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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Estudo dos óleos essenciais de espécies de *Pelargonium* (Geraniaceae) e de suplementos alimentares e compostos emagrecedores contendo 1,3-dimetilamilamina: uma abordagem química, antifúngica e forense

MAÍRA KERPEL DOS SANTOS

Porto Alegre, 2018

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Tese apresentada por **Maíra Kerpel dos Santos** para obtenção do TÍTULO DE DOUTOR em Ciências Farmacêuticas

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Epígrafe

“Persistir em meio às dificuldades e contrariedades da vida, é o preço justo daqueles que desejam vencer, crescer e se fortalecer”.

Joel Beuter

RESUMO

A 1,3-dimetilamilamina (DMAA) é um estimulante que passou a ser adicionada aos suplementos alimentares e compostos emagrecedores a partir de 2006, sendo amplamente consumida por atletas e militares americanos. No entanto, após relatos de toxicidade a DMAA foi proibida por agências regulatórias do Brasil e Estados Unidos. Porém, mesmo após a sua proibição, a DMAA ainda pode ser encontrada em suplementos alimentares. A sua origem foi relacionada ao óleo essencial de *Pelargonium graveolens*, e, no entanto, inúmeros autores questionaram os resultados originais e a sua origem natural. Adicionalmente, os óleos essenciais de espécies de *Pelargonium* tiveram a sua atividade antimicrobiana reportada frente a bactérias e fungos. Assim, considerando os aspectos abordados, este trabalho teve como objetivo determinar a presença de DMAA nos óleos essenciais de *Pelargonium* spp. por GC-MS, DART-MS/MS e LC-MS/MS; assim como nas folhas das mesmas espécies, utilizando a extração por *headspace*, previamente otimizada, seguida de análise por GC-MS. Também se propôs a investigar a atividade antifúngica dos óleos essenciais de *P. graveolens* de diferentes origens e desenvolver uma formulação contendo uma nanoemulsão do óleo para o tratamento de candidíase vaginal. Por fim, teve como objetivo desenvolver metodologia de *screening* para avaliar a presença de DMAA e outros estimulantes em suplementos alimentares apreendidos, através de DART-MS/MS. Os resultados revelaram que a DMAA não está presente nos óleos essenciais de diferentes espécies de *Pelargonium* spp. obtidos por hidrodestilação, do Rio Grande do Sul. Após a otimização através de desenho experimental, a técnica de *headspace* provou ser eficaz na extração dos constituintes voláteis presentes nas folhas e, no entanto, a DMAA não foi detectada, assim como nos óleos essenciais comerciais de *P. graveolens* do Brasil, China, Egito, África do Sul, Albânia e Ilhas Reunião. Os óleos essenciais apresentaram atividade antifúngica frente às cinco espécies de *Candida*. Ainda, este efeito antifúngico apresentou melhores resultados com a nanoformulação contendo o óleo essencial. A análise de *screening* por DART-MS/MS se mostrou eficaz na detecção de DMAA, efedrina, sinefrina, cafeína, sibutramina e metilfenidato, em amostras de suplementos alimentares apreendidos, apresentando resultados positivos para todos os estimulantes. Com base nos resultados obtidos e nos objetivos propostos, verificou-se que mesmo após a

utilização de três técnicas analíticas distintas e uma nova alternativa para extração dos constituintes voláteis, a DMAA não foi econtrada nos óleos essenciais e nas folhas das espécies de *Pelargonium*, corroborando com outros estudos realizados, e indicando que a sua origem não é natural nestas espécies. A formulação final contendo a nanoemulsão com o óleo essencial apresentou atividade antifúngica superior a do óleo essencial livre. As análises das amostras apreendidas mostraram que mesmo após a sua proibição pelas agências regulatórias, os suplementos contendo DMAA e outros estimulantes ainda são comercializados, representando um grande risco para a saúde dos seus usuários.

Palavras chave: 1,3-dimetilamilamina, *Pelargonium*, óleo essencial, suplementos alimentares, antifúngico.

ABSTRACT

1,3-dimethylamylamine (DMAA) is a stimulant that started to be added in dietary supplements and weight loss compounds since 2006 and is widely consumed by athletes and the USA army. However, after reports of toxicity DMAA has been banned by regulatory agencies in Brazil and United States. However, even after its prohibition, DMAA still can be found in dietary supplements. Its origin was related to the essential oils of *Pelargonium graveolens*, and, however, many authors questioned the results and its natural origin. In addition, the essential oils of species of *Pelargonium*, had their antimicrobial activity reported against bacteria and fungi. Considering the aspects mentioned, this work aimed to determine the presence of DMAA in the essential oils by GC-MS, DART-MS/MS and LC-MS/MS; as well as in the leaves of the same species using the headspace extraction, previously optimized, followed by analysis through GC-MS. It has also been proposed to investigate the antifungal activity of essential oils of *P. graveolens* from different origins and develop a formulation containing an oil nanoemulsion for the treatment of vaginal candidiasis. Finally, it aimed to develop a screening method to evaluate the presence of DMAA and other stimulants in seized dietary supplements by DART-MS/MS. The results showed that DMAA is not present in the Rio Grande do Sul's essential oils of *Pelargonium* spp. obtained by hydrodistillation. After optimization through experimental design, the headspace technique proved to be effective in extracting volatile constituents present in the leaves and, however, DMAA was not detected, as well as in commercial essential oils of *P. graveolens* from Brazil, China, Egypt, South Africa, Albania and Reunion Islands. The essential oils presented antifungal activity against five Candida species. Furthermore, this antifungal effect presented better results with the nanoformulation containing essential oil. DART-MS/MS screening was effective in detection of DMAA, ephedrine, synephrine, caffeine, sibutramine and methylphenidate in seized dietary supplements, showing positive results for all stimulants. Based on the results obtained and proposed objectives, it was verified that even after using three different analytical techniques and a new alternative for volatile constituents extraction, DMAA was not found in essential oils and leaves of *Pelargonium* species, corroborating with other studies carried out, and indicating that its origin is not natural in these species. The final formulation containing the nanoemulsion with the essential oil had antifungal activity superior compared to

dispersed essential oil. The analysis of seized samples showed that even after its prohibition by regulatory agencies, supplements containing DMAA and other stimulants are still commercialized, representing a major health risk for their users.

Keywords: 1,3-dimethylamylamine, *Pelargonium*, essential oil, dietary supplements, antifungal.

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DMAA	1,3- Dimetilamilamina
GC-MS	Cromatografia gasosa acoplada ao detector de massas
GC-FID	Cromatografia gasosa associada ao detector de ionização em chamas
GC-NPD	Cromatografia gasosa associada ao detector nitrogênio fósforo
LC-DAD	Cromatografia Líquida associada ao detector de arranjo de diodos
LC-MS/MS	Cromatografia Líquida acoplada ao detector de massas com análise sequencial
LC-QTOF/MS	Cromatografia Líquida acoplada ao detector de massas do tipo quadrupolo e TOF
DART-MS/MS	Análise direta em espectrometria de massa em tempo real com análise sequencial
WADA	*World Anti-doping Agency*
ANVISA	Agência Nacional de Vigilância Sanitária
FDA	Food and Drug Administration
DSHEA	*Dietary Supplement Health Education Act*
MIC	Concentração Inibitória Mínima
SPME	Microextração em fase sólida
HS	*Headspace*
BBD	*Box Behnken Design*

APRESENTAÇÃO

De acordo com as normas vigentes no Estatuto do Programa de Pós-Graduação em Ciências Farmacêuticas, da Universidade Federal do Rio Grande do Sul, a presente tese foi redigida na forma de capítulos, para a melhor organização e discussão dos resultados obtidos em cada etapa. Assim, este trabalho encontra-se dividido da seguinte forma:

1. Introdução, contendo a caracterização e justificativa do estudo proposto;

2. Objetivos, geral e específicos;

3. Revisão Bibliográfica;

4. Manuscrito I: *"Determination of 1,3-dimethylamylamine in different species of Pelargonium using a multi-analytical approach*".

5. Manuscrito II: *"DART-MS*/MS *screening for the determination of 1,3 - Dimethylamylamine and undeclared stimulants in seized dietary supplements from Brazil".*

6. *Manuscrito III: "Antifungal activity and development of a new nanoemulsion-hydrogel carrying essential oil of Pelargonium graveolens against vaginal candidiasis".*

7. Discussão geral, visando um entendimento global de todas as etapas realizadas no trabalho;

8. Conclusões;

9. Referências.

SUMÁRIO

1. INTRODUÇÃO

A falta de regulamentação e evidências científicas a respeito da eficácia e segurança dos suplementos alimentares não impediu o crescimento do seu consumo pela população na última década (HU et al., 2016; LOPEZ-AVILA; ZORIO, 2013; STICKEL; SHOUVAL, 2015). Entre as substâncias estimulantes empregadas de modo frequente nos suplementos, recentemente tem se destacado a 1,3-dimetilamilamina (DMAA). Os produtos contendo DMAA chegaram a atingir um total de 440 milhões de vendas desde 2007 nos Estados Unidos e foram largamente utilizados pelos militares americanos e atletas, até a sua proibição pelo Food and Drug Administration (FDA), em 2013 (DOLAN; GATCH, 2015; FOLEY et al., 2014; RODRICKS; LUMPKIN; SCHILLING, 2013; STRIPES, 2012; USA, 2013).

Também conhecida como 4-metil-2-hexanamina; 2-amino-4-metilhexano; 1,3-dimetilpentilamina, 2-amino-4-metillhexano, ou geranamina, a DMAA é uma amina alifática que teria possível origem natural em pequenas quantidades no óleo essencial da espécie *Pelargonium graveolens,* conhecida popularmente como gerânio (LI, J. S.; CHEN; LI, Z. C., 2012; PING Z., 1996). A DMAA surgiu na década de 40, inicialmente como descongestionante nasal patenteado pela indústria farmacêutica Eli Lilly, sob o nome "*Forthane*®" (SHONLE, H.A., ROHRMANN, 1944). Na década de 70, o medicamento foi retirado do mercado norte americano por razões desconhecidas (DOLAN; GATCH, 2015; PAWAR et al., 2014). Com propriedades estimulantes, em 2006, a DMAA reapareceu no mercado como constituinte dos suplementos alimentares (GAUTHIER, 2013; GEE et al., 2012).

A primeira evidência da presença de DMAA na espécie de *P. graveolens* foi publicada no trabalho de PING e colaboradores (1996), que realizaram a determinação dos constituintes químicos do óleo extraído das folhas e raízes da planta através de hidrodestilação. No entanto, a quantidade encontrada (0,6%) está muito abaixo dos teores expressos nos suplementos alimentares comerciais, normalmente entre 25 e 50 mg por dose de consumo (COHEN et al., 2017; SCHILLING et al., 2013). Desde então, alguns trabalhos científicos relacionados à identificação e determinação da DMAA no óleo de *P. graveolens* foram realizados, mostrando resultados controversos a respeito da sua identificação (AVULA et al., 2015; DI LORENZO et al., 2013; ELSOHLY et al., 2012; FLEMING; RANAIVO; SIMONE, 2012; GAUTHIER, 2013; LI; CHEN; LI, 2012; ZHANG et al., 2012). A

presença de DMAA não foi evidenciada além do relato de 1996 e de 2012 (LI; CHEN; LI, 2012; PING Z, 1996), ambos com espécies cultivadas na China (ATAILIA; DJAHOUDI, 2015; AVULA et al., 2015; BRASIL, 2014; ELSOHLY et al., 2012, 2015; FLEMING; RANAIVO; SIMONE, 2012; GAUTHIER, 2013). Também não foram encontrados, até o momento, dados a respeito da presença de DMAA em óleos essenciais de outras espécies de *Pelargonium* cultivadas no Brasil.

Normalmente a investigação da presença de DMAA é avaliada nos óleos essenciais obtidos através dos processos tradicionais de hidrodestilação e destilação por arraste de vapor. Estas técnicas podem proporcionar a perda de alguns constituintes voláteis e/ou compostos quimicamente instáveis como a DMAA (BICCHI et al., 2011; STASHENKO; MARTÍNEZ, 2008). A análise da constituição química das partes aéreas de *Pelargonium,* através da extração dos seus componentes voláteis pela técnica de *Headspace* (HS) e posterior análise através de cromatografia gasosa associada ao detector de massas (GC-MS), também pode ser uma alternativa para avaliação dos seus constituintes, e até o momento ainda não foi utilizada para determinar a presença de DMAA no gênero *Pelargonium*.

O óleo essencial de *P. graveolens*, conhecido comercialmente como “óleo de gerânio”, é amplamente utilizado na indústria de cosméticos, e na indústria alimentícia (ATAILIA; DJAHOUDI, 2015; BOUKHATEM; KAMELI; SAIDI, 2013; LIS-BALCHIN; STEYRL; KRENN, 2003; RAVINDRA; KULKARNI, 2015). Na medicina popular, é utilizado para diversos fins, entre eles como antiinflamatório, antibacteriano e antifúngico (ATAILIA; DJAHOUDI, 2015; BOUKHATEM; KAMELI; SAIDI, 2013; GHEDIRA; GOETZ, 2015)**.** Estudos científicos já relataram a atividade do óleo essencial frente a bactérias Gram positivas e negativas; e isolados de fungos filamentosos e leveduras (HSOUNA; HAMDI, 2012; ROSATO et al., 2010, 2008).

As leveduras do gênero *Candida* tem uma contribuição representativa em infecções oportunistas do trato genitourinário e gastrointestinal em adultos. Entre as mulheres, constituem um dos principais agentes patogênicos responsáveis pela vulvovaginite (ACHKAR; FRIES, 2010; CASSONE, 2015; PETERS et al., 2014). Apesar dos relatos da atividade antifúngica do óleo essencial de *P. graveolens* (ATAILIA; DJAHOUDI, 2015; GHEDIRA; GOETZ, 2015; HSOUNA; HAMDI, 2012),

não existem relatos desta atividade utilizando o óleo essencial proveniente do Brasil. Ainda, ressalta-se que os óleos essenciais podem sofrer degradação através de suas reações de oxidação, isomerização e hidrogenação (SHARIF et al., 2017). Uma abordagem alternativa para viabilizar o seu emprego e aumentar a sua eficácia é através do uso da nanotecnologia, que permite o aumento da sua estabilidade física, protegendo de interações com o ambiente e diminuindo a sua volatilidade, além de melhorar a eficácia da atividade antimicrobiana dos óleos essenciais por distintos mecanismos de ação e porporcionar uma melhora na absorção dos compostos ativos (BILIA et al., 2014; SHARIF et al., 2017).

Ao longo dos anos, a DMAA passou a ser utilizada predominantemente como aditivo nos compostos emagrecedores e suplementos alimentares, sendo consumida principalmente por atletas (LOPEZ-AVILA; ZORIO, 2013; WADA, 2010a). A partir do ano de 2008, a Organização Mundial dos Esportes - *World Anti-doping Agency* (WADA) relatou o consumo abusivo de DMAA e em 2010 proibiu a sua utilização pelos atletas profissionais (WADA, 2010a). Mesmo após a sua proibição pelas agências reguladoras do Brasil e Estados Unidos (BRASIL, 2012a; USA, 2013), a comercialização de suplementos alimentares contendo DMAA continua ocorrendo (COHEN et al., 2017; FDA, 2015; NEVES; CALDAS, 2017).

A DMAA apresenta também uma perspectiva de abuso em países como o Brasil (JUSTA NEVES; CALDAS, 2015a; NEVES; CALDAS, 2017) e por atletas profissionais, já que continua sendo reportada nos relatórios da WADA como um dos estimulantes mais consumidos por essa classe (WADA, 2015, 2017a). Uma alternativa para determinar a adulteração de suplementos alimentares e compostos emagracedores, identificando substâncias não declaradas, monitoradas ou proibidas, é a análise direta em espectrometria de massa em tempo real com análise sequencial (DART-MS/MS). O DART-MS/MS é capaz de uma análise rápida das amostras, à pressão atmosférica com um preparo mínimo ou sem preparo das amostras (AVULA et al., 2015; GROSS, 2014; LESIAK et al., 2014a). Esta técnica vem sendo utilizada atualmente para diferentes fins, como a determinação de ingredientes vegetais, produtos farmacêuticos e drogas de abuso, podendo ser uma alternativa para a análise de substâncias proibidas como a DMAA em amostras complexas (CODY et al., 2005b; CODY; LARAMEE; DURST, 2005b; GROSS, 2014).

Diante do exposto, esta tese visou realizar a determinação da constituição química dos óleos essenciais e das folhas de diferentes espécies de *Pelargonium*, investigar a presença de DMAA nos óleos essenciais por GC-MS, DART-MS/MS e LC-MS/MS e em suplementos alimentares apreendidos através de DART-MS/MS; além de avaliar a atividade antifúngica dos óleos essenciais obtidos de diferentes países e desenvolver uma formulação contendo o óleo essencial de *P.* gr*aveolens* para o tratamento da candidíase vaginal**.**

2. OBJETIVOS

2.1 Objetivos gerais

Realizar a análise química dos óleos essenciais e de folhas de diferentes espécies de *Pelargonium*; determinar a presença da DMAA e outros estimulantes em suplementos alimentares; avaliar a atividade antifúngica dos óleos essenciais e desenvolver uma formulação contendo o óleo essencial de *Pelargonium graveolens* para o tratamento da candidíase vaginal.

2.1.1 Objetivos Específicos

- Obter os óleos essenciais de espécies de cultivares de *Pelargonium* do Rio Grande do Sul pela técnica de hidrodestilação e determinar a sua constituição química através de cromatografia gasosa associada a detector de massas (GC-MS);

- Verificar a presença de 1,3- dimetilamilamina (DMAA) nos óleos essenciais extraídos por hidrodestilação através de GC-MS;

- Otimizar a técnica de extração por *headspace* para a determinação dos constituintes voláteis das folhas de espécies de *Pelargonium* do Rio Grande do Sul, através de desenho experimental do tipo Box–Behnken e, posteriormente, determinar a presença de DMAA através de análise por GC-MS;

- Determinar a constituição química dos óleos essenciais comerciais de *P. graveolens* provenientes do Brasil, China, Egito, África do Sul, Albânia e Ilhas Reunião através de GC-MS;

- Verificar a presença de DMAA nos óleos essenciais comerciais pelas técnicas de GC-MS, análise direta em espectrometria de massa em tempo real com análise sequencial (DART-MS/MS) e cromatografia Líquida associada ao detector de massas com análise sequencial (LC-MS/MS);

- Avaliar a atividade antifúngica dos óleos essenciais comerciais do Brasil, China, Egito, África do Sul, Albânia e Ilhas Reunião frente à espécies de *Candida;*

- Desenvolver formulação contendo o óleo essencial comercial com melhor desempenho antifúngico utilizando a nanotecnologia, e avaliar sua atividade antifúngica frente à espécies de *Candida;*

- Realizar *screening* da presença de DMAA, efedrina, sinefrina, cafeína, sibutramina e metilfenidato em 108 amostras de suplementos alimentares apreendidos pela Polícia Federal através de DART-MS/MS.

3.REVISÃO BIBLIOGRÁFICA

3.1 1,3-dimetilamilamina (DMAA)

A 1,3-dimetilamilamina é considerada um estimulante do tipo alquilamina, caracterizada pela presença de uma amina primária ligada a uma cadeia de carbono curta (Figura 1) (SARDELA et al., 2013). Conhecida popularmente como metilhexanamina (MHA) ou “geranamina”, passou a ser comercializada no mercado norte americano a partir da década de quarenta sob o nome de “Forthane” (SHONLE, H.A., ROHRMANN, 1944) e utilizada como descongestionante nasal devido as suas propriedades vasoconstritoras (COHEN, 2012; DOLAN; GATCH, 2015; ELSOHLY et al., 2012; PAWAR et al., 2013). Farmacologicamente é classificada como uma agonista α-1-adrenérgica (ELSOHLY et al., 2012). Em 2006, a DMAA reapareceu no mercado como constituinte dos suplementos alimentares, com propriedades estimulantes e atividade simpatomimética característica. Ao longo dos anos, a DMAA passou a ser utilizada predominantemente como aditivo nos compostos emagrecedores e suplementos alimentares, possuindo também uso recreativo na Europa e Nova Zelândia, sendo consumida na forma de comprimidos e usualmente em concentrações superiores (até 30 vezes maiores) às comumente adicionadas ao suplementos alimentares (GEE et al., 2012; RODRICKS; LUMPKIN; SCHILLING, 2013).

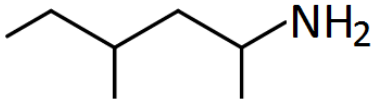

Figura 1. Fórmula estrutural da 1,3-dimetilamilamina (DMAA).

Pouco se sabe ainda sobre o mecanismo de ação da DMAA e sobre seu perfil toxicológico, mas inúmeros relatos de eventos adversos após o seu consumo, incluindo a ocorrência de mortes, indicam a sua toxicidade, principalmente associada ao aumento grave da pressão arterial e ritmo cardíaco, levando a ocorrências de infartos, toxicidade hepática e hemorragia cerebral (DOLAN; GATCH, 2015; DURGAM et al., 2013; FOLEY et al., 2014; GEE et al., 2012;

KARNATOVSKAIA; LEONI; FREEMAN, 2015). A maior parte dos casos de eventos adversos ocorreram com jovens, praticantes de atividade física e que utilizaram suplementação contendo DMAA na forma de cápsulas com o objetivo de melhorar o desempenho físico e auxiliar na perda de peso ou de forma recreativa em festas (FOLEY et al., 2014; J. PAVLETIC; PAO, 2014; KARNATOVSKAIA; LEONI; FREEMAN, 2015).

Estudo realizado com homens jovens, avaliou os efeitos fisiológicos e farmacocinéticos da DMAA após a suplementação com uma dose diária de 25 mg. Amostras de sangue foram coletadas antes e após a suplementação, e analisadas através de cromatografia líquida associada ao detector de massas (LC-MS). Os resultados mostraram que apesar do pequeno aumento na pressão arterial, ritmo cardíaco e temperatura corporal, não houveram relatos de eventos adversos. A concentração média máxima obtida foi de 70 ng/mL entre 3 e 5 horas (SCHILLING et al., 2013). Outro relato de estudo realizado com homens durante 15 dias, avaliou o efeitos causados pela ingestão de DMAA e cafeína separadamente e em combinação. O resultados demonstraram um aumento no ritmo cardíaco, indicando que os seus efeitos cardíacos são dose dependente, e, que provalmente por isso não levaram a eventos adversos graves, considerando que os realatos de eventos graves e mortes estão relacionados ao consumo de doses muito elevadas, entre 15 e 30 vezes maiores que as doses utilizadas nestes estudos (GEE et al., 2012; RODRICKS; LUMPKIN; SCHILLING, 2013).

Os principais sintomas após consumo em doses elevadas de DMAA são o aumento do ritmo cardíaco e temperatura corporal, vômito, dor de cabeça, fadiga e sudorese, podendo levar a toxicidade hepática, hemorragia cerebral e infarto (FOLEY et al., 2014; GEE et al., 2012; KARNATOVSKAIA; LEONI; FREEMAN, 2015). Ressalta-se que normalmente, juntamente com o consumo da DMAA, também ocorre o consumo de outras substâncias estimulantes como a cafeína, sinefrina, efedrina, entre outros, frequentemente presentes nas formulações de suplementos alimentares termogênicos. Sobretudo, deve-se considerar que a composição dos suplementos alimentares e compostos emagrecedores é complexa e diversa podendo haver muitas substâncias, inclusive não declaradas no rótulo, ou em concentrações distintas das informadas, contribuindo para a falta de segurança

do seu consumo e representando um sério risco para a saúde dos usários (HACHEM et al., 2016; NEVES; CALDAS, 2017; VIANA et al., 2015).

Desde o ano de 2008 cerca de 80 atletas já foram penalizados pela Agência Internacional Anti-Dopagem (WADA) devido à utilização abusiva de DMAA (LOPEZ-AVILA; ZORIO, 2013). Estas evidências estimularam a WADA a proibir o seu consumo pelos atletas profissionais em 2010 (WADA, 2010b). No ano de 2013, após algumas mortes e eventos graves indicando toxicidade (COHEN, 2012; DURGAM et al., 2013; GEE et al., 2012), e sem dados suficientes que indiquem a sua segurança e eficácia, a DMAA foi banida dos EUA (FDA, 2013) e outros países como Canadá, Nova Zelândia, Austrália e Brasil (BRASIL, 2012a; CANADA, 2011; USA, 2013).

Mesmo diante da proibição pela WADA, dados referentes às análises realizadas nos jogos de inverno olímpicos e paraolímpicos em laboratório credenciado à WADA em Moscow, “XXII Winter Olympic and XI Paralympic games”, revelaram a presença da DMAA além de outras susbtâncias proibidas e de uso controlado e monitorado, como a pseudoefedrina (SOBOLEVSKY et al., 2014). Ademais, conforme os últimos relatórios da WADA, a DMAA continua entre os estimulantes proibidos mais utilizado pelos atletas, juntamente com a anfetamina e o metilfenidato (WADA, 2015, 2017a).

Considerando o que diz respeito às proibições da presença da DMAA nos suplementos alimentares, a Agência Nacional de Vigilância Sanitária (ANVISA) suspendeu a sua distribuição, divulgação, comércio e uso no Brasil, e incluiu a DMAA na Lista F2 – Lista das Substâncias Psicotrópicas de Uso Proscrito do anexo I da Portaria SVS/MS nº. 344/98, conforme RDC nº. 37 de 2 de julho de 2012 (BRASIL, 2012a). Estes fatos alertaram também a FDA, que lançou um comunicado, enfatizando os perigos decorrentes do seu consumo. No entanto, apesar dos alertas, em abril de 2013 a FDA recebeu aproximadamente 90 notificações relacionadas a problemas de saúde e mortes decorrentes do uso de suplementos contendo DMAA, quando então passou a ser proibida (USA, 2013). Dados divulgados pela Polícia Federal mostram que apesar da proibição pelos órgãos regulamentadores, os compostos contendo a DMAA continuam sendo comercializados de forma clandestina e contrabandeados no Brasil (JUSTA NEVES; CALDAS, 2015a; NEVES; CALDAS, 2017).

A identificação da DMAA no óleo essencial de *Pelargonium graveolens* L'Her ex Ait. foi relatada pela primeira vez em 1996. O trabalho publicado no *Journal of Guizhou Institute of Technology* descreve brevemente a metodologia utilizada através de análise por GC-MS e relata a presença de 0,6% de DMAA no óleo de gerânio (PING Z, 1996). A partir deste resultado, a DMAA passou a ser adicionada aos suplementos alimentares e compostos emagrecedores, conforme regulamentado pelo "*Dietary Supplement Health and Education Act"* (DSHEA) (FDA, 1994). No entanto, diversos trabalhos foram realizados questionando o resultado apresentado em 1996 com a espécie de *P. graveolens* cultivada na China, (AUSTIN et al., 2014; AVULA et al., 2015; DI LORENZO et al., 2013; ELSOHLY et al., 2012, 2015), utilizando distintas técnicas analíticas. Apesar dos relatos científicos, a presença da DMAA não foi identificada, com exceção do primeiro relato e, posteriormente, do resultado apresentado por pesquisadores provenientes da China, que realizaram a análise dos óleos essenciais através de LC-MS/MS. O método desenvolvido e validado foi aplicado em três óleos essenciais de espécies de *P. graveolens* coletadas de diferentes locais da China, que apresentaram concentrações entre 0,167 e 13,271 µg/g de DMAA (LI; CHEN; LI, 2012).

Conforme a Tabela 1 verifica-se que a maior parte dos óleos essenciais de *P. graveolens* extraídos de flores, folhas ou caules, por hidrodestilação ou destilação por arraste de vapor de países como Algéria, África, Inglaterra, Tunísia, Alemanha, Irã e Egito não apresentaram a DMAA em sua composição, com resultados positivos apenas para as espécies da China.

Tabela 1. Métodos de extração dos óleos essenciais (OE) de *Pelargonium graveolens* e verificação da presença de DMAA.

Autor	Origem da planta	Parte da planta utilizada	Planta seca ou fresca	Método de extração do óleo essencial	Método de análise	Presença de DMAA
(PING Z, 1996)	China	Folha e raíz	Não informado	Hidrodestilação	GC-MS	Sim
(LIS-BALCHIN et al., 1998)	Inglaterra	Folha	Fresca (100g)	Arraste a vapor - Clevenger	Não informado	Não
(RANA; JUYAL; BLAZQUEZ, 2003)	Índia	Folha	Fresca (500g)	Hidrodestilação - Clevenger	GC-MS	Não
(LIS-BALCHIN; STEYRL; KRENN, 2003)	Inglaterra	Folha	Fresca (500g)	Hidrodestilação - Clevenger	GC-MS	Não
(JALALI-HERAVI; ZEKAVAT; SERESHTI, 2006)	Irã	Folha e caule	Fresca	Hidrodestilação - Clevenger	GC-MS e GC-FID	Não
(SAXENA et al., 2008)	Índia	Partes aéreas da planta	Fresca	Hidrodestilação- Clevenger	GC-FID	Não
(ROSATO et al., 2009)	Itália	Folha e caule	Seca	Hidrdestilação - Clevenger	GC-MS	Não
(ROSATO et al., 2010)	Itália	OE comercial	Não informado	Arraste a vapor	GC-MS	Não
(LISI et al., 2011)	França e Egito	Não informado	Não informado	Arraste a vapor	GC-MS e GC-NPD	Não
(BOUKHRIS et al., 2012)	Tunísia	Folha	Fresca	Hidrodestilação - Clevenger	GC-MS	Não

Tabela 1. Continuação

Autor	Origem da planta	Parte da planta utilizada	Planta seca ou fresca	Método de extração do óleo essencial	Método de análise	Presença de DMAA
(SANJA; MAKSIMOVI, 2012)	Bósnia e Hezergovina	Folha e caule	Seca	Hidrodestilação	GC-MS	Não
(ELSOHLY et al., 2012)	Estados Unidos Índia	Folha e caule OE comercial	Fresca e seca Não informado	Arraste a vapor Não informado	GC-MS e LC-MS/MS e LC-QTOF/MS	Não
(GHANNADI et al., 2012)	Irã	Partes aéreas da planta	Seca	Hidrodestilação – Clevenger	GC-MS	Não
(HSOUNA; HAMDI, 2012)	Tunísia	Folha	Seca	Extração com Sohxlet	GC-MS	Não
(LI; CHEN; LI, 2012)	China	Não informado	Fresca	Não informado	LC-MS/MS	Sim
(BOUKHRIS et al., 2013)	Tunísia	Folha	Fresca (600g)	Hidrodestilação - Clevenger	GC-MS	Não
(BOUKHATEM et al., 2013)	Algéria	Partes aéreas da planta	Fresca	Arraste a vapor	GC-MS e CG-FID	Não
(BOUKHATEM; KAMELI; SAIDI, 2013)	Algéria	Partes aéreas da planta	Seca	Hidrodestilação – Clevenger	GC-MS	Não
(DI LORENZO et al., 2013)	Itália África	Folhas e caules OE comercial	Fresca Não informado	Extração com solvente Não informado	LC-DAD	Não

Tabela 1. Continuação

Autor	Origem da planta	Parte da planta utilizada	Planta seca ou fresca	Método de extração do óleo essencial	Método de análise	Presença de DMAA
(MOHAREB; BADAWY; ABDELGALEIL, 2013)	Egito	Folha	Seca	Hidrodestilação – Clevenger	GC-MS	Não
(AUSTIN et al., 2014)	Estados Unidos	Folha e caule	Fresca	Extração com solvente orgânico	LC-MS/MS	Não
(BADAWY; ABDELGALEIL, 2014)	Egito	Folha	Seca	Hidrodestilação – Clevenger	GC-MS	Não
(NEJAD; ISMAILI, 2013)	Irã	Folha	Seca	Hidrodestilação - Clevenger	GC-MS e GC-FID	Não
(SHAROPOV; ZHANG; SETZER, 2014)	Tajiquistão	Partes aéreas da planta	Fresca	Hidrodestilação	GC-MS	Não
(SILVA et al., 2014)	Brasil	Folha	Fresca	Hidrodestilação – Clevenger	GC-MS e GC-FID	Não
(ATAILIA; DJAHOUDI, 2015)	Algéria	Partes aéreas da planta	Seca	Hidrodestilação – Clevenger	GC-MS	Não
(ELSOHLY et al., 2015)	China África do Sul	Toda a planta Óleo essencial comercial	Fresca e Seca Não informado	Extração com solvente orgânico Não informado	LC-MS/MS	Não
(RAVINDRA; KULKARNI, 2015)	França (Bourboun) India	Folha e caule	Não informado	Hidrodestilação – Clevenger	GC-MS	Não

Fatores como as variações das condições edafoclimáticas, o método de extração do óleo essencial e método de análise da planta, podem influenciar o perfil químico do óleo e explicariam, pelo menos em parte, os resultados controversos apresentados na literatura a respeito da identificação da DMAA em óleos extraídos de *P. graveolens* (LI; CHEN; LI, 2012; PING Z, 1996). No entanto, deve-se considerar as pequenas concentrações de DMAA encontradas nos óleo de gerânio, sugerindo que apesar de aparecer nos rótulos dos suplementos como de origem natural, a DMAA encontrada nesses produtos é predominantemente sintética, já que o percentual da ocorrência natural encontra-se muito abaixo dos teores expressos nos suplementos comerciais (AUSTIN et al., 2014; AVULA et al., 2015; ELSOHLY et al., 2015; LISI et al., 2011; ZHANG et al., 2012), e, portanto, não justificaria a sua adição nos suplementos alimentares, conforme regulamentação estabelecida pelo DSHEA (FDA, 1994).

3.2 *Pelargonium graveolens* L'Her ex Ait.

