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Extraction and analysis of free amino acids and 5′-nucleotides, the key contributors to the umami taste of seaweed

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ABSTRACT

Assessing the umami taste of seaweed on a chemical level can inform the use and selection of seaweed in European cuisine. Accordingly, we developed a method for the simultaneous extraction, separate clean-up and analysis of 21 free amino acids and 10 free nucleotides by reversed phase and mixed-mode HPLC respectively. Of multiple mouth emulating solvents, extracting in Milli-Q at 35 °C was found most suitable. This method showed good linearity ($R^2 > 0.9996$), resolution ($R_s \geq 1.5$) and picomole detection limits, and was successfully applied to determine the Equivalent Umami Concentration (EUC) and Taste Activity Values (TAV) of seven Dutch seaweed species. *Phaeophyceae* showed the highest EUC, followed by *Chlorophyceae* and *Rhodophyceae* (≈ 9.5, 3.7 and 1.1 g/100 g respectively). Glutamic acid always exceeded the TAV, while other umami compounds were species specific. Our method can accurately predict umami intensity and therefore contributes towards species selection for the European palette.

1. Introduction

Zero hunger is one of the sustainable development goals (SDGs) adopted by all United Nation Member States (United Nations, 2017). In order to provide enough food for the world population, the global food production needs to increase by>50 % (Searchinger, Waite, Hanson, & Ranganathan, 2019). However, many agree that due to global land limitation, our current land-based system of food production is not capable of meeting future needs (Searchinger, Waite, Hanson, & Ranganathan, 2019).

The oceans cover over 70% of the earth's surface and have a vast potential to feed the planet in a sustainable manner (Harnedy & FitzGerald, 2011). Herein, the cultivation of seaweed can aid in the development of high value, functional foods (Hafting, et al., 2015). When consumed as a whole, seaweeds provide compounds of high nutritional value such as proteins and carbohydrates, as well as bioactive compounds such as carotenoids, flavonoids and phytosterols (Fitzgerald, Gallagher, Tasdemir, & Hayes, 2011). While primary functions of food like nutritional value are of great importance, secondary functions such as palatability with respect to sensory input induced by taste, smell, texture and colour, also play a very important role in food systems. With regards to consumer acceptance, seaweeds are viewed as "natural" and promote a positive response (Fitzgerald, Gallagher, Tasdemir, & Hayes, 2011). Nevertheless, contrary to Asia, consumer acceptance of seaweed as a food application in Europe is relatively low, in part due to the negative connotations of seaweed with the unpleasant odours of beached and decomposing seaweed (Mouritsen, Duelund, Petersen, Hartmann, & Frøst, 2019). However, the characteristic taste of seaweed *i.e.* umami is often associated with deliciousness and an increased focus on seaweed umami potential may unlock its gastronomic potential and increase its acceptance as a food source (Bellisle, 2008) (Mouritsen, Williams, Bjerregaard, & Dueland, 2012). In order to produce high quality seaweed products that are compatible with European palatability, it is imperative that we develop the knowledge to chemically assess the umami taste of seaweeds.

Free amino acids have been found important contributors to the taste of seaweeds. They can impart a sweet, sour or bitter taste depending on the amino acid and its concentration (Kawai, Sekine-Hayakawa,

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Okiyama, & Ninomiya, 2012). Free amino acids are also key contributors to the characteristic umami taste of seaweed, and this taste sensation is known to be enhanced by free 5′-monophosphate nucleotides, contributing to the perceived end-taste (Mouritsen et al., 2012). In this study, non-volatile taste-active components, *e.g.* 21 free amino acids and 10 free 5′-nucleotides, were analyzed by means of reversed phase and mixed mode high performance liquid chromatography (HPLC) respectively. Assessing the umami taste implies a close approximation of the oral environment. Therefore, multiple mouth emulating solvents were tested for the simultaneous extraction of free amino acids and 5′-nucleotides from seaweeds. Subsequent work-up and analysis procedures were assessed and optimized to retain the obtained flavour profile. The developed and optimized method was then applied to 7 seaweed species from the Eastern Scheldt (the Netherlands), and the synergistic effects of their free amino acid and 5′-nucleotide content were assessed by means of the Equivalent Umami Concentration (EUC). Herein, the concentrations of umami amino acids (L-aspartic acid, L-Asp and L-glutamic acid, L-Glu) and umami-enhancing 5′-nucleotides (adenosine monophosphate, AMP; guanosine monophosphate, GMP and inosine monophosphate, IMP) were factored with their relative umami intensities to assess their combined impact on flavour (Yamaguchi, Yoshikawa, Ikeda, & Ninomiya, 1971).

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were of analytical grade unless mentioned otherwise. Ultrapure water (Milli-Q) (18.2 MΩ.cm) was provided by a Milli-Q Academic water purifier equipped with a Quantum EX Cartridge, Q-Gard 2 Purification Cartridge and a Millipak Express 20 membrane filter (Millipore Corporation, MA, U.S.A.). Sulphuric acid (96 %), ammonium hydroxide (25 %), hydrochloric acid (37–38 %), calcium chloride (95 %), boric acid, sodium hydroxide (tablets), methanol, sodium chloride (>99.5 %) and potassium chloride (≥99.9 %) were purchased from Boom B.V. (Meppel, Netherlands). DOWEX 50WX8 hydrogen form resin (50–100 mesh), disodium hydrogen phosphate, disodium tetraborate, potassium carbonate and dipotassium phosphate were obtained from Merck (Darmstadt, Germany). *Ortho*-phthaldialdehyde (OPA), 3-mercaptopropionic acid (MPA) and fluorenylmethyloxycarbonyl chloride (FMOC-Cl), sodium alginic acid (≥85 %) and α-amylase (35 units/mg solid) were purchased from Sigma-Aldrich (Steinheim, Germany). Magnesium chloride (≥99 %) was supplied by Acros Organics (New Jersey, U.S.A.).

A combined standard solution of 2.5 mM L-alanine (L-Ala), L-arginine (L-Arg), L-aspartic acid (L-Asp), L-cystine (L-Cys, exception: 1.25 mM), L-glutamic acid (L-Glu), glycine (Gly), L-histidine (L-His), L-isoleucine (L-Ile), L-leucine (L-Leu), L-lysine (L-Lys), L-methionine (L-Met), L-phenylalanine (L-Phe), L-proline (L-Pro), L-serine (L-Ser), L-threonine (L-Thr), L-tyrosine (L-Tyr) and L-valine (L-Val) in 0.1 M HCl was obtained from Sigma-Aldrich (Steinheim, Germany) and supplemented with L-asparagine (L-Asn), L-glutamine (L-Gln), L-tryptophan (L-Trp) and hydroxy-L-proline (L-Hyp) from the same supplier. Sarcosine (Sar) and disodium salts of cytidine 5′-monophosphate (≥99 %, CMP), 2′-deoxycytidine 5′-monophosphate (≥98 %, dCMP), guanosine 5′-monophosphate (≥99 %, GMP) 2′-deoxyguanosine 5′-monophosphate (≥99 %, dGMP), inosine 5′-monophosphate (≥98 %, IMP) and thymidine 5′-monophosphate (≥99 %, TMP) were obtained from Sigma Aldrich (Steinheim, Germany). Disodium salts of adenosine 5′-monophosphate (≥97 %, AMP), 2′-deoxyadenosine 5′-monophsphate (≥98 %, dAMP), 2′-deoxyinosine 5′-monophosphate (99 %, dIMP) and uridine 5′-monophosphate (>98 %, UMP) were supplied respectively by HoneyWell Fluka (New Jersey, U.S.A.), MP Biomedicals (Eschwege, Germany), Thermo Fischer GmbH (Kandel, Germany) and Acros Organics (New Jersey, U.S.A.).

2.2. Sampling and Post-Harvest handling

All seaweed samples were harvested in 2018 during low tide from the coastal region of the Eastern Scheldt in Kattendijke, the Netherlands (51°31′45.6″N, 3°57′20.7″E). *Undaria pinnatifida* was harvested on the 20th of March, *Chondrus crispus* on the 5th of April, *Sargassum muticum* and *Scytosiphon lomentaria* on the 10th of May, *Ulva* sp. on the 17th of July, *Grateloupia turuturu* on the 12th of September and *Codium fragile* on the 29th of October. Samples were transported in seawater to our laboratory, where they were gently washed in tap water, freed from visible epiphytes and stored at −20 °C. Prior to analysis samples were lyophilized (CHRIST Alpha 1–2 LDplus, Osterode am Harz, Germany) for 48 h and ground to a powder under liquid nitrogen using mortar and pestle. All samples were stored at −20 °C until analysis.

2.3. Extraction of free amino acids and nucleotides

To approximate the extraction of umami-inducing and -enhancing components as it occurs in an oral environment, several mouth-emulating solvents were tested as extraction solvents. These were tested in triplicate on *Sargassum muticum*, *Ulva* sp. and *Grateloupia turuturu*, representing one species from each of the major classes of seaweed (*i.e. Phaeophyceae, Chlorophyceae and Rhodophyceae* respectively). The solvents consisted of Milli-Q, 3 mg/mL α-amylase, stimulated saliva obtained according to Neyraud et al. (2012) and artificial saliva according to DIN 53160–1 (German Institute for Standardisation, 2010). To 50 mg of sample, 85 μL of internal standard solution (4.43 mM sarcosine) and 5 mL of either Milli-Q, α-amylase solution, stimulated saliva or artificial saliva was added followed by homogenization. Samples were then allowed to extract for 15 min in a water bath set to 35 °C, during which period they were shaken by hand at 1-minute intervals. Additional extractions were performed using Milli-Q at room temperature for 24 h under constant agitation (325 rpm). Following this, samples were centrifuged at 3700 rcf for 10 min. For each sample, 3 mL of supernatant was retrieved for free amino acid (FAA) analysis and supplied with 600 μL of 37 % HCl to precipitate large polysaccharides and proteins (*i.e.* acid precipitation), followed by a second centrifugation step at 3700 rcf for 20 min. An additional 1 mL aliquot of the original supernatant was taken for nucleotide analysis and supplied with 125 μL concentrated H_2SO_4 for acid precipitation, followed by centrifugation at 3700 rcf for 20 min. Both were stored at −20 °C until further use. To infer whether free amino acids were lost to the precipitate during the acid precipitation step, a standard mixture of amino acids (10 μM) was prepared to include sodium alginate (1.5 mg/L) and subjected to the acid precipitation in triplicate. The same standard mixture, with no added sodium alginate, was subjected to the same acid precipitation.

