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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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Supporting Information

ABSTRACT: Sensory-directed fractionation of traditional balsamic vinegar of Modena (TBV) led to the identification of the sweet-bitter tasting hexose acetates 6-*O*-acetyl-α/β-D-glucopyranose and 1-*O*-acetyl-β-D-fructopyranose as well as the previously unknown sweetness modulator 5-acetoxymethyl-2-furaldehyde. Taste re-engineering experiments and sensory time-intensity studies confirmed 5-acetoxymethyl-2-furaldehyde to contribute to the typical long-lasting sweet taste quality of TBV. Moreover, the response of the sweet taste receptor to this furaldehyde was verified by means of a functional hTAS1R2/hTAS1R3 receptor assay. Quantitative analysis of a total of 59 nonvolatile sensometabolites and taste modulators revealed higher concentrations of the sweet-modulating 5-acetoxymethyl-2-furaldehyde, nonvolatile organic acids and polyphenols such as wood-derived ellagitannins, and lower concentrations of acetic acid in the premium quality TBV when compared to balsamic vinegar of Modena (BV). Quantitative monitoring of sensometabolites throughout TBV manufacturing, followed by agglomerative hierarchical clustering and sensomics heatmapping, gave molecular insights into the taste alterations occurring during TBV maturation.

KEYWORDS: *taste modulator, sweet taste, taste receptor, 5-acetoxymethyl-2-furaldehyde, balsamic vinegar, sensometabolites, sensomics, Maillard reaction*

INTRODUCTION

Due to its mouth-watering and highly attractive taste profile centering around a well balanced sweet and sour taste as well as a long-lasting mouthfullness, traditional balsamic vinegars from Modena (TBV) and Reggio Emilia, respectively, are considered as key ingredients of top-level cuisine and are highly appreciated by consumers all over the world.

Traditional balsamic vinegars, certified with the protected designation of origin (PDO) status in the year 2000 (EC Council Regulation No. 813/2000), are produced by following a traditional but rather time-consuming manufacturing process.[1] After cooking in an open vessel, the reduced must prepared mainly from Trebbiano and Lambrusco grapes is undergoing a spontaneous alcoholic and acetic fermentation in a barrel, coined "badessa". Thereafter, the vinegar is matured in a sequence of a minimum of five casks of decreasing volume and made from different woods such as, e.g., oak, acacia, chestnut, cherry, mulberry, and ash, respectively. Once a year an aliquot of the aged vinegar is taken from the smallest barrel ("prelivio") and refilled with material of the next bigger barrel. Sequential refilling ("travaso") restores the volume in all vessels except the biggest barrel which is refilled with must from the badessa ("rincalzo"). During the maturation in this so-called "batteria", the vinegar is becoming increasingly concentrated by slow evaporation of water. Depending on their age of maturation, two different varieties of the traditional balsamic vinegar of Modena are available, namely "Affinato" and "Extravecchio", matured for at least 12 and 25 years, respectively.[1]

In comparison to these premium-quality TBV products, balsamic vinegar of Modena (BV) is produced in an industrial scale from wine vinegar and coloring and flavoring additives, and does greatly differ in price and concentration of monosaccharides, organic acids, amino acids, phenolic acids, and furan-2-aldehydes from the traditional, artisanal-type variety.[1−17] In order to differentiate premium quality traditional balsamic vinegar from balsamic vinegars, tremendous research efforts have been targeted toward the identification of marker molecules to analytically monitor quality and validate authenticity as well as the age of traditional balsamic vinegars.[13−19] For example, hexose acetates were recently reported to occur in traditional balsamic vinegars and were claimed to be favorably generated with increasing age of maturation.[18]

The volatile key odorants in traditional balsamic vinegar from Modena have recently been identified by means of gas chromatography/olfactometry, quantified by means of stable

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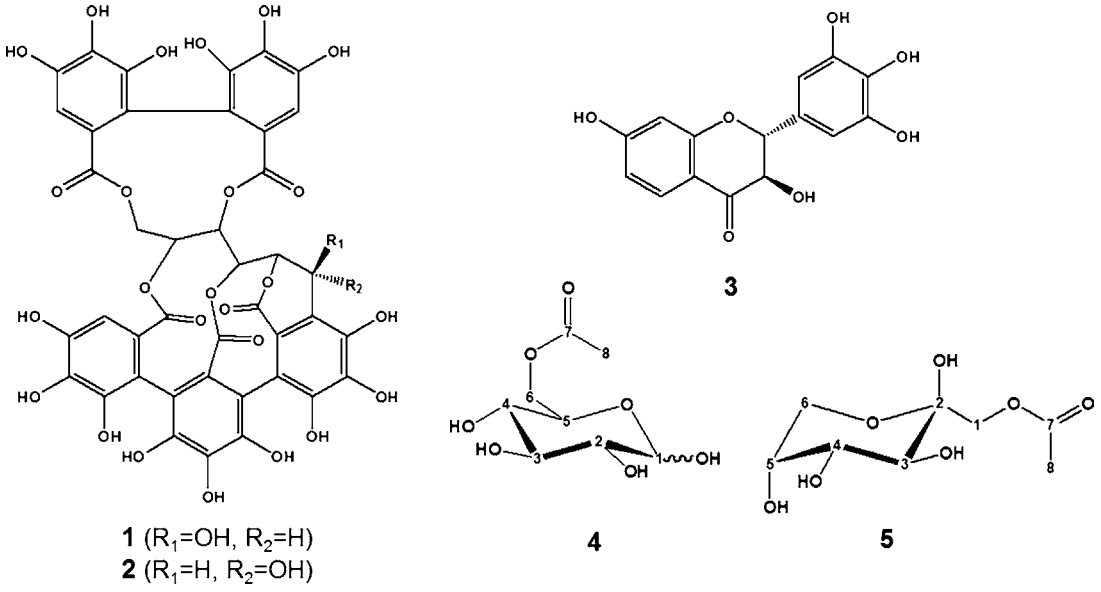

Figure 1. Structures of oak-derived ellagitannins vescalagin (**1**) and castalagin (**2**), acacia-derived (+)-dihydrorobinetin (**3**), and hexose mono acetates 6-*O*-acetyl-α/β-D-glucopyranose (**4**) and 1-*O*-acetyl-β-D-fructopyranose (**5**).

isotope dilution analysis, and validated by means of aroma reconstitution experiments.[20] Although the key players imparting the typical taste of red wine were recently identified,[21,22] any systematic studies on the nonvolatile sensometabolome coining the highly attractive orosensory profile of a traditional balsamic vinegar are lacking. Moreover, the impact of the artisanal "batteria"-type maturation on taste-active molecules in TBV has not yet been elucidated on a molecular level. Although the well-known astringent ellagitannins compounds, vescalagin (**1**) and castalagin (**2**), Figure 1, originate from oak wood,[23] their contribution to the taste of wood-matured TBV is unclear.

Driven by the need to discover the key players imparting the typical taste of foods, the research area "sensomics" has made tremendous efforts in recent years in mapping the comprehensive population of sensory active, low-molecular weight compounds, coined sensometabolome, and cataloging, quantifying, and evaluating the sensory activity of metabolites which are present in raw materials and/or are produced upon food processing and storage, respectively.[22,24] Aimed at decoding the typical taste signature of food products on a molecular level, the so-called taste dilution analysis (TDA) was developed as an efficient screening tool enabling the sensory-directed identification of dietary key tastants[22,23,25−29] as well as taste modulating compounds in foods such as, e.g., γ-glutamyl peptides in cheese and beans,[30,31] *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids in stewed beef,[32] and N^2-(1-carboxyethyl)guanosine 5′-monophosphate in yeast extract,[33] respectively.

As the entire nonvolatile sensometabolome of traditional balsamic vinegar has not yet been investigated, the objective of the present investigation was to identify and quantify taste active and taste modulatory compounds in TBV and BV, to rank them in their sensory impact based on dose-activity considerations, and to validate their sensory relevance by means of taste re-engineering experiments. Finally, quantitative monitoring of taste compounds in intermediary samples taken from the "batteria" should visualize the evolution and/or degradation of selected sensometabolites throughout a full-scale TBV manufacturing process.

MATERIALS AND METHODS

Chemicals. All chemicals used were purchased from Sigma-Aldrich (Taufkirchen, Germany) and Fluka (Neu-Ulm, Germany), respectively. Stable isotope-labeled amino acids were purchased from Cambridge Isotope Laboratories (Andover, MA). For sensory analysis, bottled water (Evian) was adjusted to pH 3.0 with hydrochloric acid (0.1 M). Solvents were of HPLC grade (Mallinckrodt Baker, Griesheim, Germany). Ultrapure water used for chromatography was purified by means of a Milli-Q-water Gradient A 10 system (Millipore, Schwalbach, Germany), and deuterated solvents for NMR spectroscopy were supplied by Euriso-Top (Gif-Sur-Yvette, France). Reference samples of vescalagin (**1**) and castalagin (**2**) were isolated from *Quercus alba* L. following a literature procedure.[34] (+)-Dihydrorobinetin (**3**) was purchased from Extrasynthese (Genay Cedex, France).

Vinegar Samples. Samples of balsamic vinegar of Modena (BV), traditional balsamic vinegar of Modena (TBV; "affinato" quality, 12 years), and eight intermediary samples of TBV manufacturing collected from a "batteria" were obtained from local producers in the region of Modena, Italy. The "batteria" consisted of a sequence of eight casks differing in wood variety and volumes as given in parentheses: barrel A (acacia, 50 L), B (chestnut, 40 L), C (cherry, 30 L), D (mulberry, 23 L), E (oak, 13 L), F (chestnut, 10 L), G (chestnut, 5 L), and H (chestnut, 5 L). Dry mass[35] and pH value of intermediary vinegar samples A (43.91%, pH 2.65), B (55.23%, pH 2.62), C (63.75%, pH 2.58), D (70.75%, pH 2.52), E (70.95%, pH 2.52), F (72.76%, pH 2.48), G (73.98%, pH 2.49), and H (74.45%, pH 2.49) were determined as given in parentheses. All samples were kept at 4 °C in the dark until used for analysis.

Ultrafiltration. An aliquot (10 mL) of the TBV was diluted with water (200 mL) and separated into a low (<5 kDa; TBV-LMW; yield: 656.3 g/L) and a high molecular weight fraction (>5 kDa; TBV-HMW; yield: 8.7 g/L) by means of a Vivacell 250 static gas pressure filtration system (Vivascience, Hannover, Germany) equipped with a 5000 MWCO PES membrane (Vivascience, Hannover, Germany) following the protocol reported recently.[21] Both fractions collected from various separations were pooled accordingly and kept at −20 °C until further analysis.

Gel Adsorption Chromatography (GAC). A portion (1.0 g) of the lyophilized TBV-LMW fraction was dissolved in MeOH/water

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(20/80, v/v; 10 mL) and transferred onto the top of a XK 50/100 glass column (Amersham Pharmacia Biotech, Uppsala, Sweden) filled with a slurry of Sephadex LH 20 (GE Healthcare, Munich, Germany) conditioned with MeOH/water (20/80, v/v; adjusted to pH 4.0 with a 1% aqueous formic acid). Chromatography was carried out at a flow rate of 1.3 mL/min by eluting the column sequentially with aliquots (400 mL, each) of MeOH/water mixtures containing 20%, 40%, or 80% methanol, respectively, followed by pure methanol (400 mL). Monitoring the effluent at 220 nm by means of an UV-2575-type UV−vis detector (Jasco, Gross-Umstadt, Germany), the individual fractions were collected every 10 min and combined to give a total of ten fractions (I−X), which were separated from solvent in vacuum, freeze-dried, and stored at −20 °C until used for further analysis.

Identification of Taste-Active Compounds in GAC Fraction VII. An aliquot (100 mg) of the GAC fraction VII was dissolved in acetonitrile/water (50/50, v/v; 5 mL) and, after membrane filtration, was analyzed by semipreparative hydrophilic interaction liquid chromatography (HILIC) on a 300 × 21.5 mm^2 i.d., 10 μm, TSKgel Amide-80 column (Tosoh Bioscience, Stuttgart, Germany) equipped with a 75 × 21.5 mm^2 i.d., 10 μm, guard column of the same type (Tosoh Bioscience). Using a flow rate of 6.0 mL/min, chromatography was performed using 1% aqueous acetic acid as solvent A, and acetonitrile containing 1% acetic acid as solvent B. Starting with 5% A and increasing A to 100% within 20 min, the effluent was monitored by means of an evaporative light scattering detector (ELSD) and fractionated into five subfractions (VII-1 to VII-5), which were separated from solvent under vacuum and then lyophilized twice. Comparison of chromatographic (HILIC) and mass spectrometric data, followed by cochromatography with the corresponding reference compounds led to the identification of 1-*O*-acetyl-β-D-fructopyranose in fraction VII-2, 6-*O*-acetyl-α- and 6-*O*-acetyl-β-D-glucose in VII-3 and VII-4, and D-glucose in fraction VII-5, respectively.

