VNIVERSITAT DE VALÈNCIA

Facultat de Farmàcia

Area de Nutrició i Bromatologia, Departament de Medicina Preventiva i Salut

Pública, Ciències de l´Alimentació, Toxicologia i Medicina Legal

INNOVATIVE EXTRACTION AND SEPARATION TECHNOLOGIES TO IMPROVE THE RECOVERY OF MICROALGAE NUTRIENTS AND BIOACTIVE COMPOUNDS WITH POTENTIAL BENEFICIAL EFFECTS ON HUMAN HEALTH

EXTRACCIÓN INNOVADORA Y TECNOLOGÍAS DE SEPARACIÓN PARA MEJORAR LA RECUPERACIÓN DE NUTRIENTES Y COMPUESTOS BIOACTIVOS DE MICROALGAS CON POTENCIALES EFECTOS BENEFICIOSOS EN LA SALUD HUMANA

TESIS DOCTORAL INTERNACIONAL

Presentada por: Jianjun Zhou

València, Julio 2023

Programa de Doctorado con Mención a la Excelencia en

CIENCIAS DE LA ALIMENTACIÓN

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CERTIFICAN QUE:

El Graduado en "Food Science and Engineering" D. Jianjun Zhou ha realizado, bajo su dirección y en los laboratorios del área, el trabajo que lleva por título: **"Innovative extraction and separation technologies to improve the recovery of microalgae nutrients and bioactive compounds with potential beneficial effects on human health/Extracción innovadora y tecnologías de separación para mejorar la recuperación de nutrientes y compuestos bioactivos de microalgas con potenciales efectos beneficiosos en la salud humana"** y autorizan su presentación para optar al título de **Doctor Internacional** por la Universitat de València

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La presente tesis doctoral ha dado lugar a 11 artículos publicados o que se publicarán en las siguientes revistas:

1. **Jianjun Zhou**, Min Wang, Celia Carrillo, Zhenzhou Zhu, Mladen Brncic, Houda Berrada, Francisco J. Barba. (2021). Impact of pressurized liquid extraction and pH on protein yield, changes in molecular size distribution and antioxidant compounds recovery from *Spirulina Foods*, 19(4), 207. https://doi.org/10.3390/md19040207. *Impact factor: 5.561*.

2. **Jianjun Zhou**, Min Wang, Houda Berrada, Zhenzhou Zhu, Nabil Grimi, Francisco J. Barba. (2022). Pulsed Electric Fields (PEF), Pressurized Liquid Extraction (PLE) and combined PEF + PLE process evaluation: Effects on *Spirulina* microstructure, biomolecules recovery and Triple TOF-LC-MS-MS polyphenol composition. *Innovative Food Science and Emerging Technologies*, 77, 102989. https://doi.org/10.1016/j.ifset.2022.102989. *Impact factor: 7.104.*

3. **Jianjun Zhou**, Min Wang, Christine Bäuerl, Erika Cortés-Macías, Joaquim Calvo-Lerma, Maria Carmen Collado, Francisco J. Barba. (2023). The impact of liquid-pressurized extracts of *Spirulina*, *Chlorella* and *Phaedactylum tricornutum* on *in vitro* antioxidant, antiinflammatory and bacterial growth effects and gut microbiota modulation. *Food Chemistry*, 401, 134083. https://doi.org/10.1016/j.foodchem.2022.134083. *Impact factor: 9.231*.

4. **Jianjun Zhou**, Min Wang, Francisco J. Barba, Zhenzhou Zhu, Nabil Grimi. (2023). A combined ultrasound + membrane ultrafiltration (USN-UF) for enhancing saccharides separation from *Spirulina* (*Arthrospira platensis*). *Innovative Food Science and Emerging Technologies*, 85, 103341. https://doi.org/10.1016/j.ifset.2023.103341. *Impact factor: 7.104*

5. **Jianjun Zhou**, Ziyang Jia, Min Wang, Qi Wang, Francisco J. Barba, Liuyu Wan, Xuedong Wang, Yang Fu. (2023). Effects of *Laminaria japonica* polysaccharides on gelatinization properties and long-term retrogradation of wheat starch. *Food Hydrocolloids,* 133, 107908. https://doi.org/10.1016/j.foodhyd.2022.107908. *Impact factor: 11.504.*

6. Paulo E S Munekata, Mirian Pateiro, Rubén Domínguez, **Jianjun Zhou**, Francisco J Barba,Jose M Lorenzo. (2020). Nutritional characterization of sea bass processing by-products. *Biomolecules*, 10 (2), 232. https://doi.org/10.3390/biom10020232. *Impact factor:6.064.* (Colaboró con otros equipos, no incluidos en la tesis).

7. **Jianjun Zhou,** Min Wang, Celia Carrillo, Abdo Hassoun, Maria Carmen Collado, Francisco J. Barba. (2022). Application of omics in food color. *Current Opinion in Food Science*, 46, 100848. https://doi.org/10.1016/j.cofs.2022.100848. *Impact factor: 9.8.*

8. **Jianjun Zhou**, Beatriz Gullón, Min Wang, Patricia Gullón, José M. Lorenzo, Francisco J. Barba. (2021). The application of supercritical fluids technology to recover healthy valuable compounds from marine and agricultural food processing by-products: A Review. *Processes*, 9, 357. https://doi.org/10.3390/pr9020357. *Impact factor: 3.352*.

9. **Jianjun Zhou,** Min Wang, Jorge A. Saraiva, Ana P. Martins, Carlos A. Pinto, Miguel A. Prieto, Jesus Simal-Gandara, Hui Cao, Jianbo Xiao, Francisco J. Barba. (2022). Extraction of lipids from microalgae using classical and innovative approaches. *Food Chemistry*, 384, 132236. https://doi.org/10.1016/j.foodchem.2022.132236. *Impact factor: 9.231*.

10. **Jianjun Zhou**, Min Wang, Nabil Grimi, Zhenzhou Zhu, Francisco J. Barba. (2023). Membrane separation process for enhancing carbohydrates recovery from ultrasonic *Phaeodactylum tricornutum* extracts. *Food Chemistry*, (under review). *Impact factor: 9.231*.

11. **Jianjun Zhou**, Min Wang, Nabil Grimi, Basharat N. Dar, Joaquim Calvo-Lerma, Francisco J. Barba. (2023). Research progress in microalgae nutrients: Emerging extraction and purification technologies, digestive behaviour, and potential effects on human gut. *Critical Reviews in Food Science and Nutrition*, (minor revision). *Impact factor: 11.208.*

Ha contado con la participación y el apoyo de:

- Plan complementario en Agroalimentación “AGROALNEXT” (Proyecto AGROALNEXT/2022/060 - Desarrollo y optimización de procesos innovadores y sostenibles de extracción de aceite y proteínas a partir de microalgas, insectos, residuos y subproductos agroalimentarios: Evaluación de propiedades biológicas (EXTRAOLIOPRO)) financiado por MCIN con fondos NextGenerationEU de la Unión Europea (PRTR-C17. I1) y la Generalitat Valenciana.
- BBI-JU through the H2020 Project AQUABIOPRO-FIT “Aquaculture and agriculture biomass side stream proteins and bioactives for feed, fitness and health promoting nutritional supplements” (Grant number 790956).
- Generalitat Valenciana: IDIFEDER/2018/046 - "Procesos innovadores de extracción y conservación: pulsos eléctricos y fluidos supercríticos" through European Union ERDF funds (European Regional Development Fund).
- China Scholarship Council (CSC) No. 201908420246.
- Red Iberoamericana de Investigación en Valorización de Residuos y Subproductos de Origen Alimentario (VALORAL), auspiciada por la Asociación Universitaria Iberoamericana de Postgrado (AUIP).
- Servei Central de Suport a la Investigació Experimental (SCSIE), Universitat de València, València.
- Service of Genomics and Proteomics, IATA-CSIC, Valencia.

“我思故我在。”

“Pienso, luego existo.”

René Descartes (1596-1650)

To all my family and
everyone who has helped me

Now, I have come to this exciting time, and these four years of life have come to my mind like a movie. I am fortunate to have the opportunity to complete my PhD in Valencia.

First, I would like to thank my supervisors, María Carmen Collado and Francisco J. Barba, for giving me the opportunity to participate in the two teams. It is also because of your patience, enthusiasm, and professional knowledge and guidance that I have been able to get to where I am today. Thank you for your continued support and help, you will be my role model in my future life and career.

Thanks to Prof. Jordi Mañes for giving me the opportunity to do my PhD, and the staff at the University of Valencia and IATA-CSIC for always helping in my experiments.

Thanks to my lab colleagues at IATA-CSIC, Marta, Erika, Cristine, Michela, Julian, Pepe, Maria, Quim, Manuel, Raul, Qiqi, they are always enthusiastic and professional, and they taught me a lot of knowledge that I had not been exposed to before, making this journey more interesting.

Thanks to the colleagues in lab at the University of València, Juan, Fran, Bea, Noelia, Manuel, Albert, Mara, Fadila, they always provided me with selfless help in my study and life, which made me from unfamiliar to familiar here, and I am very honored to know everyone.

Thanks to Nabil Grimi and his team for helping, Nadia, Yuqi, Junting, Adila, Nico, Sara, to spend six unforgettable months in Compiègne. They were always friendly and patient in telling me everything and added a wonderful journey to my PhD career.

Thanks to my partner Min. We have faced a lot together in recent years during the difficult years of Covid. Solidarity and mutual assistance can always cope with life calmly, so we overcame these difficulties and completed the mission of doctoral students.

Most importantly, thanks to my family, especially my mother. She always gives me unconditional encouragement and support, let me do everything I want to do, let me feel

loved and warm from beginning to end. Thanks to my grandmother, we lost the chance to see each other for the last time, but you will live in my heart forever.

In addition, I would also like to thank my great country, China, for providing me with a scholarship that allows me the opportunity to complete my studies here without burden. Thank the country for all contributions to world peace and progress.

Thanks to everyone I've met over the years, who helped me, who smiled at me, who said ‘Hola’ to me. Friends and life in Spain are like the summer sun here, always bright. This wonderful journey will be the greatest treasure of my life, and it will always be remembered and treasured.

Thank you.

Jianjun

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List of abbreviations

AAPH	2,2'-azobis-2-methyl-propanimidamide
ABTS	2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonic acid)
ASE	Accelerated Solvent Extraction
BCA	Bicinchoninic acid
BF	Bioaccessible fraction
BHI	Brain heart infusion medium
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	Differential scanning calorimetry
E. coli	*Escherichia coli*
EPA	Eicosapentaenoic acid
FM	Fluorescence microscope
FTIR	Fourier Transform Infrared Spectrometer
GC-MS	Gas chromatography mass spectrometry
GM	Gut microbiota
HGS	Human gastric simulator
HPLC	High Performance Liquid Chromatography
ICP-MS	Inductively coupled plasma mass spectrometer
IL-1β	Interleucina-1 beta
IL-6	Interleucina-6
ILs	Ionic liquids
L-cys	*L*-cysteine
LPS	Lipopolysaccharide
MRS	Man rogosa sharpe medium
MS	Microwave-assisted

MUFA	Monounsaturated fatty acids
MWCOs	Molecular weight cut-offs
NMR	Nuclear magnetic resonance
NRV	Relative Nutrient Values
ORAC	Oxygen Radical Absorbance Capacity Assay
PCA	Principal component analysis
PC	Phycocyanin
PE	Phycoerythrin
PEC	Phycoerythrocyanin
PEF (PE)	Pulsed electric fields (Pulsos eléctricos)
PLE (PLE)	Pressurized liquid extraction (Extracción con líquidos presurizados)
PUFA	Polyunsaturated fatty acids
PS	Polysaccharides
ROS	Reactive oxygen species
SCFAs (AGCC)	Short-chain fatty acids (Ácidos grasos de cadena corta)
SC-CO_2	Supercritical CO_2
SEM	Scanning Electron Microscopy
SFE	Supercritical fluids extraction
SFA	Saturated fatty acids
S. aureus	Staphylococcus aureus
TAG	Triacylglycerols
TEAC	Trolox equivalent antioxidant capacity
TNF-α	Tumor Necrosis Factor-α
USN	Ultrasonic
UF	Ultrafiltration

ABSTRACT

Microalgae are rich in a wide variety of bioactive compounds, including proteins, polyphenols, polysaccharides, pigments, minerals, vitamins, etc., which can be directly utilized or separated to obtain higher added-value components. Extraction and separation are key steps in the processing of microalgae-related products, being innovative technologies a useful tool to improve the utilization of microalgae. Currently, microalgae and their bioactive compounds are being used as food, medical and pharma resources, due their potentially relevant impact on human health.

In this study, the application of pulsed electric fields (PEF), pressurized liquid extraction (PLE), and ultrasound-assisted extraction (USN) improved the extraction efficiency of microalgae high-added-value compounds. *Spirulina (Arthrospira platensis)*, *Chlorella*, and *Phaeodactylum tricornutum* have been selected as the target matrices to recover high-added-value compounds due their high content in valuable components. It was observed by scanning electron microscopy (SEM) that PEF and USN broke the microalgae cell wall structure during the extraction process. Moreover, when using PEF + PLE, the yield of *Spirulina (Arthrospira platensis)* high-added-value compounds was greatly increased ($p < 0.05$), and the extraction time was significantly reduced ($p < 0.05$).

The high-added-value compounds, especially saccharide fraction (mainly polysaccharides) in microalgae, can be considered as a potential source of new prebiotics. However, the traditional separation process is complicated, with serious pollution issues and low yields. In this study, USN + membrane separation technology was used to separate crude polysaccharides from microalgae extracts. The results showed that relatively high purity polysaccharide fractions were obtained with 4 and 10 kDa membranes, while separation efficiency was higher when 100 and 150 kDa membranes were used. USN treatment increased the concentration of relatively low molecular weight

saccharides fraction (4, 10 kDa) in the permeate ($p < 0.05$) but there was not a significant ($p > 0.05$) effect on the high molecular weight saccharides fraction (300, 500 kDa).

In addition, this study explored the potential effect of microalgae extracts on the human gut health. The use of innovative extraction techniques significantly increased the antioxidant capacity of the microalgae extracts ($p < 0.05$), which was attributed to a higher yield of bioactive compounds. *Spirulina (Arthrospira platensis)* and *Phaeodactylum tricornutum* extracts significantly ($p < 0.05$) modulated the *in vitro* activation of the inflammatory NF-κB pathway on epithelial intestinal cell models. In addition, microalgae extracts inhibited the growth of foodborne and potential pathogenic bacteria (strains of *Listeria*, *Staphylococcus*, *Salmonella*, *Escherichia)*($p < 0.05$), while promoting ($p < 0.05$) the growth of potentially beneficial bacteria (including *Lactobacillus* and *Bifidobacteri*um strains). *In vitro* gut microbiota was modulated by *Spirulina* and *P. tricornutum* extracts, and after *in vitro* gastrointestinal digestion, microalgae extracts showed more evident benefits on specific microbial genera (*Bifidobacterium*) by targeted qPCR.

In addition to microalgae, macroalgae bioactive compounds-*Laminaria japonica* polysaccharides (LJP), the main extract of kelp, has been widely used in the food industry due to their health effects such as regulating blood lipids, lowering blood sugar, antiinflammatory, and antioxidation. This study also investigated the effects of *Laminaria japonica* polysaccharides (LJP) on the rheological, gelatinization, and retrogradation properties of wheat starch (WS) gels. Dynamic rheological test showed that the addition of LJP decreased the storage and loss modulus. LJP significantly decreased the peak viscosity, breakdown, and setback value of WS. Moreover, LJP incorporation significantly reduced the WS gel hardness during storage. SEM results showed that the addition of LJP improved the structure of WS gel. The short and long-range ordered

structure showed that the relative crystallinity and formation of ordered structure were reduced with the addition of LJP.

Overall, this study established a new protocol for the efficient extraction of high-added-value compounds from microalgae by using innovative approaches (PEF, PLE, and USN). The innovative extraction technology combined with membrane separation technology enhanced the separation efficiency of microalgae´ polysaccharides. Moreover, microalgae extracts obtained through innovative extraction techniques have shown potential benefits for the human gut health. In addition, this study explored the ability of macroalgae polysaccharides to improve food qualities and found that LJP polysaccharides improved the quality of baked products, suggesting that the bioactive compounds of microalgae may also have the potential to improve food qualities, which needs further investigation in the future.

This thesis provides novel data for the extraction, separation, and application of algae bioactive compounds with potential benefits to human health, which provides more ideas for the development and application of algae products.

Keywords: Microalgae, electrical pulses, pressurized liquid extraction, nutrients, bioactive compounds, health.

RESUMEN

Las microalgas son ricas en una gran variedad de compuestos bioactivos, que incluyen proteínas, polifenoles, polisacáridos, pigmentos, minerales, vitaminas, etc., que pueden utilizarse o separarse directamente para obtener componentes de mayor valor. La extracción y la separación son pasos clave en la obtención y posterior procesamiento de los productos relacionados con las microalgas, representando las tecnologías innovadoras de procesado una estrategia innovadora y eficiente para mejorar la calidad de estos productos. Actualmente, las microalgas y sus compuestos (nutrientes, compuestos bioactivos, etc.) se utilizan como alimento y también con finalidades desde el punto de vista de salud, así pues, es necesario investigar su impacto en la salud intestinal humana.

En la presente tesis, se observó como la aplicación de los pulsos eléctricos (PE), así como las extracciones con líquidos presurizados (PLE) y ultrasonidos (USN) mejoraron la eficiencia de extracción de nutrientes y compuestos bioactivos a partir de microalgas. Se ha seleccionado *Spirulina* (*Arthrospira platensis*), *Chlorella* y *Phaeodactylum tricornutum* para extraer los compuestos debido a su alto contenido en los mismos. Mediante microscopía electrónica de barrido (SEM) se observó como tanto los PEF como los USN rompieron la estructura de la pared celular de las microalgas durante el proceso de extracción. Además, cuando se usó la combinación PEF + PLE, el rendimiento de nutrientes y compuestos bioactivos a partir de *Spirulina* (*Arthrospira platensis*) aumentó considerablemente ($p < 0.05$) y el tiempo de extracción se redujo significativamente ($p < 0.05$).

La fracción de polisacáridos de las microalgas es una fuente potencial de prebióticos, sin embargo, el proceso de separación tradicional para obtenerla es complicado, con una gran contaminación y bajo rendimiento. En este estudio, se utilizó la tecnología de separación por membrana + USN para separar los polisacáridos de los extractos de

microalgas. Los resultados mostraron que se obtuvieron fracciones de polisacáridos de pureza relativamente alta con membranas de 4 y 10 kDa, mientras que la eficiencia de separación fue mayor con membranas de 100 y 150 kDa. El tratamiento con USN aumentó la concentración de la fracción de sacáridos de peso molecular relativamente bajo (4, 10 kDa) en el permeado ($p < 0.05$) pero no hubo un efecto significativo ($p > 0.05$) en la fracción de sacáridos de alto peso molecular (300, 500 kDa).

El uso de técnicas de extracción innovadoras aumentó significativamente la capacidad antioxidante de los extractos de microalgas ($p < 0.05$), lo que se atribuyó a un mayor rendimiento en la recuperación de compuestos bioactivos antioxidantes. Los extractos de *Spirulina* (*Arthrospira platensis*) y *Phaeodactylum tricornutum* redujeron significativamente ($p < 0.05$) la activación lde la ruta inflamatoria NF-κB *in vitro* en un modelo celular epitelial intestinal. Asimismo, los extractos de microalgas inhibieron el crecimiento de bacterias patógenas (*Listeria*, *Staphylococcus aureus*, *Salmonella*, *Escherichia coli*) ($p < 0.05$), al tiempo que promovieron el crecimiento de cepas potencialmente beneficiosas como *Lactobacillus* spp. y *Bifidobacterium* spp. ($p < 0.05$). La microbiota intestinal se vió modulada por los extractos de *Spirulina* y *P. tricornutum* en un modelo *in vitro* de digestión gastrointestinal seguido de una fermentación colónica donde los extractos modularon grupos bacterianos específicos (*Bifidobacterium*) analizados mediante qPCR.

Además de las microalgas, los polisacáridos de *Laminaria japonica* (LJP), el principal extracto de algas marinas, se han utilizado ampliamente en la industria alimentaria debido a sus efectos sobre la salud, como la regulación de los lípidos en la sangre, reducción de azúcar en la sangre, efecto antiinflamatorio y antioxidante. En la presente tesis se investigaron los efectos de los polisacáridos de *Laminaria japonica* (LJP) en las propiedades reológicas, de gelatinización y de retrogradación de los geles de almidón de

trigo (AT). La prueba reológica dinámica mostró que la adición de LJP disminuyó el módulo de almacenamiento y pérdida. Los LJP disminuyeron significativamente la viscosidad máxima, la descomposición y el valor de retroceso de AT. Además, la incorporación de LJP redujo significativamente la dureza del gel AT durante el almacenamiento. Los resultados de SEM mostraron que la adición de LJP mejoró la estructura del gel de AT. La estructura ordenada de corto y largo alcance mostró que la cristalinidad relativa y la formación de la estructura ordenada se redujeron con la adición de LJP.

En general, este estudio estableció un protocolo innovador para la extracción eficiente de nutrientes y compuestos bioactivos a partir de microalgas mediante técnicas innovadoras (PE, PLE y USN). La utilización de tecnologías innovadoras de extracción combinadas con la tecnología de separación por membrana mejoró la eficiencia de separación de los polisacáridos de microalgas. Además, los extractos de microalgas obtenidos a través de técnicas de extracción innovadoras han mostrado beneficios potenciales para la salud intestinal humana. Además, este estudio exploró la capacidad de los polisacáridos de las macroalgas para mejorar la calidad de los alimentos y encontró que los polisacáridos de LJP mejoraron la calidad de los productos horneados, lo que sugiere que los compuestos de las microalgas también pueden tener el potencial de mejorar la calidad de los alimentos, lo que requiere más investigación en el futuro.

Palabras clave: Microalgas, pulsos eléctricos, extracción con líquidos presurizados, nutrientes, compuestos bioactivos, salud.

RESUMEN GLOBAL DE LA TEMÁTICA, PRINCIPALES RESULTADOS Y CONCLUSIONES

En este estudio, se utilizaron diferentes técnicas de extracción innovadoras para extraer nutrientes y compuestos bioactivos a partir de microalgas, combinando el proceso con técnicas de separación por membrana para aislar los componentes específicos. También se evaluó la bioactividad de los extractos obtenidos y su impacto potencial en la salud intestinal. Además, se llevó a cabo una evaluación preliminar de los efectos de los polisacáridos de algas en la calidad de los alimentos. Para proporcionar una descripción general completa de la estructura y el enfoque del estudio, se muestra a continuación un diagrama esquemático con los principales aspectos que se han desarrollado en cada capítulo de la tesis (Figura 1).

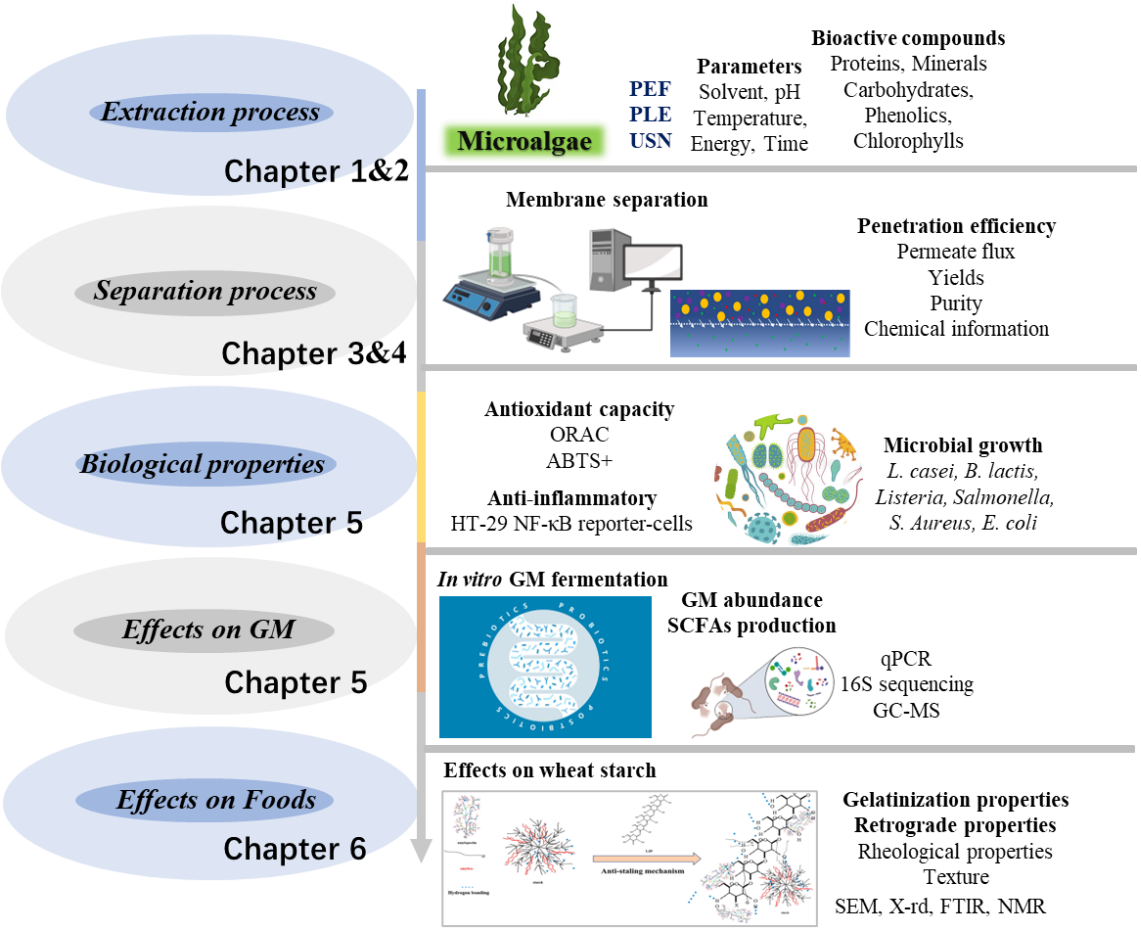

Figura 1. Contenidos de los diferentes capítulos.

1. Aplicación de tecnologías de extracción innovadoras en el proceso de recuperación de nutrientes y compuestos bioactivos de microalgas

1.1 Impacto de la extracción con líquidos presurizados (PLE) y el pH en el rendimiento de extracción de proteínas, cambios en la distribución del tamaño molecular y la recuperación de compuestos antioxidantes a partir de la microalga Spirulina.

Esta investigación tiene como objetivo extraer los nutrientes/componentes bioactivos de la microalga *Spirulina* utilizando un método no tóxico, ecológico y eficiente como la extracción con líquidos presurizados (PLE). Para ello, se utilizó la Metodología de Superficie de Respuesta (RSM)-Diseño Compuesto Central (CCD) para evaluar y optimizar el tiempo de extracción (5~15 min), temperatura (20~60 °C) y pH (4~10) durante la extracción PLE (103.4 bares). Los resultados del RSM-CCD mostraron que, bajo la presión de 103.4 bares, las condiciones óptimas para recuperar el mayor contenido de nutrientes y compuestos bioactivos fueron 10 minutos, 40 °C y pH 4. Además, los nutrientes, compuestos bioactivos y la capacidad antioxidante de las muestras obtenidas se evaluaron y compararon con los extractos de las muestras control (sin PLE). Los resultados mostraron que bajo las condiciones óptimas de extracción (10 minutos, 40 °C y pH 4), PLE mejoró significativamente la capacidad antioxidante (2870.5 ± 153.6 µM TE), rendimiento de proteínas (46. 8 ± 3.1%), clorofila a (1.46 ± 0.04 mg/g), carotenoides (0.12 ± 0.01 mg/g), polifenoles totales (11.49 ± 0.04 mg/g) y carbohidratos (78.42 ± 1.40 mg/g) de los extractos en comparación con la extracción no PLE ($p < 0.05$). La distribución molecular de proteínas de los extractos se analizó mediante SDS-PAGE y los resultados mostraron una mayor cantidad de proteínas de bajo peso molecular en los extractos de PLE frente al control. Además, los resultados de Triple TOF-LC-MS-MS mostraron que los extractos eran ricos en compuestos fenólicos, fundamentalmente en

ácido p-cumárico y el ácido cinámico, principales compuestos fenólicos en los extractos PLE.

En estudios anteriores se ha utilizado la extracción acelerada con diferentes disolventes (hexano, éter de petróleo, etanol y agua) para evaluar la extracción de compuestos antioxidantes de la *Spirulina* (Herrero et al., 2005). Los autores investigaron los efectos de diferentes temperaturas (60, 115, 170 °C) y tiempo (3, 9 15 minutos) sobre la capacidad antioxidante del extracto, obteniendo los autores muestras con mayor capacidad antioxidante a 170 °C y 15 minutos tras utilizar etanol como reactivo de extracción. En comparación con nuestro estudio (10 minutos, 40 °C y pH 4), la temperatura de extracción seleccionada en ese estudio es demasiado alta, lo que es desfavorable para obtener componentes sensibles al calor como pigmentos, polifenoles y proteínas. De acuerdo con el principio de extracción con PLE, la fuerte interacción entre el soluto y la matriz causada por las fuerzas de van der Waals o los enlaces de hidrógeno, y la atracción dipolar de las moléculas de soluto y los sitios activos de la matriz de la muestra pueden reducirse en gran medida a alta temperatura y presión. Esto acelera el proceso de extracción de las moléculas, reduce la energía de activación requerida para el proceso de análisis y reduce la viscosidad del disolvente, lo que disminuye la resistencia del disolvente a la matriz estudiada y promueve la difusión del disolvente a través de la muestra (Zhuang, McKague, Reeve, & Carey, 2004). Esto indica que la extracción PLE puede promover la extracción de compuestos bioactivos de *Spirulina*, lo cual es de gran importancia para la aplicación de esta tecnología para obtener nutrientes y compuestos bioactivos a partir de algas marinas y microalgas.

1.2 Evaluación de los pulsos eléctricos (PE), extracción con líquidos presurizados (PLE) y combinación de PE + PLE: Efectos sobre la microestructura de Spirulina, recuperación de biomoléculas y composición de polifenoles Triple TOF-LC-MS-MS.

Se analizó el impacto de diferentes procesos: pulsos eléctricos (PE), extracción con líquidos presurizados (PLE) y un proceso de varios pasos que combina PE + PLE, en el rendimiento de compuestos bioactivos (proteínas, polifenoles, clorofila a, clorofila b y carotenoides) de *Spirulina*. Los resultados mostraron que la aplicación de PE o PLE aumentó significativamente la recuperación de nutrientes y componentes bioactivos. Los resultados del proceso de extracción con PE se atribuyeron al daño inducido por esta tecnología en la estructura helicoidal de la *Spirulina*, el cual se verificó mediante microscopía de fluorescencia (FM) y microscopía electrónica de barrido (SEM). Un estudio relacionado comparó los efectos de los PE (25 kV/cm durante 150 µs) y el tratamiento de molienda con perlas en la microestructura de la *Spirulina*, mostrando los resultados microscópicos como el tratamiento con PE favoreció la separación de células de filamentos cilíndricos (tricomas), mientras que la molienda con perlas interrumpió la estructura celular intacta, lo que resultó en una extracción mayor de C-ficocianina y de mayor pureza en el extracto obtenido por PE (Martínez, Luengo, Saldaña, Álvarez, & Raso, 2017).

Para aumentar aún más el rendimiento de extracción, se utilizó PE + PLE, como un enfoque de extracción innovador, para extraer nutrientes y compuestos bioactivos de microalgas. Los resultados mostraron que PE + PLE mejoró mucho el rendimiento de extracción en comparación con los tratamientos con PE o PLE solos. En comparación con la extracción de Folch, PE + PLE redujo significativamente ($p < 0.05$) el tiempo de extracción (165 minutos) y aumentó los valores de proteína (1328%), polifenoles (979%), clorofila a (11%) y capacidad antioxidante (47%) de los extractos de *Spirulina* respectivamente. El uso combinado de PE + PLE en este estudio logró una extracción mejorada en comparación con la extracción asistida por PE o PLE sola encontrada en otros estudios. Por ejemplo, Martí-Quijal et al. (2021) aplicaron PE para obtener extractos

de *Spirulina* que contenían carotenoides (0.50 mg/g dw, 60 min), clorofila a (0.60 mg/g dw, 120 min) y polifenoles (19.75 mg GAE/g dw, 180 min) (Martí-Quijal et al., 2021), mientras que Zhou et al. (2021) aplicaron PLE para obtener extractos de *Spirulina* que contenían proteína (210 mg/g dw), clorofila a (1.46 mg/g dw), carotenoides (0.12 mg/g dw) y polifenoles (11.49 mg/g dw) (Zhou et al., 2021).

El tratamiento con PE destruyó la integridad estructurar celular de las microalgas y, por lo tanto, redujo la dependencia del tratamiento con PLE de la alta temperatura, lo que permitió que el tratamiento PLE se pudiera realizar a temperatura ambiente, lo que no solo mejoró la eficiencia de extracción, sino que también protegió a los antioxidantes. En este estudio, PE + PLE desempeñó el papel de 'uno más uno es mayor que dos', y PE-'electroporación' combinada con PLE-'fluido a presión' lo hizo más eficiente y respetuoso con el medio ambiente en comparación con los métodos tradicionales o el uso de PE o PLE solo. Además, los resultados del análisis con Triple TOF-LC-MS-MS mostraron que el tratamiento con PE + PLE aumentó tanto el tipo como el contenido de compuestos fenólicos. En concreto, los polifenoles de los extractos de PE + PLE fueron principalmente p-anisaldehído (3.07 µg/g), ácido elágico (1.40 µg/g), 3-o-glucurónido de quercetina (1.38 µg/g) y ferulato de sitostanilo (1.10 µg/g), mientras que para los extractos PE, PLE, Folch, los principales polifenoles correspondieron a p-anisaldehído (1.07 µg/g), panisaldehído (1.47 µg/g)/quercetina 3-o-glucurónido (1.10 µg/g) y 24-ferulato de metilcolestanol (1.37 µg/g) respectivamente.

2. Separación de nutrientes y compuestos bioactivos de microalgas mejorada por tecnología de extracción innovadora y proceso de ultrafiltración de membrana

2.1 Tratamiento con ultrasonidos combinado con ultrafiltración con membrana (USN-MUF) para mejorar la separación de sacáridos (carbohidratos) de la microalga Spirulina (Arthrospira platensis)

Tras utilizar la combinación de ultrasonidos (USN) + ultrafiltración por membrana (MUF) para separar los polisacáridos de *Spirulina* (SPS) se observó un aumento significativo ($p < 0.05$) del rendimiento promedio de extracción de compuestos de alto valor añadido cuando se aplicó del tratamiento por USN de 400W/10 min. Los requisitos de energía (kWh/kg) para la extracción por USN sin sistema de refrigeración para alcanzar los rendimientos máximos de recuperación de nutrientes fueron inferiores a los del sistema de USN con refrigeración, es decir, proteína (380 vs. 450 kWh/kg), polifenoles (350 vs. 450 kWh/kg), sacáridos (460 vs. 470 kWh/kg), clorofila a (350 vs. 450 kWh/kg), carotenoides (360 vs. 460 kWh/kg) y compuestos totales (350 vs. 450 kWh/kg). La mayor extracción de los diferentes compuestos se atribuyó a los efectos disruptivos de los USN en las microalgas. La cavitación acústica es una teoría aceptada respecto a la disrupción celular ultrasónica (Liu et al., 2022; Wani & Uppaluri, 2022), siendo las frecuencias ultrasónicas bajas (20~40 kHz) las más utilizadas en el proceso de extracción de compuestos a partir de microalgas (González-Balderas et al., 2020; Greenly and Tester, 2015), especialmente en aquellas con paredes celulares delgadas.

En este trabajo, también se evaluaron los efectos de la eliminación de proteínas y polifenoles por precipitación con etanol al 75% y calentamiento a 90 °C, siendo este proceso menos eficaz para eliminar proteínas, polifenoles y pigmentos en comparación con el proceso MUF. En estudios previos se ha visto como la separación de SPS crudo de las microalgas requiere la eliminación paso a paso de pigmentos, proteínas, polifenoles, etc., al tiempo que involucra una variedad de reactivos químicos y biológicos, como cloroformo, n-butanol, TCA, proteasas, carbón activado, H_2O_2, etanol, etc., lo que no solo conduce a la pérdida gradual de SPS, sino que también aumenta el costo y la contaminación (Kang et al., 2022; Qiu et al., 2022; Tang et al., 2020).

El tratamiento con USN aumentó la concentración de SPS de peso molecular relativamente bajo (4, 10 kDa) en el permeado ($p < 0.05$), pero no hubo un efecto significativo ($p > 0.05$) sobre los SPS de peso molecular alto (300, 500 kDa). En general, este estudio obtuvo sacáridos con más del 70% de pureza (50 % ↑) del extracto original de *Spirulina* (~ 20 %) mediante la tecnología USN-MUF, lo que proporcionó la base para obtener SPS de mayor pureza.

Se observó un ensuciamiento evidente de la membrana durante el proceso MUF de sacáridos, lo que indica que el proceso podría mejorarse aún más. En estudios previos otros autores han observado como si el ensuciamiento en la superficie de la membrana es dominante, el aumento en el ensuciamiento reversible conduce a una disminución en el flujo de permeado de la membrana (Marshall et al., 1993). Cuando aumenta el ensuciamiento interno, se encuentra el resultado contrario, es decir, la permeabilidad de la membrana es irreversible (Tanudjaja et al., 2022). Los resultados de este estudio mostraron que los compuestos bioactivos ingresaron en la membrana durante el proceso de separación de esta, lo que resultó en un ensuciamiento irreversible de la membrana.

2.2 Separación de sacáridos (carbohidratos) de Phaeodactylum tricornutum mediante tecnología de ultrasonido de baja frecuencia (LF-USN) y ultrafiltración (UF).

En una primera aproximación se empleó un tratamiento de ultrasonido de baja frecuencia (LF-USN, 24 kHz) para recuperar biomoléculas de *Phaeodactylum tricornutum* (*P. tricornutum*). Los resultados mostraron mayores rendimientos de extracción al utilizar USN (400 W, 20 minutos), en comparación con las potencias de 100 y 200 W. Los efectos del tratamiento por USN se definieron como KUSN, mostrando los resultados que el valor de KUSN aumentó con la potencia de USN y el tiempo de extracción. Específicamente, el KUSN de proteínas, polifenoles, sacáridos, clorofila a, clorofila b y carotenoide KUSN alcanzó valores de 3.0, 3.3, 1.6, 20.5, 18.2 y 15.0, respectivamente. El aumento en el

rendimiento de nutrientes y compuestos bioactivos de *P. tricornutum* por USN podría atribuirse al mecanismo de cavitación acústica. Estudios similares han utilizado los USN para aumentar el rendimiento de extracción de nutrientes y compuestos bioactivos, por ejemplo, Cui et al. (2022) utilizaron los USN (100 W) para extraer flavonoides de tallos y hojas de astrágalo con un rendimiento de 22.0 ± 2.6 mg/g (Cui, Ma, Wang, & Niu, 2022), mientras que Sankaran et al. (2018) utilizaron los USN (20 kHz, 200 W) para extraer proteína de *Chlorella vulgaris* con un rendimiento del 93.3 % (Sankaran et al., 2018).

La microscopía electrónica de barrido (SEM) mostró que los USN promovieron una desestructuración a nivel de las células de *P. tricornutum* durante la extracción, tal y como lo confirmaron posteriormente las pruebas de distribución del tamaño de las partículas. Otro estudio exploró el efecto de las condiciones de USN en la morfología microscópica de las cianobacterias (*Pseudomonas aeruginosa*) a través de microscopía electrónica de barrido (Huang et al., 2021). De acuerdo con los resultados de este estudio, se encontró que los USN promovieron daños graves en las células de las microalgas.

Se usaron diferentes pesos moleculares de corte de la membrana (MWCO) (4, 10, 50, 100, 150, 300, 500 kDa) para separar los sacáridos. El flujo de permeado disminuyó con el proceso de UF y el índice de ensuciamiento de la membrana (MFI) se relacionó con los MWCO. La conductividad y la concentración de compuestos bioactivos del permeado aumentaron con el proceso de UF. La mayoría de las proteínas y polifenoles, así como casi todos los pigmentos fueron retenidos durante el proceso de USN-UF, promoviendo la concentración de sacáridos del permeado. La pureza de los sacáridos se vió afectada por los MWCO de membrana en el proceso USN-UF, con una pureza de sacáridos de ~78% por membrana de 4~150 kDa y ~70% por membrana de 300~500 kDa. La UF de membrana de los sacáridos también se ha evaluado en estudios previos. Por ejemplo, Xie

et al. (2014) utilizaron membranas de 300 kDa, 100 kDa y 6 kDa para aislar polisacáridos crudos solubles en agua con una pureza del 69.5 % a partir de *Cyclocarya paliurus* (Xie et al., 2014). Sun et al. (2011) aislaron polisacáridos crudos con una pureza del 54% de la colza, porque las impurezas como las proteínas en la solución de alimentación no podían ser retenidas completamente por la membrana (Sun, Qi, Xu, Juan, & Zhe, 2011). En general, los resultados de nuestro estudio indicaron que el proceso USN-UF podría usarse como una nueva opción para la preparación de sacáridos crudos de *P. tricornutum* durante el proceso de purificación de polisacáridos.

Los componentes polisacáridos fueron identificados por FTIR, X-rd y NMR, y los resultados mostraron que, durante el proceso de separación, los cortes de peso molecular (MWCO, 4~500 kDa) no tuvieron ningún efecto significativo sobre la composición química y la cristalización de SPS o PPS. Los monosacáridos estaban principalmente en forma de β-glucosa, α/β-ramnosa, β-manano y α-fucosa en la fracción SPS, mientras que β-manano, β-glucosa y β-galactosa en la fracción PPS. Se ha informado que la composición de monosacáridos de los polisacáridos de microalgas no es concluyente, según el entorno de cultivo y el método de aislamiento. Por ejemplo, Cai et al. (2022) utilizaron secuencialmente precipitación con etanol, Sevag y ultrafiltración para aislar el polisacárido SP90-1 de *Spirulina platensis*, que contenía principalmente ramnosa, glucosa, galactosa, ácido glucurónico y una pequeña cantidad de fucoidano y xilano (Cai et al., 2022).

3. *Evaluación de las propiedades biológicas y funcionales de extractos de microalgas.*

3.1 Impacto de los extractos líquidos presurizados de Spirulina, Chlorella y Phaedactylum tricornutum sobre capacidad antioxidante, antiinflamatoria, crecimiento bacteriano in vitro y modulación in vitro de la microbiota intestinal colónica.

Se evaluó el impacto de los extractos de microalgas *Spirulina*, *Chlorella* y *Phaeodactylum tricornutum* (*P. tricornutum*) obtenidos por extracción líquida presurizada (PLE) sobre las actividades antioxidantes, antiinflamatorias, y antibacterianas, así como el impacto en la cinética de crecimiento de bacterias potencialmente beneficiosas. También se estudió el impacto en la composición de la microbiota intestinal colónica mediante un sistema de microfermentación colonica *in vitro*. Los extractos de *Spirulina* y *P. tricornutum* redujeron significativamente ($p < 0.05$) la activación *in vitro* de la ruta inflamatoria NF-κB en modelos celulares. Además, el extracto de *Spirulina*-PLE redujo significativamente ($p < 0.05$) el valor μ-max/h y el valor A-MOD de *Salmonella enterica*; para *P. tricornutum*, mientras que el extracto de *P. tricornutum*–PLE redujo significativamente el valor de μ-max/h de *S. aureus* y *Salmonella enterica* ($p < 0.05$). Más interesante aún, los extractos obtenidos por PLE de las tres microalgas redujeron significativamente ($p < 0.05$) la tasa de crecimiento de *Salmonella enterica*. De acuerdo con nuestra investigación, Plaza et al. también utilizaron PLE para obtener extractos de *Chlorella* con propiedades antibacterianas, especialmente cuando se usó etanol como agente de extracción, mostrando fuertes efectos inhibitorios sobre el crecimiento de *E. coli* y *S. aureus* (Plaza et al., 2015). Además, otros estudios también obtuvieron una actividad antibacteriana del 99.9 % contra *E. coli* y *S. aureus* multirresistente tras utilizar nanofibras de gelatina con *P. tricornutum* (Kwak et al., 2014).

No se observaron efectos de los extractos de microalgas sobre las bacterias potencialmente beneficiosas. *P. tricornutum*–PLE no tuvo ningún efecto significativo sobre el crecimiento de *L. casei* y *B. animales-lactis* ($p > 0.05$), mientras que los extractos de *Chlorella*–PLE redujeron significativamente el valor μ-max/h de *B. lactis* ($p < 0.05$). Remarcar que *Spirulina*-PLE, *P. tricornutum*-PLE aumentaron significativamente el valor A-MOD en ambas cepas potencialmente probióticas *L* ($p < 0.05$). El análisis de

componentes principales (PCA), muestra una relación negativa entre los polifenoles, los carbohidratos y las proteínas en las microalgas y *S. aureus*. Debido al elevado contenido de polifenoles y polisacáridos en los extractos de *Spirulina* y *P. tricornutum*, se evaluaron los efectos de los extractos antes y después de la digestión *in vitro* sobre la fermentación colónica. Los efectos de los extractos de *Spirulina*–PLE y *P. tricornutum*–PLE sobre la salud intestinal no pueden generalizarse, ya que no solo aumentan los niveles del género *Lactobacillus* y disminuyen los del género *Streptococcus*, sino que también disminuye los niveles de los géneros *Bifidobacterium* y *Bacteroides*. Como sabemos, algunos miembros de *Bifidobacterium* y *Lactobacillus* se consideran potencialmente probióticos y organismos beneficiosos para la salud humana, ya que permiten controlar los niveles de colesterol sérico, prevenir enfermedades intestinales, modular el sistema inmunitario y, además, presentan actividades anticancerígenas (Di Gioia, Aloisio, Mazzola, & Biavati, 2014). Las bacterias pertenecientes al género *Bacteroides* también jugarían un papel importante en la mejora de trastornos metabólicos e inmunológicos en individuos obesos, y el género *Lactobacillus* se considera una bacteria beneficiosa con efectos en la modulación de la microbiota y también, en la mejora de la función gastrointestinal, aumentando la digestibilidad de los alimentos, reduciendo el colesterol sérico, etc. (Azizian et al., 2021).

Los ácidos grasos de cadena corta (AGCC) son los productos de la actividad microbiana y contribuyen a la homeostasis fisiológica y energética del huésped. Los AGCC se refieren principalmente al ácido acético, ácido propiónico y ácido butírico, que están altamente asociados con los niveles bacterianos durante la fermentación colónica (Guo et al., 2021). Los resultados mostraron que *Spirulina*-PLE-digesto y *P. tricornutum*-PLE-digesto fueron capaces de aumentar el contenido acético, mientras que los extractos no digeridos no tuvieron efecto sobre el ácido acético dentro de las 48 h de fermentación.

Los extractos de *P. tricornutum*–PLE mejoraron la producción de ácido propanoico a las 48 h. La muestra de *Spirulina*-PLE digerida aumentó significativamente el ácido butanoico, tanto después de 24 como de 48 horas de fermentación. A partir de esto, tanto el tiempo de fermentación como la digestión *in vitro* pueden afectar los niveles de AGCC. En combinación con los resultados de qPCR, los extractos de *Spirulina* y *P. tricornutum* redujeron la mayor parte de la cantidad de bacterias durante el proceso de fermentación de 24 h, mientras que el transcurso del tiempo y la digestión *in vitro* aumentaron la cantidad de bacterias, lo que resultó en la variación de los niveles de AGCC.

4. **Aplicación de polisacáridos de algas en productos alimenticios.**

El almidón es uno de los carbohidratos más abundantes en la naturaleza y es la principal fuente de carbohidratos en la dieta humana, ya que proporciona aproximadamente el 70 % de la ingesta diaria de energía del cuerpo humano (Lin et al., 2021).

Es por ello, que resulta de un gran interés mejorar las propiedades del almidón para poder utilizarlo en la elaboración de una amplia gama de productos. En este sentido, en este estudio se investigaron los efectos de los polisacáridos de *Laminaria japonica* (LJP) en las propiedades reológicas, de gelatinización y de retrogradación de los geles de WS (almidón de trigo). La prueba reológica dinámica mostró que la adición de LJP disminuyó el módulo de almacenamiento y pérdida. Además, los LJP disminuyeron significativamente el valor de viscosidad máxima (PV), ruptura (BD) y retroceso (SB) de los geles de WS.

En estudios previos, Lin et al. (2021) mostraron que las altas concentraciones de polisacáridos sin almidón formaron más enlaces de hidrógeno con las moléculas de almidón, lo que resultó en tendencias dependientes de la concentración en los valores de PV, BD y SB (Lin et al., 2021). En general, las características de gelatinización de las

partículas de WS se vieron significativamente afectadas por la interacción entre los LJP y el almidón.

Asimismo, la incorporación de LJP redujo significativamente la dureza del gel WS durante el almacenamiento. Estudios previos han mostrado que las interacciones entre el almidón y la xantana podrían obstruir las interacciones entre las amilosas durante el almacenamiento a largo plazo, incluida la reagregación de amilosa y la recristalización de amilopectina, lo que podría ser la razón por la que la adición de LJP disminuye la dureza del gel WS en este estudio (Tang, Hong, Gu, Zhang, & Cai, 2013). Además, la retrogradación del almidón estuvo acompañada por la migración de agua, y las moléculas de LJP podían competir con el almidón por la absorción de agua e inhibir la retrogradación del almidón durante el almacenamiento del gel, lo que reduciría la dureza del gel WS (Yu, Wang, Chen, Li, & Wang, 2018). Los resultados de SEM mostraron que la adición de LJP mejoró la estructura del gel WS.

La estructura ordenada de corto y largo alcance mostró que la cristalinidad relativa y la formación de la estructura ordenada se redujeron con la adición de LJP. De lo contrario, el LJP inhibió la migración de humedad, lo que se demostró mediante LF-NMR. En resumen, la adición de LJP podría inhibir la retrogradación del gel WS, así como también disminuir la estructura de doble hélice de la amilosa o la amilopectina, lo que proporciona una nueva perspectiva teórica para la producción de estudios de alimentos basados en WS. De manera similar, otros estudios también mostraron que los polisacáridos, como la adición de inulina, pululano, podrían reducir la cristalinidad del almidón retrogradado, lo que indicó que LJP tuvo el mismo efecto al inhibir la recristalización de amilopectina y la retrogradación del almidón (Luo et al., 2017). Sin embargo, en comparación con la adición de 0.5~1.5% de LJP para inhibir la retrogradación del almidón, la proporción de polisacáridos añadidos fue mayor en estos estudios. Por ejemplo, se observó como

adicionar 5.0%~7.5% y 15% de inulina podría inhibir la retrogradación del almidón a largo y corto plazo, respectivamente (Luo et al., 2017). Además, la adición de inulina inhibió la retrogradación de amilosa, pero aceleró la retrogradación de amilopectina, mientras que no hubo evidencia de que la adición de LJP acelerara la retrogradación de amilopectina. La retrogradación del almidón (reordenamiento y ordenación) fue el proceso inverso de la gelatinización del almidón (fractura y desorden), durante el cual el almidón pasó de un estado desordenado a un estado cristalino, acompañado de cambios en la relación agua libre/agua ligada (Niu et al., 2018). Durante el proceso de retrogradación, a medida que se incorporaba agua a la estructura cristalina, el almidón tendía a formar un estado cristalino más estable. Sin embargo, la adición de LJP redujo la proporción de agua libre, lo que indica que LJP podría inhibir el comportamiento de cristalización del almidón al competir con las moléculas de almidón por el agua. En resumen, la adición de LJP podría inhibir la retrogradación del gel WS, así como también disminuyó la estructura de doble hélice de la amilosa o la amilopectina, lo que proporciona una nueva perspectiva teórica para la producción de estudios de alimentos basados en WS.

Conclusiones

De los resultados obtenidos en la presente Tesis Doctoral se puede concluir que:

1) Las tecnologías de extracción innovadoras (Pulsos eléctricos, PE; Extracción con líquidos presurizados, PLE; ultrasonidos, USN) se pueden utilizar para obtener compuestos bioactivos a partir de microalgas, ya que aumentan los rendimientos de los compuestos bioactivos en extractos de microalgas y al mismo tiempo, preservan sus propiedades biológicas y funcionales.

2) Las extracciones de PE y USN tienen un efecto dañino en la estructura de las microalgas durante el proceso de extracción, que es la razón principal del aumento en el rendimiento de nutrientes y compuestos bioactivos.

3) La tecnología de USN + separación de membrana se puede utilizar para separar fracciones de polisacáridos de microalgas, y la información química y las propiedades de cristalinidad de los polisacáridos se ven ligeramente afectadas por el peso molecular (MWCO).

4) Los extractos acuosos de *Spirulina, Chlorella* y *Phaeodoactylum tricornutum* son fuentes potenciales de agentes antiinflamatorios y antimicrobianos naturales para ser utilizados en la prevención, tratamiento y control de infecciones bacterianas, así como una estimulación potencial de bacterias benéficas, incluidos los miembros de *Bifidobacterium* y *Lactobacillus*, algunos de ellos reconocidos como probióticos.

5) Los polisacáridos de algas pueden inhibir la retrogradación a largo plazo del almidón del trigo, lo que mejora la calidad de los alimentos de panadería durante el almacenamiento.

6) Los extractos de microalgas afectan el crecimiento del microbiota intestinal y promueven la producción de AGCC, que tienen beneficios potenciales para la salud humana.

Estas conclusiones demuestran que tanto PE como PLE y USN se pueden utilizar para la extracción eficiente de nutrientes y compuestos bioactivos de diferentes microalgas, ya que aumentan la recuperación de éstos preservando a su vez las propiedades biológicas de los extractos obtenidos. Sobre la base de estas tecnologías innovadoras, será de gran importancia para la economía de la industria de las microalgas y la protección del medio ambiente si realmente es posible realizar una extracción industrial a gran escala de

nutrientes y compuestos bioactivos de microalgas y, al mismo tiempo, reducir el consumo de energía y la contaminación.

La utilización de USN combinada con la tecnología de separación por membrana se puede usar como un método libre de contaminación y fácil de operar para separar los polisacáridos de los extractos de microalgas. La pureza de separación actual es inferior al 80%, lo que se puede mejorar ajustando los parámetros de separación de membrana en el futuro.

Desde la perspectiva de las curvas de crecimiento antiinflamatorio y microbiano, los extractos acuosos de *Spirulina*, *Chlorella* y *P. tricornutum* pueden sugerirse como fuentes potenciales de agentes antiinflamatorios y antimicrobianos naturales para la prevención, tratamiento y control de infecciones bacterianas y estimulación de la actividad probiótica. Los extractos de *Spirulina* y *P. tricornutum* modularon la microbiota intestinal *in vitro*, y tras digestión gastrointestinal, los extractos de microalgas mostraron efectos sobre algunos generos bacterianos específicos obtenidos por qPCR. Sobre esta base, es necesario explorar el mecanismo adyacente a la influencia de estos nutrientes y compuestos bioactivos en la composición y actividad del microbiota intestinal.

Los experimentos preclínicos *in vivo* (ratones, nematodos, etc.) son necesarios para evaluar la bioactividad de los extractos de microalgas y sus efectos sobre la salud intestinal. Finalmente, los componentes de las microalgas, como los polisacáridos de algas, pueden mejorar la calidad de los productos horneados, lo que puede ampliar el ámbito de aplicación para mejorar la calidad de más sustratos alimentarios

1. INTRODUCTION

1. Introduction and literature review

1.1 Microalgae consumption and market overview

Growing world population, ongoing COVID-19 pandemic, political conflicts, and climate change issues make human food stability and security very challenging. Therefore, fundamental changes to the current food system and the search for new alternative food sources are required (Gohara-Beirigo, Matsudo, Cezare-Gomes, Carvalho, & Danesi, 2022; Helmy et al., 2022.). In this context, topics related to the environment, sustainability, healthy food, and quality of life, such as the SDGs (Sustainable Development Goals) proposed by the United Nations, increase the need for scientific research on better choices of food, processing, and ingredients. One of the most promising alternatives has to do with the use of microalgae. Generally, microalgae can be found as prokaryotes and eukaryotes and are classified into secondary groups according to pigments (Ferreira de Oliveira & Bragotto, 2022) (Figure 1).

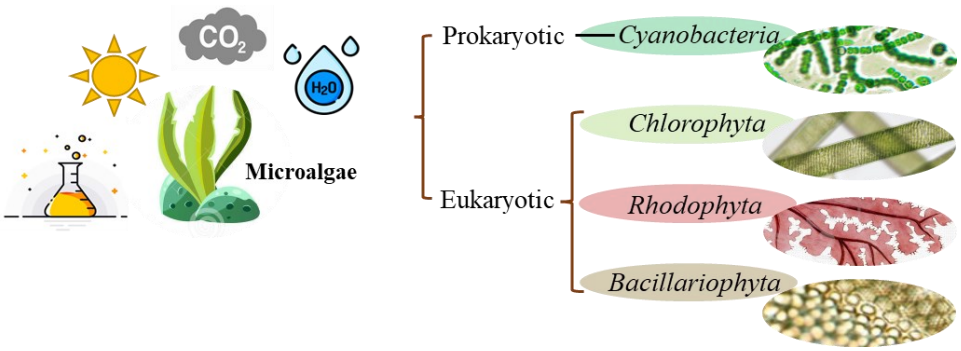

Figure 1. Microalgae represented by phylum and cell structure.

Compared with traditional terrestrial crops, microalgae have more environmentally friendly low-carbon characteristics, because microalgae can be produced on non-cultivated land with high productivity per square meter, thereby reducing the expansion of agricultural land for food production. They can capture 1.1011 kg of carbon dioxide per day and produce 50% of the earth's oxygen, and synthesize various bioactive

substances important to human health, including proteins, lipids, carbohydrates, vitamins, antioxidants, pigments, minerals, etc (Chisti, 2007).

Microalgae with GRAS (Generally Recognized As Safe) status according to the U.S. Food and Drug Administration (FDA) include *Spirulina platensis*, *Chlorella vulgaris*, *Dunaliella bardavelta*, *Chlamydomonas reinhardtii*, *Chlorella protothecoides* and *microphthalmia* (Torres-Tiji, Fields, & Mayfield, 2020). Moreover, other algae can be used to obtain specific compounds for use in health products or dietary supplements, such as β-carotene, astaxanthin, fucoidan, EPA, and DHA, among others (Abdel-Latif et al., 2022; Gohara-Beirigo et al., 2022).

During 2010-2012, the world's top three algae exporters were China, Indonesia, and South Korea, each exporting more than $125 million/year of algae products. In the same period, the main exporter to the EU was Chile, with an average annual export value of algae products of 13 million US dollars, followed by Indonesia and the United States. The global market for algal products is expected to grow from $3.2 billion in 2017 to $5.38 billion in 2025, while the market for microalgae-based products is expected to reach $3.2 billion by 2030 (Globe, 2020; Vigani et al., 2015). The microalgae market has great potential in the future, with 70~80% of the total cost arising from the downstream processing of biomass, where energy consumption is the most expensive factor in the process. Low-cost harvesting and processing of pollutants remains a potential challenge for their wider application (Helmy et al., 2022.).

1.2 *High-added-value compounds (HAVCs) from algae*

The potential high-added-value of microalgae is mainly due to its richness in nutrients and bioactive compounds. These high-added-value ingredients have been studied for their bioactivity and health properties, such as antioxidant, anti-carcinogenic, antiviral, antiinflammatory, anti-hypertensive, anti-diabetic, anticoagulant, antimicrobial,

hypocholesterolemic, cardiovascular protective, immunomodulatory, etc. (Nova et al., 2020).

1.2.1 Proteins

The protein content of microalgae is about 45%~70%, and it is considered a supplement to traditional protein products because of its amino acid composition and nutritional properties similar to animal protein (Christaki, Florou-Paneri, & Bonos, 2011). The protein content of algae varies between species and changes with the seasons and surrounding environment. In terms of general production, red algae have a higher protein content, followed by green algae and brown algae (Kadam, Tiwari, & O'Donnell, 2013). According to different absorption wavelengths, microalgae proteins can be divided into phycocyanin (PE, λ=490~570 nm), allophycocyanin (APC, λ=650~660 nm) and phycoerythrin (PC, λ=610~625 nm) and phycoerythrocyanin (PEC, λ=560~600 nm), collectively referred to as phycobiliproteins (PBP) (Wang et al., 2022). Among them, PE is located in the outermost layer of PBP, where it absorbs light energy and transfers it to PC and APC. The types of PBP vary, depending on the algae, i.e., the red and blue algae are mainly composed by PE, PC and APC. As a water-soluble pigment protein, it is a chromophore group that exists in algae, which can participate in the photosynthesis of algae, and is used for coloring foods, pharmaceuticals, and other fields such as clinical medicine (Kissoudi, Sarakatsianos, & Samanidou, 2018).

1.2.2 Lipids

The lipid content in microalgae ranges between 1% and 40% and might reach up to 85% of total dry weight based on the environmental fluctuations (Du et al., 2021; Xu, Zhao, & Su, 2021). Microalgae lipids can be divided into membrane lipids such as glycosyl glycerides and phosphor glycerides, storage lipids in the form of triglycerides (TG), and a small amount of fat-soluble pigments (chlorophyll a, carotenoids, etc.), sphingolipids,

sterols, etc. Microalgae lipids are an important source of unsaturated fatty acids (UFAs), containing a large amount of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), such as *Aurantiochytrium* and *Crypthecodinium cohnii* (Du et al., 2021; Miyashita, Mikami, & Hosokawa, 2013; Xu, Zhao, & Su, 2021). The type of microalgae and the growth environment will affect the lipid accumulation, for example, low temperature and cold water can promote the produce of UFAs in the algae, while a higher total lipid amount can be collected during the summer season (Miyashita et al., 2013). Microalgae lipids, especially UFAs, have various health benefits for humans, especially concerning the reduction of cardiovascular diseases, mainly high blood pressure, arrhythmia, and stroke, in addition to positive impact regarding asthma, rheumatoid arthritis and depression (Adarme-Vega et al., 2012). In addition, algae also contain 10%~20% phospholipids, which are the main carriers for stability of cell membrane structure and the transportation of nutrients, can act as emulsifiers in the diet and have strong antioxidant capacity (Akyüz, Ata, & Dinç, 2016).

1.2.3 Carbohydrates (polysaccharides)

As one of the most abundant resources in the marine media, microalgae are rich in polysaccharides (cell wall structural polysaccharides, storage polysaccharides, fungal polysaccharides, etc.), which account for 4% to 75% of the dry weight (Zheng, Chen, & Cheong, 2020). The main species used to produce polysaccharides from microalgae include *Chlorella*, *Tetraphytum, Porphyra*, *Porphyra, Isochrysis*, and *Rhodophylla*, and the main strategy for accumulating exopolysaccharides is to limit certain nutrients (Levasseur, Perré, & Pozzobon, 2020). Similar to proteins and lipids, the type of polysaccharide depends on the species of algae. For example, laminarin, alginate, and fucoidan are mainly found in brown algae; carrageenan, agar, xylan, and porphyrin are the main components in red algae; and sulfan is mainly found in green algae (Lim, Yusoff,

Ng, Lim, & Ching, 2021). Algae polysaccharides have been widely used in food, medicine, textile, and other industries, and have positive effects on human health such as antibacterial, antiinflammatory, and low calorie. In addition, algae polysaccharides are considered as dietary fibers, and fermented by intestinal bacteria can regulate intestinal immune function, regulate energy intake, glycemic control, and serum lipids (Villarruel-López, Ascencio, & Nunõ, 2017).

1.2.4 Polyphenols

Phenolic compounds (PCs) represent the largest group of secondary metabolites in plants, ranging from simple aromatic rings to more complex molecules including flavonoids, phenolic acids, tannins, lignans or coumarins (Del Mondo, Sansone, & Brunet, 2022). Recent studies have shown microalgae to be a source of phenolic compounds. Different growth environments lead to differences in the phenolic substances in algae and terrestrial plants, first showing stronger biological activities, such as bromophenol, thiabolol and chlorotannin. Compared with red algae and green algae, brown algae have lower phenolic content, especially epitannin mainly exists in brown algae and is also the most studied algal phenolic component (Liu, Hansen, & Lin, 2011). Modern research has proved that phenolic compounds have a wide range of nutritional functions, including anti-allergic, antioxidation, antiaging and antiinflammation (Zhou et al., 2022). In addition, studies have also shown that phenolic compounds derived from microalgae have medical functions such as anti-diabetes, regulation of lipid metabolism, and prevention of heart disease (Naveen, Baskaran, & Baskaran, 2021; Sharma & Baskaran, 2021; Singh et al., 2021).

1.2.5 Vitamins and minerals

Vitamins not only play an important role in their own metabolism, but also play an important role in human and animal health (Gohara-Beirigo et al., 2022). Microalgae are

a promising source of vitamins such as provitamin A (β-carotene), vitamins B, C and E, as well as folic acid, inositol, and biotin (Zhou et al., 2022). Functional foods from *Spirulina Arthrospira* and *Chlorella* are popular as supplements because they are rich in vitamin B, A, E and K (Karkos, Leong, Karkos, Sivaji, & Assimakopoulos, 2011). The content and type of vitamins are related to the type of algae. For example, brown and green algae are generally higher in vitamin C than red algae, such as *Enteromorpha flexuosa* and *Ulva fasciata* (300 mg/100 g and 220 mg/100 g DW, respectively) (Škrovánková, 2011). Algae such as *Ulva* and *Antarctic kelp* contain high-quality fat-soluble vitamin E (tocopherols, in the α-, β-, γ-, and Δ- forms), which have been shown to have strong antioxidant properties (Ortiz et al., 2006). *Isochrysis galbana* contains vitamin A, B1, B2, B3, B5, B6, B12, E, and biotin, while *Euglena gracilis* Z contains provitamin A, vitamin C and E (Zhou et al., 2022). Moreover, microalgae are rich in minerals, including calcium (Ca), sodium (Na), potassium (K), magnesium (Mg), etc., which are beneficial to human health. Specifically, minerals may play key roles in several physiological processes in the body, and their deficiency can lead to disorders or disease symptoms (Eggleston et al., 2022; Michos et al., 2021; Gohara-Beirigo et al., 2022; Salnikow, 2021). Additionally, microalgae-organic minerals can improve metabolic performance and health to a greater extent than inorganic minerals (Pomport, Warren, & Taylor-Pickard, 2021).

1.3 Microalgae nutrients and bioactive compounds downstream processes

Microalgae are rich in nutrients and bioactive compounds, such as protein, carbohydrates, lipids, vitamins, minerals, polyphenols, carotenoids, etc. The cell wall structure of microalgae hinders the release of biomass from the cytoplasm. Choosing proper extraction and separation techniques can improve the extraction yield and protect the biological activity of the target compound. Some traditional methods, including Soxhlet

extraction, hot water extraction, organic reagent/acid-base extraction, etc., have been used to recover bioactive compounds from microalgae. However, these methods have disadvantages such as large consumption of reagents and long extraction time (Barba, Grimi, & Vorobiev, 2014). In recent years, more and more innovative technologies have been used to extract and separate bioactive compounds from microalgae, and satisfactory results have been achieved.

1.3.1 Green Extraction Technologies

Pulsed Electric Fields (PEFs)

As a non-thermal treatment method, pulsed electric field (PEF) has been widely used in the food industry, including the extraction of bioactive compounds, food preservation and drying (Barba et al., 2015). The main principle of PEF is the "electroporation" theory, that is, applying high-pressure PEF to treat the sample for a short time, causing the cell membrane to rupture and form temporary/permanent pores (pores), thereby increasing the permeability of the membrane (Zhou et al., 2022). The application of PEF may cause changes in the electrical conductivity and rigidity of the microalgae cell wall, thereby improving the extraction rate. Our previous research used PEF to enhance the extraction efficiency of proteins, carbohydrates, polyphenols, etc. from *Spirulina. sp* and *Chlorella. sp* and found the damaging effect of PEF on microalgae cells under fluorescence microscopy (FM) and scanning electron microscopy (SEM) (Wang, Zhou, et al., 2023; Zhou, Wang, et al., 2022). At present, the application of PEF to improve the extraction rate in the food and health products industry is still in its infancy.

Pressurized Liquid Extraction (PLE)

Pressurized Liquid Extraction (PLE), also known as Accelerated Solvent Extraction (ASE), is a technique that uses a combination of temperature and pressure to extract solid or semi-solid samples, which can better retain biologically active compounds and shorten

the extraction time, has been extensively studied (Ruiz-Domínguez et al., 2021). PLE is performed under conditions of temperature and pressure high enough to keep the solvent in a liquid state above the boiling point and below the critical point. The polarity and dielectric constant of the solvent change in this state, which can achieve better extraction effect (Zhou et al., 2022). The use of pressure keeps the solvent below its boiling point and maintains a high fluid density, which aids in elution resistance, forcing the solvent to penetrate into areas normally inaccessible under atmospheric conditions, thereby facilitating extraction (Mustafa & Turner, 2011). The high temperature environment will change the physical and chemical properties of the solvent, reduce the surface tension and solvent viscosity, and increase the diffusion rate of the analyte to the solvent (Plaza & Turner, 2015).

Ultrasound extraction (USN)

Ultrasound (USN) has been recognized as a green, emerging, and non-toxic technology as it expands the use of green solvents (GRAS) by replacing conventionally used organic solvents (Rao et al., 2021). Furthermore, the chemical-free operation and the absence of toxic residues creates an environmentally friendly method that meets the goals of a clean and green extraction process. USN is widely used in the extraction of biomass, mainly relying on cavitation induced by ultrasound (Saini & Keum, 2018). When ultrasonic waves are applied, the solvent is cavitated to produce microbubbles, which rupture after absorbing the ultrasonic energy, and these microbubbles, when they contact the cells and rupture, generate a large shear force to damage the cell wall. Acoustic cavitation can cause different changes in plant cell tissue, such as matrix fragmentation, cell erosion, pore formation, increased absorption, shear force, and changes in swelling index (Rao et al., 2021). Cavitation generates shock waves, microjets, shear forces, and turbulence, which in turn cause changes in the plant matrix and accelerate extraction. USN destroys the cell

wall of microalgae through cavitation to improve the extraction efficiency of nutrients and is currently an ideal extraction technology.

Other extraction technologies

In addition, microwave extraction (MWE), supercritical fluid extraction (SFE), bead milling (BM), etc. are also extraction technologies that are used for the recovery of microalgae nutrients. MWE is considered an effective cell disruption technique and is commonly used as a pretreatment technique in the extraction of microalgae biomass. During microwave treatment, heat is transferred from the inside to the outside so that the entire sample is heated to facilitate the extraction process (Zhou et al., 2022). SFE has also been used as a promising technology to recover nutrients from microalgae. Commonly used SFE extraction reagents include carbon dioxide (CO_2), methanol, ethanol, pentane, ammonia water, etc., which are mainly used to extract fat-soluble components such as lipids and carotenoids in microalgae (Zhou et al., 2021). The BM method can achieve different degrees of destruction depending on the operating conditions, from selective deconstruction to disintegration. The bead beating method has the advantage of being very effective in destroying various microalgae without destroying the properties of the biomolecules (Liu et al., 2021).

1.3.2 Separation process

Efficient utilization of microalgae requires the separation of microalgae extracts into various useful compounds by applying different biochemical processes. In this process, the separation and purification process of specific components of microalgae is particularly important, and all biomass components need to be fractionated, which requires a multi-step purification method. At present, the separation and purification process of microalgae biomass is complex and inefficient, and there is still a long way to go from industrial-scale production to commercial product application.

Microalgae lipids purification

Microalgae lipids are quite different from traditional edible lipids. For example, microalgae lipids have high polar lipid content, high viscosity, poor fluidity, high acid value, and high pigment content (dark green) (Xue et al., 2018). Microalgae crude lipids contains some peptizing impurities, such as phospholipids, proteins, mucus, etc. The existence of these peptizing impurities will reduce the storage stability and edible value of lipids and affect the purification process (Paisan, Chetpattananondh, & Chongkhong, 2017). The purification process of microalgae crude oil usually includes degumming, deacidification, decolorization and so on. Specifically, the degumming step is mainly to remove sticky substances such as phospholipids, which will lead to a sharp decline in the nutritional value of algae lipids. There are many methods for crude oil degumming, such as hydration method, acid method, adsorption method, enzymatic method, membrane method, etc., and hydration method and acid method are the most commonly used (dos Passos et al., 2022). The deacidification step is used to remove free fatty acids and may also lose some useful lipids, while the depigmentation step is used to remove pigment and lose a lot of polar lipids, resulting in lower EPA content.

Microalgae protein purification

Microalgae protein concentrates or isolates can be used in various industrial applications such as products with gelling, foaming and emulsifying properties. However, high-quality protein purification from microalgae remains a technical challenge for the following reasons. i) the rigid cell walls of microalgae lead to limited protein availability; ii) high concentrations of anionic or non-polar polysaccharides in the extract; iii) inherent problems related to protein stability. Currently, challenge 'i' can be solved by applying techniques such as PEF, PLE, USN, etc., while for challenges 'ii' and 'iii', separating proteins while maintaining protein stability becomes a key challenge (Anjos, Estêvão,

Infante, Mantecón, & Power, 2022). Trichloroacetic acid (TCA) and low pH can be used for protein sedimentation to achieve protein separation. However, it causes protein denaturation, which eliminates proteolytic and degradative activity and makes resolubilization of precipitated proteins extremely difficult (Khanra et al., 2018). The ammonium salt precipitation method (ASP) can also be used for the separation and purification of C-phycocyanin (C-PC) from the wet biomass of *Spirulina platensis*, which is simple in operation and low in cost (Amarante, Braga, Sala, Moraes, & Kalil, 2020). In addition to TCA/low pH/ASP, there are many techniques that can be used to further purify microalgae proteins, such as column chromatography, gel chromatography, ion exchange chromatography, high performance liquid chromatography, etc. Furthermore, membrane separation of phycocyanin from *Spirulina platensis* offers several advantages over chromatography, including high volumetric throughput, high extract concentration, and lower cost (Chaiklahan, Chirasuwan, Loha, Tia, & Bunnag, 2011). However, precipitation method, chromatography technology, or membrane separation technology all have some deficiencies. For example, the precipitation method takes a long time and cannot be promoted on a large scale; the chromatography technology is costly and the protein yield is low; the membrane separation efficiency is affected by multiple factors such as membrane resistance, porosity, morphology, shear rate and hydrodynamics (Azmi et al., 2021).

Microalgae polysaccharides purification

Microalgae polysaccharide purification is an important step in enriching polysaccharide species or isolating polysaccharide fractions for usable, safe, and reproducible research. During the purification process of polysaccharides from microalgae, the appropriate technology should be selected according to the molecular weight distribution (MWD), charge characteristics, affinity characteristics and other properties of polysaccharides.

Methods for separating microalgae polysaccharides include ethanol precipitation, salt fractionation, quaternary ammonium salt coordination, ultrafiltration, ion exchange column chromatography, gel column chromatography and affinity chromatography, etc. (Beaumont et al., 2021). Ethanol precipitation is a commonly used polysaccharide separation method, which dehydrates polysaccharides by reducing the dielectric constant of aqueous solution, resulting in polysaccharide precipitation. However, the ethanol precipitation method has the disadvantage of poor separation selectivity of polysaccharides, and the precipitation process needs to be repeated to improve the purity of polysaccharides and reduce the yield of polysaccharides (Shi, Yan, Cheong, & Liu, 2018). Compared to traditional polysaccharide purification methods such as ethanol precipitation and Sevag deproteinization, the aqueous two-phase system (ATPS) has the advantages of simple operation, short time consumption, and less amount of organic reagents, and can be used as an effective way to separate water-soluble polysaccharides (α-acidic polysaccharide) from *Spirulina platensis* (Wu, Li, Zhao, & Liu, 2017).

1.4 Microalgae bioactive compounds: Effects on human gut microbiota

Microalgae biomass has attracted attention as a promising alternative source of prebiotic ingredients due to the presence of oligosaccharides, polysaccharides, and phenolic compounds (Barros de Medeiros, da Costa, da Silva, Pimentel, & Magnani, 2022). Prebiotics are biochemical compounds that can be metabolized by intestinal flora, which are resistant to digestion and absorption by acids and enzymes in the upper gastrointestinal tract. In this way, it is fermented by the gut microbiota (GM) and stimulates their growth, improving their activity and the health of the host. Consumption of prebiotics may modulate bacterial composition and metabolism through production of short-chain fatty acids (SCFAs), tryptophan, and organic acids, with beneficial effects including defense against pathogens, modulation of immune responses, mineral

absorption, improved intestinal function, metabolism, and satiety (Gibson et al., 2017). Prebiotics are originally considered nondigestible carbohydrates, but a number of biomolecules, including oligosaccharides, fermentable fibers, polyphenols, noncarbohydrate substrates, and fatty acids, have been shown to act as prebiotics (Cunningham et al., 2021). The prebiotic compounds currently studied are mainly inulin-type fructans, such as fructo-oligosaccharides (FOS), and the utilization of non-conventional prebiotic ingredients has become a new trend. For example, a recent study found that *Spirulina* PEF extracts promote the growth of *Lactobacillus rhamnosus*, whose metabolomic profile is particularly rich in SCFAs and organic acids (3-phenyllactic acid) (Ricós-Muñoz et al., 2023). Bruna et al. (2017) isolated oligosaccharide samples from *Chlorella vulgaris* and *cyanobacterium Arthrospira platensis* as a carbon source for probiotic animal *Bifidobacterium* and *Lactobacillus casei,* contributing to the growth of beneficial bacteria and SCFAs (such as lactic acid and acetic acid) generation (Leal et al., 2017).

Bruna et al. (2017) isolated oligosaccharide samples from *Chlorella vulgaris* and *Arthrospira platensis* as a carbon source for probiotic animal *Bifidobacterium* and *Lactobacillus casei,* contributing to the growth of beneficial bacteria and SCFAs (such as lactic acid and acetic acid) generation (Leal et al., 2017).

Currently, studies considering the role of microalgae prebiotics are still scarce, mainly evaluating the effect of isolated saccharides from microalgae on the growth of specific probiotic cultures, and the research on the effect of microalgae components on the human GM is far from enough.

Table 1. Studies on algae nutrients as potential prebiotics

Microalgae	Compounds	Study	Dosage	Microbiota analysis	Effects on GM and SCFAs level	Ref.
Spirulina platensis	Oligosaccharides	Human	10% (v/v)	Fecal samples 16S rRNA sequencing	Isobutyric↑, Isovaleric↑ *Dialister*↑ *Bacteroides*↑ *Megamonas*↑ *Escherichia-Shigella*↑ *Megasphaera*↑ *Blautia*↑ *Holdemanella*↑ *Collinsella*↑ *Phascolarctobacterium*↑ *Bifidobacterium*↑ *Prevotella_9*↓	(Cai et al., 2022)
Spirulina platensis	Crude polysaccharides	Mice	150 mg/kg/d	Cecal Samples qRT-PCR	Acetic acid↑, Isobutyric↑, n-butyric acid↑ *Bacteroides*↑ *Escherichia*↓ *Shigella*↓ *Corynebacterium*↑ *Alloprevotella*↑ *Romboutsia*↓ *Paraprevotella*↑ *Atopostipes*↑ *Flavonifractor*↑ *Jeotgalicoccus*↑	(Li et al., 2021)
Spirulina platensis	Phycocianin	Mice	50 mg/kg/d	Fecal samples 16S rRNA sequencing	*Bacteroidetes*↑ *Actinobacteria*↑ lipopolysaccharide level↓	(Li et al., 2020)
Undaria pinnatifida	Fucoidan	Mice	100 mg/kg/d	Cecal samples 16S rRNA sequencing	*Bacteroidetes*↑ *Firmicutes*↓ Serum total cholesterol was regulated	(Chen et al., 2019)
Red algae	κ-/ι-/λ-carrageenan	Mice	20 mg/L	Cecal samples 16S rRNA sequencing	Body weight↑ Blood sugar↑ *Akkermansia*↑ and SCFAs↑	(Shang et al., 2017)
Eisenia bicyclis	Laminaran	Mice	2% (w/w)	Cecal samples 16S rRNA sequencing	*Bacteroidetes*↑	(Takei et al., 2020)

Table 1. (cont.)

Microalgae	Compounds	Study	Dosage	Microbiota analysis	Effects on GM and SCFAs level	Ref.
Chlorella pyrenoidosa	Polysaccharide	Worms	10 mg/mL	Cell lysis 16S rRNA sequencing	*Faecalibacterium*↑ *Haemophilus*↑ *Vibrio*↑ *Shewanella*↑	(Wan et al., 2021)
Sargassum henslowianum	Polysaccharides	Human	20 mg/mL	Fecal samples 16S rRNA sequencing	Acetic↑, Propionic↑, n-butyric acids↑ *Enterobacteriaceae*↑, *Haemophilus parainfluenzae*↓ *Gemmiger formicilis*↓	(Cui et al., 2021)
Gracilaria lemaneiformis	Polysaccharides	Human	4 mg/mL	Fecal samples 16S rRNA sequencing	SCFAs↑ *Bacteroides*↑	(Sun et al., 2022)
Brown algae	Alginates	Mice and *in vitro* cells model	2% (w/v)	Liver and spleen samples HT-29-Luc cell model	The infection of mice liver and spleen of mice was reduced↓	(Kuda et al., 2017)

1.5 Safety of microalgae in food applications and their future in food and feed

Microalgae are rich in bioactive components and are a potential source of nutritionally balanced foods, which are in high demand for human diets and can be considered for use in the food production chain in the future. Microalgae with GRAS (Generally Recognized As Safe) status according to the U.S. Food and Drug Administration (FDA) include *Spirulina platensis*, *Chlorella vulgaris*, *Dunaliella bardavelta*, *Chlamydomonas reinhardtii*, *Chlorella protothecoides* and *microphthalmia* (Torres-Tiji, Fields, & Mayfield, 2020). Microalgae-based products can be widely consumed by the general public, especially vegetarians, vegans, and the elderly. Microalgae offer the possibility to develop new flavors and colors of food, as well as provide a wider choice of new food supplements (Lafarga et al., 2021). Microalgae biomass also can be used to develop different types of food, such as 3-D printed food (Uribe-Wandurraga et al., 2021), and attract consumers and industries to develop new products.

The optimization of the microalgae cultivation process, the use of reagents and the downstream processing is crucial to the quality and safety control of microalgae products. The standardization of microalgae cultivation system and processing is the requirement to establish the level of safe and toxic substances. Standardization of microalgae nutrient composition will become possible with advances in processing technology, and this is a rapidly developing field (Ferreira de Oliveira & Bragotto, 2022). The presence of potentially toxic chemicals in microalgae products may pose a risk to human health, and a broader and integrated approach, such as risk analysis, should be used to better address this issue. In addition, studies to quantify chemical contaminants in food supplements and microalgae biomass foods should be conducted to monitor the levels of these compounds, for which data remain limited or unreported.

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2. OBJECTIVE

2. Rationale and Objectives

This PhD thesis explores the application of innovative extraction technologies such as Pulse electric fields (PEF), Pressurized liquid extraction (PLE) and Ultrasonic extraction (USN) to extract nutrients/bioactives from microalgae (*Spirulina* (*Arthrospira platensis*), *Chlorella* (*Chlorella Vulgaris*), *Phaeodactylum tricornutum*). The polysaccharide fraction of the microalgae extract is further separated using ultrasound + membrane separation technology. Moreover, the biological activities of microalgae extracts, such as antioxidative, antiinflammatory, and antimicrobial properties, and the effects on the growth of human gut microbiota are also explored. Finally, the properties of algae polysaccharides to improve food quality were preliminarily explored, aiming to provide a reference for the future application of microalgae bioactive compounds in the food industry.

The **general objective** of this PhD thesis is to optimize innovative technologies (PEF, PLE, USN) to extract nutrients and bioactive compounds from microalgae and to evaluate their biological activity and their potential impact on human gut health. Moreover, to evaluate the impact of algae polysaccharides on food quality, these objectives can provide critical theoretical evidence for the application of microalgae in food, medicine, etc., and promote the sustainable development of the microalgae industry.

The specific objectives are:

i) Optimizing the microalgae bioactive compounds extraction process based on PEF, PLE and USN technology.

ii) Establish a method for separating specific bioactive compounds (polysaccharide components) from microalgae based on USN combined with membrane separation technology.

