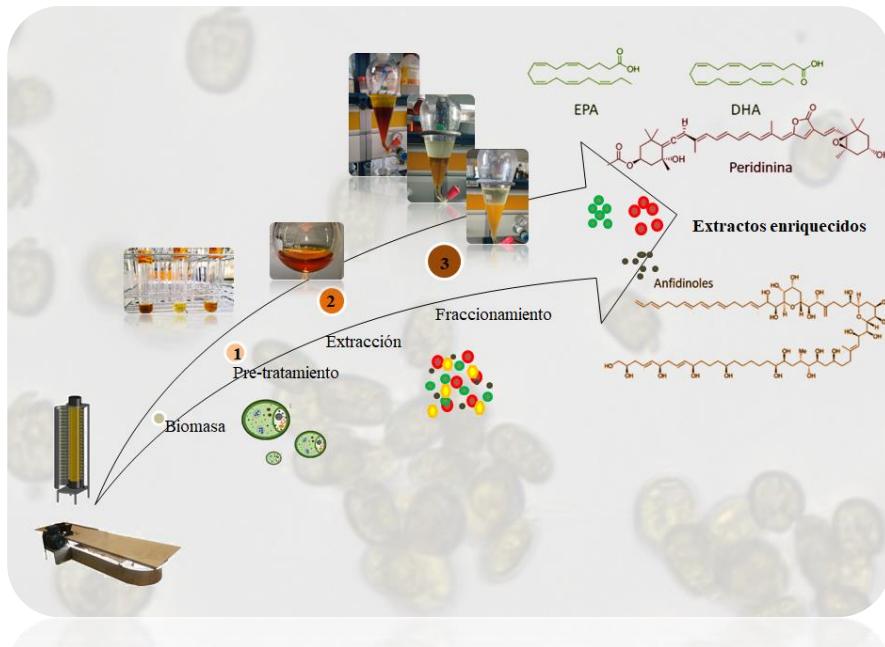




UNIVERSIDAD
DE ALMERÍA

TESIS DOCTORAL

Producción de concentrados de carotenoides, ácidos grasos poliinsaturados y compuestos bioactivos a partir de la microalga marina dinoflagelada *Amphidinium carterae*



MERCEDES LÓPEZ RODRÍGUEZ

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Producción de concentrados de carotenoides, ácidos grasos poliinsaturados y compuestos bioactivos a partir de la microalga marina dinoflagelada Amphidinium carterae

Production of carotenoids, polyunsaturated fatty acid and bioactive compounds concentrates from marine dinoflagellate microalgae Amphidinium carterae

Tesis doctoral presentada por:

Mercedes López Rodríguez

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Directora:

Dra. María del Carmen Cerón García (Catedrática Universidad de Almería)

Co-Director:

Dr. Francisco García Camacho (Catedrático Universidad de Almería)

Enero, 2023

Dra. María del Carmen Cerón García, Catedrática de Ingeniería Química de la Universidad de Almería y **Dr. Francisco García Camacho**, Catedrático de Ingeniería Química de la Universidad de Almería.

CERTIFICAN:

Que la memoria titulada “Producción de concentrados de carotenoides, ácidos grasos poliinsaturados y compuestos bioactivos a partir de la microalga marina dinoflagelada *Amphidinium carterae*”, presentada por Dña. Mercedes López Rodríguez para optar al título de **Doctora por la Universidad de Almería**, ha sido realizada bajo su dirección y tutela en las instalaciones del grupo de investigación Biotecnología de Microalgas Marinas de la Universidad de Almería.

Y, para que así conste, firmamos la presente en Almería a 28 de Octubre de 2022

Fdo: María del Carmen Cerón García

Fdo: Francisco García Camacho

Durante el periodo de elaboración de la Tesis Doctoral, Mercedes López Rodríguez disfrutó de un contrato de Personal Laboral Técnico de Apoyo y de Gestión de la I+D+I del Programa Operativo de Empleo Juvenil, código PEJ_UAL_2017/019, en el Grupo de Biotecnología de Microalgas Marinas BIO-173 (Responsable el Dr. Emilio Molina Grima, Catedrático de Ingeniería Química de la Universidad de Almería) y bajo la supervisión de la Dra. María del Carmen Cerón García. El trabajo de esta Tesis ha sido financiado por los siguientes proyectos de investigación:

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*“El valor de un acto realizado reside más en el
esfuerzo por llevarlo a cabo que en el resultado”*

Albert Einstein

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RESUMEN

Hoy en día el potencial biotecnológico de las microalgas marinas pertenecientes al grupo de los dinoflagelados está ampliamente reconocido, dada la gran variedad de compuestos bioactivos que producen. El estado del arte es tratado de forma resumida en el **Capítulo 1 de Introducción**, en el cual se introducen las líneas de investigación en las que se enmarcan las actividades desarrolladas en esta tesis. Inicialmente se muestran aspectos generales y una visión resumida de las principales aplicaciones de las microalgas. Su potencial se debe a la gran variedad de aplicaciones en distintos sectores como el farmacéutico, cosmético, biocombustibles, biofertilizantes, bioestimulantes, biopesticidas, tratamiento de aguas residuales, alimentación (tanto humana como animal) y nutracéutico. Surgen, por tanto, como una fuente alternativa y sostenible de compuestos de alto valor añadido. En la actualidad son varias las especies de microalgas de mayor relevancia, cuya producción se encuentra establecida comercialmente. Sin embargo, muchas de las investigaciones tecnológicas basadas en microalgas no han logrado alcanzar el nivel comercial, dadas las dificultades encontradas en el desarrollo de procesos eficientes de producción, extracción y purificación de productos de interés comercial. El concepto de biorrefinería, basado en la completa valorización de la biomasa microalgal, parece erigirse como principal alternativa, centrándose en variables que afectan al pretratamiento de la biomasa, extracción y purificación de metabolitos de interés, tales como carotenoides, ácidos grasos y compuestos bioactivos (principalmente metabolitos secundarios).

Especialmente se abordan, aspectos generales de las microalgas marinas dinoflageladas, así como producción y recuperación de metabolitos secundarios que producen. Estos metabolitos secundarios poseen un prometedor potencial en el

desarrollo de nuevos fármacos y agentes terapéuticos. Su demanda en diferentes áreas de investigación tales como medicina, química o ecología, se ha visto incrementada en los últimos años. Entre dichos metabolitos secundarios encontramos anfidinoles, (APDs) representativos del género *Amphidinium* con actividad anti-fúngica, anti-tumoral y hemolítica. Específicamente, la presente tesis, se centra en la especie *Amphidinium carterae*, fuente natural de APDs y también de un interesante grupo de metabolitos primarios de alto valor añadido, los cuales merecen ser recuperados para la revalorización de la biomasa de *A. carterae*. Entre ellos, carotenoides, como peridinina, con gran potencial como agente terapéutico frente a líneas celulares tumorales, y ácidos grasos poliinsaturados (PUFAs) como EPA y DHA de gran importancia nutricional y farmacológica. Brevemente se detallan los avances en cuanto a su cultivo, las adaptaciones de *A. carterae* a distintos medios de cultivo y tipo de biorreactor empleados y su potencial biotecnológico. Tras el estudio de los diferentes metabolitos de interés de *A. carterae*, se detallan algunos de los procesos de extracción y purificación de éstos, descritos hasta el momento para la valorización integral de *A. carterae*.

La línea de trabajo seguida en esta investigación aborda aspectos relacionados con el desarrollo de un bioprocreso enfocado al pretratamiento de la biomasa de *A. carterae* para la extracción y recuperación de extractos ricos principalmente en APDs, pero que también priorice la recuperación de otros dos grupos de metabolitos de gran interés comercial, carotenoides y ácidos grasos poliinsaturados, contenidos en la biomasa. El abordaje se divide en dos áreas. La primera, orientada a optimizar y adaptar diferentes estrategias de extracción y recuperación de carotenoides y ácidos grasos poliinsaturados (PUFAs), típicas de microalgas no-dinoflageladas, que permitan recuperar también APDs para mejorar las perspectivas de los dinoflagelados como

fuente de compuestos de alto valor añadido. Para ello, se trabaja en el **pretratamiento de la biomasa** con el objeto de mejorar la rotura celular y favorecer la extracción de metabolitos.

En la segunda área de abordaje se trabaja en el **fraccionamiento y la obtención de extractos enriquecidos** en los tres grupos de bioproductos con dos enfoques: uno dirigido a la recuperación de APDs y otro dirigido a una valorización integral de la biomasa de *A. carterae* procedente de un fotobiorreactor tipo *race-way* (RW-FBR) operado con reutilización del medio de cultivo agotado. La idea que subyace es la de contribuir a mejorar la economía y sostenibilidad del proceso bajo el concepto de biorrefinería.

La sección anterior da paso al **Capítulo II de Justificación y objetivos** donde se expone la justificación para la realización de la presente tesis y se detallan los objetivos perseguidos. Para la consecución de éstos últimos ha sido necesario llevar a cabo una serie de actividades, las cuales se ilustran de forma esquemática mostrando la relación entre ellas y las publicaciones derivadas de las mismas, proporcionando una visión global del trabajo realizado (Figura 1). Dichas publicaciones serán aportadas en ésta tesis agrupadas por capítulos.

Importancia Biotecnológica de los dinoflagelados

Estado del arte

Compuestos Bioactivos de *Amphidinium carterae*
Extracción y obtención de extractos enriquecidos

Capítulo III. Adaptación y optimización de diferentes metodologías originariamente diseñadas para recuperar carotenoides, ácidos grasos poliinsaturados y APDs individualmente

Etapa 1. Optimización metodologías de extracción

Enfoque
Ácidos Grasos

Enfoque
Carotenoides

Enfoque
APDs

Integración en serie de metodologías enfocadas a carotenoides y APDs: Proceso integrador

Publicación 1. Assessment of multi-step processes for an integral use of the biomass of the marine microalga *Amphidinium carterae*

Capítulo IV. Mejora de la extracción de compuestos bioactivos de *Amphidinium carterae*

Etapa 2.1 Efecto Rotura Celular

- Control Sin Rotura celular
- Ultrasonidos
- Molino de Bolas
- Molturación sin alúmina
- Molturación con alúmina 1:1

Etapa 2.2 Efecto T°/ % KOH

- Optimización de variables de extracción T° 25-80°C
- C_{KOH} 0-60%

Etapa 2.3 Extracción con disolventes

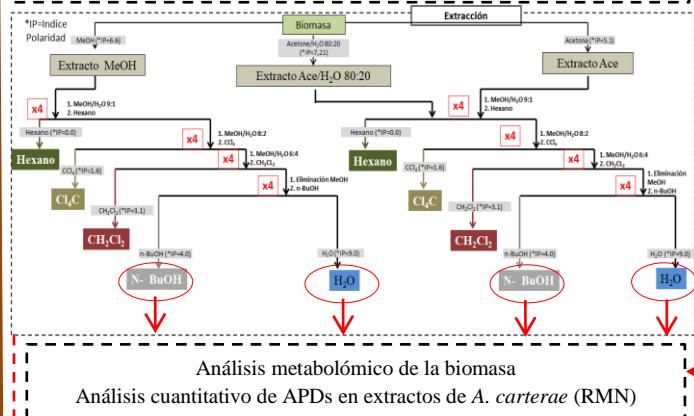
- Disolventes puros y mezclas
- Variación índices de polaridad y Parámetros solubilidad (14,11-31,3 MPa^{1/2})

Publicación 2. Improved extraction of bioactive compounds from biomass of the marine dinoflagellate microalga *Amphidinium carterae*.

Capítulo V. Fraccionamiento de la biomasa de *A. carterae* para la obtención de fracciones enriquecidas en carotenoides, PUFA y APDs

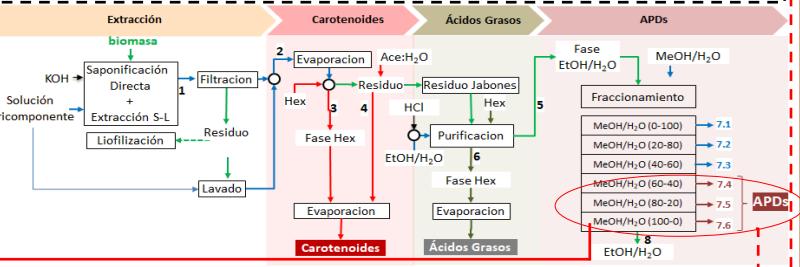
Etapa 3.1 Enfoque priorizado a APDs

Partición secuencial de extractos crudos de *A. carterae*



Etapa 3.2 Enfoque integrado dirigido a APDs, carotenoides y ácidos grasos

Método propuesto por López-Rodríguez et al., (2019)



Publicación 3. An integrated approach for the efficient separation of specialty compounds from biomass of the marine microalgae *Amphidinium carterae*.

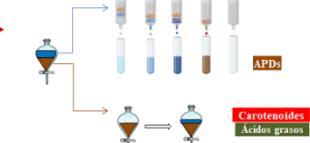
Capítulo VI. Mejora Fraccionamiento para la obtención de extractos enriquecidos en APDs

Etapa 4.1 Fraccionamiento directo

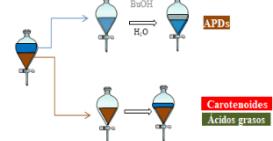
- Extracción
- Adsorción y fraccionamiento
- Rotura celular
- Ratio biomasa/agente extractante (p/v)
- Capacidad de Retención
- Volumen de elución

Etapa 4.2 Extracción líquido-líquido (ELL)

Etapa 4.2.1 Enfoque acoplado a EFS



Etapa 4.2.2 Enfoque acoplado a partición con n-BuOH



Publicación 4. The isolation of specialty compounds from *Amphidinium carterae* biomass by two-step solid-phase and liquid-liquid extraction.

Capítulo I: Introducción.

Capítulo III: Adaptación y optimización de diferentes metodologías originariamente diseñadas para recuperar carotenoides, ácidos grasos poliinsaturados y APDs individualmente.

Capítulo IV: Mejora en la extracción de compuestos bioactivos.

Capítulo V: Fraccionamiento de la biomasa de *A. carterae* para la obtención de fracciones enriquecidas en carotenoides, PUFA y APDs.

Capítulo VI: Mejora del Fraccionamiento para la obtención de extractos enriquecidos en APDs.

Relación directa proceso

Conexión entre capítulos

Seguidamente, los siguientes capítulos están destinados al desarrollo y evaluación de los diferentes objetivos que componen esta investigación. Así, la **Adaptación y optimización de diferentes metodologías, originariamente diseñadas para recuperar carotenoides, ácidos grasos poliinsaturados y APDS individualmente** se trata en el **Capítulo III**. Para el desarrollo de este objetivo se seleccionaron tres metodologías, originalmente recomendadas en la bibliografía para recuperar preferentemente cada una de las tres familias de compuestos (Figura 1, Etapa 1). Cada metodología fue optimizada en algunas de sus etapas y se evaluó su impacto en la recuperación simultánea de las tres familias de compuestos en *A. carterae*. La primera metodología, enfocada principalmente en la recuperación de ácidos grasos libres, consistió en la molturación de la biomasa y subseciente saponificación y extracción simultánea de la biomasa. Las optimizaciones realizadas, usando la biomasa de *A. carterae* ACRN03 lograron unos porcentajes de recuperación de 79%, 61% y 100% de ácidos grasos totales, carotenoides y APDs respectivamente. Sin embargo, dicha metodología no permitió recuperar carotenoides polares de mayor valor añadido de *A. carterae* como peridinina o dinoxantina, recuperando tan solo los carotenoides apolares, tales como β -caroteno, o de media polaridad diadinoxantina y diatoxantina. Con la finalidad de recuperar todos los carotenoides, una segunda metodología, consistente en la combinación de tratamiento alcalino y extracción en una única etapa y posterior etapa de purificación, fue adaptada en *A. carterae*. Esta metodología, logró, tras la etapa final de purificación, unos porcentajes totales de 80%, 97% y 100% de ácidos grasos, carotenoides y APDs (estos últimos no purificados). Finalmente, se adaptó una tercera metodología enfocada en la recuperación de compuestos bioactivos con actividad hemolítica, principalmente APDs. Ésta consistió en una primera etapa de extracción con metanol y rotura celular mediante ultrasonidos. El sobrenadante

metanólico resultante, fue sometido a extracción en fase sólida (EFS) mediante cromatografía de adsorción en fase reversa para separar APDs de otras fracciones, y posterior elución mediante el uso de mezclas metanol: agua (MeOH:H₂O) en diferentes proporciones (% v/v): 0:100, 20:80, 40:60, 60:40, 80:20 y 100:0 y con diferentes rangos de polaridad (PI) (10,2 a 6,6). Tras adsorción y fraccionamiento, las diferentes fracciones obtenidas fueron analizadas mediante actividad hemolítica. Las fracciones comprendidas entre 0:100 y 40:60 de MeOH:H₂O no contenían APDs. Por el contrario, las fracciones más activas fueron 60:40 y 80:20 con un 13% y 33% de APDs, respectivamente. El porcentaje total de recuperación de APDs, en dicha metodología fue casi de un 70%, sin embargo, los análisis de actividad hemolítica revelaron presencia de metabolitos no adsorbidos en la columna de fase sólida (C18) en un 11 %. Esto podría deberse a un alto ratio masa _{extracto}/ masa _{adsorbente}. Ninguna de las tres metodologías evaluadas permitió recuperar el 100% de los tres grupos de metabolitos presentes en la biomasa.

Para mejorar la recuperación se trabajó en la integración en serie de dos de las metodologías, concretamente aquellas que priorizan la obtención de carotenoides y APDs. Con la finalidad de desarrollar un único proceso que permita la recuperación completa de las tres familias de compuestos de interés (carotenoides, PUFA y APDs) contenidos en la biomasa de *A. carterae*, se desarrolló inicialmente un plan experimental para mejorar su extracción y cuyos resultados se describen en el **Capítulo IV (Mejora de la extracción de compuestos bioactivos de *Amphidinium carterae*)**.

Por primera vez para *A. carterae*, en la primera fase se optimizaron los procesos de rotura celular y evaluación de diferentes concentraciones de KOH en la biomasa (0%-60% sobre peso seco de biomasa (p.s.b)) y temperaturas de operación (25°C-80°C) (Figura 1, Etapas 2.1 y 2.2). La eficacia de cada factor estudiado se midió en términos

de recuperación de carotenoides. En una segunda fase se buscó maximizar los rendimientos de recuperación de las tres familias de metabolitos utilizando una amplia gama de disolventes individuales y mezclas de ellos, con rangos de polaridad (PI) y de parámetro de solubilidad de Hildebrand (δ_T) oscilando entre 0 y 7,46, y entre 14,11 y 31,30 MPa^{1/2}, respectivamente (Figura 1, Etapa 2.3). Derivado de la primera fase, se observó que la saponificación de la biomasa procedente de la cepa Dn241EHU conllevó pérdidas de carotenoides por degradación a cualquier concentración de KOH, comparado con el control (sin saponificación). Contrariamente, para la biomasa de ACRN03 se produjo un aumento en el porcentaje de recuperación de peridinina ($p<0.05$), respecto al control, hasta la concentración del 20% KOH p.s.b. No están claras las razones de la ausencia de degradación de peridinina en la cepa ACRN03 durante la saponificación; podría deberse a diferencias en la composición de la pared celular causada por el modo de cultivo, o incluso a la presencia de peridinina en forma éster. Ante cualquiera de las circunstancias planteadas, se seleccionó la cepa de *A. carterae* Dn241EHU como más prometedora dado su mayor contenido en carotenoides comparado con *A. carterae* ACRN03, bajo condiciones óptimas de extracción, 0% KOH p.s.b y 60°C. Respecto al contenido total de ácidos grasos, (p.s.b.), éste fué similar para ambas cepas con valores de $18,12 \pm 1,74\%$ p.s.b. para Dn241EHU y $18,41 \pm 2,47\%$ p.s.b. para ACRN03, siendo ácido hexadecaenoico (16:0), EPA (20:5n3) y DHA (22:6n3) aquellos con mayor proporción de contenido en peso. En la segunda fase, a través de la correlación entre la eficiencia de extracción y PI y δ_T del disolvente, se pudieron seleccionar disolventes que trajeran eficientemente carotenoides, ácidos grasos y APDs. Disolventes con δ_T entre 16,5 y 19 [MPa^{1/2}] y PI de 3 a 5,5, tales como acetona: H₂O 99:1 (v/v) y acetona: H₂O 99,5:0,5 (v/v) proporcionaron los mayores porcentajes de recuperación de carotenoides y ácidos grasos. Por el contrario,

disolventes con δ_T entre 22-31 [MPa^{1/2}] y PI de 6-7,5, como MeOH 100%, EtOH: H₂O 80:20 (v/v), acetona: H₂O 80:20 (v/v) y MeOH: H₂O 80:20 (v/v) proporcionaron los mayores porcentajes de recuperación de APDs.

Los resultados obtenidos en el **Capítulo IV** sirvieron para completar el estudio orientado al desarrollo de un único proceso que permitiera la recuperación completa de las tres familias de compuestos de interés. Los resultados se analizan en el **Capítulo V (Fraccionamiento de la biomasa de *A. carterae* para la obtención de fracciones enriquecidas en carotenoides, PUFAs y APDs)**. La finalidad era implementar una estrategia de fraccionamiento de la biomasa para obtener fracciones ricas en las tres clases de metabolitos de mayor interés. Para el desarrollo del objetivo se aplicaron dos enfoques. **(i)** Un primer enfoque basado en la priorización de APDs mediante partición secuencial de extractos crudos de *A. carterae* por gradiente de polaridad de disolventes (Figura 1, Etapa 3.1). Se utilizaron tres extractos crudos de *A. carterae* obtenidos cada uno con tres disolventes diferentes: acetona 100%, MeOH 100%, acetona: H₂O 80:20 (v/v). La selección de éstos se basó en los resultados observados en el **Capítulo IV**, en el cual se demostró que solo disolventes con PI y δ_T superior a 6 y 20 [MPa^{1/2}], respectivamente lograron la extracción de APDs en *A. carterae*. De esta forma MeOH 100% y acetona: H₂O 80:20 (v/v) reunían las características necesarias para la extracción de APDs, pero no acetona 100% (extracto utilizado como control negativo). Los tres extractos fueron sometidos a una partición secuencial en cuatro etapas con diferentes disolventes (en orden de polaridad creciente (PI entre 0,1 y 10,2): hexano, tetracloruro de carbono (CCL₄), diclorometano (DCM), n-butanol (n-BuOH) y H₂O. El proceso fue exitosamente aplicado en *A. carterae* por primera vez, separando los extractos en diferentes grupos y concentrando APDs en la fracción n-BuOH. Sin embargo, no fue suficientemente efectivo para ácidos grasos y carotenoides ya que a

pesar de concentrarlos principalmente en la fracción hexano y CCL₄, respectivamente, cantidades significantes de éstos fueron identificados en subsecuentes fracciones de mayor polaridad (DCM, n-BuOH y H₂O). Por tanto, cuando los metabolitos objetivo son APDs esta metodología puede ser empleada para concentrarlos en una única fracción y obtener extractos enriquecidos. **(ii)** Alternativamente y bajo el contexto de biorrefinería basado en dinoflagelados, se aplicó experimentalmente el proceso integrado propuesto en el **Capítulo III**. Como se dijo anteriormente, se trata de un enfoque integrador dirigido a la extracción de APDs, carotenoides y ácidos grasos (Figura 1, Etapa 3.2). Dicho enfoque consistió en la saponificación y extracción simultánea de la biomasa de *A. carterae* con el fin de saponificar los ácidos grasos en forma de jabones y separar los carotenoides posteriormente. Con una primera sección dirigida a la recuperación de carotenoides apolares y polares, seguida de una sección para liberar ácidos grasos existentes en forma de jabones, y posteriormente purificados con hexano. La fase hidroalcohólica resultante se introdujo en una sección dirigida a APDs, consistente en una extracción en fase sólida (EFS) mediante cuatro cartuchos C18-130 mg fase reversa conectados en serie. La aplicación experimental del proceso logró unos porcentajes de recuperación de 89%, 82% y 100% de carotenoides, ácidos grasos y APDs (no purificados) respectivamente.

Para evaluar la eficacia de ambos procesos, en cuanto a extracción y obtención de extractos enriquecidos en APDs, se realizó un análisis metabolómico mediante RMN. Dicha técnica nos permitió determinar la distribución porcentual de las clases de metabolitos identificados en *A. carterare* que comprende desde aquellos altamente polares a hidrocarburos completamente hidrofóbicos o incluso desde moléculas pequeñas hasta compuestos de mayor peso molecular como lípidos. Este análisis permitió valorar el grado de enriquecimiento en APDs en las fracciones obtenidas en

ambos métodos (partición secuencial y proceso integrado). El análisis se realizó para aquellas fracciones polares y moderadamente polares que contenían APDs resultantes, estas son: fracción n-BuOH procedentes de extractos crudos obtenidos con metanol 100% y Acetona: H₂O 80:20 (v/v) para el primer proceso; y las diferentes fracciones MeOH: H₂O obtenidas tras el fraccionamiento en el segundo proceso.

Del enfoque integrado, las fracciones más activas tras la adsorción y fraccionamiento por elución fueron MeOH: H₂O 60:40, 80:20 y 100:0 (v/v). En este proceso, el fraccionamiento no parte de un extracto de la biomasa, sino de una fase hidroalcohólica, cuyo material orgánico es mucho menor, facilitando una mayor capacidad de retención de APDs en la columna. Sin embargo, no se recuperó el 100% de APDs en las fracciones mencionadas, observándose pequeñas pérdidas en otras fracciones (menos del 8%). Otras clases de metabolitos polares, tales como, aminoácidos, ácidos orgánicos, polialcoholes, azúcares, compuestos de amonios cuaternarios y bases nitrogenadas también fueron recuperados en pequeñas cantidades en esas mismas fracciones activas.

Los resultados obtenidos contrastan con los obtenidos mediante partición secuencial en gradiente con disolventes orgánicos. A pesar de ser una metodología eficaz para concentrar APDs en una única fracción (n-BuOH), también se concentraron cantidades significantes de otras clases de metabolitos polares ya mencionados.

Hasta el momento, todos los procesos orientados al fraccionamiento de la biomasa de *A. carterae*, lograron obtener buenos porcentajes de recuperación de APDs. Sin embargo, la ausencia de concentración de APDs en una única fracción o pérdidas de éstos por el efluente como ocurría en el enfoque dirigido a APDs y en el proceso integrado, desarrollados en los **Capítulos III y V**, respectivamente; o el solapamiento con porcentajes significantes de carotenoides o ácidos grasos, como en el método de

partición secuencial del **Capítulo V**, condujo al abordaje de la mejora en el fraccionamiento para la obtención de extractos enriquecidos en APDs, que se desarrolla en el **Capítulo VI**. Este objetivo se centró en la investigación de la mejora del fraccionamiento de extractos en columna de adsorción de fase reversa, con el objetivo de una adsorción completa de APDs, sin pérdidas por el efluente (extracto no retenido), concentrándolos en una única fracción. De esta forma se podrían obtener extractos de APDs enriquecidos procedentes de *A. carterae*, sin interferencias con otros metabolitos, pero que además nos permitiese recuperar carotenoides y ácidos grasos. Para ello se compararon dos métodos de partición.

El primer método consistió en un fraccionamiento directo del extracto metanólico, mediante EFS en fase reversa (Figura 1, etapa 4.1). El proceso, realizado en cartuchos 1-10g C18 fue optimizado, desde **pretratamiento de la biomasa**, con el objeto de mejorar la rotura celular y favorecer la extracción de APDs, optimización del ratio biomasa/ agente extractante (p/v), y cantidad de extracto metanólico máximo que puede ser retenido por el lecho de la columna, conocido como capacidad de retención. En último lugar, el volumen de elución, además, fue optimizado para la completa desorción de metabolitos retenidos y asimismo evitar pérdidas durante el lavado de sales. La eficacia de cada variable de operación fue medida en términos de actividad hemolítica.

El segundo método estaba compuesto por dos estrategias de mejora del grado de enriquecimiento en APDs: (i) una estrategia que implicaba un etapa previa de extracción líquido-líquido (ELL) justo antes de la etapa de adsorción (ELL-EFS) (Figura 1, etapa 4.2.1). Brevemente consistió en adición de diclorometano (DCM) y agua al extracto metanólico y posterior separación mediante decantación, para obtener dos fases: una fase oscura compuesta por DCM ($\text{PI } 3,1; \delta_T 20 \text{ MPa}^{\frac{1}{2}}$) y una fase clara compuesta por

MeOH: H₂O 70:30 v/v (PI 7,7; δ_T 34,82 MPa^½). Finalmente, el volumen optimizado de fase clara fue extraído mediante EFS utilizando columna 80g C18; (ii) una segunda estrategia consistente en extracción líquido-líquido seguida de partición con n-butanol (ELL-n-BuOH) (Figura 1, etapa 4.2.2). La selección de n-BuOH se basó en la capacidad de éste para concentrar APDs, según los resultados obtenidos en el **Capítulo V**.

Según los datos obtenidos, se consiguió con éxito la correcta optimización del fraccionamiento directo, siendo las fracciones MeOH: H₂O 80:20 (v/v) y MeOH: H₂O 60:40 (v/v) las de mayor actividad hemolítica. Sin embargo, el solapamiento con ácidos grasos en dichas fracciones fue inevitable. Estos resultados fueron el motivo de la aplicación del segundo método, ELL acoplada con EFS, el cual dio lugar a una fase enriquecida en APDs (denominada fase clara) separándolos de carotenoides y ácidos grasos. En segundo lugar, el volumen de fase clara necesario para garantizar pérdidas de APDs por debajo de 5% fue optimizado, y el escalado a columnas 80g C18 resultó en una mejora del proceso, recuperando en un 100% APDs en una única fracción, MeOH: H₂O 80:20 (v/v).

Una vez más, análisis metabolómico mediante RMN fue empleado para evaluar el grado de enriquecimiento en APDs de las fracciones obtenidas del segundo método (esto es, fracción, MeOH: H₂O 80:20 (v/v) para la primera estrategia y fracción n-butanol para la segunda). En ambas estrategias la interferencia con otros metabolitos polares se repitió. Sin embargo, se obtuvo un alto grado de enriquecimiento en fracciones que contenían APDs, cercano al 70% mediante partición con n-butanol, tres veces más que aquellas fracciones más activas obtenidas mediante EFS. Respecto a los otros dos compuestos de mayor valor en *A. carterae*, carotenoides y ácidos grasos, ELL acoplada con partición con n-BuOH también proporcionó los mejores resultados.

PUBLICACIONES DERIVADAS DE LA TESIS DOCTORAL

Capítulo III

M. López-Rodríguez, M.C. Cerón-García, L. López-Rosales, C.V. González-López, A. Molina-Miras, A. Ramírez-González, A. Sánchez-Mirón, F. García-Camacho, E. Molina-Grima. Assessment of multi-step processes for an integral use of the biomass of the marine microalga *Amphidinium carterae*. *Bioresource Technology* 282 (2019) 370–377. (JCR 2019, Biotechnology & Applied microbiology, Factor de Impacto 7,53, Q1 (12 de 156)).

<https://doi.org/10.1016/j.biortech.2019.03.041>

Capítulo IV

M. López-Rodríguez, M.C. Cerón-García, L. López-Rosales, E. Navarro-López, A. Sánchez-Mirón, A. Molina-Miras, A.C. Abreu, I. Fernández, F. García-Camacho. Improved extraction of bioactive compounds from biomass of the marine dinoflagellate microalga *Amphidinium carterae*. *Bioresource Technology* 313 (2020) 123518. (JCR 2020, Biotechnology & Applied microbiology, Factor de Impacto 9,64, Q1 (12 de 159)).

<https://doi.org/10.1016/j.biortech.2020.123518>

Capítulo V

M. López-Rodríguez, M.C. Cerón-García, L. López-Rosales, E. Navarro-López, A. Sánchez-Mirón, A. Molina-Miras, A.C. Abreu, I. Fernández, F. García-Camacho. An integrated approach for the efficient separation of specialty compounds from biomass of the marine microalgae *Amphidinium carterae*. *Bioresource Technology* 342 (2021) 125922. (JCR 2021, Biotechnology & Applied microbiology, Factor de Impacto 11,889, Q1 (11 de 158)).

<https://doi.org/10.1016/j.biortech.2021.125922>

Capítulo VI

M. López-Rodríguez, L. López-Rosales, G. Diletta, M.C. Cerón-García, E. Navarro-López, J.J. Gallardo-Rodríguez, A.I. Tristán, A.C. Abreu, F. García-Camacho. The Isolation of Specialty compounds from *Amphidinium carterae* biomass by two-step solid-phase and liquid-liquid extraction. Toxins 14(9) (2022), 593. (JCR 2021, Toxicology, Factor de impacto 5,075, Q1 (20 de 94)).

<https://doi.org/10.3390/toxins14090593>

SUMMARY

Nowadays, the biotechnological potential of marine dinoflagellate microalgae has been widely recognized given the high value bioactives they produce. **Chapter 1** briefly summarises the state of the art in this field and introduces the lines of research framing the activities developed in this thesis. Initially, the general aspects are presented and an overview of the main microalgal applications are provided. They can potentially be used in a wide variety of applications in diverse sectors such as pharmaceuticals, cosmetics, biofuels, biofertilizers, biostimulants, biopesticides, wastewater treatment, food (both human and animal) and nutraceutical. They have therefore emerged as an alternative and sustainable source of high-added-value compounds. At present, there are several species of microalgae considered important that are already being produced at the large scale. However, much of the technological research looking into microalgae has not yet reached the commercial level, given the difficulties encountered in developing efficient production, extraction and product purification processes that are commercially viable. The biorefinery concept, based on the complete valorisation of the microalgal biomass, seems to be the main alternative. Such research focuses on those parameters that affect biomass pretreatment, and on extracting and purifying the metabolites of interest, such as carotenoids, fatty acids, and bioactive compounds (mainly secondary metabolites).

This thesis especially addresses the general aspects of marine dinoflagellate microalgae, as well as the production and recovery of the secondary metabolites they produce. These secondary metabolites show promise in the development of new drugs and therapeutic agents. Demand for them has increased over recent years in various research areas such as medicine, chemistry, and ecology. Among these secondary metabolites are an important group of compounds, amphidinols and amphidinolides

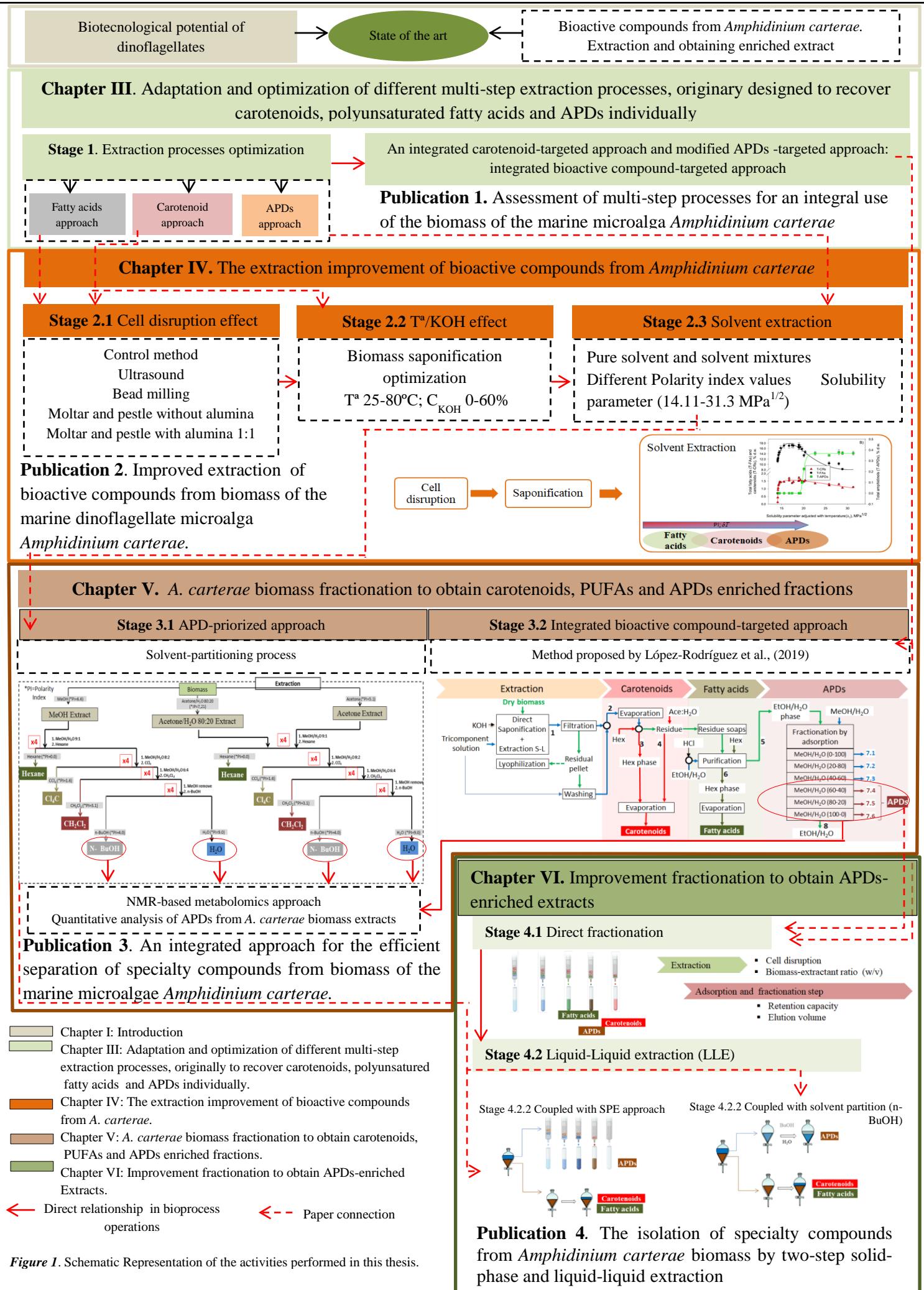
(APDs), which are synthesized by *Amphidinium* and promote potent antifungal, anti-tumour and hemolytic activity. This thesis specifically focuses on the species *Amphidinium carterae*, which, apart from APDs, also synthesize other high value compounds, namely carotenoids and polyunsaturated fatty acids (PUFAs), which need to be recovered within a specifically devised bioprocess. Among these primary metabolites are carotenoids, such as peridinin, that have great potential as therapeutic agents against tumour cell lines, and polyunsaturated fatty acids (PUFAs), such as EPA and DHA, which have great nutritional and pharmacological importance. The advances in *A. carterae* cultivation are briefly described, including its adaptation to different culture conditions, the photobioreactor design, biomass harvesting and its biotechnological potential. After studying the different compound families of interest produced by *A. carterae*, their extraction and purification processes are discussed.

This research addresses the existing scientific bottlenecks in developing a biorefinery approach focused on pre-treating the *A. carterae* biomass to enable the extraction and recovery of extracts, mainly those rich in APDs, while also considering the recovery of multiple high value co-products (i.e., carotenoids and polyunsaturated fatty acids), which are also present in the biomass. The approach is divided into two areas. The first is dedicated to assessing the impact and optimizing the different multi-step extraction processes for extracting and recovering the carotenoids and polyunsaturated fatty acids (PUFAs), originally applied in non-dinoflagellate microalgae, while also allowing the recovery of APDs, thus improving the prospects of exploiting dinoflagellates as a source of high-added-value compounds. To this end, the **biomass pretreatment** is optimized to improve cell disruption and make metabolite extraction feasible.

The second area is dedicated to the **fractionation** of the three groups of bioproducts and on **obtaining enriched extracts**. This is done using two strategies: one prioritizing APD fractionation and the other an integrated strategy targeting the bioactive compounds present in the *A. carterae* biomass, which was produced in a raceway photobioreactor (RW-FBR) by reusing the depleted culture medium. The underlying idea is to contribute to improving the economy and sustainability of the bioprocess using the biorefinery concept.

The next section, **Chapter II - Justification and objectives**, lays out the justification for carrying out this thesis and details the objectives pursued. To achieve the latter, a series of activities are necessary, which are summarised schematically showing the relationship between each of them and the publications derived from them; this provides an overview of the work done (Figure 1). These publications are presented in the thesis grouped by chapters.

Summary



The subsequent chapters develop and assess the different objectives that comprise this research. **The Adaptation and optimization of the different methodologies designed to recover carotenoids, polyunsaturated fatty acids and APDs individually** is discussed in **Chapter III**. Three multi-step extraction processes were selected for this objective; each was originally recommended in the literature for the preferential retrieval of one of the three compound families (Figure 1, Stage 1). The various steps of each methodology were optimized and then their impact on the simultaneous recovery of the three compound families present in the *A. carterae* biomass were evaluated. The first methodology focused mainly on recovering free fatty acids (PUFAs); it consisted of initially grinding the *A. carterae* biomass followed by saponification and simultaneous extraction. The optimizations carried out using the biomass from *A. carterae* ACRN03 achieved recovery percentages of 79%, 61% and 100% for fatty acids, carotenoids and APDs, respectively. However, this methodology did not allow polar carotenoids such as peridinin or dinoxanthin (which have a higher added value) to be recovered from the *A. carterae* biomass. Only non-polar carotenoids were recovered, such as β -carotene, or moderately polar diadinoxanthin and diatoxanthin. In order to recover both polar and non-polar carotenoids, a second methodology was adapted for *A. carterae*. The carotenoid-targeted approach combined direct saponification and simultaneous extraction in a single step with a final purification step. This methodology achieved total recovery percentages of 80%, 97% and 100% for fatty acids, carotenoids and APDs (the latter not purified), respectively. Finally, a third methodology focused on recovering bioactive compounds that exhibit hemolytic activity (mainly APDs) was adapted. Briefly, the biomass was sonicated with methanol and the resulting methanolic supernatant was subjected to solid-phase extraction (SPE) by reverse phase chromatography to separate the APDs from the other

compounds, followed by elution using methanol:water mixtures (MeOH:H₂O) in different proportions (% v/v): 0:100, 20:80, 40:60, 60:40, 80:20 and 100:0, and with different polarity ranges (PI) (10.2 to 6.6). After the sorption and elution protocol, the different fractions obtained were analysed for their hemolytic activity. The phases with methanolic fractions ranging between 0:100 and 40:60 contained no hemolytic compounds. In contrast, the most active fractions were 60:40 and 80:20 with 13% and 33% of APDs, respectively. The overall APD recovery percentage using this methodology was almost 70%, with around 11% not being adsorbed on the solid-phase column (C18). This could be due to a high crude extract-to-adsorbent ratio. None of the methods assessed in **Chapter III** were able to recover 100% of the three compound families.

In view of the results, a combination of the modified carotenoid-targeted approach and the modified APD-targeted approach was investigated to improve the recovery yield, the intention being to develop a biorefinery approach prioritizing the recovery of multiple high value co-products (i.e., carotenoids, PUFAs and APDs). To develop a single process that allowed the complete recovery of all three compound families of interest contained in the *A. carterae* biomass, an experimental plan was initially developed to improve their extraction, the results of which are described in **Chapter IV (Improvement in the extraction of bioactive compounds from *Amphidinium carterae*)**. The first phase aimed to optimise the cell disruption and biomass alkaline saponification step by varying the temperature (25°C-80°C) and KOH concentration (0%-60% d.w.) (Figure 1, Stages 2.1 and 2.2), the first time this has been attempted for *A. carterae*. The efficacy of each factor studied was measured in terms of carotenoid recovery. The aim of the second phase was to maximize the recovery yields of the three metabolite families using a wide range of individual solvents, and mixtures

thereof, with the polarity index (PI) and the Hildebrand solubility parameter (δ_T) ranging from 0 to 7.46, and between 14.11 and 31.30 MPa^{1/2}, respectively (Figure 1, Stage 2.3). In the first phase, it was observed that biomass saponification of the Dn241EHU strain led to carotenoid losses from degradation, at whichever KOH concentration, unlike the non-saponified control. Conversely, for the ACRN03 biomass, there was a significantly improved peridinin recovery percentage ($p<0.05$) with respect to the control at all KOH concentrations, reaching a plateau at 20% KOH d.w.. The reasons for the absence of peridinin degradation in the ACRN03 strain during saponification are unclear; it might be due to differences in the cell wall composition of each strain, to the culture mode, or even to the presence of peridinin in ester form. Whatever the circumstances, the *A. carterae* Dn241EHU strain was selected as the most promising, given its higher carotenoid content compared to *A. carterae* ACRN03 when under optimal extraction conditions, namely, 0% KOH d.w. at 60°C. Regarding the total content of fatty acids, this was similar for both strains, with values of 18.12 ± 1.74% d.w. for Dn241EHU and 18.41 ± 2.47% d.w. for ACRN03, with hexadecanoic acid (16:0), EPA (20:5n3) and DHA (22:6n3) being those with the highest dry weight content. In the second phase, the optimal solvent conditions were predicted by correlating the extraction efficiencies with the solvent polarity and solubility parameter. The optimal extraction of carotenoids and fatty acids was achieved using solvents with PI values of 3-5.5 and δ_T values of 16.5- 19 [MPa^{1/2}], such as acetone: H₂O 99:1 (v/v) and acetone: H₂O 99.5:0.5 (v/v), respectively. In contrast, solvents with δ_T between 22-31 [MPa^{1/2}] and a PI of 6-7.5, such as methanol 100%, ethanol: H₂O 80:20 (v/v), acetone: H₂O 80:20 (v/v) and methanol: H₂O 80:20 (v/v) provided the highest APD recovery percentages.

The results obtained in **Chapter IV** completed the study aimed at developing a single process that allowed the full recovery of the three compound families of interest. The results are analysed in **Chapter V (Fractionation of the *A. carterae* biomass to obtain fractions enriched in carotenoids, PUFAAs and APDs)**. The objective was to implement an APD-prioritized fractionation strategy that obtained fractions rich in the most important metabolite classes. Two approaches were applied to achieve this objective: **(i)** An APD-prioritized approach sequentially partitioning the crude *A. carterae* extracts using a sequential polarity gradient (Figure 1, Stage 3.1). Three crude *A. carterae* biomass extracts were used, each obtained with three different solvents: acetone 100%, acetone: water 80:20 (v/v), and methanol 100%. These were selected based on the results obtained in **Chapter IV**, in which it was shown that only solvents with a PI and δ_T greater than 6 and 20 [MPa^{1/2}], respectively, managed to extract the APDs present in the *A. carterae* biomass. Accordingly, methanol 100% and acetone: H₂O 80:20 (v/v) met the above requirements, but not 100% acetone (the extract used as the negative control). The crude extracts were subjected to a solvent-partitioning process based on a sequential gradient partition with solvents (hexane (Hex), carbon tetrachloride (CCl₄), dichloromethane (DCM), n-butanol (n-BuOH) and water (H₂O)) with polarity values ranging from 0.1 to 10.2. The process was successfully applied to *A. carterae* for the first time, clearly isolating the APDs in the n-BuOH fraction. However, it was not sufficiently effective for the fatty acids and carotenoids - despite concentrating them mainly in the hexane and CCl₄ fractions, respectively, small amounts were identified in subsequent higher polarity fractions (DCM, n-BuOH, and H₂O). Therefore, when the target metabolites are APDs, this methodology can be used to concentrate them into a single fraction and obtain enriched extracts. **(ii)** Alternatively, in the dinoflagellate biorefinery context, the integrated process proposed in **Chapter III**

was experimentally applied. As stated above, this is an integrated approach aimed at extracting APDs, carotenoids and fatty acids (Figure 1, Stage 3.2). The approach consisted of saponification and simultaneous biomass extraction from *A. carterae* to saponify the fatty acids in the form of soaps and separate the carotenoids. The first stream targeted the recovery of non-polar and polar carotenoids, then a second stream aimed to release the fatty acids present in the form of potassium salts followed by a subsequent purification step with hexane. The resulting hydroalcoholic phase became the input stream for the APD-targeted section. In brief, the hydroalcoholic phase was loaded into a C18-130 mg packed bed comprising four disposable C18 cartridges connected in series. The experimental application of the process achieved recovery percentages of 89%, 82% and 100% for carotenoids, fatty acids and APDs (not purified), respectively.

