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High-performance liquid chromatographic determination of α-tocopherol in macroalgae

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Abstract

A high-performance liquid chromatographic (HPLC) method for the microscale determination of α-tocopherol in macroalgae is reported. The method includes microscale saponification and extraction with *n*-hexane. The presence of α-tocopherol in macroalgae samples was confirmed by HPLC–MS. α-Tocopherol levels as determined in samples by HPLC with UV and fluorescence detection did not differ significantly; however, fluorescence detection has a higher sensitivity (detection limit 10.4 ng/ml, vs. 104 ng/ml with UV detection), as well as good precision (relative standard deviation 1.81%) and recovery (94.3%). Fluorescence detection is also faster. We used this method to determine the α-tocopherol contents of four commercial macroalgae products from northwest Spain as part of nutritional studies in dehydrated *Himanthalia elongata* and *Laminaria ochroleuca*, and also in canned *Himanthalia elongata* and *Saccorhiza polychides*.

Keywords: Food analysis; Tocopherols

1. Introduction

Macroalgae are major contributors to the total marine plant biomass [1]. A number of species are traditional food products, notably in Japan [2]. Currently, macroalgae are attracting increasing interest, in view of their low calorie content and high vitamin, mineral and dietary fibre contents [3], making them attractive to both consumers and the food industry [4].

Vitamin E is a generic term applied to the tocopherols and tocotrienols (which show similar nutritional properties to α-tocopherol) [5]. These substances are highly stable to heat and acids, and unstable to alkalis, UV light and oxygen; in addition, they are degraded on contact with rancid fats, lead and iron [6]. α-Tocopherol is a liposoluble compound that is capable of fixing free radicals via its phenol group, and is thus considered to play an antioxidant role in biological membranes, lipoproteins and fat deposits, controlling or reducing lipid peroxidation. In addition, it has been suggested that it helps protect against cancer induced by free-radical-generating contaminants, such as ozone or nitrogen dioxide [7].

HPLC methods for the determination of α-tocopherol have largely replaced direct spectrophotometric and fluorometric procedures [5]. HPLC had been used with fluorescence detection [8–13], with UV detection [14–17], and most recently with detection by evaporative light scattering [18]. Method precision is in all cases high. The current technology

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allows samples for HPLC to be obtained at the micro scale, and the procedure has very high precision; HPLC is thus widely selected as a routine method. Losses of α-tocopherol may occur as a result of oxidation, most notably due to contact with air or light during the extraction procedure [13].

In previous studies, HPLC with fluorescence detection has been used to determine the α-tocopherol content of microalgae [19], to investigate seasonal variation in the α-tocopherol content of *Laminaria japonica* [20], and to quantify antioxidant substances in Phaeophyte, Rhodophyte and Chlorophyte algae [21]. However, the available information on the α-tocopherol content of macroalgae processed for human consumption is very limited [22].

In the present study, we compared two methods of detection (UV and fluorescence) for the HPLC determination of α-tocopherol content in macroalgae. In view of our findings, we describe a validated reversed-phase HPLC method (including microscale sample preparation) suitable for routine laboratory use.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade methanol and acetonitrile (Merck, La Coruña, Spain). Analytical-grade pyrocatechol (Aldrich, Madrid, Spain), potassium hydroxide (Analema, Pontevedra, Spain), (±)-α-tocopherol (Sigma, St. Louis, MO, USA) and ethanol (Merck, Darmstadt, Germany). Ultrapure water was prepared using a Milli-Q filter system (Millipore, Bedford, MA, USA). KOH solutions were prepared in methanol (0.5 *M*) and ethanol (1 *M*). The pyrocatechol solution (1 g in 5 ml of methanol) was prepared fresh daily, and stored at about 4 °C in the dark.

2.2. Equipment

Three chromatographic systems were used [HPLC–flouresence detection (FC), HPLC–UV and HPLC–MS].

The HPLC–FC system comprised a liquid chromatograph equipped with an HP1000 quaternary pump, a 20-μl injection loop (Rheodyne, Cotati, CA, USA) and an HP 1000 fluorescence detector controlled by HP CHEM software (all from Hewlett-Packard, Waldbronn, Germany). The column was a Kromasil 100 C_{18} (25×0.4 cm I.D., 5 μm particle size) (Teknokroma, Barcelona, Spain).

