

## Supporting Information

### Sensomics Analysis of Taste Compounds in Balsamic Vinegar and Discovery of 5-Acetoxymethyl-2-furaldehyde as a Novel Sweet Taste Modulator

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### **Analysis of amino acids:**

Analysis was performed on HPLC-MS/MS system 1 equipped with a 150 x 2.0 mm i.d., 5  $\mu$ m; TSKgel Amide-80 column (Tosoh Bioscience, Stuttgart, Germany). Using acetonitrile containing 5% of an aqueous solution of ammonium acetate (5 mmol/L, pH 3.0) as eluent A and an aqueous ammonium acetate solution (5 mmol/L, pH 3.0) as eluent B, chromatography was carried out at a flow rate of 0.2 mL/min, starting with a mixture of 85% A and 15% B for 3 min, then increasing the content of B within 7 min to 25%, then within 5 min to 50% and, finally to 100% within another 3 min. The following analytes and their corresponding isotope labelled standards were analyzed in the positive electrospray ionization mode (ESI<sup>+</sup>) using the mass transitions and declustering potential (DP, in V), entrance potential (EP, in V), collision energy (CE, in V), and cell exit potential (CXP, in V) as follows:

glycine ( $m/z$  76.1 $\rightarrow$ 76.0; +31/+10/+5/+6), glycine-<sup>13</sup>C<sub>2</sub>-<sup>15</sup>N ( $m/z$  79.0 $\rightarrow$ 79.0; +41/+10/+5/+5), L-alanine ( $m/z$  90.1 $\rightarrow$ 90.0; +26/+10/+5/+6), L-alanine-<sup>13</sup>C<sub>3</sub> ( $m/z$  93.0 $\rightarrow$ 93.0; +41/+10/+5/+5), L-serine ( $m/z$  106.1 $\rightarrow$ 60.0; +26/+10/+17/+4), L-serine-<sup>13</sup>C<sub>3</sub> ( $m/z$  109.0 $\rightarrow$ 62.0; +38/+10/+16/+5), L-proline ( $m/z$  116.1 $\rightarrow$ 70.0; +21/+10/+21/+4), L-proline-<sup>13</sup>C<sub>5</sub>-<sup>15</sup>N ( $m/z$  122.0 $\rightarrow$ 75.0; +73/+10/+25/+5), L-valine ( $m/z$  118.1 $\rightarrow$ 72.1; +21/+10/+15/+6), L-valine-<sup>13</sup>C<sub>5</sub>-<sup>15</sup>N ( $m/z$  124.0 $\rightarrow$ 77.0; +64/+10/+14/+10), L-threonine ( $m/z$  120.1 $\rightarrow$ 73.9; +36/+10/+17/+6), L-threonine-<sup>13</sup>C<sub>4</sub>-<sup>15</sup>N ( $m/z$  125.0 $\rightarrow$ 78.0; +32/+10/+14/+5), L-leucine/L-isoleucine ( $m/z$  132.1 $\rightarrow$ 86.0; +41/+10/+15/+6), L-leucine-<sup>13</sup>C<sub>2</sub> ( $m/z$  134.1 $\rightarrow$ 87.9; +46/+10/+15/+6), L-isoleucine-<sup>13</sup>C<sub>6</sub> ( $m/z$  139.1 $\rightarrow$ 92.0; +39/+10/+14/+10), L-asparagine ( $m/z$  132.9 $\rightarrow$ 73.9; +46/+10/+19/+6), L-asparagine-<sup>15</sup>N<sub>2</sub> ( $m/z$  135.0 $\rightarrow$ 75.0; +39/+10/+20/+5), L-aspartic acid ( $m/z$  134.1 $\rightarrow$ 87.9; +46/+10/+15/+6), L-aspartic acid-<sup>13</sup>C<sub>4</sub>-<sup>15</sup>N ( $m/z$  139.1 $\rightarrow$ 92.0; +39/+10/+14/+10), L-glutamine ( $m/z$  147.0 $\rightarrow$ 84.0; +46/+10/+23/+6), L-glutamine-<sup>13</sup>C<sub>5</sub> ( $m/z$  152.0 $\rightarrow$ 88.0; +43/+10/+23/+10), L-glutamic acid ( $m/z$  148.1 $\rightarrow$ 84.0; +31/+10/+23/+6), L-glutamic acid-<sup>13</sup>C<sub>5</sub>-<sup>15</sup>N ( $m/z$  154.0 $\rightarrow$ 89.0; +38/+10/+23/+10), L-lysine ( $m/z$  147.0 $\rightarrow$ 84.0; +46/+10/+23/+6), L-lysine-<sup>13</sup>C<sub>6</sub>-<sup>15</sup>N<sub>2</sub> ( $m/z$  155.0 $\rightarrow$ 90.0; +44/+10/+23/+10), L-methionine ( $m/z$  150.1 $\rightarrow$ 104.0; +31/+10/+15/+8), L-methionine-d<sub>3</sub> ( $m/z$  153.1 $\rightarrow$ 107.0; +50/+10/+14/+10), L-histidine ( $m/z$  156.1 $\rightarrow$ 110.0; +41/+10/+21/+8), L-histidine-<sup>13</sup>C<sub>6</sub> ( $m/z$  162.0 $\rightarrow$ 115.0; +46/+10/+21/+10), L-phenylalanine ( $m/z$  166.0 $\rightarrow$ 120.0; +51/+10/+19/+10), L-phenylalanine-d<sub>5</sub> ( $m/z$  171.0 $\rightarrow$ 125.0; +48/+10/+19/+10), L-arginine ( $m/z$  175.1 $\rightarrow$ 70.1; +36/+10/+33/+4), L-arginine-<sup>13</sup>C<sub>6</sub> ( $m/z$  181.0 $\rightarrow$ 74.0; +78/+10/+36/+5), L-tyrosine ( $m/z$  182.1 $\rightarrow$ 136.0; +26/+10/+19/+10), L-tyrosine-d<sub>4</sub> ( $m/z$  186.0 $\rightarrow$ 140.0; +38/+10/+19/+10), L-tryptophan ( $m/z$  205.1 $\rightarrow$ 146.0; +41/+10/+25/+12), L-tryptophan-d<sub>5</sub> ( $m/z$  210.0 $\rightarrow$ 150.0; +40/+10/+26/+10). After triplicate LC-MS/MS analysis of mixtures of analytes and internal standards in six molar ratios from 0.04 to 8.0, calibration curves were calculated by plotting peak area ratios of each analyte to the respective internal standard against

