Avaliação química e toxicológica de derivados de lipídeos naturais e esteroidais sintéticos

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Pelotas, 2022.

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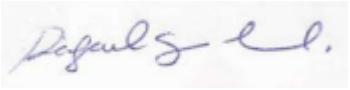

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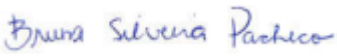

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Resumo

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Os lipídeos compreendem uma vasta classe de moléculas de origem natural (ácidos graxos, AGs) ou sintética (esteroides anabolizantes androgênicos, EAAs). O conhecimento dessas moléculas pode auxiliar no desenvolvimento de bioativos, compreensão de rotas bioquímicas e de mecanismos toxicológicos. Nesse sentido, os objetivos desse trabalho foram de avaliar os AGs e outros bioativos presentes em macroalgas sub-Antárticas e Antárticas através de Cromatografia Gasosa bem como caracterizar amostras e identificar potenciais graus de citotoxicidade de formulações apreendidas de EAAs por ensaio de viabilidade celular em cultura de células MDBK. Durante a pesquisa, também foi realizado o desenvolvimento de uma proposta para a aplicação de agentes anabólicos no ensino de Química Forense bem como também a literatura sobre métodos de extração e técnicas de análise de EAAs foi sistematicamente analisada. Os resultados mostraram que as macroalgas são ricas em AGs incluindo os ácidos palmítico, oleico e linoléico. Também foram observadas a presença de bioativos na forma de compostos orgânicos voláteis incluindo hexanal, heptadecano e furanos, por exemplo. A análise de formulações de EAAs indicou que grande parte das amostras era falsificada. Paralelamente, também se pode observar que houve efeito citotóxico relacionado não somente à substância química ativa, mas também aos outros componentes da formulação. Cabe salientar que a Espectrometria de Massas foi fundamental para a elucidação dos agentes anabólicos. Portanto, o estudo de lipídeos naturais e sintéticos permitiu avançar o conhecimento de composição e técnicas de análise as quais são importantes na Bioprospecção e na Toxicologia.

Palavras-chave: Ácidos graxos, Esteroides anabolizantes androgênicos; Cromatografia Gasosa; Viabilidade celular; Técnicas analíticas.

Abstract

BERNEIRA, Lucas Moraes. Chemical and toxicological evaluation of natural and synthetic steroidal lipids. 2022. 139p. Doctoral exam (Doctorate's degree) – Biochemistry and Bioprospection Post-Graduate Program, Food, Pharmaceutical and Chemical Sciences Center, Federal University of Pelotas, Pelotas, 2022.

Lipids comprise a wide class of molecules that include fatty acids (FAs), acylglycerols, waxes and steroids, for example. These compounds can be naturally derived from plants, animals and macroalgae as well as synthetic counterparts including anabolic androgenic steroids (AASs). The knowledge of these molecules can assist in the development of bioactives, understanding of biochemical pathways and probable toxicological mechanisms. In this sense, the aims of this work were to evaluate the FAs and other bioactives present in sub-Antarctic and Antarctic macroalgae as well as to characterize samples and identify potential degrees of cytotoxicity of seized AASs formulations. Results showed that macroalgae are rich in FAs that include, for example, palmitic, oleic and linoleic acids. In turn, the presence of bioactives in the form of volatile organic compounds including hexanal, heptadecane and furans, for example, were also observed. Analysis of AASs' formulations indicated that most of the samples were falsified, being composed of their oily component and various types of anabolic agents such as testosterone and nandrolone esters. Throughout the application of AASs in cell culture, it could be observed that there was a cytotoxic effect that may be related not only to the active chemical substance, but also to the other components of the formulation. Therefore, the study of natural lipids could show potential pharmaceutical, biochemical and biotechnological applications in the development of novel bioactive compounds while the analysis of AASs allowed a probable correlation between degrees of falsification and cytotoxicity.

Keywords: Fatty acids; Anabolic androgenic steroids; Gas Chromatography; Cellular viability; Analytical techniques.

Lista de Figuras

Lista de Tabelas

Lista de Abreviaturas

AG	Ácido Graxo
AGM	Ácido Graxo Monoinsaturado
AGP	Ácido Graxo Poli-insaturado
AGS	Ácido Graxo Saturado
CCD	Cromatografia de Camada Delgada
CG-EM	Cromatografia Gasosa-Espectrometria de Massas
CL-EM	Cromatografia Líquida-Espectrometria de Massas
EAM	Extração Assistida por Micro-ondas
EAU	Extração Assistida por Ultrassom
EASU	Extração Assistida por Sonda Ultrassônica
EABU	Extração Assistida por Banho Ultrassônico
ELL	Extração Líquido-Líquido
ESL	Extração Sólido-Líquido
EAA	Esteroide Anabolizante Androgênico
PF	Polícia Federal

Sumário

Avaliação química e toxicológica de derivados de lipídeos naturais e esteroidais sintéticos

1. Introdução

Os lipídeos compreendem uma vasta classe de compostos apolares que incluem, por exemplo, ácidos graxos (AGs), ceras, acilgliceróis e esteroides. Essas substâncias tem um papel fundamental nos organismos atuando na sua composição, estrutura e sinalização molecular (NELSON & COX, 2018, SANTOS et al., 2021). Devido a sua vasta importância, são encontrados em organismos terrestres e marinhos tais como animais, plantas, fungos e macroalgas, por exemplo (LIEBERMAN & MARKS, 2009).

Por sua vez, as macroalgas são organismos polifiléticos e pluricelulares que podem ser agrupadas em algas verdes (Chlorophyta), pardas (Ochrophyta) e vermelhas (Rhodophyta) de acordo com características morfológicas e fisiológicas (BERNEIRA et al., 2020b). As macroalgas podem ser encontradas em diversos ambientes aquáticos que variam entre habitats tropicais, temperados e polares. Isso se deve ao seu amplo potencial de adaptação por meio da ativação de seu metabolismo secundário que produz diversos bioativos (BERNEIRA et al., 2021a).

Dentre os habitats em que as macroalgas podem ser encontradas se pode citar as regiões sub-Antártica e Antártica. Esses lugares são conhecidos por apresentarem condições ambientais inóspita que incluem baixas temperaturas, fortes ventos, alta intensidade de radiação ultravioleta, por exemplo. Ambos esses habitats Antárticos e sub-Antárticos apresentam mais de 400 espécies de macroalgas sendo que a maioria ainda é pouco explorada (PEREIRA et al., 2017; SANTOS et al., 2019). No entanto, uma grande quantidade desses organismos marinho permanece desconhecido e inexplorado de forma que ainda são necessários mais estudos sobre a composição química destes organismos (PASSOS et al., 2020).

Nesse sentido, o ecossistema marinho foi reconhecido como fonte de produtos naturais com potencial terapêutico e com diversas moléculas peculiares com uma ampla diversidade de alvos moleculares (FREITAS et al., 2020). Nas

últimas décadas, foram isolados e identificados mais de 2.400 compostos biologicamente ativos com diferentes propriedades farmacológicas e atividades biológicas que incluem ações antibacteriana, antifúngica, antioxidante, antiviral, antitumoral, anti-inflamatória, por exemplo. Cabe salientar que uma quantidade considerável pode ser encontrada em macroalgas (SANTOS et al., 2017).

Por outro lado, os esteroides são compostos lipofílicos conhecidos por apresentarem caracteristicamente um núcleo de quatro anéis fundidos conhecido como ciclopentanoperidrofenantreno. No organismo, o colesterol serve como o molde para a biossíntese de todos os outros esteroides endógenos tais como mineralocorticoides, glicocorticoides, estrogênios e androgênios. Dentre as suas funções no organismo, podem ser citadas: o controle na absorção de íons inorgânicos, a regulação da gliconeogênese, a redução da resposta inflamatória e à formação das características sexuais masculinas e femininas (BERNEIRA et al., 2019).

Assim, os esteroides anabolizantes androgênicos (EAAs) compreendem uma classe de substâncias quimicamente derivadas da testosterona. Esses compostos são conhecidos devido ao seu uso ilícito por atletas e praticantes de atividades físicas visto que estão associados com aumento de performance esportiva e desenvolvimento muscular (WEBER et al., 2017). Todavia, o uso irrestrito de EAAs também está relacionado a uma série de efeitos colaterais que incluem danos no sistema cardiovascular, desenvolvimento de tumores no fígado além de danos físicos (HULLSTEIN et al, 2015).

O alto risco associado ao uso dessas substâncias levou diversos países como por exemplo o Brasil, Estados Unidos e Inglaterra a controlarem ou proibirem o uso de agentes anabólicos entre a população (WEBER et al, 2017). No entanto, o volume de apreensões de EAAs vem crescendo consideravelmente de forma que no Brasil, entre os anos de 2008 a 2011, houve um aumento de 200% nas apreensões realizadas pela Polícia Federal (PF). Cabe também salientar que houve um aumento expressivo no número de anabolizantes falsificadas, os quais representam 33% dos produtos apreendidos (NEVES et al., 2013). Cabe salientar que este é o dado mais recente visto que no Brasil pesquisas de prevalência de consumo de drogas não são realizadas com frequência adequada. Tais formulações falsificadas podem intensificar os danos causados pelo consumo de EAAs ao passo que são produzidas sem

condições sanitárias adequadas e sem controle de qualidade (NEVES et al., 2016).

Na área forense, as formulações de EAAs apreendidas pela PF são analisadas a fim de determinar sua constituição química, servindo como prova pericial em processos jurídicos. Essa etapa analítica é de considerável importância durante a análise de formulações visto que afeta diretamente os resultados obtidos pelo analista e, no caso de especialistas forenses, do laudo pericial. Inicialmente, as amostras têm seu princípio-ativo extraído por métodos que envolvem extração líquido-líquido (ELL) ou assistida por banho ultrassônico (EABU) (COOPMAN; CORDONNIER, 2012; THEVIS et al., 2008). Todavia, outras formas proeminentes de extração como a assistida por sonda ultrassônica (EASU) ou por micro-ondas (EAM) não são usualmente utilizadas durante a análise de agentes anabólicos a limitando analiticamente (GALESIO et al., 2011).

De acordo com a literatura, formulações de EAAs são geralmente analisadas através de técnicas cromatográficas, espectroscópicas e espectrométricas. Dentre essas ferramentas analíticas cabe destacar a Cromatografia Gasosa (CG-EM) e a Cromatografia Líquida (CL-EM) acoplada a Espectrometria de Massas assim como a Espectroscopia no Infravermelho (FAVRETTO et al., 2013; KRUG et al., 2015; NEVES et al, 2016). Cabe também ressaltar o uso de procedimentos de *screening* tais como a inspeção visual e testes colorimétricos empregados em menor extensão (CHIONG et al., 1992; THEVIS et al., 2008). Na última década, novas técnicas analíticas tais como Análise Direta em Ionização em Tempo Real acoplada a Espectrometria de Massas e Cromatografia de Camada Delgada (CCD) acoplada a Densitometria vêm sendo aplicadas a formulações de agentes anabólicos a fim de reduzir o preparo de amostras assim como o custo e a duração da análise (MUSHARRAF et al., 2015; PROKUDINA et al., 2015).

Com base no exposto acima, se pode notar que, para a análise de EAAs se pode notar que existe uma grande limitação no que tange a técnicas analíticas disponíveis para a avaliação de formulações. Nesse sentido, o desenvolvimento de novas técnicas analíticas para a análise de agentes anabólicos se mostra essencial a fim de responder adequadamente a alta demanda de análises requeridas. A aplicação de tais técnicas analíticas poderia auxiliar na detecção

de falsificações de acordo com a presença de contaminantes e adulterantes. Isso é importante para que sejam detectadas substâncias que podem ampliar ainda mais os danos causados pelo consumo ilícito de EAAs. Cabe ressaltar que ensaios toxicológicos e microbiológicas também podem auxiliar no processo de avaliação de toxicidade de agentes anabólicos. Dessa forma, avaliações químicas e toxicológicas podem se complementar auxiliando órgãos de segurança pública a determinar componentes e prováveis danos à saúde causados pelo consumo de agentes anabólicos.

Nesse sentido, o estudo de lipídeos tanto em sua forma natural quanto em sua forma sintética pode auxiliar na elucidação de mecanismos bioquímicos e fisiológicos em organismos marinhos bem como auxiliar na avaliação de potenciais agentes tóxicos em materiais de apreensão. Dessa maneira, o conhecimento nesses campos é crucial para a evolução do conhecimento sobre macroalgas de regiões extremas como também sobre formulações lipídicas de agentes anabólicos.

2. Objetivos

2.1. Objetivo Geral

O objetivo deste trabalho foi de avaliar a constituição química de AGs provenientes de macroalgas sub-Antárticas e Antárticas bem como avaliar técnicas analíticas e a toxicidade de componentes lipídicos de formulações de agentes anabólicos apreendidos pela Polícia Federal.

2.2. Objetivos Específicos

Os objetivos específicos foram de:

I. Detectar os componentes de AGs em macroalgas da região sub-Antártica e Antártica;
II. Identificar a presença de potenciais bioativos nas amostras algais;
III. Correlacionar e discriminar macroalgas de diferentes filos de acordo com as distintas concentrações de ácidos graxos em sua composição utilizando Análise Multivariada;
IV. Detectar os componentes de formulações de EAAs apreendidos pela PF utilizando micro-ondas e energia acústica através de CG-EM;

V. Avaliar a toxicidade de formulações de EAAs através de ensaios toxicológicos *in vitro* com cultura de células de rim bovino;
VI. Revisar a literatura acerca de métodos de extração e técnicas analíticas para a análise de formulações de agentes anabólicos.

3. Referencial teórico

3.1. Ácidos graxos

3.1.1. Lipídeos em macroalgas

De uma forma geral, os AGs são compostos que contém cadeias de carbonos ligados a um grupo terminal carboxila os quais podem apresentar ou não apresentar duplas ligações. Quando as cadeias de carbonos não apresentam duplas ligações são denominados ácidos graxos saturados (AGSs) ao passo que quando apresentam uma única dupla ligação são chamadas de ácidos graxos monoinsaturados (AGMs). Por fim, quando apresentam duas ou mais ligações duplas são chamados de poli-insaturados (AGPs) (CHRISTIE & HAN, 2012; NELSON & COX, 2018).

As fontes de AGs encontradas na natureza podem ser provenientes de diversas matrizes vegetais, como por exemplo frutos, sementes oleaginosas e algas. Atualmente, a pesquisa na área de macroalgas tem sido intensificada pela grande facilidade de coleta associada a grande diversidade de AGs que apresentam estes organismos com importância farmacológica e nutracêutica (SANTOS et al., 2021; SANTOS et al., 2017). Dentre as possíveis fontes de AGs de macroalgas podem ser citados os organismos provenientes da região sub-Antártica e Antártica. As macroalgas desses habitats apresentam uma grande concentração de AGPs, tais como o ácido linoléico (18:2*n*6) e alfa-linolênico (18:3*n*3). Esses AGs são essenciais ao metabolismo de humanos, ou seja, não são produzidos pelo organismo, sendo necessário seu consumo através da dieta (BERNEIRA et al., 2021a; FREITAS et al., 2020).

As algas são organismos fotossintéticos que existem em várias formas e tamanhos. Podem ser encontradas desde microalgas as quais são microscópicas e unicelulares até formas macroscópicas e multicelulares que são as macroalgas (PEREIRA et al., 2017). Esses organismos são conhecidos por apresentarem ausêncua de raízes, caules e folhas (sendo, assim, designados como talófitas). Além disso, seu pigmento fotossintético primário é a clorofila *a*.

As macroalgas são comumente encontradas em ambientes aquáticos das zonas tropicais, temperadas e polares (SANTOS et al., 2019).

As macroalgas estão inseridas no domínio dos eucariotos e podem ser classificadas em três grandes filos principais de acordo com características morfológicas, anatômicas e fisiológicas. Assim, podem ser as Rhodophyta (algas vermelhas), Ochrophyta (algas marrons) e Chlorophyta (algas verdes) (BERNEIRA et al., 2020b). Cabe também salientar que esses organismos apresentam uma vasta importância para o ecossistema visto que se encontram na base da cadeia alimentar e também podem auxiliar na geração de oxigênio necessário ao metabolismo dos organismos consumidores (PASSOS et al., 2020).

A diversidade de espécies de algas e a sua grande capacidade de adaptação à ambientes extremos as tornam uma importante usina biológica de compostos bioativos e complexos os quais geralmente não estão presentes em plantas terrestres (FREITAS et al., 2020). Os AGs provenientes do metabolismo primário das algas têm sido alvo de estudos nas áreas de química, nutracêutica e farmacêutica pois seu perfil lipídico tem sido associado a diversas atividades biológicas, incluindo ações antioxidantes, antiinflamatórios, antivirais e anticâncer, por exemplo (MARTINS et al., 2018; PACHECO et al., 2018).

Além disso, se sabe que as macroalgas são fontes renováveis de AGPs principalmente nas formas de ômega-3 (*n*3) e ômega-6 (*n*6) os quais são considerados AGs essenciais para a nutrição humana e que devem ser obtidos pela dieta alimentar. Dentre essas biomoléculas essenciais está o ácido linoléico e o ácido α-linolênico (BERNEIRA et al., 2020b). Os AGPs desempenham um papel importante na prevenção de doenças cardíacas, neurológicas, autoimunes e inflamatórias (PACHECO et al., 2018).

Em macroalgas, geralmente a biossíntese dos AGs do tipo ômega ocorre a partir do ácido palmítico (C16:0) cuja cadeia é alongada até o ácido esteárico (C18:0). Através da enzima Δ9 desaturase ocorre a remoção de dois átomos de hidrogênio do ácido esteárico formando uma ligação dupla (ácido oleico, C18:1). Outras duas enzimas denominadas Δ12 e Δ15 desaturase as quais atuam em organismos vegetais realizam a formação do C18:3*n*3 e do C18:2*n*6 no organismo humano. Os AGs essenciais também são precursores de outros AGPs de cadeia longa tais como os ácidos eicosapentaenoico (C20:5*n*3),

docosahexaenóico (C22:3*n*3) e araquidônico (C20:4*n*6) (GUSCHINA et al., 2006; VANCE & VANCE, 2008)

É importante ressaltar que as macroalgas são organismos de rápido desenvolvimento e fácil manipulação. Nesse sentido, estudos têm sido feitos para cultivar algas em condições controladas com o objetivo de aumentar os níveis de compostos bioativos. Portanto, esses organismos podem prover um número crescente de extratos, frações ou compostos puros naturais e renováveis (FREITAS et al., 2020; MARTINS et al., 2018; BERNEIRA et al., 2021a).

3.1.2. Região sub-Antártica e Antártica

O continente Antártico é considerado o mais frio, o mais seco, o mais alto, o mais ventoso, o mais inexplorado e o mais preservado dentre as grandes massas continentais da Terra. Devido a suas condições climáticas inóspitas que incluem baixas temperaturas, exposição fotossintética limitada pelas estações, alta nível de luz ultravioleta, altos níveis de salinidade e variações nas concentrações de nutrientes as macroalgas ativam seu metabolismo secundário para sobreviver nesta região (PACHECO et al., 2018; PEREIRA et al., 2017). Por sua vez, a região sub-Antártica compreende o extremo sul do Chile e da Argentina bem como a porção dos oceanos Atlântico, Pacífico e Índico que circundam o Círculo Polar Antártico. Esse local apresenta diversas condições ambientes semelhantes às que são encontradas na Antártica (SANTOS et al., 2017; SANTOS et al., 2019).

Embora as regiões Antárticas e sub-Antárticas sejam locais com menor potencial para o desenvolvimento de vida comparadas às regiões tropicais, há um amplo grau de endemismo nessas regiões. De acordo com estudos anteriores cerca de 130 espécies de algas foram relatadas no continente Antártico sendo 35% delas endêmicas. Dessa forma, esse habitat apresenta diversas espécies únicas que ainda são pouco caracterizadas e identificadas quimicamente (SANTOS et al., 2020; BERNEIRA et al., 2020b).

Cabe também salientar que as algas de regiões frias como a Antártica e a região sub-Antártica geralmente apresentam uma vasta produção de AGPs os quais são produzidos via metabolismo secundário a fim de manter as condições ideais de vida nesses ambientes inóspitos (PEREIRA et al., 2017, MARTINS et

al., 2018). Com base nisso, a avaliação dos constituintes químicos lipídicos nesses organismos podem ser utilizados para melhor entender sua fisiologia bem como para bioprospectar compostos para serem utilizados nas áreas farmacêuticas, médicas e biotecnológicas (FREITAS et al., 2020).

3.1.3. Avaliação Química

A análise de AGs se inicia pelo processo de extração de maneira que ocorre uma migração desses compostos para um solvente apolar. O método mais utilizado é o descrito por BLIGH & DYER (1959). Nessa metodologia, os compostos são extraídos a frio evitando uma possível degradação ou oxidação. Geralmente esses AGs não estão na forma livre, mas sim, conjugados a uma molécula de glicerol os quais formam acilgliceróis ou fosfolipídeos. Nesse contexto, a extração dos AGs, aliada a uma metodologia eficiente de derivatização é imprescindível para quantificação e identificação destes compostos nos mais diferentes tipos de matrizes (CHRISTIE & HAN, 2012; SIDIQUI et al., 2009).

A derivatização é um processo que permite a modificação de grupos funcionais em uma molécula com a finalidade de aumentar sua volatilidade, diminuir sua polaridade e melhorar sua estabilidade o que permite uma melhor interação com uma coluna capilar empregada na técnica de Cromatografia Gasosa. Nesse contexto, a análise de AGs sem sua conversão em ésteres metílicos pode levar a um acúmulo de compostos na coluna e consequentemente a uma danificação ou inutilização dessa porção do cromatógrafo gasoso (CHRISTIE & HAN, 2012, MILINSK et al., 2008).

Devido aos vários tipos de catalisadores existentes, a derivatização pode seguir uma catálise ácida, básica ou mista. A catálise ácida utiliza reagentes como ácido sulfúrico (H_2SO_4), ácido clorídrico (HCl) e trifluoreto de boro (BF_3). Por sua vez, na catálise básica são utilizados reagentes como hidróxido de sódio (NaOH) e hidróxido de potássio (KOH) (CHRISTIE & HAN, 2012). Outro método que pode ser utilizado é a chamada catálise mista, onde há uma associação de uma catálise ácida e uma básica no mesmo processo reacional (MOSS, LAMBERT & MERWIN, 1974).

A identificação de ácidos pode ser realizada através da Cromatografia a Gás por Ionização de Chama. Esta técnica de análise se apresenta ideal para

análise de AGs em vista de que esse detector é universal e de grande sensibilidade e permite a qualificação e a quantificação mais rápida e adequada dos componentes de uma amostra (BERNEIRA et al., 2020b). Dessa forma, a adaptação de metodologias de derivatização para análise em Cromatografia a Gás por Ionização de Chama se torna necessária, a fim de se obter reações mais rápidas, menos tóxicas e resultados mais confiáveis (CHRISTIE & HAN, 2012).

3.2. Esteroides e seus derivados sintéticos

3.2.1. Aspectos gerais e químicos

Os esteroides são compostos lipofílicos conhecidos por apresentarem um núcleo cíclico similar ao fenantreno (anéis A, B e C) que está ligado a um anel de ciclopentano (D) (RODWEL et al., 2021). No organismo, o colesterol serve como o molde para a biossíntese de todos os outros esteroides endógenos que incluem, por exemplo, ácidos biliares, os hormônios adrenocorticais, os hormônios sexuais, as vitaminas D e os glicosídeos cardíacos. Essas substâncias e seus derivados também desempenham papeis importantes em vegetais compreendo os sitoesteróis (NELSON & COX, 2018). A **Figura 1** representa a estrutura química de alguns esteroides endógenos.

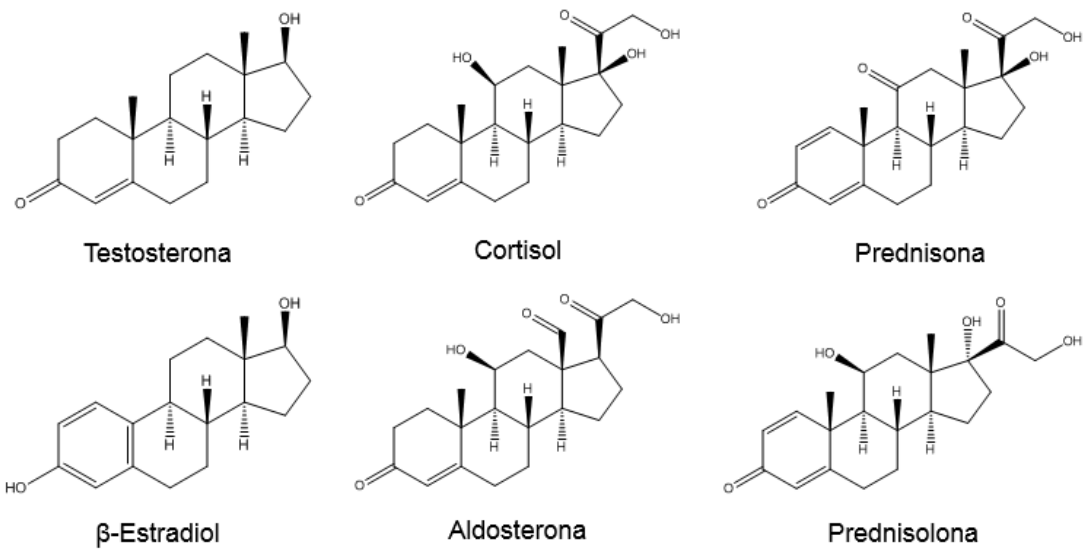

Figura 1. Estrutura química de esteroides endógenos.

Cabe salientar que cada um dos anéis que contém seis carbonos do núcleo esteroide pode ser encontrado em dois tipos de conformações tridimensionais as quais podem ser na forma de “cadeira” ou na forma de “barco” (NELSON & COX, 2018). De uma forma geral, em esteroides de ocorrência natural, os anéis estão na forma de cadeira que é a conformação mais estável para a molécula. Dependendo dos substituintes e de suas posições nos anéis que compõem o núcleo esteroide também podem haver configurações *cis* e *trans* as quais são importante para identificar a origem e a função biológica no organismo (RODWEL et al., 2021).

Dentre as várias famílias de esteroides, os andrógenos compreendem a família de compostos endógenos similares a testosterona servindo como hormônios sexuais masculinos que são produzidos principalmente nos testículos, porém também podem ser sintetizados pelas glândulas adrenais e, em menor concentração, pelos ovários. Dentre as suas funções no organismo podemos citar o crescimento, desenvolvimento e manutenção dos órgãos sexuais masculinos, diferenciação sexual, espermatogênese além de promover o desenvolvimento ósseo e muscular (NELSON & COX, 2018).

Por sua vez, os derivados sintéticos da testosterona são conhecidos como EAAs, os quais foram inicialmente desenvolvidos para terapias médicas. No entanto, devido a sua ação anabólica, seu uso de forma ilícita se tornou constante por parte de atletas e praticantes de atividades físicas (BERNEIRA et al., 2019). Devido a seus efeitos colaterais essas substâncias foram banidas por organizações esportivos ou tiveram seu uso restrito em diversos países, tais como, Brasil, Estados Unidos, Reino Unido, Austrália e Argentina (BERNEIRA et al., 2020).

Os EAAs podem ser divididos em três grupos principais sendo os análogos onde o grupo hidroxila 17β foi esterificado com grupos alquila de tamanhos distintos, análogos cuja posição 17α foi alquilada e análogos cujos anéis A ou B foram modificados. Essas alterações são utilizadas para influenciar a absorção, taxa de inativação, metabolização e a diminuição de processos de aromatização no anel A que, em altas concentrações, podem gerar efeitos adversos. Além disso, essas modificações também servem para alterar a razão

anabolizante/androgênica e, assim, diminuir os potenciais efeitos androgênicos (EVANS, 2004).

No organismo masculino, a testosterona assim como também outros andrógenos, tais como a 5α-di-hidrotestosterona, a desidroepiandrosterona e a androstenediona são sintetizados pelas células de Leydig localizadas nos testículos (NELSON & COX, 2018). De forma geral, a biossíntese é iniciada através do colesterol através de duas rotas distintas que envolvem uma série de reações enzimáticas que sucessivamente convertem a pregnolona à testosterona como pode ser visto na **Figura 2**.

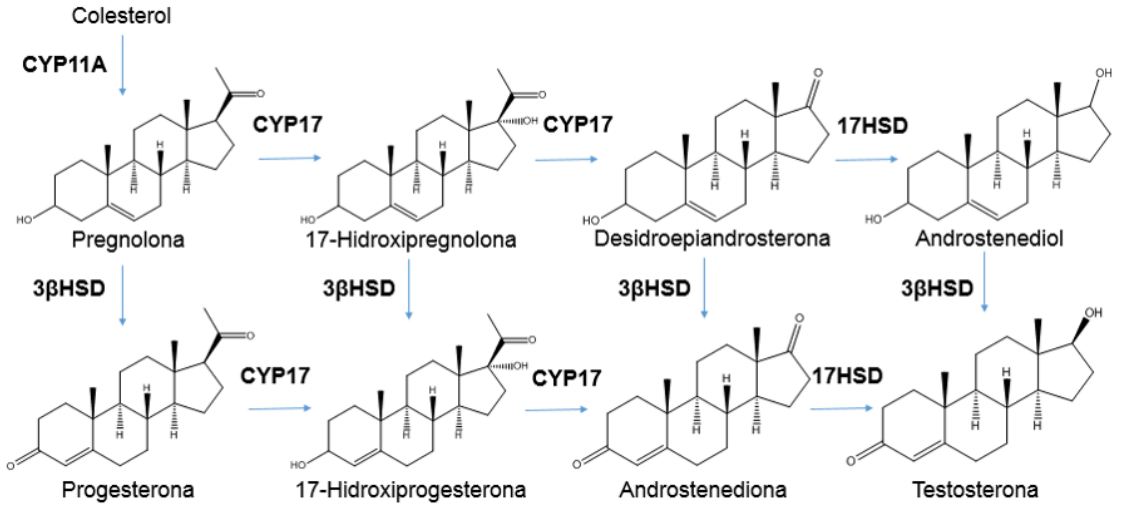

Figura 2. Mecanismo de biossíntese da testosterona no organismo (CYP17: citocromo P450 família 17; 17HSD: 17β-hidroxiesteroide desidrogenase).

3.2.2. Aspectos bioquímicos, farmacológicos e toxicológicos

De uma forma geral, os EAAs podem ser absorvidos através da via oral, via dérmica ou através da via intramuscular as quais apresentam diferentes taxas de meia-vida no organismo (BERNEIRA et al., 2019). Posteriormente a sua absorção, os EAAs são carregados na corrente sanguínea pela globulina ou pela albumina de forma que uma pequena porção desses andrógenos sintéticos permanece livre na corrente sanguínea. Nesse tecido, os esteroides β-esterificados são hidrolisados pelas carboxilesterases liberando o seu esteroide

corresponde que, na maioria dos casos, compreende à testosterona (KICMAN, 2008).

Subsequentemente à hidrólise e à liberação da proteína carreadora, os andrógenos podem se ligar a receptores androgênicos localizados no citoplasma celular formando um complexo que pode ser transportado ao núcleo celular (**Figura 3**). Tais receptores androgênicos podem ser encontrados em diversas porções do organismo dentre as quais se pode elencar o trato reprodutivo, os ossos, o músculo esquelético, o cérebro, o fígado, os rins e os adipócitos (BUTTNER; THIEMME, 2009). Uma vez no núcleo celular, o complexo do andrógeno e seu receptor se liga ao DNA iniciando diversos processos de transcrição, ligados principalmente a ações anabolizantes e androgênicas (KICMAN, 2008).

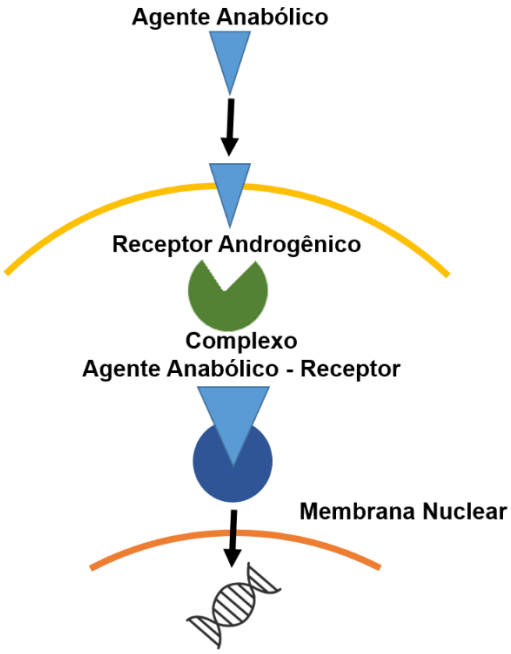

Figura 3. Mecanismo de ação de agentes anabólicos.

Dentre os metabólitos de agentes anabólicos encontrados no organismo está a di-hidrotestosterona a qual é conhecida por apresentar majoritariamente efeitos androgênicos (KIKMAN, 2008). Essa atividade está relacionada com às características sexuais masculinas, tais como, espessamento das cordas vocais, aumento de secreção de glândulas sebáceas e produção de pelos (BERNEIRA et al., 2019; EVANS, 2004). Dentre as razões que explicam o alto potencial

androgênico da di-hidrotestosterona está o fato desse composto possibilitar uma redução 5α em sua estrutura pela enzima 5α redutase (EVANS, 2004).

Por sua vez, a testosterona está relacionada principalmente a um efeito anabólico que pode ser definido como um estado fisiológico em que o nitrogênio é retido no organismo estimulando a síntese de proteínas (BERNEIRA et al., 2021b). Assim, há um aumento de massa, volume e força dos músculos esqueléticos bem como uma ampliação na síntese de colágeno e na densidade dos ossos sendo que a sua ação anabólica é dose-dependente (BERNEIRA et al., 2020a).

Em média, um adulto do sexo masculino produz aproximadamente 7 mg de testosterona diariamente através das células de Leydig. No entanto, essa quantidade não é capaz de induzir uma atividade anabólica extensa relacionada ao aumento de massa muscular. Assim, para promover esse efeito, o usuário de substâncias ilícitas consome de 10 a 100 vezes da faixa terapêutica de EAAs promovendo uma superdosagem no organismo (EVANS, 2004).

É comum o consumo dessas substâncias em ciclos de 4 a 18 semanas aumentando gradativamente a dose conhecido como pirâmide. Em alguns casos, o usuário também pode recorrer a uma mistura de anabolizantes conhecido por *stacking* ou ainda pode ocorrer o que se chama de *plateuning* processo no qual se utiliza diversos ciclos com EAAs distintos para evitar uma possível tolerância (BARCELOUX & PALMER, 2013). O consumo dessa elevada dose pode levar a vários distúrbios clínicos e bioquímicos que podem ser vistos na **Tabela 1**.

Tabela 1. Uso indiscriminado de EAAs e seus efeitos adversos

Alvo	Efeitos colaterais
Fígado	Colestase, peliose, alterações enzimáticas, adenomas hepatocelulares e tumores
Sistema cardiovascular	Infarto do miocárdio, hipertensão, arritmia, hiperoscoletemia, aumento da lipoproteína de baixa densidade, diminuição da lipoproteína de alta densidade

Sistema Nervoso	Irritabilidade, agressividade, euforia, impulsos destrutivos, mudança de humor, distúrbios de sono e psicose
Sistema Reprodutivo	Puberdade precoce, supressão da produção endógena da testosterona, amenorreia, atrofia testicular e diminuição da espermatogênese
Sistema Urinário	Aumento na creatinina do soro, cálculos renais e poliúria
Glândulas Mamárias	Atrofia em mulheres e ginecomastia em homens;
Tecido Ósseo	Fechamento precoce das epífises em crianças e adolescentes gerando parada no crescimento
Cabelo	Hirsutismo em mulheres, aceleração da calvície em homens e seborreia;
Pele	Acne, urticária e aumento da oleosidade;
Cordas Vocais	Espessamento das cordas vocais em mulheres;

Adaptado de: KICMAN, 2008; EVANS,2004.

Por meio de estudos de citotoxicidade se pode observar que a aplicação de agentes anabólicos em sua forma pura em um cultivo de células neurais gerou vários danos celulares causados pela indução direta da apoptose produzidos pela exposição a andrógenos sintéticos (BASILLE et al., 2013; CARACI et al., 2011). Além disso, foi indicado que o consumo de hormônios sexuais também pode resultar em genotoxicidade e carcinogenicidade dependendo da quantidade da dose bem como também do tempo de exposição. Além disso, fatores genéticos e epigenéticos também podem influenciar na toxicidade (ZELLEROTH et al., 2019; MEIRELES et al., 2013).

Vale ressaltar que o potencial de induzir apoptose está associado ao anabolizante utilizado de forma que geralmente ocorre na seguinte ordem: nandrolona > testosterona > estanozolol > trembolona (BASILLE et al., 2013). Tal ordem foi observada no estudo de ZELLEROTH et al. (2019) que aplicou vários agentes anabólicos em concentrações de 100, 30 e 10 µM em células corticais primárias de rato. Por fim, também se pode destacar que as pesquisas aplicando EAAs em células utilizaram apenas materiais puros de forma que a

aplicação de formulações sejam elas genuínas ou falsificadas podem apresentar uma alteração na toxicidade desses materiais. Para formulações falsificadas os riscos à saúde podem ser ainda maiores devido a componentes e a concentrações incertas, possível presença de adulterantes e risco de contaminação (COOPMAN & CORDONNIER, 2012; NEVES & CALDAS, 2017).

3.2.3. Métodos de extração e técnicas analíticas

Com o intuito de diminuir a atividade androgênica e aumentar o potencial anabólico inúmeros EAAs foram desenvolvidos (**Figura 4**) de forma que podem ser agrupados em três classes principais já descritas anteriormente (PROKUDINA et al., 2015). Tais modificações químicas também permitiram que os agentes anabólicos possam ser encontrados em diversas formulações incluindo, por exemplo soluções oleosas, comprimidos e suspensões aquosas tornando o processo de extração e de análise instrumental mais dificultoso para essa classe de compostos (EVANS, 2004).

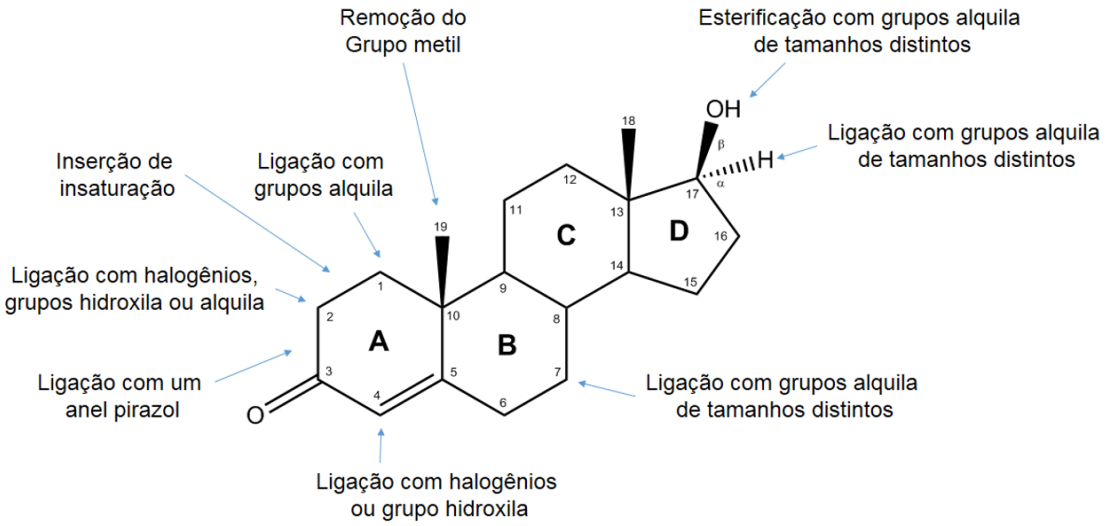

Figura 4. Modificações químicas na estrutura da testosterona para a síntese de agentes de anabólicos.

Em linhas gerais, a extração pode ser caracterizada como a migração do analito a partir de sua matriz para um solvente extrator. Este processo é um dos passos mais influentes na análise de formulações de EAAs uma vez que afeta os resultados finais obtidos por técnicas analíticas (**Figura 5**). No caso de formulações apreendidas, essa etapa pode ser desafiadora visto que as

preparações têm origem, pureza e constituição desconhecida (NEVES & CALDAS, 2017). Cabe salientar que um adequado processo de extração pode auxiliar no processo de detecção dos analitos visto que a presença de matriz pode interferir nos resultados obtidos pelas técnicas analíticas como também danificar a instrumentação dos equipamentos (CHIONG et al., 1992).

