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LUCAS CALDEIRÃO RODRIGUES MIRANDA

HERBS, INFUSIONS, AND HERBAL-BASED READY-TO-DRINK BEVERAGES: DETERMINATION OF CONTAMINANTS AND SAMPLE PREPARATION TECHNIQUES

ERVAS, INFUSÕES E BEBIDAS PRONTAS-PARA-CONSUMO À BASE DE ERVAS: DETERMINAÇÃO DE CONTAMINANTES E TÉCNICAS DE PREPARO DE AMOSTRA

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Thesis presented to the School of Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Food Science.

Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Ciência de Alimentos.

Orientadora: Helena Teixeira Godoy
Coorientadora: Sara Cristina da Silva Cunha

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A ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

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Cântico negro

"Vem por aqui" — dizem-me alguns com os olhos doces
Estendendo-me os braços, e seguros
De que seria bom que eu os ouvisse
Quando me dizem: "vem por aqui!"
Eu olho-os com olhos lassos,
(Há, nos olhos meus, ironias e cansaços)
E cruzo os braços,
E nunca vou por ali...
A minha glória é esta:
Criar desumanidades!
Não acompanhar ninguém.
— Que eu vivo com o mesmo sem-vontade
Com que rasguei o ventre à minha mãe
Não, não vou por aí! Só vou por onde
Me levam meus próprios passos...
Se ao que busco saber nenhum de vós responde
Por que me repetis: "vem por aqui!"?

Prefiro escorregar nos becos lamacentos,
Redemoinhar aos ventos,
Como farrapos, arrastar os pés sangrentos,
A ir por aí...
Se vim ao mundo, foi
Só para desflorar florestas virgens,
E desenhar meus próprios pés na areia inexplorada!
O mais que faço não vale nada.

Como, pois, sereis vós
Que me dareis impulsos, ferramentas e coragem
Para eu derrubar os meus obstáculos?...
Corre, nas vossas veias, sangue velho dos avós,
E vós amais o que é fácil!
Eu amo o Longe e a Miragem,
Amo os abismos, as torrentes, os desertos...

Ide! Tendes estradas,
Tendes jardins, tendes canteiros,
Tendes pátria, tendes tetos,
E tendes regras, e tratados, e filósofos, e sábios...
Eu tenho a minha Loucura!
Levanto-a, como um facho, a arder na noite escura,
E sinto espuma, e sangue, e cânticos nos lábios...
Deus e o Diabo é que guiam, mais ninguém!
Todos tiveram pai, todos tiveram mãe;
Mas eu, que nunca principio nem acabo,
Nasci do amor que há entre Deus e o Diabo.

Ah, que ninguém me dê piedosas intenções,
Ninguém me peça definições!
Ninguém me diga: "vem por aqui"!
A minha vida é um vendaval que se soltou,
É uma onda que se alevantou,
É um átomo a mais que se animou...
Não sei por onde vou,
Não sei para onde vou
Sei que não vou por aí!

José Régio

RESUMO

O objetivo desse trabalho foi desenvolver, otimizar e validar métodos cromatográficos para a determinação de contaminantes em ervas aromáticas (n = 20), infusão de ervas (n = 20) e bebidas prontas-para-consumo à base de ervas (n = 16). A tese foi dividida em quatro capítulos. O primeiro tratou da aplicação de um método QuEChERS-LC-MS/MS e ICP-MS para a respectiva determinação de 14 micotoxinas e 21 elementos potencialmente tóxicos em ervas e infusão de ervas. O segundo abordou uma metodologia QuEChERS-GC-MS e DLLME-GC-MS para a análise de 25 pesticidas das classes dos organoclorados, organofosforados e piretroides, também em ervas e infusões, respectivamente. O capítulo seguinte tratou do desenvolvimento e validação de um método DLLME-GC-MS/MS para a determinação de 5 ftalatos e um adipato em bebidas à base de ervas envasadas em diferentes embalagens (polipropileno, polietileno de tereftalato, alumínio e embalagem cartonada). Por fim, o último capítulo versou sobre o desenvolvimento, otimização e validação de um método LDDES-DLLME-GC-MS/MS para determinação de hidrocarbonetos policíclicos aromáticos em bebidas envasadas de base vegetal. Os métodos validados demonstraram parâmetros de seletividade, limite de quantificação, limite de detecção, linearidade, precisão e recuperação satisfatórios para análise de compostos traços, por isso, eles foram aplicados às amostras reais. Ademais, os métodos podem ser considerados rápidos e de fácil aplicação. Quanto ao teor de contaminantes nas amostras, verificou-se que apenas as micotoxinas e pesticidas apresentaram resultados acima dos limites máximos considerados seguros.

Palavras-chave: Contaminantes, alimentos – análise, cromatografia, preparo de amostra - química.

ABSTRACT

This work aimed to develop, optimize, and validate chromatographic methods intended to determine contaminants in aromatic herbs (n = 20), herbal infusions (n = 20), and ready-do-drink herbal-based beverages (n = 16). This thesis was organized into four chapters. The first one deals with the application of a QuEChERS-LC-MS/MS and an ICP-MS method to determine 14 mycotoxins and 21 toxic elements in herbs and herbal infusions. The second one intended to develop and validate a DLLME-GC-MS method for the determination of 25 pesticides (organochlorine, organophosphorus, and pyrethroid) in herbs and herbal infusions as well. The following chapter deals with the development and the validation of a DLLME-GC-MS/MS method to investigate the presence and content of 5 phthalates and one adipate in herbal-based soft drinks packaged in different packaging materials (polypropylene, polyethylene terephthalate, aluminum, and carton). Finally, the last one handled the development, optimizations, and validation of a LDDES-DLLME-GC-MS/MS to determinate polycyclic aromatic hydrocarbons in packaged herbal-based beverages. The methods showed satisfactory parameters of selectivity, limits of detection, limits of quantification, linearity, precision, and recovery for trace analysis, therefore, they were applied to real samples. Moreover, the method can be considered quick and easy to perform. About the content of contaminants in the samples, only mycotoxins and pesticides showed results above the safe maximum levels.

Keywords: Contaminants, food – analysis, chromatography, sample preparation.

LISTA DE ILUSTRAÇÕES

CAPÍTULO I: HERBS AND HERBAL INFUSIONS: DETERMINATION OF NATURAL CONTAMINANTS (MYCOTOXINS AND TRACE ELEMENTS) AND EVALUATION OF THEIR EXPOSURE

CAPÍTULO II: DETERMINATION OF PESTICIDE RESIDUES IN HERBS AND MIGRATION ASSESSMENT TO THE HERBAL INFUSIONS

CAPÍTULO III: PHTHALIC ACID ESTERS AND ADIPATE IN HERBAL-BASED SOFT DRINKS: AN ECO-FRIENDLY METHOD

CAPÍTULO IV: A NOVEL DISPERSIVE LIQUID-LIQUID MICROEXTRACTION USING A LOW DENSITY DEEP EUTECTIC SOLVENT-GAS CHROMATOGRAPHY TANDEM MASS SPECTROMETRY FOR THE DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN SOFT DRINKS

DISCUSSÃO GERAL

LISTA DE TABELAS

CAPÍTULO I: HERBS AND HERBAL INFUSIONS: DETERMINATION OF NATURAL CONTAMINANTS (MYCOTOXINS AND TRACE ELEMENTS) AND EVALUATION OF THEIR EXPOSURE

CAPÍTULO II: DETERMINATION OF PESTICIDE RESIDUES IN HERBS AND MIGRATION ASSESSMENT TO THE HERBAL INFUSIONS

CAPÍTULO III: PHTHALIC ACID ESTERS AND ADIPATE IN HERBAL-BASED SOFT DRINKS: AN ECO-FRIENDLY METHOD

CAPÍTULO IV: A NOVEL DISPERSIVE LIQUID-LIQUID MICROEXTRACTION USING A LOW DENSITY DEEP EUTECTIC SOLVENT-GAS CHROMATOGRAPHY TANDEM MASS SPECTROMETRY FOR THE DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN SOFT DRINKS

SUMÁRIO

INTRODUÇÃO GERAL

Ervas aromáticas e medicinais podem ser utilizadas pelas indústrias de alimentos, cosméticos e farmacêuticas como fonte de óleos essenciais, compostos bioativos ou mesmo como matéria-prima para produção direta de alimentos e bebidas (Filly et al., 2014; Maier, Oelbermann, Renner, & Weidner, 2017; Saeed et al., 2017). Dentre essas aplicações, a mais relevante é a produção de bebidas, seja por infusão, decocção ou maceração. Infusões de ervas podem ser obtidas a partir de diversas plantas e de suas diferentes partes como flores, folhas, cascas e raízes. Devido a sua característica sensorial, apelo natural, baixo custo de aquisição, baixo teor de açúcar e por ser fonte de compostos bioativos, as infusões de ervas vem se tornando mais populares entre consumidores, substituindo, inclusive, as bebidas de alto teor de açúcares como os refrigerantes, por exemplo (Maier et al., 2017; Marshall & Mejia, 2012). Ademais, diversas infusões de ervas são utilizadas como auxiliares no tratamento de sintomas e doenças, listadas, inclusive, nos sistemas nacionais de saúde de diversos países (The World Health Organization, 2013). Recentemente, a indústria de bebidas vem ganhando espaço com as bebidas prontas-para-consumo a base de vegetais como os chás preto, verde e branco (*Camellia sinensis*) e, especialmente no Brasil, a erva mate (*Ilex paraguariensis*). Apesar de tudo, pouco se sabe acerca da presença de contaminantes em ervas e em bebidas à base dessas plantas, especialmente o quanto desses contaminantes podem migrar da matéria-prima para a bebida e, consequentemente, qual a real ingestão desses compostos pelos consumidores (Kala, 2015; Meinhart, Caldeirão, Damin, Filho, & Godoy, 2018; Meinhart, Damin, Caldeirão, & Godoy, 2019; Morais, 2014). Dentre os contaminantes que podem estar presentes em ervas, citam-se os elementos tóxicos (metais pesados), resíduos de pesticidas, micotoxinas, hidrocarbonetos policíclicos aromáticos (PAHs) e ésteres de ftalatos (PAEs). Esses dois últimos, além de serem considerados contaminantes ambientais, podem também ser originados durante o processamento (PAHs) ou migrar de embalagens, no caso de produtos processados (ftalatos)

(Beneta, Mutavdžić Pavlović, Periša, & Petrović, 2018; Cao, Zhao, & Dabeka, 2015; Chen et al., 2016; Ciemniak, Kuźmicz, Rajkowska-Myśliwiec, & Cadena, 2019; Dalipi, Borgese, Tsuji, Bontempi, & Depero, 2018; Kabak & Dobson, 2017).

São contaminantes inorgânicos os elementos que apresentam propriedades tóxicas, alguns podem até possuir função nutricional, como o zinco, alumínio, cobre e níquel, entretanto, em concentrações elevadas, podem figurar um cenário de toxicidade. Os efeitos deletérios no organismo humano dependem do elemento em si, da concentração e da frequência de exposição. Alguns vegetais podem absorver e acumular esses elementos, sobretudo quando o solo está contaminado, atingindo, portanto, a mesa do consumidor (de Paiva, Morgano, & Arisseto-Bragotto, 2019; Hajeb, Shakibazadeh, & Sloth, 2016). As micotoxinas são metabólitos secundários produzidos por fungos filamentosos, especialmente aqueles pertencentes aos gêneros *Aspergillus*, *Penicillium*, *Alternaria*, e *Fusarium* (Santos Pereira, C. Cunha, & Fernandes, 2019). Estima-se que cerca de 300 a 400 micotoxinas foram identificadas até o momento, entretanto, poucos grupos são de importância econômica ou para saúde. Dentre esses grupos, destacam-se as aflatoxinas (AFs), fumonisinas (Fs), tricotecenos (TRCs) e a zearalenona (European Commission, 2006; Santos Pereira et al., 2019). Outras micotoxinas, denominadas "emergentes", como as eniatinas (ENs), beauvericina (BEA), moniliformina (MON) e esterigmatocistina (STE) tem recebido atenção especial devido ao aumento de sua detecção nos alimentos (Santos Pereira et al., 2019).

Os pesticidas podem chegar aos vegetais por aplicação direta, quando há a finalidade de proteção das plantas, ou então por meio do ar e água ou solo contaminados. Tais compostos configuram problemas à saúde humana quando acima dos limites máximos residuais (LMR) estabelecidos pelas autoridades de saúde. Esses contaminantes ambientais são divididos em quatro grandes famílias: organoclorados, organofosforados, carbamatos e piretroides. (Jayaraj, Megha, & Sreedev, 2016; Sulaiman, Rovina, & Joseph, 2019).

Os PAHs compõem um grupo de mais de 100 compostos contendo dois ou mais anéis benzênicos, o que dão a eles alta estabilidade e toxicidade (Koszucka & Nowak, 2019). Como já mencionado, são contaminantes ambientais majoritariamente produzidos por práticas antrópicas como a combustão incompleta de combustíveis fósseis em veículos e processos industriais. Nos alimentos, esses contaminantes podem ser originados durante a processamento em altas temperaturas, a citar: fritura, defumação e forneamento, por exemplo. Ademais, devido à alta distribuição na água, ar e solo, eles podem facilmente contaminar vegetais destinados ao consumo humano (Guo et al., 2019; Koszucka & Nowak, 2019; Mologноni, Daguer, Motta, Merlo, & Lindner, 2019). Os ftalatos, por sua vez, são uma classe de compostos orgânicos sintéticos altamente empregado como plasticizantes ou solventes em diversos produtos como embalagens, cosméticos, pesticidas e repelentes. Quimicamente, os ésteres do ácido ftálico (PAEs) são um grupo de diesteres do ácido orto-ftálico (Cao, 2010; Fasano, Bono-Blay, Cirillo, Montuori, & Lacorte, 2012; Haji Harunarashid, Lim, & Harunsani, 2017; Serrano, Braun, Trasande, Dills, & Sathyanarayana, 2014). Já os ésteres do ácido adípico (ou adipatos), que são substitutos frequentes dos ftalatos, também são bastante empregados como plasticizantes, mas apresentam menor toxicidade. Dentre os adipatos, o di-(2-etil-hexila) (DEHA) é o maior representante desse grupo (Cao, 2010).

Quanto aos métodos analíticos, com exceção dos contaminantes inorgânicos que geralmente é realizada uma digestão ácida da matéria orgânica em sistema de micro-ondas e detecção em plasma acoplado indutivamente-espectrômetro de massas (ICP-MS), os contaminantes orgânicos podem ser extraídos por diversas técnicas. As mais comuns são baseadas em extração sólido-líquido ou líquido-líquido e analisados em sistemas de cromatografia líquida de alta eficiência (HPLC) ou cromatografia a gás (GC) acoplados aos mais diversos detectores, dependendo das características físico-químicas das moléculas. Os detectores de espectrometria de massas (MS), considerados detectores universais, são muito utilizados para fins de

determinação de compostos traços devido a sua alta sensibilidade e seletividade (Guo et al., 2019; Nasiri, Ahmadzadeh, & Amiri, 2020; Plaza-Bolaños, Frenich, & Vidal, 2010; Samsidar, Siddiquee, & Shaarani, 2018; Tokalioğlu, 2012; Zhang, Dou, Zhang, Logrieco, & Yang, 2018).

Diante do exposto, o desenvolvimento e validação de métodos para a determinação de contaminantes em nível traço em ervas aromáticas faz-se necessária para o entendimento do cenário nacional quanto a esse tópico, visando, ainda, a fiabilidade dos resultados obtidos. Ademais, analisar, também, as bebidas oriundas de ervas (infusões e bebidas prontas-para-consumo) é importante para o entendimento do comportamento desses contaminantes frente a preparação e/ou processamento das matérias vegetais.

OBJETIVOS

Objetivo Geral

Validar metodologias analíticas para avaliar a presença e concentração de contaminantes orgânicos e inorgânicos em 20 ervas aromáticas, infusão de ervas e bebidas prontas-para-consumo a base de ervas.

Objetivos específicos

- Validar um método QuEChERS-LC-MS/MS e determinar micotoxinas em ervas aromáticas comercializada no Brasil e suas infusões bem como estimar a exposição dos consumidores a esses contaminantes.
- Determinar a concentração de contaminantes inorgânicos via ICP-MS em ervas aromáticas comercializadas no Brasil.
- Validar um método QuEChERS-GC-MS e determinar a concentração de pesticidas organoclorados, organofosforados e piretroides em ervas aromáticas comercializadas no Brasil.
- Avaliar a migração de pesticidas de ervas para suas respectivas infusões via DLLME-GC-MS e avaliar a exposição dos consumidores a esses contaminantes.
- Desenvolver e validar um método DLLME-GC-MS/MS para determinação de 6 ésteres de ácido ftálico e um éster de ácido adípico em bebidas prontas-para-consumo à base de ervas envasas em diferentes materiais.
- Desenvolver e validar um método LDDES-DLLME-GC-MS/MS para determinação de hidrocarbonetos policíclicos aromáticos (PAHs) em bebidas prontas-para-consumo à base de ervas.

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CAPÍTULO I: HERBS AND HERBAL INFUSIONS: DETERMINATION OF NATURAL CONTAMINANTS (MYCOTOXINS AND TRACE ELEMENTS) AND EVALUATION OF THEIR EXPOSURE

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HERBS AND HERBAL INFUSIONS: DETERMINATION OF NATURAL CONTAMINANTS (MYCOTOXINS AND TRACE ELEMENTS) AND EVALUATION OF THEIR EXPOSURE

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ABSTRACT

Herbal infusions are amongst the world's most popular and widely enjoyed beverages, due to both large variety and convenience. However, natural contaminants, such as mycotoxins and trace elements can accumulate in aromatic herbs, which may have serious food safety and public health implications. In this study, the presence of mycotoxin, as well as the content of trace elements was evaluated in herbs and herbal infusions commercialized in Brazil. For the determination of fourteen mycotoxins, including the emerging mycotoxins enniatins (EN), beauvericin (BEA), and sterigmatocystin (STE), a liquid-chromatography tandem mass spectrometry (LC-MS/MS) method was validated. Overall, 42 out of 58 herb samples (72%) were contaminated, being BEA the most usual mycotoxin, present in 43% of the samples, followed by STE and HT-2 toxin, present in 37% and 24 % of the samples, respectively. In herbal infusions, the occurrence of mycotoxin was 88% lesser than those verified in raw products. Despite these low levels, the hazard quotient (HQ) calculated revealed a potential health concern for HT-2 in two infusions. The margin of exposure values for aflatoxins (AF), and ochratoxin A (OTA) , and sterigmatocystin (STE) from six herbal infusions were below 10,000, indicating also potential health risks. The twenty-one trace elements comprising toxic elements such as arsenic (As), cadmium (Cd), and lead (Pb) were determined in herb raw materials by inductively coupled plasma-mass spectrometry (ICP-MS). The levels of trace elements in herbs were very varied, with aluminum (Al) presenting the highest amount. The levels of legislated elements (As, Cd, Pb) analyzed in herbs were lower than 3.03 µg g-1 (Pb), thus not exceeding the legal limits defined for herbal medicinal by both European and Brazilian pharmacopeia.

Keywords: Mycotoxins; emerging mycotoxin; elemental analysis; QuEChERS; Food Contaminant.

1. Introduction

Aromatic and medicinal herbs are used by food, pharmaceutical, and cosmetic industries as a source of essential oils, additives, bioactive compounds, or even as a raw material in the preparation of food and beverages (Filly et al., 2014; Maier, Oelbermann, Renner, & Weidner, 2017; Saeed et al., 2017). Among these applications, the use of raw medicinal herbs for therapeutic purposes is still representing one of the most important roles, particularly in emerging and developing countries in Asia, Africa, and Latin America. Herbs are used by indigenous or alternative systems of medicine such as Ayurveda, homeopathy, naturopathy, Oriental, and Native American Indian medicine (The World Health Organization, 2013). Herbal infusions can be obtained from several plants and different parts of the plant such as flowers, leaves, bark, and roots. Due to their sensory characteristics, natural appeal, low acquisition cost, low sugar content, and to be a source of bioactive compounds (e.g. phenolics, tannins, and essential oils), herbal infusions have become popular worldwide (Maier et al., 2017; Marshall & Mejia, 2012). In South American countries as Brazil, these herbs are frequently purchased in bulk from public markets and fairs, where no information or guarantee about their quality is given to costumers, in particular as regards the presence of contaminants (Kala, 2015; Meinhart, Caldeirão, Damin, Filho, & Godoy, 2018; Meinhart, Damin, Caldeirão, & Godoy, 2019; Morais, 2014). Among the more likely contaminants in these products, mycotoxins and heavy metals may cause relevant concerns.

Mycotoxins are secondary metabolites produced by filamentous fungi especially those belonging to the genus *Aspergillus*, *Penicillium*, *Alternaria*, and *Fusarium* (Santos Pereira, C. Cunha, & Fernandes, 2019). Until now 300 to 400 mycotoxins have been identified and reported, but only a few groups of mycotoxins are of safety and economic concern, namely, aflatoxins (AFs), fumonisins (Fs), ochratoxins (OTs), trichothecenes (TRCs), and zearalenone (ZEN) (European Commission, 2006a; Santos Pereira et al., 2019). For example, aflatoxins

(AF), especially AFB1, are classified as group 1 human carcinogen according to the International Agency of Research on Cancer (IARC, 2020). Other mycotoxins such as Ochratoxin A (OTA) and fumonisins (Fs) are classified in group 2B as possibly carcinogenic to humans. Besides, studies reveal that OTA may cause nephrotoxicity, immunotoxicity, and renal tumors in animals (Bui-Klimke & Wu, 2014; Clark & Snedeker, 2007), while fumonisins are responsible for several diseases in animals and being mainly reported as nephrotoxic and hepatotoxic (Ahangarkani, Rouhi, & Gholamour Azizi, 2014). Trichothecenes comprise more than 150 compounds, among them nivalenol (NIV), deoxynivalenol (DON), and T-2 toxin (T-2) are the most common in food supply. These mycotoxins are classified in group 3 by IARC as not carcinogenic to humans (Ostry, Malir, Toman, & Grosse, 2017), however, they can cause many other health problems as acute and chronic toxicosis, DNA and protein synthesis inhibition, and outbreaking of the digestive system (Yazar & Omurtag, 2008).

Other mycotoxins such as enniatins (ENs), beauvericin (BEA), moniliformin (MON), and sterigmatocystin (STE) have been recently received special attention from the scientific community due to its increased occurrence in food and feed (Santos Pereira et al., 2019). These mycotoxins are generally defined as “emerging mycotoxins” since they are not frequently studied or regulated by health authorities. Concerning their toxicity, *in vivo* studies data is limited, but *in vitro* studies suggest genotoxic effects of BEA, EN-A, EN-A1, EN-B1, and MON (Fraeyman, Croubels, Devreese, & Antonissen, 2017), and *in vivo* oral exposure study showed the effects of BEA and EN-B over duodenum, kidney, thymus bone marrow, liver and uterus besides immunotoxicity, hyperplasia, increase of Reactive Oxygen Species and reduce of glutathione brain levels (Maranghi, Tassinari, Narciso, Tait, & La, 2018). STE is a precursor of AFB1, and some studies reported the induction of tumors in the liver, skin and lungs, and STE is the unique emerging mycotoxin classified as 2B group by IARC (EFSA, 2013b; Ostry et al., 2017; Pfeiffer, Fleck, & Metzler, 2014).

Due to its unavoidable and unpredictable nature, mycotoxins are present in many foods including herbs. Their production can start in the field throughout the crop growing cycle and continue during harvesting, drying, processing, and storage steps, and end in our cup. The presence of mycotoxins in herbs and infusions has been reported in the literature (Borja et al., 2018; Kabak & Dobson, 2017; Ozden & Ozden, 2017; Reinholds, Bogdanova, Pugajeva, & Bartkevics, 2019) as well by official regulators. The Rapid Alert System for Food and Feed (RASFF) of the European Union released 2720 alerts on mycotoxins between 2014 and 2019, being 253 (9.3%) related to herbs and spices products, all involving AFs or OTA. Despite their prevalence and toxicity, the legal limits established for mycotoxin in herbs are scarce, only AFB1 and a total of AFs (B1, B2, G1, and G2) have maximum legal limits. European Pharmacopeia (European Pharmacopoeia, 2019) establishes, respectively, in 2 μg kg^{-1} and 4 μg kg^{-1}, while the Brazilian Pharmacopeia (Brasil, 2019) set in 5 μg kg^{-1} and 20 μg kg^{-1}, for AFB1 and AFs respectively. These limits, however, are set for herbs with medicinal purposes and there are no maximum limits for herbs intended for beverage preparation (e.g. infusions).

Inorganic contaminants are a large class of elements with toxic properties, some of them are commonly classified as ‘heavy metals’ (e.g. cadmium, arsenic, lead, and mercury) and others have nutritional importance although, in high concentration, they become toxic to humans (e.g. aluminum, copper, zinc, and nickel). The effects of toxic elements in biological systems depend on the element itself and the exposure frequency (de Paiva, Morgano, & Arisseto-Bragotto, 2019; Hajeb, Shakibazadeh, & Sloth, 2016). IARC classifies Cd, Cr(VI), As and Ni compounds as group 1 (carcinogenic to humans), Pb inorganic compounds as group 2A (probably carcinogenic to humans), Ni (metallic), Pb and Co as group 2B (possibly carcinogenic to humans), and Cr(III) and Cr (metallic) as group 3 (not classifiable as to its carcinogenicity to humans) (IARC, 2020).

Humans are exposed to inorganic contaminants mainly through the diet, herbs, and infusions included. These elements can be accumulated in plants and become an important issue in countries where these products are typically consumed. Studies that determine the content of trace elements in herbs (e.g. Al, As, Cd, Cr, and Pb) showed very variable results (Dalipi, Borgese, Tsuji, Bontempi, & Depero, 2018; Haidu et al., 2017; Milani, Silvestre, Morgano, & Cadore, 2019). It occurs because of the concentration of toxic elements in plants may vary with soil and water qualities, agricultural practices, and the characteristic of the plant to absorb and accumulate them (Hajeb et al., 2016).

Arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg) are in the top 10 chemicals or groups of chemicals of major public health concern (WHO, 2010). Therefore, Brazilian (Brasil, 2019) and European pharmacopeia (European Pharmacopoeia, 2019) set the same limits for As (0.60 mg kg^{-1}), Cd (1.0 mg kg^{-1}), Pb (5.0 mg kg^{-1}), and Hg (0.10 mg kg^{-1}) for medicinal herbs, except for Cd that is only cited in Brazilian pharmacopeia.

Therefore, taking into consideration the lack of information concerning the presence and content of mycotoxins and trace elements in herbs commercialized in Brazil, this work aimed to evaluate the presence of these contaminants in herbs sold in Brazil. For that, a method for quantification of 14 mycotoxins by quick, easy, cheap, rugged, and safe extraction followed by liquid chromatography-tandem mass spectrometry (QuEChERS-LC-MS/MS) was validated and applied to the herbs and their herbal infusions. Furthermore, 21 trace elements in herbs were determined by inductively coupled plasma – mass spectrometry (ICP-MS). The relation between the content of mycotoxins in the herbs and herbal infusions was calculated to evaluate the potential exposure and hazard risk of mycotoxins to humans resulting from the herbal infusion consumption. The selection of mycotoxins was studied to take into consideration the prevalence reported in the literature and the possibility of occurrence in Brazil.

2. Materials and Methods

2.1. Reagents and standard solutions

The standards of aflatoxin-B1 (AFB1), aflatoxin-B2 (AFB2), aflatoxin-G1 (AFG1), aflatoxin-G2 (AFG2), HT-2 toxin (HT-2), T-2 toxin (T-2), fumonisin-B1 (FB1), fumonisin-B2 (FB2), and ochratoxin A (OTA) all with purity >98% were purchased from Sigma (West Chester, PA, USA) and Fluka (West Chester, PA, USA). The emergent mycotoxins sterigmatocystin (STE), enniatin-A (EN-A), enniatin-A1 (EN-A1), enniatin-B (EN-B), enniatin-B1 (EN-B1), and beauvericin (BEA), all with purity >95% were purchased from Sigma (West Chester, PA, USA). The internal standard (IS) Ochratoxin A-(phenyl-d5) (OTAd5) was purchased from Fluka (West Chester, PA, USA). Standard stock solutions of each mycotoxin at 10 mg L^{-1} were prepared in MeOH, from this, two mix working solutions at 1000 µg L^{-1} each were prepared in MeOH, one of legislated mycotoxin (AFB1, AFB2, AFG1, HT-2, T2, FB1 and FB2) and other with emergent mycotoxins (STE, EN-A, EN-A1, EN-B, EN-B1 and BEA). A stock solution of the internal standard was prepared at 10 mg L^{-1} in dimethylsulfoxide (DMSO) and a work solution at 500 µg L^{-1} was prepared in MeOH. All solutions and standards were stored at -18 °C when not in use.

Methanol (MeOH), acetonitrile (MeCN), formic and acetic acids, all high-performance liquid chromatography (HPLC) grade, were purchased from Merck (Darmstadt, German). Ammonium acetate (p.a.) was also from Merck. Ultrapure water, purified with a “Seral” system (SeralPur Pro 90 CN), was used in the preparation of the mobile phase.

Octadecylsilica (C18, particle size 55–105 mm) was purchased from Waters (Milford, MA, USA) and Z-sep^{+} was purchased from Supelco (Bellefonte, PA, USA). Anhydrous magnesium sulfate (anhydrous $MgSO_4$) was purchased from Sigma (West Chester, PA, USA) and treated for 5 h at 500°C before use.

A multi-elemental mix standard solution (75As, 111Cd, 208Pb, 7Li, 9Be, 11B, 27Al, 48Ti, 51V, 60Ni, 85Rb, 88Sr, 90Zr, 105Pd, 107Ag, 118Sn, 133Cs, 137Ba, 205Tl, 195Pt, and 209Bi) at 10 mg L^{-1} each isotope (PlasmaCAL SCP-33-MS, SCP Science, Quebec, Canada) was used to build the calibration curves for multi-elemental analysis in ICP-MS. Nitric acid (HNO_3) ≥69% (Honeywell. Fluka) and hydrogen peroxide (H_2O_2) (30-32%, w/w) (Fischer Scientific, UK) used to sample digestion were both trace analysis grade.

2.2. Sampling

A total of 58 samples from 20 types of dried herbs (Table S1) were purchased from public markets and fairs and in Campinas (São Paulo, Brazil) and Londrina (Paraná, Brazil). The samples were grounded (1000 rpm for 10 s, Grindomix GM 200, Retsch GmbH, Germany), mixed, vacuum packed (portions of 50 g), and stored at room temperature until the analysis.

2.3. Mycotoxin analysis

2.3.1. Instrument and analytical conditions

High-performance liquid chromatography assays were performed using an HPLC system Waters Alliance 2695 (Waters, Milford) with a triple quadrupole mass spectrometer Quattro Micro (Waters, Manchester, UK) and electrospray ionization source (ESI). A Kinetex C18 2.6 μm particle size analytical column (150 × 4.6 mm) with a pre-column from Phenomenex (Tecnocroma, Portugal), maintained at 30 °C, was used for chromatographic separation. The method was similar to those previously applied in nuts by (Cunha, Sá, & Fernandes, 2018). The mobile phase was water/methanol/acetic acid [94:5:1 (v/v) and 5 mM ammonium acetate] (solvent A) and methanol/water/acetic acid [97:2:1 (v/v)] (solvent B). The elution was conducted in a gradient starting at 95% of phase A with a linear decrease to 35% in 7 min. Then the mobile phase A decreased to 25% at 11 min, decreased to 0% at 13 min, and remained constant until 25 min. Initial column conditions were reached at 25 min and remained for 2

minutes until the next injection. The flow rate was 0.3 mL min^{-1} and the injection volume was 20 µL. The optimized MS/MS parameters for each analyte are listed in Table S2.

The MS/MS acquisition was operated in positive-ion mode with multiple reaction monitoring (MRM), the collision gas was Argon 99.995% (Gasin, Portugal) with a pressure of 2.9×10^{-3} mbar in the collision cell. Capillary voltages of 3.0 kV were used in the positive ionization mode. Nitrogen was used as desolvation gas and cone gas being the flows of 350 and 60 L h^{-1}, respectively. The desolvation temperature was set to 350 °C and the source temperature to 150 °C. Dwell times of 0.1 s $scan^{-1}$ were selected. The data were collected using the software program MassLynx 4.1.

