2
3 **ESTERIFIED CAROTENOIDS AS NEW FOOD COMPONENTS IN**
4 **CYANOBACTERIA**

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ABSTRACT

Among the nutritional properties of microalgae, this study is focused in the presence of carotenoid esters in prokaryote microalgae, an event that has not been shown so far. Three carotenoid esters that accumulate in non-stressful culture conditions are identified in *Aphanotece microscopica* Nägeli and *Phormidium autumnale* Gomont, what may provide an extra value to the quality attributes of the carotenoid profile in cyanobacteria as functional foods. In addition, new data on the carotenoid characterization added quality criteria for the identification of the esterified metabolites, enabling the monitoring of these food components. Specifically, the metabolomic approach applied to the food composition analysis, has allowed to differentiate between the esters of zeinoxanthin and β -cryptoxanthin, which were undifferentiated to date during the MS characterization of carotenoids in other food sources. We propose a new qualifier product ion specific for zeinoxanthin ester, which it is not present in the MS² spectrum of β -cryptoxanthin esters.

Keywords: *Aphanotece*; cyanobacteria; carotenoid esters; esterification; mass spectrometry; *Phormidium*.

Chemical compounds studied in this article

9-cis-violaxanthin (PubChem CID: 5282218); 9-cis-neoxanthin (PubChem CID: 101052392); zeaxanthin (PubChem CID: 5280899); zeinoxanthin (PubChem CID: 5281234); echinenone (PubChem CID 5281236); α -carotene (PubChem CID: 6419725); β -carotene (PubChem CID: 5280489)

1. Introduction

Most of microalgae biotechnology companies concentrate investments and technology in low-volume high-value food products, which can be successfully allocated in lines of business with high projected returns and growing demands. This is the case of the microalgae market for production of secondary metabolites, a cluster of healthy food components with attractive selling prices and significant applications in the development of functional food products. Microalgae carotenoids represent a model of success in terms of economic viability that competes in a global market value of \$1.4 billion with an estimated annual growth rate of 2.3% (Liu, Sun & Gerken, 2016). Indeed, the alternative chemical synthesis of carotenoids presents a limited use for human consumption due to safety concerns, so that natural sources of carotenoids are the production platform for the nutraceutical market (Gong & Bassi, 2016). Microalgae present several advantages as carotenoid sources in comparison with higher plants, including faster growth rate, greater specific carotenoid content and higher versatility to different cultivation conditions. Consequently, a large group of food industries produce carotenoids from microalgae, with astaxanthin in the pool position followed by β -carotene and lutein, with promising market values in the range of \$230-\$447 million (Panis & Rosales Carreon, 2016).

Several strategies are applied to improve the carotenoid production in microalgae, from genetic modifications to the implementation of new technologies, as well as diverse environmental growing settings. Hence, for secondary carotenoids (astaxanthin, canthaxanthin, zeaxanthin or fucoxanthin) and some primary carotenoids (β -carotene) the nutrient-stressed, high light and high salt conditions are factors that promote the quantities of carotenoids accumulated in the eukaryote microalgae (Lemoine & Schoefs, 2010). Biologically, the high accumulation of specific carotenoids is a strategy to store energy and carbon supplies for future stressful conditions, and to enhance the cell resistance to oxidative stress (Lemoine et al. 2010). This spectacular carotenogenesis induced in eukaryote microalgae by stressful environment implies morphological and metabolic modifications

59 in the cells, which have been intensely studied in eukaryote microalgae considering the economic
60 value of those metabolites and their industrial applications. The most extensively studied taxa are
61 *Dunaliella salina* to produce β -carotene and *Haematococcus pluvialis* for the extraction of
62 astaxanthin.

63 An interesting biological consequence of the intense carotenogenesis in eukaryote microalgae is
64 the activation of the esterification process of the secondary carotenoids with fatty acids. In this
65 sense, only a significant accumulation of esterified carotenoids has been described for different
66 Chlorophyte species, *Haematococcus pluvialis*, *Chlamydomonas nivalis*, and *Chlorella zofingiensis*
67 (Lemoine et al., 2010). The esterification of carotenoids is one incompletely comprehended
68 metabolic process, frequently associated with the ripening of carotenogenic fruits such as peppers
69 (Breithaupt & Schwack) or oranges (Giuffrida, Dugo, Salvo, Saitta, & Dugo, 2010), flowers and
70 cereals (Ziegler, Wahl, Würschum, Longin, Carle & Schweiggert, 2015). This reaction proceeds
71 only with xanthophylls that carry at least one hydroxyl group, which reacts with the carboxylic
72 group of the fatty acid, yielding an ester bond. During the carotenogenic process, the chloroplasts
73 are transformed into chromoplasts, where the esterified carotenoids are accumulated in the
74 corresponding plastoglobules. The accumulation of esterified carotenoids is well-documented in
75 fruits and vegetables and recent reviews compile the current available information (Bunea, Socaciu
76 & Pinte, 2014; Mercadante, Rodrigues, Petry & Mariutti, 2017).

