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Quantitative Studies and Taste Reconstitution Experiments of the Sour and Lingering Mouthful Orosensation in a Debittered Extract of Traditional Japanese Dried and Fermented Skipjack Tuna (*Hongarebushi*)

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Supporting Information

ABSTRACT: *Hongarebushi*, Japanese dried skipjack tuna and a high quality ingredient of Japanese dashi, was investigated for its taste active composition. The recent investigation focused on a debittered fish fraction, which revealed a strong umami and salt impact accompanied with a pleasant and pronounced sourness. Whereas the umami and salt tastes could be correlated to monosodium glutamate (MSG), ribonucleotides, and mineral salts, the pleasant sourness was not exclusively induced by organic acids. The essential compound imparting the sour orosensation, persistence, and mouthfulness of the debittered skipjack tuna extract was investigated, and omission experiments emphasized the impact of *N*-acetylglutamic acid (NAG) on the overall taste sensation of the debittered fish extract. This metabolite, which is known to be present as a minor constituent in animal- and plant-derived foods, was quantified in this study for the first time in seafood, soybean products, dried shiitake mushrooms, and dried fish in notable amounts. Furthermore, it was described for the first time as an essential taste contributor to the nonvolatile profile of a foodstuff, in this case of a debittered extract of hongarebushi.

KEYWORDS: *skipjack tuna, hongarebushi, N-acetylglutamic acid, sourness, umami, taste reconstitution*

INTRODUCTION

For centuries, kelp, shiitake mushrooms, and dried fish were used as essential ingredients in dashi preparation to impart an attractive aroma as well as the typical savory taste to the traditional Japanese soup stock (dashi). This flavorful broth was investigated by Ikeda, who in 1909 isolated monosodium glutamate (MSG) from kelp and described this pure tastant for the first time as umami active.[1] But it was not until the late 1990s that the presence of a fifth taste umami was confirmed scientifically by the discovery of MSG-sensitive receptors.[2] The most famous and palatable umami-rich stock is prepared from kelp and dried bonito. These two ingredients are loaded with umami compounds, and combining them in dashi synergizes their umami impact, with kelp being rich in MSG and bonito having high levels of 5′-mononucleotides.[3−6] The umami taste of MSG is in fact known to be intensely enhanced by guanosine-5′-monophosphate and inosine-5′-monophosphate,[7,8] and the synergy is a hallmark of this taste quality. This powerful combination of umami molecules contributes consequently to the attractive savory taste of a range of food, such as cured meat, aged cheese, mushrooms, fish, and seafood.

In the past decade further investigation led to the discovery of several natural umami molecules, such as creatine and creatinine in Japanese dried herring fillet,[9] (*R*)-strombine in scallop,[10] *N*-(1-desoxy-D-fructos-1-yl)-L-glutamic acid in sun-dried tomatoes,[11] theogallin in mat-cha,[12] *N*-lactoylguanosine-5′-monophosphate in bonito,[13] and (*S*)-morelid in air-dried morels.[14]

Despite the sensory knowledge of nonvolatile compounds in dried and fermented food[15,4,9] the typical taste profile and in particular the essential balance between the umami, salty, sour taste and mouthfulness of the dried skipjack tuna (*hongarebushi*) is not fully understood. To comprehend the taste impact of dried fishes in a food preparation such as a dashi soup, the key taste active compounds were quantified in a Japanese dried skipjack tuna (*hongarebushi*) with chiai. The Japanese word "chiai" describes the deep red flesh representing the blood line in the center of the filet close to the spine and it imparts a particular fish flavor dimension. *Hongarebushi*, a high-quality dashi ingredient from Japan, is produced by a four-step preservation technique including boiling, smoking, mold culturing with several molds, and sun-drying. This preservation technique is used to accumulate a spectrum of flavor molecules within the fish. Not only do enzymatic and fermentation processes take place but also the fine roasted and smoked notes from sun-drying and smoking contribute to the palatable aroma and taste of the fish.

It is known that the authentic taste profile of a food is reflected by the molecular blueprint of its taste active, extractable, and low molecular weight compounds,[15−17] and much progress has been made in recent years in the field of

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sensomics to systematically identify, map, and quantitate the sensory active key metabolites that are present in foods.[17,18]

The objective of this work was therefore to perform quantitative analysis and rank the fish tastants according to their relevance. Taste re-engineering experiments should lead to the identification of key players in the taste interaction. In the case of discovery of interesting taste compounds in the selected target, a broader screening for this molecule can give more insight in the presence of tastants in other related foodstuffs.

