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DETERMINAÇAO DE METABÓLITOS LIPÍDICOS EM MICROALGAS

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos, Área de Concentração em Ciência e Tecnologia dos Alimentos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Ciência e Tecnologia dos Alimentos.**

Orientador: Prof. Dr. Roger Wagner

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RESUMO

DETERMINAÇAO DE METABÓLITOS LIPÍDICOS EM MICROALGAS

AUTORA: Mariane Bittencourt Fagundes

ORIENTADOR: Prof. Roger Wagner

Microalgas são microrganismos com um metabolôma complexo e pouco explorado, podem desta forma ser considerados uma fonte rica de material para estudos de ômica. Atualmente, asociedade busca por compostos que possuam bioatividade e possam ser aplicados como nutracêuticos e também como insumo para indústrias farmacêuticas. Dentre as microalgas catalogadas e estudadas, a cepa de Phormidium autumnale destaca-se como pouco explorada de forma geral, os últimos estudos relatam teores de carotenoides e lipídeos, no entanto, o estudo da esterolômica é algo novo dentro do campo da biotecnologia migroalgal. Em face disso, o objetivo deste trabalho foi explorar a o perfil esterolômico das microalgas e também verificar a influência da destes metabólitos frente a doenças neurodegenerativas. Como resultado foram gerados artigos e capítulos de livros (publicadosou em processo de publicação), que foram organizados neste documento em capítulos. Os capítulos de 1 á a 3 referem se ao artigo de revisao revisão e aos capítulos de livros, já e os capítulos 4 e 5 referem-se as publicaçoes adquiridasaos artigos científicos publicados em periódicos de pesquisa. Capitulo 1: “Desenvolvimentode método de esteróis em microalgas: uma breve revisão sobre o isolamento e potencial bioativo” (artigo de revisão em processo de publicação); Capítulo 2: "Sterols from microalgae” (capítulo de livro/publicação concluída); Capítulo 3: “Sterols Biosynthesis in Algae” (capítulo de livro/publicação concluída); Capítulo 4: “Green microsaponification- based method for gas chromatography determination of sterol and squalene in cyanobacterialbiomass” (artigo de pesquisa/publicação concluída); Capítulo 5: “Phytosterol-rich compressed fluids extracts from Phormidium autumnale cyanobacteria with neuroprotective potential” (artigo de pesquisa/publicação concluída). Por fim, os resultados destas produções cientificas indicam que a cianobactéria Phormidium autumnale pode ser considerada fonte de esteróis devido as concentrações encontradas, sendo elas: 5–18 mg kg−1 estigmasterol, 6 mg kg−1 colesterol, e 3 mg kg−1 de β-sitosterol, obtidas a partir de uma micro saponificação. Também,dependendo das formas de extração, o extrato pode apresentar inúmeras bioatividades, conforme foi observado utilizando-se as tecnologias emergentes de extração, como fluído supercrítico e etanol/ solvente expandido por gás (GXL). Como resultados foram obtidos comoas melhores condições de otimização: 266,3 bar de pressão, e 7% de etanol. Os valores de inibição bioquímicos (IC50) adquiridos foram de: 65,80 μg mL−1 para acetilcolinaesterase, 58,20 μg mL−1 para inibição da lipoxigenase, e 7,40 μg mL−1 para atividade antioxidante.Logo, os resultados adquiridos indicam que o extrato apresenta bioatividade frente a doenças neurodegenerativas.

Palavras-chave: *Phormidium autumnale*. Esteróis. Bioatividade. Extrações emergentes. SFE. GXL. Ultrassom. *Green Chemistry.*

ABSTRACT

DETERMINATION OF LIPID METABOLITES IN MICROALGAE

AUTHOR: Mariane Bittencourt Fagundes

ADVISOR: Prof. Roger Wagner

Microalgae are microorganisms with a complex and little explored metabolome, and can therefore be considered a rich source ofmaterialfor omicsstudies. Currently,society islookingfor compounds that have bioactivity and can be applied as nutraceuticals and also as an input for pharmaceutical industries. Among the microalgae cataloged and studied, the strain of Phormidium autumnale stands out as little explored in general, the latest studies report carotenoids and lipids, however, the study of sterolomics. Therefore, the objective of this workwas to explore the sterolomic profile of microalgae and also to verify the influence of these metabolites against neurodegenerative diseases. As a result, articles and book chapters (published or in the process of publication) were generated, which were organized in this document into chapters. Chapters 1 to 3 refer to review article and book chapters, and chapters4 and 5 refer to publications purchased in research journals. Chapter 1: “Development of a sterol method in microalgae: a brief review of isolation and bioactive potential” (review articlein the process of publication); Chapter 2:"Sterols from microalgae” (book chapter/publicationcompleted); Chapter 3: “Sterols Biosynthesis in Algae” (book chapter/publication completed);Chapter 4: “Green microsaponification-based method for gas chromatography of sterol and squalene in cyanobacterial biomass” (research article/publication completed); Chapter 5: “Phytosterol-rich compressed fluid extracts from Phormidium autumnale cyanobacteria with neuroprotective potential” (research article/publication completed). Finally, the results of these scientific productions indicate that the cyanobacterium Phormidium autumnale can be considered a source of sterols due to the concentrations found, which are: 5–18 mg kg−1 stigmasterol, 6 mg kg−1 cholesterol, and 3 mg kg−1 of β sitosterol mg kg−1, obtained from amicro-saponification, and that, depending on the forms of extraction, the extract can show bioactivity, as observed using emerging extraction technologies such as supercritical fluid and ethanol/gas-expanded solvent (GXL). The results were obtained as the best optimization conditions:266.3 bar pressure, and 7% ethanol. The biochemical inhibition values (IC50) acquired were: 65.80 μg mL−1 for acetylcholinesterase, 58.20 μg mL−1 for lipoxygenase inhibition, and 7.40 μg mL−1 for antioxidant activity. Soon, these results indicate that the extract has bioactivity against neurodegenerative diseases.

Key words: Microalgae. *Phormidium autumnale*. Sterols. Bioactivity. Emerging Extractions. SFE. GXL. Ultrasound. Green Chemistry.

APRESENTAÇÃO

Esta tese de doutorado está organizada em sete itens principais, sendo os dois primeiros compostos pela Introdução e objetivos. Os demais itens encontram-se divididos na forma de capítulos temáticos. Nesse sentido, o Capítulo 1 é composto pela revisão bibliográfica acerca dos principais tópicos que fundamentam esta pesquisa. Os capítulos 2 e 3 discorrem acerca das rotas metabólicas de formação dos esteróis e se apresentam como capítulos de livros já publicados. Enquanto os capítulos4 e 5 trazem os resultados experimentais da pesquisa realizada no formato de artigos científicos também devidamente publicados em periódicos. Por fim, o Capítulo 6 contempla a discussão e conclusão geral do trabalho e o item Referências refere-se àquelas inseridas na Introdução e discussão geral, o qual estão dispostas ao final destatese. A sequência dos trabalhos segue uma ordem cronológica de execução e não de publicação.

SUMÁRIO

1 INTRODUÇÃO

As microalgas apresentam inúmeros benefícios, os quais são consequência do seu metabolismo desempennhado para o crescimento e manutenção celular, são responsáveis por fixar o carbono orgânico e produzir O2, também são as produtoras primárias de biomassa para o sistema aquático, assim suportando a vida no planeta. Na mesma linha de raciocínio podemos inferir que as microalgas são organismos que contribuem significativamente para a economia global, através da potencialidade da aplicação de seus metabólitos secundários em indústrias farmacêuticas, utilizados como insumo farmacêutico ativo, e também em indústrias de suplementos alimentares (LA BARRE & BOROWITZKA, 2018).

O termo microalgas inclui tanto as microalgas, cepas eucarióticas, quanto as cianobactérias, consideradas células procarióticas, sendo que ambas apresentam metabolismos fotoautotrofico e heterotrófico. Logo, em função da flexibilidade do seu metabolismo, as microalgas podem ser produzidas em diferentes locais do mundo, também estes microrganismos são de crescimento acelerado, capazes de adquirir mais de 100 toneladas/ha por ano (FERNÁNDEZ et al., 2021). Neste sentido, sendo passíveis de superar as fontes convencionais de metabolitos bioativos, as quais normalmente são fontes não sustentáveis, altamente dependentes da sazonalidade.

No entanto, sabe-se que o metabolismo microalgal dependendo da cepa, ainda é desconhecido, vinculando-se esta informação ao aumento significativo na demanda por compostos bioativos, estas cepas destacam-se para serem aplicadas em estudos “ômicos” no cenário científico atual.

O estudo ômico de compostos bioativos pode ser considerado uma ferramenta para compreensão da interação desses compostos em nível molecular e celular, pois é um estudo abrangente dos metabólitos envolvidos em diversas rotas bioquímicas. Assim, permite explorar evidencias científicas dos benefícios destas moléculas frente a saúde humana (ALVAREZ RIVERA et al., 2018; GILBERT-LÓPEZ; MENDIOLA & IBÁÑEZ, 2017; IBÁÑEZ; CIFUENTES, 2014). Tendo em vista os inúmeros metabólitos passíveis de serem estudados,xiste uma linha de pesquisa cuja qual se encontra em ascensão, denominada de lipidômica,que por sua vez, constitui-se como uma ramificação dos estudos do metaboloma, o qualcompreende mais 5000 tipos de lipídeos de diversas subclasses (LEE & YOKOMIZO, 2018). O estudo ômico associado aos lipídeos (lipidômica) ainda é um campo que precisa ser explorado, pois existem muitas moléculas que não foram elucidadas e suas rotas de produção não foram bem estabelecidas (LEE & YOKOMIZO, 2018). Nesse sentido, a lipidômica

aplicada a estes microrganismos está subdividida em lipidômica de moléculas provenientes da membrana plasmática, e a lipidômica de bioativos lipídicos encontrados em baixas concentrações livres na célula (WOOD & CEBAK, 2018).

Em termos analíticos, a lipidômica pode ser dividida em global, a qual visa caracterizar o maior número de biomoléculas destafração, e as análises target, as quais compreendem apenas alguns metabólitos. Em termos de cultivos microalgais para aplicabilidade industrial destes metabólitos os cultivos fotoautotróficos possuem inúmeras vantagens, como a ausência de fontes exógenas de carbono, e assim baixo custo para a produtividade destes químicos-finos. Entretanto, para produção em alta escala, destaca-se como uma desvantagem e limitação a dispersão da luz quando utilizam-se reatores superiores a 100 L, obtendo-se como um desafio, a homogeneidade do sistema (PEREZ-GARCIA et al., 2011). Com o intuitode reverter este problema, devido ao alto investimento que seria aplicado para homogeneização do sistema, uma alternativa pode ser conduzir os cultivos microalagais em condições ambientais de modo que as mesmas desempenhem o metabolismo heterotrófico. Ou seja, algumas microalgas/cianobactérias possuem a habilidade de se multiplicar através do uso de fontes exógenas de carbono na ausência de luminosidade e transforma-las em energia (PEREZ-GARCIA et al., 2011).

As diferentes formas de cultivo podem levar a produção de metabólitos lipídicos distintos, entre eles estão os ácidos graxos, provenientes da fração saponificável, os quais têm sido amplamente estudados para biomassas microalgais. Por outro lado, dentro da fração não saponificável da biomassa, encontram-se os esteróis (FAGUNDES et al., 2019a), os quais possuem como precursor metabólico o esqualeno, um bioativo de elevado valor nutracêutico (FAGUNDES et al., 2019b). Os metabólitos da fração não saponificável são considerados de alto valor agregado, devido à benefícios que podem proporcionar a saúde humana, tais como propriedades anticarcinogenicas e antiinflamatórias, em conjunto com elevada capacidade antioxidante (FENG et al., 2018; HARI-NARAYAN et al., 2010).

O método de preparo de amostra e as ferramentas analíticas a serem utilizadas para a determinação desses metabólitos ainda necessitam de maiores investigações (VENDRUSCOLO et al., 2018). Normalmente, o procedimento analítico para análise da fração não saponificável compreende as etapas de extração de lipídeos e, subsequente a saponificação, onde se obtém além das moléculas livres, as esterificadas, bem como proporciona a limpeza do extrato, eliminando os ácidos graxos, entre outros interferentes polares (HELENO et al., 2016). Em adição, alguns trabalhos utilizam mais uma etapa para aumento do sinal analítico, a derivatização dos esteróis com agentes sililantes, após os processos de saponificação

(FLAKELAR et al., 2017). Em ambas as etapas se utiliza uma grande quantidade de solvente, tornando as analises dispendiosas e também como as técnicas requerem grandes quantidades de lipídeos, necessita-se consequentemente de grande quantidade de amostra.

Com relação aos cultivos biotecnológicos, o uso de maiores quantidades de amostra 6 tornam o processo de elucidação da lipidômica complicado, pois muitas vezes utilizam-se reatores de escala laboratorial para a produção, perfazendo, em média, um volume total de 2 L. E os métodos de preparo de amostra de referência para análise desta fração presentes na literatura requerem um elevado valor de biomassa microalgal que não é obtido através de cultivos em pequena escala (GRASSO, et al., 2016).

Neste sentido, o uso de métodos simultâneos de extração/saponificação, bem como métodos verdes para os estudos target da lipidômica microalgal podem ser uma alternativa, pois em apenas uma etapa os metabólitos de interesse são obtidos, proporcionando a redução de tempo no preparo de amostras, culminando em baixo custo de analise, atendendo também ao princípio da química verde (ARMENTA et al., 2019). Portanto, o desenvolvimento deste novos métodos vem a auxiliar na elucidação da lipidômica microalgal, permitindo a execução das etapas consecutivas do presente trabalho, que visam a compreensão da produção dos esteróis através de diferentes metabolismos.

Diante do exposto, acredita-se que os metabólitos lipídicos microalgais, podem desempenhar papeis importantes em várias funções biológicas com relação ao organismo humano. Para tal comprovação, ensaios in-vitro e estudos in-silico de bioinformática, prévios a ensaios in-vivo, são requeridos, para compreender em termos químicos quais as ligações intermoleculares que os metabólitos podem realizar para exercer a função específica no organismo humano. Logo, estudos para a compreensão da melhor extração destes compostos e sua aplicabilidade em termos de bioatividade podem auxiliar de forma significativa o campo de estudo da lipidômica, principalmente com relação a cepas pouco estudadas.

2 OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo geral deste trabalho foi desenvolver métodos miniaturizados para realizar a caracterização dos esteróis na cianobactéria Phormidium autmnale e monitorar cultivos conduzidos sob diferentes variáveis. Na sequência, avaliar o potencial bioativo destes metabólitos.

2.2 OBJETIVOS ESPECÍFICOS

Para atingir o objetivo geral foram estabelecidos os seguintes objetivos específicos:

•Realizar o estudo do metabolismo microalgal, afim de compreender as rotas metabólicas de produção dos esteróis,

•Realizar estudos de lipidômica target, através do perfil esterolômico, na biomassada cianobactéria Phormidium autmnale.

•Desenvolver métodos de extrações de esteróis verdes (miniaturizados) para a cepada cianobactéria Phormidium autmnale.

•Determinar esteróis e esqualeno produzidos em cultivos microalgais heterotróficoscom diferentes fontes de nutrientes.

•Aplicar novas tecnologias de extração e otimizar para obtenção de extratos, com afração dos esteróis, que possuem maior potencial bioativo.

• Avaliar a capacidade bioativa dos esteróis da cianobactéria, frente a doenças neurodegenerativas.

3 CAPÍTULO 1

3.1 MANUSCRITO 1 - REVISÃO BIBLIOGRÁFICA

Desenvolvimento de método para análise de esteróis em microalgas: uma breve revisão sobre o isolamento e potencial bioativo

Sterol method development in microalgae: a brief review of isolation and bioactive potential

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RESUMO

Nos últimos anos, os estudos de esterolômica tem aumentado significativamente, de maneira que fontes não convencionais dessas biomoléculas vêm sendo estudadas. As microalgas e cianobactérias apresentam-se como potenciais fontes de esteróis, no entanto ainda inexploradas. Estes químicos-finos são muito importantes para serem utilizados como insumos na indústria farmacêutica, como nutracêuticos e insumos para medicamentos. Logo, para avançar no conhecimento científico sobre estes compostos, faz-se necessário uma compreensão sobre a sua síntese, as formas de extração destes metabólitos e suas aplicabilidades. Neste sentido, essa revisão descreve os principais aspectos relacionados aos esteróis produzidos por microalgas, bem como o potencial bioativo que estas moléculas podem fornecer.

Palavras chave: Biomassa microalgal, Esterolômica, Bioatividade, Extrações verdes, Líquidos pressurizados.

ABSTRACT

In recent years, studies on sterolomics have increased significantly, so that unconventional sources of these biomolecules have been studied. Microalgae and cyanobacteria are potential sources of sterols, however still unexplored. These fine chemicals are very important to be used as inputs in the pharmaceutical industry, as nutraceltical, and for being used as income in drug development. Therefore, to advance the scientific knowledge about these compounds, it is necessary to understand their synthesis, the ways of extracting these metabolites and their applicability. In this sense, this review describes the main aspects related to sterols produced by microalgae, as well as the bioactive potential that these molecules can provide.

Keywords: Microalgae biomass, Sterolomic, Bioactivity, Green extractions, Pressurized liquids.

1. INTRODUÇÃO

Os fitosteróis, incluindo esteróis, fazem parte de uma classe de compostos semelhantes ao colesterol em estrutura e função. Fisiologicamente, eles contribuem para funções essenciais nas células vegetais, como a fluidez da membrana e a transdução de sinal celular (Nes, 2011; Hernandez-Ledesma &Herreiro, 2014; Fagundes et al., 2020). No contexto nutrição, e saúde os esteróis destacam-se devido as inúmeras funções que podem exercer no organismo humano, tais funções são altamente dependentes da sua estrutura química. A função mais conhecida dos esteróis, está associada ao auxilio na redução de doenças cardiovasculares, pois os fitoesteróis atuam dificultando a absorção intestinal do colesterol. Em resposta, diminuindo o nível plasmático do colesterol da lipoproteína de baixa densidade (LDL) (Gylling et al., 2014).

No entanto, sabe-se que inúmeras funções podem ser desempenhadas, pois estão associadas as estruturas químicas. De forma que, as microalgas por apresentarem uma gama de esteróis não convencionais, podem fornecer funções metabólicas distintas. Estes biocompostos auxiliam na prevenção e nos tratamentos de doenças como, diabetes, obesidade, também atuando como anti-aterosclerose, anti-câncer, anti-Alzheimer e hepatoprotetor (Catani et al., 2018; Hannan, 2020, Fagundes et al., 2021).

Encontrar estratégias para a determinação destes analitos em microalgas e cianobactérias é um grande desafio, pois estes são metabólitos secundários, encontrados em concentrações diminutas e suscetíveis a degradação durante processos de extração. Os métodos convencionais utilizam solventes de elevada toxicidade e assim como utilizam grandes volumes, entretanto métodos emergentes apresentam alternativas mais verdes para estudos de esterolômica em microalgas (Vendruscolo et al., 2019; Fagundes et al., 2021).

Neste sentido, entender as etapas e o que tange cada uma delas, para a partir desta finalidade construir uma pesquisa sobre os desenvolvimentos metodológicos para extração destes metabólitos é fundamental para a inovação e descoberta de novas moléculas para serem exploradas no campo da saúde. Logo, esta revisão apresenta os principais fundamentos acerca dos esteróis em microalgas e cianobactérias, afim de auxiliar na ascensão desta linha de pesquisa.

2. MICROALGAS

O termo “microalgas” engloba microorganismos pertencentes a grupos distintos. De forma que se incluem cepas procarióticas (conhecidos como cianobactéria), e outras demais conhecidas como eucarióticas. As células eucarióticas classificam-se como: Diatomacias (*Bacillariophyceae*), as algas verdes (*Chlorophyceae*), microalgas flageladas (*Euglenophyta*), microalgas douradas, (*Haptophyta*), Dinoflagelados (*Dinophyceae*), (*Eustigmatophyceae),* e microalgas vermelhas, (*Rhodophyta*). Com relação as cepas procarióticas, (*Cyanophyceae*) ou blue-green algae (conhecidas como cianobactérias microalgas azuis), e (*Prochlorophyta*), estas possuem características celulares similares as bactérias, no entanto também são consideradas microalgas (Madeira et al., 2017).

As células procarióticas foram estudadas e descobertas como os primeiros organismos unicelulares do planeta terra, foram os pioneiros na realização da fotossíntese, sendo responsáveis pela oxigenação do planeta. Outro benefício importante destes microrganismos está associado a capacidade dos mesmos de fixar o nitrogênio (Andersen, 2013).

As *blue-greens* são bactérias Gram-negativas, medindo de 2 a 5 μm de diâmetro, ocorrendo em diferentes ambientes do mundo, de aquático ao terrestre, sob diferentes climas: de tropical a climas de extremo frio (Fay, 1983). Parte da habilidade metabólica está associada a membrana plasmática destes microrganismos, pois os mesmos contêm hopanoides os quais são os responsáveis pela rigidez da membrana, mantendo o conteúdo intracelular mais protegido frente a variações extremas (Belin et al, 2018).

O compartimento celular das cianobactérias distingue-se das cepas eucarióticas, em função da organização celular, pois as cianobactérias não possuem núcleo organizado. As diferenças encontradas com relação as bactérias estão associadas a presença de pigmentos específicos como clorofila *a*, ficobiliproteínas (ficocianina, aloficocianina e ficoeritrina), em conjunto com todo um complexo de enzimas, sendo os pigmentos responsáveis pelo auxílio no captura de luz solar para a conversão em energia, e as enzimas responsáveis pelo maquinário metabólico de transporte de elétrons (Williams & Laurens, 2010; Borowitzka, 2018). As diferenças em nível celular das cepas procarióticas e eucarióticas podem ser observadas de acordo com a Figura 1.

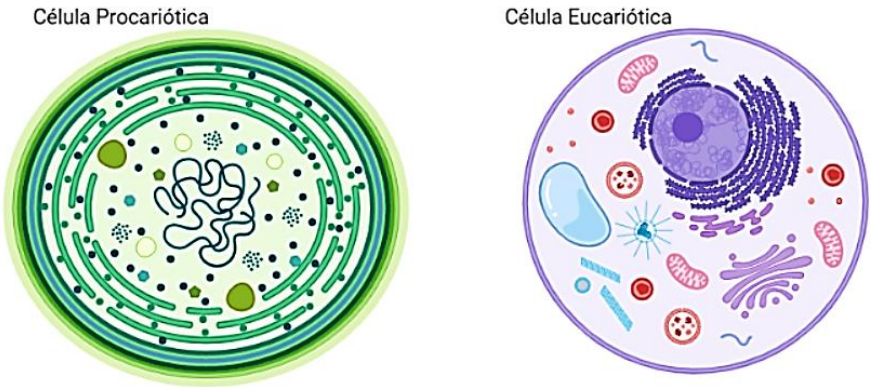

Figura 1. Diferença estrutural entre uma cianobactéria (célula procariótica), com relação as microalgas em geral (célula eucariótica). Fonte: criado utilizando o Biorender.

Conforme conseguimos observar as cepas eucarióticas possuem uma estrutura organizacional bem compartimentada, e em função disto são conhecidas como ultraestruturas, pois constituem diversas organelas, sendo estas: o núcleo, mitocôndria, cloroplastos, retículo endoplasmático, aparato de Golgi, e os lisossomos (Andersen, 2013). De modo geral, com relação a fonte de energia, as microalgas são classificadas em fotoautotróficas, heterotróficas ou mixotróficas. O metabolismo autotrófico é o responsável pela conversão da radiação eletromagnética da luz em fonte de energia celular, ou seja, em compostos orgânicos e inorgânicos responsáveis pelo metabolismo microalgal.

O uso do dióxido de carbono em conjunto com a energia solar contribui para a fixação do mesmo na biomassa microalgal. Essa conversão normalmente ocorre em duas etapas essenciais responsáveis pela fotossíntese: as reações dependentes da luz, que ocorrem nos tilacóides e o ciclo de Calvin-Benson, realizado no estroma. Na primeira etapa, são formados os compostos responsáveis pela energia (adenosina trifosfato) ATP e os redutores equivalentes (NADPH). A fase escura como conhecida, em resumo é caracterizada pelo uso de ATP e NADPH para a construção dos metabólitos (Masojídk et al., 2013).

Por outro lado, em termos de metabolismo heterotrófico, apenas algumas microalgas são capazes de realizar a absorção de fontes externas de carbono orgânico, das suas mais variadas formas, desde as estruturas químicas mais simples como a glicose até as formas mais complexas (Cheng et al.,

2019). As microalgas são capazes de armazenar energia na forma de amido, que posteriormente pode ser quebrado e utilizado pelas rotas metabólicas *Embden Mayerhoff-Parnas* (EMP ou glicólitica), e também através da rota das pentoses fosfato (PP), liberando NADH e ATP, realizando-se assim o processo de respiração (Hu et al., 2018).

O cultivo heterotrófico pode também ser uma alternativa às formas convencionais de tratamento de resíduos obtendo-se como resultado a valorização dos mesmos, que são utilizados como fonte exógena de carbono orgânico, possibilitando a produção de importantes metabólitos (Jacob-Lopes et al., 2007). Entre as espécies pesquisadas passíveis de serem aplicadas em resíduos agroindustriais a *Phormidium autumnale* destaca-se devido a sua elevada produtividade e ótima eficiência de remoção de matéria orgânica. Essa cianobactéria caracteriza-se por 3 a 4 µm de diâmetro, sendo filamentosa, a mesma possui habilidade crescer em condições com elevadas concentrações de nutrientes (Guiry & Guiry, 2014).

Com relação aos estudos nesta cepa, a literatura traz a caracterização do perfil de carotenoides (Maroneze et al., 2019), bem como o perfil das clorofilas, no qual foram separados onze compostos desta classe (Fernandes et al., 2017), ambos estudos obtidos por cultivo fotoautotrófico, utilizando diferentes quantidades de fótons. Outra classe de compostos pesquisada foram os ácidos graxos, provenientes de cultivo heterotrófico, neste estudo foram elucidados os perfis obtidos a partir de fontes exógenas de carbono distintas (Francisco et al., 2014).

A *Aphanotece microscopica* Nägeli, é outra cianobactéria que se destaca na produção tanto de carotenoides (Patias et al., 2017), quanto na produtividade lipídica (Francisco et al., 2010; Queiroz et al., 2011), demonstrando possuir um elevado teor de ácidos graxos insaturados em seu perfil (Zepka et al., 2008).

As cepas eucarióticas já foram mais intensamente estudadas em relação a composição de compostos bioativos (ao metaboloma), tornando-as mais utilizadas no mercado. De acordo com a *Algae Base* (Algaebase.org), mais de 160 mil espécies de microalgas estão catalogadas. Dentre as cepas eucarióticas a *Chlorella* é a mais utilizada no mercado, devido a sua composição, aliado aos baixos níveis de toxicidade. As cianobactérias em termos de mercado estão representadas pela microalga *Spirulina*, em que vem sendo utilizada, esta cepa é conhecida pelo elevado teor proteico e entrou no

mercado através do seu uso em suplementação alimentar Apurav et al. (2019).

3. ESTERÓIS

Na atualidade discute-se os potenciais que as microalgas e as cianobactérias possuem em produzir uma ampla gama de metabólitos com elevado valor agregado em nível industrial, substituindo-se assim as fontes não-renováveis. Dentre os compostos químicos de interesse podemos destacar: as proteínas, lipídios, carboidratos, pigmentos e os ácidos nucléicos (Maroneze et al., 2019). Embora exista este campo amplo de estudos utilizando as microlgas, tanto através do metabolismo fotoautotrófico, quanto do metabolismo heterotrófico, existe uma carência de estudos associados a produção dos esteróis. Para as cianobactérias, muitos estudos antigos difundiram a informação de que estes microrganismos não possuíam o aporte metabólico necessário para produzir tais moléculas Levin & Bloch (1964). Essa teoria foi levantada devido a presença dos hopanoides em sua estrutura da membrana celular, o qual supostamente substituiria a produção dos esteróis em sua função, sendo como uma das mais importantes a de regulação da membrana plasmática (Nes, 2011). No entanto, recentemente foram reportadas elevadas concentrações de esteróis na cianobactéria *Phormidum autumnale*, devido ao emprego apropriado de métodos analíticos associado também ao cultivo ideal para produção (Fagundes et al., 2019). Outros estudos reportaram a expressão genética de enzimas específicas produtoras de esqualeno, o precursor metabólico dos esteróis (Donia et al., 2011; Wei et al., 2016).

Os esteróis estruturalmente tem por base triterpenóides tetracíclicos, originados por um anel ciclopentanoperidrofenantreno com dois grupos metila angulares (Nes & Parish, 2012). Variações podem ocorrer no sistema dos anéis de acordo com as posições das duplas ligações, sendo estas moléculas pertencentes à classe de isoprenóides (Lu et al., 2014; Swan & Watson, 1998), conforme podemos observar na Figura 2, em conjunto com o perfil dos esteróis mais comuns encontrados em microalgas.

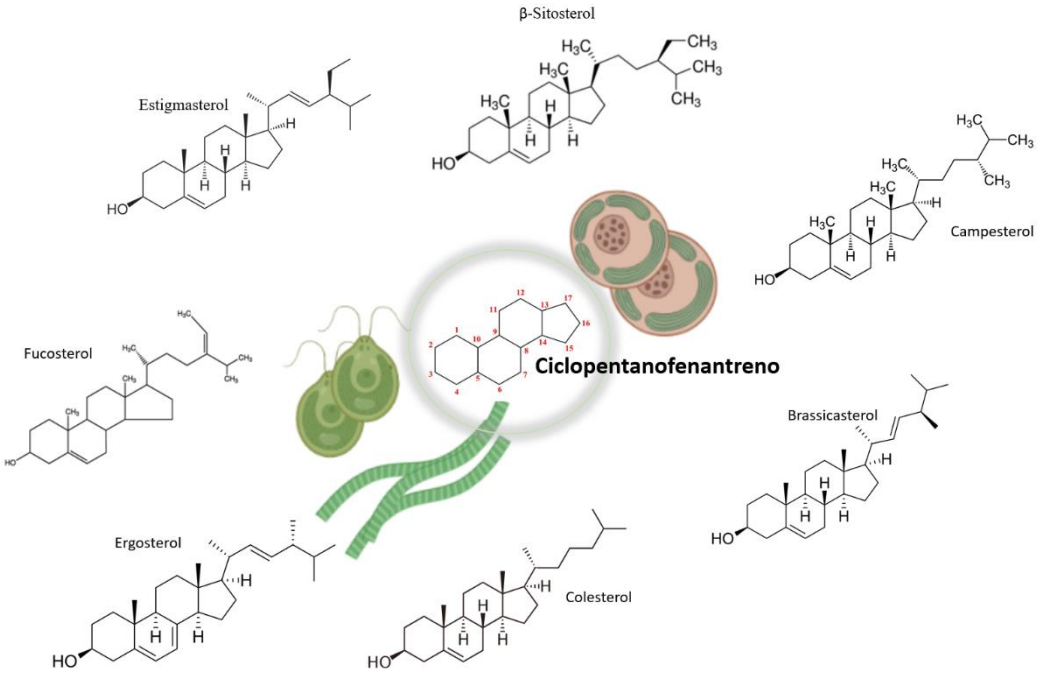

Figura 2. Esqueleto de um esterol (cicliperinofenantreno), e seus derivados (esteróis comumente encontrados em microalgas. Fonte: criado utilizando o biorender.

Funcionalmente, estas moléculas são responsáveis por manter a estrutura da membrana celular, de forma que, dependendo da composição de esteróis da membrana pode existir maior ou menor rigidez estrutural. Neste contexto, são responsáveis pela fluidez da membrana, ou seja, pelo processo de fagocitose, também responsáveis pelo *cell signaling*, e auxiliam na resistência ao estresse externo (Xu et al., 2005).

Os esteróis podem auxiliar na identificação das microalgas e cianobactérias, pois estas estruturas como muitos triterpenóides policíclicos, são bastante recalcitrantes e seus produtos de degradação, os esteranos, são facilmente preservados em sedimentos antigos, de forma que podem ser mantidos por mais de 1,6 bilhão de anos (Brocks, 2005).

Os esteróis obtidos por fontes marinhas usualmente possuem característica estrutural única, tais características não são encontradas em fontes obtidas de superfície terrestre, como o exemplo do grupo de sulfatos de topsentiasterol, proveniente da cepa de *Topsentia sp,* aliado a esta informação é interessante salientar que modificações estruturais fornecem novas funções biológicas (Pereira et al., 2013).

3. CARACTERIZAÇÃO DE ESTERÓIS E ESQUALENO EM PROCESSOS MICROALGAIS

O estudo das rotas metabólicas para a produção destas moléculas bioativas, torna-se de grande importância. Desse modo é possível identificar e obter químicos considerados intermediários com potencial bioativo, uma vez que, cada rota possui seus intermediários específicos (Panchasara et al., 2018).

O esqualeno apresenta-se como um metabólito necessário para ambas as rotas de produção dos esteróis. Neste sentido, a otimização da produção de esqualeno esta principalmente associada as modificações de cultivo como podemos observar em leveduras em que apenas modificando-se os substratos (concentração de lipídeos) tem-se concentrações superiores deste metabólito (Wei et al., 2018).

Com relação as microalgas, a cepa *Aurantiochytrium mangrovei* apresenta a maior capacidade dentre outras cepas, chegando a concentrações de 21.2 g L^{-1}. Em estudo através de um método de otimização com a inserção de terbinafina (10-100 mg L^{-1}), como agente de inibição da síntese de esteróis e glicose como fonte externa de carbono, foi possível observar um aumento de 36 e 40%, respectivamente (Fan et al., 2010). Pesquisas anteriores demonstram que a cepa *Phormidium autumnale* é produtora de esqualeno, atingindo quantidades de 1440 mg kg^{-1} de biomassa seca (Fagundes et al., 2019a). Essa cianobactéria cultivada em meio heterotrófico, em resíduo de abatedouro, foi comparada a sua fonte tradicional de obtenção o óleo do fígado do tubarão.

Essa cepa demonstrou que através da sua elevada produtividade, pode ser facilmente empregada em sistemas biotecnológicos de remoção de matéria orgânica, com simultânea produção de esqualeno, atingindo valores superiores a fonte tradicional (Fagundes et al., 2019b). Após a produção do esqualeno, ocorre a síntese de esteróis, cujo qual diferirá de acordo com o metabolismo microalgal, pois cada filo é caracterizado por uma classe específica de esteróis, vale destacar que, fatores externos ao cultivo também proporcionam modificações nas produção destes metabólitos (Volkman et al., 2016).

Um breve histórico da produção destes compostos descreve que, os primeiros estudos de

caracterização foram realizados em microalgas verdes de acordo com Patterson (1974), sendo estas: *Cladophorales*, na qual foi encontrado um alto teor de colesterol, 24-metileno colesterol e 28-isofucosterol; *Spirogyra sp*., foram encontrados os esteróis, clionasterol e poriferasterol; *Chlorella* sp. foi reportado esteróis 24-β como compostos majoritários, como sitosterol e stigmasterol.

Atualmente, ainda existem muitos esteróis sendo elucidados, de acordo com os pesquisadores Li et al. (2017) foram encontradas novas estruturas químicas através da espectroscopia de ressonância magnética nuclear 1D e 2D, sendo estas, (24R)-5,28-stigmastadieno-3β,24-diol-7-ona, (24S)-5,28-stigmastadieno-3β,24-diol-7-ona, e (24R-24S)-vinilcolesta-3β, 5-α, 6β, 24-tetraol na cepa de *Ulva australis*. Os autores Geng et al. (2017) reportaram os principais esteróis de 13 espécies de algas pertencentes aos filos: *Dinophyceae, Bacillariophyceae, Ulvophyceae* e *Pelagophyceae*. Os principais esteróis encontrados em cada filo foram: 28- isofucosterol e colesterol (Ulvophycea), colesterol, (24 E) 24-propilidenecholesterol, (24Z)-24-propilidenecolesterol, (22E)-stigmasta-5,22-dien-3β-ol, campest-5-en-3β-ol, e stigmast-5-en3β-ol (Pelagophyceae), dinosterol, colesterol, (22E)-ergosta-5,22-dien-3β-ol e (22E)-stigmasta-5,22-dien-3β-ol (Dinophyceae).

No trabalho de Belghit et al. (2017), os autores encontraram os seguintes esteróis, ergosterol, fucosterol, colesterol, campesterol, estigmasterol, e β-sitosterol, em 21 cepas de microalgas. As maiores concentrações encontradas em *Gracilaria vermiculophylla* (*Rhodophyta*) foram (4135,7 μg g^{-1} de biomassa seca) de colesterol e 731,1 μg g^{-1} de biomassa seca de fucosterol para a *Sargassum fusiforme* (*Phaeophyta*).

Para a cepa *Grateloupia asiática* Kawaguchi & Wang foi encontrado 661,0 (μg g^{-1} biomassa seca) de β-sitosterol, já com relação as cepas *Gracilaria vermiculophylla* (*Rhodophyta*), e *Undaria pinnatifida* (*Phaeophyta*), foram encontradas concentrações de ergosterol de 93,4 (μg g^{-1} de biomassa seca) e 86,9 (μg g^{-1} de biomassa seca), respectivamente.

Campesterol foi encontrado apenas na microalga *Zostera marina* Linnaeus (*Spermatophyta*), apresentando concentrações de 125, 9 μg g^{-1} de biomassa seca. Assim como estigmasterol que foi encontrado apenas na cepa *Grateloupia asiatica* (*Rhodophyta*), apresentando concentrações na faixa de 60, 7 μg g^{-1} de biomassa seca. Já com relação as cianobactérias, as classes de esteróis comumente encontradas são as 24-etilcolesterol, mas vale destacar que a síntese de esteróis vai se diferenciar de

acordo com outros fatores externos ao cultivo (Volkman, 2003).

Em estudos recentes Fagundes et al. (2019a) descreveram a produção de diferentes esteróis em *Phormidium autumnale* cultivada em sistema heterotróficos com diferentes fontes de carbono, sendo eles: estigmasterol (455,3 μg g^{-1}), β-sitosterol (279,0 μg g^{-1}), colesterol (820,6 μg g^{-1}) e ergosterol (1033,3 μg g^{-1}) todos expressos em biomassa seca.