Identification of Taste Modulating Compounds in GAC Fraction X. An aliquot (50 mg) of GAC fraction X was dissolved in MeOH/water (20/80, v/v; 1 mL), and after membrane filtration, an aliquot (200 μL) was injected into the HPLC system connected to a 250 × 21.2 mm^2 i.d., 5 μm, Microsorb RP18 column (Varian, Darmstadt, Germany). Using a flow rate of 18 mL/min, chromatography was performed using 0.1% aqueous formic acid (solvent A), and 0.1% formic acid in methanol (solvent B). Starting with 0% solvent A and increasing A to 100% within 20 min, the effluent was monitored at 280 nm and separated into subfractions X-1 and X-2, which were freed from solvent under vacuum and lyophilized. LC-MS/MS and 1D/2D-NMR studies led to the identification of 5-hydroxymethyl- (**6**) and 5-acetoxymethyl-2-furaldehyde (**7**). Comparison of chromatographic (RP-18) and spectroscopic data (UV−vis, LC−MS/MS, and NMR) with those of the corresponding reference compound confirmed the identity of these molecules.

*5-Hydroxymethyl-2-furaldehyde (**6**).* UV−vis (MeOH): λ_{max} = 280 nm. LC−MS (ESI^+): *m/z* 127 (70, $[M + H]^+$), 148 (100, $[M + Na]^+$). ^1H and ^{13}C NMR data were identical with those measured for the commercially available reference compound.

*5-Acetoxymethyl-2-furaldehyde (**7**).* UV−vis (MeOH), λ_{max} = 280 nm. LC−MS (ESI^+): *m/z* 169 (70, $[M + H]^+$), 191 (100, $[M+Na]^+$). ^1H NMR (400 MHz, MeOD, COSY): δ 2.20 [s, 3H, H−C(1)], 5.17 [s, 2H, H−C(3)], 6.71 [d, 1H, *J* = 4.0 Hz, H−C(5)], 7.39 [d, 1H, *J* = 4.0 Hz, H−C(6)], 9.59 [s, 1H, H−C(8)]. ^{13}C NMR (100 MHz, MeOD, HSQC, HMBC, DEPT): δ 19.1 [C(1)], 57.4 [C(3)], 112.2 [C(5)], 122.5 [C(6)], 153.0 [C(7)], 156.0 [C(4)], 170.5 [C(2)], 178.2 [C(8)].

Synthesis of 6-*O*-Acetyl-α/β-D-glucopyranose (4), 1-*O*-Acetyl-β-D-fructopyranose (5), and 6-*O*-Acetyl-α/β-[$^{13}C_6$]-D-glucopyranose ([$^{13}C_6$]-1). D-Glucose or D-fructose (3 mmol), respectively, was dissolved in dry pyridine/THF (4/1, v/v; 5 mL), and after addition of acetic anhydride (1.5 mmol), the solution was stirred at 0 °C. After 30 min, deionized water (1 mL) was added, pyridine was removed in vacuum, and the residue was diluted with water (50 mL), freeze-dried, and, then, separated by means of preparative HPLC on a 250 × 21.2 mm^2 i.d., 5 μm, Microsorb RP18 column (Varian, Darmstadt, Germany). Using 1% aqueous formic acid as the eluent, separation was performed isocratically, monitoring the effluent by means of an ELSD. The reaction products were collected and freeze-dried twice to afford the title compounds 6-*O*-acetyl-α/β-D-glucopyranose (0.9 mmol) and 1-*O*-acetyl-β-D-fructopyranose (0.8 mmol) as white solids with purities of >98%. For synthesis of 6-*O*-acetyl-α/β-[$^{13}C_6$]-D-glucopyranose, D-glucose-$^{13}C_6$ (1 mmol) and acetic anhydride (0.3 mmol) were reacted in dry pyridine/THF (4/1, v/v; 2 mL) and purified as detailed above for the natural ^{13}C abundant isotopologue.

*6-O-Acetyl-α/β-D-glucopyranose (**4**).* LC−MS (ESI^+): *m/z* 240 (100, $[M + NH_4]^+$), 223 (20, $[M + H]^+$). ^1H NMR (400 MHz, D_2O, COSY): δ 2.03 [s, 6H, H−C(8α/β)], 3.14 [dd, 1H, *J* = 4.0, 8.0 Hz, H−C(2β)], 3.31−3.61 [m, 6H, H−C(4α), H−C(4β), H−C(3β), H−C(2α), H−C(5β), H−C(3α)], 3.92 [ddd, 1H, *J* = 4.0, 8.0, 12.0 Hz, H−C(5α)], 4.14 [dd, 1H, *J* = 4.0, 12.0 Hz, H−C(6βa)], 4.22 [dd, 2H, *J* = 4.0, 8.0 Hz, H−C(6α)], 4.29 [dd, 1H, *J* = 4.0, 12.0 Hz, H−C(6βb)], 4.54 [d, 1H, *J* = 8.0 Hz, H−C(1β)], 5.10 [d, 1H, *J* = 4.0 Hz, H−C(1α)]. ^{13}C NMR (100 MHz, D_2O, HSQC, HMBC): δ 20.1 [C(8α/β)], 20.2 [C(8β/α)], 63.4 [C(6α/β)], 63.6 [C(6β/α)], 69.1 [C(5α)], 69.5 [C(4α/β)], 69.6 [C(4β/α)], 71.4 [C(2α)], 72.6 [C(5β)], 73.3 [C(3α)], 74.0 [C(2β)], 75.5 [C(3β)], 92.0 [C(1α)], 96.0 [C(1β)], 174.09 [C(7α/β)], 174.11 [C(7β/α)].

*1-O-Acetyl-β-D-fructopyranose (**5**).* LC−MS (ESI^+): *m/z* 240 (100, $[M + NH_4]^+$), 223 (20, $[M + H]^+$). ^1H NMR (400 MHz, D_2O, COSY): δ 2.08 [s, 3H, H−C(8)], 3.63 [dd, 1H, *J* = 4.0, 12.0 Hz, H−C(6a)], 3.71 [d, 1H, *J* = 8.0 Hz, H−C(4)], 3.84 [dd, 1H, *J* = 4.0, 12.0 Hz, H−C(5)], 3.93−3.99 [m, 2H, H−C(6b), H−C(3)], 4.12 [d, 2H, *J* = 4.0 Hz, H−C(1)]. ^{13}C NMR (100 MHz, D_2O, HSQC, HMBC): δ 20.1 [C(8)], 63.4 [C(6)], 65.6 [C(1)], 67.8 [C(4)], 68.8 [C(3)], 69.2 [C(5)], 96.8 [C(2)], 173.6 [C(7)].

*6-O-Acetyl-α/β-[$^{13}C_6$]-D-glucopyranose ([$^{13}C_6$]-**4**).* LC−MS (ESI^+): *m/z* 246 (100, $[M + NH_4]^+$), 229 (20, $[M + H]^+$). ^1H NMR (500 MHz, D_2O, COSY): δ 2.06 [s, 6H, H−C(8α/β)], 3.04 [m, 0.5H, *J* = 4.0, 12.0 Hz, H−C(2β)], 3.22−3.82 [m, 7.5H, H−C(2α), H−C(2β), H−C(3α), H−C(3β), H−C(4α), H−C(4β), H−C(5α), H−C(5β)], 4.01−4.43 [m, 4H, H−C(6α), H−C(6β)], 4.49 [d, 0.5H, *J* = 8.0 Hz, H−C(1β)], 4.75 [d, 0.5H, *J* = 8.0 Hz, H−C(1β)], 4.98 [d, 0.5H, *J* = 4.0 Hz, H−C(1α)], 5.32 [d, 0.5H, *J* = 4.0 Hz, H−C(1α)]. ^{13}C NMR (125 MHz, D_2O, HSQC, HMBC): δ 20.1 [C(8α/β)], 20.2 [C(8β/α)], 63.5 [d, *J* = 44.0 Hz, C(6α), C(6β)], 68.7−69.8 [m, C(5α), C(4α), C(4β)], 71.0−74.3 [m, C(2α), C(5β), C(3α), C(2β)], 75.5 [m, C(3β)], 92.1 [d, *J* = 46.2 Hz, C(1α)], 96.0 [dt, *J* = 3.75, 5.0, 46.2 Hz, C(1β)], 174.12/174.13 [C(7α/β)].

Synthesis of 5-[$^{13}C_2$]-Acetoxymethyl-2-furaldehyde ([$^{13}C_2$]-7), 5-(Butanoyloxy)methyl-2-furaldyhde (8), and 5-(Hexanoyloxy)methyl-2-furaldehyde (9). [$^{13}C_2$]-Acetylchloride, butanoylchloride, or hexanoylchloride (3 mmol), respectively, were added dropwise to a solution of 5-hydroxymethyl-2-furaldehyde (4 mmol) in THF/triethylamine (5/1, v/v; 6 mL). After stirring for 18 h at 20 °C, deionized water (1 mL) was added and the title compounds were isolated by means of preparative HPLC on a 250 × 21.2 mm^2 i.d., 5 μm, Microsorb RP18 column (Varian, Darmstadt, Germany). Chromatography was performed by use of 1% formic acid in water (eluent A) and acetonitrile (eluent B) at a flow rate of 18 mL/min, starting with 30% B for 1 min, then increasing the content of B to 100% within 10 min, and, finally, holding this conditions for another 2 min, while monitoring the effluent at 280 nm. 5-[$^{13}C_2$]-Acetoxymethyl-2-furaldehyde ([$^{13}C_2$]-**7**, 0.5 mmol), 5-(butanoyloxy)methyl-2-furaldehyde (**8**, 0.7 mmol), and 5-(hexanoyloxy)methyl-2-furaldehyde (**9**, 1.6 mmol) were obtained as pale yellow liquids and their structures verified by means of LC−MS/MS and NMR spectroscopy.

*5-[$^{13}C_2$]-Acetoxymethyl-2-furaldehyde ([$^{13}C_2$]-**7**).* UV−vis (MeOH): λ_{max} = 284 nm. LC−MS (ESI^+): *m/z* 271 (70, $[M + H]^+$), 291 (100, $[M + Na]^+$). ^1H NMR (400 MHz, MeOD, COSY): δ 1.95 [d, 1.5H, *J* = 8.0 Hz H−C(1)], 2.21 [d, 1.5H, *J* = 8.0 Hz, H−C(1)], 5.51 [d, 2H, *J* = 4.0 Hz, H−C(3)], 6.70 [d, 1H, *J* = 4.0 Hz, H−C(5)], 7.37 [d, 1H, *J* = 4.0 Hz, H−C(6)], 9.57 [s, 1H, H−C(8)]. ^{13}C NMR (100 MHz, MeOD, HSQC, HMBC, DEPT): δ 19.1 [d, *J* = 200 Hz, C(1)], 57.3 [C(3)], 112.1 [C(5)], 122.6 [C(6)], 153.0 [C(7)], 156.0 [C(4)], 170.5 [d, $J_{1,2}$ = 200 Hz, C(2)], 178.2 [C(8)].

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*5-(Butanoyloxy)methyl-2-furaldehyde (**8**).* LC−MS (ESI^+), m/z 219 (100, $[M + Na]^+$), 197 (60, $[M + H]^+$). ^1H NMR (500 MHz, D_2O, COSY): δ 0.93 [d, 3H, J = 8.0 Hz, H−C(10)], 1.63 [ddd, 2H, J = 8.0, 16.0, 24.0 Hz, H−C(9)], 2.32 [t, 2H, J = 8.0 Hz, H−C(8)], 5.16 [s, 2H, H−C(6)], 6.68 [d, 1H, J = 4.0 Hz, H−C(4)], 7.38 [d, 1H, J = 4.0 Hz, H−C(3)], 9.57 [s, 1H, H−C(1)]. ^{13}C NMR (125 MHz, D_2O, HMQC, HMBC): δ 13.9 [C(10)], 19.4 [C(9)], 36.6 [C(8)], 58.7 [C(6)], 113.6 [C(4)], 124.0 [C(3)], 154.4 [C(2)], 157.6 [C(5)], 174.4 [C(7)], 179.6 [C(1)].

*5-(Hexanoyloxy)methyl-2-furaldehyde (**9**).* LC−MS (ESI^+), m/z 247 (100, $[M + Na]^+$), 225 (80, $[M + H]^+$). ^1H NMR (500 MHz, D_2O, COSY): δ 0.89 [d, 3H, J = 8.0 Hz, H−C(12)], 1.31 [m, 4H, J = 4.0, 8.0 Hz, H−C(11), H−C(10)], 1.63 [dd, 2H, J = 8.0, 16.0 Hz, H−C(9)], 2.35 [t, 2H, J = 8.0, H−C(8)], 5.16 [s, 2H, H−C(6)], 6.68 [d, 1H, J = 4.0 Hz, H−C(4)], 7.37 [d, 1H, J = 4.0 Hz, H−C(3)], 9.57 [s, 1H, H−C(1)]. ^{13}C NMR (125 MHz, D_2O, HMQC, HMBC): δ 12.8 [C(12)], 21.9 [C(11)], 24.4 [C(9)], 30.9 [C(10)], 34.7 [C(8)], 58.7 [C(6)], 113.6 [C(4)], 124.0 [C(3)], 154.4 [C(2)], 157.6 [C(5)], 174.6 [C(7)], 179.6 [C(1)].