To evaluate the efficacy of both processes in terms of extraction and obtaining APD-enriched extracts, a metabolomic analysis was performed using NMR. This technique allowed us to determine the percentage distribution of the metabolite classes identified in *A. carterae*, ranging from highly polar zwitterionic metabolites to entirely hydrophobic hydrocarbons, or from small molecules up to higher-molecular-weight compounds such as lipids. Indeed, the NMR-based metabolomic approach allowed us to assess the degree of APD enrichment in the most active fractions obtained by both methods (solvent-partitioning and the integrated bioactive compound-targeted approach). The analysis focused on the NMR metabolomic profiles of the moderately polar and polar fractions that contained the resulting APDs. In the former, the data correspond to the n-BuOH fractions obtained from two crude extracts: acetone: water 80:20 (v/v) and methanol 100%, while the latter corresponds to the different fractions obtained after the fractionation step.

From the integrated bioactive compound-targeted approach, the most active fractions following adsorption and fractionation by elution were MeOH: H₂O 60:40, 80:20 and 100:0 (v/v). Due to the fractionation step being carried out using a hydroalcoholic phase, the retention of polar compounds on this non-polar surface (i.e. C18) was further favoured because the amount of organic matter in the mobile phase was small compared to the amount of sorbent. However, the aforementioned fraction did not recover 100% of the APDs since tiny percentages were found in other fractions (<8%). Regarding the other classes of polar metabolites, namely amino acids, organic acids, sugars, quaternary ammonium compounds, polyhydric alcohols and nitrogenous bases, these were also found in tiny amounts in those same APD-enriched fractions.

The results from the integrated bioactive compound-targeted process contrast with those obtained in the solvent-partitioning procedure. Even though it could be an advantage for all the APDs to be recovered in a single fraction (n-BuOH), it also swept along significant amounts of those polar metabolite classes already identified.

So far, all the processes aimed at fractionating *A. carterae* biomass managed to obtain good APD recovery percentages. However, the absence of an APD concentration in a single fraction, or APD losses in the effluent (as occurred in the bioactive compound-targeted approach and in the integrated process carried out in **Chapters III** and **V**, respectively), or the overlap with small percentages of carotenoids or fatty acids (as occurred in the APD-prioritized approach through solvent-partitioning carried out in **Chapter V**), led to an approach **to improve fractionation to obtain APD-enriched extracts**, which is developed in **Chapter VI**. In this section, the objective was to improve extract fractionation by reverse phase chromatography, completely adsorbing the APDs with no effluent losses (the extract was not retained), and then concentrating them in a single fraction. In this way, APD-enriched extracts could be obtained from *A.*

carterae without interference from other metabolites, while also allowing us to recover the carotenoids and fatty acids. For this, two partitioning methods were compared.

The first method consisted of direct fractionation of the methanolic extract using SPE in reverse phase (Figure 1, Stage 4.1). The process, carried out using 1-10g C18 cartridges, was optimized for the **biomass pretreatment**, the object being to improve cell disruption and make APD extraction feasible, as well as optimizing the biomass / extractant agent ratio (w / v) and the maximum amount of organic material that could be retained (the retention capacity) in the reverse-phase SPE device. Finally, the elution volume was also optimized to completely elute all the retained metabolites and to avoid losses during the salt washing. The efficacy of each optimized parameter was assessed by measuring the hemolytic activity.

The second method comprised two strategies for improving the level of APD enrichment: (i) a two-step strategy that involved a prior liquid-liquid extraction (LLE) step coupled with SPE. Briefly, this consisted of adding dichloromethane (DCM) and water to the crude MeOH extract and subsequent separation by decantation to obtain two phases: a dark phase composed of DCM (PI 3.1; δ_{T20} MPa^{1/2}) and a clear phase composed of MeOH: H₂O 70:30 v/v (PI 7.7; δ_T 34.82 MPa^{1/2}). Then, the loaded volume of clear phase was optimized and extracted by SPE using an 80g C18 column; (ii) a second strategy consisting of liquid-liquid extraction followed by solvent partitioning with n-butanol (LLE-n-BuOH) using the clear phase. n-BuOH was selected because of its ability to concentrate APDs, according to the results obtained in **Chapter V**.

Based on the data obtained, the direct fractionation was successfully optimized, the MeOH fractions with the highest hemolytic activity being: H₂O 80:20 (v/v) and MeOH: H₂O 60:40 (v/v). However, the overlapping of fatty acids in these fractions was inevitable. This was the reason for applying the second method, LLE coupled with SPE.

First, an APD-enriched phase was obtained (the clear phase), separating the APDs from the carotenoids and fatty acids. Secondly, the clear phase extract volume needed to ensure APD losses below 5% was optimized and the absorbent was scaled up 80-fold using 80g C18 columns – this led to an improvement in the process, with 100% of the APDs being recovered in the MeOH: H₂O 80:20 (v/v) fraction.

Once again, NMR metabolomic analysis was used to evaluate the degree of APD enrichment present in the fractions obtained from the second method (that is, the MeOH: H₂O 80:20 (v /v) fraction for the first strategy and the n-butanol fraction for the second). In both strategies, interference with other polar metabolites was inevitable. Nevertheless, a high degree of purity was obtained in the APD-enriched fractions, close to 70% using n-butanol partitioning, which is three-times greater than in the more active fractions obtained after SPE. Regarding the other two higher-value compounds present in *A. carterae* (carotenoids and fatty acids), two-step LLE coupled with the solvent-partitioning method presented the best results.

PUBLICATIONS RESULTING FROM THE THESIS

Chapter III

M. López-Rodríguez, M.C. Cerón-García, L. López-Rosales, C.V. González-López, A. Molina-Miras, A. Ramírez-González, A. Sánchez-Mirón, F. García-Camacho, E. Molina-Grima. Assessment of multi-step processes for an integral use of the biomass of the marine microalga *Amphidinium carterae*. *Bioresource Technology* 282 (2019) 370–377. (JCR 2019, Biotechnology & Applied microbiology, Impact Factor 7,53, Q1 (12 de 156)).

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Chapter IV

M. López-Rodríguez, M.C. Cerón-García, L. López-Rosales, E. Navarro-López, A. Sánchez-Mirón, A. Molina-Miras, A.C. Abreu, I. Fernández, F. García-Camacho. Improved extraction of bioactive compounds from biomass of the marine dinoflagellate microalga *Amphidinium carterae*. *Bioresource Technology* 313 (2020) 123518. (JCR 2020, Biotechnology & Applied microbiology, Impact Factor 9,64, Q1 (12 de 159)).

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Chapter V

M. López-Rodríguez, M.C. Cerón-García, L. López-Rosales, E. Navarro-López, A. Sánchez-Mirón, A. Molina-Miras, A.C. Abreu, I. Fernández, F. García-Camacho. An integrated approach for the efficient separation of specialty compounds from biomass of the marine microalgae *Amphidinium carterae*. *Bioresource Technology* 342 (2021) 125922. (JCR 2021, Biotechnology & Applied microbiology, Impact Factor 11,889, Q1 (11 de 158)).

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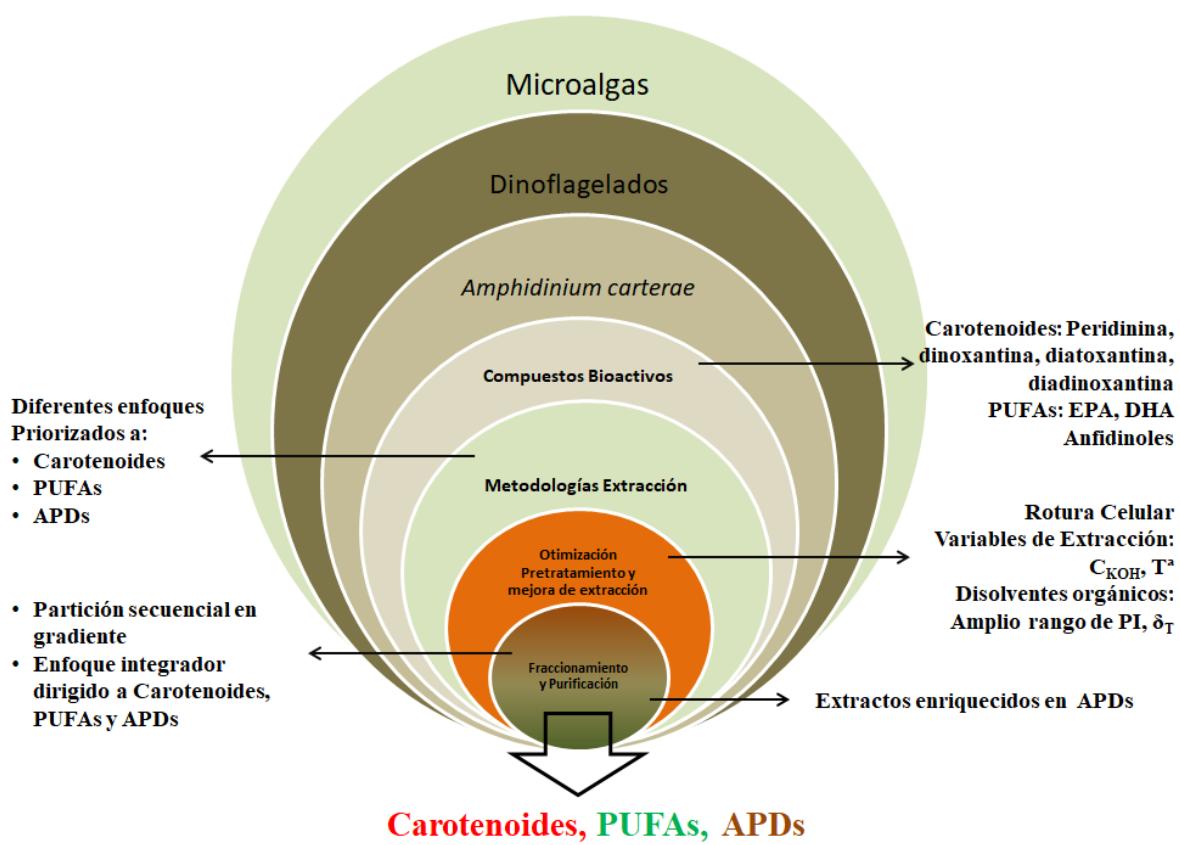
Chapter VI

M. López-Rodríguez, L. López-Rosales, G. Diletta, M.C. Cerón-García, E. Navarro-López, J.J. Gallardo-Rodríguez, A.I. Tristán, A.C. Abreu, F. García-Camacho. The Isolation of Specialty compounds from *Amphidinium carterae* biomass by two-step solid-phase and liquid-liquid extraction. Toxins 14(9) (2022), 593. (JCR 2021, Toxicology, Impact Factor 5,075, Q1 (20 de 94)).

<https://doi.org/10.3390/toxins14090593>

CAPÍTULO 1

INTRODUCCIÓN



1.1 ASPECTOS GENERALES DE LAS MICROALGAS

Las microalgas son microorganismos unicelulares, esenciales dentro del ecosistema, ya que son fuente de alimento para una gran variedad de organismos acuáticos (Loke-Show, 2022). La mayoría de las caracterizadas son fotosintéticas las cuales usan luz solar, agua y CO₂ como nutrientes para su crecimiento (Park y cols., 2022), sin embargo, en líneas generales, existen otras formas de modo de nutrición en las microalgas como son la mixotrofía o heterotrofía, entre otras. Se encuentran en casi todos los hábitats conocidos, principalmente acuáticos, tanto en ambientes marinos como de agua dulce (Dolganyuk y cols., 2020). Constituyen un grupo compuesto por numerosas especies de diferentes filos, que viven bajo un amplio rango de condiciones ambientales. El número de especies identificadas se estima en aproximadamente 100.000; sin embargo, solo unas 35.000 especies han sido caracterizadas (Sathasivam y cols., 2019; Park y cols., 2022).

Son microorganismos de rápido crecimiento frente a cultivos terrestres; capaces de lograr altas productividades de biomasa por encima de 100 t/ha año. Pueden ser cultivados sin utilizar grandes superficies de tierra fértil cultivable, empleo de productos fitosanitarios de origen químico o herbicidas comparado con los cultivos de especies vegetales (Acién-Fernández y cols., 2021; Siddiki y cols., 2022). Incluso algunas especies pueden ser cultivadas utilizando aguas residuales, suponiendo una ventaja ambientalmente sostenible (Benedetti y cols., 2018; Dolganyuk y cols., 2020). Y además son productores de metabolitos de gran interés comercial en diferentes sectores (Orejuela-Escobar y cols., 2021).

Existe gran diversidad de metabolitos producidos por las microalgas, comprendidos desde metabolitos primarios simples hasta metabolitos secundarios complejos (Cardozo y cols., 2007; Park y cols., 2022). Estos compuestos, de gran valor

añadido, incluyen carotenoides, ácidos grasos, proteínas, policétidos, macrólidos, toxinas, esteroles, vitaminas, entre otros.

Las microalgas han sido utilizadas por los humanos como suplemento nutricional durante cientos de años, pero no fue hasta el siglo 20 cuando la producción comercial de algunas especies de microalgas comenzó a darse. Especies como *Spirulina* fueron declaradas una fuente potencial futura de alimento por United Nation World Food Conference (Amaro y cols., 2018) y en 1977 estableciéndose la primera planta comercial en Tailandia. Especies como *Chlorella* también han sido utilizadas como aditivos alimentarios en Japón (Khan y cols., 2018; Camacho y cols., 2019), e incluso algunos productos comerciales como pastas o bebidas han sido suplementados nutricionalmente mediante la incorporación de biomasa microalgal (Barra y cols., 2014; Mu y cols., 2019).

El incremento de popularidad en el uso de microalgas es notable a nivel mundial. La producción anual de biomasa microalgal está estimada, aproximadamente, en unas 7000 toneladas a nivel mundial. Durante la pandemia de COVID-19 en 2020 el mercado global rondó los 3,4 mil millones USD y se prevé un alcance de hasta 4,6 mil millones para el año 2027 (Tang y cols., 2020; Loke-Show, 2022). Estos datos son resultado del, cada vez mayor, conocimiento en cuanto al potencial de los compuestos de gran valor que producen y los diversos beneficios que aportan en términos de sostenibilidad, comparado con los métodos tradicionales de producción de alimentos y/o energía (Loke-Show, 2022). Además, existe un amplio rango de aplicaciones emergentes como biofertilizantes, tratamiento de aguas residuales, productos farmacológicos cuyo mercado es enorme (Acién-Fernández y cols., 2021). Los últimos análisis de mercado indican que el interés comercial por las microalgas es impulsado principalmente por la demanda de la industria farmacéutica y nutracéutica. Ambos

sectores son espoleados por la creciente preocupación de los consumidores por su salud y por el uso de remedios más naturales, así como por el aumento de enfermedades crónicas de difícil tratamiento que necesitan nuevos tratamientos (Balasubramaniam y cols., 2021).

Actualmente, solo unas pocas especies han sido aprobadas por US Food and Drug Administration (FDA) (Balasubramaniam y cols., 2021), con una parte de ellas producidas a gran escala y comercialmente disponibles tanto para fines terapéuticos como nutricionales. En la Tabla 1 se presentan ejemplos de algunas de las especies explotadas comercialmente por diferentes compañías, producto o su área de aplicación y sus diferentes usos en la industria especialmente para fines nutracéuticos , entre ellas, *Spirulina*, *Dunaliella*, *Chlorella* y *Haematococcus* (Benedetti y cols., 2018, Balasubremaniam y cols., 2021).

Tabla 1. Listado de algunas de las especies de microalgas comercializadas, aplicaciones industriales, empresas comercializadoras y países implicados. Modificado de Balasubramaniam y cols., 2021.

GÉNERO	PRODUCTO	USOS INDUSTRIALES/ ÁREA DE APLICACIÓN	COMPAÑÍA
<i>Spirulina</i> .	Biomasa, ficocianina, proteínas, vitamina B12	Suplemento nutricional, fórmulas infantiles, biocolorantes	Cyanotech (EEUU), Panmol/Madaus (Austria) Parry Nutraceuticals (India), Phytobloom (Portugal)
<i>Chlorella</i>	Biomasa, carbohidratos, ficobiliproteinas,	Alimentos saludables, suplementos alimenticios, suplementos piensos, cosmética, nutrición humana y animal, acuicultura	Nikken Sohonsa Corp. (Japón), Earthrise Nutritionals (EEUU), Klötze (Alemania), Phytobloom (Portugal)
<i>Dunaliella</i>	β-caroteno, glicerol	Alimentos saludables, suplementos alimenticios, suplementos piensos, pigmentos, nutrición humana y animal	Nature Beta Technologies Cognis (Australia), Cyanotech (EEUU), Nikken Sohonsa Corp. (Japón), Earthrise Nutritionals (EEUU), Phytobloom (Portugal)
<i>Haematococcus</i>	Astaxantina, carotenoides, alimentos saludables	Aditivos nutricionales, biocolorantes, nutrición humana, acuicultura	Cyanotech (EEUU), BioReal (EEUU), Parry agro Industries (India)
<i>Odontella</i>	Ácidos grasos, cosmética, EPA y DHA	Suplemento nutricional, aditivos nutricionales, fórmulas infantiles	InnovalG (Francia)
<i>Porphyridium</i>	Polisacáridos, cosmética, fórmulas infantiles, B-ficoeritrina, C-ficocianina	Nutrición y actividad antioxidante	InnovalG (Francia)

Dadas las características que las definen, la gran diversidad de especies de microalgas y los recientes desarrollos tecnológicos, este grupo de organismos representa una de las fuentes más prometedoras para la obtención de nuevos productos y aplicaciones (Cerón-García y cols., 2013; De Moraes y cols., 2015). Por esta razón, la biotecnología de microalgas ha adquirido gran importancia en las últimas décadas. Sin embargo, todavía existen desafíos que comprometen el desarrollo a gran escala, tales como el alto coste de producción y procesamiento de la biomasa. Por el contrario, muchos estudios concluyen que el desarrollo biotecnológico de microalgas a gran escala puede llegar a ser económicamente viable. Basado en el concepto de biorrefinería, la coproducción de diferentes compuestos parece ser la respuesta para desarrollar procesos económicamente viables ('t Lam y cols., 2018; Chew y cols., 2017; Corrêa y cols., 2020; Ma y cols., 2020; Kiran y Venkata-Mohan., 2021).

1.1.1 Aplicaciones de las microalgas

Son múltiples los sectores dónde diversas especies microalgales encuentran su nicho de aplicación. Previo a ello, la selección de la especie así como los requerimientos nutricionales de éstas, son importantes aspectos a considerar (Figura 2). Alcanzar el máximo potencial de producción de microalgas, requiere tareas de estudio de: fluidodinámica, requerimientos nutricionales, influencia de factores abióticos, adhesión celular, modo de operación, diseño y escalado del fotobiorreactor.

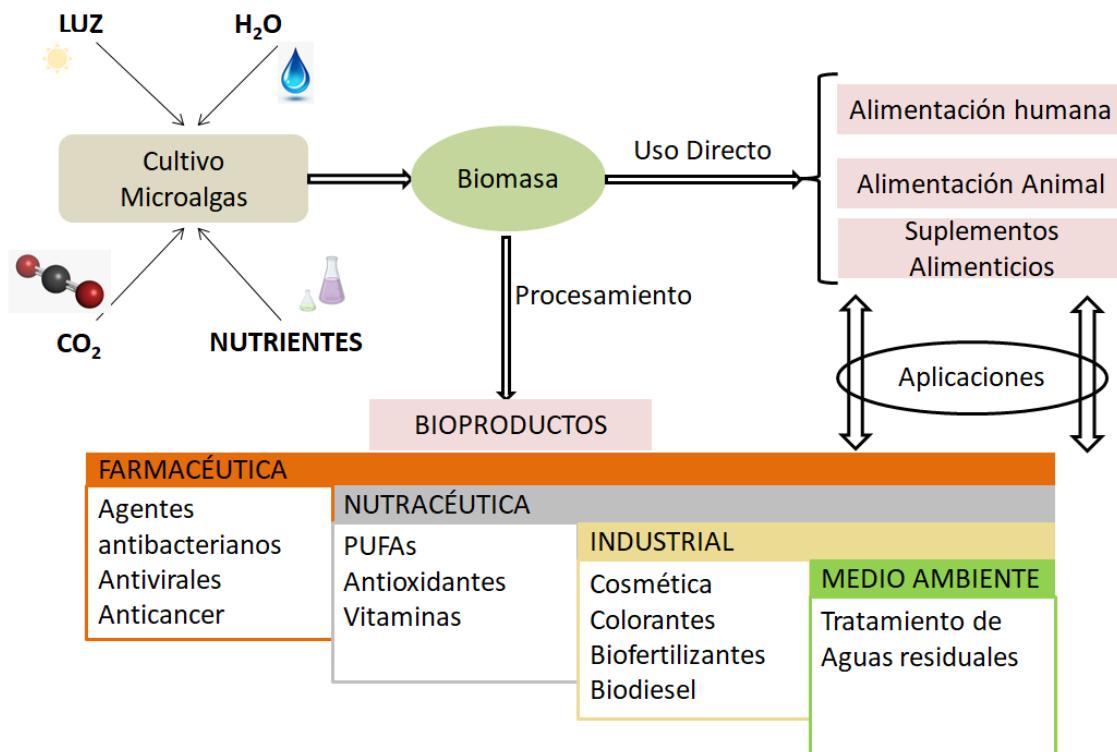


Figura 2. Representación de las principales aplicaciones de microalgas. Modificado de Mehariya y cols., 2021.

De manera global, las aplicaciones de las microalgas pueden ser agrupadas en tres bloques. El primero de ellos corresponde al uso de la propia biomasa. En cualquiera de sus formatos comerciales, como por ejemplo, grajeas, polvo, harinas u otras, las microalgas son en la actualidad empleadas en la industria alimentaria como suplementos alimentarios, alimentos funcionales o como ingredientes para la alimentación animal, dadas sus propiedades nutracéuticas (Camacho y cols., 2019) y cuya cuota en el mercado va aumentando cada año (Lafarga y Acién-Fernández, 2022). El segundo bloque de aplicación está ligado a la obtención a partir de la biomasa de productos tales como carbohidratos, proteínas, lípidos, pigmentos, vitaminas (Khan y cols., 2018; Stirk y cols., 2020). A muchos de ellos se les atribuyen efectos beneficiosos sobre la salud humana por sus actividades antioxidantes, anti-inflamatorias, o incluso protectoras de la piel, abarcando así nichos de mercado tales como el, nutracéutico o el cosmético (Tang y cols., 2020). Pero además, las microalgas pueden potencialmente satisfacer las

exigencias de otros tipos de industrias como: tratamiento de aguas residuales, producción de biofertilizantes o biodiesel (Cerón-García, 2013; Jiménez-Callejón, y cols., 2020a; Lafarga y Acién-Fernández, 2022). Por último, el tercer bloque está relacionado con metabolitos secundarios los cuales son producidos en bajas concentraciones y no son estrictamente esenciales para su supervivencia. El interés en éstos es principalmente debido a su potencial uso farmacológico (Borowitzka, 1995; Pradhan y Ki, 2022). Ejemplo de ello, policétidos y macrólidos producidos por dinoflagelados y cianobacterias dado sus efectos terapéuticos y actividad anticancerígena (García-Camacho y cols., 2007; Gallardo-Rodríguez y cols., 2012; Sathasivam y cols., 2019).

➤ Metabolitos primarios

Como organismos fotosintéticos, las microalgas contienen una serie de pigmentos naturales captadores de la luz como las clorofilas, ficoliproteínas y carotenoides. Desde un punto de vista comercial, los carotenoides, son el grupo que ha atraído mayor interés dadas las aplicaciones como suplementos alimenticios, nutrición humana y animal y elaboración de productos cosméticos (Balasubramaniam y cols., 2021; Menaa y cols., 2021). Se trata de lípidos solubles con propiedades antioxidantes confiriendo protección a las microalgas frente a los efectos de radicales libres. La mayoría de ellos comparten una estructura común de C40 unidades de isopreno y se dividen en dos grupos: carotenos y xantofilas (Gong y Bassi, 2016). A nivel celular, podemos distinguir carotenoides primarios, como violaxantina o luteína, asociados a función estructural ubicados en el aparato fotosintético; y carotenoides secundarios los cuales desempeñan funciones de protección bajo condiciones de estrés y se encuentran almacenados en cuerpos lipídicos, como astaxantina, zeaxantina o β -caroteno (Collins, y cols., 2011).

Constituyen un grupo de compuestos con gran potencial dada su actividad antioxidante, protección frente a enfermedades cardiovasculares, antiinflamatoria, anticáncer, entre otros, siendo β -caroteno, astaxantina, luteína, cantaxantina y fucoxantina, los de mayor interés comercial (Gong y Bassi, 2016; Galasso y cols., 2019). β -caroteno, precursor de la vitamina A, ha sido uno de los primeros compuestos de alto valor añadido, de origen microalgal, en ser comercializado con fines cosméticos y nutracéuticos por sus efectos antioxidantes y de protección frente a rayos UV (Gateau y cols., 2017; Galasso y cols., 2019). Astaxantina es otro de los carotenoides de alto valor añadido con aplicaciones en la industria nutracéutica, cosmética y alimentaria. Éste es utilizado en acuicultura para dar pigmentación a la carne de pescado (Olaizola y Huntley, 2003). Además se le ha atribuido una mayor capacidad antioxidante con respecto a otros carotenoides (Camacho y cols., 2019) con grandes efectos de antienvejecimiento protegiendo la piel del daño causado por la exposición a rayos UV (Olaizola y Huntley, 2003; Capelli y cols., 2012).

Otro de los componentes celulares más populares a nivel comercial son los lípidos, que dependiendo de las condiciones de cultivo, pueden suponer entre 30 y 50% del total del peso de la biomasa (Chew y cols., 2017). La fracción lipídica en microalgas está compuesta principalmente por: (i) lípidos neutros, que incluye lípidos saponificables como triglicéridos, ácidos grasos libres y ceras; lípidos insaponificables como los carotenoides; (ii) lípidos polares como fosfolípidos, galactolípidos y glucolípidos (Robles-Medina y cols., 1998; Molina-Grima y cols., 2013; Navarro-López y cols., 2016). Entre los ácidos grasos producidos por microalgas encontramos los saturados (SFA), monosaturados (MUFA) o poliinsaturados (PUFA). Estos compuestos juegan un papel fundamental en el metabolismo y crecimiento microalgal. Los SFAs y MUFA son excelentes fuentes para la producción de biodiesel (Papachristou y cols.,

2021). Por el contrario, ácidos grasos poliinsaturados (PUFAs) han sido considerados como productos de alto valor añadido dadas sus aplicaciones como nutracéuticos con numerosos beneficios sobre la salud, tales como reducción de problemas cardiovasculares, incremento de colesterol bueno (HDL) (Jacob-Lopes y cols., 2019); elaboración de formulaciones infantiles (Barkia y cols., 2019; Papachristou y cols., 2021); como fuente alimenticia y de aditivos en la cría comercial de muchas especies acuáticas (Acién-Fernández y cols., 2021; Sales y cols., 2021). En este último caso, tradicionalmente, harinas y aceites de pescado han sido empleados para cumplir los requerimientos nutricionales de especies acuáticas utilizadas en acuicultura. Sin embargo, el porcentaje de derivados de pescado en los alimentos acuícolas han disminuido progresivamente debido a su alto coste y su disponibilidad cada vez menor. Por ello, la industria de la alimentación acuícola está sustituyendo estos ingredientes por harinas y aceites de origen vegetal. A pesar de esta alternativa de origen vegetal, tiene una serie de limitaciones en comparación a los de origen marino, como presencia de factores antinutricionales, desequilibrio de aminoácidos y reducción de PUFAs (Sales y cols., 2021). Alternativamente, las microalgas surgen como fuente sostenible de PUFAs, especialmente EPA y DHA. No solamente son utilizadas como fuente de alimentación en acuicultura, sino que también mejoran la calidad de las especies animales cultivadas. Por ejemplo, especies como *Isochrysis*, *Tetraselmis* o *Nannochloropsis*, fuentes de PUFAS especialmente de EPA o DHA, han sido utilizadas para alimentación y desarrollo de varias especies larvarias empleadas en acuicultura, y además promueven pigmentación de la piel e inmunoestimulación (Sales y cols., 2021). Incluso el uso de algunas especies de microalgas en la dieta de peces disminuye su precio en un 50% e incrementa su valor nutricional (Sathasivam y cols., 2019).

Los tipos de PUFA más conocidos son EPA y DHA, no solo por su empleo en acuicultura sino también dadas las varias actividades biológicas que presentan, por los beneficios frente a enfermedades cardiovasculares y respiratorias así como por presentar actividades antimicrobianas y antiinflamatorias (Adarme-Vega y cols., 2012; Riccio y Lauritano, 2020; Macías-Sánchez y cols., 2022). Los seres humanos no son capaces de sintetizarlos por lo que necesitan incorporarlos mediante la dieta (Sales y cols., 2020). Una de las fuentes alimenticias de este grupo de ácidos grasos poliinsaturados, incluye el pescado o plantas superiores. Sin embargo, la recuperación de dichos compuestos a partir de pescado se ha visto obstaculizada debido a la elevada sobreexplotación de recursos marinos, mientras que la demanda ha seguido creciendo (Jiménez-Callejón y cols., 2020b). Por esta razón la búsqueda de fuentes alternativas se ha visto incrementada en los últimos años, y es así dónde las microalgas juegan un papel importante como fuente sostenible de este tipo de compuestos (Jiménez-Callejón y cols., 2020a; Sales y cols., 2020).

Entre la gran diversidad de compuestos producidos por las microalgas, como podemos observar, ácidos grasos poliinsaturados (PUFAs) y carotenoides son los de mayor interés en el campo de la nutrición y farmacológico (Delbrut y cols., 2018). En relación a éstos últimos, el mercado creció de 1,24 mil millones USD en 2016 a más de 1,53 mil millones USD en 2021 (Barkia y cols., 2019).

➤ Metabolitos secundarios

Además de metabolitos primarios, algunas microalgas producen metabolitos secundarios, los cuales muestran diversos efectos terapéuticos tales como anticáncer, antifúngico antibiótico y neurotóxico (Park y cols., 2022; Pradhan y Ki, 2022). Aquellos producidos por las microalgas del grupo dinoflagelados son particularmente interesantes por su diversidad y singularidad estructural y mecanismo de acción.

Estos metabolitos de alto valor añadido revalorizan la utilización de dicha biomasa, haciendo de los dinoflagelados un atractivo para la investigación en el campo farmacológico, como es el caso de las enfermedades neurodegenerativas (Sathasivam y cols., 2019). Estos compuestos naturales son incluso más efectivos a menores dosis que muchos de los competidores obtenidos por síntesis química. Para algunos de estos metabolitos secundarios se ha podido establecer su síntesis química. Sin embargo, el elevado número de etapas químicas implicadas da lugar a procesos bastante complejos e inviables desde el punto de vista tanto económico como medioambiental. Por tanto, la principal vía de producción a nivel comercial de estos metabolitos naturales pasa por el cultivo de los dinoflagelados productores, extracción de la biomasa y purificación (Assunção y cols., 2017).

1.1.2 Extracción de compuestos de alto valor añadido

Generalmente los procesos basados en microalgas están orientados a la recuperación de un único producto para una aplicación específica (Jacob-Lopes y cols., 2019). Un bioprocreso basado en microalgas implica una sección de procesamiento de la biomasa cosechada en fotobiorreactores, conocida como *downstream processing*. Dentro de esta sección se diferencian diversas operaciones: i) Pre-tratamiento de la biomasa para facilitar la accesibilidad de los sistemas extractantes a los metabolitos de interés; ii) extracción con disolventes orgánicos, altamente influenciado por las características físico-químicas del metabolito objetivo; iii) fraccionamiento y purificación del extracto crudo (Figura 3).

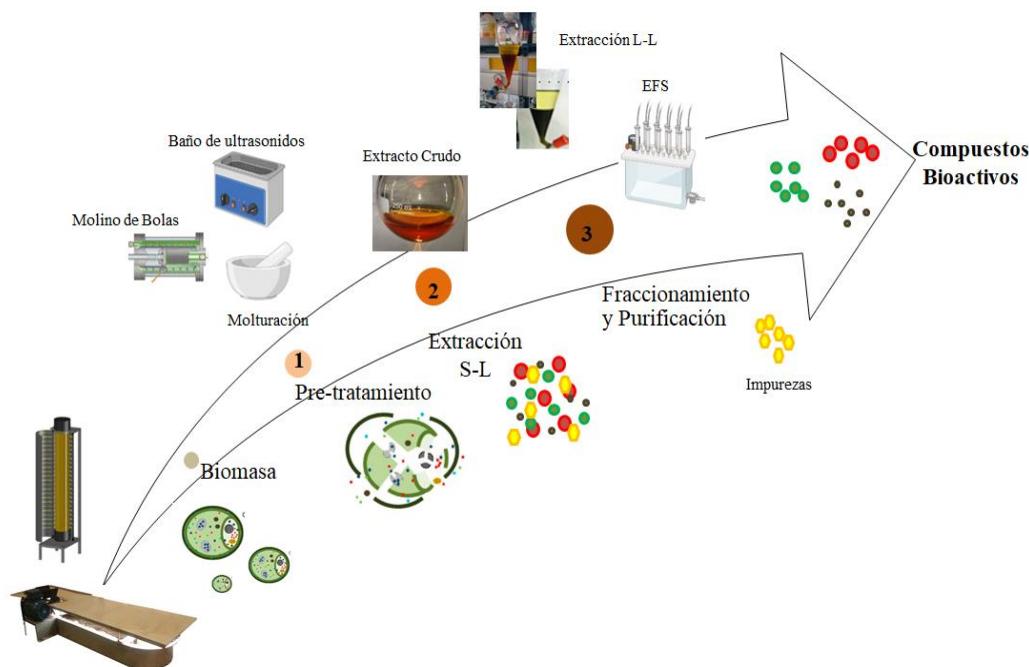


Figura 3. Resumen esquemático de un bioprocreso a partir de microalgas bajo el concepto de biorrefinería para la obtención de compuestos bioactivos. EFS: extracción en fase sólida.

La morfología celular, así como las propiedades de los metabolitos objetivo son determinantes a la hora de seleccionar aquellos métodos o técnicas que pueden ser aplicadas en cada etapa (Corrêa y cols., 2021).

La mayoría de metabolitos producidos por las microalgas se encuentran almacenados intracelularmente. Por tanto, la liberación de los mismos, para su posterior extracción, implica romper la pared celular. La robustez de la pared celular depende de la composición química y estructura de la misma, que a su vez presentan variabilidad inter- e intraespecíficas. Muchas especies de microalgas presentan paredes celulares difíciles de romper, dificultando la extracción de los metabolitos por el mero contacto con el disolvente extractante. Por tanto, se requiere una etapa de **pretratamiento** basada en la rotura de la pared celular para facilitar su recuperación (Mehariya y cols., 2021).

En la Figura 4 se clasifican los métodos de rotura celular en dos grandes grupos (Kim y cols., 2013): mecánicos y no mecánicos.

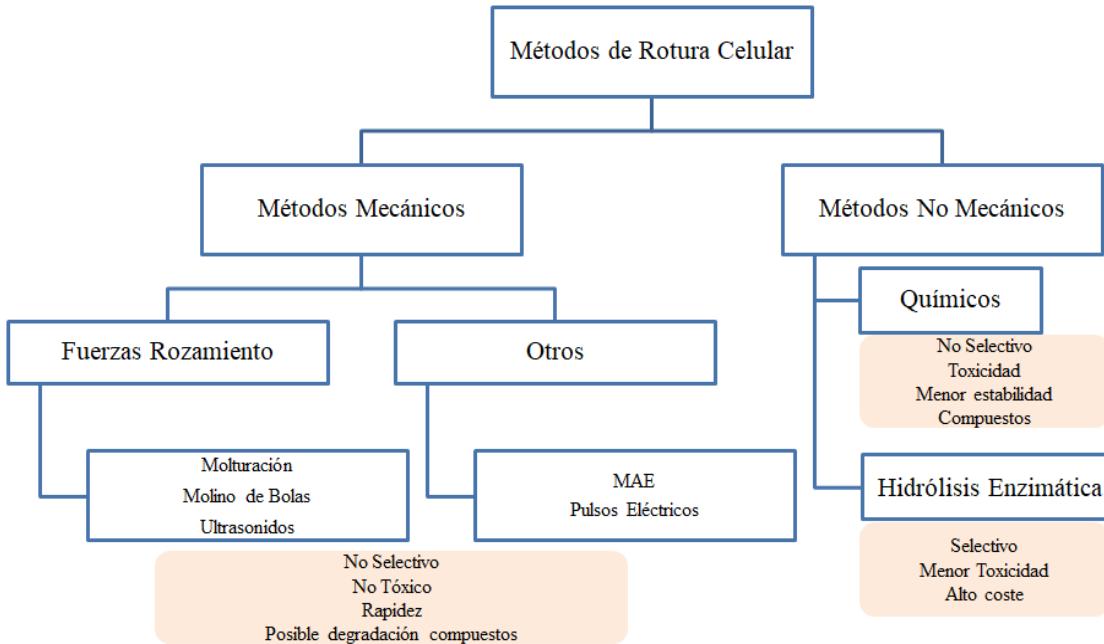


Figura 4. Clasificación, ventajas y desventajas generales de métodos de rotura celular. Adaptación de Mehariya y cols., 2021. MAE: extracción asistida con microondas (*siglas en inglés*).

La extracción mediante métodos mecánicos emplea fuerzas de rozamiento, fuerzas de impacto, fuerzas de zizalla, pulsos eléctricos, ondas electromagnéticas, ondas sónicas o calor cuya finalidad es alterar la estructura celular. Entre estos métodos, la **molturación** es uno de los más simples. La molturación es una operación escalable que se realiza en molinos de bolas que consisten en cilindros huecos rotatorios en cuyo interior se alojan bolas de material cerámico o metálico. La molturación de la biomasa seca de microalgas tiene lugar por el impacto y rozamiento entre las bolas. Este método suele destinarse a especies de microalgas con pared dura (Molina-Grima y cols., 2013).

El método basado en **ultrasonidos** produce la rotura celular a través de la transmisión de ondas sónicas que crean cavitaciones en la superficie celular desintegrando la pared celular, habitualmente empleado en microalgas con una pared celular moderadamente resistente (Günerken y cols., 2015; Corrêa y cols., 2021). Los dispositivos de **pulsos eléctricos o pulsos de campo eléctrico** (siglas en inglés PEF) utilizan un campo eléctrico externo el cual induce un potencial eléctrico crítico a través

de la membrana/pared celular causando poros en ella (electroporación) (Günerken y cols., 2015; Roux y cols., 2016). A pesar de ser un método rápido, eficiente y escalable, el equipamiento empleado tiene un alto coste y la técnica depende del medio en donde se emplee. Lo ideal es un medio libre de iones no conductores, limitando por tanto su uso en especies marinas (Günerken y cols., 2015).

Entre los métodos no-mecánicos destacan los **tratamientos químicos**, los cuales emplean el uso de ácidos, álcalis, detergentes, entre otros, que interactúan con componentes de la pared celular provocando deformaciones y promoviendo la ruptura celular. Es una técnica sencilla, pero los estudios de impacto ambiental sobre el uso de productos químicos en ocasiones cuestionan su uso para fines industriales, especialmente en el sector alimentario. El uso de la **hidrólisis enzimática**, por su parte, genera reticencias económicas dado el alto coste de las enzimas (Roux y cols., 2016).

Las diferentes técnicas de rotura celular pueden ser aplicadas con mayor o menor grado de eficacia, dependiendo de las características de la pared celular que presenta cada especie de microalgas (Gong y Bassi, 2016; Amaro y cols., 2018). Varios autores han estudiado los efectos de diferentes métodos de rotura celular sobre la recuperación de diferentes metabolitos en especies específicas. Por ejemplo, *Nannochloropsis* posee una pared celular en múltiples capas, lo que provoca un obstáculo para la extracción de compuestos y hace que en muchas ocasiones sea necesario emplear métodos de rotura celular (Molina-Grima y cols., 2013; Novoveská y cols., 2019). Otra especie con pared celular rígida es *Scenedesmus almeriensis*. Cerón-García y cols., (2008) demostraron que la rotura celular era necesaria para *S. almeriensis*. En dicho estudio se compararon tres métodos de rotura celular diferentes: molturación, molino de bolas y ultrasonidos. Como resultado, observaron que el pretratamiento consistente en rotura celular con molino de bolas y alúmina como agente

abrasivo en proporción 1:1 p/p fue la mejor opción para este tipo de especie con pared celular rígida. Por el contrario, existen especies cuya pared celular no es tan rígida, ofreciendo menor resistencia a la entrada de disolventes y por tanto no es necesario emplear una etapa de rotura celular. Por ejemplo, Kim y cols., (2012) obtuvieron aproximadamente un 95% de recuperación de fucoxantina en *Isochysis galbana* únicamente mediante extracción directa con disolventes sin necesidad de emplear una etapa de pretratamiento, dada la estructura menos rígida de su pared celular.

No sólo la eficacia de cada método puede variar significativamente en base al tipo de microalga sino también en base a las propiedades de cada compuesto. Por ejemplo, métodos mecánicos pueden producir degradación de compuestos bioactivos como carotenoides dado al estrés térmico al que puede ser sometida la biomasa (Stirk y cols., 2020). Algunos estudios mostraron que durante el proceso de rotura celular mediante sonicación se producía una gran disminución de carotenoides (Mäki-Arvela y cols., 2014). Contrariamente, son muchos los estudios que han reportado altos porcentajes de extracción de lípidos en diversas especies de microalgas tras someter a la biomasa a una etapa de rotura celular (Prabakaran y Ravindran, 2011; De Souza-Silva y cols., 2014). En este sentido se puede decir que dependiendo de las características de la pared celular y el metabolito objetivo la extracción puede ser abordada desde dos enfoques; un primer enfoque en el cual se combine una etapa previa de pretratamiento mediante rotura celular seguido de una extracción sólido-líquido o un segundo enfoque mediante extracción sólido-líquido directamente sobre la biomasa (Kim y cols., 2013). En la Tabla 2 se pueden observar las principales ventajas y desventajas de algunos de los métodos de extracción, bien sea por una extracción sólido-líquido con disolventes combinado con métodos de rotura celular o directamente sobre la biomasa.

Tabla 2. Ventajas y desventajas de diferentes métodos de extracción sólido-líquido combinados con rotura celular o directamente sobre la biomasa (Saini y Keum, 2018).

Método de extracción	Ventajas	Desventajas
Extracción sólido-líquido con disolventes	- Altos rendimientos de extracción sin utilizar instrumentos sofisticados.	- Requiere gran consumo de disolventes tóxicos y de tiempo de maceración, lo que incrementa el coste de producción.
Extracción sólido-líquido asistida con microondas	-Simple, rápida y económico.	- Puede causar degradación térmica a cis-trans-isomerización de los carotenoides.
Extracción sólido-líquido asistida con ultrasonidos	-Rápida, no térmica y eficiente extracción.	- La antigüedad de la sonda puede cambiar la eficiencia de la extracción. - Se requiere partículas pequeñas menor a 50 µm.
Extracción sólido-líquido a altas presiones (homogenización)	-Rápida, requiere mínima cantidad de solventes.	-Dificultad a aplicar a grandes volúmenes.
Extracción sólido-líquido con pulsos eléctricos	-Altamente aplicable a escala de laboratorio. - Altos rendimientos de extracción. - No es proceso térmico. - Usa poca energía.	-Alto coste de instrumentación. - Burbujas en las muestras puede provocar problemas técnicos. - Los parámetros pueden variar con la conductividad eléctrica.
Extracción sólido-líquido con fluidos supercríticos	-Uso de disolventes, no inflamables, no tóxicos y reciclables. -Útil para la extracción de compuestos termolábiles. - Proporciona carotenoides con alta pureza.	- No validas con muestras con alta cantidad de agua. - Bajo rendimiento de carotenoides polares. -Alto coste de instrumentación.
Extracción sólido-líquido asistida con enzimas	-Rápida y eficiente extracción con uso mínimo de los solventes.	-Alto coste de las enzimas.

Posterior a la etapa de pretratamiento, la **extracción con disolventes** es una de las etapas vitales para la recuperación de compuestos. En esta etapa, la extracción puede ser específica de un único metabolito objetivo, o de una co-extracción de múltiples metabolitos. Sin embargo, las tendencias actuales, dentro del contexto de biorefinería, indican que el desarrollo de una co-extracción es imperante para el buen desarrollo de un bioproceso basado en microalgas marinas (Ma y cols., 2020; Kiran y Venkata-Mohan., 2021). Y de esta forma, mejorar las perspectivas comerciales del bioproceso. En la Tabla 3, se muestran algunos de los métodos de extracción, disolventes y compuesto objetivo de diferentes especies o géneros de microalgas, más utilizados.

Tabla 3. Ejemplos de métodos de extracción y purificación, disolvente y metabolito objetivo en diferentes especies de microalgas, extraído de diferentes referencias bibliográficas.

Género/ Especie	Método Extracción	Disolvente	Producto	Referencia
<i>Karlodinium veneficum</i>	Extracción S-L y posterior extracción en fase sólida	Metanol	Karlotoxinas	Van Wagoner y cols., 2008
<i>Amphidinium carterae</i>	Extracción S-L y posterior extracción en fase sólida	Metanol	Anfidinoles	Nuzzo y cols., 2014
<i>Amphidinium carterae</i>	Extracción S-L y posterior partición secuencial en gradiente	Metanol	Anfidinoles	Satake y cols., 2017
<i>Amphidinium carterae</i>	Extracción S-L y posterior partición secuencial en gradiente	Metanol:agua (80:20)	Anfidinoles	Meng y cols., 2010
<i>Amphidinium carterae</i>	Extracción S-L y posterior extracción en fase sólida	Metanol	Anfidinoles	Cutignano y cols., 2015
<i>Phaeodactylum tricornutum</i>	Extracción S-L y posterior extracción líquido-líquido	Etanol(96%); hexano	Lípidos	Ramírez-Fajardo y cols., 2007
<i>Nannochloropsis gaditana</i>	Extracción S-L y posterior extracción líquido-líquido	Etanol(96%);hexano	Lípidos	Hita y cols., 2015
<i>Nannochloropsis sp</i>	Extracción S-L	Hexano	Lípidos	Jiménez-Callejón y cols., 2014
<i>Nannochloropsis sp.</i>	Extracción S-L	Hexano; Etanol (96%)	Lípidos	Jiménez-Callejón y cols., 2020b
<i>Acutodesmus obliquus</i>	Extracción Soxhlet	Etanol:Hexano (2:1);Etanol:Hexano (1:1) Etanol:Hexano (1:2)	Lípidos	Escorsim y cols., 2018
<i>Scenedesmus almeriensis</i>	Extracción S-L	Hexano	Luteína	Cerón-García y cols., 2008
<i>Scenedesmus almeriensis</i>	Extracción S-L	Etanol/hexano/agua 76:18:6	Luteína	Fernández-Sevilla y cols., 2012
<i>Scenedesmus almeriensis</i> <i>Haematococcus pluvialis</i>	Extracción S-L y posterior extracción líquido-líquido	Etanol/hexano/agua 84:6:10	Carotenoides Ácidos Grasos	Cerón-García y cols., 2018a
<i>Nannochloropsis gaditana</i>				
<i>Chlorella sp</i>				
<i>Symbiodinium voratum</i>	Extracción S-L	Metanol	Peridinina	Benstein y cols., 2014
<i>Isochrysis galbana</i>	Extracción S-L	Etanol	Fucoxantina	Kim y cols., 2012
<i>Chlorella sp.</i>	Extracción S-L	Acetona; Hexano; Metanol; Hexano:acetona (1:1); Hexano:etanol (7:3)	Carotenoides	Sarma y cols., 2021

La **extracción sólido-líquido** mediante el uso de disolventes orgánicos es una de las técnicas de extracción más aplicadas (Tabla 3). Frecuentemente está asociado con una etapa anterior de rotura celular que facilite la entrada del disolvente y facilite la extracción de compuestos bioactivos (Cerón-García y cols., 2008; Rammuni y cols., 2019; De Souza-Silva y cols., 2014).

Generalmente disolventes apolares como el hexano son buenos candidatos para extraer carotenoides apolares como el β -caroteno. Por otra parte, los carotenoides primarios generalmente están unidos a proteínas, mediante enlaces de hidrógeno, dentro del complejo fotosintético, por lo que son necesarios el uso de disolventes polares para romper dichos enlaces, siendo acetona o etanol los disolventes más apropiados para extracción de carotenoides polares (Saini y Keum, 2018). Cerón-García y cols., (2008) propuso un método de extracción en multietapas empleando hexano para la obtención final de extractos enriquecidos en luteína procedentes de *Scenedesmus almeriensis*. Dicho proceso condujo a un 95% de recuperación de luteína tras seis etapas de extracción con hexano. En este mismo estudio, los autores complementan la rotura celular con tratamiento alcalino, empleando hidróxido potásico (KOH), lo cual permite obtener luteína en su forma libre reteniendo ácidos grados y clorofilas en la fase acuosa. El tratamiento alcalino ha sido ampliamente utilizado para mejorar la extracción de carotenoides, ya que permite hidrolizar carotenoides en forma de ésteres y eliminación de clorofilas (Granado y cols., 2001). Posteriormente Fernández-Sevilla y cols., (2012) propone una modificación del anterior método basado en rotura celular y una extracción con mezcla tricomponente compuesta por hexano:etanol:agua (76:16:6) y simultáneo tratamiento alcalino para saponificar lípidos e hidrolizar clorofilas y permitir la recuperación de luteína libre en *S. almeriensis* (Tabla 3).

En relación al tratamiento alcalino, diversos parámetros como tiempo de tratamiento, temperatura y concentración de agente alcalino han sido investigados en muchos estudios (Inbaraj y cols., 2008; Chan y cols., 2013). El estudio realizado por Cerón-García y cols., (2018b) propone un método basado en tratamiento alcalino con KOH y simultánea extracción con disolventes con amplios rangos de polaridad usando diferentes especies de microalgas. Para ello, realizó una optimización del tratamiento alcalino para maximizar la recuperación de carotenoides en un total de ocho especies diferentes de microalgas. En dicho estudio se evaluó la influencia de diferentes concentraciones de KOH (0-60% p.s.b) y diferentes rangos de temperatura 25-80°C sobre la recuperación de carotenoides. Observaron que las condiciones de saponificación deben ser seleccionadas en función de la especie y del carotenoide objetivo. En algunos casos, excesivos porcentajes KOH producían una disminución en la recuperación de carotenoides, especialmente en xantofilas, por lo que para especies con alto contenido en xantofilas polares las condiciones de saponificación deben de ser suaves. Referente al disolvente, los autores obtuvieron buenos resultados en todas las especies de microalgas utilizadas en el ensayo, así como para todos los carotenoides (polares y apolares), a través del empleo de solución tricomponente compuesta por agua, etanol y hexano.

Por otro lado, disolventes como, hexano, metanol, etanol o mezcla metanol-cloroformo (2:1) (v/v) han sido usados para la extracción de lípidos (Robles-Medina y cols., 1998; Molina-Grima y cols., 2013; Jiménez-Callejón y cols., 2020b; Macías-Sánchez y cols., 2022) (Tabla 3). No obstante, el porcentaje de extracción puede no ser eficaz dependiendo del perfil lipídico que posea la especie en cuestión. Además, debido a la toxicidad presentada por disolventes como cloroformo o metanol su uso se restringe únicamente a la escala de laboratorio, no siendo aceptados en industrias dedicadas al

procesamiento de alimentos (Papachristou y cols., 2021). Muchos lípidos neutros pueden almacenarse en el citoplasma formando complejos con lípidos polares unidos mediante enlaces de hidrógeno a proteínas de la membrana celular. En estos casos disolventes polares son los más apropiados para romper dichos enlaces. Lípidos neutros pueden almacenarse formando glóbulos lipídicos en el citoplasma los cuales solo pueden ser extraídos por disolventes polares, mientras que aquellos que se encuentran en forma libre son mejor extraídos con disolventes apolares (Halim y cols., 2012; Ryckebosh y cols., 2014).

Otro de los procedimientos empleados, principalmente para la extracción de lípidos, es la extracción Soxhlet. Este método es dependiente de temperatura ya que la temperatura empleada durante la extracción afecta al porcentaje de recuperación lipídico. Las temperaturas varían desde 30 a 60°C, sin embargo, temperaturas superiores a 70°C provoca disminución en el porcentaje de recuperación debido a la pérdida de compuestos termolábiles por degradación (Guldhe y cols., 2016). De forma general el porcentaje de extracción suele ser bajo dada la escasez de extracción de lípidos polares. Su uso es aplicado para extraer principalmente lípidos no-polares y neutros (Escorsim y cols., 2018) (Tabla 3).

A diferencia de carotenoides y ácidos grasos, los metabolitos secundarios en microalgas suelen producirse en bastante menor cantidad, por lo que son necesarios grandes volúmenes de cultivo para obtener unos pocos miligramos de material para su posterior análisis (Meng y cols., 2010). Algunos de estos compuestos como azúcares, aminoácidos, ácidos orgánicos, toxinas, entre otros, son compuestos orgánicos solubles, los cuales son solubles en disolventes polares (Czartoski y cols., 2010). Diversos estudios basado en la identificación molecular muestran como alguno de ellos se caracterizan por poseer en su estructura grupos lipofílicos e hidrofílicos sugiriendo un

carácter polar de estos compuestos, como es el caso de anfidinoles producidos por *Amphidinium carterae* (Wellkamp y cols., 2020). Dichos estudios basan sus métodos de extracción usando como agentes extractantes metanol (Wellkamp y cols., 2020), etanol: agua (80:20) (Washida y cols., 2006) (Tabla 3) o acetona (Paul y cols., 1995), los cuales cubren los requerimientos de extracción de compuestos moderadamente polares a polares.

La gran y compleja diversidad de metabolitos producidos por las microalgas impide determinar un sistema extractante común para todos ellos. La selección de cada disolvente, debe ser acorde a la naturaleza del compuesto de interés; al menos en base a la polaridad y termolabilidad de éstos. El parámetro de solubilidad de Hildebrand (δ_T) ha sido considerado como una herramienta muy útil que permite establecer una correlación entre extracción del metabolito de interés y eficacia del disolvente para solubilizar dicho metabolito. El parámetro δ_T es una estimación numérica muy importante para evaluar la solubilidad de los compuestos en diferentes disolventes (Zhang y cols., 2018). Teóricamente, predice que si el soluto y el disolvente poseen valores de δ_T similares es probable que se disuelvan y en última instancia representa la eficiencia de extracción de este último (Saha y cols., 2015). En este sentido, la Figura 5 resume las principales variables y factores que podrían conducir a una extracción satisfactoria de los metabolitos de interés.



Figura 5. Resumen esquemático de la extracción de compuestos de alto valor añadido y principales variables de extracción.

1.1.3 Fraccionamiento y purificación

Tras la extracción, el extracto crudo obtenido se trata, por tanto, de una matriz compleja constituida por gran variedad de sustancias de diferente naturaleza química. Con la finalidad de obtener extractos enriquecidos en productos de interés, esta matriz debe ser fraccionada (Chan y cols., 2013).

Las técnicas de separación son por naturaleza más complejas debido a las dificultades en aislar compuestos objetivo a altos rendimientos y con cierto grado de pureza (Ventura y cols., 2017). De forma general, entre estas técnicas destacan la extracción en fase sólida (EFS) y la partición con disolventes orgánicos.

➤ Extracción en fase sólida (EFS)

Esta técnica surgió en los años 70 y se ha difundido rápidamente despertando gran interés en diferentes campos de investigación (Bielicka-Daszkiewicz y Voelkel, 2009). EFS se aplica con diferentes propósitos tales como obtención de fracciones enriquecidas y purificación de metabolitos de interés, o eliminación de sales en las fracciones que los contienen (Żwir-Ferenc y Biziuk, 2006). Es un excelente procedimiento incluso para retener trazas de metabolitos que normalmente requiere pequeñas cantidades de muestra (Moldoveanu y David, 2015). Esta técnica está basada en la separación de los metabolitos mediante el empleo de una fase sólida (sorbente) y una fase móvil consistente en disolventes capaces de disolver el extracto con todos los metabolitos. Generalmente se utilizan columnas o cartuchos de adsorción colocados en

un colector de vacío. La fase móvil con la muestra es introducida en los cartuchos o columnas y mediante vacío se hace pasar a través del dispositivo. Las moléculas contenidas en la fase móvil quedan retenidas en la superficie de la fase sólida para posteriormente ser eluídas a través de sistemas de disolventes generando diferentes fracciones ricas en metabolitos de interés. Las interacciones entre soluto y material adsorbente, más comunes, están basadas en fuerzas Van der Waals, enlaces de hidrógeno, fuerzas dipolo-dipolo e interacciones iónicas (Żwir-Ferenc y Biziuk, 2006).

La selección del adsorbente es un punto importante a tener en cuenta a la hora de emplear esta técnica, siendo los adsorbentes como la sílice, poliméricos o de carbón activo los más utilizados (Bielicka-Daszkiewicz y Voelkel, 2009). Sin embargo, el punto crítico es la capacidad máxima de retención del sorbente, que marca el volumen máximo de muestra que puede ser cargado en la columna o cartucho sin ocasionar pérdidas del metabolito de interés. Esto es conocido como volumen de ruptura (Poole y cols., 2000; Moldoveanu y David, 2015).

Con esta metodología se pueden recuperar diversos compuestos contenidos en el extracto crudo, sin descartar aquellos de menor valor, pero concentrando en una fracción los de mayor interés. Dicho proceso permite recuperar un amplio rango de metabolitos de diferente polaridad e incluso compuestos cuya concentración es menor frente a otros y por tanto su actividad podría quedar enmascarada. Este método de purificación y fraccionamiento ha sido empleado en muestras de origen marino debido a la presencia de sales que pueden sobreestimar el peso del extracto e inducir una mala interpretación del nivel de compuestos bioactivos. Concretamente para la extracción, fraccionamiento y purificación de metabolitos secundarios en especies dinoflageladas (Tabla 3) (Van Wagoner y cols., 2008; Cutignano y cols., 2015).

- Partición con disolventes orgánicos

La partición con disolventes también es utilizada para la separación de diferentes clases de compuestos. El principio básico implica el uso de dos disolventes inmiscibles en un embudo de decantación. Los compuestos se distribuyen entre los dos disolventes de acuerdo con sus diferentes coeficientes de partición. Generalmente el tipo de líquidos utilizados son agua y disolventes orgánicos (Tang y cols., 2021). Su principio se basa en una extracción inicial (sólido-líquido). El extracto líquido resultante es concentrado y extraído con el mismo volumen de otro disolvente inmiscible. Ambas mezclas inmiscibles son separadas en embudos de decantación. Esta técnica es conocida generalmente como extracción líquido-líquido (Otsuka, 2006). El proceso está basado en el método Kupchan (Kupchan y cols., 1973), inicialmente aplicado para aislar compuestos citotóxicos en extractos de la planta terrestre *Eupatorium cuneifolium*.

Modificaciones del método de Kupchan también han sido empleadas en muestras de origen marino con la finalidad de obtener fracciones ricas en diversos compuestos, especialmente de carácter lipo y/o hidrofílico. En una de las modificaciones (Riguera, 1997) el extracto generado es particionado en cinco fracciones: hexano, tetracloruro de carbono (CCL₄), diclorometano (DCM), n-Butanol (n-BuOH) y agua. Brevemente, el extracto crudo es resuspendido en solución hidroalcohólica con metanol 90% y posteriormente sometido a una extracción líquido-líquido con otro disolvente inmiscible, hexano, y posterior separación de ambas fracciones por decantación. La fracción metanólica es diluida con agua hasta dejarla con un 80% de metanol. A continuación se añade el siguiente disolvente inmiscible, CCL₄. Esta misma estrategia es seguida para el resto de los disolventes en orden de polaridad creciente, DCM y n-BuOH, utilizando una fracción metanólica del 60% metanol y finalmente agua 100%, respectivamente. Esta metodología es conocida también como partición secuencial en gradiente.

La partición con disolventes ha sido ampliamente utilizada para separar un amplio rango de toxinas de origen marino hidrofílicas e hidrofóbicas (Tabla 3; Satake y cols., 2017). Algunos autores afirman que entre las desventajas de esta técnica está el elevado gasto de disolventes orgánicos (Pedersen-Bjergaard y Rasmussen, 2008; Juhascik y Jenkins, 2009), incrementando el coste de la operación. Sin embargo este hecho ha sido cuestionado. Andrade-Eiroa y cols. (2016) explican la comparación entre el uso de volúmenes entre los métodos EFS y partición de dos estudios diferentes. El resultado de dicha comparación parece indicar que las necesidades volumétricas de disolventes son similares e incluso el mismo, los propios autores afirman que en cierto sentido el gasto es algo mayor en EFS.

1.2 IMPORTANCIA BIOTECNOLÓGICA DE DINOFAGELADOS MARINOS

Los dinoflagelados son un grupo filogenético de microalgas eucariotas unicelulares que habitan diversos nichos ecológicos, desde agua dulce, ambientes marinos y adaptados tanto a medios pelágicos como bentónicos. Pertenecen a la clase dinophyceas, la cual consta de 117 géneros incluyendo unas 4000 especies además de 500 especies con interacciones parásitas o simbióticas con invertebrados como corales (Manning y cols., 2021). Su adaptación a tan amplia variedad de ambientes es debido a la gran diversidad morfológica y diferentes estrategias nutricionales como fotoautotrofía, heterotrofía y mixotrofía que desarrollan (Gómez, 2012).

El interés biotecnológico de los dinoflagelados marinos ha sido puesto de manifiesto en los últimos años (García-Camacho y cols., 2007; Gallardo-Rodríguez y cols., 2012; Camacho y cols., 2019) como productores de metabolitos secundarios. Los metabolitos secundarios más estudiados que han sido descubiertos en las últimas décadas se basan en las toxinas, que algunas especies producen, por su impacto en la salud humana, a través de la cadena trófica, organismos marinos y ecosistemas

(Washida y cols., 2006; Kellmann y cols., 2010; Kobayashi y Kubouta, 2010; Cho y cols., 2020). En los últimos años el interés por sus toxinas, y otros metabolitos secundarios, ha crecido fuertemente debido al potencial que tiene su uso en aplicaciones terapéuticas dadas sus actividades anticancerígenas y antimicrobianas, o sus efectos frente a enfermedades neurodegenerativas reportadas en diferentes revisiones (Gallardo-Rodríguez y cols., 2012; Assunção y cols., 2017; Cho y cols., 2020).

La biomasa de dinoflagelados, además, contiene cantidades significantes de otros compuestos de alto valor añadido con aplicación comercial, como pigmentos, ácidos grasos o polisacáridos, los cuales han demostrado ser beneficiosos para la salud mediante la prevención del desarrollo y proliferación de células tumorales así como por sus actividades antiinflamatorias y antivirales (Benstein y cols., 2014; Ishikawa y cols., 2016).

Aunque muchos de los metabolitos secundarios activos procedentes de dinoflagelados marinos ya han sido aislados, son sólo unos pocos los que han alcanzado el nivel comercial. Por ejemplo, uno de los medicamentos existentes desarrollados a partir de dinoflagelados es Halneuron® cuyo ingrediente activo es la tetrodotoxina (TTX). Posee efecto bloqueador de los canales de sodio y es desarrollado por WEX Pharmaceuticals Inc., una compañía biofarmacéutica privada. Se trata de una potente neurotoxina y usado como analgésico en las terapias contra el cáncer (Hagen y cols., 2017). El ácido okadaico, también es otro tipo de neurotoxina, con efectos terapéuticos, comercializada por diversos laboratorios (Gallardo-Rodríguez y cols., 2012).

Algunos de los motivos de la escasa expansión comercial de estos compuestos, son la baja velocidad de crecimiento del dinoflagelado productor, complejidad del metabolismo y baja tasa de producción de compuestos de interés (Gallardo-Rodríguez y cols., 2012; García-Camacho y cols., 2014; Morales-Amador y cols., 2021). A pesar de

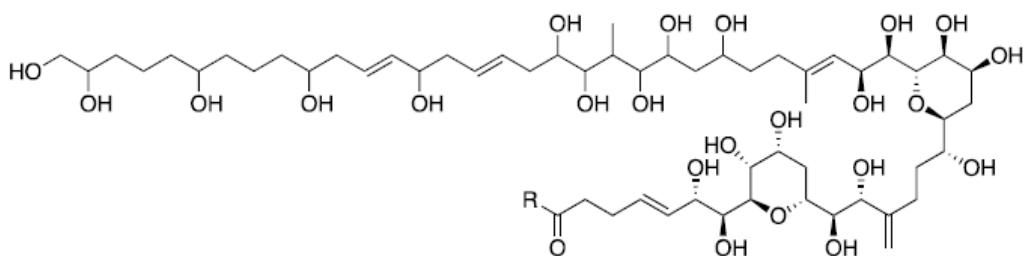
las limitaciones presentadas, las estructuras únicas y singulares, así como la funcionalidad, que presentan estos compuestos, convierten a este grupo de microalgas en interesantes tanto desde un punto de vista biológico, como farmacológico y comercial (Assunção y cols., 2017). No obstante, diversos estudios ya han proporcionado grandes avances en cuanto a formulación del medio de cultivo, optimización de las condiciones de cultivos y diseño de fotobiorreactores en dinoflagelados proporcionando una mejora, bajo el concepto de economía circular, de bioprocessos basados en dinoflagelados (Juhl y cols., 2001; López-Rosales y cols., 2015; Rahman-Sha y cols., 2016; Molina-Miras y cols., 2018a; Molina-Miras y cols., 2018b; Tsirigoti y cols., 2020).

1.3 CASO *AMPHIDINIUM CARTERAE*

Amphidinium es un género de dinoflagelados, miembro de la familia Gymnodiniaceae. Es un grupo de dinoflagelados atecados, el cual carecen de material de celulosa en sus vesículas anfisemal (Murray y cols., 2012). Se encuentran en ambientes marinos tanto en formas de vida bentónicas como estados de simbiosis.

El género *Amphidinium* produce un grupo de policétidos de cadena larga, perteneciente al grupo de metabolitos secundarios denominados anfidinoles (APDs). Estos policétidos poseen forma de horquilla, caracterizada por presentar una unidad central conformada por dos anillos tetrahidropirano separados por una cadena C6, un brazo lipofílico y otro hidrofílico (Wellkamp y cols., 2020). El interés en este tipo de metabolitos secundarios es debido a las distintas actividades que poseen, entre ellas, antifúngica, hemolítica y citotóxica (Morales-Amador y cols., 2021). En la Figura 6, se muestra una representación esquemática de la estructura química de algunos anfidinoles reportados en la bibliografía.

(A)



metabolitos como carotenoides y ácidos grasos poliinsaturados. Especial atención merecen los carotenoides como peridinina, dinoxantina y diatoxantina debido a la ausencia de otras fuentes no-microalgales de éstos. El carotenoide más representativo en dinoflagelados es la peridinina (Johansen y cols., 1974), concretamente en *A. carterae* representa entre un 0,2-0,9% de peso seco de biomasa (Molina-Miras y cols., 2018a). Sus aplicaciones tecnológicas son debidas al potencial terapéutico frente a diferentes enfermedades (Ishikawa y cols., 2016). Diversos estudios han demostrado la capacidad que posee peridinina para actuar como fuerte inhibidor de ciertos agentes virales e inducir apoptosis de ciertas células tumorales en humanos (Onodera y cols., 2014). La peridinina se encarga de transferir energía al centro de la reacción fotosintética localizado en el complejo peridinina- clorofila *a*- proteína (PCP). Este carotenoide protege a la clorofila *a* de la descomposición fotodinámica (Zigmantas, y cols., 2003). Respecto a los ácidos grasos, *A. carterae* produce elevados porcentajes de ácido eicosapentaenoico (EPA) y ácido docosapentaenoico (DHA) en su biomasa (Molina-Miras y cols., 2018b). Los beneficios del EPA y DHA en la salud humana ya han sido reportados ampliamente (Adarme-Vega y cols., 2012; Assunção y cols., 2017; Acosta-Montaño y col., 2018). En este sentido, la Figura 7 representa de forma esquemática los principales metabolitos producidos por *Amphidinium carterae*, especialmente metabolitos secundarios y su uso potencial en investigaciones biomédicas y farmacológicas.

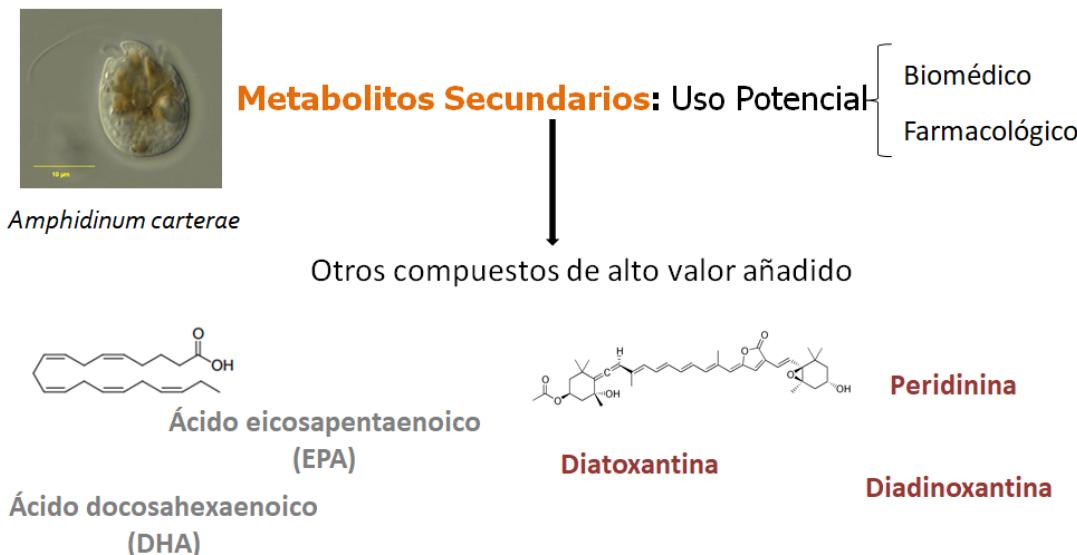


Figura 7. Importancia biotecnológica y metabolitos de interés en *A. Carterae*.

1.3.1 Producción de Biomasa

El concepto de biorrefinería en microalgas no-dinoflageladas para la producción de compuestos de alto valor añadido está bien documentado (Molina-Grima y cols., 2003; Chew y cols., 2017). Por el contrario, el cultivo de dinoflagelados presenta algunos inconvenientes, ya citados anteriormente. Así, el desarrollo de un cultivo masivo de dinoflagelados para la producción de compuestos bioactivos requiere especial atención en diversas áreas, entre las que se encuentran: i) formulación del medio de cultivo; ii) optimización de las condiciones de cultivos como la irradiancia, fotoperiodo, temperatura, pH; iii) diseño y operación de fotobiorreactores (Gallardo-Rodríguez y cols., 2012).

Generalmente el medio L1 ha sido utilizado tradicionalmente para cultivar diversas especies de dinoflagelados. Sin embargo, este tipo de medio, originalmente diseñado para el cultivo de diatomeas, mostró no ser adecuado para un cultivo de gran productividad en dinoflagelados (López-Rosales y cols., 2015). El uso de algoritmos genéticos basados en búsquedas estocásticas se presenta como una estrategia atractiva que reduce de manera significativa el número de experimentos a realizar. Por ejemplo,

en el caso de *K. veneficum* se optimizó la formulación de un medio que permitió un aumento en la concentración celular de karlotoxinas de cultivos en un 120% y en un 190% respectivamente comparado con los resultados obtenidos con un medio de cultivo inespecífico (medio L1) (García-Camacho y cols., 2016). Otro de los medios de cultivo citado en bibliografía es el medio f/2. La formulación de estos medios se basa normalmente en el enriquecimiento con agua de mar, siendo utilizado casi exclusivamente en el cultivo de dinoflagelados. El efecto de los diferentes requerimientos nutricionales sobre la tasa de crecimiento y la producción de metabolitos secundarios depende en gran medida de la especie de dinoflagelado que se trate. Es por ello que la optimización del medio del cultivo es considerada como una prioridad (Assunção y cols., 2017). Molina-Miras y cols. (2018b), cultivando *A. carterae* en un fotobiorreactor a escala piloto, optimizaron la composición del medio de cultivo f/2 aumentando la concentración de los nutrientes tres veces y variando la relación N:P hasta 5. Además, con un patrón de irradiancia sinusoidal (L/D, 24:0) y una irradiancia máxima del ciclo de $900 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, consiguieron mejorar notablemente la productividad de biomasa y su contenido de APDs en *A. carterae* respecto a lo obtenido en experimentos a escala de laboratorio en condiciones experimentales reportadas en bibliografía.

Estudios a escala de laboratorio, han demostrado la capacidad de aclimatación de *A. carterae* a diferentes fuentes de nitrógeno, así como a medio 100% reutilizado sin detectar efectos negativos sobre la productividad de la biomasa. En este último estudio tanto extractos de biomasa, como sobrenadante mostraron fuerte actividad antiproliferativa frente a células tumorales humanas (Molina-Miras y cols., 2020).

Al igual que la formulación del medio de cultivo, el tipo de fotobiorreactor que se utilice también tiene importancia. Los dinoflagelados son extremadamente sensibles

al estrés hidrodinámico y puede influir en la producción de sus metabolitos (García-Camacho y cols., 2007). De hecho, uno de los cuellos de botella en la biotecnología de microalgas es encontrar una especie robusta que pueda ser utilizada como una biofactoría celular para producir diferentes metabolitos de interés y que pueda ser cultivada durante largos períodos de tiempo (Greenwell y cols., 2010). Estudios han confirmado la capacidad de aclimatación de *A. carterae* a ser cultivada tanto en condiciones internas como en externas en fotobiorreactores cerrados tipo columna de burbujeo a largo plazo (Fuentes-Grünwald y cols., 2016). Adicionalmente, los estudios realizados por Molina-Miras y cols. (2018a) confirman la capacidad de recuperar APDs del sobrenadante en un sistema de cultivo tipo columna de burbujeo a escala piloto, en el cual no se observó daños causados por el estrés hidrodinámico. En este aspecto, *A. carterae* mostró un patrón de mayor sensibilidad frente a estrés hidrodinámico comparado con otras microalgas no-dinoflageladas, pero mayor resistencia que a otros dinoflagelados. También, recientes estudios reportaron el éxito del cultivo de *A. carterae* en fotobiorreactores tipo *raceway* durante casi un año de forma ininterrumpida y reutilizando el sobrenadante (Molina-Miras y cols., 2020).

Referente al sistema de iluminación empleado, las lámparas fluorescentes han sido la fuente de iluminación más habitualmente empleada para el cultivo de dinoflagelados. Sin embargo, la iluminación LED está ganando mayor terreno ya que a pesar de suponer un mayor coste inicial, la vida útil de este tipo de sistema es mayor y una mejor eficiencia energética con respecto a los sistemas de iluminación fluorescentes (Schulze y cols., 2014). Este tipo de sistemas permite, además de controlar con precisión el régimen, composición del espectro e intensidad de la luz. Los primeros dinoflagelados cultivados con iluminación LED fueron *K. veneficum* y *A. tamarensis* (Schulze y cols., 2014; Kwon y cols., 2013). Posteriormente diversos estudios han

demonstrado la versatilidad y robustez de sistemas de iluminación basados en LED multicolor en diferentes geometrías de fotobiorreactores y en diferentes escalas de cultivo de *A. carterae* (Molina-Miras y cols., 2018a; Molina-Miras y cols., 2018b; Molina-Miras y cols., 2020). La Tabla 4 muestra algunos de los principales resultados obtenidos sobre tipo de fotobiorreactor, modos de operación, y otras variables operacionales para el cultivo de *A. carterae*.

Tabla 4. Ejemplos de diversos resultados sobre tipo de bioreactor, modos de operación, y otras variables operacionales para producción de biomasa de *A. carterae* y/o producción de metabolitos.

Especie y cepa	Tipo de Reactor	Modo de operación	Tamaño reactor, L	T ^a , °C	Sistema de iluminación	Irradiancia ($\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	Metabolito	Referencia
<i>Amphidinium carterae</i> (Hulbert)	Flask	Discontinuo	40L	22	Lámparas fluorescentes	40	N/A	Samarakoon y cols., 2013
<i>Amphidinium sp.</i>	Flask	Discontinuo	3L	25	-	108	Anfidinólidos	Kobayashi y Tsuda, 2004
<i>Amphidinium carterae</i> ACRN03	Columna de burbujeo	Semi-continuo	540 L 320 L 48 L	23	-	158 (interno) 464 (externo)	N/A	Fuentes-Grünwald y cols., 2016
<i>Amphidinium carterae</i> (JHWAC)	Columna de burbujeo	Batch	700L	20	Lámparas fluorescentes	40-50	Antioxidantes	Rahman-Sha y cols., 2016
<i>Amphidinium carterae</i> ACRN03	Columna de burbujeo	Fed-Batch	80L	-	LED	100-40	APDs	Molina-Miras y cols., 2018a
<i>Amphidinium carterae</i> Dn241EHU	Raceway	Semi-continuo	33L	21	LED	900	APDs, Carotenoides, PUFAs	Molina-Miras y cols., 2018b

1.3.2 Producción de metabolitos de interés procedentes de *A. carterae*

Teniendo en cuenta los significantes avances alcanzados en cuanto al cultivo de *A. carterae*, la biomasa obtenida contiene cantidades bastantes significantes de compuestos de alto valor añadido como carotenoides, ácidos grasos y APDs que deben recuperarse de la forma más eficiente para facilitar la economía del bioprocreso basado en este dinoflagelado.

Diversos estudios ya han confirmado la capacidad de recuperar APDs del sobrenadante en un sistema tipo columna de burbujeo (CB-FBR) a escala piloto y posteriormente bajo cultivo en tipo *raceway* (RW-FBR) con medio 100% reutilizado (Molina-Miras y cols., 2018a; Molina-Miras y cols., 2020). Por un lado, el proceso seguido para aislar APDs a partir del sobrenadante procedente de CB-FBR se basa en someter éstos a una EFS mediante cartuchos preenvasados C18 de fase reversa (GraceTM RevelerisTM SRC, PN 5152105, 80g). El material orgánico retenido en dichos cartuchos se eluyen con metanol y posteriormente se evapora el disolvente para posterior análisis por RMN de dichos extractos. Tras la purificación cromatográfica del extracto enriquecido con APDs, se identificaron luteofanol D y lingshuiol A (>95% pureza) y AM20 (>80% pureza). Por otra parte, la presencia de APDs en extractos enriquecidos procedentes de sobrenadantes obtenidos en RW-FBR fue determinado mediante actividad hemolítica.

Recientemente, tres nuevos análogos estructurales (AM24, AM25 y AM26) han sido identificados mediante recuperación del sobrenadante en *A.carterae* cultivado a escala piloto en columna de burbujeo (80L) mediante iluminación LED con un patrón de irradiancia sinusoidal máxima a medio día de 1500 $\mu\text{mol fotones m}^{-2}\cdot\text{s}^{-1}$ y en modo *fed-batch* (Morales-Amador y cols., 2021). La recuperación extracelular de este tipo de metabolitos supone una mejora económica del proceso, puesto que en

muchas ocasiones su presencia puede quedar enmascarada por otros compuestos, dada su baja concentración.

No obstante, diversos estudios, muchos de ellos basados en modificaciones del método de Place y Deeds, (2005), se centran en la recuperación y purificación de extractos ricos en APDs almacenados intracelularmente. Por ejemplo, Nuzzo y cols., (2014) describieron la presencia de anfidinoles (AM18, AM19) en extractos metanólicos de *A. carterae*. En este estudio, la biomasa de *A. carterae* (3,6 g) fue extraída con metanol (3x10mL) mediante sonicación. El extracto metanólico (466 mg) fue fraccionado en columna de fase sólida Chromabond C18 Hydra (20g) y eluída con diferentes mezclas de MeOH: H₂O (v/v) (desde 0:100 hasta 100:0). La fracción 75:25 (v/v) fue purificada mediante HPLC obteniendo un total de 6,4 mg AM18 purificado y 2,5 mg de AM19 mediante purificación por HPLC de la fracción 50:50 (v/v). Los anfidinoles se encontraban en las fracciones MeOH: H₂O 50:50 (v/v) y MeOH: H₂O 75:25 (v/v).

Otros estudios, enfocados en la extracción y purificación de compuestos bioactivos de origen marino han empleado métodos de aislamiento y elucidación estructural de metabolitos a partir de una etapa previa de extracción de biomasa y posterior partición en gradiente secuencial con diferentes disolventes en función de la polaridad de los compuestos. Samarakoon y cols., (2013) utilizaron este método para determinar actividad antiinflamatoria y anticáncer en extractos crudos procedente de diferentes especies de microalgas, entre ellas *A. carterae*. Brevemente, la biomasa fue extraída mediante sonicación en presencia de metanol 80% para obtener extracto crudo. Posteriormente, los extractos obtenidos fueron sometidos a una partición secuencial obteniendo así cuatro fracciones n-hexano, CCL₄, acetato de etilo y agua, con incremento de polaridad. Sus resultados detectaron actividad anticáncer frente a tres

líneas celulares tumorales de las fracciones hexánica y acetato de etilo procedentes de extractos de *A. carterae*. Sin embargo, este estudio sólo demuestra la actividad en extractos de *A. carterae* sin identificar químicamente ningún compuesto en concreto.

De forma general, en la gran mayoría de estudios, el contenido de metabolitos secundarios con diferentes bioactividades, ha sido medido de forma indirecta. Esto es, mediante ensayos de actividad hemolítica, para aquellos metabolitos secundarios hemolíticos, y/o ensayos antiproliferativos. Este tipo de métodos incrementan el tiempo y operación del proceso. Contrariamente, Abreu y cols., (2019) identifica químicamente dos compuestos pertenecientes a la familia de APDs, mediante espectrofotometría RMN en extractos enriquecidos en APDs procedentes de *A. carterae* Dn241EHU producido en fotobiorreactor tipo *raceway*-LED multicolor. Por una parte, estos autores desarrollan un protocolo mediante la técnica RMN para el análisis metabolómico de la biomasa que les permite identificar las respuestas metabólicas ante diferentes condiciones de cultivo. Por otra parte, se optimizó un método para el análisis cuantitativo de APDs en dichos extractos. Los valores de concentración de APDs pueden ser determinados a partir del valor absoluto de la integral de la señal de protón δ_H 5,07 ppm en el espectro de 1H -RMN o bien mediante la correlación con la actividad hemolítica. En este caso, obtuvieron una alta correlación ($R^2=0,97$).

Hasta el momento, la gran mayoría de estudios relacionados con el procesamiento de la biomasa de *A. carterae* están enfocados a la recuperación extractos enriquecidos en metabolitos secundarios con bioactividad como anfidinoles. A pesar de haber identificado la presencia de compuestos tales como, EPA, DHA, peridinina, aminoácidos, carbohidratos, bases nitrogenadas entre otros (Abreu y cols., 2019), no se han reportado métodos dirigidos a la valorización integral de la biomasa de *A. carterae*.

En este sentido, se deben investigar métodos de extracción eficientes para metabolitos secundarios junto a su integración con procesos de recuperación múltiple de otros compuestos. Es necesario para ello explorar parámetros como rotura celular o extracción de disolventes que garantice la funcionalidad de diferentes compuestos celulares. Por ejemplo, los carotenoides poseen unas propiedades muy restrictivas las cuales pueden ser determinantes a la hora de diseñar un bioproceso basado en dinoflagelados. Con respecto a los carotenoides y los ácidos grasos la concentración de metabolitos secundarios es menor por lo que dicho proceso tiene que estar enfocado a la recuperación de éstos metabolitos minoritarios, pero sin comprometer la recuperación y/o estabilidad de otros compuestos de alto valor.

En definitiva, *A. carterae* ha demostrado tener un gran potencial como fuente natural de metabolitos con numerosas aplicaciones comerciales e industriales, especialmente de metabolitos secundarios de gran interés farmacológico, biomédico, ecológico y como fitosanitarios en agricultura. Los significantes avances alcanzados en cuanto medios de cultivo y variables de operación, diseño de fotobiorreactores, entre otros, en *A. carterae* suponen una ventaja para el desarrollo de un bioproceso basado en este dinoflagelado marino. Pero para alcanzar un alto grado de viabilidad económica de dicho bioproceso es necesario llevar a cabo un procesado posterior de la biomasa adecuado. En este sentido es necesario investigar y desarrollar métodos optimizados de extracción y fraccionamiento que generen extractos enriquecidos de metabolitos primarios y secundarios de gran valor contenidos en la biomasa del dinoflagelado marino, *Amphidinium carterae*, descritos en bibliografía.

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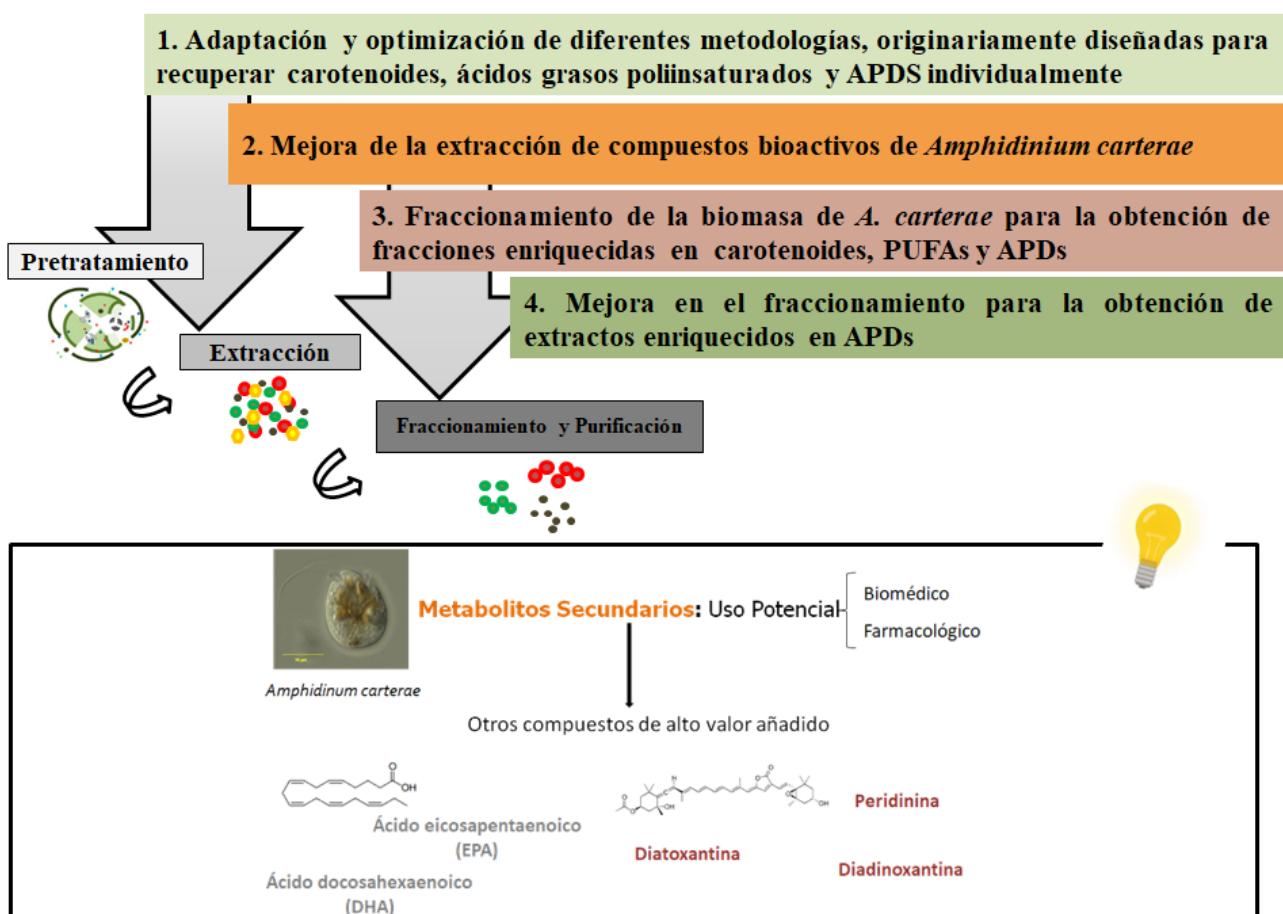
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CAPÍTULO 2

JUSTIFICACIÓN Y OBJETIVOS



Las microalgas marinas dinoflageladas son consideradas como factorías celulares por su capacidad de producir sustancias bioactivas, cuya aplicación principal en el sector farmacéutico, como alternativa a la síntesis química, ha sido ampliamente reconocida. A pesar de su potencialidad, la mayoría de dichas sustancias no han progresado más allá de la etapa de descubrimiento dadas sus bajas productividades. La síntesis química podría ser una solución; sin embargo, la complejidad estructural que presentan muchos de estos compuestos dificultan esta vía. A pesar de las dificultades reportadas, la biomasa de dinoflagelados productores se mantiene como foco de atención en estudios para la extracción y recuperación de estas sustancias. Más aún ahora, dónde la concienciación por la sostenibilidad ambiental y el uso de recursos naturales renovables prima frente las fuentes procedentes de la industria química. *Amphidinium carterae* ha demostrado ser candidato potencial a nivel industrial por ser productor de múltiples compuestos bioactivos, como carotenoides, ácidos grasos poliinsaturados y anfidinoles , entre otros. La valorización integral de la biomasa de *A. carterae* mejoraría las perspectivas de los dinoflagelados como fuente de compuestos bioactivos de gran valor. Estudios recientes han demostrado la viabilidad de su cultivo en fotobiorreactores tipo columna de burbujeo, *flat panel* y *raceway*, a escala piloto, así como su adaptación a medios de cultivo reutilizados. Como parte del bioprocreso basado en este dinoflagelado, y bajo el concepto de biorrefinería, es necesario desarrollar una tarea adicional de procesado de la biomasa mediante el estudio del pretratamiento de la biomasa que favorezca la extracción de los diversos compuestos, posterior fraccionamiento y obtención de extractos enriquecidos de éstos.

Bajo este escenario, y dados los avances en el cultivo del dinoflagelado *A. carterae* en fotobiorreactores, con esta tesis se pretende profundizar en la valorización integral de la biomasa procedente de *A. carterae* mediante el desarrollo de estrategias

para el procesamiento de ésta, que supongan una mejora en las expectativas industriales y comerciales de sus bioproductos.

El objetivo planteado se divide en diferentes objetivos técnicos específicos:

1. Adaptación y optimización de diferentes metodologías, originariamente diseñadas para recuperar carotenoides, ácidos grasos poliinsaturados y APDS individualmente. El objetivo es evaluar el impacto de tres metodologías diferentes de extracción, originalmente diseñadas para recuperar una única familia de compuestos en la recuperación múltiple de APDs, carotenoides y PUFAs de *A. carterae*. Cada metodología es optimizada, en algunas de sus etapas, para mejorar la recuperación de las tres familias de compuestos procedentes de la biomasa de *A. carterae*.

2. Mejora de la extracción de compuestos bioactivos de *Amphidinium carterae*. El objetivo es caracterizar la biomasa procedente de dos cepas de *A. carterae* y mejorar la extracción de las tres familias de compuestos bioactivos (APDs, carotenoides y PUFAs) presentes en *A. carterae*. La primera fase está orientada a optimizar la rotura celular y tratamiento alcalino, los cuales nunca han sido realizadas en la biomasa de *A. carterae*. La segunda fase comprende la mejora del porcentaje de recuperación de las tres familias de compuestos utilizando un amplio rango de disolventes y mezcla de éstos, con diferentes índices de polaridad y parámetros de solubilidad de Hildebrand.

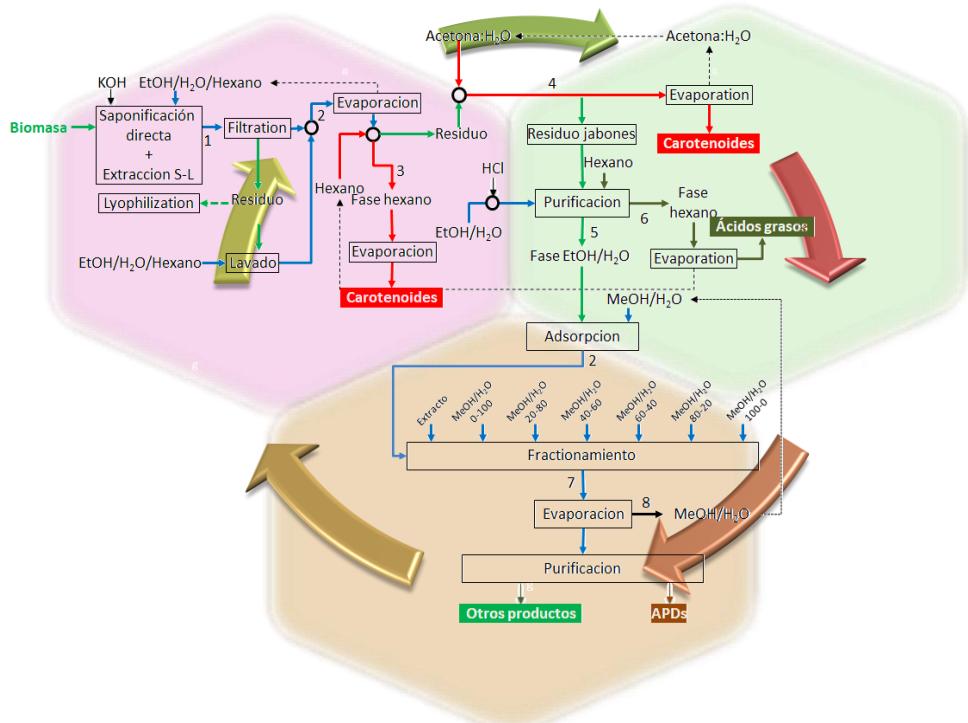
3. Fraccionamiento de la biomasa de *A. carterae* para la obtención de fracciones enriquecidas en carotenoides, PUFAs y APDs. En este apartado se implementa una estrategia de fraccionamiento del extracto crudo de la biomasa de *A. carterae*, priorizando la recuperación APDS, para separar las tres familias de compuestos (APDs, carotenoides y PUFAs). Una de las estrategias consiste en una partición secuencial en gradiente, con disolventes orgánicos inmiscibles de diferentes índices de polaridad, a diferentes extractos crudos de *A. carterae*. La otra estrategia aplicada se trata de un

proceso con un enfoque integrador, basado en los resultados obtenidos del objetivo 1, para obtener fracciones enriquecidas de APDs, carotenoides y ácidos grasos en el contexto de biorrefinería aplicada a dinoflagelados. La biomasa de *A. carterae* utilizada en este estudio fue producida reutilizando el sobrenadante agotado como base para la preparación del medio de cultivo; aspecto relevante en la aplicación de bioeconomía circular en bioprocessos.

4. Mejora en el fraccionamiento para la obtención de extractos enriquecidos en APDs. En este apartado se comparan dos métodos de partición cuyo principal objetivo es la mejorar la obtención de extractos enriquecidos de APDs, sin pérdidas de éstos ni interferencias con otros metabolitos contenidos en la biomasa de *A. carterae*. Y además se extienda a la recuperación de carotenoides y ácidos grasos. El primer método consiste en fraccionamiento directo, mediante una extracción en fase sólida (EFS) el cual es optimizado (desde rotura celular, ratio biomasa/ agente extractante, ratio masa_{extracto}/masa_{adsorbente}, y volumen de elución) para fraccionar extractos crudos metanólicos procedentes de la biomasa de *A. carterae* usando diferentes masas de adsorbentes (1-10g C18). El segundo método consiste en una extracción líquido-líquido (ELL) seguida de EFS y alternativamente como estrategia de enriquecimiento una partición con n-butanol, basado en los resultados obtenidos en el objetivo 3.

CAPÍTULO 3

ADAPTACIÓN Y OPTIMIZACIÓN DE DIFERENTES METODOLOGÍAS, ORIGINARIAMENTE DISEÑADAS PARA RECUPERAR CAROTENOIDES, ÁCIDOS GRASOS POLIINSATURADOS Y APDs INDIVIDUALMENTE





Assessment of multi-step processes for an integral use of the biomass of the marine microalga *Amphidinium carterae*



M. López-Rodríguez, M.C. Cerón-García*, L. López-Rosales, C.V. González-López,
A. Molina-Miras, A. Ramírez-González, A. Sánchez-Mirón, F. García-Camacho, E. Molina-Grima

Department of Chemical Engineering and CIAMBITAL, University of Almería, 04120 Almería, Spain

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ABSTRACT

Sustainable dinoflagellate microalgae-based bioprocess designed to produce secondary metabolites (SMs) with interesting bioactivities are attracting increasing attention. However, dinoflagellates also produce other valuable bioproducts (e.g. polyunsaturated fatty acids, carotenoids, etc.) that could be recovered and should therefore be taken into account in the bioprocess. In this study, biomass of the marine dinoflagellate microalga *Amphidinium carterae* was used to assess and optimise three different methods in order to obtain three families of high-value biochemical compounds present in the biomass. The existing processes encompassed a multi-step extraction process for carotenoids, fatty acids and APDs individually and are optimized for the integral valorization of raw *A. carterae* biomass, with SMs being the primary target compounds. Total process recovery yields were 97% for carotenoids, 80% for total fatty acids and 100% for an extract rich in APDs (not purified).

1. Introduction

Marine dinoflagellate microalgae have attracted increasing attention in recent years due to their ability to produce high value added bioactive substances (Gallardo-Rodríguez et al., 2012; Assunção et al., 2017). *Amphidinium carterae* produces an interesting group of polyketide secondary metabolites with potent anticancer, antifungal and hemolytic activities, namely amphidinolides and amphidinols (APDs), which means that they are considered to be potential sources of new drugs (Kobayashi and Kubota, 2010).

Recent studies have assessed the feasibility of recovering APDs from supernatants of pilot-plant cultures of *A. carterae* using a simple and scalable process (Molina-Miras et al., 2018a). The biomass produced in these systems also contains significant quantities of other high-value products, such as carotenoid pigments and fatty acids. The recovery of these products would improve the sustainability and economics of these bioprocesses. In particular, the carotenoid peridinin and the polyunsaturated fatty acids EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) are produced in abundance by *A. carterae* (Molina-Miras et al., 2018b; Fuentes-Grunewald et al., 2016). Peridinin, a dinoflagellate-associated apocarotenoid with unique photophysical properties, has been reported to have technological applications and to be a potential therapeutic agent against different diseases (Carbonera et al., 2014; Onodera et al., 2014; Ishikawa et al., 2016), whereas EPA

and DHA have numerous nutraceutical and pharmaceutical applications (Adarme-Vega et al., 2014). Unfortunately, the two methods currently used to obtain carotenoids and fatty acids individually (Fernández-Sevilla et al., 2012; Hita et al., 2015) cannot efficiently extract both families from microalgal biomass.

The concept of biorefining non-dinoflagellate microalgae to produce high value added products (e.g. pigments, proteins, lipids, carbohydrates, vitamins or antioxidants) is well-documented (Chew et al., 2017). However, when the priority objective of a marine dinoflagellate-based bioprocess is the production of specific secondary metabolites (SMs), such as APDs from *A. carterae*, the challenge that arises is how to recover the largest amount of the relatively minority metabolites (APDs) while minimising the loss of other valuable by-products. As a result, an efficient extraction method for APDs and its integration into a multi-product recovery process should be developed. To the best of our knowledge, there is no report in the literature of a biorefining approach that targets SMs from dinoflagellates in which the recovery of multiple high-value co-products is considered.

Procedures for extracting amphidinol-like polyketides contained in dinoflagellate biomass pellets were not originally devised to recover carotenoids and fatty acids (Place et al., 2005). Excessively high extraction temperatures, for example, could degrade carotenoids (Araujo et al., 2013). In contrast, other methods for extracting lipids are able to preserve carotenoids but are unable to extract all of them (Hita et al.,

* Corresponding author at: Department of Chemical Engineering, University of Almería, Carretera Sacramento s/n, 04120 Almería, Spain.
E-mail address: mcceron@ual.es (M.C. Cerón-García).

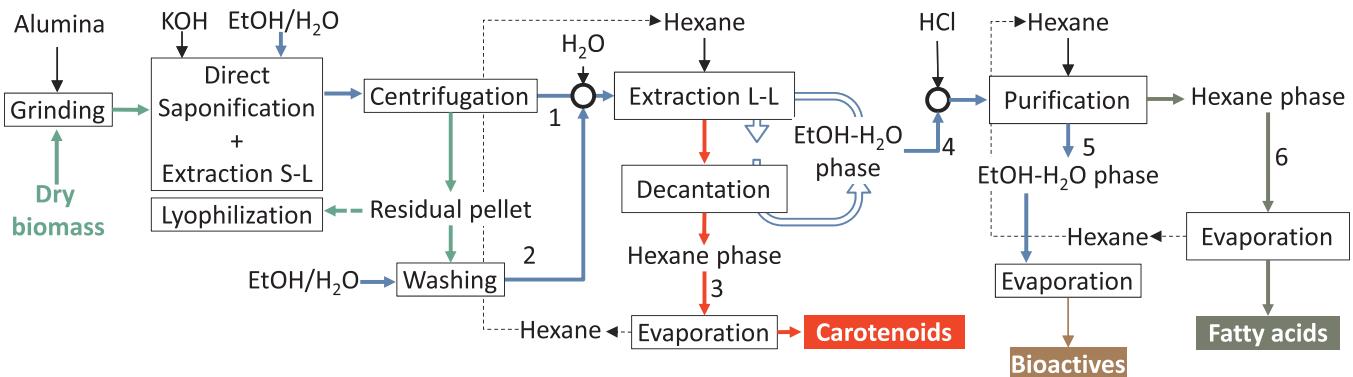


Fig. 1. Scheme of the process for recovering mainly fatty acids, as well as bioactive compounds, by modification of the method proposed by Hita et al. (2015).

2015).

With regard to the extraction solvents, studies using organic solvents (acetone, hexane, ethanol, dichloromethane, chloroform etc.) have been carried out to gauge their ability to extract lipids from microalgae cells. Due to toxicity problems (dichloromethane), the use of polar alcohols (less toxic solvents) and solvent mixtures such as hexane/methanol (3:2), hexane/isopropanol (3:2), cyclohexane/1-butanol (9:1) and hexane/ethanol/water (17:77:6), are good candidates amongst non-halogenated solvents ([Ryckebosch et al., 2014](#)). Due to their different polarities, the most suitable options are the use of hexane, acetone and ethanol, or mixtures thereof with different polarities, given that their use in food processing is already accepted ([Cerón-García et al., 2018a,b](#)).

The objective of this work is to assess the impact of three multi-step extraction processes, originally designed to recover a single family of compounds, on the recovery of APDs, carotenoids and polyunsaturated fatty acids (PUFAs) together from *A. carterae* biomass produced in a pilot-scale photobioreactor. Two of the processes have previously been used to extract carotenoids and fatty acids from non-dinoflagellate microalgae individually (Fernández-Sevilla et al., 2012; Hita et al., 2015, respectively), whereas the third has only been used to extract amphidinol-like polyketides (Place et al., 2005). These methods were optimized to maximize the recovery of these three compound families from *A. carterae* biomass.

2. Methods

2.1. Microalgal biomass

Biomass from the marine dinoflagellate microalga *Amphidinium carterae* (strain ACRN03) was produced and used as reported previously (Molina-Miras et al., 2018a). Briefly, a pilot-scale LED-illuminated (80 L) bubble column, similar to that described elsewhere (López-Rosales et al., 2016), was used as photobioreactor (PBR). This PBR was operated in fed-batch mode with a pulse feeding strategy to maintain a stationary growth phase for 10 days (Molina-Miras et al., 2018a). This approach provided a growth pattern strongly limited by the availability of phosphate content in the supernatant, thus stimulating the production of cellular APDs (Molina-Miras et al., 2018a). The biomass was harvested at the end of the culture by centrifugation. Frozen biomass pellet was lyophilized and stored at -22 °C ready for use as a raw material in different analytical techniques and different bioactive extraction methods.

2.2. Analytical procedures

The fatty acid (FA) content and profile in samples were determined by gas chromatography (Agilent Technologies 6890 N Series Gas Chromatograph, Santa Clara, CA, USA) after direct transesterification.

as described by Rodríguez-Ruiz et al. (1998). The carotenoid content and profile were determined using a photodiode-array HPLC apparatus (Shimadzu SPDM10AV) as explained by Cerón-García et al. (2018a). Measurements were carried out in duplicate. The detection of APDs in samples (i.e. biomass extracts and sub-fractions generated in the methods assayed) was bio-guided by the hemolytic activity thereof, which was determined as reported elsewhere (López-Rosales et al., 2015). A positive control, i.e. the maximum percentage of hemolysis equal to 100%, was obtained using distilled water.

2.3. Multi-step approaches for the extraction of carotenoids, PUFAs and APDs

Three different extraction protocols originally aimed at recovering a family of metabolites, namely PUFAs (Hita et al., 2015), carotenoids (Fernández-Sevilla et al., 2012), and APDs (Place et al., 2005), were explored and optimized to recover the three families of compounds. Their integration into a single, multistep process was proposed based on the results obtained.

2.3.1. PUFA-targeted approach

The extraction of PUFAs and the remaining fatty acids was based on an earlier method devised for the non-dinoflagellate microalga *Nannochloropsis gaditana* (Hita et al., 2015). The process flowsheet is shown in Fig. 1 and presents some modifications designed to improve the extraction of carotenoids and APDs. The first step is one of these modifications and consists of grinding the lyophilized *A. carterae* biomass to improve the yields of the subsequent extraction steps. Grinding with alumina has been reported to provide high fatty acid recovery percentages for non-dinoflagellate microalgae (Fernández-Sevilla et al., 2012). As such, 2.5 g of dry biomass was ground with the same mass of alumina (1:1 w/w) to break the cells.

In a second step, a direct saponification reaction with simultaneous extraction was carried out. This consisted of treating the dry biomass with aqueous ethanol (70 mL 96% EtOH and distilled water, 9.7 mL, PI: 6.8), containing 0.4 g KOH 85% per gram of dry biomass, instead of the 0.2 g KOH/g biomass reported by Hita et al. (2015) due to the higher content of saponifiable lipids in *A. carterae*. The reaction was performed at 60 °C in 250 mL Erlenmeyer flasks under an argon atmosphere. The mixture was stirred magnetically for 30 min instead of 1 h as reported by Hita et al. (2015). The mixture was then centrifuged to separate the ethanolic phase from the residual biomass pellet. The pellet was washed with 32 mL of aqueous ethanol. The two hydroalcoholic phases (streams 1 and 2 in Fig. 1) containing the dissolved fatty acid salts were combined for treatment in a third step, which consisted of a multiple liquid/liquid extraction of the unsaponifiable lipids with hexane (PI: 0). Briefly, 32 mL of water was added to the hydroalcoholic phase to increase the water content to 40% w/w, higher than the 30% recommended by Hita et al. (2015) in order to enhance the recovery of

carotenoids. The extraction of unsaponifiable lipids was carried out at 20 °C by adding several fractions of hexane to the hydroalcoholic phase in a 1:1 (v/v) proportion. The mixture was stirred magnetically for 10 min at 300 rpm, higher than the value of 250 rpm reported by Hita et al. (2015). The two immiscible phases subsequently formed were separated by decantation (hexane phase in stream 3 of Fig. 1) and fatty acids (hydroalcoholic phase in stream 4 of Fig. 1). The extraction process with hexane was repeated until the hydroalcoholic phase was virtually colourless, thus indicating the absence of carotenoids. In a fourth step, the fatty acid salts contained in the hydroalcoholic phase were purified and recovered. Briefly, the pH of the hydroalcoholic phase (stream 4) was adjusted to between 3 and 5 using 37% HCl. Fatty acids were then extracted by adding hexane in a simple liquid/liquid extraction at a 1:1 (v/v) ratio. Extraction was performed under argon atmosphere at 20 °C, stirring magnetically for 10 min. The hydroalcoholic (stream 5) and hexane phases (stream 6 Fig. 1) were separated by decantation. Streams 1, 3, 4, 5 and 6 were evaluated for carotenoids, fatty acids and hemolytic bioactivity.

2.3.2. Carotenoid-targeted approach

A method previously found to be effective for microalgae was adapted to the carotenoid profile of *A. carterae* (Fernández-Sevilla et al., 2012; Cerón-García et al., 2018b) and modified to improve the extraction of carotenoids contained in the biomass and to recover fatty acids and APDs. The process flowsheet is illustrated in Fig. 2. In contrast to Fernández-Sevilla et al. (2012), the procedure starts with a direct saponification and simultaneous extraction using 2.5 g of dry biomass not subjected to cell breakage. Subsequently, 100 mL of a three-component (EtOH/H₂O/Hexane) solvent mixture with a KOH content of 1.177 g (equivalent to 40% (w/w) relative to dry biomass) was used. This KOH proportion ensured both optimal recovery of carotenoids and removal of chlorophylls. The three-component solvent mixture (PI: 5.78) consisted of 87.5 mL 96% EtOH, 6.5 mL distilled water, and 6 mL hexane. The reaction was carried out at 60 °C for 30 min instead of 25 °C and 1 h (Fernández-Sevilla et al., 2012).

The reaction mixture was subsequently separated by filtration using a 12 cm-diameter porous glass plate (60 µm pore diameter, Pobel Madrid, Spain) and the residual pellet retained on the filter was washed with another 40 mL of fresh three-component mixture. Samples from the alkaline and washing treatments were collected for analysis of

carotenoids, fatty acids and haemolysis (stream 2 in Fig. 2). The residual pellet was lyophilized for subsequent analysis of fatty acids, carotenoids and hemolytic activity. The hydroalcoholic phase was transferred into a flat-bottomed balloon and evaporated on a rotary evaporator to remove the solvent. This operation started at a temperature of 30 °C and a pressure of 250 mbar, which were varied to a maximum of 55 °C and a minimum of 80 mbar to remove all solvents. Hexane (a non-polar solvent) was used initially to extract the non-polar carotenoids, followed by acetone:water mixtures (polar solvents) to recover the polar ones. The dry residue was first re-suspended in 50 mL of hexane at 40 °C then stirred at 150 rpm for 1 h. The hexane phase was saved for further analysis (stream 3 in Fig. 2). The residue obtained was then extracted with acetone and optimised to maximise carotenoid recovery by testing the following four acetone/H₂O mixtures (% v/v): 100:0.0 with a PI: 5.4 (used as control and corresponding to the conditions used by Fernández-Sevilla et al. (2012), 99.5:0.05 (PI: 5.47), 99:1 (PI: 5.54) and 97.5:2.50 (PI: 5.77). The extraction was carried out at 40 °C and 150 rpm for 7 h with all mixtures. The carotenoid-containing acetone extracts (stream 4 in Fig. 2) were then separated from the residue and samples saved for subsequent analysis. Hexane and acetone were recovered using a rotatory evaporator. A section for fatty acid recovery was added to Fernández-Sevilla's Original method (2012). Thus, the residue was resuspended in a mixture consisting of 18.2 mL EtOH and 6.8 mL H₂O (ethanol–water phase). Concentrated HCl was then added to the ethanol–water phase with stirring to acidify the mixture (pH 5). This pH guaranteed the integrity of any carotenoids remaining in the ethanol–water mixture, thus allowing them to be recovered from this phase (Hita et al., 2015). The mixture was subsequently acidified to pH 2 to release any fatty acids present in the form of potassium salts. The free fatty acids thus obtained were then extracted with hexane, as previously described in Section 2.3.1. Next, 25 mL of hexane was added to the mixture, which was stirred for 5 min at 150 rpm with a magnetic stirrer. The mixture was then poured into a separating funnel and left for 15 min to allow complete separation of the two phases into a lower hydroalcoholic aqueous phase (stream 5 in Fig. 2) and a lighter upper hexane phase (stream 6 in Fig. 2). Both phases were collected separately and an aliquot of each was taken for analysis of the three families of metabolites.

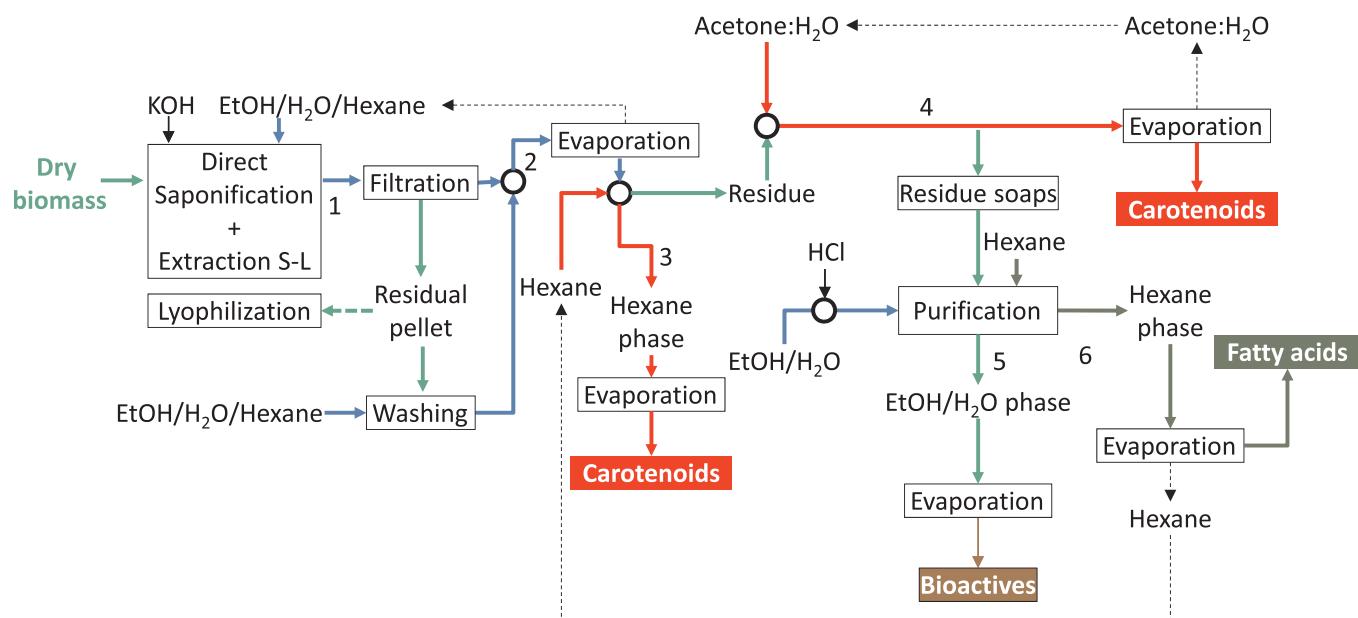


Fig. 2. Scheme of the process for obtaining mainly carotenoids, as well as fatty acids and bioactive compounds, by modification of the method proposed by Fernández-Sevilla et al. (2012).

2.3.3. Hemolytic bioactive-targeted approach

The method used to extract amphidinolides was based on the recovery of bioactive compounds from *Karlodinium* cultures described by Place et al. (2005). Thus, the biomass (10 mg dry wt) was sonicated for 15 min with 1 mL methanol and then centrifuged. The supernatant was injected into a disposable cartridge (Sep-Pak@light C18 Cartridges, 130 mg sorbent per Cartridge, 55–105 µm particle Size, Waters) attached to a vacuum collector (Supelco VISIPREP™ DL, 10–15 in-Hg). This modification of Place's original method (2005) was intended to increase the adsorbent/extract mass ratio. Thus, the extract was passed through four solid-phase extraction cartridges in series since previous results showed that a single cartridge did not adsorb all of the bioactive compounds.

Cartridges were equilibrated with 20 mL methanol followed by 20 mL distilled water. Once equilibrated, the methanolic biomass extract was diluted with deionized water to a final 20% methanol concentration (Krock et al., 2017) and loaded onto the equilibrated cartridges. The cartridges were eluted with different water-methanol mixtures with different ratios (polarities): 100:0, 80:20, 60:40, 40:60, 20:80, and 0: 100 (H₂O:MeOH), with PI values of between 10.2 and 6.6. The different fractions were dried on a rotary evaporator and the carotenoid and fatty acid contents and hemolytic activity determined.

The polarity index was calculated from the pure components for solvent mixtures as follows:

$$PI_{mix} = \sum_{i=1 \dots p} X_i \cdot PI_i \quad (1)$$

where PI_{mix} and PI_i are the polarity indices of the mixture and solvent i , respectively, and X_i is the volumetric fraction of solvent i in the mixture (Poole and Poole, 1991).

3. Results and discussion

3.1. Optimization of extraction processes

3.1.1. PUFA-targeted approach

The fatty acids present in the biomass were recovered in a higher percentage (94.37%) than the carotenoids (79.5%; see stream 1 in Fig. 1, which corresponds to the crude fatty acid extract produced in the direct saponification). This stream contains fatty acid potassium salts, proteins, APDs, pigments, other unsaponifiable lipids, such as sterols or vitamins, and other lipids. The residual biomass pellet obtained after saponification may still contain some fatty acids, which can be recovered in the ethanol/water washing step to increase the fatty acid yield. Two washes proved sufficient to recover, in total, almost 100% of both fractions (additional 4.33% and 0% in the case of fatty acids and 20.5% and 0% in the case of carotenoids) (stream 2, Fig. 1). As a result, the stream (sum of 1 and 2) that entered the liquid/liquid extraction step with hexane (see Fig. 1) contained essentially 100% of the total fatty acid salts and carotenoids. Table 1a shows the recovery percentages of carotenoids, fatty acids and active metabolites for different steps in our modified version of the process reported by Hita et al. (2015). These recovery percentages, namely Y_{carot} , $Y_{fatty\ acids}$ and $Y_{bioactive}$, are recovery yields (% d.w.), in other words percentage of carotenoids, fatty acids and bioactive compounds extracted with respect to the total amount of these compounds present in the initial biomass. The fatty acids quantified in stream 2 comprised 0.36% tetradecanoic acid (14:0), 3.64% hexadecanoic acid (16:0), 0.65% octadecanoic acid (C18:0), 1.65% oleic acid (18:1n9), 0.27% 9-eicosenoic acid (20:1n9), 2.59% stearidonic acid (SDA; 18:4n3), 0.32% arachidonic acid (ARA; 20:4n6), 2.15% EPA (20:5n3) and 4.29% DHA (22:6n3) (all percentages based on biomass dry weight). The average total fatty acid content (FA_T) was $15.94 \pm 0.8\%$ d.w., which was 22% higher than the content reported by Molina-Miras et al. (2018b). These authors concluded that FA_T was not significantly affected by the different environmental culture conditions tested, therefore there are

intrasppecies differences in FA_T between the different strains of *A. carterae*. However, the FA profiles were similar, with the exception of ARA content and another unnamed fatty acid, with a content of 0.3% d.w., which could possibly be 18:5n3 (Zhukova and Titlyanov, 2006). The PUFA fraction is higher than 60% FA_T , similar to the value obtained by Molina-Miras et al. (2018b). The total pigments content in stream 2 comprised the chlorophylls C2 (0.51% d.w.) and A (0.81% d.w.), and the carotenoid fraction comprised peridinin (0.82% d.w.), dinoxanthin (0.12% d.w.), diadinodoxanthin (0.22% d.w.), diatoxanthin (0.25% d.w.) and β-carotene (0.05% d.w.). The pigment profile is similar to that reported by Molina-Miras et al. (2018b) for this species. The chlorophylls content is 40% lower because the KOH used hydrolyzes these compounds, thereby reducing their content in the extract because of chlorophyll precipitation. With regard to carotenoids, their content (1.45% d.w.) is similar to the value previously reported by the authors (1.34% d.w., Molina-Miras et al., 2018b). This demonstrates no influence of the initial saponification step on the recovery of these molecules from the biomass, probably because this strain may contain carotenoids as esters. However, some differences were again found between strains as the ACRN03 strain contains only 0.05% d.w. of β-carotene, which contrasts with the value of 0.30% d.w. for the strain Dn241EHU (Molina-Miras et al., 2018b).

As fatty acid salts are polar, stream 2 was subjected to repetitive liquid/liquid extractions with hexane to extract the carotenoid fraction (broad range of polarities; see Fig. 1). Emulsions were formed at water contents in the hydroalcoholic phase of more than 50% (w/w), which makes extraction difficult and decreases fatty acid and carotenoid recoveries (Hita et al., 2015). No emulsions were observed at 40% (w/w) in our experiments. As the fatty acids were in the form of soaps, they remained in the hydroalcoholic phase (higher solubility), whereas the carotenoids were distributed in both phases according to their polarity: peridinin is very polar, β-carotene is non-polar and the remaining carotenoids have intermediate polarities. After six extractions, the hexane phase (stream 3 in Fig. 1) contained 62% of total carotenoids (100% β-carotene, 80% diadinodoxanthin, and 45% diatoxanthin relative to the amounts determined in the dry biomass), similar to the values reported by Hita et al. (2015). The most polar carotenoids, such as peridinin and dinoxanthin, were mixed with the fatty acid salts and thus completely washed away with the hydroalcoholic phase (stream 4 in Fig. 1). As such, this method is not suitable for the recovery of polar carotenoids.

Prior to recovery of the fatty acids contained in hydroalcoholic stream 4 (purification step), its pH was lowered in order to transform the potassium salts into free fatty acids, thus allowing them to be extracted with hexane (Fig. 1). The fatty acid extraction yield of 78.69% obtained at pH 3 is higher than the value of 67.33% achieved at pH 5. Although an improved yield may be expected upon acidification below pH 2, the risk of emulsion formation when hexane is added increases, as reported for non-dinoflagellate microalgae (González et al., 1998; Hita et al., 2015). EPA and DHA, the most representative fatty acids in *Amphidinium*, were recovered with an extraction yield of 83% and 77%, respectively similar to the total fatty acids yield. In contrast, acidification caused degradation of the carotenoids present in the hydroalcoholic phase (i.e. peridinin and dinoxanthin). As such, this PUFA-targeted approach does not seem to be appropriate for the recovery of two of the most valuable metabolite families from *A. carterae* (or other dinoflagellates containing dinoxanthin or peridinin as principal carotenoids), namely PUFAs and carotenoids.

The presence of APD compounds with hemolytic activity in the different fractions was assayed (Table 1). In the initial extract from the modified process of Hita et al. (2015), corresponding to alkaline treatment of the biomass (stream 1, Fig. 1), the recovery percentage of bioactives was 100%. In contrast, neither of the final hexane phases obtained by decantation, comprising carotenoids and free fatty acids (streams 3 and 5 Fig. 1), contained hemolytic APDs. Finally, the percentage recovery for these bioactive compounds was 100% for the

Table 1

Recovery percentages of carotenoids, fatty acids and active metabolites for different steps in the modified processes of: (a) Hita et al. (2015); b) Fernández-Sevilla et al. (2012); and c) Place et al. (2005).

Step	Y_{carot}	$Y_{\text{fatty acids}}$	$Y_{\text{bioactive}}$
<i>Yield</i>			
(a) Modification of Hita et al. (2015)			
Alkaline extraction	98 ± 2.36	98.89 ± 3.86	100 ± 2.68
Fatty acids recovery (pH 5)	–	67.33 ± 3.37	0.00 ± 0.00
Fatty acids recovery (pH 3)	–	78.69 ± 3.93	0.00 ± 0.00
Final recovery process	61.90 ± 2.36	78.69 ± 3.93	100 ± 2.68
(b) Modification of Fernández-Sevilla et al. (2012)			
Alkaline extraction	100 ± 5	97.81 ± 4.89	100 ± 0.23
Fatty acids recovery (pH 5)	–	53.87 ± 2.15	0.00 ± 0.00
Fatty acids recovery (pH 3)	–	79.83 ± 4.18	0.00 ± 0.00
Final recovery process	97 ± 4	79.83 ± 4.18	100* ± 2.10
*The hydroalcoholic phases contained all the bioactive metabolites, which was not the case for the hexane phase in both methods above			
(c) Modification of Place et al. (2005)			
Extraction	68.00 ± 2.68	61.50 ± 2.89	85.15 ± 0.30
No adsorbed	1.02 ± 0.01	37.00 ± 2.56	10.93 ± 0.30
Water:methanol (100:0)	9.71 ± 0.53	0.00 ± 0.00	0.00 ± 0.00
Water:methanol (80:20)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Water:methanol (60:40)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Water:methanol (40:60)	0.00 ± 0.00	0.00 ± 0.00	12.86 ± 0.15
Water:methanol (20:80)	36.13 ± 2.63	0.00 ± 0.00	32.71 ± 0.50
Water:methanol (0:100)	4.08 ± 0.08	12.00 ± 0.36	0.79 ± 0.10
Final recovery process	51.11 ± 0.93	49 ± 0.56	68.09 ± 0.25

Y_{carot} , $Y_{\text{fatty acids}}$ and $Y_{\text{bioactive}}$ are recovery yield (% d.w.): Percentage carotenoids, fatty acids and bioactive compounds extracted with respect to the compounds present in the initial biomass.

Table 2

Carotenoid extraction expressed as dry weight content (% d.w.) using different acetone:water mixtures as solvent.

Acetone:water (%)	Peridinin (% d.w.)	Dinoxanthin (% d.w.)	Diadinoxanthin (% d.w.)	Diatoxanthin (% d.w.)	β -carotene (% d.w.)	Total Carotenoids (% d.w.)
100:0*	0.48 ± 0.02	0.15 ± 0.01	0.09 ± 0.00	0.19 ± 0.01	0.01 ± 0.00	0.92 ± 0.05
99.5:0.05	0.64 ± 0.03	0.16 ± 0.01	0.09 ± 0.00	0.22 ± 0.01	0.01 ± 0.00	1.13 ± 0.06
99:1	0.78 ± 0.04	0.15 ± 0.01	0.10 ± 0.00	0.20 ± 0.01	0.01 ± 0.00	1.24 ± 0.06
97.5:2.5	0.76 ± 0.04	0.16 ± 0.01	0.10 ± 0.00	0.20 ± 0.01	0.01 ± 0.00	1.23 ± 0.06

*This data set corresponds to the method reported by Fernández-Sevilla et al. (2012).

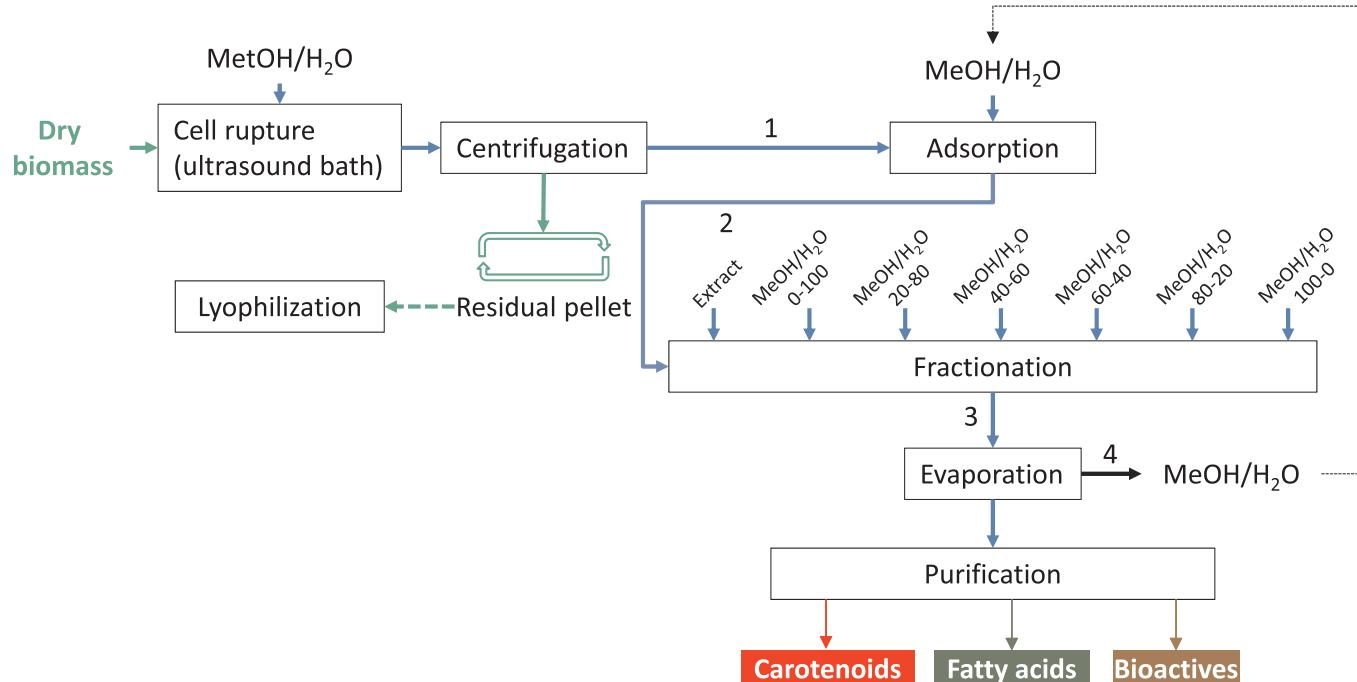


Fig. 3. Scheme of the process for obtaining mainly bioactive compounds, as well as fatty acids and carotenoids, by modification of the method proposed by Place et al. (2005).

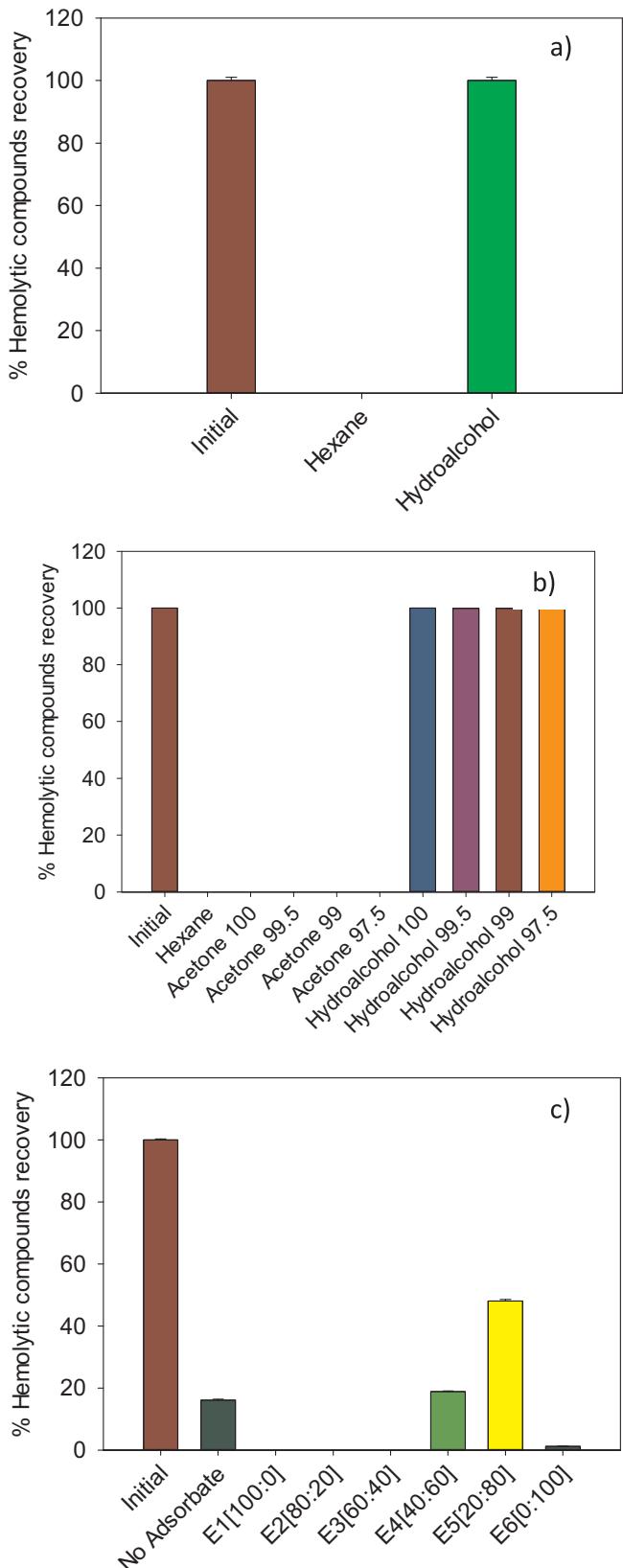


Fig. 4. Percentage hemolytic compounds recovered when using the modified methods of: a) Hita et al. (2015); b) Fernández-Sevilla et al. (2012); and c) Place et al. (2005).

hydroalcoholic phase (stream 4, Fig. 1). This means that APDs are successfully extracted by polar solvents due to their high polarity, which is a key issue as they will not contaminate fractions rich in fatty acids and carotenoids.

3.1.2. Carotenoid-targeted approach

A modification of Fernández-Sevilla's method (2012) was proposed (see Section 2.3.2) for extraction of the three families of compounds (see Fig. 2). As expected, the carotenoids present in the biomass were recovered in a high percentage (100%) compared to fatty acids (91.3%). Stream 1 in Fig. 2 corresponds to the crude fatty acid extract produced upon direct saponification (see Table 1b). This stream contains the same compounds as in the previous method (fatty acid potassium salts, proteins, amphidinolides, pigments, such as carotenoids and chlorophylls, other unsaponifiable lipids, such as sterols or vitamins, and other lipids). A simple step of washing the residual pellet allowed 3% d.w. of the carotenoid fraction and 4.65% d.w. of fatty acids to be recovered, with the sum of the two washings reaching almost 97% of the carotenoid fraction and 86.6% of the fatty acids fraction (in stream 2, Fig. 2).

To recover carotenoids and remove the solvents from stream 3, the extract from the saponification was transferred into a flat-bottomed balloon and evaporated on a rotary evaporator. This procedure differs from that reported by Fernández-Sevilla et al. (2012) (Fig. 2) in that, once the residue had been dried, 50 mL of hexane was added and magnetically stirred at 150 rpm and 40 °C for 1 h. The hexane phase was extracted and saved for further analysis (stream 3). This first extraction with hexane was intended to effectively recover the non-polar carotenoid β-carotene (96.7% of the total). The residue in the balloon was then extracted with acetone (stream 4, Fig. 2), in accordance with previous studies using other strains (Cerón-García et al., 2018b). This extract contained part of the peridinin (40% of the total), dinoxanthin (81% of the total) and diadinoxanthin (30.8% of the total). These results indicate that this non-polar solvent is not suitable for recovering all the carotenoids. Thus, the process was optimized by using different ratios of acetone/water in order to vary the polarity. Data are shown in Table 2. Two such solutions, namely 99:1 and 97.5:2.5 acetone:water (v/v), gave similar results for extraction of these pigments. The total percentage of carotenoids recovered for the first solution was 34% higher than that achieved using the original method (Fernández-Sevilla et al., 2012), whereas for the second solution it was 33% higher. Peridinin and diatoxanthin were found to be better extracted by the 99:1 solution, with these two pigments accounting for more than 3.5% d.w. of the biomass, therefore this ratio was chosen as the best alternative as it also minimized dissolution of the fatty acid potassium salts. The acetone/water solution was added to the residual pellet in the balloon and the resulting mixture kept in the bath while stirring at 150 rpm and 40 °C for 7 h. The liquid phase was then removed (stream 4, Fig. 2) and the solvents evaporated to recover the carotenoids. The recovery of carotenoids from stream 4 after evaporation was 97% (Table 2). Subsequently, 18.2 mL ethanol and 6.8 mL water were added to the residue left in the balloon (stream 5, Fig. 2) in order to solubilise the fatty acid potassium salts and recover the fatty acids in stream 6 (Fig. 2) (Hita et al., 2015). During this purification step, the pH was adjusted to 5 with HCl to free the fatty acids from the salts. This pH value prevented carotenoid degradation in the hydroalcoholic phase (Hita et al., 2015), as reported previously. The fatty acid recovery at this pH was 53.87%, thus meaning that this pH value is too high to efficiently recover free fatty acids. As such, the hydroalcoholic solution was further acidified to pH 2 and the extraction step repeated in order to determine whether more fatty acids could be recovered from the hydroalcoholic phase. With this treatment, the total recovery of fatty acids increased to 79.83%, which is similar to the value reported by other authors (Hita et al., 2015, González et al., 1998).

The percentage APD recovery (see Fig. 4b and Table 1b) was highest for the initial extract (stream 2, Fig. 2: alkaline treatment of the

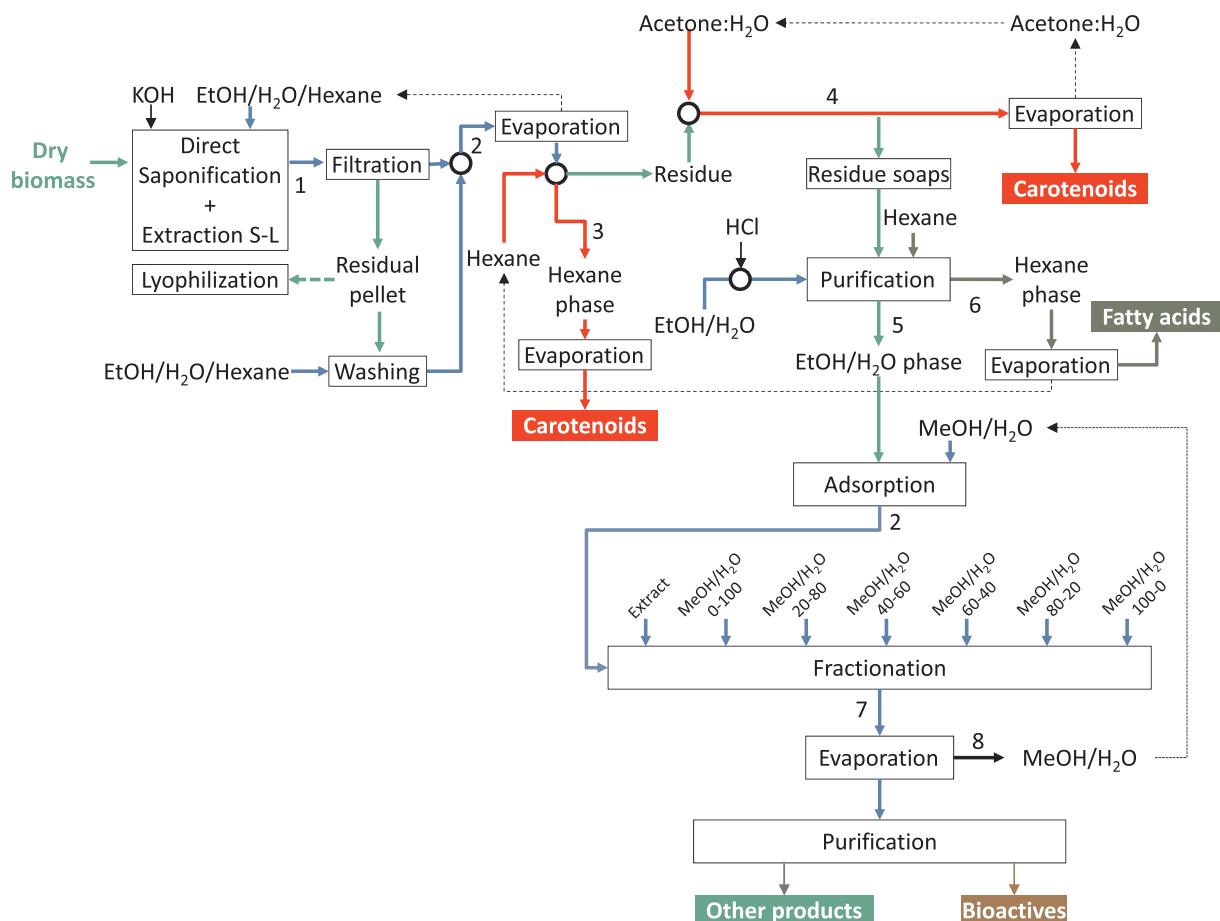


Fig. 5. Scheme of the proposed process for obtaining optimal recovery of all compounds (fatty acids, carotenoids and bioactive compounds) involving a combination of the modified methods proposed by Fernández-Sevilla et al. (2012) and Place et al. (2005).

biomass) and in the hydroalcoholic phases (stream 5, Fig. 2), whereas it was minimal (less than 0.2% of the total) for the acetone/water (stream 4, Fig. 2) and hexane phases (streams 3 and 6, Fig. 2). As mentioned above, the hemolytic bioactives are completely extracted by the aqueous phases, in contrast to the non-polar hexane and acetone ones.

3.1.3. Bioactive compound-targeted approach

The recovery results obtained for each fraction with the method described in Fig. 3 are shown in Table 1c. It is clear that methanol (stream 1, Fig. 3) is not an effective extractant for any of the families of compounds as only around 70% of the total was extracted. In the fractionation step (Fig. 3), carotenoids are distributed between the 100% 20% and 0% water phases, with the 80:20 MeOH:water mixture (v/v) being the most effective (70% extraction of total carotenoids). This is probably due to the highly polar character of these pigments. The fatty acids were found in the eluate (not adsorbate of C18 cartridges) of the methanolic biomass extract and in the 100:0 MeOH:water solution (37% and 12% of the total, respectively). This could be due to the fact that fatty acids account for a large proportion of the polar lipids (68% of total fatty acids, data not shown), and therefore they do not tend to be absorbed by the non-polar stationary phase. In this process, 51% of the carotenoids and 49% of the total fatty acids were recovered. As such, this method is less effective than the modification of the method of Fernández-Sevilla et al. (2012) and that of Hita et al. (2015) for carotenoids and fatty acids (see Table 1b and c).

As expected, the modified method of Place et al. (2005) (Fig. 3) allowed 68% of the APDs contained in stream 1 to be recovered (Fig. 4c). In the fractionation step, the phases with methanol fractions ranging from 0% to 40% did not contain hemolytic compounds. The

80:20 MeOH:water mixture contained 33% of the APDs, with the 60:40 mixture containing 13% and only a very small percentage in the 100:0 mixture. This means that some water is needed to extract and recover these water-soluble substances. The global recovery was almost 70%, with around 11% not being adsorbed on the column, which could mean that the quantity of biomass used was too high for these cartridges.

As none of the methods assessed was able to recover and purify the three families of compounds in a percentage higher than 90%, further work is needed to solve this problem. In view of the results obtained, a combination of the modified processes of Fernández-Sevilla et al. (2012) and Place et al. (2005) is proposed in Fig. 5. The two methods are performed in series and linked with the hydroalcoholic mixture (stream 5). In this combined process, carotenoids would be separated first from the hexane phase (stream 3) and then from the acetone:water mixture (stream 4, Fig. 5). Fatty acids would be concentrated in stream 6 and purified by evaporation of the hexane. The residual ethanol/water phase from the lipid purification step (stream 5) is introduced into the adsorption column, which is eluted with different MeOH/H₂O mixtures. The APDs would be recovered for the 60–80% MeOH mixtures and, thus, separated from other compounds such as chlorophylls, proteins or other undesirable lipids.

4. Conclusions

It has been possible to successfully apply and modify two methods originally developed for non-dinoflagellate biomass to recover high percentages of fatty acids and carotenoids from *A. carterae* biomass. The modified methods can be applied to other dinoflagellate species. The modified method of Fernández-Sevilla et al. (2012) allowed 100% of

the carotenoids and 98% of the fatty acids to be extracted from the *A. carterae* biomass after saponification. The total process recovery percentages were 97% for carotenoids and 80% for fatty acids after purification, with 100% of APDs also being obtained (not purified). The modified method of Hita et al. (2015) was less effective for extraction and purification of the carotenoids family. Finally, the modified method of Place et al. (2005), which is not intended for the extraction of carotenoids or fatty acids, performed worse than the modified method of Hita et al. (2015). As such, there is no unique method for recovering the three families of compounds from the biomass of *A. carterae* and further work is needed in this respect. The results obtained led us to propose a combination of the methods of Fernández-Sevilla et al. (2012) and Place et al. (2005), which will be tested in future work.

Acknowledgements

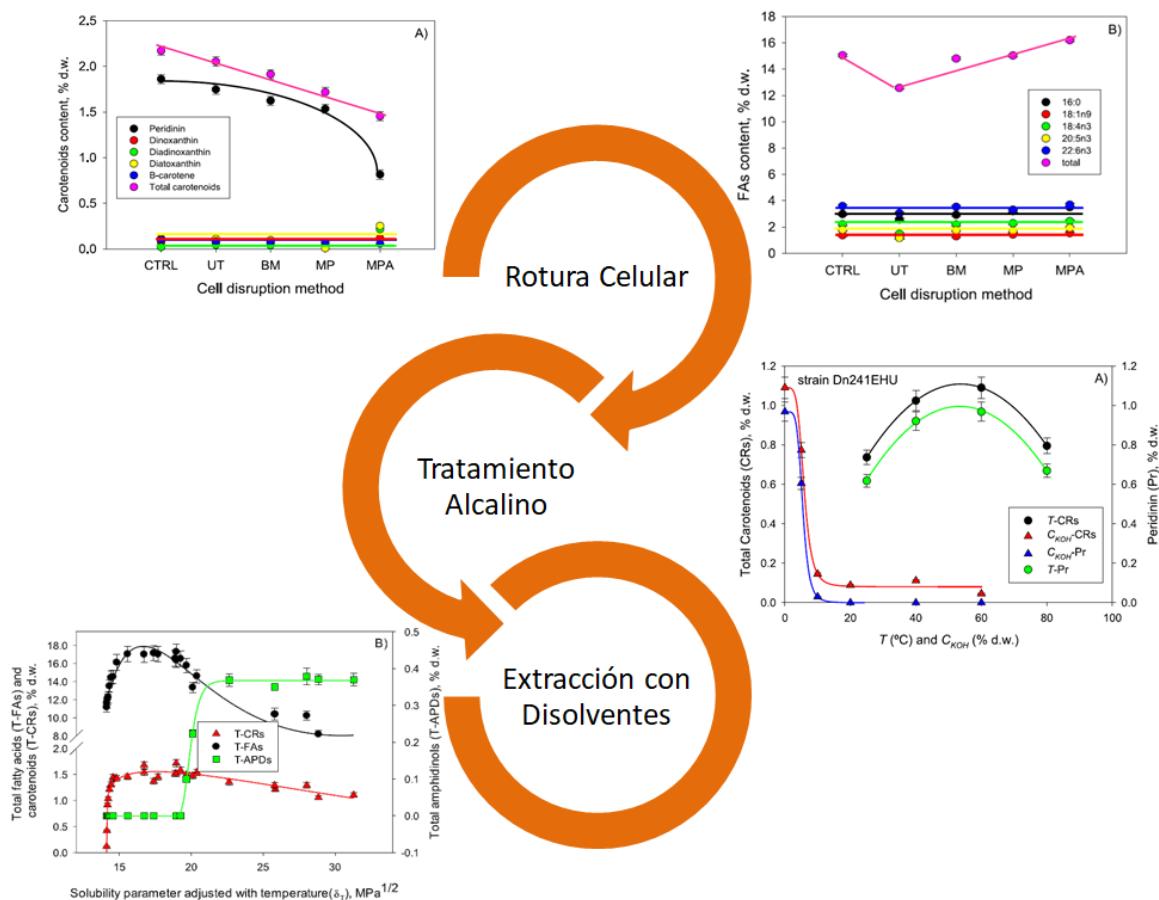
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CAPÍTULO 4

MEJORA DE LA EXTRACCIÓN DE COMPUESTOS BIOACTIVOS DE *AMPHIDINIUM CARTERAE*





Improved extraction of bioactive compounds from biomass of the marine dinoflagellate microalga *Amphidinium carterae*



M. López-Rodríguez^a, M.C. Cerón-García^{a,*}, L. López-Rosales^a, E. Navarro-López^a, A. Sánchez-Mirón^a, A. Molina-Miras^a, A.C. Abreu^b, Ignacio Fernández^b, F. García-Camacho^a

^a Department of Chemical Engineering, Research Centre CIAIMBITAL, University of Almería, Ctra. Sacramento, s/n, 04120 Almería, Spain

^b Department of Chemistry and Physics, Research Centre CIAIMBITAL, University of Almería, Ctra. Sacramento, s/n, 04120 Almería, Spain

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ABSTRACT

The extraction of three families of compounds (carotenoids, fatty acids and amphidinols) from the biomass of two strains of *Amphidinium carterae* (ACRN03 and Dn241EHU) was improved by tuning cell disruption and solvent extraction operations. The extraction of carotenoids was evaluated using alkaline saponification (0–60% KOH d.w.) at different temperatures (25–80 °C). High levels of carotenoids were obtained at 60 °C using freeze-dried biomass, not subjected to cell disruption methods. The ACRN03 strain required 20% KOH whereas the Dn241EHU strain did not require saponification since carotenoid degradation was observed. The extraction efficiencies were determined with a wide range of pure solvents and mixtures thereof. Two empirical non-linear equations were used to correlate extraction percentages for each family of compounds with the Hildebrand solubility parameter (δ_T) and the polarity index of the solvents (PI). Thresholds of δ_T and PI of around 20 MPa^{1/2} and 6, respectively, were determined for the extraction of amphidinols, consistent with antiproliferative activity measurements.

1. Introduction

The biotechnological production of microalgae biomass has been developing rapidly over recent years due to the high value of the secondary metabolites (SMs) that they can produce, such as carotenoids, polyunsaturated fatty acids, complex polyketides, antioxidants, phenolics, phytosterols, isoprenoids, non-ribosomal peptides, oxylipins and alkaloids, etc. Species selection is considered a crucial matter for this biotechnology's success. In this respect, marine dinoflagellate microalgae stand out given that they produce a wide range of bioactive compounds that are of increasing commercial, biomedical and therapeutic interest (Gallardo-Rodríguez et al., 2012). *Amphidinium carterae* is notable because it synthesizes an important group of polyketide compounds, namely amphidinolides and amphidinols (indistinctly referred to APDs). APDs promote potent anticancer, antifungal and hemolytic activity; thus, they are potentially useful in rational drug design studies (Kobayashi and Kubota, 2010). Recent works have successfully proven the viability of recovering APDs from pilot-plant cultures of *A. carterae* using a scalable process (Molina-Miras et al., 2018b). However, *A. carterae*, and marine dinoflagellates in general, also synthesize other high value compounds (e.g. polyunsaturated fatty acids (PUFAs) and

carotenoids), which need to be recovered within a specifically-devised bioprocess (Molina-Miras et al., 2018a). As far as is known, only one biorefining approach has been reported recently (López-Rodríguez et al., 2019) for obtaining APDs from dinoflagellates (in particular, *A. carterae*) that includes the recovery of different high-value co-bioproducts.

Nonetheless, there is no single process to completely recover the compound families of interest (carotenoids, polyunsaturated fatty acids and APDs) contained in the *A. carterae* biomass (López-Rodríguez et al., 2019). Carotenoids present very restrictive properties that largely determine any sustainable dinoflagellate-based bioprocess constructed to produce SMs using a biorefining approach. For example, carotenoids are more thermolabile, photosensitive and acid-sensitive than the rest of the compounds, thus limiting their potential exposure to excess heat, light and acids, respectively (Saini and Keum, 2018). The efficiency of cell disruption methods to facilitate the release of intracellular carotenoids (and other metabolites) is species-dependent (Cerón-García et al., 2018). As a result, minimizing carotenoid degradation during analysis and extraction procedures should be balanced with maximizing the recovery of carotenoids, polyunsaturated fatty acids and APDs in *A. carterae*. This compromise can be regulated by the appropriate choice of

* Corresponding author.

E-mail address: mcceron@ual.es (M.C. Cerón-García).

a single solvent, or solvent mixture, as reported for other microalgae (Cerón-García et al., 2018). Nevertheless, the diversity and profile of carotenoids with varied polarity indexes in the biomass complicates their simultaneous solvent extraction (Saini and Keum, 2018). Despite the numerous studies on carotenoid extraction using solvents reported in the literature, the generalization of their recommendations is very limited. Many of them are based on the simple rule “like dissolves like” applied only in terms of polarity, which has been found to be insufficient in complex samples. Lately, solvent selection following the Hildebrand solubility parameter approach has been reported to improve the efficiency of bioactives extraction from plants (Saha et al., 2015).

The aim of the present study was to characterize the biomass of two *A. carterae* strains and improve the extraction of the three families of bioactive compounds (mentioned above) that they contain. A first step aimed to optimize the cell disruption and biomass alkaline saponification processes; this has not been attempted before for this strain. The second step was carried out to improve the recovery yields of the three families of compounds using a wide range of single solvents, and mixtures thereof, with different polarity indexes and Hildebrand solubility parameters.

2. Materials and methods

2.1. Microalgae biomass

Biomass from two strains of the marine dinoflagellate microalga *Amphidinium carterae* was used. The Dn241EHU strain was obtained from the Microalgae Culture Collection of the Plant Biology and Ecology Department at the University of the Basque Country (Seoane et al., 2018). The ACRN03 strain was obtained from the Culture Collection of Harmful Microalgae at the IEO (Vigo, Spain). The *A. carterae* Dn241EHU biomass comes from a long-term pilot-scale semicontinuous culture (Molina-Miras et al., 2018a). The *A. carterae* ACRN03 biomass was obtained from a pilot-scale fed-batch culture (Molina-Miras et al., 2018b). Culture suspensions of the two strains were centrifuged at 700 g in 50 mL falcon tubes. The pellets were softly washed with distilled water. The supernatants were carefully removed, and the tubes containing pellets with cells were immediately frozen. Afterwards, the tubes were freeze-dried. The lyophilized biomass was stored at – 22 °C to be processed for different purposes.

2.2. Determination of the proximate chemical composition of the biomass

The protein and ash content in the biomass was determined by a method described elsewhere (López et al., 2010). The total lipid content (TLs) in the biomass was ascertained by the method described by (Kochert, 1978). The carbohydrate content was determined by the difference between 100 and the total of the other fractions, such as proteins, total lipids and ash (Camacho-Rodríguez et al., 2014).

2.3. Determination of the saponifiable lipid fractions and the fatty acid profile in the biomass

The fatty acid (FA) profile and content in the dry biomass were determined by gas chromatography following direct transesterification to obtain the fatty acid methyl esters (FAMEs), as described previously (Rodríguez-Ruiz et al., 1998). The FAMEs were used to calculate the amount of saponifiable (SLs) and transesterifiable (TLs) lipids in the biomass. The percentage of unsaponifiable lipids in the biomass was determined using the difference between the TLs (d.w. %) and SLs (d.w. %).

The SLs were fractionated in neutral lipids (NSLs) and polar lipids, specifically glycolipids (GLs) and phospholipids (PLs), as described elsewhere (Callejón et al., 2014). Briefly, the dry TL extract obtained (as detailed above; Section 2.2) was resuspended in 0.5 mL of

chloroform and then fractionated using a single-use silica gel cartridge. Sequential elution with chloroform (30 mL), acetone (30 mL) with chloroform: methanol 85:15 v/v (20 mL), and methanol (30 mL), allowed the NL, GL and PL fractions, respectively, to be collected separately. All the eluents were recuperated in a rotary evaporator and the fractions were converted into FAMEs, as described above (Section 2.2).

2.4. Cell disruption methods

Freeze-drying (FD) is the preferred process for drying microalgae in the value-added biocompounds industry (Lee et al., 2017). It is also considered to be a heat-based disruption method because the cells are damaged during the freeze-thaw cycle (Lee et al., 2017), making subsequent recovery of the intracellular products easier. In this context, all the methods tested in this section were performed with freeze-dried biomass (5 mg samples), in such a way that their effects on cell breakage were also associated with freeze-drying. The control method (CTRL) was carried out using only the freeze-dried biomass. Hence, a total of four cell disruption methods were assayed: (i) ultrasonication (UT) (Selecta, model 3000683, 110 W, frequency 50/60 Hz); (ii) bead milling (BM); (iii) mortar-and-pestle without alumina (MP); and (iv) mortar-and-pestle with alumina present as an abrasive (MPA) at a 1:1 w/w biomass/alumina ratio. The BM-based cell disruption method used a 2.5 L bead mill rotating at a speed of 120 rpm, with 27 mm-diameter ceramic beads. In the MP-assisted procedure, a laboratory mortar-and-pestle with a 250 mL volume was used to ground the lyophilized biomass sample. MP provides good compound recovery but cannot be scaled up for industrial use (Rajesh et al., 2017); consequently, it was tested for comparison purposes only. The alumina in the MPA method was obtained from Sigma-Aldrich (Type WN-3, 013K3422Inc St. Louis, MO, USA). The experiments were operated at room temperature (22–25 °C) for 2 min. The samples from the treatments were saponified and their carotenoid content and profile were determined, as explained elsewhere (Cerón-García et al., 2018). Briefly, the biomass saponification was performed in a tricomponent solution in Pyrex glass tubes at 25 °C with a KOH weight to biomass weight ratio of 0.4 (i.e. 40% d.w.). The carotenoids were analyzed by HPLC using a photodiode array detector. The efficiency of each method was discussed in terms of carotenoid recovery compared to the control. The measurements for each disruption method were carried out in duplicate.

2.5. Biomass alkaline saponification

The alkaline treatment used for biomass saponification, as described previously for other microalgae (Cerón-García et al., 2018), was optimized for *A. carterae* by varying the temperature (*T*) and KOH concentration (*C_{KOH}*). Briefly, 5 mg samples of freeze-dried biomass were placed in Pyrex glass tubes. Then, 1 mL of monophasic tricomponent solution was added to each tube and shaken in a vortex for 20 s over a 2-min period. The tricomponent solution was composed of ethanol, hexane and water in a ratio of 76:18:6 v/v/v while the specific percentage of KOH was calculated on the basis of the sample's dry biomass weight. The temperature of the mixture was controlled by submerging the tubes in a shaking water bath (Julabo® SW22, Julabo USA Inc.). The shaking frequency was set at 3 cycles per minute. After the reaction was completed (2 min), the tubes were left to cool at room temperature. Next, the samples were centrifuged at 12000 rpm for 3 min (Eppendorf® MiniSpin Plus® microcentrifuge, Hamburg, Germany). The resulting supernatants were transferred into HPLC vials to determine the carotenoid content and profile as a means of assessing the efficiency of each *T-C_{KOH}* combination. The temperature and KOH concentration were varied from 25 °C to 80 °C and from 0% to 60%, respectively. All the experiments were conducted in duplicate and each sample was analyzed in duplicate.

Table 1

Polarity index (PI), Hansen parameters (δ_d , δ_p , δ_h), solubility parameter for the solvent mixture of the different solvents and mixtures (δ), adjusted with temperature (δ_T), which was then used to extract carotenoids, fatty acids and amphidinols from *A. carterae* biomass.

Solvent (v/v)	PI	δ_d	δ_p	δ_h	δ	δ_T
Hexane	0.000	14.900	0.000	0.000	14.900	14.107
Acetone:hexane(1:99)	0.096	14.906	0.104	0.070	14.907	14.114
Acetone:hexane(5:95)	0.464	14.930	0.520	0.350	14.943	14.148
Acetone:hexane(10:90)	0.895	14.960	1.040	0.700	15.012	14.214
Acetone:hexane(15:85)	1.295	14.990	1.560	1.050	15.107	14.304
Acetone:hexane(20:80)	1.668	15.020	2.080	1.400	15.228	14.418
Acetone:hexane(25:75)	2.016	15.050	2.600	1.750	15.373	14.555
Acetone:hexane(30:70)	2.342	15.080	3.120	2.100	15.542	14.715
Hexane:ethanol(70:30)	2.581	15.530	6.160	13.580	21.530	20.385
Diethyl ether	2.850	14.500	2.900	5.100	15.642	14.810
Acetone:hexane(50:50)	3.463	15.200	5.200	3.500	16.442	15.567
Hexane:ethanol(50:50)	3.624	15.350	4.400	9.700	18.683	17.690
Acetone:hexane(70:30)	4.356	15.320	7.280	4.900	17.655	16.716
Acetone:hexane(80:20)	4.737	15.380	8.320	5.600	18.361	17.384
Absolute ethanol	5.200	15.800	8.800	19.400	26.522	25.112
Acetone	5.400	15.500	10.400	7.000	19.935	18.875
Acetone:water(99.5:0.5)	5.472	15.501	10.428	7.177	20.013	18.948
Ethanol:hexane:water (76:18:6) ¹	5.507	15.626	7.648	17.282	24.522	23.218
Acetona:water (99:1)	5.542	15.599	15.599	15.599	27.018	25.581
Ethanol:water(96:4)	5.643	15.792	9.088	20.316	27.290	25.838
Ethanol:hexane:water (84:6:10) ²	5.785	15.726	8.992	20.526	27.377	25.920
Acetone:water(97.5:2.5)	5.800	15.503	10.540	7.883	20.336	19.254
Acetone:water(95:5)	6.000	15.505	10.680	8.765	20.768	19.663
Ethanol:hexane:water (80:10:10)	6.043	15.690	8.640	20.526	27.242	25.793
Acetone:water(92.5:7.5)	6.300	15.508	10.820	9.648	21.228	20.099
Methanol	6.600	15.100	12.300	22.300	29.607	28.032
Ethanol:water(80:20)	6.880	15.760	10.240	23.980	30.468	28.847
Acetona:water(80:20)	7.217	15.520	11.520	14.060	23.901	22.630
Methanol:water(80:20)	7.462	15.200	13.040	26.300	33.057	31.299

¹ Fernández- Sevilla et al. (2009)

² Fernández-Sevilla et al. (2012)

2.6. Solvent extraction of carotenoids

As microalgae biomass contains diverse carotenoids with varying polarity levels, selecting the appropriate solvents is critical for optimizing their extraction. **Table 1** displays the range of solvents and mixtures tested as extractants of the carotenoids, fatty acids and amphidinols from *A. carterae* biomass. They cover a wide range of polarity indexes (PI). The PI values for the solvent mixtures (PI_{mix}) were calculated as follows:

$$PI_{mix} = \sum_{i=1 \dots p} X_i \cdot PI_i \quad (1)$$

where the subscript i represents the pure solvent i , and X is the volumetric fraction of the pure solvent in the mixture (Poole and Poole, 1991). The solubility parameter, originally introduced by Hildebrand, was divided into three partial components by Hansen: a dispersion force component (δ_d), a hydrogen-bonding component (δ_h) and a polar component (δ_p). The total solubility parameter (δ_T) for the pure solvent is given by the equation:

$$\delta_T = \sqrt{\delta_d^2 + \delta_p^2 + \delta_h^2} \quad (2)$$

In the case of solvent mixtures, each partial solubility parameter is calculated as in eq. (1):

$$\delta_k = \sum_{i=1 \dots p} X_i \cdot \delta_{k,i} \quad (3)$$

where i represents the solute in the mixture and k each partial solubility parameter (i.e., d , p and h). The temperature adjustment for the solubility parameter was carried out as proposed by Barton (1983):

$$\left(\frac{\delta_1}{\delta_2} \right)^2 = \frac{T_2}{T_1} \quad (4)$$

where T_1 is the reference temperature (25 °C) and T_2 the extraction temperature (60 °C).

The procedure consisted of adding 1 mL of each solvent to 5 mg of dry biomass deposited in a Pyrex glass tube. Samples were subjected to a temperature of 60 °C in a shaking water bath for 2 min and energetically mixed every 20 s over the 2 min. Next, the samples were centrifuged at 12000 rpm for 3 min (Eppendorf® MiniSpin Plus® microcentrifuge, Hamburg, Germany). The carotenoids were determined in the resulting supernatants by HPLC, as reported previously (Cerón-García et al., 2018). The experiments were carried out in duplicate. Recovery yields of carotenoids (T-CRs Recovery, %), shown below in **Table 4**, were calculated with a reference value (1.1% d.w.) that was the maximum obtained using the modified version of the traditional analytical method reported earlier (Cerón-García et al., 2018), and described briefly in **Section 2.5**.

2.7. Solvent extraction of fatty acids

To cover the extraction of both polar and non-polar lipids, a selection of the pure solvent and solvent mixtures shown in **Table 1** were selected to cover a wide range of PI values. The procedure consisted of adding 2 mL of each solvent to 10 mg of dry biomass placed in a Pyrex glass tube. Samples were subjected to a temperature of 60 °C in a shaking water bath for 2 min and energetically mixed every 20 s over the 2 min. Next, the samples were centrifuged at 3500 rpm (Heraeus Labofuge 200 Thermo Fisher Scientific, Osterode, Germany) for 4 min and the extracts separated for drying in a N₂ stream at 45 °C. After this, the fatty acid profile in the extracts was determined by GC, as described in **Section 2.2**. To calculate the recovery yields of fatty acids (T-FAs Recovery, %), displayed in **Table 4** below, the reference value used (18.2% d.w.) was that of the saponifiable fatty acids content in the biomass, measured as described briefly in **Section 2.3** (as cited by Rodríguez-Ruiz et al., 1998).

2.8. Determination of amphidinols

The *A. carterae* Dn241EHU strain has recently been shown to produce amphidinols A and B (Abreu et al., 2019). The presence of the A and B amphidinols in the different extracts obtained was preliminarily confirmed by assaying the hemolytic and antitumoral activity (López-Rosales et al., 2015; Molina-Miras et al., 2018b). The antitumoral assays were performed as described elsewhere (Abreu et al., 2019).

Extracts with pure solvent and solvent mixtures obtained from 5 mg of *A. carterae* were processed for the acquisition of the NMR spectra and the quantification of the APDs as previously described (Abreu et al., 2019). Analyses were carried out on a Bruker Avance III HD 600 spectrometer. The relative peak integral at δ_H 5.07 ppm was used in all of the acquired ¹H NMR spectra for the quantification of the APDs, as amphidinol A (MW 1338 g/mol), the value of which was based on the TSP signal integral of known concentration. Regarding the recovery yields of amphidinols (T-APDs Recovery, %), shown below in **Table 4**, these were calculated using the reference value (0.37 d.w.) that was obtained with methanol:water (80:20), according to a previous study ((Rodríguez-Ruiz et al., 1998)).

2.9. Non-lineal fittings

Two non-lineal equations were proposed for fitting the cell content of three groups of compounds (fatty acids, carotenoids and amphidinols) with the polarity index (PI) and the total solubility parameter (δ_T) (see below in **Fig. 3**). Equation (1), based on the four-parameter Weibull-type distribution, was used to fit the total carotenoid (T-CRs) and total fatty acid (T-FAs) contents:

$$T\text{-CRs, FAs} = a \cdot \left(\frac{c-1}{c} \right)^{\frac{1-c}{c}} \cdot \left[\frac{x-x_0}{b} + \left(\frac{c-1}{c} \right)^{\frac{1}{c}} \right]^{c-1} \cdot e^{-\left[\frac{x-x_0}{b} + \left(\frac{c-1}{c} \right)^{\frac{1}{c}} \right]^c} + \frac{c-1}{c} \quad (5)$$

and the following five-parameter sigmoidal equation was used to fit the total amphidinol content (T-APDs):

$$T - APDs = y_0 + \frac{a}{\left[1 + e^{-\left(\frac{x-x_0}{b} \right)^c} \right]} \quad (6)$$

where x represents PI or δ_T . The parameters y_0 , x_0 , a , b and c were estimated using non-linear regression (Sigma Plot 6.0 software, Systat Software Inc., San Jose, US). Equations (1) and (2) were provided by the SigmaPlot equations library. These do not have direct physico-chemical and biological meaning; both equations are simply mathematical tools for describing the data and, thus, avoiding subjective errors.

2.10. Statistical analysis

All experiments were performed in duplicate. The experimental results were shown as the mean values of the two independent experiments and their standard deviation. Statistical data analyses were performed using the Statgraphics Centurion XVII (version 17.2.04) statistical software (2014, Statpoint Technologies, Inc., Warrenton, VA). The normality and homogeneity tests were performed using the Kolmogorov-Smirnov and Levene tests, respectively. Statgraphics was used for a significant difference analysis with a one-way and multi-way analysis of variance (ANOVA) test.

3. Results and discussion

3.1. Proximate chemical composition

Table 2 displays the proximate compositions of both *A. carterae* strains. The profiles were comparable considering the limited percentage differences between them (below 30%). The discrepancies observed might be attributable not only to intra-specific differences, but also the type of photobioreactor and the operation mode used to produce the biomass of each strain (see details in Section 2.1). On this point, it is well-known that proximate composition is sensitive to the environmental conditions of the culture and the growth phase when the biomass is harvested. Dramatic changes in the proximate cellular components of non-dinoflagellate microalgae have been reported for the same strain when cultured under conditions of both nitrogen sufficiency and deficiency (Thomas et al., 1984). For example, N-deficient cells of *Tetraselmis suecica* and *Dunaliella primolepta* decreased their protein content by about 130%, the carbohydrate content markedly rose to about 300%, and the total lipid content decreased nearly 50%, compared to cells grown in N-sufficient medium (Thomas et al., 1984). Keeping in mind these wide variations reported within a species, the proximate compositions displayed in **Table 2** are consistent with those

Table 3

Profile of the percentages of the main fatty acids identified for the *A. carterae* ACRN03 and *A. carterae* Dn241EHU strains.

Fatty acid	ACRN03 (% d.w.)	Dn241EHU (% d.w.)
14:0	0.04 ± 0.0	0.5 ± 0.0
16:0	3.9 ± 0.6	4.4 ± 0.5
18:0	2.2 ± 0.3	2.3 ± 0.1
18:1n9	0.1 ± 0.0	1.9 ± 0.2
18:2n6	0.1 ± 0.0	0.2 ± 0.0
18:3n3	0.02 ± 0.0	–
18:4n3	2.1 ± 0.3	1.8 ± 0.2
20:1n9	0.3 ± 0.0	0.3 ± 0.0
20:4n6	0.4 ± 0.0	–
20:5n3	2.7 ± 0.1	2.4 ± 0.1
22:6n3	4.7 ± 0.4	4.4 ± 0.5
Total fatty acids	18.4 ± 2.5	18.2 ± 1.7

reported for another strain of *A. carterae* (Parsons et al., 1961).

Regarding the total lipids, the saponifiable fraction was predominant in both strains, with relative values of 59.5% and 73.2% of the total lipids for ACRN03 and Dn241EHU, respectively. The total FA contents were similar in both strains, with $18.2 \pm 1.74\%$ d.w. for Dn241EHU and $18.40 \pm 2.47\%$ d.w. for ACRN03. The FA profiles in **Table 3** are in good agreement with those recently reported for both strains when cultured in photobioreactors (López-Rodríguez et al., 2019; Molina-Miras et al., 2018a).

3.2. Comparison of cell disruption methods

Fig. 1 compares the effectiveness of the four cell breakage methods tested in terms of the amounts of recovered carotenoids and fatty acids. All the cell disruption methods, including the control, were able to disrupt *A. carterae* cells (with cytolysis visible under an optical microscope). However, the extraction capacity was dependent on both the compound family and the method. Regarding carotenoids, all methods extracted peridinin, peridinin diatoxanthin, diadinoxanthin, pyrrhoxanthin, diadinoxanthin and β-carotene. **Fig. 1A** displays the content of the different types of carotenoids in the samples. The profiles were in line with those reported for *A. carterae* Dn241EHU, and consistent with the Type 1 pattern typical of dinoflagellate with peridinin as the major carotenoid (Jeffrey and Wright, 2006). The maximum carotenoid content extracted, $2.17 \pm 0.05\%$, corresponded to the CTRL. Any of the remaining treatments led to lower carotenoid recovery ($p < 0.05$); the MPA method, in particular, involved a dramatic decrease in the total carotenoid yield of about 33%, and 56% for peridinin, compared to the CTRL. The degradation of carotenoids seemed to be the most feasible cause because UT, BM, MP and MPA are mechanical disruption procedures where the shear stresses and temperatures involved can affect the quality and structure of the carotenoids, according to a recent review study (Lee et al., 2017). With respect to the total FAs, the effect was radically different compared to carotenoids (**Fig. 1B**). Only the UT method extracted FAs in lower amounts than the control ($p < 0.05$), but to a limited percentage (< 16%). There were no statistically significant differences between BM, MP and the CTRL ($p < 0.05$). Only the MPA method slightly improved FA recovery relative to the control (< 6%). The individual fatty acids did not appear to follow the same pattern as their sum (i.e. the total FAs). No specific reasons were found to justify the selective action of each method tested. It was evident that the FD-based method (CTRL) provided the highest recovery values for both carotenoids and FAs, making it unnecessary to reinforce this pretreatment step with an additional cell disruption method. A similar conclusion was obtained in studies on other microalgae (Kim et al., 2012). However, it is well-known that the selection of a suitable cell disruption method is dependent on the cell-wall's characteristics and composition, both of which are specific to the microalga species and the culture's

Table 2

Biomass composition (total lipids, saponifiable lipids, proteins, carbohydrates and ashes (% biomass d.w.) identified for the *A. carterae* ACRN03 and *A. carterae* Dn241EHU strains.

Biomass composition	% Biomass d.w.	
	ACRN03	Dn241EHU
Total lipids	30.9 ± 0.3	23.5 ± 0.5
Saponifiable lipids	18.4 ± 2.5	18.2 ± 0.5
Proteins	25.9 ± 0.3	20.9 ± 0.2
Carbohydrates	35.6 ± 0.2	46.3 ± 0.2
Ashes	7.3 ± 0.2	7.8 ± 0.4

Table 4

Effect of the extraction solvents on the recovery yields of total carotenoids (T-CRs), fatty acids (T-FAs) and amphidinols (T-APDs). PI is the polarity index; δ_T is the total solubility parameter.

Entry	Solvents, v:v	PI	δ_T , MPa ^{1/2}	T-CRs Recovery, %	T-FAs Recovery, %	T-APDs Recovery, %			
1	Hexane HPLC, 100:0	0.00	14.11	11.9	\pm 0.6	61.4	\pm 3.6	0.0	\pm 0.0
2	Acetone:Hexane, 1:99	0.10	14.12	42.7	\pm 2.1	64.0	\pm 3.8	0.0	\pm 0.0
3	Acetone:Hexane, 5:95	0.46	14.17	93.7	\pm 4.7	66.2	\pm 3.9	0.0	\pm 0.0
4	Acetone:Hexane, 10:90	0.90	14.21	105.7	\pm 5.3	67.5	\pm 4.0	0.0	\pm 0.0
5	Acetone:Hexane, 15:85	1.30	14.30	124.2	\pm 6.2	74.2	\pm 4.4	0.0	\pm 0.0
6	Acetone:Hexane, 20:80	1.67	14.42	133.6	\pm 6.7	79.3	\pm 4.7	0.0	\pm 0.0
7	Acetone:Hexane, 25:75	2.02	14.56	147.5	\pm 7.4	79.8	\pm 4.7	0.0	\pm 0.0
8	Hexane:Acetone, 70:30	2.34	16.72	157.3	\pm 7.8	80.0	\pm 0.00	0.0	\pm 0.0
9	Hexane:Ethanol, 70:30	2.58	20.39	157.7	\pm 7.9	80.2	\pm 4.7	0.0	\pm 0.0
10	Diethyl ether, 100:0	2.85	14.81	145.9	\pm 7.3	88.6	\pm 5.2	0.0	\pm 0.0
11	Hexane: Acetone, 50:50	3.46	15.57	149.0	\pm 7.4	93.6	\pm 5.5	0.0	\pm 0.0
12	Hexane:Ethanol, 50:50	3.62	17.69	148.1	\pm 7.4	93.5	\pm 5.5	0.0	\pm 0.0
13	Acetone:Hexane, 70:30	4.36	16.72	171.7	\pm 8.6	93.4	\pm 5.5	0.0	\pm 0.0
14	Acetone:Hexane, 80:20	4.74	17.38	140.2	\pm 7.0	94.2	\pm 5.6	0.0	\pm 0.0
15	Acetone HPLC, 100:0	5.40	18.88	154.7	\pm 7.7	96.4	\pm 5.7	0.0	\pm 0.0
16	Acetone:Water, 99.5:0.5	5.47	18.95	155.0	\pm 7.8	94.9	\pm 5.6	0.0	\pm 0.0
17	Acetone: Water, 99:1	5.54	18.95	175.5	\pm 8.8	89.9	\pm 5.3	0.0	\pm 0.0
18	Ethanol: Water 96:4	5.60	25.84	124.6	\pm 6.2	57.1	\pm 3.4	0.0	\pm 0.0
19	Acetone: Water, 97.2:2.5	5.80	19.25	161.3	\pm 8.1	90.8	\pm 5.4	0.0	\pm 0.0
20	Acetone: Water, 95:5	6.00	19.66	152.6	\pm 7.6	86.7	\pm 5.1	27.0	\pm 1.4
21	Ethanol:Hexane:Water, 80:10:10	6.04	25.79	131.9	\pm 6.6	57.1	\pm 3.4	94.6	\pm 4.7
22	Acetone:Water, 92.5:7.5	6.30	20.10	150.86	\pm 7.5	73.4	\pm 4.3	60.2	\pm 3.0
23	Methanol HPLC, 100:0	6.60	28.03	132.0	\pm 6.6	56.3	\pm 3.3	102.4	\pm 5.1
24	Ethanol:Water, 80:20	6.88	28.85	108.0	\pm 5.4	45.0	\pm 2.7	100.0	\pm 5.0
25	Acetone:Water, 80:20	7.22	22.63	138.2	\pm 6.9	42.2	\pm 2.5	99.6	\pm 4.9
26	Methanol:Water, 80:20	7.46	31.30	112.9	\pm 5.6	35.7	\pm 2.1	100.0	\pm 5.0

environmental conditions (Gong and Bassi, 2016; Lee et al., 2017). Consequently, the FD-based method was used for the rest of study.

3.3. Effect of the saponification conditions on carotenoid recovery

Fig. 2 shows the effect of saponification T and C_{KOH} (4x6 levels) on the total carotenoids (CRs) recovered and the peridinin (Pr) contents in both of the *A. carterae* strains. Fig. 2 collates the results from a multi-factor ANOVA. Thus, for each T and C_{KOH} , the point represented is the average value from all the different C_{KOH} (0, 5, 10, 20, 40 and 60%) and all the temperatures assayed (25, 40, 60 and 80 °C), respectively. Both factors (T and C_{KOH}) and their interaction ($T \cdot C_{KOH}$) had a statistically significant effect on CRs and Pr at the 95.0% confidence level ($p < 0.05$). However, the contribution of the $T \cdot C_{KOH}$ interaction was substantially lower than those of T and C_{KOH} individually. Specifically, the combined $T \cdot C_{KOH}$ interaction had a contribution of 14.77%, with the weight of T (61.39%) higher than C_{KOH} (23.83%). This pattern observed in the ANOVA was irrespective of the strain, as reported in a recent study carried out on eight microalgae species of different genera (Cerón-García et al., 2018).

A closer inspection of Fig. 2 reveals intraspecific differences in the saponification effect on CR recoveries, particularly for Pr, as it is the main carotenoid. Compared to the non-saponified control ($C_{KOH} = 0$), saponification of the Dn241EHU biomass at any C_{KOH} underestimated the CRs on account of Pr degradation, the higher the C_{KOH} the higher the losses from degradation (Fig. 2A). This is consistent with the few previous studies reporting Pr as an alkali-labile carotenoid (Barańska and Kaczor, 2016). In contrast, when biomass of the ACRN03 strain was used, saponification at all C_{KOH} significantly improved the recovery of Pr ($p < 0.05$) relative to the control, reaching a plateau above 20% C_{KOH} (Fig. 2B). The optimal temperature was the same for both strains, a value of 60 °C. A plausible interpretation might be found by considering the biomass fat content. It is known from the literature that a general rule for reducing the risk of carotenoid degradation or loss is to apply milder conditions to low-lipid products in the saponification step, and to then increase the treatment severity as the lipid content increases (Rodríguez-Bernaldo de Quirós and Costa, 2006). However, the

saponifiable lipid content was similar in both strains.

Therefore, the reason why peridinin degradation was protected during the ACRN03 biomass saponification might be due to differences in the cell wall composition of each strain, caused by the culture mode, whether pneumatic stress exists or not (Gallardo-Rodríguez et al., 2016), or even due to the presence of peridinin in ester form. Peridinin can often undergo hydrolysis, losing acetic acid to convert into peridinol, and then in the presence of fatty acids convert into ester form (Sugawara et al., 2009). As a result, the extraction of carotenoids from the microalgal biomass will require an alkaline treatment to break up the ionizable lipids (acyl-glycerols) and to completely destroy the cell wall in order to release those carotenoids that might appear in esterified form. These esters would remain in the hydro-alcoholic phase; otherwise, they would remain ionized in the aqueous phase after the alkaline treatment, together with many other lipids such as fats and waxes, and other non-polar compounds (Cerón et al., 2008). As the Dn241EHU biomass presented the highest carotenoid content, it was selected for the rest of the experimental plan.

3.4. Solvent extraction of product contents

With the goal of improving the overall extraction yield of carotenoids, saponifiable lipids (fatty acids) and amphidinols from the *A. carterae* biomass, different solvent systems were used (Table 1). In general, solvent selection is not a straightforward variable since it depends on the type of microalgal species and the target metabolite (Chan et al., 2013; Cerón-García et al., 2018) that one would like to extract selectively. For instance, as far as is known, there is no unique process to simultaneously recover carotenoids, polyunsaturated fatty acids and APDs from the same type of biomass (López-Rodríguez et al., 2019).

In the total lipid profile determined in the Dn241EHU biomass, the polar constituents are dominant, with 45.1% of neutral lipids (NLs), 47.3% of glucolipids (GLs) and 7.6% of phospholipids (PLs). It is known that in eukaryotic microalgae cells, NLs associate through van der Waals forces to establish lipid globules in the cytoplasm; as a result, polar solvents such as ethanol or acetone are expected to extract them more efficiently. In contrast, non-polar solvents, such as hexane, will

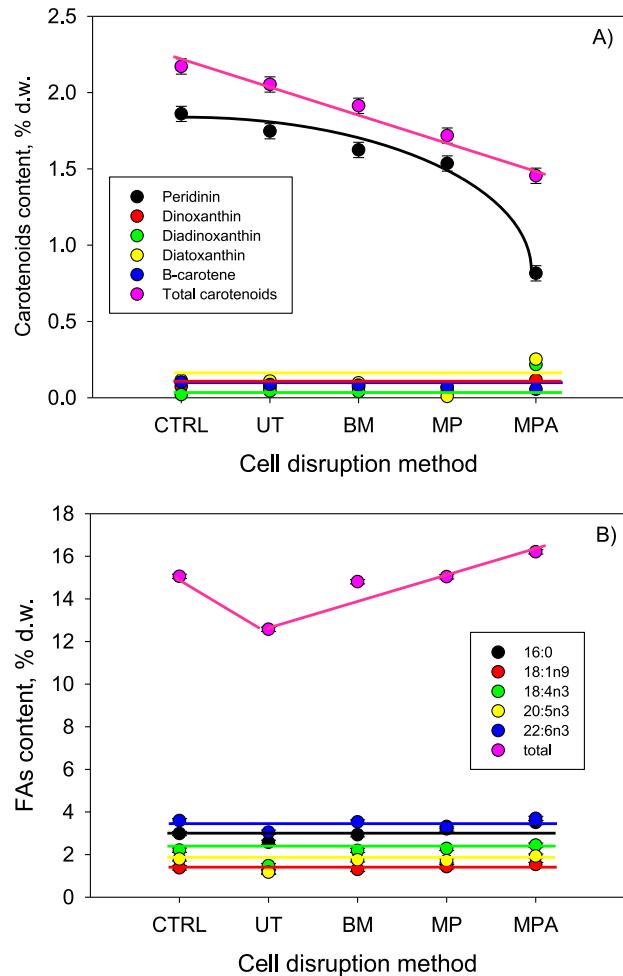


Fig. 1. Effect of different cell disruption methods on the extraction of (A) carotenoids and (B) fatty acids from lyophilized *Amphidinium carterae* biomass (Dn241EHU). CTRL: control; UT: ultrasounds; BM: bead milling ; MP: mortar-and-pestle without alumina; MPA: mortar-and-pestle with alumina present as an abrasive at a 1:1 w/w biomass/alumina ratio. Data points are averages, and vertical bars are standard deviations (SD) for duplicate samples. Points without SD bars indicate that the SD was smaller than the symbol.

extract free NLs from the cells. However, the possibility exists that some neutral lipids may form complexes with polar lipids, and that these associated architectures, which are already hydrogen bonded to the cell membrane proteins, would only be extracted by polar solvents such as acetone, water or ethanol (Ryckebosch et al., 2014a). Consequently, mixtures of polar and non-polar solvents are capable of increasing the extraction yield as they might extract both the neutral and the polar lipids that form part of these complexes, as well as the neutral lipids that are free in the cytoplasm (Ryckebosch et al., 2014a; Balasubramanian et al., 2013).

Given that the families of cellular compounds (carotenoids, polyunsaturated fatty acids and APDs) present different functional groups with variable overall dipolar moments, the polarity parameters of the solvents need to be considered for their optimum extraction. These were calculated for each solvent using eq (1) and the solubility parameter adjusted with temperature using equation (4) (Table 1). In this study, mixtures of different polarity indexes were prepared using different quantities of pure solvents (hexane, acetone, ethanol and water). Fig. 3A and B illustrate the extraction percentages obtained for the different extraction systems tested in this work against the polarity indexes and the solubility parameters adjusted with temperature, respectively, for each family of compounds analyzed. In the following

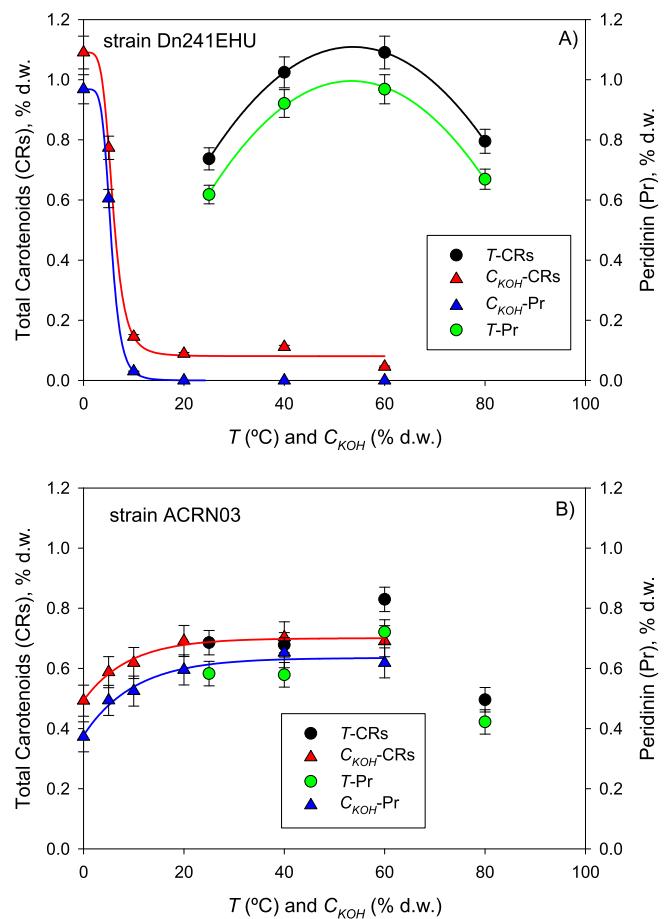


Fig. 2. Influence of temperature (°C) and KOH concentration (% d.w.) on the total carotenoid content of the different strains (*Amphidinium carterae* ACRN03 (A) and *Amphidinium carterae* Dn241EHU (B)).

subsections, the results from these three families of compounds are discussed separately.

3.4.1. Carotenoids

The optimal extraction of carotenoids was achieved using *Pi* solvent values of 3–5.5 (Fig. 3A) and δ_T values of 16.5–19.5 [MPa^{1/2}] (Fig. 3B). The maximum content of total carotenoids (T-CRs), 1.7% d.w., was obtained using acetone: water (99:1) (polarity index: 5.54). Regarding the effect of the solvent system on the recovery yields of T-CRs, Table 4 shows that the maximum value (entry 17) was 175.5 ± 8.8% higher than the control. In particular, the highest peridinin percentage was obtained with the acetone: water (99:1) solvent system (1.56% d.w. ± 0.07). This peridinin value was higher than those reported by Molina-Miras et al. (2018b); and as López-Rodríguez et al. (2019) previously described, this might be due to the fact that carotenoids such as peridinin are associated with proteins in the photosynthetic complex (Zigmantas et al., 2003) and can only be disrupted by polar organic solvents that are able to dissociate hydrogen bonding (Ryckebosch et al., 2014a). Indeed, the acetone: hexane (70:30) mixture (entry 13, Table 4), with a polarity index of 4.36, was the second most effective solvent for carotenoids, with a recovery yield 171.7 ± 8.6% greater than the control, achieving a 1.67% d.w. of carotenoids (Fig. 3A). This is in agreement with previous reports that always describe acetone and hexane as being frequently selected to extract polar and non-polar carotenoids, respectively (Saini and Keum, 2018). The hexane:acetone (70:30, entry 8, Table 4) and hexane:ethanol (70:30, entry 9, Table 4) solvent mixtures provided similar results of ca. 157% of T-CRs (1.54% d.w.). Lin & Chen (2003) also found that an ethanol:hexane mixture

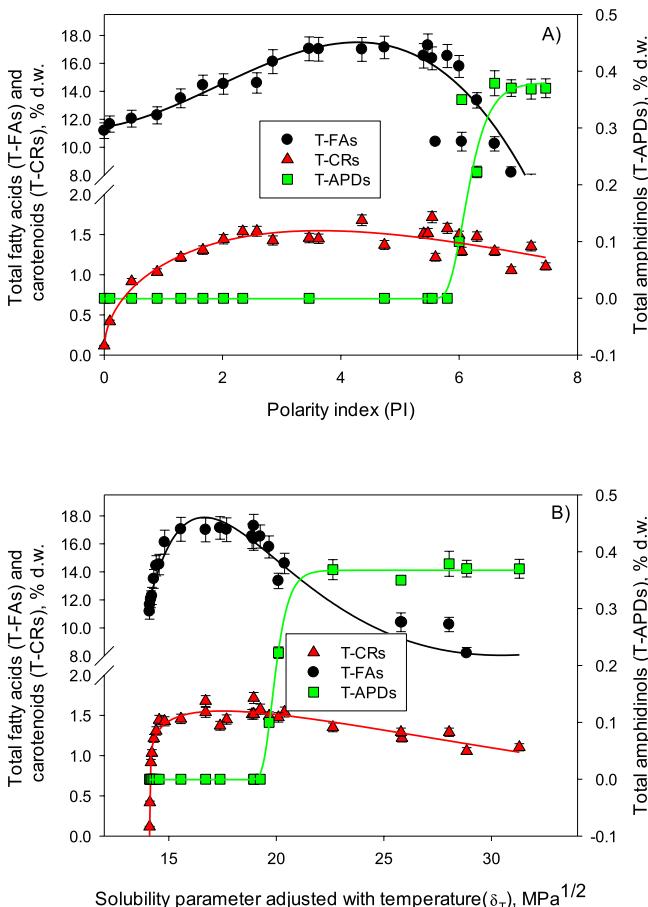


Fig. 3. Correlation between the extraction of the different compound families (carotenoids, polyunsaturated fatty acids and APDs) from *Amphidinium carterae* Dn241EHU (A) with the solvent polarity (B) and with the solubility parameter adjusted with temperature (the content of carotenoids (T-CRs), polyunsaturated fatty acids (T-FAs) and amphidinols (T-APDs).

