677	V	430.3024	413.2758		4
11	1134.6153	1117.5888	1116.6048	A	331.234	314.2074		3
12	1247.6994	1230.6729	1229.6888	L	260.1969	243.1703		2
13				K	147.1128	130.0863		1

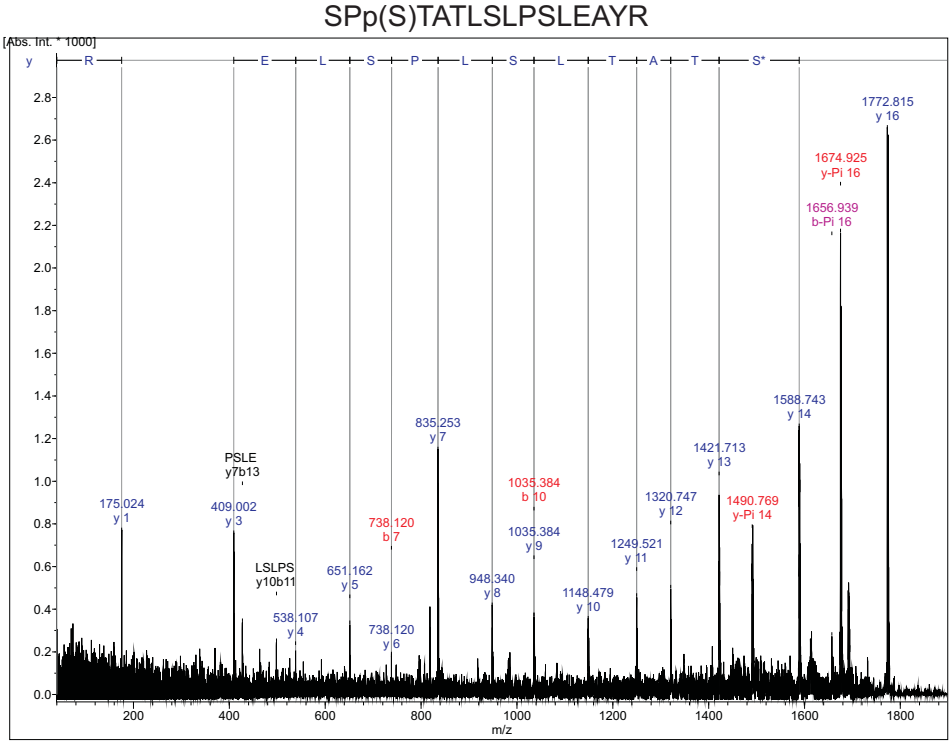
Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation from Biotools. Part C: Mascot search results and ion table.

Supplementary image 1.6: PKA phosphorylated NFAT *m/z* 1772.82

A)



B)



C)

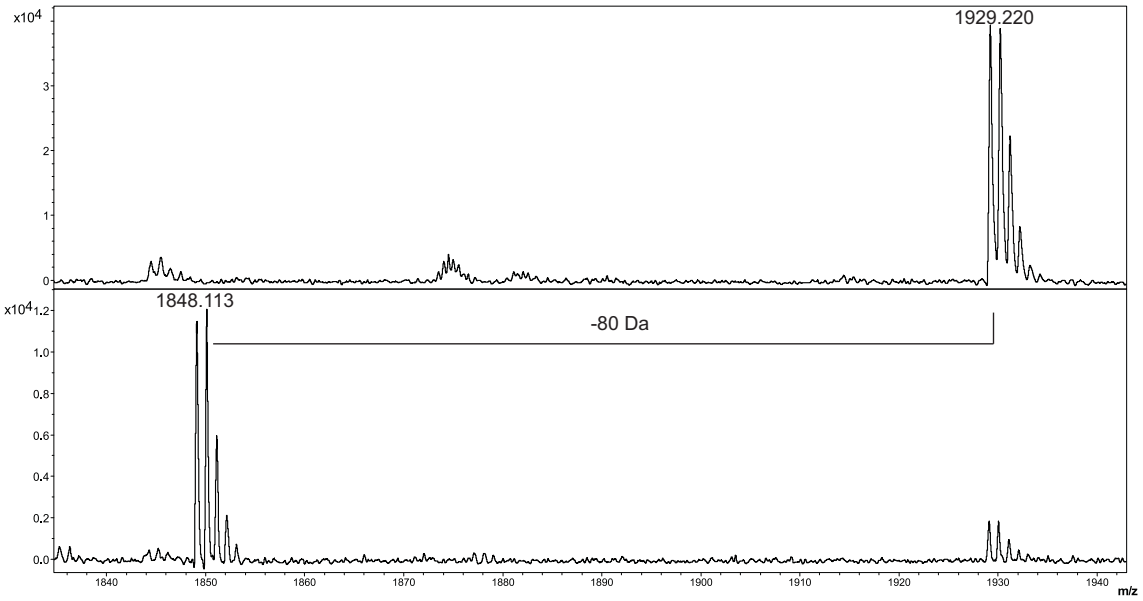
Monoisotopic mass of neutral peptide Mr(calc): 1771.8393
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
S3 : Phospho (ST), with neutral losses 97.9769(shown in table), 0.0000
Ions Score: 109 Expect: 1.4e-12
Matches (highlighted): 25/353 fragment ions using 29 most intense peaks

#	b	b^0	Seq.	y	y*	y^0	#
1	88.0393	70.0287	S				16
2	185.0921	167.0815	P	1587.8377	1570.8111	1569.8271	15
3	254.1135	236.103	S	**1490.7849**	1473.7584	1472.7744	14
4	355.1612	337.1506	T	**1421.7635**	1404.7369	1403.7529	13
5	426.1983	408.1878	A	**1320.7158**	1303.6892	1302.7052	12
6	527.246	509.2354	T	**1249.6787**	1232.6521	1231.6681	11
7	640.3301	622.3195	L	**1148.631**	1131.6045	1130.6204	10
8	727.3621	709.3515	S	**1035.5469**	1018.5204	1017.5364	9
9	840.4462	822.4356	L	**948.5149**	931.4884	930.5043	8
10	937.4989	919.4884	P	**835.4308**	**818.4043**	817.4203	7
11	1024.5309	1006.5204	S	**738.3781**	721.3515	720.3675	6
12	1137.615	1119.6044	L	**651.3461**	634.3195	633.3355	5
13	1266.6576	1248.647	E	**538.262**	521.2354	520.2514	4
14	1337.6947	1319.6841	A	**409.2194**	392.1928		3
15	1500.758	1482.7475	Y	338.1823	321.1557		2
16			R	**175.119**	158.0924		1

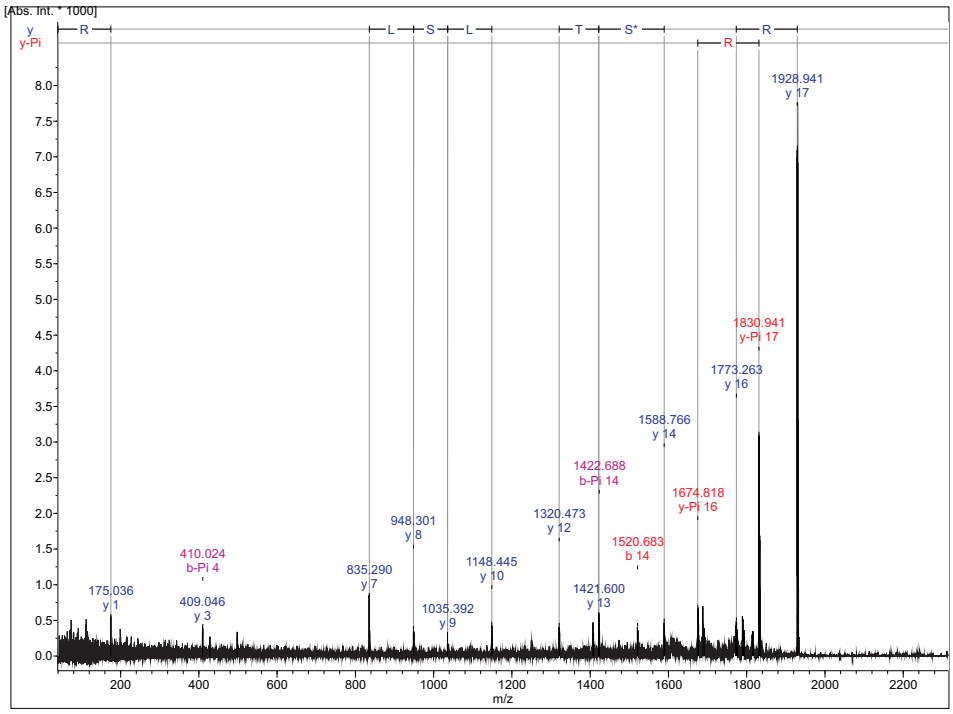
Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation from Biotools. Part C: Mascot search results and ion table.

Supplementary image 1.7: PKA phosphorylated NFAT *m/z* 1928.93

A)



B)



C)

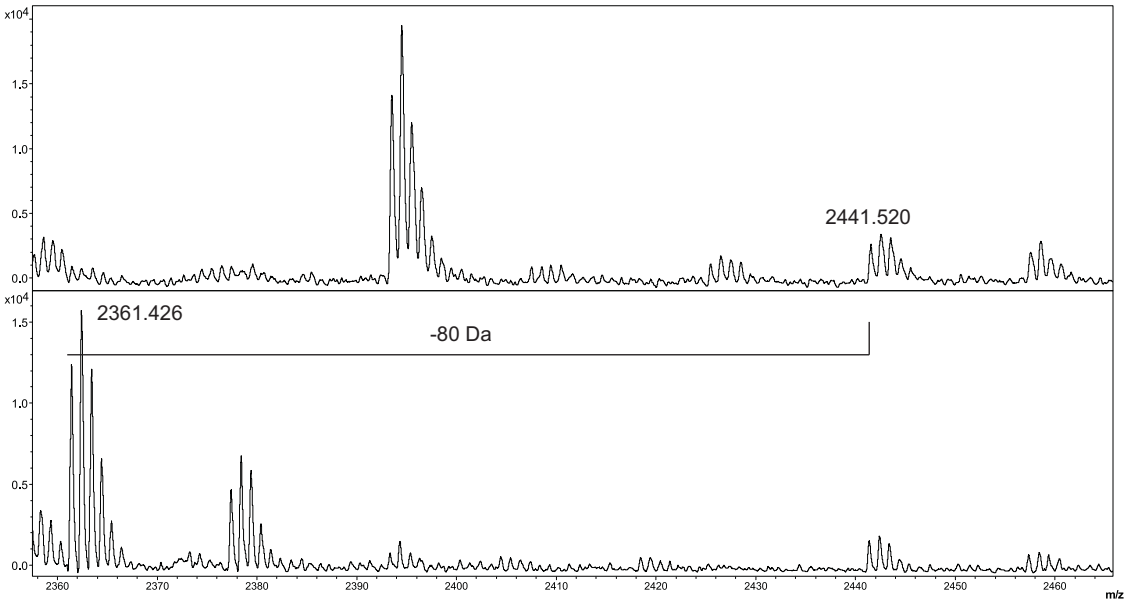
Monoisotopic mass of neutral peptide Mr(calc): 1927.9404
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
S4 : Phospho (ST), with neutral losses 97.9769(shown in table), 0.0000
Ions Score: 61 Expect: 7.9e-08
Matches (highlighted): 18/474 fragment ions using 24 most intense peaks

#	b	b*	b^0	Seq.	y	y*	y^0	#
1	157.1084	140.0818		R				17
2	244.1404	227.1139	226.1298	S	**1674.8697**	1657.8432	1656.8592	16
3	341.1932	324.1666	323.1826	P	1587.8377	1570.8111	1569.8271	15
4	**410.2146**	393.1881	392.2041	S	1490.7849	1473.7584	1472.7744	14
5	511.2623	494.2358	493.2517	T	**1421.7635**	1404.7369	1403.7529	13
6	582.2994	565.2729	564.2889	A	**1320.7158**	1303.6892	1302.7052	12
7	683.3471	666.3206	665.3365	T	1249.6787	1232.6521	1231.6681	11
8	796.4312	779.4046	778.4206	L	**1148.631**	1131.6045	1130.6204	10
9	883.4632	866.4366	865.4526	S	**1035.5469**	1018.5204	1017.5364	9
10	996.5473	979.5207	978.5367	L	**948.5149**	931.4884	930.5043	8
11	1093.6	1076.5735	1075.5895	P	**835.4308**	818.4043	817.4203	7
12	1180.6321	1163.6055	1162.6215	S	738.3781	721.3515	720.3675	6
13	1293.7161	1276.6896	1275.7056	L	651.3461	634.3195	633.3355	5
14	**1422.7587**	1405.7322	1404.7481	E	538.262	521.2354	520.2514	4
15	1493.7958	1476.7693	1475.7853	A	**409.2194**	392.1928		3
16	1656.8592	1639.8326	1638.8486	Y	338.1823	321.1557		2
17				R	**175.119**	158.0924		1

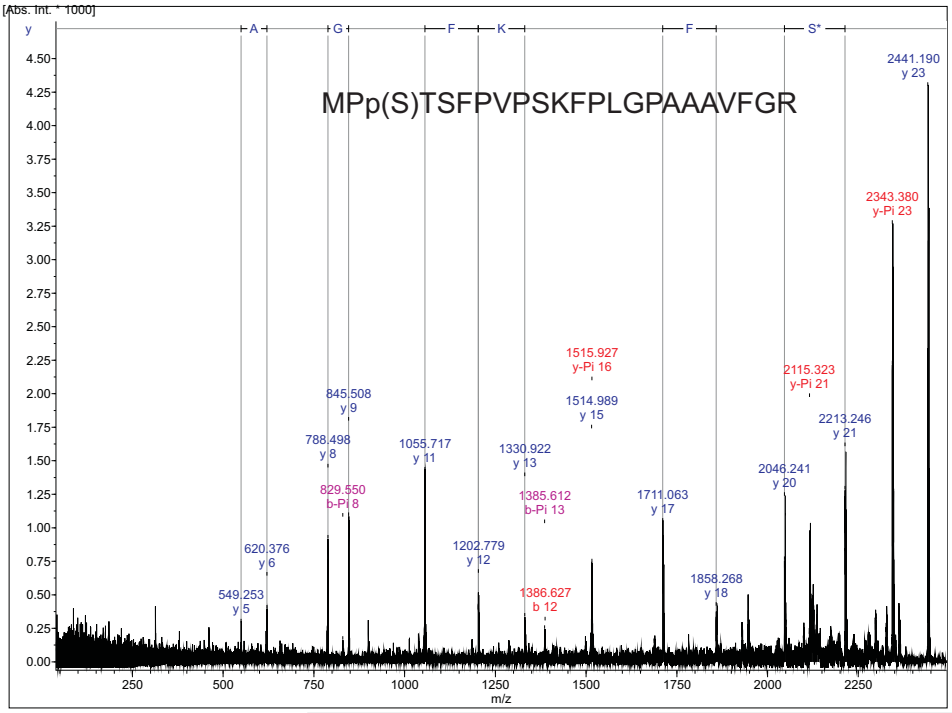
Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation from Biotools. Part C: Mascot search results and ion table.

Supplementary image 1.8: PKA phosphorylated NFAT *m/z* 2441.19

A)



B)



C)

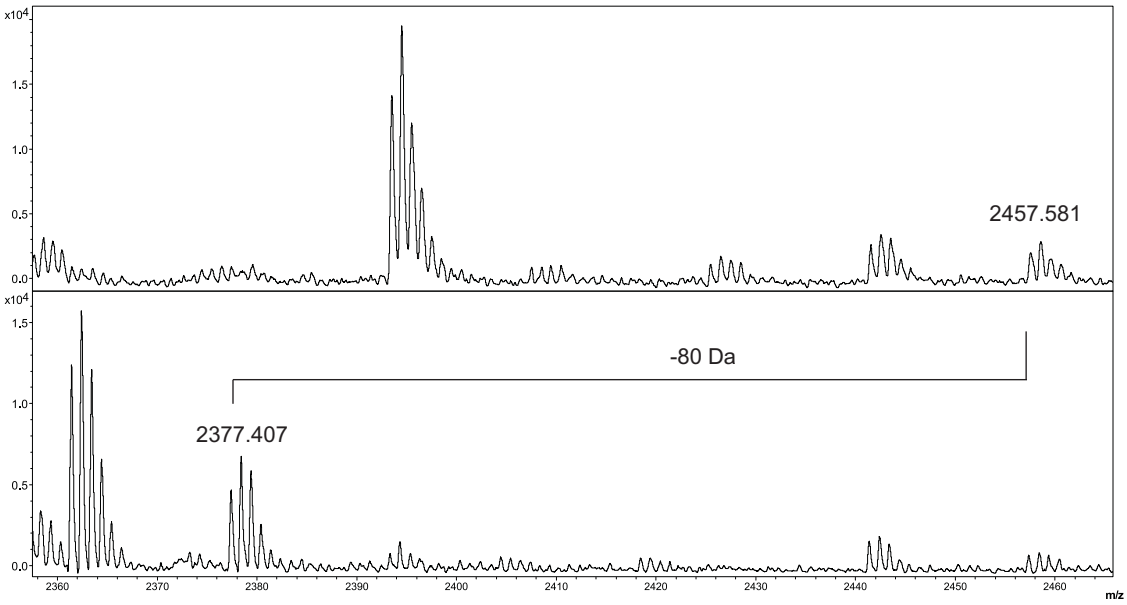
Monoisotopic mass of neutral peptide Mr(calc): 2440.2014
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
S3 : Phospho (ST), with neutral losses 97.9769(shown in table), 0.0000
Ions Score: 53 Expect: 5e-07
Matches (highlighted): 29/560 fragment ions using 50 most intense peaks

#	b	b*	b^0	Seq.	y	y*	y^0	#
1	132.0478			M				23
2	229.1005			P	2212.1913	2195.1648	2194.1808	22
3	298.122		280.1114	S	**2115.1386**	2098.112	2097.128	21
4	399.1697		381.1591	T	**2046.1171**	2029.0906	2028.1065	20
5	486.2017		468.1911	S	1945.0694	1928.0429	1927.0589	19
6	633.2701		615.2595	F	**1858.0374**	1841.0109	1840.0268	18
7	730.3229		712.3123	P	**1710.969**	1693.9424	1692.9584	17
8	829.3913		811.3807	V	1613.9162	1596.8897	1595.9057	16
9	926.444		908.4335	P	**1514.8478**	1497.8213	1496.8372	15
10	1013.4761		995.4655	S	1417.795	1400.7685	1399.7845	14
11	1141.571	1124.5445	1123.5605	K	**1330.763**	1313.7365		13
12	1288.6394	1271.6129	1270.6289	F	**1202.6681**	1185.6415		12
13	**1385.6922**	1368.6657	1367.6816	P	**1055.5996**	1038.5731		11
14	1498.7763	1481.7497	1480.7657	L	958.5469	941.5203		10
15	1555.7977	1538.7712	1537.7872	G	**845.4628**	828.4363		9
16	1652.8505	1635.824	1634.8399	P	**788.4413**	771.4148		8
17	1723.8876	1706.8611	1705.8771	A	691.3886	674.362		7
18	1794.9247	1777.8982	1776.9142	A	**620.3515**	603.3249		6
19	1865.9618	1848.9353	1847.9513	A	**549.3144**	532.2878		5
20	1965.0303	**1948.0037**	**1947.0197**	V	478.2772	461.2507		4
21	2112.0987	2095.0721	2094.0881	F	379.2088	362.1823		3
22	2169.1201	2152.0936	2151.1096	G	232.1404	215.1139		2
23				R	175.119	158.0924		1

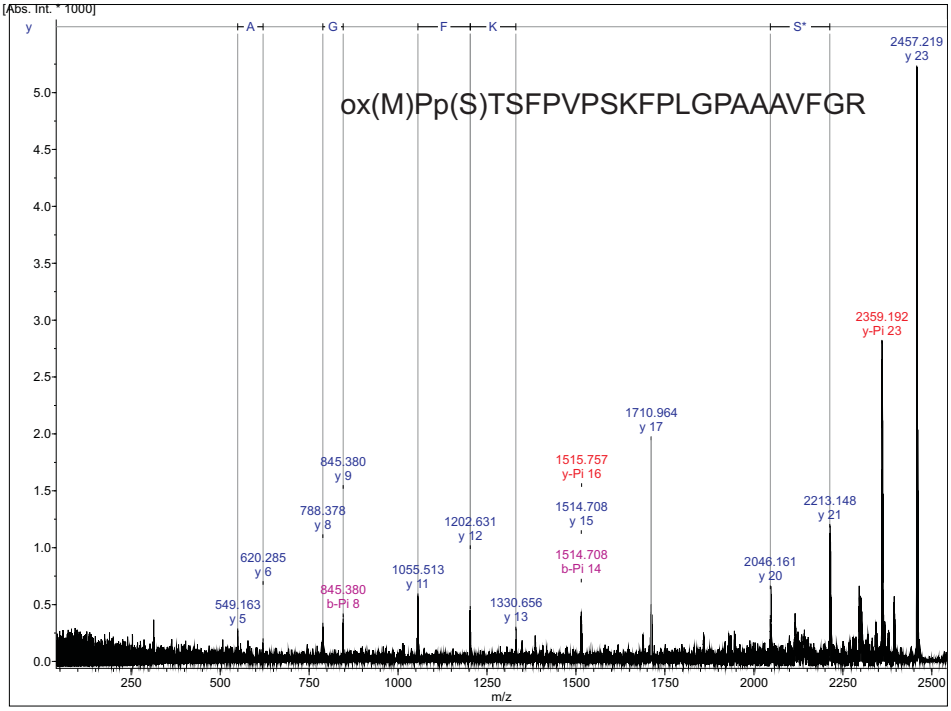
Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation from Biotools. Part C: Mascot search results and ion table.

