Ultrasound-assisted green solvent extraction of high-added value compounds from microalgae *Nannochloropsis spp.*

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Abstract

The aim of this work was to investigate ultrasound (US)–assisted green solvent extraction of valuable compounds from microalgae *Nannochloropsis*. The individual green solvents (water, ethanol (EtOH), dimethyl sulfoxide (DMSO)) and binary solvents (water-DMSO and water-EtOH) were used in extraction procedures. The maximum total phenolic compounds yield, $Y_{p,}$ obtained for 15 min at W=400 W, was Y_p≈0.33 as compared with Y_p≈0.06, for the control sample. The highest yield of total chlorophylls, Y_c, which was obtained for 7.5 min at W=400 W, was Y_c≈0.043 as compared with Y_c≈0.004, for the control sample. For US-assisted extraction in water, the absence of noticeable synergy of the application of US with simultaneous increasing of temperature, was observed. The recovery efficiency decreased in the raw DMSO>EtOH>H_2O. Moreover, when the binary mixture of solvents (water-DMSO and water-EtOH) was used, the maximum recovery was observed when concentration of organic solvent C was above 25-30%, for both cases.

Keywords: *Nannochloropsis*, ultrasound-assisted extraction, phenolic compounds, chlorophylls, ethanol, dimethyl sulfoxide.

1. Introduction

Microalgae have attracted a considerable attention as they are a good source of natural food colorants, antioxidants and antimicrobials including, among others, chlorophylls, carotenoids and polyphenols (Barba et al., 2014; Dufossé et al., 2005). These compounds are enclosed in intracellular vacuoles and chloroplasts, thus complicating their recovery by conventional heat and/or solvent extraction. For this reason, over the last years, several research groups have investigated the use of non-conventional technologies in order to improve the extraction yield of these compounds while reducing the processing temperature, the solvent consumption and shortening the treatment time.

In this line, ultrasound (US)-assisted extraction has been used over the last years to recover nutritionally valuable compounds from plant food materials and algae matters (Roselló-Soto et al., 2015). This technology has the ability to disrupt cell wall based on the cavitation phenomena, thus improving extraction yield and kinetics compared to conventional techniques, with a significant reduction in the temperature, solvent consumption and extraction time. Moreover, compared to other non-conventional methods, it is a well-known technology with low capital cost and can be easily implemented in the food and pharmaceutical industries (Barba et al., 2014).

Several works published in current litterature were devoted to US-assisted extraction of lipids from microalgae (Adam et al., 2012; Araujo et al., 2013; Bermúdez Menéndez et al., 2014; Cravotto et al., 2008; Keris-Sen et al., 2014; Ma et al., 2015; Natarajan et al., 2014; Qv et al., 2014; Sun et al., 2014). For example, it was demonstrated that US-assisted extraction of oils from a cultivated marine microalgae improved the extraction yield (+20%) as compared with conventional Soxhlet extraction (Cravotto et al., 2008).

The solvent-free US-assisted extraction was also applied to extract lipids from fresh aqueous *Nannochloropsis oculata* biomass (Adam et al., 2012). It was demonstrated that US-assisted extraction resulted in a significant increase of oil extraction from *C. vulgaris* (52.5% w/w) (Araujo et al., 2013). Application of US-treatment to mixed microalgal cultures resulted in increased concentrations of protein and carbohydrates (Keris-Sen et al., 2014). The application of US also allowed 1.5-2.0-fold increase in lipid extraction yields in the presence of two different solvents, n-hexane and chloroform/methanol mixture. US-assisted extraction from green algae *Dunaliella tertiolecta* allowed to obtain an extraction yield of lipids of 45.94% under the optimum conditions of ultrasonic power of 370 W, extraction time of 5 min and liquid/solid ratio 125 ml/g (Qv et al., 2014). The advantages of US- and microwaves (MW)-assisted lipid extraction from microalgae, both in terms of efficiency and operational costs were also demonstrated (Bermúdez Menéndez et al., 2014).

US-assisted extraction of lipids from several microalgae species (*Tetraselmis suecica*, *Nannochloropsis sp*., *Chlorella sp*.) were studied under various US conditions (Natarajan et al., 2014). It was found that for *Chlorella sp.*, with rigid cell walls, lipids were released to the aqueous phase while for *T. suecica* and *Nannochloropsis sp.*, with flexible cell membranes, lipids were retained inside cells after disruption. US-treatment allowed effective extraction of high-value metabolites (polysaccharides and lipids) from microalgae *Chlorella protothecoides* (Sun et al., 2014). The combination of US and microwave (MW) treatment was applied to assist biodiesel production from microalgae *Chlorella vulgaris* (Ma et al., 2015).