2.3.2. Sample preparation

2.3.2.1. Herbs

Mycotoxins extraction was performed by a QuEChERS method previously developed (Cunha et al., 2018) with some modifications. Briefly, 1.0 g of sample was weighed into a 50 mL centrifuge tube and 200 µL of OTAd5 (IS) at 500 µg L^{-1} was added. After leaving the samples overnight for equilibration, 5 mL of water was added, and the tube was shacked for 30 min. Thereafter, 5 mL of MeCN with 1% formic acid was added along with 2.0 g of $MgSO_4$ anhydrous salt and 1.0 g of NaCl and tubes were mixed for 1h in an orbital shaker. The tubes were then centrifuged at 4000 *g* for 15 min to induce phase separation and mycotoxins partitioning. For the dSPE clean-up procedure, exactly 1.2 mL of the organic phase was transferred to a 4 mL vial containing 100 mg C18 and 50 mg Z-sep^{+}, vortexed for 30 seconds, and centrifuged for 4000 *g* for 5 min. Then, 0.80 mL of the upper layer was transferred to an injection vial and evaporated to dryness under a stream of nitrogen (SBH CONC/1 sample concentrator from Stuart®, Staffordshire, USA). The final extract was reconstituted in 750 µL

of mobile phase B (methanol: water: acetic acid (97:2:1) with 5mM ammonium acetate) and transferred to a 2 mL glass vial for LC-MS/MS analysis. Each sample was injected twice.

2.3.2.2. Infusions

Mycotoxins extraction was performed by a QuEChERS method previously developed (Cunha et al., 2018) with some modifications. An aliquot of 1 g of the dried herb was weighed in an Erlenmeyer flask, then 50 mL of hot distilled water (~98ºC) was added and maintained in contact for 15 min. After centrifuged at 4000 *g* for 5 min, 5 mL of the supernatant was transferred to a 50 mL centrifuge tube and 100 µL of OTAd5 (IS) at 500 µg L^{-1} was added. Thereafter, 5 mL of acidified MeCN was added along with 2.0 g of $MgSO_4$ anhydrous salt and 1.0 g of NaCl. The tubes were mixed for 15 min and then centrifuged at 4000 g for 5 min to induce phase separation and mycotoxins partitioning. The organic phase was transferred to a 2 mL vial, evaporated to dryness under a stream of nitrogen, and finally reconstituted in 160 µL of mobile phase B and analyzed by LC-MS/MS. Each sample was injected twice.

2.3.3. Method validation and quality control

Initially, the matrix effect was evaluated in a sample of *assa-peixe* herb (not contaminated with mycotoxins) for all the mycotoxins included in this study and it was expressed as:

$$Matrix\ effect\ (\%) = \frac{Slope\ in\ matrix}{Slope\ in\ solvent} - 1\ x\ 100 \qquad \text{(equation 1)}$$

This herb was chosen due to the high chlorophyll content and consequently high matrix-effect. The quantification was performed using matrix-matched calibration curves for each analyte, using, at least, five calibration points, through the linear range of the compounds, as suggested by Commission Regulation (EC) No 401/2006. In herbs, quantification was performed from 40 to 380 µg kg^{-1} for AFB1, AFB2, AFG1, AFG1, FB1, FB2, OTA, HT-2, T2, and STE, from 40 to 320 µg kg^{-1} for EN-B1, and from 10 to 320 µg kg^{-1} for EN-A, EN-A1, AN-B, and BEA. For herbal infusions, it was used also five calibration points ranging from 10 to 200 µg L^{-1} for AFB1,

AFB2, FB2, OTA, HT-2, and T-2, from 20 to 200 µg L^{-1} for FB1, and, finally, from 2.5 to 100 µg L^{1} for EN-A, EN-A1, AN-B, EN-B1, and BEA.

Precision for inter and intra-day was expressed as relative standard deviation (%RSD), both parameters were determined by analysis of triplicate spiked samples, in the same day (intra-day) and three subsequent days (inter-day), at three concentration level: 40 µg kg^{-1}, 220 µg kg^{-1} and 380 µg kg^{-1} for legislated mycotoxins (AFB1, AFB2, AFG1, FB1, FB2, OTA, HT-2, and T-2), 40 µg kg^{-1}, 160 µg kg^{-1}, and 320 µg kg^{-1} for emerging mycotoxins (STE, EN-A1, EN-B, EN-B1, BEA, and EN-A) in herbs while for infusion was 10 µg L^{-1}, 100 µg L^{-1} and 200 µg L^{-1} for legislated mycotoxins and 10 µg L^{-1}, 20 µg L^{-1} and 50 µg L^{-1} for emerging mycotoxins. The limit of detection (LOD) was determined by successive analyses of chromatographic extracts of sample solutions spiked with decreasing amounts of the analytes until a signal-to-noise ratio of 3:1 was reached. The limit of quantification (LOQ) of each mycotoxin was estimated as the minimum concentration that provides suitable trueness (recovery, >70% and <120%) and precision (<20%) values according to Commission Regulation (EC) (No 401/2006) (European Commission, 2006a).

2.4. Multi-elemental-analysis

2.4.1. Digestion and ICP-MS analysis

Initially, the samples were dried at 105 ºC for 8 hours or until constant weight; then about 300 mg of dry herb raw material was weighted in a polytetrafluoroethylene (PTFE) digestor vessel, and 3 mL of HNO_3 and 1 mL of H_2O_2 were added over the sample. After this, the vessel was hermetically sealed and forwarded to a microwave digestion unit Milestone MLS 1200 1200 (Sorisole, Itália) using the following configuration: 250 W for 1 min, then 0 W for 2 min, again in a 250 W for 5 min, 100 W for 5 min and finally 600 W for 5 min, totalizing 26 min for each batch. After digestion, the sample was transferred to a 20 mL volumetric flask, and the volume

was completed with ultrapure water. For every bath, a blank sample with all reagents but without sample was prepared to evaluate possible analytical contaminants.

The multi-elemental analysis was performed in an ICP-MS iCAP^{TM} Q (Thermo Fischer Scientific, Bremen, Germany). The calibration curves were built diluting the commercial multi-elemental mix at 10 mg L^{-1} PlasmaCAL SCP-33-MS (SCP Science, Quebec, Canada) in a range from 0.5 to 200 µg L^{-1}. LODs were determined from the analysis of 10 blank samples.

Control quality of the method was assessed by analysis of a Certified Reference Material (CRM) NRCC (Ottawa, Canada). The CRM was analyzed in the same conditions as the samples.

2.5. Exposure assessment

The exposure assessment of non-carcinogenic mycotoxins in infusions was performed comparing the estimated Probable Daily Intake (PDI) for each mycotoxin with the correspondent reference value, when available, to determine the respective Hazard Quotient (HQ) showed in (equation 2). When the HQ was < 1, the exposure was considered to be within safe limits (EFSA, 2013a; Santos Pereira et al., 2019). The estimated PDI was compared with the Tolerable Daily Intake (TDI) reference value for HT-2 and T-2 toxins (0.02 µg kg^{-1} bw day) (EFSA, 2017).

$$HQ = \frac{PDI}{TDI} \quad \text{(equation 2)}$$

Where: PDI is the probable daily intake obtained from equation 3; TDI is the available oral toxicity reference value.

The PDI (µg kg^{-1} bw day) was performed considering a daily consumption of a cup of infusion (200 mL) by an adult (Milani et al., 2019).

$$\text{PDI} = \frac{C_i \text{ x } C_m}{BW_i} \quad \text{(equation 3)}$$

Where: PDI is the probable daily intake of an individual (μg kg^{-1} bw day); C_i is the estimated average dose of herbal infusion intake per day by Brazilian consumers (L day^{-1}) (Milani et al., 2019); C_m is the average mycotoxin concentration in the herbal infusion analyzed (μg L^{-1}); BW_i is the body weight of an adult person (70 kg).

The exposure assessment of carcinogenic mycotoxin, namely aflatoxins, ochratoxin A, and sterigmatocystin in herbal infusions, was calculated as Margin of Exposure (MoE) using the Benchmark Dose Lower Confidence Limit (BMDL10) as proposed by Benford, Leblanc, and Setzer (2010), according to Equation (4). In this approach, MoE less than 10,000 (MoE<10,000) indicates a high public health concern risk (EFSA, 2013a).

$$MoE = \frac{BMDL10}{PDI} \quad \text{(equation 4)}$$

Where: MoE is the Margin of Exposure; BMDL10 is the Benchmark Dose Lower Confidence Limit for each mycotoxin; and PDI is the probable daily intake of an individual (μg kg^{-1} bw day). BDML10 for AFs (individual and sum), OTA, and STE is 0.4 μg kg^{-1} bw day (EFSA, 2020a), 14.5 μg kg^{-1} bw day (EFSA, 2020b), and 160 μg kg^{-1} bw day (EFSA, 2013b), respectively.

Combined Margin of Exposure (MoET) was calculated for the sum of AFB1 and AFB2 by the equation 5, where MoEAFB1 and MoEAFB2 is the calculated Margin of Exposure for AFB1 and AFB2, respectively.

$$MoET = \left[\frac{1}{\left(\frac{1}{MoEAFB1}\right)+\left(\frac{1}{MoEAFB2}\right)}\right] \quad \text{(equation 5)}$$

2.6. Statistical analyses

Calibration curves were submitted to regression analysis by the least square method and analysis of variance (ANOVA), where the lack of fit was assessed. The results showed that all curves were statistically significant ($p<0.05$) and no lack of fit was founded ($p>0.05$), so these

results evidenced no linearity deviation. All statistical analyses were performed in Statistica 10.0 (StatSoft®).

3. Results and discussion

3.1. Mycotoxins

3.1.1. In-house method validation

Method linearity was evaluated by matrix-matched calibration curves including five concentration levels and being subjected to the entire developed procedure. In general, the matrix effects (Figure 1) in assa-peixe herb and its infusion followed the same behavior, except for FB1, FB2, BEA and EN-A. Low matrix effect (> - 20% or < + 20%) was observed for AFG1 (-16.5%), FB2 (8.5%), EN-A1 (-6.1%), EN-B (-1.0%), BEA (-9.7%), and EN-A (-6.1%) in herbs and for FB1 (-8.3%), STE ($^{-1}$1 %), EN-A1 (-1.4%), and EN-A (19.4%) in infusions, so these effects can be ignored. In contrast, high matrix effect (< - 50% or > + 50%) was observed for AFB1 (-68.3%), FB1 (55.9%), STE (-90.4%) and EN-B1 (-97.1%) in herbs and AFB1 (-81.1), AFB2 (-66.4%), OTA (-53.8%), T-2 (-63.4%), EN-B1 (-67.5%) in infusions. Hence, matrix-matched calibration standards were used for both matrices aiming to overcome these matrix effects.

Good linearity was observed for both matrices (herbs and herbal infusion), with determination coefficients (R^2) higher than 0.966 (Table 1). Additionally, linear models were submitted to F test (ANOVA, 95% confidence) and residual analysis, which did not present anomalies or evidence of linear deviation.

LODs in herbs ranged from 2.5 to 20 µg kg^{-1} while in herbal infusions ranged from 0.5 to 7 µg L^{-1}. LOQs ranged from 10 to 40 µg kg^{-1} in herbs and from 2.5 to 20 µg L^{-1} in infusions. The results here obtained are slightly higher than those achieved by other chromatographic methods reported in a literature review conducted by Zhang et al (2018). These differences in LOD and

LOQ values were expected since in a multi-mycotoxin analysis an individual optimization of instrumental conditions (LC and MS) is difficult to achieve due to the wide differences in their physicochemical properties.

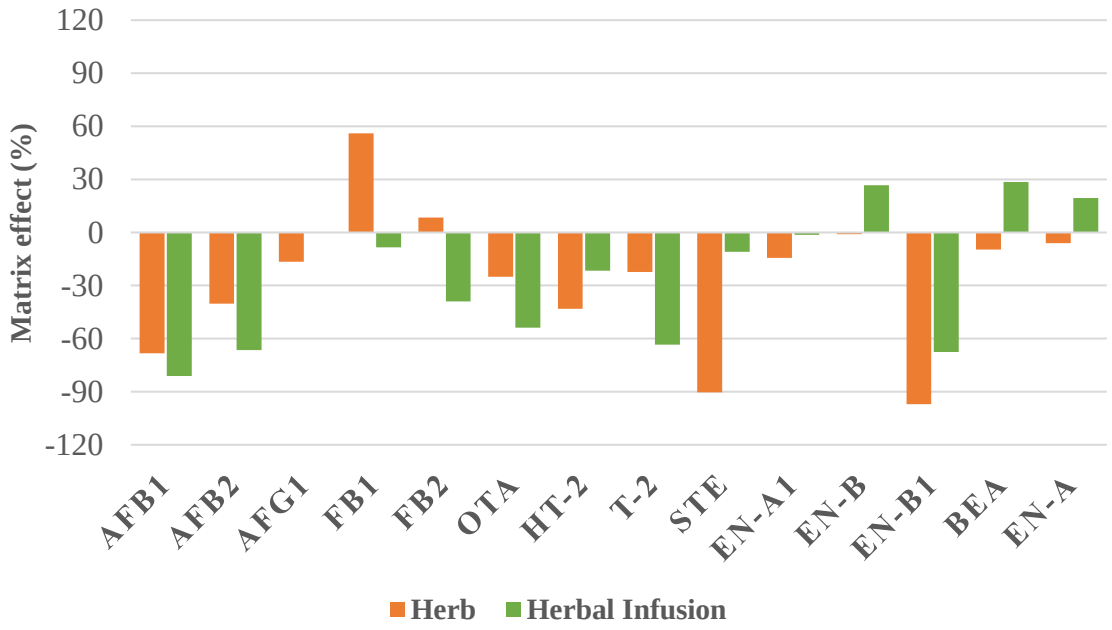

Figure 1 – Matrix Effects obtained for every mycotoxin in QuEChERS-LC-MS/MS for herbs and herbal infusions.

The percentage of recovery (%) was higher than 73% in both matrices (Table 1). The relative standard deviation (%RSD) values for inter-day and intra-day precision were lower than 20% for herbs and herbal infusions. The results obtained are within the control limits established by European Commission Regulation (European Commission, 2006b).

Table 1 - Performance parameters: limit of detection (LOD), linear range, coefficient of determination (R^2), % recovery (n=3), % relative standard deviation intra-day (%RSD, n=3) and % relative standard deviation -day (%RSD, n=9), obtained with developed QuEChERS- LC-MS/MS method for herbs and herbal infusions

	Herbs						Herbal infusions					
Mycotoxin	**LOD**	**Linear range**	**Linearity**	**% Recovery (%RSD intra-day, inter-day) Level**			**LOD**	**Linear range**	**Linearity**	**% Recovery (%RSD intra-day, inter-day) Level**		
	(µg kg^{-1})	**(µg kg^{-1})**	**(R^2)**	**1**	**2**	**3**	**(µg L^{-1})**	**(µg L^{-1})**	**(R^2)**	**1**	**2**	**3**
AFG1	2.5	40.0 - 380.0	0.989	108 (12, 18)	98 (9, 15)	97 (18, 3)	-	-	-	-	-	-
AFB2	20	40.0 - 380.0	0.995	100 (15, 15)	104 (19, 6)	102 (18, 14)	3	10.0 – 200.0	0.973	96 (16, 9)	110 (9, 7)	107 (14, 12)
AFB1	20	40.0 - 380.0	0.989	80 (21, 17)	99 (18, 16)	89 (7, 18)	3	10.0 – 200.0	0.989	104 (20, 18)	114 (17, 19)	102 (16, 20)
FB1	20	40.0 - 380.0	0.99	77 (15, 10)	91 (19, 9)	97 (11, 9)	7	20.0 – 200.0	0.994	111 (19, 20)	99 (18, 19)	111 (17, 12)
HT-2	20	40.0 - 380.0	0.991	108 (14, 11)	108 (15, 10)	100 (18, 7)	3	10.0 – 200.0	0.981	93 (17, 20)	110 (17, 16)	107 (19, 3)
T-2	20	40.0 - 380.0	0.982	115 (18, 15)	86 (14, 3)	97 (16, 8)	3	10.0 – 200.0	0.979	105 (20, 13)	102 (17, 15)	107 (14, 5)
OTA	20	40.0 - 380.0	0.997	107 (17, 9)	101 (16, 5)	97 (11, 4)	3	10.0 – 200.0	0.993	81 (19, 3)	98 (18, 18)	102 (19, 19)
FB2	20	40.0 - 380.0	0.978	104 (13, 13)	106 (11, 6)	108 (17, 8)	3	10.0 – 200.0	0.984	81 (18, 15)	98 (19, 9)	102 (20, 12)
STE	10	40.0 - 320.0	0.986	101 (10, 3)	101 (4, 6)	100 (5, 11)	2	2.5 – 100.0	0.966	101 (10, 7)	73 (14, 4)	74 (4, 3)
EN-A1	5	10.0 - 320.0	0.987	102 (20, 12)	102 (12, 7)	81 (4, 5)	1.5	2.5 – 100.0	0.998	118 (15, 9)	122 (4, 7)	102 (14, 7)
EN-B	5	10.0 - 320.0	0.983	104 (7, 17)	104 (12, 2)	101 (7, 9)	1.5	2.5 – 100.0	0.999	103 (14, 4)	106 (18, 4)	100 (9, 5)
EN-B1	10	40.0 - 320.0	0.989	115 (9, 7)	98 (11, 4)	106 (9, 10)	2	2.5 – 100.0	0.983	96 (3, 3)	100 (15, 6)	105 (9, 3)
EN-A	5	10.0 - 320.0	0.994	101 (17, 9)	99 (7, 7)	92 (7, 5)	1.5	2.5 – 100.0	0.999	113 (9, 8)	116 (4, 6)	102 (19, 8)
BEA	5	10.0 - 320.0	0.992	100 (3, 8)	106 (6, 8)	97 (3, 6)	1.5	2.5 – 100.0	0.999	98 (8, 7)	101 (3, 8)	98 (3, 7)

Levels used for precisions and recovery assays were: level 1 = 40 µg kg^{-1}, level 2 = 220 µg kg^{-1}, and level 3 = 380 µg kg^{-1} for AFB1, AFB2, AFG1, FB1, FB2, OTA, HT-2, and T-2 in herbs; level 1 = 40 µg kg^{-1}, level 2 = 220 µg kg^{-1}, and level 3 = 380 µg kg^{-1} for STE, EN-A, EN-A1, EN-B, EN-B1, and BEA in herbs; level 1 = 10 µg L^{-1}, level 2 = 100 µg L^{-1}, and = 200 µg L^{-1} for AFB1, AFB2, FB1, FB2, OTA, HT-2 in herbal infusions; level 1 = 10 µg L^{-1}, level 2 = 20 µg L^{-1}, and level 3 = 50 µg L^{-1} for STE, EN-A, EN-A1, EN-B, EN-B1 in herbal infusions.

3.1.2. Real samples

3.1.2.1. Herbs

Overall, 42 out of 58 samples (72%) presented at least one mycotoxin, as can be seen in Table 2. BEA was the most usual mycotoxin, present in 43% of the samples, followed by STE and HT-2 toxin found in 37% and 24% of the samples, respectively. FB1, FB2, and T-2 were not detected (<LOD) in any sample. The maximum number of mycotoxins found simultaneously in herbs samples was eight in *lemongrass*, followed by *sage* with seven mycotoxins, and *lemon balm* and *guaco* both with six mycotoxins each (Figure S1). Despite the high co-occurrence verified in these samples, their total averages were lower than those found in *carqueja*, *chamomile,* or *star anise* which showed a total content of 2126.79 µg kg^{-1}, 1320.14 µg kg^{-1}, and 1207.52 µg kg^{-1}, respectively. The quantification process in these and other samples required the dilution of the extracts about ten times to put mycotoxin amount in the defined range of the linearity.

Regarding aflatoxins (AF), AFB1 was found in 10 of 58 analyzed samples (17%), with the positive levels ranging from 96 to 855 µg kg^{-1}. All the positive samples for AFB1 showed levels much higher than those stipulated by both Brazilian and European pharmacopeia for medicinal herbs (2 µg kg^{-1} and 5 µg kg^{-1}, respectively). AFB2 was present in four types of herbs, from which *carqueja* herb showed the highest level followed by *malva*, *lemon balm*, and *rosemary*. AFG1 was also found in four herb samples (*chamomile, lemongrass, carqueja,* and *espinheira-santa*) with levels ranging from 2 to 1627 µg kg^{-1}. Overall, 19 samples were contaminated with AFs with levels ranging from 59.29 (*malva*) to 1987.73 µg kg^{-1} (*carqueja*). All the positive samples showed levels of total AFs higher than those laid down for medicinal herbs by both Brazilian and European pharmacopeia (4 and 20 µg kg^{-1}, respectively). Hence, it is mandatory to assess if AFs can be released to the beverages (infusions) and affect human health.

Table 2 - Levels of mycotoxins in herbs (µg kg $^{-1}$) and herbal infusions (µg L $^{-1}$) (- not detected; + positive samples).

Sample type	Samples	Nº samples	Aflatoxin G1		Aflatoxin B1		Aflatoxin B2		Sum of AF µg kg^{-1}	HT-2		Ochratoxin A		Sterigmatocystin	
			+	Average* (Range) µg kg^{-1}	+	Average (Range) µg kg^{-1}	+	Average (Range) µg kg^{-1}		+	Average (Range) µg kg^{-1}	+	Average (Range) µg kg^{-1}	+	Average (Range) µg kg^{-1}
Herbs	**Artichoke**	**3**	0	-	0	-	0	-	-	1	291.82	0	-	1	<LOQ
	Rosemary	**3**	0	-	1	96.18	3	<LOQ	126.18	0	-	0	-	3	62.07 (45.54 - 75.23)
	Star Anise	**3**	0	-	3	854.47 (187.35 - 1992.53)	0	-	854.87	0	-	1	187.34	1	66.22
	Assa-peixe	**3**	0	-	0	-	0	-	-	1	168.56	0	-	0	-
	Boldo	**3**	0	-	0	-	0	-	-	0	-	1	<LOQ	1	76.66
	Calendula	**3**	0	-	0	-	0	-	-	0	-	2	50.19 (49.54 - 50.85)	0	-
	Chamomile	**3**	3	112.30 (98.78 - 134.72)	0	-	0	-	112.30	3	1060.49 (490.92 - 1767.07)	0	-	3	147.35 (87.95 - 256.32)
	Lemongrass herb	**3**	1	<LOQ	0	-	0	-	21.25	1	208.84	2	232.87 (204.45 - 261.30)	1	61.15
	Cinnamon	**3**	0	-	0	-	0	-	-	0	-	0	-	0	-
	Carqueja	**3**	1	1627.40	1	176.55	1	183.78	1987.73	0	-	0	-	3	77.61 (56.25 - 115.16)
	Chapéu-de-couro	**3**	0	-	0	-	0	-	-	0	-	0	-	0	-
	Anise	**3**	0	-	0	-	0	-	-	2	145.07 (61.92 - 228.23)	1	404.09	3	62.95 (44.26 - 96.02)
	Espinheira-santa	**3**	1	<LOQ	0	-	0	-	21.25	2	546.80 (449.73 - 643.88)	1	153.50	0	-
	Guaco	**3**	0	-	2	74.29 -122.28	0	-	98.29	0	-	0	-	2	33.69 (25.00 - 42.38)
	Mint	**2**	0	-	1	164.22	0	-	164.22	1	554.93	1	51.03	0	-
	Malva	**3**	0	-	0	-	1	59.29	59.29	0	-	0	-	0	-
	Passion fruit	**3**	0	-	0	-	0	-	-	0	-	0	-	0	-
	Lemon balm	**3**	0	-	0	-	1	48.73	48.73	3	649.83 (98.45 - 985.33)	1	72.07	3	128.90 (55.17 - 203.41)
	Sage	**2**	0	-	2	346.40 (321.03 - 371.76)	0	-	346.40	0	-	1	45.33	2	69.85 (53.67 - 86.03)
	Roselle	**3**	0	-	0	-	0	-	-	0	-	0	-	0	-
	Positive samples (%)			**6 (10.34)**		**10 (17.24)**		**6 (10.34)**			**14 (24.14)**		**11 (18.97)**		**23 (36.66)**
Herbal infusions	**Artichoke**	**3**	0	-	0	-	0	-	0	1	<LOQ	0	-	0	-
	Rosemary	**3**	0	-	0	-	1	3.25	3.25	0	-	0	-	0	-
	Star Anis	**3**	0	-	1	6.73	0	-	6.73	0	-	0	-	0	-
	Assa-peixe	**3**	0	-	0	-	0	-	0	1	<LOQ	0	-	0	-
	Boldo	**3**	0	-	0	-	0	-	0	0	-	0	-	1	<LOQ
	Chamomile	**3**	0	-	0	-	0	-	0	1	<LOQ	0	-	2	<LOQ
	Lemongrass herb	**3**	0	-	0	-	0	-	0	0	-	0	-	1	<LOQ
	Carqueja	**3**	0	-	0	-	1	<LOQ	0	0	-	0	-	1	<LOQ
	Espinheira-santa	**3**	0	-	0	-	0	-	0	1	31.89	0	-	0	-
	Guaco	**3**	0	-	0	-	0	-	0	0	-	0	-	0	-
	Mint	**2**	0	-	0	-	0	-	0	1	15.42	1	<LOQ	0	-
	Passion fruit	**3**	0	-	0	-	0	-	0	0	-	1	<LOQ	0	-
	Lemon balm	**3**	0	-	0	-	0	-	0	2	<LOQ	1	<LOQ	0	-
	Sage	**2**	0	-	1	7.82	0	-	7.82	0	-	1	<LOQ	0	-
	Positive samples (%)					**2 (3.45)**		**2 (3.45)**			**7 (12.07)**		**4 (6.90)**		**5 (8.62)**

* For averages, when <LOQ it was considered the arithmetic mean between the limit of detection (LOD) and the limit of quantification (LOQ).

Table 2 - Levels of mycotoxins in herbs (µg kg $^{-1}$) and herbal infusions (µg L $^{-1}$) (- not detected; + positive samples) (CONT).

Sample Type	Samples	Nº samples	Beauvericin		Enniatin A		Enniatin A1		Enniatin B		Enniatin B1		Sum of EN µg kg^{-1}	Total µg kg^{-1}
			+	Average (Range) µg kg^{-1}	+	Average (Range) µg kg^{-1}	+	Average (Range) µg kg^{-1}	+	Average (Range) µg kg^{-1}	+	Average (Range) µg kg^{-1}		
Herbs	**Artichoke**	**3**	2	16.73 (<LOQ - 25.95)	0	-	0	-	0	-	2	82.56 (73.54 - 91.58)	82.56	416.11
	Rosemary	**3**	0	-	0	-	0	-	0	-	0	-	-	188.25
	Star Anise	**3**	3	99.09 (51.56 - 181.35)	0	-	0	-	0	-	0	-	-	1207.52
	Assa-peixe	**3**	1	17.43	1	81.47	1	36.12	0	-	0	-	117.59	303.58
	Boldo	**3**	0	-	0	-	1	<LOQ	0	-	0	-	7.50	114.16
	Calendula	**3**	1	<LOQ	0	-	0	-	0	-	0	-	-	57.69
	Chamomile	**3**	0	-	0	-	0	-	0	-	0	-	-	1320.14
	Lemongrass herb	**3**	3	28.62 (16.43 - 39.28)	1	<LOQ	1	10.72	2	28.18 (14.48 - 41.88)	0	-	46.40	599.13
	Cinnamon	**3**	1	62.92	0	-	0	-	0	-	0	-	-	62.92
	Carqueja	**3**	3	61.45 (37.24 - 80.53)	0	-	0	-	0	-	0	-	-	2126.79
	Chapéu-de-couro	**3**	1	27.42	0	-	1	<LOQ	0	-	0	-	7.50	34.92
	Anise	**3**	0	-	0	-	0	-	0	-	0	-	-	612.11
	Espinheira-santa	**3**	1	54.07	0	-	0	-	0	-	0	-	-	775.62
	Guaco	**3**	2	32.24 (25.81 - 38.67)	1	<LOQ	1	11.45	1	7.50	0	-	26.45	190.67
	Mint	**2**	2	13.82 (<LOQ - 20.14)	0	-	0	-	0	-	0	-	-	784.00
	Malva	**3**	1	19.11	0	-	0	-	0	-	0	-	-	78.40
	Passion fruit	**3**	2	56.93 (17.30 - 96.56)	2	<LOQ	1	25.43	1	313.61	1	239.2	585.74	642.67
	Lemon balm	**3**	2	29.64 (23.37 - 35.91)	0	-	0	-	1	38.64	0	-	38.64	787.81
	Sage	**2**	0	-	2	11.56 (<LOQ - 15.63)	2	24.43 (<LOQ - 41.36)	2	32.13 (13.33 - 50.39)	1	106	174.12	635.7
	Roselle	**3**	0	-	0	-	0	-	0	-	0	-	-	-
	Positive samples (%)			**25 (43.10)**		**7 (12.07)**		**8 (13.69)**		**7 (12.07)**		**4 (6.90)**		
Herbal infusions	**Artichoke**	**3**	0	-	-	-	0	-	-	-	-	-	-	2.00
	Rosemary	**3**	0	-	-	-	0	-	-	-	-	-	-	3.25
	Star Anis	**3**	0	-	-	-	0	-	-	-	-	-	-	6.73
	Assa-peixe	**3**	0	-	-	-	0	-	-	-	-	-	-	6.50
	Boldo	**3**	0	-	-	-	0	-	-	-	-	-	-	2.25
	Chamomile	**3**	0	-	-	-	0	-	-	-	-	-	-	4.03
	Lemongrass herb	**3**	0	-	-	-	1	<LOQ	-	-	-	-	2.00	4.25
	Carqueja	**3**	0	-	-	-	0	-	-	-	-	-	-	8.75
	Espinheira-santa	**3**	0	-	-	-	0	-	-	-	-	-	-	31.89
	Guaco	**3**	1	<LOQ	-	-	0	-	-	-	-	-	-	2.00
	Mint	**2**	0	-	-	-	0	-	-	-	-	-	-	21.92
	Passion fruit	**3**	1	<LOQ	-	-	0	-	-	-	-	-	-	8.50
	Lemon balm	**3**	0	-	-	-	0	-	-	-	-	-	-	11.00
	Sage	2	0	-	-	-	0	-	-	-	-	-	-	14.33
	Positive samples (%)			**2 (3.45)**				**1 (1.72)**						**19 (32.76)**

* When <LOQ it was considered the arithmetic mean between the limit of detection (LOD) and the limit of quantification (LOQ).

Among the classical mycotoxins, HT-2 was one of the most usual mycotoxins, present in 7 out of 20 types of herbs analyzed. This mycotoxin was found by descendent order of contamination in *chamomile*, *mint espinheira-santa*, *lemon balm*, *artichoke*, *lemongrass*, and *assa-peixe*. Among the mycotoxin analyzed, HT-2 was the one with higher mean amounts, with levels ranged from 61.92 to 1767 μg kg^{-1} (Table 2).

Eleven of 58 herbs samples analyzed were contaminated with OTA in levels ranging from 45 to 404 μg kg^{-1}; the highest levels were found in *anise* and *lemongrass*.

Among the emerging mycotoxins studied, BEA was the most usual mycotoxin, present in 25 out of 58 samples with positive levels ranging from 16.4 μg kg^{-1} (*lemongrass*) to 181.3 μg kg^{-1} (*star anise*). The second most frequent emergent mycotoxin was STE, present in eleven of the 58 analyzed samples, ranging from 33.69 μg kg^{-1} (*guaco*) to 147.35 μg kg^{-1} (*chamomile*). EN-A was found in 7 samples (12%) with positive levels from 11.56 μg kg^{-1} (*sage*) to 81.47 μg kg^{-1} (*assa-peixe*). EN-A1 was detected in 8 samples (14%) with levels ranging from 10.72 μg kg^{-1} (*lemongrass*) to 36.12 μg kg^{-1} (*assa-peixe*). Regarding, EN-B and EN-B1, they were found in 7 and 4 herbs, from 28.18 μg kg^{-1} (*lemongrass herb*) to 313.61 μg kg^{-1} (*passion fruit*) and from 82.56 μg kg^{-1} (*artichoke*) to 239.20 μg kg^{-1} (*passion fruit*), respectively.

In general, the results here obtained for the mycotoxins regulated are higher than those reported in the literature: 5.4 μg kg^{-1} for AFB1 in *green tea* (Martínez-Domínguez, Romero-González, & Garrido Frenich, 2016), not detected AFs in 48 herbs (Romagnoli, Menna, Gruppioni, & Bergamini, 2007), 6.75 μg kg^{-1} for AFB1 and <LOQ for AFB2 in one of 14 herbs (Zhang et al., 2017). However, similar values were obtained for emerging mycotoxin with a survey in 60 Chinese medicinal herbs showing EN-A, EN-A1, EN-B, and EN-B1 with maximum concentrations of 355, 253, 291, and 40 μg kg^{-1}, respectively (Hu & Rychlik, 2014).

