HEAT TREATMENT INCREASES THE PROTEIN BIOACCESSIBILITY IN THE RED SEAWEED DULSE (PALMARIA PALMATA), BUT NOT IN THE BROWN SEAWEED WINGED KELP (ALARIA ESCULENTA).

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ABSTRACT

Bioaccessibility of plant proteins has been shown to be inferior to that of proteins of animal origin. Heat treatment has been shown to positively affect this in some plants. The aim of this study was to investigate the effect of heat treatment on bioaccessibility of seaweed proteins. An *in vitro* gastrointestinal digestion model was used for evaluation of potential effects on the brown seaweed *Alaria esculenta* and the red seaweed *Palmaria palmata* proteins.

In *P. palmata*, the content of accessible amino acids increased by 86 - 109 % after heat treatment. Following a simulated *in vitro* gastrointestinal digestion the amount of liberated amino acids was 64 - 96 % higher in heat-treated samples compared to their raw counterparts. The increase was largest in samples boiled for 15 and 30 minutes. No deterioration of single amino acids was seen and hence, the amount of available essential amino acids was increased accordingly. In *A. esculenta* no equivalent changes were observed.

In conclusion, a short heat treatment may be a simple way of increasing the utilization potential of seaweed proteins in food and feed. However, there are species differences and the effects observed in the *in vitro* digestion model need to be confirmed in clinical studies.

INTRODUCTION

To meet the expected population growth there will be an increased demand for food in the coming decades. Cereals are, and probably will remain, the single most food energy source worldwide (WHO, 1995). However, the agriculture sector is already utilizing 30 % of the world's land area and 70 % of available freshwater. This sector is also a big contributor to the environmental challenge the world is facing, being responsible for nitrate and ammonia pollution of ground water, greenhouse gas emissions and deforestation (FAO, 2013). A further increase in this sector may intensify these environmental challenges and finding sustainable alternative food, in particular protein, sources should therefore be a priority (Gjedrem et al., 2012).

Marine seaweeds have previously been indicated to have great potential as alternative food sources (Fleurence et al., 2012; MacArtain et al., 2007). This is by virtue of their favorable growth conditions, including low nutrient demands, high growth rates and no need for freshwater or arable land areas. In addition, being a very diverse group of plants, they are abundant in marine environments all over the world (Bolton, 1994). In several studies, it has been shown that many seaweed species contain good quality protein in sufficient amounts to be used as biomass (substrate) for economically and environmentally justifiable large-scale protein (food) production (Kolb et al., 2004; Maehre et al., 2014; Taboada et al., 2013).

However, there are some challenges that must be addressed. Seaweeds are plants, and similar to most terrestrial plants, the digestibility of seaweed proteins is known to be inferior to proteins of animal origin. This has been attributed both to their complex polysaccharide structure, which may impede the accessibility of the proteins to the gastrointestinal enzymes

and to their content of anti-nutritional factors, such as phenolic compounds, phytic acids and protease inhibitors.

A large part of our diet is comprised of foods that are processed or heat treated. Heat treatment of foods has many rationales, such as improvement of taste and texture, food quality, safety and preservation of food products and ingredients (Finley et al., 2006). Additional positive effects of heat treatment, including increased bioavailability of certain nutrients and inhibition of anti-nutrients, have also been described (Dewanto et al., 2002; Hwang et al., 2012). However, heat treatment may also result in loss of some nutrients such as free amino acids (Dragnes et al., 2009; Larsen et al., 2007; Mierke-Klemeyer et al., 2008) and vitamins (Delchier et al., 2013; Gutzeit et al., 2008; Jakobsen and Knuthsen, 2014). For proteins, both advantages and disadvantages have been ascribed to processing and heat treatment (Meade et al., 2005). On one hand, heat treatment will lead to partially or complete denaturation of the original protein structure, making access easier for the gastrointestinal enzymes and hence, improving the utilization of the protein. On the other hand, it may result in decreased bioavailability due to amino acid racemization, protein crosslinking and increased reactivity of single amino acids, such as lysine.

The aim of this study was to investigate the effect of heat treatment on bioaccessibility of seaweed proteins. An *in vitro* gastrointestinal digestion model was used for evaluation of potential effects on the brown seaweed *Alaria esculenta* and the red seaweed *Palmaria palmata* proteins.

MATERIALS AND METHODS

Raw materials

Dried samples of the red seaweed *Palmaria palmata* and the brown seaweed *Alaria esculenta* were purchased from “Fremtidens Mat” (Oslo, Norway). According to the manufacturer, both species were harvested at the south coast of Iceland, flushed with seawater and dehydrated using electrical fans driven by geothermal energy in Iceland. The drying temperature was 40°C and the drying time was 24 hours. Flour samples (corn, rice and wheat) were purchased in a local supermarket.

Sample preparation

The dried seaweed samples (n = 5 for each species) were cut into pieces of 2x2 cm and divided into four different batches. One of the batches remained raw, while the other three were subjected to boiling in distilled water (1:20 w/v) for 15, 30 and 60 minutes. After boiling the samples were transferred to a sieve for removal of excess water and following cooling they were weighed in order to define the uptake of water during boiling. All samples were subjected to analysis of water content, amino acid composition (free and total) and a simulated gastrointestinal (GI) digestion. During the GI digestion procedure samples were collected after 5, 120 and 240 minutes, simulating the mouth, stomach and intestinal phases, respectively. These samples were subjected to analysis of amino acid composition (free and total). Samples of three different flours (corn, rice and wheat) were also subjected to the GI digestion. All chemicals used in this study were of analytical grade and purchased from Sigma Chemical Co (St.Louis, MO, USA) unless otherwise stated.

Simulated gastrointestinal digestion

The simulated GI digestion was performed according to Versantvoort et al. (2005) with an adaption, namely reducing the enzymes (amylase, pepsin and pancreatin) by 50 % due to a lower protein content in the algae samples in this study compared to the protein content of the samples in the original study. Approximately 1 g of the boiled and 0.5 g of the raw seaweed samples were mixed with 6 mL of saliva buffer (pH 6.80 ± 0.06) and homogenized with an Ultra Turrax T25 basic (IKA Werke GmbH, Staufen, Germany) for 30 seconds, followed by incubation at 37°C for 5 minutes under constant rotation. The pH of the digesta was measured, before centrifugation at 2750 x g for 3 minutes and collection of a 2 mL sample from the supernatant. To the rest of the digesta, 12 mL of gastric buffer (pH 1.30 ± 0.01) was added, followed by incubation at 37°C for 120 minutes under constant rotation. The sampling procedure was repeated, before adding 12 mL of duodenal buffer (pH 8.11 ± 0.02), 6 mL of bile buffer (pH 8.22 ± 0.04) and 2 mL of 1M $NaHCO_3$. The mixture was then incubated for another 120 minutes at the same conditions, before collection of the final sample. In order to inactivate the enzymes, all of the GI samples were heated at 90°C for 5 minutes and then put on ice. Pending analysis, the samples were kept frozen at -55°C. Samples without seaweed were subjected to the same procedure and used for adjustment of amino acid contribution from the digestive enzymes.

