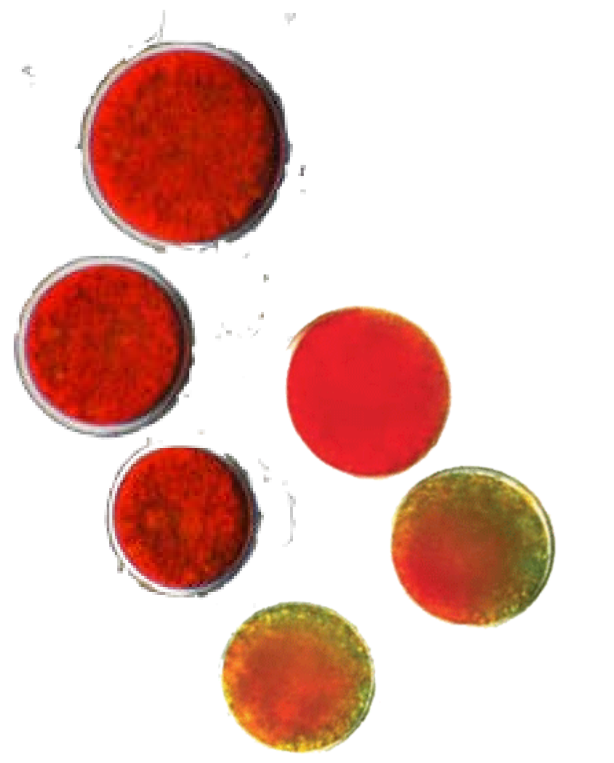
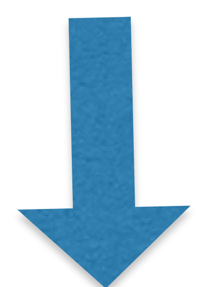
Highlights

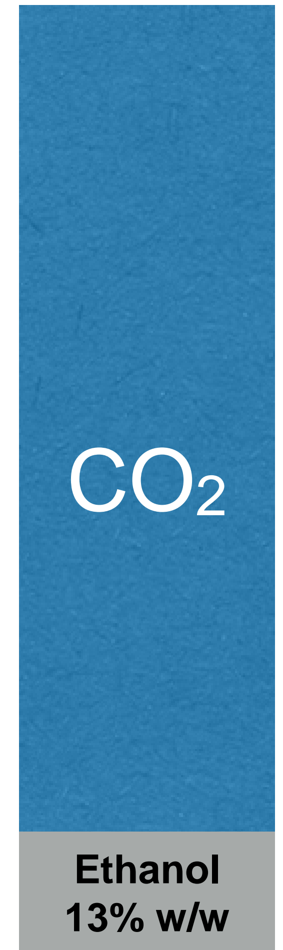
- In depth response surface analysis was done for scCO_2 extraction of *H. pluvialis.*
- Ethanol content in CO_2 was the only significant factor over pressure and temperature in astaxanthin extraction and antioxidant activity of the extracts.
- We investigated a further increase in ethanol content up to a region of Gas–expanded liquids.
- CO_2-expanded ethanol (CXE) surpassed scCO_2 extractions to match conventional extraction, maintaining high quality extracts.
- CXE extraction is revealing as an appropriate new green extraction technology for high value compounds derived from microalgae.

