Supplemental Information

Identification of Bitter Peptides in Aged Cheddar Cheese by Crossflow Filtration-based Fractionation, Peptidomics, Statistical Screening and Sensory Analysis

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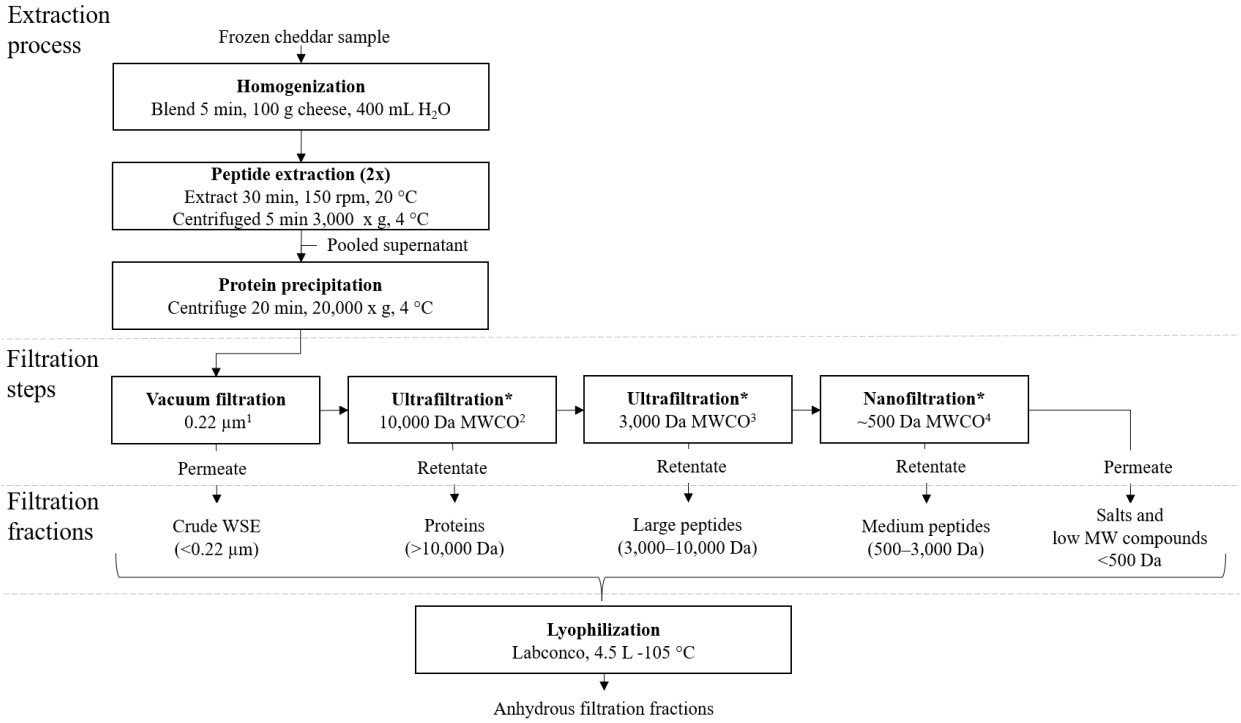

Figure S1. Water-soluble extraction (WSE) and molecular weight fractionation procedure using ultra- and nano-crossflow filtration. MWCO: molecular weight cut-off, [1]vacuum filter systems, Corning 431097, 0.22 µm, polyethersulfone. *Synder sheet filter membrane models for Sterlitech Sepa CF Med/High Foulant Cell, 316 SS, 75 mil. Membrane type: [2]ST (polyethersulfone), [3]VT (polyethersulfone), [4]NFW (polyamide).

