Application of compressed fluids-based extraction and purification procedures to obtain astaxanthin-enriched extracts from *Haematococcus pluvialis* and characterization by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry

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ABSTRACT

The green microalga *Haematococcus pluvialis* has been widely studied due to its capacity to accumulate great amounts of astaxanthin, a high-value carotenoid with biological activities. In the present work, two green compressed fluids-based processes, pressurized liquid extraction (PLE) and supercritical antisolvent fractionation (SAF) are integrated to obtain an astaxanthin-enriched extract from this microalga. PLE was carried out using pressurized ethanol as solvent, for 20 min, at 10 MPa, and 50 °C as extraction temperature. Subsequently, the obtained extract was processed by SAF to further purify the carotenoids fraction. The SAF process was optimized using a 3-level factorial experimental design and considering three experimental variables: (i) CO_2 pressure (10-30 MPa), (ii) percentage of water in the PLE extract (20-50%) and (iii) PLE extract/supercritical-CO_2 flow rate ratio (0.0125-0.05). Total carotenoids content was evaluated in both *extracts* and *raffinates*. Best results were obtained at 30 MPa, 0.05 feed/SC-CO_2 mass flow rate and 20 % (v/v) of water in the feed solution, achieving values of 120.3 mg g^{-1} carotenoids in extract (in the SAF *extract* fraction), which were significantly higher than those obtained in the original PLE extract. In parallel, a new fast two-dimensional comprehensive liquid chromatography (LC×LC) method was optimized to get the full carotenoids profile of these extracts in less than 25 min. This is the first time that the use of a C30 column is reported in an on-line LC×LC system.

Keywords: supercritical antisolvent fractionation, SAF, *Haematococcus pluvialis*, PLE, astaxanthin, LC×LC

1. INTRODUCTION

Microalgae are widely considered as a potential source of bioactive compounds with beneficial properties for human health. These photosynthetic organisms are used in several fields such as nutraceutical, cosmetic, and food industries, mainly thanks to their high content in natural pigments [1]. Biologically, carotenoids have an important role in many physiological functions, including light-harvesting, protection against oxidation and excess of light, and even they can also contribute as growth regulators. It is known that carotenoids are commonly associated with other natural compounds including fatty acids, sugars or proteins, which can also influence their chemical and biological properties [2]. Astaxanthin, β-carotene, lutein, canthaxanthin, and lycopene are the most commercially used carotenoids [1]. Among these, astaxanthin from *Haematococcus pluvialis* is one of the most demanded products due to its biological activities such as antioxidant, UV-light protection, anti-inflammatory, among others. Besides, natural astaxanthin is preferred over its synthetic counterpart. There are many natural sources of astaxanthin, such as salmon, trout, red sea bream, shrimp, lobster, and fish eggs, but *Haematococcus pluvialis* is considered the richest source of natural astaxanthin. In fact, accumulation of up to 30 mg of astaxanthin and derivatives per g of dry biomass has been reported at industrial scale [3].

Due to the huge interest in carotenoids, their extraction from natural matrices, specifically microalgae, is a hot research topic. Traditionally, the recovery of these compounds implied the use of high volumes of organic solvents and required long extraction times. Nowadays, these techniques are being replaced by more advanced and environmentally friendly processes such as supercritical fluid extraction (SFE) or pressurized liquid extraction (PLE), among others [4]. PLE is based on the use of solvents at high temperatures and pressures, which helps to maintain the solvent in its liquid state and provides a fast and efficient extraction process. Moreover, generally recognized as safe (GRAS) solvents such as ethanol, water or ethyl lactate are preferred [5]. One of the most influential parameters when dealing with PLE is the extraction temperature. It is known that high temperatures, along with high pressures,

