Maximizing carotenoid extraction from microalgae used as food additives and determined by liquid chromatography (HPLC)

Cerón- García, M.C.*, González-López C.V., Camacho-Rodríguez J., López-Rosales L., García-Camacho F., Molina-Grima E.

Chemical Engineering Area, Department of Engineering, University of Almería, E04120, Almería, Spain.

*Corresponding author: mcceron@ual.es

Address: Chemical Engineering Area, Department of Engineering, University of Almería. Carretera Sacramento s/n. E04120, Almería, Spain.

Telephone number: +34 950015981 Fax: +34 950 015484; e-mail address: mcceron@ual.es

Abstract

Microalgae are an interesting source of natural pigments that have valuable applications. However, further research is necessary to develop processes that allow us to achieve high levels of carotenoid recovery while avoiding degradation. This work presents a comprehensive study on the recovery of carotenoids from several microalgae genera, optimizing carotenoid extraction using alkaline saponification at various temperatures and KOH concentrations. Results show that *I. galbana* requires a temperature of 60ºC and less than 10% KOH, *N. gaditana* and *K. veneficum* require 60ºC and no saponification, *P. reticulatum* requires 40ºC and 10% KOH, *T. suecica* and *H. pluvialis* require 25ºC and 40% KOH while *C. sp.* and *S. almeriensis* require 80ºC and 40% KOH. The influence of the solvent on carotenoid recovery was also studied. In general terms, an ethanol:hexane:water (17:77:6 v/v/v) mixture results in good yields.

Keywords

microalgae genus; extraction, saponification; temperature; solvent; polarity

1. Introduction

Interest in functional foods has been growing over recent years. A food ingredient is functional if it can be shown to improve health or reduce the risk of disease. Amongst these additives, carotenoids have been highlighted as valuable compounds due to their antioxidant capacity, which offers protection against oxidative stress (Guedes et al., 2011). They have also been shown to exhibit immunomodulation and anti-inflammatory activity, to be antimicrobial and antiviral, as well as to prevent degenerative diseases, such as cardiovascular disease, diabetes and some types of cancer (Bernal et al., 2011;

Christaki *et al*., 2013; Buono et al, 2014). The global market for carotenoids was valued at $1.5 billion in 2014, and expected to rise beyond $1.8 billion in 2019 (Business Communications Company, 2015). Natural carotenoids are preferred to synthetic compounds because the former are a mixture of *trans* and *cis* isomers that exhibit anticancer activity, while the synthetic forms are usually all-*trans* isomers. Natural carotenoid accumulation of the algal beta-carotene isomer mixture has been shown to be tenfold higher than that of the synthetic compound all-trans-beta-carotene (Ben-Amotz et al., 1989).

Of the range of natural carotenoid sources, microalgae are those with the greatest potential (Fernández-Sevilla et al., 2010). Although several methods for carotenoid extraction and recovery have been tested (Saini and Keum, 2018), such as electrical treatment and supercritical fluid extraction, only solvent extraction seems to achieve sufficient levels of efficiency and purity to be considered for scaled-up processes (Fernández-Sevilla et al., 2010). Studies dealing with the solubility of carotenoids in organic solvents, such as acetone, petroleum ether, hexane, diethyl ether, ethanol, methanol and dichloromethane, have been carried out to gauge their ability for extracting carotenoids from microalgae cells. The use of dichloromethane, however, has been restricted due to its carcinogenic nature, volatility and corrosive power. Other non-halogenated and less toxic solvents have been proposed as alternatives. The use of polar alcohols in non-polar solvents has demonstrated efficient extraction of the desired compounds (Balasubramanian et al., 2013, Ryckebosch et al., 2012, 2013). Some of the mixtures studied such as hexane/methanol (3: 2), hexane/isopropanol (3: 2) and cyclohexane/1-butanol (9: 1) are candidates for being the best of the non-halogenated solvents, along with the tricomponent solution of hexane:ethanol:water (17: 77: 6). The

most suitable options are the use of hexane, acetone and ethanol given that their use in food processing is already accepted (Vergari et al., 2010; Minguez-Mosquera et al., 1992; Fernández-Sevilla et al., 2009). Hexane is normally used for the solvent extraction of carotenoids from algal biomass to meet commercial specifications for large-scale production (Cerón *et al*., 2008; Fernández-Sevilla *et al*., 2010). However, with hexane more than eight extraction stages are necessary, which wastes large quantities of solvent. Moreover, it is not capable of recovering all types of polar carotenoids, making it necessary to use a mixture of solvents with a different polarity. With regard to the analytical methods for carotenoid identification and quantification, HPLC analysis is the one most readily applied (Bernal et al., 2011). The aim of this work was to optimize the extraction of microalgal carotenoids to establish an easy and reliable method to analyse them by HPLC. To do this, after disrupting the cells, saponification was performed at several working temperatures and KOH concentrations. The optimized conditions are shown for each microalga and for each pigment. In addition, a step was carried out to recover and purify the carotenoids – this was done by using several solvents with different polarities in the extraction process.

2. Methods

2.1. Microalgae biomass and pretreatment

Biomass from various microalgae strains were used to perform an extensive study for each genus. Biomass from *Nannochloropsis gaditana, Chlorella sp., Haematococcus pluvialis, Scenedesmus almeriensis, Isochrysis galbana, Tetraselmis suecica* and *Karlodinium veneficum* were obtained from pilot-plant outdoor cultures, whereas *Protoceratium reticulatum* was obtained from indoor cultures; all of them were

provided by the UAL Marine microalgae biotechnology group. The group's facilities are designed to operate using seawater or fresh water in a closed circuit with recirculation of the culture medium. The cultures were cultivated at pH 8.0 by on-demand CO_2 injection and the temperature was kept at 30°C by passing water through a heat exchanger located inside the reactor. The biomass was harvested daily by centrifugation and immediately frozen, after which it was lyophilized and stored at −22°C ready for use as a raw material.

