

# Fermented soy-derived bioactive peptides selected by a molecular docking approach show antioxidant properties involving the Keap1/Nrf2 pathway in a cellular model

Federica Tonolo <sup>1, #</sup>, Laura Moretto <sup>1, #</sup>, Alessandro Grinzato <sup>1, #</sup>, Federico Fiorese <sup>1</sup>, Alessandra Folda <sup>1</sup>, Valeria Scalcon <sup>1</sup>, Stefania Ferro <sup>1</sup>, Giorgio Arrigoni <sup>1,2,3</sup>, Marco Bellamio <sup>4</sup>, Emiliano Feller <sup>4</sup>, Alberto Bindoli <sup>5</sup>, Oriano Marin <sup>1,\*</sup>, and Maria Pia Rigobello <sup>1,\*</sup>

<sup>1</sup> Department of Biomedical Sciences, University of Padova, via Ugo Bassi 58/b, 35131 Padova, Italy

<sup>2</sup> Proteomics Center, University of Padova and Azienda Ospedaliera di Padova

<sup>3</sup> CRIBI, Biotechnology Center, University of Padova

<sup>4</sup> Centrale del Latte di Vicenza, UO di Centrale del Latte d'Italia, via Alessandro Faedo, 60 Vicenza;

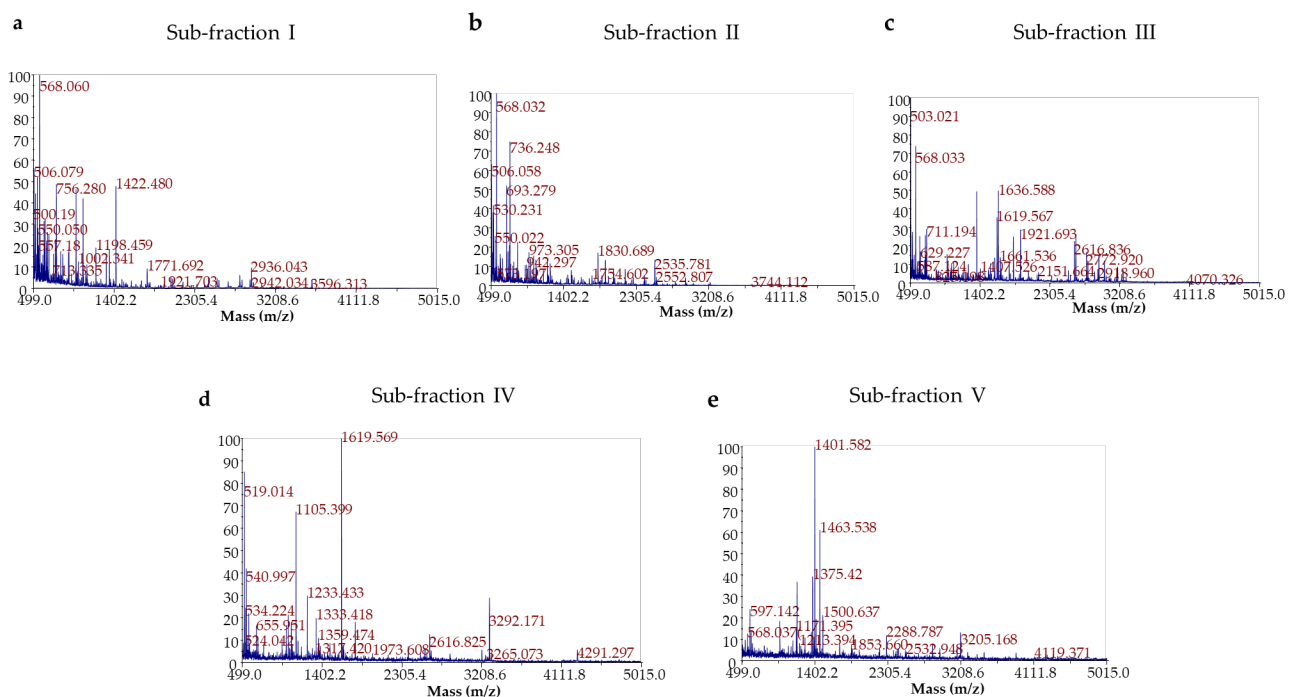
<sup>5</sup> Institute of Neuroscience (CNR), viale G. Colombo 3, 35131, Padova, Italy;

