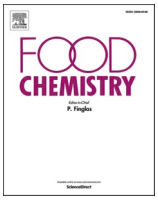
Food Chemistry 434 (2024) 137437

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Ultrasound-assisted extraction of carotenoids from phytoene-accumulating *Chlorella sorokiniana* microalgae: Effect of milling and performance of the green biosolvents 2-methyltetrahydrofuran and ethyl lactate

Ángeles Morón-Ortiz [a], Paula Mapelli-Brahm [a], Antonio León-Vaz [b], Ana M. Benitez-González [a], Rosa León [b], Antonio J. Meléndez-Martínez [a,*]

[a] Food Colour and Quality Laboratory, Facultad de Farmacia, Universidad de Sevilla, 41012 Sevilla, Spain
[b] Laboratory of Biochemistry, Faculty of Experimental Sciences, Marine International Campus of Excellence and RENSMA, University of Huelva, 21071 Huelva, Spain

ARTICLE INFO

Keywords:
Bioactive compounds
Norflurazon
2-methyloxolane
Powdering
Sonication
Sustainability

ABSTRACT

This study aimed at optimizing the accumulation of phytoene in *Chlorella sorokiniana* by using norflurazon and investigating the capacity of green and traditional solvents to extract carotenoids by ultrasound-assisted extraction with and without previous milling.

Phytoene-rich first described *C. sorokiniana* biomass was used, both fresh, freeze-dried, and encapsulated. The ideal dose of norflurazon (1 μg/mL) was selected to block the carotenoid pathway at the level of phytoene desaturase and induce the accumulation of phytoene in *C. sorokiniana*. A mill pre-treatment allowed a higher recovery of carotenoids compared to non-milled samples, in both the freeze-dried and encapsulated matrices. 2-Methyloxolane provided a higher total carotenoid content (4.75–5546.96 μg/g) compared to the other solvents tested in all the matrices, proving a promising bio-based solvent to replace traditional organic ones for the extraction of microalgal carotenoids.

1. Introduction

Current political worldwide agendas highlight the urgent need to shift to more sustainable and healthier agro-food systems. Microalgae are very important in this scenario for different reasons. Microalgae are sustainable sources due to their high productivity and growth characteristics: their production takes place all year round, they can grow under stressful conditions, their nutritional and water requirements are low, and the use of herbicides or pesticides in open lands is not necessary. In addition, they contribute to CO_2 sequestration and their culture systems require low utilization of space and do not require the use of arable land. Moreover, they accumulate valuable health-promoting compounds, and modulating their biosynthesis by modifying growth conditions is relatively easy (Caporgno & Mathys, 2018).

Microalgae are a good source of nutrients and bioactive compounds, and thus they could play an essential role in reducing poverty and hunger and improving health and well-being. A recent review highlighted that the incorporation of bioactive compounds of microalgae into different varieties of food, including bread, pasta, dairy products, emulsions, vegetarian gels, and cookies, could lead to health and techno-functional benefits (Caporgno & Mathys, 2018). Microalgae are considered a rich source of proteins, carbohydrates, vitamins, and health-promoting compounds such as carotenoids. Carotenoids are lipophilic molecules that can be obtained from different dietary sources, predominantly plant foods (fruits, vegetables, herbs, oils, cereals) but also from animal foods (dairy, egg yolk, fish, etc.), supplements, and even as additives (colorants) present in drinks and other food products (Mapelli-Brahm et al., 2020). Other dietary sources of carotenoids, such as macroalgae (seaweeds), microalgae, fungi and bacteria, are eliciting increased interest (Meléndez-Martínez et al., 2022). In. general the main carotenoids present in foods and in human fluids and tissues are α-carotene, β-carotene, lycopene, lutein, zeaxanthin, β-cryptoxanthin and the colourless carotenoids phytoene and phytofluene. Beyond the role of some carotenoids as provitamins A, the possible health benefits of carotenoids are usually attributed to direct antioxidant mechanisms (quenching, scavenging), although there is evidence that there are others (prooxidant mechanisms, enhancement of gap junctional communication between cells, modulation of gene expression,

* Corresponding author.
E-mail address: ajmelendez@us.es (A.J. Meléndez-Martínez).

https://doi.org/10.1016/j.foodchem.2023.137437
Received 26 May 2023; Received in revised form 24 July 2023; Accepted 7 September 2023
Available online 12 September 2023

modulation of immune function, light absorption). As a result, carotenoids can contribute to reduce the risk of cardiovascular diseases, cancer, eye disorders and other conditions (Meléndez-Martínez & Mapelli-Brahm, 2021; Meléndez-Martínez et al., 2022). In microalgae, these compounds contribute to membrane stabilization, light harvesting, and energy dissipation, among others actions. Some species can accumulate very high amounts of carotenoids. For example, *Dunaliella* can accumulate up to 13% of dry weight in β-carotene, while other species such as *Scenedesmus*, *Chlorella*, *Coccomyxa, Parachlorella,* or *Tetraselmis* have also been studied for their potential in lutein synthesis (Ren et al., 2021). Lutein, α-carotene, and β-carotene are usually present in a high amount in *Chlorella*. Phytoene is a colorless carotenoid that can be considered a rarity in the carotenoid's kingdom due to its lack of color. However, there is mounting evidence suggesting that colorless carotenoids may play a role in promoting health (Meléndez-Martínez et al., 2019). Phytoene is the precursor of the other carotenoids in the carotenogenesis pathway. The treatment with norflurazon causes inhibition of phytoene desaturase, resulting in inhibition of the synthesis of coloured carotenoids and the accumulation of phytoene in diverse organisms, including microalgae (León et al., 2005).

Due to their physicochemical properties, carotenoids are usually extracted using lipophilic solvents (hexane, acetone, ethyl acetate, ether, etc.) (Meléndez-Martínez et al., 2022), although edible oils are being increasingly studied for the development of carotenoid-rich products oils (Ordóñez-Santos et al., 2021). The use of green solvents for the extraction of bioactive compounds is increasingly drawing the interest of researchers and industries, due to the threat of climate change and the need to make a more sustainable use of resources nowadays. A green solvent must be totally natural, suitable for existing industrial facilities, have no affections on the safety and health of operators and consumers, have a high rate of recyclability, high bio-degradability, low volatile organic compound emissions, low energy consumption, low cost of the global process, and ensure a maximal solvent recovery (Chemat et al., 2019). Ethyl lactate has already been investigated as an extraction solvent for bioactive compounds, such as carotenoids, fatty acids, and derivates from microalgae and food (Castillo et al., 2020;Villanueva-Bermejo et al., 2017). Ethyl lactate is an agrochemical solvent with low toxicity that has GRAS (Generally Recognized as Safe) status and is approved by FDA (Food and Drug Administration) and EFSA (European Food Safety Authority) as a pharmaceutical ingredient and food additive. It has a low toxicity, is a non-corrosive, non-carcinogenic, non-teratogenic, biodegradable, and non-ozone depleting solvent (Villanueva-Bermejo et al., 2017). 2-Methyloxolane, also known as 2-methyltetrahydrofuran (MeTHF) is a biomass-derived green solvent produced from furfural or levulinic acid. There are few studies on its use as a carotenoid extraction solvent. A study performed by Yara-Varón et al. (2016) showed a higher recovery of total lipids and carotenoids from dried carrots with MeTHF compared to other green solvents, such as ethyl acetate, dimethyl carbonate, isopropyl alcohol, or *n*-hexane. Among other properties, the low boiling point (79 °C) and low enthalpy of vaporization (34 kJ/mol) of MeTHF makes it an attractive solvent for industries (Smets et al., 2021).

Besides the use of sustainable extraction solvents, industries and the society are also demanding the use of greener extraction methods, such as the ultrasound-assisted extraction (UAE). UAE has numerous advantages, including greater penetration of the solvent into the matrix, higher extraction yields, shorter processing and residence time, lower solvent expenditure, energy saving, etc. (Meléndez-Martínez et al., 2022).

The aim of this study was double. On one hand, to determine the right amount of norflurazon to prevent phytoene desaturation and promote phytoene accumulation without causing cell death. On the other hand, to investigate the different capacity of novel bio-based and common organic solvents to extract microalgal carotenoids by UAE and to assess the effect of milling in the extractions. The results of this study are therefore valuable for the production of sustainable phytoene-rich products.

2. Methodology

2.1. Reagents

Tert-butyl methyl ether (HPLC-grade) was purchased from Honeywell (Seelze, Germany); ethyl lactate from Supelco (Bellefonte, PA, USA); 2-methyloxolane (MeTHF) from Sigma-Aldrich (Steinheim, Germany); chloroform, methanol (HPLC-grade), and ethyl acetate (HPLC-grade) from VWR Chemicals (Leuven, Belgium); and ethanol, dimethyl sulfoxide (DMSO), and sodium chloride from PanReac AppliChem (Barcelona, Spain).

2.2. Plant material

Chlorella sorokiniana (211–32) was kindly provided by the algal collection of the Institute of Plant Biochemistry and Photosynthesis (IBVF-CSIC, Seville, Spain) and cultured photomixotrophically in liquid Tris-acetate phosphate (TAP) medium (Chlamydomonas Source book, 1989). Cultures were maintained in a thermostatic chamber at 25 °C under continuous white light irradiation (100 μE m^2/s photosynthetically active radiation (PAR)). To obtain high density cultures, standard cultures were subcultured into TAP medium enriched with sodium acetate and ammonium chloride (León-Vaz et al., 2019). The light intensity was measured by a Delta OHM quantum photo-radiometer equipped with a PAR sensor. When cultures acquired the desired cellular concentration, biomass was harvested by centrifugation at 4400 rpm and either frozen at −20 °C, lyophilized or entrapped in alginate beads (Section 2.3).