2.4. Cation exchange chromatography

The chromatographic separation of amino acids and the associated column life-span were impeded by contamination present in the extract. To remove salts and organic contaminants from the amino acid extracts, a cation exchange chromatography step was used employing a DOWEX 50WX8 (50–100 mesh) resin. For nucleotides this treatment was not applied as it resulted in a complete loss of analytes. This clean up step was tested in both a continuous and batch approach so as to determine the effect of contact time on the amino acid recovery of the procedure. The continuous procedure was performed according to Veuger et al. (2005). In brief, the resin was activated by rinsing sequentially with NaOH (2 M), HCl (2 M) and Milli-Q, then transferring 2 mL of activated resin onto a glass column pre-fitted with a quartz wool plug. Two mL of sample was transferred onto the column and rinsed with approximately 10 mL of Milli-Q to remove impurities, after which 6 mL of NH_4OH (2 M) was used to elute the amino acids. This eluate was evaporated to dryness on a hotplate set to 35 °C, reconstituted in 2 mL dilute HCl (0.1 M) and stored at −20 °C. The batch procedure, adapted from Abe et al. (1993),

employed a longer contact time. The resin was activated as previously mentioned and transferred (2 mL) to a falcon tube. To this, 2 mL of sample was added and the falcon tube was then agitated on a benchtop shaker for 20 min at 325 rpm. Following this, the mixture was quantitatively transferred onto the glass column and treated as described in the continuous method. The recovery was determined by subjecting a triplicate of standard mixtures containing 21 amino acids (16.13 μM) to both procedures, analysing the subject standard mixtures and comparing the resulting peak areas of each amino acid with those from directly injected standard mixtures.

Because the post-DOWEX evaporation step was previously conducted on a hotplate in full exposure to air, this step introduced possible factors of degradation of amino acids (*i.e.* heat and oxygen). Both of these were separately assessed in a factorial experiment. Standard solutions (n = 5) of 21 amino acids were evaporated to dryness on a hotplate at 35 °C with or without nitrogen blanketing, or allowed to evaporate to dryness under ambient temperatures with or without nitrogen blanketing. The reconstituted standards were subsequently analysed, and the recovery was determined by comparison with directly injected standard mixtures. Regardless of DOWEX or evaporation step, samples were filtered over 10 KDa cut-off filters (Amicon Ultra-0.5 Centrifugal Filter Unit, Millipore Corporation, MA, U.S.A.) prior to analysis. These were first conditioned by sequentially adding 500 μL of Milli-Q and 0.1 M HCl, centrifuging for 10 min at 10,000 rcf after each addition. Finally, 500 μL of sample was loaded onto the filter and centrifuged for 10 min at 10,000 rcf, the filtrate being transferred to an HPLC vial and stored at −20 °C until analysis.

2.5. Analysis of free amino acids and nucleotides

Free amino acid analysis was adapted from Long (2017). Samples were analysed by means of High Performance Liquid Chromatography – Fluorescence Detection (HPLC-FLD), using a DIONEX Ultimate 3000 HPLC system equipped with an Agilent InfinityLab Poroshell 120 HPH-C18 column (100 × 4.6 mm; 2.7 μm) and matching guard column (5 × 4.6 mm; 2.7 μm) kept at 40 °C. To produce fluorescent amino acids, samples (1 μL) were derivatized on-line with 1 μL of 10 mg/mL OPA in 0.4 M borate buffer (pH 10.2) containing 0.8 % (v/v) MPA, and 1 μL of 2.5 mg/mL FMOC-Cl in acetonitrile, all of which was homogenized with 5 μL of 0.4 M borate buffer (pH 10.2). Fresh OPA-MPA and FMOC-Cl solutions were prepared a minimum of once per day. Gradient elution was employed using a two mobile phase system (A: 10 mM Na_2H-$PO_4{\cdot}xH_2O$ / $Na_2B_4O_7{\cdot}10H_2O$, pH 8.2; B: acetonitrile/ methanol/water, 9:9:2) with a flowrate of 1 mL/min. The following gradient was used: 2 % B (v/v) until 0.35 min, followed by an increase to 52 % B (v/v) from 0.35 to 14 min using Chromeleon Chromatography Data System software (v. 7.2.8, Thermo Scientific, MA, U.S.A.), subsequently holding 52 % B (v/v) until 15.5 min, increasing to 100 % B (v/v) from 15.4 to 15.5 min, holding 100 % B (v/v) until 18.7 min and decreasing back to 2 % B (v/v) until 18.8 min. Separate excitation and emission wavelengths were employed for the primary and secondary amino acids. These were respectively 340 nm and 450 nm for the primary amino acids, and 266 nm and 305 nm for the secondary amino acids. Quantification was achieved using a 6-point external calibration curve (6.25 to 100 μM) and the results were normalised using the internal standard.

The nucleotide analysis was adapted from Moerdijk-Poortvliet et al. (2014). In brief, nucleotides were analysed on a DIONEX Ultimate 3000 HPLC system, equipped with a Ultraviolet (UV) detector and a SIELC PrimeSep D mixed-mode column (150 × 4.6 mm; 5 μm) with corresponding guard column (10 × 4.6 mm; 5 μm). The injection volume was 10 μL and elution was performed isocratically at 0.8 mL/min, with 10 mM H_2SO_4 (pH 1.95) as the mobile phase; the detection wavelength was 260 nm. Quantification of nucleotides was achieved using a 7-point external calibration curve (5 to 1000 μM).

2.6. Assessment of umami potential

To determine the impact of the umami components on taste, their taste activity values (TAVs) were determined according to Duan et al. (2020). The following equation was used, in which a TAV above 1 was considered a contribution to the overall taste:

$$TAV = C_1/C_2$$

in which C_1 denotes the concentration of a taste compound and C_2 denotes the minimum concentration at which said compound can be perceived *i.e.* the threshold concentration.

The impact of free amino acids and free nucleotides on umami taste was assessed by determining the equivalent umami concentration (EUC) according to Yamaguchi et al. (1971), using the following equation:

$$EUC = \sum a_i b_i + 1218\left(\sum a_i b_i\right)\left(\sum a_j b_j\right)$$

where the EUC is expressed in mg of monosodium glutamate (MSG)/100 g, a_i and a_j denote the concentrations (mg/100 g) of umami amino acids (L-Asp or L-Glu) and nucleotides (AMP, GMP or IMP) respectively, and b_i and b_j denote the strength of the synergy factors per umami amino acid (L-Asp, 0.077; L-Glu, 1) and nucleotide (AMP, 0.18; GMP, 2.3; IMP, 1) respectively. Using the EUC, the synergistic effect of flavour enhancing nucleotides and umami amino acids was determined and as such, the umami taste sensation was approximated.

2.7. Statistical analyses

All data were processed and analysed in Microsoft Excel 2016 (Microsoft Ltd., USA). For statistical analyses, $\alpha = 0.05$ unless mentioned otherwise. Quantitative and compositional results from the solvent tests as well as results from the evaporation tests were assessed by means of one-way ANOVA with a Tukey HSD *post hoc*. Recoveries of the continuous and batch DOWEX methods were compared by means of a two-tailed, unpaired *t*-test. Linear regressions of the calibration standards were constructed using the least squares method. All data are presented as mean ± standard deviation (S.D.).