Water content

Water content was determined using a modified version of the AOAC method 950.46B (Horwitz, 2004). Approximately 1.5 g of seaweed material was dried at 105°C until constant weight and water content was determined gravimetrically. Analyses were performed in triplicate.

Protein and amino acid analysis

Free amino acids (FAA) in the non-digested samples were extracted according to Mierke-Klemeyer et al. (2008), by homogenizing approximately 1.0 g sample with 9 mL distilled H_2O and 1 mL 20 mM norleucine (internal standard) for 15 sec using an Ultra Turrax T25 basic (IKA Werke GmbH, Staufen, Germany). One mL of 35 % sulfosalicylic acid (SSA) was added for removal of proteins and large peptides, followed by homogenizing for another 15 sec and centrifugation at 4000 x g for 10 minutes. Prior to analysis aliquots of 200 µL of the supernatants were diluted 1:5 in lithium citrate buffer at pH 2.2. The extraction of FAAs in the digested samples was performed according to Ytrebo et al. (2009), mixing 360µL of digesta with 40µL of norleucine and 40µL SSA, followed by vortexing and centrifugation at 20000 x g for 5 minutes. An aliquot of 100µL was diluted 1:1 in lithium citrate buffer at pH 2.2.

For analysis of total amino acids (TAA) in the non-digested samples, approximately 200 mg of the boiled samples and 50 mg of the raw samples were dissolved in a mixture of 0.7 mL distilled H_2O and 0.5 mL 20 mM norleucine (internal standard). Concentrated hydrochloric acid (HCl, 12 M) was added to obtain a final concentration of 6 M. In the digested samples 500µL of digesta was mixed with 50µL of norleucine and 550µL of 12M HCl. In order to minimize oxidation, samples were flushed with nitrogen gas for 15 seconds before hydrolysis at 110°C for 24 hours according to Moore and Stein (1963). Following hydrolysis, 100 µL aliquots of the hydrolysates were evaporated under nitrogen gas until complete dryness. Prior to analysis the samples were re-dissolved to a suitable concentration in lithium citrate buffer at pH 2.2.

All amino acids were analyzed chromatographically and identified as described previously (Maehre et al., 2013), using a Biochrom 30 amino acid analyzer (Biochrom Co, Cambridge, UK). Protein content was calculated from the sums of individual amino acid residues (the molecular weight of each amino acid after deduction of the molecular weight of water) as recommended by FAO (2003).

Light microscopy

Small pieces of non-cooked and 60 min cooked algae tissue were cut and prepared with razor blades and embedded in a drop of water. Preparations were examined with a Leica DM6000 B microscope.

Statistics

Statistical analysis was performed using SPSS 21 (SPSS Inc, Chicago, IL, USA). Tests of normality (Shapiro-Wilk's test) and homogeneity of variance (Levene's test) returned normal distribution with unequal variance for all species and chemical variables. Hence, one-way analysis of variance (ANOVA) was performed, followed by the Dunnet's T3 post-hoc test for evaluation of statistics. Means were considered significantly different at $p < 0.05$.

RESULTS AND DISCUSSION

Selection of raw materials

In our previous study (Maehre et al., 2014), we found that some seaweed species had both higher protein content and higher content of essential amino acids (EAAs), than flours from wheat, rice and corn and that these seaweed species therefore could be a valuable complement to cereals as protein sources in food and feed.

Of the species analyzed in the aforementioned study, the red seaweed *P. palmata* was found to have the highest protein content and a very high content of EAAs. This was the basis for choosing this alga as the primary raw material for the present study on protein bioaccessibility.

In Norway there is currently a great interest in aquaculture of seaweeds, mostly of brown seaweeds. In general, brown seaweeds contain approximately half the amount of proteins compared to red seaweeds (Dawczynski et al., 2007; Misurcova et al., 2010). One well-known exception to this is *Undaria pinnatifida* (wakame), whose protein content has been shown to be comparable to some of the red seaweeds (Dawczynski et al., 2007; Taboada et al., 2013). In our previous study also the winged kelp, *A. esculenta*, was shown to be higher in protein than the other brown algae (Maehre et al., 2014). As this alga is one of the species considered for aquaculture in Norway, we decided to include it in the present study.

As the biochemical composition of algae is known to pose significant geographical and seasonal variations, and in order to ensure a stable delivery of raw material, we decided to use commercially available seaweeds for the present study.

Water content and uptake

The water content in the provided dried samples was significantly different between the two species, being 170 g kg^{-1} in *A. esculenta* and 282 g kg^{-1} in *P. palmata*, respectively (table 1). This result is within the range given in other reports for *A. esculenta*, but it is somewhat higher for *P. palmata* (Indergaard and Minsaas, 1991; Maehre et al., 2014). Seasonal and geographical variations in the biochemical composition of seaweeds have been reported (Galland-Irmouli et al., 1999; Rodde et al., 2004) and this together with

incomplete/inconsistent drying of the commersial algae could explain the high water content in *P. palmata*.

The water content in the samples after boiling was in the range 850 - 880 g kg^{-1} seaweed, not significantly different between the different boiling times within the same species, but slightly higher in *P. palmata* than in *A. esculenta*. In order to facilitate the comparison between raw and heat treated samples, further results in this paper are reported in g kg^{-1} DW.

Accordingly, the water uptake during boiling was significantly different between the species, being around three times higher in *A. esculenta* than in *P. palmata*. The previously mentioned difference in raw material water content is one possible explanation to this. An alternative explanation is the difference in cell wall composition between brown and red seaweeds. A major constituent in all plant and algal cell walls are complex polysaccharides, mostly fibers. Polysaccharides are very heterogeneous compounds, having very different properties. In brown algae the main polysaccharide is cellulose, while red algae, in addition to cellulose, contain large amounts of different xylans (Galland-Irmouli et al., 1999; Popper et al., 2011; Rodde et al., 2004). As reviewed by Bocanegra et al. (2009), these differences could affect water-holding capacity (WHC), water-binding capacity (WBC) and swelling capacity (SWC), which are important variables for the hydration properties.

Protein and amino acid composition

The FAA and TAA compositions of the two algae species are shown in tables 2 and 3, respectively. These are variables which are known to show great seasonal and geographical variations (Galland-Irmouli et al., 1999; Rodde et al., 2004). In both species the FAAs of the raw samples were lower than previously reported (Maehre et al., 2014). In addition to the

mentioned natural variations, this may be due to different handling and processing procedures prior to analysis. In *A. esculenta*, both TAAs and the relative amount of essential amino acids (EAA), which are the nine amino acids that cannot be synthesized *de novo* by humans, was higher (Maehre et al., 2014). In *P. palmata* both TAA level and relative amount of EAAs were within the same ranges as previously reported (Galland-Irmouli et al., 1999; Maehre et al., 2014).