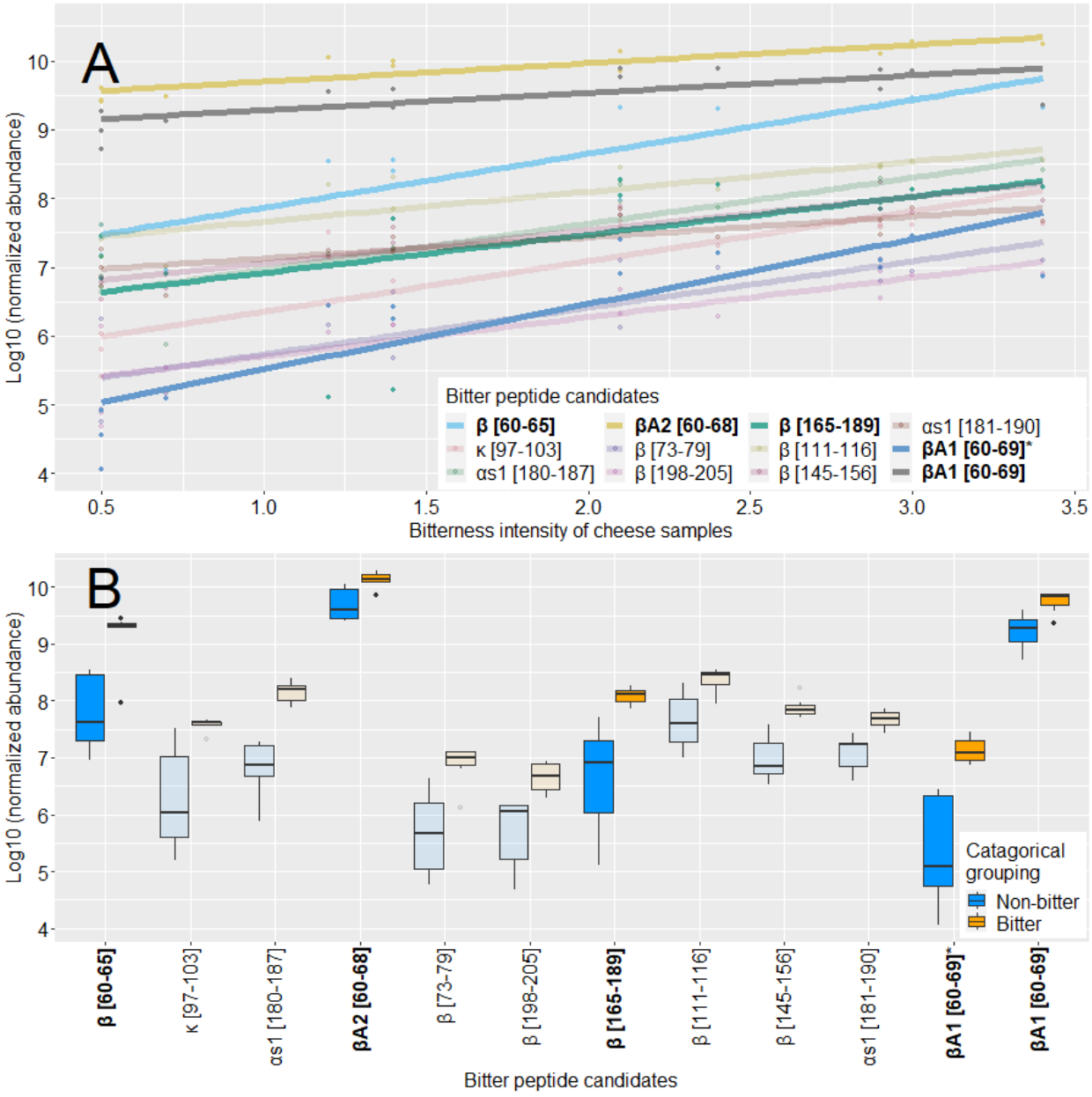

Figure S2. (A) Scatter plot illustrating peptides normalized instrument abundance compared with samples mean bitterness intensity, (B) Box plot illustrating the magnitude of change in peptide relative abundance between the categorical non-bitter group (threshold and low bitterness groups) and the bitter group (moderate and extreme groups). *Indicates phosphorylation of serine in sequence position 10. Five peptides selected identified as bitter are highlighted with bold labeling and fully opaque shading. For Figure A, cheese mean bitterness was scored on a 0–15-point universal intensity scale (Spectrum method, Meilgaard et al., 1999). For Figure B, categorical bitterness grouping: reference Table 1 for definitions.