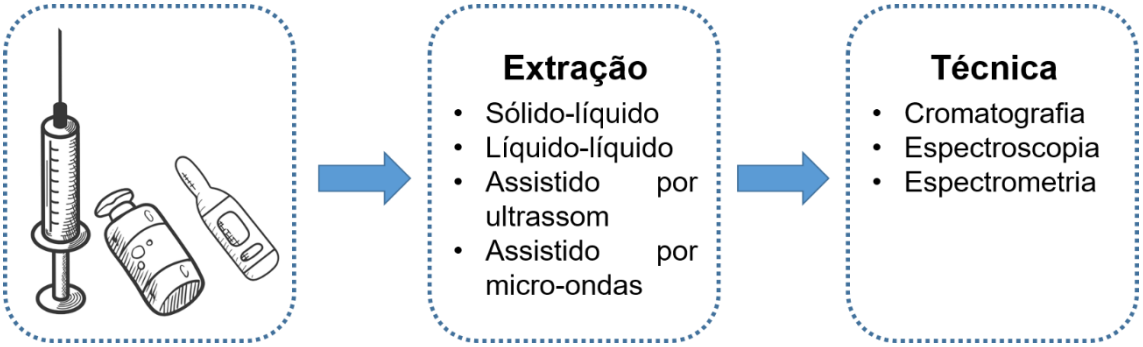

Figura 5. Etapas de extração e instrumentação analítica para a análise de formulações de agentes anabólicos.

Nesse contexto, a ELL e a ESL são métodos de extração conhecidos pela aplicação de um solvente orgânico para recuperar o analito de uma matriz líquida ou sólida, respectivamente, sem a aplicação de algum tipo de energia assistida. Tais métodos extrativos tendem a ser muito trabalhosos e demandam grandes quantidades de solventes de forma podem ser utilizados até 90 mL de metanol-água (9: 1, v/v) para extrair 1 mL de uma formulação oleosa. Geralmente, o metanol é o solvente mais empregado nas análises de forma que alguns trabalhos também utilizam acetato de etila, acetonitrila ou clorofórmio (BERNEIRA et al., 2019; CHIONG et al., 1992; KRUG et al., 2015; MUSSHOFF et al., 1997; NEVES et al., 2016; PROKUDINA et al., 2015).

Outro uso da ELL foi na análise de supostas formulações de Durateston®, em que os resultados cromatográficos indicaram a presença de óleo de amendoim, prasterona e propionato de testosterona (NEVES et al., 2016). No entanto, devido a vários pontos negativos incluindo baixo rendimento e uso de altos volumes de solventes, poucos métodos que se baseiam em ELL ou ESL foram desenvolvidos para a análise de formulações de EAAs os quais tornaram-se majoritariamente baseados no uso de ondas ultrassônicos para o processo extrativo (COOPMAN; CORDONNIER, 2012).

Com o intuito de aumentar a recuperação de analitos, diminuir o volume de solventes utilizado e o tempo de análise, métodos que se baseiam no uso de energia ultrassônica foram desenvolvidos e rapidamente se estabeleceram como a metodologia convencional para a análise EAAs. De acordo com trabalhos anteriores, a aplicação de energia acústica pode aumentar aproximadamente 5% na recuperação da substância química ativa em relação a ELL ou ESL (NEVES & CALDAS, 2017). Esse acréscimo na recuperação do analito está associado à formação de bolhas de cavitação as quais colidem com pressões e temperaturas consideráveis na amostra ampliando a interação do solvente com a formulação e, assim, favorecendo o processo extrativo do agente anabólico (CHO et al, 2015).

Semelhante ao ELL e ESL, a extração assistida por ultrassom (EAU) é realizada utilizando metanol como solvente extrator com tempos de sonicação que podem variar de 5 até 30 minutos à temperatura ambiente de forma que estudos anteriores indicaram que os EAAs podem ser extraídos de forma mais eficiente das formulações usando uma sonda ultrassônica do que um banho ultrassônico (CHO et al., 2015; FAVRETTO et al., 2013; NEVES & CALDAS, 2017; BERNEIRA et al., 2020a). Os resultados mostraram que o rendimento extrativo geralmente aumentou mais de 10% entre os analitos a partir aplicação direta de ultrassom na amostra através de uma sonda ultrassônica comparada ao banho ultrassônico devido a focalização de energia acústica na amostra (BERNEIRA et al., 2020a).

Entre os métodos de extração, o uso de EAM embora ainda não aplicado a formulações de EAAs pode ser utilizado visto que apresenta resultados promissores para outras matrizes. Essa eficácia na extração está associada ao aumento de temperatura em que é exposta no interior do equipamento. Dentre os mecanismos que podem explicar o aumento na recuperação dos analitos está a rotação de dipolo e a condução iônica que ocorre no solvente a partir da aplicação da irradiação de micro-ondas. Esses movimentos inter-moleculares aumentam a interação entre o solvente extrator polar com a formulação apolar resultando em um aumento na extração dos agentes anabólicos (VINATORU; MASON; CALINESCU, 2017).

Métodos que utilizem sonda ultrassônica ou irradiação de micro-ondas para a análise de formulações de EAAs ainda não são recorrentes na literatura.

Nesse sentido, existem apenas estudos que avaliam os procedimentos extrativistas para a recuperação de agentes anabólicos em matrizes de urina e alimentos (BARREIRO et al., 2015; GALESIO et al., 2011). Vale ressaltar que além de aumentar a eficiência da extração, o uso de irradiação de micro-ondas e ultrassom está alinhado a diversos princípios da Química Verde, pois seu uso diminui a quantidade de solvente e amostra e, assim, são capazes de reduzir o consumo de energia, geração de resíduos, exposição humana e solventes liberados para o meio ambiente (LLOMPART et al., 2019). Segundo a literatura, essas energias assistidas aumentaram a recuperação dos analitos de formulações o que também foi observado no trabalho atual na análise de formulações de EAAs (COOPMAN; CORDONNIER, 2012; NEVES & CALDAS, 2017).

Durante as últimas décadas, várias técnicas analíticas foram desenvolvidas e validadas para a análise de formulações de EAAs. Dessa forma, as ferramentas analíticas disponíveis ampliaram partindo de procedimentos convencionais que se utilizam de espectroscopia, de cromatografia e de espectrometria para uma ampla variedade de ensaios (PROKUDINA et al, 2015; MUSHARRAF et al, 2013). No entanto, o uso de técnicas cromatográficas ainda é predominante e está presente em aproximadamente 50% das publicações no campo ao passo que novas aplicações de outras técnicas compreenderam cerca de 30% dos trabalhos encontrados na literatura (**Figura 6**). Cabe ressaltar que tais ferramentas analíticas têm diferenças consideráveis em seus custos, tempo de análise e instrumentação de forma que o analista deve escolher ou combinar os procedimentos para obter resultados satisfatórios (KOVACS et al., 2014).

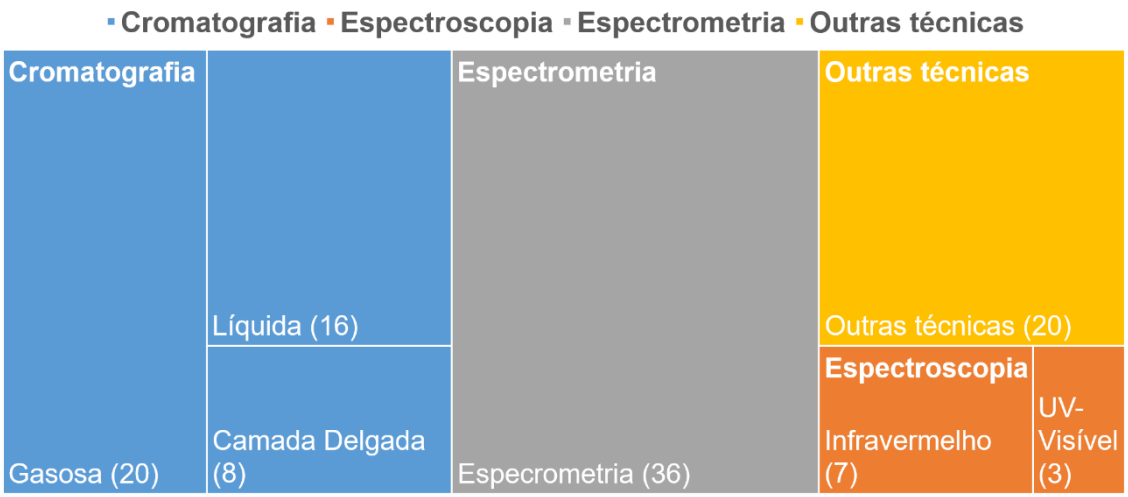

Figura 6. Técnicas analíticas empregadas na análise de formulações de EAAs.

A aplicação de técnicas analíticas é essencial na análise de formulações de EAAs uma vez que esses procedimentos são capazes de detectar os componentes da amostra incluindo a substância química ativa, excipientes, adulterantes e contaminantes (DECONINCK et al, 2013). Além disso, o uso de ferramentas analíticas pode diferenciar se uma amostra em questão é produto de falsificação ou material de contrabando. Tal conclusão é significativa para o meio jurídico visto que as sanções aplicadas ao indivíduo são distintas de acordo com os resultados dessas análises. Portanto, a aplicação de técnicas analíticas desempenha um importante papel na análise de formulações apreendidas de EAAs com os resultados servindo como uma crítica evidência forense aplicada extensivamente para fins legais (NEVES & CALDAS, 2017).

Nas etapas iniciais de análise de formulações de agentes anabólicos se pode empregar testes colorimétricos como, por exemplo, os testes de Liebermann, de Mandelin e do ácido sulfúrico (BERNEIRA et al., 2020a). Os materiais usados nesses procedimentos reagem com insaturações presentes nos anéis esteroidais resultando num produto que apresentação coloração (BERNEIRA et al., 2021b). No entanto, devido a inespecificidade desses testes para EAAs bem como o emprego de reagentes corrosivos e tóxicos, os procedimentos colorimétricos são pouco utilizados para a análise de agentes anabólicos (CHIONG et al., 1992).

Por outro lado, a Espectroscopia no Infravermelho é vastamente utilizada pois possibilita a verificação de vestígios iniciais de presença de derivados

esteroidais (DECONINCK et al., 2013). Essa técnica apresenta várias vantagens comparada a outras ferramentas analíticas convencionais visto que sua análise é não-destrutiva, rápida e de baixo custo (REBIERE et al., 2016). No entanto, a formulação pode dificultar a interpretação do espectro de infravermelho visto que estudos anteriores mostram que bandas podem ser mascaradas ou ser introduzidas nos resultados devido à presença da matriz oleosa, contaminantes e adulterantes (NEVES & CALDAS, 2017; BERNEIRA et al., 2019).

Por sua vez, as técnicas cromatográficas são as mais utilizadas na análise de formulações de agentes anabólicos. Dentre tais ferramentas analíticas, a Cromatografia Gasosa acoplada à Espectrometria de Massas é uma das principais técnicas sendo utilizada como uma maneira de separar e identificar de forma conclusiva os componentes da formulação (FAVRETTO et al., 2013). Além do espectrômetro de massas, outros equipamentos podem ser acoplados ao cromatógrafo como, por exemplo, um combustor e um espectrômetro de massa de razão isotópica (HULLSTEIN et al., 2015).

Nos últimos anos, técnicas analíticas alternativas às convencionais vêm sendo desenvolvidas e validadas. O desenvolvimento e a aplicação de tais ferramentas pode permitir ao analista um menor preparo de amostra, diminuição da interferência da matriz bem como impedir ou diminuir danos à instrumentação (DOUÉ et al., 2014; KAUPPILA et al., 2011). Nesse sentido, estudos demonstraram a aplicação de Calorimetria Exploratório Diferencial, Ressonância Magnética Nuclear e Espectroscopia Raman, por exemplo (BERNEIRA et al., 2019; REBIERE et al., 2016; RIBEIRO et al., 2018).

3.2.4. Aspectos jurídicos

O consumo de EAAs é combatido em todo o mundo com uma legislação específica a cada localidade. No caso do Reino Unido essas substâncias são caracterizadas como uma droga de classe C de acordo com o *Misuse of Drugs Act* de 1971. De acordo com tal legislação, a exportação, importação e distribuição de agentes anabólicos é ilegal. Nos Estados Unidos, os anabolizantes são drogas controladas categorizadas na *Schedule III* de forma que seu uso e distribuição para funções não-médicas é crime tipificado no *Anabolic Steroid Control Act* de 1990 (O'LEARY, 2013).

Todavia, países localizados nas regiões da África, do Oriente Médio, do Ásia e alguns países da América do Sul ainda apresentam fragilidades na lei, no controle e na distribuição de EAAs (O'LEARY, 2013). Com isto, estas regiões se tornaram centros para a produção e distribuição de EAAs para outros países. No Brasil, os agentes anabólicos são substâncias controladas encontradas na Lista C5 da Agência Nacional de Vigilância Sanitária (BERNEIRA et al., 2019). A comercialização ilegal de EAAs sem receita médica, tais como em lojas virtuais, academias e outras fontes ilícitas estão tipificadas no artigo 273 do Código Penal como crimes hediondos gerando uma pena ao distribuidor de cerca de 1 a 3 anos de reclusão e multa (BERNEIRA et al., 2020a).

O mercado de drogas ilícitas tem aumentado consideravelmente devido ao consumo excessivo destas drogas pela população o que também gera um aumento no comércio de drogas falsificadas no país (NEVES et al., 2017). Segundo a Organização Mundial da Saúde, um medicamento falsificado é aquele que pode ter um baixo padrão de qualidade, ser falsamente rotulado ou contrafeito (DE FREITAS et al., 2019). Por serem drogas falsificadas e apresentarem inúmeros compostos nocivos à saúde podem agir no organismo gerando diversos efeitos colaterais. Nesse caso, essa infração está tipificada no artigo 275 do Código Penal gerando uma pena de cerca de 10 a 15 anos de reclusão e multa (BERNEIRA et al., 2019).

4. Metodologia

4.1. Análise de ácidos graxos e bioativos de macroalgas

4.1.1. Coleta e preparação das macroalgas

As macroalgas do estudo foram coletadas na região de Punta Arenas entre 2016 e 2017 ou no continente Antártico em 2015. O material coletado foi inicialmente acondicionado em caixas tipo *cooler* contendo água do mar até seu envio ao laboratório. Subsequentemente, as macroalgas foram lavadas em água destilada sendo congeladas em sacos plásticos escuros a - 20 ºC até sua secagem. O processo de secagem foi realizado em uma estufa de circulação de ar a 35 °C (± 1 ° C) por um período de 24 a 30 h. Por fim, as amostras foram pulverizadas em um moinho de facas e armazenadas em sacos escuros a - 20 °C antes das análises. As macroalgas utilizadas nos estudos que estão compreendidos nesta tese foram *Desmarestia confervoides*, *Adenocystis*

utricularis, *Myriogramme manginii*, *Gigartina skottisbergii*, *Curdiea racovitzae*, *Georgiella confluens*, *Ulva intestinalis*, *Lessonia searlesiana*, M*acrocystis pyrifera*, *Lessonia flavicans* e *Mazzaella laminarioides*.

4.1.2. Extração de ácidos graxos

Para a extração foi utilizado um 1 g de biomassa algal sendo que as amostras foram colocadas em um balão de 100 mL seguindo a extração de BLIGH & DYER (1959). Em seguida, foram adicionados 10 mL de clorofórmio, 20 mL de metanol e 10 mL de solução aquosa a 1,5% (*m/v*) de sulfato de sódio. Posteriormente, a mistura reacional foi agitada à temperatura ambiente num agitador magnético durante 30 min. Após este tempo, foram adicionados novamente 10 ml de clorofórmio e 10 mL de solução aquosa de sulfato de sódio a 1,5% (*m/v*). A mistura foi transferida para tubos tipo *falcon* os quais foram submetidos à centrifugação durante 30 min a 5000 rpm. Após a centrifugação, a fase orgânica foi separada e evaporada sob pressão reduzida.

4.1.3. Derivatização de ácidos graxos

Os lipídeos extraídos das biomassas algais foram convertidos aos respectivos ésteres metílicos de ácidos graxos segundo o método de MOSS; LAMBERT & MERWIN (1974). Brevemente, em um balão de 50 mL, contendo os lipídeos previamente extraídos, foram adicionados 6 mL de solução de metanólica de hidróxido de potássio a 2% (*m/v*) sob agitação e aquecimento de 80 ºC por 8 min em refluxo. Após este período, foram adicionados 7 mL de trifluoreto de boro em solução metanólica a 14% (*v/v*) com agitação por 5 min sob refluxo a 80 °C. e 5 mL de solução de cloreto de sódio a 20% (m/v). A fase orgânica, contendo os ácidos graxos esterificados, foi separada utilizando 20 mL de *n*-hexano e 5 mL de solução aquosa de cloreto de sódio a 20% (*m/v*). A fase orgânica foi recuperada e posteriormente filtrada sob sulfato de sódio anidro e evaporada sob pressão reduzida.

4.1.4. Cromatografia Gasosa com Detector de Ionização por Chama

Após a derivatização, os ésteres metílicos de AGs foram analisados através de Cromatografia Gasosa com Detector de Ionização por Chama (GC-FID 2010, Shimadzu, Kioto, Japão) com injetor split/splitless, autoinjetor AOC-

20i e coluna SP 2560 (100 m x 0,25 mm x 0,20 µm). As condições operacionais para as análises de perfil lipídico foram de nitrogênio como gás carreador na vazão de 1,2 mL/min, modo split 1:100 e volume injetado de amostra de 1 µL. As condições de temperatura foram de inicialmente a 120 °C com aquecimento de 3 °C/min até 240 °C. Ambas as temperaturas do injetor e do detector foram de 250 °C.

4.1.5. Extração de compostos orgânicos voláteis

Aproximadamente 1g da biomassa algal foi colocado dentro de um *vial* de 20 mL de borossilicato e selado com um septo de silicone. O material foi introduzido em um forno e incubado por 1 h a 100 ° C. Os compostos orgânicos voláteis foram removidos após o processo de aquecimento para serem analisado por CG-EM.

4.1.6. Cromatografia Gasosa-Espectrometria de Massas

Um volume de 1000 µL dos compostos orgânicos voláteis foi injetado em um cromatógrafo a gás acoplado com um espectrômetro de massas de modelo GC-MS QP-2010 (Shimadzu, Kyoto, Japão) equipado com uma coluna capilar Rtx-5MS (30 m × 0,25 mm × 0,25 µm) e hélio como gás de arraste. A porta de injeção e a fonte de íons foram mantidas a 250 °C e 290°C, respectivamente. O forno foi operado em uma temperatura de 30°C por 2 min, aumentando 4°C/min para 180 °C e depois 20 °C/min até atingir a temperatura final de 280 °C mantendo por 5 min. O método de porcentagem de área foi usado para medir a concentração de compostos orgânicos voláteis bem como a biblioteca NIST-08 e o índice de retenção para identificação dos compostos. O índice de retenção das substâncias detectadas foi determinado usando uma solução do padrão C8-C20 (Sigma-Aldrich, St. Louis, Estados Unidos).

4.2. Análise de formulações apreendidas de agente anabólicos

4.2.1 Extração de formulação de agentes anabólicos

As análises para avaliação dos métodos extrativos foram realizadas utilizando formulações na forma de cápsulas, comprimidos, soluções oleosas ou suspensões aquosas utilizando banho ultrassônico ou micro-ondas. A quantificação dos analitos foi realizada utilizando como padrão interno colesterol.

4.2.1.1. Extração assistida por banho ultrassônico

A metodologia utilizada seguiu o procedimento desenvolvido NEVES & CALDAS (2017) onde 25 µL ou 25 mg das formulações de agentes anabólicos serão inseridos em tubos *falcon* e extraídos com 5 mL de metanol em um banho ultrassônico durante 10 min sob temperatura ambiente. Ao fim desse período, as amostras foram centrifugadas por 5 min e uma aliquota da camada superior foi injetada em um CG-EM.

4.2.1.2. Extração assistida por Micro-ondas

Aproximadamente 25 µL ou 25 mg das formulações de agentes anabólicos foram introduzidas em balões e extraídas com 5 mL de metanol em micro-ondas de sistema aberta durante 10 min a 55 °C. Ao fim desse período, as amostras foram centrifugadas por 5 min e uma aliquota da camada superior foi injetada em um CG-EM. Essa extração foi adaptada a partir da metodologia descrita por NEVES & CALDAS (2017).

4.2.2. Cromatografia Gasosa-Espectrometria de Massas

Para análise cromatográfica das formulações, 1 µL da fração metanólica foi injetada em um cromatógrafo a gás acoplado a um espectrômetro de massas em modo split (1:25). A coluna capilar utilizada foi do tipo Rtx-5MS usando hélio como gás de arraste. O injetor e a interface foram operarados a 280 °C enquanto que a temperatura programada da coluna foi de 200°C aumentando 30 °Cmin^{-1} a 250°C mantendo-se por 16 min. Após este período, a temperatura foi aumentada a 30°C.min^{-1} a 300°C mantendo-se por 14,5 min. O espectrômetro de massas operou com uma ionização de elétrons a 70 eV escaneando íons entre 30 a 550 m/z. A identificação dos componentes das amostras foi realizada através de uma biblioteca do equipamento.

4.2.3. Espectroscopia no Infravermelho

Inicialmente, a fase orgânica extraída foi evaporada sob pressão reduzida de forma que aproximadamente 3 mg do material extraído das formulações foram analisadas por Espectroscopia no Infravermelho com Transformada de

Fourier com detector de Reflexão Total Atenuada utilizando uma resolução de 4 cm^{-1} variando de 4000 cm^{-1} a 600 cm^{-1}.

4.2.4. Avaliação toxicológica de formulações de agents abólicos

O ensaio citotóxico das formulações foi realizado usando células Madin Darby de rim bovino cultivadas a 37 °C em meio essencial mínimo suplementado com soro fetal bovino (10%, v/v). As células foram cultivadas atingindo uma densidade de 3×10^4 células por poço por 24 h a 37 °C em uma atmosfera contendo 5% de gás carbônico e 95% de ar umidificado. Os experimentos foram feitos em triplicata (n=3) e repetidos duas vezes em experimentos independentes usando soluções das formulações em meio essencial mínimo de 0,5, 0,25, 0,125 e 0,062 $mg.mL^{-1}$. Os óleos de soja e de amendoim bem como células sem tratamento foram usados como controles negativos. Após este procedimento, as células foram incubadas sob temperatura e condições atmosféricas iguais por 24 h. Após este período, 50 µL de uma solução de MTT (1 $mg.mL^{-1}$) foi aplicada em placas as quais foram incubadas em condições semelhantes descrito acima por 3 h. Posteriormente, o sobrenadante foi removido e os cristais de formazan foram solubilizados em 100 µL de DMSO por 10 min. Finalmente, as placas foram analisadas em espectrofotômetro em 540 nm.

4.2.5. Avaliação sistemática da literatura

Para a avaliação sistemática da literatura, foram realizadas buscas em sítios eletrônicos do Google Acadêmico, Pubmed e do Science Direct utilizando as palavras-chave “Anabolic androgenic steroids”, “formulations”, “chemical analysis”, “extraction” e “analytical techniques” buscando trabalhos acadêmicos publicados entre os anos de 1979 a 2021.

4.3. Análise estatística

Os resultados apresentados foram apresentados na forma de média ± desvio padrão. Todas as análises foram realizar em triplicatas (n=3). A análise estatística foi realizada através de Análise de Variância de Duas Vias com Teste de Tukey ($p \leq 0,05$) utilizando o software GraphPad. A análise multivariada foi realizada utilizando o software Minitab.

5. Resultados

Os resultados obtidos para a análise de AGs em macroalgas da região Antártica bem como de outros bioativos que incluem os compostos orgânicos voláteis foram publicados na revista Journal of Applied Phycology em 2020 no trabalho intitulado “Evaluation of the volatile composition and fatty acid profile of seven Antarctic macroalgae”. Por sua vez, os resultados da análise de bioativos em macroalgas da região sub-Antártica foi publicado na revista Brazilian Journal of Botany em 2021 com o título de “Evaluation of volatile organic compounds in brown and red sub-Antarctic macroalgae”. Por fim, o manuscrito intitulado “Novel application of sub-Antarctic macroalgae as zinc oxide nanoparticles biosynthesizers” foi submetido para a revista Materials Letters.

Foi realizado o levantamento de métodos de extração e de técnicas analíticas aplicadas para formulações de esteroides anabolizantes androgênicos foi aceito para publicação na revista científica WIREs Forensic Science na forma de review intitulado “Extraction and analytical approaches in the forensic evaluation of anabolic androgenic steroids formulations”. A aplicação de métodos de extração variados em formulações apreendidas de EAAs foi publicado na revista Drug Testing and Analysis intitulado “Analytical approaches applied to the analysis of apprehended formulations of anabolic androgenic steroids”. Por sua vez, os dados em relação a análise toxicológica e de identificação das amostras foram reunidos em forma de manuscrito intitulado “Chemical and cytotoxicity evaluation of apprehended formulations of anabolic-androgenic steroids” aceito para a publicação na revista Talanta Open.

Capítulo 1

Extraction and analytical approaches in the forensic evaluation of anabolic androgenic formulations

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OVERVIEW

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Extraction and analytical approaches in the forensic evaluation of anabolic androgenic steroid formulations

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Abstract

Anabolic androgenic steroids are controlled or prohibited substances worldwide since their abusive usage can lead to extensive side effects. For forensic purposes, apprehended formulations of anabolic agents have to be analyzed to determine their components as well as possible adulterants and contaminants. Nevertheless, there are no comprehensive reviews in the field in order to compile the methods and analytical techniques that have been described so far. Therefore, the aim of this work was to review the analytical techniques and methods of extraction available for the analysis of formulations of anabolic agents. In this sense, 76 research works that analyzed formulations of anabolic agents from 1979 to 2021 were reviewed and distributed under the extraction methods and the analytical techniques employed by the authors. Generally, ultrasonic-assisted extraction associated with methanol as extractor solvent is the procedure of choice with little contribution of solid or liquid–liquid extraction. According to the literature, there are several analytical tools available for the analysis of these substances, although gas and liquid chromatography coupled to mass spectrometry as well as spectroscopic techniques are frequently employed. Nonetheless, novel techniques have been developed in order to decrease analysis time, cost, and sample preparation, which are important features in the expressive numbers of anabolic agents apprehended yearly. Therefore, the application of adequate methods of extraction coupled to suitable analytical techniques can be efficient forensic tools in order to contest the illicit supply of anabolic androgenic steroids providing consistent evidence to legal courts.

This article is categorized under:

Forensic Chemistry and Trace Evidence > Emerging Technologies and Methods

Toxicology > Drug Analysis

Toxicology > Analytical Toxicology

KEYWORDS

anabolic androgenic steroids, analytical techniques, apprehended formulations, extraction methods, forensic analysis

1 | INTRODUCTION

Anabolic androgenic steroids (AASs) are a class of synthetic derivatives of testosterone therapeutically developed to treat medical conditions such as hypogonadism, burns and osteoporosis (Van Amsterdam et al., 2010). These compounds are also known for their illicit use among athletes and the general population since their consumption is linked to enhancement of strength and increase of muscular mass (Coopman & Cordonnier, 2012). However, this indiscriminate usage can lead to several side effects such as hepatotoxicity, coronary damage, and physical impairment. In this sense, anabolic agents became controlled and prohibited worldwide (Hartgens & Kuipers, 2014).

Several AASs have been synthesized (Figure 1) in order to decrease the androgenic activity associated to its side effects and to enhance the anabolic potential that is targeted for medical or illicit use (Prokudina et al., 2015). Generally, the developed compounds can be divided into three main groups: (I) derivatives that have the 17β hydroxyl portion alkylated (e.g., nandrolone decanoate); (II) analogs that have the 17α position alkylated (e.g., oxandrolone and oxymetholone); or (III) the derivatives that have a chemical modification in the ring A, B, or C (e.g., stanozolol). However, to this day, no steroid has been developed with a complete lack of androgenic action and, therefore, side effects (Evans, 2004).

Usually, AASs are found in an oily solution that must be injected intramuscularly or in a tablet form that is orally active. The distinct forms of formulation are available because pills or encapsulated anabolic agents have a 17α metilation that enables them to overcome the first-pass metabolism. However, this modification is considerably toxic for the liver and can lead to the formation of tumors (Kanayama et al., 2010). Once in the organism, steroids in the free form act in the androgen receptors that, in turn, favor transcription and translation processes in the target cells releasing proteins (Nieschlag & Vorona, 2015).

In average, a male adult produces about 7 mg of testosterone every day—quantity that cannot exert an extensive anabolic activity (Evans, 2004). Thus, in order to promote this effect, the illicit user consumes 10–100 times of this amount in the form of AASs. However, this overdose leads to several clinical and biochemical disorders in the organism, such as: hepatic tumor (Pellegrini et al., 2012), heart attack, aggressiveness, psychosis, testicular atrophy, infertility (De Souza & Hallak, 2011), increase of low-density lipoprotein and decreased production of follicle-stimulating hormone as well as luteinizing hormone (Kicman, 2008).

Since the abusive use of these drugs leads to several side effects, AASs are rigidly controlled substances worldwide. In the United Kingdom, anabolic agents are in the Class C category, which means that their importation, exportation, or distribution without a license are forbidden within the country. However, it is not a crime to possess them, unless the product is falsified (Coomber et al., 2015). In other countries, such as the United States and Brazil, there are similar

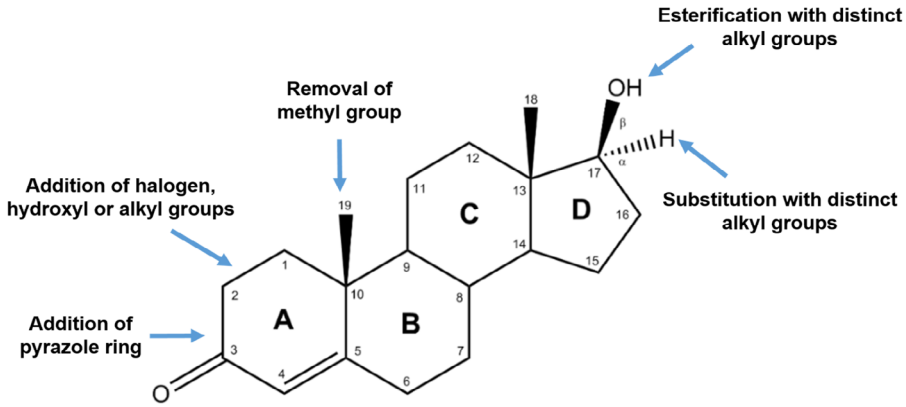

FIGURE 1 Main possible modifications in the structure of teststerone to synthetize anabolic agents

legislations about anabolic agents as their use is circumscribed to certain clinical conditions (Cordaro et al., 2011; I. R. Hullstein et al., 2015).

Although being restricted in several nations, the illicit use of AASs has been increasing to alarming levels among recreation and professional athletes (Neves et al., 2013). It is worth noting that the use of anabolic agents to enhance sportive performance is known as doping and this practice is thoroughly oversighted by anti-doping agencies which analyze bodily fluids from competing athletes to detect illicit substances (Fink et al., 2019). One possible explanation is that steroids are mainly commercialized online from which the World Health Organization (WHO) estimates that half of them are falsified (Antonopoulos & Hall, 2016; Arzamatzev et al., 2004). In general lines, a falsified medicine is a product mislabeled or with a different composition claimed in the package intentionally or not. These counterfeit drugs are a serious threat to public health since they have dubious sources, contain unknown compounds and are not regulated by sanitary agencies (Neves & Caldas, 2017).

In this context, the identification of formulations of AASs are essential for law-enforcement departments to ensure the fulfillment of the legislation (Neves et al., 2013). According to the literature, AASs can be detected by numerous techniques of which liquid (LC) or gas chromatography coupled to mass spectroscopy (GC–MS), and infrared (IR) spectroscopy are the most employed for the analyses (Chiong et al., 1992; Cho et al., 2015; Coopman & Cordonnier, 2012). The mentioned analytical tools are needed since they can provide relatively fast and accurate results to the high demand of samples (Deconinck et al., 2013).

For the detection of AASs, the extraction step is a critical phase of the analysis since these compounds are usually part of a complex matrix that can interfere considerably with the results by either masking the analyte or preventing its extraction (Deconinck et al., 2013). In order to overcome this obstacle, extraction procedures are performed under ultrasound to increase the recovery of the active ingredient in relatively short times and low solvent volumes (Yang et al., 2014). Another approach regularly used to minimize or eliminate the matrix from the extracted material is a clean-up procedure performed with nonpolar solvents, refrigeration or surfactant agents (Carignan et al., 1980; Chiong et al., 1992; Gonzalo-Lumbreras et al., 2005).

As it can be seen, the detection of AASs from formulations is of considerable importance in the forensic field mainly because these substances are controlled and yet there has been a growing number of apprehension. Despite the importance of the field, there is no comprehensive review of extraction methods or the analytical techniques for detection of anabolic agents from their formulations. Therefore, the aim of this work was to review the literature (1979–2021) concerning the analysis of apprehended formulations of AASs encompassing extraction and analytical approaches currently available.

2 | VISUAL INSPECTION

Visual inspection can be characterized as an initial analysis of the package, label and internal content of an apprehended pharmaceutical in order to visualize preliminary signs of falsification in the questioned sample (Kovacs et al., 2014). Detection of falsified products can be made through visual inspection because black market packages and letterings are generally labeled incorrectly. Besides, information such as trade name, active ingredient, address of manufacturer, name of laboratory, expiration and batch number are generally absent, adulterated or misspelled (Berneira et al., 2019; I. R. Hullstein et al., 2015). Recently, the World Health Professions Alliance released guidelines for visual inspection (Figure 2) based on the label content, providing substantial assistance for the detection of possible falsified samples (World Health Professions Alliance, 2011).

Previous reports indicated that visual inspection is a feasible tool for the identification of falsified anabolic agents produced from underground laboratories (Thevis et al., 2008). However, this procedure cannot distinguish label and package of falsified AASs from their legitimate counterparts in cases the falsification is meticulously executed to simulate the physical features of a genuine (Berneira et al., 2019; Coopman & Cordonnier, 2012). These types of falsification can be difficult to detect since faked labels can be found in high-quality printing and packages from veridical medicines can be reused by counterfeiters. In this sense, differences between falsified and genuine AASs are the size of the components, batch number or the chemical composition (Thevis et al., 2008). Besides these disadvantages, AASs may be apprehended in illicit laboratories while still under production without any form of label, packaging neither identification, which can also hamper or difficult visual inspection (I. R. Hullstein et al., 2015). Although useful, visual inspection serves only for preliminary investigation of suspected samples, and must be further supported by analytical techniques for the detection and quantification of their constituents (Berneira et al., 2019).

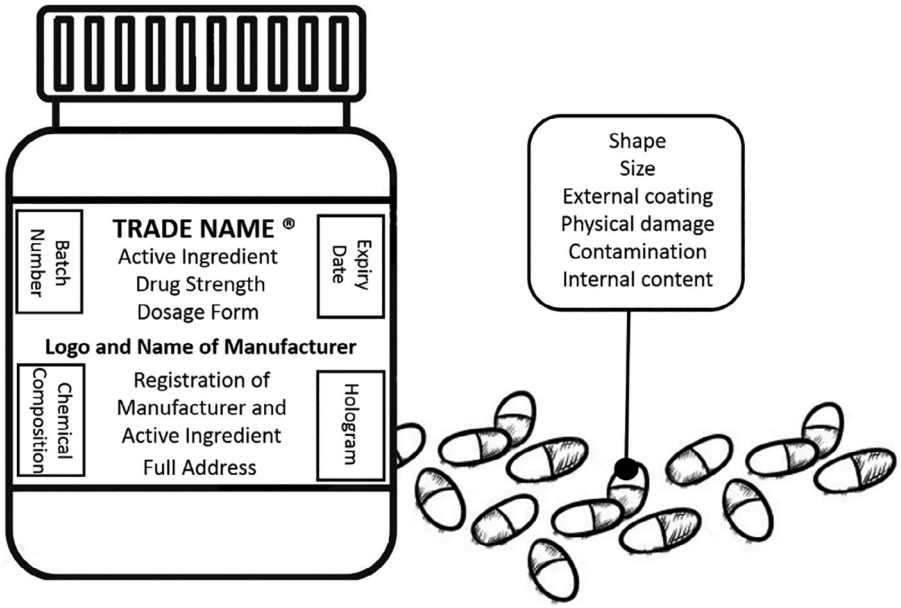

FIGURE 2 Visual elements evaluated in apprehended products

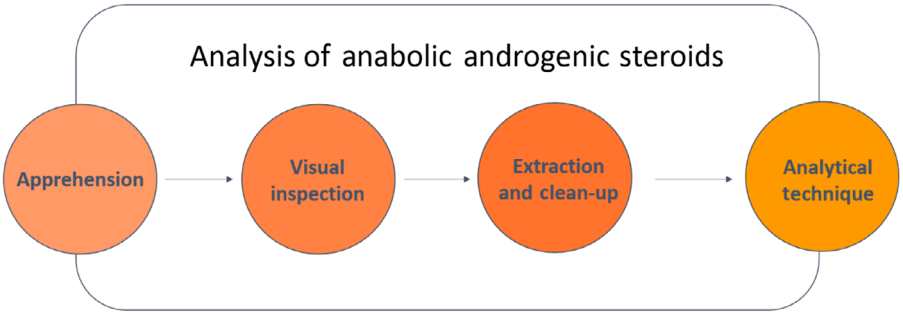

FIGURE 3 General steps in the analysis of formulation of anabolic androgenic steroids

3 | EXTRACTION AND CLEAN-UP APPROACHES

In general lines, an extraction procedure can be characterized as the migration of the analyte from the matrix to an extractor solvent. This process is one of the most influential steps in the analysis of formulations of AASs (Figure 3) since it affects the overall instrumental results. This procedure is very challenging in cases of apprehended anabolic agents because these compounds have similar structures and are distributed in distinct formulations (Chiong et al., 1992; Musshoff et al., 1997b). Besides, the matrix can interfere in several instrumental analysis including FT-IR spectroscopy and GC, in which the analysis of the crude formulation can result in weak, inconclusive spectra, co-elution of the analytes or equipment damage mainly in the column and in the detector (Chiong et al., 1992).

Liquid–liquid (LLE) or solid–liquid extraction (SLE) are analytical procedures in which an organic solvent is applied to extract a target molecule from a liquid or solid, respectively. These forms of extraction can be very laborious and demand high amounts of organic solvents with methods, for example, using up to 90 mL of methanol–water (9:1, v/v) to extract 1 mL of an oily-formulation (Carignan et al., 1980). Few research works employ SLE or LLE to the analysis of AASs due to the complex formulation in which these compounds are homogenized. Methanol (Musshoff et al., 1997a, 1997b; Neves et al., 2016; Prokudina et al., 2015) is the solvent of choice, however ethyl acetate (Prokudina et al., 2015), acetonitrile (Krug et al., 2014) or chloroform (Neves et al., 2016) have already been used in qualitative analyses (Chiong et al., 1992; Neves et al., 2016). In this sense, LLE was employed in the analysis of alleged formulations of Durateston®, in which chromatographic results indicated the presence of peanut oil, prosterna and testosterone propionate (Neves et al., 2016).

In order to increase the extraction efficiency while decreasing the amount of solvents or time required for the analysis of AASs, ultrasonic-assisted extraction (UAE) was developed and quickly established as the most conventional methodology of extraction. According to previous works, the application of acoustic energy in the analysis of formulations of AASs increased approximately in 5% the recovery of the active ingredient compared to SLE or LLE (Neves & Caldas, 2017). Enhancement in the extraction process can be linked to the formation of cavitation bubbles that arise in the solvent and explode with considerable values of pressure and temperature in the sample. Similarly to SLE and LLE, UAE is performed using methanol (Favretto et al., 2013; Musshoff et al., 1997b; Neves & Caldas, 2017) as the extractor solvent with sonication times of 5 (Gonzalo-Lumbreras & Izquierdo-Hornillos, 2003; Shi et al., 2008), 10 (Musshoff et al., 1997b; Neves & Caldas, 2017), 20 (Favretto et al., 2013; Mesmer & Satzger, 1997), or 30 min (Cho et al., 2015) at ambient temperature.