3.1.2.2. Infusions

Aware of the mycotoxin levels found in herbs, it becomes imperative to ascertain the release of these mycotoxins for the infusions to evaluate human health risk.

Seven out of the 14 mycotoxins studied were found in infusions, less 4 than those found in the herbs (Table 2). Overall, 19 samples (33%) were contaminated with mycotoxins, among them, four samples (*chamomile*, *lemongrass*, *mint*, and *sage*) were contaminated with two mycotoxins.

Regarding AFs, AFB1 was present in two samples with levels of 6.73 µg L^{-1} and 7.82 µg L^{-1} in *star anise* and *sage*, respectively. Also, AFB2 was detected in two samples, *rosemary* and *carqueja* infusions with 3.25 µg L^{-1} and <LOQ, respectively. Overall, lower levels and a lower frequency of total AFs were observed in infusions in comparison to herbs, with four positive samples, in levels ranging from <LOQ to 7.82 µg L^{-1}. Among the classical mycotoxin found, HT-2 was the most frequent in herbal infusions, similar behavior was observed in herbs, with levels ranging from <LOQ to 31.89 µg L^{-1} (*espinheira-santa*). OTA was found in four samples (*mint*, *passion fruit*, *lemon balm* and *sage*), all with levels <LOQ. Concerning emerging mycotoxin STE, EN-A1 and BEA were the only mycotoxins found in herbal infusions but all with levels <LOQ.

3.1.3. Migration of mycotoxin between herbs and infusions

As mentioned above, the levels and occurrence of mycotoxins in herbal infusion were lower than those verified for herbs. In herbal infusions, total average levels ranged from 2.00 to 31.89 µg L^{-1} and, in herbs, it was from 34.9 to 2126.79 µg kg^{-1}. The occurrence was 88% lesser in herbal infusions than those verified in herbs. This decrease can be explained by the physical-chemical properties of the mycotoxins, especially, solubility in water, polarity, octanol-water constant (K_{ow}), and the difference between chemical affinity with matrix (herb) and water.

Another explanation is the use of warm water (98 °C) in the preparation of the infusion. Similar behavior has been reported by (Serrano, Font, Mañes, & Ferrer, 2016) that showed a reduction for ENs of 100% when process pasta at 100 ºC and pH < 4. Literature also reports degradations of AFs, DON, T2, OTA, and Fs during food processing, but the complete elimination is rarely achieved (Karlovsky et al., 2016; Milani and Maleki 2014; Aiko and Mehta 2015; Kabal, 2009; Schmidit et al. 2017). Nevertheless, it shall be reminded that this pioneer exploratory study was based on a targeted analytical approach, so further processing experiments (e.g. time, temperature, pH, etc.) should be conducted.

3.1.4. Exposure assessment

As previously referred, there are not maximum allowable levels of mycotoxin for both herbs and herbal infusions, therefore an exposure assessment was performed using HQ and MoE (Table 3) for the herbal infusion samples. When the mycotoxin was detected but not quantified (<LOQ), it was considered the arithmetic mean between the LOD and LOQ (LOD+LOQ/2).

As can be observed in Table 3, exposure assessment data revealed a health potential concern by consumption infusions of *espinheira-santa* and *mint* since HQ> 1 was seen for HT2. Also, a considerable cancer risk (MoE<10,000) was observed for infusion consumers of rosemary, star anise, mint, passion fruit, lemon balm, and sage. Similar results were reported by (Franco et al., 2019) in risk characterization using occurrence data in foods and urinary biomarkers in Brazil, who described HQ and MoE values of 3.36 for OTA and 0.005 for total AFs, for residents from rural areas in São Paulo and Santa Catarina states. Despite our study has been conducted only in infusions, which constitutes a minor segment of the common diet of Brazilians, results herein show the need of improving process production and storage condition to control and reduce the contamination of food commodities by mycotoxins.

Table 3 – Risk characterization of mycotoxins through determination of Margin of Exposure (MoE) [a], Combined MoE (MoET) [b], and Hazard Quotient (HQ) [c] based on occurrence in herbal infusions and Probable Daily Intake (PDI).

Herbal infusion	Aflatoxin B1		Aflatoxin B2		∑ aflatoxins		OTA		STE		HT2	
	PDI	MoE	PDI	MoE	PDI	MoET	PDI	MoE	PDI	MoE	PDI	HQ
Artichoke	-		-		-		-		-		0.018	0.90
Rosemery	-		0.009	44.44	0.009	44.44	-		-		-	
Star anise	0.019	21.05	-		0.019	21.05	-		-		-	
Assa-peixe	-		-		-		-		-		0.018	0.90
Boldo	-		-		-		-		0.006	26,666	-	
Chamomile	-		-		-		-		0.006	26,666	0.018	0.90
Lemongrass herb	-		-		-		-		0.006	26,666	-	
Carqueja	-		-		-		-		0.006	26,666	-	
Espinheira-santa	-		-		-		-		-		0.091	4.55
Mint	-		-		-		0.018	805.55	-		0.044	2.20
Passion Fruit	-		-		-		0.018	805.55	-		-	
Lemon balm	-		-		-		0.018	805.55	-		0.018	0.90
Sage	0.022	18.18	-		0.022	18.18	0.018	805.55	-		-	

- Not detected.

[a] Indicates high concern for public health (MoE < 10,000). MoE = BMDL10/PDI. BMDL10 for AFs (individual and sum) = 0.4 μg kg^{-1} bw day; OTA = 14.5 μg kg^{-1} bw day; and STE = 160 μg kg^{-1} bw day.

[b] MoET=1/[(1/MoEAFB1) + (1/MoEAFB2)].

[c] Indicates a non-tolerable risk (HQ > 1), HQ = PDI/TDI (TDI for T-2/HT-2 = 0.02 μg kg^{-1} bw.

When the content of mycotoxin found in infusion was <LOQ, it was considered the arithmetic mean between the limit of detection (LOD) and the limit of quantification (LOQ).

3.2. Multi-elemental analysis

The accuracy of the analytical method was evaluated through the analysis of a CRM using the same condition as for samples. The measured values were in accordance with certified values (Table S3), arising an average accuracy (percentage of measured average concentration versus certified average concentration) very satisfactory (96.1 – 112.1%). The limits of detection of the method ranged from 0.001 μg g^{-1} (Ti) to 0.646 μg g^{-1} (Al); these values were calculated considering three times the signal/noise ratio of 10 blank analyses in the same conditions of the samples.

The trace elements levels obtained in herbs were very heterogeneous, what was expected since elements content is influenced by several factors such as type of plant, nature, composition and physicochemical properties (e.g. pH) of the soil, irrigating water quality, climate, and agricultural practices (Hajeb et al., 2016; Tokalioğlu, 2012). *Artichoke* showed the highest levels (average: 4222.2 μg g^{-1}), followed by *star anise* (average: 1451.7 μg g^{-1}) and *chapeu-de-couro* (average: 1196.8 μg g^{-1}) (Table 4). The elements

present at the highest average levels were Al (596.11 µg g^{-1}) followed by Sr (73.27 µg g^{-1}), Ba (62.50 µg g^{-1}), Ti (50.59 µg g^{-1}), Rb (37.11 µg g^{-1}), and B (24.50 µg g^{-1}). Ni, Li, Zr, Pd, Cs, V, and Pb were present at lower levels while As, Be, Cd, Sn, and Tl at trace levels. Finally, Ag, Pt, and Bi were below their corresponding LOD in all herb infusion samples.

Table 4 - Levels of trace elements (μg g^{-1}) (- not detected).

Herb	Element (μg g^{-1})										
	As	**Cd**	**Pb**	**Li**	**Be**	**B**	**Al**	**Ti**	**V**	**Ni**	**Rb**
Artichoke	0.24 ± 0.24	0.08 ± 0.02	3.03 ± 2.03	0.77 ± 0.58	0.09 ± 0.06	18.07 ± 4.41	3936.32 ± 2499.86	102.03 ± 38.48	4.26 ± 3.25	2.59 ± 1.28	41.62 ± 3.96
Rosemary	0.08 ± 0.07	0.01 ± 0.00	0.96 ± 0.20	0.83 ± 0.9	0.03 ± 0.01	47.68 ± 0.67	710.08 ± 107.15	69.49 ± 4.99	1.28 ± 0.14	1.44 ± 0.77	2.42 ± 0.20
Star Anise	-	0.02 ± 0.00	0.40 ± 0.04	0.08 ± 0.03	0.02 ± 0.00	7.64 ± 1.33	1369.72 ± 62.79	7.71 ± 0.56	0.30 ± 014	4.92 ± 0.47	51.21 ± 0.20
Assa-peixe	-	0.12 ± 0.06	0.63 ± 0.17	0.07 ± 0.03	0.01 ± 0.01	24.08 ± 5.61	221.09 ± 106.97	59.36 ± 27.98	0.60 ± 0.50	1.10 ± 0.44	53.55 ± 27.26
Boldo	0.07 ± 0.13	0.02 ± 0.02	0.20 ± 0.12	0.16 ± 0.02	0.01 ± 0.00	22.01 ± 1.13	225.81 ± 131.28	54;50 ± 8.05	0.57 ± 0.28	1.43 ± 0.73	8.22 ± 0.56
Calendula	0.04 ± 0.08	0.07 ± 0.01	0.46 ± 0.34	0.25 ± 0.29	0.02 ± 0.02	45.05 ± 3.91	933.52 ± 730.76	58.87 ± 32.12	2.05 ± 2.16	3.01 ± 1.73	46.13 ± 41.10
Chamomile	0.04 ± 0.07	0.11 ± 0.02	0.23 ± 0.07	0.05 ± 0.01	0.01 ± 0.01	25.96 ± 4.76	220.93 ± 43.06	34.03 ± 2.86	0.36 ± 0.12	0.74 ± 0.15	85.51 ± 29.32
Lemongrass herb	0.02 ± 0.04	0.15 ± 0.08	0.54 ± 0.24	0.05 ± 0.01	-	4.91 ± 2.64	247.63 ± 40.51	25.64 ± 6.29	0.64 ± 0.18	1.25 ± 0.66	22.73 ± 4.34
Cinnamon	-	0.46 ± 0.31	0.08 ± 0.08	-	-	17.87 ± 2.05	22.22 ± 5.61	64.94 ± 6.72	0.10 ± 0.02	1.03 ± 0.15	21.42 ± 4.49
Carqueja	-	0.20 ± 0.08	0.41 ± 0.36	0.13 ± 0.09	0.01 ± 0.01	19.26 ± 10.77	396.61 ± 217.68	24.20 ± 4.31	0.52 ± 0.17	1.33 ± 0.95	50.54 ± 17.76
Chapéu-de-couro	0.39 ± 0.18	0.01 ± 0.00	0.40 ± 0.23	0.39 ± 0.14	0.02 ± 0.01	23.03 ± 10.52	698.72 ± 489.35	54.46 ± 12.44	0.88 ± 0.67	2.35 ± 0.94	74.27 ± 62.64
Anise	-	0.05 ± 0.04	0.08 ± 0.04	0.12 ± 0.04	-	24.83 ± 0.47	106.76 ± 60.66	28.15 ± 2.98	0.36 ± 0.11	1.99 ± 0.70	19.09 ± 8.92
Espinheira-santa	-	0.15 ± 0.14	0.18 ± 0.11	0.06 ± 0.04	0.01 ± 0.02	20.63 ± 8.97	105.74 ± 52.10	59.76 ± 13.50	0.20 ± 0.09	3.54 ± 1.38	15.23 ± 9.27
Guaco	-	0.23 ± 0.05	0.48 ± 0.21	0.24 ± 0.13	-	20.30 ± 6.37	121.65 ± 25.17	29.06 ± 11.81	0.17 ± 0.06	1.04 ± 0.57	98.99 ± 39.27
Mint	0.20 ± 0.00	0.04 ± 0.00	0.67 ± 0.00	0.53 ± 0.00	0.03 ± 0.00	34.84 ± 0.00	1252.23 ± 0.00	108.89 ± 0.00	3.51 ± 0.00	2.85 ± 0.00	7.62 ± 0.00
Malva	0.08 ± 0.07	0.10 ± 0.01	0.74 ± 0.55	0.15 ± 0.05	0.07 ± 0.02	27.60 ± 5.72	687.12 ± 383.56	46.72 ± 2.99	0.88 ± 0.38	4.25 ± 1.44	25.17 ± 6.75
Passion fruit	0.06 ± 0.05	0.16 ± 0.23	0.26 ± 0.09	0.05 ± 0.00	-	20.16 ± 6.92	147.30 ± 80.23	60.47 ± 10.48	0.36 ± 0.23	1.35 ± 0.57	23.67 ± 11.17
Lemon balm	0.03 ± 0.05	0.04 ± 0.03	0.75 ± 0.70	0.14 ± 0.09	0.02 ± 0.03	20.56 3.71	867.62 ± 905.84	47.85 ± 17.69	1.32 ± 1.52	1.31 ± 0.74	17.99 ± 4.45
Sage	0.10 ± 0.14	0.03 ± 0.02	0.70 ± 0.49	0.85 ± 0.31	0.04 ± 0.02	34.28 ± 8.35	897.20 ± 540.20	81.53 ± 20.40	1.71 ± 0.63	4.14 ± 1.62	10.56 ± 3.89
Roselle	0.02 ± 0.03	0.12 ± 0.03	0.28 ± 0.06	0.44 ± 0.09	0.05 ± 0.02	37.01 ± 12.45	213.78 ± 64.25	65.25 ± 7.05	0.54 ± 0.05	3.52 ± 0.61	34.09 ± 6.54

Table 4 - Levels of trace elements (μg g^{-1}) (- not detected). (Cont.).

Herb	Element (μg g^{-1})							
	Sr	**Zr**	**Pd**	**Sn**	**Cs**	**Ba**	**Tl**	**Total**
Artichoke	66.77 ± 0.30	0.56 ± 0.06	0.10 ± 0.02	0.02 ± 0.03	0.21 ± 0.11	45.32 ± 1.73	0.12 ± 0.05	4222.21 ± 2538.90
Rosemary	80.06 ± 21.64	0.49 ± 0.09	0.12 ± 0.05	0.02 ± 0.01	0.06 ± 0.01	19.32 ± 2.16	0.01 ± 0.00	934.37 ± 101.21
Star Anise	3.01 ± 0.70	0.27 ± 0.17	0.03 ± 0.03	0.02 ± 0.01	0.39 ± 0.11	5.97 ± 3.10	0.01 ± 0.00	1451.74 ± 70.53
Assa-peixe	91.27 ± 41.29	0..41 ± 0.21	0.12 ± 0.03	0.03 ± 0.02	0.22 ± 0.16	98.64 ± 51.60	-	551.03 ± 63.07
Boldo	92.71 ± 3.49	0.35 ± 0.10	0.12 ± 0.02	0.02 ± 0.04	0.07 ± 0.04	27.97 ± 6.17	-	434.25 ± 138.26
Calendula	43.49 ± 26.31	1.06 ± 4.48	0.09 ± 0.05	0.03 ± 0.01	0.09 ± 0.05	15.06 ± 2.60	-	1149.30 ± 768.58
Chamomile	43.65 ± 4.90	0.33 ± 0.15	0.10 ± 0.02	0.02 ± 0.00	0.19 ± 0.13	15.50 ± 3.87	0.03 ± 0.02	427.77 ± 51.75
Lemongrass herb	21.70 ± 10.11	0.62 ± 0.37	0.28 ± 0.37	0.19 ± 0.13	0.18 ± 0.12	29.93 ± 19.66	0.01 ± 0.01	356.31 ± 77.42
Cinnamon	68.51 ± 24.95	0.53 ± 0.07	0.08 ± 0.04	0.03 ± 0.05	0.32 ± 0.18	60.80 ± 0.05	0.02 ± 0.01	258.44 ± 32.64
Carqueja	68.81 ± 7.02	0.34 ± 0.13	0.22 ± 0.16	0.02 ± 0.01	0.20 ± 0.14	84.79 ± 26.56	0.01 ± 0.01	647.59 ± 211.84
Chapéu-de-couro	168.49 ± 82.06	0.31 ± 0.25	0.35 ± 0.15	0.02 ± 0.01	0.15 ± 0.20	172.51 ± 67.61	0.06 ± 0.08	1196.81 ± 406.03
Anise	19.15 ± 4.93	0.19 ± 0.08	0.12 ± 0.08	0.00 ± 0.01	0.01 ± 0.01	8.02 ± 2.91	-	208.94 ± 74.47
Espinheira-santa	107.98 ± 40.72	0.12 ± 0.13	0.20 ± 0.06	0.01 ± 0.01	0.09 ± 0.09	51.36 ± 19.94	0.03 ± 0.02	365.31 ± 77.42
Guaco	77.33 ± 49.69	0.17 ± 0.11	0.18 ± 0.05	0.01 ± 0.02	0.23 ± 0.06	39.85 ± 19.00	0.02 ± 0.01	389.93 ± 45.15
Mint	158.11 ± 0.00	2.09 ± 0.00	0.21 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	23.11 ± 0.00	0.01 ± 0.00	1597.99 ± 0.00
Malva	126.11 ± 65.55	0.42 ± 0.21	0.18 ± 0.05	0.03 ± 0.03	0.11 ± 0.09	232.96 ± 164.56	0.08 ± 0.04	1152.76 ± 323.30
Passion fruit	116.61 ± 95.92	0.29 ± 0.30	0.15 ± 0.09	0.01 ± 0.01	0.16 ± 0.10	76.52 ± 50.50	0.04 ± 0.04	447.62 ± 81.69
Lemon balm	35.36 ± 15.53	0.29 ± 0.12	0.07 ± 0.07	0.03 ± 0.02	0.06 ± 0.02	38.11 ± 8.97	0.02 ± 0.02	1031.56 ± 897.85
Sage	30.89 ± 0.73	0.72 ± 0.63	0.03 ± 0.04	0.03 ± 0.02	0.10 ± 0.07	29.69 ± 10.78	0.02 ± 0.02	1092.61 ± 555.68
Roselle	84.01 ± 7.17	0.39 ± 0.15	0.11 ± 0.03	0.02 ± 0.03	0.08 ± 0.01	131.13 ± 38.46	-	570.82 ± 75.51

Among the highest trace elements, Al (average: 596 µg g^{-1}) was the most abundant probably because it is the most prevalent constituent of mineral soil and ranks third among the most abundant element on the earth's crust (Vitorello, Capaldi, & Stefanuto, 2005).

Concerning the major elements of toxicological concern such as As, Cd, and Pb, they were found in concentrations ranging from 0.02 to 0.39 µg g^{-1}, 0.01 to 0.46 µg g^{-1}, and 0.08 to 3.03 µg g^{-1}, respectively. These levels are following the maximum limits established by both Brazilian and European Pharmacopeia for As (5.0 µg g^{-1}) Cd (1.0 µg g^{-1}) and Pb (5.0 µg g^{-1}) in medicinal herbs.

By comparing the results herein to those obtained in previous studies, it is possible to verify that similar amounts of these elements were found in herb samples from Poland, Saudi Arabia, Lebanon, and Turkey (Table S4) (Dalipi et al., 2018; Filipiak-Szok, Kurzawa, Cichosz, & Szłyk, 2015; Filipiak-Szok, Kurzawa, & Szłyk, 2015; Haidu et al., 2017; Milani et al., 2019; Tokalioğlu, 2012). Concerning toxic elements particularly Cd, Pb, Al, As, Ba, and Ni, that are listed as a public health issue, anthropogenic activities from agricultural and industrial practices are the main cause of the increasing levels of these elements on food and feed chain due to the use of pesticides, chemical fertilizers, wastewater in irrigation, precipitation from heavy coal combustion and smelter wastes, and residues from metalliferous mining on the field (Filipiak-Szok, Kurzawa, & Szłyk, 2015).

4. Conclusions

The QuEChERS-LC-MS/MS method validated for herbs and infusions allowed the simultaneous quantification of 14 mycotoxins at trace levels. Analytical performance parameters of the method such as linearity, reproducibility, and sensitivity proved to be precise and accurate for all analytes, in line with the accepted standards. The analysis of

58 herbs from Brazil revealed the presence of mycotoxins in 72% of the samples, with a clear presence of emerging mycotoxin such as BEA and STE present in 43% and 37% of the samples, respectively. The occurrence in infusions was 80% lesser than those verified for the herbs. Although a low incidence of HT-2, AFs, OTA, and STE were observed in infusions. These detectable levels indicated a potential health concern when evaluated the hazard quotient and margin of exposure, respectively. The twenty–one trace elements analyzed revealed a high variability between the herbs. In all the samples Al was the highest trace element and the legislated toxic elements as As, Cd, and Pb presented levels were below legal limits defined for herbal medicinal, indicating a lower exposure risk to these toxic through the consumption of these foodstuffs.

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7. Supplementary data

DETERMINATION OF NATURAL CONTAMINANTS IN AROMATIC HERBS AND HERBAL INFUSIONS

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Table S1 - Samples

Popular name in Brazil [a]	Scientific name [a]	Suppliers (n)	Plant parts [a]
Artichoke	*Cynara scolymus*	3	Leaves and steam
Rosemary	*Rosmarinus officinalis*	3	Leaves
Star Anise	*Illicium verum*	3	Fruits
Assa-peixe	*Vernonia polyanthes*	3	Leaves
Boldo	*Peumus boldus*	3	Leaves
Calendula	*Calendula officinalis*	3	Flowers
Chamomile	*Matricaria recutita*	3	Leaves and steam
Lemongrass herb	*Cymbopogon citratus*	3	Leaves and steam
Cinnamon	*Cinnamomum verum*	3	Bark
Carqueja	*Baccharis trimera*	3	Leaves and steam
Chapéu-de-couro	*Echinodorus macrophyllus*	3	Leaves and steam
Anise	*Pimpinella anisum.*	3	Seeds
Espinheira-santa	*Maytenus ilicifolia*.	3	Leaves and steam
Guaco	*Mikania glomerata*	3	Leaves and steam
Mint	*Mentha x piperita*	2	Leaves and steam
Malva	*Malva sylvestris.*	3	Leaves
Passion fruit	*Passiflora ssp*	3	Leaves
Lemon balm	*Melissa officinalis*	3	Leaves and steam
Sage	*Salvia officinalis.*	2	Leaves and steam
Roselle	*Hibiscus sabdariffa*	3	Flowers

Table S2 - LC-MS/MS parameters.

Mycotoxin	Retention time (min)	Precursor ion (*m/z*)	Product ions (*m/z*)	Cone energy (V)	Collision energy (kV)
Aflatoxin G1	12.6	329	243	35	30
			311.2	35	30
Aflatoxin B2	13.2	315	259.2	45	33
			287.3	45	35
Aflatoxin B1	13.7	313	241.2	45	30
			285.2	45	30
Fumonisin B1	15.8	722.5	334.2	46	40
			352.4	44	36
HT-2	15.9	444.1	215.3	18	15
			263.2	18	15
T-2	17.4	484	214.9	21	18
			245.2	23	15
Ochratoxin A	18.5	404	239.1	30	20
			358.1	28	16
OTAd5 (ISTD)	18.8	409	239	32	22
			363	32	22
Fumonisin B2	18.9	706.3	318.4	42	38
			336.1	40	36
Sterigmatocystin	19.9	325	254	35	35
			310	35	25
Enniatin A1	21.2	690	232	50	70
			350	50	70
Enniatin B	21.5	663	218	60	70
			336	60	70
Enniatin B1	21.7	653	196	60	60
			214	60	60
Enniatin A	22.6	705	232	65	65
			350	65	65
Beauvericin	27.7	806	134	50	50
			384	50	50

Table S3 – ICP-MS quality control.

Element	Determined value (Mean ± standard deviation)	Certificated value (Mean ± standard deviation)	Recovery (%)
Cd a	1.71 ± 0.03	1.66 ± 0.07	103.01
Cu a	2.77 ± 0.05	2.89 ± 0.12	95.85
Mn a	13.63 ± 0.16	13.30 ± 0.50	102.48
Mo a	16.26 ± 0.14	14.80 ± 0.50	109.86
Ni a	27.01 ± 0.50	27.00 ± 0.80	100.04
Sr a	11.34 ± 0.16	11.80 ± 0.40	96.10
Zn a	18.36 ± 3.83	79.70 ± 2.70	98.95
Sb b	23.10 ± 3.92	20.60 ± 2.60	112.14
Ti b	3.08 ± 0.28	3.00 ± 0.30	102.67

a – µg g^{-1}; **b** µg kg^{-1}

Table S4 – Comparison with other works.

Elements	Country of origin (range of concentrations, expressed in μg g^{-1})					
	Unknown [a]	**Poland [b]**	**Asiatic and European [c]**	**Romania [d]**	**Brazil [e]**	**Turkey [f]**
Al	-	-	13.21 - 87.43	15.6 - 2761.2	53 - 1268	-
As	-	-	0.01 - 0.38	< 0.08	0.025 - 0.228	-
Ba	n.d. - 89	-	0.45 - 11.64	< 0.08 - 30.5	5.3 - 89.3	-
Ca	4400 - 13300	520 - 20350	-	4420 - 22233	-	-
Cd	-	0.02 - 0.25	0.02 - 0.25	< 0.08	0.006 - 0.774	-
Co	-	-	-	-	-	n.d. - 2.35
Cr	n.d. - 2.5	0.28 - 23.18	-	< 0.08 - 1.5	0.04 - 3.91	0.44 - 8.71
Cu	3.4 - 10.1	1.12 - 27.33	-	2.0 - 12.4	2.6 - 12.2	3.32 - 30.2
Fe	80 - 1670	6.33 - 2366.5	-	161.7 - 1624	60 - 565	41.9 - 3456
K	7600 - 14800	-	-	7828 - 23054	-	-
Li	-	-	-	< 0.08 - 7.0	-	-
Mg	-	580 - 10030	-	1941 - 7606	-	-
Mn	38 - 278	1.59 - 146.36	-	12.2 - 95.6	46 - 1811	3.44 - 264
Mo	-	n.d. - 0.92	-	-	-	-
Na	-	-	-	10.4 - 2095.8	-	-
Ni	n.d. - 3.4	-	0.16 - 14.21	< 0.08 - 4.2	0.4 - 4.12	0.72 - 13.1
Pb	0.8 - 4.1	0.35 - 0.82	0.35 - 0.82	< 0.08 - 1.7	0.06 - 0.82	n.d. - 3.01
Rb	1.7 - 23.3	-	-	-	-	3.46 - 56.6
Sb	-	-	0.003 - 0.10	-	-	-
Se	-	0.06 - 0.58	-	< 0.08 - 9.0	0.024 - 0.113	-
Sr	23 - 108	-	-	7.0 - 63.5	-	10.6 - 669
Ti	14-80	-	-	-	-	-
V	-	-	-	< 0.08 - 5.4	-	-
Zn	20 - 43	6.75 - 63.38	-	9.1 - 33.2	11 - 105	4.66 - 88.0

n.d., not detected

[a] Dalipi et al., 2018; [b] Filipiak-Szok, Kurzawa, Cichosz, & Szłyk, 2015; [c] Filipiak-Szok, Kurzawa, & Szłyk, 2015; [d] Haidu et al., 2017; [e] Milani et al., 2019; [f] Tokalioğlu, 2012.

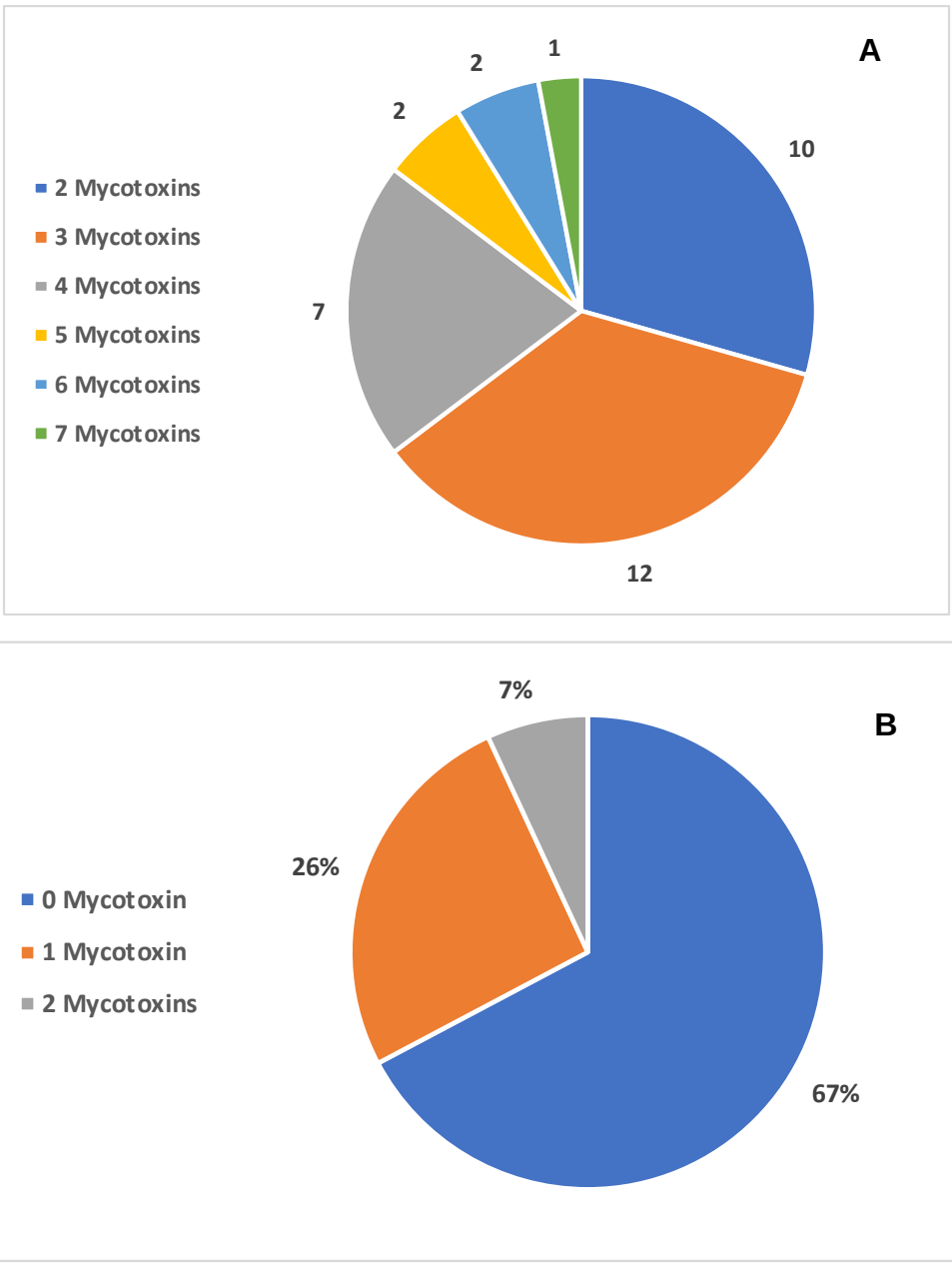

Figure S1- Number of mycotoxins found simultaneous in herbs (A) and herbal infusion (B)

CAPÍTULO II: DETERMINATION OF PESTICIDE RESIDUES IN HERBS AND MIGRATION ASSESSMENT TO THE HERBAL INFUSIONS

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DETERMINATION OF PESTICIDE RESIDUES IN HERBS AND MIGRATION ASSESSMENT TO THE HERBAL INFUSIONS

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****To be submitted to Food Chemistry**

ABSTRACT

Brazil is one of the largest pesticide consumers in the world, however, there is a deficiency in pesticide residues monitoring and surveillance in food and feedstuffs, especially for products with less economic importance such as aromatic herbs. In this work, a QuEChERS-GC-MS and a DLLME-GC-MS method were optimized and validated for the determination of pesticide residues in herbs and herbal infusions. The method's parameters of the limit of detection (LOD), limit of quantification (LOQ), linearity, precision intra- and inter-day, and recovery were assessed, and the results showed in accordance with current guidelines for pesticide residues analysis. From the 20 analyzed herbs, 80% presented at least one detectable pesticide and 62% of the samples had residual content above the maximum residual level (MRL) set by Brazilian Pharmacopoeia. It was not observed the transference of pesticides from natural contaminated herb to the herbal infusions. A control assay was conducted spiking a blank sample with pesticide analytical standard in a high concentration level (7 mg kg^{-1}), and 15 analytes were detected but not quantified (<LOQ). An exposure assessment estimation was performed considering the LOD and the LOQ of the method, and only aldrin/dieldrin showed a potential high risk to human health (100% of the ADI) at LOQ (35 µg L^{-1}) level.

Keywords: Pesticide residue, QuEChERS, DLLME, Food Contaminant, Herb, Herbal infusion.