71 increase solubility and mass transfer rates since the solvent penetrates deeper and easier into the
72 matrix. This way, a significant enhancement on the extraction rates is observed; on the other hand,
73 high extraction temperatures could directly affect the stability of thermolabile compounds. Regarding
74 carotenoids, as they are natural antioxidants, most extraction studies reveal that very high
75 temperatures may induce their degradation [6], although good results have been reported for the
76 extraction of carotenoids from *Porphyridium cruentum* using PLE with ethanol at 125 °C [7].
77 Compressed fluids-based green extraction techniques, including PLE, SFE and gas-expanded liquids
78 (GXL) have been previously reported for the extraction of astaxanthin from *H. pluvialis* [8, 9]. PLE has
79 the advantage of offering high extraction yields and faster extraction processes compared to the other
80 mentioned techniques and its usefulness for the extraction of carotenoids from other microalgae has
81 also been demonstrated [10]. However, the selectivity offered by PLE towards those compounds is not
82 extremely high. For this reason, purification protocols may be needed to obtain fractions enriched in
83 target compounds for further applications. In this regard, supercritical antisolvent fractionation (SAF)
84 has already been demonstrated to be useful for the fractionation and concentration of different
85 bioactive compounds [11-14]. Briefly, SAF is based on the selective precipitation of target compounds
86 depending on their polarity and solubility between an organic solvent and supercritical CO_2. During the
87 process, continuous contact between a relatively polar liquid extract with supercritical CO_2 is
88 established. During this period, supercritical CO_2 is able to solubilize the less polar fraction (including
89 solvents and compounds) of the liquid extract that is recovered by downstream pressure reduction
90 (called SAF *extract*). Meanwhile, more polar compounds not soluble in supercritical CO_2 precipitate in
91 the so-called *raffinate* [5]. Thus, SAF is a feasible green alternative to further enrich liquid extracts
92 thanks to the solvent properties obtained under pressurized conditions, although its use has not been
93 reported so far for natural carotenoids purification. Moreover, it provides the additional advantage of
94 producing a dried *extract*.

To monitor the performance of extraction and purification procedures, appropriate analytical methods are needed to get proper information about the composition of the generated fractions. In this regard, the use of liquid chromatography coupled to mass spectrometry (LC-MS) may be the ideal analytical tool considering the nature of carotenoids. However, there are highly complex samples for which the use of multidimensional approaches to obtain higher separation power may be justified. In this regard, on-line two-dimensional comprehensive liquid chromatography (LC×LC) has already been used for the characterization of carotenoids present in different food-related samples [15-18]. These applications benefit from the increased separation performance provided by the coupling between a normal phase separation (NP) in the first dimension and a reversed phase (RP) separation in the second dimension, using cyano and C18 columns, respectively. The separation performance of those developments was by far better than the attainable by conventional LC, although relatively long analysis times (ca. 100 min) should be assumed [15-18]. Here, a new approach is studied thanks to the use of amino and C30 columns in the first and second dimensions, respectively, looking for a significant reduction in overall 2D analysis time. C30 is the stationary phase of choice when dealing with the carotenoids analysis from complex samples by conventional LC [19], although there are no previous reports related to its use in LC×LC.

Thus, the present work aimed to develop an integrated compressed fluids-based process combining PLE and SAF to efficiently obtain for the first time an astaxanthin-enriched fraction from *H. pluvialis* microalgae. In parallel, a new LC×LC method was also established for the separation and identification of the carotenoids, both free and esterified, contained in the produced fractions.

2. MATERIALS AND METHODS

2.1. Samples and reagents

Freeze-dried *Haematococcus pluvialis*, were kindly provided by Microphyt (Baillargues, France) and stored at 4 °C until use.

HPLC-grade solvents including methyl tert-butyl ether (MTBE), methanol, acetone, and ethanol were purchased from VWR (Leuven, Belgium). Sea sand (0.25–0.30 mm particle diameter) was acquired from Panreac (Castellar del Vallés, Spain). Butylated hydroxytoluene (BHT), canthaxanthin and β-carotene (from *Anacystis nidulans* algae) were obtained from Sigma-Aldrich (St Louis, MO, USA). Lutein (from *Echinacea purpurea*) was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Astaxanthin was purchased from Acros Organics (Geel, Belgium), whereas zeaxanthin was acquired from Carbosynth Limited (Berkshire, UK). Ultrapure water used was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). For supercritical antisolvent fractionation experiments, carbon dioxide (99% purity) was supplied by Carburos Metálicos (Barcelona, Spain).

2.2. Pressurized liquid extraction (PLE)

Pressurized ethanol extractions of dried biomass were carried out using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller unit. Firstly, an optimization of PLE conditions was performed employing different extraction temperatures (50 to 200 °C, Table S1). For those extractions, 1.0 g of dried algal biomass was loaded into an 11 mL stainless steel extraction cell sandwiched between 4.0 g of sea sand. Pressure and extraction time were set at 10.5 MPa and 20 min, respectively, and pure ethanol was chosen as extraction solvent, based on previous experience. All experiments were performed in duplicate. The extracts obtained were dried using a gentle stream of nitrogen, protected from light and stored at -20 °C until further analysis. Extraction yield (% extract dry weight/ initial biomass dry weight) and total carotenoids (mg g^{-1} carotenoids in extract) were quantified to select the optimum extraction conditions. Under those extraction conditions, successive extractions were performed to obtain 500 mL of extract, to continue with the purification step.