Many efforts were also devoted to US-assisted extraction of different bioactive compounds such as carotenoids and chlorophylls from microalgae *Dunaliella salina* in organic solvents N,N′-dimethylformamide and methanol were studied (Macías-Sánchez

et al., 2009). The possibility of US-assisted extraction of bioactive compounds (carotenoids and fatty acids) from *C. vulgaris*, at analytical scale, has been demonstrated (Plaza et al., 2012). Important concentrations of extracted carotenoids, chlorophylls and essential fatty acids (among others) have been found in extracts. It was demonstrated that US-assisted extraction is the most economical method for the recovery of microalgal lutein (the main carotenoid) from *Chlorella vulgaris* (Deenu et al., 2013).

The mechanisms of US-induced disruption of microalgae species were also studied intensively (Gerde et al., 2012; Greenly and Tester, 2015; Halim et al., 2013; Ma et al., 2014). It was demonstrated that in the US process the microalgal cells can be ruptured by shock waves (Ma et al., 2014). US-treatment was evaluated for breaking heterotrophic (*Schizochytrium limacinum*) and autotrophic (*Chlamydomonas reinhardtii*) microalgae cells (Gerde et al., 2012). It was noted that the energy input required to reach the maximum disruption of cells was ≈800 J/10 ml irrespectively of cell concentration.

In addition, the impact of US-treatment on microalgae species with different sizes and cell wall compositions was studied (Greenly and Tester, 2015). It was demonstrated that the most significant disruption was observed in the initial period of sonication and at longer exposure times, differences between species were more pronounced. Disruption rate constant for US microalgal species (*Tetraselmis suecica* and *Chlorococcum sp.*) was directly proportional to US power and followed a parabolic relationship with initial cell concentration (Halim et al., 2013).

However, in most of the cases US-assisted extraction methods in applications to microalgae species were not comply with criteria of green chemistry concept. The efficient recovery of lipophilic compounds from microalgae commonly requires the use

of toxic solvents or intensive US-treatments that produce undesirable free radicals. At this stage of development, there is a lack of information about the US-assisted extraction based on application of green solvents.

This manuscript investigates the US-assisted solvent extraction of antioxidants and pigments (phenolic compounds and chlorophylls) from microalgae *Nannochloropsis spp*, which can be used as potential food additives and/or nutraceuticals. The individual green solvents (water, ethanol and dimethyl sulfoxide) or their mixtures were used as extraction media. The effects of US power, time of extraction, composition of the solvent and concentration of microalgae in suspension are discussed.

2. Material and methods

2.1. Chemicals

Sulfuric acid, methanolic HCl (3N), gallic acid, Folin–Ciocalteu reagent, and D-glucose were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). Bovine Serum Albumin (BSA) standard was obtained from Thermo Scientific (USA). Sodium bicarbonate (Na_2CO_3) was obtained from VWR (France). Ethanol (EtOH) and dimethyl sulfoxide (DMSO) were obtained from Baker (Deventer, The Netherlands).

2.2. Microalgae Nannochloropsis spp.

Nannochloropsis spp. is a marine green algae belonging to the *Eustigmataceae* family. The cells have approximately spherical shapes, and the mean diameter of the completely swelled cells was found to be about 2 μm. For the experiments, a frozen algae paste of *Nannochloropsis spp*. (12–15% solid content) was used. The biomass was first thawed at ambient temperature and then diluted with deionized water (electrical conductivity ≈2μS/cm), in order to prepare algae suspensions with a final concentration

(C_m) of 1, 3, 5 and 10 % wt. Other algae suspensions with a concentration (C_m) of 1 % wt were also prepared by diluting the initial biomass either in pure organic solvents, such as Ethanol (EtOH) and DMSO, or in binary mixture of water and organic solvents at different concentration.

2.3. Ultrasound (US)-assisted solvent extraction

High-added value compounds such as polyphenols and chlorophylls, from *Nannochloropsis spp*. cells were extracted using US-assisted extraction and results were compared with conventional solvent extraction (E). US-assisted extraction was carried out using UP 400S ultrasound equipment (Hielscher GmbH, Germany) able to provide a maximum power (*W)* of 400 W at a constant frequency (*f)* of 24 kHz. The sonication probe, acting as a wave amplifier, was plunged into a beaker containing 250±5 g of microalgae suspension in a proper extraction solvent as described above. The extraction time (*t)* was varied within 0–30 min, while three different power (*W)* of 100, 200 and 400 W were applied (Grimi et al., 2014). The experiments were carried out with the sample at the initial temperature of 291 K (18 °C), while the maximum final temperature was lower than 333 K (60 °C), as measured by a thermocouple placed into the algae suspension. For the sake of comparison, conventional solvent extractions (E) using pure water as solvent were carried out using the same protocol as for US treatment, but with the power being switched off (W=0 W). .

During both conventional and US-assisted extraction, the samples were subjected to continuous magnetic stirring in 300 ml hermetically closed flasks. Samples of algae suspension collected after each extraction process were centrifuged at 14000 rmp for 5 min using a MiniSpin Plus Rotor F-45-12-11 (Eppendorf, France) and the supernatant was taken for further analysis.

