Postprint of Journal of Functional Foods Volume 40, January 2018,
Pages 400–407 DOI: 10.1016/j.jff.2017.11.030

***In vitro* digestion of chlorophyll pigments from edible seaweeds**

Kewei Chen, María Roca*

Food Phytochemistry Department, Instituto de la Grasa, Consejo Superior de Investigaciones
Científicas (CSIC). University Campus Pablo de Olavide, Building 46, Carretera de Utrera km. 1,
Sevilla 41013, Spain.

*Corresponding author

e-mail: covichen@hotmail.com, mroca@ig.csic.es

Telephone: +00 34 954.61.15.50

Fax: +00 34 954.61.67.90

Abstract

The three most common edible seaweeds, Nori (*Porphyra umbilicalis*), Sea Lettuce (*Ulva* sp.) and Kombu (*Laminaria ochroleuca*), were subjected for the first time to an *in vitro* digestion process in order to study the digestive stability and recovery of chlorophyll pigments of the *a*, *b* and *c* series. Due to the complex and diverse chlorophyll profile in these seaweeds, new principles regarding the behaviour of chlorophylls during the *in vitro* digestion were obtained.

Thus, the *in vitro* digestion conditions favoured the pheophytinisation reaction of the *a* series in comparison with the *b* and *c* series; oxidation reactions were promoted for chlorophylls instead of pheophorbides and pheophytins; the conversion of pheophytin to pheophorbide was observed for the first time during *in vitro* digestion and only occurred when the initial chlorophyll profile contained a large proportion of pheophytins. The extracellular matrix of the seaweeds was the main determining factor in the recovery of pigments after *in vitro* digestion, with significant differences between seaweed species. The novel chlorophyll behaviour observed for digestive, stability and recovery patterns obtained in the present work are applicable to other food matrices.

Keywords: Bioavailability, chlorophyll, *in vitro* digestion, Kombu, Nori, Sea lettuce, seaweeds.

1. Introduction

Chlorophyll pigments, which allow plant species to convert light into biological energy, are the most abundant pigments on earth and exhibit a variety of biological actions (Roca, Chen, & Pérez-Gálvez, 2016), such as an antimutagenic effect (Simonich et al., 2007), antigenotoxic properties (Negishi, Rai, & Hayatsu, 1997), and a potent antioxidant capacity to scavenge free radicals, preventing lipid oxidation (Lanfer-Marquez, Barros, & Sinnecker, 2005). Additionally, natural chlorophyll pigments deriving from marine algae exhibit other potential health benefits, such as neuroprotective (Ina, Hayashi, Nozaki, & Kamei, 2007) and anti-inflammatory features (Henderson & Kincaid, 1997). Despite the fact that chlorophyll is abundant in our diet, the available information about the digestive process of chlorophyll pigments is limited. This may be due to the fact that chlorophyll pigments are very sensitive to the environmental changes and prone to chemical and enzymatic changes (Schwartz & Lorenzo, 2006). Consequently, there was an assumption in the past that chlorophyll pigments could not be absorbed by the human body. However, since Egner et al., (2000) described copper chlorin e4 and chlorin e4 ethyl ester in human serum, research began on the bioavailability of chlorophyll pigments. The assumed mechanism of chlorophyll absorption proposed by Ferruzzi and Blakeslee (2007) follows similar routes to those observed for other xenobiotic compounds that require (a) efficient release of the chlorophyll from the food matrix, (b) stability for gastric and small intestinal digestive conditions, (c) solubilisation of lipophilic derivatives (micellization), (d) uptake by small intestinal absorptive epithelial cells, and (e) secretion into circulation (basal transport).

Consequently, the first step for the large-scale research of nutrient absorption (Garrett, Failla, & Sarama, 1999) is to establish the efficiency of bioaccessibility, defined as the amount of the ingested compound that is transferred during digestion from the food matrix to the micelles,

measured through *in vitro* digestion protocols. Information on the bioaccessibility of chlorophyll derivatives is scarce and limited to higher food matrix plants, such as spinach leaves (Ferruzzi, Failla, & Schwartz, 2001) or pea purée (Gallardo-Guerrero, Gandul-Rojas, & Mínguez-Mosquera, 2008), where the chlorophyll profile is narrow: mainly chlorophyll *a* and *b* in the initial material with only tiny amounts of pheophytin *a* and pheophytin *b*. Finally, a study has been performed with several chlorophyll standards (Gandul-Rojas, Gallardo-Guerrero, & Mínguez-Mosquera, 2009) including chlorophyll *a* (*b*), pheophytin *a* (*b*), pyropheophytin *a*, pheophorbide *a* and pyropheophorbide *a*. It has been shown that de-esterification of phytol makes chlorophyll pigments more bioaccessible. This suggests the need to extend the research of the digestion process of chlorophyll pigments to a wider range of chlorophyll derivatives in a food matrix.

Marine algae or seaweeds, which have been traditionally consumed in Asian diets since ancient times, have gained popularity in European countries (Taboada, Millán, & Míguez, 2010). Currently, it is widely accepted that seaweeds are rich in vitamins, minerals, dietary fibre and other functional nutrients that can provide a necessary supplement to the modern diet, which mainly consists of refined food ingredients (Ferraces-Casais, Lage-Yusty, Rodríguez-Bernaldo de Quirós, & López-Hernández, 2012). Since the discovery of the many beneficial compounds present in algae, much effort has been expended in examining their nutritional aspects, including bioaccessibility of micronutrients such as iodine and carotenoid (Granado-Lorencio et al., 2009), and the overall nutritional effects on tested animals (Taboada, Millán, & Míguez, 2010). However, no reports were found associated with the digestive behaviour of chlorophyll pigments from seaweeds, even though it has already been discovered that content of marine algae is extremely high in these pigments and indeed the profile is very extensive (Pangestuti & Kim,

2011; Chen, Ríos, Pérez-Gálvez, & Roca, 2017).