MATERIALS AND METHODS

Chemicals and Materials. The following chemicals were obtained commercially: L-alanine, γ-aminobutyric acid, ammonium acetate, ammonium chloride, ammonium formate, 5′-AMP*2Na, L-aspartic acid, carnithine, carnosine, creatine, diammonium hydrogenphosphate, dibasic sodium phosphate, formic acid, L-glutamic acid, 5′-GMP*2Na, guanosine, L-histidine, hypoxanthine, inosine, 5′-IMP*2Na, pentane, potassium chloride, sodium chloride, sodium hydroxide (50% in solution), and taurine (Fluka, Basel, Switzerland); *N*-acetylglutamic acid, betaine, creatinine, glycine, L-isoleucine, L-lactic acid, nicotinamide, L-ornithine, L-phenylalanine, L-serine, L-threonine, uridine, and L-valine (Sigma-Aldrich, Buchs, Switzerland); L-arginine, L-leucine, and L-tyrosine (Merck, Darmstadt, Germany); *tert*-methyl butyl ether (Biosolve, Valkenswaard, The Netherlands); and acetonitrile and methanol of HPLC grade (Acros, Geel, Belgium). Deionized water used for chromatography was purified by means of a Millipore Direct-Q 3-Q system (Millipore S.A.S, Molsheim Cedex, France). For sensory analysis tap water (low mineralization) was used and adjusted to pH 6.0 with formic acid (1% in water), which is GRAS listed as a flavoring agent for food applications. The fish, kelp, abalone, and soy sauce samples (I–VIII, X–XIII, XVI, and XVII) were purchased at a local Japanese market. The dried scallop (XI) was purchased at a local Chinese market. Soy sauce XV and dried shiitake mushroom (XIV) were bought in a Swiss supermarket. The sake, mirin, kimchi, miso, and fresh tofu samples (XVIII–XXIII) were purchased at an Asian supermarket in Switzerland.

Solvent Extraction of Fish I (Hongarebushi with Chiai). In a first step the fish flakes (20 g) were defatted by extracting once with pentane (200 mL) for 60 min at room temperature. After centrifugation (10 min, 3500 rpm), the defatted residue was extracted three times for 60 min at room temperature with methanol/water (200 mL; 70:30; v/v); between the extraction steps, the solvent was removed after centrifugation using a Heraeus Sepatech Megafuge 2.0 (10 min, 3500 rpm). The combined methanol/water extracts were freed from solvent under vacuum and lyophilized to give the methanol/water extract I (yield = 12.0%; w/w). This extract was evaluated in a taste profile analysis at a dosage of 5000 ppm in water, giving a natural pH of 6.0, and because a harsh bitter taste was perceived, a debittering strategy was applied.

Debittering of Fish Extract I. An aliquot (2.0 g) of the methanol/water extract of fish I was dissolved in water (5.0 mL) and, after filtration, separated using a medium-pressure liquid chromatography system from Sepacore Chromatography (Buechi, Flawil, Switzerland) consisting of three C-605 pumps, a C-620 type pump manager, a C-660 type fraction collector, a manual injection port equipped with a 20 mL loop, and a C-635 type UV detector. Data acquisition was performed using SepacoreControl software (version 1.0, Buechi). A ternary solvent gradient consisting of 25 mM ammonium formate in water/methanol/*tert*-methyl butyl ether was used with the Easy-VarioFlash RP-18, 25–40 μm, 40 g, 58 mL column (Merck Chimie S.A.S, Fontenay Sous Bois, France) at a flow rate of 45 mL/min. Chromatography was conducted starting with 100% ammonium formate in water for 5 min, increasing to 30% methanol within 5 min and to 90% methanol in 4 min, and finally flushing the column with methanol/*tert*-methyl butyl ether (90:10; v/v) for an additional 8 min. The effluent flow was monitored at 230 nm, and the extract was separated into a polar nonbitter fraction (yield = 92.8%; w/w) and a nonpolar bitter fraction (yield = 7.2%; w/w). The nonbitter polar fraction was freed from solvent under vacuum; the residue was suspended in water and freeze-dried twice.

High-Performance Liquid Chromatography (HPLC). For analytical HPLC, an Agilent 1100 series HPLC system consisting of a binary pump, an autosampler, a column oven (at 30 °C), an online degasser, and a diode array detector (Agilent, Waldbronn, Germany) was used. Data acquisition was performed using the software HP ChemStation (Agilent, Waldbronn, Germany).

High-Performance Ion Chromatography (HPIC). The ICS-3000 ion chromatography system (Dionex, Olten, Switzerland) consisted of two ICS-300 DP pumps (isocratic and gradient), an ICS-3000 autosampler, a DC ICS-3000 thermal compartment, and an amperometric and an electrochemical detector. System control and data acquisition were performed using Chromeleon software (version 6.7, Dionex).