Previous studies indicated that AASs can be more efficiently extracted from the formulations using an ultrasonic probe than an ultrasonic bath. Results showed that the extraction generally increased more than 10% among the analytes by direct application of ultrasound waves in the sample (Berneira, dos Santos, et al., 2020). Microwave irradiation could also be a promising extraction approach as it could extract AASs with low solvent volume and little amount of time with satisfactory efficiency (Berneira, dos Santos, et al., 2020; Berneira, Silva, et al., 2020). It is worth noting that besides increasing extraction efficiency, the use of microwave irradiation and ultrasound is aligned with several green chemistry principles as their usage decreases solvent and sample amounts and, thus, they are able to reduce energy consumption, waste generation, human exposure, and solvents released to the environment (Llompart et al., 2019). The higher extraction of yield of microwave irradiation compared to conventional extraction techniques was associated with the focused energy in the sample and the increased temperatures that are formed during the extraction process (Berneira, dos Santos, et al., 2020; Berneira, Silva, et al., 2020).

The oily matrix of the formulations of AASs can hamper the detection of analytes and cause damages to specific parts of an analytical instrument including injectors and chromatographic capillary columns, for instance (Kozlik & Tircova, 2016). In this sense, the application of clean-up procedures is required after the extraction step in order to minimize or eliminate the matrix while maintaining the active ingredient. For this purpose, nonpolar solvents such as hexane, heptane, and tetrahydrofuran (THF) have been reported as efficient clean-up solvents to remove lipids (Chiong et al., 1992; Coopman & Cordonnier, 2012; Graham et al., 1979; Shi et al., 2008). Refrigeration to negative temperatures has also been used to solidify lipids with higher melting points (Carignan et al., 1980; Colman et al., 1991). Another possible approach is the use of sodium dodecyl sulfate (SDS) which increases the solubility of steroids in water without the co-extraction of other lipids due to the formation of micelles (Gonzalo-Lumbreras et al., 2005). Table 1 summarizes the methods found in the literature for the extraction and clean-up in the analysis of formulations of AASs.

4 | ANALYTICAL TECHNIQUES

Over the past years, several analytical techniques have been developed and validated for the analysis of formulations of AASs gradually shifting from conventional colorimetric and chromatographic procedures (Chiong et al., 1992) to a broad variety of spectrometric, spectroscopic, chromatographic, and thermal techniques (Berneira et al., 2019; Prokudina et al., 2015). However, the use of gas and liquid chromatography (LC) are still predominant accounting for approximately 50% of the publications in the field from 1979 to 2021. The mentioned analytical tools have considerable differences in cost, analysis time and instrumentation so that the analysts must choose the adequate procedure according to their needs and financial budget (Kovacs et al., 2014).

The application of analytical instruments is essential in the analysis of AASs formulations to detect the components of the sample, including the alleged active ingredients, adulterants as well as contaminants (Deconinck et al., 2013). Furthermore, the results can be used to classify a questioned sample as a counterfeit or a contraband material, which serves as important data in the overall outcome of judicial processes (Neves & Caldas, 2017). Therefore, the application of analytical techniques plays a major role in the analysis of apprehended AASs with the results serving as critical forensic evidence for legal purposes.

4.1 | Colorimetric procedures and ultraviolet–visible spectroscopy

Reactions that yield a characteristic color to a questioned sample encompass colorimetric procedures that can be used qualitatively to identify a target molecule or quantitatively when coupled to ultraviolet–visible (UV–vis) spectroscopy

TABLE 1 Extraction and clean-up procedures for the analysis of AASs

Technique	Analyte	Extraction and clean-up	Reference
FT-IR spectroscopy and GC–MS	TEs and PT	Approximately five droplets of the content of the injectables were diluted in methanol or chloroform	Neves et al., 2016
FT-IR spectroscopy, GC–MS, and TLC	FM, MD, MT, ND, OD, OM, ST, and TEs	Injectables were extracted with 1:2:1 of steroid–hexane–methanol under agitation. After centrifugation, the methanolic layer was retrieved for analysis	Chiong et al., 1992
GC x GC-TOF-MS	TEs, MD, and MT	Injectables were extracted with methanol while grinded tablets were extracted with ethyl acetate	Prokudina et al., 2015
GC-C-MS	TEs	Grinded tablets dissolved in methanol were incubated in a solution of sulfuric acid overnight and had the pH adjusted with a buffer and aqueous ammonium solution. The sample was extracted with hexane, evaporated, and dissolved in cyclohexane. For injectables, hexane was used for the initial dissolution instead of methanol and the subsequent procedure was kept the same	De la Torre et al., 2001
GC–HRMS and LC-HRMS	BU, DEs, MD, ME, NEs, OD, OM, ST, TEs, and TBEs	Tablets and capsules were extracted with a mixture (1:1) of acetonitrile–aqueous acetic acid (0.01%). Injectables were diluted with acetonitrile or methanol to a concentration of 10 μg mL^{-1}	Krug et al., 2014
GC–MS	MA, TE, DP, BU, NEs, TEs, MD, OD, OM, and ST	Tablets were extracted with methanol, sonicated, and centrifuged, retrieving the supernatant. Oily and aqueous solutions were extracted with 10:1 methanol–water, agitated, and centrifuged to isolate the supernatant for analysis	Musshoff et al., 1997a, 1997b
GC–MS	MT and MD	Tablets were extracted with methanol, sonicated, and filtered. The solution was dried and redissolved in methanol	Favretto et al., 2013
GC–MS	P, TEs, MD, S, OD, BU, and ND	Tablets, capsules, aqueous suspensions, and oily solutions were extracted with methanol, agitated, and sonicated. After centrifugation, the methanolic extract was diluted and analyzed with GC–MS	Neves & Caldas, 2017
GC–MS	ND, OM, OD, and ST	Extraction similar to Neves and Caldas (2017)	Berneira et al., 2019
GC–MS and GC-C-IRMS	TEs	Injectables were diluted in isopropanol and derivatized. Another aliquot of this material was also hydrolyzed in methanolic KOH solution under heating. After hydrolysis, the pH was adjusted and an extraction with tert-butyl methyl ether was performed. The dried organic fraction was derivatized. Both samples were analyzed with GC–MS	Forsdahl et al., 2011
GC–MS and GC-C-IRMS	BU and ND	The sample preparation of hydrolyzed and non-hydrolyzed steroids is similar to that of Forsdahl et al. (2011)	I. Hullstein et al., 2014
GC–MS and GC-C-IRMS	TE	The sample preparation for analysis was similar to that of Cawley et al. (2010)	Brooker et al., 2014
GC–MS and HPLC	TEs, NEs, and BEs	Samples were diluted in methanol to a final concentration of 0.1 mg mL^{-1}	Clark & Deruiter, 1990
GC–MS and LC–MS	ST, MD, TEs, OM, OD, NEs, TEs, DE, BE, MT, and FM	The sample preparation of hydrolyzed and non-hydrolyzed steroids is similar to that of Forsdahl et al. (2011)	I. R. Hullstein et al., 2015
GC–MS, GC–MS-NPD, and LC–MS–MS	TEs, OM, MD, ND, FM,, ST, BU, and TBEs	Tablets and injectables were extracted with methanol, sonicated, and centrifuged retrieving the supernatant, which was diluted for further analysis	Thevis et al., 2008

TABLE 1 (Continued)

Technique	Analyte	Extraction and clean-up	Reference
HPLC	TEs	Injectables were extracted with methanol–water (9:1) under constant agitation. The methanolic layer was retrieved, frozen to −8°C, and filtered	Carignan et al., 1980
HPLC	86 different AASs	Injectables, suspensions, and emulsions were extracted with methanol	De Beer, 1989
HPLC	MT	Injectables were extracted with methanol, sonicated, and filtered; the organic phase that was analyzed	Gonzalo-Lumbreras & Izquierdo-Hornillos, 2003
HPLC	TE and MT	Tablets were extracted with methanol, sonicated, and filtered. Injectables extracted with methanol were cleaned-up with THF	Shi et al., 2008
HPLC	26 different AASs	Tablets and injectables were extracted with methanol–water (7:3) and sonicated. After filtration, the samples were subjected to instrumental analysis	Cho et al., 2015
HPLC	TEs	Injectables were diluted in isopropanol and extracted with methanol under agitation and sonication	Kozlik & Tircova, 2016
HPLC and GC–MS	BU, TEs, and MD	Tablets, gel, and water solutions were diluted in methanol (1 mg mL^{-1} concentration) agitated, and sonicated. After centrifugation, samples were ready for GC–MS analysis. For injectables, the procedure was similar but initial dilution was made with hexane and methanol	Coopman & Cordonnier, 2012
HPLC and MEC	TEs	Tablets were extracted with a 0.1 M aqueous SDS under sonication and centrifuged. The aqueous layer was recovered and analyzed	Gonzalo-Lumbreras et al., 2005
HPLC and MS	TEs, NEs, BU, MA, MT, and FM	Tablets and injectables were extracted with methanol, sonicated, and the resulting material was filtered and analyzed	Mesmer & Satzger, 1997
GC–MS, FT-IR	TE and NEs	Tablets and injectables were extracted with methanol. Samples were extracted by SLE/LLE, sonicated or by microwave irradiation. The resulting material was filtered and analyzed	Berneira, dos Santos, et al., 2020
GC–MS, FT-IR	TP and ND	Similar procedure of Berneira, dos Santos, et al. (2020)	Berneira, Silva, et al., 2020
HPLC, GC-FID, and MEC	27 different AASs	Tablets were extracted with methanol under sonication and agitation. Injectables were agitated with methanol and filtered. For MEC analysis, an aliquot of the solution was cleaned-up with acetonitrile and SDS under sonication	Lurie et al., 1994
HPLC, GC–MS, and GC-C-IRMS	TEs	Samples were extracted with chloroform and an aliquot was dried and reconstituted in acetonitrile–water (6:4, v/v) for HPLC analysis	Cawley et al., 2010
IR spectroscopy, GC–MS, and GC-FID	MT, MD, BU, and OD	Tablets were extracted with chloroform, centrifuged, and dried. The residue was reconstituted in methanol. Injectables were extracted with methanol, centrifuged and the organic layer was frozen at −20°C. The supernatant was used for subsequent analysis	Colman et al., 1991
LC-HRMS and LC–MS–MS	33 different AASs	Sample preparation similar to that of Krug et al. (2014)	Weber et al., 2017
MEC	TEs	Samples were extracted with methanol and allowed to decant in order to retrieve the methanolic layer	Vindevogel & Sandra, 1991

(Continues)

TABLE 1 (Continued)

Technique	Analyte	Extraction and clean-up	Reference
UV–vis spectroscopy	TEs	Tablets were grinded and extracted in methanol while injectables were extracted in chloroform with both samples being subjected to a clean-up with acetonitrile-heptane (1:1)	Graham et al., 1979

Abbreviations: AASs, anabolic androgenic steroids; BE, boldenone ester; BU, boldenone undecylenate; DE, drostanolone ester; DP, drostanolone propionate; FID, flame ionization detection; FM, fluoxymesterone; FT-IR, fourier transform infrared spectroscopy; GC, gas chromatography; HPLC, high performance liquid chromatography; HRMS, high resolution mass spectrometry; IRMS, isotope ratio mass spectrometry; KOH, potassium hydroxide; LC, liquid chromatography; LLE, liquid–liquid extraction; MA, methenolone acetate; MD, methandrostenolone; ME, methenolone enanthate; MEC, micellar liquid chromatography; MS, mass spectroscopy; MT, methyltestosterone; ND, nandrolone decanoate; NE, nandrolone ester; NPD, nitrogen phosphorus detector; OD, oxandrolone; OM, oxymetholone; PT, prasterone; SDS, sodium dodecyl sulfate; SLE, solid–liquid extraction; ST, stanozolol; TBE, trenbolone ester; TE, testosterone ester; THF, tetrahydrofuran; TLC, thin-layer chromatography; UV–vis, ultraviolet–visible.

(Philp & Fu, 2018). AASs are known for undergoing colorimetric reactions with Liebermann's and Mandelin's test as well as when mixed with a solution of naphtol-sulfuric acid or concentrated sulfuric acid. This is mainly due to the presence of unsaturation in their chemical structure. Reactions with hydrazine and carbazide, for instance, also produce colored derivatives, which can be quantified by UV–vis spectroscopy in cases in which the anabolic agent has a keto group in its structure (Zivanov-Stakić et al., 1989).

Colorimetric procedures have the advantages of relatively low cost and short-time analysis, although interferences in the matrix may cause false negatives or false positives hindering a clear conclusion of the composition of a given sample (Graham et al., 1979; Philp & Fu, 2018). Previous works have shown that anabolic agents have different colors after spot tests, which could be used to initially distinguish and identify their compounds. Fluoxymesterone, for instance, turns yellow in contact with concentrated sulfuric acid, light green (if cooled) in naphtol-sulfuric acid solution, yellow in the Liebermann's test and yellow-brown in Mandelin's test color differences of an anabolic agent in distinct spot tests may be associated to different mechanisms that occur in each colorimetric analysis and to the formulation. In the Liebermann's test color is formed by the reaction of the hydroxyl group in ring A with the reagents while in the Mandelin's test color is formed due to reactions with steroidal unsaturation (Berneira, Silva, et al., 2020; Chiong et al., 1992; Moffat et al., 2011).

It should be noted that some anabolic agents do not yield colorful derivatives as it was observed for testosterone enanthate, oxandrolone, oxymetholone, and stanozolol, for example. In this sense, previous reports indicated that Mandelin's test is the most adequate colorimetric procedure compared to the other methods. Other obstacle in the analysis is that sulfuric acid may color the sample without any steroidal reaction. In this case, an experiment with more than two spot tests should be conducted in order to confirm the results (Chiong et al., 1992).

4.2 | IR spectroscopy

IR spectroscopy is a technique in which the interaction between an irradiation beam of the IR spectra region and a sample is analyzed enabling the initial elucidation of the structure of the components of the preparation. Based on the distinct absorption of the constituents of a molecule, it is possible to indicate the presence of different functional groups and, thus, initially propose organic and inorganic substances (Deconinck et al., 2013). The identification of the components of a sample can be assessed by comparing the obtained bands to compiled wavenumbers in which the functional groups are associated. The task has been gradually becoming more automatized as samples can be compared to reference materials or using appropriate mathematical algorithms (Sadlej-sosnowska & Ocios, 2007).

Initial analysis of AASs employing IR spectroscopy began in the early 1990s and to this day, the spectra of the majority of these substances are available in analytical databases (Chiong et al., 1992; Colman et al., 1991). Previous works have fully elucidated the existent vibrations in the structure of stanozolol and oxandrolone correlating the experimental results of FT-IR spectrum to calculations of density-functional theory. Theoretical and experimental values were demonstrated to be equivalent, indicating that the vibrational bands could be properly predicted and assigned to the structure of the analyzed compounds (Lemma et al., 2017).

This technique has several advantages compared to other conventional analytical analysis since it is nondestructive, fast and of low-cost, which are important features in cases when there is a large volume of samples to be analyzed (Rebiere et al., 2016). However, there are certain shortcomings in IR spectroscopy analysis since bands may be masked

or arise in the spectra by the matrix resulting in unclear or inaccurate results. Besides, AASs can exist as polymorphs, which can alter the aspect and position of bands in the spectra and hamper the detection of the AAS (Chiong et al., 1992). In this sense, IR spectroscopy is generally used as a complementary and screening technique to further analytical tools (Berneira et al., 2019).

Recently, IR spectroscopy has been combined with chemometry tools including principal component analysis (PCA) and partial least squares–discriminant analysis (PLS-DA) in order to detect possible falsifications or adulterations in a questioned sample based on previous results of supposed similar samples (Neves et al., 2016; Rebiere et al., 2016). PCA can be used as an exploratory analytical tool for the detection of outliers in a large batch of results. However, this chemometric procedure has to be associated with other analyses such as PLS-DA in order to maximize the discrimination of the results (Neves et al., 2016). In this sense, chemometry and spectroscopic techniques were associated to efficiently detect fraudulent formulations of Durateston® and anabolic tablets in previous works (Neves et al., 2016; Rebiere et al., 2016).

4.3 | Thin-layer chromatography

Thin-layer chromatography (TLC) is a type of planar chromatography in which the compounds present in a sample are separated depending on their interactions with certain stationary and mobile phases (Shewiyo et al., 2012). The stationary phase is consisted of a solid adsorbent generally coated on a support such as glass or aluminum (Siddiqui et al., 2017). On the other hand, the mobile phase is constituted of an organic solvent that can be used alone or combined with others to offer different polarities and enhance separation. This technique is known for being simple, sensitive, flexible, fast and of low-cost (Musharraf & Gulzar, 2012). It is the procedure of choice among pharmacopeias with regard to check the purity of pharmaceutical preparations including AASs (ANVISA, 2010; British Pharmacopoeia Commission, 2008).

In recent years, TLC has advanced to high performance thin-layer chromatography (HPTLC) by the use of adsorbents that are more efficient and with upgraded instrumentation (Shewiyo et al., 2012; Siddiqui et al., 2017). This enhanced technique has several advantages compared to conventional TLC such as standardization, higher resolution and possibility of sample quantification (Shewiyo et al., 2012). For quantitation strategies, densitometry has also been coupled to TLC or HPTLC (Dołowy et al., 2015). Together, the application of these features can be seen in the majority of works related to the analysis of AASs that use planar chromatography, alternatively to GC or LC (Musharraf & Gulzar, 2012; Zarzycki & Zarzycka, 2008).

Although extensively employed to analyze innumerous organic and inorganic compounds, TLC is still much unexplored to characterize AASs compared to other techniques. This can be related to the distinct chemical structures and polarities of anabolic agents that require distinct stationary and mobile phases for the adequate analysis of a particular active ingredient (Nowakowska et al., 2015). Usually, the organic solvents used in the process are nonpolar (e.g. hexane and toluene) (Dołowy et al., 2015; Musharraf et al., 2015) with a small amount of research works employing polar solvents such as methanol (Nowakowska et al., 2015; Zarzycki & Zarzycka, 2008).

Aluminum plates coated with silica gel 60F254 is the stationary phase commonly used though other types of adsorbents have been tested such as cyano-bonded and octadecyl-bonded silica gel with little success (Table 2) (Nowakowska et al., 2015). Since most of the AASs do not interact with UV-light, a spotting process is required to stain the compound in the TLC plate. This process can be achieved by the use of sulfuric or phosphomolybdic acid under heating (Dołowy et al., 2015; Musharraf et al., 2013) or with cerium sulfate and p-toluenesulphonic acid (Musharraf et al., 2015).

4.4 | Gas chromatography

Among separation techniques, GC is an analytical tool in which the separation of sample components is based on their interactions with a system composed of a chromatographic column and a carrier gas. There are certain requirements for an adequate chromatographic analysis, such as thermo stability and volatility of the analyte being the latter usually not exhibited by anabolic agents. When these requirements are not fulfilled, derivatization is needed to convert liable groups such as hydroxyl groups present in the structure of steroids into silyl derivatives enhancing interaction with the column (Favretto et al., 2013; Kauppila et al., 2011). GC is usually coupled to mass spectrometry (MS) so that the components of a questioned sample can be separated and identified based on the fragmentation pattern (Favretto et al., 2013).

TABLE 2 Thin-layer chromatography and high performance thin-layer chromatography procedures for the analysis of anabolic androgenic steroids

Sample	Mobile phase	Stationary phase	Visualization agents	Reference
Methyltestosterone	Hexane:acetone (6.5:3.5, v/v)	Aluminum plates precoated with silica gel $60F_{254}$	UV light (254 nm)	Musharraf et al., 2017
Testosterone, methyltestosterone, and trans-androsterone	Acetone:water (20:80, v/v)	Aluminum plates precoated with silica gel $60F_{254}$	Sulfuric acid:methanol (1:4, v/v) and heating	Nowakowska et al., 2015
Mesterolone	Chloroform: acetone (40:10, v/v)	Aluminum plates precoated with silica gel $60F_{254}$	Phosphomolybdic acid (10%) or concentrated sulfuric acid and heating	Dołowy et al., 2015
Mesterolone	Hexane:acetone (6.5:3.5, v/v)	Aluminum plates precoated with silica gel $60F_{254}$	Ceric sulfate and heating	Musharraf et al., 2015
Stanozolol	Ether:acetone (6:4, v/v)	Aluminum plates precoated with silica gel $60F_{254}$	Phosphomolybdic acid and heating	Musharraf et al., 2013
Testosterone esters	Hexane:ethyl acetate (8.5:1.5, v/v)	Aluminum plates precoated with silica gel $60F_{254}$	UV light	Musharraf & Gulzar, 2012
Testosterone propionate	Chloroform: diethylamine (19:1, v/v)	Aluminum plates precoated with silica gel $60F_{254}$	UV light (254 nm)	ANVISA, 2010
Testosterone enanthate	Dichloromethane: methanol (9:1, v/v)	Aluminum plates precoated with octadecylsilyl silica gel F_{254}	Alcoholic sulfuric acid solution and heating	British Pharmacopoeia Commission, 2008
Nandrolone decanoate and nandrolone phenylpropionate	Propan-2-ol: acetonitrile: water (6:4:2, v/v)	Aluminum plates precoated with octadecylsilyl silica gel F_{254}	UV light (254 nm)	British Pharmacopoeia Commission, 2008
Testosterone esters	Methanol:water (8:2, v/v)	Aluminum plates precoated with octadecylsilica (RP18W)	Phosphomolybdic acid (10%) in methanol and heating	Zarzycki & Zarzycka, 2008
Trenbolone acetate, fluoxymesterone, and oxymetholone	Chloroform: acetone (8:2, v/v)	Aluminum plates precoated with silica gel $60F_{254}$	UV light (254 nm) and *p*-toluenesulphonic acid	Leki et al., 2007

Abbreviation: UV, ultraviolet.

In general lines, derivatization is a process known for converting undesirable chemical moieties that could result in poor chromatographic analysis into derivatives with enhanced chromatographic properties. In the case of anabolic agents, hydroxyl and amine groups do not interact adequately with the chromatographic column leading to peak tailing in the chromatogram (dos Santos et al., 2014). In this sense, several authors have used silylation in order to convert these chemical groups into silyl derivatives mainly by the use of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) or trimethylsilyl iodide (TMSI) (Favretto et al., 2013). However, since this procedure is expensive and time-consuming, there are several other reports in which AASs are analyzed without a prior derivatization step (Berneira et al., 2019; Neves et al., 2016).

Techniques rather than MS may be coupled to GC such as a combustion (C) and isotope ratio mass spectrometry (IRMS). GC-C-IRMS is an analytical tool used to determine the relative ratio of isotopes of carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$), oxygen ($^{18}O/^{16}O$), and hydrogen ($^{2}H/^{1}H$) from the components of a sample. The measurement of the ratio of

TABLE 3 Conditions and parameters employed in GC

Capillary column	Flow rate (mL min^{-1})	Programed temperature	Derivatization step	Ref
HP5-MS	2.5 of helium	200–250°C (16 min) at 30°C min^{-1} to 300°C (8.5 min) at 30°C min^{-1}	–	Neves & Caldas, 2017
DB5-MS and BPX-50	–	90°C (1 min) to 300°C (3 min) at 20°C min^{-1}	–	Prokudina et al., 2015
Ultra 2	1.0 of helium	110°C (1 min) to 290°C (5 min) at 15°C min^{-1}	An aliquot of the extracted material was derivatized with MSTFA and TMSI under heating	Favretto et al., 2013
CP-SIL 8 CB	1.1 of helium	70°C (2 min) to 310°C (23 min) at 8°C min^{-1}	–	Coopman & Cordonnier, 2012
HP5-MS	1.0 of helium	100°C (2 min) to 290°C at 10°C min^{-1}	–	Pellegrini et al., 2012
DB17-MS	1.8 of helium	180°C (1 min) to 250°C at 12°C min^{-1} to 280°C at 3°C min^{-1} to 300°C (4 min) at 15°C min^{-1}	–	Cawley et al., 2010
HP5-MS	–	–	An aliquot of the extracted methanolic solution was derivatized with MSTFA under heating	Thevis et al., 2008
HP5-MS	–	100°C (2 min) to 290°C (25 min) at 30°C min^{-1}	–	Musshoff et al., 1997a, 1997b
DB1701	–	300°C (2 min) to 350°C (18 min) at 10°C min^{-1} (Method I) 275°C to 300°C (4 min) to 325°C (5 min) to 350°C (4 min) at 10°C min^{-1} (Method II)	–	Chiong et al., 1992
HP-1	1.0 of helium	180–265°C at 15°C min^{-1}	–	Colman et al., 1991
OV-1	–	70–150°C at 15°C min^{-1} to 250°C at 25°C min^{-1}	–	Clark & Deruiter, 1990

Abbreviations: GC, gas chromatography; MSTFA, N-methyl-N-trimethylsilyltrifluoroacetamide; TMSI, trimethylsilyl iodide.

isotopes can trace the source of raw materials used in synthesis of the AASs since they are related to several biological, chemical, and physical processes (Brooker et al., 2014). Distinct isotope ratios are also efficient in doping control as endogenous anabolic agents have higher values of ^{13}C than their exogenous counterparts (I. Hullstein et al., 2014). Conditions and parameters used in chromatographic analysis are describe in Table 3.

As it can be seen in Table 3, HP5-MS is the column usually chosen for the analysis of AASs although other stationary phases including OV-1, HP-1, and DB17-MS, for instance, are used for nonpolar to moderate polar analytes. The mentioned stationary phases can be used in high temperatures, which is essential in the separations of anabolic agents that elute in temperatures above 250°C. In this sense, programmed temperatures generally initiate at temperatures close to 100°C in order to detect the presence of low weight molecular compounds that could act as interferers, adulterants or contaminants and finish at approximate temperatures of 350°C to detect steroids with the highest molecular weight (Pellegrini et al., 2012). As previously discussed, derivatization is optional in the chromatographic analysis of AASs.

4.5 | Liquid chromatography

Alongside GC, LC is a technique used to separate the constituents of a sample due to interactions of the formulation with the stationary and mobile phases. Compounds with higher affinity by the mobile phase elute faster than those that

do not have similar polarity by the mobile phase. There are numerous possible combinations of stationary and mobile phases, although reversed phase LC is the most employed procedure for the analysis of AASs. After separation, the components of a sample can be detected by a diode array detector (DAD) or MS (Deconinck et al., 2013).

According to the literature, there are few available methods for the analysis of AASs employing LC techniques mainly due to chemical similarity between the compounds of this hormone class that can hamper chromatographic separation and subsequent detection. Other obstacle found is that procedures described in pharmacopeias for the analysis of anabolic agents were developed for only quality control tests. In this sense, forensic analysis of counterfeit drugs which may contain several unknown adulterants and contaminants would need different or adapted procedures (Shi et al., 2008).

Recently, optimized and validated methods to analyze AASs in oily formulations were developed in reduced time analysis (23 min to 6 min), maintaining satisfactory levels of sensitivity, precision, selectivity, and accuracy (Kozlik & Tircova, 2016; Prokudina et al., 2015). These results agree with the adequate development and optimization of analytical methods for LC analysis, which should result in peaks with reduced retention times, good resolution, shape, and sensitivity (Kozlik & Tircova, 2016). Conditions employing LC and its variations are indicated in Table 4.

As it can be noted in Table 4, C18 is the most used column in LC with small contributions of C8 and CN stationary phases. This octadecyl carbon chain bonded silica column is widely used because its nonpolar nature is ideal for the separation of AASs. On the other hand, previous research works have indicated that moderately polar stationary phases such as CN can also be used as they are more ordered and accessible than C18 (Clark & Deruiter, 1990; Gonzalo-Lumbreras et al., 2005). For mobile phases, acetonitrile and aqueous solutions of formic acid or acetic acid are used for the elution of steroids, although other authors have reported the use of water, THF, and buffers.

Generally, detection of anabolic agents is performed in DAD at wavelengths that varied from 190 to 360 nm, although over the past years, MS, MS–MS, and HRMS detectors have been also used for the identification of AASs. It should be noted that DAD is still preferable for the quantification of extracted steroids other than spectrometric approaches as it shows enhanced sensitivity, wide linear range and reproducibility (Mesmer & Satzger, 1997).

4.6 | Unconventional techniques

Illicit formulations of AASs often contain distinct concentrations than stated on the label or are found in oily forms, which make conventional chromatographic analysis difficult. Moreover, if a laborious sample preparation is not performed correctly, the complex matrix of the preparations can contaminate or damage parts of the analytical instrumentation resulting in inaccurate results and constant maintenance of the equipment (Doué et al., 2014; Kauppila et al., 2011). Therefore, novel techniques or applications have been searched and developed for the analysis of AASs (Table 5) such as new thermal, spectroscopic, or spectrometric techniques (Kovacs et al., 2014).

Thermal analysis, which includes differential scanning calorimetry (DSC) and thermogravimetric analysis (DTA), is a main area of study in the pharmaceutical industry for quality control to predict the stability of a formulation. Purity, compatibility and polymorphism of the constituents, for instance, can be evaluated efficiently by their calorimetric profile (Clas et al., 1999). In DSC analysis, the resulting thermogram is composed of curves that indicate physicochemical properties of the sample such as solid–solid transition, melting point, vaporization, crystallization and degradation, for example (Berneira et al., 2019).

For forensic purposes, thermal analysis is rarely employed as researchers use it as a complementary tool to indicate the presence of an alleged active ingredient by the calorimetric profile of the sample. In this sense, previous works have used DSC in the analysis of apprehended preparations of AASs for the detection of the melting point of the supposed active ingredient as an indicative if the sample was falsified or not, generating satisfactory results (Berneira et al., 2019). In another research paper, authors compared the effect of derivatization of anabolic agents using DSC in order to detect a potential enhancement on their thermal stability for GC–MS analysis showing that silylation prevented degradation at higher temperatures (Hadef et al., 2008).

There are other spectroscopic techniques such as proton nuclear magnetic resonance (^{1}H NMR), near-infrared (NIR), and Raman spectroscopy that have not been much employed in the analysis of anabolic agents when compared to FT-IR analysis. ^{1}H NMR spectroscopy is mainly used for structural elucidation of compounds despite the possibility of quantification as the area of the resulted peaks are proportional to the respective nuclei in the sample. The use of ^{1}H NMR could be beneficial as it shows universal detection and an adequate sensitivity (Ribeiro, Boralle, Felippe, et al., 2018; Ribeiro, Boralle, Pezza, & Pezza, 2018). Besides, the sample can be fully retrieved subsequently to the

TABLE 4 Conditions and parameters employed in LC and related techniques

Capillary column	Mobile phase	Gradient elution	Detection	Ref
C18	Acetonitrile (A) and 10 mM ammonium acetate buffer (B) at pH 4.5	80% of A until 1.5 min increasing to 100% of A and decreasing to 80% of A at 4 min	DAD at λ = 240 nm	Kozlik & Tircova, 2016
C18	0.1% aqueous formic acid (A, v/v) and acetonitrile (B)	20% of B (3 min) linearly increasing for 17 min to 100% of B (3 min) and returning to 20% of B (4.5 min)	MS	Cho et al., 2015
C18	25 mM triethyamine-phosphate buffer (A) and acetonitrile (B)	95% of A (30 min) to 30% of A (5 min)	DAD at λ of 220 and 254 nm	Coopman & Cordonnier, 2012
C18	Methanol–water (A, 62:38, v/v) and (B, 60:40, v/v) or methanol–water (C 70:30, v/v) and (D, 65:35 v/v)	–	DAD at λ of 221 and 245 nm	Shi et al., 2008
C18 or CN	Water–acetonitrile (60:40, v:v)	–	DAD at λ of 190 and 360 nm	Gonzalo-Lumbreras et al., 2005
C18	Water–acetonitrile (45:55, v/v)	–	DAD at λ of 245 nm	Gonzalo-Lumbreras & Izquierdo-Hornillos, 2003
C18	Acetonitrile (A) and water (B)	60% of A (20 min) to 90% of A (2 min) to 95% of A (7 min)	DAD	Mesmer & Satzger, 1997
C18	Water (A) and methanol (B)	45% of B to 100% of B gradually in 12 min and maintaining this proportion for 1 min	DAD at λ of 240 and 280 nm	De Beer, 1989
C18	Methanol (A) and water (B)	–	DAD at λ of 254 and 280 nm	Clark & Deruiter, 1990
C18	Methanol–THF–water (57:11:32, v/v/v)	–	DAD at λ of 240 nm	Carignan et al., 1980
C18	0.1% aqueous formic acid (A, v/v) and acetonitrile (B)	1% of B (0.1 min) to 35% gradually in 30 min to 80% of A within 5 min finishing with 1% of B	MS	I. R. Hullstein et al., 2015
C8	5 mM ammonium acetate in 0.1% aqueous acetic acid (A, v/v) and acetonitrile (B)	75% of A to 5% of A (8.6 min) gradually in 4 min (Method I) 95% of A to 5% of A (5 min) gradually in 8 min	MS–MS	Thevis et al., 2008
C8	0.1% aqueous formic acid (A, v/v) and 0.1% formic acid in acetonitrile (B, v/v)	0% of B to 100% of B gradually in 25 min	DAD at λ of 190 to 600 nm and ESI-TOFMS	Nielen et al., 2001
C18	0.2% aqueous formic acid (A, v/v) and acetonitrile (B)	0% of B to 100% of B (4 min) gradually in 5 min, decreasing to 0% of B gradually in 5 min	MS–MS	Weber et al., 2017
C18	0.1% aqueous formic acid (A, v/v) and 0.1% formic acid in acetonitrile (B, v/v)	2% of B to 100% of B (3 min) gradually in 5 min decreasing to 2% of B gradually in 5 min	HRMS	Weber et al., 2017
C18	0.1% aqueous formic acid (A, v/v) and 0.1% formic acid in acetonitrile (B, v/v)	20% of B to 100% of B (3 min) gradually in 15 min decreasing to 20% of B (3 min) in 1 min	MS/MS	Tircova et al., 2019

Abbreviations: ESI-TOF MS, electrospray ionization time of flight spectrometry; HRMS, high resolution mass spectrometry; DAD, diode array detector; LC, liquid chromatography; MS, mass spectroscopy; THF, tetrahydrofuran.

TABLE 5 Unconventional techniques used for the analysis of AASs

Technique	Compounds	Observations	Reference
DSC	Stanozolol, oxymetholone, oxandrolone, and nandrolone decanoate	Melting point was evaluated in order to detect possible fraudulent formulations	Berneira et al., 2019
DSC	Androsterone, nandrolone, testosterone propionate, and nandrolone propionate	Thermal stability of compounds was measured purely or as TMS derivatives for posterior GC–MS analysis	Hadef et al., 2008
^{1}H NMR spectroscopy	Testosterone propionate, methyltestosterone, acetate methenolone, oxandrolone, nandrolone decanoate, and stanozolol	Substances were identified and quantified using an internal reference standard	Ribeiro, Boralle, Pezza, & Pezza, 2018
^{1}H NMR spectroscopy	Testosterone esters, stanozolol, drostanolone propionate, trenbolone acetate, oxymetholone, and methandrostenolone	Substances were identified and quantified using an internal reference standard	Ribeiro, Boralle, Felippe, et al., 2018
^{1}H NMR spectroscopy	Mesterolone and oxymetholone	Substances were identified and quantified using an internal reference standard	Monakhova et al., 2013
NMR spectroscopy	Nandrolone, stanozolol, and testosterone	Apprehended formulations were identified	Chiong et al., 1992
NIR and Raman spectroscopy	Methandienone and methyltestosterone	Presence, or modification of the marketed active ingredient was evaluated as well as the chemical image of the samples	Rebiere et al., 2016
ASAP-MS	Testosterone esters, nandrolone esters, and boldenone esters	Identification of alleged AASs esters purchased from illicit websites as well as determination of their mechanisms of fragmentation	Doué et al., 2014
DAPPI-MS	Methandrostenolone, mesterolone, oxymetholone, testosterone esters, trenbolone enanthate, nandrolone decanoate, boldenone undecylenate, and stanozolol	Identification of apprehended formulations of AASs	Kauppila et al., 2011
DART-MS	Testosterone esters, nortestosterone esters, boldenone esters, methyltestosterone, mestanolone, methandienone, and oxandrolona	Identification of apprehended formulations of AASs	Prokudina et al., 2015
MEC	Testosterone esters	Resolution of the chromatogram was optimized for parameters such as buffer pH, buffer concentration, level of organic modifier, surfactant concentration, and use of mixed micelles	Vindevogel & Sandra, 1991
MEC	29 different AASs	Development of method for the analysis of AASs	Lurie et al., 1994
MEC	Methyltestosterone	Development and validation of method for the analysis of AASs	Gonzalo-Lumbreras & Izquierdo-Hornillos, 2003
MEC	Androstenedione, fluoxymesterone, methyltestosterone, testosterone, methandienone, and 17-epimetandienone	Development and validation of method for the analysis of AASs	Amundsen et al., 2004

Abbreviations: ^{1}H NMR, proton nuclear magnetic resonance; AASs, anabolic androgenic steroids; ASAP-MS, atmospheric solids analysis probe coupled to mass spectroscopy; DAPPI-MS, desorption atmospheric pressure photoionization coupled to mass spectroscopy; DART-MS, direct analysis in real time coupled to mass spectroscopy; DSC, differential scanning calorimetry; GC–MS, gas chromatography coupled to mass spectroscopy; MEC, micellar liquid chromatography; NIR, near-infrared; TMS, trimethylsilyl iodide.

analysis and further measurements in two dimensions can be made for more information about the target molecules. In this sense, a 400 MHz ^{1}H NMR was used to identify AASs and other apprehended materials as well as to determine the concentration of analytes with an internal reference standard. Satisfactory results were obtained with a detection

limit that ranged from 0.5 to 2 mg kg^{-1} (Monakhova et al., 2013). NMR spectroscopy was used in another work for the identification of apprehended AASs (Chiong et al., 1992).

At the same time, NIR and Raman spectroscopy are techniques that use the interaction of a sample with light to determine its spectral profile and the preliminary chemical structure of the active ingredient. Besides, it is possible to detect the distribution of constituents in a sample with a mathematical treatment of several obtained spectra. It should be noted that these exploratory techniques were nondestructive and had other advantages that include short time of analysis, low cost, high sensitivity, and small sample amounts (Rebiere et al., 2016).

Few research works make use of NIR or Raman spectroscopy for the analysis of AASs. Among them, supposedly methandienone tablets were analyzed in a report with NIR and Raman spectroscopy, in which the first technique was able to assess the homogeneity of the samples while the latter was able to profile the formulation. The combination of both spectroscopic techniques could detect variations in composition of the constituents of the samples as well as the presence or not of the marketed active ingredient. The authors highlighted that the information obtained by these techniques could lead to a better understanding of how illegal pharmaceuticals are manufactured and distributed through the illicit market (Rebiere et al., 2016).

Parallel to the techniques described above, there are novel ionization and spectrometric approaches including atmospheric solids analysis probe (ASAP), desorption atmospheric pressure photoionization (DAPPI), and direct analysis in real time (DART) coupled to MS. These techniques have recently been developed as variations from other ionization tools. For DAPPI and ASAP, the sample is vaporized under a hot stream of solvent gas and the material is later ionized by plasma, resulting in protonated molecules (Doué et al., 2014; Kauppila et al., 2011). In DART, helium gas is ionized in order to charge the sample that is subsequently transferred to mass analyzers (Prokudina et al., 2015). Generally, polar or nonpolar analytes can be analyzed by these techniques (Doué et al., 2014).

Previous research works employing ASAP coupled to MS have shown that esters from testosterone, nandrolone and boldenone have neutral mass loss of m/z 96.1 in common, which could allow its quick detection in the questioned sample. Moreover, identification of the active ingredient from illicit formulations of AASs purchased on the Internet was possible within 2 min with little sample preparation (Doué et al., 2014). Similarly, DAPPI was employed in another study for the analysis of several AASs formulations, in which active ingredients were detected within seconds and without any type of sample preparation. It should be mentioned that the authors also determined the minimum distance (2 and 9 mm from sample-MS inlet for liquid and solid samples, respectively) that the sample could be placed in the chamber of analysis without causing cross-contamination or memory effect in a sequential tests (Kauppila et al., 2011). Finally, DART was assessed for the analysis of confiscated AASs also demonstrating promising results (Prokudina et al., 2015).

