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Assessment of milling and the green biosolvents ethyl lactate and 2-methyltetrahydrofuran (2-methyloxolane) for the ultrasound-assisted extraction of carotenoids in common and phytoene-rich *Dunaliella bardawil* microalgae

Ángeles Morón-Ortiz [a], Paula Mapelli-Brahm [a,*], Antonio León-Vaz [b], Ana M. Benitez-González [a], Andrés N. Martín-Gómez [b], 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, Center for Natural Resources, Health and Environment (RENSMA), University of Huelva, 2110, Huelva, Spain

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ABSTRACT

This study aimed to evaluate the efficiency of green solvents (ethyl lactate and 2-methyltetrahydrofuran (MeTHF)) compared to conventional solvents (ethanol, methanol, and dimethyl sulfoxide) in the ultrasound-assisted extraction of carotenoids from *Dunaliella bardawil*. Two types of algae were evaluated, a control (common) and a phytoene-enriched algae obtained by treatment with norfluorazon. The extractions were performed on fresh, freeze-dried, and encapsulated samples, with and without ball-milling pre-treatment. The pre-treatment had no significant effect on carotenoid extraction for any of the solvents tested. The solvents that achieved the highest carotenoid yield from fresh samples were MeTHF, methanol, and ethanol (control: 2105.3, 2001.8, and 1919.1 μg/g, respectively; phytoene-rich: 3220.9, 3669.1, and 3275.0 μg/g, respectively). In freeze-dried samples, ethanol was most effective in the control strain (14337.1 μg/g), while methanol yielded the most in the phytoene-rich strain (8464.1 μg/g). For encapsulated samples, MeTHF and ethanol were the top performers (control: 421.0 and 394.0 μg/g, respectively; phytoene-rich: 186.9 and 166.4 μg/g, respectively). Overall, the green solvent MeTHF proved to be a promising alternative to traditional solvents in the food industry.

1. Introduction

Microalgae are a diverse group of microorganisms found in freshwater and marine systems that have gained significant attention due to their potential benefits to human health. These eukaryotic organisms are characterized by their ability to grow in diverse environments and extreme conditions. The metabolic adaptability of microalgae enables them to synthesize diverse compounds with potential uses in various biotechnology fields, such as food, energy, health, environment, and biomaterials. They offer other advantages, such as higher growth and productivity relative to plants; the possibility of growth under stress conditions that can induce the production of some desired metabolites; lower requirements of water and nutrients; contribution to CO_2 sequestration and wastewaters bioremediation, etc. More than 20 genera of cyanobacteria and microalgae are currently used for food or feed applications. Research in these organisms is expected to continue growing due to the need to further tap into aquatic ecosystems in order to contribute to the production of health-promoting and sustainable foods (Mapelli-Brahm et al., 2023). All microalgae biosynthesize carotenoids, which are very versatile compounds of great interest in agri-food, health, and cosmetics. Thus, some carotenoids are precursors of vitamin A and their consumption can contribute to maintaining healthy vision, skin, reproduction, brain, and immune function, among others (Meléndez-Martínez, 2019). In addition, carotenoid intake has been linked to a reduced likelihood of suffering from certain chronic diseases, such as cancer, cardiovascular diseases, eye, skin, or metabolic conditions, among others (Mapelli-Brahm et al., 2020). As a group, apart from common dietary carotenoids (lutein, β-carotene, astaxanthin, etc.), some microalgae biosynthesize uncommon carotenoids (diadinoxanthin, diatoxanthin, etc.) that have been scarcely studied. Tapping into understudied carotenoids and sources has been advocated by the COST Action EUROCAROTEN (https://www.cost.eu/actions) and the Ibero-American network IBERCAROT (http://www.cyted.org) to foster innovative developments in the field (Mapelli-Brahm et al., 2023; A. J. Meléndez-Martínez et al., 2021).

Among the most widely commercialized large-scale cultivated microalgae the genus *Dunaliella* stands out by its exceptional ability to

* Corresponding author.
E-mail address: pmapelli@us.es (P. Mapelli-Brahm).

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Abbreviations

DMSO	Dimethyl sulfoxide
MeTHF	2-Methyltetrahydrofuran
M-UAE	Samples treated with a mill and extracted by ultrasound-assisted extraction
TCC	Total carotenoid content
UAE	Ultrasound-assisted extraction

tolerate a wide range of salt concentrations (0.05–5.5 M NaCl). This genus is widely distributed across different environments and currently comprises 28 identified species, mostly marine and halotolerant (Roy et al., 2021; Sindhu et al., 2022) and many promising and profitable for various commercial applications, such as the production of proteins, biodiesel, colorants and antioxidants.

Dunaliella bardawil is one of the many species belonging to the genus *Dunaliella*. This microalga is known for its ease of cultivation on a large scale and its outstanding ability to accumulate elevated levels of β-carotene under specific environmental conditions, such as high light, nutrient deficiency, and high salinity. Thus, it can reach up to 9% of its dry weight (DW) in β-carotene (Roy et al., 2021; Xie et al., 2022). Indeed *D. salina* has been long exploited for the commercial production of β-carotene (Mapelli-Brahm et al., 2023). *D. salina* is, under certain circumstances, also amenable for the production of phytoene (Mazzucchi et al., 2022; Xu & Harvey, 2020). Carotenoids are generally known for their intense colors; however, phytoene and phytofluene are two carotenoids that have the peculiarity of being colorless. This characteristic could be one of the main reasons why these carotenoids have been largely neglected in research studies involving carotenoids in the field of agro-food, nutrition, and health until recently. Nevertheless, there is clear evidence that these carotenoids are present in widely consumed foods (tomatoes, carrots, citrus, apricots, watermelon, peppers, etc.), are easily absorbed by the body, and may contribute to promoting health. Thus, the interest in these colorless carotenoids is increasing (Mapelli-Brahm & Meléndez-Martínez, 2021).

Phytoene and phytofluene have garnered attention for their potential use in nutricosmetics, given their strong absorption of UV radiation and their possible skin-whitening properties. Besides, there is evidence that they may produce antioxidant, anti-inflammatory, and anticarcinogenic effects (Meléndez-Martínez et al., 2019).

Phytoene is the precursor of virtually all carotenoids. The biosynthesis of carotenoids from phytoene can be interrupted by inhibiting phytoene desaturase, the key enzyme involved in the conversion of phytoene to other carotenoids. The bleaching herbicide norflurazon, commonly used in agriculture, has been demonstrated to be an effective inhibitor of this enzyme both in higher plants and in several microalgal species (León et al., 2005; Molina-Márquez et al., 2019; Xu & Harvey, 2020), promoting the accumulation of phytoene in plant tissues. Thus, by optimizing the concentration of norflurazon, it is possible to promote the accumulation of this carotene (Molina-Márquez et al., 2019).

In recent years, interest in greener biosolvents has increased significantly, as they provide a more environmentally friendly alternative to conventional solvents, some of which are derived from petroleum and raise safety and environmental concerns. Among the properties for green solvents are obtaining from renewable sources (for instance carbohydrate feedstocks), high biodegradability, low toxicity and minimal environmental impact. These characteristics make them a desirable option for the extraction of compounds used in various applications including food, pharmaceuticals, nutraceuticals, and cosmetics (Chemat et al., 2019). Green chemistry is a discipline that focuses on the design, development, and implementation of chemical processes that reduce or eliminate the use and generation of hazardous substances. In addition to green solvents, green techniques, such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), or pressurized liquid extraction (PLE), among others, are an essential component of green chemistry, as they offer several advantages (including enhanced extraction, reduction of solvent or rapidity, among others) over traditional techniques (Chemat et al., 2019; Picot-Allain et al., 2021).