The levels of FAAs decreased in both species as a result of boiling in water. This is due to their high water solubility and in accordance with other studies on losses of low-molecular compounds during household preparations (Dragnes et al., 2009; Larsen et al., 2007; Mierke-Klemeyer et al., 2008).

In most studies on how heat treatment affects plant protein content, no effect or a slight decrease in protein content after cooking has been demonstrated (Avanza et al., 2013; Ee and Yates, 2013; Grewal and Jood, 2009; Lima et al., 2009; Ramirez-Moreno et al., 2013). This may be due to the choice of analytical method. The most common method for determination of crude protein content is by analyzing total nitrogen and converting it into protein by use of a nitrogen-to-protein conversion factor, the Kjeldahl method. The sample preparation used in this analytical method are very harsh compared to normal food processing, involving digestion in concentrated sulfuric acid at a very high temperature (> 400°C) for several hours. As a result of this processing the structure of the sample is completely broken down and all nitrogen present is released into the acid, whether it is available for gastrointestinal digestion or not. This is therefore not an optimal method for detecting differences in protein content as a result of processing.

As previously mentioned, the structure of plant materials is made up of cell wall polysaccharides as main constituents, giving them a rigid and hard surface. Within these structures, lipids, proteins and other nutrients interact with the complex polysaccharides that prevent accessibility to the hydrolytic (proteolytic) enzymes of the digestion. Applying heat and water normally results in a weakening of the original structure, leaving the texture softer and less rigid (Sharma et al., 2012). Increased bioaccessibility of certain nutrients, such as carotene from carrots and lycopene from tomatoes (Dewanto et al., 2002; Hwang et al., 2012), as a result of heat treatment has also been reported. Polysaccharide and protein contents and composition vary considerably between different plants and heat treatment will therefore affect each structure differently. In *A. esculenta* there were no changes in the contents of TAAs or EAAs after boiling and neither was there an apparent change in texture. In *P. palmata*, however, all of these variables were affected by the heat treatment. Both TAAs and EAAs increased significantly after boiling and also the structure was considerably softer after boiling. These differences are illustrated in figure 1, where microscopy images of raw and boiled *P. palmata* (A and B) and *A. esculenta* (C and D) are shown. The texture of P. palmate is rather mushy after cooking, and from the micrographs it is evident that *P. palmata* loose pigments, cellular and tissue integrity upon cooking, and large parts of the epidermial layer are absent from the surface. Apart from some changes in cell size *A. esculenta* on the other hand appears unaffected by cooking.

In vitro digestibility and bioaccessibility of proteins

Protein digestion *in vivo* is a complex process involving an interaction between a series of enzymes. A variety of different *in vitro* model systems mimicking this process is being and has been used in order to study protein digestibility. There are large differences between these model systems, regarding their choice of type and concentration of enzymes, reaction times, pH adjustments, endpoints etc. and care should therefore be taken when comparing results from studies using different model systems.

In this study, raw and boiled samples of *A. esculenta* and *P. palmata* were subjected to the *in vitro* simulated gastrointestinal (GI) digestion model described by Versantvoort et al. (2005), reducing the enzyme amounts in the buffers to half of the original amount due to substantially lower protein content in the seaweed raw materials compared to those used in the original study. This model includes the three main proteases involved in the protein digestibility, pepsin, trypsin and chymotrypsin. In addition, it includes enzymes involved in carbohydrate and lipid digestion, such as amylase and lipase. Due to the complexity of the raw material in this study, this method was therefore considered to be superior to methods only including proteases, although the main purpose of the study was to examine the protein digestibility.

As shown in figures 2 and 3, the amount of TAAs and FAAs liberated into the digestion fluid increased throughout the digestion process for all samples. In *P. palmata* the amount of liberated amino acids were higher at the end of the GI digestion process in the heat treated samples than in the raw sample, although significant only for 15 and 30 minutes. A similar effect could not be seen in *A. esculenta*. Among the flour samples, the liberation of amino acids was highest in the wheat samples.

The challenge of overcoming the digestibility issue of plant proteins has been focus for many studies and different processing strategies have been suggested in order to improve it. Both common dietary plants and underutilized plant species that may have potential as protein sources have been subject to these studies and by far, legumes are the best documented group of plants. Most of the studies have found that processing in general improves the digestibility. The digestibility of raw legumes has been reported to be 65-85 % and boiling in water has been shown to increase digestibility by 3-10 %. Another finding is that combining several processing techniques increases the digestibility even further. The improvement in

digestibility during processing has mostly been attributed to inhibition of anti-nutrients in the plant materials (Avanza et al., 2013; Kalpanadevi and Mohan, 2013; Shimelis and Rakshit, 2007; Vijayakumari et al., 2007).

For seaweeds, however, the results on *in vitro* digestibility are more widespread. Different studies have reported *in vitro* digestibility of red seaweeds ranging between 2 – 90 % (Cian et al., 2014; Galland-Irmouli et al., 1999; Machu et al., 2014; Marrion et al., 2005; Misurcova et al., 2010; Wong and Cheung, 2001). In studies where brown and green seaweeds have been examined, their protein digestibility has mostly been shown to be lower than for the red ones (Misurcova et al., 2010; Wong and Cheung, 2001). A thorough literature search has not revealed other studies concerning processing and digestibility of seaweeds.

Overall effects

In *P. palmata*, the results showed that the total amino acid content on a dry weight basis increased by 86 - 109 % after heat treatment (table 3). Boiling increased the liberation of total amino acids through the simulated gastrointestinal digestion process by 64 - 96 %, where the largest increase was seen in the samples boiled for 15 and 30 minutes (figure 2a). No deterioration of single amino acids was seen as a result of the heat treatment and hence, the amount of available essential amino acids was increased accordingly. In *A. esculenta* no equivalent changes were observed.

An adequate intake of EAAs is necessary in order to maintain health and when increasing the food production, ensuring this should be among the main targets. The World Health Organization (WHO) has defined a reference protein which has the required composition of EAAs and an ideal food protein source should have a composition similar to this reference

protein (WHO, 2002). Proteins of animal origin normally fulfill this pattern, whereas plant proteins often are deficient in one or more of the EAAs. In figure 4 the EAA compositions of the proteins of *P. palmata* (raw and boiled for 30 minutes), along with wheat, rice and corn flours are presented related to the reference protein. From this it is evident that both raw and boiled *P. palmata* proteins are able to cover the human requirements for EAAs and that no deterioration in single EAAs was seen as a result of the heat treatment. The flours are also able to cover the requirements of most EAAs, except for lysine, which is known to be the limiting EAA in most cereal proteins. However, also the protein content of a food item determines the total intake of EAAs in the diet. Figure 5 illustrates the amount of EAAs liberated after simulated GI digestion of equal amounts of the same five food items. Here it is evident that the increased available protein in *P. palmata* as a result of boiling improves the total dietary intake of EAAs, both compared to its raw counterpart and to the three cereal flours. Boiled *P. palmata* could therefore be a valuable protein supplement in a diet low in animal protein.