77 From the nutritional point of view, the *ester* condition is considered a positive extra attribute that
78 enhances several characteristics of the xanthophylls, and a growing number of evidences are
79 becoming available to support this point. Thus, it has been shown that xanthophyll esters are more
80 stable to UV-radiation and thermal treatment than the free forms in model systems (Bunea et al.,
81 2014), and they present an increased quenching ability that results in higher antioxidant activity in
82 both *in vivo* eukaryote microalgae organisms (Kobayashi 2000) and *in vitro* systems (Sun et al.,
83 2016). Additionally, bioavailability of carotenoid esters is relatively higher than that of the free

84 carotenoid (Mariutti & Mercadante, 2018). However, the *ester* attribute is lost during intestinal
85 digestion/cellular absorption (Pérez-Gálvez, & Mínguez-Mosquera, 2005) and only the free forms
86 have been observed in plasma (Wingerath, Stahl & Sies, 1995). Consequently, it could be assumed
87 that the extra value of the *ester* feature is to increase their bioavailability. Anyhow, a significant re-
88 esterification process has been recently discovery in human tissues (Ríos et al., 2017). Nevertheless,
89 the added benefits of xanthophylls in their ester form could be still functional during the
90 gastrointestinal transit. Thus, esterified astaxanthin from *Haematococcus pluvialis* has been shown
91 to protect from oxidative stress caused by ethanol-induced gastric ulcers in rat (Kamath, Srikanta,
92 Dharmesh, Sarada & Ravishankar, 2008) and in murine gastric ulcer models (Murata et al., 2012)
93 and attention has been focused in the possible modulation of carcinogenesis in the gastrointestinal
94 tract (Amaro, Barros, Guedes, Sousa-Pinto & Malcata, 2013).

95 Despite these progresses in knowledge, the esterification of eukaryote microalgae carotenoids
96 has been exclusively investigated in Chlorophyte, as a process resulting from stress factors
97 (Lemoine et al. 2010) and referred to both astaxanthin and fucoxanthin esters. The search of the
98 accumulation of xanthophyll esters beyond astaxanthin has recently started to be accomplished,
99 describing the presence of zeaxanthin esters in the *Coelastrella* sp. KGU-Y002 (Saeki, Aburai,
100 Aratani, Miyashita & Abe, 2017) and in other eukaryote microalgae classes. This is the case of
101 *Nannochloropsis gaditana* (Simionato et al., 2013) from the Scenedesmaceae family that
102 accumulates violaxanthin, antheraxanthin, zeaxanthin and vaucheriaxanthin esters. Therefore, this
103 scenario opens the door to the optimization process to increase the accumulation of xanthophyll
104 esters as added-value food components.

105 However, the acquisition of successful approaches to enhance the metabolism of microalgae
106 lipids requires the application of high-resolution analytical tools to obtain a clear picture of the food
107 components. The hypothesis of this study was to determine the presence of carotenoid esters in
108 prokaryote microalgae, as valuable components that have not been detected so far. Briefly, we used

109 axenic cultures of *Aphanotece microscopica* Nägeli and *Phormidium autumnale* Gomont, which
110 were grown under non-stressing conditions. We isolated the pigment fraction and the extracts were
111 analyzed by HPLC-high resolution-MSⁿ applying a data processing protocol dedicated to
112 metabolomics of carotenoids (Pérez-Gálvez, Sánchez-García, Garrido-Fernández & Ríos, 2018).
113 With these metabolomic tools we were able to identify three carotenoid esters that accumulated in
114 non-stressful culture conditions what may provide an extra value to the quality attributes of the
115 carotenoid profile in cyanobacteria as functional foods for production of added-value products.