4. DESENVOLVIMENTO ANALÍTICO: MÉTODOS VERDES PARA EXTRAÇÃO DE ESTEROIS

O desenvolvimento dos métodos analíticos são cruciais para extração de compostos bioativos, com precisão e exatidão de massa. Principalmente quando se tem poucos estudos acerca da matriz. Neste sentido, na extração de metabólitos secundários provenientes da biomassa microalgal, o desenvolvimento analítico é imprescindível, pois a partir dele podem ser gerados novos protocolos que auxiliam no dimensionamento destas biomoléculas, passíveis de serem empregadas a nível industrial (Massom et. al., 2017).

Logo, para estudos de desenvolvimento analítico, dentro do campo de biotecnologia microalgal, métodos de miniaturização de amostra são requeridos, pois normalmente reatores em escala laboratorial, possuem uma dimensão de aproximadamente 2 L. Em função disto, o dimensionamento amostral de uma menor quantidade de amostra necessita ser realizado para se ter respostas do ativo em várias fases do cultivo (Vendruscolo et al., 2019).

Não apenas com relação ao tamanho do cultivo, mas também tendo em vista que muitos métodos se baseiam em no emprego de substâncias extremamente tóxicas, principalmente, metodologias antigas, e sabe-se de seus efeitos adversos para o meio ambiente e analistas (Lenoir et al., 2020). Com essa linha de pensamento o autor Paul Anastas foi o precursor da terminologia química verde, ele foi capaz de definir as doze regras da química verde através dos trabalhos publicados: Anastas & Warner (1998) e Anastas & Williamson (1998). Essas regras tiveram grande impacto na comunidade científica, e a Química Verde tornou-se um campo próprio nas várias linhas de pesquisa e englobam os impactos no meio ambiente, financeiro e na sociedade (Płotka-Wasylka, 2021).

Os métodos modernos visam a redução/ausência de solvente orgânico, e também aliado com a redução do tempo de extração (Pena-Pereira et al., 2020). Neste sentido, na análise de esteróis, os autores Skubic et al. (2020) relataram que bons resultados de recuperação e exatidão podem ser adquiridos com métodos reduzidos de etapas. Normalmente, os métodos de determinação de esteróis envolvem processos de extração com solventes orgânicos, seguida de saponificação (Grasso, et al., 2016; Winkler-Moser, 2020). Outros métodos utilizam técnicas de extração por SPE (do inglês *solid phase extraction*) (Xu et al. 2020), outros relatam ainda hidrólises ácidas para a recuperação destes analitos (Simonetti, et al., 2020). Atualmente também existem formas de derivatização distintas, que visam a melhor resposta analítica dos ativos de interesse, tais novas características podem auxiliar no aumento da resposta analítica em detectores como exemplo ESI (do inglês *electrospray ionization*) (Kamgang, 2020).

Os métodos de extrações de esteróis em microalgas são escassos devido a elevada dificuldade do processo por serem metabólitos secundários e diferirem de concentração de acordo com o cultivo. Todavia, trabalhos como reportado por Fagundes et al. (2021) demonstram que redução de tempo, quantidade e volume de solventes tóxicos, pode auxiliar no campo da biotecnologia microalgal, descobrindo e ajudando em avanços dos estudos esterolômicos.

Por mais que ocorram reduções das quantidades de amostras, e solventes, buscar por novas alternativas, que sejam consideradas verdes faz-se necessário. Logo, extrações com solventes com base natural como: água, dióxido de carbono, etanol, e outros como alguns terpenos, são utilizados atualmente como alternativas sustentáveis. Estes solventes podem ser utilizados em métodos de extração verdes, como microondas, ultrassom (Adam et al., 2012), campo elétrico pulsado (Kumari et al., 2018) e os fluídos comprimidos. Dentre os fluídos comprimidos podemos destacar as técnicas de extração subcríticas, conhecidas como extraçoes por líquido pressurizado (do inglês, PLE).

O PLE baseia-se no uso de solvente líquido pressurizado a temperaturas acima do ponto de ebulição, permitindo extrações rápidas devido à maior difusividade, e elevada solubilização das interações analito-matriz (Alvarez-Rivera et al., 2020).

Outra extração que está ao encontro dos princípios da química verde, é a que faz o uso de fluido supercrítico (SFE). Nesta extração, o solvente é usado acima do seu ponto crítico, permitindo aumentar

a seletividade para compostos específicos no processo de extração. Essas extrações tem sido utilizadas em processos microalgais, com a terminologia de biorrefinaria, que visa trabalhar com várias etapas de extração utilizando solventes distintos afim de explorar ao máximo a extração da biomassa (global) (Herreiro et al., 2010).

Diferentes frações químicas são obtidas de acordo com as propriedades da extração. Compostos apolares são adquiridos utilizando-se solventes não polares, como por exemplo utilizar SFE-CO2, a polaridade dos analitos extraídos vai aumentando de acordo com a adição de solvente orgânico, como exemplo, o uso de um gradiente de polaridade (SFE-CO_2 + etanol). Se o emprego desses co-solventes for em concentração até 15%, considera-se extração por liquido expandido, acima pode-se dizer que está trabalhando na faixa de PLE (do inglês *pressurized liquid extraction*) (Bueno et al., 2019; Sánchez-Camargo, et al., 2020).

PLE aplicado as microalgas é altamente utilizado para extrair compostos mais polares como os fenólicos e flavanoides. Para trabalhar com a fração de esteróis deve ser otimizado método métodos como SFE-CO_2, pois estes compostos são considerados semi-polares/ apolares. Trabalhar com solventes verdes em um processo de extração de compostos bioativos é ideal devido a elevada bioatividade dos extratos, superior em comparação com solventes não convencionais (Herrero et al., 2018).

5. OTIMIZAÇÕES DE MÉTODOS DE EXTRAÇÃO E DE ANÁLISE.

Para o desenvolvimento de novas técnicas, afim de aprimorar as metodologias já existentes, os métodos de otimizações apresentam-se como alternativas ideais. Os delineamentos experimentais têm por objetivo conseguir os melhores resultados analíticos minimizando custos e tempo de análise, similarmente maximizando o rendimento do produto de estudo, alinhado a isto diversos estudos vem utilizando as técnicas de planejamento de experimentos (Di Battista et al., 2017).

A otimização de uma resposta a partir de planejamentos experimentais podem ocorrer utilizando delineamentos que inicialmente visam a seleção de variáveis. Normalmente os delineamentos Plackett-Burman (PB) são utilizados para esta finalidade, para identificar os fatores mais importantes no início da fase de experimentação. Adicionalmente, após a seleção das variáveis de influência

realizam-se as aplicações dos planejamentos experimentais com a finalidade de chegar à resposta dos parâmetros ótimos do processo em estudo (Rodrigues; Iemma, 2005).

A metodologia de superfície de resposta (RSM) é considerada uma técnica matemática e estatística com base no ajuste de modelos empíricos para os dados experimentais obtidos em relação ao desenho experimental. Em termos da aplicabilidade desta técnica analítica para o uso em otimização de técnicas cromatográficas, os modelos mais utilizados são os fatoriais de três níveis (Bezerra et al., 2008).

As aplicações dos estudos de superfície de resposta são inúmeras, tanto em relação a otimização de processo de produção de compostos, quanto dentro do desenvolvimento analítico (Li et al., 2015; Bogdanovic et al., 2016; Heleno et al., 2016; Rudke et al., 2019). Entretanto, nenhum relacionado a métodos de extração de esteróis em cianobactérias. Portanto, as técnicas de otimização levam a respostas sobre o ótimo de obtenção, tanto em termos de extração quanto em termos de produção e análise destes metabólitos alvo, resultando em uma resposta confiável com baixos níveis de detecção.

6. POTENCIAL BIOATIVO DOS EXTRADOS OBTIDOS DO ESTUDO ESTEROLÔMICO

Os extratos de esteróis microalgais podem apresentar vantagens significativas frente a fontes convencionais, não apenas por serem fontes renováveis e de elevada produtividade, mas também por essas cepas possuírem esteróis não convencionais passíveis de serem aplicados em estudos, como a anti-diabetes, anti-obesidade, anti-aterosclerose, anticancer, anti-alzheimer, e hepatoprotectora, entre outros. Tornando-os componentes essenciais de alimentos funcionais e com elevado potencial de serem utilizados como insumo farmacêutico (Hannan et al., 2020). Festa et al. (2011) encontraram esteróis não convencionais, conhecidos como solomonsterol A e solomonsterol B, com potencial de atividade de redução dos efeitos adversos encontrados em tratamentos de câncer.

Sabe-se também que os esteróis podem levar uma redução de colesterol significativa em humanos, deixando os níveis iguais aqueles obtidos com dietas pobres em gordura, tais efeitos adquiridos em função dos fitoesteróis esterificados (Moreau et al. 2002). Ao estudar a cepa de *Sargassum horneri*, Zhao et al., (2016) verificaram que o β-sitosterol proveniente da cepa apresentou

atividade antidepressiva em ratos. Outros esteróis, como o fucosterol também foram reportados com mais de uma funcionalidade, demonstrando que são propulsores do aumento dos níveis de responsáveis pelo auxilio no sistema antioxidante (Lee et al., 2003).

Lopes et al. (2011) descreveram que os esteróis (fitosteróis) possuem a habilidade de alterarem a atividade de algumas enzimas associadas as membranas celulares e modificar a transdução do sinal nas células tumorais. Em conjunto a isto, Brassicasterol, muito comum em microalgas, já tem sido usado atualmente em tratamentos de HIV, devido a seus efeitos antipiréticos (Hassan, 2020).

O estigmasterol também tem sido um esterol utilizado em terapias de doenças reumáticas e possui benefícios anticatabólicos (Gabay et al., 2010). Ademais, em estudos recentes realizados por Fagundes et al. (2021), foi observado possíveis efeitos protetores contra a neurodegeneração, através de estudos *in-sílico* da inibição da enzima acetilcolinaesterase.

7. CONCLUSÃO

As microalgas, por fim, demonstram ser fontes de esteróis. Em função disto, desenvolvimento de métodos que utilizem das mais avançadas ferramentas de extrações afim de isolar os metabólitos secundários, biologicamente ativos, da forma mais adequada para manter sua bioatividade, são imprescindíveis nos dias atuais. Pois, os esteróis derivados de algas apresentam um grande potencial de aplicações na indústria farmacêutica e de alimentos. Deste modo, fica evidente que os fitoesteróis exibem várias atividades biológicas, sendo a grande diversidade de microalgas marinhas uma fonte natural de fitoesteróis estruturalmente distintas com elevado potencial de exploração.

RECONHECIMENTOS

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4 CAPÍTULO 2

4.1 CAPÍTULO DE LIVRO 1

Chapter 21 Sterols from microalgae

Book: Fundamentals and Advances in Energy, Food, Feed, Fertilizer, and Bioactive Compounds 2020, Pages 573-596

CHAPTER 21

Sterols from microalgae

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Chapter outline

21.1 Introduction

Microalgae are well-known as a source of molecules with high bioactivity; these promising microorganisms can be used in order to increase health humans conditions (Jacob-Lopes et al., 2019). In this regard, there are many classes of bioactive compounds that could be isolated, such as carotenoids, phycobiliproteins, fatty acids, amino acids, and other important bioactives (Maroneze et al., 2019; Francisco et al., 2010; Vendruscolo et al., 2019; Pan-utai and Iamtham, 2019).However, there are a lot of compounds that need better investigation, being one of this the sterols. These molecules play an important activity in concern to microalgae cell structure, because they help in the membrane permeability (Clair and London, 2019).

Microalgae strain can present a wide range of sterols, from cholesterol to β-sitosterol, as major compounds (Fagundes et al., 2019a). Therefore, modifications in culture conditions could also change the sterol profile. This study's findings could be explored as a substantial new research area, due to the importance of sterols as antioxidant, anticarcinogenic, and antiinflammatory compounds (Volkman, 2016). This chapter discusses some aspects of microalgae sterol

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bioactivity, analytical tools for sterol determination, and sterols' potential in terms of industrial production. Based on the above, we focus on microalgae as a potential source of sterols, showing the aspects of their chemical structure, bioactivity potential, biosynthesis, and production, as well as the possible aspects of improving the concentration in microalgae strains.

21.2 Structural aspects of sterols

The ocean could be considered a new potential source of bioactive molecules; it is well known that marine chemistry currently needs to be explored further. Because many healthy human problems can be overcome by the discovery of new chemical compounds from these microorganisms, among these molecules, the sterols can be better studied. Sterols primordial structure is a cyclopentaneperhydrophenanthrene, known as a sterane ring with two angular methyl groups. The sterol structure is similar to steroids, considered their subgroup, and the major difference is the presence of a hydroxyl group (OH) at the 3-position from the A-ring. These compounds are also considered amphipathic molecules, and their primordial structure can be observed in Fig. 21.1.

Structurally, sterols have divisions built by three cyclohexanes (A, B, and C) and one cyclopentane (D). Characteristics with regards to the A section is the most important chemical part, due to the presence of the hydroxyl group (OH), which is responsible for all hydrogen-bond interactions, being active hydrogen for other chemical interaction. B is more related to structure planarity, and C is associated with the orientation of the side chain at C20. The other groups are responsible for the tilt and shape; however, depending on the chain bonded in D-cyclopentane, they can help in cell-chemical interactions (Nes, 2011).

A great diversity of sterols are found, and these variations undoubtedly influence the sterols' bioactivity and the bioaccessibility of these structures. It is important to note that sterols' configuration directly affects the cellular membrane, because the specificity of β cholesterol's 3-hydroxyl configurations is responsible for the interactions with chiral targets. Tsuchiya and Mizogami (2017) showed that the chiral sterol contained in the prokaryotic cell membranes differs from that in eukaryotic microorganisms, and also that normally these compounds in

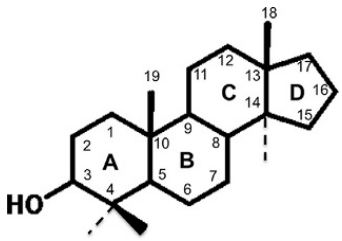

Fig. 21.1
Sterol basic chemical structure.

prokaryotic cells are replaced by hopanoids, which are similar to pentacyclic compounds. However, this is not a total substitution, because sterols can be found in some cyanobacteria (Belin et al., 2018).

Microalgae present some characteristics in sterols' structure, which is associated with the carbon 24 side-chain, with modifications in this side chain it could distinguish the microalga species. Volkman (2003) describes how 24 β-methyl sterols are located in many less-advanced organisms, including fungi and protozoa. On the other hand, the 24 α-ethyl sterols are more common in advanced organisms, being considered similar to plant metabolism. Usually, sterol configuration can present after the carbon 24 α a methyl, ethyl, or propyl groups, however, the propyl formation is more difficult to acquire them the other ones.

However, in terms of molecular phytosterols' structure, these molecules have a chain increased at carbon 24 compared to cholesterol, and this feature may provide an increase in van der Waals forces. The explanation of membrane chemicals can aid understanding of the physical cellular aspects—for example, in cell disruption studies, assisting in the comprehension of how to acquire these intracellular bioactive compounds from microalgae. In this sense, as mentioned, sterols are also essential for cell protection, being a constant group of the compound to be isolated, studied, and elucidated.

Clair and London (2019) showed the specific cell endocytosis, which is the absorption of molecules by the membrane. This situation occurs mostly according to the domains found that can be characterized as symmetric or asymmetric vesicles. In this research it can be observed differences in the membrane endocytosis, and this is associated with the membrane symmetry. Cholesterol stabilize ordered domain more than cholesten-3-one, while for symmetric vesicles the less ordered domain was epicholesterol. On this point, the endocytosis levels are closely associated with the ability of sterols to form ordered domains, and this theme has been a new approach in membrane studies.

With this in mind, it is essential to comprehend the class of sterol found, not only due to their high value, but also because these molecules are important to understand the microalgae phylogenetic organization (Leblond et al., 2010). For example, the phylum Chlorophyta (green algae) presents similarities to plant metabolism; however, there are too many species in this phylum that make difficult the sterols' determination, with the classes of 24 α, and 24 β sterols being the most common forms found (Volkman, 2016; Patterson, 1974). Abdel-Aal et al. (2015) determined the following compounds in the sterol composition of *Spirogyra longata*: stigmasterol, β-sitosterol, campesterol, ergosterol, brassicasterol, Δ7-stigmasterol, and Δ7-avenasterol. However, when analyzing Chrysophyta (gold algae), such as *Ochromonas danica* and *Ochromonas malhamensis*, these strains present mostly in their composition the 24 β, such as poriferasterol and cholesterol. Gershengorn et al. (1968) also described the same compounds in this strain. In the same line, the strain *Synura petersenii* has proven to have huge production of cholesterol (Collins and Kalnins, 1969), being the major compound described for

this phylum. The phylum Rhodophyta is known as red algae and also presents cholesterol as a major sterol, being similar to the Chrysophyta phylum (Tsuda et al., 1958). Other molecules from cholesterol metabolism such as cholestanol were observed that were found in *Gracilaria salicornia* and *Hypnea flagelliformis* (Nasir et al., 2011).

Regarding Dinophyta, dinoflagellates phylum, the major sterols present are 4-methyl, and 4-desmethyl sterols; for example, dinosterol can be found, which is considered the most important molecule from this phylum (Volkman, 2016). The phylum Euglenophyta presents ergosterol as a major compound in its composition, a typical molecule found as a fungal biomarker (Watanabe et al., 2017). Another well-known phylum is Phaeophyta; these microorganisms are known as brown algae, and present as major metabolite fucosterol (Patterson, 1971).

In the field of sterols, there are discussions among many researchers about cyanobacteria classification, because they are prokaryotic microorganisms (Volkman, 2018), and there are reports suggesting that these strains are not sterol producers (Levin and Bloch, 1964). However, the number of studies on microalgae sterol production has increased.

21.3 Sterol biosynthesis

Sterols can be synthesized by distinct pathways, as microalgae are very versatile, and capable of working with different pathways. For their production, two pathways could be used: the first one allows the formation of squalene, which is considered a key precursor for sterol formation; the other leads to the formation of specific sterols. These two pathways are shown in Figs. 21.2 and 21.3.

Microalgae cells need sources of chemical energy for the activation of metabolic pathways; the energy could be obtained by the chemical process of photosynthesis or by chemical phosphorylation in a heterotrophic culture (Perez-Garcia et al., 2011; Williams and Laurens, 2010). Usually, all microalgae by their nature are photosynthetic. However, some strains are capable of metabolizing complex or simple exogenous carbon sources (Perez-Garcia et al., 2011). In this pathway, after the obtainment of energy, the major building blocks for squalene production are glyceraldehyde-3-phosphate (GAP), pyruvate, and acetyl coenzyme A (acetyl-CoA).

Considering the photosynthetic medium, the building-block molecules are obtained in two phases (light and dark), initially in the light phase, which starts at the thylakoids lamella of the chloroplast. In the thylakoids are the photosystems, and its act capturing sunlight and assist the electrons transfer. The photosystems consist of proteins, pigments, and electron carriers. After different photons are obtained, light starts an oxidative phosphorylation, producing adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADPH); after this, the water photolysis electrons are released. These processes will be used to replace the other electrons

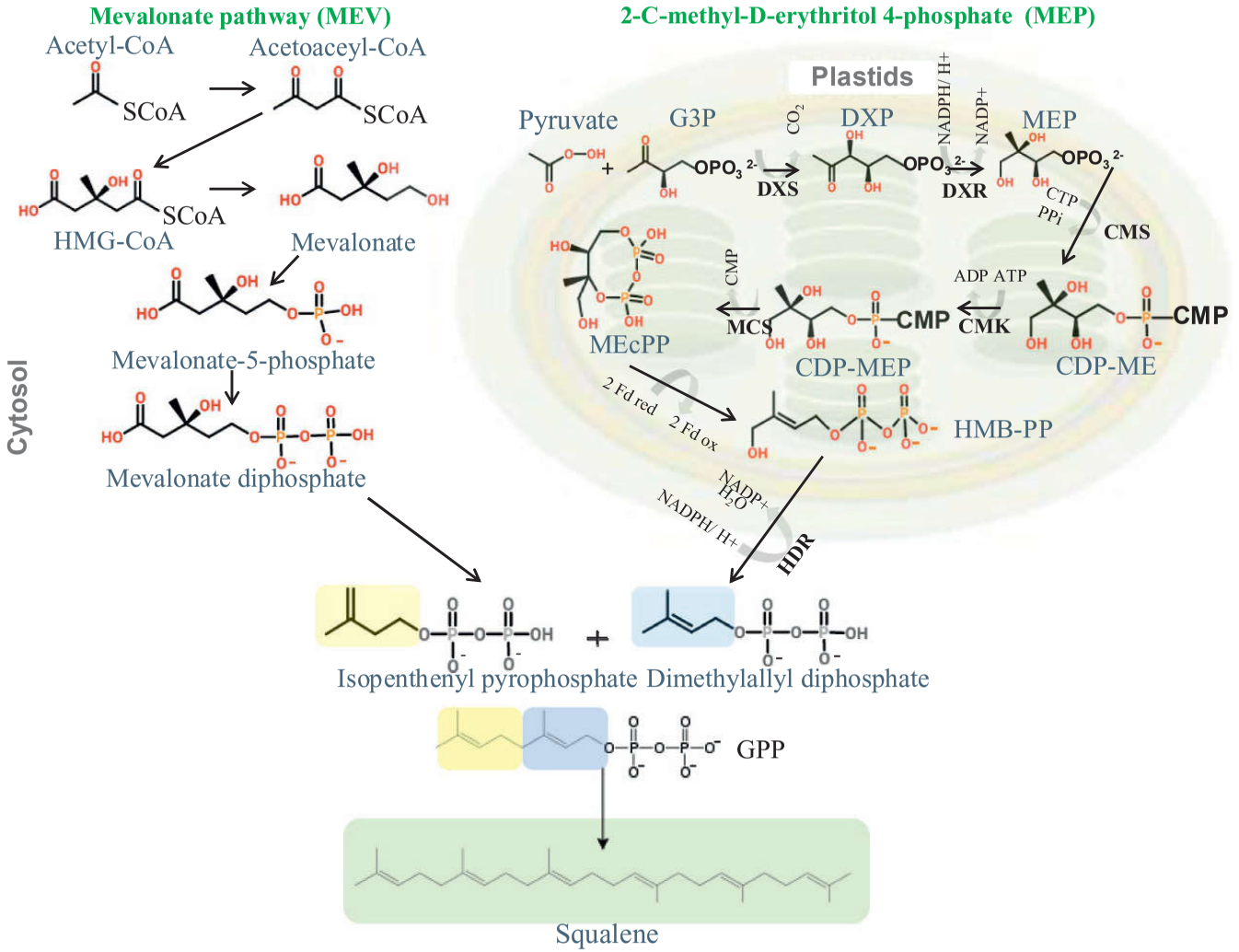

Fig. 21.2

1-Sterols biosynthesis: mevalonate (MEV) pathway and 2-C-methyl-D-erythritol-4-phosphate (MEP). Acetyl coenzyme A (acetyl-coA); 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA); 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA sintase); 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoA reductase); mevalonic acid (MEV); mevalonate kinase (MEV kinase); mevalonate phosphate (MEV phosphate); mevalonate pyrophosphate kinase (MEV pyrophosphate kinase); mevalonate pyrophosphate (MVA pyrophosphate); mevalonate pyrophosphate decarboxylase (MVA pyrophosphate decarboxylase); isopenthyl pyrophosphate (IPP); dimethylallyl pyrophosphate (DMAPP); glyceraldehyde-3-phosphate (G3P); 1-deoxy-D-xylulose-5-phosphate (DXP); 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR); 2-C-methyl D-erythritol 4-phosphate (MEP); 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (CMS); 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CDP-ME); 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK); 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (CDP-MEP); 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS); (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (MecPP); (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate synthase (HDS); 4-hydroxy-3-methylbut-2-enyl diphosphate (HMB-PP); 1-deoxy-d-xylulose 5-phosphate synthase (DXS); 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR).

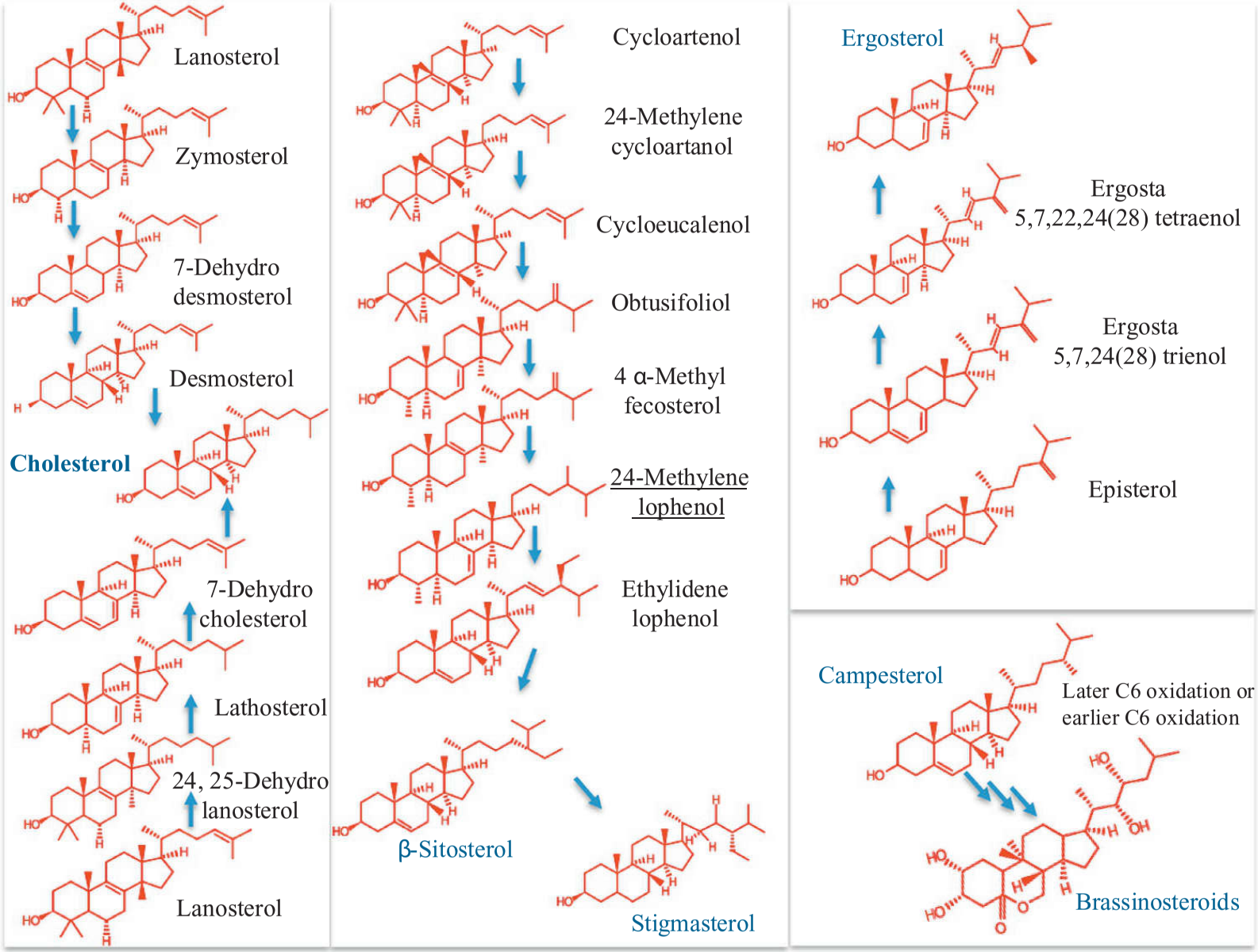

Fig. 21.3
Different sterols pathways found in microalgae metabolism.

lost by the chlorophyll in photosystem II and later to produce the oxygen molecule. The second phase is characterized by the Calvin cycle, in which several reactions lead to the formation of GAP, and two of these molecules originate glucose phosphate (Williams and Laurens, 2010). In contrast, the heterotrophic metabolism occurs in the total absence of luminosity, being known as the pentose-phosphate pathway (PPP), and also the Embden-Meyerhof pathway. At first, in these metabolisms occurs the reduction of complex carbohydrate structures to their simplest forms, which are assimilated through the metabolic process—for example, the glucose structure. After the glucose transformation, oxidative phosphorylation reactions are initiated, resulting in glucose-6-phosphate, the PPP route, in which ribulose-5-phosphate forms, and the formation of this metabolite leads to the production of glyceraldehyde-3-phosphate (Perez-Garcia et al., 2011). The sequence occurs by two possible pathways, and according to Lohr et al. (2012), it can be activated depending on the algae's evolutionary history. The route possibilities are the mevalonic acid pathway (MEV) and the methylerythritol phosphate pathway (MEP) (Fig. 21.2). The first route occurs in the cellular cytosol, whereas the second production route takes place mostly in the plastids (Gohil et al., 2019). MEV begins by condensation of acetyl-CoA with 3-hydroxy-3-methyl-glutaryl-CoA producing hydroxymethylglutaryl coenzyme A. Next, the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) forms the mevalonate (MEV) (Miziorko, 2011; Eisenreich et al., 2004). In MEP occurs the condensation of glyceraldehyde-3-phosphate and pyruvate; production of these leads to the formation of deoxy-D-xylulose-5-phosphate, followed by the enzyme 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), which produces 2-C-methyl-D-erythritol-4-phosphate (MEP).

Additionally, after the formation of MEV and MEP, the following reactions lead to the same molecule, which is considered a meeting point of both metabolic routes, the isopentenyl pyrophosphate, which is converted to farnesyl pyrophosphate by the enzyme farnesyl pyrophosphate synthase (FPPS), leading to a conversion by squalene synthase (SQS) enzyme to squalene. Then, in the presence of oxygen squalene epoxidase (SQE), the enzyme converts squalene to 2,3-oxidosqualene (Lohr et al., 2012; Bhattacharjee et al., 2001).

The sterols pathway end products, e.g., cholesterol, stigmasterol, and others is going to depend on the microalgae species, and also the environmental conditions, being the microalgae system possible metabolic transformations are shown in Fig. 21.4.

In this sense, to produce phytosterols such as stigmasterol and β-sitosterol, the enzyme cycloartenol synthase (CAS) converts 2,3-oxidosqualene to cycloartenol, which by the enzyme Δ24-sterol methyltransferase (SMT) is converted to 24-methylenecycloartenol, followed by the formation of obtusifoliol. This compound is converted to Δ8,14-sterol by the obtusifoliol-14-demethylase enzyme (ODM). Following other conversions this leads to the formation of 4-α-methylfecosterol to 24-methylene-fecosterol, to 24-methylene lupeol, to cicloestradiene, to avenasterol, and finally to β-sitosterol, and stigmasterol is synthesized from β-sitosterol by cytochrome P450 CYP710A1 through denaturation of carbon 22 (Griebel and Zeier, 2010).

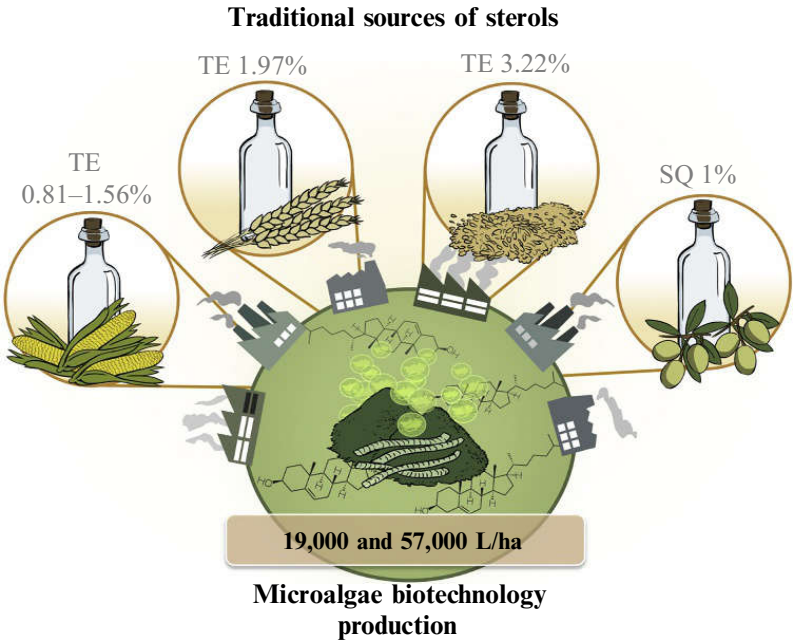

Fig. 21.4

Biotechnology production compared with traditional sources sterols and squalene, *TE*, total sterols; *SQ*, squalene; maize oil (0.81%–1.56%), germ oil (1.97%), rice bran oil (3.22%), and olive oil (1%).

In the cholesterol pathway, after 2,3-oxidosqualene, the formation of lanosterol occurs by squalene monooxygenase enzyme (SQLE) to 4,4,14-trimethylcholesta-8(9)-en-3β-ol. The conversions are as follows: 4,4-dimethylcholesta-8 (9)14-dien-3β-ol, then 4,4-dimethylcholesta-8(9)-en-3β-ol, followed by the formation of 4-α-methylcholesta-8(9)-en-3β-ol, to cholesta-8(9)-en-3β-ol, and then to cholesta-7-en-3b-ol. Finally, the cholesta-5,7-dien-3b-ol is formed as the last intermediate, and then is converted to cholesterol (cholesta-5-en-3β-ol) (Cerqueira et al., 2016).

For ergosterol, according to Brumfield et al. (2010), *Chlamydomonas reinhardtii* presents a difference compared to fungal ergosterol's metabolism; fungi metabolism starts with the lanosterol conversion. The difference pointed out by the authors is that this pathway starts with cycloartenol, followed by obtusifoliol. In this step we have the metabolism similar to the phytosterols, so, as can be observed in Fig. 21.3, the 24-methylene lophenol is converted to episterol, and a sequence of reactions then leads to ergosterol (Fagundes et al., 2019a; Brumfield et al., 2010).

Eukaryotes microorganisms present many reactions well studied in terms of sterols pathways, being methylations, unsaturations, and isomerizations, however, for prokaryotes, is not the same (Wei et al., 2016). According to Villanueva et al. (2014) there is some pathways already elucidated, e.g., ergosterol conversion to 5,7-ergostatrienol, 5,7,28-(24)-ergostatrien-3-ol, 5,7,22,28-(24)-ergostatetraen-3-ol, and ultimately to the end

product ergosta-5,7,22-trien-3-ol (ergosterol) (Fig. 21.3). So, prokaryotic microorganisms, can activate either lanosterol or cycloartenol pathways as described in a previous work with the strain *Phormidium autumnale* (Fagundes et al., 2019a).

Brassinosterols are another important end product pathway, and could be synthesized by many microalgae, being an example of the strain *Chlorella vulgaris* as described by Bajguz (2019). The possible paths involved are the C6-oxidation or earlier C6-oxidation; these two paths start with campesterol and lead to 6-deoxocathasterone to 6-deoxoteasterone, and subsequently to 6-deoxo-3-dehydroteasterone, 6-deoxotyphasterol, and 6-deoxocastasterone. The next compound is castasterone, which is found before brassinosterol; this is characterized as the later C6 oxidation. The last path also starts with campesterol, but the ensuing transformation sequences are as follows: 6-oxocampesterol, cathasterone, teasterone, 3-hydroteasterone, typhasterol, and cathasterone to brassinolide.

21.4 Sterols

The synthesis of sterols can be different among microalgae as mentioned before, since a class exists that characterizes each phylum. In addition, culture modifications can also lead to different sterol responses and composition (Volkman, 2003).

The study about green algae is not new. Patterson (Patterson, 1974) in 1974 characterized some green algae; among them in *Cladophorales* large amounts of cholesterol, 24-methylene cholesterol and 28-isofucosterol were identified, while for *Spirogyra* sp., clionasterol and poriferasterol were found. In the same study, *Chlorella* species were studied, and in their composition, the class of 24β sterols was found.

Li et al. (2017) identified by 1D and 2D nuclear magnetic resonance spectroscopy new structures of sterols compounds as (24*R*)-5,28-stigmastadiene-3β,24-diol-7-one, (24*S*)-5,28-stigmastadiene-3β,24-diol-7-one, and (24*R*) and (24*S*)-vinylcholesta-3β,5α,6β,24-tetraol from the strain *Ulva australis*. Other new sterols were found due to continuous research in this field, such as isofucoterol, (24*R*,28*S*)- and (24*S*,28*R*)-epoxy-24-ethylcholesterol, and (24*S*)-stigmastadiene-3β,24-diol, in the same strain.

Sterols from 13 species of algae belong to the phyla: Dinophyceae, Bacillariophyceae, Ulvophyceae, and Pelagophyceae were reported by Geng et al. (2017). In this work, the authors found Ulvophycea sterols, such as 28-isofucosterol and cholesterol. The Pelagophyceae presented six sterols, including cholesterol, (24*E*)-24-propylidenecholesterol, (24*Z*)-24-propylidenecholesterol, (22*E*)-stigmasta-5,22-dien-3β-ol, campest-5-en-3β-ol, and stigmast-5-en3β-ol. For Dinophyceae, in a dinoflagellate biomass, dinosterol, cholesterol, (22*E*)-ergosta-5,22-dien-3β-ol, and (22*E*)-stigmasta-5,22-dien-3β-ol were identified. In contrast, in diatoms cholesterol, (22*E*)-ergosta-5,22-dien-3β-ol, and campest-5-en3β-ol were found.

Mouritsen et al. (2017) stated that microalgae could produce only fucosterol and desmosterol; however, this cannot be confirmed, because there is a complexity of sterols that could be produced.

In other research, the sterols ergosterol, fucosterol, cholesterol, campesterol, stigmasterol, and β-sitosterol were analyzed in 21 algae from Chlorophyta, Rhodophyta, Phaeophyta, and Spermatophyte phyla (Mikami et al., 2018). In this study, the variations were huge, with a major concentration 4135.7 $\mu g\,g^{-1}$ dry biomass of cholesterol in *Gracilaria vermiculophylla* (Rhodophyta), followed by 731.1 $\mu g\,g^{-1}$ dry biomass of fucosterol, and 661.0 $\mu g\,g^{-1}$ dry biomass of β-sitosterol from *Sargassum fusiforme* (Phaeophyta). For ergosterol, 93.4 $\mu g\,g^{-1}$ of dry biomass was found in *Grateloupia asiatica* (Rhodophyta), and 86.9 $\mu g\,g^{-1}$ dry biomass in *G. vermiculophylla* (Rhodophyta) and *Undaria pinnatifida* (Phaeophyta). On the other hand, campesterol was observed in only one algae, *Zostera marina* L. (Spermatophyta), with a concentration of 125.9 $\mu g\,g^{-1}$ dry biomass; the same stigmasterol presented 60.7 $\mu g\,g^{-1}$ dry tissue in *G. asiatica* (Rhodophyta). The brown algae *Ectocarpus siliculosus* presented concentrations of fucosterol, cholesterol, and ergosterol, which ranged in their concentration according to the specimens (Mikami et al., 2018).