\* Correspondence: [mariapia.rigobello@unipd.it](mailto:mariapia.rigobello@unipd.it);

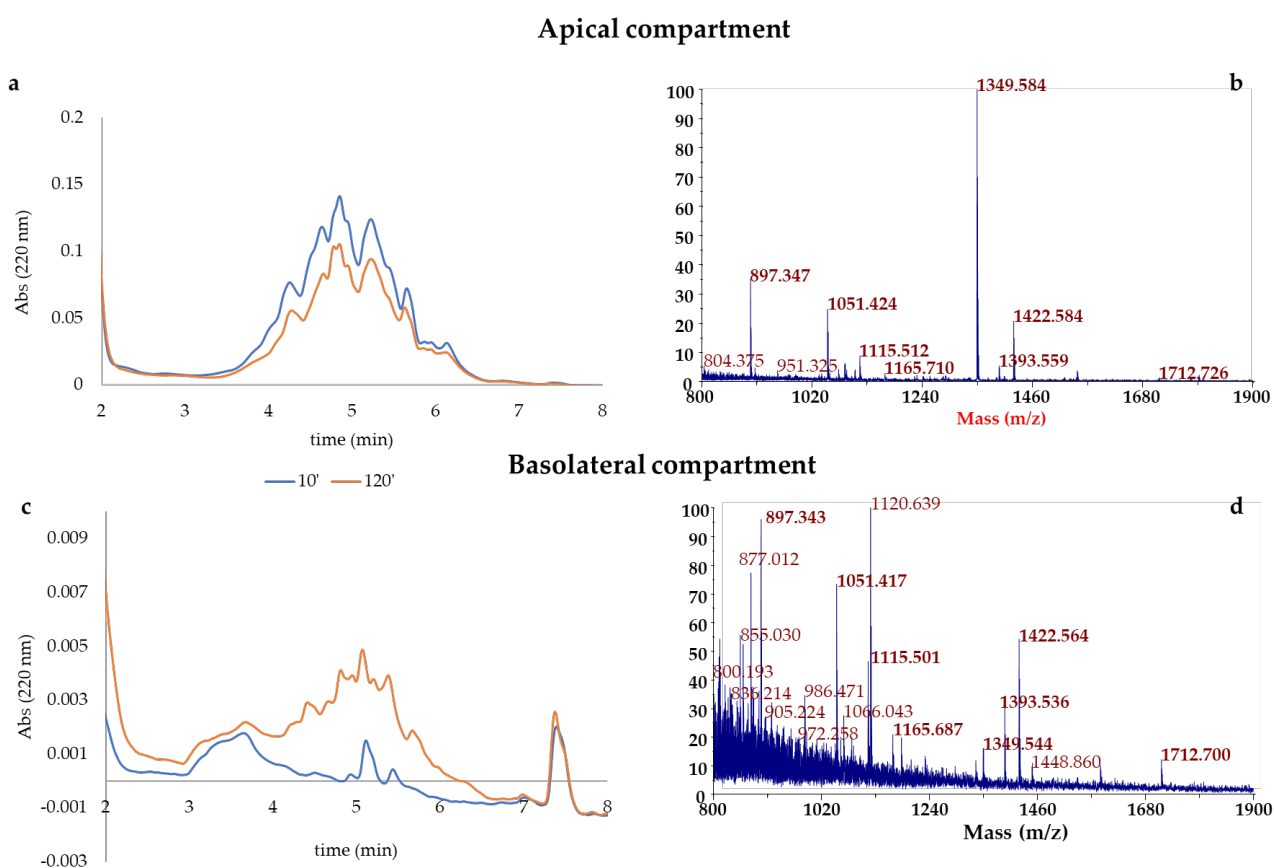
# These authors contributed equally

**Table S1.** Analysis of the antioxidant activity of 5-30% ACN *in vitro* and in a cellular model. ABTS and DPPH assays were performed for *in vitro* analysis and values were indicated respectively as TEAC ( $\mu\text{M}$  Trolox C) and percentage of DPPH scavenging inhibition. MTT test was carried out in order to evaluate cell viability in Caco-2 cells treated with the enriched peptide fraction for 24 h. Oxidative stress was induced with TbOOH (200  $\mu\text{M}$ ) for 18 h. n.d.: not detectable; \*\*\*  $p < 0.001$

