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Avaliação Química e Toxicológica de Formulações de Esteroides Anabolizantes Androgênicos Apreendidas pela Polícia Federal na Região Sul do Brasil

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Pelotas, 2020.

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Avaliação Química e Toxicológica de Formulações de Esteroides Anabolizantes Androgênicos Apreendidas pela Polícia Federal na Região Sul do Brasil

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Dissertação aprovada, como requisito parcial, para obtenção do grau de Mestre em Ciências (área do conhecimento: Bioquímica e Bioprospecção) do Programa de Pós-Graduação em Bioquímica e Bioprospecção do Centro de Ciências Químicas, Farmacêuticas e de Alimentos da Universidade Federal de Pelotas.

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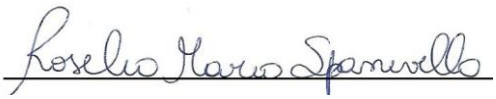

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“If we ever became the things we lost, the things we left behind I would wish for the past when we were pure suffering intertwined.”

Hippo Campus

Parte I

Resumo

BERNEIRA, Lucas Moraes. Avaliação Química e Toxicológica de Formulações de Esteroides Anabolizantes Androgênicos Apreendidas pela Polícia Federal na Região Sul do Brasil. 2020. 126f. Dissertação (Mestrado) - Programa de Pós-Graduação em Bioquímica e Bioprospecção, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Pelotas, 2020.

Esteroides anabolizantes androgênicos compreendem classe de substâncias sintéticas derivadas da testosterona desenvolvidas com um intuito terapêutico, porém utilizadas ilicitamente por atletas recreativos e profissionais. Nesse sentido, as formulações de agentes anabólicos são controladas ou proibidas e, uma vez apreendidas por órgãos de controle, necessitam ser analisadas a fim de determinar seus constituintes e sua provável toxicidade. Nesse sentido, objetivo desse trabalho foi avaliar métodos de extração, identificar analiticamente e determinar a citotoxicidade de formulações de agentes anabólicos apreendidos pela Polícia Federal na região Sul do Brasil. Os resultados indicaram que a aplicação de micro-ondas ou de ultrassom aumentaram significativamente a extração de agentes anabólicos de suas respectivas formulações. Para o Produto #1, por exemplo, o uso de micro-ondas ou de sonda ultrasônica extraiu, respectivamente, 37,46 ± 1,36 e 35,69 ± 0,98 mg/comprimido ao passo que a extração sólido-líquido resultou em 29,63 ± 0,40 mg/comprimido da substância química ativa. Por sua vez, a aplicação de técnicas analíticas permitiu a diferenciação de formulações genuínas de falsificadas ao passo que a avaliação de citotoxidade indicou que as amostras podem apresentar toxicidade. Nesse sentido, a exposição do Produto #10 e #11 em concentrações de 0,5 mg/mL apresentou uma viabilidade celular de 0%. Portanto, os métodos de extração afetaram consideravelmente os resultados e, assim, influenciam na detecção de compostos potencialmente tóxicos ao usuário.

Palavras-Chave: Esteroides anabolizantes androgênicos, Extração; Técnicas analíticas; Citotoxicidade; Formulações apreendidas.

Abstract

BERNEIRA, Lucas Moraes. Avaliação Química e Toxicológica de Formulações de Esteroides Anabolizantes Androgênicos Apreendidas pela Polícia Federal na Região Sul do Brasil. 2020. 126f. Dissertação (Mestrado) - Programa de Pós-Graduação em Bioquímica e Bioprospecção, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Pelotas, 2020.

Anabolic androgenic steroids comprise a class of synthetic substances derived from testosterone developed for therapeutic purposes, but illicitly used by recreational and professional athletes. In this sense, formulations of anabolic agents are controlled or prohibited and, once apprehended by law enforcement agencies, need to be analyzed in order to determine their constituents and their possible toxicity. Therefore, the aims of this study was to evaluate extraction methods, to identify analytically and to determine the cytotoxicity of formulations of anabolic agents apprehended by the Federal Police in the southern region of Brazil. Results indicated that the application of microwaves or ultrasound significantly increased the extraction of anabolic agents from their respective formulations. For Product #1, for example, the use of microwaves or ultrasonic probe extracted, respectively, 37.46 ± 1.36 and 35.69 ± 0.98 mg/tablet, whereas the solid-liquid extraction resulted in 29.63 ± 0.40 mg/tablet of the active chemical substance. In turn, the application of analytical techniques allowed the differentiation of genuine and counterfeit formulations while the evaluation of cytotoxicity indicated that the samples may present toxicity. In this sense, the exposure of Product #10 and #11 in concentrations of 0.5 mg / mL had minimum cell viability. Therefore, extraction methods significantly affected the results and, thus, influenced the detection of potentially toxic compounds to the user.

Keywords: Anabolic androgenic steroids, Extraction, Analytical techniques, Cytotoxicity, Apprehended formulations.

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Lista de Abreviaturas

CCD	Cromatograma de Camada Delgada
CED	Calorimetria Exploratória Diferencial
CG-EM	Cromatografia Gasosa-Espectrometria de Massas
CG-DIC	Cromatografia Gasosa-Detector de Ionização por Chama
CL-EM	Cromatografia Líquida-Espectrometria de Massas
CEBRID	Centro Brasileiro de Informações sobre Drogas Psicotrópicas
EAM	Extração Assistida por Micro-ondas
EASU	Extração Assistida por Sonda Ultrassônica
EABU	Extração Assistida por Banho Ultrassônico
ELL	Extração Líquido-Líquido
ESL	Extração Sólido-Líquido
EAA	Esteroide Anabolizante Androgênico
OMS	Organização Mundial da Saúde
PF	Polícia Federal

Sumário

Parte I

1. Introdução

Os esteroides anabolizantes androgênicos (EAAs) compreendem uma classe de substâncias as quais são quimicamente derivadas da testosterona. Esses compostos foram inicialmente desenvolvidos para o tratamento de condições clinicas associadas ao hipogonadismo (BERNEIRA et al., 2019). No entanto, os agentes anabólicos também são conhecidos pelo seu uso ilícito por atletas recreativos ou profissionais visto que estão associados com aumento de performance esportiva e desenvolvimento muscular (WEBER et al., 2017). Todavia, o uso irrestrito de EAAs 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 de agentes anabólicos levou diversos países como, por exemplo, o Brasil, os Estados Unidos e a Inglaterra a controlarem ou proibirem o uso de hormônios sintéticos entre a população (NEVES et al., 2016; REBIERE et al., 2016). 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 falsificados, os quais representam 33% dos produtos apreendidos (NEVES; MARCHETI; CALDAS, 2013). Tais formulações falsificadas podem intensificar os danos causados pelo consumo de EAAs ao passo que são produzidas em condições sanitárias inadequadas e sem o devido controle de qualidade (BERNEIRA et al., 2019; NEVES et al., 2016).

De acordo com a Organização Mundial da Saúde (OMS), formulações falsificadas compreendem quaisquer medicamentos que apresentem adulterações ou falta de informações requeridas no rótulo ou no recipiente bem como quaisquer alterações na constituição ou concentração dos componentes do material farmacêutico (DE FREITAS et al., 2019; WEBER et al., 2017). Com base em dados anteriormente reportados pela literatura, as falsificações em formulações de agentes anabólicos ocorrem principalmente pela substituição da substância química ativa pelo veículo ou pela alteração de uma substância química ativa alegada no rótulo por outro componente similar (CHIONG; CONSUEGRA-RODRIGUEZ; ALMIRALL, 1992; MESMER; SATZGER, 1997;

THEVIS et al., 2008). Nesse sentido, a avaliação dos componentes é importante a fim de determinar uma provável toxicidade dos constituintes da formulação (ZELLEROTH et al., 2019).

Na área forense, as formulações de EAAs apreendidas por órgãos governamentais de controle são analisadas a fim de determinar sua constituição química o qual serve 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 (DE FREITAS et al., 2019). Inicialmente, as amostras têm sua substância química ativa extraída por métodos que envolvem extração líquido-líquido (ELL) ou assistida por banho ultrassônico (EABU) (COOPMAN; CORDONNIER; V., 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 limitando a análise dessas formulações (GALESIO et al., 2011).

De acordo com a literatura, as 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., 2014; NEVES et al., 2016). Também se destaca o uso de procedimentos de *screening* tais como a inspeção visual e testes colorimétricos os quais são empregados em menor extensão (CHIONG; CONSUEGRA-RODRIGUEZ; ALMIRALL, 1992; THEVIS et al., 2008). Na última década, novas técnicas analíticas tais como a Calorimetria Exploratória Diferencial (CED) e a Cromatograma de Camada Delgada (CCD) 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 (BERNEIRA et al., 2019; MUSHARRAF et al., 2015).

Com base no exposto acima, se pode notar que, durante a análise de EAAs, a extração é um dos passos mais importantes visto que essa etapa afeta os procedimentos posteriores e, assim, os resultados finais acerca de amostras em

questão (DE FREITAS et al., 2019). Paralelamente, a aplicação de novas técnicas analíticas para a análise de agentes anabólicos se mostra essencial a fim de responder adequadamente a alta demanda de análises requeridas (BERNEIRA et al., 2019). Com base na associação de métodos de extração e técnicas analíticas eficientes se pode detectar falsificações de acordo com a presença de contaminantes e adulterantes. Tal avaliação de formulações falsificadas é importante visto que certas substâncias presentes em sua constituição podem gerar toxicidade e, assim, causar diversos danos à saúde do usuário (ZELLEROTH et al., 2019).

2. Objetivos

2.1. Objetivo Geral

O objetivo do trabalho foi avaliar a constituição química e a toxicidade de formulações de agentes anabólicos apreendidos pela Polícia Federal de Pelotas (RS) através de distintos métodos de extração e de ferramentas analíticas incluindo Espetroscopia no Infravermelho e Cromatografia.

2.2. Objetivos Específicos

Dentre os objetivos específicos estão:

I. Avaliar formulações de agentes anabólicos através de inspeção visual, testes colorimétricos, Espectroscopia no Infravermelho e CG-EM;
II. Determinar a composição do veículo oleoso de formulações de EAAs utilizando Cromatografia Gasosa associada ao Detector de Ionização por Chama (CG-DIC);
III. Comparar a eficácia de métodos de extração que envolvem ELL, EABU, EASU e EAM;
IV. Avaliar a toxicidade de formulações de agentes anabólicos através do ensaio de viabilidade celular em células de rim bovino.

3. Revisão da Literatura

3.1. Aspectos Químicos de Agentes Anabólicos

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 (SILVERTHORN, 2019). 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 (NELSON; LEHNINGER; COX, 2008). A **Figura 1** representa a estrutura química de alguns esteroides endógenos.

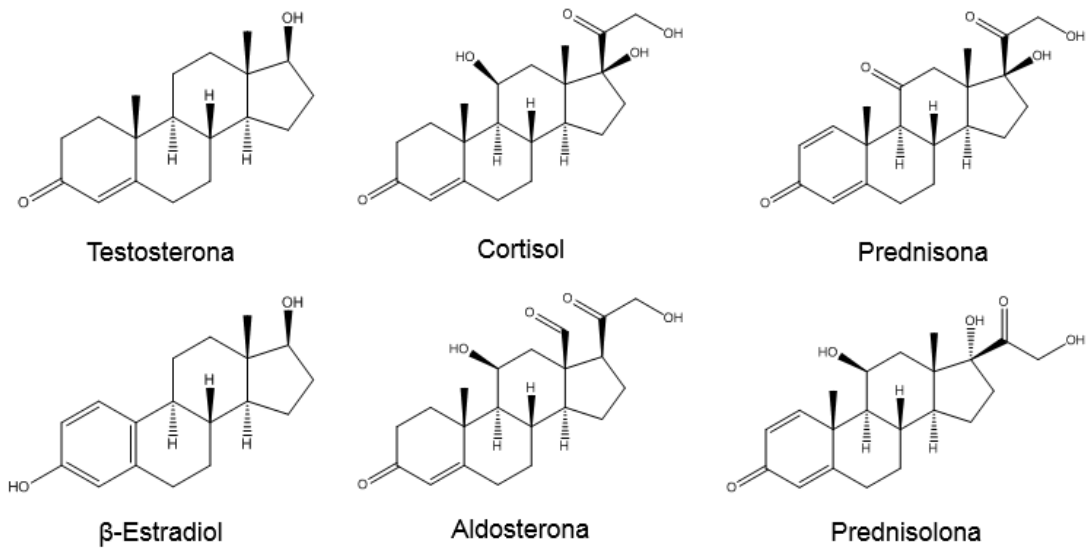

Figura 1. Estrutura química de esteroides endógenos (adaptado de NELSON; LEHNINGER; COX, 2008).

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 (KICMAN, 2008). 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 (SILVERTHORN, 2019).

Com o intuito de diminuir a atividade androgênica e aumentar o potencial anabólico inúmeros EAAs foram desenvolvidos de forma que podem ser agrupados em três classes principais (PROKUDINA et al., 2015). Dentre os grupos principais, se pode destacar: I) derivados que têm a sua porção hidroxila 17β esterificada; II) análogos que têm a sua posição 17α alquilada; bem como III) os derivados que possuem uma modificação química no seu anel A, B ou C que podem ser vistos na **Figura 2** (EVANS, 2004).

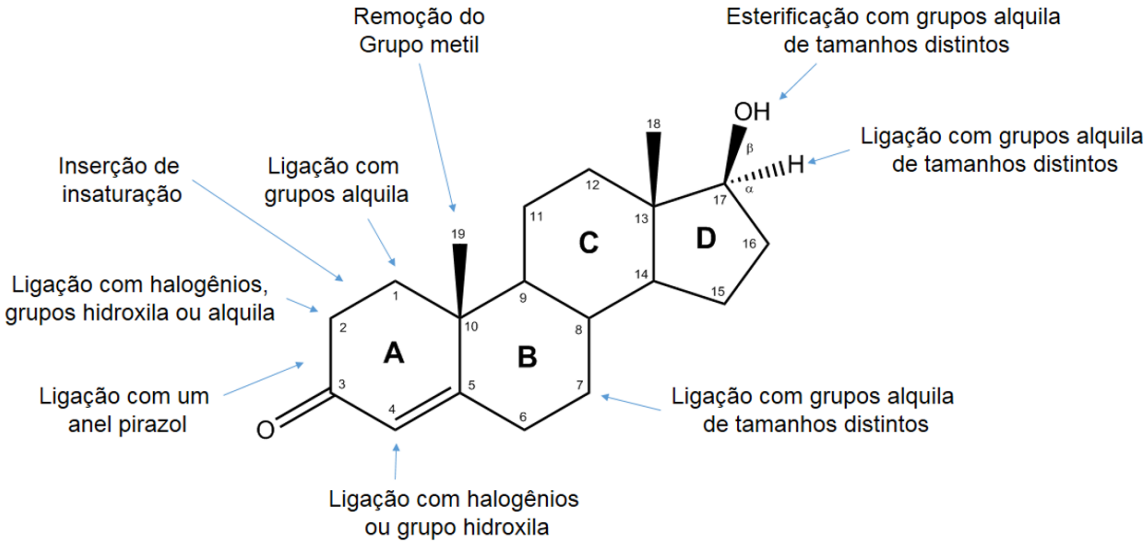

Figura 2. Modificações químicas realizadas na testosterona (adaptado de MAKIN; GOWER, 2010).

O desenvolvimento de modificações químicas na testosterona permitiu que os agentes anabólicos possam ser empregados para o tratamento de hipogonodismo, câncer de mama, estados catabólicos e insuficiência renal (ZELLEROTH et al., 2019). A alteração na estrutura química de agentes anabólicos também alterou a biodisponibilidade desses compostos bem como também sua forma de absorção de forma que podem ser encontrados em diversas formulações incluindo, por exemplo soluções oleosas, comprimidos e suspensões aquosas. Dessa forma, isso tornou o processo de extração e de análise instrumental mais dificultoso para essa classe de compostos (BERNEIRA et al., 2019; EVANS, 2004).

3.1.1. Métodos de Extração

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. No caso de formulações apreendidas, essa etapa pode ser desafiadora visto que as preparações tem origem, pureza e constituição desconhecida (NEVES; CALDAS, 2017). Nesse sentido, 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; CONSUEGRA-RODRIGUEZ; ALMIRALL, 1992).

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 modo que podem ser utilizados até 90 mL de metanol-água (9: 1, v / v) para extrair 1 mL de uma formulação oleosa (CARIGNAN; LODGE; SKAKUM, 1980). Devido a esses pontos negativos, poucas 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 ultrassônicas para o processo extrativo (COOPMAN; CORDONNIER; V., 2012; THEVIS et al., 2008).

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 nas últimas décadas se estabeleceram como a metodologia convencional para a análise EAAs (MESMER; SATZGER, 1997; WEBER et al., 2017). 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).

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 farmacêuticas e alimentícias (BARREIRO et al., 2015; DE FREITAS et al., 2019). 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 (GALESIO et al., 2011). Esses movimentos intermoleculares 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. Segundo estudos anteriores, essas energias assistidas aumentaram a recuperação dos analitos, o que também foi observado no trabalho de análise de formulações de EAAs (COOPMAN; CORDONNIER; V., 2012; NEVES; CALDAS, 2017). Dessa forma, o desenvolvimento de novas alternativas para extração se mostra crucial a fim de se obter procedimentos otimizados e precisos (DE FREITAS et al., 2019).

3.1.2. Técnicas Analíticas

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 (MUSHARRAF et al., 2015; PROKUDINA et al., 2015). 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 3**).

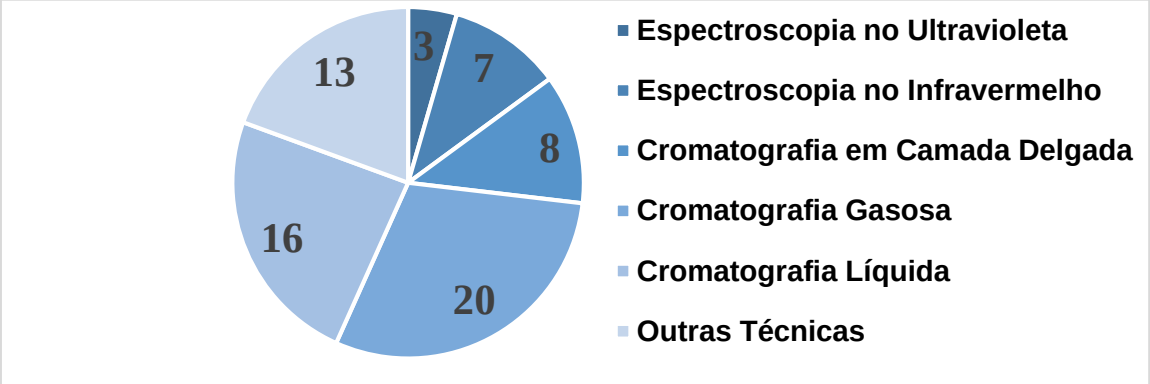

Figura 3. Técnicas analíticas empregadas na análise de formulações de agentes anabólicos.

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 (BERNEIRA et al., 2019). 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).

3.2. Aspectos Biológicos de Agentes Anabólicos

De uma forma geral, os EAAs podem ser absorvidos através da via oral a qual é caracterizada por um rápido pico de concentração plasmática em um curto período de tempo ou através da via intramuscular associada a longos tempos de meia-vida (SILVERTHORN, 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. Em tecidos-alvo, 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 4**). 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 (EVANS, 2004). 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).

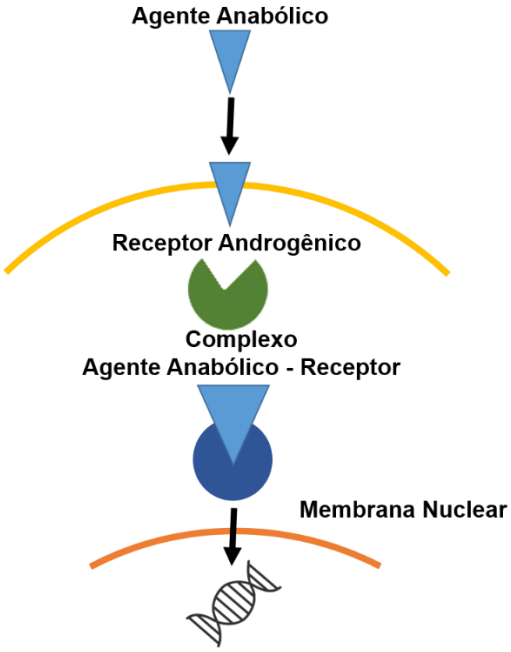

Figura 4. Mecanismo de ação de agentes anabólicos (adaptado de SILVERTHORN, 2019).