A família Geraniaceae é composta aproximadamente 800 espécies, incluindo três principais gêneros: *Erodium*, *Geranium* e *Pelargonium* (SPICHIGER et al., 2002). O gênero *Pelargonium* deriva de "Pelargos" que significa "cegonha" em grego. O nome surgiu a partir da observação do formato dos frutos, que faz alusão à um bico de uma cegonha (FOURNIER, 1961). É o segundo maior da família Geraniaceae contendo cerca 300 espécies (BAKKER et al., 2004). A espécie denominada *P. graveolens* é uma das mais importantes do gênero e pode ser caracterizada como um arbusto aromático que pode atingir até 1,3 m de altura (Figura 2) (RANA; JUYAL; BLAZQUEZ, 2003). Conhecida como *"rose scented geranium*", ou "malva-cheirosa", a espécie de *P. graveolens* é nativa da região do Cabo, na África do Sul. Atualmente é cultivada em países como Índia, China, Egito, Marrocos, Algéria, França ("Reunion Islands"), destacando-se como maiores produtores da espécie o Egito e a China, visando especialmente a produção do óleo essencial para as indústrias de cosméticos e alimentícia (ATAILIA; DJAHOUDI, 2015; SANDASI et al., 2011). Atualmente a China é responsável por cerca de 80% da produção mundial do óleo essencial de *P. graveolens,* também conhecido como óleo de gerânio (SANDASI et al., 2011; VERMA et al., 2010)

O óleo de gerânio tem um odor marcante similar ao odor de rosas e, portanto, possui grande aplicação na indústria de perfumes, com o valor e qualidade atribuídos principalmente ao conteúdo de citronelol, também o principal responsável pelo seu odor característico, juntamente com o geraniol, o linalol e seus ésteres. Devido a esta similaridade ganhou grande importância econômica, sendo um substituto mais acessível do que a *Rosa damascena* (BABU; KAUL, 2005).

O óleo de gerânio é muito empregado em cosméticos, aromaterapia e indústria alimentícia, além de possuir relatos de sua atividade anti-inflamatória, antifúngica, antioxidante e hipoglicêmica (ATAILIA; DJAHOUDI, 2015; HSOUNA; HAMDI, 2012; ROSATO et al., 2008, 2009). Ainda, possui relatos de uso na medicina popular para o tratamento de diarreia, hemorroidas, distúrbios do ciclo menstrual e menopausa, eczemas e até contra o câncer (JALALI-HERAVI; ZEKAVAT; SERESHTI, 2006) .

Figura 2. Foto das partes aéreas da espécie de *Pelargonium graveolens*. Retirado de (GHEDIRA; GOETZ, 2015).

Também foi observado o crescimento do uso dos óleos essenciais pela indústria farmacêutica, principalmente na incoproporação em formulações como cápsulas, pomadas, cremes, xaropes, supositórios, aerossóis e sprays. A inclusão

dos óleos prevalece em formulações destinadas a aplicações tópicas ou para inalação (ASBAHANI et al., 2015).

A forma de extração dos óleos também pode influenciar a sua composição química, podendo ser escolhida de acordo com o obejtivo da sua utilização. Assim, o perfil químico dos óleos essenciais difere na diversidade dos compostos que apresentam e nos tipos estereoquímicos das moléculas extraídas (BABU; KAUL, 2005; BAKKALI et al., 2008). Os óleos essenciais são biossintetizados, acumulados e armazenados em estruturas conhecidas como glândulas secretoras (BAKKALI et al., 2008). As glândulas secretoras podem estar localizadas na superfície da planta (produção exógena do óleo essencial) como as papilas epidérmicas, tricomas glandulares e não glandulares; ou nos seus órgãos internos (produção endógena) como os canais secretores e as bolsas secretoras. Também podem ser encontradas no citoplasma de algumas células secretoras, normalmente presentes nas raízes e flores (ASBAHANI et al., 2015; MAFFEI, 2010). Os tecidos glandulares são classificados de acordo com a substância que produzem, sendo os tricomas, os dutos e as cavidades, os principais responsáveis pela produção dos óleos essenciais (Tabela 2) (MAFFEI, 2010).

Os tricomas glandulares estão presentes em algumas famílias como Asteraceae, Lamiaceae e Geraniaceae. BOUKHIRIS e colaboradores (2013), realizaram a análise da morfologia, anatomia e distribuição dos tricomas através de microscopia eletrônica de varredura e microscopia eletrônica de transmissão, em folhas, flores e caules da espécie de *P. graveolens c*ultivadas na Tunísia. Os resultados mostraram que a espécie apresenta tricomas não glandulares - com superfície lisa e granulada (Figura 3) e glandulares – peltato e capitato. Os tricomas capitatos podem possuir ainda três subtipos, variando a sua forma e estrutura. Nas amostras analisadas de *P. graveolens*, os três subtipos de tricomas capitatos foram identificados (Figura 4) (BOUKHRIS et al., 2013).

Tabela 2. Estruturas especializadas no acúmulo e armazenamento dos óleos essenciais nas plantas.

ESTRUTURAS SECRETORAS	DESCRIÇÃO	ÓRGÃO DA PLANTA	EXEMPLOS	FAMÍLIA BOTÂNICA
Tecidos secretores externos				
Papilas epidérmicas	Células epidérmicas secretoras	Flores Pétalas	*Rosa damascena* *Convallaria majalis*	Rosaceae Aspargaceae Geraniaceae Lamiaceae
Vesículas secretoras ou tricomas glandulares	Células terminais dos tricomas secretores de óleos essenciais	Caules Folhas	*Pelargonium sp* *Salvia sp , Mentha sp*	
Tecidos secretores internos				
Bolsas esquizógenas	Espaço intracelular preenchido pelas células secretoras	Epicarpo do fruto	*Citrus sp.*	Rutaceae Myrtaceae
Canais secretores	Pequenos canais formados pelo agregamento de células secretoras ao longo da planta	Caules	*Petroselinum sp.* *Pimpinella sp.* *Daucus sp.*	Apiaceae
Células secretoras intracelulares	Células especializadas no acúmulo dos óleos essenciais dentro dos vacúolos	Caules Folhas	*Cinnamomum* *Ceylanicum* *Laurus nobilis*	Lauraceae

Adaptado de (ASBAHANI et al., 2015).

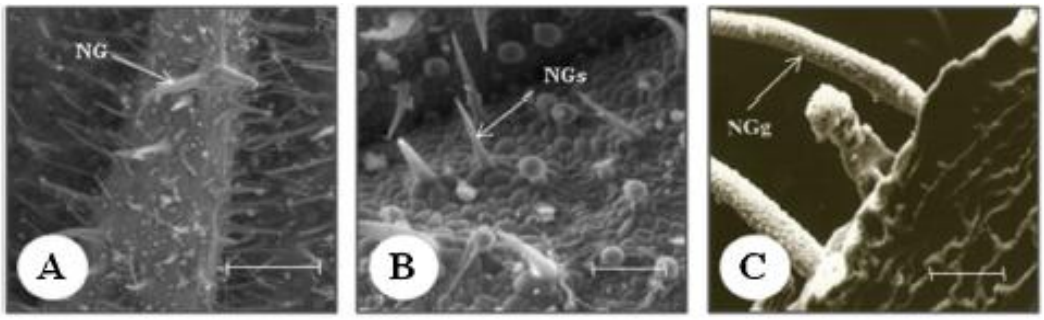

Figura 3. Tricomas não glandulares presentes na espécie de *P. graveolens* identificados pela técnica de microscopia eletrônica de varredura. (A) Vista dos tricomas não glandulares presentes na porção abaxial da folha, (B) Vista dos tricomas não glandulares com superfície lisa presentes da porção adaxial da folha e (C) Vista dos tricomas não glandulares com superfície granulada presentes da porção adaxial da folha. Retirado de (BOUKHRIS et al., 2013).

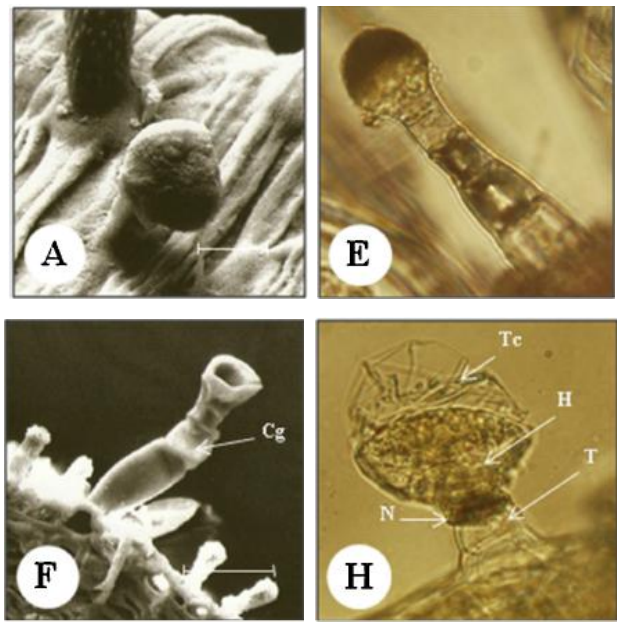

Figura 4**.** Tricomas glandulares presentes na espécie de *P. graveolens* identificados pela técnica de microscopia eletrônica de varredura (A e F) e microscopia de luz (E e H). (A) Tricoma glandular capitato tipo I presente na porção abaxial da folha, (E) Tricoma glandular capitato tipo II presente na adaxial da folha, (F) Tricoma glandular capitato tipo III na porção abaxial da folha e (H) Vista superior do tricoma glandular peltato. Retirado de (BOUKHRIS et al., 2013).

3.3 Óleos essenciais

Os compostos químicos produzidos e biotransformados pelos organismos vegetais são classificados como produtos do metabolismo primário e secundário. Os metabólitos primários constituem parte das vias de modificação e síntese de carboidratos, proteínas e lipídeos, essenciais para a manutenção da vida planta e com funções e características conhecidas. Os metabólitos secundários derivam das rotas biossintéticas dos metabólitos primários e possuem produção e distribuição limitada aos organismos vivos, sendo encontrados somente em algumas espécies de plantas (DEWICK, 2009).

Geralmente as funções e benefícios dos metabólitos secundários são desconhecidos ao organismo produtor, porém os mais comuns são a defesa contra predadores e o papel atrativo aos polinizadores (DEWICK, 2009). Ainda, são responsáveis pela maior parte dos compostos farmacologicamente ativos dos produtos naturais, sendo muito empregados na indústria farmacêutica, cosmética e química (SONG et al., 2014; DEWICK, 2009) . Como exemplo temos a artemisinina, o paclitaxel, o licopeno, o resveratrol e entre outros, que são amplamente utilizados como medicamentos ou em suplementos dietéticos (SONG et al., 2014).

Os óleos essenciais são metabólitos secundários das plantas, caracterizados por possuírem odor forte, volatilidade e composição complexa. São líquidos oleosos, aromáticos e podem estar presentes em distintas partes dos vegetais, desempenhando um papel importante na interação com outras plantas através da inibição da germinação e do seu crescimento; na interação entre as plantas e animais, atuando na atração de polinizadores e servindo como repelente de pragas, além de possuírem efeito protetivo, agindo como antivirais antibacterianos e antifúngicos. Podem ser extraídos principalmente através das técnicas de hidrodestilação, arraste de vapor ou extração com solvente orgânico (RAUT; KARUPPAYIL, 2014). Desta forma, os óleos essenciais são considerados com uma fonte de sinalizadores químicos perimitindo que a planta controle e regule o ambiente em que vive (ASBAHANI et al., 2015; BAKKALI et al., 2008; MURPHY COWAN, 1999; PINTO-ZEVALLOS; MARTINS; PELLEGRINO, 2013). Podem variar em qualidade, quantidade e na composição de acordo com o clima, composição do

solo, órgão da planta, idade e estágio ciclo vegetativo (ASBAHANI et al., 2015; BAKKALI et al., 2008).

3.3.1 Composição química dos óleos essenciais

Os óleos essenciais derivam dos metabólitos primários, das vias fundamentais do processo de fotossíntese, glicólise e do ciclo de Krebs. Estes processos básicos do metabolismo geram energia para a produção de compostos intermediários que servirão de precursores nas rotas biossíntéticas. Os metabólitos mais importantes derivam de intermediários da acetil coenzima A (acetil CoA), ácido chiquímico, ácido mevalônico e metil eritrol fosfato, que são utilizados nas vias do acetato, chiquimato, mevalonato e metileritrol fosfato (DEWICK, 2009).

Os óleos essenciais podem apresentar entre 20 e 100 compostos pertencentes a diferentes classes químicas (BAKKALI et al., 2008; BILIA et al., 2014; CARSON et al., 2002). Geralmente a sua composição é resultado de um equilíbrio de várias substâncias, embora em muitas espécies sejam encontrados compostos majoritários e demais compostos em pequenas quantidades (traços) (FINEFIELD et al., 2012; MURPHY COWAN, 1999). São principalmente constituídos de monoterpenos, sesquiterpenos e seus derivados oxigenados (álcoois, aldeídos, ésteres, éteres, cetonas, fenóis e óxidos), além dos fenilpropanoides e alguns compostos alifáticos e aromáticos, presentes em menor quantidade e que apresentam diferentes origens biossintéticas químicas (Figura 5) (BAKKALI et al., 2008; BILIA et al., 2014; CARSON et al., 2002). São extraídos de diversas plantas aromáticas que normalmente estão localizadas em locais com clima quente, como os países do Mediterrâneo e de regiões tropicais (BAKKALI et al., 2008).

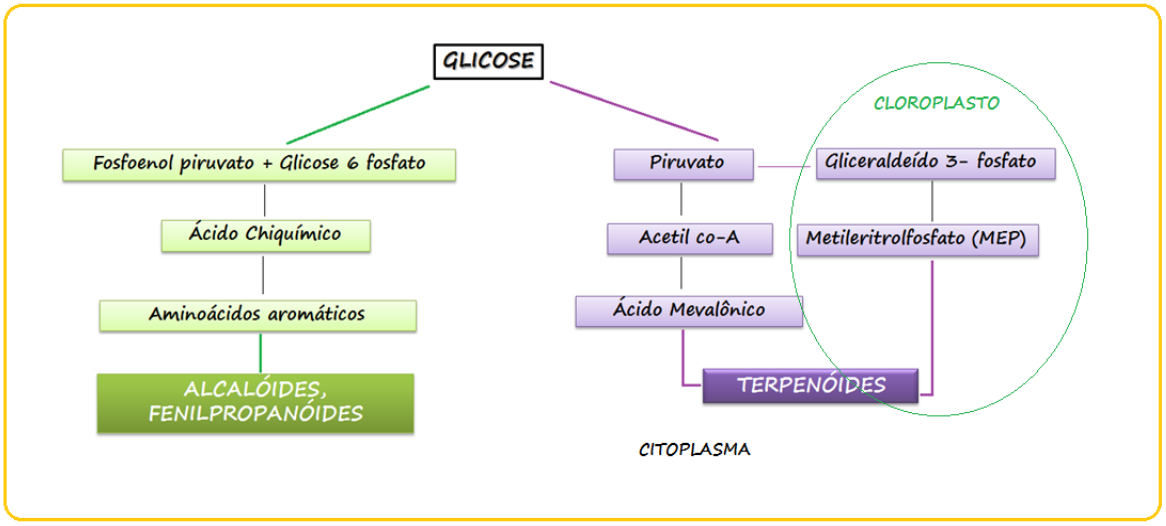

Figura 5. Representação dos precursores dos terpenóides e fenilpropanóides nos processos biossintéticos de origem vegetal. Crédito do autor.

Os terpenoides são sintetizados a partir de duas unidades de isoprenos: isopentil difosfato (IPP) e dimetilalil difosfato (DMAPP). Estes precursores são produzidos por duas vias distintas: a via do mevalonato (MVA) e a via não-mevalonato-metileritrolfosfato (MEP) (SONG et al., 2014) (Figura 6).

Os terpenoides são subdivididos em famílias com base no número de resíduos de isoprenoides. Os monoterpenos (C10) são do tipo estrutural menor, seguido pelos sesquiterpenos (C15), diterpenos (C20), sesterterpenos (C25), triterpenos (C30), tetraterpenos (C40), e politerpenos (> C40) (FINEFIELD et al., 2012). Terpenoides enantioméricos são comuns, no entanto, são geralmente limitados a monoterpenos e sesquiterpenos, e mais raramente a diterpenos (FINEFIELD et al., 2012; DEWICK, 2009). Os terpernoides, esteroides e alguns sesquiterpenos são formados pela via do mevalonato (MEV), enquanto que os o monoterpenos, diterpenos e politerpenos, são formados nos cloroplastos e derivados da via do metil eritrol fostato (MEP) (DEWICK, 2009).

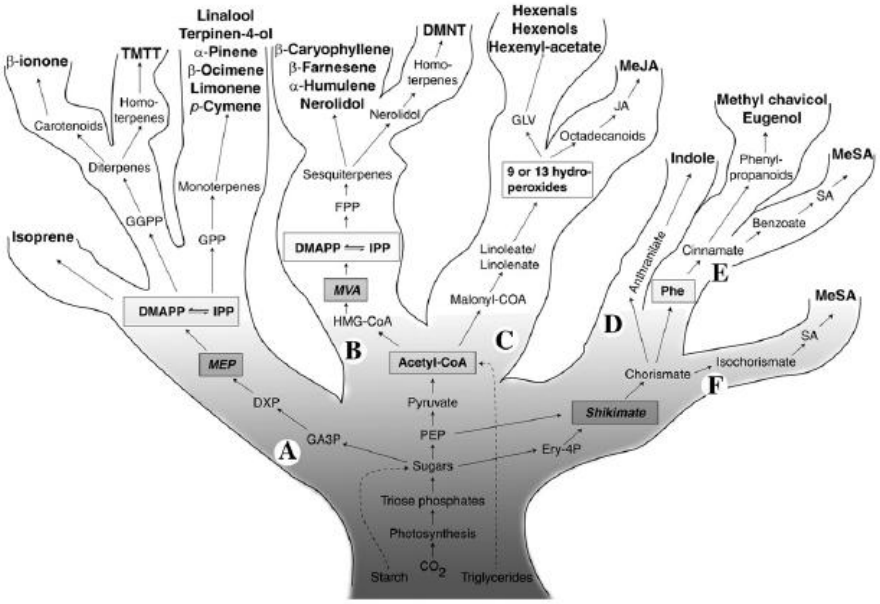

Figura 6. Vias de formação dos principais componentes dos óleos essenciais. Os compostos orgânicos voláteis presentes no óleos essenciais são produzidos por diferentes vias bioquímicas. Rota (A): as vias do MEP (metileritrolfosfato) dão origem à formação de monoterpenos e diterpenos. O isopreno é gerado a partir do dimetilalil difosfato (DMAPP). Rota (B): os sesquiterpenoides são gerados a partir do farnesil difosfato (FPP) derivado do citosol. Rota (C): as oxilipinas são geradas a partir de ácidos graxos que são clivados em derivados de derivados voláteis de folhas verdes (GLV) e ácido jasmônico (JA). Rota (D): os indóis voláteis são gerados a partir do antranilato. Rota (E): compostos como o eugenol, derivam dos fenilpropanóides, enquanto o salicilato de metila (MeSA) é derivado do ácido salicílico (SA), gerado a partir do ácido benzóico. Rota (F): alternativamente, o MeSA pode ser formado por metilação do SA decorrente do isocorismato. Retirado de (MAFFEI, 2010).

Os monoterpenos (C10) são isolados principalmente a partir de plantas superiores e compõe o aroma de muitos óleos essenciais de ervas, especiarias, frutas cítricas e coníferas. Alguns monoterpenos quirais são produzidos em ambas as formas enantioméricas, e, muitas vezes, pelas mesmas espécies de plantas. Além disso, os monoterpenos enantioméricos podem exibir atividade biológica única, e, normalmente cada um dos enantiômeros irá apresentar propriedades biológicas distintas (FINEFIELD et al., 2012). Os sesquiterpenos formam um grupo de terpenos cíclicos (C15) isolados a partir de uma grande variedade de plantas, fungos,

bactérias, espécies marinhas e de insetos. Assim como os monoterpenos estão presentes nos óleos essenciais e exibem uma vasta atividade farmacológica. São exemplos de sesquiterpenos α e β-cariofileno, β-selineno, farnesol e bisaboleno, entre outros (PROSSER et al., 2004).

Os fenilpropanoides, são uma classe de produtos naturais constituídas por uma vasta gama de diferentes metabólitos secundários derivados da via do chiquimato e formados a partir de L-fenilalanina e/ou L-tirosina (Figura 5). Os fenilpropanoides são encontrados em numerosas espécies de plantas e contribuem para a sua defesa, estrutura, produção de pigmentos e reprodução (VOGT, 2010). São exemplos de fenilpropanoides as catequinas, cumarinas, esteróis vegetais e fitoestrógenos. Estes compostos possuem propriedades terapêuticas diversas, entre elas a atividade antibacteriana, antiviral e a capacidade de proteção contra doenças cardíacas, além de atuar no combate de alguns tipos de câncer (HEMAISWARYA; DOBLE, 2013).

3.3.2 Óleos essenciais de *P. graveolens*

Os compostos majoritários encontrados nos óleos essenciais de *P. graveolens* são o citronelol e o geraniol, classificados como alcoóis monoterpênicos (ATAILIA; DJAHOUDI, 2015; GHEDIRA; GOETZ, 2015; RANA; JUYAL; BLAZQUEZ, 2003). A formação do geraniol e citronelol ocorre a partir do geranil difosfato (GPP). O GPP é formado pela condensação do isopentil difosfato e dimetilalil, que após sofrer uma adição de água ao cátion geranila, dá origem ao geraniol e posteriormente após sofrer uma redução, dá origem ao citronelol (Figura 7) (DEWICK, 2009).

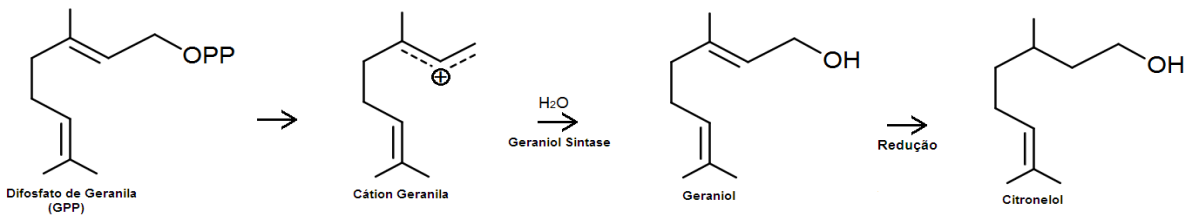

Figura 7. Formação do geraniol e citronelol a partir do difosfato de geranila. Adaptado de (DEWICK, 2009).

Também são encontrados em menor teor os compostos monoterpênicos α e β-pineno, linalol, limoneno, formato de citronelila, cis-óxido de rosa, trans-óxido de rosa, citronelal, geranial, nerol, tiglato de geranila entre outros (Figura 8) (BOUKHATEM; KAMELI; SAIDI, 2013; GHEDIRA; GOETZ, 2015; LIS-BALCHIN; STEYRL; KRENN, 2003; RAVINDRA; KULKARNI, 2015).

Os sesquiterpenos mais encontrados na espécie de *P. graveolens* são α e β-cariofileno, β-selineno, germacreno D, α-guiaeno, δ-cadineno, 10-epi-γ-eudesmol, α-copaeno, α-humuleno, entre outros (Figura 9) (BOUKHATEM et al., 2013; BOUKHRIS et al., 2012; RAVINDRA; KULKARNI, 2015).

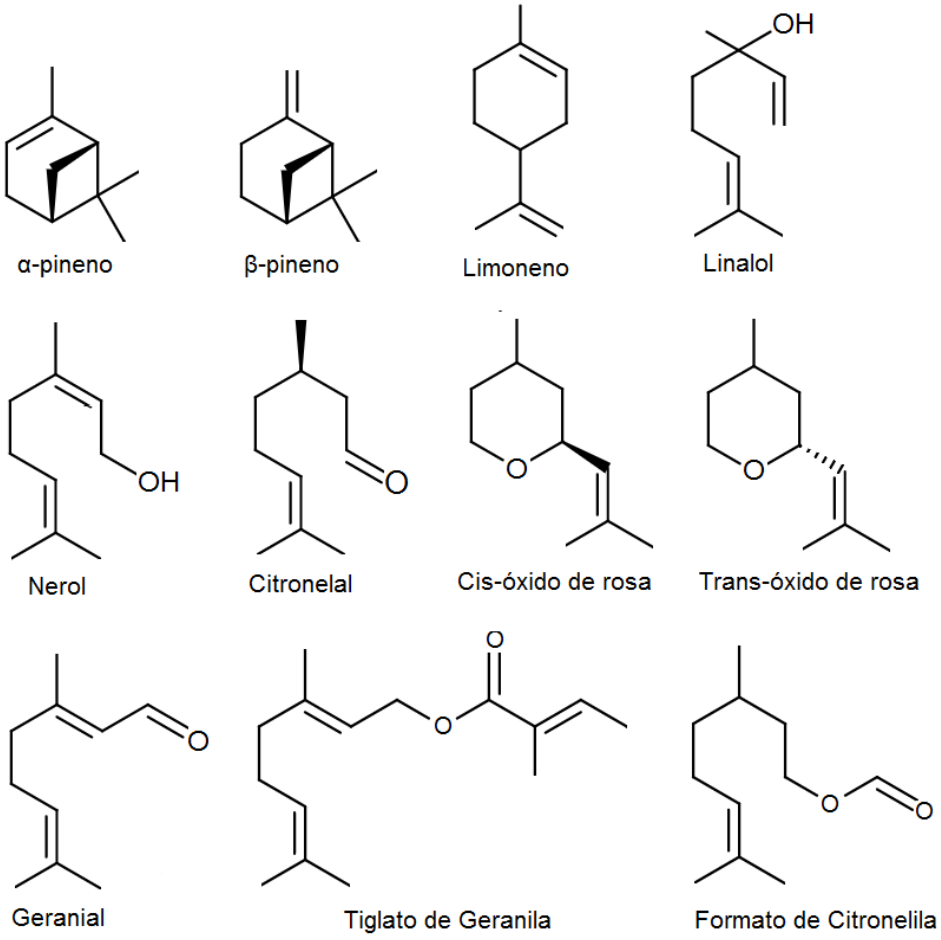

Figura 8. Exemplos de monoterpenos presentes nos óleos essenciais de *Pelargonium graveolens.*

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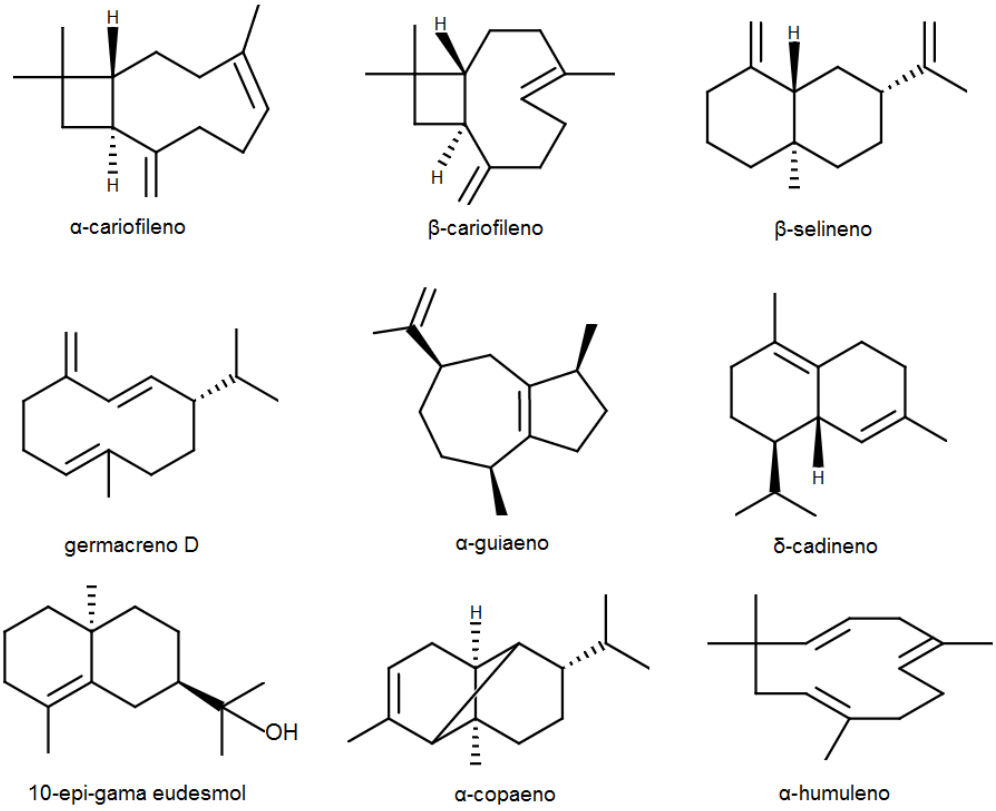

Figura 9. Exemplos de sesquiterpenos presentes nos óleos essenciais de *Pelargonium graveolens.*

3.4. Métodos de extração dos óleos essenciais de *P. graveolens*

Os óleos essenciais são obtidos das plantas através de diferentes métodos de extração. Normalmente os métodos mais empregados (convencionais), são a hidrodestilação, hidrodestilação por arraste de vapor, prensagem a frio e extração com solvente orgânico. São considerados métodos não convencionais aqueles que podem interferir na constituição química dos óleos devido às elevadas temperaturas empregadas, que podem levar a reações como hidrólise, isomerização ou oxidação. Técnicas como extração com fluido supercrítico, extração assistida por ultrassom e extração subcrítica com CO_2, são exemplos de métodos não convencionais de extração dos óleos essenciais (ASBAHANI et al., 2015).

Os óleos essenciais de *P. graveolens* são normalmente obtidos das folhas, flores ou caules (partes aéreas) através das técnicas de hidrodestilação e destilação por arraste de vapor (Tabela 1). Também foi relatada a extração das folhas de *P.*

graveolens, previamente secas, com três solventes de disitintas polaridades: hexano, acetato de etila e metanol, com intuito de avaliar o seu conteúdo fenólico e de flavonóides, além de utilizarem a técnica de hidrodestilação para análise do óleo essencial (HSOUNA; HAMDI, 2012). Os resultados mostraram que entre os solventes testados o metanol se mostrou mais eficiente na extração dos componentes fenólicos e flavonoides, compostos normalmente utilizados na avaliação da atividade antioxidante das plantas (ARDESTANI; YAZDANPARAST, 2007; DJERIDANE et al., 2006; HSOUNA; HAMDI, 2012).

O óleo essencial de *P. graveolens* possui grande importância comercial, sendo muito utilizado em perfumes e na indústria de alimentos. A composição do óleo de gerânio proveniente de diferentes países possui variações, sendo o de origem das ilhas Reunião, da França - 'gerânio Bourbon' considerado o óleo essencial de melhor qualidade, pois possui grande porcentagem de geraniol, um dos componentes mais importantes na indústria de aromas (CHEN; VILJOEN, 2010; SANDASI et al., 2011).

3.4.1 Hidrodestilação e Arraste por Vapor de Água

A destilação por arraste de vapor de água é considerada o método mais antigo e tradicional de obtenção dos óleos essenciais. Possui aparato extrator considerado simples e tempo de extração não tão longo quando comparado à hidrodestilação, além de ser barato quando comparado aos métodos tecnologicamente mais avançados, como a extração com fluido supercrítico (ASBAHANI et al., 2015; BANDONI, 2000; CASSEL; VARGAS, 2006). Neste processo, o vapor de água percola o balão contendo o material vegetal, levando à ruptura das suas células e à liberação do óleo essencial, que juntamente com a água, percorre o condensador onde ocorre seu resfriamento e, desta forma, o hidrolato e a água voltam à fase líquida, sendo separados pela sua diferença de densidade em um decantador com graduação volumétrica (Figura 10) (ASBAHANI et al., 2015; BOUKHATEM; KAMELI; SAIDI, 2013).

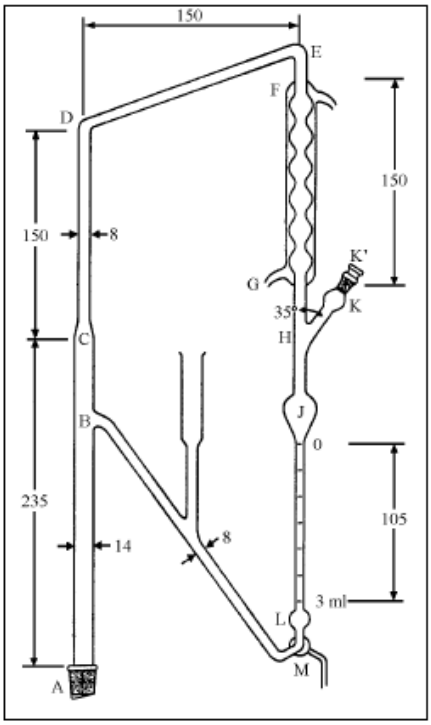

Figura 10. Aparelho para extração de óleos essenciais pelo processo de destilação por arraste de vapor. Retirado de (BRASIL, 2010).

A hidrodestilação utiliza o mesmo princípio e aparato extrativo semelhante ao da extração por arraste de vapor, diferindo na forma de contato do material vegetal com a água. Na hidrodestilação, o material vegetal fica imerso na água dentro do balão de extração, sendo mantido sob aquecimento até a ebulição (Figura 11). Desta forma os vapores gerados pela ebulição são condensados e coletados, seguindo o mesmo processo apresentado no método de destilação por arraste de vapor. O aparato extrator da hidrodestilação é conhecido como *Clevenger*. Neste método deve se considerar o tempo de extração de acordo com o perfil dos compostos presentes na planta. Desta forma, compostos que possuem um alto ponto de ebulição, irão necessitar de um maior tempo de destilação. A desvantagem deste método, quando comparado à destilação por arraste de vapor de água, reside no maior tempo necessário para extração do óleo essencial e na possibilidade de perda de compostos mais polares durante o processo, devido a sua miscibilidade na água. Ainda assim a hidrodestilação também é um método comum e muito empregado na extração de óleos essenciais (ASBAHANI et al., 2015).

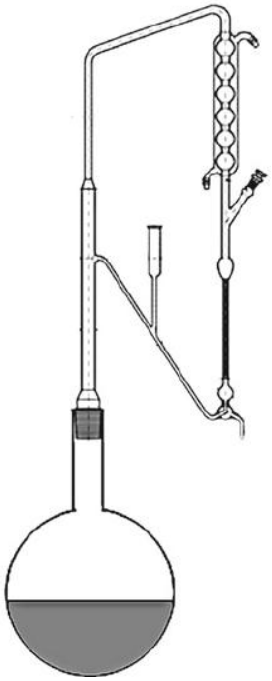

Figura 11. Aparelho de Clevenger adaptado contendo o balão para adição da água e do material vegetal. Extração de óleos essenciais por hidrodestilação. Retirado de (WALKER, 2009).