High-Performance Liquid Chromatography–Mass Spectrometry (HPLC-MS/MS). The Agilent 1200 series HPLC system, consisting of a binary pump, an online degasser, a column oven (at 30 °C), and an autosampler (Agilent), was connected to an API 3200 Q-TRAP mass spectrometer (AB Sciex Instruments, Darmstadt, Germany), which was equipped with an electrospray ionization (ESI) source and operated in the positive ionization mode. The ion spray voltage was set to 3500 or 4000 V depending on the HPLC method (HILIC, 3500 V; PFP, 4000 V), and the declustering potential and the MS/MS parameters were optimized for each substance to induce fragmentation of the pseudo molecular ion $[M - H]^+$ to the corresponding target product ions after collision-induced dissociation. The dwell time for each mass transition was 150 ms, and the declustering potential (DP), the cell exit potential (CXP), and the collision energy (CE) were optimized for each substance. Quantitative analysis was performed by means of the multiple reaction monitoring (MRM) mode using the fragmentation parameters optimized prior to analysis. Data processing and integration were performed by using Analyst software version 1.5.1 (AB Sciex Instrumentsy).

For the MRM-IDA-EPI MS experiments an information-dependent acquisition (IDA) method using these MRM survey scans to confirm the presence and identity of the NAG was applied. In the case of the presence of the target analyte, a full scan enhanced product ion (EPI) spectrum of the compound was acquired, and this mass spectrum was compared to the one of the reference compound.

Sample Preparation. The entire fishes (V–VII, 1 g), previously crushed into pieces, the fish flakes (II, III, and VIII), and the dried shrimps (IX) were extracted according to the solvent extraction procedure described above for fish I including the defatting step. The methanol/water extracts were dissolved in water and filtered prior to analysis. The canned abalone (X, 250 g) was steamed for 1 h with boiling water (1 L), and the cooking water was cooled and freeze-dried. This aqueous extract was dissolved in water and filtered prior to analysis. The dried scallops (XI, 1 kg) were soaked with cold water (1 L) for 50 min. The water was discharged, and the scallops were soaked in another 2.5 L of warm water for 1 h. Finally, the mixture was cooked for 1 h and then simmered for an additional 2 h. The filtered cooking water was cooled and freeze-dried. This water extract was dissolved in water and filtered prior to analysis. The dried kelp samples (XII and XIII, 20 g) were crushed, frozen with liquid nitrogen, ground in an analytical mill, and then extracted with methanol/water (70:30; v/v, 3 × 200 mL). The solvent was removed under vacuum, and the aqueous residues were freeze-dried twice. These extracts were dissolved in water and filtered prior to analysis. The dried shiitake mushrooms (XIV, 1 g) were crushed, frozen with liquid nitrogen, ground in an analytical mill, and then homogenized with methanol/water (30:70). This sample was adjusted to a defined volume (100 mL) and filtered and diluted before analysis. The soy sauces (XV–XVII, 1 g), sake (XVIII, 500 mg), and mirin (XIX, 700 mg) were diluted with water (100 mL) and filtered before injection. The tofu samples (XX, XXI, 5 g) and kimchi (XXIII, 5 g) were cut into small pieces and homogenized with methanol/water (30:70, 100 mL). The samples were adjusted to a defined volume and filtered before analysis. The

miso paste (XXII, 1 g) was dissolved in water (100 mL) and filtered prior to analysis.

Quantitative Analysis of Basic Taste Compounds in Nonbitter Fish Fraction by Means of HPIC. A defined amount of the methanol/water extract of the nonbitter fish fraction was dissolved in deionized water, membrane filtered (0.20 μm), and used directly for the analysis of anions and cations. Aliquots (20 μL) were injected into an ICS-3000 ion chromatography system (Dionex). For quantitation, six-point external calibration curves were recorded for the anion and cation measurements.

Anions. Anions were analyzed using an IonPac AS11-HC analytical column (4 × 250 mm, Dionex) equipped with an IonPac AG11-HC guard column (4 × 50 mm, Dionex) and self-regenerating anion suppressor ASRS-Ultra II (4 mm, Dionex) operating at 223 mA with hydroxide eluent generation. Chromatography was performed at 30 °C with a flow rate of 1.5 mL/min using aqueous potassium hydroxide as solvent and starting with a concentration of 1 mM for 1 min, increasing the ion strength to 30 mM within 14 min, then to 60 mM within 10 min, and maintaining this concentration for 7 min.

Cations. Cations were analyzed using an IonPac CS12-A analytical column (4 × 250 mm, Dionex) equipped with an IonPac CG12-A guard column (4 × 50 mm, Dionex) and self-regenerating cation suppressor CSRS-Ultra II (4 mm, Dionex) operating at 88 mA with methanesulfonic acid eluent generation. Isocratic chromatography was performed at 30 °C with a flow rate of 1.5 mL/min using aqueous methanesulfonic acid with a concentration of 20 mM for 12 min.