3. Results and observations

3.1. Chromatography of free amino acids and nucleotides

The described reversed phase chromatographic method provided excellent separation of 21 amino acid derivatives with a resolution that was consistently above 1.5 (Fig. 1). The limit of quantitation for this method ranged between 10.9 nM (L-Ile) and 199 nM (L-Glu) (10.9 and 199 fmol with an 1 μL sample injection). Peak areas were highly linear, showing an average R^2 of 0.9996 over all amino acids between 6.25 and 100 μM (equals 6.25 to 100 pmol with an injection of 1 μL sample). Similarly, the application of mixed mode chromatography provided complete separation of 6 nucleotides and 4 deoxynucleotides with a consistent resolution of 1.5 or more (Fig. 2). Here, the limit of quantitation ranged from 86.5 nM (UMP) to 584 nM (dIMP) (equals 865 and 5841 fmol with a 10 μL injection volume). Linearity was excellent, showing an average R^2 of 0.9997 over all nucleotides withing the tested concentration range of 5 to 1000 μM (equals 50 pmol to 10 nmol with an injection volume of 10 μL). The concentrations of both amino acids and nucleotides in the encountered sample extracts were at least an order of magnitude higher than their respective limits of quantitation. As illustrated in table 1, the amino acid analysis method showed good inter- and intra-day precision, averaging 3.7 % and 16 % CV respectively. The exceptions were the secondary amino acids L-pro and L-Hyp with a respective 38 % and 24 % CV. The inter- and intra-day precision of the nucleotide analysis method was excellent, averaging 0.59 % and 5.2 % CV. Both columns were stable over time and did not drop in performance over an approximately 1800 (reversed phase) and 500 (mixed-mode)

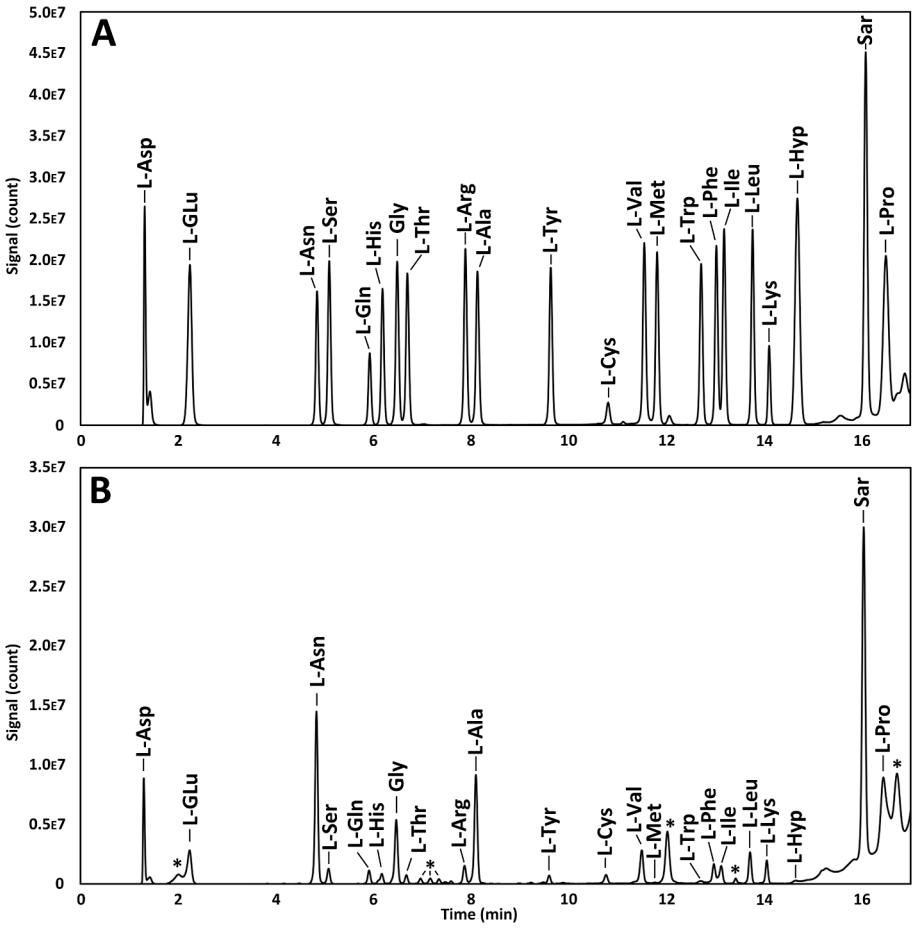

Fig. 1. HPLC-FLD chromatograms of a standard mixture (**A**) and Milli-Q extract of *Ulva* sp. (**B**) of free amino acids, derivatized with *o*-phthaldialdehyde/3-mercaptopoprionic acid/ fluorenylmethyloxycarbonyl chloride and separated over an InfinityLab Poroshell 120 high pH reversed phase column as described in section 2.5. * = unknown.

runs of samples and standards.

3.2. Efficacy of the mouth-emulating solvents

To approach the conditions under which seaweed taste is experienced, one seaweed from each of the three main classes was extracted using mouth-emulating solvents: Milli-Q (15 min and 24 h), α-amylase and stimulated saliva. Significant differences were found between the total free amino acid (FAA) content of the different solvent extracts for both *Ulva* sp. ($F_{3,8} = 42.4$, $P < 0.0001$) and *G. turuturu* ($F_{3,8} = 22.2$, $P = 0.0003$), but not for *S. muticum* (Fig. 3). Comparing individual solvents, the addition of α-amylase had no effect on the extraction of FAAs when compared to just Milli-Q under the same extraction conditions, regardless of species. However, when using Milli-Q alone, significantly more FAAs were extracted after 24 h than after 15 min for both *Ulva* sp. ($P = 0.0001$) and *G. turuturu* ($P = 0.0051$). The saliva extractions produced more varied results; whereas it produced a similar FAA content to the 24-hour Milli-Q extraction for *G. turuturu* ($P > 0.05$), for *Ulva* sp. the saliva extraction was more comparable to the 15-minute Milli-Q extraction ($P > 0.05$). The use of artificial saliva as a solvent resulted in aberrant amino acid chromatograms, and profiles could not be determined.

In contrast to the total content of FAAs extracted by the different solvents, the composition of the extracts varied widely and differently for each seaweed species. For *S. muticum*, there were no significant differences between the FAA composition of the Milli-Q extracts after 15 min or 24 h (Supplement, table S1), with the exception of L-Asn, L-Val and L-Leu ($P < 0.0010$ for all three), none of which deviated>1.60 % in composition. Similarly small differences were found when comparing the FAA composition of the 15-minute Milli-Q and the α-amylase extracts of *S. muticum*, which did not differ significantly except for L-Arg, L-Val, L-Met and L-Phe, neither of which varied>1.08 % ($P < 0.0010$ in all cases). In contrast, the saliva extractions of *S. muticum* varied significantly in FAA composition for at least 7 out of 21 FAAs regardless of which solvent it was compared to (Supplement, table S1).

The FAA composition of *G. turuturu* as extracted by Milli-Q after 15 min or 24 h differed relatively little. No significant differences were found for a majority of the FAAs; only L-Glu, L-Arg, L-Met, L-Phe and L-Pro differed significantly in composition ($P < 0.05$ for all), deviating between 0.23 and 7.68 %. Less variation was found between the FAA composition of the 24-hour Milli-Q and the α-amylase extract of *G. turuturu*. Here, L-Arg, L-Ala, L-Ile and L-Leu differed significantly in composition ($P < 0.01$ for all), at most 1.46 %. The 15-minute Milli-Q extraction and α-amylase extraction of *G. turuturu* resulted in significantly different composition percentages for 9 out of 21 FAAs, and the saliva extract differed from all other extraction solvents in resulting composition percentage for at least 10 out of 21 amino acids (Supplement, table S2). Compositional differences between the solvent extractions of *Ulva* sp. are given in Supplement table S3; this species showed the most variation between solvent extractions with a minimum of 8 amino acids differing significantly in compositional percentage, regardless of which solvents were compared. The exact concentrations of FAAs in *S. muticum, Ulva* sp. and *G. turuturu* per solvent can be found in the supplement tables S3, S4 and S5 respectively.

The use of stimulated saliva as a solvent introduced significant variation in FAA content and composition. Furthermore, use of

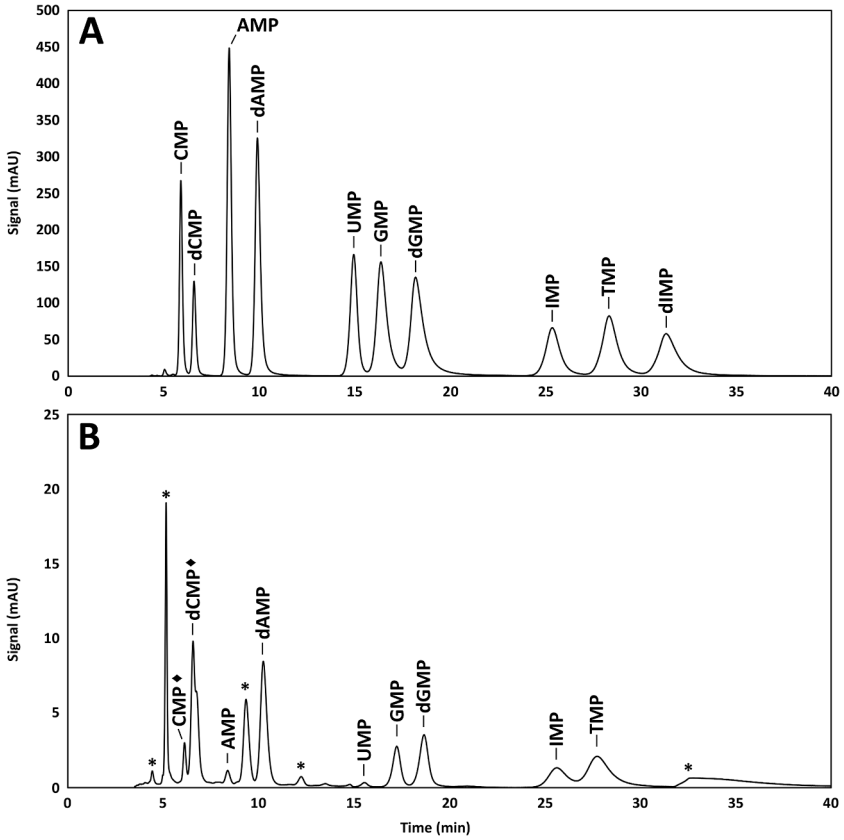

Fig. 2. HPLC-UV chromatograms of a standard mixture (**A**) and a Milli-Q extract of *Ulva* sp. (**B**) of nucleotides and deoxynucleotides, separated over a PrimeSep D mixed-mode column as described in section 2.5. Compounds marked with ◆ could not be quantified due to coelution; * = unknown.

stimulated saliva is a solvent matrix and was cumbersome to attain as an extraction solvent. Meanwhile, the α-amylase solution resulted in minimal variation from Milli-Q as an extraction solvent. Accordingly, the extraction efficacy for nucleotides was only assessed for the 24-hour and 15-minute Milli-Q extractions. There were no significant differences between the total free nucleotide content extracted by Milli-Q after 15 min or 24 h for any of the seaweed species (Fig. 4), contrary to the effect of extraction time on total FAA content. However, the extraction time did have a significant effect on the composition of free nucleotides for all investigated species (Supplement, table S7). The composition of all free nucleotides of *S. muticum* changed significantly depending on extraction time ($P < 0.05$ at least), while for *G. turuturu* only GMP and dIMP did not differ significantly between extractions. Most consistent was *Ulva* sp., wherein only the compositional percentage of dIMP changed with the different extractions ($P < 0.0001$). Free nucleotide concentrations of *S. muticum, Ulva* sp. and *G. turuturu* per solvent can be found in the supplement, table S8.