Another technique that can be used in the analysis of AASs is micellar liquid chromatography (MEC), which is a variant of LC that employs lower amounts of mobile phases and, thus, have relatively reduced toxicity and costs. Besides, MEC enables the injection of biological samples containing proteins and other biochemical molecules directly into the column as the micellar phase is able to dissolve these compounds (Gonzalo-Lumbreras & Izquierdo-Hornillos, 2003; Lurie et al., 1994). However, there are some drawbacks compared to LC mainly related to the reduced mass transfer of the sample between stationary and mobile phases, which diminishes the efficiency of chromatographic separations. Although MEC has lost space to conventional techniques in the early 2000s, several methods have been optimized and validated for the analysis of AASs.

5 | CONCLUSION

The current study reviewed research papers related to the extraction and analysis of AASs. In this sense, this review could assist forensic scientists in the area since it provided a comprehensive and critical guide of extraction methods and analytical techniques regarding the analysis of anabolic agents. According to the reviewed reports, there are several extraction methods and analytical techniques available for the analysis of formulations of AASs. Nonetheless, ultrasonic bath-based methods are the most common procedures in the extraction of anabolic agents. This is due to their low solvent use, low cost and high sample input compared to LLE or SLE. Recently, other promising extraction methods using ultrasonic probe or microwave irradiation have been developed increasing extraction efficiency compared to conventional methods. However, these procedures have yet to be analytically validated using, for instance, precision, limits of detection, limits of quantitation, and accuracy studies.

Among analytical techniques, chromatographic tools mainly as gas and liquid chromatography coupled to MS are the procedures usually chosen for the determination of the constitution of anabolic agents. Besides, novel analytical approaches have been developed and validated to assist in the identification of anabolic agents comprising of procedures that use thermal, spectroscopic, and spectrometric methods. Since illicit formulations of AASs have a complex matrix, a combination of analytical techniques is usually required in order to obtain adequate results. Thus, the availability of several techniques could assist the analyst in the forensic analysis. Therefore, the extraction and analysis of AASs formulations are important fields which require continuous development and optimization of methods and techniques in order to provide adequate analytical results to criminal and judicial cases.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Lucas Berneira: Writing - original draft. **Tais Poletti:** Methodology. **Samantha de Freitas:** Conceptualization. **Caroline da Silva:** Writing-review & editing. **Rafael Ortiz:** Project administration. **Claudio Martin Pereira de Pereira:** Funding acquisition.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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FURTHER READING

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Capítulo 2

Chemical and cytotoxicity evaluation of apprehended formulations of anabolic-androgenic steroids

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Chemical and cytotoxicity evaluation of apprehended formulations of anabolic androgenic steroids

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ABSTRACT

Apprehensions of formulations of anabolic-androgenic steroids (AASs) have been significantly increasing worldwide. It is known that the consumption of synthetic steroids can lead to several side effects including liver damage, heart failure and psychological disorders. Nonetheless, there are few studies evaluating the toxicity of anabolic agents' formulations. In this sense, the aims of this work were to determine the chemical profile of AASs apprehended in Brazil by Gas Chromatography-Mass Spectrometry as well as to determine their cytotoxicity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results showed that most formulations had their constitution adulterated or falsified by the lack of active ingredients. By the colorimetric MTT assay, it could be observed that most anabolic agents had cytotoxicity in concentrations of 0.5 mg mL^{-1} in a general dose-dependent manner. Product #3 can be cited as displaying minimum cell viability (5.67 ± 0.25%) at 0.5 mg mL^{-1} increasing to 91.82 ± 4.78% at 0.125 mg mL^{-1}. Therefore, in this study, apprehended formulations of AASs were mostly counterfeit materials with *in vitro* cytotoxicity, which shows the importance of analyzing these materials and indicating the adverse effects they pose to human health.

1. Introduction

Anabolic-androgenic steroids (AASs) comprise a vast chemical class of synthetic testosterone derivatives which are known for their illicit use by recreational and professional athletes [1]. These compounds can be found in several formulations mainly as oily solutions, aqueous suspensions or tablets depending on the intended pharmacological properties [2]. Given their high illicit use and health hazard, synthetic steroids are controlled worldwide, in which their illegal distribution and consumption are characterized as criminal offenses [3].

Over the past years, formulations of anabolic agents have been extensively apprehended by law-enforcement agencies [4,5]. Most of these formulations are falsified materials that, according to the World Health Organization, are comprised of mislabeled and/or chemically adulterated pharmaceutical agents [6]. In this sense, counterfeit AASs are known to increase the side effects associated with the use of synthetic steroids including liver damage, heart failure, psychological disorders, infection risks, multiple-organ dysfunction among other possible adverse physiological reactions [7–10].

Once apprehended, anabolic agents can be submitted to different types of analytical procedures in order to have their chemical constitution established and associated with possible health hazards [2,5]. According to the literature, techniques including Gas Chromatography, Infrared Spectroscopy and Mass Spectrometry are the most common analytical tools in order to analyze apprehended materials [6,11,12]. Furthermore, AASs can have their biological action studied by *in vitro* and *in vivo* models as described by Zeleroth et al. and Basile et al. Nonetheless, the models reported on these studies were not used to evaluate illegal formulations of anabolic agents.

As previously mentioned, analysis of the formulations o AASs are important in order to ascertain potential health hazards associated with the use of these substances. In previous studies, formulations of synthetic steroids were not analyzed, so potential health hazards associated with them were not determined [13,14]. In this sense, the aims of this study were to analyze apprehended formulations of anabolic agents provided from the Brazilian Federal Police by Gas

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Chromatography-Mass Spectrometry (GC–MS) and to determine their cytotoxicity by 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) assay.

Materials and methods

Materials and samples

Dimethyl sulfoxide (DMSO), chloroform and MTT were purchased from Sigma-Aldrich and used without further purification. For the biological tests, 96-well sterile polystyrene microplates were acquired from Kasvi (São José dos Pinhais, Brazil) as minimum essential medium and fetal bovine serum was obtained from Acumedia (Lansing, USA). Samples were obtained from the Brazilian Federal Police after an apprehension made in the Rio Grande do Sul state (Table 1) in 2019.

Gas chromatography-mass spectrometry analysis

For chromatographic analysis, 25 μL of the formulations were diluted in 1 mL of chloroform following the method of Neves et al. (2017). Samples were vortexed for 1 min and further injected in a GC–MS model QP2020 (Shimadzu, Kyoto, Japan) in split mode (1:50) using helium flow and a RTx-5MS capillary column (Restek, Bellefonte, USA). The injection port and the transfer line operated at 280 °C and 300 °C, respectively. The initial temperature of the oven was 100 °C (2 min) ramping 30 °C min^{-1} to 280 °C and 2 °C min^{-1} to a final temperature of 310 °C (10 min). Identification of the sample constituents was made by comparison of the mass spectra with the equipment's NIST-17 library. Analysis were perfomed in triplicate ($n = 3$).

Cytotoxic assay

Cytotoxic assay of the formulations was performed using Madin Darby Bovine Kidney (MDBK) cells cultured at 37 °C in minimum essential medium supplemented with fetal bovine serum (10%, *v/v*). Cells were cultured achieving a density of 3×10^4 cells per well for 24 h at 37 °C within an atmosphere containing 5% of carbonic gas and 95% of humidified air.

Experiments were made in triplicate ($n = 3$) and repeated twice in independent experiments using solutions of the formulations in minimum essential medium from 0.5, 0.25, 0.125 and 0.062 mg mL^{-1}. Soybean, peanut oil and cells without treatment were used as negative controls. Subsequent to this procedure, cells were incubated under similar temperature and atmospheric conditions for 24 h. After this period, 50 μL of a MTT solution (1 mg mL^{-1}) were applied to plates and incubated in similar conditions described above for 3 h. Afterwards, the supernatant was removed and formazan crystals were solubilized in 100 μL of DMSO for 10 min. Finally, plates were analyzed in spectrophotometer at 540 nm [15,16] .

Table 1
Main characteristics of the studied samples.

Sample	Form	Alleged Compound	Identified Compound
Product #1	Aqueous suspension	Stanozolol	Stanozolol
Product #2	Oily solution	Testosterone esters	Testosterone esters
Product #3	Oily solution	Testosterone cypionate	Testosterone cypionate
Product #4	Oily solution	Boldenone undecylenate	Nandrolone decanoate
Product #5	Oily solution	Testosterone esters	Testosterone and nandrolone esters
Product #6	Oily solution	Testosterone propionate	Testosterone and nandrolone esters
Product #7	Aqueous suspension	Stanozolol	No active ingredient detected
Product #8	Oily solution	Testosterone propionate	No active ingredient detected
Product #9	Oily solution	Trenbolone acetate	No active ingredient detected

Statistical analysis

Statistical differences in the resulting cell viabilities after exposure to anabolic agents' formulations were assessed by Analysis of Variance (ANOVA) followed by post-hoc Tukey's Test ($p < 0.05$) using Graphpad software version 7 (La Jolla, USA). Chromatographic and biological assays were performed in triplicate ($n = 3$) and results were expressed as mean ± standard deviation.

Results

Gas chromatography-mass spectrometry analysis

It was observed by chromatographic analysis (Fig. 1) that most formulations were counterfeit materials since the components stated on the label were not detected. The exceptions were for Product #1, #2 and #3 in which the labeled active ingredient (stanozolol, testosterone esters and testosterone cypionate, respectively) was found. In this sense, for Product #4 nandrolone decanote was detected instead of boldenone undecylenate while Product #5 and #6 had other synthetic steroids besides the ones stated on their label (testosterone esters and testosterone propionate, respectively). Finally, no active ingredients were found in Product #7, #8 and #9, which were only composed of excipients. Generally, benzyl alcohol, benzyl benzoate and benzyl linoate were the excipients found in most samples besides the vegetable oils. GC–MS chromatograms can be found in the *Supplementary Information* section.

Cytotoxic assay

Cell viability assay (Table 2) indicated that most anabolic agents displayed cytotoxicity towards MDBK cells at the maximum tested concentration of 0.5 mg mL^{-1}. Generally, toxicity decreased proportionally to the concentration of the sample reaching a maximum viability at the minimum tested concentration of 0.062 mg mL^{-1}. Soybean and peanut oils were used as controls as they were the basic components of the formulations and did not significantly influenced the cellular viability, which remained at its maximum value.

As it can be observed in Table 2, Product #8, #9 and #3 were the formulations that had the most cytotoxic effects at 0.5 mg mL^{-1} compared to the other samples reaching minimum levels of cellular viability for Product #8 and #9 while Product #3 had a cellular viability of 5.67 ± 0.25%. These values were gradually increasing as the concentrations were lower, maintaining maximum cell viability for Product #8 and Product #3, with a cell viability of 85.51 ± 4.60% at 0.062 mg mL^{-1} for Product #9. Regarding the other samples, a similar pattern was observed, although the cellular viability was higher compared to Product #8 and #9. Finally, maximum cellular viability was observed under exposure of cells to Product #6 at 0.5 mg mL^{-1}, which was only observed to the other formulations at lower concentrations.

Discussion

Previous studies have underlined that approximately 31.7% to 50% of illegal AASs formulations apprehended by law-enforcement agencies are comprised of counterfeit materials [1,4]. In this study, a third of the samples were falsified either by the presence of other unstated synthetic steroids in the composition or by the absence of the labeled active ingredient, which is in agreement with previous reports. These illicit formulations are known for causing hazardous effects on users because of the adverse effects its constituents may cause, and also as the manufacturing process generally does not comply with sanitary or

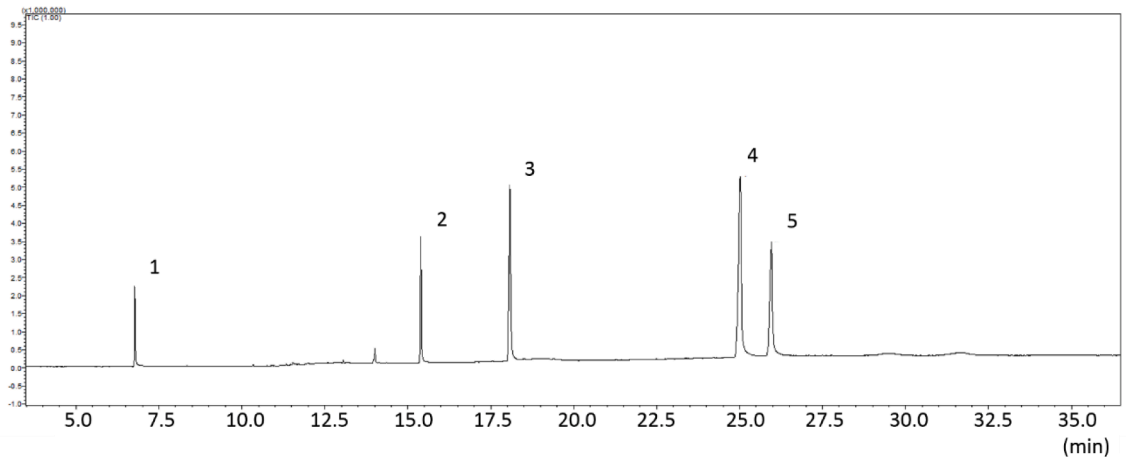

Fig. 1. GC–MS analysis of the formulation of Product #2 (1 - benzyl alcohol; 2 -testosterone propionate; 3 - testosterone isocaproate; 4 - testosterone decanoate; 5 - testosterone phenylpropionate).

Table 2
Cellular viability (%) in function of distinct concentrations of anabolic agents.

Sample	Concentration (mg. mL^{-1})	Cell viability (%)		
	0.500	0.250	0.125	0.062
Product #1	78.91 ± 1.17^{a}	100 ± 0.00^{b}	100 ± 0.00^{b}	100 ± 0.00^{b}
Product #2	94.40 ± 4.77^{a}	96.80 ± 0.24^{ab}	98.38 ± 0.62^{ab}	100 ± 0.00^{b}
Product #3	5.67 ± 0.25^{a}	62.47 ± 0.00^{b}	91.82 ± 4.78^{c}	100 ± 0.00^{d}
Product #4	86.82 ± 1.55^{a}	87.37 ± 4.95^{a}	94.17 ± 0.00^{b}	99.79 ± 0.12^{b}
Product #5	77.34 ± 2.03^{a}	97.16 ± 0.79^{b}	100 ± 0.00^{b}	100 ± 0.00^{b}
Product #6	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
Product #7	78.91 ± 1.17^{a}	85.16 ± 3.15^{b}	89.99 ± 3.73^{c}	99.30 ± 0.60^{d}
Product #8	0.00 ± 0.00^{a}	8.05 ± 3.76^{b}	63.90 ± 3.72^{c}	100 ± 0.00^{d}
Product #9	0.00 ± 0.00^{a}	3.79 ± 0.67^{a}	44.35 ± 3.54^{c}	85.51 ± 4.60^{d}
Soybean oil	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
Peanut oil	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}

Results expressed as mean ± standard deviation. Different superscripts in each row are statistically different at Tukey's Test ($p < 0.05$).

quality control guidelines [2,6].

Regarding cytotoxicity, it was found that the majority of the formulations of anabolic agents were toxic to MDBK cells at the tested concentrations. This behavior was previously highlighted by other studies that applied pure AASs to cell cultures, which suggested that cellular damage was caused by direct induction of apoptosis produced by the exposition to synthetic androgens [14,17]. Moreover, it has been indicated that consumption of sexual hormones may also result in genotoxicity and carcinogenicity depending on the dose size and exposure time as well as genetic and epigenetic factors [13,18].

It is worth noting that the potential to induce apoptosis is associated to the anabolic agent used, which, in a previous study was determined to be in the following order: nandrolone > testosterone > stanozolol > trenbolone [13]. Reasons that can explain why this cytotoxic order was not observed in the current study include but are not limited to: I) most AASs were in their respective ester derivative; II) anabolic agents were not applied isolated, but in their original formulations; and III) there may be some pharmacological differences among synthetic steroids [13].

According to previous studies conducted by Berneira et al. and Berneira et al., oily formulations of ASSs are composed of vegetable oils which may include peanut and soybean oil and other excipients such as benzyl benzoate, ethyl palmitate and ethyl oleate. It is worth noting that these components and its respective concentrations vary considerably among falsified products. These excipients are used in order to solubilize the anabolic agents and decrease the product viscosity. Nonetheless, exposure to benzyl benzoate has been associated to several adverse effects which include dermatitis, seizures and anaphylactic shock [1,2].

Moreover, the presence of medium and long-chain fatty acids as reported by Berneira et al. in the analysis of apprehended AASs could also be associated to its toxicity [2]. In this sense, Pacheco et al. evaluated the effect algal extracts composed of fatty acids to human breast cancer cell lines and observed cytotoxicity at concentrations of 100 to 200 μg/mL. The cytotoxic effect was associated to multiple mechanisms which include chances in free radicals and reactive oxygen species production, alterations in gene expression and signal transduction as well as inhibition of cell growth and increased apoptosis [19].

As it can be observed in **Table 2**, concentration and types of AASs played a major role in the cytotoxicity of the samples. A similar pattern was also indicated by Zelleroth et al., who applied several anabolic agents at concentrations of 100, 30 and 10 μM to primary rat cortical cells. On the other hand, Product #7, #8 and #9, which did not had their active ingredients detected, were also cytotoxic. In these cases, their excipients or adulterants possibly caused the reduction on the MDBK cells viability. Given this, results were in line with previous reports, which indicated that counterfeit formulations could present health hazardous due to their uncertain components and their respective concentrations [20,21].

Pomara et al. analyzed the effect of increasing concentrations of nandrolone in R2C culture cells and observed minimum levels of cell viability at 500 μg/mL which increased as the tested concentrations of nandrolone diminished reaching approximately 80% of cell viability at concentrations below 15.6 μg/mL. Comparing these results to the reported in the current study, it can be observed that cytotoxicity was lower in our research work which can be mainly attributed to the lower concentration of AASs in the samples and the distinct cell lines used in the studies [22].

We believe that the current study advanced the understanding of the cytotoxicity of distinct AASs in their original formulations. Nonetheless, there were some potential limitations in this research. Initially, MDBK cells were chosen due to their sensibility to xenobiotics, although cell lines cannot reflect the overall toxicity found in the organism as a whole. In this sense, further studies using tissues and animals could

complement the results. Moreover, concentrations of synthetic steroids may not be similar to real cases as illegal formulations of AASs have unknown concentration and composition and illicit users have distinct exposure rates depending on the use [2,9]. Nonetheless, the results showed that micromolar concentrations of anabolic agents had considerable toxicity on the tested cell culture.

Conclusion

The current study demonstrated that anabolic agents in their respective formulations caused reduction in cell viability, displaying cytotoxicity under the tested conditions. Moreover, it was determined by chromatographic analysis that most illicit formulations had distinct constituents than the ones described on the label or no active ingredient at all. In this sense, it was confirmed that illegal formulations of synthetic steroids have unknown composition that can potentially cause health hazards. These findings give support to previous works, and highlight how harmful the unadvised use of synthetic counterfeit steroids can be. Further studies may be developed with different apprehended samples or using different types of cell cultures in order to better understand the cytotoxic effects illicit formulations of anabolic agents.

Declaration of Competing Interest

None.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.talo.2022.100101.

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Capítulo 3

Extraction and identification of formulations of anabolic androgenic steroids: A forensic educational approach

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EXTRACTION AND IDENTIFICATION OF FORMULATIONS OF ANABOLIC ANDROGENIC STEROIDS: A FORENSIC EDUCATIONAL APPROACH

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The use of anabolic androgenic steroids is largely restricted worldwide since they are associated with innumerous side effects and health damage. However, apprehensions of formulations of anabolic agents have been extensively increasing throughout the world, making their analysis a common task in the forensic field. In this sense, a set of three experiments was developed and applied to undergraduate students of Forensic Chemistry dealing with visual inspection of apprehended formulations of anabolic androgenic steroids as well as chemical identification by Infrared Spectroscopy and Gas Chromatography-Mass Spectrometry. Moreover, the classroom evaluated the extraction of steroidal active ingredients by ultrasonic and microwave-assisted extraction. The experimental procedures allied with the theory behind these topics provided an important background to the students on distinct extraction methods and analytical techniques used in the analysis of anabolic agents. Finally, the classroom was led to critically evaluate and interpret the results as well as to question the adequacy of experimental methods used in forensic analysis to stimulate their critical thinking.

Keywords: chemical education; instrumentation; extraction procedures; forensic chemistry; anabolic androgenic steroids.

INTRODUCTION

Anabolic androgenic steroids (AASs) comprise a class of derivatives of testosterone that are known for their illicit use among athletes and the population in general in order to obtain a higher sportive performance and muscle mass. The abusive use of these substances is associated with several health hazards that include physical disorders, coronary damage, liver failure as well as neuropsychiatric problems.[1,2] In this sense, formulations containing AASs are controlled or prohibited worldwide in order to regulate their distribution and consumption.[3]

Despite the measures to control the consumption of anabolic agents, there has been a considerable increase in apprehensions of formulations of AASs, which are mainly supplied by illicit manufacturers. Underground suppliers are not regulated by health agencies so their products are susceptible to chemical or biological contaminations, adulterations and falsifications, making the formulations significantly more dangerous to the health of the user.[4] In Brazil, for instance, apprehension of AASs increased approximately 160% from 2009 to 2011.[3] Other researches have highlighted that counterfeit products account for about a third of the seized materials.[5,6]

Once apprehended, drug formulations have to be analyzed for their components to be detected by forensic professionals who can provide analytical evidences that are used in legal processes.[7] In this sense, it is essential for undergraduate students to be introduced to common procedures and techniques used in the analysis of drugs given that these evaluations are common for a forensic expert.[8] Moreover, reaction mechanisms, equipment features and extraction methods used in the analysis can be taught, leading to the understanding that the steps in the procedure may influence the results used in legal processes.[9]

Generally, analysis of apprehended drug formulations initiate with visual inspection for the observation of possible signs of falsification regarding aspects of the label, recipient and the internal content.[10,11] The identification of the components of the sample can be achieved by Infrared Spectroscopy and Gas Chromatography-Mass Spectrometry (GC-MS).[12,13] For GC-MS analysis, an extraction procedure is required, which is generally performed using an ultrasonic bath (UAE), however other methods can be used such as liquid-liquid or microwave-assisted extraction (MAE).[6,14] Recently, microwaves have been applied to analyze samples since it is aligned with several principles of green chemistry. These procedures are usually employed in the analysis of suspected drug samples and can be easily performed in a classroom.[15]

As it can be seen, identification of samples is considerably important in the forensic field. Besides, the increasing number of apprehensions has been making these procedures a common task for forensic experts which must be performed with the appropriate methods in order to provide precise conclusions used in legal processes.[3,16] Therefore, the aims of this work were to develop and evaluate an experimental procedure involving the analysis of two formulations of AASs employing visual inspection, Infrared Spectroscopy and GC-MS analysis using distinct forms of extraction (UAE and MAE) for an undergraduate class. Moreover, pedagogical goals were to teach several approaches to analyze formulations of AASs and the influence methods and techniques cause on the results of the analyses, stimulating the critical thinking of the students.

EXPERIMENTAL SECTION

Reagents and materials

For the experiments, HPLC-grade methanol (Sigma-Aldrich, Saint Louis, USA) was used. The Brazilian Federal Police kindly provided two formulations of AASs (Table 1) with nandrolone decanoate and testosterone propionate as the alleged active ingredients in the samples.

Module I: Visual inspection

Students were asked to evaluate the label and recipient containing the given formulations of AASs to analyze the presence, absence or

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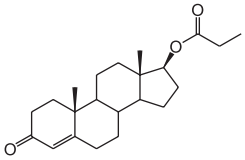
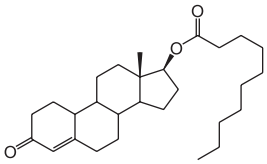
Table 1. Information about the studied samples

Structure	Supposed Active Ingredient	Exact mass (g.mol^{-1})	Physical Aspect	Sample Name
	Testosterone propionate	344.24	Dark yellow oil	Product #1
	Nandrolone decanoate	428.33	Light yellow oil	Product #2

misspelled information such as active ingredient, trade name, name and address of the manufacturer as well as expiration and batch number (Figure 1). The recipient was also evaluated according to its suitability to contain the formulation.

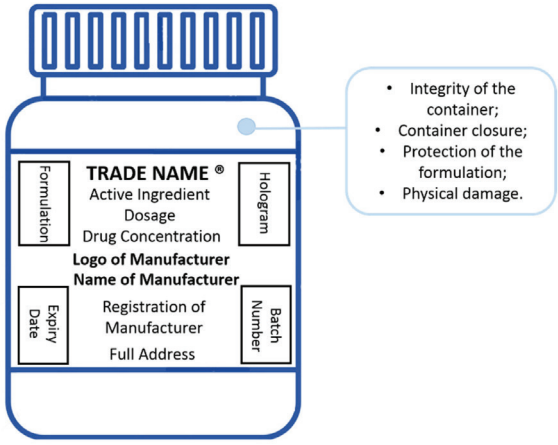


Figure 1. *General aspects of visual inspection of questioned samples*

Module II: Infrared spectroscopy analysis

One drop of each formulation was placed into the equipment and measurement was conducted in scan mode from 4000 cm^{-1} to 600 cm^{-1} with a resolution of 4 cm^{-1}. After each analysis, the equipment was cleaned with acetone and a blank measurement was made.

Module III: Extraction and GC-MS analysis

Extraction of AASs was performed using UAE and MAE. For UAE, 25 µL of the formulation and 5 mL of methanol were placed in conical tubes and vortexed for 10 s. Subsequently, samples were immerged in an ultrasonic bath at room temperature for 10 min and centrifuged at 3000 rpm for 5 min. Finally, the methanolic fraction was transferred to vials and injected in the GC-MS.[5]

For MAE, 25 µL of the formulation and 5 mL of methanol were placed in a round-bottom flask and mixed for 10 s. The sample was introduced into the microwave equipment and subjected to 150 W of power for 10 min. Afterwards, the methanolic portion was transferred to vials and analyzed by GC-MS.

Instrumentation

Spectroscopic analysis was performed on a spectrometer with Attenuated Total Reflection and Fourier Transform model Shimadzu Prestige 21 (Shimadzu, Kyoto, Japan). For chromatographic analysis, one µL of the extracted material was injected into the equipment in split mode (1:25) into the GC-MS model QP2010SE (Shimadzu, Kyoto, Japan). The capillary column was Rtx-5MS (Restek, Bellefonte, USA) and the carrier gas was helium. The injection port and the transfer line both operated at 280 °C while the programed oven temperature was 200 °C, increasing 30 °C.min^{-1} to 250 °C holding for 16 min. Then, temperature was raised from 30 °C.min^{-1} to 300 °C maintaining for 14.5 min. MS had an electron ionization of 70 eV and scanned from 30 to 550 m/z. The identification of the components of the samples was assessed by comparing the samples with the NIST-08 library included on the equipment software.

Hazards and disposal

Methanol is volatile and inhalation or contact can generate health damages, so it is recommended that students wear gloves and handle the solvent inside a fume hood. Throughout the extraction procedures, methanol can be volatilized and, thus, extraction equipment should be also placed inside a fume hood. Disposal of the methanolic solutions generated in the extraction procedures should be placed on the organic waste.

RESULTS AND DISCUSSION

The class had about 4 h of laboratory periods, which were divided into experimental modules and lectures about the techniques of identification and methods of extraction of formulations of AASs. Initially, one set of 10 undergraduate students were familiarized with the topics of the experiment by means of a 0.5 h expository lecture about steroids, mechanism of action and toxicology of anabolic agents, legislation of controlled medicines and analytical techniques for the analysis of formulations of AASs. Then, students performed Modules I and II in groups of three each, which took approximately 1.5 h.

For Module III, students were divided in two groups that would perform UAE or MAE. It should be noted that, although not experimentally performing all the extraction procedures, the groups were asked to accompany both methods while they were made. Both extractions and injection in the GC-MS were executed in parallel lasting about 1 h. As the samples were running in the equipment, there was another short lecture of 0.5 h about extraction methodologies for the analysis of AASs in order to expand the background of the students on the topic.

The combination of experimental and theoretical modules provided innumerous abilities to the students. The requirements for students in these experiments were to evaluate the veracity of

a questioned formulation, detect the active ingredient and its other components and to compare the distinct extraction procedures in relation to the quantity of extracted analytes. The class was evaluated by means of student reports on the experiments and filling of a visual inspection checklist.

Module I: Visual inspection

Visualization of the elements in the label and the recipient were feasible tools in the screening of possible falsified products. For the analyzed materials, the alleged manufacturer did not have a health license to operate in Brazil, which means they were illegal medicines.[6] Students observed that both samples displayed the trade name, active ingredient, logotype of manufacturer and expiry date. However, Product #2 had other information in the label common to genuine medicines such as batch number, hologram, registration number and address of the manufacturer, which was not noted in Product #1. Thus, visual inspection was able to initially provide signs of falsification in Product #1 while Product #2 was preliminarily designated as a genuine material.

In order to perform this exercise in the classroom, a checklist organized by the World Health Professions Alliance was given to the groups of students so that they could evaluate the label and packaging of the samples and observe possible signs of falsification or adulteration in the product. The products used in the analysis were real samples apprehended by the Brazilian Federal Police being genuine or falsified formulations. For practical purposes, the label could be developed by the teacher and inserted on a recipient to simulate apprehended samples. It would be interesting to pursue, if not existing, a partnership with local law-enforcement agencies, as they could play an important role in this type of experiment and provide further interaction of students with real case scenarios. The evaluation stimulated the students during the practical procedure since the proximity with reality made the experiment more interesting and easier to understand and correlate. Besides, it showed the importance of detailed observation and the professional responsibility that is associated with the analysis. It has been demonstrated that connection of theoretical classes with real-life situations is an important motivational method.

Module II: Infrared spectroscopy analysis

Infrared Spectroscopy analysis of the oily formulations (Figures 2 and 1S) revealed the presence of characteristic bands such as =C-H (3008 cm^{-1}), -C-H (2918 cm^{-1}), C=O (1743 cm^{-1}) and C-O (1159 cm^{-1}) in both samples. There were some differences on the spectra such as the presence of CH_2 vibrations (721 cm^{-1}) in Product #1 and the presence of aromatic C=C (1673 cm^{-1}) and Ar-H (709 cm^{-1}) in Product #2. Most of the vibrations could be associated to the alleged active ingredients in both samples, although the bands could be associated with excipients and other adulterants as well, leading to rather inconclusive results. In this sense, the use of Infrared Spectroscopy alone did not allow confirmation nor differentiation of samples, so that confirmatory analytical tools including chromatographic approaches were required in order to analyze the formulations.

The use of Infrared Spectroscopy was a feasible tool in order to introduce the student on the analytical techniques used in the detection of AAS. The spectroscopic analysis was a demonstration to the classroom that the determination of the active ingredient in a formulation can be a difficult task due to inferences of the matrix.[17,18] In this sense, the use of a sequence of analytical techniques is favored in order to achieve precise conclusions about the nature of the sample.[19] Moreover, the student could be familiarized with the instrumentation and manipulation of the equipment and interpretation of bands in the infrared spectra, which are important skills to their future careers.

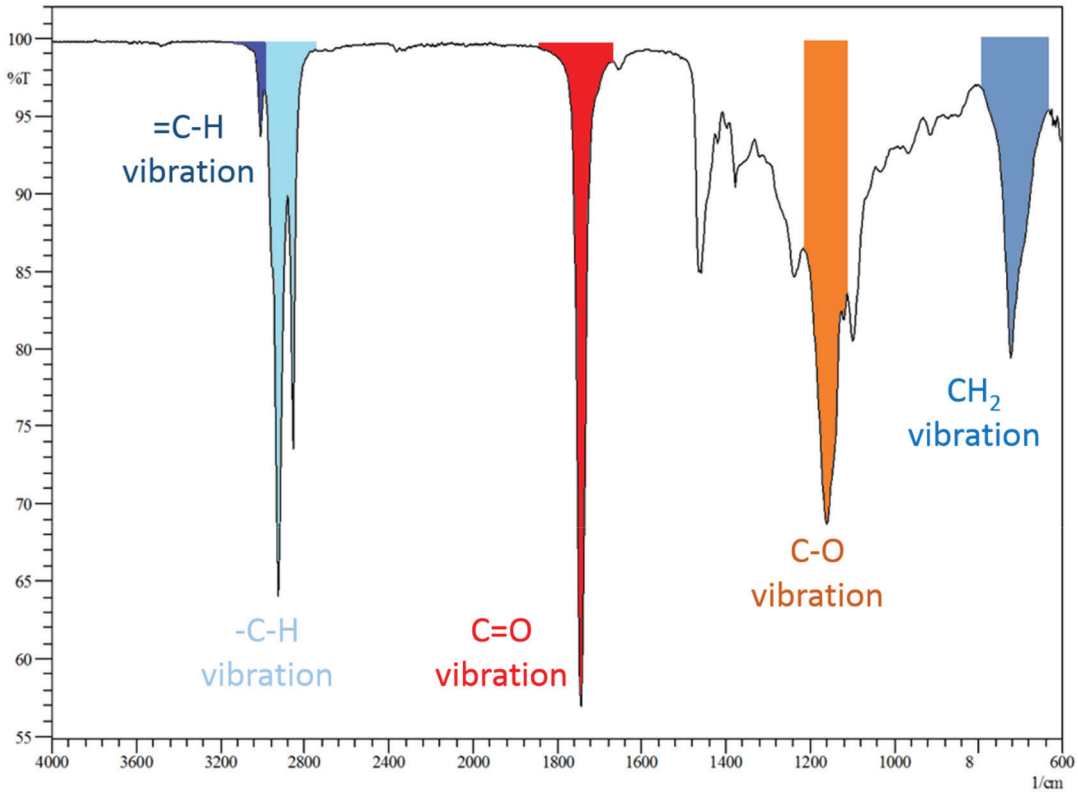


Figure 2. *Infrared spectrum of the formulation of Product #1*

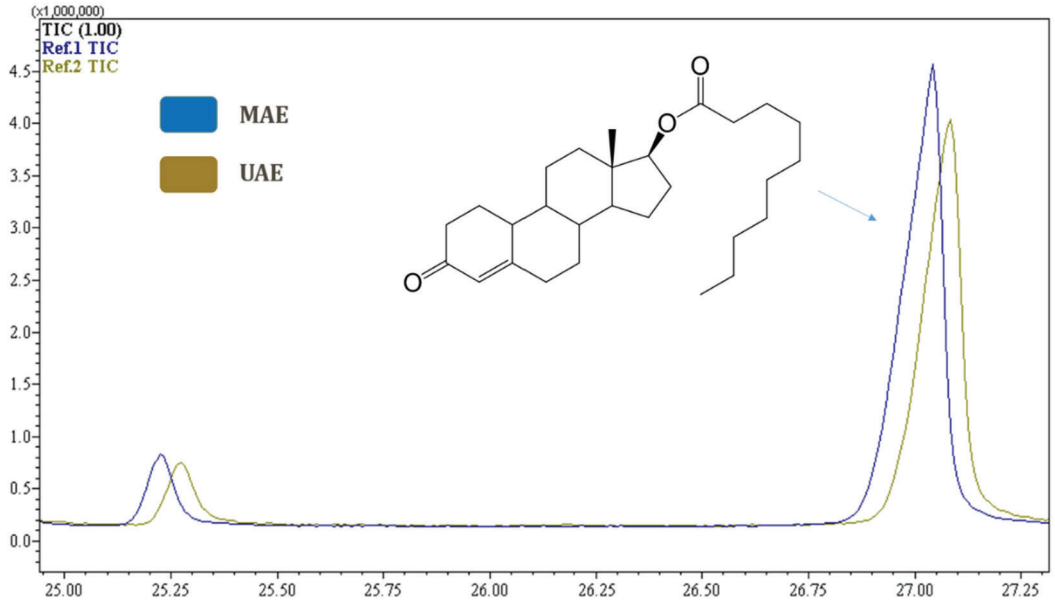


Figure 3. *GC-MS chromatogram of MAE and UAE of methanolic extracts of the formulation of Product #2*

Extraction and GC-MS analysis

GC-MS analysis allowed the student to conclude that Product #1 did not have its alleged active ingredient and was only composed of oily components. This conclusion was based on the lack of any peak in the chromatogram related to testosterone propionate, which placed the sample as a falsified material. This information was previously known and, therefore, Product #1 was not used in the comparison of extraction procedures.

On the other hand, Product #2 had its active ingredient detected (Figure 2S) and several excipients in the form of ethyl esters of fatty acids and benzyl benzoate (Figure 3S). Mass spectra fragmentation showed a molecular ion of m/z 428 and a base peak of m/z 274 which corresponds to the cleavage of the ester moiety bonded to the 17β carbon. These fragments indicated the presence of nandrolone decanoate. Given that Product #2 had the steroidal active ingredient it was used for the comparison of extraction procedures.

Comparison between the UAE and MAE (Figure 3) showed that the methods yield distinct extraction results of the sample constituents. Since analytical standards were not used in the procedure, the area of the peaks was used as an indicative of the concentration. Nandrolone decanoate had an area of 27,457.444 in UAE and 31,871.523 in MAE, indicating that microwave irradiation was more effective and that the type of extraction considerably influences on the overall results.

Regarding the proposed experiments, analysis of forensic samples by analytical techniques proved to be an important tool to enhance the learning of chemistry. On the other hand, results showed that several variables could affect the extraction process leading to the conclusion that the results should be carefully interpreted. Differences among the results can be related to the distinct mechanisms of extraction of UAE and MAE.[20,21] Finally, the students could observe the importance of conducting an adequate sample preparation and that the development and evaluation of novel procedures of extraction are important in order to obtain better results.

CONCLUSIONS

Analysis of AASs formulations was successfully used to immerge undergraduate students in the routine of a forensic chemist, showing the importance regarding the choice of methodology, data analysis and critical thinking. Students were able to be related to subjects including AASs, extraction and analytical techniques used in the analysis. Moreover, students observed that the analysis required several analytical tools in order to obtain adequate results. In conclusion, comparison between extraction procedures highlighted that the results should be carefully interpreted since in real cases the conclusions generated by the forensic expert about an unknown sample affect legal processes.

SUPLEMENTARY MATERIAL

The Supplementary Material section shows analytical data of the analyzed formulations and is freely available at http://quimicanova.sbq.org.br in pdf format.

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Capítulo 4

Analytical approaches applied to the analysis of apprehended formulations of anabolic androgenic steroids

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RESEARCH ARTICLE

WILEY

Analytical approaches applied to the analysis of apprehended formulations of anabolic androgenic steroids

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Abstract

Anabolic androgenic steroids (AASs) comprise a class of synthetic androgens resulting from chemical modifications of testosterone, known for their illicit consumption, which can result inextensive side effects. Extraction procedures applied to the analysis of their formulations are still limited to a few methodologies, despite the increasing numbers of confiscations of AASs. In this sense, the aims of this work were to evaluate the extraction of active ingredients from formulations of anabolic agents using solid-liquid or liquid-iquid, ultrasonic bath, ultrasonicprobe, and microwave-assisted extraction. The results indicated that the extraction procedures influenced the detected concentration of AASs, as the use of ultrasonic probe and microwave irradiation increased the overall extraction of anabolic agents compared with solid-liquid, liquid-liquid, and ultrasonic bath. Regarding oxymetholone, for instance, the microwave-assisted extraction and ultrasonic probe extracted, respectively, 37.46 ± 1.36 and 35.69 ± 0.98 mg/tablet, while solid-liquid extracted 29.63 ± 0.40 mg/tablet of the activeingredient. Therefore, alternative methods such as microwave-assisted extraction or theultrasonic probe could be used for the analysis of formulations of AASs assisting with the identification of illicit and toxic components.