2.3. Encapsulation of microalgae

Encapsulation in alginate beads was performed as previously described (León & Galván, 1994). Briefly, cultures of *C. sorokiniana* were harvested at the end of the exponential phase, with a biomass content of 1–1.2 g/L DW (corresponding to a OD660 of about 3.5 UA), resuspended in fresh TAP buffered culture medium and thoroughly mixed with an equal volume of a sterile alginate solution (6%, w/v) of alginate sodium salt (medium viscosity) from *Macocystis pyrifera* (SIGMA, St. Louis, Mo, USA), prepared by autoclaving for 20 min at 120 °C. Beads of about 3 mm diameter were obtained by dropping the alginate-cell mixture into a solution of 0.1 M $CaCl_2$ at 4 °C. The beads were maintained at 4 °C until their use. The whole process was performed in a laminar flow cabin under sterile conditions.

2.4. Dry weight determination

Dry weight of *C. sorokiniana* was determined by filtering an exact volume of microalgae culture (30 mL) on pre-tared glass-fiber filters (GF/F Whatman). The filter was washed with a solution of ammonium formate (0.5 M) to remove salts and then dried at 100 °C for 24 h. The dried filters were weighed in an analytical balance and the dry weight calculated by difference.

2.5. Phytoene enrichment

Cultures of *C. sorokiniana* in the middle of the exponential phase was harvested by centrifugation, resuspended in fresh TAP culture medium, divided into 50 mL-cultures, and incubated with increasing concentrations (from 0 to 20 μg/mL) of the herbicide norflurazon (NF). Growth and the content of phytoene, colored carotenoids and chlorophylls in the NF-treated cultures was followed during 72 h and compared with those of the control cultured without NF.

2.6. Experimental design

Six different matrices were evaluated: control *C. sorokiniana* (fresh, lyophilized, and encapsulated) and phytoene-rich *C. sorokiniana* (fresh, lyophilized, and encapsulated). The samples were subjected to two different treatments: 1. UAE without any prior step (Section 2.7); 2. A prior ball-mill pre-treatment (Section 2.8) followed by UAE. Five extraction solvents were tested: ethanol and methanol, which are authorized for food use in the European Union (The European Parliament and the council of The European Union, 2009), ethyl lactate and MeTHF, which are emerging green biosolvents, and DMSO, which is a common solvent for laboratory carotenoid extraction. All extractions were performed in triplicate; i.e., for each treatment and solvent, the extraction was carried out in triplicate.

2.7. Ultrasound-Assisted extraction (UAE)

0.1 g of sample (fresh, lyophilized, and encapsulated) was mixed with 2 mL of one of the extraction solvents and ultrasonicated (2 min, 30% amplitude, 20 kHz frequency) by means of a Q500 sonicator with a 1.6 mm-probe (Qsonica, EE.UU.). The samples were immersed in an ice bath during sonication to minimize carotenoid degradation. Samples were centrifuged, and the resulting supernatant was transferred to a new tube. The process was repeated until the samples were colorless.

The combined supernatants were evaporated in a rotary evaporator at a temperature below 30 °C, and the extracts were stored under a nitrogen atmosphere at −20 °C until its analysis by HPLC. Ethyl lactate and DMSO have a higher boiling point, so they were partitioned with 3 mL of trichloromethane and water. After that, they were evaporated and the extracts stored under the same conditions as the others extracts.

2.8. Milling-UAE

0.1 g of sample (fresh, lyophilized, and encapsulated) was introduced in a 2 mL-Eppendorf, and three stainless steel balls of 3 mm in diameter were introduced into the Eppendorf. The milling was applied with a ball mill (MM 400, Retsch, Germany) for 5 min with a frequency of 30 Hz. The protocol of the ball mill was based on a study carried out by Serive et al. (2012). The stainless steel balls were stored in the freezer to prevent heating during the extraction process. After ball mill pre-treatment, the samples were subjected to ultrasound under the same conditions as UAE samples (Section 2.7).

2.9. High-Performance liquid chromatography (HPLC) analysis

An HPLC system (1260 Infinity II Prime LC System, Agilent) equipped with a diode array detector and a C_{30} column (YMC 150 × 4.6 mm, 3 μm particle size) was used for the carotenoid quantification. Extracts were dissolved in approximately 500 μL of ethyl acetate and 10 μL was injected in the system. The mobile phase was composed of methanol, *tert-butyl* methyl ether and water at a rated flow of 1 mL/min, using a linear gradient and a method already validated (Stinco et al. 2019). The carotenoid content was determined using calibration curves obtained as explained elsewhere (Stinco et al. 2019).

2.10. Statistical analysis

The total carotenoid content was determined as the sum of all individual carotenoids. All the experiments were carried out in triplicate. InfoStat was used for the analysis of variances (ANOVA) with Tukeý́s *post hoc* test to evaluate differences between groups. Values of $p < 0.05$ were considered statistically significant.

3. Results

This study was aimed at determining the optimal dosage of norflurazon to prevent phytoene desaturation, promoting phytoene accumulation and avoiding cell death. Moreover, the study also aimed at comparing the capacity of biobased and common organic solvents to extract carotenoids from wild-type and phytoene-rich *C. sorokiniana* using UAE, considering a resource-saving sustainable extraction technique. The effect of a mill pre-treatment in the extraction process was also evaluated.

3.1. Induction of phytoene accumulation

To induce the accumulation of the colorless carotenoid phytoene without causing total death of the microalga, it is necessary to adjust the herbicide dosses. *C. sorokiniana* growth is severely inhibited in the presence of NF (Supplementary Fig. 1). Although the effect of NF is observed at all concentrations tested, at concentrations greater than 2 μg/mL, NF is extremely inhibitory, and *C. sorokiniana* exhibits a complete absence of growth. On the other hand, NF concentration of 0.5 or 1 μg/mL have moderate inhibitory effect, reducing the growth rate but without causing total death of the culture (Fig. 1A).

Once the maximum dose of NF that can be applied was determined, the effect of NF on the carotenoids biosynthetic pathway was evaluated. For this objective, the concentration of the most abundant carotenoids in NF-treated (0.5 to 2 μg/mL) and non-treated cultures was compared. It was observed that over the 72 h-period evaluated, NF caused a strong decrease in the content of all the colored carotenoids. The carotenoid decrease was accompanied of a strong inhibition of the biosynthesis of chlorophylls (Supplementary Fig. 2A and 2B), which is one of the reasons explaining the albinism of NF-treated cells. In addition, the colorless carotenoid phytoene was undetectable in the control culture and reached the highest levels in the cultures treated with 0.5 or 1 μg/mL of NF (Fig. 1B). At these NF concentrations, cell growth remained similar. In order to ensure the inhibition of the carotenoid biosynthetic pathway without greatly affecting cell growth, the 1 μg/mL concentration was selected as the optimal to induce the accumulation of phytoene and produce the phytoene-enriched *C. sorokiniana* biomass.

3.2. Carotenoid profile

The main carotenoids detected in control *C. sorokiniana* (fresh, freeze-dried, and encapsulated matrices) were, in decreasing order quantitatively, lutein, α-carotene, β-carotene, and (9*Z*)-β-carotene (Chromatograms in Supplementary Fig. 3A-C). In the fresh and freeze-dried phytoene-rich *C. sorokiniana* the carotenoids detected were phytoene, lutein, β-carotene, α-carotene, and (9*Z*)-β-carotene, in descending order (Chromatograms in Supplementary Fig. 4A-D). In the encapsulated matrix only lutein and phytoene were found (Chromatograms in Supplementary Fig. 4E-F). Violaxanthin was detected, in small amounts, in all control and phytoene-rich matrices, while traces of zeaxanthin were only found in the fresh and freeze-dried matrices (control and phytoene-rich matrices).

3.3. Effect of the mill

In the fresh control and fresh phytoene-rich matrices, there were no significant differences regarding the content of individual carotenoids (Fig. 2A and Fig. 3A) and the total carotenoid content (TCC) (Supplementary Fig. 5) between UAE and M−UAE in all solvents.

On the other hand, the mill pre-treatment resulted in a significant increase ($p < 0.05$) of each individual carotenoid for all the tested solvents in the freeze-dried control, encapsulated control, freeze-dried phytoene-rich and encapsulated phytoene-rich samples, from 1.14 to 1.92-fold, from 1.22 to 104.07-fold, from 1.05 to 2.60-fold, and from 1.09 to 24.16-fold, respectively, depending on the carotenoid and on the solvent (Fig. 2B and Fig. 3B). Also, significant increases in the TCC were observed when the mill treatment was applied in these samples (Supplementary Fig. 5).