Supplementary image 1.9: PKA phosphorylated NFAT *m/z* 2457.22

A)



B)



C)

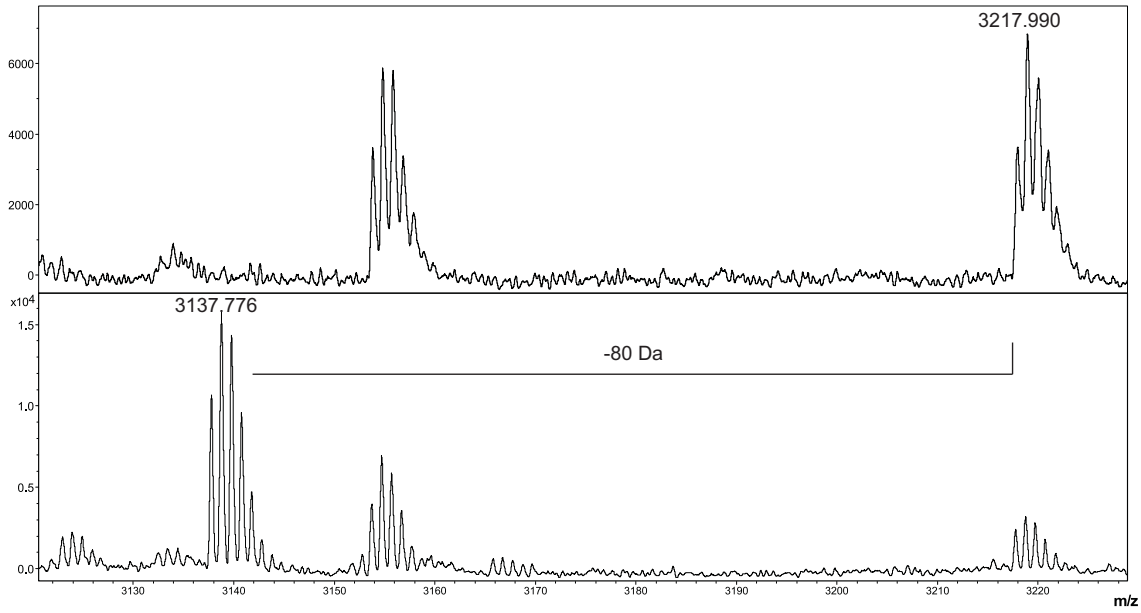
Monoisotopic mass of neutral peptide Mr(calc): 2456.1964
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
M1 : Oxidation (M), with neutral losses 63.9983(shown in table), 0.0000
S3 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
Ions Score: 51 Expect: 7.7e-07
Matches (highlighted): 26/765 fragment ions using 37 most intense peaks

#	b	b*	b^0	Seq.	y	y*	y^0	#
1	84.0444			M				23
2	181.0972			P	2310.1682	2293.1417	2292.1577	22
3	348.0955		330.0849	S	**2213.1155**	2196.0889	2195.1049	21
4	449.1432		431.1326	T	**2046.1171**	2029.0906	2028.1065	20
5	536.1752		518.1647	S	1945.0694	1928.0429	1927.0589	19
6	683.2436		665.2331	F	1858.0374	1841.0109	1840.0268	18
7	780.2964		762.2858	P	**1710.969**	1693.9424	1692.9584	17
8	879.3648		861.3542	V	1613.9162	1596.8897	1595.9057	16
9	976.4176		958.407	P	**1514.8478**	1497.8213	1496.8372	15
10	1063.4496		1045.439	S	1417.795	1400.7685	1399.7845	14
11	1191.5446	1174.518	1173.534	K	**1330.763**	1313.7365		13
12	1338.613	1321.5864	1320.6024	F	**1202.6681**	1185.6415		12
13	1435.6657	1418.6392	1417.6552	P	**1055.5996**	1038.5731		11
14	1548.7498	1531.7233	1530.7392	L	958.5469	941.5203		10
15	1605.7713	1588.7447	1587.7607	G	**845.4628**	828.4363		9
16	1702.824	1685.7975	1684.8135	P	**788.4413**	771.4148		8
17	1773.8611	1756.8346	1755.8506	A	691.3886	674.362		7
18	1844.8983	1827.8717	1826.8877	A	**620.3515**	603.3249		6
19	1915.9354	1898.9088	1897.9248	A	**549.3144**	532.2878		5
20	2015.0038	1997.9772	1996.9932	V	478.2772	461.2507		4
21	2162.0722	2145.0457	2144.0616	F	379.2088	362.1823		3
22	2219.0937	2202.0671	2201.0831	G	232.1404	215.1139		2
23				R	175.119	158.0924		1

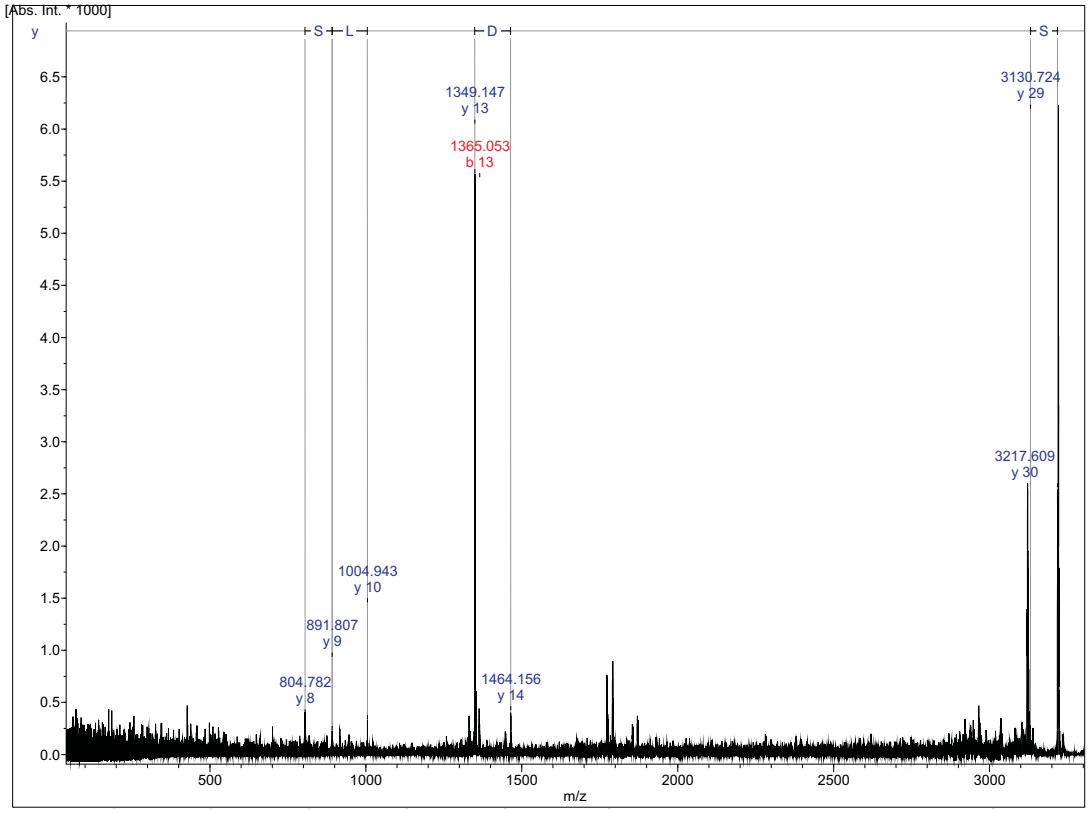
Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation from Biotools. Part C: Mascot search results and ion table.

Supplementary image 1.10: PKA phosphorylated NFAT *m/z* 3217.62

A)



B)



C)

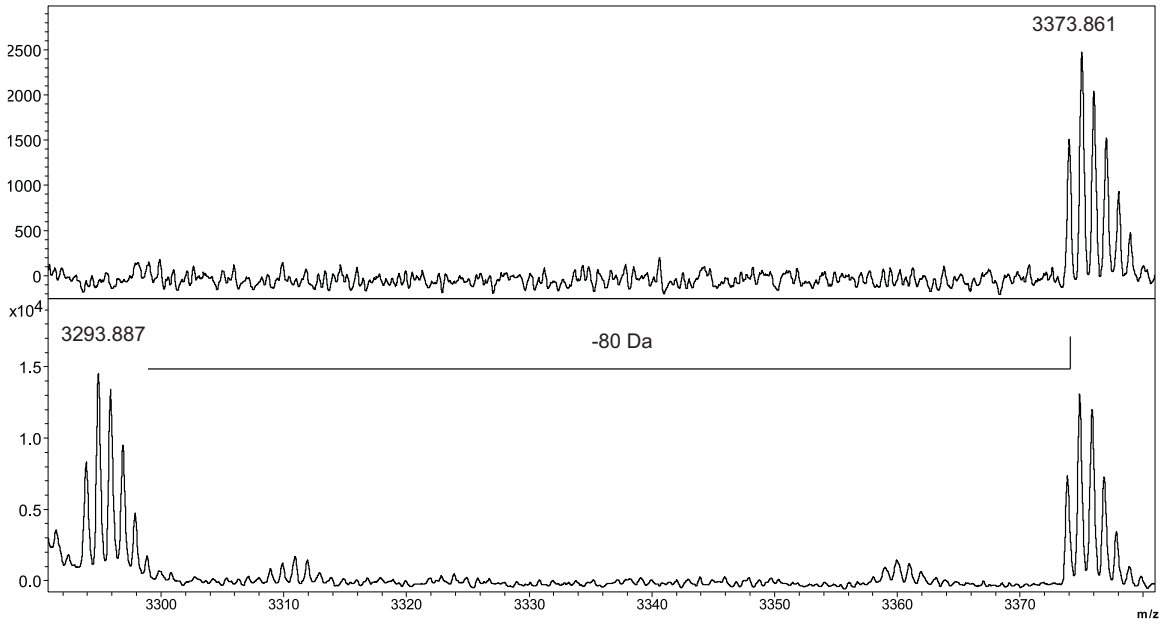
Top scoring peptides matches to query 1
Score graterh than 13 indicates identity

Score	Expect	ppm	Hit	Protein	Peptide
15.9	0.026	33.7	1	GST-NFAT	SPSTATLp(S)LPSLEAYRDPSCLSPASSLSSR
15.9	0.026	33.7	1	GST-NFAT	SPSTAp(T)LSLPSLEAYRDPSCLSPASSLSSR
15.9	0.026	33.7	1	GST-NFAT	SPSp(T)ATLSLPSLEAYRDPSCLSPASSLSSR
15.9	0.026	33.7	1	GST-NFAT	SPp(S)TATLSLPSLEAYRDPSCLSPASSLSSR
13.2	0.048	33.7	1	GST-NFAT	SPSTATLSLPp(S)LEAYRDPSCLSPASSLSSR
11	0.08	33.7	1	GST-NFAT	p(S)PSTATLSLPSLEAYRDPSCLSPASSLSSR

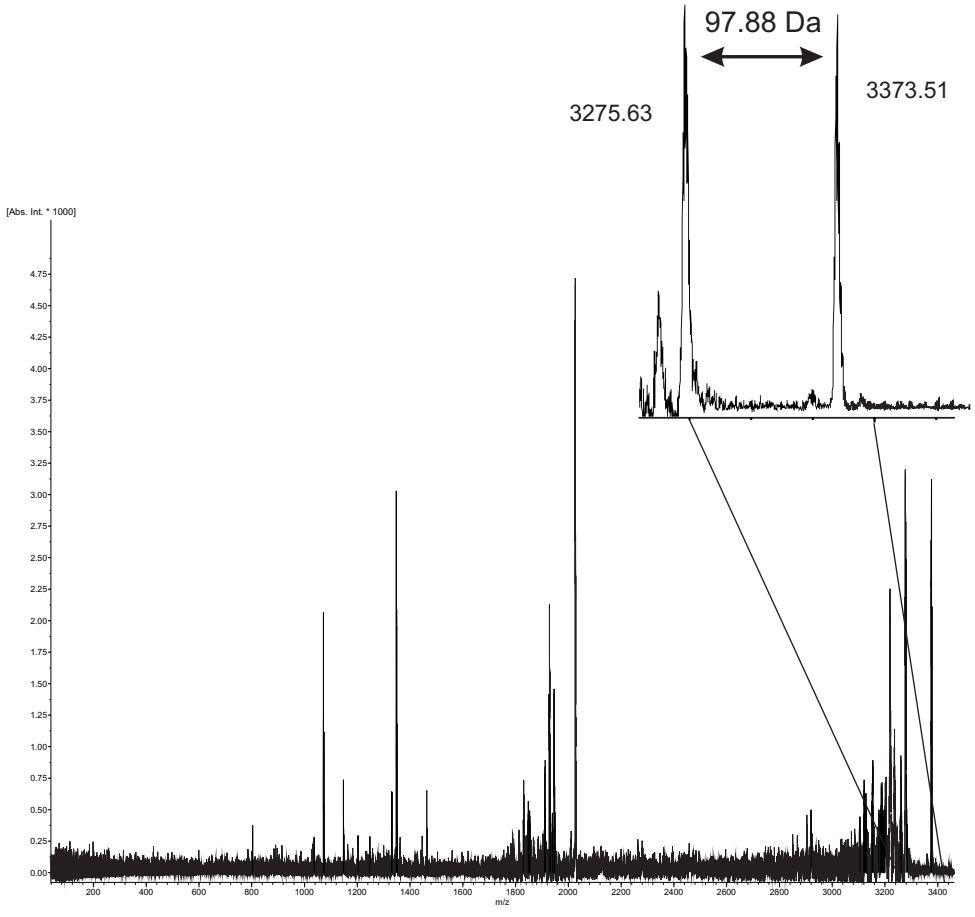
Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation from Biotools. Part C: Mascot search results and ion table.

Supplementary image 1.11: PKA phosphorylated NFAT *m/z* 3373.86

A)



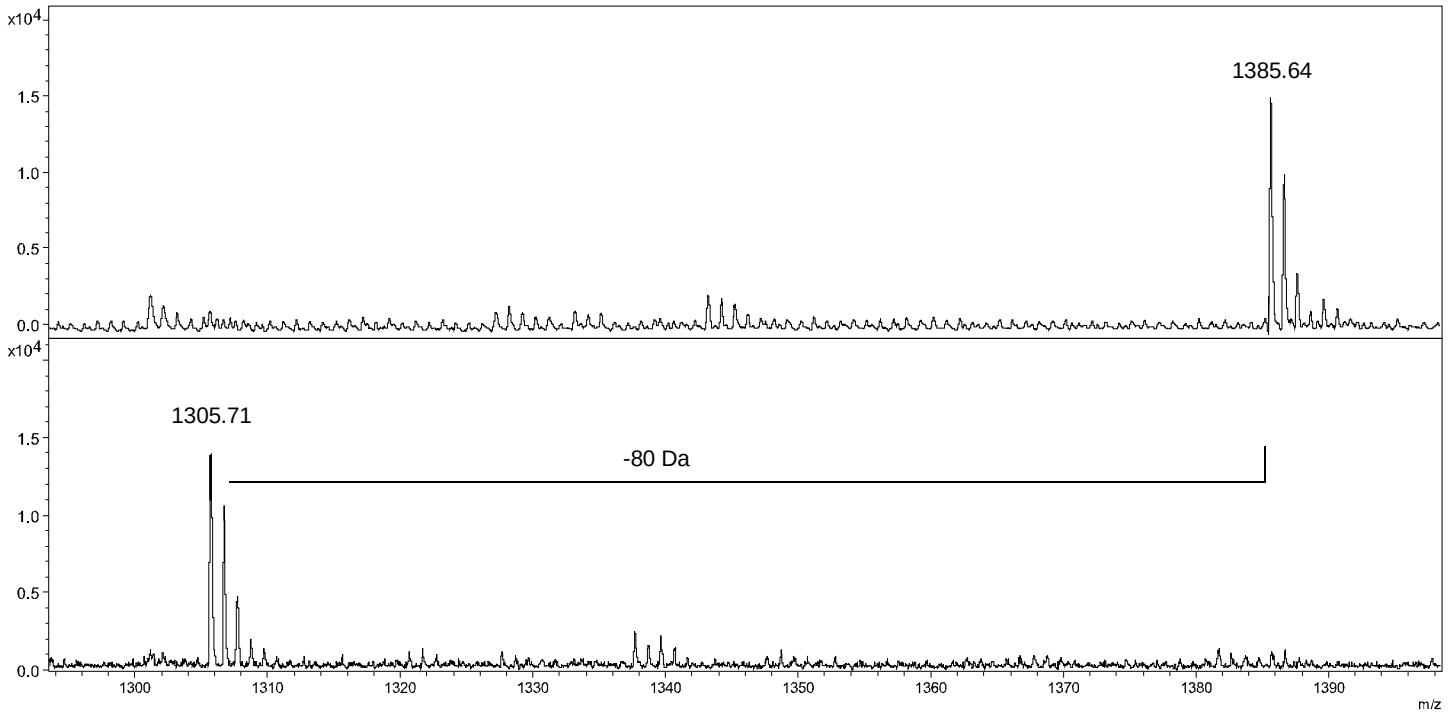
B)



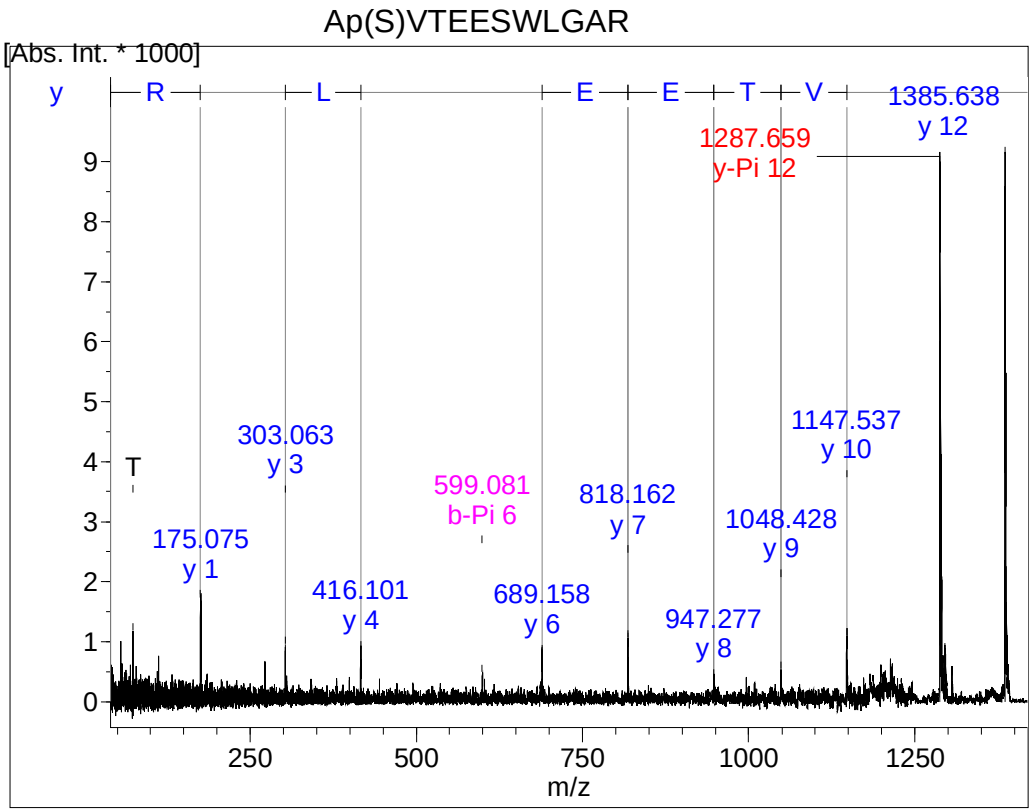
Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and indication of neutral loss.

Supplementary image 2.1: *in vivo* phosphorylated NFAT *m/z* 1385.63

A)



B)



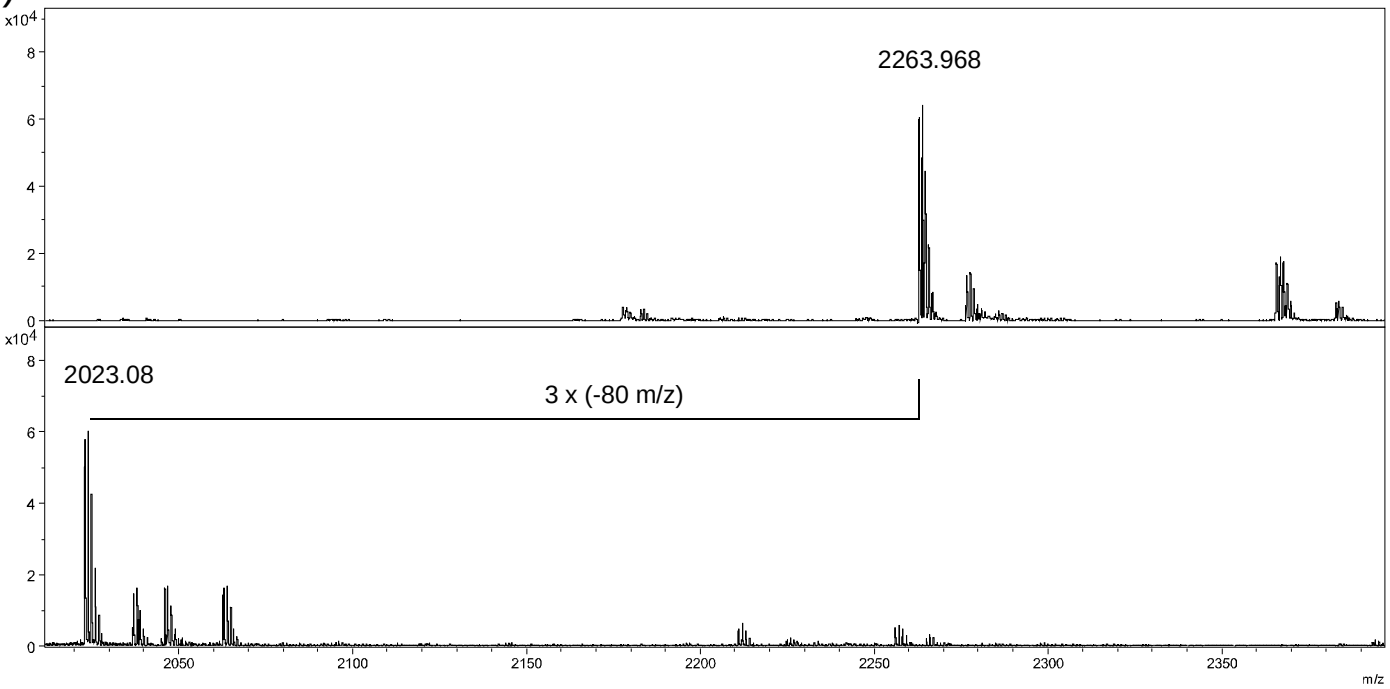
#	b	b^0	Seq.	y	y*	y^0	#
1	72.0444		A				12
2	141.0658	123.0553	S	1216.5957	1199.5691	1198.5851	11
3	240.1343	222.1237	V	1147.5742	1130.5477	1129.5636	10
4	341.1819	323.1714	T	1048.5058	1031.4793	1030.4952	9
5	470.2245	452.214	E	947.4581	930.4316	929.4476	8
6	599.2671	581.2566	E	818.4155	801.389	800.405	7
7	686.2992	668.2886	S	689.3729	672.3464	671.3624	6
8	872.3785	854.3679	W	602.3409	585.3144		5
9	985.4625	967.452	L	416.2616	399.235		4
10	1042.484	1024.4734	G	303.1775	286.151		3
11	1113.5211	1095.5105	A	246.1561	229.1295		2
12			R	175.119	158.0924		1

Monoisotopic mass of neutral peptide Mr(calc): 1384.6024
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
S2 : Phospho (ST), with neutral losses 97.9769(shown in table), 0.0000
Ions Score: 77 Expect: 4.1e-05
Matches (Bold Red): 12/220 fragment ions using 12 most intense peaks

Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation from Biotools and ion table from Mascot.