KEYWORDS

anabolic androgenic steroids, liquid–liquid extraction, microwave-assisted extraction, solid–liquid extraction, ultrasonic-assisted extraction

1 | INTRODUCTION

Anabolic androgenic steroids (AASs) comprise a broad class of compounds that are chemically derived from the natural hormone testosterone. Initially, these substances were developed for therapeutic use, being mainly applied for the clinical implications of hypogonadism. However, AASs are known for their illicit use by professional and recreational athletes due to effects such as improvement of sporting performance and an increase in muscle mass.[1] These compounds are controlled or prohibited worldwide as their abusive usage is related to several side effects that include hepatic damage, coronary failure, and physical disorders.[2]

Despite the thorough international legislation on the use of AASs as well as the health risks associated with their use, the volume of apprehensions of anabolic agents has increased considerably worldwide.[3] In Brazil, for instance, confiscation of these products by law-enforcement agencies increased by approximately 200% between 2008 and 2011. It is worth noting that falsified formulations of synthetic androgens comprised roughly a third of the analyzed materials, so increasing the risks associated with the abusive use of AASs.[4]

Apprehended formulations of anabolic agents need to undergo chemical characterization in order to identify their constituents and to distinguish genuine from falsified preparations.[5] Although there are

several methods developed for the extraction of synthetic androgens, the majority are based on the use of methanol in an ultrasonic bath in order to retrieve the alleged active ingredients of the samples.[6] In this sense, extraction of formulations of AASs can be performed with solid–liquid (SLE), liquid–liquid (LLE),[1] or ultrasonic bath-assisted extraction (UBAE).[7] Promising extractive tools such as probe ultrasound (UPAE) and microwave assisted (MAE) extraction have not been used currently in the analysis of anabolic agents.

Instrumental analysis for the identification of the extracted material from AASs formulations is generally performed using gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS).[7,8] These analytical tools provide separation and identification of the constituents of the sample, resulting in significant information about the questioned material. Besides colorimetric tests, other feasible techniques used to detect AASs include infrared (IR) spectroscopy,[9] nuclear magnetic resonance (NMR),[9] and differential scanning calorimetry (DSC).[10]

As noted previously, the identification of apprehended AASs formulations is important in the forensic field, since it can determine possible adulterations and potential cytotoxic substances. Moreover, adequate procedures are required in this type of forensic evaluation since the extraction process is a critical step that can be hampered by the complex matrix of anabolic agents and, thus, considerably influence the results of the overall analysis. Therefore, the aims of this work were to evaluate the efficiency of five distinct extraction procedures (solid–liquid, liquid–liquid, ultrasonic bath-assisted extraction, probe ultrasound, and microwave assisted) applied to 11 formulations of anabolic agents. Secondary goals included the chemical identification of the anabolic agents by means of chromatographic, spectroscopic, and spectrometric approaches.

2 | METHODOLOGY

2.1 | Chemicals, materials, and standards

HPLC-grade methanol and HPLC-grade *n*-hexane were obtained from J.T. Baker (Phillipsburg, USA). Nonadecanoic acid and a methanolic solution of boron trifluoride (14%, v/v) were purchased from Sigma-Aldrich. All other reactants were analytical grade (≥99%).

For the identification and quantitation of the components of the matrix of the oily-based formulations, a mix of 37 fatty acid methyl esters as a standard was purchased from Supelco (Bellefonte, USA) and used as a solution of 2 mg/mL in *n*-hexane. Quantitation of AASs was performed according to Berneira et al[10] using a stock solution (2 mg/mL) of cholesterol (≤99%, Sigma-Aldrich, St Louis, USA) in methanol that was used to obtain the working solution (1 mg/mL) and the calibration curve (0.15, 0.25, 0.50, 0.75, and 1 mg/mL).[10]

Eleven AASs in tablet, oily-based, or aqueous-based formulations apprehended by the local station of the Brazilian Federal Police in 2017 in Pelotas (Brazil) were kindly provided to our

TABLE 1 Main characteristics of the studied samples

Sample	Form	Appearance	Declared compound	Declared concentration	Identified compound
Product 1	Tablet	Round, white and market with an "L"	Oxymetholone	50 mg	Oxymetholone
Product 2	Tablet	Round, light-pink and market with an "L"	Unknown	Unknown	Stanozolol
Product 3	Aqueous suspension	White viscous liquid	Stanozolol	50 mg	Stanozolol
Product 4	Oily solution	Oily light yellow viscous liquid	Testosterone esters	Unknown	Testosterone esters
Product 5	Oily solution	Oily light yellow viscous liquid	Testosterone cypionate	100 mg/mL	Testosterone cypionate
Product 6	Oily solution	Oily dark yellow viscous liquid	Boldenone undecylenate	250 mg/mL	Nandrolone decanoate
Product 7	Oily solution	Oily dark yellow viscous liquid	Testosterone esters	250 mg/mL	Testosterone and nandrolone esters
Product 8	Oily solution	Oily dark green viscous liquid	Testosterone propionate	100 mg/mL	Testosterone and nandrolone esters
Product 9	Aqueous suspension	White viscous liquid	Stanozolol	100 mg/mL	No active ingredient
Product 10	Oily solution	Oily light yellow viscous liquid	Testosterone propionate	100 mg/mL	No active ingredient
Product 11	Oily solution	Oily dark yellow viscous liquid	Trenbolone acetate	100 mg/mL	No active ingredient

research laboratory (Table 1). The samples had been identified previously using GC–MS with a NIST-08 electronic mass spectra library. The study was designed in order to evaluate and to develop continuously adequate methodologies involving evidentiary cases of anabolic agents.

2.2 | Extraction

First, 25 μL of the oily solutions, 50 μL of aqueous suspensions, or 20 mg of the solid formulations was introduced into conical centrifuge tubes and partially dissolved in methanol. For ultrasonic probe-assisted extraction, the quantities used in the process were doubled to fit the capacity of the ultrasonic probe. Sequentially, 250 μL of a methanolic solution of cholesterol (2 mg/mL) was added to the tubes as an internal standard and the samples were extracted following the procedure of ultrasonic bath-assisted extraction developed by da Justa Neves and Caldas.[7] The other described extraction methods were developed in our laboratory following this line of thought, but in a design that would fit each procedure accordingly. All analyses were performed in triplicate (n = 3).

2.2.1 | Ultrasonic bath assisted extraction

Following the procedure described by da Justa Neves and Caldas,[7] the samples were vortexed vigorously for 10 s. Then, the tubes were sonicated for 10 min, using an ultrasonic bath model USC 1800A (Unique, Indaiatuba, Brazil) at ambient temperature (25°C), fixed frequency (40 Hz), and power (120 W). Then, the conical centrifuge tubes were centrifuged at 5000 × g for 5 min and the upper organic phase was retrieved for chromatographic analysis.[7]

2.2.2 | Liquid–liquid and solid–liquid extraction

After the initial process of dissolution of the materials with methanol, the samples were vortexed vigorously for 10 min. Then, the tubes were centrifuged at 5000 × g for 5 min and the upper organic phase was retrieved for chromatographic analysis.

2.2.3 | Ultrasonic probe assisted extraction

Following the dissolution step with methanol, the samples were vortexed for 10 s and, then, tubes were sonicated for 10 min using an ultrasonic probe model VC 505 (Sonics, Newtown, USA) at an ambient temperature (25°C), frequency (40 KHz), power (120 W), and amplitude (20%). Then, the conical centrifuge tubes were centrifuged at 5000 × g for 5 min and the upper organic phase was retrieved for chromatographic analysis.

2.2.4 | Microwave assisted extraction

Subsequent to the first step of dissolution with methanol, the samples were vortexed for 10 s and, afterwards, the flasks were introduced in an open vessel microwave model Discover 9,080,005 (CEM, Matthews, USA) and irradiated with microwaves for 10 min at the boiling temperature of methanol (55°C) under fixed power (150 W). Then, the material was centrifuged at 5000 × g for 5 min and the upper organic phase was retrieved for chromatographic analysis.

2.3 | Visual inspection

Visual inspection of the apprehended samples was conducted following the World Health Professions Alliance (WHPA) guidelines in which the label, the recipient, and the content of the alleged product were physically evaluated for possible signs of counterfeit.[11]

2.4 | Spot tests

2.4.1 | Sulfuric acid test

Briefly, 25 μL of the samples in solution or 20 mg of the tablets were diluted with 1 mL of chloroform and mixed with 1 mL of concentrated sulfuric acid in a test tube. Then, the samples were diluted with 1 mL of deionized water and heated in a water bath at 100°C for 2 min. The development of color was evaluated before and after the heating process as well as after the addition of distilled water. Cholesterol was used as a positive control while an analytical blank was used as a negative control for this spot test as well as for the other colorimetric tests.[9]

2.4.2 | Naphthol-sulfuric acid test

First, 25 μL of the samples in solution or 20 mg of the tablets was diluted with 1 mL of chloroform and mixed with 1 mL of a 2.5% solution of naphthol-sulfuric acid (w/v) in a test tube and heated in a water bath at 100°C for 2 min. Afterwards, the solution was cooled and 1 mL of distilled water was added to the test tube. The development of color was evaluated before and after the heating process as well as after the addition of distilled water.[9]

2.4.3 | Liebermann's test

Initially, 25 μL of the samples in solution or 20 mg of the tablets was diluted with 1 mL of chloroform, mixed with three droplets of a 10% solution of sodium nitrite-sulfuric acid (w/v) in a test tube and heated in a water bath at 100°C for 2 min. The development of color was evaluated before and after the heating process.[9]

2.5 | Formulation analysis

In order to investigate the constituents of the matrix of the oily-based materials, the formulations were derivatized in triplicate (n = 3) following the procedure described by Moss et al.[12] Briefly, 20 μL of the formulation and 5 mL of a methanolic solution (2%, w/v) of sodium hydroxide were placed in a flask and stirred under reflux for 5 min. After this period, 5 mL of a methanolic solution (14%, v/v) of boron trifluoride was added and the system was kept under reflux for another 5 min. Under stirring, the derivatized content was transferred to a separation funnel containing 3 mL of a saturated aqueous solution of sodium chloride and 20 mL of *n*-hexane. Finally, the organic phase was retrieved, filtered with anhydrous sodium sulfate, and dried under reduced pressure.[12]

2.6 | Instrumental analysis

2.6.1 | Infrared spectroscopy analysis

Infrared spectroscopy analyses of the oil-based, water suspensions, and tablets were performed on an attenuated total reflection spectrometer with Fourier Transform model Shimadzu Prestige 21 (Shimadzu, Kyoto, Japan) scanning the samples from 4000 to 600 cm^{-1}. The analysis was carried out using 3 mg of the content of the tablets or the dried material from the water suspensions or 25 μL of the oil-based formulations.

2.6.2 | Chromatographic analysis

For the comparison of extraction procedures, analyses were carried out in a gas chromatograph coupled to a mass spectrometry model QP2010 (Shimadzu, Kyoto, Japan) equipped with a Rtx-5MS capillary column (30 m × 0.25 mm × 0.25 μm) using helium as the carrier gas with a flow of 0.97 mL/min. The injection mode was split (1:25) while the temperature of the injector and the ion source were, respectively, 280°C and 230°C. The initial programmed temperature of the oven was 200°C that increased 30°C.min^{-1} to 250°C holding for 16 min and increasing 30°C.min^{-1} to a final temperature of 300°C maintained for 14.5 min giving a running time of 33.83 min.

For the analysis of the formulations, evaluation was performed on a gas chromatograph coupled to a flame ionization detector (GC-FID) model GC-2010 (Shimadzu, Kyoto, Japan) using a SP-2560 capillary column (100 m × 0.25 mm × 0.2 μm) and nitrogen as the carrier gas. The injector was kept at 260°C with the injections being performed in split mode (1:100). The oven temperature was 140°C increasing 4°C.min^{-1} to 240°C maintained for 10 min, giving a running time of 40 min.

2.7 | Statistical analysis

Statistical analysis was carried out using two-way analysis of variance (ANOVA) and Tukey's test ($P < 0.05$) using Graphpad software version 7 (La Jolla, USA), while principal component analysis was performed using Minitab software version 17 (State College, USA). The results of the triplicates (n = 3) are expressed as mean ± standard deviation.

3 | RESULTS

3.1 | Evaluation of extraction procedures

Chromatographic analysis (Figure 1) indicated that the majority of the samples had a composition different to that one stated on the label. For product 6, the alleged active ingredient boldenone undecylenate was actually nandrolone decanoate, while products 7 and 8 had nandrolone decanoate and testosterone cypionate in the formulation instead of the supposed active ingredient. In some cases, the sample was only composed of excipients as observed for products 9, 10, and 11, while product 2 had an unknown active ingredient, which was further identified as stanozolol. On the other hand, products 1, 3, 4, and 5 had the same composition as stated on their labels.

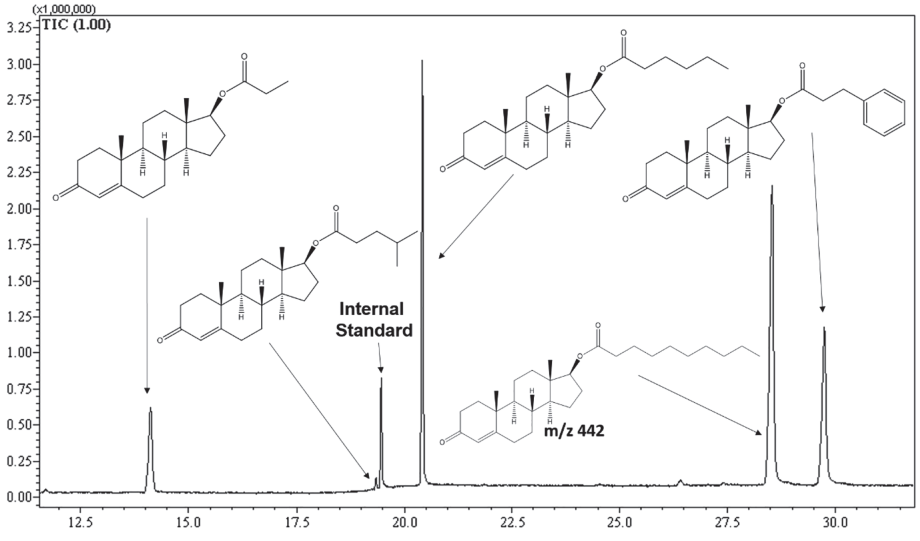

FIGURE 1 GC–MS chromatogram of the AASs found in the formulation of product 4 (allegedly containing testosterone esters)

TABLE 2 Comparison of liquid–liquid extraction (LLE) or solid–liquid extraction (SLE), ultrasonic bath-assisted extraction (UBAE), ultrasonic probe (UPAE), and microwave-assisted extraction (MAE) in the extraction of anabolic agents

Sample	LLE or SLE	UBAE	UPAE	MAE
Product 1				
OXY (mg/tablet)	29.63 ± 0.40^{a}	33.00 ± 0.43^{b}	35.69 ± 0.98^{c}	37.46 ± 1.36^{c}
Product 2				
STA (mg/tablet)	9.81 ± 0.64^{a}	9.90 ± 0.16^{a}	9.27 ± 0.38^{a}	9.93 ± 0.27^{a}
Product 3				
STA (mg/mL)	47.68 ± 0.59^{a}	47.07 ± 0.35^{a}	46.24 ± 0.93^{a}	47.39 ± 0.20^{a}
Product 4				
TPRO (mg/mL)	33.44 ± 0.71^{a}	35.48 ± 2.51^{ab}	37.47 ± 1.13^{b}	35.69 ± 0.24^{ab}
TISO (mg/mL)	12.41 ± 0.12^{a}	12.41 ± 0.25^{a}	12.44 ± 0.07^{a}	12.44 ± 0.06^{a}
TCAP (mg/mL)	61.59 ± 3.03^{a}	62.31 ± 2.29^{a}	68.18 ± 0.03^{b}	66.23 ± 1.40^{b}
TDEC (mg/mL)	97.74 ± 4.97^{a}	97.64 ± 2.15^{a}	108.59 ± 4.09^{b}	103.82 ± 2.76^{c}
TPHE (mg/mL)	55.17 ± 3.03^{a}	60.88 ± 4.42^{b}	64.49 ± 1.90^{b}	61.16 ± 1.88^{b}
Product 5				
TCYP (mg/mL)	80.05 ± 1.56^{a}	89.81 ± 2.16^{b}	99.06 ± 5.58^{c}	100.62 ± 2.56^{c}
Product 6				
NDEC (mg/mL)	49.46 ± 0.80^{a}	51.20 ± 0.17^{a}	60.78 ± 1.69^{b}	64.23 ± 1.94^{b}
Product 7				
TPRO (mg/mL)	40.47 ± 1.22^{a}	44.40 ± 1.20^{b}	48.20 ± 1.49^{c}	45.15 ± 0.70^{BC}
TCAP (mg/mL)	13.06 ± 0.08^{a}	13.25 ± 0.35^{a}	13.63 ± 0.14^{a}	13.33 ± 0.27^{a}
TDEC (mg/mL)	13.21 ± 0.11^{a}	13.45 ± 0.23^{a}	13.50 ± 0.05^{a}	13.52 ± 0.50^{a}
NDEC (mg/mL)	16.84 ± 0.09^{a}	17.25 ± 1.11^{a}	18.32 ± 0.08^{a}	18.14 ± 0.28^{a}
TPHE (mg/mL)	11.56 ± 0.18^{a}	11.95 ± 0.24^{a}	12.44 ± 0.10^{a}	11.87 ± 0.12^{a}
Product 8				
TPRO (mg/mL)	16.64 ± 0.12^{a}	15.93 ± 0.29^{a}	15.76 ± 0.45^{a}	15.98 ± 0.29^{a}
TCYP (mg/mL)	36.96 ± 1.48^{a}	35.95 ± 3.02^{a}	36.52 ± 1.12^{a}	35.46 ± 0.39^{a}
NDEC (mg/mL)	32.23 ± 0.95^{a}	32.93 ± 2.26^{a}	35.28 ± 1.23^{a}	32.26 ± 0.68^{a}
Product 9				
STA (mg/mL)	Nd	Nd	Nd	Nd
Product 10				
TPRO	Nd	Nd	Nd	Nd
Product 11				
TACE	Nd	Nd	Nd	Nd

Results expressed as mean ± standard deviation. Different superscripts in each row are statistically different at Tukey's test ($P < 0.05$)
Nd, non-detected; OXY, oxymetholone; STA, stanozolol; TPRO, testosterone propionate; TISO, testosterone isocaproate; TCAP, testosterone caproate; TDEC, testosterone decanoate; TPHE, testosterone phenylpropionate; TCYP, testosterone cypionate; NDEC, nandrolone decanoate; TACE, trenbolone acetate.

Evaluation of the extraction procedures (Table 2) showed significant differences among the tested methods. Generally, liquid–liquid or solid–liquid extraction were less efficient compared with ultrasonic and microwave assisted protocols, with the exception of stanozolol found in products 2 and 3 as well as for some constituents of products 7 and 8. In some cases, liquid–liquid or solid–liquid, and ultrasonic bath had a similar extraction of the active ingredient as can be observed in some components of products 4 and 6. Furthermore, the application of focused ultrasonic energy and microwave irradiation generally increased the extraction of the anabolic agents as can be perceived for components of products 1, 4, 5, 6, and 7. It is worth noting that this was the first time that AASs have been extracted using ultrasonic probe and microwave assisted extraction.

As can be observed in Table 2, products 1, 6, and 8 had the anabolic agent in concentrations considerably lower than that stated on the label, while products 9, 10, and 11 did not have the active ingredient. On the other hand, products 3, 5, and 6 had similar experimental concentrations to the amount reported on the label. Finally, for products 2 and 4, that did not have their drug strength labeled, the amount of anabolic agents were detected as 9 mg of stanozolol and 250 mg of testosterone esters, respectively.

3.2 | Visual inspection

Visual inspection (Table 1S) followed WHPA guidelines and was used as a preliminary analysis in order to detect possible signs of counterfeit according to the information found on the label, condition of the recipient, and appearance of tablets, oily solutions or aqueous suspensions. In this sense, it was possible to observe several indications that products 6, 7, 8 9, 10, and 11 were falsified, as the trade name and the manufacturers were not licensed to distribute the formulations in Brazil. Besides, essential information such as the address of the manufacturer, dosage form, expiry date, and batch number were lacking in some cases, reinforcing that the samples were possible counterfeit AASs.

On the other hand, product 5 was placed as a possible genuine formulation of AAS as several of the required details such as indication of the active ingredient, dosage form, drug strength, name, and logo of manufacturer were found on the label. Moreover, the manufacturer was registered to distribute the active ingredient within the country under the indicated trade name. These features were also perceived in products 1, 3, and 4. However, the manufacturer did not have a health license to operate in the country, making these pharmaceutical preparations illegal, despite possibly being genuine formulations. Since the recipient of product 2 was unlabeled, visual inspection was applied to its tablets, which did not show signs of damage, unevenness or cracks, characteristic of counterfeit preparations.

3.3 | Spot tests

Colorimetric analysis of the formulations of AASs (Table 2S) revealed a wide variety of colors within the samples that could mainly be attributed to the presence of steroidal active ingredients, although excipients could have influenced the results. Generally, the color changed during the different tests as well as when the samples were diluted or heated. It should be noted that the combination of the spot tests provided positive indications of the presence of AASs, however, it was not possible to confirm the presence of a specific one.

3.4 | Infrared spectroscopy analysis

Infrared spectroscopy analysis (Table 3S) of product 1 revealed bands that could be associated with the alleged active ingredient oxymetholone such as the hydroxyl (3523 and 3330 cm^{-1}) and carbonyl groups (1613 cm^{-1}). Furthermore, products 2 and 3 had similar spectra with characteristic bands associated with amine group (3330 to 3110 cm^{-1}) and to the C=N bonding (1657 cm^{-1}) that can be linked to stanozolol. On the other hand, product 9 that should contain stanozolol did not result in the characteristic bands seen in the other samples. In this sense, the vibrations observed in the spectrum were probably related to the excipient carboxymethylcellulose. In turn, products 10 and 11 had vibrations that could mainly be attributed to their excipients.

On the other hand, the lack of distinct chemical groups that could differentiate the constituents of products 4, 5, 6, 7, and 8 produced very similar spectra that, in most cases, only varied in transmittance values. Among the minor differences found in the results were bands associated with α-β unsaturated carbonyl group (1722 and 1723 cm^{-1} in products 5, 6, and 7) and α-β unsaturation (1617 cm^{-1} in product 4). Generally, these formulations presented vibrations related to unsaturation, carbonyl group, and aromatic moieties that can be associated with components of vegetable oil, steroidal active ingredients, and other possible vehicles used to prepare the formulations.

3.5 | Formulation analysis

Chromatographic analysis of the oily formulations and of the vegetable oil control (soybean and peanut oil, Table 4S) revealed that the vegetable oils used as excipients in the apprehended materials consisted of 9 to 14 fatty acids having carbon side chains that varied from medium to very long-chains. Generally, *cis*-linoleic (C18:2*n*6c), *cis*-oleic (C18:1*n*9c), and palmitic (C16:0) acids were the most representative compounds found in all the analyzed samples. It should be noted that minor concentrations of *trans*-fatty acids in the form of *trans*-linoleic (C18:2*n*6t) and *trans*-oleic (C18:1*n*9t), which are not usual, were also detected.

As can be observed in Table 4S, the fatty acid profile of each formulation varied significantly in concentration or number of components. Nonetheless, it could be observed that products 4, 5, 10, and 11 were very similar since the samples had the same predominant fatty acids, indicating that the formulations were composed of the same vegetable oil, although being from distinct sources. The same affirmation can be made for products 6, 7, and 8, which also displayed similar predominant fatty acids.

For products 4, 5, 10, and 11 monounsaturated fatty acids (MUFAs) were found in considerable concentrations ranging from 43.49 ± 0.62% to 63.96 ± 0.09%, while for products 6, 7, and 8 polyunsaturated fatty acids (PUFAs) were the predominant fatty acid class varying from 56.45 ± 0.05% to 59.19 ± 0.08%. For all the analyzed samples, saturated fatty acids (SFAs) were detected in lower concentrations than the other classes varying from 13.13 ± 0.37% to 23.97 ± 0.17%. It is worth noting that this was the first time that oily-based AASs had had their matrix analyzed and identified.

Comparing the resulting fatty acid profile of the formulations with the profiles of vegetable oils found in the literature, it could be noted that products 4, 5, 10, and 11 were probably constituted of peanut oil, while products 6, 7, and 8 were possibly composed of soybean oil.[13] Further analysis using soybean and peanut oil acquired from a local market and used as positive controls confirmed the presence of these vegetable oils in the formulations of the samples. The results of GC–MS, GC-FID, and FT-IR of the studied samples can be found in the Supplementary Information section.

Principal component analysis (PCA) was performed in order to verify the correspondence between the major fatty acids found in the

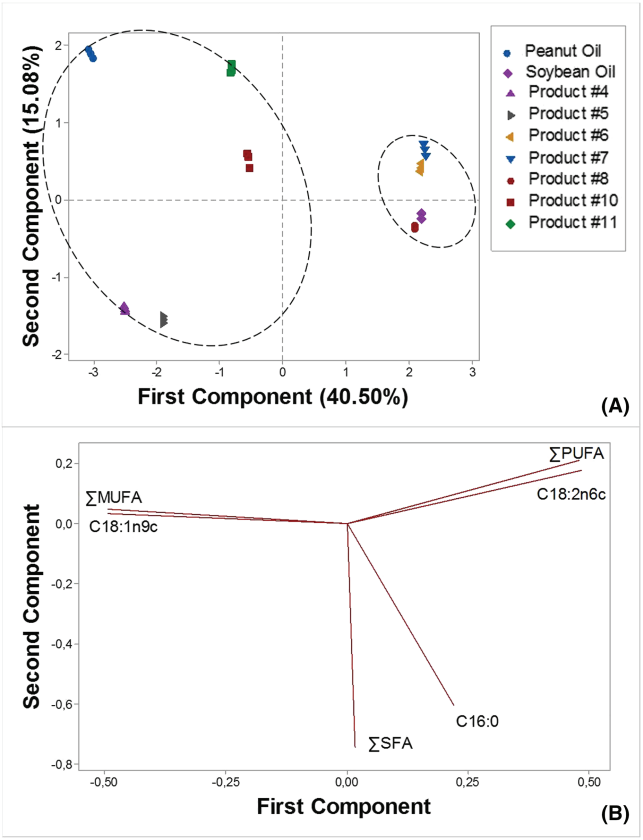

FIGURE 2 Score plot (A) and loading plot (B) of PCA applied to the formulations of AASs and the vegetable oils control [Colour figure can be viewed at wileyonlinelibrary.com]

formulations of AASs and their respective vegetable oil excipients. This statistical approach can be a feasible tool to compare similar preparations, since the ANOVA analysis could not be used efficiently to correlate the formulations to their oily excipients. In this sense, C16:0, C18:1*n*9c, and C18:2*n*6c as well as the sum of SFAs, MUFAs, and PUFAs were chosen as representative loading biomarkers (Figure 2B) to distinguish or cluster the samples. The results of the analysis showed that the model generated by PCA could explain approximately 55.58% of the differences found among the samples (Figure 2A).

As can be observed in Figure 2, the variables used in PCA could differentiate the samples and the vegetable oil controls into two distinct portions of the score plot. This was possible since the variable C16:0 and ∑SFA influenced the samples in the negative direction of the second component (PC2) as C18:1*n*9c, ∑MUFA pushed the materials to the negative direction of the principal component (PC1) while C18:2*n*6c and ∑PUFA influenced the samples to the positive axis of PC1. In this sense, formulations composed of soybean oil as the possible excipient (products 6, 7, and 8) clustered in the positive axis of PC1 since the samples were mainly composed of PUFAs and C18:2*n*6c. On the other hand, samples with peanut oil as the possible oily excipient (products 4, 5, 10, and 11) clustered generally in the negative region of PC1 as the materials were mainly constituted of MUFAs in the form of C18:1*n*9c.

4 | DISCUSSION

4.1 | Evaluation of extraction procedures

According to the results, the extraction procedures influenced the overall recovery of the active ingredients. Among the reasons that can explain the distinct extraction efficiencies are the presence or absence of assisted energies, the chemical moieties of the AASs, and their concentration in the formulation.[5] In this sense, the presence of a pyrazole ring in stanozolol possibly favored the interaction with the extractor solvent enabling its extraction with simple extraction procedures.[14] On the other hand, long carbon-chain nandrolone or testosterone esters required the application of an assisted energy as these anabolic agents are not polar enough to interact with the extractor solvent. Finally, lower concentrations of the active ingredient allowed its migration from the formulation to methanol as a chemical equilibrium could be quickly achieved.[15]

The evaluation of extraction methods is recurrent in the literature for several classes of substances. Nonetheless, few works compare the analytical procedures developed in the literature for the analysis of formulation of AASs, which is important in forensic science, given that the results are used as criminal evidence.[10] Among research studies, da Justa Neves and Caldas reported that there was a 5% increase in the recovery of testosterone, nandrolone, or trenbolone esters from oily formulations using ultrasonic bath compared with liquid–liquid or solid–liquid extraction. Comparing the reported results to the current work, it can be observed that generally there was indeed an increase in the extraction of anabolic agents varying from 1% to 10% with the use of an ultrasonic bath.[7]

Generally, acoustic energy improved the extraction of the active ingredient compared with liquid–liquid or solid–liquid extraction. The enhancement in the extraction process can directly be related to cavitation bubbles that are formed, improving solvent–sample interaction. In this sense, the recovery of anabolic agents can be associated with certain effects caused by cavitation bubbles, such as: (i) high temperatures that enhance solubility and diffusivity of the components and (ii) high pressures that enable the penetration and transport of the analyte through the matrix.[16]

As can be observed from the evaluation of extraction procedures, ultrasonic probe showed results superior to ultrasonic bath. This could be due to the dissipation of the ultrasound energy within the ultrasonic bath.[17] In this sense, the overall power to which the sample was subjected decreased, leading to a reduction in the formation of cavitation bubbles and, therefore, the effectiveness of the procedure. Although used considerably in the analysis of formulations of AASs, ultrasonic baths have poor repeatability and reproducibility compared with ultrasonic probes, which can negatively affect the results obtained by this method.[16]

The ultrasonic probe focused acoustic energy into the sample increasing the formation of cavitation bubbles and the overall recovery of the analytes.[18] Despite showing promising results when compared with the conventional methods used, the ultrasonic probe is rarely applied for the extraction of active ingredients from

formulations of anabolic agents. The higher instrumentation cost and the fact that only one sample can be extracted at a time could be reasons why ultrasonic probe is used less than the ultrasonic bath, in which multiple samples can be analyzed at the same time.[19]

Compared with solid–liquid, liquid–liquid, or ultrasonic-based methods, microwave assisted extraction also showed promising results that can be associated with the increase of temperature that the sample experiences within the equipment. Among the mechanisms that can explain the enhanced recovery of the active ingredients are the dipole rotation and ionic conduction that occur in the solvent under microwave irradiation. These interactions may enhance the interaction between a polar extractor solvent with non-polar formulations and, thus, increase their extraction.[20]

Methods that employ ultrasonic probe or microwave irradiation for the analysis of formulations of AASs have not been frequently reported in the literature.[21] In this sense, there are only studies that evaluate extraction procedures for the recovery of anabolic agents in urine and food matrices.[21,22] According to previous reports, the use of assisted energies increased the extraction of the analytes, which was also observed herein for formulations of AASs. It is worth noting that these procedures were quick, efficient, and reproducible, indicating that ultrasonic probe and microwaves could be used in the analysis of formulations of anabolic agents.[1,7]

According to the World Health Organization, falsified pharmaceutical products comprise formulations that have misspelled, absent, or incorrect label, as well as unknown or differently marketed chemical compositions/concentrations. Given that differences in the stated concentration of the active ingredients and the amounts detected in formulations also characterize signs of falsification, the extraction methods applied should retrieve the analytes quantitatively in the preparation.[5,10] Evaluation of the extractive protocols showed that generally the use of conventional methods (liquid–liquid, solid–liquid, or ultrasonic bath) had lower retrievals of the active ingredient compared with microwave assisted and ultrasonic probe. Therefore, the choice of the extraction procedure is considerably important in order to obtain adequate and accurate results.

The counterfeit pharmaceutical materials analyzed in the current work also can be a potential threat to public health as these formulations are usually manufactured under conditions that do not follow sterile guidance, increasing their susceptibility for microbiological contamination.[3,5,23] Additionally, the chemical composition of the products is uncertain as there is no overview from regulatory agencies, which could increase the side effects associated with the use of AASs and risk to the health of the user to alarming levels.[8]

4.2 | Visual inspection

The use of visual inspection allowed the initial detection of possible counterfeit formulations (products 6, 7, 8, 9, 10, and 11) that were confirmed by further instrumental analysis. It is worth noting that the label of these apprehended materials was clearly manufactured without following any guidelines, which enabled an efficient evaluation of the materials. In a previous research work visual inspection was also employed for the analysis of 70 apprehended products, but labels could not be differentiated as they were similar to the ones found in the counterpart genuine formulations.[6] In the current study, signs of falsification could not be evaluated in product 2 because it was unlabeled as similarly described by Hullstein et al. (2015) since the samples were apprehended still under manufacturing.[3] Further analysis confirmed that product 2 as well as products 1, 3, 4, and 5 were genuine formulations.

Although requiring relatively short amounts of time at no cost, visual inspection is still a largely unexplored tool for the identification of possible signs of falsification of formulations of AASs.[24] Among the reasons that can explain this are meticulous falsifications that can look very similar to genuine products differing only in the chemical fingerprint of the preparation. Besides, the formulation may be apprehended while still under manufacture without label or recipient, which could hamper or prevent the analysis.[1,3,6] However, given these details, it is believed that visual inspection could be used as a screening tool for possible counterfeit products with further confirmation by more accurate analytical techniques.

4.3 | Spot tests

The results of the spot tests indicated the possible presence of anabolic agents due to the formation of color among the several performed tests. However, interference from excipients was observed for products 1, 2, and 9, as the samples developed color despite previous indications in the literature that no reaction with the active ingredient could occur or no active ingredient was present in order to develop coloration. The interference of the matrix could easily be observed in the naphthol-sulfuric acid test as the samples developed a green color that could be related to the presence of starch in the tablets. Moreover, after the heating process, the anabolic agents had distinct colors from those indicated in the literature which could lead to false interpretations.[9]

For products 4, 5, 6, and 7, there were clear indications of the presence of AASs as the colors observed in the spot tests agreed with the results reported in the literature. Since the formulations were constituted of acylglycerols and benzyl benzoate, there were lower chances of interference from the excipients among the samples. Previous analysis of several apprehended formulations of AASs in tablet or oil-based solutions reported in the literature had similar results to those found in the current work with differences in color possibly associated with the excipients.[9] Nonetheless, the presence of multiple active ingredients in the preparations prevented the identification of the constituting anabolic agents.

4.4 | Infrared spectroscopy analysis

The application of IR spectroscopy analysis as a preliminary analytical technique indicated the presence of the stated active ingredients for products 1 and 3. It also pointed to the presence of the active ingredient stanozolol in the unknown formulation of product 2 since this synthetic androgen had particular groups and distinct chemical

transitions.[25] However, for the other samples, it was not possible to identify clear distinctions because testosterone and nandrolone esters as they differ only in the carbon length of their side chains. Moreover, the oily formulation and the vehicle interfered in the resulting spectra with additional bands, possibly masking characteristic vibrations of the anabolic agents.[23] Finally, analysis of product 9 revealed the presence of the excipient carboxymethylcellulose, which was not observed in the chromatographic analysis.

Generally, the application of IR spectroscopy generated weak or inconclusive results as, in most cases, it was not possible to identify with certainty the active ingredients based solely on their spectrum.[9] However, the presence of bands associated with conjugated carbonyl groups indicated the presence of an anabolic agent. Given these results, IR spectroscopy served as an adequate tool for the screening of samples and reinforcement of remarks previously found in the visual inspection and spot tests. Nonetheless, confirmatory analytical techniques such as GC–MS should be used in order to detect the organic components of the samples.[7]

4.5 | Formulation analysis

Since anabolic agents are mostly hydrophobic, their formulations are constituted of vegetable oils such as arachis, castor, peanut, and sesame oil and benzyl benzoate.[2] These components are known as excipients and fulfill several roles in a pharmaceutical preparation conferring solubility, permeability, coloring, and stability so that the anabolic agent is able to accomplish its biological goal.[26] Excipients can potentially be used as the sole components in counterfeit alleged pharmaceutical formulations, comprising more than 40% of the cases involving falsifications. Moreover, non-pharmaceutical preparations can be a serious health hazard as these products can be manufactured under non-sterile and non-controlled conditions.[2]

Forensic evaluation of excipients in AAS formulations is not usually performed, as the main objective in such cases is to detect the presence of the stated active ingredients in the material. Nonetheless, it can be observed that the analysis of the oily formulations provided important information about the preparation of the anabolic agents since it was possible to correlate signs of falsification with the type of oily excipient. For the analyzed samples, genuine materials were composed of peanut oil, while counterfeit formulations of AASs were composed of soybean oil probably due to its low cost and ease of acquisition.[27] Therefore, the analysis of the constituents of the oily matrix could be used to evaluate possible signs of falsification found in a sample.[28]

Vegetable oils are common constituents in formulations of anabolic agents, serving not only to solubilize the active ingredient but also to assist their pharmacokinetics. They provide a slow release of the AAS into the circulation and prolong its biological effect within the organism.[29] In this sense, factors such as length of the esterified chain of the anabolic agent and the oily constitution of the formulation are key aspects that influence the partition coefficient of the active ingredient from the intramuscular region to the plasma.

Generally, the increase of the carbon chain in the formulation or in the AAS is associated with lower rates of release of the anabolic agent into the bloodstream and a prolonged biological effect.[2] Similar to their release in the human organism, we believe that the composition of vegetable oils also influenced the extraction of the AASs from their respective matrix. This affirmation could be made since anabolic agents esterified with long carbon chains needed the application of focused energies including ultrasound probe and microwaves in order to fully be retrieved from the matrix. Therefore, the higher affinity of long-chain esterified anabolic agents to non-polar components of the matrix increased their interaction, hampering the methanolic extraction of the active ingredients.

Benzyl benzoate is another excipient used in formulations of AASs, which is an oily liquid at ambient temperature used as an additive in pharmaceutical and non-pharmaceutical preparations. In oily solutions of anabolic agents, benzyl benzoate is generally added in order to lower the viscosity of the formulation and, thus assist in the intramuscular administration of the product.[26] Besides, it is used to increase the solubility of AASs in the vegetable oil preventing crystallization during storage. It should be noted that the administration of benzyl benzoate has been associated with allergic reactions within the body. This risk is particularly higher in counterfeit formulations since there is no control over the concentrations of the excipient added to the preparation.[2]

The results of PCA showed that products 6, 7, and 8, which were falsified formulations of AASs clustered around soybean oil, while products 4 and 5 that were genuine materials clustered in the same portion of the score plot. Nonetheless, products 10 and 11, which were counterfeit materials also clustered around peanut oil. Given these results and previous statistical analysis, there was an indication that the oily excipient could be a biomarker for the discrimination of counterfeit from genuine samples. However, further parameters should be included in order to satisfactorily discriminate the samples.[23]

It should be noted that PCA has not been used currently for this purpose in the analysis of AASs, even though previous reports indicated the use of particular bands found in IR spectroscopy analysis as indicative of falsification in preparations of anabolic agents.[23] Therefore, the application of PCA provided noteworthy insights concerning the composition of the oily excipient that could efficiently be used to identify possible signs of counterfeit materials.

5 | CONCLUSION

In the present study, five extraction protocols were evaluated in formulations of anabolic androgenic steroids. The results indicated that the use of microwaves or an ultrasonic probe increased the efficiency of the extraction compared with liquid–liquid, solid–liquid, and ultrasonic bath assisted extraction. Together, visual inspection, spectroscopy, and spectrometry allowed the conclusion that products 6, 7, 8, 9, 10, and 11 were counterfeits, while products 1, 2, 3, 4, and 5 were genuine. Therefore, the application of analytical approaches

and efficient extraction procedures are required in order to provide adequate results in formulations of anabolic agents.

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DECLARATION OF INTEREST

The authors declare that they have no conflict of interest.

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Capítulo 5

Evaluation of the volatile composition and fatty acid profile of seven Antarctic macroalgae

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Evaluation of the volatile composition and fatty acid profile of seven Antarctic macroalgae

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Abstract

Fatty acids (FAs) and volatile organic compounds (VOCs) are among bioactive substances produced by macroalgae, which have important reported biological activities. The aim of this work was to determine the diversity of FAs and VOCs in brown, green, and red Antarctic macroalgae. Results showed that seaweeds contained 13 to 25 FAs with a predominance of palmitic, oleic, linoleic, and eicosapentaenoic acids. Concerning VOCs, 28 to 55 distinct compounds could be detected, distributed among aldehydes, hydrocarbons, furan derivatives, and ketones, for instance. Generally, hexanal (5.83%–25.51%), heptadecane (0.92%–49.84%) and 2-pentylfuran (3.02%–12.57%) were found in considerable amounts in the analyzed specimens. It is worth noting that, to the best of our knowledge, this was the first time that Antarctic macroalgae had their VOCs elucidated. Therefore, Antarctic seaweeds were composed of several VOCs and FAs which could assist in the elucidation of secondary metabolites from these organisms.