Em média, um adulto do sexo masculino produz aproximadamente 7 mg de testosterona diariamente através das células de Leydig (MAKIN; GOWER, 2010). No entanto, essa quantidade que não é capaz de induzir uma atividade

anabólica extensa relacionada ao aumento de massa muscular. Assim, para promover esse efeito, o usuário ilícito consome de 10 a 100 vezes da faixa terapêutica de EAAs promovendo uma superdosagem no organismo (EVANS, 2004). 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 agentes anabólicos 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 EVANS, 2004; KICMAN, 2008

Embora vários efeitos adversos associados com a superdosagem de agentes anabólicos já tenham sido descritos ainda existem poucos estudos associando a toxicidade aguda ou crônica dessas substâncias (BASILE et al., 2013; CARACI et al., 2011). De acordo com a literatura, estudos *in vitro* indicam uma citotoxicidade para a sobrevivência de células neuronais a qual pode ser gerada por indução à apoptose (BASILE et al., 2013). Além disso também já foi indicado que a nandrolona foi um dos EAAs mais tóxicos ao cultivo celular comparado a testosterona, estanozolol e trembolona os quais apresentaram uma citotoxicidade similar (ZELLEROTH et al., 2019).

No Brasil, de acordo com o Centro Brasileiro de Informações sobre Drogas Psicotrópicas (CEBRID), a prevalência do uso de EAAs é considerável e está em ascendência. Segundo o CERBRID, em 2001, pelo menos 1% da população masculina de idades entre 18 e 65 já havia utilizado algum anabolizante no decorrer da vida sendo que esse percentual saltou para 2,1% em 2005. Segundo esse órgão, o perfil do usuário de EAA brasileiro é de pertencente ao sexo masculino de idade entre 18 e 34 anos (CARLINI et al, 2005). Por sua vez, pesquisas indicam que nos Estados Unidos cerca de 2% a 12% de adolescentes do sexo masculino já utilizaram EAAs com a finalidade de aumento da massa muscular (KRUG et al., 2014).

O consumo de EAAs é combatido em todo o mundo com uma legislação específica a cada localidade. Por exemplo, no Reino Unido essas substâncias são caracterizadas como uma droga de classe C de acordo com o *Misuse of Drugs Act* de 1971. Isso indica que a exportação, importação e distribuição de anabolizantes 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 na lei *Anabolic Steroid Control Act* de 1990 (KRUG et al., 2014). Após a década de 90, essa norma sofreu ajustes de forma que também passou a abranger pró-hormônios e *designer steroids*

facilitando a entrada de novos EAAs nas listas de medicamentos controlados no país (KING, 2009).

No Brasil, os anabolizantes são controlados de maneira que estão na Lista C5 da ANVISA de forma que estão no grupo de substâncias controladas. De acordo com a Lei Federal nº 9.965 de 27 de abril de 2000, a venda de EAAs é de uso restrito e sua retirada em drogarias ou farmácias só pode ser realizada mediante a apresentação da receita médica nesses estabelecimentos. Em caso de descumprimento, pode ocorrer penalidades ao estabelecimento e responsáveis técnicos segundo a Lei Federal nº 6437 de 20 de agosto de 1977. Através dessa norma podem ocorrer sanções como advertência, multa, apreensão e inutilização do produto, cancelamento do item tal como interdição do estabelecimento (BRASIL, 1988).

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 (DA JUSTA NEVES; MARCHETI; CALDAS, 2013). Nesse caso, essa infração está tipificada no artigo 274 do Código Penal a qual inclui falsificação, corrupção, adulteração, alteração de produtos determinados para fins terapêuticos, importação, comercialização, distribuição e seu consumo. Nesse sentido, essa infração gera uma pena de cerca de 10 a 15 anos de reclusão e multa (BRASIL, 1988).

Parte III

ARTIGO CIENTÍFICO

Todos resultados dessa dissertação foram apresentados na forma de manuscrito submetido ao periódico internacional *Drug Testing and Analysis*.

BIOANALYTICAL APPROACHES APPLIED TO THE ANALYSIS OF APPREHENDED FORMULATIONS OF ANABOLIC ANDROGENIC STEROIDS

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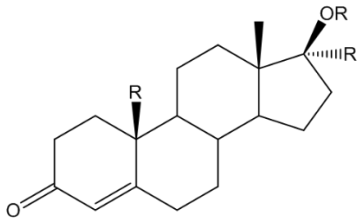
Declaration of Interest

The authors declare that they have no conflict of interest.

Supplementary Material for Review

Anabolic agents were evaluated by several methods of extraction and analytical techniques. Results showed that the use of microwave irradiation and ultrasonic probe enhanced the extraction process. Moreover, chemical evaluation showed that the oily excipient could be used as a possible marker to detect falsifications.



BIOANALYTICAL APPROACHES APPLIED TO THE ANALYSIS OF APPREHENDED FORMULATIONS OF ANABOLIC ANDROGENIC STEROIDS

Abstract

Anabolic androgenic steroids (AASs) comprise a class of synthetic androgens resulted from chemical modifications in testosterone known for their illicit consumption which can result in extensive side effects. Extraction procedures applied to the analysis of their formulations are still limited to few methodologies despite the increasing numbers of apprehensions 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 (SLE) or liquid-liquid (LLE), ultrasonic bath (UBAE), ultrasonic probe (UPAE) and microwave-assisted extraction (MAE) as well as determine the cytotoxicity of the formulations. Results indicated that 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 to SLE, LLE and UBAE. Moreover, **Product #10** and **#11**, for instance, decreased cellular viability to minimum levels at 0.5 $mg.mL^{-1}$ while the other samples generally had some toxicity to the cell culture. Therefore, alternative methods such as MAE or UPAE as could be used for the analysis of formulations of AASs assisting in the identification of components that could have toxicity to the user.

Key-words: Solid-Liquid Extraction; Liquid-Liquid Extraction; Ultrasonic-assisted Extraction; Microwave-assisted Extraction; Anabolic Androgenic Steroids; Cytotoxic Assay.

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 in clinical implications of hypogonadism. However, AASs are known for their illicit use by professional and recreational athletes due to effects such as improvement of sportive performance and 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 approximately 200% between 2008 and 2011. It is worth noting that falsified formulations of synthetic androgens comprehended roughly a third of the analyzed materials increasing the risks associated to the abusive use of AASs [4].

Apprehended formulations of anabolic agents need to undergo a 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 is based on the use of methanol under 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 currently used 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 previously noted, the identification of apprehended AASs formulations is considerably 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 four distinct extraction procedures as well as determine the cytotoxicity of eleven 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, methanolic solution of boron trifluoride (14%,

v/v), dimethyl sulfoxide (DMSO) and diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. 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 were obtained from Acumedia (Lansing, USA). All other reactants were analytical grade (≥ 99%).

For the identification and quantitation of the components of the matrix of the oily-based formulations, a 37-mix fatty acid methyl esters standard was purchased from Supelco (Bellefonte, USA) and used as a solution of 2 $mg.mL^{-1}$ in *n*-hexane. Quantitation of AASs was performed according to Berneira et al. (2019) using stock solutions (2 $mg.mL^{-1}$) of cholesterol (≤ 99%, Sigma-Aldrich, St, Louis, USA) in methanol that was used to obtain the working solution (1 $mg.mL^{-1}$) and the calibration curve (0.15, 0.25, 0.50, 0.75 and 1 $mg.mL^{-1}$) [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 research laboratory (**Table 1**). Samples were previously identified using GC-MS with a NIST-08 electronic mass spectra library. The study was designed in order to evaluate and continuously develop adequate methodologies involving evidentiary cases of anabolic agents.

[INSERT TABLE 1]

2.2. Extraction

Firstly, 25 µL of the oily solutions, 50 µL of aqueous suspensions or 20 mg of the solid formulations were introduced in 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 $mg.mL^{-1}$) were added to the tubes as an internal standard and the samples were extracted following the procedure of UBAE developed by Neves and Caldas (2017). 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 analysis were performed in triplicate (n=3).

2.2.1. Ultrasonic Bath Assisted Extraction

Following the procedure described by Neves and Caldas (2017), samples were vigorously vortexed for 10 s. Then, 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-force 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, samples were vigorously vortexed for 10 min. Then, tubes were centrifuged at 5000 G-force 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, 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 ambient temperature (25 °C), frequency (40 KHz), power (120 W) and amplitude (20%). Then, conical centrifuge tubes were centrifuged at 5000 G-force for 5 min. and the upper organic phase was retrieved for chromatographic analysis.

2.2.4. Microwave Assisted Extraction

Subsequently to the first step of dissolution with methanol, samples were vortexed for 10 s and, afterwards, flasks were introduced in an open vessel microwave model Discover 9080005 (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-force 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, 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 to the other colorimetric tests [9].

2.4.2. Naphtol-Sulfuric Acid Test

Firstly, 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 a 2.5% solution of naphtol-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 were 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. (1973). Briefly, 20 µL of the formulation and 5 mL of a methanolic solution (2%, w/v) of sodium hydroxide were placed in flasks 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 separation funnels 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 sulphate and dried under reduced pressure [12].

2.6. Cytotoxic assay

Cytotoxic assay of the formulations were 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 $mg.mL^{-1}$. Product **#1** and **#2** were not evaluated due to limited sample amount. 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 by a spectrophotometer at 540 nm [13,14] .

2.7. Instrumental Analysis

2.7.1. Infrared Spectroscopy Analysis

IR Spectroscopy analysis 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 cm^{-1} 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.

2.7.2. Chromatographic Analysis

For the comparison of extraction procedures, analysis were carried out in a gas chromatograph coupled to mass spectrometry model QP2010 (Shimadzu, Kyoto, Japan) equipped with a Rtx-5MS capillary column (30 m x 0.25 mm x 0.25 µm) using helium as the carrier gas with a flow of 0.97 $mL.min^{-1}$. 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 programed 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 totalizing 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 x 0.25 mm x 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, totalizing a running time of 40 min.

2.8. 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). Results of the triplicates (n=3) were expressed as mean ± standard deviation.

3. Results

3.1. Evaluation of Extraction Procedures

Chromatographic analysis (**Fig 1**) indicated the majority of the samples had a composition different than the one stated in the label. For **Product #6**, the alleged active ingredient boldenone undecylenate was actually nandrolone decanoate, while **Product #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 **Product #9, #10** and **#11** while **Product #2** had an unknown active ingredient, which was further identified as stanozolol. On the other hand, **Product #1**, **#3**, **#4** and **#5** had the same composition as stated in their labels.

[INSERT FIGURE 1]

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

[INSERT TABLE 2]

As it can be observed in **Table 2**, **Product #1**, **#6** and **#8** had the anabolic agent in concentrations considerably lower than the stated in the label while **Product #9**, **#10** and **#11** did not have the active ingredient. On the other hand, **Product #3**, **#5** and **#6** had similar experimental concentrations to the amount reported on the label. Finally, for **Product #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 in 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 **Product #6**, **#7**, **#8 #9, #10** and **#11** were falsified as the trade name and manufacturer were not licensed to distribute the formulations in Brazil. Besides, essential information such as address of the manufacturer, dosage form, expiry date and batch number were lacking in some cases reinforcing that the samples were possible counterfeit AASs.

[INSERT TABLE 1S]

On the other hand, **Product #5** was placed as a possible genuine formulation of AAS as several required information such as indication of active ingredient, dosage form, drug strength, name and logo of manufacturer were found in 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 **Product #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 neither 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 be mainly attributed to the presence of steroidal active ingredients, although excipients could have influenced in the results. Generally, color changed during the different tests as well as when the samples were

278 diluted or heated. It should be noted that the combination of the spot tests provided
279 positive indications of the presence of AASs, however it was not possible to confirm the
280 presence of a specific one.

281 [INSERT TABLE 2S]

282

283 **3.4. IR Spectroscopy Analysis**

284 IR Spectroscopy analysis (**Table 3S**) of **Product #1** revealed bands that could be
285 associated to the alleged active ingredient oxymetholone such as the hydroxyl (3523 and
286 3330 cm^{-1}) and carbonyl groups (1613 cm^{-1}). Furthermore, **Product #2** and **#3** had similar
287 spectra with characteristic bands associated with amine group (3330 to 3110 cm^{-1}) and to
288 the C=N bonding (1657 cm^{-1}) that can be linked to stanozolol. On the other hand, **Product**
289 **#9** that should contain stanozolol did not result in the characteristic bands seen in the
290 other samples. In this sense, the vibrations observed in the spectrum were probably related
291 to the excipient carboxymethylcellulose. In turn, **Product #10** and **#11** had vibrations that
292 could be mainly attributed to their excipients.

293 [INSERT TABLE 3S]

294 On the other hand, the lack of distinct chemical groups that could differentiate the
295 constituents of **Product #4**, **#5**, **#6**, **#7** and **#8** produced very similar spectra that, in most
296 cases, only varied in transmittance values. Among the minor differences found in the
297 results were bands associated to α-β unsaturated carbonyl group (1722 and 1723 cm^{-1} in
298 **Product #5**, **#6** and **#7**) and α-β unsaturation (1617 cm^{-1} in **Product #4**). Generally, these
299 formulations presented vibrations related to unsaturation, carbonyl group and aromatic

moieties that can be associated to 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 ramifications 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.

[INSERT TABLE 4S]

As it 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 **Product #4** and **#5** 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 **Product #6**, **#7** and **#8**, which also displayed similar predominant fatty acids.

For **Product #4** and **#5**, monounsaturated fatty acids (MUFAs) were found in considerable concentrations ranging from 54.85 ± 0.05% to 63.96 ± 0.09% while for **Product #6**, **#7, #8**, **#10** and **#11** polyunsaturated fatty acids (PUFAs) were the

predominant fatty acid class varying from 56.45 ± 0.05% to 59.19 ± 0.08%. For all of the analyzed samples, saturated fatty acids (SFAs) were detected in lower concentrations than the other classes varying from 15.05 ± 0.26 to 23.97 ± 0.17. It is worth noting that this was the first time that oily-based AASs had their matrix analyzed and identified.

Comparing the resulting fatty acid profile of the formulations to the profiles of vegetable oils found in the literature, it could be noted that **Product #4** and **#5** were probably constituted of peanut oil, while **Product #6**, **#7**, **#8, #10** and **#11** were possibly composed of soybean oil [15]. 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. 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 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 (**Fig 2b)** to distinguish or cluster the samples. Results of the analysis showed that the model generated by PCA could explain approximately 55.58% of the differences found among the samples (**Fig 2a)**.

[INSERT FIGURE 2]

As it can be observed in **Fig 2**, the variables used in PCA could differentiate the samples and the vegetable oils control 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 possible excipient (**Product #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 (**Product #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.

3.6. Cytotoxic assay

Cellular viability assay (**Table 3)** indicated that most anabolic agents had cytotoxicity to MDBK cells at the maximum tested concentration of 0.5 $mg.mL^{-1}$. Generally, toxicity decreased as the exposed amount also diminished reaching 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 component of the formulations and did not significantly influenced the cellular viability, which remained at its maximum value.

[INSERT TABLE 3]

As it can be observed in **Table 3**, **Product #10, #11** and **#5** 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 #10** and **11** while **Product #5** had a cellular viability of 5.67 ± 0.25%. These values were gradually increasing as the concentrations were diminished reaching none toxicity for **Product #10** and **Product #5** while **Product #11** had cellular viability of 85.51 ± 4.60% at 0.062 $mg.mL^{-1}$. Regarding

the other samples, it was observed a similar pattern, however the cellular viability was higher compared to **Product #10** and **11**. Finally, **Product #8** had the maximum cellular viability at 0.5 $mg.mL^{-1}$, which was only observed to the other formulations at lesser concentrations.

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 [16]. On the other hand, long carbon-chain nandrolone or testosterone esters required an 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 [17].

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 considerably important in the forensic science given that results are used as criminal evidence [10]. Among research studies, Neves and Caldas (2017) reported that there was a 5% increase in the recovery of testosterone, nandrolone or trenbolone esters from oily formulations using UBAE compared to LLE or SLE. Comparing the reported results to the current

work, it can be observed that generally there was indeed an increase on the extraction of anabolic agents varying from 1 to 10% with the use of ultrasonic bath [7].

Generally, acoustic energy improved the extraction of the active ingredient compared to LLE or SLE. The enhancement in the extractive process can be directly related to cavitation bubbles that are formed, improving solvent-sample interaction. In this sense, the recovery of anabolic agents can be associated to 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 [18].

As it can be observed from the evaluation of extraction procedures, UPAE showed results superior to UBAE. This could be due to the dissipation of the ultrasound energy within the ultrasonic bath [19]. 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 considerably used in the analysis of formulations of AASs, ultrasonic baths have poor repeatability and reproducibility compared to ultrasonic probes, which can affect negatively the results obtained by this method [18].

The ultrasonic probe focused acoustic energy into the sample increasing the formation of cavitation bubbles and the overall recovery of the analytes [20]. Despite showing promising results when compared to the conventional methods used, UPAE is rarely applied on 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 the reasons why UPAE is less used than the ultrasonic bath, where multiple samples can be analyzed at the same time [21].

Compared to SLE, LLE or ultrasonic-based methods, MAE also showed promising results that can be associated to 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 [22].

Methods that employ ultrasonic probe or microwave irradiation for the analysis of formulations of AASs have not been frequently reported in the literature [23]. In this sense, there are only studies that evaluate the extractive procedures for the recovery of anabolic agents in urine and food matrices [23,24]. 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 different 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 in most cases the use of LLE, SLE or UBAE could lead to false negatives as these methods retrieved lower concentrations of AASs compared to MAE and UPAE.

Therefore, the choice of the extraction procedure is considerably important in order to obtain adequate and truthful 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 sanitary guidance, increasing their susceptibility for microbiological contamination [3,5,25]. Additionally, the chemical composition of the products is uncertain as there is no oversight 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 (**Product #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 **Product #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 [26]. Among the reasons that can explain this are meticulous falsifications that can look very similar to genuine products differencing only in the chemical fingerprint of the preparation. Besides, formulation may be apprehended while still under manufacturing 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

Results of the spot tests indicated the possible presence of anabolic agents due to the formation of color among the several performed tests. However, interferences of excipients were observed for **Product #1**, **#2** and **#9** as samples developed color despite previous indications of 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 be easily observed in the Naphtol-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 than the indicated in the literature which could lead to false interpretations [9].

For **Product #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 acylglicerols 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 on the literature had similar results found in the current work with differences in color possibly associated to excipients [9]. Nonetheless, the presence of multiple active ingredients in the preparations prevented the identification of the constituting anabolic agents.

4.4. IR Spectroscopy Analysis

The application of IR Spectroscopy analysis as a preliminary analytical technique indicated the presence of the stated active ingredients for **Product #1** and **#3**. It also pointed out the presence of active ingredient stanozolol in the unknown formulation of **Product #2** since this synthetic androgen had particular groups and distinct chemical transitions [27]. However, for the other samples, it was not possible to identify clear distinctions because testosterone and nandrolone esters as they differ only in the carbonic length of their ramifications. Moreover, the oily formulation and the vehicle interfered in the resulting spectra with additional bands, possibly masking characteristic vibrations of the anabolic agents [25]. 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 surely identify 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,

513 confirmatory analytical techniques such as GC-MS should be used in order to detect the
514 organic components of the samples [7].

515

516 **4.5. Formulation Analysis**

517 Since anabolic agents are mostly hydrophobic, their formulations were constituted
518 of vegetable oils such as arachis, castor, peanut and sesame oil and benzyl benzoate [28].
519 These components are known as excipients and fulfill several roles in a pharmaceutical
520 preparation conferring solubility, permeability, coloring and stability so as the anabolic
521 agent is able to accomplish its biological goal [29]. Excipients can be potentially used as
522 sole components in counterfeit alleged pharmaceutical formulations, comprehending
523 more than 40% of the cases involving falsifications. Moreover, non-pharmaceutical
524 preparations can be a serious health hazard as these products can be manufactured under
525 non-sterile and non-controlled conditions [28].

526 Forensic evaluation of excipients in AASs formulations is not usually performed
527 as the main objective in such cases is to detect the presence of the stated active ingredients
528 in the material. Nonetheless, it can be observed that the analysis of the oily formulations
529 provided important information about the preparation of the anabolic agents since it was
530 possible to correlate signs of falsification to the type of oily excipient. For the analyzed
531 samples, genuine materials were constituted of peanut oil while counterfeit formulations
532 of AASs were composed of soybean oil probably due to its low-cost and easiness of
533 acquisition [30]. Therefore, the analysis of the constituents of the oily matrix could be
534 used to evaluate possible signs of falsification found in a sample [31].

535 Vegetable oils are common constituents in formulations of anabolic agents serving
536 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 [32]. 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 are associated with lower rates of release of the anabolic agent into the bloodstream and a prolonged biological effect [28]. Similarly 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 probe ultrasound and microwaves in order to fully be retrieved from 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 [29]. 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 to 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 [28].

Results of PCA showed that **Product #6**, **#7** and **#8,** which were falsified formulations of AASs clustered around soybean oil, while **Product #4** and **#5** that were genuine materials clustered in the same portion of the score plot. Nonetheless, **Product #9**, **#10** and **#11**, which were counterfeit materials also clustered around peanut oil. Given these results and previous statistical analysis, there was an indicative that the oily excipient could be a biomarker for the discrimination of counterfeit from truthful samples. However, further parameters should be included in order to satisfactory discriminate the samples [25].