3.4.2 Métodos alternativos para a obtenção dos constituintes voláteis: Micro Extração em Fase Sólida (SPME) *e Headspace* (HS)

Os métodos de extração mais comuns para a determinação dos constituintes das plantas são a hidrodestilação e destilação por arraste de vapor, no caso dos óleos essenciais; e extração com *Soxhlet* e fluido supercrítico, para determinação de outros constituintes presentes nas plantas. No entanto, estes métodos podem resultar na perda dos compostos voláteis durante o processo de extração, além de necessitarem de um tempo de preparo muito elevado (BICCHI et al., 2011; STASHENKO; MARTÍNEZ, 2008). Desta forma, as técnicas de SPME e *headspace* podem constituir uma alternativa para a extração dos componentes voláteis das plantas quando o objetivo é a identificação e caracterização da sua composição química (YANG; WANG; LI, 2013; ZHU et al., 2013). Alguns trabalhos relatam como uma alternativa para a extração de compostos voláteis de plantas, a técnica de SPME, seguida de análise por cromatografia gasosa, devido a possibilidade de

análise direta, sem pré-tratameno da amostra (LIN et al., 2013; YANG; WANG; LI, 2013). Normalmente as plantas são concentradas ou secas, dissolvidas em água e esta solução utilizada na microextração (YANG; WANG; LI, 2013). A SPME tem sido empregada para extração de componentes voláteis de folhas, flores, frutas e também para realização do controle de qualidade de ervas e temperos (LIN et al., 2013; SGORBINI et al., 2015; YANG; WANG; LI, 2013; ZHU et al., 2013).

3.4.2.1 Microextração em fase sólida (SPME)

O princípio da técnica de microextração em fase sólida baseia-se na partição entre os compostos presentes em uma matriz e uma microfibra, constituída por um filme polimérico. Os filmes que recobrem a microfibra de sílica fundida são constituídos de polidimetilsiloxano (PDMS), poliacrilato (PA) ou carboxeno (CA), e estão disponíveis em diversos tamanhos e espessuras (PRAGST, 2007).

A técnica de SPME pode ser realizada através do contato direto entre a fibra e a amostra por imersão direta (SPME-ID), onde ocorrerá o equilíbrio entre os componentes voláteis da amostra e a microfibra; ou através do *headspace*, onde a microfibra ficará somente no espaço gasoso e, desta forma, deverá ocorrer o equilíbrio entre a amostra, a microfibra e o *headspace* – (SPME-HS) (Figura 12) (STASHENKO; MARTÍNEZ, 2004).

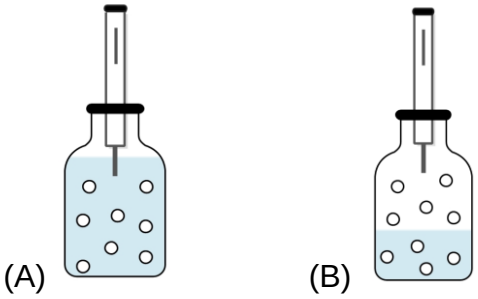

Figura 12. Representação da técnica de microextração em fase sólida através de imersão direta (A) e *headspace* (B).

Conforme o fluxograma abaixo (Figura 13), há disponibilidade de diversas formas de utilização das técnicas de microextração, incluindo a SPME, na obtenção de componentes voláteis das plantas. A sua obtenção deve levar em consideração algumas características da sua composição, trazendo então as opções mais indicadas de análise através de microextração e qual o preparo da amostra mais adequado do material vegetal que proporcionará a análise dos seus constituintes voláteis (YANG; WANG; LI, 2013).

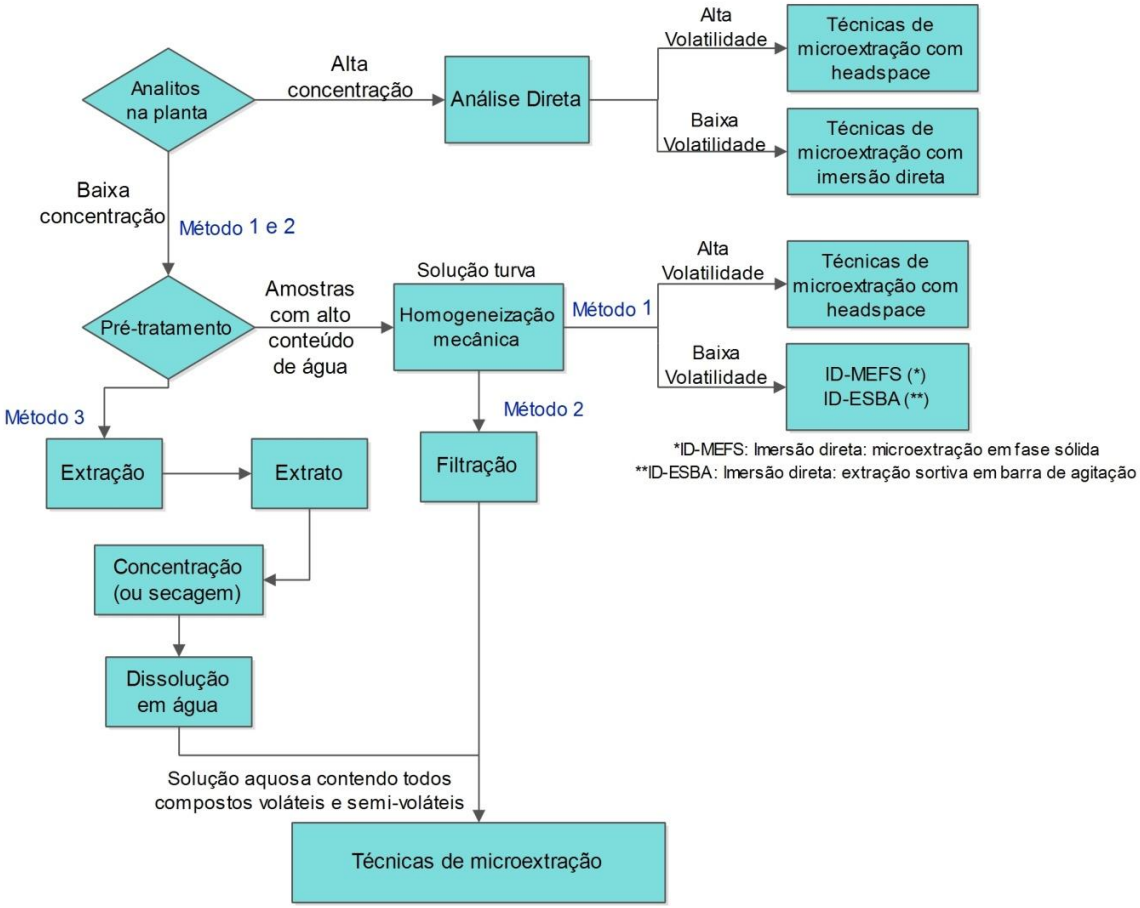

Figura 13. Fluxograma dos processos mais utilizados na obtenção dos componentes voláteis de plantas através de técnicas de microextração. Adaptado de (YANG; WANG; LI, 2013).

3.4.2.2 Headspace (HS)

A técnica de *headspace* (HS) baseia-se na análise dos componentes voláteis de uma amostra quando submetida à aquecimento e agitação. Está técnica permite a análise de compostos voláteis presentes em amostras líquidas ou sólidas, acondicionadas em frascos específicos e devidamente seladas (RESTEK, 2012). É empregada para a análise de compostos orgânicos voláteis presentes em amostras biológicas, como na determinação do etanol em sangue ou saliva, nos produtos farmacêuticos, análises industrial de monômeros em polímeros e plásticos, compostos aromatizantes em bebidas e produtos alimentícios e fragrâncias em perfumes e cosméticos (RESTEK, 2012; SANTOS et al., 2014).

É considerada uma técnica simples, que possibilita a extração e pré-concentração dos compostos voláteis da amostra, sem o seu pré-tratamento e, normalmente, sem a necessidade da utilização de solventes, diminuindo o custo e o tempo das análises (KOLB, 2000; RESTEK, 2012). Na análise por HS, os componentes voláteis da matriz são submetidos à aquecimento em frasco específico (*vial)* e devidamente selado, separando-se dos componentes não voláteis e concentrando-se no "*headspace"* ou espaço livre do *vial*. Após o estabelecimento do tempo ideal de aquecimento para que ocorra o equilíbrio e concentração dos voláteis, uma alíquota da fase gasosa é retirada e então injetada no cromatógrafo gasoso (CG) onde os compostos serão identificados (RESTEK, 2012).

A técnica de HS estático é a mais simples e mais utilizada. Neste caso o recipiente selado é submetido à aquecimento e agitação (manual ou automático) para a formação do vapor e posteriormente uma alíquota do gás é retirado do recipiente através de uma seringa específica *"gás-tight"* (Figura 14-A). Na técnica de HS dinâmico ou *purge on trap,* a amostra é levada através de um gás inerte para um *trap*, compartimento fechado, que então é submetido à aquecimento e posteriormente uma alíquota gasosa é transferida diretamente ao CG (KOLB, 2000) (Figura 14-B).

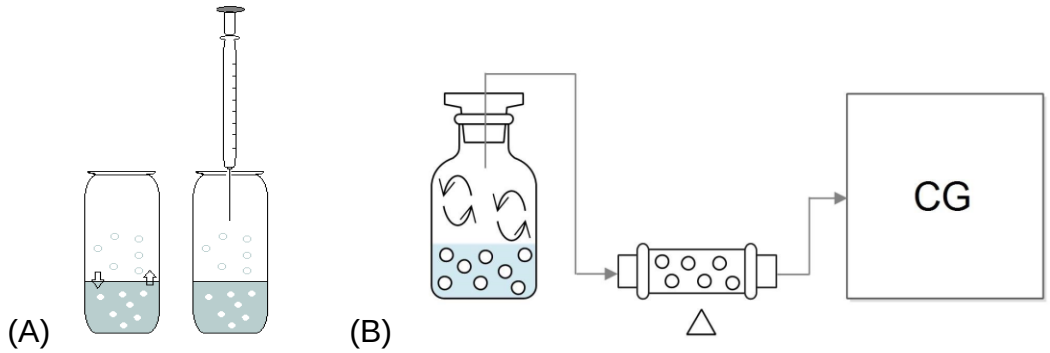

Figura 14. Representação da técnica de extração por *headspace* estático (A) e *headspace* dinâmico (B).

Dados da literatura reportaram o uso da técnica de HS associada à SPME-HS para a determinação dos constituintes voláteis das plantas (BICCHI et al., 2011; LIN et al., 2013; SGORBINI et al., 2015; YANG; WANG; LI, 2013; ZINI et al., 2002). Apesar das vantagens da extração dos constituintes voláteis pela técnica de HS, como a praticidade, simplicidade e baixo custo; quando comparado aos demais métodos de extração dos constituintes voláteis, e inclusive à SPME, há poucos relatos na literatura, e, até o momento, não foram encontrados trabalhos que realizaram a determinação dos constituintes voláteis das partes aéreas de espécies do gênero *Pelargonium* utilizando esta metodologia.

3.5 Análise Estatística Multivariada: Desenho experimental

As análises cromatográficas normalmente requerem etapas de preparação de amostra, a separação e a quantificação dos compostos que serão identificados (FERREIRA et al., 2007b). O planejamento experimental destas etapas através de técnicas estatísticas multivariadas possibilitam a sua otimização, ou seja, a descoberta das melhores condições experimentais que irão fornecer as melhores respostas para os fatores que serão testados (BRERETON, 2007; FERREIRA et al., 2007b; VERA CANDIOTI et al., 2014).

Entre as técnicas estatísticas multivariadas destacam-se as metodologias de superfície de resposta *"Response surface methodolgy"* (RSM). Estas metodologias compreendem um conjunto de técnicas matemáticas e estatísticas no qual o ajuste

dos dados experimentais é realizado através de uma equação polinomial. A equação gerada proporciona a interpretação dos dados e, desta forma, a realização dos testes estatísticos para prever a adequação dos resultados ao propósito da técnica. Sua utilização é indicada quando a resposta requerida, ou o seu conjunto, é influenciado por inúmeras variáveis, pois possibilita a análise simultânea destas variáveis em diferentes níveis (BEZERRA et al., 2008; BRERETON, 2007).

Os desenhos experimentais de superfície de resposta quadrática constituem um conjunto de experimentos que combinam diferentes variáveis em diferentes níveis. A avaliação dos resultados obtidos nos experimentos é realizada através dos valores obtidos de acordo com o tipo de metodologia e técnica empregada, como por exemplo, através dos valores de absorbância, de intensidade do sinal elétrico ou do perfil do(s) pico(s) cromatográfico(s) (BEZERRA et al., 2008)

O Box-Behnken Design (BBD) é um exemplo de desenho utilizado normalmente para otimização de parâmetros cromatográficos como a temperatura, características da coluna, fluxo, e, principalmente, de etapas críticas do preparo da amostra relacionadas à extração dos analitos. Seu objetivo, portanto, é proporcionar um aumento da sensibilidade da técnica, normalmente avaliada pelo aumento da área dos picos obtidos e pela diminuição do tempo de análise (CARINI et al., 2013; FERREIRA et al., 2007b; SANTOS et al., 2014).

No BBD, os pontos experimentais estão localizados de maneira equidistante do ponto central. Todos os fatores devem ser ajustados em três níveis (0, -1, +1) e distribuídos de forma equidistante entre eles (Tabela 3). O BBD tem como característica, enfatizar a repetição dos pontos centrais evitando análises em condições extremas e que possam levar a resultados insatisfatórios. Possui como vantagem um número reduzido de experimentos, o que leva a uma diminuição de custos e do tempo das análises (BEZERRA et al., 2008; FERREIRA et al., 2007b).

Tabela 3. Exemplo de um planejamento experimental através do Box–Behnken design considerando três variáveis (A, B e C), fornecido pelo software Minitab 17®.

A	B	C
-	+	0
0	+	+
0	-	-
+	0	+
+	0	-
0	+	-
0	-	+
0	0	0
0	0	0
+	+	0
-	-	0
0	0	0
+	-	0
-	0	+
-	0	-

As extrações normalmente otimizadas através dos desenhos experimentais são a SPME e extração assistida por microondas, também sendo aplicada para a técnica HS (FERREIRA et al., 2007b; SANTOS et al., 2014; VERA CANDIOTI et al., 2014). No caso do HS estático, variáveis como temperatura de aquecimento, tempo de aquecimento da amostra, volume (ou peso) da amostra, entre outros, são fatores determinantes para a obtenção de condições ótimas de extração (KOLB, 2000; RESTEK, 2012), e, portanto, requerem uma análise prévia das condições ideais para a obtenção de resultados satisfatórios. O BBD constitui, portanto, uma ótima alternativa para a otimização dos parâmetros que influenciam na extração dos compostos analisados pela técnica de HS, já que requer um número reduzido de experimentos, proporciona a combinação entre as diferentes variáveis em três níveis distintos e ainda fornece através da equação e da interpretação dos dados estatísticos gerados as condições ótimas da técnica (BEZERRA et al., 2008; FERREIRA et al., 2007b).

A análise dos componentes voláteis das plantas de forma alternativa à hidrodestilação ou destilação por arraste de vapor de água, através das técnicas de HS já foi reportada (LIN et al., 2013; OMAR et al., 2016; YANG; WANG; LI, 2013; ZHU et al., 2013; ZINI et al., 2002). OMAR e colaboradores (2016) realizaram a otimização de metodologia para a determinação de sete monoterpenos: *α*-pineno, camfeno, *β*-pineno, p-cimene, limoneno, eucaliptol, e *γ*-terpineno, presentes nas espécies de *Rosmarinus officinalis, Salvia officinalis, Lavandula angustifolia* e *Elleteria cardamomum,* através da técnica de HS-dinâmico, utilizando previamente a extração do material vegetal com fluido supercrítico. O método foi otimizado e validado mostrando resultados satisfatórios para precisão e exatidão na determinação dos monoterpenos avaliados.

Assim, através do BBD é possível otimizar a extração dos constituintes voláteis a serem utilzados na extração pela técnica de HS, possibilitando a análise da composição química de acordo com a espécie da planta, órgãos que serão utilizados, quimiotaxonomia, entre outros, trazendo, portanto, uma maior confiabilidade à metodologia empregada na extração dos consitutintes voláteis, que posteriromente serão identificados através das análises cromatográficas.

3.6 Método de análise dos óleos essenciais

A determinação da constituição química dos óleos essenciais é usualmente realizada através de técnicas cromatográficas, especialmente a cromatografia em fase gasosa acoplada ao detector de massas (GC-MS). Como os óleos essenciais são formados predominantemente pelos terpenos, compostos de baixo peso molecular e elevada volatilidade, a cromatografia em fase gasosa é técnica de preferência e mais utilizada na determinação dos seus constituintes, associada ao detector de ionização em chama (FID) e ao detector de massas (MS). A análise dos componentes através do espectro de massas dos compostos, junto com a identificação do índice de retenção relativo à série homóloga de hidrocarbonetos, constituem a metodologia predominantemente utilizada para a caracterização química dos óleos essenciais (ADAMS, 2007; JALALI-HERAVI; ZEKAVAT; SERESHTI, 2006).

Métodos cromatográficos como a cromatografia líquida acoplada aos detectores de massas (LC-MS) e o detector de arranjo de diodos (LC-DAD) são técnicas que também foram reportadas na caracterização química dos óleos essenciais, além da técnica de escolha GC-MS (DI LORENZO et al., 2013; ELSOHLY et al., 2012; JALALI-HERAVI; ZEKAVAT; SERESHTI, 2006). A utilização destes métodos instrumentais de análise com as técnicas cromatográficas viabilizam além da identificação dos componentes da amostra, a sua quantificação, através da determinação das áreas dos picos e/o do seu espectro de massas (JALALI-HERAVI; ZEKAVAT; SERESHTI, 2006).

Através dos dados obtidos na Tabela 1, verifica-se a maior predominância da GC-MS para a análise da composição química dos óleos essenciais de *P. graveolens*, juntamente com a análise através do índice de retenção de *n*-alcanos para a determinação da sua composição química. Alguns autores utilizaram técnicas analíticas mais avançadas para a análise do óleo de gerânio, como a LC-MS/MS e LC-QTOF/MS, a fim de determinar a presença de DMAA em amostras comerciais e extraídas (ELSOHLY et al., 2012, 2015). Apesar do desenvolvimento de metodologias através de equipamentos com maior sensibilidade e especificidade como a LC-MS/MS e LC-QTOF/MS, a DMAA não foi encontrada nos óleos essenciais de gerânio, com exceção dos relatos de PING e colaboradores (1996) e LI e colaboradores (2012).

3.7 Atividade antifúngica dos óleos essenciais de *P. graveolens*

Recentemente, a busca por moléculas naturais que tenham atividade contra bactérias, fungos e vírus levou a uma maior investigação do potencial antimicrobiano dos óleos essenciais em diversas espécies de plantas (RAUT; KARUPPAYIL, 2014; SAVIUC et al., 2015; SONG et al., 2014). Outro grande fator que contribuiu pela busca de novos ingredientes ativos é a emergente resistência dos microrganismos frente aos fármacos tradicionalmente utilizados na terapia convencional e a diminuição do descobrimento de novos fármacos que acompanhem o crescimento na incidência de infecções por microorganismos resistentes e a complexidade do

seu tratamento (KATHIRAVAN et al., 2012; MURPHY COWAN, 1999; ROSATO et al., 2009; SAVIUC et al., 2015).

Os óleos essenciais possuem grande potencial de atividade antimicrobiana principalmente devido à diversidade da sua composição. A ação antimicrobiana dos óleos essenciais pode ser atribuída aos seus compostos majoritários ou à combinação de todos os seus constituintes que contribuem para a sua ação antimicrobiana através de diferentes mecanismos de ação (BAKKALI et al., 2008; BURT, 2004; RAUT; KARUPPAYIL, 2014). Devido a sua característica lipofílica, os óleos essenciais alteram a fluidez da membrana celular dos microrganismos, permitindo a sua passagem através da parede celular e promovendo um rompimento da membrana citoplasmática, modificando a sua estrutura, afetando diferentes camadas de polissacarídeos, ácidos graxos e fosfolípidos. A alteração na permeabilidade das membranas resulta em extravazamento de radicais, íons de cálcio e proteínas, entre outros constituintes, incluindo danos às mitocôndrias, que podem levar à morte celular por apoptose e necrose (ARMSTRONG, 2006; BAKKALI et al., 2008).

A atividade antifúngica dos óleos essenciais contra patogenias humanas, contaminação de alimentos e plantas já foi relatada na literatura (BAG; CHATTOPADHYAY, 2015; SHARIFZADEH et al., 2016) . Os óleos essenciais constituem desta forma, uma alternativa aos medicamentos antifúngicos utilizados na terapêutica, que possuem relatos de efeitos adversos, interação com outros medicamentos, além de apresentarem desenvolvimento de resistência aos tratamentos mais empregados e toxicidade (BAKKALI et al., 2008; RAUT; KARUPPAYIL, 2014; SHIN; LIM, 2004).

Os óleos essenciais de *P. graveolens* foram considerados como potenciais agentes antimicrobianos devido à grande quantidade de monoterpenos oxigenados (SHIN; LIM, 2004). Presentes em elevada concentração, componentes como citronelol, geraniol, acetatos de geranila e outros monoterpenos (BOUKHATEM; KAMELI; SAIDI, 2013; BOUKHRIS et al., 2013; RAVINDRA; KULKARNI, 2015), atuam bloqueando o crescimento de leveduras como *Candida albicans*, através da inibição do seu crescimento na fase S do ciclo celular. Também podem atuar impedindo a formação de biofilmes resistentes à fármacos utilizados no tratamento

de infecções causadas por espécies de *Candida*; inibindo a produção do ergosterol da membrana celular fúngica e atuando na via de sinalização da morfogênese das hifas; podendo também alterar a permeabilidade da membrana, levando ao extravasamento do conteúdo citoplasmático e consequentemente, à morte celular da levedura (AGARWAL; LAL; PRUTHI, 2008).

Os fungos classificados como leveduras (estrutura unicelular) e os filamentosos (estrutura multicelular) são os principais responsáveis pelas infecções de interesse médico (ANDRIOLI et al., 2009; ANVISA, 2004). Dentre as leveduras, o gênero *Candida* é um dos mais importantes, possuindo uma grande contribuição nas infecções oportunistas do trato genitourinário e gastrintestinal encontradas nos adultos (FALAGAS; BETSI; ATHANASIOU, 2006; GUNTHER et al., 2014; PFALLER, 2012). Habita as superfícies das mucosas de indivíduos normais, estando bem adaptado ao corpo humano e, desta forma, contribui para o alto risco de infecções endógenas, conhecidas como candidíases (ÁLVARES; SVIDZINSKI; CONSOLARO, 2007). Entre as mulheres, as leveduras do gênero *Candida*, configuram como um dos principais patógenos responsáveis pelas vulvovaginites (infecção da vulva e vagina) e candidíase vulvovaginal recorrente (ÁLVARES; SVIDZINSKI; CONSOLARO, 2007; GUNTHER et al., 2014; RYLANDER, 2004; ŞENYIĞIT et al., 2014), infecções consideradas importantes na clínica médica devido à sua elevada ocorrência na rede de saúde pública e privada (ANDRIOLI et al., 2009). A principal espécie encontrada nas infecções vaginais é a *Candida albicans* (ÁLVARES; SVIDZINSKI; CONSOLARO, 2007; ANDRIOLI et al., 2009), no entanto, outras espécies de *Candida* podem estar presentes, como por exemplo *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, *C. stellatoidea* e *C. lusitaniae* (ANDRIOLI et al., 2009; SILVA et al., 2011; SULLIVAN; CROWLEY, 2006).

A atividade antifúngica dos óleos essenciais de *P. graveolens* já foi reportada frente a algumas espécies de leveduras e fungos filamentosos, através dos ensaios de concentração inibitória mínima (MIC), compreendida como a concentração de fármaco mais baixa que não resultou em qualquer crescimento visível do fungo em comparação com o crescimento do controle; e do ensaio de avaliação do efeito sinérgico *Checkerboard,* no qual ocorre a associação dos óleos essenciais com diferentes substâncias, incluindo medicamentos utilizados na terapia convencional

(HSOUNA; HAMDI, 2012; ROSATO et al., 2008, 2009; SABZGHABAEE et al., 2011; SHIN; LIM, 2004). Mas apesar dos relatos da atividade antifúngica frente a alguns isolados de *Candida,* até o momento não foram reportados dados da atividade do óleo de gerânio proveniente do Brasil e da correlação entre a variação da composição química dos óleos essenciais de *P. graveolens* de diferentes países, com a sua atividade antifúngica.

Ainda, os óleos essenciais podem sofrer reações químicas e enzimáticas, influenciadas pelas condições de armazenamento. Fatores como temperatura, luz e o contato com o oxigênio atmosférico podem contribuir para a sua degradação, através de diferentes tipos de reações (Figura 15) (TUREK; STINTZING, 2013). Desta forma, uma alternativa para viabilizar o seu emprego em formulações tópicas e aumentar a sua eficácia, é através do uso da nanotecnologia.

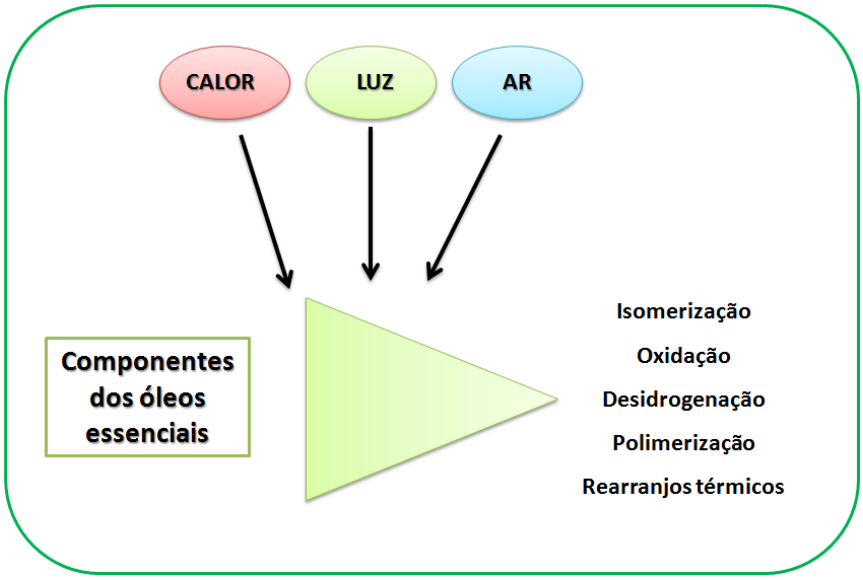

Figura 15. Possíveis reações de degradação que podem ocorrer nos óleos essenciais. Adaptado de (TUREK; STINTZING, 2013).

O desenvolvimento de formulações tópicas utilizando nanocarreadores, como uma nanoemulsão, aumentou recentemente, devido ao interesse pela encapsulação de ingredientes funcionais lipofílicos (SHARIF et al., 2017). Esta é uma abordagem alternativa para desenvolver formulações contendo óleos essenciais, uma vez que

aumenta sua estabilidade física, através da diminuição da sua volatilidade, conferindo proteção, diminuindo o contato com o meio externo e desta forma, evitando interações e reações de degradação (RAVI KUMAR, 2000; SHARIF et al., 2017; ZHAO et al., 2010). Além disso, as nanoformulações podem melhorar a absorção dos compostos ativos na mucosa vaginal ativa, melhorando a eficácia do tratamento, particularmente no caso de candidíase vaginal recorrente (BILIA et al., 2014; FRANK et al., 2014).

A metodologia de incorporação de nanopartículas em um sistema viscoso pode contribuir para o maior tempo de contato e melhor entrega dos compostos ativos no local de ação. Como vantagens, destaca-se uma maior eficácia, diminuição da toxicidade e melhor adesão ao tratamento, quando comparado aos sistemas terapêuticos convencionais (RAVI KUMAR, 2000). Ainda assim, o uso de veículos bioadesivos pode melhorar os efeitos dos compostos ativos, pois permite uma ação terapêutica de longo prazo ao prolongar o contato entre a formulação e a mucosa - mucoadesão (BALOGLU et al., 2009; FRANK et al., 2014).

A própria nanoencapsulação, ao usar um polímero policatiônico, pode melhorar a eficácia da formulação, devido ao aumento das interações entre a superfície da nanopartícula e os constituintes celulares, como no caso dos fungos e leveduras (DE MARCHI et al., 2017). Um exemplo de um polímero catiônico e biodegradável é a quitosana, de origem natural, biocompatível, bioadesivo e solúvel em água, obtido a partir da quitina, um dos polissacarídeos mais abundantes na natureza (FRANK et al., 2014). Além disso, dados da literatura demonstraram o potencial antimicrobiano da quitosana contra bactérias, fungos e leveduras, por diferentes mecanismos de ação (CHAMPER et al., 2013; CHUNG et al., 2004; RABEA et al., 2003). Assim, a quitosana apresenta inúmeras vantagens para ser utilizada durante o desenvolvimento de formulações tópicas antifúngicas, além contribuir para o aumento do espectro de ação antimicrobiano da formulação, tornando mais difícil o surgimento de resistência ao tratamento (ŞENYIĞIT et al., 2014).

Desta forma, o desenvolvimento de uma formulação contendo a nanoemulsão do óleo essencial de *P. graveolens* e quitosana, poderá melhorar a sua eficácia no sítio de ação, prolongando o seu efeito no combate à candidíase vaginal.

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4. CAPÍTULO I - MANUSCRITO I

Determination of 1,3-dimethylamylamine in different species of Pelargonium using a multi-analytical approach

4. MANUSCRITO I

A seguir encontra-se disposto o artigo intitulado ***"Determination of 1,3-dimethylamylamine in different species of Pelargonium using a multi-analytical approach"***, submetido ao periódico *Analytical Methods.*

O manuscrito apresenta uma investigação da presença de DMAA em óleos essenciais de *Pelargonium* spp. através de três técnicas analíticas distintas e nas folhas de espécies de *Pelargonium* spp. do Brasil. Além disso, também foi realizada a determinação da composição química de todos os óleos essenciais obtidos

Determination of 1,3-dimethylamylamine in different species of *Pelargonium* using a multi-analytical approach

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Abstract

The first report regarding the presence of the stimulant 1,3-dimethylamylamine (DMAA) in geranium oil was published in 1996. After this release, DMAA was added to dietary supplements, which were very popular with athletes. However, several studies have questioned the original results and have raised the question about the natural occurrence of DMAA in geranium oil. In 2013, DMAA was subsequently banned from dietary supplements by the FDA. Here, we performed the analysis of DMAA in commercial essential oils of *Pelargonium* sp through a multi-analytical approach, and we determined the chemical composition from commercial and extracted essential oils of *Pelargonium* sp from Brazil and other countries by GC-MS. The presence of DMAA was investigated in essential oils of *P. graveolens* using GC-MS, DART-MS/MS, and LC-MS/MS. Essential oils obtained by hydrodistillation from Brazilian species of *P. fragrans, P. hortorum* and *P. peltatum* were analyzed by GC-MS and its leaves by headspace extraction followed by GC-MS analysis, optimized by an experimental design. For the detection of more volatile compounds the headspace-GC-MS was more favorable than extracted oil analysis by GC-MS, but both methods showed all the samples to be absent of DMAA, indicating that DMAA is not natural to the studied plants.

Keywords: 1,3-dimethylamylamine, DMAA, *Pelargonium,* essential oil, headspace.

1. Introduction

DMAA is a common additive in weight loss compounds and dietary supplements, and it is used recreationally in Europe and New Zealand.[1,2] The frequency of DMAA abuse has recently increased in countries like Brazil, and there is some debate regarding the natural occurance of DMAA in botanic matter.[3] In 1996, a Chinese study provided the first evidence of naturally-occuring DMAA in plants in the species *Pelargonium graveolens,* also known as the rose geranium. Ping and Jun, 1996,[4] carried out the determination of the chemical constituents of the oil extracted from leaves and roots of the plant using hydrodistillation. The main components found were β-citronellol, geraniol, *p*-menthone, linalool, their respective esters of acetate and propionate, and a small amount of DMAA (0.6%).

Since the original observation of DMAA in geranium essential oil, there have been conflicting reports on the presence of DMAA in essential oils of the family Geraniaceae.[2,5–8] Several groups have now evaluated the chemical composition of hydrodistilled or steam distilled essential oils of *Pelargonium* spp., and the presence of DMAA has not been replicated in these studies .[9–14] The only remaining observations of DMAA in essential oils of Geraniaceae are those of Ping and Jun 1996,[4] and Li et *al.*, 2012,[7] who both studied oils from China. Based on the results of naturally-occuring DMAA presented by Ping and Jun, 1996,[4] DMAA could be legally added to the dietary supplements.[15] Over the years, DMAA has become predominantly used as an additive in weight loss compounds and dietary supplements, especially in Brazil.[2,3] Because of it's stimulant activity, the World Antidoping Agency (WADA) banned the consumption of DMAA by the athletes in 2010. Regulatory agencies such as the Brazil National Health Surveillance Agency (ANVISA), and the United States Food and Drug Administration (FDA) also banned the use of DMAA.[16–18] However, even after its prohibition, and despite numerous warning letters from the FDA to dietary supplement manufacturers, DMAA can still be found in dietary supplements.[3,19,20]

The determination of plant constituents is commonly employed using hydrodistillation and steam distillation. Also, because of its exhaustive capability, Soxhlet extraction and supercritical fluids have also been used for the characterization of a more extensive suite of components present in plants.[21] However, a few drawbacks of these methods include: the potential for thermal degradation of thermally labile compounds during high-temperatures extractions; the

slow nature of the extractions, which typically exceed 4 hours; the consumption of large quantities of organic solvents; and the potential to loose the most volatile organics, especially during post-extraction concentration steps.[22,23] Thus, it is hypothesized that past research in this area may have suffered from the loss of volatile and unstable compounds—like the monoterpenes and DMAA, respectively—during the hydrodistillation process.