(4:3 v/v) provided the highest carotenoids extraction yield from tomato juice.

A simple variance test (ANOVA) was used to evaluate the influence of the polarity index on carotenoids extraction. From this, target data were obtained which were fitted into a non-linear regression, adjusted to a 4-parameter Weibull-type distribution.

In Table 5, the different parameters are collected for the correlation given by eqs. (1) and (2) between the average concentration of T-CRs with both the polarity index and the solubility parameter (adjusted with temperature). By inspecting these data, it was possible to conclude that the solubility parameter theory is applicable to carotenoids extraction since it improves the recovery of the most abundant carotenoids, such

Table 5

Values of the parameters that provide the best fitting of the solvent extraction data to eq. (1) and (2), represented in Fig. 3A,B. T-FAs: total fatty acids; T-CRs: total carotenoids; T-APDs: total amphidinols; R²: determination coefficient.

Metabolites	Parameters					R ²
	y ₀	a	b	c	x ₀	
T-FAs (3A)	11.441	0.645	0.692	-0.1196	-	0.911
T-CRs (3A)	-	1.549	8.189	1.451	3.653	0.891
T-APDs (3A)	-1.144E-9	0.343	0.003	5091.850	5.978	0.964
T-FAs (3B)	-	23.416	1.953	121.388	-	0.869
T-CRs (3B)	-	1.554	0.026	58.489	17.488	0.945
T-APDs (3B)	-0.0006	0.368	0.425	14860.967	15.709	0.998

as peridinin (a polar carotenoid), from *Amphidinium carterae*. Although the solvent selection depends on the target carotenoid to be recovered, based on their polarity, the solvent mixtures reported here have demonstrated good performance when extracting both xanthophylls and carotenes.

3.4.2. Fatty acids

Regarding the extraction of fatty acids, as shown in Fig. 3A, the maximum saponifiable lipid (T-FAs) contents, of ca. 17% d.w., were obtained using solvent systems with polarity indexes ranging between 3.4 and 5.4, corresponding to values between 17 and 19 [MPa $^{1/2}$] (Fig. 3B). Most are mixtures of polar and non-polar solvents such as acetone: hexane or ethanol:hexane. As an example, a 17.28% d.w. content was obtained using acetone:water (99.5:0.5) (polarity index: 5.47), with a recovery of 95.0 ± 5.7% (entry 16, Table 4) with respect to the initial saponifiable lipid content. Very similar recoveries were obtained with other extraction mixtures that have a low polarity index, such as hexane:acetone 50:50 (entry 11, Table 4) or acetone:hexane 80:20 (entry 14, Table 4) with values of 93.6 ± 5.6 and 94.2 ± 5.6%, respectively. Mixtures with higher polarity indexes clearly recover lower amounts of FAs (Fig. 3A). For instance, solvent mixtures based on ethanol:water 80:20 (entry 24, Table 4), acetone:water 80:20 (entry 25, Table 4) or methanol:water 80:20 (entry 26, Table 4) achieved recovery yields of 45.0 ± 2.7, 42.2 ± 2.5 and 35.7 ± 2.1%, respectively, with respect to the initial saponifiable lipids. This could be due to the incapacity of very polar solvents to extract free NLs in the cytoplasm, as mentioned above (Balasubramanian et al., 2013).

On the other hand, the use of non-polar solvents, such as hexane (PI = 0), achieved a FAs content of 11.2% d.w. in the biomass, and a T-FAs recovery of 61.5 ± 3.7% (entry 1, Table 4). Other authors, such as Navarro-López et al. (2016), described similar behaviour in the extraction of saponifiable lipids from the microalgae *Nannochloropsis gaditana* when using mixtures of polar and non-polar solvents for the lipid extraction, achieving a maximum saponifiable lipids extraction yield of 85% using ethanol and hexane as solvents, and only a 36% yield if hexane was used for the lipid extraction. Fatty acids correlate well with the same Weibull-type distribution fitting, as shown in Table 5.

It is worth mentioning that among the variables analysed here, the performance of a particular solvent system strongly depends on the microalgal species. Thus, the yields obtained using hexane with *A. carterae* are higher than those obtained by Ryckebosch et al. (2014a) using the same solvent but with *Nannochloropsis gaditana*. Under these conditions, the authors reported that only 36% of the initial saponifiable lipids contained in the biomass were extracted. The differences between the extraction yields in the two species (*Nannochloropsis gaditana* vs *Amphidinium carterae*), which have similar lipid profiles (i.e. 40% NLs and 60% polar lipids for the former and 45.1% NLs and 54.9% polar lipids for the latter), might be explained by the difference in their cell permeability. The *Nannochloropsis* species possesses a thick rigid cell wall that contains a biopolymer named algaenan, making lipid extraction difficult (Ryckebosch et al., 2014b; Navarro-López et al., 2016), while *Amphidinium Carterae* belongs to a group named aethocates, as described by Lindemann (1928), which are fragile and easily disrupted, thus allowing the fatty acids to be extracted without further complication.

3.4.3. Amphidinols

With regard to APD extraction, Fig. 3A shows that the maximum extraction for T-APDs occurs at polarities between 6 and 7.5, corresponding to solubility parameter values from 22 to 31 [MPa $^{1/2}$], adjusted with temperature (Fig. 3B). Interestingly, APDs increase in a sigmoidal way at polarity values over 6, with the lowest APD content obtained using solvents that have polarities below 6, such as acetone or hexane. The maximum values, with no significant differences, corresponded to solvents with polarity indexes close to methanol, such as acetone:water, ethanol:water or methanol:water 80:20. From these,

Table 6

Antiproliferative activity of *A. carterae* biomass extracts with different solvents at polarities from 5.4 to 7.5 for the four human tumor cell lines, as mentioned in Abreu et al. 2019.

Solvent (v:v)	PI	HT-29	A549	MDA-MB-231	PSN-1
Acetone (100)	5.4	145.0	82.0	106.0	53.4
Acetone:water (99:1)	5.5	148.8	81.1	104.3	55.8
Acetone:water (97.5:2.5)	5.8	66.2	52.6	58.5	-21.3
Acetone:water (95:5)	6.0	87.4	25.8	58.5	-40.9
Acetone:water (92.5:7.5)	6.3	-77.1	-76.1	-68.3	-77.5
Methanol (100)	6.6	-86.0	-87.0	-94.0	-88.0
Ethanol:water (80:20)	6.9	-79.5	-82.2	-80.5	-80.1
Acetone:water (80:20)	7.2	-86.8	-82.2	-75.2	-86.1
Methanol:water (80:20)	7.5	-86.8	-75.1	-69.8	-85.2

target data were obtained which were fitted into a non-linear regression, adjusted to a 5-parameter Sigmoidal-type distribution, as presented in Table 5. The correlation with the polarity index, and the solubility parameter adjusted with temperature, was higher than 0.99. This is the first time that a correlation of APDs with the solvents' polarity and solubility parameters has been presented. Correlations between extraction percentages of bioactive ingredients and solvent polarity (using model solvents with a broad polarity range) have been previously described for plants (Kim et al., 2007). These correlations were demonstrated to be dependent on the compound type (Kim et al., 2007); thus, antioxidant compounds showed a similar correlation as those results for carotenoids and fatty acids presented here, but different to those of APDs.

In general, it can be appreciated that the representations using the δ_T (Fig. 3A) of the solvent system presented less results spread than those using the polarity index (see Fig. 3A). Although bioproducts solubility is commonly found to be associated with the polarity index, it is also well-known that the solubility in binary solvents mixed in a certain ratio may be greater than that of both pure solvents (Jin et al., 2017). This phenomenon, called cosolvency, is better interpreted using the δ_T parameter (Jin et al., 2017). The existence of cosolvency was a feasible explanation in several of the solvent mixtures used here (see Table 4). The δ_T -based Fig. 3B more clearly determined the maximum and threshold values.

3.5. Antiproliferative activity of the *A. Carterae* extracts

The antiproliferative properties of molecules in the amphidinol family have already been reported (Kobayashi and Kubota, 2010). To link the presence of APDs, observed in the extracts from the solvents shown in Fig. 3A, to a hypothetical antiproliferative effect, their antitumoral activity was measured against the four tumour cell lines. This was carried out on a selection of the extracts whose PI and δ_T values encompassed the abrupt change in APD recovery observed in Fig. 3A. Table 6 shows these results. The PI and δ_T thresholds, of around 6 and 20 MPa^{1/2}, respectively, can be appreciated, similar to those observed in Fig. 3A. When these thresholds are exceeded, the antitumoral bioactivity abruptly increased (< -75%). Extracts with δ_T values below 20 MPa^{1/2} (or 6 for PI) presented no bioactivity. This is consistent with the preliminary results previously reported for the same strain of *A. carterae*, where the existence of a linear correlation was reported between the APD concentration in methanolic:water (80:20) extracts, and its hemolytic activity in sheep blood erythrocytes (Abreu et al., 2019). These assays confirm the importance of using the APD metabolites produced by *Amphidinium*, a basis for their potential medium to large-scale supply in the biomedical industry.

4. Conclusions

Carotenoid extraction from *Amphidinium carterae* should be

performed using a low KOH concentration at 60 °C. An effective solvent for extracting metabolites such as carotenoids, fatty acids and amphidinols from *A. Carterae* was identified by varying the solvent species and composition. By correlating the extraction efficiencies with the solvent polarity and solubility parameter, the optimal solvent conditions could be predicted - a solubility parameter above 17, 17 and 22 [MPa^{1/2}] with polarities above 4, 4 and 6.5, respectively. Hence, the same solvent can be used to extract the carotenoids and fatty acids, but not the amphidinols. The biomass extracts exhibited potent antitumoral activities.

CRediT authorship contribution statement

M. López-Rodríguez: Methodology, Data curation, Writing - Original draft preparation. **M.C. Cerón-García:** Supervision, Writing - Reviewing and Editing, Project administration. **L. López-Rosasales:** Conceptualization, Methodology, Software. **E. Navarro-López:** Methodology, Data curation, Formal analysis. **A. Sánchez-Mirón:** Visualization, Writing - Reviewing. **A. Molina-Miras:** Methodology, Data curation, Formal analysis. **A.C. Abreu:** Methodology, Data curation, Formal analysis. **Ignacio Fernández:** Writing - Reviewing. **F. García-Camacho:** Supervision, Writing - Reviewing and Editing, Project administration.

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.

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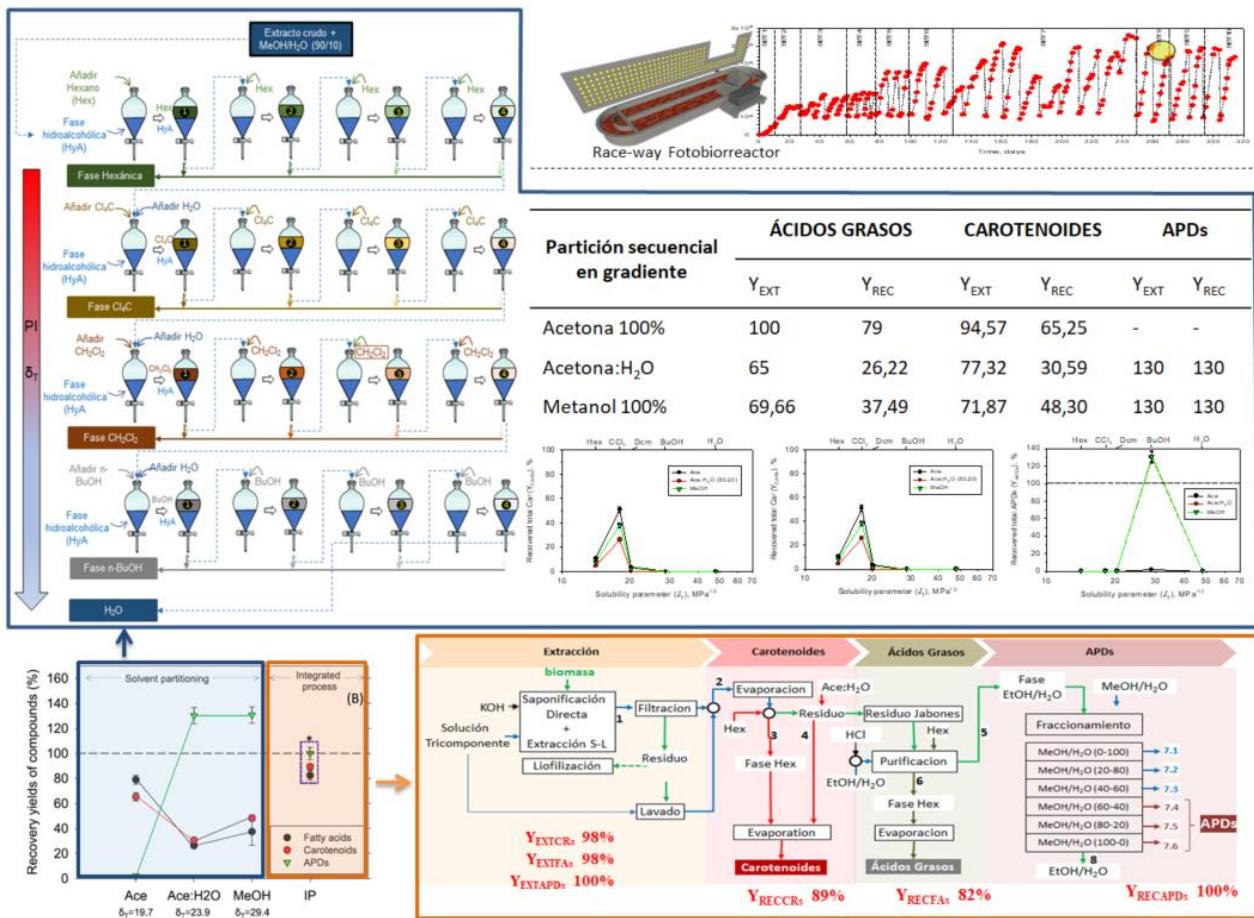
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CAPÍTULO 5

FRACTIONAMIENTO DE LA BIOMASA DE A. CARTERAE PARA LA OBTENCIÓN DE FRACCIONES ENRIQUECIDAS EN CAROTENOIDEOS, PUFAs Y APDs





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An integrated approach for the efficient separation of specialty compounds from biomass of the marine microalgae *Amphidinium carterae*

M. López-Rodríguez^a, M.C. Cerón-García^{a,c}, L. López-Rosales^{a,c}, E. Navarro-López^{a,c}, A. Sánchez Mirón^{a,c}, A. Molina-Miras^{a,c}, A.C. Abreu^{b,c}, Ignacio Fernández^{b,c}, F. García-Camacho^{a,c,*}

^a Department of Chemical Engineering, University of Almería, 04120, Almería, Spain

^b Department of Chemistry and Physics, University of Almería, 04120, Almería, Spain

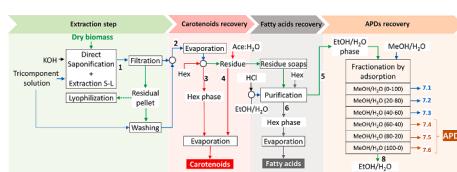
^c Research Centre CIAIMBITAL, University of Almería, 04120, Almería, Spain



HIGHLIGHTS

- First ever an integrated amphidinol-prioritized fractioning approach.
- The approach readily separates specialty metabolites of the alga *Amphidinium carterae*.
- Compared to a solvent partitioning method, the integrated approach was more efficient.
- NMR-based metabolomics is a valuable tool for screening solvent extraction methods.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Dinoflagellate
Solvent partitioning
Amphidinol
Fatty acids
Carotenoids

ABSTRACT

An amphidinol-prioritized fractioning approach was for the first time developed to isolate multiple specialty metabolites such as amphidinols, carotenoids and fatty acids using the biomass of the marine microalgae *Amphidinium carterae*. The biomass was produced in a raceway photobioreactor and the exhausted culture media were reused, thus fulfilling sustainability criteria employing a circular economy concept. The integrated bioactive compounds-targeted approach presented here consisted of four steps with which recovery percentages of carotenoids, fatty acids and amphidinols of 97%, 82% and 99 %, respectively, were achieved. The proposed process was proved to be a better extraction system for this microalga than another based on a sequential gradient partition with water and four water-immiscible organic solvents (hexane, carbon tetrachloride, dichloromethane and *n*-butanol). The proposed process could be scaled-up as a commercial solid-phase extraction technology well-established for industrial bioprocesses.

1. Introduction

The production of low-value bulk commodities from microalgae is not yet economically feasible (Sarkar et al., 2020; Vermüe et al., 2018). As a result, emphasis has to be given to valorising different biomass

fractions for the production of valuable specialty or niche products (e.g., carotenoids, phycobiliproteins or polyunsaturated fatty acids), which are often more expensive than more generic products (Sarkar et al., 2020; Vermüe et al., 2018). In this respect, the interest by compounds derived from marine dinoflagellate microalgae have experienced a

* Corresponding author at: Department of Chemical Engineering, University of Almería, Carretera Sacramento s/n. 04120, Almería, Spain.
E-mail address: fgarcia@ual.es (F. García-Camacho).

remarkable increase (Assunção et al., 2017; Chakdar et al., 2021; Cousseau et al., 2020; Karnaouri et al., 2020; Yew et al., 2019).

In the context of biorefining dinoflagellate microalgae, it has recently been shown that the complete recovery of distinct families of important compounds contained in the *A. carterae* biomass within a single process is complex (López-Rodríguez et al., 2019). In that study, different multi-step approaches, originally aimed at separately recovering each family of metabolites from non-dinoflagellate biomass, namely PUFAs, carotenoids, and amphidinols (APDs), were optimized to maximize the recovery of the three families of compounds. Although the results were particularly successful in applying a carotenoid-targeted approach (with recovery percentages of 97% for carotenoids and 80% for fatty acids), all the APDs were unfortunately recovered in a poorly enriched fraction. In contrast, the modified method of Place et al. (2005) accommodates an attractive step based on solid-phase extraction to concentrate the APDs. Consequently, it was hypothesized that integrating the two approaches into one would be a feasible way to improve the viability of the process.

For the purpose of comparison, we employed a solvent-partitioning process that is extensively used to isolate and purify natural marine products. Its usage is indicated in those crude extractions from marine organisms that produce complex matrices of compounds and salts; this is because it allows the successful fractionation of the crude extracts into different classes of natural products with minimal degradation, thus facilitating the subsequent separation stages. A wide variety of biologically active compounds (predominantly medium-to-high polarity compounds) have been isolated utilizing this strategy. Solvent partitioning mainly involves the use of multiple pairs of immiscible solvents in separatory funnels, where the compounds are distributed in each pair of solvents according to their different partition coefficients. In general, the solubility data for interesting compounds are not known so a prior selection of specific solvents is not normally applied. For this reason, the Kupchan solvent-partitioning method (and slight modifications thereof) is usually adopted as a reference method. It has been reported to be highly effective in natural product extracts (Houssen and Jaspars, 2012); however, its application to microalgae has been scarcely studied.

The present work has focused on implementing an APD-prioritized fractionation strategy to isolate important metabolite classes such as APDs, carotenoids and fatty acids, and thus advance the biorefinery concept applied to dinoflagellate microalgae that can provide niche compounds. The biomass used in this study was produced by reusing exhausted culture media based on sustainability criteria encompassing a circular economy concept (Molina-Miras et al., 2020).

2. Material and methods

2.1. The microalga

The marine microalgae used in this work was *Amphidinium carterae* Dn241EHU (Seoane et al., 2018). It is deposited in the microalgae culture collection of the Department of Plant Biology and Ecology at the University of the Basque Country. The inoculum was kept in f/2 medium, as explained elsewhere (Molina-Miras et al., 2018).

2.2. Production of microalgal biomass

The biomass utilised in this study was produced in a long-term (>270 days) culture of *A. carterae* grown in a paddlewheel-driven fiberglass raceway photobioreactor (PBR). The PBR design was previously reported elsewhere (Molina-Miras et al., 2018). Details regarding the operation mode and experimental approach have recently been published (Molina-Miras et al., 2020). Briefly, zenith lighting was distributed across the PBR surface using multicolour LED strips. The environmental conditions were as follows: (a) a sinusoidal diel variation pattern of irradiance with a maximum irradiance of 900 $\mu\text{E m}^{-2} \text{s}^{-1}$ at midday in a 24:0h L/D cycle, with a daily mean irradiance of 573 $\mu\text{E m}^{-2}$

s^{-1} ; (b) a culture medium composition of f/2 \times 3 (N:P = 5); (c) the pH and temperature maintained at 8.5 and 21 \pm 1 °C, respectively. The operation mode consisted of repeated semi-continuous cultures in which the exhausted supernatants, obtained by centrifuging each biomass harvesting stage, were completely recycled. The preparation of the reused medium has been described elsewhere (Molina-Miras et al., 2020). Harvesting was carried out once the cultures entered the stationary phase. The cell suspension samples harvested at day 272 were centrifuged at 1000 \times g (RINA model 100 U, 200 SM centrifuge). The obtained pellets were gently washed with distilled water. Lastly, the cells were repelleted, lyophilized and stored at -22 °C, ready for use in the different analytical procedures and extraction methods.

2.3. Solvent-partitioning process

Crude extracts from the lyophilized biomass were subjected to a modification of Kupchan's solvent-partitioning process based on a sequential gradient partition with solvents, as shown in Fig. 1 (Riguera, 1997). Compounds contained in the crude extract are distributed into fractions according to their polarity. The process provided five fractions, hexane (Hex), carbon tetrachloride (CCl₄), dichloromethane (DCM), *n*-butanol (BuOH) and water (H₂O) (see Fig. 1). As mentioned above, this partitioning process is thought to facilitate subsequent steps of chemical profiling and biological activity screening in natural marine extracts. The method was carried out in darkness and at a room temperature of 25 °C. The total crude extract was suspended in 80 mL of a 90:10 (v/v) MeOH/water mixture and 80 mL of hexane under a N₂ atmosphere. The mixture was vigorously stirred in a Pyrex glass bottle with a Teflon-coated magnetic stirring bar at 250 rpm for 30 min in a shaking water bath (SW22, JULABO GmbH, Seelbach, Germany), and left to decant overnight in a separating funnel placed in a refrigerated chamber at 5 °C. The hexane upper phase was recovered, and the MeOH/water layer was extracted three more times over three consecutive days with the same hexane volume in each step. Water was added to the remaining MeOH/water layer to reach an 80:20 (v/v) ratio and then 80 mL of CCl₄ were added. After 30 min of stirring, the mixture was left to decant for one night in a separating funnel and again the MeOH/water layer was extracted three more times over three consecutive days with the same CCl₄ volume each time. A similar strategy was applied to the remaining solvents, DCM and *n*-BuOH, using a 60:40 (v/v) MeOH/water ratio and 100% water, respectively. In total, four layers were obtained for each solvent partition. The layers were dried under a 20–100 mBar vacuum in a rotary evaporator (BUCHI Rotavapor™ R-210, Buchi Ibérica, Barcelona, Spain) and stored at -22 °C until further analysis. The recovery yields for the three families of compounds were determined by adding the amounts obtained in the four extractions performed for each partition (see Fig. 1).

The solvent-partitioning process described above was applied to three biomass crude extracts obtained with three different solvents: acetone (100%), methanol (100%) and acetone:water (80:20 v/v). This selection was based on a recent work in which we demonstrated that only solvents with polarity indexes (*PI*) and Hildebrand solubility parameters (δ_T) above ca. 6 and 20 MPa^{1/2}, respectively, allowed amphidinols to be extracted from *A. carterae* biomass (López-Rodríguez et al., 2020), along with different lipid classes, according to their polarity. Thus, methanol (100%; δ_T = 28.03 MPa^{1/2}; *PI* = 6.6) and acetone:water (80:20 v/v; δ_T = 22.63 MPa^{1/2}; *PI* = 7.22) met the above requirement, but not acetone (100%; δ_T = 19.7 MPa^{1/2}; *PI* = 5.4). Preparation of the extracts was carried out in darkness as follows: an 80 mL solvent volume was added to 2.5 g of lyophilized biomass placed in a Pyrex glass bottle. The samples were subjected to a temperature of 40 °C in a shaking water bath at 250 rpm for 60 min under an inert atmosphere with N₂. Then, the mixture was filtered (11 µm, Vidrafoc-Schott; Vidrafoc, Barcelona, España) to separate the spent pellet from the extracting solvent. The resulting spent pellet was re-extracted with 32 mL of fresh solvent (40% of initial volume). After removing the residue by filtration, this volume

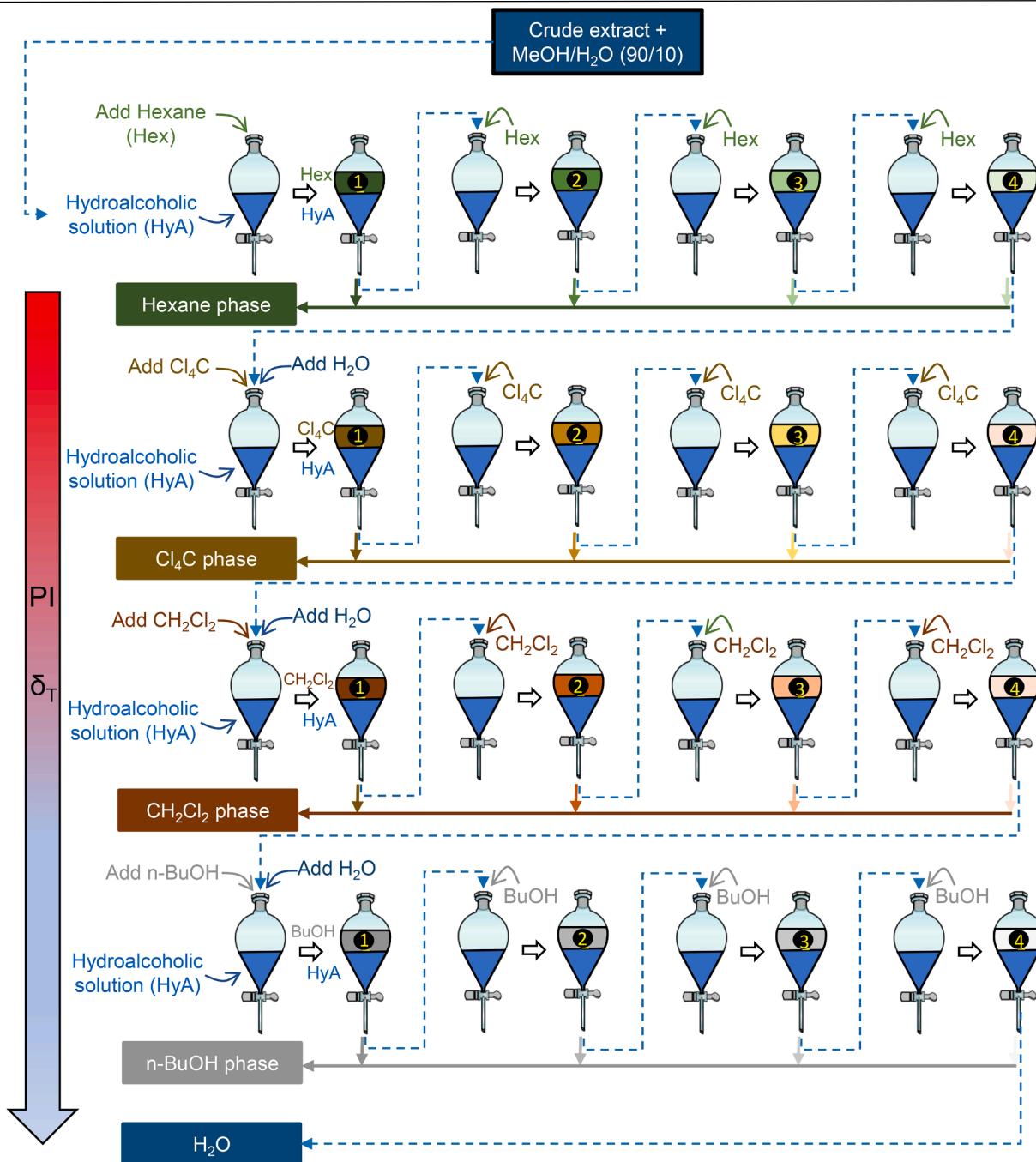


Fig. 1. Solvent-partitioning process of microalgal biomass crude extracts into five fractions (PI: polarity indexes; δ_T : Hildebrand solubility parameter).

was mixed with the solvent from the first extraction, dried under a 20–100 mBar vacuum in a rotary evaporator (BUCHI Rotavapor™ R-210, Buchi Ibérica, Barcelona, Spain) at a maximum temperature of 50 °C.

2.4. Integrating extraction approaches targeted at APDs, carotenoid and fatty acids

According to the results obtained in a recent study (López-Rodríguez et al., 2019), a combination of two processes, one targeted at extracting APDs and the other carotenoids, was hypothesized to provide enhanced extraction of the APDs, carotenoids and fatty acids contained in the biomass. Fig. 2 shows the experimental integration of the two processes, assembled in series. In brief, carotenoids are separated first from stream

3 (the hexane phase) and from stream 5 (the acetone:water mixture). Fatty acids are extracted in stream 6. The hydroalcoholic mixture (stream 5) leaves the carotenoid-targeted section to become the input stream for the APD-targeted section. Stream 5 was loaded into a C18 packed bed comprising four disposable C18 cartridges connected in series (Sep-Pak® C18 Plus Light, 130 mg of sorbent per cartridge, 55–105 µm particle size, Waters). This assembly provided a high adsorbent/sample ratio to ensure a high adsorption of organic metabolites from low to moderate polarity. The cartridges were connected to a vacuum collector (Supelco VISIPREP™ DL, 10–15 in-Hg). The conditioning step was carried out as recommended by the manufacturer. Subsequently, elutions were completed with different MeOH/H₂O mixtures ranging from 100% H₂O to 100% MeOH. In this study, the process in Fig. 2 was tested for the first time and 2.5 g samples of dry biomass, not subjected to cell

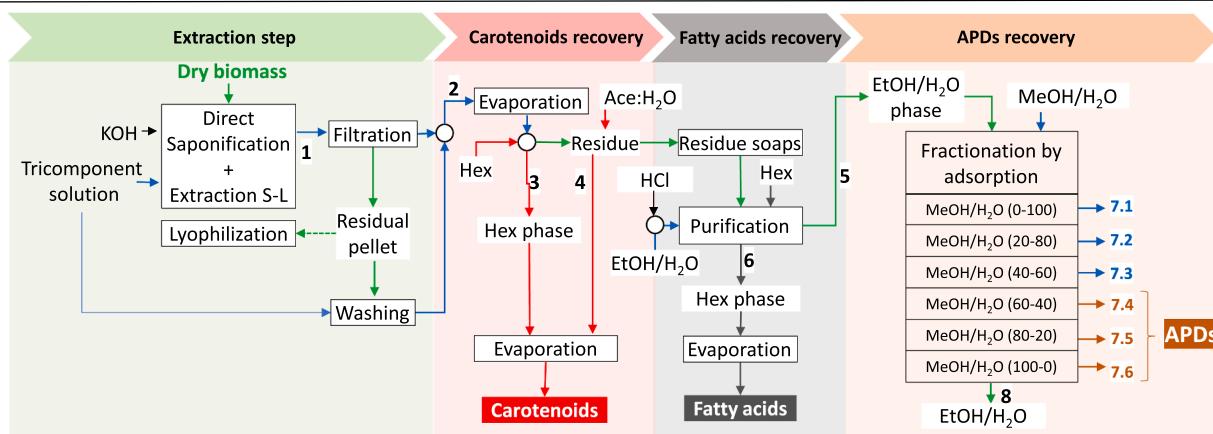


Fig. 2. Flowsheet of the proposed process for optimal extraction of fatty acids, carotenoids and APDs. For the sake of clarity, the different solvent recovery lines have been omitted. The tricomponent solution was composed of ethanol, hexane and water in a ratio of 76:18:6 v/v/v.

Adapted from López-Rodríguez et al., 2019

breakage, were used.

2.5. Analytical procedures

The following techniques were used both in the lyophilized biomass and in the resulting extracts. The carotenoid content and profile were determined using an HPLC photodiode array detector, as previously explained (Cerón-García et al., 2018). The quantified carotenoid pigments comprised diadinoxanthin, β-carotene, peridininol, diatoxanthin, dinoxanthin, pyrrhoxanthin, diadinoxanthin and peridinin. The total lipid content (TL) in the biomass was ascertained using the method described by (Kochert, 1978). Direct transesterification was used to determine the fatty acid methyl esters (FAMEs) content and profile using gas chromatography coupled to a flame ionization detector (FID) (Agilent Technologies 6890 N Series Gas Chromatograph, Santa Clara, CA, USA) as described earlier (Rodríguez-Ruiz et al., 1998). The quantified FAs comprised tetradecanoic acid (14:0), hexadecanoic acid (16:0), octadecanoic acid (C18:0), oleic acid (18:1n9), 9-eicosenoic acid (20:1n9), stearidonic acid (18:4n3), eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3). Thus, the amount of saponifiable lipids (SLs) were calculated from the FAMEs. The difference between the TLs (d.w. %) and SLs (d.w.) provided the percentage of unsaponifiable lipids in the biomass. The fractionation of the SLs into their neutral lipids (NLs) and polar lipids, specifically glycolipids (GLs) and phospholipids (PLs), was performed as described elsewhere (López-Rodríguez et al., 2020). The measurements were carried out in duplicate.

Localizing the bioactive fractions from the presence of APDs was guided by assays testing the hemolytic and antitumoral activity in the different extracts, as described elsewhere (Abreu et al., 2019; López-Rosales et al., 2015; Molina-Miras et al., 2018). Again, the measurements were carried out in duplicate.

2.6. NMR analysis

The *A. carterae* biomass contains compounds ranging from highly polar zwitterionic metabolites to entirely hydrophobic hydrocarbons, or from small molecules to higher-molecular-weight compounds such as lipids. Thus, the capability of the process described in Fig. 2 for isolating APDs, carotenoids and fatty acids, compared to that displayed in Fig. 1, was addressed using a recent untargeted and fast NMR-based metabolomics approach (Abreu et al., 2019). The high to mid-range polarity compounds were obtained by extracting 5 mg extracts with 600 µL CD₃OD/D₂O KH₂PO₄ buffer (80:20 v/v) at pH 6, containing the sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d₄ acid (TSP; 0.01% ww) and

the enzyme inhibitor sodium azide (NaN₃; 90 µM), with 20 min of sonication followed by 5 min of centrifugation (13500 rpm). The NMR-based metabolic profiles were recorded on a Bruker Avance III HD 600 spectrometer operating at a proton frequency of 600 MHz and using a 5 mm QCI quadruple resonance pulsed field gradient cryoprobe, as explained in detail elsewhere (Abreu et al., 2019). All spectra were acquired without rotation at 293 ± 0.1 K and using a NOESY presaturation pulse sequence (Bruker 1D noesygppr1d). The following metabolites were identified and quantified in the ¹H NMR spectra (Abreu et al., 2019): (i) amino acids, AA (valine, isoleucine, leucine, threonine, alanine, proline, methionine, glutamate, glutamine, glycine, lysine, aspartate, tryptophan, tyrosine, phenylalanine, histidine); (ii) organic acids, OA (lactate, acetate, succinate, fumarate, formate); (iii) sugars, SA (β-galactose, β-glucose, α-glucose, α-galactose); (iv) Quaternary ammonium compounds, QAC (choline, betaine); (v) Polyhydric alcohols, PA (glycerol); (vi) Nitrogenous bases, NB (uracil, cytosine); and (vii) APDs. The metabolite assignments in the ¹H NMR spectra are given in detail in Abreu et al. (2019). The relative peak integral of diagnostic and isolated signals of each molecule, with respect to the TSP signal integral of known concentration, were used to quantify the above-mentioned metabolites. The measurements were carried out in triplicate. Importantly, as the polar metabolites profile carried all the APDs, the focus was on the polar streams (i.e., 5 and 7 in Fig. 2; fractions n-BuOH and H₂O in Fig. 1) because these could potentially carry the APDs.

2.7. Recovery yields of the compounds

The recovery yields of the total carotenoids were calculated with a reference value (0.63 % d.w.), which was the maximum obtained using the modified version of the traditional analytical method reported earlier and cited in Section 2.3 (Cerón-García et al., 2018). To calculate the recovery yields of the total fatty acids, the reference value used (13.53 % d.w.) was that of the saponifiable fatty acid content in the biomass, measured as described earlier (Rodríguez-Ruiz et al., 1998) and cited in Section 2.3. Regarding the recovery yields of the total amphidinols, these were calculated using the reference value (0.69 % d.w.), which was obtained with methanol, following a previously published method (Place and Deeds, 2005).

2.8. Statistical analysis

Two replicates were prepared for each extraction. Each sample was analysed individually, and the data are reported as the mean ($n = 2$) ± standard deviation. The analysis of variance (ANOVA) was performed to detect any significant differences between the factors at $p < 0.05$. The

software used was Statgraphics Centurion XVI (StatPoint, Herndon, VA, USA).

3. Results and discussion

3.1. Evaluation of the solvent-partitioning process

The solvent-partitioning procedure was applied to three crude biomass extracts obtained with the following solvents, listed in order of the Hildebrand solubility parameter value (δ_T) at 25 °C: acetone (Ace), acetone:water (Ace:H₂O) 80:20 v/v, and methanol (MeOH). In each partition in Fig. 1, the hydroalcoholic phase was extracted four times with the corresponding fresh organic solvent. Table 1 displays the number of extractions needed to ensure the maximum extraction of metabolites for each of the four organic solvents and for the three crude extracts. Overall, the higher the fatty acid content extracted with a particular solvent, the greater the number of extractions needed. This observation was not repeated with the carotenoids. The low carotenoid content in the biomass compared to that of fatty acids (almost ten times lower) was probably the factor responsible for this discrepancy. The hemolytic and antitumoral activity assays performed on all the fractions revealed that only the *n*-BuOH fraction contained APDs. The NMR analysis confirmed the presence of APDs in this fraction.

Fig. 3 displays the yields of the three crude extracts, obtained as described at the end of the section 2.3, for the three target compound families (fatty acids, carotenoids and APDs) (Fig. 3A) and the corresponding recovery yield values after crude extracts were fractionated by the reference method represented in Fig. 1 based on the solvent partitioning (left side in Fig. 3B), and those coming from the process represented in Fig. 2 based on integrating extraction approaches targeting APDs, carotenoid and fatty acids (left side in Fig. 3B). In general terms, the yields of crude extracts in Fig. 3A can be divided into two groups. One consists of acetone and the other of the other two solvents. Acetone extracted a quantity of fatty acids and carotenoids, practically 100% of both, considerably higher than the other two solvents ($p < 0.05$). In contrast, acetone did not extract any amphidinols, while the other solvents did, with similar percentages ($p > 0.05$) well above the 100% corresponding to the analytical reference value. After fractionating the crude extracts (left side in Fig. 3B), this pattern of variation continued to be appreciated, although with significantly decreased recovery yields compared to those of the crude extracts (Fig. 3A). This effect of the solvents is discussed below, based on the Hildebrand solubility parameter (δ_T). The reason for this has been reported in a previous work (López-Rodríguez et al., 2020), the results of which were better interpreted in terms of δ_T than PI due to the fact that bioproduct solubility is not only associated with PI, but also with phenomena such as cosolvency, dispersive interactions, polar interactions, and hydrogen bonding. Thus, as can be seen in Fig. 3B, the three solvents were able to extract significant amounts of FAs and carotenoids; this is expected for solvents with δ_T values in the 14.5 to 31.3 [MPa^{1/2}] range, as previously reported (López-Rodríguez et al., 2020). Acetone provided the maximum extraction percentages ($p < 0.05$), which again is expected based on its δ_T value in the 16.5 to 19.5 [MPa^{1/2}] range, considered to be optimum (López-Rodríguez et al., 2020). While the APD extraction was

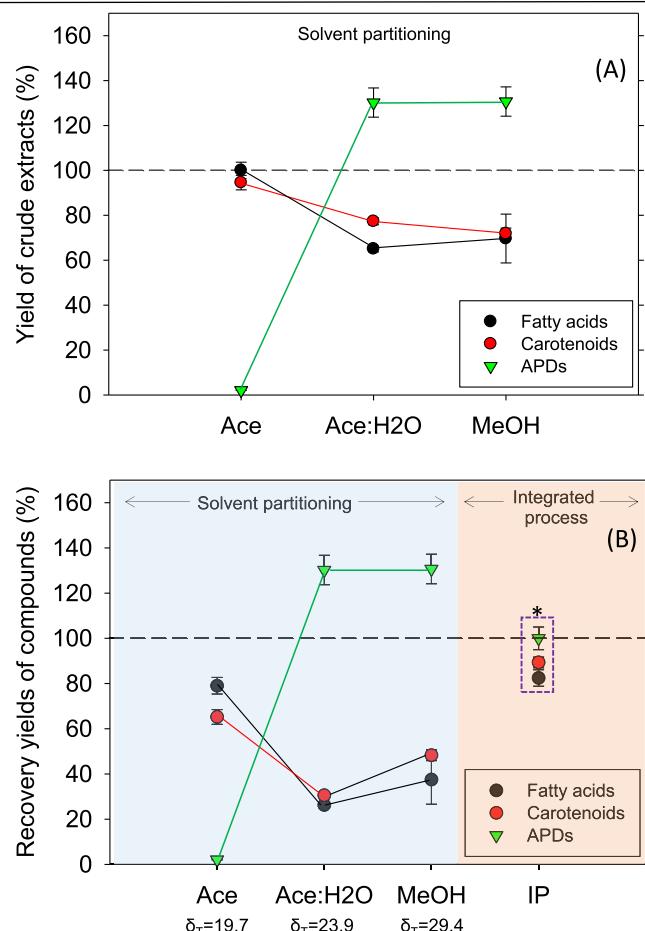


Fig. 3. (A) Influence of the solvent type used in the preparation of crude extracts on the extraction yields of the three compound families (fatty acids, carotenoids and amphidinols (APDs)). (B) Effect of the extraction system for the three compound families on the recovery yields of each of them. (Left side) Solvent-partitioning method applied to three crude biomass extracts obtained with acetone (Ace), acetone:water (Ace:H₂O) 80:20 v/v, and methanol (MeOH). δ_T represents the solubility parameter expressed in MPa^{1/2}. (Right side) Process based on integrating the extraction approaches targeting APDs, carotenoid and fatty acids. The asterisk indicates the goodness of this method. Values are the mean \pm standard deviation.

only feasible using the solvents Ace:H₂O and MeOH, both exceeded the δ_T threshold value (around 20 MPa^{1/2}), above which APDs can be extracted (López-Rodríguez et al., 2020). The recovery percentages of fatty acids and carotenoids in the three crude biomass extracts were below 100% compared to the analytical reference method (Fig. 3B). However, Ace:H₂O and MeOH recovered over 30% more than that measured in the biomass compared to the analytical reference method. Therefore, it is clear that the solvent-partitioning process is not a reference extraction system and is not sufficiently effective for fatty acids and carotenoids; nonetheless, it performed well for APDs,

Table 1

Number of extractions needed in the solvent partitioning process to ensure a maximum extraction of metabolites for each of four organic solvents at each of the three crude extracts used. Ace, acetone; Ace:w, acetone:water (80:20); MeOH, methanol; Hexane (hex); carbon tetrachloride (Ctc); dichloromethane; butyl alcohol (BuOH); water (w).

Solvent	Fatty acids			Carotenoids			APDs		
	Ace	Ace:w	MeOH	Ace	Ace:w	MeOH	Ace	Ace:w	MeOH
Hex	3	3	3	1	1	4	nd	nd	nd
Ctc	2	2	2	1	1	4	nd	nd	nd
Dcm	1	1	1	nd	nd	4	nd	nd	nd
BuOH	4	nd	nd	nd	nd	nd	nd	2	2

depending on the solvent used to produce the crude extract. The absence of APDs in the crude acetone extract showed how dramatic the effect can be (of the solvent selected to generate the crude extract) on the recovery yields of the metabolites of interest.

Solvent-partitioning, which consists of simplifying the extract composition into several groups of metabolites sharing similar physicochemical properties, has been roughly used to identify bioactive sterol-rich fractions from non-dinoflagellate microalgae in a few studies (Samarakoon et al., 2013; Sanjeewa et al., 2016). In the study presented herein, a solvent-partitioning procedure was for the first time successfully applied in a systematic way to crude extracts from marine dinoflagellate biomass, as can be seen in Fig. 4. Consequently, when APDs are the main target, separating them by solvent partitioning could be performed in a clear-cut way in the *n*-BuOH fraction (see Fig. 4C) ($p < 0.05$). The desalting capability is inherent in the method as the salts accumulated in the aqueous one.

As mentioned in previous sections, the target lipids were those saponifiable quantified in the form of FAMEs. Saponifiable lipids (SL) accounted for $13.53 \pm 0.45\%$ of the dry biomass. The polar fraction was dominant ($47.33 \pm 2.37\%$ of glycolipids and $7.63 \pm 0.38\%$ of phospholipids), with $45.06 \pm 2.25\%$ of non-polar lipids. Therefore, the SL profile in crude extracts is tuned with the polarity of solvents as reported elsewhere (López-Rodríguez et al., 2020). In this sense, acetone, the less polar of the three polar solvents used to produce crude extracts, extracted the highest amounts of fatty acids. Overall, most of the fatty acids migrated to the less polar fraction (*n*-hexane in Fig. 4B) rather than to the remaining fractions of increasing polarity ($p < 0.05$). This suggested a significant defatting capability of *n*-hexane. A small proportion of fatty acids (mainly SDA and EPA) was extracted by the next more polar solvent (CCl_4) compared to the *n*-hexane fraction ($p < 0.05$). Among FAs profile contained in the biomass, only a tiny amount of SFAs were found in DCM.

Conversely, most of the carotenoids were better extracted by CCl_4 ($p < 0.05$) (see Fig. 4A). This result is in line with a previous study that reported the poor capability of hexane to solubilize carotenoids from *A. carterae* biomass, the optimal extraction being achieved using solvents with δ_T values ranging from 16.5 to 19.5 [$\text{MPa}^{1/2}$] (López-Rodríguez et al., 2020). However, it is important to mention that some families of metabolites, i.e., fatty acids or carotenoids, are found in successive fractions of the solvent-partitioning process (see below). As a result, tiny amounts of fatty acids and carotenoids were also identified in the solvents with the higher δ_T values (DCM, *n*-BuOH and H_2O).