The yields of extraction of total phenolic compounds (Y_p) and total chlorophylls (Y_c) were defined as follows:

$$Y_p = C_p / C_p^m, \quad (1)$$

$$Y_c = C_c / C_c^m, \quad (2)$$

where C_p and C_c are the concentrations of total phenolic compounds and total chlorophylls, respectively.

The superscript *m* denotes the maximum concentration of extract obtained after application of the procedure of high-throughput homogenization (H) in 100% DMSO. Preliminary investigations have shown that this cell disintegration technique allows the extraction of almost all intracellular compounds (Koubaa et al., 2015). In this work, 20 mg of lyophilized microalgae, 1 ml of DMSO and 1 ceramic bead were mixed in screw tubes. Then, these tubes were placed to the high-throughput ball mill homogenizer (Precellys 24, Ozyme). The homogenization was carried out at 6,500 counts per min (cpm) for 3 min with 15 s pauses each minute. After homogenization the treated microalgae was washed with 1 ml of pure DMSO and centrifuged for 10 min at 14500 rpm. This procedure was repeated 8 times for reaching constant values of concentration of valuable compounds. The supernatants were used to determine the different metabolite concentrations. The maximum concentrations were C_p^m=14.9±0.8 μg/mg DW (total phenolic compounds) and C_c^m=26.34±0.9 μg/mg DW (total chlorophylls).

2.4. Chemical analysis of extracts

Absorption spectra of the extracts (supernatant) were measured by UV-spectrophotometer Libra S32 (Biochrom, Lagny-sur-Marne, France). The wavelength range was within 300–800 nm against blank (with the precision of ±1 nm). The path length of the SUPRASIL quartz cuvette was 10 mm (Hellma, Müllheim, Germany). The PeakFit program (Version 4.12, SeaSolve Software Inc.) was used for the spectral shape

194 analysis of the UV absorption bands and for their graphical deconvolution. The autofit
195 baseline option was used to remove the baseline prior to deconvolution of peaks, their
196 fitting and estimation of their intensity.

197 *2.4.1. Total phenolic compounds*

198 Concentration of total phenolic compounds, C_p, (in μg of gallic acid equivalent/mg
199 DW of biomass) was determined by the Folin–Ciocalteu method based on colorimetric
200 oxidation/reduction reaction of phenols (Singleton et al., 1999). First, 0.2 ml of extract
201 and 1 ml of Folin–Ciocalteu reagent (diluted 1:10 in water) were mixed. Afterwards, 0.8
202 ml of Na_2CO_3 (75 g/l) was added to this mixture. The sample was incubated for 10 min
203 at 323 K, followed by cooling to room temperature (T=293 K). The absorbance was
204 then measured at 750 nm. Gallic acid was used for the calibration.

205 *2.4.2. Total chlorophylls*

206 For pigments quantification, the maximum absorbancies of chlorophyll-a (A^a_c) and
207 chlorophyll-b (A^b_c) were measured at 665 and 653 nm, respectively (Kumar et al.,
208 2010). The concentrations of total chlorophylls (C_c, in mg of pigment/g DW) in the
209 extracts were calculated according to the following equations (Arnon, 1949;
210 Lichtethaler and Wellburn, 1983):

211 For EtOH extract:

212 $$C_c = 4.34\, A^a_c + 19.71\, A^b_c \quad (3)$$

213 For DMSO extract:

214 $$C_c = 0.0202\, A_c^a + 0.00802\, A_c^b \quad (4)$$

215

2.5. Statistical Analysis

All experiments and analyses were repeated using at least five replicates. One-way analysis of variance was used for statistical analysis of the data using Statgraphics plus software (version 5.1, Statpoint Technologies Inc., Warrenton, VA). For each analysis, significance level of 5% was assumed. The error bars presented in the figures correspond to the standard deviations.

3. Results and discussion

Figure 1 shows the yield of total phenolic compounds, Y_p, versus extraction time t for the extracts obtained by US-assisted and conventional extraction (E) in water. The ultrasound power W was set at 400 W while the concentration of microalgae suspensions was 1%. The initial temperature of the biomass was $T \approx 18$ °C. During the US-assisted extraction the temperature T of the sample increased owing to the input of the ultrasound energy. After ≈20 min of extraction the temperature stabilized at the level ≈68 °C that corresponded to the equilibrium for the energy exchange between the suspension and its surrounding medium. This experiment was designated as US ($T\uparrow$).

For comparison purposes, the conventional extraction in water was carried out under non-isothermal conditions using the same protocol of temperature increase as for the US-assisted extraction experiment. This hot water extraction experiment was designated as E ($T\uparrow$). At the same time, US-assisted extraction at constant temperature T=18 °C was also carried out by placing the sample in a thermostatic bath.. This “cold” water US-extraction experiment was designated as US (T=18 °C).

