Electronic Supplementary Material (ESI) for Green Chemistry.

Extraction and milking of astaxanthin from *Haematococcus pluvialis* cultures

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Lipids and other carotenoids characterization in algal extracts.

Each extract obtained with the tested solvents (except almond oil) was quantitatively and qualitatively characterized by GC-MS in terms of bounded fatty acid (BFA expressed as fatty acid methyl esters, FAMEs) and other GC-MS detectable compounds.

For BFA analysis, about 2 mg of dried extracts were dissolved in DMC (0.4 mL), and added with 2,2-dimethoxypropane (0.1 mL) and 0.5 M NaOH in MeOH (0.1 mL). The samples were placed in an incubator at 90°C for 30 min. After cooling for 5 min to room temperature, 1.3 M BF_3-methanol 10% (w/w) reagent (0.7 mL) was added before repeating the incubation for 30 min. After cooling for 5 min to room temperature, saturated NaCl aqueous solution (2 mL) and hexane (1 mL) containing methyl nonadecanoate (0.02 mg) were added and the samples were centrifuged at 4000 rpm for 1 min. The upper hexane-DMC layer, containing FAMEs, was transferred to vials for GC-MS analysis. Each analysis was repeated in duplicate.

For other GC-MS detectable compounds analysis, about 2 mg of dried extracts were dissolved in CH_3CN (0.2 mL), and added with bis (trimethylsilyl)trifluoroacetamide (BSTFA, 0.05 mL) containing 1% of trimethylchlorosilane (TMCS), pyridine (0.02 mL) and containing methyl nonadecanoate (0.02 mg). The solutions were placed in an incubator at 80 °C, stirred for 30 min, then analyzed by GC-MS.

GC-MS analyses were performed by using a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP quadrupole mass spectrometer. The injection port temperature was 280°C. Analytes were separated by a HP-5 fused-silica capillary column (stationary phase poly[5%diphenyl/95% dimethyl]siloxane, 30 m, 0.25 mm i.d., 0.25 μm film thickness), with helium as carrier gas (at constant pressure, 33 cm s^{-1} linear velocity at 200°C). Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan s^{-1} within the 12-600 m/z range.

The temperature program of the column for the analysis of TFA was the follow: from 50°C up to 180°C at 50°C min^{-1}, then from 180°C up to 300°C at 5°C min^{-1}.

The temperature program of the column for the analysis of sterols and other GC-MS detectable compounds was the follow: from 50 °C up to 180 °C at 50 °C min^{-1}, then from 180 °C up to 300 °C at 5 °C min^{-1}.

Methyl nonadecanoate was utilized as internal standard for quantification of all the compounds by assuming an unitary response factor.

Each extract obtained with the tested solvents (except almond oil) was also qualitatively characterized in terms of other carotenoids presence. About 0.5 mg of dried extracts were dissolved in HPLC-grade CH_3CN (0.5 mL), and analyzed by HPLC UV-Vis. Standards of astaxanthin, β-carotene and canthaxanthin were used for peak identification. The HPLC consisted of a 1200 series device (Agilent Technologies, Waldbronn, Germany) with a diode array detector (470 nm). The separations were performed on a 4.6 x 150 mm XBridge ® C8 column with an average pore diameter of 137 Å and a particle size of 3.5 μm at a temperature of 25°C. The separation of the carotenoids in the algae extracts was achieved using a mobile phase composition of methanol (A) and water (B). The elution proceeded from 90% A for 2 min and was followed by a linear gradient to 100% A until 5 min, 90% A until 18 min and 80% A until 20 min, with a flow rate of 0.7 mL min^{-1}. The injection volume was 5 μL.

Each extract was also analyzed by ^{1}H-NMR spectroscopy; the spectra were recorded using a 5 mm probe on a Varian Mercury 400 spectrometer in $CDCl_3$.

