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First Assessment of Feasibility of Liquid-Liquid Extraction of Xantophylls from Bittern

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Carotenoids are valuable compounds that can be naturally produced by microorganisms. Some of these grow and proliferate in hypersaline environments, such as saltworks, where essential nutrients and key stress conditions (e.g., high temperature, salinity, and solar irradiation) are naturally present. For this reason, their concentration can be significant in bittern, the waste solution generated by saltworks at the end of the salt production process. In the present work, to the best of the authors' knowledge, the direct extraction of pigments from real natural bittern is reported for the first time, to demonstrate the technical feasibility of recovering pigments from a waste solution, avoiding upstream costs and limiting the costs for cells harvesting and lysis. Following a bittern concentration step, achieved via UltraFiltration, liquid-liquid extraction was performed by adding an extracting solvent to the concentrated bittern, briefly shaking and subsequently centrifuging it. The light phase obtained after centrifugation was analysed by UV-VIS spectrophotometry and HPLC analysis. The efficacy of different solvents was assessed, namely methanol, ethyl acetate, hexane, and acetone. Acetone was identified as the most effective solvent and the extraction process was further optimized by testing different volume ratios and centrifugation settings, resulting in a xanthophyll concentration of 3.08 ± 0.03 mg/L in the acetone phase, starting from 0.41 ± 0.05 mg/L in the concentrated bittern. Based on these results liquid-liquid extraction appears to be a feasible approach, although further optimization is still required.

1. Introduction

Carotenoids are a broad class of tetraterpenoids that differ in their chemical structure. They can be divided into two groups: carotenes and xanthophylls. Carotenes are strictly hydrocarbons, whereas xanthophylls contain hydroxyl groups attached to the cyclohexane rings. An example of the first category is β-carotene, whilst antheraxanthin and zeaxanthin are well-known xanthophylls. In general, these pigments absorb light in the blue-green to violet region of visible spectrum and exhibit a wide range of health benefits. For example, they show retinal protective and reparative effects and antioxidant activity as effective free radical scavengers. β-carotene has been reported to be effective against heart disease and cancer, lutein and zeaxanthin display strong protective activity against age-related macular degeneration, cataracts, retinal nerve disease, and diabetic retinopathy (Widomska et al., 2020). Within this context, carotenoids represent a rapidly growing market, which in 2019 was estimated at 1.43 billion USD (Monte et al., 2019). In fact, they are widely used as colorants in food, feed and beverage industries, but also find applications in the pharmaceutical, cosmetic and nutraceutical sectors. For these purposes, carotenoids can be produced either naturally or synthetically. Natural carotenoids can be obtained from biowastes, such as tomato peels and seeds, or from cultivated algae and microorganisms. The synthetic production of vitamin A and its precursors, such as β-carotene, has a long industrial history and several established routes. As a representative example, Lindlar's catalyst is employed in DSM-Firmenich's industrial process for carotenoid production. Although synthetic carotenoids are generally less expensive than natural ones, they are often considered less sustainable and potentially less beneficial for human health (Ludwig et al., 2021). Several species of microalgae, bacteria and archaea can biosynthesize and accumulate

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carotenoids. Among the most prominent industrial producers are *Dunaliella salina* for β-carotene (Ortega Méndez et al., 2012) and *Nannochloropsis oculata* for lutein and zeaxanthin (Smaoui et al., 2021). Production facilities must be located in regions characterized by high solar irradiation and elevated temperatures, as carotenoids are synthesized by these microalgae as protective response to environmental stresses, such as heat, salinity, and nutrient limitation (Ortega Méndez et al., 2012). Solar saltworks represent a remarkable semi-natural system supporting carotenoid-producing microorganisms. In these environments, hypersaline brines provide a favourable habitat for halophilic and halotolerant microorganisms. Natural representatives include eukaryotic microalgae such as *Dunaliella salina* as well as prokaryotic microorganisms such as *Salinibacter ruber* and *Halobacterium salinarum* (Villanova et al., 2021). These microorganisms become particularly concentrated in bittern, i.e. the residual brine generated at the end of the salt production cycle during the hot season (Vicari et al., 2022). Cultivating carotenoid-producing microorganisms at industrial scale requires large amount of water, space, and energy. The cost of upstream processes is about the 20-30% of the total production costs (Monte et al., 2018). Conversely, bitterns are naturally rich in minerals and carotenoid-producing microorganisms making them an attractive eco-sustainable resource. Some valuable products, such as magnesium hydroxide, can be recovered from these streams, as demonstrated in the SEArcularMINE treatment chain. To enable this recovery, ultrafiltration (UF) is commonly employed as a pre-treatment to retain organic matter (Scelfo et al., 2024). As a by-product, a UF retentate enriched in microbial biomass is generated, which represents a waste stream within the SEArcularMINE magnesium hydroxide production chain. The aim of this work was to evaluate the feasibility of recovering carotenoids from concentrated bitterns produced as UF retentate. Although solid-liquid extraction from lyophilized biomass is commonly employed for carotenoid recovery, this approach cannot be applied in the present context, as cell disruption already occurs during UF step (Monte et al., 2018). For this reason, an easy and rapid liquid-liquid extraction (LLE) protocol was developed and optimized, testing various common organic solvents and different operating conditions.