	<i>In vitro</i> analysis		Cell viability in Caco-2 cells	
	TEAC ( $\mu\text{M}$ Trolox C)	DPPH scavenging inhibition (%)	None	TbOOH
Cnt	n.d.	n.d.	100.00 $\pm$ 0.00	62.11 $\pm$ 7.19
5-30% ACN fraction	0.57 $\pm$ 0.37 (***)	15.27 $\pm$ 5.98 (***)	123.26 $\pm$ 8.44	95.06 $\pm$ 10.96 (***)



**Figure S1.** Examples of mass spectrometry spectra of sub-fractions I, II, III, IV and V. **a)** Sub-fraction I. **b)** Sub-fraction II. **c)** Sub-fraction III. **d)** Sub-fraction IV. **e)** Sub-fraction V.



**Figure S2.** Transepithelial transport in Caco-2 cell monolayer of sub-fraction I. Cells were grown on Transwell inserts for 21 days to let their differentiation. Then the experiment was carried out as reported in Materials and Methods section. RP-HPLC analysis of apical (AP) compartment (a) and basolateral (BL) compartment (c) of cells treated with sub-fraction I for 10 min (blue) and 120 min (orange) are reported. Mass spectrometry analysis of AP compartment (b) and BL compartment (d) of cells treated with sub-fraction I for 120 min are depicted. Peptides that are able to cross the Caco-2 monolayer without modifications are reported in bold.

**Table S2.** Analysis of the antioxidant activity of sub-fractions I, II, III, IV and V *in vitro*. ABTS assay were performed for *in vitro* analysis and values were indicated respectively as TEAC ( $\mu\text{M}$  Trolox C). \*\*\*  $p < 0.001$

Sub-fraction	TEAC ( $\mu\text{M}$ Trolox C)
I	$0.95 \pm 1.34$
II	$1.87 \pm 1.15$ (***)
III	$1.95 \pm 1.52$ (***)
IV	$2.64 \pm 1.41$ (***)
V	$2.78 \pm 1.18$ (***)

