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LC-MS Study To Reduce Ion Suppression and To Identify *N*-Lactoylguanosine 5′-Monophosphate in Bonito: A New Umami Molecule?

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In this study a specific taste modulating flavor ingredient, *N*-lactoylguanosine 5′-monophosphate (*N*-lactoyl GMP), was determined in bonito (Japanese, Katsuobushi, dried fermented skipjack) and in powdered bonito using liquid chromatography−electrospray ionization (+) mass spectrometry−mass spectrometry (LC−ESI(+)-MS/MS) with a methanol/ammonium acetate or formate gradient. Furthermore, the influence of ion suppression due to sample matrix effect was investigated and was found to substantially influence the total MS response of *N*-lactoyl GMP; by adjusting the LC conditions the response could be approximately 5-fold-enhanced. The *N*-lactoyl GMP concentrations in different types of bonito products were between 0.2 and 2.4 μg/g.

KEYWORDS: Mass spectrometry; bonito; *N*-lactoylguanosine 5′-monophosphate; flavor; liquid chromatography; umami; triple quadrupole; ion suppression

INTRODUCTION

Next to the four commonly known basic tastes sweet, salty, bitter, and sour there is a lesser-known fifth basic taste—umami. Umami is derived from the Japanese word umai—delicious—and is characterized as savory, brothy, and meaty. Ikeda first described the principle of umami in 1909; a translation of this work was published in 2002 (*1*). Ikeda investigated konbu, a dried seaweed, used together with flaked bonito (Japanese, Katsuobushi, dried fermented skipjack) to make dashi, the flavorful broth that is used in many Japanese recipes. It was observed that next to konbu and dashi there were many foods such as asparagus, tomatoes, cheese, and meat that had a common taste different from the known basic tastes. Ikeda isolated this flavor in the broth by extracting crystals of glutamic acid, which had a distinctive character that was named umami.

Glutamic acid, or glutamate, is the most abundant amino acid in nature; it is a key molecule in cellular metabolism and exists in both the free form as monosodium glutamate (MSG), in plant and animal tissues, and in a bound form in proteins. Free glutamate plays a role in palatability and is naturally present in a number of foods, including mushrooms, aged cheese, tomatoes, and meats, fish, and poultry. Natural processes, such as ripening, drying, curing, aging, and fermenting, concentrate the umami flavor by breaking down the proteins into free amino acids, including glutamic acid (*2*).

It was not until the late 1990s, that the presence of a fifth taste was confirmed by the discovery of taste receptors for MSG (*3*−*5*). Next to amino acids, umami is also provided by IMP (inosine monophosphate) and GMP (guanosine monophosphate), acids naturally present in many protein-rich foods. Generally speaking, IMP is found primarily in meat and other animal proteins, whereas GMP is more abundant in plants. The synergizing effect between MSG, IMP, and GMP produces a strong umami taste in certain concentrations. Although the exact mechanism for this synergy is not yet known, it was found that using a 50/50 blend of MSG and IMP, for example, could result in an 8-fold increase in the umami-enhancing effect (*6*).

In our research program for taste-active compounds *N*-lactoyl GMP has been shown to provide a long-lasting umami effect. Currently we are performing a sensory study to further investigate this effect, of which the results will be published in the near future. In the current study the presence of *N*-lactoyl GMP was investigated in typical umami products: bonito (in normal (whole), powdered, flaked, and broth form), Nam Plaa fish sauce, and fresh skipjack. The bonito products were selected for their content of the precursors GMP and lactic acid (*7*, *8*), and because they are fermented, during the process *N*-lactoyl GMP may be formed. Skipjack, the starting material for bonito preparation, was selected for comparison; since it is not fermented, it does not contain any lactic acid. The fish sauces were analyzed because of their strong umami taste. The isolation

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and concentration of *N*-lactoyl GMP was done by preparative liquid chromatography (LC). Liquid chromatography−electrospray ionization (+) mass spectrometry (LC−ESI(+)-MS) in selected reaction monitoring (SRM) mode of three characteristic transitions was used for its determination in the individual samples.

Targeted analysis in complex matrices is often challenged by the interference between the analyte and the matrix. In LC−MS, one of the most common cases of loss in signal is caused by ion suppression. Ion suppression can occur when a coeluted compound suppresses the ionization of the target molecules in the MS source. Ion suppression can sometimes be eliminated by improved sample preparation, but generally it is easier to adjust the chromatographic conditions so the peaks of interest are not eluting in the suppression region (*9*). To gain maximum sensitivity for the identification of *N*-lactoyl GMP, the MS signal should be as high as possible. Therefore, an experiment was designed to test whether ion suppression might play a role under the conditions used, and the original chromatographic conditions were reviewed to maximize sensitivity.