116 **2. Materials and Methods**

117 **2.1. Microorganisms and cultivation conditions.**

118 Axenic cultures of *Aphanotece microscopica* Nägeli (rsman92) were originally isolated from the
119 Patos Lagoon Estuary, at the state of Rio Grande do Sul, Brazil (32°01's - 52°05'w). The strain is
120 within the collection of the Cyanobacteria and Phycotoxins Laboratory of the Institute of
121 Oceanography from the Federal University of Rio Grande - FURG (www.cianobacterias.furg.br).
122 Axenic cultures of *Phormidium autumnale* were originally isolated from the Cuatro Ciénegas
123 desert, Mexico (26°59' N, 102°03' W). The strains were propagated and maintained in synthetic BG-
124 11 medium (Rippka, Deruelles, Waterbury, Herdman, & Stanier, 1979). The incubation conditions
125 used were 26 °C, a photon flux density of 25 $\mu\text{mol.m}^{-2} \text{s}^{-1}$, and a photoperiod of 12:12 h light:dark.

126 **2.2. Microalgal biomass production**

127 The biomass production was carried in a bubble column photobioreactor, operating in
128 intermittent regime, fed with 2.0 L of BG11 medium (Ripka et al., 1979). The experimental
129 conditions were as follows: initial concentration of inoculum of 100 mg/L, temperature of 26 °C,
130 aeration of 1 volume of air per volume of medium per minute, a photon flux density of 25 $\mu\text{mol.m}^{-2}$
131 s^{-1} , a light cycle of 24:0 h (light:dark), and a residence time of 168 h. The microalgae biomasses
132 were separated from the culture medium by centrifugation.

133 **2.3. Reagents**

134 All the chemical compounds used for the BG-11 medium, sodium chloride, ammonium acetate
135 (98%) and tetrabutylammonium acetate were supplied by Sigma-Aldrich Chemical Co. (Madrid,
136 Spain). Acetone and diethyl ether (analysis grade) were supplied by Teknokroma (Barcelona,
137 Spain). *N,N*-dimethylformamide (DMF) and hexane (analysis grade) and methanol, methyl *tert*-
138 butyl ether and water (HPLC grade) were supplied by Panreac (Barcelona, Spain). The deionised
139 water used was obtained from a Milli-Q 50 system (Millipore Corp., Milford, MA, USA). Solutions
140 of carotenoid standards α -carotene, β -cryptoxanthin and β -cryptoxanthin esters, echinenone,
141 myxoxanthophyll, neoxanthin and violaxanthin isolated from natural sources were obtained
142 following the procedures described by Britton, Liaaen-Jensen and Pfander (1995), while zeaxanthin
143 and β -carotene standards were obtained from Extrasynthese (Genay Cedex, France). Aliquots of the
144 carotenoid standard solutions were analysed with the same experimental conditions and the UV-
145 visible and bbCID MSⁿ spectra were acquired and compared with those obtained from the
146 microalgae extracts, so that direct experimental criteria were applied for identification of known
147 carotenoids. Standards of zeinoxanthin and β -cryptoxanthin epoxides were not available, so that
148 identification was performed according to literature data.

149 **2.4. Extraction of pigments**

150 Microalgae biomass aliquots (5 mL) were filtered under vacuum through a 47-mm diameter
151 glass microfiber filter (GF/F; Whatman), and immediately frozen at -80 °C (Wright et al., 1991).
152 The filtered microalgae was mixed with liquid nitrogen and grinded into powder in a mortar. Then,
153 this powder was mixed with 10 mL of extraction solvent, DMF: deionised water (9:1) under stirring
154 at 4 °C for 15 min and centrifuged (10000 rpm, 5 min). The organic layer was accumulated in a
155 decanting funnel, while the solid residue was re-extracted with 10 mL hexane, mixed in an
156 ultrasonic bath (5 min, 720 W), vortexed (5 min), and after the addition of 10 mL NaCl solution
157 (10% w/v) the mixture was centrifuged (10000 rpm, 5 min). The supernatant was added to the
158 funnel and the pellet was re-suspended with 10 mL diethyl ether, mixed in an ultrasonic bath (5

159 min, 720 W), vortexed (5 min), and finally centrifuged (10000 rpm, 5 min) after the addition of 10
160 mL NaCl solution (10% w/v). The combined solvent fractions in the funnel were extracted with
161 diethyl ether and NaCl solution (10% w/v). The upper phase was isolated and concentrated to
162 dryness in a rotary evaporator. The residue was dissolved in acetone. Samples were stored at -20°C
163 until analysis.