Results of conventional extraction show that the $Y_p(t)$ curves were saturated after ≈20-30 min of extraction. The extraction yields for US ($T\uparrow$)-assisted method is ≈2 times

239 higher than those for conventional hot water extraction E ($T\uparrow$). However, it is worth
240 noting that the sum of extraction yields of US (T=18 °C) and E ($T\uparrow$) samples were
241 approximately the same as for those of US ($T\uparrow$) treated samples. Thus, the combination
242 of US and temperature increase gave approximately an additive contribution into
243 extraction efficiency. This reflects the absence of any synergy between the application
244 of US with simultaneous increasing of temperature.

245 Figure 2 presents the effects of US power (W=0-400 W) on the kinetics of extraction
246 of total phenolic compounds, Y_p, (Figure 2a) and total chlorophylls, Y_c, (Figure 2b) in
247 water. The values of Y_p and Y_c increased proportionally to the US power and increased
248 with the elapse of treatment time. The maximum total phenolic compounds yield was
249 obtained when using the highest power and the longer treatment time. E.g., extraction
250 for 15 min at W=400 W resulted in $Y_p \approx 0.33$ as compared with $Y_p \approx 0.06$ for the control
251 sample (conventional "cold" water extraction, W=0 W).

252 Results of Figure 2b, instead, show that the highest yield of total chlorophylls was
253 obtained after applying a US power of 400 W for 7.5 min, while longer treatment times
254 led to a decrease in chlorophyll's content. On the other hand, when US-assisted
255 extraction was carried out at the lower power values (100 and 200 W), the extraction
256 yield of chlorophyll showed an increasing trend with time. This different behaviour is
257 likely attributed to the higher increase of temperature measured in the medium when US
258 was applied at the higher power and during long treatment times, thus promoting the
259 degradation of some thermolabile pigments (Chemat et al., 2011). In fact, it is well
260 known that microalgae pigments are highly susceptible to thermal degradation which
261 results in colour changes (Pasquet et al., 2011). Moreover, for long sonication times the
262 produced free radicals can also induce degradation of extracted products (Gerde et al.,
263 2012).

Finally, in comparison with control sample (W=0 W, T=18 °C), our findings clear show that US treatments allowed a significant increase in the chlorophyll recovery. These results were in close agreement with previously reported data (Kong et al., 2012), which showed a significant yield increase (+59%) for US-assisted extraction (200 W/78.7 min/61.4 ºC) of chlorophyll from *Chlorella vulgaris* as compared with conventional extraction process. The increased recovery of chlorophyll a and b for US-assisted extraction as compared to those obtained using conventional maceration and Soxhlet extraction was also found (Kwang et al., 2010). US-assisted extraction was also found to show higher extraction efficiency as compared with either conventional or other innovative technique such as supercritical fluid extraction and microwave assisted extraction, when applied to *Dunaliella salina*, *Dunaliella tertiolecta*, and *Cylindrotheca closterium* (Macías-Sánchez et al., 2009; Pasquet et al., 2011).

Figure 3 compares results of the extraction yields of total phenolic compounds, Y_p, (Figure 3a) and total chlorophylls, Y_c, (Figure 3b) obtained during US-assisted extraction in pure solvents (H_2O, EtOH, DMSO) at a fixed US power of 400 W. For total phenolic compounds, regardless the extraction solvent, the yield Y_p increased with time reaching the saturation after about 5 min of extraction. For total chlorophylls, the kinetic of extraction followed a different path depending on the extraction solvent. While in EtOH and water, the extraction yield Y_c reached a plateau after 7.5 min of US-assisted extraction, in DMSO Y_c reached a plateau after 5 min of extraction and decreased when longer extraction times (>7.5 min) were used. Thus, to prevent chlorophyll degradation an extraction time of 5 min was selected as optimum value for further experiments. Results of Figure 3 also show that at each fixed extraction time, the extraction efficiency of total phenolic compounds and total chlorophylls decreased in the order DMSO>EtOH>H_2O. The efficiency of US-assisted extraction in binary

289 solvents (water-DMSO and water-EtOH) was also studied. This is because, although
290 results of Figures 1-3 clearly show that US-assisted extraction was rather efficient for
291 the recovery of total phenolic compounds even in pure water, the use of organic solvent
292 was required for a more effective recovery of chlorophylls. Figure 4 presents the yields
293 of total phenolic compounds, Y_p, and total chlorophylls, Y_c, versus concentration (C) of
294 organic solvent (DMSO or EtOH), for US-assisted extraction in binary solvents
295 H_2O+DMSO (Figure 4a) and H_2O+EtOH (Figure 4b). Results show that a significant
296 increase in the amount of total phenolic compounds and chlorophylls was observed both
297 in DMSO and EtOH when solvent's concentration *C* was above 25-30%.