It should be noted that PCA has not been currently used 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 [25]. Therefore, the application of PCA provided noteworthy insights concerning the composition of the oily excipient that could be efficiently used to identify possible signs of counterfeit materials.

4.6. Cytotoxic assay

Results indicated that generally the formulations of anabolic agents were cytotoxic 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[33,34]. It is worth noting that the potential to induce apoptosis is associated to the anabolic agent used having a toxicity order of nandrolone > testosterone > stanozolol > trenbolone [35]. This cytotoxic order was not observed in the current study

possibly because most AASs were in their respective ester derivative or since the anabolic agents were applied altogether with their formulations.

As it can be observed in **Table 3**, concentration of ASSs played a major role in the cytotoxicity of the samples. This behavior was also indicated by Zelleroth et al (2013) which applied several anabolic agents in concentrations of 100, 30 and 10 µM to primary rat cortical cells. On the other hand, **Product #10** and **#11** that did not had their active ingredients were also cytotoxic indicating that their excipients or adulterants possibly played a major role in their toxicity to MDBK cells. Given this, results were in line with previous reports of the literature, which indicated that counterfeit formulations could be health hazardous due to their uncertain constitutions and their respective concentrations[1,7].

Conclusion

In the present study, several extraction methods were evaluated, cytotoxic effects of formulation of anabolic agents were determined and the samples could be successfully analyzed. Results indicated that the use of microwaves or ultrasonic probe increased the efficiency of the extraction compared to liquid-liquid, solid-liquid and ultrasonic bath assisted extraction. Altogether, visual inspection, spectroscopy and spectrometry allowed the conclusion that **Product #6**, **#7**, **#8, #9**, **#10** and **#11** were counterfeits while **Product #1**, **#2**, **#3**, **#4** and **#5** were veridical. Regarding cytotoxic effects, samples generally influenced negatively cellular viability indicating that they had toxicity at the tested concentrations. Therefore, the application of analytical approaches and efficient extraction procedures were required in order provide adequate results and screen for possible health hazardous compounds in formulations of anabolic agents.

608

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615

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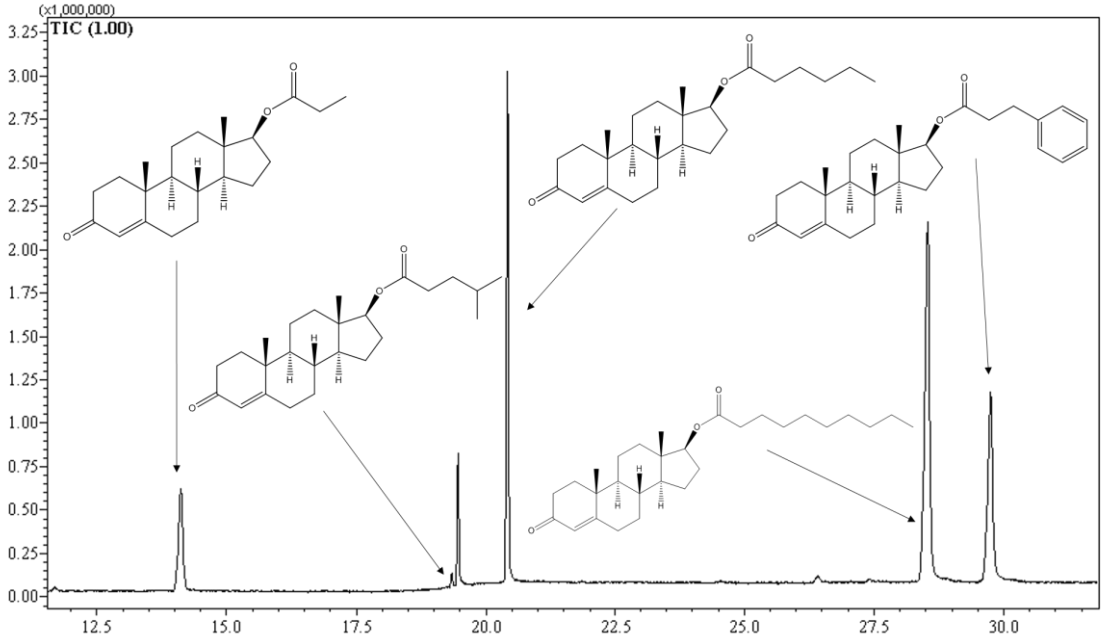
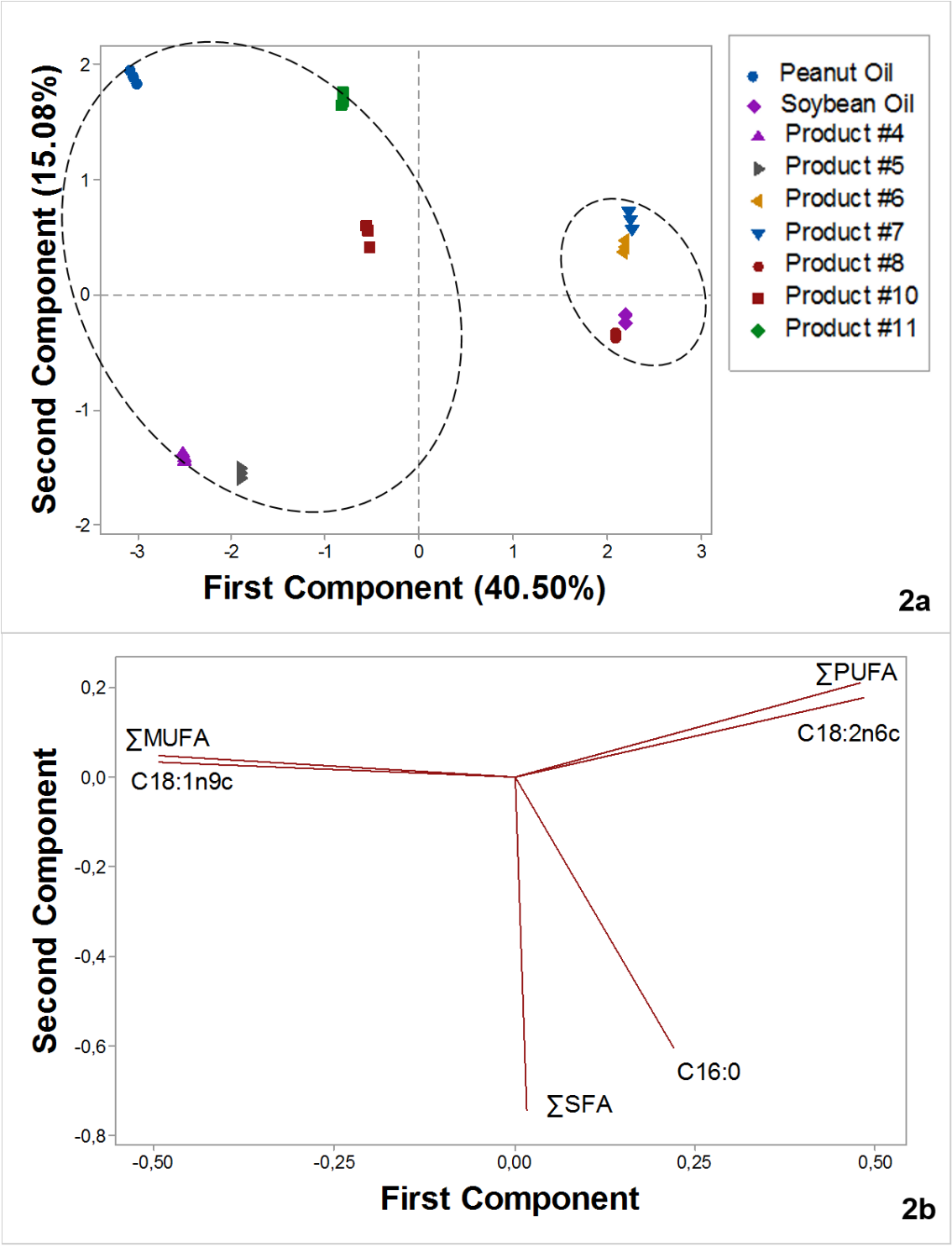


735

736 **Figure 1.** GC-MS chromatogram of the AASs found in the formulation of **Product #4**

737 (allegedly containing testosterone esters).

738



739

740 **Figure 2.** Score plot (**a**) and loading plot (**b**) of PCA applied to the formulations of AASs

741 and the vegetable oils control.

742

743 **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 $mg.mL^{-1}$	Testosterone cypionate
Product #6	Oily solution	Oily dark yellow viscous liquid	Boldenone undecylenate	250 $mg.mL^{-1}$	Nandrolone decanoate

Product #7	Oily solution	Oily dark yellow viscous liquid	Testosterone esters	250 $mg.mL^{-1}$	Testosterone and nandrolone esters
Product #8	Oily solution	Oily dark green viscous liquid	Testosterone propionate	100 $mg.mL^{-1}$	Testosterone and nandrolone esters
Product #9	Aqueous suspension	White viscous liquid	Stanozolol	100 $mg.mL^{-1}$	No active ingredient
Product #10	Oily solution	Oily light yellow viscous liquid	Testosterone propionate	100 $mg.mL^{-1}$	No active ingredient
Product #11	Oily solution	Oily dark yellow viscous liquid	Trenbolone acetate	100 $mg.mL^{-1}$	No active ingredient

749

750

Table 2. Comparison of LLE or SLE, UBAE, UPAE and 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^{-1})	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^{-1})	33.44 ± 0.71^{a}	35.48 ± 2.51^{ab}	37.47 ± 1.13^{b}	35.69 ± 0.24^{ab}
TISO (mg.mL^{-1})	12.41 ± 0.12^{a}	12.41 ± 0.25^{a}	12.44 ± 0.07^{a}	12.44 ± 0.06^{a}
TCAP (mg.mL^{-1})	61.59 ± 3.03^{a}	62.31 ± 2.29^{a}	68.18 ± 0.03^{b}	66.23 ± 1.40^{b}
TDEC (mg.mL^{-1})	97.74 ± 4.97^{a}	97.64 ± 2.15^{a}	108.59 ± 4.09^{b}	103.82 ± 2.76^{c}
TPHE (mg.mL^{-1})	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^{-1})	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^{-1})	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^{-1})	40.47 ± 1.22^{a}	44.40 ± 1.20^{b}	48.20 ± 1.49^{c}	45.15 ± 0.70^{bc}
TCAP (mg.mL^{-1})	13.06 ± 0.08^{a}	13.25 ± 0.35^{a}	13.63 ± 0.14^{a}	13.33 ± 0.27^{a}
TDEC (mg.mL^{-1})	13.21 ± 0.11^{a}	13.45 ± 0.23^{a}	13.50 ± 0.05^{a}	13.52 ± 0.50^{a}
NDEC (mg.mL^{-1})	16.84 ± 0.09^{a}	17.25 ± 1.11^{a}	18.32 ± 0.08^{a}	18.14 ± 0.28^{a}
TPHE (mg.mL^{-1})	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^{-1})	16.64 ± 0.12^{a}	15.93 ± 0.29^{a}	15.76 ± 0.45^{a}	15.98 ± 0.29^{a}
TCYP (mg.mL^{-1})	36.96 ± 1.48^{a}	35.95 ± 3.02^{a}	36.52 ± 1.12^{a}	35.46 ± 0.39^{a}
NDEC (mg.mL^{-1})	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^{-1})	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 ($p < 0.05$)

Solid-Liquid Extraction (SLE); Liquid-Liquid Extraction (LLE); Ultrasonic Bath-Assisted Extraction (UBAE); Microwave-Assisted Extraction (MAE); Non-detected (nd).

Oxymetholone (OXY); Stanozolol (STA); Testosterone Propionate (TPRO); Testosterone Isocaproate (TISO); Testosterone Caproate (TCAP); Testosterone Decanoate (TDEC); Testosterone Phenylpropionate (TPHE); Testosterone Cypionate (TCYP); Nandrolone Decanoate (NDEC); Trenbolone acetate (TACE).

Table 3. Cellular viability (%) in function of distinct concentrations of anabolic agents.

Sample	**Concentration (mg.mL^{-1})**			
	0.500	**0.250**	**0.125**	**0.062**
Product #3	78.91 ± 1.17^{a}	100 ± 0.00^{b}	100 ± 0.00^{b}	100 ± 0.00^{b}
Product #4	94.40 ± 4.77^{a}	96.80 ± 0.24^{ab}	98.38 ± 0.62^{ab}	100 ± 0.00^{b}
Product #5	5.67 ± 0.25^{a}	62.47 ± 0.00^{b}	91.82 ± 4.78^{c}	100 ± 0.00^{d}
Product #6	86.82 ± 1.55^{a}	87.37 ± 4.95^{a}	94.17 ± 0.00^{b}	99.79 ± 0.12^{b}
Product #7	77.34 ± 2.03^{a}	97.16 ± 0.79^{b}	100 ± 0.00^{b}	100 ± 0.00^{b}
Product #8	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
Product #9	78.91 ± 1.17^{a}	85.16 ± 3.15^{b}	89.99 ± 3.73^{c}	99.30 ± 0.60^{d}
Product #10	0.00 ± 0.00^{a}	8.05 ± 3.76^{b}	63.90 ± 3.72^{c}	100 ± 0.00^{d}

Cellular viability (%)

Product #11	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 ($p < 0.05$).

BIOANALYTICAL APPROACHES APPLIED TO THE ANALYSIS OF APPREHENDED FORMULATIONS OF ANABOLIC ANDROGENIC STEROIDS

Supplementary Information

Table 1S. Results of the visual inspection of the samples.

Parameter of Evaluation	Product #1	Product #2	Product #3	Product #4	Product #5	Product #6	Product #7	Product #8	Product #9	Product #10	Product #11
Container	Sealed	Not sealed	Sealed	Sealed	Sealed	Sealed	Sealed	Sealed	Sealed	Sealed	Sealed
Information legible	Yes[2]	n.a.[1]	Yes[2]	Yes[2]	Yes	Yes[2]	Yes[2]	Yes[2]	Yes[2]	Yes[2]	Yes[2]
Trade Name Registered	No[3]	n.a.[1]	No[3]	No[3]	Yes	No	No	No	No	No	No
Manufacturer Registered	No[3]	n.a.[1]	No[3]	No[3]	Yes	No	No	No	No	No	No

Spelling of the Active Ingredient	Correct[2]	n.a.[1]	Correct[2]	Correct[2]	Correct[2]	Correct[2]	Correct[2]	Correct[2]	Correct[2]	Correct[2]	Correct[2]
Active Ingredient	Legal[4]	n.a.[1]	Legal[4]	Legal[4]	Legal[4]	Legal[4]	Legal[4]	Legal[4]	Legal[4]	Legal[4]	Legal[4]
Manufacture's Name/Logo	Yes	n.a.[1]	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	No
Presence of Hologram	No	n.a.[1]	Yes	No	No	No	No	No	No	No	No
Manufacture's Address	Yes	n.a.[1]	Yes	No	No	No	No	No	Yes	No	No
Presence of Drug Strength	Yes	n.a.[1]	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Presence of Dosage Form	Yes	n.a.[1]	Yes	Yes	Yes	No	No	No	Yes	Yes	No
Number of Units Stated	Yes	No	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]

Batch Number and Expiry Date	Yes	n.a.[1]	Yes	Yes	Yes	No	Yes[5]	Yes	No	Yes	Yes
Shape of Tablets	Round	Round	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]
Size of Tablets	Uniform	Uniform	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]
Damaged or Empty Tables	No	No	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]
Content	Uniform solid	Uniform solid	Suspension	Uniform solution	Uniform solution	Uniform solution	Uniform solution	Uniform solution	Biphasic solution	Uniform solution	Uniform solution

Note: [1]Non-available (n.a.);

[2] Information written in other language (English, Spanish or German) rather than in Portuguese;

[3] Products and trade names not registered in Brazil, but registered in Paraguay;

[4] Active ingredients legal, but placed under controlled substances list in Brazil;

[5] Product had expiry date, but did not have batch number.

Table 2S. Results of the spot tests of the samples.

Sample	Sulfuric Acid Test			Naphtol-Sulfuric Acid Test		Liebermann's Test	
	Cooled	**Diluted**	**Heated**	**Cooled**	**Heated**	**Cooled**	**Heated**
Product #1	Light Yellow	Red	Dark Red	Blue-Green	Black	Orange Precipitate	Orange Precipitate
Product #2	Light Yellow	Light Red	Dark Red	Blue-Green	Dark Purple	Light Yellow	Light Yellow
Product #3	Light Yellow	Yellow	Light Orange	No reaction	Brown-Green	Orange Precipitate	Orange Precipitate
Product #4	Yellow	Orange	Brown-Blue	Dark Brown	Brown-Green	Light Orange	Dark Orange
Product #5	Yellow	Orange	Brown-Blue	Dark Brown	Brown-Green	Light Orange	Dark Brown
Product #6	Dark Brown	Light Brown	Dark Red	Dark Brown	Brown-Red	Orange	Dark Brown
Product #7	Red	Orange	Brown-Blue	Dark Brown	Dark Brown	Orange	Dark Brown
Product #8	Red	Light Brown	Dark Red	Brown-Red	Brown-Green	Dark Orange	Dark Brown

Product #9	Nil	Nil	Nil	No reaction	No reaction	Light Yellow	Light Yellow
Product #10	Nil	Brown-Red	Brown	Red	Brown	Orange	Dark Brown
Product #11	Light Yellow	Brown-Red	Brown	Red	Brown	Orange	Dark Brown
Soybean oil	Nil	Orange	Orange	Orange	Black	Light Yellow	Dark Orange
Peanut oil	Nil	Orange	Orange	Orange	Black	Light Yellow	Dark Orange
Cholesterol	Orange	Light Yellow	Yellow	Light Brown	Brown	Nil	Nil
Blank	Nil	Nil	Nil	Dark Grey	Dark Green	Nil	Nil

Table 3S. Results of the IR Spectroscopy analysis of the samples.

Bonding (cm^{-1})	**Product #1**	**Product #2**	**Product #3**	**Product #4**	**Product #5**	**Product #6**	**Product #7**	**Product #8**	**Product #9**	**Product #10**	**Product #11**
-O-H	3523 3330	3521	3469	-	-	-	-	-	3393	-	-
-N-H	-	3330 3266	3221 3178 3110	-	-	-	-	-	-	-	-
=C-H	-	-	3013	3004 866	3006	3007	3008	3008	-	3008	3008
-C=O	1613	-	-	1740	1741 1722	1740 1723	1740 1722	1742	-	1743	1742

-C=N	-	1538	1523	-	-	-	-	-	-	-	-
-N=N-H	-	1424	1413	-	-	-	-	-	-	-	-
-C=C	-	-	1598	1677	1676	-	-	-	-	1654	1654
				1617							
-N-H	-	1159	1159	-	-	-	-	-		-	-
-CH_2-	1467	1471	1471	1464	1464	1464	1464	1464	1470	1458	1460
-CH_3	1453	1453	1446	1456	1454	1454	1456	1457	-	1377	1379
	1372	1377	1374	1376	1376	1375	1375	1376			
-C-O	1217	1260	1277	1163	1268	1269	1269	1161	1411	1097	1099
	1070	1070	1080		1069	1072	1070	1098	1117	1159	1158
	1029	1030			1026	1025	1026			1236	1236
	1016										

Ar-H	-	-	-	722 698	710 695	711 695	711 695	722 697	-	-	-

Table 4S. Fatty acid composition (% of area) of the oily-based formulations of AASs and the vegetable oils control.