The freeze-dried biomass was milled in a mortar with alumina 1:1 w/w for 5 min just prior to saponification, as described by Cerón *et al*. (2008). Each test was carried out with 10 mg of total sample, containing 5 mg of dry biomass. In previous tests (data not shown) it was checked whether some microalgae were degraded as a result of milling; *Isochrysis galbana,* for example, which contains a significant amount of easily-degradable xanthophylls. All the tests were performed under an N_2 atmosphere and in darkness.

2.2. Biomass saponification

Saponification was performed in glass Pyrex tubes submerged in a water bath (Julabo SW22), which provided the required temperature and mixing. Firstly, 5 mg of dry biomass was placed in each tube. Then, 1 ml of monophasic tricomponent solution was added and shaken in the vortex for 30 sec. The tricomponent solution was composed of ethanol:hexane:water in a proportion of 17:77:6 v/v/v as described by Fernández-Sevilla et al. (2009) and contained 0-60% d.w. potassium hydroxide ((g KOH/g dry biomass)x100). At this point, the tube was submerged in the water bath with a preset temperature of between 25 and 80ºC, where it was left for 5 min. After this, the tube was taken out, vortexed again for 30 sec and left to cool for 1 h at room temperature.

Subsequently, it was centrifuged at 12000 rpm for 2 min (Mini Spin Plus, Eppendorf) and the supernatant was transferred into a vial ready to be analysed by HPLC.

2.3. Carotenoid recovery

A step was performed in order to purify the carotenoid extract. The supernatant obtained after saponification (at 25ºC and 0-60% d.w. KOH) and centrifugation was dried by a N_2 flow inside the tube. Once dried, 1 ml of a solvent was added and the sample was vortexed for 2 min. Then, the carotenoids were again analysed by HPLC. The solvents tested had different polarities allowing better extraction of more polar or more apolar carotenoids depending on their polarities. Furthermore, these solvents were amongst those listed as extraction solvents permitted for use in Europe (Directive 2009/32/CE and Directive 2010/59/UE) during the processing of raw materials, foodstuffs, food components or food ingredients. The solvents were: methanol, acetone:water (92.5:7.5 v/v), acetone:water (95:5 v/v), acetone:water (97.5:2.5 v/v), ethanol:water (96:4 v/v), absolute ethanol, acetone, monophase tricomponent solution (ethanol:hexane:water, 77:17:6 v/v/v), hexane:ethanol (50:50 v/v), hexane:ethanol (70:30 v/v), diethyl ether and hexane. The above-mentioned directives include usage in compliance with good manufacturing practice for all uses of propane, butane, ethyl acetate, ethanol, carbon dioxide, acetone (in the refining of olive-pomace oil) and nitrous oxide. They also include the use of the following solvents under specified conditions of use (for some kinds of processes and/or with maximum residue limits): hexane, methyl acetate, ethylmethylketone, dichloromethane, methanol, propan-2-ol, diethyl ether, cyclohexane, methyl acetate, butan-1-ol, butan-2-ol, propan-1-ol and 1,1,1,2-tetrafluoroethane. Therefore, when using one of these solvents, it is important to be careful not to contaminate the product.

134 *2.4. Liquid chromatography method*

135 The carotenoids were analysed by HPLC (Shimadzu SPDM10AV High Liquid
136 Performance Chromatograph) using a photodiode array detector applying the method
137 described by Mínguez-Mosquera *et al*. (1992) with the modifications proposed by Del
138 Campo *et al*. (2000) along with final modifications by Cerón *et al*. 2007. Although the
139 method followed was that proposed by Cerón et al. 2007, a different column was
140 employed - the LiChrospher© 100 RP-18 (5-µm) column (4.6×150 mm) - in which the
141 separation was performed. The injection volume of each sample was 20 µl. Two eluents
142 were used: (A) water:methanol 1:4 v/v and (B) acetone:methanol 1:1 v/v. The gradient
143 of the mobile phases was 25% B 0-8 min, 75% B 8-18 min, 90% B 18-23 min, 100% B
144 25-27 min and 25% B 27-32 min. Carotenoids were eluted at a rate of 1 ml/min and
145 detected by absorbance at 360-700 nm; to be precise, at 440, 450 and 475 nm.
146 Calibration lines were constructed (Table 1) with different carotenoid concentrations;
147 depending on the carotenoid, each has a different absorbance maximum - for example,
148 lutein at 450 nm or peridinin at 475 nm. Standards of neoxanthin, lutein, fucoxanthin
149 and β-carotene were provided by Sigma Chemical Co. (USA) while peridinin,
150 violaxanthin, zeaxanthin, vaucheriaxanthin, diatoxanthin, diadinoxanthin, gyroxanthin
151 ester and dinoxanthin standards were purchased from DHI Lab Products (Hørsholm,
152 Denmark). Vaucheriaxanthin esther and hex-fucoxanthin were calibrated relative to the
153 vacheriaxanthin and fucoxanthin curves (comparing the molar extinction coefficient of
154 both), respectively. Each standard solution was prepared in duplicate, and each sample
155 was analysed in duplicate. In this way, the average values correspond to four
156 experimental measurements.

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158 *2.5. Statistical analyses*

Statistical data analyses were performed using the Statgraphics Centurion XVI software package. Data, in percentage, were arcsin(x1/2) transformed. The normality and homogeneity analyses were performed using the Kolmogorov–Smirnov and Levene tests, respectively. Multifactor ANOVA tests were used to study the effect of the factors (temperature and potassium hydroxide) and their interactions (temperature-potassium hydroxide) at a 95% confidence level for the total carotenoid content with the aim of deciding the most influential factor in the carotenoid composition.