CONCLUSIONS

The results from this study showed that boiling of *P. palmata* increased the amount of bioaccessible protein, with no deterioration of the amino acid composition. The total amount of available essential amino acids was therefore increased accordingly. In *A. esculenta* no equivalent changes were observed, probably due to the rough texture of this alga. In conclusion, a short heat treatment may be a simple way of increasing the utilization potential of seaweed proteins in food and feed. However, there are species differences and effects observed in *in vitro* digestion models have to be confirmed in clinical studies.

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Table 1: Water content and water uptake in raw and boiled (15, 30 and 60 minutes) *Alaria esculenta* and *Palmaria palmata*. Values are reported as mean ± SD (n = 5). Units are g kg^{-1} for water content and % for water uptake, respectively. Different letters in the same row indicate significant differences ($p < 0.05$)

	Alaria esculenta				***Palmaria palmata***			
	Raw	Boiled 15 min	Boiled 30 min	Boiled 60 min	Raw	Boiled 15 min	Boiled 30 min	Boiled 60 min
Water content	17.0 ± 1.1^{a}	85.2 ± 1.6^{cd}	85.6 ± 0.8^{c}	86.8 ± 1.1^{cd}	28.2 ± 3.5^{b}	86.9 ± 0.3^{cd}	87.4 ± 0.7^{cd}	87.6 ± 0.3^{d}
Water uptake		309.0 ± 17.5^{b}	331.8 ± 14.7^{b}	365.6 ± 24.2^{b}		121.2 ± 11.8^{a}	117.4 ± 15.3^{a}	118.0 ± 11.7^{a}

Table 2: Free amino acid content in raw and boiled (15, 30 and 60 minutes) *Alaria esculenta* and *Palmaria palmata*. Values are reported as mean ± SD and in mg AA g^{-1} DW (n = 5). Different letters in the same row indicate significant differences ($p < 0.05$). bdl. = below detection limit

	Alaria esculenta				***Palmaria palmata***			
	Raw	Boiled 15 min	Boiled 30 min	Boiled 60 min	Raw	Boiled 15 min	Boiled 30 min	Boiled 60 min
Essential amino acids (EAA)								
Threonine	0.3 ± 0.0^{c}	0.1 ± 0.0^{ab}	0.1 ± 0.0^{ab}	0.1 ± 0.1^{abc}	0.1 ± 0.0^{b}	bdl.^{a}	bdl.^{a}	bdl.^{a}
Valine	0.2 ± 0.1	bdl.	bdl.	bdl.	0.1 ± 0.0	bdl.	bdl.	bdl.
Methionine	Traces	bdl.	bdl.	bdl.	bdl.	bdl.	bdl.	bdl.
Isoleucine	Traces	bdl.	bdl.	bdl.	Traces	bdl.	bdl.	bdl.
Leucine	0.1 ± 0.0^{b}	bdl.^{a}	bdl.^{a}	Traces^{ab}	0.1 ± 0.0^{b}	0.1 ± 0.0^{ab}	bdl.^{a}	bdl.^{a}
Phenylalanine	0.1 ± 0.0	bdl.	bdl.	bdl.	Traces	bdl.	bdl.	bdl.
Lysine	0.2 ± 0.0^{b}	0.2 ± 0.1^{ab}	0.1 ± 0.0^{a}	0.2 ± 0.0^{ab}	bdl.^{a}	bdl.^{a}	bdl.^{a}	bdl.^{a}
Histidine	Traces	bdl.	bdl.	bdl.	bdl.	bdl.	bdl.	bdl.
Non-essential amino acids (NEAA)								
Aspartic acid	0.6 ± 0.2^{bd}	0.2 ± 0.0^{a}	0.2 ± 0.0^{ac}	0.2 ± 0.0^{abc}	2.0 ± 0.4^{f}	0.6 ± 0.2^{be}	0.6 ± 0.2^{bc}	0.7 ± 0.1^{de}
Serine	0.2 ± 0.0^{c}	0.1 ± 0.0^{abc}	0.2 ± 0.2^{abc}	0.1 ± 0.0^{abc}	0.1 ± 0.0^{b}	bdl.^{a}	bdl.^{a}	bdl.^{a}
Asparagine	0.4 ± 0.1^{b}	bdl.^{a}	bdl.^{a}	bdl.^{a}	bdl.^{a}	bdl.^{a}	bdl.^{a}	bdl.^{a}
Glutamic acid	1.3 ± 0.2^{b}	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}	4.3 ± 0.2^{c}	1.2 ± 0.1^{b}	1.2 ± 0.1^{b}	1.3 ± 0.1^{b}
Glutamine	0.8 ± 0.2^{c}	0.3 ± 0.1^{bc}	0.3 ± 0.2^{ab}	0.1 ± 0.0^{b}	0.2 ± 0.1^{ab}	bdl.^{a}	bdl.^{a}	bdl.^{a}
Proline	0.1 ± 0.0^{ab}	bdl.^{a}	bdl.^{a}	bdl.^{a}	3.5 ± 1.4^{b}	1.1 ± 0.1^{ab}	1.1 ± 0.1^{ab}	1.3 ± 0.2^{ab}
Glycine	0.1 ± 0.0^{ab}	0.1 ± 0.0^{a}	bdl.^{a}	bdl.^{a}	0.3 ± 0.1^{c}	0.1 ± 0.0^{b}	0.1 ± 0.1^{ab}	0.1 ± 0.0^{b}
Alanine	6.0 ± 1.9^{b}	2.5 ± 0.9^{ab}	2.7 ± 1.2^{ab}	3.0 ± 1.2^{ab}	1.2 ± 0.1^{b}	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	0.4 ± 0.0^{a}
Cysthathionine	0.2 ± 0.0	bdl.	bdl.	bdl.	bdl.	bdl.	bdl.	bdl.
Tyrosine	0.1 ± 0.0	bdl.	bdl.	bdl.	Traces	bdl.	bdl.	bdl.
Arginine	0.1 ± 0.0^{b}	Traces^{ab}	Traces^{ab}	bdl.^{a}	bdl.^{a}	bdl.^{a}	bdl.^{a}	bdl.^{a}
Sum FAA	10.7 ± 2.3^{b}	3.7 ± 1.0^{a}	3.8 ± 1.7^{a}	4.2 ± 1.4^{a}	12.0 ± 1.0^{b}	2.9 ± 0.4^{a}	3.0 ± 0.3^{a}	3.3 ± 0.4^{a}

Table 3: Total amino acid content in raw and boiled (15. 30 and 60 minutes) *Alaria esculenta* and *Palmaria palmata*. Values are reported as mean ± SD and in mg AA g^{-1} DW (n = 5). Different letters in the same row indicate significant differences ($p < 0.05$).