UAE has gained popularity in recent years due to its ability to extract compounds from various natural sources with high efficiency and low environmental impact. One of the main advantages of the UAE is that it can significantly reduce the extraction time and energy consumption, while increasing extraction yield, compared to other traditional extraction methods, such as Soxhlet or solvent extraction (Mushtaq et al., 2020).

In the present work, the objective was to examine the diverse capabilities of novel bio-based (ethyl lactate and 2-methyltetrahydrofuran) and conventional organic solvents (dimethyl sulfoxide, ethanol, methanol) to extract by UAE different carotenoids from the commercially important genus *Dunaliella bardawil*, under different presentations (fresh, freeze-dried, and encapsulated), and to assess the impact of milling on these extractions.

2. Methodology

2.1. Reagents

Tert-butyl methyl ether (HPLC-grade) was purchased from Honeywell (Seelze, Germany); ethyl lactate and norflurazon from Supelco (Bellefonte, PA, USA); 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. Microalgae and culture conditions

Dunaliella bardawil (UTEX 2538, hereafter named control *D. bardawil*) was obtained from the UTEX Culture Collection of Microalgae (University of Texas, Austin). Standard cultures were grown in modified Johnson's *Dunaliella* medium as described by Johnson et al. (1968), with 2 M NaCl, under controlled conditions of temperature (25 °C) in a thermostatic chamber, bubbled with air containing 3% (v/v) CO_2, continuously agitated (100 rpm) and illuminated with cool white and daylight from fluorescent lamps (light intensity 100 μE m^2/s PAR). The light intensity was monitored using a Delta OHM quantum photo radiometer equipped with a PAR sensor.

To induce the accumulation of phytoene, *D. bardawil* was incubated with the pyridazinone herbicide norflurazon following the procedure described in León et al. (2005). Briefly, well grown standard cultures (0.5 L) were used to inoculate new cultures with fresh 2 M NaCl Johnson's medium (4 L), supplemented with 10 μg/mL of the herbicide norflurazon (optimized concentration) and cultured for 8–10 days. The microalgae thus treated will be hereafter named phytoene-rich *D. bardawil*.

Once the cultures (standard and phytoene-rich matrices) reached the desired biomass content (about 0.6–0.8 g/L DW, they were harvested by centrifugation and either lyophilized in a Telstar freeze dryer (Terrassa, Spain), encapsulated in alginate beads, as detailed below, or kept at −80 °C for further extraction treatments (Supplementary Figs. 1A–C). Once the cultures (standard and phytoene-rich matrices) reached the desired biomass content (approximately 0.6–0.8 g/L DW), the biomass content was determined by analyzing the DW of the cultures, as previously described (Morón-Ortiz et al., 2024).

2.3. Immobilization of D. bardawil by entrampment in alginate hydrogel beads

Standard and phytoene-rich cultures of *D. bardawil* were harvested

by centrifugation, resuspended in fresh Johnson's medium with reduced salinity (0.5 M NaCl) and thoroughly mixed with an equal volume of a sterile solution of sodium alginate (6%, w/v) from *Macocystis pyrifera* (medium viscosity), prepared by autoclaving for 20 min a 120 °C. Hydrogel beads of about 3 mm diameter were obtained by pumping slowly the alginate-cell mixture through a needle into a cold solution of 0.2 M $CaCl_2$ (Johnson et al., 1968). The whole process was preformed under sterile conditions in a laminar flow cabin. Beads were maintained at 4 °C until their use.

2.4. Experimental design

Each matrix, that is, *D. bardawil* and phytoene-rich-*D. bardawil* microalgae (in three different presentations: fresh, freeze-dried, and encapsulated), was divided into five groups: the control unprocessed group and four groups with different ball-mill and ultrasound treatments. The ball-mill (MM400, Retsch, Germany) was applied for 5 min, with two different frequencies (5 and 30 Hz); the ultrasound (QSonica Ultrasonic Q500, 20 kHz) was applied for 2 min with two different amplitudes (30 and 70%).

2.5. Extraction of carotenoids from microalgae

The efficiency in extracting carotenoids with five solvents (ethanol, methanol, ethyl lactate, DMSO, and MeTHF) was compared. Approximately 0.5 g of fresh and encapsulated matrices and 0.2 g of freeze-dried matrix were weighted and 2 mL of solvent was added to the sample. The different treatments (ball-mill and ultrasound) were applied. Then the samples were centrifuged and the supernatant was collected. The residue was extracted until the sample showed no color. The samples were concentrated in a rotary evaporator (Eppendorf Concentrator plus™, Eppendorf, Hamburg, Germany) for high-performance liquid chromatography (HPLC) analysis.

2.6. Chromatography analysis

Carotenoid quantification was conducted using a HPLC system, specifically an Agilent (Palo Alto, CA) 1260 Infinity II Prime LC System. This system was equipped with a diode array detector and a C_{30} column (3 μm, 150 × 4.6 mm) (YMC, Wilmington, NC). The carotenoid extracts were dissolved in 500 μL of ethyl acetate and 10 μL was injected into the system for analysis. The wavelengths monitored were 285 nm for the analysis of phytoene and 450 nm for the rest of the carotenoids. The mobile phase consisted of a mixture of methanol, *tert*-butyl methyl ether, and water, which was delivered at a flow rate of 1 mL/min through a linear gradient, as described in a study by Stinco et al. (2019). The carotenoid content was determined by external calibration as explained in that study. To determine the total carotenoid content, the sum of all individual carotenoids was calculated.

2.7. Statistical analysis

All experiments were conducted three times and statistical analysis was performed using the InfoStat software (2008 version). Analysis of variances (ANOVA) were performed and the Tukey's post-hoc test was used to evaluate differences between groups. The differences were considered statistically significant if the *P*-value was lower than 0.05.

3. Results

3.1. Carotenoid profile

The carotenoids identified in the control *D. bardawil* samples (fresh, freeze-dried, and encapsulated matrices) were lutein, (all-*E*)-β-carotene, zeaxanthin, α-carotene, (9*Z*)-antheraxanthin, and (9*Z*)-β-carotene (Chromatograms in Supplementary Figs. 2A–C).

In the case of all the phytoene-rich *D. bardawil* (fresh, freeze-dried, and encapsulated), the main carotenoids detected were (15*Z*)-phytoene, lutein, and (all-*E*)-β-carotene, but (all-*E*)-phytoene, (9*Z*)-antheraxanthin, zeaxanthin, and (9*Z*)-β-carotene were also identified in smaller amounts (Chromatograms in Supplementary Figs. 3A–F). On the other hand, violaxanthin was detected in all control and phytoene-rich matrices in minor quantities.

3.2. Effect of the mill treatment

There were no significant differences regarding individual carotenoids or total carotenoid content (TCC) between UAE group (samples extracted by UAE) and M-UAE group (samples treated with a mill and extracted by UAE) with any of the solvents and in any matrix (fresh control, freeze-dried control, encapsulated control, fresh phytoene-rich, freeze-dried phytoene-rich, and encapsulated phytoene-rich samples) (Figs. 1 and 2). This means that the mill pre-treatment did not enhance the UAE of carotenoids from *D. bardawil*.

3.3. Effect of the extraction solvent

3.3.1. Control D. bardawil

To evaluate the extraction capacity of the different solvents in the different matrices, the carotenoid concentration in the extract obtained with each solvent after applying UAE was compared (UAE group) (Fig. 3). To assess the extraction capacity of the solvents, the samples treated with the mill (M-UAE group) were not considered, as this treatment did not affect the carotenoid extraction (Section 3.2 and Fig. 1A–C).