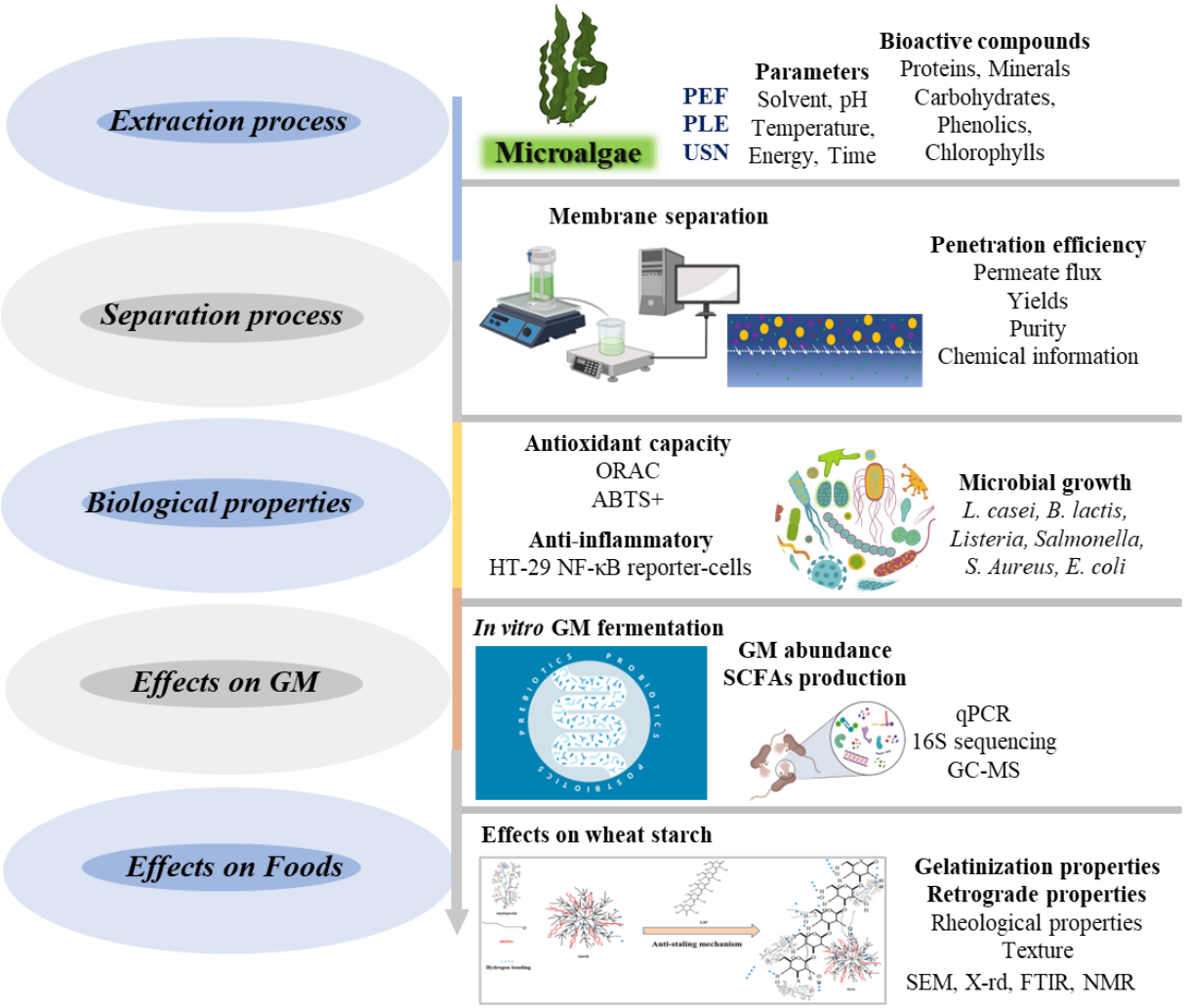
iii) Evaluate the biological and functional activities of microalgae extracts, including antioxidative, antiinflammatory, and antimicrobial properties., etc.

iv) Evaluation of microalgae extracts as potential sources of prebiotics using an *in vitro* human fecal microbial fermentation model.

v) Preliminary evaluation of the effect of algae polysaccharides on food quality (wheat starch products)

3. EXPERIMENTAL PROCESS

Experimental process



4. RESULTS

4.1 Impact of pressurized liquid extraction and pH on protein yield, changes in molecular size distribution and antioxidant compounds recovery from *Spirulina*

Journal: *Foods*, 2021, 10(9), 2153; https://doi.org/ 10.3390/foods10092153

Impact of pressurized liquid extraction and pH on protein yield, changes in molecular size distribution and antioxidant compounds recovery from *Spirulina*

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Abstract

The research aims to extract nutrients and bioactive compounds from *Spirulina* using a non-toxic, environmentally friendly, and efficient method-Pressurized Liquid Extraction (PLE). In this work, Response Surface Methodology (RSM)-Central Composite Design (CCD) was used to evaluate and optimize the extraction time (5-15 minutes), temperature (20-60 °C) and pH (4-10) during PLE extraction (103.4 bars). The multi-factor optimization results of the RSM-CCD showed that under the pressure of 103.4 bars, the optimal conditions to recover the highest content of bioactive compounds were 10 minutes, 40 °C and pH 4. Furthermore, the compounds and antioxidant capacity of PLE and non-pressurized extraction extracts were compared. The results showed that under the optimal extraction conditions (10 minutes, 40 °C and pH 4), PLE significantly improved the antioxidant capacity (2870.5 ± 153.6 μM TE), protein yield (46.8 ± 3.1 %), chlorophyll a (1.46 ± 0.04 mg/g), carotenoids (0.12 ± 0.01 mg/g), total polyphenols (11.49 ± 0.04 mg/g) and carbohydrates content (78.42 ± 1.40 mg/g) of the extracts compared with non-pressurized extraction ($p < 0.05$). The protein molecular distribution of the extracts was analysed by SDS-PAGE and the results showed that there were more small-molecule proteins in PLE extracts. Moreover, Triple TOF–LC–MS–MS was used to analyse the phenolic profile of the extracts, and the results showed the extracts were rich on phenolic compounds, such as p-coumaric acid and cinnamic acid being the predominant phenolic compounds in the PLE extract. This indicates that PLE can promote the extraction of bioactive compounds from *Spirulina*, which is of great significance for the application of PLE technology to obtain active sub-stances from marine algae resources.

Keywords: Microalgae, PLE, RSM-CCD, SDS-PAGE, triple TOF–LC–MS–MS, bioactive compounds

1. Introduction

Functional foods are generally foods rich in ingredients that can provide or promote specific beneficial effects for human health. These ingredients, such as proteins, lipids, vitamins and trace elements, can be derived from microalgae (Finkel et al., 2016; Rangel-Yagui, Danesi, De Carvalho, & Sato, 2004). There are more than 1,000 kinds of algae in the ocean, and they are used as an important food source in many countries, such as China, Japan, and South Korea. Microalgae have been also introduced into Western countries as a natural food rich in nutrients (Gomez-Gutierrez, Guerra-Rivas, Soria-Mercado, & Ayala-Sánchez, 2011; Wang, Zhang, & Fang, 2019).

Among the numerous species of algae, *Spirulina* (*Arthrospira platensis*), a cyanobacteria, has been extensively studied due to its commercial importance as a source of proteins, vitamins and fatty acids (Zhang, Zhang, & Chen, 1999). Moreover, according to recent reports, *Spirulina* has the highest protein content (70% on a dry basis) among algae organisms and contains a large amount of essential amino acids (Danesi et al., 2002; Morist et al., 2001). At present, *Spirulina* is used worldwide as a source to extract biological compounds, mainly protein, pigments and compounds with antioxidant properties (Pereira & Meireles, 2010). Therefore, *Spirulina* has the potential to provide solutions for food, pharmaceutical and cosmetic industries. However, many processes are required before microalgae can be used in these industries, involving microalgae cultivation, functional ingredients recovery, and safety evaluation of the ingredients recovered (Torres-Tiji, Fields, & Mayfield, 2020). At present, the cultivation technology of microalgae has become mature. Therefore, the main problem in practical industrial applications is how to use green, highly efficient, and low-cost extraction methods to make the bioactive compounds of *Spirulina* useful in the industry.

Traditional extraction methods include dipping (soaking), percolation, countercurrent extraction and Soxhlet extraction (Hosikian, Lim, Halim, & Bio, 2010; Lee et al., 2010).

However, the rigid cell wall structure of *Spirulina* is disadvantageous for traditional extraction methods (Parniakov et al., 2015). Traditional methods increase the extraction temperature (over 100 °C) and volume of organic reagents (chloroform, ether, etc.) and extend the extraction time to achieve the purpose of obtaining algae biomass (Wang & Weller, 2006). Although heating and organic solvents increase the yield of the target product, it has a negative impact on the substances obtained, such as destroying the structure and function of some compounds (Barba, Grimi, & Vorobiev, 2014). Therefore, a growing interest regarding the use of low-temperature, non-toxic, and efficient extraction techniques has been shown.

Compared with traditional extraction technology, the Pressure Fluid Extraction (PLE) method has advantages in protecting the functional ingredients and shortening the extraction time (Ji et al., 2020; Mustafa & Turner, 2011). PLE is a fully automatic extraction technology that uses a combination of elevated temperature and pressure to obtain bioactive compounds in a short time. In addition, PLE can process multiple samples in the same batch with a low reagent usage (Pearson, Cornish, McMahan, Rath, & Whalen, 2010). PLE has been used in the fields of food and medicine and achieved good results (Kang, Kim, & Moon, 2016; Saha, Walia, Kundu, Sharma, & Paul, 2015). At this respect, a study used Irish seaweed *Ascophyllum nodosum*, *Fucus vesiculosus* and *Fucus serratus* as the extraction raw materials, 80% ethanol aqueous solution as the extraction reagent, and pressure solvent extraction (1000 psi) as the extraction method, and obtained extracts with high antioxidant capacities, based on their ability to protect against oxidant-induced DNA damage (O'Sullivan et al., 2013). Similarly, Denery et al. used pressurized extraction solvent technology (1500 psi, 40 °C, 10 minutes) to extract astaxanthin from *Haematococcus pluvialis* and reached up to 9.5 mg/g and 8.4 mg/g of astaxanthin recovered with acetone and ethanol, respectively (Denery, Dragull, Tang, & Li, 2004).

However, to the best of our knowledge, there are few reports using PLE to obtain bioactive compounds from *Spirulina*. Research at this respect is mainly focused on ultrasound, microwave assisted extraction and pulsed electric fields (Silva et al., 2017; Herrero et al., 2005; Parniakov et al., 2015; Prabakaran, Sampathkumar, Kavisri, & Moovendhan, 2020). Therefore, using PLE as an extraction technology to obtain active substances from *Spirulina* and optimizing the extraction process to get as many functional ingredients as possible have important economic value and significance for the utilization of *Spirulina* resources.

In this study, PLE was used to extract *Spirulina* nutrients and bioactive compounds using deionized water at different pH (4-10), mild temperatures (20-60 °C), and short extraction times (5-15 minutes) with a constant pressure of 103.4 bars. Further, the compounds (protein, carbohydrate, chlorophyll a, carotenoids, polyphenol) and the antioxidant properties of the extracts obtained were analyzed.

2. Materials and Methods

2.1 Chemicals and reagents

AAPH [2,2′-Azobis(2-methylpropionamidine) dihydrochloride], Folin–Ciocalteu reagent, gallic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), D-glucose, phenol reagent, fluorescein sodium salt and potassium persulfate ($K_2S_2O_8$) were purchased from Sigma–Aldrich (Steinheim, Baden-Württemberg, Germany). Sodium carbonate (Na_2CO_3) was acquired from VWR (Saint-Prix, France). SDS (sodium dodecyl sulfate, purissimum-CODEX) was obtained from Panreac (Barcelona, Spain), Sodium hydroxide, glacial acetic acid, and sulfuric acid were supplied by Fisher Scientific (Madrid, Spain). Acetonitrile (HPLC grade), acetone, glycerol, and bromophenol blue indicator (ACS reagent) were supplied by Merck (Darmstadt, Germany). The Silica (SiO_2) was purchased from Supelco Analytical™, diatomaceous earth and other materials for generation of PLE extracts were purchased from Dionex (Dionex, Leeds, UK).

2.2 Samples

Spirulina biomass comes from *Arthrospira platensis* species, strain paracas 15016, being Paracas the lake where it originally comes from (Lima, Peru). Cultivation took place at Eco*Spirulina* company (Serra, Valencia, Spain) in raceway ponds using a greenhouse under natural sunlight without any artificial light added. Shadow nets partially covered the cultivation ponds, thus allowing to control phytopigments production. At the time of the experiment, day-time temperature was 32 °C on average, while temperature decreased to 24 °C at night. Culture's pH varied between 9.8 and 10.4. It was regulated by the addition of CO_2 at the time of harvesting, which took place daily. Then, biomass was filtered using a tambor filter of 31 micra mesh (6-10 rpm). Cultivation medium went back to the cultivation pond, while biomass was vacuum-pressed and then frozen in 50 g portions. Frozen biomass was used to carry out the experiments at the laboratory of Nutrition and Food Science, Faculty of Pharmacy, Universitat de València.

2.1. PLE extraction process

Spirulina samples had a moisture content of 74.2 ± 2.1% (w/w). After freeze-drying, they were transferred into a grinder for pulverization for subsequent experiments. Before PLE extraction, *Spirulina* was pretreated according to the experimental method previously reported by O'Sullivan et al (O'Sullivan et al., 2013). Five hundred milligrams of *Spirulina* sample were mixed with 1.5 g silica in a ratio of 1:3 (w/w) and 1.0 g diatomaceous earth in a ratio of 1:2 (w/w). After mixing well with a mortar, all the mixture was transferred into the PLE extraction cell. Before the extraction, an initial heating up of the cell was performed and the cell was rinsed by the extractant reagent. The extraction reagent was purged from the cell under a nitrogen pressure of 103.4 bars. Cleaning procedures were used between different groups to prevent residual extracts. After the extraction procedure was completed, the sample in the collection bottle was

transferred to 15 mL centrifuge tubes. The sample was stored at -20 ºC for subsequent experimental analysis.

2.2. Total protein determination

The total protein content in the sample was tested by Kjeldahl method. Briefly, 2 mL of *Spirulina* extract, 3 g of potassium thiosulfate, 6 zeolites, 5 drops of copper sulfate and 5 ml of concentrated sulfuric acid were added to the nitrification tube. Then it was heated at 120 °C until the color was clear and bright. Automatic Kjeldahl nitrogen analyzer was used to collect ammonia. Finally, the distillate was collected in a boric acid solution (2.0% w/v) and titrated using a hydrochloric acid solution (0.014 mol/L), methyl orange was used as the end point indicator of the titration. The formula previously described by de Lourdes Mendes Finete et al. was used for calculating the nitrogen contents (Finete, Gouvêa, Marques, & Netto, 2013), using the conversion factor of protein content according to Grossmann et al. (Grossmann, Lutz Ebert, Sandra Hinrichs, Jörg Weiss, 2018). The calculation formula of nitrogen and protein content are as follows:

$$\text{Nitrogen \%} = (\text{mL Standard acid- mL Blank}) \times \text{N of acid} \times 14/(\text{Weight of sample}) \quad (1)$$

$$\text{Protein \%} = 6.5 \times \text{Nitrogen \%} \quad (2)$$

2.3. Total carbohydrate content determination

The carbohydrate content was tested by the concentrated sulfuric acid-phenol method (Zhang, et al., 2020). Specifically, 1 mL of sample, 0.5 mL of phenol and 2.5 mL of concentrated sulfuric acid were sequentially added to a glass reaction flask and reacted for 30 minutes at room temperature, then the absorbance value at 490 nm was measured by spectrophotometry. D-glucose was used as a standard for calculating carbohydrate content.

2.4. Pigments determination

The determination of the pigments (chlorophyll a and carotenoids) was carried out according to the method previously described by Parniakov et al (Parniakov et al., 2015)

with light modifications. After the sample was thawed, it was centrifuged at 4 °C, 18407 g for 5 minutes, and the absorbance value was measured at the wavelengths of 665.2 and 470 nm, respectively. The calculation of chlorophyll a, and carotenoids contents were according to the equations previously described by Wellburn et al. (Wellburn, 1994).

$$C_{Cha} = 12.6 \times A665.2 \quad (3)$$

$$Ccr = (1000 \times A470 - 1.63 \times Ccha) / 221 \quad (4)$$

Where C_{Cha}, and C_{cr} are the concentrations of chlorophyll a, and carotenoids respectively.

2.5. Polyphenols determination

The total polyphenol content in the sample was analyzed using the Folin-Ciocalteu method (Parniakov et al., 2015). Specifically, 0.2 mL of *Spirulina* extract, 1 mL of Folin-Ciocalteu solution (dilution with water, 1:10, v/v) and 0.8 mL sodium carbonate solution (75 g/L) were mixed and was reacted for 10 min in a water bath at 50 °C under darkness. Finally, the absorbance value of the reaction solution at 750 nm was measured by spectrophotometry. Gallic acid was used to calibrate the total polyphenol content in the sample. The identification and quantification of the major phenolic compounds present in the *Spirulina* extracts was carried out on TripleTOF™ 5600 (ABSCIEX) LC/MS/MS system equipped with Agilent 1260 Infinity (Agilent, Waldbronn, Germany). Chromatographic separation was performed on a Waters UPLC C18 column 1.7 µm (2.1 × 50 mm) Acquity UPLC BEH.C18 from Waters (Cerdanyola del Vallès, Spain). The mobile phase was composed of water (0.1% formic acid, A) and methanol (0.1% formic acid, B). The gradient elution of the mobile phase was as follows: from 0 to 13 min, 90% (A) and 10% (B); from 13 to 15 min, 100% (B); from 15.1 to 22 min, 90% (A) and 10% (B). The flow rate and injection volume were 0.4 mL/min and 5 µL, respectively. The MS acquisition was under a mass range of 80-1200 m/z. The calibration was performed applying an external calibration delivery system, which infuses the calibrating solution before sample introduction. The MS was operated using an Information Dependent

Acquisition (IDA) method with the survey scan type (TOF-MS) and the dependent scan type (Product Ion) at -50 V of collision energy. The MS parameters were as follows, ion spray voltage of -4500 V, declustering potential of 90 V, collision energy of -50 V, temperature at 400 °C with curtain gas of 25 psi, ion source gas 1 at 50 psi and ion source gas 2 at 50 psi, IDA MS/MS was performed using the following criteria: ions that exceeded 100 CPS, ion tolerance 50 MDa, collision energy fixed at 25 V and dynamic background subtraction activated. The quantification was based on the use of standards of the polyphenols corresponding to the main groups of compounds identified.

2.6. Antioxidant capacity

The Oxygen radical absorbance capacity (ORAC) was used to determine the antioxidant capacity of the extract. The method previously described by Du et al. with slight modifications was used (Du & Xu, 2014). AAPH was used as a peroxide generator, and Trolox as an antioxidant standard. Each sample was added to 5 wells in parallel and repeated 3 times. The microplate reader was programmed to record the fluorescein fluorescence of each cycle. Kinetic readings were recorded as 45 cycles, and each cycle was set to 60 s. PBS (75 mM, pH 7.4) was used as a blank and to configure Trolox standards. Briefly, 50 µL of extract and 50 µL of fluorescein solution were added to the 96-well plates and was incubated in a microplate reader for 30 minutes, then 25 µL peroxide AAPH was added to the 96-well plates to initiate the oxidation reaction. To calculate the ORAC value, the linear equation between the Trolox standard or sample and the net area under the fluorescence decay curve was used. The calculation formula was as follows:

$$\text{ORAC (Trolox)} = (A_{Sample} - A_{Blank})/(A_{Trolox} - A_{Blank}) \quad (5)$$

Trolox equivalent antioxidant capacity (TEAC) referred to related literature and slightly modified was used to evaluate the antioxidant capacity of the extract (Agregán et al., 2017). Briefly, mix 25 mL of 7 mM 2,2'-azidobis-(3-ethylbenzothiazolin-6-sulfonate)

(ABTS) with 440 µL of 140 mM potassium thiosulfate solution and store the mixed solution in the dark at room temperature for 12 to 16 hours as working solution. Dilute the working solution with 96% ethanol to keep the absorbance value between 0.700 ± 0.020. During the test, mix 0.1 mL sample or standard solution with 2 mL working solution, and after reacting for 3 minutes in a dark room, read the absorbance value of the reaction solution at 734 nm. Trolox at different concentrations was used as the standard product for antioxidant capacity calibration.

2.7. Protein molecular size distribution (SDS-PAGE gels)

To evaluate the protein molecular size distribution, the method described by Jong et al. was used with slight modifications (Jong, Thien, Yong, Rodrigues, & Yong, 2015). Firstly, the buffer was prepared. For this purpose, 0.5 g of SDS, 2.46 g of Tris-HCl, 6.25 mL of glycerol, 2.5 mL of bromophenol blue reagent and 13 mL of water were combined in the beaker and mixed well using a magnetic stirrer under darkness conditions. Then, 8 mg of Dithiothreitol (DTT) was added to 500 µL of the sample buffer, named as 'A'. Fixed working solution was configured as follows, 400 mL methanol + 100 mL acetic acid + 500 mL water. The decolorizing solution was 200 mL methanol + 100 mL acetone + 700 ml deionized water. Moreover, to prepare the electrophoresis solution, 0.5 g SDS, 7.2 g Glycine and 1.515 g Trizma base were dissolved in 1000 mL distilled water. 100 µL of *Spirulina* extract were taken and mixed with 400 µL of acetone to prepare the gels. Then, the mixture was vortexed for 10 s and centrifuged at 11000 rpm and 4 °C for 10 minutes. The supernatant was removed and put it in a fume hood for 5 minutes to volatilize the remaining acetone reagent. Then, 100 µL of water was added to rinse the precipitate, and the mixture was immersed in an ultrasound bath at 25 °C for 20 s to fully dissolve the precipitate, named as 'B'. Then, 'A' and 'B' were mixed (20:20, v/v) and heated at 95 °C for 5 min. The Bio-Rad device for gel electrophoresis was used and the Marker and sample loading volumes were 10 µL and 25 µL, respectively. A constant

voltage of 80 V was used, and the electrophoresis was finished when the marker band reached the bottom of the gel. Soak the gel in the fixative for 30 min, then the Coomassie Brilliant Blue dye solution was added and kept for 30 min. Finally, the decolorizing solution was added and kept shaking for 24 h until the protein bands in the gel were clear. Image-J was used to calculate the area of the bands.

2.8. Experimental design and statistical analysis

Three factors (temperature, time, pH) and three levels of the response surface full factorial design experiment were used to optimize the PLE extraction. Two central points were selected to establish the experimental error, a total of 16 experiments were carried out with a random sequence. The experimental variables were temperature: 20-60 °C; time: 5-15 min and pH: 4-10. There are 7 response factors, followed by content of protein, carbohydrates, chlorophyll a, carotenoids, polyphenol and ORAC. The 16 experiments are shown in Table 1.

All results are presented as mean ± standard deviation. SPSS was used to analyze the data for variance analysis and Duncan's test was performed; $p < 0.05$ indicates that the data is significantly different, $p < 0.01$ indicates that very significant difference exists. Image-J software was used for the electrophoresis band analysis, selecting 8-bits as the image type, and the gray level 50%.

3. Results and discussion

3.1 Effect of PLE on high-added-value compounds

In this study, the protein extraction rate, carbohydrate content and antioxidant properties of the extracted samples were analyzed. Table 2 shows the compound content in the 16 extracts under different conditions respectively. The results show that the protein extraction rate ranged from 14% to 48%, observing higher protein yield at 40 °C, 10 min, pH 4 (47.87%) and 40 °C, 10 min and pH 10 (45.81%).

Table 1. Response surface methodology– central composite design

Experiment	Temperature (°C)	Time (min)	pH
1	20	10	7
2	40	10	7
3	40	5	7
4	40	15	7
5	40	10	7
6	60	10	7
7	20	5	4
8	20	15	4
9	40	10	4
10	60	15	4
11	60	5	4
12	20	15	10
13	20	5	10
14	40	10	10
15	60	15	10
16	60	5	10

This is significantly higher than the extraction rate obtained after applying PLE at 20 °C, 15 min, pH 4 (14.02%) and 20 °C, 5 min, pH 10 (14.43%) ($p < 0.05$). From the results obtained, it can be observed that 40 °C seems to be the better temperature to promote protein extraction compared to 20 °C. Moreover, from the obtained regression equation (7), higher temperatures and longer extraction times provided a higher protein recovery, while a negative effect regarding protein extraction was found when pH was high. The model F-value of 5.84 implies the model is significant and there is a 2.36% chance that a Lack of Fit F-value this large could be due to the noise. The optimized conditions for protein extraction are shown in Figure 1, obtaining the optimal extraction conditions after PLE at 42 °C, 10.7 min, pH 10.

$$\text{Protein (\%)} = -4.718 + 3.092 \times T + 6.942 \times t - 18.646 \times pH - 0.040 \times T^2 + 0.019 \times T \times t + 0.010 \times T \times pH - 0.360 \times t^2 - 0.004 \times t \times pH + 1.317 \times pH^2$$

$$(\text{Adjust } R^2 = 0.898,\ P = 0.022) \qquad (6)$$

Table 2. Results of bioactive compounds and antioxidant capacity in *Spirulina* extract

Run	Protein	Carbohydrates	Chlorophyll a	Carotenoids	Polyphenol	ORAC
	%	mg/g	mg/g	mg/g	mg/g	μM TE
1	18.32±1.10^{abc}	12.98±0.62^{bcde}	0.267±0.004^{e}	0.092±0.003^{f}	1.59±0.02^{cd}	480.1±5.5^{c}
2	25.64±0.00^{de}	23.51±0.75^{f}	0.195±0.003^{e}	0.133±0.004^{g}	3.63±0.08^{fg}	365.9±14.9^{abc}
3	22.13±0.00^{cd}	26.15±0.18^{f}	0.283±0.003^{e}	0.137±0.002^{g}	4.64±0.01^{cde}	1491.0±96.4^{d}
4	29.82±0.76^{e}	39.83±0.64^{g}	0.436±0.002^{g}	0.265±0.003^{i}	6.23±0.00^{f}	1595.4±96.4^{d}
5	25.89±0.72^{de}	21.21±0.65^{f}	0.204±0.010^{e}	0.145±0.000^{g}	3.71+±0.08^{f}	419.9±58.9^{bc}
6	19.48±0.00^{bc}	12.58±0.57^{bcd}	0.089±0.001^{b}	0.036±0.002^{d}	1.93±0.01^{b}	359.1±47.8^{abc}
7	15.21±0.49^{ab}	6.90±0.25^{a}	0.110±0.000^{c}	0.031±0.000^{cd}	0.97±0.01^{a}	148.1±7.6^{ab}
8	14.02±1.08^{a}	10.80±0.37^{abc}	0.057±0.001^{a}	0.004±0.000^{a}	0.63±0.04^{a}	129.2±16.1^{a}
9	47.87±1.20^{f}	80.95±2.90^{i}	1.406±0.007^{i}	0.648±0.002^{j}	11.61±0.39^{h}	2915.5±371.4^{e}
10	24.07±1.60^{d}	14.06±1.06^{cde}	0.061±0.001^{a}	0.013±0.000^{b}	2.79±0.17^{cde}	414.2±48.3^{bc}
11	15.15±0.68^{ab}	8.81±0.14^{ab}	0.058±0.001^{a}	0.015±0.001^{b}	1.61±0.06^{b}	247.0±19.2^{abc}
12	15.66±0.51^{ab}	15.55±0.15^{de}	0.225±0.003^{d}	0.085±0.001^{ef}	2.78±0.07^{de}	427.8±30.6^{c}
13	14.43±0.00^{a}	10.55±0.38^{abc}	0.090±0.011^{bc}	0.001±0.000^{a}	2.41±0.02^{g}	280.6±34.7^{abc}
14	45.81±0.58^{f}	58.90±3.02^{h}	0.516±0.008^{h}	0.002±0.000^{a}	10.85±0.19^{h}	2831.1±295.5^{e}
15	25.37±1.21^{de}	14.41±0.40^{cde}	0.058±0.000^{a}	0.029±0.000^{c}	2.75±0.08^{e}	468.5±53.5^{c}
16	19.33±0.00^{bc}	13.04±0.36^{bcde}	0.114±0.001^{c}	0.081±0.001^{e}	2.35±0.06^{c}	485.4±61.6^{c}

Note: The same letters in the same column of data indicate no significant difference ($p > 0.05$), and different letters indicate significant differences ($p < 0.05$).

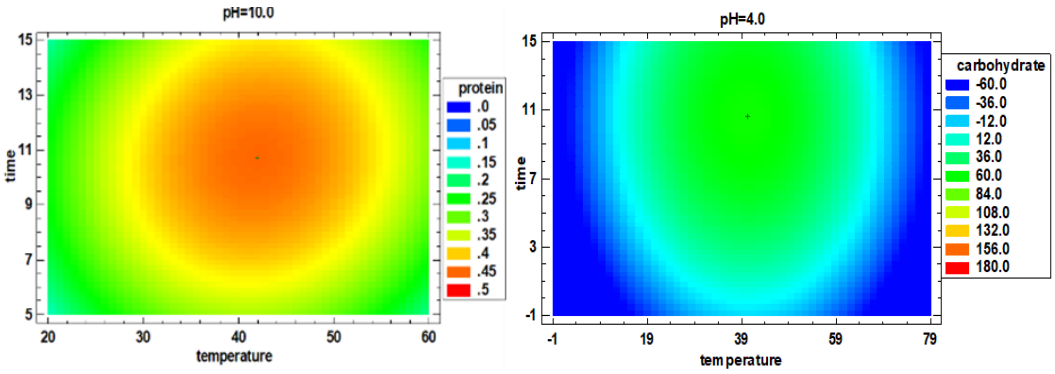

Figure 1. Estimated Response surface of protein extract efficiency (%) and carbohydrate content (mg/g).

The results in Table 2 show that the carbohydrate content of the extracts obtained ranged from 6.9 mg/g to 81 mg/g. The results showed that the extraction amount of carbohydrates at 40 °C, 10 min and pH 4 and 40 °C, 10 min and pH 10 were 80.95 mg/g and 58.90 mg/g, respectively, which was significantly higher than other experiments, especially after PLE at 5 min and pH 4 at 20 °C (6.90 mg/g) or 60 °C (8.81 mg/g) ($p < 0.05$). These results show that changing the extraction temperature and time with the same pH significantly changes the carbohydrate content of the *Spirulina* extracts ($p < 0.05$). From the obtained regression equation (8), similarly to protein recovery, it can be seen an in-creased carbohydrate extract efficiency when temperature and time were increased, while a negative effect was found for pH. The model F-value of 4.45 implies the model is significant and there is a 9.28% chance that a Lack of Fit (F-value 68.4) could be due to the noise. The optimal conditions are shown in Figure 1, being 40.3 °C, 10.6 min and pH 4 the optimal conditions to achieve the highest carbohydrate recovery.

$$\text{Carbohydrate mg/g} = -33.734 + 6.862 \times T + 11.680 \times t - 36.168 \times pH$$
$$- 0.084 \times T^2 - 0.541 \times t^2 - 0.023 \times t \times pH + 2.601 \times pH^2$$
$$(\text{Adjust } R^2 = 0.617, P = 0.026) \quad (7)$$

Moreover, the contents of chlorophyll a, and carotenoids in the extracts obtained were analyzed. For chlorophyll a, the extract content at 40 °C, 10 min and pH 4 (1.406 mg/g) was significantly ($p < 0.05$) higher than the other experiments. Likewise, the carotenoid content was lower than that observed for chlorophyll a, although similarly to chlorophyll a, the highest extraction rate for carotenoids (0.648 mg/g) was found at 40 °C, 10 min and pH 4.

From the regression equations (8-9), it can be observed a positive effect of temperature and time regarding chlorophyll a and carotenoid extraction, while pH had a negative effect. In this experiment, an aqueous solution was used as the extraction reagent, so the content of chlorophyll a/carotenoids (fat-soluble pigments) was low. And the ingredients

we consider are more water-soluble substances, so from this point of view, the equation can be used as a reference, and Figure 2 shows the optimized extraction conditions for chlorophyll a, and carotenoids were 39.7 °C, 10.0 min, pH 4 and 39.6 °C, 10.3 min, pH 4, respectively. From the above results, it could be seen that the extraction conditions of the pigments are similar, that is, almost at 40 °C, 10 min, and pH 4.

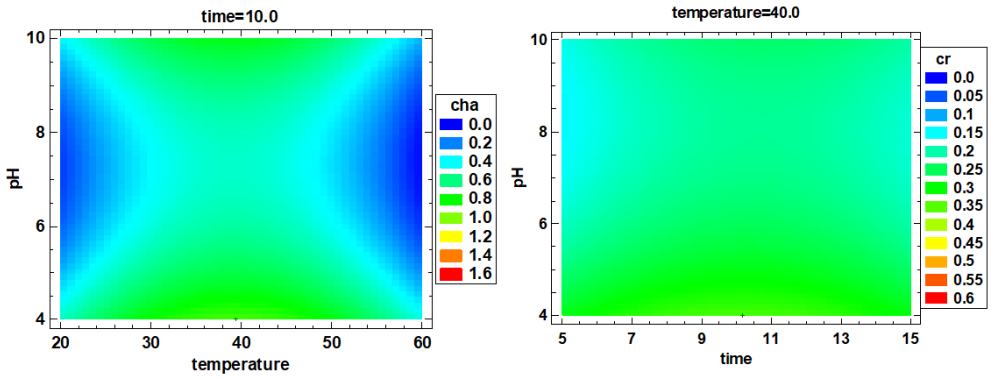

Figure 2. Estimated response surface of chlorophyll a (mg/g), and carotenoids (mg/g). cha, and cr correspond to chlorophyll a, and carotenoids respectively.

$$\text{Chlorophyll a mg/g} = -0.251 + 0.088 \times T + 0.206 \times t - 0.564 \times pH - 0.001 \times T^2 - 0.010 \times t^2 + 0.039 \times pH^2$$

$$(\text{Adjusted } R^2 = 0.608,\ P = 0.131) \qquad (8)$$

$$\text{Carotenoids mg/g} = -0.279 + 0.039 \times T + 0.048 \times t - 0.134 \times pH - 0.002 \times t^2$$

$$(\text{Adjusted } R^2 = 0.475,\ P = 0.075) \qquad (9)$$

Regarding total polyphenols extraction, the optimal recovery was achieved at 40 °C, 10 min, pH 4 and 40 °C, 10 min, pH 10, corresponding to 11.61 mg/g and 10.85 mg/g, respectively. However, when the temperature was 20 °C and the pH = 4, even if the time was increased from 5 minutes to 15 minutes, the yield of polyphenols was still very low, corresponding to 0.97 mg/g and 0.63 mg/g respectively. This indicates that for the extraction content of polyphenols, 40 °C is significantly better than 20 °C. Equation (10) shows the positive effect of increased temperatures and longer extraction times, while the negative effect of increased pH. The model F-value of 4.62 implies the model is

significant and there is a 2.90% chance that a Lack of Fit F-value could be due to the noise. The optimized conditions obtained from response surface plot for polyphenol are 39.9 °C, 10.4 min and pH 10 (Figure 3).

$$\text{Polyphenol mg/g} = -6.604 + 1.169 \times T + 1.570 \times t - 5.570 \times pH - 0.014 \times T^2 + 0.006 \times T \times pH - 0.080 \times t^2 + 0.424 \times pH^2$$

$$(\text{Adjusted } R^2 = 0.659,\ P = 0.029) \qquad (10)$$

Oxygen free radical scavenging capacity was used to evaluate the total antioxidant capacity of 16 samples. Table 2 shows that the antioxidant value of the samples varies greatly, ranging from 100 to 3000, which mainly depends on the biomass content (protein, polyphenols, carbohydrates, and pigments) analyzed previously.

The samples extracted under the conditions of 40 °C, 10 min and pH 4 had the highest antioxidant value of 2915 μM TE, which is similar to the sample obtained under 40 °C, 10 min and pH 10, which had 2831 μM TE. There was no significant difference in the antioxidant value of the two samples. It is worth noting that when the extraction conditions were 40 °C, 10 min, pH 7 and 40 °C, 15 min, pH 7, the antioxidant capacity of the sample was relatively strong, being the values 1491.0 μM TE and 1595.4 μM TE, respectively. The antioxidant value of the above four groups is much higher than other groups (between 100-500 μM TE). It can be inferred that temperature has an important influence on the antioxidant value of the extract, especially at 40 °C.

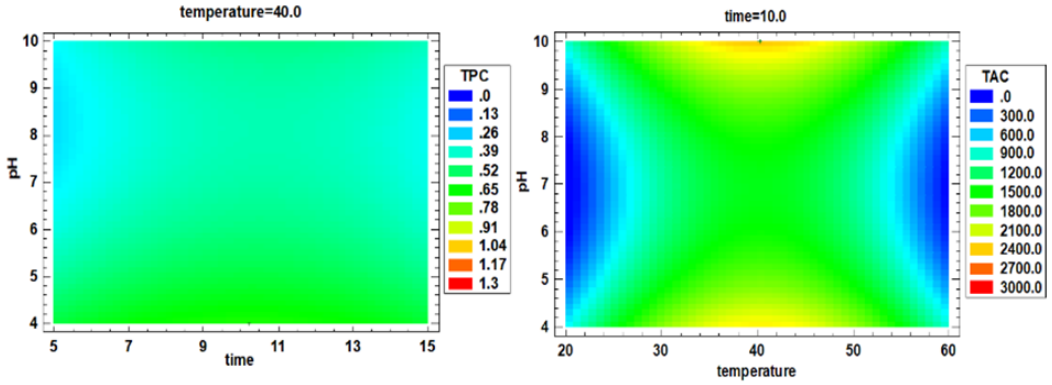

Figure 3. Estimated Response surface of total polyphenol content and total antioxidant capacity. TPC (mg/g) and TAC corresponding to total polyphenol content and total

antioxidant capacity (μM TE).

$$\text{ORAC } \mu\text{M TE} = -0.279 + 0.039 \times T + 0.048 \times t - 0.134 \times pH - 0.002 \times t^2 + 0.001 \times t \times pH + 0.008 \times pH^2$$

$$(\text{Adjusted } R^2 = 0.687,\ P = 0.047) \qquad (11)$$

Equation (11) shows a positive effect of temperature and time on ORAC values, whereas pH had a negative effect. The model F-value of 3.30 implies the model is significant and there is a 5.29% chance could be due to the noise. The optimized conditions for ORAC are 40.2 °C, 10.2 min, pH 10 (Figure 3).

3.2 Multiple factor response results and verification experiments

According to the single-factor optimization, the multi-factor optimization analysis was performed, and the response surface diagram is shown in Figure 4. According to the comprehensive optimization results of all extract indexes, the optimal extraction conditions were determined to be 40.3 °C, 10.3 min, and pH 4. The RSM-CCD predicted value is compared with the verified experimental value (predicted value vs. verified value), that is, the protein extraction rate was 43.5 ± 1.9% vs. 46.8 ± 3.1%, chlorophyll a was 0.89 ± 0.13 vs. 1.46 ± 0.04 mg/g, carotenoids were 0.12 ± 0.01 vs. 0.14 ± 0.02 mg/g, the TPC was 9.64 ± 1.75 vs. 11.49 ± 0.04 mg/g, the carbohydrate content was 69.3 ± 1.8 vs. 78.4 ± 1.4 mg/g and the ORAC value was 2297 ± 338 vs. 2870 ± 153 μM TE ($p > 0.05$). There were no significant differences of the results between the verification experiment and group 9 ($p > 0.05$). Therefore, using deionized water as extraction reagent, 40 °C, 10 min and pH 4 provides the best extraction conditions.

3.3 Effect of pressure on compounds extraction and antioxidant capacity

RSM-CCD optimization experiments showed a significant impact of temperature, time, and pH on the extraction of *Spirulina* bioactive compounds ($p < 0.05$). Throughout the extraction process, nitrogen pressure was constant at 103.4 bars. It is worth discussing

whether the constant pressure of 103.4 bars during the PLE extraction process played a key role in the extraction of *Spirulina* active substances. Therefore, a control experiment focused on the effect of pressure on the extraction of bioactive substances in *Spirulina* was carried out. Specifically, *Spirulina* powder and pH 4 deionized water were mixed in proportions (0.5 g/40 mL), and samples without pressure extraction were obtained under the conditions optimized by RSM-CCD at 40 °C and 10 min.

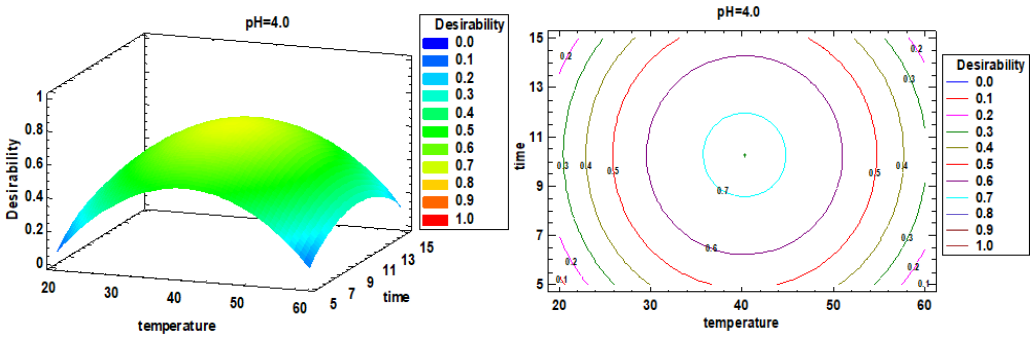

Figure 4. Estimated response surface and contour plots for multiple factor responses.

Then the protein, chlorophyll a, carotenoids, polyphenols, carbohydrates content and antioxidant capacity (ORAC and ABTS) of the control samples were analyzed. As it is shown in Figure 5, no significant differences were observed for ORAC results when the PLE sample and the non-pressurized one when compared, ranging the results from 2500~2800 μM TE. On the other hand, the ABTS results showed that the antioxidant capacity of the PLE extracts was significantly higher than the control group ($p < 0.05$), and the results were close to 2300 μM TE and 800 μM TE respectively. Obviously, ORAC and ABTS showed a different behavior, which is attributed to the different mechanism of action. Both ORAC and ABTS can test the antioxidant capacity of the sample. The former is to examine the ability of the sample to scavenge ROO·, ·OH and other free radicals, and the latter is to examine the ability of the sample to scavenge $ABTS^{+}$ free radicals. Since the type of free radicals removed by the samples are different, the results are also inconsistent (Wang et al., 2021); although differences between the two

assays, such as the corresponding end point times, could also explain the results observed.

Spirulina has been extensively studied due to its great content of various functional compounds. A previous study used accelerated solvent extraction combined with different solvents (hexane, petroleum ether, ethanol and water) to evaluate the extraction of antioxidant compounds from *Spirulina* (Herrero et al., 2005). The authors investigated the effects of different temperatures (60, 115, 170 °C) and time (3, 9 15 min) on the antioxidant capacity of the extract. The results showed that using ethanol as the extraction reagent, samples with higher antioxidant capacity can be obtained at 170 °C and 15 minutes. Compared with our study, the extraction temperature selected in that study is too high, which is unfavorable for obtaining heat-sensitive components such as pigments, polyphenols, and proteins from *Spirulina*. Unfortunately, their study only examined the total antioxidant activity of the extracts and did not analyze any components in the *Spirulina* extract. Moreover, we selected water as the extractant and analyzed a variety of functional substances, which reduced the cost of the reagent (compared to ethanol) and obtained a variety of bioactive compounds. The protein concentration in the control sample was approximately 120 mg/g, which was significantly ($p < 0.05$) lower than the protein concentration in the PLE group (138 mg/g). PLE also increased significantly ($p < 0.05$) the carbohydrate concentration (approximately 78 mg/g vs. 74 mg/g) ($p < 0.05$) under the pressure of 103.4 bars as well as the concentration of chlorophyll a, carotenoids, and polyphenols compared to control.

Regarding the effect of pressure treatment on *Spirulina* extract, another work explored that different pretreatment methods (autoclaving at 121 °C with 103.4 kPa for 30 min; ultrasonication using a probe sonication 20% maximum power for 60 min; high-pressure homogenization at 103.4 MPa) affect the extraction of phycocyanin from *Spirulina* (Parimi et al., 2015). The results showed that under optimized extraction conditions (dissolution step: pH 11.38, 35 min; precipitation step: pH 4.01, 60 min), the samples

processed by high pressure homogenization contained more proteins, essential amino acids, and other high value unsaturated fatty acid. This is consistent with our research results, that is, pressurization promotes the extraction of *Spirulina* bioactive substances.

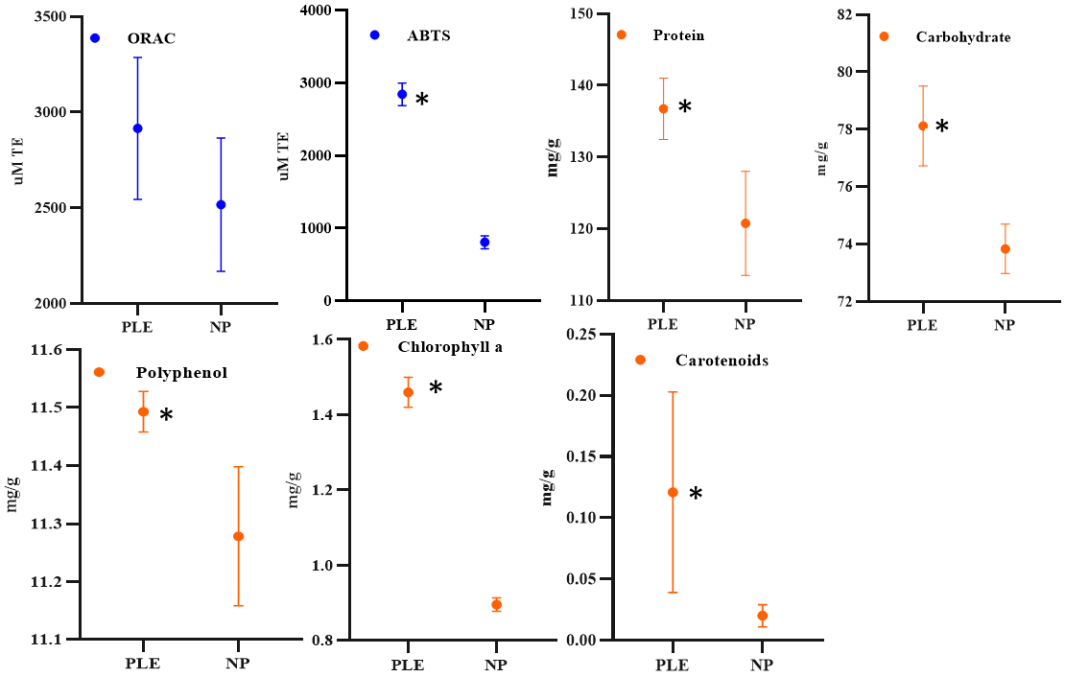

Figure 5. Biomass and antioxidant capacity of PLE and non-pressurized (NP) extracts. Note: * indicate significant differences ($p < 0.05$).

According to the principle of pressure extraction, the strong interaction between the solute and the matrix caused by van der Waals forces or hydrogen bonds, and the dipole attraction of solute molecules and the active sites of the sample matrix can be greatly reduced under high temperature and high pressure. This speeds up the extraction process of the solute molecules, reduces the activation energy required for the analysis process, and reduces the viscosity of the solvent, thereby reducing the resistance of the solvent to the sample matrix and promoting the solvent to diffuse into the sample (Zhuang, McKague, Reeve, & Carey, 2004). Therefore, compared with non-pressurized extraction, the pressure of 103.4 bars in this study significantly increased the protein, carbohydrate, chlorophyll a, carotenoids, and polyphenols in the *Spirulina* extract.

In addition to the PLE extraction technique, other green, non-toxic, and efficient extraction methods, such as ultrasonic and microwave-assisted extraction have also been used to obtain biologically active compounds from *Spirulina*. The results of a recent experiment showed that ultrasonic treatment (20 kHz) promotes the mass transfer process (efficient diffusivity) in the extraction process. Compared with traditional extraction methods, ultrasonic treatment significantly increased the extracted protein content (8.63 ± 1.15 g/100 g dw vs. 229.42 ± 1.15 g/100 g dw). Under the microscope, the perforation and fragmentation of the ultrasonic treatment samples were observed, which promoted the extraction of bioactive compounds in *Spirulina* (Vernès et al., 2019). Another study also showed that compared to the Soxhlet extraction (80 °C and 8 h), the more efficient extraction process for extracting biomolecules from *Spirulina* is to perform ultrasonic-assisted extraction in ethanol-chloroform-water-Na_2SO_4 and ethanol after 20 s of water extraction in a microwave oven. Moreover, the microwave-assisted extraction method is more efficient than the ultrasonic extraction method alone (De Sousa E Silva et al., 2017). In general, PLE, ultrasound, and microwave technology all show advantages in extracting active substances from *Spirulina*. It is worth mentioning that our research does not use organic reagents, which is cleaner and cheaper than the extraction process in the above research.

3.4 SDS-PAGE analysis of protein molecules in Spirulina extracts

Figure 6 shows the protein molecular weight distribution in *Spirulina* extracts, ranging from 2~250 kDa. The protein molecular weights of the PLE extracts are distributed in 100, 75~50, 37, 20~15 and 10 kDa, the control extracts are distributed in 100, 75, 50~37, 20 and 10 kDa. The difference of the band distribution indicates that PLE extraction could affect the protein molecule in *Spirulina* extracts. Image-J is used to calculate the area value of each band, and its relative proportion is shown in Figure 6. The results show that the proportion of bands between 20~100 kDa in the non-pressurized extracts is greater

than that of the PLE-extracted group. However, when the molecular weight is lower than 20 kDa, PLE group is more than that of the non-pressurized group. This indicates that the content of small molecular weight protein in the extracted samples of the PLE group is higher than that of the non-pressurized group.

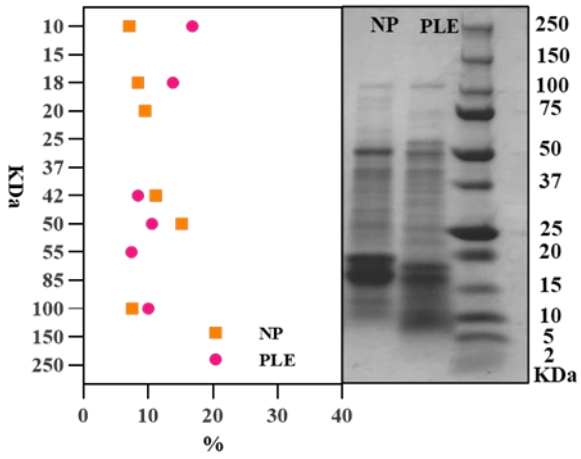

Figure 6. Protein molecular weight distribution of the extracts -SDS-PAGE.

There are two possible reasons for this phenomenon. One hypothesis is that more small-molecular-weight proteins are eluted due to the pressurizing effect during the extraction process of PLE, another hypothesis is that the pressurization of PLE may affect the protein structure, resulting in more small-molecule peptides. Moreover, some previous studies have shown that pressurization can affect the structure of protein molecules in food, that is, the pressures below 150 MPa can affect the quaternary structure of proteins, 200 MPa can affect the tertiary structure, and 300~700 MPa can change the secondary structure (Briones-Labarca et al., 2019). Obviously, the pressure in our study is close to 10 MPa, which is not enough to change any structure other than the quaternary structure of the protein.

Moreover, some related studies showed that high pressure does not affect the covalent bond of protein molecules, that is, it does not damage the primary structure of protein molecules-peptide bond structure (Zamantha Escobedo-Avellaneda, Yildiz, Lavilla, & Welti-Chanes, 2020). Therefore, the second hypothesis is invalid, that is, under 103.4

bars, PLE extraction is almost impossible to destroy the polypeptide chain of the protein by pressure. Furthermore, combining the principle of the PLE, with a certain temperature, pressure can increase the permeability of the solvent, making it easier to enter the sample matrix and increase the contact time between the sample and the solvent (Ahmad, Ahmad, Al-Anaki, Ismail, & Al-Jishi, 2020). Therefore, it can be inferred that the area change of different molecular weight bands is due to PLE changing the solubility of different molecular weight proteins in the extract, which leads to a higher content of small molecule proteins.

3.5 Polyphenol profile of the extracts: Triple TOF–LC–MS–MS

As polyphenols are active substances with strong antioxidant properties, it is valuable to analyze the specific phenol components in *Spirulina* extracts. Therefore, in this study, PLE and non-pressurized extracts were analyzed by Triple TOF–LC–MS–MS, the effect of pressure treatment on the type of polyphenols is analyzed.

Figure 7 shows the results of the type and content of polyphenol components. The results showed that different extraction processes can change the composition of polyphenols in algae extracts. For example, cinnamic acid and 4-hydroxybenzaldehyde were both detected in PLE and non-pressurized *Spirulina* biomass. However, kaempferol, *p*-coumaric acid and 24-methylcholestanol ferulate were only found in PLE extracts while quercetin, and sitostanyl ferulate were found in the non-pressurized extracts. Moreover, PLE treatment increased the content of cinnamic acid (4.0 mg/kg vs. 1.0 mg/kg) and 4-hydroxybenzaldehyde (0.8 mg/kg vs. 0.2 mg/kg) when comparing with non-pressurized extracts. Moreover, the polyphenols in the PLE extract were mainly 24-methylcholestanol ferulate (2.3 mg/kg), cinnamic acid (4.0 mg/kg) and p-coumaric acid (3.0 mg/kg), while the polyphenols in the non-pressurized extracts were mainly quercetin (4.6 mg/kg). This shows that PLE can promote the recovery of a variety of polyphenols in *Spirulina*, which

is an important reason for promoting the antioxidant capacity.

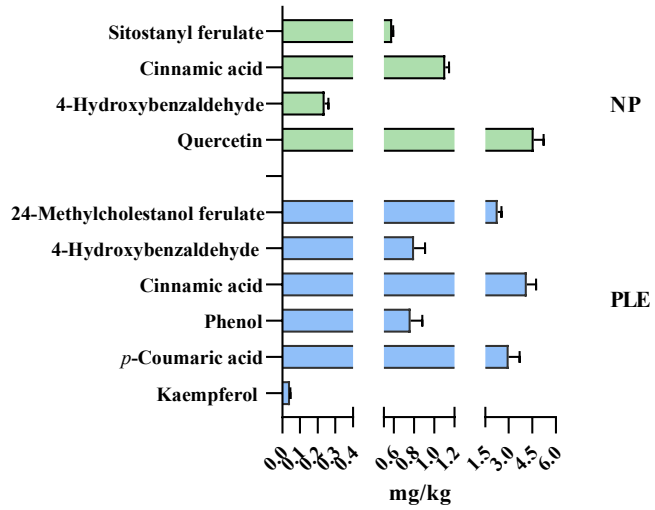

Figure 7. Concentration of phenolic components in PLE and non-pressurized extract of the extract.

Polyphenols in the biomass exist in free and bound forms, but in most cases they are bound to other molecules such as proteins, structural carbohydrates, etc., by ester bonds through their carboxyl group, or by ether bonds through their hydroxyl group (Mithul Aravind et al., 2021). In this study, it can be concluded that PLE changed the form of polyphenols in the extract. Considering the pressure treatment in the PLE extraction process, this is likely to promote the separation and release of bound polyphenols from *Spirulina*.

4. Conclusion

The results obtained from this study showed that *Spirulina* is a relevant source of nutrients and bioactive compounds with antioxidant capacity but the recovery of these valuable constituents (proteins, polyphenols, carbohydrates, pigments) was greatly influenced by the extraction conditions (particularly temperature) explored in this study. Meanwhile, this study highlights the potential of PLE as an effective green extraction methodology to obtain high-added-value compounds from *Spirulina*. Our study demonstrated that PLE may be a promising alternative for enhancing the selective ex-traction of antioxidant

bioactive compounds from microalgae, which could be interesting for industrial upscaling. Finally, this study is the initial step for PLE to obtain natural active substances from *Spirulina*, more applications of *Spirulina* protein, carbohydrate and antioxidant substances in food, medicine and other industries need to be further developed.

Acknowledgments

Authors would like to acknowledge Nicolas Mazurier (Eco*Spirulina* company) for providing the microalgae samples to carry out the experiments. Jianjun Zhou was supported by a PhD fellowship from the China Scholarship Council (CSC) (No. 201908420246). Min Wang would like to thank China Scholarship Council (CSC) for her PhD fellowship (No. 201908420245). Zhenzhou Zhu would like to acknowledge the "Outstanding young and middle-aged science and technology innovation team in Hubei Province (T2020012)". The authors also thank the Mass Spectrometry Service of Central Support Service for Experimental Research (SCSIE)-University of Valencia for technical support in polyphenol identification and quantification.

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4.2 Pulsed Electric Fields (PEF), Pressurized Liquid Extraction (PLE) and combined PEF + PLE process evaluation: Effects on *Spirulina* microstructure, biomolecules recovery and Triple TOF-LC-MS-MS polyphenol composition

Journal: ***Innovative Food Science and Emerging Technologies*****, 2022, 77, 102989; https://doi.org/ 10.1016/j.ifset.2022.102989**

Pulsed Electric Fields (PEF), Pressurized Liquid Extraction (PLE) and combined PEF + PLE process evaluation: Effects on *Spirulina* microstructure, biomolecules recovery and Triple TOF-LC-MS-MS polyphenol composition

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Abstract

This study aims at evaluating the impact of different processes-pulsed electric fields (PEF), pressurized liquid extraction (PLE) and a multistep process combining PEF + PLE on the yield of antioxidant compounds (protein, polyphenols, chlorophyll a, and carotenoids) from *Spirulina*. Firstly, the effects of PEF or PLE treatment on the extraction yield of *Spirulina* biomolecules were evaluated. To further increase the extraction yield, PEF + PLE was used, as an innovative extraction approach. The results showed that PEF + PLE greatly improved the extraction yield compared with the PEF or PLE treatments alone. Compared with Folch extraction (conventional control technique), PEF + PLE significantly ($p < 0.05$) shortened the extraction time (-165 min) and increased the protein. polyphenol, chlorophyll a content and antioxidant capacity of *Spirulina* extracts by 1328%, 979%, 11% and 47% respectively. Furthermore, Triple TOF-LC-MS-MS results showed that PEF + PLE increased both the type and content of phenolic compounds. The above results were attributed to PEF-induced damage on *Spirulina* helical structure, which was verified by fluorescence and scanning electron microscopy.

Keywords: Microalgae, pulsed electric fields (PEF), pressurized liquid extraction (PLE), biomolecules, Triple TOF-LC-MS-MS phenolic profile, cell structure

1. Introduction

In recent years, marine microalgae biomass have attracted much attention due to their high content of high-added-value compounds such as nutrients (proteins, fatty acids, carbohydrates, vitamins and minerals) as well as antioxidant compounds (polyphenols and pigments such as chlorophylls, carotenoids, etc.) with antioxidant, antibacterial and antiinflammatory properties, among others (Costa, Freitas, Moraes, Zaparoli, & Morais, 2020; Junior, Gorgich, Martins, Mata, & Caetano, 2020). Among microalgae biomasses, a growing interest has been shown over the last years in *Spirulina*, as it is included in the catalog of the European Commission as a novel food for human consumption.

Although the whole biomass can be consumed after different preparation steps, especially to avoid microbial contamination, the different biomolecules of *Spirulina* are also of a great interest and several traditional extraction methods mainly based on solid-liquid or liquid-liquid extractions (i.e., Soxhlet extraction, Folch, etc.) have been used to recover the different high-added value compounds from these microalgae (Lee, Yoo, Jun, Ahn, & Oh, 2010). However, there is a need to develop and optimize new extraction approaches as the traditional methods involve the use of high amount of solvent, which can be toxic, long extraction times and high temperatures (Chen, Liu, Song, Sommerfeld, & Hu, 2020; Mansour, Abo El-Enin, Hamouda, & Mahmoud, 2019). Moreover, some of these biomolecules are labile and can be easily destroyed under such extraction conditions. Considering the current focus on sustainability in agreement with Sustainable Development Goals (SDGs), from the perspective of improving extraction efficiency, preserving the biological activity of the compounds and environmental protection, this requires the extraction conditions to be as gentle, efficient, and environmentally friendly as possible (Zhao, de Alba, Sun, & Tiwari, 2019). Therefore, new extraction technologies are recognized in the extraction of microalgae biomass, including PEF (pulsed electric fields), PLE (pressurized liquid extraction), UAE (ultrasonic-assisted extraction), MAE

(microwave-assisted extraction), etc. (De Sousa e Silva et al., 2017; Guo et al., 2019; Kokkali et al., 2020; De Sousa e Silva, de Magalhães, Moreira, Rocha, & Bastos 2018; Vasistha, Khanra, Clifford, & Rai, 2021). The working principles of these novel extraction technologies are different, and researchers have already focused on combining multiple novel extraction techniques to improve the yield of microalgae nutrients. For example, the use of UAE in combination with MAE enhanced oil production from *Chlorella* (Ma et al., 2015), the combined US + freezing and thawing method increased the yield of phycocyanin from *Spirulina* (Tavanandi, Mittal, Chandrasekhar, & Raghavarao, 2018), etc. Therefore, combining different extraction technologies in multistep processes seem to be an interesting tool to increase the extraction efficiency, reducing consumption of toxic solvents, extraction time and the use of high temperatures. In our previous studies, PEF and PLE were used to obtain high-added-value compounds with strong antioxidant properties from microalgae *Tetraselmis chuii* and *Phaedoactylum tricornutum* (Kokkali et al., 2020) and marine fish side streams (Wang, Zhou, Collado, & Barba, 2021). PEF is a novel non-thermal technology that can be used to produce safe, high-quality, and nutritious food with excellent flavor and extended shelf life. PEF devices typically include an electrical pulse generator, a treatment chamber, and electrodes, with the electrical pulse placed between or through two electrode (Zhao et al., 2019). PEF treatment can alter cell membrane properties, which are induced by high-intensity electric field pulse discharges, resulting in increased cell membrane permeability (Blahovec, Vorobiev, & Lebovka, 2017). PLE technology is efficient and produces less waste during the extraction process, which can reduce costs and save time. In the PLE process, the solvent is below the critical point to maintain the liquid phase during extraction, and the pressure and temperature conditions are chosen to increase the mass transfer rate by reducing the solvent surface tension and viscosity and increasing

the solubility of the components, which makes the solvent more permeable into the extracted solid matrix (Andrade et al., 2021).

In this line, on the one hand, the application of short electrical pulses can control the thermal effect at a low level, which can protect the antioxidant properties of algae biomass (Gómez et al., 2019). On the other hand, the application of high-pressure of PLE greatly reduces the strong interaction force between the solute and the matrix, such as van der Waals forces, hydrogen bonds, etc., thereby promoting the diffusion of the solvent into the samples (Zhuang, McKague, Reeve, & Carey, 2004). That is, PEF treatment can lead to changes in the permeability of microalgae cell membranes, while PLE can promote the entry of solvents into the microalgae cytoplasm to dissolve bioactive substances. Therefore, PEF and PLE have a potential 'synergistic' effect in nutrients recovery of *Spirulina*, which is of great significance for the efficient recovery of *Spirulina* biomolecules with good bioactivity.

In this study, PEF, PLE and the combination of PEF + PLE were used to recover protein, polyphenols, pigments (chlorophyll a, carotenoids) from *Spirulina* and the antioxidant capacity of the extracts was evaluated. In addition, from the point of saving reagents and reducing contamination as well as the good solubility of DMSO, the application of H_2O + organic solvent mixtures (DMSO) was used for biomolecule extraction in this study. The effects of PEF on *Spirulina* microstructure were also analyzed using both fluorescent and scanning electron microscopy. Finally, the triple TOF-LC-MS-MS phenolic profile of the combined process PEF + PLE was evaluated and compared with that obtained after a conventional Folch extraction process.

2. Materials and methods

4.1. Chemicals and reagents

ABTS (2,2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid), AAPH (2,2'-Azobis(2-methylpropionamidine) dihydrochloride), Folin-Ciocalteu, gallic acid, Trolox (6-

hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), D-glucose, phenol, fluorescein sodium salt and $K_2S_2O_8$ were purchased from Sigma-Aldrich (Steinheim, Baden-Württemberg, Germany). Sodium carbonate (Na_2CO_3) was acquired from VWR (Saint-Prix, France). Sodium hydroxide, glacial acetic acid, and sulfuric acid were supplied by Fisher Scientific (Madrid, Spain). Diatomaceous earth and other materials for generation of PLE extracts were bought from Dionex (Dionex, Leeds, UK). Deionized water (resistivity > 18 MΩ cm^{-1}) was from Milli-Q SP® Reagent Water System (Millipore Corporation, Bedford, MA, USA).

4.2. Samples

Spirulina comes from *Arthrospira platensis* species, strain paracas 15016, being Paracas the lake where it originally comes from (Lima, Peru). Cultivation took place at Eco*Spirulina* company (Serra, Valencia, Spain) in raceway ponds using a greenhouse under natural sunlight. During the experiment, the day-time temperature was 32 °C and temperature decreased to 24 °C at night. The pH of the culture varied between 9.8 and 10.4, which was controlled by the addition of CO_2 at the time of harvesting daily. *Spirulina* biomass was filtered using a tambor filter of 30 micra mesh. Cultivation medium went back to the cultivation pond, while biomass was vacuum-pressed and then frozen in 50 g portions. The sample was freeze-dried at -40 °C for 72 h to reduce the degradation of poorly resistant biomolecules at higher temperatures before extraction process.

4.3. Microalgae biomolecules extraction

4.3.1. PEF extraction

Spirulina powder and water (~2 g/200 mL) were mixed in the treatment chamber ranging the conductivity between 1000~2000 µS/cm. The minimum electric field strength required to produce changes in the cell is 1 kV/cm, and when the pulse duration is milliseconds, an electric field of 3~4 kV/cm can produce electroporation. Then, the

samples were PEF-treated (3 kV/cm, 44 pulses, 99 kJ/kg) according to previous studies (Martí-Quijal et al., 2021). The temperature and conductivity of each sample were measured with a portable conductivity meter ProfiLine Cond 3310 (WTW, Xylem Analytics, Weilheim in Oberbayern, Germany). After PEF treatment, the samples were transferred to a beaker, and 200 mL H_2O or DMSO were added to make the sample-solvent system reach to 2 g algae powder/400 mL solvent. A magnetic stirrer was used to continuously stir the samples at room temperature and the samples were collected at 0, 10, 20, 30, 60, 90, 120 and 180 minutes, respectively. It should be noted that in PEF extraction, pure DMSO could not be used as an extraction reagent due to its non-conductive properties. The control experiment was carried out as 2 g algae powder/400 mL H_2O or 2 g algae powder/200 mL H_2O + 200 mL DMSO stirred at room temperature and the samples collected at the same time as PEF extraction. Finally, the samples were centrifuged (2504 g, 4 °C, 15 minutes), and the supernatants were collected and stored at -20 °C until needed for analyses.

4.3.2. PLE extraction

The PLE extraction was based on our previous studies (Zhou et al., 2021). Microalgae samples and diatomaceous earth were thoroughly mixed (0.5 g : 1.5 g) in a mortar and then placed into the PLE extraction tank. An ASE-200 Accelerated Solvent Extractor (Sunnyvale, CA, USA) was used to perform the extraction, and the operating conditions were referred to our previous study: preheating period 1 min, heating period 5 min, flush volume 60%, nitrogen purge 60 s, extraction pressure of 103.4 bars, extraction temperature of 40 °C, extraction time of 15 minutes. Referring to related studies (Parniakov et al., 2015b), different proportions of DMSO (50% DMSO, DMSO) were used for PLE extraction to observe the effect of DMSO concentration on the PLE extraction yield. For 0.5 g *Spirulina* (dw), the final extracts volume was near 20 mL. According to the PLE extracts volume, the control experiment was carried out as 0.5 g

algae powder/20 mL solvent (H_2O, 50% DMSO, DMSO) stirred at 40 °C for 15 minutes. The samples were centrifuged (2504 g, 4 °C, 15 minutes), and the supernatants were stored at -20 °C until needed for analyses.

4.3.3. PEF + PLE extraction

The PEF and PLE extraction processes were then combined in a multistep extraction process to further obtain a higher yield of biomolecules from *Spirulina*. Similar to PEF extraction (*section 2.3.1*), *Spirulina* powder and H_2O (~2 g/200 mL) were mixed in the treatment chamber ranging the conductivity between 1000~2000 µS/cm. The samples were PEF-treated (3 kV/cm, 44 pulses, 99 kJ/kg) and transferred to a beaker. Then according to the selected PEF extraction conditions (*section 2.3.1*), 200 mL H_2O were added to make the sample-solvent system reach to 2 g algae powder/400 mL solvent and continuously stirred at room temperature for 120 minutes. Collecting the sample and freeze dried at 40 °C for 72 h for further PLE extraction. Based on the selected PLE extraction conditions (*section 2.3.2*), freeze dried sample and diatomaceous earth were thoroughly mixed (0.5g:1.5g) in a mortar and then placed into the extraction tank. The operating conditions were as follows: preheating period 1 min, heating period 5 min, flush volume 60%, nitrogen purge 60 s, extraction pressure 103.4 bars, extraction temperature 40 °C, extraction time 15 min with DMSO as solvent. Finally collecting the extracts as PEF + PLE samples for further analysis. The conventional Folch extraction method was used as a control (Folch et al., 1957; Ulmer et al., 2018) and the results were compared with those obtained after PEF, PLE and PEF + PLE extractions. For Folch extraction, chloroform and methanol were mixed (5:2, v/v) to obtain the extraction reagent. Then, the microalgae powder and the extraction reagent were mixed at the ratio of 1 g/20 mL and stirred at 40 °C under magnetic stirring under darkness for 6 hours. The extract was centrifuged (2504 g, 4 °C, 15 min) and the supernatant was stored at -20 °C for subsequent experimental analysis.

4.4. Microalgae cell structure

4.4.1. FM (Fluorescence Microscope)

The samples obtained after PEF treatment (3 kV/cm, 44 pulses, 99 kJ/kg) (see *section 2.3.1.*) were centrifuged at 157 g/10 min. After centrifugation, the supernatant was removed to collect the precipitate, and it was washed with 90% methanol and centrifuged. The sample was repeatedly washed until the supernatant was colorless, and the precipitate was collected and diluted with deionized water. Then the cell structure of the microalgae was observed under a fluorescence microscope. The control group was set as a mixed extract of microalgae and H_2O without PEF treatment (see *section 2.3.1.*). The bright-field digital images were collected through an Eclipse 90i Nikon microscope (Nikon corporation, Japan) with a 3/16× objective, equipped with a digital camera (Nikon DS-5Mc). Images were processed and analyzed by the Nis Elements BR 2.32 software (Nikon corporation, Japan) software.

4.4.2. SEM (Scanning Electron Microscopy)

A scanning electron microscope (S-4800) was used to analyze the microstructure of freeze-dried samples of microalgae (Fang, Xu, Kawashima, Hata, & Kijima, 2021). Freeze dried microalgae samples in *section 2.4.1* were then mounted on specimen stubs with colloidal silver, sputter-coated with gold-palladium and imaged with a SEM (S-4800) at magnifications of 110×, 450× and 1500.

4.5. Biomolecules (protein, polyphenol, pigments) and antioxidant properties analyses

The bicinchoninic acid (BCA) method was used to analyze the protein content of the extracts (Al Khawli, Martí-Quijal, Pallarés, Barba, & Ferrer, 2021). The protein content was determined using a calibration curve (0~2000 mg/L) with bovine serum albumin (BSA) as a standard. Ten microliters of samples or BSA and 200 µL of BCA working solution were added to a 96-well plate, mixed well, and incubated at 37 °C for 30 minutes. Then, the absorbances were measured at 562 nm.

On the other hand, spectrophotometry was used to analyze the pigments concentration of *Spirulina* extracts. The absorbance values and formulas used to analyze extracts varied with solvent, for DMSO and 50% DMSO extracts, the equation was as follows (Wellburn, 1994) :

$$C_a = 12.47 \times Abs_{665.1nm} - 3.62 \times Abs_{649.1nm}$$

$$C_{Carotenoids} = (100 \times Abs_{480nm} - 1.29 \times C_a - 53.78 \times C_b)/220$$

For H_2O extracts, the equation was as follows (Kokkali et al., 2020) :

$$C_a = 16.82 \times Abs_{665nm} - 9.28 \times Abs_{653nm}$$

$$C_{Carotenoids} = (1000 \times Abs_{470nm} - 1.91 \times C_a - 95.15 \times C_b)/225$$

Where C_a is the concentrations (mg/L) of chlorophyll a. The final yield of pigments was calculated based on the dry weight of *Spirulina* (mg/g dw).

The oxygen radical antioxidant capacity (ORAC) (Gregório et al., 2020) and the Trolox equivalent antioxidant capacity (TEAC) (Sridhar & Charles, 2019) assays were used to evaluate the antioxidant capacity of the extract. In the ORAC assay, Trolox and 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) were used as antioxidants and oxygen free radicals, respectively, while phosphate buffer was used as a blank control. To carry out the assay, 50 μL of extract and 50 μL of the fluorescein sodium salt solution were added to a 96-well plate and incubated in a microplate reader at 37 °C for 10 minutes, then 25 μL AAPH solution were added, and the absorbance was recorded at 520 nm. Each group of samples was tested in 3 wells in parallel, and the experiment was repeated three times to make the coefficient of variation value less than 10%.

Regarding TEAC assay, first the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) working solution was prepared. For that purpose, 25 mL of 7 mM ABTS were mixed with 440 μL of 140 mM potassium thiosulfate solution and incubated under darkness at room temperature for 12~16 hours to obtain the working solution. The working solution was diluted with 96% ethanol to obtain an absorbance value of 0.700 ±

0.020 at 734 nm. Then, 0.1 mL of the samples or Trolox standard solution and 2 mL of the working solution were mixed, and after reacting for 3 minutes in a dark room, the absorbance at 734 nm was measured. Trolox was used as the standard solution to calculate the antioxidant capacity of the sample.

The Folin-Ciocalteu method was used to analyze the total polyphenol content in the extracts (Korzeniowska, Łęska, & Wieczorek, 2020). That is, 0.2 mL of sample, 1 mL of Folin-Ciocalteu (diluted with water at a ratio of 1:10, v/v) and 0.8 mL of a sodium carbonate solution (75 g/L) were mixed and incubated in a water bath at 50 °C for 10 minutes. Then, the absorbances were measured at 750 nm using a spectrophotometer. Gallic acid was used as a standard to prepare the calibration curve to quantify the amount of total polyphenols in the extracts.

Finally, the phenolic profile of *Spirulina* extracts was evaluated using a TripleTOF™ 5600 (ABSCIEX) LC/MS/MS system equipped with Agilent 1260 Infinity (Agilent, Waldbronn, Germany). The chromatographic separation was carried out on a Waters UPLC C18 column 1.7 μm (2.1×50 mm) Acquity UPLC BEH.C18 from Waters (Cerdanyola del Vallès, Spain). The mobile phase is composed of water (0.1% CH_2O_2, A) and methanol (0.1% CH_2O_2, B). The gradient elution of the mobile phase is as follows: 0~13 min, 90% (A) and 10% (B); 13~15 min 100% (B); 15.1~22 min, 90% (A) and 10% (B). The flow rate and injection volume are 0.4 mL/min and 5 μL. The MS acquisition is under a mass range of 80~1200 m/z. The calibration was carried out using an external calibration delivery system, which infuses the calibrating solution before samples introduction. The MS is operated using an Information Dependent Acquisition (IDA) with the survey scan type (TOF-MS) and the dependent scan type (Product Ion) at -50 V of collision energy. The MS parameters are ion spray voltage of -4500 V, decluttering potential of 90 V, collision energy of -50 V, temperature at 400 °C with curtain gas of 25 psi, ion source gas 1 at 50 psi and ion source gas 2 at 50 psi, IDA MS/MS is performed

using the following criteria: ions that exceeded 100 CPS, ion tolerance 50 m Da, collision energy fixed at 25 V and dynamic background subtraction activated. For the quantification, an external calibration curve using a representative polyphenol of each group of phenolic compounds potentially found in the samples was prepared, being the following polyphenols, the ones selected for each specific group: phenolic acids (gallic acid); flavonoids (flavones: apigenin; flavonols: kaempferol; flavanones: naringenin; flavanols: catechin); stilbenes (resveratrol); isoflavonoids (genistein); phenylethanoids: (hydroxytyrosol).

4.6. Statistical analysis

One-way ANOVA with Dunnett's multiple comparisons test was performed using Statgraphics® Centurion XV (Statpoint Technologies, Inc., USA) and it was used to detect statistically significant differences of results between different extraction technologies on the follows: yields of proteins, polyphenols, chlorophyll a, carotenoids, and antioxidant properties. Statistical significance was accepted at $p < 0.05$. All experiments were carried out with at least three replicates.

5. Results and discussion

1.6 *PEF extraction- Spirulina biomolecules yield*

The results of protein, polyphenol, chlorophylls a and carotenoids of the *Spirulina* extracts obtained at different times (0~180 min) assisted by PEF pretreatment and different solvents (H_2O, 50% DMSO) were shown in Figure 1. As it was shown in the figure, the *Spirulina* protein yield ranged between 10 and 400 mg/g dw, observing increased values of protein (up to 300 mg/g dw) when PEF was applied compared to the samples without PEF pretreatment, independently of the solvent used. It should be also noted that the protein extraction increased until 60 min, then the values reached a plateau, and it was not observed a significant increase in the protein recovery with the further extend of extraction time. Moreover, it was observed that the protein extraction was

higher when H_2O was used compared to 50% DMSO. The polyphenol yield from *Spirulina* ranged between 3~20 mg/g dw. Similar to the results found for protein, PEF pretreatment increased the polyphenol content of the extracts compared to untreated samples, independently of the solvent used. Moreover, compared with 50% DMSO, H_2O is more effective in promoting polyphenol recovery, observing a rapid increase polyphenol yield (from 7.5 to 17.5 mg/g dw) within 0~60 minutes in PEF-H_2O extracts, being the final yield near 20 mg/g dw at 180 minutes.

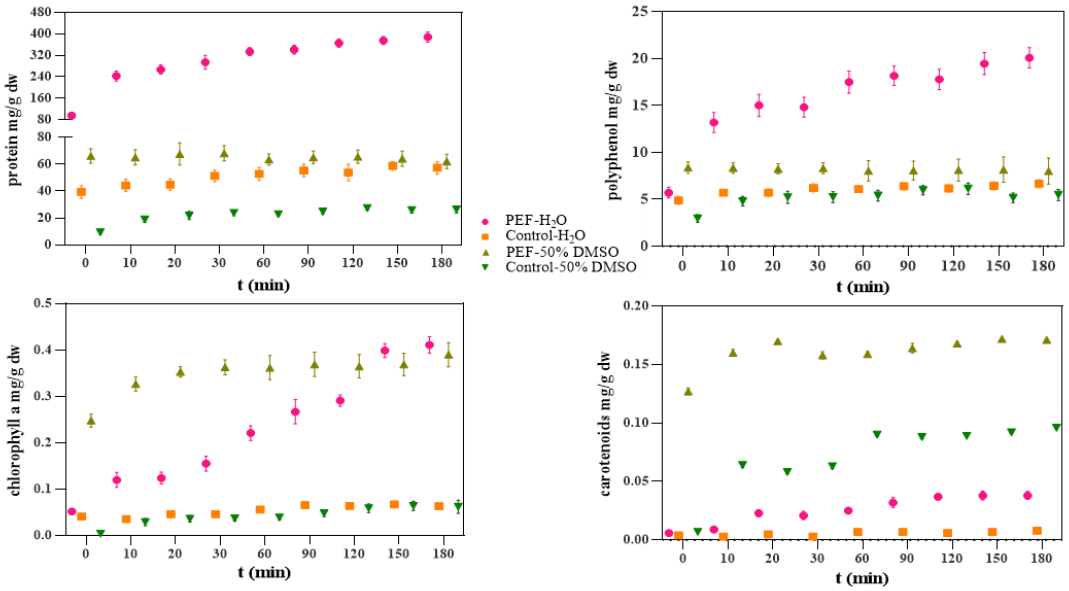

Figure 1. Protein, polyphenol, chlorophyll a, and carotenoids yield (dw) from *Spirulina* treated with PEF/without PEF (control) under different extraction times (0~180 min) and solvents (H_2O, 50% DMSO).

The results showed a low content of chlorophyll a, and carotenoids in *Spirulina* extracts, corresponded to 0.05~0.4 mg/g dw, and 0~0.2 mg/g dw, respectively. PEF treatment increased the pigment content of the *Spirulina* extract in both H_2O and 50% DMSO extracts. Considering the time effect, the yield curves of chlorophyll a, and carotenoids in the PEF group increased slowly with the extraction time extending to 180 min, while the yield in the control group remained stable, especially in the control-H_2O group, which was hardly affected by the extraction time.

Similar results for protein and polyphenol extraction kinetics curve were found in other studies. For example, Parniakov et al. (2015) explored the impact of PEF on the recovery of biomolecules of microalgae when prolonged extraction times with H_2O as a solvent, showing that the biomolecules content in microalgae extracts gradually increased over time, especially increasing rapidly before 3600 s, and observing a slow increase rate until extraction time of 10800 s (Parniakov, et al., 2015a). These results could be explained as the lower solution saturation in the early stage of extraction was conducive to the dissolution of biomolecules into the solvent. However, as the extraction time was further extended, higher solution saturation might lead to a decrease in the extraction rate.

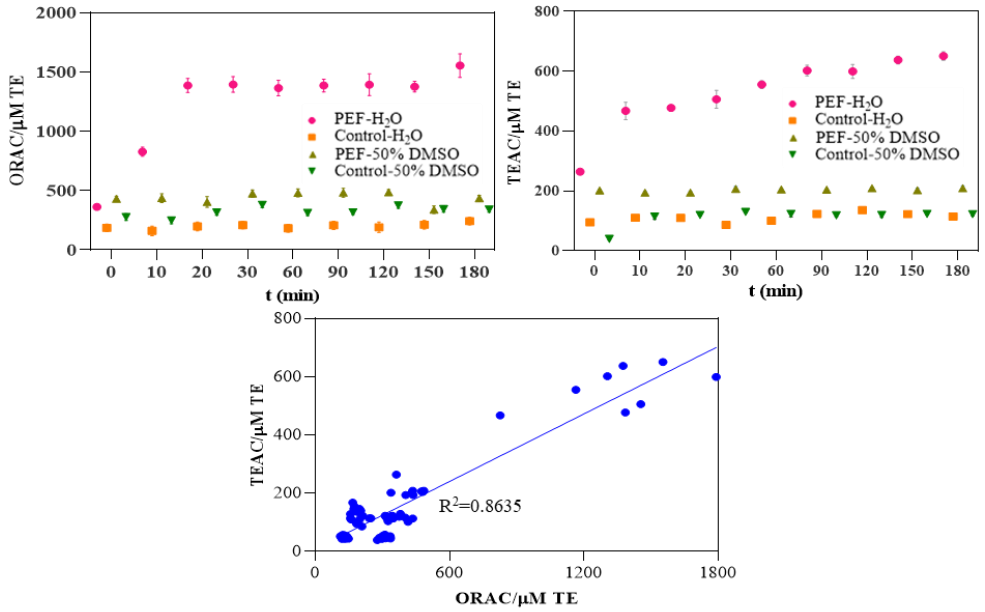

Figure 2. Antioxidant capacity results of *Spirulina* extracts treated with PEF/without PEF (control) under different extraction times (0~180 min) and solvents (H_2O, 50% DMSO). ORAC and TEAC corresponds to oxygen radical antioxidant capacity and Trolox equivalent antioxidant capacity respectively.

Obviously, the extraction solvent in this study affected the extraction results of *Spirulina* biomolecules. For the low extraction yield of protein in 50% DMSO extracts, another study attributed the phenomenon to the precipitation of proteins at high

concentration of organic solvent during the extraction process (Arakawa, Kita, & Timasheff, 2007). Previous studies have shown that polyphenols solubility decreased with the DMSO concentration increased from 0~50%, and then increased with the DMSO concentration increased from 50% to 100%, indicting the low solubility of biomolecules in 50% DMSO, which was consistent to our research (Parniakov et al., 2015b). However, the pigments extraction trend was different from protein and polyphenol. The higher contents of chlorophyll a, and carotenoids presented in 50% DMSO was related to solvent dissolution properties. DMSO $(CH_3)_2SO$) had a hydrophilic sulfinyl group and two hydrophobic methyl groups, which could dissolve both water-soluble compounds and fat-soluble compounds, resulting in the increase of fat soluble pigments content in a 50% DMSO extracts (Mueller, Trapp, & Neubert, 2019). Overall, the results indicated that both PEF and solvent had important effect on protein, polyphenol, and pigments yield, which together determined the extraction efficiency.

5.2 Effects of PEF on antioxidant properties

ORAC and TEAC assays were used to analyze the antioxidant capacity of *Spirulina* extracts. As it was shown in Figure 2, the antioxidant capacity of PEF extracts was higher than that of control group (no PEF treatment). The antioxidant capacity evaluated by ORAC and TEAC were both as PEF-H_2O > PEF-50% DMSO > control-H_2O/control-50% DMSO, which was in the same order as the polyphenol content, indicating that the polyphenols played an important role in the total antioxidant capacity of the extracts. From the perspective of the effect of extraction time on the total antioxidant capacity, PEF-H_2O extracts showed a significant increase of ORAC and TEAC value over time (0~30 min), which could be attributed to the increase of polyphenols and chlorophylls content during this process. The antioxidant value of other extraction curves did not change significantly within 0~180 minutes. Specifically, TEAC and ORAC assays evaluated the capacity of the samples to remove 2,2'-azinobis-(3-ethylbenzothiazoline-6-

sulphonate) and 2,2'-azobis-2-methyl-propanimidamide respectively (Şen, Bener, Bekdeşer, & Apak, 2021). Considering the possible evaluation differences caused by the different radical ions in ORAC and TEAC assays, we further analyzed the correlation coefficient between these two experiments. The results showed that there was a good positive correlation (R^2=0.8635) between TEAC and ORAC, indicating that they were highly consistent in the evaluation of antioxidant properties. In addition, correlation analysis based on extraction conditions (PEF, time, solvent), biomolecules (proteins, polyphenols, pigments) and antioxidant properties (ORAC, TEAC) was further performed using PCA (Principal Component Analysis), and the results were shown in Figure 3.

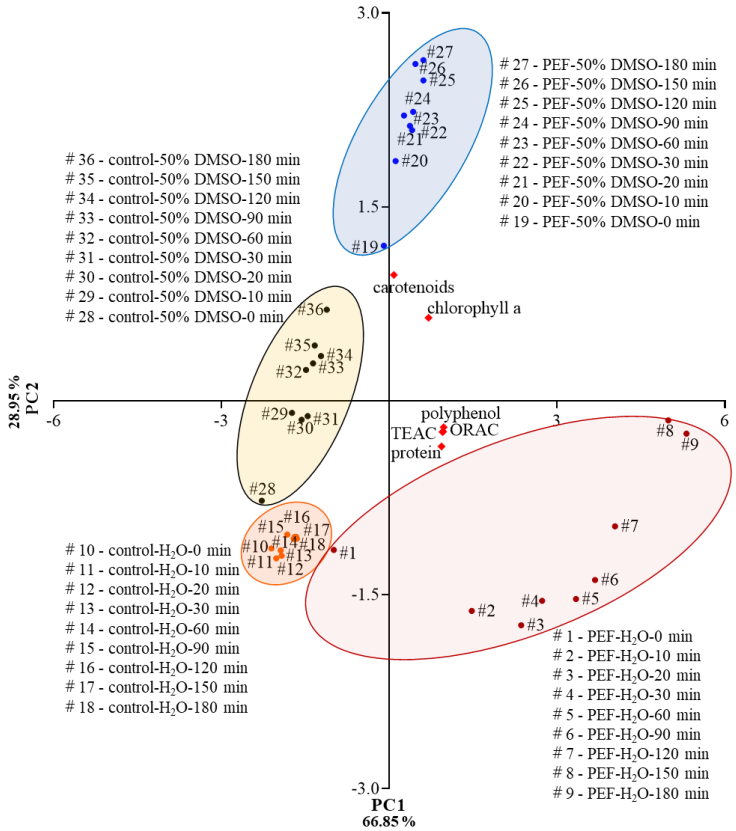

Figure 3. Principal Component Analysis (PCA) of the correlation between extraction conditions (PEF-H_2O, PEF-50% DMSO, control-H_2O, control-50% DMSO, extraction time of 0~180 minutes), biomolecules yield and antioxidant properties.