Fatty Acid	Product #4	Product #5	Product #6	Product #7	Product #8	Product #10	Product #11	Soybean oil	Peanut oil
C10:0	nd[a]	3.49 ± 0.03[b]	2.70 ± 0.12[c]	nd[a]	nd[a]	0.14 ± 0.01[a]	nd[a]	nd[a]	nd[a]
C12:0	nd[a]	5.05 ± 0.31[b]	3.78 ± 0.27[c]	3.47 ± 0.09[d]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]
C14:0	1.62 ± 0.09[a]	nd[b]	nd[b]	nd[b]	nd[b]	nd[b]	1.52 ± 0.62[a]	nd[b]	nd[b]
C16:0	9.25 ± 0.01[a]	7.64 ± 0.04[b]	7.09 ± 0.05[b]	7.77 ± 0.10[b]	10.73 ± 0.01[c]	7.94 ± 0.21[b]	11.28 ± 0.05[c]	10.79 ± 0.06[c]	10.84 ± 0.12[c]
C18:0	3.13 ± 0.03[a]	2.29 ± 0.00[b]	3.31 ± 0.01[a]	3.52 ± 0.05[a]	3.49 ± 0.02[a]	3.58 ± 0.03[a]	5.18 ± 0.09[b]	3.40 ± 0.06 [a]	2.87 ± 0.07[b]
C18:1*n*9c	61.43 ± 0.14[a]	54.54 ± 0.06[b]	24.15 ± 0.15[c]	25.33 ± 0.16[d]	27.82 ± 0.10[e]	49.29 ± 0.26[f]	43.10 ± 0.74[g]	27.46 ± 0.02[h]	44.66 ± 0.00[i]
C18:1*n*9t	1.03 ± 0.03[a]	0.30 ± 0.01[b]	0.18 ± 0.01[b]	0.14 ± 0.02[b]	nd[b]	nd[b]	nd[b]	0.13 ± 0.01[b]	nd[b]
C18:2*n*6c	16.10 ± 0.06[a]	19.55 ±0.02[b]	57.16 ± 0.21[c]	58.08 ± 0.09[d]	50.78 ± 0.15[e]	37.30 ± 0.19[f]	35.30 ± 0.24[g]	52.33 ± 0.09[h]	34.40 ± 0.35[i]
C18:2*n*6t	nd[a]	nd[a]	0.16 ± 0.02[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]
C18:3*n*6	nd[a]	1.58 ± 0.08[b]	0.07 ± 0.00[a]	nd[a]	0.29 ± 0.05[a]	nd[a]	nd[a]	0.17 ± 0.01[a]	nd[a]

C18:3*n*3	nd[a]	nd[a]	0.34 ± 0.01[a]	1.06 ± 0.01[b]	5.33 ± 0.03[c]	nd[a]	1.32 ± 0.15[b]	5.09 ± 0.05[c]	0.57 ± 0.40[a]
C20:0	1.33 ± 0.01[a]	1.12 ± 0.04[a]	0.18 ± 0.01[b]	nd[b]	0.39 ± 0.06[b]	0.28 ± 0.02[b]	0.57 ± 0.15[b]	0.26 ± 0.01[b]	1.34 ± 0.04[a]
C20:1	1.50 ± 0.01[a]	nd[b]	0.16 ± 0.00[b]	0.27 ± 0.00[b]	0.44 ± 0.09[b]	0.24 ± 0.04[b]	0.39 ± 0.11[b]	0.29 ± 0.01[b]	1.15 ± 0.01[a]
C22:0	2.99 ± 0.08[a]	2.79 ± 0.09[a]	0.31 ± 0.01[b]	0.28 ± 0.01[b]	0.47 ± 0.02[b]	0.81 ± 0.04[b]	0.79 ± 0.10[b]	nd[b]	2.97 ± 0.18[a]
C24:0	1.55 ± 0.05[a]	1.58 ± 0.01[a]	0.11 ± 0.00[b]	nd[b]	0.19 ± 0.00[b]	0.34 ± 0.08[b]	0.40 ± 0.12[b]	nd[b]	1.53 ± 0.17[a]
∑SFA	19.87 ± 0.16[a]	23.97 ± 0.17[b]	17.50 ± 0.37[c]	15.05 ± 0.26[d]	15.28 ± 0.07[d]	13.13 ± 0.37[e]	19.87 ± 0.67[a]	14.55 ± 0.20[d]	19.55 ± 0.36[a]
∑MUFA	63.96 ± 0.09[a]	54.85 ± 0.05[b]	24.49 ± 0.14[c]	25.75 ± 0.18[d]	28.26 ± 0.02[e]	49.53 ± 0.21[f]	43.49 ± 0.62[g]	27.88 ± 0.04[h]	45.81 ± 0.00[i]
∑PUFA	16.16 ± 0.07[a]	21.18 ± 0.12[b]	58.00 ± 0.23[c]	59.19 ± 0.08[d]	56.45 ± 0.05[e]	37.30 ± 0.19[f]	36.69 ± 0.19[g]	57.60 ± 0.13[c]	34.98 ± 0.76[h]

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

Saturated fatty acids (SFAs); Monounsaturated fatty acids (MUFAs); Polyunsaturated fatty acids (PUFAs); Non-detected (nd).