The best solvents for the extraction of lutein in the fresh control *D. bardawil* (Fig. 3A) were methanol, ethanol, and MeTHF, without significant differences between them (1457.69, 1402.61, and 1344.58 μg/g, respectively). Regarding extraction of (9*Z*)-antheraxanthin, the best solvents were methanol, DMSO, and MeTHF (72.25, 71.10, and 70.19 μg/g, respectively). The solvent that extracted a significantly higher amount of the other carotenoids, i.e., zeaxanthin, α-carotene, and (all-*E*)-β-carotene was MeTHF (62.97, 96.36, and 465.09 μg/g, respectively). In the case of (9*Z*)-β-carotene, the extraction with MeTHF resulted in the highest yield, although there was no significant difference with ethanol.

In the freeze-dried matrix (Fig. 3B), the best solvents for lutein extraction were ethanol and methanol, showing a concentration of 10164.25 and 9029.15 μg/g, respectively. There were no significant differences between them. The highest zeaxanthin extraction was found with methanol (329.40 μg/g), ethanol (320.14 μg/g), and MeTHF (316.30 μg/g), without statistical differences between them. For (9*Z*)-antheraxanthin extraction, DMSO resulted in being the best solvent. Ethanol and MeTHF showed the highest extraction of (all-*E*)-β-carotene (2733.75 and 2493.92 μg/g, respectively). Finally, for (9*Z*)-β-carotene, ethanol, MeTHF, and ethyl lactate were the best solvents (406.92, 388.12, and 342.32 μg/g, respectively).

In the encapsulated matrix (Fig. 3C.), MeTHF and ethanol showed the best extractions for lutein (312.27 and 291.70 μg/g, respectively), α-carotene (16.37 and 17.58 μg/g, respectively), and (all-*E*)-β-carotene (55.33 and 53.50 μg/g, respectively). (9*Z*)-β-Carotene was best extracted with ethanol (12.43 μg/g), (9*Z*)-antheraxanthin with MeTHF (22.50 μg/g, without significant differences with ethyl lactate, DMSO, and methanol), and zeaxanthin with ethyl lactate (7.20 μg/g). Regarding TCC in fresh control (Fig. 6A), MeTHF showed the highest carotenoid extraction (2105.31 μg TCC/g), showing no statistical differences with methanol and ethanol (2001.80 and 1919.13 μg/g). The TCC recovered with MeTHF in the fresh control sample was 1.3 times higher than that of ethyl lactate, and 1.4 times higher than that of DMSO. In the freeze-dried control sample (Fig. 6B), the best solvent was ethanol (14337.13 μg/g), being a TCC 1.2, 1.3, 1.3, and 2-fold higher than that of methanol, ethyl lactate, MeTHF, and DMSO, respectively. Finally, in the

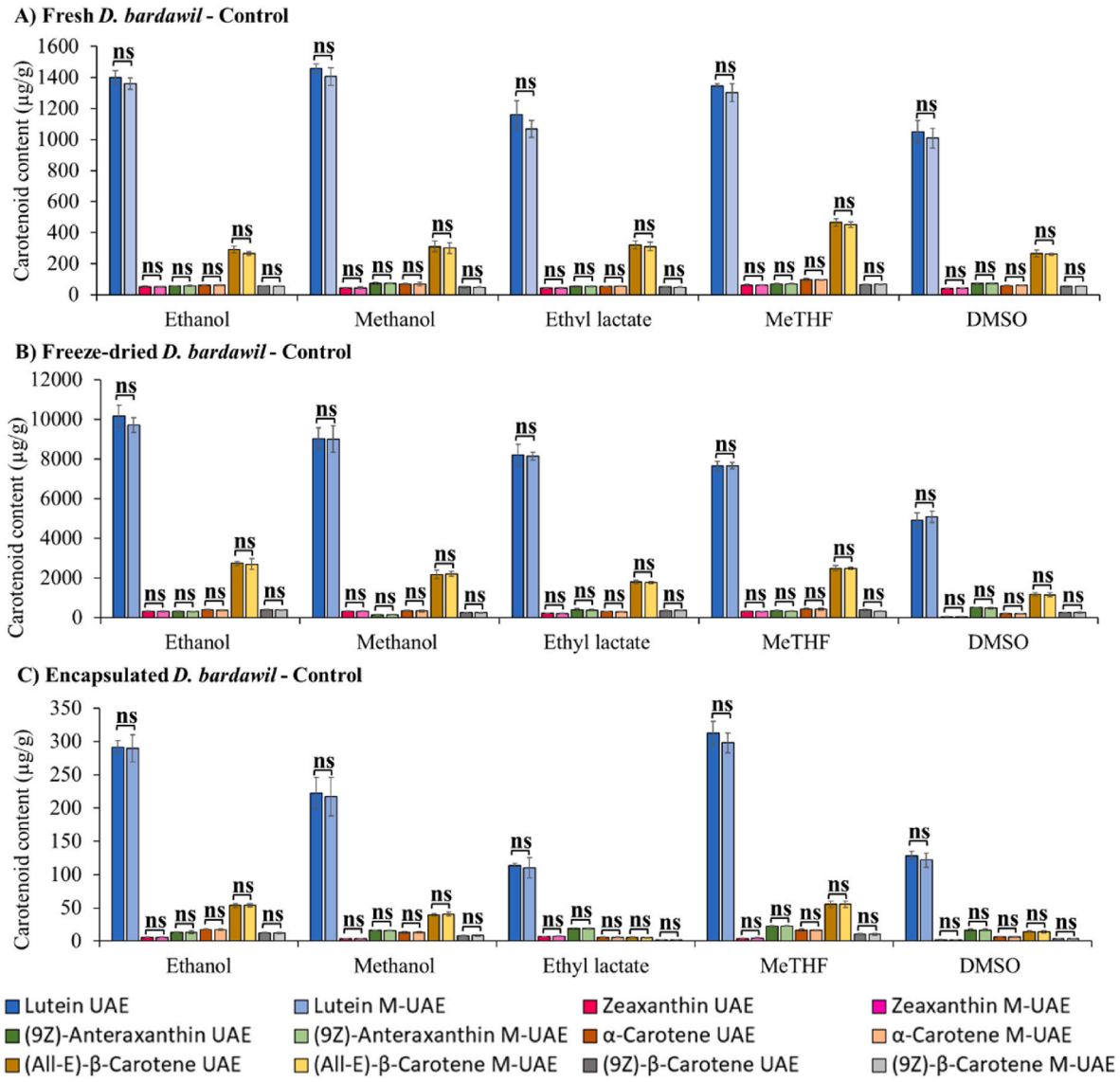

Fig. 1. Differences in individual carotenoid content in control *Dunaliella bardawil* between UAE (ultrasound-treated samples) and M-UAE (mill + ultrasound-treated samples), for each evaluated solvent. Figures A, B, and C depict the carotenoid content in fresh, freeze-dried, and encapsulated control matrices, respectively. The error bars in the figures represent the standard deviation (SD). NS: Not significant. MeTHF: 2-methyltetrahydrofuran; DMSO: dimethylsulfoxide.

encapsulated matrix (Fig. 6C), the best extraction solvent was MeTHF (421.00 µg/g), followed by ethanol (394.04 µg/g), with no significant differences between them.