**Table S3. Files from Orbitrap analysis of sub-fraction I**

	Sequence	# PSMs	# Proteins	# Protein Groups	Protein Group Accessions	Modifications	$\Delta Cn$	q-Value	PEP	IonScore	Exp Value	Charge	MH+ [Da]	$\Delta M$ [ppm]	RT [min]
High	VNPESQQGSPR	3	2	1	P02858		0.0000	0	0.00001754	79	3.29476E-05	2	1198.58220	0.88	13.23
High	REQDEDEDEDEDKPRPSRPSQG	2	2	1	P02858		0.0000	0	0.0000027	54	0.004150825	4	2614.12905	1.58	13.60
High	KQGQHQQEEEEEGGSV	2	2	1	P02858		0.0000	0	0.00003189	51	0.010716809	2	1798.78740	1.95	12.92
High	NNQLDQTPR	2	2	1	P02858		0.0000	0	0.0005204	50	0.019479559	2	1085.53459	1.05	14.78
High	NALEPDHRVESEGG	1	3	2	P02858; A0A0R0GMV1		0.0000	0	0.005443	49	0.031992697	2	1509.69512	1.47	15.69
High	EQDEDEDEDEDKPRPSRPSQG	1	2	1	P02858		0.0000	0	0.001727	47	0.009999094	3	2458.02976	2.42	13.95
High	WQEQQDEDEDE	1	2	1	P02858		0.0000	0	0.0003587	44	0.002887334	2	1450.52458	0.30	16.65
High	DEQIPSHPPR	1	2	1	P02858		0.0000	0	0.006591	37	0.572071635	2	1175.58232	1.63	15.86
High	DEDEDEDEDEQIPSHPPRRPSHG	1	2	1	P02858		0.0000	0	1.509E-08	36	0.169319411	4	2557.08559	1.28	16.00
High	EQDEDEDEDEDKPRPSRPS	1	2	1	P02858		0.0000	0	0.0002238	33	0.296062992	3	2272.94785	1.80	14.01
High	QGQHQQEEEEEGGSV	1	2	1	P02858		0.0000	0	0.002631	27	1.37252143	2	1670.69341	2.68	14.27
High	KEQQQEQQQEEQPLE	1	1	1	P0DO15		0.0000	0	0.000009733	74	9.53075E-05	2	1898.87700	2.26	15.63
High	IGINAENNQRN	1	1	1	P0DO15		0.0000	0	0.01234	67	0.000469711	2	1242.61943	0.68	15.67
High	LFSREEGQQQGEQ	1	1	1	P0DO15		0.0000	0	0.01571	61	0.001976832	2	1535.71123	1.75	15.96
High	SREEGQQQGEQRLQE	2	1	1	P0DO15		0.0000	0	7.355E-08	59	0.003467165	3	1801.84696	2.52	14.14
High	FSREEGQQQGEQ	2	1	1	P0DO15		0.0000	0	0.002445	59	0.002160545	2	1422.62663	1.52	13.31
High	KEQQQEQQQEEQPL	1	1	1	P0DO15		0.0000	0	0.01296	58	0.004813334	2	1769.83599	3.32	15.84
High	EQQQEQQQEEQPLE	1	1	1	P0DO15		0.0000	0	0.0003122	54	0.005880889	2	1770.78081	1.73	16.99
High	SLVNNDDRDS	3	1	1	P0DO15		0.0000	0	0.05486	48	0.026660789	2	1134.50237	0.13	16.34
High	SREEGQQQGEQRLQES	1	1	1	P0DO15		0.0000	0	0.00000703	46	0.063416736	3	1888.87552	0.57	14.20
High	VGLKEQQQEQ	1	1	1	P0DO15		0.0000	0	0.0264	44	0.091111039	2	1186.60820	1.61	14.25
High	LKEQQQEQQQEEQPLE	2	1	1	P0DO15		0.0000	0	0.00008758	39	0.42622471	2	2011.96257	2.88	16.58
High	VGLKEQQQEQQ	1	1	1	P0DO15		0.0000	0	0.02885	37	0.542579939	2	1314.66655	1.28	14.36
High	QREEQEWPRKEEK	1	1	1	P0DO15		0.0000	0	0.00003053	25	10.75979545	4	1771.87368	0.79	13.47