Figure 1S. Example of ^{1}H NM

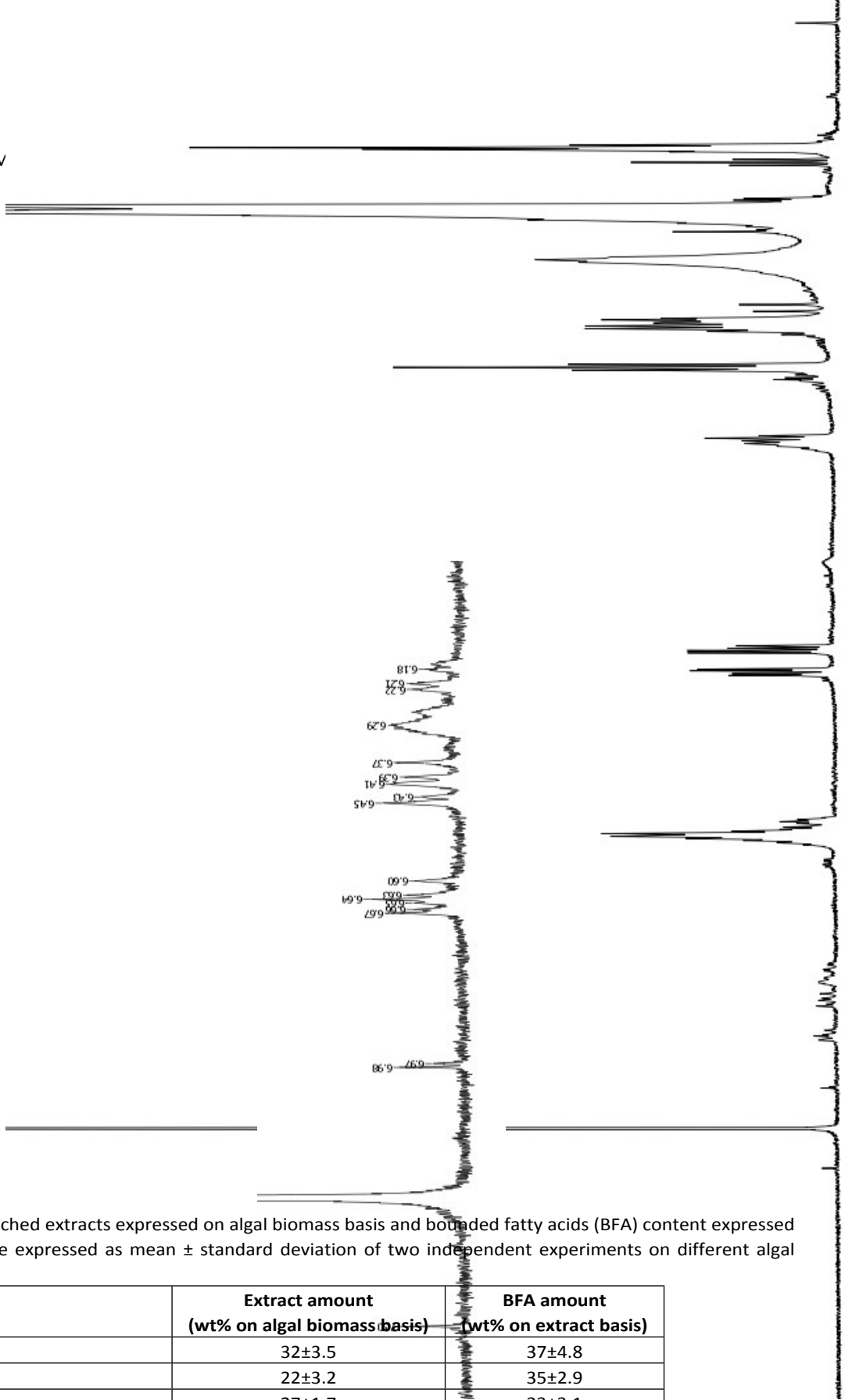

Table 1S. Carotenoid-enriched extracts expressed on algal biomass basis and bounded fatty acids (BFA) content expressed on extract basis. Data are expressed as mean ± standard deviation of two independent experiments on different algal cultures.