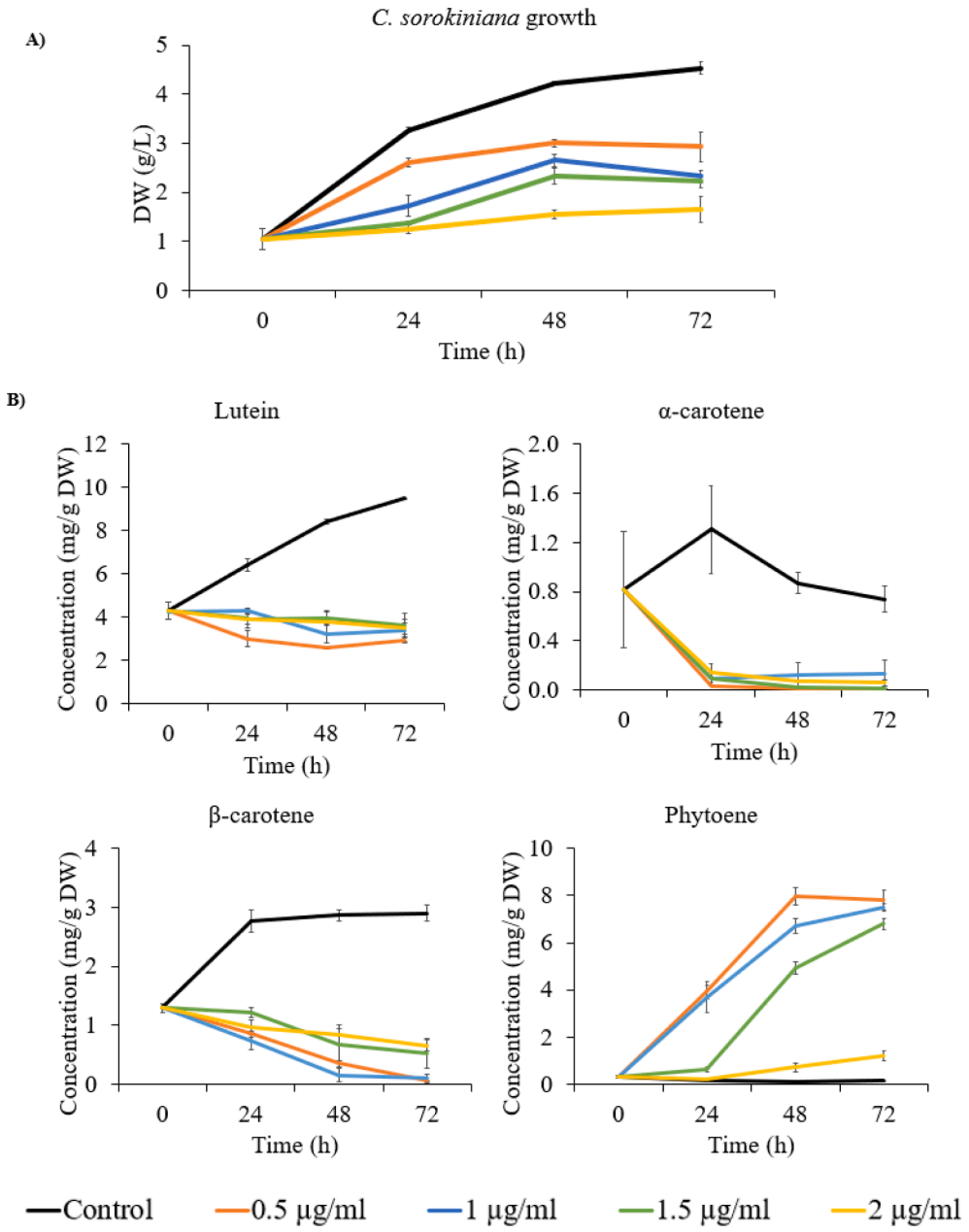


Fig. 1. A) Growth of *C. sorokiniana* treated with increasing concentrations of the herbicide norflurazon (0 – 2 µg/mL). Control refers to non-treated samples (0 µg/mL). B) Carotenoid content in *C. sorokiniana* cultures treated with increasing concentrations of the herbicide norflurazon (0 – 2 µg/mL). Control refers to non-treated samples (0 µg/mL). The error bars in the figures represent the standard error of the mean (SEM). DW: Dry Weight.

3.4. Effect of the extraction solvent

3.4.1. Control C. sorokiniana

To evaluate the extraction capacity of the different solvents in the fresh control matrix, the carotenoid content obtained with each solvent after applying UAE was compared; in this case, the use of the mill was not considered as it had no effect on carotenoid content in this matrix (Section 3.3 and Fig. 2A). The best solvents for the extraction of lutein, α-carotene, and β-carotene were methanol (1316.26, 326.56, and 208.71 µg/g, respectively) and ethanol (1262.29, 310.05, and 203.78 µg/g, respectively), showing no significant differences between them, followed by MeTHF (1098.62, 283.42, and 180.20 µg/g, respectively). Ethanol and MeTHF extracted a significantly higher ($p < 0.05$) amount of (9Z)-β-carotene (27.07 and 23.90 µg/g, respectively), compared to the other solvents (Fig. 4A). The best solvents regarding TCC in the fresh control matrix were ethanol and methanol (with no significant differences between them), followed by MeTHF (Fig. 6A).

In the freeze-dried and encapsulated control matrices there was a significant increase in the carotenoid extraction with the application of the mill (Section 3.3, Fig. 2B and 2.C). Thus, the carotenoid concentration values obtained after the application of UAE with the mill pretreatment were used to evaluate the extraction capacity of the different solvents. In the freeze-dried matrix the best solvent for the extraction of all the carotenoids evaluated, was methanol (2456.05, 821.20, 428.70, and 64.26 µg/g, respectively); however, no significant differences ($p < 0.05$) between this solvent and ethanol, ethyl lactate, and MeTHF were found (Fig. 4B). In the encapsulated control matrix, the best extraction solvents for lutein were methanol, ethyl lactate, and MeTHF (without significant differences between them); for α-carotene and β-carotene were ethanol, methanol, and MeTHF; finally, all solvents had similar extractability regarding the (9Z)-β-carotene (Fig. 4C).

Regarding TCC, the best extraction solvents for freeze-dried control

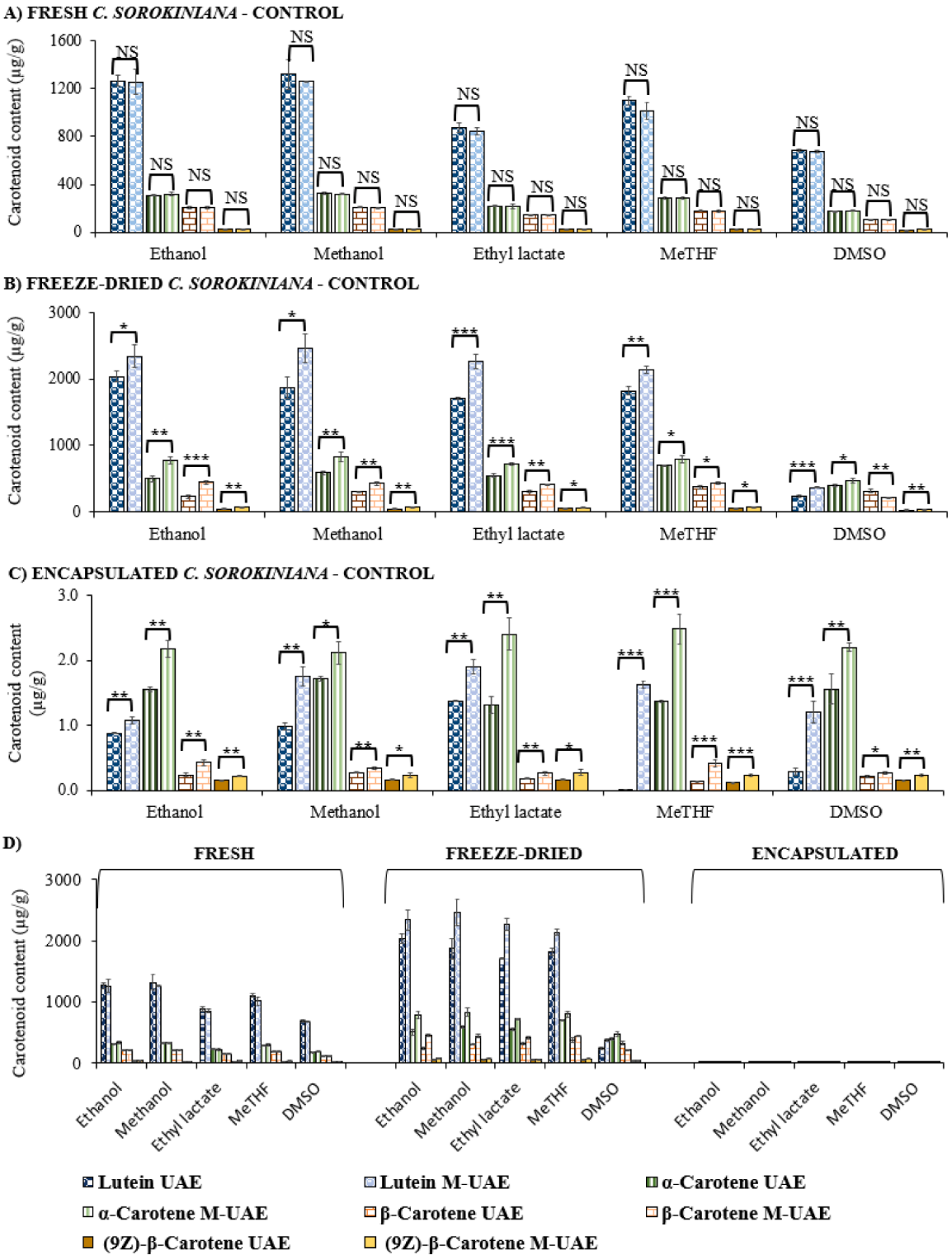


Fig. 2. Differences in individual carotenoid content in *C. sorokiniana* control between UAE (ultrasound-treated samples) and M−UAE (mill + ultrasound-treated samples), for each evaluated solvent. Figures A, B, and C depict the carotenoid content in fresh, freeze-dried, and encapsulated control matrices, respectively. Figure D illustrates the carotenoid content in the three matrices on a standardized scale, showing the very low amount of carotenoids in the encapsulated matrix as compared with the fresh and freeze-dried samples. The error bars in the figures represent the standard deviation (SD). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns: Not significant. MeTHF: methyloxolane; DMSO: dimethylsulfoxide.

matrix were ethanol, methanol, ethyl lactate, and MeTHF ($p > 0.05$). In addition, in the encapsulated control matrix there were no significant differences between methanol, ethyl lactate, and MeTHF, being the last one, the solvent leading to the highest extractability (Fig. 6A).