21.5 Sterols in cyanobacteria

In the literature, many studies have described how cyanobacteria cannot produce sterols, providing a huge discussion among researchers. According to Levin and Bloch (1964), the class of microalgae that is well-known as blue-green algae could not produce these biomolecules, but this knowledge is considered out of date. In 1968, only some years after the publication of an article describing the absence of sterol in cyanobacteria, the authors De Souza and Nes (De Souza and Nes, 1968) published a study reporting seven unsaturated sterols produced by cyanobacteria *Phormidium luridurn*. Reitz and Hamilton (1976) described the profile from two strains: *Anacystis nidulans* and *Fremyella diplosiphon*.

In addition, some authors define the strain *Synechocystis* sp. (Kaneko and Tabata, 1997) as a protein gene producer responsible for sterol synthesis. Forin et al. (1972) showed the principal class of sterols as 24-ethyl.

The cyanobacteria *Spirulina platensis* Geitler also presented similar profiles, producing several sterols, such as cycloartenol, 24-methylene-cycloartanol, cycloeucalenol, obtusifoliol, 4α-methyl-Δ8-ergostenol, Δ7-cholestenol, cholesterol, Δ7-ergostenol, Δ5-ergostenol, isofucosterol, Δ7-chondrillastenol, clionasterol, and poriferasterol. Therefore, after these findings, Loeschcke et al. (2017) proved the presence of the enzyme 2,3-oxidosqualene; this enzyme signifies a principal step in the transformation of squalene to sterols. The expression of this enzyme was also observed in the cyanobacteria *Synechocystis* sp. Despite all

this evolution, many cyanobacteria need to be elucidated concerning this enzyme expression (Volkman, 2003).

21.6 Environmental influence on microalgae sterols production

According to Volkman (2016), environmental variations influence sterol production by microalgae significantly, such as temperature and nutrients. Concerning sterol metabolic pathways, the first important metabolite acquired is squalene; as described earlier, this compound presents many bioactive proprieties, so many studies have provided information about the accumulation of this compound. Nevertheless, when squalene accumulation occurs in the cell sterols are not produced.

The temperature affects the plant sterols' metabolism, because the ethyl groups branched at C24 are produced in order to protect the cell from thermal shocks (Beck et al., 2007). With this in mind, squalene could also be produced by varying the temperature in *Thraustochytrid aurantiochytrium*, to acquire it by biotechnology production. Nakazawa et al. (2012) studied the temperatures 10°C, 15°C, 20°C, 25°C, 30°C, and 35°C in the culture system, and the best response acquired from this study was 25°C. Additionally, they evaluated the diverse glucose concentrations, in the range of 2%–6%, and the addition of seawater was investigated, varying from 25% to 50%. A squalene concentration of 171 $mg\,g^{-1}$ dry biomass was obtained using 2% glucose and 50% seawater concentrations in the medium at 25°C.

Zhang et al. (2017), studying the strain *Schizochytrium limacinum*, evaluated the influence of butanol addition in the cultivation system. The use of this molecule was capable of increasing the squalene content from 0.65 to 20.09 $mg\,g^{-1}$. In the same line, Naziri et al. (2011) performed an optimization by using the strain *Aurantiochytrium mangrovei* cultured with distinct concentrations of terbinafine and methyl jasmonate, and the authors reported that the major concentration obtained was 10.02 $mg\,g^{-1}$ of dried biomass. In addition, in other studies using distinct terbinafine concentrations in the same strain, the authors observed an increase of 60% in squalene content (Fan et al., 2016).

In terms of sterol production, few studies describe the influence of external nutrients on the microalgae profile. Fagundes et al. (2019a) showed the differences between exogenous carbon sources in a heterotrophic cultivation system and their influence on sterol profile. In this research, exogenous carbon source glucose, sucrose, and wastewater were explored. Glucose presented in squalene in a concentration of 1440.4 $\mu g\,g^{-1}$ of dry biomass, and ergosterol 1033.3 $\mu g\,g^{-1}$ of dry biomass; for sucrose a concentration of 225.4 $\mu g\,g^{-1}$ of dry biomass of squalene was found, and by using wastewater it was capable of producing a diversity of sterols, being 425.6 $\mu g\,g^{-1}$ of dry biomass of squalene, 820.6 $\mu g\,g^{-1}$ of dry biomass of cholesterol, and 455.3 $\mu g\,g^{-1}$ of stigmasterol, and β-sitosterol in a concentration of 279.0 $\mu g\,g^{-1}$ of dry biomass. In the same study, it was also possible to characterize many intermediary pathway sterols.

In the same line, considering the increase in sterol production, Ahmed and Schenk (2017) reported the increase in sterol content by using UV-C radiation on the strain *Pavlova lutheri*. In this study, higher total sterol contents ($20.3\,mg\,g^{-1}$) were found in an irradiation of $100\,mJ\,cm^{-2}$. In addition, the authors evaluated a treatment by using H_2O_2; the concentrations explored were: 1, 5, 10, 50, 100, and $500\,\mu mol\,L^{-1}$, and the major concentration was obtained by using $100\,\mu L\,L^{-1}$, being the total sterol $400\,\mu g\,g^{-1}$ of dry biomass.

21.7 Sterols analysis

In complex matrices such as microalgae, analyses of sterols and their precursor squalene may involve several steps, generally extraction, saponification and identification, and quantification by chromatographic techniques (Lv et al., 2015). The extraction of free and esterified sterols is usually carried out as lipid extraction due to the polarity of the compounds, but it is worth noting that some sterols such as glycosylated sterols (steryl glycosides and acylated steryl glycosides) may be neglected because they have superior polarity (Feng et al., 2015).

The extraction with reflux of organic solvents, known as the Soxhlet method, is a reference in lipid extractions and other nonpolar substances such as squalene, free sterols, and esterified sterols, and uses solvents such as petroleum ether (Abdallah et al., 2015), methanol-chloroform mixture (Hidalgo et al., 2015), hexane (Kozłowska et al., 2016), and dichloromethane (Martins et al., 2016). Although it is simple and efficient, the Soxhlet method presents long periods of extraction, consumes high volumes of toxic and flammable solvents, and involves high-energy usage for distillation and recovery of organic solvents (Mubarak et al., 2015).

The partitioning by the solvents methanol, chloroform, and water used in the method of Folch (1957) and Bligh and Dyer (1959) has shown great relevance in extracting the nonpolar fraction of microorganisms to biological tissues (Breil et al., 2017). Optimal extraction occurs by homogenizing the sample with the mentioned solvents to form a single-phase solution. Afterwards, a bi-phasic system is intentionally induced, and the nonpolar substances are solubilized in the chloroform phase and the polar compounds in the methanol-water phase (Odeleye et al., 2019). Although is not used higher temperatures in the conventional methods, as in Soxhlet method, Folch and Bligh and Dyer they are responsible to expend large volumes of organic solvents, with this in mind, current works bring as an alternative the miniaturization of these methods (Vendruscolo et al., 2018).

Supercritical fluid mainly using carbon dioxide (CO_2) has been used for extraction of lipids and sterols (Attard et al., 2018; Li et al., 2016); its advantages include efficiency, environmental friendliness by being free of organic solvents, and maintaining thermolabile compounds. Studies evaluated the squalene and β-sitosterol extraction using supercritical fluids of CO_2 and Liquefied Petroleum Gas (LPG); although the LPG has extractive capacity of nonpolar compounds, the highest concentrations of the sterol and its precursor were obtained with the use

of CO_2 (Scapin et al., 2017a,b). Despite its advantages, when compared with extraction with organic solvents, supercritical fluid extraction is a process of high cost and operational complexity (Mubarak et al., 2015).

In microalgae, free and bound sterols are found. However, normally is used a saponification step after the lipid extraction to obtain free sterols from esterified sterols is recommended, as well as to eliminate interferers saponifiable such as triacylglycerols (Vendruscolo et al., 2019). Potassium hydroxide in methanol or ethanol is the most common saponification agent, with concentrations ranging from 0.35 to 2.7 M (Albuquerque et al., 2016). The saponification reaction with alkaline reagent can be carried out at room temperature or under heating at temperatures that can reach close to 100°C; usually higher temperatures require shorter reaction times (Uddin et al., 2018; Menéndez-Carreño et al., 2016). Some studies, including those on microalgae, have performed saponification directly on the sample, without the need for extraction first as mentioned previously, reducing steps and analysis time (Fagundes et al., 2019a; Pereira et al., 2016).

After the saponification, the free forms of the esterified sterols are obtained; however, the acetal bond between the sterol hydroxyl group and the sugar cannot be hydrolyzed in alkaline conditions as in the case of glycosylated sterols (Yu et al., 2018). In addition, in lipid extractions prior to saponification, due to superior polarity the glycosylated sterols (steryl glycosides and acylated steryl glycosides) are not extracted (Feng et al., 2015). Thus, for determination of the glycosylated sterols, acid or enzymatic hydrolysis is recommended to cleave the glycosidic bond (Munger et al., 2015). Acid hydrolysis can degrade compounds and isomerize some sterols; enzymatic hydrolysis is recommended to obtain true concentrations of glycosylated sterols (Moreau et al., 2018). Munger et al. (2015) performed acidic hydrolysis with 6 M HCl at 85°C for 30 min. and enzymatic hydrolysis with commercial mixture of exo- and endoinulinases at 40°C for 18 h in different samples; the total glycosylated sterol values were at least 65% higher in the enzymatic hydrolysis.

Gas chromatography (GC) coupled with mass spectrometry is the most common technique for analysis of sterols and squalene (Yuan et al., 2017). Before GC analysis, derivatization reactions can be performed to obtain products with better chromatographic conditions—for example, improving volatility, sensitivity, selectivity, and thermal stability (Poojary and Passamonti, 2016). Acylating agents and silylants are used to derivatize sterols (Gachumi and El-Aneed, 2017). The acylation reaction consists of introducing an acyl group into an organic molecule with active hydrogen using reagents such as acyl halides, acid anhydrides, or reactive acyl derivatives such as acylated imidazoles (Segura et al., 1998). The acyl halides are highly reactive and during the reaction hydrogen acid is formed; a basic compound is then required for neutralization. Therefore, it is recommended to eliminate excess reagent in order not to damage the chromatographic system (Gachumi and El-Aneed, 2017).

The silylation reaction is the most prevalent among derivatization reactions. A second-order nucleophilic attack occurs (SN2), where active hydrogen of an acid, alcohol, thiol, amine,

amide, aldehyde, and enolizable ketone is replaced with a trimethylsilyl group (Miyagawa and Bamba, 2019). Several reagents are used as silylating agents, including trimethylchlorosilane (TMCS), trimethylsilylimidazole (TMSI), *N*-methyl-trimethylsilyltrifluoroacetamide (MSTFA), *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), and *N*-(*t*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA). The obtained products can be injected directly into the chromatographic system; no purification step is required as in the acylation (Schummer et al., 2009).

Gas chromatography with a flame ionization detector (GC-FID) is also widely used in the analysis of sterols, and is described in several protocols of the American Oil Chemists' Society (AOCS) (Alberici et al., 2016). As the FID is nondiscriminatory and the identification is performed by a reference retention time, misunderstandings and co-elution of compounds with similar affinity to the chromatographic column may occur. In these cases, it is possible to use columns with different phases or to confirm the identity by GC/MS through spectrum comparison of the substances, although this detector is less indicated for quantifications than the FID (Duong et al., 2016).

GC/MS may also not be robust enough in cases of complex samples with multiple sterols because these compounds and interfering may have the same ionic fragments. Thus, higher sensitivity and resolution can be obtained with comprehensive two-dimensional gas chromatography (GC × GC) (Xu et al., 2015). The GC × GC has the objective to submit chromatographic bands where there is co-elution of compounds for further separation, in a continuous and sequential mode (Tranchida et al., 2015). Two chromatographic columns with stationary phases of different polarities are used; they are separated by an interface called a modulator, so the column effluent of the first dimension enters the column of the second dimension (Muscalu and Górecki, 2018). As in GC, GC × GC can be used in different detectors, usually FID and MS for sterol analysis, and also flight-of-time mass spectrometry (ToF/MS) (Xu et al., 2014, 2015, 2018). The ToF/MS presents greater mass accuracy and higher acquisition rates compared to single quadrupole MS, so it is preferable in GC × GC, but it is expensive, which limits accessibility (Keppler et al., 2018). Although GC with FID and MS was the most used separation technique for quantification and identification of sterols and squalene, it has the following disadvantages: complex sample preparation, requirement of high temperatures, and destructive detection technique (Feng et al., 2015). Thus, some methods have been developed using high performance liquid chromatography (HPLC), which has the main advantage of analyzing these compounds in both their free and bound forms without the steps of hydrolysis and derivatization used in GC; only the steps of extraction, saponification, or direct saponification, as already discussed, are necessary. In general, the methods utilize normal-phase HPLC for determination of sterol classes, and reverse-phase HPLC for separation of free sterols and conjugated sterols analysis (Moreau et al., 2018).

Different detectors can be used for determination of squalene and sterols by HPLC. As these compounds exhibit absorption in the ultraviolet (UV) region, some works were carried out

using a UV detector or diode array detector (Feng et al., 2015; Yuan et al., 2017; Delgado-Zamarreno et al., 2016; Novak et al., 2018). Identifications using a UV and DAD detector are based on reference retention times obtained from commercial standards or by mass spectra using the HPLC-MS (Villegas et al., 2018). In HPLC with UV or DAD, there are limitations of sensitivity and also selectivity—for example, squalene and sterols absorb UV light in the 190–210 nm range, which allows absorption by other substances (Moreau et al., 2018). Different detectors based on mass spectrometry were used to detect and identify squalene and sterols in order to solve problems of sensitivity, selectivity, and particularly cases of co-elutions: tandem mass spectrometry (MS/MS) (Flakelar et al., 2017; Jauković et al., 2017; Pereira et al., 2016) and quadrupole time-of-flight mass spectrometry (QToF) (Millan et al., 2016).

Methods used more recently in sample preparation for analysis of squalene and sterols in microalgae were presented and discussed, as well as the analytical techniques used for their detection, separation, and identification. Table 21.1 lists the studies that carried out these analyses on microalgae samples.

21.8 Bioactivity of sterols

Bioactivity is defined by obtaining specific effects after exposure to a particular substance; these effects may include tissue uptake, metabolism, or physiological response (Karaś et al., 2017). The bioactivity can be assessed from methodologies in vivo (animal or human studies), ex vivo (gastrointestinal organs in laboratory conditions), and in vitro (simulated gastrointestinal digestion, artificial membranes, cell cultures, isolated and reconstituted cell membranes, using chambers). However, only in vivo assays can provide accurate bioactivity responses of a specific compound (Carbonell-Capella et al., 2014).

Just as sterols do, the precursor squalene has some human health benefits. Reduced cholesterol levels, protection against coronary heart disease, antioxidant properties, potential anticarcinogenic activity, and tumor reduction were effects that were observed in different sources of this compound (Chhikara et al., 2018). However, the literature has reported the great potential for sustainable production of squalene from microalgae, but the bioactivity of this compound obtained from the microalga *P. autumnale* was not evaluated (Fagundes et al., 2019a,b). Fernando et al. (2018) evaluated the bioactivity of squalene isolated from the alga *Caulerpa racemosa*, where it was possible to observe potent antioxidant activity and a wide range of antiinflammatory function in a cell assay.

Sterols are indicated as compounds with high bioactivity, and have already presented several effects in humans including antiinflammatory, antioxidant, anticancer, acting in immunomodulation to reduce the effects of neurological diseases like Parkinson's and Alzheimer's, antihypercholesterolemic, and antidiabetic (Luo et al., 2015; Khan et al., 2018;

Table 21.1 Recent studies analyzing squalene and/or sterols in microalgal biomass.

Strain	Compounds	Objective	Sample preparation	Tool	Ref
Nannochloropsis oceanica	SQU/STE	Characterization/ biosynthetic pathway determination	SE/SAP	GC/MS	Lu et al. (2014)
4 Microalgae strains	STE	Lipid extraction method/ characterization	SAP/DER	GC/FID-GC/MS	Ryckebosch et al. (2014a)
8 Microalgae strains	STE	Nutritional value	SE/SAP/DER	GC/FID-GC/MS	Ryckebosch et al. (2014b)
Schizochytrium sp.	SQU/STE	Characterization/ effect on cholesterol reduction	SE/SAP/DER	GC/MS	Chen et al. (2014)
5 Microalgae strains	SQU/STE	Characterization	SE/SAP	GC/FID-GC/MS	Yao et al. (2015)
Schizochytrium aggregatum	STE	Oxidative stability of microalgae oil/ bioaccessibility/ antioxidant ability of digested	SE/SAP	GC/MS	Lv et al. (2015)
17 Microalgae strains	STE	Characterization	SE/SAP/DER	GC/FID-GC/MS	Martin-Creuzburg and Merkel (2016)
Pavlova lutheri	STE	Effects of UVeC radiation and hydrogen peroxide	DSAP/DER	GC/MS	Ahmed and Schenk (2017)
Phormidium autumnale	SQU	Production	SE/DER	GC/FID-GC/MS	Fagundes et al. (2019b)
Phormidium autumnale	SQU/STE	Distinct sources of carbon	DSAP	GC/FID-GC/MS	Fagundes et al. (2019a)

SQA, squalene; *STE*, sterols; *SE*, solvent extraction; *SAP*, saponification; *DER*, derivatization; *DSAP*, direct saponification; *GC/MS*, gas chromatography mass spectrometry; *GC-FID*, gas chromatography with flame ionization detector; *Ref*, reference.

Moreau et al., 2018). Some studies have verified the activities of sterols extracted from microalgae; Chen et al. (2014) compared the effect of β-sitosterol only with a *Schizochytrium* sp. sterol extract (lathosterol, ergosterol, stigmasterol, 24-ethylcholesta-5,7,22-trienol, stigmasta-7,24(24^1)-dien-3β-ol, and cholesterol) and squalene, in the reduction of total cholesterol in hamster plasma. Although less effective than β-sitosterol alone, sterol extract and squalene at the 0.06 and 0.30 g kg^{-1} diet doses reduced the total plasma cholesterol by 19.5% and 34%, respectively, when compared to the control.

The lipid fraction of the microalgae *Schizochytrium aggregatum* was analyzed and presented in its composition 42% of polyunsaturated fatty acids (PUFA) and the sterols: cholesterol, campesterol, 24-methylene cholesterol, 24-methyl-cholest-7-en-3b-ol, ergosterol, stigmasterol, and Δ7,24-stigmastadienol. After characterization, the bioactivity property of this microalga was evaluated by in vitro gastrointestinal simulation, and the results showed good bioaccessibility and moderate antioxidant activity (Lv et al., 2015). The bioactivity of a sterol-rich fraction extracted from the microalga *Nannochloropsis oculata* was also evaluated in macrophages and cancer cells. This fraction, containing about 64% of total sterols, was shown to be viable in the development of drugs for the treatment of diseases associated with inflammatory processes and also cancer of promyelocytic leukemia (Sanjeewa et al., 2016).

21.9 Industrial sterol production

In terms of the first bioactive compound obtained, squalene is known for being traditionally extracted from the liver of deep-sea sharks. This compound can represent up to 80% of the oil extracted from that organ. However, the growth and slow reproductive cycle of these animals, in addition overfishing for different purposes allied to the growing environmental concern and restrictive laws, have reduced this practice to obtain squalene (Zhuang and Chappell, 2015). Vegetable sources may be alternatives, and amaranth oil and olive oil present about 7% and 1%, respectively, of squalene in its composition; however, the slow development of these plants, seasonality, and edaphoclimatic dependence are restrictive factors. Thus, biotechnology routes attract attention; different strains of the *Aurantiochytrium* microalga present 3.3%–31.8% of squalene in their oil and strains of *Schizochytrium* 1.1%–8.4%. These values are associated with a high productivity of biomass and lipids (minimum of 30% in dry mass) (Aasen et al., 2016).

The main sources of total sterols (TE) for human consumption are plant species, as shown in Fig. 21.4, such as maize oil (0.81%–1.56%), germ oil (1.97%), and rice bran oil (3.22%) (Khan et al., 2018). However, as already mentioned, microalgae biotechnology presents advantages in the potential production of these compounds. Thus, the oil of *Isochrysis galbana* presented 0.37%, *S. aggregatum* 0.52%, *Schizochytrium* sp. 0.66%, *Phaeodactylum tricornutum* 1.13%, *Nannochloropsis* sp. 2.47%, and *Nannochloropis gaditana* 2.52% (Ryckebosch et al., 2014a, 2014b; Chen et al., 2014; Lv et al., 2015). It is estimated that the annual production of

microalgal oil can reach between 7000 and 23,000 L/ha, representing 60–200 times more production than high-performance plant species (Luo et al., 2015).

At the end of microalgae cultivations, harvesting techniques are based on solid-liquid separations, the most common being filtration, flotation, centrifugation, and, although slower, gravity sedimentation. After obtaining the biomass, processes of thermal drying or lyophilization are required (Grima et al., 2003). Thermal drying can be artificial (spray-drying, drum drying), where the drying temperature must be controlled so that the compounds of interest are not degraded, or natural, by using solar energy, but this process depends on the weather conditions and can be extended for days (Khanra et al., 2018).

The extraction of squalene and sterols can be facilitated with a prior cell disruption. Some procedures are performed for this purpose, such as freezing and thawing cycles, chemical disruption (acid hydrolysis), enzymatic treatment, high-pressure homogenization, and bead-milling. Extraction with organic solvents is widely used due to its high recovery efficiency of intracellular metabolites; chloroform, hexane, and petroleum ether may be cited. However, with the current tendency of not using these organic solvents due to their toxicity, it is worth highlighting the supercritical fluid, mainly with CO_2 supercritical (Grima et al., 2003). For obtaining these compounds, saponification processes are required; as already mentioned in the sterol analysis section, the process is performed with an alkaline alcohol solution (Albuquerque et al., 2016). If the goal is to obtain fine chemicals, in the case of purified sterols, subsequent chromatography steps are required (Khanra et al., 2018).

The squalene and sterols can be consumed from the ingestion of dried biomass. In this sense, the process of separation of the culture medium and drying are performed to obtain this product, and these steps represent the major cost of production. In the case of the strain *Schizochytrium*, these costs represent about 38% of the market value, for example. However, the cost of production can be higher if the target products are microalgae oil or even squalene and sterols isolated. In these cases, depending on the purity degree, the cost can reach 90% of the sale value (Jacob-Lopes et al., 2019). Despite the great productive potential, the cost of obtaining fine chemicals from microalgal cultivation is still considered high, but this can be overcome by increasing the production scale (Caporgno and Mathys, 2018).

21.10 Conclusion

In this chapter we discussed aspects related to sterol structure, and production by diverse microalgae (eukaryotic and prokaryotic), plus reports about the occurrence in diverse phyla and the strategies for their production. This chapter presented new attempts for microalgae exploring sterols and their derivate squalene as a potential high-value chemical to be employed in diverse industry areas.

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5 CAPÍTULO 3

5.1 CAPÍTULO DE LIVRO 2

Chapter- Sterols Biosynthesis in Algae

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Chapter

Sterols Biosynthesis in Algae

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Abstract

Sterols are secondary metabolites, they are considered bioactive, due to their recognized activity as antioxidants, anticarcinogenic, cardiovascular protectors, and antiviral capacity. These triterpenoids can be found in a wide range of concentrations in different algae strains, being the variations related to external factors. In the world, there are millions of algae, some strains have the ability to produce high-value phytosterols, like stigmasterol, and sitosterol, however, others could lead to cholesterol production. For this reason, understand the principal factors involved in sterols biosynthesis, allows us to appoint the algae strain for industrial application and escalating these specific compounds production. Some algae are capable to produce sterols from mevalonic acid pathway, other strains present the methylerythritol 4-phosphate (MEP), or 1-deoxy-D-xylulose-5-phosphate (DOXP) as the main pathway, each one is responsible for the production of plans of intermediary compounds. In this sense, this chapter summarizes current knowledge of the biosynthetic pathways responsible for different sterols formation, as well as, describe main sterols that could be isolated from algae metabolism.

Keywords: macroalgae, microalgae, cyanobacteria, phytosterols

1. Introduction

Marine biota has a diversified metabolism, possessing worldwide most complex and unexplored organisms, and maybe the richest source of important compounds, bioactive molecules, that could lead in benefits for distinct areas in human life [1]. In this way, exploring these microorganisms in the context of their biochemistry is an important step, not only for drug discovery, or nutraceuticals, but also to understand their evolution. This affirmative comes from a question never totally elucidated about the molecular origin, and its association with algae sterol metabolism, named as "sterolomic". This approach could present important information's about the cell membrane, without them does not exist cellular protection and organization [2].

Cellular membrane composition is major composed by phospholipids, and between sterols cholesterol, in terms of animal cell organization, however, plants possess phytosterols replacing cholesterol, and the most interesting information in the microalgae metabolic system is associated with the capability of some strains producing both classes of sterols. In this chapter, we are going to synthesize aspects about algae principal sterols metabolic pathways, and the ways that they can be manipulated to produce specific compounds.

2. Algae metabolism: sterols discovery

The literature brings information's about diverse algae sterolomic profile, so in this chapter let us begin with the most curious and strong algae, considered the earliest life forms in the world, the prokaryotes microalgae (cyanobacteria). These strains are also known as blue-green algae, they are widely distributed in the world, due to their robustness. Cyanobacteria are considered by biologists a variation from bacteria and eucaryotic strains, which could lead in a production of sterols related with vegetal, and also animal kingdom [3].

Cyanobacteria for this reason, can occur in marine environments with a huge salt variation, in cold waters as Antarctic system, and hot waters, could also proliferate in desert sand and rocks, providing a major response from their metabolic systems modifications according to the natural evolution. These cyanobacteria can produce different metabolites according to the habitat that they are living, for this reason, merging the information's we can understand that they can present many metabolic pathways leading to different end-sterols products. Their resistance comes from their plasmatic membrane associated mostly with structures named hopanoids, that are very similar to sterols, and are responsible for the flexibility of cyanobacteria cellular membrane [4].

The major discussion on the literature is the unknown ability of these organisms producing sterols. Many years ago, some researches described the possibility to exist only hopanoids in their structure, in fact, with the advance in tandem mass spectrometry, nuclear magnetic resonance analysis associated with new extraction techniques it was discovered the presence of sterols in their membrane. Thus, metabolism involved in sterols biosynthesis by cyanobacteria are not totally elucidated.

In the history context, the first works showing sterols production in cyanobacteria were in a filamentous cyanobacteria named as *Phormidium luridum* in 1968 [5], in this study it was isolated unsaturated sterols, like as 24 ethyl sterols, following this research's other studies investigated a way to produce this metabolite in large scale, considering the fact that this cyanobacterium has resistance in front of other microorganisms, inferring a remarkable capability for industrial application.

In the ninety's the researchers Sallal, Nimer, and Radwan [6] studied other cyanobacteria strains, and verified that after dark incubation, sterols concentration increased. In in agreement to this study, Fagundes et al. [7] showed higher concentrations of sterols (β-sitosterol, stigmasterol, and cholesterol), for *Phormidium autumnale* cultured in heterotrophic system, being the inoculum without the presence of these compounds. In general, cyanobacteria are manly photosynthetic, but some strains can growth in heterotrophic conditions, in this context, it can be concluded that more studies on this particular area are necessary for further acquire more comprehension for biotechnological application.

Eukaryotic microalgae are reported in the literature as the most prominent strains for sterols production, and they are important to make feasible membrane cell permeability, and maintain structural protection [8, 9]. In this sense, the study of sterols biosynthesis started in eukaryotic cells, standing out in numerous hypothesis, and one of them is related to life adaptation on earth, showing that these molecules were produced in this cell as a protective response to reactive species of oxygen [10]. The first study in eukaryotic microalgae was in 1960 with *Scenedesmus*, showing as the major compound chondrillasterol [11], years later the same researchers Iwata and Sakurai [12] reported ergosterol as the most abundant sterol for *Chlorella.* In terms of macroalgae, the (brown) species *Ulva lactuca*, and *Cytoceira adriatica* from Adriatic Sea, were analyzed by the authors Kapetanovic et al. [13], showing that these species were the main sterols cholesterol and fucosterol for both algae.

In summary algae strain choice directly reflects in their potential for commercial application, for this reason, the knowledge of algal productivity, and the biotechnological treatment applied for each alga is important. So, understand the metabolic pathways for the full comprehension of sterols, and their intermediary metabolites formed provides important information for future culture modifications enhancing specific compounds [14]. For this, depending on the triterpenoid produced they can be applied for medical proposes, which is a great alternative since in the last decade we have the challenge for the isolation of new compounds, in front of many problems associated nowadays with diseases' outbreaks. Algae possess a diverse metabolic system; their sterol composition is interesting due to the fact that they show in their composition unconventional structural variations [15]. The main structure consist of a tetracyclic, with a fused-ring skeleton, with the presence of a hydroxyl group at the carbon 3 (head group- 3β), and biochemical modifications at the carbon C24 (in sterol side chain), besides modifications found in the tetracyclic nuclei, and also their side chain with different alkylation's patterns [15].

Nowadays, there are studies focusing on unconventional sterols bioactivity like the sterols isolated from *Isochrysis galbana*, being cholest-5-24-1,3-(acetyloxy)-3β-ol, ergost-5-en-3-β-ol, and 24-oxocholesterol acetate. Other study identified unconventional sterols in *Sargassum fusiforme:* saringosterol, 24-hydroperoxy-24-vinyl-cholesterol, 29-hydroperoxy-stigmasta-5,24 (28)-dien-3β-ol, 24-methylene-cholesterol, 24-keto-cholesterol, and 5α, 8α-epidioxyergosta-6,22-dien-3β-ol all associated with anti-atherosclerotic function [16].

Industrial initiative for algae biomass application started in 20 centuries with the investment in many programs for algae research. The principal countries producing algae biomass and their products are shown in the **Figure 1**. Their major focus are on biofuels, or commercializing the biomass powder, and in terms of fine-chemicals the market is based on pigments, being only two sterols commercially produced from algae, fucosterol and desmosterol [17]. With this in mind, is important highlight that sterols are important bioactive metabolites that are normally isolated from non-renewable source, comprehend the metabolic sterols pathways and the ways to modify their production, presenting algae as a new source of sterols to the world, could lead to a sustainable sterols production.

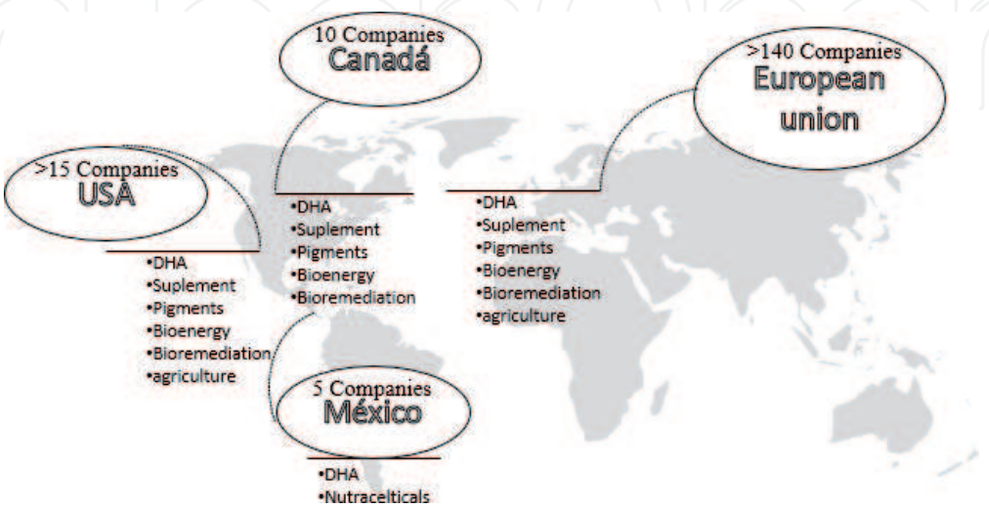

Figure 1.
Principal countries with important algae biotechnology companies' and their products. DHA - docosahexaenoic acid.

3. Algae sterols metabolic pathways

Sterols biosynthesis started by two main pathways the mevalonic acid (MVA), and by the 1-deoxy-D-xylulose-5-phosphate/2-C-methyl-D-erythritol-4-phosphate (MEP), recently discovered [18], also known as non-mevalonate pathway. The objective of these two pathways is produce an isoprenoid structure, a molecule of 5 carbons isopentenyl diphosphate (IPP), and dimethylallyl pyrophosphate (DMAPP), that are considered the sterol building block. MVA pathway occurs in cytosol, when MEP in the plastids, however the pathways activation are different according to the algae classification, being that some algae with the presence of both pathways' biochemical machinery MEP and MVA and others with only one of them active [19].

Understand the pathways involved for sterols production in algae is difficult, due to a huge phylogenetic heterogeneity found in strains. Since today still have research's showing for the first time the active pathway in some algae, like the observed by Scodelaro Bilbao et al. [20] studying *Haematococcus pluvialis*. A deeper discussion about numerous algae and the two possible active pathways can be found at the review from the authors Lohr et al. [21].

The prokaryotic cell, are known for possess MEP as the active isoprenoid producer, and for the ancestor reason, probably they were responsible for introducing this metabolism in eukaryotic strains. The MEP pathway is described as the major used for sterols production in algae, being green algae (*Chlorophyta*), with only MEP active for sterol production due to the loss of MVA pathway in the algae cellular evolution [21], as in many algae system both pathways occur, for this reason the pathways are depicted in the **Figure 2**.

The pathways are divided in two segments, the first one can be observed at the **Figure 2A**, which represents the transformation of DMAPP and IPP to squalene, this step consists in the MVA, and MEP. MVA pathway occurs in the cell cytosol until a condensation of two molecules of acetyl-CoA with the catalysis of acetoacetyl-CoA thiolase, after occurs other condensation forming 3-(*s*)-hydroxy-3-methylglutaril-CoA (HMG-CoA) by the action of 3-(s)-hydroxy-3-methylglutaril coenzyme A synthase. After that, the conversion to 3-(*R*)-mevalonate trough a reduction occurred by a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reductive diacylation by the enzyme HMG-CoA reductase [22]. The following steps consist in the conversion of MVA to mevalonate-5-diphosphate, catalyzed by mevalonate kinase (MK), and mevalonate-5-diphosphate kinase (MVADP), with the insertion of two ATP molecules, being the last step the conversion by isopentenyl diphosphate isomerase to the formation of DMAPP.

In terms of MEP pathway, the first step is a thiamin diphosphate-dependent condensation between D-glyceraldehyde 3-phosphate and pyruvate forming 1-Deoxy-D-xylulose-5-phosphate by the enzyme 1-deoxy-d-xylulose-5-phosphate synthase (DXS), following an isomerization to 2-*C*-methyl-o-erythritol-4-phosphate (MEP) by the enzyme 1-Deoxy-D-xylulose-5-phosphate (DXR) reducto-isomerase [18]. After, MEP and cytidine 5′-triphosphate are coupled, being catalyzed by 4-diphosphocytidyl-2-C-methylerythritol (MCT) synthetase, forming methylerythritol cytidyl diphosphate. The other enzymes involved in MEP pathway are in the sequence: 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase (CMK) for 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol 2-phosphate formation. After, 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate synthase (MDS) which forms the 2-*C*-methyl-D-erythritol-2,4-cyclic diphosphate, 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HMBPP- synthase), leading to (2E)-4-hydroxy-3-methylbut-2-enyl diphosphate, and 4-hydroxy-3-methylbut-2-enyl diphosphate

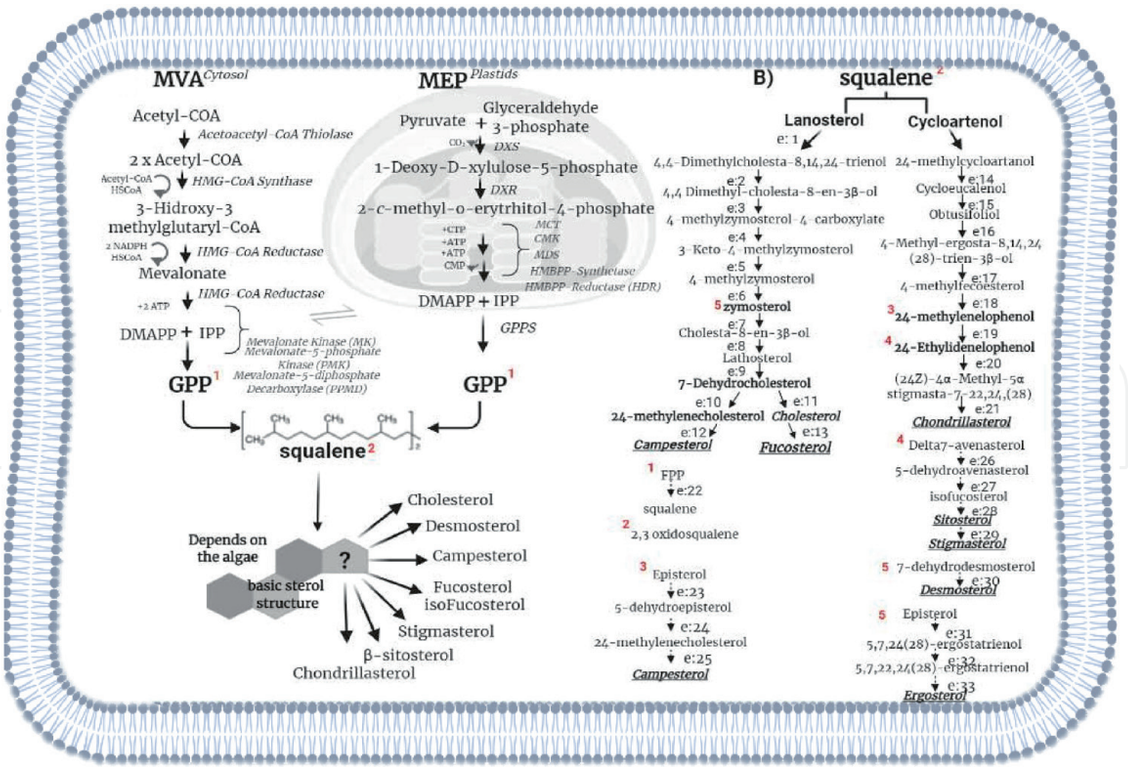

Figure 2.
Algae sterols different pathways, a: Mevalonic acid pathway, and B: Non-mevalonic acid pathway (methylerythritol 4-phosphate): HMG-CoA: Beta-Hydroxy-beta-methylglutaryl-coenzyme a, ATP: Adenosine triphosphate, GPP: Geranyl pyrophosphate, FPP: Farnesyl pyrophosphate, e:1: Lanosterol synthase, e:2: Sterol 14alpha-demethylase, e:3: Methylsterol monooxygenase, e:4: Sterol-4alpha-carboxylate 3-dehydrogenase, e:5: 3-keto steroid reductase, e:6: Methylsterol monooxygenase, e:7: Cholestenol Delta-isomerase, e:8: Cholestenol delta-isomerase, e:9: Delta7-sterol 5-desaturase, e:10: Delta7-sterol C5 desaturase, e:11: 7-dehydrocholesterol reductase, e:12: Delta-24-sterol reductase, e:13: 24-sterol reductase, e:14: 3-beta-hydroxysteroid 3-dehydrogenase, e:15: Cycloeucalenol cycloisomerase, e:16: Sterol 14alpha-demethylase, e:17: Delta14-sterol reductase, e:18: Cholestenol Delta-isomerase, e:19: 24-methylenesterol C-methyltransferase, e:20: 4-alpha-monomethylsterol monooxygenase, e:21: 7-dehydrocholesterol reductase, e:22: Farnesyl-diphosphate farnesyltransferase, e:23: Delta24(24(1))-sterol reductase, e:24: 7-dehydrocholesterol reductase, e:25: Delta24-sterol reductase, e:26: 3-beta-hydroxysteroid 3-dehydrogenase, e:27: 7-dehydrocholesterol reductase, e:28: Delta24-sterol reductase, e:29: Sterol 22-desaturase, e:30: 7-dehydrocholesterol reductase,e:31: Delta7-sterol 5-desaturase, e:32: Sterol 22-desaturase, e:33: Delta-24(24(1))-sterol reductase.

reductase (HMBPP-Reductase) being formed (2E)-4-hydroxy-3-methylbut-2-enyl diphosphate. The last step consists in the building blocks IPP and DMAPP and their coupling through isopentenyl-diphosphate isomerase [18, 23].