Supplementary image 2.2: *in vivo* phosphorylated NFAT *m/z* 2262.96

A)

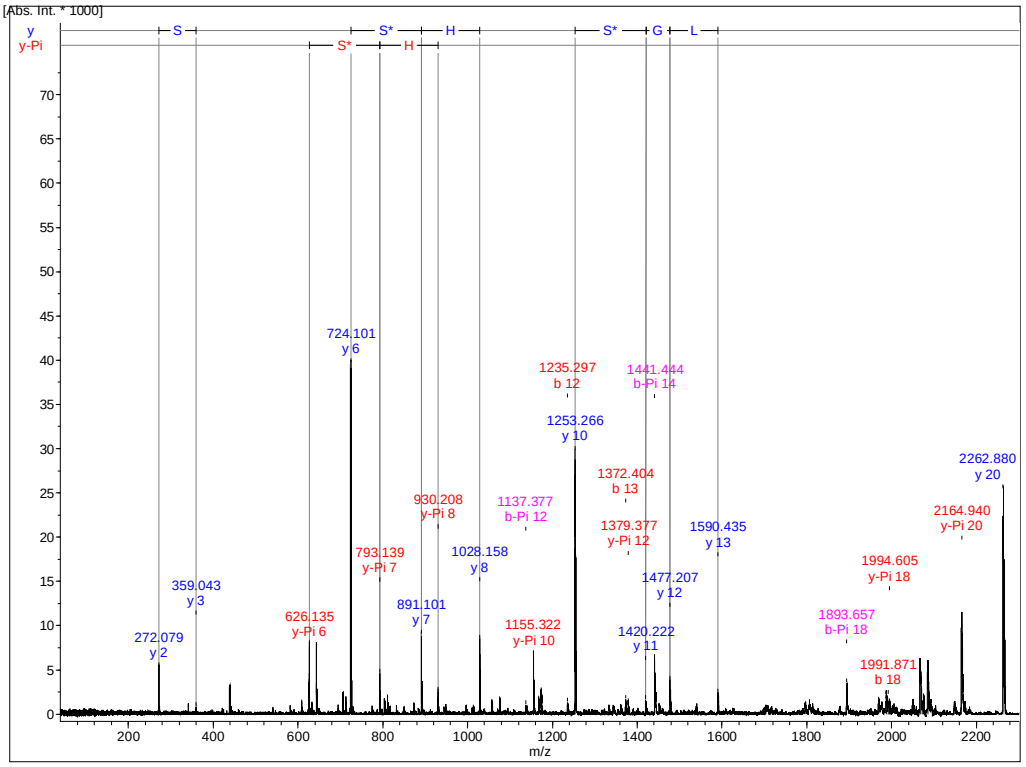


B)

224-GLGAcarb(C)TLLGp(S)PQHp(S)Pp(S)TSPR-243

Monoisotopic mass of neutral peptide Mr(calc): 2261.8943
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
S10 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
S14 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
S16 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
Ions Score: 31 Expect: 18
Matches (highlited): 23/532 fragment ions using 46 most intense peaks

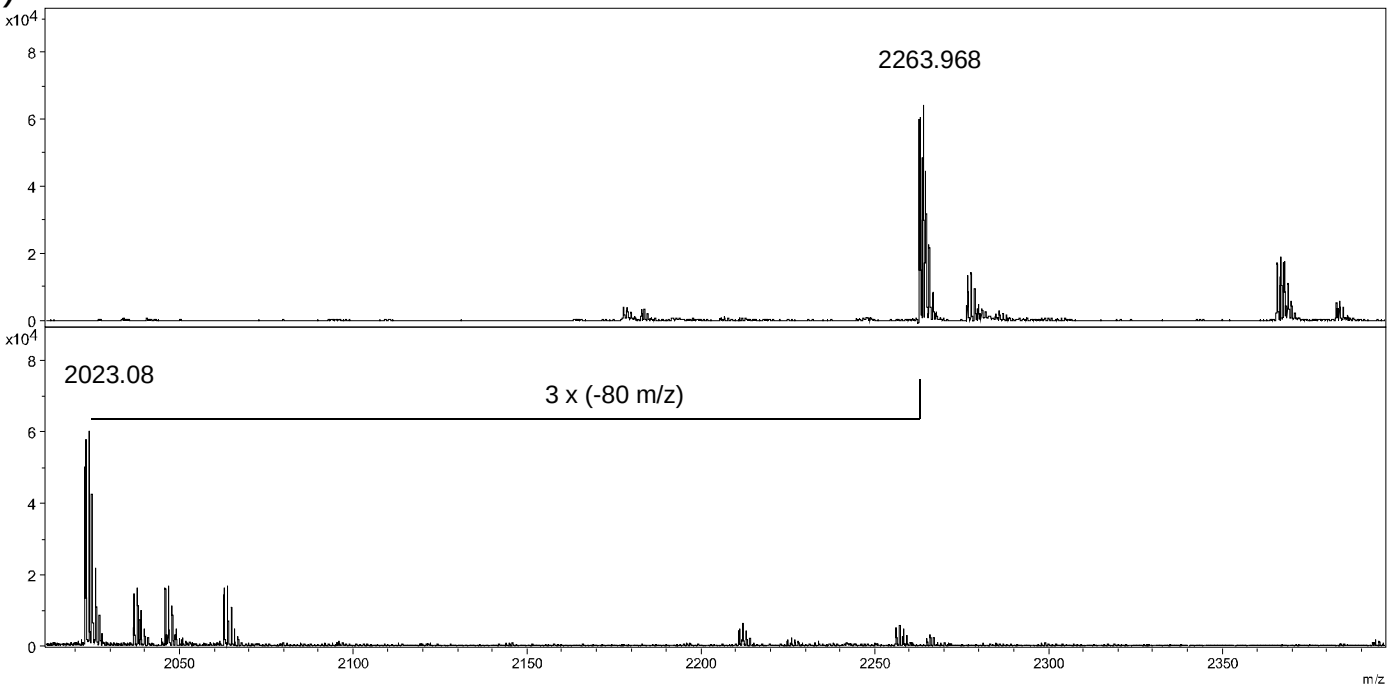
#	b	Seq.	y	y^0	#
1	58.0287	G			20
2	171.1128	L	2205.88	2187.87	19
3	228.1343	G	2092.796	2074.785	18
4	299.1714	A	2035.775	2017.764	17
5	459.202	C	1964.737	1946.727	16
6	560.2497	T	1804.707	1786.696	15
7	673.3338	L	1703.659	1685.649	14
8	786.4178	L	1590.575	1572.565	13
9	843.4393	G	1477.491	1459.48	12
10	1010.438	S	1420.47	1402.459	11
11	1107.49	P	1253.471	1235.461	10
12	1235.549	Q	1156.418	1138.408	9
13	1372.608	H	1028.36	1010.349	8
14	1539.606	S	891.3009	873.2903	7
15	1636.659	P	724.3025	706.292	6
16	1803.657	S	627.2498	609.2392	5
17	1904.705	T	460.2514	442.2409	4
18	1991.737	S	359.2037	341.1932	3
19	2088.79	P	272.1717		2
20		R	175.119		1



Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation and ion table from Mascot.

Supplementary image 2.3: *in vivo* phosphorylated NFAT *m/z* 2262.96

A)

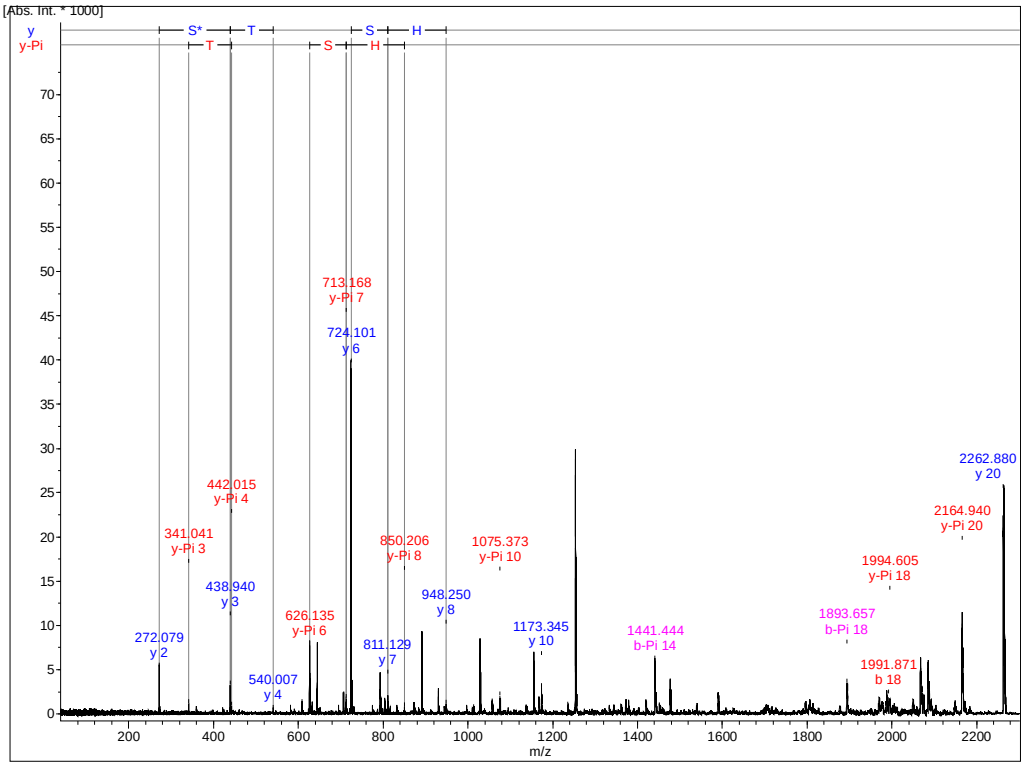


B)

224-GLGAcarb(C)p(T)LLGp(S)PQHSPSTp(S)PR-243

Monoisotopic mass of neutral peptide Mr(calc): 2261.8943
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
T6 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
S10 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
S18 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
Ions Score: 15 Expect: 7.2e+02
Matches (Highlited): 25/575 fragment ions using 35 most intense peaks

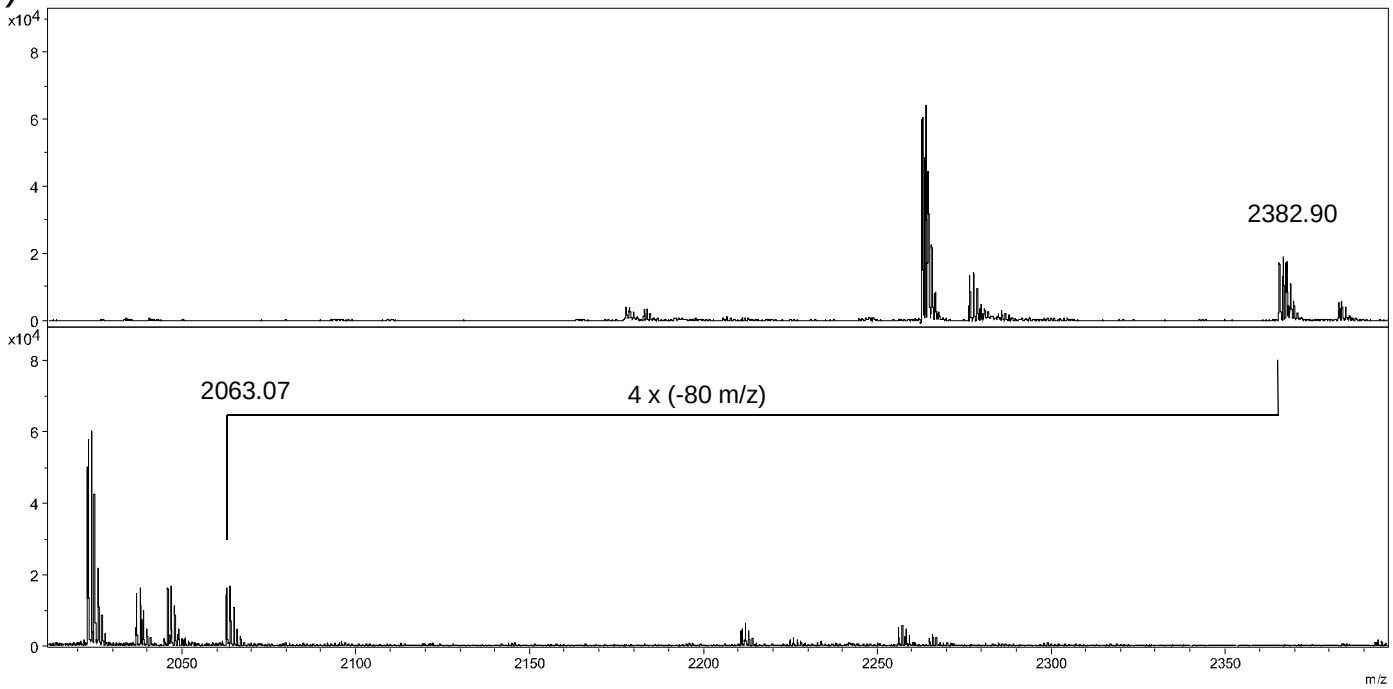
#	Seq.	y	y^0	#
1	G			20
2	L	2205.88	2187.87	19
3	G	2092.796	2074.785	18
4	A	2035.775	2017.764	17
5	C	1964.737	1946.727	16
6	T	1804.707	1786.696	15
7	L	1623.693	1605.682	14
8	L	1510.609	1492.598	13
9	G	1397.525	1379.514	12
10	S	1340.503	1322.493	11
11	P	1173.505	1155.494	10
12	Q	1076.452	1058.442	9
13	H	948.3935	930.3829	8
14	S	811.3346	793.324	7
15	P	724.3025	706.292	6
16	S	627.2498	609.2392	5
17	T	540.2177	522.2072	4
18	S	439.1701	421.1595	3
19	P	272.1717		2
20	R	175.119		1



Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum, peak annotation from Biotools and ion table from Mascot.

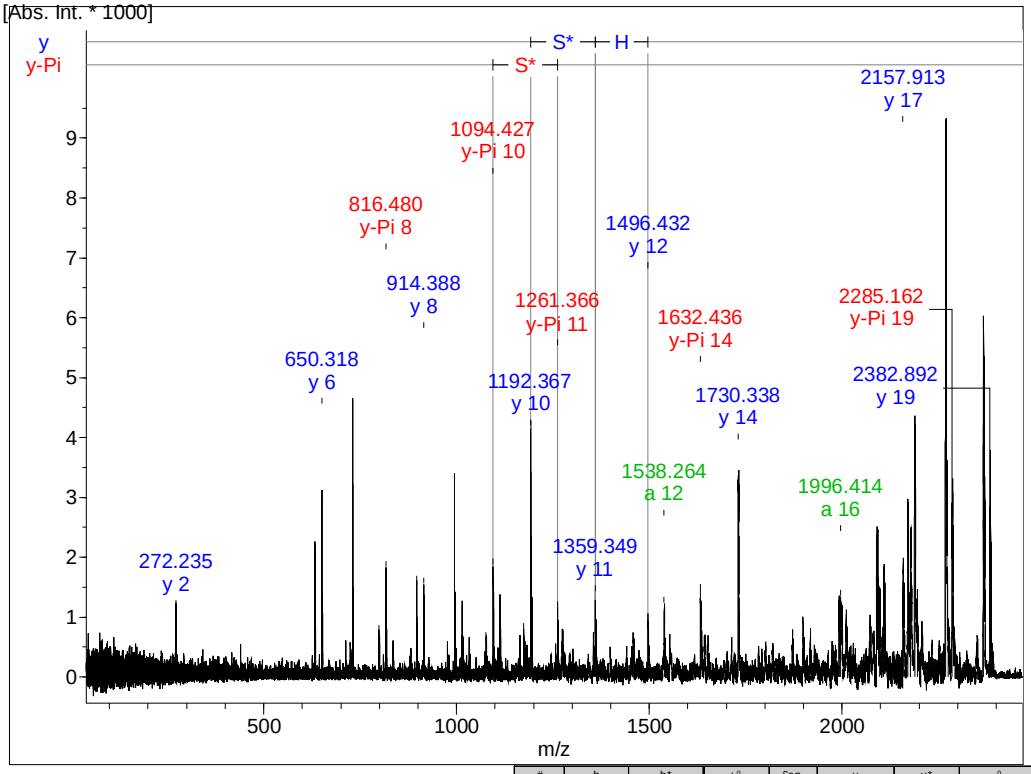
Supplementary image 2.4: *in vivo* phosphorylated NFAT *m/z* 2382.90

A)



B)

QPPYp(S)PHHp(S)Pp(T)Pp(S)PHGSPR



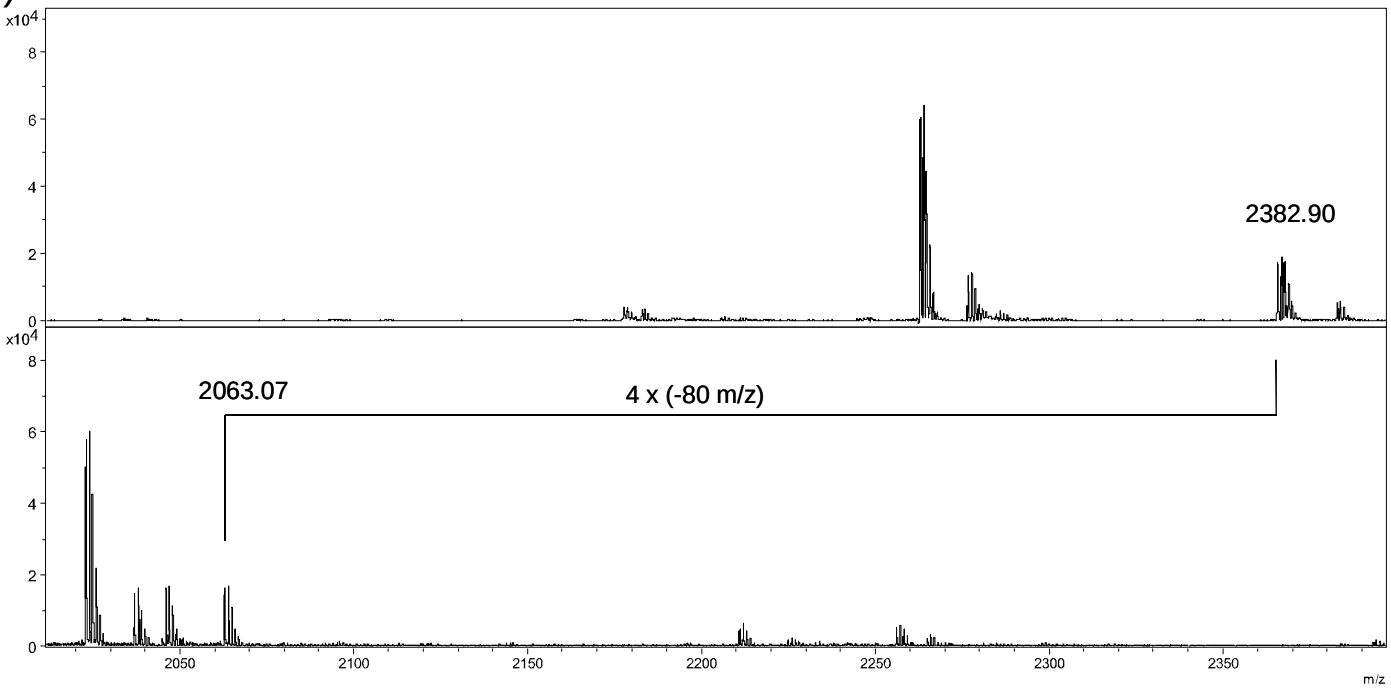
Monoisotopic mass of neutral peptide Mr(calc): 2381.8422
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
S5 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
S9 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
T11 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
S13 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
Ions Score: 26 Expect: 0.00054
Matches (highlighted): 17/546 fragment ions using 39 most intense peaks

#	b	b*	b^0	Seq.	y	y*	y^0	#
1	129.0659	112.0393		Q				19
2	226.1186	209.0921		P	2254.7909	2237.7644	2236.7804	18
3	323.1714	306.1448		P	2157.7382	2140.7116	2139.7276	17
4	486.2347	469.2082		Y	2060.6854	2043.6588	2042.6748	16
5	653.2331	636.2065	635.2225	S	1897.6221	1880.5955	1879.6115	15
6	750.2858	733.2593	732.2753	P	1730.6237	1713.5972	1712.6131	14
7	887.3447	870.3182	869.3342	H	1633.5709	1616.5444	1615.5604	13
8	1024.4037	1007.3771	1006.3931	H	1496.512	1479.4855	1478.5015	12
9	1191.402	1174.3755	1173.3914	S	1359.4531	1342.4266	1341.4426	11
10	1288.4548	1271.4282	1270.4442	P	1192.4548	1175.4282	1174.4442	10
11	1469.4688	1452.4422	1451.4582	T	1095.402	1078.3755	1077.3914	9
12	1566.5215	1549.495	1548.511	P	914.388	897.3615	896.3774	8
13	1733.5199	1716.4933	1715.5093	S	817.3352	800.3087	799.3247	7
14	1830.5727	1813.5461	1812.5621	P	650.3369	633.3103	632.3263	6
15	1967.6316	1950.605	1949.621	H	553.2841	536.2576	535.2736	5
16	2024.653	2007.6265	2006.6425	G	416.2252	399.1987	398.2146	4
17	2111.6851	2094.6585	2093.6745	S	359.2037	342.1772	341.1932	3
18	2208.7378	2191.7113	2190.7273	P	272.1717	255.1452		2
19				R	175.119	158.0924		1

Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation from Biotools and ion table from Mascot.

Supplementary image 2.5: *in vivo* phosphorylated NFAT *m/z* 2382.90

A)

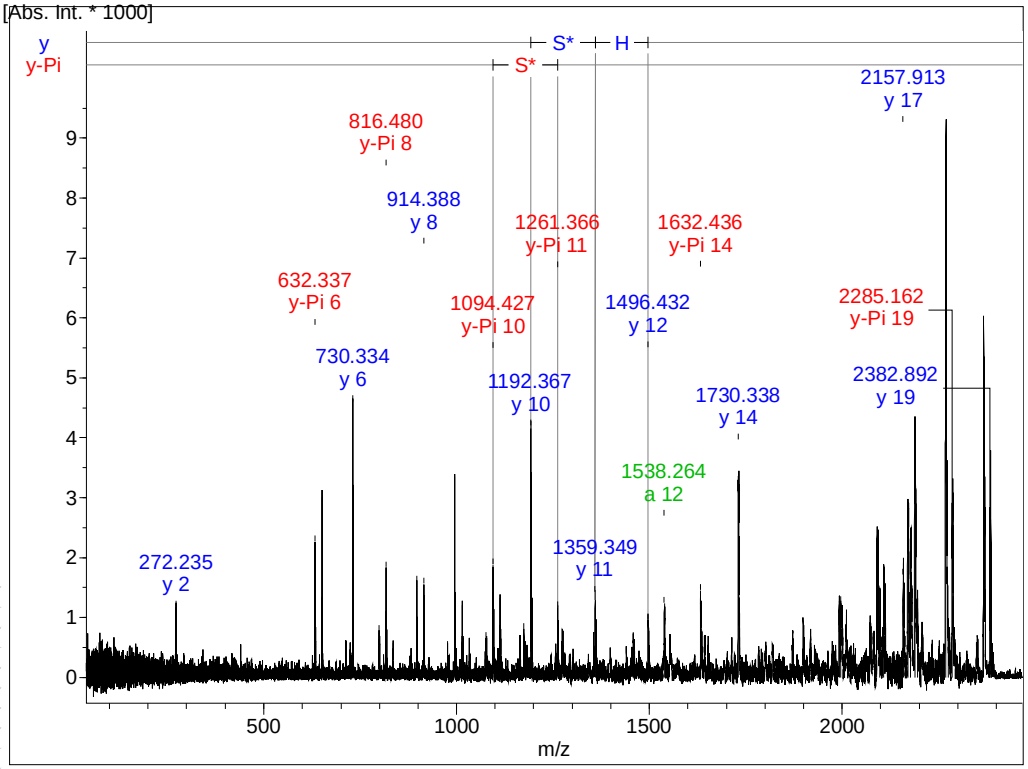


B)

QPPYp(S)PHHp(S)Pp(T)PSPHGp(S)PR

#	Seq.	y	y^0	#
1	Q			19
2	P	2254.7909	2236.7804	18
3	P	2157.7382	2139.7276	17
4	Y	2060.6854	2042.6748	16
5	S	1897.6221	1879.6115	15
6	P	1730.6237	1712.6131	14
7	H	1633.5709	1615.5604	13
8	H	1496.512	1478.5015	12
9	S	1359.4531	1341.4426	11
10	P	1192.4548	1174.4442	10
11	T	1095.402	1077.3914	9
12	P	914.388	896.3774	8
13	S	817.3352	799.3247	7
14	P	730.3032	712.2926	6
15	H	633.2504	615.2399	5
16	G	496.1915	478.181	4
17	S	439.1701	421.1595	3
18	P	272.1717		2
19	R	175.119		1

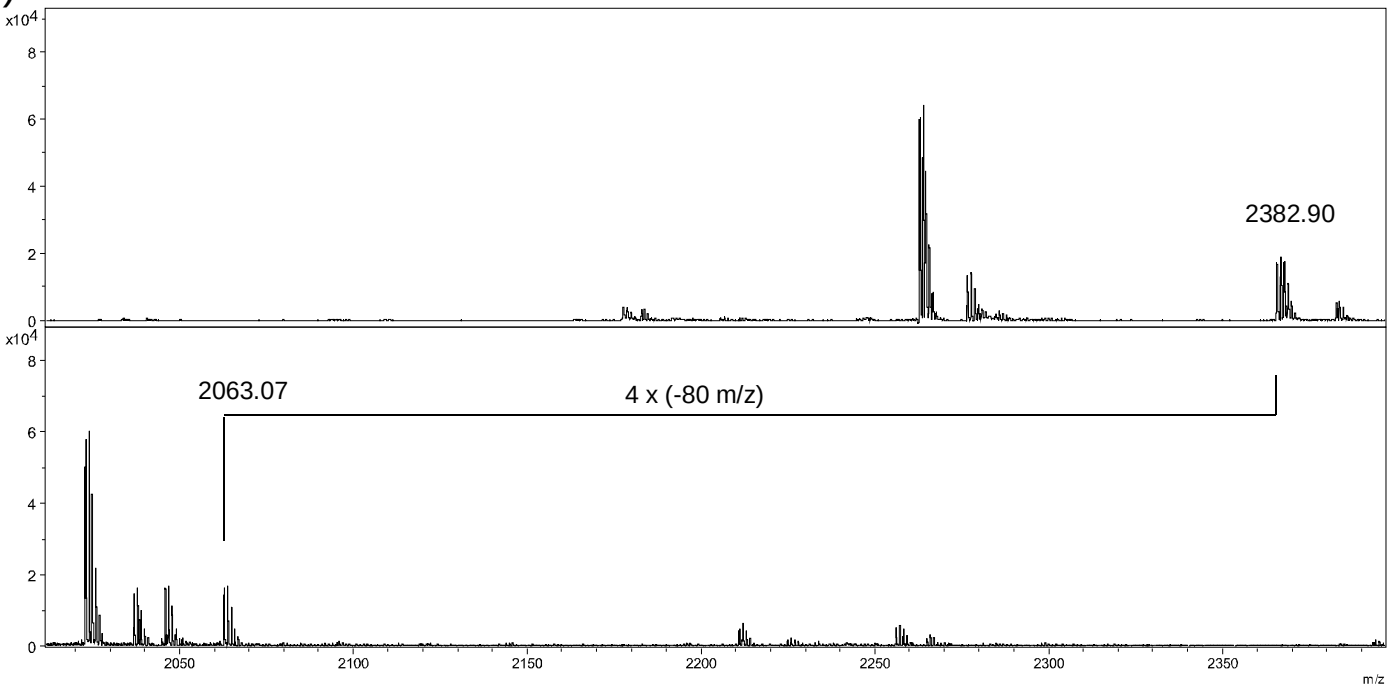
Monoisotopic mass of neutral peptide Mr(calc): 2381.8422
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
S5 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
S9 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
T11 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
S17 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
Ions Score: 23 Expect: 0.0011
Matches (highlighted): 18/575 fragment ions using 39 most intense peaks



Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation from Biotools and ion table from Mascot.