MATERIALS AND METHODS

Chemicals. Prepsolv and lichrosolv methanol, guanosine, pyridine, chlorotrimethylsilane, trimethylphosphate, formic acid, and acetic acid were purchased from Merck (Darmstadt, Germany); ammonium acetate, ammonium formate, lactic acid, phophorylchloride, and guanosine 5′-monophosphate were from Sigma−Aldrich (Steinheim, Germany); and charcoal (Norit Rox 0.8) was obtained from Norit (Amersfoort, The Netherlands). Water for preparative HPLC (0.5 MΩ) was prepared with a Millipore-DirectQ system (Etten-Leur, The Netherlands). 5-Methyl-1,3-dioxolane-2,4-dione was prepared as described in ref *10*.

Preparative LC Isolation and Characterization of *N*-Lactoylguanosine 5′-Monophosphate. *Reaction.* A 1000 g amount of lactic acid and 250 g of GMP were mixed together in a flask and heated in a reaction block to 100 °C for 4 h while being stirred. The reaction mixture was cooled to 60 °C, 4500 g of ethyl acetate was added, and the mixture was stirred for 1 h. The precipitate was washed with 1000 mL of ethyl acetate, filtered (filtrate was discarded), and dried in a vacuum oven. The obtained reaction product was subjected to preparative LC fractionation (see next paragraph).

Preparative LC Fractionation. For preparative HPLC an Amersham Biosciences (Piscataway, NJ) BioPilot prep station equipped with a UV detector and controlled by UNICORN software was used. The separation was carried out on a Source 30 RPC 135 × 50 mm i.d., 20 μm column (Amersham Biosciences). A sample load of 20 mL/run was achieved. The system was operated at a flow rate of 150 cm/h; taking the column dimensions into account, this corresponds to 294 mL/h. The solvents used for separation were (A) 1% formic acid in water and (B) methanol. After an isocratic step of 100% A for 15 min, a linear gradient from 0 to 15% B in 30 min was followed by a 10 min regeneration step of 15−50% B and held for 12 min; subsequently, the percentage of B was lowered to 0% in 6 min, and the column was re-equilibrated at 100% A for 6 min. The sample was injected 200 times. Fractions were collected by time slots. Each fraction was freeze-dried, redissolved in water, and organoleptically evaluated by our flavorists. The fraction collected in the time range between 45.5 and 46.5 min showed a strong umami character. This fraction was freeze-dried. By use of a pure *N*-lactoyl GMP reference sample (for synthesis, see the next paragraph), this molecule was identified as the active umami principle in the fraction by LC−MS (data not shown).

Preparation of N-Lactoylguanosine 5′-Monophosphate. A 33 g amount of chlorotrimethylsilane (9 equiv) was added slowly at room temperature to a mixture of 9.9 g of guanosine (35 mmol) in 150 mL of pyridine and 600 mL of methylene chloride. The mixture was stirred for 2 h until it was clear. A solution of 5 g (43 mmol) of 5-methyl-1,3-dioxolane-2,4-dione (as prepared in ref *10*) in 60 mL of tetrahydrofuran was added slowly at 10−15 °C. The mixture was stirred overnight, while the reaction flask was cooled by ice water. Methanol (150 mL) was added, and the solution was allowed to stand for another night. After evaporation of the solvents, the semisolid residue was taken up in methanol and the solids were filtered and dried at 60 °C/(20 mbar) to yield 3 g of product. Subsequently 1.8 g (11.8 mmol) of phosphoryl chloride was mixed with 7 mL of trimethyl phosphate and cooled to −10 °C under nitrogen. While stirring, 1.8 g of the product was added to this solution in small portions. The mixture was stirred for 3 h at −5 °C. The reaction was quenched by pouring the mixture in 300 mL of ice water, and the pH was adjusted to 2 with sodium hydroxide solution. The solution was percolated over a column (diameter = 3 cm) of activated charcoal (30 g) and washed with water, and the product was collected by elution with 200 mL of a 50:48:2 ethanol−water−28% NH_4OH solution. This solution was evaporated to dryness. The semisolid was taken up in 20 mL of methanol, and the solids were filtered and dried at 80 °C/(10 mbar) to yield 0.8 g of *N*-lactoyl guanosine monophosphate. Its identity was confirmed by NMR; the purity was >95%.

NMR. The NMR spectra were measured on a JEOL (Tokyo, Japan) Eclipse 600 NMR spectrometer operating at 600 and 150 MHz for ^1H and ^{13}C, respectively. The instrument is equipped with a three-channel 5 mm autotune Z-gradient NMR probe. D_2O (99.9% D) was used as a solvent, trimethylsilyl-d_4 propionate (sodium salt) as chemical shift reference material, both purchased at Buchem B. V. (Apeldoorn, The Netherlands). ^1H NMR spectra were recorded with 32 768 points covering a frequency range of 9.0 kHz and an acquisition time of 3.6 s and a relaxation delay time of 1 s. ^{13}C NMR spectra were recorded covering a frequency range of 47.3 kHz with an acquisition time of 0.69 s and a relaxation delay of 2 s. A set of 32 768 data points was registered. The obtained synthesis product was identified as the *N*-lactoylguanosine 5′-monophosphate. ^1H and ^{13}C NMR spectra including signal assignments are depicted in **Figure 1**.