We therefore used a variety of analytical methods to compare and contrast the effectiveness for identifying different classes of chemicals in the botanic matter and essential oils of different botanic matter of interest. The current work investigated the presence of DMAA in commercial essential oils from Brazil, Egypt, South Africa, China, Reunion Island-Bourbon and Albania, by GC-MS, DART-MS/MS, and LC-MS/MS. We also report on the comparison of headspace GC-MS analysis and GC-MS analysis of essential oils from three from cultivars of *Pelargonium* from Brazil. The headspace extraction of the volatile components of the cultivars was optimized through experimental design using GC-MS as a measure of efficacy. The results demonstrate that DMAA is uncommon or absent in the plants and extracts studied and that fast screening methods using Direct Analysis in Real Time-tandem mass spectrometry (DART-MS/MS) provided all true negatives and no false positives for DMAA in plant matter.

2. Experimental

2.1 Chemicals and materials

Ethyl acetate (99.8%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The HPLC grade solvents Methanol, Formic Acid, Water LC-MS and the Hydrochloric acid were obtained from Fischer Optima (Pittsburgh, PA, USA). Reference compound 1,3-dimethylamylamine hydrochloride (DMAA) was purchased from LGC GmbH (Wesel, Germany) and Sigma-Aldrich. Headspace vials (10 mL) and aluminum screw caps with PTFE–silicone septa and two mL vials were purchased from Agilent Technologies (Agilent J&W Scientific, Folsom, CA, USA).

2.2 Plant material and essential oils

Botanic arterial parts were obtained from three *Pelargonium* cultivars collected at Nova Petrópolis, RS, Brazil, in June 2016, on the same time and day. The species were authenticated by the botanical expert Dr. Mara Ritter and deposited at the

Herbarium of the Federal University of Rio Grande do Sul. The *Pelargonium* species were identified as *Pelargonium hortorum*, ICN 195251, *Pelargonium peltatum* (L.), ICN 195252, *Pelargonium fragrans* (*Pelargonium odoratissimum* x *P. extipulatum*), ICN 195294. Also, eight commercial essential oils of *P*. *graveolens* were obtained by a donation from Laszlo Aromaterapia LTDA (Egypt, South Africa, China, Reunion Island-Bourbon, Albania), Ferquima (Africa) and Verbena (Brazil and Africa).

2.3 Essential oils (EO) extraction

2.3.1 Hydrodistillation. One hundred grams of fresh leaves of *Pelargonium* species from the Rio Grande do Sul, Brazil, was chopped and weighed. Extraction was conducted through Clevenger apparatus by hydrodistillation for 4 hours.[24] The hydrodistilled essential oils were then reconstituted in ethyl acetate, diluted and analyzed by GC-MS. Due to the relatively low yield (less than 1%), the essential oils obtained by the hydrodistillation were analyzed using only GC-MS.

2.4 Headspace analysis and Experimental Design

The experimental conditions of the headspace sampling method were optimized using the Box–Behnken design (BBD).[25] Headspace analyses were performed with fresh leaves that were chopped and weighed (0.5, 1.0 and 1.5 g), before extraction. The headspace volume was 10 ml and the headspace gas volume injected on the GC-MS was 1.0 mL.

The designs were programmed considering the preliminary tests trough headspace analysis of leaves and the major compounds found in the essential oil liquid injection. Three different experimental designs, one for each *Pelargonium* specie, were performed for three factors at three levels: heating temperature 80 °C (-1), 115 °C (0) and 150 °C (+1); stirring time 10 min (-1), 20 min (0) and 30 min (+1) weight of leaves 0.5 g (-1), 1.0 g (0) and 1.5 g (+1) (Table 1).

Table 1

The three experiments were carried out in random order totaling 15 runs for each design (Table 1). Three major compounds of each specie (Table 2) were selected as outcome variables based on prior knowledge obtained by the analysis of essential

oils. All headspace analyses of the essential oils were performed in the same chromatographic method.

Experimental data were fitted following a second-order polynomial model (equation). **Yi** generically represents each response, **n** is the number of factors or variables, $\mathbf{b_0}$ is the regression coefficient of the intercept, and $\mathbf{b_i}$, $\mathbf{b_{ii}}$, and $\mathbf{b_{ij}}$ are the regression coefficients for the linear, quadratic and interaction of each factor **Ai**, respectively.

$$Yi = b_0 + \sum_{i=1}^{n} biAi + \sum_{i=1}^{n} biiA^2i + \sum_{1\leq i\leq j}^{n} bijAij$$

The validity and predictive capacity of the mathematical model were evaluated under optimal conditions to compare the optimum responses obtained by the model with the experimental results. The model considered the results obtained for each compound (total of nine) that were carefully analyzed with the residual analysis and other statistical parameters offered by Minitab 17.0 software.

Table 2

2.5 Sample preparation

Before the GC-MS liquid injections, the hydrodistilled essential oils and the commercial essential oils were diluted to 2% in ethyl acetate. For the LC-MS/MS analysis, the commercial essential oils were extracted following the methodology described by Elsohly *et al*., 2015.[26] The method used a liquid-liquid extraction with hexane and 0.5 M hydrochloric acid (HCl), following centrifugation. The aqueous layer was then filtered and analyzed. For the DART-MS/MS analysis, the commercial essential oils were diluted to 0.1% in methanol, and two microliters of each sample were pipetted onto a clean capillary tube and allowed to dry before analysis.

2.6 Analytical procedure

2.6.1 GC-MS. The GC-MS analyses were performed on a 7890A gas chromatograph coupled with a 5975C mass spectrometer (Agilent Technologies, CA, USA), equipped with an automatic headspace auto-sampler (CTC Analytics Combipal, Basel, Switzerland). A fused-silica DB-5 column (30 m x 0.25 mm x 0.25 µm) was

employed for chromatographic separation. The mass detector was operated using 70 eV electrons with a source temperature, transfer line and injection port set at, 150 °C, 300 °C, and 220 °C, respectively. The oven temperature was programmed to start at 60 to 300 °C, with an increase of 3 °C /min. Ultrapure helium was utilized as the carrier gas at a flow rate of 1 mL/min. Compounds were identified using a combination of their retention indices—using *n*-alkanes as external standards—and their mass spectral similarities to literature and NIST database entries.[27]

2.6.2 DART-MS/MS. DART analyses were performed using a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer. The DART source was attached to a standard Vapur® interface (Ionsense, Saugus, MA, USA) and fitted to the mass spectrometer using a custom-built 3D-printed flange. All the experiments were performed in triplicate. A blank of methanol was run between each sample. The commercial essential oils were analyzed using both full scan mode, from *m/z* 40-350, and selected reaction monitoring (SRM) mode using compound-specific transitions. For DMAA, the precursor and product ions transitions for [M+H] were *m/z* 116→57 and *m/z* 116→43. The collision energy selected for DMAA was 18 V. The DART ion source was operated with the helium gas at 300 °C, a flow rate of 2 L/min, and a grid voltage of 530 V. Xcalibur 2.0.7 software was used for data processing. Ultra high purity helium (DART gas) and nitrogen (collision gas) were from Matheson.

2.6.3 LC-MS/MS. LC-MS/MS analyses were performed using a Shimadzu Liquid Chromatograph LC-20AD (Columbia, MD, USA) coupled to Applied Biosystems MDS Sciex 3200 QTRAP (Foster City, CA, USA) equipped with a turbo V^{TM} ion source. A Luna® Omega Polar C_{18} column (50 x 2,1mm ID, 3.0 µm), Phenomenex® (Torrance, California, USA) and a line filter KrudKatcher Phenomenex® were used for the chromatographic analysis. The column oven was maintained at 35 °C, and the autosampler was cooled to 5 °C. The injection volume was five microliters, and flow was continuously set at 0.5 mL/min. The mobile phase consisted of A: Milli-Q water with 0.1% of Formic Acid and B: Methanol with 0.1% of Formic Acid. The MS system was operated in positive electrospray ionization mode (ESI+) and the multiple reaction monitoring (MRM) mode. For DMAA confirmation, the same precursor and product ions used in the DART-MS/MS analysis were monitored. Analyst 1.6.1 software was used for data acquisition and analysis. The DMAA linearity range was

established using concentrations between 25 and 1000 ppb. The lower limit of quantification for DMAA was 25 ppb and was estimated at a signal-to-noise ratio (S/N) ratio of ten.

3 Results and Discussion

3.1 Chemical Characterization of *P. hortorum, P. peltatum*, and *P.fragrans*

The chemical composition of the hydrodistilled essential oils from *P. hortorum*, *P. peltatum*, and *P. fragrans* were determined by comparison of mass spectra and retention indices of n-alkanes with the literature (ESI 1). The essential oils of *P. hortorum* and *P. peltatum* showed fewer monoterpenes and more sesquiterpenes than expected, with the sesquiterpenes representing 79.0 and 90.0 % of their composition in *P. hortorum* and *P. peltatum*, respectively. Citronellol and geraniol were absent in both these species. Monoterpenes were practically absent in hydrodistilled essential oils species of *P. hortorum* and *P. peltatum* (Fig. 1).

DMAA was not found in any of the three *Pelargonium* species studied by using the traditional hydrodistillation method. Because the monoterpenes were less abundant than expected, one concern was that the extraction method was based against volatile components. Therefore alternative approaches involving the headspace extraction directly from the leaves obtained from *P. hortorum*, *P. peltatum*, *P. fragrans* were attempted to enable the determination of DMAA, if it was present. Multivatiate design of experiments was used to ensure that the best extraction conditions were used in headspace analysis *P. hortorum* and *P. peltatum.*

Figure 1

3.2 Headspace analysis

The objective of the Box Behnken design (BBD) was to investigate the influence of the extraction variables for compounds present in the leaves of different *Pelargonium* cultivars. Therefore, the purpose of the BBD was both to maximize the response of nine compounds of interest and to evaluate the presence of DMAA. The parameters studied were temperature, stirring time and weight of leaves. The BDD optimization approach provides maximum sensitivity (largest peak areas) for the studied analytes. The three variables were weighted with the same degree of importance.

The selected compounds were first evaluated to see if the model was adequate. ANOVA was used to examine the coefficients of correlation, the lack-of-fit and the p-values for various factors (Table 3). In assessing the adequacy of the model, outliers were also considered—as was the type and significance of any interactions between variables (compounds). For example, one assumption was wether or not the mathematical model could describe the relationship between responses and factors evaluated. In this model, x_1 represents the heating temperature, x_2 the stirring time, and x_3 the weight of the leaves (Table 3). From the results obtained for each of analyzed compound, optimization was performed by the software and evaluated by the desirability value, which informed the best extraction conditions. Extraction conditions were seleced that gave the highest probabilities of providing the optimal values for each variable (Table 3). Furthermore, the predictive capacity of the software was tested by comparing predicted and experimental responses and by analyzing the relative standard deviation (RSD) obtained from the analysis of five replicates under the optimized conditions. An RSD less than 15% was considered acceptable.

Table 3

3.2.1 Extraction optimization of *P. fragrans.* For limonene and fenchone, the extraction model gave p-values<0.05, which demonstrates that there is a lack-of-fit of the model. The lack of fit can be explained by the presence of outliers for both compounds. However, the R^2 obtained by ANOVA for the two compounds was higher than 0.80, which shows the significant influence of temperature on the extraction. The model for limonene and fenchone can be classified as a quadratic polynomial ($p < 0.05$) (Fig. 2A). In this case, there was no significant interaction between the variables tested (time and weight). Also, the temperature is significant in the extraction of both limonene and fenchone. Methyl-eugenol presented $p > 0.05$, which demonstrates the suitability of the model to parameters tested, in agreement with the value of R^2 that was higher than 0.95. There were no outliers, and ANOVA analysis showed values of $p < 0.05$ for all variables when evaluated independently, which then contributed significantly to the extraction (Fig. 2A). The model also considered a quadratic polynomial. For methyl-eugenol, the variables of weight, temperature and extraction time significantly influenced the extraction, but independently (Table 3).

For the contour plots of methyl-eugenol (Fig. 2A), the largest peak area—and therefore best extraction efficiencies—were obtained around the intermediate conditions of time (15-25 mins), temperature (110-130 °C) and sample size (0.75-1.25 g). In contrast, the optimum extraction conditions for germacrene-B were using 1.5 g of the sample for only 10 minutes. As might be expected, the least volatile compound, 10-epi-gamma-eudesmol, was best extracted using 1.0 g samples at the highest temperatures (150 °C) and most extended extraction times (30 mins).

The model generated the following optimum conditions of analysis for the three studied compounds: temperature (113 °C), stirring time (19.5 min) and weight (1.07 g). Through the predictive capacity, RSDs less than 5% were obtained, which provides the reliable evaluation of the best extraction conditions for *P. fragrans* leaves.

3.2.2 Extraction optimization of *P. peltatum.* For beta-caryophyllene and germacrene-B compounds, the model was appropriate ($p > 0.05$). However, the model was not appropriate for gamma-cadinene, as demonstrated by the lack-of-fit of the model ($p < 0.05$). Furthermore, for the three compounds studied, the R^2 values were found to be less than 0.80. ANOVA analysis did not show any significant differences in the models (quadratic, linear and interaction), so it was concluded that there was minimal influence of tested variables in extraction (Table 3). Thus, the optimization result evaluated by the desirability value, below 0.8, can be considered weak (Table 3). The values of RSD found were all higher than 15%, so the best conditions of temperature (148 °C), stirring time (10 min) and weight (1.5 g) are not strict requirements. Through the germacrene-B contour plot graph, the best responses were obtained for the optimization of the extraction, despite the non-adequacy of the model. The contour plot graph (Fig. 2B) showed that with intermediate conditions of time and temperature the most significant values of the area were obtained. In this way, the weight can be considered as the determining factor in the improvement of extraction conditions of germacrene-B.

3.2.3 Extraction optimization of *P. hortorum.* For all evaluated compounds—beta-caryophyllene, alpha-humulene, and 10-epi-gamma-eudesmol—the model was appropriate with values of $p>0.05$. Also, an R^2 below 0.80 and the presence of outliers were found. Using ANOVA, no significant differences were found in any

model (quadratic, linear and interaction). Therefore, no influence of the variables tested was verified in the extraction (Fig. 2C). Thus, the optimization result, evaluated by the desirability value, was below 0.5, which is considered to be poor (Table 3). The values of RSD found for the predictive capacity were all higher than 15%. Combining all three compounds, the optimum conditions were found to be an extraction temperature of 123 °C, an extraction time of 30 min and a mass of 0.82

Figure 2

Considering the lack-of-fit of the model, the absence of interactions, and that there was minimal influence of the variables on the extraction efficiencies; hindsight demonstrates that the ranges used for the variables tested in these two species were not sufficiently broad. Despite the results, the contour plots indicated that the most significant parameters in both cases are temperature and weight. In future work, better limits of detection could presumably be achieved through larger sample sizes and higher extraction temperatures.

Table 3

As previously discussed, the difference in chemical composition and chemical profile of the classes of compounds of each species should be considered. *P. hortorum* and *P. peltatum* have higher levels of sesquiterpenes—compounds with a higher boiling temperature than monoterpenes—which therefore require a higher temperature extractions. Monoterpenes were very abundant in the leaves extracted by headspace extraction, but almost non-existent in the essential oil of *P. hortorum*, which was obtained by hydrodistillation (Fig. 3). The extreme range of volatilities of extractable compounds makes it challenging for one technique to extract all the compounds with uniform efficiency. Headspace extraction favors volatile organics like the monoterpenes, and hydrodistillation prefers semi- and non-volatiles like the sesquiterpenes. Through the surface contour graphs of these species (Fig. 2), the results show that the amount of plant material used in the design has a significant influence on the resulting peak areas. So, to obtain a more efficient extraction of these compounds, it is necessary to adjust the weight values (e.g., to above 1.5 g).

Nevertheless, the BBD can be considered a useful tool to determine the best conditions for headspace extraction, which proves to be sensitive to the more volatile compounds in plants, like the monoterpenes and DMAA. At higher temperatures and longer extraction times, it is possible to obtain better results for the less-volatile compounds such as the sesquiterpenes. After the optimization results, the best conditions were selected, and the presence of DMAA was evaluated. DMAA was not present in hydrodistilled extracts, either because DMAA is not present, ot because it's volatility makes it difficult to extraction using hydrodistillation.[22,23] However, DMAA was also not found in any *Pelargonium* sp. after headspace, even though headspace was more amenable to DMAA extraction. The absence of DMAA in headspace-extracted matter confirms its absence in Brazilian species of *Pelargonium*.

Figure 3

3.3 *P. graveolens*

3.3.1 GC-MS. More than 70 compounds were identified in the commercial essential oils of *P. graveolens* analyzed through the methodology previously described (ESI 2). Through a combination of retention time matching and mass spectral comparisons to database spectra, between 97.7 and 99.8% of the GC-MS peaks were identified in the eight commercial essential oils (Fig. 4).

In the essential oils of *Verbena* from Brazil and Africa, geraniol appears as the primary compound, with relative concentrations of about 40% while citronellol reaches less than 12% . Similar results are presented elsewhere for essential oils obtained from India and the United Kingdom using analogous extraction methods.[28,29] In the essential oils from Brazil, the oxygenated monoterpenes represent nearly 60% of the essential oil composition (Fig. 4). The essential oils of *Lazslo* from Egypt, South Africa and China presented the highest percentage of oxygenated monoterpenes, greater than 70% (Fig. 4), with a total of monoterpenes of about 90%. Geranium oil from Reunion Island "Bourboun", which the cosmetic industry touts as having the best quality of the *P. graveolens* essential oils, was comprised of almost 70% of monoterpenes, which makes the monoterpenes more abundant in *P. graveolens than in* to geranium oils from Egypt, South Africa and China.[30,31]

Figure 4

Although the essential oils of the four species analyzed by GC-MS belong to the same genus (*Pelargonium*), there is a significant difference between the chemical profile of the essential oil of *P. graveolens*, *P. fragrans*, *P. hortorum* and *P. peltatum,* especially in the relative abundance of monoterpenes and sesquiterpenes.

None of the extracted oils or commercial oils, including the sample from China, contained DMAA (Fig. 5), despite the fact that some exemplars from China have previously been reported to contain DMAA.[4,7] The headspace GC-MS and hydrodistillation GC-MS results are therefore in agreement with the bulk of the literature in this regard.[2,5,6,8,13,32,33] The employed extraction methods were verified for DMAA by extracting a spiked analytical standard of DMAA in the same way used for the analysis of essential oils. The results show that DMAA has a retention time (RT) of 3.677 min (Fig. 5). Besides the proposed headspace analysis of leaves and the essential oil analysis by GC-MS, two non-traditional analytical techniques were performed specifically to detect the presence of DMAA in the essential oils; these methods included DART-MS/MS and LC-MS/MS.

Figure 5

3.3.2 DART-MS/MS of essential oils. DART-MS is an analytical tool for the rapid analysis of samples at atmospheric pressure.[33] DART-MS can be used for solid or liquid materials deposited or adsorbed onto surfaces in the open atmosphere, without sample pre-treatment. DART-MS can be performed in both positive and negative ion modes and can detect compounds with a range of polarities and low to medium molecular masses.[34,35] These advantages have contributed to DART's rising popularity in different fields, such as quality control, clinical and pharmaceutical applications, forensics, biological studies, food quality and food safety.[36–42] DART-MS has also been reported for the analysis of essential oils.[26,37,43] DART-MS is most commonly performed using an accurate-mass time of flight (ToF) mass spectrometer, and any fragmentation analysis is typically performed using in-source CID to achieve pseudo-tandem mass spectra. In such experiments, there is less confidence that a given fragment belongs to a given precursor because the precursor ions cannot be

isolated before activation. In the present work, a triple quadrupole mass spectrometer is used to obain true tandem mass spectra of DART-generated precursor ions.

A DMAA standard was used to obtain optimum conditions for ionization and selected reaction monitoring (SRM) transitions at 1 ppm. After the selection of most abundant ions and best source parameters, the essential oils were analyzed in product ion scan mode and SRM scan mode (Fig. 6). The SRM transitions were *m/z* 116→57 and 116→43, which are the primary fragmentation pathways for the protonated precursor of DMAA $[M+H]^+$ ion at *m/z* 116. The DART-MS/MS results showed that DMAA was not detected in any of the essential oils (e.g., Figure 6), in agreement with the DART-MS/MS results [26] and the GC-MS results presented in this study.

Despite the absence of DMAA in the essential oils, DART-MS/MS allowed their rapid analysis (less than 1 min per sample) without pre-treatment, using a minimal amount of sample (2 µL), which makes DART-MS/MS a good alternative for the confirmation of substances in plant materials. Also, DART-MS/MS can be useful for the quality control purpose of the essential oils, allowing a simple and clear mass spectra analysis, even with the presence of multiple compounds, with an excellent sensitivity response.[26,37,44]

Figure 6

3.3.3 LC-MS/MS. A 25 ppb DMAA standard was used to spiked the essential oil matrix to assess recovery efficiency. For the spiked sample, MRM transitions for DMAA were observed at the retention time of 4.37 min (Fig. 7). Although DMAA could be recovered at 90%, DMAA was not identified in any of the unspiked essential oil samples (Fig. 7). These results concur with other LC-MS/MS published results, [5,13] and with the data obtained in this study using GC-MS and DART-MS/MS. DMAA was not found in the essential oils from Brazilian *Pelargonium* sp. or *Pelargonium* from any countries, including China—the only nation to date to have demonstrated the presence of DMAA in essential oils.[4,7]

Figure 7

4 Conclusions

DMAA was not identified in any geranium oil, whether evaluated using GC-MS, DART-MS/MS or LC-MS/MS, which suggests that DMAA is not natural in these species. The significant contribution of this study was to assess the presence of DMAA in essential oils from *Pelargonium* species from Brazil and to facilitate the search of DMAA in leaves of *Pelargonium* spp. using a headspace technique followed by GC-MS analysis. Despite the absence of DMAA in leaves and essential oils, DART-MS/MS and headspace-GC-MS can be considered as an excellent alternative to the traditional methods of plants extraction—like hydrodistillation—when the identification of the chemical composition and the determination of the chemical profile of volatile components in plants are the primary objective.

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Conflicts of interest

There are no conflicts to declare.

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Table 1. Box-Behnken Design showing the three factors evaluated and their levels.

Run Order	Temperature (ºC)	Time (min)	Weight (g)
1	80	30	1
2	115	30	1.5
3	115	10	0.5
4	150	20	1.5
5	150	20	0.5
6	115	30	0.5
7	115	10	1.5
8	115	20	1
9	115	20	1
10	150	30	1
11	80	10	1
12	115	20	1
13	150	10	1
14	80	20	1.5
15	80	20	0.5

Table 2. Major compounds selected for the optimization parameters of each Box–Behnken design.

Plant species	**Major compounds**		
P. fragrans	fenchone	limonene	methyl-eugenol
P. hortorum	beta-caryophyllene	alpha-humulene	10-epi-gamma-eudesmol
P. peltatum	beta-caryophyllene	gamma-cadinene	germacrene-B

Table 3. ANOVA *p-values* from Box Behnken Design for three species of *Pelargonium.*

	Compounds of *P. Fragrans*			**Compounds of *P. hortorum***			**Compounds of *P. peltatum***		
Terms	limonene	fenchone	methyl-eugenol	beta-caryophyllene	alpha-humulene	10-epi-gama-eudesmol	beta-caryophyllene	gamma-cadinene	germacrene-
X_1	0.037*	0.120	0.312	0.934	0.974	0.157	0.175	0.146	0.827
X_2	0.649	0.911	0.654	0.783	0.463	0.121	0.283	0.776	0.205
X_3	0.062	0.086	0.462	0.783	0.414	0.945	0.056	0.133	0.285
X_1^2	0.012*	0.005*	0.000*	0.028*	0.045*	0.570	0.395	0.462	0.091
X_2^2	0.148	0.157	0.009*	0.748	0.477	0.438	0.672	0.406	0.895
X_3^2	0.182	0.143	0.007*	0.755	0.470	0.332	0.411	0.294	0.898
$X_1 X_2$	0.713	0.751	0.216	0.946	0.917	0.070	0.822	0.941	0.920
$X_1 X_3$	0.931	0.999	0.627	0.938	0.944	0.951	0.102	0.065	0.684
$X_2 X_3$	0.568	0.203	0.086	0.136	0.539	0.745	0.917	0.874	0.508
Lack-of-fit	0.007*	0.032*	0.207	0.988	0.805	0.068	0.783	0.012*	0.738
R^2	0.868	0.878	0.953	0.719	0.656	0.737	0.763	0.748	0.637

X_1 = temperature (°C); X_2 = time (min); X_3 = weight (g).
* Significant difference at *p-value*<0.05.

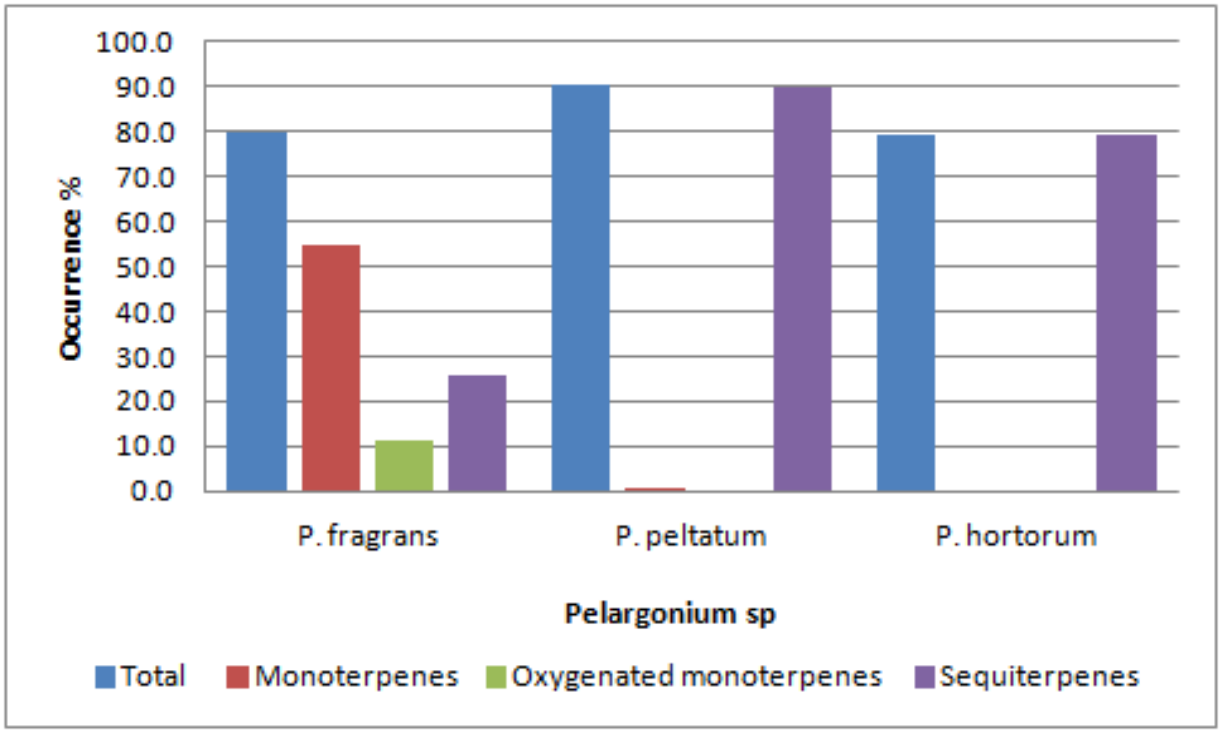

Figure 1. Classes of compounds identified in the hydrodistilled essential oils of *P. fragrans*, *P. hortorum* and *P. peltatum* (%).

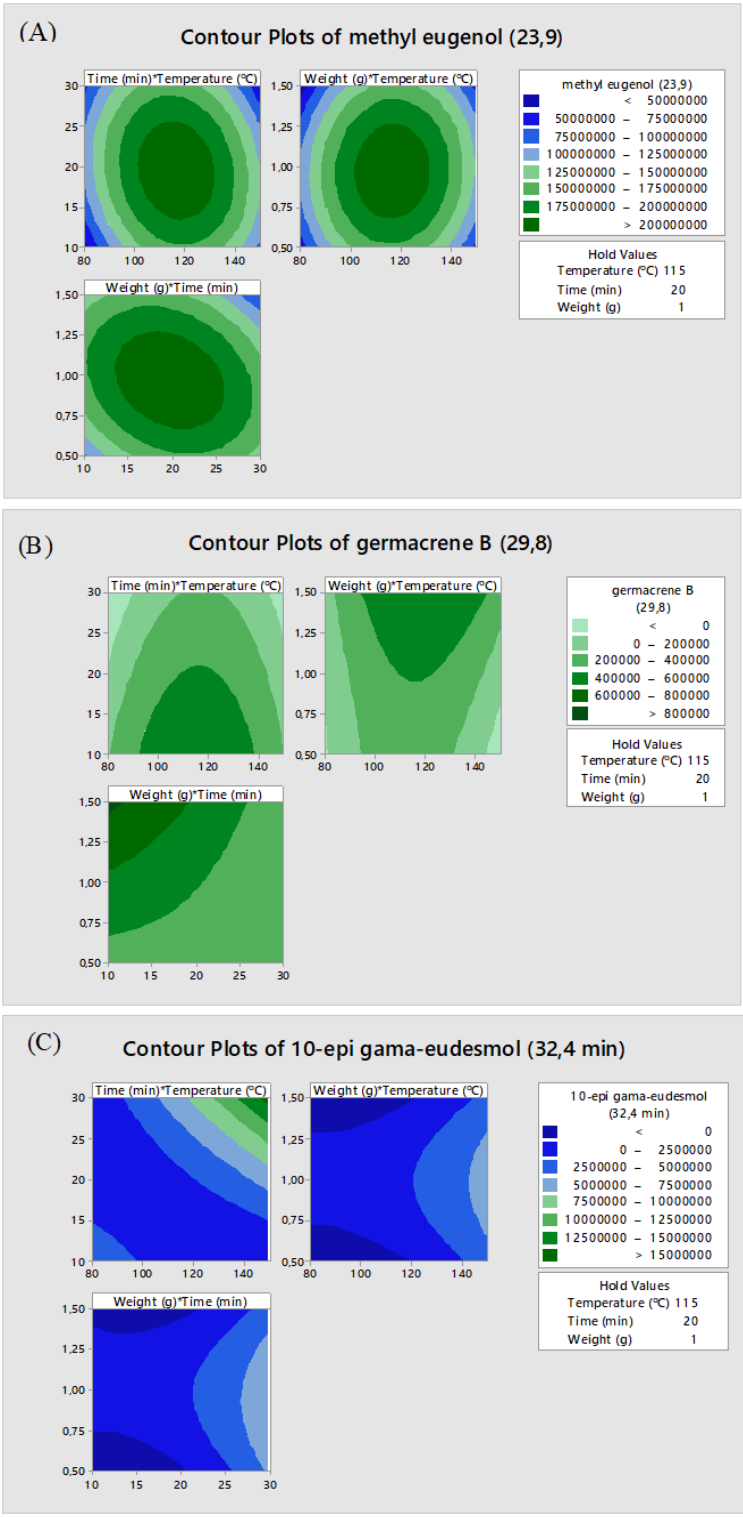

Figure 2. Contour plots of methyl-eugenol (A) from *P. fragrans*; 10-epi-gamma-eudesmol (B) from *P. peltatum* and germacrene B (C) from *P. hortorum,* obtained by BBD for the three factors evaluated in the headspace: heating temperature, stirring time and weight of the leaves.

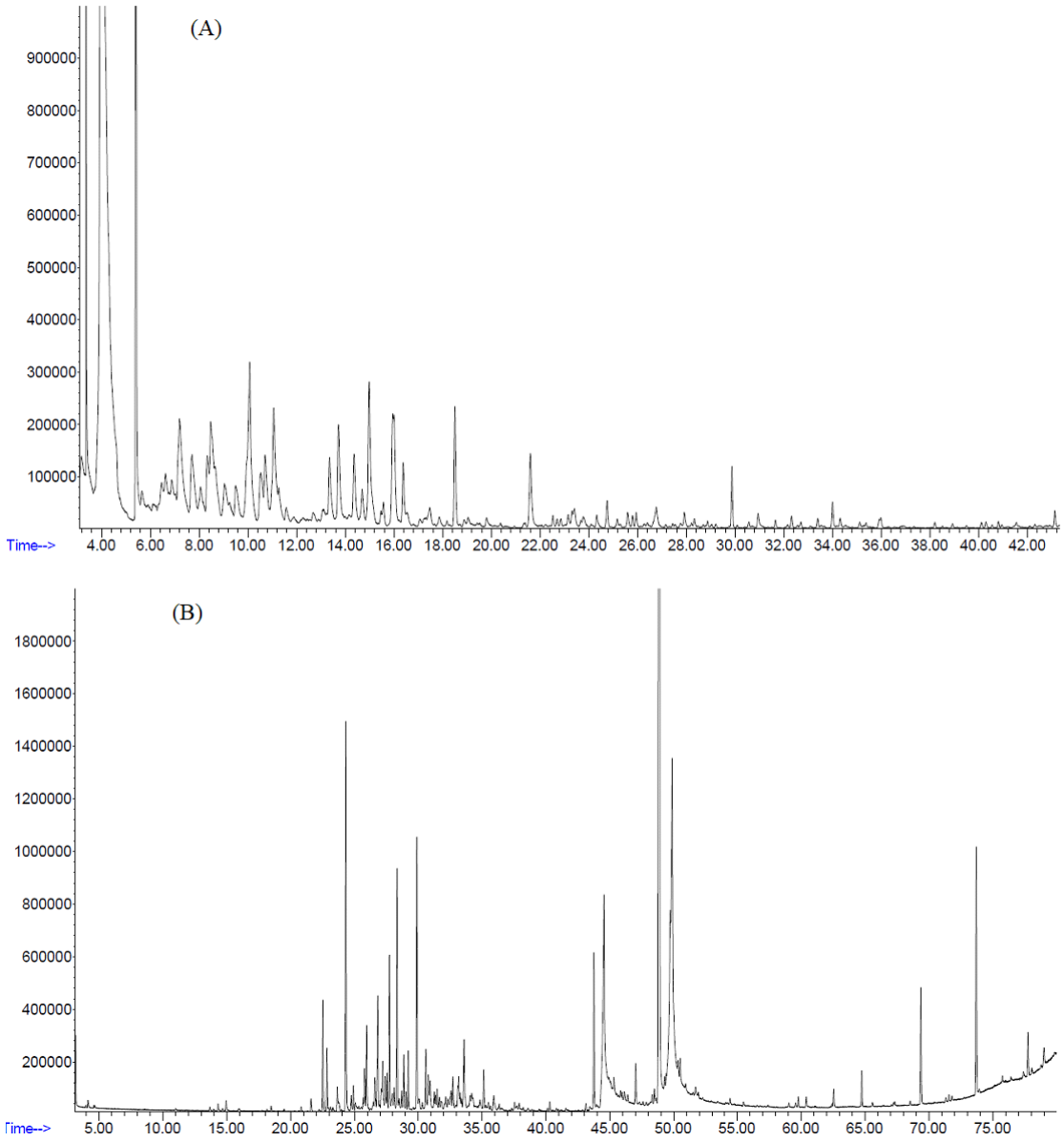

Figure 3. Chromatograms of leaf headspace (A) and hydrodistilled essential oil (B) samples of the same botanic matter of *P. hortorum*.

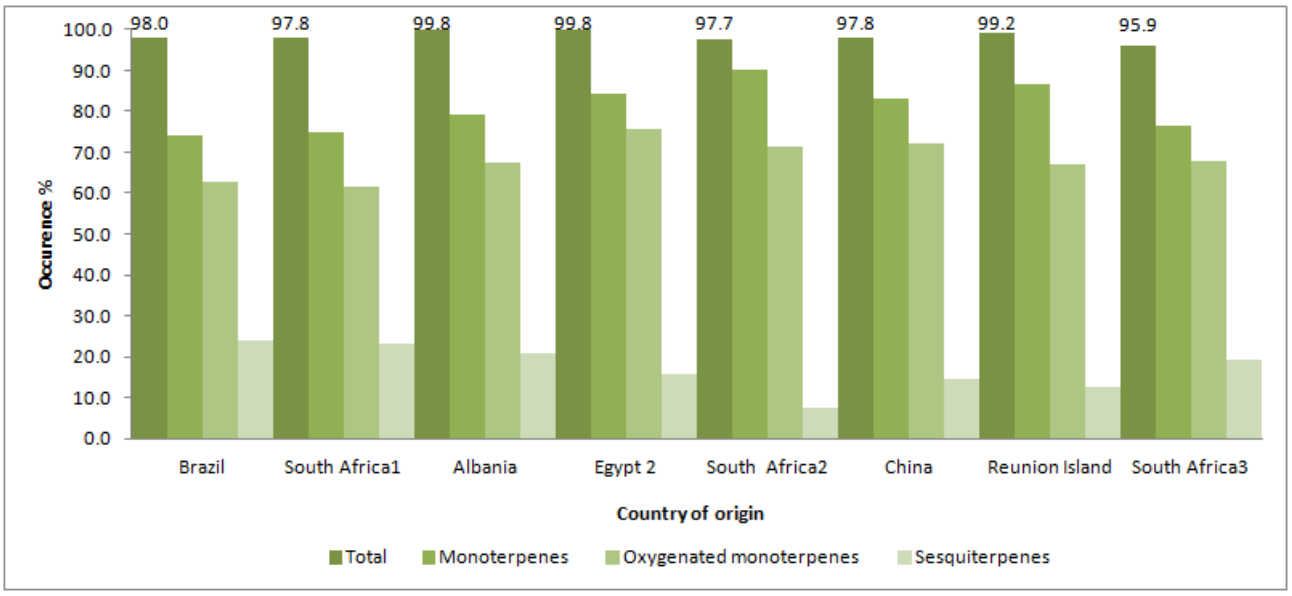

Figure 4. Percent composition of classes of compounds identified in the essential oils of *P. graveolens* (%)* (1 Verbena, 2 Laszlo Aromaterapia and 3 Ferquima). *Numbers do not sum to 100% because oxygenated monoterpenes are a sub-class of monoterpenes.

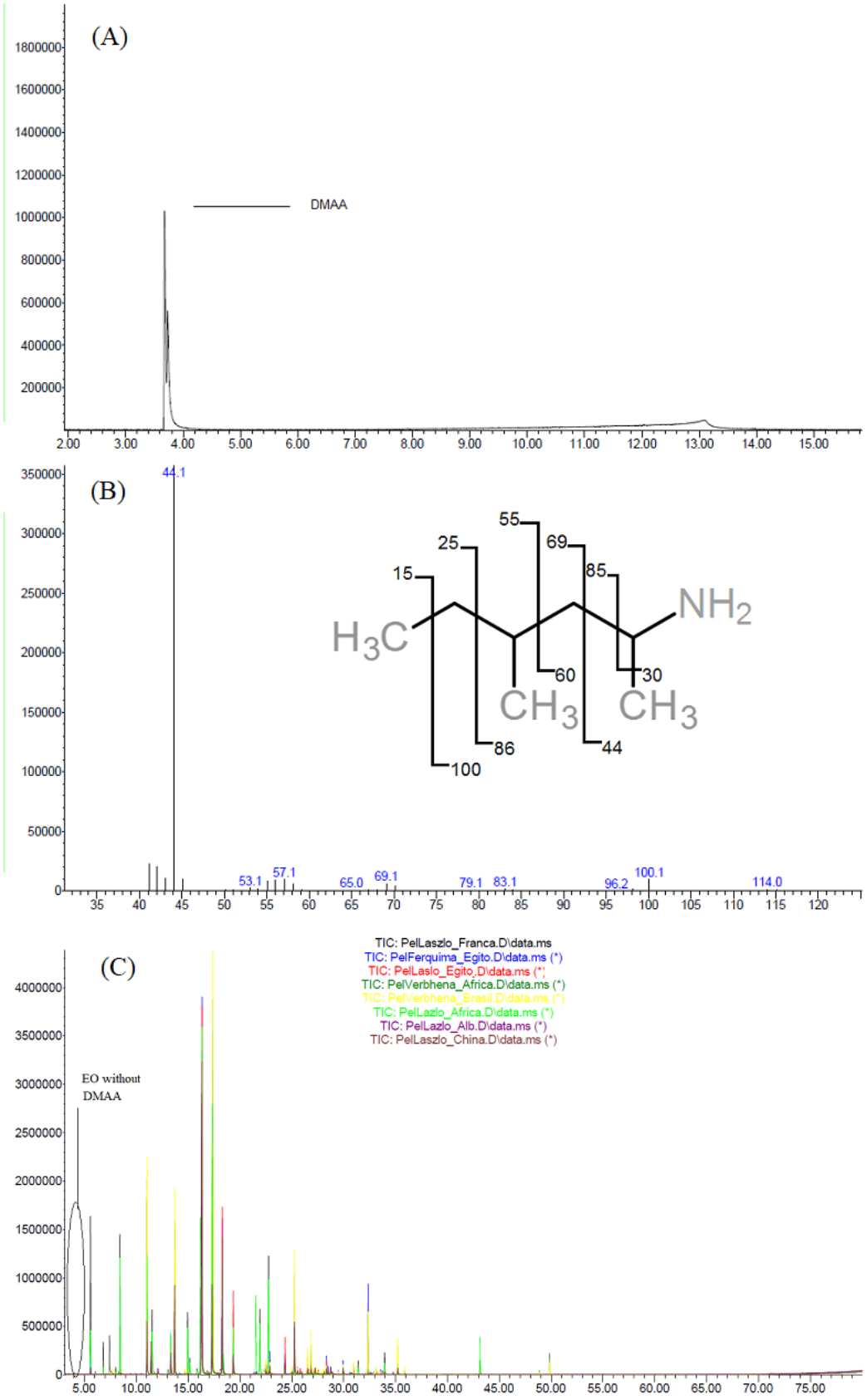

Figure 5. GC-MS Chromatogram (A) and mass spectra (B) of DMAA and overlaid chromatograms obtained from the eight commercial essential oils of *P. graveolens* showing the absence of DMAA (C).

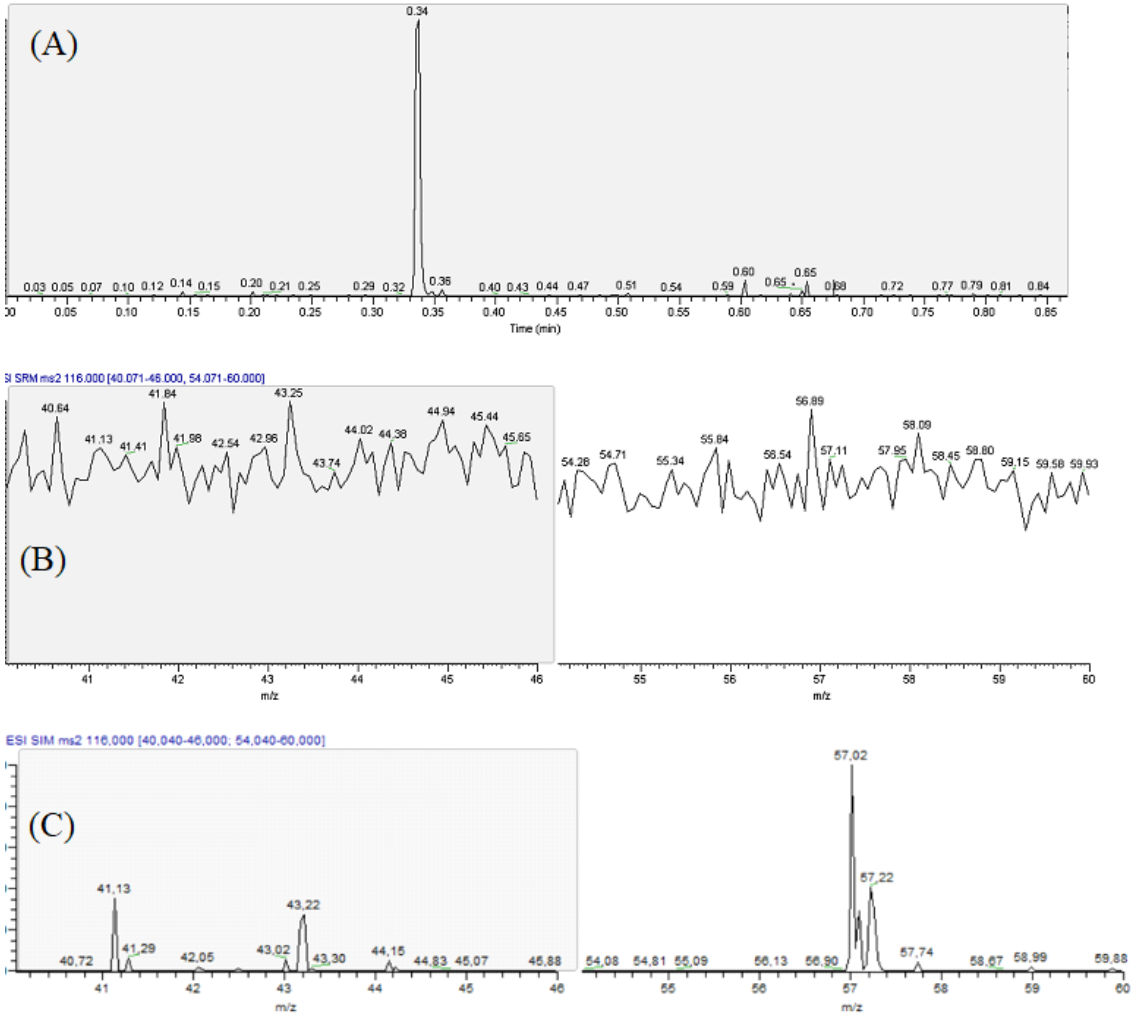

Figure 6. DART-MS/MS analysis of essential oils of *P. graveolens* from China. (A) Chromatogram of the TIC showing the essential introduction at 0.34 minutes; (B) background product ion scan of *m/z* 116 at 0 minutes; (C) Product ion scan of a DMAA standard *m/z* 116 at 0.34 minutes showing DMAA-specific product ions at *m/z 57* and *m/z* 41.

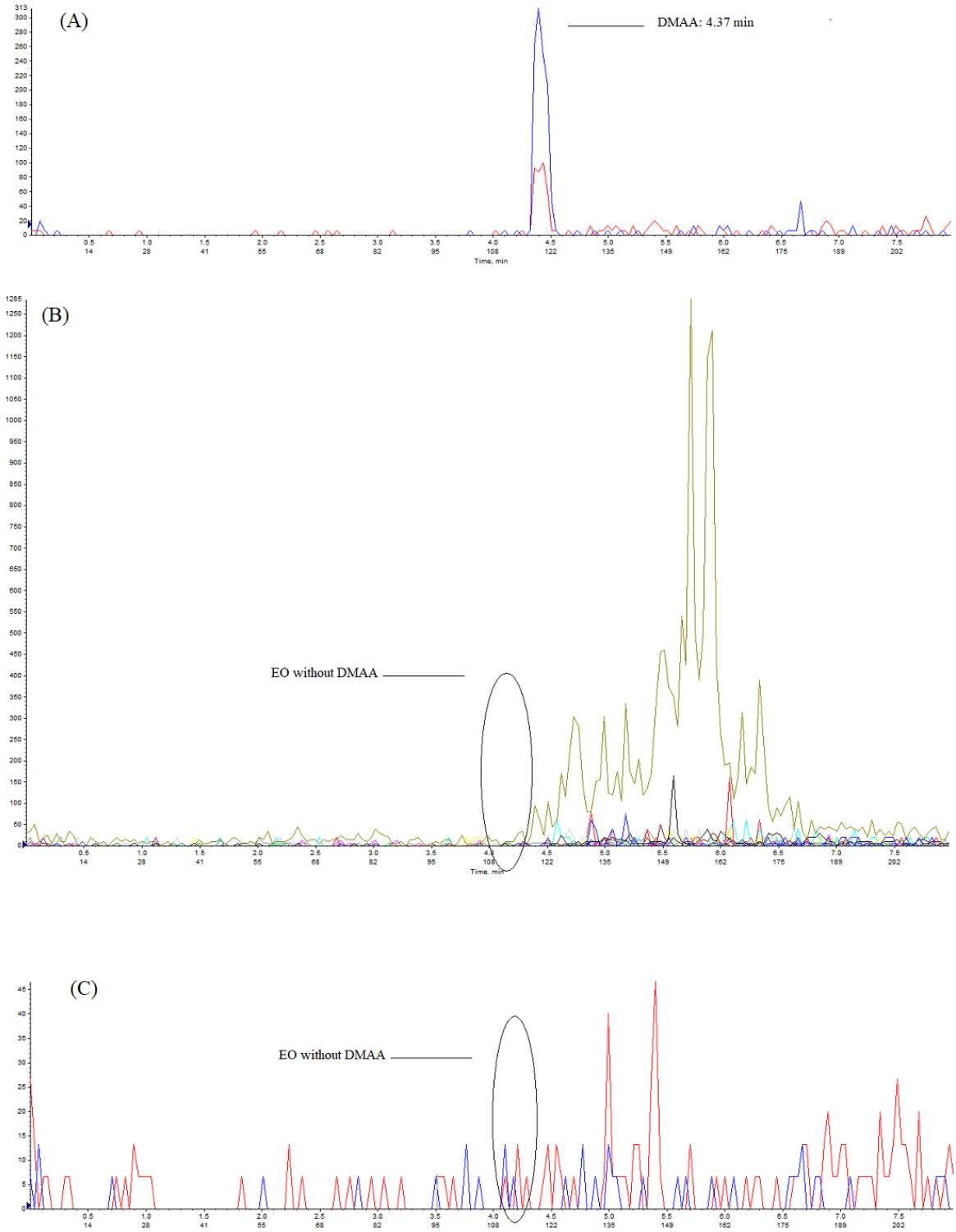

Figure 7. LC-MS/MS analysis of essential oils showing the absence of DMAA in *P. graveolens:* (A) DMAA-selective MRM transitions for a standard of DMAA; (B) essential oil from China indicating the lack of DMAA; (C) absence of DMAA transitions for the same essential oil.

Eletronic Supllementary Material

ES1 and ES2

ESI 1. Chemical composition of essential oils of *P. fragrans, P. peltatum e P. hortorum* obtained by hidrodistillation.

			P. fragrans	***P. peltatum***	***P. hortorum***
		plant part	leaves	leaves	leaves
		origin	Brazil	Brazil	Brazil
	RI	**compound %**			
1	925	tricyclene	3.9	nd	nd
2	933	alpha-pinene	6.1	nd	nd
3	942	1,4,4-trimethyl-cyclopentene-3,5-dimethylene	0.9	nd	nd
4	948	alpha-fenchene	0.1	nd	nd
5	949	camphene	0.5	nd	nd
6	953	thuja-2,4-(10)diene	0.1	nd	nd
7	972	sabinene	1.0	nd	nd
8	978	beta-pinene	2.1	nd	nd
9	988	myrcene	0.5	nd	nd
10	1005	3E-hexenyl acetate	0.3	nd	nd
11	1017	alpha-terpinene	1.2	nd	nd
12	1025	para-cymene	1.6	nd	nd
13	1029	limonene	6.3	nd	nd
14	1032	1,8 cineole	1.8	nd	nd
15	1044	beta-ocimene	0.2	nd	nd
16	1057	gamma-terpinene	2.1	nd	nd
17	1085	para-mentha-2,4(8)-diene	0.6	nd	nd
18	1091	fenchone	10.4	nd	nd
19	1100	linalool	2.0	nd	nd
20	1122	exo-fenchol	1.7	nd	nd
21	1127	trans-rose-oxide	0.1	nd	nd
22	1143	trans-sabinol	0.4	nd	nd
23	1156	menthone	0.2	nd	nd
24	1166	iso-menthone	2.6	nd	nd

nd: not detected

ESI 1. Continuation

			P. fragrans	*P. peltatum*	*P. hortorum*
		plant part	Leaves	leaves	leaves
		Origin	Brazil	Brazil	Brazil
	RI	**compound %**			
25	1173	borneol	0.9	nd	nd
26	1181	terpine-4-ol	4.2	nd	nd
27	1196	gamma-terpineol	1.5	0.7	nd
28	1217	trans-carveol	0.4	nd	nd
29	1231	cis-carveol	0.1	nd	nd
30	1243	carvone	0.5	nd	nd
31	1331	delta-elemene	0.3	nd	nd
32	1373	alpha-copaene	1.8	4.2	0.4
33	1380	beta-bourbonene	1.1	2.8	0.7
34	1396	methyl-eugenol	9.6	2.1	nd
35	1416	beta-caryophyllene	5.7	15.6	12.5
36	1426	beta-copaene	0.2	0.8	0.1
37	1427	trans-beta-bergamotene	0.3	1.1	0.3
38	1432	alpha-guaiene	0.1	nd	nd
39	1438	aromadendrene	0.2	nd	0.5
40	1447	6,9-guaiadiene	0.2	nd	0.8
41	1450	muurola-3,5-diene-cis	0.6	nd	nd
42	1452	alpha-humulene	nd	1.9	12.6
43	1456	allo-aromadendrene	1.1	4.0	nd
44	1472	gamma-gurjunene	nd	1.2	0.4
45	1477	gamma-murolene	3.2	5.1	2.8
46	1487	cis-beta-guaiene	1.4	1.7	1.1
47	1492	bicyclogermacrene	1.4	1.7	1.1
48	1495	alpha-muurolene	0.3	1.4	0.7
49	1500	not identified	nd	6.3	7.1

nd: not detected

ESI 1. Continuation.

			P. fragrans	*P. peltatum*	*P. hortorum*
		plant part	leaves	leaves	leaves
		origin	Brazil	Brazil	Brazil
	RI	**compound %**			
50	1505	beta-bisabolene	nd	nd	2.7
51	1509	gamma-amorphene	nd	0.8	nd
52	1511	delta-amorphene	2.2	nd	nd
53	1515	gamma-cadinene	nd	8.5	3.5
54	1519	delta-cadinene	nd	nd	1.4
55	1524	E-gamma-bisabolene	0.3	0.6	0.5
56	1528	gamma-cuprenene	nd	2.1	0.5
57	1533	alpha-cadinene	2.0	0.7	nd
58	1537	alpha-calacorene	3.3	2.5	0.4
59	1552	germacreno-B	2.1	10.2	nd
60	1560	E-Nerolidol	nd	nd	0.6
61	1572	spathulenol	3.1	3.4	nd
62	1577	caryophylene oxide	2.0	2.1	0.4
63	1582	2-phenyl-ethyl-tiglate	nd	nd	4.6
64	1604	cedrol	nd	nd	3.2
65	1618	10-epi-gamma-eudesmol	nd	nd	8.6
67	1632	gamma-eudesmol	nd	nd	0.6
68	1638	10-epi-alpha-cadinol	0.4	nd	3.5
69	1640	alpha-muurolol	nd	1.0	nd
70	1649	alpha-cadinol	1.0	4.2	6.5
71	1664	eudesmol-7-epi-alpha	nd	2.3	nd
73	1684	alpha-bisabolol	0.2	nd	nd
74	1693	eudesm-7(11)-en-4-ol	nd	1.5	nd
76	1788	not identified	nd	nd	1.6
74	1693	eudesm-7(11)-em-4-ol	nd	1.5	nd
75	1779	not identified	nd	nd	0.4
76	1788	not identified	nd	nd	1.6

nd: not detected

ESI 2. Chemical composition of the essential oils of *P. graveolens* obtained by hydrodistillation.

			Pelargonium graveolens							
		origin	**Brazil**	**South Africa[1]**	**Albania**	**Egypt [2]**	**South Africa[2]**	**China**	**Reunion Island**	**South Africa[3]**
	RI	**compound %**								
1	933	alpha-pinene	0.06	0.06	0.5	0.2	1.5	0.3	5.7	0.5
2	958	camphene	nd	nd	nd	nd	nd	nd	0.1	nd
3	978	beta-pinene	nd	nd	nd	nd	0.3	nd	1.2	nd
4	989	myrcene	0.1	0.1	0.2	nd	nd	nd	nd	nd
5	997	2-gamma-carene	0.03	0.02	nd	nd	nd	nd	nd	nd
6	1008	3-gamma-carene	0.3	0.3	nd	nd	nd	nd	nd	nd
7	1017	alpha-terpinene	nd	nd	nd	nd	0.2	nd	0.4	nd
8	1025	para-cymene	nd	0.1	nd	nd	0.6	nd	0.2	0.04
9	1029	limonene	0.13	0.1	0.3	nd	5.0	nd	6.3	0.1
10	1031	beta-phellandrene	0.2	0.2	nd	nd	nd	nd	nd	nd
11	1035	beta-Z-ocimene	0.1	0.1	nd	nd	nd	nd	nd	nd
12	1045	E-beta-ocimene	0.1	0.1	nd	nd	nd	nd	nd	nd
13	1070	cis-linalool-oxide	nd	nd	nd	nd	nd	nd	nd	0.01
14	1085	trans-linalool-oxide	nd	nd	nd	nd	nd	nd	nd	0.4
15	1100	linalol	10.5	10.8	3.6	9.9	5.7	4.3	8.8	4.9
16	1110	cis-rose-oxide	0.04	nd	2.6	1.4	0.2	3.1	0.2	1.1
17	1112	pentyl ethyl alcohol	nd	nd	nd	nd	2.7	nd	4.0	nd
18	1126	trans-rose-oxide	nd	nd	0.8	0.2	nd	0.6	0.1	0.4
19	1149	cis-beta-terpineol	nd	nd	nd	nd	0.2	nd	4.0	nd
20	1156	menthone	0.5	1.6	2.3	1.8	2.3	2.3	0.5	2.4
21	1165	iso-menthone	9.7	10.0	8.2	6.5	1.1	8.4	1.5	5.8
22	1176	2-phenyl ethyl formate	nd	nd	nd	nd	nd	nd	0.2	nd
23	1189	neo-iso-menthol	0.4	0.3	nd	nd	nd	nd	nd	nd
24	1196	gamma-terpineol	0.3	0.3	0.3	0.2	0.8	nd	3.7	0.3

1: Verbena, 2: Laszlo Aromaterapia 3: Ferquima nd: not detected

ESI 2. Continuation.

			Pelargonium graveolens							
		origin	**Brazil**	**South Africa[1]**	**Albania**	**Egypt[2]**	**South Africa[2]**	**China**	**Reunion Island**	**South Africa[3]**
	RI	**compound %**								
25	1200	alpha-terpineol	nd	nd	nd	nd	2.8	nd	1.1	nd
26	1224	nerol	nd	nd	nd	nd	8.9	nd	5.0	nd
27	1227	citronellol	11.4	10.8	40.0	28.8	24.2	39.3	22.0	31.7
28	1240	citronellal	nd	nd	nd	0,7	nd	nd	0.1	0,3
29	1250	geraniol	39.8	38.7	5.5	18.9	17.5	9.4	9.6	15.3
30	1268	geranial	0.3	0.2	0.2	0.4	nd	nd	nd	0,5
31	1273	citronellyl formate	nd	nd	13.0	10.1	4.2	13.2	4.8	9.5
32	1276	neryl formate	nd	nd	nd	nd	1.4	nd	1.1	nd
33	1298	geranyl formate	nd	0.3	1.4	5.0	2.8	2.3	2.4	3.3
34	1345	gamma-ylangene	nd	nd	nd	nd	nd	nd	nd	nd
35	1350	citronellyl acetate	nd	nd	0.3	nd	4.6	nd	0.2	nd
36	1358	neryl acetate	nd	nd	nd	nd	3.1	nd	3.5	nd
37	1369	decanoic acid	nd	0.7	nd	nd	nd	nd	nd	nd
38	1372	alpha-ylangene	1.6	nd	nd	nd	nd	nd	nd	0.2
39	1373	alpha-copaene	0.7	0.6	0.5	0.5	nd	0.2	nd	0.6
40	1378	geranyl acetate	0.3	nd	nd	nd	5.4	nd	7.3	nd
41	1381	beta-bourbonene	0.7	0.6	3.4	0.9	nd	0.8	nd	1.6
42	1393	phenyl-ethyl-isobutanoate	0.1	nd	nd	nd	nd	nd	nd	nd
43	1416	beta-caryophyllene	0.4	0.6	1.5	2.3	0.2	1.6	0.5	1.4
44	1426	beta-copaene	nd	nd	nd	nd	nd	nd	nd	0.1
45	1432	alpha-guaiene	0.5	0.5	0.4	0.4	0.3	0.4	0.7	nd
46	1439	6,9-guaiadiene	7.1	7.3	7.5	nd	nd	nd	nd	0.9
47	1446	alpha-neo-clovene	0.4	0.4	0.6	0.4	nd	nd	nd	0.5
48	1452	alpha-humulene	nd	nd	0.3	0.4	nd	nd	nd	nd

1: Verbena, 2: Laszlo Aromaterapia 3: Ferquima nd: not detected

ESI 2. Continuation

			Pelargonium graveolens							
		Origin	**Brazil**	**South Africa[1]**	**Albania**	**Egypt [2]**	**South Africa[2]**	**China**	**Reunion Island**	**South Africa[3]**
	RI	**compound %**								
49	1456	allo-aromadendrene	nd	nd	0.3	nd	nd	5.4	nd	nd
50	1469	geranyl propanoate	1.5	1.3	nd	1.1	nd	0.7	0.2	0.5
51	1472	gamma-gurjunene	2.5	1.8	0.8	nd	nd	nd	nd	0.1
52	1477	gamma-muurolene	nd	nd	nd	nd	nd	nd	nd	0.8
53	1481	germacrene D	0.3	nd	nd	1.9	nd	0.8	0.2	nd
54	1485	beta-selinene	nd	0.2	nd	0.3	nd	nd	nd	nd
55	1487	cis-beta-guaiene	nd	nd	1.2	nd	nd	1.0	0.2	nd
56	1492	bicyclogermacrene	0.5	0.2	nd	0.3	nd	nd	nd	0.4
57	1507	beta-bisabolene	0.3	nd	nd	nd	nd	nd	nd	nd
58	1509	gamma-amorphene	0.3	0.3	nd	nd	nd	nd	nd	nd
59	1514	gamma-cadinene	0.5	0.6	0.8	0.8	nd	0.6	0.2	1.2
60	1519	trans-calamenene	nd	nd	0.4	0.3	nd	0.2	nd	0.7
61	1525	citronellyl butanoate	nd	nd	1.3	0.2	nd	0.9	nd	0.6
62	1529	trans-cadina-1,4-diene	nd	nd	nd	nd	nd	nd	nd	0.2
63	1556	geranyl butanoate	0.6	0.6	0.5	0.4	nd	0.8	0.1	1.1
64	1582	2-phenyl-ethyl tiglate	0.9	0.8	0.2	0.4	nd	nd	1.0	0.8
65	1589	guaiol	nd	nd	nd	nd	0.4	nd	nd	nd
66	1618	10-epi-gama-eudesmol	3.6	4.5	nd	3.3	0.3	nd	0.3	6.1
67	1651	valerianol	0.2	0.2	nd	0.1	nd	nd	0.2	0.7
68	1661	allohimachol	nd	nd	0.5	nd	0.8	0.3	1.5	nd
69	1695	geranyl tiglate	2.1	2.1	0.4	1.7	nd	nd	0.1	1.0
70	1712	farnesol-2-E-6-Z	0.5	0.4	nd	nd	nd	0.8	nd	nd

1: Verbena, 2: Laszlo Aromaterapia 3: Ferquima nd: not detected

5. CAPÍTULO II- MANUSCRITO II

DART-MS/MS screening for the determination of 1,3-dimethylamylamine and undeclared stimulants in seized dietary supplements from Brazil

5. MANUSCRITO II

A seguir encontra-se disposto o artigo intitulado ***"DART-MS/MS screening for the determination of 1,3-dimethylamylamine and undeclared stimulants in seized dietary supplements from Brazil"***, publicado no periódico *Forensic Chemistry.*

O manuscrito apresenta o desenvolvimento de metodologia e posterior análise da presença de DMAA e outros estimulantes em 108 amostras de suplementos alimentares apreendidos pela Polícia Federal do Brasil.

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DART-MS/MS screening for the determination of 1,3-dimethylamylamine and undeclared stimulants in seized dietary supplements from Brazil

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ABSTRACT

1,3-dimethylamylamine (DMAA) is an alkylamine with stimulating properties that has been used predominantly as an additive in dietary supplements. DMAA is mostly consumed by professional athletes, and several doping cases reported since 2008 led to its prohibition by the World Anti-Doping Agency (WADA) in 2010. Adverse effects have indicated DMAA toxicity, and there is few data regarding its safety, so it was banned by regulatory agencies from Brazil and the United States. Ambient ionization methods such as Direct Analysis in Real Time Tandem Mass Spectrometry (DART-MS/MS) are an alternative for dietary supplements analysis, because they enable the analysis of samples at atmospheric pressure in a very short time and with only minimal sample preparation. Therefore, the aim of this work was to develop a methodology by DART-MS/MS to detect the presence of DMAA, ephedrine, synephrine, caffeine, sibutramine, and methylphenidate in 108 dietary supplements seized by the Brazilian Federal Police (BFP). The results show that DART-MS/MS screening was successfully employed to simultaneously detect the six substances in casework samples with rapid and reliable results and with minimum sample preparation. DMAA was present in 20% of the seized dietary supplements, being more prevalent along with sibutramine and caffeine. Out of the 108 samples, almost 50% were positive for sibutramine and 10% for methylphenidate. It appears that even after prohibition, dietary supplements and weight-loss products containing DMAA are still being commercialized.

DART-MS/MS screening for the determination of 1,3-Dimethylamylamine and undeclared stimulants in seized dietary supplements from Brazil

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Abstract

1,3-dimethylamylamine (DMAA) is an alkylamine with stimulating properties that has been used predominantly as an additive in dietary supplements. DMAA is mostly consumed by professional athletes, and several doping cases reported since 2008 led to its prohibition by the World Anti-Doping Agency (WADA) in 2010. Adverse effects have indicated DMAA toxicity, and there is few data regarding its safety, so it was banned by regulatory agencies from Brazil and the United States. Ambient ionization methods such as Direct Analysis in Real Time Tandem Mass Spectrometry (DART-MS/MS) are an alternative for dietary supplements analysis, because they enable the analysis of samples at atmospheric pressure in a very short time and with only minimal sample preparation. Therefore, the aim of this work was to develop a methodology by DART-MS/MS to detect the presence of DMAA, ephedrine, synephrine, caffeine, sibutramine, and methylphenidate in 108 dietary supplements seized by the Brazilian Federal Police (BFP). The results show that DART-MS/MS screening was successfully employed to simultaneously detect the six substances in casework samples with rapid and reliable results and with minimum sample preparation. DMAA was present in 20% of the seized dietary supplements, being more prevalent along with sibutramine and caffeine. Out of the 108 samples, almost 50% were positive for sibutramine and 10% for methylphenidate. It appears that even after prohibition, dietary supplements and weight-loss products containing DMAA are still being commercialized.

Keywords: 1,3-dimethylamylamine, DART-MS/MS, dietary supplements, stimulants, adulterants.

1. Introduction

Dietary supplements and herbal dietary supplements are very popular worldwide, showing an increase in consumption in recent decades [1–4]. 1,3-dimethylamylamine (DMAA) is considered to be an alkylamine-type stimulant, characterized by the presence of a primary amine attached to a short carbon chain [5]. Pharmacologically, DMAA is classified as an α-1-adrenergic agonist [6]. Known popularly as methylhexanamine, 2-amino-4-methylhexane and geranamine, DMAA began to be marketed in North America in the 1970s under the name "Forthane" and was used as a nasal decongestant because of its vasoconstricting properties [6–8]. In 2006, DMAA reappeared on the market as a constituent of dietary supplements, with stimulating properties. Over the years, DMAA has been used predominantly as an additive in dietary supplements [9,10].

Since 2008, dozens of athletes have been excluded of sports activities by the World Anti-Doping Agency (WADA) due to the abuse of DMAA. [11]. The continuous use of laced sports supplements by professional athletes encouraged WADA to ban DMAA in 2010 [12]. After cases of deaths and toxicity events [10,13], and no sufficient data indicating its safety and efficacy, DMAA was banned in the United States [14] and in other countries such as Canada and Brazil in recent years [15,16]. In 2014, Foley and contributors [17] reported eight cases of military personnel (men and women) who consumed DMAA and had liver damage. Even after its prohibition; by WADA [18] the analyses carried out at the XXII Winter Olympic and XI Paralympic games; revealed the presence of DMAA along with other controlled or monitored substances, such as pseudoephedrine [19]. According to the latest reports published by WADA, DMAA is still the most widely used banned-stimulant among athletes [20–22].

Considering the prohibition of DMAA, the US Food and Drug Administration (FDA) issued an alert to manufacturers, banning its addition to dietary supplements, emphasizing the dangers arising from consumption of DMAA in dietary supplements. However, despite the alerts, in April 2013 the FDA received 86 notifications related to health problems and deaths resulting from the use of supplements containing DMAA [14]. The National Agency of Sanitary Surveillance (ANVISA) suspended its distribution, disclosure, trade and use in Brazil. ANVISA also included DMAA in the List F2 - List of Psychotropic Substances of outlawed use in annex I of Portaria SVS/MS n^{o} 344/98, according to RDC n^{o}. 37 of July 2, 2012 [16]. In Brazil, studies

conducted with dietary supplements seized by the Brazilian Federal Police (BFP) show that compounds containing DMAA continue to be clandestinely marketed and smuggled [2,23].

A good alternative to determine the adulteration of dietary supplements, identifying unlabeled or forbidden substances, is using Direct Analysis in Real Time Tandem Mass Spectrometry (DART-MS/MS) [24–26]. DART-MS/MS is capable of rapid analysis of samples at atmospheric pressure with only minimum sample preparation [24,27]. This technique is currently being used for different purposes, such as the determination of plants ingredients, pharmaceutical products and drugs of abuse [24,28]. Thus, DART-MS/MS can be considered a good screening test to analyze emerging drugs by identifying a large spectrum of substances simultaneously, through a combination of ambient ionization and tandem mass spectrometry [26,29].

This study presents a fast screening method by DART-MS/MS to detect the presence of DMAA and other stimulants found in dietary supplements such as ephedrine, synephrine, caffeine, sibutramine and methylphenidate in 108 samples of dietary supplements seized by the BFP. A select number of casework samples that tested positive with this DART-MS/MS method were later confirmed using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS).

2. Material and Methods

2.1 Chemicals and materials

HPLC optima grade methanol and LC-MS grade water was purchased from Fisher Scientific (Pittsburgh, Pennsylvania). 1S, 2R (+) ephedrine hydrochloride (1.0 mg/mL), methylphenidate hydrochloride (100 µg/mL) and sibutramine hydrochloride (1.0 mg/mL) in methanol were purchased from Cerilliant Corporation (Round Rock, Texas). Methylhexanamine (DMAA), caffeine and synephrine hydrochloride 10 mg, were purchased from Sigma Aldrich (St Louis, Missouri); JT Baker (Phillipsburg, New Jersey) and Apex Bio Technology (Fannin St, Houston), respectively. Capillary tubes, 1.5-1.8x90 mm, Pyrex, were purchased from Corning Life Sciences (Tewksbury, Massachusetts).

2.2 Dietary Supplements

Dietary supplements (capsules with solid contents, capsules with liquid contents and tablets) were purchased at a local retailer located in Morgantown, West Virginia

to develop the analytical method. Also, 108 Brazilian samples were seized according to the criteria established by BFP and constitute a group of the samples that were described by Neves and Caldas [2]. The seized dietary supplements included solid capsules, liquid capsules and tablets.

2.3 Sample preparation

The analytical standards used for the mass spectrometer optimization parameters and method development were diluted in methanol to a concentration of 1 ppm. The solid tablets were tested by holding the tablet directly between the ionization source and the inlet of the mass spectrometer. Extracts of the tablets were also analyzed on glass capillary tubes by first diluting 1/200 of the median weight of three capsules/tablets with 8 mL of methanol and 2 mL of Milli-Q water, depositing 2 µL of the extracted solution into a capillary tube for sampling. Liquid capsules were diluted using only 10 mL of methanol due to their oily content. After dilution, solutions were vortexed for 1 min and 2 µL of sample was pipetted into a glass capillary tube and analyzed.

2.4 Analytical procedure

2.4.1 Instrumentation

DART analyses were performed using a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer. The DART source was attached to a standard Vapur® interface from IonSense (Saugus, Massachusetts) and fitted to the mass spectrometer using a custom-built 3D-printed flange. The DART ion source was operated with helium gas at 300 °C, at a flow rate of 2 L/min, and a grid voltage of 530 V. Xcalibur 2.0.7 software was used for data processing. Ultra-high purity helium (DART gas) and argon (collision gas) were purchased from Matheson TRIGAS (Fairmont, West Virginia). The capillary tube temperature for the mass spectrometer was set at 350 °C. The data acquisition consisted of 3 seconds (s) for sample analysis and 20 or 30 s for initial and final background, totaling less than 1 min per analysis. The precursor and product ions were determined using full scan mode from *m/z* 40-*m/z* 350 and the precursor to product ion transitions were monitored using selected reaction monitoring (SRM) mode using compound-specific transitions. The values monitored for each compound were: DMAA = *m/z* [M+H] 116→57 and *m/z* 116→43; Caffeine = *m/z* [M+H] 195→138 and *m/z* 195→110; Ephedrine = *m/z* [M+H]

166 →133 and *m/z* 166→115; Methylphenidate= *m/z* [M+H] 234→84 and *m/z* 234→56; Sibutramine = *m/z* [M+H] 280→ 125 and *m/z* 280→103; Synephrine [M+H] *m/z* 168→ 134 and *m/z* 168→ 108. The decision to use two transitions per compound maximized the number of data points acquired during the relatively short signal durations of the DART ion source. However, the use of two transitions per compound may not offer the highest degree of specificity for DART-MS/MS, and until all the samples are confirmed using an independent method, like LC-MS/MS, the possibility of some false positives cannot be ruled out.

2.4.2 DART-MS/MS optimization parameters

The values of Collision Energy (CE), gas temperature (250 °C, 300 °C, 350 °C, 400 °C and 450 °C) and scan times (0.20 s, 0.10 s, 0.05 s, 0.02 s and 0.01 s) were optimized for DMAA, ephedrine, synephrine, caffeine, sibutramine and methylphenidate. The optimum values were chosen considering a signal to noise ratio of three (S/N = 3) (Figure 1). All the optimization experiments were analyzed in triplicate with the standards solutions prepared as described at section 2.3.

3 Results and Discussion

To ensure the use of the best conditions for detection of all selected compounds in seized dietary supplements by DART-MS/MS, an optimization of collision energy (CE), gas temperature, and scan times was performed. The CE determines the amount of internal energy gained through collisions with the argon collision gas. CE optimization is important and very common in MS/MS methods, as optimized CE results for the specific target molecule can increase product ion intensities [30,31]. The optimum CE was obtained for each analyte considering the two product ions with the most stable relative abundances observed in the MS/MS spectra (Figure 1) using the product ion scan mode. The final values for each standard were: DMAA = 18 V; Caffeine = 30 V; Ephedrine = 35 V; Methylphenidate and Synephrine = 37 V and Sibutramine = 40 V.

Figure 1

The DART gas temperature and flow rate are also important parameters affecting the desorption and ionization process of the substances, thus influencing the

sensitivity of detection [32]. Gas temperatures were tested from 250 °C to 450 °C. Low intensities were observed at 250 °C for the six substances analyzed, indicating poor desolvation or desorption. When the temperature was raised to 300 °C, significantly better results were obtained. However, at temperatures greater than 300 ^{o}C, lower signal intensities were observed for DMAA and ephedrine, probably due the degradation of the molecules [33,34]. 300 °C provided the best results for the six analytes, considering our multianalyte method development, so this temperature was selected for the remainder of the study. After the gas temperature optimization, the capillary temperature was raised to 350 °C, to ensure that the compounds would not condense inside the capillary transfer tube entrance of the mass spectrometer.

Scan time or dwell time is the time spent acquiring a specific SRM transition during each cycle. A very short dwell time can be used for each analyte; however, a longer dwell time is desirable for better signal/noise and sensitivity. Scan times of 0.20 s, 0.10 s, 0.05 s, 0.02 s, and 0.01 s were tested. The optimum scan time was determined to be 0.05 s since it allowed for the detection of each compound with a low standard deviation regarding the signal-to-noise ratio..

After optimization, the method was tested on commercial dietary supplements acquired at a local retailer store located in Morgantown, West Virginia. Direct analysis of the solid tablets/capsules allowed for the identification of analytes without the need for sample preparation. The samples were also diluted as described in section 2.3. The results of the direct analysis were then compared to the results of diluted samples to ensure that the dilution was suitable for the identification of the target substances. However, after running the first few solid samples, prolonged carryover was observed for certain direct analysis of solid samples. To prevent the need to clean the transfer optics in the atmospheric sampling interface, we therefore decided to continue the analysis on diluted samples only. Blank samples of methanol were run between the analyses.

The limit of detection was established using the standard definition of S/N = 3. However, analytes cannot be accurately quantified near the threshold levels because the limit of quantitation is considerably higher [35,36]. The minimum relative abundance for all analytes was on the order of 1 x 10^4, except for caffeine, which was usually very concentrated when it was present and has a higher background level in the DART-MS/MS method. The lower limit of detection (LLOD) was between 25 to 50

pg/μL for the six substances, which is more than sufficient to detect the six analytes at their relevant levels in the dietary supplements.

The 108 samples of dietary supplements seized by the BFP were categorized according to different types of matrices: solid capsules (65 samples), liquid capsules (33) and tablets (10). After dilution, samples were analyzed to assess presence of DMAA, caffeine, ephedrine, synephrine, sibutramine and methylphenidate. DMAA was present in 20% of the samples (Figure 2) and the majority of positive results were found in solid capsules (25%), but positive results were also obtained from liquid capsules (Figure 3). For tablet form samples, only one test sample showed presence of DMAA, synephrine, ephedrine, sibutramine and caffeine. Regarding the positive samples, when DMAA was present, it was always present with other stimulants. For example, DMAA was found with caffeine in 95% of the samples. DMAA was found with synephrine, sibutramine and ephedrine in almost 30% of the samples and methylphenidate in 5% of the samples (Figure 4). These results agree with previous data reported that found DMAA along with other stimulants as well as caffeine, synephrine and sibutramine [2,37], a very common combination in these products mainly due their thermogenic activity and appetite suppressant properties [37–39].

Figure 2

The results presented in our study supports that DMAA presence is still highly troublesome [19,22]. For example, based on the last two WADA doping monitoring list, DMAA is one of the most widely used stimulant among athletes [20,22]. According to the anti-doping report from 2013, DMAA was the most commonly found stimulant detected in doping cases [20]. The adverse effects of DMAA were already described and include tachycardia, nausea, vomiting, dizziness, headache, chest pain and in some cases, death, which led to its prohibition in Brazil and United States, in 2012 and 2013 respectively [13,17,40,41]. However, our results show that DMAA can still be found in the dietary supplements, either declared or undeclared, because it is still clandestinely commercialized and smuggled in Brazil [2] and other countries [42].

Figure 3

DMAA can also be declared in the formulations as “geranamine”, due to its possible natural origin from the *Geraniaceae* family [43,44]. The presence of DMAA was initially attributed to the essential oils from *Pelargonium* sp., in 1996 [45]. After this initial report, several research groups conducted independent studies debating over the synthetic or natural origin of DMAA. The conclusions from these research efforts discarded the natural presence of DMAA in the essentials oil [6,43,44,46–48]. Furthermore, it is important to emphasize that DMAA was proscribed by ANVISA, which included DMAA at the F2 List of Psychotropic substances of outlawed use, the same category as ecstasy (3,4-methylenedioxymethamphetamine) and LSD (lysergic acid diethylamide); DMAA and its commerce is therefore completely illegal in Brazil [16].

Figure 4

Methylphenidate is the preferred drug commonly prescribed to Brazilian patients diagnosed with attention deficit hyperactivity disorder (ADHD). Because of its stimulating properties, there are also reports of its recreational use, generating concern to the authorities [49,50]. Moreover, MPH is one of the most frequently detected stimulants in doping test according to the last WADA reported data [20,22]. From our dataset of tested specimens, we highlight the trace occurrence of methylphenidate (MPH) in 10% of the samples analyzed (Figure 2). Solid capsules were the tested samples with the most part of positive results for MPH (Figure 5). Additionally, the seized samples that showed positive results for MPH also presented other stimulants, being the most prevalent caffeine, followed by sibutramine and synephrine. The fact that MPH appeared as one of the most widely use stimulants among athletes, might be due to its addition in some dietary supplements, rather than being consumed as a medication, deserving attention of the authorities.

Figure 5

Sibutramine was detected in 44% of the formulations (Figure 2). Solid and liquid capsules were the most prevalent samples with a positive result (Figure 6). Likewise, other stimulants were detected alongside sibutramine, specially, synephrine,

ephedrine and caffeine. The known toxicological effects for sibutramine are cerebrovascular accidents, myocardial infarction and psychiatric disorders, and so, its consumption should be firmly restricted and followed by medical monitoring [51–53]. However, in accordance with our data and other reports, sibutramine is one of the most common substances added as a undeclared compound to dietary supplements and weight loss compounds [2,54,55]. This represent a major health risk, especially if consumed with caffeine, ephedrine and synephrine due to their synergistic effects [51,56]. Sibutramine is considered a psychotropic anorectic drug, and is the oldest allowed weight loss medicine in Brazil; it is being commercialized since 1998. Control of sibutramine was reinforced since 2011, it was submitted for special prescription for its commercialization [57], and recently updated by the Brazilian authorities under the RDC 133 of 2016 [58].

Figure 6

Synephrine and ephedrine were also found in a significant portion of the seized samples, at rates of 46% and 39%, respectively (Figure 2). Liquid capsules were the most prevalent (Figure 7), along with tablets, considering both compounds. Ephedrine is a sympathomimetic amine belonging to *Ephedra* genus that is used in weight loss compounds and dietary supplements, having thermogenic and stimulant properties [59,60]. Its adverse effects are manifested mostly to as cardiac symptoms, with consequences such as stroke, myocardial infarction and sudden death [51,61,62]. Ephedrine consumption was restricted by WADA in 2007 [63], and replaced by synephrine over the years in the formulations of dietary supplements and weight loss products. However, considering the results and reported data, ephedrine can still be found in these products, and is predominantly associated with other stimulants such as caffeine [39,64,65]. Synephrine is the most active substance naturally found in C*itrus aurantium*. The addition of synephrine to dietary supplements and weight loss products is very common due to its stimulant and weight-loss properties [38,64]. Synephrine consumption has been regulated by WADA since 2009 [66]. We detected synephrine in 49 samples (~45%), which agrees with previous data reported, confirming that its addition is a common practice, particularly together with other stimulants such as ephedrine, sibutramine and caffeine [39,56,64,65].

Figure 7

Caffeine was the most prevalent substance, present in 105 analyzed samples (Figure 2), in agreement with data already reported by Neves and Caldas [2]. During the method development with the commercial samples, a carryover due the high concentration of caffeine reported in these products was detected. Thus, it was decided that a higher threshold value for caffeine of 1×10^6, instead of 1×10^4, was required in addition to considering the signal to noise ratio of three for positive results. Caffeine is the most common stimulant known [59] and its consumption is allowed by WADA, in established concentrations [18]. Nevertheless, caffeine can be toxic when consumed in high doses and particularly in association with other stimulants [59], which is very common in dietary supplements according to these results (Figure 8) and data already published [2,56,67].

Figure 8

The 108 seized samples analyzed showed the presence of DMAA in most of the solid capsules, and approximately 20% of the liquid capsules and tablets. The DART-MS/MS screening results of were validated using was confirmed using multiple reaction monitoring on an HPLC-MS/MS triple quadrupole mass spectrometer, and Considering these results and other reported [42], it can be assumed that clandestine commercialization of DMAA is still occurring. Also, it was noted that all the six stimulants analyzed were frequently found in the seized samples, and occasionally, with five of the compounds together in different combinations (Figure 9). Therefore, the consumption of stimulants simultaneously represents a major health risk for the consumer, leading to toxicity and even death [8,17,41,51,56]. Fast and simple methods are required for routine screening analysis of adulterants, which are present on numerous dietary supplement samples, therefore, ambient ionization methods simplify screening analyses by eliminating the need for clean-up steps and provide mass spectral data without the sample preparation required by the chromatography based methods [26,27,29].

Figure 9

4 Conclusions

The DART-MS/MS approach used in this study was able to rapidly detect six different stimulants in dietary supplements. DART-MS/MS detected DMAA in 20% of the seized dietary supplements, and DMAA was usually found together with other stimulants such as sibutramine, which is used for appetite suppressant. Sibutramine and methylphenidate—which is used for attention deficit hyperactivity disorder—were found in almost 50% of the samples that contained DMAA. These findings highlight the concern about the composition of clandestine dietary supplements, which represent a serious health risk for the users, due to the simultaneous consumption of anorectics and stimulants, which certainly deserves attention of regulatory agencies.

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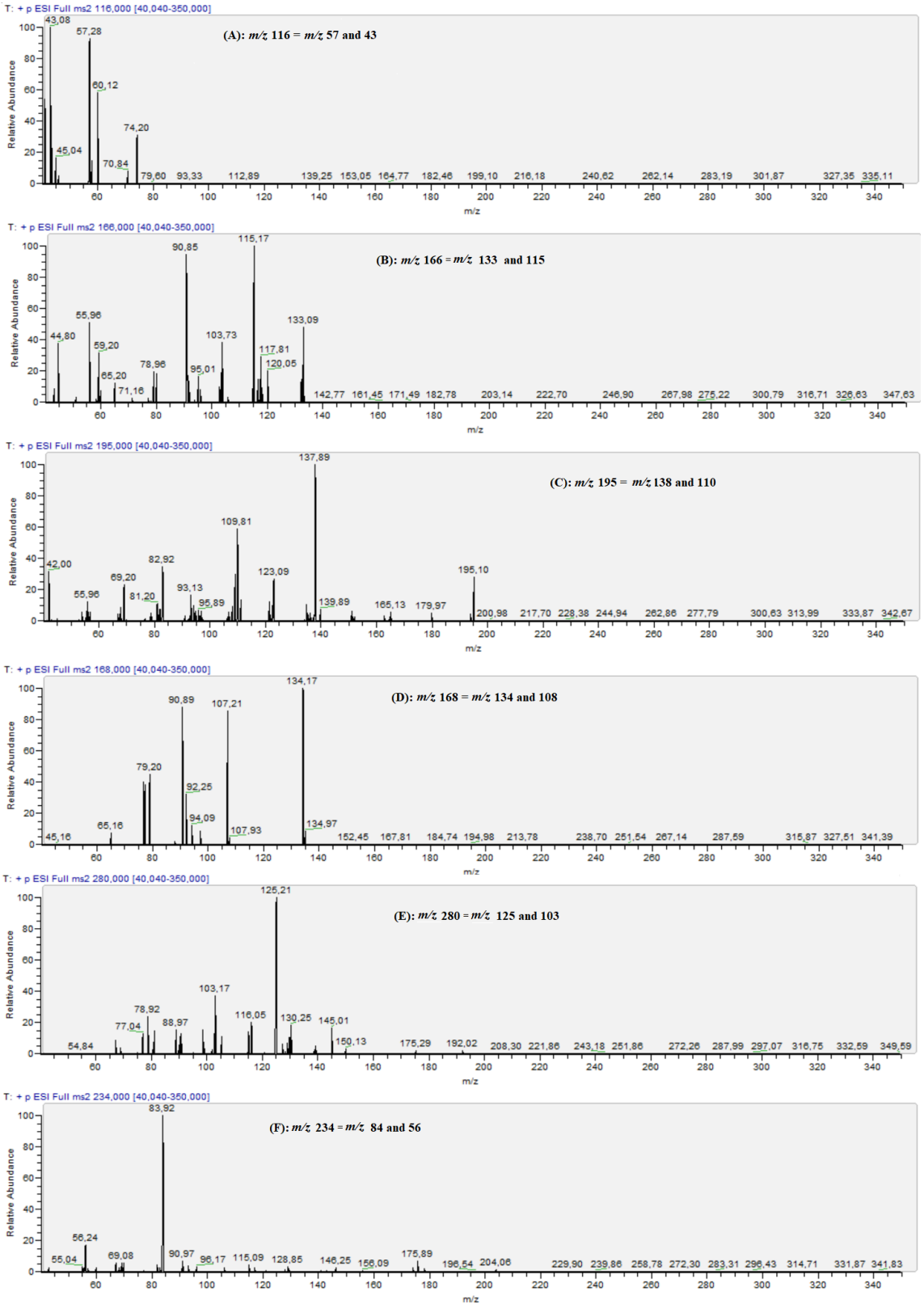


Figure 1. Product ions observed postcollision energy optimization. (A) DMAA, (B) ephedrine, (C) caffeine, (D) synephrine, (E) sibutramine and (F) methylphenidate.

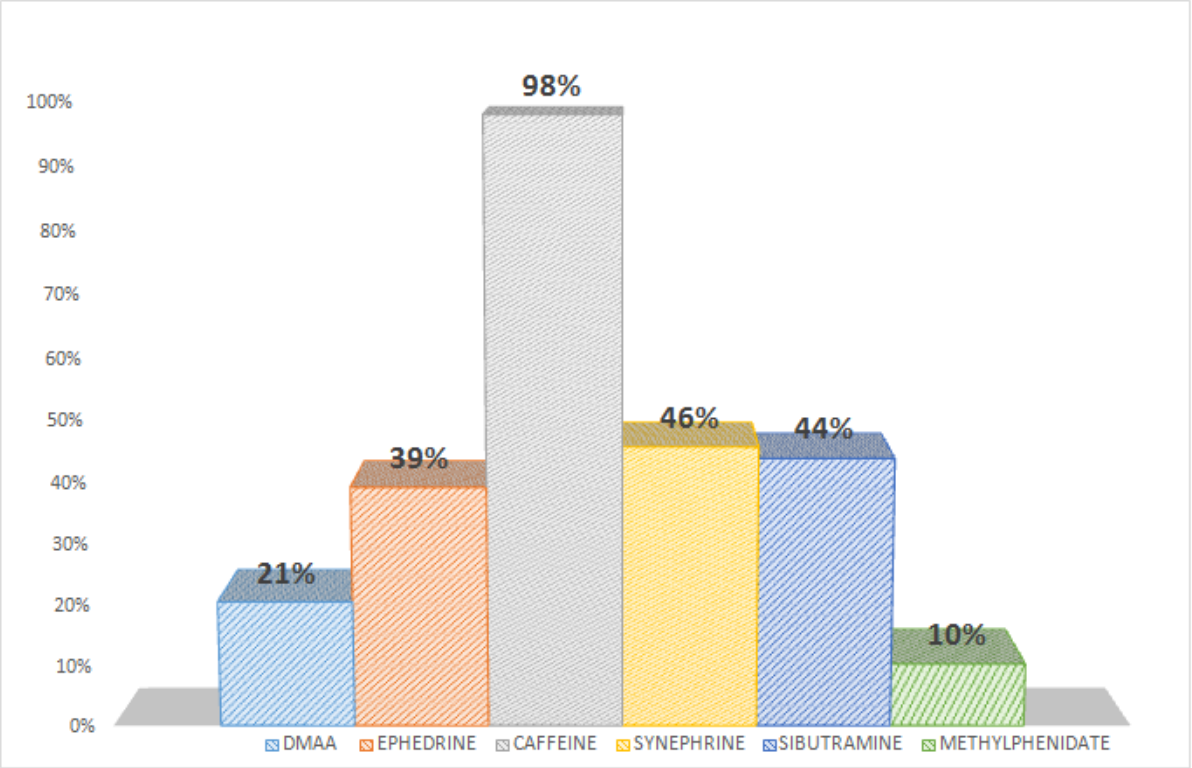


Figure 2. Incidence of stimulants in 108 samples of dietary supplements after DART-MS/MS analysis.

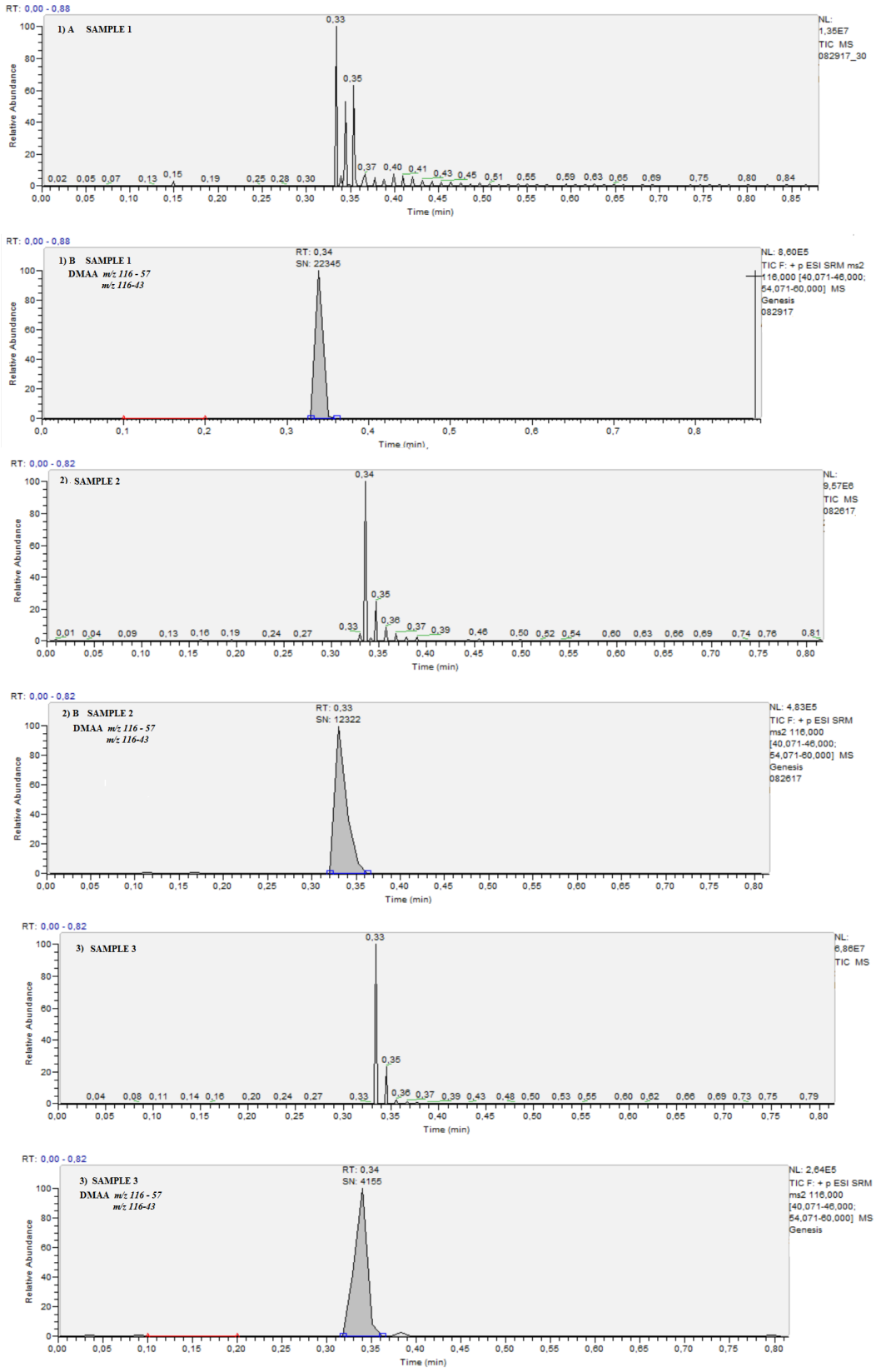
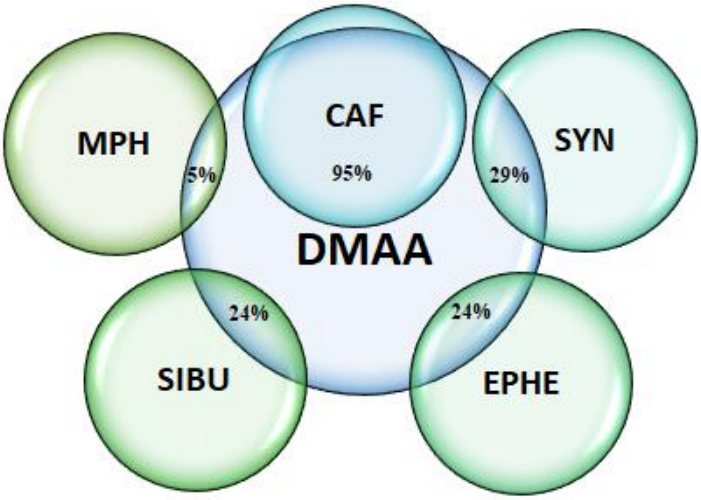


Figure 3. DART-MS/MS chromatograms and SRM spectra of samples with positive results for DMAA. (1A and 1B) Sample 1, (2A and 2B) Sample 2 and (3A and 3B) Sample 3.



CAF: Caffeine SYN: Synephrine EPHE: Ephedrine SIBU: Sibutramine MPH: Methylphenidate

Figure 4. Correlation between positive samples of DMAA along with other stimulants.

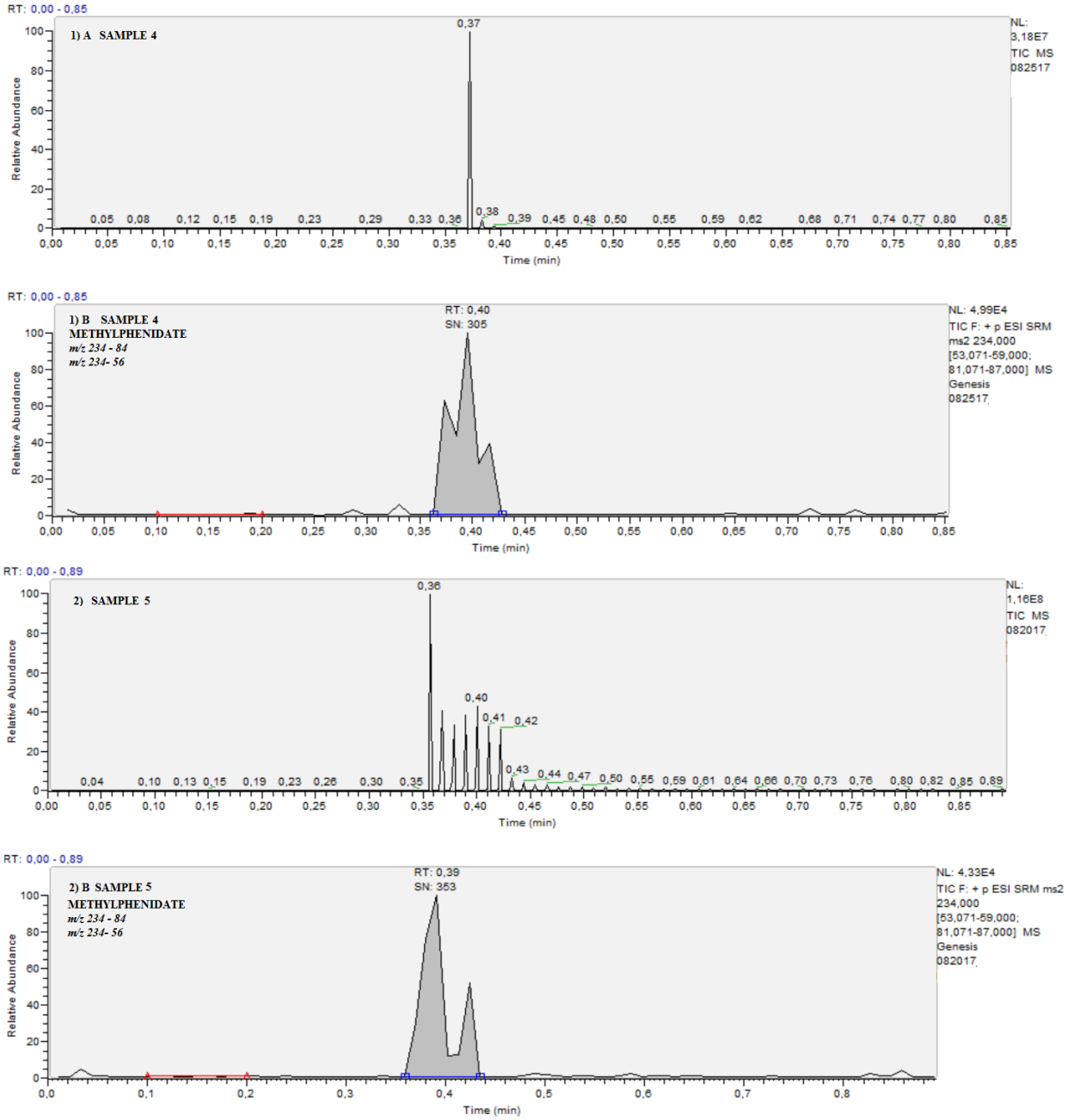


Figure 5. DART-MS/MS chromatograms and SRM spectra of samples with positive results for methylphenidate. (1A and 1B) Sample 1 and (2A and 2B) Sample 2.

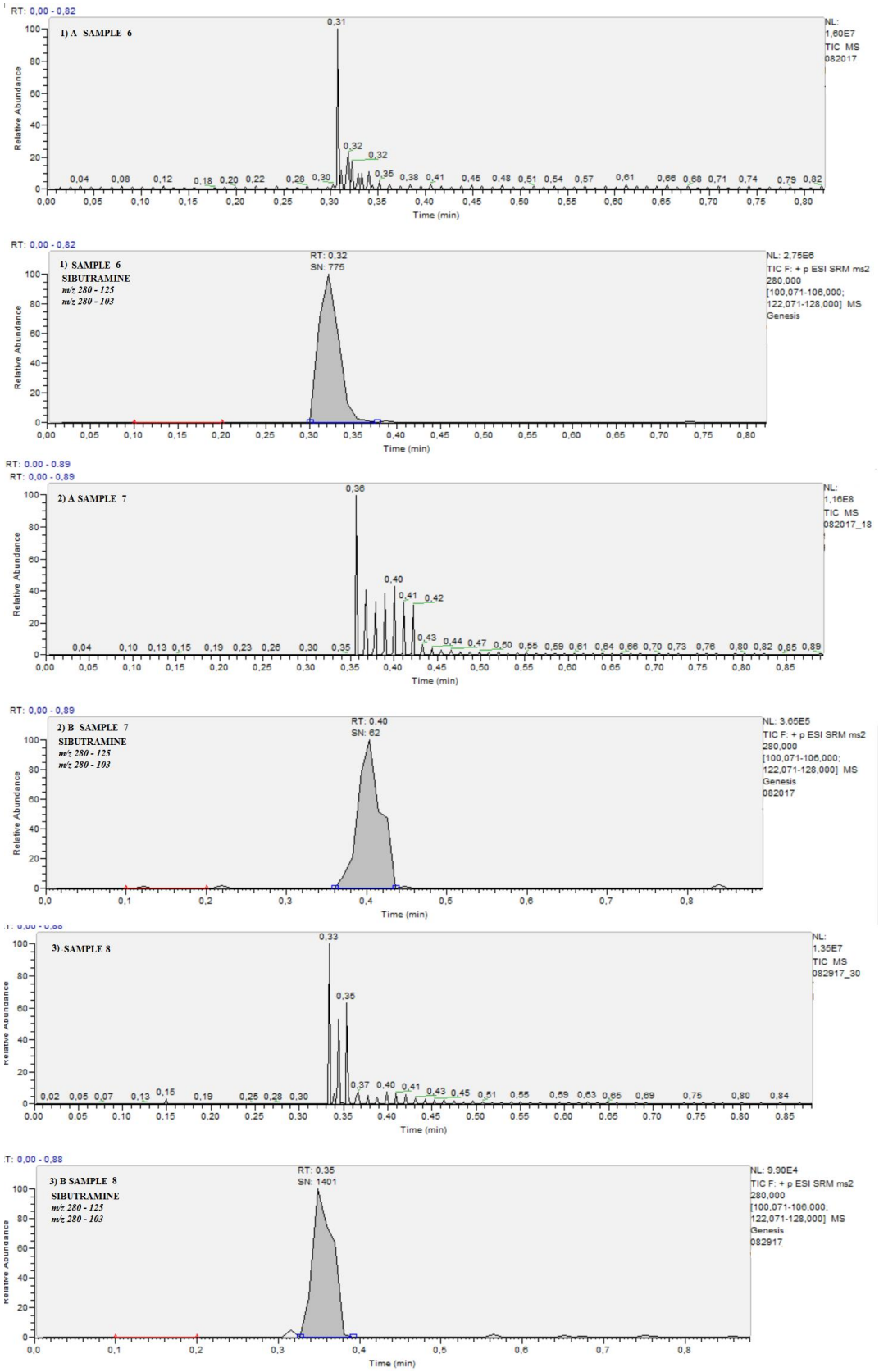


Figure 6. DART-MS/MS chromatograms and SRM spectra of samples with positive results for sibutramine. (1A and 1B) Sample 1, (2A and 2B) Sample 2 and (3A and B) Sample 3.

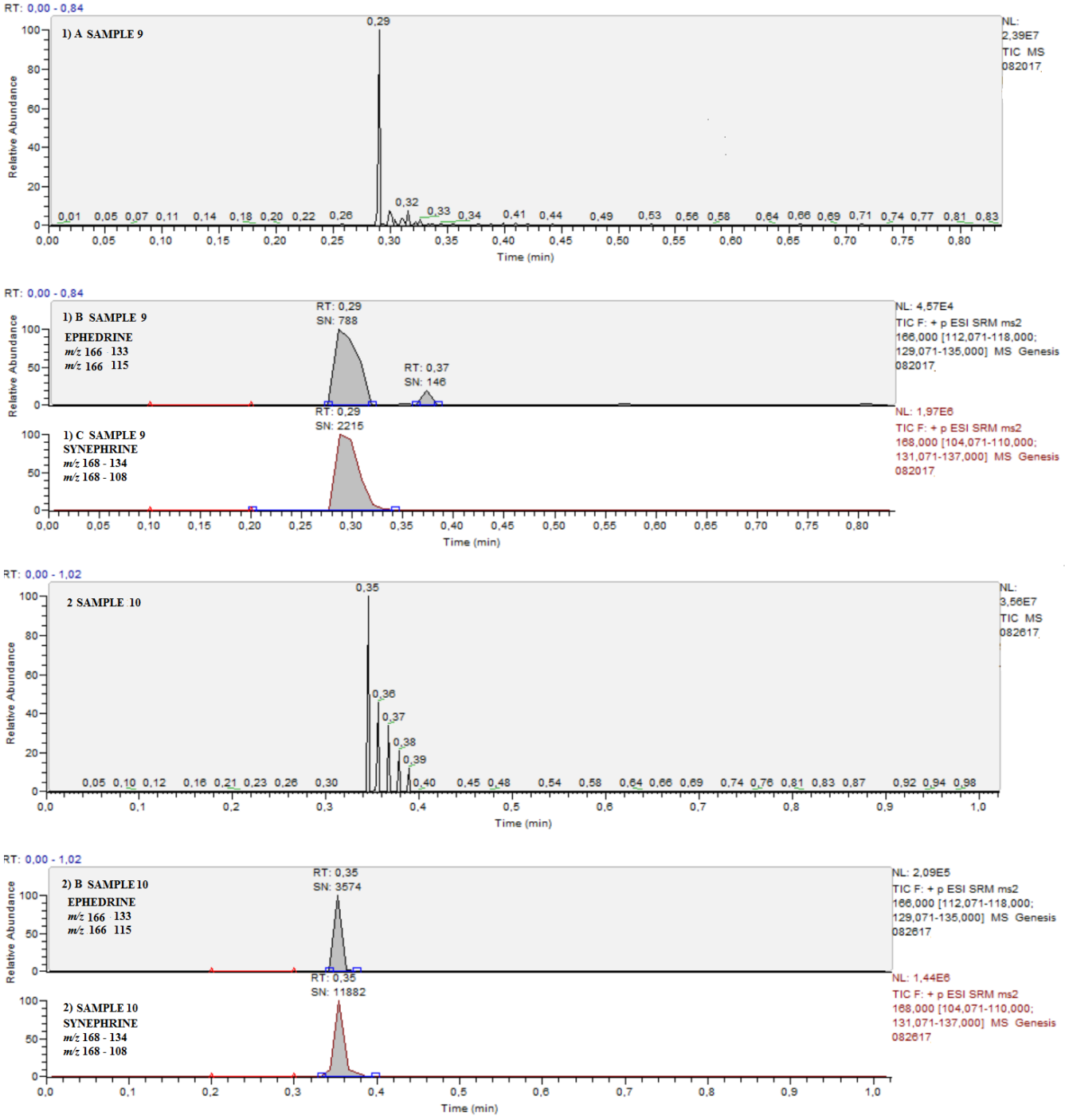


Figure 7. DART-MS/MS chromatograms and SRM spectra of samples with positive results for ephedrine and synephrine. (1A, 1B and 1C) Sample 1 and (2A, 2B and 2C) Sample 2.

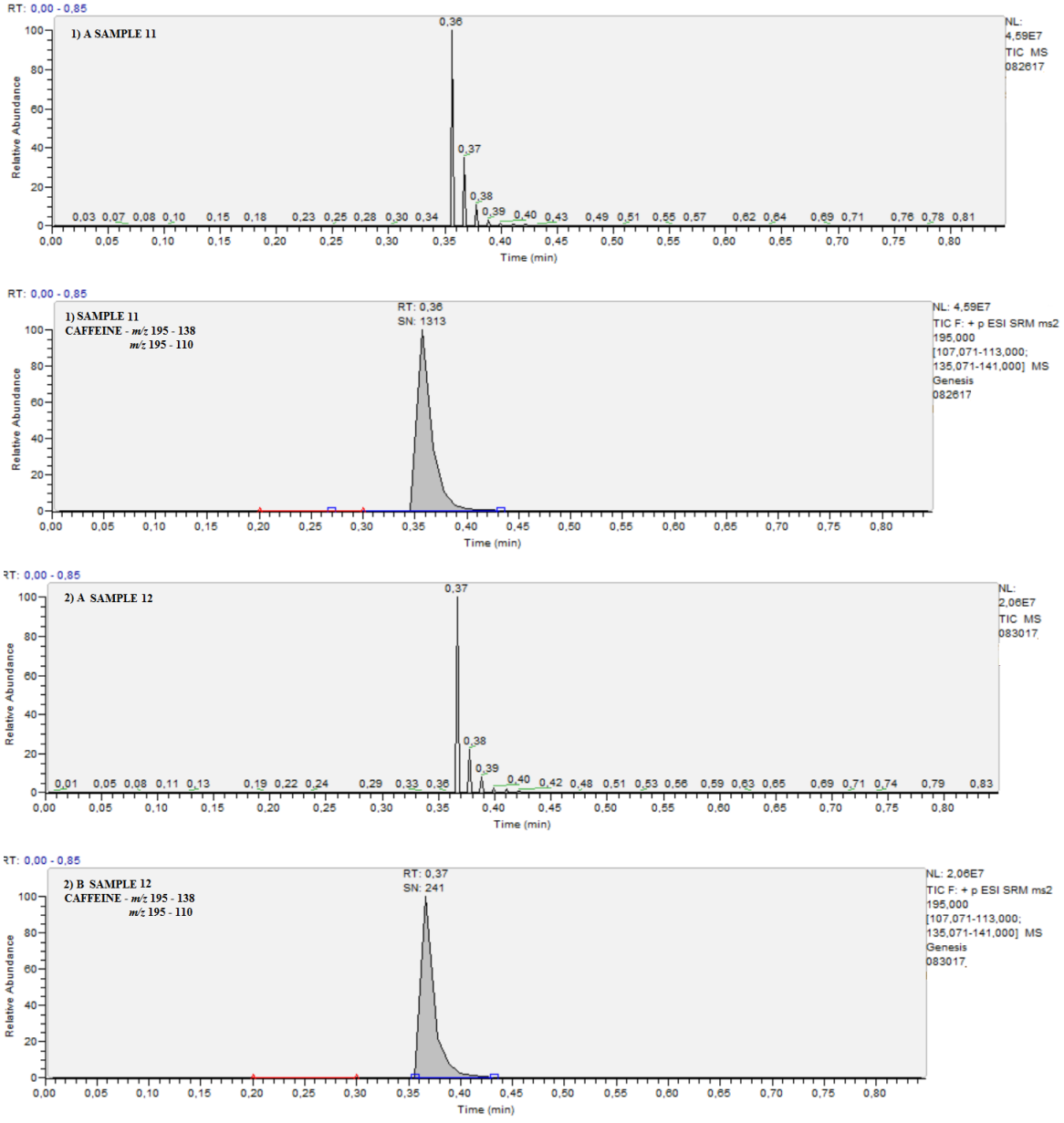


Figure 8. DART-MS/MS chromatograms and SRM spectra of samples with positive results caffeine. (1A and 1B) Sample 1 and (2A and 2B) Sample 2.

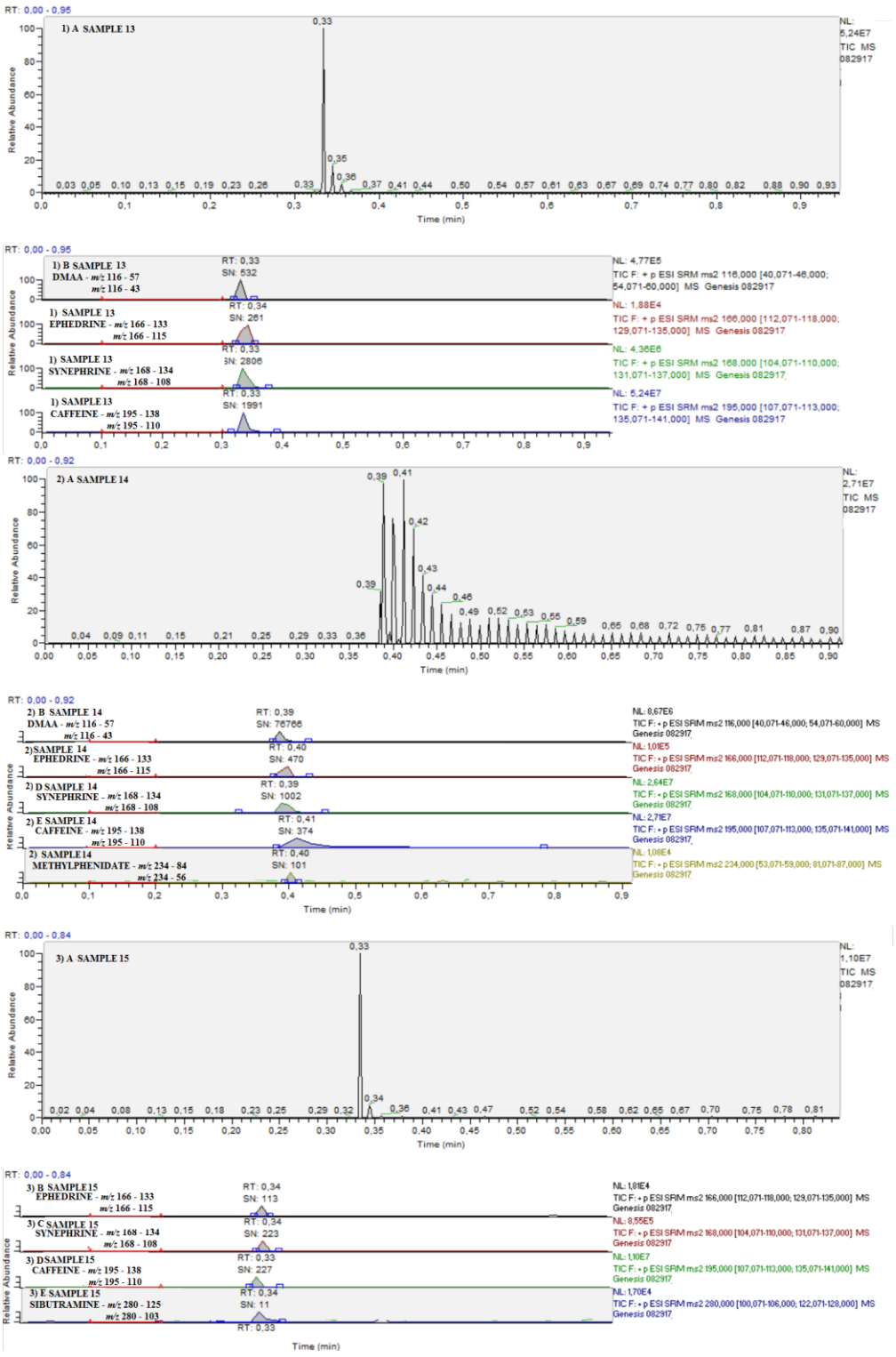


Figure 9. DART-MS/MS chromatograms and SRM spectra of samples with positive results for DMAA, ephedrine, synephrine, caffeine, sibutramine and methylphenidate. (1A, 1B, 1C, 1D and 1E) Sample 1 (2A, 2B, 2C, 2D, 2E and 2F), Sample 2 (3A, 3B, 3C, 3D and 3E) and Sample 3.

6. CAPÍTULO III- MANUSCRITO III

"Antifungal activity and development of a new nanoemulsion-hydrogel carrying essential oil of Pelargonium graveolens against vaginal candidiasis"

6. MANUSCRITO III

A seguir encontra-se disposto o artigo intitulado ***"Antifungal activity and development of a new nanoemulsion-hydrogel carrying essential oil of Pelargonium graveolens against vaginal candidiasis"***, a ser submetido no periódico *Pharmaceutical research.*

O manuscrito apresenta a atividade antifúngica de óleos essenciais de *Pelargonium graveolens* de diferentes países contra diferentes espécies de *Candida* e compara com a atividade de uma formulação desenvolvida contendo uma nanoemulsão com o óleo essencial, com objetivo final de uma alternativa ao tratamento da candidíase vaginal.

Antifungal activity and development of a new nanoemulsion-hydrogel carrying essential oil of *Pelargonium graveolens* against vaginal candidiasis

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ABSTRACT

Purpose: This work reports an evaluation of antifungal activity of essential oils of *Pelargonium graveolens* from different countries and the development of a chitosan hydrogel containing a nanoemulsion with the essential oil to treat vaginal candidiasis.

Methods: The antifungal activity of essential oils of *P. graveolens* was evaluated against five species of *Candida* and Minimum Inhibitory Concentration (MIC) was determined. After, a chitosan hydrogel containing nanoemulsion with the most active essential oil was produced, characterized and had its MIC determined.

Results: Eight essential oils from *P. graveolens* showed antifungal activity against *Candida* sp. South African essential oil showed the best activity and was selected to the development of a nanoformulation. The chitosan hydrogel containing the essential oil nanoemulsion showed higher antifungal activity with lower MIC values when compared to dispersed essential oil and to the nanoemulsion. **Conclusions:** The chitosan hydrogel containing the essential oil nanoemulsion can be considered a promising alternative formulation to treat vaginal candidiasis, once it uses biodegradable materials and improves treatment efficacy.

KEYWORDS *Pelargonium graveolens, Candida* spp., hydrogel, nanoemulsion, candidiasis

ABBREVIATIONS

MIC Minimum Inhibitory Concentration

NPG *P. graveolens* Nanoemulsion

NB Blank Nanoemulsion

HNPG *P. graveolens* Nanoemulsion Based-Hydrogels

HCHI Blank Hydrogels

PDI Polydispersity Index

ZP Zeta Potential

EO Essential oils

INTRODUCTION

Recently, the search for natural molecules with activity against bacteria, fungi and viruses has led to an increase of investigations of essential oils' (EO) potentials in several species of plants (1–3). An important factor that contributed to this searching of new active ingredients, is the emerging resistance of microorganisms to drugs used in conventional therapy, its toxicity and the reduction of development of new formulations for this purpose (4,5). The inclusion of EO prevails in formulations intended for topical or inhalation applications, having a great potential of antimicrobial activity mainly due to diversity of its composition and its physical chemical characteristics (6,7).

The EO of *P. graveolens* is known commercially as geranium oil and is widely used in cosmetics industry, mainly in perfumes, and in food industry as a flavoring and preservative compound (8–10). In pharmaceutical field it is used for several purposes, among them as anti-inflammatory, antibacterial and antifungal (9,11,12). Studies reported its activity against Gram positive and negative bacteria and strains of filamentous fungi and yeasts, from extracts of roots and leaves (11–13).

Nevertheless, EO can suffer degradation through oxidation, isomerization and hydrogenation reactions, enzymatically or chemically, influenced by the conditions during its processing and storage (14,15). The use of nanosystems to develop skin formulations, as nanoemulsions containing oil/water, increased recently, due to interest in encapsulation of lipophilic functional ingredients (16). This is an alternative approach to develop essential oils formulation, since it increases its physical stability, protect them from interactions with environment and decrease their volatility (16–18). Also, nanoformulations may improve the absorption of active compounds into the vaginal mucosa, promoting a better interaction between the oil and the microorganism, enhance the delivery of active compound and consequently increase the efficacy of treatment (19).

The methodology of incorporation of nanoparticles into a viscous system can contribute to the correct delivery of the active compounds at the site of action. Moreover, the nanoencapsulation itself, when using a polycationic polymer, can improve formulation mucoadhesivity and its efficacy, due to the increase of interactions between the surface of nanoparticles and cell constituents (19–21).

Candida is one of the most important yeasts having a representative contribution in opportunistic infections of genitourinary and gastrointestinal tract in adults. Among women, constitute one of the main pathogens responsible for vulvovaginitis (vulva and vagina infection) and recurrent vulvovaginal candidiasis (22–24). The specie most prevalent in vaginal infection is *Candida albicans*, however other species such as *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata, C. stellatoidea* and *C. lusitaniae* (25,26), also can be found (22,27–29). Traditionally, the treatment of candidiasis is made with azoles and amphotericin B. Nevertheless, when submitted to a long-term treatment, these drugs have toxic effects and result in yeasts resistance, which has increased over years for different species of *Candida* (22,25,29,30).

Considering the high number of resistant microorganisms, the limited therapeutic arsenal available and the high toxicity of some antifungal drugs used in treatment of vulvovaginal candidiasis, an alternative formulation containing essential oils with bioadhesive polymers can be promising. This formulation could facilitate skin penetration, increasing EO absorption, and improving the treatment efficacy (3,18,31).

Therefore, the aim of this work was to evaluate antifungal activity of commercial essential oils of *P. graveolens* from Brazil, Egypt, South Africa, China, Reunion Island-Bourbon and Albania against five *Candida* species, and to develop a biodegradable and mucoadhesive formulation capable of combating vaginal candidiasis with the commercial essential oil of *P. graveolens.*

MATERIALS AND METHODS

Chemicals and materials

Pelargonium graveolens essential oils were obtained by donation from Laszlo Aromaterapia LTDA, Espírito Santo, Brazil (Egypt, South Africa, China, Reunion Island-Bourbon, Albania); Ferquima, São Paulo, Brazil, (South Africa) and Verbena, Rio Grande do Sul, Brazil (Brazil and South Africa). Tween 20® and low molecular weight chitosan were purchased from Sigma-Aldrich, St. Louis, MO, USA. Medium chain triglycerides (MCT) were purchased from Delaware (Porto Alegre, Brazil). Latic acid was purchased from Synth (São Paulo, Brazil). Sodium lauryl sulfate, NaOH.

Ultrapure water was obtained from a Milli-Q® apparatus (Millipore, Billerica, USA). All other chemicals or reagents were of analytical grade.

Preparation of formulations

Preparation of nanoemulsions

Composition of *P. graveolens* nanoemulsion (NPG) and blank nanoemulsion (NB) are described in Table I. NB was prepared without *P. graveolens* essential oil (only MCT 10% w/w). Aqueous (Milli-Q® water and Tween 20®) and oily phases (*P. graveolens* essential oil or MCT and Span 80®) were weighed (w/w) and prepared separately. Then, the aqueous phase was poured into the oily phase, under magnetic stirring, to form a coarse emulsion. To decrease the droplet size, this coarse emulsion was submitted to Ultra-turrax (Ultra-Turrax® T18, IKA, Germany) for 1 min at 13500 rpm followed by high-pressure homogenization (Emulsiflex-C®, Avestin, Canada) for 6 cycles at 750 bar. All steps were performed at under room temperature. All formulations were prepared in triplicate and stored from light, under refrigerated temperatures.

Table I. Final composition of the different formulations (g).

Formulation	*P. graveolens* essential oil	MCT	Span 80®	Tween 20®	Water	Chitosan
NPG	1		0.1	0.1	8.8	
NB		1	0.1	0.1	8.8	
HCNPG	1		0.1	0.1	8.8	0.2
HCHI					10	0.2

NPG: *P. graveolens* nanoemulsion; NB: blank nanoemulsion; HCNPG: *P. graveolens* nanoemulsion; based-hydrogel; HCHI: blank hydrogel thickened with chitosan. MCT: medium-chain triglycerides

Preparation of hydrogels

P. graveolens nanoemulsion based-hydrogels (HCNPG) were prepared by adding low molecular weight chitosan (2%, w/w) to nanoemulsion (NPG), stirring at 500 rpm during 45 min and left to swell overnight. Later, lactic acid was added for adjusting pH value and formation of gel. Blank hydrogels with chitosan were produced as described for HCNPG, but replacing NPG by water (HCHI). All formulations are described in Table I and were prepared in triplicate and stored protected from light, under refrigerated temperatures.

Physico-chemical characterization

Essential oil

The essential oil from South Africa had its chemical composition determined using a GC-MS by a combination of their retention indices—using *n*-alkanes as external standards (32), according Santos M.K , 2018 (paper submitted).

In addition, the essential oil of *P. graveolens* from South Africa was characterized through the refractive index, performed with a refractometer Carl Zeiss (Germany), and density measured with a 5-mL calibrated pycnometer, both at 23 ± 0.5 °C. All measurements were carried out in triplicate and represented as mean ± SD (standard deviation)

Nanoemulsions and hydrogels

Citronellol and geraniol content in nanoemulsion (NPG) and hydrogel (HCNPG) were evaluated using a headspace extraction followed by GC-MS analysis. The major compounds content were evaluated for 30 days - short stability purposes. All formulations were analyzed in triplicate.

The analyzes were performed on a 7890A gas chromatograph coupled with a 5975C mass spectrometer (Agilent Technologies, CA, USA), equipped with an automatic headspace auto-sampler (CTC Analytics Combipal, Basel, Switzerland). A fused-silica DB-5 column (30 m x 0.25 mm x 0.25 µm) was employed for chromatographic separation. The mass detector was operated using 70 eV electrons with a source temperature, transfer line and injection port set at, 150 °C, 300 °C, and 220 °C, respectively. The oven temperature was programmed to start at 60 to 180 °C, with an increase of 3 °C/min. Helium was utilized as the carrier gas at a flow rate

of 1 mL/min. The experimental conditions of headspace (HS) extraction were stirring time of 10 min and heating temperature of 70 °C, under 500 rpm of agitation. The HS gas volume injected on the GC-MS was 1.0 mL.

pH and density measurements

NPG, NB, HCHI and HCNPG had their pH directly determined at 23 ± 0.5 °C using a Denver UltraBasic digital pH meter (Denver Instruments, New York, USA) previously calibrated and shown as mean ± standard variation (SD). Density was also measured with a 5-mL calibrated pycnometer at 23 ± 0.5 °C and expressed as mean ± standard variation (SD).

Droplet size, polydispersity index and zeta potential

Droplet size (DS) and size distribution (polydispersity index = PDI) of NB, NPG and HCNPG were accessed at 25 °C after 1000x dilution in ultrapure water by photon correlation spectroscopy using a Malvern Zetasizer Nanosizer® ZS90 (Malvern Instruments Ltd, Worcestershire, UK). Zeta potential (ZP) of formulations were also measured at 25 °C in the same equipment by electrophoretic mobility after diluting 1000x in NaCl 0.1 mM solution filtered in 0.45 µM filter. All measurements were carried out in triplicate and represented as mean ± SD (standard deviation).

Rheological behavior

Rheological profiles of HCHI and HCNPG were performed in a Brookfield Rotational Viscometer, model DV-II+ (Brookfield Engineering Laboratories, Middleboro, USA), and were analyzed at rotational speed 8, 10, 15, 20, 30 and 40 rpm with spindle 18. Rheological determinations were carried out with 10 g of samples, performed in triplicate at 24.0 ± 1 °C and respecting the limits of values of torque (above 10% and less than 100%). Rheograms are shown as shear stress (D/cm²) *vs* shear rate (1/sec) and viscosity (cP) *vs* shear rate (1/sec).

Antifungal assay

Fungal strains

Clinical isolates and/or standard strains of *C. albicans* (ATCC 18804, CA01), *C. krusei* (CK02, ATCC 6258), *C. tropicalis* (CT56, CT72A), *C. parapsilosis* (RL01 e RL 20) and *C. glabrata* (RL24, CG40039) belonging to the mycology collection of the Applied Mycology Laboratory, School of Pharmacy, from Federal University of Rio Grande do Sul (UFRGS), Brazil.

2.5.2 Assessment of Minimum Inhibitory Concentration (MIC)

Eight commercial essential oils of *P. graveolens* were diluted in dimethyl sulfoxide (DMSO; Synth). Fungal strains were grown on Sabouraud agar with chloramphenicol (24h, at 35°C). First, an antifungal screening of EO was tested (512 µg/mL) against all clinical isolates mentioned above. MIC was determined only in cases where antifungal activity was found in the screening test, by the broth microdilution method according to the M27-A3 protocol (33). MICs values were defined as the lowest concentration of compounds at which the microorganisms tested did not demonstrate visible growth in 48 h. The experiments were conducted in RPMI-MOPS culture (RPMI 1640; without sodium bicarbonate and pH 7.0 with morpholinepropansulfonic acid). EO samples were tested in concentrations ranging 2-512 µg/mL. Tween 20 and RPMI were used as negative controls and fluconazole as positive control test.

MIC was also evaluated for twelve different interactions between the EO of *P. graveolens* based on the difference of your major compounds. Were tested the following combinations: Brazil and Albania; Albania and Africa; Brazil and China; Egypt and Brazil; Egypt and Africa; Albania, China and Egypt; Albania, China and Brazil. Finally, MIC was tested for nanoemulsions with the EO (NPG) and without the EO blank nanoemulsion (NB). The final formulation, hydrogel containing the essential oil (HCNPG) and chitosan, blank hydrogel (HCHI) were also tested. Formulations were first diluted in RMPI medium to reach the same test conditions of the EO. All the experiments were performed in triplicate

RESULTS AND DISCUSSION

Physico-chemical characterization of *P. graveolens* essential oil, nanoemulsions and hydrogels

Chemical characterization of South African essential oil of *P. graveolens showed* geraniol and citronellol as major compounds, reaching 17.5 and 24.5% and presenting a total amount of oxygenated monoterpenes of 71.5% (Santos M.K et al, 2018, paper submitted). Citronellol and geraniol were then used as markers during development of nanoemulsion and hydrogel formulations. The refractive index and density of the essential oil were also measured with a quality control purpose, at 23 ± 0.5 °C, with values of 1.4662 ± 0.0003 and was 0.8784 ± 0.0049 $g.mL^{-1}$, respectively.

Density measurements performed at 23 ± 1 °C were 1.0123 ± 0.0038, 1.0043 ± 0.0023, 1.0007 ± 0.0042 and 1.0087 ± 0.0026 g/mL for NB, NPG, HCHI and HCNPG, respectively. Furthermore, the mean pH values for all formulations were between 4.32 – 4.48, without significant difference when analyzed by ANOVA ($p > 0.05$), indicating its adequate physico-chemical stability during 30 days. Thus, the final formulation – HCNPG, is compatible with vaginal pH, which requires pH values between 3.5 and 4.5, naturally acidic due to the presence of lactobacilli responsible for the conversion of glycogen in lactic acid and that offers natural resistance to the colonization of various microorganisms (34,35).

Nanoemulsions and hydrogels showed nanometric diameters according to droplet size (DS) (Table II), showing a mean value lower than 230 nm. *P. graveolens* nanoemulsion-based hydrogel (HCNPG), presented the highest droplet size, presenting a mean value lower than 297 nm. This could be explained due to the incorporation of chitosan to nanoemulsion, creating the hydrogel, which can increase the particle size (Table II). This effect is possible due to the different charges between nanoemulsion (negatively charged) and the cationic polymer (chitosan, positively charged), which interact forming an external layer, as can be seen by the increase in the droplet diameter (36,37).

The polydispersity index (PDI), reflects the uniformity of particle diameter. The lower PDI value is related to homogeneous size distribution of droplets (38). PDI values for NB, NPG and HCNPG were between 0.142 to 0.345 (Table II).

Homogeneous distribution of droplets remained adequate for all formulations during the 30-days storage (Fig. 1).

Regarding zeta potential, the values obtained before chitosan-coating (NPG) were negative (Table II). After addition of chitosan, the surface charge of NPG changed from - 32.21 to + 53.43 (day 0) at HCNPG formulations (Table II). The charge value of NPG and HCNPG maintained similar even after 30 days. Due to the chitosan positive charge, its addition to formulation may alter the zeta potential, interacting with negatively charged molecules from nanoformulations (39). Despite that, formulations containing chitosan, a biocompatible and biodegradable polymer with low toxicity, can increase the release of antimicrobial agents due its bioadhesion, safety and controlled release, thus being a good alternative to development of vaginal formulations (40–43).

Table II. Physico-chemical characterization of nanoemulsions and hydrogels.

	NB				NPG				HCHI				HCNPG			
	T0	T7	T15	T30	T0	T7	T15	T30	T0	T7	T15	T30	T0	T7	T15	T30
DS (nm)	226.06 ± 6.25	229.54 ± 4.36	220 ± 3.56	221 ± 4.06	141.83 ± 2.16	140.93 ± 2.64	135.06 ± 9.05	141.63 ± 5.85					281.16 ± 5.78	296.7 ± 6.39	283.96 ± 8.88	276.15 ± 6.42
PDI	0.150 ± 0.02	0.147 ± 0.04	0.142 ± 0.05	0.145 ± 0.06	0.229 ± 0.04	0.227 ± 0.05	0.24 ± 0.07	0.195 ± 0.05					0.321 ± 0.09	0.335 ± 0.08	0.326 ± 0.05	0.345 ± 0.07
ZP (mV)	-36.98 ± 2.44	-38.15 ± 5.98	-39.67 ± 7.16	-38 ± 3.51	-32.21 ± 11.95	-34.16 ± 1.13	-36.18 ± 5.72	-33.85 ± 2.53					53.43 ± 3.85	48.26 ± 5.69	55.56 ± 6.43	51.25 ± 7.52
pH	4.36 ± 0.05	4.38 ± 0.03	4.37 ± 0.04	4.32 ± 0.06	4.39 ± 0.08	4.42 ± 0.09	4.45 ± 0.06	4.43 ± 0.07	4.44 ± 0.04	4.48 ± 0.02	4.41 ± 0.08	4. 38 ± 0.07	4.42 ± 0.04	4.45 ± 0.08	4.43 ± 0.06	4.40 ± 0.05

Values displayed as mean ± standard deviation; NB: blank nanoemulsion; NPG: nanoemulsion of *P. graveolens*; HCHI: blank chitosan hydrogel; HCNPG: *P. graveolens* nanoemulsion-based chitosan hydrogel. DS: droplet size; PDI: polydispersity index; ZP: zeta potential. T0: time zero; T7: time 7 days; T15: time 15 days; T30: time 30 days.

Short-stability evaluation

Considering the citronellol and geraniol content (%), it was observed that its concentration remain stable until 15 days after the preparation of formulations (NPG and HNPG). Beyond that, its content decrease, mainly for geraniol (Table III). It is possible that the major compounds or the essential oil itself remain in the external phase of the droplets instead the oily core, leading to its volatilization.

Table III. Short-stability of Citronellol and Geraniol in nanoemulsion and hydrogel. Values displayed as mean ± standard deviation; NB: blank nanoemulsion; NPG: nanoemulsion of *P. graveolens*; HCHI: blank chitosan hydrogel

Content (%)	NPG				HCNPG			
	T0	T7	T15	T30	T0	T7	T15	T30
Citronellol	68.20 ± 6.94	79.76 ± 12.29	63.15 ± 7.49	52.29 ± 6.67	74.49 ± 12.73	72.63 ± 1.07	72.13 ± 14.20	70.43 ± 4.32
Geraniol	70.19 ± 8.63	67.28 ± 13.99	45.49 ± 7.88	25.22 ± 3.99	71.54 ± 2.20	46.67 ± 3.03	44.03 ± 3.75	39.57 ± 6.78

Hydrogels rheological profile

Rheological and viscosity profile of HCHI and HCNPG presented non-Newtonian flow, since the relation between shear stress and shear rate was not directly proportional. Furthermore, a pseudoplastic behavior was observed in both hydrogels, characterized by a decrease in viscosity with an increase in shear rate. Also, it was observed a thixotropic behavior for HCNPG, since ascendant and descendant curves are not overlapping, the opposite of HCHI. This may occur due to the time-dependent change in viscosity, considering that a decrease occurs over time under a constant shear (44).

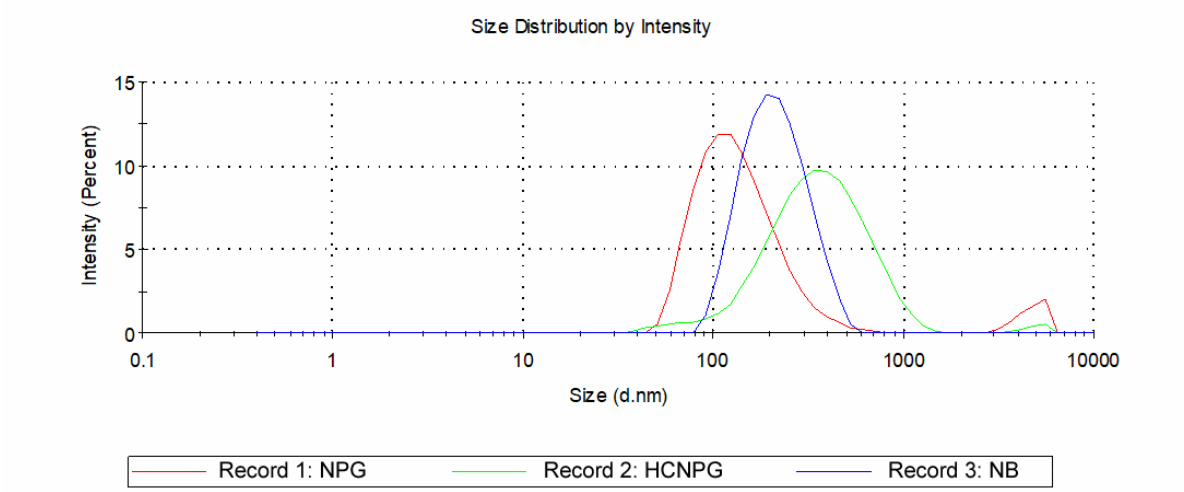

Fig. 1. Size distribution (diameter in nm) by intensity (%) of blank nanoemulsion (NB), *P. graveolens* nanoemulsion (NPG) and *P. graveolens* nanoemulsion-based chitosan hydrogel (HCNPG).

Also, the presence of NPG along with chitosan polymer influenced the viscosity; once the results of HCHI were lower (Fig. 2). This effect related to the higher viscosity of NPG might be due to the interaction between the nanoemulsion and chitosan chains, as well as the presence of essential oil and surfactants in the formulation, increasing the viscosity of the hydrogel, in agreement with studies that also used chitosan (42,45).

Additionally, different rheological flow models (Bingham, Ostwald, Casson and Herschel-Bulkley) were applied to predict flow behavior based on the shear rate and shear stress data. The best-fit model selected from the coefficient of determination values was found to be the Ostwald model ($T = Ky^n$, where T = shear stress, y = shear rate, K = consistency and n = flow index) for both HCHI and HCNPG formulations. (Table IV). The flow index values (n), obtained by Ostwald equation, were lower than 1 (0.8568 and 0.7189 for HCHI and HCNPG, respectively), which indicate that both hydrogels have non-Newtonian flow with pseudoplastic behavior, corroborating with the rheological profile and the previous discussion (Fig. 2).

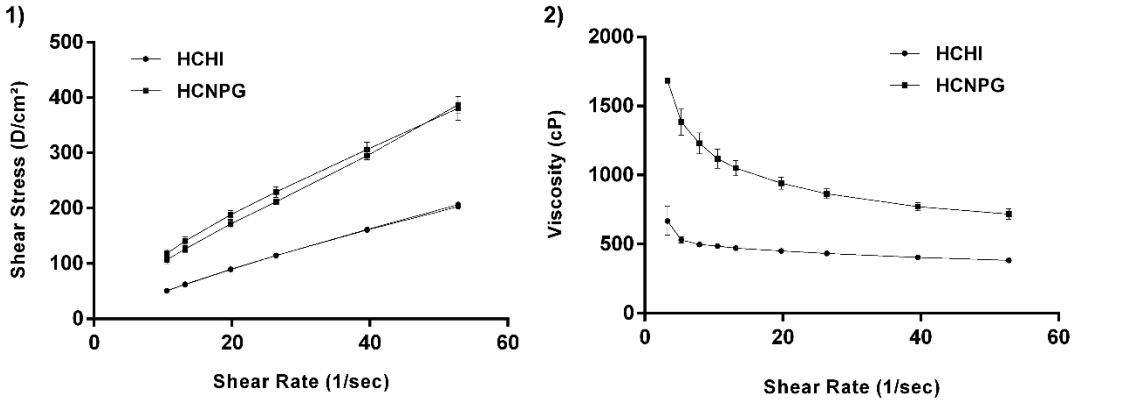

Fig. 2. Rheological profile (1) and viscosity profile (2) from HCNPG and HCHI (n = 3).

Table IV. Coefficients of determination (r^2) for rheological flow models based on shear rate and shear stress curves from hydrogels.

	Coefficient of determination (r^2)	
	HCHI	HCNPG
Bingham	0.9975	0.9970
Ostwald	0.9996	0.9998
Casson	0.9987	0.9990
Herschel-Bulkley	0.9667	0.9630

HCHI: blank hydrogel and **HCNPG:** hydrogel containing the essential oil.

Antifungal activity

All the EO of *P. graveolens* showed antifungal activity against five species of *Candida* sp in agreement with data reported in the literature (13,46–48). Were obtained MIC values slightly different for the eight EO against *Candida* species (Table V), probably due to their difference in chemical composition, since the essential oils come from different countries and variations in edaphoclimatic conditions may lead to changes in their chemical profile (49,50). Antifungal potential of essential oils is attributed in part to the large presence of monoterpene alcohols in its composition (SHIN, LIM, 2004; SILVA et al, 2012), that are the main constituents

of the essential oils of *P. graveolens.* Their major compounds are citronellol and geraniol, followed by linalool, nerol, geranyl acetate among others (11,51,52).

The most active EO against *Candida* sp. was from South Africa (Laszlo), with a MIC, between 128 and 256µg/mL, considering the best results for all isolates and strains tested. Similar results were found in EO from France and China. The EO from Brazil and the EO from South Africa, provided by Verbhena showed a small capacity to inhibit the growth of *Candida* species and showed a MIC of 512 µg/mL for the majority of isolates and strains (Table V). So far, the Brazilian EO of *P. graveolens* did not have its antifungal activity and its chemical composition evaluated, and compared to EO from different origins.

Regarding the difference between antifungal activity of total essential oil and its isolated major compounds citronellol and geraniol, in a study carried out by SHIN, LIM, 2004, the evaluation of MIC against *Trichophyton* sp strains showed that there was no significant difference between MICs of isolated compounds and total essential oil. Thus, it can be considered that antifungal activity should be attributed to the total oxygen compounds present in essential oils, which act in a complementary way inhibiting *Candida* growth (SILVA et al, 2012). ROSATO et al, 2008 evaluated the MIC of Tunisian geranium essential oil, extracted by hydrodistillation (Clevenger). The OE activity was evaluated against *C. albicans, C. krusei, C. tropicalis, C. guillermondi, C. parapisolis* and *C. glabrata*. The results showed inhibition of growth of isolates with MICs of 350 µg/mL, proving antifungal activity for the OE from Tunisia.

However, MIC values obtained in this study showed a higher activity for most of the EO (China, Africa, France, Egypt and Brazil) against *Candida* sp. *C. parapsilosis* was the most sensitive specie for EO of *P. graveolens* followed by *C. tropicalis* (Table V), with MIC values of 128 µg/mL and 256 µg/mL, respectively. Also, despite having the same country of origin, essential oils from South Africa, from three suppliers, presented difference in values obtained from MIC for most species, with exception of *C. albicans*, probably due to differences in their chemical compositions. Twelve different combinations of OE were also evaluated, and however none of them presented a significant response when compared to obtained values with each EO tested separately.

Table V. Comparison of inhibitory activities of *P. graveolens* essential oils against *Candida* sp trough Minimum Inhibitory Concentration (MIC).

	Minimum inhibitory concentration (µg/mL)									
	C. albicans		***C. tropicalis***		***C. krusei***		***C. glabrata***		***C. parapsilosis***	
	ATCC18804	**CA01**	**CT72A**	**CT56**	**ATCC6258**	**CK02**	**CG40039**	**RL24**	**RL01**	**RL20**
Essential oils										
Brazil	512	512	256	> 512	512	256	512	512	512	128
Africa[1]	512	512	256	> 512	512	512	512	> 512	512	256
Albania	512	512	512	512	512	512	512	512	512	128
Egypt	256	256	256	256	256	512	512	256	256	128
Africa[2]	512	256	256	256	256	256	256	256	256	128
France	512	256	256	512	512	512	512	256	256	128
China	256	512	256	512	256	256	512	512	256	256
Africa[3]	512	256	256	512	256	512	512	512	256	128
Controls										
Fluconazol	4	1	4	4	NT	NT	32	32	-	1
Tween 20	> 512	> 512	> 512	> 512	> 512	> 512	> 512	> 512	> 512	> 512

1: Verbhena, 2: Laszlo Aromaterapia e 3: Ferquima; Fluconazol: positive control; Tween 20: negative control; NT: not tested

The nanoemulsion containing essential oil (NPG) and a blank nanoemulsion (NB) were evaluated against the same especies of *Candida* tested f*or the EO,* along with hydrogel containg essential oil (HCNPG) and blank hydrogel (HCHI). According to Table VI, the NPG did not show difference in MIC values against *Candida*, when compared to dispersed EO. However, the opposite was found for the hydrogel containing *P. graveolens* nanoemulsion (HCNPG), produced with chitosan, wich allow better application via vaginal route . The results showed that MIC values obtained for HCNPG were lower for the majority of isolates tested, reaching 8 µg/mL for *C. albicans* and *C. glabrata*, indicating an improvement of up to 64 times in antifungal activity when compared to dispersed essential oil and to nanoemulsion (Table VI). C*. krusei*, and *C. parapsilosis* also presented greater susceptibility for the final formulation, with a reduction in MIC of up to 32 times for *C. krusei*; and of up to 16 times for *C. parapsilosis* This might be attributed to the fact that the addition of chitosan, a polycationic polymer, can improve drug delivery directly to *Candida* cells by allowing an electrostatic interaction between nanoparticle and microorganism improving its antifungal activity (19).

The influence of chitosan in antifungal activity was also observed through MIC values obtained from HCHI (formulation without the essential oil), when compared to values obtained for the nanoemulsions (NB and NPG). However, better results were obtained with the hydrogel containing the essential oil of *P. graveolens* (HCNPG), as mentioned before. Despite antifungal activity of chitosan, probably it was not the main reason why HCHI improved NPG efficacy, once chitosan itself has a MIC reported as 1 mg/mL against *Candida albicans* (53,54). So, it can be suppose that the inversion of potential zeta promoted by chitosan (from -32.21 to +53.43) improved electrostatic interaction between negative cell membrane and positive droplets, as seen for bacterias and positive nanocapsules, increasing the drug delivery to *Candida* cells (19).

Until now, there are few studies that comprise the development of formulations containing *P. graveolens* essential oil. Giongo et al, 2016 developed a nanoemulsion containing *P. graveolens* essential oil to evaluate its activity after biofilm formation against *Candida spp* in hospital medical supplies (55). Furthermore, a nanoemulsion-based hydrogel with Carbopol® containing *P. graveolens* was developed to topical

delivery and antifungal activity against *Candida albicans*, *Candida glabrata* and *Candida tropicalis* (56). However, the results presented did not show any improvement in antifungal activity against the *Candida* species when compared to the dispersed essential oil or to the nanoemulsion containing essential oil, with no advantages in its application. So, this reinforces our hypothesis of electrostatic interaction between cationic nanoparticles and *Candida*'s cell membrane, once Carbopol® has negative charge.

Table VI. Minimum Inhibitory Concentration of nanformulations containing the essential oil from *Pelargonium graveolens.*

	Minimum inhibitory concentration (µg/mL)									
	C. albicans		***C. tropicalis***		***C. krusei***		***C. glabrata***		***C. parapsilosis***	
	ATCC18804	**CA01**	**CT72A**	**CT56**	**ATCC6258**	**CK02**	**CG40039**	**RL24**	**RL01**	**RL20**
Formulations										
NB	> 512	> 512	> 512	> 512	> 512	> 512	> 512	> 512	> 512	> 512
NPG	512	512	256	512	512	256	256	512	512	128
HCHI	16	> 512	512	512	64	128	64	8	64	64
HCNPG	8	256	256	64	16	16	16	8	16	32

NB: blank nanoemulsion; **NPG**: nanoemulsion with the EO; **HCHI:** blank hydrogel and **HCNPG:** hydrogel containing the essential oil.

CONCLUSIONS

The eight dispersed-essential oils of *P. graveolens* demonstrate antifungal activity against five species of *Candida,* being the South African essential oil the most active*.* So, a chitosan hydrogel containing nanoemulsion of South African *P. graveolens* essential oil was developed. This formulation had adequate physico-chemical characteristics and a convenient pharmaceutical form for vaginal delivery. The nanoemulsion-based hydrogel showed an increase in antifungal activity against *Candida* spp with a significant reduction of MIC (up to 64 times), when compared to dispersed-essential oil. Considering that traditional antifungal therapy presents high toxicity, resistance and requires a long-term treatment, the promising results obtained, allied to the formulation constitution with biodegradable materials, make the nanoemulsion-based hydrogel of *P. graveolens,* an interesting alternative to treat vaginal candidiasis.

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7.DISCUSSÃO GERAL

A presente tese trouxe uma abordagem diversificada a respeito da DMAA. Procurou-se contemplar desde a investigação química em material vegetal, até em suplementos alimentares apreendidos; e a investigação biológica, através da avaliação da atividade antifúngica dos óleos essenciais. Também teve uma abordagem farmacotécnica, com o desenvolvimento de uma formulação utilizando a nanotecnologia.

O Manuscrito I trata da investigação da presença da DMAA em óleos essenciais extraídos por hidrodestilação de espécies de *Pelargonium* obtidas no Rio Grande do Sul. Também foram apresentados dados da constituição química destes óleos essenciais através de análise por GC-MS, que até o momento não havia sido relatada para espécies cultivadas no Brasil. A ausência de DMAA em espécies brasileiras, levantou a hipótese da possível perda da DMAA durante o processo de extração, já que durante o processo de obtenção dos óleos por hidrodestilação, que inclui altas temperaturas e elevado tempo do processo, poderiam ocorrer perdas dos constituintes mais voláteis, como os monoterpenos e até mesmo da DMAA, justificando então, a sua ausência nas espécies de *Pelargonium* (BICCHI et al., 2011; STASHENKO; MARTÍNEZ, 2008). Esta hipótese não havia sido levantanda até momento nos inúmeros trabalhos que realizaram a investigação acerca da presença da DMAA em espécies de *Pelargonium* (AUSTIN et al., 2014; AVULA et al., 2015; DI LORENZO et al., 2013; ELSOHLY et al., 2012, 2015; GAUTHIER, 2013; ZHANG et al., 2012). Para isso, foi empregada a técnica de extração por *headspace* seguido de análise por GC-MS. Para garantir a maior eficiência da extração por *headspace*, realizou-se um desenho experimental para cada espécie. Considerando o método proposto de extração por *headspace* estático, foi constatado que não houve a necessidade do uso de fibra de microextração em fase sólida, solventes ou adição de qualquer outro constituinte químico para realizar a identificação dos compostos voláteis presentes nas folhas das espécies de *P. fragrans*, *P. hortorum* e *P. peltatum*.

Normalmente os trabalhos que fizeram a determinação dos constituintes voláteis das plantas através de métodos alternativos de extração, como a microextração em fase sólida ou *headspace* dinâmico, realizaram um preparo do material vegetal, como a concentração ou secagem das plantas, sua dissolução em água e,

posteriormente, sua submissão à microextração (LIN et al., 2013; SGORBINI et al., 2015). Considera-se desta forma, o método proposto como uma alternativa mais simples e com custo inferior aos apresentados (JIROVETZ et al., 2002; LIN et al., 2013; SGORBINI et al., 2015; STASHENKO; MARTÍNEZ, 2004; ZINI et al., 2002). Ainda assim, a DMAA não foi identificada através da extração por *headspace* seguida da análise por GC-MS.

Este Manuscrito também apresentou a avaliação da presença de DMAA em óleos comerciais obtidos do Brasil, China, África do Sul, Egito, Albânia e Ilhas Reunião, além da determinação da sua composição química, através de GC-MS. Se estivesse presente nos óleos, sua identificação poderia ter sido realizada a partir da sua fragmentação gerada pelo detector de massas, associada à análise cromatrográfica, quando comparada ao espectro do padrão analítico e também identificada através do cálculo do índice de retenção, assim como foi realizado para os demais constituintes dos óleos essenciais. Ainda assim, decidiu-se empregar outras técnicas analíticas de elevada sensibilidade para confirmar a sua ausência nos óleos comerciais. A análise direta em espectrometria de massa em tempo real com análise sequencial (DART-MS/MS) constitui uma alternativa para a análise de substâncias presentes em matérias vegetais, incluindo os óleos essenciais (AVULA et al., 2015; KIM; JANG, 2009). Suas principais vantagens são a rapidez, sensibilidade e o mínimo (ou ausente) preparo da amostra (CODY; LARAMEE; DURST, 2005a; GROSS, 2014; KIM; JANG, 2009; LESIAK et al., 2014b). O método teve os parâmetros da DMAA previamente otimizados, através do padrão analítico, e, no entanto, não revelou a sua presença em nenhum dos óleos comerciais avaliados, confirmando o resultado encontrado por investigação anterior (AVULA et al., 2015).

A possibilidade do monitoramento do espectro de massas das transições dos íons, o que confere maior sensibilidade às análises, a ausência da necessidade de preparação das amostras, além da sua rapidez e praticidade, a tornam uma alternativa para avaliação qualitativa da composição química dos óleos essenciais, quando comparada às técnicas convencionais, como a cromatografia gasosa (GROSS, 2014; SINGH et al., 2012). Os óleos essenciais comerciais também foram analisados por LC-MS/MS. A metodologia desenvolvida, não detectou a presença de

DMAA nos óleos essenciais de *P. graveolens* (DI LORENZO et al., 2013; ELSOHLY et al., 2012, 2015). Os resultados obtidos neste trabalho corroboram com relatos científicos apresentados a respeito da ausência da DMAA nos óleos essenciais de *P. graveolens* obtidos de outros países, e outras espécies provenientes do Brasil e que mesmo utilizando métodos analíticos avançados, não encontraram a DMAA (AUSTIN et al., 2014; AVULA et al., 2015; BABU; KAUL, 2005; BOUKHATEM et al., 2013; BOUKHRIS et al., 2012; DI LORENZO et al., 2013; ELSOHLY et al., 2012, 2015; LIS-BALCHIN; STEYRL; KRENN, 2003). Os resultados também confirmaram a sua ausência em óleos essenciais provenientes da China, os únicos a apresentarem resultados positivos para a DMAA até o momento (LI; CHEN; LI, 2012; PING Z, 1996), e nos óleos essenciais comerciais e extraídos do Brasil, os quais não haviam sido reportados.

Assim, com base nos resultados encontrados e nos dados já relatados, entende-se que a adição da DMAA nos suplementos alimentares e compostos emagrecedores dos produtos comercializados livremente, apesar da proibição, não possui origem natural (AVULA et al., 2015; ELSOHLY et al., 2012). Ainda, deve ser considerado que mesmo que estivesse presente no óleo de gerânio, seria necessária uma grande quantidade da planta para extração da DMAA, e, desta forma, possibilitar a sua adição aos suplementos alimentares .

Após a investigação da presença da DMAA nos materiais vegetais, por distintas técnicas analíticas, foi realizada a investigação da sua presença, juntamente com cafeína, sibutramina, sinefrina, efedrina e metilfenidato em amostras de suplementos alimentares apreendidos pela Polícia Federal. O método proposto se mostrou simples e eficaz para a detecção de todas as substâncias simultaneamente, proporcionando uma análise de menos de um minuto por amostra e com um simples preparo, após a sua diluição em metanol e água. Todas as amostras analisadas (cápsulas sólidas, líquidas e comprimidos), apresentaram a presença de pelo menos um dos estimulantes investigados. A DMAA foi encontrada em 20% das amostras, juntamente com a cafeína, presente e 98% das amostras e outros estimulantes como a efedrina, sinefrina e sibutramina. Os resultados apresentados confirmam que mesmo após a proibição pela Agência Nacional de Vigilância Sanitária (ANVISA) em 2012 (BRASIL, 2012a), a DMAA continua sendo comercializada nos suplementos

alimentares, conforme dados já reportados no Brasil e em outros países (COHEN et al., 2017; JUSTA NEVES; CALDAS, 2015a; NEVES; CALDAS, 2017), e também amplamente consumidas pelos atletas profissionais, conforme os últimos relatórios da WADA (WADA, 2015, 2017a).

A DMAA é uma alquilamina alifática, com efeito simpatomimético, que quando consumida em altas doses pode levar a um grave aumento da pressão arterial e morte, e efeitos com leves e moderados quando consumida em doses inferiores (COHEN, 2012; FOLEY et al., 2014; KARNATOVSKAIA; LEONI; FREEMAN, 2015). No entanto o seu consumo concomitante ao de outros estimulantes, conforme os resultados obtidos, também podem oferecer um grande risco à saúde dos usuários, com um potencial aumento na toxicidade e na ocorrência de eventos adversos graves (GURLEY; STEELMAN; THOMAS, 2015; NEVES; CALDAS, 2017). Também, ressalta-se a presença simultânea de outros estimulantes, como a efedrina, sibutramina e o metilfenidato, tornando ainda mais grave o seu consumo, com um grande risco à saúde dos seus usuários, aumentando a possibilidade da ocorrência de efeitos adversos e até a morte (CALAHAN et al., 2016; GURLEY; STEELMAN; THOMAS, 2015; JUSTA NEVES; CALDAS, 2015a; STICKEL; SHOUVAL, 2015).

Métodos rápidos e simples são necessários para a análise de rastreamento de rotina de adulterantes que estão presentes em inúmeras amostras de suplementos alimentares, portanto, os métodos de ionização ambiente, como o DART-MS/MS simplificam as análises de triagem, eliminando a necessidade de etapas de limpeza e fornecendo dados espectrais de massa sem a preparação da amostra exigida nos métodos cromatográficos, por exemplo, sendo uma ótima alternativa aos laboratórios forenses.

Além da investigação da DMAA e análise da composição química, utilizou-se os óleos essenciais comerciais de *P. graveolens* adquiridos de diferentes países, para a avaliação da sua atividade antifúngica, frente a cinco espécies de *Candida* e, posteriormente o desenvolvimento de uma formulação para o tratamento da candidíase vaginal, considerando dados da literatura sobre a sua potencial atividade antifúngica (CARMEN; HANCU, 2014; HSOUNA; HAMDI, 2012; SESENI et al., 2015), conforme apresentado no Manuscrito III.

Desta forma, através dos resultados obtidos, comprovou-se a ação antifúngica do óleo de gerânio em todas as espécies de *Candida* testadas, através do ensaio da CIM, demonstrando maior atividade antifúngica contra a espécie de *C. parapsilosis*. Os óleos que apresentaram maior atividade frente à *Candida* foram os provenientes da África do Sul e Egito (fornecidos pela Laszlo), com valores de CIM de 256 µg/mL chegando até 128 µg/mL, considerando todas as espécies testadas, possivelmente devido à sua maior quantidade de monoterpenos oxigenados, quando comparado aos demais óleos essenciais (SHIN; LIM, 2004), conforme resultados obtidos no Manuscrito I. Ao contrário, o óleo essencial do Brasil, mostrou uma menor atividade antifúngica quando comparada aos óleos dos outros países, que pode ser atribuída à sua menor quantidade proporcional de monoterpenos oxigenados.

Além da diferença nas espécies de *Candida* testadas, devem ser considerados como fatores importantes para a avaliação dos resultados de CIM entre os OE a diferença na sua composição química, atribuídas às partes da planta utilizada para a extração, o local de cultivo, o país de origem e a metodologia e extração e análise. Ainda assim, os resultados encontrados com os óleos essenciais de gerânio corroboram com os dados da literatura (HSOUNA; HAMDI, 2012; ROSATO et al., 2008, 2009; SABZGHABAEE et al., 2011; SHIN; LIM, 2004), inclusive o que diz respeito aos valores encontrados para a CIM.

Ainda, considerando o potencial antifúngico a partir dos resultados apresentados, o aumento no número de microorganismos resistentes e o limitado arsenal terapêutico disponível (KATHIRAVAN et al., 2012; SAVIUC et al., 2015), foi desenvolvida uma formulação contendo o óleo essencial de *P. graveolens.*

O desenvolvimento de nanoemulsões contendo óleo/água vem sendo muito empregado recentemente, pois possibilita a inclusão de sistemas lipofílicos nas formulações, como os óleos essenciais, trazendo vantagens como menor tamanho de partícula, elevada área superficial, com consequente aumento da sua estabilidade e eficácia (SHARIF et al., 2017). Os resultados apresentados no Manuscrito III revelaram que o hidrogel de quitosana branco apresentou atividade antifúngica, com resultados superiores à nanoemulsão, porém 2 à 8 vezes menor que o hidrogel contendo a nanoemulsão de óleo essencial (formulação final). Ainda, quando comparada à atividade antifúngica do óleo essencial, a formulação final

apresentou uma diminuição de até 64 vezes na CIM de uma das cepas de *C. albicans*, e 32 vezes para uma das cepas de *C. glabrata*. A sua maior atividade pode ser atribuída à presença das cargas positivas conferidas (DE MARCHI et al., 2017) pela quitosana, as quais proporcionam uma maior interação eletrostática entre a nanoemulsão e o microorganismo (carregado negativamente), corroborando com resultados obtidos em estudos anteriores, os quais avaliaram o potencial antifúngico da quitosana (RABEA et al., 2003; SEYFARTH et al., 2008; WANG; DU; LIU, 2004). Outra hipótese é que o hidrogel facilita o contato do óleo essencial com o microorganismo, devido a sua retenção na matriz (gel), facilitando a entrega do fármaco ao local de ação e, portanto, aumentando a eficácia da formulação (LBOUTOUNNE et al., 2002). Também, deve ser ressaltado que a formulação desenvolvida promove a co-administração de duas substâncias ativas, no mesmo local de ação (quitosana e óleo essencial), com dois mecanismos de ação distintos, tornando pouco provável o desenvolvimento de resistência frente à formulação (AGNIHOTRI; MALLIKARJUNA; AMINABHAVI, 2004; BALOGLU et al., 2009; JOHAL et al., 2016)

Uma das vantagens de utilização da quitosana em formulações tópicas, inclusive vaginais, é que além de ser um polímero biodegradável, biocompatível, e melhorar a eficácia da atividade antimicrobiana dos óleos essenciais, ainda confere uma maior adesão entre a formulação e à mucosa vaginal, aumentando o tempo de contato da formulação com a área afetada e, consequentemente a sua eficácia, justificando as vantagens da sua aplicabilidade em formulações vaginais e o seu emprego para o tratamento de distintas doenças, conforme dados relatados na literatura (FRANK et al., 2014; JOHAL et al., 2016; ŞENYIĞIT et al., 2014; VALENTA, 2005).

Até o momento, poucos trabalhos foram reportados contendo nanoemulsões com o óleo essencial de *P. graveolens*. Mesmo assim, os resultados da CIM encontrados na literatura foram superiores ao deste estudo, com atividade antifúngica inferior. Além disso, ao nosso conhecimento, somente um trabalho propôs o desenvolvimento de um hidrogel contendo a nanomulesão com o óleo essencial de *Pelargonium* (IAN DA SILVA PATIAS et al., 2016), porém com uma formulação diferente da desenvolvida e que não apresentou melhora na atividade antifúngica frente às espécies de *Candida* quando comparada ao óleo essencial

isolado ou à nanoemulsão contendo o óleo essencial, sem apresentar, portanto, vantagens para a sua aplicabilidade.

8. CONCLUSÕES

A partir da obtenção dos óleos essenciais das espécies de *P. fragrans, P. peltatum* e *P. hortorum* cutlivadas no Rio Grande do Sul, pela técnica de hidrodestilação, pode ser confirmada a ausência da DMAA. A análise da constituição química das partes aéreas de *Pelargonium* através da extração por h*eadspace* e posterior análise através de GC-MS, mostrou-se como uma alternativa viável para a determinação dos constituintes voláteis de plantas, quando o objetivo final é a determinação qualitativa dos seus constituintes.

Além disso, este trabalho investigou a possível perda da DMAA nos óleos essenciais, que poderia possivelmente ocorrer durante o processo de extração por hidrodestilação. No entanto, a DMAA não foi encontrada na folhas das espécies de *Pelargonium* cultivadas no Brasil. Não obstante, os resultados apresentados a respeito da constiuição química das espécies de *Pelargonium* cultivadas no Brasil e as hipóteses levantadas pela perda na hidrodestilação, contribuíram com os dados já apresentados por outros autores que realizaram investigações sobre a presença da DMAA nas espécies de *Pelargonium*.

A determinação da presença de DMAA em óleos essenciais de diferentes países foi realizada utilizando três técnicas analíticas distintas e de elevada confiabilidade e sensibilidade, incluindo a análise por DART-MS/MS. Esta mostrou ser uma técnica rápida e simples para a análise dos óleos essenciais, também podendo ser considerada uma alternativa às técnicas tradicionais de análise, como a cromatografia gasosa. Porém, mesmo assim, a DMAA não foi encontrada nos óleos essenciais comerciais, corroborando com outros estudos realizados, e confirmando a ausência em óleos do Brasil, que ainda não haviam sido reportados e em óleos da China, os únicos que apresentaram resultados positivos até o momento.

Ainda, a respeito da DMAA, a sua presença também foi investigada em suplementos alimentares apreendidos pela Policia Federal do Brasil. Estas amostras foram analisadas por DART-MS/MS, técnica que se mostrou eficaz na determinação simultânea da DMAA, juntamente com, cafeína, efedrina, sinefrina, sibutramina e metilfenidato. Além disso, a metodologia desenvolvida por DART-MS/MS apresentou vantagens como rapidez (menos de 1 minuto por análise) e simplicidade, além de especificidade, podendo ser considerada como alternativa para a utilização na rotina dos laboratórios forenses, na detecção de substâncias lícitas e ilícitas em amostra

complexas como os suplementos alimentares, requerendo um mínimo preparo de amostra e tempo de análise, e demonstrando grande confiabilidade, conforme requerido nas análises forenses.

Os dados obtidos a partir das análises dos suplementos alimentares, corroboram com outros dados da literatura, demonstrando que os suplementos contendo DMAA continuam sendo comercializados de forma clandestina e contrabandeados, mesmo após à restrição do seu comércio e uso pelas agências regulatórias como a FDA e ANVISA.

A respeito da utilização dos óleos essenciais para investigação da sua atividade antifúngica, os ensaios que determinaram a CIM frente à diferentes espécies de *Candida,* comprovaram a atividade dos óleos de *P. graveolens* em todos os isolados testados, apresentando dados da atividade antifúngica do óleo essencial do Brasil, que não haviam sido reportados, e, que no entanto, foi inferior quando comparada a atividade dos óleos essenciais de outros países.

Ainda, a formulação final proposta, um hidrogel contendo a nanoemulsão com o óleo essencial de *P. graveolens*, provou ser mais eficaz frente às espécies de *Candida* comparada ao mesmo óleo essencial testado isoladamente. Assim, a formulação proposta pode ser considerada uma alternativa ao tratamento da candidíase vaginal quando comparada aos fármacos já existentes e aos recentemente desenvolvidos.

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10. ANEXOS

ANEXO 1

A seguir encontra-se a representação da ata referente à Tese de Doutorado apresentada:

..uCF

ATA PARA ASSINATURA Nº _____

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Programa de Pós-Graduação em Ciências Farmacêuticas
CIÊNCIAS FARMACÊUTICAS - Doutorado
Ata de defesa de Tese

Aluno: Maíra Kerpel Dos Santos, com ingresso em 01/04/2014
Título: **Estudo dos óleos essenciais de espécies de Pelargonium (Geraniaceae) e de suplementos alimentares e compostos emagrecedores contendo 1,3-dimetilamilamina: uma abordagem química, antifúngica e forense.**
Orientador: Profª Drª Renata Pereira Limberger

Data: 16/03/2018
Horário: 14:00
Local: Anfiteatro

Banca Examinadora	Origem
Miriam Anders Apel	UFRGS
Mirna Bainy Leal	UFRGS
Luciana Grazziotin Rossato	UPF

Porto Alegre, 16 de março de 2018.

Membros	Assinatura	Conceito	Indicação de Voto de Louvor
Miriam Anders Apel	Miriam Anders Apel	Aprovado	Sim
Mirna Bainy Leal	Mirna Bainy Leal	Aprovado	Sim
Luciana Grazziotin Rossato	Luciana	Aprovado	Sim

Conceito Geral da Banca: (Aprovado) Correções solicitadas: (X) Sim ( ) Não
Indicação de Voto de Louvor: (X) Sim ( ) Não

Observação: Esta Ata não pode ser considerada como instrumento final do processo de concessão de título ao aluno.
O Diploma somente será emitido após terem sido preenchidos todos os demais requisitos para a concessão do mesmo, o que deve ocorrer no prazo máximo de 90 (noventa) dias após esta data.

Maíra K. Santos
Aluno

Orientador

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