1. Introduction

Brazil is essentially an agricultural country and to support the high production, chemical inputs such as pesticides and fertilizers are intensively used (Santos, Piccoli, Cremonese, & Freire, 2019). This scenario, jointly with the lack of state surveillance over agricultural practices, made Brazil one of the largest pesticide consumers in the world, causing serious toxicological issues for humans and the environment (Pignati et al., 2017; Santos et al., 2019). The national "Pesticide Residue Analysis Program", for instance, reported in 2017/2018 that 23% of the monitored samples had some nonconformity with Brazilian law. Furthermore, this program follows only 14 vegetables, which represent only 31% of the variety of vegetables consumed in the country (ANVISA, 2019).

Pesticides can be divided into four families, namely, organochlorines, organophosphorus, carbamates, and pyrethroids (Jayaraj, Megha, & Sreedev, 2016; Sulaiman, Rovina, & Joseph, 2019). Organochlorines are super-persistent chemicals, their half-lives are about 10-30 years, and they have high toxicity, lipophilicity, bioaccumulation, and biomagnification (Jayaraj et al., 2016; Pardío et al., 2012). Fortunately, organochlorine pesticides were banished in Europe, North America, and many counties in South America, including Brazil (Brasil, 1985; Jayaraj et al., 2016; Samsidar, Siddiquee, & Shaarani, 2018). Owing to the high toxicity, organochlorines were replaced by organophosphorus and carbamates which are biodegradable and less toxic, so, they turned widely applied in agriculture as insecticides, fungicides, and herbicides (Samsidar et al., 2018; Sulaiman et al., 2019). Pyrethroids are synthetic pesticides derived from pyrethrin, a naturally existing chemical found in the chrysanthemum plant. They present low toxicity and short half-lives and, because of this, they have become one of the major insecticides used not only in agriculture but in households as well (Samsidar et al., 2018; Sulaiman et al., 2019). When applied in large monocultural areas, pesticides are sprinkled

by tractors or airplanes over the crops, affecting soil, water, and especially the air which can carry these products through the wind to the adjacent area, polluting all the surrounding environment. Thus, these compounds are considered important pollutants and environmental contaminants (Pignati et al., 2017).

Despite herbs and herbal infusions are commonly associated with health benefits (Meinhart, Damin, Caldeirão, & Godoy, 2019; Milani, Silvestre, Morgano, & Cadore, 2019) and being part of the traditional medicine in several countries, including Brazil (Brasil, 2019a; The World Health Organization, 2013), some studies around the world showed the presence of pesticides in herbs and herbal infusions. Even the banished organochlorine pesticides residues were found in these products (Beneta, Mutavdžić Pavlović, Periša, & Petrović, 2018; Rodrigues, Reyes, Magalhães, & Rath, 2007; Storelli, 2014). Likewise, organophosphorus pesticides were found in herbs commercialized in China (X. Wang, Cheng, Zhou, Wang, & Cheng, 2013), India (Saha, Makwana, & Manivel, 2019; Yadav et al., 2017), Italy (Di Bella et al., 2019), and Iran (Moinfar & Hosseini, 2009). Some studies also detected pyrethroids in herbs from India (Saha et al., 2019; Yadav et al., 2017), Italy (Di Bella et al., 2019), and China (Zhao et al., 2019), for example. In Brazil, the legislation for contaminants and pesticide residues in herbs commercialized as traditional and complementary medicine was released in 2019, but, until now, there is no maximum residual level (MRLs) for pesticides in aromatic herbs and herbal infusions (Brasil, 2019b). On the other hand, European Community (European Commission, 2020) and Codex Alimentarius (FAO/WHO, 2019) establishes MRLs for herbs and several other foodstuffs, that can easily found in the EU and Codex Alimentarius pesticide database.

Regarding the analytical method and, particularly, extraction techniques, several protocols for the determination of pesticides can be found in literature based on liquid-

liquid extraction (LLE) (Y. Wang, Jin, Ma, Lu, & Lin, 2011), liquid-solid extraction (LSE) (Łozowicka et al., 2014), solid-phase microextraction (SPME) (Rodrigues, Reyes, Rehder, & Rath, 2005), matrix solid-phase dispersion (MSPD) (Łozowicka et al., 2014), pressurized liquid extraction (PLE) (Du et al., 2012), dispersive liquid-liquid microextraction (DLLME) (Ho, Tsoi, & Leung, 2013), and *Quick, Easy, Cheap, Effective, Rugged, and Safe* (QuEChERS) (Abbas, Soliman, El-Gammal, Amer, & Attallah, 2017; Rutkowska, Łozowicka, & Kaczyński, 2018). QuEChERS approach is the official and most common extraction procedure applied to extracting pesticides from herbs (Abbas et al., 2017; AOAC International, 2011; European Commission, 2015; Parrilla Vázquez, Ferrer, Martínez Bueno, & Fernández-Alba, 2019; Saha et al., 2019). Numerous modifications in the original QuEChERS protocol are observed, but the main adjustments are in the buffering during the extraction or in the dispersive solid-liquid extraction cleanup (dSPE) (Parrilla Vázquez et al., 2019; Villaverde, Sevilla-Morán, López-Goti, Alonso-Prados, & Sandín-España, 2016). Acetate (Besil et al., 2017) and citrate (Malinowska & Jankowski, 2015; Rutkowska et al., 2018; Słowik-Borowiec, Szpyrka, & Walorczyk, 2012) are the mainly buffers applied in the QuEChERS procedure. Buffered QuEChERS is unusually important to extract pH-sensitive pesticides (Lehotay, Maštovská, & Lightfield, 2005). For the dSPE step, the main sorbents applied to pesticide analysis in herbs are octadecyl bonded silica (C18) (Abbas et al., 2017), primary secondary amine (PSA) (Malinowska & Jankowski, 2015; Słowik-Borowiec et al., 2012), graphitized carbon black (GCB) (Abbas et al., 2017; Beneta et al., 2018; Rutkowska et al., 2018), and strong anion exchange (SAX) (Molina-Ruiz, Cieslik, & Walkowska, 2015). Due to the high matrix interferents in dried herbs, a combination of two sorbents is usually employed to reach an effective cleanup. C18 acts removing non-polar interferents (e.g. lipids), while PSA and SAX retain polar components (e.g. fatty acids,

polar organic acids, polar pigments, and sugars) through chemical interaction such as Van der Waals forces and anion exchange, respectively (Beneta et al., 2018; Lawal, Wong, Tan, Abdulra'Uf, & Alsharif, 2018; Li et al., 2008; Villaverde et al., 2016). GCB, on the other hand, works by exclusion principle and it is used in samples with a high content of chlorophyll and steroids. GCB, however, can remove planar analytes such as hexachlorobenzene, thiabendazole, or chlorothalonil, consequently, its use is more restrict (Li et al., 2008; Molina-Ruiz et al., 2015; Villaverde et al., 2016).

Regardless of the QuEChERS approach is the most employed extraction procedure for pesticide analysis, some protocols as DLLME is a good alternative especially for liquid samples such as water and beverages (Moinfar & Hosseini, 2009). In this technique, few microliters of a binary mixture of an extractor and a dispersive solvent are rapidly added to an aqueous sample and tiny droplets are instantaneously formed. The droplets of extractor solvent are joined after centrifugation, collected, and then the extract is injected into the analytical system. This technique is solventless, eco-friendly, quick, and provides a good enrichment factor leading to high method sensitivity and consequently low LOD and LOD (Almeida, Fernandes, & Cunha, 2012). Studies have reported the use of acetonitrile (Ho et al., 2013; Moinfar & Hosseini, 2009), ethanol (Hou et al., 2014), and methanol (Yang et al., 2016) as the dispersive solvent, and n-hexane (Moinfar & Hosseini, 2009), 1-dodecanol (Hou et al., 2014), ionic liquid-based nanofluid (Yang et al., 2016), and carbon tetrachloride (Ho et al., 2013) as the extractor solvent in DLLME protocol to extract pesticides from herbs and herbal infusions.

The separation and the detection of pesticides from herbs and herbal infusions are usually performed by chromatographic techniques, being gas chromatography (GC) the favorite, since this procedure provides high resolution (separation), low solvent waste, and short-time analysis. Additionally, most pesticides are volatile and thermostable, so GC had

become the main separation technique applied to multi-residue analysis (Beneta et al., 2018; Parrilla Vázquez et al., 2019; Rutkowska et al., 2018). Nevertheless, liquid chromatography (LC) has been successfully applied to pesticide multi-residue analysis, mainly for high polar, non-volatile, and/or thermally labile compounds (Abbas et al., 2017; Parrilla Vázquez et al., 2019; Yang et al., 2016). For both GC and LC techniques, detectors based on mass spectrometry (MS) are predominantly employed (Abbas et al., 2017; Parrilla Vázquez et al., 2019; Rutkowska et al., 2018; Yang et al., 2016). For GC, however, other detectors such as electron capture detector (ECD) (Attallah, Barakat, Maatook, & Badawy, 2012; Malinowska & Jankowski, 2015; Słowik-Borowiec et al., 2012), nitrogen phosphorus detector (NPD) (Malinowska & Jankowski, 2015; Słowik-Borowiec et al., 2012), and flame photometric detector (FPD) (Moinfar & Hosseini, 2009; Saha et al., 2019) are found in the literature.

Finally, beyond the pesticide residue monitoring and the improvement of the analytical methods, it is important to evaluate the impact of home processing over pesticide residues. These studies are essential to evaluate the real consumers' exposure to these contaminants. Thus, this study aimed to optimize and validate a QuEChERS-GC-MS and a DLLME-GC-MS method to determine residues of organochlorine, organophosphorus, and pyrethroids in herbs commercialized in Brazil, assess the transference of these contaminants into the herbal infusions and estimate the exposure assessment when contaminated herbs are used to prepare beverages (herbal infusions).

2. Material and methods

2.1. Chemicals and solutions

The analytical standards of organochlorine (Appendix IX Organochlorine Pesticide Mix 2000 mg mL^{-1}), organophosphorus (Organophosphorus Pest Mix A 2000 mg L^{-1}), and pyrethroid pesticides (Pyrethroid standard mixture 1000 mg L^{-1}) were purchased from

Supelco (Sigma-Aldrich, EUA), and a mix working solution was prepared in acetonitrile:toluene (1:1) at 50 mg L^{-1} by combining appropriate aliquots of individual standard mixture solutions. The internal standard (IS) triphenyl phosphate (TPP, 99% purity) was also purchased from Supelco (Sigma-Aldrich, EUA). The IS stock and working standard solutions were prepared in acetonitrile at 1000 mg L^{-1} and at 50 mg L^{-1}, respectively. All the solutions were kept refrigerated (~ 4ºC) until the analysis.

Sorbents C18-bounded silica (DSC-18, particle size 55-105 µm), primary secondary amine (PSA bounded silica, particle size 50 µm), and graphite carbon black (GCB Superclean ENVI-carb ≥37µm) were purchased from Supelco® (Sigma-Aldrich, Pennsylvania, EUA).

Anhydrous magnesium sulfate ($MgSO_4$) (JT Baker, Japan) and sodium chloride (NaCl) (Synth, Brazil) were heated at 400 ºC for 5h and kept under vacuum before use. Organic solvents acetonitrile, (JT Baker, EUA), toluene (Sigma-Aldrich, Germany), and chloroform (Sigma-Aldrich, Germany) used in this study were all HPLC grade. Ultrapure water (18.2 mΩ cm^{-1}) was purified by a Milli-Q gradient system from Millipore (Milford, MA, USA).

2.2. Sampling

A total of fifty-eight samples from twenty different dried herbs (**see supplement**) were purchased from public markets and fairs in the cities of Campinas (São Paulo, Brazil) and Londrina (Paraná, Brazil), between November/2017 and January/2018. The samples were grounded (1000 rpm for 10 s, Grindomix GM 200, Retsch GmbH, Germany), mixed, vacuum packed (portions of 50 g), and stored at room temperature until the analysis.

2.3. Sample Preparation

A QuEChERS (*Quick, Easy, Cheap, Effective, Rugged, and Safe)* approach with some modifications was used to extract pesticides from dried herb samples. This technique was chosen since it is the main method used for pesticide residues analysis and the dSPE step promotes a good cleanup in a complex matrix, even those rich in chlorophyll. Meanwhile, a dispersive liquid-liquid micro-extraction (DLLME) was chosen to extract the pesticides from herbal infusions (beverage) due to the easiness of operation and the achievement of low limits of detection (LOD) and quantification (LOQ) with no extra efforts such as concentration under nitrogen flow or other additional steps. QuEChERS and DLLME procedures were optimized for the dSPE cleanup and the volume of extraction solvent (chloroform), respectively.

2.3.1. Herbs (QuEChERS)

Optimized QuEChERS extraction was performed weighing 1.0 g of dried herb in a 50 mL polypropylene (PP) centrifuge tube. The sample was spiked with 100 µL of internal standard (TPP) at 10 mg mL^{-1}, then 5 mL of ultrapure water was added, and the mixture was vortexed for 30 s, 5 mL of MeCN was added and vortexed for 30 s. After this, 2 g of anhydrous magnesium sulfate and 0.5 g of sodium chloride were added to the tube and homogenized in a vortex for 30s. The sample was centrifuged for 15 min at 3000 *g* and exactly 1 mL of the upper layer was transferred to a 15 mL PP centrifuge tube containing 150 mg of SAX and 5 mg of GCB. The sample was vortexed for 30s and centrifuged at 1000 *g* per 10 min. Finally, 500 µL of the organic phase was transferred to an injection vial and exactly 1 µL of the extract was injected in a GC-MS system.

2.3.2. Herbal infusions preparation and analysis (DLLME)

The herbal infusions were prepared with 1 g of dried herb weighed in Erlenmeyer flask, then 50 mL of hot distilled water (~98ºC) was added and maintained in contact for 15

min. After centrifuged at 3000 *g* for 5 min, 10 mL of the supernatant was transferred to a 15 mL centrifuge tube. 50 µL of internal standard (TPP) at 20 mg L^{-1} was added to the sample and vortexed for 30 s. Here after, a mixture of MeCN and chloroform was prepared adding 200 µL of each solvent and the mixture was rapidly added to the sample with help of a micropipette. At this time, an emulsion was formed, and the sample was homogenized in a vortex for 10 s, sonicated for 5 min and centrifuged at 1000 *g* for 5 min. The upper layer (aqueous) was discarded and 130 µL of the lower layer (chloroform) was transferred to an injection vial with a glass insert. Finally, exactly 1 µL of the extract was injected in a GC-MS system.

2.4. Chromatographic and mass-spectrometric conditions

A 7890 gas chromatograph (Agilent, Little Falls, DE, USA), equipped with an autosampler (Agilent 7693A) and electronically controlled split/splitless injection port, coupled with a single quadrupole inert mass selective detector (5975C, Agilent Technologies Inc., Palo Alto, CA, USA) with an electron ionization (EI) chamber, was used to pesticide analyses. CG separation was achieved on a Supelco® SLB-5ms fused silica capillary column (30m × 0.25 mm ID × 0.25 µm film thickness (Sigma-Aldrich, USA). The oven temperature was programmed initially at 60 ºC for 2 min, increased to 170 ºC at 20 ºC min^{-1}, then ramped to 230 ºC at 3 ºC min^{-1}, increased to 280 ºC at 20 ºC min^{-1} and finally increased to 300 ºC at 30 ºC min^{-1} and held for 5 min, with a total run of 35.7 min. Ultra-high purity helium (99.999%; Praxair, Brazil) was used as carrier gas at 1.0 mL min^{-1}. The injector was maintained at 280 ºC in pulsed spitless mode (0.85 min purge-off) and 1.0 µL of the sample extract was injected. The quadrupole MS was operated in selective ion monitoring (SIM) mode (**see supplement**), the electron energy was 70 eV, and the temperatures of transfer line, ion source and analyzer were 280 ºC,

230 ºC, and 180 ºC, respectively. System control and data acquisition were performed in ChemStation software.

2.5. Method validation and quality control

First, a blend of pesticide-free samples was prepared mixing equal parts of *Peumus boldus*, *Cymbopogon citratus,* and *Matricaria recuttita*. These samples were chosen due to the high content in pigments and phenolic compounds (Meinhart et al., 2017), simulating the worst-case scenario in terms of matrix complexity. The methods were validated for the limit of detection (LOD), the limit of quantification (LOQ), linearity, precision intra- and inter-days, recovery, and matrix effects as suggested by SANTE/12682/2019 (European Commission, 2019). LOD was defined as the lowest concentration in a spiked blank sample that gave a signal/noise of 3, while LOQ was set as the lowest concentration in the sample that could be quantified with precision (set by Horwitz relative standard deviation) and recovery (>70% and <120%).

For herbs, a QuEChERS-GC-MS method was proposed, and a seven-point matrix-matched curve was built for every analyte from 0.10 mg kg^{-1} to 1.60 mg kg^{-1}. In herbal infusions, a DLLME-GC-MS method was chosen, and a five-point calibration curve was performed from 35 µg L^{-1} to 135 µg L^{-1}. The linearity was assessed through the coefficient of determination (R^2) of linear regression (external standard area/internal standard versus external standard concentration). Precision for intra- and inter-day were carried out in spiked samples at three concentration levels (0.10 mg kg^{-1}, 0.85 mg kg^{-1}, and 1.60 mg kg^{-1} for herbs, and 35 µg L^{-1}, 85 µg L^{-1}, and 135 µg L^{-1} for infusions), using five replicates for each level on the same day and during three consecutive days, respectively. The precision's results were expressed as relative standard deviation (%RSD). A satisfactory %RSD was set when it was lower than RSDr calculated by the Horwitz equation ($RSDr = 2^{(1-0.5\log C)}$). Recovery (%) assays were also performed at the same three concentration

levels for herbs and herbal infusions using five replicates for each level and the result expressed as average (%).

Matrix effects were investigated by comparing the slope of the matrix-matched calibration curves with the slope of solvent calibration curves in the same concentration range and it was expressed in percentage (%ME = [{Matrix Slope/Solvent Slope}*100]-100).

2.6. Exposure assessment

Exposure assessment was performed calculating the estimated daily intake using the following equation (Food and Drug Administration, 2006):

$$EDI_{(mg.kg^{-1}\ bw)} = \frac{Cp*Ci}{bw}$$

Where ***Cp*** is the concentration of pesticide in infusion (mg L^{-1}), ***Ci*** is the average daily intake portion of herbal infusion consumed by Brazilians, set as 0.2 L (200 mL) (Milani et al., 2019), and ***bw*** is the bodyweight of an adult human (70 kg). The EDI was compared to the acceptable daily intake (ADI) pesticides (FAO/WHO, 2019) and the results were expressed as a percentage of the ADI (% ADI). Values above 100% indicate ingestion exceeding the ADI, characterizing risk for human health.

2.7. Statistical analysis

Calibration curves for each pesticide were submitted to linear regression significance by the least-squares method (LSM) and analysis of variance (ANOVA). The results showed the calibration curves were significant ($p<0.05$) and no linearity deviation was observed. The statistical tests were performed on software Statistica 10.0 (Statsoft). When necessary, ANOVA and Tukey's test were applied to compare means at 95% confidence level.

3. Results and discussion

3.1. Extraction optimization

3.1.1. QuEChERS

The blank sample was spiked with pesticides at 0.5 mg kg^{-1} and the extraction was followed as mentioned in section **2.3.1 Herbs (QuEChERS).** For dispersive solid-phase extraction (dSPE), 15 different conditions were tested to evaluate the effect of the sorbent phases (**Figure 1**). Every experiment was injected twice, and the tested response was the area of the analyte after the GC-MS analysis. The results were expressed as the normalized area by the control experiment (without cleanup). The results showed that except for heptachlor, dieldrin, and endrin-aldehyde (**see supplement**), the signal area was higher for the combination of SAX (150 mg) and GCB (5 mg), so this assay was chosen to validate the method.

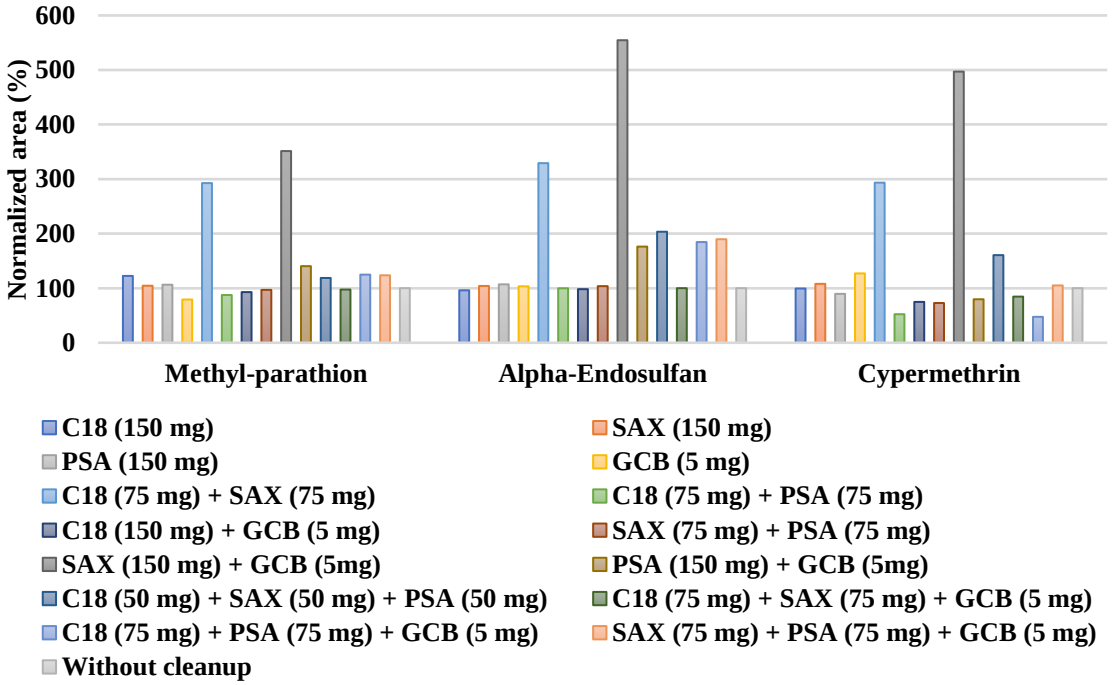

Figure 1. Results for selected analytes for every tested sorbent, normalized area by without cleanup test.

3.1.2. DLLME

The DLLME was optimized for extraction solvent (chloroform) in four different volumes (150 µL, 200 µL, 250 µL, and 300 µL) and the tested response was the area of every pesticide after GC-MS analysis. As shown in **Figure 2**, the assay applying 250 µL had the highest responses (normalized area by 150 µL chloroform assay), however, it was not statistically significant ($p > 0.05$, Tukey's test) from the assay using 200 µL of extraction solvent. Furthermore, aiming the reduction of chloroform use, the authors selected the condition of 200 µL of extraction solvent to follow the study.

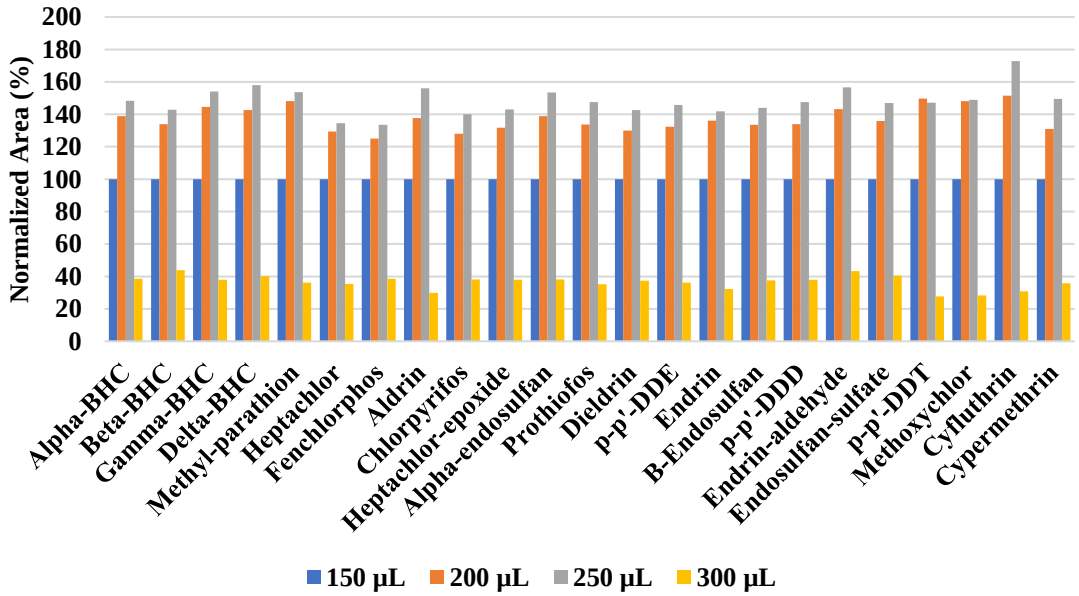

Figure 2. Normalized area for every pesticide in different volumes of extractor solvent (chloroform) for DLLME-GC-MS method.

3.2. In-house method validation

Matrix effects (**Figure 3)** for the QuEChERS method were from -75% (p-p'-DDD) to 123% (methyl-parathion) and all of the analytes showed a high matrix effect (≤-50% and ≥50%) (Saha et al., 2019). As expected, matrix effects for QuEChERS in herbs were higher than those observed to the DLLME in infusions. The explanation of this behavior

is on the fact that herbal infusions are an aqueous extraction product from the herbs and, consequently, less complex. For DLLME, the matrix effects were between -19% to 236%, however most analytes (56%) had a low matrix effect (≥-20 and ≤20%) (Saha et al., 2019).

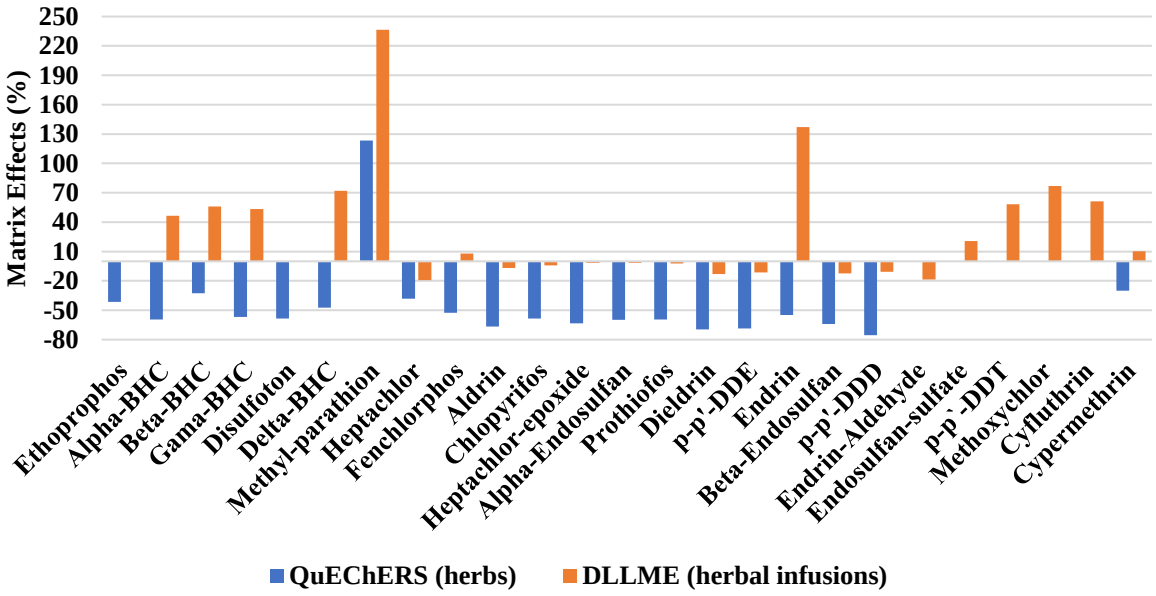

Figure 3. Matrix effects (%) for both QuEChERS (herbs) and DLLME (herbal infusions) methods. Not showed data indicates the analyte was not validated for the method.

Linearity, assessed by determination coefficient (R^2) of the calibration curves, was above 0.981 for the QuEChERS approach (endrin-aldehyde) and higher than 0.977 for DLLME (alpha-Endosulfan). The validated linear range (**Table 1**) for all analytes for QuEChERS and DLLME procedures were from 0.10 mg kg^{-1} to 1.60 mg kg^{-1} and from 35 µg L^{-1} to 135 µg L^{-1}, respectively. The limit of detection (LOD) reached for QuEChERS was between 0.04 mg kg^{-1} (cypermethrin) and 0.06 mg kg^{-1} (ethoprophos) and limit of quantification (LOQ) was from 0.08 mg kg^{-1} (cypermethrin) to 0.10 mg kg^{-1}. In the DLLME procedure, the limits were quite lower, ranging from 1.0 µg L^{-1} to 20 µg L^{-1} (LOD) and from 10 µg L^{-1} to 35 µg L^{-1} (LOQ). Comparing these results, they are similar to limits found in the literature for pesticides applying extraction techniques such as QuEChERS (Besil et al., 2017; Petrarca, Fernandes, Godoy, & Cunha, 2016; Taha &

Gadalla, 2017), DLLME (Petrarca et al., 2016), and solid-liquid extraction with low-temperature purification (SLE-LTP) (Morais, Rodrigues, Queiroz, Neves, & Morais, 2014) in several food matrixes, including herb samples (Besil et al., 2017; Taha & Gadalla, 2017). Intra-day and inter-day precision were lower than 23.4% (cypermethrin, DLLME) and 26.6% (p-p'-DDE, DLLME), respectively (**Table 1**). These results are below the predicted by the Horwitz equation (RSDr). Recoveries (**Figure 4**) were from 84.9% (aldrin) to 115.4% (disulfoton) for QuEChERS and from 77% (heptachlor-epoxide) to 143.6% (beta-BHC) for DLLME. Moreover, all of the analytes in QuEChERS were between 70 and 120% and 83% (19 analytes) are in this rage for DLLME procedure. So, these results suggest the methods are fitted for analytical purposes as established by the SANTE/12682/2019 (European Commission, 2019).

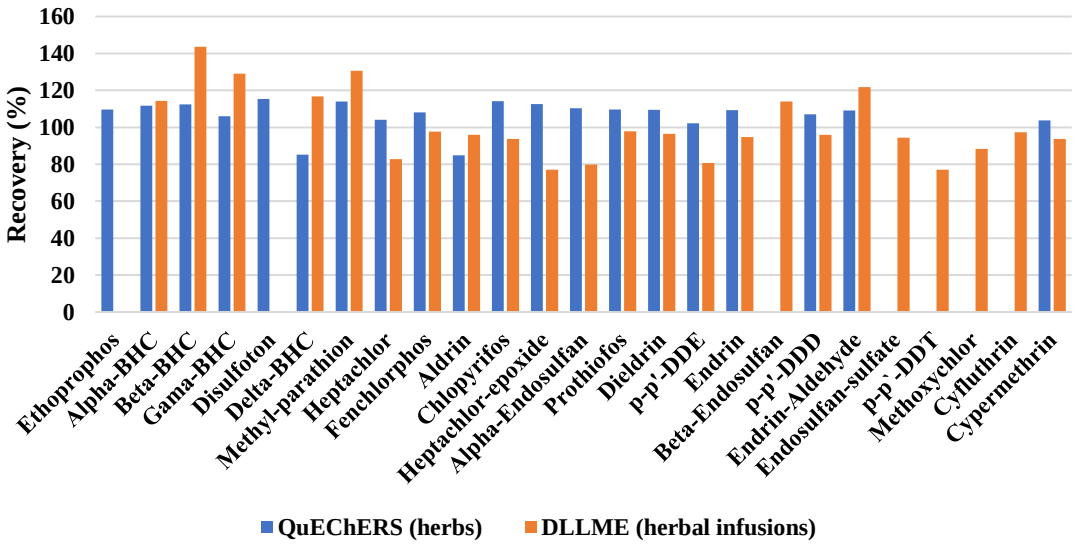

Figure 4. The average recovery for both QuEChERS-GC-MS (herbs) and DLLME-GC-MS (infusions) methods. Not showed data indicates the analyte was not validated for the method.

Beta-endosulfan, endosulfan-sulfate, p-p'-DDT, methoxychlor, and cyfluthrin showed no good linearity and/or unsatisfactory results for precision and/or recovery in the QuEChERS approach, thus they were excluded from herb analysis. Likewise,

ethoprophos and disulfoton were not validated for the DLLME method (herbal infusion), so these results are not reported in this paper.

Table 1. Validation parameters for QuEChERS-GC-MS (herbs) and DLLME-GC-MS (herbal infusions) methods.