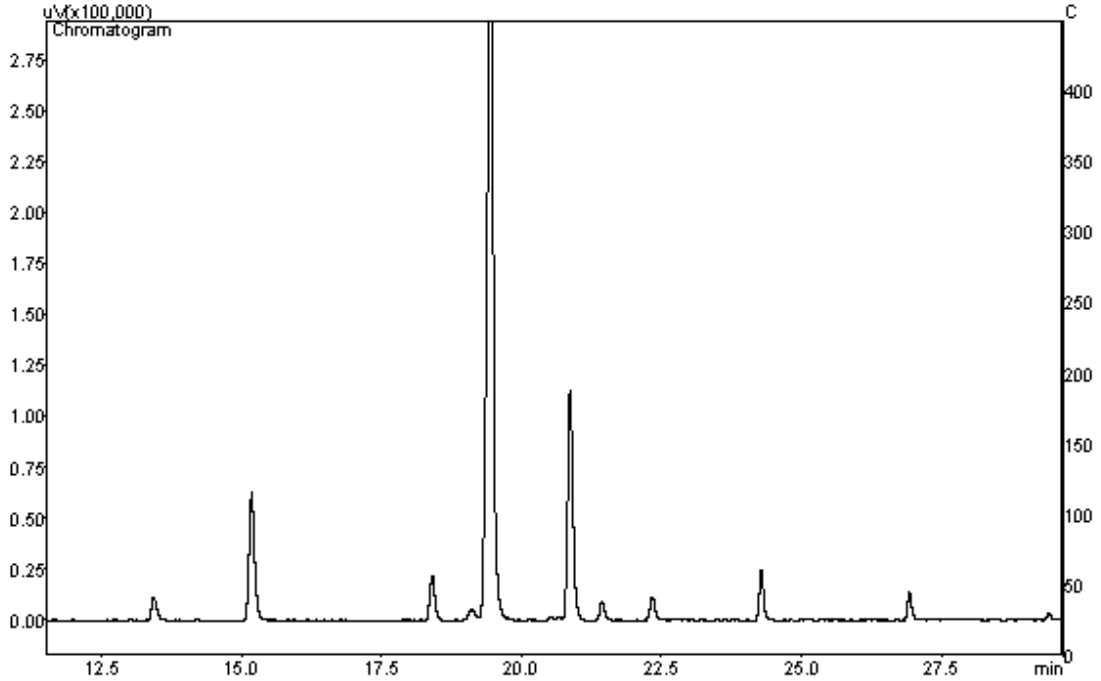


Figure 1S. GC-FID chromatogram of the oily formulation of **Product #4.**

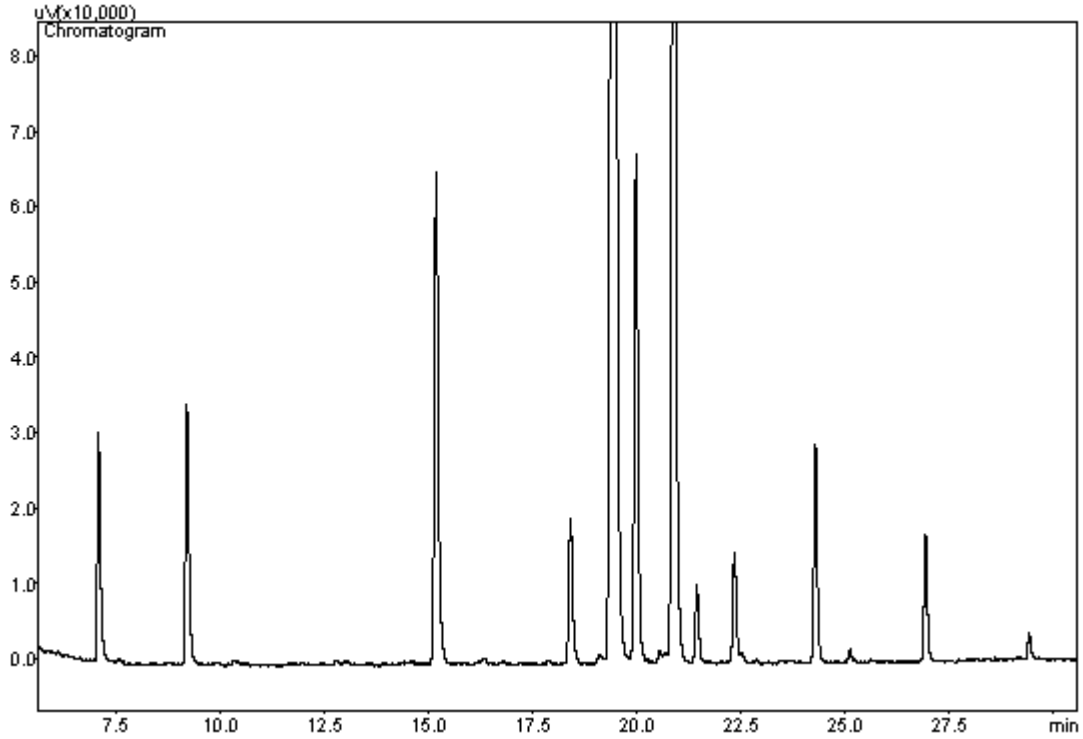


Figure 2S. GC-FID chromatogram of the oily formulation of **Product #5.**

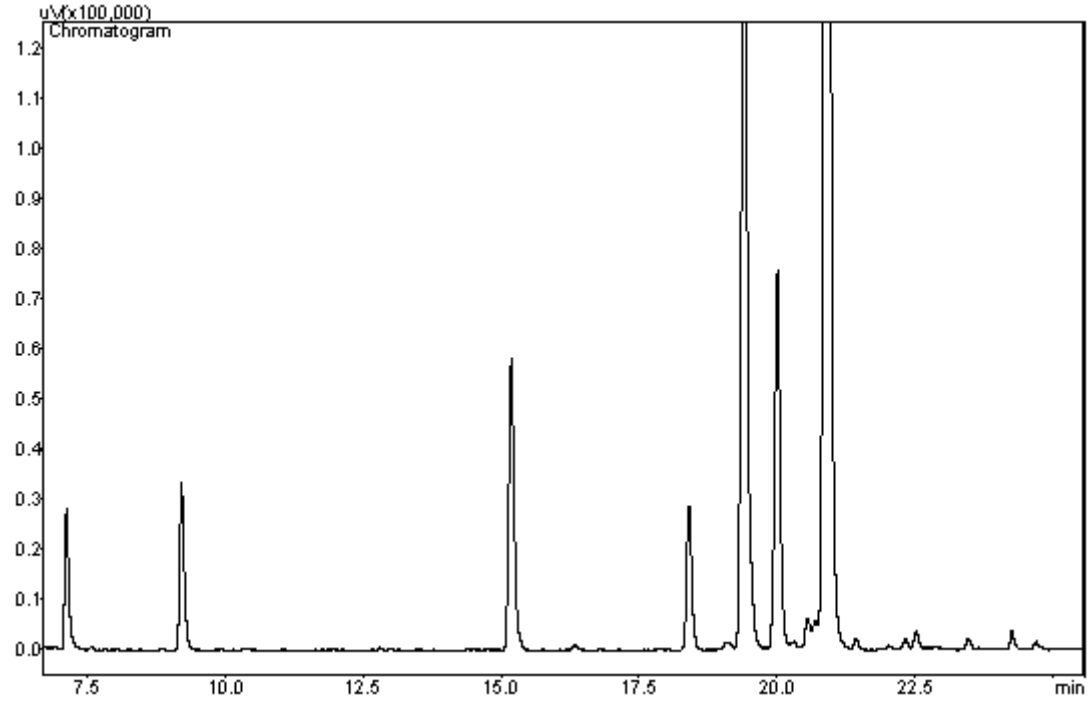


Figure 3S. GC-FID chromatogram of the oily formulation of **Product #6.**

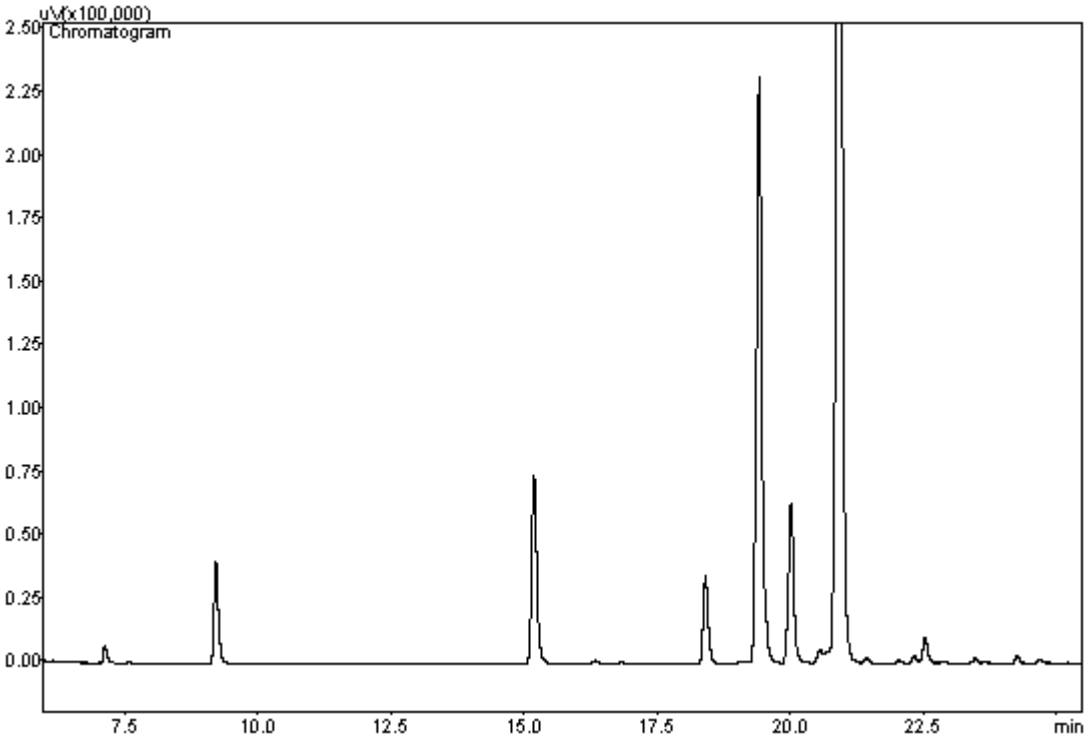


Figure 4S. GC-FID chromatogram of the oily formulation of **Product #7.**

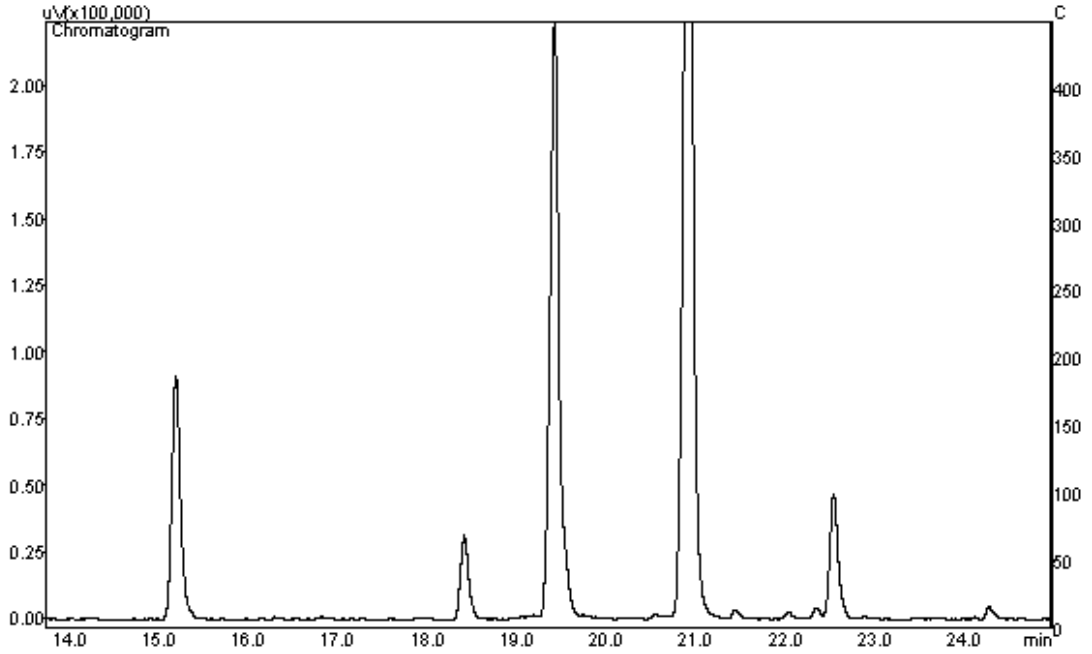


Figure 5S. GC-FID chromatogram of the oily formulation of **Product #8.**

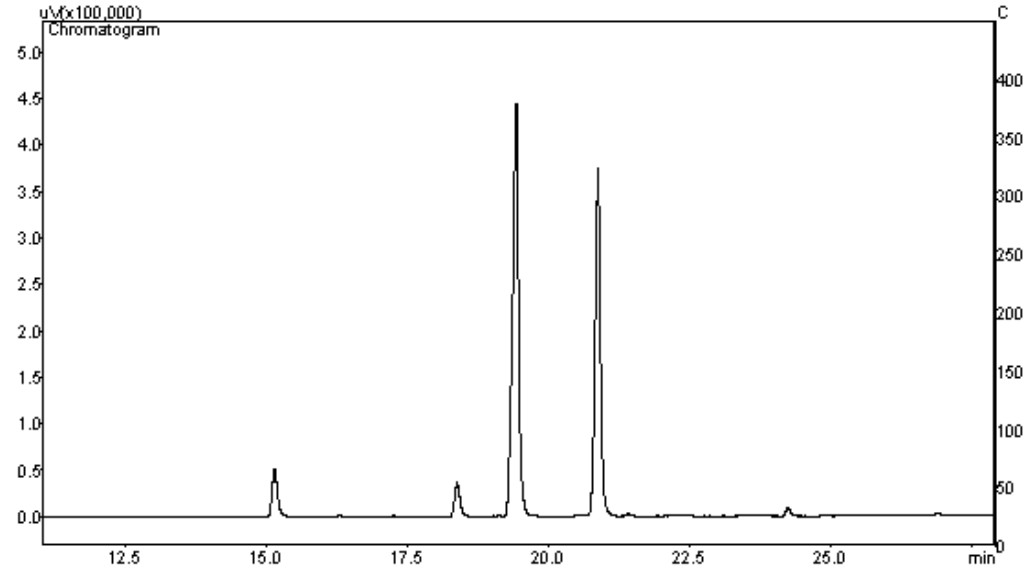


Figure 6S. GC-FID chromatogram of the oily formulation of **Product #10.**

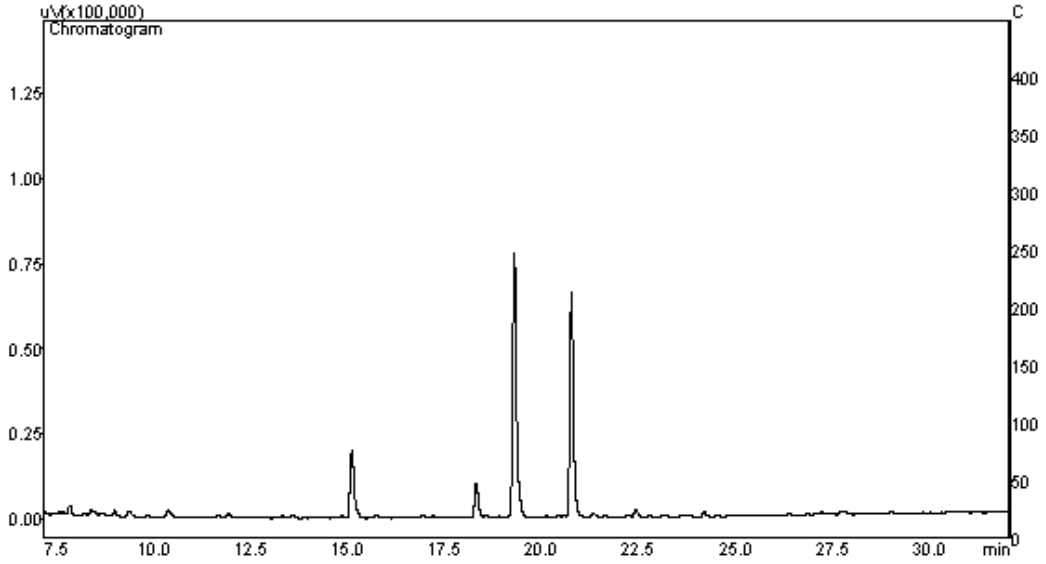


Figure 7S. GC-FID chromatogram of the oily formulation of **Product #11.**

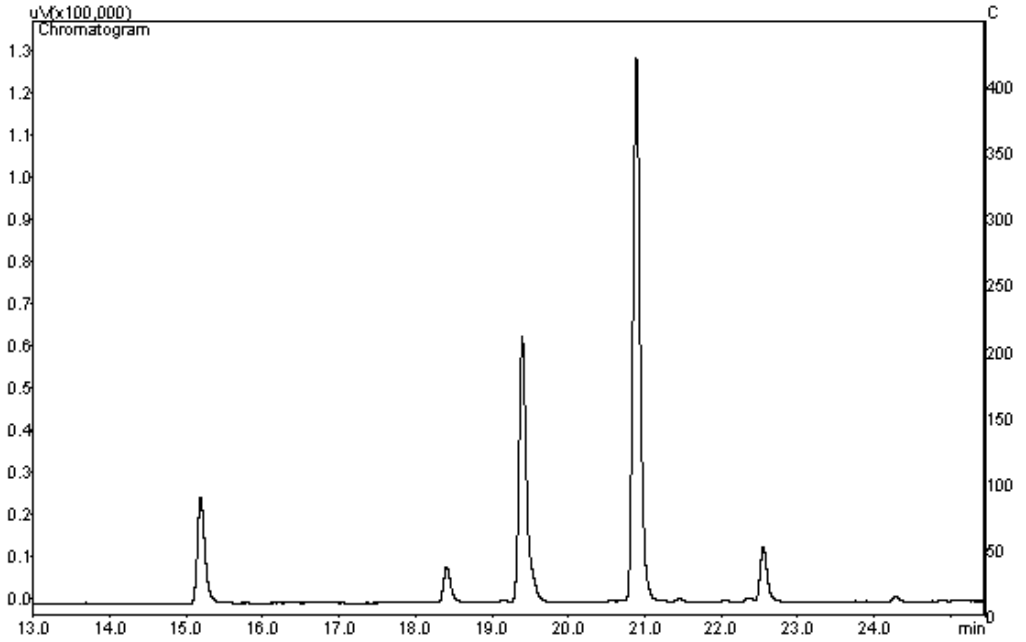


Figure 8S. GC-FID chromatogram of soybean oil**.**

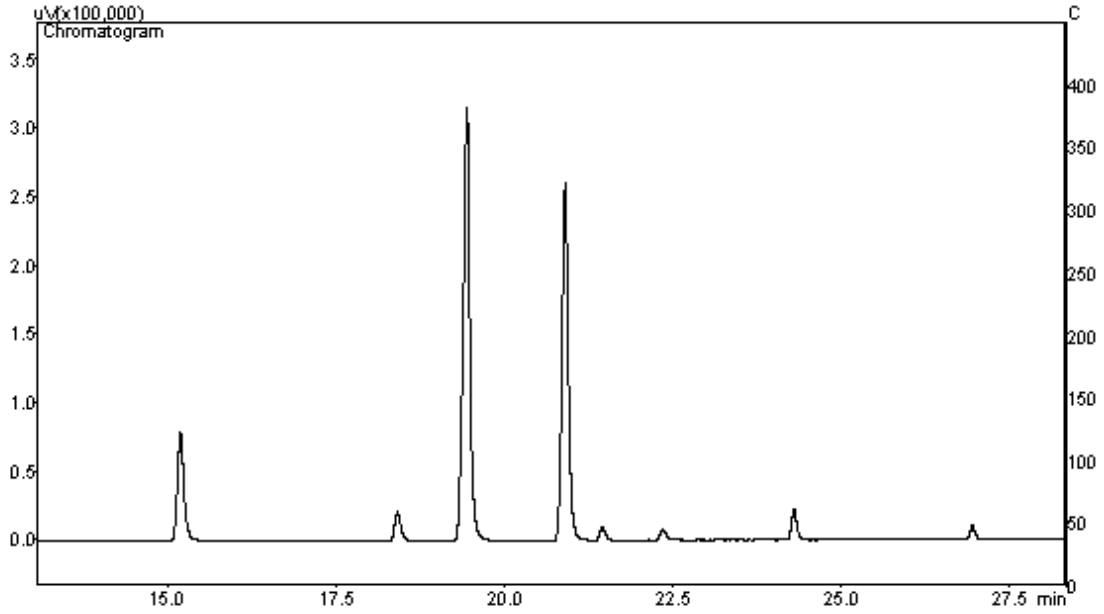


Figure 9S. GC-FID chromatogram of peanut oil.