298 Moreover, the efficiency of US-assisted extraction versus concentration of
299 microalgae in suspension, C_m, was also investigated. Figure 5 presents of the extraction
300 yields of total phenolic compounds, Y_p, and total chlorophylls, Y_c, versus concentration
301 (C_m) of microalgae in suspension for US-assisted extraction in binary solvents
302 H_2O+EtOH(*C*=50% wt). In the same graph also the energy input per kg of microalgae
303 (DW) has been reported. From the results it can be seen that both yields Y_p and Y_c
304 follow a similar trend showing a minimum value for a biomass concentration $C_m \approx 5\%$
305 wt. This behaviour is surprising and we have no reasonable explanation of this
306 phenomenon. However, it is likely that the concentration dependence can reflect the
307 changes in the efficiency of cell damage by US waves related with the intensity of
308 cavitating gas bubbles. The mechanisms of interaction of US with microalgae cells, of
309 cell destruction and cell precipitation were recently discussed (Faerman et al., 2002). It
310 was demonstrated that US could precipitate the cells in highly concentrated
311 suspensions. The phenomenon of precipitation was explained by dissolution of micro-
312 bubble carbon dioxide and removal of gas bubbles.

313 Finally, it should be noted that US-assisted recovery from concentrated suspension is
314 less power consuming. E.g., the increase of concentration C_m from 1% wt to 10% wt
315 resulted in ≈10-fold decrease of US power consumption at approximately the same
316 efficiency of extraction of total phenolic compounds and total chlorophylls.

317 **4. Conclusions**

318 Ultrasound-assisted solvent extraction was shown as a promising tool to recover
319 high-added value compounds from microalgae *Nannochloropsis.* The extraction yields
320 for US-assisted method was ≈2 times higher than for conventional hot water extraction.
321 Degradation of chlorophylls was observed at long treatment time. The 5 min duration of
322 US-assisted extraction was selected as optimal to prevent degradation of chlorophylls.
323 The recovery efficiency of phenolic compounds and chlorophylls decreased in the raw
324 DMSO>EtOH>H_2O. In addition, when binary solvents (water-DMSO and water-EtOH)
325 were used, the highest recovery of valuable compounds was observed when
326 concentration of organic component was above 25-30% for both solvents.

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Figure captions:

Fig. 1. Yields of total phenolic compounds, Y_p, versus extraction time, t, for extracts obtained by US-assisted and conventional extraction (E) in water. The ultrasound power was W=400 W; the concentration of microalgae suspensions was C_m=1% wt. The data are presented for non-isothermal protocol ($T\uparrow$) and isothermal protocol (T=18 °C). The symbol T in the legend refers to the evolution of temperature during either US ($T\uparrow$)-assisted extraction or conventional extraction E($T\uparrow$). Dashed line corresponds to the sum of the extraction yields obtained after US (T=18 °C) and E($T\uparrow$).

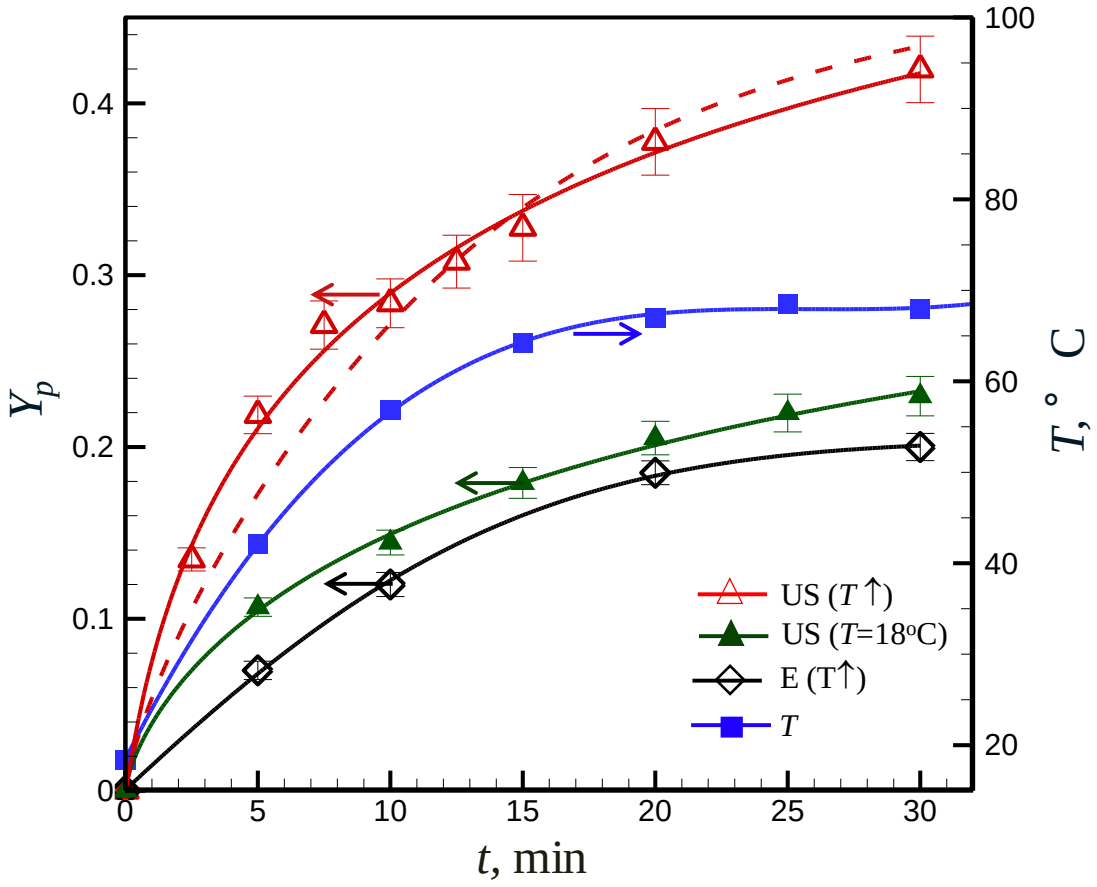
Fig. 2. (a) Yield of total phenolic compounds, Y_p, and (b) total chlorophylls, Y_c, versus time of US-assisted extraction in water at different applied ultrasound powers (0-400 W). C_m=1% wt. "Cold" water extraction for control sample (W=0 W) was done at the fixed temperature, T=18°C.