Method	Analyte	LOD	LOQ	Linear Range	Regression Model	Linearity	Precision intra-day (%) (n = 5)			Precision inter-day (%) (n = 5)		
		mg kg^{-1}	mg kg^{-1}	mg kg^{-1}		(R^2)	0.10 mg kg^{-1}	0.85 mg kg^{-1}	1.60 mg kg^{-1}	0.10 mg kg-1	0.85 mg kg-1	1.60 mg kg-1
QuEChERS (herbs)	**Ethoprophos**	0.06	0.10	0.10 - 1.60	y = 1.780x+0.009	0.991	8.37	7.21	8.09	11.08	4.56	8.92
	Alpha-BHC	0.05	0.10	0.10 - 1.60	y = 0.785x-0.019	0.991	5.69	3.60	2.18	5.20	4.14	7.52
	Beta-BHC	0.05	0.10	0.10 - 1.60	y = 0.708x-0.025	0.991	3.68	7.09	1.18	3.93	6.33	4.47
	Gamma-BHC	0.05	0.10	0.10 - 1.60	y = 0.653x-0.031	0.992	0.31	6.63	3.64	3.83	4.67	6.30
	Disulfoton	0.05	0.09	0.10 - 1.60	y = 2.477x-0.151	0.993	1.23	4.80	2.38	3.69	3.43	5.66
	Delta-BHC	0.05	0.10	0.10 - 1.60	y = 0.406x+0.014	0.990	6.44	2.93	4.65	8.37	9.26	6.98
	Methyl-parathion	0.05	0.10	0.10 - 1.60	y = 1.136x-0.02	0.991	8.16	3.11	5.19	13.69	5.07	6.49
	Heptachlor	0.05	0.10	0.10 - 1.60	y = 0.682x-0.032	0.992	3.42	11.74	3.46	1.86	6.66	3.97
	Fenchlorphos	0.05	0.10	0.10 - 1.60	y = 4.261x-0.147	0.992	0.59	13.00	7.16	2.27	6.48	6.18
	Aldrin	0.05	0.10	0.10 - 1.60	y = 0.436x-0.020	0.991	6.39	4.61	3.72	9.67	4.89	7.34
	Chlopyrifos	0.05	0.10	0.10 - 1.60	y = 1.523x-0.083	0.992	1.45	6.53	3.06	2.24	4.68	5.77
	Heptachlor-epoxide	0.05	0.10	0.10 - 1.60	y = 0.826x-0.037	0.991	1.22	5.63	2.51	2.62	4.23	5.74
	Alfa-Endosulfan	0.05	0.10	0.10 - 1.60	y = 0.206x+0.001	0.990	1.75	7.82	7.35	11.00	7.00	4.06
	Prothiophos	0.05	0.10	0.10 - 1.60	y = 1.626x-0.101	0.992	2.22	6.23	2.83	4.17	3.88	4.36
	Dieldrin	0.05	0.10	0.10 - 1.60	y = 0.295x-0.013	0.990	2.93	4.05	1.63	4.41	3.09	4.92
	p-p'-DDE	0.05	0.10	0.10 - 1.60	y = 1.426x-0.053	0.991	2.91	3.93	1.76	3.89	3.48	4.87
	Endrin	0.05	0.10	0.10 - 1.60	y = 0.369x-0.010	0.991	2.84	13.25	6.20	2.46	5.89	3.77
	p-p'-DDD	0.05	0.09	0.10 - 1.60	y = 3.665x-0.076	0.992	1.19	4.93	3.55	3.99	4.29	6.85
	Endrin-aldehyde	0.07	0.10	0.10 - 1.60	y = 0.225x-0.010	0.981	7.57	9.76	4.29	5.67	4.86	3.87
	Cypermethrin	0.04	0.08	0.10 - 1.60	y = 0424+0.055	0.995	8.25	3.69	9.15	10.89	5.44	4.56
Method	**Analyte**	**LOD**	**LOQ**	**Linear Range**	**Regression Model**	**Linearity**	**Precision intra-day (%) n = 5**			**Precision inter-day (%) n = 5**		
		µg L^{-1}	**µg L^{-1}**	**µg L^{-1}**		**(R^2)**	**35 µg L^{-1}**	**85 µg L^{-1}**	**135 µg L^{-1}**	**35 µg L^{-1}**	**85 µg L^{-1}**	**135 µg L^{-1}**
DLLME (herbal infusions)	**Alpha-BHC**	1.0	35	35 - 135	y = 0.0027x+0.0583	0.994	10.11	8.33	10.11	15.05	12.27	18.57
	Beta-BHC	1.0	35	35 - 135	y = 0.0032x+0.0636	0.991	5.07	9.49	3.54	16.05	8.59	14.97
	Gamma-BHC	5.0	35	35 - 135	y = 0.0027x+0.0353	0.991	4.40	7.95	6.94	15.72	11.33	17.55
	Delta-BHC	5.0	35	35 - 135	y = 0025x+0.0563	0.993	4.07	8.28	7.72	13.05	8.13	15.79
	Methyl-parathion	5.0	35	35 - 135	y = 0.0067x+0.1425	0.991	8.09	6.14	7.80	15.64	1.29	15.21
	Heptachlor	5.0	10	35 - 135	y = 0.0016+0.0031	0.982	11.06	11.98	22.14	10.10	20.87	20.94
	Fenchlorphos	1.0	35	35 - 135	y = 0.0076x-0.0625	0.989	4.00	12.44	16.52	11.09	11.16	7.25
	Aldrin	1.0	35	35 - 135	y = 0.0013x-0.0001	0.994	4.55	10.96	17.23	1.27	17.84	4.19
	Chlorpyrifos	5.0	35	35 - 135	y = 0.0031x-0.0492	0.992	4.38	14.45	16.16	5.19	20.19	10.87

Heptachlor-epoxide	1.0	10	35 - 135	y = 0.0019x-0.0102	0.991	4.32	23.41	15.62	17.45	20.43	18.41
Alpha-endosulfan	1.0	35	35 - 135	y = 0.005x+0.0373	0.977	5.76	12.52	7.69	8.95	16.32	19.48
Prothiofos	10.0	35	35 - 135	y = 0.0047x-0.1388	0.993	6.73	20.79	17.23	19.61	21.57	21.47
Dieldrin	5.0	35	35 - 135	y = 0.0008x-0.0141	0.990	9.85	20.10	15.84	10.50	22.89	20.37
p-p'-DDE	10.0	35	35 - 135	y = 0.0044x-0.1292	0.998	9.25	12.22	18.26	22.74	17.72	26.60
Endrin	5.0	35	35 - 135	y = 0.001x-0.0130	0.992	9.32	17.31	16.92	13.27	20.26	17.95
Beta-Endosulfan	5.0	35	35 - 135	y = 0.0007x-0.0013	0.989	5.70	12.20	11.20	16.78	17.67	9.67
p-p'-DDD	10.0	35	35 - 135	y = 0.0141x-0.3746	0.993	8.28	18.47	15.33	22.99	22.40	23.34
Endrin-aldehyde	1.0	35	35 - 135	y = 0.0012x+0.0162	0.996	5.46	7.79	3.44	7.70	12.18	15.91
Endosulfan-sulfate	1.0	35	35 - 135	y = 0.0017x+0.0142	0.991	7.60	8.59	7.48	7.69	9.79	2.97
p-p'-DDT	5.0	35	35 - 135	y = 0.0072x-0.2237	0.996	4.46	16.70	17.18	2.10	15.54	21.79
Methoxychlor	5.0	35	35 - 135	y = 0.0127x-0.2900	0.992	4.45	12.71	15.70	6.12	11.00	16.83
Cyfluthrin	20.0	35	35 - 135	y = 0.0017x-0.0577	0.990	11.32	17.96	12.03	16.23	24.33	19.63
Cypermethrin	20.0	35	35 - 135	y = 0.0044x-0.1463	0.992	10.48	17.45	13.52	11.06	25.20	20.73

"LOD" – limit of detection; "LOQ" – limit of quantification.

3.3. Real samples

3.3.1. Pesticides in herbs

Brazilian pharmacopeia (Brasil, 2019b) sets maximum residue limits (MRLs) for 69 substances in herbs and herbal extracts, including 23 out of the 25 pesticides analyzed in this study. From the 20 analyzed herbs, 16 (80%) presented at least one detectable pesticide and 10 samples (62%) had residual values above the MRLs (Brasil, 2019b). Additionally, 15 pesticides were present in samples. (**Table 2)**.

Methyl-parathion was detected in six samples, which *Peumus boldus* (0.29 mg kg^{-1}), *Baccharis trimera* (0.65 mg kg^{-1}), *Pimpinella anisium* (0.24 mg kg^{-1}) and *Passiflora ssp* 0.35 (mg kg^{-1}) extrapolated the MRL (0.20 mg kg^{-1}) (Brasil, 2019b). These results are higher than those reported by Di Bella et al., (2019) who found 0.015 mg kg^{-1} of this pesticide in *Rosmarinus officinalis* samples. Chlorpyrifos was detected in five samples but only *Rosmarinus officinalis* and *Mentha x piperita* were above the MRL (0.20 mg kg^{-1}) (Brasil, 2019b). The residual content in these samples were 0.37 mg kg^{-1} and 0.29 mg kg^{-1}, respectively. Previous works detected chlorpyrifos in herbs sold in China, from 0.045 to 0.121 mg kg^{-1}, (Fu et al., 2019) and in India (<0.025 mg kg^{-1}) (Saha et al., 2019).

The set MRL for the sum of aldrin and dieldrin is 0.05 mg kg^{-1} (Brasil, 2019b). Only aldrin was detected in herbs with concentrations from 0.37 mg kg^{-1} (*Cymbopogon citratus*) to 0.77 mg kg^{-1} (*Maytenus ilicifolia*), these values are seven and fifteen times higher than the allowed by Brazilian pharmacopeia (Brasil, 2019b). Again, these values are quite higher than those found in previously reported results, which determined 0.023 mg kg^{-1} of aldrin (Dai, Ren, He, & Huo, 2011), and 0.15 mg kg^{-1} of dieldrin (Gondo, Obuseng, Mmualefe, & Okatch, 2016; Rodrigues et al., 2007) in herbs.

Cypermethrin was detected in seven samples and, from these, *Peumus boldus* (1.15 mg kg^{-1}) and *Calendula officinalis* (2.10 mg kg^{-1}) were above the MRL (1.0 mg kg^{-1}) (Brasil, 2019b). Once Again, the content of cypermethrin in samples was higher than the value presented by Fu et al., (2019) (0.020 – 0.047 mg kg^{-1}) in herbal samples. *Calendula officinalis* extract needed dilution to be properly quantified.

One sample was positive for heptachlor (*Calendula officinalis*, <0.10 mg kg^{-1}) and one for endrin-aldehyde (*Melissa officinalis*, <0.10 mg kg^{-1}), how the content was higher than LOD (0.05 and 0.07 mg kg^{-1}, respectively) and the MRL established in Brazilian Pharmacopoeia is exactly 0.05 mg kg^{-1} for both analytes, can be inferred that these samples are not accordance with Brazilian legislation (Brasil, 2019b).

Finally, samples were also detected for residues of alpha, beta, and gamma-BHC, fenchlorphos, alpha-endosulfan, and p-p'-DDE (**Table 2**), however, the values were not above the MRL (Brasil, 2019b). These results are similar to those presented by Dai et al., (2011) and Storelli (2014), who found residues of alpha-BHC and p-p'-DDT (a p-p'-DDE precursor) in herbs commercialized in China and Italy, respectively. Moreover, ethoprophos and disulfoton were detected in three samples, but, in Brazil, there is no limits for these pesticides (Brasil, 2019b).

Table 2. Level of pesticides in herbs (mg kg^{-1}).

Herb	Pesticide							
	Ethoprophos (mg kg^{-1})	**Beta-BHC (mg kg^{-1})**	**Gamma-BHC (mg kg^{-1})**	**Disulfoton (mg kg^{-1})**	**Delta-BHC (mg kg^{-1})**	**Methyl-Parathion (mg kg^{-1})**	**Heptachlor (mg kg^{-1})**	**Fenchlorphos (mg kg^{-1})**
Rosmarinus officinalis	-	-	-	-	0.18 ± 0.02	<LOQ	-	-
Illicium verum	-	-	-	<LOQ	-	-	-	-
Peumus boldus	0.13 ± 0.08	0.11 ± 0.03	-	-	0.10 ± 0.00	0.29 ± 0.02	-	-
Calendula officinalis	-	-	-	-	-	-	<LOQ	-
Matricaria recutita	0.37 ± 0.08	-	-	-	-	-	-	-
Cymbopogon citratus	-	-	-	-	<LOQ	0.15 ± 0.06	-	-
Baccharis trimera	-	0.10 ± 0.01	-	-	<LOQ	0.65 ± 0.30	-	-
Echinodorus macrophyllus	-	-	-	-	-	-	-	-
Pimpinella anisum		-	-		-	0.24 ± 0.02		<LOQ
Maytenus ilicifolia	-	-	-	-	-	-	-	-
Mikania glomerate	-	<LOQ	0.27 ± 0.13	-	-	-	-	-
Mentha x piperita	-	-	-	-	-	-	-	-
Malva sylvestris	-	-	-	-	-	-	-	-
Passiflora ssp	-	-	-	<LOQ	-	0.35 ± 0.03	-	-
Melissa officinalis	-	-	-	<LOQ	-	-	-	-
Salvia officinalis	<LOQ	-	-	-	-	-	-	-

"<LOQ" – Indicates values above limits of detection (>LOD) but below the limit of quantification (<LOQ); "-" – indicates not detected (<LOD).

Table 2. Level of pesticides in herbs (mg kg^{-1}) (CONT).

Herb	Pesticide						
	Aldrin (mg kg^{-1})	**Chlorpyrifos (mg kg^{-1})**	**Alpha-Endosulfan (mg kg^{-1})**	**Dieldrin (mg kg^{-1})**	**p-p'-DDE (mg kg^{-1})**	**Endrin-aldehyde (mg kg^{-1})**	**Cypermethrin (mg kg^{-1})**
Rosmarinus officinalis	-	0.37 ± 0.09	0.43 ± 0.13	-	-	-	0.19 ± 0.07
Illicium verum	-	-	0.17 ± 0.02	-	-	-	-
Peumus boldus	-	-	1.17 ± 0.35	-	-	-	1.15 ± 0.22
Calendula officinalis	-	-	-	-	-	-	2.10 ± 0.73
Matricaria recutita	-	-	0.29 ± 0.25	-	-	-	
Cymbopogon citratus	0.37 ± 0.21	-	-	-	-	-	
Baccharis trimera	0.70 ± 0.07	-	-	-	-	-	0.49 ± 0.07
Echinodorus macrophyllus	-	-	0.96 ± 0.15	-	-	-	-
Pimpinella anisum		-	-	-	-	-	<LOQ
Maytenus ilicifolia	0.77 ± 0.04	-	-	-	-	-	-
Mikania glomerata	-	-	-	-	-	-	-
Mentha x piperita	-	0.29 ± 0.02	-	-	-	-	0.50 ± 0.40
Malva sylvestris	0.43 ± 0.30	<LOQ	-	-	-	-	0.15 ± 0.02
Passiflora ssp	-	0.10 ± 0.00	-	-	-	-	-
Melissa officinalis	-	<LOQ	-	0.12 ± 0.00	<LOQ	<LOQ	-
Salvia officinalis	-	-	0.53 ± 0.15	-	-	-	-

"<LOQ" – Indicates values above limits of detection (>LOD) but below the limit of quantification (<LOQ); "-" – indicates not detected (<LOD).

Considering the legislation for pesticide residue in herbs in the European Community, which is more demanding than the Brazilian one, all the positive samples are above the established MRLs. Furthermore, it is important to highlight that, from the analyzed pesticides, only cypermethrin is permitted in Europe (European Commission, 2020) and only ethoprophos, disulfoton, chlorpyrifos, cyfluthrin, and cypermethrin are authorized to be used in Brazil (ANVISA, 2020).

3.3.2. Pesticides transference to the beverages and exposure assessment

The herb samples with residual pesticide were submitted to infusion extraction, applying boiling distillate water (~98ºC) in a proportion of 1 g of sample to 50 mL water and kept soaking for 15 min. After this time, the samples were centrifuged at 3000 *g*/5 min and 10 mL of herbal infusion was used for DLLME extraction and further GC-MS analysis. No pesticide was detected in infusions prepared from dried samples. Intended to assess the transference ratio from herb to the beverage, a control experiment using the blank matrix spiked with a very high concentration (7 mg kg^{-1}) of pesticides was carried out. The spiked samples (n = 5) were submitted to the herbal infusion preparation, pesticide extraction (DLLME), and GC-MS analysis as mentioned before. From the 23 validated pesticides for herbal infusions, 15 (gamma-BHC, methyl-parathion, heptachlor, fenchlorphos, aldrin, chlorpyrifos, heptachlor-epoxide, prothiofos, dieldrin, p-p'-DDE, endrin, beta-endosulfan, p-p'-DDD, p-p'-DDT, methoxychlor) were above the LOD but below the LOQ. These results show a transference rate below 25% (<35 µg L^{-1}). The authors did not test above spiking concentrations, since 7 mg kg^{-1} is considered very high contamination and it is almost unlikely to happen in real conditions..

Table 3. Exposure assessment for pesticides in infusions.

Pesticide	ADI (mg kg^{-1} bw)	Scenario 1 (LOD)		Scenario 2 (LOQ)	
		EDI (mg kg^{-1} bw)	% ADI	EDI (mg kg^{-1} bw)	% ADI
BHC [1]	0.0050	0.00001	0.3	0.00010	0.3
Methyl-parathion	0.0030	0.00001	0.5	0.00010	3.3
Heptachlor [2]	0.0001	0.00001	14.3	0.00003	28.6
Aldrin/Dieldrin	0.0001	0.00001	14.3	0.00010	100.0
Chlorpyrifos	0.0100	0.00001	0.1	0.00010	1.0
Endosulfan [3]	0.0060	0.00001	0.2	0.00010	1.7
Endrin [4]	0.0002	0.00001	7.1	0.00010	50.0
p-p'-DDT [5]	0.0100	0.00001	0.1	0.00010	1.0
Cyfluthrin	0.0400	0.00006	0.1	0.00010	0.3
Cypermethrin	0.0200	0.00006	0.3	0.00010	0.5

"ADI" – acceptable daily intake (FAO/WHO, 2019); "EDI" – estimated daily intake using the limit of detection (LOD) (scenario 1) and limit quantification (scenario 2); "%ADI" – percentage of acceptable daily intake; "1" – ADI refers to the sum of isomers alpha-, beta-, gamma-, and delta-BHC; "2" – ADI refers to the sum of heptachlor and heptachlor-epoxide; "3" - ADI refers to the sum of alpha- and beta-endosulfan, and endosulfan-sulfate; "4" - ADI refers to the sum of endrin and endrin-aldehyde; "5" - ADI refers to the sum of p-p'-DDD, p-p'-DDT, and p-p'-DDE.

Exposure assessment (**Table 3**) was performed calculating the estimated daily intake (EDI) for two scenarios, one using the limit of detection (Scenario 1), and another using the limit of quantification (Scenario 2) of the DLLME-GC-MS method. The EDI was compared to the acceptable daily intake collected from Codex Alimentarius Pesticide Database (FAO/WHO, 2019), and the result was expressed as a percentage of the ADI. When in LOQ residual level, only aldrin/dieldrin configures a high risk to human health (100% of the ADI), and endrin a moderate risk in long term exposure (50% of the ADI). However, to be present in this concentration (35 µg L^{-1}) and considering 25% of the rate transference from herb to the herbal infusion, the herb material should be contaminated with more than 7 mg kg^{-1} what is extremely high for pesticide residues.

4. Conclusion

This study proposed the optimization and the validation of a QuEChERS and a DLLME extraction protocol to analyze pesticides in herbs and infusions by GC-MS. The best cleanup condition for QuEChERS was achieved using the mixture of SAX 150 mg and GCB 5 mg, and the best volume of chloroform (extractor solvent) for the DLLME

procedure was reached at 200 µL. Validated methods showed low limits and appropriated parameters of linearity, recovery, and intra- and inter-day precisions.

Pesticides were detected in 16 herbs, all above the maximum residual level (MRL) established by the European Community, and 10 above the MRL established by Brazilian legislation. When herbal infusions were prepared with herbs naturally contaminated, no pesticides were detected in the beverages, however, when spiked blank samples were used for infusion preparation, analytes were detected but not quantified (<LOQ). This result suggests a low transference ratio from herb to the beverage which, in normal circumstances, shall not represent a health issue. The authors recommend monitoring the presence and content of other pesticides in herbs and infusions, as well as the development of an extraction protocol aimed to detect a lower concentration of these contaminants.

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7. Supplementary data

HERBS AND HERBAL INFUSIONS: DETERMINATION OF NATURAL CONTAMINANTS (MYCOTOXINS AND TRACE ELEMENTS) AND EVALUATION OF THEIR EXPOSURE

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Table S1. Herbs name (common and scientific), sample units, and commercialized parts of the plants.

Common name in Brazil [a]	Scientific name [a]	Sample units (n)	Plant parts [a]
Artichoke	*Cynara scolymus*	3	Leaves and steam
Rosemary	*Rosmarinus officinalis*	3	Leaves
Star Anise	*Illicium verum*	3	Fruits
Assa-peixe	*Vernonia polyanthes*	3	Leaves
Boldo	*Peumus boldus*	3	Leaves
Calendula	*Calendula officinalis*	3	Flowers
Chamomile	*Matricaria recutita*	3	Leaves and steam
Lemongrass herb	*Cymbopogon citratus*	3	Leaves and steam
Cinnamon	*Cinnamomum verum*	3	Bark
Carqueja	*Baccharis trimera*	3	Leaves and steam
Chapéu-de-couro	*Echinodorus macrophyllus*	3	Leaves and steam
Anise	*Pimpinella anisum.*	3	Seeds
Espinheira-santa	*Maytenus ilicifolia*.	3	Leaves and steam
Guaco	*Mikania glomerata*	3	Leaves and steam
Mint	*Mentha x piperita*	2	Leaves and steam
Malva	*Malva sylvestris.*	3	Leaves
Passion fruit	*Passiflora ssp*	3	Leaves
Lemon balm	*Melissa officinalis*	3	Leaves and steam
Sage	*Salvia officinalis.*	2	Leaves and steam
Roselle	*Hibiscus sabdariffa*	3	Flowers

[a] Information acquired with suppliers and/or on sample label.

Table S2. GC-MS parameters for every pesticide QuEChERS protocol (herbs).

Analyte number	Analyte	Ion *(m/z)*				Retention time	Time window
		1[a]	2	3	4		
1	**Ethoprophos**	**158**	139	242		**11.2**	1
2	**Alpha-BHC**	**183**	219	254		**12.3**	2
3	**Beta-BHC**	**183**	219	254		**13.1**	3
4	**Gamma-BHC**	**183**	219	254		**13.3**	3
5	**Dissulfoton**	**88**	142	274	186	**14.1**	3
6	**Delta-BHC**	**183**	219	254		**14.2**	3
7	**Methyl-parathion**	**125**	233	263		**15.7**	4
8	**Heptachlor**	**272**	274	337		**16.0**	4
9	**Fenchlorphos**	**285**	287	125	109	**16.2**	4
10	**Aldrin**	**66**	263	91	293	**17.5**	5
11	**Chlopyrifos**	**197**	199	97	314	**17.9**	5
12	**Heptachlor-epoxi**	**353**	237	263		**19.3**	6
13	**Alpha-endosulfan**	**195**	241	170	339	**21.0**	7
14	**Prothiophos**	**267**	309	162		**22.0**	8
15	**Dieldrin**	**263**	277			**22.3**	8
16	**p-p'-DDE**	**248**	318	176		**22.3**	8
17	**Endrin**	**263**	317	281	345	**23.3**	9
18	**p-p'-DDD**	**235**	237	165	199	**24.3**	9
19	**Endrin-aldehyde**	**345**	250	173		**24.8**	9
20	**TPP (IS)**	**326**	215	170	232	**27.6**	10
21	**Cypermethrin**	**163**	181	127		**32.0**	11

[a] Quantification ion

Table S3. GC-MS parameters for every pesticide DLLME protocol (herbal infusions).

Analyte number	Analyte	*m/z*				Retention Time	Time window
		1[a]	2	3	4		
1	**Alpha-BHC**	**183**	219	254		**13.5**	1
2	**Beta-BHC**	**183**	219	254		**14.5**	2
3	**Gamma-BHC**	**183**	219	254		**14.8**	2
4	**Delta-BHC**	**183**	219	254		**15.8**	2
5	**Methyl-Parathion**	**125**	233	263		**17.3**	3
6	**Heptachlor**	**272**	274	337		**17.7**	3
7	**Fenchlorphos**	**285**	287	125	109	**17.7**	3
8	**Aldrin**	**66**	263	91	293	**19.2**	4
9	**Chlopyrifos**	**197**	199	97	314	**19.1**	4
10	**Heptachlor-epoxi**	**353**	237	263		**21.2**	5
11	**Alpha-Endosulfan**	**195**	241	170	339	**23.0**	6
12	**Prothiophos**	**267**	309	162		**23.7**	7
13	**Dieldrin**	**263**	277			**24.4**	7
14	**p-p'-DDE**	**248**	318	176		**24.2**	7
15	**Endrin**	**263**	317	281	345	**25.5**	8
16	**Beta-Endosulfan**	**195**	237	159		**26.1**	8
17	**p-p'-DDD**	**235**	237	165	199	**26.5**	8
18	**Endrin-aldehyde**	**345**	250	173		**26.9**	8
19	**Endosulfan-sulfate**	**272**	229	387		**28.1**	9
20	**p-p'-DDT**	**235**	165	199		**28.4**	9
21	**TPP (IS)**	**326**	215	170	232	**29.0**	10
22	**Methoxychlor**	**227**	228	152	274	**30.1**	11
23	**Cyfluthrin**	**163**	226	206		**32.4**	12
24	**Cypermethrin**	**163**	181	127		**32.9**	12

[a] Quantification ion

Table S4. Results for every tested sorbent, normalized area by without cleanup assay.

Assay	Ethoprophos	Alpha-BHC	Dissulfoton	Delta-BHC	Methyl-parathion	Heptachlor	Fenchlorphos	Aldrin	Chlorpyrifos	Heptachlor-epoxide
C18 (150 mg)	104.3	114.4	107.5	120.8	122.6	91.9	105.3	88.6	100.9	97.7
SAX (150 mg)	115.5	119.3	108.5	92.7	104.6	115.8	119.4	119.5	121.9	120.5
PSA (150 mg)	122.4	121.9	**106.1**	**56.9**	106.4	130.8	118.7	59.4	127.9	131.3
GCB (5 mg)	98.6	96.6	79.8	79.8	79.3	90.8	89.6	95.4	90.8	95.0
C18 (75 mg) + SAX (75 mg)	167.5	173.4	173.0	146.4	292.7	196.4	239.8	223.5	214.5	255.4
C18 (75 mg) + PSA (75 mg)	107.1	106.2	87.1	57.5	87.5	97.7	98.2	78.7	105.9	106.6
C18 (150 mg) + GCB (5 mg)	92.1	93.6	73.2	83.2	92.9	72.7	81.2	63.3	81.4	82.8
SAX (75 mg) + PSA (75 mg)	104.2	103.3	85.4	54.0	96.9	105.4	99.7	84.9	107.2	109.1
SAX (150 mg) + GCB (5mg)	186.9	198.1	210.8	169.8	351.4	190.7	306.0	271.8	293.0	364.4
PSA (150 mg) + GCB (5mg)	142.4	141.3	153.3	65.6	140.4	165.6	160.1	183.5	176.1	186.3
C18 (50 mg) + SAX (50 mg) + PSA (50 mg)	132.8	137.5	153.2	99.2	118.8	156.9	155.6	171.9	183.6	219.0
C18 (75 mg) + SAX (75 mg) + GCB (5 mg)	110.0	108.3	94.3	72.9	97.6	97.6	101.1	96.5	105.4	105.4
C18 (75 mg) + PSA (75 mg) + GCB (5 mg)	98.5	110.5	129.4	83.3	124.9	152.1	146.9	127.1	156.6	194.8
SAX (75 mg) + PSA (75 mg) + GCB (5 mg)	104.4	108.6	124.5	79.7	123.6	168.6	158.5	171.3	163.9	203.8
Without cleanup	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table S4 - Results for every tested sorbent, normalized area by without cleanup assay (Cont.).

Assay	Alpha-Endosulfan	Prothiofos	Dieldrin	p-p`-DDE	Endrin	p-p'-DDD	Endrin-Aldehyde	Endosulfan-sulfate	p-p`-DDT	Cypermethrin
C18 (150 mg)	96.2	86.5	23.1	84.7	78.4	97.3	92.4	112.8	90.6	99.5
SAX (150 mg)	104.1	124.5	88.0	123.6	120.9	131.7	150.0	121.6	119.1	108.0
PSA (150 mg)	107.1	**131.9**	32.6	140.9	128.7	121.0	194.3	67.2	196.4	89.6
GCB (5 mg)	103.4	99.7	27.2	104.2	96.9	107.5	107.5	87.3	125.9	127.1
C18 (75 mg) + SAX (75 mg)	329.3	241.6	61.5	224.8	216.3	340.0	270.4	397.4	292.1	293.5
C18 (75 mg) + PSA (75 mg)	99.8	104.3	28.1	108.6	103.4	95.8	176.7	56.3	149.4	52.3
C18 (150 mg) + GCB (5 mg)	98.2	66.4	21.4	72.9	74.2	81.1	92.5	87.7	108.6	74.9
SAX (75 mg) + PSA (75 mg)	103.8	116.1	29.6	122.9	116.9	106.9	216.5	64.5	183.2	72.9
SAX (150 mg) + GCB (5mg)	554.6	401.9	89.0	367.7	284.3	590.2	247.2	526.4	180.9	497.0
PSA (150 mg) + GCB (5mg)	176.3	188.7	50.6	211.8	176.4	176.7	280.3	110.2	238.4	79.7
C18 (50 mg) + SAX (50 mg) + PSA (50 mg)	203.5	177.5	49.8	190.6	204.0	234.0	401.9	142.9	299.6	160.6
C18 (75 mg) + SAX (75 mg) + GCB (5 mg)	100.0	99.2	27.4	99.1	98.5	104.1	136.7	81.5	119.5	84.6
C18 (75 mg) + PSA (75 mg) + GCB (5 mg)	184.6	139.0	377.9	150.4	132.4	117.4	224.9	56.8	142.8	47.5
SAX (75 mg) + PSA (75 mg) + GCB (5 mg)	189.8	169.1	47.9	197.8	149.2	176.7	360.3	115.3	280.3	105.1
Without cleanup	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

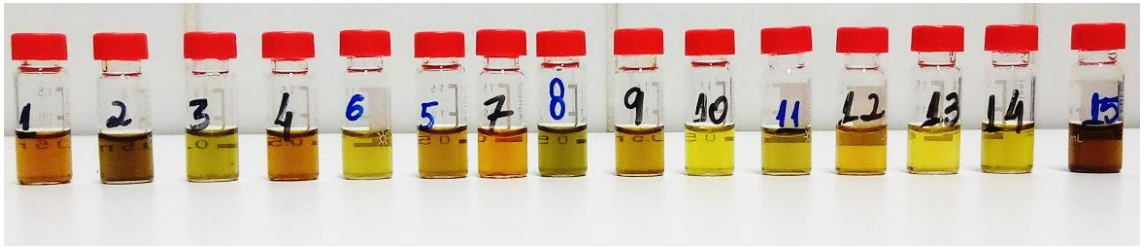

Figure S1. Visual aspect of the QuEChERS extracts after cleanup procedure with different sorbents.
From left to the right: 1 - C18 (150 mg), 2 - SAX (150 mg), 3 - PSA (150 mg), 4 - GCB (5 mg), 5 - C18 (75 mg) + SAX (75 mg), 6 - C18 (75 mg) + PSA (75 mg), 7 - C18 (150 mg) + GCB (5 mg), 8 - SAX (75 mg) + PSA (75 mg), 9 - SAX (150 mg) + GCB (5mg), 10 - PSA (150 mg) + GCB (5 mg), 11 - C18 (50 mg) + SAX (50 mg) + PSA (50 mg), 12 - C18 (75 mg) + SAX (75 mg) + GCB (5 mg), 13 - C18 (75 mg) + PSA (75 mg) + GCB (5 mg), 14 - SAX (75 mg) + PSA (75 mg) + GCB (5 mg), 15 - Without cleanup.

