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Quantitation of Key Tastants and Reengineering the Taste of Parmesan Cheese

Hedda Hillmann[‡] and Thomas Hofmann[‡,§*]

[‡]Chair for Food Chemistry and Molecular Sensory Science, Technische Universität München, Lise-Meitner-Straße 34, D-85354 Freising, Germany, and

[§]Bavarian Center for Biomolecular Mass Spectrometry, Gregor-Mendel-Straße 4, 85354 Freising, Germany.

[*] **To whom correspondence should be addressed**

PHONE +49-8161/71-2902

FAX +49-8161/71-2949

E-MAIL thomas.hofmann@tum.de

ABSTRACT

Targeted quantitation of 65 candidate taste compounds and ranking on the basis of dose-over-threshold (DoT) factors, followed by taste re-engineering and omission experiments in aqueous solution as well as in a cheese-like model matrix led to the identification of a total of 31 key tastants (amino acids, organic acids, fatty acids, biogenic amines, and minerals) with DoT-factors ≥1.0 and a total of 15 sub-threshold, but kokumi-enhancing γ-glutamyl peptides in extraordinarily high concentrations of 20468 µmol/kg. Amongst the γ-glutamyl peptides, γ-Glu-Gly, γ-Glu-Ala, γ-Glu-Thr, γ-Glu-Asp, γ-Glu-Lys, γ-Glu-Glu, γ-Glu-Trp, γ-Glu-Gln, and γ-Glu-His have been identified for the first time in Parmesan cheese. Excellent match of the sensory profile of the taste recombinants and the authentic cheese demonstrated the identified taste compounds to be fully sufficient to create the characteristic taste profile of the Parmesan cheese. This molecular blueprint of a Parmesan's chemosensory signature might be a useful molecular target for visualizing analytically the changes in taste profiles throughout cheese manufacturing and opens new avenues for a more scientifically directed taste improvement of cheese by tailoring manufacturing parameters ("molecular food engineering").

KEYWORDS:

Parmesan, cheese, taste, glutamyl peptides, biogenic amines, SIDA, kokumi

INTRODUCTION

Due to its characteristic flavor and nutritional value, Parmesan cheese (*Parmigiano Reggiano*) is highly appreciated by consumers all over the world. This semi-fat, extra hard cheese made from cows' raw milk by a traditional and well-controlled procedure that solely takes place in Northern Italy, namely in the regions of Parma, Modena, Emilia, and Bologna, respectively. During the extraordinary long ripening period, lasting for a minimum of 13 up to 36 months, Parmesan cheese develops its characteristic taste profile centering around umami, salty, bitter-sweet and burning notes as well as its typical long-lasting and complex mouthfulness (kokumi).

Representing the molecular blueprint of a food's chemosensory signature, the knowledge of the key tastants ("sensometabolome") is supposed to open new avenues for a more scientifically directed taste improvement of foods by tailoring processing parameters. Therefore, numerous studies have been performed in recent years to define the taste compounds of various cheese types on a molecular level, suggesting free amino acids, lactic acid, mineral salts, bitter peptides, and biogenic amines as the main contributors to cheese taste.[1-6]. By means of taste recombination and omission experiments, the contribution of the single components to the taste of a matured Gouda cheese have been elucidated: $CaCl_2$, $MgCl_2$, the amino acids L-leucine, L-tyrosine, L-isoleucine, and L-tryptophane, and the peptides YPFPGPIHNS, YPFPGPIPN, YPFPGPIHN, assigned to the casein sequence ß-CN(60-69) and ß-CN(60-68), respectively, as well as the tetrapeptide LPQE released from αs_1-CN(11-14) were found with the highest bitterness impact.[4,5] L-Glutamate and sodium lactate were found as main contributors to the umami taste, lactic acid and hydrogen

phosphate were demonstrated to be responsible to the sour taste, while the salty taste was due to sodium chloride, sodium phosphate, and the amino acid L-arginine.[4] Moreover, a series of γ-glutamyl dipeptides, namely γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met, γ-Glu-Leu, and γ-Glu-His, were reported to be generated upon cheese maturation in a γ-glutamyl transferase catalyzed reaction from the γ-glutamyl-donor amino acid L-glutamine and other acceptor amino acids and to contribute, due to their kokumi enhancing activity, to the long-lasting mouthfulness of matured Gouda cheese.[6,7]

Compared to our knowledge on the odor-active volatiles contributing to the typical aroma of ripened Parmesan cheese,[8-11] accurate data on the concentrations of non-volatile components and their contribution to the characteristic, long-lasting taste of Parmesan cheese are still scarce.[12-16] Some studies have been performed to correlate overall sensory properties of Parmesan cheese with its chemical constituents and rheological characteristics, however, any systematic investigations on the impact of individual tastant groups are lacking.[17,18] Although, the occurrence of some γ-glutamyl dipeptides in Parmesan cheese has been reported, however, to the best of our knowledge, accurate quantitation of individual α- and γ-glutamyl peptides and studies on their sensory impact in Parmesan cheese have not yet performed.[19]

In order to get a comprehensive picture on the concentrations and taste contribution of putative taste compounds earlier reported in cheeses, the objective of the present investigation was to quantitate all candidate taste compounds and to rank them in their taste impact in Parmesan cheese on the basis of dose/activity considerations and, finally, to validate the data by means of taste recombination experiments in an aqueous solution as well as in a cheese-like matrix. In order to carefully clarify the sensory contribution of α- and γ-glutamyl peptides and biogenic amines, a stable isotope dilution analysis (SIDA) should be developed.

MATERIALS AND METHODS

Chemicals. All chemicals used were purchased from Sigma-Aldrich (Steinheim, Germany) and Fluka (Neu-Ulm, Germany), respectively. Histamine-d_4 and tyramine-d_4 were obtained from CDN Isotopes (Quebec, Canada). Reference material of the α- and γ-L-glutamyl peptides α-/γ-Glu-Gly (**1**, **2**), α-/γ-Glu-Val (**5**, **6**), α-/γ-Glu-Thr (**7**, **8**), α-/γ-Glu-Asp (**9**, **10**), α-/γ-Glu-Lys (**11**, **12**), α-/γ-Glu-Glu (**13**, **14**), α-/γ-Glu-Trp (**15**, **16**), γ-Glu-Leu/Ile (**17**, **18**), γ-Glu-Gln (**19**), γ-Glu-Met (**20**), γ-Glu-His (**21**), γ-Glu-Phe (**22**), γ-Glu-Tyr (**23**) were obtained from Bachem (Weil am Rhein, Germany) or synthesized as detailed below (**8**, **10**, **18**). Solvents were of HPLC grade (Mallinckrodt Baker, Griesheim, Germany). Ultrapure water used for chromatography was purified by means of a MilliQ-water Gradient A 10 system (Millipore, Schwalbach, Germany) and deuterated solvents were supplied by Euriso-Top (Gif-Sur-Yvette, France). Parmesan cheese samples, ripened for 24 months and labelled with a stamp authorized by the “Consorzio del Formaggio Parmigiano-Reggiano", were obtained from a local Italian producer, delivered in 1 kg packages and stored at -20°C until use.

Preparation of the Water Soluble Extract (WSE). According to a previously published protocol,[5] a defined amount (50 g) of Parmesan cheese (PC) was cut into small pieces, placed into a centrifuge beaker with deionized water (300 mL), homogenized for 5 min by means of an Ultra-Turrax T 25 digital (Ika Labortechnik, Staufen, Germany), and then centrifuged at 9000 rpm for 20 min at 4 °C by use of a Avanti J-E (Beckman- Coulter, Krefeld, Germany). The obtained upper solid fat layer as well as the protein pellet was removed to afford the liquid layer including the

cheese water-solubles (pH 5.3). Protein pellet as well as fat layer were re-extracted with deionized water (300 mL) as described above, the aqueous layers were pooled, and soluble casein was precipitated upon adjusting the pH value to 4.6 by addition of formic acid (1%, v/v; in water). After centrifugation at 9000 rpm at 4 °C for 20 min, followed by paper filtration (Macherey-Nagel, 615-1/4) and freeze-drying (GAMMA 1/2-16LSC, Christ, Osterode, Germany), a casein-free water soluble extract (WSE) was obtained which was stored at -20 °C until further chemical and analysis (**Table 1**).

Synthesis of γ-L-Glutamyl Dipeptides 8, 10, and 18. By means of solid phase peptide synthesis,[21] γ-L-glutamyl-L-threonine (**8**) γ-L-glutamyl-L-aspartic acid (**10**), and γ-L-glutamyl-L-isoleucine (**18**) were synthesized starting from Fmoc-Thr(tBu)-Wang resin (extent of labeling: 0.4-0.6 mmol/g), Fmoc-Asp(OtBu)-Wang resin (extent of labeling: 0.6-0.9 mmol/g), and Fmoc-Ile-Wang resin (extend of labelling: 0.6 mmol/g), respectively. Wang resin (1.0 g) was placed into a fritted reaction vessel and washed with dimethylformamide (DMF) twice (3 mL, each). Then a solution of piperidine (20% in DMF, 20 mL) was added and the reaction mixture was agitated for 5 min by bubbling nitrogen through the resin bed. After removing the solvent, resin was washed with DMF (3 × 3 mL). *N,N*-Diisopropylethylamine (DIPEA, 9 mmol) was added to a solution of Fmoc-Glu-OtBu (4.5 mmol), hydroxybenzotriazol (HOBt, 4.5 mmol), and benzotriazol-1-yloxytris(pyrrolidino) phosphoniumhexafluorophosphate (PyBOP, 4.5 mmol) in DMF (1 mL) and was, then, given to the resin. After agitating for 1 h, the solvent was discarded, the resin was washed with DMF (2 × 3 mL, each), a solution of piperidine (20% in DMF, 20 mL) was added and the reaction mixture was agitated for 5 min by bubbling nitrogen through the resin bed. After removing the solvent, resin was washed with DMF (3 × 3 mL). Aqueous trifluoroacetic acid (TFA/water, 95/5, v/v; 5 mL) was added to the resin and, after agitation overnight, the

resin was removed by filtration under reduced pressure and washed twice with TFA (5 mL). Filtrates were pooled, freeze dried, the residue was dissolved in water and membrane-filtered (0.45 µm), and the target glutamyl peptides were purified by HPLC. Aliquots (0.3 - 1 mL) were repeatedly injected into a preparative HPLC system (Jasco, Groß-Umstadt, Germany) connected to a 250 x 21.0 mm i.d., 5 µm, Monochrom column (Varian, Germany) equipped with a guard column of the same type and using 1% aqueous formic acid as the eluent (18 mL/min). After freeze drying, γ-L-glutamyl-L-threonine (**8**, 0.32 mmol), γ-L-glutamyl-L-aspartic acid (**10**, 0.43 mmol) and γ-L-glutamyl-L-isoleucine (**18**, 0.5 mmol) were obtained as white amorphous powders (purity: 99%, HPLC-ELSD).

γ-L-Glutamyl-L-threonine, ***8***, **Figure 1**: LC-MS (ESI^+), *m/z* 249 (100, $[M+H]^+$), 271 (60, $[M+Na]^+$); ^{1}H-NMR (500 MHz, D_2O, COSY) δ 1.10 [d, 3H, *J* = 8.0 Hz, H-C(8)], 2.10 [ddd, 2H, *J* = 4.0, 8.0, 16.0 Hz, H-C(3)], 2.50 [ddd, 2H, *J* = 4.0, 8.0, 16.0 Hz, H-C(4)], 3.85 [t, 1H, *J* = 8.0, 12.0 Hz, H-C(2)], 4.28 [m, 1H, *J* = 4.0, 8.0 Hz, H-C(7)], 4.35 [d, 1H, *J* = 4.0 Hz, H-C(6)]; ^{13}C-NMR (125 MHz, D_2O, HMQC, HMBC) δ 18.8 [C-8], 25.9 [C-3], 31.1 [C-4], 53.1 [C-2], 58.2 [C-6], 67.0 [C-7], 172.6 [C-1], 173.9 [C-9], 174.9 [C-5].