	Alaria esculenta				***Palmaria palmata***			
	Raw	Boiled 15 min	Boiled 30 min	Boiled 60 min	Raw	Boiled 15 min	Boiled 30 min	Boiled 60 min
Essential amino acids (EAA)								
Threonine	5.3 ± 0.7^{a}	6.5 ± 2.1^{a}	5.9 ± 0.7^{a}	5.7 ± 0.9^{a}	6.0 ± 0.7^{a}	12.0 ± 0.8^{b}	12.6 ± 1.9^{b}	12.2 ± 0.4^{b}
Valine	5.9 ± 0.4^{a}	7.2 ± 3.1^{ab}	6.8 ± 1.4^{ab}	6.6 ± 1.0^{ab}	7.8 ± 0.8^{b}	15.8 ± 2.7^{c}	17.5 ± 2.5^{c}	16.4 ± 1.4^{c}
Methionine	2.6 ± 0.4^{a}	3.1 ± 1.1^{a}	3.0 ± 0.9^{a}	3.0 ± 0.8^{a}	2.8 ± 0.4^{a}	5.9 ± 0.6^{b}	6.4 ± 0.6^{b}	6.1 ± 0.2^{b}
Isoleucine	4.2 ± 0.6^{a}	5.6 ± 2.6^{ab}	4.9 ± 1.1^{a}	4.7 ± 1.3^{a}	5.1 ± 0.9^{a}	9.9 ± 2.0^{b}	11.3 ± 2.1^{b}	11.0 ± 2.1^{b}
Leucine	8.1 ± 1.2^{a}	11.1 ± 3.9^{ab}	9.6 ± 0.9^{a}	9.3 ± 1.5^{a}	9.6 ± 1.2^{a}	19.6 ± 2.5^{bc}	21.8 ± 2.5^{c}	20.4 ± 1.6^{c}
Phenylalanine	5.2 ± 0.3^{a}	6.6 ± 2.8^{ab}	5.3 ± 0.9^{a}	5.8 ± 1.2^{a}	5.9 ± 0.6^{a}	12.1 ± 1.8^{bc}	13.6 ± 1.7^{c}	12.6 ± 1.0^{bc}
Lysine	9.2 ± 1.1^{a}	11.2 ± 4.1^{ab}	10.6 ± 1.6^{a}	9.7 ± 1.4^{a}	10.4 ± 0.8^{a}	20.7 ± 1.6^{bc}	22.9 ± 1.7^{c}	20.7 ± 1.7^{bc}
Histidine	2.8 ± 0.4^{a}	3.0 ± 1.2^{ab}	3.1 ± 0.6^{a}	2.8 ± 0.5^{a}	2.3 ± 0.2^{a}	5.2 ± 0.5^{bc}	6.2 ± 0.8^{c}	5.6 ± 0.4^{bc}
Non-essential amino acids (NEAA)								
Aspartic acid*	7.3 ± 1.1^{a}	8.8 ± 2.5^{ab}	7.7 ± 1.0^{ab}	7.9 ± 1.1^{ab}	10.3 ± 1.0^{b}	16.7 ± 1.0^{c}	18.4 ± 1.2^{c}	17.3 ± 0.8^{c}
Serine	5.2 ± 0.8^{a}	6.4 ± 1.9^{a}	5.9 ± 0.8^{a}	5.8 ± 1.1^{a}	7.3 ± 0.9^{a}	15.1 ± 1.0^{b}	16.7 ± 1.4^{b}	15.2 ± 0.7^{b}
Glutamic acid*	14.6 ± 1.7^{ab}	15.9 ± 4.8^{abcd}	14.0 ± 1.4^{ac}	13.9 ± 1.8^{ab}	17.8 ± 1.2^{b}	26.5 ± 1.9^{d}	30.0 ± 2.7^{e}	27.8 ± 1.3^{e}
Proline	4.2 ± 2.0	4.4 ± 1.5	4.5 ± 2.7	5.1 ± 3.1	7.2 ± 2.7	8.5 ± 2.4	9.6 ± 2.2	9.1 ± 2.5
Glycine	6.5 ± 0.7^{a}	8.2 ± 2.8^{b}	7.2 ± 0.7^{b}	7.3 ± 0.8^{b}	8.8 ± 0.6^{a}	16.4 ± 1.5^{c}	18.4 ± 1.4^{c}	16.9 ± 1.0^{c}
Alanine	15.5 ± 3.2^{ab}	13.5 ± 4.5^{abc}	12.3 ± 1.6^{a}	12.7 ± 2.5^{a}	12.5 ± 1.2^{a}	22.9 ± 2.7^{bcd}	25.5 ± 2.4^{d}	23.6 ± 1.2^{c}
Cysteine	0.2 ± 0.0^{a}	0.5 ± 0.3^{ab}	1.2 ± 1.4^{abc}	0.5 ± 0.2^{ab}	0.7 ± 0.2^{b}	2.9 ± 0.1^{c}	3.4 ± 0.3^{c}	3.0 ± 0.5^{c}
Tyrosine	3.0 ± 0.5^{a}	4.3 ± 1.4^{ab}	4.5 ± 1.5^{ab}	3.4 ± 1.0^{ab}	4.9 ± 0.6^{b}	11.2 ± 1.3^{c}	12.4 ± 0.7^{c}	11.6 ± 0.9^{c}
Arginine	6.4 ± 0.5^{a}	9.1 ± 3.3^{ab}	7.5 ± 0.6^{a}	7.6 ± 1.0^{a}	10.4 ± 1.0^{b}	22.3 ± 1.7^{c}	24.8 ± 1.9^{c}	22.6 ± 1.7^{c}
Sum	106.1 ± 9.1^{a}	125.4 ± 41.4^{a}	113.9 ± 10.6^{a}	111.4 ± 15.6^{a}	129.8 ± 11.4^{a}	243.7 ± 21.2^{b}	271.5 ± 22.1^{b}	252.0 ± 13.6^{b}
Sum EAA	43.3 ± 4.6^{a}	54.3 ± 20.7^{ab}	49.2 ± 5.3^{a}	47.5 ± 7.9^{a}	49.9 ± 5.1^{a}	101.3 ± 12.3^{bc}	112.3 ± 12.1^{c}	104.9 ± 7.8^{c}
Relative amount EAA (%)	40.7 ± 1.2	42.8 ± 2.6	43.2 ± 1.7	42.6 ± 2.5	38.4 ± 1.9	41.5 ± 1.4	41.3 ± 1.3	41.6 ± 1.8

* Aspartic acid and Glutamic acid represent the sums of Aspartic acid + Asparagine and Glutamic acid + Glutamine, respectively, as Asparagine and Glutamine are present in their acidic form after acidic hydrolysis.

