Research Article

New approach for vitamin E extraction in rainbow trout flesh: Application in fish fed commercial and red seaweed-supplemented diets

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A vitamin E extraction method for rainbow trout flesh was optimized, validated, and applied in fish fed commercial and *Gracilaria vermiculophylla*-supplemented diets. Five extraction methods were compared. Vitamers were analyzed by HPLC/DAD/fluorescence. A solid-liquid extraction with *n*-hexane, which showed the best performance, was optimized and validated. Among the eight vitamers, only α- and γ-tocopherol were detected in muscle samples. The final method showed good linearity (>0.999), intra- (<3.1%) and inter-day precision (<2.6%), and recoveries (>96%). Detection and quantification limits were 39.9 and 121.0 ng/g of muscle, for α-tocopherol, and 111.4 ng/g and 337.6 ng/g, for γ-tocopherol, respectively. Compared to the control group, the dietary inclusion of 5% *G. vermiculophylla* resulted in a slight reduction of lipids in muscle and, consequently, of α- and γ-tocopherol. Nevertheless, vitamin E profile in lipids was maintained. In general, the results may be explained by the lower vitamin E level in seaweed-containing diet.

Practical applications: Based on the validation results and the low solvent consumption, the developed method can be used to analyze vitamin E in rainbow trout. The results of this work are also a valuable information source for fish feed industries and aquaculture producers, which can focus on improving seaweed inclusion in feeds as a source of vitamin E in fish muscle and, therefore, take full advantage of all bioactive components with an important role in fish health and flesh quality.

Keywords: Extraction method / Feed / *Gracilaria vermiculophylla* / Rainbow trout flesh / Vitamin E

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1 Introduction

In Europe, rainbow trout (*Oncorhynchus mykiss*) is the second most consumed species, representing about 69% of freshwater fish-farming in the European Union [1]. The high demand for and consumption of this fish may be explained by its nutritional profile, which includes a high content of long-chain *n*-3 polyunsaturated fatty acids (PUFA). They are valuable components in the human diet due to their well-known health benefits, such as protection against cardiovascular diseases, type II diabetes, and cancer [2, 3]. However, lipid peroxidation reactions can contribute to a decrease in *n*-3 PUFA in fish flesh. Freezing or modified atmosphere packaging are commonly used by the food industry in order to reduce oxidation and increase shelf-life of fillets. Nevertheless, they do not completely hinder these reactions [4]. In aquaculture, the supplementation of diets with antioxidants such as vitamin E can minimize the formation of free radicals during frozen storage [5, 6].

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Abbreviations: CTRL, control diet; **GRA,** diet with 5% of seaweed inclusion; **PUFA,** polyunsaturated fatty acids

Vitamin E is comprised by a group of eight molecules, α, β, γ, and δ-tocopherols and the respective tocotrienols. α-Tocopherol has the highest antioxidant activity, reacting with radical products of lipid peroxidation [7]. In flesh, α-tocopherol is the main vitamer found and, in aquaculture, its deposition in flesh depends on the ingredients or synthetic forms of this vitamer that are included in aquafeeds [8], for example commercial supplements of α-tocopherol or DL-α-tocopherol acetate [6, 9, 10].

Over the years, the effects of dietary seaweed inclusion on fish growth and health have been extensively studied [11–14]. For instance, *Gracilaria vermiculophylla*, a red seaweed, has been considered a good ingredient in rainbow trout diets due to its high digestibility and protein content [15]. Nevertheless, few studies have evaluated the dietary inclusion of *Gracilaria* sp. and other seaweeds with regard to improvement of quality parameters in fish flesh, such as sensory traits [14], iodine [16], and lipid content [17]. Antioxidant capacity is also a valuable quality parameter in this tissue, in which vitamin E (mainly α-tocopherol) plays a relevant role [18]. To our knowledge, no data exist evaluating the effects of dietary seaweed inclusion on vitamin E content of fish muscle.

Diversified methods can be used to extract vitamin E from animal tissues and some of them are described in Table 1. Several authors use saponification followed by *n*-hexane extraction [19–21], whereas others choose the traditional lipid extraction techniques such as modified Folch procedure [22, 23] or soxhlet method [24]. Thus, there is no common procedure to extract vitamin E from flesh of salmonids.