Solvent	Extract amount (wt% on algal biomass basis)	BFA amount (wt% on extract basis)
DMC	32±3.5	37±4.8
DEC	22±3.2	35±2.9
MIBK	27±1.7	33±3.1
2-MeTHF	54±4.9	40±4.5
Isoamyl acetate	23±1.4	36±2.5
EtOAc	44±1.1	32±3.1
BuOH	27±2.4	31±3.7
Cyclohexane	24±2.8	31±3.0
Cyclohexane/EtOH/acetone	46±2.1	29±2.3

Figure 2S. Bounded fatty acids (BFA) relative composition (%) in each extract.

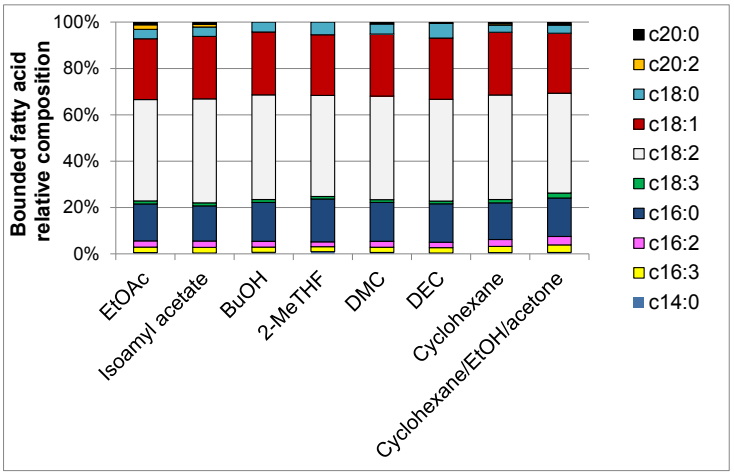

Figure 3S. Example of chromatogram obtained by HPLC UV-Vis at 470 nm: peaks of a) astaxanthin in the free form, b) canthaxanthin, c) β-carotene and d) EtOAc extract. UV-Vis spectra are reported in the inserts in the chromatograms.

