ELECTRONIC SUPPORTING INFORMATION

Evaluation of different isotope dilution mass spectrometry strategies for the characterisation of naturally abundant and isotopically labelled peptide standards

Jesús Nicolás Carcelén[1], Helí Potes Rodríguez[1], Adriana González-Gago[1], Juan Manuel Marchante-Gayón*[1], Alfredo Ballesteros[2], José Manuel González[2], José Ignacio García Alonso[1] and Pablo Rodríguez-González[1]
[1]Department of Physical and Analytical Chemistry. Faculty of Chemistry. University of Oviedo. Spain.
[2]Department of Organic and Inorganic Chemistry. Faculty of Chemistry. University of Oviedo. Spain.
*To whom correspondence should be addressed: marchant@uniovi.es

Number of Pages: 15

Number of Figures: 7

Number of Tables: 6

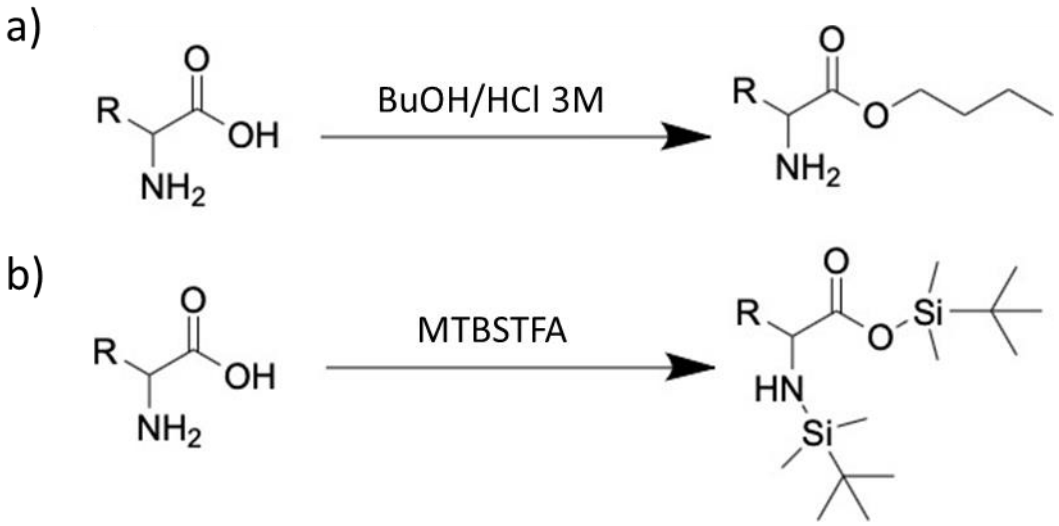

Figure S1.- Amino acids derivatization reactions: a) esterification for their determination by LC-MS/MS and b) silanization for their determination by GC-MS/MS.

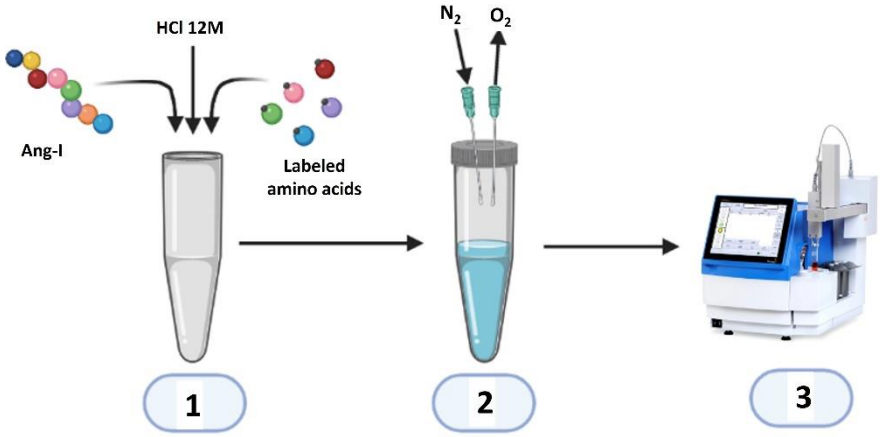

Figure S2.- Sample preparation procedure for the focused microwave assisted peptide hydrolysis.