γ-L-Glutamyl-L-aspartic acid, ***10***, **Figure 1**: LC-MS (ESI^+), *m/z* 263 (100, $[M+H]^+$), 286 (25, $[M+Na]^+$); ^{1}H-NMR (500 MHz, D_2O, COSY) δ 1.71 [m, 2H, *J* = 4.0, 8.0 Hz, H-C(3)], 2.18 [m, 2H, *J* = 4.0, 8.0 Hz, H-C(4)], 2.36 [dd, 1H, *J* = 4.0, 16.0 Hz, H-C(7a)], 2.55 [dd, 1H, *J* = 4.0, 16.0 Hz, H-C(7b)], 3.11 [dd, 1H, *J* = 4.0, 8.0 Hz, H-C(2)], 4.26 [dd, 1H, *J* = 4.0, 12.0 Hz, H-C(6)]; ^{13}C-NMR (125 MHz, D_2O, HMQC,HMBC) δ 30.9 [C-3], 32.3 [C-4], 39.7 [C-7], 53.1 [C-6], 55.5 [C-2], 175.4 [H-C5], 178.8 [C-8/C-9], 178.9 [C-9/C-8], 182.8 [C-1].

γ-L-Glutamyl-L-isoleucine, ***18***, **Figure 1**: LC-MS (ESI^+), *m/z* 261 (100, $[M+H]^+$), 284 (30, $[M+Na]^+$); ^{1}H-NMR (500 MHz, D_2O, COSY) δ 0.89 [t, 3H, *J* = 4.0, 8.0 Hz, H-

C(9)], 0.93 [d, 3H, *J* = 8.0 Hz, H-C(10)], 1.22 [m, 1H, *J* = 4.0, 8.0, 12.0, 16.0 Hz, H-C(8a/b)], 1.45 [m, 1H, *J* = 4.0, 8.0, 12.0, 16.0 Hz, H-C(8b/a)], 1.89 [m, 1H, *J* = 4.0, 8.0, 12.0 Hz, H-C(7)], 2.13 [ddd, 2H, *J* = 4.0, 8.0, 12.0 Hz, H-C(3)], 2.50 [dd, 2H, *J* = 4.0, 8.0 Hz, H-C(4)], 3.79 [t, 1H, *J* = 4.0 Hz, H-C(2)], 4.22 [d, 1H, *J* = 4.0 Hz, H-C(6)]; ^{13}C-NMR (125 MHz, D_2O, HMQC, HMBC) δ 13.7 [C-9], 18.0 [C-10], 27.6 [C-8], 29.3 [C-3], 34.3 [C-4], 39.3 [C-7], 57.1 [C-2], 61.6 [C-6], 176.8 [C-1], 177.7 [C-5], 179.7 [C-11].

Synthesis of γ-L-Glutamyl-L-alanine-[$^{13}C_3$] (24). Unlabelled Wang resin (1 g) was placed into a fritted reaction vessel, kept with dichloromethane (DCM) (5 mL) for 30 min, then washed with DCM (3 × 3 mL), and left for further use in DCM (5 mL). Fmoc-L-alanine-$^{13}C_3$ (0.8 mmol) was placed in a round bottom flask, DCM (3 mL) was added and the reaction mixture was stirred until complete dissolution. After addition of a solution of 1,3-diisopropylcarbodiimid (0.4 mmol) in DCM (3 mL) and stirring for 20 min at 0 °C, the solvent was evaporated under reduced pressure, the residue was taken up in DMF (1 mL), transferred into the resin-containing reaction vessel and, then, a solution of 4-dimethylaminopyridine (DMAP) (0.2 mmol) in DMF (3 mL) was added. After agitating for 1 h by bubbling nitrogen through the resin, the solvent was removed and the resin was washed with DMF (5 × 3 mL). Then, a solution of benzoic anhydride (0.8 mmol) and pyridine (0.2 mmol) was added, the mixture again agitated for 30 min and, then, washed with DMF (3 × 3 mL). *N,N*-Diisopropylethylamine (DIPEA, 9 mmol) was added to a solution of Fmoc-Glu-OtBu (4.5 mmol), hydroxybenzotriazol (HOBt, 4.5 mmol), and benzotriazol-1-yloxytris(pyrrolidino) phosphoniumhexafluorophosphate (PyBOP, 4.5 mmol) in DMF (1 mL) and was, then, given to the resin. After agitating for 1 h, the solvent was discarded, the resin was washed with DMF (2 × 3 mL, each), a solution of piperidine (20% in DMF, 20 mL) was added and the reaction mixture was agitated for 5 min by

bubbling nitrogen through the resin bed. After removing the solvent, resin was washed with DMF (3 × 3 mL). Aqueous trifluoroacetic acid (TFA/water, 95/5, v/v; 5 mL) was added to the resin and, after agitation overnight, the resin was removed by filtration under reduced pressure and washed twice with TFA (5 mL). Filtrates were pooled, freeze dried, and the target glutamyl peptide (**24**, 0.21 mmol; purity: 99%, HPLC-ELSD) was purified by means of HPLC as detailed above.

γ-*L*-*Glutamyl*-*L*-*alanine*-[$^{13}C_3$], ***24***, **Figure 1**: LC-MS (ESI$^+$), *m/z* 222 (100, [M+H]$^+$), 244 (10, [M+Na]$^+$); ^{1}H-NMR (500 MHz, D_2O, COSY) δ [ppm] 1.25 [dddd, 3H, *J* = 4.0, 8.0, 12.0 Hz, $J_{C,H}$ = 160,0 Hz, H-C(8)], 2.03 [m, 2H, *J* = 4.0, 8.0, 16.0 Hz, H-C(3)], 2.36 [dd, 2H, *J* = 8.0, 16.0 Hz, H-C(4)], 3.67 [dd, 1H, *J* = 4.0, 8.0 Hz, H-C(2)], 4.09 [dddd, 1H, *J* = 4.0, 8.0, 12.0 Hz, $J_{C,H}$ = 180,0 Hz, H-C(6)]; ^{13}C-NMR (125 MHz, D_2O, HMQC, HMBC) δ [ppm] 16.7 [d, *J* = 43.8 Hz, C(8)], 26.2 [C(3)], 31.2 [C(4)], 50.2 [dd, *J* = 43.8, 68.8 Hz, C(6)], 54.2 [C(2)], 173.9 [C(1)], 174.1 [C(5)], 178.9 [d, *J* = 68.8 Hz, C(7)].

Quantitation of α- and γ-Glutamyl Dipeptides (1-24) by Means of SIDA. *Sample Preparation.* A portion (1 g) of Parmesan cheese was placed in a centrifuge tube (50 mL, Roth, Karlsruhe, Germany), spiked with an aqueous solution (325 µL) of γ-L-glutamyl-alanine-[$^{13}C_3$] (0.2 mg/L) and equilibrated for 30 min whilst light shaking. After addition of hydrochloric acid (10 mL; 0.1 mmol/L), the cheese sample was homogenized for 2 min by means of an Ultra-Turrax and centrifuged (9.000 rpm; 20 min, 4 °C). The obtained cheese extract was filtered into a volumetric flask (100 mL) with following washing of protein and fat residue with water. Prior to injection into HPLC-MS/MS-system 1, the cheese extract was 1:4 diluted with water.

LC-MS/MS Analysis. An aliquot (5 µL) of the prepared sample was injected into LC-MS/MS system 1 connected to a 150 x 2.0 mm i.d., 3 µm, Luna PFP column (Phenomenex, Aschaffenburg, Germany) equipped with a guard column of the same

type. Eluent A consisted of 0.1% formic acid in acetonitrile and eluent B was 0.1% formic acid in water. Using a flow rate of 200 µL/min, chromatography was performed starting with 100% B and 0% A for 5 min, then increasing the content of A to 100% linearly within 9 min, which was followed by isocratic elution of 100% A for 3 min. Analytes and the internal standard were analyzed in the ESI^+ mode using the mass transition 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: α-/γ-Glu-Gly (**1**, **2**): (*m/z* 205.2→76.0; +26/+10/+19/+6); α-/γ-Glu-Ala (**3**, **4**): (*m/z* 219.2→90.0; +46/+10/+21/+8); α-/γ-Glu-Val (**5**, **6**): (*m/z* 247.1→118.2; +61/+10/+19/+10); α-/γ-Glu-Thr (**7**, **8**): (*m/z* 249.2→119.9; +51/+10/+23/+10); α-/γ-Glu-Asp (**9**, **10**): (*m/z* 263.2→134.0; +36/+10/+29/+6); α-/γ-Glu-Lys (**11**, **12**): (*m/z* 276.2→129.9; +76/+10/+25/+12); α-/γ-Glu-Glu (**13**, **14**): (*m/z* 277.2→130.0; +41/+10/+25/+10); α-/γ-Glu-Trp (**15**, **16**): (*m/z* 334.2→188.0; +56/+10/+25/+6); γ-Glu-Leu/Ile (**17**, **18**): (*m/z* 261.2→85.9; +61/+10/+27/+4); γ-Glu-Gln (**19**): (*m/z* 276.1→130.0; +71/+10/+27/+10); γ-Glu-Met (**20**): (*m/z* 279.1→150.1; +61/+10/+21/+4); γ-Glu-His (**21**): (*m/z* 285.2→156.0; +46/+10/+33/+8); γ-Glu-Phe (**22**): (*m/z* 295.2→166.1; +66/+10/+19/+14); γ-Glu-Tyr (**23**): (*m/z* 311.2→136.0; +66/+10/+25/+14); γ-Glu-Ala-[$^{13}C_3$] (**24**): (*m/z* 311.2→136.0; +46/+10/+17/+14).

Calibration. Internal standard γ-Glu-Ala-[$^{13}C_3$] (**24**) and the analytes **1**-**23** were mixed in ten molar ratios from 0.04 to 16 keeping a constant concentration of the internal standard. After triplicate LC-MS/MS analysis calibration curves were prepared by plotting peak area ratios of each analyte to the internal standard against concentration ratios of each analyte to the internal standard using linear regression, showing linear responses with correlation coefficients of > 0.99, each.

Recovery Experiments. For determination of recovery, cheese samples were spiked with aqueous solutions containing defined amounts of **1** - **23** (**Table 2**) and

analyzed as detailed above. Basic values for calculation of recovery were obtained by analysis of unspiked cheese samples (n = 5).

Investigation of Matrix Effects during LC-MS/MS Analysis. In order to investigate the effect of matrix components in LC-MS/MS analysis the same LC-MS/MS parameters were used as detailed above. An aliquot of cheese extract was injected, but in addition a constant flow (10 µL/min) of an aqueous solution containing γ-Glu-Ala-[$^{13}C_3$] (**24**) (1 µmol/L) was introduced by means of a PHD 4400 Hpsi syringe pump (Harvard Apparatus, Massachusetts, USA) connected to the solvent flow via a mixing tee.

Quantitation of Biogenic Amines by Means of SIDA. *Sample Preparation.* Biogenic amines were analyzed as their corresponding dansyl derivatives by means of LC-MS/MS, following the derivatization procedure reported earlier.[21] Cheese samples (5 g) were spiked with the internal standards histamine-d_4 and tyramine-d_4 (1 mL, 10 mg/L, each) and extracted with a mixture (50 mL, 1/1, v/v) of acetonitrilel and perchloric acid (0.2 mol/L). An aliquot (10 mL) of the suspension was centrifuged, an aliquot (200 µL) of the supernatant was mixed with acetonitrile (800 µL), water (700 µL) and an aqueous solution (200 µL) of Na_2CO_3 (200 mg/mL). Dansyl chloride (100 µL, 50 mg/mL in acetone) was added and incubated for 30 min at 40 °C in the dark. After addition of sodium L-glutamine (200 µL, 50 mg/mL), the mixture was kept another 60 min under the same conditions and, then, extracted with ethyl acetate (1 mL). The organic layer was separated from solvent in vacuum and the residue taken up in acetonitrile (200 µL) prior to LC-MS/MS analysis.