3.2. Evaluation of the integrated bioactive compounds-targeted approach

Any microalgal strain is a versatile cellular factory for producing value metabolites (Koller et al., 2014). Co-extraction of multiple bioactive compounds commonly found in marine non-dinoflagellate microalgae has been comprehensively reviewed recently (Ma et al., 2020). Co-extraction techniques, taking advantage of similar physicochemical properties of the extracted compounds (e.g. polarity, solubility or molecular weight), are part of microalgae-based integrated bio-refinery processes (Gilbert-López et al., 2015; Zhang et al., 2018). However, the case of the dinoflagellates is peculiar within microalgae considering that they can produce unique marker metabolites that are not found in other microalgae groups (Assunção et al., 2017), with physicochemical properties such that specie-specific thorough studies are required for their recovery. *Amphidinium carterae*, species producer of APDs, is an example of that as shown below.

As hypothesized in a previous work (López-Rodríguez et al., 2019), Fig. 2 summarizes the integrated bioactive compounds-targeted approach presented here and which has proven effective for *A. carterae*. Thus, an APD recovery yield close to 100% was feasible in combination with high recovery percentages of the carotenoids and fatty acids present in the biomass, of nearly 100% and 80%, respectively (see Fig. 3B). Stream 2 in Fig. 2 carried almost $98 \pm 5\%$ of the carotenoid

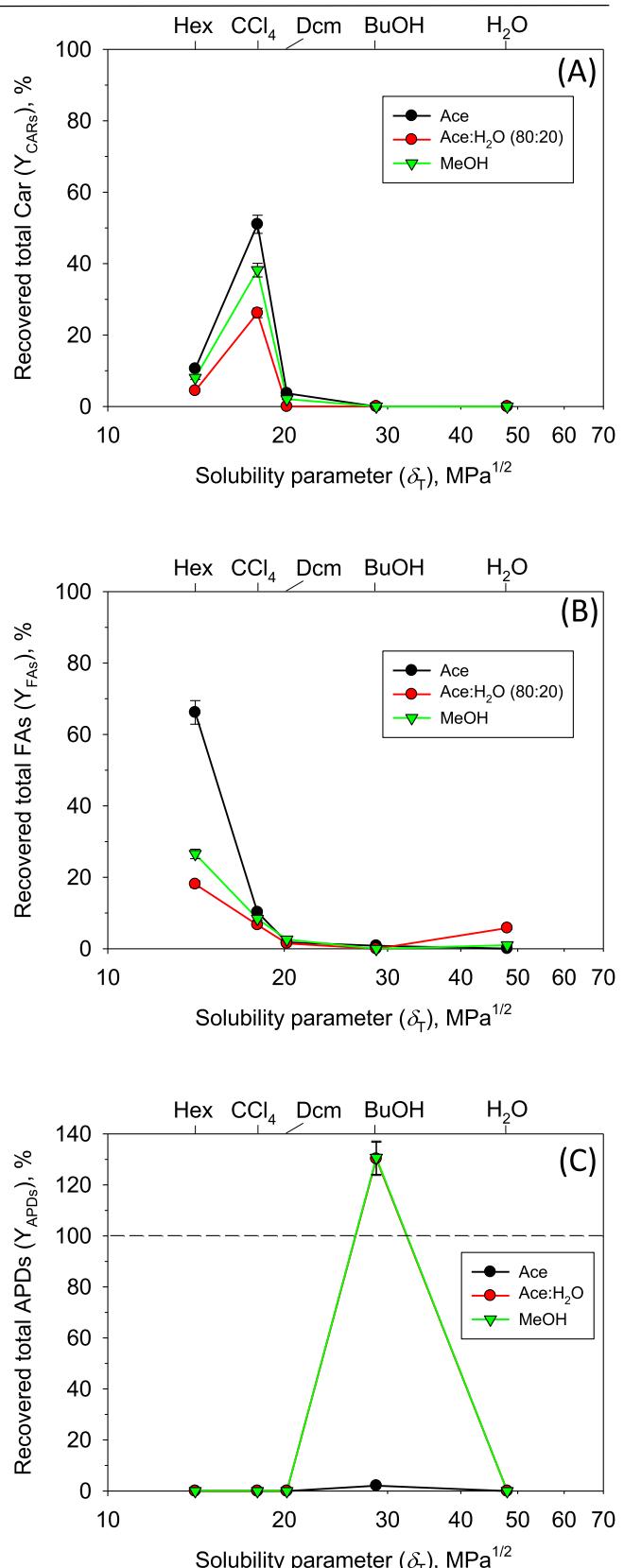


Fig. 4. Distribution of the three metabolite groups throughout the five solvents used in the fractionation of the crude extract. Values are the mean \pm standard deviation.

fraction, $98 \pm 3\%$ of the fatty acids and $100 \pm 5\%$ of the APDs; the rest of both were found in the residual pellets. The practically complete extraction of carotenoids in stream 2 is indicative of the absence of degradation of them in the saponification step, because of using an optimal KOH-to-biomass ratio (w/w) of 0.4 according to López-Rodríguez et al. (2019). Carotenoids were recovered from the treatments of streams 3 ($27.5 \pm 1.4\%$) and 4 ($69.5 \pm 3.5\%$), as shown in Fig. 2. A tiny fraction of fatty acids ($1.8 \pm 0.1\%$) was swept along with stream 3 and another $1.0 \pm 0.1\%$ with stream 4. Stream 6 allowed us to recover the richest fatty acid fraction ($82.2 \pm 3.6\%$), while it contained an insignificant amount of carotenoids (<1%).

Once again, a bio-guided search for hemolytic activity revealed that only stream 7 was active, with increased performance compared to the crude methanolic extract of the biomass. APDs were recovered in stream 7 with a recovery percentage of $99 \pm 5\%$. More specifically, the sub-fractions 7.4 to 7.6 were the most active, with 19.0, 47.9 and 33.1 % of total APDs, respectively. In the present study, fractions were obtained that were more highly enriched in APDs than those obtained in a previous work (López-Rodríguez et al., 2019).

3.3. Metabolomic approach to analyse the solvent extraction systems

Microalgal cultures are an interesting resource for evaluating how a given biosystem responds to changes in abiotic factors (Palwal et al., 2017). It has been demonstrated that untargeted metabolomics is a valuable approach for increasing our understanding of the metabolic changes appearing in such biological systems (Ding et al., 2019; Hoyos et al., 2021), and it has proven particularly useful in evaluating solvent extraction systems applied to plants (Martin et al., 2014), although it has not yet been applied to microalgae. Consequently, as indicated in Section 2.6, an NMR-based metabolomic approach, previously reported for *A. carterae*, was used to assess the processes described in Fig. 1 and Fig. 2. Here, we focus on the NMR metabolomic profiles of the moderately-polar and polar streams containing APDs. As a result, Fig. 5 displays the percentage distribution of the predominantly polar metabolite classes already identified (AA, OA, PA, SA, QAC, NB, and APDs) in both the APD-carotenoid-targeted and solvent-partitioning processes. In the latter, the data correspond to two crude extracts containing APDs: i) MeOH and ii) Ace(80):H₂O(20). The acetone crude extract is not relevant because it did not retrieve any APDs.

For the APD-carotenoid-targeted process (Fig. 5A), the percentages are relative to the compound contents in stream 5, expressed in dry biomass weight (d.w.). In this sense, stream 5 carried 0.49% d.w. of AA, 0.42% d.w. of PA, 0.68% d.w. of APDs, and <0.1% d.w. of OA and QAC; SA and NB were undetected indicating that they were retrieved in previous steps. Fig. 5A shows that the first elution (stream 7.1, MeOH (0%)/H₂O (100%)) swept along >93% of PA, QAC and AA; tiny amounts of these were detected in each of the remaining streams. Organic acids (OA) were eluted in all the streams in significant proportions, without a

clear distribution pattern being appreciable. This specific recovery profile, i.e., 23.5, 31.1, 10.5, 12.2, 7.1, and finally 15.2%, is due to the fact that the MeOH/H₂O mixture is not an optimized solvent system, and that the most efficient one is obtained in stream 7.2 (31.1%). Interestingly, increasing or reducing the water in the above-mentioned ratio does not lead to any improvement. The different pKa of the various organic acids involved, together with their distinct equilibria within the different proportions of water present, could explain why the recovery is maximized in one particular stream but is less efficient in the others.

Based on the data given in Fig. 5A, fractions 7.4 to 7.6 recovered most of the APDs while a small amount of APDs was retrieved from stream 7.1. These results are in agreement with a previous study using the same microalga (López-Rodríguez et al., 2019). A similar distribution pattern was reported for the recovery of karlotoxins from cultures of *Karlodinium*, compounds structurally similar to APDs (López-Rosales et al., 2018). This may be caused by differences in the hydrophobicity of the structural APD analogues, which *A. carterae* DN241EHU is able to synthesize (Wellkamp et al., 2020), due to differences in the functional groups of the molecule (e.g., sulphated APDs). The relatively less hydrophobic APDs would desorb with pure H₂O. Nonetheless, as Fig. 5A shows, the fractions 7.4 to 7.6 are quite enriched in APDs, which facilitates any subsequent purification step if required for a given application. Regarding the hydroalcoholic stream 8, obtained after passing stream 5 through the C18 solid phase (see Fig. 2), no metabolites were detected. The C18 solid phase can retain not only non-polar and moderately polar compounds but also highly polar compounds from a large variety of samples (Andrade-Eiroa et al., 2016). The retention of polar compounds on this non-polar surface (i.e., C18) was further favoured because the amount of organic matter in the mobile phase was small compared to the amount of sorbent (four C18 130 mg cartridges placed in series), and the flow rate through the cartridge below that was 1 mL per minute, as recommended by the manufacturer.

The results from the APD-carotenoid-targeted process contrast with those obtained in the solvent-partitioning procedure (Fig. 5B, C). Even though it could be an advantage for all the APDs to be recovered in one of the fractions (*n*-BuOH), it also swept along significant amounts of AA, OA, PA, SA, QAC and NB.

APDs are polyketide-like secondary metabolites. In principle, the APD-carotenoid-targeted process developed in this work can be extended to prioritized production of other valuable polyketides from other marine dinoflagellate microalgae, such as *Karlodinium veneficum* (López-Rosales et al., 2015). A raceway photobioreactor can be effectively used to grow massive amounts of microalgae such as *A. carterae*. The feasibility of cultivating *A. carterae* in a larger scale has been reported elsewhere for both open pond photobioreactors (Molina-Miras et al., 2018; Molina-Miras et al., 2020) and closed pneumatically agitated bubble column photobioreactors (Fuentes-Grünwald et al., 2016). The pros and cons of both culture systems, widely documented in the literature for other microalgae, are essentially applicable to



Fig. 5. Percentage distribution of the predominant groups of polar metabolome components in the adsorption step of the APD-carotenoid process outlined in Fig. 2 (A) and in the solvent-partitioning process (B, C) outlined in Fig. 1. Percentages are relative to the content in input stream 5 (A) and the initial crude extracts (B, C). AA: amino acids; OA: organic acids; SA: sugars; QAC, quaternary ammonium compounds; PA: polyhydric alcohols; NB, nitrogenous bases.

Capítulo 5. Fraccionamiento de la biomasa de *A. carterae* para la obtención de fracciones enriquecidas en carotenoides, PUFA's y APDs

A. carterae. Special attention should be paid to the low tolerance to shear stress of this alga (López-Rosales et al., 2019). The downstream extraction-purification steps of the APDs process allow one to obtain other different families of important compounds such as fatty acids and carotenoids. The process can be scaled-up to a multi-kilogram level as commercial solid-phase extraction technology is well-established in industrial bioprocesses. Given that the materials and solvents used in this process are readily available and relatively inexpensive, it could be technically and economically viable.

4. Conclusions

The feasibility of using a simple solvent-partitioning method to isolate the amphidinols produced by *A. carterae* in a clear-cut way in the *n*-BuOH fraction has been demonstrated. While defatting and desalting was shown to be effective using this approach, the overlapping of metabolites other than amphidinols was inevitably observed. Alternatively, and in the context of biorefining dinoflagellate microalgae, an integrated bioactive compound-targeted approach has proven suitable for readily separating amphidinols, carotenoids and fatty acids. In addition, the NMR-based metabolomics approach was found to be a valuable tool for screening the solvent extraction methods used.

CRediT authorship contribution statement

M. López-Rodríguez: Investigation, Conceptualization, Methodology, Writing - original draft, Data curation. **M.C. Cerón-García:** Conceptualization, Methodology, Data curation, Supervision, Writing - original draft, Project administration, Funding acquisition. **L. López-Rosales:** Investigation, Data curation. **E. Navarro-López:** Investigation, Data curation. **A. Sánchez Mirón:** Investigation, Writing - original draft. **A. Molina-Miras:** Investigation, Data curation. **A.C. Abreu:** Investigation, Methodology, Data curation. **Ignacio Fernández:** Investigation, Methodology, Writing - original draft. **F. García-Camacho:** Conceptualization, Methodology, Formal analysis, Visualization, Supervision, Writing - review & editing, Project administration, Funding acquisition.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biortech.2021.125922>.

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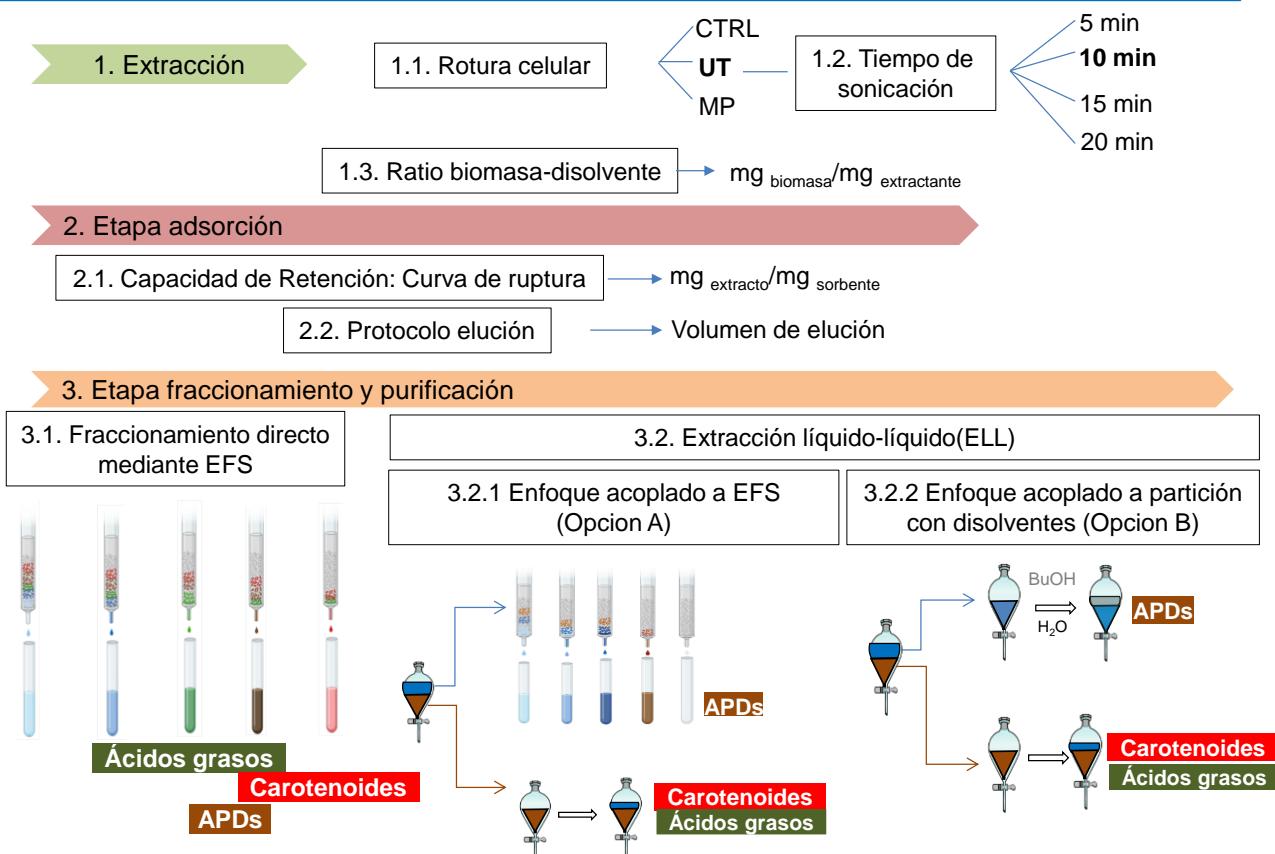
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CAPÍTULO 6

MEJORA EN EL FRACCIONAMIENTO PARA LA OBTENCIÓN DE EXTRACTOS ENRIQUECIDOS EN APDs

Purificación de compuestos de alto valor de *Amphidinium carterae*





Article

The Isolation of Specialty Compounds from *Amphidinium carterae* Biomass by Two-Step Solid-Phase and Liquid-Liquid Extraction

Mercedes López-Rodríguez ¹, Lorenzo López-Rosales ^{1,2}, Giullia Diletti ¹, María del Carmen Cerón-García ^{1,2,*}, Elvira Navarro-López ^{1,2}, Juan José Gallardo-Rodríguez ^{1,2}, Ana Isabel Tristán ^{2,3}, Ana Cristina Abreu ^{2,3} and Francisco García-Camacho ^{1,2}

¹ Department of Chemical Engineering, University of Almería, 04120 Almería, Spain

² Research Centre CIAIMBITAL, University of Almería, 04120 Almería, Spain

³ Department of Chemistry and Physics, University of Almería, 04120 Almería, Spain

* Correspondence: mcceron@ual.es

Abstract: The two main methods for partitioning crude methanolic extract from *Amphidinium carterae* biomass were compared. The objective was to obtain three enriched fractions containing amphidinols (APDs), carotenoids, and fatty acids. Since the most valuable bioproducts are APDs, their recovery was the principal goal. The first method consisted of a solid-phase extraction (SPE) in reverse phase that, for the first time, was optimized to fractionate organic methanolic extracts from *Amphidinium carterae* biomass using reverse-phase C18 as the adsorbent. The second method consisted of a two-step liquid-liquid extraction coupled with SPE and, alternatively, with solvent partitioning. The SPE method allowed the recovery of the biologically-active fraction (containing the APDs) by eluting with methanol (MeOH): water (H₂O) (80:20 v/v). Alternatively, an APD purification strategy using solvent partitioning proved to be a better approach for providing APDs in a clear-cut way. When using n-butanol, APDs were obtained at a 70% concentration (w/w), whereas for the SPE method, the most concentrated fraction was only 18% (w/w). For the other fractions (carotenoids and fatty acids), a two-step liquid-liquid extraction (LLE) method coupled with the solvent partitioning method presented the best results.

Keywords: dinoflagellate; solid-phase extraction; liquid-liquid extraction; amphidinol; fatty acids; carotenoids

Key Contribution: SPE was optimized for the first time in *A. carterae* biomass. The LLE method coupled with solvent-partitioning using n-butanol proved successful for APD purification.



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1. Introduction

Marine microalgae are a promising feedstock replete with fascinating bioactives. However, industrial-scale production of high-value bulk commodities is still a long way off, unless both bulk and specialty co-products can be obtained from the cultivated microalgae; only in this way can the production of bulk commodities become economically viable [1]. Species from classes, dinoflagellate and raphidophyceae, are known to produce metabolites with interesting bioactivities. For example, *Amphidinium carterae* is a dinoflagellate microalgae producer of amphidinols (APDs). In the context of biorefining dinoflagellate microalgae, the APD targeted approach, based on recovering the bioactive compounds from the *Karlodinium* cultures described [2], was used to extract APDs and assess the recovery of the three families of compounds from *A. carterae*, namely polyunsaturated fatty acids (PUFAs), carotenoids, and APDs [3]. The overall APD recovery obtained in that study was almost 70%, the main enriched fractions being MeOH-H₂O (80:20 v/v and 60:20 v/v) with 33% and 13% of the APDs present in the crude extract, respectively. Although the

results of that study were initially successful, around 11% of the APDs were not adsorbed on the column due to the quantity of biomass being too high for the sorption capacity of the cartridges used. In contrast, an integrated bioactive compound-targeted approach has proven suitable for readily separating APDs, carotenoids, and fatty acids contained in *A. carterae* biomass [4]. In that study, an APD–carotenoid-targeted process involving a fractionation step using solid-phase extraction (SPE) provided two distinct fractions that were quite APD-enriched, with no metabolites detected after passing the extract through the reverse-phase C18 solid phase. Although high APD recoveries were obtained, the process did not achieve clear-cut APD purification in which the fractions had no interferences from other metabolites. Indeed, the nuclear magnetic resonance (NMR) analysis showed tiny amounts of compounds, namely organic acids, which were eluted in these fractions. In addition, a small percentage (almost 8%) of the APDs was detected in the 100% water elution fraction.

SPE is a general technique for separating, concentrating, and purifying crude extracts from complex matrices. It is widely applied as a fractionation step for multiple purposes, including purification, trace enrichment, and class fractionation, among others [5,6]. This technique involves three main steps: sample preparation, column equilibration and retention, and the elution gradient. The sample volume is one of the primary factors that determines the retention capacity of the analyte sorbent (milligrams of analyte per gram of sorbent) [6,7]. A useful parameter for characterizing an SPE device is the breakthrough volume (V_B), which is established from the breakthrough curve, i.e., the point on the curve where some arbitrary amount of sample is detected at the outlet of the sampling device during the retention step [8]. In brief, the concentration of the solute in the effluent begins to increase at a starting point that is denoted as the breakthrough volume (V_B). The corresponding concentration for measuring V_B is usually taken as the minimal percentage of the initial analyte concentration (C_0). As the solution continues to pass through the sorbent, the value of the effluent concentration of the analyte (C) tends to its maximum, reaching the point of inflection that is denoted as the retention volume (V_R). Therefore, V_R is defined as the maximum loading capacity of the column [6,8]. The extracts from *A. carterae* biomass comprise a complex matrix containing a large amount of potentially interfering compounds that are generally present at higher concentrations than other relatively minor compounds, such as APDs.

Liquid-liquid extraction (LLE) is widely used to separate target compounds; it is based on the relative solubilities of these compounds in two different immiscible liquids, typically water and organic solvents [9]. As a prior isolation step, the use of a suitable organic solvent (determined by the analyte's partition coefficient) can be an effective tool for subsequent SPE purification procedures [10,11]. In addition, LLE has been used as a method for sample pre-concentration and purification to detect marine toxins, given their expected concentration and the complexity of the samples [9,12].

The present work focuses on optimizing an SPE-based method using C18 as the reverse-phase adsorbent to select and effectively fractionate extracts from *A. carterae*; this is done to obtain a rough separation of the three families of bioactive compounds. Since APDs are the main target, the process uses methanol–water mixtures as the eluent in an increasing order of polarity, as well as combining solid-liquid extraction as a pre-treatment followed by solid-phase extraction. The first step aims to optimize the biomass cell disruption and then to optimize the biomass-to-extractant ratio to obtain the maximum amount of APDs. The following determination of the breakthrough volume provides the adsorbent-to-biomass extract ratio, which ensures the adsorbent's maximum adsorption capacity of the organic metabolites. The process was initially scaled to a 10-g C18 cartridge and then scaled up to an 80-g C18 column using two approaches: (i) direct fractionation of the crude extract and (ii) LLE based on organic solvent extraction and subsequent two-way purification—by SPE and by solvent partitioning with n-butanol (BuOH)—as an APD-prioritized fractionation strategy to develop a biorefinery procedure for the integral

valorization of this dinoflagellate. The flowsheet of the processes undertaken is shown in the graphical abstract.

2. Results and Discussion

2.1. Evaluation of Biomass Pre-Treatments and Extraction Optimization

The optimization of the extraction procedure to determine the APDs was carried out by considering the cell disruption method, the pre-treatment duration, and the crude extract concentration (see Graphical Abstract and Sections 4.2.2 and 4.2.3 for more details). Figure 1A compares the effectiveness of the two cell breakage methods tested in terms of the hemolytic activity related to the content of APDs [13]. The relative hemolytic activity, expressed as the equivalent saponin potency of the extract obtained after the pre-treatment ($ESP_{\text{pretreatment}}$) relative to control ESP_{ctrl} , was observed with all three cell disruption methods: ultrasound (UT), the absence of a pre-treatment (CTRL), and milling (MP) (Figure 1A). UT was the most efficient disruption method, followed by the control (CTRL) and MP (in decreasing order). UT has been widely used for rupturing microalgae cells, particularly to improve the extraction of these types of secondary metabolites from dinoflagellates, the structures of which seem to play a role in binding to the lipid bilayer membrane [14]. Combining UT with methanol as the solvent has also proven to be a very efficient extraction method in various biological systems [5,15]. An earlier study using *A. carterae* biomass compared the effectiveness of different cell breakage methods in terms of their carotenoid and fatty acid recovery [16]. In that study, although UT was the second most suitable method for recovering carotenoids, it recovered relatively low amounts of fatty acids. In our study, UT significantly increased the hemolytic activity compared to the CTRL, indicating that cell disruption methods are necessary for APD recovery. The ESP value remained constant for sonication times from 15 to 30 min (p -value < 0.05) (Figure 1B), indicating that maximum APD extraction was achieved after 15 min of treatment. Conversely, the ESP value decreased for sonication times under 45 min. Therefore, 15 min was used for the rest of the study.

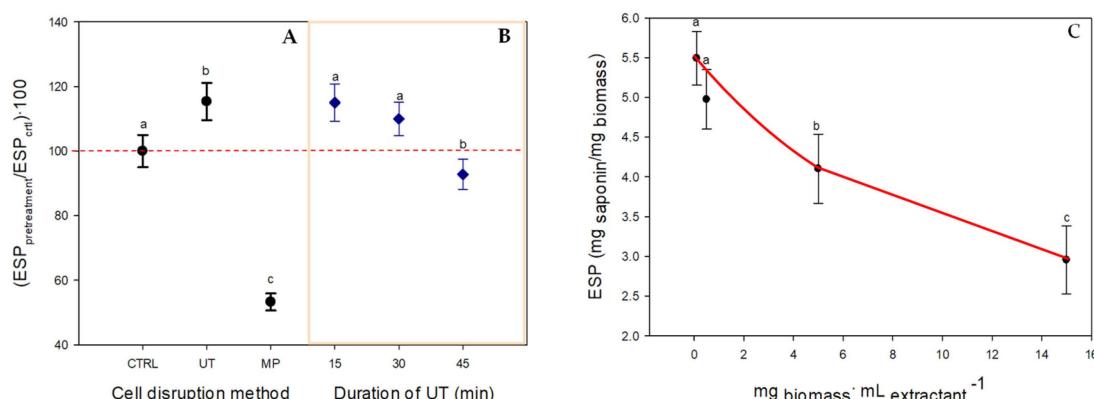


Figure 1. (A) Effect of different cell disruption methods on the extraction of APDs from *A. carterae* biomass, expressed in terms of hemolytic activity relative to the control. CTRL: control; UT: ultrasound; MP: mortar and pestle without alumina. (B) Effect of the ultrasound time (min) on the extraction of APDs. (C) Influence of the biomass-to-extractant ratio on hemolytic activity (ESP : equivalent saponin potency) of methanolic extracts produced from *A. carterae* biomass and treated with UT for 15 min. Data points are averages and vertical bars are the standard deviations for triplicate samples. The lowercase letters represent significant differences, with a p -value < 0.05.

The extraction of microalgal bioproducts was chiefly conducted using dried biomass treated with organic or aqueous solvents, depending on the polarity of the target compound [17]. With regard to the APDs, their maximum extraction takes place at polarity indexes and solubility parameter values that are close to methanol (above 6 and 20 MPa^{1/2}, respectively) [16]. In addition, sample preparation requires the optimal quantity of biomass

to be established in order to achieve a preliminary characterization of these minority metabolites compared to the carotenoids and fatty acids. As can be seen in Figure 1C, a greater hemolytic power ($\text{ESP} = 5.49 \text{ mg saponin} \cdot \text{mg biomass}^{-1}$) was reached at the lowest biomass-to-extractant ratios of 0.1 and $0.5 \text{ mg} \cdot \text{mL}^{-1}$ (equivalent to $0.06 \text{ mg extract} \cdot \text{mL}^{-1}$ and $0.3 \text{ mg extract} \cdot \text{mL}^{-1}$ in the hemolytic activity assay, respectively). Above $0.5 \text{ mg} \cdot \text{mL}^{-1}$, the ESP decreased as the biomass-to-extractant ratio increased, with the lowest ESP value ($2.96 \text{ mg saponin} \cdot \text{mg biomass}^{-1}$) being reached at a ratio of $15 \text{ mg} \cdot \text{mL}^{-1}$. In this respect, as explained elsewhere [18], high biomass-to-extractant ratios imply high analyte concentrations in the extract, which might compromise the selectivity and cause a drop in sensitivity for the analyte of interest in the sample matrix [18].

2.2. Evaluation of Breakthrough Curves

The maximum amount of material that can be retained in an SPE device must be optimized to ensure a theoretical retention percentage of 95–99% [19]. This is known as the breakthrough volume, which depends on the concentration of analytes in the solution loaded onto the sorbent. For this, the sample concentration that can be loaded onto the sorbent bed must be optimized. One approach to determine the retention capacity is the equilibrium method, in which a given volume of sample solution with a known concentration of analyte is circulated through the SPE device until a steady state is reached [20]. A lack of retention on an SPE device can be caused by the addition of too much mass in the load. In this scenario, analytes are not quantitatively retained by the sorbent and are thus detected in the effluent (the unretained volume sample) [6]. With the goal of minimizing the loss of APDs in the effluent fraction (a maximum of 5% relative to the loaded crude extract amount), the retention capacity of the 1-g reverse-phase C18 cartridge was evaluated, as detailed in Section 4.2.4 below.

Figure 2A displays the breakthrough curves obtained, represented as the $\text{ESP}_{\text{effluent}}\text{-to-}\text{ESP}_{\text{crude}}$ ratio—defined in Section 4.2.4—against the amount of crude MeOH extract and clear phase that were loaded in the 1-g and 10-g C18 cartridges (expressed in terms of the crude extract-to-adsorbent and clear phase extract-to-adsorbent ratios), respectively. One can observe that APD losses in the effluent (i.e., the $\text{ESP}_{\text{effluent}}\text{-to-}\text{ESP}_{\text{crude}}$ ratio) above 5% appeared at crude extract-to-adsorbent and clear phase extract-to-adsorbent ratios above 1.20×10^{-4} and 2×10^{-4} ($\text{mg extract} \cdot \text{mg adsorbent}^{-1}$) for the 1-g and 10-g C18 cartridges, respectively (Figure 2A). APD losses in effluents were reported in a previous study [3]; the authors concluded that they might be due to the quantity of biomass used being too high. In our findings, only about 5% of losses (in terms of ESP) were detected when a crude extract-to-adsorbent ratio of 1.20×10^{-4} was used, whereas about 80% were detected at the highest ratios (Figure 2A). Typical chemically-bonded sorbent has a capacity of about 1–10% of their weight [6]. In some cases, the sorbent bed fails to retain the target compound during the charge step or even during washing. Complex matrixes where other majority metabolites also bind to sorbent can cause this. Concretely, *A. carterae* biomass crude extracts contain a large number of compounds that are generally present at higher concentrations than APDs [3]. In this respect, to retain the largest amount of the target metabolites, a crude extract-to-adsorbent ratio of 1.20×10^{-4} was selected.

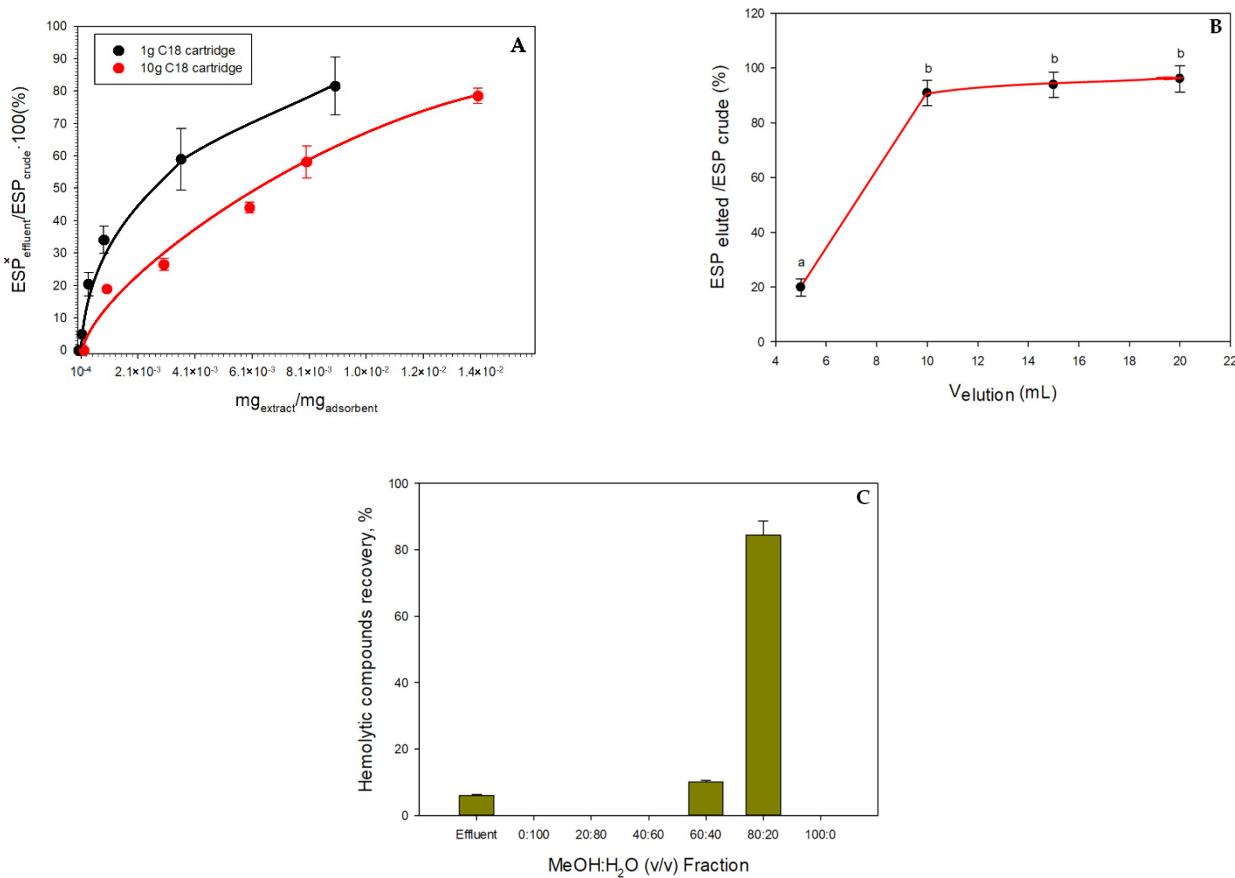


Figure 2. (A) Determination of the breakthrough curves in terms of the $\text{ESP}_{\text{effluent}}/\text{ESP}_{\text{crude}}$ ratio versus the extract-to-adsorbent ratios for 1-g reverse-phase C18 cartridges (black points) using crude MeOH extracts, and for 10-g reverse-phase C18 cartridges (red points) using an extract from the clear phase obtained in Figure 4, Step 2. (B) Optimization of the elution volume with 100% MeOH to completely elute all the compounds adsorbed. (C) Distribution of the hemolytic compounds recovered in the fractionation process using a 1-g reverse-phase C18 cartridge. The recovery of hemolytic compounds was calculated from the $\text{ESP}_{\text{eluted}}/\text{ESP}_{\text{crude}}$ measurements. Data points and bars are averages and vertical bars are standard deviations for triplicate samples. The lowercase letters in Figure 2B represent significant differences, with a p -value < 0.05 .

2.3. Optimization of the Elution Volume

With the goal of desorbing the entire mass of the metabolites (particularly the APDs) retained on the C18 bed, 1-g C18 cartridges were eluted with different volumes of MeOH (100%). The eluted solutions were collected, and their hemolytic activity was measured (see Section 4.2.4). Figure 2B shows the results obtained, indicating that a 10-mL volume of MeOH was sufficient to desorb more than 90% of the target compounds. There were no statistically significant differences between using 10, 15 or 20 mL of MeOH (p -value < 0.05). Therefore, 10 mL was chosen as the elution volume.

2.4. Evaluation of SPE-Based Fractionation

Based on the previous results, a crude MeOH extract volume corresponding to the crude extract-to-adsorbent ratio of 1.20×10^{-4} (w/w) was loaded into a 1-g C18 cartridge (see the adsorption step in Figure 3) and subjected to a six-step elution protocol, as described in Section 4.2.5 (see the fractionation step in Figure 3). The results are presented in Figure 2C. Hemolytic activity was only observed in the fractions obtained by eluting with MeOH:H₂O 80:20 (v/v) and MeOH:H₂O 60:40 (v/v). The ESP value of the MeOH:H₂O 80:20 (v/v) fraction was $7 \pm 0.35 \text{ mg saponin} \cdot \text{mg extract}^{-1}$, which is equivalent to $84.35 \pm 4.21\%$ of

the APDs in the crude extract (Figure 2C)—whereas the MeOH:H₂O 60:40 (*v/v*) fraction contained $10 \pm 0.5\%$ of APDs (equivalent to $0.83 \pm 0.04 \text{ mg saponin} \cdot \text{mg extract}^{-1}$). A similar distribution pattern was observed in previous studies [3,4], but with an APD recovery percentage of around 70% [3]—a value lower than that achieved in this study ($94.35 \pm 4.71\%$). Furthermore, the APD losses were only detected in the effluent, accounting for around 5% of the amount of APDs contained in the crude MeOH extract (see Figure 2C)—in contrast to the 11% reported elsewhere [3]. A few significant differences between our work and the previous studies mentioned above [3,4] seem to be the reason for this discrepancy: the extract carrying the APDs consisted of a hydroethanolic phase that might have contained traces of lipids, amino acids, sugars, or other compounds, and the biomass-to-adsorbent ratio was not optimized accurately [3,4]. However, an NMR analysis showed that the fraction with the APDs contained tiny amounts of amino acids (AA), organic acids (OA), polyhydric alcohols (PA), and sugar (SA). Indeed, the APDs were eluted between MeOH fractions that ranged from 60% to 100% MeOH, with no appreciable concentration pattern.

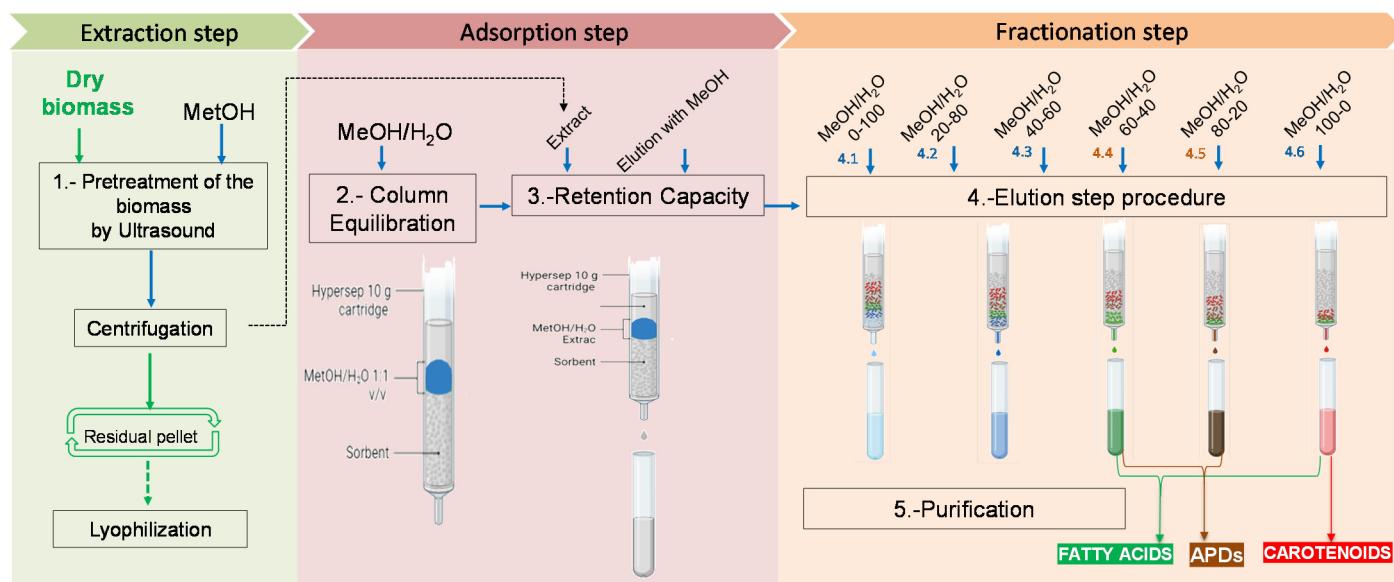


Figure 3. The direct fractionation by SPE approach using 1–10-g reverse-phase C18 cartridges.

2.5. Scale-Up of the SPE to Obtain APDs, Carotenoids and Fatty Acids

The results above were used to scale up (10-fold and 80-fold) the amounts of extracted biomass and C18 adsorbent. As explained in Section 4.3 of Materials and Methods, two approaches were evaluated for minimizing the target compound losses in the different fractions and for improving the APD purification, providing a clear-cut fraction with no interferences from other metabolites. These approaches were: (i) direct fractionation by SPE (see Figure 3) and (ii) liquid-liquid extraction (LLE) coupled with SPE, including an alternative purification step involving solvent-partitioning (see Figure 4).

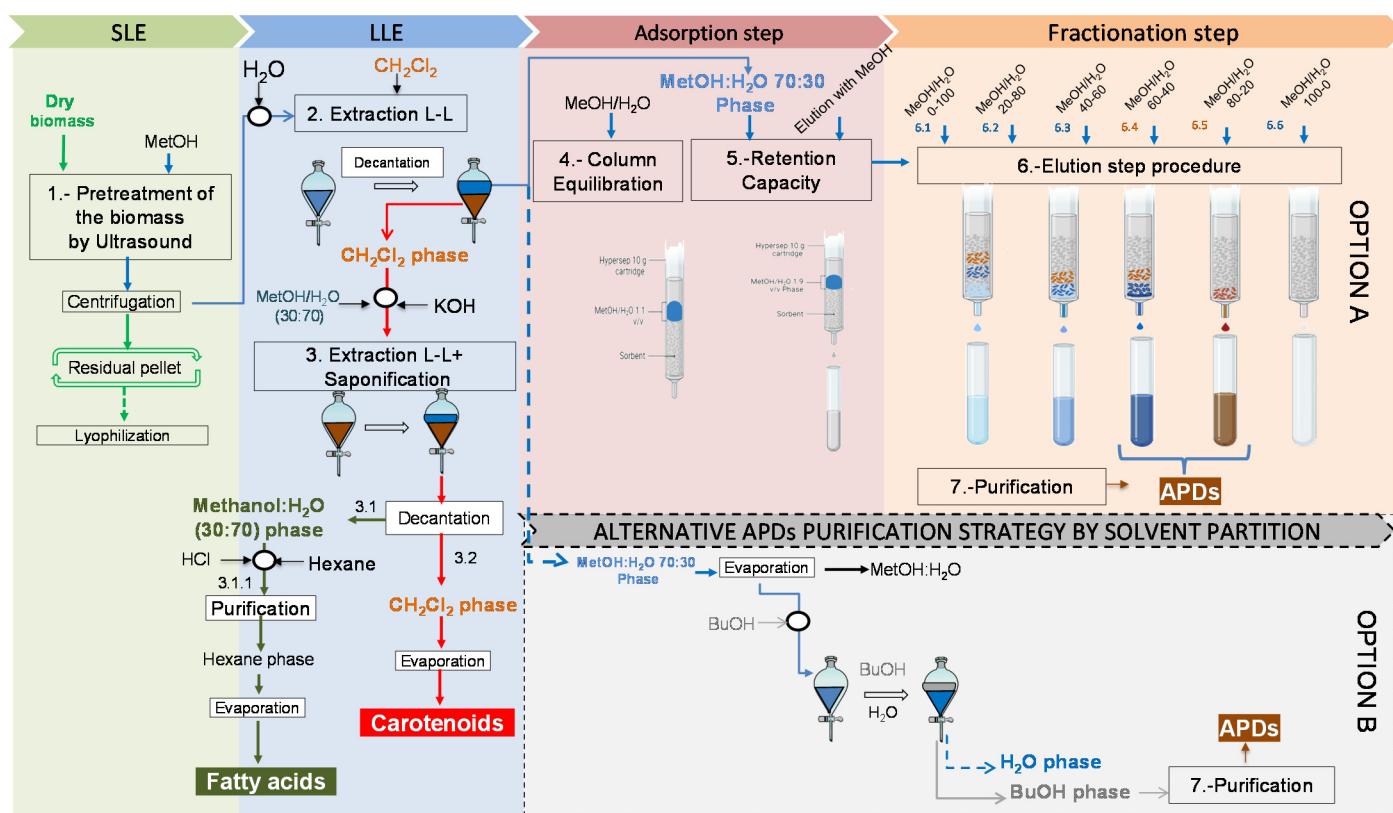


Figure 4. Flowsheet of the proposed process based on liquid-liquid extraction coupled with the SPE approach (Option A) using a reverse-phase 10-g C18 cartridge and 80-g C18 column, and an alternative APD purification strategy by solvent partitioning (Option B).

2.5.1. Evaluation of the Direct Fractionation by SPE Approach

Section 4.3.1 describes this approach and Figure 3 shows a scheme for the process. A 12-mL volume of crude MeOH extract ($0.3 \text{ mg} \cdot \text{mL}^{-1}$) from $0.5 \text{ mg}_{\text{biomass}} \cdot \text{mL}^{-1}$ extracted with methanol was mixed with 108 mL of H₂O to obtain a 10% MeOH concentration. The final hydromethanolic extract was fractionated following the previous optimizations. This volume of crude MeOH extract was intentionally selected based on the data from the rupture curves displayed in Figure 2A. This volume involved APD losses of around 20% relative to the crude MeOH extract. The reason for the increase in APD losses in the effluent from 5% (as explained in Section 4.2.4) to 20% relates to the detection limit of the NMR analysis for the different polar metabolites that are potentially present in the collected fractions. To characterize the crude MeOH extract, a 41-mL sample was analyzed using the NMR-based metabolomics approach (see Section 4.5) to determine the APD content as well as other polar metabolite classes (i.e., amino acids, AA; organic acids, OA; sugars, SA; quaternary ammonium compounds, QAC; polyhydric alcohols, PA; and nitrogenous bases, NB).

The results of this approach are shown in Figure 5. The percentage distribution of the three main metabolite groups (APDs, carotenoids, and fatty acids) represented in Figure 5A demonstrates that APDs were separated in the MeOH-H₂O (80:20 v/v) and MeOH-H₂O (60:20 v/v) fractions with recovery yields of $65.25 \pm 3.26\%$ and $14.33 \pm 0.72\%$, respectively. As expected, the APD losses in the effluent accounted for $20.42 \pm 1.21\%$. The distribution of the remaining polar metabolites analyzed by NMR can be observed in Figure 5B. The OA, PA, and SA groups were swept along on the most polar fraction. NB was completely recovered in the effluent. The QAC group was concentrated in the 60:40 fraction. The AA group was detected in all the fractions, except for the 100:0 fraction with no clear distribution pattern. AA were found in a significant proportion in the most enriched APD

stream ($\text{MeOH-H}_2\text{O}$ 80:20 v/v), along with a tiny percentage of QAC. All the carotenoids (100%) were found in the 100% MeOH fraction. The fatty acids were concentrated in the fractions from 60:40 to 100:0 (Figure 5A). This pattern was also observed by other authors [5], where it was explained that polar lipids, including glycolipids, were eluted in the same fraction as the APDs. This may be caused by the interactions between lipids and APDs mentioned above [14], which might enhance the APD desorption in the 60:40 fraction. These results indicate that some clean-up steps coupled with the solid-phase step are necessary to improve the procedure, as has been suggested [21–23].