The proportion of variance of the two principal components was 66.85% for PC1 and 28.95% for PC2, respectively. Different extraction condition groups were divided into four categories by extraction technology (PEF, control) and solvent (H_2O, 50% DMSO), indicating that the extraction results were mainly affected by extraction technology and solvent in this study. Moreover, TEAC, ORAC, proteins and polyphenols were closely distributed in the same quadrant, indicating that the antioxidant properties were mainly related to the protein and polyphenol contents in the extracts. Finally, in terms of the biomolecules yields and antioxidant properties, PEF-H_2O-120 minutes was selected as the parameters in the further PEF extraction process.

5.3 Effects of PEF on microstructure of Spirulina

5.3.1 Fluorescence microscope

Figure 4 showed the effect of PEF treatment on the microstructure of *Spirulina*. When comparing Figure 4A and 4C, it could be observed that the PEF treatment destroyed the microstructure of *Spirulina*. The fragmented *Spirulina* filaments were clearly observed in Figure 4A, while the complete algae filament structure was shown in Figure 4C. The length of *Spirulina* filaments was usually about 400~600 μm, which was observed in Figure 4C (499.63 μm), and it was significantly longer than the fragments (4.08 μm) in Figure 4A.

After magnifying the partial of Figure 4A/4C, the classic spiral structure of *Spirulina* could be observed. As it was shown in the scale of Figure 4D, the spiral length was 41.56 μm and the bottom spiral diameter was 33.09 μm, which was consistent with previous reports (Akao et al., 2019). Fluorescence microscopy images also showed that PEF treatment could only destroy partially *Spirulina* filaments, because complete *Spirulina* filaments and helical structures were still found after PEF treatment, which indicated that expanding the voltage intensity and the number of pulses of PEF might further break the *Spirulina* filaments and increase the yield of biomolecules.

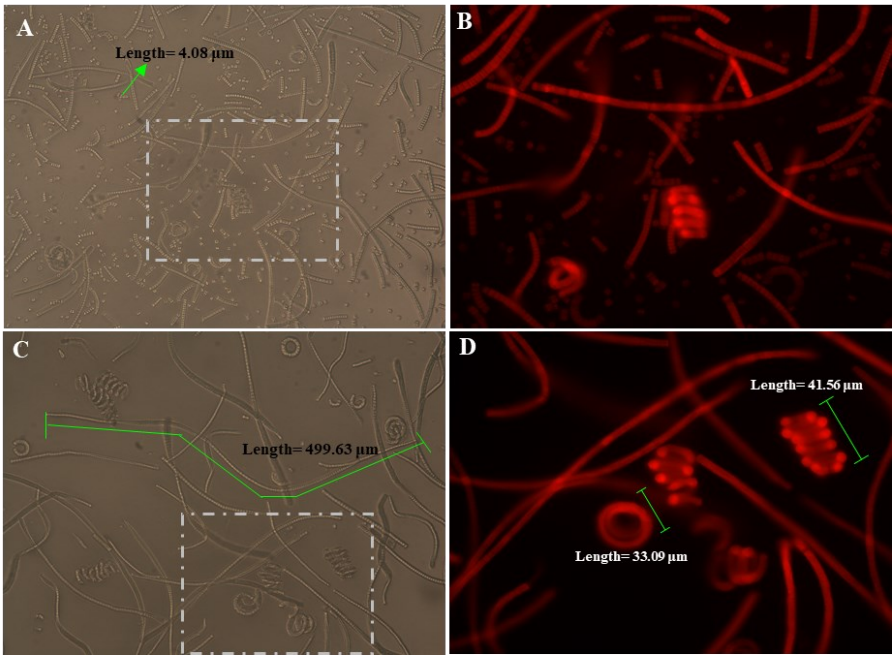

Figure 4. The effect of pulsed electric fields (PEF) on the microstructure of *Spirulina*. Figure 4A (16X)/4B (32X)-PEF-H_2O extracts, Figure 4C (16X)/4D (32X)-control-H_2O extracts.

A relate study compared the effects of PEF (25 kV/cm for 150 µs) and bead-milling treatment on the microstructure of *Spirulina*, and the microscopic results showed that PEF treatment resulted in the separation of cylindrical filament cells (trichomes), while bead-milling disrupted the intact cell structure, which resulted in a higher purity C-phycocyanin in the PEF extract (Martínez, Luengo, Saldaña, Álvarez, & Raso, 2017). This was consistent with the results of Figures 4A, 4B in our study, that was, PEF treatment dispersed the *Spirulina* 'filament' structure, resulting in an increase in the yield of biomolecules. In addition, another study has shown that PEF could cause damage to the microalgae (*Chlorella vulgaris*) cell membrane under the microscope. Scherer et al (2019) stained PEF-treated *Chlorella vulgaris* cells with Evans blue, a fuel that did not penetrate intact cells, and found that Evans blue was able to penetrate cells immediately after PEF treatment, indicating that PEF treatment penetrated *Chlorella vulgaris* cells (Scherer et al., 2019). According to Parniakov et al (2015), these results could be attributed to the

electroporation or electro-permeabilization effect of PEF, i.e., the action of short-duration and high-field-strength electrical pulses caused the microstructure damage or formation of pores of microalgae (Parniakov et al., 2015a).

5.3.2 Scanning Electron Microscope

To observe the effect of PEF on the cell surface structure of *Spirulina*, this study further used SEM to analyze the freeze-dried *Spirulina* samples. In Figure 5A/5D, when the magnification was 110 × (500 microns), the PEF-treated sample exhibited a more fragmented sheet structure, while the control group had a relatively complete block structure.

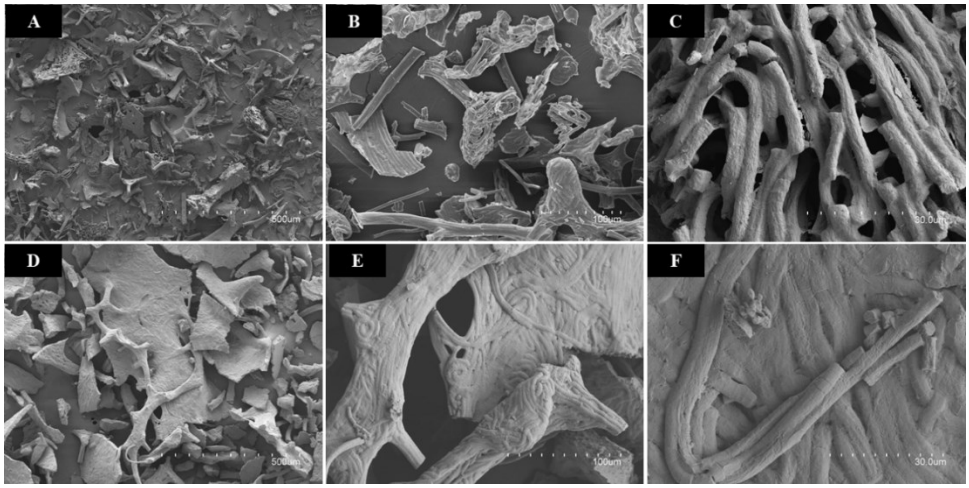

Figure 5. The effect of pulsed electric fields (PEF) on the microstructure of *Spirulina*. Figure 5A (110X)/5B (450X)/5C (1500X)-PEF-H_2O extracts, Figure 5D (110X)/5E (450X)/5F (1500X)-control-H_2O extracts.

In Figure 5B/5E, when the magnification was 450×(100 microns), it could be clearly observed that the fragmentation of *Spirulina* was more obvious after PEF treatment. Moreover, in the control group, complete filaments of *Spirulina* were observed, which appeared to be closely arranged together with a smooth surface. In Figure 5C/5F, rod-shaped *Spirulina* filaments could be observed in both PEF and non-PEF treated samples,

and the surface structure of non-PEF treated samples was still smooth and tightly arranged (Fig. 5F).

However, after PEF treatment, some of the rod-shaped *Spirulina* appeared to be broken, and the surface structure appeared loose and porous (Fig. 5C). Carullo et al (2021) conducted a similar study and their results showed that PEF treatment resulted in a rough surface structure of *Spirulina* with the formation of cracks and depressions, which was caused by electroporation and subsequent leakage of intracellular material (Carullo, Donsì, Ferrari, & Pataro, 2021). In addition, another study carried out by Käferböck et al (2020) showed that the trichome structure of *Spirulina* was damaged after PEF treatment, which caused an increase in the extraction yield of valuable components, such as C-phycocyanin, etc. (Käferböck et al., 2020). Combined with the results of fluorescence microscopy and SEM experiments, the increase in the yield of *Spirulina* biomolecules in this study was related to the electroporation effects of PEF.

5..4. Effects of PLE on Spirulina biomolecules yield

To further apply PEF + PLE to recovery biomolecules from microalgae, PLE extraction process was then determined. Figure 6 showed that PLE extraction significantly increased the protein, polyphenol, chlorophyll a, and carotenoids content when compared with the control group ($p < 0.05$). Specifically, the yield of protein was between 5-60 mg/g dw, among which the highest content was in PLE-H_2O group (60 mg/g dw), followed by the PLE-DMSO group (45 mg/g dw).

Previous studies showed that using H_2O as the extraction solvent could recover proteins, polyphenols, and pigments from microalgae assisted by PLE (Zhou et al., 2021), but there were few reports about using DMSO as a solvent in PLE extraction process. In this study, using H_2O or DMSO as the solvent could significantly increase the protein yield assisted by PLE ($p < 0.05$). For polyphenols, compared with H_2O or 50% DMSO, using DMSO

as a solvent significantly increased the content of polyphenols ($p < 0.05$). Moreover, the polyphenol content of PLE-DMSO and PLE-H_2O group corresponded to 12.5 mg/g dw and 12.0 mg/g dw respectively, which was almost three times that of control-DMSO and control-H_2O group respectively ($p < 0.05$).

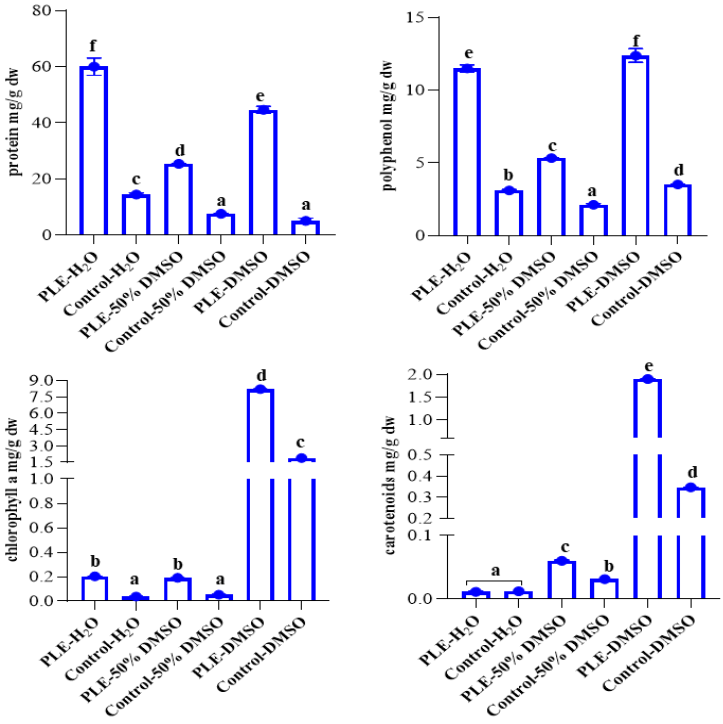

Figure 6. Effect of PLE, control (without PLE) and different solvents (H_2O, 50% DMSO, DMSO) on the yield (mg/g dw) of protein, polyphenol, chlorophyll a, and carotenoids. Same lowercase letters indicate no significant differences ($p > 0.05$) while different lowercase letters indicate significant differences ($p < 0.05$).

Therefore, both PLE and solvent had an important effect on the yield of protein and polyphenols. For pigments yield, the solvent had a greater impact than PLE effects, ie., regardless of whether PLE was used or not, the pigments yield with DMSO as the extraction solvent was significantly higher than that of H_2O and 50% DMSO extracts. Among them, the best results were shown in PLE-DMSO extracts, corresponded to 7.5 mg/g dw of chlorophyll a, and 2.0 mg/g dw of carotenoids respectively. Moreover, higher content of pigments was obtained in 50% DMSO extracts than H_2O extracts, which was

similar to the results of PEF extraction that the increase proportion of DMSO (50% DMSO) was conducive to the extraction of pigments.

5.5. Effects of PLE on antioxidant properties

As shown in Figure 7, the results of ORAC (oxygen radical antioxidant capacity) and TEAC (Trolox equivalent antioxidant capacity) presented a good correlation (R^2=0.9346). The antioxidant capacity of PLE extract was 2~3 times that of the control group, which was due to the higher biomolecules content in PLE extracts. Both ORAC and TEAC results showed that the antioxidant capacity of PLE-H_2O and PLE-DMSO extracts were stronger than PLE-50% DMSO extracts, which could be attributed to the results that PLE-H_2O extracts contained more protein/polyphenols and PLE-DMSO extracts contained more chlorophyll a and carotenoids.

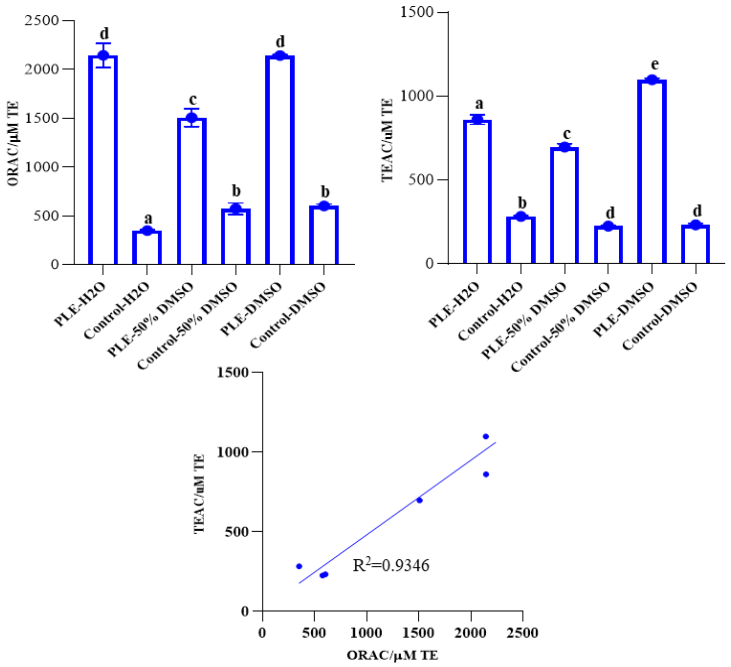

Figure 7. Effect of PLE, control (without PLE) and different solvents (H_2O, 50% DMSO, DMSO) on the antioxidant capacity of *Spirulina* extracts. Same lowercase letters indicate no significant differences ($p > 0.05$) while different lowercase letters indicate significant differences ($p < 0.05$). ORAC and TEAC corresponds to oxygen radical antioxidant capacity and Trolox equivalent antioxidant capacity respectively.

On this line, the correlation between antioxidant properties and biomolecules was analyzed using PCA, the results were shown in Figure 8. The proportion of variance of the two principal components in PCA was 77.14% for PC1 and 20.92% for PC2, respectively. All biomolecules were distributed on the same side of PC1 with PLE-H_2O/PLE-DMSO, and on the opposite side with PLE-50% DMSO, indicating that 50% DMSO as a solvent is not suitable for recovering biomolecules from *Spirulina* when using PLE, which was consistent with previous studies (Parniakov et al., 2015b). Moreover, the biomolecules were distributed on the same side of PC1 as TEAC/ORAC, indicating that all these biomolecules had a positive effect on the antioxidant properties. Among them, proteins and polyphenols were closely distributed with ORAC/TEAC, which indicated that proteins and polyphenols were more strongly associated with the antioxidant capacity of the extracts in this study.

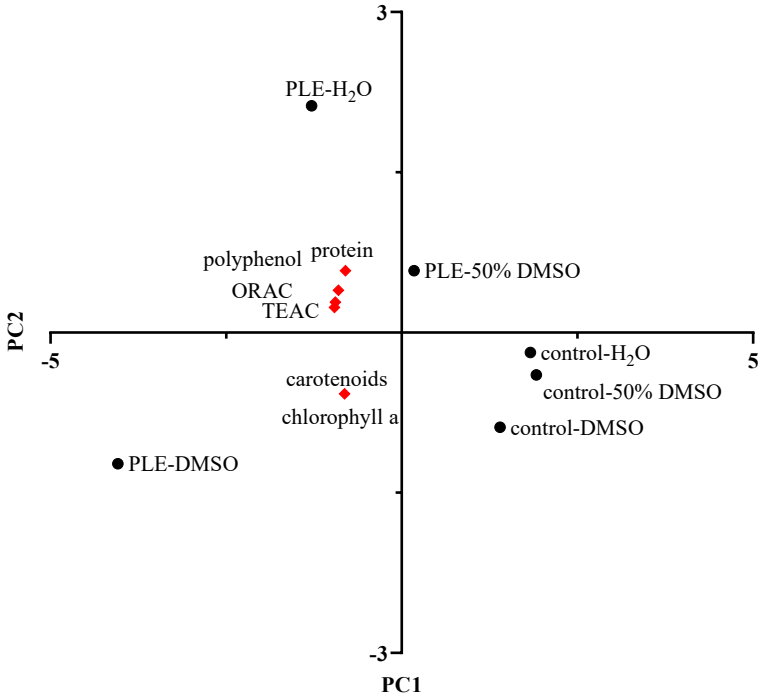

Figure 8. Principal component analysis (PCA) of the correlation between extraction conditions (PLE-H_2O, PLE-50% DMSO, PLE-DMSO, control-H_2O, control-50% DMSO, control-DMSO), biomolecules yield and antioxidant properties.

Previous studies have used ethanol and hexane to extract antioxidant compounds from *Spirulina* and found that polar reagents were more conducive to obtaining *Spirulina* biomass, which was due to the high protein and carbohydrate content of *Spirulina*, corresponding to 50~70% and ~15% respectively (Herrero, Martín-Álvarez, Señoráns, Cifuentes, & Ibáñez, 2005). Moreover, the DMSO used in this study could dissolve both water-soluble and fat-soluble compounds (Mueller et al., 2019). Therefore, in addition to water-soluble proteins and carbohydrates, fat-soluble pigments were also recovered by DMSO, which further increased the antioxidant capacity of *Spirulina* extracts. Combined the results of biomolecules content and antioxidant properties, DMSO was more suitable for the recovery of *Spirulina* biomolecules in PLE extraction process.

5.6. Effects of PEF + PLE on Spirulina biomolecules yield and antioxidant properties

The previous results showed that both PEF and PLE extraction recovered a certain proportion of proteins, polyphenols, and pigments from *Spirulina*. However, compared with the total biomass content of *Spirulina*, 17~57% protein (Lafarga, Fernández-Sevilla, González-López, & Acién-Fernández, 2020), 6.0~20.0 mg/g chlorophyll (Rangel-Yagui, Danesi, De Carvalho, & Sato, 2004) and 25~33.2 mg GAE/g polyphenol (Alberto, Francesco, Aliakbarian, Converti, & Perego, 2015), the yield of extracts obtained by using PEF or PLE alone could be further increased. Therefore, PEF + PLE, a novel extraction technology which has not been reported yet was used to obtain biomolecules from *Spirulina*, and the conventional extraction method-Folch extraction was used as a control. As it was shown in Figure 9, the biomolecules obtained by PEF + PLE corresponded to 400 mg/g dw protein, 9.2 mg/g dw polyphenols, 16.3 mg/g dw chlorophyll a and 1.7 mg/g dw carotenoids. It was obvious that the application of PEF + PLE further increased biomolecules yield compared to use PEF or PLE extraction alone in Figure 9. It was worth noting that, compared with traditional extraction methods-Folch extraction, PEF + PLE increased the protein, polyphenol, chlorophyll a and ORAC values

of *Spirulina* extracts by 1328%, 979%, 11% and 47% respectively. In addition, PEF + PLE greatly shortened the extraction time of Folch extraction by 165 minutes. On this line, PEF + PLE met the requirements of short extraction time with high biomolecules content in microalgae nutrients recovery industry.

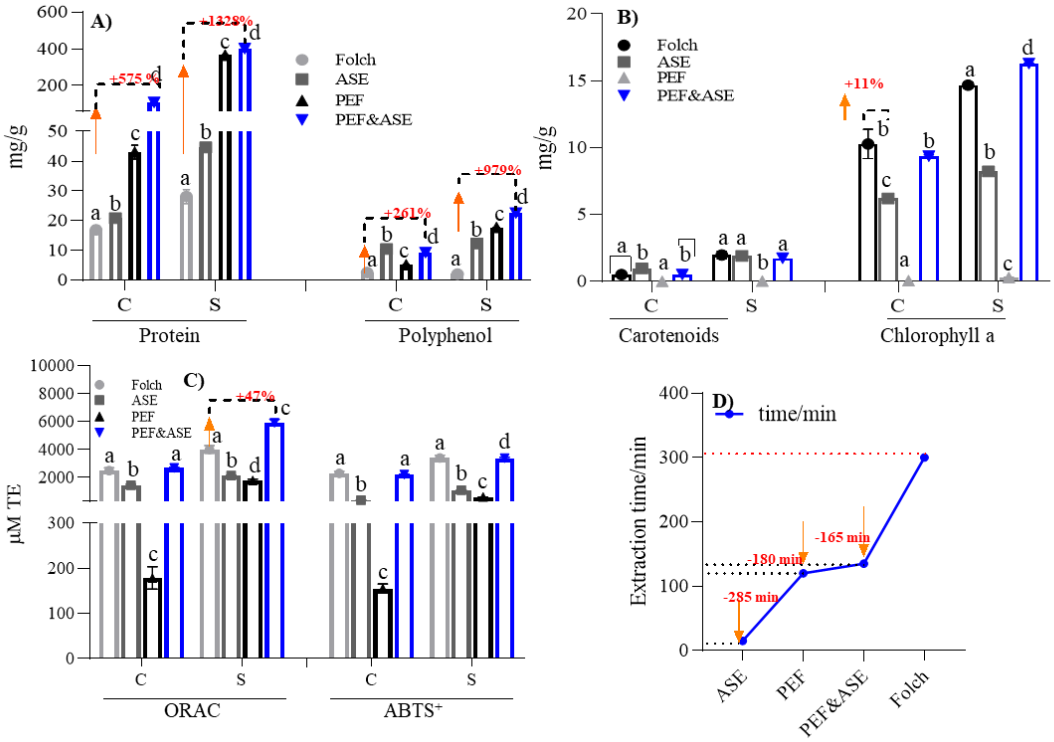

Figure 9. Protein, polyphenol, carotenoids, chlorophyll a, carotenoids content and antioxidant properties of *Spirulina* extracts as well as extraction time (refers to the extraction time for the above results, not the extraction time under the same yield) required for PEF + PLE, PEF, PLE and Folch extraction processes. Same lowercase letters indicate no significant differences ($p > 0.05$) while different lowercase letters indicate significant differences ($p < 0.05$).

Moreover, the combined use of PEF + PLE in this study achieved an improved extraction compared to the extraction assisted by PEF or PLE alone found in other studies. For example, Martí-Quijal et al. (2021) applied PEF to obtain *Spirulina* extracts containing carotenoids (0.50 mg/g dw, 60 min), chlorophyll a (0.60 mg/g dw, 120 min)

and polyphenol (19.75 mg GAE/g dw, 180 min) (Martí-Quijal et al., 2021). Zhou et al. (2021) applied PLE to obtain *Spirulina* extracts which contained protein (210 mg/g dw), chlorophyll a (1.46 mg/g dw), carotenoids (0.12 mg/g dw) and polyphenols (11.49 mg/g dw) (Zhou et al., 2021). These studies showed that the biomolecules recovery effect of using PEF or PLE alone was not as good as PEF + PLE. Moreover, the biomolecules recovered by PEF + PLE from *Spirulina* in this study was also higher than other extraction techniques. For example, Vernès et al. used ultrasound technology (probe, 20 kHz) to obtain 229% higher protein content (28.42 ± 1.15 g/100 g dw) from *Spirulina* than the traditional extraction method (8.63 ± 1.15 g/100 g dw) (Vernès et al., 2019), which was still relatively lower to the content of 400 mg/g dw protein in the PEF + PLE *Spirulina* extract.

In addition, PEF + PLE existed a potential protective effect on the antioxidants. In general, the feature of PLE came from the destructive effect of high pressure/high temperature on the raw materials during the extraction process, therefore, the biological activity of biomolecules in microalgae could inevitably damage at high temperature (Ji et al., 2020). PEF destroyed the microalgae cell structure and thus reduced the dependence of PLE on high temperature, allowing PLE could be performed at room temperature, which not only improved the extraction efficiency, but also protected the antioxidants. In this study, PEF + PLE played the role of 'one plus one was greater than two', and PEF-'electroporation' combined with PLE-'pressure fluid' made it more efficient and environmentally friendly compared with traditional methods or using PEF or PLE alone. Briefly, PEF + PLE had great potential in the development of microalgae industry.

5.7. Effects of PEF + PLE on Spirulina phenolics composition

Polyphenols had an important impact on the antioxidant capacity of biomass in this study, this was attributed to the ability of polyphenol to capture free radicals, active oxygen and chelate metal ions, which was depend on both content and type of polyphenols (Da Silva

Port's, Chisté, Godoy, & Prado, 2013). The above experimental results showed that PEF or PLE could increase the polyphenol content in the extract, which was not equal to the increase of phenolic types. Therefore, Triple TOF-LC-MS-MS was used to analyze the polyphenol components of PEF + PLE, PEF, PLE and Folch extracts, to further analyze whether extraction technology affected the polyphenol composition of *Spirulina* extract, the results were shown in Figure 10.

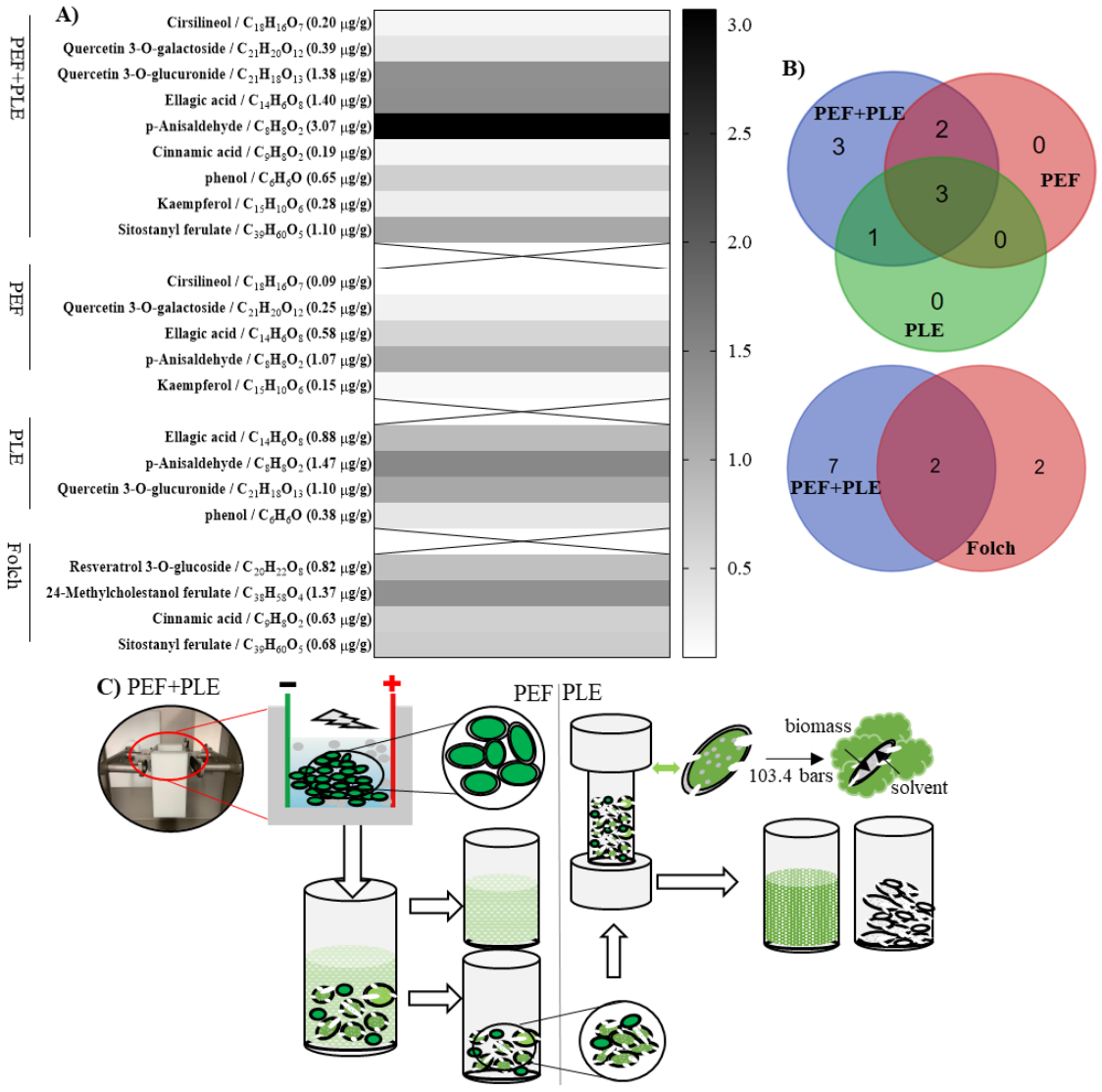

Figure 10. A) Phenolic profile and content of extracts obtained from *Spirulina* biomass by PEF + PLE and Folch extraction; B) Venn diagram-same (overlapping)/different (non-overlapping) polyphenol types; C) Schematic diagram of the effect of PEF + PLE on *Spirulina*.

The results showed that the polyphenol type identified from the PEF + PLE, PEF, PLE and Folch extracts of *Spirulina* in this study corresponded to 9, 5, 4 and 4, respectively. Specifically, the polyphenols of PEF + PLE extracts were mainly p-anisaldehyde (3.07 μg/g), ellagic acid (1.40 μg/g), quercetin 3-o-glucuronide (1.38 μg/g) and sitostanyl ferulate (1.10 μg/g), while for PEF, PLE, Folch extracts, the main polyphenols were corresponding as *p*-anisaldehyde (1.07 μg/g), *p*-anisaldehyde(1.47 μg/g)/quercetin 3-o-glucuronide (1.10 μg/g) and 24-methylcholestanol ferulate (1.37μg/g) respectively. Figure 10B showed the amount of same (overlapping) and different (non-overlapping) polyphenol types obtained by different extraction methods through Venn diagram. It could be seen from Figure 10B that the type of polyphenols obtained by PEF PLE, PEF + PLE and Folch was different, which was dependent on both extraction technology and the solvent. In general, the results of Triple TOF-LC-MS-MS confirmed that PEF + PLE extraction not only increased the content of polyphenols, but also increased the type of polyphenol profile, which was valuable for the utilization of microalgae polyphenols.

According to related reports, there were more than 1,100 kinds of algae in the ocean, which were perfect natural sources of polyphenols (Jimenez-Lopez et al., 2021). Researchers classified polyphenols in microalgae according to different molecular structures: simple phenols (phenols, catechol, hydroquinone, phloroglucinol, etc.), C6-C1 (phenolics acids, aldehydes), C6-C2 (phenylethanoids, phenylacetic, acetophenone, phenethyl) alcohol), C6-C3 (hydroxycinnamic acids, cinnamic aldehydes, monolignols, phenyl propenes, coumarins, etc.), C6-C1-C6 (xanthonoids), C6-C2-C6 (stilbenoids, anthraquinones, anthrones), C6-C3-C6 (flavonoids), C6-C7-C6 (diarylheptanoids), lignans, lignins, tannins and phenolic terpenoids (Jimenez-Lopez et al., 2021). This study identified several above typical polyphenols, such as phenol (simple phenol), cinnamic acid (C6-C3) and quercetin (C6-C3-C6). Moreover, polyphenols with relatively complex structures were also detected, such as syringaresinol, which could resist oxidation and

regulate the intestinal flora, however, reports on the extraction and application of syringaresin from microalgae were rare (Cho, Song, Yoon, Park, & Kim, 2018). Based on the results of this study, the principle of PEF + PLE improving the yield of microalgae biomolecules and polyphenol types was shown in Figure 10C. PEF treatment could cause an increase of cell membrane permeability and consequently caused an increase of the cell membrane and tissue conductivity (Maza et al., 2020). Combined FM/SEM results, the PEF extraction process destroyed the *Spirulina* filaments and causes damages to the cell structure, which helped to the diffusion of DMSO into microalgae during the PLE high-pressure extraction process, thereby to achieve efficient recovering biomolecules from *Spirulina*. It should be noted that different microalgae, such as green algae, brown algae, and cyanobacteria had different cell structures, cell wall thickness, and conductivity of the cell suspension, which might lead to different effects of PEF treatment. Therefore, considering the diversity of microalgae, it is necessary to study the optimal extraction parameters of PEF, PLE and PEF + PLE when applied to different types of microalgae.

6. Conclusions

This study is the first report regarding the combined application of pulsed electric fields (PEF) and pressurized liquid extraction (PLE) in a multistep process to recover biomolecules from *Spirulina* observing promising results regarding high yield of biomolecules obtained. The main effect of PEF in the extraction of *Spirulina* is attributed to its ability to destroy the intact filaments of the microalgae and damaging the cell wall structure, thus promoting a further PLE efficient extraction. The PEF + PLE extraction not only increases the content of polyphenols, but also increases the types of polyphenols in the extract, which has potential economic value for the industrial application of microalgae polyphenols. Compared with other traditional extraction reagents, such as chloroform, methanol, n-hexane, etc., DMSO has the advantages of low toxicity, high

fluidity, and good selectivity at normal temperature and pressure. The final extract contains DMSO, which can be removed by vacuum distillation, freeze-drying, etc. Besides, the extract in this study contains complex biomolecules, which can be further separated and purified by organic reagent precipitation, membrane separation, ion exchange column, etc. to obtain a purified single component. Overall, the application of PEF + PLE to the recovery of different microalgae biomass and the formation of industrialization is a topic worthy of further consideration.

Acknowledgements

This research was funded by the University of Valencia through the projects OTR2021-21736INVES and OTR2021-21570INVES supported by the University of Vigo. Moreover, it was partially funded by the EU Commission and BBI-JU Horizon H2020, through the AQUABIOPRO-FIT project (aquaculture and agriculture biomass side stream proteins and bioactives for feed, fitness and health promoting nutritional supplements) grant number 790956. Jianjun Zhou and Min Wang were supported by a PhD fellowship from the China Scholarship Council (CSC) (No. 201908420246 and No. 201908420245, respectively). Authors would also like to acknowledge Generalitat Valenciana for financial support (IDIFEDER/2018/046—Procesos innovadores de extracción y conservación: pulsos eléctricos y fluidos supercríticos) through the European Union ERDF funds (European Regional Development Fund) and the team of the Institute of Agrochemistry and Food Technology, National Research Council (IATA-CSIC), lead by Prof. María Carmen Collado, and Pepe Coll for their help in the experiments carried out, especially fluorescence microscopy.

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4.3 A combined ultrasound + membrane ultrafiltration (USN-UF) for enhancing saccharides separation from *Spirulina* (*Arthrospira platensis)*

Journal: ***Innovative Food Science and Emerging Technologies*****, 2023, 85, 103341; https://doi.org/10.1016/j.ifset.2023.103341.**

A combined ultrasound + membrane ultrafiltration (USN-UF) for enhancing saccharides separation from *Spirulina* (*Arthrospira platensis*)

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Abstract

In this study the combination of ultrasound (USN) + membrane ultrafiltration (UF) was used to separate *Spirulina* saccharides (SPS). The results showed that USN significantly ($p < 0.05$) increased the average high-added-value compounds yield (mainly protein 572.8 mg/g, saccharides 133.6 mg/g and polyphenols 33.6 mg/g, dw) when the setting of 400W/10 min was applied. Scanning electron microscope (SEM) results showed that USN treatment effectively destroyed *Spirulina* microstructure. The extraction efficiency differed according to USN total energy consumption, with higher extraction efficiency observed when a cooling system was not used. The total bioactive compounds yield of 741.9 mg/g (dw) was obtained when an energy of 470.6 kWh/kg was used. Different molecular weight cut-offs (MWCOs) membranes were used to separate SPS. Relative high purity SPS (~70%) were obtained when 4 and 10 kDa membranes were used, while a higher separation efficiency occurred when using 100 and 150 kDa membranes. USN treatment increased the concentration of relatively low molecular weight SPS (4, 10 kDa) in the permeate ($p < 0.05$) but there was no significant ($p > 0.05$) effect on the high molecular weight SPS (300, 500 kDa). Membrane fouling was observed during the saccharides UF process, thus indicating that the process could be further improved.

Keywords: *Spirulina*, ultrasound extraction, membrane ultrafiltration, saccharides, microscopic morphology

1. Introduction

As the global population increases so will the demand for food consumption. According to the Food and Agriculture Organization of the United Nations (FAO) it will be difficult to meet the food needs of nearly 10 billion people worldwide by 2050 (FAO, 2021). In addition, the COVID-19 pandemic has disrupted and hampered food industry production, distribution, and consumption, which has triggered a food crisis for humanity (Rivera-Ferre et al., 2021). Furthermore, adverse environmental impacts such as increased greenhouse gas emissions, overuse of pesticides, and intensified use of land and fresh water have affected traditional crop-based food supply systems (Chen et al., 2022). Within this context, the exploitation of edible marine resources, especially microalgae, is being promoted by the food industry because of their ability to survive in harsh conditions and capture solar energy, water, and incorporate inorganic nutrients into biomass, and their assimilation efficiency exceeds that of terrestrial plant (Pramanik et al., 2019).

Spirulina is a type of cyanobacteria most used in animal and human nutrition due to a high protein content from biomass and the biological activity of components such as saccharides, vitamins, minerals, and fatty acids (Costa et al., 2019). The optimization of the extraction conditions of *Spirulina* bioactive compounds has been widely studied, such as traditional extraction methods, including hot water extraction, Soxhlet extraction, Folch, etc (Zhou et al., 2021). However, innovative extraction methods need to be developed and optimized, since traditional extraction involves the usage of large amounts of toxic solvents, long extraction times, and high extraction temperatures (Zhou et al., 2022). Compared to traditional extraction techniques, ultrasound (USN) extraction has several advantages (i.e., high efficiency, mild operating conditions, low toxicity, and time efficient), Moreover, it has been used in microalgae biorefinery research (Sivaramakrishnan et al., 2018). USN cell disruption is attributed to acoustic cavitation, where ultrasound waves create molecular motion through a series of rarefaction and

compression cycles, creating small gas or vapor-filled bubbles in a liquid (Liu et al., 2022). When the bubbles reach a size where the USN energy is not sufficient to retain the vapor inside, they collapse violently during the compression cycle, releasing a large amount of energy, which produces mechanical/physical and chemical effects of microalgae cell rupture (Wang et al., 2020).

Spirulina contains a variety of high-added-value compounds (HAVCs) such as proteins, polyphenols, pigments, polysaccharides, etc. (Zhou et al., 2023), the separation of these compounds being of great significance for their potential applications. Among the HAVCs from *Spirulina*, polysaccharides from *Spirulina*. *platensis* has shown potent immunomodulatory, anti-tumor, antioxidant, and anti-fatigue activities (Liu et al., 2022). The separation of crude saccharides from microalgae extracts is a critical step in polysaccharide purification, as it is related the removal of macromolecular impurities such as proteins, pigments, and lipids. At present, some studies have carried out impurity removal before polysaccharide extraction, including the removal of proteins (Sevag, Trichloroacetic acid (TCA), protease), pigments (active carbon, H_2O_2 oxidation), lipids (ethanol soaking), etc., but each step promotes polysaccharide loss, and has a high economic cost (Kang et al., 2022; Qiu et al., 2022; Tang et al., 2020).

Ultrafiltration (UF) technology is considered a green operation technique, with a high separation efficiency and low cost, making it a good candidate for polysaccharide purification compared to common organic reagents used to remove impurities in multiple steps (Tang et al., 2020). Furthermore, the operating conditions of ultrafiltration are conducted under mild temperatures, thus reducing possible denaturation, inactivation, or degradation of biomolecules of interest (Balti et al., 2021).

The separation theory of ultrafiltration is based on the molecular weight cut-offs (MWCOs) of the membrane pore size, that is, under a certain hydrostatic pressure and flow rate, molecules of different sizes and shapes in an aqueous solution are separated

through the membrane (Feng et al., 2019). Some previous studies have used ultrafiltration membranes with different MWCOs (1, 5, 10, 100 kDa) to obtain crude saccharides of different molecular weights from *Spirulina*, *Chlorella*, etc., and finally obtained purified microalgae saccharides fraction (Cai et al., 2022; Sheng et al., 2007; Wang et al., 2020; Yuan et al., 2020). Based on this, in this study, USN extraction parameters were first selected for the most efficient release of *Spirulina* bioactive compounds into aqueous solutions, and then ultrafiltration technology was further applied using membranes of different MWCOs (4, 10, 50, 100, 150, 300, 500 kDa) to separate the saccharides in the extracts, that is, to obtain the crude saccharides efficiently by ultrasound + membrane ultrafiltration (USN-UF) technology, with the aim to provide a reference for the current microalgae *Spirulina* saccharides (SPS) refining industry.

2. Materials and methods

2.1 Materials

For the *Spirulina* culture conditions, during the experiment, the day-time temperature was 32 °C and temperature decreased to 24 °C at night. The pH of the culture varied from 9.8 to 10.4 controlled by the addition of CO_2. *Spirulina* biomass was filtered using a tambor filter of 30 micra mesh. Cultivation medium went back to the cultivation pond, while biomass was vacuum-pressed and then frozen in 50 g portions. The sample was freeze-dried (-40 °C for 72 h) to reduce the degradation of poorly resistant before use. The nutrients, microbiology and contaminants information of *Spirulina* (*Arthrospira platensis*) were as follows on a dry weight basis (dw), protein 652 ± 13 mg/g, saccharides 205 ± 4 mg/g, chlorophyll a 12 ± 1 mg/g, carotenoids 6 ± 1 mg/g, polyphenols 38 ± 3 mg/g, Ferric 1497 ± 15 mg/kg, β-carotene 1339 ± 10 mg/kg, mesophilic aerobic flora < 2000 UFC/g, *Enterobacteria* < 100 UFC/g, *E.Coli* ND, *Staph aureus* < 10 UFC/g, *Salmonella* ND, Yeasts/molds < 100 UFC/g, pesticides ND, cadmium 0.032 ± 0.001 mg/kg, arsenic 0.78 ± 0.03 mg/kg, mercury < 0.005 mg/kg, benzopyrene < 0.5 μg/kg.

Folin-Ciocalteu, gallic acid and phenol were purchased from Sigma-Aldrich (Steinheim, Baden-Württemberg, Germany). Sodium carbonate (Na_2CO_3) was acquired from VWR (Saint-Prix, France). D-glucose (98%) and bovine serum albumin (BSA, 98%) standard were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). Sodium hydroxide, glacial acetic acid, and sulfuric acid were supplied by Fisher Scientific. Polyether sulfones (PES) membranes with MWCOs of 4, 10, 100, 150, 300 and 500 kDa were purchased from Microdyn-Nadir Company (Germany).

2.2 Ultrasound (USN) extraction

2.2.1 USN extraction process

A UP-400S ultrasound processor (Hielscher GmbH, Germany) with a constant frequency of 24 kHz was used for ultrasound extraction (Zhang et al., 2020). The ultrasound probe (diameter: 14 mm, length: 100 mm) was submerged in a beaker, containing a 2.0% suspension (Tavakoli et al., 2021). The total treatment time of USN extraction was 5, 10, 15, 20 minutes, and the amplitude was fixed at 25%, 50%, 75%, 100%, which corresponded to the power of 100, 200, 300, 400 W. The temperature increase was recorded every minute and USN-cooling system (USN-CS) extraction was carried out to evaluate the thermal effects during ultrasound extraction. The control group extraction (2.0%, dw/volume, L) at different temperatures (20/30/40/50/60/70 °C) and time (5/10/15/20 min) using a magnetic stirrer was set to calculate the yield increase after USN treatments. After the extraction process, the extracts were centrifuged using a MiniSpin Plus Rotor F-45-12-11 (Eppendorf, France) at 14,100 *x*g for 15 min, then the supernatants were collected for further analysis.

2.2.2 Specific energy analysis

Specific energy consumption, W (kWh/kg suspension), was calculated for ultrasound treatment according to the following formula (Zhang et al., 2020):

$$W(kWh/kg) = (P(w)*t(s))/(m)\ (kg) \qquad (1)$$

where P (W) is the ultrasound power, t (s) is the time of USN extraction process, m is the mass of 2.0% suspension (kg).

2.2.3 Protein quantification

The bicinchoninic acid (BCA) method was used to measure the protein content in the extracts (Balti et al., 2021; Zhou et al., 2022). Protein content was determined using a calibration curve (0~2000 mg/L) using bovine serum albumin (BSA) as a standard. During the measurement, 10 μL of samples or BSA and 200 μL of BCA working solution were added and incubated at 37 °C for 30 min, the absorbance was then measured at 562 nm using an Ultraviolet-*visible* (UV-*Vis*) spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA).

2.2.4 Saccharides quantification

The saccharidess content in the extracts was measured with the concentrated sulfuric acid-phenol method (Dubois et al., 1956). Specifically, 1 mL of sample, 0.5 mL of phenol and 2.5 mL of H_2SO_4 (98%) were sequentially added to a glass reaction flask and reacted for 30 min at room temperature. The absorbance value at 490 nm was measured using a UV-*Vis* spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA). D-glucose was used as a standard for calculating saccharides content.

2.2.5 Polyphenols quantification

The Folin-Ciocalteu method was used to analyze the polyphenol content in the extracts (Li et al., 2022; Kokkali et al., 2020). That is, 0.2 mL of sample, 1 mL of Folin-Ciocalteu (diluted with water at a ratio of 1:10, v/v) and 0.8 mL of Na_2CO_3 solution (75 g/L) were mixed and incubated in a water bath at 50 °C for 10 min. Then, the absorbances were measured at 750 nm using a UV-*Vis* spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA). Gallic acid was used as a standard to prepare the calibration curve for quantifying the polyphenol content in the extracts.

2.2.6 Pigments quantification

Pigments analysis was carried out according to the method proposed by (Safi et al., 2015) with some minor modifications. Specifically, 200 µL of algae extract was mixed with 1300 µL of pure methanol and incubated in the dark for 1 h at 45 °C. Samples were then centrifuged at 10,000 *x*g for 10 min at 20 °C. The organic phase containing the pigments was recovered and the absorbance was measured using a UV-*Vis* spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA). The chlorophyll *a* and carotenoid content was calculated using the following equations (Safi et al., 2015; Wellburn, 1994):

$$\text{Chlorophyll a (mg/L)} = 16.72 * A665 - 9.16 * A652 \quad (2)$$

$$\text{Carotenoids (mg/L)} = 4 * A480 \quad (3)$$

The final yields of chlorophyll *a* and carotenoids were calculated as mg/g (dw) in microalgae, the equation was as follows:

$$\text{Yield (mg/g dw)} = (\text{Cpigments (mg/L)}) * (\text{Vextracts (L)}) / \text{W (microalgae (g))} \quad (4)$$

Where C, V and W are the abbreviation of concentration, volume, and weight respectively.

2.2.7 Ultrasound increment analysis

The yield increase after ultrasound treatments was calculated using the following equations:

$$\text{Yield increase after ultrasound (mg/ (g dw))} = \text{Yields}_{USN} - \text{Yields}_{E} \quad (5)$$

Where USN is ultrasound extraction, E is control extraction.

2.2.8 Component ratio calculation

$$\text{Dynamic proportion (\%)} = ((X)/(T)) * 100\% \quad (6)$$

Where 'X' is the content of specific bioactive compounds (protein, polyphenol, chlorophyll a, carotenoids, mg/g dw), 'T' is the total amount of the compounds in the extracts (the sum of protein, polyphenol, chlorophyll a, carotenoids content, mg/g dw).

2.2.9 Scanning Electron Microscope (SEM) analysis

The precipitate of the extracts after centrifugation was used to analyze the effects of USN on *Spirulina* microscopic morphology (Fang et al., 2021). Scanning electron microscopy (SEM Quanta 250 FEI Company, Eindhoven, The Netherlands) was used to analyze the microstructure of samples after freeze drying (MUT 002A pilot freeze-drier (Cryotec, France)) for 72 hours at a cold trap temperature of -65 °C. Freeze-dried samples were then mounted on specimen stubs with colloidal silver, sputter-coated with gold-palladium and imaged with an SEM microscope at magnifications of 1000×.

2.3 Membrane ultrafiltration (UF) of Spirulina saccharides

2.3.1 UF process

Membranes with different MWCOs were used to separate saccharides from the extracts, following the study of (Tang et al., 2020). Filtration was performed in a stirred cell Amicon 8200 (Millipore, Billaica, USA) with a membrane diameter of 63.5 mm and maximal volume of 200 mL. Compressed air was used to supply a transmembrane pressure (TMP) of 2 bars. The temperature of the extracts was kept at 20 °C during the filtration process. Seven kinds of polyether sulfones (PES) membranes (Microdyn-Nadir Company (Germany)) with MWCOs of 4, 10, 100, 150, 300 and 500 kDa were used to purify the extracts. According to the reference of Microdyn-Nadir Company (Germany), the PES membranes were soaked with deionized water for 30 min prior to use. A magnetic stirrer fixed over the membrane surface provided a constant stirring speed of 500 rpm.

Prior to ultrafiltration, 100 mL of deionized water was ultrafiltered using a soaked membrane, and the volume change of the permeate was recorded. Then, 100 mL of *Spirulina* USN extract was added into the ultrafiltration container and the ultrafiltration process was performed until the permeate volume was 90 mL and the change of the permeate volume was also recorded. The permeate of *Spirulina* extracts was collected for heat concentration (95 °C, 3 h), and the concentrated solution was centrifuged (14,100 *x*g for 15 min) and freeze-dried to obtain crude saccharides powder for subsequent analysis.

After the ultrafiltration process of the *Spirulina* extract, deionized water was used to clean the cake layer on the surface of the membrane, and the cleaned membrane was again used for deionized water (100 mL) ultrafiltration with the change in the volume of the permeate was recorded. The membrane ultrafiltration process followed control extraction (E) was abbreviated as E-UF.

2.3.2 pH and conductivity analysis

The electrical conductivity (μS/cm) and pH of *Spirulina* membrane permeates were measured with a conductivity meter InoLab pH/cond Level 1 (WTW, Weilheim, Germany). The volume reduction ratio (VRR) was defined as follows (Zhu et al., 2015):

$$VRR(mL) = V\_initial/V\_concentrate \quad (7)$$

where V initial is the initial extracts volume (mL) and Vconcentrate is the concentrate volume (mL).

2.3.3 Permeation flux analysis

The permeate flux ($mL.min^{-1}.m^{-2}$) was calculated according to the change of permeate volume over time, which was used to evaluate the filtration efficiency of the PES membrane, including the water flux, permeate flux of extracts during ultrafiltration and water flux after extracts ultrafiltration. The calculation formula is as follows (Zhu et al., 2015):

$$J(mL.min^{-1}.m^{-2}) = dv/dt.\ A^{-1} \quad (8)$$

Where dv is the derivative of volume (mL) and dt is the derivative of time (min), A is membrane area (m^2).

3. Statistical analysis

The results of bioactive compounds yield at different extraction parameters (100/200/300/400 W, 5/10/15/20 min) were analyzed using an ANOVA test followed by Tukey post-test. The results were expressed as mean ± standard deviation, and results were considered significant when the p-value was lower than 0.05. Different lowercase

letters represent significant differences ($p < 0.05$), and ns represent no significant differences ($p > 0.05$). Those parameters of interest were subjected to principal component analysis (PCA) using the Graph 9 software to measure the correlation matrix. The inputted variables amongst different samples were mapped as a biplot where the loadings and scores were distributed in two-dimensional section determined by the first two principal components (PCs) with eigenvalues > 1.0.

4. Results and discussion

4.1 Yield of high-added-value compounds and specific energy consumption

Figure 1 showed the yield and specific energy consumption of *Spirulina* USN extraction of HAVCs and evaluation of the impact of thermal effect on USN extraction efficiency. From Figure 1, it could be seen that the temperature of USN-no cooling system (USN-NCS) increased from 18 °C to 40~80 °C (100~400 W) from 5 to 20 min, and the median temperature of USN-CS was below 30 °C (100~400 W), which indicates that USN produced a strong thermal effect. Based on this, the extraction efficiency and energy consumption of USN-NCS and USN-CS was compared. Overall, it was found that the combination of USN-NCS is beneficial to recover *Spirulina* HAVCs. Specifically, USN-NCS with prolonged extraction time (up to 20 min) allowed for obtaining around 550~600 mg/g protein, 35~40 mg/g polyphenols, 130~140 mg/g saccharides, 1.1~1.3 mg/g of chlorophyll a, and 0.25~0.28 mg/g of carotenoids. Correspondingly, USN-CS could yield 500~550 mg/g protein, 35~40 mg/g polyphenols, 115~125 mg/g saccharides, 1.1~1.3 mg/g mg/g of chlorophyll a, and 0.25~0.27 mg/g of carotenoids. From the perspective of energy consumption, the biomass extraction yield, upon reaching a plateau, will no longer depend on power intensity, but on total energy consumption. The energy requirements (kWh/kg) for USN-NCS extraction to reach the maximum recovery yields of HAVCs were lower than those for USN-CS, i.e., protein (380 vs. 450 kWh/kg), polyphenols (350 vs. 450 kWh/kg), saccharides (460 vs. 470 kWh/kg), chlorophyll a (350 vs. 450 kWh/kg),

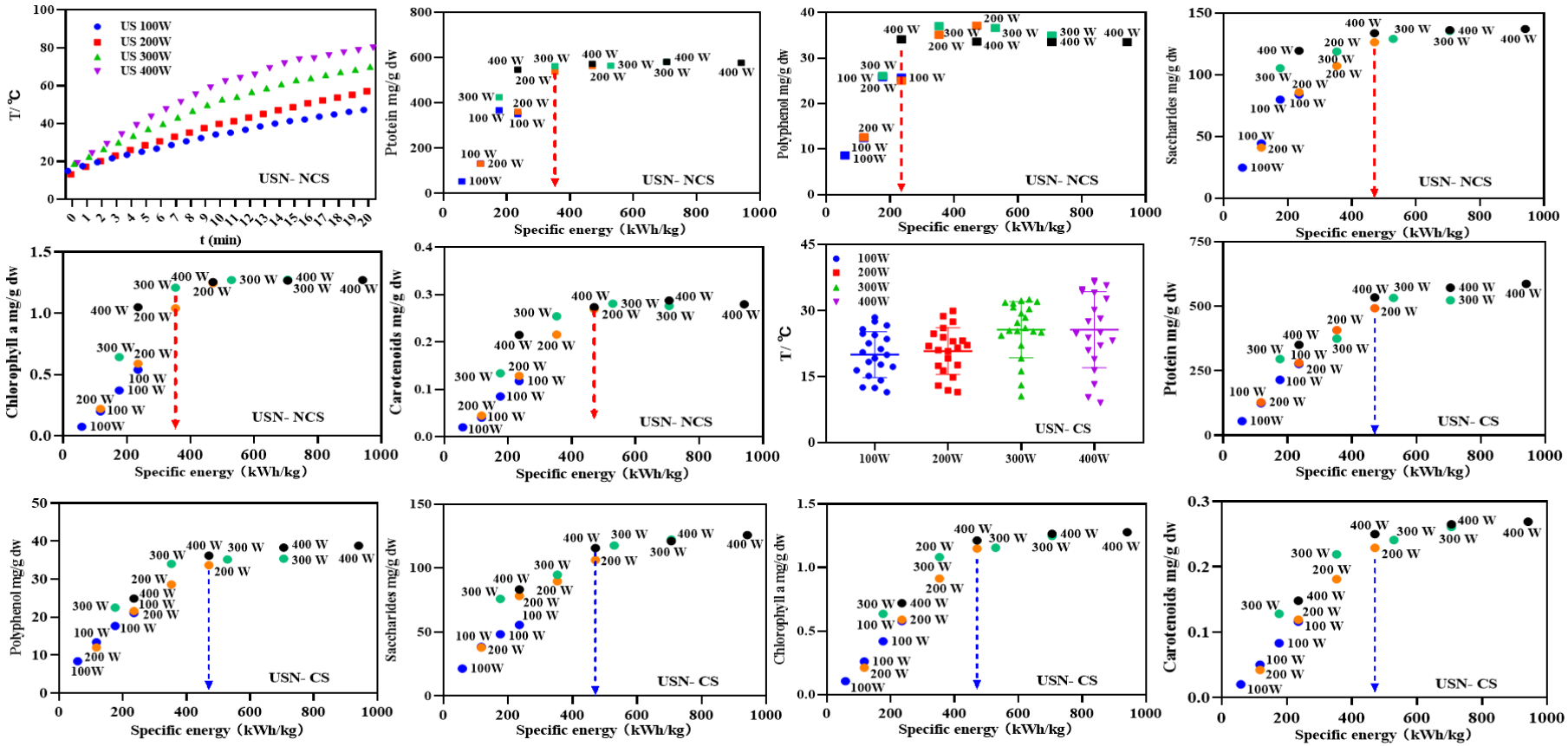

Figure 1. The thermal effects and specific energy consumption (kWh/kg) in terms of various bioactive compounds yields (mg/g dw) during ultrasound (USN) extraction. USN-NCS, Ultrasound-Not using cooling system. USN-CS, Ultrasound-Using cooling system.

carotenoids (360 vs. 460 kWh/kg) and total compounds (350 vs. 450 kWh/kg) suggesting that ultrasound extraction without a cooling system is beneficial for improved extraction yields and energy savings. The increased yields of the bioactive compounds were due to the disruption effects of USN on microalgae. Acoustic cavitation was an accepted theory of ultrasonic cell disruption (Liu et al., 2022; Wani & Uppaluri, 2022), and low ultrasonic frequencies in the 20-40 kHz range was the most used frequencies in the extraction process of microalgae (González-Balderas et al., 2020; Greenly and Tester, 2015), especially those with thin cell walls. When the intensity of ultrasonic waves was high enough, it generated severe shear forces, high pressures, and temperatures, thus promoting microalgae cell disruption and the release of HAVCs (Al Khawli et al., 2021), resulting in a higher yield of USN-NCS than US-CS (Chen et al., 2018). The high temperature generated by USN-NCS was detrimental to the properties of proteins and some pigments. Since one of the purposes of this study was to separate the crude saccharides from *Spirulina* (stable at 80 °C), considering the time efficiency, the USN-NCS extraction method (400 W, 10 min) was selected for further analysis.

4.2 USN-NCS extraction yields increase

The thermal effect in the USN-NCS extraction process can promote the extraction of the HAVCs of *Spirulina*, and it is necessary to evaluate the increase attributed to ultrasonic extraction in addition to the thermal effect. The results in Figure 2 showed that compared with the hot water extraction method, the USN-NCS greatly increased the extraction yield of *Spirulina* biomass, especially when the ultrasonic power was 200 W, 300 W and 400 W. When the ultrasonic power was 400 W and the extraction time was 20 min, the USN-NCS increases were 354.6 ± 18.2 mg/g dw for protein, 15.8 ± 2.6 mg/g dw for polyphenols, 59.8 ± 3.8 mg/g dw for saccharides, 1.2 ± 0.1 mg/g dw for chlorophyll a, and 0.25 ± 0.02 mg/g dw for carotenoids. Some related studies have demonstrated results similar to this study. For instance, Vernès et al. (2019) obtained an

increased protein recovery up to 28.42 ± 1.15 g/100 g dw after using ultrasonic isothermal extraction (20 kHz, 1000 W, 20 min) (Vernès et al., 2019), while Tavakoli et al. (2021) significantly increased ($p < 0.05$) the yields of chlorophyll a, and total carotenoids of ultrasound obtained *Spirulina* extracts (30 kHz, 100 W, 5~30 min) (Tavakoli et al., 2021).

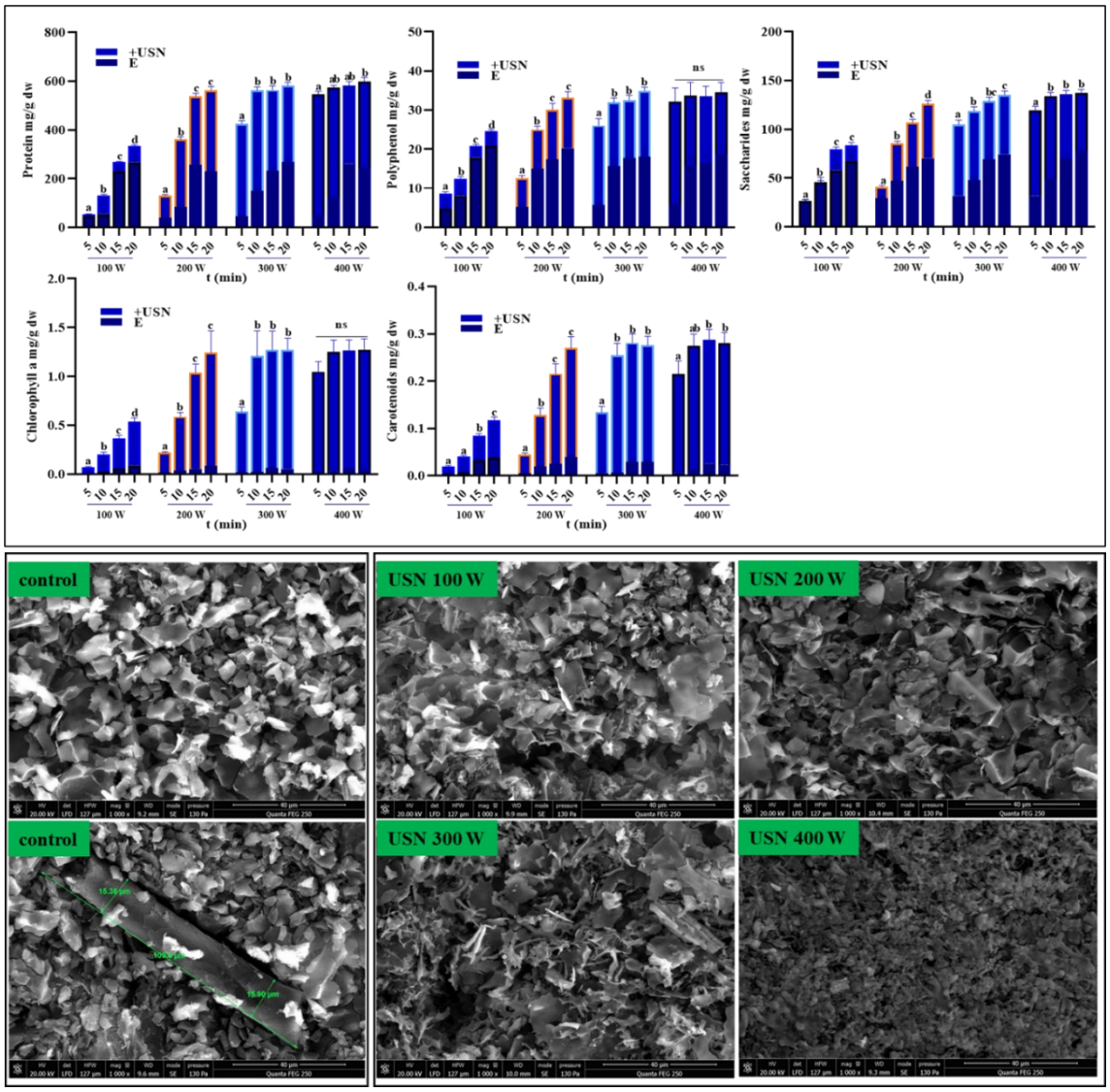

Figure 2. Increment of specific bioactive compounds yields by ultrasound extraction under different extraction parameters (100/200/300/400 W, 5/10/15/20 min) and Scanning electron microscope (SEM) analysis of *Spirulina* extraction residue microstructure (control /USN100 W/ USN 200 W/ USN 300 W/ USN 400 W, 10 min). USN-ultrasound, E-aqueous extraction. Different lowercase letters represent significant differences ($p < 0.05$), and ns represent no significant differences ($p > 0.05$).

When evaluating the ultrasonic extraction yields at different extraction times, the results showed that the extraction yields increased significantly ($p < 0.05$) with the elapse of time when the extraction powers were 100 W and 200 W. However, when the extraction powers were 300 W and 400 W, no significant increase ($p > 0.05$) were found with the elapse of extraction time, especially when the extraction power was 400 W. This result was attributed to the working principle of USN. When the USN intensity was large enough (300~400 W), the attractive forces between the molecules in the liquid phase were exceeded by the expelling force, and the cavities were generated into the liquid (Chemat et al., 2017). The high energy intensity generated severe shearing forces, high pressures, and high temperatures (Chen et al., 2018), which results in the extraction process being carried out efficiently, thus reaching the extraction target in a relatively short time.

4.3 Effects of USN process on Spirulina morphology

The effect of different USN conditions (100/200/300/400 W, 10 min) on the microscopic morphology of *Spirulina* was shown in Figure 2. The results showed that the morphology of *Spirulina* in the control group was complete, and a complete rod-like structure could be observed, with a diameter of 15.38~15.90 μm, the length being 109.8 μm. When using USN extraction, the morphology of *Spirulina* became fragmented with increasing USN power. Relatively intact structures remained after ultrasonic extraction with low power (100 and 200 W), while the *Spirulina* morphology became more fragmented when high-power ultrasonic extraction (300 and 400 W) was used, especially at 400 W. There are three mechanisms for ultrasonic-induced *Spirulina* cell rupture. First, the acoustic cavitation generated by the mechanical effect can generate enough shear force to directly rupture the microalgae cells and lead to cell fragmentation (Yao et al., 2018). Secondly, if the size of the oscillating bubbles in the ultrasonic system is comparable to that of microalgae cells, mechanical resonance can be excited effectively, thereby inducing cell rupture (Kurokawa et al., 2016). Finally, the cavitation bubbles explode rapidly during

sonication, which can lead to local temperature and pressure increases as high as 5,000 K and 100 MPa, respectively (Peng et al., 2020). When using 24 kHz with 400 W USN power, the *Spirulina* cells are disrupted by both mechanical and thermal effects, resulting in a rapid release of intracellular bioactive compounds, which accelerates the extraction process.

4.4 Membrane ultrafiltration process (UF)

4.4.1 Membrane permeability analysis

All membrane ultrafiltration finished with the collection of 90 mL of permeate (total volume of the feed: 100 mL), with the membrane separation fluxes (J, $mL.min^{-1}.m^{-2}$) of different MWCOs calculated (Figure 3). Figures 3A, B, C correspond to the ultrafiltration permeate fluxes of deionized water, ultrafiltration permeates fluxes of *Spirulina* extract, and permeate fluxes of deionized water of the cleaned membrane after ultrafiltration process of *Spirulina* extract. The results in Figure 3A showed that the permeate flux of membrane (deionized water) differed according to membrane MWCOs, that was, 500>300>150>100>10>4 kDa, and the permeation fluxes were 0.955, 0.786, 0.658, 0.547, 0.537 and 0.377 $mL.min^{-1}.m^{-2}$, respectively.

The results in Figures 3B showed the ultrafiltration flux curves of *Spirulina* USN extracts. The results showed that it took 170~280 minutes to concentrate 100 mL of USN extract to 10 mL, and the order of ultrafiltration was 500, 300, 150, 100, 10 and 4 kDa, which was related to the size of the membrane MWCOs. With the elapse of ultrafiltration time, the permeate flux value gradually decreased. The apparent decrease in membrane ultrafiltration flux with the elapse of ultrafiltration time was attributed to the occurrence of membrane fouling (Xu et al., 2022). In general, the fouling on ultrafiltration membranes was mostly affected by the filter ‘cake layer’ or aggregates (Tanudjaja et al., 2022), and the pH value of the ultrafiltration liquid and the concentration of HAVCs, as well as the membrane material and operating variables (Marson et al., 2021).

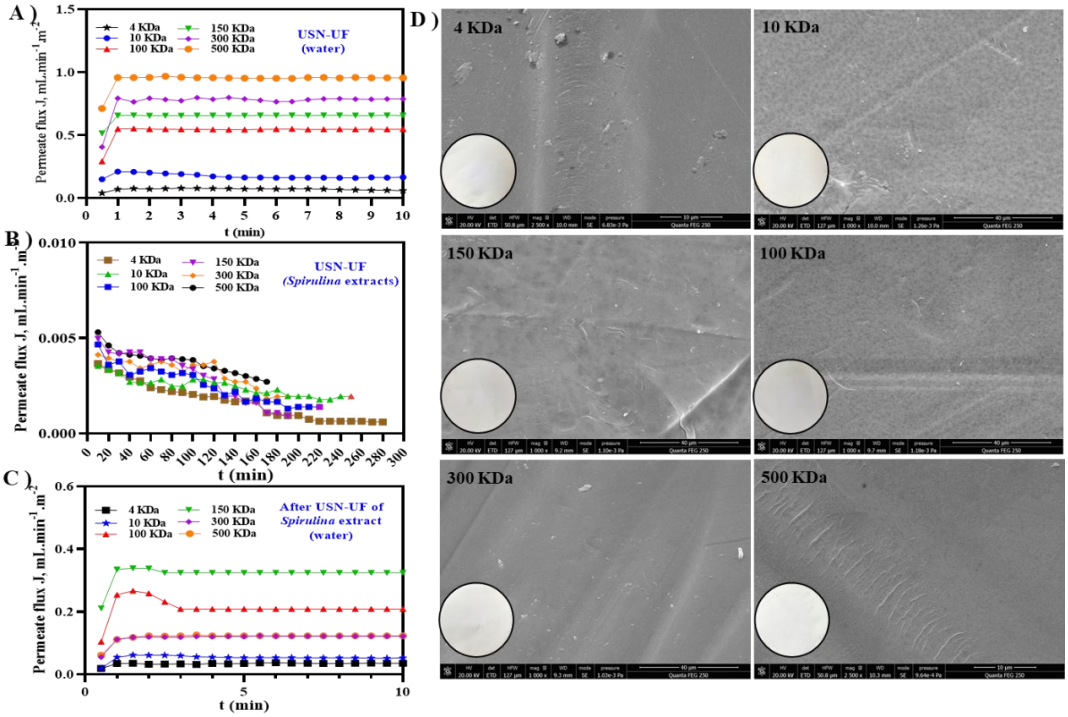

Figure 3. Membrane ultrafiltration (4/10/100/150/300/500 kDa) permeate flux. A) USN-MUF of water, B) USN-UF of *Spirulina* extracts, C) After USN-UF of *Spirulina* extracts (water). D) is the micrograph of membrane surface after ultrafiltration.

Figure 3B also showed that the membrane permeation flux of *Spirulina* extracts did not always depends on the size of the membrane MWCOs. For example, in the first 70 minutes of ultrafiltration, the permeate flux of 150 kDa membrane was higher than 300 kDa, and after 120 minutes, the permeate flux of 10 kDa membrane was higher than 100 kDa and 150 kDa. Similar results were reported by Chaiklahan et al (2011), who used a membrane with cut offs of 50 kDa, 70 kDa and 100 kDa to isolate *Spirulina* phycocyanin, with permeation flux of 70 kDa being less than 50 kDa at 85 min (Chaiklahan et al., 2011).

Membrane cleaning and surface 'cake layer' removal was performed after the ultrafiltration of *Spirulina* extracts, and the membrane permeation flux was tested again with deionized water. The results in Figure 3C showed that the membrane permeation flux was not completely dependent on the MWCOs of the membrane, which was, 150>100>500>300>10>4 kDa, and the final stable permeation fluxes corresponded to

0.324, 0.209, 0.123, 0.122, 0.050 and 0.035 mL.min^{-1}.m^{-2}, respectively. The difference between Figure 3A and Figure 3C indicated that an irreversible membrane fouling occurred during the *Spirulina* extracts UF process. Related previous reports showed that if the fouling on the membrane surface was dominant, the increase in reversible fouling led to a decrease in membrane permeate flux (Marshall et al., 1993). When the internal fouling increases, the opposite result is found, that is, the membrane permeability is irreversible (Tanudjaja et al., 2022). The results of this study showed that the HAVCs entered the membrane during the membrane separation process, resulting in an irreversible membrane fouling. Figure 3D shows a microscopic view of the surface of the ultrafiltration membrane after cleaning the 'cake layer'. It is obvious that there are 'granular' black spots on the 10 kDa, 100 kDa and 150 kDa membranes, these being HAVCs that penetrated the membrane layer, thus confirming that an irreversible fouling occurred in the UF process.

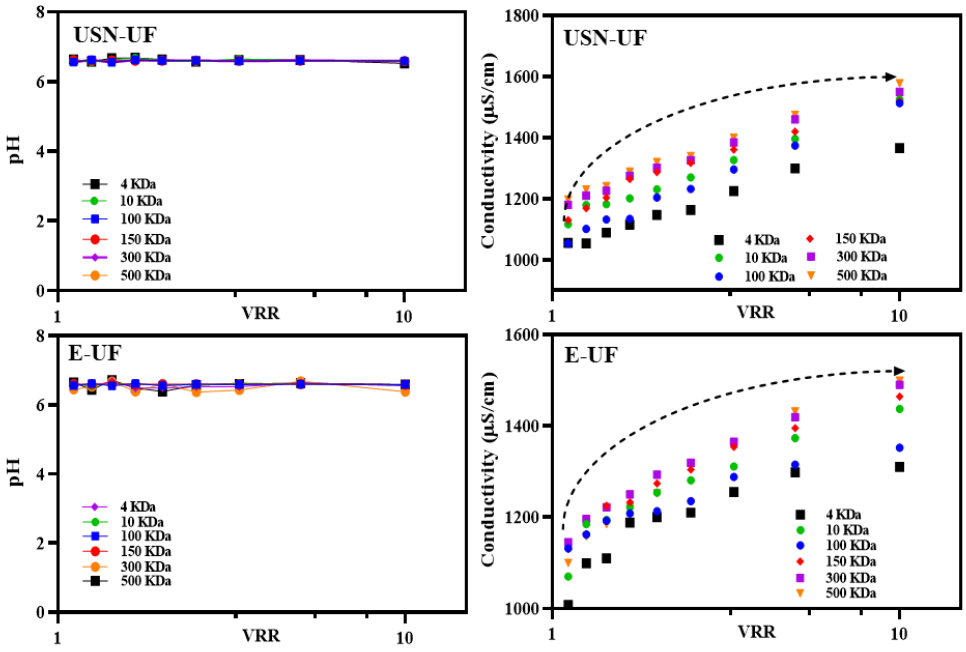

Figure 4. Evaluation of pH and conductivity (μS/cm) change of permeates with volume reduction rate (VRR). USN-UF, ultrasound extraction + ultrafiltration. E-UF, aqueous extraction + ultrafiltration.

4.4.2 pH and conductivity (μS/cm)

Permeate was collected every 10 mL during each ultrafiltration process, and the pH, conductivity (μS/cm) and resistivity (Ω/cm) of the permeate were monitored. The results in Figure 4 showed that the pH of the ultrafiltration permeate in the USN-UF and E-UF groups remained stable at around, ~7.0. With the ultrafiltration process, the conductivity of the permeate exhibited an upward trend. The initial conductivities of USN-UF and E-UF were 1055~1197 μS/cm and 1008~1145 μS/cm, respectively, and the final conductivities were 1366~1578 μS/cm and 1310~1499 μS/cm, respectively. Balti et al. (2021) reported similar results, showing a decrease in the conductivity of the retentate as diafiltration progressed while the pH remained relatively constant between 7.1 and 7.4 during diafiltration (Balti et al., 2021). The changes in conductivity should theoretically be related to the type and concentration of molecules contained in the permeate, which could indirectly reflect the content of bioactive components in the permeate.

4.4.3 Analysis of permeate components

The original extract of microalgae typically contains a complex mixture of HAVCs, such as proteins, polyphenols, saccharides, pigments, etc (Zhou et al., 2023). Therefore, removal of impurities in a clean and efficient way to obtain the targeted HAVCs is an extremely important step. The results in Figure 5 showed that the removal rates of both proteins and polyphenols increased with decreasing membrane MWCOs, and the removal rates of pigments were close to 100%. After the application of USN-UF, the removal rates of proteins and polyphenols were 88.35% (4 kDa)~79.88% (500 kDa) and 87.66% (4 kDa)~82.14% (500 kDa), respectively. For E-UF, the removal rates of proteins and polyphenols were 70.41% (4 kDa)~60.70% (500 kDa) and 80.56% (4 kDa)~73.77% (500 kDa), respectively. The results showed that the protein, polyphenol, and pigment removal efficiency of USN-UF was higher than that of E-UF.

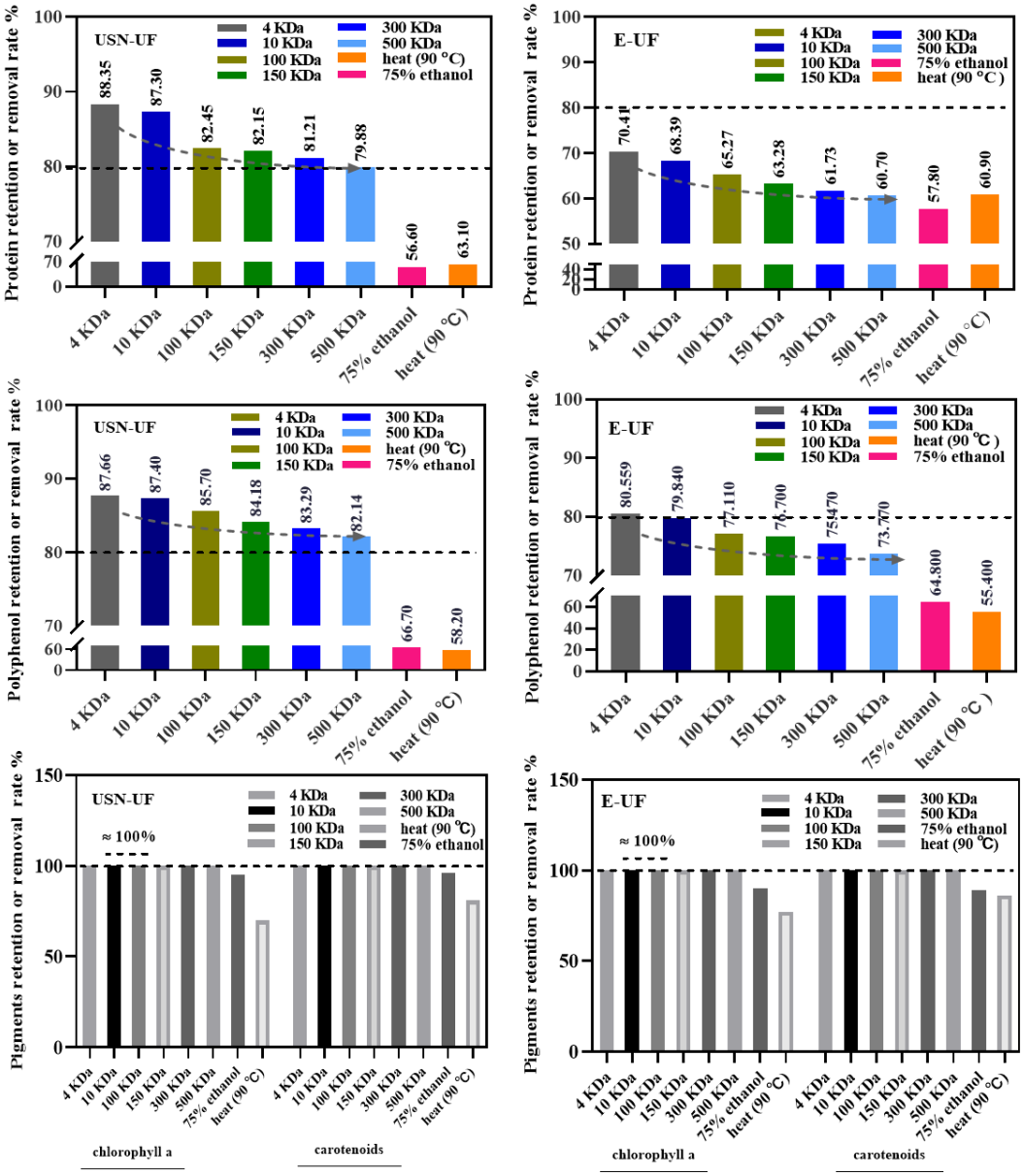

Figure 5. Retention rate of proteins, polyphenols, and pigments through different cut-off membranes during polysaccharides separation process. USN-UF, ultrasound extraction + ultrafiltration. E-UF, aqueous extraction + ultrafiltration.

On the other hand, the effects of protein and polyphenol removal by 75% ethanol precipitation and 90 °C heating concentration were also evaluated. The results showed that compared with UF process in this study, 75% ethanol precipitation and 90 °C heating concentration was less effective in removing proteins, polyphenols, and pigments. Figure 6 showed the results of the concentration of *Spirulina* saccharides in different MWCOs

permeates (Fig. 6A, B, C) as well as the appearance (Fig. 6F, G) and purity (Fig. 6I) of the final crude saccharide products.

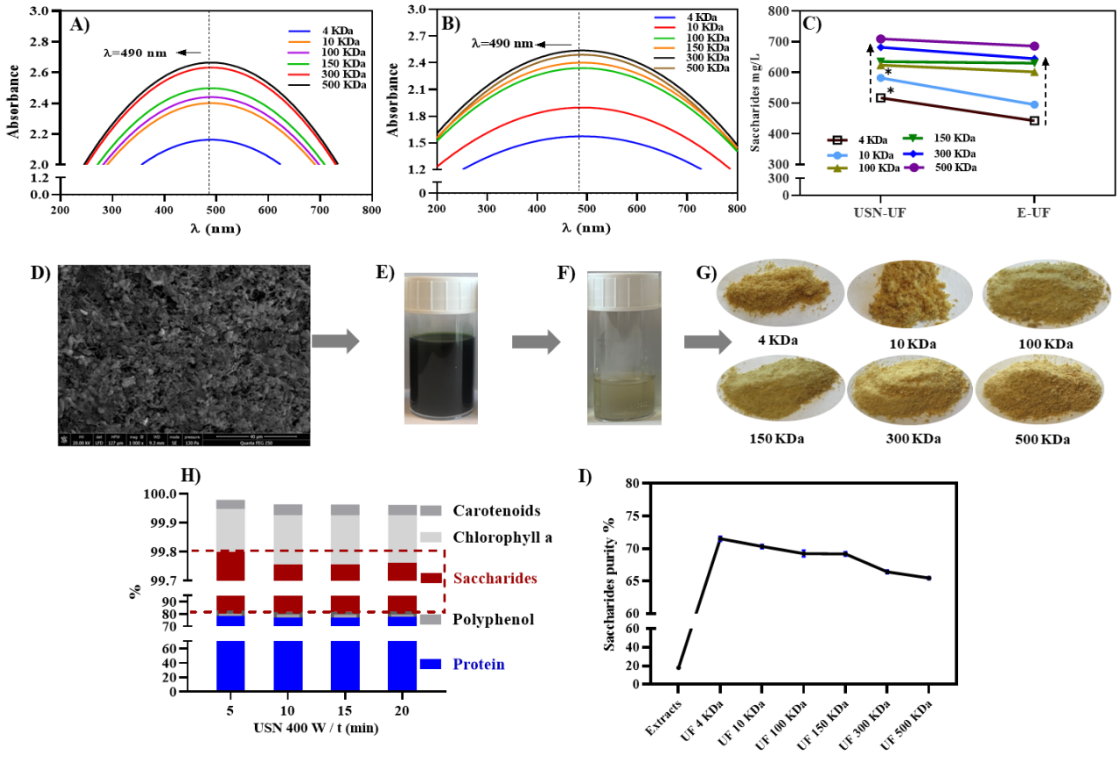

Figure 6. Saccharides concentration of membrane ultrafiltration permeates and appearance and purity of final crude saccharide. Fig. 6A) and 6B) corresponds to the UV-Vis (490 nm) absorbance of USN-UF and E-UF permeates during sulfuric acid-phenol analysis. Fig. 6C) is the saccharides concentration in USN-UF and E-UF permeates. Figure. 6D), E), F), G) corresponds to the *Spirulina* microscopic morphology after USN, *Spirulina*-USN extracts, *Spirulina*-USN-UF permeates and final saccharides powder. Figure. 6H) is the initial ratio of bioactive compounds in the *Spirulina*-USN extracts, Figure. 6I) is the final saccharides purity.

From the results obtained, whether it was USN-UF or E-UF, the saccharide concentration in the permeate increased as membrane MWCOs increased. The saccharide concentration in the USN-UF group was similar to that in the E-UF group when 100 kDa and 150 kDa membranes were used. A slightly higher saccharide concentration was

obtained in the USN-UF permeate than the E-UF permeate when using 300 kDa and 500 kDa membranes, while the saccharide concentration was significantly higher in USN-UF permeate when the 4 kDa and 10 kDa membranes were used in the UF process. This indicates that USN extraction promoted the separation of *Spirulina* saccharides with molecular weight less than 4 or 10 kDa, and slightly promoted the separation of *Spirulina* saccharides less than 300 or 500 kDa, whereas the saccharides with molecular weight less than 100 or 150 kDa were not significantly affected. The crude saccharide permeate from *Spirulina* was further concentrated at a high temperature (95 °C, 3 h), centrifuged (14,100*x*g, 15 min), and finally freeze-dried to obtain the light-yellow powder Figure 6G. The saccharides ratio in the initial extract (Fig. 6E) was less than 20% (Fig. 6H), while the purity of the final crude saccharides powder was close to 70% (Fig. 6I). Furthermore, the results in Figure 6I showed that the purity of the final crude saccharides gradually decreased with the increase of membrane MWCOs.