Fig. 3. (a) Yields of total phenolic compounds, Y_p, and (b) total chlorophylls, Y_c, versus time of US-assisted extraction in individual solvents (H_2O, EtOH, DMSO). The ultrasound power was W=400 W; the concentration of microalgae suspensions was C_m=1% wt; the initial temperature was 18°C.

Fig. 4. Yields of total phenolic compounds, Y_p, and total chlorophylls, Y_c, versus concentration of organic solvent, C, for US-assisted extraction in binary solvents H_2O+DMSO (a) and H_2O+EtOH (b). The ultrasound power was W=400 W; the concentration of microalgae suspensions was C_m=1% wt; the initial temperature was 18°C; and the time of extraction was t=5 min.

Fig. 5. Yields of total phenolic compounds, Y_p, and total chlorophylls, Y_c, versus concentration of microalgae in suspension, C_m, for US-assisted extraction in binary solvents H_2O+EtOH (C=50% wt). The ultrasound power was W=400 W; the initial temperature was 18°C; and the time of extraction was t= 5 min. The upper horizontal axis presents the energy input per kg of microalgae (DW).

465 Figure 1



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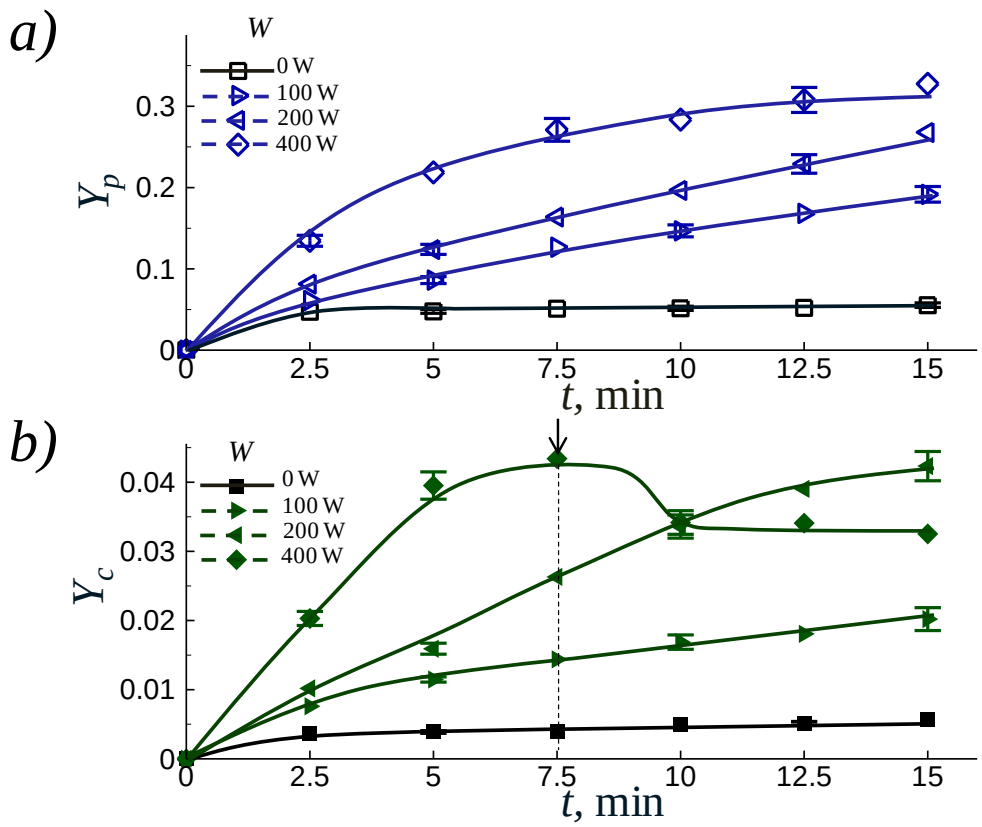
468 Figure 2