Sample Preparation. *Bonito (Dried Fermented Skipjack*). A 2.5 g amount of grated bonito was extracted with 20 mL of a 1:1 methanol−water mixture. The volume of the extract was reduced to 1 mL on a rotation vaporizer under reduced pressure at 40 °C and filtered over a 0.45 μm filter prior to injection onto the LC−MS system.

Bonito Flakes. Three different types of bonito flakes (or katsuo-bushi) were used: (1) 100 g packs of thick-sliced bonito (used for, e.g., dashi) (of a major brand sold in Japan), (2) 40 g packs of thin-sliced bonito (which is the most common form of bonito and mainly used as topping for certain dishes, from a major brand sold in Japan), and (3) 5 × 10 g packs of thin sliced bonito from a less common Japanese brand. Extracts were prepared from 42.85 (type 1), 42.42 (type 2), and 35.12 (type 3) g bonito flakes, with 500 mL 1:1 methanol−water. They were stirred for 1 h and filtered over a Buchner funnel. The extracts were dried on a rotation vaporizer under reduced pressure at 40 °C and re-dissolved in 15 mL of water. The extracts were filtered over a 0.45 μm filter prior to injection.

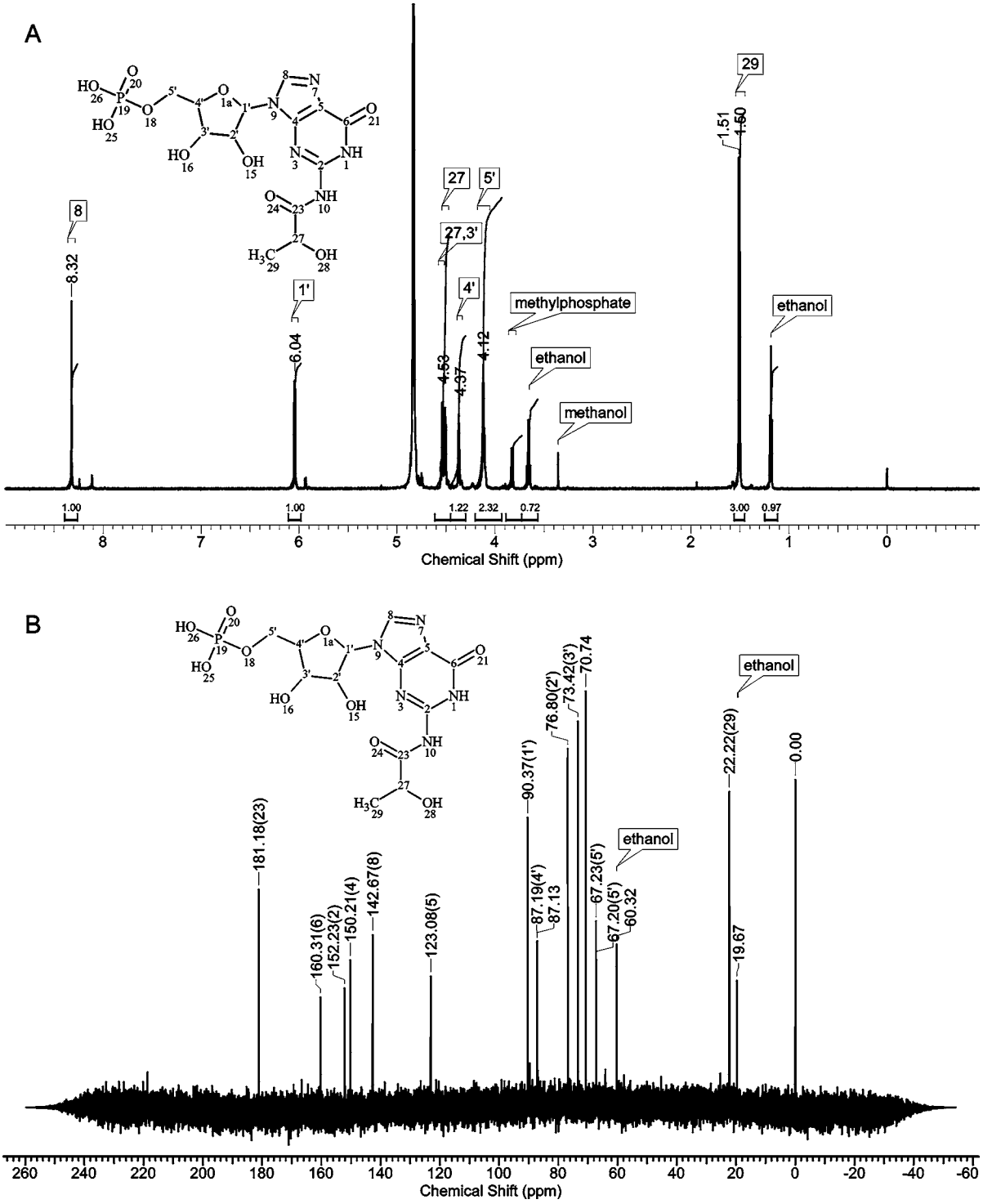

Figure 1. ^{1}H (**A**) and ^{13}C NMR (**B**) spectra of *N*-lactoyl GMP including signal assignments.

Bonito Powder. Three different types of bonito powder were used: (1) bonito powder (Givaudan, Naarden, The Netherlands) from dried skipjack (Katsuo Plamis), which has been produced from katsuo-bushi, (2) a spray-dried bonito extract powder made of finely ground 100% bonito extract (type 1600 Nikken foods, St. Louis, MO), and (3) a second spray-dried bonito extract powder made of finely ground 100% bonito extract (type 1601 Nikken foods). Extracts were prepared from 2.03 (type 1), 2.04 (type 2), and 2.03 (type 3) g bonito powder with 20 mL of 1:1 methanol−water, ultrasonicated for 10 min, and filtered over a buchner funnel. The extracts were filtered over a 0.45 μm filter prior to injection.

Fresh Skipjack. A 441.5 g amount of skipjack was cut into small cubes and ground in a food processor. A 1 L aliquot of 1:1 methanol−water was added, and it was stirred for 3 h. The mixture was filtered with a Buchner funnel, and the extract was evaporated to dryness with a rotation vaporizer under reduced pressure at 40 °C. The residue was re-dissolved in 100 mL of 1:1 methanol−water and transferred to a separatory funnel. A 25 mL aliquot of ethyl acetate was added, and the extract was thoroughly shaken for 5 min. The mixture was left standing for 30 min, and the ethyl acetate layer was separated from the water phase and discarded. The water phase was centrifuged at 10 000