Keywords Fatty acids · Volatile organic compounds · Antarctic macroalgae · Gas chromatography

Introduction

Seaweeds comprise a diverse group of approximately 10,000 aquatic organisms that can be divided depending on their pigmentation and biochemical and morphological aspects into three main groups that include the Rhodophyta, Ochrophyta, and Chlorophyta (Rodrigues et al. 2015; dos Santos et al. 2019). The biological potential of macroalgae has led to the use of seaweeds as food, fertilizers, bioenergy resources, and additives in cosmetics, for instance, mainly in Asiatic countries (Hamid et al. 2015; Passos et al. 2020). Despite their high potential, macroalgae can still be considered largely unexplored as less than 5% of the known species have been used commercially, making them feasible sources of novel biological applications (Andrade et al. 2013; Rodrigues et al. 2015).

The high adaptive capacity of seaweeds to the most adverse environments enabled their survival in severe habitats that include Polar, sub-Antarctic, and Antarctic regions of the planet (Graeve et al. 2002; Becker et al. 2010). Conditions, such as water temperature, light exposure, nutrient availability, and water salinity, can be extreme in the Antarctic region inducing macroalgae to biosynthesize secondary metabolites as survival mechanisms (Santos et al. 2017; Pacheco et al. 2018; Pacheco et al. 2018). Fatty acids (FAs), sterols, vitamins, carbohydrates, and volatile organic compounds (VOCs) are among the secondary metabolites produced by seaweeds (Santos et al. 2015; Maruti et al. 2018; Schmid et al. 2018).

Although constituting generally less than 5% of the chemical composition of macroalgae, FAs are key secondary metabolites produced by seaweeds in order to help maintain their membrane fluidity, beyond other metabolic functions (Santos et al. 2017). It is known that this biochemical mechanism is one of the possible responses to extreme environmental conditions, which leads to the production of essential FAs that cannot be synthesized by humans (Martins et al. 2016). The consumption of omega-3 (*n*3) FAs is associated to several

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biological activities, and the health benefits include antibacterial, antitumor, antioxidant, and antiinflammatory effects as well as prevention of coronary and neurologic diseases (Larsen et al. 2011; Pereira et al. 2012).

VOCs are another vast group of bioactive substances produced by seaweeds that have low molecular weight and are mostly lipophilic compounds, including, for instance, aldehydes, ketones, hydrocarbons, furan derivatives, and alcohols (Hosoglu 2018). Despite early studies dating back to the late 80s regarding VOCs, there are still few research works concerning their determination in macroalgae (Hamid et al. 2015). It is worth noting that VOCs have shown antimicrobial activity, highlighting their biological potential (Paul and Pohnert 2011). However, their mechanism of biosynthesis and action in seaweeds and environment are still largely unknown up to this date.

According to the literature, seaweeds can be a vast source of bioactive compounds, such as VOCs and FAs, that are still largely unexplored to this date. Previous research works indicated that Antarctic seaweeds contain important phytochemicals including FAs (Martins et al. 2016; Santos et al. 2017) and sterols (Pereira et al. 2017; Pacheco et al. 2018). Considering that it is important to continuously develop studies regarding the extraction of compounds from Antarctic macroalgae, the study aimed to highlight novel potential bioactive substances and determine possible chemotaxonomic relationships among the analyzed samples. Therefore, the objectives of this work were to evaluate the volatile composition and the FA profile of seven Antarctic macroalgae (Rhodophyta, Chlorophyta, and Ochrophyta) as well as to differentiate species by their metabolites using statistical approaches.

Materials and methods

Sampling

Ochrophyta representatives *Desmarestia confervoides* and *Adenocystis utricularis*, Rhodophyta species *Myriogramme manginii*, *Gigartina skottisbergii*, *Curdia racovitzae*, and *Georgiella confluens*, as well as the Chlorophyta seaweed *Ulva intestinalis* were collected in several points of the Antarctic Peninsula or in the South Shetland Islands during November and December of 2015 (Table 1). Generally, 6 to 10 individuals of representatives from each macroalgae were manually collected at low tide. Subsequent to collection, the samples were washed with seawater. Morphological identification was performed by expert phycologists by comparing the samples to a database of the Botanic Institute of the University of São Paulo. Finally, individuals were lyophilized, milled, and placed into dark plastic bags, stored at − 20 °C.

Chemicals and materials

A methanolic solution of boron trifluoride (14%, *v/v*) and a C8–C20 alkane solution were obtained from Sigma-Aldrich (USA), while standards methyl nonadecanoate and a 37-mix fatty acid methyl esters (FAMEs) were acquired from Sigma-Aldrich and Supelco (USA), respectively. Lastly, 20 mL boron-silicate vials and silicone septum were from Shimadzu (Japan) and HPLC-grade *n*-hexane was obtained from J.T. Baker (USA). All solvent and other chemical reactants were of analytical grade (≤ 98.5%).

Analysis of fatty acids

Extraction

Extraction of FAs from the samples was carried out in triplicate ($n = 3$) and followed the modified methodology proposed by Bligh and Dyer (1959). Briefly, 1 g of algal biomass, 10 mL of chloroform, 20 mL of methanol, and 10 mL of an aqueous solution of sodium sulfate (1.5%, *w/v*) were mixed and stirred for 30 min at ambient temperature. Subsequently, 10 mL of chloroform and 10 mL of an aqueous solution of sodium sulfate (1.5%, *w/v*) were added to the system, and the samples were centrifuged at 3000 rpm for 30 min and, afterwards, had their non-polar organic layer separated and filtered under anhydrous sodium sulfate and evaporated under reduced pressure.

Table 1 Specimens, collection data, and phylum of the studied Antarctic macroalgae

Species	Collection site	Geographical coordinates	Phylum
Desmarestia confervoides	Hennequim Point	62° 7′ S × 58° 23′ W	Ochrophyta
Adenocystis utricularis	Greenwich Island	62° 29′ S × 59° 45′ W	Ochrophyta
Myriogramme manginii	Snow Island	62° 46′ S × 61° 17′ W	Rhodophyta
Gigartina skottisbergii	Hennequim Point	62° 7′ S × 58° 23′ W	Rhodophyta
Curdia racovitzae	Punta Plaza	62° 5′ S × 58° 24′ W	Rhodophyta
Georgiella confluens	Hennequim Point	62° 7′ S × 58° 23′ W	Rhodophyta
Ulva intestinalis	Robert Island	62° 22′ S × 59° 41′ W	Chlorophyta

Derivatization

Derivatization of the extracted FAs was performed in triplicate ($n = 3$), and the procedure described by Moss et al. (1974) was followed. Briefly, the extracted material and 5 mL of a methanolic solution of sodium hydroxide (2%, *w/v*) were refluxed and stirred for 5 min. Subsequently, 5 mL of a methanolic solution of boron trifluoride (14%, *v/v*) was added, and the system was allowed to stir under reflux for another 5 min. Then, 20 mL of *n*-hexane was used to retrieve the non-polar organic layer that was further evaporated to dryness under reduced pressure and nitrogen flow (Moss et al. 1974).

Analysis of volatile organic compounds

Extraction

Headspace extraction of VOCs followed the method described by de Alencar et al. (2017). Briefly, 1 g of algal biomass was introduced into 20 mL boron-silicate vials and wrapped using silicone septum. Samples were incubated at 100 °C for 1 h, and, subsequently, VOCs were injected into a gas chromatograph coupled to a mass spectrometer.

Chromatographic analysis

Chromatographic analysis of FAs was carried out using gas chromatography coupled to a flame ionization detector (GC-FID) model GC-2010 (Shimadzu, Japan). The capillary column used was SP-2560 (100 m × 0.25 mm × 0.2 μm; Supelco, USA), while nitrogen at a gas flow of 1.20 mL min^{-1} was used as the carrier gas. For the analysis, 1 μL of the samples was injected in split mode (1:100) and subjected to an initial oven temperature of 140 °C increasing 4 °C min^{-1} to a final oven temperature of 240 °C maintained for 10 min. The injector port was retained at 260 °C. Identification and quantification of the detected FAs were made using a 37-mix standard of FAMEs Mix and the GC Solution software (Shimadzu, Japan).

Chromatographic analysis of VOCs was conducted by Gas Chromatography-Mass Spectrometry (GC-MS) using an equipment model GCMS-QP2010 (Shimadzu). The capillary column used was Rtx-5MS (30 m × 0.25 mm × 0.25 μm; Restek, USA) with helium at a gas flow of 1.28 mL min^{-1} as carrier gas. For the analysis, 1000 μL of the extracted VOCs was injected in direct mode in the GC–MS system using an initial oven temperature of 30 °C for 2 min growing 4 °C min^{-1} to 180 °C and then increasing to 20 °C min^{-1} until reaching the final oven temperature of 280 °C, maintaining this condition for 5 min. The ion source and the injection port were operated at 290 and 250 °C, respectively. Fragments were analyzed from m/z 30 to 450, and identification of the compounds was carried out using NIST-08 library comparing retention indexes to a C8–C20 alkane standard.

Statistical analysis

Two-way analysis of variance (ANOVA) was carried out using GraphPad version 7 (USA), and Tukey's test ($p < 0.05$) was used to differentiate FAs from the studied samples. Principal component analysis (PCA) and Ward hierarchical clustering using squared Euclidean distance were conducted by means of Minitab software version 17 (State College, USA) in order to evaluate similarity patterns among FAs and VOCs produced by the studied macroalgae.

Results

Fatty acids

Chromatographic analysis of the FAs extracted from brown, green, and red Antarctic macroalgae (Table 2) revealed that the samples were constituted of 13–25 distinct compounds that varied in aliphatic chain length from 10 to 24 carbons. Qualitatively, *D. confervoides* had the most diverse FA constitution than the other seaweeds being comprised of 10 saturated FAs (SFAs), 6 monounsaturated FAs (MUFAs), and 9 polyunsaturated FAs (PUFAs). It is worth noting that *A. utricularis* and *G. confluens* had more different types of PUFAs and MUFAs, respectively, than *G. skottisbergii* which was composed of lesser types of FAs than the other species.

As can be observed in Table 2, palmitic acid (C16:0) was the prevalent FA in the majority of the studied samples reaching as much as 65.87 ± 0.97% in *G. skottsbergii*. On the other hand, *D. confervoides* mostly contained oleic acid (C18:1*n*9c) in the concentration of 24.50 ± 0.31% while the other samples had amounts that ranged from 4.64 ± 0.62% to 22.11 ± 0.32%. Other FAs can also be highlighted from the results, including linoleic (C18:2*n*6c), α-linolenic (C18:3*n*3), arachidonic (C20:4*n*6), and eicosapentaenoic acid (C20:5*n*3). Several macroalgae also had unusual FAs in their constitution, such as heptadecanoic (C17:0), heptadecenoic (C17:1), and heneicosanoic acid (C21:0), as well as trans-FAs elaidic (18:1*n*9t) and linolenaidic acid (18:2*n*6t).

Generally, the studied macroalgae mainly contained C18-FAs (19.25 ± 0.74%–37.92 ± 0.45%), except for *C. racovitzae* that had a dominance of C20-FAs (15.26 ± 1.16%). It is worth noting that C20-FAs were also found in higher amounts in brown macroalgae (26.83 ± 0.48%–26.62 ± 1.14%) than the other samples (0.22 ± 0.06%–15.26 ± 1.16%), while C22-FAs were found in minor concentration in the studied seaweeds (0.36 ± 0.05%–2.97 ±

Table 2 Fatty acid profile (% of area) of brown, green, and red Antarctic macroalgae

Fatty acid	Ochrophyta		Chlorophyta	Rhodophyta			
	D. confervoides	*A. utricularis*	*U. intestinalis*	*M. manginii*	*G. skottisbergii*	*C. racovitzae*	*G. confluens*
10:0	1.04 ± 0.74^{a}	0.70 ± 0.06^{a}	0.15 ± 0.05^{a}	nd^{a}	1.12 ± 0.04^{a}	0.46 ± 0.24^{a}	1.48 ± 0.49^{a}
12:0	0.23 ± 0.06^{a}	0.16 ± 0.04^{a}	0.88 ± 0.34^{a}	0.59 ± 0.04^{a}	nd^{a}	5.74 ± 0.18^{b}	0.61 ± 0.10^{a}
14:0	8.04 ± 0.17^{c}	8.34 ± 0.33^{c}	2.22 ± 0.20^{d}	5.29 ± 0.13^{ab}	4.09 ± 0.05^{a}	5.84 ± 0.24^{b}	3.84 ± 0.05^{a}
14:1	0.09 ± 0.02^{a}	nd^{a}	0.27 ± 0.01^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
15:0	0.39 ± 0.03^{a}	0.38 ± 0.04^{a}	nd^{a}	0.70 ± 0.09^{a}	0.99 ± 0.02^{a}	0.76 ± 0.05^{a}	0.76 ± 0.17^{a}
16:0	20.80 ± 0.69^{e}	26.42 ± 1.11^{d}	53.06 ± 1.06^{bc}	38.61 ± 1.31^{d}	65.87 ± 0.97^{a}	53.46 ± 1.44^{b}	51.62 ± 1.31^{c}
16:1	2.71 ± 0.02^{ae}	1.76 ± 0.11^{be}	10.29 ± 1.55^{d}	6.78 ± 1.87^{f}	2.12 ± 0.07^{a}	0.50 ± 0.06^{b}	16.58 ± 0.63^{c}
17:0	0.25 ± 0.06^{a}	0.55 ± 0.05^{a}	0.15 ± 0.01^{a}	0.25 ± 0.07^{a}	nd^{a}	0.30 ± 0.02^{a}	0.48 ± 0.11^{a}
17:1	0.20 ± 0.07^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	0.73 ± 0.17^{a}
18:0	nd^{c}	1.93 ± 0.11^{b}	2.27 ± 0.24^{b}	2.28 ± 0.30^{b}	8.10 ± 0.06^{a}	3.10 ± 0.63^{b}	2.69 ± 0.34^{b}
18:1*n*9t	nd^{a}	nd^{a}	0.40 ± 0.18^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
18:1*n*9c	24.50 ± 0.31^{e}	14.89 ± 0.33^{a}	22.11 ± 0.32^{c}	20.93 ± 0.39^{c}	13.91 ± 0.06^{ad}	4.64 ± 0.62^{b}	12.94 ± 0.19^{d}
18:2*n*6t	0.26 ± 0.07^{a}	nd^{a}	1.02 ± 0.01^{a}	0.89 ± 0.24^{a}	nd^{a}	nd^{a}	0.96 ± 0.17^{a}
18:2*n*6c	8.16 ± 0.07^{c}	9.63 ± 0.04^{c}	1.13 ± 0.01^{ab}	3.08 ± 0.18^{d}	nd^{a}	2.47 ± 0.12^{bc}	1.30 ± 0.24^{ab}
18:3*n*3	4.46 ± 0.11^{c}	6.34 ± 0.11^{b}	3.28 ± 0.19^{c}	1.03 ± 0.15^{a}	nd^{a}	0.32 ± 0.14^{a}	1.35 ± 0.14^{a}
18:3*n*6	0.52 ± 0.04^{a}	0.54 ± 0.03^{a}	nd^{a}	0.48 ± 0.09^{a}	nd^{a}	0.41 ± 0.11^{a}	nd^{a}
20:0	2.28 ± 0.08^{b}	0.64 ± 0.16^{a}	0.22 ± 0.06^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
20:1	0.42 ± 0.03^{a}	nd^{a}	nd^{a}	0.62 ± 0.24^{a}	0.35 ± 0.02^{a}	0.50 ± 0.15^{a}	0.44 ± 0.10^{a}
20:2	2.70 ± 0.17^{b}	7.21 ± 0.22^{c}	nd^{a}	nd^{a}	nd^{a}	1.64 ± 0.14^{b}	nd^{a}
20:3*n*6	0.91 ± 0.06^{a}	0.69 ± 0.14^{a}	nd^{a}	0.68 ± 0.19^{a}	nd^{a}	1.37 ± 0.11^{a}	nd^{a}
20:3*n*3	0.52 ± 0.02^{a}	0.32 ± 0.12^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
20:4*n*6	14.47 ± 0.16^{c}	5.68 ± 0.22^{c}	nd^{a}	2.77 ± 0.27^{b}	0.67 ± 0.10^{a}	4.25 ± 0.24^{c}	nd^{a}
20:5*n*3	5.54 ± 0.39^{e}	12.06 ± 0.30^{d}	nd^{a}	7.95 ± 0.31^{b}	0.47 ± 0.23^{a}	7.48 ± 0.54^{b}	2.20 ± 0.18^{c}
21:0	0.12 ± 0.02^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
22:0	0.36 ± 0.05^{a}	nd^{a}	0.82 ± 0.08^{a}	0.63 ± 0.29^{a}	nd^{a}	0.27 ± 0.16^{a}	0.24 ± 0.14^{a}
22:1*n*9	$0.31 \pm 0.04a^{b}$	nd^{a}	1.61 ± 0.10^{b}	0.55 ± 0.22^{ab}	0.37 ± 0.01^{ab}	1.06 ± 0.19^{ab}	0.36 ± 0.09^{ab}
22:2	nd^{a}	1.26 ± 0.20^{ab}	nd^{a}	nd^{a}	nd^{a}	1.63 ± 0.24^{b}	nd^{a}
24:0	0.52 ± 0.02^{a}	nd^{a}	nd^{a}	0.97 ± 0.17^{a}	0.34 ± 0.16^{a}	0.73 ± 0.12^{a}	0.42 ± 0.16^{a}
24:1	nd^{a}	nd^{a}	nd^{a}	4.47 ± 0.26^{b}	1.50 ± 0.35^{ab}	2.86 ± 0.26^{b}	0.61 ± 0.20^{a}
∑C18	37.92 ± 0.45^{a}	33.34 ± 0.25^{b}	30.22 ± 0.43^{c}	28.70 ± 0.32^{c}	22.01 ± 0.12^{d}	10.96 ± 0.22^{e}	19.25 ± 0.74^{f}
∑C20	26.83 ± 0.48^{b}	26.62 ± 1.14^{b}	0.22 ± 0.06^{a}	12.58 ± 0.99^{d}	1.49 ± 0.31^{a}	15.26 ± 1.16^{c}	2.64 ± 0.28^{a}
∑C22	0.36 ± 0.05^{ad}	1.26 ± 0.20^{cd}	2.43 ± 0.13^{bce}	1.19 ± 0.51^{ade}	0.37 ± 0.01^{a}	2.97 ± 0.59^{b}	0.63 ± 0.24^{d}
∑SFA	35.81 ± 0.16^{a}	42.47 ± 4.58^{b}	59.78 ± 1.31^{c}	49.23 ± 1.37^{d}	79.87 ± 1.19^{e}	70.70 ± 2.05^{f}	62.16 ± 1.15^{c}
∑MUFA	27.94 ± 0.30^{e}	16.66 ± 0.23^{a}	34.70 ± 1.57^{b}	33.36 ± 1.85^{b}	17.75 ± 0.91^{a}	9.57 ± 0.68^{c}	31.67 ± 0.36^{d}
∑PUFA	36.13 ± 0.18^{f}	40.83 ± 4.40^{d}	5.51 ± 0.25^{c}	17.40 ± 2.46^{e}	2.37 ± 0.29^{a}	19.72 ± 1.61^{b}	6.16 ± 0.93^{c}
∑*n*6	24.33 ± 0.23^{e}	16.55 ± 0.36^{b}	2.15 ± 0.10^{ac}	7.91 ± 0.92^{d}	0.67 ± 0.10^{c}	8.52 ± 0.56^{b}	2.26 ± 0.41^{c}
∑*n*3	10.53 ± 0.31^{e}	18.73 ± 0.50^{d}	3.28 ± 0.19^{c}	9.18 ± 0.51^{be}	0.47 ± 0.23^{a}	7.81 ± 0.69^{b}	3.55 ± 0.32^{c}
∑*n*6/∑*n*3	2.30 ± 0.09^{b}	0.87 ± 0.00^{ab}	0.65 ± 0.04^{a}	0.85 ± 0.06^{ab}	1.63 ± 0.66^{ab}	1.09 ± 0.02^{ab}	0.62 ± 0.06^{a}
∑SFA/∑PUFA	0.98 ± 0.00^{d}	1.07 ± 0.22^{d}	10.85 ± 0.26^{c}	2.86 ± 0.43^{b}	34.07 ± 5.12^{a}	3.60 ± 0.38^{b}	11.98 ± 2.06^{c}

Results in triplicate ($n = 3$) represented as mean ± standard deviation

Results with distinct superscript letters within the same macroalgae development phases are significantly different ($p < 0.05$)

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; nd, not detected

0.59%). Analyzing the classes of FAs (Fig. 1S), it can be observed that SFAs showed predominance in most of the samples varying from 35.81 ± 0.16% to 79.87 ± 1.19% with the exception of *D. conferovoides* that was dominated by PUFAs (36.13 ± 0.18%). MUFAs were found in middle-ranged concentrations (9.57 ± 0.68%–34.70 ± 1.57%).

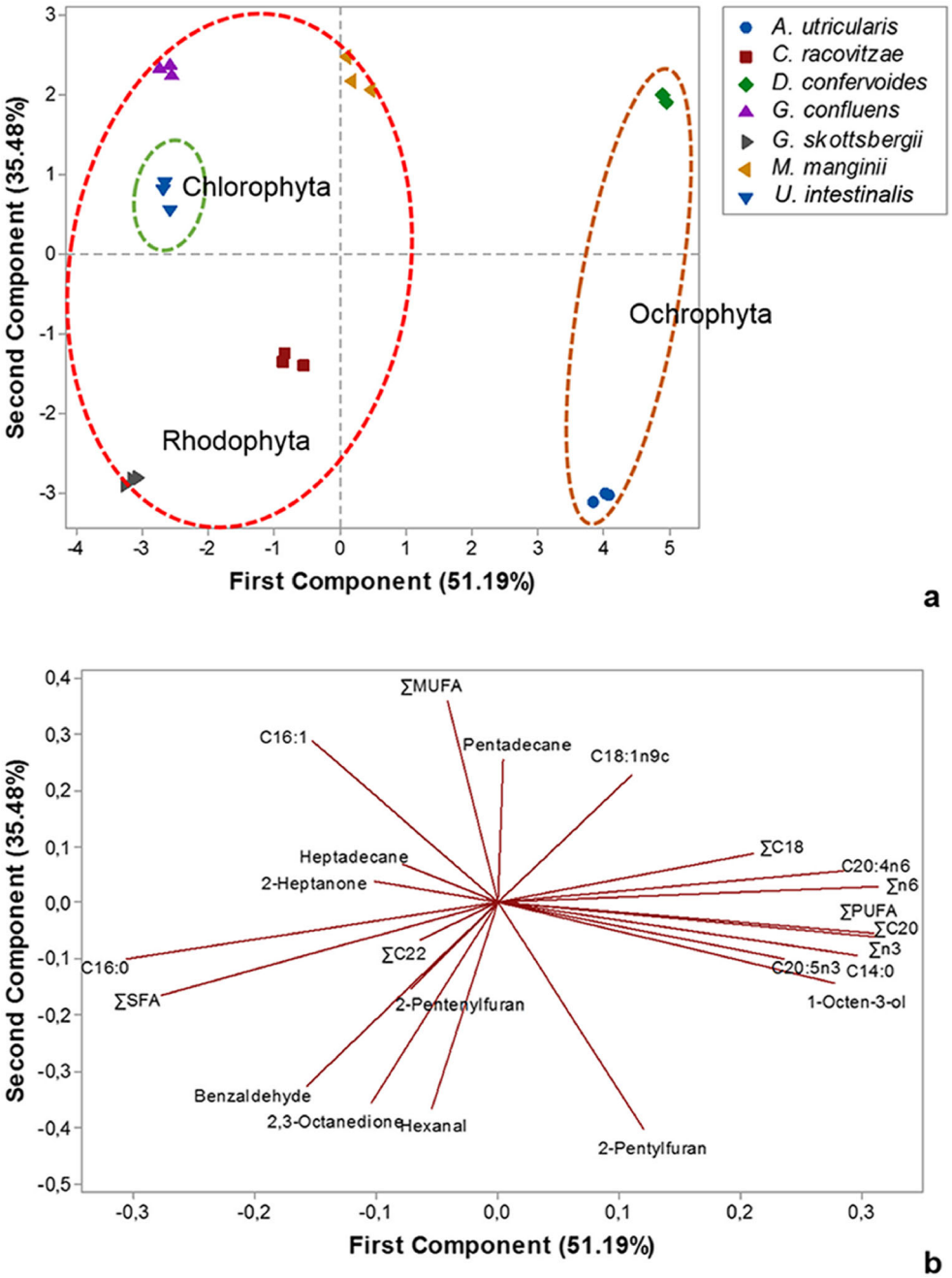

Fig. 1 Results of score (**a**) and loading (**b**) plot of multivariate analysis

Moreover, *n*3-PUFAs were found in considerable concentrations in *D. confervoides* (10.53 ± 0.31%), *A. utricularis* (18.73 ± 0.50%), and *M. manginii* (9.18 ± 0.51%) while the other samples had amounts that varied from 0.47 ± 0.23% to 7.81 ± 0.69%. Similarly, *n*6-PUFAs also had their highest concentrations in the brown macroalgae reaching as much as 24.33 ± 0.23% in *D. confervoides*, while *G. skottsbergii* only had 0.67 ± 0.10%. The ∑*n*6/∑*n*3 ratio is an important parameter to evaluate the nutritional value of a sample, in which the World Health Organization recommends a ratio of less than 10:1. In this sense, results showed that all the studied macroalgae had acceptable ∑*n*6/∑*n*3 ratios varying from 0.62 ± 0.06 to 2.30 ± 0.09.

Volatile organic compounds

Chromatographic analysis of the VOCs from brown, green, and red Antarctic macroalgae (Table 1S) indicated the presence of several chemical classes among the samples varying from 28 (*A. utricularis*) to 55 (*G. confluens*). Aldehydes (8–12), ketones (5–12), alcohols (2–6), furan

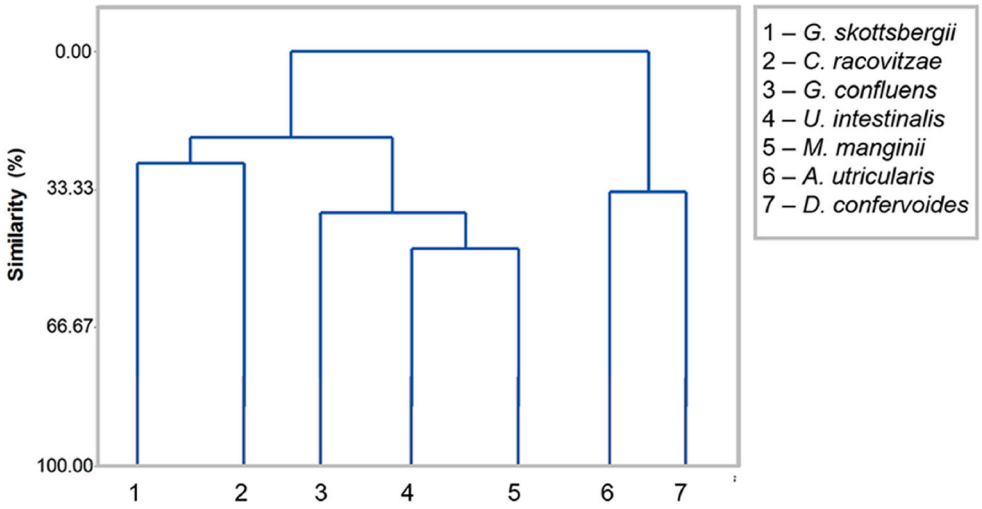

Fig. 2 Dendrogram of the hierarchical analysis of red, green, and brown Antarctic macroalgae

derivatives (2–3), hydrocarbons (7–16), and FAs (0–5) were among the chemical classes detected (Fig. 2S). VOCs were mainly found in the form of hydrocarbons (7.28–51.52%) or aldehydes (15.61–45.64%) although ketones (4.86–32.49%) and furan derivatives (4.11–18.51%) were also found in considerable amounts.

Hexanal (5.83%–25.51%), 2-pentylfuran (3.02%–12.57%), and heptadecane (0.92%–49.84%) were the compounds detected in higher concentrations in the studied samples. It is worth noting the presence of heptanal (0.30%–13.07%), 2-heptanone (2.21%–7.41%), 2-pentenylfuran (0.53%–8.65%), and palmitic acid (nd–10.02%) found in the macroalgae. Each seaweed had heptadecane as a major compound although other samples, including *A. utricularis*, for instance, had hexanal as the predominant VOC.

The VOC profile of the studied samples revealed that seaweeds had particular compounds in their constitution, such as 2-propyl-2-heptenal, β-homocyclocitral, and hexan-2-one in *G. confluens*, 2,5-dimethyl-5-heptenal, and 6-methyl-5-heptene-2-one in *D. confervoides*, and dodecanol in *A. utricularis*. Besides, some VOCs were particular to each phylum as 5-methyl-2-furaldehyde was only found in Ochrophyta samples, 4-oxoisophorone was present in the Chlorophyta representative, and 2-octenal, α-tolualdehyde, and 1-octen-3-one in the Rhodophyta species.

Multivariate analysis

Differences or similarities among the constituents of the Antarctic macroalgae were assessed using PCA (Fig. 1). In this sense, FAs (e.g., C16:0, C18:1*n*9c, and C20:5*n*3) that were significantly different in the profile of the samples and VOCs (e.g., heptadecane, hexanal, and 2-pentylfuran) found in noticeable amounts in the seaweeds were chosen as variables. Moreover, parameters, such as ∑C18, ∑PUFAs, and ∑*n*3, were also used in the statistical analysis due to their significant variations among the samples. The multivariate analysis showed that the chosen variables generated a statistical model that explained 51.19% of differences in the first component (PC1) and 35.48% in the second component (PC2) corresponding to 86.67% of the overall distinction found in the seaweeds.

From Fig. 1b, it can be observed that FAs and their corresponding classes were reasonably distributed in the loading plot as SFAs and PUFAs were found in the negative or positive axis of PC1 while MUFAs were in the positive axis of PC2. As for VOCs, similar patterns could be noticed since compounds of the same class were generally found in the same positions of PC1 and PC2. Regarding the score plot, Ochrophyta representatives were placed in positive regions of PC1 differencing in PC2 as *D. confervoides* and *A. utricularis* were in the corresponding positive or negative axis. On the other hand, Rhodophyta species clustered in the negative axis of PC1 with the exception of *M. manginii* found in the origin of PC1. Similarly to the other phylum, red macroalgae had distinct positions in PC2. *U. intestinalis* was found near Rhodophyta samples along the negative axis of PC1 and positive PC2.

Hierarchical analysis (Fig. 2) was used to assess possible similarities among the macroalgae based on their FA and VOCs profile following the same variables used in the PCA analysis. The five species were differentiated into two main clusters that were each composed of green and red seaweeds or brown macroalgae. Besides phyla, macroalgae were generally further subdivided up to the same subclass (*G. skottsbergii* and *C. racovitzae* of the Rhodymeniophycidae subclass; *A. utricularis* and

D. confervoides of Fucophycidae subclass) or order (*G. confluens* and *M. manginii* of the Ceramiales order).

Discussion

Fatty acids

The FA profile of Antarctic macroalgae showed significant differences among their constituents. Genetic and environmental factors, such as species, water temperature, photoperiod, nutrient availability, salinity, and pH, are among the reasons that can explain variations in the concentrations and types of FAs between algae species (Rautenberger and Bischof 2006; Becker et al. 2010). Indeed, analysis of the FA content of Antarctic macroalgae *Iridaea cordata*, *Palmaria decipiens*, *Plocamium cartilagineum*, and *Pyropia endiviifolia* by Santos et al. (2017) also revealed significant variations among the samples, which highlighted the influence of abiotic parameters in the production of biomolecules in seaweeds.

Given the extreme environmental conditions found in the Antarctic continent, it is thought that macroalgae adjust their biosynthesis metabolism accordingly with a higher production of PUFAs. Although susceptible to lipid peroxidation, PUFAs are essential molecules to maintain membrane's fluidity of seaweeds in cold temperatures, allowing their survival (Becker et al. 2010; Pacheco et al. 2018). It is worth noting that PUFAs can also participate in the photosynthetic mechanism as they can act as electron carriers assisting in the endogenous production of energy (Sanina et al. 2008). The occurrence of this phenomenon was observed in the Ochrophyta representatives, as well as in *M. manginii* and *C. racovitzae* in lesser proportions.

Comparing the results for the red macroalgae *G. skottsbergii*, *G. confluens*, and *C. racovitzae* to the reported in the literature, it can be noted that, qualitatively, the composition of the samples did not vary considerably. Nonetheless, the concentration of PUFAs found in the current work was lower than that indicated by Graeve et al. (2002) and Pacheco et al. (2018), who also reported the analysis of Antarctic seaweeds. Variations of environmental conditions from 2002 to 2015 may be associated to differences in the FA profile, since aspects regarding sample preparation, extraction, and derivatization methods were similar. To the best of our knowledge, this was the first time that the profile of *M. manginii* has been described with the results being similar to other representatives from the Ceramiales (Schmid et al. 2018).

Regarding brown macroalgae, *A. utricularis* had similar patterns to that observed in the literature, in which high concentrations of PUFAs were also reported. The presence of other types of FAs is worth noting, for instance, capric (C10:0), lauric (C12:0), and docosadienoic acid (C22:2) that were not observed in a previous study (Pacheco et al. 2018). The profile of *D. confervoides* was not described in previous works; however, other specimens from Desmarestiales, such as *Desmarestia muelleri* and *Desmarestia antarctica*, also had a similar FA composition (Graeve et al. 2002).

Concerning Chlorophyta, the FA composition of *U. intestinalis* partially agreed with the results reported by Horincar et al. (2014), who analyzed the same alga collected in the Romanian coast. Qualitatively, it can be observed that the Antarctic representative had a higher diversity of detected FAs although the same majoritarian components, such as C16:0 and C18:1*n*9c, were observed in both seaweeds. The foremost difference among the samples regarded the overall content of PUFAs as one third of the total composition of FAs were PUFAs for the Romanian representative, while the Antarctic representative was only composed of 5.51 ± 0.25%. Fluctuations in the results could be associated to several factors, including environmental conditions, sample treatment, extraction, and derivatization methods. Martins et al. (2016) reported the analysis of *U. intestinalis* from the sub-Antarctic region resulting in a FA profile similar to the current work.

Generally, the PUFAs found in the studied macroalgae were mainly C18:3*n*3, C20:5*n*3, and C20:4*n*6 with minor contributions of linoleic (C18:2*n*6c), dihomo-linoleic (20:2), and dihomo-γ-linolenic acid (C20:3*n*6). The presence of these compounds highlights the importance of seaweeds as natural reservoirs of nutraceutical substances since some PUFAs and their long-chain counterparts cannot be synthesized by humans or terrestrial plants (Pacheco et al. 2018). Moreover, PUFAs also have several pharmaceutical and biotechnological applications for the treatment of coronary, inflammatory, autoimmune, and tumor-related conditions as well as assisting in drug delivery (Pereira et al. 2012; Santos et al. 2016).

α-Linolenic acid (C18:3*n*3) was among the PUFAs found in greater amounts and is an important compound with reported biological activities reported in the literature, including antioxidant, neuronal protective, anticancer, and antiosteoporotic, for instance (Pereira et al. 2012; Wang et al. 2017). This FA serves as substrate to the synthesis of other relevant PUFAs, such as C20:5*n*3 and C20:4*n*6, by mechanisms of elongation and desaturation. Alongside C18:3*n*3, FAs with higher carbon-chain also have important biological roles acting on brain development, maintenance of cardiovascular health, and inflammatory processes (Larsen et al. 2011; Santos et al. 2016).

MUFAs detected mostly in the forms of palmitoleic (C16:1) and oleic (18:1*n*9c) acids were among other lipid classes of interest. Generally, MUFAs were found in intermediate concentrations (9.57 ± 0.68–34.70 ± 1.57) of PUFAs and SFAs in the studied macroalgae, reaching higher amounts in *U. intestinalis*, *M. manginii*, and *G. confluens*. These

compounds are building blocks to the further production of PUFAs and have biological activities associated to human health on the regulation of insulin sensitivity and decrease of inflammatory responses (Pacheco et al. 2018).

Finally, SFAs were also important contributors in the profile of the Antarctic macroalgae detected as the main types of FAs in almost all of the studied samples, except for *D. confervoides*. Higher uptakes of SFAs have been associated with increased risks for the development of coronary diseases, such as myocardial infarction, angina, and arteriosclerosis (Sánchez-Machado et al. 2004). However, since C16:0 was the SFA detected in higher quantity, further risks of consuming these macroalgae would be low as the enzyme Δ9-desaturase found in humans can convert it to C16:1, which itself is not associated with such biological complications (Martins et al. 2016).

Summarizing the obtained results, there is nutraceutical, pharmacological, and biotechnological potential in the studied seaweeds given that they are constituted of important *n3* and *n6*-PUFAs within their FA profile that are linked to health benefits (dos Santos et al. 2019). Pereira et al. (2012) reported that PUFAs could act as drug deliverers as they can penetrate membranes due to their lipophilicity. Moreover, lipophilic extracts of these organisms had biological potential highlighting that FAs could play important roles in antimicrobial and anticancer activities. Even though Antarctic macroalgae have not been explored for commercial purposes, their chemical composition demonstrates the effects of the natural habitat on them, providing a better understanding on the development of these organisms under extremely cold, inhospitable environments (Martins et al. 2018; Pacheco et al. 2018).

Volatile organic compounds

For the first time, macroalgae collected in the Antarctic region had their VOCs profile determined, revealing the presence of several chemical classes that included mostly aldehydes, hydrocarbons, ketones, alcohols, and furan derivatives. The occurrence of these components in seaweeds has been previously reported in organisms analyzed in other regions of the planet (Le Pape et al. 2004; Kamenarska et al. 2006; El Hattab et al. 2007). Concerning the number of compounds detected by the association of headspace extraction with GC-MS, there was a similarity to the ones found in the literature as *Osmundaria obtusiloba* and *Ceramium virgatum* were composed of 21 and 36 VOCs, respectively (Horincar et al. 2014; de Alencar et al. 2017). It is worth noting that the application of headspace sorptive extraction could enhance the total number of substances detected as previous research works indicated the presence of 152 VOCs in a mixture of *Ulva* sp. and *Gracilaria* sp. (Maruti et al. 2018).

Aldehydes in the forms of hexanal, heptanal, nonanal, and benzaldehyde were found in major proportions among the chemical classes prevalent in the profile of the studied samples. Previous works have also highlighted higher concentrations of these compounds in seaweeds, agreeing with the results found for the Antarctic samples (Nor Qhairul Izzreen and Vijaya Ratnam 2011; Ferraces-Casais et al. 2013). The occurrence of aldehydes in seaweeds can be attributed either to the metabolization of MUFAs and PUFAs (mainly linoleic acid or arachidonic acid) by the enzymes lipoxygenase/fatty acid hydroperoxide lyase or to the biosynthesis process of amino acids (Yamamoto et al. 2014; Balbas et al. 2015). Indeed, *U. intestinalis*, *G. skottsbergii*, and *G. confluens*, for instance, had decreased concentrations of C18:2*n*6 and C20:4*n*6 while higher concentrations of aldehydes compared to the other samples possibly indicating the consumption of FAs to produce VOCs.

Aldehydes are known for being part of the many constituents that influence odor and flavor aspects in macroalgae. In this sense, hexanal and benzaldehyde, for instance, have grassy-green and almond odor, respectively, conferring pleasant aromas to them. In this sense, these components could potentially be used in the flavor industry (Horincar et al. 2014). The presence of unsaturated aldehydes, including 2,4-decadienal and hexadecenal, is associated with undesirable flavors due to their fishy aroma (Peinado et al. 2014). On the other hand, aldehydes extracted from seaweeds have been reported for their antimicrobial activity against *Erwinia carotovora* and *Escherichia coli* indicating these substances could also have biological applications (Kamenarska et al. 2006).