Based on all the results, we analyzed the yields of USN-, E-, UF- on bioactive compounds using multifactorial analysis (principal component analysis, PCA). The two principal components explained the 97.5% and 1.4 % of variability, respectively, in total about 98.9%. The results showed that the energy of 352.9~942.1 kWh/kg was close to the distribution of bioactive compounds, indicating that this energy range was suitable for USN extraction of *Spirulina* nutrients. However, due to the loss of bioactive substances caused by the ultrafiltration process, the ultrafiltration conditions, and the yield of bioactive substances in the permeate are distributed in different quadrants. In previous related reports, the separation of crude SPS from microalgae requires step-by-step removal of pigments, proteins, polyphenols, etc., while involving a variety of chemical and biological reagents, such as chloroform, n-butanol, TCA, protease, activated carbon, H2O2, ethanol, etc., which not only led to the gradual loss of SPS but also increased cost and pollution (Kang et al., 2022; Qiu et al., 2022; Tang et al., 2020). In contrast, no

complicated steps or contaminating reagents were used during the UF process to separate crude saccharides in this study, which show the green and high-efficiency advantages of UF process.

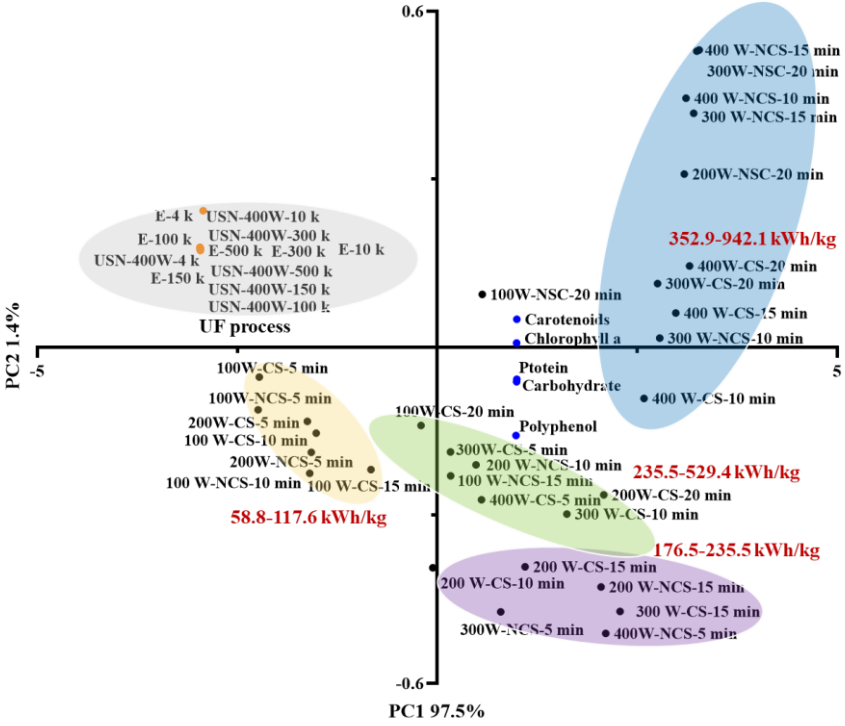

Figure 7. Principal Component Analysis (PCA) based on the extraction parameters, energy consumption, bioactive compounds yield and membrane ultrafiltration process. USN-NCS, Ultrasound-Not using cooling system. USN-CS, Ultrasound-Using cooling system. UF- ultrafiltration process.

Membrane separation techniques have been used to separate saccharides from different plant tissues. For example, Tang et al. (2020) isolated crude saccharides from Lentinus edodes using UF with MWCOs of 2.5 kDa (GH2540F30, GE, USA), 5 kDa (PE5, SEPRO, USA) and 10 kDa (PE10HR, SEPRO, USA). The final saccharide purity ranged from 22.4% to 86.1%, which contained some amino acid components derived from polypeptide or protein moieties (Tang et al., 2020).

Wang et al. (2020) used Sevag, Sephadex G-200 column chromatography combined with ultrafiltration membrane technology to separate SPS from *Spirulina*, and finally

obtained total saccharides with a purity of 92.60 ± 2.01%, which contained 1.67 ± 0.35% of protein (Wang et al., 2020). Therefore, these studies demonstrated that membrane technology could be used for the separation of HAVCs due to its advantages of high product yield and separation efficiency, simple scale-up and equipment cleaning (Marson et al., 2021). Similar to our study, these related studies failed to obtain pure saccharides, due to the presence of protein-saccharide conjugates (naturally occurring hydrogen bonds and interactions between saccharides and proteins) in the *Spirulina* extract (Jackson, 1977), which was extremely difficult for saccharides separation.

Combined with the membrane permeate flux and the final saccharide purity, under the USN extraction conditions (400 W, 10 min), choosing a 4 or 10 kDa membrane could obtain *Spirulina* saccharide with a relatively higher purity, while choosing 100 or 150 kDa membranes was more efficient in UF process. In general, this study obtained saccharides with over 70% purity (50% ↑) from *Spirulina* original extract (~ 20%) by USN-UF technology, which provided the basis for further obtaining higher purity SPS. Furthermore, we are working on the structural and compositional analysis of polysaccharides isolated from different MWCOs membranes, as well as their effects on human gut health, which will be reported in the future.

5. Conclusions

Ultrasound is an efficient extraction technique for obtaining high-added-value compounds from *Spirulina*. During the ultrasound extraction process, not using the cooling system can increase the extraction efficiency and decrease the energy consumption. This study suggests that the ultrasound extraction parameters can be set to 400 W/10 min to achieve the ideal extraction target with a 2.0% *Spirulina* suspension. When water is used as the extraction solvent, ultrasound combined with membrane separation technology can be used as a solvent-free and simple-technology process to separate crude saccharides from *Spirulina* extract. When using a 4 kDa membrane, crude

saccharides with relatively high purity can be obtained, which can be used as a reference for the purification process of algae polysaccharide. Membrane fouling occurs during membrane separation process, which reduces permeate flux. Therefore, in industrial applications, many factors such as the concentration of the extract, the pressure value, and the speed of magnetic stirring should be considered to mitigate the occurrence of membrane fouling.

Acknowledgements

This study forms part of the AGROALNEXT programme and was supported by MCIN with funding from European Union NextGenerationEU (PRTR-C17.I1) and by Generalitat Valenciana. Authors would also like to acknowledge University of Valencia through the projects OTR2021-21736INVES and OTR2021-21570INVES supported by the University of Vigo. Moreover, it was partially funded by the EU Commission and BBI-JU Horizon H2020, through the AQUABIOPRO-FIT project (Aquaculture and agriculture biomass side stream proteins and bioactives for feed, fitness and health promoting nutritional supplements) grant number 790956. Francisco J. Barba is member of the CYTED network "P320RT0186—Aprovechamiento sostenible de recursos biomásicos vegetales iberoamericanos en cosmética (BIOLATES)". Jianjun Zhou and Min Wang were supported by a PhD fellowship from the China Scholarship Council (CSC) (No. 201908420246 and No. 201908420245, respectively). Thanks to the team of the Institute of Agrochemistry and Food Technology, National Research Council (IATA-CSIC), led by Prof. María Carmen Collado for their help in the manuscript revision.

Declaration of competing interest

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

Data availability

Upon the authors' agreement data can be made available on request.

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4.4 Membrane separation process for enhancing carbohydrates recovery from ultrasonic *Phaeodactylum tricornutum* extracts

(Under review)

Membrane separation process for enhancing polysaccharides recovery from ultrasonic *Phaeodactylum tricornutum* extracts

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Abstract

Polysaccharides from algae have been used to improve the rheological and textural properties of foods, therefore recovering carbohydrates through innovative process from non-traditional sources is very promising. In this study, low-frequency ultrasound (LF-USN, 24 kHz) was firstly used to recover biomolecules from *Phaeodactylum tricornutum* (*P. tricornutum*), and then membrane separation (MS) technology was used to separate the polysaccharides fraction. The results showed that LF-USN greatly increased the yield of bioactive compounds ($p < 0.05$). The scanning electron microscopy (SEM) results showed that USN disrupted *P. tricornutum* cellular structures during extraction, as confirmed by particle size distribution tests. Different molecular weight cut-offs (MWCOs) membranes (4~500 kDa) were used to separate carbohydrates. The permeate flux decreased with the MS process, and the membrane fouling index (MFI) was related to MWCOs. The conductivity and bioactive compounds concentration of the permeate increased with the MS process. Most of the protein and polyphenols and almost all pigments were retained during the USN-MS process, promoting the carbohydrates concentration of permeate. The carbohydrates purity was affected by membrane MWCOs, with a purity of ~78% by 4~150 kDa membrane and ~70% by 300~500 kDa membrane.

Key words: Algae, Carbohydrates, ultrasound, ultrafiltration, microstructure

1. Introduction

The bioactive components in microalgae can be used as raw materials for the production of functional foods, medicines, cosmetics, etc., because of their beneficial effects such as antioxidant, antiinflammatory, antibacterial, prebiotic, etc.(Wang et al., 2022). Moreover, polysaccharides from microalgae can be used to improve the nutritional and functional properties of food products (Zhou et al., 2023).

Phaeodactylum tricornutum (P. tricornutum) is a marine pinnate diatom belonging to the *Phaeodactylum* family of the *Heterocoral* algae. *P. tricornutum*, growing in brackish to saline water in several locations of the world, owns the peculiar feature to be polymorphic, thus exhibiting three principal morphotypes: oval, triradiate, and fusiform (Celi, Fino, & Savorani, 2022). *P. tricornutum* is reported to contain high content of polysaccharides (16.8~26.1%) (dry weight, dw) under common culture conditions (Castro-Ferreira et al., 2022). However, in terms of microalgae polysaccharides recovery, solvent extraction is a traditional extraction method that has been widely used, but it is time-consuming and environmentally unfriendly due to the use of a large amount of toxic reagents (Zhou et al., 2022). Therefore, innovative extraction technologies should be used to efficiently extraction polysaccharides from microalgae and to reduce the use of toxic reagents (Greenly & Tester, 2015).

Ultrasound (USN) treatment consists of the application of mechanical sound waves that oscillate at frequencies above the upper limit of the normal human hearing range, approximately from 20 kHz to 10 MHz (Wang & Yuan, 2016). Acoustic cavitation is a well-established theory of ultrasonic cell disruption (Liu, Liu, Cui, & Yuan, 2022), i.e., ultrasound generates molecular motion through a series of sparse and compressive cycles. Negative pressure is created during the lean cycle, and when the negative pressure is lower than the vapor pressure of the liquid, small gas or vapor-filled bubbles are created in the liquid. These bubbles grow during subsequent cycles of thinning and compression,

and when the bubbles reach a size where the ultrasonic energy is not sufficient to retain the vapor inside, they collapse violently during the compression cycle, releasing a large amount of energy that produces mechanical/physical and chemical cell rupture effect (Khadhraoui, Ummat, Tiwari, Fabiano-Tixier, & Chemat, 2021; Wang, Chen, Zhou, Yuan, & Wang, 2020).

However, several studies related to *P. tricornutum* are focused on fucoxanthin (Sun et al., 2022), being relatively few studies about the *P. tricornutum* polysaccharides separation process. The extracts obtained from *P. tricornutum* assisted by USN contain multiple components, such as proteins, pigments, polyphenols, etc., which are considered undesired impurities in the saccharide's separation process. Ultrafiltration (UF) is a physical separation process with several advantages such as energy saving, high efficiency, modular design, and environmental friendliness. The principle of UF is to separate components according to the membrane pore size, which is being widely used to separate polysaccharides from 3 kDa to 500 kDa (Shao et al., 2019). For example, Sheng et al. (2007) used membranes with molecular weight cut-offs (MWCOs) of 1~100 kDa to separate polysaccharides from *Chlorella pyrenoidosa* extracts and found that the polysaccharides components could be separated by 30 kDa membranes (Sheng et al., 2007). However, the structural differences of *P. tricornutum* and *Chlorella pyrenoidosa* could lead to differences in the extraction and separation processes of polysaccharides fraction. In this line, UF technology with different molecular weight cut-offs (MWCOs) (4, 10, 50, 100, 150, 300, 500 kDa) was used in this study to separate polysaccharides from the *P. tricornutum* extract after USN extraction. The effect of USN treatment on the bioactive compounds yields and purity of polysaccharides separated by ultrafiltration (UF) were evaluated, and the changes of *P. tricornutum* microstructure and penetration performance were analyzed, which aimed to provide an innovative process to obtain polysaccharides from non-traditional sources with commercial potential in foods.

2. Materials and methods

2.1 Material

The microalgae *P. tricornutum* used in this study was provided by Algosource Saint-Nazaire, France. Samples were obtained as frozen algal paste (≈ 67.5 ± 1.7% moisture content) and stored at -20 °C. Before extraction process, the sample was thawed at a room temperature (20 ± 2 °C) and then diluted with deionized water to prepare a 2% suspension (w/v, dw) for further use.

2.2 P. tricornutum extracts preparation

USN extraction process was carried out using an UP-400S ultrasound processor (Hielscher GmbH, Germany) with a 24 kHz constant frequency. The USN probe with 14 mm diameter and 100 mm length was plunged into a beaker which contains 2.0 % *P. tricornutum* (w/v, dry basics) in 500 mL distilled water. The extraction time of USN process was 5, 10, 15, 20 minutes, and the amplitude was fixed at 25%, 50%, 75%, 100%, which corresponded to the power of 100, 200, 300, 400 W. The temperature variance was recorded to carried out the control experiments at different temperatures. The aqueous extraction with different temperatures (20/30/40/50/60/70 °C) was used to extraction the *P. tricornutum* bioactive compounds, which was control group. After extraction, the extracts were centrifuged using a MiniSpin Plus Rotor F-45-12-11 (Eppendorf, France) at 14,100 *x*g for 15 minutes and the supernatants were collected for analyzing.

2.3 Particle size distribution

The particle size of the microalgae precipitate after extraction was analyzed to evaluate the disruption of microalgae cells by USN (Zhang, Grimi, Marchal, Lebovka, & Vorobiev, 2019). The equipment used for the particle size analysis was a Mastersizer 3000 Malvern laser particle sizer equipped with a liquid dispersion cell (hydroLV). Each analysis corresponds to the accumulation of 10,000 measurements (1000 measurements per second for 10 seconds) of the diffraction of red and blue light on the detector (blue light

allows high-angle diffraction of fine particles). The device measured the diffracted light intensity at different angles, and the associated software allowed the conversion to a particle size distribution considering the optical properties of the sample and dispersant. The analysis was performed in the liquid phase (18 Mohm.cm of demineralized water) at a stirring speed of 1500 to 2000 rpm. The particle size distribution was calculated according to the Mie theory, and the refractive index of algae was 1.74 and that of water was 1.33. The sample was introduced into water to achieve approximately 15% laser obscuration.

2.4 Chemical composition analysis

The bicinchoninic acid (BCA) method was used to determine the protein concentration of the extract (Wang et al., 2023). Briefly, 10 μL of sample or BSA and 200 μL of BCA working solution was added to a 96-well plate, mix well, and incubated at 37 °C for 30 minutes before measuring absorbance at 562 nm (Thermo Electron Corporation, MA). The extract needed to be properly diluted with deionized water before analysis to achieve an absorbance value between 0.2~0.7. The protein content of the extract was calculated using a linear standard curve (0~2000 mg/L) of bovine serum albumin (BSA). The yield (mg/kg, dw) calculation formula was as follows **:**

$$Y_{(mg/kg,\ dw)} = C_{(mg/L)} * V_{(L)} / m_{(kg)} \quad (1)$$

Where Y, C, V, and m corresponded to the protein yields (mg/kg, dw), protein concentration (mg/L), extracts volume (0.5 L) and *P. tricornutum* mass (2.0%, 0.01 kg).

The polysaccharides content in the extract was analyzed by the sulfuric acid-phenol method (Zhou et al., 2021). Specifically, 1 mL of extracts or D-glucose, 0.5 mL of phenol (5.0%, w/v), and 2.5 mL of concentrated H_2SO_4 (98%) were sequentially added to a glass reaction flask and reacted at 25 °C (room temperature) for 30 minutes. After the reaction, the absorbance of 490 nm was measured using a UV-*Vis* spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA). The polysaccharides co;ncentration

was calculated according to the linear standard curve of D-glucose, and the yield (mg/kg, dw) calculation was the same as formula (1).

The Folin-Ciocalteu method was used for the determination of polyphenol content in the extract (Al Khawli, Martí-Quijal, Pallarés, Barba, & Ferrer, 2021). Specifically, 0.2 mL of sample or gallic acid standard solution, 1 mL of Folin-Ciocalteu (diluted 1:10, v/v with water), and 0.8 mL of Na_2CO_3 solution (75 g/L) was mixed and incubated in a water bath at 50 °C for 10 minutes. Absorbance was measured at 750 nm using a UV/Vis spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA). The extract needed to be properly diluted with deionized water before analysis to achieve an absorbance value between 0.2~0.7. The polyphenol concentration in the extract was calculated according to the linear standard curve of gallic acid, and the yield (mg/kg, dw) calculation was the same as formula (1).

The contents of pigments (chlorophyll a, carotenoids) were measured according to a previous study (Safi et al., 2015). Briefly, 200 μL of *P. tricornutum* extract were mixed with 1300 μL of methanol (100%) and incubated for 1 h at 45 °C in the dark, then samples were centrifuged at 10,000 *x*g for 10 min at room temperature (~25 °C) for pigments quantification.

2.5 USN extract efficiency

The USN extract efficiency was defined as K_{USN}, the formula is :

$$K_{USN} = Y_{USN}/Y_E \quad (2)$$

Where Y_{USN} and Y_E corresponds to the specific compounds yields of USN and aqueous extraction.

2.6 Membrane ultrafiltration (MUF) process

P. tricornutum crude polysaccharides were isolated from extracts using membranes with different MWCOs (Tang, Liu, Liu, et al., 2020). Ultrafiltration process was performed in a stirred cell Amicon 8200 (Millipore, Billaica, USA) with a membrane diameter of 63.5

mm and a maximum volume of 200 mL. Compressed air was used to provide a transmembrane pressure (TMP) of 2 bars. The temperature of the extract was kept at room temperature (~20 °C) during filtration. *P. tricornutum* extracts were purified using polyethersulfone (PES) membranes (Microdyn-Nadir (Germany)) with different MWCOs. According to Reference (Microdyn-Nadir, Germany), PES membranes were soaked in deionized water for 30 minutes before use, and a magnetic stirrer fixed on the membrane surface provided a constant stirring speed of 500 rpm. In this study, the microalgae extracts obtained from USN (400W, 10 min) and control (50 °C, 10 min) was use for the separation process. The feed composition of USN vs. control was protein 3.57 vs. 1.20 mg/mL, polyphenol 0.20 vs. 0.08 mg/mL, polysaccharides 1.11 vs. 0.53 mg/mL, chlorophyll a 4.2 vs. 0.4 mg/L, and carotenoids 7.2 vs. 0.6 mg/L. For each filtration, 100 mL of extract were finally concentrated to 10 mL, and permeate samples were collected every 10 mL for compositional analysis. The weight change of the permeate was recorded over time by a computer during filtration. The filtered permeate (each batch 10 mL, total 90 mL) and the retentate (10 mL) were collected for subsequent analysis. After ultrafiltration, the permeate was collected and further heat concentrated at 95 °C for 3 h, the concentrate was centrifuged at 14,100 *x*g for 15 min and freeze-dried to obtain the crude polysaccharides.

2.6.1 pH, conductivity measurement

During ultrafiltration process, the permeate was collected each 10 mL, and the electrical conductivity (μS/cm) and pH of *P. tricornutum* permeates were measured with a conductivity meter InoLab pH/cond Level 1 (WTW, Weilheim, Germany).

2.6.2 Permeate flux analysis

To evaluate the UF efficiency, the permeate flux was calculated based on the variance of permeate volume over time. The calculation formula is as follows (Shao et al., 2019):

$$J\ (L*min^{-1}*m^{-2}) = dv/dt*A^{-1} \quad (3)$$

Where *dv* and *dt* corresponds to the derivative of volume (L) and time (min) respectively, A is membrane area (m^2).

The volume reduction ratio (VRR) was calculated as follows:

$$VRR(mL) = V_{initial} / V_{concentrate} \quad (4)$$

where $V_{initial}$ and $V_{concentrate}$ corresponds to the initial extracts volume (mL) and concentrate volume (mL) respectively.

The purity *of the polysaccharides in the final products was calculated as follows:*

$$Purity\ (\%) = m_{polysacchrides}/m_{Freeze\ dried\ permeates} \quad (5)$$

where $m_{polysaccharides}$ and $m_{Freeze\ dried\ permeates}$ corresponds to the weight of polysaccharides and freeze dried permeates respectively.

2.6.3 Membrane fouling index

The membrane fouling index (MFI) was analyzed according to the equation as follows (Hebert, Mhemdi, & Vorobiev, 2021):

$$Fouling\ index = 1 - MPa/MPb \quad (6)$$

Where MPa and MPb corresponds to the membrane permeate after UF process (MPa) and membrane permeate before (MPb) UF process respectively.

2.6.4 Scanning Electron Microscope (SEM)

Scanning electron microscopy (SEM Quanta 250 FEI Company, Eindhoven, The Netherlands) was used to analyze the microstructure of samples after freeze drying (MUT 002A pilot freeze drier (Cryotec, France)) for 72 h at a cold trap temperature of -65 °C as well as the membrane surface structure after UF process. Freeze-dried samples or membranes were then mounted on specimen stubs with colloidal silver, sputter-coated with gold-palladium and imaged with an SEM microscope at magnifications of 1000×.

3. Statistical analysis

One-way ANOVA with Dunnett's multiple comparisons test was performed using Statgraphics® Centurion XV (Statpoint Technologies, Inc., USA) and it was used to detect

statistically significant differences of results on the yields of proteins, polysaccharides, polyphenols, chlorophyll a, and carotenoids. Statistical significance was accepted at $p < 0.05$.

4. Results and discussion

4.1 USN thermal effect and chemical composition analysis

The effects of extraction time (0, 5, 10, 15 and 20 minutes) and power (100, 200, 300 and 400 W) on the chemical composition of *P. tricornutum* extracts are shown in Figure 1. The yields of proteins, polysaccharides, polyphenols, and pigments (chlorophyll a, carotenoids) in *P. tricornutum* extracts augmented with the increase of USN power.

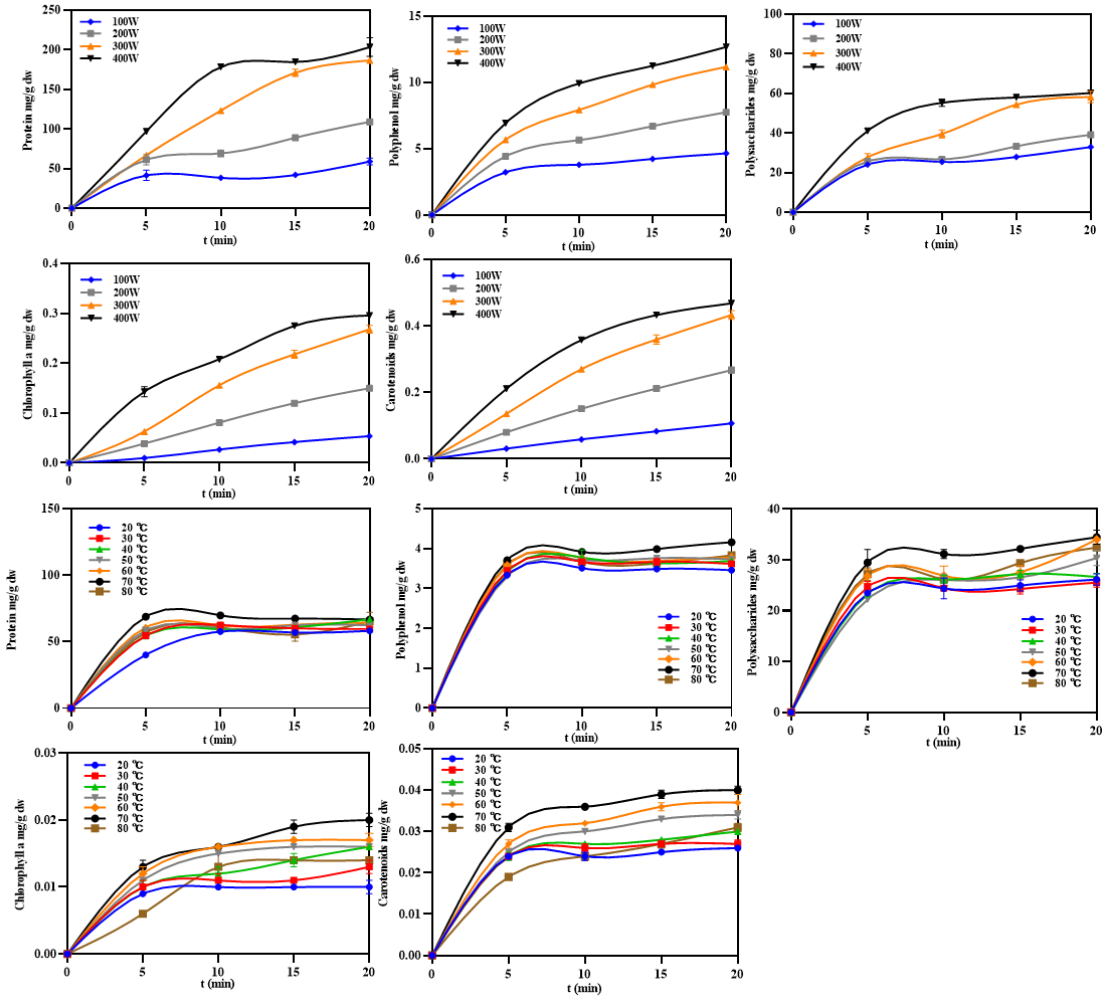

Figure 1. Bioactive compounds yield of ultrasound (USN, 100~400 W, 5~20 minutes) and control (E, 20~80 °C, 5~20 minutes) extraction.

The results showed that about 200 mg/g protein, 13 mg/g polyphenols, 60 mg/g polysaccharides, 0.3 mg/g chlorophyll a, and 0.45 mg/g carotenoids were obtained when using USN with a parameter of 400 W and 20 minutes, which was much higher than using 100 and 200 W extraction power. The yields of bioactive components in *P. tricornutum* extracts increased with USN time, especially from 0~15 minutes, but the increase was no longer evident over 15 minutes (15~20 minutes). From the perspective of yield and saving time and energy, 400 W and 10 minutes could be determined as the proper extraction conditions.

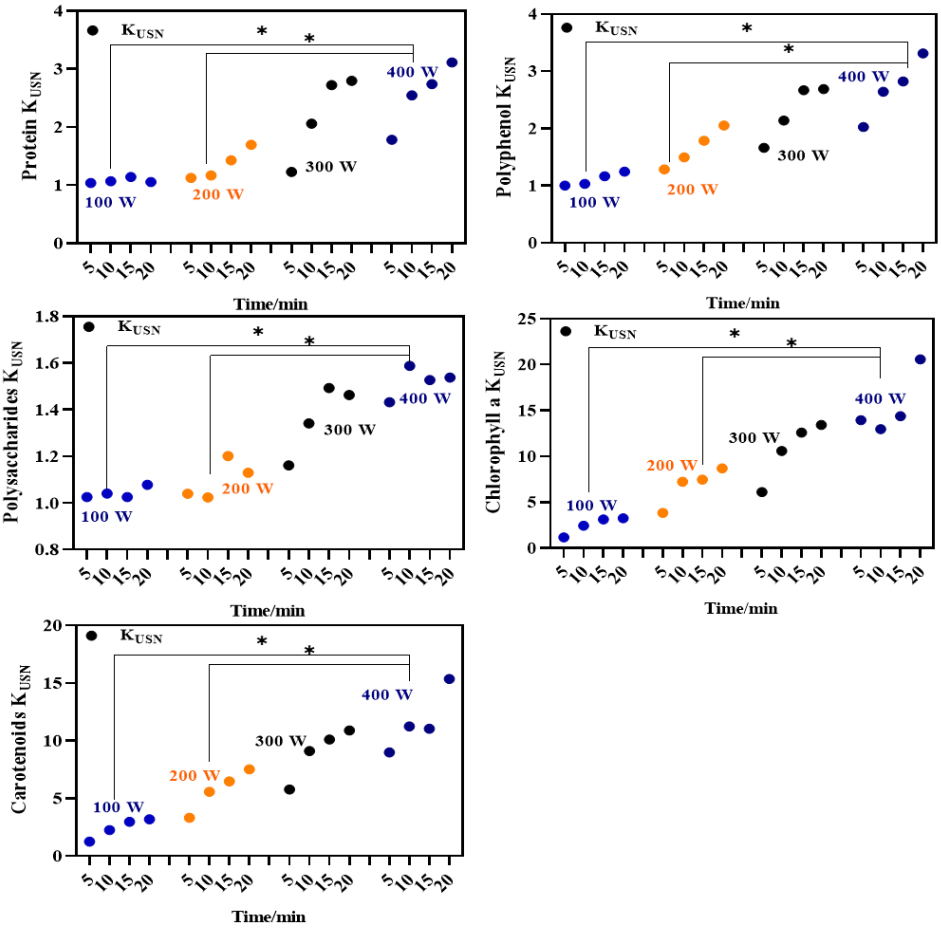

Figure 2. Ultrasound extraction efficiency (K_{USN}) of different bioactive compounds.* means significant differences at a $p < 0.05$ level.

The yields of the extracts without USN treatment at different temperatures (20~80 °C) were analyzed to evaluate the influence of ultrasonic thermal effect on the extraction

effect. As shown in Figure 1, about 65 mg/g protein, 4.2 mg/g polyphenols, 35 mg/g polysaccharides and less than 0.1 mg/g pigments (chlorophyll a, carotenoids) were obtained when the extraction time was 20 minutes. However, there was no significant yield increase when the extraction exceeded 10 minutes.

In general, compared with the control group, USN significantly increased the yield of bioactive compounds in the extract. The USN effects were defined as K_{USN}, which are shown in Figure 2. The results showed that K_{USN} value increased with USN power and extraction time. Specifically, the K_{USN} of protein, polyphenols, polysaccharides, chlorophyll a, and carotenoid K_{USN} could reach 3.0, 3.3, 1.6, 20.5, and 15.0 respectively. The increase in the yield of *P. tricornutum* bioactive compounds by USN could be attributed to the mechanism of acoustic cavitation. Similar studies used USN to increase the yield of bioactive compounds, for example, Cui et al. (2022) used USN (100 W) to extract flavonoids from *Astragalus* stems and leaves with a yield of 22.0270 ± 2.5739 mg/g (Cui, Ma, Wang, & Niu, 2022), Sankaran et al. (2018) used ultrasound (20 kHz, 200 W) to extract protein from *Chlorella vulgaris* with a yield of 93.33% (Sankaran et al., 2018). Consistent with our study, the increased yields of these bioactive compounds were closely related to acoustic cavitation during sonication process. USN cavitation resulted in shear waves, microjets, turbulence, and shock waves that enhanced extraction by altering the microalgae matrix (Rao, Sengar, C K, & Rawson, 2021). The propagation of ultrasonic waves created a negative pressure in the microalgae solution, and when higher-intensity sonic pressure propagated through the solvent, microscopically small voids or bubbles were formed. When these voids or bubbles were filled with gas or water vapor, the bubbles grew and shrank until they collapsed, which resulted in extreme mechanical shear forces that could damage *P. tricornutum* cells (Ojha, Aznar, O'Donnell, & Tiwari, 2020). In addition, the chemical mechanism of ultrasonic cavitation was also a potential reason for the increased yield of microalgae bioactive substances. Specifically,

the ultrasonic process produced hydroxyl radicals (•OH), hydrogen radicals (H•) and perhydroxyl radicals (HO_2•), which could oxidize microalgae cell membrane lipids, change the permeability of microalgae cell membranes, and promoted the release of intracellular substances in microalgae (Liu et al., 2022).

In this study, considering the yields, thermal effects, and extraction time consuming, USN extraction with 400 W /10 minutes was selected to recover *P. tricornutum* bioactive compounds. Certainly, it was worth considering the USN treatment parameters for different microalgae, such as power, frequency, time, solvent etc., depending on the properties of microalgae, such as cell size, cell wall thickness, etc.

4.2 Effects on morphology

4.2.1 Scanning Electron Microscopy (SEM) -microstructure

The sonicated microalgae cells were analyzed by scanning electron microscopy (SEM) and particle size analyzer to demonstrate that USN promoted the release of bioactive components by disrupting the microalgae cells. Figure 3 showed the results of non-sonication (control) and USN (100 W, 200 W, 300 W, 400 W) extraction on the microstructure and particle size distribution of *P. tricornutum*. The results showed that USN significantly changed the cellular structure of *P. tricornutum* compared with the control group. Moreover, with the increase of USN power, the *P. tricornutum* cell structure became uniform and finely divided. In particular, the intact *P. tricornutum* morphology could hardly be seen by ultrasonic treatment at 400 W for 10 minutes, which indicated that the USN wave destroyed the *P. tricornutum* structure through acoustic cavitation, thereby promoting the release of intracellular substances and increasing the extraction yield. Similarly, a related study used SEM to analyze the mechanism by which USN increased the extraction yield of red algae *Gracilaria gracilis* pigment (Pereira et

al., 2020).

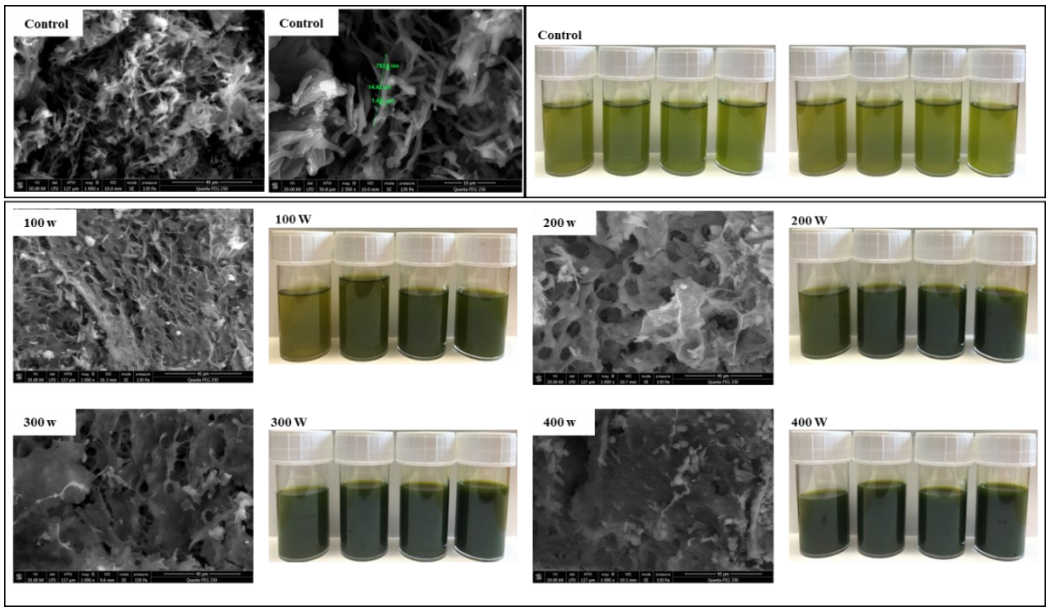

Figure 3. The microstructure of *P. tricornutum* after ultrasound (USN) extraction, the control group was the *P. tricornutum* microscopic morphology without USN extraction.

The results showed that the use of an USN probe disrupted the microalgae structure and improved the exposure of the pigment to the solvent, thereby enhancing the extraction of chlorophyll compounds and phycobiliproteins. Another study explored the effect of USN conditions on the microscopic morphology of cyanobacteria (*Pseudomonas aeruginosa*) through SEM (Huang et al., 2021). Consistent with the results of this study, it was found that ultrasonication caused serious damage to microalgae cells, and the physical damage followed the reciprocal of low frequency > high frequency sequence, ie 29.4 kHz > 470 kHz > 780 kHz, which promoted cytoplasmic dissolution. Since LF-USN (24 kHz) was used in our study, the physical effects produced by the ultrasound dominated rather than the chemical effects (generating free radicals, etc.), which was beneficial for protecting bioactive compounds from decomposition (Wang, Wang, Vieira, Wolfson, & Pingtian, 2019).

4.2.2 *Particle size distribution*

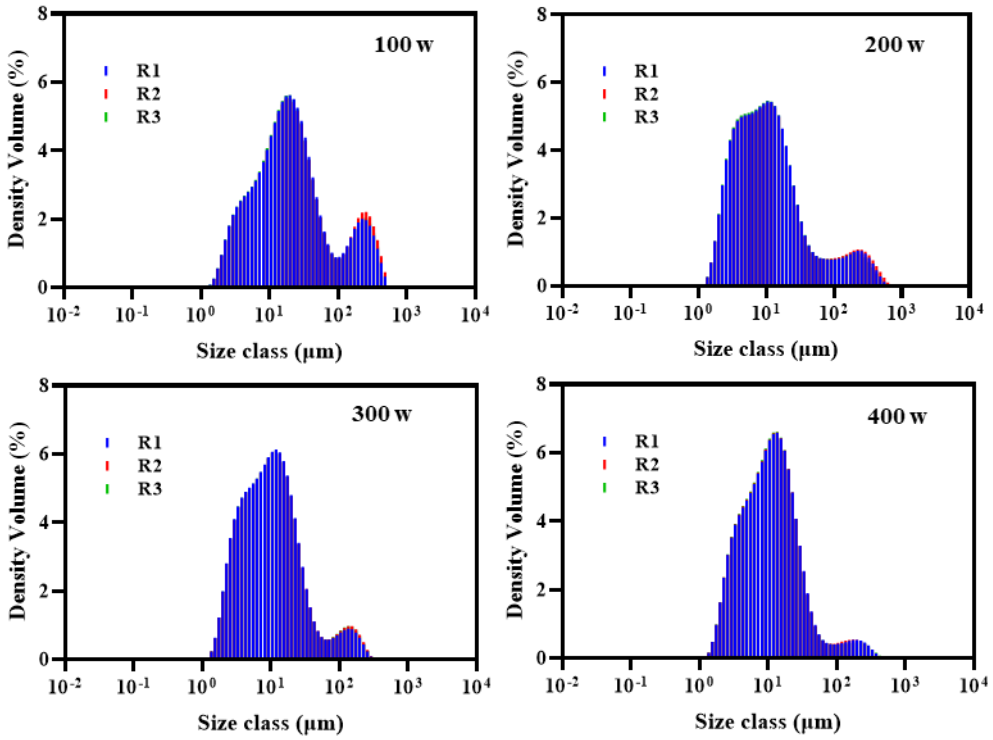

Figure 4. The particle size distribution of *P. tricornutum* after ultrasound (USN) extraction, the control group was the *P. tricornutum* microscopic morphology without USN extraction.

Furthermore, the particle size distribution of the sonicated microalgae fragments was analyzed, and the size class and corresponding density volume were also shown in Figure 4. The results showed that the size class of *P. tricornutum* after USN extraction was mainly distributed around ~25 μm and ~224 μm. With the increase of ultrasonic intensity, the proportion of the area distributed at ~224 μm decreased, which again proved that the increase in the yield of *P. tricornutum* bioactive compounds was highly related to the USN power. Moreover, with increasing USN power, the particle size distribution tends to be smaller, which is consistent to the SEM results. Similarly, a study by Zhang et al. (2019) showed that ultrasonication treatment decreased *ParaChlorella kessleri* cell size to increase the yield of biomolecules, and USN combined with ultrahigh pressure could further improve the microalgae cell disruption efficiency (Zhang et al., 2019).

4.3 Membrane ultrafiltration (MUF) evaluation

4.3.1 Permeation flux analysis

Membrane separation was used to separate polysaccharides from *P. tricornutum* extracts, and the membrane permeation fluxes of different MWCOs in the UF process were evaluated, and the results were shown in Figure 5. Figures 5A), 5B), and 5C) showed the results of permeation flux with deionized water, *P. tricornutum* extracts and deionized water (after UF of *P. tricornutum* extracts and membrane cleaning process), respectively. The results showed that when UF with deionized water, the membrane permeation flux was determined by the membrane MWCOs, and the permeation flux decreased sequentially from 500 kDa to 4 kDa, and the permeation flux of 4 kDa and 10 kDa membrane was similar. Figure 5B showed that the UF efficiency of the *P. tricornutum* extract did not show an absolute positive correlation with the MWCOs of the membrane. The 4 kDa membrane had the lowest permeation flux and the 50 kDa membrane had the highest permeation flux, corresponding to 220 and 130 minutes for 90 mL of permeate collected from 100 mL of *P. tricornutum* USN extract respectively. However, the 150 kDa membrane permeation flux was slightly lower than 10, 50 and 100 kDa membranes, which indicated that the USN process was affected by the composition and concentration of the *P. tricornutum* extract. The permeation flux decreased with the prolongation of UF time, which was due to the molecules larger than the membrane pore size aggregated on the membrane surface to hinder the permeation, including proteins, pigments, polysaccharides, etc. (Tanudjaja, Ng, & Chew, 2022). In addition, since the UF process was accompanied by continuous pressure (2 bars), molecules slightly larger than the membrane pore size could be deformed by the pressure and entered into the membrane, resulting in a decrease in membrane permeation efficiency and causing membrane fouling (Xu et al., 2022). The membrane permeation performance was re-evaluated with deionized water after the UF process of *P. tricornutum* extract, Figure 5C showed that the

permeation properties of the membrane changed, which no longer followed the initial permeation law with MWCOs, indicating that membrane fouling occurred.

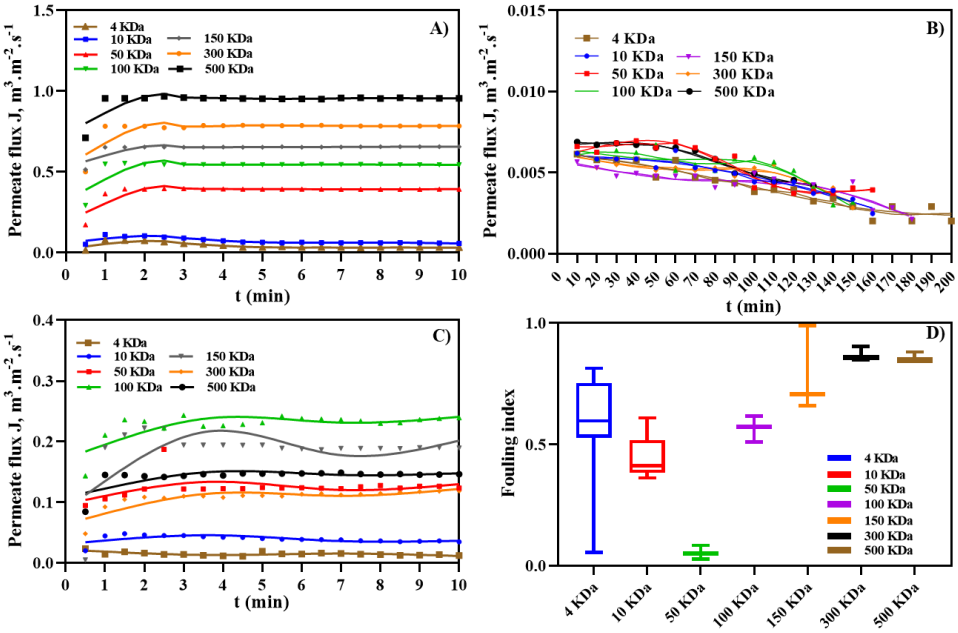

Figure 5. Permeate flux with deionized water (A), *P. tricornutum* extracts (B), and deionized water after *P. tricornutum* extracts UF process (C), and membrane fouling index (D).

In terms of the membrane fouling index (MFI) (Figure 5D), it was found that the MFI of the 150 kDa, 300 kDa and 500 kDa membranes was higher, indicating the serious fouling. Similarly, Miller et al (2019) used different MWCOs membranes to separate benzophenone and xanthone from *Cyclopia genistoides* and the fouling index in their study was ~0.3, which was lower than the 150 kDa, 300 kDa and 500 kDa membrane fouling index in our study, since it was also related to the extracts composition, concentration, pH, etc (Miller, Bosman, Malherbe, De Beer, & Joubert, 2019). Membrane fouling also occurred in the study of Hebert et al. (2021) using PES membrane to separate glucosinolates from mustard defatted meal (Hebert et al., 2021). They evaluated the effects of agitation on membrane fouling during UF process. The results showed that UF caused irreversible membrane fouling with agitation, because agitation reduced the cake

formation on the membrane surface, which made it easy for biomolecules enter the membrane and caused blockage. The use of stirring in our study reduced the cake formation on the membrane surface, thereby reducing the adverse effect of the filter cake during the UF process, but irreversible membrane fouling still occurred. Figure 6 showed a SEM view of the ultrafiltration membrane surface after cleaning the 'cake layer'. It was obvious that there are 'granular' black spots on the 10 kDa, 50 kDa, 100 kDa and 150 kDa membranes, these being bioactive compounds that penetrated into the membrane layer, thus confirming that an irreversible fouling occurred in the UF process.

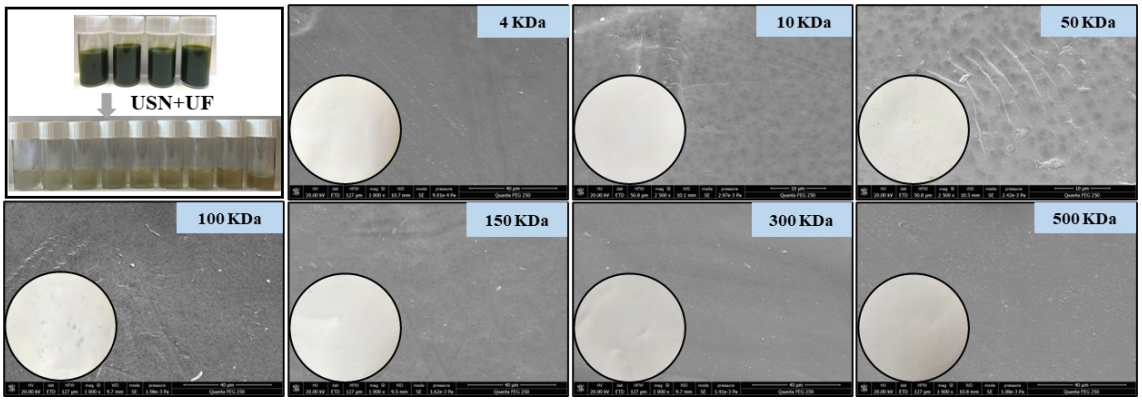

Figure 6. The microstructure of membranes after ultrafiltration (UF) process.

4.3.2 pH and conductivity

The results in Figure 7 showed that the pH of the USN-UF and control (E-UF) permeate were constant, ~7.0. The conductivity of the permeate increased with the UF time. The initial conductivities of USN-UF and E-UF are 2775~2859 μS/cm and 2558~2655 μS/cm, respectively, and the final conductivities are 3010~3100 μS/cm and 2777~2877 μS/cm, respectively. A related study used membrane UF to isolate proteins from microalgae and found that the conductivity of the retentate decreased with the UF process, while the pH remained constant (Balti, Zayoud, Hubert, Beaulieu, & Massé, 2021), which was consistent with our results. In general, the conductivity was related to the bioactive compounds' concentration in the solution, and the significant increase of the conductivity

of the permeate suggested that the composition of the permeate changed dynamically with UF process.

4.3.3 Permeate composition dynamic analysis

As shown in Figure 7, the polyphenols, polysaccharides, and protein concentrations in each permeate were analyzed. From the results, the constituent concentrations in the permeate gradually increased with UF processes. Specifically, the initial concentrations of polyphenols, polysaccharides, and proteins in the USN-UF permeate were 46.6~62.78 mg/L, 339.28~443.97 mg/L, and 384.75~569.75 mg/L, respectively. The final polyphenols, polysaccharides, and proteins concentrations were 73.41~115.66 mg/L, 412.24~539.82 mg/L, 673.50~748.50 mg/L, respectively.

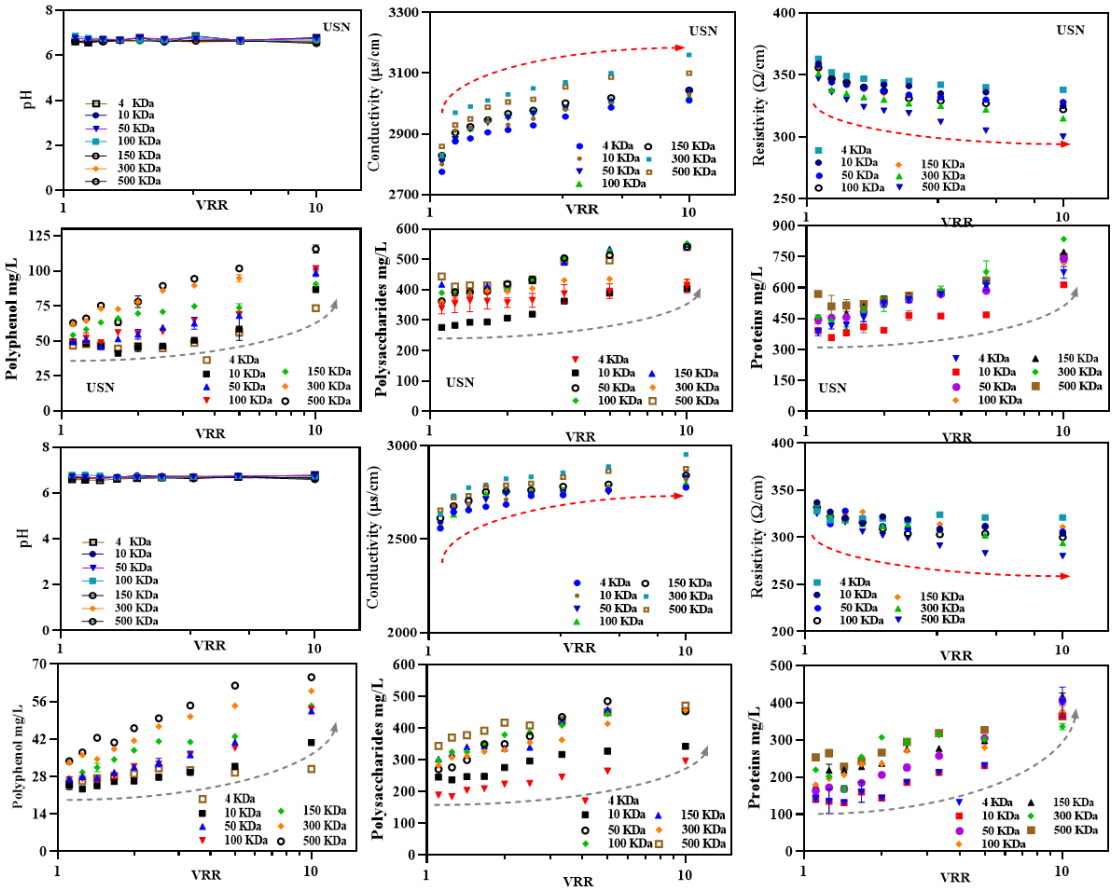

Figure 7. Changes in pH, conductivity, and bioactive compounds concentration of the permeate during the UF process. USN-ultrasound extraction, E-control extraction. VRR is the volume reduction ratio.

Correspondingly, the initial concentrations of polyphenols, polysaccharides, and proteins in the E-UF permeate were 25.32~33.69 mg/L, 188.71~343.36 mg/L, 142.25~253.50 mg/L, and the final concentrations were 30.77~65.15 mg/L, 296.06~470.11 mg/L, 402.25~363.50 mg/L, respectively. In contrast, USN-UF process resulted in higher yields of bioactive compounds than that of E-UF. In addition, the concentration of bioactive compounds increased with the increase of MWCOs of membrane.

The dynamic changes of the permeate compounds content could reflect the specific bioactive compounds ratio in the permeate at different UF time points, which provided options for the purification of specific components. For example, in the USN-UF process, the proportion of polysaccharides was relatively higher in the early UF stage, which was beneficial for further separation of polysaccharides fractions, such as polysaccharides, oligosaccharides, etc. Conversely, as UF proceeded, the fraction of permeate collected at a later stage contained relatively higher protein content, which was favorable for further purification of microalgae proteins.

4.3.4 Impurity retention and crude polysaccharide purity

This study further compared the effects of UF and traditional methods (heat concentration and ethanol precipitation) in the separation of polysaccharides from *P. tricornutum* USN extracts, and the impurity retention rate or removal rate was calculated, the results were shown in Figure 8.

The results showed that the retention rate of protein and polyphenols by USN-UF was significantly higher than that of E-UF, the retention rate of protein was higher than 80%, and the retention rate of polyphenols could reach 75%. The protein retention rate decreased slightly with the increase of membrane MWCOs (from 4 kDa to 500 kDa), while the polyphenols retention rate decreased significantly with the increase of

membrane MWCOs.

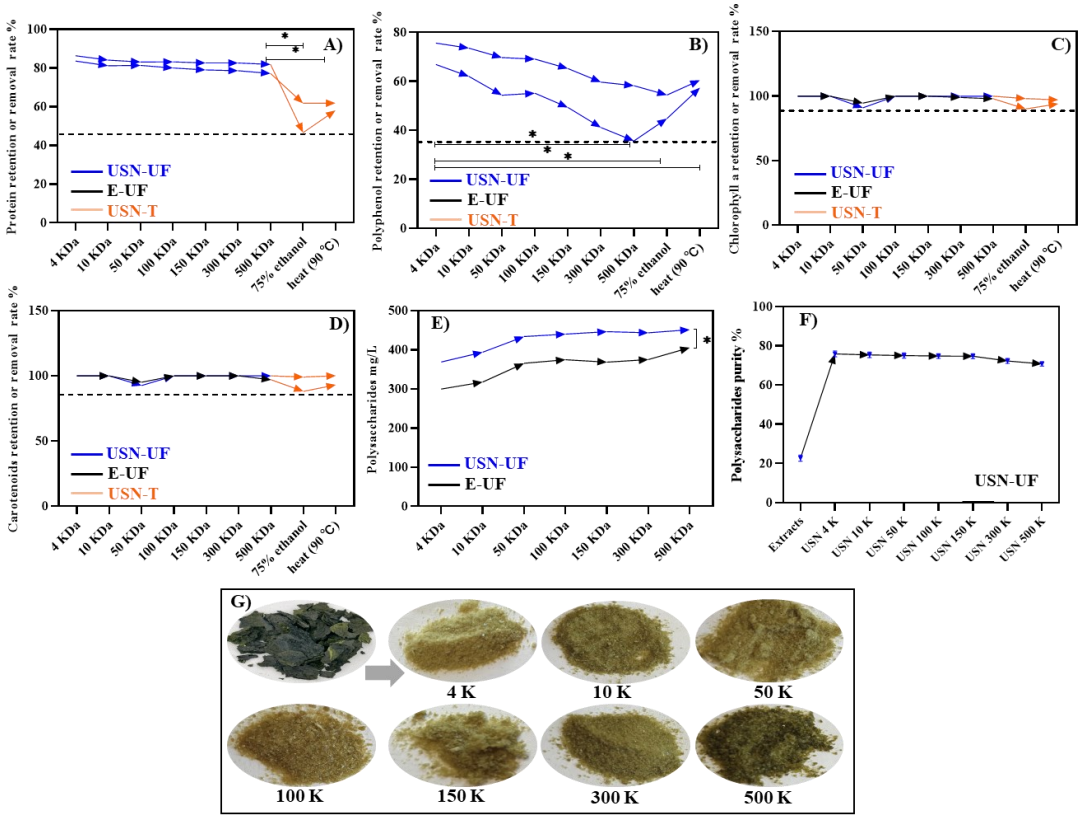

Figure 8. Impurity retention or removal ratio during polysaccharide separation process (A, B, C, D), polysaccharide concentration in permeate (E), final crude polysaccharide purity (F) and morphology (G). USN-UF, ultrasound-ultrafiltration, E-UF, control-ultrafiltration, USN-T, ultrasound-traditional separation process. * Means significant differences at a $p < 0.05$ level.

The retention rate of pigments (chlorophyll a, carotenoids) by UF was over 92%, indicating that the retentate contained almost all the pigments in the extract. The removal of proteins and polyphenols by the traditional method was lower efficiency that of UF process, and the removal rate of proteins and polyphenols was ~60%. The 75% ethanol precipitation and the heat concentration method (95 °C) could not completely remove the pigments. The above results showed that UF had a good impurity removal effect in the separation of polysaccharides. The concentrations of polysaccharides in the USN-UF and E-UF permeates were 368.99~451.35 mg/L and 299.80~405.68 mg/L, which were

positively correlated with the membrane MWCOs.

Finally, the obtained permeate by USN-UF was thermal concentrated and freeze-dried to obtain crude saccharide powder. Figure 8 showed the appearance of the final products. The color of the 4~300 kDa polysaccharides sample was light yellow, while the 500 kDa polysaccharides sample presented yellow-green mixed color. Color differences were related to polysaccharides purity. The results showed that the purity of polysaccharides showed a slight downward trend with membrane MWCOs increased, of which the purity of 4-150 kDa was ~77%, and the purity of 300 kDa and 500 kDa was 72% and 70%, respectively.

In general, the preparation of high-purity microalgae polysaccharides in previous studies required cumbersome pre-treatment processes, including depigmentation, de-lipidation, and deproteinization, etc., and a large number of organic reagents were used, including ethanol, methanol, chloroform, n-butanol, etc., which had the disadvantages of polluting the environment, complex procedures, and large loss of polysaccharides (Kang et al., 2022; Qiu et al., 2022; Tang, Liu, Yin, & Nie, 2020).

Membrane UF of polysaccharides also has been reported in previous studies. For example, Xie et al. (2014) used 300 kDa, 100 kDa and 6 kDa membranes to isolate water-soluble crude polysaccharides with a purity of 69.5% from *Cyclocarya paliurus* (Xie et al., 2014). Sun et al. (2011) isolated crude polysaccharides with a purity of 54% from rapeseed, because impurities such as proteins in the feed solution could not be completely retained by the membrane (Sun, Qi, Xu, Juan, & Zhe, 2011). Similarly, we could not directly separate high-purity *P. tricornutum* polysaccharides from USN extracts only using membrane UF process, because the raw extracts contained various impurities such as proteins, pigments, and polyphenols, etc. In addition, Sun et al. (2011) reported that different MWCOs (3, 8, 12 kDa) did not improve the purity of crude polysaccharides (Sun et al., 2011). On the contrary, our results showed that the polysaccharide purity

decreased with the increase of membrane MWCOs, which indicated that the purity of crude polysaccharide would be affected when the membrane MWCOs differed greatly. In general, the results in our study indicated that USN-UF could be used as an innovative option for the *P. tricornutum* polysaccharides recovery.

Conclusion

As an important raw material for improving the properties of food matrix such as rheology, water holding capacity and texture, the preparation process of polysaccharide is very critical. Low-frequency ultrasound (LF-USN) could be used as an efficient extraction technique to recover polysaccharides from *P. tricornutum*, which enhanced cytoplasmic release by disrupting *P. tricornutum* cellular structure. USN extraction combined with UF technology could effectively remove impurities such as proteins, polyphenols, and pigments to obtain polysaccharides from *P. tricornutum* extracts. What needs to be improved in the future is to avoid the occurrence of membrane fouling during ultrafiltration, which will reduce UF efficiency and is detrimental to the separation process. In general, the use of LF-USN+UF to enhance the separation of *P. tricornutum* polysaccharides is desirable, and the structure and chemical information of polysaccharides as well as their effects on bakery goods properties are being studied currently, which will lead to report in the near future.

Acknowledgements

This study forms part of the AGROALNEXT program (AGROALNEXT/2022/060 - Desarrollo y optimización de procesos innovadores y sostenibles de extracción de aceite y proteínas a partir de microalgas, insectos, residuos y subproductos agroalimentarios: Evaluación de propiedades biológicas (EXTRAOLIOPRO)) and was supported by MCIN with funding from European Union NextGenerationEU (PRTR-C17.I1) and by Generalitat Valenciana. Moreover, it was partially funded by the EU Commission and BBI-JU Horizon H2020, through the AQUABIOPRO-FIT project (Aquaculture and

agriculture biomass side stream proteins and bioactives for feed, fitness and health promoting nutritional supplements) grant number 790956. Francisco J. Barba is member of the CYTED network "P320RT0186—Aprovechamiento sostenible de recursos biomásicos vegetales iberoamericanos en cosmética (BIOLATES)". Jianjun Zhou and Min Wang were supported by a PhD fellowship from the China Scholarship Council (CSC) (No. 201908420246 and No. 201908420245, respectively).

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4.5 The impact of liquid-pressurized extracts of *Spirulina*, *Chlorella* and *Phaedactylum tricornutum* on *in vitro* antioxidant, antiinflammatory and bacterial growth effects and gut microbiota modulation

Journal: *Food Chemistry*, 2022, 401, 134083; https://doi.org/10.1016/j.foodchem.2022.134083.

The impact of liquid-pressurized extracts of *Spirulina*, *Chlorella* and *Phaedactylum tricornutum* on *in vitro* antioxidant, antiinflammatory and bacterial growth effects and gut microbiota modulation

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Abstract

The impact of *Spirulina*, *Chlorella* and *Phaeodactylum tricornutum* (*P. tricornutum*) microalgae extracts obtained by pressurized liquid extraction (PLE) on antioxidant and antiinflammatory activities, microbial growth and *in vitro* gut microbiota composition was evaluated. PLE, compared to conventional extraction, led to a significant ($p < 0.05$) increase in proteins, carbohydrates, polyphenols, and antioxidant capacities of the three microalgae extracts. Moreover, *Spirulina* and *P. tricornutum* extracts significantly ($p < 0.05$) reduced the *in vitro* activation of the inflammatory NF-κB pathway. The microalgae extracts had also an inhibitory effect on the pathogenic bacteria while potential beneficial *Lactobacillus* and *Bifidobacterium* strains increased growth. The effects of microalgae extract on specific bacterial groups were analyzed by quantitative PCR technology, and bacterial gene copy numbers were affected by *in vitro* digestion process and colonic fermentation time. GC–MS results showed that microalgae biomolecules' digestion promoted the release of short-chain fatty acids (SCFAs) during *in vitro* colonic microbiota fermentation, particularly acetic, butanoic and propanoic, indicating that the biomolecules in microalgae extracts have potential health benefits for human gut.

Keywords: PLE; microalgae; polyphenol; anti-bacterial; probiotics; gut microbiota; SCFAs.

1. Introduction

Over the last decades, marine algae have been widely used in the food, pharmaceutical and cosmetic industries due to their wide range of potential applications, especially attributed to their high content in antioxidant compounds including pigments, peptides, polyphenols, and polysaccharides (Terriente-Palacios & Castellari, 2022). Among the large number of available microalgae species, *Spirulina*, *Chlorella* and *P. tricornutum* have been intensively studied.

Spirulina belongs to blue-green autotrophic microalgae, which contains about 70% of protein, being also rich in unsaturated fatty acids and pigments, such as linolenic acid and linoleic acid, carotenoids and chlorophylls (Bezerra et al., 2020; Li et al., 2019). On the other hand, *Chlorella* is a single-celled green microalgae that can exist in fresh and sea water (Eckardt, 2010). Relevant studies have shown that the biologically active ingredients in *Chlorella* show positive effects as anti-hypertensive, anti-allergic, anti-asthmatic, anti-diabetic, anti-tumor and preventing heart disease (Barboríková et al., 2019; Horii et al., 2019). Moreover, *Chlorella* belongs to the "Generally Recognized As Safe" (GRAS) category of the US Food and Drug Administration being rated as a green and healthy food by the Food and Agriculture Organization of the United Nations (FAO) (Song et al., 2018). *P. tricornutum* is a unique diatom with three different morphological types (fusiform, triradial and elliptical), which can be changed by stimulating environmental conditions. *P. tricornutum* has antibacterial activity, which depends on its morphology (Kwak et al., 2014). However, the current research on the biomass of *P. tricornutum* mainly focuses on long-chain unsaturated fatty acids, and the available literature on other compounds, like polyphenols, and its corresponding functional properties is scarce (Cui, Thomas-Hall, & Schenk, 2019).

As the microalgae extracts are being more and more widely used in the food and pharmaceutical industries, their impact on food safety, such as the ability to inhibit the

growth of harmful bacteria, and the impact on human health, including antiinflammatory and antioxidant capabilities, and how they affect the human gut microbiota is a topic that needs to be further studied.

In terms of obtaining high-added-value compounds (i.e. nutrients and bioactive compounds) from marine algae, there are still many studies using traditional extraction methods such as soaking and Soxhlet extraction (Sarkar, Manna, Bhowmick, & Gayen, 2020). Although this type of method facilitates the recovery of the total amount of biomass, the extraction process takes long times and uses expensive or toxic organic reagents, such as ether, chloroform, methanol, ethanol, etc. From an economic, health and environmental point of view, there is a need to improve these limitations. In this line, pressurized liquid extraction (PLE) can greatly shorten the extraction time and improve the extraction efficiency of biomass compared with the traditional extraction methods (Tejedor-Calvo et al., 2020), obtaining promising results regarding the extraction of active plant substances, such as polysaccharides, polyphenols, and oils (Ferro, Mayer, Oliveira Müller, & Ferreira, 2020).

Therefore, this research aims to evaluate PLE as an innovative approach to recover extracts rich in high-added-value compounds from *Spirulina*, *Chlorella* and *P. tricornutum* microalgae. Furthermore, the functional properties such as antioxidant and antiinflammatory activities as well as the impact on the growth of both pathogenic and probiotic bacteria are discussed. Finally, the *in vitro* effects of the microalgae extracts on colonic fermentation will be evaluated to ascertain their potential gut health benefits.

2. Materials and methods

2.1 Chemicals and reagents

The ABTS (2,2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid), Folin–Ciocalteu reagent, gallic acid, Trolox (6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid), BCA kits, D-glucose, phenol reagent, DTT (DL-Dithiothreitol), Trizma® base, SiO_2,

diatomaceous earth (Hyflo® Super Cel®), fluorescein sodium salt and potassium persulfate ($K_2S_2O_8$) were purchased from Sigma-Aldrich (Steinheim, Baden Württemberg, Germany). Sodium carbonate (Na_2CO_3) was acquired from VWR (Saint-Prix, France). Deionized water (resistivity >18 MΩ cm^{-1}) was obtained through a Milli-Q SP® Reagent Water System (Millipore Corporation, Bedford, MA, USA).

2.2 Microalgae biomasses

Spirulina biomass came from *Arthrospira platensis* species, strain paracas 15016, being Paracas the lake where it was originally isolated (Lima, Peru). Cultivation took place at Eco*Spirulina* company (Serra, Valencia, Spain) in raceway ponds using a greenhouse under natural sunlight without any artificial light added. At the time of the experiment, day-time temperature was 32 °C on average, while temperature decreased to 24 °C at night. Culture's pH varied between 9.8 and 10.4. Frozen biomass was used to carry out the experiments at the laboratory of Nutrition and Food Science, Faculty of Pharmacy, Universitat de València. *P. tricornutum* was produced in four 800 L GemTube (LGEM, Rotterdam, The Netherlands) photobioreactors at the National Algae pilot plant in Mongstad (NAM), Norway. The photobioreactors were in a greenhouse exposed to natural light and additionally equipped with artificial illumination (EAX 170W LED lights, Evolys AS, Oslo, Norway) with an average incident artificial light of 200 µmol $m^{-2}\cdot s^{-1}$. The reactors of *P. tricornutum* were operated at pH 7.8 by CO_2 addition, and temperatures were maintained between 15~35 °C by heating the greenhouse or spraying the reactors with water to cool them down. *Chlorella* was cultivated in Hainan Island (China). *Chlorella* culture took place in open raceway ponds. Climatology of this area is continental, with average temperatures varying from 21 °C to 33 °C and precipitations of 1600 mm per year. At the time of harvesting, biomass is washed and spray-dried at 160 to 180 °C, the final product is a fine green powder with characteristic smell and taste.

2.3 PLE extraction process

Freeze-dried microalgae powders were transferred to a grinder for pulverization in order to apply further experiments. Before PLE extraction, microalgae samples were pretreated according to the experimental method previously reported by O'sullivan et al. (2013). That is, 0.5 g of algae samples were mixed with 1.5 g of silica at a ratio of 1:3 (w/w), and 1.0 g of diatomaceous earth was mixed at a ratio of 1:2 (w/w). After mixing thoroughly with a mortar, all the mixture was transferred to the PLE extraction cell. Before extraction, the extraction cell was preheated and cleaned under a nitrogen pressure of 103.4 bar with distilled water to ensure that there was not any residue in the extraction cell. The samples were extracted according to the optimized conditions (40 °C/10 minutes) previously studied in our laboratory. After the extraction procedure was completed, the sample in the collection bottle was transferred to a centrifuge tube, and the sample was stored at -20 °C for subsequent experimental analysis.

2.4 Nutrient profile and antioxidant bioactive compounds

Protein, carbohydrate, carotenoids, chlorophylls, total polyphenols and total antioxidant capacity (Trolox Equivalent Antioxidant Assay (TEAC) and Oxygen Radical Antioxidant Capacity (ORAC)) assays were carried out according to previous study (Kokkali et al., 2021).

Moreover, the phenolic profile was evaluated using a TripleTOF™ 5600 (ABSCIEX) LC/MS/MS system and an Agilent 1260 Infinity (Agilent, Waldbronn, Germany) equipment. Chromatographic separation was carried out on a Waters UPLC C18 column 1.7 µm (2.1 × 50 mm) Acquity UPLC BEH.C18 from Waters (Cerdanyola del Vallès, Spain). The mobile phase contained water (0.1% CH_2O_2, A) and methanol (0.1% CH_2O_2, B). The gradient elution of the mobile phase was 0~13 min with 90% (A) and 10% (B),13~15 min with 100% (B),15.1~22 min with 90% (A) and 10% P.E.S. The flow rate and injection volume were 0.4 mL/min and 5 µL respectively. The mass range of MS acquisition was 80~1200 m/z. An external calibration delivery system was used as

calibration. The MS is processed by an Information Dependent Acquisition (IDA) with the survey scan type (TOF-MS) and the dependent scan type (Product Ion) at -50 V of collision energy. The MS conditions were ion spray voltage of -4500 V, delustering potential of 90 V, collision energy of -50 V, temperature at 400 °C with curtain gas of 25 psi, ion source gas 1 at 50 psi and ion source gas 2 at 50 psi. IDA MS/MS was performed as follows: ions that exceeded 100 CPS, ion tolerance 50 m Da, collision energy fixed at 25 V and dynamic background subtraction activated. For the quantification, an external calibration curve using a representative polyphenol of each group of phenolic compounds potentially found in the samples was prepared, being the following polyphenols, the ones selected for each specific group: phenolic acids (gallic acid); flavonoids (flavones: apigenin; flavonols: kaempferol; flavanones: naringenin; flavanols: catechin); stilbenes (resveratrol); isoflavonoids (genistein); phenylethanoids: (hydroxytyrosol).

2.5 Antiinflammatory activity

The antiinflammatory potential of the extracts was determined by evaluating the inhibitory effect on the activation of the Nuclear Factor κappa B (NF-κB) pathway as previously described (Rocchetti et al., 2020). In brief, HT-29 NF-κB reporter-cells were seeded in a 96-well plate (65.000 cells/well) and incubated for 24 h. To investigate the activation of NF-κB, cells were stimulated with pro-inflammatory substances [tumor necrosis factor α (TNF-α), 10 ng/mL, Immunotools, Germany] in the presence or absence of microalgae PLE extract (10% v/v) and extracts without PLE treatment (10% v/v). After cell stimulation for 24 h, cell supernatant was collected and cells were lysed in PBS containing 0.1% Triton, 1 mM PMSF and 1 mM EDTA. Protein content of each well was determined using the Bradford Protein Assay (Biorad). The secreted alkaline phosphatase (SEAP) activity was determined by using p-nitrophenyl phosphate, as a phosphatase substrate according to the manufacturer's instructions (Thermo Scientific, Ref.34047) and the resulting absorbance value was normalized to the protein content of each well and

expressed as a percentage of inflammation (100% considered to be NF-κB activation induced by TNF-α).

2.6 Impact on bacterial growth kinetics

The impact of the *Spirulina*, *Chlorella* and *P. tricornutum* extracts on beneficial bacteria and their action against potential foodborne bacteria was determined following the procedures previously described (Rocchetti et al., 2020). For this experiment, the selected probiotic strains were *Lactobacillus casei* ATCC 393 (*L. casei*), and *Bifidobacterium lactis* ATCC 27536 (*B. lactis*) and the potential foodborne bacteria were *Listeria innocua* CECT 910 (*Listeria*), *Salmonella enterica* CECT 4138 (*Salmonella*), *Staphylococcus aureus* CECT 86 (*S. aureus*) and *Escherichia coli CECT 99* (*E. coli*).

Probiotic strains were grown in MRS medium with 0.05% L-Cysteine in anaerobic and static conditions at 37 °C during 20 h while the pathogens are grown aerobically in BHI (Brain Heart Infusion) medium at 37 °C overnight. The growth pattern was monitored in the presence or absence of 20 µL of the algae extracts. Overnight cultures for each strain were collected and inoculated to a final optical density (OD) at 595 nm = 0.05 in 200 µL of medium in 96 well microtiter plates and incubated at 37 °C in a POLARStar plate reader (BMG). Changes in OD595 nm were monitored and growth curves were modelled by use of the Gompertz equation (Rocchetti et al., 2020). GraphPad Prism 5 software was used to fit the Gompertz equation and Growth rate (µmax, h^{-1}) and MOD (maximal optical density) value were finally used to evaluate the bacteria growth. The Gompertz equation with slight modification is as follows (Chatterjee, Chatterjee, Majumdar, & Chakrabarti, 2015)：

$$y = K + A \exp\left[-\exp\left(-\frac{\mu_{max^e}}{A}(\lambda - t) + 1\right)\right] \qquad (1)$$

Where y is the extent of growth at time t (h), K means initial cell number, A is the change in the number of cells between the inoculum and the stationary phase, µmax is

the maximum growth rate (the variation in number of cells per unit of time), λ is the length of the lag phase (h) and e is a constant (2.7182).

2.7 Salivary-gastric-intestinal in vitro digestion and colonic fermentation

The *in vitro* simulated digestion was performed based on the method previously described by (Wu et al., 2021) with some minor modifications. The compositions of the simulated salivary fluid (SSF) simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) are shown in Table 1. During the salivary digestion, 2.5 mL sample, 2 mL SSF, 12.5 μL $CaCl_2$ and 487.5 μL water were mixed at 37 °C for 2 min. In gastric digestion process, 4.55 mL SGF, 8 mg pepsin and 2.5 μL $CaCl_2$ were added, shaking for 1 min, adjusted the pH to 3.0, added water to keep the total volume at 10 mL and agitated 2 h at 37 °C. In intestinal digestion process, 5.5 mL of SIF, 2.5 mL of pancreatin and 1.25 of bile were added, adjusted the pH to 7.0, then water was added to keep the total volume at 20 mL and shake 2 h. Finally, the extracts without digestion and the indigestible part of the salivary-gastric-intestinal juice were collected and resuspended with distilled water for observing their effects on human gut microbiota fermentation.

Table 1. Composition of the simulated salivary-gastric-intestinal juices

Constitute	Concentration	SSF	SGF	SIF
KCl	3.7 g/ 100 mL	7.55 mL	3.45 mL	3.4 mL
KH_2PO_4	6.8 g/ 100 mL	1.85 mL	0.45 mL	0.4 mL
$NaHCO_3$	8.4 g/ 100 mL	3.4 mL	6.25 mL	21.25 mL
NaCl	11.7 g/ 100 mL	---	5.9 mL	4.8 mL
$MgCl_2$	3.05 g/ 100 mL	0.25 mL	0.2 mL	0.55 mL
NaOH	1 M	---	---	---
HCl	6 M	---	---	---
$(NH_4)_2CO_3$	0.5 M	0.03 mL	0.25 mL	---
pH	---	7	3	7
Total volume	---	200 mL	200 mL	200 mL

SSF: Simulated salivary fluid. SGF: Simulated gastric fluid. SIF: Simulated intestinal fluid.

In vitro colonic fermentation was performed following the procedure of Yousi et al. (2019) with some minor modifications. The basal nutrient medium was composed of yeast extract (0.1 g), peptone (0.1 g), $NaHCO_3$ (0.1 g), bile salt (0.025 g), cysteine-HCl (0.025 g), NaCl (0.005 g), KH_2PO_4 (0.002 g), hemin (0.0025 g), $MgSO_4$ (0.0005 g), $CaCl_2$ (0.0005 g), resazurin (0.025% w/v), vitamin K1 (0.50 µL), tween 80 (100 µL), and distilled water (50 mL), the pH of basal nutrient medium was adjusted to pH 7.0 and sterilized for later use. Fresh fecal samples were collected from six healthy volunteers (two females and four males, aged from 18 to 30) who did not have digestive diseases or any treatment of antibiotics in the past three months. Written informed consent was obtained from all volunteers, and the study protocol was approved by the local ethics committee of the Atención Primaria-Generalitat Valenciana (CEIC-APCV).

The fecal samples were diluted with sterilized modified saline solution (NaCl 0.9%) to obtain 10% of fecal slurry (w/v). After homogenization for 30 min and resting for 15 min, 10.5 mL supernatant were taken and injected into a new tube containing 5 mL of Nycodenz, then the mixture was centrifuged at 4000 rpm/4 °C /60 min. The supernatant was discarded, and the bacterial phase (white phase) was transferred into a 2 mL centrifuge tube and washed with 0.9% NaCl twice (13000 rpm/5 min/4 °C). Before gut fermentation, 1235 µL of basal nutrient medium were added into a 1.5 mL sterile vial and boiled for 5 min to take out oxygen. After cooling down, 15 µL of bacterial (dilution 25 times with 0.9 % NaCl) were added and kept at 37 °C under anaerobic atmosphere for 20 h. Then, 175 µL of sample and 325 µL of sterile basal medium were injected into vials for anaerobic fermentation at 37 °C and the sample was collected at 24 h and 48 h, respectively. Finally, after centrifugation, the pellet and the supernatant were used for 16S rRNA and SCFAs profile analysis, respectively.

2.8 Targeted microbial quantification by qPCR analysis

Total DNA was isolated from bacterial pellets after colonic fermentation according to previous studies of our research team (Selma-Royo et al., 2021). In detail, 900 µL CTAB were added to the bacterial pellet obtained after *in vitro* colonic fermentation (*section 2.7*), vortexed 25s and transferred into 1.5 mL tubes containing one spoon of glass beads and bead-beating was carried out by using a FastPrep-24^{TM}5G equipment (Lab Innovations, UK). Then, samples were incubated at 95 °C for 2.5 min and vortexed for 10 s, repeating the process once. Then, 40 µL of proteinase K and 20 µL of lysozyme were added, vortexed 10 s and heated at 70 °C for 10 min, then centrifuged at 1300 rpm for 5 min. The further DNA extraction process was carried out according to manufacturer's instructions of Maxwell RSC Pure Food GMO and Authentication Kit (Promega). DNA concentration in samples was measured using a Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, USA).

Table 2. Primer sequence information and qPCR parameters

Target	Primers	Sequence from 5'to 3'	T/ºC	Size
Bifidobacterium genus	Bifido5'	GATTCTGGCTCAGGATGAACG C	60	232 bp
	Bifido3'	CTGATAGGACGCGACCCCAT		
Lactobacillus spp.	Lacto F	AGCAGTAGGGAATCTTCCA	58	341 bp
	Lacto R	CACCGCTACACATGGAG		
Enterobacteriaceae family	Enterobact F	CATTGACGTTACCCGCAGAAGAAGC	63	195 bp
	Enterobact R	CTCTACGAGACTCAAGCTTGC		
Total bacteria	16S_U515F	GTGCCAGCMGCCGCGGTAA	62	274 bp
	16S_U789R	GCGTGGACTACCAGGGTATCT		
Streptococcus	Strep-1	GTACAGTTGCTTCAGGACGTATC	55	197 bp
	Strep-2	ACGTTCGATTTCATCACGGTTG		
Enterococcus spp.	Enterococ F	CCCTTATTGTTAGTTGCCATCATT	61	144 bp
	Enterococ R	ACTCGTTGTACTTCCCATTGT		

The number of specific fragment gene copies were measured by qPCR in 96-well plates using a LightCycler 480 equipment (Roche). Briefly, the reaction mixture consisted of 1

µL DNA, 5 µL of SYBR Green Master Mix (Roche), 0.25 µL of primer 1, 0.25 µL of primer 2 and 3.5 µL of H_2O. Copy numbers of the specific gene-fragment were derived from respective standard curves. In this study, we measured specific gene-fragment copies of total bacteria, *Bifidobacterium* genus, *Lactobacillus spp*., *Enterobacteriaceae* family, *Streptococcus*, and *Enterococcus spp*., being the primer sequence and qPCR parameters shown in Table 2.

2.9 Determination of short chain fatty acids (SCFAs) by GC-MS

The concentration of SCFAs were determined by using an Agilent GC 7890B-5977 GC-MS with a multipurpose sampler (Gerstel MPS). The GC column (Agilent DB-FATWAX) of 30 m×0.25 mm ×0.25 µm was operated in split mode. The oven temperature program was established as 100 °C for 3 min, ramped to 100 °C at a rate of 5 °C min^{-1}, to 150 °C for 1 min, to 200 °C at a rate of 20 °C min^{-1}, and finally held at 200 °C for 5 min. Helium was used as a carrier gas at a flow rate of 1 mL min^{-1}. In brief, 200 µL of supernatant samples (*section 2.7*) was mixed with 800 µL of standard solutions, 1 mL of diethyl ether, one spoon of Na_2SO_4 and vortexed 10 s for GC-MS analyses.

2.10 Statistical analysis

One-way ANOVA with Dunnett's multiple comparisons test was performed using Statgraphics® Centurion XV (Statpoint Technologies, Inc., USA). It was used to detect statistically significant differences between the control and the sample-supplemented groups on the following parameters: bioactive compounds yield, antioxidant and antiinflammatory capacities, number of total and specific bacterial groups and SCFAs. Statistical significance was accepted at $p < 0.05$.

3. Results and discussion

3.1 Biomolecules of microalgae extracts and antioxidant capacity

The protein, carbohydrate, polyphenol, chlorophyll a, and carotenoid contents of *Spirulina*, *Chlorella* and *P. tricornutum* extracts obtained under pressurized liquid

extraction (PLE) or conventional non-PLE treatments are shown in Figure 1. The PLE extraction were carried out based on the optimized extraction conditions previously obtained in our laboratory using *Spirulina* as a matrix.

As shown in Figure 1, compared to non-PLE extraction, the results obtained showed that PLE extraction significantly ($p < 0.05$) increased the protein content of the three algae extracts (*P. tricornutum*: 93.3 vs. 68.6 mg/g; *Chlorella*: 30.8 vs. 21.3 mg/g; *Spirulina*: 136.7 vs. 120.8 mg/g), polyphenols (*P. tricornutum*: 4.6 vs. 3.4 mg/g; *Chlorella*: 1.7 vs. 1.3 mg/g) and carbohydrate content (*P. tricornutum*: 39.0 vs. 28.5 mg/g; *Chlorella*: 25.6 vs. 20.4 mg/g) ($p < 0.05$). However, inconsistent results appeared regarding the recovery of pigments.

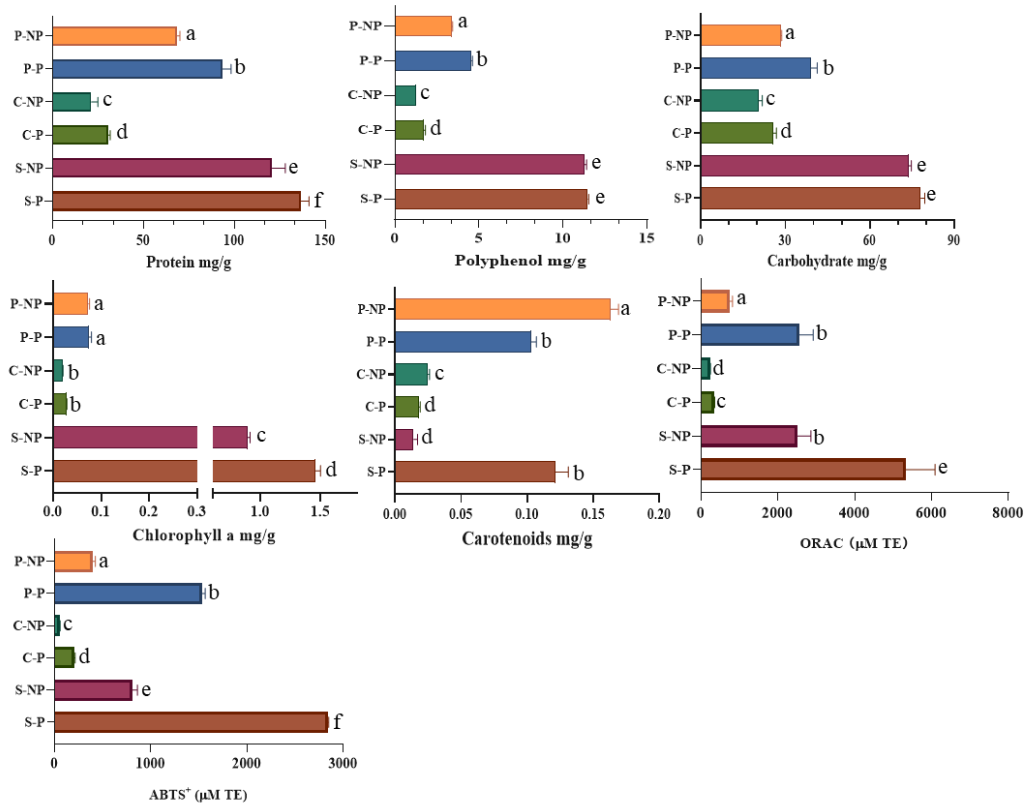

Figure 1. Protein, polyphenol, carbohydrate, chlorophyll a, carotenoids and antioxidant capacity of *Spirulina*, *Chlorella* and *P. tricornutum* extracts. *P*-NP, *P*-P, *C*-NP, *C*-P, *S*-NP, and *S*-P corresponds to *P. tricornutum*-Non-PLE, *P. tricornutum*-PLE, *Chlorella*-Non-PLE, *Chlorella*-PLE, *Spirulina*-Non-PLE and *Spirulina*-PLE respectively. Different

letters mean significant differences between the different groups ($p < 0.05$) while similar letters mean non-significant differences among the different groups ($p > 0.05$).