High	PQHPEREPQQPGEKEEDE	1	1	1	P0DO15		0.0000	0	0.001634	24	6.904048974	3	2158.96762	1.83	13.66
High	FGREGQQQGEE	1	1	1	P11827		0.0000	0	0.002668	60	0.001233267	2	1393.59844	0.36	14.21
High	GREGQQQGEERLQE	2	1	1	P11827		0.0000	0	3.254E-08	59	0.002900604	3	1772.81980	2.21	14.58
High	FVDAQPQQKEEGN	3	1	1	P11827		0.0000	0	0.008778	59	0.00288809	2	1489.69463	1.88	15.57
High	FVDAQPQ	1	3	2	P11827;F7J077		0.0000	0	0.004484	51	0.023412219	2	804.38951	1.03	16.56
High	GREGQQQGEERLQES	1	1	1	P11827		0.0000	0	1.368E-07	50	0.020554019	3	1859.85184	2.11	14.59
High	FVDAQPQQKEEG	1	3	2	P11827;F7J077		0.0000	0	0.001301	48	0.039871276	2	1375.65141	1.83	15.56
High	TLVNNDDRDS	2	3	2	P11827;F7J077		0.0000	0	0.003046	45	0.057454064	2	1148.51921	1.17	14.72
High	DEEQDKESQESEGSQREPR	2	1	1	P11827		0.0000	0	0.000002046	39	0.074475433	4	2479.04726	0.81	13.72
High	EREHPRPHQPHQKEEEKHE	2	1	1	P11827		0.0000	0	0.00008161	34	1.664037557	4	2457.18374	2.85	8.26
High	ERQQHGEKEEDEGEQPRP	1	1	1	P11827		0.0000	0	3.237E-07	31	1.395967223	4	2177.98086	0.06	12.24
High	EEEDQDEDEEQDKESQESEGSQREPR	1	1	1	P11827		0.0000	0	0.000007023	27	0.226686836	4	3353.33632	2.44	14.76
High	REHPRPHQPHQKEEEKHE	1	1	1	P11827		0.0000	0	8.73251E-09	26	10.51995116	5	2328.14069	2.82	8.23
High	EQDKESQESEGSQREPR	1	1	1	P11827		0.0000	0	0.04485	23	6.231339126	3	2234.97910	1.52	12.34
High	DEREHPRPHQPHQKEEEKHE	1	1	1	P11827		0.0000	0	0.0001336	16	82.67284865	4	2572.21938	6.10	8.38
High	QPHQEEEHEQKEEHEWHRKEE	1	1	1	P11827		0.0000	0	0.02159	16	34.39622416	5	2808.23835	0.92	13.10
High	HGGKGSEEEQDEREHPRPHQPHQKEEEKHE	2	1	1	P11827		0.0000	0	0.002019	14	92.62774546	7	3610.64238	0.55	8.53
High	HEQKEEHEWHRKEE	1	1	1	P11827		0.0000	0	0.05024	12	167.7327612	4	1930.88540	3.24	8.47
High	VVAEQGGEQGLE	2	1	1	A0A0R0GMV1		0.0000	0	5.523E-07	82	1.49779E-05	2	1215.58672	1.21	17.41
High	NNQLDQNPR	3	1	1	A0A0R0GMV1		0.0000	0	0.02175	59	0.002769075	2	1098.52983	1.03	13.71
High	VAEQGGEQGLE	1	1	1	A0A0R0GMV1		0.0000	0	0.003627	57	0.00395427	2	1116.51775	0.83	16.44
High	NALEPDHRVESEGG	1	3	2	P02858; A0A0R0GMV1		0.0000	0	0.005443	49	0.031992697	2	1509.69512	1.47	15.69
High	AGNPDIEHPET	1	1	1	A0A0R0GMV1		0.0000	0	0.03474	45	0.053652047	2	1179.52861	0.76	16.24
High	FVDAQPQQKEEGSKG	1	2	1	F7J077		0.0000	0	1.466E-08	67	0.000658928	2	1647.80388	3.95	14.47
High	FVDAQPQQKEEGS	1	2	1	F7J077		0.0000	0	0.01407	53	0.011572444	2	1462.68462	2.52	15.54
High	FVDAQPQ	1	3	2	P11827;F7J077		0.0000	0	0.004484	51	0.023412219	2	804.38951	1.03	16.56
High	FVDAQPQQKEEG	1	3	2	P11827;F7J077		0.0000	0	0.001301	48	0.039871276	2	1375.65141	1.83	15.56