2. Materials and Methods

The bittern was sampled from crystallizer ponds in Margi saltworks (Trapani, Italy) and ultrafiltered as described in Scelfo et al. (2024). Starting from 500 L of bittern, a retentate 10-times more concentrated was obtained. The latter was ultrafiltered a second time to obtain a final retentate with a 50-fold concentration factor relative to the original bittern. The maximum pressure reached by the booster pump was 2.7 bar and the maximum temperature was 50 °C. The final product was stored in a dark tank at room temperature.
LLE was performed in triplicate adding to retentate acetone, hexane, methanol and ethyl acetate, using the volumes and settings indicated in Table 1.

Table 1: LLE tested conditions. VR and VS represent the volumes of the retentate and of the solvent, respectively.

Condition	VR (mL)	Solvent	VS (mL)	Solvent-to-sample volume ratio	Speed (× g)	Time (min)	Temperature (°C)
A	15	Ethyl acetate	7.5	1:2	2700	10	15
B	15	Hexane	7.5	1:2	2700	10	15
C	15	Methanol	7.5	1:2	2700	10	15
D	15	Acetone	7.5	1:2	2700	10	15
E	15	Acetone	15	1:1	2700	10	15
F	20	Acetone	5	1:4	2700	10	15
G	15	Acetone	5	1:3	2700	10	15
H	15	Acetone	5	1:3	2700	15	8
I	15	Acetone	5	1:3	4800	10	8
L	15	Acetone	5	1:3	4800	10	15

The first tests (i.e., A-D) have been done to select the most suitable solvent for carotenoids extraction. The following tests have been carried out to identify the best solvent-to-sample volume ratio (i.e., E-G) and to identify optimal centrifugation settings and separation conditions (i.e., H-L).

2.1 Experimental set-up

The process steps are schematically illustrated in Figure 1. After the UF treatment, pigments extraction from the UF concentrate was carried out by vigorously mixing the sample with the extracting solvent for 1 minute using a MIX ARGOlab Vortex Mixer (Giorgio Bormac S.r.l., Modena, Italy). Hexane (VWR Chemicals, Leuven, Belgium), methanol (Carlo Erba Reagents, Milan, Italy), ethyl acetate (Carlo Erba Reagents, Milan, Italy) and acetone (Honeywell, Philadelphia, PA, USA) were used as extracting solvents. Phase separation was achieved by centrifugation, and the light (i.e., organic) phase was recovered with a Pasteur pipette and analyzed. The resulting extracts were stored in the dark at −20 °C until analysis; preliminary spectrophotometric screening (Fig. 1, analytical step 1) was performed, and only the most promising samples were subsequently analyzed by HPLC (Fig. 1, analytical step 2).