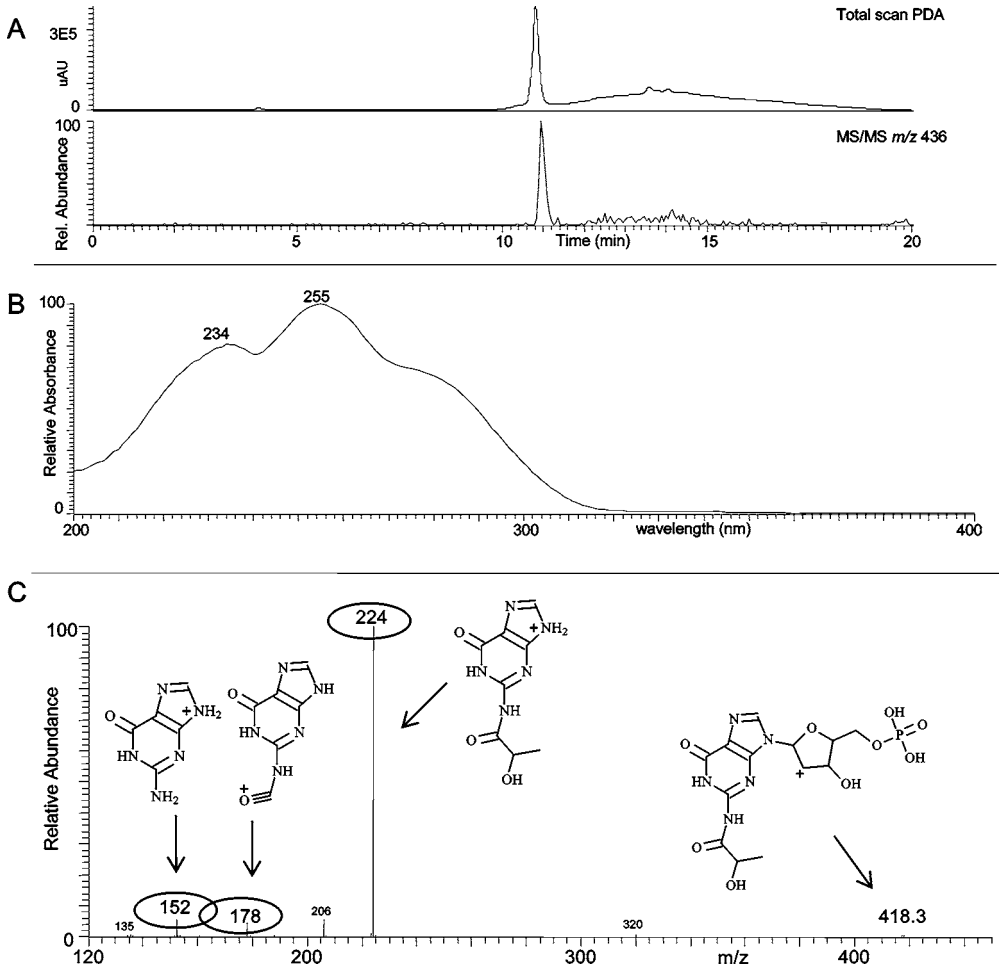

Figure 2. (**A**) LC–DAD UV and LC–ESI(+)-MS/MS traces and (**B**) PDA UV and (**C**) MS/MS spectra of 10 mg/L *N*-lactoyl GMP standard.

rpm for 10 min. The super-natant (50 mL) was filtered over a 0.45 μm filter prior to injection.

Nam Plaa (Thai Fish Sauce)**.** Five different good-quality (long fermentation time) brands were used: (1): Thipparos (market leader), containing 60% anchovy fish, 37% concentrated salt solution, and 3% sugar; (2) Plamuk (second largest), containing 60% anchovy fish, 38% salt, and 2% white sugar; (3) Razorclam, containing 70% anchovy fish, 28% salt, and 2% sugar; (4) Oyster, containing 75% fish, 24% salt, and 1% sugar; and (*5*) Cook & Lobster, containing 60% anchovy fish, 39% salt, and 1% sugar. The Nam Plaa samples were injected directly onto the LC−MS after filtration over a 0.45 μm filter.

Bonito Broth. A 20 g amount of bonito flakes or 10 g of bonito powder was added to 500 mL of boiling water. The solution was removed from the heating source and left standing for 2 min and filtered over a tea sieve (flakes) or paper filter (powder).

LC−ESI-MS. For LC−MS a ThermoFinnigan (San Jose, CA) Surveyor LC system was used equipped with an MS pump and a PDA/UV detector. The setup was coupled to a ThermoFinnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer. The separation was carried out on a 150 × 2.1 mm i.d., 5 μm Atlantis dC_{18} column (Waters, Milford, MA). The LC eluent consisted of (A) 10 mM aqueous ammonium acetate buffer (pH 4) and (B) methanol. Gradient elution starting with an isocratic step of 100% A over 5 min, followed by a linear gradient from 100% A to 100% B