3.3. Extract Purification

To prepare the seaweed extracts for analysis while maintaining the accuracy with which they represent the flavour profile of seaweed, the recovery of the various clean-up steps were characterized and optimized. Preliminary investigations found that the presence of alginate during the acid precipitation step resulted in no significant losses of amino acids. Comparing the amino acid recovery of the continuous and batch DOWEX methods revealed that the batch method produced an average recovery of 65.0 ± 11 %, while the continuous method yielded a recovery of 60.3 ± 20 %, being both lower and less consistent. Using the batch method significantly improved the recovery of L-Glu, L-Tyr, L-Met, L-Trp and L-Ile ($P < 0.05$ at least) with 16.2 to 21.0 %, while the same method loss of recovery for Gly of 14.6 % ($P = 0.010$). See figure S1 (supplement) for the complete dataset on DOWEX recoveries. Four variations in the post-DOWEX evaporation step were compared for their recovery of FAAs: evaporation under a nitrogen or air blanket, both tested at ambient temperature and at 35 °C. The evaporation temperature had little influence on the FAA recovery. When evaporating under nitrogen, only L-Lys, L-Met and L-Trp had a significantly lower recovery (at least 8 %, $P < 0.01$) at ambient temperature than at 35 °C. When no nitrogen blanket was used, evaporation at ambient temperature resulted in a lower recovery of the same amino acids (at least 6 %, $P < 0.01$) when compared to 35 °C. Out of these, significant interaction between temperature and atmosphere effects was only observed for L-Met ($F_{1, 16} = 10.28$, $P = 0.006$). Comparatively, evaporation in the presence or absence of a nitrogen blanket at 35 °C resulted in a significantly different recovery for 13 out of 21 amino acids ($P < 0.05$ at least). At this temperature, using a nitrogen blanket resulted in an average recovery of 66 ± 8 %, which is significantly higher ($P < 0.0001$) than the recovery of 60 ± 8 % that is obtained without a nitrogen blanket. In fact, evaporation at 35 °C under a nitrogen blanket yielded the highest observed recovery, as using nitrogen at ambient temperature resulted in a recovery of 62 ± 9 % and allowing the post-DOWEX extracts to evaporate at ambient temperature with no blanket resulted in an amino acid recovery of 57 ± 9 %. An overview of the recoveries for each evaporation step can be found in figure S2 (supplement).

3.4. Umami components of seven Eastern Scheldt seaweeds

Distinct patterns were found in the metabolite composition of the investigated seaweed species. The FAA content of *Phaeophyceae* was characterized by a majority of L-Gln, L-Glu and L-Ala, though not always in the same ratio. The FAA profile of *S. muticum* ~~contained~~ consisted of as much as 47.2 ± 0.55 % L-Gln, and 12.7 ± 0.43 % L-Ala, while that of *U. pinnatifida* contained an almost opposite 18.4 ± 1.5 % L-Gln and 47.9

Table 1
Validation parameters for the amino acid and nucleotide analyses. Inter- and intra-day precision values are given as mean, standard deviation (S.D.) and coefficient of variance (CV) of replicate measurements (n = 8). Intraday precision was determined from the repeated injection of a 50 μM (amino acids) and 500 μM (nucleotides) standard. The interday precision was determined from the same concentration of standards, injected bi-monthly over a 4-month period. The limit of detection and quantitation are denoted respectively by "LOD" and "LOQ" and are derived from the fraction between the standard error of a calibration curve and its slope, multiplied respectively by 3.3 or 10, produced from 5-point calibration curves for the amino acids, and 9-point calibration curves for the nucleotides. Peak area values are given in units of counts (amino acids) and mAU (nucleotides).

Compound	Intraday precision		Interday precision		Limits (fmol)	
	Area ± S.D.	CV%	Area ± S.D.	CV%	LOD	LOQ
	Amino acids					
L-Asp	1030126 ± 31386	3.0	983619 ± 134228	14	16.1	48.7
L-Glu	1711051 ± 62479	3.7	1461368 ± 181450	12	65.5	199
L-Asn	903864 ± 36742	4.1	953432 ± 170573	18	13.3	40.2
L-Ser	1241988 ± 48900	3.9	1199365 ± 146060	12	52.8	160
L-Gln	427906 ± 17984	4.2	536165 ± 120245	22	15.1	45.7
L-His	968324 ± 39935	4.1	945848 ± 110584	12	15.1	45.8
Gly	1326755 ± 53141	4.0	1237930 ± 154835	13	64.4	194
L-Thr	1186618 ± 44600	3.8	1138872 ± 144914	13	14.3	43.2
L-Arg	1364268 ± 53055	3.9	1308334 ± 160955	12	8.18	24.8
L-Ala	1241275 ± 48305	3.9	1196604 ± 149343	12	46.2	140
L-Tyr	1239886 ± 48342	3.9	1185167 ± 147112	12	20.8	63.1
L-Cys	214954 ± 9597	4.5	213906 ± 45260	21	47.0	143
L-Val	1483927 ± 52367	3.5	1408362 ± 183411	13	14.8	44.9
L-Met	1373481 ± 51552	3.8	1313611 ± 168165	13	7.61	23.1
L-Trp	1201664 ± 46528	3.9	1199198 ± 173561	14	13.1	39.8
L-Phe	1415941 ± 55003	3.9	1320325 ± 174925	13	9.04	27.4
L-Ile	1445274 ± 55439	3.8	1402565 ± 171462	12	3.59	10.9
L-Leu	1430611 ± 54373	3.8	1372796 ± 170863	12	17.4	52.7
L-Lys	429847 ± 18215	4.2	459291 ± 54720	12	26.4	80.0
L-Hyp	2978196 ± 46749	1.6	2835339 ± 677386	24		
L-Pro	2120890 ± 43337	2.0	2738712 ± 1048486	38		
	Nucleotides					
CMP	27.7 ± 0.17	0.61	29.2 ± 0.96	3.3	355	1185
dCMP	17.2 ± 0.08	0.46	17.2 ± 0.66	3.8	270	901
AMP	72.1 ± 0.39	0.54	70.5 ± 2.79	4.0	370	1234
dAMP	65.4 ± 0.34	0.52	65.5 ± 2.53	3.9	573	1910
UMP	47.1 ± 0.29	0.61	46.4 ± 1.90	4.1	260	865
GMP	61.0 ± 0.32	0.52	59.3 ± 2.25	3.8	468	1559
dGMP	54.5 ± 0.30	0.55	53.3 ± 6.25	12	295	984
IMP	35.5 ± 0.21	0.60	32.8 ± 1.62	5.0	658	2192
TMP	43.9 ± 0.29	0.67	47.9 ± 3.92	8.2	824	2746
dIMP	38.4 ± 0.33	0.85	38.4 ± 1.61	4.2	175	5841

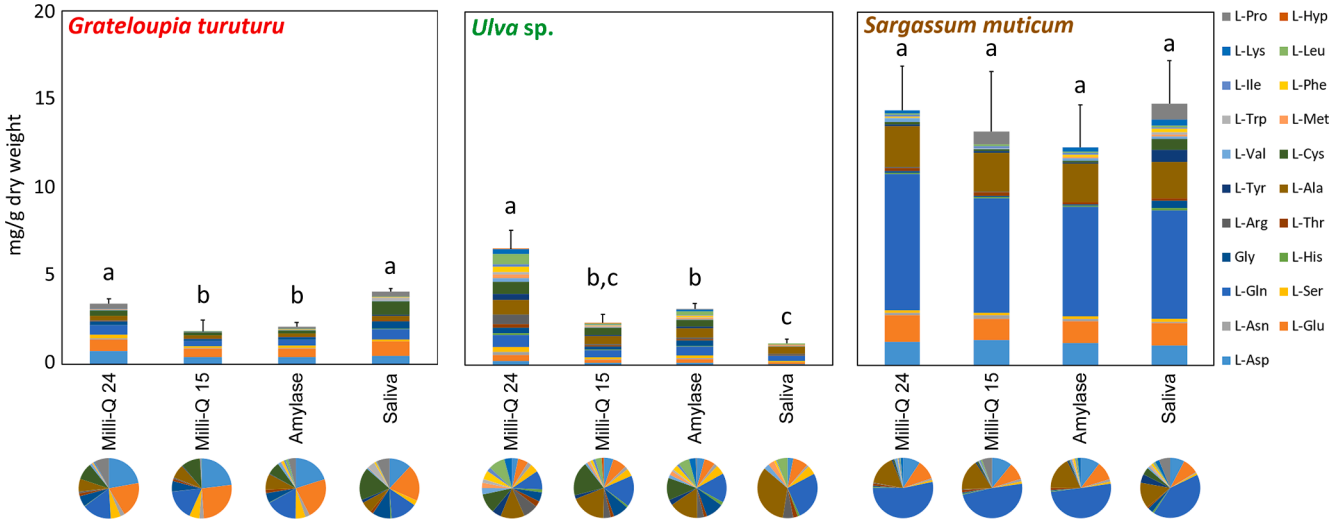

Fig. 3. Absolute and relative extraction amount of free amino acids from mouth-emulating solvents as applied to *Grateloupia turuturu* (*Rhodophyceae*), *Ulva* sp. (*Chlorophyceae*) and *Sargassum muticum* (*Phaeophyceae*). Data are given as mean ± S.D. (n = 3) in the bar graphs and as mean only in the pie charts. Means with different letters are significantly different from one another according to a Tukey HSD post-hoc following a one-way ANOVA. See supplement tables S4, S5 and S6 for exact concentrations.