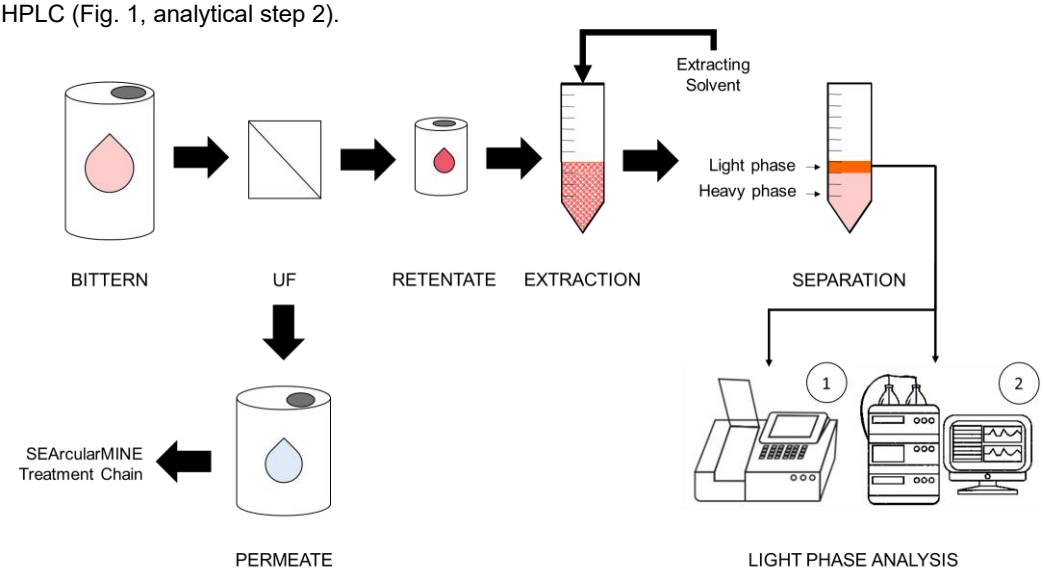

Figure 1: Schematic representation of the adopted experimental set-up and procedure.

2.2 Analytical methods

All extract samples were immediately analyzed using the spectrophotometer Onda V-11 Scan (Giorgio Bormac S.r.l., Modena, Italy) recording the UV-VIS spectra in the 350 and 700 nm range. Carotenes concentrations were calculated using Wellburn equations (Ortega Méndez et al., 2012). Total xanthophylls concentration was estimated using the Eq (1), modified from Bulda et al. (2008) as follows:

$$Xanthophyll\ concentration\ (\mathrm{mg/L}) = 3.84\ Abs_{480} - 6.87\ Abs_{495} \quad (1)$$

Selected samples were further analysed by High-Performance Liquid Chromatography (HPLC) using a 1220 Infinity (Agilent Technologies, Santa Clara, CA, USA) equipped with an UV-DAD detector and the column FlexFire Fusion C30 (250 × 4.6 mm, 5 µm). The mobile phases consisted of HPLC-grade ethyl acetate 100% (Carlo Erba Reagents, Milan, Italy) and a mixture of acetonitrile Chromasolv (Sigma-Aldrich, St. Louis, MO, USA) and HPLC-grade water (Sigma-Aldrich, St. Louis, MO, USA) (9:1, v/v). The gradient was as follow: i) 0-16 min 0-60% ethyl acetate 100%, ii) 16-30 min 60% ethyl acetate 100%, iii) 30-32 min 100% ethyl acetate 100%, iv) 32-35 min 100% acetonitrile:water (9:1). Injection volume was 10 µL. Chromatograms were recorded at 450 nm. β-carotene, zeaxanthin and antheraxanthin standards (Sigma-Aldrich, St. Louis, MO, USA) were used for identification and quantification. Calibration curves were established over the concentration range of 0.5–50 mg/L and are described by Eqs. 2, 3 and 4. Peak areas (mAU·s) used in the equations indicated as Area were obtained by integration of the corresponding chromatograms.

$$\beta carotene\ concentration\ (\mathrm{mg/L}) = 0.035\ \mathrm{Area} - 2.832 \quad (2)$$

$$Zeaxanthin\ concentration\ (\mathrm{mg/L}) = 0.0054\ \mathrm{Area} + 1.098 \quad (3)$$

$$Antheraxanthin\ concentration\ (\mathrm{mg/L}) = 0.0102\ \mathrm{Area} + 0.771 \quad (4)$$

3. Results and discussion

Extraction yield is usually expressed as the ratio between the mass of extracted pigments and the biomass content. However, in this case, it was not possible to estimate the cellular content in the bittern. In fact, at the indicated conditions of UF, concentration and lysis step occur together most probably because of shear stress (Monte et al., 2018). Further investigations will be needed to better address this aspect by quantifying the cellular content in the bittern, in order to properly define extraction yield on a biomass basis. Once the pigments were most likely in the medium, LLE was implemented and investigated. Under none of the tested conditions were carotenes detected. This result is consistent with the literature, and with the fact that some of the microorganisms found in the bittern are known to be high producers of xanthophylls, especially under stress conditions such as high salinity, presence of reactive oxygen species (ROS) and high light intensity (Smaoui et al., 2021).