The HPLC–UV system comprised a liquid chromatograph (Spectra-Physics, San Jose, CA, USA) equipped with a PV-1589 intelligent pump (Jasco, Tokyo, Japan), a 20-μl injection loop (Rheodyne), a column-thermostatting system (Spectra-Physics 8792, San Jose, CA, USA), and a UV–vis Spectra-Focus forward optical scanning detector, controlled by PC-1000 software (Thermo Separation Products, Fremont, CA, USA). The column was a Tracer Extrasil ODS2 (25×0.4 cm I.D., 5 μm particle size) (Teknokroma).

The HPLC–MS system comprised a Spectra-Physics Series P200 liquid chromatograph equipped with a 50-μl injection loop Rheodyne, and a Fisons VG Platform mass detector (VG Biotech, Altrincham, UK), controlled by VG Mass Lynx software (all from SP Thermo Separation Products, Altrincham, UK). The column was a Kromasil 100 C_{18} (15×0.4 cm I.D., 5 μm particle size) (Teknokroma).

2.3. Chromatographic conditions

2.3.1. Fluorescence detection

The mobile phase was methanol–acetonitrile (50:50 v/v) at a flow-rate of 1.3 ml/min; excitation wavelength 288 nm, emission wavelength 329 nm, ambient temperature.

2.3.2. UV detection

The mobile phase was methanol–acetonitrile (30:70 v/v), flow-rate 1.0 ml/min, detection wavelength 205 nm, temperature 30±0.2 °C.

2.3.3. MS detection

The mobile phase was methanol–acetonitrile (30:70 v/v) at a flow-rate of 1.0 ml/min. Detector settings—positive atmospheric pressure chemical ionization ($APCI^+$) mode, probe temperature 500 °C, ionization source temperature 125 °C, cone voltage 30 V, electron multiplier voltage 650 V, drying gas nitrogen at 175 l/h, APCI sheet gas nitrogen at 225 l/h, full-scan-mode detection range m/z 50–500,

selected ion recording (SIR) m/z 430 (α-tocopherol$^+$) and m/z 165 ($C_{10}H_{13}O_2^+$).

2.4. Samples

2.4.1. Dehydrated algae

We analysed dried *Himanthalia elongata* and dried *Laminaria ochroleuca* as produced by the manufacturer Algamar (Redondela, Pontevedra, Spain) in June 2001. *Himanthalia elongata* is collected on the coast at Finisterre (La Coruña, Spain). *Laminaria ochroleuca* is collected on the coast at Bayona (Pontevedra, Spain). The samples are transported in plastic mesh baskets to the factory, where they are dried at 45 °C for 24 h, maintained at ambient temperature for 3 days, then packed in polypropylene bags.

2.4.2. Canned algae

We analysed canned *Saccorhiza polychides* and *Himanthalia elongata* (a single product containing approximately equal parts of each species) as produced by the manufacturer Conservas y Ahumados Lou (Ribeira, La Coruña, Spain) in August 2001. *Saccorhiza polychides* is collected on the coast close to the Ría de Arousa (La Coruña, Spain). *Himanthalia elongata* is collected on the coast in the Ría de Arousa. After transporting to the factory, the algae are prepared, canned and sterilized at 112 °C for 40 min. For the present study, the two species were manually separated for analysis.

2.5. Standards and quantification

The α-tocopherol stock was prepared by dissolving 30 mg of α-tocopherol in 100 ml of methanol–acetonitrile (30:70 v/v), giving a final concentration of 300 μg/ml. The stock was used to obtain working solutions of 0.75, 1.5, 3.0 and 6.0 μg/ml, which were stored at −10 °C in the dark. For determination of α-tocopherol in samples, the stock solution was in all cases analysed together with the samples, and analyte concentrations in samples were estimated on the basis of peak areas. All samples were analysed in duplicate.

2.6. Sample preparation

2.6.1. Determination of water content

In the case of the canned algae, excess water was drained off, and the algae were partially dried in an oven at 48 °C for 50 h. The water content of the partially dried canned algae and of the dehydrated algae was determined by weighing before and after drying to constant mass in a vacuum oven at 62 °C.