In this study, the three most consumed seaweeds were selected for an *in vitro* digestion process, including Nori, Sea Lettuce and Kombu. These three species are representative of the three different classes of algae including the red, green and brown kinds, which are Rhodophytes, Chlorophytes and Phaeophytes, respectively. The aim of the study was to analyse the influence of different food matrices on the bioaccessibility of chlorophylls, as the different classes of algae show very different extracellular matrix features (Synytsya, Čopíková, Kim, & Yong, 2015). In addition, these materials provide unique, complex and complete chlorophyll profiles that allow a systematic approach to digestive properties. Nori contains only *a* series chlorophyll, Sea Lettuce contains *a* and *b* series, and Kombu, *a* and *c* series (Ferraces-Casais, Lage-Yusty, Rodríguez-Bernaldo de Quirós, & López-Hernández, 2012; Chen, Ríos, Pérez-Gálvez, & Roca, 2017). Indeed, seaweeds contain a large amount of dephytylated chlorophyll such as pheophorbide (Ferraces-Casais, Lage-Yusty, Rodríguez-Bernaldo de Quirós, & López-Hernández, 2012), magnesium-free chlorophyll such as pheophytin and its oxidised derivatives (Koseki, Muranaka, Sakai, & Nakajima, 2002) as well as chlorophyll *a* and *b*. This allows a comparison to be made between different structures of chlorophyll related to their digestive behaviour and the effect of food matrices from different seaweed species during the digestive metabolism.

2. Materials and methods

All the following procedures were carried out under green light to avoid the photooxidation of chlorophyll pigments.

2.1 Raw material.

Sea Lettuce (*Ulva* sp.) was provided by Suralgae (Cádiz, Spain) while Nori (*Porphyra umbilicales*) and Kombu (*Laminaria ochroleuca*) were provided by Algamar (Pontevedra,

Spain). The three seaweed species were collected on the Atlantic littoral region on the south western part (Cádiz) and the north western part (Pontevedra) of Spain. The dried material (25-45°C for 30-45 hours) is supplied in vacuum sealed bags.

2.2 Chemicals and reagents

Sunflower oil was purchased in a local supermarket. α -Amylase (porcine pancreas, VI-B), pepsin (porcine), bile extract (porcine), lipase pancreatic (porcine), sodium chloride, sodium bicarbonate, tetrabutylammonium acetate and ammonium acetate (98%) were supplied by Sigma-Aldrich Chemical Co. (Madrid, Spain). Pancreatin (porcine) was provided by Fluka (Zwijndrecht, The Netherlands). Other reagents (acetone, potassium chloride, calcium chloride, analysis grade) were supplied by Teknokroma (Barcelona, Spain). *N, N*-dimethylformamide (DMF) PAR grade and LC/MS grade solvents and water were supplied by Panreac (Barcelona, Spain), while acetone HPLC grade was supplied by Merck. The deionized water used was obtained from a Milli-Q 50 system (Millipore Corp., Milford, MA, USA).

2.3 Sample preparation

To warranty the consistency of each sample for the independent *in vitro* digestion, samples of seaweeds were prepared according to the following procedure. Amounts of fresh dried seaweeds (15 g for Nori and Kombu, 7.5 g for Sea Lettuce) were weighed and combined with saline solution (140 mM NaCl, 5 mM KCl and 6 mM CaCl₂) in a ratio of 1:20 (w/v). The mixture was homogenized to obtain the consistent puree. Aliquots corresponding to ca. 0.1 g of fresh dried seaweeds for Sea Lettuce and ca. 0.2 g for Nori and Kombu were blanketed with nitrogen and stored at -20°C before used.

2.4 *In vitro* digestion

The method was adopted from Garrett, Failla, & Sarama (1999) and Gallardo-Guerrero, Gandul-Rojas, & Mínguez-Mosquera (2008) with some modifications. Samples were subjected to the oral, gastric and intestinal phase of digestion to reproduce the physiological process. Initially, 80 μ L of sunflower oil free of chlorophyll pigments was added into the defrosted sample to achieve the requirement of food matrix in daily intake (around 4% of the fresh wet weight of seaweeds). The three digestive mixtures were incubated in a water bath (37°C) where the samples were horizontally shaken at constant rate (85 rpm) for the oral (10 min), gastric (1 h) and intestinal (2 h) phase. From the first phase, the simulated saliva was obtained by dissolving α -amylase (2041 U/g fresh wet weight of raw material) in saline solution (140 mM NaCl, 5 mM KCl, pH 7.0). The gastric phase was initiated by adding saline solution to reach a final concentration of 2.4 mg/mL pepsin and adjusting the pH to 2.0 by adding some drops of 0.1 M HCl. Subsequently, the pH was adjusted to 6.0 by adding NaHCO₃ (0.9 M) to initiate the intestinal phase including bile salts (2.4 mg/mL), pancreatin (0.4 mg/mL) and lipase (0.2 mg/mL) dissolved in NaHCO₃ (0.1 M) solution. The final volume of the digesta was around 35 mL. Reactions were stopped by placing tubes in ice bath and aliquots (2 $\times$ 5 mL) of digesta were collected. All the samples were blanketed with nitrogen and placed at -20°C until analysis.

2.5 Pigment extraction

The raw fresh dried seaweeds were grinded with liquid nitrogen and passed through 576 meshes/cm² sieves (particle size equivalent to 0.5 mm (Φ)), and the moisture content was measured. 0.2 grams of seaweed powder were extracted with 30 mL of DMF:water (9:1), vortex mixed (10 s), centrifuged (6000 rpm, 3 min), filtrated (nylon, 0.22 μ m) and stored at -20°C until HPLC analysis (Chen, Ríos, Pérez-Gálvez, & Roca, 2017).

The digesta samples were lyophilized (Virtis, Benchtop K) before pigment extraction.