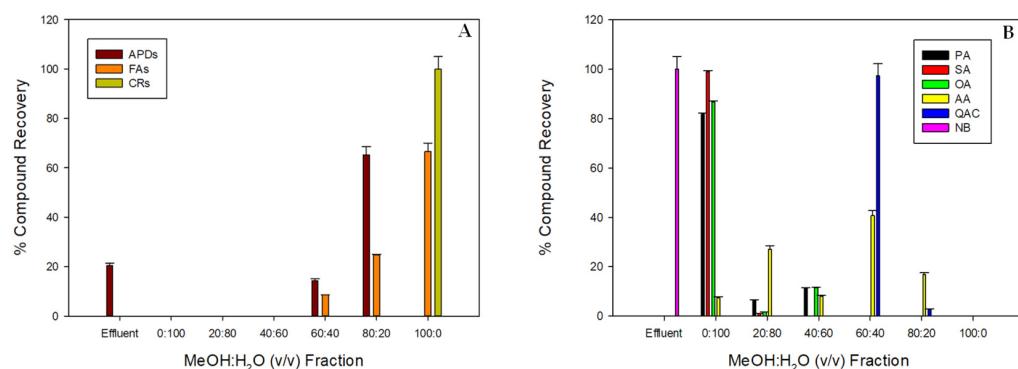


Figure 5. (A) Percentage distribution of the three metabolite groups and (B) the predominant groups of polar metabolome components in the fractionation step (direct fractionation by SPE) outlined in Figure 4, using a 10-g C18 cartridge. APDs: amphidinols; FAs: Fatty acids; CRs: Carotenoids; AA: amino acids; OA: organic acids; SA: sugars; QAC: quaternary ammonium compounds; PA: polyhydric alcohols; NB: nitrogenous bases. Percentages are relative to the content in the initial crude methanolic extracts.

2.5.2. Evaluation of the Liquid-Liquid Extraction Coupled with SPE Approach

Section 4.3.2 explains the methodology that was followed to carry out this approach, and the scheme of the process is illustrated in Figure 4. The LLE step was applied to the crude MeOH extract. After the MeOH extract was extracted by LLE and separated by decantation (Figure 4, Step 2) and two phases were formed (the LLE step in Figure 4): the clear phase (i.e., the 70:30 v/v MeOH: H_2O phase) and the dark phase (i.e., the CH_2Cl_2 phase). Figure 6 displays the distribution patterns of the recovery percentages of the three main compound families (APDs, fatty acids, and carotenoids) for both phases as well as the other polar metabolites. As expected, the carotenoids and fatty acids were swept along with the dark phase (δ_T 20 MPa $^{1/2}$) (Figure 6) while the APDs were found in the clear phase (δ_T 34.83 MPa $^{1/2}$), as well as the AA, PA, SA, NB, and QAC at percentages close to 100%. The OA distribution pattern was divided between the two phases. Based on this data, an enriched-APD fraction was obtained, making the subsequent fractionation step feasible.

For the APD determination, a 120-mL sample from the clear phase ($\text{MeOH:H}_2\text{O}$ 70:30 v/v), corresponding to $0.1 \text{ mg}_{\text{clear phase}} \cdot \text{mL}^{-1}$, was dried and analyzed using an NMR-based metabolomics approach for the same purpose as that explained in Section 2.5.1. The breakthrough volume was determined by loading different volumes of clear phase, which corresponded to different clear-phase extracts, ranging from 1 mL to 100 mL, in different reverse-phase 10-g C18 cartridges and eluted with the new optimized MeOH elution volume, in this case 200 mL. Subsequently, the hemolytic activity was determined in the different eluates obtained, in the same way as explained in Section 4.2.4. The retention volume (V_R) of the analyte is defined as the inflection point of the curve where the retention capacity is reached [19]. As shown in Figure 2A, for the reverse-phase 1-g C18 cartridges, this point occurred at a 3.6×10^{-4} crude extract-to-adsorbent ratio, reaching a level of 20% ESP_{effluent} over ESP_{crude}. This compares with the 1×10^{-3} clear-phase extract-to-adsorbent ratio that occurred in the new isolation strategy using the reverse-phase 10-g C18 cartridges (Figure 2A). This means that the sorbent retention capacity has improved by 1.77 times.

The separation of the main target, the APDs, from the remaining compounds, such as carotenoids and fatty acids, was mainly due to the LLE that was applied as a first step. In this respect, LLE has been used for sample pre-concentration to detain marine lipophilic toxins [9]. Based on the data given in Figure 2A, no metabolites were detected in the effluent when a 2×10^{-4} clear-phase extract-to-adsorbent ratio was used. Therefore, the retention of APDs was favored because of the LLE used as a first step and the optimization of the clear-phase volume load to the 10-g C18 cartridge.

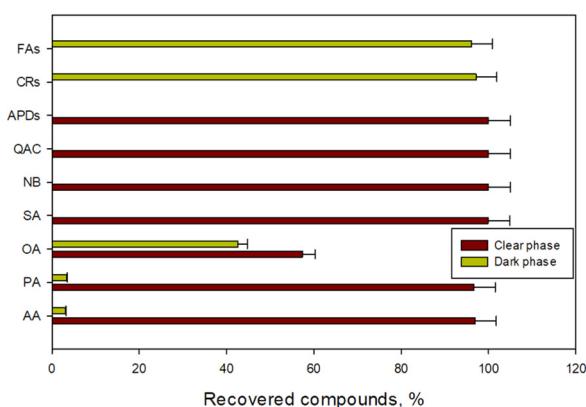


Figure 6. Distribution of the main metabolite groups throughout the two phases formed (the clear phase; 70:30 *v/v* MeOH: H₂O, and the dark phase; CH₂Cl₂) following the liquid-liquid extraction of the crude methanolic extract outlined in Figure 4, Step 2. APDs: amphidinols; FAs: fatty acids; CRs: carotenoids; AA: amino acids; OA: organic acids; SA: sugars; QAC: quaternary ammonium compounds; PA: polyhydric alcohols; NB: nitrogenous bases. Data bars are averages and vertical bars are standard deviations for duplicates samples.

To quantify (by NMR) the APDs contained in both the clear phase and in the different elution gradient fractions explained above, the clear-phase extract volume (0.1 mg·mL⁻¹) that was loaded in the 10-g C18 cartridge was optimized assuming a 20% loss of APDs in the effluent. For this, 100 mL of clear phase was used. This clear-phase volume was first suspended in 570 mL of H₂O to reach a 10% MeOH concentration. After following the fractionation and elution protocol, a sub-fraction of 6.5 (Figure 4, Option A) was found to be the most active with $84.48 \pm 4.22\%$ of the total APDs (Figure 7A). The remaining APDs ($15.52 \pm 0.77\%$) were detected in the effluent (not adsorbed), and a percentage lower than the 20% of APD losses was assumed for the clear-phase volume that was optimized for loading into the column.

Once the MeOH:H₂O 80:20 (*v/v*) fraction was determined by hemolysis as the most active fraction, the process was scaled up to an 80-g C18 column. In this case, the optimization was performed to avoid the loss of APDs to the effluent. Then, 160 mL of clear phase was loaded into the column. This volume was suspended in 888.5 mL of H₂O to again reach a 10% MeOH concentration. After carrying out the fractionation and elution protocol, a bio-guided search for hemolytic activity revealed that only the MeOH:H₂O 80:20 (*v/v*) fraction was active, having an increased performance compared to the direct fractionation process and the clear-phase fractionation process using the 10-g C18 cartridge. APDs were recovered in Stream 6.5 with a recovery percentage of $100\% \pm 5\%$, and no APDs were found in the effluent (not adsorbed) (Figure 7A). This distribution pattern is an improvement on the patterns obtained in Section 2.5.1, as the APDs were concentrated in a clear-cut way in just one fraction.

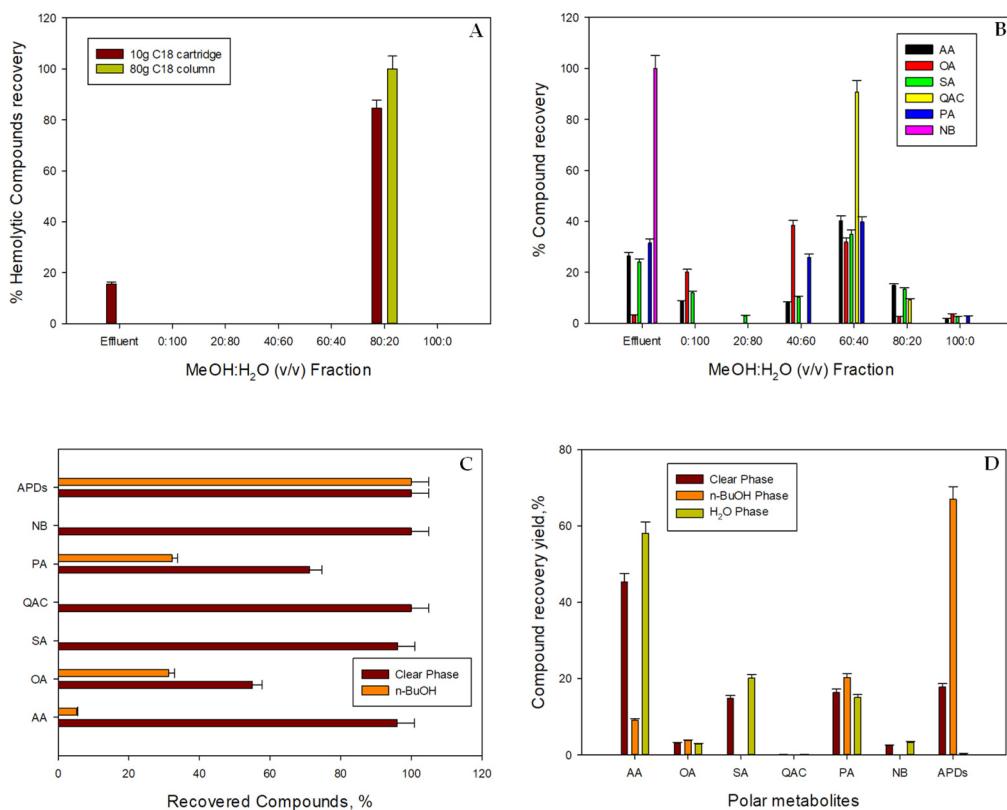


Figure 7. (A) Distribution of hemolytic compounds recovered by the fractionation process using the reverse-phase 10-g C18 cartridge (red bars) and the 80-g C18 column (green bars) and (B) the main polar metabolite groups recovered by the fractionation process using the 10-g reverse-phase C18 cartridges via the process outlined in Figure 4, Step 6, Option A. Percentages are relative to the content in the initial clear phase. (C) Distribution of the main metabolite groups over the two phases obtained from the two purification options outlined in Figure 4 (red bars, clear phase fractionation step procedure; orange bars, solvent-partitioning with n-BuOH). (D) Percentage of each compound with respect to the total of compounds present in the clear phase, n-BuOH phase, and H₂O phase. AA: amino acids; OA: organic acids; SA: sugars; QAC: quaternary ammonium compounds; PA: polyhydric alcohols; NB: nitrogenous bases; APDs: amphidinols. Data bars are averages and vertical bars are standard deviations for duplicates samples.

2.5.3. NMR-Based Metabolomics Approach to Assess the APD Purification Strategies

A deeper NMR-based metabolomics analysis approach was used to assess the processes described in Figure 4 (Option A of Step 6 and Option B), the aim being to fractionate the clear phase. The focus was on streams and fractions containing APDs. Figure 7B shows the compound recovery percentages from the Option A fractionation relative to the compound content in the crude MeOH extract (Figure 4, Step 2). The only fraction that recovered APDs (Stream 6.5; MeOH:H₂O 80:20 (*v/v*)) carried moderately small amounts of the compounds, AA, OA, SA, and QAC, all of them below 16%. In contrast, Stream 6.4 (MeOH:H₂O 60:40 (*v/v*)), which recovered no APDs, swept along significant amounts of all the compound groups, except NB; the recovery percentages ranged from the 32% minimum for OA to the maximum of around 90% for QAC (Figure 7B). NB was completely recovered in the effluent. This pattern differs from that observed in the direct fractionation process (Figure 5B). In those results, because the amounts of loaded compounds were higher (i.e., carotenoids and fatty acids), they could presumably force other polar metabolites to desorb in a more aqueous eluted fraction. Indeed, the resolution of the SPE device was lower than that of the new disposable SPE used here (an 80-g C18 column). Briefly, the SPE device resolution improves the higher the number of theoretical plates obtained by

decreasing the particle diameter [6,19]—being 20–35 µm in the 80-g C18 column compared to 40–63 µm in the 10-g C18 cartridge.

The above results from Option A of the fractionation step (Figure 4) contrast with those obtained in Option B using a simple solvent-partition with n-butanol (Figure 4). From the total of compounds contained in the clear phase, one can see that all the APDs were partitioned in the n-BuOH fraction and only tiny amounts of AA, OA, and PA were detected in this fraction (Figure 7C); the remaining compounds were swept along in the H₂O fraction, especially the 100% of QAC and NB that were not detected in the n-BuOH fraction (Figure 7C). Even though both options achieved complete recovery of all the APDs, Option B could be a rapid and advantageous methodology for recovering all the APDs in just one fraction (n-BuOH), in which the majority of the other polar metabolites were not swept along. While Option A requires large amounts of solvents and is time-consuming, Option B uses fewer materials and solvents, all of which are readily available and relatively inexpensive. Therefore, Option B might be technically more feasible and less time-consuming.

Option B is based on a recently described solvent-partitioning process that has proven highly effective at defatting and desalting [4], providing APDs in a clear-cut way in the n-BuOH fraction. Nonetheless, tiny amounts of fatty acids and carotenoids were found to be present in that fraction, along with significant amounts of AA, OA, PA, SA, QAC, and NB [4]. In the study presented here, the LLE process provided a suitable tool to effectively remove fatty acids and carotenoids from the streams feeding Options A and B before being treated. Secondly, even though other polar metabolite interferences were inevitable, their proportion (expressed as the relative percentage of each metabolite with respect to the total of compounds contained in each phase) in Option B was lower than in Option A (33% versus 81%, respectively) (Figure 7D). In particular, <22% of AA, OA, and PA were swept along in the n-BuOH. Briefly, the n-BuOH fraction carried 70% of the APDs that were purified three times more than those in the clear phase (Figure 7D).

2.5.4. Evaluation of the Free Carotenoid Isolation and the Fatty Acid Purification

To separate the carotenoids and fatty acids contained in the dark phase obtained in Section 2.5.2, a second solvent-partitioning was carried out, coupled with saponification extraction (Figure 4, Step 3). Alkaline treatment is commonly used to release carotenoids from their naturally-occurring ester form [24]. In some cases, peridinin undergoes hydrolysis, losing acetic acid to convert into peridinol, and then, in the presence of fatty acids, converts it into its ester form [16]. Table 1 shows the results when saponification extraction was carried out at 5% KOH, resulting in 98% of the carotenoids being recovered. A closer inspection of Table 1 shows the different effects of saponification on carotenoid recoveries. The maximum carotenoid value obtained was 129. 58 ± 6.47% in the dark phase using 20% KOH. This result was higher than for the compounds present in the initial dark phase (Figure 4, Step 2). In contrast, no carotenoids were detected in the MeOH:H₂O 30:70 v/v phase. This effect is due to the saponification step, which is commonly used for hydrolyzing carotenoid esters [25,26]. These esters would otherwise remain in the initial dark extract together with many other lipids. For the same mg KOH/mg SLs ratio, the fatty acids recovered in the form of soaps in the hydroalcoholic phase (higher solubility) were 20.24 ± 3.98%, meaning that this KOH value was not high enough to efficiently solubilize the fatty acid salts. Accordingly, the mg KOH/mg SLs ratio was increased and the liquid-liquid extraction step was repeated to determine whether more fatty acids could be recovered from the hydroalcoholic phase.

Table 1. Recovery percentages of the free carotenoids and fatty acids recovered by liquid-liquid extraction and the simultaneous saponification procedure outlined in Figure 4, Stream 3. Recovery yield (% d.w.): Percentage of carotenoids and fatty acids extracted with respect to the compounds present in the initial dark phase (Figure 4, Step 2).

KOH (% w/w)	Fatty Acids (% , Recovery Yield)		Carotenoids (% , Recovery Yield)	
	CH ₂ Cl ₂ Phase	MeOH:H ₂ O 30:70 Phase	CH ₂ Cl ₂ Phase	MeOH:H ₂ O 30:70 Phase
5% KOH	100 ± 5	-	98 ± 5	2 ± 0
10% KOH	91 ± 5	9 ± 0	127 ± 6	-
20% KOH	80 ± 4	20 ± 1	130 ± 6	-
40% KOH	60 ± 3	40 ± 2	86 ± 4	-
60% KOH	40 ± 2	60 ± 3	46 ± 2	-
80% KOH	19 ± 1	81 ± 4	6 ± 0	-

Although improved recovery of fatty acid salts was observed when saponification was carried out at 40% KOH in the hydroalcoholic phase, carotenoid degradation increased, with their recovery percentage in the dichloromethane (CH₂Cl₂) phase being 43% lower. A higher mg KOH/mg SLs ratio might enhance the recovery of fatty acids in the hydroalcoholic phase but, at the same time, the risk of carotenoid degradation in the CH₂Cl₂ phase would greatly increase.

From Stream 3.2, the overall free carotenoid and fatty acid recoveries were 129.58 ± 6.47% and 79.75 ± 3.98%, respectively, using an optimal KOH-to-SLs ratio (w/w) of 0.67 mg KOH/mg SLs (20% KOH). Stream 3.1 allowed us to recover 20.24 ± 3.98% of the fatty acid salts, facilitating their purification by adjusting the pH using HCL, followed by extraction by adding hexane at a 1:1 (v/v) ratio.

3. Conclusions

It was possible to successfully optimize a direct fractionation process using SPE to isolate the amphidinols produced by *A. carterae*; almost 80% of these were recovered from the MeOH:H₂O 80:20 (v/v) and MeOH:H₂O 60:40 (v/v) fractions. For the direct fractionation of the crude extract, a 12-mL load is needed, which is equivalent to a 3.4×10^{-4} mg extract/mg_{adsorbent} ratio. The overlapping of fatty acids in the same fractions was inevitably observed, but this was not the case for carotenoids. As an alternative, an approach coupling liquid-liquid extraction with SPE has been demonstrated for separating the APDs, carotenoids, and fatty acids. The clear-phase extract volume needed to ensure APD losses were below 5% was optimized and the adsorbent was scaled up 80-fold using 80-g reverse-phase C18 columns—this improvement led to a 100% recovery of the APDs in the MeOH:H₂O 80:20 (v/v) fraction. For the LLE coupled with SPE in an 80-g reverse-phase C18 column, a 160-mL load of clear phase is needed, which is equivalent to 2×10^{-4} mg_{extract}/mg_{sorbent}. Indeed, the NMR-based metabolomic approach proved the high level of purity of the APD-enriched fractions, obtaining close to 70% by solvent-partitioning using n-butanol—a level never attained before.

4. Materials and Methods

4.1. The Microalgae and the Production of Biomass

Strain Dn241EHAU of the marine dinoflagellate microalgae, *Amphidinium carterae*, was used [27]. It was obtained from the microalgae culture collection of the Plant Biology and Ecology Department of the University of the Basque Country. The biomass used in this study comes from a long-term (>270 days) culture grown in a pilot-scale, LED-illuminated raceway photobioreactor [28]. Details regarding the operation mode and experimental approach have recently been published [29]. Harvesting was carried out once the cultures entered the stationary phase. The cell suspension samples were harvested on day 260 and were centrifuged at 1000× g (RINA model 100 U, 200 SM centrifuge). The cell suspension contained 5×10^6 cell·mL⁻¹ with a 0.6 g·L⁻¹ biomass concentration. The obtained pellets

were gently washed with distilled water. Lastly, the cells were re-pelleted, lyophilized, and stored at -22°C so that they would be ready for use in the different analytical procedures and extraction methods.

4.2. Optimization of the SPE-Based Fractionation

4.2.1. Hemolytic Activity

A. carterae Dn241EHU contains amphidinols (APDs) that exhibit hemolytic activity [13]. Their content in extracts can be expressed in terms of their hemolytic activity on erythrocytes from defibrinated sheep blood, as described elsewhere [29]. Briefly, EC50 values for *A. carterae* (i.e., the number of cells per well giving 50% hemolysis) and a saponin control were calculated from dose-response Hill curves. Saponin was supplied by Sigma Aldrich (47036, CAS n. 8047-15-2, Saint Louis, MO, USA) and the corresponding EC50 was $8.5 \pm 0.6 \times 10^6$ pg per well. An equivalent saponin potency (ESP) was expressed in terms of mg saponin per *A. carterae* biomass and was calculated by dividing the EC50 for saponin by the EC50 for *A. carterae*. Knowing the $\text{mg} \cdot \text{mL}^{-1}$ (1.2×10^{-7}) and the crude methanolic extract mass ($0.3 \text{ mg} \cdot \text{mL}^{-1}$) from the $0.5 \text{ mg} \cdot \text{mL}^{-1}$ biomass concentration in methanol, the ESP can be calculated, and is expressed as mg saponin per mg *A. carterae* crude methanol extract.

As described in Section 4.5 below, APDs can be detected and quantified by NMR analysis [13]. Briefly, the assignment of APD metabolites is possible with the help of 2D NMR experiments, HRMS, and tandem MS. The two-dimensional ^1H - ^{13}C HMBC spectrum of the extract itself allows for the identification of key fragments that are common in most of the amphidinolides that are already known [13]. Since ESP is linearly correlated with the absolute integral of the peak at δ_{H} 5.07 ppm assigned to APDs, ESP can be used as a proxy of the levels of APDs in cells or extracts [13].

4.2.2. Pre-Treatment of Biomass Using Cell Disruption Methods

Two cell disruption methods were tested for pre-treating the biomass: (i) ultrasound (UT) using a probe-type device (UP200S, Hielscher Ultrasonics™, Teltow, Germany) in cycles of 50% and with an amplitude of 80% (UT); and (ii) milling based on a mortar and pestle without alumina (MP). All the assays, pre-treated (UT and MP) and not pre-treated (CTRL), were performed with freeze-dried biomass (5-mg samples). The biomass was directly extracted in 10 mL of methanol and shaken at room temperature ($22\text{--}25^{\circ}\text{C}$) in a vortex mixer (for tubes) for 1 min and then centrifuged for 8 min at $2500 \times g$ (Heraeus Labofuge 2000, Osterode, Germany). The control (CTRL) consisted of biomass without pre-treatment. For the MP method, the biomass was first placed in a mortar, and then pestled. In the UT method, after being extracted with methanol, the samples were sonicated for different lengths of time ranging from 15–45 min. The hemolytic activity was determined in the resulting supernatants, as described above. The efficiency of each method in recovering APDs was assessed in terms of the hemolytic activity compared to the control.

4.2.3. Optimization of the Biomass-to-Extractant Ratio

It has been recently demonstrated that only solvents with polarity indexes (PI) and Hildebrand solubility parameters (δ_T) above ca. 6 and $20 \text{ MPa}^{1/2}$, respectively, can extract APDs from *A. carterae* biomass [4–16]. Thus, based on those studies, methanol was chosen as the extractant for the biomass used. To optimize the biomass-to-MeOH ratio (w/v), different amounts of lyophilized biomass (ranging from 2 to 300 mg d.w.) were suspended in 20 mL of HPLC-quality MeOH to obtain ratios ranging from 0.1 to $15 \text{ mg} \cdot \text{mL}^{-1}$. The biomass–MeOH mixtures were sonicated for the time and selected as the optimum in Section 4.2.2. Each biomass-to-MeOH ratio was tested in triplicate. The hemolytic activity of each extract was analyzed in triplicate.

4.2.4. Sorption Capacity of the Reverse-Phase C18 Cartridge: The Breakthrough Curve

The maximum amount of organic material that can be retained in the reverse-phase SPE device (i.e., the C18 cartridge) was determined. For this purpose, different volumes of the same crude MeOH extract obtained in Section 4.2.3, equivalent to extracted biomass amounts ranging from 0.02 to 45 mg, were prepared by diluting them with deionized HPLC-quality water to a final 10% methanol concentration [30]. To carry out the SPE step, 1-g reverse-phase C18 cartridges (Hypersep, 40–63 µm, 100 Å, Thermo Scientific, Rockwood, TN, USA) were previously conditioned and equilibrated with 10 mL of a 50% MeOH:H₂O solution 1:1 *v/v* (Figure 3, Step 2). Each sample volume of the crude MeOH extract was then loaded into different 1-g reverse-phase C18 cartridges (Figure 3, Step 3). Sorption was performed with negative pressure using a vacuum collector (Supelco Visiprep™ DL, 10–15 in-Hg, Saint. Louis, MO, USA). The vacuum intensity was adjusted to allow the desired flow (2.4 mL·min⁻¹), which was below the maximum recommended by the manufacturer (3 mL·min⁻¹). Once the sample volume was extracted, the cartridge was washed with 10 mL of HPLC-quality water to remove any salts retained in the sorbent. The elution step was carried out with 100% MeOH (Figure 3, Step 4). The hemolytic activity of the different methanolic eluates was analyzed to establish the breakthrough curve. Breakthrough curves show the loading behaviour of the target analyte to be removed from solution in a fixed bed and is usually expressed in terms of the ratio of effluent analyte concentration (*C*) to inlet analyte concentration (*C*₀), then defined as the *C/C*₀ ratio [6,8,10,21]. The concentration of APDs in the inlet (i.e., the crude methanolic extract loaded in the C18 cartridge) and the eluate were expressed in terms of ESP and named as ESP_{crude} and ESP_{effluent}, respectively. Therefore, the ESP_{effluent}/ESP_{crude} ratio which corresponds to the ratio of effluent analyte concentration (*C*) to inlet analyte concentration (*C*₀) explained above and was used to measure the APDs concentration variation in the inlet to outlet solution. The breakthrough point was defined as the point when the ESP_{effluent}-to-ESP_{crude} ratio dropped below 5%, the quantity of analyte not adsorbed being effluent. This point allowed us to determine the maximum extract amount that can be loaded in the C18 cartridge, resulting in APD losses below 5%. Next, the C18 cartridges that were operated up to the breakthrough point were eluted with four different volumes of MeOH 100% (4, 10, 15, and 20 mL) to determine the MeOH volume that causes complete desorption of the compounds adsorbed on the sorbent. Again, the hemolytic activity measurements were taken. All the adsorption assays were conducted in triplicate, and, in each, the hemolytic activity measurements were performed in triplicate.

4.2.5. SPE-Based Fractionation

The reverse-phase C18 cartridges (1 g) were operated up to the breakthrough point, as described in Section 4.2.4, and sequentially eluted with different MeOH:H₂O (*v/v*) mixtures at the following proportions: 100:0, 80:20, 60:0, 40:60, 20:80, and 0:100. The polarity indexes (PI) and Hildebrand solubility parameters (δ_T) of the mixtures ranged from 10.2 to 6.6 and from 47.8 to 29.6 MPa^{1/2}, respectively (Figure 3, Step 4). The elution volume (10 mL) of each mixture used was determined, as explained in Section 4.2.4. The different eluted fractions were collected, including the effluent (not adsorbed), and their hemolytic activity was determined. All the assays were conducted in triplicate, and, in each, the hemolytic activity measurements were performed in triplicate.

4.3. Scale-Up of the APD-Prioritized SPE-Based Fractionation

Two APD-prioritized fractionation approaches were explored for separating the three valuable families of compounds present in the crude extracts: APDs, carotenoids, and PUFAs. The scale of adsorbent was increased 10-fold and 80-fold using reverse-phase 10-g C18 cartridges (Hypersep, 40–63 µm, 100 Å, Thermo Scientific, Rockwood, TN, USA) and 80-g C18 packed columns (Spherical, 20–35 µm, 100 Å, Agela Technologies, Torrance, CA, USA), respectively. The first approach consisted of direct fractionation of the crude extract, as described in Section 4.2.5, increasing the crude methanolic extract volume needed

10-fold to ensure APD losses were below 5% (Figure 3). The second approach is based on a three-step sequential process: (i) liquid-liquid extraction (LLE) of the crude methanolic extracts, followed by SPE and fractionation, which was aimed at enhancing the purification of the APDs (Figure 4, Option A). To compare our results with other authors, the loaded volumes were optimized to assume close to 20% of APD losses in the 10-g C18 cartridge and 0% APD loss in the 80-g C18 column by detecting them in the effluent. For comparison purposes, a second APD purification strategy was employed based on solvent-partitioning with n-butanol; this was proven to be suitable for APD isolation (Figure 4, Option B) [4].

4.3.1. The Direct Fractionation by SPE Approach

Direct fractionation using a 10-g reverse-phase C18 cartridge (Hypersep C18 10 g, Thermo Scientific) was assessed by loading an optimized volume of crude MeOH extract ($0.5 \text{ mg} \cdot \text{mL}^{-1}$) (Figure 3, Step 3). The volume of crude MeOH extract was first diluted with distilled water until a 10% MeOH concentration was reached. Following this, column calibration (Figure 3, Step 2) was performed, and then the organic materials retained on the cartridge were eluted with different $\text{H}_2\text{O}:\text{MeOH}$ mixtures, as explained in Section 4.2.5 (Figure 3, streams from 4.1 to 4.6). In this case, solid-phase extraction was always conducted in darkness to avoid carotenoid degradation. All measurements were carried out in triplicates.

4.3.2. The Liquid-Liquid Extraction Coupled with SPE Approach

In this approach, a liquid-liquid extraction (LLE) step was introduced just before the adsorption step (Figure 4). The novelty of this LLE is that it combines two procedures reported in the literature [4,31]. One of them was used to recover karlotoxins (polyketides similar to APDs) that extracted the crude methanolic extract with CH_2Cl_2 to remove most of the lipids (fatty acids and pigments) [31]. The other one used a solvent-partitioning method to isolate the amphinidins produced by *A. carterae* in a clear-cut way in the n-BuOH fraction [4]. The LLE was carried out as follows:

Briefly, 500 mL of CH_2Cl_2 and 250 mL of distilled water were added to 500 mL of crude MeOH extract ($0.3 \text{ mg} \cdot \text{mL}^{-1}$) (Figure 4, Step 2). The mixture was vigorously stirred at 250 rpm for 30 min and left to decant overnight in a refrigerated chamber at 5°C . Two immiscible phases formed, namely the dark phase (487 mL) containing CH_2Cl_2 (PI 3.1; $20 \text{ MPa}^{1/2}$) and the clear phase (763 mL) containing $\text{MeOH}:\text{H}_2\text{O}$ 70:30 v/v (PI 7.7; $34.82 \text{ MPa}^{1/2}$). Both were separated by decantation in a 2-L glass separating funnel. The dark phase carried the carotenoids and fatty acids, whereas the clear phase carried the APDs and the other polar metabolites (i.e., amino acids, AA; organic acids, OA; sugars, SA; quaternary ammonium compounds, QAC; polyhydric alcohols, PA; nitrogenous bases, NB). It is believed that the virtual absence of lipids and pigments in the clear phase improves the APD sorption capacity of the C18 sorbent.

The dark phase was expected to contain carotenoids and fatty acids; therefore, this phase was subjected to a saponification reaction with simultaneous liquid-liquid extraction using a $\text{MeOH}:\text{H}_2\text{O}$ (30:70 v/v) mixture (Figure 4, Step 3). Briefly, KOH, 50 mL of the $\text{MeOH}:\text{H}_2\text{O}$ solvent, and 50 mL of the dark phase were mixed and stirred magnetically for 30 min at 250 rpm. The amount of added KOH was calculated based on the saponifiable lipid (SL) concentration in the dark extract. Thus, different assays were carried out by varying the KOH concentration from 5% to 80% w/w (mg KOH/mg SLs). Saponification was performed at 25°C . Two immiscible new phases formed, namely the CH_2Cl_2 phase and the $\text{MeOH}:\text{H}_2\text{O}$ phase. These were separated by decantation in a 2-L separating funnel. While the CH_2Cl_2 phase (Figure 4, Stream 3.2) sweeps along the carotenoids, the saponifiable lipids are swept along by the $\text{MeOH}:\text{H}_2\text{O}$ phase (Figure 4, Stream 3.1). In a final step (Figure 4, Stream 3.1.1), the fatty acid salts contained in the hydroalcoholic phase were purified and recovered using 37% HCL to adjust the pH to 2. The fatty acids were then extracted by adding hexane at a 1:1 (v/v) ratio. The hexane phase was separated by decantation and evaluated for fatty acids, as explained elsewhere [4]. The recovery yields

were calculated as the percentage of carotenoid and fatty acid contents with respect to the compounds present in the initial dark phase, which was obtained from Figure 4, Step 2.

After the LLE step in Figure 5, different volumes of clear phase were loaded into 10-g C18 cartridges (Figure 4, Step 5, Option A) to determine the breakthrough point volume and therefore establish the maximum extract amount that can be loaded in the 10-g C18 cartridge, leading to APD losses below 5%. Elution was carried out with the optimized volume of MeOH (200 mL).

The optimized clear phase volume was diluted with distilled water until a 10% (*v/v*) MeOH proportion was reached. Once again, column equilibration (Figure 4, Step 4, Option A) was performed before loading the methanolic extract. The sorption and fractionation steps were carried out as explained in Sections 4.2.4 and 4.2.5 (Figure 4, Step 5 and Streams 6.1 to 6.6, Option A). Fractionation was performed using different C18 sorbent masses (i.e., 10 g and 80 g). The hemolytic measurements of the resulting water–methanol fractions were carried out in triplicate.

As an alternative, a purification strategy was applied on the clear phase using a simple solvent-partition with n-butanol (Figure 4, Option B). In short, 100 mL of clear phase was transferred into a flat-bottomed balloon and evaporated on a rotary evaporator to remove the solvent. The dry residue was firstly re-suspended in 100 mL of H₂O and 100 mL of n-butanol (n-BuOH); then, the mixture was stirred magnetically for 30 min at 250 rpm and left to decant, as explained above (at the beginning of this section). Two immiscible phases formed and the n-BuOH phase was analyzed to determine the APDs.

4.4. Analytical Procedures

The following techniques were used both in the lyophilized biomass and in the different resulting extracts. The carotenoid content and profile were determined using an HPLC photodiode array detector, as previously explained [32]. Direct transesterification was used to determine the fatty acid methyl esters content and profile using gas chromatography coupled to a flame ionization detector (Agilent Technologies 6890 N Series Gas Chromatograph, Santa Clara, CA, USA), as described earlier [33]. The measurement was carried out in duplicate. The localization of the bioactive fraction from the presence of APDs was conducted by assay testing the hemolytic activity in the different fractions, as described above.

4.5. NMR Analysis

The capability of the process described in Figure 4 for isolating and purifying the APDs, compared to that shown in Figure 3, was analyzed using a recent untargeted and rapid NMR-based metabolomics approach [13]. NMR metabolic profiles were recorded on a Bruker Avance III HD 600 spectrometer operating at a proton frequency of 600 MHz and using a 5-mm QCI quadruple resonance pulsed field gradient cryoprobe. Acquisition was carried out with rotation at 293 ± 0.1 K and using a NOESY pre-saturation pulse sequence (Bruker 1D noesygppr1d). Details regarding the analytical procedure and metabolite quantification have recently been explained [4]. The following metabolites were identified and quantified: (i) amino acids, AA (valine, isoleucine, leucine, threonine, alanine, proline, methionine, glutamate, glutamine, glycine, lysine, aspartate, tryptophan, tyrosine, phenylalanine, histidine); (ii) organic acids, OA (lactate, acetate, succinate, fumarate, formate); (iii) sugars, SA (β-galactose, β-glucose, α-glucose, α-galactose); (iv) Quaternary ammonium compounds, QAC (choline, betaine); (v) Polyhydric alcohols, PA (glycerol); (vi) Nitrogenous bases, NB (uracil, cytosine); and (vii) APDs.

Considering the aim of the APD isolation method, first it had to establish the amount of each fraction (crude MeOH extract, clear phase, dark phase, and n-BuOH phase) needed for screening the products by NMR. For this, different volumes of fractions were dried to obtain a final quantity in a range between 10–20 mg of extracts from the crude phase, or the other different phases, to ensure the presence of APDs and to validate the results. The measurements were carried out in triplicate. All the phases (i.e., crude MeOH extract,

clear phase and dark phase, n-BuOH phase, and water phase) and fractions were analyzed after the elution step procedure. However, the focus was on those fractions whose δ_T met the APD extraction requirements as reported earlier [15] (i.e., the clear phase and MeOH:H₂O fractions ranged from 60% to 80% MeOH, obtained after fractionation of this phase (Figure 4, Option A) and the n-BuOH phase (Figure 4, Option B); the crude MeOH extract and MeOH:H₂O fractions ranged from 60% to 80% MeOH (Figure 3) given that these fractions could potentially carry the APDs.)

4.6. Statistical Analysis

Statgraphics Centurion XVII (version 17.2.04) statistical software (2014, Statpoint Technologies, Inc., Warrenton, VA, USA) was used for: (a) a significant difference analysis with a one-way analysis of variance (ANOVA) test and (b) a significant difference analysis with a multi-way ANOVA test.

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Nomenclature

AA: amino acids; APDs: amphidinols; SPE: solid-phase extraction; V_B: breakthrough volume; BuOH: n-butanol; C18: silica-based octadecyl bonded phase; CH₂Cl₂: Dichloromethane; CRs: Carotenoids; CTRL: control extraction in absence of a pre-treatment; EC50: the number of cells per well giving 50% hemolysis; ESP: hemolytic activity of a given extract or fraction measured as equivalent saponin potency; FAs: Fatty acids; H₂O: water; LLE: liquid-liquid extraction; MeOH: methanol; MP: pre-treatment with mortar and pestle without alumina; NB: nitrogenous bases; NMR: nuclear magnetic resonance; OA: organic acids; PA: polyhydric alcohols; PI: polarity index; PUFA: polyunsaturated fatty acids; QAC: quaternary ammonium compounds; V_R: retention volume; SA: sugars; UT: pre-treatment with ultrasound; δ_T : Hildebrand solubility parameter.

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CAPÍTULO 7

CONCLUSIONES



1. La recuperación de forma simultánea de las tres familias de metabolitos de mayor interés (carotenoides, PUFAs y APDs), presentes en la biomasa cosechada del dinoflagelado marino *Amphidinium carterae*, ha sido satisfactoria mediante adaptación de diferentes metodologías originalmente diseñadas para asegurar la recuperación de solo una de ellas (Figura 1, Etapa I). (López-Rodríguez et al., 2019. *Bioresour. Technol.* 282 (2019) 370–377).
2. Se propone un único proceso integrado basado en la combinación de dos metodologías, concretamente aquellas que priorizan la obtención de carotenoides y APDs, para permitir extraer prácticamente la totalidad de carotenoides, PUFAs y APDs presentes en la biomasa de *Amphidinium carterae*. (López-Rodríguez et al., 2019. *Bioresour. Technol.* 282 (2019) 370–377).
3. La optimización de la etapa del proceso basada en el pretratamiento de la biomasa demuestra que la intensidad del tratamiento de saponificación alcalina es dependiente de la especie. No obstante, los mejores resultados se han obtenido a concentraciones de KOH por debajo del 20% p.s.b. y a una temperatura de 60°C (Figura 1, Etapa 2.2). La incorporación de procedimientos de rotura celular no mejora la extracción de carotenoides y ácidos grasos respecto al control llevado a cabo con biomasa liofilizada (Figura 1, Etapa 2.1). El notable incremento de degradación de carotenoides observado, bajo cualquier método de rotura celular, es plausiblemente provocado por el estrés mecánico y elevadas temperaturas a los que son sometidos, por el empleo de métodos mecánicos (López-Rodríguez et al., 2020. *Bioresour. Technol.* 313 (2020) 123518).
4. La eficiencia de extracción de las tres familias de compuestos está relacionada con el índice de polaridad (PI) y el parámetro de solubilidad de Hildebrand (δ_T , MPa^{1/2}) del disolvente extractor (Figura 1, Etapa 2.3). Los mayores porcentajes de

recuperación de carotenoides, ácidos grasos y APDs se obtienen con los siguientes rangos de PI y δ_T , respectivamente: PI=3-5,5 y δ_T =16,5-19,5; PI=3,4-5,4 y δ_T =17-19; PI=6-7,5 δ_T =22-31. Por tanto, se puede utilizar el mismo disolvente para extraer carotenoides y ácidos grasos, pero no los anfidinoles. Los extractos de biomasa más polares, como los obtenidos con MeOH (100), acetona:H₂O 80:20 (v/v) y MeOH:H₂O 80:20 (v/v) exhiben potentes actividades antitumorales (López-Rodríguez et al., 2020. Bioresour. Technol. 313 (2020) 123518).

5. El método basado en la extracción líquido-líquido secuencial permite, por un lado, desgrasar y desalar extractos crudos de *A. carterae* y, por otro, concentrar todos los APDs en una única fracción (n-Butanol) (Figura 1, Etapa 3.1). Esta fracción no llega a ser pura ya que arrastra cantidades significantes de carotenoides, ácidos grasos y otros metabolitos polares. (López-Rodríguez et al., 2021. Bioresour. Technol. 342 (2021) 125922).
6. Alternativamente, y bajo el contexto de biorrefinería basada en dinoflagelados, el proceso integrado (Figura 1, Etapa 3.2), propuesto en la conclusión 2, resulta un método eficaz para separar las tres familias de compuestos de interés con porcentajes de recuperación cercanos al 100%. El porcentaje de extracción de carotenoides, próximo al 100%, en la etapa de saponificación, es indicativo de la ausencia de degradación de éstos, dado el uso optimizado del tratamiento alcalino. (López-Rodríguez et al., 2021. Bioresour. Technol. 342 (2021) 125922).
7. La evaluación, mediante análisis metabolómico por RMN, del porcentaje de distribución de APDs y otros metabolitos polares obtenidos en el proceso integrado, muestra, por un lado, tres fracciones bastante enriquecidas en APDs (MeOH:H₂O 60:40; MeOH:H₂O 80:20; MeOH:H₂O 100:0) (Figura 1, Etapa 3.2) y por otro lado, cantidades significantes de otros metabolitos polares permanecen retenidos en la

- fracción MeOH:H₂O 0:100, junto con una pequeña cantidad de APDs. (López-Rodríguez et al., 2021. Bioresour. Technol. 342 (2021) 125922).
8. Para cuantificar APDs contenidos en la biomasa de *A. carterae*, se fracciona extracto metanólico mediante extracción en fase sólida (EFS). La aplicación de ultrasonidos como procedimiento de rotura celular, durante el pretratamiento es necesaria para mejorar la extracción de APDs, respecto al control realizado con biomasa liofilizada, si el objetivo es obtener el máximo rendimiento de APDs (Figura 1, Etapa 4.1). Pero si se quiere recuperar los carotenoides y ácidos grasos el efecto es diferente ya que la incorporación procedimientos de rotura celular no es el sistema de extracción de referencia, según la conclusión 3 (Figura 1, Etapa 2.1). (López-Rodríguez et al., 2021. Toxins 14(9) (2022) 593).
9. En el fraccionamiento del extracto metanólico basado en EFS, los ratios masa biomasa/agente extractante (p/v), ratio masa_{extracto}/masa_{adsorbente} (p/p) y volumen de elución con valores de 0,5 mg·mL⁻¹, 1,20 x 10⁻⁴ mg_{extracto}/mg_{adsorbente} y 10 mL, respectivamente, conducen a los mejores porcentajes de recuperación de APDs. Concretamente, las fracciones MeOH:H₂O 60:40 (v/v) y MeOH:H₂O 80:20 (v/v) acumulan cerca del 100% de recuperación (Figura 1, Etapa 4.1). Sin embargo, estas fracciones nuevamente no llegan a ser puras ya que cantidades significantes de ácidos grasos se concentran en estas mismas fracciones. (López-Rodríguez et al., 2021. Toxins 14(9) (2022) 593).
10. La inclusión de una etapa de extracción líquido-líquido (MeOH:H₂O y CH₂Cl₂) seguida de saponificación alcalina de la fase CH₂Cl₂, previas al fraccionamiento del extracto metanólico mediante EFS, mejora notablemente la separación de APDs respecto de carotenoides y ácidos grasos (Figura 1, Etapa 4.2). Proporciona una fase CH₂Cl₂ (δ_T 20 MPa ^{1/2}), denominada fase oscura, en la cual carotenoides y ácidos

grasos son arrastrados; mientras que APDs permanecen retenidos en una fase MeOH:H₂O 70:30 (v/v) (δ_T 34,83 MPa $^{1/2}$), denominada fase clara. El patrón de distribución, de las tres familias de compuestos, es el esperado acorde con el parámetro de solubilidad de cada fase. (López-Rodríguez et al., 2021. Toxins 14(9) (2022) 593).

11. Se hace necesario el escalado del fraccionamiento de la fase clara a columnas de fase reversa de 80g C18 para ser satisfactorio y evitar pérdidas de APDs. Tras la etapa de fraccionamiento se recupera el total de APDs en una única fracción (MeOH:H₂O 80:20), lo que conlleva a una mejora en el patrón de distribución y sin pérdidas con respecto al fraccionamiento directo del extracto metanólico mediante EFS (Figura 1, Etapa 4.1) de la conclusión 9 y proceso integrado (Figura 1, Etapa 3.2) de la conclusión 6 . (López-Rodríguez et al., 2021. Toxins 14(9) (2022) 593).
12. Si el fraccionamiento de la fase clara, basado en EFS de la conclusión 11, se sustituye por una extracción L-L entre la fase clara (MeOH:H₂O) y n-Butanol (Figura 1, Etapa 4.2.2) mejora el rendimiento de recuperación en peso de APDs. Permite de una manera rápida y ventajosa concentrar todos los APDs en una única fracción (n-Butanol), con el ahorro de tiempo de operación y menor empleo de disolventes. Se consigue obtener el mayor grado de enriquecimiento descrito, entorno a un 70% p/p, en APDs, obteniéndose extractos tres veces más enriquecidos en APDs que aplicando el método extracción líquido-líquido seguida de EFS (Figura 1, Etapa 4.2.1). (López-Rodríguez et al., 2021. Toxins 14(9) (2022), 593).

NOMENCLATURA

APD	Anfidinol
CB-FBR	Fotobioreactor tipo columna de burbujeo
CCL₄	Tetracloruro de carbono
DCM	Diclorometano
DHA	Ácido docosahexaenoico
EC50	Número de células por pocillo que produce 50% de hemólisis
EFS	Extracción en fase sólida
ELL	Extracción líquido- líquido
EPA	Ácido eicosapentaenoico
EtOH	Etanol
FDA	Food and Drug Administration
HDL	Lipoproteínas de alta densidad
HCL	Ácido clorhídrico
HPLC	Cromatografía líquida de alta resolución
KOH	Hidróxido potásico
LED	Diodo emisor de luz
MAE	Extracción microondas asistida (siglas en inglés)
MeOH	Metanol
MUFA	Ácido graso monoinsaturado
n-BuOH	n-Butanol
PCP	Complejo peridinina- clorofila <i>a</i> - proteína
PI	Índice de polaridad
PUFA	Ácido graso poliinsaturado
RMN	Resonancia magnética nuclear

RW-FBR	Fotobioreactor tipo <i>raceway</i>
SFA	Ácido graso saturado
TTX	Tetrodotoxina
δ_T	Parámetro Solubilidad Hildebrand