Haematococcus pluvialis

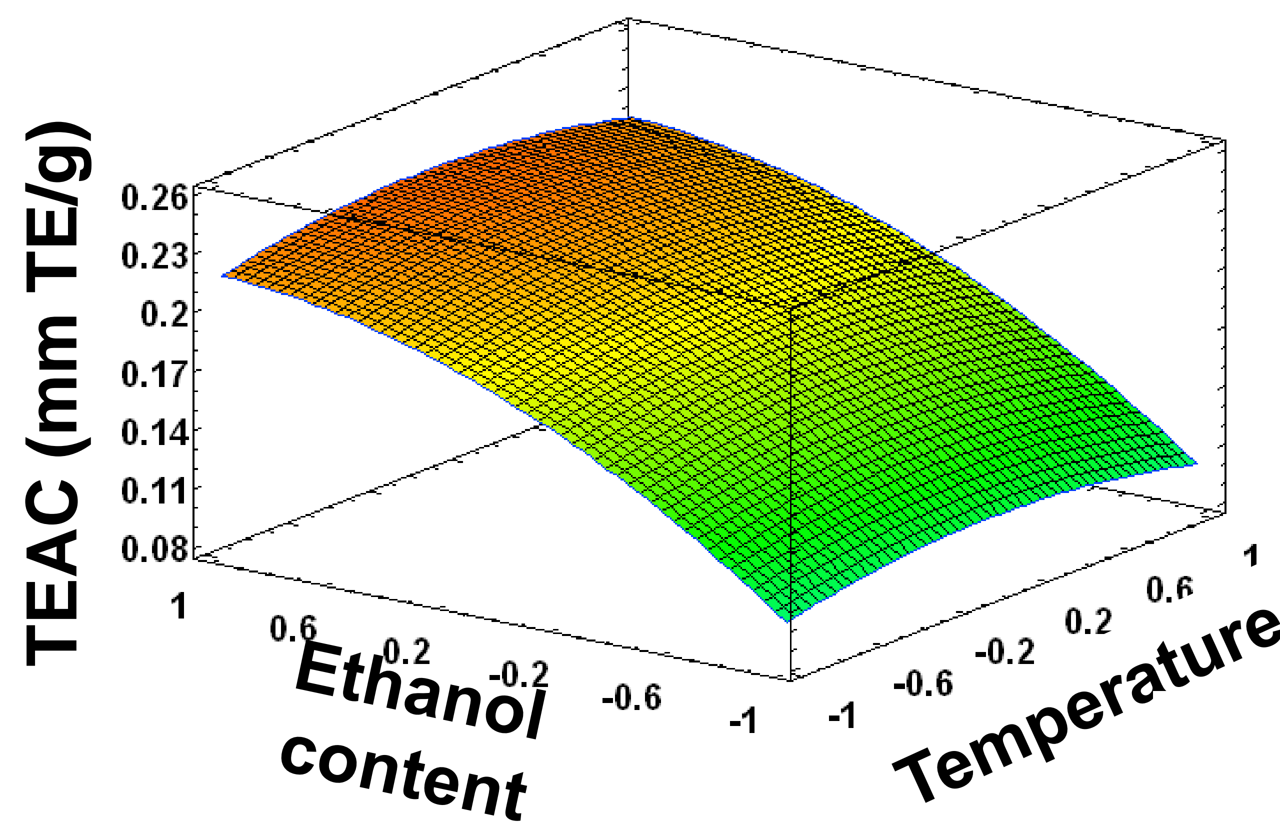




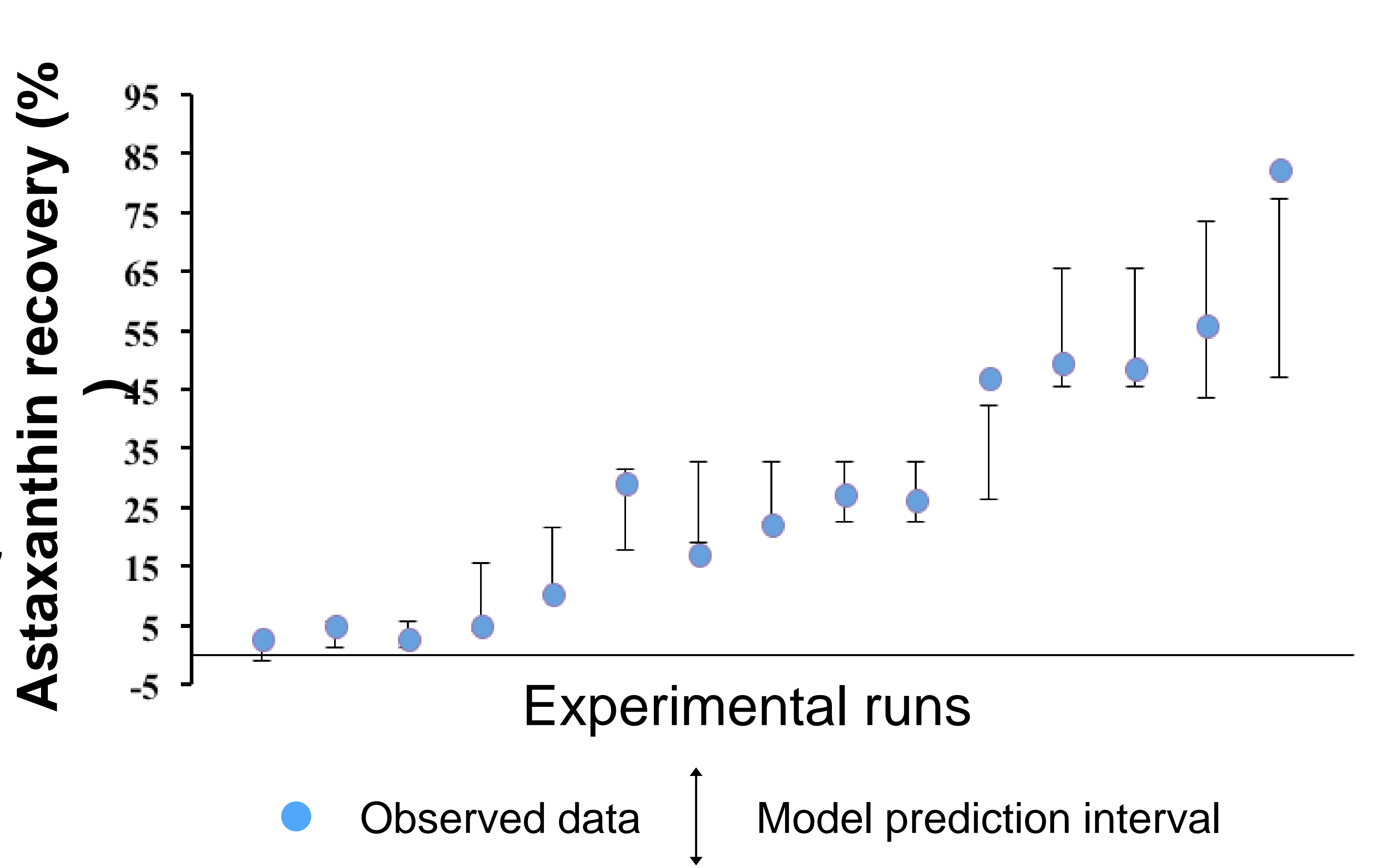
Supercritical CO_2 extraction



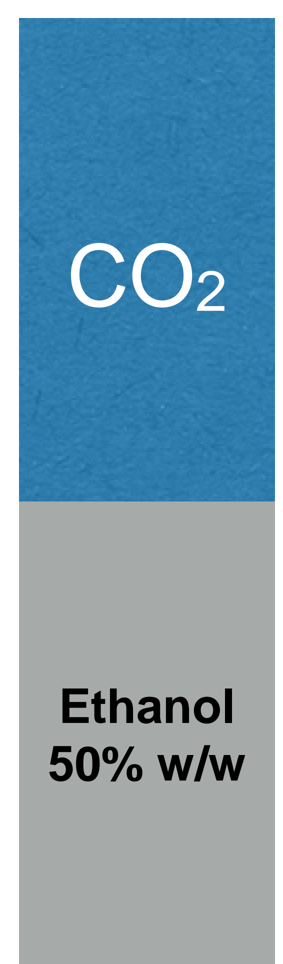
Antioxidant Activity

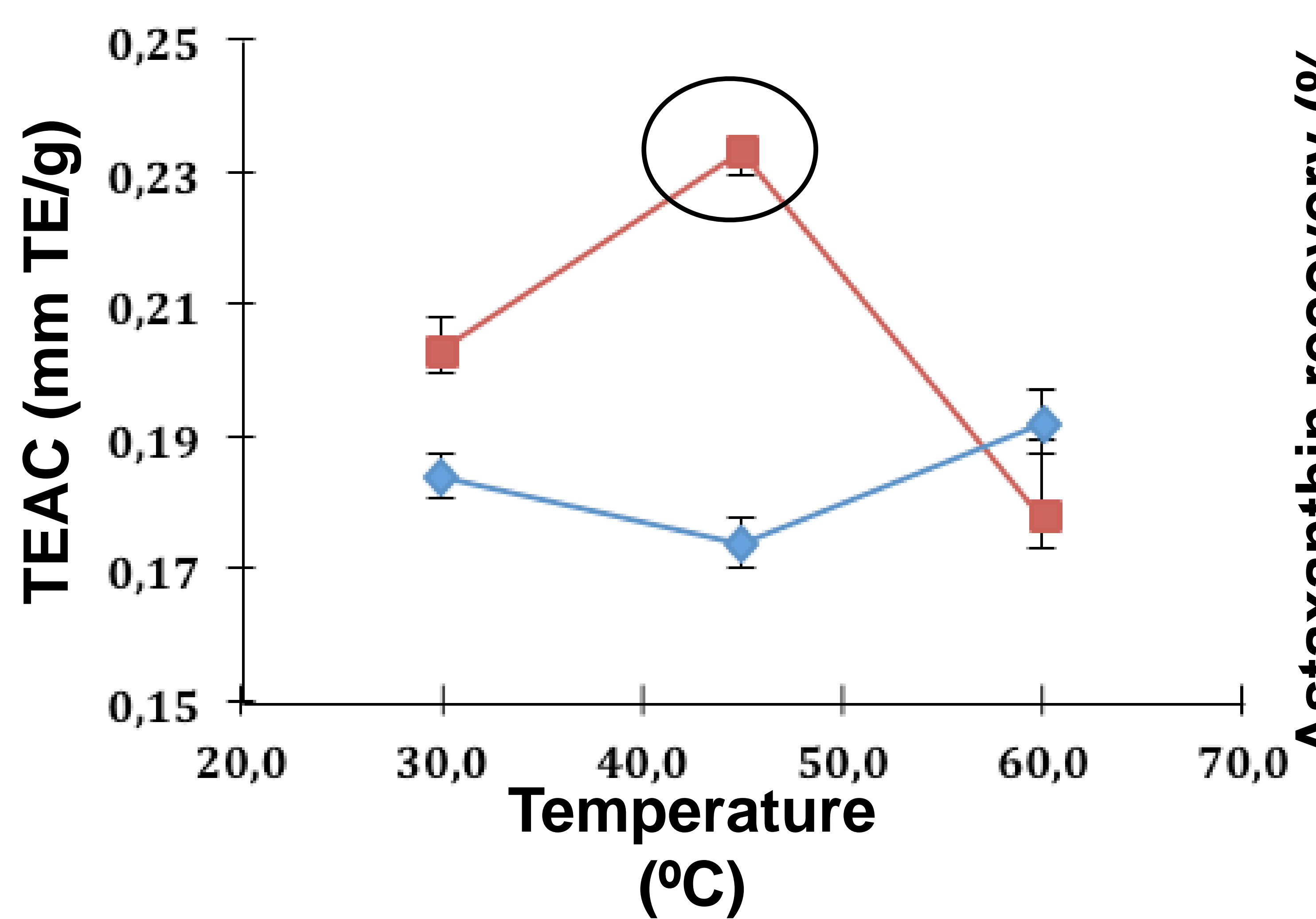


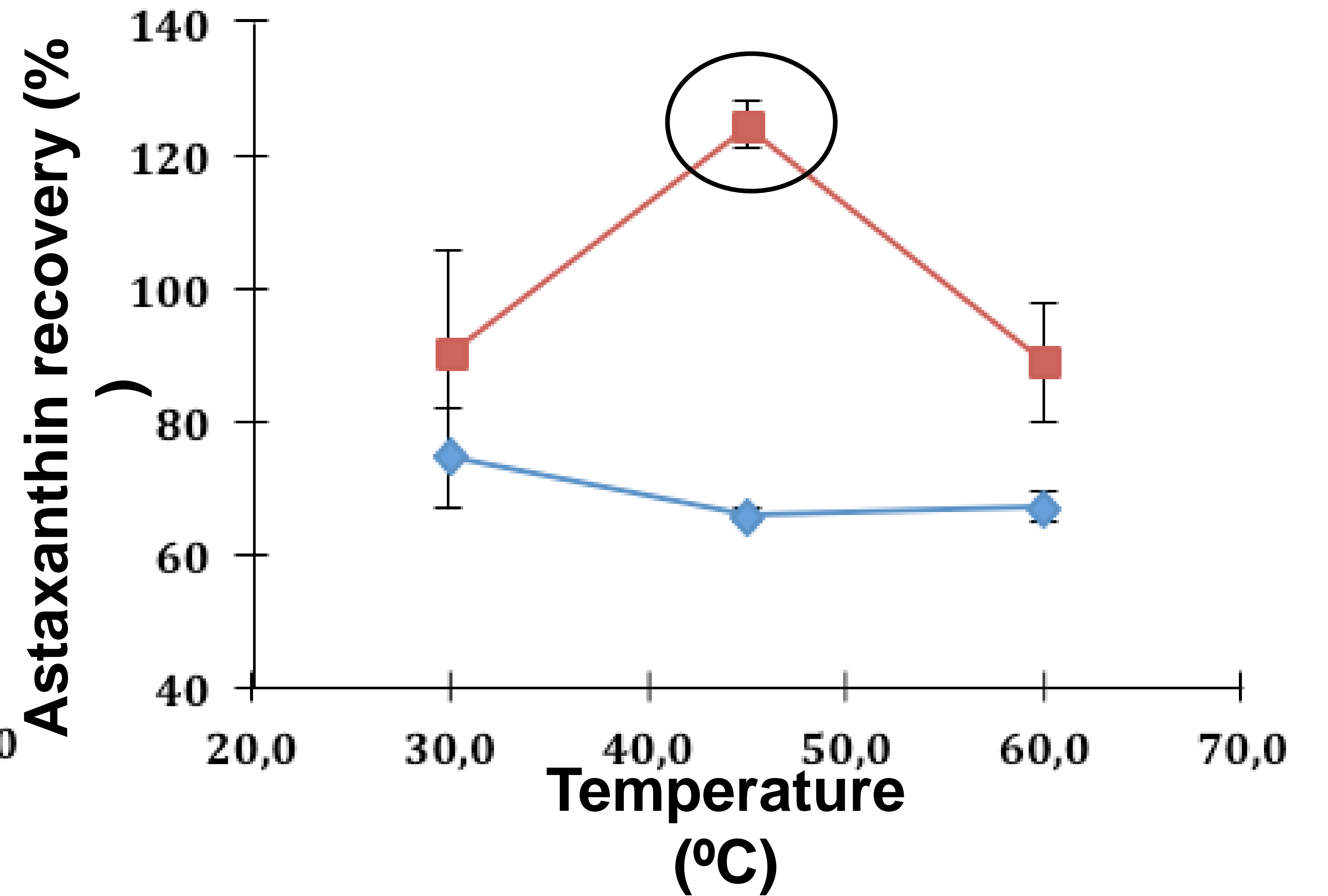
Astaxanthin Recovery



CO_2-expanded liquids







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Astaxanthin extraction from *Haematococcus pluvialis* using CO_2-expanded ethanol

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1 **Abstract**

2 Microalgae represent diverse branch of microorganism that can produce a wide range of unique
3 functional ingredients that can be used in food, cosmetics, pharmaceuticals and energy. Within them,
4 *Haematococcus pluvialis* is known for accumulating the highest levels of a potent natural antioxidant,
5 astaxanthin, which has demonstrated positive health effects. Therefore, numerous studies have focused
6 on the development of novel and efficient extraction techniques that are in agreement with the strong
7 demands in terms of quality of the extracts (purity and antioxidant activity), while complying with the
8 Green Chemistry Principles.

9 Supercritical CO_2 ($scCO_2$) emerges as an alternative to organic solvents because of its high selectivity
10 and bioactivity-preserving qualities. Nevertheless, astaxanthin is a large molecule with low solubility in
11 $scCO_2$ that usually requires high pressures, long extraction times and a necessary pretreatment (*i.e*
12 grinding) of the microalgae. Ethanol has been used as a green co-solvent for greatly improving
13 astaxanthin content by avoiding the problems faced by the use of pure $scCO_2$. In this work, a Box
14 Behnken experimental design (BBD) was employed to study the effects of operating pressure (20-35
15 MPa), temperature (40-70 ºC) and content (0-13 % w/w ethanol) on $scCO_2$ extraction of *Haematoccocus*
16 *pluvialis'* extract yield, astaxanthin content, and antioxidant activity. Results showed that for all
17 response variables the ethanol content in CO_2 effect was the most significant factor over pressure and
18 temperature. These results lead us to investigate the effects of a further increase in the ethanol content,
19 up to the region of gas-expanded liquids (GXLs), which is a new promising green alternative for
20 bioactives extraction. It was studied the effect of temperature (30-60 ºC) and ethanol content (50-70%
21 w/w) at a fixed pressure (7 MPa) on the same response variables using CO_2 expanded ethanol (CXE).
22 Results showed that temperature and CO_2-expanded ethanol had a significant influence in astaxanthin
23 extraction and antioxidant activity. Also, the overall responses of CXE surpassed $scCO_2$ extractions to
24 match acetone conventional extraction, maintaining high quality extracts (both in purity and antioxidant

1 activity), thus validating the use of this new type of green technology for extraction of high valued
2 compounds.
3

4 *Keywords*: Antioxidant activity, Astaxanthin, Gas expanded liquid extraction, CO_2 expanded liquids,
5 *Haematococcus pluvialis*, Supercritical carbon dioxide.
6

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1 **1. Introduction**

2 Microalgae are photosynthetic prokaryote or eukaryote microorganisms that can be found in both marine
3 and freshwater environments. Despite similarities in photosynthetic mechanisms, microalgae convert
4 solar energy into biomass more proficiently than terrestrial plants due to their simpler cellular structure
5 and easier access to basic nutrients (in aquatic environments) [1]. Initial widespread interest in
6 microalgae was related to the production of biofuels, but they can also synthesize a wide range of
7 unique, higher-value substances that can be used in pharmaceuticals, cosmetics, and foods [2-4].

8 *Haematococcus pluvialis* is a microalgae that accumulates large amounts of astaxanthin, a potent natural
9 antioxidant [5]. This xanthophyll can be used as a food coloring agent in aquaculture [6] and as an
10 antioxidant in human nutrition [7,8]. Astaxanthin has an antioxidant activity ten times higher than other
11 carotenoids such as zeaxanthin, lutein, and β-carotene, and over 500 times greater than tocopherol
12 [5,9,10]. There is growing evidence gathered by both *in vitro* and *in vivo* studies, that astaxanthin has the
13 potential to be a health-promoting agent in the prevention and treatment of free-radical associated
14 diseases such as oral, skin, liver, and gastrointestinal cancers; degenerative ailments such as Parkinson's
15 and Alzheimer's diseases; chronic inflammatory diseases; metabolic disorders such as diabetes; and
16 cardiovascular diseases [8,11-13].