3.4.2. Phytoene-rich C. sorokiniana

In the fresh phytoene-rich *C. sorokiniana*, the mill did not affect the carotenoid extraction (Section 3.3, Fig. 3A). Thus, the carotenoid concentration values after the UAE without previous mill treatment were used to evaluate the capacity of the solvents for the carotenoid

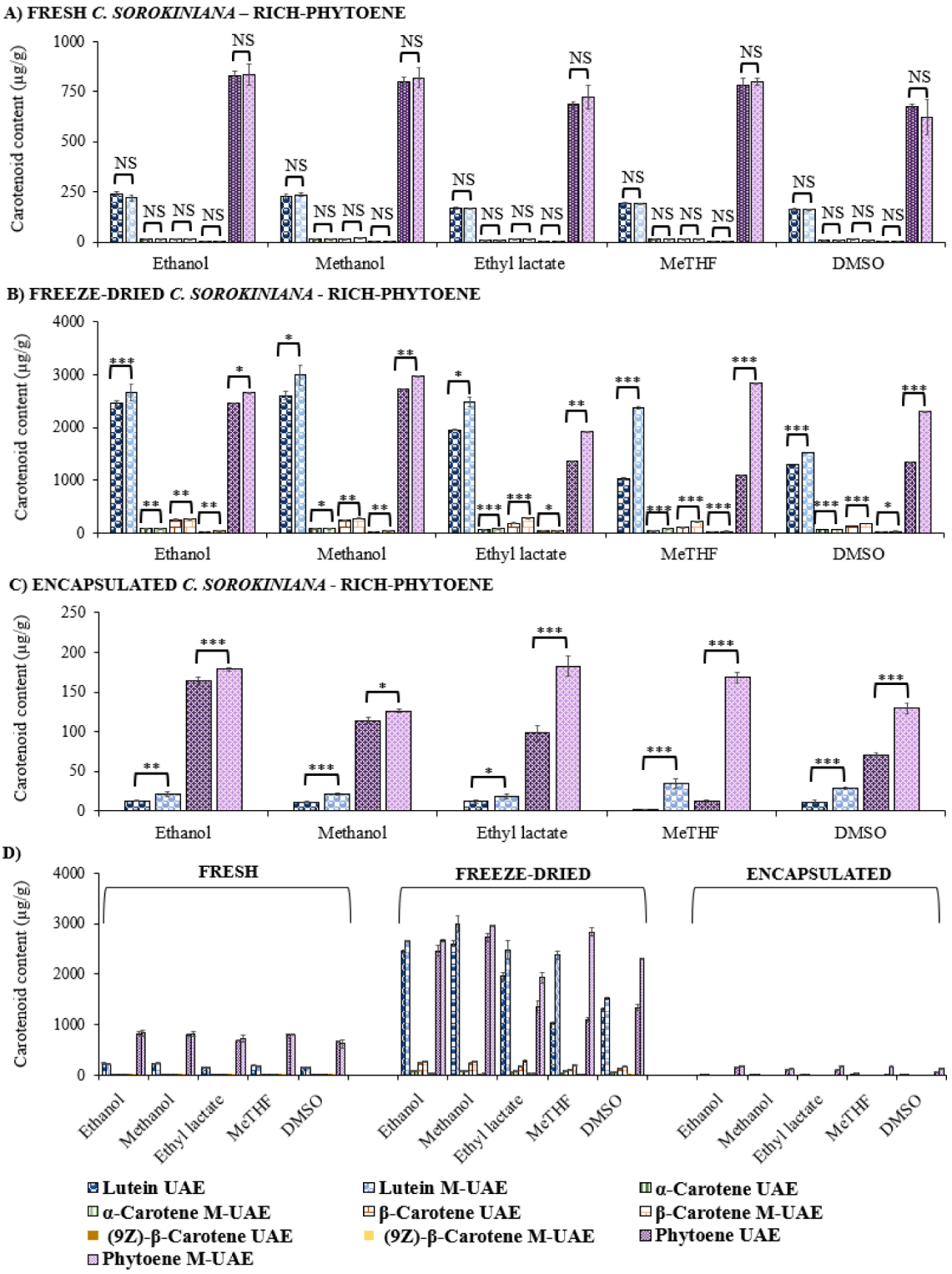


Fig. 3. Differences in individual carotenoid content in *C. sorokiniana* rich-phytoene between UAE (ultrasound-treated samples) and M−UAE (mill + ultrasound-treated samples), for each solvent evaluated. Figures A, B, and C depict the carotenoid content in fresh, freeze-dried, and encapsulated rich-phytoene matrices, respectively. Figure D illustrates the carotenoid content in the three matrices on a standardized scale, showing the very low amount of carotenoids in the encapsulated matrix as compared with the fresh and freeze-dried samples. The error bars in the figures represent the standard deviation (SD). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns: Not significant. MeTHF: methyloxolane; DMSO: dimethylsulfoxide.

extraction. The best solvents for the extraction of phytoene were ethanol (832.63 µg/g), methanol (819.51 µg/g), and MeTHF (799.57 µg/g), showing no significant differences between them ($p < 0.05$) (Fig. 5A).

In freeze-dried and encapsulated phytoene-rich *C. sorokiniana,* the mill pre-treatment resulted in a significant increase in the carotenoid extraction (Section 3.3, Fig. 3B and 3C). Thus, the carotenoid concentration values after the UAE with the previous mill treatment were used to evaluate the capacity of the solvents for the carotenoid extraction. In the freeze-dried matrix, both methanol (2967.73 µg/g) and MeTHF (2841.39 µg/g), were the best extraction solvent, showing no significant

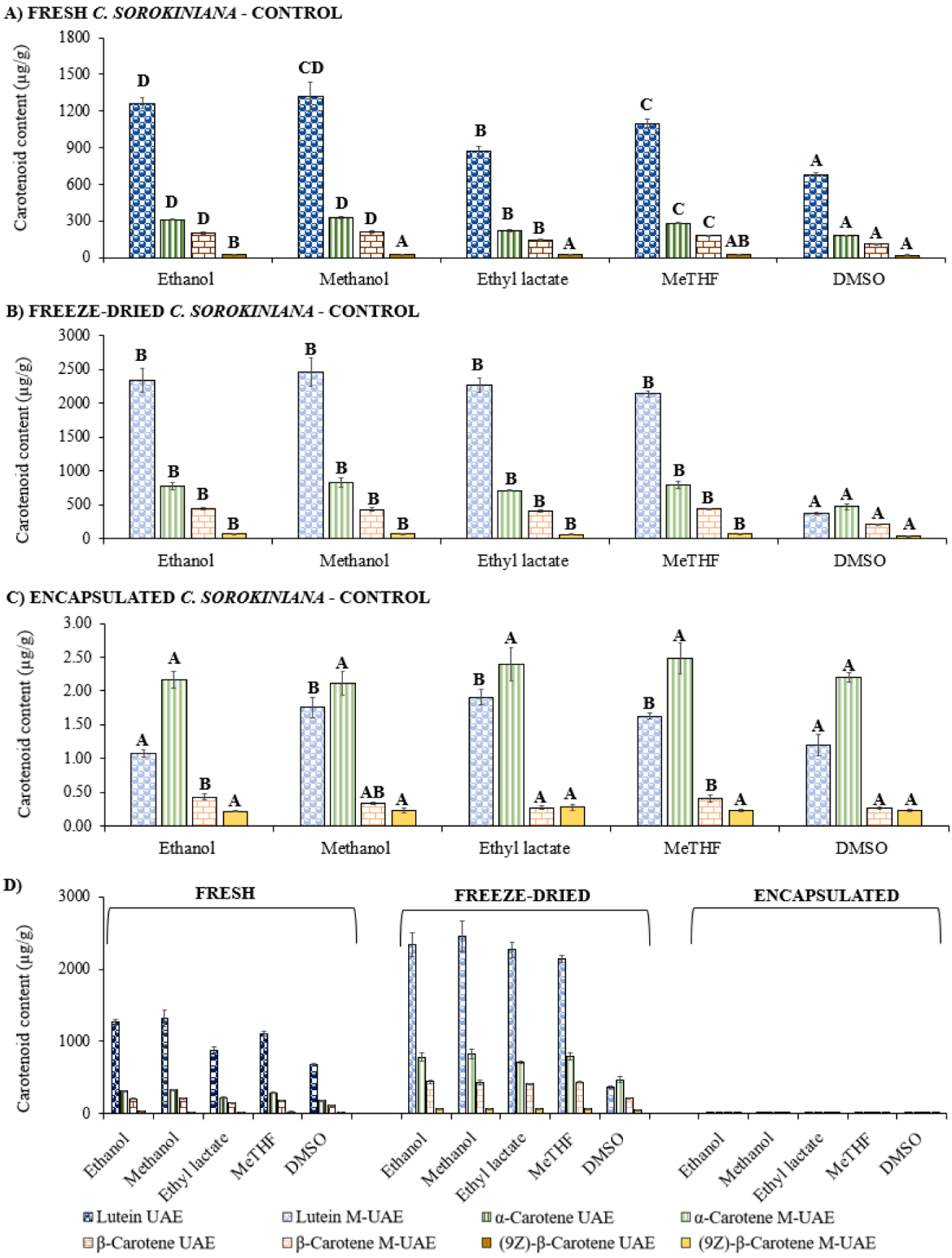


Fig. 4. Differences in the content of each carotenoid in control *C. sorokiniana* extracted with different solvents. For the fresh sample, the values after the extraction without the previous mill treatment are shown, since this treatment does not improve the extractability of the solvents in this matrix (as shown in Fig. 2.A); For the freeze-dried and encapsulated samples, the values after the extraction with the previous mill treatment is shown, since this treatment improve the extractability of the solvents in these matrices (as shown in Fig. 3.B and 3.C). Figures A, B, and C depict the carotenoid content in fresh, freeze-dried, and encapsulated control matrices, respectively. Figure D illustrates the carotenoid content in the three matrices on a standardized scale, showing the very low amount of carotenoids in the encapsulated matrix as compared with the fresh and freeze-dried samples. The error bars in the figures represent the standard deviation (SD). For the same carotenoid, different capital letters indicate statistically significant differences ($p < 0.05$) between solvents. MeTHF: methyloxolane; DMSO: dimethylsulfoxide.