Quantitative Analysis of L-Lactic Acid. A defined amount of the nonbitter fish fraction was dissolved in deionized water and membrane filtered (0.20 μm), and aliquots were used for determination of L-lactic acid. By means of an enzymatic test kit (R-biopharm, Darmstadt, Germany) L-lactic acid was determined following the protocol supplied by the vendor. For detection, a PerkinElmer Lambda 40 UV−vis spectrometer was used set at 340 nm.

Quantitative Analysis of Nucleotides. 5′-AMP, 5′-GMP, and 5′-IMP were analyzed using an RP-HPLC-DAD method. Therefore, a defined amount of the nonbitter fish fraction was dissolved in deionized water and membrane filtered (0.45 μm). Aliquots were injected onto an RP18 column (Zorbax Eclipse, 150 × 4.6, 5 μm, Agilent, Santa Clara, CA, USA) and separated with a gradient of methanol/acetonitrile (5:4; v/v; solvent A) and 23 mM $(NH_4)_2HPO_4$ in water (pH 6.0, solvent B). Using a flow rate of 1.0 mL/min, chromatography was started with 100% B; in 25 min the content of A was increased to 30% and maintained for 15 min at that solvent ratio. Detection was performed by means of a DAD set at 254 nm. For quantification six-point external calibration curves were recorded.

Quantitative Analysis of Amino Acids. Amino acids analysis was conducted via an amino acid analyzer (L-8900, Hitachi, Pleasanton, CA, USA) with postcolumn derivatization using ninhydrin. For the analyses the sample (nonbitter fish fraction) was dissolved in 0.02 N hydrochloric acid and filtered (0.45 μm) prior to injection. The analyses were carried out on an AAA Hi Speed PF column, 6 × 60 mm, 3 μm resin (Hitachi) equipped with a guard column AN0-9256. Using a gradient of AAA physiological fluids buffer (Hitachi) and 5% ethanol in water, the amino acids were separated and reacted at 135 °C to their aldehydes. The reaction was monitored at 440 and 570 nm using a diode array detector. For quantitation six-point external calibration curves were recorded.

Quantitative Analysis of Other Taste Active Compounds. To quantitate some additional taste compounds, two LC-MS/MS multimethods based on two orthogonal HPLC columns (PFP-RP18 and ZIC-HILIC) were developed. A defined amount of the nonbitter fish fraction was dissolved in deionized water and membrane filtered (0.20 μm). Aliquots were analyzed on a SeQuant ZIC-HILIC column (150 × 4.6 mm, 5 μm, SeQuant, Umeå, Sweden) and on a Phenomenex Luna PFP column (250 × 4.6 mm, 3 μm, Phenomenex, Aschaffenburg, Germany) via MS/MS. In both cases the target compounds were analyzed by means of HPLC-MS/MS operating in the MRM with positive electrospray ionization.

HILIC Measurements. Using a flow rate of 0.8 mL/min, chromatography was performed starting with 20% 10 mM NH_4HCO_3 in water (pH 6.3, solvent A) and 80% acetonitrile (solvent B) for 10 min, then decreasing the content of B to 55% within 15 min, and maintaining at this concentration for 5 min. The following compounds were analyzed using the mass transitions given in parentheses: betaine (m/z 118.1→58.1), carnithine (m/z 162.2→103.1), creatine (m/z 132.1→90.1), creatinine (m/z 114.1→44.1), guanosine (m/z 284.1→135.1), inosine (m/z 269.1→137.1), and uridine (m/z 245.1→113.0). To enable the quantitation of the given compounds six-point external calibration curves were recorded.

PFP Measurements. Using a flow rate of 0.5 mL/min, chromatography was performed starting with 95% water (0.1% formic acid, solvent A) and 5% acetonitrile (0.1% formic acid, solvent B) for 5 min, then increasing the content of B to 15% within 7.5 min, rising within 5 min to 95% B, and maintaining at this concentration for 10 min. The following compounds were analyzed using the mass transitions given in parentheses: carnosine (m/z 227.2→110.2), hypoxanthine (m/z 137.1→110.1), nicotinamide (m/z 123.1→80.0), and taurine (m/z 126.1→108.1). For quantitation of the given compounds six-point external calibration curves were recorded.

Quantitative Analysis of *N*-Acetylglutamic acid (NAG). To quantitate NAG in selected samples, the HILIC method described above was applied analyzing the target compound using the mass transitions given in parentheses: NAG (m/z 190.1→130.2). A six-point external calibration curve was recorded for quantitation of NAG, and detection and quantitation limits were determined.