TABLE S1. Descriptive sensory analysis of fundamental flavors in the 14 Cheddar samples and correlations with bitterness and age.

Sample ID[1]	Bitter[2]	Sour	Salty	Sweet	Umami
T_0.2a	0.7	2.7	3.6	2.4	2.7
T_0.2b	ND	2.4	3.5	2.4	2.5
T_0.2b	ND	2.9	3.3	2.3	2.9
T_0.2d	ND	2.6	3.2	2.5	3.0
L_3.3	1.2	2.8	4.0	2.6	4.0
L_2.9	1.4	2.9	4.3	2.7	3.8
L_6.0	1.4	3.0	4.4	2.8	4.1
M_7.3	2.1	3.1	4.5	2.9	4.5
M_6.2	2.1	2.8	4.7	3.0	4.5
M_5.6	2.4	3.0	4.5	2.7	4.2
E_7.2	2.9	2.9	4.3	2.9	4.4
E_8.7a	2.9	3.4	4.9	2.9	4.0
E_5.7	3.0	3.0	4.3	2.9	4.1
E_8.7b	3.4	3.2	4.8	2.7	4.1
Descriptive statistics					
LSD[3]	0.3	0.3	0.3	0.3	0.2
Bitterness (Pearson's *r*)[4]	1.00	0.75	0.86	0.78	0.78
Age (Pearson's *r*)[4]	0.92	0.82	0.94	0.86	0.88

[1]Reference Table 1 for Sample ID definition.

[2]Data represent average values from seven panelists performing quantitative descriptive analysis on a 0–15-point universal intensity scale in duplicate (Spectrum method, Meilgaard et al., 1999; Drake et al., 2001). ND, non-detected.

[3]LSD, least significant difference ($p < 0.05$). Data were analyzed by a general linear model analysis of variance with Fisher's LSD as a post hoc test.

[4]Pearson's correlation coefficient of mean bitterness intensity and cheese age (see Table 1) to descriptive sensory results. ND values were substituted with 0.5, the panels' limit of quantification for calculating the correlation coefficients.

TABLE S2. Descriptive sensory analysis of aromatics in the 14 Cheddar samples and correlations with bitterness and age.

Sample ID[1]	Cooked[2]	Caramel	Whey	Diacetyl	Milkfat	Fruity	Sulfur	Brothy	Nutty	Catty	Cowy barny
T_0.2a	3.6	ND	2.3	1.2	3.5	ND	ND	0.7	ND	ND	ND
T_0.2b	3.7	ND	1.9	ND	3.6	ND	ND	0.7	ND	ND	ND
T_0.2c	3.7	ND	2.8	0.9	3.6	ND	ND	ND	ND	ND	ND
T_0.2d	3.8	ND	2.4	1.0	3.5	ND	ND	ND	ND	ND	ND
L_3.3	3.5	1.7	ND	ND	3.5	0.8	3.0	3.7	2.0	ND	0.8
L_2.9	3.4	1.0	ND	ND	3.5	0.6	2.4	3.4	1.1	ND	ND
L_6.0	3.0	2.5	ND	ND	3.5	1.8	2.9	3.7	2.2	1.3	ND
M_7.3	3.2	1.7	ND	ND	3.5	1.5	3.0	3.8	2.7	1.7	ND
M_6.2	3.4	1.2	ND	ND	3.5	1.5	3.2	3.9	2.2	1.0	ND
M_5.6	3.3	2.9	ND	ND	3.5	1.2	3.0	3.7	2.9	1.1	0.6
E_7.2	3.4	2.4	ND	ND	3.5	1.1	3.2	4.0	2.6	0.8	ND
E_8.7a	3.4	3.2	ND	ND	3.5	1.3	3.2	3.8	2.3	1.2	0.6
R_5.7	3.3	2.1	ND	ND	3.5	0.9	2.7	3.5	2.3	1.1	1.2
E_8.7b	3.2	3.2	ND	ND	3.5	1.0	3.2	4.0	2.4	1.3	0.6
Descriptive statistics											
LSD[3]	0.3	0.3	0.2	0.3	0.2	0.3	0.3	0.3	0.3	0.2	0.3
Bitterness (Pearson's *r*)[4]	-0.66	0.86	-0.77	-0.61	-0.53	0.54	0.82	0.81	0.85	0.69	0.39
Age (Pearson's *r*)[4]	-0.80	0.89	-0.85	-0.69	-0.56	0.79	0.92	0.90	0.92	0.83	0.18