CAPÍTULO III: PHTHALIC ACID ESTERS AND ADIPATE IN HERBAL-BASED SOFT DRINKS: AN ECO-FRIENDLY METHOD

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PHTHALIC ACID ESTERS AND ADIPATE IN HERBAL-BASED SOFT DRINKS: AN ECO-FRIENDLY METHOD

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ABSTRACT

Phthalic acid esters (PAEs) and adipates are plasticizers with high applicability in several consumer products and building materials (e.g. cosmetics, packing) very persistent in the environment, features which turned them ubiquitous pollutants. These substances can contaminate food through the environment (water, air, and soil) and/or migration from packaging materials, being a health concern due to their toxicity. This paper describes an eco-friendly dispersive liquid-liquid microextraction (DLLME) procedure to extract five phthalates and di-2-ethylhexyladipate (DEHA) from bottled herbal-based beverages followed by GC-MS/MS quantification. The method showed low limits of detection (5.0 – 13 μg L^{-1}) and quantification (20 – 35 μg L^{-1}), good inter- and intra-day precisions (RSD<19%), and recoveries ranging from 82 to 111%. It was applied to 16 real samples, of which 13 showed the presence of at least one of the analytes under study. Additionally, an exposure assessment was performed, resulting in a hazardous quotient less than 1 (HQ<1) for all analytes, so PAEs and DEHA found in samples do not pose a health issue.

Keywords: Food contaminants, Phthalates, GC-MS/MS, DLLME, herbal infusion

1. Introduction

Phthalates or phthalic acid esters (PAEs) are a group of synthetic molecules widely used as plasticizers or solvents in diverse products such as cosmetics, pesticides, and repellents. Chemically, PAEs are a group of diesters of ortho-phthalic acid (dialkyl or alkyl aryl esters of 1,2-benzenedicarboxylic acid) [1–4]. Adipates, also applied as plasticizers in replacement of PAEs, are esters of adipic acid (hexanedioic acid), being di-2-ethylhexyl adipate (DEHA) the major representant of these group of molecules [1] (**Figure 1**). Around 8 million tons of PAEs are produced every year [5], much of which ends up in the environment due to their volatility and leaching properties. Therefore, PAEs are ubiquitous present in the environment and also in biota, food and feed [1, 2]. Numerous experimental studies reported the toxicological impact of PAES in humans, most of which were able to confirm that these substances can act as endocrine-disruptors [6], mimicking or blocking the action of natural hormones [7], prompting concerns on the development of reproductive systems, and possibly cause of some kind of cancers [8]. Additionally, epidemiological studies suggest a negative relation between phthalates exposure and the cognitive development of children [9]. The International Agency for Research on Cancer (IARC) classifies bis(2-ethylhexyl) phthalate (DEHP) as group 2B (possibly carcinogenic to humans), while benzyl butyl phthalate (BBP) and DEHA, the only adipate on IARC list, are listed in group 3 (not classifiable as to its carcinogenicity to humans) [10]. Furthermore, PAEs are classified as “chemicals of concern” by the United States Environmental Protection Agency categorizes PAEs [11] and as “substances of very high concern” by the European Comission [6] due to the mentioned human health and environmental effects [9].

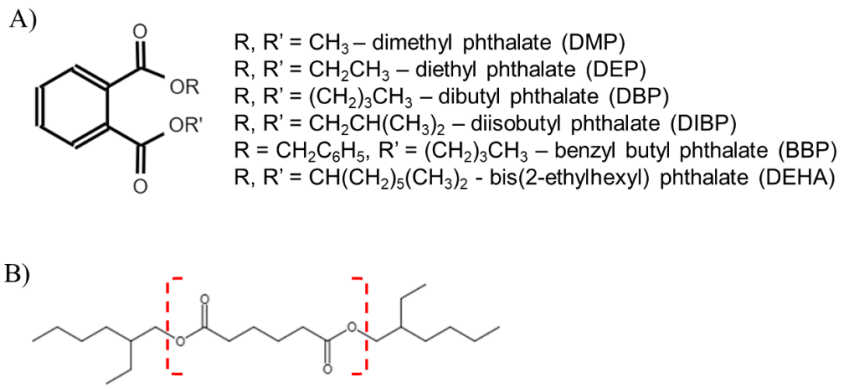

Figure 1. Phthalic acid esters (A) and di-2-ethylhexyl adipate (B).

The main source of PAEs and adipates in foodstuffs are the plastic materials extensively applied to packed food. Plastic films used to wrap food can be made of different materials such as polyvinyl chloride (PVC), polyvinylidene chloride (PVDC), polypropylene (PP), polyethylene (PE), regenerated cellulose film (RCF), and cellulose acetate [1, 2]. From these, PVC is the most employed either by the industry and domestic purposes, being bis-DEHP and DEHA the main plasticizers used in this kind of film [1]. Polyethylene terephthalate (PET) is a polymer-based on para- and/or meta-phthalic acid applied to make up bottles for bottled water, soft drinks, and other products. Although PET bottles are made to be rigid and resistant, so, in theory, they should be free of plasticizers, phthalates are constantly found in bottled water [1, 4, 12–14]. Cellulose acetate films are also often plasticized with diethyl phthalate (DEP), while some studies observed that these compounds can migrate from cardboard packages into food, due to its high porosity [1]. Beyond plastics, PAEs such as DBP, dicyclohexyl phthalate (DCHP), and DEHP can be applied in printing ink to improve adhesion and hold color. and plastic films [1, 2]. Therefore, several countries including UE [15] and Brazil [16] regulate plastics and other materials intended to come into contact with food, setting limits to the amount of phthalates and adipates that can be used in these materials as well as specific migration limits (SML). DEHP, for instance, can be applied as technical support agent at 0.1% of

the final product, and the SML cannot exceed 1.5 mg kg^{-1}. Likewise, dibutyl phthalate (DBP) and benzyl butyl phthalate (BBP) can be applied at 0.05% and 0.1% as additives, respectively, and must not exceed 0.3 and 30 mg kg^{-1} of SML in food. For DEHA the SML is quite higher, 18 mg kg^{-1}, and no usage specification is defined [15, 16].

Regarding analytical methods, official sample preparation protocols are based on classical procedures such as liquid-liquid extraction followed by a solid-phase clean-up (LLE-SPE) [14]. However, these procedures usually spend a great amount of organic solvents and are time-consuming [2], so they are gradually being replaced by procedures that consume less solvents such as QuEChERS (Quick, Cheap, Easy Rugged and Safe) [17], SPE [18], or even miniaturized protocols such as dispersive liquid-liquid microextraction (DLLME) [19] and solid-phase microextraction (SPME) [20]. The most appropriate separation and detection technique is gas chromatography coupled with mass spectrometry (GC-MS and GC-MS/MS) [18, 21–23]. Meantime, high-performance liquid chromatography (HPLC) with diode array detector (HPLC-DAD) or mass spectrometry (LC-MS) can be also applied to PAEs analysis [19, 24]. Taking into account the low levels found in food samples, techniques such as GC coupled to tandem mass spectrometry (GC-MS/MS) can be an added value, due to its highest separation power, selectivity, and sensitivity. Additionally, the use of solventless extraction techniques, and miniaturization are envisaged in the current era of “green chemistry”, but still far from optimized in phthalate analysis.

Hence, this work aimed to validate a quick, sensitive, and eco-friendly method based on dispersive liquid-liquid microextraction (DLLME) and gas chromatography tandem mass spectrometry (GC-MS/MS) to determine six phthalic acid esters (PAEs) and one adipate in herbal-based soft drinks (yerba mate and black tea). Additionally, real samples commercialized in Brazil and Portugal, stored in different packing material, were

analyzed using the developed method and the dietary exposure was assessed from the results obtained.

2. Material and Methods

2.1. Sampling

A total of 42 unit samples of 16 different herbal-based (*Camellia sinensis* or *Ilex paraguariensis*) soft drinks sold on different packaging materials [polypropylene (PP), polyethylene terephthalate (PET), aluminum (Al), and carton (CA)] were purchased in local markets in Campinas (Brazil) and Porto (Portugal). The description of the samples is provided in **Table 1**. Each sample unit was analyzed in duplicate.

Table 1. Sample code, brand, number of sampling units, package material and flavor of the samples.

Sample code	Brand	n	Package material	Herb based/Flavor
MO	A	3	Polypropylene	Yerba mate extract
ML	A	3	Polypropylene	Yerba mate extract with lemon
LF	A	3	Polypropylene	Yerba mate extract
CL	B	3	Polyethylene terephthalate	Yerba mate extract w/ açai and guarana
GM	C	3	Polyethylene terephthalate	Yerba mate extract w/ lemon
QL	D	3	Polyethylene terephthalate	Black tea extract w/ peach
LP	E	3	Polyethylene terephthalate	Black tea extract w/ lemon
LL	E	3	Aluminum	Black tea extract w/ lemon
DA	F	3	Aluminum	Black tea extract w/ hibiscus and blueberry
NE	G	1	Polyethylene terephthalate	Black tea extract
LPT	E	1	Polyethylene terephthalate	Black tea extract
CT	H	1	Carton	Black tea extract
LPL	E	3	Aluminum	Black tea extract w/ lemon
CTP	H	3	Carton	Black tea extract
LT	E	3	Carton	Black tea extract
PL	I	3	Aluminum	Black tea extract

* Information on product label

2.2. Chemicals and solutions

Analytical standards of dimethyl phthalate (DMP), diethyl phthalate (DEP), diisobutyl phthalate (DIBP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), bis(2-ethylhexyl) adipate (DEHA), bis(2-ethylhexyl) phthalate (DEHP), and the internal standard dioctyl phthalate-d4, all with a standard purity of ≥99%, were obtained from

Supelco (Sigma-Aldrich, Germany). The working solutions at 10 µg L^{-1} and 100 µg L^{-1} were prepared in ethanol (EtOH, HPLC grade) and kept refrigerated (~ 4ºC) until the analysis. In this experiment ultrapure water (18.2 mΩ cm^{-1}) purified by a Milli-Q gradient system from Millipore (Milford, MA, USA) was used. Ethanol and hexane used as dispersive and extraction solvents (both HPLC grade) were also purchased from Sigma (Sigma-Aldrich, Germany).

2.3. Dispersive liquid-liquid microextraction (DLLME)

Since n-hexane is a common solvent applied to PAEs extraction in classical protocols [2], this work proposed a dispersive liquid-liquid microextraction (DLLME) applying n-hexane as extractor solvent. In this procedure, a mixture of 100 µL of ethanol (dispersive solvent) and 200 µL of hexane (extractor solvent) was rapidly added to 10 mL of sample previously spiked with 50 µL of deuterated internal standard solution (ISTD, dioctyl phthalate-d4) at 10 µg L^{-1}. After this, the tube was homogenized in a vortex for 15 s, sonicated for 5 min, and centrifuged at 1690 *g* for 5 min. Then, 150 µL of the upper layer was transferred to a vial containing a conical insert and 1 µL of the extract was injected into the GC-MS/MS system.

To reduce possible contamination, every material used during the extraction procedure was rinsed with hexane HPLC grade and then heated at 350 ºC for 4 hours. Plastic micropipette tips were soaked in ethanol HPLC grade at 60 ºC overnight then dried before using. Furthermore, together with every batch, blanks were prepared replacing the sample with ultrapure water being the extraction performed in the same conditions of the samples. This protocol was accomplished to assess the background conditions and possible contamination during the sample preparation batch.

2.4. Chromatographic conditions and mass spectrometry (GC-MS/MS)

An Agilent 7890B gas chromatograph, equipped with an autosampler (Agilent 7693A) and electronically controlled split/splitless injection port, coupled with a triple quadrupole 7000C (Agilent Technologies Inc., Palo Alto, CA, USA) mass spectrometer with an electron ionization (EI) chamber was used to PAEs and DEHA analysis. GC separation, previously developed by Oliveira [25], was achieved on an Agilent J&W DB-5ms (30m × 0.25 mm × ID 0.25 µm film thickness, Agilent J&W, Netherlands). The oven temperature was programmed initially at 90 ºC, held for 1 min, increased to 300 ºC at 20ºC $min^{-1,}$ and held for 5 min, with a total run of 16.5 min. Ultra-high purity helium (99.999%; Gasin, Portugal) was used as carrier gas at 1.0 mL min^{-1}. The injector was maintained at 300 ºC in pulsed spitless mode (0.5 min purge-off, 35 psi) and 1.0 µL of the extract was injected. To avoid silicone rubber contamination on analysis due to the sept degradation through the repeated injections, a Merlin Microseal® sept (Agilent) was used. The triple quadrupole MS was operated in multiple reaction monitoring (MRM) mode detecting three transitions per analyte (**Table 2**), the electron energy was 70 eV and the temperatures of transfer line, ion source, and quadrupole were 300 ºC, 230, and 150 ºC, respectively. Helium was used as quench gas (2.25 $mL.min^{-1}$) and nitrogen as collision gas (1.5 $mL.min^{-1}$). System control and data acquisition were performed in MassHunter® software. The collision energies for MRM were optimized injecting the individual PAE and DEHA analytical standards in order to achieve the highest sensitivity and selectivity.

2.5. In-house validation and quality control

The method was in-house validated by determining the following figures of merit: limit of detection (LOD), limit of quantification (LOQ), linearity, intra- and inter-day precision, recovery, and matrix effects.

LOD and LOQ were defined as the lowest concentration in a spiked blank sample that gave a signal/noise of 3 and 10, respectively. Linearity was assessed through the coefficient of determination (R^2) of linear regression (external standard area/internal standard area ratio versus external standard concentration) from five points matrix-matched calibration curves constructed from 35 to 135 µg L^{-1}. Additionally, calibration curves were submitted to ANOVA, lack of fit, and residual analyses to evaluate the linearity of the mathematical models.

Table 2. GC-MS/MS conditions.

Analyte	Precursor ion (m/z)	Product ion (m/z)	Collision energy (kV)	Rt (min)	Time window
Dimethyl phthalate	164	78	20	6.3	1
	163	135	10		
	133	105	5		
Diethyl phthalate	177	149	5	7.1	2
	176	149	5		
	150	122	10		
	149	121	10		
Diisobutyl phthalate	223	149	5	8.6	3
	167	149	5		
	149	121	15		
Dibutyl phthalate	223	149	5	9.1	4
	205	149	5		
	149	121	15		
Benzyl butyl phthalate	206	149	5	10.9	5
	206	105	25		
	149	121	15		
Bis(2-ethylhexyl) phthalate	279	149	15	11.0	5
	167	149	5		
	149	121	15		
Bis(2-ethylhexyl) phthalate	129	111	60	11.6	6
	129	101	5		
Dioctyl phthalate-d4 (ISTD)	283	153	10	12.5	7
	153	153	5		
	153	125	10		

Intra and inter-days precision assays were conducted in spiked samples, at three concentration levels (35, 85, and 135 µg L^{-1}) using five replicates (n = 5), on the same day and during three consecutive days, respectively. Both intra- and inter-day precisions were expressed as relative standard deviation (%RSD). Recovery (%) assays were also performed in three concentration levels (35, 85, and 135 µg L^{-1}) using five replicates (n = 5).

Matrix effects (ME) were investigated by comparing the slope of the matrix-matched calibration curve with the slope of solvent calibration curves in the same concentration range and it was expressed in percentage (Equation 1).

$$\%\text{ME} = \left\{\left(\frac{\text{Matrix Slope}}{\text{Solvent Slope}}\right) * 100\right\} - 100$$

2.6. Exposure assessment

Exposure assessment was performed according to European Food Safety Authority recommendations [26, 27]. For estimated daily intake (EDI, µg kg^{-1} bw day) a middle-bound scenario (MB) was performed, in this approach, values <LOD and <LOQ are assigned as LOD/2 and LOQ/2, respectively. EDI was calculated according to Equation 2:

$$EDI = \frac{Ci * Di}{BW}$$

Where *Ci* is the phthalate content in samples (µg L^{-1}), *Di* is the daily consumed dosage of soft drink (L), in this case was considered the largest individual container of 0.5 L (500 mL), and *BW* is the body weight of an adult (70 kg). EDI for DBP, BBP, and DEHP was expressed as DEHP equivalent [26], so a "Group EDI" was calculated by Equation 3:

$$Group\ EDI = \left(\frac{mw\ DEHP}{mw\ DBP} * Ci_{DBP}\right) + \left(\frac{mw\ DEHP}{mw\ BBP} * Ci_{BBP}\right) + \left(\frac{mw\ DEHP}{mw\ DEHP} * Ci_{DEHP}\right)$$

Where *mw DEHP* is the molecular weight of DEHP (360.60 g mol^{-1}), *mw DBP* is the molecular weight of DBP (278.34 g mol^{-1}), *mw BBP* is the molecular weight of BBP (312.40 g mol^{-1}), and Ci_{DBP}, Ci_{BBP}, and Ci_{DEHP} are, respectively, the average concentration (µg L^{-1}) of DBP, BBP, and DEHP found in real samples.

The EDI was compared to the tolerable daily intake (TDI) reference values through the hazard quotient, calculated as showed in Equation 4:

$$HQ = \frac{EDI}{TDI}$$

The reference values of TDI (µg kg^{-1} bw day) for DEP, recommended by WHO, is 5000 µg kg^{-1} bw day [28] and the TDI for Group-DEHP and for DEHA, both indicated by EFSA, is 50 µg kg^{-1} bw day [26] and 300 µg kg^{-1} bw day [29], respectively. Values of HQ >1 indicates consumption exceeding the TDI and HQ<1 below the TDI.

3. Results and discussion

3.1. Procedure Optimization

3.1.1. GC-MS/MS System

GC system optimization included the choice of column configuration, separation conditions, and optimization of MS/MS parameters all with a major impact on precision and sensitivity of phthalates determination. The choice of a capillary column with a 5 % phenyl methylpolysiloxane stationary phase was based on previous studies [25, 30]. Column oven programming was optimized to obtain a good compromise between chromatographic resolution and band broadening. The optimization of MS/MS parameters was made using analytical standards firstly recorded in total ion current (TIC) mode, after what the precursor and the ion products were chosen taking in order to achieve the highest selectivity and sensitivity.

3.1.2. Extraction

Aware that hexane is an extraction solvent commonly used in phthalate extraction [2] this solvent was immediately chosen as extraction solvent in DLLME. The dispersive solvent, ethanol, was selected based on their miscibility with the sample, low cost as well as due to the fact of being considered more environmentally friendly than other usually

dispersive solvents such as acetonitrile and methanol. The protocol was optimized in terms of the volume of extraction solvent and sample/dispersive solvent volume ratio. Thus, different volumes of extraction solvent (ranging from 100 to 300 µL), sample:dispersive solvent volume ratio (from 100:1 to 100:3, v/v) were tested. Optimal conditions (10 mL of sample, 100 µL of ethanol and 200 µL of hexane) were selected in terms of the ones that could result in higher extraction yields of target analytes.

3.2. In-house method validation and quality control

Regarding matrix effects (**Figure 2**), it was observed a moderate to high signal suppression to all analytes, from -99.4 (DEHP) to -41.6% (DIBP). The main advantage of DLLME procedure is the high enrichment factor (EF) what lead to high method sensitivity. However, together with the analytes, matrix interferents may be concentrated, causing high matrix effects as observed in these results. Two main strategies are usually taken to overcome these effects: adding cleanup steps to the extraction protocol such as solid phase extraction (SPE) [31] or dispersive solid phase extraction (dSPE) [32], for example; and/or perform a matrix-matched calibration. In matrix-matched calibration, matrix extract are used to build the calibration curve, compensating these effects [33].

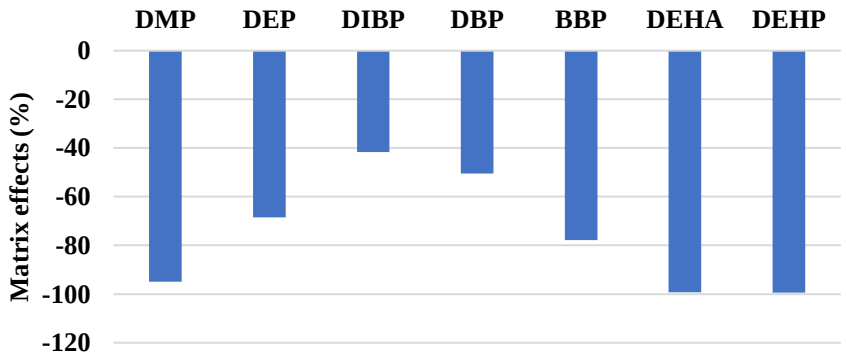

Figure 2. Matrix effects of the DLLME-GC-MS/MS method for each phthalate.
DMP - dimethyl phthalate; DEP - diethyl phthalate; DIBP - diisobutyl phthalate, DBP - dibutyl phthalate; BBP - benzyl butyl phthalate; DEHA - dis(2-ethylhexyl) adipate; DEHP - bis(2-ethylhexyl) phthalate.

Table 3. Validation's criteria for DLLME-GC-MS/MS method.

Analyte	LOD	LOQ	Linear Range	Linearity	Regression model	Precision intra-day (%) (n = 5)			Precision inter-day (%) (n = 5)			Recovery (n = 5)		
	µg L^{-1}	µg L^{-1}	µg L^{-1}	R^2		35 µg L^{-1}	85 µg L^{-1}	135 µg L^{-1}	35 µg L^{-1}	85 µg L^{-1}	135 µg L^{-1}	35 µg L^{-1}	85 µg L^{-1}	135 µg L^{-1}
DMP	11	35	35 - 135	0.993	y = 1.142x-33.604	17	4	11	13	8	11	102	92	82
DEP	12	35	35 - 135	0.992	y = 11.326x-365.227	19	7	10	15	10	11	111	93	85
DIBP	10	35	35 - 135	0.994	y = 6.881x-207.739	17	8	6	11	9	12	107	107	102
DBP	8	30	35 - 135	0.996	y = 4.878x-146.035	17	10	6	11	11	11	101	95	92
BBP	13	35	35 - 135	0.990	y = 2.519x-72.377	14	16	12	15	13	11	95	80	82
DEHA	7	22	35 - 135	0.998	y = 0.329x-3.534	9	8	8	10	9	9	91	91	85
DEHP	5	20	35 - 135	0.998	y = 0.061x-0.559	8	9	5	9	13	9	102	92	92

LOD – Limit of detection; LOQ – Limit of quantification; DMP - dimethyl phthalate; DEP - diethyl phthalate; DIBP - diisobutyl phthalate, DBP - dibutyl phthalate; BBP - benzyl butyl phthalate; DEHA - dis(2-ethylhexyl) adipate; DEHP - bis(2-ethylhexyl) phthalate.

Since the objective of this work was to develop a quick, sensitive, feasible and eco-friendly method, the matrix-matched calibration was adopted to overcome the matrix effects.

The linearity was estimated from the coefficient of determination (R^2) of five points of the matrix-matched calibration curves, which was always greater than 0.990 (BBP) (**Table 3**). LODs were from 5 µg L^{-1} (DEHP) to 13 µg L^{-1} (BBP) while LOQs ranged from 20 µg L^{-1} (DEHP) to 35 µg L^{-1} (DMP, DEP, DIIBP, and BBP). Recovery results ranged from 80% (BBP) to 111% (DEP) and intra- and inter-day precisions were lower than 19% (DEP) and 15% (DEP), respectively. The obtained results (**Table 3**) are within the expected for modern analytical procedures, thus, the method can be applied to quantitative purposes [34, 35].

Table 4. Penalty points for PAEs determination by DLLME-GC-MS/MS

	Penalty Points
Reagents	
Ethanol	2
n-Hexane	8
Instruments	
Energy	
Hotte	1
Vortex	0
Ultrasound	0
Centrifuge	1
GC-MS/MS	3
Occupational Hazard	3
Waste	3
Recycling	0
Total Penalty Points	21
Analytical Eco-Scale score	**79**

Analytical Eco-Scale score = 100 – total penalty points. When >75 represents excellent green analysis, >50 represents acceptable green analysis and <50 represents inadequate green analysis. According to Gałuszka et al (2012).

This method has the main advantages of being i) fast - all procedure, from sample preparation to data analysis, takes only 30 min for each sample; ii) low solvent consumption (300 µL), and iii) usage of very cheap and common solvents (ethanol and hexane). When compared to the United States Environmental Protection Agency (EPA) official method [14] and similar [36], which applied LLE-SPE protocol, these approaches spend 13 times more organic solvent and

it takes at least twice as long. Even when compared to other DLLME procedures, our sample preparation spends 70% less solvent and no additional steps such as pH adjustment or salting-out [19] were needed.

An assessment of the method greenness was performed according to Gałuszka et al., [37] recommendations. In this evaluation (Table 4), penalty points are assigned for each parameter of an analytical process that is not in accordance with the 12 principles of green chemistry. The method reached 79 points on Analytical Eco-Scale, so it can be considered an "excellent green analysis".

3.3. Application to real samples

Results from application of the method to real samples are shown in Table 5. Phthalates were detected in 13 out of 16 samples (81%), two of which were above the LOQ (35 μg L^{-1}), with levels of 36 μg L^{-1} and 63 μg L^{-1} for DEP. DIBP and DEP were detected in 56% samples, followed by DMP, DEHP, and DBP detected in 50%, 31%, and 19% of the samples, respectively. BBP and DEHA were not detected in any sample. Total PAEs levels ranged from <33 μg L^{-1} to 89.9 μg L^{-1}. Total PAEs were calculated summing the individual PAE for each sample, considering the LOD/2 when <LOD, and LOQ/2 when <LOQ. These results are slightly higher than others recently published. Amin et al. [38] analyzed 4 PAEs (DEP, DEHP, DBP, and BBP) in white, green, and black teas infusions and did not found values above the LOD (0.02 – 0.11 μg L^{-1}). The same result was reported by Liang et al., [20] when they searched for 5 PAEs (diallyl phthalate, DIBP, DBP, BBP, and DEHP) in jasmine based beverages, and only one sample was detected for BBP at 0.09 μg L^{-1}.

Table 5. Results for phthalates in soft drinks.

Sample	Analyte (µg L^{-1})							
	DMP	**DEP**	**DIBP**	**DBP**	**BBP**	**DEHA**	**DEHP**	**Total PAEs**
MO	<LOQ	N.D.	<LOQ	N.D.	N.D.	N.D.	N.D.	57
QL	<LOQ	<LOQ	<LOQ	<LOQ	N.D.	N.D.	<LOQ	87
ML	<LOQ	N.D.	<LOQ	N.D.	N.D.	N.D.	<LOQ	65
LP	<LOQ	<LOQ	<LOQ	N.D.	N.D.	N.D.	<LOQ	76
CL	<LOQ	<LOQ	N.D.	<LOQ	N.D.	N.D.	N.D.	67
GM	<LOQ	N.D.	<LOQ	<LOQ	N.D.	N.D.	<LOQ	76
LL	N.D.	<LOQ	<LOQ	N.D.	N.D.	N.D.	N.D.	57
LF	<LOQ	<LOQ	<LOQ	N.D.	N.D.	N.D.	N.D.	69
DA	N.D.	<LOQ	<LOQ	N.D.	N.D.	N.D.	N.D.	57
PL	<LOQ	N.D.	N.D.	N.D.	N.D.	N.D.	<LOQ	52
LT	N.D.	63 ± 29	N.D.	N.D.	N.D.	N.D.	N.D.	90
CTP	N.D.	<LOQ	N.D.	N.D.	N.D.	N.D.	N.D.	44
NE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	33
LTP	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	33
CT	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	33
LPL	N.D.	36 ± 1	<LOQ	N.D.	N.D.	N.D.	N.D.	75
Frequency (%)	50	56	56	19	0	0	31	81

<LOQ - lower than the limit of quantification; N.D. = lower than the limit of detection (<LOD); DMP - dimethyl phthalate; DEP - diethyl phthalate; DIBP - diisobutyl phthalate, DBP - dibutyl phthalate; BBP - benzyl butyl phthalate; DEHA - dis(2-ethylhexyl) adipate; DEHP - bis(2-ethylhexyl) phthalate, Total PAEs = for the sum of phthalates, it was considered the LOD/2 when N.D. (<LOD), and LOQ/2 when <LOD.

Regarding DEP, the unique PAE above the LOQ in this study, the content is in the same range found by Wu et al., [36] (<10 - 76 µg L^{-1}) and Lo Turco, et al., [18] (10.5 to 33.0 µg L^{-1}) in tea drinks. For the other PAEs analyzed by these authors, they found concentrations higher than those demonstrated in this study. DEP was also detected in foodstuffs such as nonalcoholic beverages [36] (<10 – 76 µg L^{-1}), juices and vinegar (0.03 – 0.40 µg L^{-1}) [39], tea infusion, (45 µg L^{-1}) [17], fruit jellies (450 – 1200 µg kg^{-1}) [40], and several other foods (grain and grain products, milk and dairy products, meat and meat products, fish and fish products, and beverages) from <1.5 to 9.3 µg kg^{-1} [13].

Concerning the four different package materials covered by this study, samples packaged in PET showed the highest number of detected PAEs, summing five out of the seven analytes, while DMP had the highest frequency in this package, being present in 67% of the analyzed samples (**Figure 3**). The package that showed the highest occurrence was, however, PP, in

which 100% of the samples showed the presence of DMP and DIBP. Moreover, DEP and DEHP were also detected in some of the PP stored samples. The samples conditioned in aluminum cans showed the presence of DEP, DIBP, DMP, and DEHP in the frequency of 75% for DEP and DIBP, and 25% for DMP and DEHP. Finally, only DEP was detected in 67% of the samples stored in carton packaging.

Serrano et al., [3] analyzed seventeen food monitoring surveys published in between 1990 and 2013, concluding that DIBP, DBP, BBP, and DEHP were the most frequent PAEs found in food. DEP, DMP, and di-n-octyl phthalate (DNOP), on the other hand, had the lowest occurrence. However, this scenario is not applicable for China, where DMP and DEP were found in 82% and 81% of the samples. Additionally, DEP was present in 57% of the samples from the US. Despite the difference in the occurrence of PAEs in different countries, there are a consensus that DEHP is the PAE most frequent in food being considered a public health issue, especially in developed countries [2, 3].

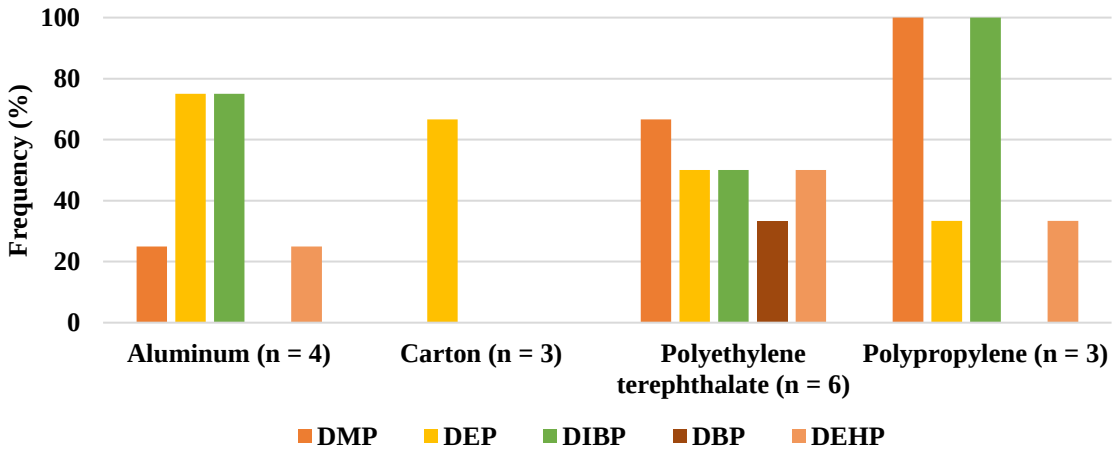

Figure 3. Frequency of individual phthalic esters (PAEs) and bis(2-ethylhexyl) adipate (DEHA) by package material.
DMP - dimethyl phthalate; DEP - diethyl phthalate; DIBP - diisobutyl phthalate, DBP - dibutyl phthalate; BBP - benzyl butyl phthalate; DEHA - dis(2-ethylhexyl) adipate; DEHP - bis(2-ethylhexyl) phthalate. BBP and DEHA were not detected in samples.

Taking into consideration the current legal framework in Europe [15], and Brazil [16], that establish specific migration limits (SML) for DBP (300 µg kg^{-1}), BBP (30000 µg kg^{-1}), DEHP (1500 µg kg^{-1}), and DEHA (1800 µg kg^{-1}) for food materials contact, all the samples analyzed here were under the legal limits. Nevertheless is important to emphasize that, despite food contact materials are the main contamination source of PAEs in food, they are not exclusive [1].