2.3. Supercritical antisolvent fractionation (SAF)

145 SAF of the optimum PLE extract was carried out in a Speed Helix supercritical fluid extractor (Applied
146 Separations, Allentown, PA, USA). A scheme of the SAF process is shown in Figure 1. The PLE extract
147 obtained under optimum conditions was diluted properly to obtain different percentages of water
148 needed according to the planned experimental design (20, 35 and 50% v/v or 24, 40.5 and 55.8% w/w,
149 respectively). These extract solutions were kept in the dark at −20 °C to avoid degradation until their
150 use for the antisolvent fractionation experiments.
151 The fractionation process was accomplished as follows (Figure 1): the feed (PLE extract with water)
152 from the extract reservoir (1), was continuously pumped by a high-pressure pump (2) at a selected
153 flow rate (0.1 to 0.5 mL min^{-1}), reached a T-tube device where it was mixed with CO_2. CO_2 was provided
154 from a pressurized cylinder (3), subcooled and pumped using another high-pressure pump (4) at a
155 constant flow rate (8.244 mL min^{-1} or 8 g min^{-1}). Then, the mixture (feed and CO_2) reached the
156 separation chamber (6), in which the compounds that were not soluble in supercritical CO_2 + EtOH
157 mixture precipitated and were collected at the bottom of the separator (this non-soluble fraction is
158 called *raffinate*). During the separation process, the temperature was fixed at 40 °C and it was
159 controlled by an oven (5). An upstream backpressure valve (7) kept constant the fractionation pressure
160 throughout the experiment. Finally, the compounds soluble in supercritical CO_2+ EtOH mixture
161 proceeded to the next vessel where CO_2 pressure was decreased to turn CO_2 into a gas to allow the
162 recovery of those components in the second separation chamber (the soluble fraction is called *extract*)
163 (8), which was kept at room temperature (25 °C). Both *raffinate* and *extract* were collected separately
164 in plastic bottles and dried. The SAF process time was set at 120 min for each experiment. All dried
165 fractions were stored at −20 °C until analysis. The recovery was determined gravimetrically, as the ratio
166 of the mass of dry *extract* or *raffinate* recovered and the mass of dry PLE extract fed and expressed as
167 a percentage.
168
169 **2.4. Experimental design**

An experimental design was applied to optimize the supercritical antisolvent fractionation process. A 3-level factorial experimental design 2^3 (including three center points) was proposed based on three factors: pressure (10 to 30 MPa), flow rate ratio (w/w) of feed (PLE extract) and supercritical CO_2 (0.01–0.05) and percentage of water in feed (20 to 50%, v/v). The response variables studied were recovery (% dry weight of *extract* or *raffinate*/dry weight of PLE extract) total carotenoids (mg g^{-1} carotenoids in *extract or raffinate*) and total astaxanthin content (mg g^{-1} astaxanthin in *extract or raffinate*). The described experimental design involved 11 experimental runs (Table S2). Both *raffinate* and *extract* from the application of each process conditions, as well as the original PLE extract, were studied. Data analysis was performed using response surface methodology (RSM) using Statgraphics Centurion XVI software (StatPoint Technologies, Inc., Warrenton, VA, USA). The influence of independent parameters on the response variables was studied at a 95% level of confidence. Moreover, a linear regression model for each parameter (Y_i) was proposed. The equation was

$$Y_i = \kappa_0 + \kappa_1 \times P + \kappa_2 \times F + \kappa_3 \times W + \kappa_{1,2} \times P \times F + \kappa_{1,3} \times P \times W + \kappa_{2,3} \times F \times W + \kappa_{1,2,3} \times P \times F \times W + error \quad (1)$$

where P is the pressure, F is the feed/supercritical-CO_2 flow rate ratio, W is the percentage of water in feed (v/v), κ_0 is a constant, κ_1, κ_2, κ_3 are the independent linear effects, $\kappa_{1,2}$, $\kappa_{1,3}$, $\kappa_{2,3}$ are the linear effects of two factors and $\kappa_{1,2,3}$ is the effect of three factors interaction. The effect of each factor and its statistical significance, for each of the response variables, were analyzed from the standardized Pareto chart. The response surfaces of the respective mathematical models were also obtained, and the significances were accepted at $p \leq 0.05$. A multiple response optimization was performed by the combination of the three experimental factors to maximize the desirability function for the response variables in the *extract* since the goal was to obtain an astaxanthin-enriched fraction. Nevertheless, both *extracts* and *raffinates* were analyzed in terms of recovery (% dry weight of *extract* or *raffinate*/dry weight PLE extract), total carotenoids content (mg carotenoid per g *extract or raffinate*)

by using a spectrophotometric method and total astaxanthin content (mg astaxanthin per g *extract or raffinate*) determined by HPLC.