LC-MS/MS Analysis. Aliquots (5 µL) of prepared sample solutions were injected into LC-MS/MS-system 2 connected to a 150 x 2 mm i.d., 5 µm, Synergi Fusion RP 80 column (Phenomenex, Aschaffenburg, Germany) equipped with a guard column of the same type. Eluent A consisted of ethanol (450 mL), water (100 mL),

acetonitrile (450 mL) and a buffer solution (2 mL; pH 8.0) containing a mixture (2/1/1, v/v/v) of tris(hydroxymethyl)aminomethane (0.1 mmol/L), acetic acid (0.1 mmol/L), and water, and solvent B containing ethanol (100 mL), acetonitrile (300 mL), water (470 mL) and an aliquot of the buffer solution (30 mL; pH 8.0) detailed above. Chromatography was performed using a flow rate of 250 µL/min starting with 5% solvent B and 95% solvent A for 1 min, then increasing the content of solvent B to 63% within 24 min and, then, up to 100% within another 3 min. Analytes and internal standards were analyzed as their dansyl derivatives 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: *N*,*N*-bisdansyl putrescine (*m/z* 555.2→170.1; +61/+7.5/+45/+4); *N*,*N*-bisdansyl cadaverine (*m/z* 569.2→170.1; +71/+8/+47/+4); *N*,*N*-bisdansyl histamine (*m/z* 578.2→170.1; +81/+7/+43/+4); *N*,*O*-bisdansyl tyramine (*m/z* 604.2→170.1; +66/+12/+43/+4); *N*,*N*,*N*-trisdansyl spermidine (*m/z* 845.3→170.1; +88/+12/+83/+4); *N*,*O*-bisdansyl histamine-d_4 (*m/z* 582.2→170.2; +71/+8.5/+47/+4); *N*,*O*-bisdansyl tyramine-d_4 (*m/z* 608.0→170.1; +81/+10/+47/+4).

Calibration. The deuterated standards and the analytes were mixed in six molar ratios from 0.5 to 5 keeping constant concentrations of the internal standards. After triplicate LC-MS/MS analysis, calibration curves were prepared by plotting peak area ratios of each analyte to the internal standard against concentration ratios of each analyte to the internal standard using linear regression, showing linear responses with correlation coefficients of > 0.99, each. Calibration curves for putrescine, histamine, and spermidine, were determined by use of histamine-d_4 and those for tyramine and cadaverine by using tyramine-d_4 as the internal standard.

Quantitation of Basic Taste Compounds. *Soluble Carbohydrates, Organic Acids and Minerals.* A defined amount of lyophilized WSE (25 mg) was dissolved in

de-ionized water (50 mL) and membrane-filtered (0.45 µm). Aliquots of the redissolved WSE (5 - 25 µL) were then analyzed by means of high-performance ion chromatography using an ICS 2500 system (Dionex, Idstein, Germany) following the method reported previously.[22]

Amino Acids. Free amino acids were quantified by means of HILIC-MS/MS with stable isotope dilution analysis, following a modified literature protocol.[23] A defined amount of lyophilized WSE (20 mg) was dissolved in de-ionized water (50 mL) and membrane-filtered (0.45 µm). After spiking the sample solution (990 µL) with a solution of the internal standards (10 µL), containing the isotope-labeled amino acids (1 mg/L, each), an aliquot (10 µL) was injected into HPLC-MS/MS-system 1 equipped with a 150 x 4.6 mm i.d., 3 µm; 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, adjusted with acetic acid) as solvent A and an aqueous ammonium acetate solution (5 mmol/L, pH 3, adjusted with acetic acid) as solvent B, chromatography was carried out at a flow rate of 1 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. After chromatographic separation, the effluent was split in a ratio of 1:5 to reduce the effluent entering the mass spectrometer and the following amino acids and their corresponding labeled standards were analyzed in the positive electrospray ionization mode (ESI^+) 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) given in parentheses: glycine (*m/z* 76.1→76.0; +31/+10/+5/+6), glycine-$^{13}C_2{}^{15}N$ (*m/z* 79.0→79.0; +41/+10/+5/+5), L-alanine (*m/z* 90.1→90.0; +26/+10/+5/+6), L-alanine-$^{13}C_3$ (*m/z* 93.0→93.0; +41/+10/+5/+5), L-serine (*m/z* 106.1→60.0; +26/+10/+17/+4), L-serine-$^{13}C_3$ (*m/z* 109.0→62.0; +38/+10/+16/+5), L-proline (*m/z*

116.1→70.0; +21/+10/+21/+4), L-proline-$^{13}C_5{}^{15}N$ (*m/z* 122.0→75.0; +73/+10/+25/+5), L-valine (*m/z* 118.1→72.1; +21/+10/+15/+6, L-valine-$^{13}C_5{}^{15}N$ (*m/z* 124.0→77.0; +64/+10/+14/+10), L-threonine (*m/z* 120.1→73.9; +36/10/+17/+6), L-threonine-$^{13}C_4{}^{15}N$ (*m/z* 125.0→78.0; +32/+10/+14/+5), L-leucine/L-isoleucine (*m/z* 132.1→86.0; +41/+10/+15/+6), L-leucine $^{13}C_2$ (*m/z* 134.1→87.9; +46/+10/+15/+6), L-isoleucine-$^{13}C_6$ (*m/z* 139.1→92.0; +39/+10/+14/+10), L-asparagine (*m/z* 132.9→73.9; +46/+10/+19/+6), L-asparagine-$^{15}N_2$ (*m/z* 135.0→75.0; +39/+10/+20/+5), L-aspartic acid (*m/z* 134.1→87.9; +46/+10/+15/+6), L-aspartic acid-$^{13}C_4{}^{15}N$ (*m/z* 139.1→92.0; +39/+10/+14/+10), L-glutamine (*m/z* 147.0→84.0; +46/+10/+23/+6), L-glutamine-$^{13}C_5$ (*m/z* 152.0→88.0; +43/+10/+23/+10), L-glutamic acid (*m/z* 148.1→84.0; +31/+10/+23/+6), L-glutamic acid-$^{13}C_5{}^{15}N$ (*m/z* 154.0→89.0; +38/+10/+23/+10), L-lysine (*m/z* 147.0→84.0; +46/+10/+23/+6), L-lysine-$^{13}C_6{}^{15}N_2$ (*m/z* 155.0→90.0; +44/+10/+23/+10), L-methionine (*m/z* 150.1→104.0; +31/+10/+15/+8), L-methionine-d_3 (*m/z* 153.1→107.0; +50/+10/+14/+10), L-histidine (*m/z* 156.1→110.0; +41/+10/+21/+8), L-histidine-$^{13}C_6$ (*m/z* 162.0→115.0; +46/+10/+21/+10), L-phenylalanine (*m/z* 166.0→120.0; +51/+10/+19/+10), L-phenylalanine-d_5 (*m/z* 171.0→125.0; +48/+10/+19/+10), L-arginine (*m/z* 175.1→70.1; +36/+10/+33/+4), L-arginine-$^{13}C_6$ (*m/z* 181.0→74.0; +78/+10/+36/+5), L-tyrosine (*m/z* 182.1→136.0; +26/+10/+19/+10), L-tyrosine-d_4 (*m/z* 186.0→140.0; +38/+10/+19/+10), L-tryptophan (*m/z* 205.1→146.0; +41/+10/+25/+12), L-tryptophan-d_5 (*m/z* 210.0→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).

Fatty Acids. Free fatty acids were quantitated following the procedure reported in the literature.[24]

Determination of Dry Matter Content, Fat and Protein Content. The dry matter content was determined in a vacuum drying oven kept at 65 °C as reported in the literature.[25] The total fat content was determined using the gravimetric micro method reported earlier.[26] The protein content, calculated as total nitrogen compounds, was determined using the Kjeldahl method.[27]

Sensory Analyses. *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 weekly training sessions with purified reference compounds by using the sip-and-spit method as reported earlier.[28,29] For training of a burning sensation, evoked by biogenic amines, tyramine (5 mmol/L) was used. The sensory sessions were performed at 21 °C in an air-conditioned room with separated booths in three independent sessions. To prevent cross-modal interactions with odorants, the panelists used nose-clips. Prior to sensory analysis, the compounds isolated or synthesized were analytically confirmed to be essentially free of solvents and buffer compounds.

Taste Recognition Threshold Concentrations. The recognition threshold concentrations for intrinsic taste of γ-L-glutamyl-L-threonine (**8**), γ-L-glutamyl-L-aspartic acid (**10**), γ-L-glutamyl-L-tryptophan (**16**), γ-L-glutamyl-L-isoleucine (**18**), α-L-glutamyl-L-lysine (**11**) and γ-L-glutamyl-L-lysine (**12**) were determined in bottled water (pH 5.3) using triangle tests with ascending concentrations of the stimulus as

379 reported earlier.[6] Values between individuals and separate sessions did not differ
380 more than plus or minus one dilution step; as a result, a threshold value of, e.g. 900
381 µmol/L for γ-L-glutamyl-L-threonine (**8**) represents a range 450-1800 µmol/L.

382 *Taste Profile Analysis*. Parmesan cheese (PC) was cut into cubic pieces (1 cm)
383 and presented to the trained sensory panel in order to evaluate the taste qualities
384 bitter, sour, sweet, salty, burning, kokumi and umami on an intensity scale from 0 (not
385 detectable) to 5 (strongly detectable). For taste profile analysis of the water soluble
386 cheese extract (WSE), the WSE lyophilisate was dissolved in bottled water in
387 "natural" cheese concentration and the pH value was adjusted to that of the authentic
388 cheese (pH 5.3), by adding trace amounts of a 1% aqueous solution of formic acid.
389 An aqueous 1:3 dilution of this stock solution was then presented to the sensory
390 panelists who were asked to rate the intensity of the individual taste qualities (see
391 above) on a scale from 0 (not detectable) to 5 (strongly detectable).

392 *Re-engineering of the Sensometabolome in Aqueous Solution*. To reconstitute
393 the taste profile of WSE, the "natural" concentrations of the tastants in groups I-VI
394 judged with a DoT-factor ≥0.5 and all peptides summarized in group VII (**Table 3**),
395 were taken up in bottled water and the pH value of this solution was adjusted to 5.3
396 by the addition of trace amounts of a 1% aqueous formic acid solution. The overall
397 taste profile of the taste recombinant (rWSE) was evaluated by means of taste profile
398 analysis using nose-clips.

399 *Taste Omission Experiments.*[29] To determine the individual taste contribution of
400 the α- and γ-glutamylpeptides and biogenic amines, partial taste recombinants were
401 prepared by omitting individual tastants or tastant groups from the total taste
402 recombinant, rWSE. Each of the partial recombinants was evaluated from the
403 panelists in comparison with the total taste recombinant, using a triangle test.
404 Panelists were asked to evaluate whether the solutions were identical in the overall

taste or not. Those panelists who detected the odd sample correctly were asked to rate the intensity of the given taste descriptors of that sample on a scale from 0 (not detectable) to 5 (strongly detectable).

Taste Re-engineering of the Sensometabolome in Parmesan-like Matrix. In order to confirm the influence of the cheese matrix to the overall taste perception, a recombinant in Parmesan matrix (rPC) was generated by addition of the mixture of the tastants in groups I-VI judged with a DoT-factor ≥0.5 and all γ-glutamyl peptides summarized in group VII (**Table 3**), in their "natural" concentrations, taken up in the "natural" water content of Parmesan cheese (PC), to the fat layer and protein pellet, obtained whilst preparation of WSE. After homogenization in a mortar, the resulting solid recombinant was wrapped in cling film, pressed into shape, and physically matured overnight at 4 °C. The matrix recombinant (rPC) was then evaluated by means of the taste profile analysis in comparison to the authentic cheese (PC) as reference.