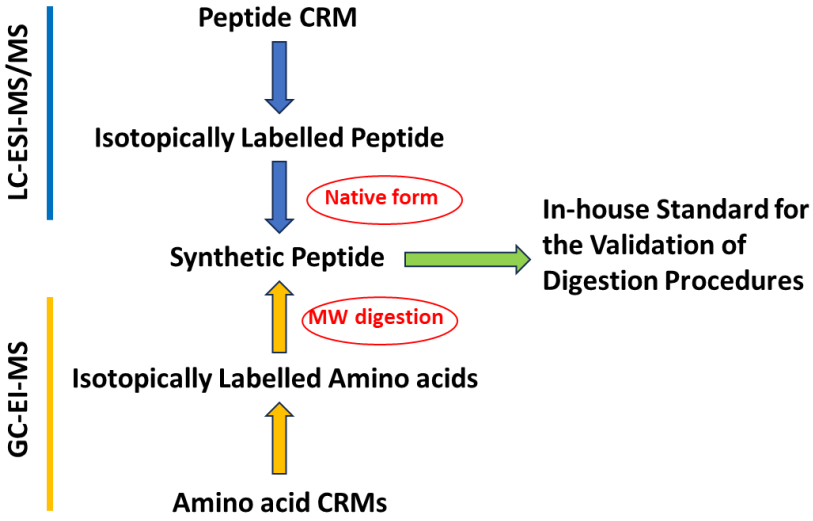

Figure S3. Study design carried out in this work

Table S1.- Chromatographic and mass spectrometric conditions for the analysis of esterified amino acids and angiotensin I by LC-MS/MS.

Liquid chromatograph	**Agilent Infinity 1290**
Column	Zorbax Eclipse Plus C18 (50 x 2.1mm x 1.8µm)
Flow	0.4 mL min^{-1}
Mobile phase A	H_2O, 0.1 % formic acid
Mobile phase B	ACN, 0.1 % formic acid
Separation program	0 min (5 %B) 1 min (5 %B) 8 min (40 %B) 9 min (60 %B) 10 min (5 %B) 12 min (5 %B)
Injection volume	1 µL
Column temperature	25ºC
Mass spectrometer	**Agilent 6460**
Ionization Source	Electrospray jet stream
Ionization mode	Positive
Gas temperature	250 °C
Gas flow	8 mL/min
Sheath gas T	300 °C
Sheath gas flow	10 mL/min
Nebulizer pressure	50 psi
Capillary voltage	2000 V
Nozzle voltage	500 V
Collision Energy	15 eV

Table S2.- Chromatographic and mass spectrometric conditions for the analysis of silanized amino acids by GC-MS.

Gas chromatograph	**Agilent 7879A**
Column	DB-5MS, 5%phenyl-95%dimethylpolysiloxane (30m x 0.25 mm x 0.25 µm)
Helium flow	2 mL min^{-1}
Separation program	40ºC (5 min) 40 - 105ºC (3.5 min) a 5ºC min^{-1} 105ºC - 120º (3 min) a 5 ºC min^{-1} 120º - 145º a 10 ºC min^{-1} 145º - 185º a 5 ºC min^{-1} 185 - 200º (0.5 min) a 10ºC min^{-1} Total time = 40 min
Injection volume	1 µL
Injection temperature	280ºC
Injection mode	Splitless
Mass spectrometer	**Agilent 7000**
Temperature of Transfer Line	250 ºC
Temperature of Ion Source	230ºC
Temperature of quadrupole analyzer	150ºC

Table S3.- Parameters used for the separation by semipreparative liquid chromatography of the synthesized peptides: natural and isotopically labelled angiotensin I.