164 **2.5. Pigments identification by HPLC-PDA-APCI(+)-hrTOF-MSⁿ**

165 The chromatographic separation was performed using a liquid chromatograph Dionex Ultimate
166 3000RS U-HPLC (Thermo Fisher Scientific, Waltham, MA, USA). For carotenoid pigments a C₃₀ 3
167 µm particle size reversed-phase YMC analytical column (250×4.6 mm, YMC Europe, Dinslaken,
168 Germany) was used. The elution gradient applied for this group of pigments was based on the
169 method published by Breithaupt, Wirt and Bamedi (2002), and it has been recently modified (Ríos,
170 Roca & Pérez-Gálvez, 2015). The injection volume was 30 µL with 1 mL/min as the flow rate. UV-
171 visible spectra were recorded by a PDA detector at the 300-700 nm wavelength range. A split post-
172 column of 0.4 mL/min was introduced directly on the mass spectrometer ion source. Mass
173 spectrometry was performed using a micrOTOF-QI™ High Resolution Time-of-Flight mass
174 spectrometer (UHR-qTOF) with Qq-TOF geometry (Bruker Daltonik, Bremen, Germany). The
175 instrument equipped with an APCI source was operated in positive ion mode using a scan range of
176 *m/z* 50-1200 Da. The full scan mode was applied for initial characterizations, while extracted ion
177 chromatogram mode was employed during the study of the MSⁿ of the new carotenoids. The mass
178 spectra acquisition was a broad-band collision induced dissociation mode (bbCID), providing at the
179 same time MS and MS² spectra. The software used for the instrument control was Hyphenation Star
180 PP (version 3.2, Bruker Daltonik, Bremen, Germany) and DataAnalysis (version 4.1, Bruker
181 Daltonik, Bremen, Germany) for evaluation of MS spectra. The software TargetAnalysis™ 1.2
182 allowed the automated peak detection on the extracted ion chromatograms and the identification
183 according to mass accuracy and in combination with the isotopic pattern in the SigmaFit™

algorithm (Ríos et al., 2015). The tolerance limits were set at 5 ppm for mass accuracy and 50 for SigmaFit values. The SmartFormula3DTM software module allowed the interpretation of the MS² spectra (Ríos et al., 2015) as well as for checking the consistency of the product ions with the same criteria for mass accuracy and isotopic pattern established for the corresponding protonated molecule. *In silico* MS² spectra were obtained for target compounds with the MassFrontierTM software (Thermo ScientificTM version 4.0, Waltham, USA) to perform the evaluation of experimental MS² and the acquisition of both analogue and diverging theoretical product ions when different structural isomers were possible for a single bbCID spectrum.

3. Results and discussion

3.1. Carotenoid profile

Table 1 shows the chromatographic and spectrometric parameters that have allowed the identification of the carotenoid profile in the cyanobacteria: the maxima wavelength value of each UV-visible spectrum, exact mass (Da), error mass (ppm) and the mSigma matching factor between the theoretical and experimental isotopic patterns (Pérez-Gálvez et al., 2018) that fulfil the established tolerance limits, as well as the main experimental characteristic product ion(s), which have been described in literature (van Breemen, Dong & Pajkovic, 2012; Rivera, Christou & Canela-Garayoa, 2014). These product ions correspond with the loss of water [M+H-18]⁺, toluene [M+H-92]⁺, [M+H-124]⁺, [M+H-40]⁺, [M+H-56]⁺, [M+H-80]⁺, [M+H-84]⁺, or in-chain losses. Table 2 contains the percent area values for the identified carotenoids in both cyanobacteria species that present the typical carotenoid pattern of the cyanobacteria phylum, rich in β -carotene, echinenone and zeaxanthin and absence of lutein. Specifically, the carotenoid profile is dominated by the group of carotenes that account more than 70% of the total carotenoids in both species. Echinenone (*trans* and *cis* isomers) is the first signifying xanthophyll (15-18%) followed by zeaxanthin (around 4%). Other minor xanthophylls (neoxanthin, violaxanthin, zeinoxanthin, 5,6-epoxy- β -cryptoxanthin and 5,8-epoxy- β -cryptoxanthin) have been also identified in both strains

209 previously (Patias, Fernandes, Petry, Mercadante, Jacob-Lopes & Zepka, 2017). The most known
210 species of *Aphanotece* is *Aphanotece halophytica* (Berland, Le Campion & Campos, 1989) with a
211 similar carotenoid content as the one described here for *Aphanotece microscopica* Nägeli.

212 3.2. Esterified carotenoids

213 In addition to the carotenoids identified in Table 1, the HPLC chromatogram of the pigments
214 extracted from cyanobacteria (Figure 1) showed three signals with the characteristic UV-visible
215 pattern of carotenoids (peaks 6, 10, 11) that eluted at the apolar region of the chromatogram.
216 Recently, it has been summarized the main MS² characteristic product ions formed using APCI in
217 positive mode for xanthophyll esters: loss of toluene (92 Da) as the featured neutral loss from the
218 polyene chain, the backbone of the xanthophyll and the loss of the fatty acid (Mercadante,
219 Rodrigues, Petry & Mariutti, 2017).