Quantitation of Candidate Taste-Active Compounds. *Soluble Carbohydrates, Alditols, Organic Acids, and Minerals.* TBV, BV, as well as intermediate samples A−H were diluted with deionized water for quantification of carbohydrates (1/10 000, v/v), alditols (1/1000, v/v), organic acids (1/200, v/v), anions and cations (1/50, v/v), respectively. After membrane filtration (0.45 μm), aliquots (5−25 μL) were analyzed by means of high-performance ion chromatography using an ICS 2500 system (Dionex, Idstein, Germany) following literature protocols.[28] Gluconic acid was determined enzymatically (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions.

Amino Acids. Free amino acids were quantified by stable isotope dilution analysis by means of HILIC−MS/MS using a modified literature protocol.[32] Vinegar samples were diluted with water (1/50; v/v) and membrane-filtered (0.45 μm), an aliquot (990 μL) of each sample was spiked with an aliquot (10 μL) of the internal standard solution containing the isotope-labeled amino acids (1 mg/L, each), and aliquots (10 μL) were analyzed on HPLC−MS/MS system 1 equipped with a 150 × 2.0 mm^2 i.d., 5 μm, TSKgel Amide-80 column (Tosoh Bioscience, Stuttgart, Germany) using the chromatographic conditions and ESI^+ instrument settings given in the Supporting Information.

*Phenols and 5-Hydroxymethyl-2-furaldehyde (**6**).* An aliquot (1 mL) of the vinegar samples was applied onto a Strata C18-E Giga Tube, 55 μm, 70 Å, RP-18 cartridge (10 g/60 mL, Phenomenex, Aschaffenburg, Germany), which was equilibrated with methanol, followed by water (100 mL, each). After flushing the cartridge with water (100 mL), the target analytes were eluted with methanol (100 mL), and after removing the solvent in vacuum, the residue was taken up in acetonitrile/water (20/80, v/v; 500 μL). Analysis was performed on LC−MS/MS-system 2 equipped with a 150 × 2 mm^2 i.d., 5 μm, Synergy Fusion RP18 column (Phenomenex, Aschaffenburg, Germany) using the chromatographic conditions and ESI^+ instrument settings given in the Supporting Information.

*Vescalagin (**1**), Castalagin (**2**), and (+)-Dihydrorobinetin (**3**).* Aliquots (1 mL) of vinegar samples were applied on a Strata C18-E Giga Tube, 55 μm, 70 Å, RP-18 cartridge (10 g/60 mL, Phenomenex, Aschaffenburg, Germany), which was equilibrated with methanol, followed by water (100 mL, each). After flushing the cartridge with water (100 mL), the target analytes were eluted with methanol (100 mL), and after removing the solvent in vacuum, the residue was taken up in acetonitrile/water (20/80, v/v; 500 μL) and an aliquot (15 μL) was analyzed on LC−MS/MS-system 1 equipped with a 150 × 2 mm^2 i.d., 5 μm, Luna Phenylhexyl column (Phenomenex, Aschaffenburg, Germany) using the chromatographic conditions and ESI^+ instrument settings given in the Supporting Information.

*6-O-Acetyl-α/β-D-glucopyranose (**4**) and 1-O-Acetyl-β-D-fructopyranose (**5**).* The balsamic vinegar samples were diluted with water (1/50; v/v) and membrane-filtered (0.45 μm), and an aliquot (990 μL) of the sample was then spiked with an aliquot of an internal standard solution (10 μL) containing 6-*O*-acetyl-α/β-[$^{13}C_6$]-D-glucopyranose (50 mg/L). Aliquots (10 μL) were injected into LC−MS/MS-system 2 connected to a 150 × 2.0 mm^2 i.d., 3 μm, TSKgel NH_2-100 column (Tosoh Bioscience, Stuttgart, Germany). Using acetonitrile containing 5% of an aqueous ammonium acetate solution (5 mmol/L) and 1% formic acid as eluent A and an aqueous ammonium acetate solution (5 mmol/L) containing 1% formic acid as eluent B, chromatography was performed isocratically with 89% solvent A at a flow rate of 0.2 mL/min for 10 min. 6-*O*-Acetyl-α/β-D-glucopyranose, 1-*O*-acetyl-β-D-fructopyranose, and the corresponding isotope-labeled standard were analyzed in the ESI^+ mode 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: 6-*O*-acetyl-α/β-D-glucopyranose (m/z 240.1→205.1; +11/+7/+20/+13/+4; 240.1→187.0; +11/+7/+20/+17/+4); 1-*O*-acetyl-β-D-fructopyranose (m/z 240.1→205.1; +11/+7/+20/+13/+4; 240.1→187.0; +11/+7/+20/+17/+4); 6-*O*-acetyl-α/β-[$^{13}C_6$]-D-glucopyranose (m/z 246.1→211.1; +11/+3/+28/+15/+4). After triplicate LC−MS/MS analysis of mixtures, containing analytes and internal standards in six molar ratios from 0.4 to 8.0, calibration curves were prepared 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, showing linear responses (correlation coefficients of >0.99 each).

*5-Acetoxymethyl-2-furaldehyde (**7**).* Vinegar samples were diluted 1:200 (v/v) with deionized water and membrane-filtered (0.45 μm), and after spiking a sample aliquot (990 μL) with the internal standard solution (10 μL) containing 5-[$^{13}C_2$]-acetoxymethyl-2-furaldehyde (200 μg/L), aliquots (10 μL) were injected into the LC−MS/MS system 2 connected to a 150 × 2 mm^2 i.d., 5 μm, Luna PFP column (Phenomenex, Aschaffenburg, Germany). Using aqueous 1% formic acid as solvent A and acetonitrile containing 1% formic acid as solvent B, chromatography was performed with a flow rate of 0.2 mL/min starting with 0% of solvent A for 2 min, then increasing solvent A to 100% within 15 min, followed by an isocratic elution for additional 2 min. 5-Acetoxymethyl-2-furaldehyde (**7**) and 5-[$^{13}C_2$]-acetoxymethyl-2-furaldehyde were analyzed in the ESI^+ mode, using the mass transitions and declustering potential (DP, in V), entrance potential (EP, in V), cell entrance potential (CEP, in V), collision energy (CE, in V), and cell exit potential (CXP, in V) given in parentheses: 5-acetoxymethyl-2-furaldehyde (m/z 168.9→109.0; +16/+7/+14/+17/+4), 5-[$^{13}C_2$]-acetoxymethyl-2-furaldehyde (m/z 171.0→109.0; +11/+8/+14/+17/+4). After triplicate LC−MS/MS analysis of mixtures containing analytes and internal standards in eight molar ratios from 0.1 to 8.9, calibration curves were prepared 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 coefficient >0.99).

Analytical Sensory Experiments. *General Conditions, Panel Training.* In order to familiarize the subjects with the taste language used by our sensory group and to get them trained in recognizing and distinguishing different qualities of oral sensations in analytical sensory experiments, 12 assessors (eight women and four men, age 26−39 years), who gave the informed consent to participate the sensory tests of the present investigation and had no history of known taste disorders, participated for at least two years in sensory training sessions with purified reference compounds by using the sip-and-spit method as reported earlier.[23,26,29] For intensity scaling, the test solutions, containing a tastant in defined concentrations, were used to calibrate the panel for judging the intensities 0, 2.5, and 5.0. Prior to sensory analysis, the fractions or compounds isolated were analytically confirmed to be essentially free of solvents and buffer compounds.

Taste Recognition Threshold Concentrations. Threshold concentrations were determined in bottled water adjusted to pH 3.0 with hydrochloric acid (0.1 M), using triangle tests with ascending concentrations of the stimulus as reported.[23] To overcome carry-over effects, astringent compounds were evaluated by means of the half-tongue test.[36] Values between individuals and separate sessions did not differ more than plus or minus one dilution step; as a result, a threshold value of, e.g., 900 μmol/L for gluconic acid represents a range 450−1800 μmol/L.

Taste Profile Analysis. Aqueous 1 + 2 dilutions of vinegar samples TBV and BV as well as the taste recombinant solutions rTBV and rBV, respectively, were presented to the panelists, who wore nose clips in order to prevent cross-modal interactions, and were asked to evaluate the taste qualities bitter, sour, sweet, salty, umami, astringent, and mouthfulness/viscosity on an intensity scale from 0 (not detectable) to 5 (strongly detectable) (Table 1). For taste profile analysis of vinegar fractions, the freeze-dried samples were taken up in bottled water in their "natural" concentrations, and after adjusting the pH value to 3.0 by adding trace amounts of hydrochloric acid (0.1 M), aqueous 1 + 2 dilutions (v/v) were presented to the panelists who were asked to rate the intensity of the individual taste qualities.

Table 1. Taste Profile Analysis of Aqueous Dilutions (1 + 2) of Traditional Balsamic Vinegar of Modena (TBV), Aqueous Solutions of the Low-Molecular Weight (TBV-LMW, <5 kDa), and the High-Molecular Weight Fraction (TBV-HMW, >5 kDa) Obtained by Ultrafiltration, and of Balsamic Vinegar of Modena (BV)

	intensity for individual taste quality[a]			
taste quality	TBV	TBV-LMW	TBV-HMW	BV
sweet	2.3	2.4 (±0.3)	0.2 (±0.2)	1.5 (±0.2)
sour	3.6	1.8 (±0.3)	0.3 (±0.3)	4.3 (±0.3)
bitter	0.6	0.5 (±0.1)	0.0 (±0.0)	0.8 (±0.2)
astringent	2.2	1.4 (±0.3)	0.9 (±0.3)	2.5 (±0.3)
mouthfulness	1.7	1.1 (±0.3)	0.2 (±0.2)	1.0 (±0.3)
umami	0.2	0.2 (±0.1)	0.0 (±0.0)	0.2 (±0.1)
salty	0.2	0.2 (±0.1)	0.2 (±0.2)	0.2 (±0.1)

[a]Intensities were judged on a linear scale from 0 (no taste impression) to 5 (strong taste impression) by 12 trained panelists. The data is given as mean of triplicates; std dev is given in parentheses.

Preparation of Taste Recombinants. A basic taste recombinant ($rTBV_{I-VI}$) was prepared by dissolving the tastants of TBV summarized in groups I–VI (Table 2) in their "natural" concentrations in water and adjusting the pH-value of this solution to 3.0 with trace amounts of hydrochloric acid (0.1 M). In addition, an extended taste recombinant ($rTBV_{I-VII}$) with the compounds given in groups I–VI (Table 2) and the TBV-HMW fraction (group VII in Table 2), each in its "natural" concentration, and a total taste recombinant ($rTBV_{total}$) containing all tastants (groups I–VIII) of TBV were prepared. In addition, a total taste recombinant (rBV_{total}) was prepared containing the taste compounds of BV, each in its "natural" concentration (Table 2). After equilibration for 12 h, the taste profiles of $rTBV_{I-VI}$, $rTBV_{I-VII}$, $rTBV_{total}$, and rBV_{total} were evaluated by means of comparative taste profile analysis.

Comparative Taste Profile Analysis. Lyophilized GAC fractions (I–X) were taken up in their "natural" concentrations either in water (10 mL), or in basic taste recombinant solution ($rTBV_{I-VI}$; 10 mL), and the pH-value was adjusted to 3.0 using trace amounts of hydrochloric acid (0.1 M). These solutions were then presented to the sensory panel, which was asked to rate the intensity of the individual taste qualities on a linear scale from 0 to 5 in comparison to the nonspiked recombinant $rTBV_{I-VI}$ (control).

Taste Dilution Analysis (TDA). Aliquots of the GAC fractions were taken up in "natural" ratios in water (10.0 mL), adjusted to pH 3.0 with trace amounts of hydrochloric acid (0.1 M), diluted stepwise 1 + 1 (v/v) with water (pH 3.0), and then used for the determination of the taste dilution (TD) factor.[25,36]

Determination of Sweetness Modulatory Activity. The sweetness modulating activity of **6** and **7** was determined in 4% sucrose solution (pH 3.0) containing 1% ethanol using a three-alternative forced-choice test.[37] Concentrations of the stimuli ranged from 0.2 to 10.0 mmol/L for **6** and from 0.2 to 2.0 mmol/L for **7**.

Time-Intensity Analysis. For time intensity recordings, a reference solution containing 4% sucrose and 1% ethanol and a sample solution containing 4% sucrose, 1% ethanol, and the test compound were rated randomly in alternating order on a horizontal nonstructured 15 cm line scale from 0 (not detectable) to 10 (strongly detectable). After a short rest (10 s), panelists were instructed to take up an aliquot (10 mL) of the test solution at once and, after swirling around in the oral cavity, to instantaneously start rating of the sweet taste intensity. After another 5 s, the panelists were asked to swallow the solution and, then, to go on with rating of the sweet intensity until the taste impression was no longer detectable. Thereafter, the panelists rinsed their mouth with water (20 mL) and, then, waited 1 min until the second sample was evaluated as given above. To familiarize the panelists with the experimental setup as well as with handling of the touch screen, training sessions with aqueous solutions of 4% sucrose and 1% ethanol in the absence and presence of sodium saccharin (5 and 10 mg/L), respectively, were performed. Data collection was performed by means of FIZZ sensory software (Version 2.46 A; Biosystems, Couternon, France). After starting the evaluation by touching the scale on the screen at a freely chosen point, the panelists moved their fingers according to the increase and decrease of the taste intensity, respectively. Data recording was automatically stopped after reaching the lower end of the intensity scale.