Chromatograph	Agilent Infinity 1260	
Column	AerisPeptide XB-C18 (Phenomenex)	
Column dimensions	250x4.6mm x5µm	
Temperature	25 °C	
Detection wavelenght	280 nm	
Injection volume	100 µL	
Flow	2 mL/min	
Phase A	0.1% formic acid in water	
Phase B	ACN, 0.1% formic acid	
Separation	Time (min)	- % B
	0	8
	5	8
	24	70
	29	70
	29.5	8
	49.5	8

Table S4.- Measured transitions, theorical and experimental values of the isotopic abundances of these transitions and standard deviation of the measurements for the reported amino acids obtained from n=5 injections in the LC-MS/MS system.

Amino acid	MRM Transition Protonated molecules→Product ions	Theoretical value	Experimental value	Standard deviation (n=5)
Arginine	231→214	0.8819	0.8828	0.0013
	232→215	0.1078	0.1070	0.0014
	233→216	0.0097	0.0096	0.0002
	234→217	0.0006	0.0006	0.0001
$^{15}N_4$-Arginine*	235→217	0.8916	0.8916	0.0014
	236→218	0.0991	0.0996	0.0011
	237→219	0.0087	0.0083	0.0004
	238→220	0.0006	0.0004	0.0001
Proline	172→116	0.9385	0.9367	0.0001
	173→117	0.0560	0.0575	0.0001
	174→118	0.0052	0.0055	0.0000
	175→119	0.0002	0.0003	0.0000
$^{13}C_1$-Proline	172→116	0.0000	0.0004	0.0001
	173→117	0.9489	0.9482	0.0003
	174→118	0.0463	0.0464	0.0002
	175→119	0.0048	0.0050	0.0001
Valine	174→118	0.9383	0.9371	0.0014
	175→119	0.0562	0.0594	0.0016
	176→120	0.0053	0.0035	0.0003
	177→121	0.0002	0.0000	0.0000
$^{13}C_1$-Valine	174→118	0.0012	0.0005	0.0006
	175→119	0.9485	0.9494	0.0013
	176→120	0.0465	0.0454	0.0016
	177→121	0.0048	0.0047	0.0008
Tyrosine	238→182	0.8966	0.8972	0.0019
	239→183	0.0928	0.0928	0.0016
	240→184	0.0099	0.0096	0.0005
	241→185	0.0007	0.0005	0.0001
$^{13}C_2$-Tyrosine	238→182	0.0001	0.0222	0.0004
	239→183	0.0199	0.0189	0.0006

	240→184	0.9057	0.8815	0.0005
	241→185	0.0742	0.0696	0.0006
Leucine	188→132	0.9281	0.9227	0.0013
	189→133	0.0658	0.0698	0.0014
	190→134	0.0058	0.0073	0.0005
	191→135	0.0003	0.0001	0.0001
$^{13}C_1$-Leucine	188→132	0.0096	0.0002	0.0001
	189→133	0.9293	0.9355	0.0008
	190→134	0.0559	0.0593	0.0007
	191→135	0.0052	0.0050	0.0004
Isoleucine	188→69	0.9467	0.9472	0.0003
	189→70	0.0522	0.0517	0.0002
	190→71	0.0012	0.0011	0.0000
	191→72	0.0000	0.0000	0.0000
$^{13}C_1$-Isoleucine**	189→69	0.9467	0.9476	0.0017
	190→70	0.0522	0.0525	0.0015
	191→71	0.0012	0.0011	0.0001
	192→72	0.00001	0.00004	0.00003
Phenylalanine	222→166	0.8988	0.9001	0.0007
	223→167	0.0927	0.0917	0.0007
	224→168	0.0080	0.0078	0.0001
	225→169	0.0005	0.0004	0.0001
$^{13}C_1$-Phenylalanine	222→166	0.0049	0.0055	0.0009
	223→167	0.9054	0.9039	0.0010
	224→168	0.0828	0.0834	0.0008
	225→169	0.0069	0.0071	0.0007

*Loss of $^{15}N_1$

**Loss of $^{13}C_1$

Table S5.- Measured transitions, theorical and experimental values of the isotopic abundances of these transitions and standard deviation of the measurements for the reported amino acids obtained from n=5 injections in the GC-MS/MS system.