Even with the several published works with PAEs in food (the research of the keywords "*phthalate* and *food*" on Scopus returns more than 1400 documents), fewer of them correlate the presence of these chemicals in food with the packaging material [41, 42]. Most of the articles only cite generically the packaging material without details (e.g. 'plastic') what hamper the comparison of the results [13, 43, 44].

3.4. Exposure assessment

Tolerated daily intake (TDI) values are available for Group-DEHP equivalent (50 µg kg^{-1} bw day) which comprehends the sum of DBP, BBP, DEHP, and DINP (diisononyl phthalate) expressed as DEHP equivalent [26], DEHA (300 µg kg^{-1} bw day) [29] and DEP (5000 µg kg^{-1} bw day) [28]. The estimated daily intake calculated for the average content of PAEs and DEHA in samples were between 0.03 µg kg^{-1} bw day (DEHA) and 0.14 µg kg^{-1} bw day (Group-DEHP equivalent) (**Table 6**). Comparing TDI with EDI through the hazardous quotient (HQ), all the results were less than 1 (HQ<1), from 8.33E-5 (DEHA) to 2.89E-3 (Group-DEHP equivalent), considering a middle bound approach [26]. Hence, the results suggest that the content of PAEs and DEHA found in herbal-based soft drinks samples do not pose a potential health concern. These results are similar to those reported by Dobaradaran et al., [45] which analyzed bottled milk and the HQs were from 1.11E-4 (DEHP) to 2.47E-1 (DEP).

Table 6. Estimated daily intake (EDI) and hazard coefficient (HQ) for phthalates in soft drinks.

Phthalate	TDI μg.kg^{-1} bw day (Reference)	EDI (HQ) μg.kg^{-1} bw day
DMP	-	0.08 (-)
DEP	5000 (WHO, 2003)	0.12 (2.35E-5)
DIBP	-	0.09 (-)
Group-DEHP equivalent	50 (EFSA, 2019)	0.14 (2.89E-3)
DEHA	300 (EFSA, 2005)	0.03 (8.33E-5)

DPM – dimethyl phthalate; DEP – diethyl phthalate; DIBP – diisobutyl phthalate; Group-DEHP equivalent for dibutyl phthalate (DBP), benzyl butyl phthalate (BBP) and DEHP - bis(2-ethylhexyl) phthalate; DEHA - dis(2-ethylhexyl) adipate; TDI – tolerable daily intake; EDI – estimated daily intake; HQ – hazard quotient.

4. Conclusions

The DLLME-GC/MS/MS method developed in this study showed to be suitable for the determination of phthalic acid esters (PAEs) and DEHA in herbal-based ready-to-drink beverages. Low values of LOD and LOQ, good linearity, precision, and recovery were achieved. The main advantages of the method are simplicity, low cost, quickness, and greenness, due to the low amounts of solvents involved. The application of the method to real samples showed that all were below the specific migration limits (SML) recommended by European and Brazilian legislation, and the hazardous quotients (HQ) were less than 1. These results show that the presence of PAEs in herbal-based soft drinks commercialized in Brazil and Portugal is not a health public issue of concern; nevertheless, more samples must be monitored.

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CAPÍTULO IV: A NOVEL DISPERSIVE LIQUID-LIQUID MICROEXTRACTION USING A LOW DENSITY DEEP EUTECTIC SOLVENT-GAS CHROMATOGRAPHY TANDEM MASS SPECTROMETRY FOR THE DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN SOFT DRINKS

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A NOVEL DISPERSIVE LIQUID-LIQUID MICROEXTRACTION USING A LOW DENSITY DEEP EUTECTIC SOLVENT-GAS CHROMATOGRAPHY TANDEM MASS SPECTROMETRY FOR THE DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN SOFT DRINKS.

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ABSTRACT

Ready-to-drink teas can provide, if properly packaged, the taste and wellness character of traditional teas. Nevertheless, in tea processing, there may be several contaminations, among which polycyclic aromatic hydrocarbons **(**PAHs), anthropogenic contaminants that can present carcinogenic and mutagenic properties. In this work, a novel low-density deep eutectic solvent-based dispersive liquid-liquid microextraction (LDDES-DLLME) procedure followed by gas chromatography tandem mass spectrometry (GC-MS/MS) was optimized for analysis of 15 polycyclic aromatic hydrocarbons (PAHs) in ready-to-drink herbal-based beverages. The new deep eutectic solvent (DES) was synthesized with natural compounds (camphor and hexanoic acid). Several parameters of the extraction procedure such as type and volume of extraction solvent, type, volume of dispersive solvent, and time of extraction were evaluated to achieve the highest yield and to attain the lowest detection limits. The validated method showed very low limits of detection (0.01 μg L^{-1}) and quantification (0.2 μg L^{-1}), good inter- and intra-day precisions (RSD<16.87%), and recoveries higher than 69%. The method was applied to 16 real samples (42 sample units) and it was found total PAHs levels ranging from 0.20 to 1.82 μg L^{-1}.

Keywords: Food contaminant; Green chemistry; Tea; Environmentally friend Deep, Miniaturized techniques

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of over 100 different organic compounds with two or more benzene rings, which give them high stability and toxicity [1]. They are environmental contaminants, mainly produced by anthropogenic practices such as incomplete burning of fossil fuels in vehicles and industrial processes. In food, these contaminants are originated mainly from cooking processes like frying, smoking, or baking. Moreover, due to the wide distribution in water, air, and soil, they can easily contaminate vegetables for human consumption [1–3]. Several studies have shown that many PAHs can cause mutations and cancer in some animals and humans, many organization including the United States Environmental Protection Agency, International Agency for Research on Cancer (IARC), and European Union (EU) classify them as potentially genotoxic and carcinogenic to humans, being diet the main exposure route [4]. The IARC have characterized 17 PAHs as priority among them benzo[a]pyrene (BaP) is classified in group 1 (carcinogenic to humans) while dibenzo[a,h]anthracene (DBahA) and dibenzo[a,l]pyrene (DBalP) are categorized in group 2A (probably carcinogenic to humans), other PAHs are classed in group 3 (not classifiable as to its carcinogenicity to humans), but some of these has shown deleterious effects over immunological and endocrinal systems [1,2,5]. EU established maximum levels of BaP and sum of BaP, benz[a]anthracene (BaA), benzo[b]fluoranthene (BbF) and chrysene (CHR) (4PAHs) for several food classes such as oils and fats, cacao and related products, smoked products, processed food for infant and young children, dietary food for special medical purposes and dried herbs and spices in values that range from 1.0 to 10.0 μg kg^{-1} and from 1.0 to 50.0 μg kg^{-1} for BaP and 4PAHs, respectively [6]. In Brazil, the current legislation sets a maximum limit of BaP for olive pomace oil, artificial smoke flavoring, and potable water in levels of 2.0 μg kg^{-1}, 0.03 μg kg^{-1}, and 0.70 μg L^{-1}, respectively [7–9].

For dried herbs and spices, the limits set by Europe Union for BaP and 4PAHs are 10 μg kg^{-1} and 50 μg kg^{-1}, respectively [10]. Some papers reported the presence of these contaminants in traditional tea (*Camellia sinensis*), yerba mate (*Ilex paraguariensis*), and other herbs used in the preparation of infusions [11–14]. PAHs can contaminate these products through i) atmospheric depositions. PAHs can be carried by air and deposited on leaves where they are impregnated due to wax cuticle in leave surface, or ii) thermal processing; during drying and toasting steps, high temperatures can be employed particularly in yerba mate production promoting PAHs formation [13,14]. Regarding drink infusions, no maximum limits are stablished and only few studies have been performed PAHs assessment [11,13,15,16]. Reported PAHs in tea infusions are in the range of 0.006 - 0.009 μg L^{-1} (n=1) [15], 0.5 μg L^{-1} (n=1) [13] and 0.10-0.15 μg L^{-1} (n=7) [11].

Owing to the low maximum tolerated levels for PAHs in food, it becomes imperative the use of sensitive and selective analytical techniques to meet the criteria established by regulatory agencies such as European Food Safety Authority (EFSA) and the Brazilian Health Regulatory Agency. PAHs are usually analyzed by high-performance liquid chromatography with fluorescence (HPLC-FLD) or mass spectrometer (HPLC-MS) detection, or by gas chromatography coupled to mass spectrometer (GC-MS) or tandem mass spectrometry (GC-MS/MS) [17–19]. These methods comprise always a previous extraction and cleanup procedures in order to avoid as much as possible the presence of interferents in the injected extracts. The classical techniques for extraction of PAHs are based on solid-liquid extraction (SLE) for solid samples and liquid-liquid extraction (LLE) for liquid samples such as oils and beverages [20,21]. Both techniques, however, use plenty of organic solvents and are time-consuming. On other hand, these procedures imply the use of further cleanup procedures such as solid-phase extraction (SPE) to remove interferences [20]. Aiming to overcome these limitations, solid-phase microextractions (SPME) (Li et al., 2019), liquid-phase

microextractions (LPME) such as in-tube-SPME, hollow-fiber-LPME (HF-LMPE) [22], and dispersive liquid-liquid microextraction (DLLME) [23–25] have gained ground in PAHs analysis, a clear trend of reducing costs and waste generation promoting the grow of environmental-friendly method.

Taking into account the principles of green chemistry, several LPME techniques have been developed in recent years, with the aim of minimizing the consumption of organic solvents, with emphasis on techniques based on the use of ionic liquids [26] and deep eutectic solvents (DESs) [27,28]. Deep eutectic solvents are eutectic mixtures that can be formed by a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), so that mixture has a melting point below their components. These solvents present several advantages when compared to organic solvents like low cost, easy preparation, low toxicity, and show good biodegradability [27]. Recently, DESs have been employed as extraction solvent in classical procedures such as SLE and LLE and miniaturized techniques as DLLME and HF-PLME for analysis of several bioactive compounds, metals, and contaminants [28]. Despite the growing tendency to the application of DES in extraction protocols, only a few have been applied in PAHs analysis, all of them in environmental samples [18,19,29,30]. The reported DES included choline chloride with phenol [29], or *p*-chlorophenol [30], tetra-n-butyl ammonium bromide with carboxylic acids, and thymol with camphor [18] all od them used as extractoion solvent in liquid-liquid microextraction procedures. Because ready teas have a somewhat different composition than environmental samples, that affects both PAHs types and levels therefore their extraction also merits further examination.

Overall, the use of green DES as extraction solvent in microextraction procedures such as DLMME not only reduces waste generation in the laboratory but also lessens environmental pollution, therefore, this study aimed to develop and validate a new method based on low-

density deep eutectic solvent-dispersive liquid-liquid microextraction (LDDES-DLLME) followed by gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis for trace determination of 15 PAHs in commercial herbal ready-to-drink beverages. The present study reports for the first time the synthesis and application of a DES made with camphor and hexanoic acid to determine PAHs in soft drinks.

2. Material and methods

2.1. Chemicals and solutions

Analytical standards of naphthalene, 2-methylnaphthalene, 1-methylnaphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, chrysene, benzo[a]anthracene, benzo[k]fluoranthene, benzo[b]fluoranthene, benzo[a]pyrene, Indene[1,2,3-cd]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene and the deuterated internal standard (chrysene-d12), all with a standard purity of >98%, were obtained from Supelco (Sigma-Aldrich, Germany). Working solutions of 20 µg L^{-1} and 400 µg L^{-1} were prepared in acetonitrile (MeCN, HPLC grade) and kept refrigerated (~ 4ºC) until the analysis. The components for the synthesis of DES, camphor (CAM) (purity ≥95%), hexanoic acid (C6) (purity ≥98%), heptanoic acid (C7) (purity ≥99%), octanoic acid (C8) (purity ≥99%), nonanoic acid (C9) (purity ≥96%), decanoic acid (C10) (purity ≥98%), dodecanoic acid (C12) (purity ≥98%) and thymol (Th) (purity ≥98%) were also purchased from Sigma-Aldrich. In this experiment, ultrapure water (18.2 mΩ cm-1) purified by a Milli-Q gradient system from Millipore (Milford, MA, USA) was used. All analytical reagents used are at or above the level of “analytical grade”.

2.2. Sampling

A total of 16 real samples (in a 42 sample units), including 9 different brands of ready-to-drink herbal drinks (*Camellia sinensis* or *Ilex paraguariensis*) were purchased from the local market

in Campinas (Brazil) and Porto (Portugal). The description of the samples is provided in **Table 1**. Each sample unit was analyzed in duplicate.

Table 1. Brand, flavor, number of sampling units and code for samples.

Sample code	Brand	Herb based/Flavor	Carbohydrates (g/100mL)	Sodium (mg/100mL)
MO	A	Yerba mate extract	8.5	0.006
ML	A	Yerba mate extract with lemon	8.5	0.006
LF	A	Yerba mate extract	8.5	0.006
CL	B	Yerba mate extract w/ açai and guarana	9.0	0.000
GM	C	Yerba mate extract w/ lemon	8.7	0.000
QL	D	Black tea extract w/ peach	12.0	0.012
LP	E	Black tea extract w/ lemon	7.5	0.006
LL	E	Black tea extract w/ lemon	7.5	0.006
DA	F	Black tea extract w/ hibiscus and blueberry	4.8	0.000
NE	G	Black tea extract	4.5	0.030
LPT	E	Black tea extract	4.6	0.057
CT	H	Black tea extract	5.0	0.030
LPL	E	Black tea extract w/ lemon	4.6	0.057
CTP	H	Black tea extract	5.0	0.030
LT	E	Black tea extract	4.6	0.057
PL	I	Black tea extract	4.5	0.000

Information acquired from the product label

2.3. Synthesis and characterization of DES

All the DESs assayed were synthesized by mixing the respective components in the appropriate molar ratio and heating the mixtures at 50 ºC with continuous stirring until a homogeneous colorless liquid was obtained. The different DESs obtained were cooled and left under vacuum until use.

Afterward, the selected DES was characterized by infrared spectroscopy, thermogravimetry (TG), also density, and viscosity. The infrared spectra of the synthesized DES and the initial materials were obtained by Fourier-transform infrared spectrometry (FTIR) (Spectrum Two, PerkinElmer, Shelton, CT, USA) operated in attenuated total reflectance (ATR) mode, with a spectral resolution of 4 cm^{-1}, in the range of 4000 to 400 cm^{-1}, and an accumulation of 30 scans. Thermal decomposition analyses of the initial reagents and the synthesized DES were performed by thermogravimetry (TGA) (PerkinElmer®, model TGA 4000) with the heating of approximately 10 mg of the solvent under the dynamic flow of N 2 (20 mL min^{-1}) in the

temperature range of 50 °C to 500 °C, at the rate of 10°C min^{-1}. Exploratory differential scanning calorimetric analyses of the synthesized NADESs were performed using a Differential Scanning Calorimetry (DSC) instrument (PerkinElmer, DSC 8000, Shelton, CT, USA), with heating of approximately 10 mg of the sample in an aluminum holder, under a flow of N 2 (50 mL. min−1), in the temperature range -30°C to 210°C, at a rate of 10°C min^{-1}.

The densities of DESs were determined volumetrically, using a pycnometer calibrated with water and an analytical balance with an accuracy of ± 0.0001 g (Model AG200, Gehaka, São Paulo, Brazil), and the viscosities were performed using a Cannon-Fenske viscometer calibrated with water. The analyses were carried out in triplicate, at a controlled temperature of 24 °C.

2.4. Low-density deep eutectic solvent-based-dispersive liquid-liquid-microextraction (LDDES-DLLME)

In the optimized method, a mixture of 175 μL of DES solvent [CAM:C6 (1:1)] and 100 μL of MeCN was rapidly added to 10 mL of sample previously spiked with 50 μL of deuterated internal standard solution (ISTD, crhysen-D12) at 200 μg L^{-1}. Subsequently, the tube was homogenized in a vortex for 10 s, sonicated for 1 min, and centrifugated at 1690 *g* for 5 min. Then, 35 μL of the upper layer was collected into a vial containing a conical insert and added with 35 μL of MeCN. Exactly 1.2 μL of the extract was injected in a GC-MS/MS system.

2.5. Chromatographic conditions and mass spectrometry (GC-MS/MS)

An Agilent 7890B gas chromatograph, equipped with an autosampler (Agilent 7693A) and an electronically controlled split/splitless injection port, coupled with a triple quadrupole 7000C (Agilent Technologies Inc., Palo Alto, CA, USA) mass spectrometer with an electron ionization (EI) chamber was used to PAHs analyses. GC separation was achieved on an Agilent J&W Select PAH capillary column (30m × 0.25 mm ID × 0.25 μm film thickness, Agilent

J&W, Netherlands), and the oven temperature was programmed initially at 70 ºC for 0.75 min, increased to 180 ºC at 60 ºC.min^{-1}, ramped to 230 ºC at 5 ºC.min^{-1} and held for 7 min, increased to 280 ºC at 22 ºC.min^{-1}, held for 8 min, and finally ramped to 300 ºC at 25 ºC.min^{-1} and held for 8.9 min, with a total run of 39.5 min. Ultra-high purity helium (99.999%; Gasin, Portugal) was used as carrier gas at 2.0 mL.min^{-1}. The injector was maintained at 300 ºC in pulsed spitless mode (0.75 min purge-off) and 1.2 µL of the extract was injected. The triple quadrupole MS was operated in multiple reaction monitoring (MRM) mode detecting two transitions per analyte (**Table 2**), the electron energy was 70 eV and the temperatures of transfer line and ion source were 300 ºC and 250 ºC, respectively. Helium was used as quench gas (2.25 mL.min^{-1}) and nitrogen as collision gas (1.5 mL.min^{-1}). System control and data acquisition were performed in MassHunter software.

Table 2. GC-MS/MS conditions.

Analyte	PAH	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (kV)	Rt (min)	Time window
		152	126	30		
1	**Acenaphthylene**	152	150	35	4.6	1
		152	151	25		
		153	127	30		
2	**Acenaphthene**	153	151	35	4.8	1
		153	152	25		
		165	139	32		
3	**Fluorene**	165	163	40	5.6	2
		165	164	25		
4	**Phenanthrene**	178	174	35	8.0	3
		178	176	20		
		178	152	28		
5	**Anthracene**	178	176	35	8.2	3
		178	177	22		
		202	152	42		
6	**Fluoranthene**	202	200	45	12.4	4
		202	201	27		
		202	151	42		
7	**Pyrene**	202	200	45	13.6	5
		202	201	27		
		228	202	35		
8	**Benzo[a]anthracene**	228	226	40	21.5	6
		228	227	25		
9	**Chrysene-D12**	240	236	38	21.7	6
		240	238	20		
		228	202	35		
10	**Chrysene**	228	226	40	21.8	6
		228	227	25		
		252	224	60		
11	**Benzo[b]fluoranthene**	252	226	35	26.1	7
		252	248	60		
		252	250	45		
		252	224	60		
12	**Benzo[k]fluoranthene**	252	226	35	26.2	7
		252	248	60		
		252	250	45		
		252	224	60		
13	**Benzo[a]pyrene**	252	226	35	28.5	8
		252	248	60		
		252	250	60		
		276	272	60		
14	**Indene[1,2,3-cd]pyrene**	276	273	60	34.6	9
		276	274	50		
		278	250	60		
15	**Dibenzo[a,h]anthracene**	278	276	42	34.6	9
		278	277	25		
		276	272	60		
16	**Benzo[g,h,i]perylene**	276	273	60	36.7	10
		276	274	50		

2.6. In-house validation and quality control

The method was validated for the limit of detection (LOD), the limit of quantification (LOQ), linearity, precision intra- and inter-days, absolute recovery, and matrix effects. LOD was defined as the lowest concentration in a spiked blank sample that gave a signal/noise of 3, while LOQ was set as the lowest concentration in the sample that could be quantified with precision (RDS <20%) and recovery between 60 and 120%.

Seven points matrix-matched calibration curves were constructed from 0.20 to 2.00 µg L^{-1}. The linearity was assessed through the coefficient of determination (R^2) of linear regression (external standard area/internal standard *versus* external standard concentration). Additionally, the calibration curves were submitted to ANOVA, lack off fit and residual analyses to evaluate the linearity of the mathematical models [31].

Inter and intra-days precision assays were carried out in spiked blank sample, at three concentration levels (0.20, 1.05, and 2.00 µg L^{-1}) using six replicates, on the same day and during three days, respectively. Both intra- and inter-day precisions were expressed as relative standard deviation (%RSD). A satisfactory %RSD was set when it was lower than %RSD calculated by the Horwitz equation ($RSD = 2^{(1-0.5*\log C)}$). Recovery (%) assays were also performed in three concentration levels (0.20, 1.05, and 2.00 µg L^{-1}) using six replicates.

Matrix effects were investigated by comparing the slope of the matrix-matched calibration curve with the slope of solvent calibration curves in the same concentration range and it was expressed in percentage (%ME = [{Matrix Slope/Solvent Slope}*100]-100) [32].

2.7. Statistical analyses

The Central Composite Design (CCD) was applied to evaluate the main factors affecting the LDDES-DLLME process. The independent variables were the volume of extraction solvent

(DES, µL), the volume of dispersive solvent (MeCN, µL), and time of ultrasound (US, min), studied in five levels (**Table 3**). The employed design consisted of 17 experiments, including three replicates in the central point. The variable-responses (Y) were the areas obtained for each PAH after GC-MS/MS analysis. For every variable-response, it was generated a statistical model that was submitted to analysis of variance (ANOVA), regression analysis, and the lack of fit test ($p<0.05$). The statistical analyses were performed on software Statistica 10.0 (Statsoft).

Table 3. Matrix for Central Composite Design experiment (CCD).

Run	DES (µL)	MeCN (µL)	US (min)
1	-1.00 (205)	-1.00 (180)	-1.00 (4)
2	-1.00 (205)	-1.00 (180)	1.00 (12)
3	-1.00 (205)	1.00 (420)	-1.00 (4)
4	-1.00 (205)	1.00 (420)	1.00 (12)
5	1.00 (295)	-1.00 (180)	-1.00 (4)
6	1.00 (295)	-1.00 (180)	1.00 (12)
7	1.00 (295)	1.00 (420)	-1.00 (4)
8	1.00 (295)	1.00 (420)	1.00 (12)
9	-1.68 (175)	0.00 (300)	0.00 (8)
10	1.68 (325)	0.00 (300)	0.00 (8)
11	0.00 (250)	-1.68 (100)	0.00 (8)
12	0.00 (250)	1.68 (500)	0.00 (8)
13	0.00 (250)	0.00 (300)	-1.68 (1)
14	0.00 (250)	0.00 (300)	1.68 (15)
15	0.00 (250)	0.00 (300)	0.00 (8)
16	0.00 (250)	0.00 (300)	0.00 (8)
17	0.00 (250)	0.00 (300)	0.00 (8)

Additionally, calibration curves for each PAH were submitted to linear regression significance by the least-squares method (LSM), analysis of variance (ANOVA), and lack of fit tests ($p = 0.05$). The results showed the calibration curves were significant ($p<0.05$) and no linearity deviation was observed. The statistical tests were performed on software Statistica 10.0 (Statsoft).

3. Results and discussion

3.1. Selection of DES

Initially, different DES composed of short chain saturated fatty acids (C6, C7, C8, and C9), camphor (CAM), and thymol (Th), namely Th-CAM (1:1), Th-CAM-C9 (1:1:1), CAM-C9 (1:1), CAM-C8 (1:1), CAM-C7 (1:1), CAM-C6 (1:1), and CAM-C6-C7 (1:1:1) were tested as extraction solvent in DLLME. Briefly, a mixture of 200 μL of DES and 500 uL of MeCN was rapidly injected in 10 mL spiked sample (150 μg L^{-1}). Then, after vortexed for 10 s and sonicated for 5 min the mixture was centrifugated at 1690 *g* for 5 min and 35 μL of the upper layer was collected and added in a vial with a conic insert containing 35 μL of MeCN. The criteria for selection of DES were based on peak intensity, peak height, peak shape, and numbers of PAHs extracted (see supplementary material).

The choice of DES components was based on the hydrogen bond donor (thymol and fatty acids) and acceptor (thymol, camphor, and fatty acids) features to achieve a hydrophobic DES able to extract of nonpolar analytes [33,34] including PAHs [18]. These previous developed DES-based protocols show high sensitivity (low LOD and LOQ) and selectivity, but they demand additional steps such as *salting out* and pH adjustment [18] or addition of the individual DES precursors over the sample [34] increasing the analysis timing, so the main challenge was to develop a simple and practical procedure for daily laboratory routine without losing sensitivity and selectivity. Another important point is the application of hydrophobic DESs for trace analysis in food samples, which are known as complex matrices. From the qualitative analysis of the chromatograms in MRM mode, it was observed that thymol-based DES were not able to extract most analytes such as naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, acenaphthylene, acenaphthene, and fluorene. Additionally, several compounds presented chromatographic peaks with tailing, fronting, or low chromatographic resolution (coelution), particularly the most nonpolar, in the final portion of the chromatogram. Among camphor DES-based tested, CAM:C8 showed the highest intensity of the analytical signal for extracted PAHs

despite not be able to extract some polar PAHs namely naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, acenaphthylene, and acenaphthene.

Figure 1 shows MRM chromatograms of blank spiked samples in the same conditions obtained from different extraction solvent composition: CAM-C8 (1:1) (A), CAM-C7 (1:1) (B), CAM-C6 (1:1) (C) and CAM-C6-C7 (1:1:1) (D). It was noticed that mixture C provided the highest analytical signal for all analytes, especially for acenaphthylene and acenaphthene that were not extracted when C8 was used. Additionally, benzo(b)fluoranthene and benzo[k]fluoranthene exhibited better resolution when compared to the other assays. Naphthalene, 1-methylnaphthalene, and 2-methylnaphthalene were not extracted with any tested DES in this study, thus, these compounds were excluded from the study. Given these results, the CAM-C6 (1:1) DES was chosen as the extraction solvent.

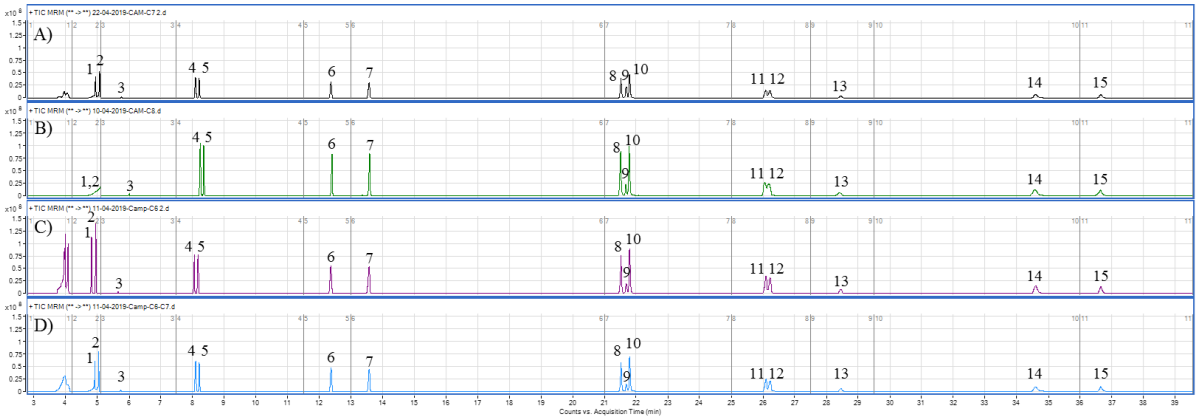

Figure 1. Total Ion Chromatogram (TIC) of different DES composition. A) CAM-C7 (1:1), B) CAM-C8 (1:1), C) CAM-C6 (1:1) and D) CAM-C6-C7 (1:1:1). 1 – Acenaphthylene, 2 – Acenaphthene, 3 – Fluorene, 4 – Phenanthrene, 5 – Anthracene, 6 – Fluoranthene, 7 – Pyrene, 8 – Benzo[a]anthracene, 9 – Chrysene-d12 e Chrysene, 10 – Benzo[b]fluoranthene, 11 – Benzo[k]fluoranthene, 12 – Benzo[a]pyrene, 13 – Indene[1,2,3-cd]pyrene, 14 – Dibenzo[a,h]anthracene, 15 – Benzo[g,h,i]perylene.

3.2. DES characterization

The formation of an eutectic solvent is characterized by intermolecular interactions as Van der Waals and electrostatic forces, and mainly hydrogen bonding [35]. In order to identify the functional groups of the synthesized DES, the FITR spectra of precursors and obtained DES were performed (**Figure 2).** In camphor (CAM) spectra, it was observed an absorption band at 1735 cm^{-1}, corresponding to stretching of carbonyl (C=O), and peaks at 2879 cm^{-1} and 2950 cm^{-1}, characteristic to symmetric and asymmetric CH_2 groups, respectively [36]. The hexanoic acid (C6) spectra presented an absorption band between 2500 and 3600 cm^{-1}, corresponding to O-H bonds and intense absorption at 1701 cm^{-1} due to C=O stretching and vibration [37]. When compared to the DES solvent with the precursors (CAM and C6), the C=O band was shifted from approximately 1734 cm^{-1}, which may indicate the formation of hydrogen bonding interaction. The increase observed in DES of an absorption band corresponding to C=O suggested a higher electron density of carbonyl oxygen, characteristic of the formation of hydrogen bonds [38]. The FTIR results confirm the formation of DES as shown in the spectra for the functional groups of the components.

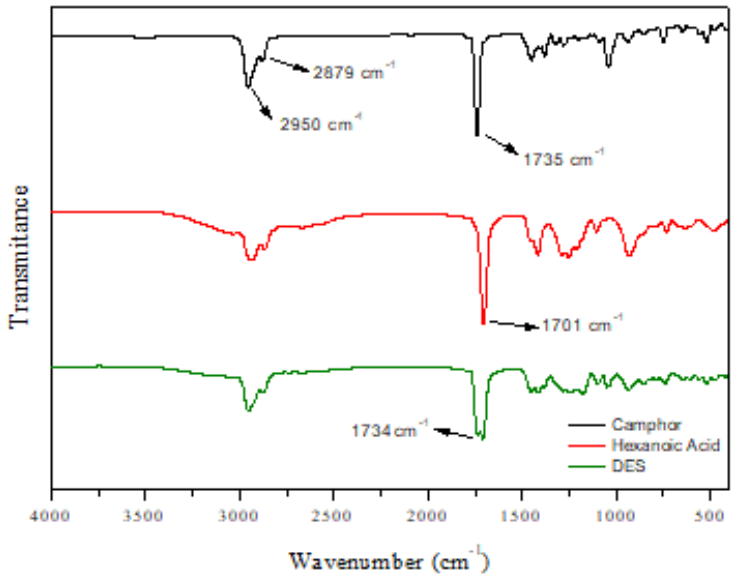

Figure 2. Infrared spectra for DES and its precursors (camphor and hexanoic acid).

The thermal stability is an important parameter to evaluate in a new solvent, providing information about the temperature at which the solvent can be used without alteration [39]. The thermal properties of the chosen DES were assessed by thermogravimetry (TG) and its derivative differential thermal analysis (DTA) to describe the decomposition temperature (Td) and weight loss for the solvent **(Figure 3)**. The thermal decomposition occurred near 100 ºC, and the mass loss event, with approximately 99 % was observed at temperatures around 190 ºC. Thus, the maximum working temperature recommended for this DES is up to 100 ºC.

Information about density and viscosity are dependents on the physicochemical properties, chemical nature, and intermolecular interaction of their components and DES [40]. DES density and viscosity were, respectively, 1.1077 ± 0.0001 $g.cm^{-3}$ (average ± standard deviation, n = 3) e 4.38 ± 0.02 (n = 3). These properties can be altered by the addition of different volumes of solvent, and the physicochemical properties adjusted for desirable application [41].

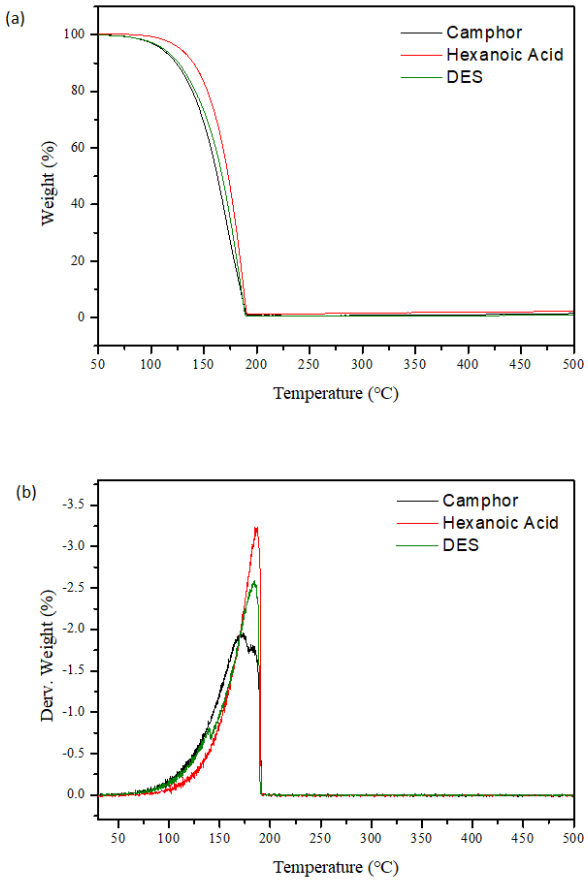

Figure 3. Thermogravimetric curves (TG) (a) and derivative thermogravimetric curves (DTA) (b) of DES and its precursors (camphor and hexanoic acid).