Then, 200 μ L distilled water was added to soften the porous residue and the mixture was shaken for 5 min. Additional 200 μ L of DMF was added to the mixture and shaken again 5 min. Next, 1.6 mL acetone was added and the mixture immersed in an ultrasonic bath (10 min, 720 W). The solvent layer after filtration was directly analyzed by HPLC.

2.6 Pigment identification and quantification by HPLC-UV-Visible

The pigments were separated by reversed-phase HPLC using a Hewlett-Packard HP 1100 liquid chromatograph. A Mediterranea Sea18 column (200 $\times$ 4.6 mm, 3 μ m particle size) was used (Teknokroma, Barcelona, Spain) protected by a guard column (10 $\times$ 4.6 mm) packed with the same material. Separation was performed using the elution gradient described by Roca, Gandul-Rojas, & Mínguez-Mosquera (2007) with the mobile phases: A, water/ion pair reagent/methanol (1/1/8, v/v/v) and B, methanol/acetone (1/1, v/v). The ion pair reagent was 0.05 M tetrabutylammonium and 1 M ammonium acetate in water. The on-line UV-visible spectra were recorded from 350 to 800 nm with the photodiode-array detector and sequential detection was performed at 410, 430, 450 and 666 nm. Data were collected and processed with a LC HP ChemStation (Rev.A.05.04). Identification of chlorophyll derivatives was made based on co-chromatography with authentic samples (previously identified by MS/MS) and from their spectral characteristics (Chen, Ríos, Pérez-Gálvez & Roca, 2015; Chen, Ríos, Roca & Pérez-Gálvez, 2015), except for oxidized pheophorbide *c*, which identification is tentative only based in spectroscopic and chromatographic characteristics (Jeffrey & Wright, 1987). Quantification of pigments was performed with the corresponding calibration curves (amount versus integrated peak area). The calibration equations were obtained by least-squares linear regression analysis over a concentration range according to the observed levels of these pigments in the analysed samples, obtaining R^2 values always higher than 0.9997. Duplicate injections were made for five

different volumes at each standard solution.

2.7 Statistical analysis

For comparison and measurement of the significant differences on the *in vitro* digestion procedure, a minimum of 4 independent digestions were made for each sample, and each digesta was analysed for pigment content in duplicate. Percentage of chlorophyll recovery in digesta was calculated as $\text{nanograms in digesta} \times 100 / \text{nanograms in the starting material used for digestion}$. Differences were compared by one-way analysis of variance (ANOVA, StatSoft, Inc., 2001) with student's *t*-test as test for comparison. Differences were considered significant at *P* values < 0.05.

3. Results and discussion

3.1 Chlorophyll structures present in seaweeds

Fig. 1 illustrates all the structures and names of chlorophyll derivatives found in the three edible seaweeds, Nori, Sea Lettuce and Kombu. Different phyla contain different chlorophyll series. Rhodophyta plastids (Nori) have phycobiliproteins and chlorophyll *a* derivatives, with the characteristic methyl group at C7 (R_1 in Fig. 1). Chlorophyta plastids (Sea Lettuce) in addition to *a* series also contain chlorophyll *b* derivatives, with the formyl group at C7 (R_1 in Fig. 1). And finally, Phaeophyta plastids (Kombu) contain chlorophyll derivatives of the *c* series, which mostly contain an acrylic acid side chain at C17 and they are not usually esterified, in addition to the chlorophyll *a* series. Seaweeds belonging to Chlorophyta phylum contain mainly chlorophylls (Chen, Ríos, Pérez-Gálvez, & Roca, 2017) while the photosynthetic apparatus of seaweeds from Rhodophyta and Phaeophyta phylum is formed mainly from pheophytins (chlorophylls with the central magnesium substituted by hydrogens, R_5 at Fig. 1) and pheophorbides (de-metalled chlorophylls de-esterified at C17³, Fig. 1) (Ferraces-Casais, Lage-Yusty, Rodríguez-Bernaldo de Quirós, & López-Hernández, 2012). However, the complete

characterisation of the chlorophyll profile in seaweeds has only been performed recently (Chen, Ríos, Pérez-Gálvez, & Roca, 2017), describing chlorophyll derivatives with different oxidation degrees. In this sense, several enzymatic activities have been proposed as being responsible for hydroxylation at C13², yielding the 13²-hydroxy derivatives (R₂ in Fig. 1). A second structural feature described in the chlorophyll profile of seaweeds (Chen, Ríos, Pérez-Gálvez, & Roca, 2017) is the rearrangement of the isocyclic ring, establishing a lactone group (Fig. 1, structure B), the 15¹-hydroxy-lactone derivatives (15¹-hydroxy-lactone chlorophylls, 15¹-hydroxy-lactone pheophorbides and 15¹-hydroxy-lactone pheophytins). A different oxidation process that also involves the isocyclic ring is the formation of the purpurin-18 derivatives (Fig. 1, structure D), with the characteristic cyclic anhydride. This catabolite is a product of the enzymatic oxidative transformation during senescence (Ma & Dolphin, 1996). Finally, pyropheophytin *a* is formed by the loss of the carboxymethoxy group at C13² probably due to the pheophorbidease enzyme (Shioi, 2006). Seaweeds certainly represent an ideal raw material for studying the structural stability of chlorophyll compounds, not only because of the high variety of chlorophyll derivatives at different oxidative statuses, but also due to the different quantitative and proportional content of them among different phyla.

3.2 Stability of chlorophyll pigments during *in vitro* digestion

Table 1 shows the modifications of each chlorophyll pigment in Nori, Sea Lettuce and Kombu, while Table 2 shows the modifications of the chlorophyll series, as a consequence of the oral, gastric and intestinal *in vitro* digestion. Numerous factors affect chlorophyll structures during digestion, mainly pH, the oxidative environment and enzymatic reactions.