Amino acid	MRM Transition Protonated molecules→Product ions	Theoretical value	Experimental value	Standard deviation (n=5)
Proline	286 → 258	0.7306	0.7421	0.0003
	287 → 259	0.1838	0.1771	0.0006
	288 → 260	0.0737	0.0700	0.0006
	289 → 261	0.0118	0.0109	0.0001
$^{13}C_1$-Proline*	287 → 258	0.7421	0.7309	0.0007
	288 → 259	0.1771	0.1829	0.0006
	289 → 260	0.0700	0.0743	0.0003
	290 → 261	0.0109	0.0019	0.0001
Valine	288 → 260	0.7419	0.7283	0.0005
	289 → 261	0.1772	0.1840	0.0003
	290 → 262	0.0700	0.0753	0.0003
	291 → 263	0.0109	0.0124	0.0001
$^{13}C_1$-Valine*	289 → 260	0.7419	0.7279	0.0003
	290 → 261	0.1772	0.1843	0.0003
	291 → 262	0.0700	0.0758	0.0002
	292 → 263	0.0109	0.0121	0.0001
Tyrosine	466 → 438	0.6155	0.6072	0.0009
	467 → 439	0.2461	0.2481	0.0006
	468 → 440	0.1105	0.1154	0.0004
	469 → 441	0.0280	0.0293	0.0002
$^{13}C_2$-Tyrosine	468 → 440	0.6285	0.6266	0.0010
	469 → 441	0.2377	0.2377	0.0007
	470 → 442	0.1076	0.1090	0.0008
	471 → 443	0.0262	0.0266	0.0003
Leucine	302 → 274	0.7339	0.7177	0.0010
	303 → 275	0.1834	0.1914	0.0004
	304 → 276	0.0712	0.0778	0.0005
	305 → 277	0.0115	0.0131	0.0001
$^{13}C_1$-Leucine*	303 → 274	0.7339	0.7185	0.0009
	304 → 275	0.1834	0.1907	0.0009
	305 → 276	0.0712	0.0777	0.0003

	306 → 277	0.0115	0.0131	0.0004
Isoleucine	302 → 274	0.7339	0.7141	0.0006
	303 → 275	0.1834	0.1931	0.0010
	304 → 276	0.0712	0.0795	0.0005
	305 → 277	0.0115	0.0132	0.0003
$^{13}C_1$-Isoleucine*	303 → 274	0.7339	0.7171	0.0019
	304 → 275	0.1834	0.1919	0.0014
	305 → 276	0.0712	0.0778	0.0004
	306 → 277	0.0115	0.0132	0.0002
Phenylalanine	336 → 308	0.7110	0.6971	0.0007
	337 → 309	0.2006	0.2079	0.0003
	338 → 310	0.0749	0.0803	0.0006
	339 → 311	0.0135	0.0147	0.0003
$^{13}C_1$-Phenylalanine*	337 → 308	0.7110	0.7064	0.0022
	338 → 309	0.2006	0.2038	0.0026
	339 → 310	0.0749	0.0760	0.0012
	340 → 311	0.0135	0.0137	0.0006
Arginine	213 → 171	0.7912	0.7734	0.0008
	214 → 172	0.1389	0.1487	0.0004
	215 → 173	0.0629	0.0699	0.0003
	216 → 174	0.0070	0.0080	0.0001
$^{15}N_4$-Arginine**	215 → 173	0.7962	0.7934	0.0006
	216 → 174	0.1350	0.1367	0.0005
	217 → 175	0.0622	0.0632	0.0002
	218 → 176	0.0066	0.0067	0.0001

*Loss of $^{13}C_1$

**Loss of $^{15}N_2$

Table S6.- Measured transitions, theorical and experimental values of the isotopic abundances for the three transitions measured and standard deviations of the measurements by LC-MS/MS for natural abundance and isotopically labelled angiotensin I.