3.3.2. *Phytoene-rich D. bardawil*

Norflurazon treated cells show an important decrease in all colored carotenoids, and an important accumulation of phytoene, mainly the isomer (15*Z*)-phytoene, followed by the all-*E*-phytoene. As in the control matrices, in the phytoene-rich matrices, the application of the mill did not have a significant effect on the extraction of individual carotenoids and TCC (Figs. 2 and 5); thus, the UAE group was chosen to compare the extraction capacity of the solvents.

In the fresh phytoene-rich matrix (Fig. 4A), (15*Z*)-phytoene was best extracted with methanol (1763.66 µg/g), MeTHF (1720.93 µg/g), and ethanol (1674.71 µg/g), with no significant differences between them. Regarding (all-*E*)-phytoene, the best solvent was methanol (442.59 µg/g).

In the freeze-dried phytoene-rich *D. bardawil* (Fig. 4B) the extraction of (15*Z*)-phytoene showed the highest concentration when methanol (3259.55 µg/g), MeTHF (3145.27 µg/g), or ethanol (2953.30 µg/g) were used as solvents, showing no significant differences between them. (All-*E*)-phytoene was best extracted with methanol (253.84 µg/g), ethanol (249.59 µg/g), ethyl lactate (237.90 µg/g), and MeTHF (237.90 µg/g) with no significant differences between them.

Finally, in the encapsulated phytoene-rich matrix (Fig. 4C), (15*Z*)-phytoene showed the best extraction concentration with DMSO (83.32 µg/g), MeTHF (82.07 µg/g), and ethanol (81.14 µg/g), with no significant differences between them. (All-*E*)-phytoene showed the highest concentration when DMSO or ethanol was applied, showing an extracted concentration of 6.87 µg/g and 6.35 µg/g, respectively.

The main carotenoids in the phytoene-enriched matrices were phytoene and lutein, although their concentrations varied between matrices. In the fresh phytoene-rich matrix, all the solvents and treatments showed a higher (15*Z*)-phytoene concentration compared to lutein. In addition, the encapsulated phytoene-enriched *D. bardawil* also contained a higher (15*Z*)-phytoene concentration compared to lutein, but in the group UAE by using ethyl lactate. Contrary to the phytoene-enriched fresh and encapsulated matrices, the concentration of lutein in the freeze-dried matrix was higher than that of (15*Z*)-phytoene in all solvents but ethyl lactate and DMSO, that extracted a similar

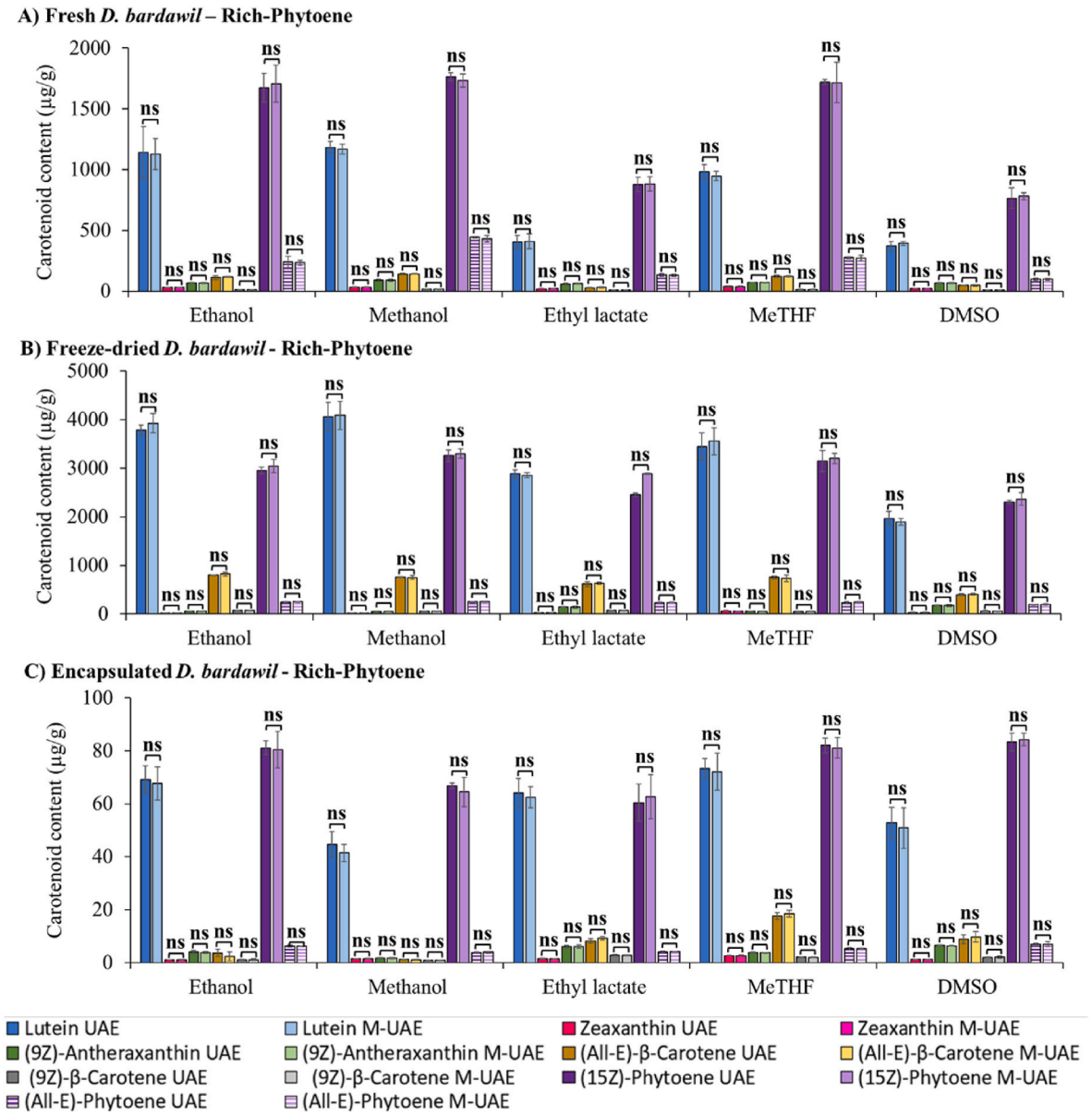

Fig. 2. Differences in individual carotenoid content in phytoene-rich *Dunaliella bardawil* between UAE (ultrasound-treated samples) and M-UAE (mill + ultrasound-treated samples), for each evaluated solvent. Figures A, B, and C depict the carotenoid content in fresh, freeze-dried, and encapsulated phytoene-rich matrices, respectively. The error bars in the figures represent the standard deviation (SD). MeTHF: 2-methyltetrahydrofuran; DMSO: dimethylsulfoxide.

concentration of both carotenoids.

Regarding TCC in the fresh phytoene-rich matrix (Fig. 6D), the best extraction solvents were methanol (3669.07 µg/g), ethanol (3274.98 µg/g), and MeTHF (3220.88 µg/g), with no statistic differences between them. Methanol was the best solvent for the total carotenoid extraction in the freeze-dried phytoene-rich matrix (8464.11 µg/g) (Fig. 6E), followed by ethanol (7936.34 µg/g), and MeTHF (7757.47 µg/g), with significant differences with methanol, resulting a TCC 1.1-fold higher in both cases compared to methanol. Regarding the encapsulated phytoene-rich matrix (Fig. 6F), MeTHF resulted in the highest TCC (181.65 µg/g), which was 1.1-fold higher than that of ethanol (166.44 µg/g), although there were no significant differences between them.

