Effect directed assay guided fractionation of marine microalgae based ethanolic extracts by centrifugal partition chromatography

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Abstract

Marine microalgae are a great source of high-value compounds such as polyunsaturated fatty acids, polyphenols, and carotenoids. These compounds possess bioactive properties (e.g., antioxidative) and have potential applications in life sciences. However, selective extraction of these molecules is difficult and requires multiple purification steps. Centrifugal partition chromatography (CPC) is a preparative, support-free, liquid-liquid chromatographic technique that can overcome this problem. Therefore, this study aimed to develop a simple bioactivity-guided isolation procedure using CPC. Crude ethanolic extracts of four microalgae species were screened for antioxidative activity using effect-directed analysis (EDA) coupled with high-performance thin-layer chromatography (HPTLC). Following the detection of biological activity, the chemical class of the most potent bioactive zones were identified. Subsequently, biphasic solvent systems with varying compositions were tested and optimized for the separation and recovery of the most potent molecules with antioxidative properties, using preparative-scale CPC.

1. Introduction

Microalgae are a diverse group of unicellular, phototrophic organisms. Owing to this one-cell-concentrated complex metabolism, microalgae are considered as suitable candidates for producing multiple products (e.g., carotenoids, polyunsaturated fatty acids (PUFAs), and polyphenols) with diverse biological activities (e.g., antioxidant, anti-inflammatory, antitumor, antimicrobial) (Bhattacharya & Goswami, 2020; Slegers et al., 2020). Among these cellular products, lead compounds are of the highest priority, as they have shown sufficient potential (measured by potency, therapeutic efficacy, etc.) to progress into a full product development program in the field of life sciences (e.g., cosmeceuticals, nutraceuticals, pharmaceutical industries) (Costa et al., 2021; de Vera et al., 2018; O’connor et al., 2022). However, obtaining bioactive molecule-rich fractions with a high purity is a complex process. This is due to the fact that these bioactive compounds are typically extracted as a complex mixture of other molecules, therefore, multiple purification steps are usually necessary. Furthermore, in the currently dominating biorefinery approach, single products are primarily targeted instead of recovering a spectrum of biologically active substances, which neglects the potential of multiproduct value chains (Bhattacharya & Goswami, 2020; Li et al., 2021; Slegers et al., 2020).

CPC is a separation technique that uses two immiscible liquid phases to partition components. One phase is held stationary by centrifugal force, whereas the other phase is forced to flow through the stationary phase and dissolved components are separated based on their distribution coefficient (Kd) between the two phases. Because CPC does

not require a solid support, unlike other chromatographic techniques, it eliminates the risk of irreversible sample adsorption. CPC also provides a high injection capacity with a recovery rate of over 95% and a purity rate of over 99%, making this technique an efficient DSP unit operation in the field of natural product isolation (Bojczuk et al., 2017; Lorántfy et al., 2020). HPTLC-EDA is a powerful technique used in the analysis of complex mixtures. This technique involves the separation of compounds on an HPTLC plate, followed by detection using biological or chemical assays. This technique combines the separation performance of HPTLC with the detection and identification capabilities of bioassays, thus allowing the identification of unknown and known compounds that may have a specific biological effect (Morlock, 2021). Therefore, CPC was coupled with HPTLC-EDA to develop a straightforward and effective method for generating multiple bioactive-rich fractions from crude microalgae-based ethanolic extracts.

2. Materials and methods

Crude lipid extracts from four microalgae species of commercial interest (*P. tricornutum, N. granulata, P. purpureum* and *T. tetrathele*) were screened for bioactive molecules using HPTLC-EDA. Antioxidative capacity (DPPH-assay) was chosen as an indicator of bioactivity. To detect antioxidative zones, the plates were immersed in a methanolic DPPH (0.2 w/v%). The most potent bioactive zones were further analyzed by spectrophotometry, HPTLC-GC-MS, and HPLC-DAD. Subsequently, biphasic solvent systems with varying compositions were screened in a scaled-down model to select the most suitable solvent system for the separation of the bioactive compounds of interest. The optimized biphasic system was then tested in a semitechnical-scale CPC for the generation of antioxidant-rich polar and medium-polar molecules from ethanolic extracts with high purity.

3. Results and Discussion

To explore the antioxidative biomolecules in the microalgae extract first HPTLC-EDA was applied. For the separation of the microalgal extracts, a medium polar mobile phase n-hexane/acetone/propan-2-ol (80/20/5 in v/v/v) was used. The bands with the most intense decolorization of the purple reagent showed the highest antioxidative capacity (*Figure 1*). The more apolar molecules with higher affinity to the mobile phase also exerted antioxidative properties; however, the zones showing the highest antioxidative capacity were observed closer to or at the application line of the HPTLC plate (*Figure 1*, *row 17*). This finding suggests that molecules with the highest antioxidative capacity were found in the more polar and medium-polar fractions of the microalgal extracts. Further analysis of the bioactive zones revealed the abundant presence of polyphenols, polyunsaturated fatty acids in galacto- and phospholipid-esterified forms and some microalgae species-specific predominant carotenoids (e.g., fucoxanthin from *P. tricornutum* (*Figure 1*) or violaxanthin from *N. granulata*). Bands that showed more moderate decolorisation were identified also as carotenoids (e.g., β-carotene, diadinoxanthin), although these contribute only to a minor part to the microalgal lipidome of the selected species.