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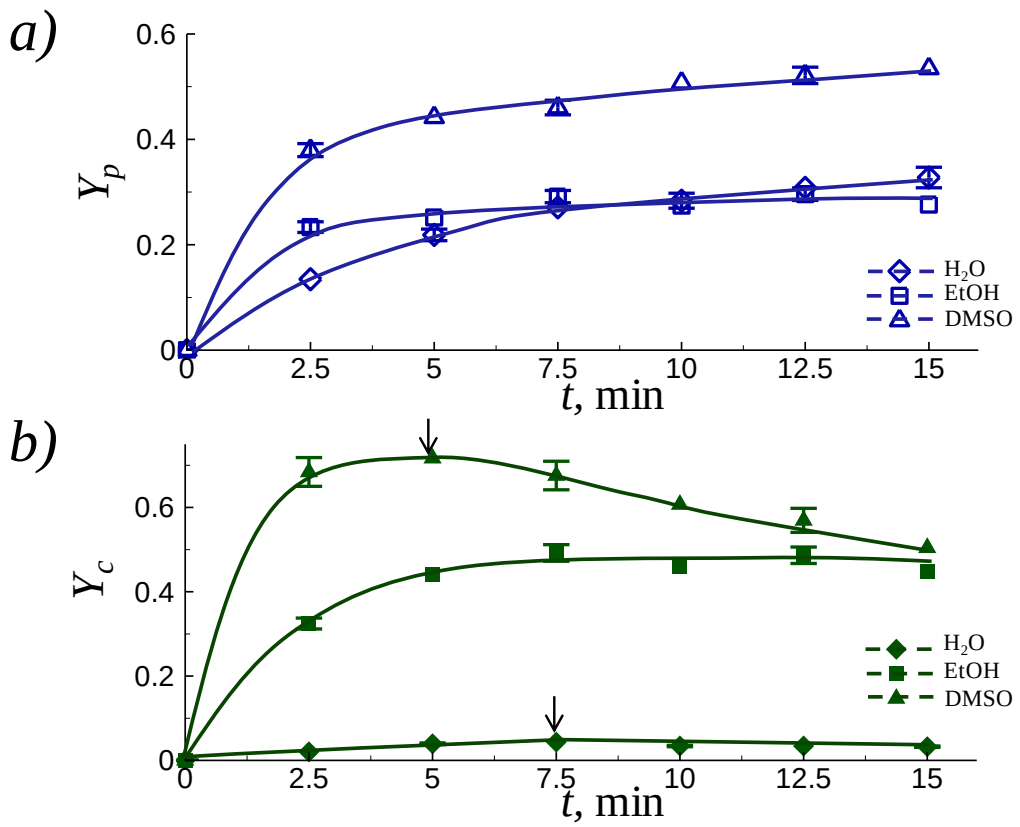