220 The first unknown pigment (peak 6, at 26.5 min.) was exclusively observed in *Aphanotece*
221 *microscopica* and exhibits the maximum absorbance value at 472 nm wavelength in the UV-Visible
222 spectrum (Figure 1), which is limited to few carotenoids. Specifically, in the carotenoid pigment
223 profile of this cyanobacteria (Patias et al., 2017), the only carotenoid identified with similar
224 absorbance spectrum is myxoxanthophyll. In this carotenoid kind, which it is exclusive of
225 cyanobacteria, the carotenoid (myxol) is bonded to different glycosides at the hydroxyl group of the
226 C2' of the ψ -end group (Takaichi, Mochimaru & Maoka, 2006). Particularly, in *Aphanotece*
227 *microscopica*, the accurate mass from the $[M+H-18]^+$ protonated molecule was observed at $m/z =$
228 829.6506 Da corresponding to the elemental composition C₅₈H₈₆O₂. Four characteristic product
229 ions were obtained in the bbCID spectrum of this pigment (Table 1, Figure 2, Fig S1). The product
230 ion at $m/z = 567.4214$ Da (C₄₀H₅₅O₂) arises from the release of the fatty acid (280 Da, Figure 2a)
231 and the product ion at $m/z = 475.3568$ Da (C₃₃H₄₇O₂) could be assigned to the combined release of
232 both the fatty acid and one in-chain unit of toluene (Figure 2b), features that match with the
233 characteristic MS² profile of an esterified xanthophyll. Two additional product ions were new

234 fragmentations. The product ion at $m/z = 607.4155$ Da corresponds to an atypical fragmentation of
235 the fatty acid that generates a compound with $C_{42}H_{55}O_3$ as elemental composition (Figure 2c).
236 Finally, the neutral loss of 354 Da ($C_{21}H_{38}O_4$) agreed with the fragmentation at the 12,13-carbon
237 bond remaining the charge in the originally esterified 2' carbon atom (Figure 2d). These
238 experimental product ions were predicted *in silico* by the MassFrontierTM software, and they
239 fulfilled the quality criteria for the mass error and mSigma value. The combination of all the MS
240 data obtained for the peak 6, allowed us to tentatively identify this carotenoid as 2'-linolenoyl-
241 myxol-ester. Esterification at the 3-hydroxy- β -ring was discarded by the *in silico* prediction of the
242 fragmentation pattern of the 3-linolenoyl-myxol-ester that did not include the product ion observed
243 at $m/z = 493.3881$ Da (Figure 2d) that it is exclusive of the unimolecular decomposition profile of
244 the 2'-linolenoyl-myxol-ester. This carotenoid has not been previously identified, and even the
245 detection of the free form (myxol) is scarce (Takaichi et al., 2006). The presence of similar
246 structures has been reported in few species of bacteria (*Rhodococcus* and *Gordonia*), the so-called
247 glucosyl mycoloyl esters (Takaichi, Maoka, Akimoto, Carmona & Yamaoka, 2008).

248 In *Phomidium autumnale*, the peak 10 at 31.2 min showed a UV-visible spectrum with three
249 maxima at 420, 448 and 472 nm (Figure 1) and the HPLC-APCI(+)-hrTOF-MSⁿ analysis
250 determined an experimental monoisotopic mass at $m/z = 763.6385$ Da corresponding with the
251 elemental composition $C_{54}H_{82}O_2$. The fragmentation of the protonated molecular ion yielded
252 different product ions (Table 1, Fig. S2), which contain information about the significant structural
253 features of this compound. The main product ion was observed at $m/z = 535.4298$ Da ($C_{40}H_{56}$) that
254 corresponds with the loss of myristic acid (228 Da) from a monohydroxylated xanthophyll (the
255 fragmentation mechanism is depicted in Figure 3a). Other characteristic product ion was observed
256 at $m/z = 443.3657$ Da with the elemental composition $C_{33}H_{47}$ generated after the loss of the fatty
257 acid and elimination of one in-chain unit of toluene (Figure 3b). The other three product ions, which
258 have not been reported before, were created *in silico* by the predictive software MassFrontierTM, and