The aim of this work was to develop and validate an extraction method for vitamin E quantification in rainbow trout muscle. Then, the selected method was applied to muscle samples of rainbow trout fed an experimental diet containing *Gracilaria vermiculophylla* (5%). Vitamin E profile and content was determined by normal-phase HPLC/diode-array/fluorescence detection.

2 Materials and methods

2.1 Standard solutions and reagents

For vitamin E analysis, standard solutions of tocopherols (α, β, γ, and δ) (Calbiochem, La Jolla, CA, USA) were prepared and diluted in *n*-hexane. Tocol (2-methyl-2-(4,8,12-trimethyltridecyl) chroman-6-ol) (Matreya Inc., PA, USA) was used as internal standard, at a concentration of 1 mg/mL in *n*-hexane with 0.01% of BHT (butylated hydroxytoluene) (Sigma–Aldrich, Madrid, Spain). All standards were stored at −20°C. *n*-Hexane, dichloromethane, chloroform, and ascorbic acid were obtained from Sigma–Aldrich. Ethanol, methanol, and anhydrous sodium sulfate (Na_2SO_4) were purchased from Merck (Darmstadt, Germany). Sodium chloride (NaCl) and petroleum ether (range 40–60°C) were purchased from Panreac (Barcelona, Spain). Potassium hydroxide (KOH) and 1,4-dioxane were obtained from Prolabo (VWR, Lisbon, Portugal) and Fluka (Madrid, Spain), respectively.

2.2 Experimental diets

A control diet (CTRL) was formulated according to nutritional requirements of rainbow trout (*O. mykiss*). An experimental diet (GRA) was formulated by replacing fishmeal by 5% of *Gracilaria* meal, being fish oil and vegetable ingredients adjusted to ensure isonitrogeneous (42%) and isoenergetic (22 kJ g^{-1}) diets (Table 2). Proximate composition analyses of experimental diets were conducted according to Association of Official Analytical Chemists (AOAC) [25]. Diets were ground, homogenized, and analyzed in terms of: Moisture (105°C for 24 h); ash content (combustion at 550°C, for 6 h); crude protein (N × 6.25; Leco N Analyzer, Model FP-528, Leco Corporation, USA); crude lipid (petroleum ether extraction, Soxtherm Multistat/SX PC, Germany), and gross energy (adiabatic bomb calorimeter, Werke C2000, IKA,

Table 1. Vitamin E extraction and quantification procedures from different matrices

Sample	Extraction	Quantification	Reference
Low-protein dishes	Soxhlet apparatus with petroleum ether (2.5 h).	Normal phase HPLC with diode array and fluorescence detectors.	[24]
Hazelnuts, adipose tissue, and coffee beans	Solid-liquid extraction with *n*-hexane.	Normal phase HPLC with diode array and fluorescence detectors.	[26]
Fish muscle	Solid-liquid extraction with methanol/chloroform (1:2).	Normal phase HPLC with fluorescence detection.	[23]
Fish and fish products	Saponification with 50% KOH solution (overnight, at room temperature) followed by extraction with *n*-hexane.	Normal phase HPLC with fluorescence detection.	[19]
Animal tissues	Saponification with 50% KOH solution (70°C in a water bath for 30 min) followed by extraction with *n*-hexane.	Reverse phase HPLC with UV detection.	[20]
Beef muscle	Saponification with 11% KOH solution (80°C in a water bath for 15 min) followed by extraction with isooctane.	Normal phase HPLC with fluorescence detection.	[21]

Table 2. Formulation and proximate composition of the experimental diets

	Dietary treatments	
	CTRL	GRA
Feed ingredients (%)		
Fishmeal 70 LT[a]	18.0	13.0
Fishmeal 65[b]	10.0	10.0
Gracilaria meal[c]	0.0	5.0
Soycomil PC[d]	8.0	10.0
Wheat gluten	0.0	3.0
Corn gluten	7.0	7.0
Soybean meal[e]	14.0	14.0
Rapeseed meal[f]	7.6	5.3
Sunflower meal[g]	4.0	0.0
Wheat meal	8.0	7.4
Whole peas	5.0	4.9
Fish oil	8.5	9.0
Rapeseed oil	3.0	3.0
Linseed oil	1.0	1.0
Palm oil	2.0	2.0
Vit[h] & Min[i] premix	1.5	1.5
Binder (guar gum)[j]	1.0	1.0
Binder (Kieselghur)[k]	1.0	1.0
DCP	0.0	1.0
L-Lysine	0.2	0.5
DL-Methionine	0.2	0.3
L-Threonine	0.0	0.1
Proximate composition		
Dry matter (DM, %)	95.89	96.25
Ash (% DM)	9.31	10.49
Crude protein (% DM)	42.79	42.33
Crude fat (% DM)	20.49	21.12
Gross energy (kJ/g DM)	22.35	21.89
α-Tocopherol	6.49	3.52
γ-Tocopherol	25.92	19.01