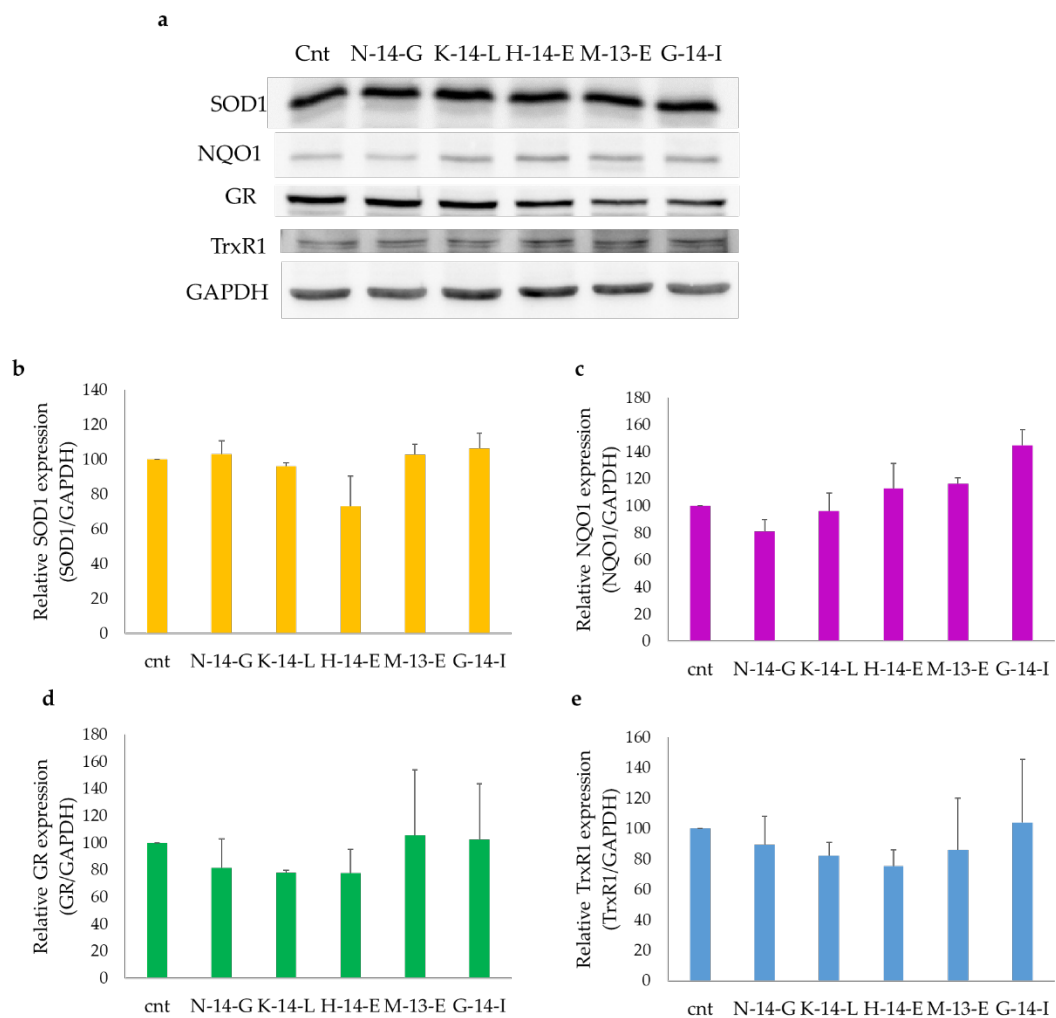
High	TLVNNDDRDS	2	3	2	P11827;F7J077		0.0000	0	0.003046	45	0.057454064	2	1148.51921	1.17	14.72
High	QRIPAGTT	1	2	1	F7J077		0.0000	0	0.01148	31	1.315264888	2	843.47093	3.09	14.46
High	LKVREDENNP	1	2	1	F7J077		0.0000	0	0.05486	30	2.954393028	2	1213.61785	0.53	14.19
High	IMSDESTESETEQ	1	2	1	O64458		0.0000	0	0.000002172	88	6.47571E-07	2	1485.59160	1.22	16.37
High	IMSDESTESETEQA	1	2	1	O64458		0.0000	0	0.00009654	63	0.000252387	2	1556.62859	1.08	17.15
High	YQEPQESQQRG	1	1	1	P04405		0.0000	0	0.0009055	55	0.006528817	2	1349.61199	2.90	14.13
High	YLAGNQEQE	17	2	2	P04776;P04405		0.0000	0	0.0109	54	0.007138189	2	1051.46929	0.17	22.14
High	RNLQGENEEDSGA	1	1	1	P04405		0.0000	0	0.0000809	52	0.005817199	2	1547.66057	2.36	14.54
High	MRKPQQEEDDDDE	1	1	1	P04405		0.0000	0	0.02409	41	0.03625453	2	1634.66533	3.28	12.65
High	RQNIGQNSSPD	2	1	1	P04405		0.0000	0	0.02188	41	0.180812407	2	1215.57109	-0.17	13.18
High	FNENESGDQV	1	1	1	P05046		0.0000	0	0.006334	61	0.000571086	2	1138.46489	0.11	17.17
High	NKVDENGTPKPS	4	1	1	P05046		0.0000	0	0.002978	57	0.005876098	2	1285.63957	0.97	12.00
High	GGTGGTDYGTGGTG	1	1	1	K7LEQ5		0.0000	0	0.0057	78	1.279E-05	2	1258.52141	2.49	15.19
High	YGNVEKQTDE	1	1	1	K7LEQ5		0.0000	0	0.0003858	47	0.030024099	2	1182.52995	2.19	14.13
High	YGTNTADTGTGPR	1	1	1	K7LEQ5		0.0000	0	0.001024	41	0.187711656	2	1310.60063	2.63	14.68
High	NLQGENEGEDKGA	1	1	1	P04776		0.0000	0	0.007443	54	0.005853614	2	1360.59941	1.33	13.99
High	YLAGNQEQE	17	2	2	P04776;P04405		0.0000	0	0.0109	54	0.007138189	2	1051.46929	0.17	22.14
High	IKPPTDEQQQRPQE	2	1	1	P04776		0.0000	0	0.0218	41	0.273058321	2	1693.85539	2.91	15.43
High	VIKPPTDEQQQRPQE	1	1	1	P04776		0.0000	0	0.00006892	39	0.449881374	3	1792.91965	0.43	15.98
High	IKPPTDEQQQRPQEE	1	1	1	P04776		0.0000	0	0.0004184	32	2.554844876	3	1822.89640	1.83	15.51
High	VIKPPTDEQQQRPQEE	1	1	1	P04776		0.0000	0	0.000217	28	5.829024329	3	1921.96305	0.81	16.17
High	IKPPTDEQQQRPQEEEE	1	1	1	P04776		0.0000	0	0.001827	25	9.864223021	3	2080.98337	2.46	15.83
High	GKHQQEENEENEGGSI	1	1	1	P04776		0.0000	0	0.04737	23	8.540962679	2	1541.68645	2.42	13.54
High	IKPPTDEQQQRPQEEEE	1	1	1	P04776		0.0000	0	0.03119	20	34.28549271	3	1951.93778	1.08	15.76
High	QAALQTPK	1	3	1	I1L860		0.0000	0	0.04134	42	0.075457449	2	856.48949	0.88	15.05
High	QAALQTPKG	1	3	1	I1L860		0.0000	0	0.02992	31	1.144109738	2	913.51079	0.64	15.38