then targeted in the experimental mass spectrum with the extracted ion chromatogram function. These product ions as well as the former ones fulfill the criteria for exact mass, elemental composition and isotope pattern described in the section 2.4. Hence, the product ion at $m/z = 507.4211$ Da and elemental composition $C_{35}H_{55}O_2$ corresponds with the fragmentation at the 14,15-carbon bond, remaining the 3-hydroxy- β -ring in its ester form (Figure 3c). The formation of the product ion at $m/z = 731.6136$ Da ($C_{53}H_{79}O$) that preserves the myristic acid but the decyclization process involves the 3,4- and 4,5-carbon bonds (Figure 3d). Considering the structural features of these product ions; that free zeinoxanthin has been identified in the pigment profile of *P. autumnale* (Rodrigues, Menezes, Mercadante, Jacob-Lopes & Zepka, 2015) (Table 1); the UV-visible wavelength maxima and the chromatographic behavior (Table 1), this peak is assigned as myristoyl-zeinoxanthin ester. Although it has been proposed that the esters of zeinoxanthin and β -cryptoxanthin show the same MS behavior (Petry and Mercadante, 2016) the *in silico* fragmentation of the myristoyl-zeinoxanthin ester with the MassFrontierTM software predicted a product ion at $m/z = 399.3041$ Da ($C_{30}H_{39}$) that was also experimentally observed. This product ion is produced from the elimination of the fatty acid moiety and fragmentation at the polyene chain at 7,8-carbon bond (Figure 3e). In the experimental MS acquired the signal at $m/z = 399.3046$ Da was consistent with the elemental composition and formulae of the *in silico* product ion, which fulfil the filtering rules applied to mass error and isotopic pattern. As this product ion is not generated in the simulated fragmentation pattern of the myristoyl- β -cryptoxanthin ester, we propose the product ion at $m/z = 399.3046$ Da to distinguish by MS the identified zeinoxanthin ester from its β -cryptoxanthin ester isomer. The former esterified pigment is a scarce carotenoid, not present in typical esterifying/carotenogenic organisms. Indeed, myristoyl-zeinoxanthin ester has only been described in very low amounts in Arazá fruits (Garzón, Narváez-Cuenca, Kopec, Barry, Riedl & Schwartz, 2015) and more recently in tangor fruits (Petry et al., 2016). The MS² fingerprint described for this carotenoid (Petry et al., 2016) contains two typical product ions, one corresponding with the fatty

284 acid elimination, the corresponding one arising from the loss of toluene from the polyene chain
285 coupled with the loss of fatty acid, coincident with the data obtained in Table 1.

286 Concurrently, the chromatographic profile of *A.microscopica* presents at 32.1 min a signal with a
287 similar UV-visible pattern to the myristoyl-zeinoxanthin ester (Figure 1, peak 11). In fact, this
288 carotenoid showed a MS protonated molecule at $m/z = 763.6389$ Da corresponding with the same
289 elemental composition as myristoyl zeinoxanthin ester ($C_{54}H_{82}O_2$). In this case, the bbCID spectrum
290 of the protonated ion at $m/z = 763.6389$ Da did not show the signal at $m/z = 399.3046$ Da.
291 Otherwise, two typical product ions were determined (Fig. S3), one corresponding with release of
292 the fatty acid (observed at $m/z = 535.4305$ Da, $C_{40}H_{56}$, Figure 4a) and the characteristic loss of the
293 myristic acid in combination with the in-chain yield of toluene (observed at $m/z = 443.3663$ Da,
294 $C_{33}H_{47}$, Figure 4b). An additional product ion was observed at $m/z = 507.4202$ Da that corresponds
295 with the fragmentation at the 15,16-carbon bond remaining the 3-hydroxy- β -ring in its ester
296 configuration (Figure 4c). Additionally, the formation of the product ion at $m/z = 731.6130$ Da
297 ($C_{53}H_{79}O$) that preserves the myristic acid but the decyclization process involves the 3,4- and 4,5-
298 carbon bonds (Figure 4d) was also observed. With these data, this signal was assigned as myristoyl-
299 β -cryptoxanthin ester. This pigment is a common carotenoid in vegetables and fruits rich in
300 esterified carotenoids such as chili, tangerine, clementine or pepper (Breithaupt & Bamedi, 2001)
301 and recently it has been found as the main monoester in peach fruit (jam and juice) (Giuffida, Torre,
302 Dugo & Dugo, 2013) or in Physalys fruits (Wen, Hempel, Schweiggert, Ni, & Carle).