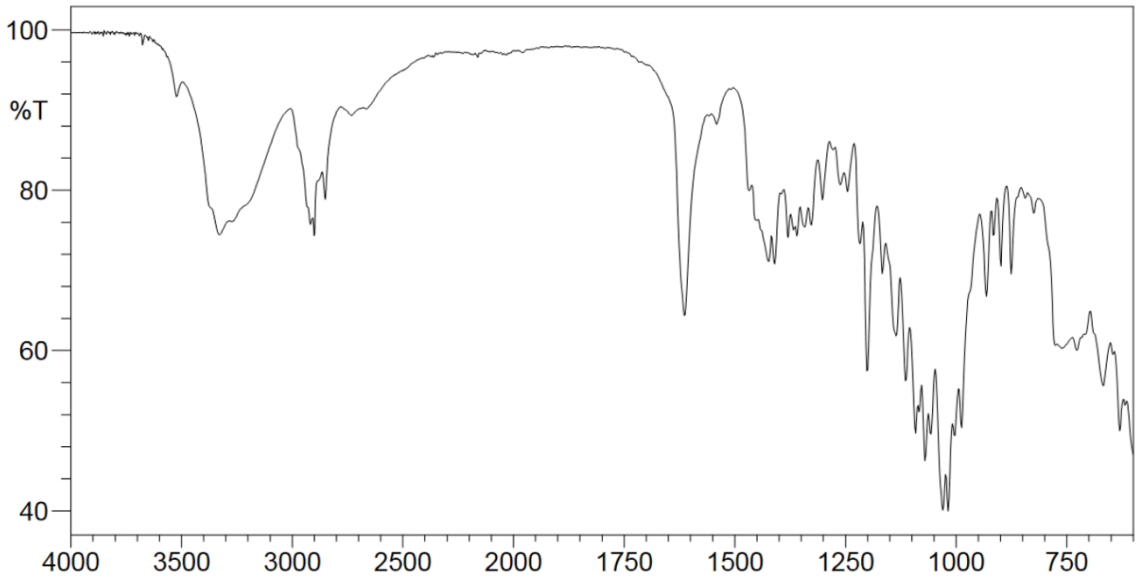


Figure 10S. FT-IR spectrum of the formulation of **Product #1.**

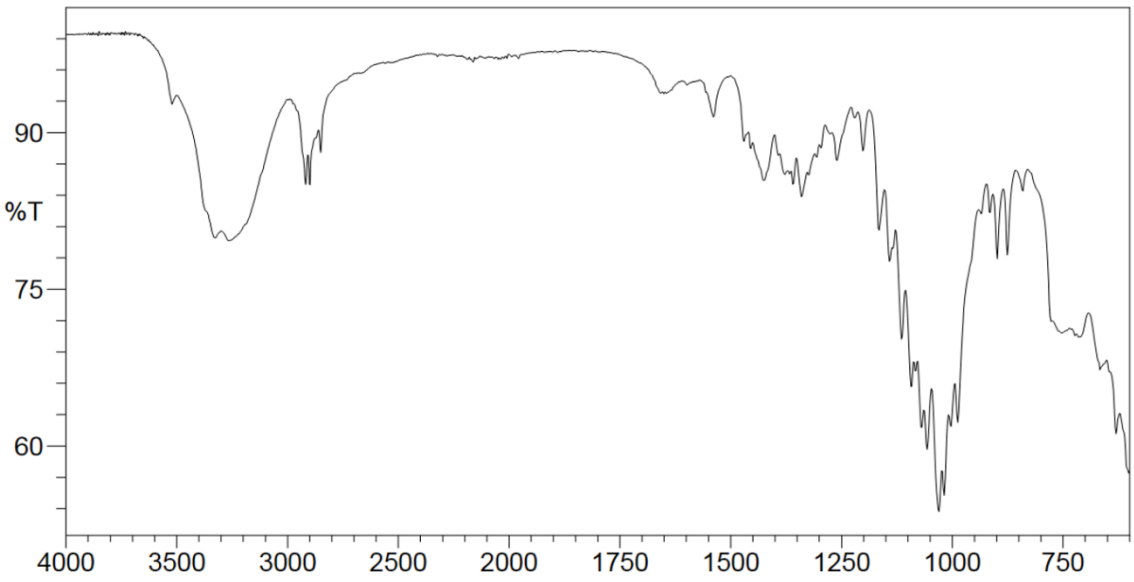


Figure 11S. FT-IR spectrum of the formulation of **Product #2.**

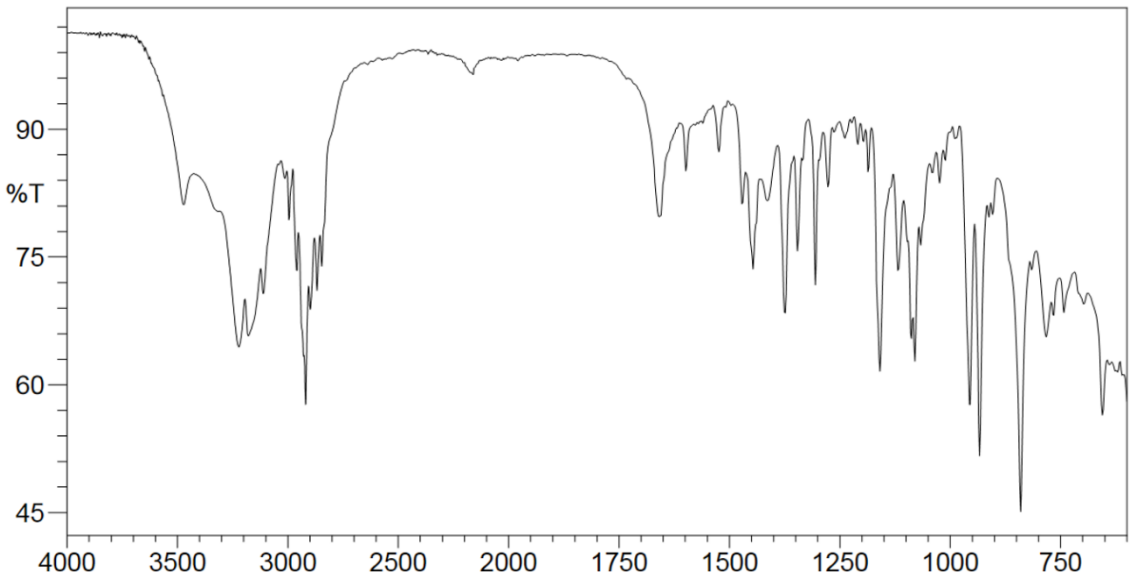


Figure 12S. FT-IR spectrum of the formulation of **Product #3.**

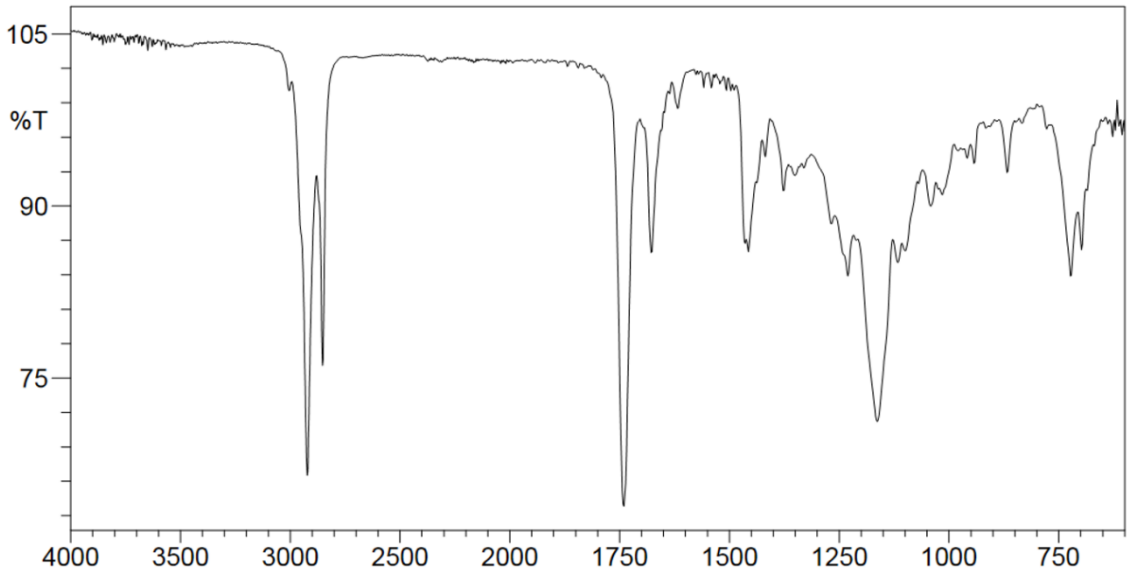


Figure 13S. FT-IR spectrum of the formulation of **Product #4.**

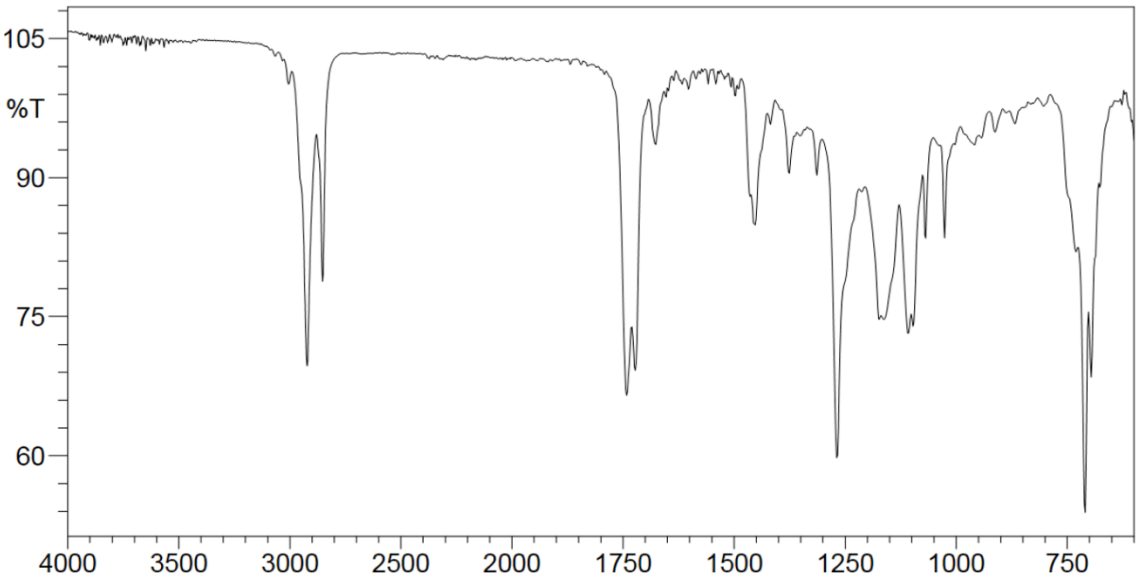


Figure 14S. FT-IR spectrum of the formulation of **Product #5.**

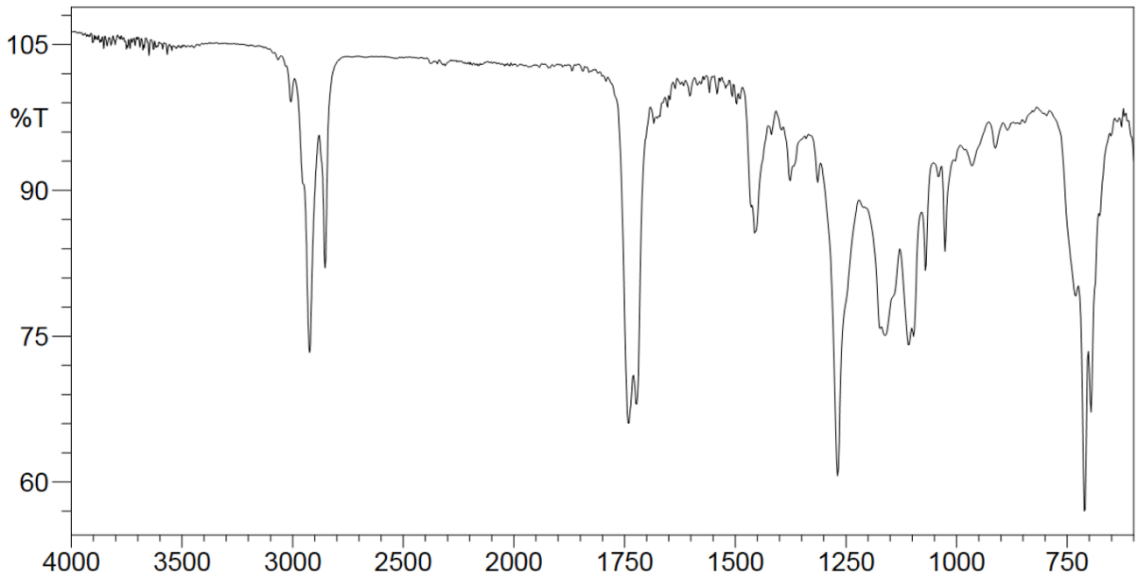


Figure 15S. FT-IR spectrum of the formulation of **Product #6.**

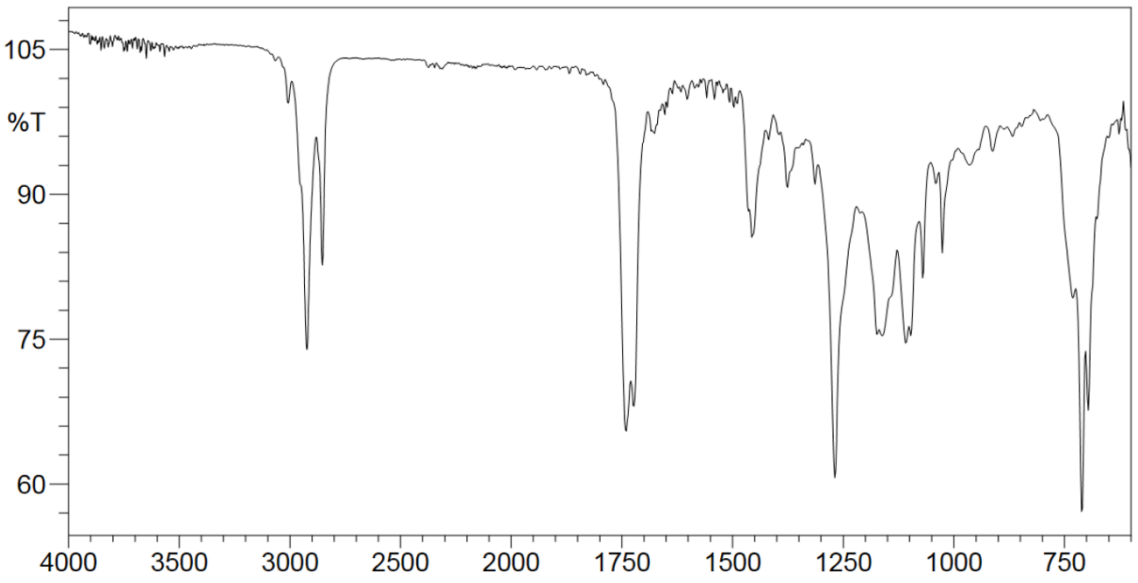


Figure 16S. FT-IR spectrum of the formulation of **Product #7.**

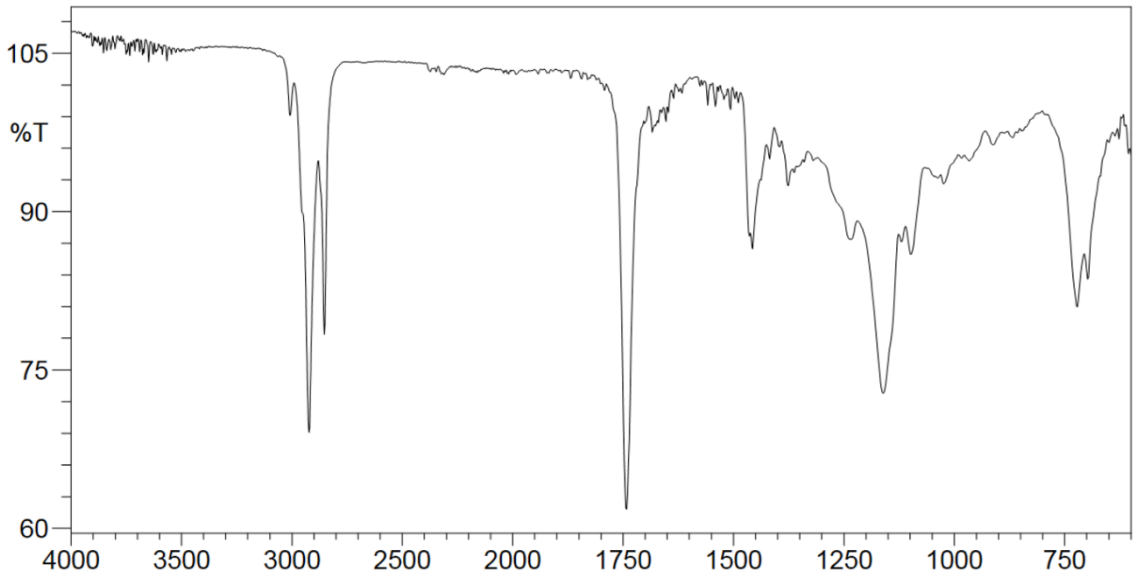


Figure 17S. FT-IR spectrum of the formulation of **Product #8.**

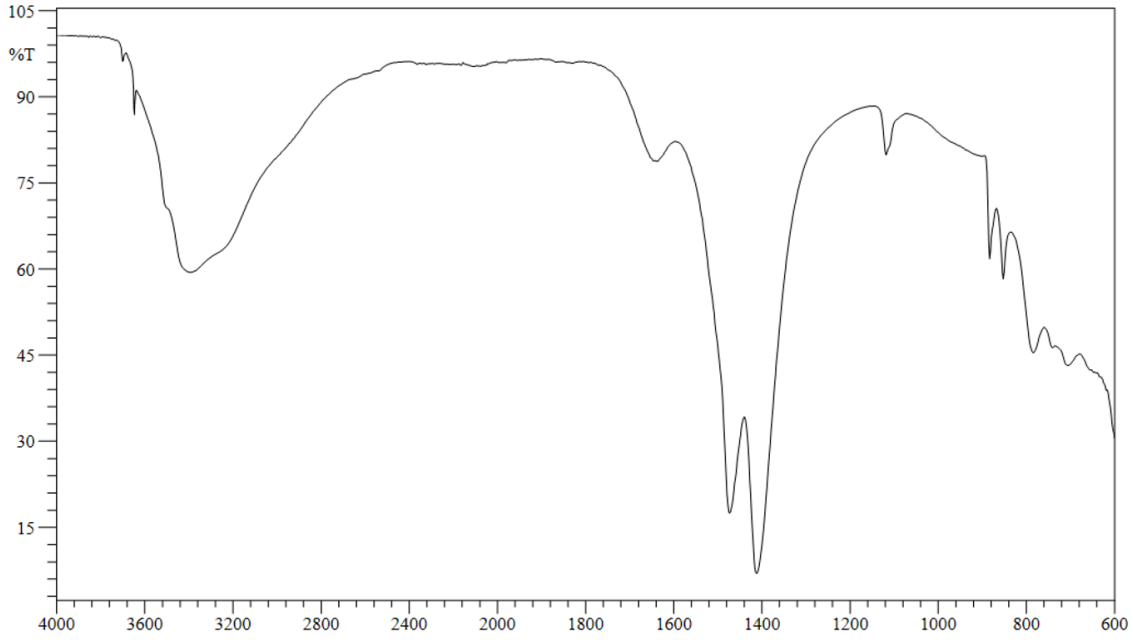


Figure 18S. FT-IR spectrum of the formulation of **Product #9.**

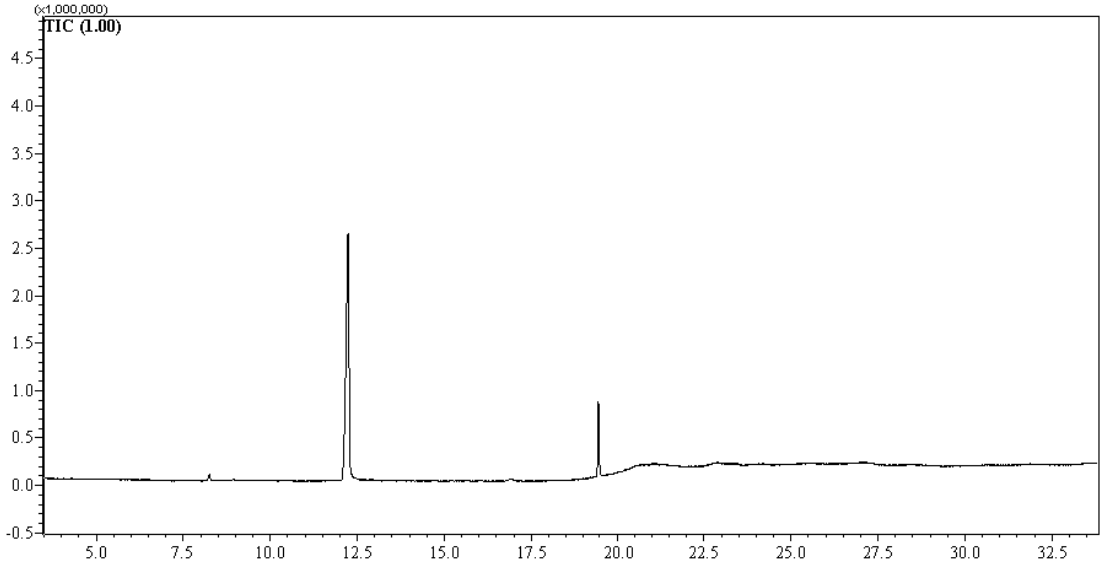


Figure 19S. GC-MS chromatogram of the formulation of **Product #1.**

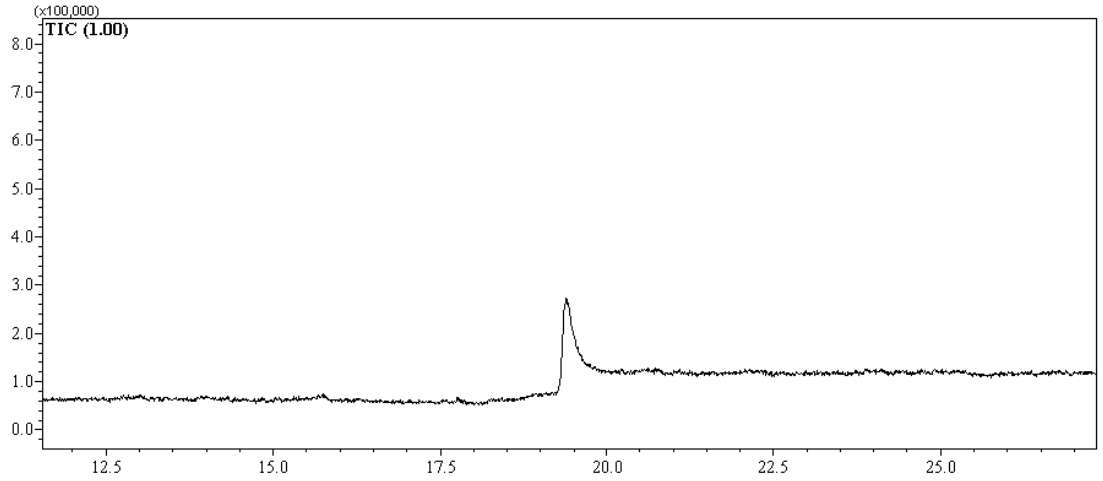


Figure 20S. GC-MS chromatogram of the formulation of **Product #2.**

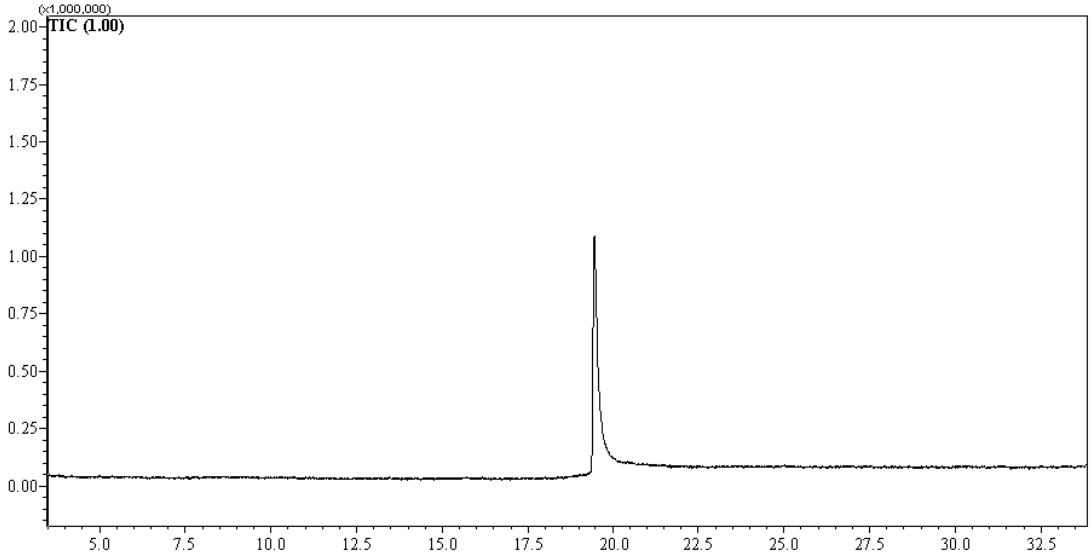


Figure 21S. GC-MS chromatogram of the formulation of **Product #3.**

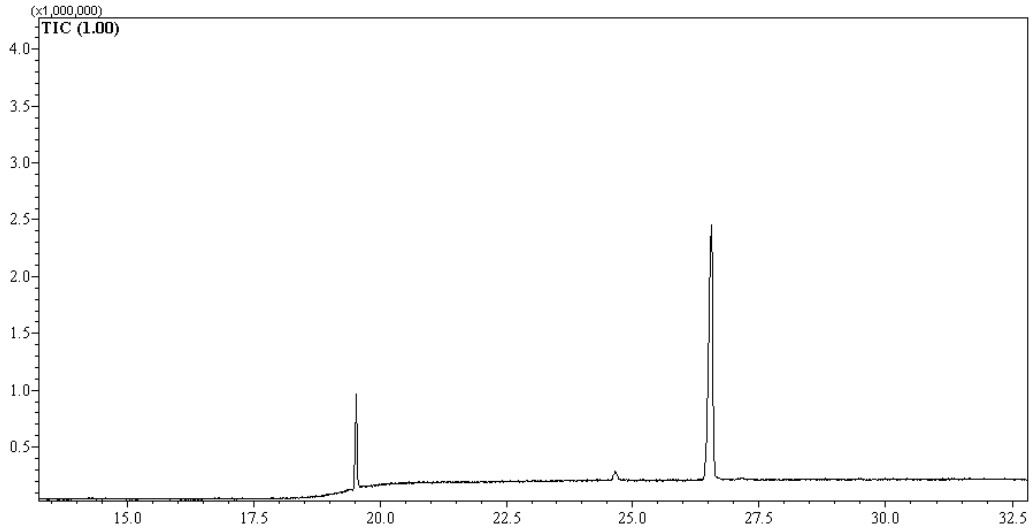


Figure 22S. GC-MS chromatogram of the formulation of **Product #5.**

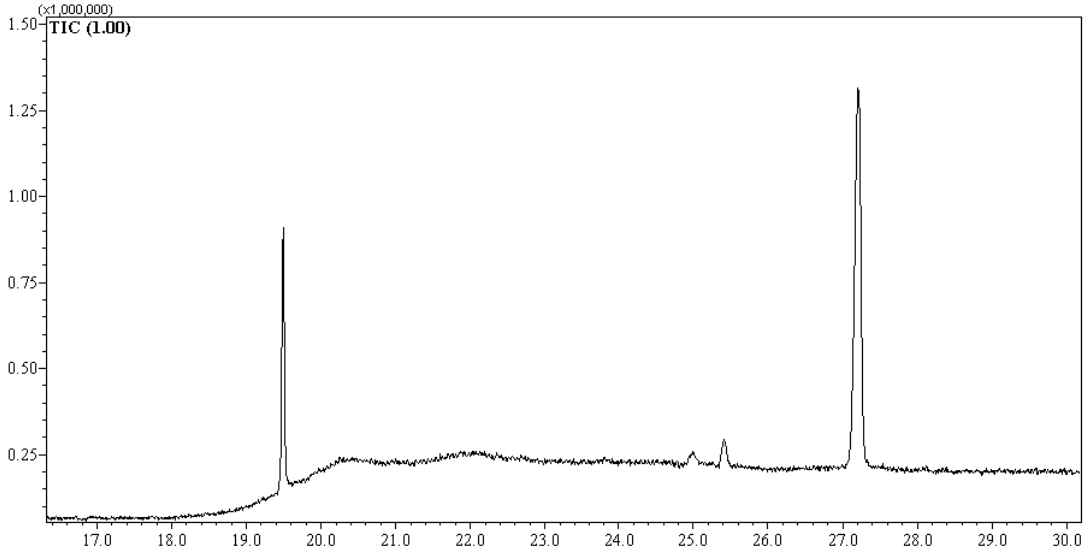


Figure 23S. GC-MS chromatogram of the formulation of **Product #6.**

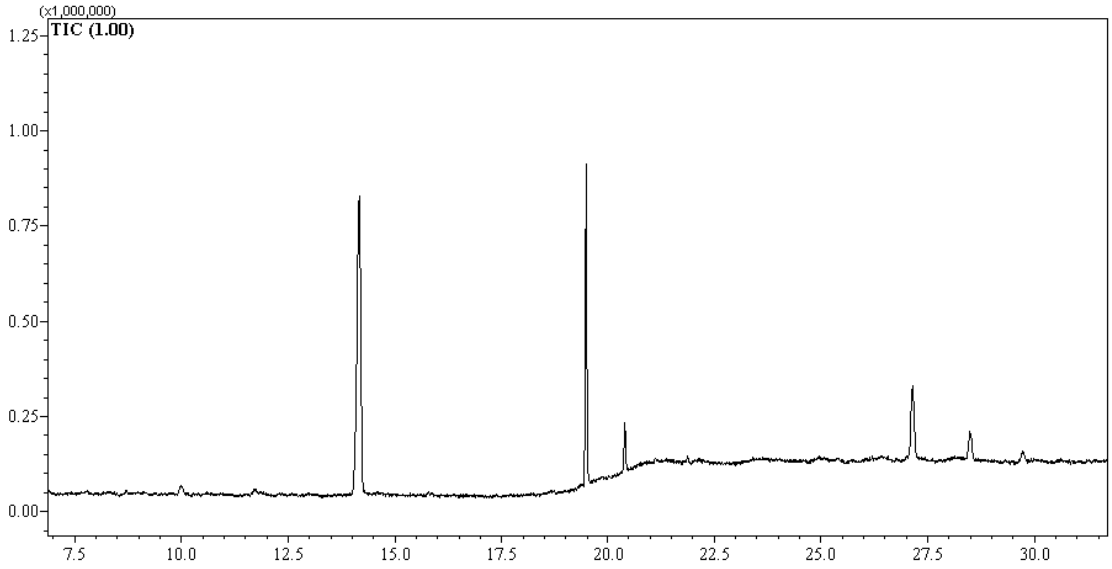


Figure 24S. GC-MS chromatogram of the formulation of **Product #7.**

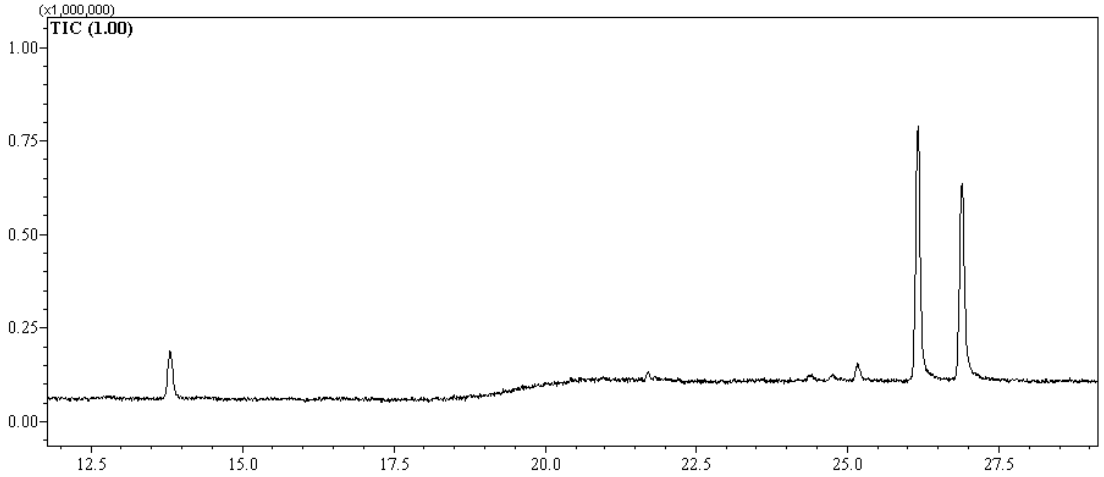


Figure 25S. GC-MS chromatogram of the formulation of **Product #8.**

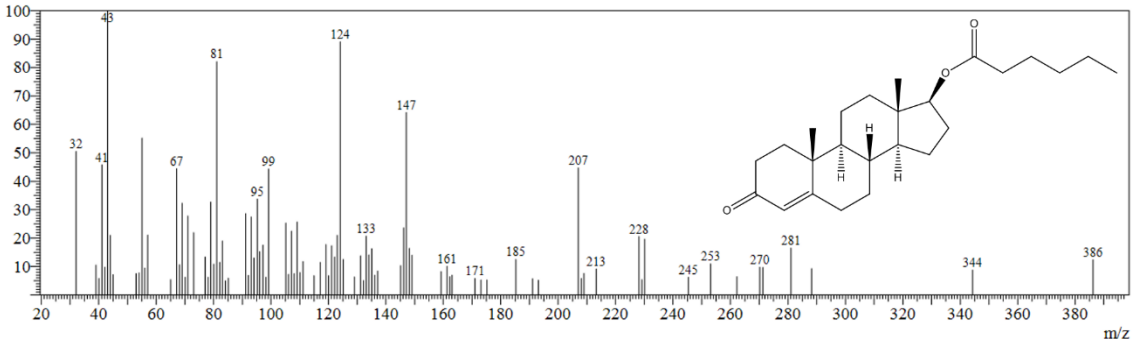


Figure 26S. Mass spectrum of testosterone caproate.

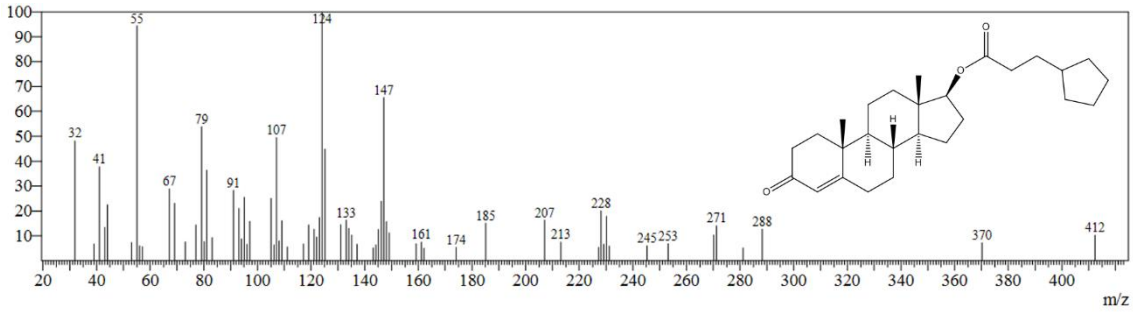


Figure 27S. Mass spectrum of testosterone cypionate.

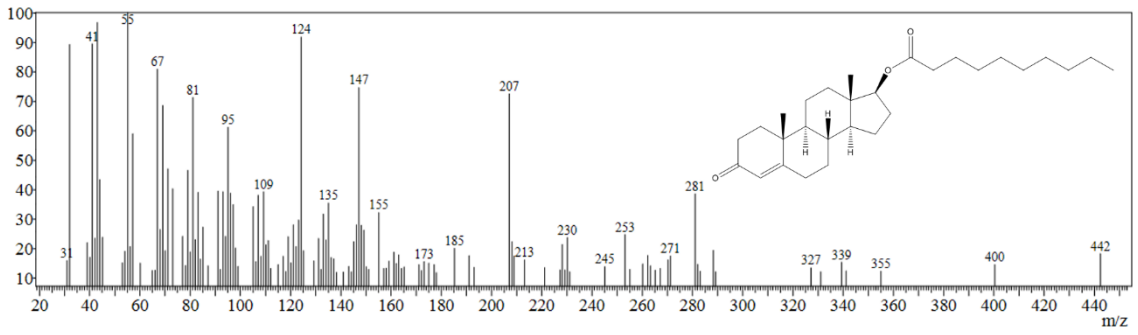


Figure 28S. Mass spectrum of testosterone decanoate.

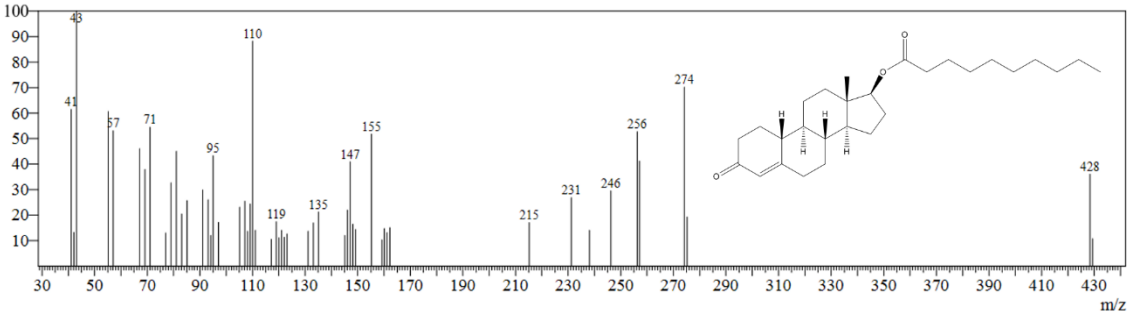


Figure 29S. Mass spectrum of nandrolone decanoate.