Supplementary image 2.6: *in vivo* phosphorylated NFAT *m/z* 2382.90

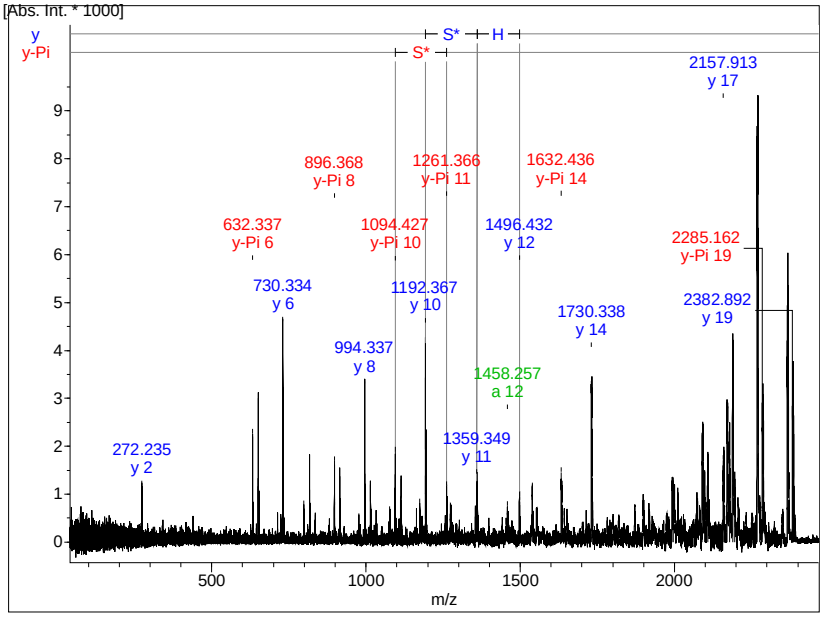
A)



B)

#	Seq.	y	#
1	Q		19
2	P	2254.7909	18
3	P	2157.7382	17
4	Y	2060.6854	16
5	S	1897.6221	15
6	P	1730.6237	14
7	H	1633.5709	13
8	H	1496.512	12
9	S	1359.4531	11
10	P	1192.4548	10
11	T	1095.402	9
12	P	994.3543	8
13	S	897.3016	7
14	P	730.3032	6
15	H	633.2504	5
16	G	496.1915	4
17	S	439.1701	3
18	P	272.1717	2
19	R	175.119	1

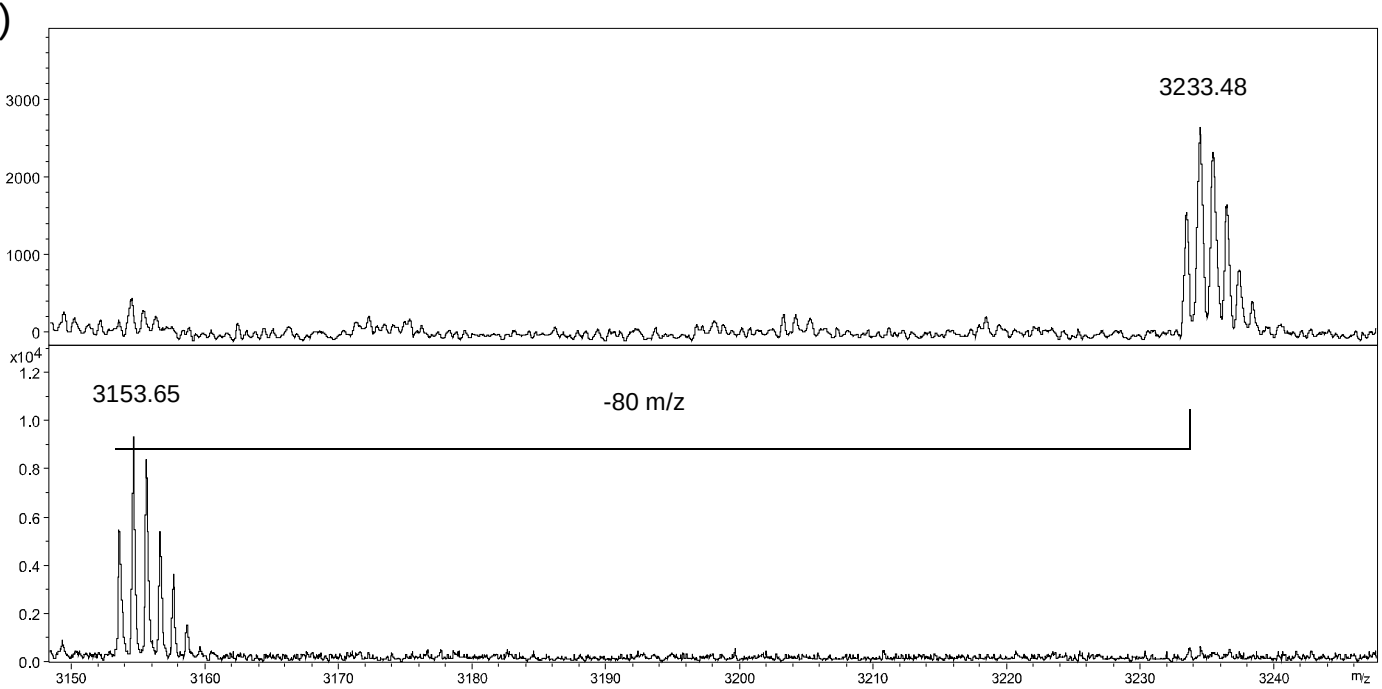
Variable modifications:
S5 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
S9 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
S13 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
S17 : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769
Ions Score: 23 Expect: 0.0011
Matches (highlighted): 12/581 fragment ions using 23 most intense peaks



Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation from Biotools and ion table from Mascot.

Supplementary image 2.7: *in vivo* phosphorylated NFAT *m/z* 3233.47

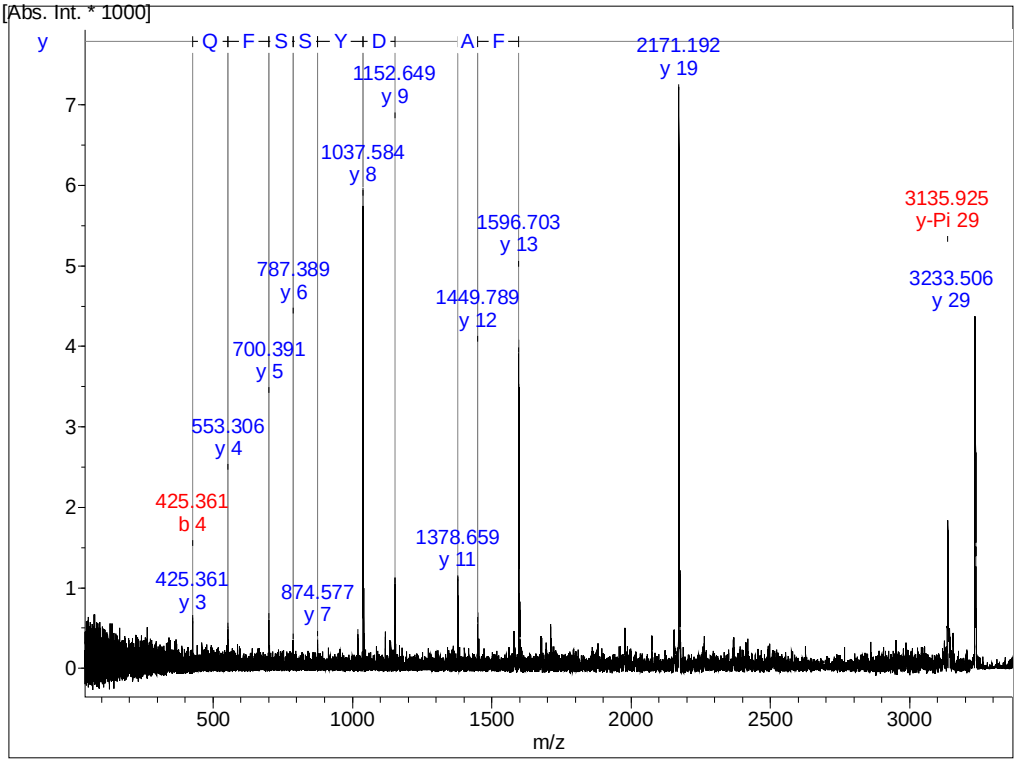
A)



B)

VEPVGEDLGp(S)PPPPADFAPEDYSSFQHIR

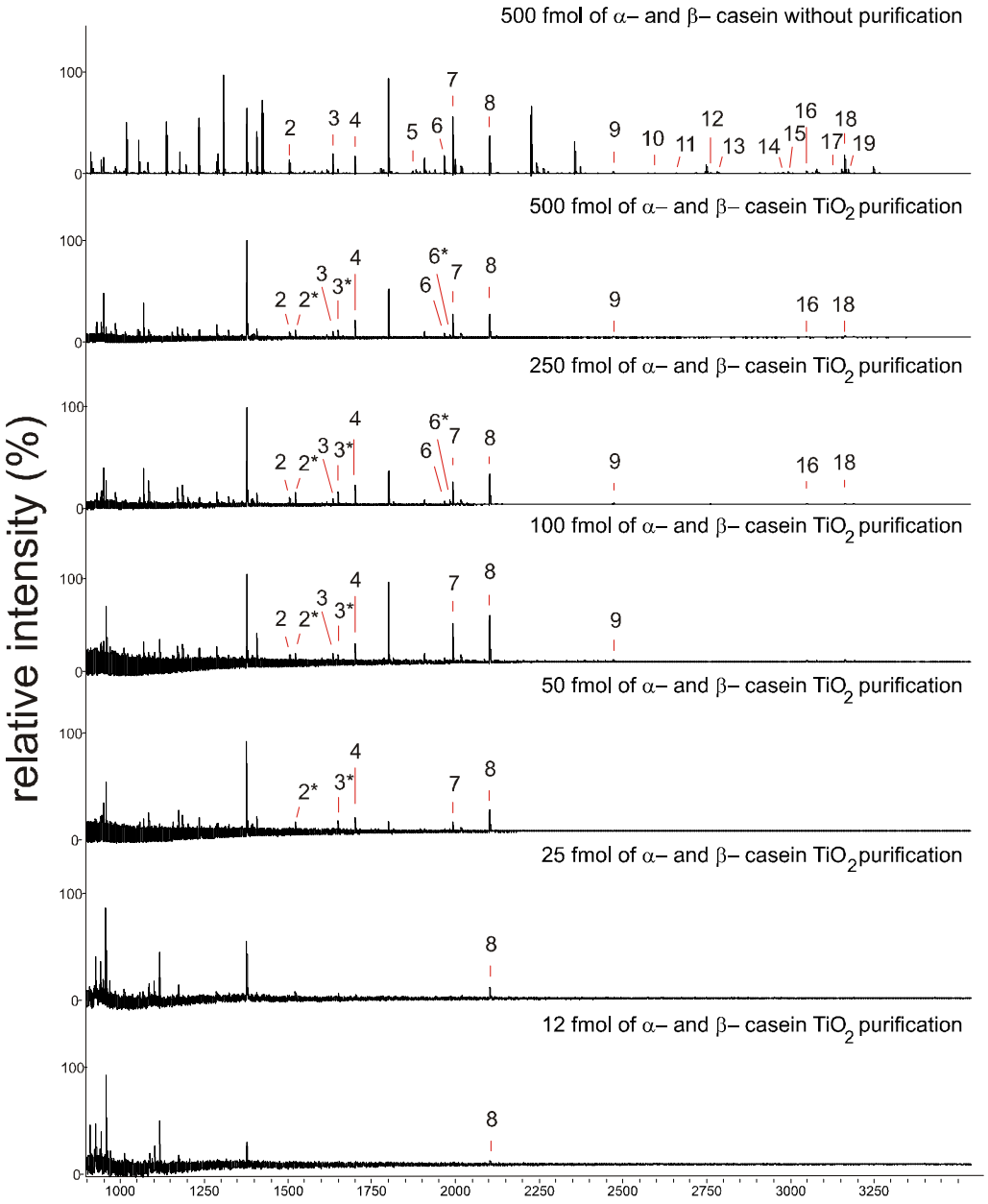
#	Seq.	y	#
1	V		29
2	E	3036.401	28
3	P	2907.358	27
4	V	2810.306	26
5	G	2711.237	25
6	E	2654.216	24
7	D	2525.173	23
8	L	2410.146	22
9	G	2297.062	21
10	S	2240.041	20
11	P	2171.019	19
12	P	2073.967	18
13	P	1976.914	17
14	P	1879.861	16
15	A	1782.808	15
16	D	1711.771	14
17	F	1596.744	13
18	A	1449.676	12
19	P	1378.639	11
20	E	1281.586	10
21	D	1152.543	9
22	Y	1037.516	8
23	S	874.453	7
24	S	787.4209	6
25	F	700.3889	5
26	Q	553.3205	4
27	H	425.2619	3
28	I	288.203	2
29	R	175.119	1



Monoisotopic mass of neutral peptide Mr(calc): 3232.4390
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
S10 : Phospho (ST), with neutral losses 97.9769(shown in table), 0.0000
Ions Score: 106 Expect: 4.1e-12
Matches (highlighted): 17/706 fragment ions using 13 most intense peaks

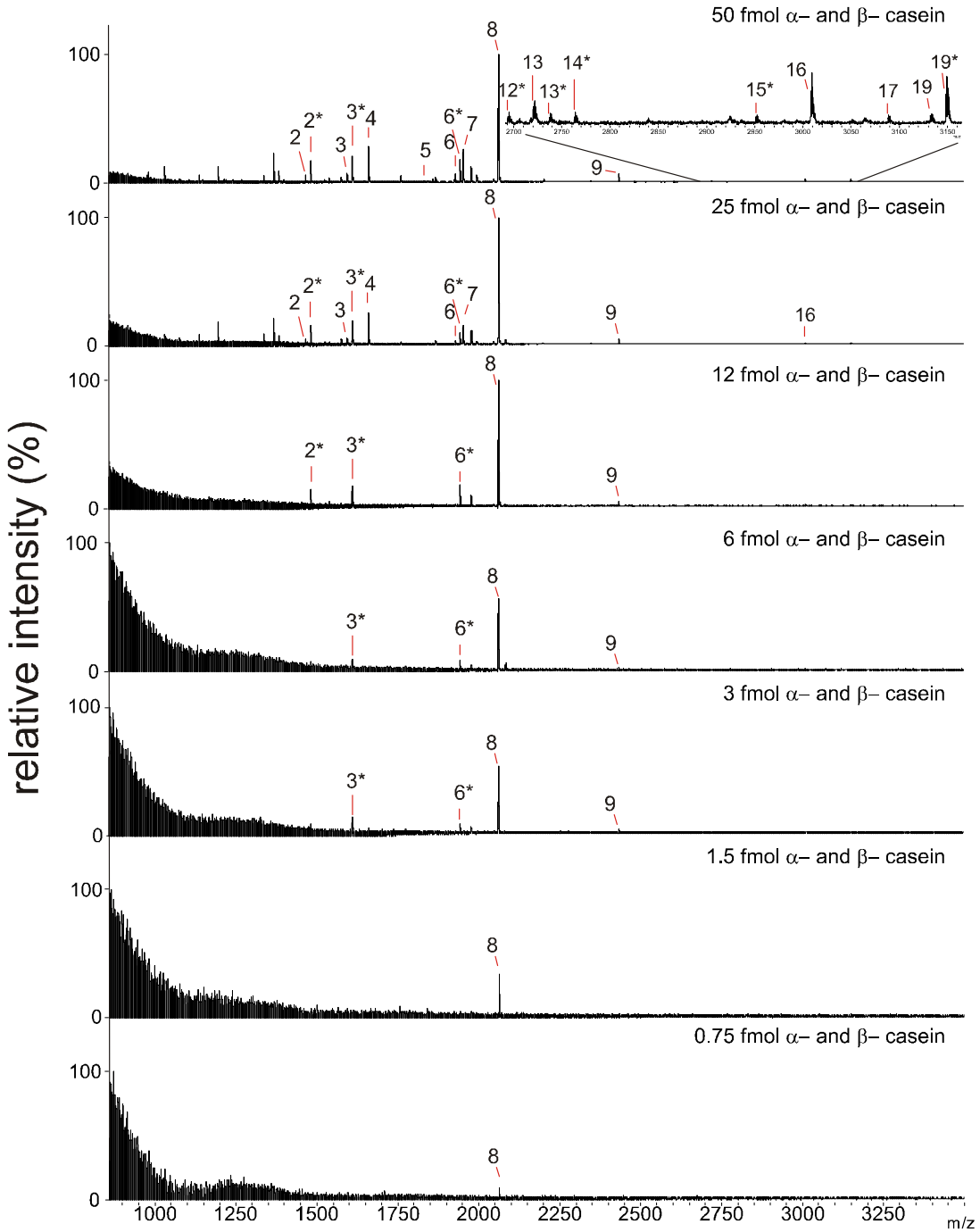
Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and peak annotation from Biotools. Ion table from Mascot.

Supplementary image 3: TiO_2 purification of α– and β– casein dilution series



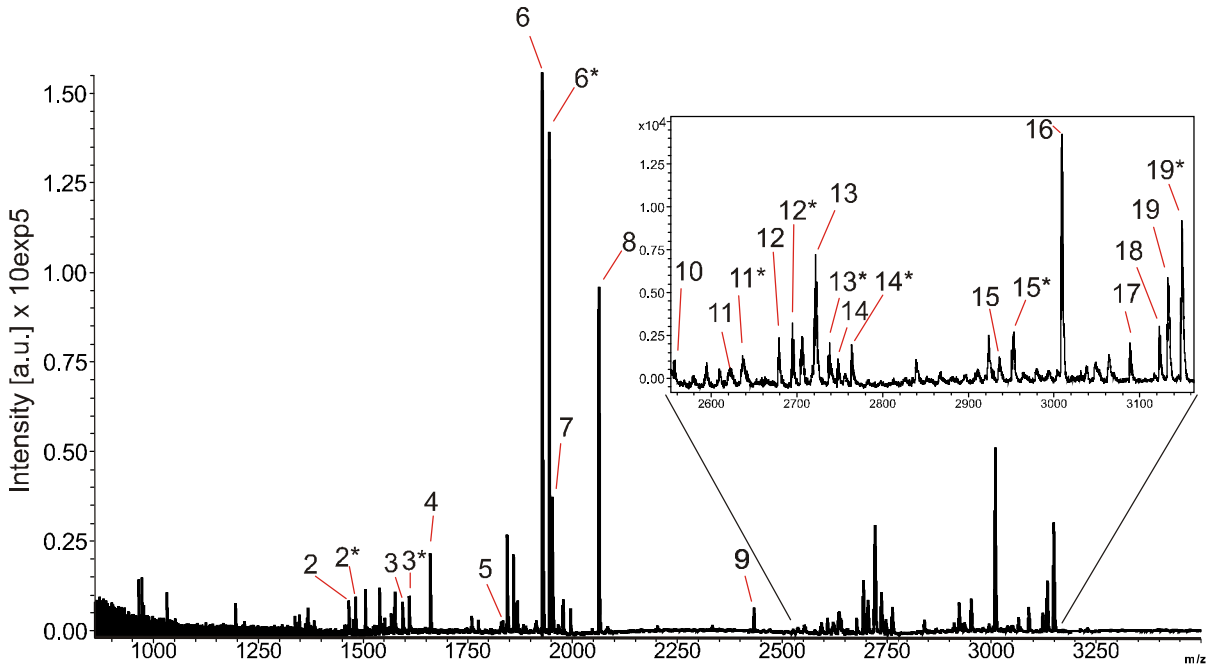
Supplementary image3: 3000 laser shots were accumulated with the following laser power settings: sample without TiO_2 treatment at 31%; TiO_2 treated samples from 500 fmol to 50 fmol at 40%; 25 and 12 fmol samples at 56% and 61% respectively. In all other cases 2000 laser shots were accumulated. The ***** denotes the oxidized phosphopeptides, and the numbers indicate phosphpopeptides, which are also detailed in Supplementary Table 3.

Supplementary image 4: ITO purification of α– and β– casein dilution series



Supplementary image 4: A dilution series of α-and β-casein peptides were spotted on ITO-coated glass slides and treated according to the optimized protocol for selective retention of phosphopeptides. Numbers indicate detected phosphopeptides described in detail in Supplementary Table 3. Two thousand laser shots were accumulated in each spectrum under the following laser settings: 36% for 50 and 25 fmol; 40 % for 12, 6, 3 and 1.5 fmol; and 48 % for 0.75 fmol.