In the literature, there are numerous data, in which sometimes contrast about the biosynthesis of the isoprene units. MEP pathway was detected for the first time in bacteria, however further evidence has shown that in eukaryotes which performs photosynthesis found compounds from this metabolic pathway [24]. Normally a cyanobacteria which possess a metabolic system similar to bacteria produce phytosterols by MEP pathway, and also other authors describe that photosynthetic eukaryotic strain produce phytosterols only from MEP pathway [25]. On the other hand, MVA pathway normally is used for the production of cholesterol in animals, and also the green macroalgae sterols, in last case it occurs due to their metabolic similarity with higher plants, differently occurred with green microalgae from Chlorophyceae as described by Volkman [8, 9].

Geranyl pyrophosphate (GPP) is formed by the isoprenoids DMAPP and IPP, and through the diverse condensations leading to a presqualene compound, followed by the formation of squalene trough farnesyl-diphosphate farnesyltransferase, and trough squalene monooxygenase, or an alternative squalene epoxidase newly discovered [26]. These two pathways transform squalene into squalene

2,3-epoxide which is the lanosterol or cycloartenol intermediary, formed when squalene is oxidized by the enzyme squalene monooxygenase.

The following stages for different sterols isolated in algae are presented at the **Figure 2B**, being considered the anaerobic postsqualene pathway step. The biosynthesis occurs through cycloartenol pathway, however some strains produce cholesterol by lanosterol pathway. In the case of ergosterol the same pathway is activated for other microorganisms, but it is different for algae, starting their pathway by cycloartenol as observed in a study performed with *Chlamydomonas reinhardtii* [27]. Fucosterol is produced manly by lanosterol pathway as observed by Gallo et al. [28] in diatoms, and sitosterol followed by a C22 desaturation leading to stigmasterol both produced until cycloartenol pathway, the same occurs with desmosterol and chondrillasterol. Cholesterol is represented in the pathway figure produced by lanosterol, however there is research proving that this compound production also occurs by cycloartenol-dependent pathway [29].

4. Ways to manipulate sterol biosynthesis

Algae sterols can be easily manipulated to enhance their concentration, however, only few studies show the culture manipulation for this objective. In the algae metabolism commonly, the major changes occur when algae are cultured by nutrient limitation/modification. Photosynthetic system modifications consists in changing light intensity, and carbon dioxide amount, in terms of heterotrophic culture the exogenous carbon source can be considered the most important influence in sterols biosynthesis activation, salinity can be other factor important to sterol enhancer in algae [14].

For this reason, algae culture nutrient changes for phytosterols production have been mostly reported as phosphorous and nitrogen concentration. In relation to nitrogen, Zhang, Sachs, & Marchetti [30] analyzed freshwater and marine algae and they showed a reduction of 20% in sterols production when observed a nitrogen limitation for *Eudorina unicocca* and *Volvox aureus*, the reduction was similarly was observed in *Botryococcus braunii* [31], and for *Schizochytrium* sp. [32]. On the other hand, phosphorous modifications in the culture lead to a different result, the authors Piepho et al. [33] studied concentrations of 50 mM as the highest phosphorous concentration, and 10 mM as the lowest phosphorous amount. However, the phosphorus concentration was different according to the strain, being the low phosphorous concentration 1 mM for *Scenedesmus*, 5 mM for *Cryptomonas* and *Chlamydomonas* and 10 mM P for *Cyclotella,* due to each specie requirements, being the major sterol concentration found in a high-phosphorous culture system [33].

In the same line, the authors Chen et al. [34], verify for the strains *Thalassiosira oceanica*, *Rhodomonas salina*, *Isochrysis galbana*, and *Acartia tonsa*, the effect of different iron concentration added to the culture system, in fact in this experiment it was observed that the highest levels of Fe were capable to increase the total sterols, with the exception of *Isochrysis galbana.*

The effect of salt stress showed that the concentration of total free sterols increased with higher levels of NaCl in *Nitzschia laevis* [35], being the same observed in *Dunaliella salina* [36, 37]. The same comportment was observed in *Pavlova lutheri*, the changes were not observed in their total sterol composition, but in the individual sterols concentration, the enhance of salt modify the algae membrane, avoiding an excessive flux of Na + and Cl − ions into cells by increasing the membrane rigidity, helping the microorganism increasing high salt concentrations [38]. The nutrient composition from the culture as already mention has a huge influence on sterols, in another study the authors Fagundes et al. [7, 39], showed

that *Phormidium autumnale* cultured with different carbon sources, glucose, sucrose, and different industrial wastes can accumulate more sterols, compared to the inoculum, and that each culture system shows a diverse composition.

Other factor of influence in sterols composition is the UV–C radiation doses, Ahmed and Schenk [40] proved that for *Pavlova lutheri* algae the sterols increase occurred by treating the algae with UV–C radiation, however the insertion of hydrogen peroxide does not show any effect. With regards to the photosynthetic system, there is few studies showing that after high light intensities the cell sterols content increase in three microalgae [33, 41].

The authors Pereira et al. [42], also showed that light intensities of 30, 60, 140, 230, and 490 mmol photons m^{-2} s^{-1} were tested for two Chlorophyceae *Scenedesmus quadricauda*, *Chlamydomonas globose*, *Cryptophyceae Cryptomonas ovata*, and the Mediophyceae (Bacillariophyta) *Cyclotella meneghiniana,* showing the best production in the highest sterol intensity. The authors explained this increase by some theories, being correlated with the algae species, as described in the biosynthesis topic some algae produce sterols from MVA pathway, and others from MEP, according to the study green algae that uses only MEP for sterols synthesis, being MEP linked to the chloroplast. For this reason, hypothetically related to the photosynthesis, being the explanation for the higher intensities of sterols found in *S. quadricauda* and the diatom *C. meneghiniana,* for this more studies needs to be performed with different strains to understand sterols metabolism.

Genetically modify strains to produce sterols are gaining attention, but also is a new strategy to turn these metabolic rich systems a source of sterols. According to D'Adamo et al. [43], they introduced in *Phaeodactylum tricornutum* three enzymes from a plant *Lotus japonicus*, the modifications were responsible for mRNA expression levels, increasing the expression of the native mevalonate and, consequently sterol biosynthesis pathway was estimuled, being responsible for the expression of important triterpenoids.

5. Final considerations

Algae sterols are a new segment for being studied, they are different according to the strain, and their environment, due to the fact that external factors affect the cellular membrane, as so, the sterol concentration. In this chapter, the most important sterols end-pathway products described are: Fucosterol, β-sitosterol, stigmasterol, ergosterol, cholesterol, chondrillasterol, and desmosterol. Still today there are research's discovering pathways for algae, due to the fact that algae are spread through the world, and can be isolated in simple access places or complex ones, being responsible for the metabolic variations. The studies involving algae sterols are ascending for industrial application, so, understand their origin is an important factor for future prospective.

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6 CAPÍTULO 4

6.1 ARTIGO CIENTÍFICO 1

Green microsaponification-based method for gas chromatography determination of sterol and squalene in cyanobacterial biomass

Mariane Bittencourt Fagundes, Gerardo Alvarez-Rivera, Raquel Guidetti Vendruscolo, Mônica Vossa, Patricia Arrojo da Silva, Juliano Smanioto Barin, Eduardo Jacob-Lopes, Leila Queiroz Zepka, Roger Wagner.

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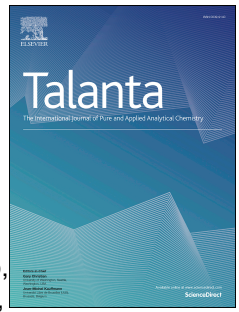

Green microsaponification-based methodology followed by gas chromatography for cyanobacterial sterol and squalene determination

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CRediT authorship contribution statement

Mariane Bittencourt Fagundes: Methodology, Formal analysis, Data curation, Writing - original draft, Writing – review & editing, Visualization, Supervision, Project administration,

Gerardo Alvarez-Rivera: Data curation, Writing - review & editing.

Raquel Guidetti Vendruscolo: Formal analysis, Investigation, Data curation, Writing - review & editing.

Mônica Voss: Investigation, Data curation.

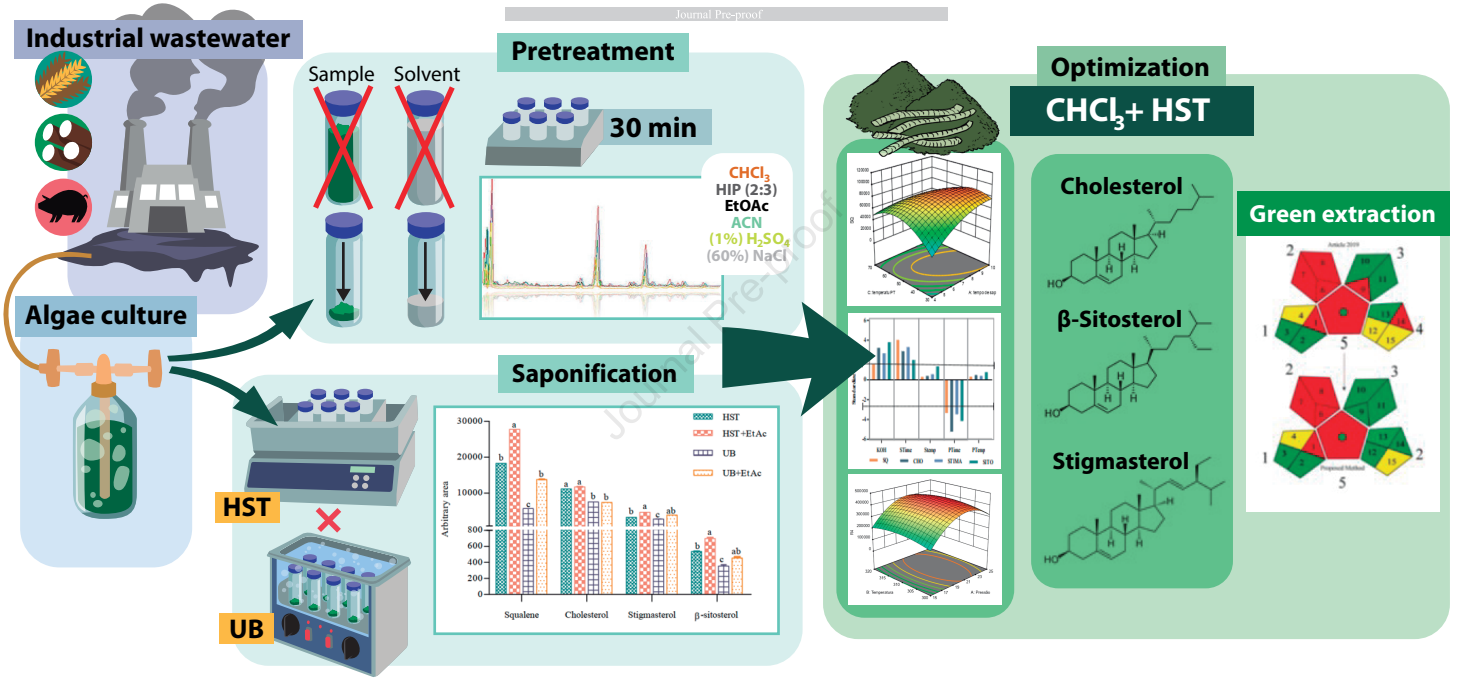
Patricia Arrojo da Silva: Formal analysis, Investigation, Data curation.

Juliano Smanioto Barin: Resources, Data curation.

Eduardo Jacob-Lopes: Validation, Resources, Investigation, Data curation.

Leila Queiroz Zepka: Validation, Resources, Writing - review & editing.

Roger Wagner: Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.



Green microsaponification-based methodology followed by gas chromatography for cyanobacterial sterol and squalene determination

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ABSTRACT

Sterol analysis of complex matrices can be very laborious. To minimize the existing drawbacks, a new micro-method of sterols and squalene determination in cyanobacteria was developed and applied to monitor their production of *Phormidium autumnale* cultured heterotrophically. Sample extraction/saponification and GC analysis of the target compounds were optimized separately using Plackett-Burman design (PB) followed by a central composite rotational design (CCRD). The most influential variables were identified to maximize compound recovery. Chloroform presented the highest capability to extract all target compounds with a horizontal shaker table (HST) for homogenization in the saponification step. For the pretreatment, a small amount of chloroform was used for 90 min at 50 °C and 6 min for the saponification time. The sample introduction in the GC injector was studied by evaluating pressure and injector temperature. High response for sterols and squalene were obtained between 19 and 23 psi and at 310 °C of injection temperature. The new method was able to determine different sterol concentrations: 0.2-0.6 mg kg^{-1} of squalene, 5-18 mg kg^{-1} of stigmasterol, 6 mg kg^{-1} of cholesterol, and 3 mg kg^{-1} of β-sitosterol, showing high analytical performance and fulfilling all steps, thus proving to be a promising technique.

Keywords: Green chemistry; Gas chromatography; Method optimization; Industrial wastewater; Sterols; Cyanobacteria; Saponification.

1. Introduction

Sterols are secondary metabolites of the terpenoid pathways known for presenting bioactive properties. They are classified as membrane lipids in

microorganisms due to their importance in cellular membrane regulation, stabilizing phospholipids, and are responsible for extracellular compound permeability [1,2,3,4]. Eukaryotic microorganisms are the most important sources of sterols. For many years, researchers have reported that only eukaryotic cells were capable of producing these molecules [5,6]. Nevertheless, with the advance of technology and the development of new methods, sterol compounds began to be emphasized in many prokaryotic cells, including cyanobacteria [7,8].

More recently, cyanobacteria have been highlighted as a new food factor due to their ability to produce large amounts of metabolites, which can be applied in biotechnology and industrial fields, such as the food industry [9,10,11]. Prochazkova et al. [12] reported the sterol production of cyanobacterial blooms with non-estrogenic power and indicated a great alternative source of sterols to replace conventional sources that are mostly based on plants and nuts, which present significant concentrations despite drawbacks arising from production seasonality [13].

Phytosterols such as β-sitosterol, stigmasterol, campesterol, and ergosterol are considered the main sterols in terms of bioactivity and known for their healthy properties. These benefits are associated with anti-obesity, anti-Alzheimer's, anti-diabetes, antioxidant, anticancer, and hepatoprotection activity, among others [14,15, 16].

Phormidium autumnale can also produce cholesterol, which is classified as a zoosterol. According to Nes [1], cholesterol is a vital molecule and a building block for other important molecules in the metabolism, including steroids (e.g., estrogen, testosterone, and cortisol). Furthermore, cholesterol is a building block for vitamin D and bile salt biosynthesis in humans. These compounds are from squalene, their metabolic precursor, which is known for its bioactive and antioxidant properties [17].

In this regard, microalgae biotechnology has proven to be a new and promising field to obtain these compounds [18]. In this context, *P. autumnale* is considered a microorganism with great biotechnological potential due to its production of numerous bioactive compounds, including squalene and sterols [7,19]. Considering the growth capacity of cyanobacteria in complex exogenous carbon sources, such as agro-industrial wastewater, the production of high added-value phytochemicals in this heterotrophic culture media may be a helpful contribution to treat industrial wastewater [20].

There are several known methods to extract sterols and squalene from microalgae in the literature, although most of them have various steps and high solvent consumption. The most common technique employs lipid extraction followed by a saponification step that consists of transforming glyceride into fatty acid soaps. In some techniques, derivatization after saponification is still necessary, resulting in a laborious process with significant waste accumulation [21], as 500 mg of lipids are normally required to perform sterol analysis [22]. Considering that *P. autumnale* produces an average of 12% of lipids, 2.5 g of biomass is required to process a single sample. Hence, this approach is not suitable for lab-scale studies in this field of research, since laboratory-scale reactors (~2 L of volume) and final production of ~1.5 g L^{-1} do not provide the required yield of biomass [23].

Therefore, there are several sample preparation strategies for microalgae phytosterol analysis that can be applied to reduce sample size, reagent amount, and time [24,25]. The addition of a cell disruption step, as described by Byreddy et al. [26], is another important point for a faster saponification procedure and essential to recover sterols and squalene since they are mainly located in the cell membrane. To that end, some studies have demonstrated that cell disruption leads to higher lipid

yields in eukaryotic strain, such as *Scenedesmus* sp. and *Chlorella* [27, 28]. Chemical disruption is a very common cell disruption technique that employs different solvents, being the solvent choice a critical step for extraction process development [29].

Thus, new methods to determine sterols in prokaryotic microorganisms are still necessary. Therefore, the present study aimed to develop a simple micro-method for sterol extraction/saponification in cyanobacterial biomass by using a reduced amount of samples and solvents. For this, a systematic experimental design was proposed to screen the main variables involved in sample preparation and introduction into the GC injection port. Therefore, the method was validated and evaluated in terms of the principles of green chemistry.

2. Materials and methods

2.1. Chemicals

Cholesterol (CHO) (5-cholesten-3β-ol; purity >99%), β-sitosterol (SITO) (5-stigmasten-3β-ol; purity >79.7%), squalene (SQ) (purity >98%), and standards were acquired from Sigma-Aldrich (USA). Potassium hydroxide (KOH 85%), sodium chloride (NaCl), and hexane were obtained from Alphatec (SP, Brazil).

2.2. Standard solutions

The CHO, SQ, and SITO stock solutions were prepared in the same concentration of 2000 mg L^{-1} by dissolving 10 mg of each analyte in 5 mL of hexane. For the calibration curve, standard working solutions covering ranges of 15-90 mg L^{-1} were used for SQ, while 90-400 mg L^{-1} were used for CHO and SITO. All curves were prepared in hexane. All solutions were stored in amber bottles in the dark and at 5 °C until analyses.

126 2.3. Cyanobacterial strain and culture medium

127 Axenic cultures of *P. autumnale* were acquired from the Cuatro Ciénegas
128 desert (26°59'N, 102°03'W - Mexico). The stock cultures were propagated and
129 maintained in a synthetic BG-11 medium according to Rippka et al. [30]. The medium
130 was composed of (mg L^{-1}): K_2HPO_4 (3.0), $MgSO_4$ (75.0), $CaCl_2 \cdot 2H_2O$ (36.0),
131 ammonium citrate and iron (0.6), Na_2EDTA (1.0), NaCl (0.72), $NaNO_3$ (150.0), citric
132 acid (0.6), Na_2CO_3 (15.0), H_3BO_3 (2.8), $MnCl_2.4H_2O$ (1.8), $ZnSO_4 \cdot 7H_2O$ (0.22),
133 $Na_2MoO_4 \cdot 2H_2O$ (0.39), and $CoSO_4 \cdot 6H_2O$ (0.04). The initial pH was adjusted to 7.6
134 and cultivation conditions were: 25 °C, a photon flux density of 15 µmol m^{-2} s^{-1},
135 incident light intensity, and a photoperiod of 12:12 h (L:D).

136

137 2.4. Cyanobacterial biomass production

138 *P. autumnale* was cultured heterotrophically and the experiments were
139 developed with distinct exogenous carbon sources by using complex sources of
140 carbon. The biomass chosen for the optimization process was obtained from the
141 slaughterhouse wastewater used as exogenous carbon source due to the variety of
142 sterols found there [7]. The other wastes were acquired and characterized according to
143 the following studies: cassava wastewater was obtained from the cassava flour
144 industry [23] and the brewery wastewater was characterized according to Santos et al.
145 [31]. The experimental conditions were initial inoculum concentration of 100 mg L^{-1}
146 and isothermal reactor operating at 26 °C with continuous aeration of 1 VVM (volume
147 of air per volume of culture per min). The carbon/nitrogen (C/N) ratios were adjusted
148 to 30 with glucose (Sigma-Aldrich, USA) in all experiments. The biomass was
149 acquired in a bubble column bioreactor and the cultures were produced under a bath

regime and fed with 2 L of each medium. All experiments were conducted for 168 h of residence time.

2.5. Biomass sample preparation for sterol extraction

The sterols were extracted from the biomass by direct saponification with modifications to allow biomass reduction and work with 50 mg of sample [7,32]. The flowchart of our newly proposed method and its modifications are shown in Fig. 1.

[Insert Fig. 1]

The method was performed in the sequence presented with two extraction screening tests: the first one evaluated the type of agitation (ultrasonic bath, UB; horizontal shaker table, HST) using ethyl acetate (EtOAc), whereas the second one, which was the pretreatment (PT), was performed before the saponification by adding different solvent combinations to the biomass for 30 min. The UB (Schuster l-100, USA) conditions were 50 kHz of frequency, 200 W of output, and temperature of 40 °C, while the HST consisted of 150 rpm of agitation and temperature of 40 °C. The samples were submitted to saponification using 1 mL of KOH solution (10% w/v) in methanol under agitation for 30 min. Then, 1 mL of salt-saturated solution (36%) was added and 1 mL of hexane was added twice in order to finish the saponification procedure. The organic fraction was dried under nitrogen, the analytes suspended in 100 μL of 3:2 HIP (hexane:isopropanol), and submitted to GC analysis, as described below to monitor CHO, SITO, STIGMA, and SQ.

The second assay consisted of using the following solvents: chloroform ($CHCl_3$), 1% of sulfuric acid aqueous solution (H_2SO_4), acetonitrile (ACN), hexane:isopropanol solution (HIP, 2:3), 60% of sodium chloride in aqueous solution

(NaCl), and ethyl acetate (EtOAc) for 30 minutes. Analyte saponification and partition were carried out as described above. The best solvent in the pretreatment was chosen for further optimization analysis.

2.6. Optimization strategy for sample preparation

The potential variables that could affect sterol extraction were evaluated in sample pretreatment and saponification steps using a Plackett-Burman screening design [33]. The experimental design was coded and shown in Table S1 (Supplementary Material). The 16 experimental runs were randomly evaluated in duplicate and analyzed twice by gas chromatography. The independent variables screened were: pretreatment temperature (PTemp; 30 to 60 °C), pretreatment time (PTime; 10 to 60 min), saponification time (STime; 5 to 30 min), saponification temperature (STemp; 30 to 60 °C), and KOH concentration (KOH; 3 to 10%), being the sterols and squalene peak areas the responses. The variables with significant effects on sterol responses were submitted to a full factorial model by a central composite rotational design (CCRD). The CCRD was performed with a three-level factorial design to determine the optimal levels of the PB design and significant variables are shown in Table S2 (Supplementary Material). Optimization was done by the highest arbitrary area of each compound obtained from the best condition of extraction/saponification.

2.7. Chromatography conditions

Sterol extracts from biomass were analyzed by gas chromatography equipped with a flame ionization detector (GC-FID) and Varian 3400 (Palo Alto, USA). The injection port of the GC was operated in splitless mode (splitter valve off by 0.8 min;

50:1). Hydrogen was used as the carrier gas at a constant pressure of 15 psi. The separation was performed in a non-polar column BPX-5MS SGE (Sydney, AUS; 25 m × 0.22 mm id; 0.25 μm-film thickness). The column temperature was initially established at 50 °C, increased to 280 °C (15 °C min^{-1}), and then to 330 °C (5 °C min^{-1}), maintaining the isothermal conditions for 5 min. The temperature of the detector was 280 °C. The peak area for each analyte from the chromatograms was used for optimization procedures.

2.8. Optimization of GC sample introduction

An optimization to obtain higher sterol and squalene arbitrary areas by studying the sample introduction was performed. In this way, the variables injector temperature and injector port pressure were evaluated using an experimental design CCRD (2^2) and the explored levels are shown in Table S3 (Supplementary Material).

2.9. Method validation

The performance of the extract obtained by the optimized extraction/saponification and GC methods was evaluated by the following parameters: recovery, precision, linearity, linear range, limits of detection (LOD), and limits of quantification (LOQ). For recovery analysis, 20% of CHO, SQ, and SITO sample concentrations were spiked to 50 mg of cyanobacterial biomass. To evaluate linearity, a linear correlation coefficient (R^2) curve was determined and the LOD and LOQ were determined according to the signal-to-noise ratios of 3 and 10, respectively. Stigmasterol was quantified as the equivalent cholesterol due to the curve angle similarity after consecutive sample dilutions. Therefore, after the validation and optimization process, the same procedure was applied to biomasses

cultured with different wastes to evaluate the potential of the method in determining these analytes.

2.10. Greenness evaluation of the analytical methods (GAPI)

The greenness of the proposed method was evaluated using the Green Analytical Procedure Index (GAPI), which was recently proposed by Plotka-Wasylka [34] as a new tool to evaluate the green aspects of an analytical method. A specific symbol with five pentagrams subdivided internally into three or four regions can be used to evaluate and quantify the environmental impact involved in each step of an analytical methodology using the colors green, yellow, and red, which represent low, medium, and high impact, respectively. The information used to evaluate the green character of the analytical method uses aspects related to sample collection through the disposal of the waste into the environment, in addition to the energy efficiency of the method. The values used in GAPI for each analytical method can be observed in Table S4 (Supplementary Material).

2.11. Statistical analysis

Solvent screening in the pretreatment process, the modifications in the saponification step, and the method application with other wastes such as exogenous carbon source were carried out in triplicate. The experimental results were evaluated by Analysis of Variance (ANOVA) using Statistica 8.0 software [35] and the means difference by the Tukey test ($P < 0.05$). Data normality was tested according to the Shapiro-Wilk test. Protimiza Experimental Design software for Plackett-Burman design and Design Expert 11.0 for CCRD were used for the optimization designs [36].

3. Results and discussion

3.1. Sterol and squalene extraction

Saponification is a chemical reaction widely studied and applied as a clean-up step when preparing high lipid-content samples. The main aim of this step is to obtain the unsaponifiable fraction by removing the fatty acids and triacylglycerols, selectively extracting unsaponified compounds [37]. In this study, a saponification treatment was optimized to remove lipid interference and maximize the recovery of the main sterols and their precursor squalene from *P. autumnale* cyanobacterial biomass. This strain was chosen for the optimization process and cultured heterotrophically in slaughterhouse wastewater as an exogenous carbon source culture medium. *P. autumnale* was characterized by the presence of SQ, CHO, STIMA, and SITO in our previous study by using 500 mg of biomass in the sample preparation [7]. In the present work, our efforts were focused on reducing the sample amount to approximately 50 mg to monitor the bioreactors at laboratory-scale. Such small-scale work can produce low amounts of biomass, which requires compatible methods.

The first results showed that the amounts of sterols obtained with different extraction/saponification treatments, by combining physical and chemical cell disruption, were significantly affected (Fig 2A). The extraction using HST with EtOAc showed higher recovery rates for the target compounds compared to the other UB-based treatment, promoting higher agitation intensity and better efficiency in the extraction/saponification step.

[Insert Fig. 2]

The EtOAc was confirmed as an important extracting agent in both HST and UB techniques, improving the recovery values for STIGMA, SITO, and SQ. The

EtOAc was selected due to its characteristic as a green solvent and capacity to extract carotenoids from various matrices [38]. According to Angles et al. [39], EtOAc acts as a chemical cell disruption agent by recovering high lipid content from the microalga *Nannochloropsis* sp., therefore, the first step for sterol extraction was carried out using this reagent.

To choose the best saponification treatment with the HST method, further experiments were carried out to test the extraction capacity of different types of solvents, including $CHCl_3$, H_2SO_4 solution, ACN, HIP, NaCl solution, and EtOAc. These solvents were chosen according to their polarity (polar and non-polar nature) and possible influence as microalgae cellular disruptors [40]. The results for the solvents tested are shown in Fig. 2B. According to Yoo et al. [41], osmotic shock can provide higher cellular disruption in microalgae *Chlamydomonas reinhardtii*, although this was contrary to what was obtained in our study in terms of sterol recovery, being fewer sterols and lower SQ recovery rate in the pretreatment with H_2SO_4 solution. According to Steriti et al. [42], H_2SO_4 (1% m/v) is the most prominent disruptor reactant to recover lipid compounds. However, the H_2SO_4 solution did not show good behavior compared to the other solvents for this cyanobacterium. Other solvents presented different behavior that may be associated with the affinity for the extracted compounds. For instance, acetonitrile has higher polarity index (5.8) compared to isopropanol (4.0) and hexane (0.1), which exhibited good recovery for STIGMA with similar performance compared to $CHCl_3$ (2.7). The HIP mixture (3:2 v/v) showed a similar extraction capacity to EtOAc. The use of EtOAc was reported as the best solvent to extract polar carotenoids [43], although it was also a great solvent for SQ in the present study. In fact, the studied solvents demonstrated lower efficiency when compared to $CHCl_3$, which was shown to be, in

general, the best solvent for the recovery of the target compounds and likely due to the high dielectric constant and polarity. Moreover, $CHCl_3$ breaks the hydrophobic interactions of phospholipids and other lipids in the cell membrane, and for this reason, it was the most efficient solvent and selected for further studies in the experimental design.

3.2. Experimental design

3.2.1. Variable screening for sterol and SQ extraction

The PB design was used to evaluate the effects of the following variables (independent variables) on sterol and SQ (dependent variables) extraction: pretreatment temperature (PTemp), pretreatment time (PTime), saponification temperature (STemp), saponification time (STime), and KOH concentration. The experimental matrix of the PB design can be seen in Table S1 (Supplementary Material). The Pareto Charts are shown in Fig. 3, where the length of each bar is proportional to the positive and negative influence that each independent variable has on the recovery of the target analytes.

[Insert Fig. 3]

These variables exceeding the horizontal dashed-line limit are considered to significantly affect the analyte recoveries ($p < 0.05$ in ANOVA; Table S5, Supplementary Material).

In general, the most significant parameter observed was STime for the three sterols and SQ, presenting a negative effect in the recoveries. This response indicates that there is no need to use longer saponification periods after the pretreatment. Other important effects observed in the pretreatment were time and temperature, which were strongly associated with cell hydrolysis. The chemical cell disruption is related to the

membrane rupture by the solvent, which facilitates molecule extraction. Thus, sterol extractability can be dependent on the contact time and solvent temperature, which was shown to influence cell chemical disruption [44]. The cell disruption performed with chloroform showed an important influence on sterol recovery. The effect of PTime was in the following order: SQ (3676)> CHO (1538)> STIGMA (901)> SITO (102), whereas the influence of Ptemp was as follows: CHO (1715)> SQ (1465)> STIGMA (731)> SITO (190); all of them presented positive effects. Therefore, the results indicate that sterol and SQ extractability is influenced by the extraction time and solvent temperature. Thus, pretreatment temperature and time and saponification time were selected as the most significant variables to be evaluated in a CCRD study.

The KOH concentration was not significant for sterol and SQ extractions. However, by integrating all chromatograms and excluding only the target molecules, a significant cleanup effect in the chromatogram was observed (Fig. S1, Supplementary Material). For this reason, the concentration chosen for the following experiment was 6.5% of KOH. For STemp, since no significant effects were observed, we chose to use the temperature of 30 °C because of the possible thermal degradation of these compounds.

3.2.2. Optimization of the experimental variables for sample preparation

A 2^3 central composition rotational design (CCRD) was used to optimize sterol and SQ extraction from cyanobacteria. The experimental matrix is presented in Table S2 (Supplementary Material), including the main variables studied at different levels (PTime, PTemp, and STime) and the experimental responses obtained for sterols and SQ. The strong curvature observed in the PB design indicated that the optimum point is very close to the range studied in the screening procedure, as

described in the literature [32]. Therefore, the following ranges for the studied variables were selected: 30 to 90 min for PTime, 30 to 70 °C for PTemp, and 4 to 10 min for STime. Eighteen experiments were performed including four central points. The CCRD was run first, including the three variables, and the models observed were significant (p<0.05; Table S6, Supplementary Material). However, not all variables were influent in the statistical model. The CCRD was performed again, this time including only the significant terms for each compound, generating a new model equation (Fig. 4). In the present work, no lack of fit was observed, suggesting that all the responses obtained were well fitted in the models predicted.

[Insert Fig. 4]

The SQ presented the most significant terms, namely quadratic STime, PTemp (p < 0.001), the interaction STime × PTemp (p < 0.001), and STime (p = 0.02). As for CHO, the significant terms were the interaction STime × PTemp (p = 0.01) and PTemp quadratic term (p = 0.04). STIGMA showed the quadratic and linear STime as significant terms with p values 0.06, and <0.0001, respectively. However, SITO was significantly affected by all variables. Thus, CCRD analysis revealed different behaviors among the analytes. The contour plots of Fig. 4 show that, for instance, to obtain the highest SQ levels, it is necessary to move towards longer periods for both PTime and STime (Fig. 4A). However, when analyzing PTemp, moderate temperatures increase SQ recovery by up to 94%. Similar behavior was observed for CHO, which showed optimal saponification time ranging from 8 to 10 min, whereas the only variable affecting STIGMA response (STime) exhibited optimal values between 6 and 7 min.

In summary, long pretreatment time (90 min), soft pretreatment temperature of 50 °C, and a saponification time of 6 min were the selected conditions that maximize

the recovery of the major compounds, mainly SITO, as their response was chosen as the most important one due to their lowest response. A sample prepared with these conditions was used for further optimization of the GC-injection conditions.

3.2.3. Optimization of GC-injection variables for sterol and SQ analysis

The chromatographic signal of the analytes was improved by evaluating the influence of the injection variables into the GC. The effects of the injector temperature (IT) and pressure (P) were evaluated through a full 2^2 CCRD. Results from ANOVA are shown in Table S7 (Supplementary Material) and the contour plots are detailed in Fig. 5.

[Insert Fig. 5]

Analysis of the variables for SQ showed that the most significant was the IT×P interaction, with a $p < 0.02$ P quadratic ($p= 0.03$), followed by the P of 0.05. Furthermore, CHO was also highly affected by P, being significant the linear, quadratic pressure (P^2), and P×IT interaction (0.05, 0.03, and 0.02 of p values, respectively). In the sample introduction, STIGMA was significantly affected in all variables, being $p<0.0001$. However, in SITO, the parameters of influence were P and P quadratic, with the quadratic IT being $p= 0.0002$.