in 10 min, and consequently an isocratic step of 100% B over 5 min, was performed at a flow rate of 0.2 mL/min. ESI spectra were obtained in the positive ionization mode in the *m*/*z* 50−1000 range. The capillary temperature was set at 275 °C, the capillary voltage at 5000 V; the sheath and auxiliary gas (nitrogen) flow rates were 80 and 40 U, respectively.

RESULTS

LC−MS. To obtain good MS/MS spectra of the analyte, the collision energy was optimized between 10 and 50 eV by direct infusion of 10 mg/L aqueous solutions of *N*-lactoyl GMP at 20 μL/min. The source temperature and spray voltage were optimized to 275 °C and 5000 V, respectively. The highest signal in selected reaction-monitoring (SRM) acquisition was obtained at 15 eV. The obtained MS/MS and PDA UV spectra of *N*-lactoyl GMP and proposed structures of the MS/MS fragments are shown in **Figure 2**. Suggested structures of the fragments *m*/*z* 418, 224, 178, and 152 are shown in the MS/MS spectrum. The characteristic fragments *m*/*z* 224, 178, and 152 in the MS/MS spectrum of *N*-lactoyl GMP were selected for monitoring in the extracts.

All extracts were analyzed in full scan (scan range *m*/*z* 50−1000), MS/MS (of *m*/*z* 436) and SRM (transitions: *m*/*z* 436 → *m*/*z* 224; *m*/*z* 436 → *m*/*z* 178; *m*/*z* 436 → *m*/ *z* 152), and the results were compared to the extracts spiked with 10 mg/L *N*-lactoyl GMP. In the two top traces of **Figure 3A** the LC−PDA UV and LC−MS/MS chromatograms are shown. In the third trace of **Figure 3A**, the corresponding LC−MS/MS extracted ion (EI) chromatogram of *m*/*z* 224 is shown, and in the fourth trace the LC−MS/MS EI chromatogram of the same