concentration ratios of each analyte to the internal standard using linear regression (correlation coefficients > 0.99 for each compound).

#### **Analysis of Phenolic Acids/Esters:**

Analysis was performed on LC-MS/MS-system 2 equipped with a 150 x 2 mm i.d., 5  $\mu$ m, Synergy Fusion RP18 column (Phenomenex, Aschaffenburg, Germany). Using acetonitrile containing 1% formic acid as solvent A and 1% aqueous formic acid as solvent B, chromatography was performed with a flow rate of 0.25 mL/min starting with 0% of solvent A for 1 min, then increasing solvent A to 50% within 20 min and to 100% within 1 min and, finally, keeping solvent A at 100% for 2 min. Phenolic acids and esters were analyzed in the positive electrospray ionization mode (ESI<sup>+</sup>) using the mass transitions and declustering potential (DP, in V), entrance potential (EP, in V), collision energy (CE, in V), and cell exit potential (CXP, in V) as follows:

caffeic acid ( $m/z$  181.1 $\rightarrow$ 89.0; +21/+6/+16/+41/+4), gallic acid ( $m/z$  171.1 $\rightarrow$ 108.9; +31/+12/+24/+25/+4), ferulic acid ( $m/z$  195.1 $\rightarrow$ 145.1; +21/+6/+18/+21/+4), gentisinic acid ( $m/z$  155.1 $\rightarrow$ 125.0; +46/+12/+14/+27/+4), quinic acid ( $m/z$  193.1 $\rightarrow$ 147.0; +26/+9/+16/+13/+4), vanilline ( $m/z$  167.1 $\rightarrow$ 92.9; +26/+8/+12/+19/+4), *p*-coumaric acid ( $m/z$  165.1 $\rightarrow$ 119.2; +21/+6/+14/+25/+4), protocatechuic acid ( $m/z$  155.1 $\rightarrow$ 123.2; +41/+12/+12/+15/+4), syringic acid ( $m/z$  199.2 $\rightarrow$ 140.2; +31/+9/+18/+21/+4), vanillic acid ( $m/z$  169.1 $\rightarrow$ 93.0; +21/+11/+14/+19/+4), syringaldehyde ( $m/z$  183.2 $\rightarrow$ 123.0; +26/+4/+16/+17/+4), 5-hydroxymethyl-2-furaldehyde (6) ( $m/z$  126.9 $\rightarrow$ 109.0; +26/+9/+10/+15/+4), gallic acid ethyl ester ( $m/z$  199.2 $\rightarrow$ 127.1; +31/+6/+16/+17/+4), vanillic acid ethyl ester ( $m/z$  167.2 $\rightarrow$ 111.0; +26/+10/+16/+17/+4).