As shown in the contour plot (Fig. 5), the region with the most responses was between 19 to 23 psi. Thus, 20 psi was the selected injector pressure that showed the highest phytosterol (SITO, STIGMA) signals, which entails a significant increase in the detectability of these analytes considering their lower response (minor compounds) compared to other sterols. Analysis of the injector temperature showed that the results demonstrated a higher response at 310 °C for STIGMA and SITO and intermediate response for SQ and CHO. However, as we focused on several

399 compounds at the same time, the injector port was set at 310 °C, because losses in
400 CHO areas were observed at higher temperatures, which is most likely due to
401 degradation in the injection port.
402
403 3.3. Method validation
404 The performance of the developed analytical method was evaluated in terms
405 of linearity, accuracy, precision, LOD, and LOQ. All validation parameters for the
406 target analytes are summarized in Table 1. Linearity was acquired by constructing the
407 following curves: 15 to 900 μg mL^{-1} for SQ, 25 to 400 μg mL^{-1} for CHO, and 90 to
408 400 μg mL^{-1} for SITO. All determination coefficients were higher than 0.99. The
409 LOD and LOQ showed values of 0.1 μg mL^{-1} for SITO and CHO and 0.05 μg mL^{-1}
410 for SQ.
411 [Insert Table 1]
412 The accuracy for SQ, CHO, and SITO was: 91, 100, and 109%, respectively,
413 which is similar to other methods that employed high sample amounts [22,45]. To
414 develop the accuracy assay, it was necessary to dilute the samples (1:1) in the same
415 solvent used for the extract dilution (HIP) in order to adapt the studied concentration
416 on the calibration curves. The repeatability of the method (n = 6) was expressed as a
417 relative standard deviation (RSD, Table 1). The present micro-extraction
418 saponification method developed for cyanobacterial biomass demonstrated
419 satisfactory figures of merit and is suitable to determine a mix of sterols and their
420 precursors.
421 The concentrations obtained in *P. autumnale* biomass were: 0.6, 6.1, 18.2, and
422 3 mg kg^{-1} for SQ, CHO, STIGMA, and SITO, respectively. A high concentration of
423 STIGMA was found, which is in accordance with Pereira et al. [45] who reported a

424 concentration range of 2.69 to 14.84 mg kg^{-1} for STIGMA and 5.29 to 16.49 mg kg^{-1}
425 of SITO in *Cystosphaera jacquinotii*, *Ascoseira mirabilis*, *Desmarestia anceps*,
426 *Adenocystis utricularis*, *Desmarestia antarctica*, and *Himantothallus grandifolius*.
427 Kim, Li, Kang, Ryu, and Kim [46] studied *Navicula incerta* and proved that STIGMA
428 present in this strain induces apoptosis in human hepatoma HepG2 cells, presenting
429 anti-cancer effects. In the same line, SITO was found in some strains, including
430 *Nostoc commune var. sphaeroides Kützing* (*N. commune*) and *Schizochytrium* [47].
431 This phytosterol was shown to play an important role in the removal of CHO
432 excess from plasma in humans [48].
433
434 3.4. SQ and sterol determination in *P. autumnale* cultured with different industrial
435 waste
436 The developed method was applied to evaluate sterol production by
437 microalgae cultured heterotrophically with an alternative exogenous source of carbon.
438 The different sterols obtained are shown in Fig. 6. Wastewater culture medium from
439 the cassava flour industry, brewery industry, and slaughterhouse was selected as the
440 exogenous carbon source for *P. autumnale*.
441 [Insert Fig. 6]
442 Cultures in the slaughterhouse, as previously described, showed values of 0.6,
443 6.1, 18.2, and 3 mg kg^{-1} for SQ, CHO, STIGMA, and SITO, respectively. Cultures in
444 the brewery waste presented 0.43 mg kg^{-1} for SQ at a concentration of 13.93 mg kg^{-1}
445 STIGMA, while waste from cassava yielded concentrations of 0.16 and 5.65 mg kg^{-1}
446 for SQ and STIGMA, respectively.
447 These results demonstrate the applicability of the proposed method to
448 determine the target sterols and SQ in *P. autumnale* biomass growing in different

449 culture systems. The culture in slaughterhouse wastewater revealed the presence of
450 high sterol concentrations, mainly STIGMA and SITO, whereas SQ was found as a
451 minor compound due to its sterol precursor character. Interestingly, cassava
452 wastewater presented higher concentrations of phosphorous (166 mg kg^{-1}) and
453 nitrogen (250 mg kg^{-1}) than the brewery industry wastewater (17.38 mg kg^{-1} of
454 nitrogen and 9.98 mg g^{-1} of phosphorous) and slaughterhouse wastewater (2.8 mg kg^{-1}
455 of phosphorous and 1.9 mg kg^{-1} of nitrogen). In this regard, Volkman [35] reported
456 that variations in phosphorous and nitrogen concentrations may lead to the formation
457 of sterols, which can explain the different sterol profiles obtained.
458
459 3.5. Evaluation of compliance with green chemistry
460 The microsaponification-based method developed in this work was evaluated
461 according to the green analytical chemistry standardized attributes of the Green
462 Analytical Procedure Index (GAPI). This tool is used to evaluate the green character
463 of analytical methods from sample collection for the final determination of analytes
464 and waste disposal. This new sterol protocol was also compared with the sterol
465 extraction described by Fagundes et al. [7] using the same strain. All data considered
466 for this evaluation are presented in Table S4 (Supplementary Material). The results
467 from our first work in 2019, with regards to the newly proposed method here are
468 shown in Fig. 7.
469 [Insert Fig. 7]
470 The first pictogram expressed as number 1 is related to sample collection,
471 preservation, transport, and storage, which are similar for all methods. The second
472 pictogram (2) was used to evaluate sample preparation. Both evaluated methods
473 required two steps for sample preparation, first saponification, and then solvent

evaporation, thus both are considered indirect methods with GC determination. However, in the proposed method, a microextraction approach was implemented to reduce sample size and solvent volume. Therefore, the new methodological proposal makes use of lower amounts of solvents, yielding higher sterol recovery.

The third pictogram (3) is related to the reagent amount, sample mass, and health and safety risks. The proposed method uses lower reagent volume (5.1 mL per replicate and 0.05 g of sample), which is 10 times lower than what was used in the previous article.

The fourth pictogram (4) is related to energy consumption, occupational hazard, waste volume, and treatment. The proposed method presented reduced energy consumption per replicate for sample preparation (0.10 kWh) compared to the previous method (0.67 kWh). It should be emphasized that the present method generated 4.6 mL of waste, which is around 10 times less compared to the previous method.

Therefore, the proposed analytical method can be considered a suitable alternative to fulfill green analytical chemistry principles in sterol and SQ determination from cyanobacterial biomass by gas chromatography.

5. Conclusion

An innovative micro-saponification method was proposed for sterol determination in cyanobacterial biomasses. The improved method involves reducing the sample amount, including solvents and time, giving a satisfactory comprehension of sterols and their precursor determination by GC-FID. The optimization strategy in sample preparation and sample introduction in GC significantly increased compound signals. Furthermore, our study proved that the new micro-method offers a good

performance by providing satisfactory parameters of merit. In addition, the same strain cultured with different exogenous carbon sources was tested, and our method proved to be capable of evaluating a wide range of concentrations. Hence, the developed method can contribute not only to the biotechnology advance but also to determine these bioactive molecules. This method shows significant advantages over conventional methods, such as reducing time consumption of sample preparation and significant sample and solvent amount, thus being characterized as a greener method.

Conflicts of interest

The authors declare no conflict of interest.

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Captions

Table 1. Validation parameters for squalene and sterols performed in the new optimized method.

Figure 1. Step-by-step of the proposed green microsaponification method applied for our sterolomic study. The first test was performed by using different mechanical agitation types in the saponification step UB: Ultrasonic bath, HST: Horizontal shaker table. The second test was based on the solvent required for the pretreatment; the solvent contact was also performed in 30 minutes. $CHCl_3$: Chloroform, HIP: Hexane: Isopropyl alcohol (2:3), EtOAc: Ethyl acetate, NaCl: Sodium sulfide (60%), and sulfuric acid (1%).

Figure 2. Effects of different pretreatments on sterol and squalene extraction. A) comparison of different saponification techniques; (HST- Horizontal shaker Table); (HST EtOAc- Horizontal Shaker Table + Ethyl Acetate); (UB- Ultrasonic Bath); (UB EtOAc- Ultrasonic Bath + Ethyl Acetate). B) Comparison of different solvents with the HST pretreatment technique; ($CHCl_3$-Chloroform); (1% H_2SO_4-Sulfuric acid);

(ACN-Acetonitrile); (HIP-Hexane:Isopronanol, 2:3); (60% NaCl); (EtOAc-Ethyl Acetate).

Figure 3. Standardizing Pareto chart of Plackett-Burman design showing the effects of the variables (STime: Saponification Time), (STemp: Saponification Temperature), (PTime: Pre-treatment Time), (PTemp: Pre-treatment temperature), and (KOH: KOH concentration) on sterol and squalene extraction.

Figure 4. Contour plot results for sample preparation techniques and direct influence on sterols, being A: squalene (SQ), pretreatment time × saponification Time; B: squalene (SQ), pretreatment temperature x saponification time; C: cholesterol (CHO), pretreatment temperature × saponification time; D: stigmasterol (STIGMA) saponification time; E: β-sitosterol (SITO), pretreatment time x saponification time F: β-sitosterol (SITO) Pretreatment temperature x saponification time.

Figure 5. Contour plot results for sample introduction, direct influence on stigmasterol (STIGMA), cholesterol (CHO), β-sitosterol (SITO), and squalene (SQ) responses, being the variables studied: injector temperature and pressure.

Figure 6. Sterols concentration in different industrial wastes.

Figure 7. The greenness pictogram from the method developed in 2019 (Fagundes et al., 2019b) and the proposed method.

Table 1. Validation parameters for squalene and sterols performed in the new optimized method

Compounds	Equations	Linear Range	R^2	Repeatability (%)	Recovery (%)	LOD	LOQ
SQ	y=6631x-16100	15-90	0.9998	11.97	91	0.05	0.50
CHO	y=960.5x-22075	70-1000	0.9936	3.72	100	0.10	1.00
STIGMA*	y=960.55x-22075	70-1000	0.9936	6.62	-	0.10	1.00
SITO	y=2221.4x-150395	25-90	0.9932	4.66	109	0.10	1.00

R^2: coefficient of determination, LOD (μg mL^{-1}): limit of detection, LOQ (μg mL^{-1}): limit of quantification; SQ: Squalene; CHO: Cholesterol; STIGMA: Stigmasterol; SITO: β-Sitosterol. STIGMA was calculated from the CHO curve due to its angle similarity after a sequence of solutions. *The parameters equations, R^2, LOD, and LOQ for STIGMA were the same as the CHO.

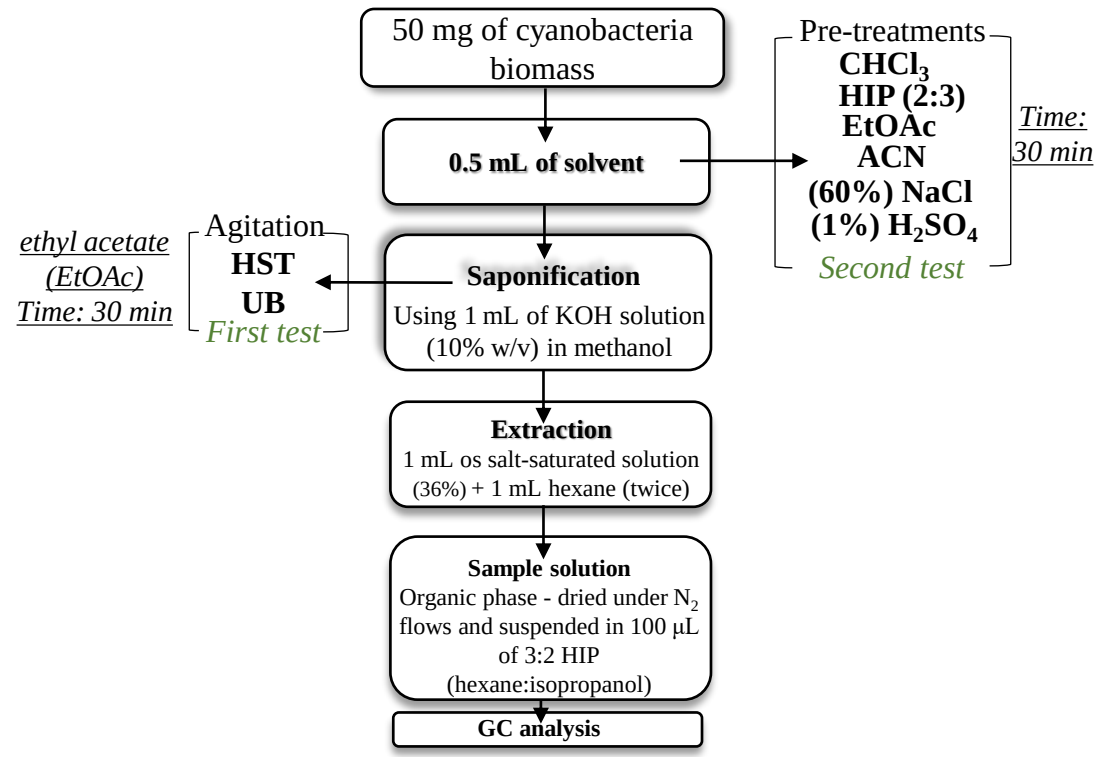
HIGHLIGHTS

- A simple miniaturized method was developed for sterol determination in cyanobacteria
- The GC optimized method provided higher sterol recovery with a reduced sample amount
- The greener improved method was validated showing satisfactory parameters of merit
- The method was efficient for sterol and squalene determination in different biomasses

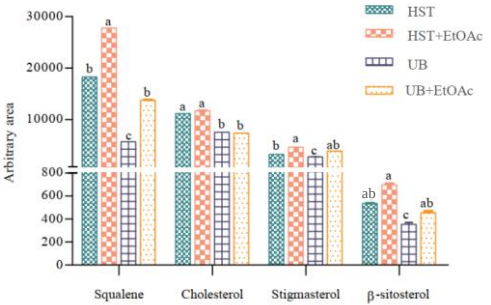
Declaration of interests

☒ 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.

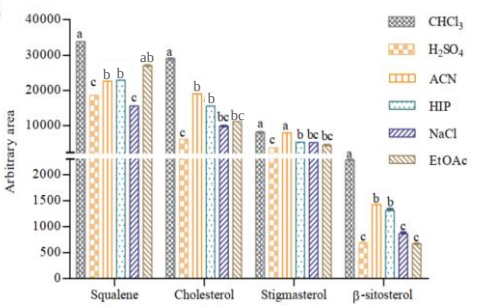
☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

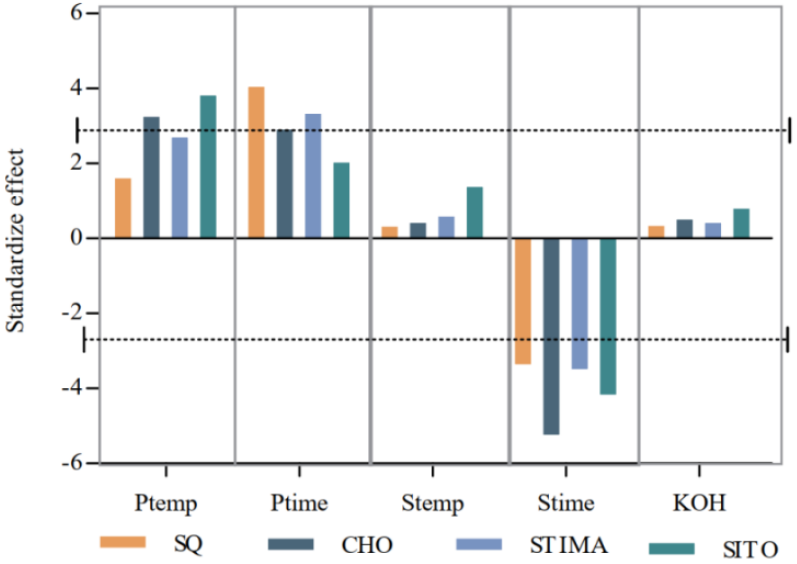

50 mg of cyanobacteria biomass
0.5 mL of solvent
Pre-treatments
CHCl3
HIP (2:3)
EtOAc
ACN
(60%) NaCl
(1%) H2SO4
Second test
Time: 30 min
Agitation
HST
UB
First test
ethyl acetate (EtOAc)
Time: 30 min
Saponification
Using 1 mL of KOH solution (10% w/v) in methanol
Extraction
1 mL os salt-saturated solution (36%) + 1 mL hexane (twice)
Sample solution
Organic phase - dried under N2 flows and suspended in 100 µL of 3:2 HIP (hexane:isopropanol)
GC analysis

A


HST
HST+EtOAc
UB
UB+EtOAc
Arbitrary area
30000
20000
10000
800
600
400
200
0
a
b
c
b
a
a
b
b
b
a
c
ab
ab
a
c
ab
Squalene
Cholesterol
Stigmasterol
β-sitosterol

B


CHCl3
H2SO4
ACN
HIP
NaCl
EtOAc
Arbitrary area
40000
30000
20000
10000
2000
1500
1000
500
0
a
c
b
b
c
ab
a
c
b
b
bc
bc
a
c
a
b
bc
bc
a
c
b
b
c
c
Squalene
Cholesterol
Stigmasterol
β-sitosterol


Standardize effect
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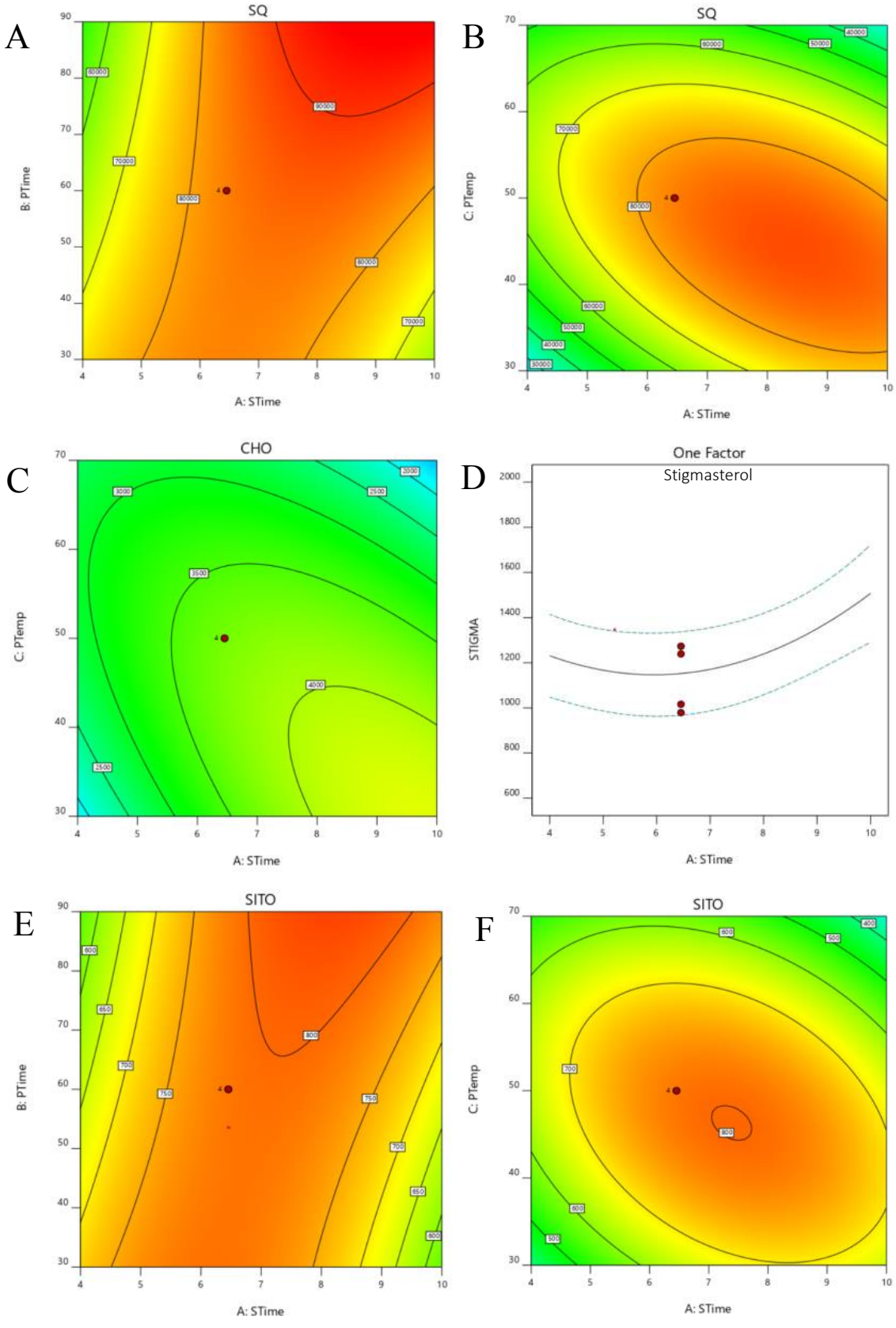
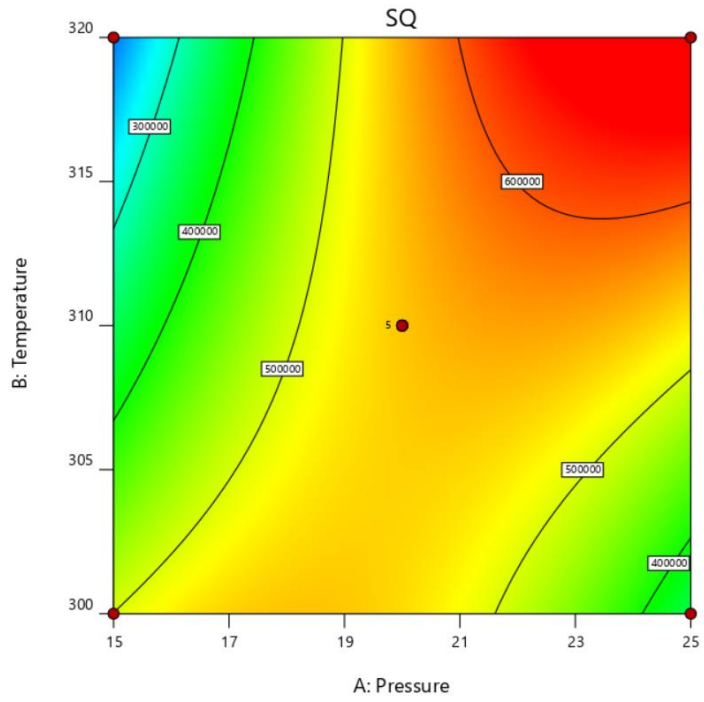
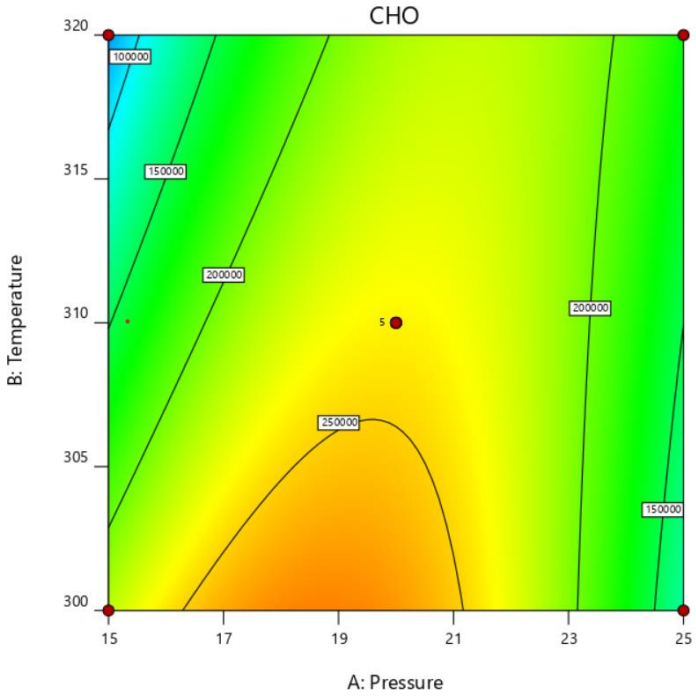
4
2
0
-2
-4
-6
Ptemp
Ptime
Stemp
Stime
KOH
SQ
CHO
STIMA
SITO


A
SQ
B: PTime
A: STime
B
SQ
C: PTemp
A: STime
C
CHO
C: PTemp
A: STime
D
One Factor
Stigmasterol
STIGMA
A: STime
E
SITO
B: PTime
A: STime
F
SITO
C: PTemp
A: STime


SQ
320
315
310
305
300
B: Temperature
15
17
19
21
23
25
A: Pressure
300000
400000
500000
600000
500000
400000
5


CHO
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315
310
305
300
B: Temperature
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17
19
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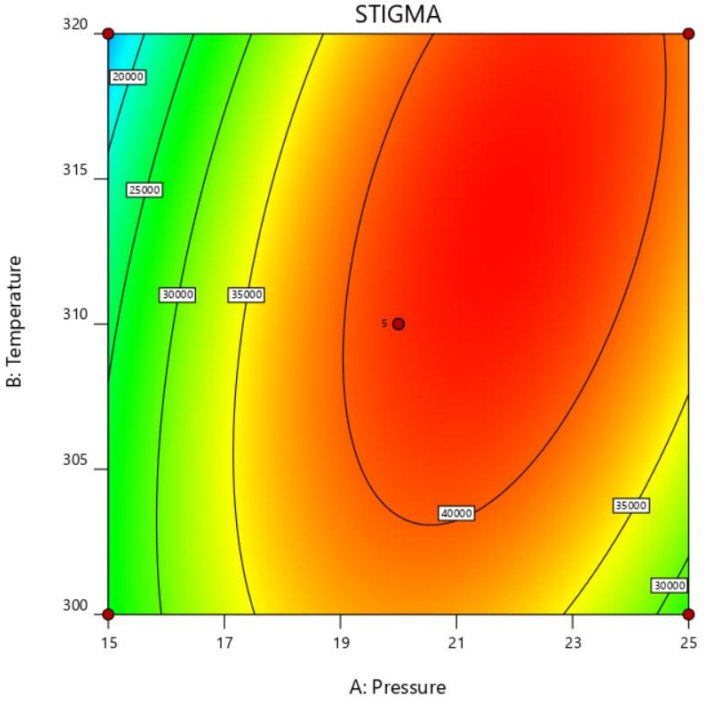
23
25
A: Pressure
100000
150000
200000
250000
200000
150000
5


STIGMA
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315
310
305
300
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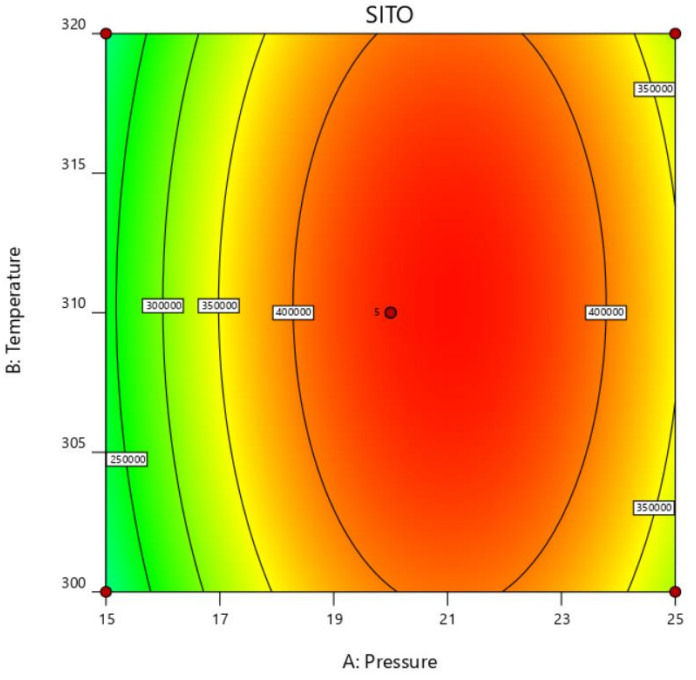
15
17
19
21
23
25
A: Pressure
20000
25000
30000
35000
40000
35000
30000
5


SITO
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315
310
305
300
B: Temperature
15
17
19
21
23
25
A: Pressure
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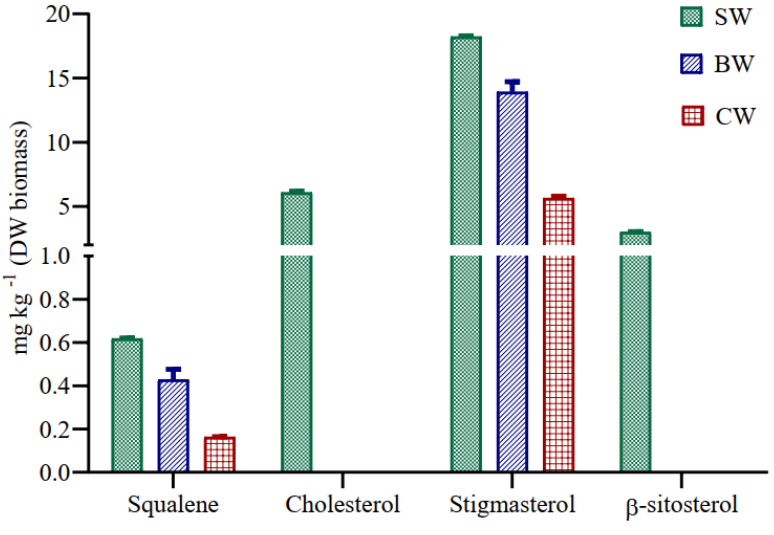
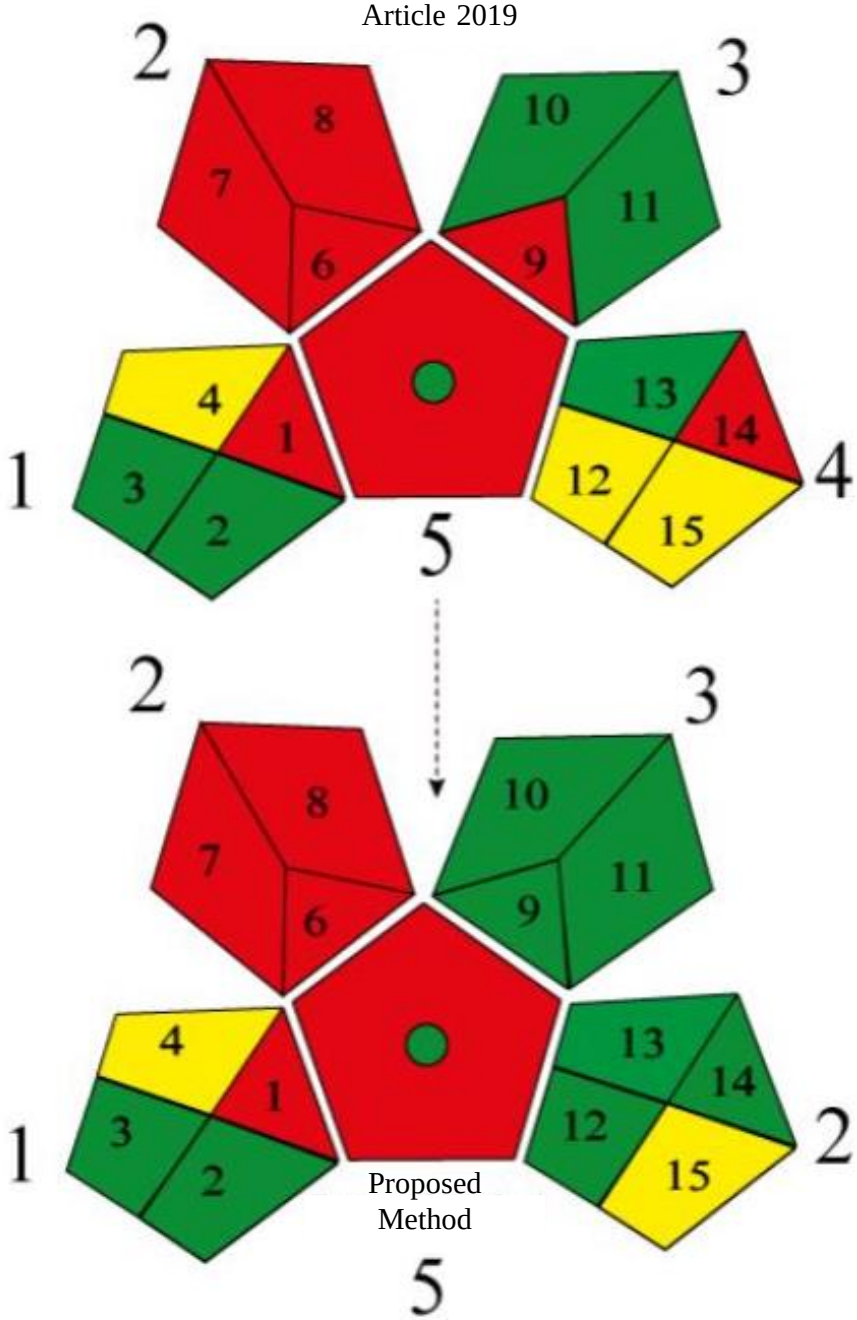
300000
350000
400000
350000
350000
5


20
15
10
5
1.0
0.8
0.6
0.4
0.2
0.0
mg kg -1 (DW biomass)
SW
BW
CW
Squalene
Cholesterol
Stigmasterol
β-sitosterol



Proposed Method

Supplementary information to manuscript:

Table S1. A Plackett-Burman design matrix with 16-run for five variables applied to sample preparation.

Run no.	*Variables*					*Experimental responses (arbitrary area)*			
	PTemp	**PTime**	**STemp**	**STime**	**KOH**	**SQ**	**CHO**	**STIGMA**	**SITO**
1	60	10	60	30	3	7620	5759	3026	693
2	60	10	60	5	3	7114	7477	3367	754
3	60	60	60	5	10	12344	9726	4316	911
4	60	10	30	5	10	9125	7813	3170	655
5	60	60	30	30	3	9441	7550	3581	644
6	45	35	45	17.5	6.5	11622	8712	4596	680
7	45	35	45	17.5	6.5	11542	8758	4573	641
8	45	35	45	17.5	6.5	10516	9550	4340	713
9	30	60	30	5	3	12659	7380	3930	631
10	60	60	30	30	10	7630	5339	1536	328
11	30	10	60	30	10	4903	3113	1959	403
12	30	60	60	30	3	6071	4120	1915	343
13	30	60	60	5	10	11761	9019	4227	862
14	45	35	45	17.5	6.5	11172	8706	4184	707
15	30	10	30	5	3	5007	5385	2136	530
16	30	10	30	30	10	4081	4354	1946	377

The variables were: PTemp: Pretreatment temperature. PTime: Pretreatment time. STemp: Saponification Temperature. STime: Saponification time. and KOH: KOH concentration. SQ: Squalene; CHO: Cholesterol; STIGMA: Stigmasterol; SITO: β-Sitosterol.

Table S2. Levels of extraction/saponification variables to CCRD design and the response of the sterols and squalene

Run no.	*Variables*			*Experimental responses (arbitrary area)*			
	PTemp	**PTime**	**STime**	**SQ**	**CHO**	**STIGMA**	**SITO**
1	30	30	3	4989	1034	1086	202
2	30	30	10	67289	4684	1866	433
3	30	90	3	8670	1016	1665	268
4	30	90	10	94593	5668	1769	878
5	70	30	3	68942	3006	1752	680
6	70	30	10	6758	1356	1522	346
7	70	90	3	9568	1008	1371	204
8	70	90	10	50583	2472	197	349
9	50	60	0.5	3456	611	1657	110
10	50	60	12	52343	1011	1824	356
11	50	10	6	86062	4111	875	823
12	50	110	6	79968	4186	872	769
13	16	60	6	6407	1585	1525	345
14	84	60	6	7714	1825	727	201
15	50	60	6	87264	3271	1239	789
16	50	60	6	77644	2711	979	754
17	50	60	6	88971	3510	1273	788
18	50	60	6	77327	3867	1016	795

The variables were: (PTemp: pretreatment temperature), (PTime: pretreatment time), and (STime: saponification time). SQ: Squalene; CHO: Cholesterol; STIGMA: Stigmasterol; SITO: β-Sitosterol.

Table S3. Level and response of variables optimized in sample introduction into GC injector port of sterols and squalene.

	Variables		*Experimental responses (arbitrary area)*			
Run no.	**P**	**PIT**	**SQ**	**CHO**	**STIGMA**	**SITO**
1	15	300	533074	256678	26086	203884
2	25	300	266016	129530	28081	307752
3	15	320	243661	68125	15634	228463
4	25	320	619394	130411	39208	317875
5	13	310	144345	35634	10562	55672
6	27	310	564609	86172	27653	248173
7	20	296	432054	251892	34739	367687
8	20	324	447120	243771	35967	355433
9	20	310	442736	305696	41131	434368
10	20	310	636674	217635	41198	429873
11	20	310	625633	215662	41213	426638
12	20	310	641762	223333	41233	447763
13	20	310	635658	235535	42993	443278

Variables: IT - injector temperature, and P - pressure; Analytes: SQ - Squalene; CHO -Cholesterol; STIGMA - Stigmasterol; SITO - β-Sitosterol.

Table S4. Greenness evaluation of analytical methods for the article of 2019 and the new proposed method for a new sterolomic study

Category	Artigo 2019	Proposed method
Sample Preparation		
Collection	The microalgae are grown in reactor and then transformed into biomass (sample)	The microalgae are grown in reactor and then transformed into biomass (sample)
Preservation	Unnecessary	Unnecessary
Transport	Unnecessary	Unnecessary
Storage	Storage under normal conditions	Storage under normal conditions
Type of method: direct or indirect	Requires a extraction	Requires a extraction
Scale of sample preparation	Macroextraction	Microextraction
Solvents/reagents used	Non green solventes/ reagentes were used	Non green solventes/ reagentes were used
Additional treatments	Saponification	Saponification
Reagent and Solvents		
Amount	0.5 g of sample 15 mL of salt-satured solution 20 mL of hexane 0.2 mL of hexane: isopropanol (3:2) 20 mL of ethanolic KOH solution (10% w/v)	0.05 g of sample 1 mL of salt-satured solution (36%) 2 mL of hexane 0.1 mL of hexane: isopropanol (3:2) 0.5 mL of ethyl acetate 1 mL of ethanolic KOH solution (10% w/v)
Health hazard	Slight toxic and irritant	Slight toxic and irritant
Safety hazard	No special hazard	No special hazard
Instrumentation		
Energy (kWh)	0.67	0.10
Occupational hazard	The microtube was use closed	The microtube was use closed
Waste	55. 2 mL + 0.5 g of biomass	4.6 mL + 0.05 g of biomass
Waste treatment	Requires passivation	Requires passivation
Quantification	Yes	Yes

Table S5. Regression coefficients, *t* and *p*-values of PB design of sample preparation

Variables	Codes	Coefficient	Standard error	*t*-value	*p*-value
SQ	Ptemp	1465	900.31	1.63	0.13
	Ptime	3676	900.31	4.08	0
	Stemp	311	900.31	0.35	0.73
	Stime	-3044	900.31	-3.38	0.01
	KOH	322	900.31	0.36	0.73
CHO	Ptemp	1715	524.36	3.27	0.01
	Ptime	1538	524.36	2.93	0.02
	Stemp	232	524.36	0.44	0.67
	Stime	-2760	524.36	-5.27	0
	KOH	208	524.36	0.54	0.6
STIGMA	Ptemp	731	269.13	2.72	0.02
	Ptime	901	269.13	3.35	0.01
	Stemp	167	269.13	0.62	0.55
	Stime	-946	269.13	-3.52	0.01
	KOH	117	269.13	0.44	0.67
SITO	Ptemp	190	49.73	3.84	0
	Ptime	102	49.73	2.05	0.07
	Stemp	82.5	49.73	1.66	0.13
	Stime	-208	49.73	-4.19	0
	KOH	41	49.73	0.83	0.43

The variables were: PTemp: Pretreatment temperature, PTime: Pretreatment time, STemp: Saponification Temperature, STime: Saponification time, and KOH: KOH concentration; SQ: Squalene; CHO: Cholesterol; STIGMA: Stigmasterol; SITO: β-Sitosterol.