303 As stated before, the esterification of carotenoids in eukaryote microalgae is associated with
304 environmental stress conditions, when the vegetative cell is transformed into red cysts (Lemoine et
305 al., 2010). As a reaction to the stressful conditions, the eukaryote microalgae synthesize new
306 carotenoids with the aim of protecting the cellular tissues from oxidative reactions. A hypothesis is
307 that, in similarity with eukaryote microalgae, cyanobacteria could respond to the environmental
308 stress not only increasing the quantity of secondary carotenoids but also esterifying them. The

309 presence of a fatty acid could facilitate the integration of the carotenoid in the different membranes,
310 as carotenes and xanthophylls affect the viscosity of the membranes (Zakar, Laczko-Dobos, Toth &
311 Gombos, 2016), allowing the acclimation of cyanobacteria to different temperatures. In fact, Zakar
312 et al. (2017) have pointed out the cooperative roles between xanthophylls and polyunsaturated
313 lipids, providing evidence for the interdependence of lipid and carotenoid contents in the thylakoid
314 membranes. Further investigations will be required to analyze the behavior of *Aphanotece* and
315 *Phormidium* under different stress conditions with the aim to potentiate the esterification of
316 carotenoids and go into detail about the physiological meaning.

317 **4. Conclusion**

318 The novelty of this study is the identification in cyanobacteria by first time of esterified
319 carotenoids as a result of the use of an exhaustive extraction procedure of the pigment profile, and
320 through the application of high resolution metabolomic tools. Specifically, we observed myristoyl-
321 zeinoxanthin and myristoyl- β -cryptoxanthin esters, which were identified previously in several
322 fruits, and 2'-linolenoyl-myxol-ester, which it is a new MSⁿ identification as myxoxanthophylls are
323 unique in cyanobacteria. The identification of esterified carotenoids in cyanobacteria generates new
324 possibilities for the food industry (previous authorizations) as it will be possible to utilize
325 cyanobacteria as a new bio-factories for esterified carotenoids. At the end, the *ester* condition is a
326 valuable character for the design of new food formulae, as it has been shown that esterified
327 carotenoids are more stable, possess higher antioxidant characteristic and indeed their
328 bioavailability is higher. The next development should be establishing the optimal stressing
329 conditions to maximize the reaction of esterification affecting the secondary carotenoids. However,
330 this would not be a handicap considering that microalgae show a high biosynthetic plasticity for
331 secondary metabolites (carotenoids) in comparison with higher plants, and the food industry has
332 taken the most of this feature previously with other microalgae strains. Finally, the application of
333 accurate hyphenated techniques and powerful post-processing software to the analysis of food has

334 allowed to propose a new qualifier product ion to distinguish between the esters of zeinoxanthin and
335 β -cryptoxanthin (with different pro-vitamin A values), a problem not determined so far in the
336 characterization of esterified carotenoids in fruits.

337 **Acknowledgements**

338 The authors would like to thank to Sergio Alcañiz for his technical assistance.

339 **Conflict of interest statement**

340 The authors declare that they have no conflict of interest.

341 **Appendix A. Supplementary data**

342 Supplementary data to this article can be found online at

343 **Funding**

344 This work was supported by the Comisión Interministerial de Ciencia y Tecnología (CICYT-EU,
345 Spanish and European Government, grant number AGL 2015-63890-R). MM was supported with a
346 fellowship from CAPES.

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472 **Figure captions**

473 Figure 1: Chromatogram traces at 450 nm of *Aphanotece microscopica* Nagéli (A) and *Phormidium*
 474 *autumnale* (P) extracts. UV-visible spectra of the esterified carotenoids. Peak number identification
 475 is detailed in Table 1. “C” means chlorophyll pigment.

476 Figure 2: Experimental product ions acquired by APCI(+)-bbCID of 2'-linolenoyl-myxol ester.

477 Figure 3: Experimental product ions acquired by APCI(+)-bbCID of myristoyl-zeinoxanthin ester.

478 Figure 4: Experimental product ions acquired by APCI(+)-bbCID of myristoyl- β -cryptoxanthin
479 ester.

Table 1. Carotenoids identified in *Aphanotece microscopica* Nagëli (A) and *Phormidium autumnale* Gomont (P) by HPLC-PDA-APCI(+)-hrTOF-MS^a.