Liquid Chromatography/Mass Spectrometry (LC-MS/MS). Analyses were acquired either on the LC-MS/MS system 1 comprising of an API 4000 Q-Trap LC-MS/MS system (Applied Biosystems, Darmstadt, Germany) connected to a 1200 HPLC-system (Agilent, Waldbronn, Germany), or the LC-MS/MS system 2 comprising of a API 3200 LC-MS/MS system (Applied Biosystems, Darmstadt, Germany) connected to a 1100 HPLC-system (Agilent, Waldbronn, Germany), both running in the positive electrospray ionization (ESI^+) mode. Zero grade air served as the nebulizer gas (45 psi) and as turbo gas for solvent drying (55 psi). Nitrogen served as the curtain (20 psi) and collision gas ($4.5x10^{-5}$ Torr). Ion spray voltage was set at 5500 V and the source temperature at 400 °C. Both quadrupoles were set at unit resolution. ESI^+ mass and product ion spectra were acquired with direct flow infusion. The declustering potential, entrance potential, collision energy, and cell exit

potential as well as the MS/MS parameters for measuring in the MRM mode were optimized for each compound, detecting the fragmentation of the $[M+H]^+$ molecular ions into specific product ions after collision with nitrogen ($4.0x10^{-5}$ Torr). For instrumentation control and data acquisition Sciex Analyst software v1.5 (Applied Biosystems, Darmstadt, Germany) was used. Detection of α- and γ-glutamyl peptides, free amino acids, and biogenic amines was performed in the multiple-reaction monitoring (MRM) mode using the characteristic mass transitions given elsewhere in the paper.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^1H, ^{13}C, COSY, HMQC and HMBC experiments were performed on an Avance III 500 MHz spectrometer (Bruker, Rheinstetten, Germany). Data processing was carried out by using Mestre-C software (Mestrelab Research, Santiago de Compostella, Spain). D_2O was used as solvent and trimethylsilylpropionic acid-d_4 (TMSP) as the internal standard.

RESULTS AND DISCUSSION

In order to gain a first insight into the taste profile of Parmesan cheese, cubic pieces of the cheese were presented to the trained sensory panelists who were asked to rate the intensities of the taste descriptors sour, bitter, umami, salty, sweet, burning and kokumi on a linear scale from 0 (not detectable) to 5 (strongly detectable). As given in **Table 1**, salt taste (2.8), burning (2.5) and kokumi sensation (2.2) were rated with the highest intensities, followed by sour (1.7), bitter (1.5), umami (1.2) and sweet taste (1.0).

As the taste active compounds in cheese were expected to be water soluble,[30] the tastants were extracted with water from Parmesan cheese matrix and separated from the protein fraction and the milk fat. The obtained water-soluble extract (WSE) was taken up in bottled water in the same concentrations as present in cheese (on mass basis) and adjusted to the pH value of the cheese (pH 5.3) with trace amounts of aqueous formic acid. Because of its strong taste, the extract was diluted 1:3 with water prior taste profile analysis with the aim to allow a good distinction between the single taste descriptors. Sensory analysis revealed that the highest intensity was found for bitterness (3.5), followed by salty taste (3.0), umami (2.5), kokumi (2.3) and burning sensation (2.2). Both, the sour and the sweet taste modality were evaluated with a score of 1.4 (**Table 1**). The deviations in the taste profile between the authentic Parmesan cheese and the water soluble extract (WSE) indicate strong matrix effects in taste perception and confirm similar observations reported ealier.[4,31,32]

For the planned taste reconstitution experiments, quantitative data on the basic taste components were needed. Therefore, the mineral cations sodium, calcium, magnesium and potassium and the anions lactate, phosphate and chloride as well as citric acid were quantified in the WSE by means of ion chromatography, 19 free amino acids were determined by means of HILIC-MS/MS and 9 free fatty acids by GC/FID (**Table 3**). As next to these basic taste compounds, kokumi-active and burning taste molecules should be considered in the taste reconstitution experiments, α- and γ-glutamyl peptides as well as biogenic amines should be quantitatively studied.

Screening for and Quantitation of α- and γ-Glutamyl Peptides. To comprehensively investigate the α- and γ-glutamyl peptides in Parmesan cheese, the WSE was screened by means of LC-MS/MS for the peptides recently reported in Gouda cheese.[6] A total of seven α-glutamyl peptides, namely α-Glu-Gly (**1**), α-Glu-Ala

(**3**), α-Glu-Val (**5**), α-Glu-Thr (**7**), α-Glu-Asp (**9**), α-Glu-Glu (**13**), and α-Glu-Trp (**15**) and eleven γ-glutamyl peptides, namely γ-Glu-Gly (**2**), γ-Glu-Ala (**4**), γ-Glu-Val (**6**), γ-Glu-Lys (**12**), γ-Glu-Glu (**14**), γ-Glu-Leu (**17**), γ-Glu-Gln (**19**), γ-Glu-Met (**20**), γ-Glu-His (**21**), γ-Glu-Phe (**22**), and γ-Glu-Tyr (**23**) have been successfully identified by comparing mass fragmentation pattern and retention time with the data obtained for reference substances, followed by co-chromatography (**Figure 1**). Except for peptides **6**, **17**, **20**, **22**, and **23**,[19] none of the other α- or γ-glutamyl peptides have been earlier reported in Parmesan cheese.

Interestingly, five additional peaks could be detected in the mass transition traces recorded for α-Glu-Thr (**7**), α-Glu-Asp (**9**), α-Glu-Trp (**15**), γ-Glu-Lys (**12**) and γ-Glu-Leu (**17**), however with different retention times (**Figure 2**). Higher retention times of the unknown peaks with the characteristic mass transitions of α-Glu-Thr (**7**), α-Glu-Asp (**9**), and α-Glu-Trp (**15**) indicated the presence of the corresponding γ-peptides γ-Glu-Thr (**8**), γ-Glu-Asp (**10**), γ-Glu-Trp (**16**), respectively (**Figures 1 and 2**). The unknown peaks in the mass traces recorded for γ-Glu-Lys (**12**) and γ-Glu-Leu (**17**) eluted earlier than the corresponding reference compounds and suggested the presence of α-Glu-Lys (**11**) and either α-Glu-Leu, or γ-Glu-Ile (**18**), the latter of which had already been identified in Parmesan cheese.[19] Whereas reference compounds of the peptides α-Glu-Lys (**11**) and γ-Glu-Trp (**16**) have been commercially available, reference compounds for γ-Glu-Thr (**8**), γ-Glu-Asp (**10**), and γ-Glu-Ile (**18**) had to be synthesized using solid state peptide chemistry using Wang resin-bound and Fmoc- and OtBu-protected amino acids. The target peptides **8**, **10**, and **18** were isolated by HPLC in a purity of >99% and their chemical structures confirmed by means of LC-MS/MS and NMR experiments. The identity of peptides γ-Glu-Thr (**8**), γ-Glu-Asp (**10**), α-Glu-Lys (**11**), γ-Glu-Trp (**16**) and γ-Glu-Ile (**18**) was confirmed by comparing mass fragmentation pattern and retention time with the data obtained for the corresponding

reference substances, followed by co-chromatography. Whereas the presence of peptide **18** could be confirmed,[19] γ-Glu-Thr (**8**), γ-Glu-Asp (**10**), α-Glu-Lys (**11**), and γ-Glu-Trp (**16**) have been identified for the first time on Parmesan cheese.

To evaluate their sensory activity, human taste threshold concentrations for the intrinsic taste of the peptides **8**, **10**-**12**, **16**, **19** were determined in water by use of an ascending triangle test. All peptides, except γ-Glu-Lys (**12**) imparted an unspecific, slightly astringent orosensation with thresholds concentrations of 300 (**8**), 900 (**10**), 1990 (**16**), 1300 (**11**) and 5000 µmol/kg (**16**), respectively (**Table 3**), fitting well with the range of taste thresholds of other glutamyl peptides reported earlier.[6] In comparison, γ-Glu-Lys (**12**) imparted a slightly umami-like taste with a threshold of 2000 µmol/L.

To accurately quantitate the glutamyl peptides identified by means of a stable isotope dilution assay (SIDA), γ-Glu-Ala-[$^{13}C_3$] (**24**) was synthesized as the internal standard (**Figure 1**). Comparing the MS/MS spectrum of γ-Glu-Ala (**Figure 3**, **A**) and γ-Glu-Ala-[$^{13}C_3$] (**Figure 3**, **B**) clearly points out the mass shift resulting from ^{13}C-labeling. γ-Glu-Ala-[$^{13}C_3$] shows a pseudo molecular ($[M+H]^+$) with *m/z* 222.2, confirming the targeted molecular mass of 221 Da. Fragmentation of the $[M+H]^+$ ion revealed the fragment series of *m/z* 130.1 and 83.9 which could be assigned to the presence of the *N*-terminal glutamyl residue (**Figure 3, B**). The C-terminal end of the peptide containing the $^{13}C_3$-alanine moiety was identified in the fragment y_1 with *m/z* 93.0. The ions *m/z* 159.1 and 158.1 resulted from the loss of an ammonium and either the C-terminal or the N-terminal carboxyl residue.

Prior to quantitative analysis, it was necessary to confirm that the co-eluting cheese matrix components did not affect differently the ionization of the analytes. To visualize such matrix effects, a constant flow of γ-Glu-Ala-[$^{13}C_3$] (**24**) was introduced into the LC-MS/MS system via a syringe pump during the LC-MS/MS analysis of a

blank injection (**Figure 4, A**) as well as an injection of the aqueous extract of the Parmesan cheese (**Figure 4, B**). As given in **Figure 4, A**, infusion γ-Glu-Ala-[$^{13}C_3$] at a constant flow rate into the LC-MS/MS system whilst blank injection, only the influence of the solvent gradient on the ionization rate could be detected, whereby increase of the acetonitrile ratio induced an increase of the signal intensity. The infusion of γ-Glu-Ala-[$^{13}C_3$] (**24**) during analysis of a cheese sample revealed a matrix dependent suppression of the ionization of γ-Glu-Ala-[$^{13}C_3$] between 1 and 5 min only to a marginal extend. In this elution window most of the polar glutamyl peptides as well as the internal standard can be detected. After 5 min the signal intensity was stable again and after 11 min it increased constantly until the end of the gradient, similar to the matrix-free analysis of the ionization of γ-Glu-Ala-[$^{13}C_3$]. In the elution time from 11 to 13 min the less polar peptides were detected. Therefore, quantitation of α- and γ-glutamyl peptides using γ-Glu-Ala-[$^{13}C_3$] (**24**) as the internal standard seemed suitable and to be an improvement compared to external calibration.

To check the accuracy of the HPLC-MS/MS method, recovery experiments were performed in the following (**Table 2**). To achieve this, samples of the Parmesan cheese were spiked with reference peptides **1** - **23** in three different concentration levels prior to extraction with aqueous hydrochloric acid and LC-MS/MS analysis, and the amounts determined were compared with those found in the non-spiked cheese sample. The average recovery rates ranged between 91.3% for γ-Glu-Trp (**16**) and 110.6% for α-Glu-Glu (**13**), showing good reliability and accuracy of the method (**Table 2**).

Using the developed SIDA method, compounds **1** - **23** were quantified in Parmesan cheese (**Figure 5**). γ-Glu-His (**21**), γ-Glu-Glu (**14**), and γ-Glu-Thr (**8**) were found as the major peptides in concentrations of 6204 , 3299, and 2538 µmol/kg (fresh weight), respectively, followed by γ-Glu-Leu (**17**), γ-Glu-Lys (**12**), γ-Glu-Phe

559 (**22**), γ-Glu-Val (**12**), and γ-Glu-Gly (**2**) present in concentrations above 1000 µmol/kg
560 (**Table 3**). Interestingly, the overall amount (20895 µmol/kg) of glutamyl peptides and,
561 in particular, the total amount of γ-glutamyl peptides (20468 µmol/kg) in the
562 Parmesan cheese was much higher than in any other cheeses investigated so far
563 including blue-veined cheese with concentrations of 3590 µmol/kg.[7] Compared to the
564 γ-glutamyl peptides, the amounts of the α-glutamyl peptides were rather low with α-
565 Glu-Lys (**11**) found as the major representative in a concentration of 175 µmol/kg
566 (**Table 3**).