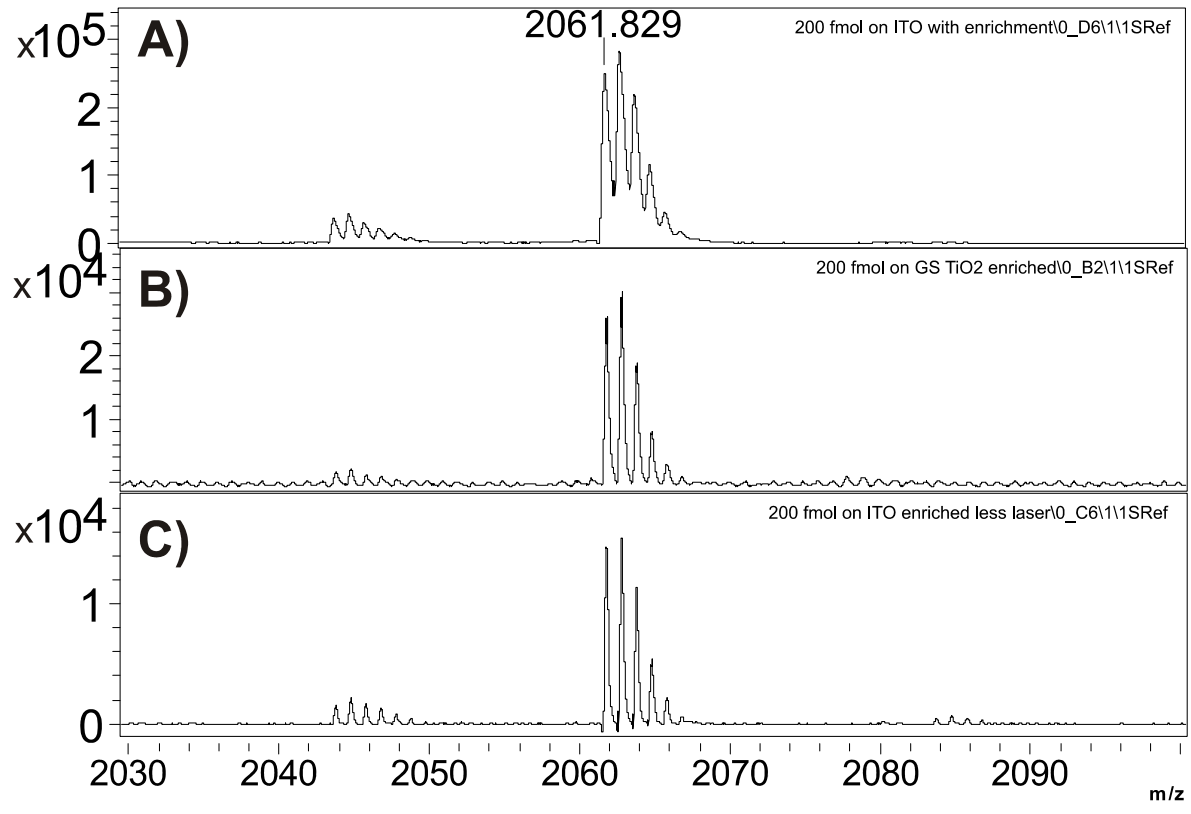
Supplementary image 5: ITO purification of 500 fmol α– and β– casein



Supplementary image 5: A mixture of α-and β-casein peptides (500 fmol each) was analyzed using the phosphopeptide purification optimized protocol in this study. Two thousand laser shots were accumulated with 40 % laser power setting. 18 different phosphopeptides were detected, as well as 9 additional oxidized forms. The ***** denotes the oxidized phosphopeptides, and the numbers indicate phosphopeptides, which are also detailed in Supplementary Table 3.

Supplementary image 6 and 7

For comparison of the different enrichment platforms (ITO vs TiO_2-enrichment & AnchorChip) the sensitivity of the laser power setting was kept constant (2000 laser shots, 50 % laser power, 64 % offset and 15 % range). Spectral acquired from the ITO plate at two different laser settings, after identical the planar-enrichment procedure, are also shown in Supplementary image 6, panels A and C. The spectra acquired from the AnchorChip plate after TiO_2-enrichment is shown in panel B. We observed best measurements of the resolution for the peptides from ITO (6C) when the laser power was at 2000 laser shots, 46% laser power, 64 % offset, 15% range. However, to facilitate comparisons between measurements, the laser settings were kept constant throughout the entire dilution series test (Supplementary image 6A and B; manuscript figure 3).



Supplementary image 6: β-casein peptide (48-FQSEEQQQTEDELQDK-63) was analyzed after the enrichment on ITO target (A) and on AC-target (B). For higher resolution the laser intensities had to be lowered for ITO (C). The laser settings were kept identical to facilitate comparison throughout the sensitivity test (manuscript figure 3). For the results of the entire α- and β-casein digestion mixture, see Supplementary images 3 and 4.. KEY: AC= AnchorChip-target, ITO=indium tinoxide

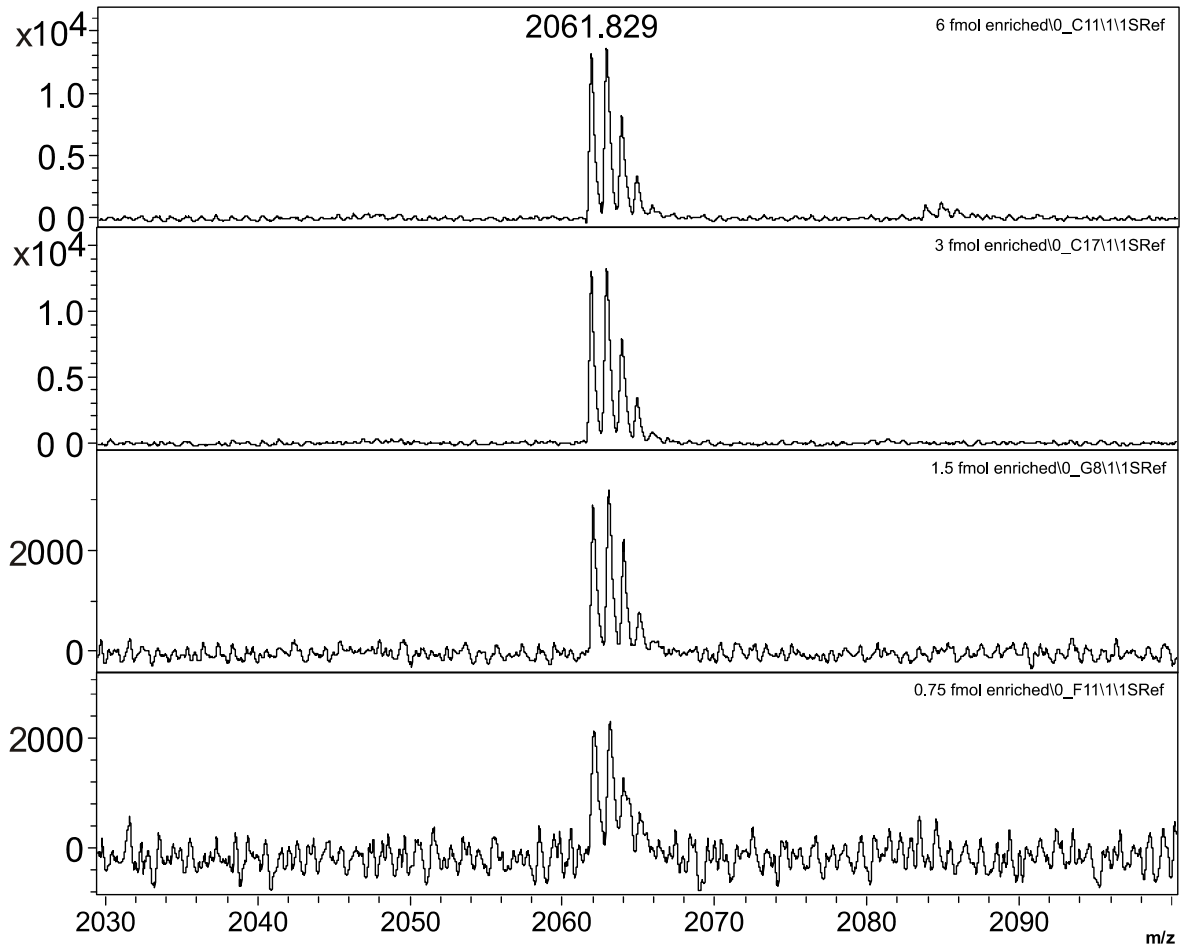
Optimising the laser intensity for each surface, to facilitate optimal ionization of the peptides can be due to several factors that we can only speculate on. Obviously the surface chemistry the ITO plate is different to the AnchorChip. This could have a direct impact on the ionization efficiency of peptides in general. On the other hand the effect

Supplementary image 6 and 7

may be more specific and only related to the ionization of (phospho)-peptides. One might consider that the ITO surface retains phosphopeptides more efficiently and therefore requires different laser settings. Another possibility is that the flat surface method (ITO) does not adhere phosphopeptide samples efficiently chromatographic techniques, and there is more left to analyse ultimately. Of course there could be a combination of the above reasons and more that we are not sure of at this point. Nevertheless, we noticed this phenomenon that appeared to be ionization efficiency when trying to compare the planar surface enrichment with chromatographic method (Figure 3 in manuscript and Supplementary images 3 and 4). In the sensitivity test on a single peptide (Manuscript figure 3) the laser intensity was kept constant, which lead to low resolution for samples analyzed from ITO. However, when analyzing the mixture of α-and β-casein the laser intensity was adjusted to produce best resolution for both of the samples (Supplementary image 3 and 4). These results also show that less laser power is required for the ITO method.

Finally, the dilution series presented in Supplementary image 4 (α-and β-casein mixture enrichment on ITO) are presented here as a “zoomed images”. The 6 fmol loading compares with the loading in figure 3 of the manuscript, but with optimal laser settings.

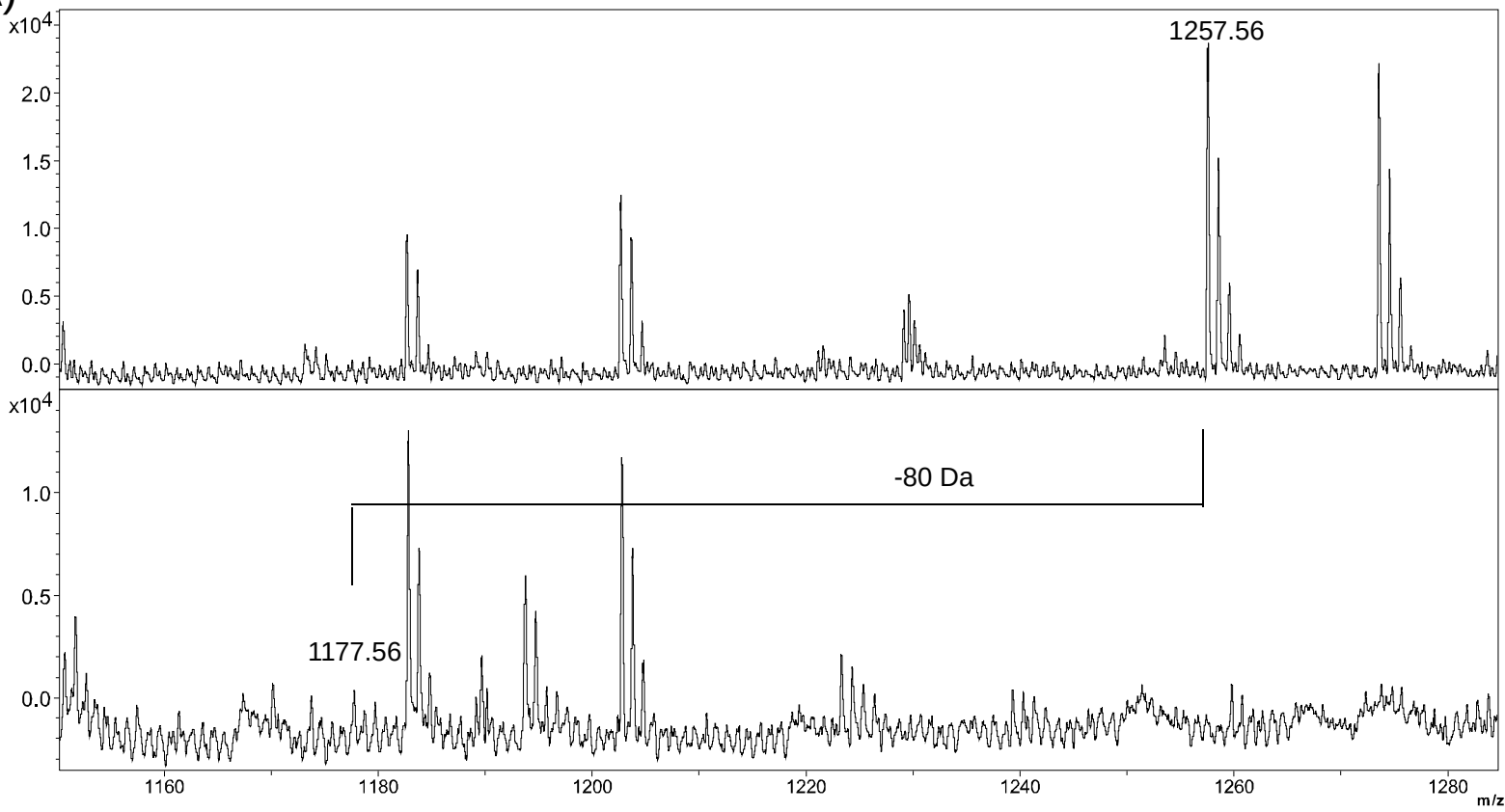
Supplementary image 6 and 7

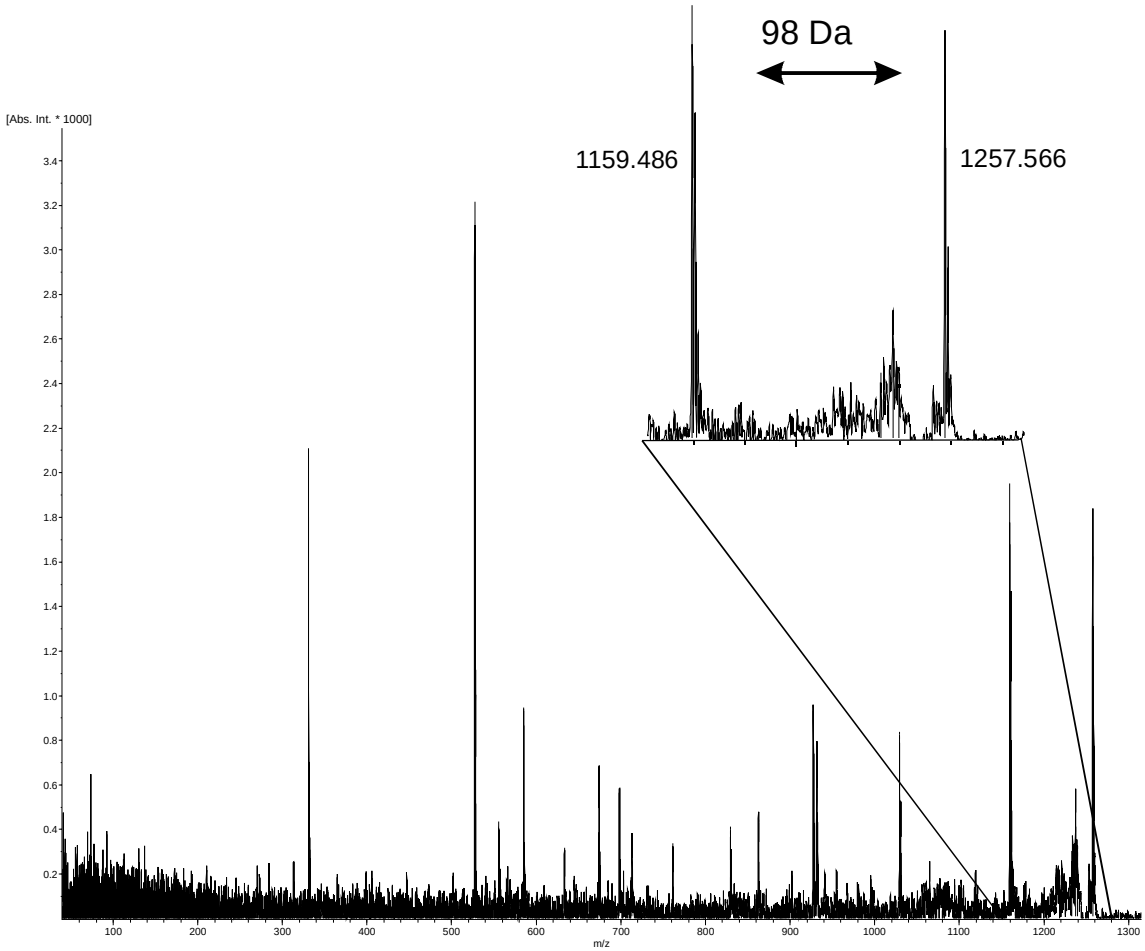


Supplementary image 7: Zoomed spectra from the Supplementary image 4. The ions are generated from an ITO glass slide. The resolution of the ions are comparable to the resolution of the TiO_2-enriched samples presented in the manuscript figure 3.

Supplementary image S1: PIM phosphorylated NFAT *m/z* 1257.5

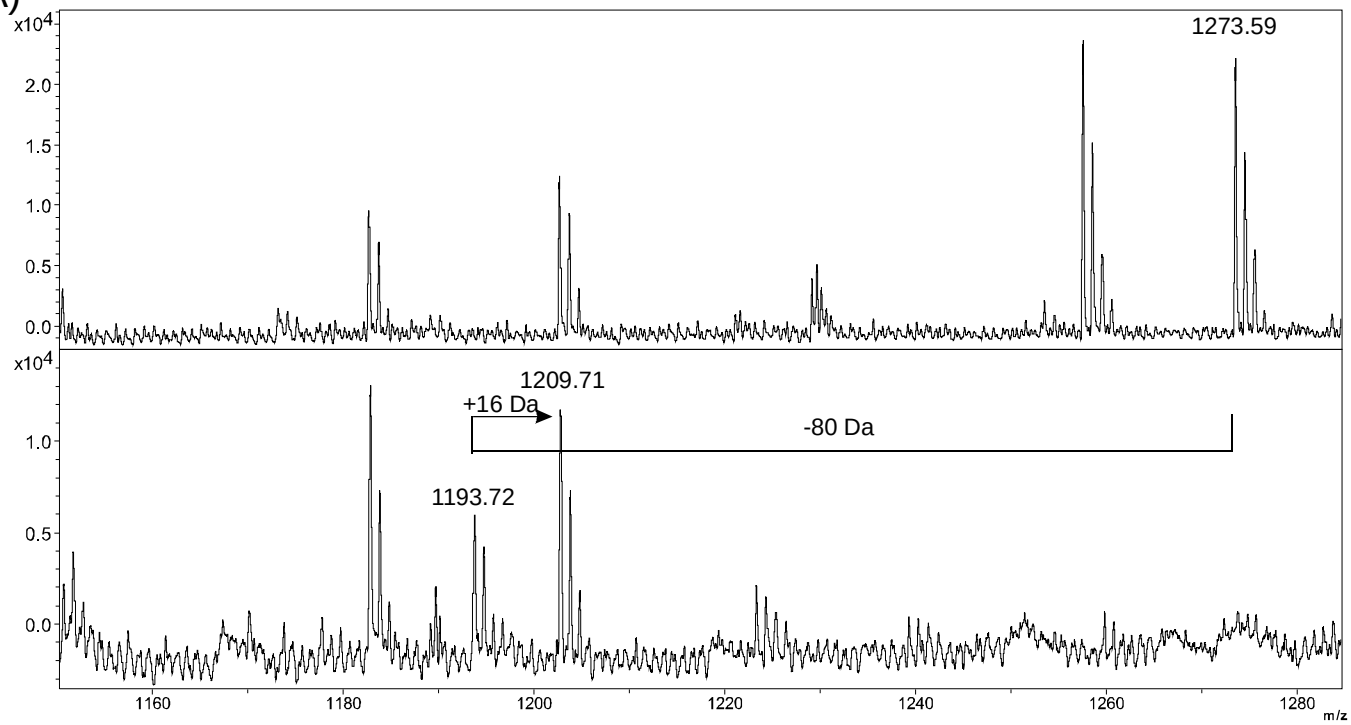
A)



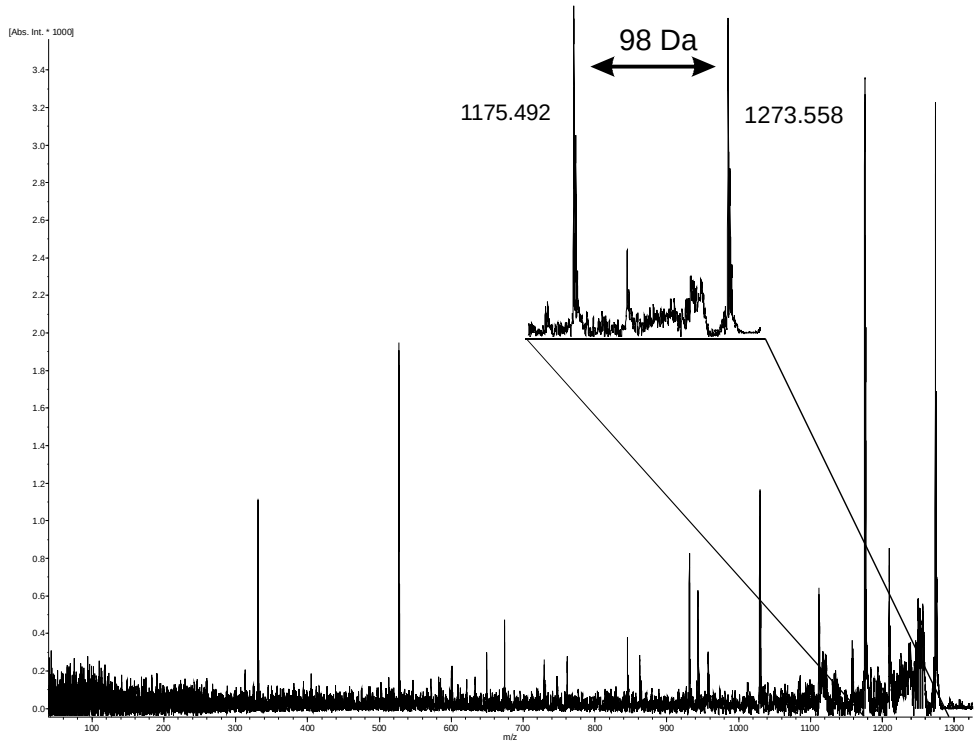


Supplementary image S1*: PIM phosphorylated NFAT *m/z* 1273.59

A)

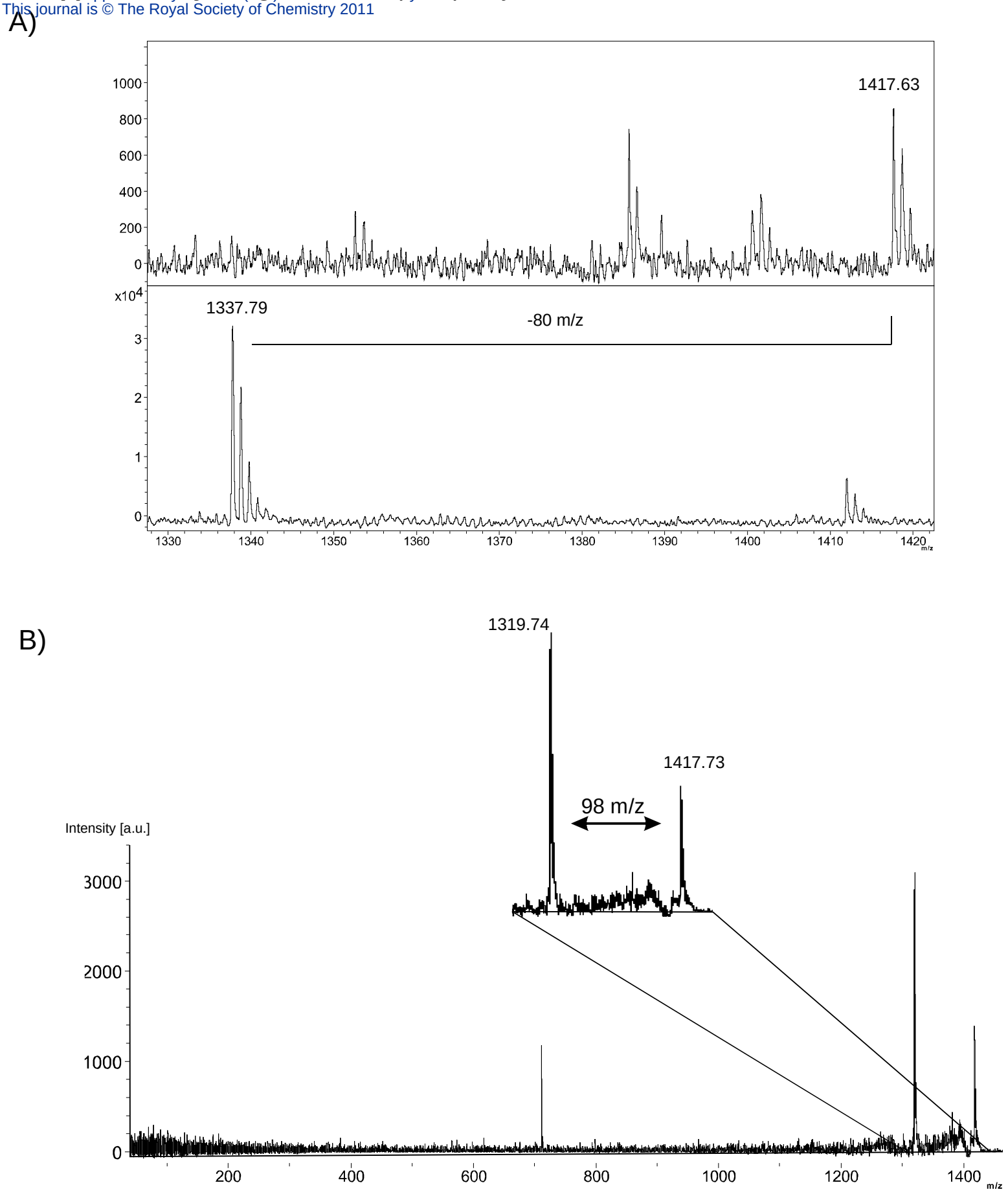


B)



Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and indication of neutral loss.