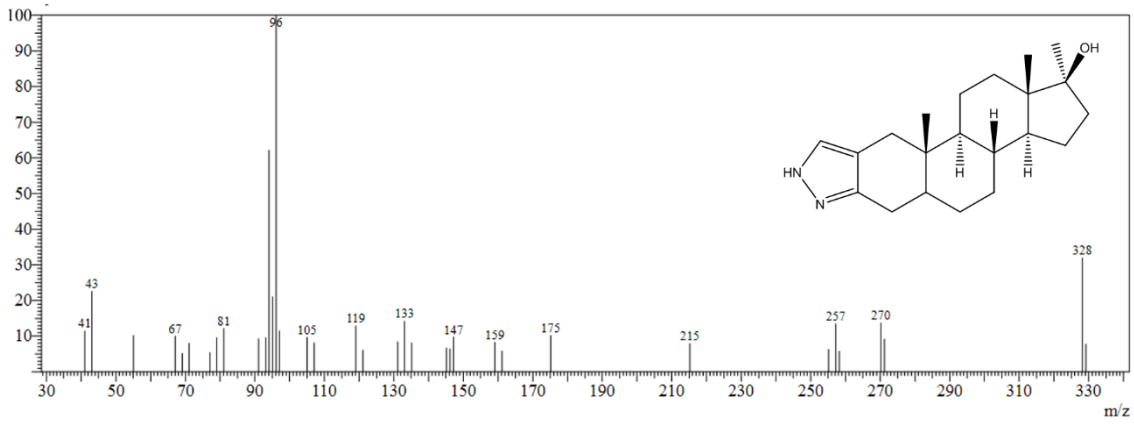


Figure 30S. Mass spectrum of stanozolol.

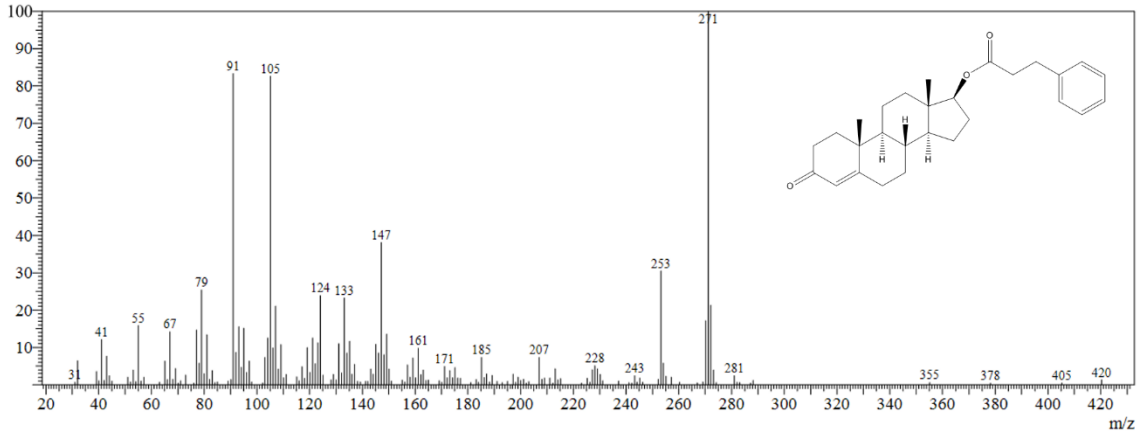


Figure 31S. Mass spectrum of testosterone phenylpropionate.

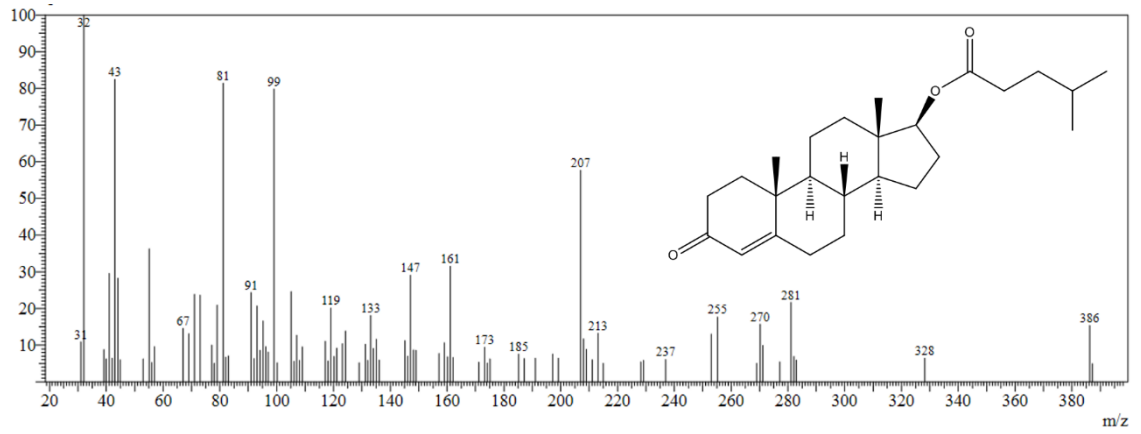


Figure 32S. Mass spectrum of testosterone isocaproate.

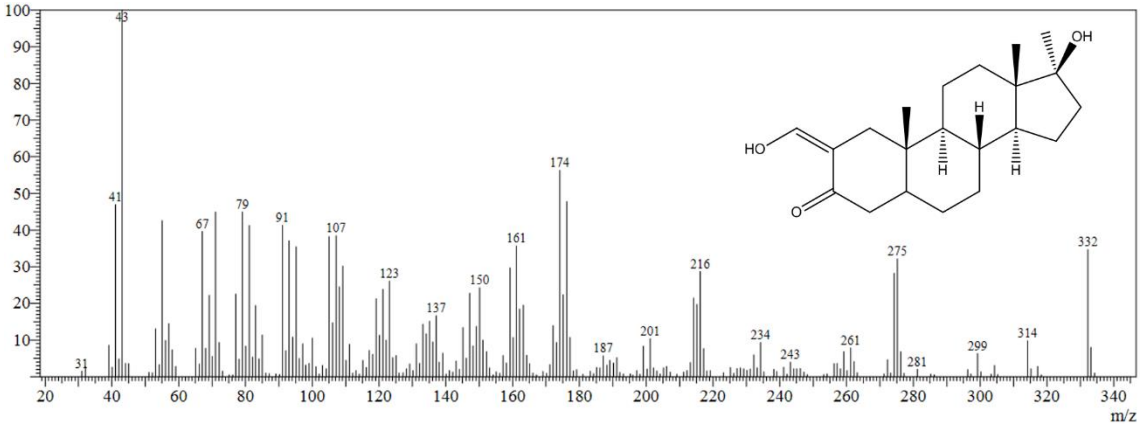


Figure 33S. Mass spectrum of oxymetholone.

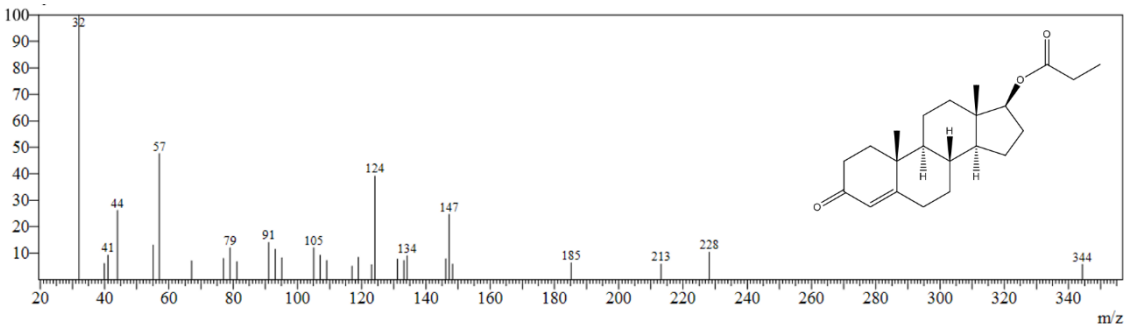


Figure 34S. Mass spectrum of testosterone propionate.

Parte IV

4. Considerações Finais

Com base nos dados expostos, se pode verificar que formulações de agentes anabólicos foram analisados através de diferentes formas de extração (líquido-líquido, assistida por banho ultrassônico, assistida por sonda ultrassônica e assistida por micro-ondas) e por ferramentas analíticas distintas (Cromatografia Gasosa, Espectrometria de Massas e Espectroscopia no Infravermelho) bem como sua toxicidade foi determinada através de testes de viabilidade celular. Nesse sentido, foi possível verificar que os procedimentos de extração influenciam consideravelmente na quantificação dos constituintes das amostras afetando sua concentração final. Além disso, se mostrou necessária a aplicação de uma sequência de técnicas analíticas para a determinação dos componentes de uma formulação a fim de se obterem resultados adequados. Também cabe salientar que a avaliação toxicológica mostrou que as formulações de agentes anabólicos podem apresentar toxicidade em concentrações iguais ou menores que 0,5 $mg.mL^{-1}$. Dessa forma, o desenvolvimento e avaliação de métodos de extração aplicados a agentes anabólicos é consideravelmente importante a fim de se obter resultados precisos em laudos periciais, por exemplo, como também para determinar potenciais efeitos tóxicos gerados pelo consumo de formulações de esteroides anabolizantes androgênicos.

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6. Anexos

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Application of differential scanning calorimetry in the analysis of apprehended formulations of anabolic androgenic steroids

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ABSTRACT

Over the past ten years, there has been a significant increase in the amount of formulations containing anabolic androgenic steroids apprehended worldwide. A considerable amount of these illicit preparations is falsified imposing a series of challenges for the analytical identification of alleged active ingredients due to the presence of interferers. In this sense, the aim of this work was to identify and quantify the active ingredient using cholesterol as internal standard in eight apprehended formulations of anabolic androgenic steroids in either tablet, capsule or injectable forms employing visual inspection and instrumental analysis of Fourier Transform Infrared Spectroscopy, Gas Chromatography — Mass Spectrometry and Differential Scanning Calorimetry. The assessed samples were kindly provided by the Brazilian Federal Police as representative samples from an apprehension made in July of 2017. Qualitatively, 25% of the analyzed materials were determined to be falsified as they were composed of excipients only while the others had the alleged active ingredient confirmed. However, after quantitative analysis, the majority of samples were placed as counterfeit materials as the active substance was found in concentrations lower than stated in the label. Preliminary visual inspection provided important information to distinguish genuine from falsified samples. It should be noted that this work was one of the few available reports to employ Differential Scanning Calorimetry in the analysis of anabolic agents, which proved to be an important complementary tool for the detection of the active ingredient, when present, along with the calorimetric profile of the formulations studied. Fourier Transform Infrared Spectroscopy and Gas–Chromatography — Mass Spectrometry were also efficient analytical tools in order to identify and to characterize substances present in fraudulent preparations.

1. Introduction

Anabolic androgenic steroids (AASs) comprise a class of numerous synthetic compounds derived from testosterone that were developed for the treatment of conditions associated to hypogonadism in males [1]. Nonetheless, these compounds are known for its illicit use among professional and recreational athletes in order to obtain enhanced sports performance and muscle development [2]. The illegal usage of steroids is associated with side effects such as cardiovascular system damage [3], liver intoxications [4] among other possible severe physical damage, and so, their commercialization is strictly controlled or prohibited in many countries [2,5].

Over the past decade, the illicit consumption of anabolic agents is increasing worldwide leading to an increase in the apprehension of these materials by law enforcement agencies. In Brazil, for instance, apprehensions of AASs triplicated between the years of 2008 and 2011. At the same time, falsifications involving formulations have also been growing and reached roughly a third of the analyzed samples [6]. Fraudulent preparations can impose health hazards associated with the abuse of anabolic agents since the formulations are manufactured under poor sanitary conditions [7], exposing illicit users to unpredictable side effects [5] or even death [8].

Generally, analysis of AASs involves visual inspection for a verification of signs of falsification followed by chemical determination using a suitable analytical technique [9]. In this sense,

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several analytical tools require an extraction procedure which may be performed employing methanol in ultrasonic bath [2,7,9]. For Gas Chromatography — Mass Spectrometry (GC–MS), a derivatization step may be required to enable the detection and increase the resolution of the chromatogram, which requires the use of derivatizing agents [8]. However, this process is optional and, in several reports [7,10,11], derivatization is not employed since it is expensive and time consuming [11].

According to previous research works, formulations of AASs are generally analyzed using GC–MS [10], High Performance Liquid Chromatography (HPLC) [2] or Fourier Transform - Infrared Spectroscopy (FT-IR) [7]. There are still other techniques that can be employed such as Direct Analysis in Real Time Ionization — Mass Spectrometry (DART-MS) [11] and Thin-Layer Chromatography [12]. It should be noted that the application of novel analytical tools with reduced sample preparation, cost and analysis time is crucial for the characterization of illicit compounds. In this sense, Differential Scanning Calorimetry (DSC) is a promising technique rarely used for the forensic determination of apprehended drugs, which may be worth exploring as it can be used to measure several physico-chemical properties of a sample with minimum work-up [13–15].

As previously discussed, the higher incidence of apprehensions of AASs formulations demands solid forensic analysis in order to determine the chemical fingerprint of the material as well as to detect falsifications. Nonetheless, the analysis of anabolic agents is still limited to chromatographic and spectroscopic techniques, which can hamper forensic determination in countries with different infrastructure, making it necessary to develop alternative techniques. Therefore, the aim of this work was to identify and quantify the active ingredients of eight formulations of AASs in oily solutions, water suspensions, tablets and capsules forms apprehended by the Brazilian Federal Police by means of visual inspection, GC–MS, FT-IR and DSC.

2. Method

2.1. Chemicals and materials

HPLC-grade methanol was obtained from J.T. Baker (Phillipsburg, USA), cholesterol ($\leq$ 99%) was purchased from Sigma-Aldrich (St. Louis, USA) and aluminum crucibles were acquired from Netzsh (Selb, Germany). The Brazilian Federal Police provided the samples (Table 1) from an apprehension made in July of 2017 in the city of Rio Grande, localized in the south region of Brazil.

2.2. GC–MS analysis

GC–MS analysis was performed following the procedure described by Coopman and Cordonnier [10]. Briefly, an aliquot of tablets finely grounded or injectables was diluted in methanol in order to achieve a nominal concentration of 1 mg/mL [10]. In cases in which the concentration of the tablets or the capsules was unknown, a fraction of 50 mg of the AASs was used for analysis. For water suspensions, the formulation was dried under nitrogen flow and the extraction was carried similarly to the other formulations.

After the extraction procedure, one μL of the samples was injected in split mode (1:25) in a GC–MS QP2010SE (Shimadzu, Kyoto, Japan) equipped with an Rtx-5MS capillary column (Restek, Bellefonte, USA) employing helium as the carrier gas. The temperature of the injection port and the transfer line were 280 °C and 300 °C, respectively. In the oven, the programmed temperature was initially 100 °C for 2 min increasing 30 °C min^{-1} to 280 °C and gradually increasing 2 °C min^{-1} to a final temperature of 310 °C, holding for 10 min. The mass spectrometer operated with ion electron ionization at 70 eV in the ion source scanning from m/z 30 to m/z 550. Identification of compounds was performed by comparison of the resulting mass spectra with the NIST-08 library while the quantitation of the analysis was performed using a calibration curve of cholesterol in concentrations of 1.25 mg/mL, 1 mg/mL, 0.75 mg/mL, 0.50 mg/mL and 0.25 mg/mL in methanol.

2.3. FT-IR analysis

For the FT-IR analysis, tablets and capsules had their content extracted following the procedure of Neves and Caldas (2017). Briefly, 50 mg of the grounded sample was dissolved in 5 mL of methanol, vortexed for 10 s, then sonicated in an ultrasonic bath (USC-1800A, Unique) for 10 min, and centrifuged for 5 min at 3000 RPM. Finally, the organic phase was recovered and evaporated under reduced pressure [9]. FT-IR analysis were performed on an Attenuated Total Reflection spectrometer with Fourier Transform (Shimadzu Prestige 21, Shimadzu, Kyoto, Japan) with a resolution of 4 cm^{-1} ranging from 4000 cm^{-1} to 600 cm^{-1}. Approximately 3 mg of the extracted material from tablets or capsules and the crude oily formulations or dried fraction of water suspensions were analyzed. For water suspensions, a preliminary drying under nitrogen flow was required.

2.4. DSC analysis

DSC analysis was carried out on a 200 F3 Maia equipment (Netzsh, Selb, Germany) previously calibrated with a standard containing indium. Initially, 3 mg of the extracted samples from tablets or capsules as well as the crude material of oily based or water suspensions were placed in aluminum crucibles and the analysis was conducted from 30 to 300 °C under constant heating of 10 °C min^{-1} and nitrogen flow at 50 mL min^{-1}. The extraction of tablets and capsules followed the same procedure described in the *FT-IR Analysis* section.

3. Results and discussion

3.1. Visual inspection

Visual inspection of the apprehended samples (Table 2) was an important preliminary procedure in order to distinguish genuine

Table 1
Details of the confiscated anabolic androgenic steroids.

Sample	Sample form	Sample appearance	Declared compound	Batch number
Product #1	Tablet	Round, white and marked with an “L”	Oxymetholone	15135
Product #2	Tablet	White and blue capsules	Unknown	Unknown
Product #3	Tablet	Round, light-pink and marked with an “L”	Unknown	Unknown
Product #4	Water suspension	White liquid	Stanozolol	416092
Product #5	Water suspension	White liquid	Stanozolol	Unknown
Product #6	Oily solution	Light yellow viscous liquid	Nandrolone decanoate	416087
Product #7	Oily solution	Light yellow viscous liquid	Testosterone propionate	0141725
Product #8	Oily solution	Dark yellow viscous liquid	Trenbolone acetate	TRE081094

Table 2
Visual inspection of the apprehended samples.

Parameter	Product #1	Product #2	Product #3	Product #4	Product #5	Product #6	Product #7	Product #8
Container	Sealed	Sealed	Sealed	Sealed	Sealed	Sealed	Sealed	Sealed
Legible information	Yes[b]	n.a.[a]	n.a.[a]	Yes[b]	Yes[b]	Yes[b]	Yes[b]	Yes[b]
Registration of trade name	No[c]	n.a.[a]	n.a.[a]	No[c]	No[c]	No[c]	No	No
Registration of manufacturer	No[c]	n.a.[a]	n.a.[6]	No[c]	No[c]	No[c]	No	No
Spelling of the active ingredient	Correct[b]	n.a.[a]	n.a.[a]	Correct[b]	Correct[b]	Correct[b]	Correct[b]	Correct[b]
Status of the active ingredient	Legal[d]	n.a.[a]	n.a.[a]	Legal[d]	Legal[d]	Legal[d]	Legal[d]	Legal[d]
Logotype and name of manufacturer	Yes	n.a.[a]	n.a.[a]	Yes	Yes	Yes	Yes	Yes
Hologram of manufacturer	No	n.a.[a]	n.a.[a]	Yes	Yes	Yes	No	No
Address of manufacturer	Yes	n.a.[a]	n.a.[a]	Yes	Yes	Yes	No	No
Statement of drug strength	Yes	n.a.[a]	n.a.[a]	Yes	Yes	Yes	Yes	Yes
Statement of dosage form	Yes	n.a.[a]	n.a.[a]	Yes	Yes	Yes	No	No
Statement of number of units	Yes	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]
Batch number and expiry date	Yes	n.a.[a]	n.a.[a]	Yes	Yes[e]	Yes	Yes	No
Size of solid materials	Round	Ellipsoid	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]
Shape of solid materials	Uniform	Uniform	Uniform	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]
Damaged or empty solid materials	No	No	No	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]
Content	Uniform solid	Uniform solid	Uniform solid	Uniform suspension	Uniform suspension	Uniform solution	Uniform solution	Uniform solution

[a] Non-available (n.a.).
[b] Information written in other language (English, Spanish or German) rather than in Portuguese.
[c] Products and trade names not registered in Brazil, but registered in Paraguay.
[d] Active ingredients legal, but placed under controlled substances list in Brazil.
[e] Product had expiry date, but did not have batch number.

from falsified samples. Generally, the format of the label information vary worldwide but, according to the World Health Organization (WHO), it should contain, for instance, trade name, active ingredient, manufacture's name, logotype and full address, batch and expiration date. In counterfeit samples, such data may be absent, adulterated or incomplete indicating that the product under review may be falsified. Moreover, other components of the sample including the recipient and its content can also be evaluated assisting in this process [16].

Throughout visual inspection, it was noted that **Product #1**, **#4**, **#5** and **#6** were manufactured by a Paraguayan laboratory that did not have a health license to distribute anabolic agents in Brazil, which means that these preparations were illegal, even if the formulation was genuine [17]. According to the Brazilian Penal Code, the illicit importation and distribution of these materials can be typified as a crime punishable from 10 to 15 years of incarceration [18]. Observation of the physical presentation of the aforementioned AASs did not show signs of falsification as several required information typical of genuine formulations were found in the packaging. It should be noted that the informed batch numbers, expiration date and physical form of the samples agreed with those found in the website of the Paraguayan laboratory indicating that the formulations were genuine.