567 **Screening for and Quantification of Biogenic Amines.** As biogenic amines
568 are considered to be the cause of the burning orosensation induced by some
569 cheeses,[3] a cheese extract was derivatized with dansyl chloride and the dansylated
570 biogenic amines were analyzed by means of LC-MS/MS to afford tyramine,
571 histamine, putrescine, cadaverine, and spermidine as biogenic amines. Using
572 standards tyramine-d_4 and histamine-d_4 as internal standards, a stable isotope
573 dilution assay was developed for accurate quantitation of these biogenic amines
574 (**Figure 6**). Cheese was spiked with defined amounts of the internal standards
575 tyramine-d_4 and histamine-d_4 prior to aqueous extraction, the obtained suspension
576 was centrifuged, and an aliquot of the clear supernatant taken for derivatization with
577 dansyl chloride, followed by LC-MS/MS analysis. Quantitative analysis of the
578 Parmesan cheese revealed histamine (1170 µmol/kg), tyramine (540 µmol/kg), and
579 cadaverine (304 µmol/kg) as the main biogenic amines, whereas putrescine and
580 spermidine were less abundant and found in concentrations of 89 and 16 µmol/kg,
581 respectively (**Table 3**).

582 **Dose-over-Threshold (DoT) Factors of Taste Compounds in Parmesan**
583 **Cheese.** To rank the taste compounds identified in their taste impact, the dose-over-
584 threshold factor was calculated for each compound as the ratio of the concentration

585 in the food sample and the taste threshold concentration. Depending on their taste
586 qualities, the taste compounds were grouped into seven classes, namely I - VII
587 (**Table 3**).

588 The bitter tasting group I consisted of the bivalent cations calcium and
589 magnesium as well as the bitter amino acids L-leucine, L-tyrosine, L-isoleucine,
590 L-tryptophan, L-lysine, L-valine, L-phenylalanine, L-arginine, and L-histidine (**Table 3**).
591 Amongst these bitter compounds, calcium chloride showed by far the highest DoT-
592 factor with a value of 23.0, followed by the amino acids L-leucine, L-isoleucine,
593 L-valine, L-tyrosine and L-lysine, for which DoT-factors of 8.0, 7.6, 2.9, 2.3, and 1.3
594 were calculated, respectively. With a calculated DoT-factor of 2.4, also magnesium
595 chloride exceeded its bitter taste threshold, whereas the remaining bitter amino acids
596 showed DoT-factors below 1.0.

597 The umami tasting group II consisted of the amino acids L-glutamic acid,
598 L-aspartic acid, L-glutamine, and L-asparagine (**Table 3**). Very high DoT-factors of
599 138.7 and 80.7 were determined for L-glutamic acid and L-aspartic acid, whereas the
600 corresponding amides were present below their threshold concentrations.

601 Tastant group III comprised sour and salty compounds, namely the cations
602 sodium and potassium, the anions lactate, phosphate, chloride, and the organic acids
603 acetic acid and citric acid. High DoT-factors of 59.6 and 26.8 were determined for
604 sodium and chloride. Also potassium (2.2), phosphate (1.9), lactate (1.5) and acetate
605 (1.2) were found above their threshold concentrations and were expected to have a
606 direct impact to the taste of the parmesan cheese.

607 The sweet tasting compounds were summarized in tastant group IV, comprising
608 the amino acids L-methionine, L-alanine, L-serine, glycine, L-proline, and L-threonine.
609 Although all compounds were present in concentrations exceeding their sweet taste

610 threshold, the highest DoT-factors were found for L-proline (5.1), L-methionine (4.5),
611 and L-alanine (3.9), respectively (**Table 3**).

612 In the group of free fatty acids (group V), only butyric acid, evoking a sour taste,
613 and oleic acid, eliciting an astringent mouthfeel as well as a fatty mouth coating,
614 showed DoT-factors above 1 (**Table 3**).

615 Tastant group VI contained the biogenic amines histamine, tyramine, putrescine,
616 cadaverine, and spermidine. DoT-factors of 2.3, 2.0, and 1.1 found for cadaverine,
617 histamine and tyramine indicated their contribution to the characteristic burning taste
618 of parmesan cheese, whereas the other biogenic amines were below their
619 orosensory thresholds.

620 The kokumi-active α- and γ-glutamyl peptides were classified into tastant group
621 VII. Without any exception, α-glutamyl peptides were present in concentrations far
622 below their intrinsic taste thresholds (**Table 3**). Although the content of the γ-glutamyl
623 peptides was much higher than that of their corresponding α-glutamyl peptides, only
624 γ-Glu-Thr (**8**) (8.5) and γ-Glu-His (**21**) (2.4) revealed DoT-factors >1.0 for their
625 intrinsic taste qualities. However, the thresholds for the kokumi enhancing activity of
626 γ-glutamyl peptides are much lower (5 - 20 µmol/kg) and depend on the matrix.[7,33]
627 Therefore, the impact of these peptides on the long-lasting taste perception should
628 be studied by recombination and omission experiments in the following.

629 **Taste Re-Engineering and Omission Experiments in Aqueous Solution.** In
630 order to sensorially validate the results of quantitative analysis and to study whether
631 the compounds already identified could create the characteristic taste of the water
632 soluble extract made from Parmesan cheese, taste re-engineering experiments were
633 performed in the following.

634 First, an aqueous taste recombinant (rWSE) of the water soluble cheese extract
635 was prepared containing all taste compounds in groups I-VI judged with a DoT-factor

≥0.5 and all peptides summarized in group VII (**Table 3**), each in the "natural" concentration as determined in Parmesan cheese. The taste compounds were dissolved in bottled water in their "natural" concentrations, and the pH value was adjusted to that of the WSE by addition of trace amounts of formic acid. After aqueous dilution (1:3) of the taste recombinant solution (rWSE) as well as of the WSE, the trained sensory panel was asked to evaluate the taste profiles of rWSE in comparison to that of the authentic WSE by scoring the taste descriptors given in **Table 4** on a scale from 0 (not detectable) to 5 (strong detectable). The intensities of the bitter (3.5/3.6±0.2), salty (3.0/3.1±0.1), kokumi (2.3/2.2±0.3), burning (2.2/2.1±0.3), and sour taste (1.4/1.5±0.1) in WSE and rWSE were rather close and not significantly different, whereas a complete match was found for the umami (2.5) and the sweet note (1.5). On the basis of these data, it was concluded that the components in groups I – VII are sufficient to create the characteristic taste profile of an aqueous extract made from Parmesan cheese.

In order to investigate the sensory contribution of γ-glutamyl peptides and biogenic amines, respectively, a series of taste omission experiments were performed by preparing partial taste recombinants, lacking one or the other tastant group, followed by sensory evaluation by means of a triangle test using two samples of rWSE and one sample of the partial recombinant. Those panelists who detected any difference in the taste profile were asked to rate the intensity of the taste descriptors as given in **Table 4** on a scale from 0 to 5. In a first experiment, all α- and γ-glutamyl peptides were omitted from the taste recombinant, leading to $rWSE^{-\alpha/\gamma}$. Taste profile analysis of $rWSE^{-\alpha/\gamma}$ showed that the lack of α- and γ-glutamyl peptides (group VII, **Table 3**) led to a drastic decrease of the intensity of kokumi perception (2.1→1.3) as well as a slight bitterness increase (3.6→3.9). Sour, umami, salty and

sweet taste modalities of rWSE$^{-\alpha/\gamma}$ were evaluated with the same scores as in rWSE (**Table 4**).

In order to verify the recent observation in Gouda cheese that only the γ-glutamyl peptides show kokumi enhancing activity,[6] partial recombinants were prepared by omitting either the α-glutamyl peptides (rWSE$^{-\alpha}$), or the γ-glutamyl peptides (rWSE$^{-\gamma}$). Sensory analysis of rWSE$^{-\alpha}$ in comparison to rWSE did not reveal any significant difference in the kokumi sensation, nor in any other taste quality (**Table 4**). In comparison, a strong decrease of kokumi intensity (2.2→1.3) as well as a slight decrease in bitter intensity (3.6→3.8) was reported by the sensory panel when rWSE was tested against rWSE$^{-\gamma}$. These findings nicely match previous data found for Gouda cheese,[6,33] and clearly demonstrated the key impact of γ-glutamyl peptides on the complex and long lasting taste perception of Parmesan cheese, while the α-glutamyl peptides can be neglected.

In order to validate the role of biogenic amines in the cheese's burning impact, a partial recombinant was prepared by omitting the group of biogenic amines (group VI), thus leading to rWSE^{-BA}. This partial recombinant (0.5) was judged significantly lower in comparison to the total recombinant (2.1) (**Table 4**), thus clearly pointing out the key role of the biogenic amines cadaverine, histamine, tyramine, and putrescine for the characteristic burning taste of the Parmesan cheese.

Taste Re-Engineering Experiments in a Cheese-Like Matrix. In order to investigate the influence of the cheese matrix on taste perception, the aim of the final re-engineering experiment was to perform a taste recombinant in a cheese-like matrix. To achieve this, first, dry matter (71.9 g/100 g), water content (28.1 g/100 g), fat (26.6 g/100 g), and protein content (31.2 g/100 g) of the Parmesan cheese (PC) were determined. Based on these data, a recombinant in Parmesan-like matrix (rPC) was generated by adding the taste compounds from groups I-VI judged with a DoT-

factor ≥1 and all γ-glutamyl peptides from group VII (**Table 3**), dissolved in water (28.1 g/100g), to the tasteless protein and fat fraction, both isolated from Parmesan cheese, each in its natural concentration ratio. After homogenization, the resulting material was wrapped in cling film, pressed into shape, and physically matured overnight at 4 °C. The taste recombinant in the cheese-like matrix (rPC) was then evaluated by means of a taste profile analysis in comparison to the authentic cheese (PC) as the reference. As given in **Table 5**, sensory analysis revealed the taste recombinant (rPC) to match well the taste profile of the authentic cheese (PC), thus demonstrating that the identified taste compounds are fully sufficient to create the characteristic taste profile of the Parmesan cheese.

In summary, quantitative studies on the nonvolatile sensometabolites of ripened Parmesan cheese, led to the identification of 31 primary tastants with DoT-factors above 1.0 and 15 kokumi-enhancing γ-glutamyl peptides, amongst which γ-Glu-Gly (**2**), γ-Glu-Ala (**4**), γ-Glu-Thr (**8**), γ-Glu-Asp (**10**), γ-Glu-Lys (**12**), γ-Glu-Glu (**14**), γ-Glu-Trp (**16**) γ-Glu-Gln (**19**), γ-Glu-His (**21**) have been identified for the first time in Parmesan cheese. The extraordinarily high concentration of 20468 µmol/kg found for the group of γ-glutamyl peptides raises the question as to which parameters control the generation of these taste enhancing peptides during cheese manufacturing. Elucidation of the key factors governing the formation of these peptides would open a new avenue to tailor the taste profile of cheeses.

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FIGURE LEGEND

Figure 1. Chemical structures of α-Glu-Gly (**1**), γ-Glu-Gly (**2**), α-Glu-Ala (**3**), γ-Glu-Ala (**4**), α-Glu-Val (**5**), γ-Glu-Val (**6**), α-Glu-Thr (**7**), γ-Glu-Thr (**8**), α-Glu-Asp (**9**), γ-Glu-Asp (**10**), α-Glu-Lys (**11**), γ-Glu-Lys (**12**), α-Glu-Glu (**13**), γ-Glu-Glu (**14**), α-Glu-Trp (**15**), γ-Glu-Trp (**16**), γ-Glu-Leu (**17**), γ-Glu-Ile (**18**), γ-Glu-Gln (**19**), γ-Glu-Met (**20**), γ-Glu-His (**21**), γ-Glu-Phe (**22**), γ-Glu-Tyr (**23**), and the stable-isotope labeled internal standard γ-Glu-Ala-[$^{13}C_3$] (**24**). ^{13}C labeled atoms are marked with an square.

Figure 2. HPLC-MS/MS (MRM) chromatograms (ESI$^+$) of a α-Glu-Asp (**9**) reference (**A**), α-Glu-Asp (**9**) in cheese extract (**B**), a α-Glu-Thr (**7**) reference (**C**), α-Glu-Thr (**7**) in cheese extract (**D**), a α-Glu-Trp (**15**) reference (**E**), α-Glu-Trp (**15**) in cheese extract (**F**), a reference of α-Glu-Lys (**12**) and γ-Glu-Gln (**19**) (**G**), α-Glu-Lys (**12**) and γ-Glu-Gln (**19**) in cheese extract (**H**), a γ-Glu-Leu (**17**) reference (**I**), and Glu-Leu (**17**) in cheese extract (**K**), respectively.