3. Results and discussion

3.1. Optimization of biomass saponification

Carotenoids are contained inside the microalgal cells. Therefore, it is necessary to develop a process that improves cell-wall disruption and facilitates their extraction. Carotenoid extraction might be up to 10 times greater when effective cell wall disruption is performed (Michelon *et al*., 2012; Uquiche *et al*., 2016). Furthermore, carotenes are in free form whereas xanthophylls are usually joined with fatty acids as mono- or di-esters (Mercadante *et al*., 2016), so saponification is required to separate them out (and the fatty acids are thus converted into their basic salts or soaps). Saponification has been widely used to enhance carotenoid extraction as it allows the removal of lipids and the destruction of chlorophylls; however, at high temperature, KOH concentration and contact time, it can cause carotenoid degradation (Inbaraj *et al.*, 2008). Saponification also helps to solubilize large quantities of proteins and carbohydrates, which would otherwise interfere with extraction (Clark, 2011). Moreover, saponification will hydrolyse carotenoid esters and is therefore to be avoided when attempting to determine esterified carotenes such as the astaxanthin mono- and di-esters contained in *Haematococcus pluvialis*. Rodríguez-Bernaldo de Quirós and Costa

(2006) determined carotenoids in tropical leafy vegetables and recommended that samples with a low fat content should be saponified under gentler conditions and high fat samples under stronger conditions.

In this work, the saponification of the previously milled microalgal biomass has been studied at several temperatures and KOH concentrations (6x4 levels) and the data are presented in surface response (3D) (Figure 1). In order to simplify the analysis of the data, we compiled Figure 2 using ANOVA; for each temperature, the value presented is the average total carotenoid concentration obtained at all the different KOH concentrations tested (0, 5, 10, 20, 40 and 60%); and for each KOH concentration, the value is the average total carotenoid concentration obtained at all the different temperatures tested (25, 40, 60 and 80ºC). Figure 2 shows the effect of temperature (T) and KOH (K) on the loss of total carotenoid content whereas Table 2 provides a statistical analysis of the above-mentioned factors in order to study both factors and their interaction on total carotenoids. With regard to Table 2, temperature and KOH are important factors affecting carotenoid content, but no significant differences were observed regardless of the microalgae species used. Depending on the strain, the contribution of both factors was different; or in any case, the effect of the temperature-KOH (T-KOH) interaction was the most important (Table 2). Both temperature and KOH led to significant differences as the p-value was below 0.05 in all cases. Higher F-ratios mean a higher contribution from this factor. Thus, the effect of temperature was higher for *T. suecica*, *C. sp.*, *N.gaditana*, *P. reticulatum* and *S. almeriensis*; although for the latter, the KOH concentration effect was also high. Nonetheless, the effect of KOH concentration was higher for *I. galbana*, *H. pluvialis* and *K. veneficum*.

The presented data allow us to select the most appropriate temperature and KOH concentration to use in the saponification step for each microalga in terms of total carotenoid extraction. It is important to select the temperature that provides the highest extraction (the higher the temperature, the lower the solvent viscosity, hence its diffusion into the sample is made easier) and avoids damage to the thermolabile carotenoids, which may occur at high temperatures (Saini and Keum, 2018). *I galbana, N. gaditana* and *K. veneficum* needed a temperature of 60ºC and KOH concentrations below 10% d.w. for *I. galbana* and no saponification for the others due to carotenoid degradation under stronger conditions. The same KOH behaviour was observed for *P. reticulatum* although a temperature of 40ºC was sufficient to achieve higher carotenoid extraction. Carotenoid extraction from *T. suecica* and *H. pluvialis* hardly changed with temperature so 25ºC was the temperature selected as it involved lower energy consumption. Regarding KOH concentration, the best values were obtained at 10 and 40% d.w. for *T. suecica* and *H. pluvialis*, respectively; although for *T. suecica,* there were significant differences at the different KOH concentrations tested. The contents generally decreased by up to 20% even when different maximal values (Figure 1b) were observed; this was due to interactions between temperature and %KOH, obtaining similar values at 60ºC with 0 % or 20% KOH, and 25 ºC with 10 % KOH. The high value obtained for *H. pluvialis* was due to the extraction of an amount of carotenoid that had not been extracted under the other conditions tested; *H. pluvialis* contains mono- and di-esters that require potash consumption to quantify them. Lastly, the microalgae *C. sp.* and *S. almeriensis* have stronger cell walls so they required a temperature of 80ºC to achieve maximal carotenoid extraction. For *S. almeriensis*, 20% d.w. KOH was sufficient whereas for *C. sp.*, no statistically significant differences were found. Other

230 authors have carried out carotenoid extraction using a variety of organic solvents.
231 Working with pink shrimp, Mezzomo *et al*., 2011, performed maceration in acetone,
232 Soxhlet extraction with hexane/isopropanol as well as ultrasound-assisted extraction.
233 They concluded that maceration was the best method because the absence of heating
234 avoided carotenoid degradation. On the other hand, when Soxhlet extraction was used,
235 the low viscosity and surface tension at the boiling point temperature improved the
236 diffusion and solubilization of the carotenoids. However, in our study, we confirmed
237 that at such high temperatures, xanthophyll degradation generally occurred. Poojary and
238 Passamonti (2015) obtained a 94.7% recovery of the carotene lycopene at 20°C for 40
239 min with 40 ml/g of acetone/hexane (1:3 v/v). Heffernan *et al*. (2016) obtained the
240 highest xanthophyll extraction from the macroalgae *Fucus serratus* using
241 hexane/acetone 7:3 at 50°C for 24 h. When hot saponification is carried out, the high
242 temperature can cause isomerization and degradation of the carotenoids. Our study
243 shows a decrease in carotenoid extraction with temperature (Figure 2) for *T. suecica* and
244 *P. reticulatum.* In general, the use of a gentler temperature was favourable in most
245 cases.
246 These results present only the total carotenoid values; nonetheless, the study was
247 performed in a way that accounted for each of the carotenoids analysed (data not
248 shown). This is because the data were used to select the most appropriate temperature
249 for each microalga. Subsequently, the most appropriate KOH concentration was
250 selected taking into account each of the extracted carotenoids. Figure 3 shows the
251 extraction of carotenoids at the temperature selected for the KOH concentration of each
252 microalga. To achieve the maximum total carotenoid extraction, *I. galbana* required a
253 KOH concentration below 10% d.w. This was due to the fucoxanthin content as this