4. Discussion

The objective of this study was to compare the efficacy of biobased solvents (ethyl lactate and MeTHF) and conventional organic solvents (ethanol, methanol, and DMSO) in extracting carotenoids from common (control) and phytoene-rich *D. bardawil* through the utilization of UAE. The impact of a mill pre-treatment on the extraction process was also assessed. The study included fresh, freeze-dried, and encapsulated samples. Encapsulation of microalgae offers several advantages, such as improved stability and protection against contamination, which facilitates their handling and harvesting at an industrial level (Han et al., 2023). Among the different methods, encapsulation in calcium alginate hydrogel stands out for its low cost, biocompatibility and sustainability, in addition to being carried out under mild conditions without the need for high temperatures or aggressive chemicals (Adamiak & Sionkowska, 2023; Jia et al., 2024). In the case of *Dunaliella*, immobilization in polymeric matrices has been studied to increase stability, but its effect on carotenoid extraction efficiency had not yet been investigated (León et al., 2001; Thakur & Kumar, 1999).

4.1. Carotenoid profile

The carotenoids detected in the control matrices were mainly lutein,

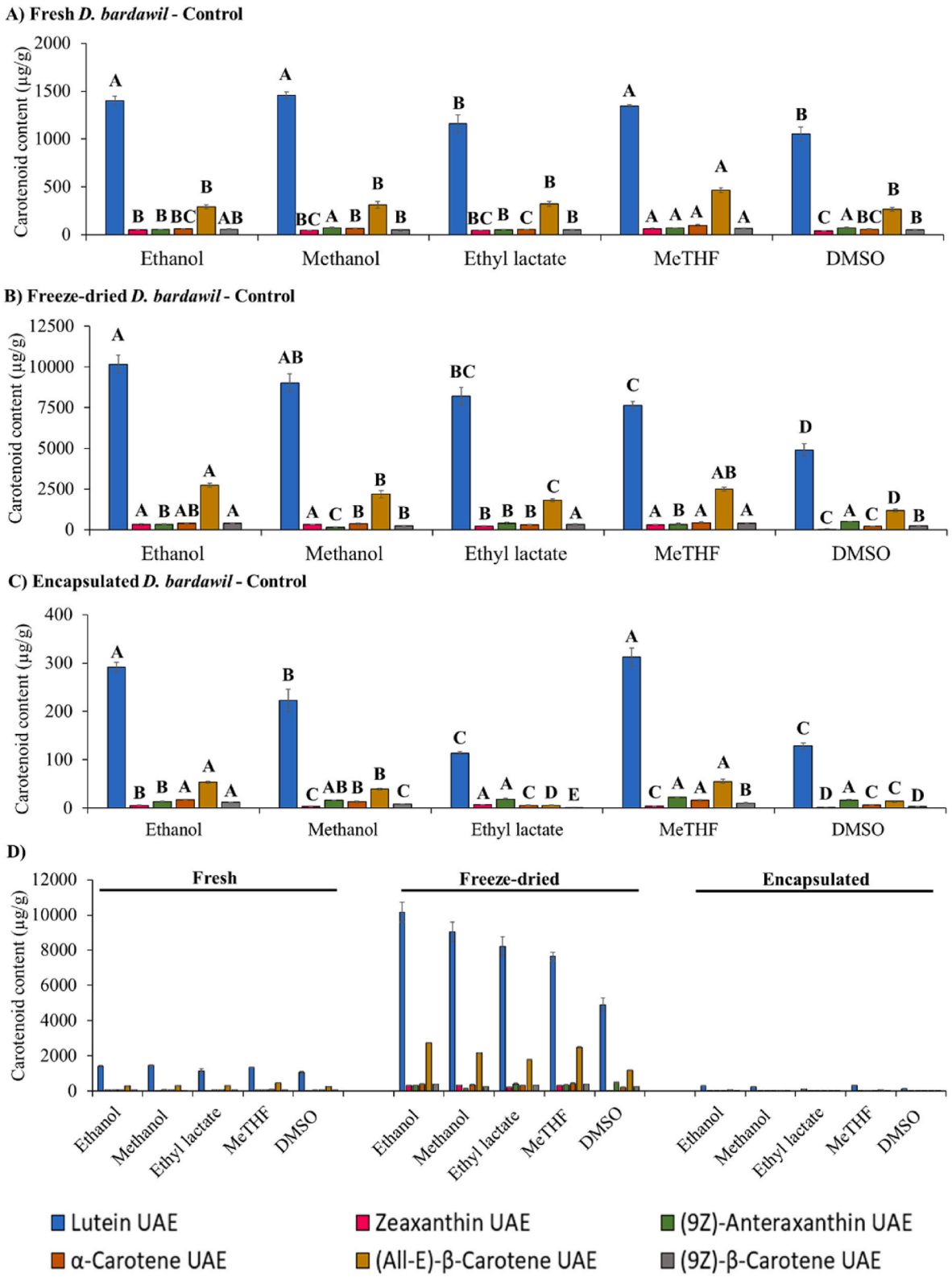

Fig. 3. Concentration of each carotenoid in control *Dunaliella bardawil* extracted with different solvents. For all the matrices, the values without the previous mill treatment are shown, since the mill pre-treatment does not improve the extractability of the solvents (as shown in Fig. 1). Figures A, B, and C depict the carotenoid concentration in the fresh, freeze-dried, and encapsulated control matrices, respectively. Figure D illustrates the carotenoid concentration in the three matrices on a standardized scale, showing the very low concentration 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: 2-methyltetrahydrofuran; DMSO: dimethylsulfoxide.