17 The increasing legislative restrictions on the presence of organic solvents in food products [14] coupled
18 to their negative effects on the nutritional and functional properties of compounds such as carotenoids
19 [15,16] have driven the search for "greener" alternatives than hexane, which is commonly used to
20 extract valuable lipophilic compounds from microalgae. High-pressure extractions using supercritical
21 and pressurized fluids have emerged as an alternative to normal pressure extractions using conventional
22 organic solvents. These high-pressure processes can reduce the demand of food grade solvents, increase
23 the selectivity towards valuable bioactive compounds, and preserve their bioactivity [17]. The
24 physicochemical properties (density, diffusivity, viscosity, and dielectric constant) of compressible

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1 fluids and fluids mixtures (carbon dioxide, water, and/or ethanol) can be easily tuned by changing the
2 operating conditions (pressure, temperature, composition) making them versatile and efficient solvents
3 for the extraction of natural compounds [17-19].

4 Carbon dioxide (CO_2) at supercritical conditions has been already used to extract astaxanthin from
5 *Haematococcus pluvialis* (Table 1). Because cells must be disrupted to extract astaxanthin from the
6 microalgae cyst [20,21], which has a tough cell wall [27], most studies in Table 1 experiment with
7 disrupted cell cyst. Typically, extraction of astaxanthin has been investigated as a function of extraction
8 pressure, temperature, and/or time but few authors [23,28] studied the effect of these variables on the
9 antioxidant activity of the $scCO_2$ extracts, which is in fact one of its main attributes. Astaxanthin
10 recovery generally improved as pressure and extraction time increased. Temperature generally enhances
11 astaxanthin recovery at high pressures, but this not always results in an increase in antioxidant activity
12 [23]. At closer look, it appeared that above 50 ºC there is a reduction in the net gain of the antioxidant
13 recovery [24,26] this could be explained by thermal degradation of astaxanthin, which could imply a
14 reduction in the antioxidant activity.

15 The supercritical extraction of *H. pluvialis* is limited because of the low solubility of astaxanthin (a
16 heavy and polar solute) in $scCO_2$ [29]. This has led to recommend increasing extraction pressures (above
17 50 MPa) to improve extraction rate and/or yield with a foreseeable increase in processing costs. This
18 drawback can be overcome by increasing astaxanthin solubility by using a polar co-solvent such as
19 ethanol [20-22,26,28]. Ethanol has also been used in pressurized liquid extractions (PLEs) leading to
20 extracts with a high yield of astaxanthin and antioxidant activity [30,31]. The present work used a Box-
21 Behken design (BBD) to systematically study the effects of pressure, temperature, and ethanol content in
22 $scCO_2$ in the yield, astaxanthin content, and antioxidant activity of *H. pluvialis* extracts using response
23 surface methodology (RSM). The results showed that ethanol content was by far the most important and
24 significant factor when extracting astaxanthin from *H. pluvialis*. These results led us to explore a new
25 region of pressurized liquids in which ethanol is the primarily solvent and CO_2 serves as an aid in the

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1 extraction. This so called gas-expanded liquid (GXL) region consist generally of a mixture between a
2 liquid solvent and a compressible gas where the properties of the liquid phase(s) are substantially
3 different from those at atmospheric pressure [32]. Moreover, GXLs have the advantage of requiring mild
4 working pressures, reducing the energy consumption hence the cost of the process [33]. Similar to
5 supercritical fluids, GXL have shown to improve mass transfer by decreasing interfacial tension,
6 reducing viscosity and improving diffusivity [17,32,34]. Although GXLs have been proposed as a
7 promising media for extraction of valuable bioactives from natural sources, only one work has been
8 published dealing with food applications; in this study, Golmakani *et al.* [35] extracted gamma-linolenic
9 acid from *Arthrospira platensis* (Spirulina) using CO_2 expanded-ethanol (CXE) that was similar to PLE
10 but better than $scCO_2$.
11 Therefore, the aim of this work was to assess and validate CXE as a new promising media for extraction
12 of bioactives from microalgae (such as astaxanthin from *H. pluvialis*). Moreover, results and analysis of
13 the experimental design employed for studying $scCO_2$ extraction are described as a baseline to compare
14 to the results obtained by using CXE.