Sensory Analysis. *General Conditions.* The sensory panel consisted of 8−10 assessors (depending on the sensory test) with no history of known taste disorders (5 women and 5, ages 23−57 years). The panel was trained with reference solutions for the basic taste attributes sweet, salty, sour, bitter, umami, and, furthermore, astringent and lingering/mouthfeel. To prevent cross-modal interactions with odorants, the panelists used nose clips. For the training of the individual gustatory modalities, the panelists participated in sensory training session with purified compounds, as reported in detail in the literature.[20] The following compounds were dissolved in water (pH 6.0) and used in these sessions: sucrose (50 mmol/L) for sweet taste, lactic acid (20 mmol/L) for sour taste, NaCl (20 mmol/L) for salty taste, caffeine (1 mmol/L) for bitter taste, MSG (3 mmol/L) for umami taste, tannic acid (0.05%) for astringency, and a solution of reduced glutathione (5 mmol/L) in sodium chloride (30 mmol/L) and L-glutamic acid (10 mmol/L) for lingering/mouthfeel.

Recognition Threshold Determination. Ten panelists determined the threshold concentrations of the commercially purchased pure compounds in tap water (pH 6.0) using a duo test with ascending concentrations of the stimulus, as reported in detail in the literature.[19] The panelists were asked to distribute the solution in the oral cavity for 10 s prior to expectoration. The individual threshold concentration of each panelist is calculated as the geometric mean between the last incorrect and the first correctly identified sample solution. The bitter recognition threshold of the panel was calculated from the geometric means of all individual threshold concentrations.

Comparative Taste Profile Analysis. The total fish extract was dosed at 5000 ppm in water for sensory assessment. The debittered fish extract I was dissolved in water at 4640 ppm, taking the yield of the debittering step into consideration. The tasting solution was presented to the trained sensory panel of eight panelists, and the intensities of the descriptors bitter, sweet, sour, salty, astringent, umami, and lingering/mouthfeel were rated on a structured scale from 0 (not detectable) to 5 (intensely perceived).

Re-engineering of the Nonvolatile Fingerprint of Fish I. To reconstitute the nonvolatile fingerprint of the fish extract I, the "natural" concentrations of tastants (Table 1) were dissolved in tap water. The taste profile of the debittered fish recombinant was compared to those of the original debittered fish extract I.

Taste Omission Experiment. A reconstitution sample, with NAG, was used as a reference for a duo test. The panel was asked to evaluate the taste profile of the full reconstitution with NAG (control) and without this compound.

Table 1. Taste Qualities, Taste Thresholds, Concentrations, and Dose-over-Threshold (DoT) Factors of Nonvolatile Taste Active Compounds in Dried Japanese Skipjack Tuna (Hongarebushi) with Chiai

taste compound	TC[a] (ppm)	concentration[b] (ppm)	DoT[c]
Group I: Bitter-Tasting Compounds			
L-histidine	6171 [d]	10771	1.7
nicotinamide	275	265	1.0
inosine	5365 [e]	1614	0.3
creatinine	2036 [e]	494	0.2
L-leucine	1278 [d]	145	0.1
creatine	11146 [e]	48	<0.1
L-isoleucine	1132 [d]	36	<0.1
L-lysine	10254 [d]	253	<0.1
L-valine	2974 [d]	133	<0.1
L-phenylalanine	6623 [d]	96	<0.1
L-arginine	11714 [d]	12	<0.1
hypoxanthine	5989 [e]	289	<0.1
taurine	18773 [e]	843	<0.1
Group II: Sweet-Tasting Compounds			
glycine	1426 [d]	96	0.1
L-ornithine	463 [e]	24	0.1
L-threonine	3539 [d]	120	<0.1
betaine	4680 [f]	229	<0.1
Group III: Umami-Tasting Compounds			
5′-IMP	871 [e]	3506	4.0
L-glutamic acid	142 [d]	157	1.1
5′-GMP	125 [g]	72	0.6
L-aspartic acid	69 [d]	36	0.5
5′-AMP	1389 [e]	301	0.2
carnosine	514 [e]	108	<0.1
Group IV: Salty-Tasting Compounds			
ammonium[d]	90 [h]	10843	120.2
sodium[d]	90 [h]	4096	45.7
chloride[e]	138 [h]	5301	38.3
potassium[d]	493 [h]	5060	10.3
phosphate[e]	712 [h]	6867	9.6
Group V: Sour-Tasting Compounds			
L-lactic acid	1394 [h]	17505	12.6
N-acetylglutamic acid	70	95	1.4
Group VI: Astringent-Tasting Compounds			
uridine	6	36	6.3

[a]Taste threshold concentrations (TC) were determined in water (pH 6.0) by means of a duo test or are taken from the literature. Values are taken from the literature: [d] 18; [e] 21; [f] 26; [g] 27; [h] 1717. [b]Concentration in Japanese skipjack tuna (hongarebushi) with chiai. [c]DoT factor is calculated as the ratio of concentration and taste threshold. [d]Taste threshold and DoT factor determined for the corresponding chloride salt. . [e]Taste threshold and DoT factor determined for the corresponding sodium salt.