For evaluation of time intensity behavior of 5-acetoxymethyl-2-furaldehyde (**7**), reference solutions (10 mL) and sample solutions (10 mL) containing 5-acetoxymethyl-2-furaldehyde (**7**) (200, 1000, 2000, or 2500 μmol/L, respectively) were presented in randomized order to the panelists and evaluated as given above. After calculating the average time intensity (TI) curves using the FIZZ Calculations software and determining area-under-the-curve, duration, and duration of increasing and decreasing phase from the individual TI curves, the trapezoidal method[38] was applied after extraction of data points of 5% and 95% of the respective maximum intensity on the increasing as well as decreasing part of the individual curves (Figure 5A). The following parameters were extracted for further data analysis: maximum intensity, total area under the curve, area under increasing part of the curve, area under the plateau, area under the decreasing part of the curve, total duration of taste perception.

For data analysis, the data analysis and visualization platform R (version 2.15.0) was used.[39] Within R, repeated-measures ANOVA and sphericity tests were performed using the ez package (version 3.0-1), while the ggplot2 package was applied for result visualization.[40] The extracted parameters from samples with additive were normalized on the corresponding sample without additive from the same evaluation session. First a one-way repeated-measures ANOVA was conducted with the additive concentration as factor to detect significant changes of sensory perception depending on the additive concentration. Sphericity was assessed using Mauchly's test,[41] and Bonferroni correction was used as post hoc procedure. In a second step, a factorial repeated-measures design was used to evaluate the influence of the additive on the 3 phases of the time-intensity curve (increasing, plateau, decreasing) depending on the additive concentration. ANOVA was calculated by means of the ezANOVA fuction of R with post hoc tests and tests for sphericity as reported above.

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 chimeric G protein subunit Gα15Gαi3 following the protocol reported recently[42] (see also Supporting Information). Raw fluorescence signals of hTAS1R2/hTAS1R3-expressing cells were corrected for the fluorescence signals of control cells (software FLIPR^{tetra}, Molecular Devices, Bieberach, Germany) 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** (Figure 4) was performed with linear dilutions rows (1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 50 μmol/L). $\Delta F/F$ values were subtracted by solvent control. Finally, the concentration of test substance, evoking a fluorescence ratio significantly higher compared to the solvent control, was defined as threshold concentration for hTAS1R2/hTAS1R3 receptor activation.

Table 2. Taste Qualities, Taste Recognition Thresholds, Concentrations, and Dose-over-Threshold (DoT) Factors of Nonvolatile Sensometabolites in Traditional Balsamic Vinegar of Modena (TBV) and Balsamic Vinegar of Modena (BV)[a]

taste compound	TC[b] [μmol/L]	conc [μmol/L][c] (RSD in %)		DoT[d]	
		TBV	BV	TBV	BV
Group I: Sweet Tasting Compounds					
fructose	10 200[e]	1 601 111 (±0.6)	642 583 (±0.0)	157.0	69.1
glucose	18 000[e]	1 818 333 (±0.3)	704 760 (±0.0)	101.0	35.7
glycerol	81 100[e]	134 377 (±0.6)	55 191 (±1.2)	1.7	0.7
L-proline	25 000[f]	10 830 (±6.2)	12 458 (±0.5)	0.4	<0.1
inositol	17 700[e]	5372 (±0.4)	1298 (±3.8)	0.3	0.1
sorbitol	33 800[e]	10 534 (±3.2)	490 (±9.7)	0.3	<0.1
erythritol	36 300[e]	6612 (±9.7)	1638 (±9.6)	0.2	<0.1
xylitol	12 500[e]	1766 (±5.7)	358 (±6.3)	0.1	<0.1
mannitol	40 000	2900 (±3.8)	2316 (±4.0)	<0.1	0.1
arabitol	43 100	2200 (±3.4)	536 (±3.5)	<0.1	<0.1
ribitol	45 300	1400 (±4.9)	456 (±8.9)	<0.1	<0.1
L-methionine	5000[g]	17 (±3.9)	54 (±3.0)	<0.1	<0.1
L-alanine	12 000[f]	563 (±8.9)	1042 (±7.8)	<0.1	<0.1
L-serine	25 000[f]	575 (±5.5)	4722 (±5.4)	<0.1	<0.1
glycine	25 000[f]	268 (±10.3)	837 (±1.7)	<0.1	<0.1
L-threonine	35 000[f]	19 (±6.9)	61 (±4.8)	<0.1	<0.1
Group II: Sour Tasting Compounds					
tartaric acid	292[e]	31 450 (±1.2)	10 466 (±0.3)	107.7	35.8
gluconic acid	900	60 302 (±3.4)	4342 (±5.7)	67.0	4.8
glycolic acid	600	23 946 (±4.3)	3325 (±9.2)	39.9	5.5
malic acid	3690[e]	107 315 (±0.4)	19 366 (±3.5)	29.0	5.2
acetic acid	19 900[e]	371 161 (±1.0)	649 564 (±0.8)	18.7	32.6
citric acid	2600[e]	11 929 (±1.3)	2282 (±8.0)	4.6	0.9
succinic acid	900[e]	2420 (±8.7)	n.d.	2.7	n.d.
lactic acid	15 480[e]	7302 (±3.9)	9786 (±4.4)	0.5	0.6
Group III: Bitter Tasting Compounds					
calcium	6200[i,j]	24 578 (±3.8)	10 101 (±0.2)	4.0	1.6
magnesium	6400[i,j]	20 024 (±1.5)	8335 (±2.6)	3.1	1.3
L-arginine	75 000[g]	7691 (±0.7)	14 322 (±5.4)	0.1	0.8
L-leucine	11 000[g]	143 (±7.3)	531 (±2.3)	<0.1	<0.1
L-tyrosine	4000[g]	77 (±6.0)	281 (±5.2)	<0.1	<0.1
L-isoleucine	10 000[g]	193 (±2.7)	453 (±8.4)	<0.1	<0.1
L-valine	30 000[i]	226 (±6.0)	410 (±4.9)	<0.1	<0.1
L-phenylalanine	45 000[g]	88 (±4.2)	336 (±1.4)	<0.1	<0.1
L-histidine	45 000[g]	29 (±2.9)	175 (±1.2)	<0.1	<0.1
Group IV: Astringent Compounds					
castalagin (**2**)	1.1[l]	51 (±1.1)	n.d.	46.4	n.d.
vescalagin (**1**)	1.1[l]	38 (±4.4)	n.d.	34.5	n.d.
5-hydroxymethyl-2-furaldehyde (**6**)	10 000[l]	29 419 (±1.8)	8681 (±5.1)	2.9	0.9
trans-caffeic acid	72[l]	29 (±2.7)	23 (±0.5)	0.4	0.3
gentisic acid	122[l]	38 (±1.9)	10 (±1.4)	0.3	<0.1
coumaric acid	139[l]	35 (±2.5)	23 (±0.9)	0.2	0.2
gallic acid	292[l]	32 (±10.6)	17 (±5.7)	0.1	<0.1
p-hyroxybenzoic acid	665[l]	17 (±1.8)	9.1 (±0.0)	<0.1	<0.1
quinic acid	579	15 (±2.5)	1.3 (±1.5)	<0.1	<0.1
protocatechuic acid	206[l]	10 (±3.8)	0.7 (±3.5)	<0.1	<0.1
vanillic acid	315[l]	20 (±8.2)	6.3 (±4.9)	<0.1	<0.1
ferulic acid	67[l]	2.4 (±9.4)	1.8 (±2.4)	<0.1	<0.1
vanilline	829[l]	7.2 (±1.7)	0.7 (±9.8)	<0.1	<0.1
gallic acid methyl ester	232[l]	3.3 (±3.5)	1.6 (±1.1)	<0.1	<0.1
gallic acid ethyl ester	185[l]	2.9 (±5.8)	46 (±9.3)	<0.1	<0.1
syringaldehyde	330[l]	8.7 (±3.4)	0.4 (±1.5)	<0.1	<0.1
(+)-dihydrorobinetin (**3**)	23	1.1 (±1.8)	n.d.	<0.1	n.d.
Group V: Salty Compounds					
potassium	13 000[i,j]	58 025 (±3.6)	71 311 (±4.6)	3.1	5.5
phosphate	5000[h,k]	12 230 (±2.9)	7366 (±2.1)	2.4	1.5
sodium	3900[i,j]	7608 (±9.8)	14 667 (±2.1)	2.0	3.8

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Table 2. continued

taste compound	TC[b] [μmol/L]	conc [μmol/L][c] (RSD in %)		DoT[d]	
		TBV	BV	TBV	BV
Group V: Salty Compounds					
chloride	3900[k,i]	6025 (±2.0)	3640 (±1.1)	1.5	0.7
Group VI: Umami-like Compounds					
L-aspartic acid	600[i]	528 (±12.8)	1776 (±9.5)	0.9	3.0
L-glutamic acid	1100[i]	179 (±8.3)	591 (±0.6)	0.1	0.5
Group VII: Astringent Polymers					
HMW-fraction (>5 kDa)	n.d.	8.7 g/L	n.d.	n.d.	n.d.
Group VIII: Acetylated Compounds					
4	12 300[n]	8329 (±7.4)	2468 (±8.7)	0.7	0.2
4	12 300[o]	8329 (±7.4)	2468 (±8.7)	0.7	0.2
5	21 200[n]	13 823 (±4.8)	5643 (±7.5)	0.7	0.2
5	16 900[o]	13 823 (±4.8)	5643 (±7.5)	0.8	0.3
7	1500[m]	315 (±3.1)	76 (±6.9)	0.2	<0.1

[a]Taste-active compounds were determined in TBV and BV, if not stated otherwise. [b]Taste threshold concentrations (TC) are given as the mean of triplicates in bottled water and were determined by means of a three alternative forced choice test, or were taken from literature. [c]Concentration (μmol/L) in TBV and BV. [d]Dose-over-threshold (DoT) factor is calculated as the ratio of concentration and taste threshold. [e]Value taken from ref 26. [f]Value taken from ref 59. [g]Value taken from ref 60. [h]Value taken from ref 61. [i]Value taken from ref 24. [j]TC determined for the corresponding chloride salt. [k]TC determined for the corresponding sodium salt. [l]Value taken from ref 21. [m]TC for sweet taste enhancement determined in the 4% sucrose solution. [n]TC for bitter taste. [o]TC for sweet taste.

High Performance Liquid Chromatography (HPLC). The HPLC system (Jasco, Gross-Umstadt, Germany) consisted of two PU-2087 Plus pumps, a DG-2080-53 degasser, a LG-2080-02 gradient unit, a AS-2055 Plus autosampler with a 100 μL loop, a Rh 7725i injection valve with a 1000 μL loop (Rheodyne, Bensheim, Germany), a MD-2010 Plus multiwavelength detector, and a 85 Sedex LT-ELSD (Sedere, Alfortville, France).

Liquid Chromatography/Mass Spectrometry (LC−MS/MS). LC−MS/MS analyses were performed on two different systems: LC−MS/MS system 1 was a API 4000 Q-Trap LC−MS/MS system (Applied Biosystems Sciex Instruments, Darmstadt, Germany) connected to a 1200 HPLC-system (Agilent, Waldbronn, Germany); LC−MS/MS system 2 was a API 3200 LC−MS/MS system (Applied Biosystems) connected to a 1100 HPLC-system (Agilent, Waldbronn, Germany). Both mass spectrometers were operated in the full scan mode for monitoring of positive or negative ions using the following instrument settings: ion spray voltage (5500 V for ESI^+, −4500 V for ESI^- mode), source temperature (425 °C), curtain gas (nitrogen, 20 psi), and declustering potential (+25 V for ESI^+, −25 V for ESI^- mode). Fragmentation of $[M + H]^+$ or $[M - H]^-$ pseudomolecular ions into specific product ions was induced by collision with nitrogen (4×10^{-5} Torr) and a collision energy of +25 (ESI^+) and −25 V (ESI^-), respectively. Declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE), and cell exit potential (CXP) were tuned for each compound by flow injection (10 μL/min) via syringe pump injection, detecting the fragmentation of the $[M + H]^+$ or $[M - H]^-$ pseudomolecular ions into specific product ions after collision with nitrogen. Control of LC−MS/MS instruments was performed using Analyst software (version 1.5; Applied Biosystems).

Nuclear Magnetic Resonance Spectroscopy (NMR). ^1H, ^{13}C, COSY, HSQC, and HMBC experiments were performed on a Bruker DRX-400 and an Avance-III-500 spectrometer, respectively, the latter of which was equipped with a Cryo-CTCI probe (Bruker, Rheinstetten, Germany). MeOD or D_2O were used as solvents, and trimethylsilylpropionic acid-d_4 (TMSP) was used as the internal standard. Data processing was performed by using Topspin software (version 2.1; Bruker) as well as Mestre-C software (version 4.8.6; Mestrelab Research, Santiago de Compostella, Spain).