2.6.2. Saponification and α-tocopherol extraction

α-Tocopherol was extracted from the dried algae by the method of Botsoglou et al. [23] with minor modifications. The sample was ground immediately before extraction, in a light-protected laboratory. A subsample of 0.25 g (±0.001 g) was weighed out in a screwtop assay tube. A 200-μl volume of pyrocatechol solution and 5 ml of KOH solution (0.5 *M* in methanol) was added, and immediately vortexed for 20 s. The tubes were placed in a waterbath at 80 °C for 15 min, removing them every 5 min and vortexing again for 15 s. After cooling in iced water, 1 ml of distilled water and 5 ml of hexane was added, and the mixture was rapidly vortexed for 1 min, then centrifuged for 2 min at 373 *g*. A 3 ml volume of the upper phase was transferred to another test tube and dried under nitrogen. The residue was redissolved in 3 ml of the HPLC mobile phase (methanol–acetonitrile, 30:70 v/v), then membrane-filtered (pore size 0.50 μm; Millipore, Bedford, MA, USA). Finally, a 50-μl aliquot was injected into the HPLC column. Before injection, the extracts were maintained at −10 °C in the dark.

2.7. Statistical analysis

α-Tocopherol contents are cited as means±standard deviation. Means obtained by the different detection methods (fluorescence and UV) were compared by a *t* test for related samples ($P < 0.05$) as performed by the statistical package SPSS 9.0.

3. Results and discussion

3.1. Sample preparation

There have been many studies of optimum saponi-

fication conditions for extraction of α-tocopherol from foods of different types [10,16,18,19,23,24], but fewer studies have considered the effect of alkaline conditions on α-tocopherol stability [19,23,25,26]. To establish optimal saponification conditions, we performed preliminary assays using the method of Rodríguez-Bernaldo de Quirós [8], varying the amount of 1 *M* ethanolic KOH (10, 33 and 50 ml per gram of sample) and maintaining reflux time constant. These assays indicated that the best extraction was achieved with 33 ml of ethanolic KOH per gram of sample.

The above procedure is however expensive in terms of the apparatus required, the cost of solvents, and time. We therefore decided to develop a microscale method of the type described by Botsoglou et al. [23]. To determine optimal sample volume, preliminary assays were performed with 0.2, 0.4 and 0.6 g of sample; the results indicated that good results are achievable with 0.25 g of sample. We also assessed the stability of α-tocopherol in the HPLC mobile phase, by repeated analysis of a single extract-in-mobile-phase: during the first 24 h 13% of the analyte was lost, and the analyte content then remained roughly constant until the end of the assay (72 h). To investigate the effect of sample grinding and time elapsed before α-tocopherol extraction, we performed four assays on a single sample, in each case with quantification immediately, and 24 h later: the results indicate an α-tocopherol loss of 69.5%, attributable to the increased particle surface as a result of the grinding of the sample, favouring photolysis. Tocopherols are sensitive to air [27], and light accelerates their breakdown [28].

3.2. α-Tocopherol identification

After optimization of the HPLC conditions, peaks were observed at 13.92 min (fluorescence detection) and 9.19 min (UV detection). In HPLC analysis of α-tocopherol standards, the peak likewise eluted at these times, and showed very similar excitation and emission spectra (fluorescence detection) and absorbance spectra (UV detection) (Fig. 1), indicating that the peaks corresponded to α-tocopherol. The presence of α-tocopherol in the samples was confirmed by HPLC–MS analysis: SIR chromatograms for the fragments corresponding to α-tocopherol (m/z 430, α-tocopherol, and m/z 165, $C_{10}H_{13}O_2^+$) are shown in Fig. 2A and B (retention time, 11.89 min), and the mass spectrum for the α-tocopherol is shown in Fig. 2C.

In preliminary studies with HPLC–MS we optimized fragmentation temperature over the range 200–500 °C, and cone voltage over the range 10–70 V. To determine the limit of detection we assayed solutions with α-tocopherol concentrations over the range 0.75–300 μg/ml. Optimal conditions were fragmentation temperature 500 °C and cone voltage 30 V; the limit of detection was 750 ng/ml. The predominant masses were m/z 430 (α-tocopherol) and m/z 165 ($C_{10}H_{13}O_2^+$).