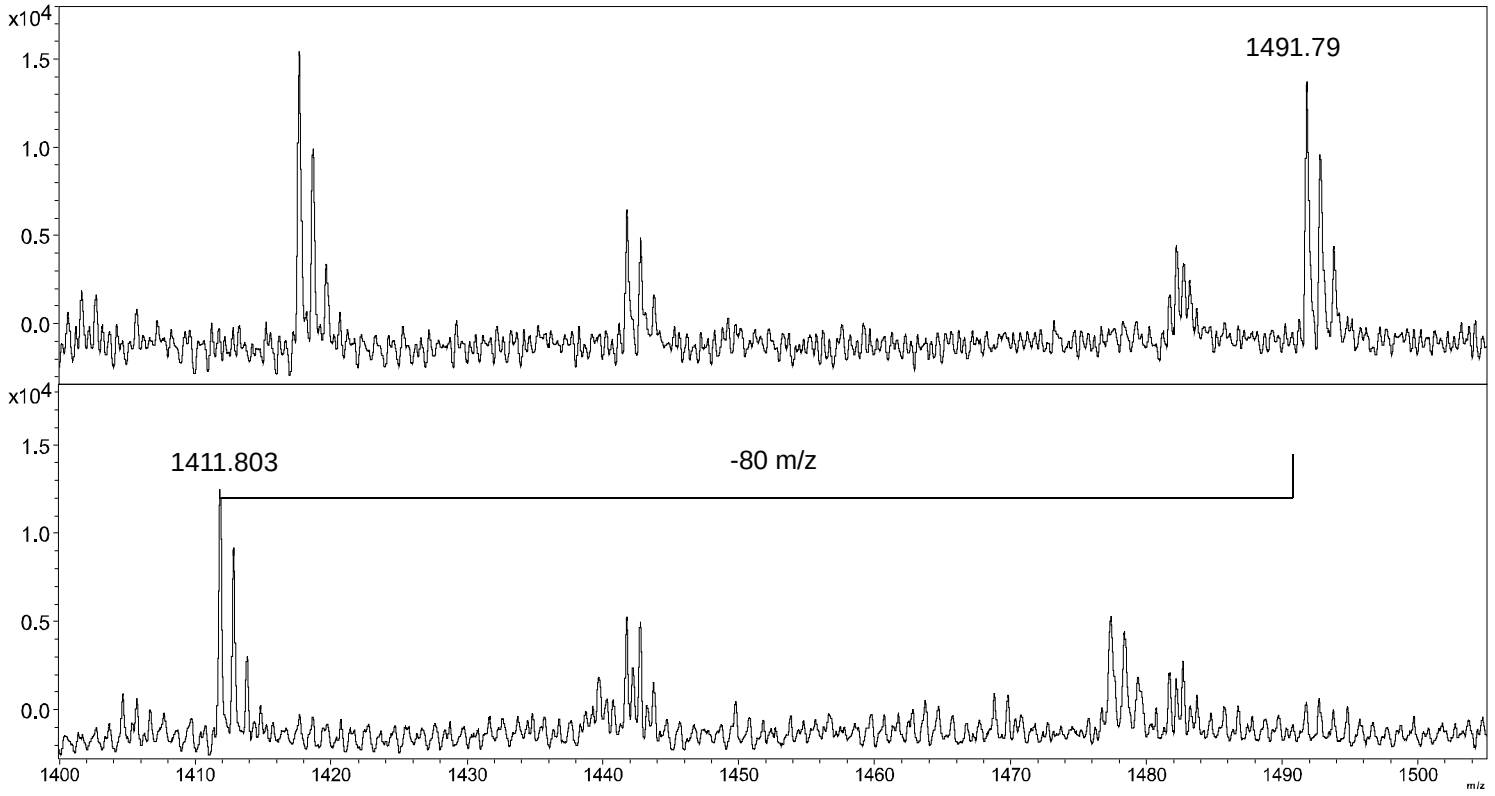
Supplementary image S2*: PIM phosphorylated NFAT *m/z* 1417.61



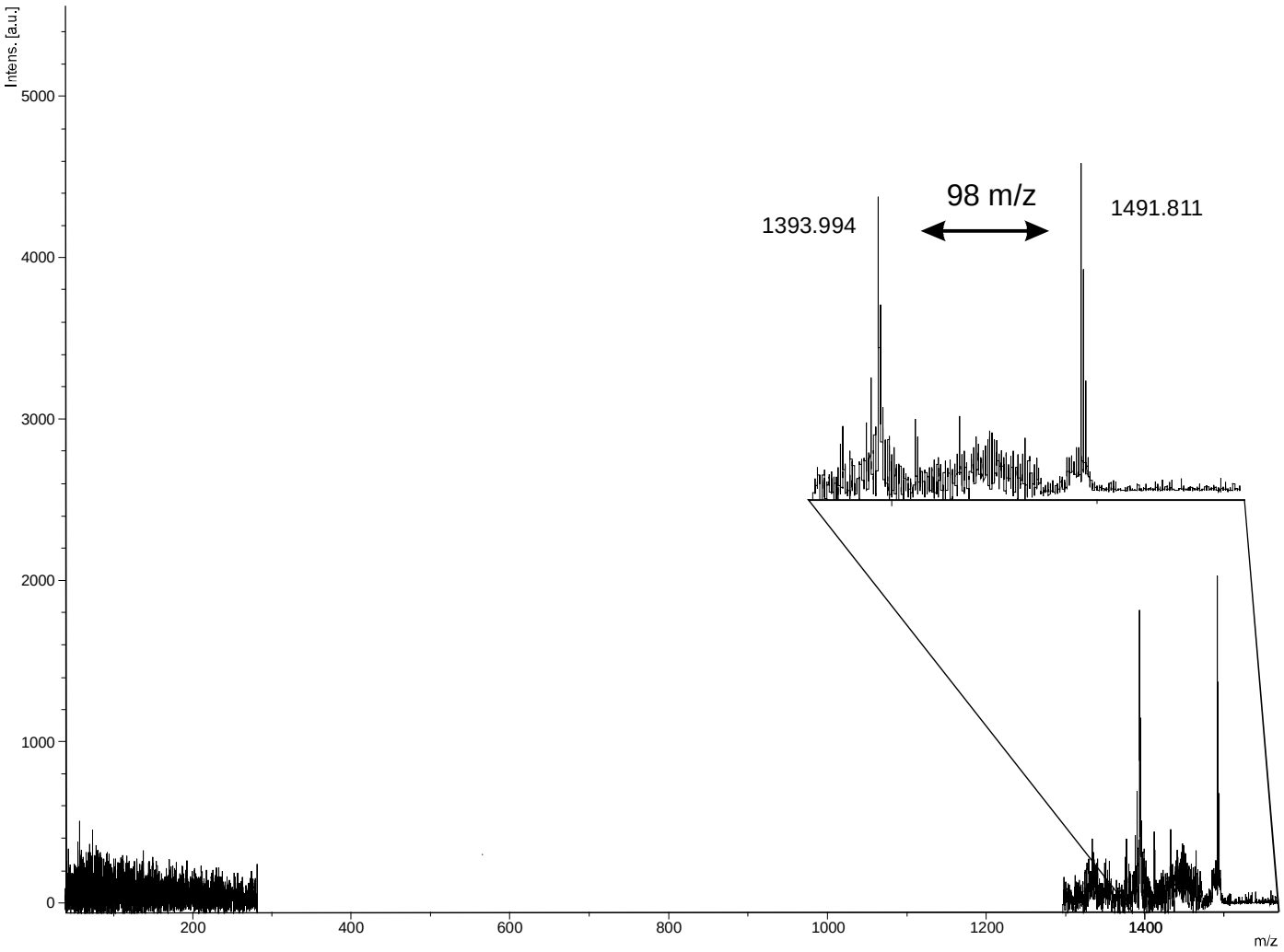
Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and indication of neutral loss.

Supplementary image S3: PIM phosphorylated NFAT *m/z* 1491.77

A)

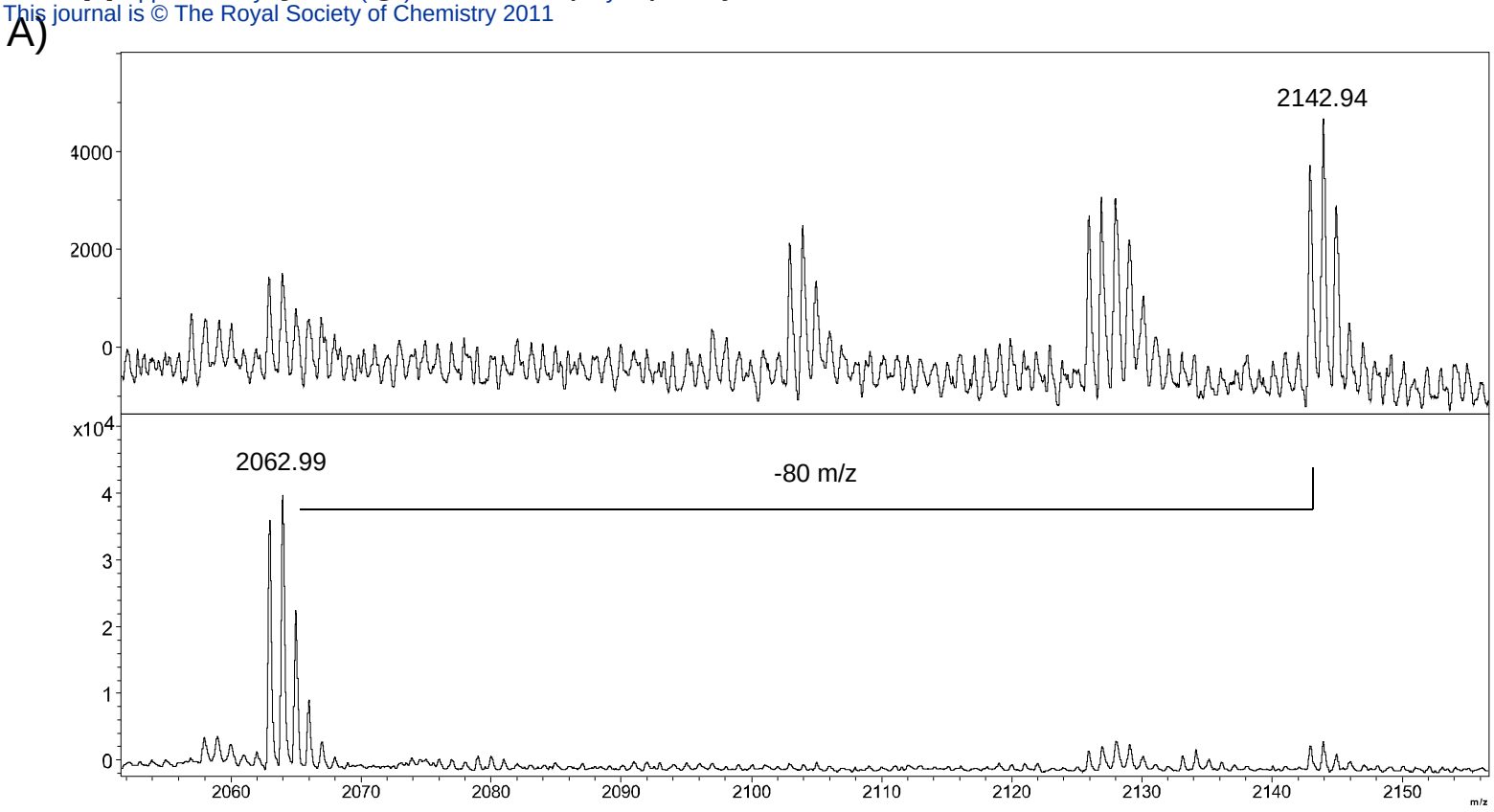


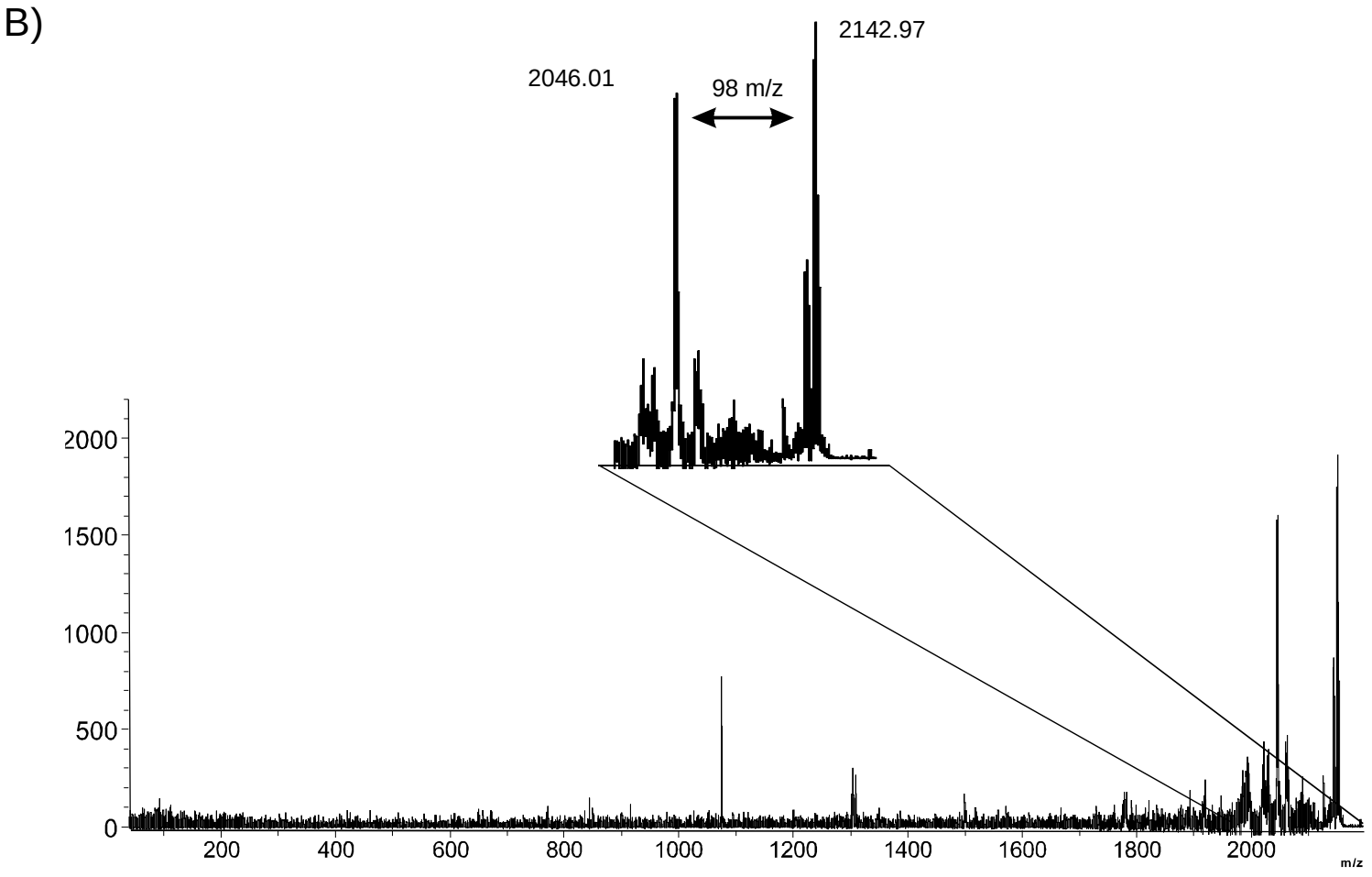
B)



Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and indication of neutral loss.

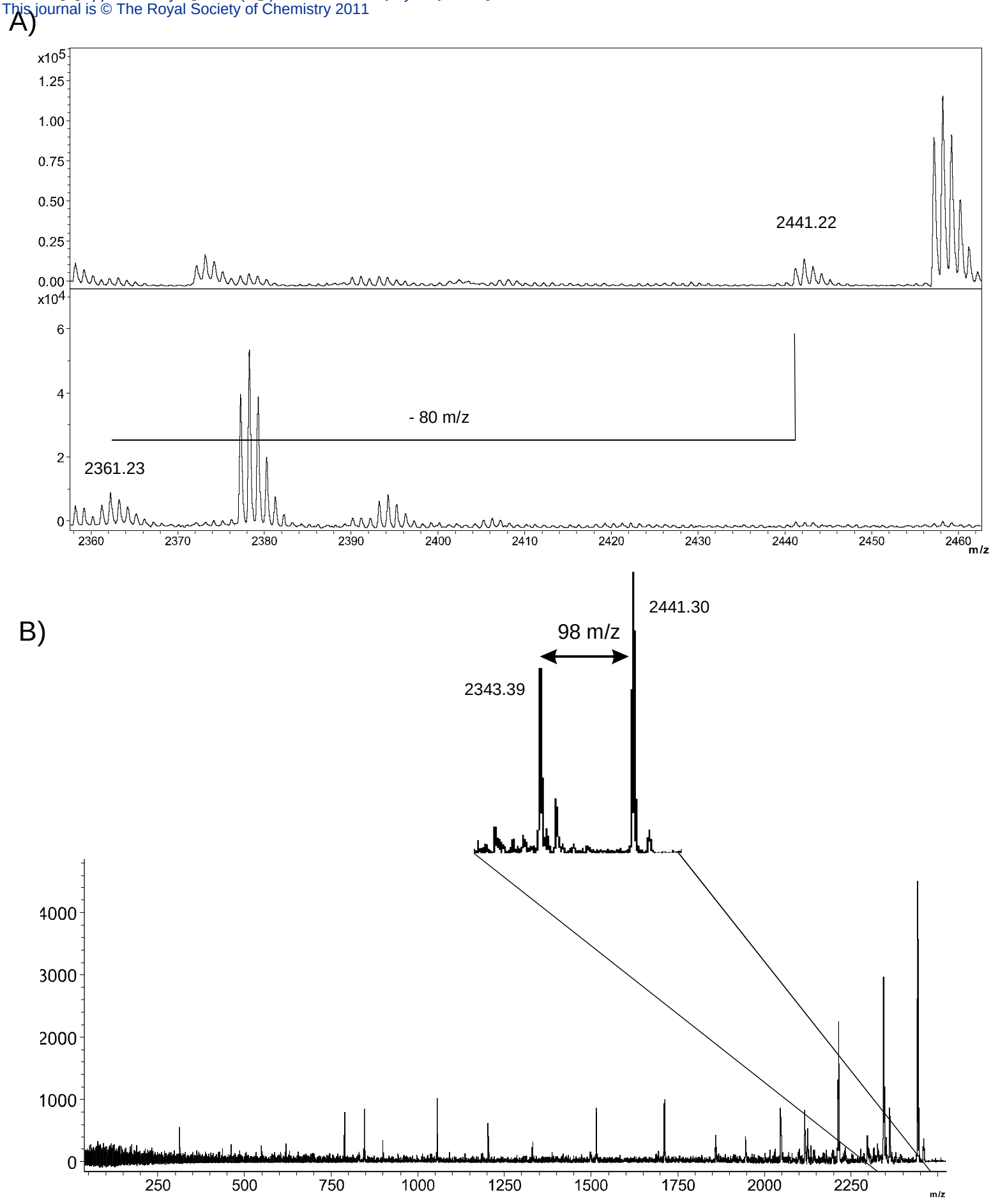
Supplementary image S4: PIM phosphorylated NFAT *m/z* 2142.95





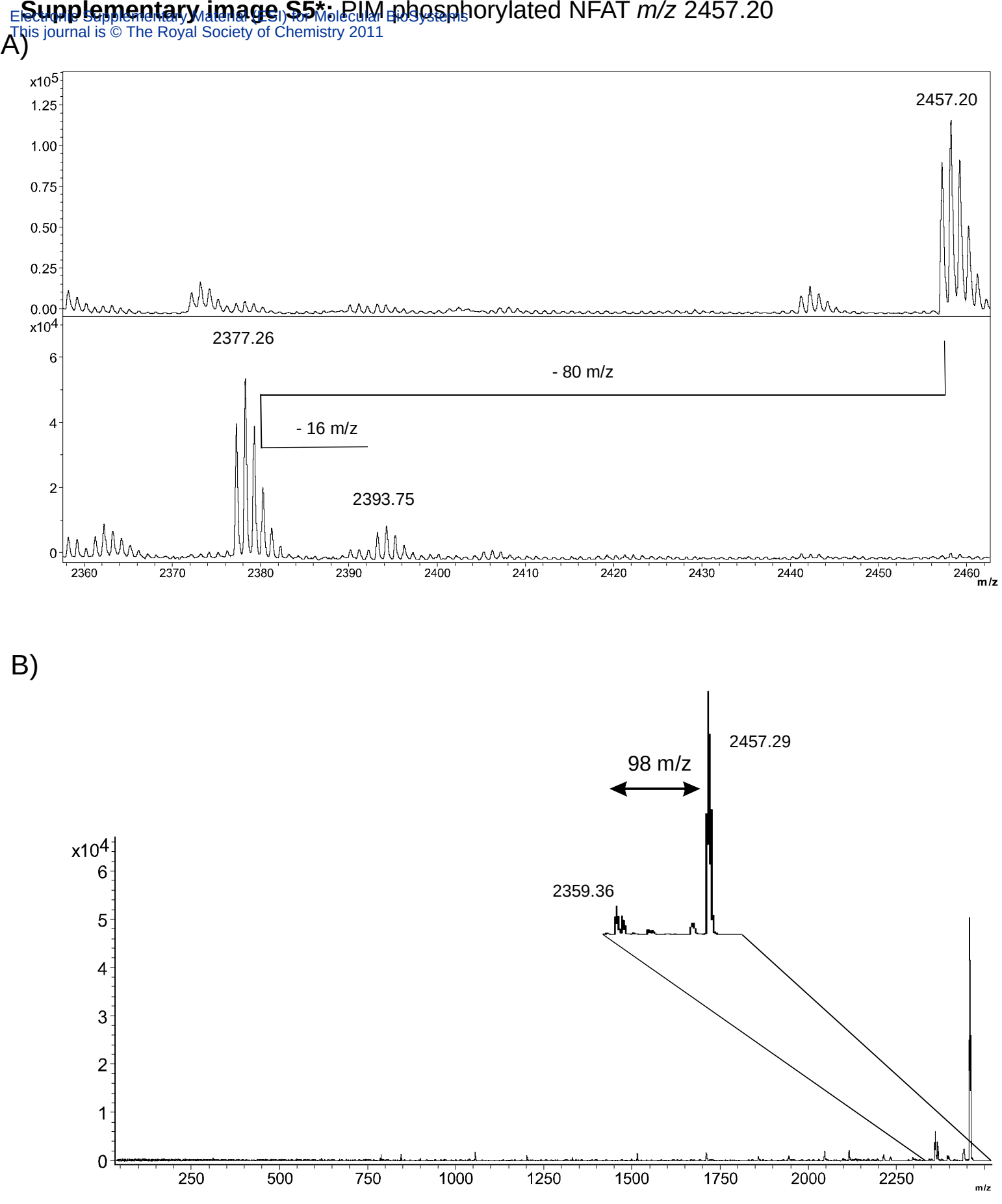
Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and indication of neutral loss.