Compound	Transition	Theoretical value	Experimental value	Standard deviation (n=5)
Angiotensin I	433→534	0.7378	0.7311	0.0006
	433→535	0.2154	0.2225	0.0007
	433→536	0.0410	0.0399	0.0005
	433→537	0.0058	0.0065	0.0002
$^{13}C_1$-Angiotensin I	433→534	0.0057	0.0253	0.0010
	433→535	0.7416	0.7377	0.0042
	433→536	0.2084	0.1983	0.0035
	433→537	0.0389	0.0387	0.0015
Angiotensin I	433→647	0.6871	0.6852	0.0013
	433→648	0.2464	0.2519	0.0014
	433→649	0.0556	0.0530	0.0005
	433→650	0.0094	0.0100	0.0002
$^{13}C_1$-Angiotensin I	433→647	0.0062	0.0075	0.0003
	433→648	0.6904	0.7087	0.0031
	433→649	0.2399	0.2351	0.0029
	433→650	0.0532	0.0487	0.0014
Angiotensin I	433→619	0.6962	0.6938	0.0011
	433→620	0.2419	0.2404	0.0009
	433→621	0.0523	0.0504	0.0005
	433→622	0.0084	0.0080	0.0002
$^{13}C_1$-Angiotensin I	433→619	0.0063	0.0093	0.0003
	433→620	0.6995	0.6978	0.0031
	433→621	0.2352	0.2342	0.0033
	433→622	0.0499	0.0482	0.0001

Figure S4. Separation of amino acids mixtures at 10 µg·g^{-1} detected in MRM mode: A) LC and B) GC.