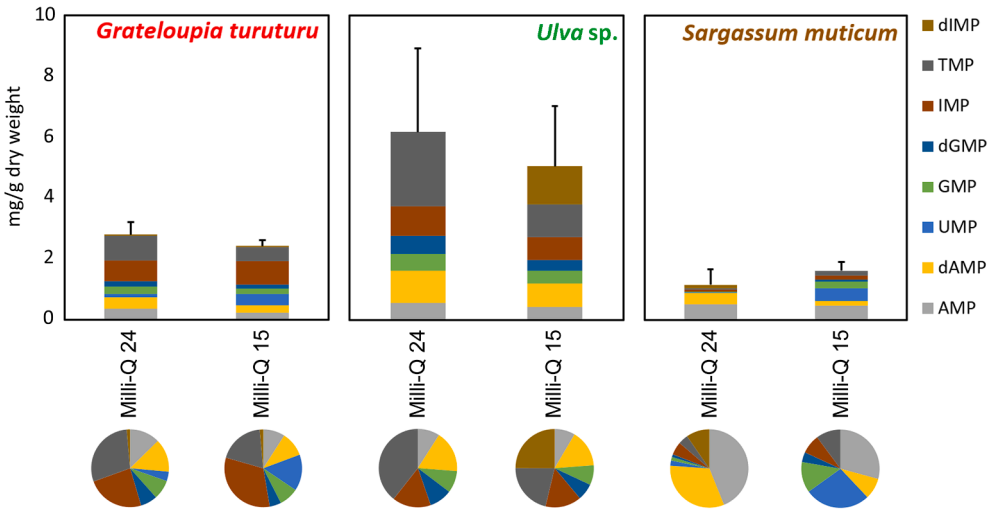

Fig. 4. Absolute and relative extraction amount of free nucleotides from Milli-Q extractions (15 min at 35 °C and 24 hr at room temperature) as applied to *Grateloupia turuturu* (*Rhodophyceae*), *Ulva* sp. (*Chlorophyceae*) and *Sargassum muticum* (*Phaeophyceae*). CMP and dCMP could not be quantified due to coelution. Data are given as mean ± S.D. (n = 3) in the bar graphs and as mean only in the pie charts. See supplement table S8 for exact concentrations.

± 3.7 % L-Ala. Major FAAs of *S. lomentaria* were more evenly distributed, constituting a composition of 14.4 ± 0.23 % L-Glu, 18.0 ± 0.12 % L-Gln and 13.6 ± 0.22 % L-Ala (Fig. 5A). Similarly, the *Rhodophyceae* species showed distinct FAA patterns, always containing at least 25 % L-Glu, 13 % L-Gln and 10 % L-Asp. A notable exception was L-Phe, which constituted 24.6 ± 1.2 % of the FAA profile of *C. crispus* and only 1.84 ± 0.19 % of *G. turuturu*. The FAA patterns of the investigated *Chlorophyceae* were less similar. While both *Ulva* sp. and *C. fragile* were characterized by a majority of L-Gln (19.2 ± 0.34 % and 37.7 ± 0.40 % respectively), other major constituents differed greatly. Whereas *Ulva* sp. contained large amounts of L-Ala (15.8 ± 0.35 %) and L-Cys (13.0 ± 0.58 %), major FAAs in *C. fragile* consisted of L-Glu (17.8 ± 0.64 %) and L-Asn (12.4 ± 0.14 %), amongst others.

Less patterns were discernible in the free nucleotides of the investigated seaweed species (Fig. 5B). While all species of *Phaeophyceae* contained large amounts of UMP (over 20 %), other nucleotides diverged more in composition. The main free nucleotide of *S. muticum* and *S. lomentaria* was IMP (45.7 ± 1.6 % and 37.1 ± 1.9 % respectively), while the free nucleotides of *U. pinnatifida* predominantly consisted of AMP (38.2 ± 2.0 %) and only little IMP (4.96 ± 0.67 %). Concerning *Chlorophyceae*, both *Ulva* sp. and *C. fragile* were found to contain over 16 % of TMP and at least 15 % dCMP, while no patterns could be discerned from the other free nucleotides of this class. The same was true for species of *Rhodophyceae*, of which both *C. crispus* and *G. turuturu* contained large amounts of UMP (23.8 ± 2.7 % and 23.3 ± 0.49 % respectively) and CMP (17.1 ± 0.55 % and 59 ± 1.6 % respectively), all other free nucleotides diverged in composition. However, while little patterns could be derived from the present free nucleotides of the *Rhodophyceae*, there was a consistent absence of GMP, dGMP, IMP and dIMP in all investigated species of this class. The consistent absence of certain free nucleotides was not found for the other classes.

There were significant differences between the mean concentration of all umami components, *i.e.* L-Asp, L-Glu, AMP, GMP, and IMP, of all analysed seaweed species ($P < 0.0001$ for all components). While all seaweed species contained L-asp and L-Glu, AMP could not be found in *S. lomentaria* and *Ulva* sp.. Moreover, of the *Phaeophyceae* only *U. pinnatifida* contained GMP and none of the examined *Rhodophyceae* contained GMP nor IMP. The latter nucleotide could not be found in *Ulva* sp. either. Comparing the mean concentration of free umami amino acids and nucleotides per seaweed class, significant differences were found for all compounds ($P < 0.0001$ in all cases) except for AMP. L-Asp was found to be significantly lower in *Chlorophyceae* (319 ± 144 mg/kg

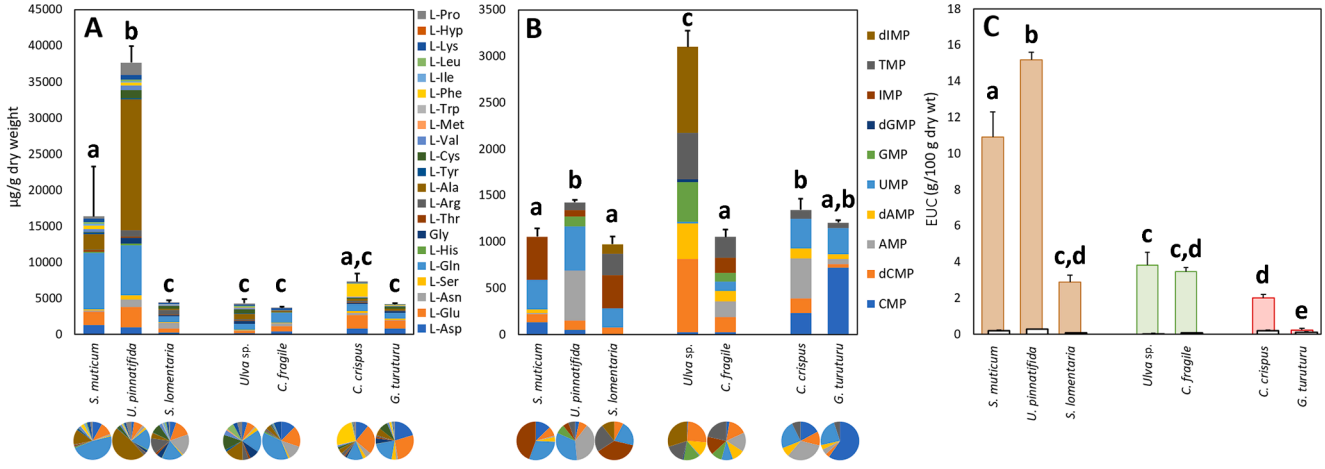

Fig. 5. Absolute and relative extraction amount of free amino acids (A), free nucleotides (B) and corresponding Equivalent Umami Concentration (C) of selected seaweed species from the Eastern Scheldt. EUC values (C) determined without nucleotide synergy are marked in white. Values are given as mean ± S.D. (n = 3). Means with different letters are significantly different from one another according to a Tukey HSD post-hoc following a one-way ANOVA. See supplement tables S9 and S10 for exact concentrations and table S11 for the exact EUC values.

DW) than in both *Phaeophyceae* (776 ± 479 mg/kg DW, $P = 0.042$) and *Rhodophyceae* (825 ± 63 mg/kg DW, $P = 0.035$). L-Glu was also found to be higher on average in species of *Phaeophyceae* than in those of *Chlorophyceae* (1754 ± 973 vs. 478 ± 197 mg/kg DW, $P = 0.0074$) while no significant differences were present between the average L-Glu content of *Rhodophyceae* and *Chlorophyceae* species, nor of *Phaeophyceae* and *Rhodophyceae* species. Contrary to these observations, species of *Chlorophyceae* contained on average more GMP (261 ± 182 mg/kg DW) than *Phaeophyceae* species ($P = 0.0017$) of which only *U. pinnatifida* contained GMP (108 ± 2.8 mg/kg DW). GMP could not be found in *Rhodophyceae* species, nor could IMP, while all *Phaeophyceae* species contained IMP; the average concentration in *Phaeophyceae* (301 ± 181 mg/kg DW) was significantly higher than in *Chlorophyceae* ($P = 0.012$), of which only *C. fragile* contained IMP (160 ± 19 mg/kg DW). A complete dataset of the free amino acid and nucleotide content of the seven seaweed species can be found in the supplement in tables S9 and S10.