Hydrocarbons represented mainly as heptadecane were another important group of VOCs detected, although other aliphatic, branched, and cyclic alkanes, alkenes, and aromatic compounds, for instance, were found in minor concentrations. Higher concentrations of heptadecane have also been reported in *Ulva prolifera* and *Ulva linza*, while the presence of alkanes has been highlighted in *Undaria pinnatifida* (Yamamoto et al. 2014; Balbas et al. 2015). Generally, hydrocarbons are produced from degradation processes that occur in FAs and carotenoids. Nonetheless, abiotic factors that include water temperature, photoperiod, and species also play crucial roles in the production of hydrocarbons (López-Pérez et al. 2017). It is thought that these compounds are biosynthesized to act as chemical messengers of male gametes to assist on the reproductive cycle of the organism (de Alencar et al. 2017).

Several ketones were also detected in red, brown, and green Antarctic macroalgae, in which the most predominant substances found in the studied species were 2-heptanone and 2,3-octanedione. 2-Heptanone is produced naturally in several foodstuffs, and it is used as additive in food for human consumption (de Alencar et al. 2017). The presence of ketones was also detected in *Ceramium virgatum* and *Monostroma nitidum* (Horincar et al. 2014; Yamamoto et al. 2014). Similarly to the other chemical classes, ketones are mainly

produced from metabolization processes of FAs, carotenoids, and amino acids within the seaweed (Horincar et al. 2014; Balbas et al. 2015). In aquatic organisms, the role of ketones has still been unknown, although they can act as repellent or attractive compounds to insects in terrestrial plants (Kamenarska et al. 2006). This can be associated to the low odor thresholds of most ketones that, alongside aldehydes, are important contributors to the aroma of macroalgae, in which α-ionone and 6,10-dimethyl-5,9-undecadien-2-one can be cited as examples linked to violet-like and green odors, respectively (Yamamoto et al. 2014; Balbas et al. 2015).

In diminished concentrations when compared to the other classes, alcohols were detected in the studied seaweeds mainly as 1-octen-3-ol and *p*-ethylphenol. These compounds were also present in the macroalgae *Dictyopteris membranacea* and *Palmaria palmata* from Algeria and France, respectively (Le Pape et al. 2004; El Hattab et al. 2007). Alcohols can be biosynthesized by several mechanisms that include secondary decomposition of PUFAs, glycolysis of carbohydrates, reduction of aldehydes, and from amino acids (Zhou et al. 2017; Jerković et al. 2018). The main role of alcohols is still little understood; however, it is thought that these compounds can assist in defense mechanisms of macroalgae (Kamenarska et al. 2006; Sun et al. 2012). Moreover, alcohols, such as 1-octen-3-ol, can also contribute to the aroma of the aquatic organism as they have low odor thresholds (Peinado et al. 2014).

Previous works regarding halogenated VOCs from Antarctic macroalgae highlighted the presence of diiodomethane, bromoform, dibromomethane, dibromochloromethane, chloroiodomethane, and bromodichloromethane in seaweeds (Laturnus et al. 1996, 1998). Although *G. skottsbergii* contained decyl chloride and α-chlorotoluene, generally halogenated hydrocarbons were not observed for the studied macroalgae. Among the reasons that could explain differences between the results are the distinct harvest approaches, algae treatment, and chromatographic settings. In general lines, halogenated VOCs are produced by the enzyme haloperoxidase that can fix halide ions into organic molecules. This leads to the production of halogenated metabolites that assess defensive mechanisms in macroalgae against microorganisms and competitive seaweeds (Kamenarska et al. 2006; Sun et al. 2012).

Other types of VOCs were also detected in Antarctic macroalgae, including sulfur metabolites, furan derivatives, FAs (low carbon-chain), and phthalates. Compounds containing sulfur have often been reported in seaweeds, mainly as dimethyl trisulfide, dimethyl sulfide, and dimethyl sulfoxide (Hosoglu 2018; Maruti et al. 2018). Furan derivatives compose several chemicals that influence the aroma of macroalgae, being produced from the degradation of linoleic acid or carbohydrates (Sun et al. 2012; de Alencar et al. 2017). Phthalates mainly as diisobutyl phthalate, diethyl phthalate, and dimethyl phthalate were also observed in the studied organisms. These substances are not associated to endogenous processes in macroalgae, but they could be found in seaweeds as a result of environmental pollution, such as those reported for *Capsosiphon fulvescens* collected in South Korea (Sun et al. 2012).

According to reports from the literature, VOCs produced by macroalgae are considerably affected by environmental factors and species, which could also be observed in the present work. Nonetheless, few seaweeds have had their profile of VOCs elucidated, indicating further research studies need to be conducted for the identification of aquatic components (Maruti et al. 2018). In this manner, it would be possible to detect chemicals with commercial or biological applications in the food, biotechnological, and pharmaceutical industries, for instance, as it has been demonstrated that seaweeds comprehend a vast and biorenewable source of interesting substances (Horincar et al. 2014; de Alencar et al. 2017).

Multivariate analysis

According to the PCA results, FA and VOCs profiles could be used to differentiate macroalgae in their respective phylum. In this sense, higher concentrations of variables that included C18-FAs, C20-FAs, PUFAs (C20:4*n*6 and C20:5*n*3), and 1-octen-3-ol assisted in the distinction of Ochrophyta representatives from the other phyla. Moreover, distinct amounts of 2-pentylfuran and C18:1*n*9c differentiated the brown algae *D. confervoides* and *A. utricularis*. Similar patterns were observed for Rhodophyta as samples of this phylum could be distinguished by the presence of SFAs (mainly C16:0), MUFAs (mainly C16:1), 2-heptanone, 2-pentenylfuran, benzaldehyde, 2,3-octanedione, and hexanal. Further differences in the concentrations of these variables also allowed the distinction of species on the score plot.

Using PCA, Van Durme et al. (2013) also observed that distinct concentrations of VOCs could differentiate several species of microalgae. Multivariate analysis was also employed by Bravo-Linares and Mudge (2009) who showed that physico-chemical and environmental conditions, including wind speed, water temperature, and seasoning, influenced the biosynthesis of VOCs in marine organisms. Moreover, the use of FAs as means to establish distinctions among seaweeds and their phyla has also been reported in the literature (Kumari et al. 2009).

The application of hierarchical analysis reinforced the results obtained in the PCA analysis as similar macroalgae tended to cluster in the dendrogram. In this sense, it was observed that the distinct FA and VOC profiles present mostly in brown and red seaweeds could differentiate them into two main groups. Similarities among the variables also caused the clustering of red and green macroalgae, which could be associated to their genetic closeness compared to brown

seaweeds. Analyzing the FA profile of 27 macroalgae, Kumari et al. (2009) successfully differentiated or clustered samples based on their chemical composition agreeing with the results of the current research work.

Conclusions

In the present study, the FA and VOC profiles of red, brown, and green Antarctic macroalgae were successfully analyzed. Results showed that palmitic, oleic, linoleic, and eicosapentaenoic acids were the predominant FAs while hexanal, heptadecane, and 2-pentylfuran were the major types of VOCs in the samples. Furthermore, statistical analysis of the algal chemical components indicated distinct degrees of similarities among them. Therefore, this study elucidated that Antarctic macroalgae had a wide range of constituents which are associated to defense mechanisms and could lead to the development of novel bioactive compounds for applications in food, pharmaceutical, and biotechnological industries.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Capítulo 6

Evaluation of volatile organic compounds in brown and red sub-Antarctic macroalgae

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BIOCHEMISTRY & PHYSIOLOGY - ORIGINAL ARTICLE

Evaluation of volatile organic compounds in brown and red sub-Antarctic macroalgae

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Abstract

Volatile organic compounds (VOCs) comprise a wide range of compounds with low molecular weight that are released from seaweeds as secondary metabolites. Therefore, the aim of this work was to evaluate the profile of VOCs from six sub-Antarctic macroalgae by means of gas chromatography–mass spectrometry. Results indicated that the studied seaweeds contained approximately 40 different compounds found as aldehydes, ketones, hydrocarbons and alcohols, for instance. For brown macroalgae, hexanal was found in greater amounts (12.87–29.46%) followed by 2-pentylfuran (5.52–11.61%) and pentadecane (4.29–10.28%), while red seaweeds were mainly composed of heptadecane (27.79–85.12%), hexanal (1.78–11.62%) and benzaldehyde (1.36–2.87%). Further evaluation using principal component analysis showed that distinct compositions of VOCs could differentiate brown and red seaweeds. Therefore, sub-Antarctic macroalgae had their VOCs elucidated for the first time assisting in the efforts for understanding their biochemical constitution as well as supporting in further potential pharmacological and biotechnological applications.

Keywords Gas chromatography–mass spectrometry · Ochrophyta · Rhodophyta · Sub-Antarctic region · Volatile organic compounds

1 Introduction

Algae encompass a complex group of unicellular or multicellular organisms that inhabit innumerous aquatic environments throughout the planet (Peinado et al. 2014) which can be divided into Ochrophyta, Rhodophyta and Chlorophyta phyla according to certain patterns that include pigmentation, energy storage and physiological features (Horincar et al. 2014; Passos et al. 2020). Macroalgae can be used for several purposes including, for instance, human consumption, soil fertilizers and animal feed, due to their diverse range of metabolites (Maruti et al. 2018; de Freitas et al. 2020). Moreover, they have biological potential as previous works showed their antimicrobial, antifungal and antitumor activities (Martins et al. 2018; Pacheco et al. 2018).

The enhanced capacity of macroalgae to adapt to diverse habitats allowed these organisms to succeed at inhospitable environments such as the sub-Antarctic region (Rozzi et al. 2012). Although known for low temperatures, high exposure to ultraviolet radiation, limited photoperiod (winter season) and restricted nutrient availability, there are several species of macroalgae that inhabit the sub-Antarctic region. In order to overcome extreme abiotic factors, seaweeds produce several compounds that include fatty acids, proteins, vitamins and volatile organic compounds (VOCs) (Mansilla et al. 2012; Peinado et al. 2014; Berneira et al. 2020).

Among metabolites synthesized by macroalgae, VOCs comprehend a broad group of compounds that generally have low molecular weight, little miscibility with water and high vapor pressure, which enable these substances to

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cross membranes and interact with the external environment (de Alencar et al. 2017). In this sense, VOCs can be found most notably as aldehydes, ketones, hydrocarbons, alcohols, terpenes, halocompounds and esters (López-Pérez et al. 2017). The role of VOCs in aquatic organisms is still largely unknown; however, it is speculated that these substances act as mechanisms of attraction, communication and defense in macroalgae (Jerković et al. 2018).

According to the literature, there are several extraction-developed procedures for the analysis of VOCs that include, for instance, methods involving headspace, solvent, hydrodistillation and supercritical fluid extraction (Gressler et al. 2009; Berneira et al. 2020). It is worth noting that most methodologies are time-consuming and rely on the use of organic solvents and a thorough sample preparation step (Izzreen and Ratnam 2011; Ferraces-Casais et al. 2013). Given this, the use of headspace extraction associated with detection by gas chromatography–mass spectrometry (GC–MS) can overcome these disadvantages serving as a simple and efficient procedure for the analysis of VOCs (de Alencar et al. 2017).

Despite extensive studies of VOCs produced by terrestrial plants, there are still few works dealing with the profile of these compounds in marine organisms. Moreover, to the best of our knowledge, there are no studies of the composition VOCs regarding sub-Antarctic seaweeds. The determination of these compounds could be an interesting approach to uncover their biochemical constitution and to search for potential bioactive substances. Therefore, the aims of this work were to evaluate VOCs produced by six brown or red sub-Antarctic macroalgae by means of headspace extraction with detection by GC–MS.

2 Materials and methods

Sampling – Brown macroalgae *Lessonia searlesiana* Asensi & de Reviers, *Macrocystis pyrifera* (Linnaeus) C .Agardh, *Lessonia flavicans* Bory and *Durvillaea antarctica* (Chamisso) Hariot as well as red macroalgae *Mazzaella laminarioides* (Bory) Fredericq and *Iridaea cordata* (Turner) Bory were collected in the sub-Antarctic region in locations near Punta Arenas, Chile, between December of 2016 and November of 2017 (Table 1). Generally, six to ten individuals were manually collected at low tide in the intertidal zone or in the lower littoral zone. After collection, samples were washed with seawater and distilled water in order to remove epiphytes. Subsequently, samples were identified, lyophilized and pulverized being maintained within dark plastic bags under − 20 °C before analysis. Approximately 10 g of each specimen was obtained after this sample preparation process. Morphological identification was performed by the sub-Antarctic Marine Ecosystems Laboratory while exsiccates were stored under specified numbering in the Herbarium of the Antarctic.

Extraction – After samples were lyophilized and pulverized, their volatile components were extracted following the modified procedure described by de Alencar et al. (2017) where 1 g of the algal biomass was placed within 20-mL borosilicate vials and sealed with a silicone septum. The material was introduced in an oven and incubated for 1 h at 100 °C, and VOCs were removed after the heating process to be further analyzed by GC–MS.

Chromatographic analysis – Briefly, 1000 µL of the VOCs was injected into a GC–MS model QP-2010 (Shimadzu, Kyoto, Japan) equipped with a Rtx-5MS capillary column (30 m × 0.25 mm × 0.25 µm) and helium as the carrier gas. The injection port and the ion source were kept at 250 °C and 290 °C, respectively. The oven was operated at an initial temperature of 30 °C for 2 min, increasing 4 °C/min to 180 °C and then 20 °C/min until reaching a final temperature of 280 °C maintaining for 5 min. Percentage of area (% of area) was used to measure concentration of VOCs as well as NIST-08 s and retention index for the identification of the compounds. The retention index of the detected substances was determined using a solution of the C8–C20 standard (Sigma-Aldrich, St. Louis, USA).

Table 1 Sampling information about the brown and red sub-Antarctic macroalgae

Species	Collection site	Sampling date	Marine habitats	Development phase	Herbarium number
L. searlesiana	Puerto del Hambre	November 2017	Infralittoral zone	Gametophytic	1303r
M. pyrifera	Puerto del Hambre	December 2016	Littoral zone	Gametophytic	518p
L. flavicans	Puerto del Hambre	December 2016	Infralittoral zone	Gametophytic	522p
D. antarctica	Seno Otway	April 2017	Lower mesolittoral zone	Gametophytic	582p
I. cordata	San Juan	December 2016	Middle intertidal zone	Gametophytic	1307r
M. laminarioides	San Juan	November 2017	Higher intertidal zone	Gametophytic	1298r

Statistical analysis Principal component analysis (PCA) was used to evaluate and discriminate macroalgae based on selected VOCs that were employed as variables using Minitab software version 17 (State College, USA).

3 Results

The majoritary VOCs profile of the sub-Antarctic macroalgae from the sub-Antarctic region (Table 2) revealed that the samples were composed of 28 (*M. laminarioides*) to 43 (*D. antarctica*) compounds. The detected substances could be divided into several classes that included, for instance, aldehydes (8–10), ketones (4–13), hydrocarbons (5–10), alcohols (3–8) and furan derivatives (1–3). Generally, aldehydes (5.68–38.16%) and hydrocarbons (16.61–88.94%) dominated the VOCs profile followed by furan derivatives (1.37–14.63%) and ketones (1.15–15.95%). Online Resource 1 displays the complete profile of VOCs found in the studied samples.

As it can be observed in Table 2, hexanal (1.78–29.46%) was the component found in major concentrations in all the studied samples except for *M. laminarioides* in which heptadecane (85.12%) was detected in enhanced amounts. Besides heptadecane (0.29–85.12%), pentadecane (1.22–10.28%) and 8-heptadecene (0.13–1.77%) were the most representative hydrocarbons in the macroalgae. Other components found in considerable amounts were 2-pentyl furan (0.12–11.61%), 2,3-octanedione (0.13–4.94%) and 2-pentenyl furan (0.21–4.94%).

It is worth noting that the macroalgae had particular compounds in the VOCs profile, such as 2-octenal and *p*-tolylmethylcarbinol in *M. laminarioides*. Moreover, some VOCs were particular to each phylum as 2,5-dimethylundecane, 2-dodecenol and 1-octen-3-one, for instance, were generally found in representatives from the Ochrophyta phyla, while 3,4-dihydro- β-ionone, α-ionone and 3-methylfuroate were only found in Rhodophyta samples.

PCA was used to evaluate the distinct compositions of VOCs in brown and red sub-Antarctic macroalgae (Fig. 1). In order to establish possible similarities within the samples, VOCs that had noticeable concentrations in the studied seaweeds were chosen as variables for the statistical evaluation. Therefore, 17 compounds of distinct chemical classes were selected including, for instance, benzaldehyde, pentadecane, dimethyl trisulfide, 1-octen-3-ol, 2-pentenylfuran and 4-oxoisophorone. Results showed that these variables generated a statistical model that explained approximately 50% of variances between the samples.

4 Discussion

Generally, the chemical classes highlighted in this study were previously observed in the literature regarding macroalgae from other environments (López-Pérez et al. 2017; Jerković et al. 2018). Moreover, it was possible to observe that qualitatively the number of compounds determined by the use of headspace extraction associated with GC–MS was in the range reported by previous works, which varied from 21 VOCs in *Pterocladiella capillacea* (S.G.Gmelin) Bornet 1876 to 46 compounds in *Ulva intestinalis* 1753 (Horincar et al. 2014; de Alencar et al. 2017).

Previous studies indicated the prevalence of aldehydes in the brown macroalgae *Halopteris filicina* (Grateloup) Kützing and *Dictyota dichotoma* (Hudson) J.V.Lamouroux collected from the Adriatic Sea as well as red seaweed *Kappaphycus alvarezii* (Doty) L.M.Liao from Borneo Island (Izzreen and Ratnam 2011; Jerković et al. 2018). These compounds are derived from the metabolization of polyunsaturated fatty acids (PUFAs) or from the biosynthesis pathway of amino acids (Yamamoto et al. 2014). The presence of aldehydes is known for considerably influencing flavor aspects of seaweeds (López-Pérez et al. 2017; Berneira et al. 2020).

Table 2 Concentration (% of area) of the VOCs from the sub-Antarctic macroalgae

Compound	Literature RI	Experimental RI	Ochrophyta				Rhodophyta	
			L. searlesiana	*M. pyrifera*	*L. flavicans*	*D. antarctica*	*M. laminarioides*	*I. cordata*
Hexanal	790	< 800	29.46	19.25	19.99	12.87	1.78	11.62
Benzaldehyde	960	964	1.09	2.04	1.74	1.55	1.36	2.87
2-Heptanone	892	912	3.97	2.03	2.09	nd	nd	0.70
2,3-Octanedione	995	995	3.59	4.94	3.25	2.20	0.13	0.78
1-Octen-3-ol	985	990	2.97	4.40	2.67	9.15	0.05	1.85
2-Pentylfuran	1000	1001	11.61	5.52	8.58	6.64	0.12	3.93
Pentadecane	1500	1500	8.34	4.29	10.28	4.92	1.76	1.22
Heptadecane	1700	1700	0.29	7.79	15.30	4.65	85.12	27.79

RI Retention Index; *nd* non-detected

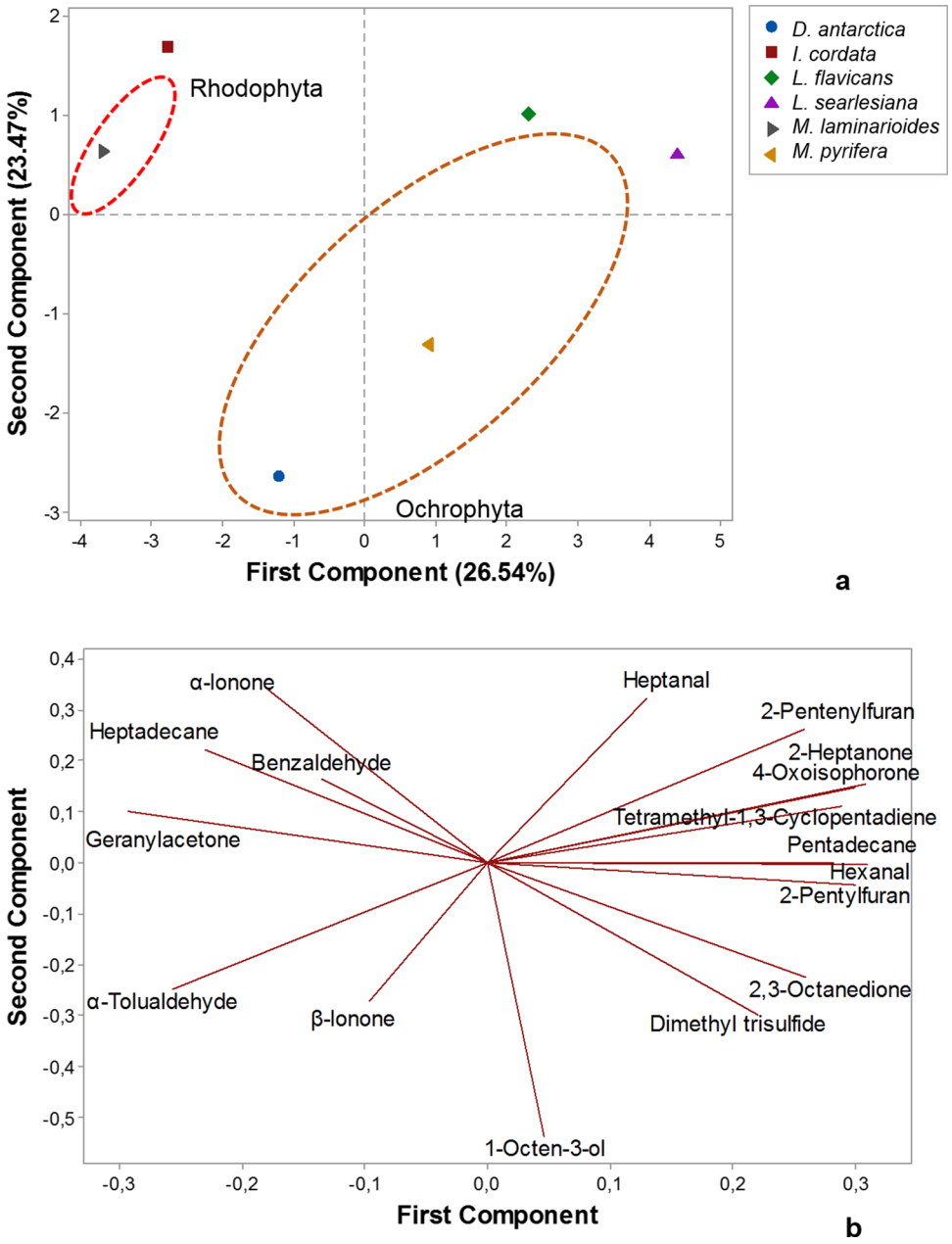

Fig. 1 Loading (**b**) and score (**a**) plots of PCA of red and brown sub-Antarctic macroalgae

Ketones were also found in considerable amounts in the studied macroalgae and are derived from the metabolization of fatty acids, carotenoids and amino acids (Horincar et al. 2014; Balbas et al. 2015). They are important aroma contributors to macroalgae with low odor thresholds with *β*-ionone and *α*-ionone being related to wood and violet-like scent (Yamamoto et al. 2014). In general, carbonyl compounds including ketones and aldehydes are known for their roles as attractants or repellents in terrestrial plants, but their exact function in marine organisms is still little understood (Kamenarska et al. 2006).

The presence of alcohols can be associated with the secondary decomposition of hydroperoxides of PUFAs, from the glycolysis of carbohydrates, from amino acids via the Ehrlich pathway or from reduction of aldehydes contributing to the aroma of seaweeds (Peinado et al. 2014; López-Pérez et al. 2017; Zhou et al. 2017; Jerković et al. 2018). It is worth noting that this is the first time that 2,4-di-tert-butylphenol and *p*-tolylmethylcarbinol are reported in macroalgae. Similar alcohols to those were detected in previous studies and are related defense mechanisms of seaweeds (Kamenarska et al. 2006; Sun et al. 2012).

Hydrocarbons were another representative group of VOCs from sub-Antarctic macroalgae reaching as much as 88.94% of the composition of *M. laminarioides*. According to the literature, hydrocarbons can be originated from

degradations of hydroperoxides of fatty acids and carotenoids. These compounds act in the reproductive process of organism serving as chemical signaling to male gametes (de Alencar et al. 2017; Zhou et al. 2017). According to López-Pérez et al. 2017, the production of hydrocarbons is considerably influenced by species, which could be observed in the results as each macroalgae had distinct concentrations and types of hydrocarbons.

Other classes of substances could also be detected in the profile of VOCs from the sub-Antarctic macroalgae-included furan derivatives that are associated with the oxidative degradation of linoleic acid or sugar dehydration (Le Pape et al. 2004; Sun et al. 2012; de Alencar et al. 2017). Free fatty acids were observed in *L. searlesiana* as arachidonic and docosahexaenoic acid as macroalgae from the Laminariales order generally have higher concentrations of these biomolecules (Santos et al. 2017; Schmid et al. 2018).

As it can be noticed in Fig. 1, there were no clear separations among the chosen representatives of the chemical classes used as variables for the loading plot. Nonetheless, generally aldehydes were observed in the negative axis of the first component (PC1), furan derivatives were seen in the positive axis of PC1, while alcohols were found in the negative axis of the second component (PC2). Despite the distinct influences of the variables of the same chemical class, samples of different phylum could be discriminated as red macroalgae clustered in negative PC1 and positive PC2, while brown seaweeds were usually observed in positive PC1 and in both regions of PC2.

Among the reasons that explained the phylum discrimination were, for instance, higher amounts of heptadecane in Rhodophyta and hexanal in Ochrophyta, although other variables also influenced the overall results. Brown macroalgae were more spread across the plot than red seaweeds which could be attributed to distinct concentrations of β-ionone, geranylacetone and 1,2,3,4-tetramethyl-1,3-cyclopentadiene within Ochrophyta.

The PCA as a statistical tool to discriminate macroalgae based on their distinct chemical composition has been successfully used in the literature. Kumari et al. (2010) evaluated differences among the constitution of fatty acids in tropical macroalgae and observed that red, green and brown seaweeds could be discriminated based on distinct concentrations and types of fatty acids in their constitution. Furthermore, Peinado et al. (2014) used VOCs associated with physicochemical parameters and sensory attributes and also efficiently differentiated macroalgae. Similarly, Balbas et al. (2015) discriminated wakame samples based on their distinct profile of VOCs.

As it can be observed, sub-Antarctic macroalgae were composed of several VOCs representing a renewable and natural source of potential bioactive substances (de Alencar et al. 2017). Previous research works have indicated that VOCs could be applied to several industrial areas that include agricultural, pharmaceutical, cosmetics and food (Rodríguez-Meizoso et al. 2008; Borik 2014; Horincar et al. 2014; Berneira et al. 2020). Indeed, VOCs have been shown to have antimicrobial activity as demonstrated in strains of *Erwinia carotovora* (Jones) and *Escherichia coli* (Migula) (Kajiwara et al. 2006). The presence of certain ketones can also lead to the use of VOCs as repellents in agricultural fields. Moreover, Kamenarska et al. (2006) and Van Durme et al. (2013) indicated that the volatile content could also have chemotaxonomical applications for the identification of species and phyla of macroalgae and microalgae.

5 Conclusion

Summarizing, the profile of VOCs from red and brown sub-Antarctic macroalgae was elucidated for the first time indicating the presence of approximately 40 components in their constitution. The presence of these compounds can be associated with biochemical mechanisms of seaweeds in order to survive the inhospitable environment of the Strait of Magellan. Moreover, VOCs could have economical applications to be used in biotechnological, pharmaceutical and food areas.

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Author's contribution LMB contributed to writing—original draft; CCS contributed to writing—review and editing; LFP administrated the project; AM helped in conceptualization; MAZS contributed to methodology; and CMPP contributed to funding acquisition.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Capítulo 7

Novel application of sub-Antarctic macroalgae as zinc oxide nanoparticles biosynthesizers

Manuscrito submetido para a revista
Materials Letters.

Novel application of sub-Antarctic macroalgae as zinc oxide nanoparticles biosynthesizers

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Abstract

The search for novel starting materials in the biological synthesis of zinc oxide nanoparticles (ZnO NPs) is crucial to develop synthetic procedures using environment-friendly approaches. In this sense, the aim of this study was to synthesize and evaluate ZnO NPs using green approaches produced from three sub-Antarctic macroalgae. It is worth noting that this was the first time that sub-Antarctic macroalgae were used for this purpose. Results showed that band intensity of polar bands that included hydroxyl (3779-3002 cm^{-1}) and carbonyl groups (1770-1639 cm^{-1}) decreased with the formation of ZnO NPs while the band associated to the zinc-oxygen bond appeared at 450 cm^{-1} in the synthesized nanomaterials. Further analysis confirmed the formation of ZnO NPs as the product was mostly composed of zinc (≥ 97.27%) in the form of nanoparticles. Finally, X-Ray Diffraction indicated the zincite phase present in the hexagonal structures of ZnO NPs while SEM analysis confirmed that the nanomaterials were homogeneous and well-compacted. Therefore, sub-Antarctic could be promising sources of phytochemicals to produce nanomaterials under environment-friendly approaches.

Key-words: Biomaterials; Nanoparticles; Spectroscopy; X-Ray techniques

1. Introduction

Over the past years, there has been an increasing interest regarding zinc oxide nanoparticles (ZnO NPs) due to its extensive range of applications in biomedical, electronics, optics and optoelectronics areas [1,2]. In this sense, it has been reported their use in ointments, bio-imaging agents, drug-delivery structures, antimicrobial materials and dye degradation processes [3,4]. Conventional chemical and physical methods rely on chemical precipitation, hydrothermal and solvothermal approaches. Nonetheless, conventional synthesis of ZnO NPs is costly and use toxic reagents that can damage environment equilibrium and human health. Given this, eco-friendly and alternative approaches have been proposed, including the use of microorganisms, enzymes and plants to synthesize NPs [5].

Among feasible alternatives to NP synthesis are macroalgae, which comprise multicellular organisms differentiated into several phyla due to their distinct physiological, morphological and biochemical patterns [6,7]. They can inhabit several marine environments ranging from polar, sub-Antarctic and tropical waters [8]. Moreover, these organisms are a source of several phytochemicals that include carbohydrates, proteins, vitamins and volatile organic compounds [9]. Previous research works indicated their potential to act as biosynthesizers of NPs, however most specimens remain unexplored [2,10].

As it can be observed, green, low-cost and fast synthesis of NPs are important aspects in order to obtain materials with little to none environmental impact and reduced human toxicity [5,11]. Given this, macroalgae can be a rich,renewable and, yet, unexplored reservoir to act as biosynthesizers of NPs. In this sense, the aim of the study was to evaluate the potential of three sub-Antarctic macroalgae to biosynthesize ZnO NPs using Fourier-Transform Infrared (FT-IR) Spectroscopy, Ultraviolet-Visible (UV-Vis)

Spectroscopy, Energy-Dispersive X-Ray Spectroscopy (EDS), X-ray Diffraction (XRD), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

2. Materials and Methods

Sub-Antarctic macroalgae *Lessonia flavicans*, *Gigartina skottsbergii* and *Mazaella laminarioides* were collected from the Punta Arenas region (Chile) in 2018. Samples were washed with water, morphologically identified, dried, pulverized and stored in dark plastic bags at – 20 °C before analysis [6].

Extraction was performed using 2 g of the biomass and 100 mL of deionized water which were heated to 100 °C for 1 h and filtered in order to obtain an aqueous extract. Samples were further mixed with a 0.1 M aqueous solution of zinc acetate di-hydrate (5:2, *v/v*) under heating and stirring for 2 h at 70 °C. pH was adjusted to 11 using a 1 M aqueous solution of sodium hydroxide. Samples were centrifuged at 4,000 rpm for 10 min and the solid product was isolated, washed with deionized water and dried at 100 °C for 48 h. ZnO NPs were characterized by FT-IR Spectroscopy model Spirit (Shimadzu, Japan), UV-Vis Spectroscopy model UV M90 (Bel, Italy), EDS (EDX-720, Shimadzu), SEM model JSM-6010LA (Jeol, USA), TEM model JEM-1400 (Jeol, USA) and XRD model Shimadzu XRD 2000 [12].

3. Results and Discussion

Throughout visual assessment it was possible to observe that the nanomaterials were synthesized since the reaction changed from light green to pale white similarly to what was described by Azizi et al. (2014) and Sanaeimehr et al. (2018). Infrared

Spectroscopy analysis (**Fig 1a**) indicated that sub-Antarctic macroalgae were composed of several phytochemicals including carbohydrates, proteins and lipids due to the presence of hydroxyl groups (3779-3002 cm^{-1}), aliphatic carbons (2999-3039 cm^{-1}), carbonyl groups (1770-1639 cm^{-1}) and carbon-oxygen bonds (1329-956 cm^{-1}) agreeing with the observed by Passos et al (2020).

Formation of ZnO NPs from macroalgae (**Fig 1b**) decreased the intensity of bands associated to polar groups, showing that hydroxyl and sulfate groups commonly present in polysaccharides are important in the biosynthesis of NPs. It was also observed the presence of a broad (NH) C=O stretch associated to proteins at 1568 cm^{-1}, indicating that these biomolecules may act as stabilizers of NPs. Moreover, ZnO NPs had a characteristic Zn-O stretch at 450 cm^{-1} indicating that the nanostructures were formed. Regarding UV-Vis Spectroscopy, it was observed bands between 300 and 400 nm associated to the absorption of ZnO crystals [1,2]. Results regarding the macroalgae and their synthesized nanomaterials can be seen in the *Supplementary Information* section.

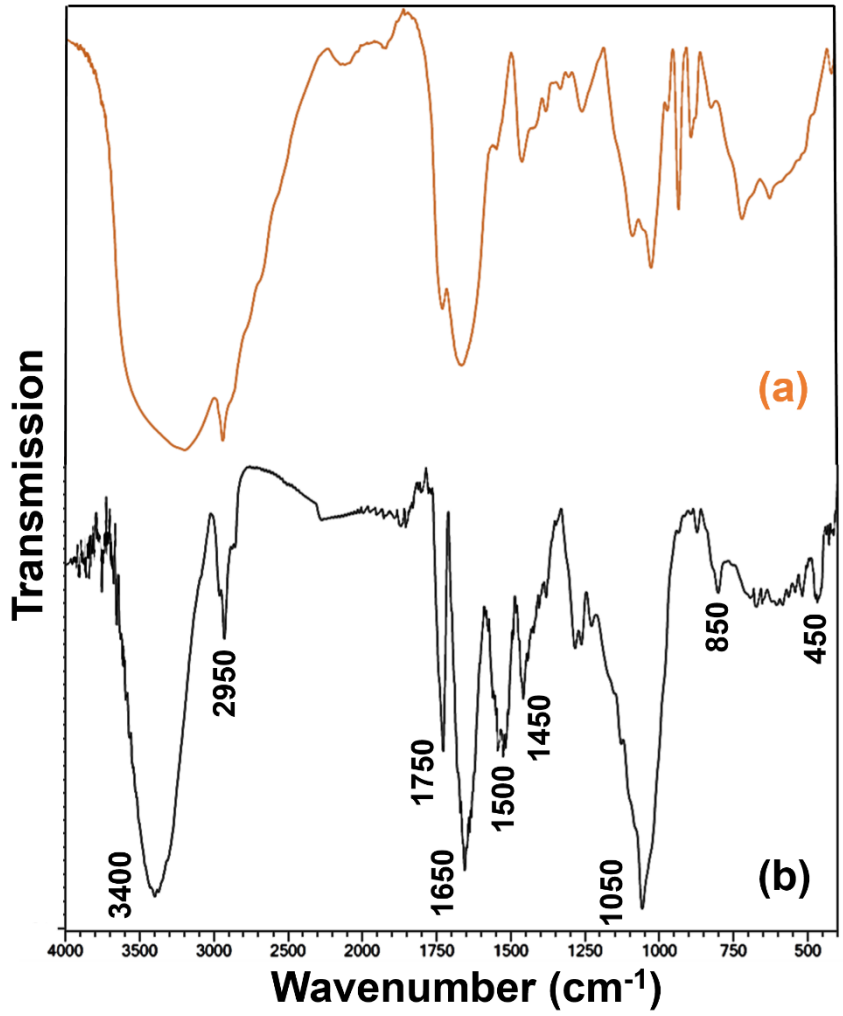

Figure 1. FT-IR analysis of *L. flavicans* extract (**a**) and ZnO NPs synthesized from *L. flavicans* extract (**b**).

EDS analysis indicated the NPs formation as zinc was the main constituent in the samples (≥ 97.27%) agreeing with previous studies in the area [1,2]. Other metals found in the nanomaterials were, for instance, sulfur (1.16% – 0.22%), potassium (0.15%) and calcium (1.39% - 0.62%) probably sourced from the algal biomass [1,8].

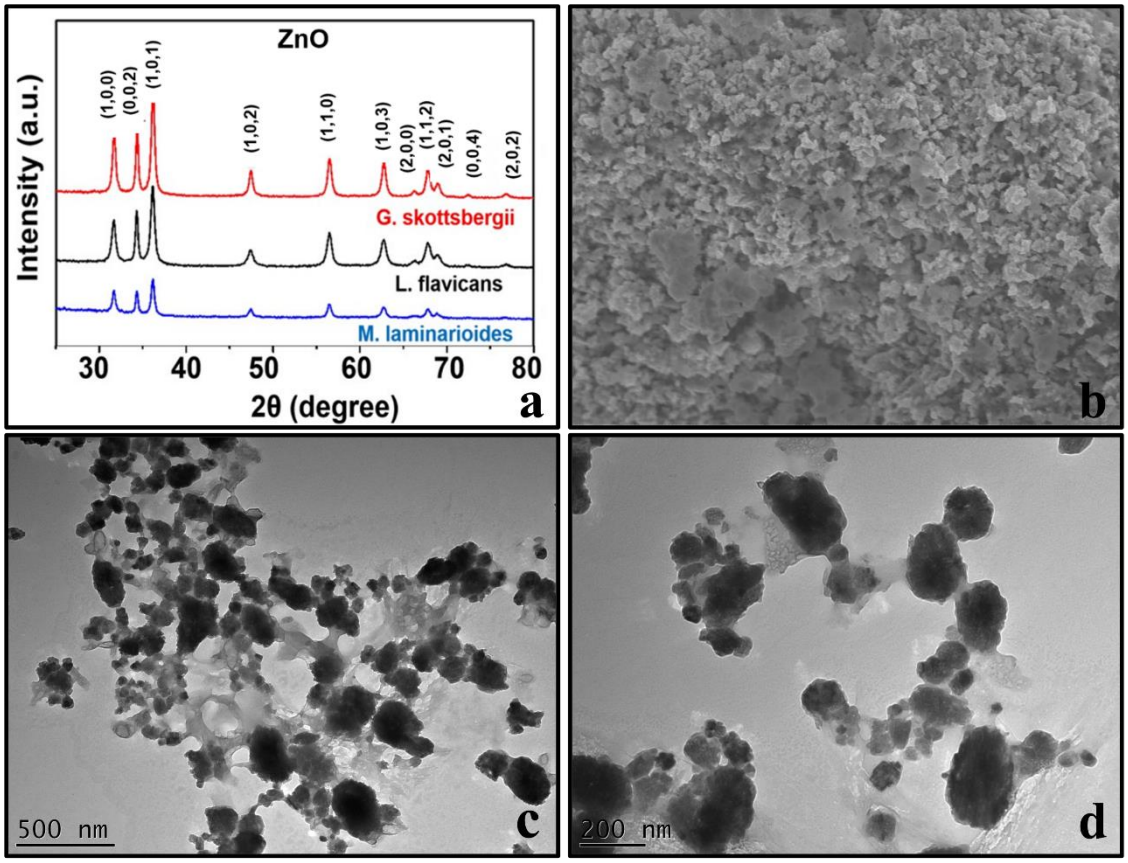

Figure 2. XRD patterns of ZnO NPs biosynthesized from sub-Antarctic macroalgae (**a**); SEM analysis of ZnO NPs from *L. flavicans* (**b**); TEM analysis of ZnO NPs from *L. flavicans* (**c, d**).

XRD analysis (**Fig. 2a**) confirmed the presence of ZnO NPs formed from sub-Antarctic macroalgae according to the patterns observed in the spectra. The presence of peaks related to the reflections of zincite phase of hexagonal structure of ZnO NPs could be observed for all samples, according to the Joint Committee on Powder Diffraction Standard (JCPDS) 36-1451 crystallography database. The main peaks were noticed at 31°, 34° and 36° for all samples, corresponding to the planes (1,0,0), (0,0,2) and (1,0,1), respectively, indicating that the biosynthesis of ZnO NPs was effective.