Supplementary image S5: PIM phosphorylated NFAT *m/z* 2441.21



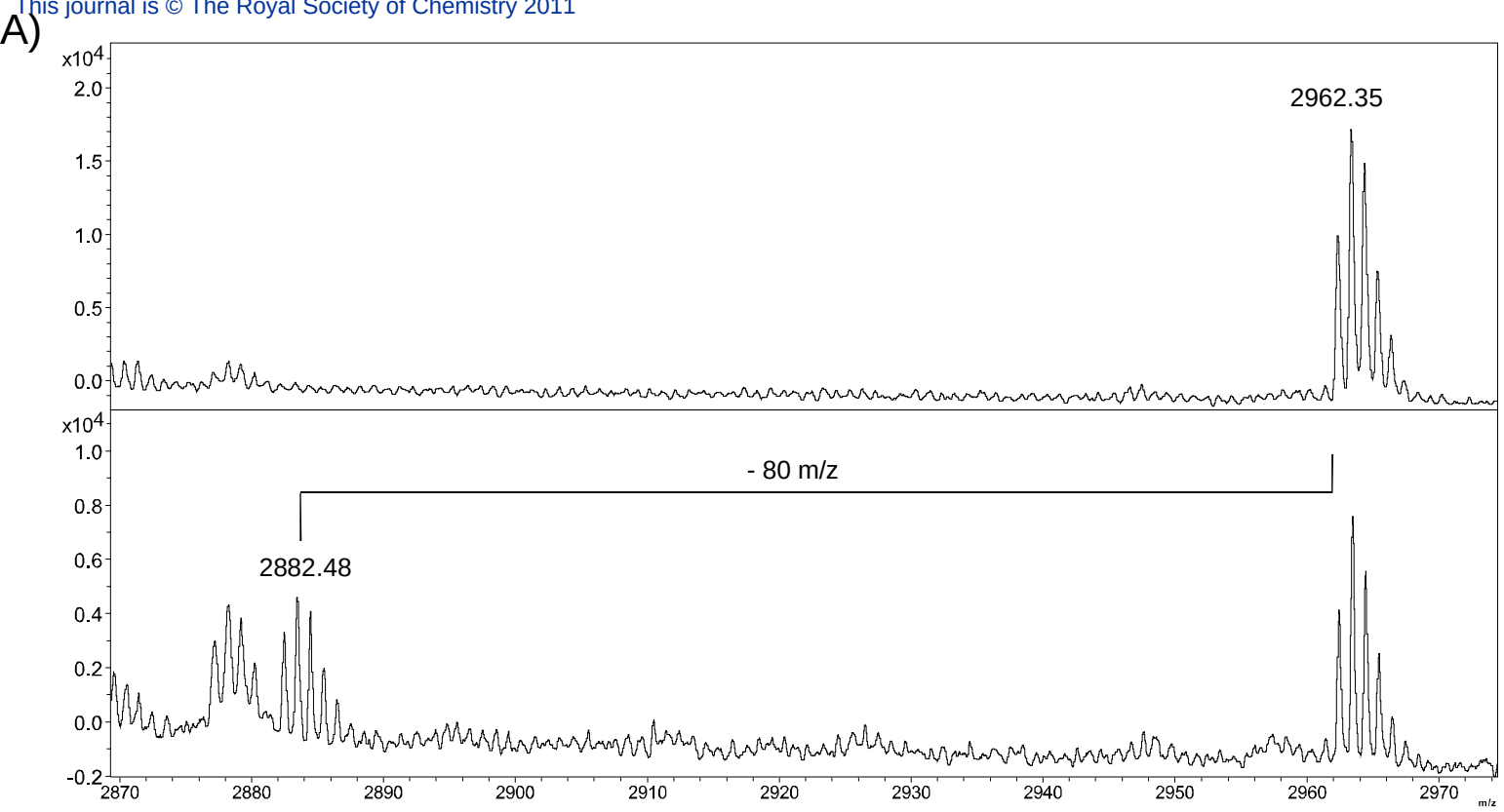
Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and indication of neutral loss.

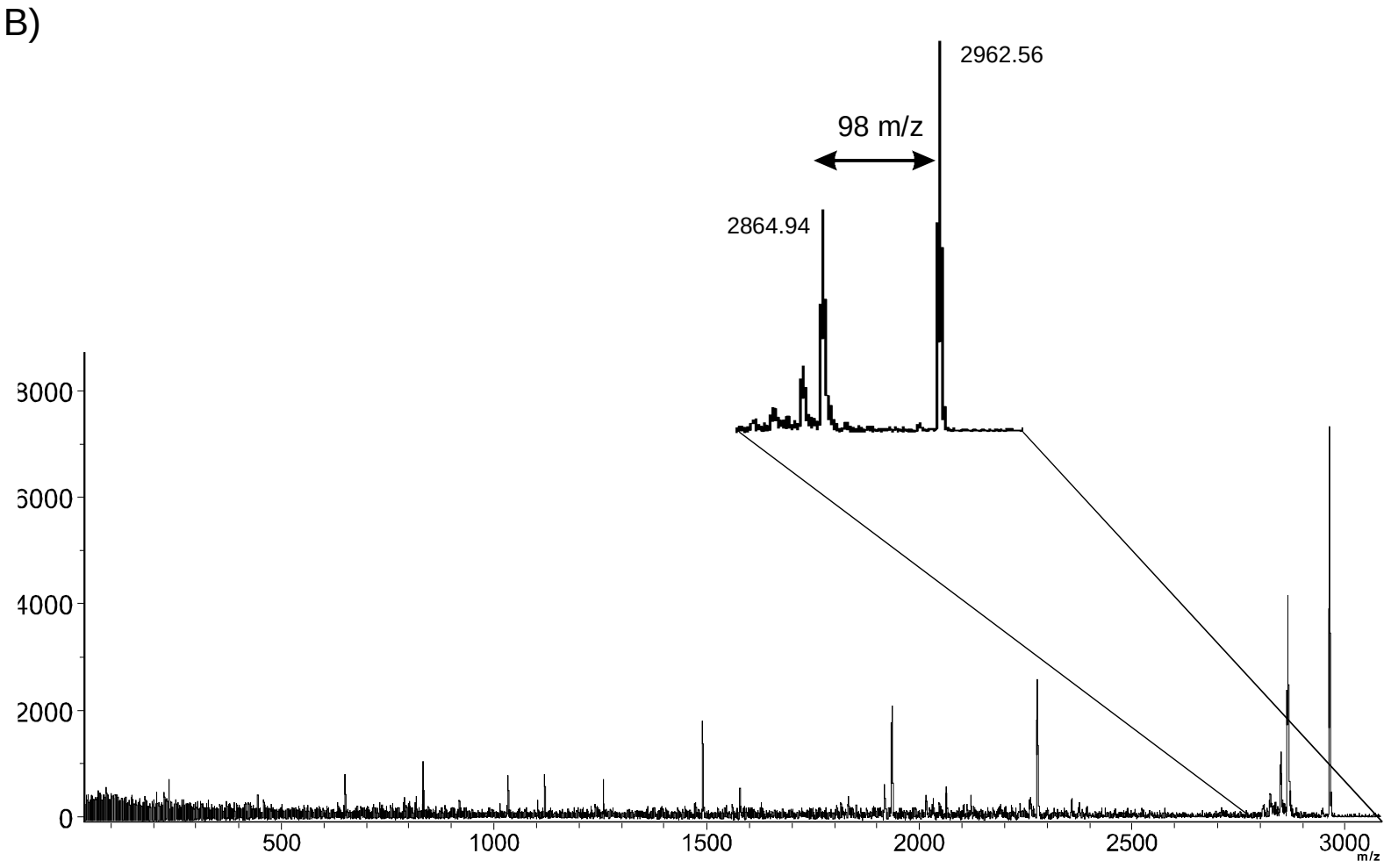
Supplementary image S5*: PIM phosphorylated NFAT *m/z* 2457.20



Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and indication of neutral loss.

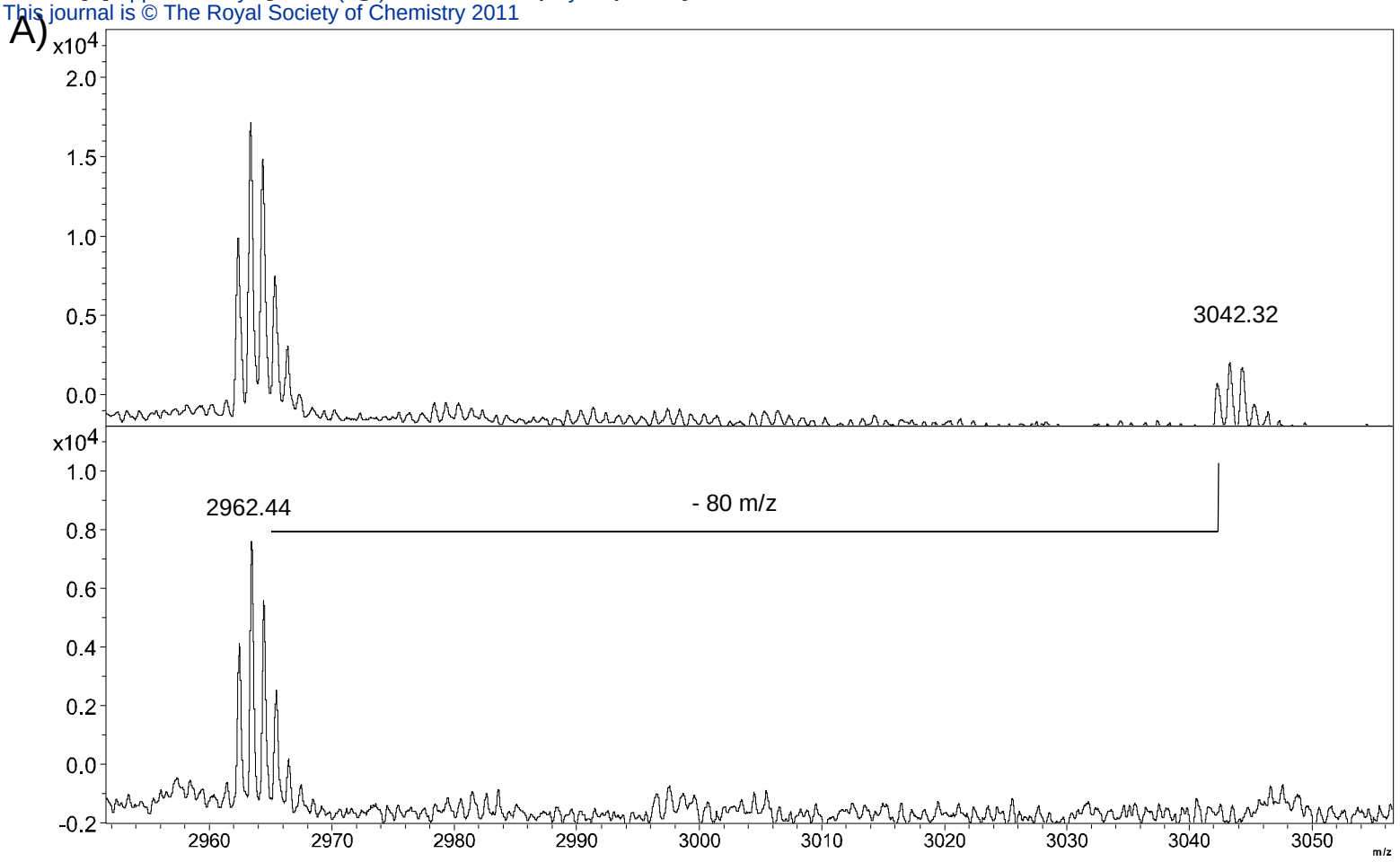
Supplementary image S6: PIM phosphorylated NFAT *m/z* 2962.35

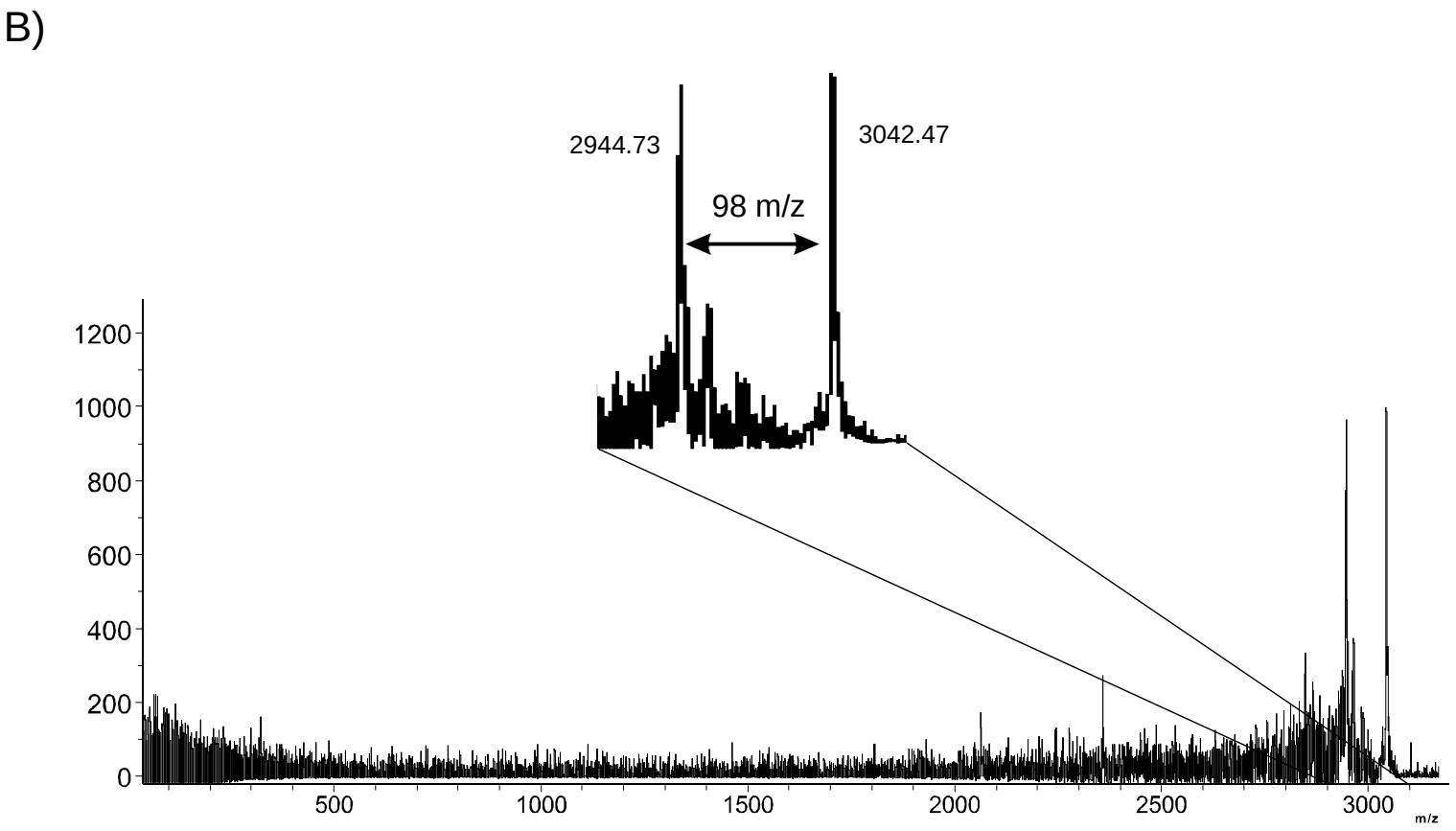




Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and indication of neutral loss.

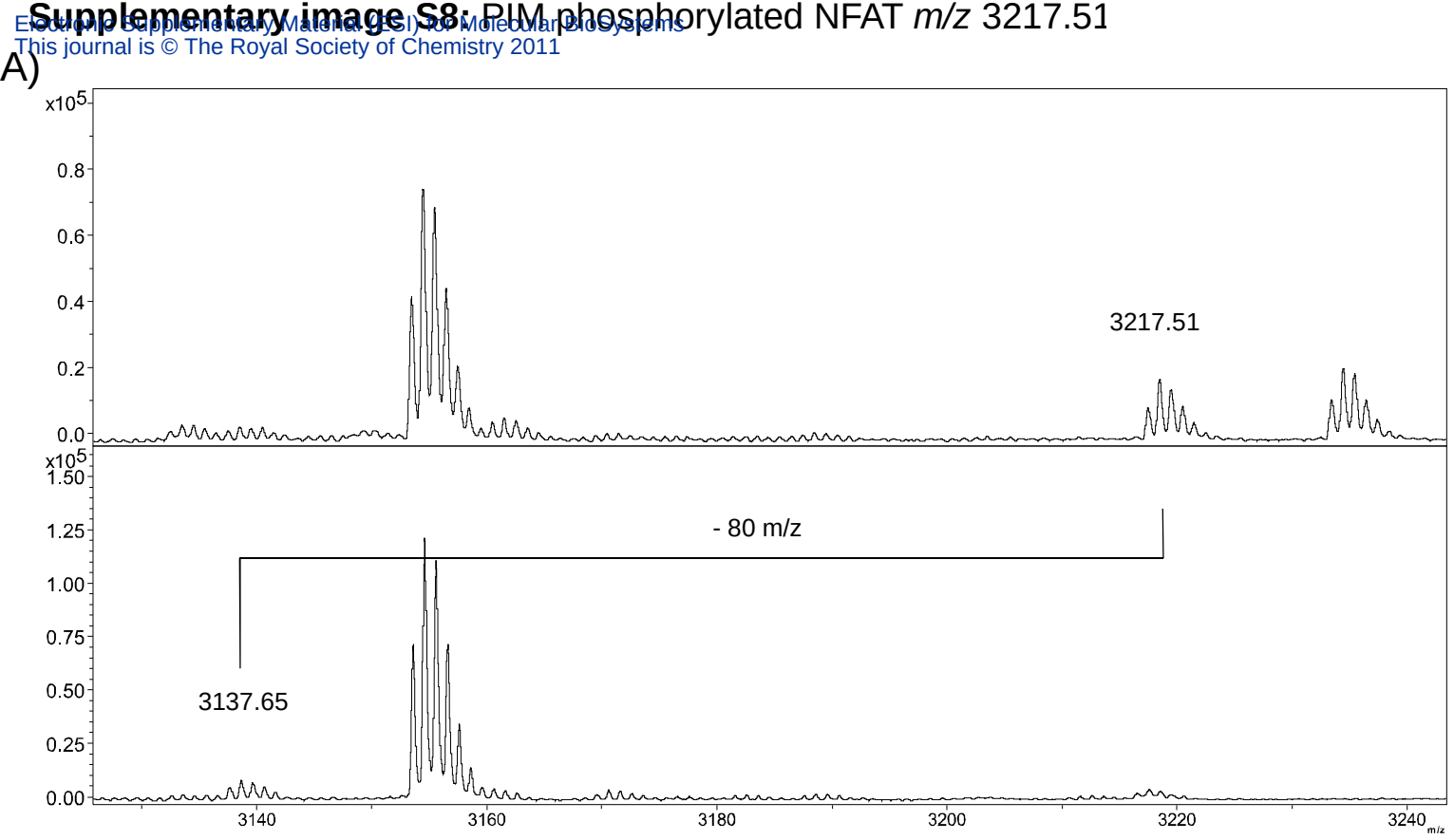
Supplementary image S7: PIM phosphorylated NFAT *m/z* 3042.47

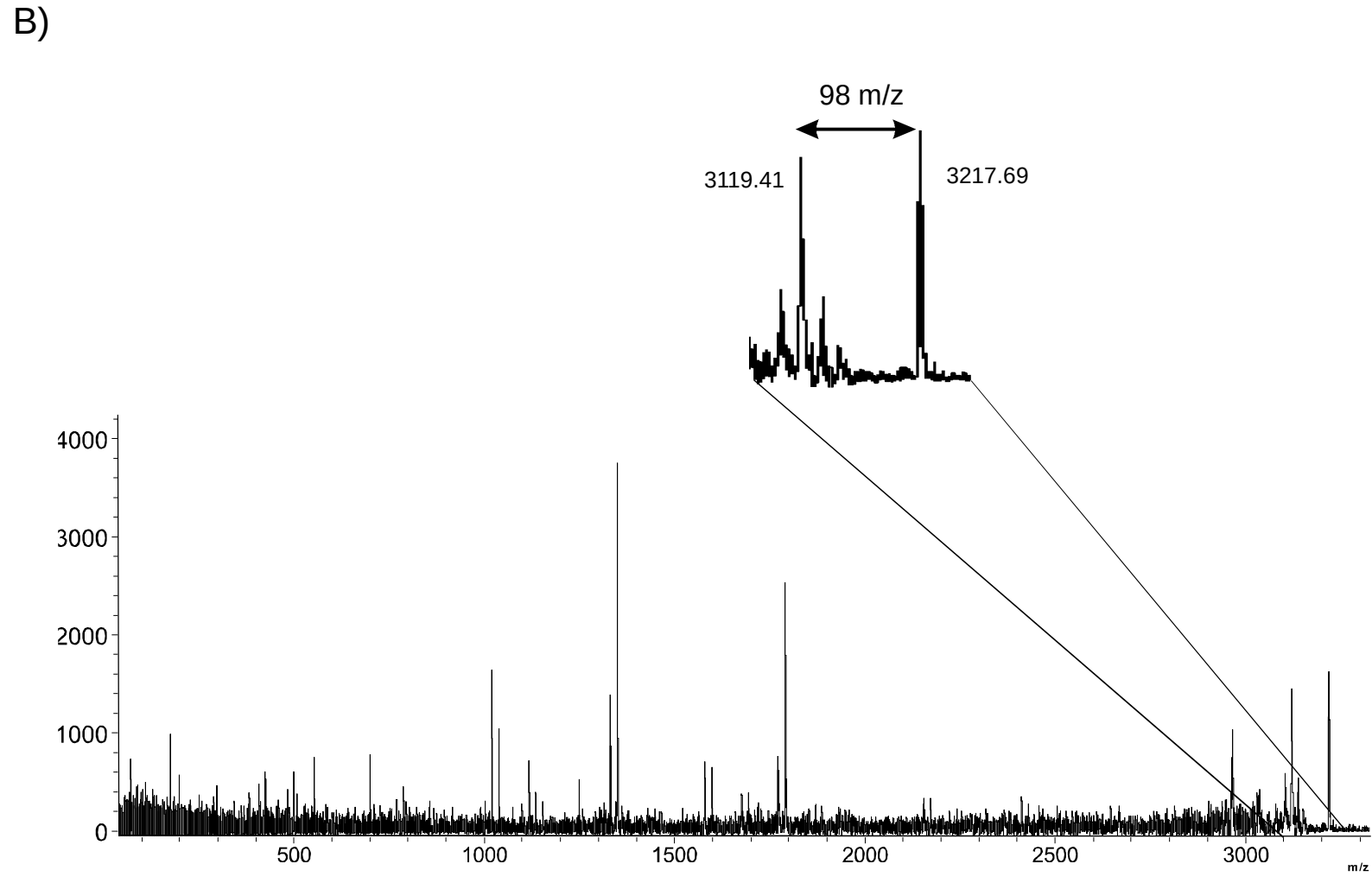




Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and indication of neutral loss.