Quantitative analysis was performed by external calibration in triplicate by comparing the peak areas obtained for the corresponding mass traces with those of defined standard solutions (0.05 - 5.0 mg/L) of each reference compound in 20% aqueous methanol.

#### **Analysis of Ellagitannins**

Analysis was performed on the LC-MS/MS-system 1 equipped with a 150 x 2 mm i.d., 5  $\mu$ m, Luna Phenylhexyl column (Phenomenex, Aschaffenburg, Germany). Using methanol containing 1% formic acid as solvent A and aqueous 1% formic acid as solvent B, chromatography was performed with a flow rate of 0.25 mL/min starting with a mixture of 10% solvent A and 90% solvent B for 1 min, then increasing the content of solvent A within 12 min to 100%, which was then kept isocratic for additional 5 min. Compounds **1 - 3**

were analyzed in the positive electrospray ionization mode (ESI<sup>+</sup>) using the mass transitions and declustering potential (DP, in V), entrance potential (EP, in V), collision energy (CE, in V), and cell exit potential (CXP, in V) as follows:  
vescalagin (*m/z* 933.0→301.0; -150/-10/-70/-7; *m/z* 933.0→631.0; -150/-10/-40/-29), castalagin (*m/z* 933.0→301.0; -150/-10/-70/-7; *m/z* 933.0→631.0; -150/-10/-40/-29), (+)-dihydrorobinetin (*m/z* 303.0→174.9; -75/-10/-26/-11; *m/z* 303.0→108.9; -75/-10/-44/-5)  
For quantitation of compounds **1-3**, aliquots of the BV (1 mL) were spiked with different amounts of stock solutions of compounds **1-3** (0.5 - 5.0 mg/L, each), followed by sample-work up as detailed above. After triplicate LC-MS/MS analysis, calibration curves were calculated by plotting the peak area of each analyte against the concentration using linear regression, showing linear responses with correlation coefficients of > 0.99 each. Quantitative analysis was performed in triplicate by comparing the peak areas obtained for the corresponding mass traces in the samples and the standard solutions of the references.

### **Functional hTAS1R2/hTAS1R3 Sweet Receptor Experiments**

Functional experiments were carried out in the human embryonic kidney cell line HEK293 (Invitrogen, Karlsruhe, Germany) stably expressing the human sweet taste receptor subunits hTAS1R2 and hTAS1R3 and the G protein subunit Gα15Gai3. Cells were cultured under regular conditions at 37°C, 5% CO<sub>2</sub> and 95% humidity in Dulbecco's Modified Eagle Medium (D-MEM) containing 4.5 g/L glucose (Invitrogen) supplied with 10% fetal calf serum (tetracycline-free), 1% L-glutamine, 1% penicillin G (10000 units/mL), streptomycin (10 mg/mL), G418 (200 µg/mL), hygromycin (200 µg/mL), blasticidin (15 µg/mL) and puromycin (1 µg/mL). For functional experiments, cells were seeded into 96-well plates (Greiner Bio-One, Frickenhausen, Germany) coated with poly-D-lysine (10 µg/mL) to improve cell adhesion. Expression of the hTAS1R3 sweet receptor subunit was induced 24 h prior to the experiment using tetracycline (0.5 µg/mL) in D-MEM GlutaMAX (1g/L glucose) supplied with 10% dialyzed fetal calf serum (tetracycline-free), penicillin G (10000 units/mL) and streptomycin (10 mg/mL). Prior to the experiment, cells were incubated for 1 h with Fluo-4-AM (2 µmol/L, Molecular Probes) in serum-free D-MEM low Glucose GlutaMAX (Invitrogen) containing 2.5 mM probenecide to block active transport of the fluorescent dye out of the cell. Cells were washed with a bath solution (130 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L HEPES, 2 mmol/L CaCl<sub>2</sub>, 1 mmol/L Na-Pyruvat, pH 7.4) three times and incubated at room temperature for 40 min to allow complete de-esterification of the fluorescent dye. Test substances were dissolved in the bath solution. For co-application experiments, the individual substances were premixed prior application. Intracellular calcium levels were recorded during automated application of the test solutions using a fluorometric imaging plate reader (FLIPR<sup>tetra</sup>, Molecular Devices, Munich, Germany). To control for cell vitality