For instance, PLE significantly increased the content of chlorophyll a and carotenoids in *Spirulina* extract, while a significant reduction ($p < 0.05$) in carotenoid content of *Chlorella* and *P. tricornutum* extracts compared to control samples. The low yield of chlorophyll a, and carotenoids in this study is attributed to the use of water as the extraction reagent, and it was less affected by PLE extraction. The *Spirulina* extracts had the strongest antioxidant capacity, followed by *P. tricornutum* and *Chlorella* (Figure 1). Compared to control group, PLE significantly ($p < 0.05$) improved the ability of microalgae extracts to scavenge oxygen free radicals, independently of the antioxidant assay used. For instance, some previous studies have shown that the antioxidant capacity of algae extracts mainly comes from polyphenols and pigments, other compounds such as proteins as well as polysaccharides also have important antioxidant activities (Jimenez-Lopez et al., 2021; Alkhalaf, 2021). The magnitude of the value was different according to the method used, which is attributed to the different mechanisms of action of both methods having different types of free radical scavenging.

Briefly, PLE-assisted water extraction is shown as a promising tool for the extraction of microalgae biomass, which is mainly attributed to the extraction principle of PLE, reducing the strong interaction forces (van der Waals forces, hydrogen bonds, etc.) between the solute and the matrix caused under high pressure and the viscosity of the solvent, thus promoting the diffusion of the solvent into the sample (Wang, Zhou, Collado, & Barba, 2021). Overall, in this part, PLE promotes the extraction efficiency of the bioactive compounds, especially protein and polyphenols, which can be a reliable way to recover the biomolecules from microalgae.

3.2 Phenolic profiles -Triple TOF-LC-MS

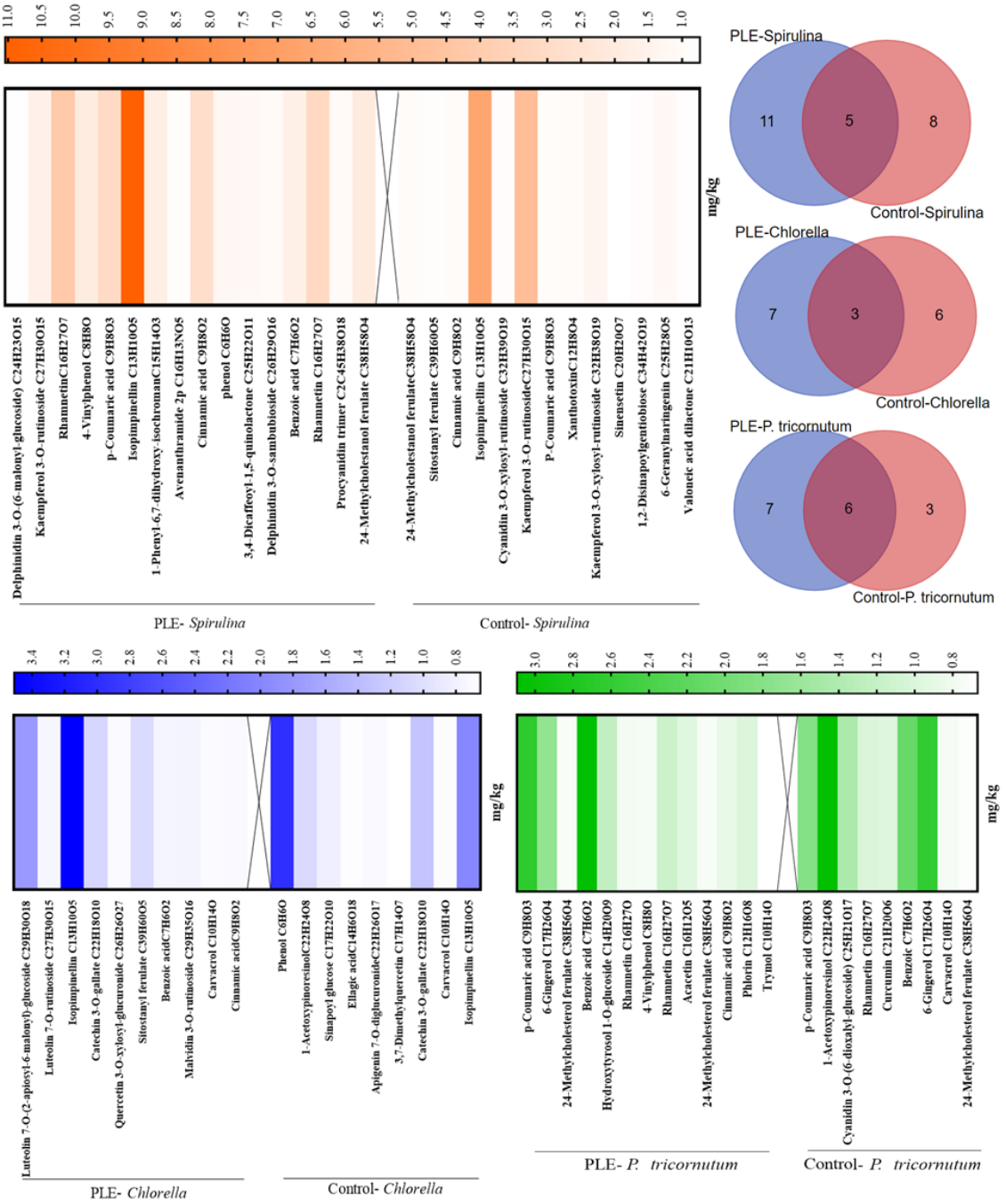

Figure 2. Phenolics profiles identification (Triple TOF-LC-MS-MS) of microalgae extracts assisted by PLE and conventional extraction methods.

After analyzing total polyphenols using the spectrophotometric method it was observed that microalgae contained a considerable amount of polyphenols, especially in *Spirulina* and *P. tricornutum* extracts. However, considering the interferences of that method it is

of paramount importance to identify and quantify the individual profile due to the different biological activities of the individual compounds.

When the phenolic profiles of the samples were evaluated (Figure 2), 16, 13, 10, 9, 13 and 9 different phenolic compounds were identified corresponding to the group of PLE-*Spirulina*, control-*Spirulina*, PLE-*Chlorella*, control-*Chlorella*, PLE-*P. tricornutum* and control-*P. tricornutum,* respectively.

It should be noted that both extraction processes and microalgae strain determined the profile and amount of phenolic compounds found in the microalgae extracts obtained. More specifically, the predominant phenolic compounds in the PLE-*Spirulina* were isopimpinellin (11.04 μg/g), rhamnetin (4.15 μg/g), p-coumaric acid (3.117 μg/g) and cinnamic acid (2.798 μg/g), while for control-*Spirulina* isopimpinellin and (6.493 μg/g) and kaempferol 3-O-rutinoside (4.910 μg/g) were the main phenolic acids. For *Chlorella*, PLE extracts mainly contained isopimpinellin (3.508 μg/g), luteolin 7-O-(2-apiosyl-6-malonyl)-glucoside (1.789 μg/g) and catechin 3-O-gallate (1.101 μg/g), being the predominant phenolics in control samples phenol (2.886 μg/g) and isopimpinellin (1.987 μg/g). Benzoic acid (3.085 μg/g), p-Coumaric acid (2.666 μg/g), 6-gingerol (1.701 μg/g) and hydroxytyrosol 1-O-glucoside (1.255 μg/g) were the main phenolic compounds in the PLE-*P. tricornutum* extracts, while for the control group, higher contents in 1-acetoxypinoresinol (3.095 μg/g), 6-gingerol (2.621 μg/g), benzoic acid (2.110 μg/g) and p-coumaric acid (1.820 μg/g) were found. Obviously, in this study, more phenolic compounds were determined in *Spirulina* extracts, followed by *P. tricornutum* and *Chlorella*, and PLE process increased the abundance of the phenolic profiles.

Among all these phenolics, isopimpinellin, p-coumaric acid and cinnamic acid are frequently present in these samples, and such phenolics are beneficial to human health. For example, isopimpinellin has been demonstrated to be effective as a vasodilative and antioxidants, being an interesting tool to improve gynecological and andrological

disorders (Li et al., 2014). On the other hand, p-coumaric acid was found to reduce the cholesterol ester levels, thus providing a protective mechanism against atherosclerosis (Roy & Prince, 2013). Moreover, cinnamic acid has anti-diabetic and hypoglycemic functions, through the modulation of glycogenesis and gluconeogenesis and amelioration of glucose tolerance and insulin secretion (Babaeenezhad, Nouryazdan, Nasri, Ahmadvand, & Moradi Sarabi, 2021). Some other phenolics, such as carvacrol and ellagic acid, etc., though the content is not so high, present many healthy properties, such as antioxidant, antimicrobial, antihypertensive, immunomodulatory and anticancer, etc. (Larrosa, García-Conesa, Espín, & Tomás-Barberán, 2010; Rathod, Kulawik, Ozogul, Regenstein, & Ozogul, 2021). Considering the above-mentioned information, it can be verified that *Spirulina*, *Chlorella* and *P. tricornutum* is a high-added-value material source to human life due to their abundant phenolic compounds.

3.3 *Impact on antiinflammatory capacities*

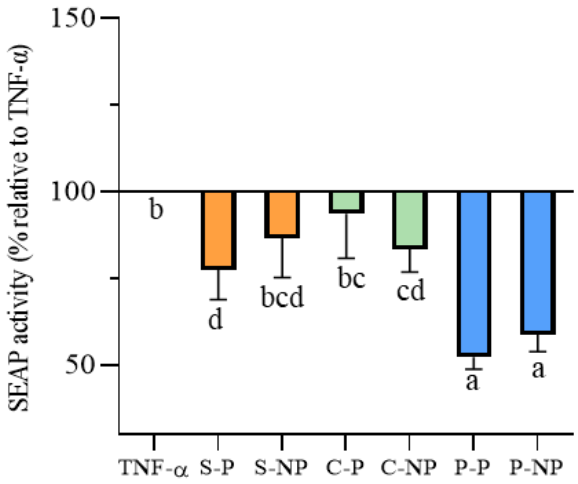

Figure 3. SEAP activity (% relative to TNF-α) of algae extracts and TNF-α. Bars represent the reduction of TNF-α-induced SEAP activity. *P*-NP, *P*-P, *C*-NP, *C*-P, *S*-NP, and *S*-P corresponds to *P. tricornutum*-Non-PLE, *P. tricornutum*-PLE, *Chlorella*-Non-PLE, *Chlorella*-PLE, *Spirulina*-Non-PLE and *Spirulina*-PLE respectively. Similar letters

represent non-significant differences ($p > 0.05$) while different letters show significant differences ($p < 0.05$).

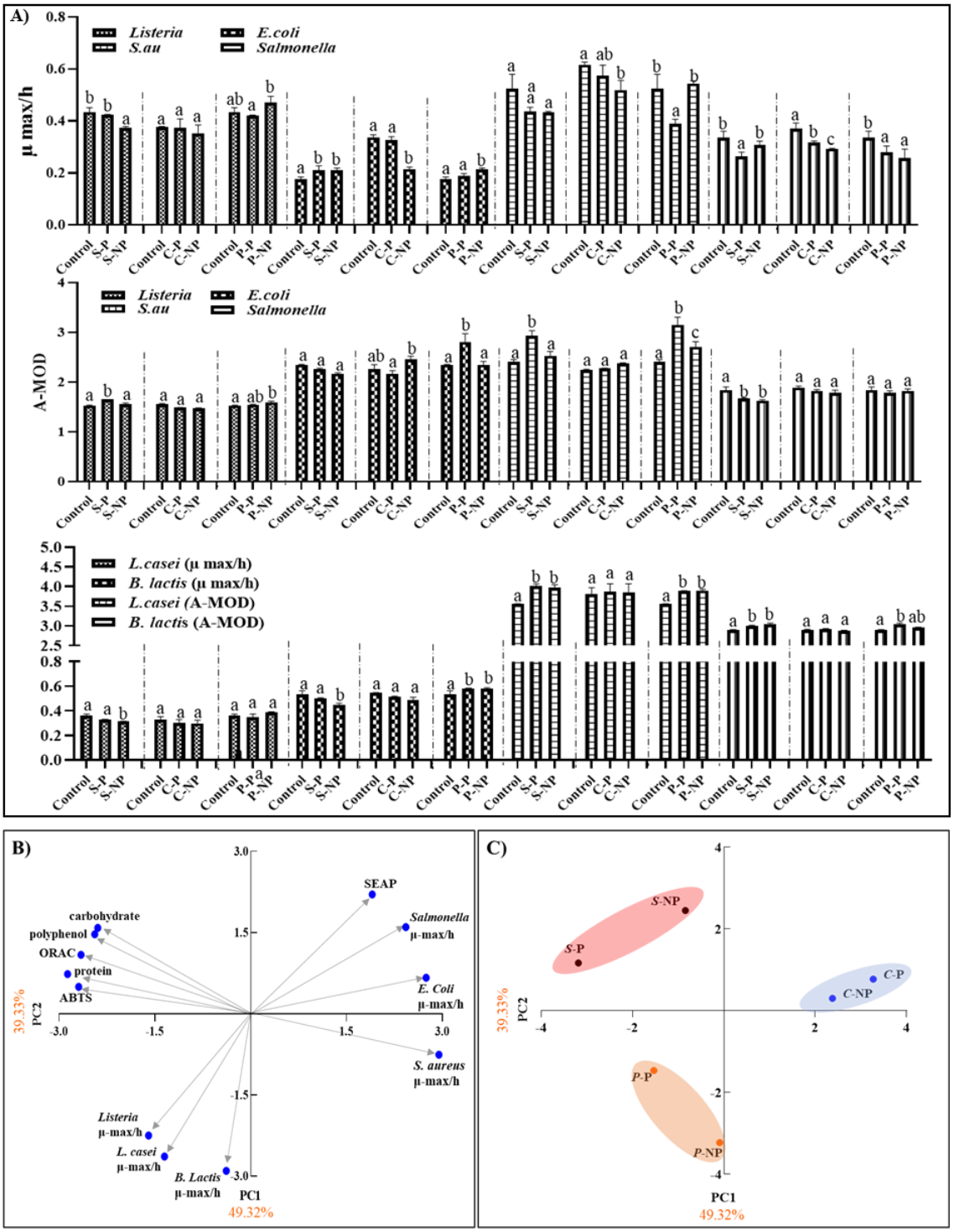

Figure 4. A) Effects of microalgae extracts on the growth of *Salmonella*, *E. coli*, *S. aureus*, *Listeria*, *L. casei* and *B. lactis.* B) and C) corresponds to loading plot and score plot of principal component analysis (PCA) based on the compounds content, antioxidant, antiinflammatory, specific bacterial growth rate and microalgae extracts. P-NP, P-P, C-

NP, C-P, S-NP, and S-P corresponds to *P. tricornutum*-Non-PLE, *P. tricornutum*-PLE, *Chlorella*-Non-PLE, *Chlorella*-PLE, *Spirulina*-Non-PLE and *Spirulina*-PLE respectively. Different letters mean significant differences between the same bacterial groups of specific microalgae ($p < 0.05$) while similar letters or no letters means non-significant differences ($p > 0.05$).

All the extracts were able to reduce the TNF-α-induced activation of NF-κB pathway (Figure 3). Differences were observed according to the algae specie evaluated, having *P. tricornutum* the strongest antiinflammatory effect among the three different algae extracts and the SEAP activity of *P*-NP and *P*-P extracts were 58.8 ± 5.0% and 52.3 ± 3.4% relative to TNF-α respectively. Furthermore, *C*-NP and *S*-P also showed that SEAP activity was significantly lower than that of the TNF-α control group ($p < 0.05$). Both *P. tricornutum* and *Spirulina* PLE extracts showed a higher antiinflammatory potential compared to the non-PLE extracts, albeit not reaching significant differences ($p > 0.05$) between extraction methods.

3.4 Impact on microbial bacterial growth

As an edible energy source, it is very meaningful to investigate the inhibition of food-borne microorganisms by microalgae compounds. *Listeria*, *E. coli*, *S. aureus* and *Salmonella* are key bacteria that cause food-related diseases; therefore, they were selected in many studies to assess the antibacterial activity of food components (Alvarez-Ordóñez, Broussolle, Colin, Nguyen-The, & Prieto, 2015). The effects of microalgae compounds on probiotics, *L. casei* and *B. lactis*, were also studied in this study. The μ-max/h and A-MOD values according to Gompertz equation represent the growth rate and the difference between the final and initial absorbance values of the bacterial solution (Rocchetti et al., 2020).

As it is shown in Figure 4A, for *Spirulina*, the *S*-P extract significantly ($p < 0.05$) reduced the μ-max/h value and A-MOD value of *Salmonella*, and the *S*-NP extract significantly reduced the *Listeria* μ-max/h value and the *Salmonella* A-MOD value ($p < 0.05$); for *P. tricornutum*, *P*-P extract significantly reduced the μ-max/h value of *S. aureus* and *Salmonella* ($p < 0.05$), while *P*-NP extract significantly reduced the μ-max/h value of *Salmonella* ($p < 0.05$); for *Chlorella*, *C*-P extract significantly reduced the μ-max/h value of *Salmonella* ($p < 0.05$), and *C*-NP extract significantly reduced the A-MOD value of *E. coli*, *S. aureus* and *Salmonella* ($p < 0.05$).

The results indicate that the inhibitory effect on bacteria differs according to both microalgae specie and extraction method, which affects the biomass composition in the extract, and consequently the proliferation of bacteria. However, the results showed that not all extracts can inhibit bacterial growth. For example, *S*-NP and *P*-P extracts increased the μ-max/h value of *E. coli* ($p < 0.05$), indicating that some extracts can increase the growth rate of *E. coli* in the logarithmic phase, which is unfavorable.

More interestingly, the PLE extracts of all three microalgae as well as the non-PLE extracts from *Chlorella* and *P. tricornutum* were able to significantly reduce the growth rate of *Salmonella*. *Listeria* was less affected by the microalgae extracts and the growth rate was significantly ($p < 0.05$) reduced by the *S*-NP extracts. Similar studies recovered extracts with different polyphenol content from *Spirulina*, named fraction A (7.80 ± 0.15 mg GAE/g) and fraction B (44.48 ± 1.71 mg GAE/g) (Mohammad, Rajapandiyan, & Wahida, 2021). Among them, fraction B exhibited significant antibacterial activity against *E. coli* and *S. aureus*, while fraction A did not exhibit antibacterial properties, and they attributed the result to the differences in polyphenol content (Mohammad, Rajapandiyan, & Wahida, 2021). Consistent with our research, Plaza et al. also used PLE to obtain *Chlorella* extracts with antibacterial properties, especially when ethanol was used as extraction regents, showing strong inhibitory effects on *E. coli* and *S. aureus*

growth (Plaza et al., 2015). In addition, other studies also reported that the *P. tricornutum*-loaded gelatin nanofiber mat showed 99.9% antibacterial activity against *E. coli* and multi-drug resistant *S. aureus*, which was related to the fatty acid of *P. tricornutum* because it was detected to combine with the nanofibers (Kwak et al., 2014).

Regarding the probiotic potential of microalgae extracts, *P*-P and *P*-NP groups did not have any significant effect on the growth of *L. casei* and *B. lactis* ($p > 0.05$), while *S*-NP significantly reduced the μ-max/h value of *L. casei* ($p < 0.05$), *C*-P and *C*-NP significantly reduced the μ-max/h value of *B. lactis* ($p < 0.05$). It is worth noting that *S*-P, *S*-NP, *P*-P significantly increased the A-MOD value of *L. casei* and *B. lactis* ($p < 0.05$), and *P*-NP significantly increased the A-MOD value of *L. casei* ($p < 0.05$). This result indicates that some algae-extracts reduced the maximum growth rate value of the probiotics in the logarithmic growth phase, but as the culture time was elapsed, A-MOD values increased, and higher amounts of bacteria were reached in the stationary growth phase. For example, it can be seen from Figure SA (supplementary files) that after 10 h, the absorbance difference between *S*-P/*S*-NP and the control group becomes larger, and it is obvious that the control groups enter earlier in the stationary growth phase than *S*-P and *S*-NP. Moreover, as seen from Figure SG (supplementary files) the μ-max/h value of *L. casei* in the *S*-P group was significantly higher than that in the *S*-NP group ($p < 0.05$), thus indicating that the extraction method had a significant impact on the growth of *L. casei*.

To reveal the potential correlation between the biomass and functional properties, the results, except for the pigment (the water-extracted pigment content is very low and can be ignored), were used to perform a principal component analysis (PCA) (Figure 4B, 4C). The two principal components explained the 49.32% and 39.33% of variability, respectively, in total about 88%. The score plot clearly shows that same microalgae are distributed in the same quadrant, suggesting that the composition and functional similarity mainly depends on the type of microalgae. The loading plot shows that the bioactive

compounds (polyphenols, carbohydrates, proteins) in microalgae are closely distributed with ORAC and ABTS, indicating the positive contribution of the antioxidants.

In the first principal component, polyphenols, carbohydrates, and proteins content are distributed in the opposite direction of the maximum growth rate of *E. coli* and *Salmonella* as well as SEAP, and forwardly distributed in the maximum growth rate of *B. lactis* and *L. casei*, which indicates that they can inhibit the foodborne bacteria and inflammation but are beneficial to probiotics. Interestingly, whether it is the PC1 or PC2, polyphenols, carbohydrates, and proteins in microalgae are distributed negatively with *S. aureus*, which is an important reason to explain why the growth of *S. aureus* was inhibited by algae extracts. From loading plot, it can be depicted that *Spirulina* was more related to polyphenol, carbohydrate, protein, and antioxidant capacity, while *P. tricornutum* is associated to *L. casei* and *B. lactis* growth, which shows that the content of bioactive compounds does not directly determine its prebiotic effect, considering that the total polyphenols, proteins, and carbohydrates content of *P. tricornutum* are lower than that of *Spirulina*.

3.5 Impact of microalgae extracts and in vitro gastrointestinal transit *on colonic fecal fermentation*

In this study, the nutrient content, antioxidant and antiinflammatory properties, antibacterial ability, and probiotic properties of *Spirulina* and *P. tricornutum* extract are superior to *Chlorella*, and finally, the extracts of *Spirulina* and *P. tricornutum* assisted by PLE were selected to explore their effects on colonic fermentation. It is worth noting that a previous study showed that some bioactive components in edible microalgae, such as polyphenols, are an excellent source of prebiotics (Fatima, Akhtar, & Sheikh, 2017). However, dietary polyphenols are mostly of low bioavailability, thus their absorption in the small intestine is limited. For example, only 5~10% of the ingested unbound phenolics can be absorbed in the small intestine and the unabsorbed phenolics and their derivatives

in the colon have been found to exert prebiotic-like effects (González-Sarrías, Espín, & Tomás-Barberán, 2017).

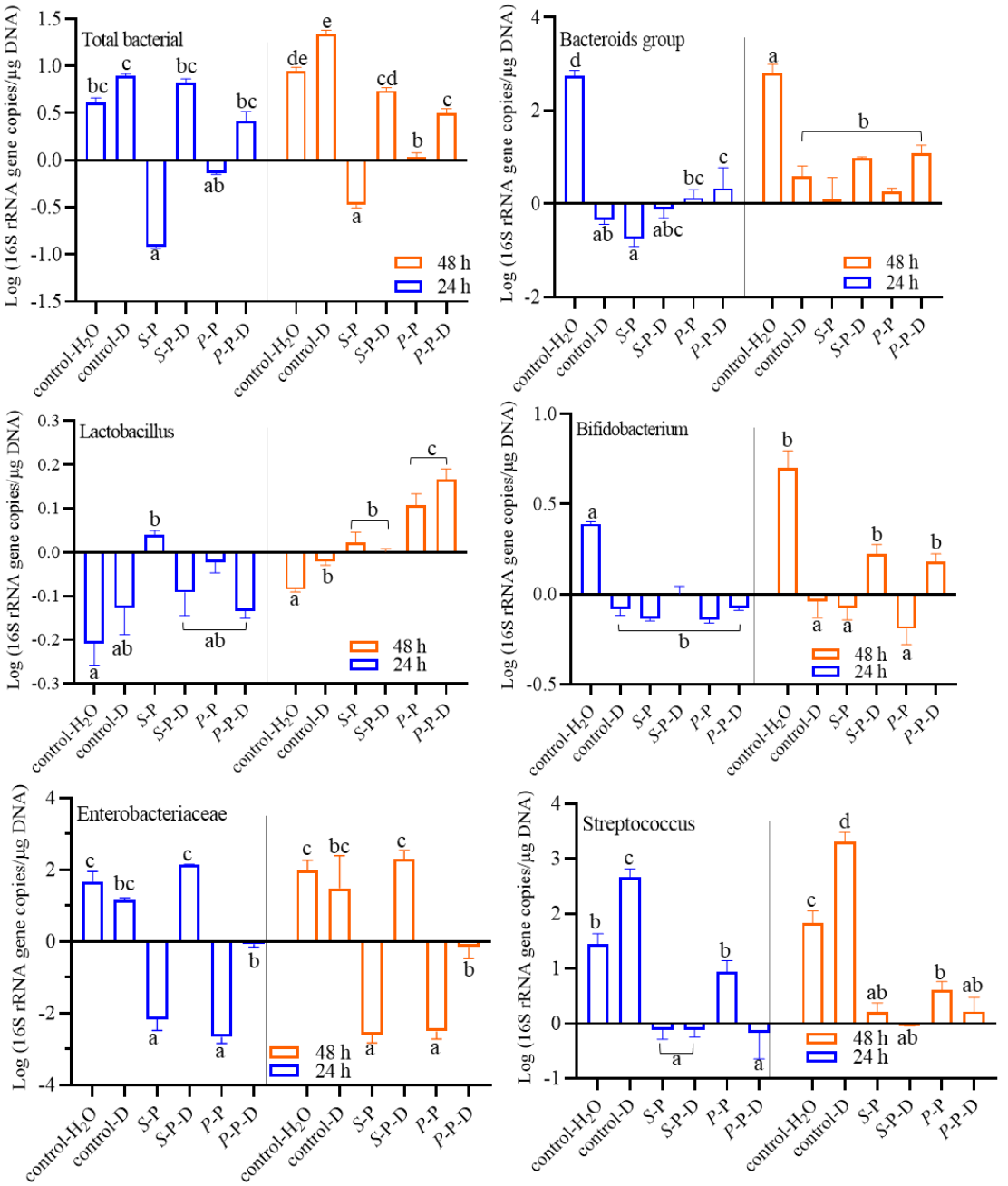

Figure 5. Total and specific bacterial levels measured with qPCR in cultures with fecal adult's microbiota supplemented with *S*-P (*Spirulina*-PLE), *P*-P (*P. tricornutum*-PLE) extracts, *S*-P-D (*Spirulina*-PLE-digest extracts), *P*-P-D (*P. tricornutum*-PLE-digest extracts). The baseline is set as the 16s rRNA gene copies/µg DNA of total or specific bacterial groups at 0 h colonic fermentation. Control groups are without extracts added and instead of the same volume of distilled water (control-H_2O) and digest juice (control-

D) respectively. Different letters mean significant differences between 24- or 48-h fermentation groups ($p < 0.05$) while similar letters do not mean significant differences ($p > 0.05$).

Taking into account the considerable amount of polyphenols and carbohydrates in *Spirulina* and *P. tricornutum* extracts, the effects of the extracts before and after the *in vitro* digestion on colonic fermentation were evaluated. Microbiota fermentation was observed by analyzing the 16s rRNA gene copies/μg DNA of total bacteria, *Bacteroides* group, *Lactobacillus*, *Bifidobacterium*, *Enterobacteriaceae* and *Streptococcus*, during the human fecal microbial fermentation process with *Spirulina* and *P. tricornutum* extracts before/after *in vitro* digestion.

S-P and *P*-P extracts decreased the number of total bacteria, *Bacteroides*, *Bifidobacterial*, *Enterobacteriaceae* and *Streptococcus* at both 24 and 48 h of the microbiota fermentation compared with control group (control-H_2O, same volume distilled water instead of microalgae extracts) (Figure 5).

Contrary to that, *S*-P and *P*-P extracts increased the number of *Lactobacillus*. Therefore, the effects of *S*-P and *P*-P extracts on intestinal health cannot be generalized, since they not only increase *Lactobacillus* and decrease *Streptococcus*, but also decrease the number of *Bifidobacterium* and *Bacteroides*. As we know, *Bifidobacterium* is an important probiotic in the intestinal tract for human health, as it controls serum cholesterol levels, prevents intestinal diseases, modulates immune system, and possesses anti-cancer activities (Gioia, Aloisio, Mazzola, & Biavati, 2014), *Bacteroides* play an important role in improving metabolic and immune disorders in obese individuals (Gioia, Aloisio, Mazzola, & Biavati, 2014), and *Lactobacillus* is considered a beneficial bacteria with effects on microbiota modulation and also, on the improvement of the gastrointestinal function, increasing food digestibility, reducing serum cholesterol, etc (Azizian et al.,

2021). Considering that the special nutrient requirements and catabolic pathways of different flora, such as *Bacteroidetes* is one of the major gut bacteria responsible for the degradation of polysaccharides (Patnode et al., 2019), the growth and establishment of *Bifidobacterium* and *Lactobacillus* can be promoted by polyphenols (Morais, Rosso, Estadella, & Pisani, 2016), it can be speculated that the regulation of *S*-P and *P*-P extracts on gut health is affected by multiple nutrients, which leads to differences in the effects on colonic fermentation. In this line, a recent study associated the probiotic effects of *Spirulina* to several nutrients, such as amino acids, minerals, and vitamins, which determined the colonic fermentation behavior (Çelekli, Alslibi, & Bozkurt, 2019). In addition, the fermentation time showed certain effects on microbiota regulation, for instance, compared with 24 h fermentation, *S*-P, *P*-P/*P*-P-D significantly increased the *Lactobacillus* number at 48 h.

Furthermore, *in vitro* digestion altered the effects of microalgae extracts on colonic fermentation. Under the exclusion of the effect of control-D on colonic fermentation, compared with *S*-P, *S*-P-D significantly increased the number of *Bifidobacterium* fermented for 48 h, and compared with *P*-P, *P*-P-D significantly increased the *Bifidobacterium* number fermented for 48 h and significantly reduced the *Streptococcus* number fermented for 24 h. In this line, the *in vitro* digestion enhances the beneficial effects of microalgae extracts on human fecal microbiota regulation. Some previous related studies pointed out that the digestive enzymes, bile salts, and pH during digestion can affect physicochemical properties, such as chemical composition, molecular weight, and chain conformation of some nutrients, such as polysaccharides, proteins, carbohydrates in microalgae extracts, which can affect their effects on human gut microbiota (Yuan et al., 2020). In addition, the indigestible polysaccharide, polyphenol, etc., can interact with gut microbiota to generate energy benefiting the primary microbial

consumers, their syntrophic partners, and the host, which could explain the beneficial effects of *in vitro* digestion extracts on human gut fermentation (Han et al., 2020).

3.6 Short Chain Fatty Acids (SCFAs) production from human colonic fermentation

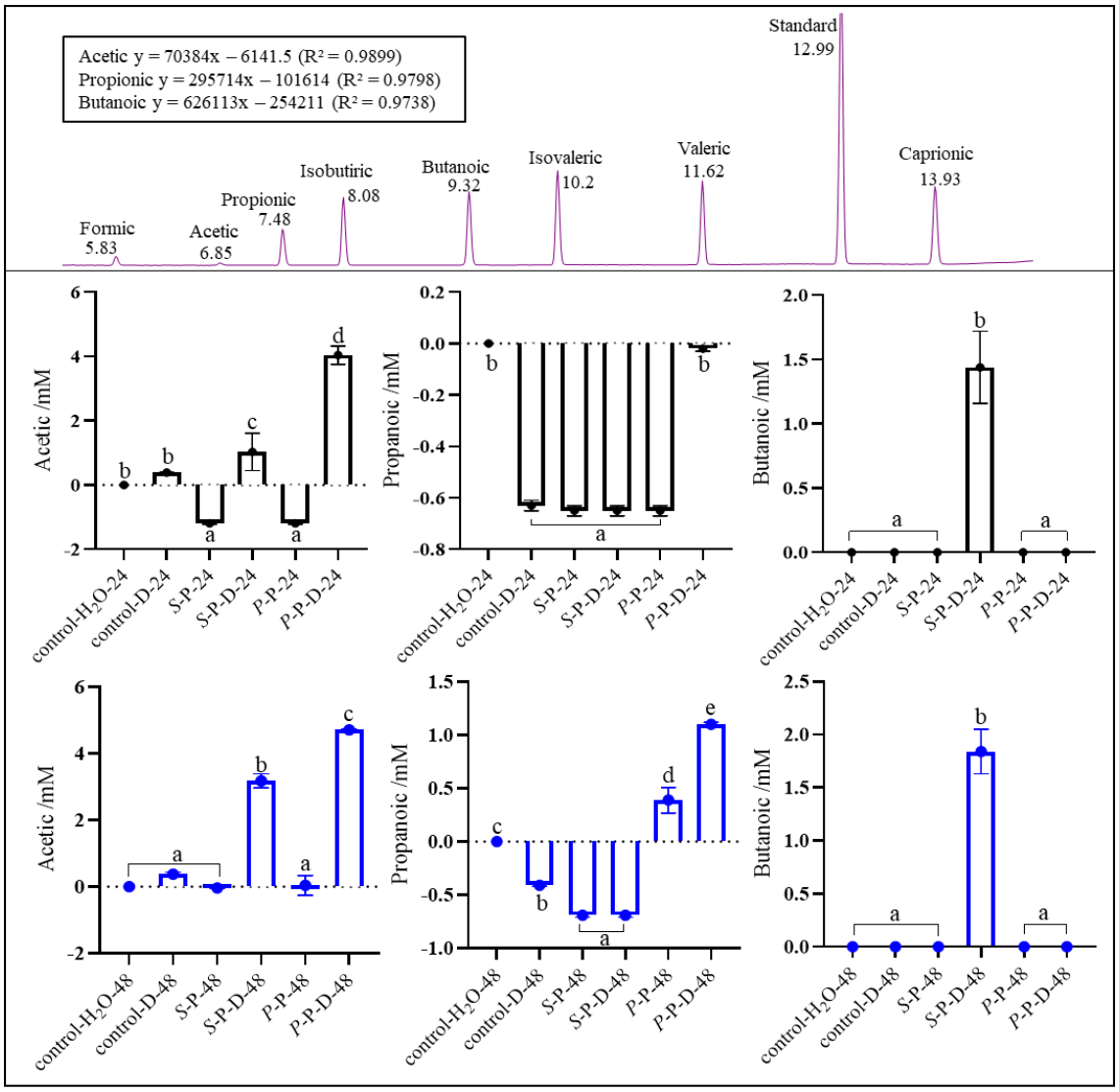

Figure 6. Short Chain Fatty Acids (SCFAs) production in fecal adult's microbiota supplemented with S-P (*Spirulina*-PLE), P-P (*P. tricornutum*-PLE), S-P-D (*Spirulina*-PLE-digest), P-P-D (*P. tricornutum*-PLE-digest) after 24 and 48 h. The baseline is set as the fatty acids amount at 0 h colonic fermentation. Control groups are without extracts added and instead of the same volume of distilled water (control-H_2O) and digest juice (control-D) respectively. Different letters mean significant differences between the 24- or 48-h fermentation groups ($p < 0.05$) while similar letters do not show significant differences ($p > 0.05$).

SCFAs are the products of the microbial activity and can contribute to host physiology and energy homeostasis (Wu et al., 2021). In this study, SCFAs levels were analysed by GC–MS, being acetic, propanoic and butanoic the dominant SCFAs in the fermentation samples. From Figure 6, it can be observed that *S*-P-D and *P*-P-D sample were able to increase acetic content, while undigested extracts had no effect on acetic acid within 48 h fermentation. While *S*-P and *S*-P-D sample had a negative impact on propanoic acid at both 24 and 48 h, both *P*-P extracts enhanced propanoic acid production at 48 h. *S*-P-D sample significantly increased the butanoic acid, both after 24- and 48-h fermentation. From this, both fermentation time and *in vitro* digestion can affect the levels of SCFAs.

SCFAs mainly referring to acetic acid, propionic acid and butyric acid, which highly associated with the bacterial levels during colonic fermentation (Guo et al., 2021). Combined with qPCR results, *Spirulina* and *P. tricornutum* extracts decreased most of the bacterial amount during the fermentation process, while the elapse of time and *in vitro* digestion increased the bacterial account, which resulted in the SCFAs levels variation.

Considering the beneficial effects of SCFAs, such as acetic acid is an important energy source for tissues and substrate for cholesterol synthesis (Yousi et al., 2019), propanoic can reduce the serum cholesterol level and protect against diet- induced obesity, also benefit the tissue insulin sensitivity (Fu et al., 2019), butanoic is important as an energy source for colonic epithelial cells and can also alleviate insulin resistance and diabetes (Lin et al., 2012). Therefore, it can be concluded that the *in vitro* digestion can promote the beneficial effects of microalgae extracts because of the increased number of SCFAs. Overall, microalgae extracts have potential benefits to in terms of the effects on SCFAs. The raw material in our study is microalgae extracts, which contain a wide variety of nutrients and bioactive compounds. Therefore, to clarify the specific compounds of microalgae that perform beneficial functions, it is necessary to explore the health effects

of pure compounds, such as polysaccharides, polyphenols, peptides, etc., which is meaningful for the use of microalgae resources in the future.

4. Conclusions

In this study, PLE showed an excellent extraction efficiency in terms of recovering protein, carbohydrates and phenolics from *Spirulina*, *Chlorella* and *P. tricornutum*. From the perspective of antiinflammatory and microbial growth curves, the aqueous extracts of *Spirulina*, *Chlorella* and *P. tricornutum* may be suggested as potential sources of natural antiinflammatory agents and antimicrobials for the prevention, treatment and control of bacterial infections and stimulation of probiotic activity. Human fecal microbiota fermentation can be affected by *Spirulina* and *P. tricornutum* extracts, and after the gastrointestinal digestion, microalgae extracts showed more obvious benefits to the microbiota according to the results of 16S rRNA gene copies/μg DNA and SCFAs. Overall, the work presented here is one of the few studies that identified phenolic compounds from different microalgae and evaluated the effects of *in vitro* digestion on the healthy properties of the microalgae nutrients on human gut microbiota. In view of the diversity of microalgae biomass and the uniqueness of its specific bioactive substance functions, more research is needed to be carried out in this line in the future.

CRediT authorship contribution statement

Jianjun Zhou: Conceptualization, Methodology, Formal analysis, Data curation, Software, Writing – original draft. Min Wang: Conceptualization, Methodology, Formal analysis, Data curation, Software, Writing – original draft. Software. Christine Bäuerl: Methodology, Formal analysis, Data curation, Software, Writing – review and editing. Erika Cortés-Macías Methodology, Formal analysis, Writing – review and editing. Joaquim Calvo-Lerma: Methodology, Formal analysis, Writing – review and editing, Maria Carmen Collado: Conceptualization, Investigation, Validation, Project administration, Funding acquisition. Supervision, Writing – original draft, Writing –

review and editing. Francisco J. Barba: Conceptualization, Investigation, Validation, Project administration, Funding acquisition. Supervision, Writing – original draft, Writing – review and editing.

Declaration of Competing Interest

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

Acknowledgements

This research was funded by the University of Valencia through the projects OTR2021-21736INVES and OTR2021-21570INVES supported by the University of Vigo. Moreover, it was partially funded by the EU Commission and BBI-JU Horizon H2020, through the AQUABIOPRO-FIT project (Aquaculture and agriculture biomass side stream proteins and bioactives for feed, fitness and health promoting nutritional supplements) grant number 790956. Jianjun Zhou and Min Wang were supported by a PhD fellowship from the China Scholarship Council (CSC) (No. 201908420246 and No. 201908420245, respectively). We also thank the Spanish Ministry of Science and Innovation for the Juan de la Cierva Formación grant awarded to Joaquim Calvo-Lerma (Ref. FJC2019-041730-I). IATA-CSIC is a Centre of Excellence Severo Ochoa (CEX2021-001189-S MCIN/AEI/10.13039/ 501100011033) funded by MICIN/AEI/FEDER, UE.

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4.6 Effects of *Laminaria japonica* polysaccharides on gelatinization properties and long-term retrogradation of wheat starch

Journal: *Food Hydrocolloids,* 2022, 133, 107908; https://doi.org/10.1016/j.foodhyd.2022.107908.

Effects of *Laminaria japonica* polysaccharides on gelatinization properties and long-term retrogradation of wheat starch

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Abstract

This study investigated the effects of LJP (*Laminaria japonica* polysaccharides) on the rheological, gelatinization, and retrogradation properties of WS (wheat starch) gels. Dynamic rheological test showed that the addition of LJP decreased the storage and loss modulus. Furthermore, LJP significantly decreased the peak viscosity, breakdown, and setback value of WS. Moreover, LJP incorporation significantly reduced the WS gel hardness during storage. SEM results showed that the addition of LJP improved the structure of WS gel. The short and long-range ordered structure showed that the relative crystallinity and formation of ordered structure were reduced with the addition of LJP. Otherwise, the LJP inhibited moisture migration, which was demonstrated by LF-NMR. In summary, the addition of LJP could inhibit the retrogradation of WS gel, as well the double helix structure of amylose or amylopectin was decreased, which provides a new theoretical insight to produce WS-based food study.

Keywords: *Laminaria japonica* polysaccharides, rheological, gelatinization, retrogradation

1. Introduction

Starch is one of the most abundant carbohydrates in nature, and it is the primary source of human dietary carbohydrates, providing about 70% of the human body's daily energy intake (Liu et al., 2021a). Starch is used as a gelling agent, stabilizer, thickener, and water-retaining agent in the food industry due to its renewable, natural, inexpensive, and biodegradable advantages (Ma et al., 2022). However, natural WS has problems such as low solubility and easy retrogradation after starch gelatinization, which limits its application in food, materials, and other industries (Chen, Fu, & Luo, 2015; BeMiller 2011).

In this line, improving starch properties to suit a wide range of application markets better is required. At present, the methods of starch modification mainly include physical, chemical, and biological enzyme method (Lee, Park, & Lim, 2019). Among them, the physical mixing of hydrophilic colloids such as non-starch polysaccharides and starch is a green and environmentally friendly method, which can significantly improve the gel quality, such as starch gelatinization and texture properties (Ma, Tian, Chen, Cai, & Jin, 2019; Ren et al., 2020). Starch retrogradation is very common in food processing and has a great impact on food quality, such as bread deterioration, loss of freshness, rice soup precipitation, etc. are the results of starch retrogradation. In fact, gelatinized starch during storage would become opaque and even coagulate precipitation, that is, starch retrogradation occurs. It has been reported that polysaccharides can improve the gel properties and hydrophilic properties of starch, and the polysaccharides produced industrially for food are safe, non-toxic, and inexpensive (Liu et al., 2021b). On this basis, if polysaccharide could improve the quality of starch products (texture, storage period, etc.), its application in starch products will have great potential. Therefore, it is valuable to explore more natural non-starch polysaccharides to improve WS quality.

Laminaria japonica polysaccharides (LJP), the main extract of kelp, has health effects

such as regulating blood lipids, lowering blood sugar, antiinflammatory, antioxidation, and has been widely used in the food industry (Yin et al., 2021). Studies have shown the positive effects of LJP on starch gel quality, such as the addition of LJP could reduce the apparent gelatinization viscosity by inhibiting the swelling of starch granules (Kim & BeMiller, 2012). In addition, LJP combined with stearic acid could delay the retrogradation of wheat starch and changed the crystallization mode of wheat starch retrogradation (Yu, Wang, Chen, Li, & Wang, 2018), which showed that the potential capacity of LJP in improving starch retrogradation.

Starch retrogradation can be divided into short-term retrogradation and long-term retrogradation, caused by molecular rearrangement of amylose or amylopectin under different leading role conditions during different storage periods, respectively. Recent study has focused on the effect of LJP on short-term retrogradation of starch, but in fact, starch-related foods tend to have a long shelf life, such as some commercially available bakery products lasting several weeks (Liu et al., 2020). Therefore, long-term retrogradation has an essential impact on the quality of starch products, and it is valuable to evaluate the effect of LJP on the long-term retrogradation of WS. We speculated that LJP may be a stabilizer and retrogradation inhibitor, could enhance the hydrogen bond cooperation and restrain the formation of starch double helix, thus inhibiting WS staling.

This study systemically explored the effect of LJP on the gel properties of WS, including dynamic rheological properties, gelatinization properties, and microstructure. In addition, starch relative crystallinity, gel hardness, and moisture distribution were also investigated to explore the effects of LJP on the long-term retrogradation of WS. Meanwhile, the correlation between gel properties and starch retrogradation by principal component analysis (PCA) was analyzed to comprehensively evaluate the mechanism of LJP in the long-term retrogradation of WS gels. This study provided a new direction for LJP to inhibit WS retrogradation and provided a certain research basis for the application

of LJP in WS foods.

2. Materials and methods

2.1. Materials

The WS (99% purity) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. without further purification. The amylose content, moisture content, lipid content, protein content, and ash content of WS were 24.25%, 13.05%, 0.51%, 0.72%, and 0.42%, respectively. The LJP (food-grade, 90%) was purchased from Xi'an BaiChuan Biological Technology Co., Ltd. (Xi'an, China). All other chemical reagents were analytical grade.

2.2. Preparation of retrograded starch gels

WS (6%, w/v)-LJP (0, 0.5, 1.0, 1.5%, w/w, the percentage was calculated on the basis of the WS) gels were prepared as follows. The solution was stirred at room temperature (25 ℃) for 30 min to obtain the mixture. Then, it was heated in a water bath at 95 ℃ for 30 min with constant stirring. Then the gels were stored at 4 ℃ for 0, 7, 14, 21, and 28 d.

2.3. Dynamic rheological properties

Rheological properties were analyzed according to previous study with slight modification (Jia et al., 2022). The rheological properties were measured by a DHR-2 rheometer (TA Instruments, New Castle, DE, USA) using a parallel-plate geometry with 1 mm of gap and 40 mm of diameter. The prepared starch gel was placed in the middle of the plate. In the linear viscoelastic region (1% strain), the dynamic scanning was performed at an angular frequency of 1-100 rad s^{-1}. The storage modulus (G'), loss modulus (G") of the samples were measured.

2.4. Rapid viscosity analysis (RVA)

A rapid viscosity analyzer (RVA-Super 4, Perten Instruments, Sweden) was used to investigate the viscosities of WS and WS/LJP mixtures (Samutsri & Suphantharika, 2012). The samples were evenly heated at 50 ℃ for 1 min, heated to 95 ℃ at a constant rate of 12℃/min and maintained at 95 ℃ for 2.5 min, then cooled back to 50 ℃ at the same rate

and maintained for 2 min. The rotational speed was set at 960 rad/min for the first 10 s and maintained at 160 rad/min for the rest of the test. The gelatinization curves and parameters of the samples were obtained by RVA software.

2.5. Hardness analysis

The hardness properties of WS and WS/LJP gels were evaluated by the texture analyzer (TA. XT plus, Stable Microsystems, *Shanghai*, China). The gels were placed under the TA/50 probe center for testing. The settings were referenced in our previous study (Fu et al., 2021): the pre-test rate was 1.0 mm/s, the side-center rate was 3.0 mm/s, the trigger force was 5 g, the down-pressure variable was 50%, and the interval time was 5 s. The hardness of gels was recorded, and the experiment was repeated 3 times for each group of gels.

2.6. X-ray diffraction (XRD)

The XRD method was adjusted appropriately compared with our previous methods (Fu et al., 2021). XRD patterns of lyophilized gel powders were measured by an X-ray diffractometer (XRD, Empyrean, PANalytical B.V, Netherlands) with 40 kV energy, and 40 mA current, using Cu-Kα irradiation (λ=1.54056 Å). The samples were scanned at room temperature with a scanning frequency of 4°/min ranging from 4 to 40°. The peaks and relative crystallinity of the image were analyzed by Jade 6.5 software.

2.7. Fourier-transform infrared spectroscopy (FTIR)

FTIR analysis was carried out based on previous study with slight modification (Yu, Wang, Chen, Li, & Wang, 2018). The freeze-dried gel powders were mixed with dried potassium bromide at a mass ratio of 1:100. The grinding samples were taken and pressed, then placed on the sample rack of Fourier transform infrared spectroscopy (FTIR, Thermo Nicolet NEXUS670, Thermo Fisher Science Inc., Waltham, USA). The samples were scanned over the wavenumber range from 4000 to 400 cm^{-1}. Each measurement was scanned 25 times with a resolution of 4 cm^{-1}. Spectra analysis was performed by OMNIC

and Peakfit software.

2.8. Low-field nuclear magnetic resonance (LF-NMR)

The water migration of starch gels was determined using the LF-NMR (NMI20-015V, Niumag Electric Corporation, China). The instrument was equipped with a 40 mm diameter glass tube. The relaxation curves were acquired using a CPMG pulse sequence. The water distribution (free water and bond water ratio) was analyzed referred to Yang et al. (Yang, Dhital, Shan, Zhang, & Chen, 2021).

2.9. Scanning electron microscope analysis (SEM)

The gels microstructure was analyzed according to the study carried out by Ren et al. (2021) with some minor modifications (Ren et al., 2021). The gels with different storage time were freeze-dried using a freeze dryer (FD-1A, Beijing Boyiakang Experimental Instrument Co., Ltd, China). After gold spraying, the samples were photographed using a SEM (Sigma 300, Zeiss) with the magnification of 500 ×.

2.10. Statistical analysis

The obtained data were statistically analyzed by using one-way Analysis of Variance (ANOVA), and means were compared by Duncan's multiple range test ($p < 0.05$). Statistical analyses were performed using SPSS19.0 analysis software.

3. Results and discussion

3.1. Dynamic rheological properties

In this study, dynamic rheological analysis was used to detect the shear properties and rheological behaviors of WS gels, and the main parameters, G', G", were measured. Among them, G' was used to reflect the solid-like properties and formation of network structure of WS gels, while G" was used to reflect the liquid-like properties and relative motion of linear molecules of WS gels.

The trends of G' and G" of the WS gels with frequency were shown in Figure 1. For all samples, G' was always over G", indicating a characteristic solid-gel-like behavior,

and there was no crossover in the tested angular frequency range (0.1~100 rad/s). The G' and G" values decreased as the concentration of LJP increased, which indicated that the elastic properties of gels were reduced and related to the inhibition of regeneration, probably because the interaction between LJP and amylose reduced the leaching of amylose.

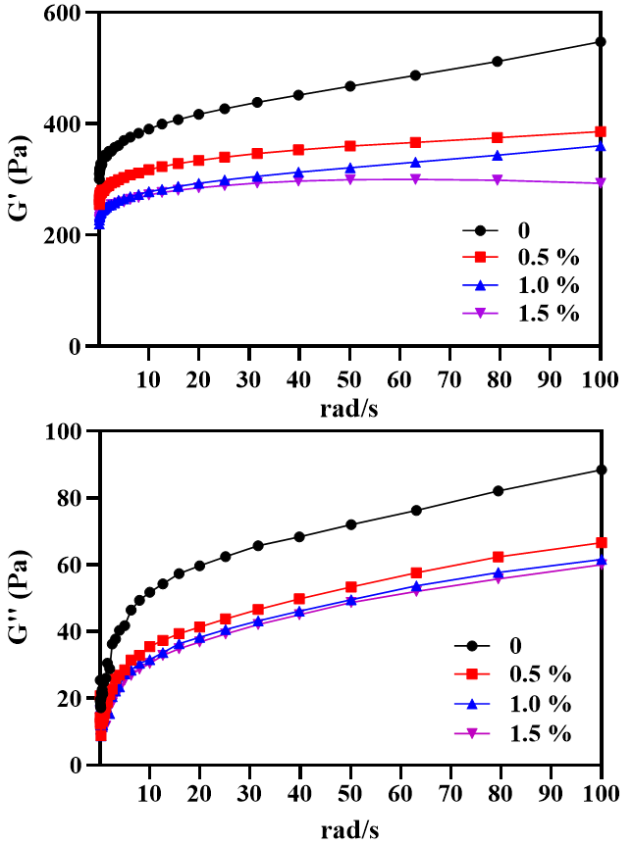

Figure 1. Frequency sweep measurement of WS gels containing different concentrations of LJP (0-1.5%, w/w).

In addition, the introduction of numerous hydroxyl groups in the system, which inhibited the crosslinking of hydrogen bonds between starch chains and thus reduced the crosslinking orderliness of the gel network. Similar study had shown that other polysaccharides, such as tamarind seed polysaccharides and cordyceps polysaccharides, could reduce the viscoelasticity of starch, which was consistent with this study (Xie et al., 2020). Furthermore, similar to the LJP effect, adding konjac glucomannan decreased G' values, while the increase of G' was observed with the addition of agar and xanthan gum.

The phase separations resulting from the thermodynamic incompatibility of agar and xanthan gum with starch are thought to result in rigid gels.

3.2. Pasting properties

Starch gelatinization, including starch granules swelling with water, destruction of starch crystal structure, and leaching of amylose, which is very important for starch products quality (Li, Cao, Cao, & Li. 2022). The gelatinization behavior of WS gels containing different concentrations (0, 0.5%, 1.0%, and 1.5%) of LJP was investigated using RVA, and the gelatinization parameters of WS gels were shown in Table 1.

Table 1. Effects of LJP on pasting properties of WS

LJP (% w/w)	PV	BD	SB	PT
0	2788±10^{d}	449±15^{d}	819±16^{d}	92.85±0.08^{a}
0.5	2728±16^{c}	376±21^{c}	793±13^{c}	93.00±0.09^{b}
1.0	2659±32^{b}	286±25^{b}	642±22^{b}	93.70±0.15^{c}
1.5	2597±22^{a}	206±26^{a}	594±25^{a}	94.40±0.25^{d}

Data are expressed as means ± SD of duplicate assays. Values followed by different superscripts in the same column present significantly different ($p < 0.05$).

Compared with the control group, the addition of 0.5% LJP slightly increased the gelatinization temperature of WS (+0.15 °C), while the addition of 1.0% LJP and 1.5% LJP significantly ($p < 0.05$) increased the gelatinization temperature of WS by 0.85 °C and 1.55 °C, respectively. Previous study has shown that the addition of polysaccharides increased the starch gelatinization temperature by competing for moisture with starch granules (Li, Wang, Chen, Liu, & Li, 2017). Therefore, in this study, the increased gelatinization temperature of WS could be attributed to the competition of the LJP with the starch for the free water in the system, causing the swelling and expansion of WS granules more difficult and a higher pasting temperature.

Peak viscosity (PV) is the viscosity of the starch granules when its volume is expanded

to the maximum level during heating process, representing the swelling degree of the starch granules during the pasting process (Ren et al., 2021). The results showed that the addition of LJP reduced the PV of WS, and the PV value decreased to 2597 when 1.5% LJP was added, which was similar to the previous reports that carboxymethyl chitosan could decrease the PV of WS (Liu et al., 2021b). However, the study by Liu et al. showed that xanthan gum and sodium alginate increased the peak starch viscosity, which was opposite to the effect of LJP on starch gel viscosity in this study (Liu et al., 2021b). From a specific comparison, in this study, when LJP was added from 0.5 to 1.5%, the peak viscosity decreased from 2788 to 2597, but when 0.1~0.5% of xanthan gum and sodium alginate were added, the peak viscosity increased from 326 and 292 to 682 and 429, respectively. It should be noted that the structure and function of different polysaccharides are specific, and their effects on starch gel will not be totally the same.

Theoretically, in the WS-LJP system, the reduction of starch PV was related to the adhesion of LJP to starch granules, thereby limiting the swelling of starch granules and inhibiting the leaching of amylose (Gul, Riar, Bala, & Sibian, 2014). Compared with LJP, similar studies have found that the low viscosity and lubricity of spherical molecular structure of soybean soluble polysaccharide could reduce the PV of starch, as well as corn fiber gum and acacia gum were able to have the same effect. Breakdown viscosity (BD) is the difference between peak viscosity and valley viscosity, which can be used to represent the degree of destruction of starch granules during gelatinization (Liu et al., 2021b). Furthermore, BD reflects the stability of starch granules against heat and shear, and generally, low BD values indicate better shear resistance (Karwasra et al., 2017). Compared with the control group, the addition of significantly ($p < 0.05$) reduced the BD value of WS, indicating LJP reduced the expansion and rupture of WS and enhanced the resistance of WS to thermal and mechanical forces.

Retrogradation is the process that starch molecules transform from a high-energy

disordered state to an ordered state, and the setback value (SB) indicates the degree of short-term retrogradation, which is more tightly related to the degree of reassociation of gelatinized starch molecules (particularly amylose) during cooling process (Li, Wang, Chen, Liu, & Li, 2017). Compared with the control group, the incorporation of LJP significantly ($p < 0.05$) reduced the SB value of WS, especially when 1.5% LJP was added, the SB was reduced by 27.4%, suggesting that the degree of starch recrystallization was reduced by LJP, potentially inhibiting the short-term retrogradation of WS. The short-term retrogradation of WS is mainly caused by the recrystallization and gelation of amylose. In the mixture gels, the hydrogen bonding which between LJP with amylose or water molecules was formed and increased with the addition of LJP. This newly formed hydrogen bonding impeded the binding of amylose-amylose to some extent and reduced the gelation degree of amylose, thus inhibiting the retrogradation of amylose.

Indeed, the addition of LJP in this study reduced the PV, BD, and SB of WS and showed a concentration-dependent trend. Lin et al. (2021) reported that high concentrations of non-starch polysaccharides formed more hydrogen bonds with starch molecules, resulting in concentration-dependent trends in PV, BD, and SB values (Lin et al., 2021). Overall, the gelatinization characteristics of WS particles were significantly affected by the interaction between LJP and starch. Moreover, it is particularly noteworthy that the addition of LJP significantly reduced the SB value, which is beneficial for starch products to delay retrogradation during storage.

3.3. Gels hardness

LJP possessed potential anti-starch retrogradation effects (SB value was significantly reduced), as well the increase of gel hardness during storage was positively correlated with starch retrogradation. Therefore, the hardness of WS gel with or without the addition of LJP after stored for 0, 7, 14, 21, and 28 days at 4 °C was further analyzed, as shown in Fig. 2. The results showed an increase in the hardness of WS gel upon the extent of storage

time, which was due to amylose reassociation and amylopectin crystallization during the cooling and storage process, causing the gel structure to become tight and stiff (Tang & Copeland, 2007).

Compared with control group, the addition of 1.0% and 1.5% LJP slightly decreased the hardness of the WS gel at 0 d (fresh gel), which was attributed to the inhibition effect of LJP on the short-term retrogradation of WS. Furthermore, the addition of 0.5~1.5% LJP significantly ($p < 0.05$) reduced the hardness of WS gel stored for 21/28 d, indicating that LJP could delay the hardness increase of WS gel during the long-term storage. Previous studies showed that the interactions between starch and xanthan could obstruct the interactions between amyloses during the long-term storage, including amylose reaggregation and amylopectin recrystallization, which could be the reason that the addition of LJP decreased the hardness of WS gel in this study (Tang, Hong, Gu, Zhang, & Cai, 2013). In addition, starch retrogradation was accompanied by water migration, and LJP molecules could compete with starch for water absorption and inhibited starch retrogradation during gel storage, thereby reducing WS gel hardness (Yu, Wang, Chen, Li, & Wang, 2018).

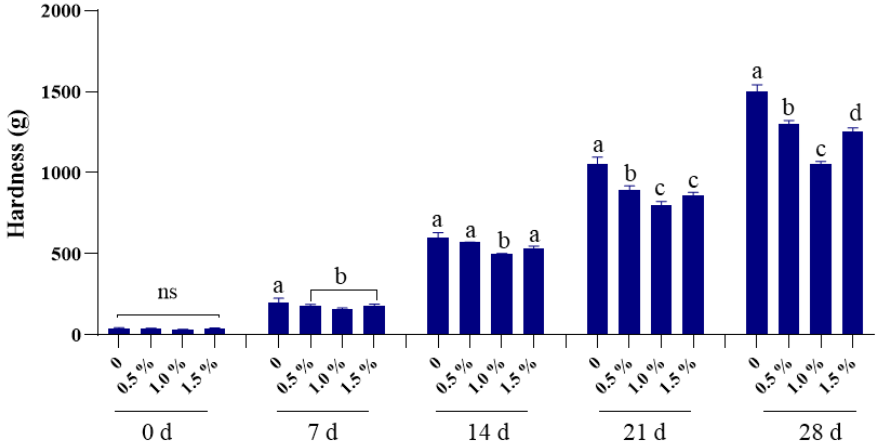

Figure 2. Effects of LJP on gels hardness (g) during different storage times (0/7/14/21/28 d). The same letter or 'ns' represents no significant difference ($p > 0.05$), and different lowercase letters represent significant difference ($p < 0.05$).

Figure 2 further showed that the decrease in the hardness of WS gel was potentially related to the amount of LJP. For example, when stored for 14 days, only adding 1.0% LJP significantly ($p < 0.05$) reduced the hardness of WS gel, and when stored for 21 days, the hardness of the WS gels in 1.0% LJP group was significantly ($p < 0.05$) lower than the 0.5% group, and with the time extended to 28 days, the hardness of the WS gel in the 1.0% group was significantly ($p < 0.05$) lower than that in the 0.5% and 1.5% groups, indicating that the addition of 1.0% LJP reduced the hardness of the WS gel to the greatest extent. The WS gelatinization results showed that adding 1.5% LJP reduced the SB value of WS to the greatest extent, while the results in this part showed that 1.0% LJP reduced the hardness of WS to the greatest extent, which indicated that the effect of LJP on the hardness of WS gels was not solely dependent on delaying WS retrogradation. That was, the addition of a small amount of LJP (0.5~1.0%) could reduce the gel hardness by inhibiting starch retrogradation or reducing the amount of retrograded starch, but when the addition amount exceeded 1.5%, it might effectively change the WS gels network structure, indicating that the addition of LJP could affect the WS gel hardness through inhibiting the retrogradation and changing the network structure. LJP with polyhydroxy structure may compete with starch to form hydrogen bonds, thus inhibiting starch swelling. This can lead to changes in gel networks and a reduction in gel hardness, as gel hardness may be related to starch expansion and water absorption.

3.4. FTIR

To further observed the effects of LJP on WS long-term retrogradation, the WS gel containing different concentrations of LJP stored for 0 d and 28d was chosen for further analysis. FTIR was used to reflect the short-range order of WS structure in this study, and the results were shown in Figure 3. A pronounced peak could be observed at 3392.3~3416.2 cm^{-1}, which was related to the stretching vibration of O-H, reflecting the interactions between H_2O and WS, as well as H_2O-H_2O interactions (free water) via

hydrogen bonds (Zhao et al., 2020). The peak at 1638.9~1644.1 cm^{-1} was due to the bending vibration of O-H of water absorbed in the amorphous regions of WS. The peaks of 1019~1020 cm^{-1} and 1080.1~1080.5 cm^{-1} were attributed to the vibration of C-H and C-O-H bending. In addition, peaks at 708.1~930.5 cm^{-1} were observed in WS-LJP gel samples, attributed to skeletal vibrations of the pyranoid ring (Karwasra et al., 2017). Compared with control group, the addition of LJP did not show any new peaks, which implied that no new covalent bonds were generated between WS and LJP, mainly due to hydrogen bonds.

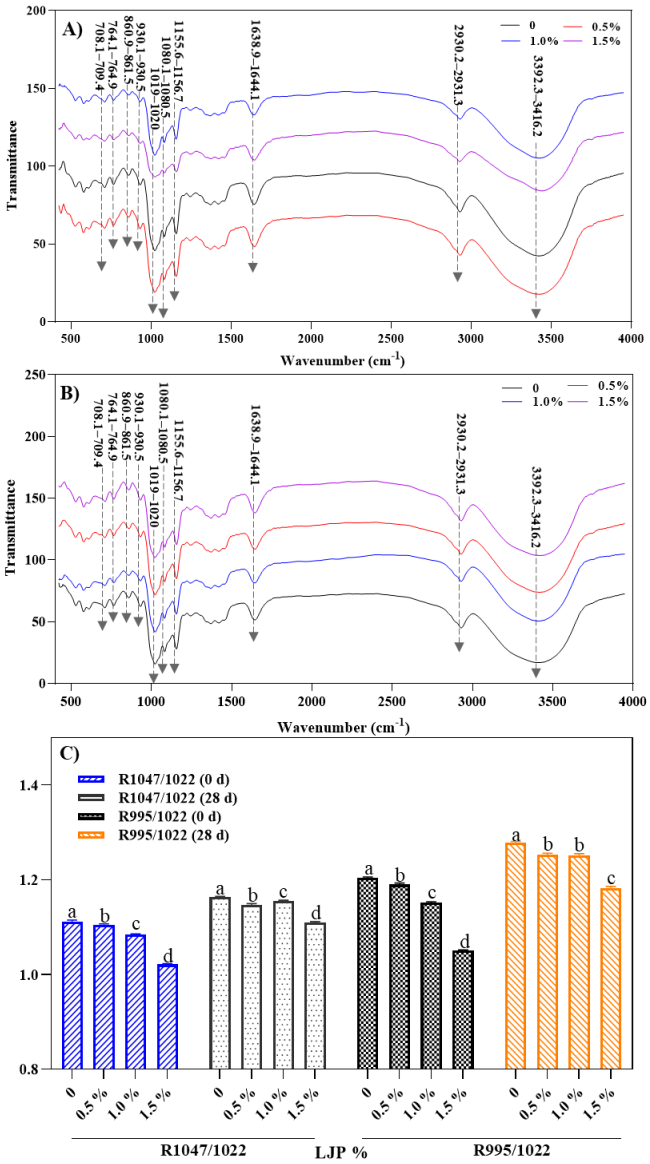

Figure 3. Effects of LJP on chemical bond of WS gels. (A) and (B) corresponds to FTIR spectrum of 0 d and 28 d samples, respectively. (C) represents the results of $R_{1047/1022}$ and

$R_{995/1022}$. The same letter or 'ns' represents no significant difference ($p > 0.05$), and different lowercase letters represent significant difference ($p < 0.05$).

According to related reports, the peaks at 1047 and 1022 cm^{-1} are ascribed to ordered (e.g., double-helices formation) and amorphous structures of starch and the peaks at 995 cm^{-1} are more related to hydrated crystalline samples (Sevenou, Hill, Farhat, & Mitchell, 2002). In addition, $R_{1047/1022}$ is generally considered to quantify the short-range order of starch structures while the ratio $R_{995/1022}$ reflects the alignment of helices in short order range (Garcia-Valle et al., 2021). $R_{1047/1022}$ and $R_{995/1022}$ were shown in Fig. 3C. Compared with fresh WS gel, the $R_{1047/1022}$ and $R_{995/1022}$ values increased when the storage time was 28 days, especially when 1.0% and 1.5% LJP were added. The increase in $R_{995/1022}$ of WS gel reached a significant level ($p < 0.05$). The results indicated that amylopectin molecules rearranged during storage of WS gels, the number of ordered structures increased, and starch gels underwent retrogradation. When 0.5~1.5% LJP was added, both $R_{1047/1022}$ and $R_{995/1022}$ were significantly reduced ($p < 0.05$) in fresh or stored for 28 days WS gels, indicating that both short-term and long-term retrogradation of WS gels were affected by LJP, and showed an inhibitory effect, which corresponded to the decreased SB value in the gelatinization properties. This may be attributed to the fact that the addition of LJP increased the disorder degree of the gel system during storage, and the introduction of hydroxyl group inhibited the molecular rearrangement of amylopectin. In addition, the reduction in $R_{995/1022}$ may be due to the loss of the double helix. Related studies attribute the reduction of the $R_{1047/1022}$ value of starch gels by additives to the interaction between the hydrocolloid and the leached amylose via hydrogen bonding or the inhibition of water loss during the retrogradation of amylopectin, thereby delaying the formation of ordered starch structures (Tang, Hong, Gu, Zhang, & Cai, 2013). In this study, FTIR further verified that LJP has potential anti-retrogradation ability, which was valuable for improving the long-term preservation quality of starch products.

3.5. X-Ray diffraction pattern

Different from FTIR, XRD can reflect long-range WS structural information (López et al., 2012). In this study, XRD was used to analyze the effect of LJP on WS crystallization during retrogradation, and the XRD patterns of WS gels containing 0~1.5% LJP stored for 0 d/28 d were shown in Figure 4.

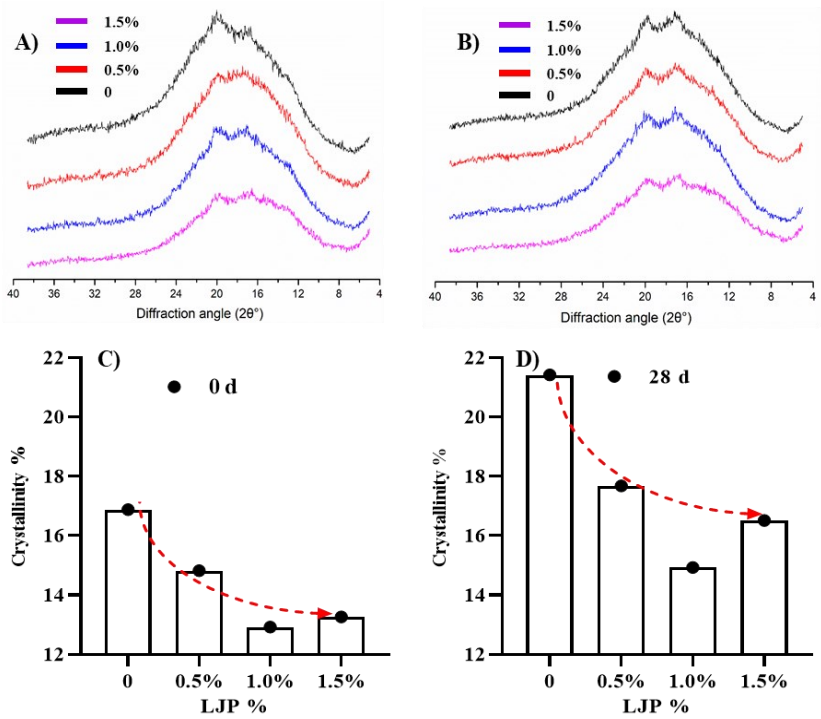

Figure 4. Effects of LJP on starch crystallinity in fresh (0 d) and storage (28 d) WS gel samples. (A) and (B) corresponds to the X-rd patterns of 0 d and 28 d samples, respectively. (C) and (D) corresponds to the crystallinity of WS gel stored for 0 d and 28 d samples, respectively.

The results in Figure 4 showed no new diffraction peaks after adding LJP, indicating that no new starch crystals were generated. However, compared with 0 d, the peak intensity at 2θ=17.5° increased after storage for 28 d, which was a typical B-type starch crystal structure. B-type starch crystallization was a double helix structure formed between amylose and amylopectin (Li, Wang, Chen, Liu, & Li, 2017), which could reflect the starch retrogradation extent. In this study, the increase in peak intensity at 2θ=17.5°

verified the retrogradation phenomenon of 28 d WS gel. Figure 4A and 4B corresponded to the XRD patterns of gelatinized WS stored for 0 d and 28 d, respectively. Both of them contained a peak at 2θ=20°, which was V-starch, indicating the presence of amylose-lipid complexes (Maphalla & Emmambux, 2016). Furthermore, the crystallinity of gels decreased with LJP, which may be due to the interaction between hydroxyl groups of LJP and amylose, thus preventing the binding of amylose with lipids and other small molecules and inhibiting the formation of V-shaped crystals.

The gelatinized starch granules were automatically rearranged and recrystallized during the cooling process, and the crystallinity could reflect the level of starch retrogradation. Figure 4C and 4D were the crystallinity of WS stored for 0 d and 28 d. Compared with WS of 0 d (Figure 4C), the crystallinity was significantly increased (Figure 4D) after 28 d of storage, which was due to the transformation of starch crystals from an amorphous state to polycrystalline state during long-term storage (Luo et al., 2017). Moreover, the addition of LJP reduced the crystallinity of WS regardless of whether it was stored for 0 d or 28 d, indicating that the addition of LJP inhibited the transformation of starch crystallization from amorphous state to polycrystalline state, which was mainly the recrystallization of amylopectin. On the other hand, some hydroxyl groups of LJP interacted with WS and water molecules through hydrogen bonding, which interfered with the interaction between starch chains, leading to the reduction of relative crystallinity of starch. Similarly, other studies also showed that polysaccharides, such as adding inulin, pullulan, could reduce the crystallinity of retrograded starch, which indicated that LJP had the same effect on inhibiting amylopectin recrystallization and starch retrogradation (Luo et al., 2017; Chen, Ren, Zhang, Tong, & Rashed, et al., 2015). However, compared with adding 0.5~1.5% LJP to inhibit starch retrogradation, the proportion of polysaccharides added was higher in these studies. For example, adding 5.0~7.5% and 15% inulin could inhibit starch retrogradation in the long-term and short-

term, respectively (Luo et al., 2017). Moreover, the addition of inulin inhibited amylose retrogradation but accelerated amylopectin retrogradation, while there was no evidence that LJP addition accelerated amylopectin retrogradation. Furthermore, related studies attributed the effects of polysaccharides reduced starch crystallinity to the interaction between polysaccharides and the side chains of amylose or amylopectin, thus delaying the formation of double-helical structures, which could be the reason for LJP decreased crystallinity of WS in this study (Niu, Zhang, Xia, Liu, & Kong, 2018).

3.6. SEM

After gelatinization, gels were fully developed during cooling process, and molecules reaggregated with each other to form a dense three-dimensional network structure. These complex structural networks can be observed by SEM to study the effect of LJP on the changes of gel microstructure.

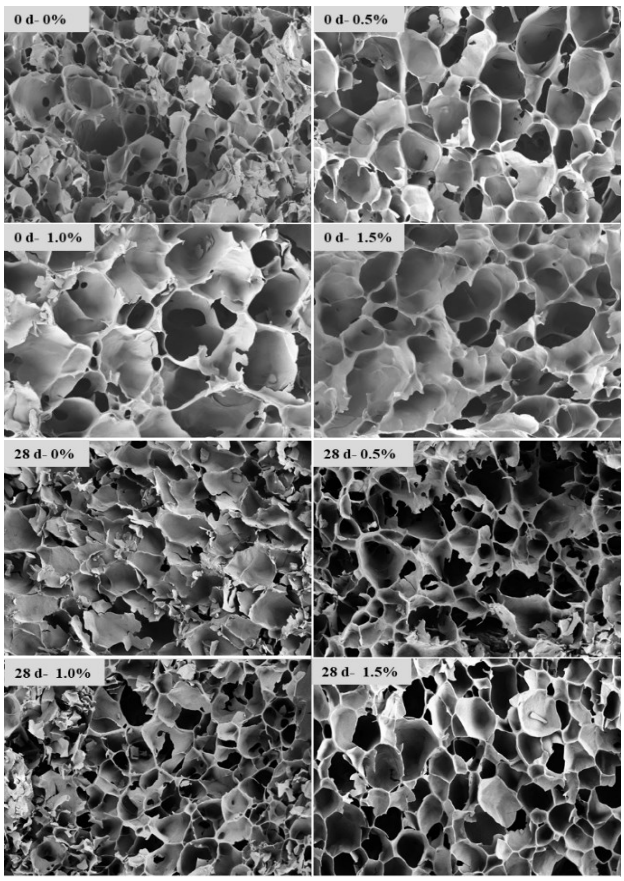

Figure 5. Effects of different concentrations of LJP (0-1.5%, w/w) on the microstructure of WS gel stored for 0 d and 28 d.

The micromorphology of freeze-dried WS gels stored at 4 °C for 0 d and 28 d were shown in Figure 5. Overall, all the gels showed a "honeycomb" network structure, which may be due to forming a dense three-dimensional network with branches in WS molecules during gelation. In addition, the gel network structure also was destroyed by the loss of water during storage to some extent, such as some flake shedding. WS gels stored for 28 d had a more compact network structure and smaller pore size than the 0 d WS gel, which was due to the rearrangement of amylose and amylopectin of gelatinized starch during long-term storage (Zhou et al., 2019). Moreover, this dense structure could increase gel hardness, which validated the conclusions in the hardness analysis.

Furthermore, the results showed that the addition of LJP changed the structure of the WS gel. When stored for 0 days, compared with the WS gel without LJP, the WS gel with 0.5~1.5% LJP added had larger pores and a more uniform pore distribution. The gel pores formed after gel cooling were related to the aggregation of starch molecules and water distribution, and LJP was rich in hydroxyl and hydrophilic groups (Fu et al., 2021), which favored amylose to form aggregates through hydrogen bonding, thereby improving the WS gel structure. On the one hand, LJP with hydrophilic groups can compete with WS for water molecules and improve the water holding capacity of gels. This explains the enlargement of gel pores during freeze-drying. On the other hand, the hydroxyl group can also interact with amylose and amylopectin to enhance intermolecular hydrogen bonding (starch-starch or starch-water), resulting in a loose matrix structure of gels.

The long-term storage (28 d) WS gels structure was mainly affected by amylopectin rearrangement, which was usually accompanied by water migration and redistribution. Gels in the control group showed apparent gel network growth and mutual coverage, indicating that a large number of WS gel retrogradation occurred after 28 days of storage. It was worth noting, adding 0.5~1.5% LJP improved the network structure of WS gels stored for 28 days, especially after adding 1.0% and 1.5% LJP, the pores of WS gels

became more uniform and neater. This result implied that LJP could affect water dynamics through hydrophilic groups during gel network formation (Donmez, Pinho, Patel, Desam, & Campanella, 2021). In addition, compared with the control group, the number of pores in the gel network increased with the addition of LJP, especially at 1.0% and 1.5%. This can be attributed to the spatial barrier effect of macromolecular polysaccharides and the hydrogen bonding cooperation between LJP and starch or water molecules to reduce the degree of gel retrogradation. A related study showed that the interaction between polysaccharides and starch molecules could promote water separation and change the number of pores in starch gels, which was consistent with the results of this study (Luo et al., 2020).

3.7. LF-NMR and multifactor analysis

Starch retrogradation is accompanied by moisture migration and redistribution, and LF-NMR is a fast and convenient method for analysing moisture distribution in food systems (Ma, Zhu, & Wang, 2019). In this study, LF-NMR was used to further analyse the water signal intensity in the WS gel during long-term storage. P_{21} and P_{22} corresponded to the ratio of bound water and free water in the WS gel system. The effect of LJP addition and storage time on the moisture distribution of WS gels as well as relaxation time was shown in Table 2.

Table 2. Effects of LJP and storage time on the moisture distribution of WS gels

LJP	0 d				28 d			
(% w/w)	T_{21}	P_{21}	T_{22}	P_{22}	T_{21}	P_{21}	T_{22}	P_{22}
0	2.01^{a}	3.379^{a}	231.0^{a}	96.621^{d}	1.75^{a}	2.806^{a}	231.0^{a}	97.194^{c}
0.5	1.75^{b}	3.623^{c}	215.2^{b}	96.377^{b}	1.52^{b}	2.982^{b}	231.0^{a}	97.018^{b}
1.0	1.75^{b}	3.650^{d}	212.2^{b}	96.350^{a}	1.15^{c}	2.999^{b}	200.9^{b}	97.001^{b}
1.5	1.95^{c}	3.488^{b}	225.0^{c}	96.512^{c}	1.15^{c}	3.228^{c}	200.9^{b}	96.772^{a}

Note: The P_{21} and P_{22} represented the ratio value of the bound water and free water to the

total water, respectively. The T_{21} and T_{22} represented the relaxation time, respectively. Values followed by different superscripts in the same column present significantly different ($p < 0.05$).

The results showed that the addition of LJP increased the P_{21} and decreased the P_{22} value of WS gel, which corresponded to the increase of bound water and decrease of free water in WS gel. A similar study showed that polysaccharides could decrease the free water ratio in starch gels due to their hydrophilicity, and their competition for water with starch molecules could increase the gelatinization temperature of starch (Ma, Zhu, & Wang, 2019), which was consistent with the results in pasting properties of this study. Starch retrogradation (rearrangement and ordering) was the reverse process of starch gelatinization (fracture and disorder), during which starch transitioned from a disordered state to a crystalline state, accompanied by changes in the free water/bound water ratio (Niu, Zhang, Xia, Liu, & Kong, 2018). During the retrogradation process, as water was incorporated into the crystal structure, starch tended to form a more stable crystalline state (Yang et al., 2021). However, the addition of LJP reduced the proportion of free water, indicating that LJP could inhibit the crystallization behaviour of starch by competing with starch molecules for water.

To explain the potential mechanism of WS gel retrogradation, this study further applied PCA (principal component analysis) to comprehensively analyse the correlation between LJP/storage time and retrogradation factors. The results were shown in Figure 6. The results showed that the two principal components, PC1 and PC2, were able to explain 75.89% and 14.93% of the variable, respectively, for a total of 90.82%. The score plot in Fig. 6A showed WS gel stored at 0 d and 28 d clustered, respectively, in the left and right sides of PC1. This indicated that storage time had a significant impact on the retrogradation of WS gels, that is, a large amount of retrogradation occurred during storage. Moreover, combined with loading plot (Figure 6B), the indexes related to starch

retrogradation ($R_{995/1022}$, crystallinity, hardness, and P22) were distributed on the right side of PC1, indicting their positive relationship with 28 d WS gel, which was in line with the law of starch retrogradation.

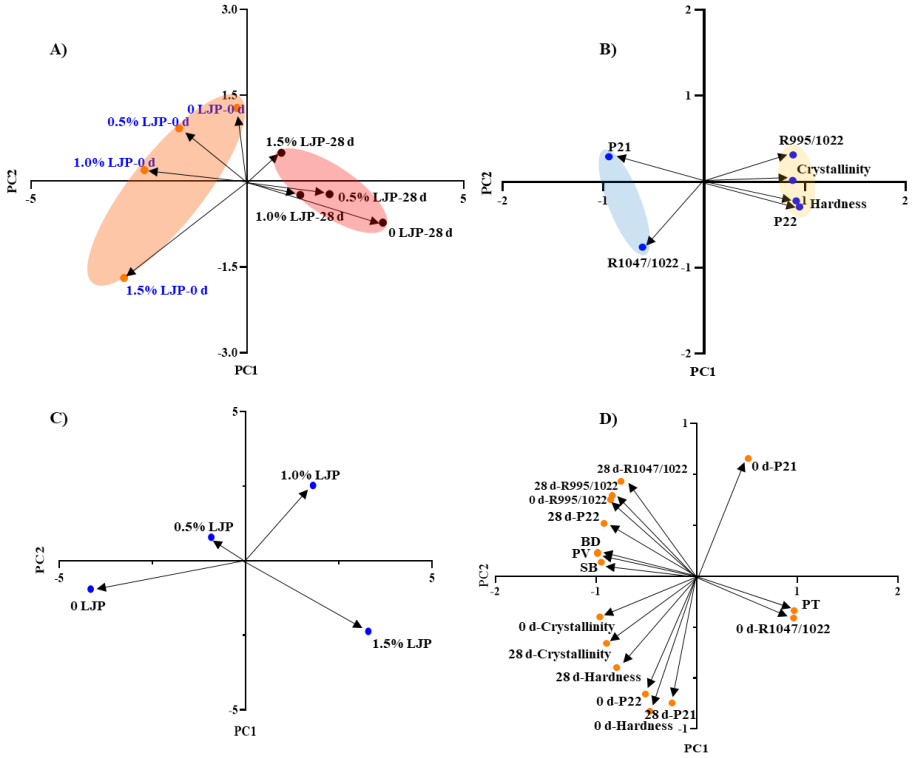

Figure 6. Principal component analysis (PCA) - Correlation analysis based on storage time (A, score plot. B, loading plot) and LJP addition (C, score plot. D, loading plot) with WS gel retrogradation (hardness, crystallinity, moisture distribution, amorphous region).

Figure 6C score plots showed that different amounts of LJP were distributed in different quadrants, and combined with the loading plot (Figure 6D), it could be found that the factors related to starch retrogradation (mainly crystallinity, P22) were distributed in the same quadrant with 0% LJP (the third quadrant), and was opposed to the 1.0% LJP group (the first quadrant), which indicated that the optimum addition amount of LJP to retard WS retrograde in this study was 1.0%, and LJP inhibited WS retrogradation mainly by affecting starch crystallinity and water distribution. In addition, the loading plots in Figure 6B and 6D both showed that the vectors of crystallinity, gel hardness, P22, and short-range order were close to each other, indicating that these indicators were positively

correlated during the retrogradation of WS gels, which was consistent with other studies (Li, Wang, Chen, Liu, & Li, 2017; Zhao et al., 2020; Luo et al., 2020).

4. Conclusion

This study showed that the addition of LJP altered the rheological, gelatinization, and retrogradation properties of WS gels. The addition of LJP improved the rheological properties and microstructure of the WS gel and decreased the hardness of the WS gel during long-term storage. FTIR and XRD results showed that hydrogen bond as the main force promoted the formation and stability of gels. The decrease in the relative crystallinity of WS gels after the addition of LJP can be attributed to the fierce competition between LJP and amylose or amylopectin for water molecules in the process of retrogradation and recrystallization. In addition, the long-term retrogradation of WS was inhibited by LJP, which delayed WS retrogradation by reducing WS rearrangement and crystallization. Multiple factor analysis showed that adding 1.0% LJP could effectively resist WS retrogradation. At this point, more hydrogen bonds can be formed with starch, thus delaying the formation of amylose or amylopectin double helix structure. In conclusion, the inhibitory effect of LJP on WS retrogradation may have broad application prospects in WS foods.

Acknowledgment

This work was supported by financial support from the Scientific Research Project of the Education Department of Hubei Province (Project No: B2020065). Authors also thank the support of the Open Project Fund of the Key Laboratory for Deep Processing of Major Grain and Oil (Wuhan Polytechnic University) (Project No: 2019GYBQGDKFB02) and the Open Project Fund of Hubei Key Laboratory for Processing and Transformation of Agricultural Products (Project No: NJZ202103).

Declaration of competing interest

The authors have no conflict of interest to declare.

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4.7 Application of omics in food color

Journal: Current Opinion in Food Science, 2022, 46: 10848. https://doi.org/10.1016/j.cofs.2022.100848.

Application of omics in food color

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Abstract

Color can reflect the food quality and influence people's sensory evaluation of food. Regulation mechanisms of the natural color of fruits, vegetables and meat products and the use of synthetic colors to improve food acceptability are effective ways to provide a consistent appearance in foods. In recent years, consumers have become more favorable for natural colors due to the increased demand for healthy foods. However, natural coloring has not been able to completely replace artificial coloring due to their poor stability and high production costs. Omics technologies, including genomics, transcriptomics, proteomics, and metabolomics, could interpret the biosynthesis, accumulation, degradation, and metabolic mechanisms of food natural colors from different perspectives. Moreover, recent advances in these technologies make it possible to protect food color and increase the production of natural colors that can be used for food additives, providing more benefits for the food industry.