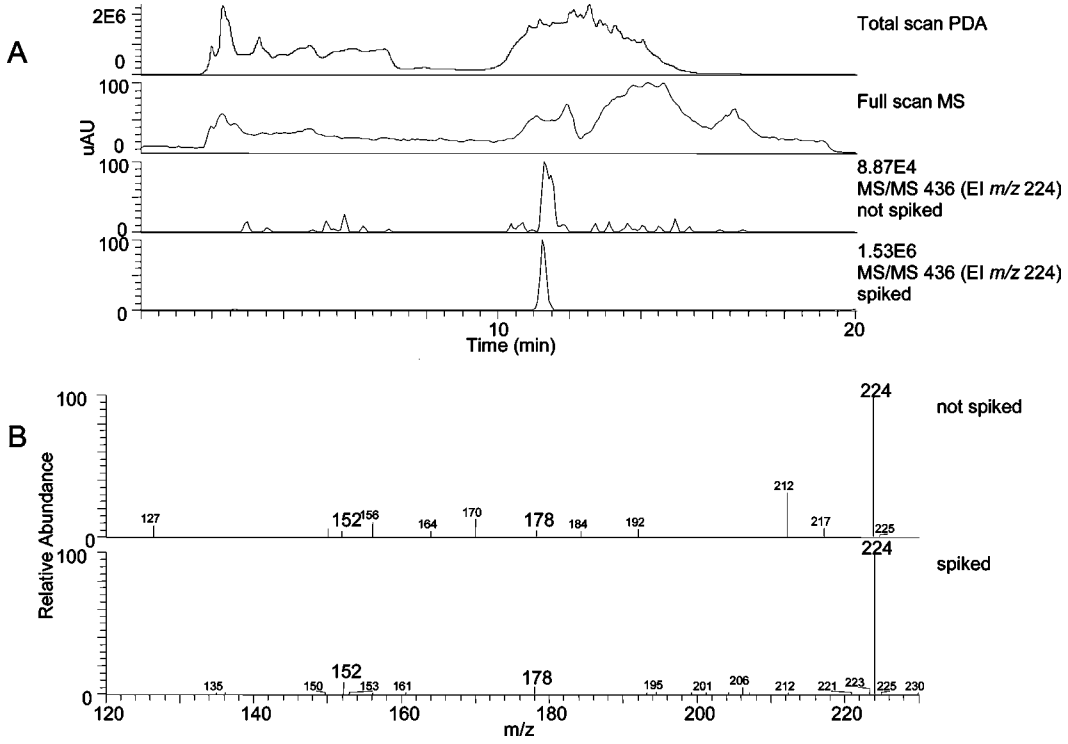

Figure 3. LC–ESI(+)-MS/MS traces of bonito extract not spiked and spiked with 10 mg/L *N*-lactoyl GMP standard. For LC–MS conditions see text.

bonito extract spiked with 10 mg/L *N*-lactoyl GMP. The MS/MS spectra of the not-spiked and spiked peaks at 11.5 min are shown in **Figure 3B**, respectively. In both spectra the same fragments *m*/*z* 224, 178, and 152 are observed as in the MS/MS spectrum of the standard (**Figure 2**). As can be seen in **Figure 3A**, the relative abundance of the peak in the spiked extract at 11.5 min increases relative to the not-spiked extract, and no other peaks show up in the LC−MS/MS trace. On the basis of these results, the peak was identified as *N*-lactoyl GMP. The relative abundance of the fragments of the analyte *m*/*z* 178 and *m*/*z* 152 to the most intensive fragment *m*/*z* 224 is 4.5 and 3.4%, respectively. This is in good agreement with the fragmentation pattern of the spiked sample (7.8 and 4.5%) and the reference standard (4.4 and 5.2%). In our previous work (*11*) it was described that for a valid identification the variation in relative ion intensities of the characteristic fragments compared to the standard should be within ±30%; this is true in all cases. Full-scan and SRM results are not shown here, because they do not add any extra information.

In the same way, *N*-lactoyl GMP was identified in the three bonito powder extracts: the result of the SRM experiment of one of the extracts is shown in **Figure 4**. The *N*-lactoyl GMP peak has the same retention time in the spiked and not-spiked bonito powder extract, and both show the same fragments in SRM (*m*/*z* 436 → *m*/*z* 224, 178, and 152) with similar relative abundance to *m*/*z* 224 (100%): 17 (*m*/*z* 152) and 25% (*m*/*z* 178) for the spiked, and 12 (*m*/*z* 152) and 30% (*m*/*z* 178) for the not-spiked sample. The somewhat higher abundances of the fragments in the bonito powder extracts compared to the bonito extract are due to different matrix effects, as will be explained below. Full-scan and MS/MS results are not shown.