Taste activity values were determined to assess these differences in umami compounds in relation to their minimally perceivable concentration. All species contained L-Glu in excess of the minimum threshold. The TAV for L-Glu was largest in *U. pinnatifida* (9.5 ± 0.09), followed by *C. crispus* (6.2 ± 1.1), *S. muticum* (5.9 ± 0.7), *G. turuturu* (3.7 ± 0.2), *C. fragile* (2.1 ± 0.1), *S. lomentaria* (2.1 ± 0.1) and *Ulva* sp. (1.0 ± 0.2). Contrasting this, only one species contained L-Asp in excess of the threshold concentration, being *S. muticum* (TAV = 1.3 ± 0.05). Only the *Phaeophyceae* contained perceivable amounts of IMP, with *S. muticum* and *S. lomentaria* showing a respective TAV of 1.8 ± 0.1 and 1.4 ± 0.2. While the TAV for IMP of *U. pinnatifida* was lower than 1, it was the only species of *Phaeophyceae* where the TAV for AMP exceeded 1 (4.3 ± 0.2). Of the investigated species, only *C. fragile* and *C. crispus* showed a similarly perceivable TAV for AMP, being 1.3 ± 0.2 and 3.5 ± 0.3 respectively. None of the species contained GMP concentrations exceeding the thresholds, and neither *Ulva* sp. nor *G. turuturu* were able to exceed the thresholds for any of the 5′-nucleotides.

The differences in free amino acids and nucleotides between the analysed seaweed species were reflected in the observed EUC values. As can be seen in Fig. 5C (supplement table S11), the highest synergistic EUC belonged to *U. pinnatifida* with a mean value of 15.2 ± 0.42 g/100 g DW, while the lowest EUC, 0.265 ± 0.066 g/100 g DW was found in *G. turuturu*. These species belong to the class of *Phaeophyceae* and *Rhodophyceae* respectively, whose average EUC mirrored the highest and lowest of the individual species. The average EUC of the *Phaeophyceae* species was significantly higher than that of the *Rhodophyceae* species (9.52 ± 5.8 vs. 1.14 ± 0.96 g/100 g DW, $P = 0.0019$) and that of the *Chlorophyceae* species (3.65 ± 0.51 g/100 g DW, $P = 0.026$); the mean EUC of the latter two species did not differ significantly. Comparing the synergistic umami concentration of the Dutch seaweed species with the umami concentration derived from their FAAs alone (basal umami) reveals substantial differences between the two. The synergistic EUC of *U. pinnatifida* is 15.2 ± 0.42 g/100 g DW, being the highest of the investigated species; the basal EUC of this same species is two orders of magnitude smaller at 0.293 ± 0.0025 g/100 g DW. This pattern repeats for nearly all species, *S. muticum* for instance decreases from 10.9 ± 1.38 to 0.194 ± 0.025 g/100 g DW and *Ulva* sp. from 3.82 ± 0.71 to 0.0316 ± 0.0056 g/100 g DW. Only the *Rhodophyceae* showed less substantial differences. The synergistic EUC of *C. crispus* is 2.01 ± 0.20 g/100 g DW while the basal EUC drops to 0.192 ± 0.033 g/100 g DW, and *G. turuturu* drops only half in EUC, from a synergetic 0.228 ± 0.11 g/100 g DW to a basal 0.119 ± 0.0058 g/100 g DW.

4. Discussion

4.1. Efficacy of the mouth-emulating solvents

Seaweed can play an important role in taking on the Zero Hunger Sustainable Development Goal, but in order to realise the potential of seaweed in the European cuisine, the characteristic taste of seaweed *i.e.* umami must be characterized. For this reason, we developed and validated an analytical procedure to analyse the free amino acids and 5′-nucleotides in seaweed, starting with a mouth emulating solvent system. Comparing the total amount of FAAs extracted by Milli-Q after 15 min and 24 h shows that the latter results in a significantly higher FAA content for *Ulva* sp. and *G. turuturu*. However, previous investigations using water as a solvent for green tea extractions reported FAA amounts reaching an optimum within a 20 to 40 min extraction window for both conventional (Xu, et al., 2018) and novel (*e.g.* ultrasonic) extraction methods (Das & Eun, 2018). Seaweeds exhibit endogenous proteolytic activities that are significantly higher than their microalgal counterparts, being comparable to terrestrial plants (Pérez-Lloréns, Benítez, Vergara, & Berges, 2003), and it has previously been shown that uninhibited proteolytic activity can lead to an accumulation of additional FAAs during sample treatment (Carrea, et al., 1993). It is therefore likely that the increase in FAA content following the 24-hour Milli-Q extraction originates from endogenous peptides that were enzymatically degraded during the extraction procedure. There were no differences in the total FAA content extracted by Milli-Q and the α-amylase solution after 15 min, illustrating that the presence of α-amylase in the mouth environment does not affect the extraction of bulk FAA's. This is to be expected, considering the non-proteolytic nature of α-amylase (Ramasubbu, Paloth, Luo, Brayer, & Levine, 1996). Despite this, the addition of α-amylase did yield significant changes in composition for all three seaweed species. Contrary to α-amylase, saliva as a whole is characterised by a large amount of endogenous and exogenous proteolytic activities (Helmerhorst & Oppenheim, 2007) which are capable of releasing free amino acids from larger peptides (Lightfoot & Coolidge, 1959). This is reflected in the saliva extractions of both *Ulva* sp. and *G. turuturu*, where saliva produced highly deviating FAA profiles, and for *G. turuturu* resulted in a significantly higher FAA content relative to the Milli-Q extraction of the same time. This combined with the cumbersome production of saliva and the FAA's native to its matrix, prevent saliva from being a viable mouth-emulating extraction solvent. Artificial saliva was unusable as a solvent, because the abundant divalent ions in the artificial salvia likely inhibited the derivatization of the FAAs through complexion of the $–NH_2$ groups (Yin, Liu, Yi, Wang, & Zhang, 2017).

Contrary to the FAAs, there were no significant differences between the total free nucleotide content extracted by Milli-Q after 15 min and 24 h, for any of the seaweed species. Instead, the different timeframes were characterized by widely varying nucleotide compositions. Several mechanisms for altered nucleotide profiles have been previously proposed, such as the enzymatic degradation of RNA into 5′-nucleotides (Peinado, Girón, Koutsidis, & Ames, 2014) or the dephosphorylation of nucleotide triphosphates and diphosphates to form monophosphates (Nguyen & Sporns, 1985). However, such mechanisms are accompanied by an increase in nucleotide concentration, and the lack thereof between the 24-hour and 15-minute Milli-Q extractions suggest that the change in composition is not a result of such mechanisms, but rather of interconversion of free nucleotide species. Similar mechanisms have previously been reported in red seaweed species, where deaminase activity led to the conversion of IMP from AMP (Nakamura, Akagawa, Ikawa, & Kawanobe, 1968). As such, the 15-minute Milli-Q extraction confers less nucleotide interconversion as the 24-hour extraction and thereby produces a free nucleotide profile that is truer to what would be experienced in a tasting environment. This is in line with previous investigations using the same solvent, wherein the extraction optimum for brown algae was found to be 30 min (Cao, Duan, Guo, Guo, & Zhao, 2014). As both free amino acid and free nucleotide profiles are enzymatically altered during the 24-hour extraction process in a way that deviates from what would be experienced in the mouth environment, this extraction method is not viable for an unbiased taste assessment of seaweed. Taking the above into account, it is clear that the Milli-Q extraction for 15 min presents the best choice for the extraction of free amino acids and nucleotides.

4.2. Recovery of the cation-exchange chromatographic steps

To minimize analyte loss during the cation-exchange step, we compared two methods and characterized their amino acid recoveries. Employing the DOWEX batch method resulted in a significantly higher recovery than when the continuous DOWEX method was used, suggesting the former is better suited for the clean-up of FAA extracts. This may be due to the finite time available in a continuous system for equilibration to take place between the FAAs and the cation exchange resin. These times differ per amino acid (Hirs, Moore, & Stein, 1954) and the discrepancy between the continuous and batch FAA recovery may be due to premature elution of some FAAs in the continuous method. However, four out of five amino acids showing significantly improved recoveries are hydrophobic. These are retained more strongly on the DOWEX resin than acidic or basic amino acids and when aromatic, may even undergo additional interaction with the resin, depending on concentration (Lee & Hong, 1995). It is therefore unlikely that of all amino acids, these would prematurely elute during the continuous DOWEX method. Previously reported recoveries vary widely, ranging from 43 % (Veuger, Middelburg, Boschker, & Houtekamer, 2005) to over 90 % (Takano, Kashiyama, Ogawa, Chikaraishi, & Ohkouchi, 2010). The average recovery of the batch method (65.0 ± 11 %,) falls within this range. Still, the recovery does not approach the 90 % reported elsewhere and accordingly, requires active monitoring through the use of reference standards. Correcting with only the internal standard does not suffice due to the differences in the recovery of each individual FAA.