3.3. Method validation

The principal validation data for the fluorescence and UV detection methods are summarized in Table 1. The relationship between known and measured concentrations was assessed by linear regression, and the strong linearity obtained indicates that both methods are appropriate for quantification of α-tocopherol. Method precision was evaluated on the basis of the relative standard deviation (RSD) of α-tocopherol determinations in six samples of dehydrated *Himanthalia elongata*, with very good results for both UV detection (2.12%) and fluorescence detection (1.81%); both values are low by comparison with those obtained in related previous studies [8,16]. Determination of detection limits on the basis of signal-to-noise ratio (3:1) as per American Chemical Society guidelines [29] indicated that fluorescence detection (detection limit 10.4 ng/ml) is ten times more sensitive than UV detection (detection limit 104 ng/ml). Mean estimated α-tocopherol concentration did not differ significantly ($P > 0.05$) between the two methods for any of the six samples analysed. Fluorescence detection can be considered preferable in view of its higher sensitivity, shorter chromatographic time (16 min), and lower likelihood of interference by other sample components, in view of the high specificity of the wavelengths used; however, UV detection permits simultaneous detection of phytosterols [30], which elute later under the same chromatographic conditions.

The recovery of the fluorescence detection method

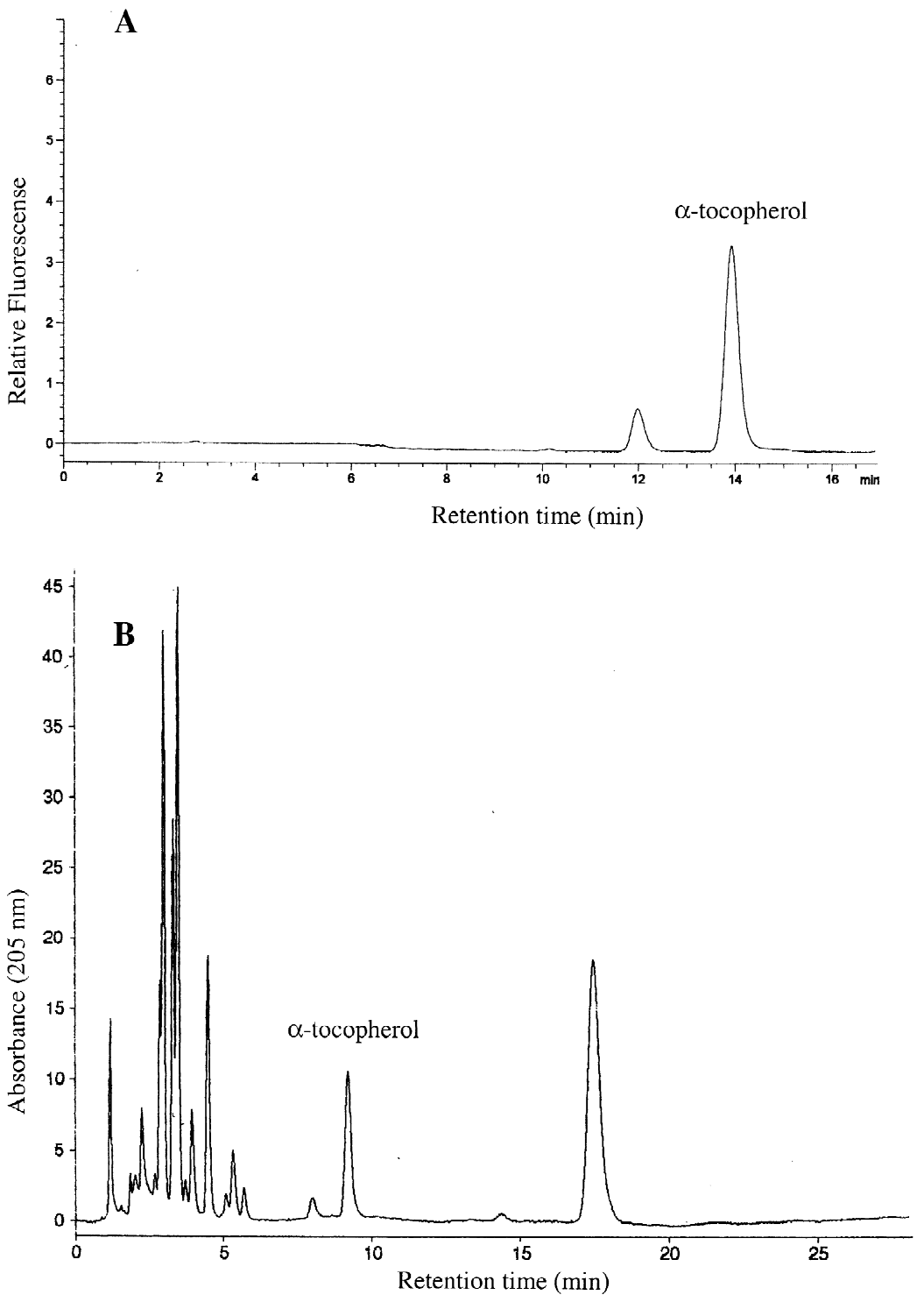

Fig. 1. HPLC chromatograms for determination of α-tocopherol in dehydrated *Himanthalia elongata*. (A) Fluorescence detection; (B) UV detection.