isoproterenol, agonist of endogenously expressed  $\beta$ -adrenergic receptors, was applied subsequently to test compounds. Cells not induced with tetracycline, and thus lacking the functional sweet taste receptor heteromer, served as a control for unspecific responses of the cellular background to the test compounds. To control for the specificity of the sweet receptor activation, test substances were coapplied with the selective antagonist of hTAS1R2/hTAS1R3, 2-(4-methoxy-phenoxy)-propionic acid (*lactisole*) (1 mM). Data were collected from three independent experiments carried out in duplicates. Raw fluorescence signals of hTAS1R2/hTAS1R3-expressing cells were reduced by fluorescence signals of non-induced cells (software FLIPR<sup>tetra</sup>, Molecular Devices) and normalized to baseline fluorescence ( $\Delta F/F$ , SigmaPlot 9.0, Systat Software GmbH, Erkrath, Germany). Determination of threshold concentrations for receptor activation for compounds **6** - **11** was performed with linear dilutions rows (1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 50  $\mu\text{mol/L}$ ).  $\Delta F/F$  values obtained for cells reacted with buffer solution were subtracted from  $\Delta F/F$  values obtained for solutions containing the test substances. Finally, the concentration of test substance, resulting in a “buffer-corrected”- $\Delta F/F$  value, being significantly higher than the  $\Delta F/F$  of the buffer solution was defined as threshold concentration for hTAS1R2/hTAS1R3 receptor activation.

**Table S1.** Concentrations [ $\mu\text{mol/l}$ ] of Taste Active Compounds in Intermediate Levels from Barrels A-H

compound	Concentrations [ $\mu\text{mol/l}$ ]							
	A	B	C	D	E	F	G	H
glucose	1243918	1684921	2027658	2267333	2315269	2346427	2379981	2240969
fructose	1252128	1696042	2041041	2282299	2330550	2361914	2395690	2255760
glycerol	99435	95993	109773	123466	127071	124769	132186	124585
erythritol	692	1431	1727	1721	1490	2047	1865	1835
sorbitol	713	1634	2558	3297	3041	3912	3724	3468
arabitol	1288	988	848	693	628	594	603	570
xylitol	0	255	453	637	603	702	751	751
mannitol	623	756	698	706	709	741	736	730
ribitol	458	423	459	438	442	430	462	441
6- <i>O</i> -acetyl-glucose ( <b>4</b> )	5623	6857	9286	12814	12357	15492	13960	12844
1- <i>O</i> -acetyl-fructose ( <b>5</b> )	10854	11970	14252	19375	19703	26168	23905	20832
acetic acid	750286	646131	558477	466612	485249	436519	432857	417775
citric acid	12722	16462	19905	21667	21603	23801	22939	24161
tartaric acid	39135	40397	47557	54883	57209	59644	62362	65352
glycolic acid	7651	6342	6126	8742	7851	6676	7380	7767
malic acid	88708	107498	118512	119942	127333	128553	128520	129864
lactic acid	2589	3490	3735	3578	3531	3871	3386	3617
gluconic acid	79116	79116	71397	74291	84905	78151	76221	80080
castalagin ( <b>2</b> )	59.4	23.5	30.5	28.8	29.6	34.4	33.3	42.5
vescalagin ( <b>1</b> )	22.6	13.2	22.1	22.4	22.3	23.1	24.5	34.5
(+)-dihydrorobinetin ( <b>3</b> )	2.6	0.1	0.0	0.3	0.1	0.4	0.3	0.4
trans-caffeic acid	10.1	6.6	5.3	4.0	4.4	3.6	3.8	4.3
gentisic acid	10.1	8.1	8.1	7.8	7.4	7.1	6.6	7.0
p-coumaric acid	8.6	6.9	6.6	6.1	6.0	5.7	5.6	5.7
gallic acid	1.0	0.8	1.0	1.3	1.3	1.4	1.5	1.4
p-hydroxybenzoic acid	5.2	4.2	4.5	4.9	4.3	4.2	4.2	4.3
protocatechuic acid	11.4	8.6	8.6	8.0	7.7	7.1	6.9	7.1
vanillic acid	4.0	3.1	3.4	3.4	3.2	3.0	3.1	3.1
vanilline	3.5	2.4	2.1	1.9	1.9	1.9	1.9	1.8
syringaldehyde	0.15	0.07	0.04	0.03	0.03	0.03	0.02	0.02

5-hydroxymethylfurfural (6)	4430	10075	17950	24279	27496	19652	28948	29052
5-acetoxymethyl-2 furaldehyde (7)	103.3	178.1	248.4	349.3	340.3	404.5	407.3	394.4
gallic acid methyl ester	0.8	0.5	0.5	0.4	0.4	0.4	0.3	0.4
gallic acid ethyl ester	0.9	0.4	0.3	0.2	0.2	0.2	0.2	0.2
quinic acid	0.3	0.2	0.3	0.3	0.3	0.3	0.4	0.3
syringic acid	6.3	5.3	5.5	5.4	5.1	4.9	4.6	4.7
ferulic acid	1.1	0.7	0.6	0.4	0.5	0.4	0.4	0.4