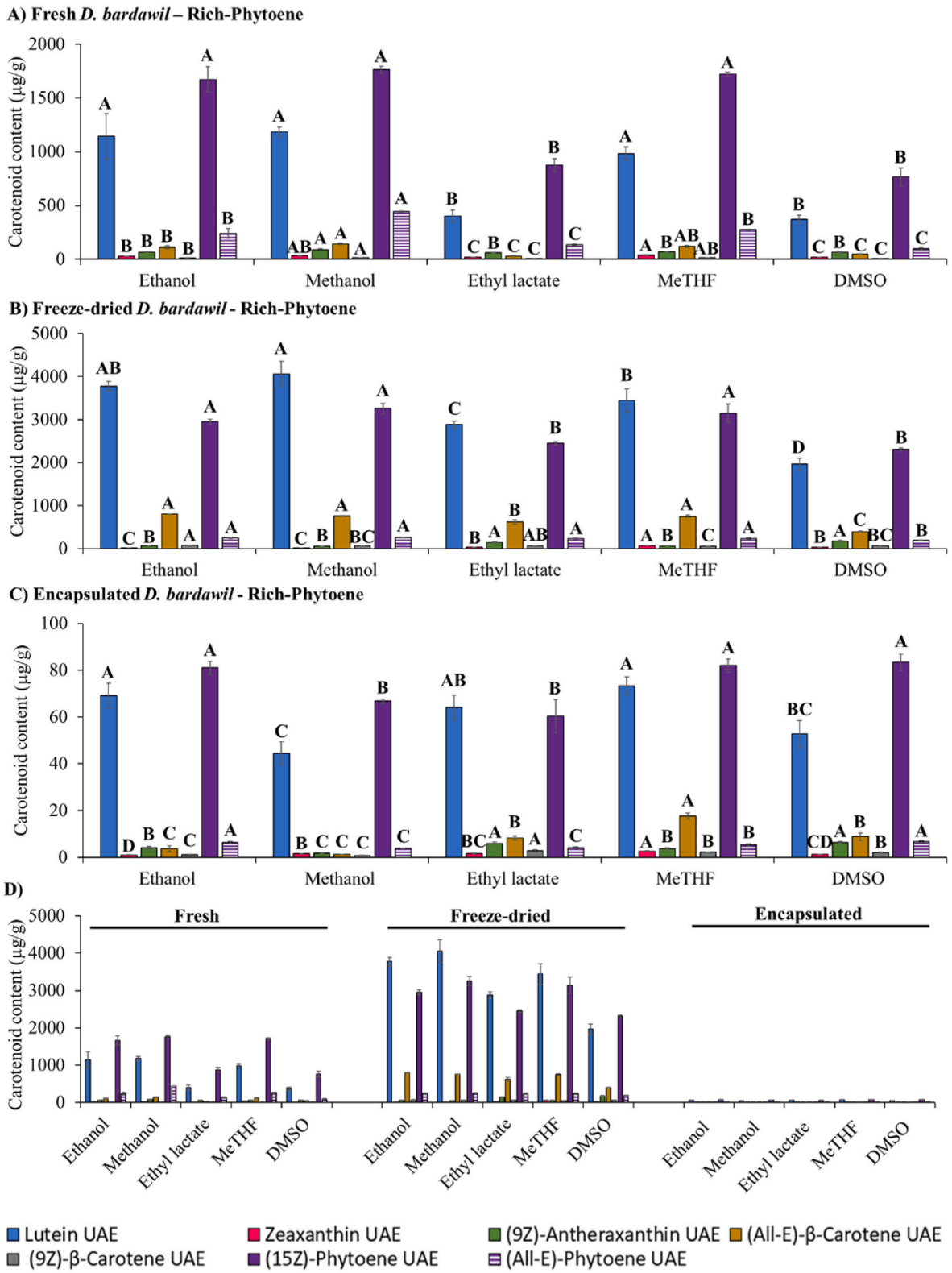

Fig. 4. Concentration of each carotenoid in phytoene-rich *Dunaliella bardawil* extracted with different solvents. For all the matrices, the values after the extraction without the previous mill treatment are shown, since this treatment does not improve the extractability of the solvents (as shown in Fig. 2). 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: 2-methyltetrahydrofuran; DMSO: dimethylsulfoxide.

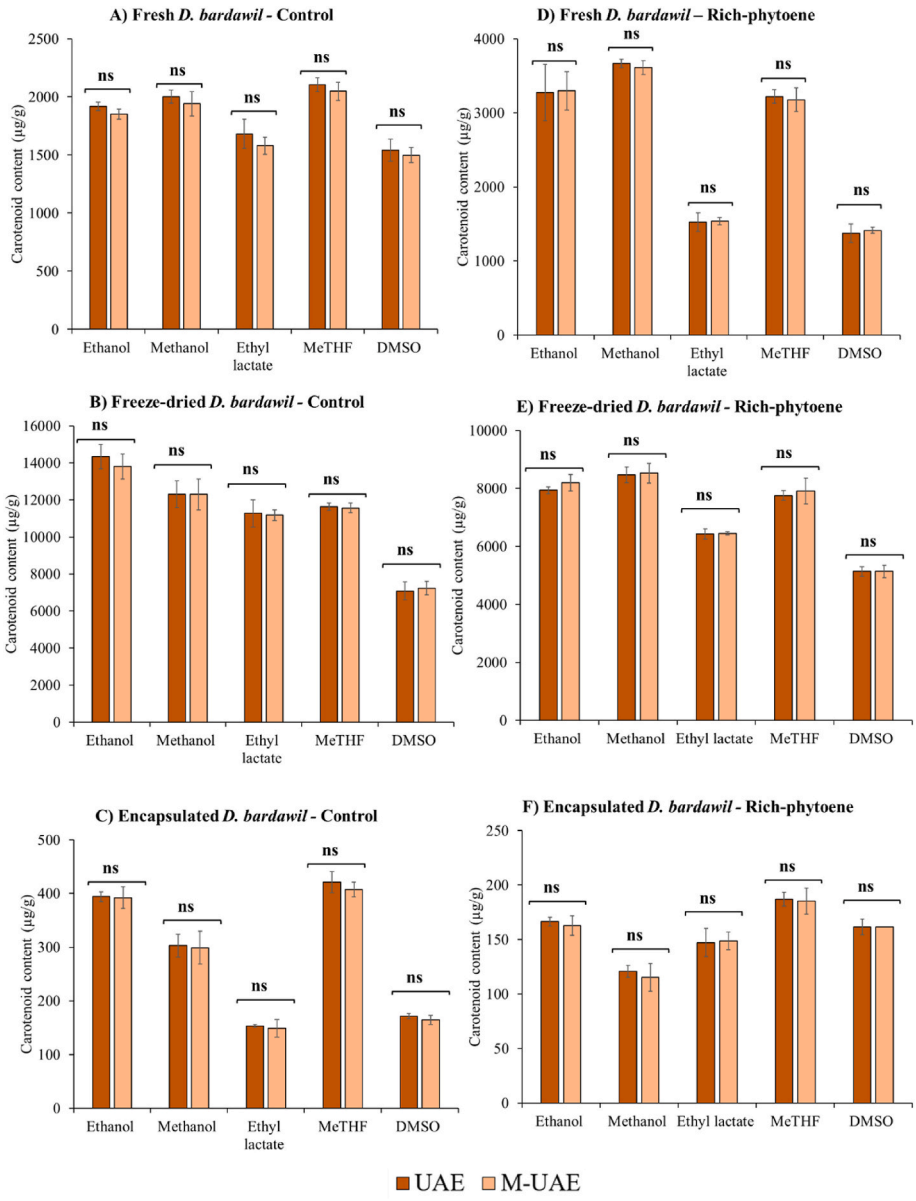

Fig. 5. Total carotenoid content in control *Dunaliella bardawil* (A: fresh; B: freeze-dried; C: encapsulated) and in phytoene-rich *Dunaliella bardawil* (D: fresh; E: freeze-dried; F: encapsulated). Ultrasound-assisted extracted samples with and without the previous mill treatment (M-UAE and UAE, respectively) are shown. The error bars in the figures represent the standard deviation (SD). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; NS: Not significant. MeTHF: 2-methyltetrahydrofuran; DMSO: dimethylsulfoxide.

followed by zeaxanthin, (9*Z*)-antheraxanthin, α-carotene, (all-*E*)-β-carotene, and (9*Z*)-β-carotene. The intermediate carotenoid phytoene, was not present in standard-grown control *D. bardawil*. These results are in accordance with other studies, in which lutein, zeaxanthin, α-carotene, and β-carotene were identified in *D. bardawil* (Xie et al., 2021). In the fresh control sample, the optimum solvent for the extraction of total carotenoid content was MeTHF, which was able to extract 465.09 µg/g of (all-*E*)-β-carotene and 1344.58 µg/g of lutein. It is noteworthy that these concentrations were higher than those found in other common foods. For example, in the case of fresh carrots, conventional solid-liquid extraction using tetrahydrofuran resulted in an extraction of 66.28 µg/g of (all-*E*)-β-carotene and 2.88 µg/g of lutein. Similarly, fresh spinach, subjected to the same conventional extraction method, exhibited contents of 42.29 µg/g of lutein (Granado et al., 1992). Taking into account the classification of dietary sources of carotenoids based on their respective carotenoid content by Britton and Khachik (2009), fresh control *D. bardawil* can be considered a food source with a very high total carotenoid content (>2 mg/100 g). In the phytoene-rich matrices, those carotenoids were found, in addition to (15*Z*)-phytoene and (all-*E*)-phytoene, the (15*Z*)-isomer clearly predominating over the (all-*E*)-counterpart, as is common for this carotenoid (Mapelli-Brahm & Meléndez-Martínez, 2021).