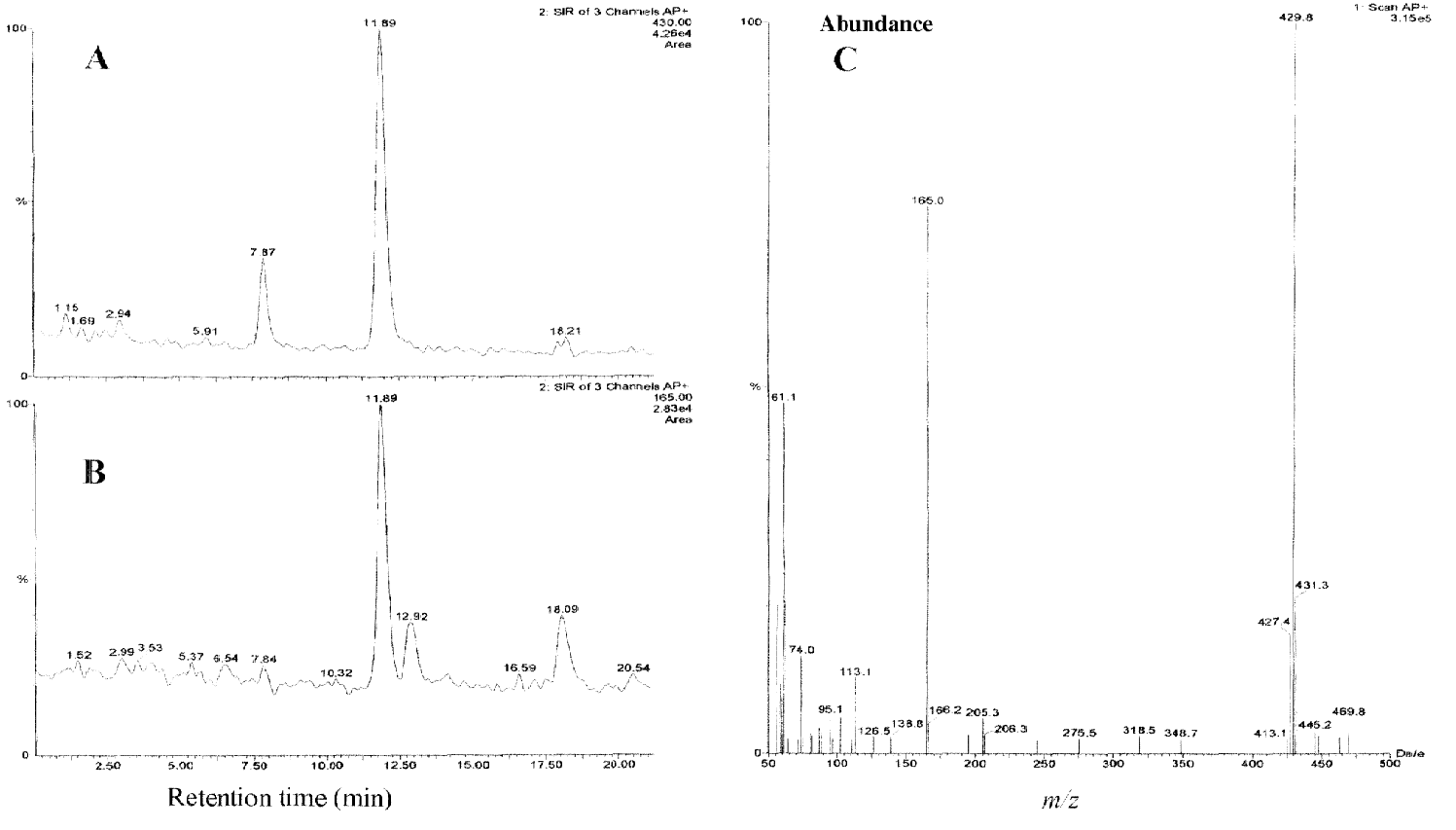

Fig. 2. HPLC chromatogram with MS detection (SIR mode) for the m/z 430 (A) and m/z 165 (B) fragments of the dehydrated *Himanthalia elongata* sample at 11.89 min. (C) Full-scan mass spectra of the α-tocopherol.

Table 1
Method validation parameters for determination of α-tocopherol in macroalgae

	Detection	
	UV	Fluorescence
Standard linearity		
Intercept	−4574.5	−1.9167
Slope	55 323	63.928
r	0.9994	0.9998
Range (μg/ml)	0.75–6.0	0.75–6.0
Method precision ($n=6$)		
Mean (μg/g)	36.9	37.7
Relative SD (%)	2.12	1.81
Detection limit (ng/ml)	104	10.4

was evaluated by analysis of six samples of dehydrated *Himanthalia elongata* spiked before saponification with α-tocopherol (51.6 μg/g of sample), and estimated as 94.3%.

3.4. α-Tocopherol in canned algae and dehydrated algae

The practical applicability of the method was assessed by analysis of ten samples of processed algae (Table 2). The α-tocopherol content of the samples showed very marked variation among the four types of sample. Mean content in dehydrated *Himanthalia elongata* (33.3±4.2 μg/g dry mass) was about thrice that in canned *Himanthalia elongata* (12.0±2.0 μg/g dry mass). This difference may be attributable to processing-related differences, seasonal variation and/or variation among harvesting sites [5]. α-Tocopherol losses in plant foods as a result of cooking may be up to 55% [15], and even the slightest contact with oxygen may lead to oxidation and increased losses [31].

Table 2
α-Tocopherol content in processed macroalgae