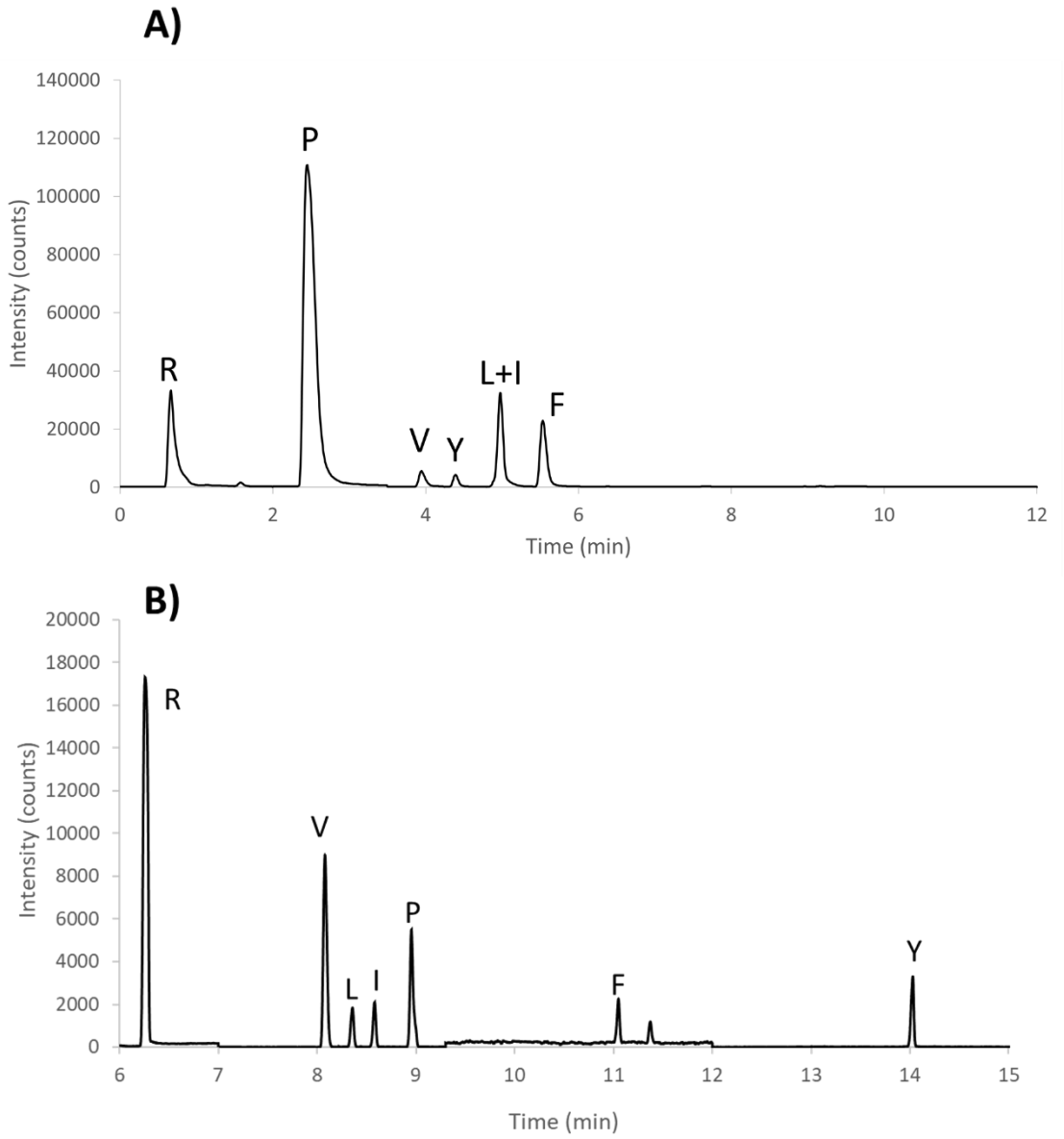

Figure S5.- Mean recoveries obtained for the CRM SRM 998 Angiotensin I by classical acid hydrolysis measuring the amino acids cleaved by GC-MS/MS.