FIGURE CAPTIONS

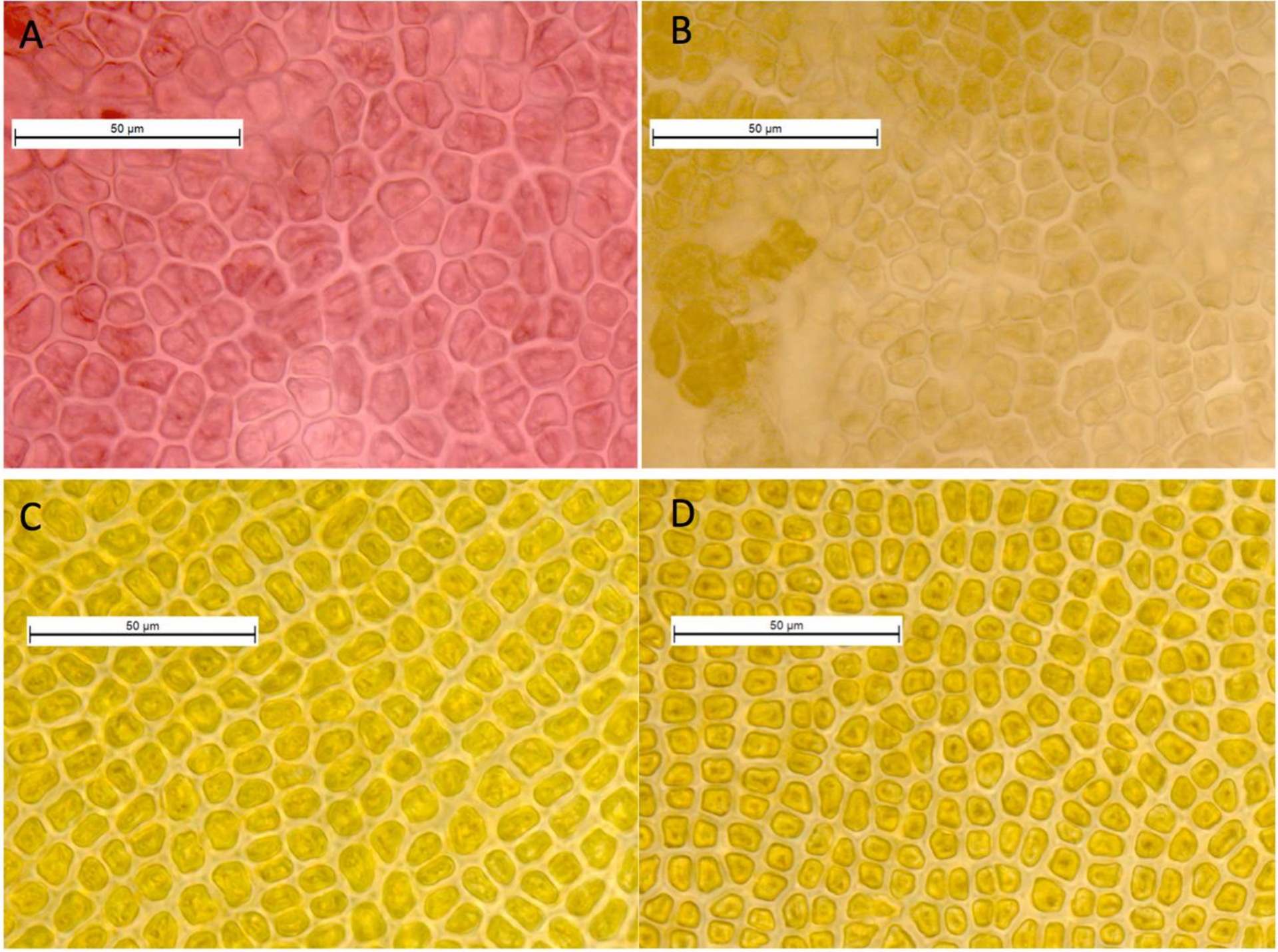
Fig 1: Microscopy images of raw and boiled (60 min) *Palmaria palmata* (A and B) and *Alaria esculenta* (C and D).

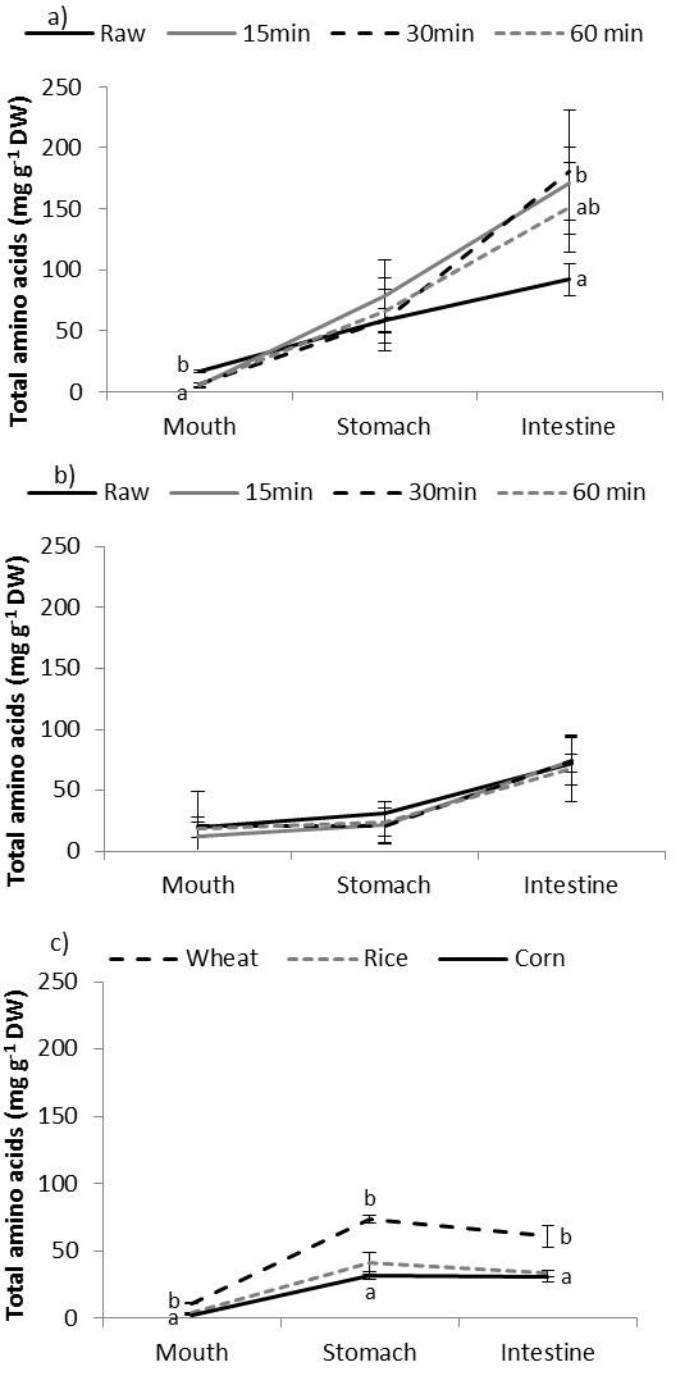
Fig 2 a-c: Total amino acids liberated in the mouth, stomach and intestinal fluids during gastrointestinal digestion of (a) *Palmaria palmata* (raw and boiled for 15, 30 and 60 minutes), (b) *Alaria esculenta* (raw and boiled for 15, 30 and 60 minutes) and (c) flours of wheat, rice and corn. Values are reported as mean ± SD (n = 5) and in mg AA g^{-1} DW. Different letters indicate significant differences ($p < 0.05$) within the same GI stages between treatments (algae) and type (flours).