pigment is degraded at higher KOH concentrations. However, it should be noted that when KOH concentrations below 40% d.w. were used, chlorophylls were present in the samples; this might interfere with chromatographic analysis by overlapping with the most polar xanthophylls, such as neoxanthin or peridinin. Similar behaviour was observed for *N. gaditana*, *K. veneficum* and *P. reticulatum* because of their vaucheriaxanthin and peridinin contents, respectively. *T. suecica* was hardly influenced by KOH concentration at the selected temperature, so 10% d.w. KOH was the best concentration to choose as it avoided the presence of chlorophylls. The same occurred for *C. sp.* given that carotenoid extraction does not significantly increase at higher KOH concentrations for the selected temperature. In the case of *H. pluvialis*, astaxathin was only detected in tests carried out at 40% d.w. KOH. Likewise, *S. almeriensis* demonstrated slightly better carotenoid extraction at 20% d.w. KOH but 40% d.w. also produced good extraction so this was preferred as it avoided the presence of chlorophylls. The conditions selected for each microalga are summarized in Table 3. These data (Figure 3) can be analysed for each carotenoid; in this way, the KOH concentration providing the maximum extraction for each carotenoid is determined (Table 4). When there were no statistically significant differences between different KOH concentration values, we selected the one that allowed other pigments to be extracted at the same time from the particular microalgae. The data show that for many of the pigments, a KOH concentration of 40% d.w. was the best alternative as it provided greater extraction and avoided the presence of chlorophylls. This is in accordance with similar procedures such as that of Cerón *et al.* (2008) and Granado *et al.* (2001). However, pigments such as fucoxanthin, vaucheriaxanthin (and ester), peridinin and gyroxanthin require low KOH concentrations to avoid degradation. This

means that when a microalga contains one of these pigments, the carotenoid analysis has to be performed twice: one at a low KOH concentration to determine the content of these easily degradable xanthophylls and the other under stronger conditions to determine the rest of the pigments (while avoiding chlorophyll interference) thus achieving the best extraction results.

3.2. Optimization of carotenoid recovery

Having optimized the saponification step, the recovery of the carotenoids was studied. Carotenoids are complex molecules of different polarities, from xanthophylls (containing oxygen as a functional group) to carotenes (containing only a hydrocarbon chain with no functional group) (Guedes *et al*., 2011). Once they have been extracted from inside the microalga, they can be recovered using different solvents. Carotenoids that are more polar, such as neoxanthin, are better solvated using polar solvents, such as acetone, whereas more apolar carotenoids, such as β-carotene, are better solvated in apolar solvents, such as hexane. Amorim-Carrilho *et al.* (2014) claimed that hexane, acetone and ethanol/hexane (4:3) are the most used solvents for plant-derived samples. The selection of the appropriate solvent (or solvent mixture) is difficult because many factors affect the extraction: the carotenoid's polarity and its chain length, the sample matrix, and the moisture content (Saini and Keum, 2018). In this work, carotenoid recovery optimization was performed with different microalgae to check the behaviour of the main carotenoids analysed. Figure 4 shows the carotenoid recovery for each of the tested solvents, ordered by their polarity calculating the P-index (Molina *et al*., 2013), which were amongst those listed as extraction solvents permitted for use in the production of food ingredients in Europe (Directive 2009/32/CE and Directive

2010/59/UE). The study found that using the tricomponent solution allows one to achieve good results for all the microalgae and carotenoids; this is because it is composed of a water, ethanol and hexane mixture. If only one solvent can be chosen, tricomponent solution would be the most appropriate. However, if the aim is to isolate only one, or only some carotenoids, other solvents would provide better results. In this case, the microalga strain should be taken into account and a more polar or apolar solvent should be chosen depending on the particular carotenoid to be recovered (Figure 4). Other authors have performed solvent extractions using different solvent combinations with different polarities. For example, a mixture of ethyl acetate (polar) and hexane (apolar) provided the greatest carotenoid recovery from tomato waste, compared to other solvent combinations (Strati and Oreopoulou, 2011). Chuyen *et al.* (2017) obtained greater carotenoid extraction from the lyophilized peel of Gac fruit (54 % β-carotene, 31% lycopene and 13% lutein) with ethyl acetate compared to acetone, ethanol and hexane due to its higher polarity thus allowing the extraction of both carotenes and xanthophylls. Ryckebosh et al. (2014) evaluated the use of hexane/isopropanol (3:2; HI) and hexane (H) with *I. galbana*, *N. gaditana*, *N. sp*. and *P. tricornutum* and obtained better results with HI - the highest general recovery was from *Isochrysis*, the lowest from the two *Nannochloropsis* species, and an intermediate level from *Phaeodactylum*. They claimed that these carotenoid recoveries could be explained by a combination of two factors - the first being the similar polarity of the carotenoid and the solvent, the second dealing with the dual location of the carotenoids. Primary carotenoids are mostly associated with the photosynthetic membrane complexed to proteins, so it is necessary to use polar organic solvents capable of forming hydrogen bonds to disrupt the complexes; secondary carotenoids, however, can be localized in oil

bodies. As mentioned above, it is not possible to recommend a specific solvent or solvent mixture for all samples as it depends on the specific carotenoid composition. In general, the use of a mixture of solvents such as the tricomponent solution used in this study demonstrates good results for a sample containing a mixture of xanthophylls and carotenes.