[a]Peruvian fishmeal—low tempearture (LT), EXALMAR, Peru.
[b]Fair Average Quality (FAQ) fishmeal (61% crude protein, 11% crude fat, COFACO, Portugal).
[c]*Gracilaria* meal (*Gracilaria vermiculophyllla*).
[d]Soycomil-PC (soy protein concentrate, 65% crude protein, 0.7% crude fat, ADM, the Netherlands).
[e,j]SORGAL SA, Portugal (Soybean meal—solvent-extracted dehulled soybean meal).
[f,g]Premix, Portugal.
[h]Vitamins (mg or IU kg^{-1} diet): Vitamin A (retinyl acetate), 20.000 UI; vitamin D3 (DL-cholecalciferol), 2000 UI; vitamin E (Lutavit E50), 100 mg; vitamin K3 (menadione sodium bisulfitete), 25 mg; vitamin B1(thiamine hydrochloride), 30 mg; vitamin B2 (riboflavin), 30 mg; calcium pantothenate, 100 mg; nicotinic acid, 200 mg; vitamin B6 (pyridoxine hydrochloride), 20 mg; vitamin B9 (folic acid), 15 mg; vitamin B12 (cyanocobalamin), 100 mg; vitamin H (biotin), 3000 mg; vitamin C (Lutavit C35), 1000 mg; inositol, 500 mg; colin chloride, 1000 mg; betaine (Betafin S1), 500 mg;
[i]Minerals (mg or % kg^{-1} diet): Co (cobalt carbonate), 0.65 mg; Cu (cupric sulphate), 9 mg; Fe (iron sulphate), 6 mg; I (potassium iodide), 0.5 mg; Mn (manganese oxyde), 9.6 mg; Se (sodium selenite), 0.01 mg; Zn (zinc sulphate) 7.5 mg; Ca (calcium carbonate), 18.6%; KCl, 2.41%; NaCl, 4.0%.
[k]Sopropeche, France.

Germany). α- and γ-Tocopherol were determined as described by Alves, et al. [26]. Samples were analyzed in duplicate.

2.3 Samples

Rainbow trout (*O. mykiss*) fish (average body weight 67.04 ± 0.35 g) were fed experimental diets for 91 days (Table 2). This trial was performed in fibre-glass tanks at the Experimental Research Station (Vila Real, Portugal) of University of Trás-os-Montes e Alto Douro (UTAD) and conducted by trained scientists following category C recommendations from Federation of European Laboratory Animal Science Associations (FELASA) and the European Directive 2010/63/EU of European Parliament and of the Council of European Union on the protection of animals used for scientific purposes. Fish were sacrificed by a sharp blow on the head. Eighteen muscle samples were collected from the dorsal body area for vitamin E analysis from each dietary treatment, frozen in liquid nitrogen and stored at −80°C until further use.

To select the best method of extraction (section 2.4), muscle samples from rainbow trout fed a commercial diet were used.

2.4 Method selection

2.4.1 Comparison of different methods of extraction

In order to select the best methodology to extract vitamin E, five different methods frequently applied to foodstuffs (Table 3) were tested in muscle samples of rainbow trout and compared in relation to extractability of compounds, time, and reagents consumption. For each method, muscle samples were weighted (1 g) and analyzed in quadruplicate. A 1 mg/mL tocol solution (20 μL) was added to samples. The selected methods were performed according to the respective references (with some modifications) as described in Table 3.

Method A, a Soxhlet extraction, was performed by homogenization of muscle samples with anhydrous sodium sulfate and extraction with petroleum ether for 2.5 h. A solid-liquid extraction with dichloromethane/methanol (0.01% BHT) (2:1), a Folch-based extraction procedure (method B), was applied to muscle samples. After homogenization, the mixture was agitated for 15 min on an orbital shaker, at room temperature. After centrifugation (2500 rpm, for 10 min), it was filtered and re-extracted. After a new filtration, phase separation was achieved by adding a 0.7% NaCl solution. Samples were centrifuged and the lower layer was recovered. Finally, the solvent was removed using a rotatory evaporator to obtain the lipid extract (Büchi R-114 Rotavapor, Switzerland). Method C consisted of a solid-liquid extraction with *n*-hexane and the final method is fully described in section 2.5. A solid-liquid extraction was applied to muscle samples as described for method C,

Table 3. Summary of the five extraction methods tested

Method	Description	References
A	Soxhlet extraction. Homogenization with anhydrous sodium sulfate and extraction with petroleum ether for 2.5 h.	[24]
B	Folch method. Extraction with dichloromethane/methanol-BHT (0.01%) (2:1).	[23]
C	Solid-liquid extraction with *n*-hexane (0.01% of BHT).	[26, 46]
D	Solid-liquid extraction with methanol-BHT (0.01%) similar to method C.	[32]
E	Homogenization and overnight saponification using KOH solution and magnetic agitation, at room temperature. Extraction with *n*-hexane as described in method C.	[19]

replacing *n*-hexane by methanol (method D). Method E was performed by homogenization with ascorbic acid (0.12 g), 4 mL of distilled water, and 10 mL of absolute ethanol, under nitrogen stream (for 30 min). The mixture was saponified using 1 mL of 50% KOH solution and magnetic agitation, at room temperature. An extraction with *n*-hexane was then performed as described in method C. All final extracts obtained from tested methods were dried and dissolved in 1 mL of *n*-hexane (0.01% BHT) before HPLC analysis.

2.5 Evaluation of vitamin E levels in the flesh of rainbow trout fed experimental diets

The selected extraction method was optimized, validated (see "Results and discussion" section), and applied to analysis of muscle samples from rainbow trout fed experimental diets. For each dietary treatment, three pools of six muscle samples ($n = 3$) were homogenized in a grinder (Retsch GM200 Grindomix, Germany) and stored at −80°C until further use. Sample aliquots of approximately 1 g were weighted, spiked with the internal standard (10 μL of a 1 mg/mL tocol solution), and extracted with 5 mL of absolute ethanol and 10 mL of *n*-hexane (0.01% of BHT). After vortexing and constant agitation (30 min), 5 mL of 1% NaCl solution was added. The mixture was centrifuged and the residue was re-extracted twice with 10 mL of *n*-hexane. Organic layers were pooled and Na_2SO_4 was added. After a new centrifugation, the supernatant was collected and the solvent was dried under nitrogen stream. The residue was re-dissolved in 500 μL of *n*-hexane and filtered before injection into the HPLC system. All extractions were performed in amber glassware and protected from light.

2.6 Chromatographic analysis

The HPLC analysis was conducted using an AS-2057 automated injector, a PU-2089 pump, and a MD-2018 multi-wavelength diode array detector (DAD) coupled to a fluorescence detector FP-2020 (Jasco, Japan). Chromatographic separation was performed using a normal phase Supelcosil^{TM} LC-SI (75 cm × 3 mm, 3 μm) column (Supelco, Bellefonte, PA) and an isocratic system with *n*-hexane/1,4-dioxane as mobile phase (98:2), at a flow rate of 0.7 mL/min. Vitamin E was identified according to their UV absorption spectra and the retention time of commercial standards. Quantification was achieved using the fluorescence detector set for a gain of 10, with excitation and emission wavelengths of 290 and 330 nm, respectively. Data were analyzed with JASCO-Chrom NAV Chromatography Software (Jasco, Japan).

2.7 Statistical analysis

In order to test normality and homogeneity of variances, data were submitted to Kolmogorov–Smirnov test and Levene's test, respectively. Data were then analyzed with one-way analysis of variance (ANOVA) to test differences between dietary treatments. Significant differences were considered when $p < 0.05$ and individual means were compared using Tukey's test. All statistical analyses were performed using the program IBM SPSS STATISTICS, 21.0 version, IBM Corporation, New York, USA (2011).