	α-Tocopherol (μg/g dry mass) (mean±SD)
Dehydrated	
Himanthalia enlongata	33.3±4.2
Laminaria ochroleuca	8.9±2.1
Canned	
Himanthalia enlongata	12.0±2.0
Saccorhiza polychides	5.7±1.3

Mean α-tocopherol level determined in the present study in dehydrated *Laminaria ochroleuca* was 8.9 μg/g dry mass, somewhat lower than in a previous study of summer-harvested *Laminaria ochroleuca* from Japan [20]. The mean level determined in the present study in dehydrated *Himanthalia elongata* (33.4 mg/g dry mass) markedly exceeds those reported for other plant foods, such as grass silage (20.8 μg/g dry mass) [14], garlic (9.4 μg/g dry mass) [32], tomatoes (3.15 μg/g dry mass) [25] and lentils (1.01 μg/g dry mass) [15]. It has been reported that Phaeophyte algae contain more α-tocopherol than Rhodophytes and Chlorophytes [21], and that α-tocopherol levels in plant foods vary depending on the plant part consumed [33].

In this study, we have developed a convenient method for the determination α-tocopherol in processed macroalgae, however minor amount of β-, γ- and δ-analogues occur in some algae species [21], although, in brown algae α-tocopherol is the principal tocopherol.

In conclusion, the method presented here is sensitive, selective, precise, fast, simple, cheap and convenient for routine determination of α-tocopherol in macroalgae intended for human consumption. Macroalgae collected for human consumption on the coasts of Galicia (northwest Spain) show high α-tocopherol levels.

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