Keywords: Color, genomics, transcriptomics, proteomics, metabolomics.

1. Introduction

Food colors, including natural or artificial colors, are being widely used in the food industry to enhance many beyond eye-pleasing visuals of foods such flavor, safety, and nutritional quality. Compared with artificial food colorants, natural food colorants are becoming more and more popular worldwide due to their health effects and low toxicity (Lu et al., 2021). According to statistics, the economic value of the food coloring market is expected to reach US$3.75 billion by 2022, of which natural food colorants will account for the largest share, and the global economic value of natural food colorants in 2025 will reach US$2.34 billion (Zielinski et al., 2021).

Moreover, the visual perception of the color of food by consumers usually affects the pricing and sales of food. Therefore, protecting the natural food color and adding food coloring to enhance sensory evaluation have become hot research topics. Researchers have used a variety of omics techniques to conduct extensive research on natural colors and these high-throughput methods combine different cutting-edge analysis platforms, big data output, and bioinformatics to comprehensively analyze the gene expression, protein synthesis pathways, and metabolic pathways (Ayda Ghahary & Abiri, 2021). This review summarized the development of food color and the application of omics technology in the research of different natural food colors, aiming to provide a reference for the food industry.

2. Food color classification and natural food color extraction

According to the chemical structure, natural food colors can be divided into the following categories: anthocyanins, carotenoids, chlorophyll, betalains, iridoids, curcumin, carminic acid, astaxanthin and phycocyanin, which is shown in Table 1 (Zielinski et al., 2021). Natural food color is mainly derived from plants, such as carrots, purple potatoes, grapes, etc., while animal-derived colors are mainly extracted from shrimp, salmon, scallops, etc. (Cristina et al., 2021; Ghosh, Sarkar, Das, & Chakraborty, 2021; Zhang et al., 2014; Zielinski et al., 2021).

Traditional extraction methods including Soxhlet extraction, immersion, and water distillation that are usually used to obtain food colors are simple and economical, but they have disadvantages such as the use of toxic reagents or long extraction time, among others (Luxsika, Sakamon, & Chiewchan, 2017). In order to reduce the losses caused by the extraction process, some new extraction technologies, such as ultrasonic extraction, high-voltage pulsed electric field, pressure fluid extraction, supercritical extraction, etc., have been extensively studied, increasing the yield and protecting color and pigments bioactive properties (Shahin Roohinejad et al., 2016).

Table 1. Common sources of natural food colorants

Food color	Source
anthocyanin	grape skin extract/ berry fruit juice/ carrot juices…
annatto	*Bixa orellana* L.,
astaxanthin	*Paracoccus carotinifaciens*/ *Phaffia rhodozyma*
β-carotene	Carrots/ corn endosperm
chlorophylls	alfalfa (*Medicago sativa*)
curcuminoids	turmeric (*Curcuma longa* L.)
betalains	beet (*Beta vulgaris* L.)
carminic acid	cochineal (*Dactylopius coccus*)
caramel	heating of sugars
phycocyanin	*Spirulina*

3. Current application of omics in food color research

Omics technology, from genomics, transcriptomics, proteomics to metabolomics, gradually reveals the key genetic information and protein factors related to the formation of natural food color, as well as the metabolic pathways in the process of color synthesis, accumulation, and

degradation. Since a single omics technology is usually unable to characterize the production and degradation mechanisms of natural color fully and accurately, the use of multi-omics technologies has become an important way for studying color information, as it is shown in Figure 1.

3.1 Genomics and transcriptomics

Genomics is a biological technology applied for the collective characterization and quantitative study of organism genes, and for the comparison of different genomes, with next-generation sequencing (N-GS) being the main research method (Haynes, Jimenez, Pardo, & Helyar, 2019). Transcriptomics is used to analyse all types of ribonucleic acid (RNA) molecules (including mRNA and non-coding RNA) expressed in specific cell stages. The corresponding technologies include RNA sequencing (RNA-seq), microarrays, and high-throughput real-time polymerase chain reaction (RT-PCR) (Chen, He, Xu, & Huhu Wang, 2021). In genetic informatics, transcription is the first step in gene expression and regulation and a key link in gene expression regulation, leading to the inseparability of genomics and transcriptomics in the research of food color formation.

Researchers have used genomics and transcriptomics technologies to carry out a series of studies to investigate biosynthesis mechanisms of anthocyanins and carotenoids in fruits and vegetables. Among them, the regulation mechanism of the color of fruits and vegetables by specific genes seems to be an important point of such investigations. For example, asparagus is a highly nutritious vegetable, and its common colors are purple and green. It was shown that most of the anthocyanin′ biosynthesis genes and at least 5 transcription factors are significantly different between green and purple varieties (Dong et al., 2019). Moreover, in the same study

it was also shown that anthocyanin´ biosynthesis and regulatory genes such as phenylalanine ammonia-lyase (*PAL*), cinnamate 4-hydroxylase (*C4H*), 4-coumarateCoA ligase (*4CL*), etc. are all down-regulated under darkness (Dong et al., 2019). This means that the color difference of asparagus is restricted by the expression of anthocyanin genes and the accumulation of these pigments is dependent on light, which provides useful information for the cultivation of asparagus rich in anthocyanins.

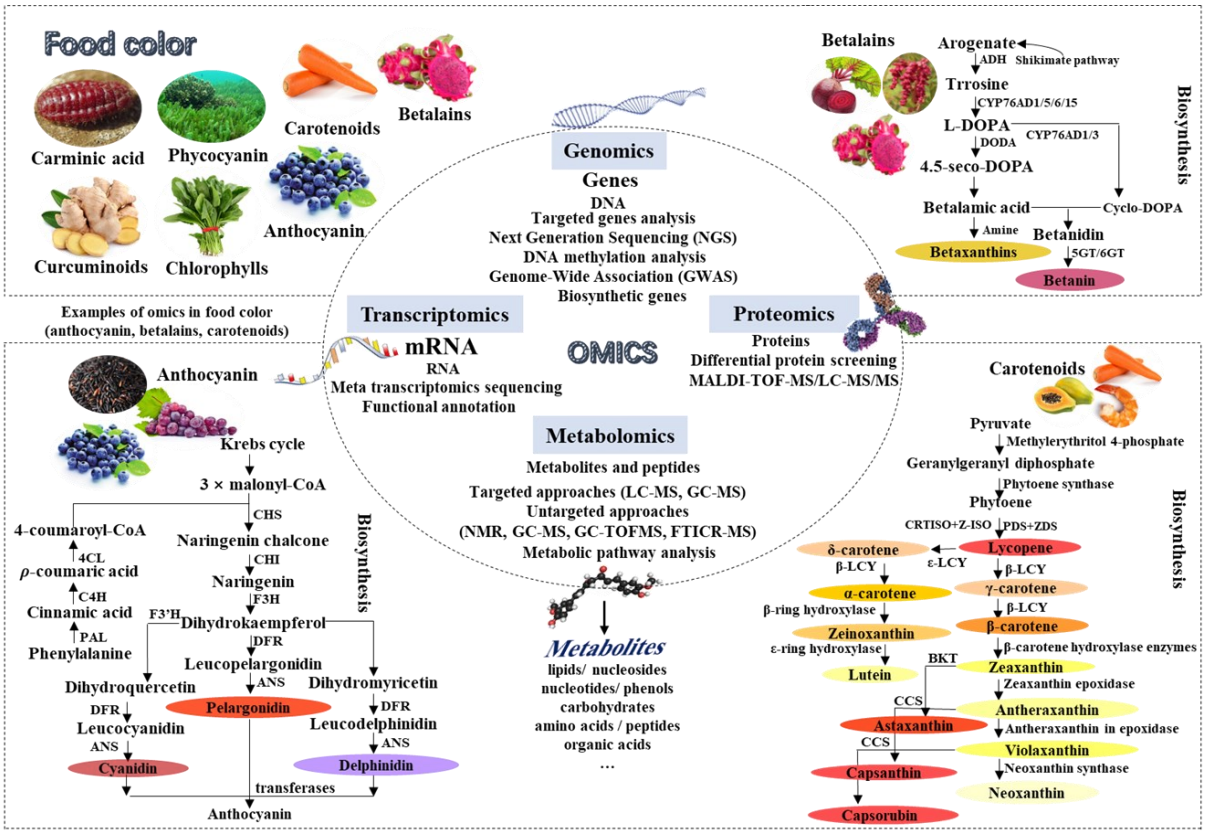

Figure 1. Genomics, transcriptomics, proteomics, and metabolomics in food color research.

Similarly, mature peppers contain a wide variety of carotenoids. For example, capsanthin is a class of carotenoids that is commonly used by the food industry as a natural color enhancer. However, the pepper color at different developmental stages is significantly different due to the accumulation of carotenoids. A recent transcriptome study showed that β-carotene hydroxylase 1 (*CHY1*) and capsanthin-capsorubin synthase (*CCS*) genes are significantly different in red and orange pepper fruits, especially *CCS* genes, presenting extremely low and

high levels in orange and red fruits, respectively, thus indicating that the difference in *CCS* gene regulation affects the carotenoids formation of pepper fruits (Li et al., 2021).

The color degradation during the storage of fruits and vegetables that leads to the reduction of product quality remains also an important problem. Some previous works in the available literature have explored the molecular mechanism of lychee peel browning at the transcription level (Qu, Li, Wang, Yu, & Zhu, 2021). The results showed that compared with fresh lychees, there were 4769 differentially expressed genes (DEGs) in lychees stored for 5 days, of which 2357 were up-regulated, 2412 were down-regulated. In addition, some DEGs related to anthocyanin synthesis were found to be up regulated. This study showed that the biosynthesis and degradation of anthocyanins simultaneously occurred during the browning of the lychee peel, which has a practical value for the preservation of lychee.

Genomics and transcriptomics technology not only can analyze the mechanism of pigment accumulation, but also can be used to integrate key color genes to increase production. In a previous research study, a strategy for the astaxanthin biosynthesis strain -'Kluyveromyces marxianus Sm23' was developed, and the key astaxanthin synthesis genes hydroxylase (Hpchyb) and β-carotene ketolase (bkt) were further integrated. Finally, 4 improved strains were obtained and the astaxanthin production increased up to 9972 μg/g DCW in a 5 L fermentor (Lin et al., 2017). Overall, it can be concluded that genomics and transcriptomics are of great significance for the analysis of food color synthesis/degradation genes, thus acquiring natural food color through the modification of biological genes. More relevant studies are shown in Table 2.

Table 2. Application of omics in food color evaluation

Food color	Omics	Methodology	Results	Ref
Pepper carotenoids	Transcriptomics	qRT-PCR, RNA-seq	the CHY1 and CCS genes were significantly different in red and orange pepper fruits	(Li et al., 2021)
Jaboticaba anthocyanin		qRT-PCR, RNA-seq	ethylene and abscisic acid related genes were positively correlated with anthocyanin accumulation	(Zhang et al., 2018)
Cherry fruits anthocyanin		qRT-PCR	light induced the expression of the key anthocyanin-related structural genes CHS, CHI, F3H, and F3′H	(Guo et al., 2018)
Barley anthocyanin	Proteomics	LC-PRM/MS, LC-MS/MS	proteins involved in amino acid biosynthesis, carbon metabolism, metabolic pathways, and phenylpropanoid biosynthesis were related to barley color	(Zhang et al., 2019)
Lycium anthocyanin		iTRAQ	differentially expressed proteins related to anthocyanin synthesis and accumulation are enriched and increased in ripening Lycium	(Zeng et al., 2020)
Purple sweet potato anthocyanin		2-DE, MALDI-TOF/TOF-MS	starch degradation may provide precursors of anthocyanin biosynthesis	(Wang et al., 2016)

Table 2. (cont.)

Food color	Omics	Methodology	Results	Ref
Peach fruit peel color	Proteomics	iTRAQ, nano LC-MS/MS	shortening the bagging time could improve the peach red color and anthocyanin content	(Zhou, Yu, & Ye, 2019)
Goat *Longissimus* thoracis color		iTRAQ, LC-MS/MS	MYLPF, MYL1/3 and IDH3B could be considered as biomarkers for meat color	(Gu et al., 2020)
Peach peel color		iTRAQ, nano LC-MS/MS	Mg-protoporphyrin IX chelatase and PAO are the key proteins for regulating chlorophyll metabolism	(Zhou et al., 2018)
Yesso scallop carotenoids		2-DE, qTOF-MS	carotenoid-rich Yesso scallops produced seven differentially expressed proteins in comparison with ordinary individuals	(Zhang et al., 2014)
Kadsura coccinea anthocyanin	Metabolomics	ESI-Q TRAP-MS/MS LIT	color difference is explained by the abundance of anthocyanin 3-O-rutin, anthocyanin 3-O-glucoside and delphinium 3-O-glucoside	(Ding et al., 2021)

Table 2. (cont.)

Food color	Omics	Methodology	Results	Ref
Wine fermentation anthocyanin		UHPLC-QE-MS	six anthocyanin secondary metabolites were identified in the main fermentation process and two in the post-fermentation process	(Ai et al., 2021)
Pepper carotenoids		UHPLC-QE Orbitrap/MS	lipids and their derivatives and phenylalanine could be the key factors differentiating red pepper	(Feng, Yu, Li, & Kan, 2022)
Curcuminoids		LC-MS/MS	the contents of numerous minor curcuminoids increased significantly after fermentation	(Xiang et al., 2020)
Zingiberaceae curcuminoids		HPLC, ESI-Q TRAP-MS/MS	*C. longa* has a greater diversity flavonoid associated with the curcumin biosynthesis	(Ye et al., 2022)
Multi-Omics application				
grape anthocyanin	metabolomics transcriptomics	HPLC-ESI-MS/MS, RNA-seq	MYBA1 upregulated in Yan73 skin and flesh at the late stages of ripening	(Xie, Liu, Chen, Zhang, & Ge, 2021)

Table 2. (cont.)

Food color	Omics	Methodology	Results	Ref
Asparagus anthocyanin		qRT-PCR, RNA-seq	exhibiting much higher anthocyanin accumulation in the peels of light-treated purple asparagus	(Dong et al., 2019)
Haematococcus astaxanthin		RNA-seq	carotenoid and astaxanthin content in reddish cells were 1.4-fold and 15-fold higher, respectively, than those in green cells	(Cristina et al., 2021)
Zucchini carotenoids		qRT-PCR, RNA-seq, LC-MS/MS	27 genes in chlorophyll biosynthesis and degradation and carotenoid synthesis were upregulated in yellow and orange zucchini	(Xu et al., 2021)
Blood orange anthocyanin	genomics transcriptomics	qRT-PCR	curing enhanced upregulation of the main transcription factor genes regulating the flavonoid pathway	(Carmona et al., 2021)
Brassica juncea anthocyanin		qRT-PCR, RNA-seq	overexpression of BjTT8 and target genes involved in late anthocyanin biosynthesis and transport might account for the increase of anthocyanin	(Zhang et al., 2020)

Table 2. (cont.)

Food color	Omics	Methodology	Results	Ref
Litchi anthocyanin	transcriptomic proteomic	RNA-seq, iTRAQ, LC-MS	anthocyanins biosynthesis pathway is functioning in litchi pericarp during storage	(Qu et al., 2021)
Monascus purpureus FAFU618 color	genomics	RT-qPCR, 2-DE UPLC-QTOF-MS/MS	Color significantly different between soluble starch and glycerol groups	(Huang et al., 2018)
Yeast astaxanthin	metabolomics	qRT-PCR, qPCR, HPLC	produce the 3S, 3′S-astaxanthin at 9972 μg/g DCW in a 5 L fermentor	(Lin et al., 2017)
Banana pulp carotenoids	metabolomics proteomics	qRT-PCR, nano LC-MS/ MS, GC-MS	upregulated of carotenogenesis-associated genes alongside elevated carbohydrate accumulation contribute to high carotenoid content	(Ding et al., 2021)

3.2 Proteomics

Proteomics is a powerful tool commonly used to study the protein composition and dynamic states of cells, tissues, and organisms. For instance, it has been utilized for analysing hundreds of proteins expressed in complex mixtures, which is of a great relevance in food research. When analysing proteins related to natural pigments, two-dimensional electrophoresis (2DE) is the most used method to obtain a global map of protein expression levels, while mass spectrometry (MS) is the preferred technique for protein identification. A study on the mechanism of anthocyanin accumulation in purple sweet potato tubers used 2DE & matrix assisted laser desorption ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF-MS) technology to repeatedly detect 800 proteins. The technique used was able to identify 50 proteins related to starch metabolism and glycolysis (Wang et al., 2016). Starch phosphorylase and glucose phosphate mutase in purple sweet potato promote the synthesis of precursors for anthocyanin synthesis, indicating that starch degradation may contribute to the accumulation of anthocyanins in purple sweet potato. Proteomics is also used to analyse the accumulation mechanism of marine mollusk color, such as carotenoids. A related study used 2DE+MS technology to compare and analyse the proteome of common scallop and carotenoid-enriched Yesso scallop. The results showed 7 different proteins, such as proteasome subunit alpha type-1 (PS), fructose-bisphosphate aldolase (FBA), etc., that may be involved in carotenoid absorption, transportation, metabolic transformation and deposition (Zhang et al., 2014).

Despite the rapid development of proteomics studies, the 2DE technology has shown some shortcomings, such as the difficulty in identifying protein molecules that are too large, too small, extremely hydrophobic, and low in abundance. To overcome these limitations, isobaric tags for relative and absolute quantitation (iTRAQ) technology are being used to analyse food color. For example, a related study identified 1848 unique

proteins from peach peel (*Prunus persica* 'Hujingmilu') using this technology, 25 of which were related to color (Zhou, Yu, & Ye, 2018). The results showed that the light-harvesting complex chlorophyll a/b binding protein (CAB) and proto-chlorophyll reductase (POR) were key functional proteins in regulating chlorophyll metabolism, while magnesium protoporphyrin IX chelator and pheophytin an oxygenase (PAO) were key functional proteins in regulating chlorophyll metabolism after harvesting. The results of this study could facilitate further understanding regarding the behaviour of key-colored compounds of peach. Moreover, iTRAQ could also be used to study changes in total proteome related to meat quality traits, such as color difference to identify meat biomarkers. A recent study used iTRAQ to analyze the effect of repeated freezing and thawing cycles on the color of goat longest pectoral muscle (Gu, Wei, Zhang, & Liu, 2020). Proteomics analysis showed that there is a potential relationship between flesh color and myosin regulatory light chain 2 (MYLPF), myosin light chain 1/3 (MYL1/3) and isocitrate dehydrogenase [NAD] subunit beta (IDH3B) proteins, which can be related to meat color biomarkers. In addition, proteomics is often combined with other omics technologies to enable a more comprehensive molecular characterization. Some of the most recent relevant studies are listed in Table 2.

3.3 Metabolomics

Metabolomics is used to analyse the response of organisms to various changes in specific internal or external conditions. Metabolomic analysis could be combined with multivariate statistics to reveal not only the activities of organisms, but also their diversity by comparing metabolic profiles (Aranha, Hoffmann, Barbieri, Rombaldi, & Chaves, 2017). A variety of analytical tools have been used in metabolomics, including nuclear magnetic resonance (NMR), liquid chromatography-mass spectrometry (LC-MS), and gas chromatography-mass spectrometry (GC-MS), among others. (Liu et al., 2019). Metabolomics could be divided into non-targeted and targeted analysis strategies. Non-

targeted analysis is the most commonly used method in MS-based metabolomics research, and its analytes are unknown (Xiang et al., 2020). For example, during the wine making process, the composition of anthocyanins is modified, leading to changes in the sensory quality of wine, including color, aroma, taste, and mouthfeel, but the metabolites related to fermentation are unknown (Han et al., 2019). A recent study established an ultra-high performance liquid chromatography system coupled with the Q-Exactive mass spectrometry method (UHPLC-QE-MS) by using untargeted metabolite profiling in combination with multivariate statistical tools to analyse wine fermentation metabolites. The study identified a total of 154 metabolic pathways involving 99 different metabolites, including lipids, nucleosides and nucleotides, phenols, etc. The results showed that the metabolism of anthocyanins mainly occurs in the main fermentation process as 6 secondary metabolites of anthocyanins were identified (Ai, Wu, Battino, Bai, & Tian, 2021). Unlike non-targeted metabolomics, targeted metabolomics is used to analyse known metabolites of food color. For example, a study based on liquid chromatography quadrupole time of flight mass spectrometry (LCQTOF-MS/MS) analysis used targeted metabolomics to comprehensively characterize 115 curcumin molecules in crude and fermented turmeric. The results showed that the content of predominant curcumin forms in the fermented samples decreased while the amount of the minor curcumin molecules increased. The findings of this study could be helpful to understand the potential molecular mechanisms involved in fermented turmeric and screen curcuminoids with better bioavailability (Xiang et al., 2020). Moreover, metabolomics is often used together with transcriptomics and proteomics to identify the relationship between metabolites´ changes and discoloration as well as to analyse the differences in color metabolites between different species, thus providing valuable ideas for preventing discoloration of fruits, vegetables, meat products, etc. Some related research studies are shown in Table 2.

4. Conclusion and future perspectives

Food natural colors are preferred by consumers because of their health benefits, hence the increasingly general trend to replace artificial colors with natural ones. Omics technology is being extensively used in recent years for research on the retention and production of food color, offering many benefits for the food color protection. However, the entry of natural food color additives into the market is still limited due to some obstacles. With the development of omics and fermentation engineering, exploring new microbial strains to produce microbial color has great potential in the commercial production of food color. Moreover, the use of systems biology to integrate all omics, mathematical models, artificial intelligence, and machine vision will have prospects in the food coloring industry to improve food quality, food nutritional value and food safety.

Acknowledgements

Francisco J. Barba is member of the CYTED network "P320RT0186 - Aprovechamiento sostenible de recursos biomasicos vegetales iberoamericanos en cosmética (BIOLATES). Jianjun Zhou and Min Wang were supported by a PhD fellowship from the China Scholarship Council (CSC) (No. 201908420246 and No. 201908420245, respectively.

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4.8 The application of Supercritical Fluids Technology to recover healthy valuable compounds from marine and agricultural food processing by-products: A Review

Journal: *Processes*, 2022, 9: 357. https://doi.org/ 10.3390/pr9020357.

The application of supercritical fluids technology to recover healthy valuable compounds from marine and agricultural food processing by-products: A Review

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Abstract

Food by-products contain a remarkable source of bioactive molecules with many benefits for humans; therefore, their exploitation can be an excellent opportunity for the food sector. Moreover, the revalorization of these by-products to produce value-added compounds is considered pivotal for sustainable growth based on a circular economy. Traditional extraction technologies have several drawbacks mainly related to the consumption of hazardous organic solvents, and the high temperatures maintained for long extraction periods which cause the degradation of thermolabile compounds as well as a low extraction efficiency of desired compounds. In this context, supercritical fluid extraction (SFE) has been explored as a suitable green technology for the recovery of a broad range of bioactive compounds from different types of agri-food wastes. This review describes the working principle and development of SFE technology to valorize by-products from different origin (marine, fruit, vegetable, nuts, and other plants). In addition, the potential effects of the extracted active substances on human health were also approached.

Keywords: Green extraction; supercritical fluid extraction; agro-industrial by-products; bioactive compounds; health effects

1. Introduction

During the production and processing of aquatic products, fruit, vegetables, and cereals, a huge amount of by-products are generated in the food industry. Although these by-products are rich in high-added-value biologically active substances, including proteins, oils, vitamins, and polyphenols, they are usually only employed for feed processing, biological fertilizers, etc., and even a considerable part is directly discarded (Gullón et al., 2020). This not only reduces the use value of these by-products, but also causes severe pollution problems for the environment (Gullón et al., 2020; Rico, Gullón, Alonso, & Yáñez, 2020b).

For the purpose of recycling waste and protecting the environment, researchers have evaluated the suitability of by-products generated during the food industry′s production process to recover their biologically active ingredients by various extraction methods (Ameer, Shahbaz, & Kwon, 2017; Rico et al., 2020b). Extracting nutrients and bioactive compounds from agricultural and sideline raw materials and processing them into different products is an important part of food production, whereas extraction technology is the key factor to determine the quality of the extracted compounds. Due to the shortcomings of traditional extraction technologies, such as low extraction efficiency, high temperatures, which can promote the degradation of thermolabile compounds, and residual toxic reagents, researchers have continuously explored the application of new extraction technology in the recovery of by-products in the agri-food industry over recent years (Durante et al., 2017).

Among the green technologies to extract bioactive compounds, different studies have shown that supercritical fluid extraction (SFE) technology is simple to operate, has low extraction temperature, and is non-polluting (Baldino, Della Porta, & Reverchon, 2017). SFE has been widely applied in the extraction of different nutrients and bioactive compounds from several types of food processing by-products, such as polyunsaturated fatty acids from marine fish by-products (Kuvendziev, Lisichkov, Zekovi, Marinkovski, & Musliu, 2018), carotenoids from

vegetable wastes and marine microalgae (Abrahamsson, Cunico, Andersson, Nilsson, & Turner, 2018; Matrices, 2019), as well as polyphenol antioxidant bioactive substances from fruit processing by-products (Kelly, Kelly, & O'Mahony, 2019). In addition to being used alone, SFE is also combined with other extraction technologies, such as mechanical expression, to extract phenolic substances and oils from olive kernels, and improve the extraction efficiency, further protecting the biological activity of the extracted substances (Misra et al., 2017).

Besides the physical and chemical properties of recovered extracts, it is necessary to take into account their positive impact on human health, since the main objective of their extraction is their incorporation into functional foods. Precisely, the feasibility of SFE technology is not only reflected in improving the extraction efficiency, but also has the effect of preserving the bioactivity of the recovered substances as well as to improve the functional characteristics of the extract. The low-temperature extraction conditions of supercritical fluids are very suitable for the recovery of biologically active compounds from agri-food by-products, allowing the recovery of thermolabile or easily oxidizable molecules (Fabrowska, Ibañez, & Herrero, 2016). In this sense, a recent study showed the superiority of supercritical CO_2 extraction to obtain highly bio-accessible α-mangostin from mangosteen pericarp extract in comparison with conventional extraction using ethanol (91% vs. 30%) (Pimentel-Moral et al., 2019). Other research also demonstrated that the application of SFE technology can enhance the extraction efficiency of fish oil and protect its antioxidant activity (Melgosa, Sanz, Benito-román, Illera, & Beltrán, 2019).

At this time of fast technological development, the relationship between SFE technology and the food industry will become closer in the future. From the perspective of social economic benefits, consumer′s requirements regarding the biological activity of extracted products from natural resources and their impact on human health will also increase. This review aims to summarize the application of supercritical extraction technology in the recovery of natural

active substances from food processing by-products and underutilized biological resources, and their beneficial effects on human health. The main aspects reported in this review are shown in Figure 1.

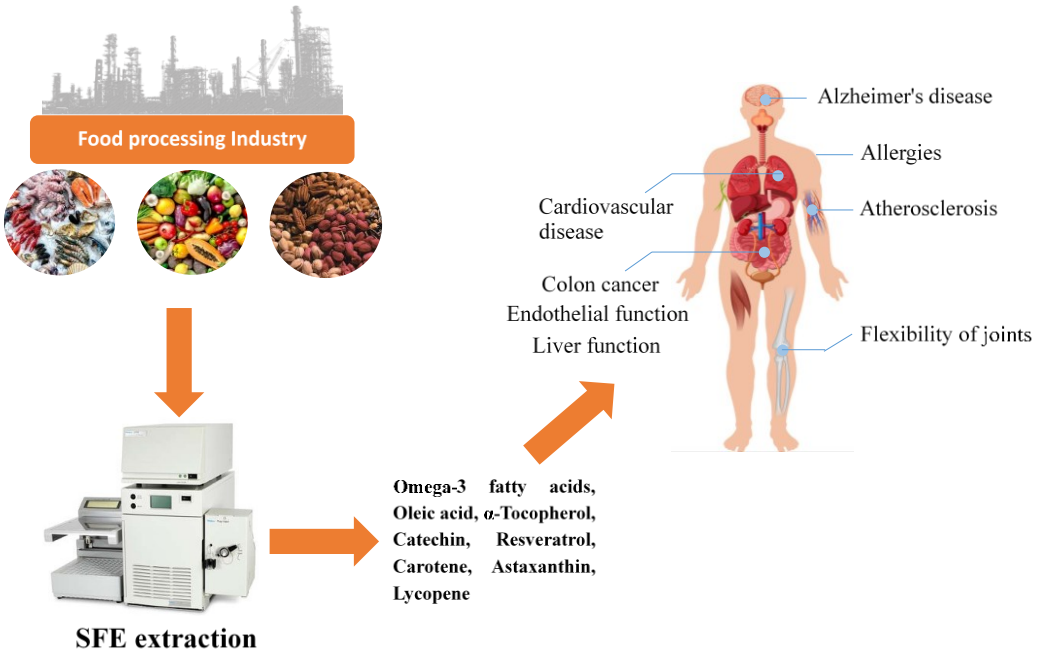

Figure 1. Application of supercritical fluid extraction (SFE) in the recovery of natural active substances from food processing by-products and their beneficial effects on human health.

2. General aspects of SFE process

Supercritical fluid (SF) is defined as any substance that presents properties of both gas and liquid above its critical temperature and pressure (Figure 2). Under these conditions, SF has lower viscosity and higher diffusivity than traditional solvents, which allows increasing of the penetration of the solvent through the solid matrix and, therefore, enhancing of the extraction yields of a broad spectrum of bio-compounds (Ameer et al., 2017; Gallego, Bueno, & Herrero, 2019). Another important characteristic of this type of fluids is the possibility of modifying its density by changing its pressure and/or temperature in a manner that facilitates the extraction of the desired compounds (Pimentel-Moral et al., 2019). Various solvents can be used as SF; however, when selecting the most suitable solvent for a particular application, aspects such as

the critical conditions of pressure and temperature, toxicity, cost, and solvation power should be considered (Pereira & Meireles, 2010). Carbon dioxide (CO_2) has become the most commonly used gas in SFE due to its multiple advantages such as its innocuous nature to human health, environmentally friendly, inexpensive, non-corrosive, readily available, and its reusability (Wrona, Rafińska, Możeński, & Buszewski, 2017). CO_2 also has interesting physicochemical properties, since it is an inert, nonpolar, nonflammable, odorless, and tasteless gas. Moreover, it has low-value critical parameters (temperature of 31.1 °C and pressure of 73.8 bar). Its moderate critical temperature makes it especially suitable for the extraction of thermolabile bioactive molecule (Pimentel-Moral et al., 2019). Another notable advantage of CO_2 is that it is gaseous at room temperature and pressure, so once the extraction is finished and by decompression of the system, the total removal of CO_2 is achieved, obtaining a solvent-free extract without the need for further expensive purification treatments (Herrero, Cifuentes, & Ibañez, 2006).

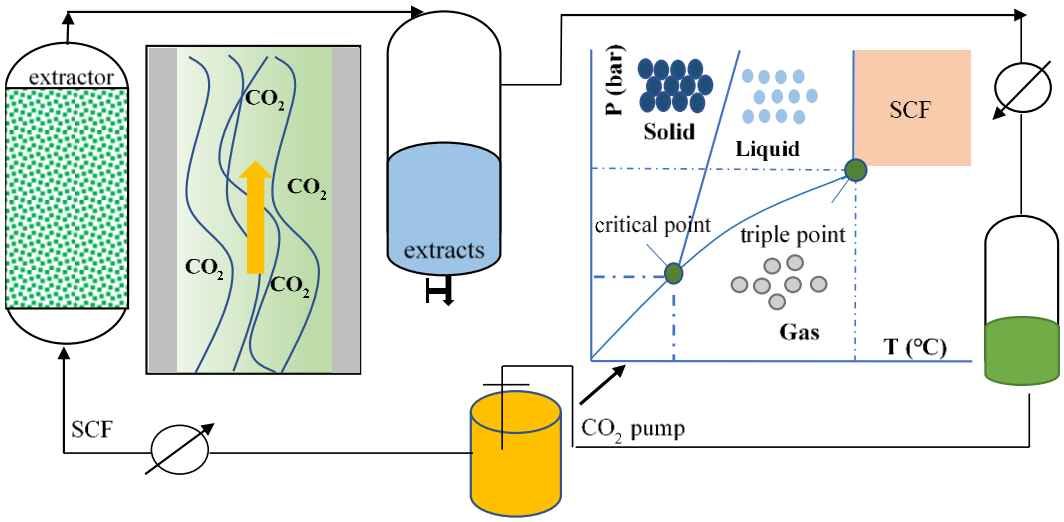

Figure 2. Schematic diagram of the supercritical fluid phase transition curve.

Nevertheless, the low polarity of CO_2 makes it less effective in extracting more polar phytochemicals from natural sources. In order to overcome this limitation, co-solvents (also called modifiers or entrainers) are used and added in small quantities that can improve the

solubilizing capacity of CO_2 and increase the extraction of more polar compounds (Wrona et al., 2017). Ethanol is commonly used as a modifier due to its low toxicity and enhanced extraction capacity of polar compounds such as polyphenols (Ameer et al., 2017).

The extraction of phytochemicals from several natural sources by SFE is a complex process that is influenced by several operating variables: process parameters (temperature and/or pressure), extraction time, factors related to mass transfer (solvent to solid ratio and particle size), percentage of modifiers, and solvent flow rate (Tita, Navarrete, Martín, & Cocero, 2021). Therefore, proper optimization of all of them is necessary in order to improve the extraction yields and selectivity of the extracted biomolecules.

In fact, a great number of reviews and original works have focused on the optimization of the SFE process to recover valuable compounds from plant matrices (Casas et al., 2010; Kehili et al., 2017; Porto & Natolino, 2017; Trabelsi et al., 2016). The next sections discuss the more relevant information derived from these studies.

3. Use of SFE Technology for Aquatic By-Products

3.1. Current Status of Consumption of Aquatic Products and Utilization of the By-Products

Aquaculture is the fastest growing food production sector, representing about 50% of the fish that is used as food worldwide (FAO, 2018). According to a recent report by the Food and Agriculture Organization of the United Nations (FAO), world fish production increased from 19 million metric tons (MMT) in 1950 to 171 MMT in 2016 (FAO, 2018). In per capita terms, fish consumption has also risen from 9.0 kg in 1961 to 20.2 kg in 2015 (Kokkali et al., 2020).

As a consequence of industrial fish processing, a great amount of waste is generated each year, which can represent up to 75% of the total weight of the catch, although it differs according to the species, size, or the processing type (Rustad, Storrø, & Slizyte, 2011). The fishery by-products include skin, scales, bones, thorns, and internal organs, among others (Khawli et al., 2019). As shown in Figure 3, these marine by-products are generally discarded

without trying to take advantage of them, causing serious environmental problems. However, several studies have indicated that they are a magnificent source of different active biomolecules with interesting health-promoting properties (Gullón et al., 2020; Khawli et al., 2019).

Figure 3. The benefits of supercritical fluid technology to recover by-products from aquatic products.

3.2. Main Biologically Active Ingredients in Aquatic By-Products

According to the characteristics of SFE, by-product extracts from aquatic industries are mainly non-polar components. Unsaturated fatty acids rich in omega-3, which is one of the main extracted compounds from aquatic fish and shrimp by-products, are recognized for their outstanding human health benefits (Atef et al., 2017). Omega-3 fatty acids, mainly containing two types of fatty acids—eicosapentaenoic (EPA) and docosahexaenoic acids (DHA)—present multiple biological activities on lipoprotein, blood pressure, heart function, cancer, endothelial function, vascular reactivity, and cardiac electrophysiology, as well as effective antiinflammatory effects (Zheng et al., 2017). Recently, Bettadahalli et al. demonstrated the retinoprotective potential of oleic acid and EPA + DHA in hyperlipidemia-induced retinal dysfunction (Bettadahalli et al., 2020). Some authors have also reported that omega-3 PUFAs

supplementation has a positive impact on the prevention and/or treatment of neurological diseases, mainly depression and anxiety (Robertson et al., 2017). An epidemiological study conducted by Kim et al. demonstrated that the consumption of long-chain omega-3 PUFAs had beneficial effects in preventing distal large bowel cancer (Kim et al., 2010). More recently, Zheng et al. reported that Antarctic krill oil rich in EPA and DHA exhibited a notable inhibitory effect on the growth of several tumor cells (U937, K562, SMMC-7721, PC-3, MDA-MB-231, HL60, and MCF-7) (Zheng et al., 2017). The role of omega-3 on the gut microbiota has also been studied by many researchers. For instance, omega-3 acids have been evaluated by their capacity to attenuate clinical colitis and colonic immunopathology in animal models through decreasing proinflammatory cytokine synthesis and improving epithelial barrier function (Balbas et al., 2015). In different clinical studies, it has been shown that supplementation with omega-3 PUFAs led to a remarkable increase in *Bifidobacterium*, *Roseburia*, and *Lactobacillus* and decreases in *Firmicutes*/*Bacteroidetes* ratio as well as levels of *Coprococcus* and *Faecalibacterium* (Balfegò et al., 2016; Watson et al., 2018).

Astaxanthin, a biologically active substance that can be obtained from shrimp and crab by-products, has a very strong antioxidant capacity, even 6000 times higher than vitamin C (Iwata et al., 2018). Astaxanthin has a high potential and promising applications in nutrition as it presents multiple beneficial effects on human health. This compound is effective to treat chronic inflammation, cardiovascular disease, diabetes, metabolic syndrome, neurodegenerative diseases, cancer, and skin and eyes diseases (Zhang, Sun, Sun, Chen, & Chen, 2014). Hence, the valorization of this waste to obtain high-added-value compounds is an interesting strategy, since it satisfies environmental concerns and improves the viability of the fishing industry.

3.3. Application of SFE in Extraction of Aquatic By-Products

Up to now, most of the research studies that have assessed the potential of SFE to recover valuable molecules from fish wastes have mainly brought into focus the extraction of polyunsaturated fatty acids and antioxidants compounds (Khawli et al., 2019; Sánchez-Camargo, Martinez-Correa, Paviani, & Cabral, 2011; Vázquez et al., 2019). Some relevant works based on SFE for the isolation of bioactive compounds from these by-products are summarized in Table 1 and commented on in the following paragraphs. A survey of the literature showed that the efficiency of the SFE process depends on several operating variables (type of supercritical fluid and CO_2 pressure, temperature, time, entrainer, and flow rate) that need to be optimized to reach high extraction rates as well as to ensure the quality of the recovered compounds. For instance, Ferdosh et al. evaluated the effects of pressure (20–40 MPa), temperature (45–65 °C), and flow rate (1–3 mL/min) on the efficient recovery of oil from tuna head using a central composite rotatable design (Ferdosh et al., 2013). The SFE process at the combined conditions of 65 °C, 40 MPa, and 3 mL/min resulted in an optimal oil yield of 35.5%. The authors also reported that the fatty acid profile was formed by 41.6, 24.7, and 26.8% of saturated, monounsaturated, and polyunsaturated fatty acids (PUFAs), respectively. Among PUFAs, 22.3% corresponded to omega-3 fatty acids. Lisichkov et al. applied an experimental design based on response surface methodology to study the effect of some working parameters on the extraction of PUFAs from common carp (*Cyprinus carpio* L.) viscera. Analysis of the 3D response surfaces revealed that the increase in pressure, CO_2 flow rate, and extraction time had a positive impact on the extraction yield; however, the temperature displaying different behaviors relied upon the operating pressure values at isobaric conditions. SFE using 400 bar, 60 °C, at a CO_2 flow rate of 0.354 kg/h for 180 min resulted in an optimal yield of PUFAs (25.24%) (Lisichkov, Kuvendziev, Zeković, & Marinkovski, 2014). Rubio-Rodríguez et al. assessed the efficiency of SFE to recover omega-3 rich oil from hake (*Merluccius capensis*, *and Merluccius paradoxus*) by-products in comparison with

conventional solvent (Rubio-rodríguez et al., 2008). Under optimized extraction conditions (25 MPa, 10 kg CO_2/h, and 40 °C), more than 96% of the total oil present in the raw material (determined by Soxhlet extraction) was extracted after 3 h. The results indicated that the employment of SFE at low temperature resulted in a higher content of total fatty acids, mainly unsaturated, than the oil extracted with hexane. This behavior can be attributed to the fact that the Soxhlet extraction is carried out at high temperatures (69 °C), which can lead to thermal degradation of fatty acids. In another work, Sahena et al. compared various SFE techniques (co-solvent extraction, soaking, and pressure swing), using different pressures (20–35 MPa) and temperatures (45–75 °C) for oil extraction from ground skin of Indian mackerel (*Rastrelliger kanagurta*) (Sahena et al., 2010). The results indicated that the oil extractability raised with pressure and temperature, achieving for all extraction modes values between 52.3 and 53.2% at 35 MPa and 75 °C, which are very close to those obtained with the Soxhlet method (53.6%). In this study, the authors also stated that the addition of small amounts of an entrainer like ethanol to CO_2 increased the solubility of the oil, improving the extraction of relatively more polar unsaturated triglycerides. Compared with traditional methods, SFE shows extraordinary advantages in fish oil extraction (Rubio-Rodríguez et al., 2012).

Table 1. Application of SFE in aquatic by-products or unexploited resources

	Aquatic By-Products	Extract Conditions	Extracts/Effects	Ref.
Fish	Carp (*Cyprinus carpio* L.) caviar and viscera	400 bars, 60 °C	MUFA and PUFA	(Kuvendziev et al., 2018)
	Hake (*Merluccius capensis–Merluccius paradoxus*) skin with some stuck muscle	25 MPa, 10 kg CO_2/h, 40 °C	96 wt.% fish oil (6% EPA, 14% DHA)	(Rubio-rodríguez et al., 2008)
	Indian mackerel skin	35 MPa and 75 °C	Fish oil 52.3/100 g sample	(Sahena et al., 2010)
	Thunnus tonggol head	40 MPa, 65 °C, and 3 mL/min	24.7% monounsaturated acids; 26.8% PUFA; 22.3% omega-3 fatty acids	(Ferdosh et al., 2013)
	Sardine (*Sardina pilchardus*) muscle	18.0 MPa, 321 K	increase in PUFA content	(Esqu et al., 1997)
	Cod liver	12% ethanol; 333 K; 300 bars	Increased the oil and squalene content	(Catchpole et al., 2000)
Shrimp	Northen shrimp	35 MPa and 40 °C	Deep red oil, rich in ω-3 PUFA; 7.8 ± 0.06% EPA and 8.0 ± 0.07% DHA	(Treyvaud et al., 2012)
	Pink shrimp (head, carapace, and tail)	300 bar, 333.15 K and 13.3 g/min CO_2 flow rate	high yield of carotenoids, astaxanthin, cryptoxanthin, and flavonoid components	(Mezzomo et al., 2013)
	redspotted shrimp waste (head, shell and tail)	43 °C/370 bars	High astaxanthin extraction yield	(Sánchez-Camargo et al., 2011)
Algae	*Aurantiochytrium* sp.	40 °C/300 bars	39.3 wt.% DHA; AOC = 1.4 mg TEAC g^{-1} extract; TPC = 2.24 mg TEAC g^{-1} extract	(Melo et al., 2020)

Table 1 (cont.)

	Aquatic By-Products	Extract Conditions	Extracts/Effects	Ref.
Algae	Freshwater green algae	40 °C/300 bars and 11.4% ethanol as co-solvent	24.90 mg fucoxanthin equivalents g^{-1} extract and 30.20 mg gallic acid equivalents g^{-1} extract	(Fabrowska et al., 2016)
	Cyanobacteria	42.5 MPa, 55 °C and 120 min	α-linolenic acid (27% of total fatty acids) and α-tocopherol (293 μg/g)	(Syrpas et al., 2018)
	Algae *Chlorella vulgaris*	40 °C, 300 bars	69% pigment recovery rate	(Gouveia, 2007)
Scallop	Pecten maximus	45 °C	Increased the phospholipids yield	(Cansell et al., 2015)

It was found that SFE under mild conditions (25 MPa and 313 K) is a suitable technology for obtaining fish oil with a high content of omega-3 PUFAs, whether compared with conventional extraction methods (cold extraction, wet reduction, enzymatic extraction). The results revealed that SFE prevented lipid oxidation and considerably reduced the levels of certain pollutants like arsenic. Similar findings were also reported by Taati et al. who indicated that SFE led to high oil extraction yields and reduced the damage of PUFAs and vitamins, resulting in a better quality product (Taati, Shabanpour, & Ojagh, 2017). The authors attributed these results to the absence of atmospheric oxygen in the SFE process and the mild temperatures used during extraction. Kuvendziev et al. (Kuvendziev et al., 2018) proved that SFE (400 bar, 60 °C, and CO_2 flow rate of 0.194 kg/h) was more selective to isolate mono- and polyunsaturated fatty acids from carp (*Cyprinus carpio* L.) by-products (viscera) than solid–liquid extraction by Soxhlet.

In addition to fish, industrial shrimp processing also produces large amounts of waste (in particular heads, shells, and tails) that correspond with approximately 50–60% of the catch (Treyvaud Amiguet et al., 2012). Several studies have reported that these by-products are an

excellent source of omega-3 PUFAs, carotenoids, and astaxanthin that can be extracted by SFE (Gullón et al., 2020; Khawli et al., 2019). In this context, Treyvaud Amiguet et al. applied SFE at moderate conditions (35 MPa, 40 °C) to extract deep red oil rich in omega-3 PUFAs from these by-products (Treyvaud Amiguet et al., 2012). In this research, conventional extraction resulted in higher oil extraction yields (206 mg oil/g for acetone and 178 mg oil/g for hexane) than SFE (137 mg oil/g). However, the extract obtained by SFE had a higher content of total fatty acids (TFA: 795 mg/g) including eicosapentaenoic acid (EPA: 78 mg/g) and docosahexaenoic acid (DHA: 79.7 mg/g), as compared to extractions with acetone (TFA: 627 mg/g; EPA: 69.9 mg/g; DHA: 66.8 mg/g) and hexane (TFA: 705 mg/g; EPA: 72 mg/g; DHA: 69.7 mg/g). In addition, SFE presented two important advantages over Soxhlet extraction: reduction in extraction time and avoidance of additional solvent removal. Similarly, Nguyen et al. also highlighted the potential of SFE to recover lipids with a high level of PUFAs and low concentration of heavy metals from the livers of Australian Rock Lobsters (*Jasus edwardsii*) compared with Soxhlet extraction (Nguyen et al., 2015). More interestingly, Mezzomo et al. analyzed the cost of the SFE to extract carotenoids from pink shrimp (*P. brasiliensis* and *P. paulensis*) by-products and their report showed this technology is suitable for the valorization of pink shrimp wastes, since it allows obtaining of an extract with high yield and quality, and with a low cost of the target product (Mezzomo et al., 2013).

In summary, we can suggest that SFE is a suitable technology to develop sustainable and efficient extraction processes compared to conventional extraction not only for waste management from the aquatic industries but also to satisfy the high demand for valuable compounds such as PUFA.

4. Use of SFE Technology for Fruits and Vegetables By-Products

4.1. Current Status of Consumption of Fruits and Vegetables and Utilization of the By-Products

Fruit demand has raised significantly during the last decade, mostly due to several epidemiological studies that have linked the dietary intake of fruits with a reduction in the incidence of cancer and cardiovascular disease mortality (Rico, Gullón, Alonso, & Yáñez, 2020a; Sagar, Pareek, Sharma, Yahia, & Lobo, 2018). According to FAO data, the global production of fruits consists of 27.4 MMT of melon, 86.1 MMT of apples, 115.7 MMT of bananas, 138.6 MMT of citrus (including oranges, lemons, limes, grapefruit, and tangerines and mandarins), 79.12 MMT of grapes, 55.38 MMT of mangoes, mangosteens, and guavas, and 28.25 MMT of pineapples (FAO, 2018). In industrial fruit processing, an important amount of waste is generated as skins, pulp, seeds, stones, and pomace, which accounts for 10% to 35% of processed material (Majerska, Michalska, & Figiel, 2019). Vegetable processing also produces a large amount of waste that is an exceptional natural source of phytochemicals, including phenolic compounds, flavonoids, carotenoids, and vitamins (Sagar et al., 2018). For example, tomato is one of the most consumed worldwide, and its processing to produce ketchup and sauce generates great amounts of by-products including peels, pulp, and seeds, which corresponds to 40% of the net weight of tomatoes (Machmudah, Winardi, Sasaki, Goto, & Kusumoto, 2012). Tomatoes present a high content of carotenoids (5.1~6.3 mg/100 g), lycopene being the main component (accounting for 70%~80% of the total carotenoids) and it is responsible for the deep-red color of ripe fruit (Vági et al., 2007). It is important to note that the discarded tomato peel contains up to five times more lycopene than pulp (Machmudah et al., 2012). Onion is also an important crop worldwide, and its annual production in Europe is estimated at about 6 MMT, generating each year approximately 500,000 tons of waste. These by-products are a high-quality source of active biomolecules such as flavonoids, phenolic acids, anthocyanins, and thiosulfates, among others (Campone et al., 2018; Gullón et al., 2020). As shown in Figure 4, there is also a huge waste of other fruit and vegetable by-products. These by-products lack value-added industrial applications and are often discarded, causing not only

serious environmental problems due to their high biodegradability, but also important economic loss.

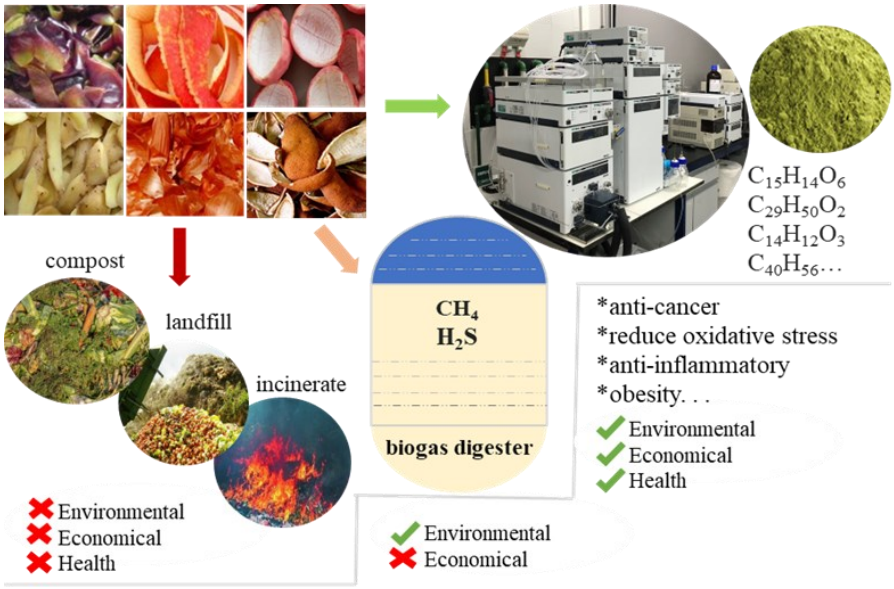

Figure 4. The benefits of supercritical fluid technology to recover by-products from fruit and vegetable products.

4.2. Main Biologically Active Ingredients in Fruit and Vegetable By-Products

At present, SFE has been applied in the extraction of fruits and vegetables by-products. There are some reported bioactive components with added value for human health, such as catechin, α-tocopherol, resveratrol, and various natural pigments. α-tocopherol is the substance with the strongest antioxidant capacity among the eight forms of vitamin E, and is widely used in the treatment of illness related to human oxidative damage (Tucker & Townsend, 2005). Besides its antioxidant properties, recent studies have confirmed the important role of α-tocopherol in other health problems, such as obesity (Hamułka, Górnicka, Sulich, & Frąckiewicz, 2019), fatty liver (Bartolini et al., 2017), heart disease (Wallert et al., 2019), lung function (Kumar et al., 2020), inter alia. In addition, the results of some animal studies have shown that α-tocopherol reverses neurodegeneration by reducing oxidative stress, which promotes brain health (Morris et al., 2015). Some studies have also revealed that α-tocopherol intake reduces

the risk of colon, pancreatic, and bladder cancer (Ranard & Erdman, 2018). Catechins are present in several fruits and herbs including tea, apples, cacaos, grapes, and berries. Catechins exhibit a plethora of pharmacological effects that have been demonstrated by both epidemiological and *in vitro* studies, and including antioxidants, antiinflammatory, antibacterial, neuroprotective, cardiac protection, and anticancer activities (Isemura, 2019). For instance, Alshatwi stated apoptotic effects of catechin on MCF-7 breast cancer cells and Shahid et al. reported its protective effect in the lungs against toxins such as benzo (a) pyrene (Alshatwi, 2010; Shahid et al., 2016). Due to their high antioxidant potential, catechins also have shown promising results in reducing neurotoxicity and oxidative stress in neurodegenerative disorders as in Alzheimer's disease (AD) (Ejaz Ahmed et al., 2013) and Parkinson's disease (PD) (Teixeira et al., 2013). Resveratrol (3,5,40-trihydroxy-trans-stilbene) is another important biomolecule that can be obtained from fruit by-products. Evidence *in vitro*, *in vivo*, and even clinical trials have shown that resveratrol possesses numerous therapeutic benefits, namely antioxidant, neuroprotective, cardioprotective, antiinflammatory, anticancer, and antidiabetic properties (Salehi et al., 2018). Related research results show that resveratrol protects different organs from ischemia-reperfusion injury by up-regulating antioxidant enzymes, reducing cell death induced by hypoxia and oxidative stress (Cheng et al., 2015; Guan et al., 2019). However, a recent study revealed that the continuous administration of resveratrol (1000 mg/day or higher) in overweight older adults increases the levels of cardiovascular risk, which indicated high doses of resveratrol may also have a negative impact on human health (Mankowski et al., 2020).

Natural pigments, including liposoluble pigments such as β-carotene, astaxanthin, lycopene, and lutein, and water-soluble pigments such as anthocyanins are also present in several by-products. β-carotene is a natural pigment abundant in plants and fruits responsible for the yellow and orange colorations of these products. β-carotene is the major carotenoid in the human diet and is the main precursor of Vitamin A (Gul et al., 2015). Several epidemiological

studies have confirmed the myriad of health benefits related to the consumption of this compound including antioxidant activity, prevention of some types of cancer, cardiovascular disease, age-related macular degeneration, cataracts, and enhancement of immunological function (Gul et al., 2015). Unlike β-carotene, lycopene is less stable because it is extremely sensitive to oxidation reactions. However, the antioxidant capacity of lycopene is much higher than other carotenoids and has superior physiological functions. Some epidemiological studies have indicated an inverse relationship between lycopene consumption and the risk of certain types of cancer (Caseiro et al., 2020). Lycopene reduces the aggression of prostate tumors and prevents lung tumorigenesis (Kelkel, Schumacher, Dicato, & Diederich, 2011; Palozza, Simone, Catalano, & Mele, 2011). Lycopene also has a key role in preventing cardiovascular diseases and arteriosclerosis, strengthening the human immune system, delaying aging, and can reverse neurobehavioral deficits. Besides its health-promoting properties, lycopene has been widely used in the food industry as a natural colorant due to its strong red color and non-toxicity (Caseiro et al., 2020).

Lutein is present in leafy green vegetables and brightly colored fruits, presenting an antioxidant activity 10 times more powerful than β-carotene and 15 times stronger than that of lycopene. Studies have shown that lutein has a positive effect on protecting eyesight, inhibiting oxygen free radical activity in the body, preventing tumor angiogenesis and cell proliferation, slowing the development of early arteriosclerosis, and decreasing the risk of diabetes (Hajizadeh-Sharafabad, Ghoreishi, Maleki, & Tarighat-Esfanjani, 2019). The daily intake of lutein is well below the recommended dose (1.7 vs. 6~14 mg/dL) necessary to exert its health-promoting effects. Therefore, the development of foods enriched in lutein recovered from natural sources can be an interesting strategy for the modern food industry.

4.3. Application of SFE in Fruit By-Products

SFE is recognized as an efficient extraction method to selectively isolate valuable molecules from fruit by-products, mainly phenolic compounds and essential oils, such as in grape by-products. Casas et al. evaluated the impact of SFE on the isolation of resveratrol from grape pomace, an industrial residue from the wine process, consisting of grape seeds, skin, and stems (Casas et al., 2010). In this case, the examined parameters were pressure (100, 400 bar) and temperature (35, 55 °C), using 5% (v/v) of ethanol as the modifier. The authors proved that the addition of co-solvent improves the solubility of resveratrol, and hence increases the extraction performance. On the other hand, Marqués et al. applied SFE to extract antioxidants (catechins, epicatechin, gallic acid, and resveratrol) from grape seeds (Marqués et al., 2013). The results showed that operating under optimal conditions (15 MPa, 40 °C, and a molar fraction of CO_2 of 0.98) from 1 kg of seeds, 51 mg of gallic acid, 49 mg of catechin, 53 mg of epicatechin, and 667 mg of resveratrol were recovered. In addition, the solids obtained after extraction showed remarkable values of total polyphenol content (TPC), ranging from 15.60 to 22.56 g equivalent gallic acid (GAE)/kg seed. This solid could be used as a source of antioxidant dietary fiber for the formulation of innovative foods, such as functional meat products. Furthermore, Farías-Campomanes et al. compared conventional extraction and supercritical CO_2 to recover phenolic compounds, including syringic acid, vanillic acid, gallic acid, and p-hydroxybenzene formic acid, protocatechuic acid, p-coumaric acid, and quercetin, from grape bagasse (Farías-campomanes et al., 2013). Although the classic Soxhlet extraction led to a higher extraction yield (10.4%) than the SFE process (5.5%), analysis of the extracts' composition evidenced that SFE containing 10% ethanol (w/w) at 313 K and 20 MPa was more efficient to extract the target compounds (23 vs. 1.8 g/kg of extract). In addition, the authors carried out an economic analysis of the process, indicating that it is possible to design an industrial plant based on SFE to recover antioxidant compounds from grape bagasse. With the purpose to enhance the recovery of desired compounds from grape waste, some researchers have proposed the

simultaneous combination of supercritical fluids with other extraction methods. Passos et al. evaluated the effect of an enzymatic pre- treatment, containing cellulase, protease, xylanase, and pectinase, on the extraction yield of grape seed oil using supercritical CO_2 (Parimi, Naga Sirisha Singh, Manjinder Kastner, James R. Das, Keshav C. Forsberg, Lennart S. Azadi, 2015). The results indicated that total extraction rate augmented in the pre-treated seeds, attaining 16.5%, as compared 11.5% with untreated seeds, which represents a rise of 44%. These better results are due to the enzymatic action that causes the degradation of the cellular structure improving the subsequent extraction of the oil by supercritical CO_2. Porto et al. applied a combined process based on ultrasound-assisted extraction followed by supercritical CO_2 to isolate polyphenols from grape marc. The finding of this study revealed that this strategy allows for an increase in the recovery of polyphenols and total antioxidant activity by 28% and 62%, respectively, in comparison with extraction using only SFE (Porto et al., 2015). Orange processing by-products have also been widely used to recover biologically active compounds by SFE. Benelli et al. compared Soxhlet, ultrasonic-assisted extraction, hydrodistillation, and SFE to obtain bioactive extracts of orange pomace (Benelli et al., 2010). The results showed that although the ultrasonic and Soxhlet extraction provided the highest yield, the extract obtained by SFE (200 bar and 50 °C) exhibited better antioxidant and antibacterial properties.

Table 2. Application of SFE in fruit by-products

Fruit by-products		Extract Conditions	Extracts/Effects	Ref.
Grape	seeds	15 MPa and 40 °C	151 mg (gallic acid), 49 mg (catechin), 53 mg (epicatechin) and 667 mg (resveratrol)/kg of seeds; TPC ranges from 15.60 to 22.56 g GAE/kg seed	(Marqués et al., 2013)
	seeds	80 bar, 6 kg/h carbon dioxide, and 20 w/w% entrainer	The highest extraction efficiency of polyphenol reached 7132 mg GAE/100 g DM	(Porto & Natolino, 2017)
	pomace	20 MPa, 40 ºC, 180 min, and supercritical CO_2 containing 10% ethanol (w/w)	Syringic acid, vanillic acid, gallic acid, p-hydroxybenzene formic acid, protocatechuic acid, p-coumaric acid, and quercetin obtained.	(Farías-campomanes et al., 2013)
	seeds	313.15 K, solvent flow rate 1.7×10^{-4} kg s^{-1}; 160, 180, and 200 bars	The maximum extraction yield was 16.5%, which was 44% higher than the 11.5% yield obtained with untreated seeds	(Passos et al., 2009)
	marc	Ultrasound (80 °C, 4 min) pretreat grape waste combined with SFE	Polyphenols (3493 mg GAE/100 g DM) and antioxidant activity (7503 mg α-tocopherol/100 g DM)	(Porto et al., 2015)
	pomace	400 bar, 35 °C, and 5% ethanol	Increased the yield of resveratrol	(Casas et al., 2010)
	pomace	300 bars, 50–60 °C	The SFE extracts presented the highest antimicrobial effectiveness compared to the other grape pomace extracts due to the presence of antimicrobial active compounds	(Oliveira et al., 2013)
	seeds	500 bar and 50 °C	Oil yields from SFE resulted in the range 10.9–15.0%	(Fiori et al., 2014)
	marc	6 kg/h CO_2 flow rate, 313.15 K and 8 MPa	The highest extraction yields were obtained (2600 mg GAE/100 g DM)	(Porto et al., 2014)

Table 2. (cont.)

	Fruit by-products	Extract Conditions	Extracts/Effects	Ref.
Orange	peels	120 min, 170 bar, and 2.7 kg/h	Obtained fatty acid esters (FAE), phenols, coumarin derivatives, terpene derivatives, isogeijerin, hexadecane, and squalene	(Trabelsi et al., 2016)
	peel	15–35 MPa, 40–60 °C, Pure ethanol,	The phenolic compounds ranged from 2.01 to 2.62%, and TPC (18–21.8 mg GAE/g)	(Espinosa-pardo et al., 2016)
	pomace	313.15 and 323.15 K and 100 to 300 bars	The main extracts were l-limonene, palmitic and oleic acids, n-butyl benzenesulfonamide, and β-sitosterol	(Benelli et al., 2010)
Passion fruit	seeds, Pulp	35 MPa and 40 °C	19.1 g oil/100 g feed	(Hatami et al., 2020)
	seed	25 MPa and 323 K	Oil obtained presented an estimation of 30% of the triacylglycerols	(Cristina, Gabrí, Tarso, & Martínez, 2019)
Apples	peels	25 MPa and 50 °C using CO_2 and ethanol (96%) in 75:25 mol ratio	The highest phenolics yield was 800 mg/100 g dry peels; the antioxidant capacity values up to 5–6 mg equivalent ascorbic acid/g extract	(Massias, Boisard, Baccaunaud, Leal, & Subra-paternault, 2015)
	pomace	30 MPa, 45 °C, 2 h, and ethanol (5%)	Antioxidant activity (5.6 ± 0.1 mg TEA/g)	(Ferrentino et al., 2018a)
	seeds	1300 bar and 336 k	The maximum solubility achieved was ~ 191 g extract/kg CO_2	(Montañés et al., 2018)
Mango	peel	25.0 MPa, 60 °C, and 15% w/w ethanol	The carotenoids extraction yield was 1.9 mg all-trans-β-carotene equivalent/g dried mango peel	(Sánchez-camargo et al., 2019)
	peel	40 °C and 10 MPa	The extracts with an antioxidant activity of 851.9 mol TE/g and a half inhibition concentration of DPPH radical of 90 g/mL	(Meneses et al., 2015)
	leaves	30 MPa and 323 K	obtained alkaloids, flavonoids and terpenoids	(Prado et al., 2013)

Table 2. (cont.)

	Fruit by-products	Extract Conditions	Extracts/Effects	Ref.
Guava	seed	30 MPa, 313 K, and 30 min	High content of PUFAs	(Castro-vargas et al., 2011)
Umbu	seed	15–30 MPa and 40 °C	Applying SFE and UAE as a combined process is a promising and useful tool to selectively recover hydrophilic (phenolic-rich fraction) and lipophilic compounds (oil-rich fraction) from *umbu* seeds	(Dias et al., 2019)
Blackcurrant	pomace	60 °C, 45 MPa, 120 min	Lipophilic extracts were rich in PUFAs (linoleic 46.89%, γ-linolenic 14.02%) and tocopherols (in total 2468 μg/g oil)	(Kraujalien, 2017)
Berry	raspberry seed	35 MPa, 0.4 kg/h, and 40 °C	The highest initial mass transfer rate 0.11779	(Pezo, 2020)
	rowanberry pomace	45 MPa, 60 °C, and 180 min	The recovery of total carotenoids was up to 49.7% linoleic (59%), oleic (27%), and palmitic (9%)	(Kraujalis et al., 2020)
	blueberry	40 °C, 20 MPa, and 10 mL/min	The highest antioxidant activities and phenolic contents were found in the extracts obtained with pure ethanol and ethanol + water	(Paes et al., 2014)
	cranberry pomace	42.4 MPa, 53 °C, and 158 min	Linoleic (36.58%), linolenic (32.44%), oleic (21.79%), and palmitic (4.36%) acids were obtained	(Tamkut, 2020)
	bilberry seed	20 MPa and 60 °C	The extracted bilberry seed oils exhibited high contents of vitamin E and PUFAs	(Gustinelli et al., 2018)
	elderberry pomace	53 °C, 35 MPa, 45 min	polyunsaturated linoleic (42.0%) and α-linolenic (34.1%) fatty acids	(Kitryt et al., 2020)

In a more recent study, Espinosa-Pardo et al. also investigated the extraction efficiency of SFE versus conventional Soxhlet extraction to isolate phenolic compounds from orange pomace (Espinosa-pardo et al., 2016). It was found that despite the SFE enabling the acquirement of only half of the phenolic compounds obtained by Soxhlet, it is more cost-effective in terms of energy, time, and solvent consumption, since it requires 78% less time and 10 times less ethanol than Soxhlet.

Other fruits that are consumed in large quantities, such as apples, mangoes, and passion fruits, generate by-products that have also been valorized by supercritical extraction, and the main isolated biomolecules include polyphenols, tocopherols, and carotene, among others (Ferrentino, Morozova, Mosibo, Ramezani, & Scampicchio, 2018b; Hatami et al., 2020; Sánchez-Camargo et al., 2011). Hatami et al. (Hatami et al., 2020) proposed, for the first time, a process based on the integration of SFE and supercritical adsorption (SESA) to obtain tocols from passion fruit by-products. Under optimized extraction conditions (35 MPa and 40 °C), SESA led to a yield of 19.1 g oil/100 g feed. In addition, the authors carried out the economic analysis and the results indicated that in order to obtain 1 kg of the extract, the cost of production would range from 35 to 54 USD, suggesting that the cost of raw materials (by-products and solvents) is the main factor that influences these costs. In another study, Ferrentino et al. applied SFE for the extraction of phenolics compounds from freeze-dried apple pomace (Ferrentino et al., 2018b). According to the authors, SFE operating under optimized condition (30 MPa and 45 °C) resulted in extracts with an antioxidant activity approximately 2.74 times greater than the Soxhlet extraction in a much shorter time (2 vs. 6 h). Sánchez-Camargo et al. optimized the SFE conditions for the isolation of carotenoids from mango peel using a Box–Behnken design (Sánchez-camargo et al., 2019). SFE at 25.0 MPa, 60 °C, and 15% w/w ethanol resulted in optimal carotenoids yield of 1.9 mg/g dry sample. In this study, the

authors also highlighted the potential of this extract to protect the sunflower oil against lipid oxidation, suggesting that this application could be an interesting alternative for mango peel valorization. Valuable lipophilic constituents extraction from elderberry juice processing by-products was optimized by Kitryte et al. and they reported a recovery of this fraction of 14.05 g/100 g pomace when SFE was performed under optimal conditions (53 °C, 35 MPa, 45 min) (Kitryt et al., 2020). In addition, this extract presented health-beneficial polyunsaturated linoleic (42.0%) and α-linolenic (34.1%) fatty acids. The authors also reported that the efficiency of SFE-CO_2, in terms of time and of extraction yields, was higher compared to conventional Soxhlet and maceration (up to 4% higher yield in an 8-fold shorter time).

However, compared to UAE, SFE turned out to be a less efficient technique (up to 29% lower yield in 3-fold longer time). Similarly, the use of SFE to recover bioactive compounds, mainly carotenoids and unsaturated fatty acids from raspberry, rowanberry, elderberry, and cranberry, has also been proposed. Table 2 details more studies about SFE for the recovery of several value-added molecules from fruit by-products.

4.4. Application of SFE in Vegetables By-Products

Several studies have applied SFE to obtain lycopene from tomato by-product. Kehili et al. extracted lycopene from tomato peels at a temperature of 50~80 °C, pressure of 300~500 bar, and flow rate of 3~5 g CO_2/min for 105 min (Kehili et al., 2017). The extraction performance of lycopene and carotene was between 32.0~60.9% and 28.4~58.8%, respectively, and the results suggested that only the temperature had a significant influence on the extraction process. Moreover, SFE was compared to conventional maceration extraction using different solvents (hexane, ethyl acetate, and ethanol). The authors found that SFE (400 bar, 80 °C, and 4 g CO_2/min) led to a higher lycopene recovery (728.98 mg/kg of dry tomato peels) compared to conventional

extraction (hexane: 608.94 mg/kg; ethyl acetate: 320.35 mg/kg; and ethanol: 284.53 mg/kg of dry tomato peels). In a previous study, Vági et al. evaluated the effect of the temperature and pressure on the recovery of lycopene from tomato by-products (Vági et al., 2007). The extraction efficiency was higher at a temperature of 80 °C and a pressure of 460 bar, obtaining an extract with a high concentration of lycopene (90.1%). In this study, the authors also studied the effect of different storage conditions on supercritical extraction of carotenoids from tomato by-products. The results revealed that the recovery of carotenoids from deep-frozen tomato waste was ten-fold higher in comparison with those of air-dried samples, indicating that the deep-frozen storage is the most suitable choice to guarantee a greater recovery of valuable biocompounds from tomato waste.

Extensive research has also been conducted on SFE from onion by-products. Devani et al. evaluated the effects of temperature (50~90 °C), pressure (150~450 bar), dynamic time (30~150 min), and particle size (0.4~1.2 mm) on oleoresin extraction yield, sulfur content, and pyruvate content from rotten onion waste through a central composite rotatable design (Devani et al., 2020). Under optimized extraction conditions (80 °C, 400 bar, particle size 0.53 mm for 60 min) 1.012% oleoresin, 31 g of sulfur/kg of oleoresin, and 10.41 μmol pyruvate/g fresh weight of onion were recovered. Furthermore, the authors also compared the efficiency of SFE with conventional extraction using hexane as the solvent. The results displayed that SFE permitted the yield of twice the sulfur content (responsible for the characteristics of the flavor of onion and some of the bioactivities associated with its consumption) in the oleoresin if compared to Soxhlet extraction. In another study, Campone et al. applied SFE to recover flavonoids from brown onion peels (Campone et al., 2018). The extract obtained under optimized conditions (40 °C, 100 bar, and 85% ethanol) presented high content of quercetin and quercetin derivatives. The SFE extract exhibited stronger antioxidant capacity (measured

by DPPH and ABTS) than that obtained using ultrasound-assisted extraction. Table 3 collects more studies about the extraction of valuable compounds from other vegetable processing by-products using SFE. Soybean is one of the most important leguminous crops worldwide, and nowadays, it is mainly used for the production of vegetable oil (Campone et al., 2018). During soybean oil processing, a residue called soybean cake is generated, which is used as animal feed due to its high protein content. However, important amounts of polyphenols and other biologically active ingredients remain in the solid residue after oil extraction (Alvarez et al., 2019). Several studies have shown that SFE is a good technique for the recovery of active substances from soybean by-products. Kao et al. compared the extraction efficiency of isoflavones from soybean cake using SFE and conventional solvent extraction (Kao et al., 2008). The results showed that solvent extraction enabled a higher yield of malonylglucoside and glucoside from soybean cake, while SFE operating at 350 bar and 80 °C led to a higher amount of acetylglucoside and aglycone using this same by-product. In another study, Fang et al. used SFE to concentrate tocopherols from methyl esterified oil deodorizer distillate (ME-DOD) (Shen, Fang, Gao, & Guo, 2017). The authors found that a pressure of 20 MPa led to an extract with a high content of tocopherols (>50%) and with high recovery (>80%). Taking these results into account, the authors concluded that SFE fractionation is suitable for concentrating natural tocopherols from by-products from the soybean oil refining process. Recently, Alvarez et al. studied the effectiveness of SFE and the use of ethanol as a co-solvent for the recovery of phytochemicals with antioxidant capacity from soybean oil extraction by-products (soybean expellers) (Alvarez et al., 2019). The best results in terms of yield, maximum extraction of polyphenols and flavonoids, and maximum antioxidant activity were achieved using CO_2 as solvent (5 kg CO_2/kg expeller) at 40 MPa and 35 °C using ethanol (25% w/w expeller), as modifier.

Table 3. Application of SFE in vegetables and beans by-products.

Vegetables/ Beans By-Products		Extract Conditions	Extracts/Effects	Ref.
Vegetables	Tomato peels	50~80 °C, 300~500 bar, and 3~5 g CO_2/min.	The final extraction rate of lycopene and carotene was between 32.0~60.9% and 28.4~58.8%, respectively	(Kehili et al., 2017)
	Tomato	80 °C and 460 bars	The lycopene content in extracts is 90.1%	(Vági et al., 2007)
	Tomato pomace	77.4 ± 13.7 °C, 37.3 ± 7.0 MPa, 11 ± 2.5 kg CO_2/h, and 211 ± 10.3 min	SFE acts as an efficacious system to increase fiber biodegradability (+ 64%)	(Scaglia et al., 2020)
	Tomato peel	90 °C and 40 MPa	56% of lycopene was extracted	(Machmudah et al., 2012)
	Rotten onions	80 °C, 400 bar, particle size 0.53 mm, and 60 min	The final onion oleoresin extraction rate was 1.012%, 31 g sulfur/kg oil, and 10.41 μmol pyruvate/g fresh onion	(Devani et al., 2020)
	Brown onion peels	40 °C and 100 bars	The SFE extracts showed strong antioxidant capacity	(Campone et al., 2018)
	Spinach waste	56 °C, 3.6 h, 39 MPa, and 10% ethanol	Obtained 72% lutein and 50% chlorophyll	(Derrien, et al., 2018)
	Red pepper seeds, skin leftovers and stems	60 °C, 24 MPa, 0.2–0.5 mm particle size	Obtained high-yield red pepper oil	(Romo-Hualde, 2012)
	Pumpkin	80 °C and 250 bars	Obtain carotene from pumpkin waste	(Shi et al., 2013)
	Beetroot	250 bar, 40 °C, 0.5 kg CO_2/h, and 4 h	A certain proportion of ethanol/water mixture increased the extraction efficiency of phenols	(Fabian et al., 2019)

Table 3. (cont.)

Vegetables/ Beans By-Products		Extract Conditions	Extracts/Effects	Ref.
Beans	Carrot peel	58.5 °C, 306 bar, 14.3% ethanol; 59.0 °C, 349 bar, and 15.5% ethanol	The highest carotene extraction rate (86.1%) at 59.0 °C, 349 bar, and 15.5% ethanol	(Lima et al., 2018)
	Soybean residue	40 MPa and 35 °C	Total phenol content of 10.6 and 16.0 mg GAE/100 g d.m; carotenoid content of 65.0 and 31.3 QE/100 g d.m; DPPH values of 9.7 and 12.0 μmol TE/100 g d.m, respectively	(Alvarez et al., 2019)
	Soybean oil by-products	60 °C/80 °C and 350 bars	Improve the extraction of acety glucoside and aglycone	(Kao et al., 2008)
	Soybean oil	16 MPa/313~348 K	Natural tocopherols (>50%) could be obtained	(Fang et al., 2007)
	Defatted soy hypocotyls	35 MPa, 2 h, 45°C, 5 L/min CO_2	β-glycosides, glycitin, daidzin, and genistin accounted for about 83.7% of the total isoflavones	(Yu et al., 2007)
	Lentinus edodes sing stipe	50 °C, 15~30 MPa, and 40 min	improved the color quality of the soybean oil	(Li et al., 2020)

The authors concluded that SFE is a safe and green technology to recover phytochemicals from soybean expellers. As shown in this section, the use of the SFE process presents a lot of potential for the recovery of a wide range of valuable compounds from vegetable by-products. However, optimization of extraction parameters is key not only to maximize extraction performance but also to achieve greater selectivity of the target compounds isolated from this food waste.

5. Use of SFE technology for nuts and other plant by-products industries

Edible nuts have excellent effects on human and development, enhance physical fitness, and prevent diseases. In some nut processing industries, a huge amount of by-products is

also produced. For example, tiger nuts, in the process of making "horchata", generate waste rich in oleic acid, polyunsaturated fatty acids (linoleic acid and linolenic acid), vitamins C and E (especially α-tocopherol), and minerals (such as potassium, calcium, and magnesium) (Roselló-Soto et al., 2019; Roselló-soto et al., 2019). Therefore, the recovery of these heat-sensitive substances by SFE is an interesting alternative, since the oxidation potential of the extracted solutes is reduced.

Table 4. Application of SFE in nuts by-products and other plants

By-Products		Extract Conditions	Extracts/Effects	Ref.
Nuts	Horchata	10~40 MPa, 40 °C, 2 h, and CO_2 flow 20 g/min with 30 mL ethanol	The main phenolic compound obtained by SFE was isohydroxymatrine, especially at 30 and 40 MPa	(Roselló-soto et al., 2019)
	Horchata	10~40 MPa, 40 °C, 2 h	The content of α-tocopherol after SC-CO_2 treatment was significantly higher than that of traditional extraction methods	(Roselló-Soto et al., 2019)
	Chañar almonds	60 °C and 40 MPa	Obtained high amounts of monounsaturated fatty acids and PUFAs, which were 363 ± 4 and 468 ± 13 mg/g oil, respectively	(Salinas et al., 2020)
	Colombian coffee beans waste	33.1 MPa and 35.9 °C	The main fatty acids identified were palmitic acid (46.1%), linoleic acid (32.9%), oleic acid (8.0%), stearic acid (6.6%), and arachidic acid (1.9%)	(Hurtado-benavides et al., 2016)
Other plants	Cocoa bean hulls	20 MPa and 40 °C	Pressurized liquid extraction extracted from SC-CO_2 residue had higher total phenol content and antioxidant properties, TPC from 35 to 51 mg GAE/g and EC_{50} values from 115 to 177 μg/mL, respectively	(Mazzutti et al., 2018)

Table 4. (cont.)

By-Products		Extract Conditions	Extracts/Effects	Ref.
Other plants	Jabuticaba	323 K, 20 MPa, 20% ethanol	High antioxidant compounds	(Cavalcanti et al., 2011)
	Agave salmiana	150~450 bar, 40~60 °C	Antioxidant capacity increased from 12.18 ± 1.01 to 20.91 ± 1.66 μmol TE/g; and saponins from 19.05 ± 1.67 to 61.59 ± 1.99 μg/g when used SFE + Ultrasound	(Santos-zea et al., 2019)
	Lycium barbarum	30 MPa, 45 °C, 60 min and CO_2 flow 25 kg/h	Make the extraction more efficient	(Guoliang et al., 2011)
	Euterpe edulis Mart	10 MPa and 40, 60, and 80 °C	Obtained an extract rich in anthocyanins and heat resistant phenolic compounds	(Garcia-mendoza et al., 2017)

In this context, Roselló-Soto et al. investigated the effect of SFE pressure (10–40 MPa) on the recovery of oil from "horchata" by-products (Roselló-Soto et al., 2019). Through component analysis, it was found that monounsaturated fatty acids (MUFA) were the main compounds in the by-products of "horchata", accounting for about 70% of total fatty acids. The content of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) was higher and MUFA was lower in the oil extracted at 10 MPa compared to that obtained at 20, 30, or 40 MPa. The results showed that the oil yield increased from 0.61 g/100 g to 7.36 g/100 g of "horchata" by-products applying 10 and 40 MPa, respectively. In addition, in contrast with conventional extraction, the content of α-tocopherol after SC-CO_2 treatment was significantly higher than that of the traditional method, regardless of the pressure applied. The level of polyphenols and total antioxidant activity raised with the increase in SC-CO_2 extraction pressure, and the data showed a linear correlation. Furthermore, they analysed the phenolic profile of oils, and the results revealed that the predominant compound in the oil extracted by SC-CO_2 was isohydroxymatrine, especially at 30 and 40 MPa, while 3-vinylphenol was the main phenolic compound in

the oil recovered by traditional method. Overall, the increase in SC-CO_2 extraction pressure improved both the recovery of phenolic compounds as well as the antioxidant potential and oxidation quality of the extracted oil (Roselló-soto et al., 2019).

Recently, Salinas et al. optimized SFE to extract oil from chañar almonds, a residue from "arrope" production (Salinas et al., 2020). In this study, the influence of pressure (20, 30, and 40 MPa) and temperature (40 and 60 °C) on the extraction yield and oil composition was tested. The results indicated that the best conditions to obtain a high oil yield (40 %) from chañar almonds were 60 °C and 40 MPa. With these conditions, high amounts of MUFAs (363 mg/g oil) and PUFAs (468 mg/g oil) were recovered. More recently, Mazzutti et al. combined SFE with the pressurized liquid extraction (PLE) method to extract antioxidant compounds from cocoa bean hulls (Mazzutti et al., 2018). Compared with single separation method extracts, PLE extracted from SC-CO_2 residue had higher total phenol content and antioxidant properties. TPC values ranged from 35 to 51 mg GAE/g, and EC50 (concentration that gives half-maximal response) determined by DPPH values range from 115 to 177 μg/mL. The combined application of SC-CO_2 and PLE could selectively recover biologically active extracts from cocoa bean shells, which represented a green method that was promising and could provide consumables for the cosmetics and food industries. Table 4 collects more studies about the extraction of valuable compounds from nuts and other food processing by-products using SFE.

6. Conclusions and some Technical Consideration

This review paper summarizes the application of supercritical fluid technology in the extraction of active substances with potential effects on human health from comprehensive agricultural food processing by-products. The rational use of agricultural and sideline products, using green advanced technologies, could allow for the implementation of a circular economy in the food industry in the future. New green

extraction technologies are indispensable tools in the food industry nowadays. The extraction technology represented by SFE not only improved the efficiency of food processing, but also saved energy and reduced pollution.

Despite the advantages reported in the literature for the extraction of bioactive molecules from agricultural food processing by-products using SFE, there are still some challenges to overcome. The first is related to the scaling up of this technology and a better understanding of the kinetic mechanisms involved in the extraction process to improve the process yield of the bioactive compounds. The second is regarding a deeper assessment of the interaction of these molecules with food components if they are incorporated into a food matrix as well as their bioavailability in the human body and the main mechanisms employed to exert their beneficial effect on health. Third, the solvent commonly used in supercritical fluid technology is carbon dioxide fluid, and the corresponding target substances are mainly non-polar components. Therefore, how to efficiently match the entrainer to extract more polar substances is still a topic worth discussing. Fourth, due to the density of the fluid itself, the extraction of macromolecular substances from by-products, such as proteins, is still restricted. Therefore, whether it is possible to extract substances with relatively large molecular masses through supercritical fluid technology in the future is also a challenge.

Author Contributions: Conceptualization, J.M.L. and F.J.B.; writing—original draft preparation, J.Z. and M.W.; writing—review and editing, J.Z., B.G., M.W., P.G., J.M.L. and F.J.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data sharing not applicable.

Acknowledgments: Thanks to GAIN (Axencia Galega de Innovación) for supporting this review (grant number IN607A2019/01). Jose M. Lorenzo is a member of the HealthyMeat network, funded by CYTED (ref. 119RT0568). B. Gullón would like to express their gratitude to the Spanish Ministry of Economy and Competitiveness for financial support (Grant reference RYC2018-026177-I). Jianjun Zhou was supported by a PhD fellowship from the China Scholarship Council (CSC) (No. 201908420246). Moreover, Min Wang would like to thank China Scholarship Council (CSC) for her PhD fellowship (No. 201908420245). In addition, Francisco J. Barba thanks Generalitat Valenciana for support (IDIFEDER/2018/046-Procesos innovadores de extracción y conservación: pulsos eléctricos y fluídos supercríticos) through European Union ERDF funds (European Regional Development Fund).

Conflicts of Interest: The authors declare no conflict of interest.

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4.9 Extraction of lipids from microalgae using classical and innovative approaches

Journal: *Food Chemistry*, 2022, 384: 132236. https://doi.org/10.1016/j.foodchem.2022.132236.

Extraction of lipids from microalgae using classical and innovative approaches

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Abstract

Microalgae, as a photosynthetic autotrophic organism, contain a variety of bioactive compounds, including lipids, proteins, polysaccharides, which have been applied in food, medicine, and fuel industries, among others. Microalgae are considered a good source of marine lipids due to their high content in unsaturated fatty acid (UFA) and can be used as a supplement/replacement for fish-based oil. The high concentration of docosahexaenoic (DHA) and eicosapentaenoic acids (EPA) in microalgae lipids, results in important physiological functions, such as antibacterial, antiinflammatory, and immune regulation, being also a prerequisite for its development and application. In this paper, a variety of approaches for the extraction of lipids from microalgae were reviewed, including classical and innovative approaches, being the advantages and disadvantages of these methods emphasized. Further, the effects of microalgae lipids as high value bioactive compounds in human health and their use for several applications are dealt with, aiming using green(er) and effective methods to extract lipids from microalgae, as well as develop and extend their application potential.

Keywords: Microalgae, Lipids, Extraction, Biological activity

1. Introduction

In recent years, the rapid growth of the global population has been accompanied by an increase in human demand for food, water, and various energy sources. With the continuous economic development, the sustainability of agriculture will be closely related to food safety in the next decades since more and more people pay attention to the impact of diet on health. For this, more functional foods are being developed to satisfy the people's needs. However, excessive agricultural production also threatens a sustainable development concerning climate and ecology. Therefore, it has become more urgent to reduce greenhouse gas emissions and ensure the sustainable development of resources under the premise of ensuring sufficient food supplies for the growing human population (Campi et al., 2021). Researchers have explored the bioactive compounds in a variety of animals and plants foods, which have been proven to have an impact on human health as components of functional foods (Görgüç et al., 2020).