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**Table S2.** Concentrations [ $\mu\text{mol/kg}$  dry matter] of Taste Active Compounds in Intermediate Levels from Barrels A-H

compound	Concentrations [ $\mu\text{mol/kg}$ dry matter]							
	A	B	C	D	E	F	G	H
glucose	2832725	3050642	3180484	3204906	3263182	3224914	3217191	3010079
fructose	2851422	3070777	3201476	3226059	3284719	3246199	3238426	3029946
glycerol	226440	173801	172185	174521	179097	171482	178685	167343
erythritol	1575	2591	2710	2432	2100	2813	2520	2465
sorbitol	1624	2959	4013	4661	4285	5377	5035	4658
arabitol	2933	1788	1329	980	885	816	815	766
xylitol	0.0	462.3	711.3	900.7	849.4	965.2	1014.6	1008.6
mannitol	1419	1369	1095	998	998	1019	995	981
ribitol	1044	766	720	620	623	591	625	592
6- <i>O</i> -acetyl-glucose (4)	12805	12416	14566	18113	17416	21292	18871	17252
1- <i>O</i> -acetyl-fructose (5)	24716	21672	22356	27387	27770	35965	32314	27981
acetic acid	1708596	1169855	875999	659562	683919	599948	585124	561157
citric acid	28970	29805	31222	30627	30447	32711	31008	32453
tartaric acid	89120	73140	74596	77578	80632	81974	84300	87780
glycolic acid	17423	11483	9609	12357	11065	9176	9976	10433
malic acid	202010	194630	185892	169540	179466	176683	173729	174434
lactic acid	5895	6318	5859	5057	4976	5321	4576	4858
gluconic acid	180167	143243	111990	105012	119666	107410	103034	107564
castalagin (2)	135.2	42.6	47.8	40.8	41.7	47.2	44.9	57.1
vescalagin (1)	51.5	24.0	34.6	31.6	31.4	31.8	33.1	46.4
(+)-dihydrorobinetin (3)	6.0	0.2	0.0	0.5	0.1	0.5	0.5	0.5
trans-caffeic acid	23.1	11.9	8.2	5.6	6.2	5.0	5.1	5.8
gentisic acid	23.1	14.7	12.7	11.1	10.5	9.7	9.0	9.4
p-coumaric acid	19.6	12.5	10.3	8.7	8.4	7.8	7.5	7.7
gallic acid	2.3	1.5	1.6	1.9	1.8	1.9	2.0	1.9
p-hydroxybenzoic acid	11.9	7.6	7.1	7.0	6.0	5.8	5.7	5.8
protocatechuic acid	25.9	15.6	13.5	11.4	10.9	9.8	9.4	9.5
vanillic acid	9.1	5.7	5.3	4.9	4.4	4.1	4.2	4.1
vanilline	7.9	4.3	3.3	2.7	2.7	2.6	2.5	2.5



syringaldehyde	0.3	0.1	0.1	0.0	0.0	0.0	0.0	0.0
5-hydroxymethylfurfural (6)	10089	18241	28156	34319	38753	27009	39132	39023
5-acetoxymethyl-2 furaldehyde (7)	235.3	322.4	389.6	493.8	479.6	555.9	550.6	529.8
gallic acid methyl ester	1.8	0.9	0.8	0.6	0.6	0.5	0.5	0.5
gallic acid ethyl ester	2.1	0.8	0.5	0.3	0.3	0.3	0.2	0.3
quinic acid	0.6	0.3	0.5	0.4	0.4	0.5	0.5	0.4
syringic acid	14.3	9.6	8.7	7.6	7.2	6.7	6.2	6.3
ferulic acid	2.6	1.3	0.9	0.6	0.7	0.6	0.5	0.5

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**Figure S1.** LC-MS (MRM) analysis of (A) vescalagin (1) and castalagin (2) in balsamic vinegar (BV), (B) (+)-dihydorobinetin (3) in BV, (C) vescalagin (1) and castalagin (2) in traditional balsamic vinegar (TBV), (D) (+)-dihydorobinetin (3) in TBV, (E) vescalagin (1) and castalagin (2) in balsamic vinegar (BV) spiked with 1 and 2 prior to analysis, (F) (+)-dihydorobinetin (3) in BV spiked with 1 and 2 prior to analysis.