15 ## 2. Materials and methods

16 ### *2.1 Samples*

17 Disrupted, dried *H. pluvialis* (maximum of 3% w/w astaxanthin feed grade powder) was kindly provided
18 by Atacama Bio Natural Products Inc. (Iquique, Chile). To prevent degradation, this substrate was
19 placed in a tightly sealed aluminum bag and stored at -20 ºC until use.

20 ### *2.2 High-pressure extractions*

21 All high-pressure extractions were carried out in a PrepMaster supercritical fluid extractor from Suprex
22 (Pittsburgh, PA). For each extraction, 0.5 g samples of *H. pluvialis* were mixed with 1 g of washed sea
23 sand (0.25–0.30 mm diameter) from Panreac Quimica S.A. (Barcelona, Spain) into a 20 mL stainless-
24 steel extraction cell sandwiched between glass wool plugs at the entrance and exit of the cell. This setup
25 prevented caking of the fine *H. pluvialis* powder and associated channeling of the solvent or solvent

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1 mixture flowing through the cell, on one hand, and plugging of tubing lines connected to the cell, on the
2 other. The PrepMaster unit pressurizes the CO_2 to the required set value and maintains the cell at the
3 required set temperature. In experiments with ethanol-modified CO_2 or CO_2-expanded ethanol, ethanol
4 (Panreac Quimica S.A., Barcelona, Spain) was fed by a PU2080 HPLC pump from Jasco (Tokyo, Japan)
5 set at the required volumetric flow rate, and the solvent mixture in the feed tubing was preheated to the
6 extraction temperature in a thermostated bath. In all experiments, a constant mass flow rate (0.06 g/min)
7 of premier quality CO_2 (Carburos Metálicos, Air Products Group, Madrid, Spain) was adjusted at the
8 exit of the extraction cell using two stop valves in tandem as a variable restrictor. Extracts (and ethanol)
9 were collected in a Falcon that was cooled by immersion in dry ice. Extraction time was fixed at 120
10 min. upon completion of each extraction experiment, ethanol and excess moisture were fully removed
11 from the Falcon by blowing gently technical quality N_2 (Carburos Metálicos, Air Products Group,
12 Madrid, Spain). Extraction yield (expressed in $mg \cdot g^{-1}$ dry extract/dry microalgae) was determined
13 gravimetrically by weighing the Falcon. Astaxanthin content (expressed in $mg \cdot g^{-1}$ astaxanthin/dry
14 extract) was estimated based on HPLC analysis of extract samples. Finally, antioxidant activity
15 (expressed as $mM \cdot g^{-1}$ trolox equivalent/dry extract) was estimated by TEAC assays of extract samples.
16 To avoid dry extract degradation, Falcons were protected from light with aluminum foil and stored at -
17 18 ºC prior to HPLC and TEAC analyses.

18 *2.3 Experimental design*

19 Response surface methodology (RSM) was used to unveil the functional relationship between extraction
20 responses (extract yield, astaxanthin content, and antioxidant activity of extracts) and the variables:
21 extraction pressure, extraction temperature, and ethanol content in $scCO_2$ extractions. The experimental
22 design was of Box-Behnken type, which are a class of rotatable or nearly rotatable second-order designs
23 based on three level incomplete factorial designs [36,37]. A total of 15 experiments were done which
24 involved 13 three-level experimental points and two repetitions of the central point. The extraction
25 pressure ranged from 20 to 35 MPa, temperature ranged from 40 to 70 ºC, and ethanol content in CO_2

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1 ranged from 0 to 13% (w/w). For experiments using ethanol, the flow of the co-solvent pump was fixed
2 at 0.05 mL/min (0.04 g/min of ethanol at 20 ºC) and 0.1 mL/min (0.08 g/min at 20ºC) to obtain mixtures
3 containing 6.5 and 13.0% (w/w) ethanol, respectively. Extract samples in scCO_2 extractions were
4 collected in 15 mL Falcons.

5 In CO_2-expanded ethanol (CXE) extractions pressure was fixed at 7 MPa, temperature was adjusted to
6 30, 46, or 60 ºC, and the co-solvent pump was operated at 0.469 mL/min (0.6 g/min of ethanol at 20 ºC)
7 or 0.657 mL/min (0.83 g/min at 20ºC) to obtain mixtures containing 50 and 70% (w/w) ethanol,
8 respectively, leading to six experimental combinations which were done in duplicates. Extract samples
9 in CXE extraction were collected in 50 mL Falcons.

10 *2.4 Analyses*

11 A conventional acetone extraction was performed to determine the total extractable compounds in *H.*
12 *pluvialis* using the method of Castro-Puyana et al. [38] with minor modifications. A microalgal sample
13 (200 mg) was mixed with 20 mL of HPLC-grade acetone (LabScan, Gliwice, Poland) containing 0.1%
14 (w/v) butylated hydroxytoluene (Sigma-Aldrich, Saint Louis, MO, USA) in a 50-mL Falcon that was
15 protected from light using aluminum foil, and the mixture was shaken for 24 h in an agitated
16 thermostatic bath (Selecta, Barcelona, Spain) operating at 500 rpm and 20 ºC. Following extraction, the
17 exhausted substrate was precipitated out in a refrigerated centrifuge (Hettich, Tuttlingen Germany)
18 operating at 10000 rpm and 4 ºC (10 min treatment). Following a 10 min centrifugation, the supernatant
19 was collected, and solvent was removed by blowing gently technical N_2 as done for the crude extracts in
20 scCO_2 and CXE extractions. Dry acetone extracts were weighed and stored at -18 ºC after weighting.
21 Conventional extractions were quadruplicated.

22 HPLC analysis were performed in an Agilent HP 1100 Series (Palo Alto, CA, USA) apparatus equipped
23 with a diode array detector (DAD) following a previously published method [31] with minor
24 modifications. Separation of carotenoids was performed in a YMC-C30 reversed-phase column (250
25 mm × 4.6-mm inner diameter, 5-μm particle size (YMC Europe, Schermbeck, Germany) using as the

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1 mobile phase HPLC-grade acetone from (LabScan, Gliwice, Poland) and water purified using a Milli-Q
2 system (Millipore Corporation, Billerica, MA). The program consisted of an isocratic elution of 84:16
3 (v/v) acetone/water for the first 10 min, and a gradient elution to 97:3 (v/v) acetone/water in 50 min and
4 then back to 84:16 (v/v) acetone/water in 52 min. The flow rate was 1 mL/min, the injection volume was
5 10 μL, and detection was done at 480 and 660 nm (spectra recorded between 240 to 770 nm by DAD).
6 Standard calibration curves were prepared using solutions in acetone of astaxanthin monopalmitate
7 (ranging from 40 to 1.25 μg/mL) from CaroteNature (Lupsingen, Switzerland), and free astaxanthin (10-
8 0.32 μg/mL), cantaxanthin (10-0.3125 μg/mL), and lutein (30.0-0.938 μg/mL) from Sigma-Aldrich
9 (Saint Louis, MO). Major carotenoids in the extracts were identified by their retention times and spectra
10 in comparison to their standards and literature data. Concentration of astaxanthin esters were quantified
11 as a whole from the standard calibration curve prepared from astaxanthin monopalmitate. Total
12 astaxanthin was obtained as the sum of free astaxanthin and astaxanthin esters. All analyses were done
13 in duplicates.

14 The antioxidant activity of extracts was measured using a Trolox Equivalent (TE) Antioxidant Capacity
15 (AC) assay described previously by Jaime et al. [31]. Briefly, 2,2-azinobis (3-ethyl-benzothiazoline-6-
16 sulfonic acid) di-ammonium salt ($ABTS^{\bullet+}$) radical cation was generated by reacting 7 mM ABTS
17 (Sigma-Aldrich, Saint Louis, MO) with 2.45 mM potassium persulfate after 16-h incubation at room
18 temperature and in the dark. The $ABTS^{+}$ radical solution was diluted with ethanol to an absorbance of
19 0.7 ± 0.2 at 734 nm. Extracts (10 μL) at five different concentrations were added to 990 μL of diluted
20 $ABTS^{+}$ radical solution. The reaction was measured after the absorbance reached a plateau at 45 min. 6-
21 hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (Trolox) from Fluka Chemie AG (Buchs,
22 Switzerland) was used as a reference standard. All analyses were done at least in duplicates.