2.5. Total carotenoids determination

A spectrophotometric method was used to determine the total carotenoids content, based on their characteristic absorbance, as previously described [7]. PLE extracts and fractions obtained from the SAF process were dissolved in ethanol at a concentration of 0.1 mg mL^{-1} and their absorbance was recorded at a specific wavelength (470 nm). The calibration curve was constructed using astaxanthin as an external standard (0.16-10.00 µg mL^{-1}). Results were expressed as mg carotenoids per g *extract* or *raffinate*.

2.6. Chemical characterization of *H. pluvialis* extracts and SAF fractions by liquid chromatography coupled to diode array detection (HPLC-DAD)

The profiles of carotenoids of the *H. pluvialis* extracts and the different SAF fractions were firstly determined using HPLC-DAD, according to a previously developed method [20], with some modifications. An Agilent 1100 series liquid chromatograph (Santa Clara, CA, USA), coupled to a diode-array detector (DAD) was used to analyze all samples. The analytical conditions included the use of a YMC-C30 reversed-phase column (250 × 4.6 mm, 5 µm; YMC Europe, Schermbeck, Germany) and a YMC-C30 pre-column (10 × 4 mm, 5 µm), using the following mobiles phases: methanol–MTBE–water (90:7:3, v/v/v) as solvent A and methanol–MTBE (10:90, v/v) as solvent B. A linear gradient was employed for elution as follows: 0 min, 0% B; 20 min, 30% B; 35 min, 40% B; 45 min, 80% B; 50 min, 100% B; 52 min, 0% B. The injection volume was 10 µL while the flow rate was 0.8 mL min^{-1}. The diode array detector was set at 280, 450 and 660 nm, although spectra from 240 to 770 nm were recorded (peak width 0.1 min (2 s), slit 4 nm). The instrument was controlled by LC ChemStation 3D Software Rev. B.04.03 (Agilent Technologies, Santa Clara, CA, USA).

This method was also employed to estimate astaxanthin amounts in the extracts and SAF fractions. All samples were dissolved in pure ethanol at an appropriate concentration (1–10 mg mL^{-1}) and filtered using 0.45 µm nylon filters before analysis. External standard calibrations were performed using at least five different concentrations of astaxanthin (3.1 to 50.0 µg mL^{-1}) dissolved in ethanol that were analyzed in triplicate. Good linearity was obtained in the mentioned range (R^2 = 0.986). Moreover, appropriate limits of detection (LOD = 0.28 µg mL^{-1}) and limits of quantification (LOQ = 0.93 µg mL^{-1}) calculated as a concentration giving a signal-to-noise ratio equal to 3 and 10, respectively, were obtained. Results were expressed as mg g^{-1} astaxanthin in *extract* or *raffinate*.

2.7. Chemical characterization of *H. pluvialis* extracts and SAF fractions by comprehensive two-dimensional liquid chromatography (LC×LC DAD-APCI-MS/MS)

For the characterization of the specific carotenoids contained in *H. pluvialis* extracts and SAF fractions, a new LC×LC method was developed. The instrumentation consisted of a first dimension (^{1}D) composed by an Agilent 1200 series liquid chromatograph (Agilent Technologies, Santa Clara, CA) equipped with an autosampler. The second dimension (^{2}D) separation was performed using an additional LC pump (Agilent 1290 Infinity). Both dimensions were connected by an electronically-controlled two-position ten-port switching valve (Rheodyne, Rohnert Park, CA, USA) acting as modulator equipped with two identical 30 µL injection loops. The modulation time of the switching valve was 1.0 min. The separation was recorded through the DAD at 450 nm (maximum sampling rate selected, 20 Hz), although the system was also connected to an Agilent 6320 Ion Trap mass spectrometer equipped with atmospheric pressure chemical ionization (APCI) interface working under positive ionization mode. MS detection was performed using the following settings using positive ionization mode: capillary voltage, -3.5 kV; drying temperature, 350 °C; vaporizer temperature, 400 °C; drying gas flow rate, 5 L min^{-1}; nebulizer gas pressure, 60 psi; and corona current, 4000 nA; *m/z* 150 to 1300. The LC data were elaborated and visualized using LC Image software (version 1.0, Zoex Corp., Houston, TX).

The finally optimized normal phase × reversed phase (NP×RP) method involved the use of the following separation conditions:

^{1}D separation: A Hypersil Gold Amino column (150 x 1.0 mm, 3 µm, Thermo Scientific, Waltham, MA, USA) was used, eluted under isocratic conditions using hexane as mobile phase at 20 µl min^{-1}.