Figure 3. MS/MS (ESI$^+$) spectra of (**A**) γ-Glu-Ala (**4**) and (**B**) γ-Glu-Ala-[$^{13}C_3$] (**24**).

Figure 4. LC-MS/MS (MRM) chromatograms (ESI$^+$) recorded for an injection of water (**A**) and aqueous cheese extract (**B**) whilst a continuous flow of γ-Glu-Ala-[$^{13}C_3$] (**24**) (10 µL/min) was introduced into the LC-MS/MS-system system by means of a syringe pump.

Figure 5. HPLC-MS/MS (MRM) analysis of α- and γ-glutamyl peptides in freshly prepared cheese extract (numbers of peaks refer to **Figure 1**).

Figure 6. HPLC-MS/MS (MRM) chromatograms (ESI$^+$) of dansyl chloride (DCl) derivatized biogenic amines in freshly prepared cheese extract.

Table 1. Sensory Evaluation of Parmesan Cheese (PC) and of the Water Soluble Extract (WSE) Prepared from PC.

taste quality	intensities for individual taste qualities[a] in	
	PC	WSE
sour	1.7	1.4±0.2
bitter	1.5	3.5±0.1
umami	1.2	2.5±0.3
salty	2.8	3.0±0.2
sweet	1.0	1.4±0.1
burning	2.5	2.2±0.2
kokumi	2.2	2.3±0.3

[a] Intensities were judged on a scale from 0 (not detectable) to 5 (strongly detectable) by trained sensory panelists. Data are given as the mean of triplicates.

Table 2. Determination of Recovery Rates for α- and γ-Glutamyl Peptides (**1** - **23**) in Parmesan Cheese.

	amount added [µmol/kg][a] at spiking level			recovery [%] at spiking level			mean value
peptide no.	I	II	III	I	II	III	[%]
1	1.8	4.4	8.9	101.4	114.0	110.1	108.5
2	83.7	209.1	418.3	98.9	102.2	99.1	100.1
3	1.3	3.3	6.6	100.6	97.3	103.8	100.6
4	107.1	267.7	535.5	99.9	105.7	101.9	102.5
5	2.5	6.3	12.5	102.5	114.1	103.8	106.8
6	51.2	127.9	255.9	99.5	110.0	110.8	106.7
7	1.8	4.6	9.1	102.9	109.7	114.6	109.1
8	69.8	174.6	349.2	98.4	106.3	107.4	104.0
9	0.7	1.9	3.7	96.6	108.3	101.4	102.1
10	21.4	53.5	107.0	97.1	94.8	93.4	95.1
11	38.9	97.3	194.5	103.5	107.9	106.2	105.9
12	184.5	461.4	922.7	101.6	108.8	116.9	109.1
13	6.8	16.9	33.8	100.3	112.7	118.8	110.6
14	123.3	308.3	616.7	96.0	98.0	101.8	98.6
15	1.5	3.7	7.4	103.7	107.1	104.2	105.0
16	7.6	18.9	37.8	97.2	90.9	85.6	91.3
17	79.9	199.7	399.5	103.5	110.1	110.8	108.1
18	123.3	308.0	616.0	102.2	93.2	88.6	94.7
19	7.0	17.5	34.9	97.5	104.1	108.7	103.4
20	26.3	65.6	131.3	101.4	109.6	114.1	108.4
21	149.9	374.7	749.4	101.6	113.7	116.3	110.5
22	43.7	109.2	218.5	98.8	106.9	108.2	104.6
23	6.5	16.2	10.0	98.5	110.7	111.4	106.9

[a] Peptides **1** - **23** (**Figure 1**) were added to Parmesan cheese in three different concentrations (calculated on fresh weight) prior to quantitative analysis, and the values determined (n = 3) after spiking were compared with those of the non-spiked cheese (**Table 3**).

Table 3. Taste Qualities, Taste Recognition Thresholds, Concentrations, and Dose-over-Threshold (DoT) Factors of Non-Volatile Sensometabolites and Mineral Salts in Parmesan Cheese.

taste compound[a]	TC [b] [μmol/L][b]	conc. [μmol/L][c] (±RSD in %)	DoT[d]
group I: bitter tasting minerals and amino acids			
calcium	6200[p,w]	142653 (±3.1)	23.0
L-leucine	11000[k]	88212 (±7.9)	8.0
L-isoleucine	10000[k]	76479 (±5.9)	7.6
L-valine	30000[w]	87377 (±4.0)	2.9
magnesium	6400[p,w]	14775 (±2.3)	2.3
L-tyrosine	4000[k]	9151 (±3.1)	2.3
L-lysine	80000[k]	103486 (±3.7)	1.3
L-tryptophan	4000[k]	3823 (±3.6)	0.9
L-histidine	45000[k]	21700 (±0.7)	0.5
L-arginine	75000[k]	24790 (±0.0)	0.3
L-phenylalanine	45000[k]	3378 (±8.6)	<0.1
group II: umami-like compounds			
L-glutamic acid	1100[g,w]	152708) (±6.6)	138.7
L-aspartic acid	600[g,w]	48449 (±1.3)	80.7
L-asparagine	50000[g,w]	31763 (±1.5)	0.6
L-glutamine	50000[g,w]	5956 (±1.3)	0.1
group III: sour/salty compounds			
sodium	3900[p,w]	232601(±2.0)	59.6
chloride	3900[q,w]	104429 (±0.4)	26.8
potassium	13000[p,w]	28917(±1.1)	2.2
phosphate	5000[h,q]	9643 (±3.1)	1.9
lactate	11890[f,q,]23770[g,q]	17726 (±0.4)	1.5/0.7
acetate	3100[l,w]	3602 (±4.2)	1.2
citric acid	2600[m]	314 (±9.3)	0.1
group IV: sweet tasting compounds			
L-proline	25000[i]	126621 (±7.8)	5.1

L-methionine	5000[k]	22570(±1.1)	4.5
L-alanine	12000[i]	46486 (±2.1)	3.9
L-serine	25000[i]	89787 (±3.9)	3.6
glycine	25000[i]	51781(±3.9)	2.1
L-threonine	35000[i]	39028 (±3.3)	1.1
group V: fatty[s]			
oleic acid	670[l,n,w]/2650[l,o,w]	6447 (±2.8)	9.8/2.5
butyric acid	4000[l,m,w]	4639 (±0.2)	1.2
caproic acid	3400[l,m,w]	1815 (±1.8)	0.5
caprylic acid	5200[l,n,w]	1236 (±1.5)	0.2
capric acid	15500[l,n,w]	1720 (±1.3)	0.1
lauric acid	12000	1031 (±1.2)	<0.1
myristic acid	15000	3744 (±0.1)	<0.1
palmitic acid	15000	8576 (±3.4)	<0.1
stearic acid	12000	1611(±3.6)	<0.1
group VI: burning[s]			
histamine	600[h,r]/10000[e,x]	1170 (±13.7)	2.0
tyramine	500[h,r]/2000[e,x]	540 (±5.7)	1.1
putrescine	100[e,y]	89 (±6.6)	0.9
cadaverine	130[e,y]	304 (±5.6)	2.3
spermidine	130[t]	16 (±9.3)	<0.1
group VII: kokumi[s]			
α-Glu-Gly (**1**)	2500[u,z]	27 (±9.8)	<0.1
α-Glu-Ala (**3**)	10000[u,z]	8 (±5.3)	<0.1
α-Glu-Val (**5**)	5000[u,z]	33 (±7.8)	<0.1
α-Glu-Thr (**7**)	2500[u,z]	35 (±7.8)	<0.1
α-Glu-Asp (**9**)	1250[u,z]	11 (±7.1)	<0.1
α-Glu-Lys (**11**)	1300[u]	175 (±2.3)	0.1
α-Glu-Glu (**13**)	2500[v,z]	131(±9.4)	<0.1
α-Glu-Trp (**15**)	5000[e,z]	3 (±18.5)	<0.1
γ-Glu-Gly (**2**)	1250[u,z]	1055 (±5.7)	0.8
γ-Glu-Ala (**4**)	900[u,z]	216 (±5.9)	0.2

γ-Glu-Val (**6**)	3300[u,z]	1290 (±7.0)	0.4
γ-Glu-Thr (**8**)	300[u]	2538 (±5.1)	8.5
γ-Glu-Asp (**10**)	900[u]	276 (±7.4)	0.3
γ-Glu-Lys (**12**)	2000[v]	1156 (±4.6)	0.6
γ-Glu-Glu (**14**)	5000[u,z],10000[v,z]	3299 (±4.7)	0.6/0.3
γ-Glu-Trp (**16**)	2000[u]	60 (±4.2)	<0.1
γ-Glu-Leu (**17**)	9400[u,z]	1296 (±4.2)	0.1
γ-Glu-Ile (**18**)	5000[u]	952 (±6.8)	0.2
γ-Glu-Gln (**19**)	2500[u,z]	152 (±8.3)	<0.1
γ-Glu-Met (**20**)	2500[u,z]	626 (±5.1)	0.2
γ-Glu-His (**21**)	2500[u,z]	6204 (±6.2)	2.4
γ-Glu-Phe (**22**)	2500[u,z]	1146 (±4.2)	0.5
γ-Glu-Tyr (**23**)	2500[u,z], 5000[e,z]	200 (±5.9)	<0.1

[a] Taste-active compounds were determined in the water-soluble extract (WSE), if not stated otherwise; [b] Taste threshold concentrations (TC) were determined in bottled water by means of a triangle test and are given as the mean of triplicates, if not stated otherwise or taken from literature; [c] Concentration (µmol/kg) in cheese; [d] Dose-over-threshold (DoT) factor is calculated as the ratio of concentration and taste threshold; [e] Taste threshold concentration for bitter taste; [f] Taste threshold concentration for sour/salty taste; [g] Taste threshold concentration for umami taste; [h] Value taken from (*3*); [i] Value taken from (*39*); [k] Value taken from (*40*); [l] Taste threshold determined in the emulsifier Emultop (0.02% in water); [m] Taste threshold for sourness; [n] Threshold for astringent mouthfeel; [o] Threshold for fatty mouth-coating; [p] Threshold concentration determined for the corresponding chloride salt; [q] Threshold concentration determined for the corresponding sodium salt; [r] Taste threshold for burning sensation [s] Fatty acids, biogenic amines, glutamyl-peptides determination in cheese. [t] threshold of cadaverine was used to estimate the DoT-factor of spermidine; [u] Taste threshold for an unspecific, slightly astringent mouthfeel; [v] Taste threshold for an umami-like taste; [w] Value taken from (*4*); [x] Value taken from (*37*); [y] Value taken from (*38*); [z] Value taken from (*6*)

Table 4. Sensory Evaluation of Taste Recombinant (rWSE), Containing the Tastants of Groups I-VI (DoT ≥0.5) and All Peptides (group VII), Partial Taste Recombinant ($rWSE^{-\alpha/\gamma}$) Lacking α- And γ-Glutamyl Peptides, Partial Taste Recombinant ($rWSE^{-\alpha}$) Lacking α-Glutamyl Peptides, Partial Taste Recombinant ($rWSE^{-\gamma}$) Lacking γ-Glutamyl Peptides, and Partial Taste Recombinant ($rWSE^{-BA}$) Lacking the Biogenic Amines.

taste quality	intensities for individual taste qualities[a] in					
	WSE	rWSE	$rWSE^{-\alpha/\gamma}$	$rWSE^{-\alpha}$	$rWSE^{-\gamma}$	$rWSE^{-BA}$
sour	1.4	1.5±0.1	1.5±0.2	1.5±0.3	1.3±0.3	1.7±0.2
bitter	3.5	3.6±0.2	3.9±0.2	3.5±0.4	3.8±0.3	3.4±0.3
umami	2.5	2.5±0.2	2.5±0.2	2.5±0.2	2.5±0.2	2.5±0.2
salty	3.0	3.1±0.1	3.1±0.1	3.1±0.4	3.0±0.4	3.3±0.3
sweet	1.5	1.5±0.3	1.5±0.1	1.5±0.1	1.5±0.2	1.5±0.1
burning	2.2	2.1±0.3	2.4±0.2	2.1±0.2	2.1±0.2	0.5±0.1
kokumi	2.3	2.2±0.3	1.3±0.4	2.2±0.1	1.3±0.2	2.2±0.2