23 *2.6 Statistical analysis*

24 RSM design was used to evaluate the effects of the pressure (P, MPa; coded variable X_1, Eq. (1a)),
25 temperature (T, ºC; coded variable X_2, Eq. (1b)), and ethanol content in CO_2 (E, % w/w; coded variable

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X_3, Eq. (1c)) on extraction yield, astaxanthin content, and antioxidant activity of extracts (response variable Z).

$$X_1 = \frac{P - 27.5}{7.5} \tag{1a}$$

$$X_2 = \frac{T - 45}{15} \tag{1b}$$

$$X_3 = \frac{E - 6.5}{6.5} \tag{1c}$$

A second-order polynomial equation was used to express the response variable Z as a function of the independent dimensionless variables X_1, X_2, an X_3, using Eq. (2):

$$Z = \sum_{i=0}^{3} \sum_{j=i}^{3} a_{ij} X_i X_j , \tag{2}$$

where X_0 (dummy variable) equals one; a_{00} is a constant; a_{01}, a_{02}, and a_{03} are linear coefficients; a_{12}, a_{13}, and a_{23} are cross-product coefficients; and a_{11}, a_{22} and a_{33} are quadratic coefficients. Model parameters of the model were estimated by multiple regressions analysis using Design-Expert Software, version 7.0 (Stat-Ease, Inc., Minneapolis). The statistical significance of the models was evaluated with a multi-factor ANOVA with a 95% confidence level.

The statistical analysis for CXE extraction experiments was done by a 2-Factor ANOVA using Statgraphics Centurion XVI software, version 16.1.11 (Statpoint Technologies, Warrenton, VA).

3. Results and discussion

The maximum extraction yield of *H. pluvialis* microalgae obtained by a conventional extraction with acetone was 360.2 mg/g. Figure 1 shows the chromatographic profile of the extract obtained by HPLC. As expected, most of the carotenoids (95 ± 2% w/w) corresponded to different forms (free, mono-, and di-esters) of astaxanthin, being astaxanthin esters the predominant form (90-95% of all astaxanthin forms) in the microalgae cyst. This agrees with reports in literature stating that 70% of all astaxanthin is in mono-esters form, 25% in di-esters form, and 5% in free form [13,47]. Our sample also contained minor amounts of lutein (1.02 mg/g extract) and cantaxanthin (0.4 mg/g extract). The total (free plus

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1 esterified) astaxanthin concentration in the acetone extract was 51.02 mg/g that correspond to 1.83 ±
2 0.17% (w/w) of the dry microalgae.

3 *3.1 scCO_2 extractions of* H. pluvialis

4 Table 2 shows experimental conditions and results of the BBD design for the scCO_2 extraction of
5 *Haematococcus pluvialis* with ethanol modified CO_2. As a reference, astaxanthin recovery results are
6 also presented for each experimental run. Extraction yield ranged from 73.4 to 282.5 (mg/g), astaxanthin
7 content from 5.98 to 53.48 (mg/g), and TEAC values from 0.084 to 0.243 (mM TE/g). Astaxanthin
8 recovery (expressed in % mg astaxanthin/mg total astaxanthin) was estimated as a percentage of the
9 total astaxanthin extracted in each sample to the total astaxanthin extracted in a conventional acetone
10 extraction, which was assumed to be 100%. The total astaxanthin extracted is in fact calculated from
11 extraction yield and astaxanthin content. The recovery of astaxanthin in scCO_2 experiments ranged from
12 2.5% when using pure CO_2 to 82.3% when using CO_2 modified with 13% (w/w) ethanol, both at 55 ºC
13 and 20 MPa.

14 Table 3 presents the statistical analysis of the BBD for the different responses considered (extraction
15 yield, astaxanthin content, and antioxidant activity. Ethanol content is the only factor that contributes
16 significantly ($p < 0.05$) to all responses. For each RSM model a stepwise removal of the least
17 significant term (that contributes the least to explain the response) was applied. The removal criteria
18 considered was to maximize adjusted-R^2 and minimize the difference between R^2 statistic and adjusted-
19 R^2. Lack-of-fit test was also considered in the removal criteria. The statistical significance of each term
20 eliminated in the model was assessed as described previously del Valle et al [39,40]. Briefly, a F-value
21 test was used to evaluate term-by-term elimination in comparison with the complete model. For each
22 term removal a corrected sum of square was generated for the new model until it reached the R^2 criteria
23 described before. When one reduces a statistical model (*i.e.* Eq. 2) by this methodology it is possible to
24 unveil the effect of other significant factors such as extraction pressure or temperature. The resulting
25 equation, which is simpler than Eq. (2), has a non-significant lack-of-fit ($p < 0.05$) and eliminates noise

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1 related terms, thus improving the signal-to-noise ratio (recommended signal-to-noise > 4) and adjusted-
2 R^2 statistics (Table 4). This is illustrated for extraction yield, as follows: Eq (3) was obtained using this
3 procedure and is written in term of coded variables (Eq. (1a)-(1c)),

4 $$Y\ (mg/g) = 183.0 + 75.0\left(\frac{E-6.5}{6.5}\right) - 14.6\left(\frac{P-27.5}{75}\right)\left(\frac{E-6.5}{6.5}\right) - 18.3\left(\frac{T-55}{15}\right)^2 - 23.4\left(\frac{E-6.5}{6.5}\right)^2 \quad (3)$$

5 For example, at the center of the experimental region the extraction yield only increases with an increase
6 of ethanol content in CO_2 (positive a_{03} coefficient). Because of negative *P-E* interaction (negative a_{13}
7 coefficient), the positive effect of ethanol content in CO_2 on extraction yield decreases as pressure
8 increases. Furthermore, since this interaction term is the only one that includes pressure then it is
9 convenient to analyze the response surface for extraction yield at the lowest studied pressure (20 MPa in
10 our case). At P = 20 MPa the slope describing the effect of ethanol content on extraction yield for E =
11 6.5% is [10 x (75.0 + 14.6)/6.5] 137.8 mg/g per 10 % of ethanol. The negative contribution of the
12 second-order term E^2 (a_{33} coefficient) indicates that the surface is convex, or that the slope describing
13 the positive effect of ethanol concentration on the yield decreases continuously as this concentration
14 increases. Likewise, second-order T^2 term (a_{22} coefficient) contributes negatively but in a smaller scale
15 than a_{33}. Therefore, the surface plot is flatter when moving along an axis for pressure changes than an
16 axis for changes in ethanol content. Moreover, the absence of any other term dependent on temperature
17 indicates an optimal at the midpoint value experimentally studied (55 ºC). These features are
18 exemplified in Fig. 2A. The response surface indicates that any eventual optimal in extraction yield is
19 beyond the studied experimental region for ethanol concentration. At P = 20 MPa the slope describing
20 the effect of ethanol concentration on extraction yield for E = 13.0% ethanol is 101.8 mg/g per 10 % of
21 increase of ethanol [10 x (7.50 + 1.46 – 2.34)/6.5]. In fact, extraction yield keeps on increasing with
22 ethanol concentration for the maximal value studied.

23 Eq. (4) was obtained applying the same stepwise procedure to eliminate least significant terms from the
24 equation describing the effect of the independent variables on astaxanthin content in the extracts. This

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1 response relates to the selectivity of the extraction process, thus when this variable increases the extract
2 is enriched with astaxanthin.

3 $$S\,(mg/g) = 27.35 + 4.78\left(\frac{P-27.5}{75}\right) + 19.23\left(\frac{E-6.5}{6.5}\right) - 5.42\left(\frac{P-275}{75}\right)\left(\frac{T-55}{15}\right) - 3.34\left(\frac{P-275}{75}\right)\left(\frac{E-6.5}{6.5}\right) \quad (4)$$

4 Table 4 shows that Eq. (4) is adequate to describe the response surface for astaxanthin content. In
5 addition, pressure term becomes a significant ($p < 0.05$) contributor, as with ethanol content, in the
6 astaxanthin content response. At the center of the experimental region astaxanthin content in the extract
7 increases with extraction pressure and even more with ethanol concentration in CO_2 (a_{03} is larger than
8 a_{01}, which is positive). Eq. (4) shows that the only term dependent on temperature that affects
9 astaxanthin content is the negative *P-E* interaction (a_{12} coefficient). Hence, the positive effect of
10 pressure on this response starts decreasing as temperature increases for the entire experimental region.
11 For instance, astaxanthin content decreases 0.85 mg/g as temperature increases 10 ºC at 35 MPa [10
12 (5.42 – 4.78)/7.5]. Therefore, the extract reaches its highest astaxanthin content at the lowest studied
13 temperature (40 ºC in our case). Similarly, the negative interaction between *P-E* (negative a_{13}
14 coefficient) decreases its contribution on astaxanthin content as pressure increases. Likewise, as ethanol
15 content increases, *P-E* contribution on astaxanthin content is continuously reduced for the entire
16 experimental region. Consequently, the largest slopes in the response surface of Fig. 2B can be observed
17 in the front corner (for *P* = 20 MPa, *T* = 40 ºC, and *E* = 0% ethanol**).** These slopes are 18.05 mg/g per 10
18 MPa increase in pressure [10 (5.42 + 4.78 + 3.34)/7.5], and 34.72 mg/g per 10% increase in ethanol
19 concentration in CO_2 [10 (19.23 + 3.34)/6.5]. Thana *et al.* [28] [23] RSM statistical model shows that
20 astaxanthin extraction depends significantly on positive *P* term as in our Eq. (4). However, their model
21 also depends on positive *P-T* terms, which in our case was negative. Solubility of compounds in $scCO_2$
22 depends on a balance between solute vapor pressure and fluid density, both properties in which pressure
23 and temperature have important influence. Complex *P-T* interactions may emerge at different
24 experimental conditions, thus the outcome is not always clear unless the interaction is evaluated within
25 an experimental region. Thana *et al.* [23] experimented within 30-50 MPa and 40-80 ºC, which is a

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1 broader experimental range than our study, therefore it can explain the difference with our *P-T*
2 interaction term. Furthermore, our study included ethanol modified CO_2 and its presences can reduce the
3 contribution interaction terms such as *P-T*.