Ion-Suppression Experiments. *N*-Lactoyl GMP could not be detected in the skipjack extract, the Nam Plaa fish sauce or any of the bonito flakes or broth samples. Therefore, an experiment was designed to test whether ion suppression might play a role under the conditions used. First, a 100 ppm solution of the *N*-lactoyl GMP standard was infused at a flow rate of 10 μL/min into the LC effluent (200 μL/min), creating an elevated but constant baseline. Consequently, blank bonito extract was injected onto the LC system. Any eluted material that suppresses ionization in the MS will cause a drop in the baseline. In **Figure 5A**,**B** the PDA UV trace and extracted ion MS trace of *m*/*z* 436 from full scan are shown, using the LC conditions as described in Materials and Methods. As can be seen in **Figure 5B**, the *m*/*z* 436 trace drops to zero at $t = 2$ min due to the peak of unretained material at the beginning of the chromatogram (see PDA UV trace). This does, however, not interfere with the analyte (*N*-lactoyl GMP) because it elutes at 11.6 min—as can be seen in **Figure 5C**, which shows the MS/MS (of *m*/*z* 436) trace of a spiked bonito powder extract. However, another drop in the baseline is observed at $t = 11.8$ min (**Figure 5B**)—that corresponds to the peak at 11.8 min in the PDA UV trace (**Figure 5B**)—which partly overlaps with the analyte of interest. This means that ion suppression plays a role in the bonito powder extract, resulting in a decrease of signal and loss of sensitivity.

To compensate for this loss in signal, the chromatographic conditions were adjusted to move the peak of interest away from the suppression region. The ammonium acetate buffer was replaced with a 10 mM ammonium formate buffer (pH 4.0) using the same gradient; results are shown in **Figure 5D**,**E**. Under these LC conditions, the interfering peak at 11.8 min in **Figure 5A** shifted to 12.1 min (**Figure 5D**), while the MS/MS peak of *m*/*z* 436 of *N*-lactoyl GMP of the spiked bonito extract shifted to 11.5 min (**Figure 5E**), thus eluting before the ion-suppression region. As can be seen in **Figure 5E** the peak height increased 5-fold under these chromatographic conditions resolving the loss of sensitivity. The same ion-suppression experiments were performed for the other selected samples, resulting in an increase of the *N*-lactoyl GMP signal of the spiked samples. However, under the optimized conditions, still no *N*-lactoyl GMP could be detected in the skipjack extract, the fish sauce, or any of the bonito flakes or broth samples. This means that *N*-lactoyl GMP could not be detected under the experimental

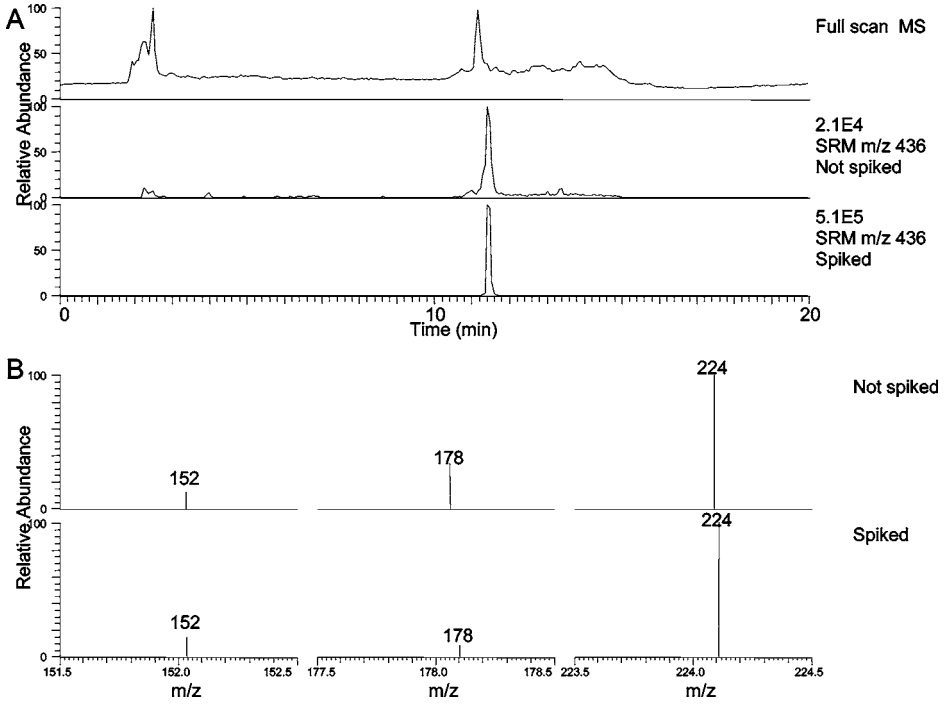

Figure 4. LC–ESI(+)-MS/MS in SRM mode of bonito powder extract not spiked and spiked with 10 ppm *N*-lactoyl GMP. For LC–MS conditions, see text.