The seaweed Nori, being a Rhodophyta phylum, only contains chlorophyll *a* series derivatives and consequently structural changes of the chlorophyll pigments develop within the *a*

series (Tables 1-2). The chlorophyll profile is quite particular, presenting 60% of pheophytin derivatives, 37% of pheophorbide-related compounds and less than 2% of chlorophylls (Chen, Rios, Pérez-Gálvez, & Roca, 2017). In the fresh dried Nori, chlorophyll *a* is completely transformed into pheophytin *a* during digestion because of the acidic environment in the gastric phase. Another significant change is the formation of 15¹-hydroxy-lactone pheophorbide *a* after digestion; nevertheless, a small increase in 15¹-hydroxy-lactone pheophytin *a* content is also observed, derived from the oxidising ambient attained during the digestive process that favoured isocyclic ring oxidation. In line with this observation, a slight increase can be observed for 13²-hydroxy pheophorbide *a* in all the samples after digestion, which correlates again with an increase in the oxidative environment during *in vitro* digestion. Pyropheophytin *a* and purpurin-18 *a* series were degraded to some extent during simulated digestion, and purpurin-18-*a* is completely degraded. The *in vitro* digestion of the fresh and dried Nori sample produced a significant increase in pheophorbide *a* (15%) and conversely decreased the percentage of pheophytin *a* (12%). This means that pheophytin is partially transformed into pheophorbide during *in vitro* digestion by the loss of the phytol chain.

The green seaweeds (Sea Lettuce) contain both chlorophyll *a* and *b* series, introducing a new variable in the study, and the relative proportions of chlorophyll derivatives is also different. Thus, most of the content is chlorophyll (74%), while pheophytins (19%) and pheophorbides (7%) are lower contributors to the pigment profile. After *in vitro* digestion, relatively high percentages of chlorophyll *a* (43%) and chlorophyll *b* (24%) were observed, and a fraction of chlorophyll *a* plus 13²-hydroxy-chlorophyll *a* was entirely transformed (into pheophytin *a* and 13²-hydroxy pheophytin *a*), while chlorophyll *b* is more steady in the digestion environment with the same percentage (14%) remaining in the digesta. Conversion from chlorophyll *a* to

pheophytin *a* is also associated with chlorophyll *a* degradation to non-coloured derivatives, so that the *a/b* ratio decreased from 2.97 to 1.87, as the degradative reaction does not take place for the *b* series. It is interesting that no significant increase after digestion was observed for the pheophorbide compounds present in fresh material as observed in the Nori seaweed samples. Calculation of total oxidised derivatives (13²-hydroxy pheophytin *a*, 13²-hydroxy chlorophyll *b* and 13²-hydroxy pheophytin *b*) that were quantified as 13²-hydroxy derivatives experienced a great increase after the digestion process, which means that oxidative reactions in the digestion process were favoured. The conversion reactions from 13²-hydroxy chlorophyll *a* and 13²-hydroxy chlorophyll *b* to 13²-hydroxy pheophytin *a* and 13²-hydroxy chlorophyll *b*, respectively cannot explain that increase sufficiently. The percentage content of 15¹-hydroxy-lactone chlorophylls, including 15¹-hydroxy-lactone pheophorbide *a*, 15¹-hydroxy-lactone pheophytin *a* and 15¹-hydroxy-lactone chlorophyll *b* is similar before and after the digestion process. Purpurin-18 *a* derivatives are also prone to degradation in Sea Lettuce as they were completely degraded during the *in vitro* digestion.

Kombu, the brown algae contains both chlorophyll *a* and *c* series and a profile fashioned by pheophytin (68%), pheophorbides (5%) and chlorophylls (27%) (Chen, Rios, Pérez-Gálvez, & Roca, 2017). The digestion of Kombu samples implies the complete transformation of chlorophyll *a* series derivatives, as observed in the other seaweeds. Chlorophyll *c* is more resistant to digestion; although a portion of the original compound is transformed into pheophorbides, some amount (3%) is still present in the final digesta as the original chlorophyll *c*. The absence of the phytol chain in the structural arrangement of chlorophyll *c* (Fig. 1) facilitates the conversion between chlorophyll *c* and pheophorbide *c* by just the removal of the magnesium atom in the centre of the pyrrole ring, a process easily afforded in the acidic conditions of

digestion. Thus, the digestion of Kombu samples produced a proportional conversion of chlorophyll *c* to pheophorbides. And following the same outline as observed for the Nori sample, the main modification during the digestion of Kombu is the significant increase in the percentage of pheophorbide *a*, from 5% to 23%, a concomitant process with the decrease of pheophytin *a*, from 55% to 45%. In addition, the presence of 15¹-hydroxy-lactone pheophorbide *a* is detected after digestion, as observed in Nori samples. Once again, the transformation of chlorophyll *a* to pheophytin *a*, due to the magnesium dechelation process, cannot explain the decline of pheophytin *a*. As denoted for Nori samples, some amounts of pheophytin *a* are degraded to pheophorbide *a*. Finally, the percentages of oxidised derivatives significantly rise, mainly due to the outstanding presence of oxidised pheophorbide *c*. The content of the rest of the chlorophyll derivatives is maintained at levels similar to starting levels, except for purpurin-18-*a* derivatives, which are completely degraded. In summary, the *in vitro* digestion in brown seaweeds implies the modification in the ratio of pheophorbide to pheophytin, from 5:68 to 36:61, the total depletion of chlorophyll *a*, the loss of most of chlorophyll *c*, and an enhanced level of oxidised chlorophyll derivatives.

In general, the reactions that change the chlorophyll profile are the same for the three seaweeds (pheophytinisation, “pheophorbidation” and oxidation), but the intensity of the transformation depends on the initial characteristic chlorophyll profile of each type of seaweed. The low pH conditions that govern the gastric digestion phase caused the transformation of chlorophylls to their corresponding magnesium-free derivatives. Among the green chlorophylls including chlorophyll *a*, *b*, and *c* and their 13²-hydroxy or 15¹-hydroxy-lactone counterparts, chlorophyll *a*, 13²-hydroxy chlorophyll *a* and 15¹-hydroxy-lactone chlorophyll *a* are the most prone to pheophytinisation since they are totally transformed after the digestion process. Most of

the other two series of chlorophyll (chlorophyll *b*, 13²-hydroxy chlorophyll *b*, 15¹-hydroxy-lactone chlorophyll *b* and chlorophyll *c*) are also converted to their corresponding magnesium-free derivatives, while small amounts are still present in the final digesta.