Table S6. Analysis of variance for the experimental results from sampre preparation, sterols and squalene.

	Sum of squares	df	mean square	*f*-value	*p*-value
Squalene					
Model	2.10E+10	7	3.00E+09	2.23E+01	< 0.0001
A-STime	1.11E+09	1	1.11E+09	8.29E+00	2.00E-02
B-PTime	1.04E+08	1	1.04E+08	7.80E-01	4.00E-01
C-PTemp	4.73E+08	1	4.73E+08	3.52E+00	9.00E-02
AB	2.01E+09	1	2.01E+09	1.50E+01	< 0.0001
AC	3.59E+09	1	3.59E+09	2.67E+01	< 0.0001
A^2	4.80E+09	1	4.80E+09	3.57E+01	< 0.0001
C^2	9.23E+09	1	9.23E+09	6.87E+01	< 0.0001
Residual	1.34E+09	10	1.34E+08		
Lack of Fit	1.23E+09	7	1.76E+08	4.60E+00	1.20E-01
Pure Error	1.15E+08	3	3.82E+07		
Cholesterol					
Model	2.88E+07	5	5.76E+06	6.91E+00	0.00E+00
A-STime	1.70E+06	1	1.70E+06	2.04E+00	1.80E-01
C-PTemp	2.87E+06	1	2.87E+06	3.45E+00	9.00E-02
AC	9.01E+06	1	9.01E+06	1.08E+01	1.00E-02
A^2	1.05E+07	1	1.05E+07	1.26E+01	0.00E+00
C^2	4.35E+06	1	4.35E+06	5.22E+00	4.00E-02
Residual	1.00E+07	12	8.33E+05		
Lack of Fit	9.29E+06	9	1.03E+06	4.38E+00	1.30E-01
Pure Error	7.07E+05	3	2.36E+05		
Stigmasterol					
Model	1.13E+06	2	5.64E+05	7.33E+00	1.00E-02
A-STime	3.32E+05	1	3.32E+05	4.31E+00	6.00E-02
A^2	1.05E+06	1	1.05E+06	1.36E+01	< 0.0001
Residual	1.15E+06	15	7.69E+04		
Lack of Fit	1.09E+06	12	9.05E+04	3.99E+00	1.40E-01
Pure Error	6.81E+04	3	2.27E+04		
β-sitosterol					
Model	1.23E+06	8	1.54E+05	2.43E+02	< 0.0001
A-STime	4.22E+03	1	4.22E+03	6.66E+00	3.00E-02
B-PTime	2.09E+03	1	2.09E+03	3.31E+00	1.00E-01
C-PTemp	3.59E+04	1	3.59E+04	5.68E+01	< 0.0001
AB	9.20E+04	1	9.20E+04	1.45E+02	< 0.0001
AC	1.33E+05	1	1.33E+05	2.10E+02	< 0.0001
BC	1.21E+05	1	1.21E+05	1.91E+02	< 0.0001
A^2	4.97E+05	1	4.97E+05	7.85E+02	< 0.0001
C^2	4.27E+05	1	4.27E+05	6.75E+02	< 0.0001
Residual	5.70E+03	9	6.33E+02		
Lack of Fit	4.66E+03	6	7.76E+02	2.25E+00	2.70E-01

Pure Error	1.04E+03	3	3.46E+02

The variables were: (STime: saponification time). (PTime: pretreatment time). and (PTemp: pretreatment temperature). SQ: Squalene; CHO: Cholesterol; STIGMA: Stigmasterol; SITO: β-Sitosterol.

Table S7. Analysis of variance for the sample introduction experimental results from sterols and squalene.

	Sum of squares	df	mean square	*f*-value	*p*-value
Squalene					
Model	2.47E+11	4	6.17E+10	5.03E+00	3.00E-02
A-P	6.18E+10	1	6.18E+10	5.04E+00	5.00E-02
B-IT	9.09E+08	1	9.09E+08	7.00E-02	7.90E-01
AB	1.03E+11	1	1.03E+11	8.43E+00	2.00E-02
A^2	8.07E+10	1	8.07E+10	6.58E+00	3.00E-02
Residual	9.80E+10	8	1.23E+10		
Lack of Fit	6.83E+10	4	1.71E+10	2.30E+00	2.20E-01
Pure Error	2.97E+10	4	7.42E+09		
Cholesterol					
Model	2.47E+11	4	6.17E+10	5.03E+00	3.00E-02
A-P	6.18E+10	1	6.18E+10	5.04E+00	5.00E-02
B-IT	9.09E+08	1	9.09E+08	7.00E-02	7.90E-01
AB	1.03E+11	1	1.03E+11	8.43E+00	2.00E-02
A^2	8.07E+10	1	8.07E+10	6.58E+00	3.00E-02
Residual	9.80E+10	8	1.23E+10		
Lack of Fit	6.83E+10	4	1.71E+10	2.30E+00	2.20E-01
Pure Error	2.97E+10	4	7.42E+09		
Stigmasterol					
Model	1.32E+09	5	2.64E+08	6.20E+02	< 0.0001
A-P	3.09E+08	1	3.09E+08	7.26E+02	< 0.0001
B-IT	7.27E+05	1	7.27E+05	1.71E+00	2.30E-01
AB	1.16E+08	1	1.16E+08	2.73E+02	< 0.0001
A^2	8.75E+08	1	8.75E+08	2.05E+03	< 0.0001
B^2	6.66E+07	1	6.66E+07	1.56E+02	< 0.0001
Residual	2.98E+06	7	4.26E+05		
Lack of Fit	3.86E+05	3	1.29E+05	2.00E-01	8.90E-01
Pure Error	2.60E+06	4	6.49E+05		
β-Sitosterol					
Model	1.66E+11	4	4.15E+10	2.07E+02	< 0.0001
A-P	2.71E+10	1	2.71E+10	1.35E+02	< 0.0001
B-IT	3.77E+07	1	3.77E+07	1.90E-01	6.80E-01
A^2	1.37E+11	1	1.37E+11	6.81E+02	< 0.0001
B^2	8.75E+09	1	8.75E+09	4.35E+01	0.00E+00
Residual	1.61E+09	8	2.01E+08		
Lack of Fit	1.29E+09	4	3.23E+08	4.05E+00	0.1021
Pure Error	3.19E+08	4	7.96E+07		

The variables were: (IT: injector temperature). and (P: pressure). SQ: Squalene; CHO: Cholesterol; STIGMA: Stigmasterol; SITO: β-Sitosterol.

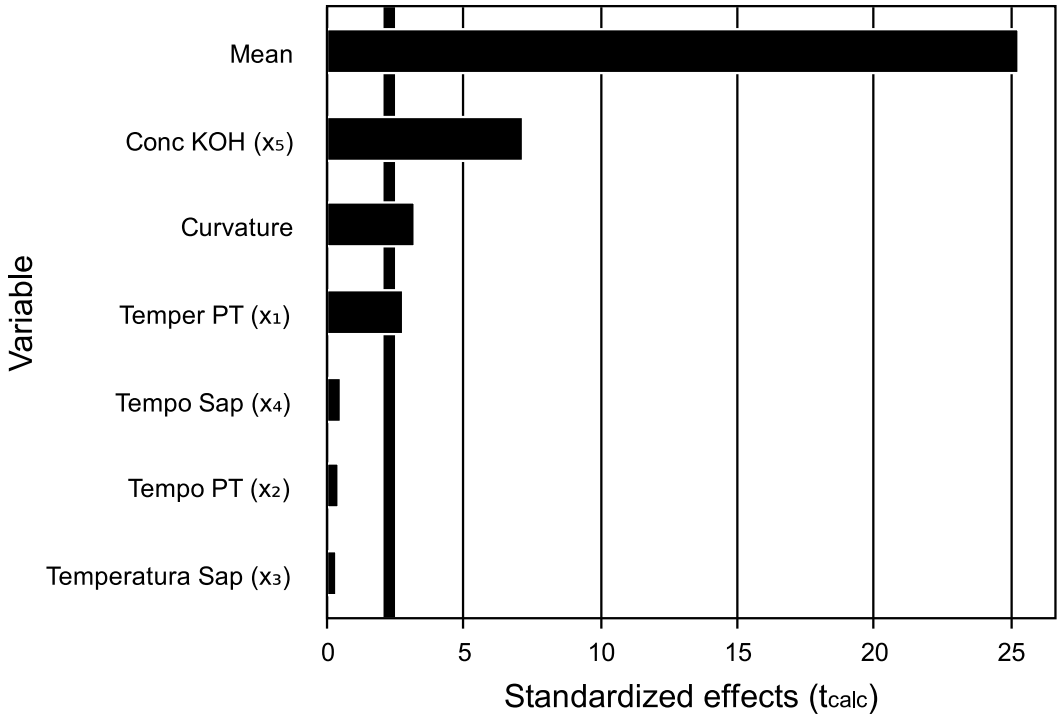


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Figure S1 Significant effects from KOH concentration at the clean-up in sample preparation.

7 CAPÍTULO 5

7.2 ARTIGO CIENTÍFICO 2

Phytosterol-rich compressed fluids extracts from Phormidium autumnale cyanobacteria with neuroprotective potential

Mariane Bittencourt Fagundes, Gerardo Alvarez-Rivera, Jose A.Mendiola, Mónica Bueno, José David Sánchez-Martínez, Roger Wagner, Eduardo Jacob-Lopes, Leila Queiroz Zepka, Elena Ibañez, Alejandro Cifuentes.

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Phytosterol-rich compressed fluids extracts from *Phormidium autumnale* cyanobacteria with neuroprotective potential

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ABSTRACT

Phormidium autumnale (*P. autumnale*) is a cyanobacteria with an unknown metabolic system, since only few studies have been reported. Among the produced metabolites, sterols profile is important considering the ability to this cyanobacterium in producing such compounds and their important benefits for human health. In this study, compressed fluid technologies were evaluated to obtain phytosterol-rich extracts from *P. autumnale* cyanobacteria for investigating their potential neuroprotective properties. A preliminary study was performed by comparing gas-expanded liquid extraction and supercritical fluid extraction using ethanol as a co-solvent. The following steps were optimized using a two-level factorial design and considering solvent composition and pressure as experimental factors. The bioactive potential of the optimized compressed fluid extract was tested using *in vitro* bioactivity assays, including acetylcholinesterase, lipoxygenase inhibition, and antioxidant capacity. The supercritical fluid multi-optimization response presented optimum phytosterol enrichment conditions at 266.3 bar of pressure and 7% of ethanol. Moreover, the optimized extract had higher *in-vitro* neuroprotective activity than the non-enriched extract, presenting the biochemical half maximal inhibitory concentration (IC_{50}) values of 65.80 μg mL^{-1} for acetylcholinesterase, 58.20 μg mL^{-1} for lipoxygenase inhibition, and 7.40 μg mL^{-1} for antioxidant activity. *In-silico* molecular docking analyses showed the specificity of sterol interaction with acetylcholinesterase active sites. These results provide evidence for further exploring *P. autumnale* as a source of bioactive phytosterols, demonstrating that compressed fluid technologies are powerful tools to obtain phytosterol-rich extracts.

1. Introduction

Neurodegenerative diseases (ND) may result from the progressive degeneration of the structure and function of the central nervous, and peripheral nervous systems. The leading cause of ND is aging, and as the lifespan of populations increases in developed countries, the prevalence of these diseases is also expected to increase. Alzheimer's disease (AD) is the main cause of dementia; it is associated with numerous phenomena, including neuroinflammation, extensive oxidative/nitrosative damage, and cognitive impairment [1,2]. Since the discovery of cholinergic deficit in AD, in function of the rapid hydrolysis of acetylcholine, the neurotransmitter, by acetylcholinesterase enzymes (AChE), their inhibition are being studied to increase the acetylcholine levels in the brain [3]. Typically, the inhibition is induced by synthetic drugs, such as physostigmine, galantamine, and huperzine, although these substances may have adverse effects, (*e.g.*, hepatotoxicity, bradycardia, and nausea) [2]. Therefore, developing alternative compounds or extracts from natural sources is paramount to preventing AD on set and development.

In ND research, phytosterols are important compounds since large amounts of circulating plant sterols can cross the blood-brain-barrier (BBB) [4] and act as AChE inhibitors [5]. Given the above, cyanobacteria may be potential sources of high-value chemicals in the nutraceutical and pharmaceutical industries [6,7,8].

Our research group recently extracted and established the sterolomic profile of heterotrophically cultured *P. autumnale*. In this study, different exogenous carbon sources, such as glucose, sucrose, and also

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slaughterhouse wastewater were employed in the cultivation process. Slaughterhouse wastewater showed in this previous study the most prominent result for sterols production, being selected for this study [9] The use of this cyanobacterium is quite advantageous from the industrial point of view, given its high production rates, also its capability to convert organic matter into valuable products, and its resistance to numerous external conditions, thus standing out from conventional non-renewable sources such as vegetable oil sources and nuts, which are dependent on seasonality.

Several methods have been used to obtain sterols, including lipid extraction and saponification, which is considered a clean-up step that only isolates non-saponifiable compounds [10] using organic solvents (*e.g.*, chloroform [11]). Nevertheless, these extractions use solvents that are not generally recognized as safe (GRAS). Additionally, one concern regarding microalgae extraction is the rigid cell wall hindering the mass transfer of intracellular compounds [12]. Thus, green extraction techniques such as supercritical fluid extraction (SFE) and gas-expanded liquid extraction (GXL), are alternatives that can isolate valuable compounds, reduce solvent use and associated hazards, and preserve molecular bioactivity [13]. These advantages are mostly associated to the low temperatures applied in the system, maintaining the chemical structure of these thermolabile compounds, as already observed by Uddin et al. [14] who described the effects of using SFE for phytosterol extraction. The major differences between these technologies (GXL, SFE) are the solubility of the metabolites since changing the solvent used alters the dielectric constant, reaching simultaneously different intermolecular bounds. Moreover, changes related to mass transfer are expected that depends on some factors including convection and diffusion, in which each molecule present a different response [15,16].

Gas-expanded liquid extraction has one advantage over SFE since it can employ a wide range of solvents and extract numerous compounds. For instance, more polar compounds are typically extracted when using ethanol as a solvent in GXL. However, SFE is considered the most environmentally friendly technique because it uses only CO_2, selectively extracting more apolar compounds, although a co-solvent must be used to increase the polarity of the metabolites extracted, the addition occurs in lower amounts [15].

The importance to study both technologies for phytosterols extraction is related to the fact that these metabolites can be found differently chemically bonded into cell membrane (*e.g.*, sterified, glycosylated or others), thus, it is necessary to understand the physico chemical proprieties changes for these compounds solubilization [14].

Given the aforementioned data, the present study aims to obtain phytosterol-rich extracts from *P. autumnale* cyanobacteria using compressed fluid technologies employing chemometric tools to optimize the extraction process. Hence, different compressed fluid technologies and pretreatments for cell hydrolysis were initially tested, and the neuroprotective potential of the extract with the highest enrichment was analyzed using different *in vitro* bioactivity tests, such as acetylcholinesterase (AChE), lipoxygenase inhibition (LOX), and antioxidant capacity (ORAC). Additionally, the phytosterol binding affinity for the structures of the active sites of the AChE amino acid residues was evaluated by *in silico* molecular docking simulations.

2. Materials and methods

2.1. Cyanobacterial biomass production

P. autumnale axenic culture was originally isolated from the Cuarto Cienegas desert (26°59′N, 102°03′W - Mexico). Cultures were propagated and maintained in a synthetic BG-11 medium prepared according to Rippika et al. [17]. The following conditions were established for the propagation: pH of 7.6, temperature of 25 °C, photon flux density of 15 mol^{-2} s^{-1}, and a 12/12 h dark/light cycle. Therefore, the strain was transferred for the cultivation performed heterotrophically, using slaughterhouse wastewater, as exogenous carbon source, due to its high sterols concentrations, as found in our previous study [9]. The carbon/nitrogen (C/N) ratio was adjusted to 30. The biomass was acquired in a bubble column bioreactor and the cultures were produced under a bath regime and fed with 2 L of medium. All experiments were conducted until 168 h of residence time. The samples were then separated from the cultivation medium by centrifugation for 10 min at 11200 ×*g* using a Hitachi (Tokyo, Japan), and subsequently freeze-dried for 24 h at −50 °C under −175 mmHg. The cultivations were performed twice.

2.2. Reagents

Conventional extractions were carried out using chloroform, hexane, heptane, methanol, potassium hydroxide (KOH), and sodium chloride (NaCl), which were obtained from Sigma–Aldrich (Missouri, United States). Ethanol (99.8%) (VWR Chemicals, Fontenay-sous-Bois, France), and CO_2 (99.9%; Carburos Metálicos, Air Products Group, Madrid, Spain) were used for green extractions. Total phenol assay required: Folin–Ciocalteu's phenol reagent (2 N) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent (99.9% purity), which were obtained from Sigma–Aldrich (Missouri, United States). For the bioactivity assays, the following compounds were used: 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), fluorescein, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) (Derbyshire, United Kingdom), randomly methylated β-cyclodextrin (RAMEB) from TCI Chemicals (Tokyo, Japan), acetone, potassium dihydrogen phosphate (KH_2PO_4), quercetin, tris hydrochloride, acetylthiocholine iodide (ACth), 4-fluoro-7-sulfamoylbenzofurazan (ABD-F), and galantamine from TCI Chemicals (Tokyo Japan). The standards used were: (fucoxanthin; purity >99%), (chlorophyll *a*; purity >90%), (chlorophyll *b*; purity >95%), cholesterol (CHO) (5-cholesten-3β-ol; purity >95%), β-sitosterol (SITO) (5-stigmasten-3β-ol; purity >79.7%), linoleic acid (purity >99%), gallic acid (99.9% purity), and ascorbic acid (99.9% purity), which were all obtained from Sigma–Aldrich (Missouri, United States). The enzymes lipoxygenase (LOX) and acetylcholinesterase (AChE) were purchased from Sigma–Aldrich (Missouri, United States).

2.3. Conventional extractions

Conventional extractions were performed to compare global yields. In this study, standard saponification and heptane extraction were carried out. For comparison, the extractions were performed with the same amount of biomass (100 mg). Heptane (Treatment 1 - T1) extraction was done by adding 4 mL of heptane for 24 h at 20 °C, and the slurry was mixed in a thermomixer (Eppendorf AG, Hamburg, Germany), as described by Bueno et al. [18]. Heptane extraction is considered a greener solvent compared to others [19], therefore, a standard saponification procedure (Treatment 2 - T2) was also investigated.

The T2 was conducted by direct biomass saponification without lipid extraction [20]. Chloroform (0.5 mL) was added to the cyanobacteria and mixed for 90 min, followed by an ethanolic KOH solution (1 mL; 6% w/v). Fast saponification was then performed in a thermomixer for 6 min. Afterward, a salt-saturated solution was added to finish the saponification step. The partition was achieved with 4 mL of hexane; the entire extraction process was performed at 25 °C. The fractions corresponding to those with esterified sterols were isolated once converted into their free form. The T1 and T2 extracts were then submitted to derivatization for its sterolomic profile (see Section 2.5), for gas chromatography analysis. For the global yield (%) (X_0), the liquid extracts were dried in an evaporator under nitrogen flows and, after completely dry, they were weighed. Therefore, the equation was determined by a relationship between the extracted mass (Em) and cyanobacterial biomass used (100 mg; mc), as observed in Eq. (1).

$$X_0 = \frac{Em}{mc} 100 \quad (1)$$

After the extractions, the extracts were preserved in sealed dark vials at −18 °C until further analysis.

2.4. Preliminary studies using green compressed fluid technologies: gas-expanded liquids extraction (GXLs) and supercritical fluid extraction (SFE)

Preliminary studies were conducted to select the best compressed fluid extraction technique: GXL (carbon dioxide expanded ethanol, CXE) or SFE with ethanol as co-solvent for phytosterol isolation. The conditions used in this study for SFE and CXE were adapted from Abrahamsson et al. [21] and Gilbert-López et al. [22], respectively. Ethanol was selected as co-solvent due to the prominent results obtained in the literature for sterols extraction [23,24]. Extractions were carried out using the same equipment that consisted of a homemade compressed fluid extractor coupled to a high-pressure pump (PU-2080 Plus CO_2; Jasco, Hachioji, Japan) and solvent pump (PU-2080; Jasco Plus, Hachioji, Japan), as depicted in Fig. 1. For extraction, 100 mg of *P. autumnale* mixed with 2 g of sea sand (0.25–0.30 mm, Panreac, Barcelona, Spain) were used. The collection vessel was weighed before and after to determine the yield (Eq. (1)). The flow rate was established at 4 mL min^{-1}.

The SFE with ethanol as co-solvent was performed using 0.4 mL min^{-1} of EtOH and 3.6 mL min^{-1} of CO_2 (10% of ethanol) while operating at 275 bar and 50 °C. To determine the extraction time, a kinetic study was performed for 200 min collecting fractions every 20 min in 15-mL falcon tubes placed in an ice bath and protected from light to avoid degradation.

The CXE extracts were collected in 100-mL bottles and followed the same sample protection procedure. The extraction cell temperature was set at 50 °C. The co-solvent pump was operated at 70 bar, obtaining mixtures of 50% (w/w) of ethanol. A kinetic study was also performed, collecting fractions every 5 min for a total extraction time of 50 min. The solvents from all extracts were removed under a stream of N_2. Each extraction was done in duplicate. The extraction yield (%) was calculated according to Eq. (1). After the extractions, the samples were preserved in sealed dark vials at −18 °C until further sterol analysis.

2.5. Pretreatment before extraction

Four PT were chosen for cyanobacterial cell disruption after selecting the green compressed technology. Cyanobacteria were prepared at a concentration of 100 mg mL^{-1} in deionized water, except for bead-beating (BB). After the PT time evaluation and cell disruption observation in the microscope, the extracts were freeze-dried for further sterol analysis by applying the T1 extraction (heptane extraction). The PT procedures were as follows:

1) Microwave heating (MW) at 400 W (Saivod-MSG1428, Madrid, Spain) was performed according to Silva et al. [25], with modifications, using deionized water as a solvent and different cycles (5, 15, 30, and 60 s).
2) Ultrasonic bath (US) (Elma D-78224, Singen, Germany) using parameters adapted from Joyce et al. [26], being the temperature of 25 °C, 40 kHz, and 45 min of sonication;
3) Freeze and thaw (F/T) was performed in a frost-free freezer with air circulation for three 30-min cycles at −18 °C. Thirty minutes were necessary for the sample to reach the temperature.
4) Bead-Beating (BB) was carried out in cryogenic milling (Mixer Mill CryoMill Retsch, Haan, Germany) and three cycles, as described by Castro-Puyana et al. [27], which consisted in the use of dried biomass, on following conditions: pre-cooling, grinding, and intermediate cooling, being the frequencies: 1/s (5 during 2 min), 1/s (20 during 5 min), and 1/s (5 during 1 min), respectively.

2.6. Co-solvent supercritical carbon dioxide (SC-CO_2) optimization

For sterols response optimization, a previous selection of the best pressurized technology and pretreatment was done. Subsequently, the SFE conditions for *P. autumnale* biomass extraction were optimized. For this purpose, two experimental factors at three levels were selected: extraction pressure (200, 275, and 350 bar) and ethanol percentage (0, 6, and 12%), whose ranges were established based on previous research [14] and includes the screening conditions (Section 2.5). Factorial 2^3 design was constructed, and the experimental design involved 11 runs conducted in a randomized order (Table 1), including two axial points and three experimental replicates in the central point. Response variables, which corresponded to sterol abundance in the extracts, were

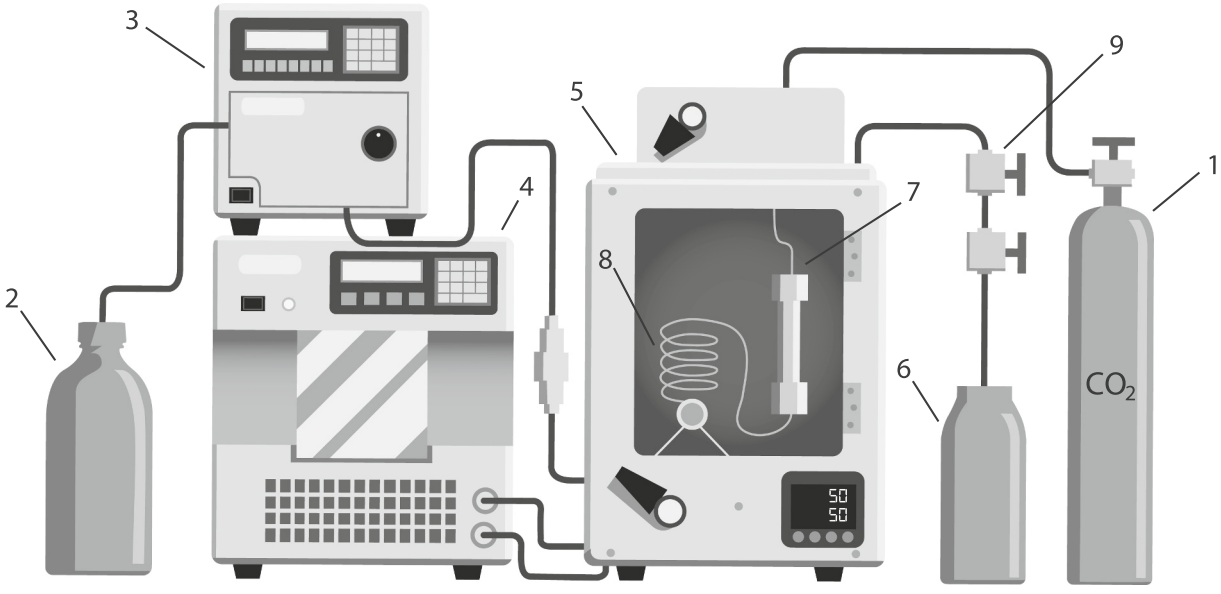

Fig. 1. A representation of the supercritical fluid extractor responsible for both green extractions: carbon dioxide expanded liquid and supercritical fluid extraction. The scheme of the extractor is as follows: (1) CO_2 cylinder; (2) Ethanol solvent bottle; (3) High-pressure liquid pump; (4) High-pressure CO_2 pump; (5) Oven with temperature control; (6) Collection flask; (7) Extraction cell; (8) Microfluid mixer with a serpentine; (9) micro-metering needle valves.

Table 1
Experimental factorial design conditions for supercritical fluid dioxide extraction optimization. Levels of selected variables: Pressure, and the percent of ethanol as co-solvent, and experimental responses based on the sterol abundance in the extracts.

Run no.	Variables		Experimental responses[a]				
	Pressure (Bar)	EtOH (%)	STIGMA	SITO	CHO	Total sterol	Total Phyto
1	200	0	107.1[b]	0.9	91.5	199.5	144.2
2	350	0	83.4	0.5	15.6	99.5	88.2
3	200	12	98.9	10.0	15.6	124.4	84.0
4	350	12	93.9	4.3	46.0	144.2	109.2
5	200	6	130.9	9.4	42.6	182.9	136.3
6	350	6	117.0	6.3	47.6	170.9	122.3
7	275	0	142.3	0.9	122.5	265.7	194.6
8	275	12	140.8	10.3	51.9	203.0	147.1
9[c]	275	6	171.5	11.1	145.9	328.5	186.0
10[c]	275	6	172.5	9.8	120.6	302.9	180.5
11[c]	275	6	173.5	8.5	95.3	277.3	175.0

[a] Sterols found in *P. autumnale*: STIGMA: Stigmasterol; SITO: Sitosterol; CHO: Cholesterol; Total Phyto: Total Phytosterols.
[b] Sterols arbitrary area ($\times 10^4$): arbitrary units of chromatography area/10000.
[c] Central point of the experimental design.

expressed in arbitrary area ($\times 10^4$). Experiments were also done using 100 mg of *P. autumnale* biomass mixed with sand (2 g). Extraction temperature was maintained at 50 °C due to compound thermosensitivity. A kinetic study of the extraction process at the central conditions of the experimental design was carried out to select the extraction time, which was set to 120 min.

Extracts were collected in 15-mL falcon tubes, transferred to a previously weighed glass vial, and evaporated using a gentle nitrogen stream (N_2). For the yield calculation, the vials were weighed in triplicate. The thermophysical properties of the supercritical CO_2 systems were calculated using the software NIST Chemistry Webbook [28]. After the optimum point acquisition this treatment (Treatment 3 - T3) was compared with the conventional methods in terms of sterols, and bioactivity assays.

2.7. Sterol determination by gas chromatography coupled to high-resolution quadrupole time-of-flight mass spectrometry (GC-q-TOF/MS)

For the sterolomic profile, the samples were derivatized according to the method described by Fienh [29]. The cyanobacterial extracts (10 μL) were dried with a nitrogen stream at 25 °C. The residue was re-dissolved in 10 μL of methoxamine hydrochloride (40 mg mL^{-1}) in pyridine and vortexed for 1 min, derivatized at 60 °C for 15 min, and performed under agitation using a thermomixer (Eppendorf AG, Hamburg, Germany). Then, 90 μL MSTFA (*N*-methyl-N-(trimethylsilyl) trifluoroacetamide) with 1% TMCS (trimethylchlorosilane) was added, followed by 2 μL of d27-myristic acid. The mixture was shaken at 750 rpm for 30 min at 37 °C (Eppendorf AG, Hamburg, Germany), and 1 μL of the derivatized extracts were injected in split mode (10:1) into a gas chromatography–mass spectrometry (GC-q-TOF/MS) (Agilent 7890B GC/7200 q-TOF/MS) equipped with an electronic impact (EI) ionization interface, at 280 °C. Helium at constant pressure (15 psi) was used as the carrier gas. Sterol separation was performed in a non-polar column DB5-MS + 10 m Duragard Capillary Column (30 m × 250 μm × 0.25 μm) (Agilent technologies, 122-5532G).

The temperature program was initially 60 °C and increased to 325 °C at 10 °C min^{-1}, maintaining isothermal conditions for 10 min. The temperature of the detector was set at 250 °C. The transfer line and quadrupole temperatures were 290 and 150 °C, respectively. The EI ionization source was operated at 70 eV, scanning from *m/z* 50 to 600 amu 5 spec/s at 250 °C.

After the mass spectra deconvolution and the tentative unknowns, identification was performed for minor sterols by Agilent Mass Hunter Unknown Analysis tool and mass spectral databases (NIST MS Search, and Fiehn Library). Quantification was performed for cholesterol (CHO), stigmasterol (STIGMA), and β-sitosterol (SITO) by an external calibration curve. The curve was prepared in a concentration ranging from 5 to 200 μg mL^{-1}. The standards were first diluted in ethanol, and 10 μL were transferred, being prepared in the same way as the samples by adding MSTFA (*N*-methyl-N-(trimethylsilyl) trifluoroacetamide) with 1% of TMCS (trimethylchlorosilane). The sterols were expressed as μg g^{-1} of dried biomass.

2.8. Anti-cholinergic (AChE) activity

AChE inhibitory capacity of all extracts was based on Ellman's method, which consists of fluorescent enzyme kinetics [30], and some adaptations, according to Sánchez-Martinez et al. [31]. Briefly, the extracts were diluted in water-ethanol (50:50, v/v), 100 μL Tris-HCl buffer at pH 8 and 25 μL of AChE diluted in buffer (0.8 U/mL), and then incubated for 10 min. Then, 25 μL of 125 μM ABD-F in the buffer and 50 μL of 1.4 mM ATCI in water were added to each well. Excitation and emission wavelengths (389 nm and 513 nm, respectively) were recorded in a fluorescence reader (Synergy HT, BioTek Instruments, Winooski, VT, USA) Gen5TM (version 2.0), and recorded every minute for 10 min at 37 °C. Readings were performed at λ excitation 389 nm and λ emission 513 nm every min for 10 min at 37 °C. Data analysis was performed using the software.

2.9. Lipoxygenase inhibitory capacity

The LOX inhibition activity was measured by fluorescence assay based on enzyme kinetics and according to the method by Whent et al. [32] and adapted by Sánchez-Martinez et al. [31]. The assay solution consists in 100 μL of extract sample at the different concentrations (100 μg–1000 μg mL^{-1}) in EtOH/H2O (1:1, v/v), 75 μL of fluorescein (1 μM) in buffer (150 mM Tris-HCl pH 9), 60 μL of LOX 208 U/μL in buffer and Linoleic acid, in a concentration studied in KM, prepared in EtOH/H2O (1:1, v/v), in each well. The reference inhibitor used was Quercetin. KM is the concentration of substrate which permits the enzyme to achieve half Vmax, and their value was measured by mixing 100 μL of LA (6.5 mM) in EtOH/H_2O (1:1, v/v), 100 μL of EtOH/H_2O (1:1, v/v), 75 μL of fluorescein (1 μM) in buffer and 60 μL of LOX 208 U/μL in buffer, in each well. Fluorescence was measured at λ excitation of 485 nm and λ emission of 530 nm every min for 15 min at 25 °C. Readings were measured using the software Gen5TM (version 2.0).

2.10. Oxygen radical absorbance capacity (ORAC)

The ORAC method was carried out according to Ou et al. [33], with some modifications. The extract was prepared in the 10–167 μg mL^{-1} range using a randomly methylated β-cyclodextrin (RMCD) to increase lipophilic antioxidant solubilization. For this reason, a prepared solution containing 7% of RMCD in 50% of acetone: H_2O (w/v) was used; this mixture was found to sufficiently solubilize the extracts. Afterward, 25 μL of fluorescein (0.012 μM) were added (prepared in the same buffer solution) in a 96-well microplate (SPL Life Science, Gyeonggi-do, Korea) and incubated for 10 min at 37 °C using a microplate reader (Gen5TM version 2.0). Then, 25 μL of AAPH solution (150 μmol L^{-1}) were added as a peroxyl radical generator, and the fluorescence measurement was monitored every 5 min at 37 °C for 1 h. The antioxidant activity was determined through the area under the curve of the fluorescence decay, and the results were compared to the standard curve of Trolox and expressed as μmol of Trolox equivalent per grams of biomass extract.

2.11. Total phenols determination

Total phenolic content (TPC) was estimated according to Singleton &

Rossi [34] and based on the Folin–Ciocalteu assay, with some modifications. First, ultrapure water (600 μL) was added and mixed with the extract solution (10 μL; 10 mg mL^{-1}). Then, the Folin–Ciocalteu reagent was added (50 μL) (Merck, Darmstadt, Germany). The reactional medium was then left to rest for 1 min. Afterward, 150 μL of 20% (w/v) sodium carbonate Na_2CO_3 was added and the final volume of the reactional medium was adjusted to 1 mL of water. The mixture was agitated and incubated for 2 h at room temperature in the dark. Later, 300 μL of each reaction mixture was transferred to a 96-well microplate. The readings were performed in the software Gen5TM (version 2.0), and absorbance was measured at 760 nm. A standard curve of gallic acid was employed in the range of 0.031 to 2.00 mg mL^{-1}, and used for calibration. Data were expressed as mg gallic acid equivalents (GAE) g^{-1} extract. Results were presented as the average of triplicate analyses.

2.12. Total carotenoid and chlorophyll determination

The total chlorophyll and carotenoids were determined by a spectrophotometric method described by Gallego et al. [35]. All T1, T2, and T3 extracts were dissolved in methanol at a concentration of 0.05 mg mL^{-1}. The absorbance of the solutions was recorded at two specific wavelengths (470 and 665 nm) for the carotenoids and chlorophylls, respectively. External standard calibration curves of fucoxanthin (0.5–10 μg mL^{-1}) and chlorophyll-a (0.5–7.5 μg mL^{-1}) were used to calculate the total carotenoid and chlorophyll content, respectively. Total carotenoids were expressed as μg carotenoids g^{-1} of dried biomass by interpolating the absorbance of the extract at 470 nm in the fucoxanthin calibration curve. For chlorophyll determination, the results were obtained by interpolating the absorbance of the extract at 665 and 652 nm in the calibration curve of chlorophyll-a plus chlorophyll-b, and the results were expressed as μg chlorophyll g^{-1} of dried biomass.

2.13. Molecular docking studies

An *in silico* molecular docking study between stigmasterol and galantamine ligands and human acetylcholinesterase (AChE) was performed using 1° A Samson 2020 (software for adaptive modeling and simulation of nanosystems) and Autodock Vina (free software available at http://vina.scripps.edu) [36]. The AChE crystal structure was acquired from the protein data bank (PDB ID: 4EY6). In order to prepare the protein for the docking model, co-factors (ligands, water, and chain B) were removed using the software Chimera (version 1.14). Moreover, the three-dimensional structures of the ligands were obtained from PUBCHEM (https://pubchem.ncbi.nlm.nih.gov/). The first step of the docking simulation's was the grid box generation to obtain the best protein binding site. For our model, 44 × 45 × 90 Å was applied, and the docking was performed by applying the empirical free energy function together with the Lamarckian genetic algorithm. The visualization and data export was performed using the software Discovery Studio (v.20.1.0), and the results are expressed in energy affinity, being the energetically lowest conformations the most desirable, indicating a larger binding affinity with the enzyme.

2.14. Statistical analyses

Data sets obtained from the pretreatment screening were evaluated using one-way analysis of variance (ANOVA) and Tukey's *post hoc* analysis ($p > 0.05$). Data normality was verified by using the Shapiro-Wilk test, and trends were considered significant only when the means were different. All the statistical analyses were performed by Statistica 7.1 (StatSoft Inc., Oklahoma, United States). The surface response was constructed with the Design Expert 11.0 software to generate the overlapped contour plots for the optimization process.

The models were validated by the analysis of variance (ANOVA) at the 95% confidence level. The optimum conditions for phytosterols were then determined by examining the response contour graphs and using the multi-criteria response technique using a desirability value (DV), which was established for each response combined into their recommended global desirability function according to Derringer & Suich [37]. The individual desirability was defined to maximize phytosterol concentrations in the extract. Data treatment was carried out using the Design Expert 12.1 (Minneapolis, United States) software. The predicted conditions were submitted to experimental verification and carried out in triplicate. A principal component analysis (PCA) was applied to the data obtained for the optimized method (T3) and conventional extractions (T1 and T2). The data were auto-scaled before performing PCA, using R Studio (v. 3.4.4), with Factomine R package (v.1.34).