RESULTS AND DISCUSSION

To identity the key compounds that impart the typical taste sensation of dried and fermented Japanese skipjack tuna flakes (*hongarebushi*, fish I), the flakes were extracted and the freeze-dried extract was dissolved in water (pH 6.0, 5000 ppm) to determine the intrinsic taste profile. Because an intense bitter off-note was perceived influencing the perception of other taste attributes such as saltiness or umami taste (data not shown), a debittering protocol was developed. By means of a medium-pressure liquid chromatography system, the extract of the skipjack tuna (fish I) was separated on a preparative RP18 column giving a polar nonbitter fraction and a bitter nonpolar fraction The sensory evaluation of the nonbitter fraction (debittered extract) revealed a decrease of the bitterness and a clearly pleasant savory, mouthfeel sensation accompanied with a clear mild sourness (Figure 1A). This palatable sourness is familiar for several savory foods such as meat or fermented fishes. Recently, β-alanylglycine, β-alanyl-*N*-methyl-L-histidine, and β-alanyl-L-histidine were reported as key contributors to the thick−sour orosensation of chicken broth,[20] whereas *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids induce the pleasant thick−sourness in stewed beef juice.[21]

To discover molecules dominating the taste of the selected dried and fermented, debittered skipjack tuna extract, the concentrations of amino acids, anions, cations, L-lactic acids, 5′-nucleotides, and further fish-derived taste active molecules such as creatine, creatinine,[9] and nicotinamide[22] were measured using a set of analytical methods. In our research program for taste-modulating savory compounds NAG has been discovered as a potential tastant and was therefore included in the routine analysis via HILIC-HPLC-MS/MS.

Concentrations and Dose-over-Threshold (DoT) Factors of Taste Compounds in Debittered Skipjack Tuna Extract. In the nonbitter fish fraction 31 putative taste active compounds were identified. In addition, the taste threshold concentrations of these compounds were determined or taken from the literature to calculate the DoT factors for each compound as the ratio of the concentration and the threshold.[23]

In the group of bitter compounds (group I), mainly L-histidine was quantified in a notable amount of 10771 ppm, resulting in a DoT factor of 1.7 (Table 1). Inosine was also found in high concentrations (1614 ppm), but due to the high threshold concentration (TC) (5365 ppm), the resulting DoT value was below 1.0. All other bitter compounds were present in the nonbitter fish fraction in concentrations below 500 ppm (Table 1). Besides L-histidine, only nicotinamide was found to contribute to the bitter taste of the nonbitter fish fraction, as this compound possessed a strong bitter taste (TC 275 ppm) to give a DoT of 1.0.

Group II contained all sweet compounds such as glycine with concentrations between 463 and 4680 ppm (Table 1). Generally in the tasting of debittered fish fraction I only a weak sweetness was perceived. This fact was emphasized with a low concentration of sweet molecules and the correspondingly low DoT factors in the range of $\leq$0.1.

The debittered fish fraction exhibited a strong umami taste, which is mainly induced by compounds such as L-glutamic acid in combination with 5′-nucleotides. These compounds are summarized in group III. Overall concentrations of 36−3506 ppm were quantified, and only 5′-IMP and L-glutamic acid showed DoT values above 1.0 (Table 1). All other compounds (5′-GMP, 5′-AMP, L-aspartic acid, and carnosine) had DoT values below 0.6. It is known from the literature that 5′-nucleotides and L-amino acids such glutamic and aspartic acid have synergistic effects enhancing the overall umami perception.[6,7] Certainly this taste-enhancing effect could elicit the intense umami taste of the nonbitter fish fraction.

The key contributors to the saltiness (group IV) of the nonbitter fish fraction are the anions chloride and phosphate in combination with the cations ammonium, sodium, and potassium with high DoT values of >9 (Table 1). Outstanding are high concentrations of ammonium (10843 ppm) and

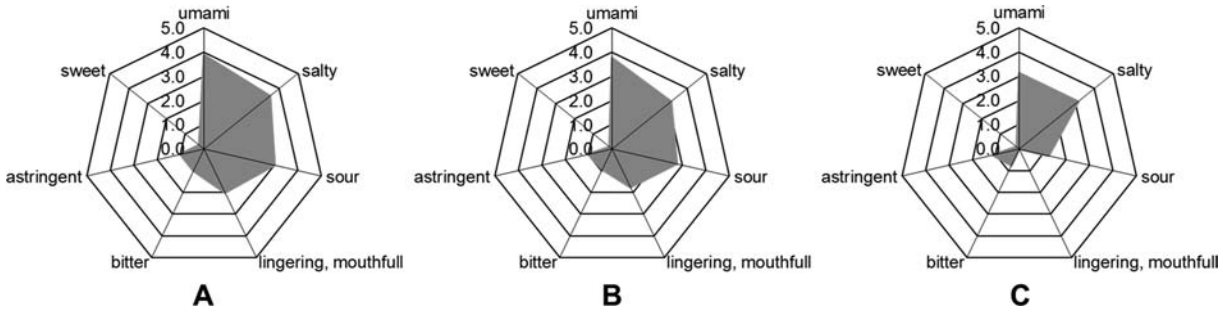

Figure 1. Comparison of taste profiles of (A) debittered fish extract I, (B) recombinant, and (C) omission experiment showing the influence of omission of *N*-acetylglutamic acid on the sourness and saltiness of fish taste.

phosphate (6867 ppm), whereas potassium and sodium have concentrations (≤5100 ppm).