The comparison of these results with chlorophyll behaviour during digestion in different food matrices only concerns chlorophylls of series *a* and *b* as previous studies performed the *in vitro* digestion with plant species. While the complete pheophytinisation is reported for chlorophylls in spinach puree (Ferruzzi, Failla, & Schwartz, 2001), allomerised chlorophyll *b* was resistant to digestion in fresh peas (Gallardo-Guerrero, Gandul-Rojas, & Mínguez-Mosquera, 2008). A clear resistance of chlorophyll *b* to digestion has only been observed (Gandul-Rojas, Gallardo-Guerrero, & Mínguez-Mosquera, 2009) with an oily mixture of chlorophyll *a* and chlorophyll *b* standards. These reports point to the higher stability of chlorophyll *b* than chlorophyll *a* due to the presence of the formyl group at C7 site (Fig. 1), which should have some incidence in the pheophytinisation process during digestion process. It has been widely observed that chlorophyll *a* transformation to pheophytin *a* is faster than for chlorophyll *b*, 2.5-9-fold according to different reports (Lajollo, Tannenbaum, & Labuza, 2010). Although theoretically, the exchange of magnesium for protons in the chlorophyll molecule is energetically favoured (Kania et al., 2014), the reactivity is not only associated with the porphyrin macrocycle but also with the constitution of the peripheral groups. Chlorophyll *a* and *b* share the same macrocycle arrangement and only the coupling of the formyl group at the C7 position with the π -electron system of the porphyrin ring in chlorophyll *b* is different. This group exerts an electron withdrawal effect that makes the core nitrogens in the macrocycle less susceptible to an electrophilic attack in comparison with the effect of the methyl group at C7 in chlorophyll *a* (Mazaki, Watanabe, Takahashi, Struck, & Scheer, 1992).

This is the first time that the chemical behaviour of chlorophyll *c* during *in vitro* digestion has been reported. The chlorophyll *c* identified in Kombu is chlorophyll *c_I*, which does not present the phytol chain. Conversion to a magnesium-free derivative is not affected by the steric hindrance between the C17 chain and the β -keto ester group (accounted for in dephytylated chlorophyll structures) (Schanderl & Chichester, 1962), such that other structural arrangements should be reasonable causes. Chlorophyll *c_I* shows a different unsaturation arrangement in the porphyrin macrocycle with different C=C and C=N locations in comparison with *a* or *b* series (structure C, Fig. 1), combined with the additional double bond between C17¹ and C17², making the chlorophyll molecule less flexible and fully conjugative (Shishkin, 1997). Thus, the π -electron density in the planar porphyrin ring of the chlorophyll *c_I* molecule is decreased by the higher tendency of electron withdrawal to the double bond at C17¹, and it makes the molecule more stable than chlorophyll *a* (Mazaki, Watanabe, Takahashi, Struck, & Scheer, 1992).

The second major modification that occurs during *in vitro* digestion is the transformation of pheophytins to pheophorbides, which means the loss of the phytyl chain at C17³ (Fig. 1). Although it is accepted that pheophytin could be dephytylated to pheophorbide under strong acid treatment (Sievers & Hynninen, 1977), no significant increase of the pheophorbide content was observed after *in vitro* digestion of spinach purée (Ferruzzi, Failla, & Schwartz, 2001), peas (Gallardo-Guerrero, Gandul-Rojas, & Mínguez-Mosquera, 2008) and pure standard of pheophytin (Gandul-Rojas, Gallardo-Guerrero, & Mínguez-Mosquera, 2009). In this study, a significant increase in the pheophorbide proportion was observed in the samples of Nori and Kombu after digestion but not in the Sea Lettuce samples. The “pheophorbidation” reaction (pheophytin is dephytylated to pheophorbide) does not significantly occur in Sea Lettuce because the chlorophyll profile of this seaweed contains large amounts of green chlorophylls (chlorophyll

a or *b*), and, in acidic conditions, the dechelation reaction is the first one to take place (Sievers & Hynninen, 1977). Consequently, the transformation processes in the acidic environment is dominated by the pheophytinisation reaction during the *in vitro* digestion of Sea Lettuce, and there was no increase in the pheophorbide proportion in the fresh dried sample. Actually, the chlorophyll profile of Sea Lettuce is very similar to the green plant species previously used, spinach purée and peas (Ferruzzi, Failla, & Schwartz, 2001; Gallardo-Guerrero, Gandul-Rojas, & Mínguez-Mosquera, 2008), where the increase in the pheophorbide proportion is not obvious as observed in green seaweed. Chlorophyll *a* or *b* are prone to transformation into pheophytin *a* or *b* in the acidic environment, thus preventing pheophytin from de-esterification into pheophorbide. Consequently, in those seaweeds where most of the initial chlorophyll derivatives are pheophytins (Nori and Kombu), the yield of pheophorbide from pheophytin increases after the digestion process. The only report that performed *in vitro* digestion with the pheophytin *a* standard (Gandul-Rojas, Gallardo-Guerrero, & Mínguez-Mosquera, 2009), dissolved that pigment in oil and consequently it was protected from digestion to some degree, as the authors stated.