4 As with previous responses, Eq. (5) was obtained applying the stepwise procedure to eliminate least
5 significant terms from the equation describing the effect of the independent variables on antioxidant
6 activity (response surface for TEAC) of the extract.

7 $$TEAC\ (mM\ TE/g) = 0.19 + 0.008\left(\frac{P-27.5}{75}\right) + 0.059\left(\frac{E-6.5}{6.5}\right) - 0.014\left(\frac{T-55}{15}\right)^2 - 0.025\left(\frac{E-6.5}{6.5}\right)^2 \quad (5)$$

8 Table 4 shows that Eq. (5) is adequate to describe the response surface for TEAC from a statistical
9 standpoint. Second-order E^2 term (negative a_{33} coefficient) in Eq. (5) is a significant ($p < 0.05$)
10 contributor in the antioxidant activity as with pressure in astaxanthin content. Applying the same
11 analysis as with extraction yield and astaxanthin content, Figure 2C plots this response surface for an
12 extraction pressure of 35 MPa for which TEAC is highest. The surface will move vertically down 0.016
13 mM TEAC/g when reducing extraction pressure to 20 MPa as indicate by the linear term on pressure
14 (a_{01}) in Eq. (5). Thana *et al.* [23] study showed that only *P-T* interaction was significant contributor in
15 antioxidant activity. Their statistical model suggested a minimum point at 67ºC, 40.3 MPa, and 1.86 h of
16 extraction, in which an IC_{50} of 2.57 mg/l was obtained. The highest antioxidant activity was estimated to
17 be at the lowest temperature (40 ºC), highest pressure (50 MPa) and longest extraction time (4 h). In our
18 study, ethanol content in CO_2 greatly influences antioxidant activity, reducing the contribution of other
19 factors such as pressure and temperature. The absence of *P-T* term in Eq. (5) indicates that, under these
20 experimental conditions, it has no contribution in antioxidant activity. Moreover, temperature second-
21 order term T^2 (negative a_{22} coefficient) suggests that the highest antioxidant activity can be approached
22 at the midpoint value experimentally studied (55 ºC).

23 As a way to validate our models it was estimated the astaxanthin recovery from the predicted values of
24 extraction yield, Eq. (3), and astaxanthin content, Eq. (4), with a 95% confidence limits for the mean

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1 response. Figure 3 plots the predicted interval values for each run ordered at increasing astaxanthin
2 recovery and the observed data. Only 2 of the 15 runs lie considerably outside the predicted interval,
3 which implies that our models combine together, have at least 87% prediction for the astaxanthin
4 recovery. Most of the values are predicted for low concentration of ethanol in CO_2. This implies that as
5 ethanol content increases the predicted intervals grow larger.

6 *3.2 CXE extractions of* H. pluvialis

7 Results obtained after analyzing the experimental design for $scCO_2$ of *H. pluvialis* confirmed that
8 ethanol content in the solvent was the most important factor contributing to the different responses
9 considered such as extraction yield, astaxanthin content, and antioxidant activity. Therefore, it seemed
10 reasonable to explore the experimental region considering ethanol content above 13% and low pressures.
11 This selection was also supported by previous data obtained by Santoyo, *et al* (2009) [30] and Jaime, *et*
12 *al* [31] using pressurized ethanol at pressures of 10 MPa. In this sense, GXLs offer several advantages
13 compared to $scCO_2$ and pressurized ethanol: it allows working at low pressures (compared to $scCO_2$)
14 and using less amount of organic solvent (compared to PLE). Therefore, to carry out the present study, a
15 low pressure was selected (7 MPa), lower than the critical pressure of CO_2 (7.38 MPa). Also, three
16 levels of temperature were chosen: 30ºC (low), 45ºC (moderate) and 60ºC (high). On the other hand,
17 since results obtained by Golmakani *et al.* [35] demonstrated that extraction rate increased when using
18 ethanol content up to 50%, it was decided to explore further ethanol content by selecting CO_2-expanded
19 70% (w/w) ethanol and compare it to CO_2-expanded 50% (w/w) ethanol.

20 Table 5 shows the results obtained by performing the experiments with CO_2-expanded (50-70% w/w)
21 ethanol (CXE extractions); these extractions generally resulted in higher extraction yield (333.1 to 450.0
22 mg/g), astaxanthin content in the extracts (28.69 to 62.57 mg/g), astaxanthin recovery (65.5% to
23 124.2%) and TEAC values (0.174 to 0.233 mM TE/g) than $scCO_2$ extractions, this behavior is due to the
24 positive influence of ethanol content in the solvent mixture on these responses.

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1 Table 6 shows the statistical analysis through a 2-factor ANOVA for the 4 responses. On one hand,
2 ethanol composition shows non-significant ($p > 0.05$) influence on extraction yield on CXE neither its
3 interaction with temperature, within the experimental range. On the other hand, both astaxanthin content
4 and antioxidant activity showed a significant influence of these variables besides the temperature. Yet
5 astaxanthin recovery, in contrast to the other responses, showed significance to ethanol content and its
6 interaction with temperature, but not temperature alone.

7 By observing Figure 4A, as temperature increases so does the extraction yield for both groups (CO_2-
8 expanded 50% and 70% w/w ethanol). As expected, as temperature rises so does the extraction kinetics.
9 Since there is non-significant ($p < 0.05$) difference between both groups, a mean average was obtained
10 from the results and a linear regression was applied and plotted in Figure 4A. Unlike extraction yield,
11 the temperature related behavior is not replicated in the astaxanthin content (Figure 4B). There is a
12 maximum obtained at moderate temperatures at CO_2-expanded 50% (w/w) ethanol, while at CO_2-
13 expanded 70% (w/w) ethanol there is a decrease of the concentration of astaxanthin in the extracts as
14 temperature increases. In the case for astaxanthin recovery (Figure 4C), a similar behavior is observed at
15 50% (w/w) when comparing to astaxanthin content. Interestingly, a maximum is obtained at moderate
16 temperatures that exceed the maximum determined by conventional acetone extraction (recovery in
17 experiment #2 is 124.2% w/w). Nevertheless, at 70 % (w/w) the curve is almost a straight line showing
18 no influence of temperature in the recovery. This opposite behavior may be explained because of its *T-E*
19 interaction. TEAC follow the same trend as astaxanthin content in CO_2-expanded 50% ethanol, which
20 demonstrates the dependence of TEAC values on the astaxanthin content of the extracts (Figure 4D)
21 under these experimental conditions. On the contrary, at CO_2-expanded 70% ethanol the values do not
22 decrease. In present study, high-temperature showed to be detrimental in antioxidant activity of $scCO_2$
23 and CXE extracts of *H. pluvialis*. This may be due to the poor stability that antioxidant have at high
24 temperatures, but Wang *et al* [25] have shown that this is worsened when astaxanthin is solubilized in
25 ethanol under high-temperature and oxygen-involved environment.

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1 Comparing scCO_2 with CXE extractions at optimum extraction conditions (20 MPa, 55 ºC, 13% w/w for
2 scCO_2 and 50% w/w ethanol, 7 MPa, 45°C for CXE), CXE showed better results in terms of extraction
3 yield (333.1 mg/g), astaxanthin content (62.57 mg/g) and astaxanthin recovery (124.2% w/w) than
4 scCO_2 extraction (282.5 mg/g, 53.48 mg/g and 82.3% w/w respectively), while antioxidant activity
5 showed similar figures (0.233 mM TE/g extract for CXE and 0.243 mM TE/g extract under supercritical
6 conditions). On the other hand, the achievement of CXE extraction over conventional extractions is
7 interesting since, for example, Soxhlet extractions takes between 24-48 hours to be completed instead of
8 the 2 hours that it took CXE extraction.