Only two sour-tasting compounds (group V) were determined in the debittered fish with high levels of lactic acid with 17505 ppm and comparable lower concentrations of NAG (95 ppm), respectively (Table 1). Lactic acid has a DoT value of 12.6, whereas NAG exhibited in water a very low perception threshold with 70 ppm and should therefore contribute according to the DoT concept as well to the overall sourness of the fish.

The only astringent compound (group VI) present in the nonbitter fish fraction was uridine, with a concentration of 36 ppm and a DoT value of 6.3.

Re-engineering of the Nonvolatile Fingerprint of Debittered Skipjack Tuna Extract. To confirm the results of the instrumental quantitative analyses and to demonstrate that the compounds determined can create the typical taste sensation of the fish, a taste recombinant was prepared and compared to the original fish extract.

An aqueous solution of all 31 quantified tastants (Table 1) was prepared, and the sensory panel was asked to evaluate the taste profile of this recombinant in direct comparison to the solution of nonbitter fish fraction by scoring the taste descriptors umami, salty, sour, sweet, bitter, astringent, and lingering/mouthful on a structured linearscale from 0 (not detectable) to 5 (strong taste sensation). The original nonbitter fish fraction was evaluated as strong umami (3.9) followed by a distinct saltiness (3.5), sourness (3.0), and a long-lasting lingering and mouthfulness (2.0). The bitter taste and astringency were ranked with intensities of 1.0, whereas the sweetness was very weak (0.3) (Figure 1A; Table 2). In comparison, the recombinant was scored with slightly lower intensities of umami taste (3.9vs 3.8), saltiness (3.5 vs 3.1), sourness (3.0 vs 2.8), and lastingness/mouthfulness (2.0 vs 1.8). The bitterness (1.0 vs 0.9) and astringency (both 1.0) were ranked comparable as well as the sweetness (0.3 vs 0.1) (Figure 1B; Table 2). As the taste profile of the recombinant was very close to that of the original fish, the panel concluded that the aqueous blend of 31 compounds succeeded in mimicking the typical taste of a debittered skipjack tuna extract.

Taste Omission Experiment. To investigate the remarkable and typical sour taste of the dried and fermented, debittered skipjack tuna, a simple omission experiment was performed comparing the full recombinant (Figure 1B; Table 2) with a recombinant excluding NAG (Figure 1C; Table 2). This compound had an extremely low threshold for sourness (70 ppm, Table 1), and by removing this molecule from the taste solution, the sourness decreased from 2.8 to 1.3 accompanied with minor decreases in the saltiness (3.1 vs 2.9) and umami impact (3.8 vs 3.1) and the entire loss of the lingering/mouthful taste (1.8 vs 0.0). This experiment shows clearly the impact of this molecule on the sourness of the debittered skipjack tuna (fish I) and its necessity for the overall savory sensation and especially the lingering mouthfulness.

Table 2. Taste Profile Analysis of Debittered Fish Extract I, Recombinant, and Omission Experiment

	intensities for individual taste qualities[a]		
taste descriptor	debittered fish extract I	recombinant[b]	omission experiment[c]
umami	3.9 (±0.4)	3.8 (±0.5)	3.1 (±0.4)
salty	3.5 (±0.5)	3.1 (±0.4)	2.9 (±0.4)
sour	3.0 (±0.8)	2.8 (±0.5)	1.3 (±0.5)
lingering, mouthful	2.0 (±0.5)	1.8 (±0.5)	0.0 (±0.0)
bitter	1.0 (±0.5)	0.9 (±0.7)	0.8 (±0.5)
astringent	1.0 (±0.5)	1.0 (±0.5)	1.1 (±0.4)
sweet	0.3 (±0.5)	0.1 (±0.4)	0.1 (±0.4)

[a]The intensity of the individual taste qualities was rated on a structured scale from 0 (not detectable) to 5 (intensely perceived). The standard deviations are given in parentheses. [b]The recombinant contained the tastant groups I–VI in the concentrations given in Table 1. [c]The omission experiment solution was prepared by omitting *N*-acetylglutamic acid from recombinant.