During the evaporation step of care must be taken to minimize the loss of FAAs. We found that contrary to the use of nitrogen blanketing, the evaporation temperature had relatively little effect. Both with and without nitrogen blanketing, only L-Met, L-Lys and L-Trp had significantly worse recovery at ambient temperature than at 35 °C. Methionine is especially susceptible to oxidation, forming methionine sulfoxide (Hu, Qin, Xue, Fink, & Uversky, 2008). This compound is known to further react with other amino acids, particularly with L-Trp and L-Tyr (Lamp, Kaltschmitt, & Lüdtke, 2018). It is therefore likely that the increased evaporation time at ambient temperature presented a greater opportunity for oxidation of these amino acids to take place, resulting in their lower recovery. The small effect of temperature is in line with the literature, where temperatures up to 40 °C are considered mild for this purpose (Hirs, Moore, & Stein, 1954) and temperatures in excess of 60° C have been reported (Veuger, Middelburg, Boschker, & Houtekamer, 2005). Conversely, the use of nitrogen blanketing is of bigger influence; regardless of evaporation temperature, the use of nitrogen blanketing improved the recovery of every amino acid by at least 0.9 % and up to 12.7 %. All amino acids are susceptible to oxidation (Hu, Qin, Xue, Fink, & Uversky, 2008), and clearly the introduction of oxygen during the evaporation step is detrimental.

4.3. Umami components of seven Eastern Scheldt seaweeds

The free amino acid content of the Eastern Scheldt samples were in agreement with, and shared a similar composition to previously reported values for the same species of *Phaeophyceae* (Tsekos, Margaris, & Haritonidis, 1978) (Cao, Duan, Guo, Guo, & Zhao, 2014) (Vieira, et al., 2018), *Chlorophyceae* (Tsekos et al., 1978) and *Rhodophyceae* (Vieira, et al., 2018). There are notable differences between the FAA content observed in this study and those previously reported such as for *S. lomentaria*, where we found the highest FAA concentration to belong to L-Asn, while Tsekos et al. (1978) report this to be L-Glu, followed by L-Asn. However, these differences are mostly subtle and can be attributed to seasonal and regional changes (Sun, et al., 2012). The total FAA content of the Eastern Scheldt seaweeds was within the same order of magnitude as previously reported values with the exception of *C. crispus* (7229 μg/g DW versus the 11252 μg/g reported by Vieira et al. (2018)). While far less literature is available on the free nucleotide content of seaweed, total content and composition values that *are* available are within the same order of magnitude as measured in this study (Peinado, Girón, Koutsidis, & Ames, 2014).

The FAA profiles of the analysed seaweed species can give valuable information on the flavour of the seaweed. The amino acids which characterize the FAA profiles of the *Phaeophyceae*, being L-Glu, L-Gln and L-Ala, are known to impart an umami (L-Glu) or sweet (L-Gln, L-Ala) taste (Kawai, Sekine-Hayakawa, Okiyama, & Ninomiya, 2012). As such, both *S. muticum* and *U. pinnatifida* are expected to have a sweet quality due to their high L-Gln and L-Ala content, while the balance of L-Glu to L-Gln and L-Ala in *S. lomentaria* may impart an even distribution of sweet and umami taste. Similarly, the *Rhodophyceae* are characterizable by their high L-Glu and L-Asp content, both of which impart an umami taste. While both *C. crispus* and *G. turuturu* contain moderate amounts of L-Glu and are thus expected to be partially sweet, the FAA profile of *C. crispus* contains almost 25 % L-Phe, which imparts a bitter taste (Kawai, Sekine-Hayakawa, Okiyama, & Ninomiya, 2012). The *Chlorophyceae* are difficult to group together based on probable taste. *C. fragile* is very similar to the *Rhodophyceae* in its FAA profile and is expected to have similar flavour qualities because of it, while *Ulva* sp. is particularly low in umami amino acids and contains relatively large percentages of L-Gln, L-Ala and L-Cys, leading to a bittersweet quality (Kawai, Sekine-Hayakawa, Okiyama, & Ninomiya, 2012).

While umami was first described as a fifth primary taste after the isolation of L-Glu from seaweed (Ikeda, 2002), subsequent investigations into the EUC of seaweeds are rare. A number of *Phaeophyceae* have been investigated for their EUCs, ranging from approximately 2 to 74 g MSG/100 g (Peinado, Girón, Koutsidis, & Ames, 2014), illustrating the range of concentrations that umami components can take within a single phylum. The Eastern Scheldt seaweeds display EUC values within this range with the exception of *G. turuturu* (0.26 ± 0.066 g MSG/100 g), albeit with less disparity (supplement table S11). More recently, Milinovic et al. (2020) investigated EUC values for Portuguese seaweeds, describing values substantially lower than those we describe here. This may be attributable to regional or seasonal changes, or the fact they did not detect any free 5′-nucleotides and thus did not capture the synergistic effect between free amino acids and 5′-nucleotides on the umami intensity (Yamaguchi, Yoshikawa, Ikeda, & Ninomiya, 1971). Comparing the synergistic umami concentration of the Dutch seaweed species with the basal umami from their FAAs alone reveals the substantial effect the free 5′-nucleotides have on the umami intensity of seaweed. For nearly all investigated species, the synergy between FAAs and FNs leads to an EUC that is approximately two orders of magnitude larger than the EUC from FAAs alone. This is especially remarkable considering the TAV of the umami components in the investigated species; while the nucleotide concentrations in the analyzed seaweeds rarely exceed the taste threshold concentration, they substantially increase the equivalent umami concentration value in all species. One of the more striking examples is that of *Ulva* sp., none of whose nucleotides exceed the taste threshold concentration, yet whose basal EUC is 81 % lower than its synergistic one.

Of further interest is that there were distinct patterns in EUC per phylum. The EUC (synergistic) increased in the order *Rhodophycea* (≈ 1.1 g MSG/100 g), *Chlorophyceae* (≈ 3.7 g MSG/100 g) and *Phaeophyceae* (≈ 9.5 g MSG/100 g) and it follows that different phyla may find specific applications for different food applications. A prevalent umami taste may contribute to the increased culinary use of seaweeds (Mouritsen, Duelund, Petersen, Hartmann, & Frøst, 2019) and to their acceptance as a novel food item in general (Bellisle, 2008). Thus, selected cultivation of species with a high EUC such as *U. pinnatifida* or *S. muticum* may result in the increased use of seaweed as a sustainable, locally sourced ingredient. Species such as *Ulva* sp. or *S. lomentaria* may be preferable when a milder umami taste is needed, with *S. lomentaria* having particular potential for those applications requiring sweetness.

5. Conclusion

We have developed and validated a versatile methodology for the extraction, clean-up and complete separation of 21 free amino acids and 10 free nucleotides from seaweed. Optimal extraction conditions for free amino acid and nucleotide extraction were found by agitating seaweed in Milli-Q for 15 min, followed by acid treatment and a 10 kDa filtration step. For the amino acid clean up the highest recovery was achieved by using the described batch DOWEX treatment followed by evaporation at 35 °C under a nitrogen blanket. This methodology was successfully applied to determine the EUC values of seven seaweed species native to the Netherlands. The accurate prediction of umami intensity plays an important role in tailoring seaweed cultivation and species selection towards a product that is readily accepted as a food source. Achieving the Zero Hunger Sustainable Development Goal requires the development of high value, functional foods; seaweed that is tailored to the European palette would be an excellent candidate to fill this role.

Statement of informed consent, human/animal rights

Informed consent, or human or animals are not applicable to this study.

CRediT authorship contribution statement

Tanja C.W. Moerdijk-Poortvliet: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Writing - original draft. **Dylan L.C. Jong:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing - original draft. **Roy Fremouw:** Formal analysis, Investigation, Methodology, Validation, Writing - review & editing. **Sandra Reu:** Conceptualization, Formal analysis, Methodology, Writing - review & editing. **Jose M. Winter:** Conceptualization, Writing - review & editing. **Klaas Timmermans:** Conceptualization, Writing - review & editing. **Geert Mol:** Conceptualization, Writing - review & editing. **Norbert Reuter:** Conceptualization, Methodology, Writing - review & editing. **Goverdina C.H. Derksen:** Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.131352.

References

Abe, I., Nakamura, K., Toyonaga, T., Kobara, Y., & Wasa, T. (1993). Determination of D, L-Amino Acids in Natural Water by Gas Chromatography with Enantiomer Labeling. *Analytical Sciences, 9*(6), 775–778. https://doi.org/10.2116/analsci.9.775

Bellisle, F. (2008). Experimental studies of food choices and palatability responses in European subjects exposed to the Umami taste. *Asia Pacific Clinical Nutrition, 17*, 376–379.

Cao, Y., Duan, J., Guo, J., Guo, S., & Zhao, J. (2014). Rapid determination of nucleosides, nucleobases and free amino acids in brown seaweeds using ultra-performance liquid chromatography coupled with triple quadrupole mass spectrometry. *Journal of Applied Phycology, 26*(1), 675–686. https://doi.org/10.1007/s10811-013-0079-3

Carrea, A., Canepa, A., Perfumo, F., Ancarani, P., Verrina, E., & Gusmano, R. (1993). Proteolytic activity and free amino acid concentrations in polymorphonuclear leucocytes. *Annals of Clinical Biochemistry, 30*(6), 559–564. https://doi.org/10.1177/000456329303000606

Das, P. R., & Eun, J.-B. (2018). A comparative study of ultra-sonication and agitation extraction techniques on bioactive metabolites of green tea extract. *Food Chemistry, 253*, 22–29. https://doi.org/10.1016/j.foodchem.2018.01.080

Duan, W., Huang, Y., Xiao, J., Zhang, Y., & Tang, Y. (2020). Determination of free amino acids, organic acids, and nucleotides in 29 elegant spices. *Food Science and Nutrition, 8*(7), 3777–3792. https://doi.org/10.1002/fsn3.v8.710.1002/fsn3.1667

Fitzgerald, C., Gallagher, E., Tasdemir, D., & Hayes, M. (2011). Heart health peptides from macroalgae and their potential use in functional foods. *Journal of Agriculture Food and Chemistry, 59*(13), 6829–6836. https://doi.org/10.1021/jf201114d

German Institute for Standardisation. (2010). *DIN 53160–1: 2010–10, Determination of the colourfastness of articles for common use - Part 1: Test with artificial saliva*. Berlin: German Institute for Standardisation.