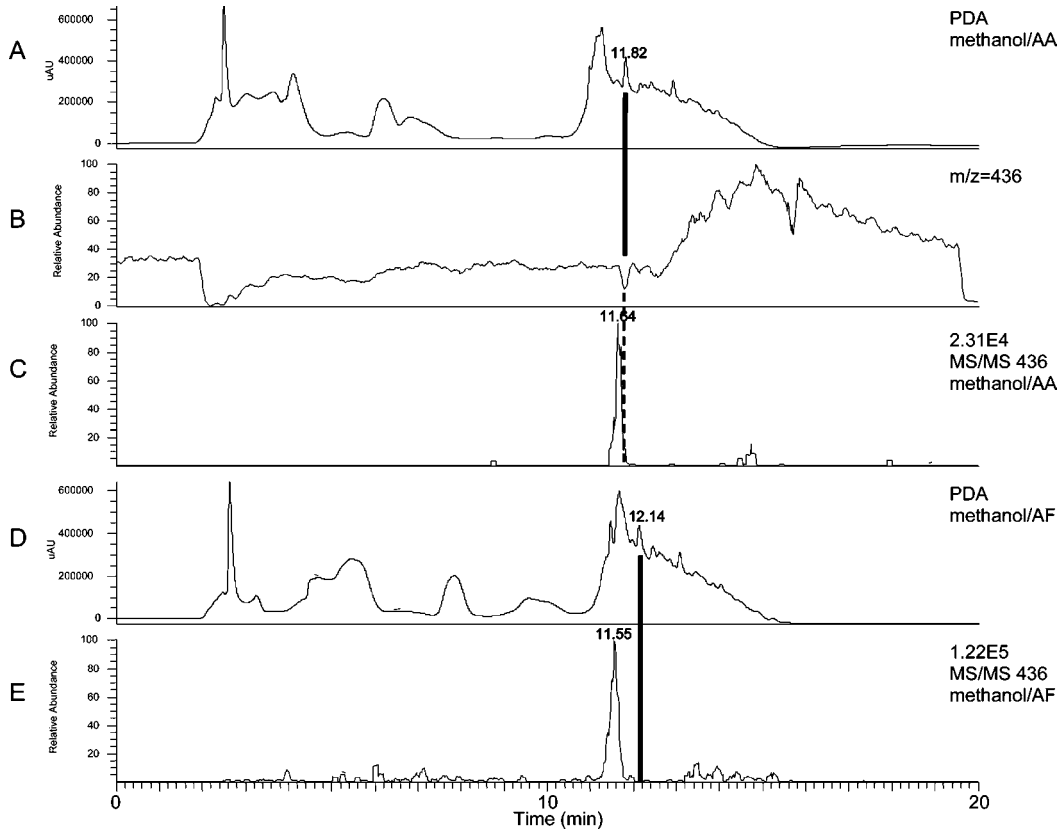

Figure 5. Effect of the eluent composition on ion suppression of *N*-lactoyl GMP in bonito powder extract: (**A**–**C**) original LC conditions; (**D**, **E**) optimized LC conditions. For details see text.

conditions used, and the concentrations are below the limit of detection (LOD).

Analytical Performance. The linearity of MS detection was tested in MS/MS mode. R^2 values and LODs ($S/N = 3$) were determined by injecting standard solutions containing between 0.01 and 10 mg/L (four data points in triplicate) *N*-lactoyl GMP. R^2 values of the calibration plots were 0.9999 in all cases, and LODs were found to be 10 μg/L. This external calibration plot was used for the quantification of *N*-lactoyl GMP in MS/MS mode. The concentration in the bonito extract was determined to be 1 ppm, corresponding to 0.4 μg *N*-lactoyl GMP/(g of bonito), and 0.02, 0.21, and 0.24 ppm in the bonito extract powders, corresponding to 0.2, 2.1, and 2.4 μg/g, respectively.

DISCUSSION

Umami is one of the principal qualifiers for the deliciousness of savory foodstuff of the Asian kitchen. Therefore we investigated widely used typical Asian food ingredients (bonito and Nam Plaa) for the presence of *N*-lactoyl GMP, a molecule which has a long-lasting umami effect. In bonito the concentration of the target compound was 0.4 μg/g, and in the bonito powders, the concentrations were 0.2−2.4 μg/g.

It was shown in this study, that ion suppression due to sample matrix effects can substantially influence the total MS response—and in this way jeopardize the sensitivity of the whole assay. Using the approach described in this study, ion suppression cannot only be discovered but also by adjusting the LC conditions it can be prevented. In this way the response for *N*-lactoyl GMP could be enhanced approximately 5-fold.

Even under these optimized conditions *N*-lactoyl GMP was not detected in the Thai fish sauce, bonito flakes, broth samples, and fresh skipjack. It could be speculated that *N*-lactoyl GMP is a characteristic constituent of bonito—formed during its production from fresh skipjack—and its concentration correlates with the quality of the product. This would explain why *N*-lactoyl GMP was not found in fresh skipjack (not present), not in bonito flakes (low quality, below LOD), and not Thai fish sauce (contains no bonito)—but the data available so far are very limited, and further research is required.

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