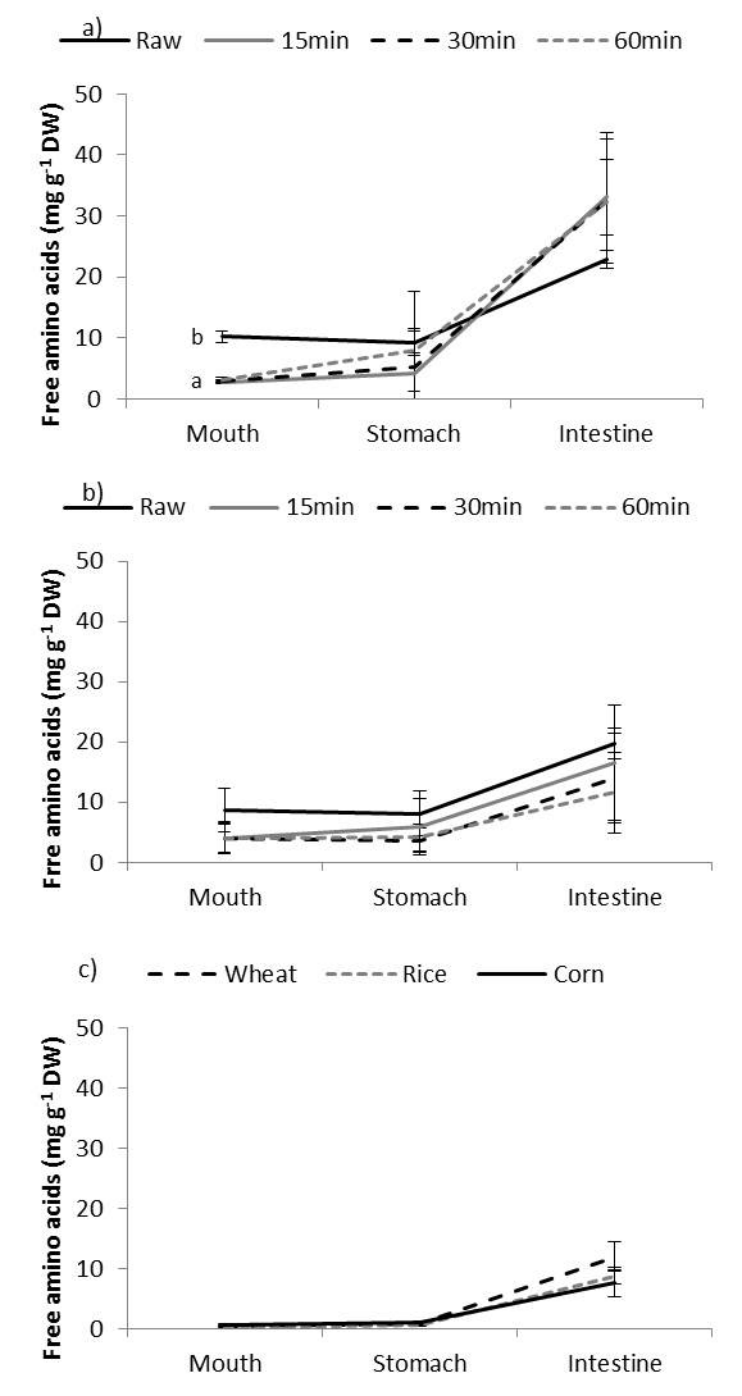
Fig 3 a-c: Free amino acids liberated in the mouth, stomach and intestinal fluids during gastrointestinal digestion of (a) *Palmaria palmata* (raw and boiled for 15, 30 and 60 minutes), (b) *Alaria esculenta* (raw and boiled for 15, 30 and 60 minutes) and (c) flours of wheat, rice and corn. Values are reported as mean ± SD (n = 5) and in mg AA g^{-1} DW. Different letters indicate significant differences ($p < 0.05$) within the same GI stages between treatments (algae) and type (flours).

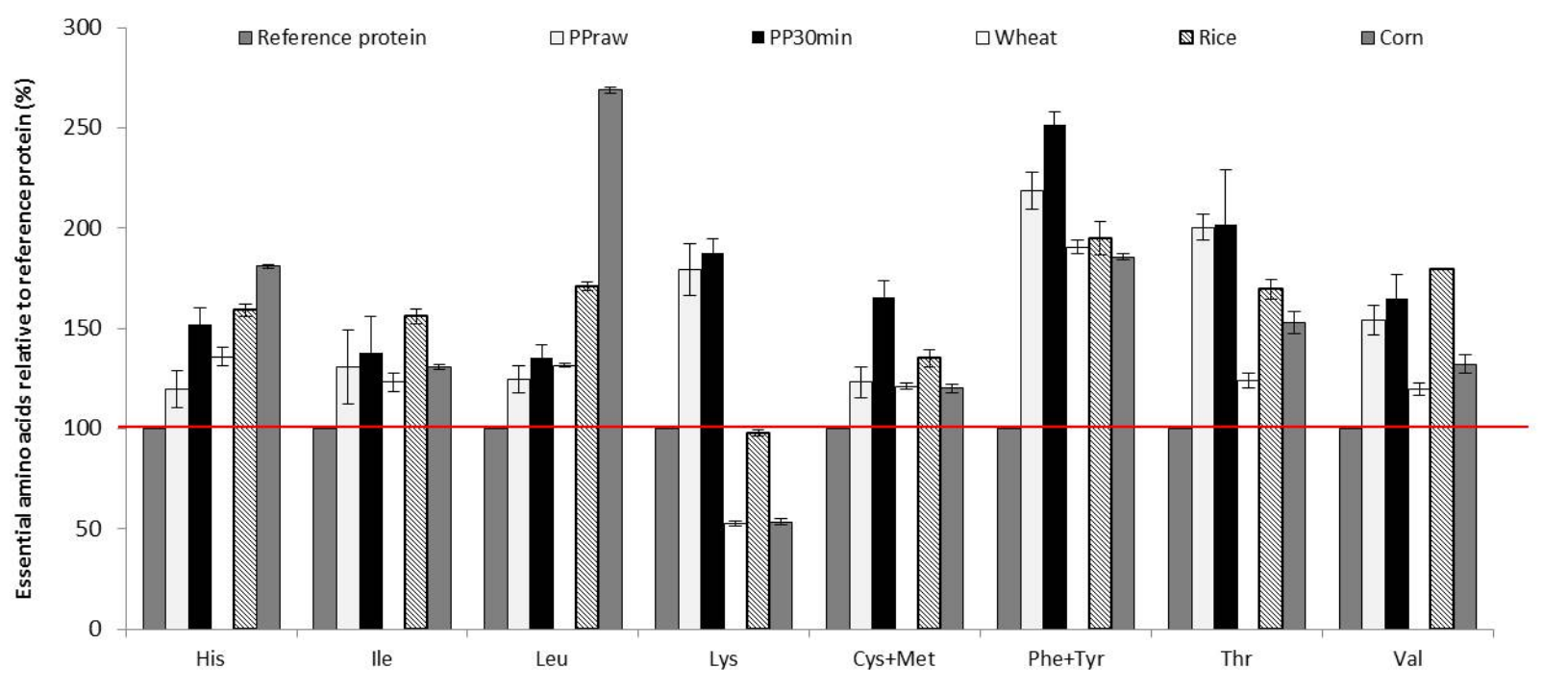
Fig 4: Essential amino acid composition in *Palmaria palmata* (raw and boiled for 30 minutes), wheat, rice and corn proteins related to the reference protein set by the WHO. The values are given as mean ± SD (n = 5) and in % of the reference protein.