On the other hand, **Product #8** lacked several information in its label such as expiration and manufacturing date, composition as well as origin, indicating that the sample was falsified. These preliminary observations were reinforced since the manufacturer does not officially exist and, therefore, the formulation was from a clandestine laboratory. It is worth noting that there are no manufacturers with a health license to distribute the alleged active ingredient trenbolone acetate in Brazil and, thus, the potentially falsified product is illegal within the country [17].

Nevertheless, identifying falsified pharmaceuticals throughout visual inspection may not be an easy task because fraudulent preparations can be meticulously designed to look like as genuine medicine [16]. This was the case of **Product #7** whose label had several information common to non-falsified products. Its label was written in German while the logotype and name of the supposed manufacturer were very similar to the Bayer laboratory, which could give the user a false impression that the formulation was imported. However, similarly to **Product #8**, the manufacturer of **Product #7** does not exist leading to the conclusion that the sample was counterfeit despite having an adequate label and recipient.

Product #2 and **#3** were not labeled as the samples were found in a blank recipient. Notwithstanding the absence of labeling, visual inspection of the recipient and its contents did not reveal particularities found in counterfeit formulations such as damages, cracks or unevenness of the constituents. **Product #3** had an "L" inscription on its surface, which is characteristic of a Paraguayan manufacturer that also produced **Product #1**, **#4**, **#5** and **#6,** while the **Product #2** did not have any distinguishable features that could indicate its possible origin. Nonetheless, a chemical characterization was conducted in order to confirm the presence or absence of anabolic agents in **Product #2** and **#3** as well as in the other samples visually inspected [16].

Table 2. Visual inspection of the apprehended samples.

As it could be observed in the results of visual inspection, the procedure allowed an adequate initial screening to discriminate possible falsified products from genuine formulations. However, visual inspection is not commonly used in the analysis of AASs among the literature as labeling of counterfeit and veridical samples can be very similar as well as some apprehended materials are not labeled hampering the procedure. These obstacles were observed in the current work as well as in previous research works that dealt with the visual inspection of apprehended anabolic agents. Nonetheless, a clear distinction between falsified and genuine materials was possible for most samples of this work based in the information of the label which was not observed in the previous publications found in the literature [19,20].

3.2. Chemical characterization

FT-IR analysis of **Product #7** and #**8** oil droplets (Fig. 1S) showed the same patterns of bands with similar intensities that correspond to = C—H (3008 cm^{-1}), C=O (1743 cm^{-1}), C—O stretching (1159 cm^{-1}) and CH_2 rocking vibration (721 cm^{-1}). The identified chemical groups matched several of the possible molecular vibrations of the

indicated active ingredients of both samples. However, the indicated vibrations may also be related to the oily excipient, which is in the form of long-chain alkyl esters normally found in vegetable oils [21]. Therefore, it was not possible to confirm or exclude the presence of the active ingredient employing FT-IR and further analysis were required in order to identify the formulation properly. Infrared spectrum of all samples can be seen in the *Supplementary Information* section. Based upon the spectroscopic analysis that did not clearly established the presence of the supposed active ingredients, DSC analysis of both samples (Fig. 1) was conducted. The use of DSC allowed the identification of a certain compound due to its distinctive melting point (MP) and other physico-chemical properties in the thermogram [13].

Similarly to the FT-IR results, thermograms from both **Product #7** and **#8** were identical, strongly indicating that these materials were constituted of the same compounds. As shown in Fig. 1, there were no events related to the MPs of testosterone propionate (118–122 °C) or trenbolone acetate (94–97 °C) indicating that these compounds were not present in any of the samples [22]. The first exothermic event occurred at 175.0 °C, which was probably related to the partial oxidation of the oily formulation while the exothermic event at 232.1 °C can be associated with the vaporization of the sample. These results are very similar to a previous work reported in the literature [23] related to the analysis of long-chain alkyl esters of soybean oil performed under identical DSC conditions. Comparison of the results strongly indicated that none of the samples had active ingredients but were composed only of vegetable oil.

GC–MS analysis confirmed the results indicated by visual inspection and DSC as both testosterone propionate and trenbolone acetate were not detected in the formulations of **Product #7** and **#8**, respectively. In this order, both apprehended samples were considered falsifications categorized as formulations only composed of excipients according to the WHO guidelines. It should be noted that this is one of the most common form of counterfeit accounting for approximately 40% of falsifications involving pharmaceutical products [24]. Although only composed of vegetable oil, the samples were manufactured in inadequate sanitary conditions and poor quality control which could implicate in several health hazards to a user that include, for instance, generalized infections and muscular atrophy [2].

On the other hand, spectroscopic analysis of **Product #3, #4** and **#5** (Fig. 2S) indicated the presence of stanozolol due to the occurrence of characteristic vibrations associated with the compound including N—H (3473 cm^{-1} and 1523 cm^{-1}), C=N (1660 cm^{-1}), O—H (3112 cm^{-1}) and =C—H (3014 cm^{-1}). The presence of stanozolol in the samples was also observed in DSC analysis (Fig. 2), in which it was possible to observe the MP of one of its crystals forms (151.2 °C) in agreement with the literature (155 °C). Other calorimetric characteristics such as dehydration (85.9 °C) and degradation of the molecule (219.4 °C) were also observed in the thermogram [22].

GC–MS analysis of **Product #3, #4** and **#5** showed only one peak that was identified as stanozolol according to the library of the equipment. In the mass spectra of these AASs (Fig. 3S) the molecular ion of m/z 328.25 proximate to the exact mass of stanozolol (m/z: 328.25) was identified and, thus, confirming its presence in the samples. GC–MS results of all analyzed materials can be seen in the *Supplementary Information* section. Stanozolol entered the US market in the 1960s as aqueous suspensions or tablets and was mainly used for the treatment of osteoporosis and protein deficiency disorders [12]. The anabolic agent was later banned in the US due to its hepatotoxicity, however its illicit use continued since it cannot be converted to estrogen in the organism and, thus, avoid some common anabolic disorders such as water retention and ginecomastia [25].

For **Product #6**, FT-IR analysis of the oily formulation (Fig. 4S) indicated the presence C=O (1719 cm^{-1}), C—O (1174 cm^{-1}), C=C (1673 cm^{-1}), C=C related to the presence of monosubstituted aromatic rings (709 cm^{-1}), and stretches of long carbon chains (606 cm^{-1}) which would be in agreement either with the presence of active ingredient or its excipients. DSC analysis of the oily solution (Fig. 3) revealed one endothermic event at the onset temperature of 201.8 °C that was related to the melting and vaporization of the oily formulation. It was not possible to observe the MP of nandrolone decanoate (118 °C) as well as other particular physico–chemical properties due to the high affinity of the components in the formulation [22].

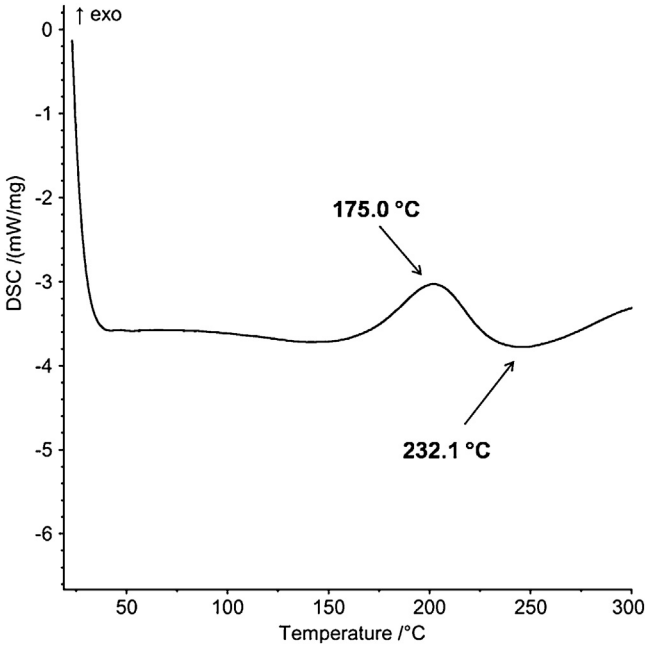

Fig. 1. DSC thermogram of the oily formulation of **Product #7** and **#8**.

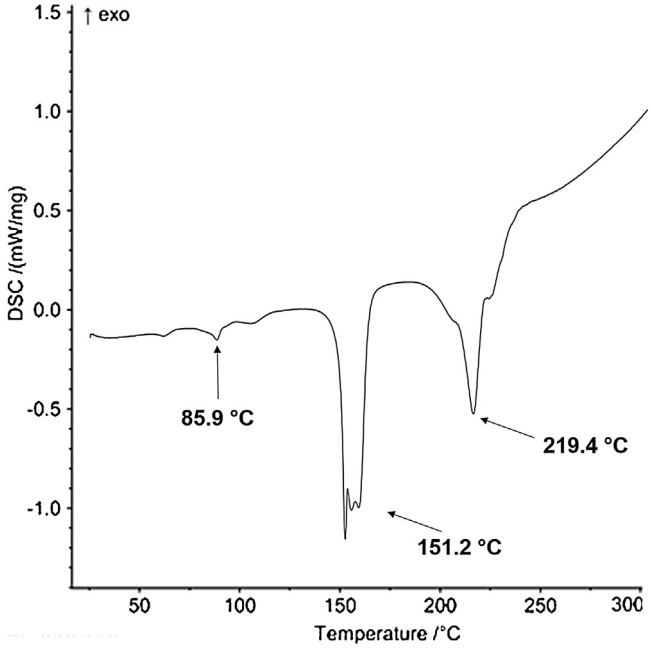

Fig. 2. DSC thermogram of the content from **Product #3, #4** and **#5.**

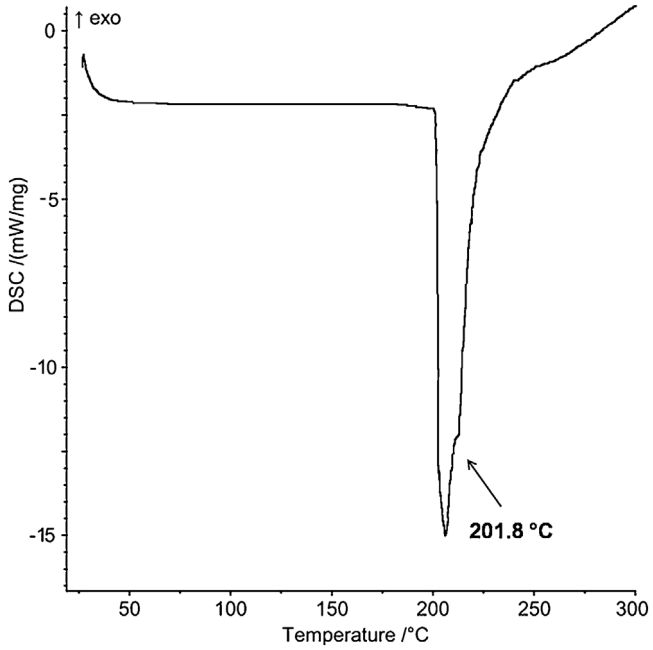

Fig. 3. DSC thermogram of the oily formulation of **Product #6**.

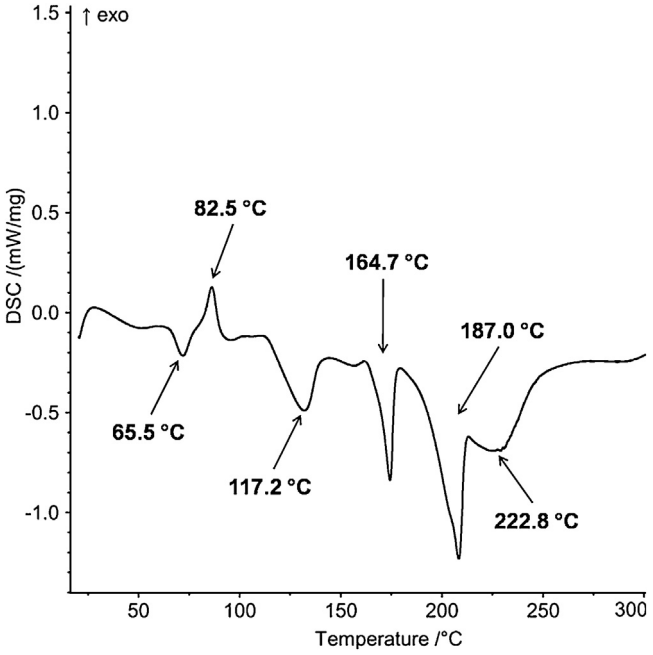

Fig. 4. DSC thermogram of the extracted content of **Product #1**.

GC–MS analysis (Fig. 5S) detected eight peaks for **Product #6** with a predominance of benzyl benzoate, ethyl oleate and nandrolone decanoate, and a peak confirming (**Fig 6S**) the presence of the active ingredient (nandrolone decanoate; m/z 428) and the excipients. Qualitatively, the sample was not considered a counterfeit, which was further confirmed by a quantitative analysis. Nandrolone decanoate acts as a prodrug releasing nandrolone in the organism up to 3 weeks after injection and is therapeutically used to treat osteoporosis and sarcopaenia. However, due to a higher ratio of anabolic: androgenic activity compared to other AASs, nandrolone decanoate is widely used illicitly [26,27].

Benzyl benzoate is extensively used as an excipient in injectable anabolic agents in order to decrease the viscosity of the oily solution as well as to prevent crystallization of steroids. The substance is also used in food preservatives, perfume solvents, flavorings, repellents and insecticides, but it can cause serious adverse effects such as skin irritation, dermatitis, anaphylactic shock and seizures when used in high concentrations. Ethyl oleate was found among other substances in the formulation, which is a common vehicle for injectables although not approved by the Food and Drug Administration since there are no studies on its biosafety [27]. In addition to this compound, other fatty acid ethyl esters such as ethyl palmitate, stereate and laurate are commonly used in oily-based AASs in order to prolong the effect of the steroid in the organism [26].

FT-IR analysis of the extracted content from **Product #1** (**Fig 7S**) indicated the presence of oxymetholone due to distinguishing bands of O—H (3331 cm^{-1}), C=O (1613 cm^{-1}), C—O (1199 cm^{-1}) and C = C (931 cm^{-1}) bands. It should be noted that the C=O stretch of this compound is very characteristic since it undergoes tautomerization, which shifts the appearance of the carbonyl group to lower wavenumbers. DSC analysis (Fig. 4), indicated endothermic events at 65.5 °C and 117.2 °C associated with solid-solid transitions followed by an exothermic event related to crystallization (82.5 °C) as well as endothermic events related to the MP at 164.7 °C and 187.0 °C possibly due to the polymorphism in the material. These results were close to the reported in the literature (172–180 °C) and also indicated the presence of oxymetholone [22]. It can also be observed that in the endothermic event found at 222.8 °C the solid begins to suffer a degradation process.

GC–MS analysis of **Product #1** showed the presence of one peak whose mass spectra (**Fig 8S**) revealed a molecular ion of 332.25 related to the active ingredient. Oxymetholone belongs to the 17α alkylated group of AASs and, therefore, cannot be inactivated by the first-pass liver metabolism when consumed orally. However, the alkylation in the 17α position also enhanced the risk of hepatotoxicity of oxymetholone which made the compound controlled worldwide [26,28].

Lastly, for **Product #2** bands such as O—H (3512 cm^{-1}) and C = O (1716 cm^{-1}) were observed (**Fig 9S**). Since the apprehended sample was unlabeled, the observed bands could not be associated to the stated anabolic agent as these vibrations can be seen in innumerous AASs as well as other compounds. DSC analysis (Fig. 5) showed an initial exothermic event at the onset temperature of 82.2 °C related to the crystallization of the solid. Furthermore, an endothermic event at 238.0 °C was related to a MP that was close to the reported in the literature for oxandrolone (235 °C) indicating the presence of the active ingredient in the sample [22]. GC–MS analysis (**Fig 10S**) confirmed oxandrolone (m/z 306).

The overall results of the spectroscopic analysis showed that the identification of the active ingredients was difficult due to the interference of excipients. Moreover, in cases in which the active ingredient was not stated in the label, it was not possible to associate the bands to a particular anabolic agent. In this sense, it was necessary to complement the results with an evaluation by DSC in which it was possible to identify most of the AASs stated or not in the label with little sample preparation, low cost and quick analysis [29]. Given the results, the association of DSC with another screening technique can be used to identify the active ingredient in questioned formulations which could serve as an is important tool for a wider range of laboratories with different instrumentation capacity [16].

For quantitation purposes, an analytical curve containing cholesterol (R^2 = 0.9999) was employed for the procedure. The use of cholesterol can be a feasible approach to quantify synthetic androgens due to their chemical structure similarity overcoming certain limitations or the inability to acquire pure standards. It is

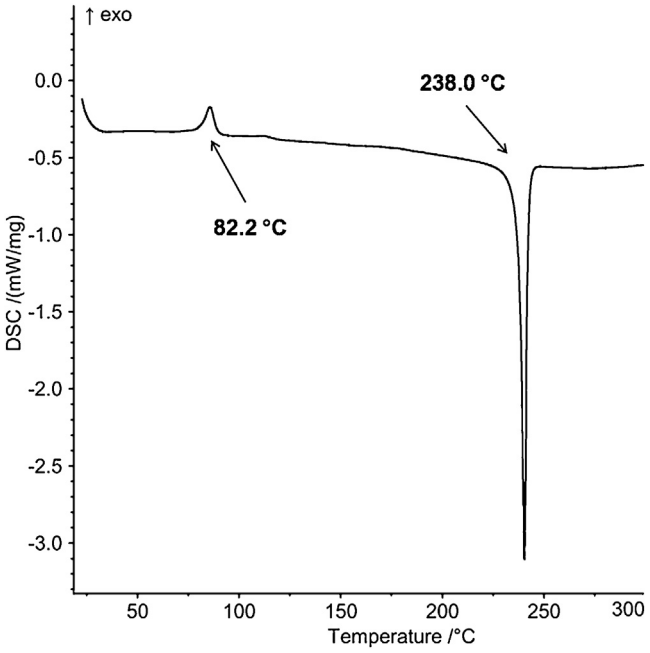

Fig. 5. DSC thermogram of the content of **Product #2**.

worth noting that there could be minor differences in the obtained concentration of the studied active ingredients as cholesterol and anabolic agents do not share the same chemical structure and chromatographic characteristics.

Quantification of active ingredients in the samples (Table 3) showed that most of the apprehended formulations of AASs were counterfeit as **Product #6** was the only formulation to achieve the concentration stated in the label. For the other anabolic agents, the active substance, when present, was generally found in concentrations equal to half or less than declared on the label. Neves and Caldas (2017) also observed that, after a quantitation step, the rate of counterfeit formulations reached approximately 50% and was higher than the falsifications detected by qualitative analysis that were found in 25% of the preparations [9]. It should be noted that the falsification rate found after quantitative analysis was higher in the current work than the previous research, which can be associated to the limited number of samples evaluated.

4. Conclusion

According to the obtained results, it was observed that two of the eight apprehended products were a counterfeit composed of excipient-only, while for the other six, the active ingredients were detected. Among the samples with the active ingredient, 5 out of 6 formulations did not reach the concentrations stated in label, which classify these preparations as falsified materials. Moreover, the application of sequential analytical tools was required for the adequate identification of the samples. It was demonstrated that visual inspection allied with an instrumental characterization of the AASs were crucial procedures in order to characterize and detect falsifications. Finally, DSC proved to be a feasible complementary analysis by providing events related to characteristic physico-chemical properties that assisted in the identification of the alleged active ingredients in the samples. Preliminary visual inspection associated to FT-IR, DSC or GC–MS successfully allowed for the detection of anabolic agents and, therefore, could be used in laboratories of different infrastructure for the forensic analysis of apprehended formulations.

Table 3
Results of the quantitative analysis of the apprehended samples.

Sample	Stated concentration	Experimental concentration
Product #1	50 mg/tablet	14.14 ± 0.29 mg/tablet
Product #2	Unknown	9.56 ± 1.05 mg/capsule
Product #3	Unknown	6.11 ± 0.64 mg/tablet
Product #4	50 mg/mL	23.51 ± 0.47 mg/mL
Product #5	50 mg/mL	18.19 ± 1.42 mg/mL
Product #6	200 mg/mL	205.69 ± 15.85 mg/mL
Product #7	100 mg/mL	nd
Product #8	100 mg/mL	nd

Non-detected (nd); Results expressed as mean ± standard deviation (n = 3).

Conflict of interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Lucas Moraes Berneira: Conceptualization. **Samantha Coelho de Freitas**: Conceptualization. **Alexandre de Mattos Machado**: Conceptualization, Methodology. **Claudio Martin Pereira de Pereira**: Conceptualization, Funding acquisition. **Marco Aurélio Ziemann dos Santos**: Conceptualization, Supervision.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.forsciint.2018.12.022.

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