^{2}D separation: A Thermo Accucore C30 partially porous column (50 x 4.6 mm, 2.6 µm, Thermo Scientific, Waltham, MA, USA) was used using water/acetonitrile (20:80, v/v, solvent A) and 2-propanol (solvent B) as mobile phases eluted following full-in-fraction repetitive gradients as follows: 0 min, 10% B; 0.6 min, 90% B; 0.75, 90% B; 0.76 min, 10%B; 1.0 min, 10% B. The flow rate employed was 3.0 mL min^{-1} and the column temperature was held at 60 °C. The effluent from the ^{2}D column was split before entering the MS instrument so that the flow rate introduced in the MS detector was *ca*. 0.6 mL min^{-1}. Practical peak capacity values were determined using the approach proposed by Li et al [21] whereas method orthogonality was estimated following the asterisk equations [22]. Details about these calculations are provided as electronic supplementary information.

3. RESULTS AND DISCUSSION

3.1 Extraction of carotenoids from *H. pluvialis* and purification by supercritical antisolvent fractionation process

Astaxanthin is present in *H. pluvialis* both in its free form as well as in a more stable esterified form bound to different fatty acids [3]. Moreover, astaxanthin can form mono- and di-esterified derivatives, thus, increasing the complexity of the whole carotenoids composition naturally present in the microalgae (Figure S1). In this sense, the extraction of carotenoids from *H. pluvialis* is challenging considering that the complex chemical composition also implies very different polarities of its carotenoids. Based on our previous experience, PLE using ethanol as a solvent was considered as the most suitable GRAS alternative to extract carotenoids from *H. pluvialis*. The combined use of PLE and SAF provides higher selectivity towards the compounds of interest whereas the use of PLE in the first

step allows obtaining high extraction yields. Fast optimization of the extraction temperature at constant pressure (10.5 MPa) and time (20 min) was performed. As shown in Table S1, the use of 50 °C was considered optimum since it allowed working with a more carotenoids-enriched extract.

To track the effect of the subsequent SAF process on the chemical composition of the extract, an LC-DAD method previously employed to reveal the carotenoids pattern of other microalgae was applied to the optimum PLE extract. Figure S2 shows the profile obtained. As can be observed, the extract was relatively complex with multiple peaks belonging to carotenoids according to their UV-Vis spectra. Astaxanthin, canthaxanthin, lutein, and β-carotene could be identified in the extract by coelution with commercial standards. Moreover, a high number of peaks possessed typical UV-Vis spectra with a maximum around 474 nm, compatible with astaxanthin. According to their retention windows [23], these compounds were tentatively considered as astaxanthin monoesters and astaxanthin diesters.

Once the most suitable conditions for the pressurized extraction of carotenoids from *H. pluvialis* were selected, a relatively high volume of extract was generated to be employed as feed for the SAF process optimization. Up to 500 mL of extract were prepared with a total concentration of solids of 3.12 g L^{-1} of extract solution. To study the most important variables influencing the SAF process using supercritical CO_2 (sc-CO_2) as antisolvent, an experimental design was devised and applied. Feed to supercritical CO_2 ratio, process pressure and the amount of water in the feed have repeatedly been shown as the factors that have the greatest impact on fractionation performance [13,14,24]. The performance of the process was monitored in terms of recovery, total carotenoids and astaxanthin content in both SAF *extract* and *raffinate*. Astaxanthin content was determined as the sum of the concentration of all the peaks contained in the LC-DAD chromatograms possessing the typical astaxanthin UV-Vis spectra. Table 1 shows the results obtained under each process condition for *extracts* and *raffinates*. As can be observed, the recovery of total carotenoids in the *raffinates* was generally lower than in the extracts, as could be expected from the diverse solubility of carotenoids between the water-soluble fraction (*raffinate*) and the ethanol + SC-CO_2 fraction (*extract*). For this