3. Results and discussion

3.1. Green compressed fluid technology: a comparison between GXL and SFE with co-solvent for phytosterols isolation

A preliminary study was carried out to select the most appropriate technology to obtain extracts with the highest phytosterol concentrations. Hence, carbon dioxide expanded ethanol (CXE) and supercritical fluid extraction (SFE) plus ethanol as co-solvent were studied for their potential extractability by evaluating the total yield of extract (%) and sterol. An increase of ethanol percentage from SFE with co-solvent to the range of gas expanded ethanol may lead to higher phytosterol extraction because ethanol percentage and pressure modify solvent properties. This preliminary comparative study is essential due to the lack of data on the effects of ethanol percentage on cyanobacterial matrix.

According to Jafarian et al. [38], SFE is more selective for apolar compounds, although the authors noted that the highest percentage of ethanol (5% wt) used as co-solvent produced the best phytosterol extraction. The temperature used for both technologies was the same. To the best of our knowledge, no other study has attempted to extract phytosterols from cyanobacteria using compressed fluids, and our choice of 50 °C was based on CXE and SFE data from microalgae [22]. In addition, Temelli et al. [39] reported that phytosterol solubility in CO_2-expanded ethanol increased with temperature, although the authors only checked until 35 °C.

The details from CXE and SFE + ethanol conditions are described in Section 2.5. Both technologies were compared to two conventional extraction procedures: heptane extraction (T1) and saponification (T2), as described in Section 2.3. The CXE (employing 50% of ethanol) presented a better extraction yield (18.83 ± 3.84%) compared to SC-CO_2 with co-solvent (10% of ethanol) presenting 7.27 ± 3.43% (Fig. 2A). This result supports the idea that ethanol in gas expanded condition favors matrix penetration, showing better intracellular compound extraction [40]. Indeed, not only ethanol but other solvents also increase microalgae yields, as observed for *Spirulina* [35] and *Scenedesmus* [41]. Reyes et al. [42] also described similar behavior for *Haematococcus pluvialis* and reported increases of up to 75% in global yields.

Compared to traditional extraction with heptane (T1) and standard saponification (T2) procedures, no differences in yields were obtained by SC-CO_2 with co-solvent (10% of ethanol). Nonetheless, similar yields were acquired, and conventional procedures use more toxic extraction solvents, such as chloroform, that should be avoided compared to the SFE-based approaches. Conventional extractions typically demand longer analysis times and may result in losses by oxidation/degradation from the target analytes. With regards to compressed fluids, solvent extractions present high viscosity, which leads to low intracellular metabolite extractions [43]. Martins et al. [44] evaluated 2.5–5% of ethanol addition and noted that 5% was responsible for 91% of sterol extraction, showing that ethanol percentage is the most important factor for sterol extraction.

The kinetic behavior of the SFE and CXE extraction procedures is shown in Fig. 2B. For SFE using ethanol as co-solvent (10%), the maximum extraction yield was achieved at 120 min, whereas CXE reached stabilization at 30 min. Although the global extraction yield is

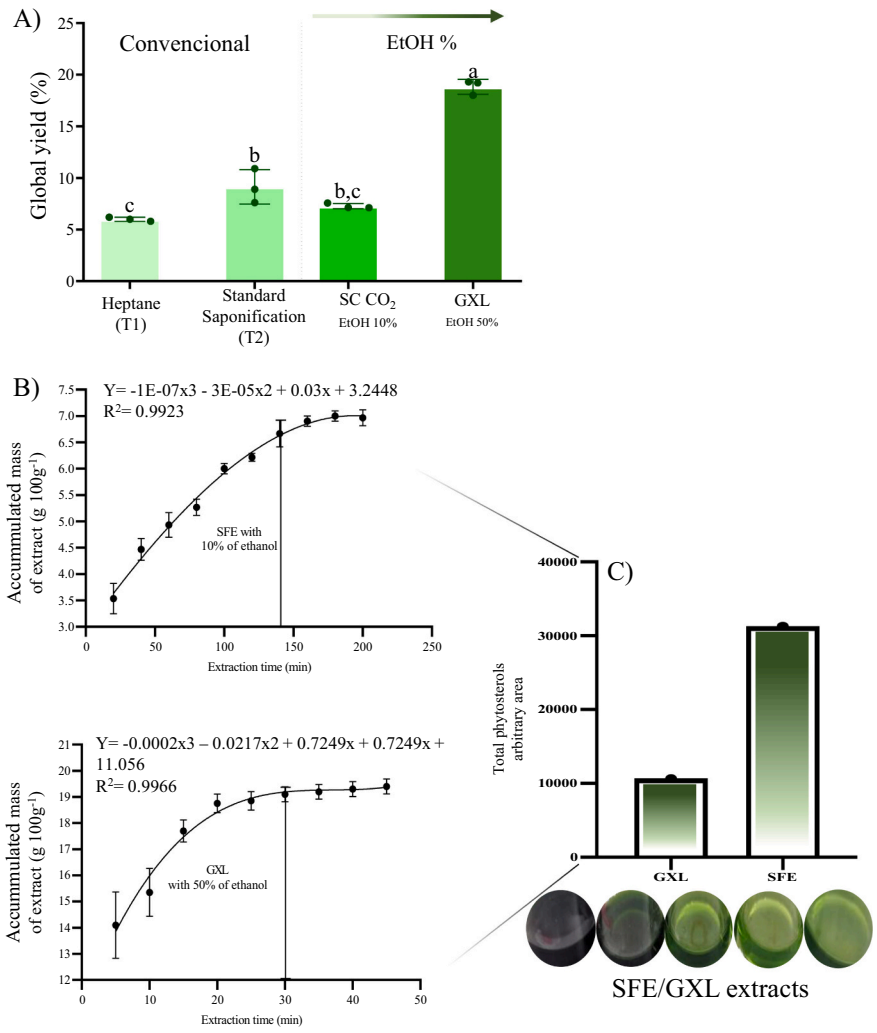

Fig. 2. Preliminary studies using green compressed fluid technologies applied to *P. autumnale*, global yield (%) from conventional techniques: Heptane extraction (T1) and standard saponification (T2) and from compressed fluid technologies: Gas expanded with 50% of ethanol (GXL $_{EtOH\ 50\%}$), and supercritical carbon dioxide extraction with 10% of ethanol as co-solvent (SC $CO_{2\ EtOH\ 10\%}$) (A), Kinect behavior of the extraction yield (g 100 g^{-1} of biomass), accumulated mass of extract regarding the initial dry amount of microalgae biomass for SC $CO_{2\ EtOH\ 10\%}$, and GXL $_{EtOH\ 50\%}$ (B), and total sterol arbitrary area from both compressed technologies GXL $_{EtOH\ 50\%}$ compared to SC $CO_{2\ EtOH\ 10\%}$ (C). Error bars are standard deviations based on $n = 3$ extraction replicates, means the same letter are not significantly different based on Tukey's HSD test ($\alpha = 0.05$).

higher for CXE than for SFE (and the extraction time is shorter), the total amount of sterols recovered from the biomass using SFE + ethanol is around 30 times higher than the CXE extract (Fig. 2C). This increase in sterol concentration was also observed by Sharif et al. [45]. This phenomenon is explained by the changes in the polarity of the compressed fluids due to modifications in their composition [40]. In our case, SFE with a higher CO_2 proportion increased non-polar compound extraction, including sterols.

Considering the CO_2/Ethanol mixture density, as stated by the Soave-Redlich-Kwong equation of state, SFE with co-solvent density is 792.2 kg·m^{-3}, while for CXE, the value obtained is 732.8 kg·m^{-3}. This lower density obtained by CXE may be another factor responsible for low phytosterol solubilization. Additionally, the solvent-matrix contact time may be another factor since sterols are commonly found esterified at the cellular matrix, making long solvent contact times necessary. Temelli et al. [39] proved these theories using different ethanol concentrations for sterol recovery and showing that low ethanol amounts can solubilize phytosterols. Hence, SFE was selected as the best choice to follow with the phytosterol rich extract optimization.

3.2. Cyanobacterial cell disruption pretreatments

To improve phytosterol recovery, different sample pretreatments, including ultrasonication (US), microwave (MW), freeze/thaw (F/T), and bead beating (BB) were tested to promote the disruption of the cellular structures. In Fig. S1 (Supplementary material), microscope optic images show the fragments released from cyanobacterial cells after the disruption treatments, proving the cell fragmentation; the treatment applied after the disruption method was the conventional method (T1) using heptane. The control sample (no pretreatment applied) images show the filamentous structure of cyanobacterial cells without any fragmentation. Furthermore, F/T was performed in 3 cycles of 30 min at a temperature of −18 °C. Fragmentations were observed after the third cycle. The US treatment was visualized every 10 min, and the best sonication time was selected at 40 min due to the number of generated fragments. The fastest PT technology was MW, which showed good disruption results at 60 s, followed by BB, which consisted of 3 cycles of 5 min.

Total extract yield (%) and total phytosterol concentrations (μg g^{-1} dried biomass) were determined for each sample pretreatment procedure (Fig. 3). In terms of extract yield, BB showed higher values (11%) than the other PT. The total extract yield of the tested PT decrease as follows BB > US > F/T > MW. Moreover, BB had the highest phytosterol concentration (1323.00 μg g^{-1}), being more than 7 times higher than the control sample (175.40 μg g^{-1}). Notably, BB is well known for achieving good results, as it extracts metabolites at low temperatures and processing times [46]. Hence, BB was chosen as a previous step before the extraction process optimization. The cell disruption images after BB pretreatment can be observed in Fig. S2 (Supplementary material).

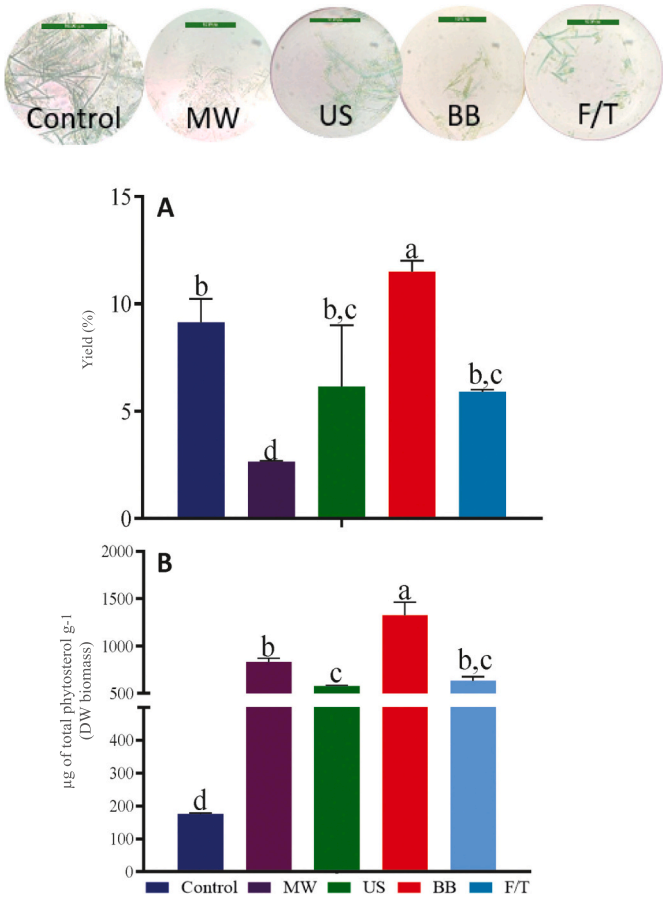

Fig. 3. Pretreatment methods selection applied to *P. autumnale* biomass, control: without cell disruption; US: ultrasonication (40 kHz, 40 min, and 25 °C); MW: microwave (400 W, for 60 s); F/T: Freeze/Thaw (three cycles at −18 °C), BB: bead beating (steel spheres in cryogenic grinding, conditions: pre-cooling 1/s (5 for 2 min), grinding 1/s (20 for 5 min), and intermediate cooling 1/s (5 for 1 min). Global yield of extract (g 100 g^{-1} of dried biomass) (A), and Total phytosterol (µg g^{-1} dry weight biomass) extraction efficiencies (B) from each treatment. Error bars are standard deviations based on n = 3 extraction replicates, means with the same letter are not significantly different based on Tukey's HSD test (α = 0.05).

3.3. Sterol extraction optimization

For the response surface methodology (RSM) the extraction temperature was set at 50 °C to avoid phytosterol degradations, as suggested in previous studies for stigmasterol and β-sitosterol molecules [47,48]. Regarding the pressure, Marzorati et al. [49] described that pressures up to 300 bar are important to achieve better bioactive compound extraction from cyanobacteria. For this, higher pressures were also chosen to enable triterpenoid structure solubilization. Imbimbo et al. [50] reported a working pressure of 350 bar for bioactive intracellular metabolite extraction from *Galdieria phlegre* cyanobacteria, while for *Arthrospira platensis* and *Arthrospira maxima*, pressures of 300 and 350 bar were required [51,52].

Individual responses corresponding to target major sterols in the *P. autumnale* biomass (STIGMA, SITO, and CHO) were evaluated. Analysis of variance for the different response variables showed no lack of fit ($p > 0.05$); hence, the models obtained were well fitted to our data (Table S1, Supplementary material). Each variable presented different behavior; for instance, stigmasterol extraction was significantly affected by the linear and quadratic effects of pressure (P), the quadratic effects of ethanol (EtOH), and the P × EtOH interaction. For β-sitosterol, all factors were significant except P × EtOH interaction, while cholesterol was only affected by P^2. Fig. S3 (Supplementary material) shows the Pareto charts and surface responses obtained for the different response variables studied.

The highest abundance obtained considering the sum of stigmasterol and β-sitosterol (total phytosterols) was observed in run 4, as shown in Table 1 (275 bar and 6% of EtOH, central point). Nevertheless, cholesterol showed a different behavior, with higher concentrations in run 9 (275 bar of pressure, 0% of EtOH). The predicted values for STIGMA, SITO, and CHO showed satisfactory correlation with the experimental data obtained (Fig. S4, Supplementary material).

The desirability function was built to optimize all the responses simultaneously. As shown in Fig. 4A, multiple response optimization was calculated to obtain a maximum concentration of stigmasterol and β-sitosterol. The desirability function reached a value of 0.92, which is very close to the highest possible value (1) for the model fitting (Fig. 4B). The profile for the predicted values of the target compounds can be observed in Fig. 4C, highlighting that the values for the factors that maximize phytosterol extraction were predicted close to the central point (266.3 bar and 7% EtOH); Table 2 shows the match between experimental and predicted values for sterols achieved under optimum SFE conditions (T3).

Comparing the results obtained using conventional extraction protocols such as heptane (T1) and saponification (T2) with SFE under optimum conditions (T3), SFE provided not only the safest and most sustainable process but also the highest sterol concentration (2093.4 µg g^{-1} for stigmasterol µg g^{-1}, and 243.5 µg g^{-1} for β-sitosterol), as shown in Fig. 5. In another study, *P. autumnale* using US-assisted extraction recovered 734.30 µg g^{-1} of total phytosterols [9], which is three times lower than the developed SFE method with BB pretreatment. Phytosterol extraction yields reported in the literature are substantially lower ranging from 23.90 to 83.40 µg g^{-1} [53], than the yields achieved with our optimized SFE method. According to Martins [44], high pressures and 5% ethanol were the optimum conditions for stigmasterol extraction; no influence of the extraction temperature was observed.

Our results demonstrate the importance of an SFE optimization process for sterol enrichment, showing a significant increase in sterol concentration, specifically in stigmasterol content, for *P. autumnale* cyanobacteria.

3.4. Chlorophyll, total phenol, carotenoid content, and phytosterol profiling analysis

The content of several groups of bioactive phytochemicals, including chlorophylls, carotenoids, polyphenols, and phytosterols, was obtained under optimum supercritical fluid extraction conditions (T3) and compared to the conventional treatments (T1 and T2). As shown in Table 3, higher chlorophyll and total phenol concentrations were obtained in T3 compared to conventional treatments, except for total carotenoids, which also presented a similar concentration in T2 (916.4 µg g^{-1} of dried biomass). This behavior was expected because saponification eliminates interference and has a selective unsaponifiable lipid fraction determination. Therefore, as already discussed in the optimization section, total sterols presented a notable increase of 92% regarding T2 and 34% regarding T1.

A comprehensive GC-q-TOF/MS analysis of the optimized *P. autumnale* SFE extract revealed eleven phytosterols (Fig. S5, Supplementary material), the biosynthetic precursor squalene, and α-tocopherol. The identification was based on the positive match of experimental mass spectra with theoretical MS data in the NIST MS database. The identified terpenoids, including retention time, match factor given by the NIST database, molecular formula, exact mass, calculated mass error, and MS/MS fragments, are summarized in Table 4. These compounds were positively identified in their derivatized form (trimethylsilyl derivatives) by characteristic [M-H + TMS] ions, except for squalene as it does not undergo derivatization. Cholesterol, stigmasterol, and β-sitosterol were the major compounds previously used for

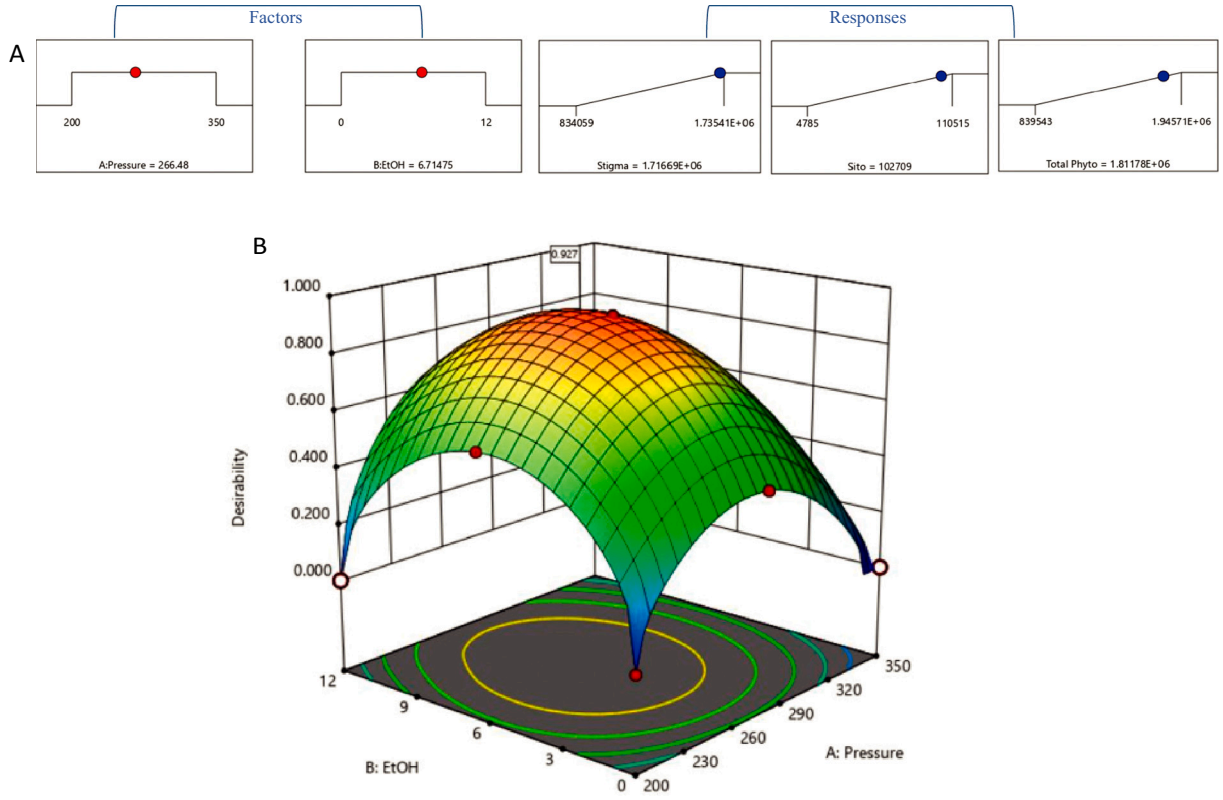

Fig. 4. Desirability function graph, the effects of the factors: pressure, and ethanol (EtOH) on supercritical carbon dioxide phytosterols extraction applied to *Phormidium autumnale*; (A) the desirability ramp, graphical representation of numerical optimization results, with the optimum point for each factor, obtained from the adjust to maximize the responses: Stigma: stigmasterol, Sito: sitosterol, and Phyto: total phytosterol; (B) contour plot of desirability function.

Table 2
Data model confirmation, results from experimentally observed responses (sterols arbitrary areas) for the predicted variables, obtained from supercritical carbon dioxide optimum extraction point (266.3 bar of pressure and 7% of ethanol).

Response	Observed responses (arbitrary area)[a]	Predicted responses	95% PI low[b]	95% PI high[c]
STIGMA[d]	161.03 ± 4.22[e]	171.94	169.20	174.67
SITO	9.67 ± 1.88	9.72	7.50	11.94
CHO	121.97 ± 5.27	114.18	66.48	161.88
Total Sterol	272.09 ± 7.86	295.84	246.92	344.76
Total Phyto	193.79 ± 0.25	183.32	164.91	201.72

[a] Sterols arbitrary area ($\times 10^4$): arbitrary units of chromatography area/10000.
[b] Prediction interval (lower limit, with 95% of confidence level).
[c] Prediction interval (upper limit, with 95% of confidence level).
[d] Sterols found in *P. autumnale*: STIGMA: Stigmasterol; SITO: Sitosterol; CHO: Cholesterol; Total Phyto: Total Phytosterols.
[e] Error bars are standard deviations based on n = 3 optimal point extractions replicates, means with the same letter are not significantly different based on Tukey's HSD test (α = 0.05).

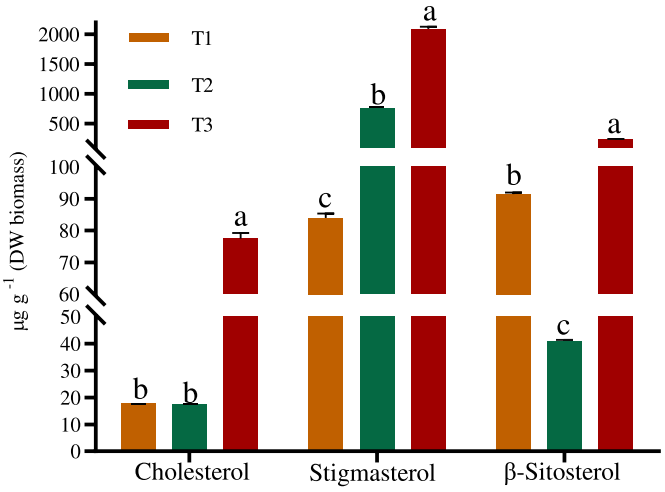

Fig. 5. Major sterol concentrations (µg g^{-1} Dry Weight) from different extraction techniques, conventional extractions: (T1) heptane extraction, and (T2) standard saponification compared to optimized supercritical extract (266.3 bar of pressure and 7% of ethanol) (T3). Error bars are standard deviations based on n = 3 extraction replicates, means with the same letter are not significantly different based on Tukey's HSD test (α = 0.05).

extraction procedure optimization. Among the minor phytosterols, lanosterol, ergosterol, and epicholesterol were the most abundant constituents, playing an essential role as intermediates in biosynthetic phytosterol pathways [9].

3.5. *In vitro* neuroprotective potential assessment

The phytosterol-rich extract obtained under optimal SFE conditions was tested against a set of *in vitro* bioactivity assays to test the neuroprotective potential. For comparative purposes, extracts obtained by conventional extraction approaches were also submitted to bioactivity assessment. Thus, extraction with heptane (T1) and standard saponification processes (T2), which are normally used for phytosterol determination, were considered reference methods to evaluate the enhanced bioactive potential of the optimized SFE process. The results

Table 3
Bioactive compounds found in *P. autumnale* under different extractions conditions.

Class of compounds	Treatments		
	T1[a]	T2	T3
Total Phenols (mg GAE g^{-1} of extract)[b]	31.2 ± 2.8b[d]	13.4 ± 2.8c	39.7 ± 4.9a
Total Carotenoids (μg g^{-1} of DW biomass)[c]	714.3 ± 4.1b	916.4 ± 4.1a, b	1007.8 ± 7.4a
Chlorophyll a (μg g^{-1} of DW biomass)	70.0 ± 6.0b	365.4 ± 0.1b	2586.2 ± 0.3a
Chlorophyll b (μg g^{-1} of DW biomass)	60.2 ± 1.8b	149.4 ± 9.3b	1162.9 ± 3.4a
Total Sterols (μg g^{-1} of DW biomass)	193.3 ± 1.6c	824.4 ± 0.1b	2414.4 ± 2.8a

[a] Different extractions, treatment 1 (T1): Heptane extraction, treatment 2 (T2): Standard saponification, and treatment 3 (T3): Supercritical fluid optimized extract (266.3 bar of pressure and 7% of ethanol).
[b] Total phenolics expressed as milligrams of gallic acid equivalents per gram of *P. autumnale* extract.
[c] DW: dry weight biomass.
[d] Values are expressed as mean ± standard deviation ($n = 3$). The levels of significant difference between the mean values were determined using the Tukey's HSD test ($\alpha = 0.05$).

obtained from the different bioactivity assays performed in the tested extracts are expressed in Table 5. The optimized SFE extract presented the highest inhibition capacity IC_{50} value of 65.80 μg mL^{-1}, whereas lower inhibitory activity was observed for the T2 and T1 extract (IC_{50} of 292.70 and 1399.00 μg mL^{-1}, respectively), IC_{50} value indicates the concentration of extract causing 50% inhibition, which means that the extract with the lowest IC50 is the one with the highest enzymatic inhibition activity. AChE inhibition potential can be classified into three categories: high ($IC_{50} < 20$ μg mL^{-1}), moderate ($20 < IC_{50} < 200$ μg mL^{-1}), and low ($200 < IC_{50} < 1000$ μg mL^{-1}) [3].

Thus, the optimized SFE extract exhibited a satisfactory (moderate-high) anticholinergic potential. Interestingly, data from the AChE enzymatic assay revealed that conventional extraction methods using saponification yielded *P. autumnale* extracts with higher inhibition capacity than heptane extracts, most probably due to the clean-up effects of the saponification process, reducing the fatty content of the terpenoid fraction.

The number of studies on AChE inhibitory potential of algae extracts is limited, and the reported IC_{50} values vary significantly between different algae. Some researchers such as Stirk et al. [53] reported algae extracts with low anticholinergic potential, as shown for *S. boveanum* (IC_{50} 1.00 mg mL^{-1}), *S. oligocystum* (IC_{50} 2.50 mg mL^{-1}), *G. corticate* (IC_{50} of 9.50 mg mL^{-1}), and *G. salicorni* (IC_{50} 8.70 mg mL^{-1}). Meanwhile, Erdogan et al. [54] noted notably lower values for *Ecklonia stolonifera* ethanolic extract (IC_{50} of 100.00 μg mL^{-1}) and *Gelidiella acerosa* (IC_{50} of 434.61 μg mL^{-1}). Several studies suggested that microalgae extract AChE inhibitory activity is strongly associated with the phytosterol content [55–58]. Thus, by analyzing algae from different phyla, distinct results were observed by Custódio et al. [59], including higher inhibition activities from *C. minutissima* (79.30%), *T. chuii* (85.70%), and *R. salina* (81.50%) extracts; compared to our SFE optimized extract, similar values of inhibition were found (85.00%), although with a

Table 5
Enzymatic inhibition (AChE and LOX), and antioxidant capacity (ORAC) of *P. autumnale* extracts.

Bioactivities[b] (μg mL^{-1})	Treatments		
	T1[a]	T2	T3
IC_{50} (AChE)[c]	1399.0 ± 10.15a[d]	292.7 ± 9.39b	65.8 ± 1.09c
IC_{50} (LOX)	122.8 ± 1.55a	119.2 ± 0.05b	58.2 ± 1.05c
IC_{50} (ORAC)	11.9 ± 2.51a	5.8 ± 0.99c	7.4 ± 2.7c

[a] T1: Heptane extraction, T2: Standard Saponification, T3: Supercritical fluid optimized extract (266.3 bar of pressure and 7% of ethanol).
[b] AChE: acetylcholinesterase, LOX: Lipoxygenase, ORAC: Oxygen radical absorbance capacity.
[c] IC_{50} for Galantamine standard on AChE inhibition assay presenting value of 1.34 ± 0.02 μg mL^{-1}.
[d] Values are expressed as mean ± standard deviation (n = 3). The levels of significant difference between the mean values were determined using the Tukey's HSD test ($\alpha = 0.05$).

Table 4
Tentatively identified terpenoids from *P. autumnale* optimum SFE extract by GC-q-TOF/MS analysis.

Compound	Common synonym	Peak no	RT (min)	Match factor (%)	Formula	Monoisotopic mass	*m/z* $[M + R]^+$ (measured)[a]	*m/z* $[M + R]^+$ (theoretical)[b]	Error (ppm)	Main fragments (*m/z*)
Cholestan-5α-en-3β-ol	β-Cholestanol	1	23.23	50.8817	$C_{27}H_{46}O_2$	402.3497	474.3901	474.3897	−0.84	370, 75, 73
Squalene	–	2	24.84	87.6182	$C_{30}H_{50}$	410.3912	482.4316	482.4312	−0.83	69, 81, 95, 121
Cholestan-3-ol	Dihydrocholestenol	3	26.69	81.3213	$C_{27}H_{48}O$	388.3705	460.4127	460.4105	−4.78	147, 215, 370
5-Cholesten-3α-7β-diol	7 alpha-Cholesterol	4	26.85	74.9110	$C_{27}H_{46}O_2$	402.3497	474.3899	474.3897	−0.42	57, 73, 129,
α-Tocopherol	Vitamin E	5	27.02	78.3455	$C_{29}H_{50}O_2$	430.3811	502.4225	502.4211	−2.79	165, 205
(3β)-Cholest-5-en-3-ol	Cholesterol	6	27.17	82.1259	$C_{26}H_{46}O$	374.3548	446.3956	446.3948	−1.79	105, 145, 255, 386
Epicholesterol	–	7	27.24	82.3645	$C_{27}H_{46}O$	386.3548	458.3955	–	–	–
Ergosta-7-22-dien-3-ol	–	8	27.42	71.6498	$C_{28}H_{46}O$	398.3548	470.3954	470.3948	−1.28	69, 255, 470
Ergosta-5,7,22E-trien-3β-ol	Ergosterol	9	27.66	85.3001	$C_{28}H_{44}O$	396.3392	468.3802	468.3792	−2.14	69, 253, 363
Stigmastanol	Fucostanol	10	27.77	60.9166	$C_{29}H_{52}O$	416.4018	488.4422	488.4418	−0.82	215, 398
Campest-5-en-3β-ol	Campesterol	11	27.83	78.9912	$C_{28}H_{48}O$	400.3705	472.4115	472.4105	−2.12	129, 343, 382
Stigmasta-5,22-dien-3β-ol	Stigmasterol	12	28.01	84.9540	$C_{29}H_{48}O$	412.37051	484.4105	484.4102	−0.62	83, 129, 255
5-Stigmasten-3β-ol	β-Sitosterol	13	28.41	84.1049	$C_{29}H_{50}O$	414.3861	486.4275	486.4261	−2.88	396, 357, 145

[a] Trimethylsilyl (TMS) derivative: R = (−H + TMS).
[b] Identification was performed using the Agilent Mass Hunter Unknown Analysis tool, being the measured *m/z* compared to theoretical *m/z* from mass spectral database NIST MS Search v.2.0, with a match factor > 50%.

higher IC_{50}. Besides phytosterols, other co-extracted molecules in the SFE extract, such as polyphenols, other terpenoids and chlorophylls might also have some effect in the observed *in-vitro* bioactivity. Phenolic compounds are powerful radical scavengers with demonstrated antioxidant activity that might have some influence in ORAC results. In addition, the influence of phenolic compounds in the observed enzymatic inhibition activities cannot be discarded. However, these hypotheses need to be confirmed in further research works.

The low IC_{50} value obtained from our optimized extract may be associated to the presence of phytosterols; this observation is in agreement with Czernicka et al. [60], who proved that sesquiterpene-enriched SFE extracts showed the best bioactivity. Together with the anticholinergic activity, antioxidant and anti-inflammatory capacity are important factors when evaluating the potential of bioactive extracts against AD. Since reactive oxygen species (ROS) generation can be particularly relevant in the central nervous system due to high oxygen consumption [61], antioxidants may help control ND effects. Thus, ROS scavenging capacity was evaluated by a lipophilic ORAC assay considering the polarity of the phytosterol enriched extracts. The ORAC assay results showed the lowest IC_{50} values for T2 (5.83 ± 0.99 μg mL^{-1}) and SFE extract (T3) (7.13 ± 1.02 μg mL^{-1}) compared to T1 extract (11.95 ± 2.4 μg mL^{-1}). As for the anti-inflammatory capacity, LOX is an essential enzyme responsible for the inflammatory response progression during ND processes. Results reveal that SFE extracts showed the best IC_{50} values (58.21 ± 1.05) compared to conventional T2 (119.21 ± 0.05 μg mL^{-1}) and T1 (122.80 ± 1.55 μg mL^{-1}) extracts. In fact, our optimal SFE extract was shown as an effective LOX inhibitor, with IC_{50} values of 58.21 ± 1.05 μg mL^{-1}. Therefore, this data proves that *P. autumnale* extracted by supercritical fluid, using ethanol as a co-solvent can be a natural source of compounds proven benefits against ND effects.

3.6. Linking in vitro bioactivity and chemical composition

In an attempt to establish a relationship between the chemical composition of *P. autumnale* extracts and their *in vitro* neuroprotective bioactivity, a multivariate data analysis based on principal components analysis was carried out. The PCA was performed, including the content of identified sterols, total carotenoids, and total phenolic composition as the main variables, and the tested *in vitro* bioactivities (AChE, LOX, ORAC) as auxiliary variables for correlation. Thus, the association was established by proximity between samples and variables distributed in the multivariate space. As illustrated in the PCA biplot graph (Fig. 6), the first two dimensions (PC1 and PC2) can explain 91% of the total variance. The PC1, which carries 77% of the variance, differentiated the conventional treatments from the SFE optimized extract, evidencing the high phytosterol presence obtained under supercritical conditions. Moreover, these compounds are involved in numerous phytosterols pathways as intermediary compounds, as previously described by Fagundes [9], and other end-pathway sterols, including campesterol and fucosterol, although in minor quantities. On the other hand, PC2 is related to the general *in vitro* neuroprotective activity. Thus, samples and compounds distributed along with the PC2 axis exhibit higher (positive value in PC2) or lower (negative value in PC2 axis) neuroprotective potential according to the IC_{50} results from the *in vitro* assays (displayed as $1/IC_{50}$ for positive correlation). AChE inhibitory activity is strongly associated with the optimized SFE procedure, suggesting that the more phytosterol concentrations in the extract, the higher the anticholinergic potential [62].

Nonetheless, the capacity of phenolic compounds to go through the BBB is controversial since it is a complex and highly effective barrier that favors the transport of lipophilic compounds. Figueira et al. [63] demonstrated that many phenolics could cross the BBB and remain active concerning neurological protection, although lipophilic antioxidant capacity was associated with the presence of specific carotenoids, as deduced from the relative position of both variables in the biplot.

The correlation matrix of all variables involved in PCA

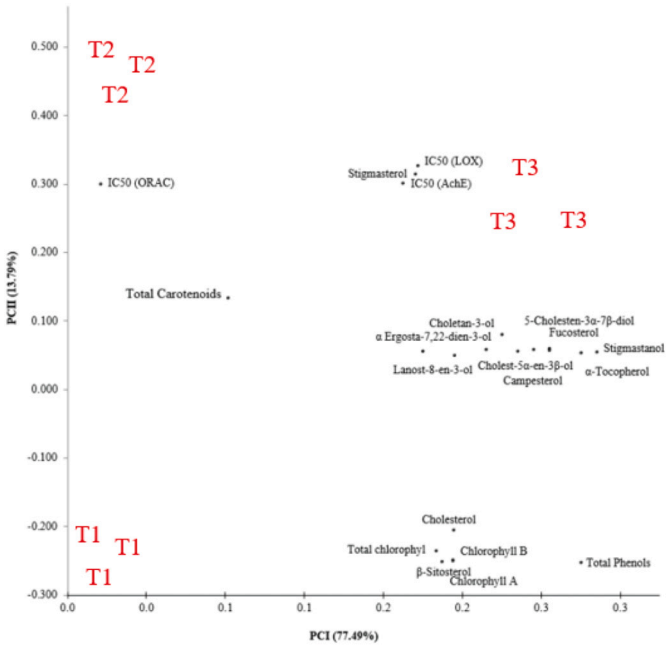

Fig. 6. Principal component analysis, scores and loadings biplot from the extraction treatments: heptane extraction (T1), standard saponification (T2), and the supercritical carbon dioxide optimal point (266.3 bar of pressure and 7% of ethanol) (T3). For the IC_{50} values obtained from the bioactivity analysis: Acetylcholinesterase enzyme (AChE), and lipoxygenase enzyme (LOX) inhibitory capacities, as well as the antioxidant capacity (ORAC), these were used as the inverse of IC_{50} ($1/IC_{50}$) in the data matrix to facilitate the graphical analysis of PCA.

(Supplementary material Table 2) showed a significant positive correlation between stigmasterol and AChE inhibitory activity ($r = 0.95$). Our results agree with *in vitro* studies demonstrating a high AChE inhibitory activity of stigmasterol isolated from an indigenous medicinal plant (*Rhazya stricta*) extract [64]. This evidence is also supported by *in vivo* studies showing stigmasterol as responsible for ameliorated scopolamine-induced memory dysfunction [65]. Similar behavior was observed for LOX activity that exhibited significant positive correlations with the stigmasterol content ($r = 0.917$).

3.7. In-silico simulation of stigmasterol - AChE interaction

In this study, a molecular docking simulation was performed to evaluate the binding mode and binding affinity of a complex between the major phytosterol, stigmasterol, and acetylcholinesterase enzyme. Human AChE protein 4Ey6:PDB complexed with galantamine (GNT) was chosen as a reference docking model.