3 Results and discussion

3.1 Selection of the extraction method

When comparing the five methods of extraction, it was possible to observe that vitamin E in rainbow trout muscle consisted of α-tocopherol (the main vitamer) and γ-tocopherol, whose levels were below the detection limit or sometimes not found. These findings are in general accordance with literature since α-tocopherol is usually stored in lipid membranes, whereas γ-tocopherol is mainly deposited in adipose tissues, which may explain its low concentration in trout muscle [27–29].

The concentrations of α-tocopherol recovered in methods B, C, D, and E were 11.76, 16.11, 13.56, and 15.09 mg/kg of flesh, respectively. The best extraction rates for α-tocopherol were achieved with method C ($p < 0.05$). Besides, this method presented a clean chromatographic profile of α- and γ-tocopherol. The same result was not obtained with Soxhlet extraction (A). Although method A was previously used in different food matrices such as deserts, beef, and salmon flesh [24, 30], in the current study, this method extracted several interfering substances, which contributed to less pure peaks, thereby affecting the identification of tocopherols. Other traditional extraction methods for fat-soluble compounds, for example Folch (B) or Bligh and Dyer [22] methods, have been used for vitamin E extraction in salmonids [27, 28, 31]. However, in this case,

α-tocopherol levels were significantly lower than those obtained in methods C, D, and E.

The replacement of *n*-hexane by methanol (method D) affected both α- and γ-tocopherol extraction. This method has been previously validated and used to extract tocopherols from aquatic organisms [32]. Nevertheless, methanol exhibits higher polarity then *n*-hexane, which may explain its reduced efficiency for vitamin E extraction from lipid-rich matrices as rainbow trout muscle.

Saponification is a common step for vitamin E extraction from flesh of salmonids [19, 33, 34]. However, in the current study, the addition of a saponification step before *n*-hexane extraction (method E) did not contributed to a more effective extraction of vitamin E content when compared to method C. Additionally, and as referred in literature [26, 35], tocol was only added after neutralization of KOH since it is degraded by alkaline agents and converted in interfering compounds.

3.2 Optimization of experimental conditions

After the selection of the extraction procedure, optimization was performed in order to reduce extraction times and reagents consumption (Table 4).

The best extraction rates were achieved with volumes of 5 and 10 mL of ethanol and *n*-hexane, respectively, and triplicate extractions with a longer agitation time (30 min). Although reduction of ethanol volume did not affect α-tocopherol extraction, these experimental conditions may have contributed to the decrease of γ-tocopherol levels. The reduction of ethanol and *n*-hexane (to 2.5 and 5 mL, respectively) negatively influenced α- and γ-tocopherol quantification since tocol peak area was slightly lower than that obtained with the initial conditions. The replacement of ethanol by ethyl acetate also decreased the extraction of both tocopherols. Additionally, their recovery was also affected by the use of lower volumes of organic solvents and shorter agitation times.

Ethanol seems to be critical to vitamin E extraction since it increases the polarity of non-polar solvents, such as *n*-hexane. Additionally, ethanol dissolves the lipids of cell membrane, increasing the extraction efficiency [36]. Although a previous study have successfully used ethyl acetate to extract vitamin E from espresso coffee [37], this solvent may not be as suitable as ethanol to extract vitamin E from this food matrix. The reduction of *n*-hexane also affected extraction since tocol, which is more polar than tocopherols, may require a higher volume of *n*-hexane to be efficiently recovered. Likewise, agitation time seems to be crucial to extract tocopherols since its reduction may decrease the contact time between the matrix and the solvent, and thereby the extraction efficiency. Finally, in order to increase γ-tocopherol signal, the final extracts were re-dissolved in a lower solvent volume (500 μL). Figure 1 shows a fluorescence chromatogram of α- and γ-tocopherol extracted from muscle samples of rainbow trout using the final extraction method.

Table 4. Relative areas of α- and γ-tocopherol obtained from muscle samples under different experimental conditions

		Relative area (%)	
Experimental conditions		α-Tocopherol	γ-Tocopherol
Solvent amount (mL)			
5 absolute ethanol + 10 *n*-hexane		100	100
2.5 absolute ethanol + 10 *n*-hexane		97	83
2.5 absolute ethanol + 5 *n*-hexane		96	83
10 *n*-hexane/ethyl acetate (90:10)		54	51
Number of extractions	Agitation time (min)		
2	5 + 2 + 2	78	73
	15 + 2 + 2	80	73
3	5 + 2 + 2	80	75
	15 + 2 + 2	80	75
	30 + 2 + 2	100	100

3.3 Method validation

The validation parameters were calculated for α- and γ-tocopherol since those were the only vitamin E vitamers present in the sample.