294 reason, the analysis of the fractionation performance is focused on the extracts, since this fraction was
295 the most suitable to maximize the recovery of carotenoids.
296 As can be seen in Table 1, the total recoveries obtained in the *extract* fractions were very variable,
297 from 17.8 to 88.4%, which implies that there is a significant effect of the studied parameters on the
298 solubility of the components present in the PLE extract on the supercritical CO_2 + ethanol mixture.
299 Figure 2 shows the standardized Pareto charts for the three response variables studied, together with
300 their corresponding response surfaces. Different bar shadings indicate if the effect is positive or
301 negative over the response variables, whereas the vertical line marks the significance of the effects at
302 the 95% confidence level.
303 As can be observed, for the total recovery (Figure 2A), the individual influence of the percentage of
304 water in feed and the PLE extract/SC-CO_2 ratio showed a negative effect, although only the first one
305 was significant. The pressure showed a non-significant positive effect. It can be noted that the
306 percentage of water in feed was the most influencing factor, followed by its interaction with the
307 feed/SC-CO_2 ratio, as it can be also observed in their corresponding response surfaces (Figures 2B and
308 C). In terms of total carotenoids content as well as total astaxanthin content, both pressure and PLE
309 extract/SC-CO_2 ratio exhibited a positive and significant effect, with small differences in terms of
310 influence, whereas the content of water in the feed showed a negative effect, as it is shown in Figure
311 2D and G. These two factors, pressure, and PLE extract/SC-CO_2 flow rate ratio, are strongly related to
312 mass transfer and, thus, the use of higher pressures and feed ratios meant that more carotenoids
313 would be available to be extracted. Figures 2E and H and Figures 2F and I show the response surfaces
314 of total carotenoids and total astaxanthin content, respectively, when % of water in feed is fixed. In
315 terms of percentage of water in feed, as expected, compounds from PLE extract were more soluble in
316 ethanol than in water; thus, as the proportion of ethanol (and less % water) in the feed was increased,
317 a higher amount of compounds were recovered in the mixture SC-CO_2 + EtOH (*extract*).

In general, the increase in fractionation pressure always increased the recovery of carotenoids in the *extracts*, as can be deduced from Table 1. Moreover, when the pressure was fixed, higher feed/SC-CO_2 ratios were more favorable. Lastly, the percentage of water in the feed influenced the total amount of carotenoids recovered depending on the extraction pressure. At higher pressures (30 MPa) smaller ratios provided better carotenoids recoveries, whereas at lower pressures (10 MPa) the opposite trend was found. An ANOVA analysis of the experimental design was employed to statistically assess the recovery of total carotenoids in the SAF *extract*.

Apart from the confirmation of the statistical influence of pressure and feed to SC-CO_2 ratio at the 95% confidence level, the model presented an R-squared (R^2 = 0.97) and adjusted R-squared (R^2 = 0.90) values that indicated a close agreement between the experimental results and theoretical values.

To determine the extraction conditions to obtain the most purified SAF fraction with the highest possible recovery, a multiple response optimization was performed to maximize all the studied variables simultaneously. The estimated response surface obtained for the multiple optimization can be observed in Figure 3. Additionally, the optimum conditions and the estimated responses proposed by the model, along with those obtained experimentally at those conditions are shown in Table 2. Consistently, the experimental results for the extraction performed according to the optimum conditions were similar to those predicted.

Overall, the SAF fraction obtained in SC-CO_2+ethanol mixture (*extract*) was effectively enriched in carotenoids (up to 1.3-fold compared to the PLE original extract) when the process was performed at 30 MPa, and 20% (v/v) of water in the mixture and 0.05 PLE extract/SC-CO_2 flow rate ratio. Under those conditions, the carotenoid content was 120.3 mg g^{-1} carotenoids in extract. These results show the first application of SAF for the purification of natural carotenoids.

3.2. Characterization of extracts using comprehensive two-dimensional liquid chromatography (LC×LC) coupled to mass spectrometry detection

Up to now, the characterization of carotenoids present in microalgae extracts has been carried out by conventional HPLC using C30 columns. However, even if this type of column is very well suited for the separation of carotenoids, very complex samples cannot be completely resolved. As can be observed in Figure S2, the chemical composition present in the optimum *H. pluvialis* PLE extract (50 °C) could be considered complex enough to justify the use of multidimensional approaches to improve the separation of its components and to resolve some of the coelutions found. In this line, the use of on-line comprehensive two-dimensional LC (LC×LC) has been previously been demonstrated as very powerful for the separation of carotenoids from other natural sources [15-18, 25]. For this reason, the use of a new LC×LC-MS method for the separation of carotenoids contained in H*. pluvialis* is proposed here for the first time.

The proposed method was optimized combining NP in the ^{1}D and RP in the ^{2}D. A new column combination, not used before for carotenoids analysis by LC×LC, was selected involving the use of a microbore amino column in the ^{1}D and a short partially porous C30 column in the ^{2}D. A separate optimization of the separation conditions of each dimension was performed. ^{1}D separation conditions were studied including different mobile phases and gradients although, finally, the isocratic elution using hexane as mobile phase provided with proper retention and rapid elution of the carotenoids contained in the sample into the ^{1}D amino column. On the other hand, considering the lack of previous reports including the use of a C30 column in LC×LC set-ups, different mobile phase compositions and gradients were also tested in the ^{2}D. One min was established as the target modulation time. The finally selected separation conditions are reported in Section 2.7. Figure 4A shows the separation attainable of the *H. pluvialis* PLE extract using this configuration. Peak assignment is shown in Table 3. As can be observed, a good resolution between the different components was obtained, being able to separate free carotenoids (including carotenes and xanthophylls) as well as other mono- and di-esterified carotenoids derived from astaxanthin. The main compound found was the Astaxanthin-$C_{18:0}$ monoester (peak 19), although relevant amounts were also found of its $C_{18:1}$ monoesters (peaks 14,

20, 21), presenting different isomers. Regarding astaxanthin-diesters, the $C_{18:1}/C_{18:1}$ was the most intense (peak 8) together with the $C_{18:0}/C_{18:0}$ diester (peak 7). Free carotenoids were also detected and tentatively identified, including astaxanthin (peak 24), canthaxanthin (peak 15), lutein (peak 16) and β-carotene (peak 3).