The validated models show root mean square deviation (RMSD) values within 2 Å. From the docking procedure, ten positions were analyzed for galantamine and stigmasterol complexed to 4Ey6:PDB. Thus, the binding interactions between the ligands (GNT and stigmasterol) and binding pocket atoms were comparatively evaluated. The different ligand-protein active site interactions are shown in Fig. 7.

Similar binding energies were obtained from the docking model for GNT (-8.5 kcal mol^{-1}) and stigmasterol (-8.1 kcal mol^{-1}) ligands. As illustrated in Fig. 7A–B, GNT interacts with characteristic amino acid residues sites, such as HIS447 by hydrogen bonds, at a distance of 2.52 Å, and SER203 (2.82 Å) by a carbon-hydrogen bond. These two amino acid residues are involved in the catalytic triad located in the deep gorge, a significant AChE binding site. Furthermore, GNT interacts with PHE338 (5.33 Å) by Pi-Alkyl bonding and with TRP86 (3.98 Å) by Pi-sigma bonding. These residues are located at the AChE peripheric anionic site (PAS), which is a site associated with acetylcholine

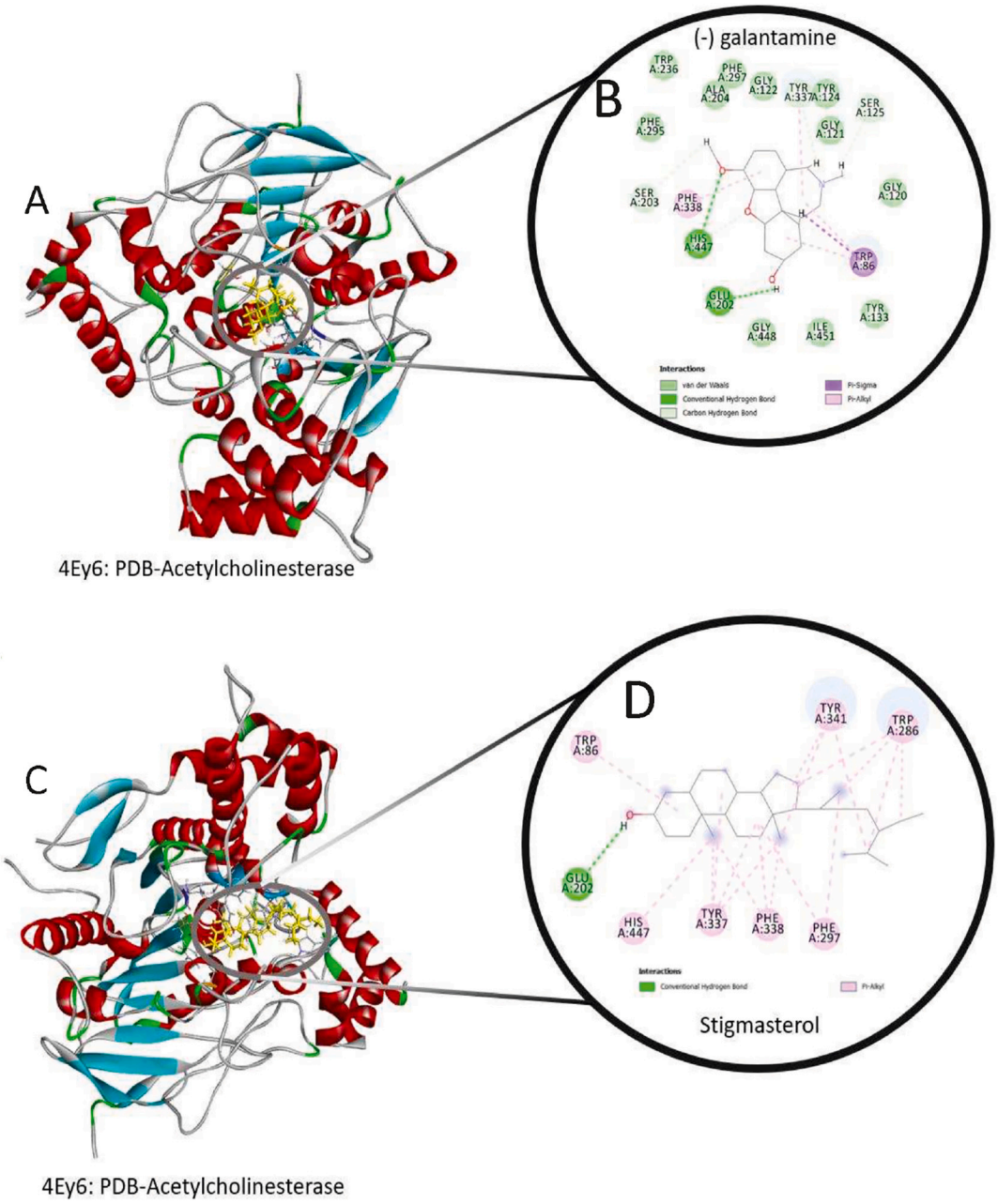

Fig. 7. *In silico* study, diagram of interactive 3D and 2D of galantamine (A, B) and stigmasterol (C, D) with acetylcholinesterase human enzyme complexation, the enzyme obtained from Protein Data Bank archive, coded as (4Ey6:PDB); the residual amino acids from the acetylcholinesterase enzyme: phenylalanine (PHE), tryptophan (TRP), alanine (ALA), tyrosine (TYR), serine (SER), glycine (GLY), isoleucine (ILE), histidine (HIS), glutamine (GLU).

sequestration and entrance into a deep narrow gorge [66]. Nonetheless, stigmasterol interacts with HIS447 residue by hydrophobic interaction at 2.84 Å of distance and with PHE338 from the anionic site (Fig. 7C–D). The major difference was in Pi-Alkyl binding with PHE297 and TRP86, which are associated with the acyl pocket, and PAS, respectively, involving hydrophobic interactions.

A more considerable binding affinity was obtained for the GNT-AChE complex, with a higher abundance of hydrogen bonds between the ligand and the enzyme. However, the applied structure-based approach reveals the potential anticholinergic activity of stigmasterol at the molecular level, acting in more than one AChE binding site and with similar binding free energy results than the reference inhibitor (GNT).

4. Conclusion

The results obtained in this study demonstrate the potential of the proposed SFE procedure to obtain a phytosterol-rich extract from *P. autumnale* biomass with enhanced neuroprotective properties compared to conventional extraction. The optimal SFE conditions (266.3 bar, and 7% EtOH) were obtained by maximizing the extraction yield and phytosterol content. Before SFE optimization, different pre-treatment methods were evaluated to enhance phytosterols recoveries. The optimal SFE extract was submitted to a chemical characterization through phytosterol profiling and determination of total phenolic, carotenoid, and chlorophyll content. Further characterization of *in vitro* bioactivity revealed the high antioxidant capacity and enhanced AChE and LOX inhibitory potential of the obtained SFE due to the phytosterol enrichment. Both *in silico* results obtained by molecular docking and

AChE *in vitro* assay suggest that phytosterols can be valuable anticholinergic agents.

To our knowledge, this is the first time that *P. autumnale* is extracted by supercritical fluid, focusing on a sterolomic study and evaluating their biological potential. These findings significantly contribute to an improved understanding of the SFE-EtOH phytosterol extract, suggesting that *P. autumnale* is a promising source of bioactive compounds with neuroprotective properties.

CRediT authorship contribution statement

Alejandro Cifuentes: Conceptualization, methodology, writing, reviewing and editing, supervision, Funding acquisition; **Elena Ibañez**: Conceptualization, methodology, writing, reviewing and editing, supervision, Funding acquisition; **Leila Queiroz Zepka**: Reviewing, Funding acquisition; **Eduardo Jacob-Lopes**: Reviewing, Funding acquisition; **Roger Wagner**: Reviewing and Editing; **José David Sánchez-Martínez**: Data analysis; **Mónica Bueno**: Data analysis, Visualization; **Jose A. Mendiola**: Data analysis, Visualization; **Gerardo Alvarez-Rivera**: Data analysis, Visualization, Writing, Reviewing and Editing; **Mariane Bittencourt Fagundes**: Investigation, Original draft writing, Reviewing and Editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2021.102264.

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Supplementary material

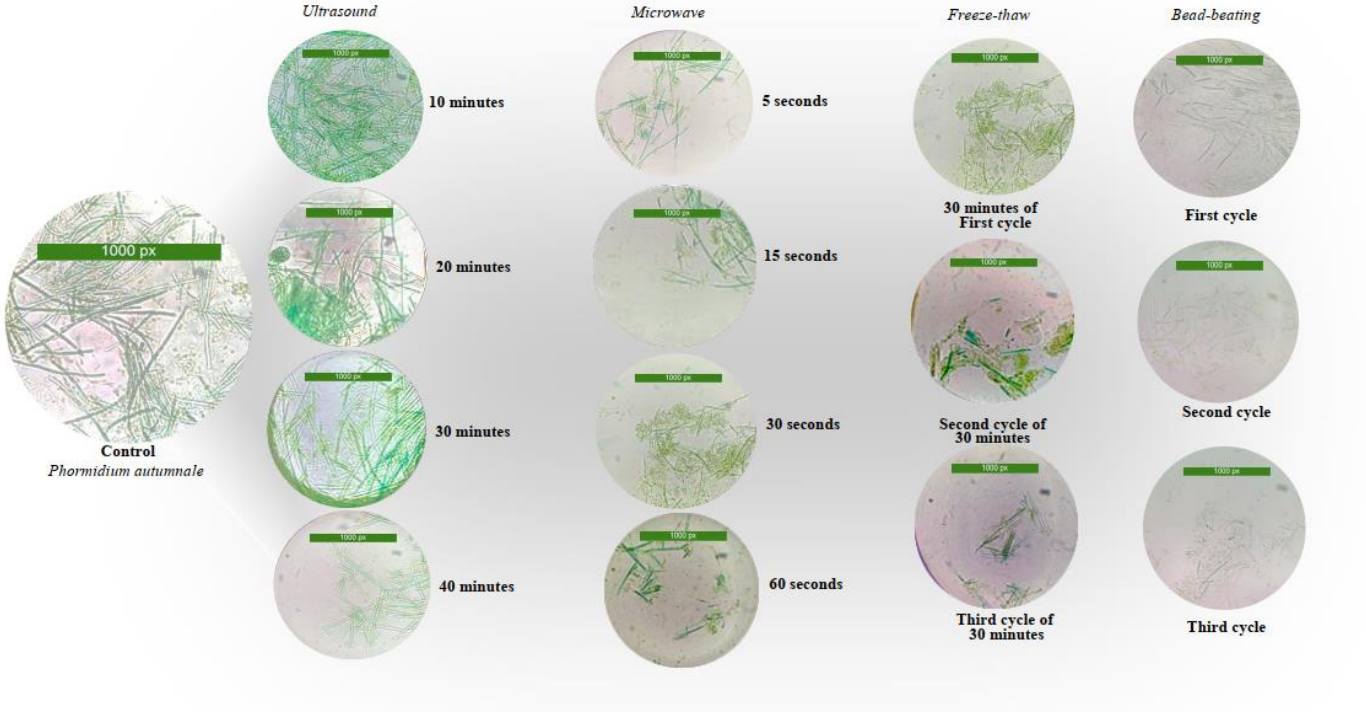

Figure S1. Images from the cyanobacteria strain before (control), and after cell hydrolysis, consisting in four pretreatments: ultrasound (25 °C, 40 kHz), microwave (400 W), freeze-thaw with cycles of freeze at -18 °C, and bead-beating using steel spheres in a cryogenic grinding, each cycle with the following conditions: pre-cooling 1/s (5 during 2 min), grinding 1/s (20 during 5 min), and intermediate cooling 1/s (5 during 1 min), all observed at a Magnification of 100×.

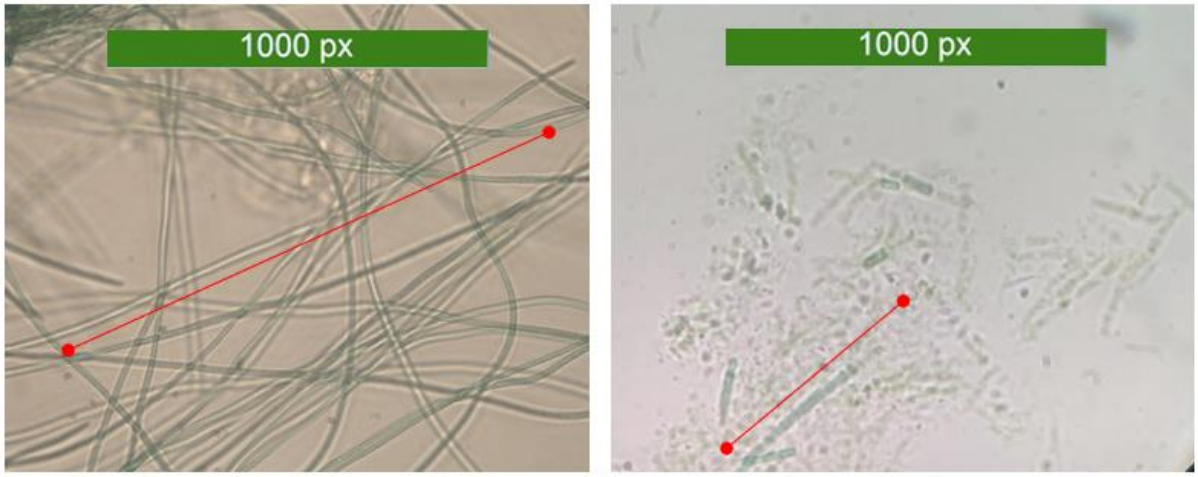

Figure S2. Bead beating (BB) showing the *Phormidium autumnale* cell segments formed after three cycles of BB, consisting in a pre-cooling 1/s (5 during 2 min), grinding 1/s (20 during 5 min), and intermediate cooling 1/s (5 during 1 min), at a Magnification of 100×.

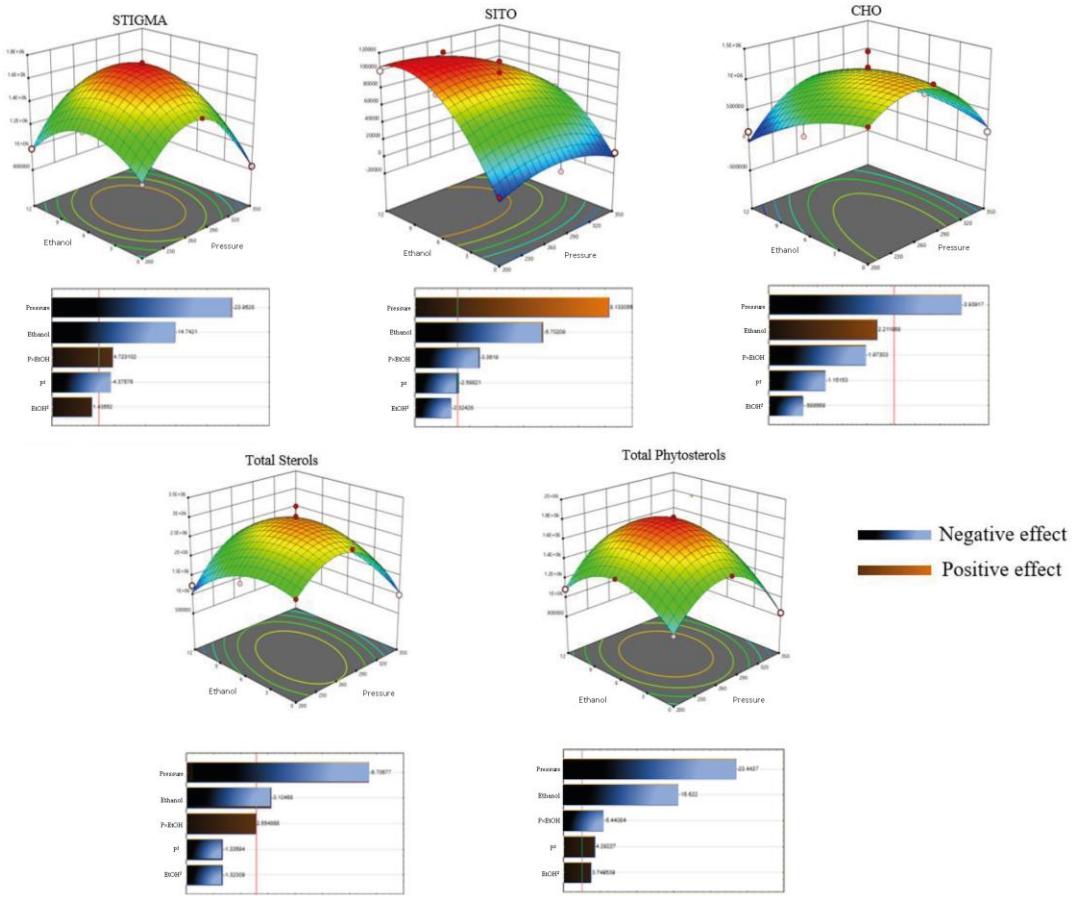

Figure S3. Response surface results, and standardized pareto charts for supercritical carbon dioxide fluid extraction, direct influence of pressure (P), and ethanol percentage (EtOH), in sterols response (arbitrary area $\times 10^4$)*;* STIGMA: Stigmasterol, SITO: β- sitosterol, CHO: Cholesterol.

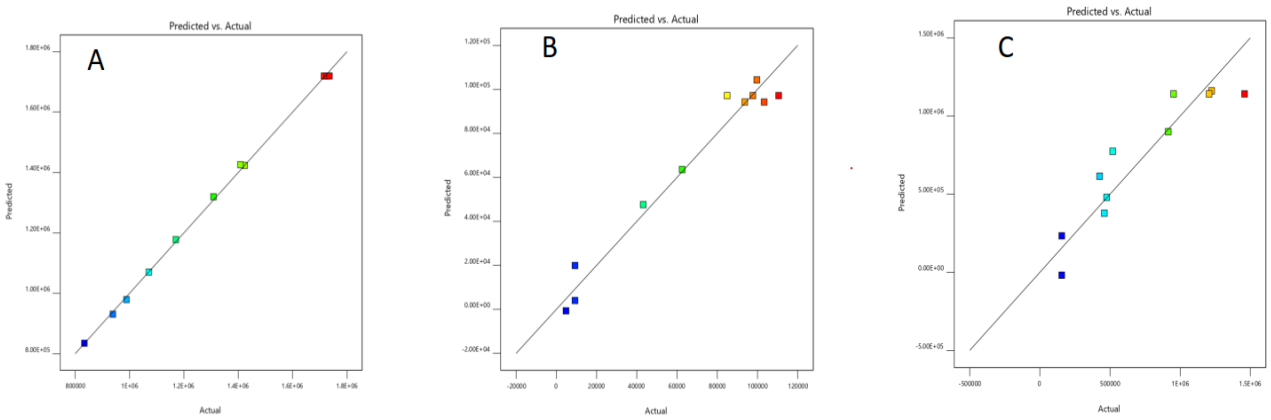

Figure S4. Comparison graph of actual and predicted phytosterols response; (A) stigmasterol, (B) β-sitosterol, and (C) cholesterol.

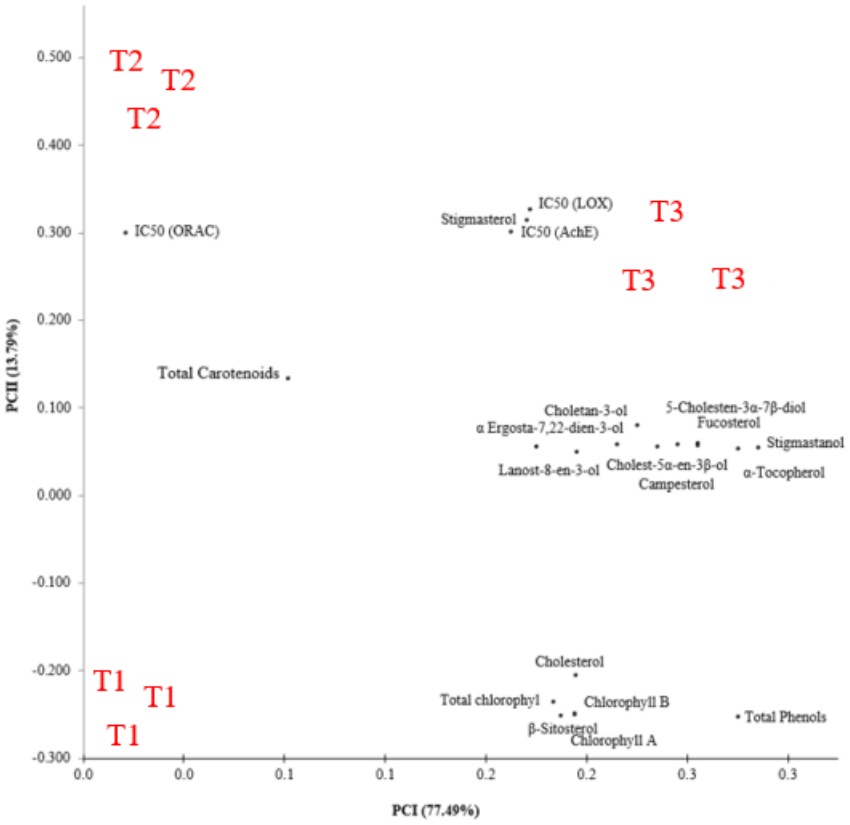

Figure S5. Principal component analysis, scores and loadings biplot from the extraction treatments: heptane extraction (T1), standard saponification (T2), and the supercritical carbon dioxide optimal point (266.3 bar of pressure and 7% of ethanol) (T3). For the IC_{50} values obtained from the

bioactivity analysis: Acetylcholinesterase enzyme (AChE), and lipoxygenase enzyme (LOX) inhibitory capacities, as well as the antioxidant capacity (ORAC), these were used as the inverse of IC_{50} ($1/IC_{50}$) in the data matrix to facilitate the graphical analysis of PCA.

Table S1. Analysis of variance (ANOVA) for response surface modelling showing linear, quadratic, and interaction relations of each variable from sterols arbitrary area experimental results.

Stigmasterol	Sum of squares	Df[1]	mean square	*F*-value	*p*-value
Model	1.08E+12	5	2.17E+11	1140.88	< 0.0001[2]
P-Pressure	3.02E+10	1	3.02E+10	159.36	< 0.0001
EtOH- Ethanol	7.98E+06	1	7.98E+06	0.04	0.8456
P×EtOH	8.76E+09	1	8.76E+09	46.19	0.0010
P ²	5.61E+11	1	5.61E+11	2959.10	< 0.0001
EtOH ²	2.20E+11	1	2.20E+11	1160.39	< 0.0001
Residual	9.49E+08	5	1.90E+08		
Lack of Fit	7.44E+08	3	2.48E+08	2.43	0.3048
Pure Error	2.04E+08	2	1.02E+08		
β-Sitosterol					
Model	1.67E+10	5	3.35E+09	26.73	0.0013
P-Pressure	1.42E+09	1	1.42E+09	11.30	0.0201
EtOH-Ethanol	8.28E+09	1	8.28E+09	66.15	0.0005
P×EtOH	6.76E+08	1	6.76E+08	5.40	0.0677
P ²	8.45E+08	1	8.45E+08	6.75	0.0484
EtOH ²	4.07E+09	1	4.07E+09	32.51	0.0023
Residual	6.26E+08	5	1.25E+08		
Lack of Fit	2.97E+08	3	9.91E+07	0.60	0.6724
Pure Error	3.28E+08	2	1.64E+08		
Cholesterol					
Model	1.73E+12	5	3.46E+11	6.00	0.0357
P-Pressure	2.74E+10	1	2.74E+10	0.47	0.5217
EtOH-Ethanol	2.25E+11	1	2.25E+11	3.89	0.1055
P×EtOH	2.82E+11	1	2.82E+11	4.89	0.0779
P ²	8.96E+11	1	8.96E+11	15.52	0.0110
EtOH ²	7.65E+10	1	7.65E+10	1.33	0.3016

Residual	2.89E+11	5	5.77E+10		
Lack of Fit	1.60E+11	3	5.35E+10	0.83	0.5857
Pure Error	1.28E+11	2	6.41E+10		
Total Sterol					
Model	5.36E+12	5	1.07E+12	17.64	0.0034
P-Pressure	1.42E+11	1	1.42E+11	2.34	0.1866
EtOH- Ethanol	1.45E+11	1	1.45E+11	2.38	0.1835
P×EtOH	3.59E+11	1	3.59E+11	5.91	0.0593
P^2	2.98E+12	1	2.98E+12	48.99	0.0009
$EtOH^2$	6.56E+11	1	6.56E+11	10.80	0.0218
Residual	3.04E+11	5	6.07E+10		
Lack of Fit	1.73E+11	3	5.75E+10	0.88	0.5712
Pure Error	1.31E+11	2	6.55E+10		
Total Phytosterol					
Model	1.46E+12	5	2.93E+11	34.06	0.0007
P-Pressure	3.32E+10	1	3.32E+10	3.87	0.1064
EtOH- Ethanol	1.25E+11	1	1.25E+11	14.57	0.0124
P×EtOH	1.65E+11	1	1.65E+11	19.17	0.0072
P^2	8.58E+11	1	8.58E+11	99.87	0.0002
$EtOH^2$	7.01E+10	1	7.01E+10	8.16	0.0355
Residual	4.30E+10	5	8.59E+09		
Lack of Fit	3.69E+10	3	1.23E+10	4.06	0.2038
Pure Error	6.06E+09	2	3.03E+09		

[1]Df: degree of freedom

[2] *p*-value: significant $p<0.05$

Table S2. Correlations between sterols, and bioactivity analyses; lipoxygenase (LOX), acetylcholinesterase (AChE) inhibition, and antioxidant capacity (ORAC)

Variables[1]	IC50 (AChE)	IC50 (LOX)	IC50 (ORAC)	Total PHE	CAR	CLO A	CLO B	Total CLO	SITO	STIGMA	CHO	CHOLES	SQ	CHOLEST	CHOLES7	TOCO	ERGO	CAM	STIGMAST
[2]IC_{50} (AChE)	**1**																		
[3]IC_{50} (LOX)	**0.867**	**1**																	
[4]IC_{50} (ORAC)	0.427	0.427	**1**																
Total Phenols	0.427	0.427	-0.458	**1**															
Carotenoids	0.283	0.283	0.36	0.117	**1**														
chlorophyll A	0.469	0.435	-0.441	**0.945**	0.276	**1**													
chlorophyll B	0.502	0.485	-0.366	**0.87**	0.427	**0.975**	**1**												
Total chlorophyll	0.483	0.45	-0.452	**0.929**	0.267	**0.996**	**0.979**	**1**											
β-Sitosterol	0.533	0.433	-0.46	**0.912**	0.217	**0.954**	**0.937**	**0.967**	**1**										
Stigmasterol	**0.95**	**0.917**	0.377	0.427	0.233	0.469	0.502	0.483	0.483	**1**									
Cholesterol	0.467	0.5	-0.427	**0.879**	0.183	**0.887**	**0.887**	**0.9**	**0.933**	0.417	**1**								
Cholest-5α-en-3β-ol	**0.762**	**0.842**	-0.02	**0.825**	0.287	**0.825**	**0.825**	**0.822**	**0.782**	**0.822**	**0.782**	**1**							
Squalene	**0.762**	**0.842**	-0.02	**0.825**	0.287	**0.825**	**0.825**	**0.822**	**0.782**	**0.822**	**0.782**	**1**	**1**						
Cholestan-3-ol	**0.762**	**0.842**	-0.02	**0.825**	0.287	**0.825**	**0.825**	**0.822**	**0.782**	**0.822**	**0.782**	**1**	**1**	**1**					
5-Cholesten-3α-7β-diol	**0.762**	**0.842**	-0.02	**0.825**	0.287	**0.825**	**0.825**	**0.822**	**0.782**	**0.822**	**0.782**	**1**	**1**	**1**	**1**				
α-Tocopherol	**0.762**	**0.842**	-0.02	**0.825**	0.287	**0.825**	**0.825**	**0.822**	**0.782**	**0.822**	**0.782**	**1**	**1**	**1**	**1**	**1**			
Ergosta-7,22-dien-3-ol	**0.762**	**0.842**	-0.02	**0.825**	0.287	**0.825**	**0.825**	**0.822**	**0.782**	**0.822**	**0.782**	**1**	**1**	**1**	**1**	**1**	**1**		
Campesterol	**0.762**	**0.842**	-0.02	**0.825**	0.287	**0.825**	**0.825**	**0.822**	**0.782**	**0.822**	**0.782**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	
Stigmastanol	**0.762**	**0.842**	-0.02	**0.825**	0.287	**0.825**	**0.825**	**0.822**	**0.782**	**0.822**	**0.782**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**

[1] Bolded value indicate significant correlation between parameters ($p<0.05$); Total PHE: Total Phenols; CAR: Carotenoids; CLO A: chlorophyll A; CLO B: chlorophyll B; Total CLO: Total chlorophyll; SITO: β-Sitosterol; STIGMA: Stigmasterol; CHO: Cholesterol; CHOLES: Cholest-5α-en-3β-ol; SQ: Squalene; CHOLEST: Cholestan-3-ol; CHOLES7: 5-Cholesten-3α-7β-diol; TOCO: α-Tocopherol; ERGO: Ergosta-7,22-dien-3-ol; CAM: Campesterol; STIGMAST: Stigmastanol.

[2] Acetylcholinesterase (AChE) inhibitory capacity, the value was used as the inverse of IC_{50} ($1/IC_{50}$) for the correlation.

[3] Lipoxygenase (LOX) inhibitory capacity, the value was used as the inverse of IC_{50} ($1/IC_{50}$) for the correlation.

[4] Antioxidant capacity (ORAC), the value was used as the inverse of IC_{50} ($1/IC_{50}$) for the correlation.

8 DISCUSSÃO GERAL

O estudo "*target*" de lipidômica em cianobactérias, principalmente correlacionado com esterolômica é escasso, pois conforme previamente mencionado as cianobactérias eram designadas como "*lack of sterols*", ou seja, cepas sem a presença destes metabólitos (LEVIN & BLOCH, 1964). As investigações nestes microrganismos existem a muitos anos conforme podemos ver para o gênero *Phormidium* (DE SOUZA & NES, 1968). No entanto, ainda assim as pesquisas indicavam que estas cepas não possuíam o aporte para ter tais compostos, estas teorias foram superadas por alguns estudos conforme MARTIN-CREUZBURG et al. (2008) e WEI et al. (2016).

Nesta mesma linha de raciocínio, o trabalho de FAGUNDES et al., (2019) foram iniciados os primeiros *insigths* na determinação dos esteróis em *P. autumnale*, a partir da verificação da presença do precursor esqualeno. Os estudos evoluíram então no desenvolvimento trabalhos que quantificaram estes analitos em meio a um cultivo heterotrófico, o que pode estar associado a presença de nitrogênio e fósforo nos cultivos microalgais.

Os esteróis estão em evidência na pesquisa científica, devido a sua elevada potencialidade no retardo de certas doenças, ou seja, os mesmos destacam-se como uma fonte a ser explorada em ensaios de inibição, tanto em estudos de inibição enzimáticos, quanto em docagens associadas a vírus. De acordo com os autores Khan et al. (2020), através de estudos *in-silico*, foi possível verificar a correlação dos fitoesteróis com a aplicação dos mesmos em estudos de docagem para inibição da proteína principal do corona vírus (SARS COV-2).

Neste sentido, podemos observar que estamos frente a novas possibilidades de exploração dos metabólitos secundários, não apenas das cianobactérias, mas também das microalgas de modo geral. Assim, os estudos esterolômicos podem tangenciar descobertas para uso em indústrias farmacêuticas e os mesmos podem ser aplicados como nutracêuticos, de forma que, o que está correlacionado a isto é a elevada produtividade das microalgas. Pois, embora muitas vezes as concentrações destes ativos sejam baixas nas células, há uma elevada produtividade de biomassa, acarretando em aplicabilidade comercial.

A cepa da cianobactéria *P. autumnale*, destaca-se em termos de produtividade de e outro fator importante previamente estudado, foi a forma de cultivo que esta cepa foi submetida, pois cultivos com fonte de carbono simples, em comparação com fontes de carbono complexas modificam a resposta final. Também observou-se que apenas com o inóculo não se teve resposta adequada a produção de esteróis, entretanto, a rota metabólica heterotrófica promoveu conversão das fontes de nutrientes externas para a biomassa assistindo a biossíntese

de esteróis, os quais possuem como funcionalidade a proteção da membrana plasmática, ou seja a proteção do material celular. Ademais, foi descoberto que a partir dos estudos realizados no grupo, diferentes resíduos agroindustriais foram responsáveis por modificar a resposta do perfil de esteróis, obtendo-se compostos majoritários distintos (FAGUNDES et al., 2021).

A importância de trabalhar com microalgas para estudos de esterolômica não é proveniente apenas do fato das cepas possuírem um metaboloma desconhecido e a presença de esteróis não convencionais, mas também do potencial de trabalhar com novas substâncias dentro do contexto da sustentabilidade. Os autores Savio et al. (2021) trouxeram uma discussão interessante acerca do potencial de utilizar as cepas como fontes (insumos) para indústria, pois destacam alguns pontos como desafios para implementar estes bioativos de microalgas na indústria. Outro ponto interessante para implementação do uso das cepas microalgais, como fonte destes metabólitos, é a elucidação dos mesmos por cromatografia em fase gasosa (do inglês GC), os autores Vendruscolo et al. (2019), trazem em sua discussão crítica diversas formas de extração para determinação destes metabólitos por GC.

A GC se apresenta como uma poderosa ferramenta para a determinação do perfil de esteróis, os mesmos podendo ser derivatizados ou não, a alta sensibilidade da GC permite a fácil realização de fórmulas nutracêuticas, no caso de se trabalhar com suplementos alimentares.

Em um primeiro momento, foi avaliada a possibilidade de reduzir a quantidade de biomassa e solventes orgânicos tóxicos sem influenciar na exatidão e precisão cromatográfica (FAGUNDES et al., 2021). Atualmente, dentro do campo de estudo dos esteróis a redução do uso de solvente orgânico está em alta, e também do uso de alternativas para agentes saponificantes e derivatizantes, que influenciam diretamente na resposta analítica, e também velocidade de análise (NZEKOUE et al., 2020).

Neste sentido, fez-se necessário verificar a possibilidade de realizar extrações que utilizem tecnologias verdes, e que fossem a partir delas adquiridas frações de esteróis com maior potencial de bioatividade. Nesta linha de raciocínio, muitos estudos comprovam que o uso das extrações emergentes, como os fluídos pressurizados são capazes de prover metabólitos com elevadas propriedades bioativas (POLLETO et al., 2020). No grupo *foodomics,* a linha de pesquisa faz do uso da ferramenta metabolômica, aplicada a extratos provenientes das tecnologias verdes de extrações, descobertas correlacionando extratos naturais com diferentes potenciais bioativos (*in-vitro* e *in-vivo*). A partir destes estudos o pesquisador Sánchez-

Martinez et al. (2021) fizeram descobertas acerca do potencial uso dos esteróis em ensaios de inibição das enzimas colinérgicas como as acetilcolinérgicas, Acetilcolinesterase

(AChE) e a Butirilcolinesterase (BuChE), e também da capacidade dos mesmos de ultrapassar a barreira hemato-encefálica, do inglês *Blood Brain Barrier* (BBB).

Neste sentido, a sequência de trabalhos visou verificar as diferentes extrações convencionais, em comparação com as extrações com redução de solvente (Fagundes et al., 2021a), bem como, com as extrações por líquidos pressurizados, liquido expandido por gás (do inglês GXL), e também fluído supercrítico (do inglês SFE) (Fagundes et al., 2021b).

Neste estudo, foi possível verificar que além das maiores concentrações de fitoesteróis, otimizadas com a extração por SFE, foi possível compreender os melhores percentuais de etanol aplicados no processo extrativo para obter um extrato rico em fitoesteróis. Ademais, as correlações dos fitoesteróis com as atividades de inibição das enzimas (AChE), e também enzima do sistema anti-inflamatório (LOX), bem como, a capacidade antioxidante dos mesmos (ORAC). Os resultados adquiridos demonstraram maior efetividade com os extratos provenientes das tecnologias emergentes, como a SFE.

Em consonância a isto, através de estudos *in-silico*, foi possível verificar que ocomposto majoritário da extração adquirida por SFE, e também o composto de maior potencialde inibição da enzima (AChE), o estigmasterol foi capaz de realizar acoplagens de baixa energia, ou seja, tornando-se uma molécula ligada a AChE de forma estável. Esta característica,foi possível obter devido a ponte de hidrogênio que o grupo hidroxila do composto estigmasterol realiza com a enzima, conforme destacado no artigo. Portanto, tal estudo mostrao potencial dos extratos naturais adquiridos da *Phormidium autumnale* frente a estudos de neurodegeneração.

9 CONCLUSÃO GERAL

Os estudos esterolômicos nos dias atuais tornam-se necessários, devido a demanda incessante por compostos que podem vir a ser aplicados nas doenças, conhecidas como doenças do mal do século. Como conclusão foi possível verificar as diversas rotas metabólicas que levam a construção destes compostos, nestas rotas verificamos que muitos esteróis não convencionais são produzidos.

Foi possível verificar que a cepa da *Phormidium autumnale* foi capaz de produzir diferentes classes de compostos, variando seu perfil de acordo com as fontes exógenas de carbono utilizada no metabolismo heterotrófico. A partir destas biomassas, a obtenção dos esteróis também foi realizada por diferentes processos de extração, e verificou-se a importância dodesenvolvimento de métodos miniaturizados e com elevada resposta analítica utilizando-se a GC.

Portanto, neste trabalho foi demostrado estratégias que permitiram ampliar o conhecimento sobre o esterolôma da cianobactéria *Phormidium autumnale*, e também reduziu-se substancialmente os tempos de extração com economia de energia e menoruso de solventes orgânicos, quando comparado aos métodos convencionais de extração. Adicionalmente, foi avaliado o potencial da intensificação do processo extrativo baseado em tecnologias fluidos pressurizados, obtendo extratos mais enriquecidos de fitosteróis, passíveis de serem aplicados como insumo para indústria farmacêutica em medicamentos associados a doenças neurodegenerativas.

Em face disso, é possível dizer o uso das tecnologias emergentes possibilitam a extração da fração de esteróis, simultaneamente apresentando alternativas mais eficientes e sustentáveis de obtenção de compostos naturais.

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