Supplementary image S8: PIM phosphorylated NFAT *m/z* 3217.51



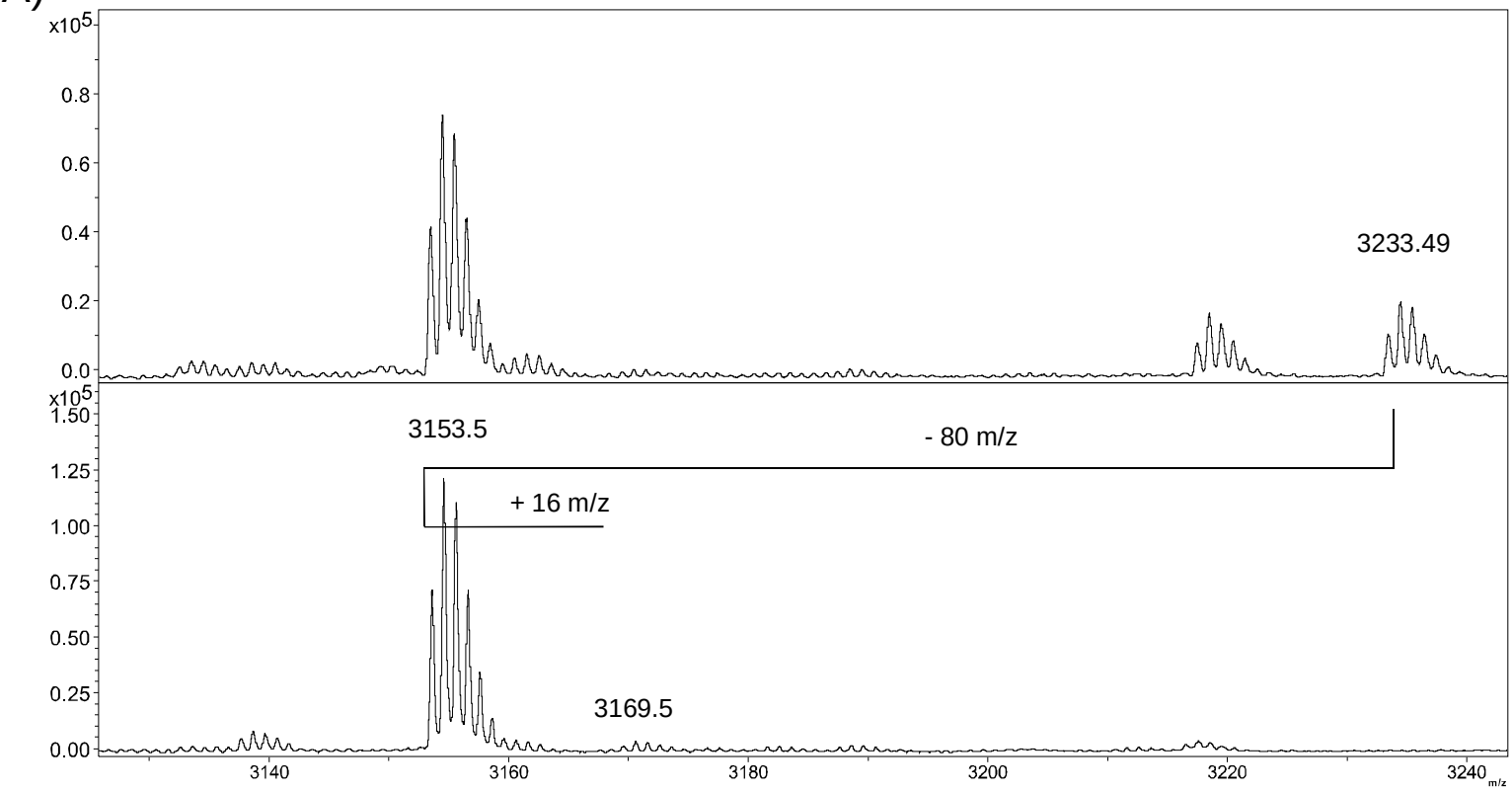


Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and indication of neutral loss.

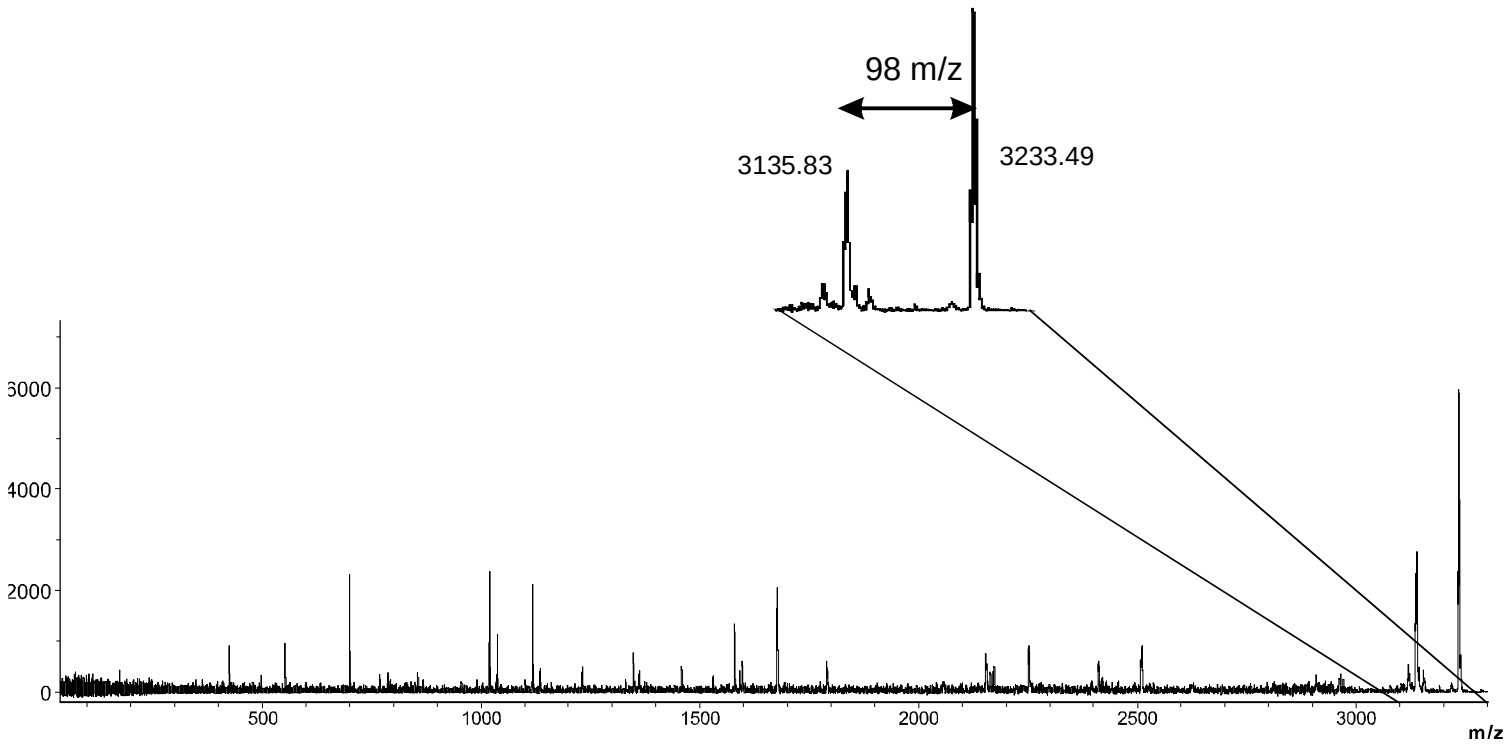
Electronic Supplementary Material (ESI) for Molecular BioSystems

Supplementary image S8*: PIM phosphorylated NFAT *m/z* 3233.45

A)



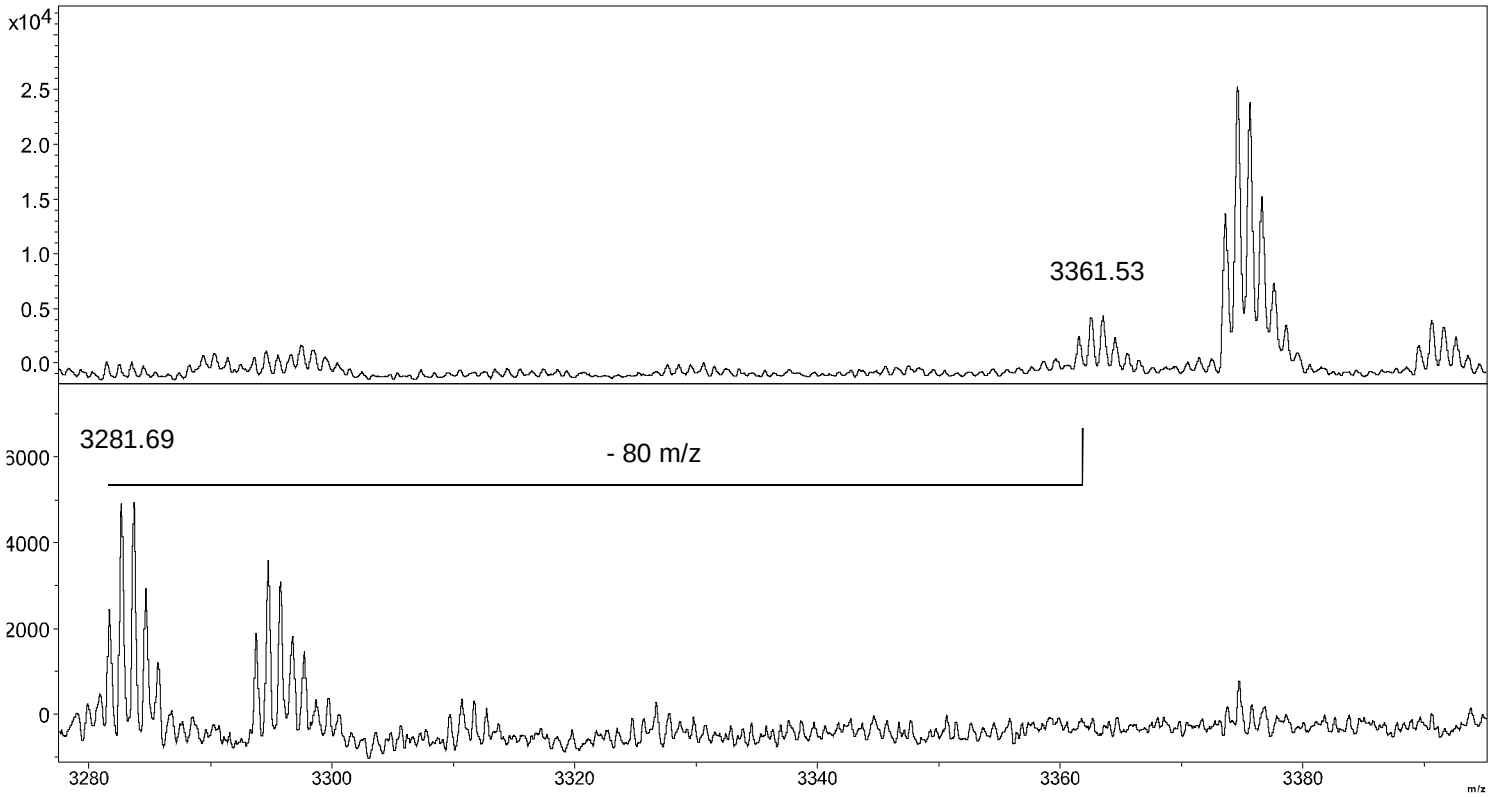
B)



Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and indication of neutral loss.

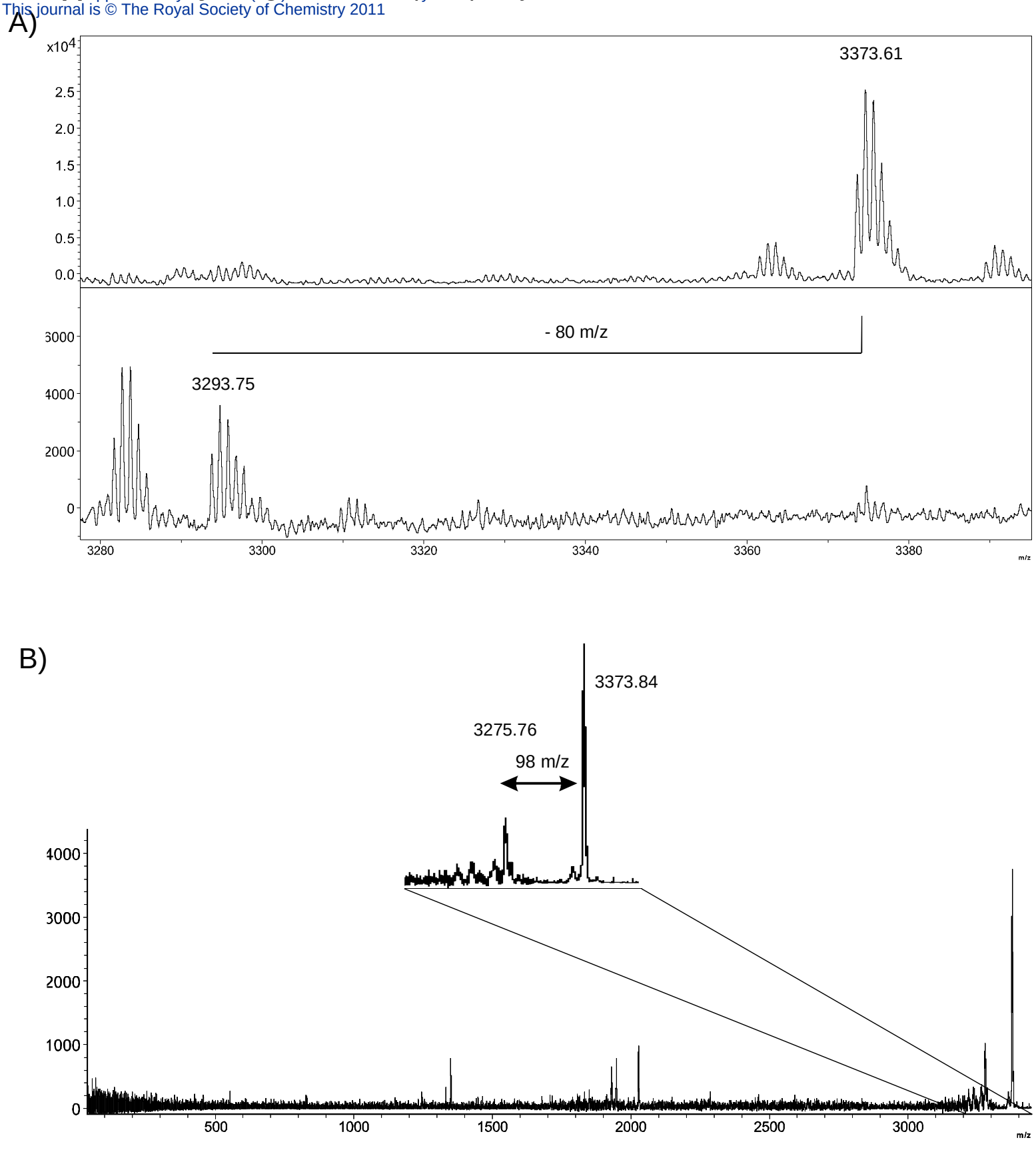
Supplementary image S9: PIM phosphorylated NFAT *m/z* 3361.68

A)



Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.

Supplementary image S10: PIM phosphorylated NFAT *m/z* 3373.58



Part A: Mass shift of 80 m/z after the alkaline phosphatase treatment.
Part B: MS/MS spectrum and indication of neutral loss.

Supplementary table 1. Annotated peaks for PKA phosphorylated NFATc1

N:o	m/z	start	Sequence	end	Modifications (Mascot)	Mascot score	*)
1	1257.55	1	MPp(S)TSFPVPSK	11	phos@3	77	1.1
1*	1273.54	1	ox(M)Pp(S)TSFPVPSK	11	Ox@1, phos@3	42	1.2
2	1385.61	244	Ap(S)VETEESWLGAR	255	phos@245	106	1.3
2*	1417.59	244	Ap(S)VETEESox(W)LGAR	255	phos@245, Ox@252	79	1.4
3	1491.78	337	Kp(TT)LEQPPSVALK	349	phos@338 or 339	24 24	1.5
4	1772.82	151	SPp(S)TATLSLPSLEAYR	166	phos@153	109	1.6
5	1928.93	150	RSPp(S)TATLSLPSLEAYR	166	phos@153	61	1.7
6	2441.19	1	MPp(S)TSFPVPSKFPLGPAAAVFGR	23	phos@3	53	1.8
6*	2457.22	1	ox(M)Pp(S)TSFPVPSKFPLGPAAAVFGR	23	Ox@1, phos@3	51	1.9
7	3217.62	151	p(S)Pp(ST)Ap(T)Lp(S)LPp(S)LEAYRDPS carb(C)LSPASSLSSR	180	phos@151,154,156,158 or 161; carb@169	10 to 15	1.10
8	3373.86		unknown		1 x phos		1.11

NFATc1 was phosphorylated with PKA and four different phosphorylation sites were detected (amino acids 3, 153, 245 and 338 or 339) in nine different peptides (peptides 1, 1*, 2, 2*, 3, 4, 5, 6 and 6*). Serine phosphorylation at site three was detected in four different peptides (peptides 1, 1*, 6, 6*) due to methionine oxidation (peptides 1* and 6*) and missed cleavage (peptides 6 and 6*). Phosphorylation at S-153 was detected two times due to missed cleavage (peptides 4 and 5). Serine 245 was detected to be phosphorylated in peptide 2 and its doubly oxidized from (peptide 2*). Peptide number 3 was singly phosphorylated from sites T-338 or T-339. No reliable phosphorylation site determination could be done for peptides 7 and 8. Phos = phosphorylation, carb = carbamidomethylation, Ox = oxidation, *) = supplementary image number.

Supplementary table 2

Pim-1 phosphorylated peptides used in method optimization (figure 2)

m/z	neutral loss in MS/MS	Mass shift after alkaline phosphatase treatment	*)
1257.56	-98 m/z	-80 m/z	S1
1273.55	-98 m/z	-80 + 16 m/z	S1*
1385.61	-98 m/z	-80 m/z	1.3
1417.63	-98 m/z	-80 m/z	S2*
1491.77	-98 m/z	-80 m/z	S3
2142.95	-98 m/z	-80 m/z	S4
2441.21	-98 m/z	-80 m/z	S5
2457.20	-98 m/z	-80 + 16 m/z	S5*
2962.60	-98 m/z	-80 m/z	S6
3042.32	-98 m/z	-80 m/z	S7
3217.51	-98 m/z	-80 m/z	S8
3233.45	-98 m/z	-80 m/z	S8*
3361.68		-80 m/z	S9
3373.58	-98 m/z	-80 m/z	S10

Supplementary Table 3. Observed phosphopeptides derived from 500 fmol of α- and β-caseins

Number	Peptide sequence	Protein (Swiss-Prot)[a]	Sequence Start-End	Number of phosphoryl groups	$[M+H]^+$ (*m/z*)[b]	Observed phosphopeptides with TiO_2 coated glass slide[c]	Observed phosphopeptides with ITO coated glass slide[d]
1	EQL<u>S</u>T<u>S</u>EENSK	CASA2_BOVIN	141-151	2	1411.5	x	
2	TVDME<u>S</u>TEVFTK	CAS2_BOVIN	153-164	1	1466.61	x	x
2*	(*Oxidized*)				1482.61	x	x
3	TVDME<u>S</u>TEVFTKK	CAS2_BOVIN	153-165	1	1594.71	x	x
3*	(*Oxidized*)				1610.70	x	x
4	VPQLEIVPN<u>S</u>AEER	CAS1_BOVIN	121-134	1	1660.79	x	x
5	YLGEYLIVPN<u>S</u>AEER	CAS1_BOVIN		1	1832.85	x	x
6	DIG<u>S</u>E<u>S</u>TEDQAMEDIK	CAS1_BOVIN	58-73	2	1927.69	x	x
6*	(*Oxidized*)				1943.69	x	x
7	YKVPQLEIVPN<u>S</u>AEER	CAS1_BOVIN	119-134	1	1951.95	x	x
8	FQ<u>S</u>EEQQQTEDELQDK	CASB_BOVIN	48-63	1	2061.83	x	x
9	IEKFQ<u>S</u>EEQQQTEDELQDK	CASB_BOVIN	45-63	1	2432.05	x	x
10	FQ<u>S</u>EEQQQTEDELQDKIHPF	CASB_BOVIN	48-67	1	2556.09		x
11	NTMEHV<u>SSS</u>EE<u>S</u>IISQETYK	CAS2_BOVIN	17-36	4	2618.90		x
11*	(*Oxidized*)				2634.90		x
12	VNEL<u>S</u>KDIG<u>S</u>E<u>S</u>TEDQAMEDIK	CAS1_BOVIN	52-73	3	2678.02		x
12*	(*Oxidized*)				2694.02	x	x
13	QMEAE<u>S</u>I<u>SSS</u>EEIVPN<u>S</u>VEQK	CAS1_BOVIN	74-94	5	2720.91	x	x
13*	(*Oxidized*)				2736.91		x
14	NTMEHV<u>SSS</u>EE<u>S</u>IISQETYKQ	CAS2_BOVIN	17-37	4	2746.96		x
14*	(*Oxidized*)				2762.96	x	x
15	EKVNEL<u>S</u>KDIG<u>S</u>E<u>S</u>TEDQAMEDIK	CAS1_BOVIN	50-73	3	2935.16		x
15*	(*Oxidized*)				2951.16		x
16	NANEEEYSIG<u>SSS</u>EE<u>S</u>AEVATEEVK	CAS2_BOVIN	61-85	4	3008.03	x	x
17	NANEEEYSIG<u>SSS</u>EE<u>S</u>AEVATEEVK	CAS2_BOVIN	61-85	5	3088.00	x	x
18	RELEELNVPGEIVE<u>S</u>L<u>SSS</u>EESITR	CASB_BOVIN	16-40	4	3122.27	x	x
19	KNTMEHV<u>SSS</u>EE<u>S</u>IISQETYKQEK	CAS2_BOVIN	16-39	4	3132.20		x
19*	(*Oxidized*)				3148.19	x	x

[a]P02662 CAS1_BOVIN, P02663 CAS2_BOVIN, P02666 CASB_BOVIN

[b]Known phosphopeptides detected by MALDI-MS (Stensballe 2004, Larsen 2005).

[c]Phosophopeptides reported in Imanishi *et al.* (2009)

[d]Phosphopeptides reported in this study