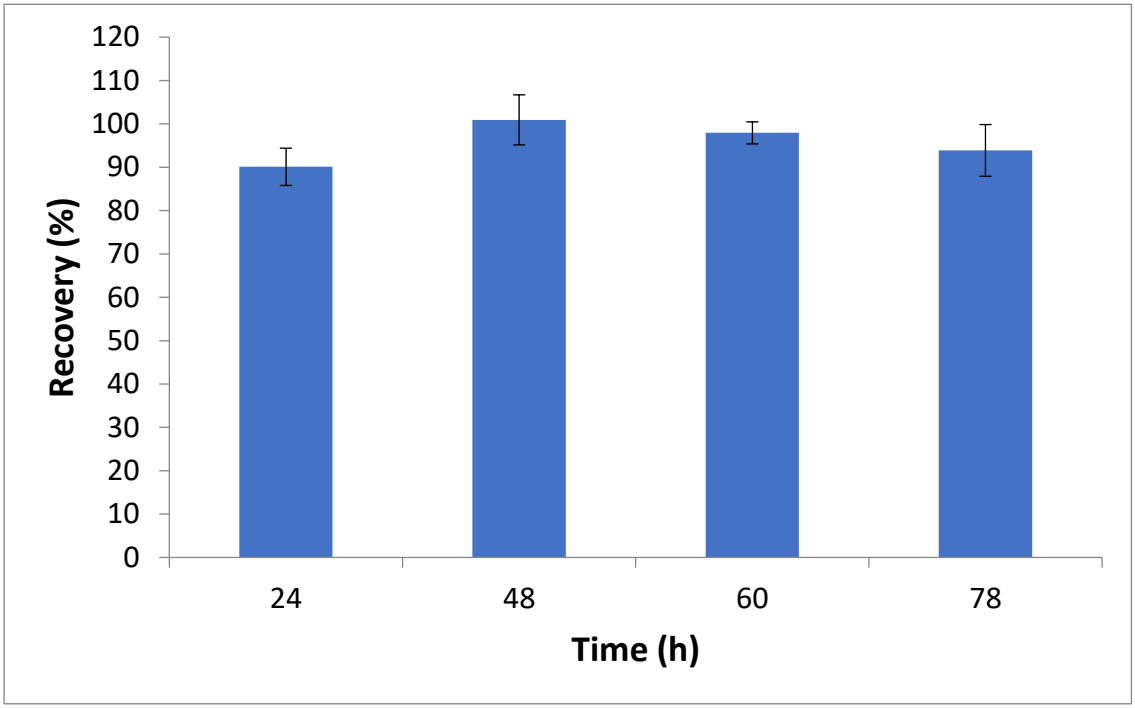

Figure S6. Mass spectra of the product ions for the precursor ion $[M+H_3]^{3+}$ at mass 432.8 (natural angiotensin, top) and 433.2 (labelled angiotensin, bottom).

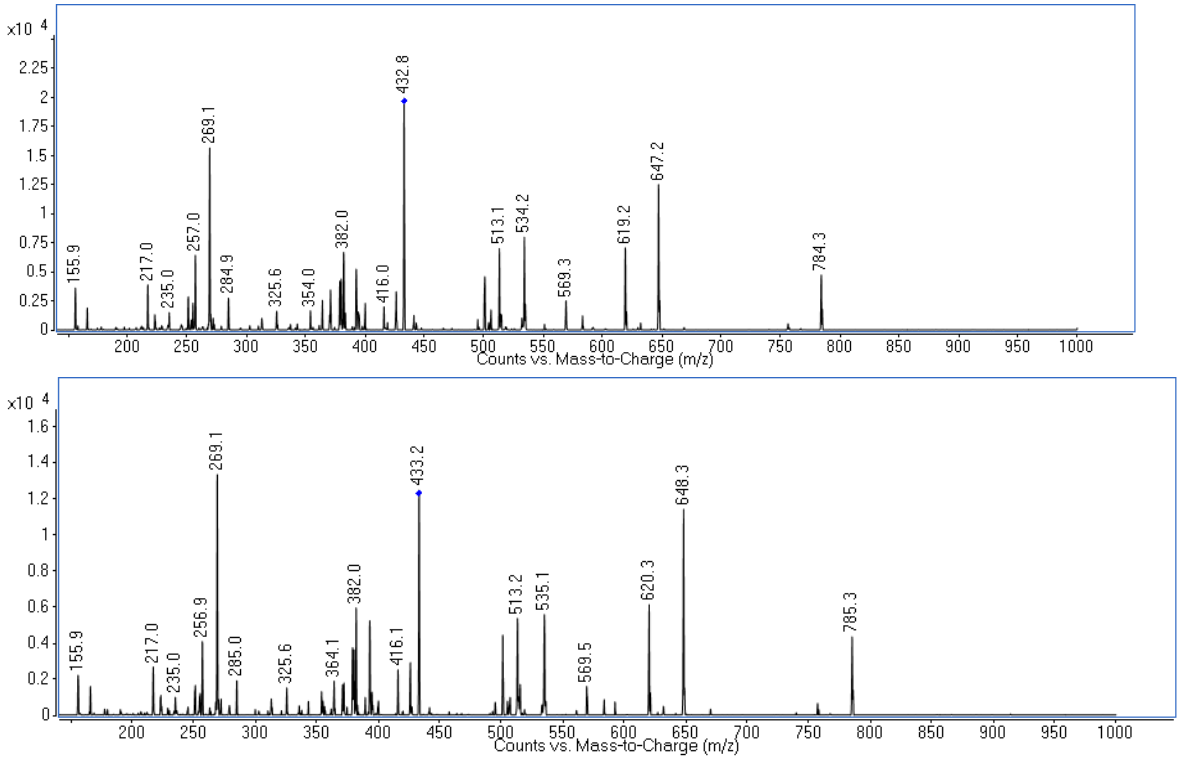

Figure S7.- Precursor ion scan of the mixture of natural and isotopically enriched angiotensin for the product ion 647.7, 648.7, 649.7 and 650.7 corresponding to ion b5+ in four transmission conditions: G=20, W=4, B) G=10, W=4, C) G=10, W=2, D) G=10, W=1. (G= gain offset, W= width offset)