Peak	Carotenoid	Strains	t _R (min)	Wavelength maxima (nm)	[M+H] ⁺ (m/z)	Mass error (ppm)	mSigma	Main product ions (m/z)
1	<i>cis</i> -violaxanthin	A, P	5.9	412, 434, 463	599.4126 ^a	4.3	29.5	581.4002 [M+H-18] ⁺ , 477.3559 [M+H-3-hydroxy-ring] ⁺
2	<i>cis</i> -neoxanthin	A	6.3	412, 434, 462	599.4113 ^a	2.2	32.9	583.4400 [M+H-18] ⁺
3	<i>all-trans</i> -zeaxanthin	A, P	17.2	425, 450, 476	569.4368	2.6	18.1	551.3379 [M+H-18] ⁺ , 477.3571 [M+H-92] ⁺
4	5,6-epoxy- β -cryptoxanthin	A	20.0	420, 444, 470	569.4373	3.5	32.1	551.3251 [M+H-18] ⁺
5	<i>all-trans</i> -zeinoxanthin	P	26.0	420, 448, 472	553.4386	3.3	19.0	535.4295 [M+H-18] ⁺ , 461.3349 [M+H-92] ⁺ , 377.2892 [M+H-9,10C] ⁺ , 337.2524 [M+H-12,13C] ⁺
6	2'-linolenoyl-myxol ester	A	26.5	446, 470, 502	829.6506 ^b	3.4	14.6	607.4155 [M+H-C ₁₅ H ₂₆ -18] ⁺ , 567.4214 [M+H-FA] ^{tc} , 493.3881 [M+H-FA-56] ⁺ , 475.3568 [M+H-FA-92] ⁺
7	<i>all-trans</i> -echinenone	A, P	27.7	461	551.4257	1.8	10.4	533.4121 [M+H-18] ⁺ , 495.3632, 459.3641 [M+H-92] ⁺
8	<i>cis</i> -5,8-epoxy- β -cryptoxanthin	A	28.3	402, 426, 450	569.4325	4.9	32.1	551.3237 [M+H-18] ⁺
9	<i>cis</i> -echinenone	A, P	29.4	455	551.4236	2.0	13.0	533.4153 [M+H-18] ⁺ , 495.3641, 459.3690 [M+H-92] ⁺
10	myristoyl-zeinoxanthin ester	P	31.2	420, 448, 472	763.6385	1.0	14.2	731.6136 [M+H-CH ₄ O] ⁺ , 535.4298 [M+H-FA] ⁺ , 507.4211 [M+H-14,15C] ⁺ , 443.3657 [M+H-FA-92] ⁺ , 399.3046 [M+H-FA-7,8 C] ⁺
11	myristoyl- β -cryptoxanthin ester	A	32.1	424, 450, 472	763.6389	1.0	16.4	731.6130 [M+H-CH ₄ O] ⁺ , 535.4305 [M+H-FA] ⁺ , 507.4202 [M+H-14,15 C] ⁺ , 443.3663 [M+H-FA-92] ⁺
12	9- <i>cis</i> - α -carotene	A, P	32.3	330, 420, 444, 472	537.4443	2.2	18.5	453.3683 [M+H-84] ⁺
13	<i>all-trans</i> - β -carotene	A, P	36.6	424, 451, 477	537.4436	3.6	16.6	457.33770 [M+H-80] ⁺ , 413.3107 [M+H-124] ⁺
14	9- <i>cis</i> - β -carotene	A, P	38.0	340, 420, 446, 472	537.4455	0.0	28.2	457.3765 [M+H-80] ⁺ , 413.3155 [M+H-124] ⁺

^aProtonated ion arising from the M⁺⁺ radical ion. ^b[M+H-18]⁺. ^cFA means fatty acid.

Table 2. Carotenoids quantified (percent area at 450 nm) in *Aphanotece microscopica* Nagëli and *Phormidium autumnale* Gomont by HPLC-PDA.

Peak	Carotenoid	<i>Aphanotece microscopica</i>	<i>Phormidium autumnale</i>
1	<i>cis</i> -violaxanthin	0.82	0.67
2	<i>cis</i> -neoxanthin	0.69	0.66
3	<i>all-trans</i> -zeaxanthin	4.35	3.70
4	5,6-epoxy- β -cryptoxanthin	1.37	-
5	<i>all-trans</i> -zeinoxanthin	-	0.41
6	2'-linolenoyl-myxol ester	0.91	-
7	<i>all-trans</i> -echinenone	7.96	6.00
8	<i>cis</i> -5,8-epoxy- β -cryptoxanthin	0.72	-
9	<i>cis</i> -echinenone	10.1	9.52
10	myristoyl-zeinoxanthin ester	-	0.79
11	myristoyl- β -cryptoxanthin ester	1.46	-
12	9- <i>cis</i> - α -carotene	0.70	0.22
13	<i>all-trans</i> - β -carotene	67.5	69.4
14	9- <i>cis</i> - β -carotene	3.42	8.63

Figure 2

Figure 3