**Table S4.** Residues involved in the binding of Keap1 with the analyzed peptides. The residues involved in the binding between Keap1 and Nrf2 are highlighted in red

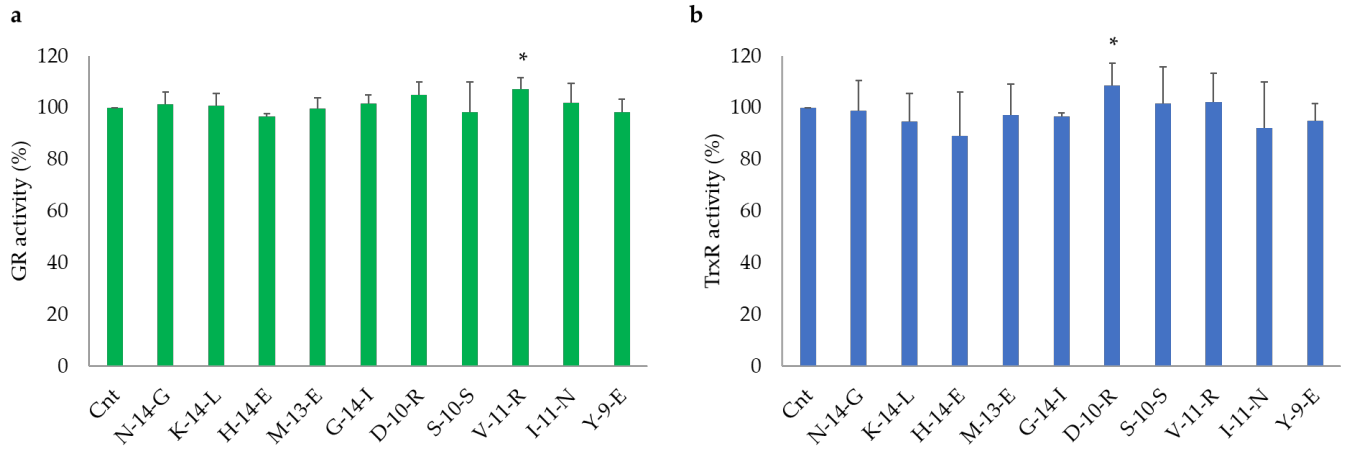
Keap 1	N-14-G	K-14-L	H-14-E	M-13-E	G-14-I	D-10-R	S-10-S	F-12-G	V-11-R	I-11-N	F-12-E	Y-9-E
Y 334	H7	E2					N5	P6	R11			
R 336			E5								K9	
S 363			R11		G12					Q9	E10	
R 380	E10, E12	L14	K4	E8	E10	S6	D9	D3, Q5, Q8			E10	Q7, E9
N 382			E5			H7		Q5		R10	Q8	
D 385			Q3							R10		
N 387	E12		H1, E5					D3				
D 389			Q3	Q6	K2							
N 414	R8	E6	E14		E10		R8					
R 415	D6	E6	E8, E14	D9, D11		E2	N5, D7	D3, G12	Q7	E6, N11	F1, Q5, E12	E9
S 431		E6		E7								
H 432	G 14											
H 436				E7								
R 483		E10; E11	E8	D9, D10				E11	E4	E6	F2	
S 508										E6		
Y 525							V3				D3	
Q 530								E10		E6		
Y 572					E6				R11		K9	
S 602	D6	Q12	R11	D12	S13					N7	G10	