This separation was characterized by a practical peak capacity of 268 while orthogonality degree was estimated at 40%. Although these figures-of-merit are interesting by themselves, it is worth to mention that the whole analysis took less than 25 min, which was nearly the half than the original conventional HPLC method, as can be inferred from a comparison between Figure S2 and Figure 4. Thus, the peak capacity achieved is rather high for a 25 min analysis (*ca*. 11 peaks min^{-1}). This analysis time can be considered as very fast compared to other LC×LC methods applied for carotenoids analysis which are typically around 90-100 min [15-18, 25], and it is, indeed, a significant analytical advantage over the use of conventional LC.

The application of this method also allowed establishing a comparison between the original PLE extract used as feed for the SAF process and the enriched *extract* obtained after purification. As can be observed in Figures 4A and B, the chemical composition was not the same comparing both fractions, although astaxanthin derivatives were clearly the main components. Although astaxanthin-monoesters were still the main compounds found, the proportions between the whole carotenoid pattern were modified, indicating a possible difference in the solubility of individual components under the SAF processing conditions. This fact could be expected considering the large differences in relative polarity among the carotenoids present in the original PLE extract. In this regard, fewer amounts of β-carotene, as well as astaxanthin diesters, were evident. Moreover, the possibility of different reactions, including isomerization or even degradation, taking place during the SAF process might not be ruled out. Indeed, astacin-derivatives were more prominent in the SAF extract compared to the PLE extract (peaks 9-11). Besides, new peaks appeared (peaks 26, 27) that could not be properly assigned although they could correspond to newly formed compounds. Unfortunately, due to the lack of commercial

393 standards, no precise quantification of each separated compound was possible either by LC×LC or
394 conventional LC.
395
396 **4. CONCLUSIONS**
397 The combination of compressed fluids-based processes for extraction and purification of target
398 fractions has been demonstrated as a feasible environmentally green alternative for the recovery of
399 astaxanthin and other carotenoids from *H. pluvialis* microalgae. Specifically, a PLE process performed
400 with ethanol at 50 °C and 10.5 MPa for 20 min was demonstrated to be useful for the extraction of
401 carotenoids from *H. pluvialis* biomass. Subsequently, this extract was used as feed for a SAF process
402 targeting the further purification of carotenoids. An experimental design, studying different process
403 parameters, including CO_2 pressure (10-30 MPa), percentage of water in the PLE extract (20-50%) and
404 PLE extract/SC-CO_2 flow ratio (0.0125-0.05) was useful to select the optimum purification conditions
405 allowing the recovery of a carotenoids-enriched fraction as SAF *extract*. By using 30 MPa, 0.05 feed/SC-
406 CO_2 mass flow rate and 20% (v/v) of water in the feed solution, a purified *extract* containing 120.3 mg
407 g^{-1} carotenoids in the extract was produced. Thus, the possibility of using SAF to purify carotenoids
408 from natural extracts was demonstrated. Moreover, a new fast LC×LC method based on the coupling
409 of NP and RP separations has been optimized and applied in order to get the full carotenoids profile of
410 these extracts in less than 25 min. Astaxanthin mono- and diesters were the most important
411 compounds present, although other free carotenoids including canthaxanthin, lutein, and β-carotene
412 were also detected. Interestingly, although the SAF process was able to produce an enrichment on
413 total carotenoids, the application of this LC×LC method confirmed that the profiles obtained were
414 qualitatively different compared to the original *H. pluvialis* PLE extract.
415
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Conflicts of Interest

The authors would like to declare no conflict of interest in the publication of this research.

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FIGURE LEGENDS.

Figure 1. Scheme of the supercritical antisolvent fractionation equipment: (1) PLE extract reservoir, (2) high-pressure liquid pump, (4) high-pressure CO_2 pump, (5) oven, (6) separator 1 (*raffinate* collection cell), (7) back pressure regulator, (8) separator 2 (*extract* collection cell).