4. Conclusions

It is not possible to establish a widely applicable method for carotenoid extraction. Saponification conditions must be selected for each strain and the recovery solvent needs to be selected based on the carotenoid to be purified. When a microalga is composed of easily degradable polar xanthophylls, the analysis should be performed under gentle conditions. To sum up, *I galbana* needs a temperature of 60ºC and less than 10% KOH; *N. gaditana* and *K. veneficum* require 60ºC and no saponification; *P. reticulatum* needs 40ºC and 10% KOH; *T. suecica* and *H. pluvialis* require 25ºC and 10% or 40% KOH, respectively, while *C. sp.* and *S. almeriensis* require 80ºC and 40% KOH. The use of a mixture of solvents, such as the tricomponent solution, demonstrates generally good results for a sample with xanthophylls and carotenes.

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447 **Table and Figure Captions**

448 **Table 1.** Concentration ranges of standard solutions as a calibration method for
449 carotenoid determination. Channel indicates the maximum absorption wavelength (nm).
450 **Table 2.** Multifactor ANOVA testing the effect of temperature (T) and KOH (K) on the
451 total carotenoid content in each microalgae biomass. The data variability is attributable
452 to the main effect of each factor and interaction found, as indicated by the p value. The
453 contribution of each factor was expressed as the percentage variation in the response
454 (the F ratio of each factor relative to the sum of all F ratios).
455 **Table 3.** Extraction conditions for each microalgae species.
456 **Table 4.** Extraction conditions for each carotenoid from the different microalgae species
457
458 **Figure 1.** Surface response (3D) of the variation in total carotenoid content as a
459 function of the extraction conditions (temperature (ºC) and KOH content (%, d.w.)) for
460 the biomasses of the different microalgae species (*Isochrysis galbana* (A), *Tetraselmis*
461 *suecica* (B), *Haematococcus pluvialis* (C), *Chlorella sp.* (D), *Nannochloropsis gaditana*
462 (E), *Scenedesmus almeriensis* (F) *Karlodinium veneficum* (G) and *Protoceratum*
463 *reticulatum* (H).
464 **Figure 2**. Influence of the extraction conditions on the total carotenoid content of the
465 different microalgae species (*Isochrysis galbana* (A), *Tetraselmis suecica* (B),
466 *Haematococcus pluvialis* (C), *Chlorella sp.* (D), *Nannochloropsis gaditana* (E),
467 *Scenedesmus almeriensis* (F) *Karlodinium veneficum* (G) and *Protoceratum reticulatum*
468 (H)) as a function of temperature (ºC) and KOH content (%, d.w.). Values are the
469 mean±standard deviation in the different microalgae. Average temperature (dark circles)

and average KOH (red triangles) are the average values of the total carotenoids under each condition.

Figure 3. Influence of the KOH percentage on the carotenoid content of the different microalgae species (*Isochrysis galbana* (A), *Tetraselmis suecica* (B), *Haematococcus pluvialis* (C), *Chlorella sp.* (D), *Nannochloropsis gaditana* (E), *Scenedesmus almeriensis* (F) *Karlodinium veneficum* (G) and *Protoceratum reticulatum* (H)) as a function of temperature (ºC) and KOH content (%, d.w.). Values are the mean±standard deviation in the different microalgae.

Figure 4. Influence of the different solvents (sorted by polarity index: hexane: 0, hexane-ethanol (70:30): 2.6, ethyl ether: 2.9, hexane-ethanol (50-50): 3.6, absolute ethanol: 5.2, acetone: 5.4, ethanol-hexane-water: 5.5, ethanol (96%): 5.6, acetone-water (97.5:2.5): 5.7, acetone-water (95:5): 6, acetone-water: 6.3 and methanol 6.6) on the carotenoid content of the different microalgae species (*Nannochloropsis gaditana* (A), *Chlorella sp.* (B), *Isochrysis galbana* (C), *Tetraselmis suecica* (D), and *Protoceratum reticulatum* (E)). Values are the mean±standard deviation in the different microalgae.

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Table 1

Concentrations ranges of standard solutions as a calibration method for carotenoid determination. Channel indicates the maximum absorption wavelength (nm).

Pigment	Concentration range, $\mu g \cdot ml^{-1}$	Calibration levels	Channel (λ), nm
Neoxanthin	0 – 75	5	440
Lutein	0 – 30	5	440
Fucoxanthin	0 – 90	5	440
Peridinin	0 – 6.5	5	475
Violaxanthin	0 – 3	5	440
Zeaxanthin	0 – 30	5	450
Vaucheriaxanthin	0 – 30	5	450
Diatoxanthin	0 – 4	5	450
Diadinoxanthin	0 – 3.5	5	440
Gyroxanthin ester	0 – 2	5	440
Dinoxanthin	0 – 2	5	440
β-carotene	0 – 37	5	450

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Table 2

Multifactor ANOVA testing the effect of temperature (T) and KOH (K) on the total carotenoid~~s~~ content in each microalgae biomass~~es~~. The data variability is attributable to the main effect of each factor and interaction~~s~~ found, as indicated by the p value. The contribution of each factor was expressed as the percentage variation of the response (F ratio of each factor relative to the sum of all F ratios).

Microalgae	Statistics	T	KOH	T-KOH
Isochrysis	%	8.24	87.38	4.39
galbana	*p*-value	<0.05	<0.05	<0.05
Tetraselmis	%	41.99	10.01	48.00
suecica	*p*-value	< 0.05	< 0.05	< 0.05
Haematococcus	%	14.34	80.89	4.79
pluvialis	*p*-value	<0.05	<0.05	<0.05
Chlorella sp.	%	97.21	2.32	0.47
	p-value	<0.05	<0.05	<0.05
Nannochloropsis	%	78.65	18.14	3.21
gaditana	*p*-value	< 0.05	< 0.05	< 0.05
Scenedesmus	%	47.74	34.09	18.17
almeriensis	*p*-value	< 0.05	< 0.05	< 0.05
Karlodinium	%	5.32	88.89	5.79
veneficum	*p*-value	<0.05	<0.05	<0.05
Protoceratum	%	89.30	9.77	0.93
reticulatum	*p*-value	<0.05	<0.05	<0.05

Red values mean significant differences (*p*-value < 0.05)

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Table 3. Extraction conditions for each microalgae species.