[1]Reference Table 1. for Sample ID definition.

[2]Data represent average values from seven panelists performing quantitative descriptive analysis on a 0–15-point universal intensity scale in duplicate (Spectrum method, Meilgaard et al., 1999; Drake et al., 2001). ND, non-detected. Flavors not listed were not detected in cheeses.

[3]LSD, least significant difference ($p < 0.05$). Data were analyzed by a general linear model analysis of variance with Fisher's LSD as a post hoc test.

[4]Pearson's correlation coefficient of mean bitterness intensity and cheese age (see Table 1) to descriptive sensory results. ND values were substituted with 0.5, the panels' limit of quantification for calculating the correlation coefficients.

TABLE S3. Compositional data for the 14 Cheddar samples

Sample ID[1]	pH[2]	%, w/w				
		Moisture[3]	Salt[4]	Fat[5]	FDB[6]	Protein[7]
T_0.2a	5.10	35.3	1.86	34.7	53.6	24.0
T_0.2b	5.06	35.5	1.72	33.5	52.0	24.2
T_0.2c	5.10	35.6	1.75	32.4	50.3	24.3
T_0.2d	5.10	35.4	1.74	32.5	50.4	24.1
L_3.3	5.06	32.8	1.70	37.2	55.4	25.2
L_2.9	5.14	32.8	1.59	36.5	54.3	24.7
L_6.0	4.99	33.6	1.72	34.8	52.4	24.3
M_7.3	5.24	32.7	1.66	37.6	55.9	24.5
M_6.2	5.03	33.0	1.79	38.2	57.0	24.4
M_5.6	5.10	35.4	1.75	37.3	57.7	24.3
E_7.2	5.12	33.3	1.63	35.1	52.6	24.5
E_8.7a	4.98	32.2	1.80	34.5	50.9	24.5
E_5.7	5.10	35.5	1.82	37.4	58.1	24.7
E_8.7b	4.98	32.3	1.67	32.4	47.9	24.1

[1]Reference Table 1 for Sample ID definition.
[2]Duplicate analysis, pH at 25 °C
[3]Triplicate analysis by forced air oven method (Hooi et al., 2004)
[4]Duplicate analysis by Volhard (titratable chloride) method (Hooi et al., 2004)
[5]Duplicate analysis by Mojonnier method (Barbano et al., 1988)
[6]Fat dry basis (FDB), duplicate analysis by Mojonnier method (Barbano et al., 1988)
[7]Triplicate analysis by Kjeldahl method (Barbano and Clark, 1990)

TABLE S4. Peptides identified in the 14 cheeses that were documented as bitter in previous literature, their standard mean difference in abundance between bitter and non-bitter groups, their linear correlation with cheese bitterness intensity, their bitterness threshold value, mean bitterness intensity and literature source (*continue on next page*).