3.3.1 Linearity

In order to assess linearity range, six different concentrations of α- and γ-tocopherol standard solutions were prepared (0.14–27.57 μg/mL for α-tocopherol and 0.13–25.65 μg/mL for γ-tocopherol). Calibration curves were obtained using standard concentration (μg/mL) and the ratio between peak areas of individual tocopherols and the internal standard. The resulting calibration curves displayed high correlation coefficients (>0.999).

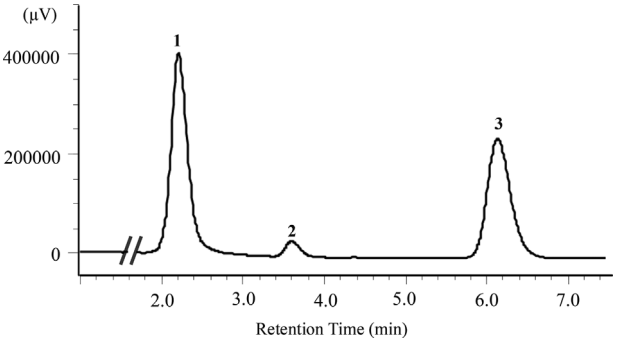

Figure 1. Fluorescence chromatogram of rainbow trout muscle. α-Tocopherol (1). γ-tocopherol (2). and tocol (as internal standard) (3).

3.3.2 Detection and quantification limits

The detection and quantification limits were determined using the ratio between the standard deviation of the blank signal and the slope of the calibration curve, and multiplying it by 3.3 and 10, respectively. The detection limits were 39.9 and 111.4 ng/g of fish muscle for α- and γ-tocopherol, respectively. The quantification limits were 121.0 ng/g (for α-tocopherol) and 337.6 ng/g (for γ-tocopherol).

3.3.3 Precision

The intra-day precision was calculated by analyzing the same muscle sample four times during the same day. The resulting coefficients of variation were 1.33 and 3.03% for α- and γ-tocopherol, respectively. The inter-day precision, which was determined by analyzing the same muscle sample in four different days, showed coefficients of variation of 1.38 and 2.50% for α- and γ-tocopherol, respectively.

3.3.4 Accuracy

Accuracy was assessed by the standard addition method, carried out in duplicate and expressed as percentage of recovery (Table 5). Before extraction, α- and γ-tocopherol standards were added in increasing concentrations to the muscle sample. High recovery values were achieved for α- tocopherol (> 94%) and for γ-tocopherol (> 97%).

3.4 Vitamin E quantification in samples

The selected method was applied to muscle samples of rainbow trout fed experimental diets. The extraction was conducted in quadruplicate and the results are described in Fig. 2. Average α- and γ-tocopherol values in flesh of CTRL group were 18.3 and 1.0 mg/kg, respectively. These values are in general accordance with those reported in previous studies for flesh of this species [19, 28, 38, 39], ranging from 11.2 to 30.5 mg vitamin E/kg flesh. Only one work reports higher values: 152.26–185.5 mg α-tocopherol /kg of fillet in fish fed control diets [33]. These variations in vitamin E content found for the same species (taking into account that, in general, the animals were supplemented with similar levels of vitamin E) may reflect differences, not only in the growth conditions and/or diet composition, but also in extraction and quantification methods used to determine vitamin E levels in rainbow trout muscle.

G. vermiculophylla was selected based on its feasibility as ingredient in rainbow trout [15]. In this work, 5% of this seaweed was included in a rainbow trout diet and the remaining ingredients were adjusted in order to keep diets isonitrogeneous and isoenergetic. However, GRA diet contained lower levels of α- and γ-tocopherol (3.52 and 19.01 mg/kg of feed, respectively) when compared with CTRL diet (6.49 and 25.92 mg/kg) (Table 2).