Microalga	Temperature, ºC	KOH, %d.w.
I galbana	60	10
N. gaditana, K. veneficum	60	0
P. reticulatum	40	10
T. suecica,	25	10
H. pluvialis		40
C. sp., S. almeriensis	80	40

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Table 4. Extraction conditions for each carotenoid from the ~~of~~ different microalgae species.

Carotenoid	Microalga	Temperature, ºC	KOH, %d.w.
Fucoxanthin	*I. galbana, K. veneficum*	60	0
Diadinoxanthin	*I. galbana*	60	0
Diadinoxanthin	*P. reticulatum*	40	10
Diatoxanthin	*I. galbana, K. veneficum*	60	40
Diatoxanthin	*P. reticulatum*	40	10
Dinoxanthin	*P. reticulatum*	40	10
Lutein	*T. suecica,*	25	10
	H. pluvialis		40
Lutein	*S. almeriensis*	80	40
Violaxanthin	*T. suecica*	25	10
	, H. pluvialis		40
Violaxanthin	*C. sp., S. almeriensis*	80	40
Violaxanthin	*N. gaditana*	60	20
Vaucheriaxanthin	*N. gaditana*	60	20
Vaucheriaxanthin ester	*N. gaditana*	60	20
Zeaxanthin	*C. sp.*	80	40
Neoxanthin	*N. gaditana*	60	40
Astaxanthin	*H. pluvialis*	25	40
β-carotene	*N. gaditana, K. veneficum*	60	40
β-carotene	*K. veneficum*	60	0
β-carotene	*T. suecica,*	25	10
	H. pluvialis		40
β-carotene	*C. sp., S. almeriensis*	80	60
β-carotene	*P. reticulatum*	40	10
Gyroxanthin	*K. veneficum*	60	0
Peridinin	*P. reticulatum*	40	10