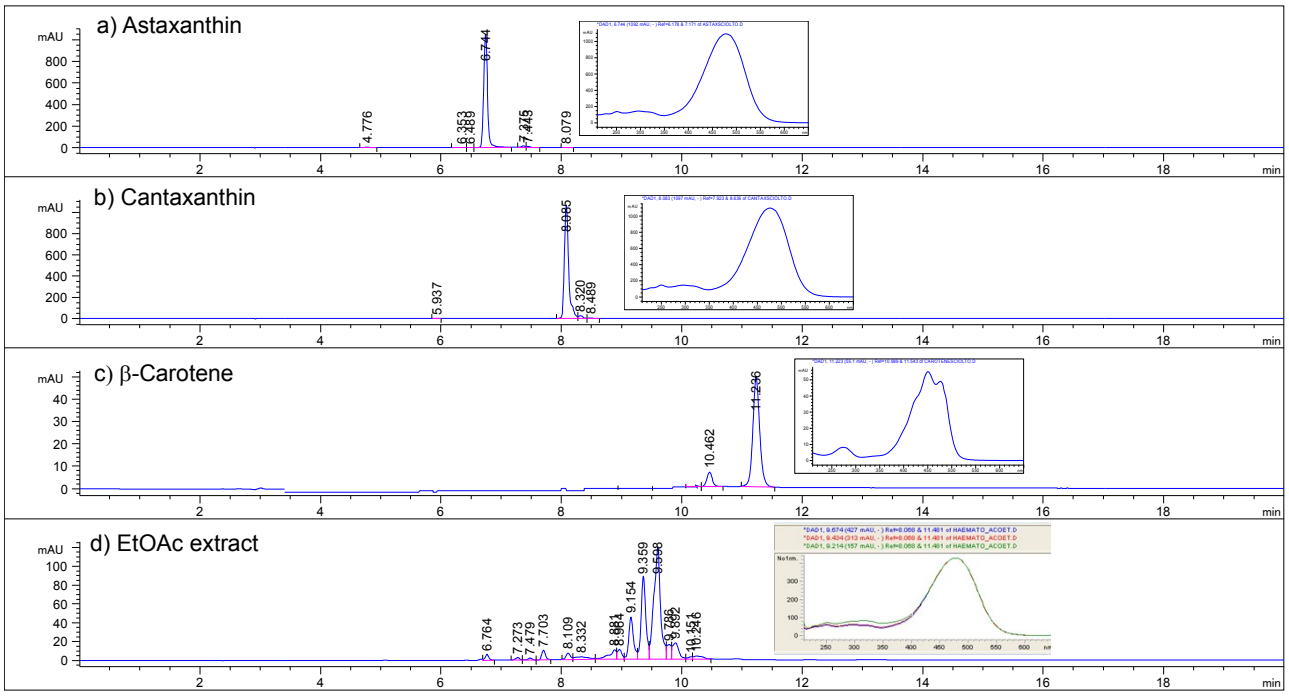

Figure 4S. *H. pluvialis* cells observed under light microscope (320x) after the extraction (30 minute) with each solvent.

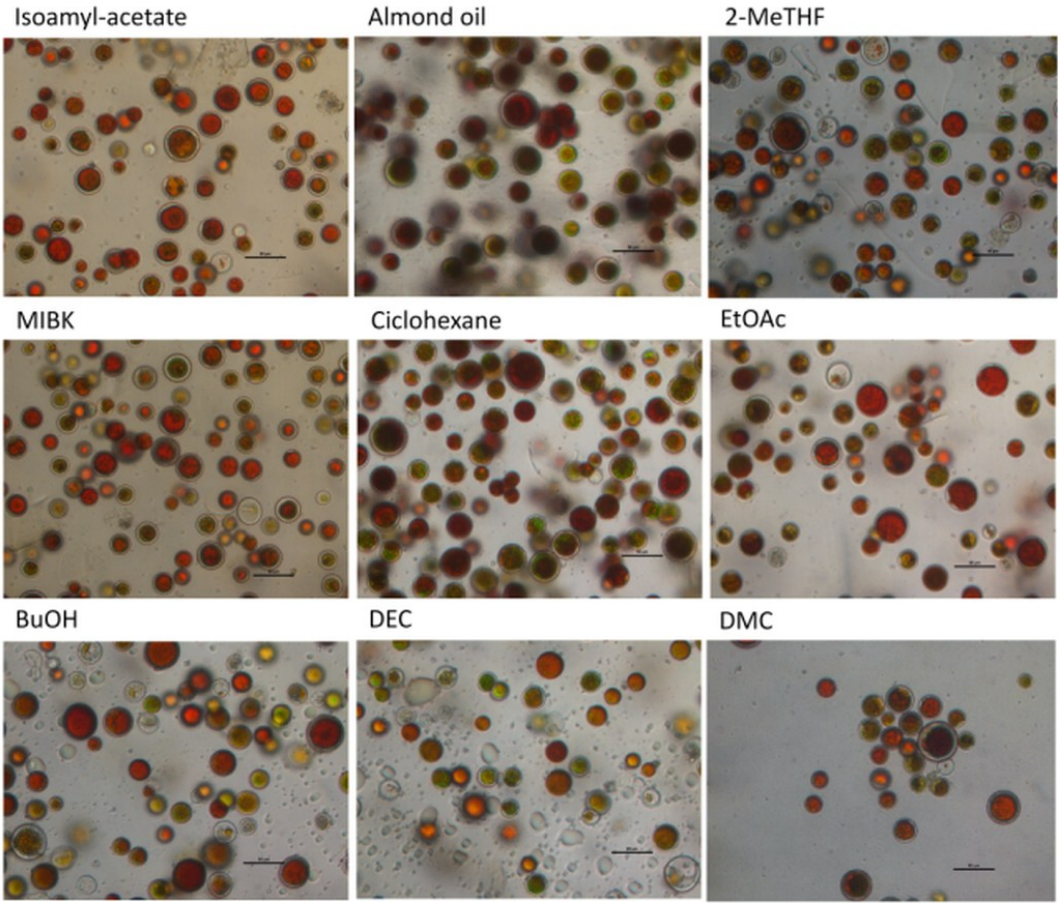

Figure 5S. Total UV-Vis spectra of the acetone extracts obtained from *H. pluvialis* in the green phase (in green) and in the red one (in red). Chlorophylls a and b were evident in the extract from the green phase with their identifying maxima absorption at 430 and 662 nm (a) and 453 and 642 nm (b).