Peptide sequence	Peptide's origin[1]	Rank order mean[2]	Standard mean difference[3]	Bitterness linear correlation (R value)[4]	Threshold value ($\mu mol \cdot L^{-1}$)[5]	Mean bitterness intensity[5] (0–15)	Literature Reference[6]
YPFPGPIPN	βA2 [60–68]	4	2.10	0.91	230	-	Toelstede & Hofmann, 2008a
YPFPGPIHNS	βA1 [60–69]	12	2.26	0.66	50	-	Toelstede & Hofmann, 2008a
YPFPGPIHN	βA1 [60–68]	16	1.45	0.76	100	-	Toelstede & Hofmann, 2008a
KPWIQPK	α_{s2} [191–197]	19	1.79	0.64	-	-	Lee et al., 1996
RPKHPIK	α_{s1} [1–7]	30	1.94	0.58	-	-	Lee et al., 1996
VLPVPQ	β [170–175]	38	1.52	0.57	310	-	Sebald et al., 2018, 2020
LVYPFPGPIHN	βA1 [58–68]	68	1.11	0.50	80	-	Toelstede & Hofmann, 2008a
YPFPGPI	β [60–66]	79	0.73	0.67	160	-	Shinoda et al., 1986a; Lemieux & Simard, 1992
EMPFPKYPVEPF	β [108–119]	101	0.74	0.51	-	NB	Karametsi et al., 2014
VYPFPGPIPN	βA2 [59–68]	118	1.11	0.40	170	-	Toelstede & Hofmann, 2008a
TDVENLHLPLPL	β [128–139]	129	0.73	0.44	-	-	Visser et al., 1975; Lemieux & Simard, 1992
MPFPKYPVEPF	β [109–119]	132	0.54	0.55	-	0.56	Karametsi et al., 2014
HLPLPLLQ	β [134–141]	143	0.60	0.48	440	-	Sebald et al., 2018, 2020
QEPVLGPVRGPFPII	β [194–208]	144	0.60	0.48	330	-	Sebald et al., 2020
YPFPGPIPNS	βA2 [60–69]	170	1.06	0.32	330	-	Toelstede & Hofmann, 2008a
TPVVVPPFLQPEVM	β [80–93]	174	0.82	0.35	>1600	-	Sebald et al., 2020
RDMPIQAFLLY	β [183–193]	199	0.75	0.32	-	-	Monnet et al., 1986; Lemieux & Simard, 1992

YQEPVLGPVRGPFPI	β [193–207]	209	0.45	0.43	-	0.56	Karametsi et al., 2014;
QEPVLGPVRGPFPI	β [194–207]	211	0.31	0.48	-	NB	Monnet et al., 1986; Karametsi et al., 2014; Lemieux & Simard, 1992
YQQKPVAL	κ [43–50]	218	0.75	0.28	500	-	Sebald et al., 2018, 2020
SLVYPFPGPIHNS	βA1 [57–69]	241	0.48	0.34	60	-	Toelstede & Hofmann, 2008a
AMAPKHKEMPFPKYPVEPF	β [101–119]	260	0.29	0.37	250	-	Sebald et al., 2020
APKHKEMPFPKYPVEPF	β [103–119]	269	0.28	0.36	-	0.36	Karametsi et al., 2014
LGPVRGPFP	β [198–206]	352	0.02	0.28	-	NB	Karametsi et al., 2014
LHLPLPLLQS	β [133–142]	368	0.13	0.17	160	-	Sebald et al., 2020
VVVPPFL	β [82–88]	373	0.50	-0.05	140	-	Shinoda et al., 1986b; Lemieux & Simard, 1992
TQTPVVVPPFLQPE	β [78–91]	399	0.13	0.09	280	-	Sebald et al., 2018, 2020
QSKVLPVPQ[7]	β [167–175]	453	0.04	-0.03	-	-	Monnet et al., 1986; Lemieux & Simard, 1992
IAKYIPI	κ [22–28]	457	-0.09	0.01	160	-	Sebald et al., 2020
LHLPLPLL	β [133–140]	477	-0.12	-0.04	110	-	Sebald et al., 2018, 2020
VLPVPQKAVPYPQ	β [170–182]	495	-0.47	0.04	340	-	Sebald et al., 2020
GPVRGPFP	β [199–206]	558	-0.70	-0.05	1180	-	Toelstede & Hofmann, 2008a
FALPQYLK	αs2 [174–181]	561	-0.54	-0.19	-	-	Matoba et al., 1969; Lemieux & Simard, 1992
TQTPVVVPPFL	β [78–88]	566	-0.34	-0.34	200	-	Sebald et al., 2020
VPPFLQPE	β [84–91]	567	-0.75	-0.03	>1730	-	Sebald et al., 2020