Figure 1

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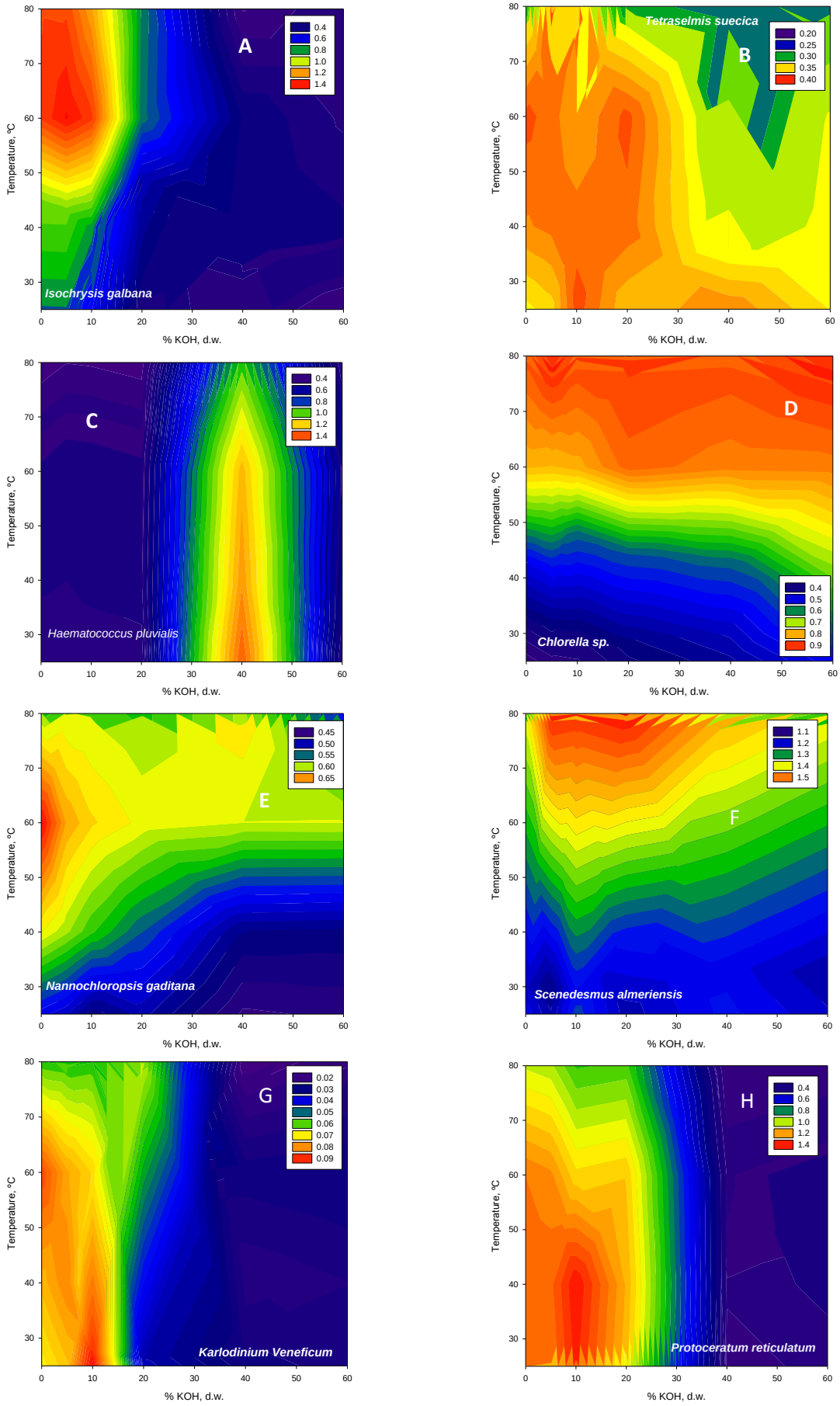
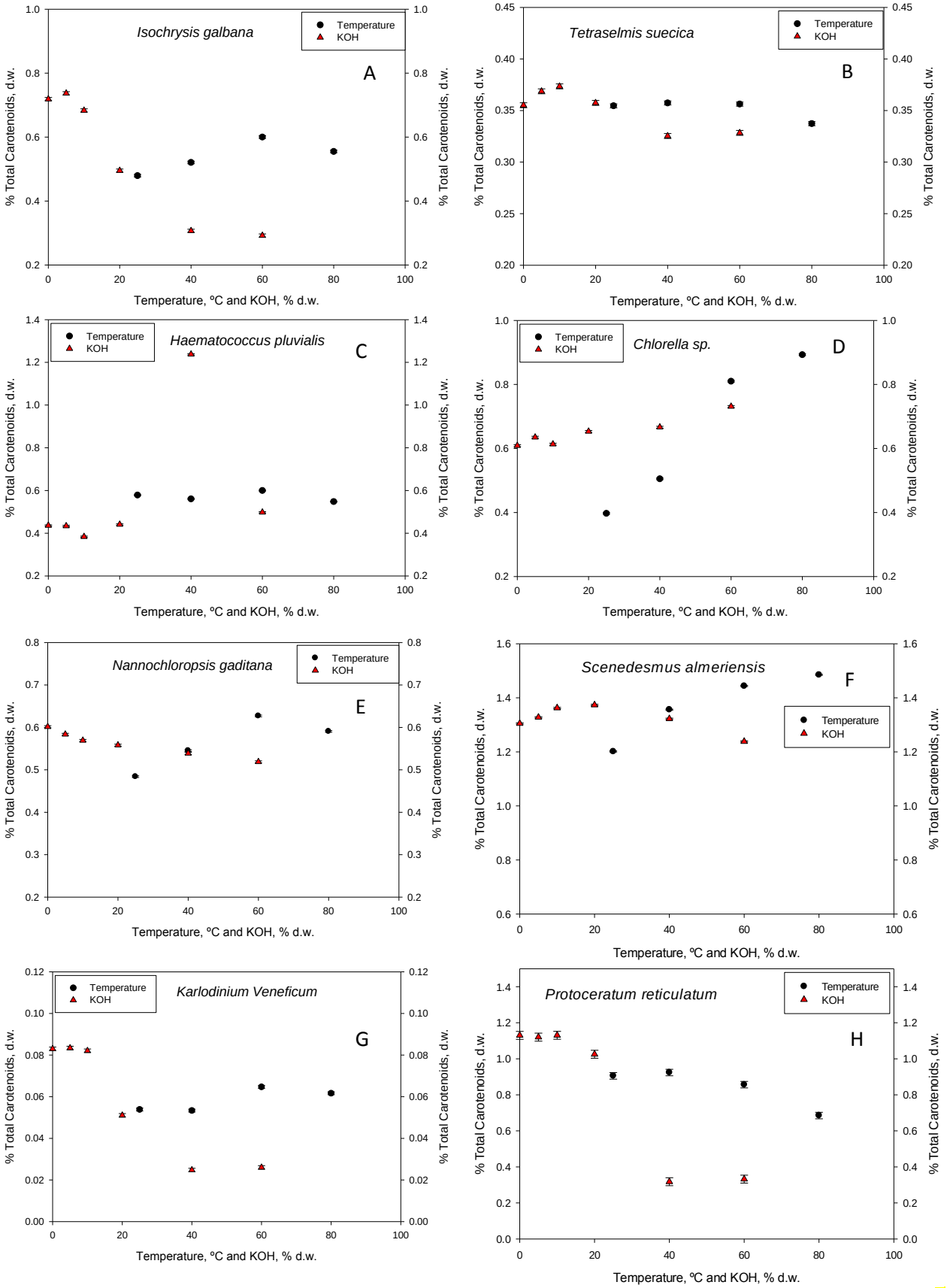