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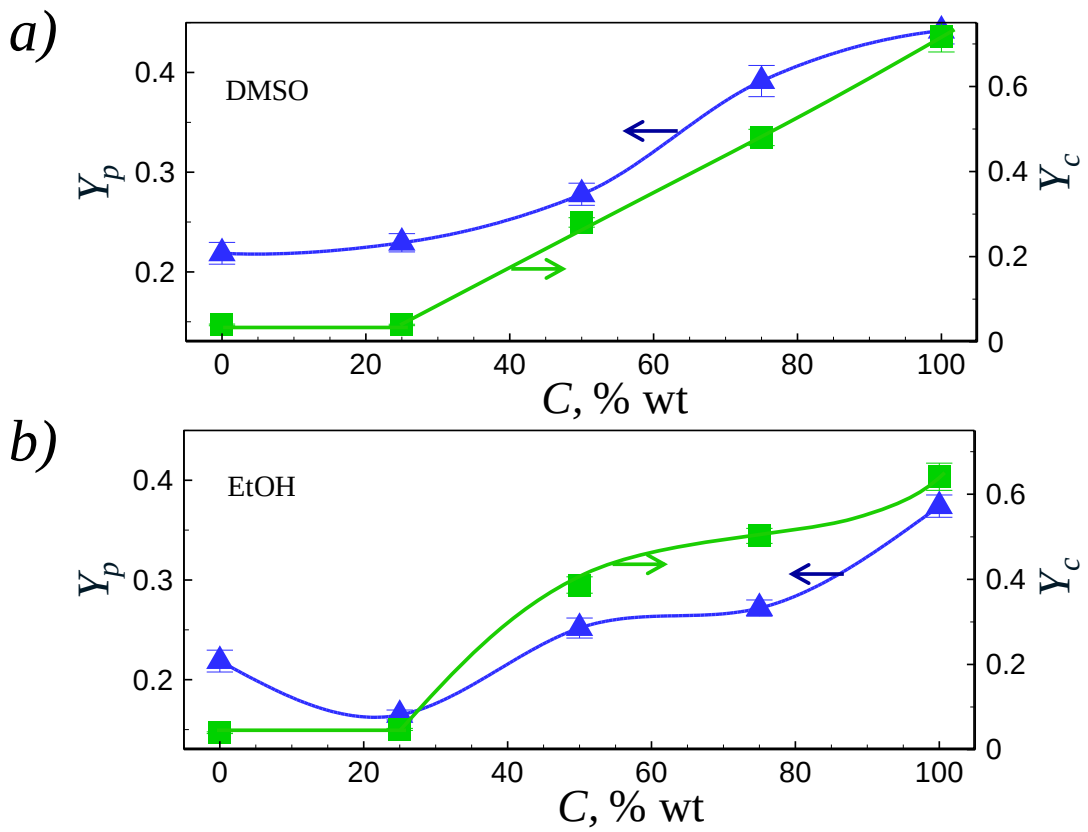
474 Figure 4



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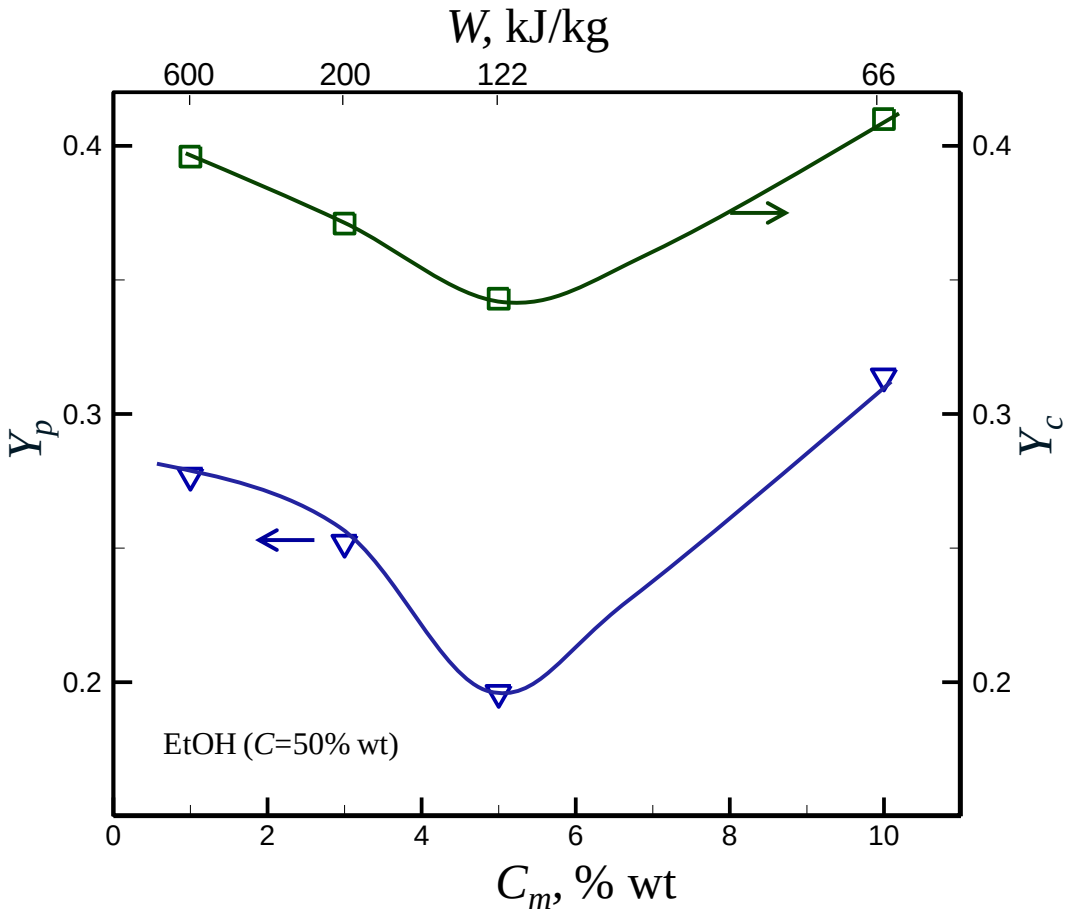
477 Figure 5



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