Oxidised reactions are observed during the digestion process, since a variety of oxidative derivatives (13²-hydroxy and 15¹-hydroxy-lactone) are found to increase in percentage or appear in the final digesta. Previous results have shown one-off increases in allomerised chlorophylls and pheophytins during digestion explained with different hypotheses, either enzymatic activity, such as peroxidase (Ferruzzi, Failla, & Schwartz, 2001; Gallardo-Guerrero, Gandul-Rojas, & Mínguez-Mosquera, 2008), or due to chemical origin (Gandul-Rojas, Gallardo-Guerrero, & Mínguez-Mosquera, 2009). Our results show that the oxidative process is unselective, yielding, after digestion, 13²-hydroxy and 15¹-hydroxy-lactone derivatives of chlorophylls, pheophytins,

351 pheophorbides and occurring in a similar way to the *a*, *b* and *c* series. This result is consistent
352 with a purely chemical origin of the oxidative reactions. Likewise, oxidation reactions were also
353 observed for other phytochemical compounds such as dietary vitamin and carotenoids during the
354 *in vitro* digestion process (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso,
355 2013). In addition, the wide distribution of chlorophyll derivatives in the initial digestive
356 seaweeds allows the comparison of the behaviour of magnesium-containing derivatives
357 (chlorophyll) and magnesium-free ones (pheophytin and pheophorbide) through the oxidative
358 reactions. The highest increases of 13²-hydroxy derivatives during the *in vitro* digestion are for
359 Sea Lettuce (9%) mainly observed for 13²-hydroxy-pheophytin *a* and 13²-hydroxy-pheophytin *b*,
360 and for Kombu (8%), mainly observed for oxidised pheophorbide *c*. The initial chlorophyll
361 profile consists of large amount of magnesium-containing chlorophylls, so it could be inferred
362 that for magnesium-containing chlorophylls (chlorophyll *a*, chlorophyll *b* and chlorophyll *c*) the
363 oxidation reactions are promoted in comparison with the pheophytinisation, and then oxidised
364 derivatives were pheophytinised. It is difficult to develop a theory to explain such results
365 considering the different reaction mechanisms that may take place. For oxidation reactions, the
366 main limiting factors is the generation of reactive oxygen species that induce allomerisation
367 (Gandul-Rojas & Mínguez-Mosquera, 1996), while pheophytinisation reactions mainly depend
368 on the pH value of the environment (Gunawan & Barringer, 2000). Indeed, the magnesium
369 chelation in the centre of the macrocycle could increase the ability to donate electrons from the
370 conjugated porphyrin system, such that metal-chelated chlorophyll derivatives also show a
371 stronger antioxidant ability (Ferruzzi, Böhm, Courtney, & Schwartz, 2002). This may explain the
372 preference of the oxidative process by chlorophylls instead of pheophytin.

Finally, the chemical conditions reached during the *in vitro* digestion completely degrade the purpurin-18 *a* derivatives in the three species tested, probably due the characteristic configuration of the isocyclic ring that confers a major liability to this structure towards nucleophilic reactions (Wang, Yin, & Yang, 2013).

3.3 Recovery rate of chlorophyll derivatives from fresh dried seaweeds after *in vitro* digestion

The recovery rate is measured by the content of chlorophyll pigments in the digesta with respect to the initial chlorophyll content of the starting seaweed material. This index is expressed as a percentage and allows the determination of the extension of the degradative reactions that transform chlorophylls into non-coloured products, and the effect of the food matrix on the release of food nutrients. Fig. 2 shows the recovery rate of the chlorophyll derivatives in the three seaweeds.

The recovery rate of the *a* series in Nori samples, represents the recovery rate of the total chlorophyll content. In the fresh dried material of Nori, the recovery rate of chlorophyll is *ca.* 74%, a result significantly higher than the values observed in Sea Lettuce and Kombu, regarding both the *a* series and total chlorophyll recovery rates. The green algae Sea Lettuce has chlorophylls of *a* and *b* series and the recovery rate of total chlorophyll is around 37%. The recovery rate of the *a* series is significantly lower than the *b* series, because some fraction of the chlorophyll *a* content is degraded during the *in vitro* digestion, at higher levels than chlorophyll *b*, as shown by *a/b* ratio values before and after digestion (Table 2). Consequently, chlorophylls of *b* series are more resistant to digestion and their recovery rate is higher. Kombu mainly contains chlorophylls of the *a* and *c* series and according to the recovery values depicted in Fig. 2, both series show similar stability during the digestion process. The fresh dried Kombu sample

presents the lowest recovery rate in all the tested algae with a 15% recovery rate value for total chlorophyll.

The percentages of recovery rates of chlorophyll of Nori and Sea Lettuce are similar to the observed values in peas (60-87%, Gallardo-Guerrero, Gandul-Rojas, & Mínguez-Mosquera, 2008) and in an oily mixture of chlorophyll standards (50-82%, Gandul-Rojas, Gallardo-Guerrero, & Mínguez-Mosquera, 2009). The loss of chlorophyll pigments during *in vitro* digestion may be due to the activity of peroxidase and lipoxygenase, which contributes to degrading chlorophyll pigments, as stated previously (Gallardo-Guerrero, Gandul-Rojas, & Mínguez-Mosquera, 2008) since peroxidase (Murthy, Sharma, & Rao, 1988) and lipoxygenase (Kuo, Hwang, Hsu, & Pan, 1996) has been identified to be widely distributed in seaweed. However, Kombu shows lower recovery rate values compared with those data showing that differences in the food matrix are significant contributors for the different releases of chlorophyll pigments. Despite the fact that marine algae generally contain large amounts of amorphous embedding matrix polysaccharides in their cell walls (Wehr & Sheath, 2003; Davis), there are many differences related with polysaccharide components and distribution among Rhodophyta, Chlorophyta and Phaeophyta. Red algae contain composite cell walls made of cellulose, xylan, or mannan fibrils and sulphated galactans such as agars and carrageenans; green algae also present various sulphated and/or carboxylic polysaccharides with a specific structure in cell walls, such as ulvans and sulphated glycans (Synytsya, Čopíková, Kim, & Yong, 2015). Indeed, the cell walls of brown algae contain cellulose fibrils that consist of highly branched sulphated heteropolysaccharides (xyloglucans, xylofucoglucuronans, etc.) and special sorts of sulphated and carboxylic polysaccharides called fucoidans and alginates, respectively. These highly branched polysaccharides may interact with proteins, polymeric phenolics, metal cations and

other molecules making the cell walls of brown algae rigid and stable (Synytsya, Čopíková, Kim, & Yong, 2015). Compared with Nori (red algae) and Sea Lettuce (green algae), Kombu (brown algae) is extensively filamentous, multicellular, complex and has additional mucus cavity (Kumar, 2009), which makes the access of digestion enzymes more difficult for the release of chlorophyll pigments, leading to the lowest recovery rate values being observed in Kombu.