3.1. Evaluation of solvents

Extraction experiments were performed using different solvents. When ethyl acetate or hexane were employed, phases separation was clearly achieved, but the corresponding UV–VIS spectra were indistinguishable from those of the respective blanks. This observation indicates that the pigments were not transferred to the organic phase, which remained transparent. Methanol, instead, formed a single homogeneous phase with the sample, preventing phase separation. Acetone, conversely, showed good extractive properties, although solvent recovery was limited due to its partial miscibility with water. The color of the heavy phase obtained at the end of the process with acetone is a off-white very similar to the color of spent pellets obtained at the end of solid-liquid extractions with acetone or other solvents in pure cultures (Eilers et al., 2024). Thus, it can be assessed that the extraction phase is completed and there is no more recoverable pigment in the heavy phase. According to literature, acetone is not usually the most suitable solvent to recover apolar compounds like β-carotene, which should have higher solubility in solvents like ethyl acetate or hexane (Hladnik et al., 2024). These results suggest a higher concentration of polar carotenoids (e.g., xanthophylls) than carotene (e.g., β-carotene) in the retentate (Sajilata et al., 2008). Another possible explanation is that carotenoids were not present as freely extractable apolar species but were likely associated with polar cellular components or dispersed as colloidal complexes within the highly saline matrix (Monte et al., 2018).

3.2. Evaluation of volume ratios and centrifugation settings

Each sample was then analysed in triplicate by spectrophotometry. The mean concentrations of total xanthophylls obtained from the different acetone extracts (D-L) are shown in Figure 2.

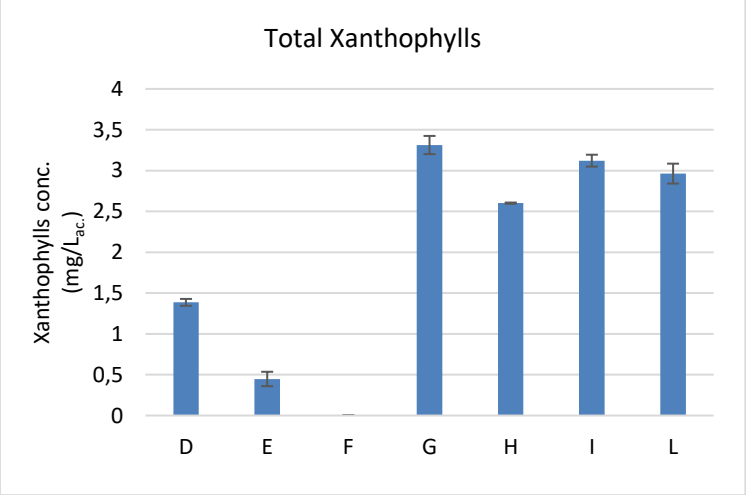

Figure 2: Quantification of total xanthophylls according to UV-VIS spectra in samples obtained with a solvent (acetone) to sample volume ratio 1:2 (D), 1:1 (E), 1:4 (F) and 1:3 (G, H, I, L). Note that tests (G, H, I, L) were performed at different centrifugation condition.

A marked dependence of extraction efficiency on the solvent to sample volume ratio was observed. Conditions D and E, corresponding to 1:2 and 1:1 volume ratio respectively, resulted in lower xanthophyll concentrations. Condition F, corresponding to a volume ratio of 1:4, could not be analyzed because the light phase is too small to be recovered with a Pasteur pipette without also collecting some of the heavy phase.
By contrast, conditions G-L with a solvent-to-sample volume ratio of 1:3, showed the highest extraction yields, with total xanthophyll concentrations close to or above 3 mg/L. The effect of centrifugation speed and time was