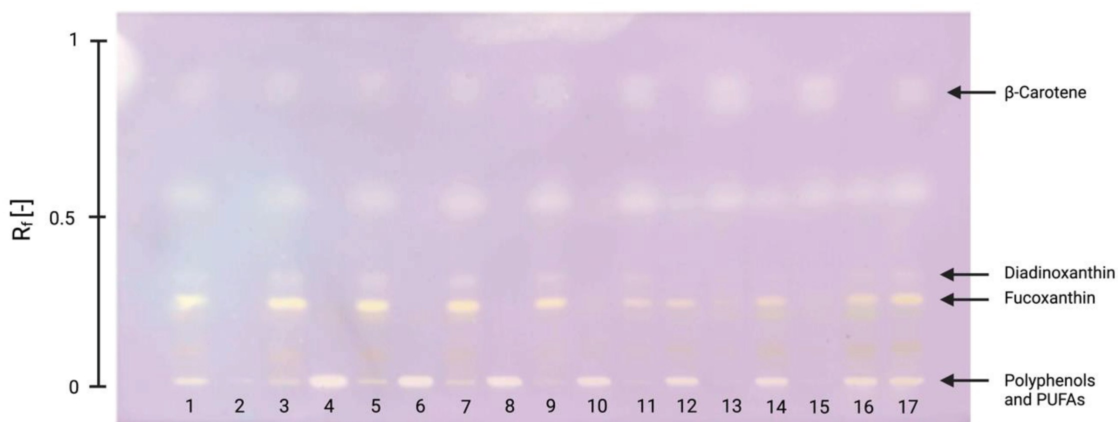

Figure 1:Mobile phase: n-hexane/acetone/propan-2-ol 80:20:5 (v/v/v/v/v). Solid phase: SG60-F254 glass plate (20x10 cm). Application line was at 8 mm. Frontline at 90 mm derivatized with 0.2% w/v) ethanolic DPPH solution and dried at 22°C for 1 h in the absence of light. The rows numbered 1, 3, 5, 7, 9, 11, 13, and 15 are samples taken from the upper phase of the biphasic solvent system of n-hexane/ethyl acetate/ethanol/water with increasing ratio of ethanol and parallel decrease in the water ratio from 1 to 8 (v/v) and 9 to 2 (v/v), respectively. The ratio of n-hexane to ethyl acetate was kept constant with ratio of 5/5 (v/v). Rows numbered 2, 4, 6, 8, 10, 12, 14, and 16 are the respective lower phases of the biphasic systems. 17: Crude ethanolic lipid extract from P. tricornutum, which was also used to test the biphasic systems.

Based on the physicochemical properties and chemical identity of the bioactive compounds, n-hexane/ethyl acetate/ethanol/water was chosen as the biphasic solvent system, since it is generally applied – in varying volumetric ratios – to separate polar and medium polar compounds in counter current chromatography (Han et al., 2022). The volumetric ratio of the most polar solvent constituents of the system, ethanol and water, was changed stepwise between 1:9 and 9:1 (v/v). By decreasing the polarity of the aqueous phase, the affinity of the most antioxidative molecules towards the upper organic phase decreased and they were predominantly present in the aqueous phase, as confirmed by visual observation (*Figure 1, 1-16*) and the calculated Kd values of the chemically identified species (data not shown). Based on the evaluation of the Kd values of the bioactive molecules, the results indicated that the biphasic solvent system consisting of n-hexane/ethyl acetate/ethanol/water at a volumetric ratio of 5/5/6/4 was the most suitable for separation. This is because the Kd values of the compounds exerting the highest antioxidative capacity, independently from the microalgae species, ranged from 0.3 and 3.5, which is close to the recommended range of 0.5 to 3.0. in counter current chromatographic techniques (Bojczuk et al., 2017).

Based on small-scale screening experiments, the n-hexane/ethyl acetate/ethanol/water (5/5/6/4 v/v/v/v) biphasic system was applied to a preparative scale CPC (250 mL). Crude lipid extracts (500 mg) were redissolved in 10 mL of the aqueous ethanol phase and injected into the CPC, which had been previously loaded and equilibrated with the biphasic solvent system (the aqueous phase was used as the mobile phase). The extracts were separated at a flow rate of 2 mL min^{-1}, and the processing time for separation was set to 120 min. The fractions from the separation were collected and analyzed individually. Based on the analytical results, the samples were pooled at a limit of 95% purity. The total polyphenol content of all microalgae species was recovered with a purity value above 99.5% and a recovery rate between 98.12% and 99.53%. On average, 78.67% of the PUFA-containing galacto- and phospholipids could be recovered to maintain the purity of the fraction above the set limit. Therefore, further optimization of

the process (solvent system, flow rate during separation, etc.) is required to increase the recovery of GL and PL. Fucoxanthin was found only in *P. tricornutum* and was separated by recovery rates and purities above 98%. Similarly, recovery rates higher than 99% were achieved for violaxanthin produced by *N. granulata* and *T. tetrathele*. During the CPC separation, no elution of major carotenoid fractions from the *P. purpureum* extract was obtained. This is most likely due to the presence of more apolar carotenoids such as zeaxanthin or β-carotene, which would elute later owing to their high Kd values. Based on the obtained results from *N. granulata*, *P. tricornutum* and *T. tetrathele* three, while from *P. purpureum* two chemically distinguished high-value fractions could be obtained, with the here presented method. These results clearly indicate the applicability and highlight the enormous potential of microalgae for the production of multiple products.

4. Conclusion

In conclusion, this study demonstrates the potential of bioactivity-guided isolation via CPC for the effective separation and isolation of antioxidant-rich fractions from crude microalga-based ethanolic extracts. The optimized biphasic solvent system consisting of n-hexane/ethyl acetate/ethanol/water in a volumetric ratio of 5/5/6/4 was found to be the most suitable biphasic solvent system. The recovery rate and purity of the extracted compounds were high, making this technique a promising DSP unit operation. Furthermore, this study highlights the enormous potential of microalgae to produce multiple bioactive products, along with the applicability of CPC in the separation and recovery of these high-value compounds.

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