4. Conclusions

The diverse and complex chlorophyll profile of edible seaweeds allowed the study of different digestive behaviour of chlorophyll derivatives with different structural arrangements. During the *in vitro* digestion process, chlorophylls of the *a* series are more prone to pheophytinisation than *b* and *c* series chlorophylls; oxidation reactions commonly occur in the digestion process and have a preference for chlorophylls rather than pheophytins; the “pheophorbidation” reaction was first observed in the digestion of Nori and Kombu seaweeds as they provided large amounts of pheophytins and small amounts of chlorophylls in the initial materials. And it is assumed that oxidation reactions took place more easily for metal-chelated chlorophylls than for metal-dechelated pheophorbides or pheophytins. According to the recovery rate values, it is clear that the extracellular matrix of the seaweeds is a determinant factor for the recovery rate of chlorophylls after simulated digestion. The results suggest that both the chemical structure of phytochemicals and the nature of the food matrix are decisive for the digestive stability and recovery of chlorophylls after *in vitro* digestion.

Acknowledgements

This work was supported by the Comisión Interministerial de Ciencia y Tecnología (CICYT-EU, Spanish and European Government, AGL 2015-63890-R).

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Table 1: Quantitative distribution (percentage) of individual chlorophyll pigments in fresh dried Nori, Sea Lettuce and Kombu seaweeds previous (pre-d) and posterior (post-d) to *in vitro* digestion (means $\pm$ SE).

	Nori		Sea Lettuce		Kombu	
	pre-d	post-d	pre-d	post-d	pre-d	post-d
Chlorophyll <i>a</i>	1,81 $\pm$ 0,15		43,41 $\pm$ 0,33		7,21 $\pm$ 0,22	
13 ² -hydroxy chlorophyll <i>a</i>			5,14 $\pm$ 0,38			
15 ¹ -OH-lact. chlorophyll <i>a</i> ^a					2,41 $\pm$ 0,11	
Pheophorbide <i>a</i>	35,70 $\pm$ 4,04	48,30 $\pm$ 2,70	4,57 $\pm$ 0,10	3,58 $\pm$ 0,37	4,63 $\pm$ 0,46	22,57 $\pm$ 3,70
13 ² -hydroxy pheophorbide <i>a</i>	1,60 $\pm$ 0,24	3,82 $\pm$ 0,67	2,10 $\pm$ 0,01	2,77 $\pm$ 0,29	0,44 $\pm$ 0,17	1,26 $\pm$ 0,11
15 ¹ -OH-lact. pheophorbide <i>a</i>		0,43 $\pm$ 0,10	0,29 $\pm$ 0,01	0,17 $\pm$ 0,05		0,53 $\pm$ 0,38
Purpurin-18 <i>a</i>	0,19 $\pm$ 0,04					
Pheophytin <i>a</i>	44,85 $\pm$ 2,58	32,97 $\pm$ 1,18	11,35 $\pm$ 0,03	42,87 $\pm$ 0,37	54,98 $\pm$ 0,02	45,25 $\pm$ 6,71
13 ² -hydroxy pheophytin <i>a</i>	8,24 $\pm$ 1,30	7,80 $\pm$ 0,63	6,64 $\pm$ 0,52	14,85 $\pm$ 0,33	11,80 $\pm$ 0,25	13,39 $\pm$ 1,53
15 ¹ -OH-lact. pheophytin <i>a</i>	1,24 $\pm$ 0,11	2,83 $\pm$ 1,15	1,16 $\pm$ 0,37	0,93 $\pm$ 0,08	1,19 $\pm$ 0,01	1,53 $\pm$ 0,17
Pyropheophytin <i>a</i>	5,81 $\pm$ 0,37	3,42 $\pm$ 0,38			0,32 $\pm$ 0,01	1,00 $\pm$ 0,33
Phytol purpurin-18 <i>a</i>	0,56 $\pm$ 0,05	0,45 $\pm$ 0,18	0,15 $\pm$ 0,00		0,34 $\pm$ 0,05	
Chlorophyll <i>b</i>			23,67 $\pm$ 0,61	14,47 $\pm$ 0,20		
13 ² -hydroxy chlorophyll <i>b</i>			1,23 $\pm$ 0,21	2,15 $\pm$ 0,05		
15 ¹ -OH-lact. chlorophyll <i>b</i>			0,30 $\pm$ 0,12	0,95 $\pm$ 0,12		
Pheophytin <i>b</i>				13,15 $\pm$ 0,14		
13 ² -hydroxy pheophytin <i>b</i>				4,10 $\pm$ 0,14		
Chlorophyll <i>c</i>					16,68 $\pm$ 0,92	2,85 $\pm$ 0,79
Pheophorbide <i>c</i>						5,57 $\pm$ 1,81
Oxidized pheophorbide <i>c</i>						6,05 $\pm$ 1,40

^a15¹-OH-lact.: 15¹-hydroxy-lactone.

Table 2: Quantitative distribution (percentage) of chlorophyll pigments in fresh dried Nori, Sea Lettuce and Kombu seaweeds previous (pre-d) and posterior (post-d) to *in vitro* digestion (means $\pm$ SE).