The ocean accounts for 70% of the earth's surface and according to statistics, there are nearly 30,000 kinds of metabolites of marine organisms, which have become a treasure-house that can provide high-added-value compounds. The exploration and development of these compounds have laid the foundation for their application in for medicine, food, materials, and in other fields (Ali et al., 2021). Among these marine-derived bioactive compounds, the contribution of algae is about 30%.

Algae can be divided into macroalgae and microalgae, with macroalgae being usually termed seaweeds (Alvarez et al., 2021). In Asian countries, such as Japan and Korea, macroalgae are already used as foods on a widespread basis. Compared with macroalgae and other marine organisms (fish, shellfish and so on), the utilization rate of microalgae is relatively low in food industry and it has development potential (Yamagata, 2021).

Microalgae are protist that can perform photosynthesis, including prokaryotic

cyanobacteria and eukaryotic organisms such as green algae and grow mainly in fresh water and seawater (Mehariya et al., 2021). According to statistics, there are more than 1×10^5 to a 1×10^6 species of microalgae in nature that contain many macro and micro metabolites, including proteins, carbohydrates, lipids, phenols, and minerals. The bioactive compounds present in microalgae have been proven to have the potential to be used in medicine, food, bioenergy, and other fields (Jimenez-Lopez et al., 2021). Among them, the most abundant components are polysaccharides, such as carrageenan and fucoidans, which all show a variety of biological activities and are not degraded by enzymes in mammals. They are considered to be good dietary fibres, showing anti-tumour, anti-coagulation, and immune regulation effects (Carina et al., 2021). Proteins in microalgae are also present in a large proportion and the high content of amino acids provides another basis for application of microalgae. In addition, compounds such as phycocyanin in *Spirulina* can be used as natural food colorants. The polyphenols of microalgae also make microalgae exhibit stronger antioxidant properties (Martelli et al., 2014). In the ocean, fish is the most important source of lipids and can be used as a source of high-quality lipids. The lipids content of microalgae is lower than in fish, but due to its high content of unsaturated fatty acids (UFAs), it is considered a potential source of marine lipids. Indeed, microalgae can be an alternative source of lipids and terpenes that are usually obtained from animals, such as whales and codfish, which raises increasing ethical and environmental issues.

The lipids in microalgae include polar lipids such as phospholipids and glycolipids, and neutral lipids such as triacylglycerol and UFAs. Polar lipids are mainly found in cell membranes and organelles whereas glycerol and UFAs are used for energy storage (Lupette & Benning, 2020). The type of microalgae, light, growth environment and temperature affect the lipids contents in microalgae. However, despite these variations,

microalgae are still a source of high amount of polyunsaturated fatty acids (PUFAs) including docosahexaenoic (DHA) and eicosapentaenoic acid (EPA). These UFAs have also been proven to have antioxidant properties, prevent hypertension and showing immune regulation effects (Nascimento et al., 2020).

In addition to its high content in bioactive compounds, microalgae have other advantages that enable them to be widely used: a) microalgae grow in water, so they can save the land currently used for cultivation and relieve land pressure; b) compared with other crops, microalgae grow and reproduce extremely fast and can survive under harsher conditions; c) in agriculture production, microalgae can also be used to improve soil fertility by promoting soil nutrient cycling, which plays a positive role in crop growth (Alvarez et al., 2021). Overall, microalgae are regarded to have great normous potential further development and utilization.

Although algae resources are abundant, the current lipids extraction approaches are mostly based on animal-derived foods and for microalgae lipids still need to be further explored and summarized. Some high-efficiency and low-cost extraction methods have been explored to obtain and recover high-added value lipids in microalgae and greatly improve the utilization rate, which is also in line with the needs of sustainable development (Chen et al., 2020a).

This review aims providing an overview of classical and innovative approaches for lipids extraction from microalgae, based on existing studies, while aims to explore the effects of different approaches on its yield and biological activity, so as to develop its application potential.

2. Microalgae lipids-Extraction technology

Microalgae have thick-walled cell wall, and the release of their bioactive compounds is limited by the rigidity of the microalgae matrix. Therefore, appropriate pre-treatment and extraction methods should be selected to obtain lipids from microalgae and maintain their bioactivity becomes critically important.

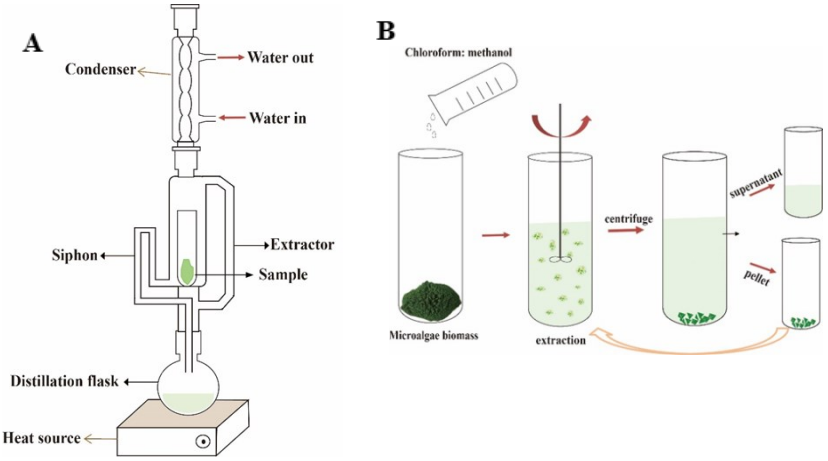

Figure 1. Schematic diagram of classical approaches: A) Soxhlet extraction, B) Folch/Bligh-Dyer extraction

For a long time, some classical approaches, including Soxhlet extraction, Folch and Bligh-Dyer extraction have been used for lipids extraction (Figure 1), but while these methods are usually simple to operate and low budget, what promotes their use, they also have disadvantages like large consumption of organic reagents and environmental pollution, in addition to the prolonged extraction times (several hours). In recent years, many innovative approaches have been explored for lipids extraction, such as supercritical fluid extraction (SFE), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) and others (Figure 2).

Table 1. Advantages and disadvantages of different extraction methods.

	Method	Advantages	Disadvantages
Traditional extraction methodologies	Soxhlet	Low cost; simple operation; high extraction rate.	Long extraction time, large reagent, and energy consumption.
	Folch	Fast, easy to handle large number of samples, the complete process is gentle.	Toxic reagents are used, which is harmful to human health and environment.
	Bligh-Dyer	Lipid extraction and separation can be achieved at the same time.	Extractive reagents are toxic and have few substitutes, the cost is high.
	Super/subcritical fluids/pressurized lipids extraction	High extraction efficiency, less use of toxic reagents and easy separation of lipids; protect bioactive compounds, reduce energy consumption and pollution.	It has selectivity to lipids of different polarity and the equipment is more expensive.
Emergent extraction methodologies	Pulsed electric fields	The operation is simple and pollution-free; processing of large number of samples.	It is necessary to control the proper electric field strength. Electric field too high may adversely affect the extraction.
	Ultrasound-assisted extraction	The temperature in the process is low, and the energy required is less. High extraction rate can be achieved in a short time.	The intensity and time of ultrasound need to be controlled to avoid negative effects.

Table 1. (cont.)

	Method	Advantages	Disadvantages
	Microwave-assisted extraction	Reduced extraction time and energy consumption, improve the extraction efficiency.	The polarity of the solvent has an impact on the extraction and is not suitable for treatment of heat sensitive substances.
	Ionic liquids	Low toxicity and high stability, with adjustable physical and chemical properties.	Possibility of pollution during synthesis.
	Enzyme-assisted extraction	Selective to substrate, pre-treatment can be completed at room temperature and pressure to reduce energy consumption.	The price of enzyme preparation is high, it is necessary to optimize the conditions to get the highest extraction rate.

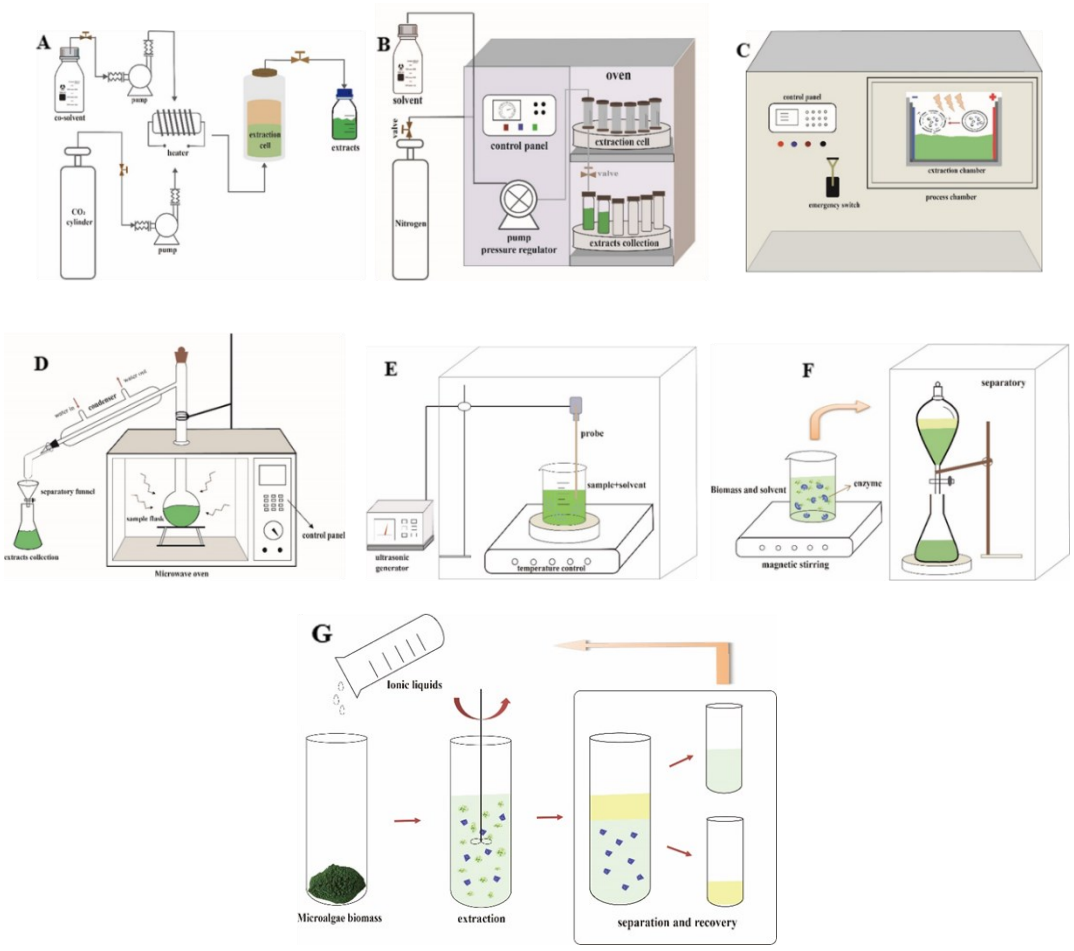

Figure 2. Schematic diagram of innovative approaches: A) supercritical fluid extraction (SFE), B) pressurized liquid extraction (PLE), C) pulsed electric fields (PEF), D) microwave-assisted extraction (MAE), E) ultrasound-assisted extraction (UAE), F) enzyme-assisted extraction (EAE), G) ionic liquids (ILs). These innovative approaches overcome the shortcomings of classical approaches to a certain extent and at the same time, they are also beneficial to improve the extraction rate of lipids and maintain the bioactive activity of the extracts (Table 1-2).

2.1 Traditional extraction approaches

2.1.1 Soxhlet extraction

Soxhlet extraction method was proposed as early as 1879 and was originally used to

quantify the total lipids amount in milk (Soxhlet, 1879) and was further gradually promoted in the fields of food, pharmaceutical and other industries. Soxhlet extraction is a technique in which the sample is repeatedly in contact with the extractant during the extraction process, thereby increasing the yield of the extract. Although the traditional Soxhlet extraction has low cost and simple operation, it also has disadvantages such as long extraction time and large reagent consumption (Luque de Castro & García-Ayuso, 1998). In recent decades, based on the principle of Soxhlet extraction, some new methods have been used, such as high-pressure Soxhlet extraction, automatic Soxhlet extraction, microwave integrated assisted Soxhlet extraction technology and others (Luque de Castro & Priego-Capote, 2010).

As a solvent-based lipid extraction method, Soxhlet extraction is still one of the common methods for extracting lipids form microalgae. In order to evaluate the effect of Soxhlet extraction on the lipids´ recovery from microalgae, Ramluckan et al. (Ramluckan et al., 2014) studied 13 types of solvents with different polarities (including hexane, chloroform, toluene, acetone, methanol, etc.) on *Chlorella sp*. lipids extraction. When compared with other reagents, ethanol, chloroform, and hexane showed the higher extraction rates (≈10.78%), and 3 h was the best extraction time. In addition, the mixture of chloroform: ethanol (1:1) also showed a high extraction rate with a value of about 11.76 %. Aravind et al. (Aravind et al., 2021) used n-hexane for *Spirulina sp.* lipid's extraction and found that completely dry and fine samples had a higher extraction rate, and the optimal extraction time was also 3 h. This may be because there is more contact between the smaller size of the *Spirulina* sp., and the solvent, thereby increasing the extraction rate.

Table 2. Application of various innovative extraction methods in the extraction of microalgae lipids.

Microalgae	Solvent	Conditions	Results	Reference
Supercritical carbon dioxide (SC-CO_2)				
Nannochloropsis oculata, Chlorella vulgaris	Ethanol (10 %, as cosolvent)	T: 50 °C P: 250/450/750 bar CO_2 flow: 25 g/min Solvent rate: 0.35~1.02 g/g·min^{-1} Ethanol flow rate: 1.9 g/min	The neutral lipids extraction rate of *C. vulgaris* was 97 %; the extraction rate of *N. oculate* was 83 %.	(Obeid et al., 2018)
Chlorella protothecoides		T: 70 °C P: 150~300 bar CO_2 flow: 75 CO_2 h^{-1} kg $biomass^{-1}$	Oil extraction yield was 21%.	(Viguera et al., 2016)
Schizochytrium sp.		T: 40/55 °C P: 25/40/55/70 MPa CO_2 flow: 10 kg/h	Compared with n-hexane extraction (Soxhlet extraction), SC-CO_2 has higher extraction rate, and the product has higher nutritional value (high ratios ω3/ω6 and DHA/EPA).	(Zinnai et al., 2016)

Table 2. (cont.)

Microalgae	Solvent	Conditions	Results	Reference
Scenedesmus obliquus, Scenedesmus obtusiusculus	Ethanol (5 %, co-solvent)	T: 20~200 °C P: 7~80 MPa CO_2 flow: 10 kg/h CO_2 to biomass ratio: 20–200	The lipids extraction rate was higher than Bligh-Dyer method, and the recovery rate of lipids reached 92 % (*w/w*).	(Lorenzen et al., 2017)
Scenedesmus obliquus, Chlorella protothecoides, Nannochloropsis salina		T: 45 °C P: 15/20/25/30 MPa CO_2 flow: 0.4±0.05 kg/h	Compared with Soxhlet extraction (methanol: chloroform 2:1 was used as solvent), SC-CO_2 has higher extraction rate and is beneficial to the percentage of ω-3.	(Solana et al., 2014)
Subcritical fluid extraction				
Phaeodactylum tricornutum, Nannochloropsis oculata, Porphyridium cruentum	n-butane	T: 40 °C P: 15 bar Solvent flow: 3 mL/min	Compared with SC-CO_2, more PUFA (ω-3 and ω-6) can be obtained by subcritical n-butane extraction.	(Feller et al., 2018)

Table 2. (cont.)

Microalgae	Solvent	Conditions	Results	Reference
Nannochloropsis salina	water	T: 220/205 °C Time: 25 min	Compared with the classical method (Folch extraction), the extraction rate of lipids under subcritical water method is 70 %, with microwave assistance, the extraction rate can achieve 100 %. meanwhile, the energy was reduced by 2-8 times.	(Reddy et al., 2014)
T. obliquus	Dimethyl ether	T: 20 °C P: 0.55 MPa	Microwave, ultrasound, and heat assisted extraction of subcritical dimethyl ether can improve the extraction rate of lipid by changing the permeability of cell wall.	(Wang et al., 2021)
Pressurized fluid extraction (PLE, traded as accelerated solvent extraction, ASE)				
Scenedesmus sp., *Chlorella zofingiensis, Isochrysis galbana*	methanol/DMSO (9:1 *v/v*, 1 cycle) hexane/diethyl ether (1:1 *v/v*, 2 cycles)	T: 120 °C P: 1500 psi	Compared with classical method (Folch extraction), ASE has a higher extraction rate and reduces lipid oxidation.	(Chen et al., 2020b)

Table 2. (cont.)

Microalgae	Solvent	Conditions	Results	Reference
Nannochloropsis oculata	n-hexane, n-hexane/2-PrOH (2:1 vol.%) ethanol (96 %)	T: 60 °C P: 10-12 MPa Time: 0.8 h	Among the three solvents, ethanol had the highest extraction rate (36±4 mass%), while n-hexane had the lower extraction rate (6.1±0.3 mass%).	(Pieber et al., 2012)
Pulsed electric field (PEF)-assisted extraction				
Ankistrodesmus falcatus	chloroform ethyl acetate	Pulse repetition frequency: 240 Hz Captured pulses: 24	PEF-assisted green solvent ethyl acetate extraction resulted in higher extraction rate of lipids than chloroform, which could be attributed to the fact that 90 % of cells were lysed under PEF.	(Zbinden et al., 2013)
Chlorella		Voltage: 35 kV Pulse repetition rate: 1~1k Hz Square wave pulse: 2~99 μS	PEF treatment increased the extraction yield of lipids to 166 %.	(Zhang et al., 2021b)
Chlorella pyrenoidosa	hexane/ethanol	Voltage: 20 kV Pulse frequency: 150 Hz Pulse width: 0~10 μs	PEF pre-treatment leads to defects on the cell surface and release of intracellular water-soluble substances, increasing lipid extraction.	(Han et al., 2019)

Table 2. (cont.)

Microalgae	Solvent	Conditions	Results	Reference
Ultrasound-assisted extraction (UAE)				
Scenedesmus sp.	hexane	Ultrasonic power: 0~50 W Time interval: 0~5 s Time: 0~10 min	The optimal treatment condition was 20 W, 2 s time interval, 4 min treat time, the total lipid yield increased from 0.76 h/L to 1.31 g/L.	(Sivaramakrishnan & Incharoensakdi, 2019)
Nanoochloropsis sp.	hexane	Time: 1/3/5 min Intensity of ultrasonic: 20 kHz	High-intensity ultrasonication can increase lipids recovery by destroying microalgae cells, the yield is about 6.5 times higher than of high-pressure homogeneity.	(Yao et al., 2018)
Microwave-assisted extraction (MAE)				
Nannochloropsis sp., *Tetraselmis* sp.		Thermocouple: 65 °C Microwave radiation: 500 W	For the two species of microalgae, the maximum lipid extraction rates were: microwave-assisted Hara-Radin extraction (8.19 %) and microwave-assisted Folch extraction (8.45 %).	(Teo & Idris, 2014)

Table 2. (cont.)

Microalgae	Solvent	Conditions	Results	Reference
Chlorella PY-ZU1	Chloroform: methanol (1:1 *v/v*)	T: 80~120 °C Time: 5/10 min	Microalgae were treated at 80 °C for 26 minutes, and the cell walls were destroyed at the maximum curvature. The increase in microwave temperature also increases the degree of damage to the cell wall. In addition, the microwave electromagnetic effect also led to the increase of short chain and saturated fatty acid in the extract.	(Cheng et al., 2013)
Ionic liquids extraction (ILs)				
Chlorella sorokiniana, Nannochloropsis salina, Galdieria sulphuraria	1-butyl-3-methylimidazolium hydrogen sulphate [BMIM][HSO_4]	[BMIM][HSO_4]-microwave: T: 120 °C, Power: 800 W Time: 10~60 min [BMIM][HSO_4]-ultrasound: T: 120 °C, Power: 800 W Time : 60 min	Compared with the classical Bligh-Dyer method, [BMIM][HSO_4]-microwave extraction could improve the lipid extraction rate to 1990 %, 370 % and 1170 %. The addition of [BMIM][HSO_4] makes the lipids easier to extract.	(Pan et al., 2016)

Table 2. (cont.)

Microalgae	Solvent	Conditions	Results	Reference
Nannochloropsis oculata, Chlorella salina	Ten carboxylate protic ionic liquids (PILs) with lactam and ammonium cations	PIL-sonication treatment: Intensity of ultrasonic: 20 kHz Power: 100 W Time: 1 min	Hexanoate and formate PILs exhibited enhanced lipids recovery, PILs also showed an inhibitory effect on lipase.	(Mukund et al., 2019)
Enzyme-assisted extraction				
Chlamydomonas reinhardtii	lysozyme, collagenase, trypsin, autolysin		Enzymatic pre-treatment can degrade the cell walls of microalgae, thereby facilitating the extraction of lipids and proteins from biomass.	(Sierra et al., 2017)
Photoelectrochemical system				
Chlorella	hexane, isopropanol	Cathode: P-Pd Photoelectric anode: 0.5 M, 1 M, and 1.5 M nitrogen (N)-doped TiO2 nanotube Anode electrolyte: Na_2SO_4	The •OH produced by the photochemical system attacks the cell walls and membranes of microalgae, facilitating lipid extraction.	(Wu et al., 2021)

Table 2. (cont.)

Microalgae	Solvent	Conditions	Results	Reference
		Osmotic shock		
Chlamydomonas reinhardtii	methanol: n-hexane (10:0, 7:3, 4:6 *v/v*)	T: 20 °C Speed: 20~25 rpm (1 d)	After osmotic shock treatment, lipids recovery of microalgae biomass can be increased by more than three times.	(Yoo et al., 2012)

After many years of development, Soxhlet extraction has become a standard technique to measure the efficiency of lipid extraction as well as its shortcomings are gradually being overcome due to continuous development by researchers. However, there are still some disadvantages, including long extraction time, large reagent consumption and unfavourable environment.

2.1.2 Folch extraction

The Folch method was proposed in 1957 using a mixture of chloroform: methanol (2:1 v/v) to extract lipids from animal fat (Jordi et al., 1957). In this method, a mixture of chloroform and methanol are first used to extract the lipids, then water is added to achieve phase separation, and finally the extracted lipids can be obtained after rotary evaporation. This method does not need high temperature and/or high pressure during the extraction process and it is one of the reliable methods for lipids extraction.

As a classical method, Folch is often compared with Soxhlet extraction. For instance, Onay et al. (Onay et al., 2016) evaluated the effects of various extraction methodologies (including Soxhlet, Folch and Bligh-Dyer, assisted by cell lyophilization, homogenization and ultrasound), on the lipids extraction of different thermo-resistant microalgae (*Hindakia*, *Scenedesmus* and *Micractinium Species*). The three extraction methods showed different extraction rates on microalgae and among them Soxhlet proved to have a higher extraction rate for *Micractinium spp.*, while Folch extraction was more suitable for *Scenedesmus spp*. In addition, when other assisted extraction technologies were applied, such as lyophilization and ultrasound, a higher lipids extraction rates were achieved.

Similarly, based on green solvents, Jesus et al. (Jesus et al., 2019) also compared the effects of Soxhlet, Folch and Bligh-Dyer on the extraction of *Chlorella pyrenoidosa* lipids. The results showed that when the solvent was a mixture of chloroform: methanol (2:1

v/v), Folch and Bligh-Dyer showed higher extraction rates, corresponded to 113.47±7.58 and 115.05 ± 5.32 (mg lipids/g biomass) respectively (the fatty acid content was also higher than with other solvents). When traditional solvents were used (singly), Folch and Bligh-Dyer also showed high lipids extraction capabilities.

In order to achieve better extraction results, Kumari et al. (Kumari & Singh, 2019) improved Folch process by using a combination of different polar reagents (chloroform and methanol) the results showed that not only the extraction rate of lipids was improved, but also promoted the release of other components from microalgae. As a simple and fast extraction method, Folch can be used for lipids extraction of a large number of samples, but it uses more toxic reagents, which is harmful to human health and environment.

2.1.3 Bligh-Dyer extraction

The Bligh-Dyer extraction method is a method based on a two-phase solvent extraction and it can be considered as a variant of Folch (Bligh & Dyer, 1959) in which a mixture of chloroform-methanol-H_2O is used as extraction solvent. Furthermore, Bligh-Dyer can separate lipids from the chloroform phase while allowing proteins to be precipitated between different phases.

This methodology has been widely used in the extraction of microalgae lipids. Ellison et al. compared the effects of standard Bligh-Dyer and hexane (non-polar solvent) extraction on the extraction of total lipids of *Chlorella vulgaris/Cyanobacteria leptolyngbya* (Ellison et al., 2019). Compared with the extraction method using the non-polar solvent, the average lipids mass extracted by the Bligh-Dyer is twice that of hexane extraction, which makes it a promising method for lipids extraction. Dejoye Tanzi et al. (Dejoye Tanzi et al., 2013) compared the effects of Soxhlet, Bligh-Dyer and a new technology called Simultaneous Distillation and Extraction Process (SDEP) on the extraction of two different microalgae (*Nannochloropsis oculata* and *Dunaliella salina*)

lipids. The results showed that for *Nannochloropsis oculata*, SDEP-cymene and Bligh-Dyer methods have similar lipids extraction yields of 21.45 and 23.78%, respectively, while for *Dunaliella salina*, SDEP-pinene and Bligh-Dyer provided the highest lipids extraction yields at 3.29 and 4.03%. As well as in the previous study of Onay et al. (Onay et al., 2016), the extraction rate (52.5% *w/w*) of *Chlorella vulgaris* lipids using the ultrasound-assisted Bligh-Dyer method was the highest compared with Soxhlet and Folch extraction. As a method close to Folch, Bligh-Dyer also uses toxic reagents, which generates a large amount of environmentally harmful waste. Therefore, waste recovery and cost need to be considered when it is used for large-scale extraction.

The above mentioned three lipids extraction methods commonly use toxic solvents such as methanol, chloroform, n-hexane, etc. Indeed, these solvents have negative impacts on both human health and environment and is in inconformity with the requirements for sustainable development. Therefore, in order to reduce the pollution caused by toxic reagents, some green extraction reagents are being explored to replace traditional toxic reagents (cyclopentyl methyl ether, 2-methyltetrahydrofuran, etc.), aiming to achieve a greener lipids extraction method (Jesus et al., 2019).

2.1.4 Other extraction techniques

Regarding traditional extraction processes, in addition to solvent-based extraction methods, mechanical-based methods can also be used in microalgae lipid's extraction, such as mechanical pressing, bead beating (also known as bead milling) and homogenization.

Extracting lipids from raw materials by pressing and compressing is an ancient and simple method. Chemical reagents are unnecessary in this method, instead, uniaxial high pressure is used directly to rupture the cell walls of biomass to extract lipids. In this method choose a suitable pressure for lipids extraction is a key factor because excessive

pressure or heat production will reduce the extraction rate of lipids and have a negative impact on the quality of the extracts. Compared with solvent extraction, the extraction efficiency of pressing is lower, which accounts about 75% of solvent extraction (Topare et al., 2011). Bead beating is also an assisted lipids extraction method, which allows the sample cells to be destroyed when passing through high-speed moving beads. The density of the beads determines the final state of the sample being processed but a large amount of energy needs to be consumed during the processing, which increases production costs (Günerken et al., 2015). Similar to bead beating, homogenization is also an effective method to rupture microalgae cell walls. The high-energy bubbles produced by homogeneous cavitation can cause cell rupture, which is a simple and rapid method for cell destruction. Mulchandani et al. (Mulchandani & Kar, 2015) used high-pressure homogenization to extract lipids from *Chlorella saccharophila*. Compared with the chloroform-methanol solvent extraction method, the lipids recovery rate of high-pressure homogenization was nearly 89.91 ± 3.69% (*w/w*). The main disadvantage of this method is that the considerable temperature increase caused by homogenization, which will destroy/alter bioactive compounds in the biomass. However, with the improvement and development of technology, now these technologies are mostly used for the pre-treatment of microalgae, and they need to be combined with other technologies to achieve better extraction results.

2.2 Innovative approaches

2.2.1 Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) has been proven to be one of the effective methods for extracting lipids. Supercritical fluids mainly refer to compounds in a critical state, where its pressure and temperature are above the critical point (Nagappan et al., 2019). The unique intermediate characteristics make supercritical fluids to have transportability

properties like gases and the solubility like liquids and several. As a kind of non-toxic solvent for lipids extraction, several supercritical fluids have the characteristics of environmental-friendly solvents.

Substances that can be used as supercritical extractants include carbon dioxide (CO_2), methanol, ammonia, and others. Among them, supercritical carbon dioxide (SC-CO_2) is the most widely used, because of low costs and non-toxicity and low critical temperature and pressure, which can avoid the degradation of heat-sensitive compounds during the extraction process and reduce operation cost (Bhargavi et al., 2018). In addition, under normal temperature and pressure, CO_2 is gaseous and can be separated directly from the extracts easily, thus disregarding the need of solvent separation (by distillation, etc.).

In terms of microalgae lipids extraction, SC-CO_2 can be used as an alternative to organic solvent extraction to increase the extraction rate. Obeid et al. (Obeid et al., 2018) used SC-CO_2 combined with ethanol as co-solvent to extract neutral lipids from freeze-dried *Nannochloropsis oculata* and *Chlorella vulgaris*. Under the optimal condition, the lipids extraction rates of *Nannochloropsis oculata* and *Chlorella vulgaris* were 83 % and 97%, respectively. Similarly, Viguera et al. (Viguera et al., 2016) also optimized the conditions for SC-CO_2 extraction of lipids from microalgae (*Chlorella protothecoides*), showing the highest lipids extraction rate at 300 bars and 70 °C. In addition to improving the extraction rate, SC-CO_2 also affects the fatty acids composition in the extract. Zinnai et al. (Zinnai et al., 2016) used SC-CO_2 and n-hexane to extract lipids from *Schizochytrium* sp. and the long-chain PUFAs were analysed. Compared with n-hexane, the lipids obtained by SC-CO_2 extraction have higher nutritional value, which is mainly represented by a high ratio of ω-3/ω-6 and DHA/EPA. Lorenzen et al. (Lorenzen et al., 2017) compared SC-CO_2 and Bligh-Dyer extraction to extract and purify lipids from *Scenedesmus obliquus* and *Scenedesmus obtusiusculus* biomass and found that at 12 MPa

(120 bar) and 20 °C, the lipids extraction rate can reach 92%, indicating the potential application of SC-CO_2 technology for industrial use, due to the high extraction rate and mild operational conditions (12 MPa and 20 °C). Because of the non-polarity of CO_2, it is easier for SC-CO_2 to extract neutral lipids that are not combined with polar lipids. In order to increase extraction rate and improve the solubility of more polar lipids, some co-solvents can be applied to the extraction process. For example, the above cited work of Obeid et al. (Obeid et al., 2018) used ethanol as co-solvent to increase the extraction rate. De Melo et al. (De Melo et al., 2020) also used the ethanol as the co-solvent and set different processing conditions to recover lipids from *Aurantiochytriu*m sp. biomass. Under the optimal extraction conditions, the DHA content in the extract is about 3.5 times that of the ordinary fish oil. Anyway, still high-priced equipment limits the large-scale use of SC-CO_2 to a certain extent.

2.2.2 Pressurized liquid extraction (PLE)

Pressurized Liquid Extraction (PLE), also known as Accelerated Solvent Extraction (ASE), is a technology to extract solid or semi-solid samples using the combination of temperature and pressure, which can better retain bioactive compounds and shorten the extraction time as well as has been widely studied (Ruiz-Domínguez et al., 2021). As a clean and green solvent, water is often used for subcritical extraction, since when water is heated over the boiling point and below the critical point, as well as the pressure is controlled to keep it in a liquid state, water reaches a state that is called a subcritical state. In this state, the polarity and dielectric constant of water change, with potential to achieve a possible better extraction effect. The use of water as a solvent is also called subcritical water extraction (Wani, 2021). Reddy et al. (Reddy et al., 2014) used subcritical water and microwave-assisted subcritical water to extract lipids from wet *Nannochloropsis salina* algal biomass. The results showed that the extraction efficiency of subcritical water

is much higher than that of the traditional Folch method, about 70% vs. 30%. In addition, when using microwave assistance, all lipids can be extracted, and the energy consumption is reduced by 2~8 times. In addition to water, several other solvents are also used in subcritical extraction, such as dimethyl ether, n-butane, propane and others (Wang et al., 2021). Wang et al. (2021) used subcritical dimethyl ether, a solvent that is not harmful to the environment, to extract lipids from *T. obliquus*. Microwave and ultrasounds are sometimes combined with subcritical extraction enhance cells disruption to facilitate extraction. The results showed that, compared with the Bligh-Dyer extraction, subcritical dimethyl ether can effectively extract lipids from microalgae, and the assisted extraction techniques such as microwave or ultrasound can enhance the permeability of the cell wall, thereby improving the extraction efficiency. Nowadays, some bio-based solvents are also used in PLE to replace traditional reagents because of their green and degradable properties. Golmakani et al. (Golmakani et al., 2014) used limonene, ethanol, and hexane as solvents to extract lipids from a variety of microalgae *(Spirulina*, *Phormidium*, *Anabaena* and *Stigeoclonium*) using PLE, and the fatty acids composition was also analysed. The results showed that the extraction of *Spirulina* lipids with limonene: ethanol (1:1, v/v) resulted in the highest extraction rate and the higher content of omega-3 fatty acids. Similarly, Ruiz-Domínguez et al. (Ruiz-Domínguez et al., 2021) used ethanol, water and limonene extracts as solvents and designed response surface experiments to explore the effect of PLE on the extraction rate of bioactive compounds from *Geitlerinema* sp. When water and ethanol were used as solvents, the extraction rate of phycobiliproteins is improved, while ethanol: limonene extract is more effective for the recovery of lipids and methyl palmitate. The application of these bio-based solvents makes the extracts easier to be applied to the food and pharmaceutical industries.

2.2.3 Pulsed electric field (PEF)

As a nonthermal processing method, pulsed electric field (PEF) has been widely used in the food industry, including the extraction of bioactive compounds, food preservation and drying (Barba et al., 2015). The main principle of PEF is the "electroporation" theory, that is, high voltage PEF is applied to treat the sample during a short period of time, which causes the cell membrane to rupture and form temporary/permanent holes (pores). In this way, the nutritional content and sensory quality of the food will be retained to a greater extent. The application of PEF may cause changes in the conductivity and rigidity of the microalgae cell wall, thereby increasing the extraction rate (Gómez et al., 2019). In Silve's et al. (Silve et al., 2018) research, the effects of PEF-assisted processing combined with ethanol-hexane blends solvent extraction on the lipid extraction from *AuxenoChlorella protothecoides* were explored. The results showed that the PEF treatment may lead to cell permeabilization, thereby causing the release of intracellular ions, increasing the conductivity of the sample and the lipids recovery rate could reach 90%. Moreover, Nile red staining was applied to verify that the cell structure is still intact under the PEF treatment. Zhang et al. (Zhang et al., 2021a) also used PEF to extract *Chlorella* lipids and explored the relationship between lipids extraction and cell breakdown under PEF treatment. Han et al. (Han et al., 2019) also explored the effect of PEF-assisted treatment on the extraction of *Chlorella* lipids, with the results showing that PEF-assisted Bligh-Dyer extraction can effectively enhance the lipids extraction rate of *Chlorella pyrenoidosa*, which was 12% higher than ultrasonic pre-treatment. The PEF-assisted technology causes surface defects in the cell wall and promotes the contact between the extraction solvent and the lipids to increase the extraction rate. In general, PEF is used to extract microalgae lipids mainly by increasing the permeability of the cell wall to increase the extraction rate, and it can process a large amount of sample, since it can operate, and usually operates in continuous, and it is considered a low-polluting

extraction technology.

2.2.4 Ultrasound-assisted extraction (UAE)

Ultrasounds are mechanical waves that propagates through compression and sparseness in a media. In the process of lipid-assisted extraction technology, it mainly destroys cells through cavitation and acoustic effects (Saini & Keum, 2018). The solvent generates bubbles under the action of ultrasonic waves, which burst due to the reaction force when contacting the cells and generate local shock waves to destroy the cells, resulting in an increase in the lipids extraction rate (Sallet et al., 2019). Compared with classical methods, UAE is simple to operate, requires less energy and is a fast and effective assisted extraction technology (Li et al., 2018). There are both reports on the use of high-power and low-power ultrasound to extract lipids from microalgae. Sivaramakrishnan et al. (Sivaramakrishnan & Incharoensakdi, 2019) used low-power ultrasound-assisted hexane to extract lipids from *Scenedesmus* sp. biomass. Under optimized conditions, the lipids yield increased from 0.76 to 1.31 g/L. In addition to increasing the yield of lipids, the assistance of ultrasound also increased the release rate of carotenoids and oxygen, resulting in higher photosynthesis efficiency. Yao et al. (Yao et al., 2018) also confirmed that high-power ultrasound can destroy *Nannochloropsis* sp. cells. The results showed that when the treatment time is within 1 to 3 minutes, the cell rupture increases with time, and the rupture effect on the cells decreases after the treatment time exceeds 5 minutes, which effectively increases the lipid yield in a short time, from 11 to 70%. Araujo et al. (Araujo et al., 2013) also confirmed that UAE can improve the extraction rate of microalgae lipids, by using ultrasound combined with five methods including Bligh-Dyer, Folch and Soxhlet to extract lipids from *Chlorella vulgaris*. The results showed that the extraction rate of lipids under ultrasound Bligh-Dyer (52.5%) was higher than other methods, and this might be due to the increased weakness of the cell wall promoted by

ultrasounds, leading to cell rupture. Additionally, Pez et al. (Pez et al., 2017) used UAE with ethanol to extract lipids and carotenoids from *HeteroChlorella luteoviridis* and found that when the ultrasound intensity is 40~80% and the ethanol concentration is 60~75%, the lipids extraction only increases with the increase of ethanol concentration, which was an expected result, as a higher ethanol content should dissolve higher amounts of lipids, thus increasing the extraction rate. It can be inferred that the intensity and frequency of ultrasound will affect the extraction rate, although a proper selection of the extraction solvent is of upmost importance.

2.2.5 Microwave-assisted extraction (MAE)

Microwave mainly refers to non-contact electromagnetic radiation with an energy of 0.3~3000 GHz. In the process of microwave treatment, heat will be transferred from the inside to the outside through the medium to heat the entire samples. When microwave is used to extract bioactive compounds, the pressure rises after the cells absorb energy and the cell wall is destroyed so that the bioactive compounds can be extracted into the solvent (Paré et al., 1997). Solvents of different polarities have different microwave absorption capabilities, and strong polar solvents have better microwave absorption capabilities (Virot et al., 2008). MAE has been used to extract a variety of bioactive compounds from microalgae, such as polysaccharides and lipids (Yao et al., 2018; Zhao et al., 2019) and it can significantly increase the yield of lipids, but the most suitable method for different microalgae is microalgae dependent. For example, microwave assisted Folch extraction can significantly increase the lipids recovery rate of *Nannochloropsis* sp. (Teo & Idris, 2014). Moreover, the extraction time and temperature can have an important influence on the microwave extraction effect. Cheng et al. (Cheng et al., 2013) used the microwave-assisted Bligh-Dyer method to extract lipids from microalgae and explored the effects of processing conditions on the extraction rate. When the microwave processing temperature

is within 80~120 °C, the increase of temperature leads to the increase of the fractal dimension of cell, which also reflects the increase in the degree of damage to the cell wall. On the other hand, as the processing time increases, the pore size on the cell wall gradually increases, making lipids extraction easier to achieve. Many studies have used MAE to extract biologically active substances from microalgae, but considering its working principle, the biomass is heated under microwave action to cause cell wall rupture, so the disadvantage of this method is that it is not suitable for the extraction of heat-sensitive compounds.

2.2.6 Ionic liquids extraction (ILs)

ILs are a low-melting salt with anions and cations (generally below 100 °C), some of which can be liquid at room temperature. By selecting different ions solvents for specific purposes can we obtain it, which makes ionic liquids customizable (in tailor made way) and leads to a large number of ILs, about 10^6 types currently (Skoronski et al., 2020). Common cations in ionic liquids, such as alkylammonium, alkylnitride, etc., have asymmetry that restricts the accumulation of ILs crystals, which leads to the generation of low melting-point solvents (Prusty et al., 2021). In general, ILs shows high stability and non-flammability. Moreover, because ILs have good solubility for lignocellulose, one of the main components of cell walls, ILs are potential good disruptors of the cell wall, thus being also potential to extract lipids from microalgae, since they can be useful to overcome the obstacles of the hard and thick cell walls of microalgae during the extraction process (Kilpeläinen et al., 2007).

Pan et al. used ILs-assisted ([BMIM]HSO_4) microwave/ultrasound to extract lipids from different microalgae and compared with traditional methods, microwave ILs can increase the lipid extraction rate of *Chlorella sorokiniana*, and that of *Nannochloropsis salina* by 10-fold. ILs can effectively promote the extraction of microalgae lipids without

affecting fatty acids composition (Pan et al., 2016). Mukund et al. (Mukund et al., 2019) also used ILs to pre-treat two types of microalgae (*Nannochloropsis oculata.* and *Chlorella salina*) and recover lipids, using ten different ILs, with Butyrolactm hexanoate showing strong cell disruption ability, being able to destroy about 84% of cells, and the lipids recovery rate for the *Nannochloropsis oculata* and *Chlorella salina* reached 134.9% and 85.4%, respectively, compared with Bligh-Dyer method. In addition, ILs also inhibits enzyme activity inhibition and prevent lipolysis during wet extractions. However, not all ILs are green and non-toxic, while some ILs may be accompanied by the production of toxic substances during the synthesis process, which may affect the environment.

2.2.7 Enzyme-assisted extraction

As aforementioned, breaking the cell wall of microalgae is the core step in the extraction of its biologically active ingredients. Therefore, in order to improve the extraction rate, researchers have adopted a variety of methods to destroy the cell wall of microalgae (such as PEF, UAE, bead milling, etc.), but this usually requires the use of expensive equipment (Günerken et al., 2015). To overcome these difficulties, some biochemical methods have been used to improve the extraction rate, such as enzyme-assisted extraction. Compared with other mechanical treatments, enzyme-assisted cell destruction has a higher substrate selectivity and can be carried out under normal pressure and low temperature, effectively reducing the energy consumption (Zhang et al., 2018). Enzyme-assisted extraction is also considered to be a method that can be explored to replace classical methods to obtain a variety of bioactive compounds from microalgae, as normally these enzymes target the microalgae cell walls. Previous studies have shown that the use of enzymes to extract lipids from microalgae not only improve the extraction rate, but also facilitate the fractionation of lipids (Alavijeh et al., 2020). He et al. (He et al., 2020) used four different hydrolases (cellulase, hemicellulase, papain and pectinase) to disrupt *Nannochloropsis*

cells and combined the third phase partitioning to extract lipids. The results showed that comparing the extraction effects of four hydrolytic enzymes on microalgae lipids, the extraction rate after cellulase treatment was higher, and the combination of the four enzymes increased the extraction rate of microalgae lipids by 2-fold. Generally, enzyme-assisted extraction combined with other extraction methods can achieve better extraction results. Alavijeh et al. (Alavijeh et al., 2020) combined bead milling and enzymatic hydrolysis to separate and extract lipids, proteins, and carbohydrates from *Chlorella vulgaris*. The results showed that the lipid extraction rates of bead milling, and enzymatic hydrolysis were as high as 75% and 88%, respectively, and the recovery rates of protein and carbohydrates were also improved. Moreover, the combined use of the two extraction methods could successfully separate different biologically active compounds and minimize losses and shorten the extraction time. Similarly, Sierra et al. (Sierra et al., 2017) combined the Bligh-Dyer method with enzymatic hydrolysis to extract lipids and proteins from *Chlamydomonas reinhardtii*, and found that enzymatic pre-treatment can degrade the cell walls of microalgae, thereby facilitating the extraction of lipids and proteins.

Obviously, the assistance of enzymatic hydrolysis can significantly increase the lipid extraction rate, but enzymes have specific reaction conditions and therefore, it is very important to select suitable enzymes and control enzymatic hydrolysis conditions to promote the destruction of microalgae cell walls and increase the extraction rate.

2.2.8 Other methods

In addition to the above methods, other new technologies have also been explored for the extraction of microalgae lipids. For example, based on the principle of photoelectron-chemistry, Wu et al. (Wu et al., 2021) constructed a photoelectric system that uses TiO_2-based photoanode and UV-Vis light to generate •OH at a phosphate-palladium cathode, which was used to extract *Chlorella* lipids. After the pre-treatment of the photoelectric

system, the extraction rate of *Chlorella* lipids can reach 96%, which is higher than that of samples without pre-treatment. It also can be considered as an environmentally friendly, low-energy processing method. Similarly, osmotic shock treatment has also been explored for the extraction of microalgae lipids. For example, Yoo et al. (Yoo et al., 2012) used NaCl to adjust the intensity of solvent osmotic shock to extract lipids from *Chlamydomonas reinhardtii* biomass and found that osmotic shock can double the lipids recovery rate, indicating that it is also a promising technology in microalgae lipid extraction.

In general, choosing the appropriate cell disruption process and extraction method can increase the extraction rate, and the ingenious combination of different technologies can also show great application value in the extraction of microalgae lipids.

3. Bioactivity of microalgae lipids

As one of the main producers of marine lipids, the high content of (PUFAs, especially EPA and DHA, is believed to have a variety of biological activities, including antibacterial, antifungal, antiinflammatory, alleviating cardiovascular diseases and modulating gut microbiome (Figure 3). In addition, microalgae also contain other essential fatty acids, such as linoleic acid, linolenic acid, arachidonic acid, etc., which also endow the microalgae lipids with more functions, so that the microalgae can be used in animal feed, biodiesel and so on (Das, 2018).

3.1 Antimicrobial activity

The antimicrobial activity of PUFAs has been well known and PUFAs are used as an antibacterial food additive, like having an inhibitory effect on the growth of *Staphylococcus*, *Streptococcus*, *Bacillus*, etc. The main contribution to these antibacterial activities is linoleic, linolenic, and oleic acids, while some fatty acids derivatives may also have the potential to exert antibacterial activity (Kabara et al., 1972; Zheng et al.,

2005). Considering that microalgae can be used as a natural source of fatty acids, its antibacterial activities has also been explored and the antibacterial effects of microalgae lipids and their extracts against a variety of Gram-positive and negative bacteria have been reported (Table 3).

As early as 1996, scientists isolated a mixture of fatty acids from algae and found that it showed inhibitory effects on both Gram-positive and Gram-negative bacteria (Dellar et al., 1996). On this basis, some microalgae with high fatty acids content have been studied. Najdenski et al. (Najdenski et al., 2013) selected nine species of blue-algae (cyanobacteria) and explored the antibacterial and antifungal activities of their intracellular and extracellular compounds, including polysaccharides, fatty acids, phycocyanin, etc.

In terms of lipids and fatty acids, a variety of microalgae (*Aphanizomenon flos-aquae*, *Scenedesmus obliquus* and *Rhodella violacea*) have shown an inhibitory effect on *Streptococcus pyogenes* and *Staphylococcus aureus*, while the lipids of *Trachidiscus minutus* and *Nostoc* sp. only had an inhibitory effect on *Streptococcus pyogenes*. In addition, the lipids isolated from *Chlorella* sp., *Coelastrella* sp. and *Rhodellales violacea* showed obvious inhibitory effects on *Staphylococcus aureus*. Asthana et al. (Asthana et al., 2006) extracted γ-linolenic acid (GLA) from the *Fischerella* sp. and found that it is active against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Enterobacter aerogenes*. Among them, the effect on *Staphylococcus aureus* was the most significant, followed by *Salmonella typhi* and showing a lower inhibitory effect on *Enterobacter aerogenes*, which also reflects the difference in the effect of fatty acids on different bacteria.

Table 3. Studies on antibacterial activity of microalgae lipids.

Microalgae	Active compounds	Bacteria	Reference
Phaeodactylum tricornutum	EPA (20:5)	*Bacillus cereus,*	(Desbois et al., 2009)
		Staphylococcus epidermidis	
		Staphylococcus aureus,	
		Listonella anguillarum	
Rhodella maculata, Phaeodactylum tricornutum, Boekelovia hooglandii, Goniochloris sculpta, and Chloridella simplex	Capric acid (10:0), palmitoleic acid (16:1), γ-linolenic acid [18:3 (n-6)], arachidonic acid [20:4 (n-6)], and docosadienoic acid [22:2 (n-6)]	*Escherichia coli*	(Ruffell et al., 2016)
		Staphylococcus aureus	
Scenedesmus intermedius	Fatty acid methyl esters	*Escherichia coli*	(Davoodbasha et al., 2018)
		Pseudomonas aeruginosa	
Fischerella sp.	γ-linolenic acid [18:3 (n-6)]	*Staphylococcus aureus*	(Asthana et al., 2006)
		Escherichia coli,	
		Salmonella typhi (local strain)	
		Pseudomonas aeruginosa Enterobacter aerogenes	

Table 3. (cont.)

Microalgae	Active compounds	Bacteria	Reference
Aphanizomenon flos-aquae, Scenedesmus obliquus, Rhodella violacea	Intracellular extract (fatty acid)	*Streptococcus pyogenes*	(Najdenski et al., 2013)
Chlorella sp., Coelastrella sp., rviolacea		*Staphylococcus aureus*	
Dunaliella salina	Hexane, methanol, and DCM extracts	*Micrococcus luteus*	(Yavuz et al., 2014)
		Escherichia coli	
		Pseudomonas aeruginosa	
	Ethanol extracts	*Candida albicans*	
	Methanol, and ethanol extracts	*Vibrio alginolyticus,*	
Phaeodactylum tricornutum Bohlin	EPA, secondary algal metabolites	*Vibrio vulnificus,*	(Wang et al., 2018)
		Vibrio parahaemolyticus	

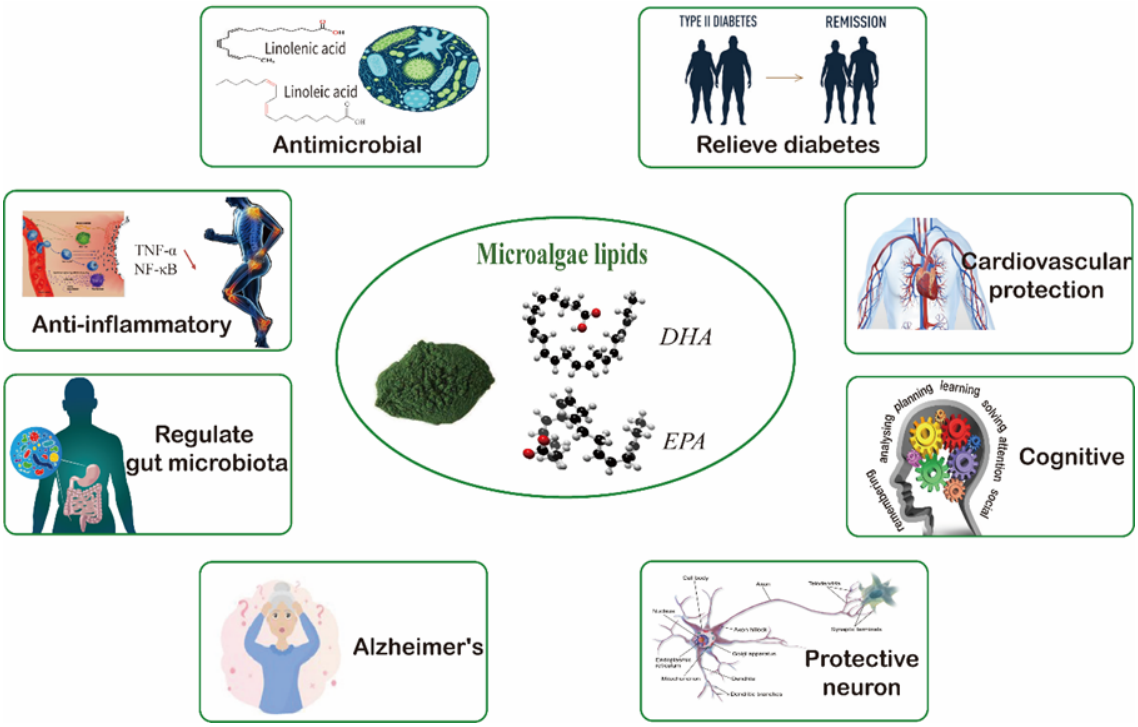

Figure 3. Bioactivity of microalgae lipids

The potential of microalgae fatty acids as antibiotics was also evaluated. Ruffell et al. (Ruffell et al., 2016) explored the effects of 29 fatty acids present in different types of microalgae on *Escherichia coli* and *Staphylococcus aureus*. Five fatty acids including palmitic and γ-linolenic acid showed great antibacterial activity. Since fatty acids content are affected by growth conditions, the results can be used as basis for microalgae cultivation and screening, so that high-concentration and high-quality fatty acids under specific growth condition can we promote it, becoming a good source of antibiotics. Davoodbasha et al. (Davoodbasha et al., 2018) further explored the high content of palmitic acid (C16:0) fatty acid methyl esters in *Scenedesmus* sp. and determined its antimicrobial abilities against a variety of bacteria and fungi. The fatty acid methyl esters showed higher inhibitory activities against Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) compared with Gram-positive bacteria, with the lowest inhibitory concentration being about 12~24 μg/mL. Furthermore, *Phaeodactylum*

tricornutum, *Chlamydomonas reinhardtii*, *Anabaena*, etc. have also been shown to have antimicrobial properties against *Staphylococcus aureus*, *Salmonella typhimurium* and other bacteria (Desbois et al., 2009; Svircev et al., 2008).

Due to the increased use of antibiotics, drug resistance of bacteria has increased and the search for new natural antimicrobial compounds from nature has attracted the attention of researchers, and in this context, microalgae lipids and fatty acids are considered to have great development potential because their antibacterial abilities.

3.2 Antiinflammatory activity

Inflammation is one of the common reactions of the human innate immune system, which can protect the body from damage by parasites, microorganisms, or viruses. When the body is injured by inflammation, uncontrolled inflammation in the body may be linked to the occurrence of some diseases, including cardiovascular disease, intestinal inflammation, obesity, diabetes and so on. If there is no inflammatory at all, the body's immune system will be weakened, so it is very important to maintain a balance between inflammatory and antiinflammatory processes (Liu, 2021). Research has indicate that omega-3 fatty acids related lipids have antiinflammatory effects and omega-6 fatty acid have the opposite effect, which can be attributed to the products of lipids metabolism (Kumar et al., 2019). In addition, high concentrations of EPA and DHA have also been extensively studied for their antiinflammatory effects (Itariu et al., 2012). As one of the main sources of PUFAs in the marine, microalgae are being explored for their antiinflammatory potential (Table 4).

Table 4. Effects of microalgae lipids on health.

Microalgae	Model	Active compounds	Dosage	Result	Reference
Antiinflammatory					
Chlorella	Human peripheral blood mononuclear cells	Lipid extracts	10~20 μg/mL	It can alleviate the acne vulgaris by inhibiting the activity of *Propionibacterium acnes.*	(Sibi, 2015)
Chlamydomonas debaryana, Nannochloropsis gaditana	THP-1 macrophages and HT-29 colon cells	Bioactive molecules generated by the oxidation of fatty acids	100 μM	Inhibition of the activation of NF-κB pathway, reduce the production of inflammatory markers and contribute to the regression of acute inflammation.	(Ávila-Román et al., 2018)
Anti-diabetes					
Chlorella pyrenoidosa, Spirulina platensis	High-fat, high-sucrose mice	Ethanol extracts	150 mg/kg per day	Work against hypoglycaemia and regulate gut microbiota	(Wan et al., 2019)
Spirulina platensis	Mice	Ethanol extracts	150 mg/kg per day	Reduces blood lipid, regulate gut microbiota and relieves diabetes	(Li et al., 2018)

Table 4. (cont.)

Microalgae	Model	Active compounds	Dosage	Result	Reference
Isochrysis galbana, Nannochloropsis oculata	Mice	Fatty acids	5 µg/5 mg/50 mg	Increased low density lipoproteins and decreased high density lipoproteins in healthy/diabetic mice.	(Nuño et al., 2013)
Cardiovascular disease					
Odontella aurita	Mice	PUFAs	12 % (*w/w*)	Reduces platelet aggregation and oxidative stress, as well as prevents CVD.	(Haimeur et al., 2016)
Spirulina	Washed rabbit platelets	Lipid extracts		Exhibit inhibitory effect on PAF and thrombin.	(Koukouraki et al., 2020)
Microalgae	Persons without coronary heart disease	DHA supplementation	~1.68 g/d	Decreased serum TG and increase HDL/LDL-cholesterol in persons without coronary heart disease	(Bernstein et al., 2012)

Table 4. (cont.)

Microalgae	Model	Active compounds	Dosage	Result	Reference
Others					
Nannochloropsis, Schizochytrium	Mice	EPA and DHA	1.8/2.4 %	It is beneficial for the protection of neurons.	(Lopes et al., 2017)
Botryococcus braunii, Nannochloropsis oculata	Human neuroblastoma cell line (SH-SY5Y)	Organic reagent extract		Protect SH-SY5Y cells from H2O2-induced cytotoxicity.	(Custódio et al., 2015)
Turbinaria ornata	Human colon cancer cells (HT-29)	Hexadecanoic acid (HA)		The extract exhibits potential antioxidant and anticancer activity	(Bharath et al., 2021)

In order to explore the influence of microalgae lipids on inflammation, Sibi (Sibi, 2015) explored the inhibitory effects of six *Chlorella* lipid extracts on inflammation, inhibition of pro-inflammatory cytokines and tumor necrosis factor-α (TNF-α) produced by human peripheral blood mononuclear cells. It was also evaluated the extract's inhibition of lipase and active oxygen to explore the antiinflammatory mechanism. The inhibitory range of various lipid extracts on TNF-α was about 58.39~78.67%, with *Chlorella vulgaris* showed the lowest inhibitory concentration of about 10 μg/mL, followed by *Chlorella ellipsoidea*, *Chlorella protothecoides* and *Chlorella pyrenoidosa* with about 20 μg/mL. At the same time, *Chlorella ellipsoidea, Chlorella vulgaris* and *Chlorella protothecoides* showed also high lipase inhibitory activity (58.9~61.73%). Further analysis of fatty acid methyl esters revealed that of the 19 fatty acids in the extract, 14 were C14 to C24 unsaturated fatty acids. The results showed that the lipid extract of *Chlorella* can inhibit the activity of *Propionibacterium acnes* by inhibiting the activity of lipase, thereby alleviating the inflammatory disease acne vulgaris. Furthermore, oxylipins produced by the oxidation of fatty acids have also been found to have the effect of relieving inflammation. Ávila-Román et al. (Ávila-Román et al., 2018) explored the *in vitro* antiinflammatory mechanism of the oxylipins in *Chlamydomonas debaryana* and *Nannochloropsis gaditana*, since oxylipins can reduce the production of pro-inflammatory factors in THP-1 macrophages and HT-29 colon cells, thereby acting as an activator of PPAR-γ to inhibit the activation of the NF-κB pathway, showing the potential in the treatment of inflammatory diseases. The EPA and DHA abundant in microalgae can inhibit the pro-inflammatory factors and change the phospholipid composition of the cell membrane, reducing the expression of inflammatory genes and other potential antiinflammatory capabilities.

3.3 Regulating gut microbiota and alleviating diabetes

Diabetes is a metabolic disease with high morbidity and mortality. The distinctive feature of diabetes is the high blood glucose levels, so the control of these levels has become an important part of the treatment of diabetes (Wang et al., 2021). With the improvement of people's living standards and the extension of life expectancy, diabetes has gradually become a disease with increasing prevalence around the world and at the present, diet combined with medicine treatment are effective methods to alleviate the diabetes (Kgosidialwa et al., 2015). However, medicine treatment will more and less bring certain side effects, so the development of healthier products that can be introduced in the diet has also attracted people's attention. Previous studies have shown that the gut microbiota may be related to the occurrence of various metabolic syndromes, including diabetes. Therefore, it is possible to regulate the structure of the gut microbiota through diet to alleviate the occurrence of diabetes (Arora et al., 2021).

Marine-derived PUFAs have been applied to a variety of disease models due to their bioactivity, among them, microalgae as an important source of fatty acids and others bioactive compounds. These compounds are popular in the food and pharmaceutical industries (Jia et al., 2014). Wan et al. (Wan et al., 2019) explored the anti-diabetes activity of PUFAs in two common microalgae (*Chlorella pyrenoidosa* and *Spirulina platensis*), with ethanol extracts of microalgae being used to feed mice on the high-fat, high-sucrose diet. After 8 weeks, DNA from the cecum of the mice was collected and extracted, and 16S rRNA sequencing was performed, with the results showing that both microalgae extracts can supplement and maintain the beneficial bacteria in the intestines, including *Oscillibacter*, *Parasutterella*, and *Ruminococcus*, and reduce the abundance of *Blautia* and *Turicibacter*. At the same time, *Chlorella pyrenoidosa* showed more obvious effects than *Spirulina platensis* in the fight against hypoglycaemia in mice. Based on the correlation analysis between blood glucose level and intestinal flora, it is speculated that

Ruminococcus may be a bacteria related to the regulation of diabetes, so it can provide a supplement for further exploration of the treatment of diabetes and the regulation of the mechanism of lowering blood glucose. Similarly, studies carried out by Li et al. (Li et al., 2018) with *Spirulina platensis*, also explored the effects of PUFAs on the regulation of gut microbiota and lipid metabolism in high-fat mice. In order to alleviate metabolic disorders and reduce blood lipids, a 95% ethanol extract was fed to mice and the protective effect of the extract on the liver was evaluated. RT-PCR analysis of liver DNA found that the extract increased the abundance of beneficial bacteria (including *Prevotella* and *Alloprevotella*) which were positively correlated with low-density-lipoprotein cholesterol levels, triglycerides, etc., and the decrease of the abundance of microbes such as *Romboutsia* and *Clostridium XVIII*, which were negatively correlated with the serum high-density-lipoprotein cholesterol levels. The extract had regulating effects on the patient's gut microbiota to alleviate diabetes and shown the potential as functional foods. An increased number of studies have shown that biologically active compounds derived from microalgae, including PUFAs can alleviate and treat diabetes by regulating the structure of the gut microbiota of patients.

3.4 Impact on cardiovascular disease

Cardiovascular disease (CVD) has become one the main causes of damage to health in non-communicable diseases, hypertension, high cholesterol, and oxidative stress and may also lead to coronary heart disease and atherosclerosis (Krijger et al., 2021). Among them, hypertension is widely concerned as a controllable risk factor in CVD and its impact on health. Diet and lifestyle will all influence the occurrence of CVD, at the same time, medication has long been used to relieve chronic diseases such as CVD. Considering the possible side effects of long-term medication, the role of some natural active ingredients in alleviating CVD is being explored (Ejike et al., 2017).

Researcher have confirmed that PUFAs, including EPA and DHA, as dietary supplements also show certain advantages in the prevention of CVD in addition to antiinflammatory activity. Several studies on fish oils with high PUFAs content in marine sources and showed that EPA and DHA can effectively lower the incidence of CVD and play a certain protective effect on the cardiovascular system (Yamagata, 2020). Meanwhile, microalgae as one of the effective alternative sources of PUFAs, in which high content of PUFAs and other high-value compounds have shown great development potential in human health. Haimeur et al. (Haimeur et al., 2016) compared the effects of fish oil with rich in omega-3 PUFA and microalgae oil on CVD. Fish oil and freeze-dried *Odontella aurita* were fed to mice on a high-fat diet. After 8 weeks, the plasma insulin, tissue lipids, platelet activity were analysed. The results showed that although the addition of microalgae and fish oil can reduce high-fat levels in mice with serum lipid levels and insulinemia, *Odontella aurita* showed a more pronounced effect in preventing lipid denaturation and in the reduction of triglyceride levels, showing a great biological effect, which may be due to microalgae with high PUFAs content and other bioactive compounds. In addition, the lipid extracts of microalgae can also affect the formation of thrombus. Koukouraki et al. (Koukouraki et al., 2020) explored the relationship between *Spirulina* lipid extracts and thrombosis through the effects of platelet activating factor (PAF) and thrombin, and found that the lipid extracts of *Spirulina* exhibit a strong inhibitory effect on PAF and thrombin, showing anti-thrombotic properties. Microalgae oil can be considered as a substitute of fish oil as a supplement for dietary nutrition.

3.5 Other bioactivities

In addition to the above-mentioned bioactivities that have been investigated, microalgae lipids have also been found to have an impact in other ways and are being further studied. Long-chain PUFAs, especially EPA are associated with the occurrence of some

neurological diseases, such as Alzheimer's disease, cognitive decline, depression, and other diseases. Because microalgae are rich in DHA and EPA, it has been found that it can alleviate the imbalance of plasma metabolites in mice, and has a protective effect on the plasma lipid profile, which is beneficial to the structure and functions of neurons (Lopes et al., 2017). The highly PUFAs extracts of microalgae showed also inhibitory effects on human colon cancer cells (HT-29 cells) and may be developed into a potential anti-cancer material in the future (Bharath et al., 2021). Compared with the waste of resources and fishy smell that may occur during the processing of fish, microalgae can be regarded as a good substitute for PUFAs due to its large yield and fast growth, which has great development value.

4. Application potential of microalgae lipids

4.1 Food and medicine industry

Due to changes in modern lifestyles, some high-caloric or unhealthy foods are consumed by people, which has led to a series of health problems, such as diabetes and heart disease mentioned above (Sathasivam et al., 2019). As a source of high-value bioactive compounds, microalgae can be used to fortify human diet, to obtain more nutrition and functional foods, especially because PUFAs in microalgae have high nutritional value. PUFAs are used as dietary supplements in health products, beverages, jam, pasta products and other daily foods with no or low content of PUFAs (Lafarga, 2019; Robertson et al., 2016). Foods containing high-value fatty acids can reduce the risk of some diseases by adjust cholesterol and other indicators, and can also reduce the accumulation of toxins and heavy metals that may be due to the consumption of fish products (Shahidi & Ambigaipalan, 2018). In addition to PUFAs, microalgae also contain many other bioactive compounds, such as protein, minerals, polysaccharides, which also contribute to the increase of nutrients that have potential to be added to food products (Paula et al.,

2013).

4.2 Biofuel

The extensive use of traditional fossil fuels produces harmful substances while also polluting the environment, leading to global warming. Obtaining new plant biofuels from some renewable raw materials to replace part of fossil fuels can reduce the release of CO_2 during the combustion process and reduce the damage to the environment (Chhandama & Satyan, 2021; Xue et al., 2021). At present, traditional plants or crops such as corn and rapeseed can be used as biofuels, but most of them also can be used as edible oils. Microalgae not only have faster growth and reproduction ability, but also have strong lipid production and accumulation ability of inedible triglycerides that can be converted into biofuels, making it possible for microalgae to be used as a biofuel (Xue et al., 2021). The advantage of microalgae biofuels is that it will not have a negative impact on arable land, but can also alleviate the problem of energy shortage to a certain extent, which has broad prospects (Peng et al., 2020).

4.3 Aquaculture and animal feed

The bioactivity of PUFAs present in microalgae has been explored and found to have a positive effect on the health of animals. Furthermore, microalgae products have also been explored to be applied to animals and aquaculture in different forms as feed. Common microalgae used to feed include: *Spirulina*, *Chlorella*, *Lobosphaera incisa*, *Isochrysis*, *Schizochytrium* sp., *Phaeodactylum*, *Nannochloropsis* and others (Lu et al., 2021; Sathasivam et al., 2019). Feeds rich in PUFAs can improve the ratio of fatty acids in meat products while improving the immunity of animals, further affecting the flavour or mouthfeel of the products (Medeiros et al., 2020).

4.4 Cosmetic products

In addition to above, PUFAs-rich microalgae are also used in the cosmetic industry. The

PUFAs contained in microalgae provide a variety of potential benefits to the products due to their biological activity. For example, PUFAs has antioxidant properties and can be used in anti-aging and sunscreen products; microalgae rich in PUFAs have also antiinflammatory effects and eliminates inflammation and regulate the balance of water and oil (Ying et al., 2020). At present, products containing microalgae are produced for skin care, hair care and water supplies (Lourdes et al., 2017).

5. Conclusions and future perspectives

Over the past few decades, concerns about food safety and agricultural sustainability have increased and microalgae have gained interest as natural substances rich in a variety of bioactive compounds, including lipids, proteins, amino acids, and polysaccharides. Among them, lipids are a good replacement for fish oil because of its high PUFAs content. Microalgae lipids extraction has been studied more specifically considering the effects on the cell walls. This paper summarizes the techniques and assisted techniques of microalgae lipids' extraction in order to provide more comprehensive information about the topic, and to identify opportunities and challenges to overcome. Classical extraction techniques (Soxhlet, Folch, Bligh-Dyer, etc.) have been widely used and fully explored. Although most of them are simple to operate, they also cause damage to the environment due to the extensive use of toxic reagents and the extraction time is long. Therefore, it is necessary to explore efficient and environmentally friendlier methods. A series of innovative techniques have been applied to the extraction of microalgae lipids, such as SFE, PLE, PEF, UAE, MAE, ILs, enzyme-assisted extraction and others. These methods have proven to have positive effects in improving extraction rate and reducing pollution. As a valuable bioactive compounds, microalgae lipids have also been found to have physiological functions such as antimicrobial and antiinflammatory effects and the capability to alleviating diabetes and cardiovascular diseases. Based on the bioactivity of

microalgae lipids, its applications in various industries are also summarized, including in food, medicine, cosmetics, animal feed, biofuels, and other industries. As an important source of PUFAs in the ocean, microalgae have attracted more and more attention of researchers. Therefore, is of great interest to explore appropriate techniques to improve the extraction rate and reduce the cost on the premise of ensuring the bioactive abilities of lipids, so that microalgae can be better applied to industries. In the future, the application of microalgae on a larger scale is worth expecting. In addition to combining existing technologies and develop new technologies, it is also necessary to further explore the use of bio-based non-toxic extractants in the extraction process. This makes it easier for the target components to be extracted to be applied, as well as recycling of the remaining material after extraction. Moreover, algae biomass fractionation, in the scope of algae biorefinery approach for high-value compounds extraction, is expected to boost in the next years,

Declaration of competing interest:

The authors declare no conflict of interest.

Acknowledgments:

Thanks are due to the University of Aveiro and FCT/MCT for the financial support of LAQV-REQUIMTE research Unit (UIDB/50006/2020) through national funds and, where applicable, co-financed by the FEDER, within the PT2020 Partnership Agreement, and for financing the PhD grant of Carlos A. Pinto (SFRH/BD/137036/2018) and Ana P. Martins (SFRH/BD/146369/2019). Jianjun Zhou and Min Wang were supported by a PhD fellowship from the China Scholarship Council (CSC) (No. 201908420246 and No. 201908420245, respectively).

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4.10 Research progress in microalgae nutrients: Emerging extraction and purification technologies, digestive behaviour, and potential effects on human gut

Journal: ***Critical Reviews in Food Science and Nutrition*** **(minor revision).**

Research progress in microalgae nutrients: Emerging extraction and purification technologies, digestive behaviour, and potential effects on human gut

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Abstract

Microalgae are rich in a wide variety of high-added-value compounds which can be directly used or fractionated into higher-added-value components. Extraction, separation, and purification are key processes in the industrial application of microalgae, being innovative technologies and a key tool for their efficient utilization. In addition, due to the use of microalgae for food and medical purposes, there is a growing interest in their digestive properties and impact on human gut health. In this review, a comprehensive literature review was performed to highlight the main high-added-value microalgae components. Moreover, innovative technologies used to extract and purify microalgae high-added-value compounds were evaluated. Finally, the digestive behaviour of microalgae nutrients and their health effects on the human gut microbiota were discussed. The technologies for obtaining bioactive compounds from microalgae are being developed rapidly, and various innovative, efficient, and green separation and purification technologies are emerging, thus helping in the scaling-up and subsequent commercialization of microalgae products. Microalgae nutrients exhibit favourable digestive properties and certain components have been shown to benefit gut microbes. The reality that must be faced is that multiple processes are still required for microalgae raw materials to final usable products, involving energy, time consumption and loss of ingredients, which still face challenges.

Keywords: Microalgae, bioactive compounds, extraction, purification, digestibility, gut microbiota.

1. Introduction

The global population is estimated to increase by more than a third (2.3 billion people) by 2050, while the demand for food production will increase by about 70% (Prosekov & Ivanova, 2018). Unfortunately, the ongoing COVID-19 pandemic and the Russian-Ukrainian war have triggered a global food and energy crisis. Humans need to develop and apply new biological resources to deal with the impending existential crisis (Adekoya et al., 2022; Bakalis et al., 2020). In addition, the currently used intensive farming methods will soon cease to be an option due to their adverse environmental impacts. Intensive farming contributes to the destruction of natural habitats and threats biodiversity, and the production of greenhouse gases from land clearing, fertilizer and livestock production, and nutrient loss from chemical fertilizers, can damage marine, freshwater, and terrestrial ecosystems (Bleakley, 2017).

Taking a chance on marine resources is expected to alleviate the strong dependence on land resources of human beings (Zhu et al., 2021). In recent years, the high-added-value components of microalgae have been widely used in the fields of food, medicine, cosmetics, and fuel, which potentially relieves the pressure of global resource shortage (Wang et al., 2022; Zhuang et al., 2022).

For instance, over the last five years, farming, harvesting, extraction and biorefining are reported as hot topics related to microalgae research according to statistics (Scopus, from 2017 to 2023, Figure 1). At the same time, in the downstream microalgae processing process, the extraction, separation and purification of bioactive compounds are the key points to perform an efficient utilization of microalgae biomass.

Most of the bioactive components of microalgae, including proteins, lipids, carbohydrates, polyphenols, minerals, etc., are distributed in the cytoplasm (Zhou et al., 2022). Nutrients in microalgae are not readily available due to the tough cell walls (Ahmed & Kumar,

2022). The cell wall structure of microalgae (Figure 2) is very complex, with multiple layers, consisting of polysaccharides, proteins, lipids, and inorganic salts (Bernaerts et al., 2019). Therefore, the first issue to be considered is how to efficiently, safely, and environmentally 'break through' the cell wall barrier of microalgae to obtain high-added-value components (Mehariya et al., 2021; Wang et al., 2019).

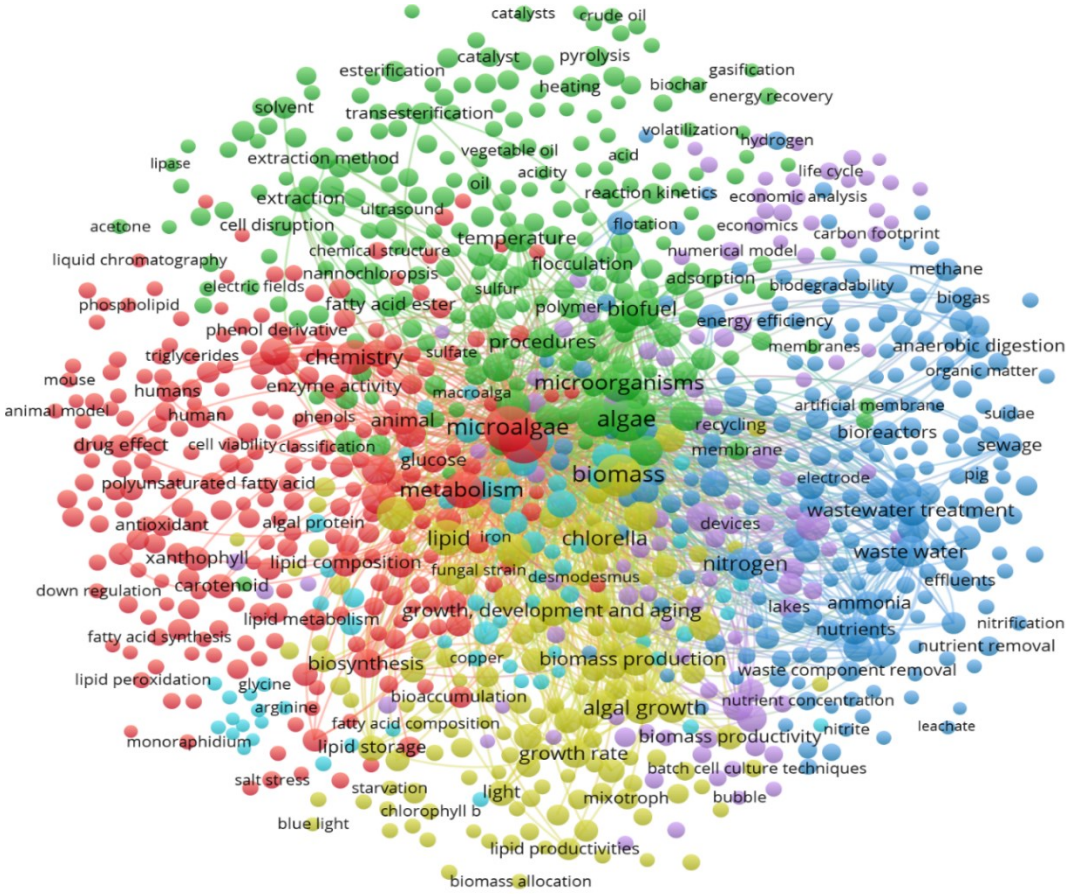

Figure 1. 'Key words' related to microalgae research in the latest five years (Scopus, from 2018 to 2022). Article and review papers were retrieved from a Scopus Collection database search for "microalgae" from 2018 to 2023 in the topic field (which includes title, abstract, author keywords, and keywords plus). A total of 2000 results were processed using VOS viewer® software.

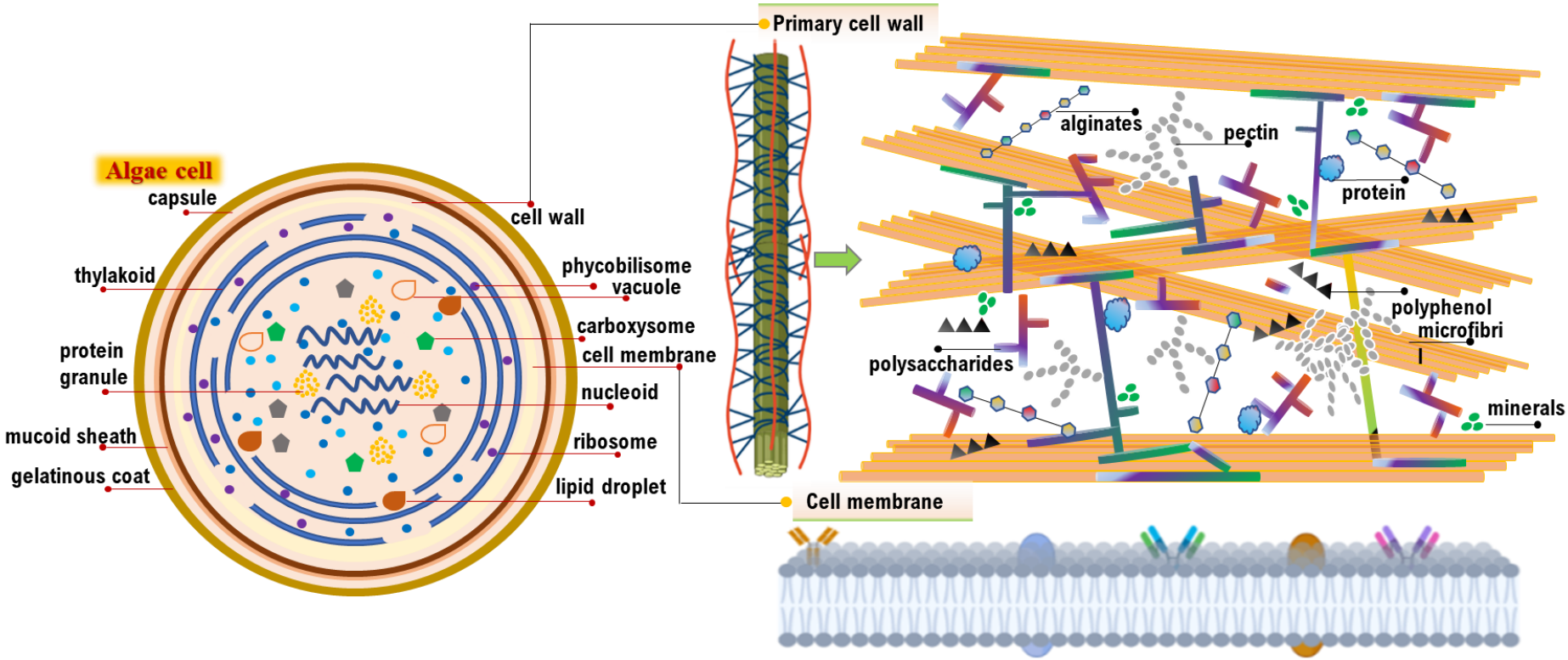

Figure 2. Microalgae cell and cell wall structure.

With the emergence of novel and sustainable extraction technologies, great progress has been made in the efficient recovery of microalgae resources (Chandrasekhar et al., 2022). Compared to traditional extraction technologies (Soxhlet extraction, Folch extraction, hot water extraction, etc.), the innovative extraction technologies, including ultrasound extraction (USN), pressurized liquid extraction (PLE), pulsed electric fields (PEF), supercritical fluid extraction (SFE), microwaves (MW), etc., are highly efficient. With these new technologies, the extraction process greatly reduces the use of organic solvents, and protects the biological function of the microalgae bioactive substances, which protects the environment and saves the cost of the whole process (Gallego et al., 2019; Tavakoli et al., 2021; Zhou, Wang, Berrada, et al., 2022; Zhou, Wang, Saraiva, et al., 2022).

Considering the different properties, functions, and applications of microalgae bioactive substances, processes such as fractionation and purification are essential. In recent years, researchers have conducted numerous experiments on the fractionation of proteins, polysaccharides, lipids, polyphenols, pigments and minerals based on the differences in the physicochemical properties (solubility, polarity, molecular weight and isoelectric point, etc.) of these microalgae components, and some impressive results have been achieved (Antecka et al., 2022; Li et al., 2019; Orr & Rehmann, 2016; Wang et al., 2020). In addition, the digestive behaviour of microalgae nutrients and its impact on human intestinal health is also being researched to a great extent, which lays the foundation for providing healthy microalgae products to humans in the future.

This review summarizes the research progress related to microalgae, including its nutrient composition, extraction and purification technology, digestibility, and impact on human intestinal health, to provide a reference for the future development of the microalgae industry.

2. Microalgae nutrients and biological activities

The potential high-added-value of microalgae is mainly attributed to their large number of nutrients and bioactive compounds. These high-added-value components have been studied for their bioactivity and health properties, such as antioxidant, antiinflammatory, antihypertensive, etc. Figure 3 shows the macronutrients and bioactive compounds ratios of different microalgae based on a dry weight basis.

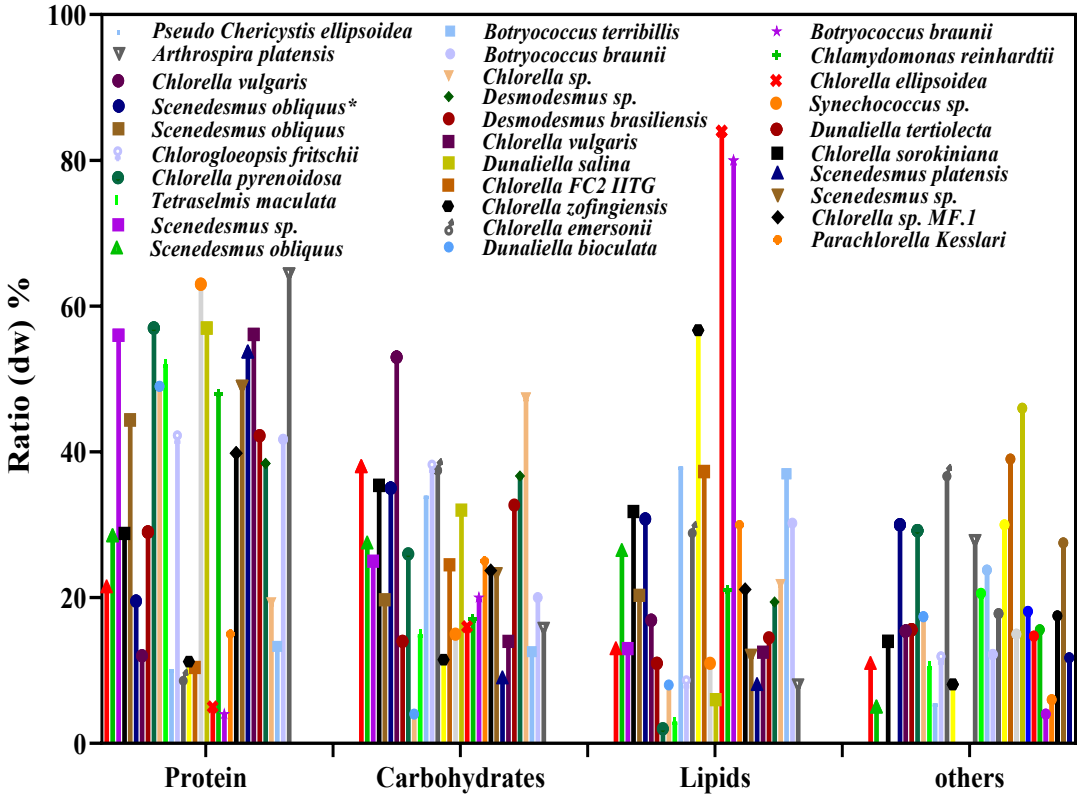

Figure 3. Main bioactive compounds (protein, carbohydrates, lipids, and other compounds) content of several microalgae species.