The TCC in the fresh matrix, averaged across all solvents used, was 1849.9 µg/g, while in the freeze-dried sample, it amounted to 11322.3 µg/g. When taking into account the humidity percentage of the sample (80.7%), the expected TCC of the freeze-dried sample (expressed on a fresh weight basis) would be 2185.22 µg/g. Therefore, considering the average values obtained with all the solvents evaluated, the extraction of total carotenoids in the freeze-dried microalgae was 1.2 times higher than that of the fresh samples. Consequently, the freeze-drying process, when applied before extraction, allows for a higher recovery of carotenoids compared to the fresh sample in *D. bardawil*. It is important to note that using 0.5 g of freeze-dried samples, comparable to the fresh sample amount, would have significantly increased the required solvent

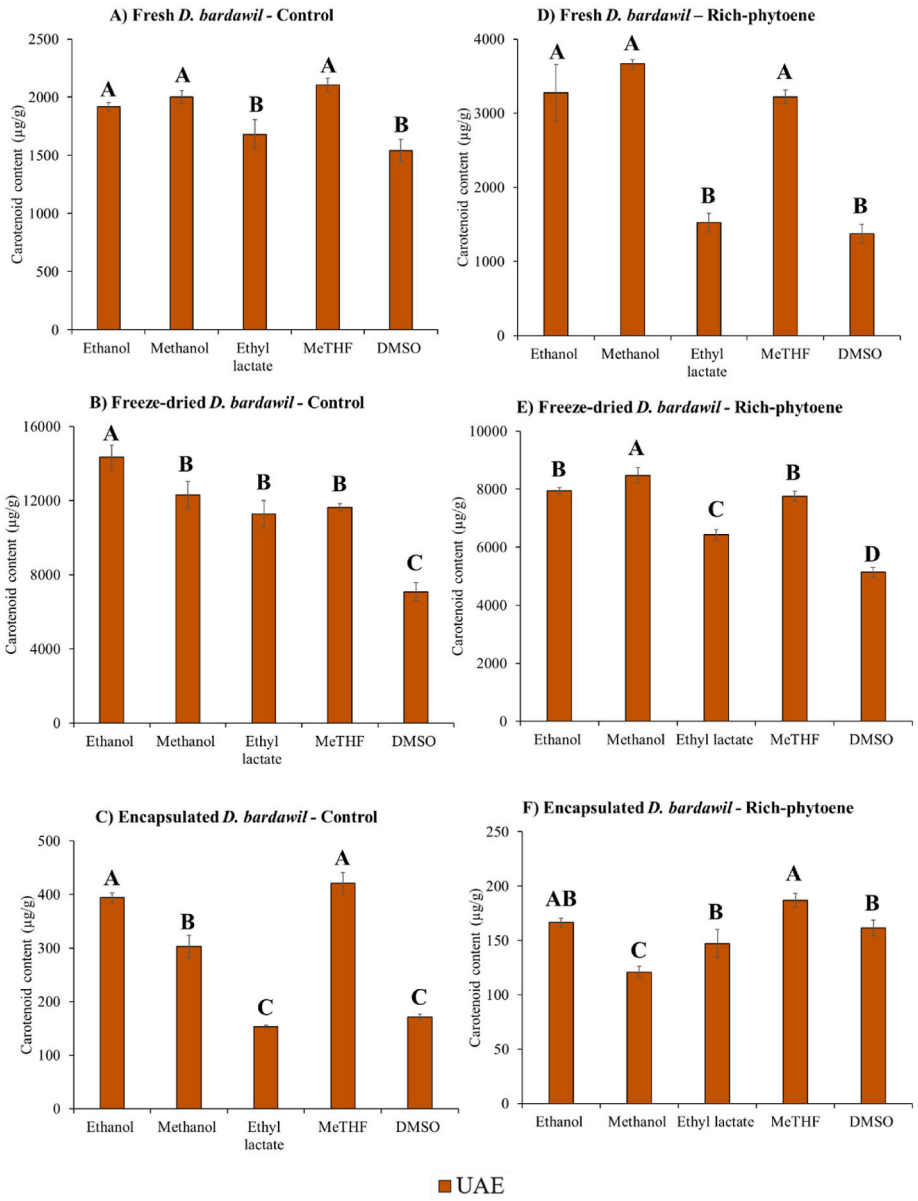

Fig. 6. Differences in TCC in control *Dunaliella bardawil* (A: fresh; B: freeze-dried; C: encapsulated) and phytoene-rich *Dunaliella bardawil* (D: fresh; E: freeze-dried; F: encapsulated) between solvent. The error bars in the figures represent the standard deviation (SD). The error bars in the figures represent the standard deviation (SD). Different capital letters indicate statistically significant differences ($p < 0.05$) between solvents. MeTHF: 2-methyltetrahydrofuran; DMSO: dimethylsulfoxide. Data from UAE is shown, as the ball mill (M-UAE) did not affect carotenoid extraction.

volume and, consequently, the duration of the extraction process. This would have conflicted with the sustainability objective of the study, which focused on minimizing solvent usage. The 0.2 g amount for freeze-dried samples was, therefore, a compromise that ensured accurate detection of carotenoids, extraction efficiency, and adherence to the sustainability goals of the present research.

4.2. Effect of the mill

The mill pre-treatment did not have a significant effect on the extraction of carotenoids in any of the *D. bardawil* evaluated matrices (control fresh, control freeze-dried, control encapsulated, phytoene-rich fresh, phytoene-rich freeze-dried, and phytoene-rich encapsulated). It can be presumed that, depending on the conditions and the matrix, ball-mill pre-treatment can lead to higher carotenoid releases by causing cell disruptions. For instance, in other species of microalgae, such as *Chlorella* (*C. vulgaris, C. protothecoides,* and *C. zofingiensis*), the application of ball mill (25 Hz, 3.5 min) enhanced carotenoid release due to the breaking of the cell wall, allowing a 1.2–12- fold higher carotenoid release compared to glass bead vortexing (Araya et al., 2014). In addition, in a previous study conducted in our group, the pre-treatment of the ball mill (5 min, 30 Hz) in *Chlorella sorokiniana* allowed up to ~15-fold higher carotenoid release (Morón-Ortiz et al., 2024). Certain microalgae species, such as *Chlorella*, are characterized by the presence of a cell wall. In contrast, others, such as *D. bardawil* lack such structure, which could explain the results observed (Barbosa et al., 2023; Morón-Ortiz et al., 2024). Thus, in the *D. bardawil* matrices tested, the ball mill treatment applied did not enhance significantly the extractions achieved by UAE alone. The usefulness of ultrasounds for the extraction of cellular components is attributed to the phenomenon of acoustic cavitation, which causes the disintegration of solid materials (such as membranes and cell walls). This enhances the contact between the solvent and the cell content and favours and accelerates mass transfer. The technique has been applied successfully to the extraction of diverse

carotenoids from fruits, vegetables, agricultural by-products and microbes including microalgae, as mentioned above (Araya et al., 2014; A. J. Meléndez-Martínez et al., 2021; Morón-Ortiz et al., 2024).

4.3. Effect of the extraction solvent

Green analytical chemistry continues to grow and there is a need for more sustainable eco-friendly solvents to replace other solvents, such as toxic and/or petroleum-derived solvents (Tobiszewski & Namieśnik, 2017). Some alternatives to these solvents are the green solvents ethyl lactate and MeTHF (Hashemi et al., 2018). The choice of an appropriate solvent for carotenoid extraction is crucial for an efficient yield extraction, and the selection of this solvent will depend on different factors, such as the attributes of carotenoids (e.g. the polarity or the chain length of the carotenoids), or the nature of the matrix (e.g. the water content). Some solvents commonly used for carotenoid extraction, such as hexane, dichloromethane, chloroform, or diethyl ether, raise concerns in terms of sustainability and/or safety (Saini & Keum, 2018).