Flesh from rainbow trout fed diets fed *G. vermiculophylla*, in this study, resulted in a significant decrease of α-tocopherol content from 18.3 ± 0.9 (CTRL group) to 15.1 ± 0.9 mg/kg of flesh in GRA group. γ-Tocopherol concentration from CTRL dietary treatment (1.01 mg/kg of flesh) also differed significantly from levels found in muscle of trout fed GRA (0.70 mg/kg). The values of vitamin E found in trout muscle may reflect the values present in the experimental diets. The different ratios of vegetable ingredients in fish feeds may have affected their vitamin E content [27, 28]. In the current study, the inclusion of vegetable oils was similar among dietary treatments, but the different inclusion levels of vegetable proteins may explain the observed results. Sunflower meal and wheat meal exhibit good levels of vitamin E (9.1 and 23.9 mg/kg, respectively), being α-tocopherol the main vitamer [40, 41]. Rapeseed meal also exhibits interesting levels of vitamin E (13.4 mg/kg) and α- and γ-tocopherol are the main vitamers. Additionally, fishmeal contributes with vitamin E, mainly α-tocopherol (5 mg/kg) [40]. On the other hand, *G. vermiculophylla*

Table 5. Method accuracy for α- and γ-tocopherol tested in spiked muscle of rainbow trout

	Original levels (mg/mL)	Added (mg/mL)	Detected levels (mg/mL)	Recovery (%)
α-Tocopherol	18.90 ± 0.13	4.73	23.13 ± 0.16	97.9
		9.46	28.24 ± 0.22	99.7
		14.19	31.06 ± 0.29	93.9
γ-Tocopherol	1.05 ± 0.01	0.26	1.42 ± 0.00	109.2
		0.52	1.66 ± 0.03	106.1
		0.78	1.78 ± 0.01	97.3

Mean value ± standard deviation.

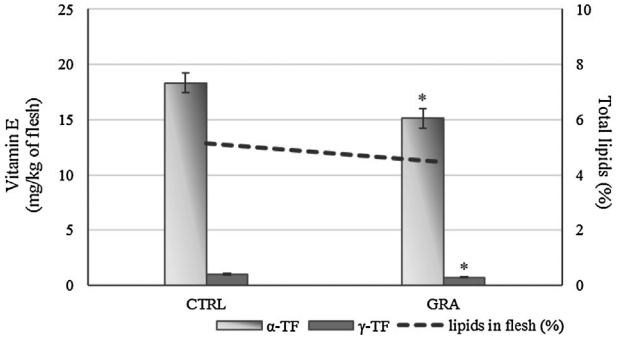

Figure 2. Vitamin E and lipid content in rainbow trout muscle fed experimental diets. Values are mean ± SD ($n = 18$).α-TF and γ-TF represent α and γ-tocopherol, respectively. Asterisks indicate significant differences between the two experimental groups (<0.05).

may not exhibit substantial levels of α- and γ-tocopherol to balance or increase the vitamin E content of experimental diets. Previous studies show that *G. vermiculophylla* present a lower levels of α-tocopherol (28.4 mg/kg of extract) [42] when compared to other *Gracilaria* sp., such as *G. chilensis* and *G. bursa-pastoris* (86.5 mg/kg lipid and 570 mg/kg, respectively) [43, 44].

Vitamin E is closed related to lipid content as it is a fat-soluble vitamin. In the current study, when expressing levels of α- and γ-tocopherol by the percentage of total muscle lipids, no significant differences ($p > 0.05$) were observed between the two experimental groups. Although levels of tocopherols were different, their profile in lipid matrix is equal between dietary treatments. Seaweed inclusion may have altered lipid absorption, transport and/or metabolism [45], which can affect lipid deposition in muscle and, consequently, vitamin E levels in this tissue. Since final body weight was similar in the two experimental groups (~220 g), it would be interesting to study other factors that could have influenced this difference in the total lipid content of muscle, such as potential compounds that may act as anti-nutritional factors. Studies in this regard are already being conducted in order to better understand the effects of this seaweed on nutrient absorption and metabolism, and flesh quality in rainbow trout.

4 Conclusions

The selected method was shown to be precise and accurate for quantification of vitamin E in rainbow trout muscle. It was possible to obtain clean chromatograms of tocopherols, without a saponification step to eliminate interfering compounds.

The adjustment of vegetable ingredients and/or the potential alterations in lipid metabolism associated with *G. vermiculophylla* dietary inclusion may have contributed to the decrease of lipid content and therefore the reduction of vitamin E in flesh from fish fed this experimental diet.

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