Hafting, J. T., Craigie, J. S., Stengel, D. B., Loureiro, R. R., Buschmann, A. H., Yarish, C., … Graham, M. (2015). Prospects and Challenges for Industrial Production of Seaweed Bioactives. *Journal of Phycology, 51*(5), 821–837. https://doi.org/10.1111/jpy.12326

Harnedy, P. A., & FitzGerald, R. J. (2011). Bioactive proteins, peptides, and amino acids from macroalgae. *Journal of Phycology, 47*, 218–232. https://doi.org/10.1111/j.1529-8817.2011.00969.x

Helmerhorst, E. J., & Oppenheim, F. G. (2007). Saliva: A Dynamic Proteome. *Journal of Dental Research, 86*(8), 680–693. https://doi.org/10.1177/154405910708600802

Hirs, C. H. W., Moore, S., & Stein, W. H. (1954). The Chromatography of Amino Acids on Ion Exchange Resins. Use of Volatile Acids for Elution. *Journal of the American Chemical Society, 76*(23), 6063–6065. https://doi.org/10.1021/ja01652a059

Hu, D., Qin, Z., Xue, B., Fink, A. L., & Uversky, V. N. (2008). Effect of Methionine Oxidation on the Structural Properties, Conformational Stability, and Aggregation of Immunoglobulin Light Chain LEN. *Biochemistry-US, 47*(33), 8665–8677. https://doi.org/10.1021/bi800806d

Ikeda, K. (2002). New Seasonings. *Chemical Senses, 27*, 847–849. https://doi.org/10.1093/chemse/27.9.847

Kawai, M., Sekine-Hayakawa, Y., Okiyama, A., & Ninomiya, Y. (2012). Gustatory sensation of L- and D-amino acids in humans. *Amino Acids, 43*(6), 2349–2358. https://doi.org/10.1007/s00726-012-1315-x

Lamp, A., Kaltschmitt, M., & Lüdtke, O. (2018). Improved HPLC-method for estimation and correction of amino acid losses during hydrolysis of unknown samples. *Analytical Biochemistry, 543*, 140–145. https://doi.org/10.1016/j.ab.2017.12.009

Lee, K., & Hong, J. (1995). Nonionic adsorption of aromatic amino acids on a cation-exchange resin. *Reactive & Functional Polymers, 28*(1), 75–80. https://doi.org/10.1016/1381-5148(95)00085-X

Lightfoot, L. H., & Coolidge, T. B. (1959). The role of acidity of saliva in the nutrition of oral acidogens. *Journal of Dental Research, 38*(1), 96–100. https://doi.org/10.1177/00220345590380013001

Long, W. (2017). Automated Amino Acid Analysis Using an Agilent Poroshell HPH-C18 Column (Application Note no. 5991-5571EN). USA: Agilent Technologies, Inc.

Milinovic, J., Campos, B., Mata, P., Diniz, M., & Noronha, J. P. (2020). Umami free amino acids in edible green, red, and brown seaweeds from the Portuguese seashore. *Journal of Applied Phycology, 32*(5), 3331–3339. https://doi.org/10.1007/s10811-020-02169-2

Moerdijk-Poortvliet, T. C. W., Brasser, J., de Ruiter, G., Houtekamer, M., Bolhuis, H., Stal, L. J., & Boschker, H. T. S. (2014). A versatile method for simultaneous stable carbon isotope analysis of DNA and RNA nucleotides by liquid chromatography/isotope ratio mass spectrometry. *Rapid Communication in Mass Spectrometry, 28*(12), 1401–1411. https://doi.org/10.1002/rcm.6919

Mouritsen, O. G., Williams, L., Bjerregaard, R., & Duelund, L. (2012). Seaweeds for umami flavour in the new Nordic cuisine. *Flavour, 1*, Article 4. https://doi.org/10.1186/2044-7248-1-4

Mouritsen, O. G., Duelund, L., Petersen, M. A., Hartmann, A. L., & Frøst, M. B. (2019). Umami taste, free amino acid composition, and volatile compounds of brown seaweeds. *Journal of Applied Phycology, 31*(2), 1213–1232. https://doi.org/10.1007/s10811-018-1632-x

Nakamura, S., Akagawa, H., Ikawa, T., & Kawanobe, H. (1968). Separation and Identification of Nucleotides in Some Seaweeds. *Botanical Magazine Tokyo, 81*, 556–565. https://doi.org/10.15281/jplantres1887.81.556.

Neyraud, E., Palicki, O., Schwartz, C., Nicklaus, S., & Feron, G. (2012). Variability of human saliva composition: Possible relationships with fat perception and liking. *Archives of Oral Biology, 57*(5), 556–566. https://doi.org/10.1016/j.archoralbio.2011.09.016

Nguyen, T. T., & Sporns, P. (1985). Decomposition of the Flavor Enhancers, Monosodium Glutamate, Inosine-5'-Monophosphate and Guanosine-5'-Monophosphate during

Canning. *Journal of Food Science, 50*, 812–814. https://doi.org/10.1111/j.1365-2621.1985.tb13802.x

Peinado, I., Girón, J., Koutsidis, G., & Ames, J. (2014). Chemical composition, antioxidant activity and sensory evaluation of five different species of brown edible seaweeds. *Food Research International, 66*, 36–44. https://doi.org/10.1016/j.foodres.2014.08.035

Pérez-Lloréns, J. L., Benítez, E., Vergara, J. J., & Berges, J. A. (2003). Characterization of proteolytic enzyme activities in macroalgae. *European Journal of Phycology, 38*(1), 55–64. https://doi.org/10.1080/0967026031000096254

Ramasubbu, N., Paloth, V., Luo, Y., Brayer, G. D., & Levine, M. J. (1996). Structure of Human Salivary α-Amylase at 1.6 Å Resolution: Implications for Its Role in the Oral Cavity. *Acta Crystallographica D, 52*(3), 435–446. https://doi.org/10.1107/S0907444995014119

Searchinger, T., Waite, R., Hanson, C., & Ranganathan, J. (2019). *World Resources Report - Creating a Sustainable Food Future: A Menu of Solutions to Feed Nearly 10 Billion People by 2050*. Washington: World Resources Institute.

Sun, S.-M., Cho, S.-Y., Shin, T.-S., Chung, G.-H., Ahn, C.-B., & Kim, D. (2012). Variation in the chemical composition of Capsosiphon fulvescens with area and during the harvest period. *Journal of Applied Phycology, 24*(3), 459–465. https://doi.org/10.1007/s10811-012-9823-3

Takano, Y., Kashiyama, Y., Ogawa, N. O., Chikaraishi, Y., & Ohkouchi, N. (2010). Isolation and desalting with cation-exchange chromatography for compound-specific nitrogen isotope analysis of amino acids: Application to biogeochemical samples. *Rapid Communication in Mass Spectrometry, 24*(16), 2317–2323. https://doi.org/10.1002/rcm.4651

Tsekos, I., Margaris, N. S., & Haritonidis, S. (1978). Pools of Free Amino Acids in Greek Marine Algae. *Biochemical and Physiological Pfl, 167*(2), 165–172. https://doi.org/10.1016/S0015-3796(17)30773-4

United Nations. (2017). *World Population Prospects: The 2017 Revision, Key Findings and Advance Tables. Working Paper No. ESA/P/WP/248*. New York: United Nations.

Veuger, B., Middelburg, J. J., Boschker, H. T. S., & Houtekamer, M. (2005). Analysis of ^{15}N Incorporation into D-alanine: A new method for tracing nitrogen uptake by bacteria. *Limnology and Oceanography, Methods, 3*(5), 230–240. https://doi.org/10.4319/lom.2005.3.230

Vieira, E. F., Soares, C., Machado, S., Correia, M., Ramalhosa, M. J., Oliva-teles, M. T., … Delerue-Matos, C. (2018). Seaweeds from the Portuguese coast as a source of proteinaceous material: Total and free amino acid composition profile. *Food Chemistry, 269*, 264–275. https://doi.org/10.1016/j.foodchem.2018.06.145

Xu, Y.-Q., Ji, W.-B., Yu, P., Chen, J.-X., Wang, F., & Yin, J.-F. (2018). Effect of extraction methods on the chemical components and taste quality of green tea extract. *Food Chemistry, 248*, 146–154. https://doi.org/10.1016/j.foodchem.2017.12.060

Yamaguchi, S., Yoshikawa, T., Ikeda, S., & Ninomiya, T. (1971). Measurement of the relative taste intensity of some L-α-amino acids and 5'-nucleotides. *Journal of Food Science, 36*, 846–849. https://doi.org/10.1111/j.1365-2621.1971.tb15541.x

Yin, L.-H., Liu, X.-P., Yi, L.-Y., Wang, J., Zhang, Y.-J., & Feng, Y.-F. (2017). Structural characterization of calcium glycinate, magnesium glycinate and zinc glycinate. *Journal of Innovative Optical Health Sciences, 10*(03), 1650052. https://doi.org/10.1142/S1793545816500528