	Nori		Sea Lettuce		Kombu	
	pre-d	post-d	pre-d	post-d	pre-d	post-d
Pheophytin <i>a</i> series	60,14 $\pm$ 3,94	47,47 $\pm$ 2,29	19,15 $\pm$ 0,12	58,65 $\pm$ 0,58	68,29 $\pm$ 0,23	61,17 $\pm$ 7,81
Pheophytin <i>b</i> series				17,25 $\pm$ 0,21		
Total pheophytins	60,14$\pm$3,94	47,47$\pm$2,29	19,15$\pm$0,12	75,90$\pm$0,46	68,29$\pm$0,23	61,17$\pm$7,81
Pheophorbide <i>a</i> series	37,31 $\pm$ 4,27	52,55 $\pm$ 2,24	6,96 $\pm$ 0,10	6,52 $\pm$ 0,62	5,07 $\pm$ 0,63	24,36 $\pm$ 4,13
Pheophorbide <i>c</i> series						11,63 $\pm$ 3,19
Total pheophorbides	37,31$\pm$4,27	52,55$\pm$2,24	6,96$\pm$0,10	6,52$\pm$0,62	5,07$\pm$0,63	35,98$\pm$7,21
Chlorophyll <i>a</i> series	1,81 $\pm$ 0,33		48,55 $\pm$ 0,71		9,62 $\pm$ 0,11	
Chlorophyll <i>b</i> series			25,20 $\pm$ 0,94	17,57 $\pm$ 0,24		
Chlorophyll <i>c</i> series					16,68 $\pm$ 0,92	2,85 $\pm$ 0,79
Total chlorophylls	1,81$\pm$0,33		73,75$\pm$0,23	17,57$\pm$0,24	26,31$\pm$0,81	2,85$\pm$0,79
<i>a</i> series	100,00 $\pm$ 0,00	100,00 $\pm$ 0,00	74,65 $\pm$ 0,94	65,17 $\pm$ 0,35	83,32 $\pm$ 0,92	85,53 $\pm$ 3,87
<i>b</i> series			25,20 $\pm$ 0,94	34,82 $\pm$ 0,34		
<i>c</i> series					16,68 $\pm$ 0,92	14,48 $\pm$ 3,88
Ratio <i>a/b</i> ^a			2,97 $\pm$ 0,15	1,87 $\pm$ 0,03		
Ratio <i>a/c</i> ^a					5,00 $\pm$ 0,33	6,29 $\pm$ 1,75
13 ² -hydroxy derivatives	9,84 $\pm$ 1,12	11,62 $\pm$ 0,26	15,11 $\pm$ 0,70	23,87 $\pm$ 0,23	12,24 $\pm$ 0,42	20,70 $\pm$ 1,21
15 ¹ -hydroxy-lactone series	1,24 $\pm$ 0,11	3,27 $\pm$ 1,06	1,75 $\pm$ 0,49	2,05 $\pm$ 0,14	3,60 $\pm$ 0,10	2,06 $\pm$ 0,38
Pyro derivatives	5,81 $\pm$ 0,37	3,42 $\pm$ 0,38			0,32 $\pm$ 0,01	1,00 $\pm$ 0,33
Purpurin-18 <i>a</i> series	0,75 $\pm$ 0,03	0,45 $\pm$ 0,18	0,15 $\pm$ 0,00		0,34 $\pm$ 0,05	

a, numbers were expressed with values.

Compound	Structure	R ₁	R ₂	R ₃	R ₄	R ₅
Series <i>a</i>						
Chlorophyll <i>a</i>	A	CH ₃	H	COOCH ₃	phytyl	Mg
13 ² -hydroxy chlorophyll <i>a</i>	A	CH ₃	OH	COOCH ₃	phytyl	Mg
15 ¹ -hydroxy-lactone chlorophyll <i>a</i>	B	CH ₃	OH	(15 ²)COOCH ₃	phytyl	Mg
Pheophorbide <i>a</i>	A	CH ₃	H	COOCH ₃	H	2H
13 ² -hydroxy pheophorbide <i>a</i>	A	CH ₃	OH	COOCH ₃	H	2H
15 ¹ -hydroxy-lactone pheophorbide <i>a</i>	B	CH ₃	OH	(15 ²)COOCH ₃	H	2H
Purpurin-18 <i>a</i>	D	CH ₃	-	-	H	2H
Pheophytin <i>a</i>	A	CH ₃	H	COOCH ₃	phytyl	2H
13 ² -hydroxy pheophytin <i>a</i>	A	CH ₃	OH	COOCH ₃	phytyl	2H
15 ¹ -hydroxy-lactone pheophytin <i>a</i>	B	CH ₃	OH	(15 ²)COOCH ₃	phytyl	2H
Pyropheophytin <i>a</i>	A	CH ₃	H	H	phytyl	2H
Phytyl purpurin-18 <i>a</i>	D	CH ₃	-	-	phytyl	2H
Series <i>b</i>						
Chlorophyll <i>b</i>	A	CHO	H	COOCH ₃	phytyl	Mg
13 ² -hydroxy chlorophyll <i>b</i>	A	CHO	OH	COOCH ₃	phytyl	Mg
15 ¹ -hydroxy-lactone chlorophyll <i>b</i>	B	CHO	OH	(15 ²)COOCH ₃	phytyl	Mg
Series <i>c</i>						
Chlorophyll <i>c₁</i>	C	CH ₃	H	COOCH ₃	H	Mg

Fig. 1: Names and structures of chlorophyll derivatives found in the fresh dried Nori, Sea Lettuce and Kombu seaweeds.

Fig. 2: Recovery of chlorophyll derivatives from tested Nori, Sea Lettuce and Kombu seaweeds after *in vitro* digestion. Total Chl stands for total chlorophylls. Data represent mean $\pm$ SEM for 8 independent measurements. Nori contains *a* series; Sea Lettuce contains *a* and *b* series; Kombu contains *a* and *c* series.