MAPKHKEMPFP KYPVEPF	β [102–119]	571	-0.55	-0.21	90	0.9	Karametsi et al., 2014; Sebald et al., 2018, 2020
QEPVLGPVRGPF PIIV	β [194–209]	582	-0.56	-0.25	-	-	Gordon & Speck, 1965; Lemieux & Simard, 1992
ERYLGYLEQ	αs1 [89–97]	610	-0.66	-0.28	460	-	Sebald et al., 2020
IPPLTQTPVVVPP	β [74–86]	632	-0.86	-0.22	>6,000	-	Toelstede & Hofmann, 2008b
RPKHPIKHQGLP Q	αs1 [1–13]	648	-0.80	-0.34	-	-	Lee et al., 1996
APFPEVFG	αs1 [26–33]	652	-0.64	-0.42	-	-	Matoba et al., 1970; Lemieux & Simard, 1992
SITRINK	β [22–28]	664	-0.92	-0.29	>6,000	-	Toelstede & Hofmann, 2008a
RPKHPIKHQ	αs1 [1–9]	669	-0.65	-0.45	-	-	Broadbent et al., 1998, 2002
EPVLGPVRGPFP	β [195–206]	674	-1.06	-0.22	-	NB	Karametsi et al., 2014
PVLGPVRGPFP	β [196–206]	676	-1.06	-0.22	-	NB	Karametsi et al., 2014
PVRGPFPIIV	β [200–209]	685	-0.76	-0.46	4	-	Shinoda et al., 1985
APFPEVF	αs1 [26–32]	700	-0.78	-0.51	-	-	Richard & Creamer, 1973; Lemieux & Simard, 1992
NLHLPLPLLQS	β [132–142]	703	-0.88	-0.47	180	-	Sebald et al., 2020
YQEPVLGPVRGP FP	β [193–206]	730	-1.03	-0.44	-	-	Karametsi et al., 2014; Visser et al., 1975, 1983; Lemieux & Simard, 1992
KAVPYPQ	β [176–182]	766	-0.98	-0.58	-	-	Monnet et al., 1986; Lemieux & Simard, 1992
VAPFPEVFGKE	αs1 [25–35]	769	-1.02	-0.57	570	-	Sebald et al., 2018, 2020
KVLPVPQKAVPY PQ	β [169–182]	776	-1.09	-0.56	140	-	Sebald et al., 2018, 2020
GPVRGPFPIIV	β [199–209]	809	-1.33	-0.55	-	4.56	Karametsi et al., 2014
YLGYLEQLLR	αs1 [91–100]	810	-1.23	-0.58	-	-	Hill and Van Leeuwen, 1974; Lemieux & Simard, 1992

QEPVLGPVRGPFP	β [194–206]	842	-1.82	-0.60	-	NB	Karametsi et al., 2014
VFGKEKVNEL	αs1 [31–40]	862	-1.53	-0.77	110	-	Sebald et al., 2018, 2020

[1]Casein variants (bovine origin) are α_{s1}-, α_{s2}-, β- and κ-casein. βA1 is the A1 variant of β-casein in which position 67 is Pro. βA2 is the A2 variant of β-casein in which position 67 is His.

[2]Ranked mean of the ascending order of average standard mean difference and R-values for the bitterness linear correlation.

[3]Standard mean difference calculated in accordance with Cohen's d value formula.

[4]R-value, correlation of cheese mean bitterness score (see Table 1) to each peptide's normalized abundance.

[5]Reference literature sources for information on sensory analysis methodology. NB indicates non-bitter, "-" means no data is available.

[6]Full literature references are found in the following article: Kuhfeld, R. F., H. Eshpari, Z. Atamer & D. C. Dallas. 2023. A comprehensive database of cheese-derived bitter peptides and correlation to their physical properties, Critical Reviews in Food Science and Nutrition.

[7]The dotted line distinguishes peptides according to their standard mean difference, with positive values located above the line and negative values situated below it.