Figure 2. Standardized Pareto charts obtained for recovery (A), total carotenoids content (D) and total astaxanthin content (G) for SAF *extract* fraction (white bars and grey bars show positive and negative effects, respectively), and their corresponding response surfaces, keeping constant the response variable that influences the least (pressure for recovery and % water in feed for carotenoid and astaxanthin content).

Figure 3. Estimated response surface obtained for the multiple response optimization for SAF *extract* fraction.

Figure 4. 2D plots (450 nm) of the NP×RP separation of the *H. pluvialis* PLE extract obtained at 50 °C (A) and the fraction obtained after SAF purification (B). Peak assignment as in Table 3. For detailed separation conditions, see section 2.7.

Table 1. Experimental design of the factors and values of the response variables studied for the SAF *extract* and *raffinate*.

Exp.	P (MPa)	Feed/SC-CO_2	Water in feed	*Extract*			*Raffinate*		
				Recovery	Total carotenoids	Astaxanthin	Recovery	Total carotenoids	Astaxanthin
			(% v/v)	(wt %)	(mg g^{-1})	content (mg g^{-1})	(wt %)	(mg g^{-1})	content (mg g^{-1})
1	10	0.05000	20	36.5	31.5	29.0	63.5	11.2	6.0
2	30	0.05000	20	42.4	120.3	103.3	57.6	9.8	5.6
3	10	0.01250	20	66.5	9.7	9.6	33.5	11.5	6.8
4	30	0.01250	20	88.4	47.7	39.8	11.6	2.3	0.4
5	20 (CP)	0.03125	35	31.6	47.8	24.9	68.4	3.7	0.7
6	20 (CP)	0.03125	35	47.8	62.7	43.1	52.2	1.3	0.6
7	20 (CP)	0.03125	35	31.7	52.6	30.1	68.3	1.1	0.5
8	10	0.05000	50	25.6	13.7	10.9	74.4	48.9	47.4
9	30	0.05000	50	24.7	69.7	63.3	75.3	48.1	47.5
10	10	0.01250	50	17.8	12.4	8.2	82.2	18.2	17.1
11	30	0.01250	50	24.6	54.7	45.7	75.4	20.6	20.2

CP: experimental design central point.

Table 2. Optimum and experimental factor and response values obtained for the multiple response optimization for SAF *extract* fraction.

Factors			**Responses**		
Factor	*Optimum*	*Experimental*	*Response*	*Optimum*	*Experimental*
Pressure (MPa)	30	30	Recovery (%)	50.5	42.4
Feed/SC-CO_2 flow rate ratio	0.046	0.05	Total carotenoid content (mg g^{-1})	97.8	120.3
% Water in feed	20.0	20.0	Total astaxanthin content (mg g^{-1})	81.2	103.3

Table 3. Peak assignment, UV–Vis maxima and MS information of the separated peaks and contents of the quantified compounds in the *H. pluvialis* PLE extract and SAF fractions.

Peak #	Identification	UV-Vis maxima (nm)	$[M + H]^+$ *m/z*
1	Ast DE ($C_{18:0}/C_{18:1}$)	474	1127.0
2	Ast DE ($C_{18:1}/C_{18:1}$)	474	1125.9
3	β-carotene*	420s, 450, 480	537.0
4	Ast DE ($C_{18:1}/C_{18:2}$)	474	1123.9
5	Ast DE ($C_{18:2}/C_{18:3}$)	474	1119.9
6	Zeaxanthin	420s, 445, 476	569.0
7	Ast DE ($C_{18:0}/C_{18:0}$)	478	1129.2
8	Ast DE ($C_{18:1}/C_{18:1}$)	478	1125.0
9	Astacin-$C_{18:2}$	478	855.9
10	Astacin-$C_{18:3}$	478	853.0
11	Astacin-$C_{18:4}$	478	851.0
12	Ast ME ($C_{18:3}$)	478	857.0
13	Ast ME ($C_{18:0}$)	475	863.3
14	Ast ME ($C_{18:1}$)	475	861.7
15	Canthaxanthin*	474	565.7
16	Lutein*	422, 446, 474	568.9
17	Pheophytin a	412, 666	872.1
18	n.i.	478	865.3
19	Ast ME ($C_{18:0}$)	478	863.3
20	Ast ME ($C_{18:1}$)	478	861.7
21	Ast ME ($C_{18:1}$)	478	861.0
22	Ast ME ($C_{18:2}$)	478	859.8
23	Ast ME ($C_{18:2}$)	478	859.0
24	Astaxanthin*	478	597.6
25	n.i.	420s, 446, 474	
26	n.i.	475	
27	n.i.	420s, 446, 474	

* Identified with a commercial standard; s, spectral shoulder; n.i., not identified; Ast: astaxanthin; ME: monoester; DE: diester.