differences between them (Fig. 5B). It is noteworthy that despite phytoene is the major carotenoid in the norflurazon-treated *C. sorokiniana* fresh samples, the amount of lutein extracted in the freeze-dried biomass is comparable and even higher in some cases than that of phytoene.

Finally, in the encapsulated phytoene-rich *C. sorokiniana* the solvents that extracted the highest amount of phytoene were ethanol (178.90 μg/g), ethyl lactate (182.73 μg/g), and MeTHF (168.14 μg/g) ($p > 0.05$) (Fig. 5C).

In the fresh phytoene-rich *C. sorokiniana*, TCC was significantly higher with ethanol (1098.28 μg/g), followed by methanol and MeTHF, without significant differences between methanol and MeTHF (Fig. 6B). The best solvent in the freeze-dried phytoene-rich matrix, was methanol (TCC = 6357.98 μg/g) ($p < 0.05$), followed by ethanol and MeTHF ($p > 0.05$). Finally, the highest TCC in the encapsulated phytoene-rich matrix were obtained with MeTHF (202.74 μg/g), ethyl lactate, and ethanol, without differences between them ($p < 0.05$) (Fig. 6B).

4. Discussion

4.1. Carotenoid profile and induction of phytoene accumulation

In general, microalgae are environmentally friendly, as they are able to grow under a wide range of nutrients sources and conditions, protect against pathogens, and can even be used as biofuel feedstocks (Alvarez et al., 2021). Microalgae are a rich source of health-promoting carotenoids, with important qualitative and quantitative differences across species (Zhou et al., 2022). *C. sorokiniana* has been proposed as alternative to food enrichment in pasta and gluten-free bread, as it is considered a rich source of proteins, carbohydrates, vitamins, and carotenoids (lutein, α-carotene, and β-carotene, among others) (Bazarnova et al., 2021; Diprat et al., 2020).

In this study, the main carotenoids detected in control *C. sorokiniana* were lutein, α-carotene, β-carotene, (9*Z*)-β-carotene, violaxanthin, and zeaxanthin.

In the fresh, freeze-dried, and encapsulated matrices stressed with norflurazon (*C. sorokiniana* phytoene-rich), phytoene was also found. Although phytoene is the precursor of the rest of the carotenoids, this carotenoid has been largely ignored by the scientific community, probably due to its lack of color. However, in recent years, this carotenoid has gained more importance, as it is now clear that they are present in considerable amounts in widely consumed fruits and vegetables (including tomatoes, carrots, citrus, apricots, watermelon, etc.), they are major circulating carotenoids in humans and have been associated to diverse health-promoting biological actions (Meléndez-Martínez et al., 2019). Chlorophyte microalgae do not accumulate in normal conditions phytoene, which is rapidly metabolized into other downstream carotenoids (Supplementary Fig. 1). The bleaching herbicide norflurazon inhibits phytoene desaturase by competition with its redox cofactors, as has been demonstrated for higher plants and some microalgae species (Breitenbach et al., 2001). In *C. sorokinina*, as expected, norflurazon stress resulted in blockage of the carotenogenic pathway at the phytoene desaturase level, allowing a high accumulation of phytoene. As explained above, the optimum concentration of norflurazon, which facilitated partial inhibition of PDS and consequently, both cellular growth and phytoene accumulation, was determined to be 1 μg/mL. The results indicate that herbicide stress may serve as a promising method for generating phytoene-rich matrices. Phytoene is a carotenoid that has been reported in studies of diverse nature to exhibit immunological, cosmetic, cardiovascular, metabolic, photoprotective, and anticarcinogenic properties, which could be attributed to different actions (UV radiation absorption, antioxidant activity, modulation of gene expression, anti-inflammatory action) (Meléndez-Martínez & Mapelli-Brahm, 2021).

Commonly, carotenoids are presented in their *E* configuration, which is usually considered the most stable form, although evidence is accumulating that this might not apply to acyclic carotenoids such as phytoene, phytofluene and lycopene (Meléndez-Martínez et al., 2014). In all *C. sorokiniana* matrices, most of the identified carotenoids were presented in their *E* form, with the exception of phytoene, which is usually found in the *Z* configuration in most known matrices (Mapelli-Brahm et al., 2018). In agreement with our data, that show that (*Z*)-phytoene was the predominant isomer in the three *C. sorokiniana* matrices studied, The *E/Z* ratio of β-carotene (average between all solvents) in the fresh control and phytoene-rich *C. sorokiniana* was 6.90 and 10.68, respectively. In the freeze-dried control matrix, the *E/Z* ratio was 6.86, and in the freeze-dried phytoene-rich *C. sorokiniana* was 6.94. In a recent study, this value was 2.1 in freeze-dried *C. sorokiniana* (Fernandes et al., 2021), far from our results. Finally, the encapsulated control matrix resulted in a *E/Z* ratio of 1.43, and 1.49, which could indicate that the dehydration and encapsulation in alginate beads could have led to the isomerization of β-carotene, allowing the formation of a higher proportion of (*Z*)isomers (Sampaio et al., 2019).

4.2. Mill treatment

The mill pre-treatment (M−UAE) resulted in a significant increase in TCC in the freeze-dried and encapsulated samples compared with the UAE samples without mill pre-treatment: 1.12 – 1.35-fold in freeze-dried control, 1.38 – 2.88-fold in encapsulated control, 1.08 – 2.44-fold in freeze-dried phytoene-rich, and 1.14 – 14.85-fold in encapsulated phytoene-rich. The disruptions in the matrix caused by the milling adds to the microstructural changes caused by ultrasounds. The mill pre-treatment reduces of the particle size due to particle fractures, allowing a greater contact surface, as well as the disruption of the robust cell wall of *C. sorokiniana*, thus, allowing a higher yield extraction (Stirk et al., 2020). Furthermore, the ultrasound application after the mill pre-treatment allows a higher cell disruption, and hence, a higher carotenoid release from the matrix. Other studies have also shown a significant ~ 1.2-fold and 1.5-fold higher extraction yield from freeze-dried *C. vulgaris* with the milling application compared to untreated and sonicated samples, respectively (Stirk et al., 2020). However, in the fresh samples, both control and phytoene-rich, the mill did not have the same effect, which could be related to the water content and structure of the matrix. These findings were expected, as in previous studies in our group, it has been seen that the application of the mill (5 min, 30 Hz) or the ultrasound (2 min, 20 kHz, 30%) resulted in the same carotenoid recovery ($p > 0.05$) in the fresh control *C. sorokiniana* (data not shown), while a higher 1.3-fold recovery of TCC was found in the mill-treated samples (30 Hz), in both freeze-dried control and encapsulated control matrices, compared to the ultrasound-treated samples (30% of amplitude). Thus, the prior step of the mill does not affect total carotenoid release in the fresh control matrix, but affects to freeze-dried control and encapsulated control *C. sorokiniana*. Further studies on the effect of the mill on the carotenoid extraction from wet biomass are required, as most studies are conducted on dry biomass.

4.3. Extraction efficiency of different solvents

The election of an appropriate solvent is crucial for the extraction of bioactive compounds. The extraction capacity of a solvent depends on the dielectric constant, polarity, viscosity, solvent penetration, and its interaction with the matrix (Patil & Akamanchi, 2017). More sustainable extraction methodologies and greener solvents are needed in connection with the growing importance of green analytical chemistry. In this context, the replacement of unsustainable petroleum-derived solvents whose toxicity raise concerns is desirable (Pacheco-Fernández & Pino, 2019). Thus, safer, sustainable and environmentally-friendly biobased solvents, such as MeTHF and ethyl lactate, are being sought (Hashemi et al., 2018).