9 It is important to highlight the important dependence of antioxidant activity with astaxanthin content; as
10 can be seen in Figure 5, the TEAC value increases as astaxanthin content does. This trend is followed
11 independently on the type of extraction process applied (supercritical fluid extractions (SFE) and CXE).
12 Nevertheless, trend line does not pass through the origin, which suggests astaxanthin is not the single
13 contributor to this antioxidant activity. Coupled with the observation that all xanthophylls and
14 xanthophyll-esters in *H. pluvialis* were jointly extracted in our experiments, the trend line in Figure 5
15 means this microalgae contains additional bioactive compounds (other than carotenoids) that can be
16 extracted using ethanol-modified CO_2 or CO_2-expanded ethanol that are not necessarily carotenoids.
17 Goiris *et al* [41] suggest that phenolics contribute also to the antioxidant activity of several microalgae
18 including *H. pluvialis*.

19 In conclusion, carbon dioxide expanded extractions (CXE) are suitable for fast and efficient withdrawal
20 of high quality (both composition and antioxidant activity) *H. pluvialis* extracts. By comparing the best
21 result obtained in the present work (124.2% recovery) with previous reported data (see Table 1) results
22 obtained by CXE are much better than those obtained previously. Thus, CXE extraction is revealing as
23 an appropriate new green extraction technology for high value compounds derived from microalgae.

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1

2 # List of Figures

3

4 **Figure 1.** Representative HPLC-DAD chromatogram of acetone extract obtained by conventional
5 extraction of *Haematococcus pluvialis*. Peak number relate to the identified carotenoids (480 nm) being
6 free astaxanthin (1), lutein (2), cantaxanthin (3), astaxanthin mono-esters (4), and astaxanthin di-esters
7 (5).

8

9 **Figure 2.** Response surface plots of (A) extract yield as a function of ethanol content and temperature
10 (pressure fixed at 20.0 MPa); (B) Astaxanthin content as a function of ethanol content in CO_2 and
11 pressure (temperature fixed at 40 ºC); (C) TEAC as a function of ethanol content in CO_2 and
12 temperature (pressure fixed at 35.0 MPa). The level of fixed factor for each plot was adjusted to give the
13 highest response surface plot.

14

15 **Figure 3.** Predicted values (interval bars) versus observed values (dots) of astaxanthin recovery for each
16 run. Values are ordered in increasing astaxanthin recovery. A 95% confidence limits were assigned for
17 the predicted values.

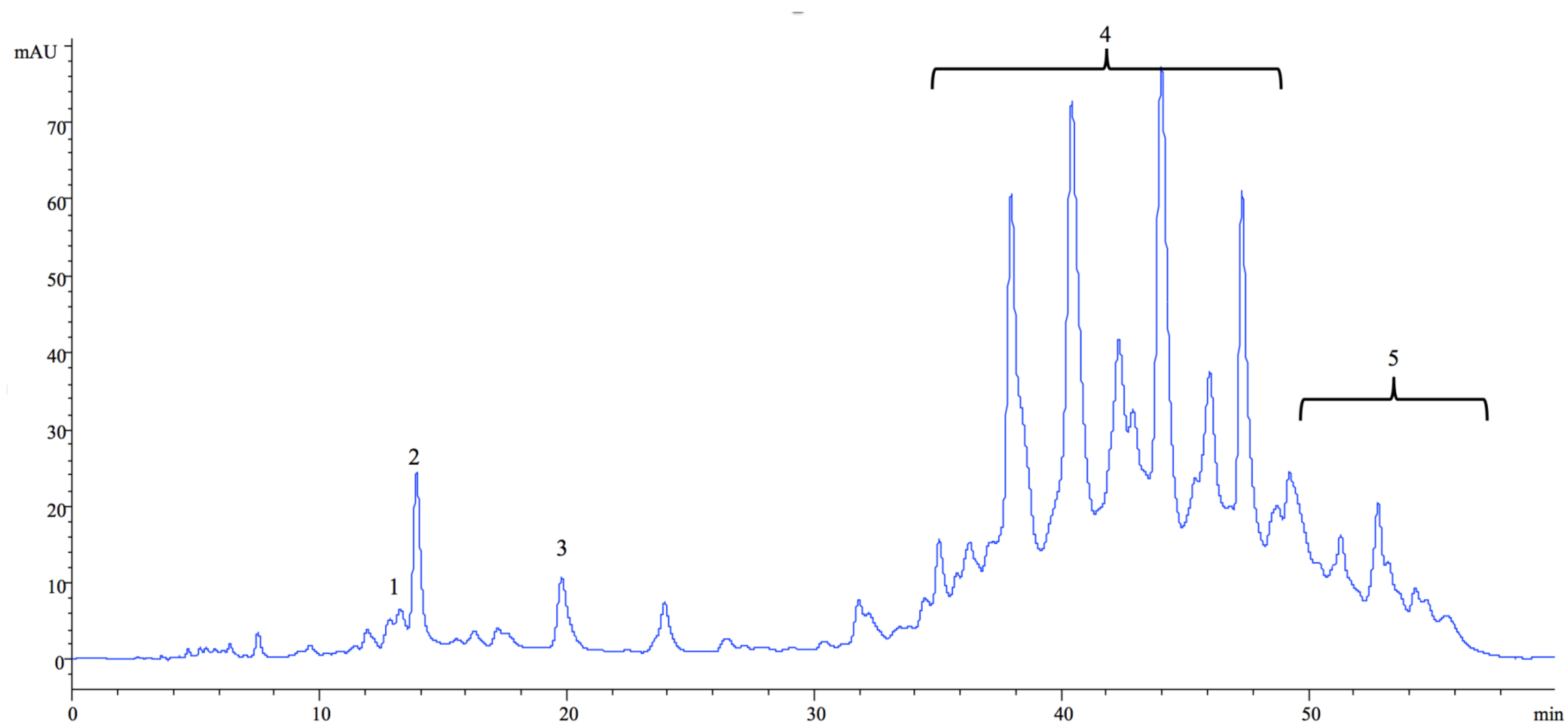
18

19 **Figure 4.** Effect of temperature and ethanol content in CO_2 in the extract yield (%, w/w), astaxanthin
20 content (mg/g), TEAC (mM TE/g), and astaxanthin recovery (%) of CXE extractions.

21

22 **Figure 5.** Antioxidant activity dependence to astaxanthin content in the extract of *H. pluvialis*. Data of
23 both types of extraction was plotted and a combined linear regression was applied.

24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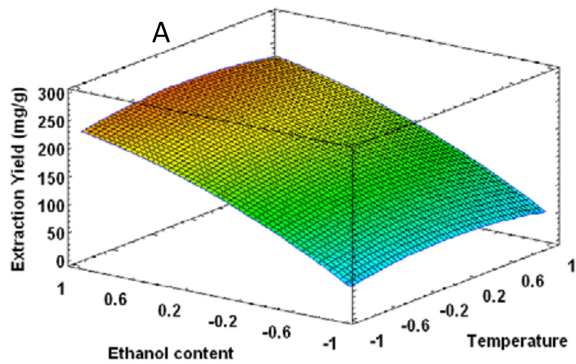