Figure 1

Figure 2



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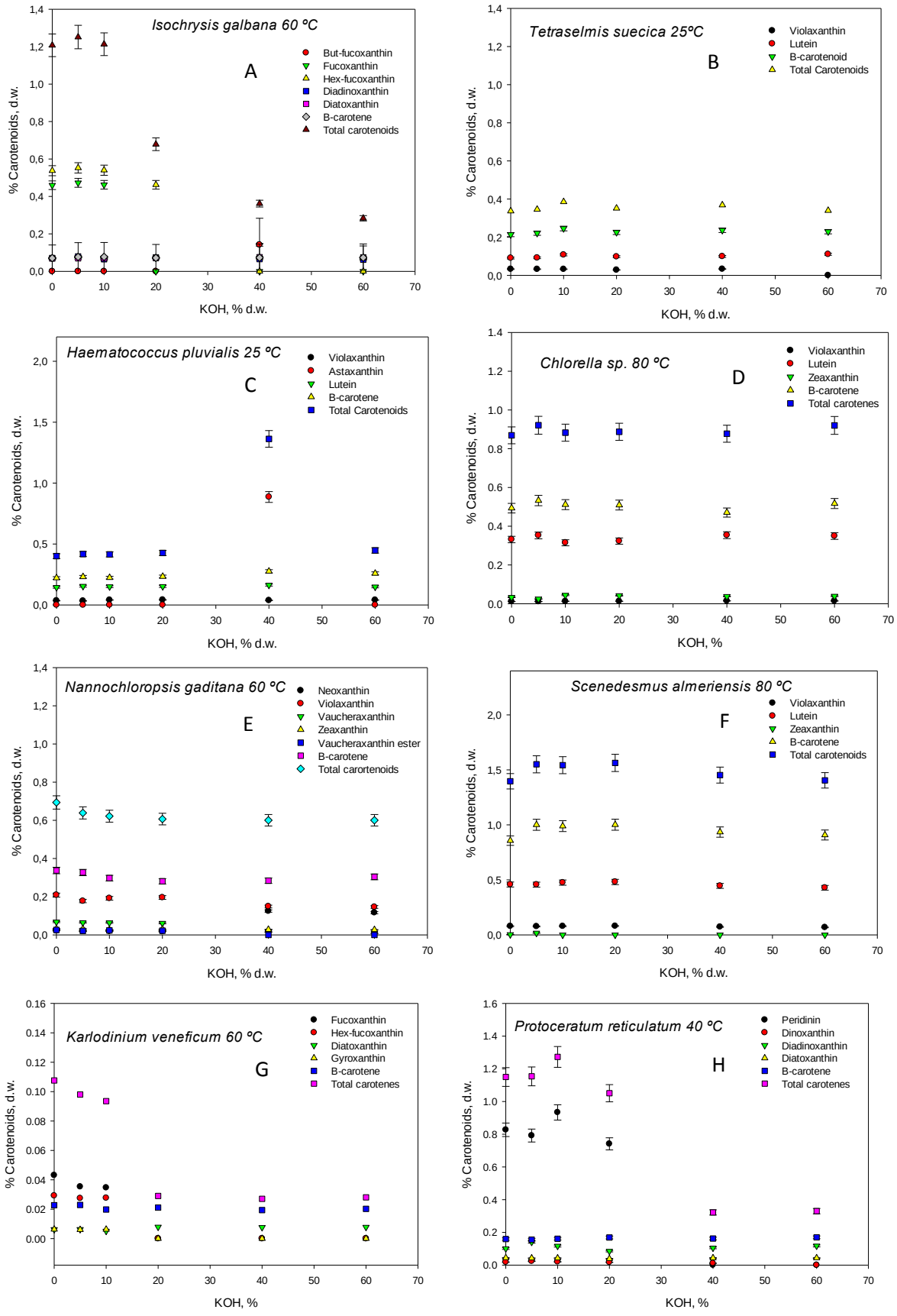

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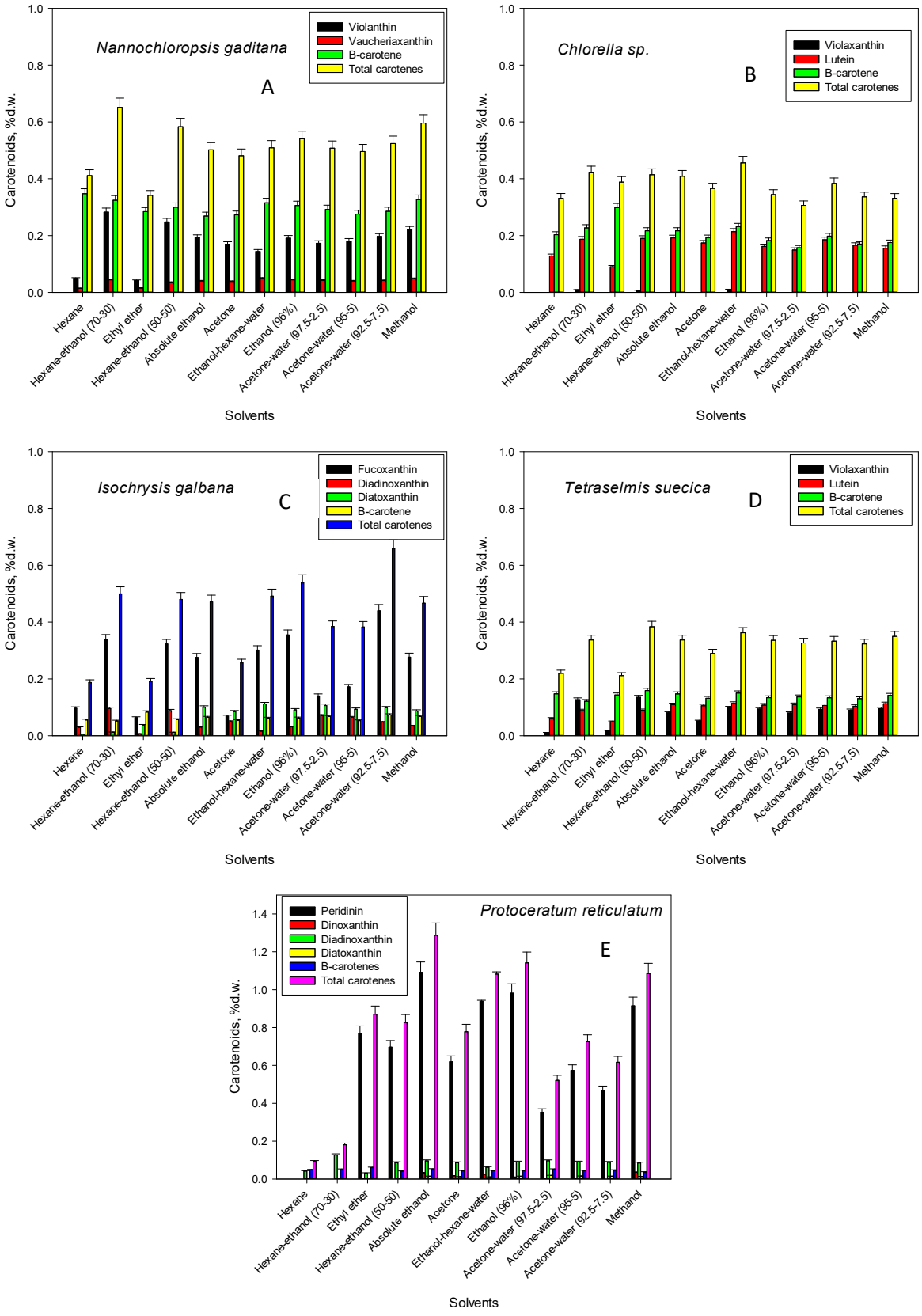

Figure 4.