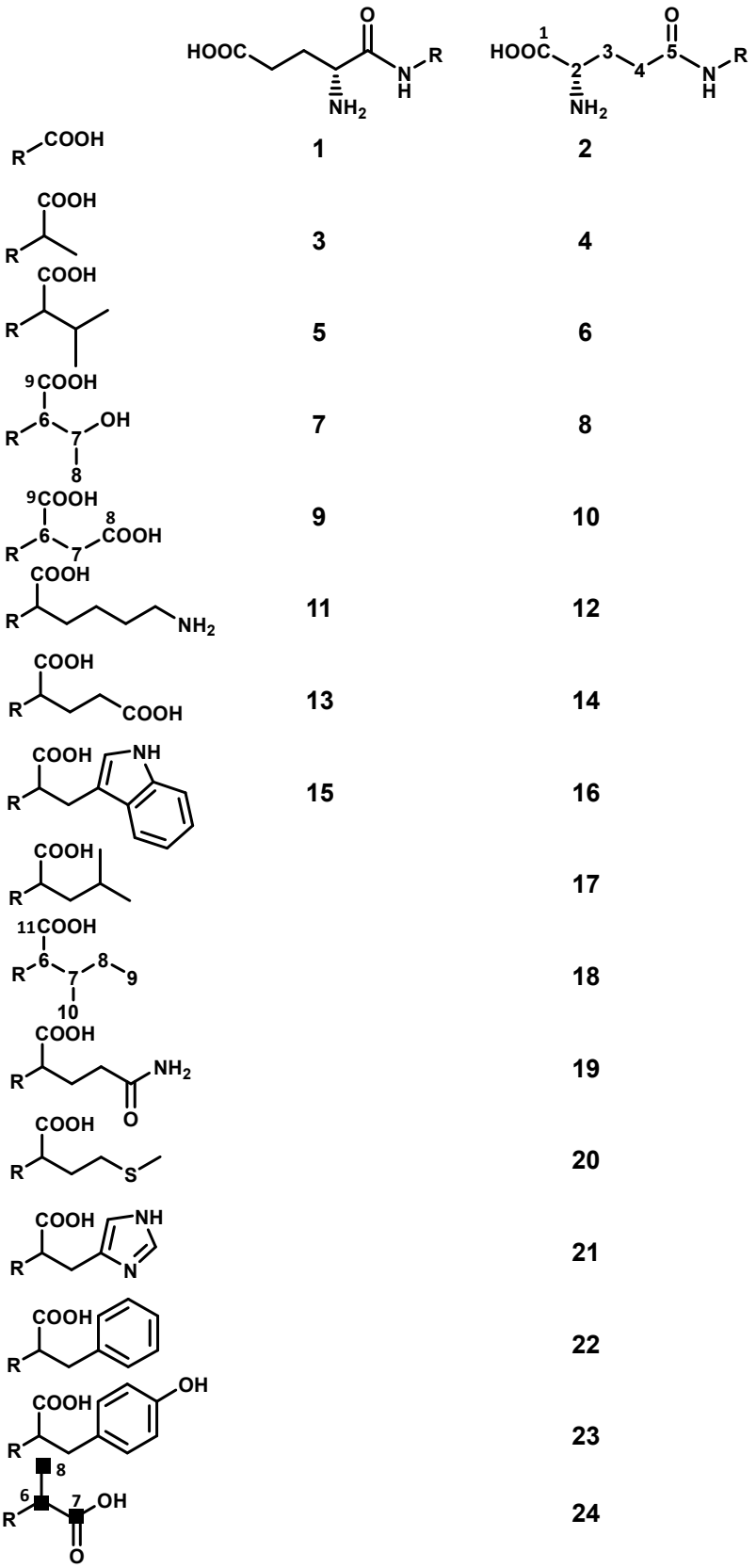
[a] Intensities were judged by the panel on a scale from 0 (not detectable) to 5 (strongly detectable). Values and standard deviations are given as the mean of triplicates.

Table 5. Sensory Evaluation of Parmesan Cheese (PC) and the Taste Recombinant in Cheese-Like Matrix (rPC) Containing the Tastants (Groups I-VI, DoT-factor ≥0.5) and the γ-Glutamyl Peptides (group VII)

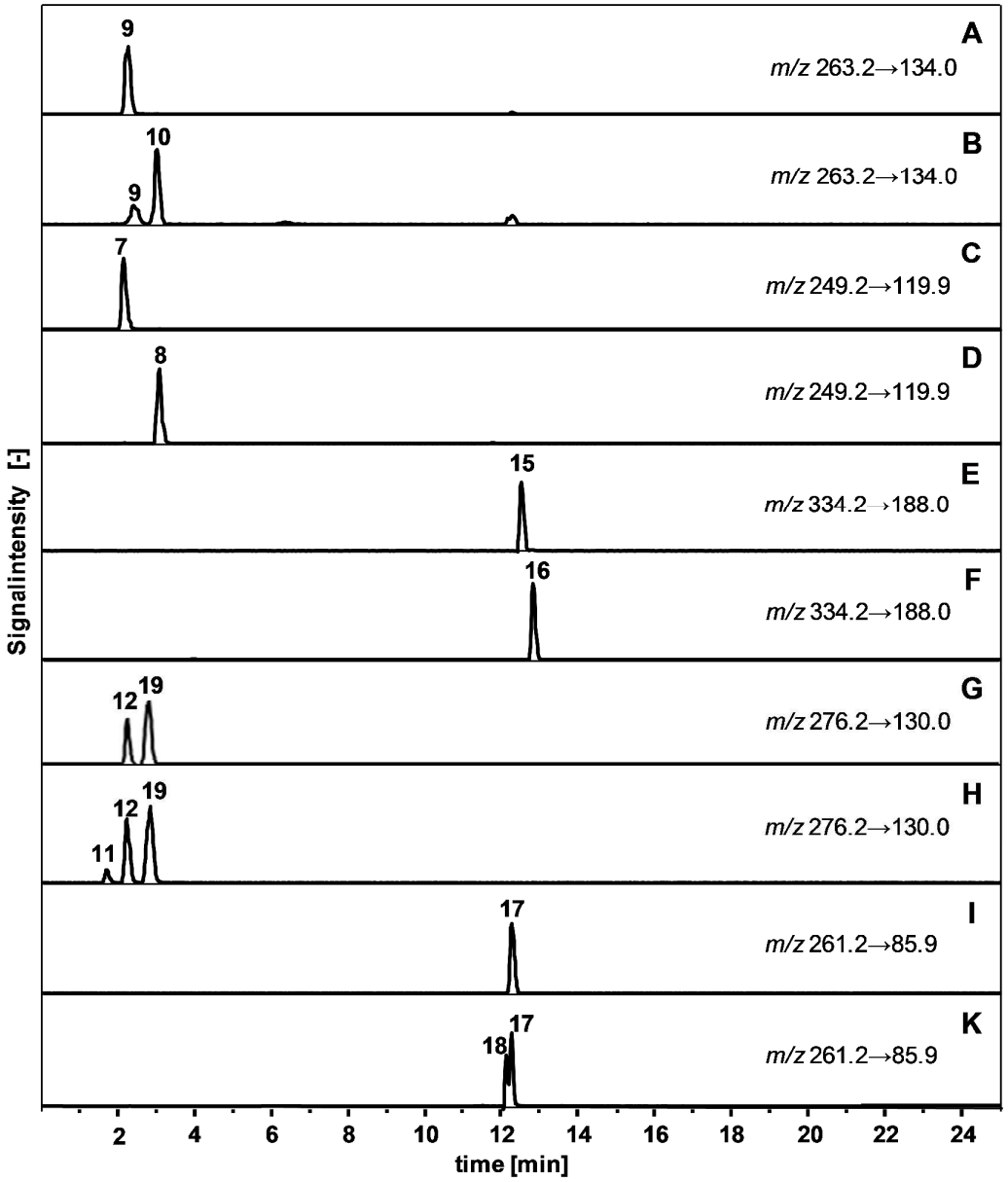
taste quality	intensities for individual taste qualities[a]	
	PC	rPC
sour	1.7	1.7±0.1
bitter	1.5	1.4±0.2
umami	1.2	1.2±0.1
salty	2.8	2.8±0.2
sweet	1.0	1.1±0.2
burning	2.5	2.4±0.2
kokumi	2.2	2.2±0.1

[a] Intensities were judged by the panel on a scale from 0 (not detectable) to 5 (strongly detectable). Values and standard deviations are given as the mean of triplicates.