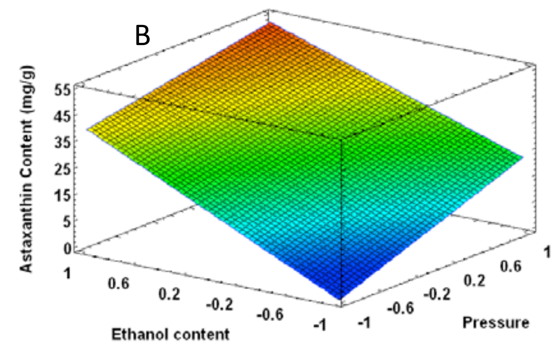

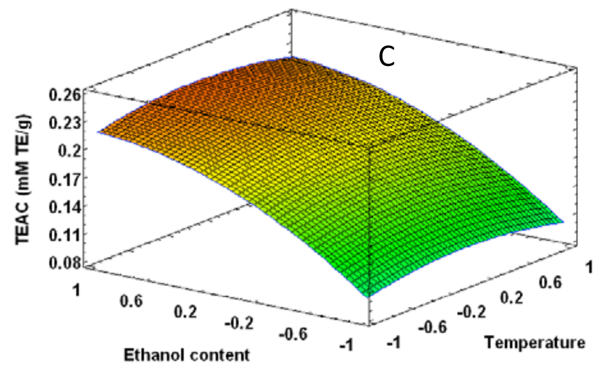

Response level

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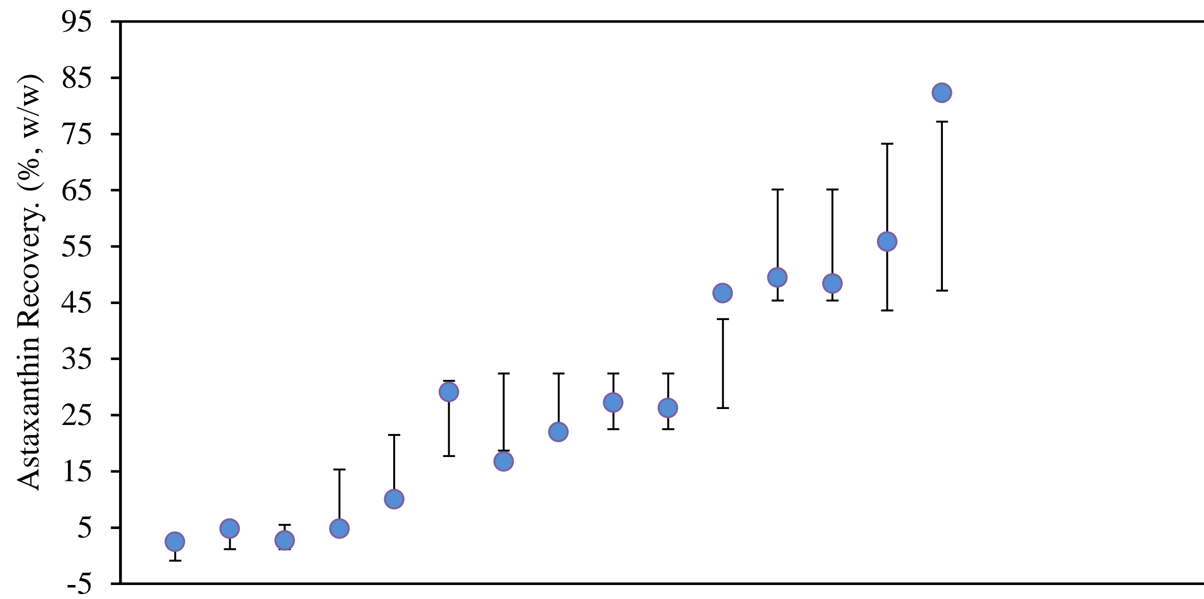
- Mean
- High


Astaxanthin Recovery. (%, w/w)
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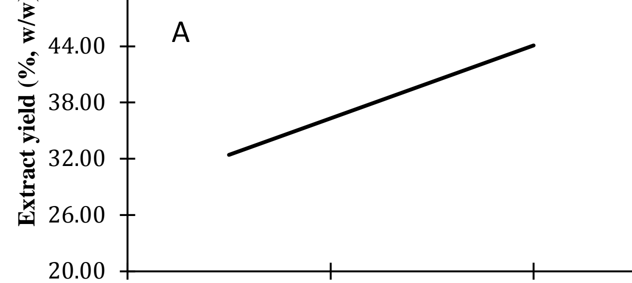
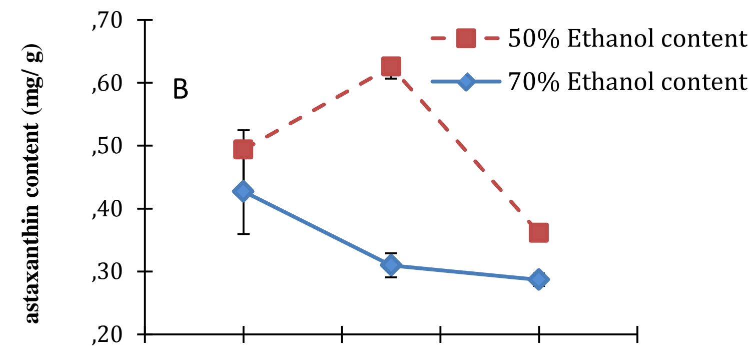
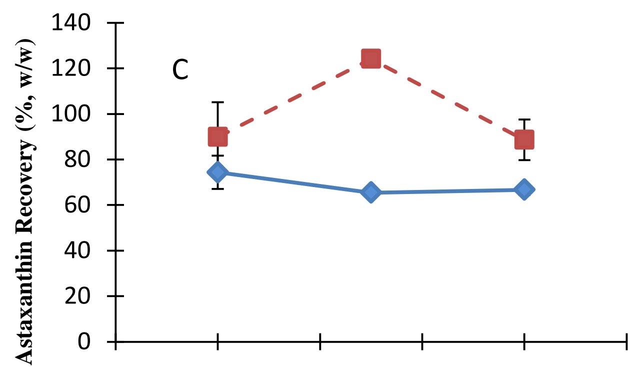


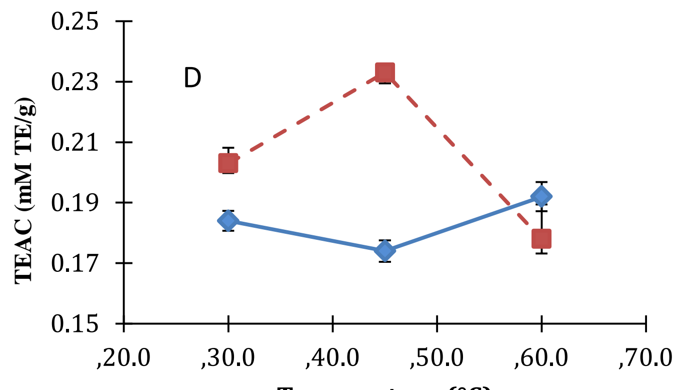
A
Extract yield (%, w/w
44.00
38.00
32.00
26.00
20.00



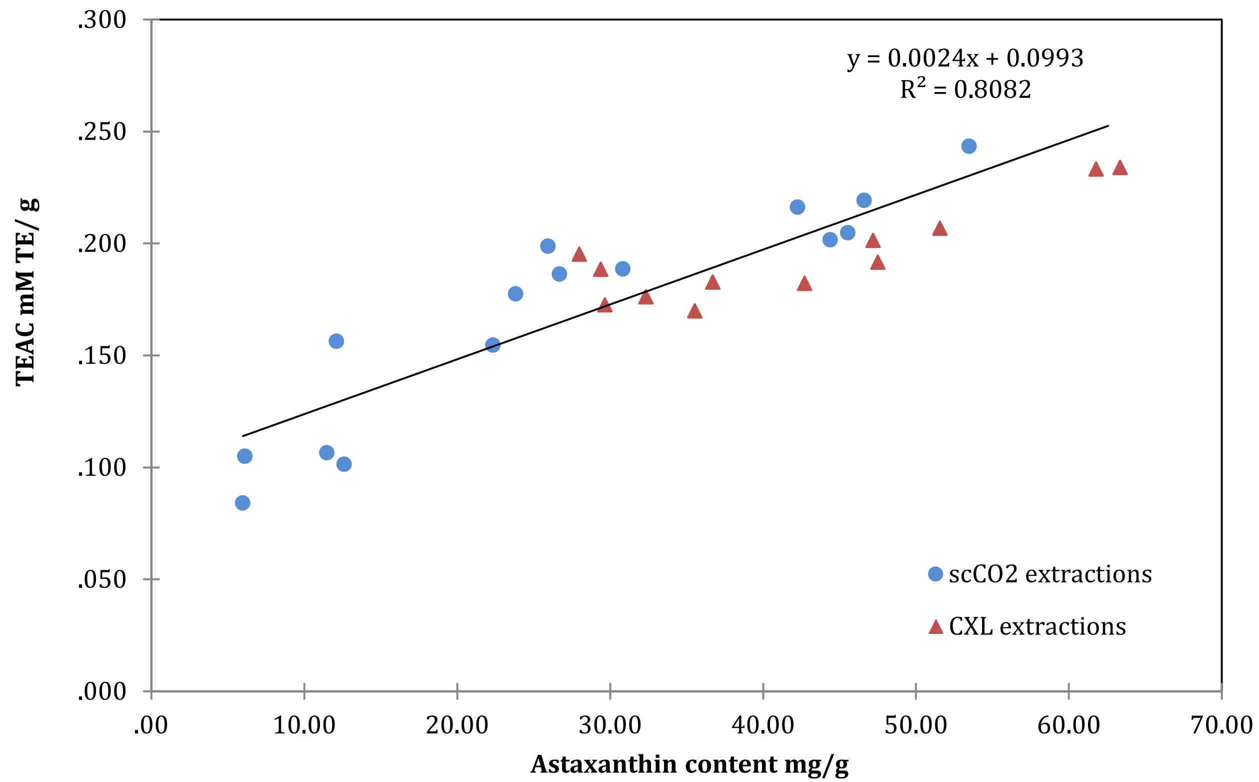
B
astaxanthin content (mg/ g)
,70
,60
,50
,40
,30
,20
50% Ethanol content
70% Ethanol content



C
Astaxanthin Recovery (%, w/w)
140
120
100
80
60
40
20
0



D
TEAC (mM TE/g)
0.25
0.23
0.21
0.19
0.17
0.15
,20.0
,30.0
,40.0
,50.0
,60.0
,70.0



TEAC mM TE/ g
.300
.250
.200
.150
.100
.050
.000
.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
Astaxanthin content mg/g
y = 0.0024x + 0.0993
R2 = 0.8082
scCO2 extractions
CXL extractions