The protein content of microalgae ranges from 45~70%, and it is considered a substitute to traditional protein products because of its amino acid composition and nutritional properties similar to animal protein (Christaki et al., 2011). The protein content of algae is affected by many factors, such as microalgae-species, growth environment, growth temperature, etc. (Becker, 2007). According to different absorption wavelengths,

microalgae proteins can be divided into phycocyanin, allophycocyanin and phycoerythrin. The bioactivities of microalgae proteins are mainly attributed to their amino acid sequence (Ovando et al., 2018). Meanwhile, more health-promoting properties of algae protein have been confirmed, such as antibacterial, neuroprotective, immune regulation, etc., which have great development and application prospects (Ashaolu et al., 2021). Microalgae is also an important source of lipids, mainly including triglycerides, glycosylglycerides, sphingolipids and lipid-soluble pigments (Nascimento et al., 2020). Microalgae lipids are an important source of Unsaturated Fatty Acids (UFAs), containing a large amount of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), especially *Aurantiochytrium* and *Crypthecodinium cohnii* (Du et al., 2021; Miyashita et al., 2013; Xu et al., 2021). The bioactivities of microalgae lipids from different sources have been confirmed, including antibacterial, antiinflammatory, antioxidant and immunomodulatory (Lupette & Benning, 2020). Microalgae is rich in polysaccharides, the content of this structure ranging from 20~75% (dry basis), depending on the species. For example, brown algae have higher content of alginate and fucoidan, while carrageenan and agar are unique to red algae (Jesumani et al., 2019). Studies have shown that the microalgae-derived polysaccharides are only a little or no degraded at all by digestive enzymes and reach the colon without being absorbed in the upper gastrointestinal tract (de Medeiros et al., 2021). This is relevant due to the large number of microorganisms present in the colon, which can take this undegraded polysaccharides as substrate and, through anaerobic fermentation, produce metabolites, including short-chain fatty acids (SCFAs) that can participate in the body's immunity and metabolism (Gibson et al., 2017). Furthermore, microalgae polysaccharides have been found to have a variety of bioactivities, including anticoagulation, antithrombosis, microbiota modulation , etc., and have been widely used in food, medicine, packaging and other

industries (Diêgo et al., 2020).

In addition, microalgae are also important polyphenols sources, such as catechol, gallic acid, tannins, terpenoids, flavonoids, etc. (Jimenez-Lopez et al., 2021). Due to the unique growth environment of microalgae, the polyphenols they contain show stronger bioactivities, including antiinflammatory, antioxidant, antibacterial, neuroprotective, etc. (Vuolo et al., 2019). Moreover, microalgae also contain pigments, minerals, vitamins, and other bioactive compounds, which has been widely used as food supplements (Afonso et al., 2021).

3. Traditional and emerging extraction techniques

The extraction and recovery of microalgae biomass is a crucial part of exploring the value of microalgae. Selecting the appropriate pre-treatment and extraction techniques can improve the extraction rate while protecting the bioactivity of the target compounds to the greatest extent. Some traditional methods, including Soxhlet extraction, hot water extraction, organic reagent/acid-base extraction, etc., have been used to recover high-value biomass from microalgae. However, these methods have disadvantages, such as high reagent consumption and long extraction time. The thick cell walls of microalgae will also have a negative impact on the release of biomass during the extraction process. In recent years, more and more innovative extraction/pre-treatment techniques have been developed/applied to recover high-value compounds from microalgae with satisfactory results.

3.1 Pulsed electric fields (PEF)

Pulsed electric field as a treatment technology for biomass recovery mainly relies on its short-term instantaneous high-voltage pulse to rupture the cell membrane and generate membrane pores. The disruption of the cell membrane, thereby, increases the permeability of the cell membrane, and at the same time promotes the dissolution of the

intracellular biomass, improving the extraction efficiency (Gómez et al., 2019; Silve et al., 2018). It is worth noting that PEF is a non-thermal treatment method, which is not accompanied by high temperature during the process so it can protect the bioactivity of the target biomass very well and will not affect the sensory quality of the product (Barba et al., 2015). The application of PEF as an assisted technique in the extraction of microalgae biomass has been explored, with studies mainly focussing on the extraction of protein and lipids (Table 1). Zhang et al. (Zhang et al., 2021) and Canelli et al. (Canelli et al., 2022) both explored the effect of PEF treatment on lipid extraction from *Chlorella vulgaris*. The former found that PEF treatment could increase the lipid extraction rate by 166.67%, and the latter also proved that PEF-treatment could improve the lipid bioaccessibility of *Chlorella vulgaris* while maintaining oxidative stability. Zhang et al. (Zhang et al., 2020) and Zhou et al. (Zhou et al., 2022) explored the effect of PEF combined with different solvents on the recovery of microalgae biomass. The results showed that the assistance of PEF could improve the extraction rate of microalgae biomass and shorten the extraction time, including protein, pigments, and polyphenols. In addition, Martínez et al. (Martínez et al., 2019) evaluated the effect of PEF on the extraction of astaxanthin from the Nordic microalga *Haematococcus pluvialis*. In this study, PEF treatment triggered esterase activity, resulting in more astaxanthin in the extracts, which improved the extraction rate.

3.2 Ultrasound-assisted extraction (USN)

Ultrasound (USN) is widely used in the extraction of biomass, which mainly relies on the cavitation caused by ultrasonic waves (Saini & Keum, 2018). When ultrasonic waves are applied, the solvent is cavitated to create microbubbles that rupture after absorbing the ultrasonic energy. These microvesicles generate a large shearing force to destroy the cell wall when they contact the cells and burst, which is considered a simple and low-energy

cost extraction technology (Sallet et al., 2019). Numerous studies have focused on the effects of USN on the extraction of microalgae biomass. Tavakoli et al. (Tavakoli et al., 2021) used USN-assisted combined with food-grade reagents to extract high-value-added compounds in *Spirulina*. Studies have shown that different sonication times can affect the extraction rate of biomass, including the optimal extraction of chlorophyll *a* at 10 min and the highest extraction rate of carotenoids at 20 min. At the same time, these high-value compounds also have the highest antioxidant capacity and antibacterial activity. The effect of continuous sonication on lipid release from microalgae was explored by Natarajan et al. (Natarajan et al., 2014), finding that sonication favoured the release in rigid-walled microalgae and that the rupture of cells was closely related to ultrasonic energy. In addition to biomass extraction, the US has also been found to promote the accumulation and dissolution of microalgae biomass, with the potential for multiple applications.

3.3 Microwave-assisted extraction (MW)

Microwaves (MW) are also considered to be an effective technology for cell disruption and are often used as a pre-treatment technique in biomass extraction. Under the MW treatment, the heat is transferred from the inside to the outside, so that the entire sample is heated. The use of microwave-assisted treatment has been demonstrated to efficiently extract high-value biomass from microalgae, including protein, lipid and carbohydrate. For instance, Zhang et al. (Zhang et al., 2022) found that MW assistance could increase lipid yield from fresh microalgae, from 3.81% to 38.43%, and showed a positive correlation with MW energy input. Sivaramakrishnan et al. (Sivaramakrishnan et al., 2020) explored the effect of microwave treatment on the biomass yield of *Scenedesmus* sp. and found that MW not only increased the yield of total lipids by 1.8-fold but also increased the yield of polysaccharides. Focusing on protein, Chew et al. (Chew et al.,

2019) enhanced the extraction and purification of *Chlorella* proteins by a technique of MW-assisted multiphase partitioning. Under the optimal conditions, the protein recovery and fractionation efficiencies were 63.2% and 67.2%, respectively, which could be attributed to the MW-assisted destruction of the cell wall and enhanced release of biomolecules. In addition to its application in extraction, MW treatment for the transformation and accumulation of microalgae biomass is also being explored (Gan et al., 2020; Hu et al., 2018). It is worth noting that MW also has disadvantages. When MW is used as an extraction technique, the temperature can reach 80~120 °C, which is not suitable for the extraction of heat-sensitive compounds (Nisoa et al., 2022).

3.4 Supercritical fluids extraction (SFE)

Supercritical fluids are also used as a promising technology for the recovery of biomolecules. Common extraction reagents are carbon dioxide (CO_2), methanol, ethanol, pentane, ammonia, etc. Because of the low toxicity and low cost of CO_2, more than 90% of SFE choose this extraction reagent. At the same time, under supercritical conditions, CO_2 has high osmotic and diffusivity to destroy the cell wall, which is helpful for the dissolution of biomass (Cavalcanti et al., 2011; Lorenzen et al., 2017). The application of SFE in microalgae is mostly used to extract lipids, carotenoids, and other fat-soluble components. Conde et al. (Conde et al., 2015) used SFE to extract fatty acids, phenols and fucoxanthin from *Sargassum muticum*. The results showed that SFE combined with 15% ethanol extraction significantly increased the yields of fucoxanthin about 90-fold and had higher proportion of omega-3 fatty acids. Molino et al. (Molino et al., 2018) recovered astaxanthin and lutein from *Haematococcus pluvialis* using ethanol as a co-solvent in conjunction with SFE. The optimal extraction conditions obtained in this study were 65 °C, 550 bar, under which the amounts of astaxanthin and lutein were 18.5 mg/g and 7.15 mg/g, respectively. Moreover, the assistance of SFE can also reduce the use of

organic reagents, which are environmentally friendly.

3.5 Pressurized liquid extraction (PLE)

Pressurized liquid extraction (PLE) is also referred to in the literature as accelerated solvent extraction (ASE), and when water as the extraction reagent, it is also referred to as subcritical water extraction (SWE) (Zhou et al., 2022). When the water is in a subcritical state, its dielectric constant gets gradually closer to the value of organic reagents such as ethanol, which helps to achieve the optimal/maximum extraction effect and is considered to be one of the most efficient extraction technique (Herrero et al., 2015; Pieber et al., 2012). PLE has been used to recover a variety of biomass from microalgae, including carotenoids, lipids, polyphenols, etc. Golmakani et al. (Golmakani et al., 2014) used food-grade reagents combined with PLE to extract carotenoids from *Neochloris oleoabundans* and analysed their profiles. The content of carotenoids increased from 57.4 mg to 120.2 mg, mainly lutein, canthaxanthin, zeaxanthin, and astaxanthin monoesters. Zhou et al. (Zhou et al., 2022) explored the effect of the combined process of PEF and PLE on the recovery of *Spirulina* biomass. Based on PEF extraction, continuous application of PLE assistance can significantly shorten the extraction time, improve the antioxidant capacity of the extract, as well as increase the type and content of phenolic compounds. At present, the application of PLE in the recovery of microalgae biomass is mostly focused on fat-soluble compounds, while the extraction of carbohydrates and proteins is still less applied.

3.6 Other technologies

In addition to the most explored technologies described above, several other techniques have been also used to facilitate the extraction of microalgae biomass. Bernaerts et al. (Bernaerts et al., 2017) used high-pressure homogenization as a mechanical treatment to disrupt microalgae cells and promote the release of intracellular substances. Alavijeh et

al. (Alavijeh et al., 2020) separated protein, carbohydrates and lipids from *Chlorella* using a combination of bead milling and enzymatic (lipase, phospholipase, protease, and cellulase) hydrolysis. When bead milling was used, the recoveries of lipids, carbohydrates and protein were 75%, 31% and 40%, respectively. The enzymatic treatment can significantly improve the recovery of these biomasses. Therefore, selecting an appropriate technique to extract biomass from microalgae can not only improve the extraction efficiency, but also shorten the extraction time and reduce the consumption of reagents.

4. Microalgae nutrients biorefinery and purification

4.1 Microalgae biorefinery

Microalgae biomass can be used as raw materials for bio-based products since they are rich in a variety of bioactive components, even though this idea is currently difficult to achieve on a large scale. To increase the economic value of microalgae, all biomass components need to be valorised, which requires a multi-product biorefinery approach (Lam et al., 2017). The microalgae biorefinery is the process of fractionating microalgae extracts into various useful compounds by applying different biochemical processes (Mutanda et al., 2020). In the complete chain process of microalgae biorefinery, the separation and purification process of a specific component is particularly important. As far as the current reports are concerned, the microalgae biomass biorefinery process is complex and inefficient, and there is still a long way to go from industrial-scale production to commercial product application (Kumar et al., 2022). In recent years, studies have reported separation and purification of microalgae proteins, polysaccharides, lipids, etc., which provided some ideas for the refining process and high-value application of microalgae biomass (as shown in Figure 4). Among the valuable compounds in the microalgae cells, proteins and polysaccharides are hydrophilic, while lipids, and pigments (chlorophylls, carotenoids) are hydrophobic (Alavijeh et al., 2020).

Table 1. Application of innovative technologies in microalgae biomass extraction

Technique	Microalgae	Target compounds	Results	Ref.
Pulsed electric fields	*Chlorella vulgaris*	Lipid	Improves lipid yield and bioavailability, preserves oxidative stability of biomass	(Canelli et al., 2022)
Pulsed electric fields	*Chlorella vulgaris*	Lipid	Lipid extraction rate increased by 166.67%	(Zhang et al., 2021)
Pulsed electric fields	*Chlorella vulgaris*	Protein	Improved protein extraction rate	(Buchmann et al., 2019)
Pulsed electric fields and high-shear homogenization	*Arthrospira platensis*	Water-soluble compounds	The tandem use of the two technologies significantly improves the recovery of the target compound, showing a synergistic effect	(Carullo et al., 2021)
Pulsed electric fields and high-pressure homogenization	*Chlorella vulgaris*	Intracellular compounds	Pulsed electric field can improve the extraction rate of carbohydrates, and high-pressure homogenization has a more significant effect on the extraction rate of protein.	(Pataro et al., 2018)

Table 1. (cont.)

Technique	Microalgae	Target compounds	Results	Ref.
Ultrasound	*Spirulina platensis*	Carotenoids, chlorophyll	Improves biomass yields and has higher antioxidant and antibacterial activities	(Tavakoli et al., 2021)
Ultrasound	*Tetraselmis suecica, Nannochloropsis* sp.	Lipid	Accelerates cell rupture and promotes lipid release	(Natarajan et al., 2014)
Ultrasound	*Spirulina platensis*	Protein	Under the optimized conditions, the content of protein and total phenols were significantly increased, and the digestibility of protein was improved	(Yucetepe et al., 2018)
Ultrasound	*Phaeodactylum tricornutum*	Nutrients and antioxidant Bioactive Compounds	Positive effects on protein, carbohydrate and pigment recovery	(Al Khawli et al., 2021)
Ultrasound and buoyant beads	*Chlorella vulgaris*	Biomass	The combination of the two methods increases the content of saturated fatty acids and gives them better oxidative stability	(Zou et al., 2021)

Table 1. (cont.)

Technique	Microalgae	Target compounds	Results	Ref.
Microwave	*Scenedesmus* sp.	Lipid, biomass and exopolysaccharides	Increased biomass content	(Sivaramakrishnan et al., 2020)
Microwave	*Chlorella*	Lipid	Increased content of short-chain and saturated fatty acids	(Cheng et al., 2013)
Microwave	*Chlorella vulgaris*	Lutein	Increases lutein yield and purity	(Mary Leema et al., 2022)
Microwave	*Chlorella vulgaris*	Protein	Increased protein yield by 2.54 folds	(Chew et al., 2019)
Microwave	*A. Protothecoides*	Lipid	Lipid extraction rate increased to 90%	(Zhang et al., 2022)
Supercritical CO_2	*Sargassum muticum*	Fatty acids, phenolics, fucoxanthin	Increased biomass content and free radical scavenging capacity	(Conde et al., 2015)
Supercritical CO_2	*Scenedesmus obliquus, Scenedesmus obtusiusculus*	Lipid	The extraction rate of lipids reached 90%	(Lorenzen et al., 2017)

Table 1. (cont.)

Technique	Microalgae	Target compounds	Results	Ref.
Supercritical CO_2	*Haematococcus pluvialis*	Polyphenols	Maximizes the extraction rate of astaxanthin and lutein, avoids thermal degradation of carotenoids	(Molino et al., 2018)
Supercritical CO_2	*Chlorella vulgaris, Nannochloropsis oculata*	Lipid	The lipid extraction efficiencies of the two microalgae were 97% and 83%, respectively.	(Obeid et al., 2018)
Supercritical CO_2	*H. pluvialis*	Astaxanthin and triacylglycerol	The triglyceride content in the extract exceeds 99%, and the recovery rate of astaxanthin is about 72~85%.	(Kwan et al., 2018)
Pressurized liquid extraction	*Spirulina*	Protein, polyphenol, chlorophyll	Improved biomass extraction and increased types of phenolic compounds	(Zho et al., 2022)
Pressurized liquid extraction	*Haematococcus pluvialis*	Carotenoids	Significantly increases the antioxidant activity of the extract	(Jaime et al., 2010)
Pressurized liquid extraction	*Isochrysis*	lipid	Improved lipid and total fatty acid recovery	(Bueno et al., 2020)
Super-high hydrostatic pressure	*Chlorella sp.*	Lipid	Obtain high-quality lipids	(Xu et al., 2021)

Table 1. (cont.)

Technique	Microalgae	Target compounds	Results	Ref.
Ultra-high-pressure extraction	*Haematococcus pluvialis, Porphyridium cruentum*	Bioactive compounds	Promotes the extraction of carotenoids in *Haematococcus pluvialis* and the extraction of B-phycoerythrin, carotenoids in *Porphyridium cruentum*	(Bueno et al., 2020)

According to recent studies, during microalgae biomass fractionation, lipids or lipid-soluble pigments could generally be recovered by organic solvent extraction (Soxhlet, Folch, etc.) or supercritical fluid extraction technology (Zhou et al., 2022). After the removal of lipids, proteins and polysaccharides could be further recovered by aqueous extraction (Amorim et al., 2020). It is worth mentioning that the use of organic reagents during the biorefinery of microalgae multi-products may lead to protein denaturation, so it is recommended to separate proteins before lipid extraction with organic solvents to achieve full utilization of biomass (Lam et al., 2018). A lot of research has been addressed the separation and purification of protein and polysaccharide components in microalgae extracts, and it is known that the yield and purity of the final product is affected by microalgae species, protein and polysaccharide types, and separation and purification methods (reagents, separation principles, etc.) (Sierra et al., 2017).

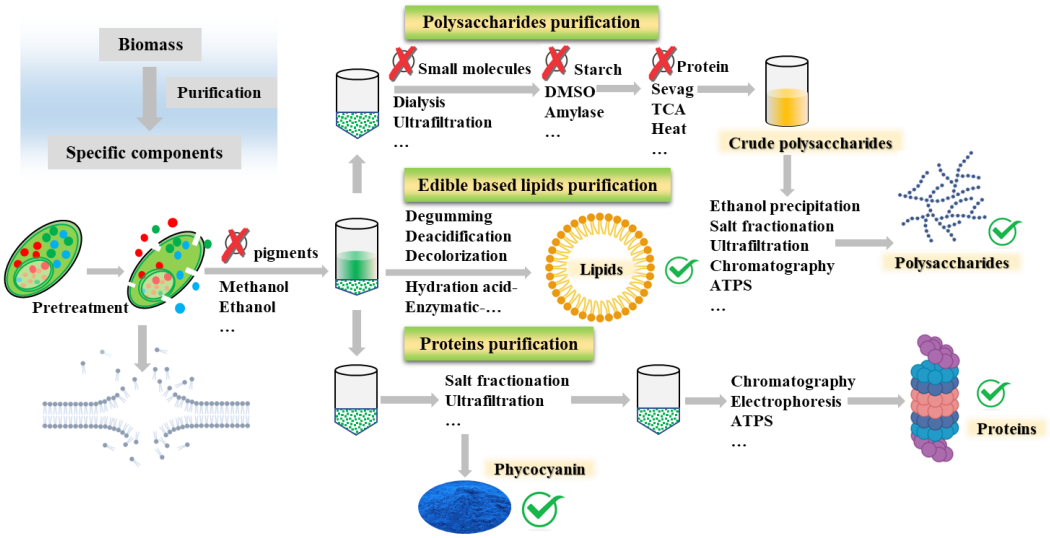

Figure 4. Purification process of proteins, polysaccharides, lipids, etc. from microalgae. ✗ and ✓ means remove or recovery specific compounds from microalgae respectively.

Moreover, various studies only emphasized the fractionation of a certain bioactive substance, and there are few studies on the biorefinery of more than two microalgae

substances.

4.2 Edible-based lipids purification

Biorefinery of microalgae lipids in the past was more focused on the production of biofuels, including the production of products such as bio-oil, syngas, bioethanol, and biogas (Koyande et al., 2019). In recent years, the extraction and purification of microalgae lipids are also expected to be used in the food industry. As described in section 2 and section 3, the Soxhlet, Folch and Bligh-Dyer methods have been used to extract microalgae lipids in the past, which rely on the use of organic solvents. The new extraction technologies developed in recent years, including ultrasound, pulsed electric field, microwave, high pressure, etc., can destruct the cell wall of microalgae and promote the contact between organic solvents and intracellular substances, so as to efficiently extract microalgae lipids (Zhou et al., 2022).

Regardless of traditional or novel extraction techniques, extracted lipids from microalgae usually contain not only triglycerides, but also various other lipidic species, such as free fatty acids, phospholipids, sterols, amino acids, and pigments (Paisan et al., 2017). The presence of impurities in crude algae oil can reduce its quality as biodiesel or edible oil, such as the presence of phospholipids can reduce the yield of fatty acid methyl esters or biodiesel (Iyer, 2016). In recent years, the frequently reported concept of algae biorefinery describes the sequential bioprocessing of algae biomass for biodiesel and other high-value-added products in an effort to utilize the entire biomass biodiesel production in a more sustainable and cost-effective manner (Khoo et al., 2019). Therefore, in the refining process of microalgae lipids, purification processes are required to improve their quality as edible oil.

Crude microalgae oil contains some peptizing impurities, such as phospholipids, proteins, mucilage, etc (Paisan et al., 2017). The existence of these peptizing impurities

reduces the storage stability and edible value of oils and affects the refining of oils. There are many methods of oil degumming, such as hydration-, acid-, adsorption-, enzyme-, membrane-, etc. Hydration degumming and acid degumming are commonly used in the oil industry (dos Passos et al., 2022). Hydratable phospholipids can be easily removed from oils by water degumming. During this process, phospholipids absorb water and lose their lipophilic properties, become insoluble in oil and agglomerate into a colloid, which is precipitated and then separated by centrifugation or gravity (Nikolaeva et al., 2020). The factors that impact on the effect of hydration and degumming are mainly related to the operating temperature, time, and the amount of added water. Acid degumming consists on adding a certain amount of acid to the crude oil, such as citric acid, oxalic acid, phosphoric acid, etc., to convert non-hydratable phospholipids into hydrated phospholipids, and then add a small amount of water or alkali to further remove phospholipids (Paisan et al., 2017). Paisan et al. used Response surface methodology (RSM)-Box-Behnken design (BBD) to optimize the effects of hydration degumming and acid degumming on *Chlorella* oil refining (Paisan et al., 2017). The results showed that under the optimum conditions of acid degumming temperature of 90 °C, time of 60 min, and phosphoric acid of 0.42 wt.%, the maximum phospholipid removal rate reached 82.5%. However, under optimal conditions for water degumming (temperature 100 °C, 30 min, water 80 wt.%) only 19.4% of the phospholipids were removed, indicating the main phospholipids in *Chlorella* are non-hydrated phospholipids.

Microalgae oil is quite different from traditional edible oil, as it has high polar lipid content (including EPA), which leads to high viscosity, poor fluidity, high acid value, and high pigment content (dark green) (Xue et al., 2018). In particular, long-chain polyunsaturated fatty acids such as EPA or DHA imply the high nutritional value of microalgae lipids. However, the refining process of microalgae lipids may cause huge

losses throughout the different steps (Borowitzka & Moheimani, 2013). The degumming step mainly removes gummy substances such as phospholipids, which leads to a sharp decrease in the nutritional value of algae lipids. Following, the deacidification step is used to remove free fatty acids, which may imply the loss of some other useful lipids. In addition, during the decolorization step, which is used to remove pigments, many polar lipids are lost, resulting in a decrease in EPA content. At present, although the refining process has been widely used in the production of animal and vegetable lipids, the particularity of microalgae lipids makes the refining method of edible lipids not directly applicable, so there are few research reports related to microalgae lipids refining (Xue et al., 2018). Therefore, the refining process in microalgae lipids production deserves more attention, and we should explore refining processes that help retaining the beneficial components of microalgae lipids while having a minimal impact on fatty acid profiles.

4.3 Protein purification

Generally, protein is the main component of the crude aqueous extract after microalgae cell disruption, which can have a direct application on protein substitution in the food industry (such as meat products) or can be further purified (Caporgno et al., 2020). Compared to the application of crude microalgae extracts, protein purification processes involve the use of solvents and chemical components, such as acids, which add economical costs and are potentially environmentally unfriendly (Böcker et al., 2021). However, protein concentrates or isolates are products that are expected to have absorbent, gelling, foaming and emulsifying properties for a variety of industrial applications (Soto-Sierra et al., 2018). Therefore, for concentrates and isolates to be cost-competitive, downstream processing should recover functional proteins in the highest possible yield and purity. However, purifying high-quality proteins from microalgae remains a technical challenge for the following reasons: a. the rigid cell walls of microalgae lead to limited

protein availability; b. the high concentration of anionic or non-polar polysaccharides in the extract; c. inherent problems related to protein stability (Anjos et al., 2022). Currently, challenge 'a' can be solved by relying on different microalgae cell disruption techniques, including bead milling, high pressure, ultrasound, microwave, pulsed electric field, cavitation, thermal and chemical disruption methods, or an integration of multiple methods (Zhou et al., 2022). For challenges 'b' and 'c', the removal of impurities in microalgae extracts or the direct isolation of proteins under the premise of preserving protein stability becomes a key strategy.

After obtaining the crude extracts, the protein can be isolated by precipitation using trichloroacetic acid (TCA)/acetone reagent and removing the supernatant (Deatherage Kaiser et al., 2015). Low pH and negative charge on TCA and the presence of organic acetone in the environment can cause protein denaturation, which eliminates proteolytic and degradative activities and makes re-solubilization of precipitated proteins extremely difficult (Khanra et al., 2018a). In addition, ammonium salt precipitation (ASP) has also been used for the separation and purification of microalgae proteins. As we know, the isolation and purification of phycocyanin have been extensively studied for its many health benefits (e.g., immunomodulatory, antiinflammatory, anti-cancer, anti-diabetic, neuroprotective, hepatoprotective and antioxidant effects, etc.) and wide range of applications (biomedical, diagnostic, beverages, food, nutraceuticals, and medicines, etc.) (Ashaolu et al., 2021).

ASP is commonly used for phycocyanin purification due to its simplicity and low-cost (Amarante et al., 2020). For example, the research of Gorgich et al. showed a significant increase in phycocyanin yield when using AS concentrations above 60% and obtained purified phycocyanin content of more than 92% when using AS at 60% saturation (Gorgich et al., 2020).

In addition to the use of TCA/acetone/AS precipitating proteins, there are many techniques currently available for further purification of microalgae proteins, including chromatography (column chromatography, gel chromatography, ion exchange chromatography, high performance liquid chromatography), membrane separation, and ultrafiltration, etc. Amarante et al. purified C-phycocyanin from *Spirulina* by ion exchange chromatography (Amarante et al., 2020). The results showed that the chromatographic condition resulting in high recovery and purity consisted of equilibration and washing with 0.025 mol/L Tris-HCl buffer (pH 6.5) and elution combining a step with 0.08 mol/L NaCl in 0.025 mol/L Tris-HCl buffer (pH 6.5) and a pH gradient elution with 0.05 mol/L citrate buffer (pH 6.2~3.0). Finally, this process resulted in C-phycocyanin with purities of 4.2 and 3.5 with recoveries of 32.6% and 49.5%, respectively, in one purification step. The algae-derived biological protease is mainly obtained through chromatographic purification technology. For example, Wang et al. (2014) purified the d-galactose-6-sulfurylase enzyme from the microalge *Betaphycus gelatinus* using ion-exchange chromatography and hydrophobic interaction chromatography, enabling the isolation of 65 kDa monomeric protein molecules (Wang et al., 2014). Han et al. isolated a 21.4 kDa protein molecule (N-acetyl-d-galactosamine-specific protein) from the filamentous red alga *Aglaothamnion oosumiense* using affinity chromatography (Han et al., 2012). Verdel et al. isolated a 43 kDa monomeric haloperoxidase from the freshwater microalgae strain *Chaetoceros* sp. by using ion exchange and gel filtration chromatography (Verdel et al., 2000).

Compared to chromatography, membrane separation offers several advantages, including high volumetric flux, high extract concentration, and lower cost. Therefore, membrane separation technology is also an effective method for microalgae protein purification. In this sense, Chaiklahan et al. purified phycocyanin by membrane

separation and showed that membrane with MWCO at 50 kDa, 69 kPa and 75 mL/min of flow rate with a mean permeate flux 26.8 L/h m^{-2} and a retention rate of 99% was found to be optimal, giving phycocyanin recovery of 88.6% and 82.9%, respectively (Chaiklahan et al., 2011). *Chlorella vulgaris* protein was purified by Ursu et al. using a PES membrane with a 300 kDa molecular weight cut-off and a 0.1 m^2 filter surface. The results showed that microalgae proteins with higher emulsifying capacity (1780 ± 20 and 3090 ± 50 mL oil/g protein) and higher stability (72% ± 1% and 79% ± 1%) were obtained when using membrane ultrafiltration (Ursu et al., 2014).

Either it is precipitation methods, chromatography technology, or membrane separation process, there are some disadvantages. The precipitation method takes a long time, it cannot be expanded on a large scale, the cost of chromatography technology is high, the protein yield is low, and the membrane separation efficiency is affected by multiple factors such as membrane resistance, porosity, morphology, shear rate, and hydrodynamics (Azmi et al., 2021). In addition to these techniques, some novel techniques, such as ionic liquid-based-aqueous two-phase system, have also been used to purify microalgae proteins in recent years. Garcia et al. used an aqueous two-phase system (ATPS) to isolate proteins from crude microalgae extracts of *Neochloris oleoabundans* and *Tetraselmis suecica*, and experimental data showed that proteins and sugars were selectively fractionated in the top and bottom phases, respectively (Suarez Garcia et al., 2018). In general, the majority of microalgae protein purification processes do not yield individual protein fractions, and even after protein isolation, multiple protein fractions as well as some polysaccharides are still present (Bertsch et al., 2021). Moreover, the microalgae species and the culture conditions will inevitably affect the amount and form of proteins, which increases the difficulty in formulating a large-scale purification process of microalgae protein.

4.4 Polysaccharide purification

Microalgae-derived polysaccharides are widely used in food, medicine, cosmetics and other products (Tang et al., 2020). The most studied microalgae polysaccharides include agar (agarose and agaropectin), carrageenan (*κ*-, *λ*-, and *ι*-), alginate and fucoidan, etc. (Khanra et al., 2018a). Microalgae polysaccharide purification is an essential step to enrich polysaccharide species or isolate polysaccharide components for usable, safe, and reproducible studies. The purification process of microalgae polysaccharides is based on the obtained crude polysaccharide extract or the crude polysaccharide product after removing impurities (lipid, pigment, protein, small molecular substances, etc.). Direct extraction of microalgae without removing impurities will obtain an extract containing various components (protein, lipid, pigment, small molecule, etc.), which is unfavourable for further separation and purification of polysaccharides (Tang et al., 2020). Removing these impurities in advance will obtain relatively high-purity polysaccharides for further purification, however, the process is cumbersome and will cause the loss of polysaccharides in the sequential stage of removing small molecules (ultrafiltration, dialyzed), pigments (active carbon, H_2O_2 oxidation), starch (enzymatic hydrolysis, DMSO soaking) and proteins (Sevag, TCA, protease) (Kang et al., 2022; Qiu et al., 2022; Tang et al., 2020).

Compared to removing impurities, polysaccharide purification is a more delicate process. In the process of purification of microalgae polysaccharides, suitable techniques should be selected according to the properties of polysaccharides, such as molecular weight distribution (MWD), charge characteristics, affinity characteristics, etc. The methods of separation and purification of polysaccharides include ethanol precipitation, salt fractionation, coordination by quaternary ammonium salt, ultrafiltration, ion exchange column chromatography, gel column chromatography and affinity

chromatography, etc (Beaumont et al., 2021). Microalgae polysaccharide purification usually includes two main processes, namely, obtaining relatively high-purity crude polysaccharides by solvent precipitation, ultrafiltration, etc., and further fractionating pure polysaccharides by chromatography technology.

The principle of ethanol precipitation of polysaccharides is to dehydrate the polysaccharides by reducing the dielectric constant of the aqueous solution, resulting in polysaccharide precipitation, which is often combined with column chromatography to purify polysaccharides from microalgae extracts. Ma et al. (2019) used 80% ethanol to precipitate the ultrasonic extract of *Spirulina*, and finally separated it with a gel column chromatography to obtain polysaccharide components with an average molecular weight of 29.6 kDa, and the monosaccharides were mainly rhamnose (24.7%), glucose (16.15%) and galactose (13.32%) (Ma et al., 2019). Shi et al. used pure ethanol to precipitate the ultrasonic extract of *Chlorella* (water bath at 100 °C uing TCA precipitation to remove proteins in advance), and finally separated polysaccharides with molecular weights of 81.877 kDa and 1.749 kDa by column chromatography respectively, and the monosaccharides were mainly mannose and glucose (Shi et al., 2007).

However, the ethanol precipitation method has the disadvantage of poor separation selectivity of polysaccharides, which requires repeated precipitation process to improve the purity, resulting in large loss and reduced recovery rate of polysaccharides. Compared with the reagent precipitation method, the ultrafiltration technology has the advantages of easy industrialization, environmental friendliness, and low consumption. The principle of ultrafiltration technology is to separate components according to the membrane pore size, which can be used for the separation of crude polysaccharides or the classification of refined polysaccharides. For example, Wang et al. obtained *Spirulina* extract using ultrasound combined with hot water extraction, and further purified the bioactive

polysaccharide SPS-3-1 (623.02 kDa) using ultrafiltration centrifugation (100 kDa, 4000 r/min, 20 min) and gel filtration chromatography (Wang et al., 2020). Further, SPS-3-1 was identified as a homogeneous β-pyran polysaccharide with 1→2, 1→3 and 1→4 glycosyl bonds, mainly composed of D-ribose, L-rhamnose, L-arabinose, L-fucose and D-glucose composition. Similarly, Sheng et al. (2007) used 100 kDa, 30 kDa, 10 kDa, 3 kDa, 1 kDa cut-off ultrafiltration membranes to separate polysaccharides from *Chlorella pyrenoidosa* extracts and found that the main polysaccharide components could be separated by 30 kDa membranes (Sheng et al., 2007). Further, two-component polysaccharides, CPPS Ia (69.658 kDa) and CPPS IIa (109.406 kDa), were purified by anion-exchange chromatography and size exclusion chromatography, and the monosaccharides were mainly rhamnose, mannose, glucose, and galactose.

In addition to the above-mentioned microalgae polysaccharide purification methods, ATPS, as a new liquid-liquid extraction technology, can also be used as a polysaccharide purification technology. The aqueous two-phase system constitutes a more environmentally friendly and efficient pre-treatment solution for the one-step separation and purification of polysaccharides (Wu et al., 2017). In recent years, the aqueous two-phase system (ATPS) has been used for the separation and purification of polysaccharides due to its mild liquid environment (Chen et al., 2016). In fact, Wu et al. isolated 12.45 mg/g acidic polysaccharide from *Spirulina platensis* using ethanol-ammonium sulfate ATPS, which consisted mainly of glucose and a small amount of rhamnose and mannose (Wu et al., 2017). Compared with traditional polysaccharide purification methods, such as ethanol precipitation and Sevag method for deproteinization, ATPS has the advantages of a simple procedure, short time and less use of organic reagents, and can be used as an effective means for polysaccharide purification (Shi et al., 2018).

In general, there is no uniform method for the purification of microalgae

polysaccharides, which is attributed to the diversity of microalgae and polysaccharide species, and each method of polysaccharide isolation and purification has advantages and disadvantages. There are many studies based on the purification of microalgae polysaccharides, but most of the existing techniques are still not suitable for large-scale industrial production.

4.5 Other compounds

As secondary metabolites of microalgae, phenols have complex and diverse chemical structures and have a wide range of physiological functions, including antioxidant, anti-inflammation, anti-allergy, etc. These physiological functions make them play an excellent role as a food supplement or in the prevention of various diseases. Based on this, researchers are focusing on obtaining stable and pure phenolic compounds from different matrices, while further developing them into more valuable products.

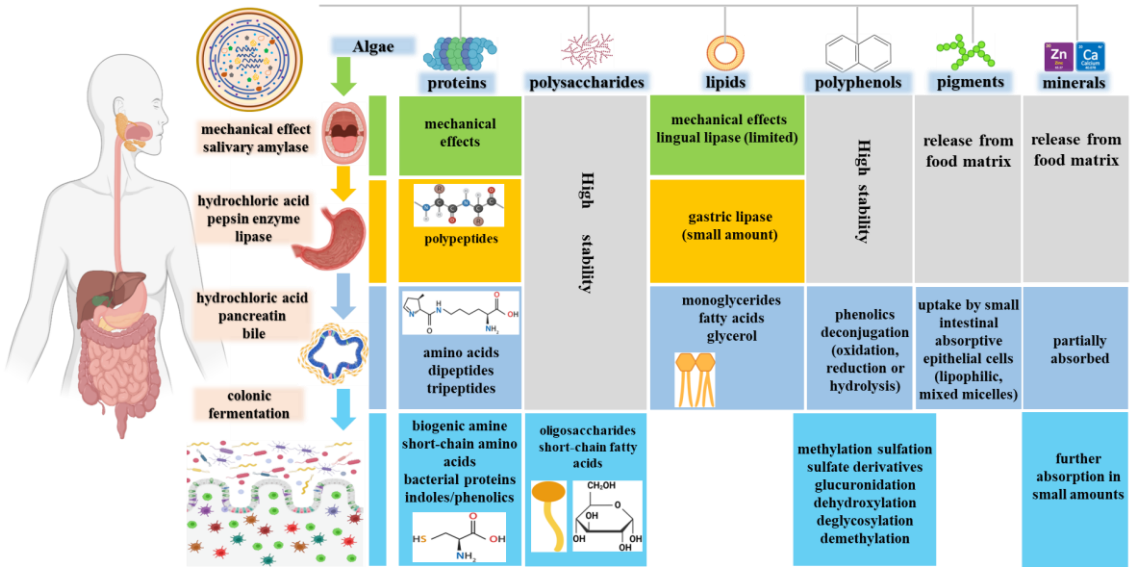

Figure 5. Microalgae nutrients (proteins, polysaccharides, lipids, polyphenols, pigments, and minerals) digestive behaviors in human Oral-gastrointestinal-colon tract.

A variety of classical and innovative techniques have been used for the extraction of phenols from microalgae, such as PEF, SFE, MW, US, etc. However, after these

technologies are used to obtain phenols from microalgae compounds, they are combined with other technologies to separate and purify phenols to obtain specific target compounds that can maximize their physiological functions (Al Daccache et al., 2022; Castro-Muñoz et al., 2016).

For example, in the study of Klejdus et al. (Klejdus et al., 2017), US combined with PLE was used to extract phenolic compounds from various microalgae. Then, micro-elution solid-phase extraction and elution solid-phase extraction were used to further separate and identify phenolic compounds. In *Cystoseira abies-marina*, 14 phenolic compounds were isolated and identified including gallic acid, salicylic acid, and protocatechuic acid etc. Mahendran et al. (Mahendran et al., 2021)extracted crude polyphenols from red microalgae using Soxhlet extraction and further purified the crude polyphenols using DEAE-cellulose 52 column to obtain 15 kinds of phenolic compounds such as flavonoids, saponins, tannic acid, etc. Furthermore, the application of membrane separation technology to separate and purify phenolic compounds from various matrices is being explored, but the application in microalgae is less reported (Castro-Muñoz et al., 2016).

In addition to the various high-value compounds mentioned above, pigments are also one of the important compounds for microalgae to perform biological functions. Pigments in microalgae such as chlorophyll, carotenoids, and chromoproteins have been found to be used as antioxidants or colorants in different fields such as food, medicine and cosmetics (Silva et al., 2020; Zhou et al., 2022). Most of the microalgae pigments are intracellular substances, choosing a suitable extraction method can release the pigments better and increase the extraction rate. In recent years, in addition to the traditional solvent extraction methods, some innovative techniques such as SFE, PLE, MW, and enzyme-assisted extraction have been used for the extraction of microalgae pigments. These

techniques showed the advantages of high efficiency, environmental protection, and easy operation. Similar to phenolic compounds, in order to obtain high-purity pigments, it is necessary to further purify the crude extraction combined with column chromatography, ultrafiltration, membrane separation and other technologies (Shen et al., 2023). For example, Gonçalves et al. (Gonçalves et al., 2020) used ultrasonic-assisted extracted pigments from *Tisochrysis lutea* and analysed the composition. Then the fucoxanthin in the extract was purified using a two-step purification method of centrifugal partition chromatography and PE-C18 column chromatography. The study found that this process can effectively separate fucoxanthin with a purity greater than 99%. These fucoxanthins from *Tisochrysis lutea* also exhibited anti-proliferative potential in melanoma cells.

Fábryová et al. (Fábryová et al., 2019) reported on lutein, three methods including US-assisted, maceration extraction and grinding-assisted extraction were used to extract lutein from *Chlorella* and the content was calculated by high-performance liquid chromatography with Diode-Array Detection (HPLC-DAD). Then, the lutein was purified by high-performance counter-current chromatography (HPCCC). It was found that the extraction rate of lutein was higher when n-heptane/ethanol/H_2O was used as solvent combined with US-assisted. Moreover, combined with HPCCC, lutein with a purity of 92% can be obtained, and it can be further purified to 97% by gel dialysis, which is considered a new mode of microalgae pigment extraction and purification. In addition, the separation and purification of various carotenoids and pigment proteins have also been reported (Chen et al., 2019; Shen et al., 2023).

5 Digestibility properties and effects on gut health

5.1 Proteins

As shown in **Figure 5**, the digestion process of protein begins in the stomach, which generates large peptides under the action of pepsin, which are afterwards hydrolysed into

smaller peptides and free amino acids under the action of small intestinal trypsin, chymotrypsin, elastase, carboxypeptidase, and other pancreatic enzymes. The action of the brush border membrane enzymes finally generate short peptides and aminoacids that can be absorbed (Kazir et al., 2019). Generally, there are three main ways of peptide uptake, namely, the peptide transporter peptide PepT1, which allows co-transport of dipeptides or tripeptides with H^+ ions; or the cell-penetrating peptide (CPP) of cell membrane cargo proteins, which allows peptide entry by endocytosis, or by tight junctions paracellular pathways which increase permeability (Peretti & Mas, 2022).

The protein quality (in terms of content, ratio, and availability of amino acid profiles) of microalgae such as *Chlorella* and *Arthrospira*, etc., according to the FAO/WHO guidelines, have beneficial essential amino acids required by humans (Matos, 2019). Human digestive proteases are specific for bonds near certain amino acids, making itcritical to evaluate the gastrointestinal digestibility of microalgae proteins (Kazir et al., 2019). The INFOGEST static *in vitro* simulation of food digestion in the gastrointestinal tract is one of the most reliable and standardized protocols for analysing protein bioaccessibility (Minekus et al., 2014; Brodkorb et al., 2019). Kazir et al. (2019) evaluated the protein bioaccessibility of two algae (*Ulva sp*. and *Gracilaria sp*.) and found that under simulated gastrointestinal digestion, algal protein concentrates (APCs) could reach proteolysis extents of at least 89% (Kazir et al., 2019).

Marrion et al. (2005) evaluated the protein bioaccessibility of two red algae and showed that the *in vitro* protein digestibility of *P. palmata* and *G. verrucosa* were 4.9% and 42.1%, respectively, and that protein digestibility was positively affected by the presence of soluble fiber content in red algae (Marrion et al., 2005). Niccolai et al. (2019) assessed the protein bioaccessibility of 12 species of microalgae and found that *A. platensis*, *C. sorokiniana IAM-C212* and *C. vulgaris* had the highest bioaccessibility, while *T. suecica*,

P. tricornutum and *P. purpureum* has the lowest bioaccessibility, and the microalgae's strong cell walls or extracellular polysaccharides were found to limit the action of digestive enzymes (Niccolai et al., 2019).

In order to improve the protein bioaccessibility, Maehre et al. (2015) explored the effect of heat treatment on microalgae protein bioaccessibility and found that the content of accessible amino acids in *Palmaria palmata* increased by 86%~109% after heat treatment, because heat treatment resulted in partial or complete denaturation of the original protein structure, making it easier for gastrointestinal enzymes hydrolysis, thereby improving protein utilization (Maehre et al., 2016).

Furthermore, one of the main reasons for low protein bioaccessibility is that microalgae contain rigid cell walls composed of silicates and ceramides, so cell disruption can be pre-treated to improve microalgae protein digestibility (Demarco et al., 2022).

Evaluating protein digestibility is relatively easy with *in vitro* systems, since most of the proteins in the digested sample are derived from the sample, however, it remains questionable whether simulated digestion with *in vitro* models can perfectly reflect the physiological reality (Dupont et al., 2017). Until now, there are still only a few *in vivo* studies of algae protein digestion, which make the detection of dietary peptides more complicated and difficult due to the presence of endogenous proteins secreted from different regions of the digestive tract.

Overall, unlike some carbohydrates and fibres, most proteins are digested and absorbed during the gastrointestinal digestion stage, and only a small fraction enter the large intestine or colon for fermentation by the gut microbiota, so related studies are rarely available.

5.2 Carbohydrates

Digestion of carbohydrates begins in the mouth, where starch undergoes initial hydrolysis

by chewing and salivary alpha-amylase, but the enzymatic hydrolysis is terminated in the stomach due to the low pH value. Then the hydrolytic process of starch is resumed in the duodenum, thanks to the action of amylase contained in pancreatin. Further, monosaccharides or disaccharides are produced under the action of pancreatic amylase, lactase, sucrase and maltase in the small intestine, which are finally absorbable (Qadir & Wani, 2022).

Digestive enzymes are specific for the digestion of polysaccharides, such as salivary and pancreatic amylase for the cleavage of α-(1→4) bonds, lactase for the cleavage of β-(1→4) bonds in lactose, and isomaltase for cleavage of α-(1→6) bond in isomaltose residues (Aguiar & Cazarin, 2021). However, the polysaccharides in microalgae generally have large molecular weights and complex structures, which are difficult to be decomposed by human gastrointestinal digestive enzymes. In fact, different studies have documented that microalgae polysaccharides cannot be degraded by simulated oral, gastric, and small intestine conditions. Focusing on the oral stage, a study incubating sulfated polysaccharide with human saliva solution at room temperature for 2 hours found that the molecular weight of the polysaccharide remained unchanged and no free monosaccharide were released, indicating that salivary amylase could not cleave the polysaccharide glycosidic bond (Di et al., 2018). Further, Chen et al. (2018) incubated *Ascophyllum nodosum* polysaccharides in *in vitro* gastric simulated digestive juice at a constant temperature of 37 °C for 6 hours and found that polysaccharides were not degraded in acidic gastric juice (electrolyte solution with pepsin, gastric lipase and mineral salts, pH 3.0), indicating that algae polysaccharides were not digested by the stomach (Chen et al., 2018). Finally, the microalgae polysaccharides from *Gracilaria chouae* in the small intestine environment (a mix of sodium bicarbonate, phospholipids, bile salts, and enzymes secreted by the pancreas, liver, and gallbladder (Zheng et al.,

2020)) showed to be structurally stable after incubation for 6 hours at pH 7, indicating that the polysaccharides were not digested in the small intestine (Li et al., 2021).

To sum up, microalgae polysaccharides cannot be digested by the upper gastrointestinal tract and enter the colon, and finally, they are utilized by gut microbiota and begin to exert their health benefits. The gut microbiota is closely linked to human health, as it has been related to relevant functions, such as protecting the intestinal epithelial barrier, regulating the immune system, or producing metabolites (Zhang et al., 2015). Studies have shown that microalgae polysaccharides have the function of altering the abundance of probiotic and harmful bacteria in the gut microbiota to maintain gut homeostasis. A recent study found that *Spirulina* polysaccharides (PSP) increased the abundance of *Lactobacillus*, *Xenobacterium*, *Xenobacterium* and *Olsenella* and decreased the abundance of *Bacteroides* and *Acinetobacter* in the gut microbiota of mice with lung cancer, which suggested that PSP could be used to protect against lung cancer disease (Lu et al., 2022). In addition, the utilization of polysaccharides by gut microbiota can produce beneficial metabolites-short-chain fatty acids, such as butyrate, acetate, and propionate, which are beneficial for the regulation of immunity, glucose homeostasis, lipid metabolism, etc (Morrison & Preston, 2016). A previous study in our laboratory found that microalgae (*Spirulina*, *Chlorella* and *Phaedactylum tricornutum*) extracts (rich in carbohydrates) promoted the release of SCFAs such as acetate, butyrate, and propionate during colonic microbiota *in vitro* fermentation (Zhou et al., 2023).

Studies have also shown that the molecular weight (MW) of polysaccharides have an impact on colonic fermentation. A recent study found that algae polysaccharides with lower MW are beneficial for human faecal bacteria to produce more SCFAs and lower branched-chain fatty acids (BCFAs) (Li et al., 2022). Moreover, low MW polysaccharides could inhibit the growth of protein-fermenting bacteria (*Parabacteroides*,

Lachnoclostridium, and *Phascolarctobacterium*) and promote the growth of butyrate-producing bacteria (*Anaerostipes*, *Eubacterium*, *Roseburia*, *Ruminococcus* and *Lachnospira*), thereby promoting intestinal health. From this point of view, depolymerization of natural microalgae polysaccharides can be used as an effective means to enhance their health value in colonic fermentation.

5.3 Lipids

Lipids undertake important functions such as energy storage, cell membrane formation, and signal transmission. Similar to protein and carbohydrates, after ingestion, these lipids need to be digested by the digestive system to be released from the cell, hydrolysed and absorbed by the body (McClements, 2018; Nascimento et al., 2020). Lipids are ingested as part of the food matrix, which undertakes structural changes in the oral cavity due to the physical chewing and saliva components. The disruption of the food matrix, enables lipid release, which promotes digestion in the subsequent stages (Wang et al., 2022).

During the gastric digestion stage, the food matrix can be further disintegrated by the mechanical action of peristalsis and the digestive enzymes, which can aid in the release of lipids. At this stage, about 10~30% of lipids can be hydrolysed under the action of the gastric lipase (Barros et al., 2016). Next, the small intestine is the main location for lipid digestion, where the absorption rate can reach 95%. The pathways of digestion and absorption are specific depending on lipid structure. Small-molecular fatty acids (C<12) are mainly responsible for small intestinal epithelial cells, while large-molecular fatty acids (C>12) enter the lymphatic system to form lipoproteins (Pereira et al., 2023).

Microalgae are one of the main sources of long-chain polyunsaturated fatty acids (PUFA), including linoleic acid (C18:2), linolenic acid (C18:3), eicosapentaenoic acid (C20:5), and docosahexaenoic acid (C22:6). Understanding the absorption and metabolism of microalgae lipids in the body could support improving the physiological functions they

exert (Lupette & Benning, 2020).

Some researchers have explored the behaviour of microalgae lipids in the digestive system through *in vitro* models. Francisco et al. (Francisco et al., 2020) evaluated the bioaccessibility and antioxidant capacity of fatty acids and various bioactive compounds from *Fucus Spiralis* by establishing a static *in vitro* digestion model from the mouth to the small intestine. The results showed that EPA in the *Fucus Spiralis* lipids was about 7.5 ± 0.1%, while the bioaccessibility was 13 ± 1.0%, as well as the bioaccessibility of total lipids was about 12.1 ± 0.1%. On the other hand, Canelli et al. (Canelli et al., 2022) also explored microalgae lipid digestion, including the effects of different treatment technologies (including PEF, enzymatic hydrolysis and high-pressure homogenization) on *Chlorella* lipids by establishing an *in vitro* digestion model. This study found that high-pressure homogenization and PEF treatment can promote lipid digestion, increasing lipid bioaccessibility by about 17.0% and 56.5%, respectively.

In addition to investigating the behaviour of lipids in the upper gastrointestinal tract, more and more studies have begun to investigate the interaction of microalgae lipids and gut microbiota. Li et al. (Li et al., 2019) explored the regulatory effect of PUFAs from *Spirulina* on the gut microbiota of rats fed with high-fat diet. The results showed that PUFAs from *Spirulina* had a regulatory effect on lipid metabolism. Specifically, they decreased the abundance of some microbiota populations, which was positively correlated with lipid metabolism, including *Turicibacter*, *Clostridium_XlVa*, and *Romboutsia*, as well as increased the abundance of *Alloprevotella*, *Prevotella*, *Porphyromonadaceae*, and *Barnesiella*. The process of lipids in the digestive system is extremely complex, so a comprehensive understanding of its digestion and absorption process and its impact on gut microbiota is required to understand the potential beneficial effect on alleviating obesity, improving cognitive ability, inhibiting inflammatory

diseases, etc.

5.4 Polyphenols

Microalgae are the main source of marine polyphenols, and most of them have been proven to have positive effects on human health, including antioxidation, inhibition of fat accumulation, and alleviation of cardiovascular diseases. Evaluating the digestive behaviour of polyphenols can be used to estimate the bioactivity in the human body (Plaza et al., 2017). In general, microalgae polyphenols mainly exist in the form of polymers or glycosides, making them less bioaccessibile. About 45~50% of the phenols are digested and absorbed in the small intestine, and the phenols undergo sulfation or methylation reactions in the small intestine to be released from the food matrix and become bioaccessible. The remaining unabsorbed phenols reach the colon, which can participate in the fermentation of gut microbiota, and then undertake conversion into phenolic acids to be absorbed (Jakobek & Matić, 2019; Williamson & Clifford, 2017).

The digestion and absorption of microalgae polyphenols and their biotransformation in the colon have aroused the attention of researchers. Vázquez-Rodríguez et al. (Vázquez-Rodríguez et al., 2021) used *in vitro* digestion and colon fermentation models to explore the effect of phlorotannin and polysaccharides fraction from *Silvetia compressa* on the composition of human gut microbiota. This study found that hydroethanolic extract rich in phlorotannin can maintain healthy of human gut microbes. Specifically, the addition of the extract promotes the growth of *Bifidobacterium* and *Lactobacillus* and increases the content of total SCFAs, which has the potential as a prebiotic.

De Medeiros et al. (de Medeiros et al., 2021) also evaluated the potential effect of microalgae biomass (*Chlorella vulgaris*, *Desmodesmus maximus*, *Chlorococcum sp. cf hypnosporum*, and *Spirulina platensis*) on human health through an *in vitro* gastrointestinal digestion model and a human faecal fermentation model. These

microalgae biomass had a significant modulatory effect in the gut microbiota, such as increasing the relative abundance of *Bifidobacterium* and *Lactobacillus–Enterococcus*, as well as decreasing the abundance of undesirable bacteria (*Eubacterium rectale group*, *Clostridium coccoides*, and *Prevotellaceae*, etc.), showing a better effect than fructooligosaccharides. In microalgae biomass, insoluble dietary fibre, and phenolic compounds both play a role in modulating the gut microorganism, thereby producing SCFAs.

Moreover, some studies have also found that appropriate processing technologies can help improving the absorption efficiency of phenols, such as heat treatment, high pressure, physical grinding, etc. so that phenols can exert greater value (Lorenzo et al., 2019).

5.5 Other compounds

Pigments such as chlorophyll and carotenoids are important bioactive compounds in microalgae, which have various physiological activities such as colouring, antioxidant, anti-tumour, and immune regulation. In order to apply microalgae pigments to food, medicine, feed and other industries, some researchers have explored their digestion and absorption characteristics (García-Vaquero et al., 2021; Khanra et al., 2018b).

Fernandes et al. (Fernandes et al., 2021) simulated the digestion behaviour of *Scenedesmus obliquus* chlorophyll with the INFOGEST *in vitro* digestion model and explored the absorption of chlorophyll by Caco-2 cells. Meanwhile, the effects of different treatments on the bioaccessibility of chlorophyll were also compared. Among them, the extract rich in chlorophyll obtained by ethyl acetate/methanol extraction had the highest bioaccessibility, about 33.45%. Furthermore, hydroxypheophytin a and pheophytin are the most abundant chlorophyll pigments in Caco-2 cells. Chlorophyll in the form of the extract is more digestible and absorbed than biomass chlorophyll (wet/dry). Muszyńska et al. (Muszyńska et al., 2018) also evaluated the digestive properties of

Chlorella vulgaris Beijerinck biomass including lutein and other bioactive compounds in a similar *in vitro* digestion model. The bioaccessibility of lutein was found to be about 4~7%. In addition, the digestibility of phenolic compounds was also measured, and was about 1~2%. Overall, the absorption rate of pigments in the digestive system is low, and there is little research on the modulation of microalgae pigments on gut microorganisms.

Minerals and vitamins are also necessary for living organisms and need to be obtained from the diet, so their absorption rate in the host body is worthy of attention. For example, Gómez-Jacinto et al. (Gómez-Jacinto et al., 2020) evaluated the bioaccessibility of selenium in Se-enriched microalga (*Chlorella sorokiniana*) and its potential as a functional product through *in vitro* digestion model and *in vivo* experiments (as mice diets). According to the gastrointestinal digestion experiment, the bioaccessibility of selenium can reach 81%, which is mainly contributed by selenoproteins. The levels of selenium and selenoproteins were both increased in the mice serum which was fed high-selenium supplements. It shows that selenium in microalgae has high bioaccessibility and can be used as a dietary selenium supplement.

Comprehensive and objective evaluation of the bioaccessibility of bioactive compounds from microalgae, and selection of appropriate treatment methods increase the absorption rate of target substances, which is conducive to the acquisition of high-quality products.

6. Future perspectives, industrial development prospects and limitations

Microalgae are rich in lipids, proteins, carbohydrates, and other bioactive compounds, which are green and sustainable resources, and are being used as a new source of nutrients in various industries. In particular, the natural compounds from microalgae are expected to be used as functional substances in food, health care products and medicine. However, most of these compounds are trapped within the microalgae cell wall, which must be

disrupted so they can get released. This review comprehensively summarized the physiological functions of microalgae bioactive compounds, extraction and purification technologies, digestion, absorption characteristics, and the effect on gut microorganisms. From the perspective of extraction and recovery of bioactive compounds, the innovative extraction technology improves the extraction efficiency and reduces the dependence on organic reagents, long-term and high-temperature conditions compared with the traditional extraction technology, which is an important achievement in the recovery of microalgae biomass. Different microalgae cells have different cell sizes and cell wall thicknesses, and appropriate extraction conditions should be selected according to specific conditions. At present, most extraction studies have obtained products with multiple components, which can be directly used in the food industry, or can also be further fractionated to obtain higher-value products. The conversion from the original extract to specific component products requires appropriate and multiple impurity removal and purification processes, which stills face challenges to industrial production. In addition, the absorption efficiency of microalgae bioactive compounds in the human body is also worthy of attention, which will affect the applicability of microalgae as food and medicine supplements.

The current market for selling microalgae and its derivatives can be further expanded. The contribution of this review to make a better use of microalgae resources, includes: 1) the need of disrupting the cell wall to improve nutrient and bioactive release; 2) the lack of existing technologies to efficiently separate compounds of different nature from microalgae extracts; and 3) the need of studying the behaviour of microalgae and their compounds under digestion conditions, as the fate after this process will define their true impact on human health. To overcome the difficulties faced in the development of microalgae from many aspects, including designing more optimized extraction and

purification processes, clarify the functional potential of bioactive compounds in the host, so that microalgae can be better utilized in the future.

Acknowledgments

Thanks are due to the University of Aveiro and FCT/MCT for the financial support of LAQV-REQUIMTE research Unit (UIDB/50006/2020) through national funds and, where applicable, co-financed by the FEDER, within the PT2020 Partnership Agreement, and for financing the PhD grant of Carlos A. Pinto (SFRH/BD/137036/2018) and Ana P.Martins (SFRH/BD/146369/2019). Jianjun Zhou and Min Wang were supported by a PhD fellowship from the China Scholarship Council (CSC) (No. 201908420246 and No. 201908420245, respectively). IATA-CSICis a Centre of Excellence Severo Ochoa (CEX2021-001189-S MCIN/AEI/10.13039/ 501100011033) funded by MICIN/AEI/FEDER, UE.

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5. GENERAL DISCUSSION

5. Summary and General Discussion

In this study, we utilized innovative extraction techniques to extract bioactive substances from microalgae and utilized membrane separation techniques to isolate specific components. The extract's bioactivity and its potential impact on gut health were also assessed. Additionally, we conducted a preliminary evaluation of the effects of algae polysaccharide on food quality. To provide a comprehensive overview of the study's structure and approach, we created a schematic diagram of the research content covered in each chapter (results part), as illustrated in the Figure 1.

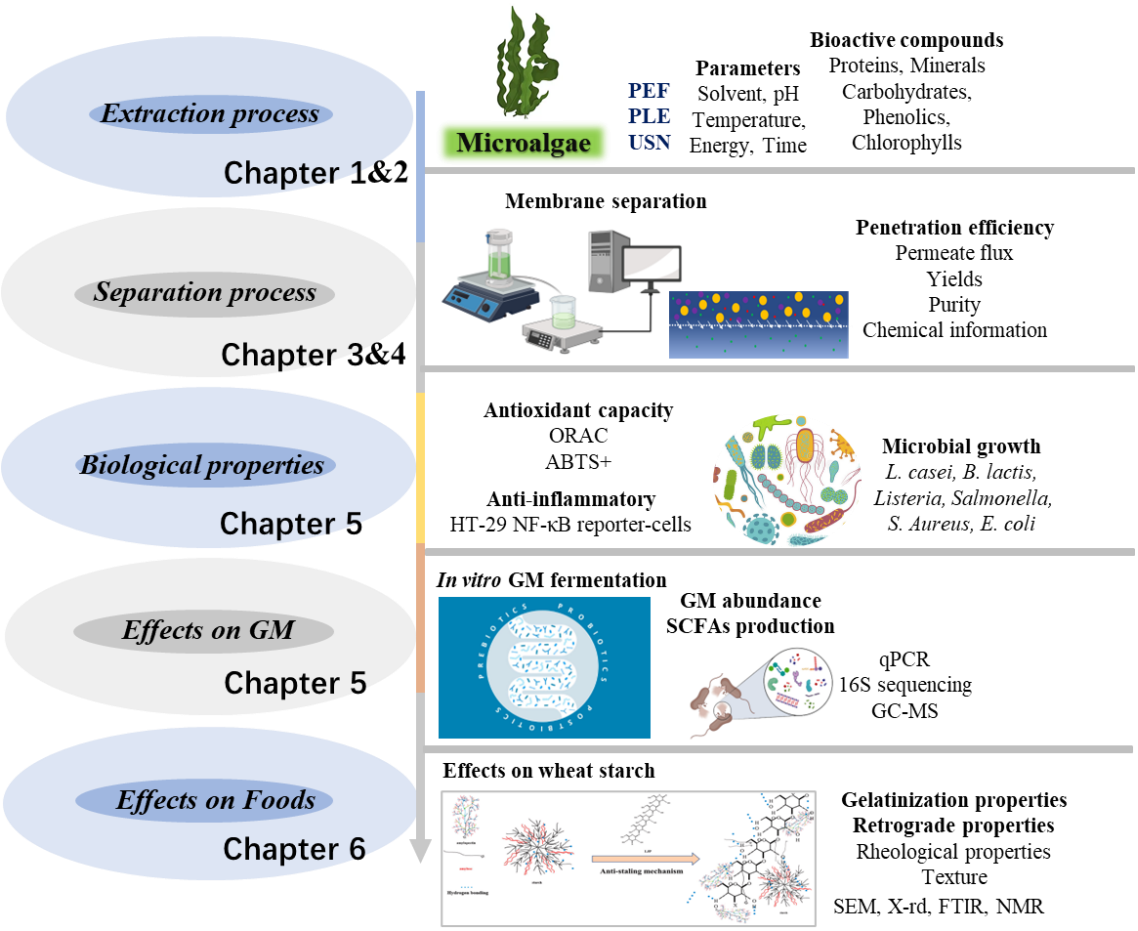

Figure 1. Research contents of different chapters.

5.1 Application of innovative extraction technologies in microalgae bioactive compounds recovery process

5.1.1 Impact of pressurized liquid extraction (PLE) and pH on protein yield, changes in molecular size distribution and antioxidant compounds recovery from Spirulina

The research aims to extract nutrients from *Spirulina* using a non-toxic, environmentally friendly, and efficient method-Pressurized Liquid Extraction (PLE). In this work, Response Surface Methodology (RSM)-Central Composite Design (CCD) was used to evaluate and optimize the extraction time (5-15 min), temperature (20-60 °C) and pH (4-10) during PLE extraction (103.4 bars). The results of the RSM-CCD showed that under the pressure of 103.4 bars, the optimal conditions to recover the highest content of bioactive compounds were 10 minutes, 40 °C and pH 4. Furthermore, the bioactive compounds and antioxidant capacity of PLE and non-pressurized extraction extracts were compared. The results showed that under the optimal extraction conditions (10 minutes, 40 °C and pH 4), PLE significantly improved the antioxidant capacity (2870.5±153.6 µM TE), protein yield (46.8 ± 3.1 %), chlorophyll a (1.46 ± 0.04 mg/g), carotenoids (0.12 ± 0.01 mg/g), total polyphenols (11.49 ± 0.04 mg/g) and carbohydrates content (78.42 ± 1.40 mg/g) of the extracts compared with non-pressurized extraction ($p < 0.05$). The protein molecular distribution of the extracts was analysed by SDS-PAGE and the results showed that there were more small-molecule proteins in PLE extracts compared with control group. Moreover, Triple TOF–LC–MS–MS results showed the extracts were rich on phenolic compounds, such as *p*-coumaric acid and cinnamic acid being the predominant phenolic compounds in the PLE extract.

A previous study used accelerated solvent extraction combined with different solvents (hexane, petroleum ether, ethanol, and water) to evaluate the extraction of antioxidant compounds from *Spirulina* (Herrero et al., 2005). The authors investigated the effects of different temperatures (60, 115, 170 °C) and time (3, 9 15 min) on the antioxidant capacity of the extract. The results showed that using ethanol as the extraction reagent, samples with higher antioxidant capacity are obtained at 170 °C and 15 minutes. Compared with our study, the extraction temperature selected in that study is too high, which is un-

favorable for obtaining heat-sensitive components such as pigments, polyphenols, and proteins. According to the principle of PLE extraction, the strong interaction between the solute and the matrix caused by van der Waals forces or hydrogen bonds, and the dipole attraction of solute molecules and the active sites of the sample matrix can be greatly reduced under high temperature and high pressure. This speeds up the extraction process of the solute molecules, reduces the activation energy required for the analysis process, and reduces the viscosity of the solvent, thereby reducing the resistance of the solvent to the sample matrix and promoting the solvent to diffuse into the sample (Zhuang, McKague, Reeve, & Carey, 2004).This indicates that PLE can promote the extraction of bioactive compounds from *Spirulina*, which is of great significance for the application of PLE technology to obtain bioactive substances from marine algae resources.

5.1.2 Pulsed electric fields (PEF), pressurized liquid extraction (PLE) and combined PEF + PLE process evaluation: Effects on Spirulina microstructure, biomolecules recovery and Triple TOF-LC-MS-MS polyphenol composition

The results in chapter 1 show that PLE can be an effective method for the extraction of bioactive compounds from microalgae. However, compared with the total bioactive components in microalgae, the extraction yield can be further improved. Therefore, in this work, the impact of different processes-pulsed electric fields (PEF), PLE and a multistep process combining PEF + PLE on the yield of antioxidant compounds (protein, polyphenols, chlorophyll a, and carotenoids) from *Spirulina* was investigated. The results showed the application PEF or PLE significantly increased the nutrients yields. The results of PEF extraction process were attributed to PEF-induced damage on *Spirulina* helical structure, which was verified by fluorescence microscopy (FM) and scanning electron microscopy (SEM). A related study compared the effects of PEF (25 kV/cm for 150 μs) and bead-milling treatment on the microstructure of *Spirulina*, showing the

microscopic results that PEF treatment resulted in the separation of cylindrical filament cells (trichomes), while bead milling disrupted the intact cell structure, which resulted in a higher purity C-phycocyanin in the PEF extract (Martínez, Luengo, Saldaña, Álvarez, & Raso, 2017).

To further increase the extraction yield, PEF + PLE was used, as an innovative extraction approach to extract bioactive compounds from microalgae. The results showed that PEF + PLE greatly improved the extraction yield compared with the PEF or PLE treatments alone. Compared with Folch extraction, PEF + PLE significantly ($p < 0.05$) shortened the extraction time (165 min) and increased the protein (1328%), polyphenol (979%), chlorophyll a (11%) and antioxidant capacity values (47%) of *Spirulina* extracts respectively. The combined use of PEF + PLE in this study achieved an improved extraction compared to the extraction assisted by PEF or PLE alone found in other studies. For example, Martí-Quijal et al. (2021) applied PEF to obtain *Spirulina* extracts containing carotenoids (0.50 mg/g dw, 60 min), chlorophyll a (0.60 mg/g dw, 120 min) and polyphenol (19.75 mg GAE/g dw, 180 min) (Martí-Quijal et al., 2021). As the results of chapter 5.1.1, we applied PLE to obtain *Spirulina* extracts which contained protein (210 mg/g dw), chlorophyll a (1.46 mg/g dw), carotenoids (0.12 mg/g dw) and polyphenols (11.49 mg/g dw) (Zhou et al., 2021).

PEF destroyed the microalgae cell structure and thus reduced the dependence of PLE on high temperature, allowing PLE could be performed at room temperature, which not only improved the extraction efficiency, but also protected the antioxidants. In this study, PEF + PLE played the role of 'one plus one was greater than two', and PEF-'electroporation' combined with PLE-'pressure fluid' made it more efficient and environmentally friendly compared with traditional methods or using PEF or PLE alone. Furthermore, Triple TOF-LC-MS-MS results showed that PEF + PLE increased both the

type and content of phenolic compounds. Specifically, the polyphenols of PEF + PLE extracts were mainly p-anisaldehyde (3.07 μg/g), ellagic acid (1.40 μg/g), quercetin 3-o-glucuronide (1.38 μg/g) and sitostanyl ferulate (1.10 μg/g), while for PEF, PLE, Folch extracts, the main polyphenols were corresponding as p-anisaldehyde (1.07 μg/g), panisaldehyde (1.47 μg/g)/quercetin 3-o-glucuronide (1.10 μg/g) and 24-methylcholestanol ferulate (1.37 μg/g) respectively.

5.2 Microalgae bioactive compounds separation enhanced by innovative extraction technology and membrane ultrafiltration process

5.2.1 A combined ultrasound + membrane ultrafiltration (USN-MUF) for enhancing saccharides separation from Spirulina (Arthrospira platensis)

From chapters 1 and 2, microalgae extracts contain a variety of bioactive compounds, including proteins, carbohydrates, pigments, lipids, etc., while the increase of the economic value of the microalgae industry requires the further separation process. In this work, the combination of ultrasound (USN) + membrane ultrafiltration (MUF) was used to separate *Spirulina saccharides* (SPS) from the crude extracts. The results showed that USN significantly ($p < 0.05$) increased the average high-added-value compounds yield when the setting of 400W/10 min was applied. The energy requirements (kWh/kg) for USN-no cooling system extraction to reach the maximum recovery yields of nutrients were lower than those for USN-cooling system, i.e., protein (380 vs. 450 kWh/kg), polyphenols (350 vs. 450 kWh/kg), saccharides (460 vs. 470 kWh/kg), chlorophyll a (350 vs. 450 kWh/kg), carotenoids (360 vs. 460 kWh/kg) and total compounds (350 vs. 450 kWh/kg). The increased yields of the bioactive compounds were due to the disruption effects of USN on microalgae. Compared with PEF (chapter 2), USN disrupted the cell structure of *Spirulina* more significantly, so the content of bioactive compounds in USN extract was higher. Acoustic cavitation was an accepted theory of ultrasonic cell

disruption (Liu et al., 2022; Wani & Uppaluri, 2022), and low ultrasonic frequencies in the 20-40 kHz range was the most commonly used frequencies in the extraction process of microalgae (González-Balderas et al., 2020; Greenly and Tester, 2015), especially those with thin cell walls.

In this study, the effects of protein and polyphenol removal by 75% ethanol precipitation and 90 °C heating concentration were also evaluated. The results showed that compared with MUF process in this study, 75% ethanol precipitation and 90 °C heating concentration was less effective in removing proteins, polyphenols, and pigments. In previous related reports, the separation of crude SPS from microalgae requires step-by-step removal of pigments, proteins, polyphenols, etc., while involving a variety of chemical and biological reagents, such as chloroform, n-butanol, TCA, protease, activated carbon, H_2O_2, ethanol, etc., which not only led to the gradual loss of SPS but also increased cost and pollution (Kang et al., 2022; Qiu et al., 2022; Tang et al., 2020). USN treatment increased the concentration of relatively low molecular weight SPS (4, 10 kDa) in the permeate ($p < 0.05$) but there was no significant ($p > 0.05$) effect on the high molecular weight SPS (300, 500 kDa). In general, this study obtained saccharides with over 70% purity (50% ↑) from *Spirulina* original extract (~ 20%) by USN-MUF technology, which provided the basis for further obtaining higher purity SPS.

Membrane fouling was observed during the saccharides MUF process, thus indicating that the process could be further improved. Related previous reports showed that if the fouling on the membrane surface was dominant, the increase in reversible fouling led to a decrease in membrane permeate flux (Marshall et al., 1993). When the internal fouling increases, the opposite result is found, that is, the membrane permeability is irreversible (Tanudjaja et al., 2022). The results of this study showed that the bioactive compounds entered the membrane during the membrane separation process, resulting in an

irreversible membrane fouling.

5.2.2 Saccharides (carbohydrates) separation from Phaeodactylum tricornutum through low-frequency ultrasound (LF-USN) and ultrafiltration (UF) technology

We also separated the saccharides fraction of *P. tricornutum* using USN combined with membrane separation technology. In this work, Low-frequency ultrasound (LF-USN, 24 kHz) was firstly used to recover bioactive compounds from *Phaeodactylum tricornutum* (*P. tricornutum*). The results showed that about 200 mg/g protein, 13 mg/g polyphenols, 60 mg/g saccharides, 0.3 mg/g chlorophyll a, and 0.45 mg/g carotenoids were obtained when using USN with a parameter of 400 W and 20 minutes, which was much higher than using 100 and 200 W extraction power.

The USN effects were defined as K_{USN}, the results showed that K_{USN} value increased with USN power and extraction time. Specifically, the K_{USN} of protein, polyphenols, saccharides, chlorophyll a, and carotenoid K_{USN} could reach 3.0, 3.3, 1.6, 20.5, and 15.0 respectively. The increase in the yield of *P. tricornutum* bioactive compounds by USN could be attributed to the mechanism of acoustic cavitation. Similar studies used USN to increase the yield of bioactive compounds, for example, Cui et al. (2022) used USN (100 W) to extract flavonoids from *Astragalus* stems and leaves with a yield of 22.027 ± 2.574 mg/g (Cui, Ma, Wang, & Niu, 2022), Sankaran et al. (2018) used USN (20 kHz, 200 W) to extract protein from *Chlorella vulgaris* with a yield of 93.33% (Sankaran et al., 2018).

Scanning electron microscopy (SEM) results showed that USN disrupted *P. tricornutum* cellular structures during extraction, as confirmed by particle size distribution tests, which is similar to the results in chapter 3. Another study explored the effect of USN conditions on the microscopic morphology of cyanobacteria (*Pseudomonas aeruginosa*) through SEM (Huang et al., 2021). Consistent with the results of this study, it was found that USN caused serious damage to microalgae cells.

Different molecular weight cut-offs (MWCOs) membranes (4, 10, 50, 100, 150, 300, 500 kDa) were used to separate saccharides. The permeate flux decreased with the UF process, and the membrane fouling index (MFI) was related to MWCOs. The conductivity and bioactive compounds concentration of the permeate increased with the UF process. Most of the protein and polyphenols and almost all pigments were retained during USN-UF process, promoting the saccharides concentration of permeate. The saccharides purity was affected by membrane MWCOs in USN-UF process, with a saccharide's purity of ~78% by 4~150 kDa membrane and ~70% by 300~500 kDa membrane. Membrane UF of saccharides also has been reported in previous studies. For example, Xie et al. (2014) used 300 kDa, 100 kDa and 6 kDa membranes to isolate water-soluble crude polysaccharides with a purity of 69.5% from *Cyclocarya paliurus* (Xie et al., 2014). Sun et al. (2011) isolated crude polysaccharides with a purity of 54% from rapeseed, because impurities such as proteins in the feed solution could not be completely retained by the membrane (Sun, Qi, Xu, Juan, & Zhe, 2011). In general, the results in our study indicated that USN-UF could be used as a new option for the preparation of *P. tricornutum* crude saccharides during the polysaccharides purification process.

5.3 Biological properties of microalgae extracts evaluation: in vitro antioxidant, antiinflammatory and bacterial growth effects, and gut microbiota modulation

Based on the extract's composition analysis in chapter 1-2, in this study, the impact of *Spirulina*, *Chlorella* and *Phaeodactylum tricornutum* (*P. tricornutum*) extracts obtained by PLE on antioxidant and antiinflammatory activities, microbial growth and *in vitro* gut microbiota composition was evaluated. *Spirulina* and *P. tricornutum* extracts significantly ($p < 0.05$) reduced the *in vitro* activation of the inflammatory NF-κB pathway. Moreover, the *Spirulina*–PLE extract significantly ($p < 0.05$) reduced the μ-max/h value and A-MOD value of *Salmonella*; for *P. tricornutum*, *P. tricornutum*–PLE

extract significantly reduced the μ-max/h value of *S. aureus* and *Salmonella* ($p < 0.05$). More interestingly, the PLE extracts of all three microalgae were able to significantly reduce the growth rate of *Salmonella* ($p < 0.05$). Consistent with our research, Plaza et al. also used PLE to obtain *Chlorella* extracts with antibacterial properties, especially when ethanol was used as extraction regents, showing strong inhibitory effects on *E. coli* and *S. aureus* growth (Plaza et al., 2015). In addition, other studies also reported that the *P. tricornutum*-loaded gelatin nanofiber mat showed 99.9% antibacterial activity against *E. coli* and multi-drug resistant *S. aureus* (Kwak et al., 2014).

Regarding the effect of microalgae extracts on potential probiotic strains, *P. tricornutum*–PLE did not have any significant effect on the growth of *L. casei* and *B. lactis* ($p > 0.05$), *Chlorella*–PLE significantly reduced the μ-max/h value of *B. lactis* ($p < 0.05$). It is worth noting that *Spirulina*–PLE, *P. tricornutum*–PLE significantly increased the A-MOD value of *L. casei* and *B. lactis* ($p < 0.05$). Interestingly, based on the principal component analysis (PCA), whether it is the PC1 or PC2, polyphenols, carbohydrates, and proteins in microalgae are distributed negatively with *S. aureus*, which is an important reason to explain why the growth of *S. aureus* was inhibited by algae extracts.

Considering the considerable content of polyphenols and polysaccharides in *Spirulina* and *P. tricornutum* extracts, the effects of the extracts before and after the *in vitro* digestion on colonic fermentation were evaluated. The effects of *Spirulina*–PLE and *P. tricornutum*–PLE extracts on gut health cannot be generalized, since they not only increase the levels of *Lactobacillus* members and decrease *Streptococcus* levels, but also decrease the number of *Bifidobacterium* and *Bacteroides*. As we know, some *Bifidobacterium* members are considered probiotics and potentially beneficial organisms in the human gut, as they would control serum cholesterol levels, prevent intestinal

diseases, modulate immune system, and also, possess anti-cancer activities (Di Gioia, Aloisio, Mazzola, & Biavati, 2014). *Bacteroides* would also play an important role in improving metabolic and immune disorders in obese individuals, and *Lactobacillus* genus is considered a beneficial bacteria with effects on microbiota modulation and also, on the improvement of the gastrointestinal function, increasing food digestibility, reducing serum cholesterol, etc (Azizian et al., 2021).

Short chain fatty acids (SCFAs) are the products of the microbial activity and can contribute to host physiology and energy homeostasis. SCFAs mainly referring to acetic acid, propionic acid, and butyric acid, which highly associated with the bacterial levels during colonic fermentation (Guo et al., 2021). The results showed that *Spirulina*–PLE–digest and *P. tricornutum*–PLE–digest sample were able to increase acetic content, while undigested extracts had no effect on acetic acid within 48 h fermentation. *P. tricornutum*–PLE extracts enhanced propanoic acid production at 48 h. *Spirulina*–PLE–digest sample significantly increased the butanoic acid, both after 24- and 48-h fermentation. From this, both fermentation time and *in vitro* digestion can affect the levels of SCFAs. Combined with qPCR results, *Spirulina* and *P. tricornutum* extracts decreased most of the bacterial amount during the 24-h fermentation process, while the elapse of time and *in vitro* digestion increased the bacterial amount, which resulted in the SCFAs levels variation.

5.4 Algae polysaccharides application in food products.

Starch is one of the most abundant carbohydrates in nature, and it is the primary source of human dietary carbohydrates, providing about 70% of the human body's daily energy intake.

In this line, improving starch properties to suit a wide range of application markets better is required. This study investigated the effects of *Laminaria japonica* polysaccharides (LJP) on the rheological, gelatinization, and retrogradation properties of

WS (wheat starch) gels. Dynamic rheological test showed that the addition of LJP decreased the storage and loss modulus. Furthermore, LJP significantly decreased the peak viscosity (PV), breakdown (BD), and setback (SB) value of WS. Lin et al. (2021) reported that high concentrations of non-starch polysaccharides formed more hydrogen bonds with starch molecules, resulting in concentration-dependent trends in PV, BD, and SB values (Lin et al., 2021). Overall, the gelatinization characteristics of WS particles were significantly affected by the interaction between LJP and starch.

Moreover, LJP incorporation significantly reduced the WS gel hardness during storage. Previous studies showed that the interactions between starch and xanthan could obstruct the interactions between amyloses during the long-term storage, including amylose reaggregation and amylopectin recrystallization, which could be the reason that the addition of LJP decreased the hardness of WS gel in this study (Tang, Hong, Gu, Zhang, & Cai, 2013). In addition, starch retrogradation was accompanied by water migration, and LJP molecules could compete with starch for water absorption and inhibited starch retrogradation during gel storage, thereby reducing WS gel hardness (Yu, Wang, Chen, Li, & Wang, 2018). SEM results showed that the addition of LJP improved the structure of WS gel.

The short and long-range ordered structure showed that the relative crystallinity and formation of ordered structure were reduced with the addition of LJP. Otherwise, the LJP inhibited moisture migration, which was demonstrated by LF-NMR. In summary, the addition of LJP could inhibit the retrogradation of WS gel, as well the double helix structure of amylose or amylopectin was decreased, which provides a new theoretical insight to produce WS-based food study. Similarly, other studies also showed that polysaccharides, such as adding inulin, pullulan, could reduce the crystallinity of retrograded starch, which indicated that LJP had the same effect on inhibiting

amylopectin recrystallization and starch retrogradation (Luo et al., 2017). However, compared with adding 0.5~1.5% LJP to inhibit starch retrogradation, the proportion of polysaccharides added was higher in these studies. For example, adding 5.0%7.5% and 15% inulin could inhibit starch retrogradation in the long-term and short-term, respectively (Luo et al., 2017). Moreover, the addition of inulin inhibited amylose retrogradation but accelerated amylopectin retrogradation, while there was no evidence that LJP addition accelerated amylopectin retrogradation. Starch retrogradation (rearrangement and ordering) was the reverse process of starch gelatinization (fracture and disorder), during which starch transitioned from a disordered state to a crystalline state, accompanied by changes in the free water/bound water ratio (Niu et al., 2018). During the retrogradation process, as water was incorporated into the crystal structure, starch tended to form a more stable crystalline state. However, the addition of LJP reduced the proportion of free water, indicating that LJP could inhibit the crystallization behaviour of starch by competing with starch molecules for water. In summary, the addition of LJP could inhibit the retrogradation of WS gel, as well the double helix structure of amylose or amylopectin was decreased, which provides a new theoretical insight to produce WS-based food study.

Conclusions

From the results obtained in the present PhD thesis it can be concluded that:

1) The innovative extraction technologies (Pulsed electric fields, PEF; Pressurized liquid extraction, PLE; Ultrasonic extraction, USN) can be used to obtain bioactive compounds from microalgae, as they increase the yields and preserve the biological properties of bioactive compounds in microalgae extracts.

2) The PEF and USN extractions have a damaging effect on the microalgae structure during the extraction process, which is the main reason for the increase in the yield of

bioactive compounds.

3) The aqueous extracts of *Spirulina, Chlorella* and *Phaeodoactylum tricornutum* are potential sources of natural antiinflammatory agents and antimicrobials to be used in the prevention, treatment, and control of bacterial infections as well as a potential stimulation of benefical bacteria including *Bifidobacterium* and *Lactobacillus* members, some of them recognized as probiotics.

4) The USN + membrane separation technology can be used to separate polysaccharides fraction form microalgae, and the chemical information and crystallinity properties of polysaccharides are slightly affected by molecular weight cut-offs (MWCOs).

5) The algae polysaccharides inhibit the long-term retrogradation of wheat starch, which improves the bakery foods quality during storage.

6) Microalgae extracts affect the growth of gut microbiota and promote the production of SCFAs, which have potential benefits for human health.

These conclusions demonstrate that PEF, PLE, and USN can be used to efficient extraction bioactive compounds from different microalgae as they both increase the bioactive compounds yields and preserve the biological properties of the extracts. Based on these innovative technologies, it will be of great significance to the microalgae industry economy and environmental protection if it can truly realize large-scale industrial extraction of microalgae bioactive compounds, and at the same time reduce energy consumption and pollution. USN combined with membrane separation technology can be used as a pollution-free and easy-to-operate method to separate polysaccharides from microalgae extracts. The current separation purity is less than 80%, which can be further improved by adjusting membrane separation parameters in the future.

From the perspective of effects on antiinflammatory and microbial growth, the aqueous extracts of *Spirulina*, *Chlorella* and *P. tricornutum* can be suggested as potential sources

of natural antiinflammatory and antimicrobial agents for the prevention, treatment and control of bacterial infections and stimulation of probiotic activity. *In vitro* gut microbiota is modulated by *Spirulina* and *P. tricornutum* extracts, and after gastrointestinal digestion, microalgae extracts showed more evident benefits for the microbiota. On this basis, it is necessary to explore the mechanism of these bioactive compounds in the growth of intestinal microbiota, such as which specific microbes can metabolize microalgae bioactive compounds and what are the other downstream metabolites besides SCFAs during the colonic microbial fermentation process. Moreover, *in vivo* animal experiments (mice, nematodes, etc.) are necessary to assess the bioactivity of microalgae extracts and their effects on intestinal health. Finally, algae components such as polysaccharides can improve the quality of baked goods, which can broaden the scope to improve the quality of more food substrates.

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