Sensomics Heatmaps and Hierarchical Cluster Analysis. Heatmap analysis of the taste-active compounds during TBVM aging was performed within the data analysis and visualization platform R (version 2.15.1)[39] using the pheatmap package (version 0.6.1). Sensomics heatmaps were calculated on the basis of scaled fresh weight concentrations (Table S1, Supporting Information) as well as on the basis of scaled dry matter data (Table S2, Supporting Information), to highlight the concentration changes during TBV storage in the barrels A−H. For cluster analysis, squared Euclidean distances were applied as distance measure while clusters were formed according to Ward's minimum variance method.[43]

RESULTS AND DISCUSSION

To identify the compounds responsible for the typical taste of traditional balsamic vinegar of Modena (TBV), a 1 + 2 dilution of the vinegar was presented to 12 trained panelists who were asked to rate the intensities of the taste descriptors sweet, sour, bitter, astringent, umami, and salty, as well as the impression of mouthfulness on a linear scale from 0 (not detectable) to 5 (strongly detectable). In comparison, taste profile analysis was performed with balsamic vinegar of Modena (BV). Sourness and sweetness of TBV were rated with the highest intensities of 3.6 and 2.3, respectively, followed by astringency (2.2) and mouthfulness (1.7), whereas bitterness was perceived with a lower score of 0.6 only (Table 1). In comparison, the BV sample exhibited a significantly more sour (4.3) and less sweet (1.5) taste profile with also lower scores judged for mouthfulness (1.0). In both samples, umami and salty taste were hardly perceivable with an average intensity of 0.2. The mean values obtained for each descriptor in triplicate analysis of TBV was used to calibrate the sensory panel for the precise sensory evaluation of the vinegar samples as well as the fractions isolated therefrom in the following.

In order to separate the taste-active compounds based on molecular weight differences, TBV was separated by means of ultrafiltration to obtain the low (TBV-LMW, < 5 kDa) and high (TBV-HMW, > 5 kDa) molecular weight fraction. To evaluate their sensory impact, both fractions were taken up in bottled water in their "natural" concentrations, 1 + 2 diluted with table water, and, after adjusting the pH to 3.0, were evaluated by means of taste profile analysis (Table 1). The intensities of the orosensory descriptors sweet, bitter, salty, and umami judged for the TBV-LMW fraction were rather close to those of the TBV sample, thus demonstrating low-molecular weight molecules to imparting these taste sensations. In comparison

Table 3. Sensory Evaluation of Traditional Balsamic Vinegar of Modena (TBV, 1 + 2 Diluted) and Balsamic Vinegar of Modena (BV, 1 + 2 Diluted) and the Corresponding Taste Recombinants rTBV and rBV (Each 1 + 2 Diluted)

	intensity for individual taste quality[a]					
taste quality	TBV	$rTBV_{I-VI}$[b]	$rTBV_{I-VII}$[b]	$rTBV_{total}$[b]	BV	rBV_{total}[b]
sweet	2.3	2.0 (±0.3)	2.1 (±0.2)	2.4 (±0.2)	1.5	1.3 (±0.2)
sour	3.6	3.8 (±0.3)	3.4 (±0.2)	3.6 (±0.2)	4.3	4.2 (±0.3)
bitter	0.6	0.5 (±0.1)	0.5 (±0.1)	0.5 (±0.1)	0.8	0.8 (±0.2)
astringent	2.2	2.0 (±0.1)	2.1 (±0.1)	2.2 (±0.2)	2.5	2.2 (±0.3)
mouthfulness	1.7	1.1 (±0.2)	1.6 (±0.2)	1.6 (±0.3)	1.0	1.0 (±0.2)
umami	0.2	0.2 (±0.1)	0.2 (±0.1)	0.2 (±0.1)	0.2	0.2 (±0.1)
salty	0.2	0.2 (±0.1)	0.2 (±0.1)	0.2 (±0.1)	0.2	0.2 (±0.1)

[a]Intensities were judged on a scale from 0 (not detectable) to 5 (strongly detectable) by 12 trained panelists. The data is given as the mean of triplicates. [b]$rTBV_{I-VI}$ contained the tastants in groups I–VI in concentrations given in Table 2 in water (pH 3.0); $rTBV_{I-VII}$ was prepared by dissolving the HMW fraction (8.7 g/L) in $rTBV_{I-VI}$; $rTBV_{total}$ was prepared by spiking $rTBV_{I-VII}$ with tastant group VIII (compounds **4**, **5** and **7**) in concentrations given in Table 2; rBV_{total} contained the tastants in groups I–VIII in concentrations given in Table 2 in water (pH 3.0).

to TBV, sourness, astringency, and mouthfulness were judged with lower scores in the TBV-LMW fraction. The comparatively low impact of sourness in the TBV-LMW fraction might be explained by the loss of the volatile acetic acid upon sample lyophilization. The low astringency score of 1.4 reported for the TBV-LMW fraction indicated that the overall astringency perception of TBV (2.2) is only partially due to low molecular weight compounds and is complemented by astringent macromolecules in the TBV-HMW fraction (0.9). This is well in line with recent findings on the equally important contribution of low (<5 kDa) and high molecular weight compounds (>5 kDa) to the astringency of red wine.[22]

Identification and Quantitation of Basic Tastants. In order to evaluate the sensory impact of basic taste active compounds to the taste profile of TBV, 2 monosaccharides, 8 alditols, 8 organic acids, 4 cations, and 2 inorganic anions were identified and quantitatively analyzed by means of high-performance ion chromatography (Table 2). In addition, gluconic acid was quantified by means of an enzymatic assay. Moreover, 8 phenolic acids, 2 phenolic acid esters, vanilline, and 5-hydroxymethyl-2-furaldehyde were identified and quantified by means of RP-HPLC–MS/MS and a total of 15 free amino acids by means of HILIC–MS/MS.

As TBV is aged in wooden barrels, the vinegar sample was analyzed for the presence of vescalagin (**1**; Figure 1) and castalagin (**2**), both ellagitannins have been reported as astringent molecules migrating from oak wood into wine and whiskey upon barrel maturation.[23] As sensory evaluation revealed an intense astringent impression above the recognition threshold concentration of 23 μmol/L (Table 2), (+)-dihydrorobinetin (**3**) was analyzed in TBV as it is reported as a marker molecule for storage of vinegars in acacia barrels.[44] After SPE cartridge cleanup, compounds **1**–**3** were analyzed by means of RP-HPLC–MS/MS in TBV, and for comparison also in the BV sample, the latter of which is not matured in wooden barrels. As expected, recording the characteristic mass transitions of vescalagin (**1**), castalagin (**2**), and (+)-dihydrorobinetin (**3**) in the BV sample did not reveal any signal (**A**/**B**; Figure S1, Supporting Information). In contrast, the analysis of the TBV sample clearly demonstrated the presence of the wood-derived polyphenols **1**–**3** (**C**/**D**; Figure S1, Supporting Information). Finally, matrix calibration by spiking the BV sample with the reference compounds, followed by HPLC–MS/MS analysis, confirmed the absence of **1**–**3** in BV and led to the identification of these polyphenols in TBV (**E**/**F**; Figure S1, Supporting Information). Matrix-calibrated HPLC–MS/MS quantitation revealed concentrations of 51 and 38 μmol/L for the ellagitannins **1** and **2**, respectively, and 1 μmol/L for (+)-dihydrorobinetin (**3**).

After quantitative analysis, the taste recognition threshold concentrations of the compounds were determined and a dose-over-threshold (DoT)-factor was calculated for each compound from the ratio of the concentration and the threshold concentration.[26] As we aimed to elucidate the key metabolites for each individual taste quality, the single taste compounds identified in TBV were grouped into classes differing in their taste qualities (Table 2).

Among the sweet tasting molecules (group I) in TBV, fructose and glucose were evaluated with the highest DoT-factors of 157.0 and 101.0, followed by glycerol with a value of 1.7, and L-proline, inositol, sorbitol, erythritol, and xylitol evaluated with DoT-factors between 0.1 and 0.4 (Table 2). Tartaric acid, gluconic acid, glycolic acid, malic acid, acetic acid, citric acid, and succinic acid exceeded their taste threshold concentrations with tastant group II comprising the sour tasting molecules. Group III consisted of bitter tasting amino acids and minerals, among which only calcium and magnesium ions exceeded their thresholds by a factor of 4.0 and 3.1, respectively (Table 2). All astringent molecules of TBV were summarized in group IV, but only the ellagitannins castalagin (**2**), vescalagin (**1**), as well as 5-hydroxymethyl-2-furaldehyde showed high DoT-Factors of 46.4, 34.5, and 2.9, respectively. (*E*)-Caffeic acid, gentisinic acid, *p*-coumaric acid, and gallic acid were evaluated with DoT-factors between 0.1 and 0.4, whereas all the other polyphenols were more than 10-fold below their taste threshold concentrations (Table 2). Among the group of salty tasting components (group V), the cations sodium and potassium and the anions chloride and phosphate were judged with DoT-factors between 1.5 and 3.1 in TBV, whereas none of the amino acids in group VI exceeded their recognition threshold for umami taste.

Re-engineering the Taste Profile of TBV. To confirm the analytical data and to check whether the compounds identified can already create the typical taste of TBV, an aqueous taste recombinant containing 54 tastants, each in its "natural" concentration (Table 2), was prepared, and after pH adjustment (pH 3.0), the taste profile of this basic taste recombinant ($rTBV_{I-VI}$) was compared to that of the TBV (Table 3). The intensities detected for sweetness, sourness, bitterness, and astringency matched rather well those determined for TBV, whereas the mouthfulness was judged significantly less intense in the tastant cocktail. In addition, the

sensory panel reported the sweetness of TBV to be more long lasting when compared to $rTBV_{I-VI}$. In order to investigate the impact of the high-molecular weight components of vinegar on the mouthfulness perception, an additional taste recombinant ($rTBV_{I-VII}$) was made by spiking $rTBV_{I-VI}$ with the TBV-HMW fraction in its "natural" concentration. Comparative taste profile analysis of $rTBV_{I-VII}$ revealed an increase in mouthfulness from 1.1 to 1.6, thus demonstrating the macromolecular components to play an important role in mouthfulness perception. Despite the presence of the TBV-HMW fraction, the recombinant $rTBV_{I-VII}$ induced a less long-lasting sweetness perception when compared to the authentic TBV or the TBV-LMF fraction, respectively, thus indicating that the basic taste recombinant is lacking compounds modulating the sweetness perception.

Sensory-Directed Fractionation of the TBV-LMW Fraction. In order to locate the molecules responsible for the long-lasting sweet taste of TBV, the TBV-LMW fraction was fractioned by means of gel absorption chromatography (GAC) on Sephadex LH-20 material using a methanol/water gradient. Monitoring the effluent by means of UV−vis detection, the TBV-LMW fraction was separated into fractions I−X, which were individually collected and freeze-dried twice (Figure 2).

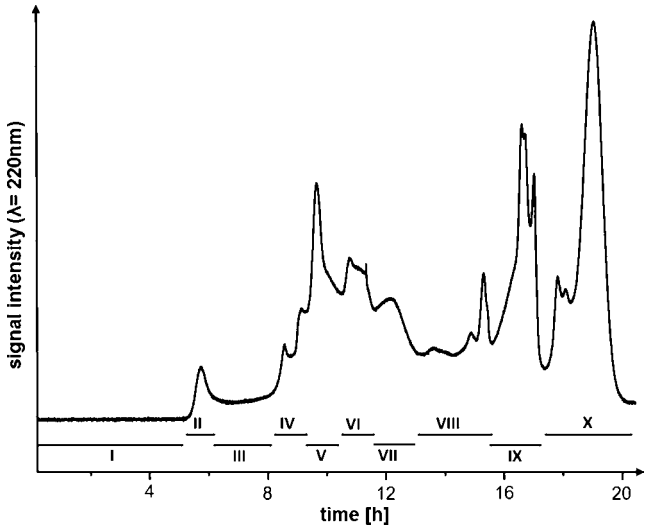


Figure 2. Gel absorption chromatogram (λ = 220 nm) of the low molecular weight fraction (TBV-LMW, <5 kDa) isolated from TBV by means of ultrafiltration.

In a first set of experiments, an aliquot of each GAC fraction was dissolved in bottled water in its "natural" concentration, which means in the amounts obtained from the GAC column, and evaluated by means of a taste dilution analysis (TDA) to evaluate their intrinsic taste impact (Table 4). The highest TD-factors of 256 and 128 were found for sweetness in fraction VII and sourness in fraction IX, respectively, followed by astringency in fraction IX and sourness in fraction III, both judged with a TD-factor of 64 (Table 4). Besides sour taste (16), bitterness (4), and astringency (2), the sensory panel reported on a sweet taste in fraction VI but with a low TD factor of 1. Except for the sensory inactive fraction I, the remaining fractions revealed some sour, bitter, and astringent taste impressions or combinations thereof with TD-factors ranging from 1 to 32 (Table 4).