The morphology of the nanomaterials was investigated by SEM as can be seen at **Fig. 2b** that exhibits the micrographs at different magnifications. It can be observed that the proposed methodology resulted in predominantly homogeneous and well-compacted of ZnO NPs. Among the reasons that can explain this behavior is the electrostatic and polarization of the nanomaterials which formed agglomerations [2]. TEM was used to examine the particle size of the ZnO NPs and revealed a predominance of irregular spherical shaped nanoparticles with size ranging from 50 nm to agglomerates reaching 200 nm, as can be seen in **Fig.2c** and **Fig. 2d**.

Jeyabharathi et al. (2017) synthesized ZnO NPs from the aqueous extract of *Amaranthus caudatus* and indicated that the samples had antimicrobial activity in gram positive and gram negative bacteria [3]. Moreover, Govarthanan et al. (2020) made ZnO NPs from the aqueous extract of *Candelabra cactus* and tested the nanomaterials for their cytotoxic action indicating that the samples decreased viability of MCF-7 cells as their concentration increased from 25 to 100 mg/mL[4]. Therefore, ZnO NPs produced from biological sources can have important biological activities with diminished toxicity.

It is worth noting that, to this date, it is still unknown the complete mechanism of ZnO NPs formation [2,13]. Nonetheless, it is recognized that polar components are associated to their production. The severe environment conditions found in the sub-Antarctic region activate the secondary metabolism within macroalgae forcing these organisms to synthesize carbohydrates, proteins and other components linked to ZnO NPs formation [6,9,14]. Therefore, sub-Antarctic macroalgae are a reservoir of several bioactive molecules with increased potential to synthesize nanomaterials.

Conclusion

The study successfully synthesized ZnO NPs from sub-Antarctic macroalgae using green approaches. Samples were characterized by FT-IR, SEM, XRD and TEM indicating that the product was satisfactorily synthesized. Therefore, sub-Antarctic macroalgae could be a potential green, renewable and low-cost agent in the phycosynthesis of NPs.

Acknowledgments

The authors are thankful to the Forensic INCT, CNPq, CAPES (PGCI-99999.002378/2015) and FAPERGS.

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Figure Captions

Figure 1. FT-IR analysis of *L. flavicans* extract (**a**) and ZnO NPs synthesized from *L. flavicans* extract (**b**).

Figure 2. XRD patterns of ZnO NPs biosynthesized from sub-Antarctic macroalgae (**a**); SEM analysis of ZnO NPs from *L. flavicans* (**b**); TEM analysis of ZnO NPs from *L. flavicans* (**c, d**).

Novel application of sub-Antarctic macroalgae as zinc oxide nanoparticles biosynthesizers

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Supplementary Information

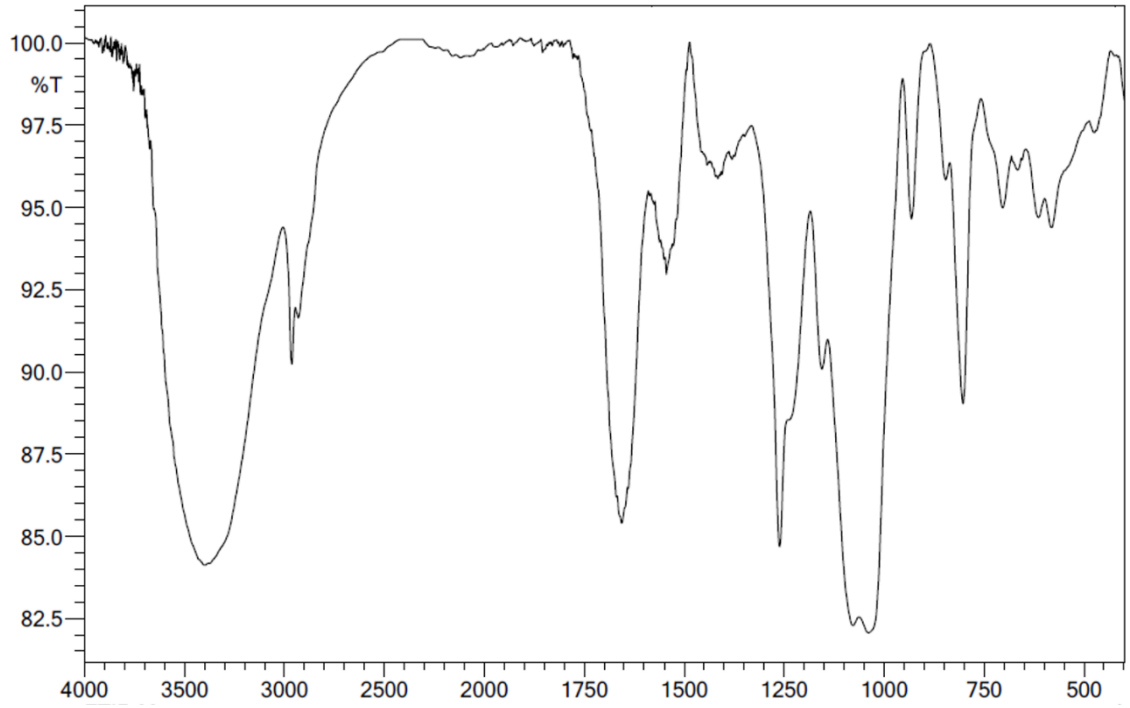

Figure 1S. FT-IR spectrum of ZnO NPs synthesized from *G. skottisbergii* extract.

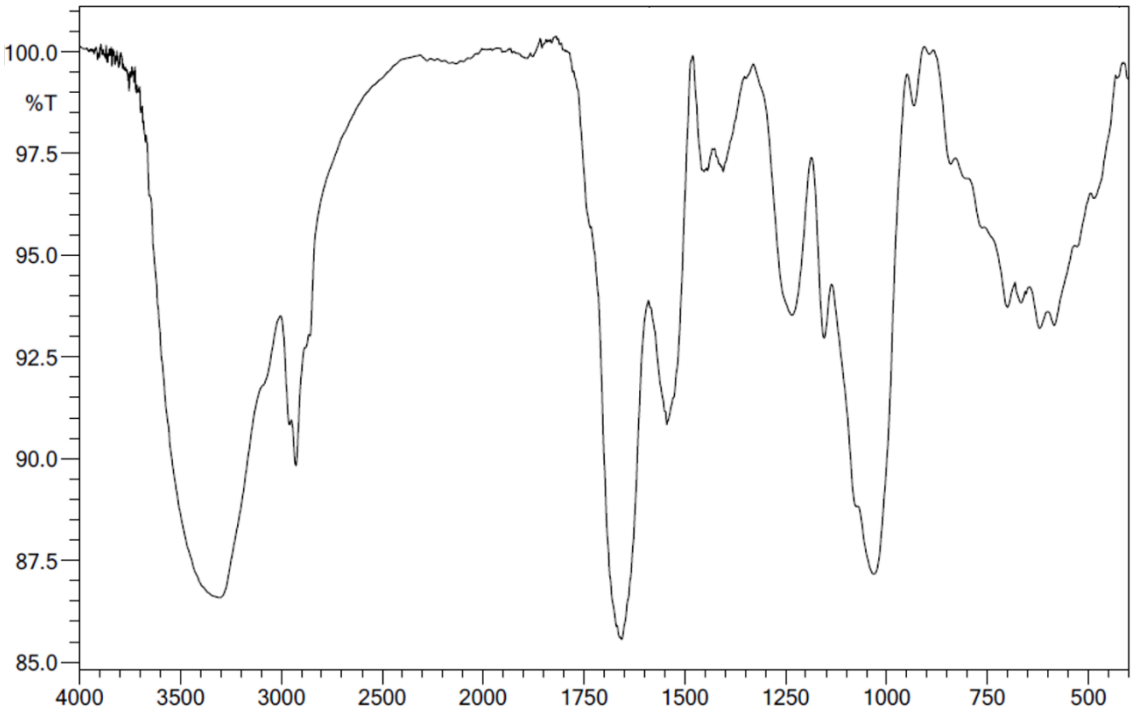

Figure 2S. FT-IR spectrum of ZnO NPs synthesized from *M. laminarioides* extract.

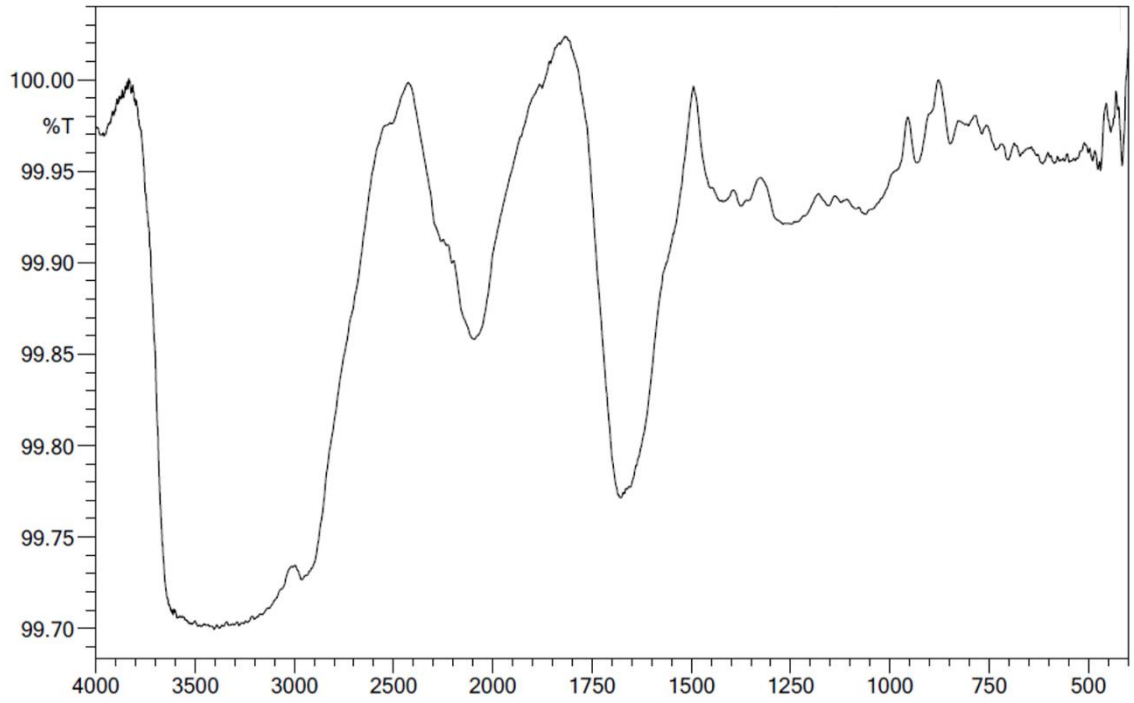

Figure 3S. FT-IR spectrum of the *G. skottsbergii* aqueous extract.

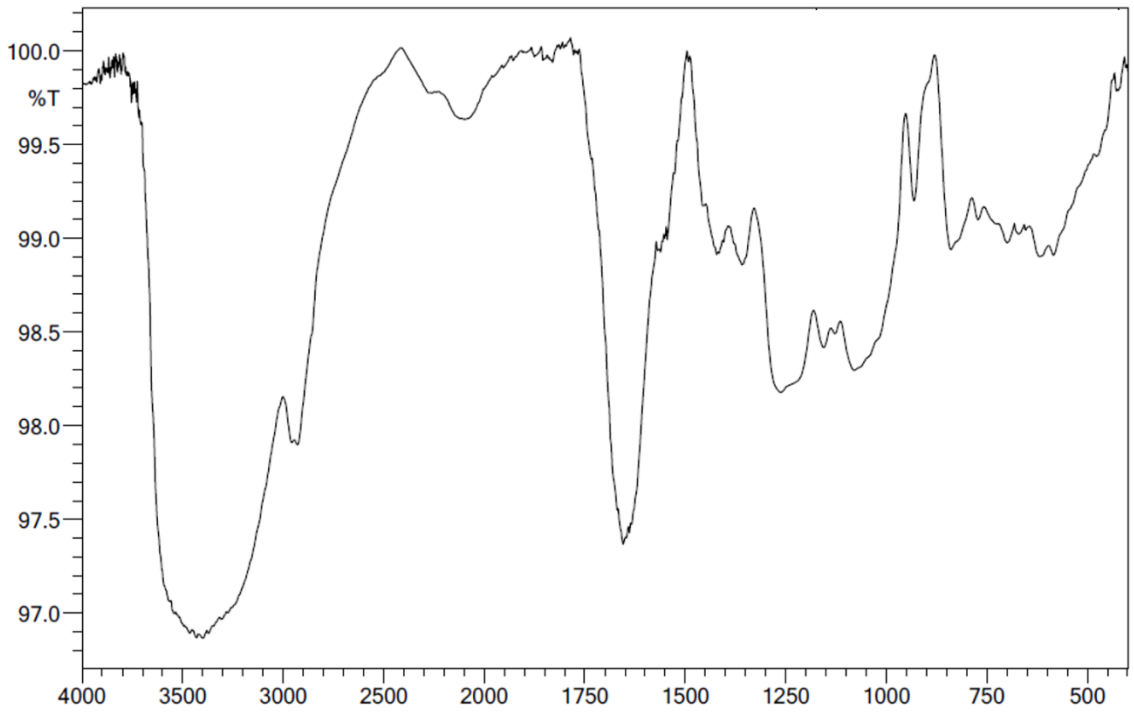

Figure 4S. FT-IR spectrum of the *M. laminarioides* aqueous extract.

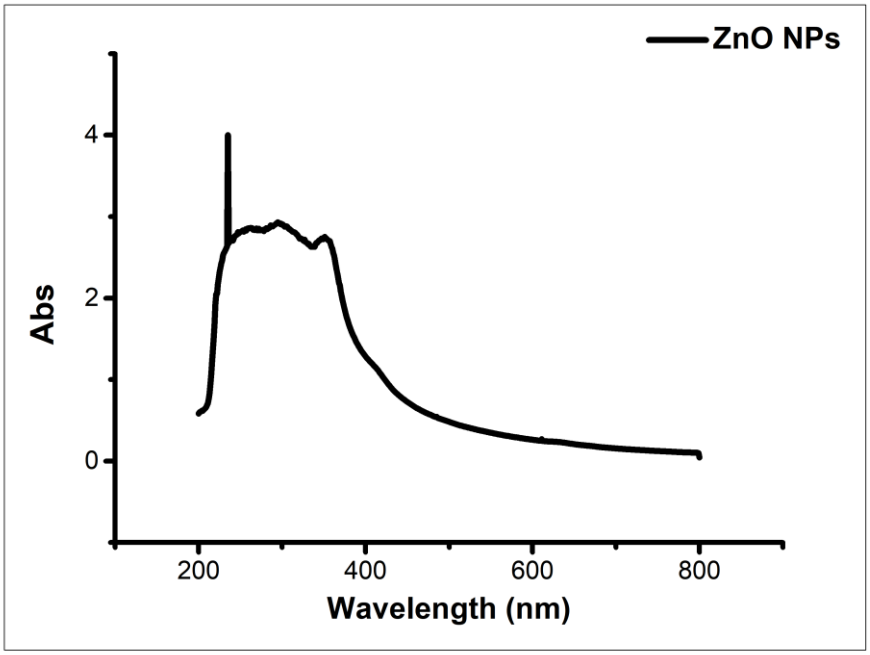

Figure 5S. UV-Vis Spectroscopy analysis of the ZnO NPs obtained from the aqueous extract of *L. flavicans*.

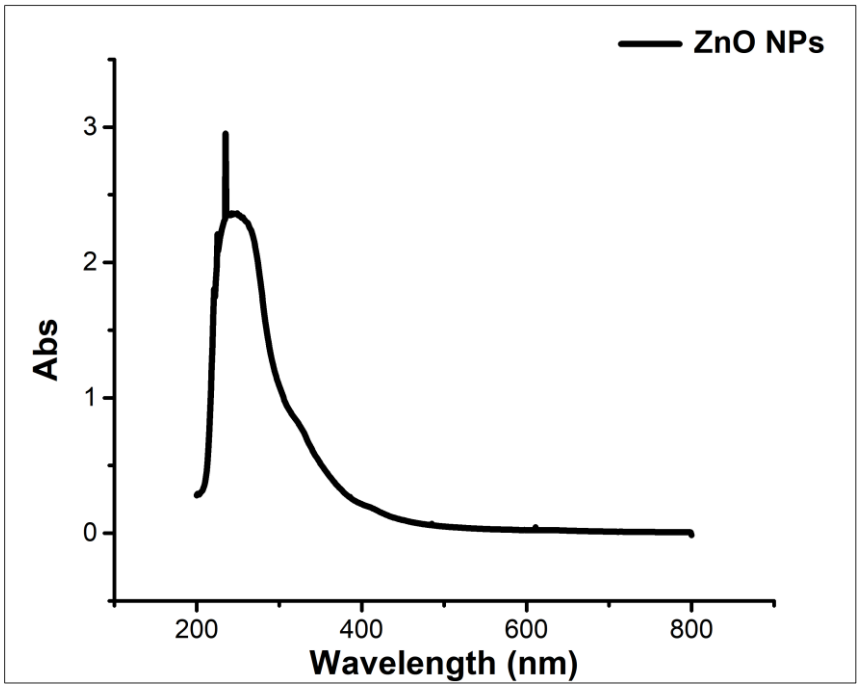

Figure 6S. UV-Vis Spectroscopy analysis of the ZnO NPs obtained from the aqueous extract of *G. skottsbergii*.

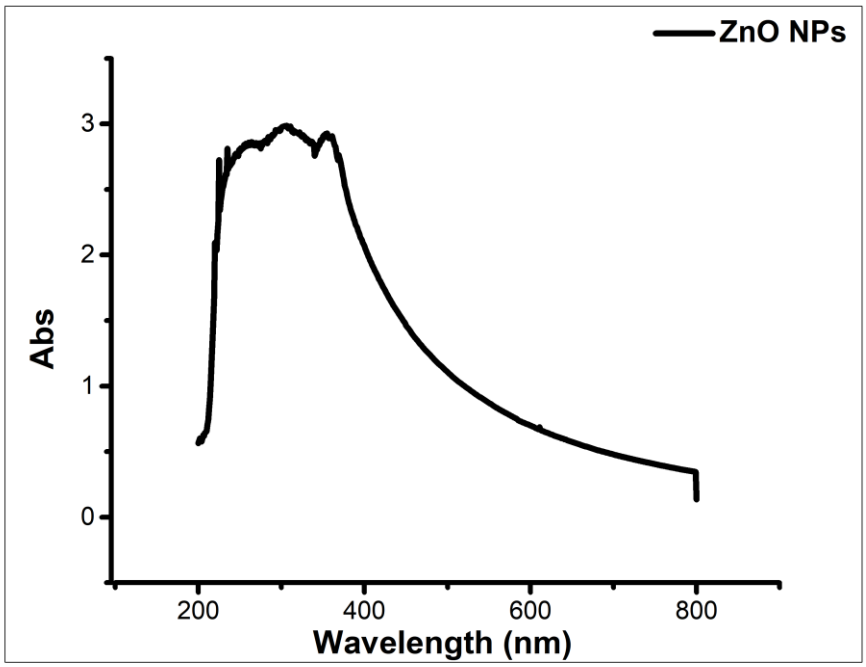

Figure 7S. UV-Vis Spectroscopy analysis of the ZnO NPs obtained from the aqueous extract of *M. laminarioides*.

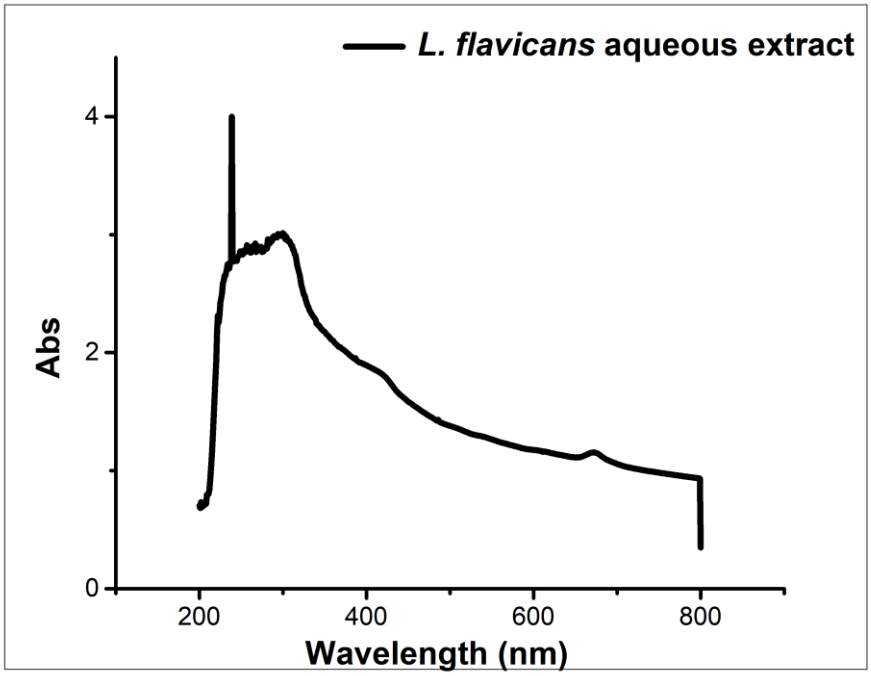

Figure 8S. UV-Vis Spectroscopy analysis of the aqueous extract of *L. flavicans*.

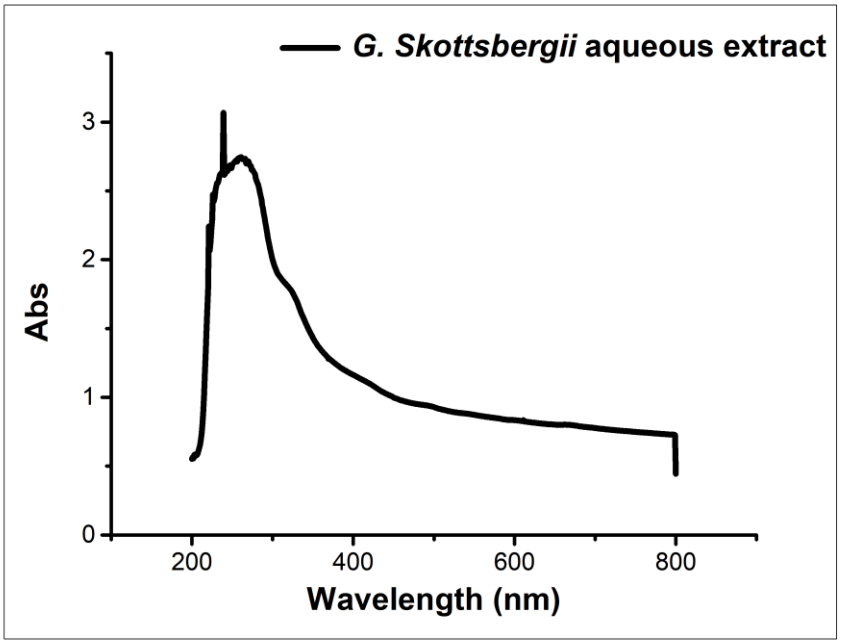

Figure 9S. UV-Vis Spectroscopy analysis of the aqueous extract of *G. skottsbergii*.

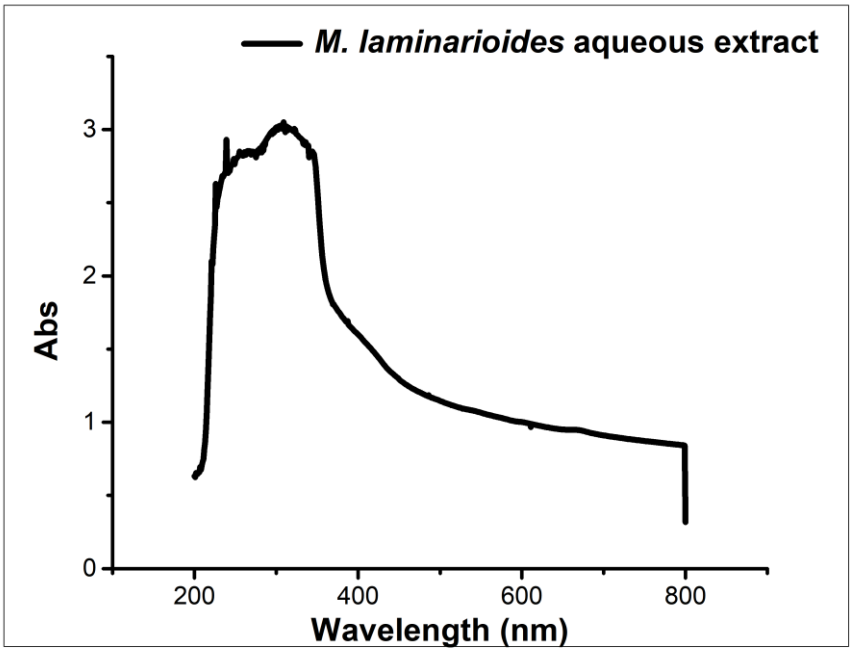

Figure 10S. UV-Vis Spectroscopy analysis of the aqueous extract of *M. laminarioides*.

Table 1S. Metal composition (%) of ZnO NPs synthesized from distinct sub-Antarctic macroalgae.

Metal	**Macroalgae**		
	L. flavicans	*M. laminarioides*	*G. skottsbergii*
Zinc	98.75	97.27	97.44
Nickel	0.03	-	-
Potassium	-	0.15	-
Calcium	0.62	1.39	1.17
Sulfur	0.22	1.01	1.16
Others	0.35	0.14	0.21

6. Discussão

De acordo com estudos anteriores, cerca de 1/3 à metade das formulações de EAAs apreendidas por órgãos da segurança pública são falsificadas. Na execução dos trabalhos, cerca um terço das amostras foram falsificadas devido a presença de outros esteroides sintéticos não declarados na composição ou pela ausência do princípio ativo rotulado (NEVES et al., 2016). Essas formulações ilícitas são conhecidas por causar efeitos nocivos aos usuários devido aos efeitos adversos que seus constituintes podem causar e também porque o processo de fabricação geralmente não obedece às normas sanitárias ou de controle de qualidade (COOPMAN & CORDONNIER, 2012; CHO et al., 2015).

Em relação à citotoxicidade, constatou-se que a maioria das formulações de anabolizantes foi tóxica para as células de rim bovino nas concentrações testadas. Esse comportamento foi previamente destacado por outros estudos que aplicaram EAAs puros em culturas de células os quais sugeriram que o dano celular era causado pela indução direta da apoptose desencadeada pela exposição à andrógenos sintéticos (BASILE et al., 2013; CARACI et al., 2011). Além disso, foi indicado que o consumo de hormônios sexuais também pode resultar em genotoxicidade e carcinogenicidade dependendo do tamanho da dose e do tempo de exposição bem como de fatores genéticos e epigenéticos (BASILE et al., 2013).

Vale ressaltar que o potencial de induzir apoptose está associado ao anabolizante utilizado que em um estudo anterior, foi determinado ser na seguinte ordem de toxicidade: nandrolona> testosterona> estanozolol> trembolona (ZELEROTH et al., 2013). As razões que podem explicar porque essa ordem citotóxica não foi observada nos trabalhos realizados provavelmente devido a fatores que incluem: I) a maioria dos EAAs estavam em seus respectivos derivados de éster e II) os anabolizantes não foram aplicados isolados, mas em suas formulações (BERNEIRA et al., 2020a; MEIRELES et al., 2013).

Como pode ser visto nos resultados, a concentração de EAAs desempenhou um papel importante na citotoxicidade das amostras. Um padrão

semelhante também foi indicado por ZELLEROTH et al. (2013), que aplicou vários agentes anabólicos em concentrações de 10, 30 e 100 µM em células corticais primárias de rato. Também foi observado que provavelmente os excipientes e os adulterantes causaram redução na viabilidade das células de rim bovino. Diante disso, os resultados foram condizentes com estudos anteriores, os quais indicaram que as formulações falsificadas podem apresentar riscos à saúde devido aos seus componentes incertos e suas respectivas concentrações desconhecidas (BASILE et al., 2013; CARACI et al., 2011).

Em relação aos os procedimentos de extração, se pode observar que os métodos influenciaram no rendimento extrativo das substâncias químicas ativas. Entre as razões que podem explicar as diferenças de eficiência da extração são a presença ou ausência de energias assistidas, o tipo de classes químicas dos EAAs e as concentrações dos agentes anabólicos na formulação (NEVES et al., 2016; BERNEIRA et al., 2020a). A avaliação dos métodos de extração é recorrente na literatura para várias classes de substâncias. No entanto, poucos trabalhos comparam os procedimentos analíticos desenvolvidos na literatura para a análise da formulação de EAAs, que é importante na ciência forense, dado que os resultados são usados como evidências criminais (BERNEIRA et al., 2019; GALESIO et al., 2011)

De acordo com NEVES & CALDAS (2017) houve um aumento de 5% na recuperação de ésteres de testosterona, de nandrolona ou de trenbolona em formulações oleosas comparando banho ultrassônico com ELL ou ESL. Comparando os resultados reportados aos do trabalho, pode-se observar que houve um aumento na extração dos agentes anabólicos variando de 1% a 10% o que está de acordo com o reportado na literatura (NEVES & CALDAS, 2017; BERNEIRA et al., 2020a)

Geralmente, a energia acústica melhorou a extração da substância química ativa em comparação com ELL ou ESL. Esse aprimoramento no processo de extração pode ser relacionado a formação de bolhas de concentração que melhoram a interação solvente-amostra. Nesse sentido, a recuperação de um agente metabólico pode ser associada a certos efeitos causados por bolhas de cavitação, como: (i) altas temperaturas que aumentam a solubilidade e a difusividade dos componentes e (ii) altas pressões que

permitem a penetração e transporte do analito através da matriz (BERNEIRA et al., 2020a; BERNEIRA et al., 2021b; GALESIO et al., 2011)

Como pode ser observado a partir da avaliação dos procedimentos de extração, a sonda ultrassônica apresentou resultados superiores ao banho ultrassônico. Isso pode ser devido a dissipação do ultrassom dentro do ultrassom banho sônico levando a redução na formação de bolhas de cavitação e, assim, na eficácia do procedimento (BERNEIRA et al., 2021b). Por sua vez, a EAM também apresentou resultados superiores na eficiência extrativa comparado com outros métodos de extração. Dentre os mecanismos que podem esse aumento está a rotação de dipolos e a condução iônica que ocorrem no solvente sob ação da radiação de micro-ondas. Tais processos podem melhorar a interação entre a amostra e o solvente extrator aumentando a eficiência da extração (GALESIO et al., 2011)

Métodos que empregam a sonda ultrassônica ou a irradiação de micro-ondas para a análise das formulações de EAAs não tem sido frequentemente relatados na literatura (BERNEIRA et al., 2021b) Nesse sentido, apenas há estudos que avaliam procedimentos de extração para a recuperação de anabolizantes em urina e em outras matrizes alimentares (BARREIRO et al., 2015; GALESIO et al., 2011). De acordo com relatórios anteriores, o uso de energias assistidas aumentou a extração dos analitos o que foi também observado para as formulações de agentes anabólicos. Cabe ressaltar que esses procedimentos são rápidos, eficientes e reproduzíveis indicando que a sonda ultrassônica e a irradiação de micro-ondas podem ser usadas na análise de formulações de agentes anabolizantes (COOPMAN & CORDONNIER, 2012; NEVES & CALDAS, 2017).

Com base nos resultados se pode observar que a avaliação dos protocolos extrativos mostrou que geralmente o uso de métodos convencionais tiveram menores recuperações do componente ativo quando comparado com a sonda ultrassônica e irradiação de micro-ondas. Portanto, o a escolha do procedimento de extração é consideravelmente importante para obter resultados adequados e precisos que posteriormente podem ser usados como provas perícias na esfera jurídica (BERNEIRA et al., 2020a; BERNEIRA et al., 2021b).

O perfil de AGs de macroalgas da Antártica mostrou diferenças entre seus constituintes. Fatores genéticos e ambientais como espécie, temperatura da água, fotoperíodo, disponibilidade de nutrientes, salinidade e pH, estão entre as razões que podem explicar variações nas concentrações e tipos de AGs entre espécies de algas (RAUTENBERGER & BISCHOF, 2006; BECKER et al. 2010). Dadas as condições ambientais extremas encontradas no continente Antártico, acredita-se que as macroalgas ajustem seu metabolismo de biossíntese de acordo com uma maior produção de AGPs. Embora suscetíveis à peroxidação lipídica, os AGPs são moléculas essenciais para manter a fluidez da membrana de algas em temperaturas frias, permitindo sua sobrevivência (BECKER et al. 2010; PACHECO et al. 2018).

Comparando os resultados para as macroalgas vermelhas, verdes e pardas ao relatado na literatura, pode-se notar que qualitativamente a composição das amostras não variou consideravelmente. No entanto, a concentração de AGPs encontrados nos trabalhos realizados foram inferiores ao indicado por GRAEVE et al. (2002) e PACHECO et al. (2018) que também analisaram algas marinhas da Antártica. Variações de ambiente condições de 2002 a 2015 podem estar associadas a diferenças no perfil de AGs uma vez que aspectos relativos à amostra métodos de preparação, extração e derivatização foram semelhantes.

Os AGSs também contribuíram de forma importante no perfil das macroalgas Antárticas sendo preponderantes na maioria das amostras, exceto para *Desmarestia confervoides*. O consumo de AGSs geralmente é associada com maiores riscos para o desenvolvimento de doenças coronárias, como infarto do miocárdio, angina e arteriosclerose (SÁNCHEZ-MACHADO et al., 2004). No entanto, o C16:0 era o AGS geralmente detectado em maior quantidade. Assim, riscos associados ao consumo dessas macroalgas seriam baixos porque a enzima Δ9-desaturase encontrada em humanos pode convertê-la em ácido palmitoleico (C16:1) o qual não está associado a tais complicações biológicas (MARTINS et al. 2018).

Por outro lado, os principais AGMs detectados nas amostras foram o C16:1 e o C18:1. Geralmente, AGMs foram encontrados em concentrações intermediárias a outras classes de de AGs. As maiores quantidades de AGMs nas macroalgas estudadas foram em *Ulva intestinalis*, *Myriogramme manginii* e

Georgiella confluens. Case ressaltar que esses compostos são blocos de construção de AGPs e têm atividades biológicas associadas na regulação da sensibilidade à insulina e na diminuição de respostas inflamatórias (FREITAS et al., 2020; PACHECO et al., 2018).

Geralmente, os AGPs encontrados nas macroalgas estudadas eram principalmente C18:3*n*3, C20:5*n*3 e C20:4*n*6 com menor contribuições do C18:2*n*6c, do ácido di-homolinoléico (C20:2) e do ácido dihomo-γ-linolênico (C20:3*n*6). A presença destes compostos destaca a importância das algas marinhas como naturais reservatórios de substâncias nutracêuticas visto que alguns AGPs não podem ser sintetizadas por humanos ou plantas terrestres (PACHECO et al., 2018). Além disso, AGPs também têm vários produtos farmacêuticos e biotecnológicos aplicações para o tratamento de doenças coronárias, inflamatórias, doenças autoimunes e relacionadas ao tumor, bem como auxiliar na entrega de medicamentos (PEREIRA et al., 2012; SANTOS et al., 2017).

O AG C18:3*n*3 estava entre os AGPs encontrados em maiores quantidades e é um composto importante com atividades biológicas relatadas na literatura, incluindo atividades antioxidante, anticâncer e antiosteoporótico, por exemplo (PEREIRA et al.; 2012; PACHECO et al. 2018). Este AG serve como substrato para a síntese de outros AGPs relevantes, como C20:5*n*3 e C20:4*n*6, por mecanismos de alongamento e dessaturação. Ao lado do C18:3*n*3, AGs com maior cadeia de carbono também têm importantes papéis biológicos atuando no desenvolvimento do cérebro, manutenção de saúde cardiovascular e processos inflamatórios (MARTINS et al., 2018; FREITAS et al. 2020).

Resumindo os resultados obtidos, destaca-se o potencial nutracêutico, farmacológico e biotecnológico das algas marinhas uma vez que são constituídas por importantes AGPs do tipo *n*3 e *n*6 em seu perfil de AGs (SANTOS et al., 2019). PEREIRA et al. (2012). Além disso, os extratos lipofílicos desses organismos também podem apresentar potencial biológico visto que os AGs podem desempenhar papéis importantes em atividades antimicrobiana e anticâncer (MARTINS et al., 2018; PACHECO et al., 2018).

Em relação a outros bioativos, cabe ressaltar que pela primeira vez foram estudos com componentes orgânicos voláteis em macroalgas coletadas na região da Antártica e na região sub-Antártica. De acordo com o perfil de

bioativos, foi observado a presença de várias classes químicas que incluíam principalmente aldeídos, hidrocarbonetos, cetonas, álcoois e derivados de furano. A ocorrência desses componentes em algas marinhas foi anteriormente relatada em organismos analisados em outras regiões do planeta e os compostos detectados apresentaram semelhança com os reportados em estudos anteriores (LE PAPE et al., 2004; KAMENARSKA et al., 2006; EL HATTAB et al., 2007).

De acordo com os resultados de análise multivariada, os perfis de AGs e de componentes orgânicos voláteis podem ser usados para diferenciar macroalgas em seus respectivos filos. Em nesse sentido, maiores concentrações de variáveis que incluíam C18-AGs, C20-AGs, AGPs (C20:4*n*6 e C20:5*n*3), e 1-octen-3-ol auxiliou na distinção de representantes Ochrophyta dos outros filos. Além disso, quantidades distintas de 2-pentilfurano e de C18:1 diferenciaram as algas marrons *Desmarestia confervoides* e *Adenocystis utricularis*. Padrões semelhantes foram observados para Rhodophyta como as amostras deste filo podendo ser distinguidas pela presença de AGSs (principalmente C16:0), AGMs (principalmente C16:1), 2-heptanona, 2-pentenilfurano, benzaldeído, 2,3-octanediona e hexanal. Outras diferenças nas concentrações dessas variáveis também permitiu a distinção de espécies no gráfico de pontuação.

Usando Análise de Componente Principal, VAN DURME et al. (2013) também observou que concentrações distintas de componentes orgânicos voláteis podem diferenciar várias espécies de microalgas. A análise multivariada também foi empregada por BRAVO-LINARES & MUDGE (2009) que mostrou que as condições físico-químicas e ambientais, incluindo velocidade do vento, temperatura da água influenciava a biossíntese de bioativos em organismos marinhos. Além disso, o uso de AGs como meio para estabelecer distinções entre algas marinhas e seus filos também foram relatados na literatura (KUMARI et al., 2009).

Assim, no presente estudo, os perfis AGs e de bioativos de macroalgas vermelhas, pardas e verdes foram elucidados com sucesso. Os resultados mostraram que os ácidos palmítico, oleico, linoléico e eicosapentaenóico foram os AGs predominantes enquanto hexanal, heptadecano e 2-pentilfurano foram os principais tipos de componentes orgânicos voláteis encontrados nas amostras. Além disso, a análise multivariada dos componentes químicos de

algas indicaram graus distintos de semelhanças entre elas. Portanto, este estudo elucidou que as macroalgas Antárticas e sub-Antárticas tinham uma ampla gama de constituintes que estão associados a mecanismos de defesa e podem levar ao desenvolvimento de novos compostos bioativos. A **Figura 7** esquematiza os principais resultados e conclusões das pesquisas conduzidas no decorrer da tese.

Figura 7. Esquema dos principais resultados e conclusões obtidas no andamento de estudos contidos na tese.

Resumindo os dados e estudos compilados nesta tese, se pode observar que o desenvolvimento a avaliação de métodos de extração e técnicas analíticas aplicadas para formulações de agentes anabólicos é importante para que esses materiais sejam adequadamente analisados conferindo provas periciais mais robustas e confiáveis. Além disso, a análise toxicológica pode indicar prováveis graus de citotoxicidade de agentes anabólicos em células MDBK mostrando que EAAs e seus excipientes podem estar ligados com tais danos celulares. Por fim, a avaliação de macroalgas mostrou a prevalência de diversos tipos de ácidos graxos e de bioativos na forma de compostos orgânicos voláteis auxiliando no conhecimento de mecanismos fisiológicos e bioquímicos desses organismos bem como indicando potenciais usos desses materiais nas áreas farmacêuticas, biotecnológicas e alimentícias.

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