Ethanol and methanol are one of the most common solvents used for the extraction of bioactive compounds from food as, they have been authorized by the European Food Safety Authority (EFSA) for food-grade applications. However, the use of methanol should be reduced due to its high toxicity (Geow et al., 2021; Nekoukar et al., 2021). In the present study, both solvents, above all ethanol, exhibited high extraction yields. The solvents leading to the highest extractions of the main individual carotenoids in the samples (lutein and (15*Z*)-phytoene) and total carotenoids will be discussed here. In brief, ethanol was among the best solvents to extract lutein in all matrices, (15*Z*)-phytoene in all phytoene-rich matrices and total carotenoids in fresh matrices, encapsulated matrices, and the control freeze-dried matrix (Figs. 3, 4 and 6). Methanol was among the best solvents to extract lutein in the fresh and freeze-dried matrices, (15*Z*)-phytoene in the fresh and freeze-dried phytoene-rich samples and total carotenoids in both types of fresh samples and the phytoene-rich freeze-dried sample. MeTHF was among the best solvents to extract lutein in all fresh and encapsulated samples, (15*Z*)-phytoene in all phytoene-rich samples and total carotenoids in all fresh and encapsulated samples. MeTHF is a green solvent that has received attention due to its unique combination of physical and chemical properties. MeTHF is a cyclic ether that is structurally similar to tetrahydrofuran (THF), but with a methyl group attached to the ring (Hu et al., 2022). MeTHF is an excellent solvent for a wide range of polar and nonpolar compounds and has a higher boiling point than THF, making it useful for high-temperature applications (Englezou et al., 2020). The solvent is obtained from lignocellulose materials, more specifically, from pentosans. These are hemicelluloses, a type of fiber found in by-products such as corn cobs, hulls, bagasse, among many others. MeTHF is being recommended for the extraction of bioactive compounds from natural products as an alternative to other petroleum solvents, notably *n*-hexane, which is raising growing concerns due to their toxicity and negative environmental impacts. Moreover, the safety of MeTHF has been recently assessed by EFSA (Lambré et al., 2022).

MeTHF has been used for carotenoid extraction in different matrices, such as carrots or microalgae (Rapinel et al., 2020). MeTHF has also shown a high carotenoid extraction in some *C. sorokiniana* matrices analogous to the ones tested in the present study (Morón-Ortiz et al., 2024). It is to be noted that, in the present study, MeTHF has been among the best extraction solvents for both carotenes ((15*Z*)-phytoene, α-carotene, (all-*E*)-β-carotene, (9*Z*)-β-carotene) and xanthophylls (lutein, zeaxanthin, (9*Z*)-antheraxanthin) (that is, for several carotenoids with important differences in conformation, polarity and other physico-chemical properties) in certain matrices.

Overall, ethyl lactate and, above all, DMSO were not the most suitable solvents for carotenoid extraction in the matrices tested. Ethyl lactate is a green solvent that can be found naturally in food in small quantities, such as chicken, wine, or some fruits. This environmentally friendly solvent, derived from corn and other carbohydrate feedstocks, stands out for being 100% biodegradable and renewable. Approved by the Food and Drug Administration as a flavor additive and endorsed by the Environmental Protection Agency as a Significant New Alternative Policy solvent, it is non-carcinogenic, non-corrosive, and has excellent penetration properties (Pereira et al., 2011).

Similarly, in our previous study on *C. sorokiniana* (Morón-Ortiz et al., 2024), ethyl lactate did not recover a remarkable carotenoid amount in most matrices (fresh and freeze-dried matrices, both control and phytoene-rich), however, the total carotenoid extraction from the encapsulated matrix was significantly high with this solvent compared to the other solvents (Morón-Ortiz et al., 2024). Ethyl lactate has been satisfactorily used as a green solvent for the extraction of carotenoids from tomato peels (Pataro et al., 2020).

The content of (15*Z*)-phytoene was higher than that of lutein in the fresh matrix; contrarily, in the freeze-dried matrix, the amount of (15*Z*)-phytoene was lower or similar to lutein in most of the solvents tested. These results are in concordance with other study performed in our group, where the amount of (15*Z*)-phytoene in fresh *Chlorella sorokiniana* was significantly higher than that lutein; however, in the freeze-dried *C. sorokiniana* the amount of lutein was higher than or similar to that of (15*Z*)-phytoene (Morón-Ortiz et al., 2024). These results might suggest that (15*Z*)-phytoene is more prone to degradation during the freeze-drying process compared to lutein. Another possible explanation could be that the microstructural changes produced as a result of drying favour the extraction of lutein over (15*Z*)-phytoene (Ma et al., 2023). At this point it is important to note that there are important differences in polarity between the two compounds as lutein is a xanthophyll containing two hydroxyl groups and phytoene is a hydrocarbon. Additionally, their location in the microalgal cells is also expected to be markedly different as, unlike phytoene, lutein participates in photosynthesis and is therefore assembled into the photosynthetic apparatus. Specifically, in land plants and green algae the xanthophylls that participate in photosynthesis are bound to the light-harvesting complexes that constitute the peripheral antenna of the photosystems (Caferri et al., 2022).

5. Conclusions

The present study aimed to investigate the efficacy of five solvents for ultrasound-assisted extraction of carotenoids from *D. bardawil* and to evaluate the effect of grinding on extraction efficiency. Ball milling pretreatment did not produce significant improvements in carotenoid extraction in any of the matrices tested, probably due to the probably due to the absence of a rigid cell wall in *Dunaliella* species. On average, freeze-drying resulted in 1.2 times higher extraction of total carotenoids compared to fresh samples. In general, ethanol, methanol and MeTHF were the solvents that produced the highest carotenoid extractions. MeTHF, in particular, showed excellent recovery of individual and total carotenoids in different conformations, polarities and properties, significantly outperforming the other biosolvent, ethyl lactate. These results support the potential of MeTHF as an environmentally friendly biosolvent to replace petroleum-derived solvents, in line with recent safety assessments. The findings contribute to the development of sustainable food production methods and the application of environmentally friendly solvents for the extraction of carotenoids from microalgae. However, the study's findings are specific to *Dunaliella bardawil* and may not be generalizable to other microalgae or plant matrices; further studies with different species are needed to establish optimal treatments and solvents. Additionally, while this study evaluated various matrices of *Dunaliella bardawil* (phytoene-rich, freeze-dried, fresh, and encapsulated), future research should focus on comparing the bioavailability of these matrices to assess whether higher carotenoid concentrations in the matrix translate to greater carotenoid concentrations in the organism. The fact that, regardless the solvent, the extraction of lutein in the phytoene-rich freeze-dried matrix was higher or very similar to that of (15*Z*)-phytoene is noteworthy and merits further research.

CRediT authorship contribution statement

Ángeles Morón-Ortiz: Writing – original draft, Investigation, Formal analysis. **Paula Mapelli-Brahm:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Antonio León-Vaz:** Writing – original draft, Investigation, Formal analysis. **Ana M. Benitez-González:** Formal analysis. **Andrés N. Martín-Gómez:** Formal analysis. **Rosa León:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Antonio J. Meléndez-Martínez:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

AJMM carries out consultancy work for companies. 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.117007.

Data availability

No data was used for the research described in the article.

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