Table 4. Taste Dilution Analysis (TDA) of GAC-Fractions I−X Dissolved in Bottled Water and Comparative Taste Profile Analysis (cTPA) of GAC Fractions I−X Dissolved in Basic Taste Recombinant ($rTBV_{I-VI}$)

	TDA in water[b]		
fraction no.[a]	TD factor	taste quality	cTPA in $rTBV_{I-VI}$[c] change in taste quality
I	<1	n.d.	n.d.
II	16	sour	n.d.
II	16	astringent	
II	1	bitter	
III	64	sour	n.d.
III	2	bitter	
IV	32	astringent	n.d.
IV	16	sour	
IV	16	bitter	
V	32	astringent	n.d.
V	16	sour	
V	16	bitter	
VI	16	sour	n.d.
VI	4	bitter	
VI	2	astringent	
VI	1	sweet	
VII	256	sweet	increased sweetness[d]
VII	16	sour	
VIII	16	sour	increased sourness[d]
VIII	8	astringent	
VIII	2	bitter	
VIII	1	sweet	
IX	128	sour	increased sourness and
IX	64	astringent	astringency[d]
X	16	sour	more long-lasting sweetness[d]
X	8	astringent	
X	4	bitter	

[a]Numbering of GAC fractions corresponds to Figure 2. [b]Taste dilution analysis (TDA) was carried out after dissolving the individual GAC fractions in bottled water (pH 3.0) in their "natural" concentration ratios. [c]The individual GAC fractions were dissolved in a 1 + 2 dilution of the basic taste recombinant solution $rTBV_{I-VI}$ (pH 3.0) containing all tastant groups I−VI given in Table 2. The descriptors given by each panelist were collected, and those given by at least 9 out of the 12 panelists are presented here. The $rTBV_{I-VI}$ solution lacking any GAC fractions was used as control. n.d. no difference detectable. [d]$p < 0.05$.

A second set of experiments were aimed at the discovery of taste-modulating molecules in the individual GAC-fractions. Therefore, a solution of the basic taste recombinant $rTBV_{I-VI}$ was used as the matrix solution for the localization of candidate sweetness modulators. To achieve this, aliquots of the individual GAC-fractions were dissolved in the 1 + 2-diluted $rTBV_{I-VI}$ solution in their "natural" concentrations and were then evaluated by means of a comparative taste profile analysis using the likewise diluted, blank $rTBV_{I-VI}$ as control. Out of the fractions I−X, spiking the $rTBV_{I-VI}$ solution with fractions VII and X induced an increased sweetness intensity and a more long-lasting sweetness perception, respectively, when compared to $rTBV_{I-VI}$ alone (Table 4). Therefore, further experiments were targeted toward the sensory active compounds imparting the enhanced and more long-lasting sweetness perceived in fractions VII and X, respectively.

Identification of Taste and Taste Modulatory Compounds in TBV. Fraction VII was separated by means of

hydrophilic liquid interaction chromatography (HILIC) to afford the subfractions VII-1 to VII-5 (Figure 3A), all of which

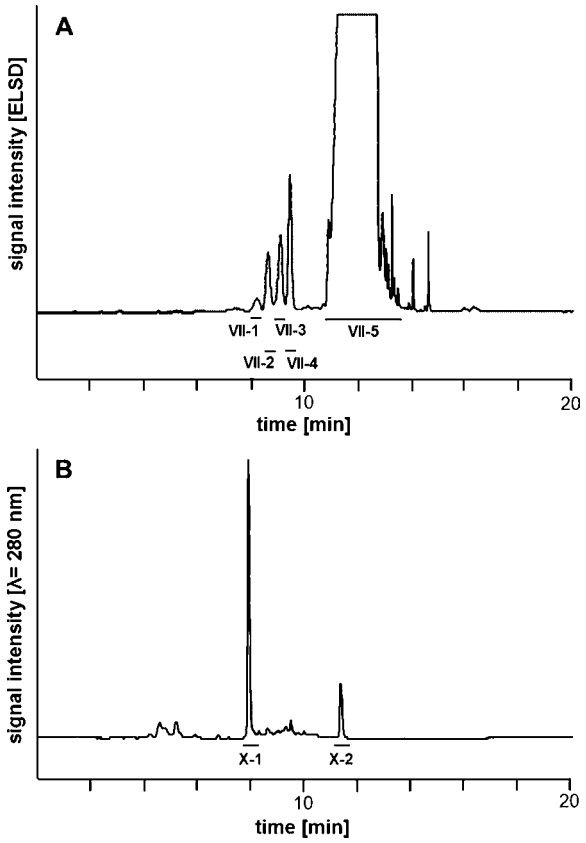
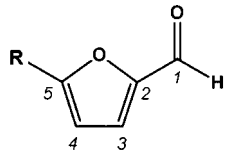


Figure 3. (A) HILIC-ELSD chromatogram of fraction VII and (B) RP-HPLC-DAD chromatogram of fraction X isolated from the TBV-LMW fraction.

imparted sweet and/or bitter taste. MS and HPIC analysis revealed fructose and glucose as the main components in subfraction VII-5. In addition, LC−MS (ESI^+) analysis revealed m/z 223 ($[M + H]^+$) and 240 ($[M + NH_4]^+$) as the pseudomolecular ions of the compounds eluting in four fractions VII-1 to VII-4, thus indicating the presence of isomers. As the molecular weight of 220 Da was well in agreement with those of monosaccharide acetates reported in literature,[18] reference compounds of 6-*O*-acetyl-α/β-D-glucopyranose (**4**, Figure 1) and 1-*O*-acetyl-β-D-fructopyranose (**5**, Figure 1) were synthesized by acetylation of D-glucose and D-fructose, respectively, with acetic anhydride in pyridine/THF, followed by structure verification by means of LC−MS/MS and 1D/2D-NMR experiments. Comparison of MS data and retention times (RP-HPLC, HILIC), followed by cochromatography with the corresponding reference compound revealed 6-*O*-acetyl-α-D-glucopyranose and 6-*O*-acetyl-β-D-glucopyranose (**4**, Figure 1) as bitter-sweet compounds eluting in fractions VII-3 and VII-4, respectively, and 1-*O*-acetyl-β-D-fructopyranose (**5**, Figure 1) as the bitter-sweet compound eluting in fraction VII-2. Sensory evaluation of the mixture of 6-*O*-acetyl-α/β-glucopyranose showed a taste threshold concentration of 12.3 mmol/L for sweet and bitter taste, whereas 1-*O*-acetyl-β-D-fructopyranose revealed a threshold of 16.9 mmol/L for sweet and 21.2 mmol/L for bitter taste.

Separation of fraction X by means of RP-HPLC and monitoring the effluent at 280 nm resulted in two peaks, namely X-1 and X-2 (Figure 3B). Comparison of MS data and retention time with those of the reference compound, followed by cochromatography, led to the identification of peak X-1 as 5-hydroxymethyl-2-furaldehyde (**6** in Figure 4). LC−MS/MS



no.	R
10	-H
11	$-CH_3$
6	$-CH_2-OH$
7	$-CH_2-O-CO-CH_3$
8	$-CH_2-O-CO-(CH_2)_2-CH_3$
9	$-CH_2-O-CO-(CH_2)_4-CH_3$

Figure 4. Structures of 5-hydroxymethyl-2-furaldehyde (**6**), 5-acetoxymethyl-2-furaldehyde (**7**), 5-(butanoyloxy)methyl-2-furaldehyde (**8**), 5-(hexanoyloxy)methyl-2-furaldehyde (**9**), 2-furaldehyde (**10**), and 5-methyl-2-furaldehyde (**11**).

analysis of peak X-2 showing sweet modulating activity in $rTBV_{I-VI}$ in a degustation experiment revealed a pseudomolecular ion ($[M + H]^+$) of m/z 168 as well as the fragment ions m/z 109 and m/z 81 for the compound eluting in fraction X-2, thus suggesting the presence of a 5-hydroxymethyl-2-furaldehyde moiety. The ^1H and ^{13}C NMR spectra revealed five proton resonance signals integrating for eight protons and a total of eight carbon signals. The proton signals H−C(3), H−C(5), H−C(6), and H−C(8) resonating at 5.17, 6.71, 7.39, and 9.59 ppm were assigned as the protons of the 5-hydroxymethyl-2-furaldehyde moiety. In addition, the singlet signal resonating at 2.02 ppm and integrating for three protons indicated the presence of a methyl group. The ^{13}C NMR spectrum exhibited the resonance signal at 178.2 ppm as expected for the aldehyde carbonyl atom C(8) and, in addition, another carbon signal C(2) at 170.5 ppm, thus suggesting the presence of an acetyl ester moiety. Heteronuclear couplings (HMBC) were observed between the carbonyl carbon C(2) and the methyl residue H−C(1) as well as the methylene protons H−C(3), thus leading to the identification of that compound as 5-acetyoxymethyl-2-furaldehyde (**7** in Figure 4).

Sensory Analysis of and Sweet Taste Receptor Responses to 5-Acetoxymethyl-2-furaldehyde. 5-Acetoxymethyl-2-furaldehyde (**7**) and, in comparison, 5-hydroxymethyl-furaldehyde (**6**) were sensorially evaluated for their intrinsic taste in water (pH 3.0, 1% ethanol) and in a 4% aqueous sucrose solution (pH 3.0, 1% ethanol) for sweet taste modulating properties. Neither **6** nor **7** showed any intrinsic taste up to a concentration of 10 and 2.0 mmol/L (water), respectively. However, the presence of 5-acetoxymethyl-2-furaldehyde (**7**) induced a significant change in sweet taste quality as well as a more long-lasting sensation of the 4% sucrose solution with a recognition threshold of 1.5 mmol/L (α-level: 0.05). It is interesting to notice that the nonacetylated compound **6** did not show any sweetness modulating activity.

In order to provide temporal information on the perceived sweetness modulating activity, time-intensity (TI) evaluations

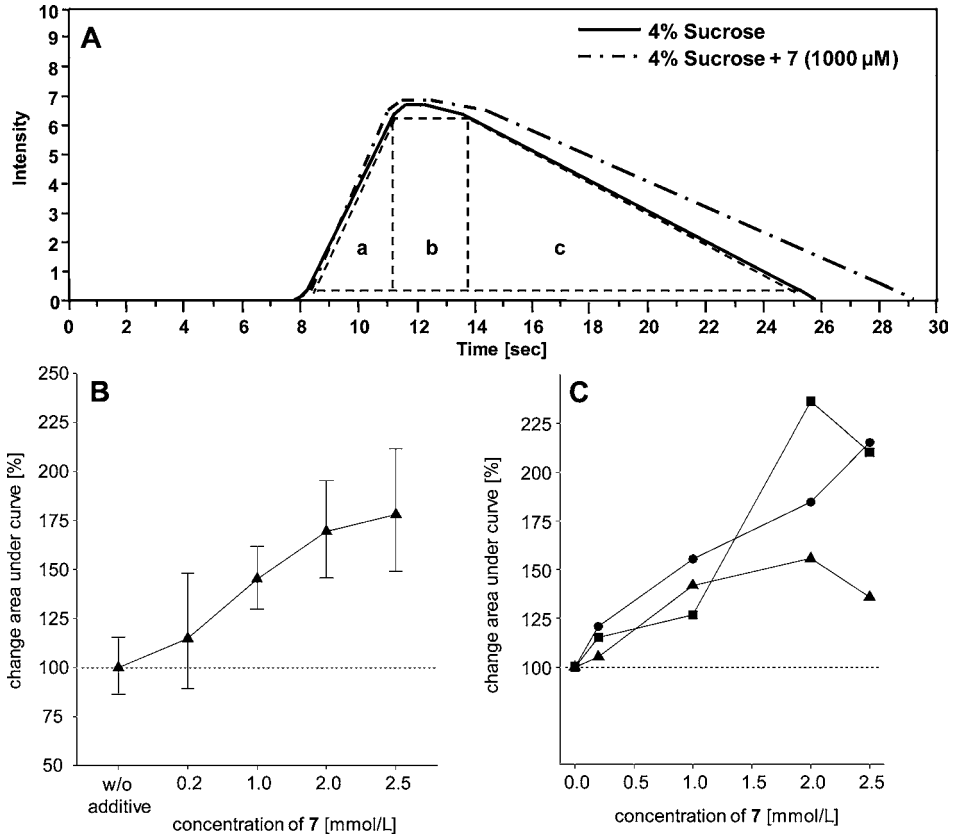


Figure 5. (A) Average time-intensity curves (n = 16) of 4% sucrose in the absence (control) and presence of 5-acetoxymethyl-2-furaldehyde (7; 1.0 mM), respectively. The area under the curve in the increasing period (a), the plateau period (b), and the decreasing period (c) was calculated from the labeled trapeze and was used for data analysis. (B) Change of mean total area (panel size n = 16) under the curve depending on the concentration of 7, error bars display 95% confidence interval. (C) Change of mean areas (n = 16) for the increasing (▲), plateau (●), and decreasing (■) segment under the time-intensity curve.

were performed with 4% sucrose solutions containing 1% ethanol in the absence (blank solution) and presence of 0.2, 1.0, 2.0, and 2.5 mmol/L of 5-acetoxymethyl-2-furaldehyde (test solution). The resulting average curves recorded for the blank solution and the test solution containing 7 at a level of 1.0 mM indicate a more long lasting sweet taste for the solution containing 5-acetoxymethyl-2-furaldehyde, whereas the maximum intensities were nearly the same for both solutions (Figure 5A). Besides the maximum intensity, total area under the curve and duration of perception, additional information was extracted from the resulting curves, namely area before and after maximum intensity as well as the area under the plateau.[45] However, with regard to individual differences in curve shape for each panelist,[46,47] mere calculation of mean values and standard deviations for the different parameters is not appropriate but requires statistical analysis for significance of differences.[45]

Therefore, data analysis was performed by means of the trapezoidal method reported in literature,[38] Figure 5A, followed by an analysis of variance. One-way repeated-measures ANOVA of total areas for blank and the test solutions demonstrated an increase of the area when compound 7 was present at levels of 1.0, 2.0, and 2.5 mM, whereas the presence of 0.2 μM of 7 did not affect the sweet taste perception of the 4% sucrose solution (Figure 5B). As Mauchly's test indicated that the assumption of sphericity was not violated (p = 0.163), further tests are reported uncorrected. The results show that the area under the curve (AUC) was significantly affected by the concentration of spiked 7 ($F(4, 60) = 5.71$, $p < 0.001$, $\eta^2 = 0.265$). Bonferroni tests revealed a nonsignificant difference for the lowest concentrations of 7 (0.2 mmol/L), but higher concentrations of 1.0 mmol/L ($p < 0.01$), 2.0 mmol/L ($p < 0.01$) or 2.5 mmol/L ($p < 0.01$) increased AUC significantly.