Identification of NAG in Food. NAG was described in the literature as a substance of content in a set of foodstuffs such as soybean, maize, grain, coffee, tea, and meat, but it was not described to be present in fermented foods associated with Asian cuisine.[25] To investigate the occurrence of NAG in Asian foods by means of HPLC-MS/MS operating in the MRM mode, characteristic mass transitions were selected and instrument settings such as declustering potential, collision energy, and cell exit potential were optimized for the target compound in tuning experiments. Furthermore, an MRM-IDA-EPI MS approach was used to confirm the identity of the target molecule in the samples. The unique MRM transitions of NAG were monitored and followed by an information-dependent acquisition (IDA) strategy based on the MRM as survey scan. If the analyte was detected, a full scan enhanced product ion (EPI) spectrum of the compound was acquired. The resulting mass spectrum was compared to those obtained for the reference compound for confirmation. In Figure S1 (Supporting Information) exemplarily the MS spectra of fish I is compared to that of the reference substance. Identical fragmentation pattern and almost equal intensities of the fragments lead to the unequivocal identification of NAG in fish I. These MS experiments were conducted for all samples, representing a selection of 23 food samples including 11

fermented and nonfermented seafood samples, 4 vegetable samples, 6 soy-derived samples, and 2 rice wines. Using the MS techniques described above, NAG was determined in 7 of the 23 samples screened (Table 3).

Table 3. Concentrations of *N*-Acetylglutamic Acid in Asian Samples I–XXIII

sample no.	sample name	concentration (ppm)
DL[a] (ppm)		0.0015
QL[a] (ppm)		0.0046
I	skipjack tuna (hongarebushi) with chiai[b]	95
II	skipjack tuna (hongarebushi) without chiai[b]	4
III	skipjack tuna (yatakuch) with chiai[b]	nd[e]
IV	frigate tuna (soda) with chiai[b]	nd
V	horse mackerel (muroaji) with chiai[b]	nd
VI	white anchovy (yatakuchi iwashi)[c]	nd
VII	black anchovy (yatakuchi iwashi)[c]	nd
VIII	flying fish (ago)[c]	nd
IX	dried shrimp	nd
X	abalone[d]	nd
XI	dried scallop	nd
XII	kelp (rishiri konbu)	nd
XIII	kelp (rishiri konbu, kuragakoi)	nd
XIV	dried shiitake mushroom	127
XV	soy sauce 1	13
XVI	soy sauce 2 (royal)	26
XVII	soy sauce 3 (supreme)	7
XVIII	sake	nd
XIX	mirin	nd
XX	tofu 1	nd
XXI	tofu 2	1
XXII	miso	nd
XXIII	kimchi	nd

[a]Limits of detection (DL) and quantitation (QL) were determined in fish containing no natural *N*-acetylglutamic acid in spiking experiments according to the signal-to-noise ratio of 1:3 and 1:5, respectively. [b]Fish was purchased as dry petal shavings. [c]Entire dried fish was purchased. [d]Abalone were purchased canned. [e]nd, not detected (concentration ≤ 0.00015 ppm).

Quantitation of NAG in Food. To check the performance of the existing HPLC-MS/MS method, detection limits and quantitation limits of the target molecule were determined in a 1000 ppm fish solution (fish VIII), which contained, according to the previous identifications experiment, no NAG. The detection and quantitation limits were determined by analyzing the prevailing signal-to-noise ratio (DL, 1:3; QL, 1:5) for the individual MRM chromatograms. Using the HPLC-MS/MS technique developed, a very low detection limit of 0.0015 ppm and a quantitation limit of 0.0046 ppm were established in the fish matrix (Table 3). Furthermore, the occurrence of matrix effects was investigated and excluded, to prove the suitability of the chosen external calibration.

By applying the HPLC-MS/MS method, levels between 7 and 26 ppm were quantified in the soy sauces (XV–XVII) and in one of the tofu samples (XXI) the tastant was detected in very low concentrations (1 ppm). In the seafood samples (I–XI), NAG was found in quantifiable concentrations only in two different hongarebushi fishes (I, 95 ppm; II, 4 ppm). Overall, the highest amounts were measured in dried shiitake mushrooms with 127 ppm (Table 3). These values correlate with previous research in which levels from 1 to 60 ppm were determined in a range of foodstuffs.[24,25]

On the basis of the data obtained, it might be concluded that besides the well-known umami enhancement inducing blend of glutamic/aspartic acid in combination with 5′-nucleotides, NAG is a key contributor not only to the sourness but also to the overall savory balanced profile as well as to the lingering/mouthfeel sensation in a nonbitter fraction of traditional Japanese dried and fermented skipjack tuna (*hongarebushi*). NAG was described for the first time to be taste-active and furthermore to possess taste-modulating properties. Quantitative studies revealed its presence also in soy sauce and in dried shiitake mushroom, indicating that NAG might also contribute to the authentic nonvolatile fingerprint of other foods.

■ ASSOCIATED CONTENT

Supporting Information

Figure 1S. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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