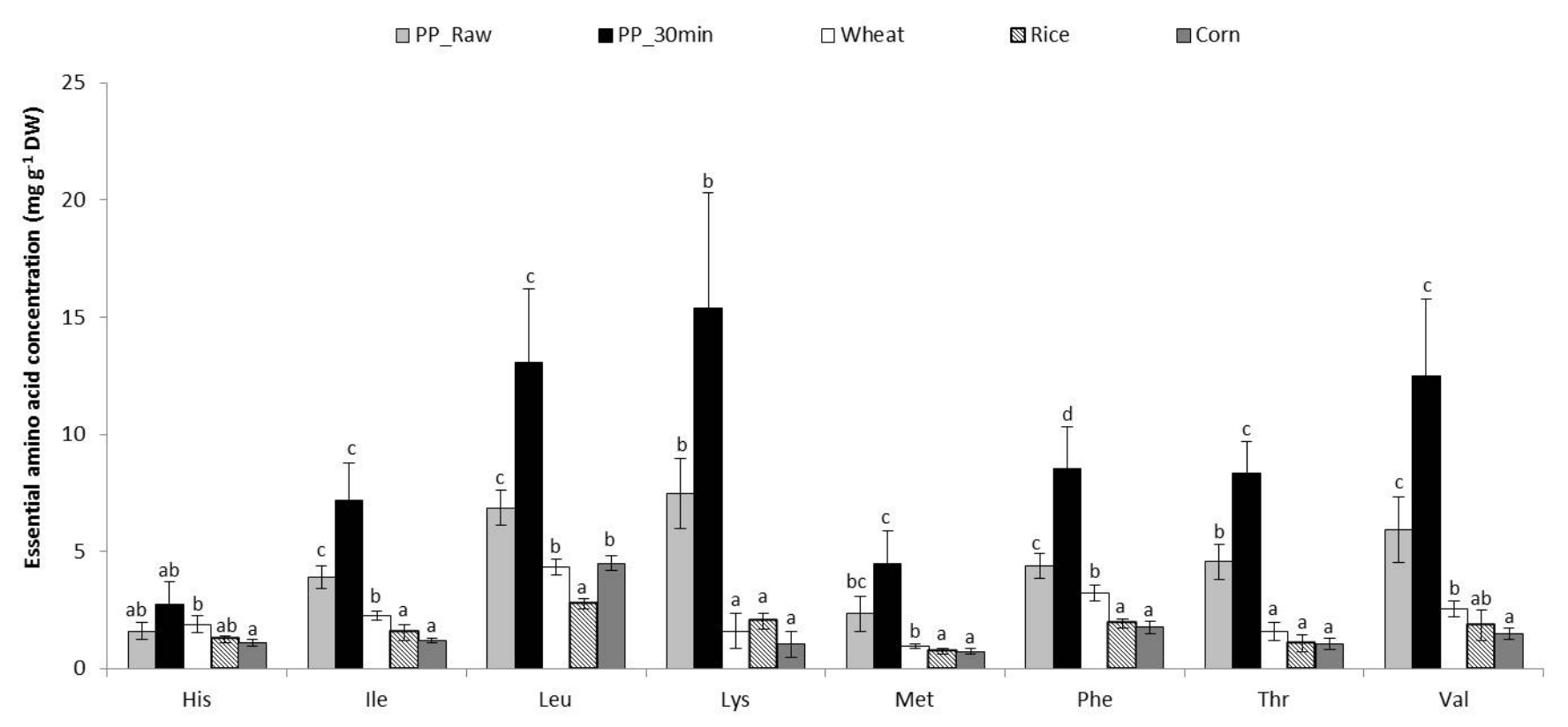
Fig 5: Liberated essential amino acids after digestion of 1 gram DW of *Palmaria palmata* (raw and boiled for 30 minutes), wheat, rice and corn flours. Values are given as mean ± SD (n = 5) and in mg g^{-1} DW. Different letters in each amino acid indicate significant differences between species ($p < 0.05$).


A
50 µm
B
50 µm
C
50 µm
D
50 µm


a)
Raw
15min
30min
60 min
Total amino acids (mg g-1 DW)
250
200
150
100
50
0
b
a
b
ab
a
Mouth
Stomach
Intestine
b)
Raw
15min
30min
60 min
Total amino acids (mg g-1 DW)
250
200
150
100
50
0
Mouth
Stomach
Intestine
c)
Wheat
Rice
Corn
Total amino acids (mg g-1 DW)
250
200
150
100
50
0
b
a
b
b
a
Mouth
Stomach
Intestine


a)
Raw
15min
30min
60min
Free amino acids (mg g-1 DW)
50
40
30
20
10
0
b
a
Mouth
Stomach
Intestine
b)
Raw
15min
30min
60min
Frre amino acids (mg g-1 DW)
50
40
30
20
10
0
Mouth
Stomach
Intestine
c)
Wheat
Rice
Corn
Free amino acids (mg g-1 DW)
50
40
30
20
10
0
Mouth
Stomach
Intestine


Reference protein
PPraw
PP30min
Wheat
Rice
Corn
Essential amino acids relative to reference protein (%)
300
250
200
150
100
50
0
His
Ile
Leu
Lys
Cys+Met
Phe+Tyr
Thr
Val


PP_Raw
PP_30min
Wheat
Rice
Corn
Essential amino acid concentration (mg g-1 DW)
25
20
15
10
5
0
His
Ile
Leu
Lys
Met
Phe
Thr
Val