The greatest differences in the extraction capacity of the solvents between samples extracted by UAE and samples extracted by UAE and milled were found when using MeTHF as a solvent in the case of

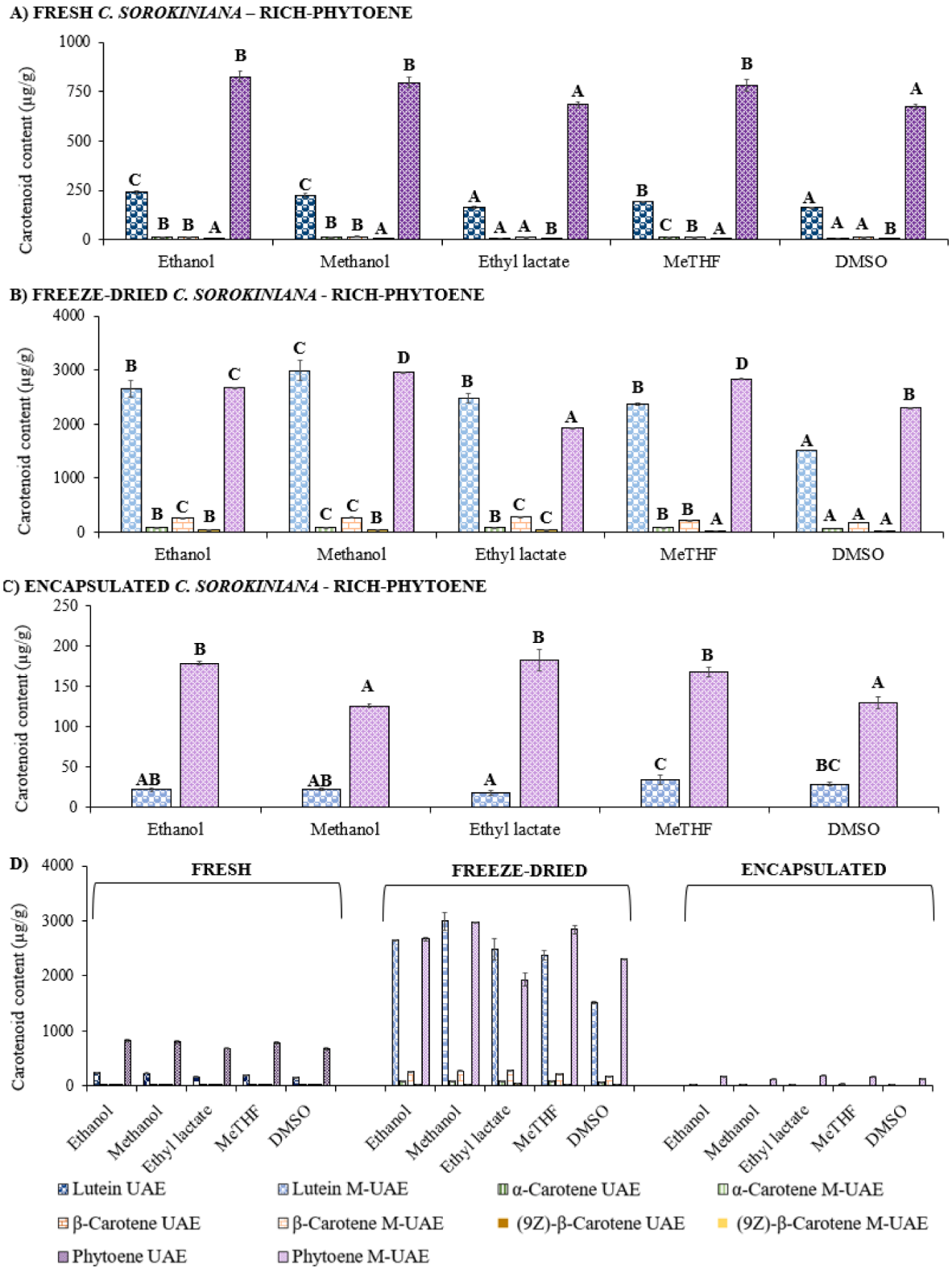


Fig. 5. Differences in the content of each carotenoid in *C. sorokiniana* phytoene-rich extracted with different solvents. For the fresh sample, the values after the extraction without the previous mill treatment are shown, since this treatment does not improve the extractability of the solvents in this matrix (as shown in Fig. 3.A); For the freeze-dried and encapsulated samples, the values after the extraction with the previous mill treatment is shown, since this treatment improve the extractability of the solvents in these matrices (as shown in Fig. 3.B and 3.C). Figures A, B, and C depict the carotenoid content in fresh, freeze-dried, and encapsulated phytoene-rich matrices, respectively. Figure D illustrates the carotenoid content in the three matrices on a standardized scale, showing the very low amount of carotenoids in the encapsulated matrix as compared with the fresh and freeze-dried samples. The error bars in the figures represent the standard deviation (SD). For the same carotenoid, different capital letters indicate statistically significant differences ($p < 0.05$) between solvents. MeTHF: methyloxolane; DMSO: dimethylsulfoxide.

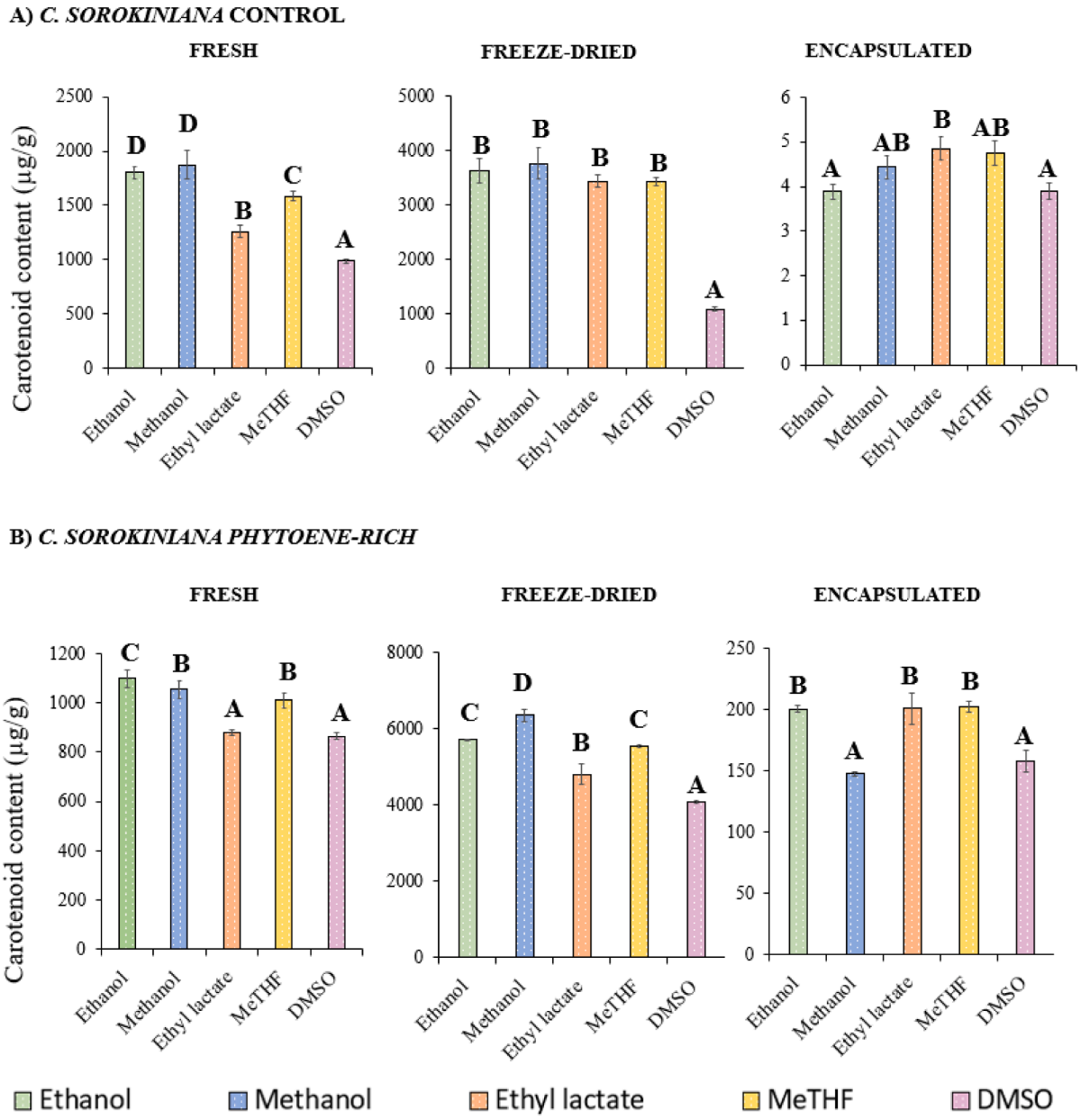


Fig. 6. Differences in the total carotenoid content (TCC) obtained with the different solvents in control and rich-phytoene *C. sorokiniana* samples. For the fresh samples, the values after the extraction without the previous mill treatment are shown, since this treatment does not improve the extractability of the solvents in these matrices (as shown in Fig. 2.A and 3.A); For the freeze-dried and encapsulated samples, the values after the extraction with the previous mill treatment is shown, since this treatment improve the extractability of the solvents in these matrices (as shown in Fig. 2.B, 2.C, 3.B, and 3.C). Different capital letters indicate statistically significant differences ($p < 0.05$) between solvents. MeTHF: methyloxolane; DMSO: dimethylsulfoxide.

encapsulated control, freeze-dried phytoene-rich and encapsulated phytoene-rich, with increases of 105-fold, 3-fold, and 25-fold, respectively.

DMSO is a solvent used for carotenoid extraction in different sources including microalgae (Wang et al., 2022). In the present study, DMSO has consistently been found to be one the least efficient extraction solvent. Contrastingly, in *Dunaliella parva*, carotenoid extraction with DMSO resulted in ~ 1.1-fold higher than with ethanol (Gan et al., 2022), suggesting that the structural (for instance presence or not of cell wall) and compositional characteristics of different microalgae can lead to important differences in carotenoid and other compounds extractability.