comparatively less pronounced. Increasing the rotational speed from 2700 × g (G) to 4800 × g (L) did not result in a substantial improvement in xanthophyll recovery. Condition H, combining low centrifugation speed and temperature, despite a longer centrifugation time, led to a slight decrease in recovered pigments, confirming that insufficient phase separation negatively affects extraction performance. Overall, the results demonstrate that the solvent-to-sample volume ratio is the dominant parameter influencing xanthophyll extraction efficiency, while centrifugation settings mainly affect solvent recovery and phase stability rather than pigment partitioning. The recovered solvent volume was consistently 2 mL from an initial 5 mL, corresponding to an approximate recovery of 40%. This is probably due to the high miscibility of acetone and water. It is known that salts in solution, as well as its temperature, affect the miscibility of these fluids, and these factors may be predominant in the present experiments. Salting-out affects both extraction and separation of the two phases (Bourayou and Meniai, 2005). The samples that, according to the quantification of the spectrophotometer, showed a concentration of carotenoids above 2.5 mg/L were further analysed by using the HPLC. The results are summarized in Figure 3. These data identify condition G as the one with the highest concentration of zeaxanthin (1.88 ± 0.16 mg/L) and I for antheraxanthin (1.34 ± 0.01 mg/L). However, only small differences were detected in the concentration of xanthophylls in G, I, and L conditions. By contrast, condition H showed xanthophyll concentrations lower than the detection limit. In the other conditions (i.e., G, I, and L) spectrophotometer quantifications were consistent to those estimated with HPLC analysis. These values were also comparable with those found in literature, even though improvable (Ahmad et al., 2021). In this case the concentration of zeaxanthin was 4.78 ± 0.12 mg/g obtained with a solid-liquid extraction using ethanol 90% starting from *Chlorella luteoviridis*. Other studies in the literature have shown xanthophyll extraction from maize using ethanol, followed by filtration and concentration of the extract by a factor of 1 to 10 through consecutive ultrafiltration and nanofiltration units, yielding final pigment concentrations of 3.11 – 22 mg/L (Tsui and Cheryan, 2007).

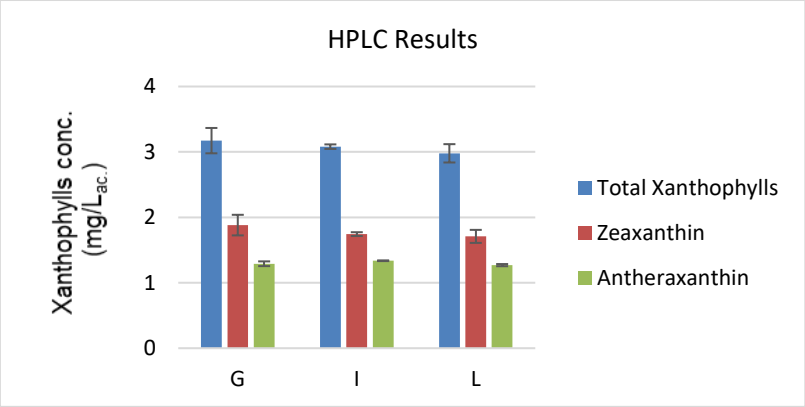

Figure 3: Results of characterization and quantification performed with the HPLC when feasible and reliable.

4. Conclusions and outlook

In this study, the efficacy of LLE of carotenoids from semi-natural bittern was assessed, starting from a pre-treated bittern, concentrated 50-fold by means of an UF treatment step. The UF retentate was then treated via LLE to recover the pigments present in the sample. Acetone proved to be the most effective solvent, particularly keeping a solvent-to-bittern ratio 1:3, resulting in a final xanthophylls concentration of 3.08 ± 0.03 mg/L. The process is strongly affected by the solvent to sample volume ratios. However, as demonstrated by condition H, a low centrifugation speed, even when combined with low temperature, results in poor pigment recovery. These results indicate that direct LLE of pigments is theoretically feasible starting from waste bitterns UF retentates, limiting the costs to the extraction process only, as biomass cultivation, harvesting and lysis are already occurring during the previous independent process phases. Starting from a waste material, the proposed process not only valorizes an otherwise discarded resource but also offers a cost-effective alternative to conventional protocols reported in the literature. It is worth noting that, to the best of the authors' knowledge, no established protocols for this approach are currently available in the literature. Accordingly, the present study constitutes a significant milestone, providing a foundational framework for future investigations and methodological advancements. Antioxidant properties of the extracts will be assessed to verify their integrity. This information will also be useful for assessing their potential applications. Observed solvent loss suggests that the separation phase may constitute a process bottleneck. Alternative extraction configurations should be explored, including solvents mixtures and different extraction techniques in order to overcome these limitations. For example, the use of supercritical CO_2 may be particularly promising. Finally, an additional experimental

campaign should be performed to evaluate the influence of seasonal variation, replicability and reproducibility of the process.

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