**Table S5.** ABTS assay with identified soy peptides (0.05 mg/mL). Values were indicated as TEAC ( $\mu\text{M}$  Trolox)

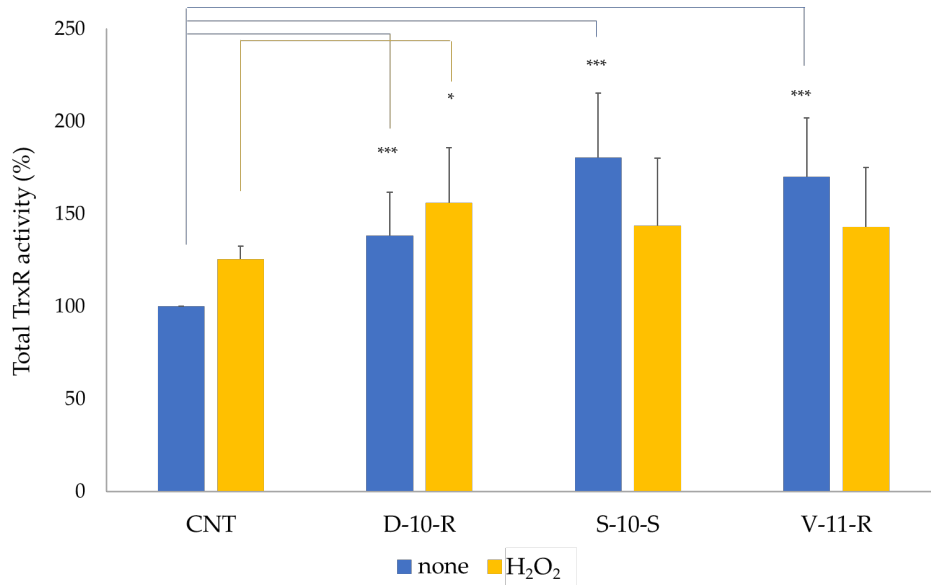
Peptide	TEAC ( $\mu\text{M}$ Trolox)
N-14-G	$0.12 \pm 0.22$
K-14-L	$0.34 \pm 0.20$
H-14-E	$0.63 \pm 0.27$
M-13-E	$0.25 \pm 0.13$ (***)
G-14-I	$0.14 \pm 0.43$
D-10-R	n.d.
S-10-S	n.d.
F-12-G	n.d.
V-11-R	n.d.
I-11-N	n.d.
F-12-E	n.d.
Y-9-E	n.d.
F-12-Q	n.d.



**Figure S3.** Western Blot analysis of antioxidant and phase II enzymes in Caco-2 cell lysates treated with N-14-G, K-14-L, H-14-E, M-13-E and G-14-I (0.05 mg/mL) for 24 h (a). **b-e)** Quantification of the above reported Western Blot.



**Figure S4.** GR (a) and TrxR activity (b) in Caco-2 cell lysates treated with soy peptides (0.05 mg/mL) for 24 h.



**Figure S5.** Total TrxR activity (%) in Caco-2 cells treated with **D-10-R**, **S-10-S** and **V-11-R** (0.05 mg/mL) in the presence or absence of 2.5 mM H<sub>2</sub>O<sub>2</sub> for 48 h.