On the other hand, ethyl lactate and MeTHF resulted to be good extraction solvents for carotenoids in *C. sorokiniana* in most matrices, both in terms of individual carotenoids and TCC. The TCC obtained with MeTHF was from 1.2 to 3.2 times higher than that with DMSO, depending on the matrix. Compared to other solvents, MeTHF had a great extraction capacity for both polar (xanthophylls: lutein) and non-polar carotenoids (carotenes: α-carotene, β-carotene, and phytoene). MeTHF resulted in one of the best solvents for the extraction of phytoene in all the matrices, both fresh phytoene-rich (799.57 µg/g), freeze-dried phytoene-rich (2841.39 µg/g), and encapsulated phytoene-rich (202.74 µg/g), compared to the other four solvents. Phytoene recovery from methanol was similar to that of MeTHF in all of the matrices. Methanol and ethanol are solvents authorized by EFSA for food-grade applications, although methanol can produce toxicity in humans if ingested in large amounts, causing acidosis or retinal damage (Joshi & Adhikari, 2019). MeTHF is produced from lignocellulosic biomass, which is the most abundant biomass resource on earth and does not compete for land space. Furthermore, it can also be produced form starch-enriched algal biomass (Rengel et al., 2022). In addition, this solvent satisfy the seven

principles for being considered a green solvent (Rapinel et al., 2020). EFSA has recently evaluated the toxicity of MeTHF (also known as 2-methyloxolane), concluding that it does not raise important safety concern and establishing a tolerable daily intake of 1 mg of MeTHF per kg of body weight (Lambré et al., 2022). Based on the above-mentioned considerations and the obtained results, MeTHF could be considered as a promising sustainable solvent for the extraction of carotenoids from microalgae as an alternative to other organic solvents such as methanol, ethanol, and DMSO. Another positive feature of this solvent that makes it interesting for its use as a green solvent for carotenoid extraction is that it has some similarities with hexane, which has been used for many years by the industry for the extraction of lipophilic compounds. Thus, those solvents with properties similar to those of hexane could be easier introduced into the industry (Rapinel et al., 2020). Similarities between MeTHF and hexane include molecular weight (86.1 and 86.2 g/mol, respectively), boiling point (80 and 69 °C, respectively), or vaporization enthalpy (34 and 31 kJ/mol, respectively). Moreover, their physical properties could be interesting for industry due to its boiling point and its low enthalpy of vaporization (Smets et al., 2021). There are limited studies about the use of MeTHF as a carotenoid extraction solvent, and to the best of our knowledge, there are no studies on the extraction of carotenoids from microalgae using MeTHF.

Finally, ethyl lactate has shown similar or significantly higher (depending on the matrix) carotenoid extraction capacity for the (9*Z*)-β-carotene compared to MeTHF. Ethyl lactate has been proposed as an extraction solvent of carotenoids from tomato by-products (Szabo et al., 2022). In our study, ethyl lactate resulted in one of the best solvents for TCC in the freeze-dried control, encapsulated control, and encapsulated phytoene-rich matrices. However, ethyl lactate is less desirable for industry as the cost for solvent elimination is high, due to its high boiling point (154 °C) and high enthalpy of vaporization (49.2 kJ/mol) (Smets et al., 2021). Thus, MeTHF may be more desirable for industries than ethyl lactate, as it is a bio-based solvent and their elimination cost is lower (Smets et al., 2021), although life cycle assessments (LCA) studies need to be conducted to have more information about the environmental impact derived from the use of both solvents.

The observation that the amount of lutein extracted in the phytoene-rich freeze-dried samples was comparable if not higher than that of the colorless carotenoid phytoene, depending on the solvent is noteworthy and intriguing. However, in the phytoene-rich fresh and encapsulated, the amount of lutein was much lower than the amount of phytoene extracted. It might be that their release and/or stability in these matrices is higher than that of phytoene. From a chemical point of view lutein is a bicyclic xanthophyll containing two hydroxy groups whilst phytoene is a linear (without rings) carotene. Besides whereas lutein is in the (all-*E*) configuration, phytoene is largely present as the 15*Z* isomer. Further research in this respect is required as it can have important economic implications for the microalgal industry.

5. Conclusions

The optimal concentration of norflurazon of 1 μg/mL was chosen to stimulate phytoene accumulation and produce *C. sorokiniana* biomass with elevated phytoene levels. The ball mill pre-treatment did not affect at carotenoid release in fresh matrices, but it led to a higher carotenoid extraction from freeze-dried and encapsulated *C. sorokiniana*. MeTHF showed high TCC in all the matrices and it was a good solvent for the extraction of lutein, α-carotene, β-carotene and phytoene, which are carotenoids with different chemical structures and polarities. MeTHF is considered a promising green solvent for food applications due to its safety and physico-chemical characteristics, and it could be a more sustainable alternative to commonly used solvents such as methanol, ethanol, or DMSO for the extraction of carotenoids from microalgae. Further studies about the extraction of carotenoids with MeTHF are necessary.

CRediT authorship contribution statement

Ángeles Morón-Ortiz: Investigation, Formal analysis, Writing – original draft. **Paula Mapelli-Brahm:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Antonio León-Vaz:** Investigation, Formal analysis, Writing – original draft. **Ana M. Benitez-González:** Supervision. **Rosa León:** Conceptualization, Methodology, Supervision, Resources, Writing – review & editing. **Antonio J. Meléndez-Martínez:** Conceptualization, Methodology, Supervision, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Upon request the data agreed by the authors could be shared

Acknowledgments

This study was financially supported by grants PID2019-110438RB-C21 (NEWCARFOODS) and PID2019-110438RB-C22 (BESTALGAE4-CAROT), funded by MCIN/AEI/ 10.13039/501100011033. PMB was supported by a posdoc fellowship from Consejería de Transformación Económica, Industria, Conocimiento y Universidades de la Junta de Andalucía (PAIDI 2020). AMO was supported by a grant associated to grant PID2019-110438RB-C21 (NEWCARFOODS). A.L-V also thanks Next Generation European Funds and the Ministry of Universities of Spain for funding the Recualificación del Profesorado Universitario system. AMO, PMB, AMBG, and AJMM are members of the Spanish Carotenoid Network (CaRed), grant RED2022-134577-T, funded by MCIN/AEI/10.13039/501100011033.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137437.

References

Alvarez, A. L., Weyers, S. L., Goemann, H. M., Peyton, B. M., & Gardner, R. D. (2021). Microalgae, soil and plants: A critical review of microalgae as renewable resources for agriculture. In *Algal Research* (Vol. 54). https://doi.org/10.1016/j.algal.2021.102200.

Bazarnova, J., Nilova, L., Trukhina, E., Bernavskaya, M., Smyatskaya, Y., & Aktar, T. (2021). Use of microalgae biomass for fortification of food products from grain. *Foods, 10*(12). https://doi.org/10.3390/foods10123018

Breitenbach, J., Zhu, C., & Sandmann, G. (2001). Bleaching herbicide norflurazon inhibits phytoene desaturase by competition with the cofactors. *Journal of Agricultural and Food Chemistry, 49*(11), 5270–5272. https://doi.org/10.1021/jf0106751

Caporgno, M. P., & Mathys, A. (2018). Trends in Microalgae Incorporation Into Innovative Food Products With Potential Health Benefits. In *Frontiers in Nutrition* (Vol. 5). https://doi.org/10.3389/fnut.2018.00058.

Castillo, A., Pereira, S., Otero, A., Fiol, S., Garcia-Jares, C., & Lores, M. (2020). Matrix solid-phase dispersion as a greener alternative to obtain bioactive extracts from: Haematococcus pluvialis. Characterization by UHPLC-QToF. *RSC Advances, 10*(47). https://doi.org/10.1039/d0ra04378h

Chemat, F., Vian, M. A., Ravi, H. K., Khadhraoui, B., Hilali, S., Perino, S., & Tixier, A. S. F. (2019). Review of alternative solvents for green extraction of food and natural products: Panorama, principles, applications and prospects. In *Molecules* (Vol. 24, Issue 16). https://doi.org/10.3390/molecules24163007.

Diprat, A. B., Silveira Thys, R. C., Rodrigues, E., & Rech, R. (2020). Chlorella sorokiniana: A new alternative source of carotenoids and proteins for gluten-free bread. *LWT, 134*. https://doi.org/10.1016/j.lwt.2020.109974

Fernandes, A. S., Nascimento, T. C., Pinheiro, P. N., Vendruscolo, R. G., Wagner, R., de Rosso, V. V., Jacob-Lopes, E., & Zepka, L. Q. (2021). Bioaccessibility of microalgae-based carotenoids and their association with the lipid matrix. *Food Research International, 148*, Article 110596. https://doi.org/10.1016/J.FOODRES.2021.110596

Gan, S., Liang, S., Zou, Q., & Shang, C. (2022). Optimization of carotenoid extraction of a halophilic microalgae. *PLoS ONE, 17*(8 August), 1–12. https://doi.org/10.1371/journal.pone.0270650

Hashemi, B., Zohrabi, P., & Dehdashtian, S. (2018). Application of green solvents as sorbent modifiers in sorptive-based extraction techniques for extraction of environmental pollutants. In *TrAC - Trends in Analytical Chemistry* (Vol. 109). https://doi.org/10.1016/j.trac.2018.09.026.