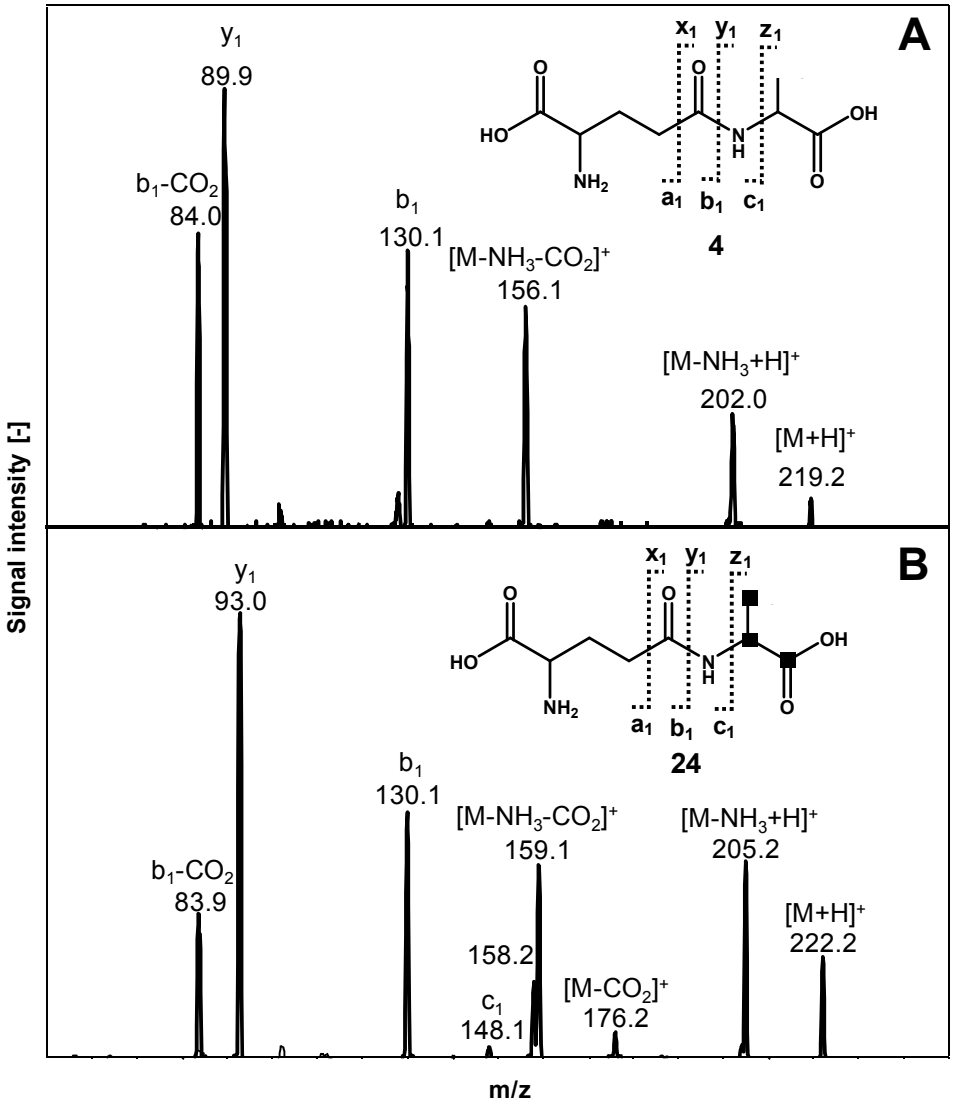
Hillmann and Hofmann, Figure 1



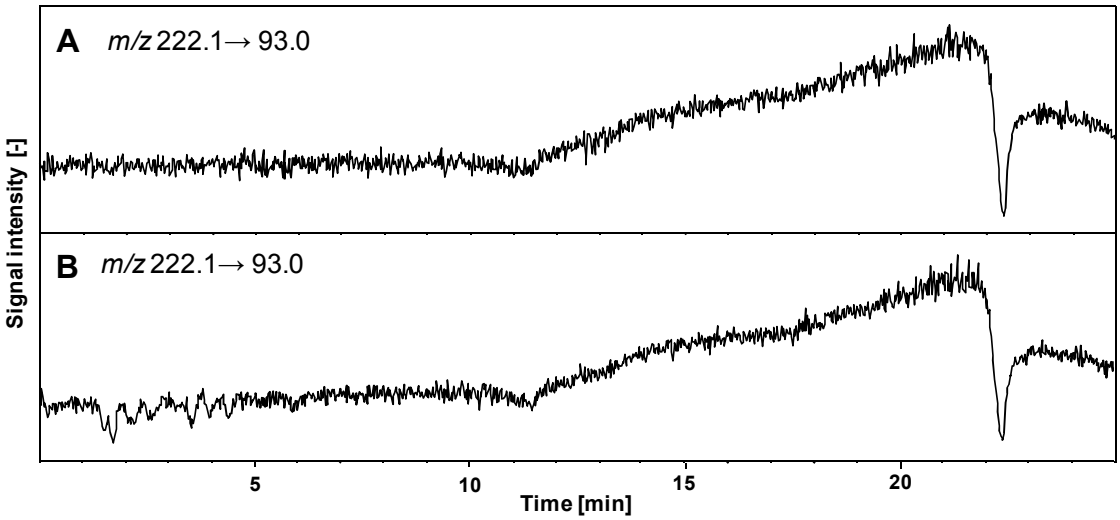
Hillmann and Hofmann, Figure 2



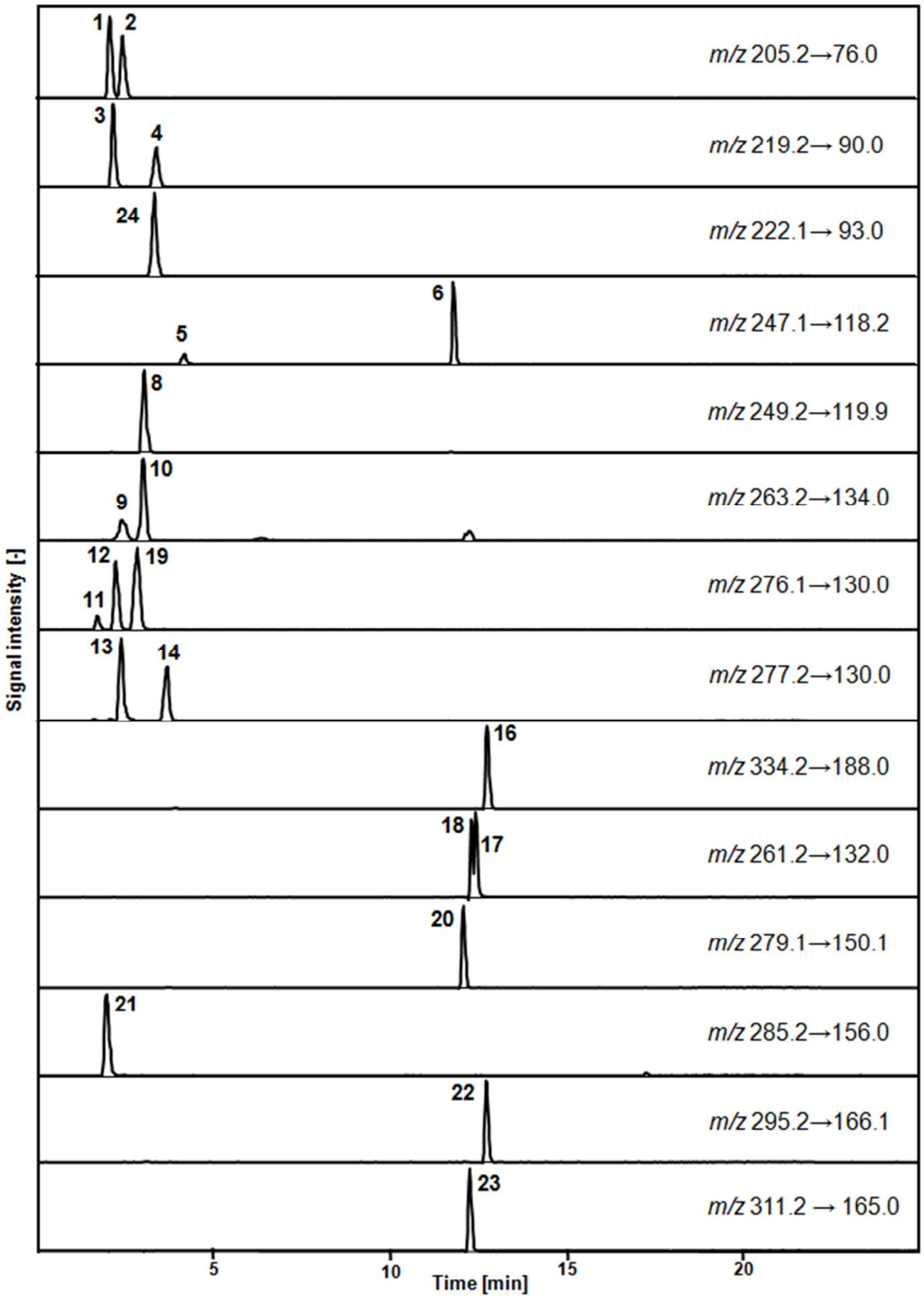
Hillmann and Hofmann, Figure 3



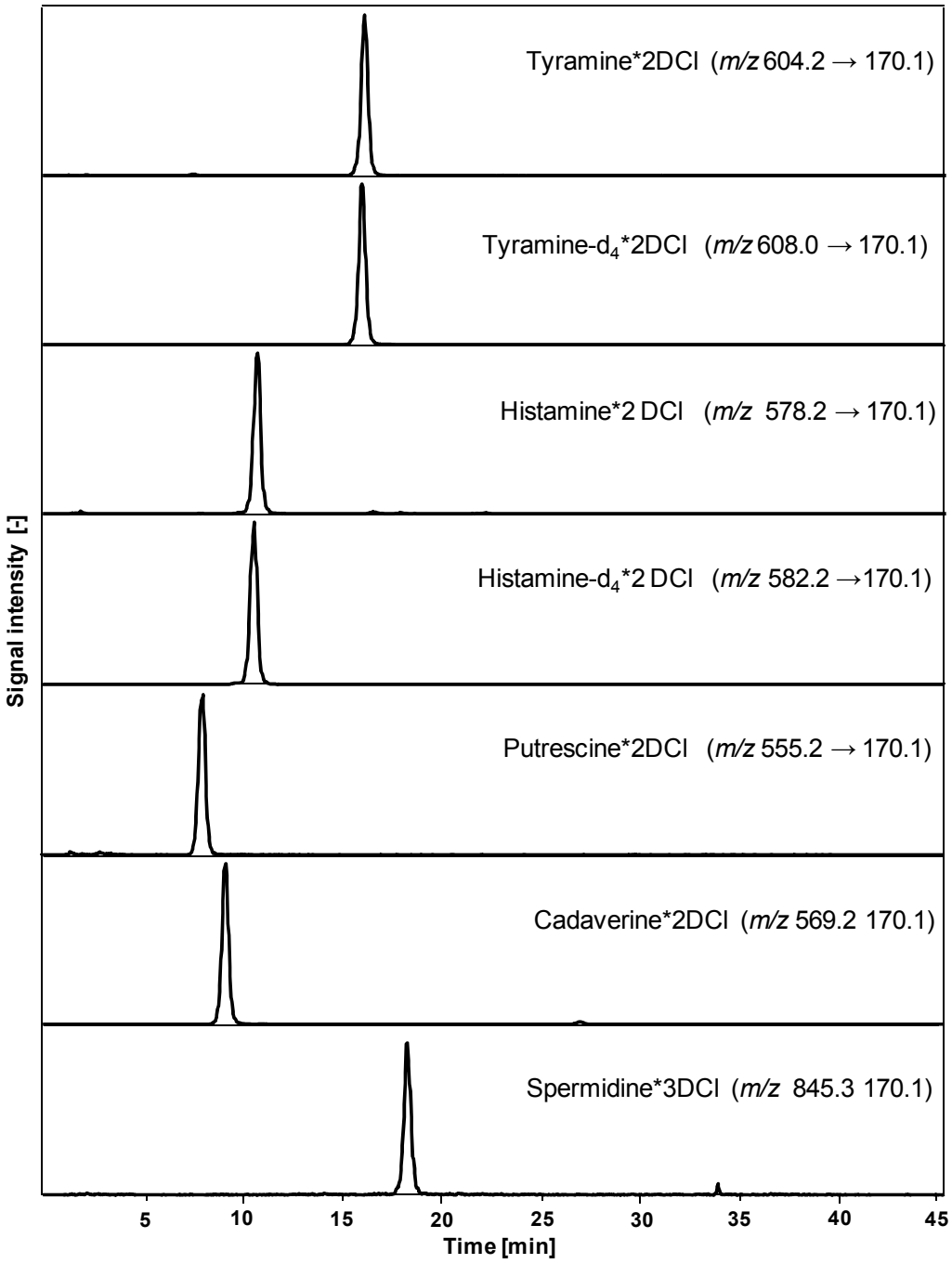
Hillmann and Hofmann, Figure 4

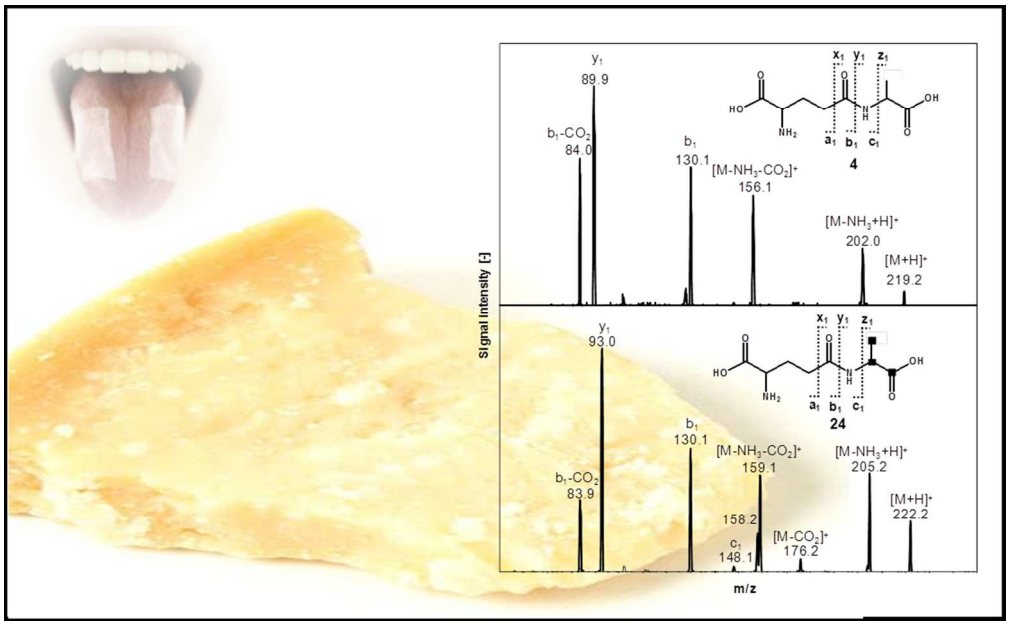


Hillmann and Hofmann, Figure 5



Hillmann and Hofmann, Figure 6





TOC
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