Further analysis of the different phases of the TI curves revealed the increase to be settled at the plateau and decreasing phase, whereas areas of the increasing phase were the same for reference and sample (Figure 5C). Sphericity was not violated for the main effects of concentration of 7 or the phase of the TI curve, but Mauchly's test indicated a violation for the interaction of TI phase and spiking concentration (W = 0.01, $p < 0.05$, ε = 0.03). Therefore, degrees of freedom were corrected using Greenhouse−Geisser estimates of sphericity. Besides the already detected significant main effect of the concentration of 7, the area under the TI curves was significantly different for the different phases [$F(2, 30)$ = 4.41]. On the other hand, the interaction effect between the concentration of 7 and the curve phase was not significant [$F(3.64, 54.69)$ = 1.04]. This indicates that the AUC in the different phases of the TI curves was affected independently from the spiking level of 7. Bonferroni post hoc tests revealed that the area under the increasing part of the curves was significantly smaller compared to the plateau ($p < 0.05$) and the decreasing part ($p < 0.01$), but not between the two latter curve segments. These findings suggest that 7 is able to increase particularly the perceived intensity and the duration after reaching the maximum intensity. Although reported earlier in

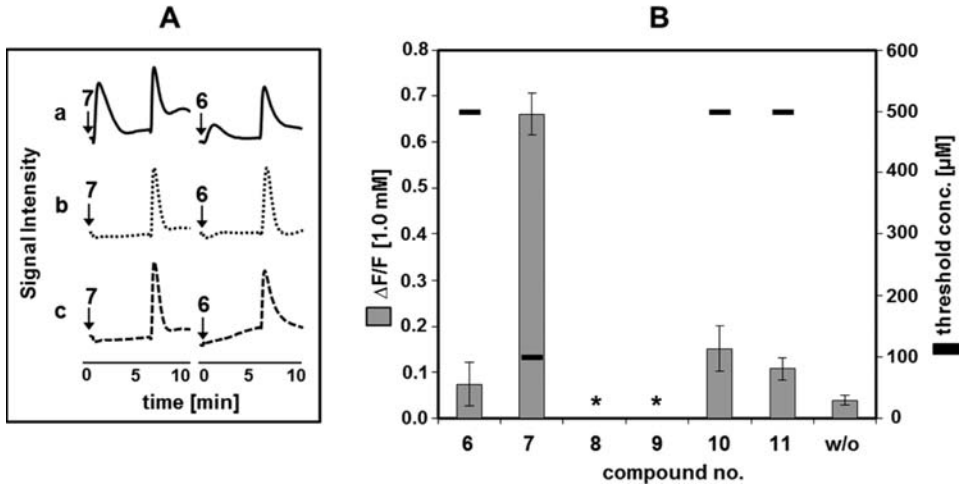


Figure 6. Representative calcium traces of cells expressing the human sweet taste receptor hTAS1R2/R3 (a) and of mock cells (b) upon stimulation (↑) with 5-acetoxymethyl-2-furaldehyde (**7**; 1.0 mM) and 5-hydroxymethyl-2-furaldehyde (**6**; 1.0 mM), respectively. Specificity of the fluorescence signals was controlled by coapplication with the selective hTAS1R2/R3 antagonist lactisole (c; 1.0 mM). Cell vitality was controlled by application of isoproterenol (a–c; 2nd peak). (B) $\Delta F/F$ ratios (left axis) and threshold concentrations for taste receptor activation (right axis) upon bath application of **6**–**11** (1.0 mM each). Error bars represent standard deviation (n = 3). Compounds labeled with an asterisk (**8**, **9**) did not elicit any signal from hTAS1R2/hTAS1R3 expressing cells up to a concentration of 1.0 mM.

literature,[10,14] the sweetness modulating impact of 5-acetoxymethyl-2-furaldehyde (**7**) has not been reported until now.

Humans possess a single sweet taste receptor that is an obligatory heterodimer composed of two subunits, taste 1 receptor family member 2 (TAS1R2) and taste 1 receptor family member 3 (TAS1R3).[48,49] Both subunits are G protein-coupled receptors with large aminoterminal domains which form orthosteric venus flytrap binding motifs and seven transmembrane segments that contain allosteric binding sites.[50] All sweet tasting substances appear to activate the TAS1R2/TAS1R3 heterodimer.[42] In order to investigate if the human sweet taste receptor heteromer is sensitive to the 2-furaldehydes, functional calcium imaging experiments were performed with **7** and **6** in HEK293 FlpIn T-Rex $G\alpha_{15}G_{i3}$/hTAS1R2 cells. This cell line stably expresses the chimeric G protein $G_{\alpha 15}G_{i3}$ to couple activation of the sweet taste receptor to cytosolic calcium levels that can be monitored via a calcium-sensitive fluorescence dye. The functional sweet taste receptor heteromer is implemented by stable expression of the subunit hTAS1R2, and inducible expression of the second subunit, hTAS1R3, through a tetracycline-responsive element.[51,52]

Cells expressing hTAS1R2/hTAS1R3 responded with a strong transient increase of calcium fluorescence to application of **7** (1.0 mM). The equimolar concentration of 5-hydroxymethyl-2-furaldehyde (**6**) also induced calcium signals though with smaller amplitudes. In mock experiments no signals were observed (Figure 6A). The cellular signals induced by the test substances were blocked completely in the presence of the selective and potent sweet taste receptor antagonist lactisole (1 mM) confirming the specificity of the responses. In conclusion, the observed 2-furaldehyde-induced signals are mediated by activation of the hTAS1R2/hTAS1R3 sweet taste receptor. The data from the receptor assay contrasts with the results of the human sensory studies, in which **6** and **7** exhibited no intrinsic taste. Moreover, in the receptor assay we did not observe modulatory effects of **6** and **7** on the sucrose-mediated cellular responses. Neither were the signal amplitudes changed nor was the time elapsed altered. However, the rank order of the compounds was the same in vitro and in vivo.

Due to their intrinsic ability to stimulate responses from hTAS1R2/hTAS1R3 in vitro, the molecular mechanism underlying the sweet-modulating effect of 2-furaldehydes observed in vivo could not be elucidated. It is known that the heterologous expression of G protein coupled receptors might lead to alterations in receptor trafficking, function and pharmacology. The function of chemosensory receptors in heterologous systems has already been reported to be influenced by accessory proteins and chaperones, as well as coupling to different G proteins.[54−58] Native taste receptor cells are likely equipped with other accessory proteins than the heterologous cells used in the receptor assays, which explains the difference between the in vitro and in vivo observations. However, the response of hTAS1R2/hTAS1R3 in vitro proves that 2-furaldehydes directly interact with the human sweet taste. Thus, we conclude that the prolonged sweetness of sucrose in the presence of 5-acetoxymethyl-2-furaldehyde (**7**) which we observed in our sensory experiments is based on prolonged sweet taste receptor signaling.

In order to gain first insight into structure/activity relationships of 2-furaldehydes, hTAS1R2/hTAS1R3-expressing cells were challenged with the synthetically prepared, higher homologues 5-(butanoyloxy)methyl-2-furaldehyde (**8**; Figure 4) and 5-(hexanoyloxy)methyl-2-furaldehyde (**9**), the Maillard reaction product 2-furaldehyde (**10**) reported in balsamic vinegar,[10,13,16] as well as 5-methylfurfural (**11**).

As the limited solubility of the 2-furaldehydes did not allow the recording of dose−response functions, threshold concentrations for hTAS1R2/hTAS1R3 receptor activation were determined by application of linear dilutions of each test compound (50−1000 μmol/L). As depicted in Figure 6B, the lowest threshold concentration was found for 5-acetoxymethyl-2-furaldehyde (**7**; 100 μmol/L), whereas compounds **6**, **10**, and **11** showed receptor activation at threshold levels of 500 μmol/L. In addition, at a concentration of 1 mM, 5-acetoxymethyl-2-furaldehyde (**7**) turned out to be by far the most efficient among the test compounds. It induced the highest fluorescence signals from hTAS1R2/hTAS1R3-expressing cells with a ratio of 0.66 (Figure 6B). The capability to stimulate the human

sweet taste receptor was eliminated by elongation of the alkanoyl side chain in **7**. The compounds 5-(butanoyloxy)-methyl-2-furaldehyde (**8**) and 5-(hexanoyloxy)methyl-2-furaldehyde (**9**) did not evoke any response up to a concentration of 1.0 mmol/L (Figure 6B). In conclusion, these data pinpoint the importance of the acetoxymethyl moiety in **7** for activation of the human sweet taste receptor in vitro.

Quantitative Analysis of 6-*O*-Acetyl-α/β-D-glucopyranose (4), 1-*O*-Acetyl-β-D-fructopyranose (5), and 5-Acetoxymethyl-2-furaldehyde (7) in TBV. To determine the concentrations of compounds **4**, **5**, and **7** in TBV, 6-*O*-acetyl-α/β-[$^{13}C_6$]-D-glucopyranose and 5-[$^{13}C_2$]-acetoxymethyl-2-furaldehyde were synthesized and used as internal standards for the development of stable isotope dilution analyses. After spiking the vinegar samples with defined amounts of the 6-*O*-acetyl-α/β-[$^{13}C_6$]-D-glucopyranose and 5-[$^{13}C_2$]-acetoxymethyl-2-furaldehyde, followed by sample clean-up, the natural [^{13}C]-abundant and [$^{13}C_6$]-6-*O*-acetyl-α/β-D-glucopyranose (**4**) and 1-*O*-acetyl-β-D-fructopyranose (**5**) were analyzed by means of HILIC−MS/MS, whereas natural [^{13}C]-abundant and [$^{13}C_2$]-labeled 5-acetoxymethyl-2-furaldehyde (**7**) were analyzed by means of RP-HPLC−MS/MS as depicted in Figure 7.

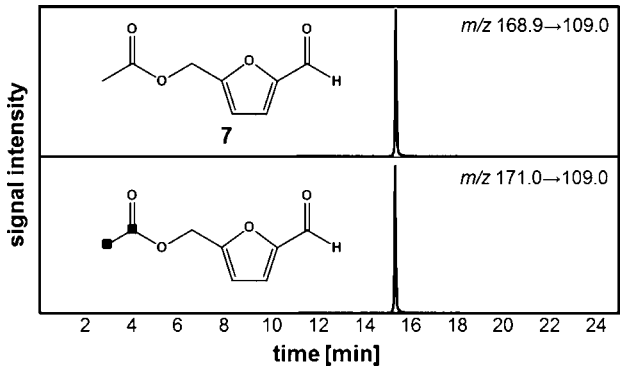


Figure 7. HPLC-MS/MS chromatogram showing the analysis of 5-acetoxymethyl-2-furaldehyde (**7**) and [$^{13}C_2$]-5-acetoxymethyl-2-furaldehyde.

The TBV sample contained 13 823 and 8329 μmoL/L 1-*O*-acetyl-β-D-fructopyranose (**5**) and 6-*O*-acetyl-α/β-D-glucopyranose (**4**), respectively, as well as 315 μmoL/L of 5-acetoxymethyl-2-furaldehyde (**7**) (Table 2). Calculation of DoT-factors revealed values of 0.7 and 0.8 for the intrinsic sweetness of the hexose acetates **5** and **4**, respectively, and a value of 0.2 for 5-acetoxymethyl-2-furaldehyde based on its threshold concentration in a 4% sucrose solution (Table 2).

In order to answer the question as to whether the differences in sweet taste quality of the taste recombinants ($rTBV_{I-VI}$, $rTBV_{I-VII}$) and the authentic TBV is due to compounds **4**, **5**, and **7**, a total taste recombinant ($rTBV_{total}$) was prepared by spiking the taste recombinant $rTBV_{I-VII}$ with 1-*O*-acetyl-β-D-fructopyranose, 6-*O*-acetyl-α/β-D-glucopyranose, and 5-acetoxymethyl-2-furaldehyde, each in its "natural" concentration. By means of a three-alternative forced choice test, $rTBV_{I-VII}$ and $rTBV_{total}$ could be significantly differentiated by the sensory panel ($p < 0.1$). Comparative taste profile analysis did not reveal any significant increase in sweet taste intensity from $rTBV_{I-VII}$ to $rTBV_{total}$, but the panelists reported on a change in sweet taste quality as well as an increase in the duration of the sweetness perception of $rTBV_{total}$, the taste profile of which closely matched that of the authentic TBV (Table 3). Omission of the hexose acetates **4** and **5** from $rTBV_{total}$ could not be significantly differentiated from $rTBV_{total}$ containing compounds **4**, **5**, and **7** by means of a three-alternative forced choice test (data not shown). Taking all these data into consideration, 5-acetoxymethyl-2-furaldehyde (**7**) might be considered a previously unkown, natural sweet taste modulator in the TBV.

Comparison of Taste Compounds in TBV and BV. In order to answer the question as to whether the differences in the taste profiles of TBV and BV is reflected by the differences in the concentrations of individual tastants, the total of 59 compounds were quantitated in the BV sample and DoT-factors were calculated (Table 2). First, an aqueous taste recombinant (rBV_{total}) containing all taste-active molecules, each in its "natural" concentration (Table 2), was prepared and its taste profile compared to that of the authentic BV sample in order to functionally confirm the analytical results (Table 3). As the taste profile of rBV_{total} matched rather well that of BV, the taste molecules summarized in Table 2 were considered the key molecules imparting the taste profile of BV.

Comparing the concentrations of the taste molecules in TBV and BV revealed 2-fold higher values for the sour tasting acetic acid, but 2−3 times lower values for the sweet tasting monosaccharides and glycerol (group I, Table 2) and the bitter-sweet tasting hexose acetates 6-*O*-acetyl-α/β-D-glucopyranose (**4**) and 1-*O*-acetyl-β-D-fructopyranose (**5**), as well as 4 times lower levels of the sweet-modulating 5-acetoxymethyl-2-furaldehyde (**7**), thus being well in line with the observed differences in sweet/sour balance between samples TBV and BV. On the other hand, the concentration of the major acids gluconic acid, glycolic acid, malic acid, and tartaric acid were 14, 7, 6, and 3 times lower when compared to TBV. Within the group of bitter and astringent compounds, BV showed significantly lower DoT-factors for the minerals as well as the polyphenolic acids, and the wood-derived ellagitannins **1** and **2** as well as (+)-dihydrorobinetin (**3**) were not detectable at all in BV, thus indicating the lack of any wood maturation in industrial BV manufacturing.

Sensomics Profiling of Storage Levels During TBV Aging. In order to gain more detailed insight into the taste development in TBV production, each intermediary sample collected from the "batteria" of barrels A−H throughout a full-scale TBV manufacturing process was analyzed by means of comparative taste profile analysis using the final TBV sample (Table 1) as the reference. With increasing degree of maturation, a slight decrease in sourness and an increase of sweetness and mouthfulness were observed (Figure 8). In comparison, perceived astringency did not seem to be significantly influenced by maturing.

Aimed at correlating the sensory data with the presence of the individual tastants, a total of 37 selected sweet, sour, or astringent sensometabolites were quantitatively determined in the barrel samples A−H. The concentrations determined for each compound on a fresh weight (A) and dry matter basis (B), respectively, were centered and scaled, and after hierarchical agglomerative clustering of the normalized data, the results were visualized in a sensomics heatmap (Figure 9).

On the basis of fresh weight calculation, the hierarchical analysis arranged the sensometabolites into the two large clusters *1* and *2* subdivided into the smaller clusters *1a* and *1b*, as well as *2a* and *2b* (Figure 9A). Cluster *1a* comprised the sensometabolites increasing in concentration upon maturation,

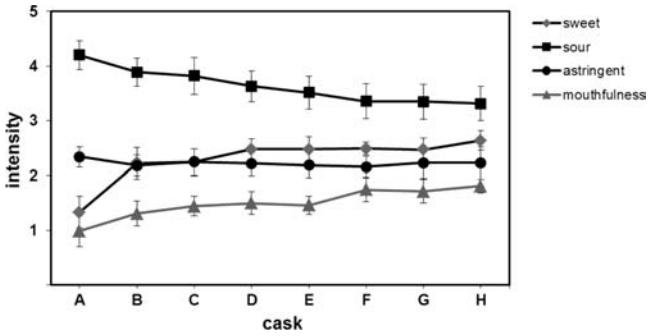


Figure 8. Influence of maturation on the taste profile of intermediary vinegar samples collected from casks A–H of the "batteria" of TBV manufacturing; data are given as the mean of triplicates.

among them the sweet tasting fructose, glucose, 1-*O*-acetyl-*β*-D-fructopyranose (**5**), 6-*O*-acetyl-*α*/*β*-D-glucopyranose (**4**), glycerol, sorbitol, xylitol, and the sweetness modulating 5-acetoxymethyl-2-furaldehyde (**7**), thus being well in line with the increased sweetness impact developing from sample A to H (Figure 8). In addition, the sour tasting malic acid, citric acid, and tartaric acid as well as the astringent gallic acid and 5-hydroxymethyl-2-furaldehyde (**6**) increased with maturation age. Cluster *1b* consisted of vescalagin (**1**), glycolic acid, ribitol, and gluconic acid, following a mixed trend throughout the maturation procedure. For example, gluconic acid went through a maximum in barrel E, whereas highest levels of vescalagin (**1**) were observed in barrel H made from fresh chestnut collaborating well with its release from chestnut.[53] The large cluster *2b* (Figure 9A) consisted of sensometabolites decreasing in concentration during maturation of the vinegar, among these, the sour tasting acetic acid, the sweet tasting arabitol, as well as the astringent phenolic compounds gentisic acid, protocatechuic acid, gallic acid methyl ester, gallic acid ethyl ester, *p*-coumaric acid, syringic acid, ferulic acid, caffeic acid, syringaldehyde, and vanilline, respectively. Cluster *2a* grouped the astringent tasting (+)-dihydrorobinetin (**3**), castalagin (**2**), vanillic acid, and *p*-hydroxybenzoic acid, all of which were present in highest concentrations in sample A taken from the acacia barrel, thereafter decreasing strongly throughout the maturation cascade.

In order to remove concentration effects by the evaporation of water throughout vinegar maturation in the "batteria" cascade, another hierarchical analysis was performed using the quantitative data based on dry matter of barrel samples A–H (Figure 9B). The sensometabolites were arranged into two main clusters with two and three subclusters, respectively. Cluster *3a* comprises the sensometabolites which are partially degraded with increasing degree of maturation, namely the astringent phenolic compounds, the sour tasting acetic acid, gluconic acid, malic acid, and lactic acid, as well as mannitol, ribitol, and arabitol. Cluster *3b* contains sensometabolites which were detected in highest concentrations in barrel sample A and dropped drastically already in barrel B such as, e.g., (+)-dihydrorobinetin (**3**). In contrast, cluster *4* summarizes the sensometabolites which are increasing in concentration. The dry matter data in cluster *4* (Figure 9B) clearly demonstrate that these taste compounds are generated with increasing maturation time. Besides citric acid, cluster *4* contained only compounds contributing to the sweet taste such as the monosaccharides fructose and glucose (cluster *4c*),

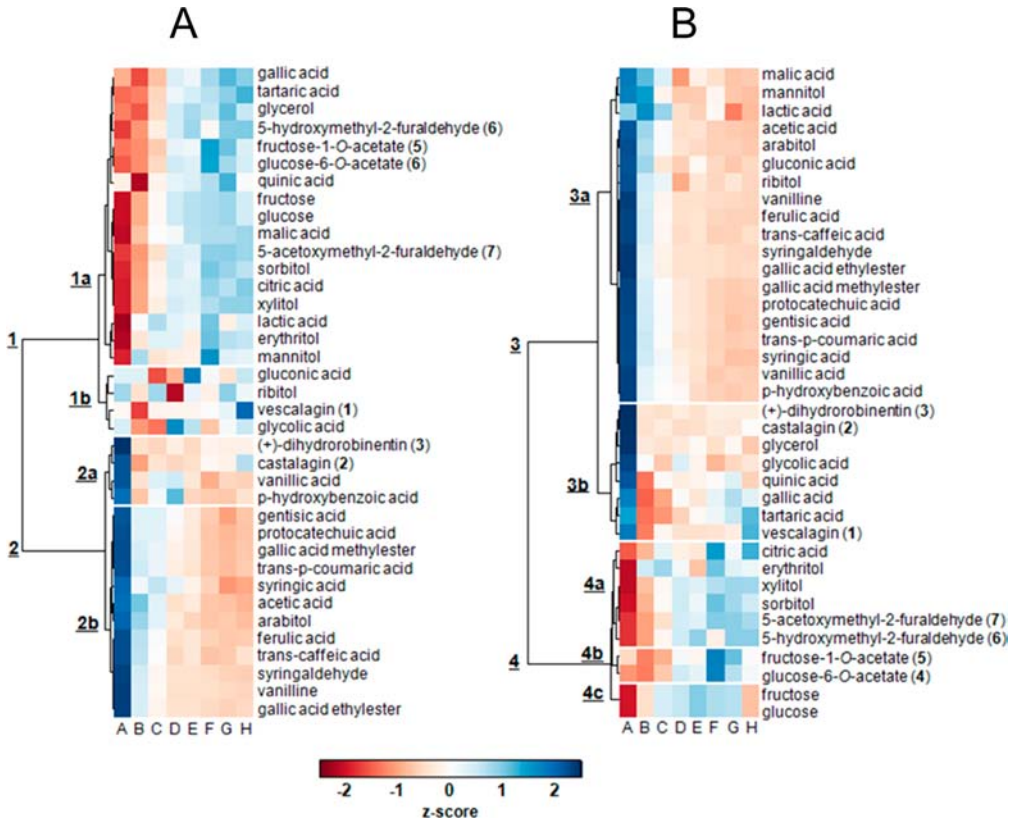


Figure 9. Sensomics heatmap calculated from quantitative data of selected sensometabolites in intermediary vinegar samples A–H collected from the "batteria" after normalizing fresh weight (A) and dry weight concentrations (B). For original data, see Supporting Information.

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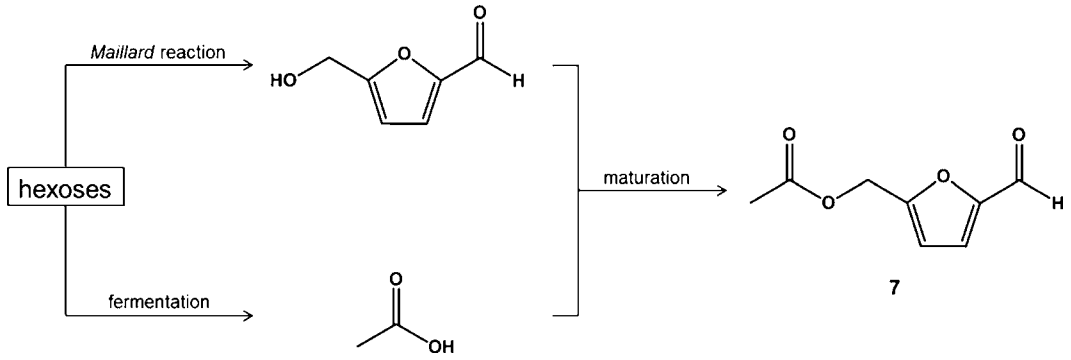

Figure 10. Reaction scheme showing the formation of the sweet taste modulator **7** via the Maillard reaction product 5-hydroxymethyl-2-furaldehyde (**6**) and acetic acid generated upon fermentation.

their monoacetylated derivates **4** and **5** (cluster *4b*) as well as the polyols erythritol, sorbitol, and xylitol, the Maillard reaction product 5-hydroxymethyl-2-furaldehyde (**6**), and its acetylation product 5-acetoxymethyl-2-furaldehyde (**7**, cluster *4a*), respectively.

In conclusion, sensory-guided fractionation, quantitative analysis, and taste recombination experiments led to the identification of the key sensometabolites in traditional balsamic vinegar from Modena (TBV). Among the sensory active nonvolatiles, 5-acetoxymethyl-2-furaldehyde (**7**) was discovered for the first time as a natural sweet taste modulator and validated to activate the human sweet taste receptor by means of functional expression studies. Compared to TBV, balsamic vinegar of Modena (BV) differed significantly by the increased concentration of acetic acid, the significantly lower concentrations of the sweet-modulating 5-acetoxymethyl-2-furaldehyde (**7**), the nonvolatile organic acids and polyphenols, and the lack of wood-derived ellagitannins. Moreover, quantitative profiling of 37 sensometabolites contributing to sweetness, sourness, and astringency of balsamic vinegar revealed a comprehensive insight into the process-induced evolution of sensometabolites throughout a full-scale TBV manufacturing process including a "batteria" of eight casks, e.g., the sweet modulating 5-acetoxymethyl-2-furaldehyde (**7**) is proposed to be generated by the esterification of the Maillard reaction product 5-hydroxymethyl-2-furaldehyde with the fermentation product acetic acid upon maturation in the "batteria" (Figure 10). These data offer the scientific basis for a knowledge-based optimization of the taste profile of TBV by technological means.

■ ASSOCIATED CONTENT

Supporting Information

Additional details, tables, and figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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