Joshi, D. R., & Adhikari, N. (2019). An Overview on Common Organic Solvents and Their Toxicity. *Journal of Pharmaceutical Research International*. https://doi.org/10.9734/jpri/2019/v28i330203

Lambré, C., Barat Baviera, J. M., Bolognesi, C., Chesson, A., Cocconcelli, P. S., Crebelli, R., Gott, D. M., Grob, K., Lampi, E., Mengelers, M., Mortensen, A., Steffensen, I. L., Tlustos, C., Van Loveren, H., Vernis, L., Zorn, H., Bignami, M., Fürst, P., Tard, A., & Van Haver, E. (2022). Safety assessment of 2-methyloxolane as a food extraction solvent. *EFSA Journal, 20*(3). https://doi.org/10.2903/j.efsa.2022.7138

León-Vaz, A., León, R., Díaz-Santos, E., Vigara, J., & Raposo, S. (2019). Using agro-industrial wastes for mixotrophic growth and lipids production by the green microalga Chlorella sorokiniana. *New Biotechnology, 51*. https://doi.org/10.1016/j.nbt.2019.02.001

León, R., & Galván, F. (1994). Halotolerance studies on Chlamydomonas reinhardtii: Glycerol excretion by free and immobilized cells. *Journal of Applied Phycology, 6*(1). https://doi.org/10.1007/BF02185898

León, R., Vila, M., Hernánz, D., & Vílchez, C. (2005). Production of phytoene by herbicide-treated microalgae Dunaliella bardawil in two-phase systems. *Biotechnology and Bioengineering, 92*(6). https://doi.org/10.1002/bit.20660

Mapelli-Brahm, P., Barba, F. J., Remize, F., Garcia, C., Fessard, A., Mousavi Khaneghah, A., Sant'Ana, A. S., Lorenzo, J. M., Montesano, D., & Meléndez-Martínez, A. J. (2020). The impact of fermentation processes on the production, retention and bioavailability of carotenoids: An overview. In *Trends in Food Science and Technology* (Vol. 99). https://doi.org/10.1016/j.tifs.2020.03.013.

Mapelli-Brahm, P., Desmarchelier, C., Margier, M., Reboul, E., Meléndez Martínez, A. J., & Borel, P. (2018). Phytoene and Phytofluene Isolated from a Tomato Extract are Readily Incorporated in Mixed Micelles and Absorbed by Caco-2 Cells, as Compared to Lycopene, and SR-BI is Involved in their Cellular Uptake. *Molecular Nutrition and Food Research, 62*(22). https://doi.org/10.1002/mnfr.201800703

Meléndez-Martínez, A. J., Mandić, A. I., Bantis, F., Böhm, V., Borge, G. I. A., Brnčić, M., Bysted, A., Cano, M. P., Dias, M. G., Elgersma, A., Fikselová, M., García-Alonso, J., Giuffrida, D., Gonçalves, V. S. S., Hornero-Méndez, D., Kljak, K., Lavelli, V., Manganaris, G. A., Mapelli-Brahm, P., … O'Brien, N. (2022). A comprehensive review on carotenoids in foods and feeds: status quo, applications, patents, and research needs. In *Critical Reviews in Food Science and Nutrition* (Vol. 62, Issue 8). https://doi.org/10.1080/10408398.2020.1867959.

Meléndez-Martínez, A. J., & Mapelli-Brahm, P. (2021). The undercover colorless carotenoids phytoene and phytofluene: Importance in agro-food and health in the Green Deal era and possibilities for innovation. In *Trends in Food Science and Technology* (Vol. 116). https://doi.org/10.1016/j.tifs.2021.07.028.

Meléndez-Martínez, A. J., Paulino, M., Stinco, C. M., Mapelli-Brahm, P., & Wang, X. D. (2014). Study of the time-course of cis/trans (Z/E) isomerization of lycopene, phytoene, and phytofluene from tomato. *Journal of Agricultural and Food Chemistry, 62*(51). https://doi.org/10.1021/jf5041965

Meléndez-Martínez, A. J., Stinco, C. M., & Mapelli-Brahm, P. (2019). Skin carotenoids in public health and nutricosmetics: The emerging roles and applications of the UV radiation-absorbing colourless carotenoids phytoene and phytofluene. In *Nutrients* (Vol. 11, Issue 5). https://doi.org/10.3390/nu11051093.

Ordóñez-Santos, L. E., Esparza-Estrada, J., & Vanegas-Mahecha, P. (2021). Ultrasound-assisted extraction of total carotenoids from mandarin epicarp and application as natural colorant in bakery products. *LWT, 139*. https://doi.org/10.1016/j.lwt.2020.110598

Pacheco-Fernández, I., & Pino, V. (2019). Green solvents in analytical chemistry. In *Current Opinion in Green and Sustainable Chemistry* (Vol. 18). https://doi.org/10.1016/j.cogsc.2018.12.010.

Patil, D. M., & Akamanchi, K. G. (2017). Microwave assisted process intensification and kinetic modelling: Extraction of camptothecin from Nothapodytes nimmoniana plant. *Industrial Crops and Products, 98*. https://doi.org/10.1016/j.indcrop.2017.01.023

Rapinel, V., Claux, O., Abert-Vian, M., McAlinden, C., Bartier, M., Patouillard, N., Jacques, L., & Chemat, F. (2020). 2-methyloxolane (2-MeOx) as sustainable lipophilic solvent to substitute hexane for green extraction of natural products. Properties, applications, and perspectives. In *Molecules* (Vol. 25, Issue 15). https://doi.org/10.3390/molecules25153417.

Ren, Y., Sun, H., Deng, J., Huang, J., & Chen, F. (2021). Carotenoid production from microalgae: Biosynthesis, salinity responses and novel biotechnologies. In *Marine Drugs* (Vol. 19, Issue 12). https://doi.org/10.3390/md19120713.

Rengel, R., Giraldez, I., Díaz, M. J., García, T., Vigara, J., & León, R. (2022). Simultaneous production of carotenoids and chemical building blocks precursors from chlorophyta microalgae. *Bioresource Technology, 351*. https://doi.org/10.1016/j.biortech.2022.127035

Sampaio, G. L. A., Pacheco, S., Ribeiro, A. P. O., Galdeano, M. C., Gomes, F. S., & Tonon, R. V. (2019). Encapsulation of a lycopene-rich watermelon concentrate in alginate and pectin beads: Characterization and stability. *LWT, 116*. https://doi.org/10.1016/j.lwt.2019.108589

Serive, B., Kaas, R., Bérard, J. B., Pasquet, V., Picot, L., & Cadoret, J. P. (2012). Selection and optimisation of a method for efficient metabolites extraction from microalgae. *Bioresource Technology, 124*. https://doi.org/10.1016/j.biortech.2012.07.105

Smets, R., Goos, P., Claes, J., & Van Der Borght, M. (2021). Optimisation of the lipid extraction of fresh black soldier fly larvae (Hermetia illucens) with 2-methyltetrahydrofuran by response surface methodology. *Separation and Purification Technology, 258*. https://doi.org/10.1016/j.seppur.2020.118040

Stinco, C. M., Benítez-González, A. M., Meléndez-Martínez, A. J., Hernanz, D., & Vicario, I. M. (2019). Simultaneous determination of dietary isoprenoids (carotenoids, chlorophylls and tocopherols) in human faeces by Rapid Resolution Liquid Chromatography. *Journal of Chromatography A, 1583*. https://doi.org/10.1016/j.chroma.2018.11.010

Stirk, W. A., Bálint, P., Vambe, M., Lovász, C., Molnár, Z., van Staden, J., & Ördög, V. (2020). Effect of cell disruption methods on the extraction of bioactive metabolites from microalgal biomass. *Journal of Biotechnology, 307*. https://doi.org/10.1016/j.jbiotec.2019.10.012

Szabo, K., Teleky, B. E., Ranga, F., Roman, I., Khaoula, H., Boudaya, E., Ltaief, A. B., Aouani, W., Thiamrat, M., & Vodnar, D. C. (2022). Carotenoid Recovery from Tomato Processing By-Products through Green Chemistry. *Molecules, 27*(12). https://doi.org/10.3390/molecules27123771

The Chlamydomonas Sourcebook. (1989). In *The Chlamydomonas Sourcebook*. https://doi.org/10.1016/c2009-0-02778-0.

The European Parliament and the council of The European Union. (2009). 2009/32/Ec. *Official Journal of the European Union, 10*, 3–11.

Villanueva-Bermejo, D., Reglero, G., & Fornari, T. (2017). Recent advances in the processing of green tea biomolecules using ethyl lactate. A review. In *Trends in Food Science and Technology* (Vol. 62). https://doi.org/10.1016/j.tifs.2016.12.009.

Wang, M., Morón-ortiz, Á., Zhou, J., Benítez-gonzález, A., Mapelli-brahm, P., Meléndez-martínez, A. J., & Barba, F. J. (2022). Effects of Pressurized Liquid Extraction with dimethyl sulfoxide on the recovery of carotenoids and other dietary valuable compounds from the microalgae Spirulina, Chlorella and Phaeodactylum tricornutum. *Food Chemistry, 134885*. https://doi.org/10.1016/j.foodchem.2022.134885

Yara-Varón, E., Fabiano-Tixier, A. S., Balcells, M., Canela-Garayoa, R., Bily, A., & Chemat, F. (2016). Is it possible to substitute hexane with green solvents for extraction of carotenoids? A theoretical versus experimental solubility study. *RSC Advances, 6*(33). https://doi.org/10.1039/c6ra03016e

Zhou, J., Wang, M., Saraiva, J. A., Martins, A. P., Pinto, C. A., Prieto, M. A., Simal-Gandara, J., Cao, H., Xiao, J., & Barba, F. J. (2022). Extraction of lipids from microalgae using classical and innovative approaches. *Food Chemistry, 384*, Article 132236. https://doi.org/10.1016/j.foodchem.2022.132236