3.3. Optimization of the LDDES-DLLME

A Central Composite Design (CCD) was chosen to optimize the volume of the extraction solvent (DES, µL), the volume of dispersive solvent (MeCN, µL), and ultrasound time (US, min). For this purpose, 17 experiments were performed being the GC-MS/MS peak areas for each analyte used as the response variables (Y) (**Table 3**). The significances of empirical models were assessed by Analysis of Variance (ANOVA), lack of fit, residual analysis, and correlation coefficient (r) so it was not found linearity deviations, lack of fit, or any hindrance to use the models. The results (**Table 4**) showed that only the DES volume variable was significant ($p<0.05$) and it had a linear fitting and negative effect for all PAHs, which means

the lower DES volume, the greater the area of the peaks. This result was expected since the lower volume of extraction solvent in DLLME, the greater is the sample concentration rate and therefore higher is the analytical response, up to the solvent saturation limit. The lowest volume of DES used in this technique was 175 µL since a lower volume resulted in an insufficient up layer recovery. The volume of dispersive solvent and ultrasound extraction time was not statistically significant, so they were fixed in the lowest values, 100 µL, and 1 min, respectively, to simplify and reduce the use of organic solvent on the extraction procedure. Finally, five assays were conducted in the optimized conditions (175 µL of DES, 100 µL of MeCN, and 1 min of US) to validate the models and the averages were used to calculate the prediction errors that were between 1.40 and -29.0% (**Table 4**).

Table 4. CCD results for selected PAHs.

Analyte	Intercept	Coefficient DES (L)	R^2	Regression Significance (p<0.05)	Model Adjust (p>0.05)	Predicted	Validation (n = 5)	Prediction Error (%)
Acenaphthylene	31026933	-8407255	0.8251	<0.0001	0.4856	45217539	49422079	-8.5
Acenaphthene	47308040	-12673309	0.8278	<0.0001	0.4813	68699318	67771173	1.4
Fluorene	1292741	-353364	0.8180	<0.0001	0.4607	1889184	1847703	2.2
Anthracene	41747643	-11101773	0.8424	<0.0001	0.5050	60486326	62218705	-2.8
Phenanthrene	40135623	-10559507	0.8480	<0.0001	0.5072	57959015	60659807	-4.5
Fluoranthene	64241246	-17106376	0.8510	<0.0001	0.4836	93115099	106841396	-12.8
Pyrene	75217316	-19776862	0.8391	<0.0001	0.4668	108598682	125391501	-13.4
Chrysene	95037840	-25426381	0.8820	<0.0001	0.5253	137955028	183021471	-24.6
Benzo[a]anthracene	105814143	-27610461	0.8688	<0.0001	0.3445	152417840	192139723	-20.7
Benzo[k]fluoranthene	48970466	-15803074	0.9000	<0.0001	0.6964	75644474	89337898	-15.3
Benzo(b)fluoranthene	80145667	-17447265	0.8009	<0.0001	0.2628	109594906	133282027	-17.8
Benzo[a]pyrene	16951183	-4781498	0.8785	<0.0001	0.4394	25021873	24187554	3.4
Indene(1,2,3,cd)pyrene	22970843	-6840217	0.8945	<0.0001	0.9244	34516446	41193140	-16.2
Dibenzo[a,h]anthracene	19361205	-6470551	0.8881	<0.0001	0.6363	30282847	42681729	-29.0
Benzo[g,h,i]perylene	20124815	-6544743	0.8382	<0.0001	0.5775	31119984	40386944	-22.9

DES (L) = linear coefficient for volume of deep eutectic solvent (µL) used in CCR design.

3.4. In-house method validation quality control

Matrix effects (**Figure 4)** for eight compounds (acenaphthylene, acenaphthene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, and benzo[a]anthracene) can be ignored because there were lower than 20%; on the other hand, an increasing signal was observed to the other analytes in the range between 26.6% (chrysene) and 743.4% (dibenzo[a,h]anthracene). These results highlight the significance of the assessment of the matrix effects and to show the relevance of the method validation for a specific matrix. Enrichment factor (EF) was calculated for method through the ratio of analyte concentration in DES phase after extraction procedure (C_{DES}) and the initial concentration in sample phase (C_0) ($EF = C_{DES}/C_0$) [25]. Enrichment values were from 50.6 (Indene(1,2,3-cd)pyrene) to 64.0 (benzo(a)anthracene).

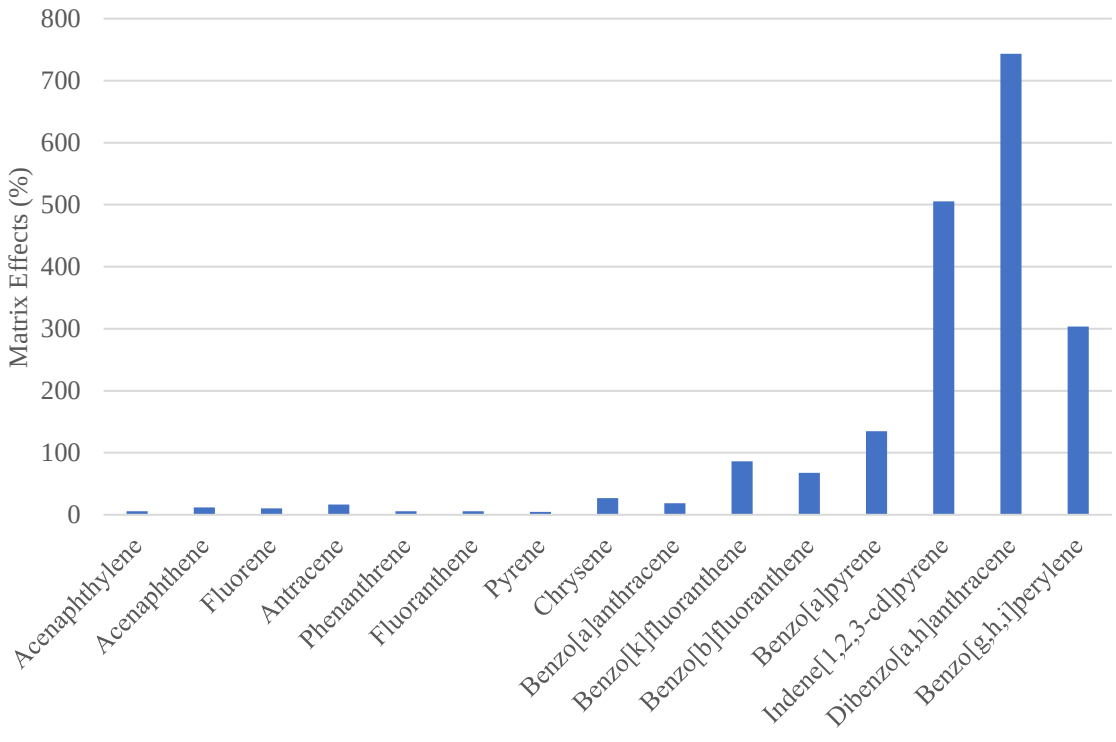

Figure 4. Matrix effects for DLLME-GC-MS/MS method for every PAH.

The limits of detection (LOD) and quantification (LOQ) were 0.01 and 0.20 µg L^{-1}, respectively, for all PAHs under study (**Table 5**) and linearity, estimated by the coefficient of determination (R^2) of calibration curves, were greater than 0.989 (anthracene). The values for intra-day and inter-day precisions were lower than 16.8% (dibenzo[a,h]anthracene) and 15.1% (Indene[1,2,3-cd]pyrene), respectively. The results for recovery assays were between 69% (Dibenzo(ah)anthracene) and 125.1% benzo[a]anthracene; validation guidelines indicate values between 60% and 120% for the range of concentration of this work and 93% of the results were among this range. So, these results suggest the method is fitted for analytical purposes [42].

Table 5. Validation's criteria for DLLME-GC-MS/MS method.

Analyte	LOD	LOQ	Linear Range	Linearity	Regression Model	Precision intra-day (%)			Precision inter-day (%)			Recovery (%)		
	µg L^{-1}	µg L^{-1}	µg L^{-1}	R^2		1	2	3	1	2	3	1	2	3
Acenaphthylene	0.01	0.20	0.20 - 2.00	0.991	y = 5.715x + 0.296	4.13	1.47	3.95	4.45	4.88	13.18	100.83	100.95	92.95
Acenaphthene	0.01	0.20	0.20 - 2.00	0.992	y = 7.790x + 0.305	4.49	1.08	4.58	4.60	4.40	11.92	102.52	102.53	89.61
Fluorene	0.01	0.20	0.20 - 2.00	0.991	y = 0.175x + 0.015	4.99	1.19	5.52	5.22	5.08	14.59	106.52	106.08	107.61
Anthracene	0.01	0.20	0.20 - 2.00	0.989	y = 3.234x + 0.377	5.10	1.07	6.10	5.17	4.51	10.99	109.33	106.50	111.71
Phenanthrene	0.01	0.20	0.20 - 2.00	0.993	y = 0.128x + 0.001	5.98	1.94	4.93	5.90	4.75	8.10	108.02	100.96	116.44
Fluoranthene	0.01	0.20	0.20 - 2.00	0.991	y = 0.876x + 0.088	6.13	1.57	6.00	5.63	4.72	6.44	109.86	102.85	112.73
Pyrene	0.01	0.20	0.20 - 2.00	0.991	y = 0.746x + 0.022	6.69	2.11	5.85	5.72	4.54	6.64	110.87	104.40	111.66
Chrysene	0.01	0.20	0.20 - 2.00	0.992	y = 11.307x - 0.872	12.15	9.55	4.33	10.03	6.69	4.44	103.99	88.33	123.23
Benzo(a)anthracene	0.01	0.20	0.20 - 2.00	0.987	y = 11.926x - 0.738	12.30	10.13	4.52	10.24	7.34	4.41	111.27	89.37	125.11
Bezo(k)fluoranthene	0.01	0.20	0.20 - 2.00	0.995	y = 5.794x - 0.278	15.15	12.76	6.63	12.26	9.24	8.08	112.83	87.15	114.39
Bezo(b)fluoranthene	0.01	0.20	0.20 - 2.00	0.991	y = 7.465x - 1.041	14.06	12.30	4.15	12.06	8.12	3.69	94.77	76.69	113.46
Bezo(a)pyrene	0.01	0.20	0.20 - 2.00	0.993	y = 1.772x - 0.188	14.39	13.30	6.55	12.37	9.19	6.75	93.44	73.75	118.38
Indene(1,2,3-cd)pyrene	0.01	0.20	0.20 - 2.00	0.994	y = 2.707x - 0.336	15.99	14.26	6.45	15.15	11.90	7.38	91.10	78.12	106.44
Dibenzo(ah)anthracene	0.01	0.20	0.20 - 2.00	0.993	y = 2.952x - 0.419	15.83	16.87	3.39	15.14	11.30	8.56	85.62	69.03	115.44
Benzo(ghi)perylene	0.01	0.20	0.20 - 2.00	0.993	y = 0.709x - 0.085	16.10	13.06	4.69	14.25	10.17	4.62	83.24	74.43	113.44

LOD = limit od detection; LOQ = limit of quantification; Precisions and Recovery levels: 1 = 0.20 µg L^{-1}, 2 = 1.05 µg L^{-1} e 3 = 2.00 µg L^{-1}.

To date, few studies have determined PAHs in ready-to-drink herb-based beverages. Usually, the works in this area are focused on the analysis of herbs and/or homemade infusions. Some of these studies are presented in **Table 6.** All selected studies have applied organic solvents during the extraction procedure, such as n-hexane, acetonitrile, ethyl acetate, acetone, cyclohexane, 1-octanol, and methylene chloride, and traditional techniques such as liquid-liquid extraction (LLE) and QuEChERS with clean-up by solid-phase extraction (SPE) followed by concentration under nitrogen flow [15,17,43]. Only one work applied a miniaturized technique (DLLME) [44]. By comparing our LODs and LOQs to those obtained in previous studies, it is possible to verify similar results. Therefore, the method developed could be suitable as an alternative for the determination of PAHs. Besides, solvent consumption is reduced by 99 % in comparison with classical solvent extraction [15,17,43]. Moreover, the use of a green solvent at a low cost comprises a pronounced advance in comparison to common approaches.

Table 6. Comparison with other methods.

Conventional solvent-based methods								
Sample (Volume)	**Extraction solvent (Volume)**	**Extraction**	**Observation**	**Separation and detection**	**LOD (µg L^{-1})**	**LOQ (µg L^{-1})**	**PAHs (n)**	**Reference**
Tea infusion (100 mL)	HEX (100 mL)	LLE	Concentration under nitrogen flow. Injection of 1 µL.	GC-MS/MS	0.023-0.097	0.0743 – 0.323	15	(Gao et al., 2017)
Herbal and fruit infusion (500 mL)	LLE: CHEX (100 mL) SPE: EtAc + CHEX (4 mL)	LLE-SPE	Concentration after and before SPE procedure. Injection of 50 µL.	GC-MS	-	0.5 – 1.0 µg.Kg^{-1}	16	(Schulz et al., 2014)
Tea infusion (10 mL)	QuEChERS: MeCN + AC (10 mL) SPE 1: MeCN + AC (5 mL) SPE 2: HEX + MtCl (2 mL)	QuEChERS -SPE-SPE	Concentration after and before SPE procedure.	GC-MS	0.1	0.2 – 0.4	4	(Pincemaille et al., 2014)
Herbal infusion (3 mL)	OCT (50 µL)	DLLME	Dilution (2x) before injection. Injection of 10 µL.	HPLC-FLD	0.001 – 0.3	0.005 – 0.4	3	(Loh et al., 2016)

LOD – Limit od detection; LOQ – Limit of quantification; PAHs – Polycyclic aromatic hydrocarbons; HEX – n-hexane; CHEX – Cyclohexane; EtAc – Ethyl acetate; MeCN – Acetonitrile. AC – Acetone; MtCl – Methylene chloride; - OCT – 1-octanol; LLE – liquid-liquid extraction; LLE-SPE – Liquid-liquid extraction-Solid phase extraction; QuEChERS-SPE-SPE – 'Quick, easy, cheap, effective, rugged and safe' followed by double Solid-phase extraction; DLLME – Dispersive liquid-liquid microextraction; GC-MS/MS - Gas chromatography-tandem mass spectrometry; GC-MS - Gas chromatography-mass spectrometry; HPLC-FLD – High performance liquid chromatography-fluorescence detector.

3.5. Application to real samples

The application of the developed method to real samples showed the presence of PAHs in all analyzed samples (**Table 7**). However, only in 4 samples (25%) the levels found were above LOQ (0.20 µg L^{-1}), with levels ranging from 0.20 µg L^{-1} to 0.30 µg L^{-1}. The total of PAHs in each sample was calculated summing the average values for every analyte, considering zero when <LOD and 0.10 when LOD<value<LOQ. Total PAH levels ranged from 0.20 µg L^{-1} (PL, NE, CT, and LPL) to 1.82 µg L^{-1} (MO). Among the samples studied, yerba mate-based showed a higher average level of PAHs (1.11 µg L^{-1}) than black tea-based (0.89 µg L^{-1}). This difference can be related to the toasting process that yerba mate is submitted before the infusion preparation [12].

Table 7. Results for real samples, in µg L^{-1}.

Analyte	Sample							
	MO	**QL**	**ML**	**LP**	**CL**	**GM**	**LL**	**LF**
Acenaphthylene	-	-	-	-	-	-	-	-
Acenaphthene	-	-	-	-	-	-	-	-
Fluorene	-	-	-	-	-	-	-	-
Anthracene	-	-	-	-	-	-	-	-
Phenanthrene	-	-	-	-	-	-	-	-
Fluoranthene	<LOQ	<LOQ	-	<LOQ	-	<LOQ	<LOQ	-
Pyrene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	-
Chrysene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Benzo[a]anthracene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Benzo[k]fluoranthene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Benzo(b)fluoranthene	0.26 ± 0.06	0.25 ± 0.04	0.20 ± 0.02	0.24 ± 0.03	<LOQ	<LOQ	<LOQ	<LOQ
Benzo[a]pyrene	0.23 ± 0.07	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Indene[1,2,3-cd]pyrene	0.27 ± 0.05	0.25 ± 0.04	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[a,h]anthracene	0.30 ± 0.04	0.27 ± 0.04	0.22 ± 0.03	0.22 ± 0.03	<LOQ	<LOQ	<LOQ	<LOQ
Benzo[g,h,i]perylene	0.26 ± 0.05	0.26 ± 0.05	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	-
Σ 4PAHs	0.69	0.55	0.50	0.54	0.40	0.40	0.40	0.40
Σ 15PAHs	1.82	1.63	1.12	1.26	0.90	1.00	1.00	0.70

Table 7. Results for real samples, in µg L^{-1} (Cont)

Analyte	Sample							
	DA	**PL**	**LT**	**CTP**	**NE**	**LTP**	**CT**	**LPL**
Acenaphthylene	-	-	-	-	-	-	-	-
Acenaphthene	-	-	-	-	-	-	-	-
Fluorene	-	-	-	-	-	-	-	-
Anthracene	-	-	-	-	-	-	-	-
Phenanthrene	-	-	-	-	-	-	-	-
Fluoranthene	<LOQ	-	-	-	-	-	-	-
Pyrene	-	-	<LOQ	<LOQ	-	<LOQ	-	-
Chrysene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Benzo[a]anthracene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Benzo[k]fluoranthene	<LOQ	-	-	-	-	-	-	-
Benzo(b)fluoranthene	<LOQ	-	-	-	-	-	-	-
Benzo[a]pyrene	<LOQ	-	-	-	-	-	-	-
Indene[1,2,3-cd]pyrene	<LOQ	-	-	-	-	-	-	-
Dibenzo[a,h]anthracene	<LOQ	-	-	-	-	-	-	-
Benzo[g,h,i]perylene	<LOQ	-	-	-	-	-	-	-
Σ 4PAHs	0.40	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Σ 15PAHs	0.90	0.20	0.30	0.30	0.20	0.30	0.20	0.20

Σ 4PAHs is the sum of benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene. For Σ 4PAHs and Σ 15PAHs, when <LOQ was considered the LOD and <LOD (-) was considered zero. Results were not corrected by recovery.

In general, the results herein are in accordance with Gao et al., [17] with a sum of 15 PAHs in green tea infusions ranging from 0.53 µg L^{-1} to 1.21 µg L^{-1} (acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[ghi]perylene, indeno[1,2,3-cd] pyrene, and dibenzo[a,h]anthracene) and with Pincemaille et al., [43] that reported levels ranging from <0.4 µg L^{-1} to 2.7 µg L^{-1} (benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[a]pyrene) in infusions of smoked and non-smoked black teas. Lower levels, however, are reported by Shulz et al., [15] with levels of PAHs < 0.005 µg L^{-1} for most of the sample of herbal infusions, including yerba mate and black tea.

Currently, no maximum tolerated level (MTL) for PAHs in ready-to-drink beverages have been defined. Although, the results obtained are following those recommended by the European Community for other foods that establishes values for benzo[a]pyrene (from 1.0 µg L^{-1} to 6.0 µg L^{-1}) and for the sum of benzo[a]pyrene, benzo[a]anthracene, benzo[b]fluoranthene, and chrysene (4PAHs) (from 1.0 µg L^{-1} to 35.0 µg L^{-1}) [6]. The results for benzo[a]pyrene in samples were from <LOD (PL, LT, CTP, NE, LTP, CT, and LPL) to 0.23 µg L^{-1} (MO) and for 4PAHs were from 0.20 µg L^{-1} (PL, LT, CTP, NE, LTP, CT, and LPL) to 0.69 µg L^{-1} (MO).

4. Conclusions

For the very first time, a novel hydrophobic deep eutectic solvent was synthesized using camphor and hexanoic acid and applied in the DLLME technique to extract 15 polycyclic aromatic hydrocarbons (PAHs) from ready-to-drink beverages. The LDDES-DLLME-GC-MS/MS method developed in this study provides several advantages over traditional extraction techniques such as simplicity of extraction procedure, high-speed sample preparation, use of natural and low-cost reagents, and low amount of sample (10 mL) and solvents (totally 310 µL for each sample are required). Such advantages can characterize the technique as “green”,

without losing analytical performance. In fact, the method complies with the expectations for modern analytical methods such as low limits of detection and quantification, and appropriate precision and recovery. The method was successfully applied to real samples and the presence of PAHs in ready-to-drink yerba-mate and black tea-based beverages seems not to be a toxicological issue of concern.

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7. Supplementary data

A novel dispersive liquid-liquid microextraction using a low density deep eutectic solvent-gas chromatography tandem mass spectrometry for the determination of polycyclic aromatic hydrocarbons in soft drinks.

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Table S1. Results for qualitative analysis in DES selection.

Analyte/DES	Thymol-Camphor (1:1)	Thymol-Camphor-C9 (1:1:1)	Camphor-C9 (1:1)
Naphthalene	×	×	×
2-Methylnaphthalene	×	×	×
1-Methylnaphthalene	×	×	×
Acenaphthylene	✓	×	×
Acenaphthene	✓	×	×
Fluorene	✓	×	×
Phenanthrene	✓	✓	✓
Anthracene	✓	✓	✓
Fluoranthene	✓	✓	✓
Pyrene	✓	✓	✓
Benzo[a]anthracene	✓	✓	✓
Chrysene	✓	✓	✓
Benzo(b)fluoranthene	✓	✓	✓
Benzo[k]fluoranthene	✓	✓	✓
Benzo[a]pyrene	✓	✓	✓
Indene[1,2,3-cd]pyrene	✓	✓	✓
Dibenzo[a,h]anthracene	✓	✓	✓
Benzo[g,h,i]perylene	✓	✓	✓

× = Missing analyte. ✓ = Peak coelution, tailing, fronting, asymmetry or low intensity. ✓ = Acceptable peak

DISCUSSÃO GERAL

Nesse trabalho, ervas aromáticas, infusão de ervas e bebidas prontas-para-consumo à base de ervas foram submetidos à determinação de 21 contaminantes inorgânicos, 14 micotoxinas, 25 pesticidas, 6 ftalatos, um adipato e 15 hidrocarbonetos policíclicos aromáticos (PAHs). Para tal, técnicas de preparo de amostra como digestão em micro-ondas, QuEChERS (quick, cheap, effective, rugged and safe) e DLLME (dispersive liquid-liquid microextraction) foram empregadas (**Figura 1**) para extração dos analitos; a separação e analise ocorreu em sistemas de cromatografia líquida de alta eficiência (HPLC), cromatografia a gás (GC) ou plasma indutivamente acoplado (ICP), todos em conjunto com detectores de espectrometria de massas (MS) e espectrometria de massas em tandem (MS/MS) de baixa resolução (quadrupolo e triplo quadrupolo). Os métodos aplicados demonstraram ser uma excelente ferramenta para análise desses contaminantes, portanto, eles foram validados de acordo com guias de validação tais quais: "*Harmonized guidelines for single-laboratory validation of methods of analysis*" (Thompson, Ellison, & Wood, 2002), "*Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics*" (Magnusson & Örnemark, 2014), e "*Analytical quality control and method validation procedures for pesticide residues analysis in food and feed*" (European Commission, 2019).

Inicialmente, amostras de 20 ervas comercializadas nas cidades de Campinas (São Paulo) e Londrina (Paraná) foram submetidas à digestão ácida em micro-ondas e à determinação de elementos potencialmente tóxicos em um ICP-MS. Observou-se que o elemento mais abundante nas amostras foi o alumínio, o que é explicado pela alta concentração desse elemento no solo (Vitorello, Capaldi, & Stefanuto, 2005) que pode ser absorvido pelos vegetais. Quanto aos elementos tóxicos legislados (As, Cd e Pb), as amostras apresentaram valores em concordância com os limites máximos preconizados pela Farmacopeia Brasileira (Brasil, 2019).

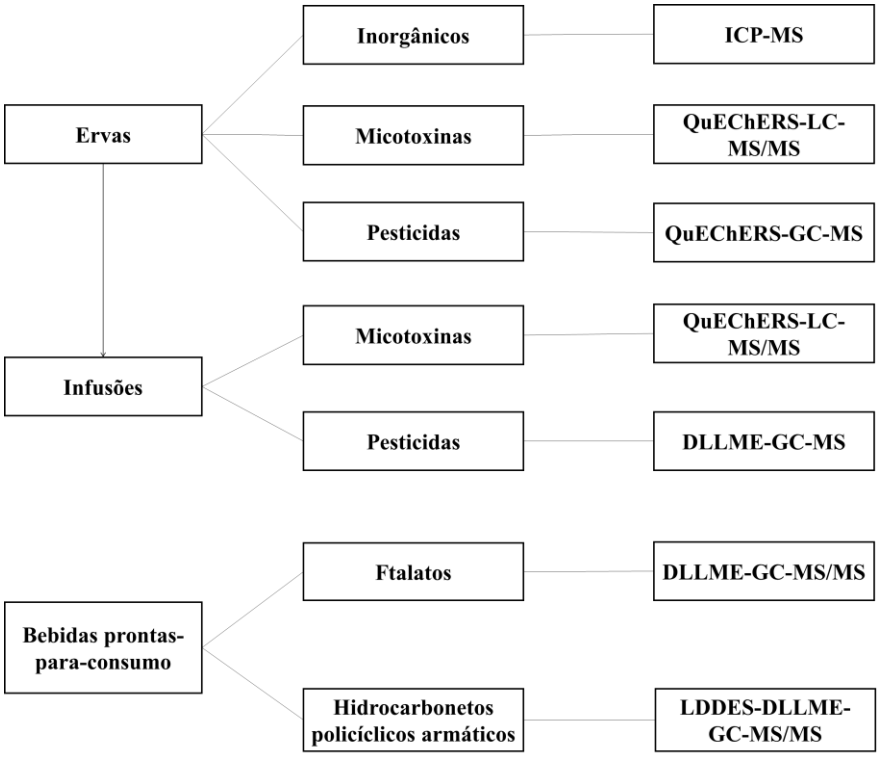

Figura 1. Determinações realizadas no trabalho.

Posteriormente, tanto as ervas quanto as infusões dessas ervas foram avaliadas quanto à presença e concentração de contaminantes orgânicos, nomeadamente, micotoxinas e pesticidas. Para tanto, um método QuEChERS-GC-MS foi otimizado para os pesticidas analisados nas ervas, um DLLME-GS-MS foi desenvolvido e otimizado para determinação de pesticidas nas infusões e um método QuEChERS-LC-MS/MS empregado para determinação de micotoxinas nas ervas e infusões de ervas. Os resultados apontaram que, dentre as 52 amostras de ervas, 72% estavam contaminadas com ao menos uma micotoxina, com destaque para beauvericina (BEA), esterigmatocistina (STE) e HT-2. Já para as infusões, 33% das amostras jaziam contaminadas, ou seja, houve evidência de transferência das micotoxinas presentes nas ervas para as respectivas bebidas. A HT-2 foi a micotoxina com maior ocorrência nas infusões de ervas e, destaca-se migração das aflatoxinas (AFs), devido ao seu potencial carcinogênico, e das micotoxinas emergentes, nomeadamente STE, BEA e eniantina A1 (EN-A1). Um estudo

de exposição foi estimado para o consumo de infusões e demonstrou um quociente de perigo (HQ) elevado para HT-2 (HQ<1) e uma margem de exposição (MoE) abaixo de 10.000 para AFs, ocratoxina A (OTA) e STE, revelando, portanto, que a ingestão de infusões de ervas contaminadas com essas micotoxinas podem figurar um risco à saúde dos consumidores.

Para os pesticidas, 15 dos 25 analitos foram encontrados nas amostras e 62% delas estavam com limite máximo de resíduo (LMR) acima do permitido pela legislação (Brasil, 2019), sendo a cipermetrina (CYP) o pesticida encontrado com a maior concentração (2 mg kg^{-1}). Ademais, não foi observada a transferência desses compostos para as infusões de ervas nos níveis encontrados nas amostras. Entretanto, quando uma amostra isenta foi fortificada com 7 mg kg^{-1} de pesticidas, os analitos foram detectados, mas não quantificados, sugerindo, portanto, que a transferência de pesticidas ocorre em taxas menores que >25% (LOQ do método).

Por fim, amostras de bebidas prontas-para-consumo à base de ervas (erva mate e chá preto) adquiridas nas cidades de Campinas (SP, Brasil) e Porto (Portugal) foram testadas quanto à presença de ftalatos, um adipato e PAHs. Para esses últimos, todas as amostras apresentaram ao menos um analito detectável, já para os ftalatos, apenas o ftalato de dietila (DEP) foi quantificado em somente duas amostras. Portanto, conclui-se que a concentração de PAHs e ftalatos próxima ao limite dos métodos não caracterizam um risco aos consumidores, haja vista que ambos os métodos (DLLME-GC-MS/MS e LDDES-GC-MS/MS) atingiram limites razoavelmente baixos.

Os resultados desse trabalho alertam para o monitoramento de contaminantes em ervas aromáticas e seus produtos, especialmente no que diz respeito ao conteúdo de pesticidas e micotoxinas.

CONCLUSÃO GERAL

Os métodos propostos foram satisfatoriamente otimizados e validados nesse trabalho. Os critérios de validação evidenciaram excelente performance analítica na determinação dos diversos grupos de contaminantes, nomeadamente: elementos tóxicos (metais pesados), micotoxinas, pesticidas, ftalatos e hidrocarbonetos policíclicos aromáticos. De modo geral, os métodos podem ser considerados rápidos, factíveis e efetivos.

Quanto à concentração de contaminantes nas amostras, destacam-se as micotoxinas e os pesticidas os quais apresentaram valores residuais acima do estipulado pela legislação. Ademais, os resultados figuram um risco potencial à saúde dos consumidores, como estimado nos estudos de exposição. Contudo, faz-se importante ressaltar que mais amostras devem ser analisadas a fim de se obter resultados estatisticamente representativos. Os resultados aqui apresentados são exploratórios e visam estimular o monitoramento e controle de contaminantes em ervas aromáticas e produtos à base de ervas.

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ANEXOS

ANEXO I – Certificado de Cadastro de acesso ao patrimônio genético ou conhecimento tradicional associado.

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO
SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Certidão

Cadastro nº A24DF85

Declaramos, nos termos do art. 41 do Decreto nº 8.772/2016, que o cadastro de acesso ao patrimônio genético ou conhecimento tradicional associado, abaixo identificado e resumido, no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado foi submetido ao procedimento administrativo de verificação e não foi objeto de requerimentos admitidos de verificação de indícios de irregularidades ou, caso tenha sido, o requerimento de verificação não foi acatado pelo CGen.

Número do cadastro:	**A24DF85**
Usuário:	**UNICAMP**
CPF/CNPJ:	**46.068.425/0001-33**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie
Cynara scolymus L.
Rosmarinus officinalis L.
Illicium verum Hook F.
Vernonia polyanthes Less
Peumus boldus Molina Calendula officinalis L.
Matricaria recutita L.
Cymbopogon citratus (DC.) Stapf
Cinnamomum verum J. Presl
Baccharis trimera (Less.) DC.
Echinodorus macrophyllus (Kunth) Micheli
Pimpinella anisum
Maytenus ilicifolia
Mikania glomerata Mentha piperita L.
Citrus aurantium L.
Malva sylvestris L.

Passiflora ssp

Achyrocline satureioides (Lam.) DC.

Melissa officinalis L.

Punica granatum L.

Sambucus nigra L.

Salvia officinalis L.

Hibiscus sabdariffa

Título da Atividade: **PLANTAS TRADICIONALMENTE EMPREGADAS COMO INFUSÃO: TÉCNICAS DE PREPARO DE AMOSTRA E DETERMINAÇÃO DE CONTAMINANTES**

Equipe

Lucas Cadeirão Rodrigues Miranda	**UNICAMP**
Helena Teixeira Godoy	**Unicamp**

Parceiras no Exterior

Universidade do Porto

Data do Cadastro: **24/10/2018 17:45:49**

Situação do Cadastro: **Concluído**

Conselho de Gestão do Patrimônio Genético

Situação cadastral conforme consulta ao SisGen em **18:55** de **14/09/2020**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN