João Victor Meirelles Leite

Development of optimized analytical methods for chemical profile assessment of *Cannabis* herbal extracts

Dissertação de Mestrado

Dissertation presented to the Programa de Pós-Graduação em Química of PUC- Rio in partial fulfillment of the requirements for the degree of Mestre em Química.

Advisor: Tatiana Dillenburg Saint' Pierre

Co-advisor: Monica Costa Padilha

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To all those who found welfare

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Abstract

Leite, João Victor Meirelles; Saint'Pierre, Tatiana Dillenburg. **Development of optimized analytical methods for chemical profile assessment of Cannabis herbal extracts.** Rio de Janeiro, 2024. 149p. Dissertação de Mestrado – Departamento de Química. Pontificia Universidade Católica do Rio de Janeiro.

Cannabis herbal extracts (CHE) are one of the most interesting and sought products for therapeutic approaches of a diverse number of clinical conditions. Regulated quality control parameters and risk assessment methods are needed for *Cannabis*-based products. Advanced analytical techniques portray powerful alternatives to *Cannabis*-based products monitoring. However, further analytical steps need to be critically optimized to keep up with instrumental performance. In this work, analytical methods for phytocannabinoids quantification by UHPLC-HRMS/MS and multielement determination by ICP-MS, both in CHE, were developed and optimized by Design of Experiments. For UHPLC-HRMS/MS, an ultrasound-assisted liquid-liquid extraction with methanol:hexane 9:1 v/v was proposed, analytical performance was successfully validated by gold-standard brazilian pharmaceutical guidelines, proving its efficiency in question. For multielement determination, three sample preparation methods were explored (acid decomposition in open-vessel, acid decomposition in closed-vessel, and organic solvent direct dilution). Their performances were critically evaluated regarding analytical metrics, ecological impact and user-friendliness. The open-vessel method with diluted HNO_3 provided the overall best performance and was applied to analyze 6 CHEs and one sesame oil sample. The phytocannabinoids quantification suggested a major discrepancy between CHE label description and quantified content, as CBD was over 10,000-times lower and both THC and CBN could not be determined. Also, in general, low metal and metalloid contents were determined, but significant potentially toxic metals content was found. By comparing with sesame oil, statistically significant differences were identified only for Au, Cu, K, Li, Mg, Mn, Ni, Pb, Ti, and Zn. Lead were found at higher levels for all *Cannabis* samples in a range 11.7 to 12.4 μg g^{-1}, in disagreement with FDA guidelines for potentially toxic elements (10 μg g^{-1}). Li, Mg, Mn, Ni, Ti and Zn were found at discordant levels between samples, suggesting a relevant heterogeneity and non-

standardized quality control for these products.

Keywords

Cannabis; ICP-MS; LC-MS; DoE.

Resumo

Leite, João Victor Meirelles; Saint'Pierre, Tatiana Dillenburg. **Desenvolvimento de metodologias analíticas otimizadas para avaliação do perfil químico de extratos herbais de Cannabis.** Rio de Janeiro, 2024. 149p. Dissertação de Mestrado – Departamento de Química. Pontificia Universidade Católica do Rio de Janeiro.

A *Cannabis* é um insumo medicinal histórico e em atual expansão para as mais diversas aplicações medicinais, cosméticas, recreativas e têxteis. O mercado medicinal de *Cannabis* se encontra em destaque no cenário global, principalmente na forma de apresentação de extratos oleosos. Os extratos herbais de *Cannabis* (CHE) são um dos produtos de maior interesse e mais procurados para abordagens terapêuticas de uma diversidade de condições clínicas. Parâmetros de controle de qualidade regulados e métodos padronizados de avaliação de risco são atualmente demandados para produtos à base de *Cannabis*. O potencial medicinal da *Cannabis* é atribuído principalmente à biossíntese de uma classe especial de metabólitos: os fitocannabinoides. O canabidiol (CBD), o tetrahidrocanabinol (THC) e o canabinol (CBN) são destacados como os principais fitocanabinoides alvos de preocupação farmacêutica. Além disso, o monitoramento de impurezas e adjuvantes, como o teor de metais e metaloides, também é fundamental para garantir a segurança e a integridade destes produtos. Técnicas analíticas avançadas retratam alternativas poderosas para o monitoramento de produtos à base de *Cannabis*. No entanto, etapas analíticas adicionais precisam ser otimizadas criticamente para acompanhar o desempenho instrumental e o Design de Experimentos (DoE) fornece uma abordagem rápida, simples, confiável e eficaz para alcançar otimizações multivariadas bem-sucedidas. Neste trabalho apresentamos o desenvolvimento de dois métodos otimizados por DoE para análise de CHE: um método de quantificação de CBD, THC e CBN por UHPLC-HRMS/MS e três métodos de determinação multielementar por ICP-MS. Para a quantificação de fitocannabinoides, as condições instrumentais foram otimizadas frente a um planejamento do tipo Plackett-Burman para 7 variáveis, buscando-se otimizar a reprodutibilidade do fenômeno de ionização. Valores de desvio-padrão relativo de 2%, 2% e 5% foram alcançados para CBD, THC e CBN, respectivamente. Além

disso, planejamentos do tipo Fatorial Completo e Box-Behnken foram utilizadas para propor um protocolo otimizado de extração líquido-líquido assistida por ultrassom com 6,9 mL de metanol:hexano 9:1 v/v, 18 min de tempo de agitação e 25 min de tempo de sonicação. O modelo preditivo construído foi validado, apresentando valores de acurácia entre 86 e 120%. O desempenho analítico foi validado por diretrizes farmacêuticas brasileiras de referência (ANVISA RDC 166/2017) frente a três diferentes abordagens de calibração: calibração externa, adição-padrão e *Matrix Matching*. Valores satisfatórios de exatidão, precisão, sensibilidade, linearidade e efeito de matriz foram alcançados com a utilização deste último, sendo representativo de uma alternativa eficiente, de maior custo-benefício e de maior frequência analítica. A aplicação desta metodologia em um lote de 4 amostras reais revelou uma preocupação significativa em relação à avaliação de risco desses produtos, sendo observada uma discrepância significativa entre a descrição do rótulo e o conteúdo quantificado de CBD (mais de 10.000 vezes menor). THC e CBN não foram encontrados acima do Limite de Quantificação para nenhuma das amostras. Fenômeno que compromete não só seu potencial terapêutico, mas também revelando um ponto cego da segurança do consumidor. Para a determinação multielementar, parâmetros instrumentais atrelados ao plasma e à introdução de amostra foram otimizados por planejamentos do tipo Composto Central para maximização de sensibilidade e minimização de interferências, alcançando-se condições de compromisso com taxas de otimização globais superiores a 80%. Quanto ao preparo de amostra, três métodos foram explorados: digestão ácida aberta em chapa de aquecimento, digestão ácida em vaso fechado e diluição direta com solvente orgânico, assim como três diferentes abordagens de calibração: calibração externa, *Matrix Matching* e adição-padrão. O desempenho de todos os métodos foi criticamente avaliado em relação à exatidão, precisão, sensibilidade, efeito de matriz e impacto ecológico. O método empregando decomposição em chapa de aquecimento com 6,9 mL de HNO_3 diluído 10% v/v com aquecimento por 60 min àa 100 ºC e uma abordagem de calibração por *Matrix Matching* forneceu o melhor desempenho geral e foi aplicado para analisar um lote de 6 amostras de *Cannabis* em comparação com óleo de gergelim, um óleo vegetal de consumo comum e muito utilizado como veículo farmacotécnico nos extratos de *Cannabis*. Em geral, foram determinados baixos teores de metais e metaloides e,

comparando os extratos de *Cannabis* com o óleo vegetal, diferenças estatisticamente significativas foram identificadas apenas para Au, Cu, K, Li, Mg, Mn, Ni, Pb, Ti e Zn. O Pb foi encontrado em níveis mais altos em todas as amostras de *Cannabis*, variando de 11,7 a 12,4 μg.g-1, em desacordo (teor quase 3 vezes maior) com as diretrizes da FDA para elementos potencialmente tóxicos. Por sua vez, Li, Mg, Mn, Ni, Ti e Zn foram encontrados em níveis discordantes entre as amostras, sugerindo uma heterogeneidade relevante na produção dos óleos e um controle de qualidade não padronizado para esses produtos.

Palavras-chave:

Cannabis; ICP-MS; LC-MS; DoE.

Executive Summary

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Abbreviations

AGC Automatic gain control

ANOVA Analysis of Variance

ANVISA Agência Nacional de Vigilância Sanitária

APEPI Apoio à Pesquisa e Pacientes de *Cannabis* Medicinal

BBD Box-Behnken Design

cAD Closed acid decomposotion

CBD Cannabidiol

CBN Cannabinol

CCD Central Composite design

CHE *Cannabis* herbal extracts

CID Collision-induced dissociation

CV Coefficient of Variation

D&S Dilute-and-Shoot

DoE Design of Experiments

DRC Dynamic Reaction Cell

ECS Endocannabinoid system

ES External standard

ESI Electrospray ionization

FFD Full Factorial design

FWHM Full width at half maximum

HCD Higher-energy Collisional Dissociation

HPLC High performance liquid chromatography

ICH International Council for Harmonisation

ICP-MS Inductively coupled plasma-mass-spectrometry

IS Internal standard

IT Injection time

LC Liquid chromatography

LOD Limit of Detection

LOQ Limit of Quantification

MM Matrix Matching

(N)CE Normalized collision energy

oAD Open acid decomposition

PB Plackett-Burman

RF Radio frequency

SA Standard addition

THC Tetrahydrocannabinol

THC-COOH Carboxy-tetrahydrocannabinol

UA-LLE Ultrasound assisted liquid-liquid extraction

UHPLC-HRMS/MS Ultra high-performance liquid chromatography coupled to high resolution tandem mass spectrometry

USP United States Pharmacopeia

1. Introduction

1.1. The *Cannabis*

Throughout human history, the unique potential of plants has been explored, especially in the medicinal field. Evidence dating back over 60,000 years shows the safety and the effectiveness of these natural resources. Their contribution to human development cannot be ignored and must be held in high regard (FORDJOUR et al., 2023).

In this context, *Cannabis sativa* L. (Linnaeus, 1753) is a plant species belonging to the *Cannabaceae* family, with highly complex, polymorphic, and variable characteristics, known and praised since ancient times (ALIFERIS; BERNARD-PERRON, 2020a; FORDJOUR et al., 2023). Being largely employed by various groups for over 5,000 years, it is the oldest plant source of fiber and food (FORDJOUR et al., 2023). Its origin is uncertain, but evidence favors temperate regions of Asia, specifically the southern Caspian, Siberia, China, or the Himalaya (FORDJOUR et al., 2023). Being widely distributed plant worldwide, C. *sativa* can be found in various environments, including different habitats, altitudes, soils, and climatic conditions (ALIFERIS; BERNARD-PERRON, 2020a; FORDJOUR et al., 2023).

The botanical types of *Cannabis* differ from each other by the type of habitat in which they grow, their chemical composition, and plant height (BONINI et al., 2018; FORDJOUR et al., 2023). *Cannabis* is a general term commonly used to refer mainly to C. *sativa* and its genre analogues. There are three main so-called species of *Cannabis* (i.e*., C. sativa, C. indica,* and *C. ruderalis*) and four subspecies (i.e., C. *sativa var. sativa, C. sativa var. spontanea*, C. *indica var. indica, and C. indica var. kafiristanica*) that differ in morphological and chemical characteristics, such as fruit shape and metabolites content (POLLIO, 2016).

The species belonging to the genus *Cannabis* are often with unstable taxonomic foundations. The nomenclature of *Cannabis* has been the object of numerous nomenclatural treatments: Linnaeus (1753) described a single

species, *Cannabis sativa*, while Lamarck (1785) proposed two species: C. *sativa* and C. *indica.* In the second decade of 1900's, a new species *C. ruderalis* was suggested by Schultes et al. (1975). Nowadays, a biphasic approach, combining morphological and chemical characters was adopted by Small and Cronquist, who recognized four subspecies, all belonging to the single species *C. sativa*, that coexist dynamically by means of natural and artificial selection (POLLIO, 2016).

The subspecies *C. sativa var. sativa* and *C. sativa var. spontanea* have a low psychoactive metabolites content, while the *C. indica var. indica* and *C. indica var. kafiristanica* a higher biosynthesis potential (ALIFERIS; BERNARD-PERRON, 2020a; FORDJOUR et al., 2023). Both variants of the *sativa* subspecies are extensively cultivated in North America, Europe, and Asia and show a low intoxicating potential compared to other variants. In contrast, the *indica* subspecies variants have a high intoxicating potential and are substantially found in the Asian continent. In Latin America and Brazil, there is a predominance in the cultivation of the *sativa* species, as it is the most common worldwide (FORDJOUR et al., 2023; POLLIO, 2016).

Cannabis carries on a negative and historical stigma, associated with its psychoactive effect. The term "marijuana" is commonly used to refer to *Cannabis* inputs with high psychoactive content and, in counterpart, the classification "hemp" is commonly coined when is strategic to highlight its capacity as a fiber source or as a non-psychoative output (CARVALHO et al., 2020a; NIE; HENION; RYONA, 2019).

1.2. Biosynthesis and mechanisms

Cannabis is an extremely versatile plant. The flowers and leaves, for example, have a specific aroma. The plant extract contains a wide variety of flavonoids, terpenes, and other substances that can act as insecticides, fungicides, and therapeutic agents. The flowers, leaves, oil, and trichome of the plant, in turn, have been shown to be antioxidants, antimicrobial, cytotoxic, appetite stimulants, antipyretic, and antihypertensive agents (FORDJOUR et al., 2023).

In addition to the rich phytochemical spectrum of *Cannabis*, a phenomenon that attracts significant research interest is the fact that non-psychoactive metabolites of *Cannabis* can act synergistically with psychoactive and potentially therapeutical molecules, boosting their action. This “Entourage Effect” contributes to *Cannabis* clinical applications and generate unlimited potential implications.

Despite having over 400 substances, the phytocannabinoid group stands out as the major metabolites that are naturally biosynthesized in the plant (DE BRITO SIQUEIRA et al., 2023; FORDJOUR et al., 2023). Chemically, phytocannabinoids are characterized by meroterpenes and alkylresorcinols groups in their molecules (BONINI et al., 2018; DE BRITO SIQUEIRA et al., 2023). They are primarily found in the resin secreted by the trichomes of female plants, while male *Cannabis* leaves have few glandular trichomes capable of producing small amounts of these substances.

Phytocannabinoids have medicinal properties attributed to their interaction with the CB1 and CB2, which are specific endocannabinoid G protein-coupled type receptors. These receptors are part of the endocannabinoid system, a set of neuromodulatory lipids that play a role in various physiological processes such as memory, sleep, appetite, learning, hormonal release, neuroprotection, and neurogenesis. By interacting with these receptors as exogenous activators, phytocannabinoids promote the activation of metabolic reactions cascade, strongly linked to neurotransmitters such as dopamine, glutamate, serotonin, and gamma-aminobutyric acid (GABA) (ALIFERIS; BERNARD-PERRON, 2020a; BONINI et al., 2018), resulting in a variety of coordinated biological reactions that regulate cellular homeostasis (AGHAZADEH TABRIZI et al., 2016).

The endocannabinoid system is natural to the human body. Endogenous metabolites analogues such as anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG) are naturally present and have a modulatory role. A brief example of this cascade pathway is illustrated in Figure 1. From a pharmacological perspective, AEA and 2-AG act as agonists of

cannabinoid receptors, (AGHAZADEH TABRIZI et al., 2016).

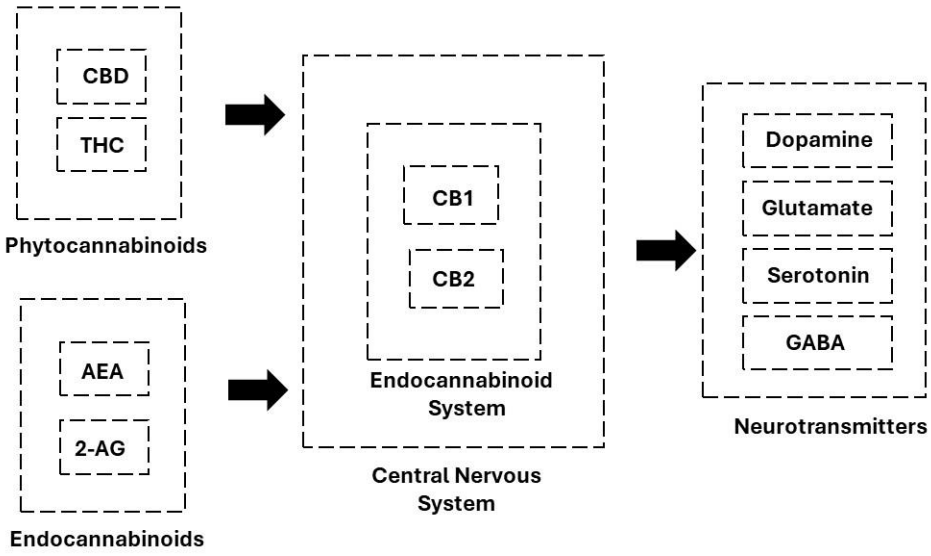

Figure 1. Phytocannabinoids pharmacological stimulation cascade.

The human brain has high levels of cannabinoid receptors, about 10 times more than opioid receptors. They are mainly located in brains of mammals and throughout the central and peripheral nervous systems, including tissues associated with the immune system. The extensive presence of endocannabinoid receptors in the components of the nervous and immune systems, with strong modulatory roles associated with motor control, cognitive functions, pain processing, emotional regulation, and inflammatory responses, well describes the extensive and multiple therapeutic potential of the *Cannabis* mechanism of action (AGHAZADEH TABRIZI et al., 2016; BONINI et al., 2018; MAROON; BOST, 2018).

1.3. Commercial, academic, and social expansion

Despite not reflecting its effects as a whole, *Cannabis* is currently classified as a "narcotic" in popular, legal, and scientific contexts, primarily due to the presence of its psychoactive ingredient Δ-9-tetrahydrocannabinol (Δ9-THC). Additionally, *Cannabis* and opioids are legally grouped together, although they are pharmacologically distinct. In this context, *Cannabis* has

been criminalized and stigmatized since World War II due to its widespread recreational use, specially by marginalized groups, leading to a lack of research and development in the field for most of the 20th century (FORDJOUR et al., 2023; NIE; HENION; RYONA, 2019)

With the isolation of Δ9-THC in 1964 (GAONI; MECHOULAM, 1964) and its complete synthesis in 1965 (MECHOULAM; GAONI, 1965), the interest in *Cannabis* research and innovation expanded (ALIFERIS; BERNARD-PERRON, 2020a). Elucidation of its therapeutic use and chemical potentials made *Cannabis* a hot topic not only at an academic scenario but also as an economical player worldwide.

Cannabis is widely used globally, by more than 4 % of the world's population aged between 15 and 64 years (approximately 209 million people) in 2020, an increase of 23 % compared to 170 million in 2010 (FORDJOUR et al., 2023). Currently, more than 47 countries cultivate *Cannabis* for research and/or commercial purposes, and approximately 25,000 products based on *Cannabis* exist in the global market. In this context, countries have different regulatory models for its legalizations: 36 countries have already adopted practical measures, and in 20 other countries, these models are still in development (ARYAL et al., 2024). The *Cannabis* market in Brazil moves around R$ 130 million per year, and it is expected that, after the medical, industrial, and recreational regulation, the sector will generate R$ 26.1 billion for the country's economy and create more than 328,000 formal and informal jobs in 4 years. The market continues to heat up, with almost 100 patent applications related to *Cannabis* and its cannabinoids in Brazil, 5 % of which have been sent for approval by the Brazilian Health Regulatory Agency (ANVISA) (DE BRITO SIQUEIRA et al., 2023).

Currently, in Brazil, patients in need of *Cannabis* -based products for treatment can obtain them in four ways: (i) from pharmacies, (ii) by importation, (iii) through associations, or (iv) by self-manufacture (DE BRITO SIQUEIRA et al., 2023). The beginning of the commercialization of medications containing cannabinoids in Brazil started at 2017, when ANVISA approved the registration of the medication Mevatyl®, a drug for

spasticity control in multiple sclerosis and composed of a hydroalcoholic extract of *C. sativa* L. containing 27 mg mL^{-1} of Δ9-THC and 25 mg mL^{-1} of CBD (CARVALHO et al., 2020a; DE BRITO SIQUEIRA et al., 2023). Acquiring this medication from pharmacies is the simplest choice. However, it is also one of the most expensive options, along with direct importation, a process authorized since 2015 through ANVISA RDC (from Brazilian Portuguese, *Resolução da Diretoria Colegiada***)** No. 17/2015 (BRASIL, 2015). Despite the approval by the regulatory body, these products are costly and not easily accessible to most of the population in need.

Besides that, non-governmental associations and some patients have been seeking legal authorization for the cultivation of *C. sativa* L. and the artisanal production of its medicinal extracts, providing its medication at a lower cost but with compromised quality control (CARVALHO et al., 2022).

In 2019, ANVISA published RDC No. 327/2019, which establishes requirements for the commercialization, prescription, dispensing, monitoring, and inspection of *Cannabis* products in Brazil. The prescription of these products is strictly restricted to professionals registered with the Federal Council of Medicine (CFM, from Brazilian Portuguese, *Conselho Federal de Medicina*). The resolution states that *Cannabis* products must predominantly contain CBD and no more than 0.2% of Δ9-THC, except in cases they may contain quantities exceeding 0.2% of Δ9-THC, which should be exclusively designated for palliative care (BRITO SIQUEIRA, DE et al., 2023; BRASIL, 2019).

1.4. *Cannabis*-based products

Although the *Cannabis* industry is still in its early stages, it is already a sector that moves millions of dollars annually worldwide, with a forecast of moving US$ 197 billion in the global industry by 2028 (NIE; HENION; RYONA, 2019; MATOS, 2023). Various *Cannabis*-derived products are available in the global market currently. For instance, hemp fibers are used in textiles, yarns, and fabrics, paper, carpets, decorative items, construction materials and insulation, automobile parts, and composites (JOHNSON,

2017). Furthermore, the leaves and flowers of the *Cannabis* contain a variety of beneficial compounds that can act as effective insecticides, fungicides, and therapeutic agents (FORDJOUR et al., 2023). In the food and beverage industry, some wine brands are already using *Cannabis* infusions (NIE; HENION; RYONA, 2019). Meanwhile, seeds are used to make oils, milk, flours, spices, and sauces (FORDJOUR et al., 2023). In the cosmetics industry, there is also a growing use of *Cannabis* due to its numerous benefits. It has been reported that it is used in skincare and hair care products (FORDJOUR et al., 2023).

Within the medical field, *Cannabis* herbal extracts (CHE) stand out. These are oily extracts obtained from the female flowers of the *C. sativa* L. plant through a solid-liquid extraction process, which can be performed using different approaches, such as hydrodistillation, steam distillation, maceration, Soxhlet extraction, among others (CARVALHO et al., 2020a).

Due to the lipophilic nature of cannabinoids, at the end of the extraction process, the active compounds are dissolved in an oily matrix known as a "vehicle" (CASIRAGHI et al., 2022). The most common vehicle employed in imported *Cannabis* products is medium-chain triglyceride, while in artisanal preparations, the *Cannabis* resin is generally dissolved in vegetable oils such as olive, coconut, or sunflower (CARVALHO et al., 2020a).

CHE has broad antioxidant and anti-aging activity and can be used to treat a variety of chronic and metabolic disorders, such as glaucoma, pain, depression, multiple sclerosis, nausea, and vomiting related to cancer and acquired immunodeficiency syndrome (AIDS) patients, among others (ALIFERIS; BERNARD-PERRON, 2020a; DE BRITO SIQUEIRA et al., 2023; FORDJOUR et al., 2023). Many studies have already been conducted and are ongoing to evaluate CHE use in the treatment of various clinical conditions (DE BRITO SIQUEIRA et al., 2023; FORDJOUR et al., 2023).

1.5. Quality Control of *Cannabis* products

Due to the recent legalization of medical *Cannabis* products in several countries and the important properties of the substances present in the plant, the need to intensify research and development in the area is increasingly urgent. *Cannabis* represents an incalculable source of bioactives to be exploited in the most diverse areas, with emphasis on the medical field (ALIFERIS; BERNARD-PERRON, 2020a).

As the *Cannabis* industry seeks to maximize its therapeutic potential, it faces challenges related to the standardization of CHE formulations, quality monitoring, and risk assessment that meet the standards established by regulatory agencies. Agricultural practices, plant growth conditions, and extraction processes play key roles in the consistency of extract content (ALIFERIS; BERNARD-PERRON, 2020a).

Approximately 566 substances have been identified and isolated from *Cannabis* samples to date, found in high abundance in the flowers and leaves of the plant and constituting more than 18 distinct classes of primary and secondary metabolites found in the plant (ALIFERIS; BERNARD-PERRON, 2020a; FORDJOUR et al., 2023). From the identified substances, 198 are non-cannabinoids, 125 are cannabinoids, 120 are terpenes, 34 are flavonoids, 42 are phenols, and some are steroids and alkaloids (BONINI et al., 2018; FORDJOUR et al., 2023). Multiple groups have bioactive and/or psychotropic endowments, contributing as interesting targets for a quality control and risk assessment scenario. Besides, residual solvents, pesticides, herbicides, mycotoxins, and many other substances can be present in the CHE and other *Cannabis*-based products as contaminants (NIE; HENION; RYONA, 2019). Among them, we can highlight two groups of interest that were evaluated in this study: (i) phytocannabinoids and (ii) metals and metalloids.

1.5.1. Phytocannabinoids analysis

Phytocannabinoids are 21-carbons polyphenolic structures, constituted by a derivated- ciclohexane-tetrahydropyran-benzene system (ANTÓNIO; RIBEIRO; FERNANDO PESSOA, 2014). Some examples are

illustrated in Figure 2. More than 100 phytocannabinoids have been identified so far, mainly found in *C. sativa* and *C. indica* and classified into 11 distinct categories (FORDJOUR et al., 2023).

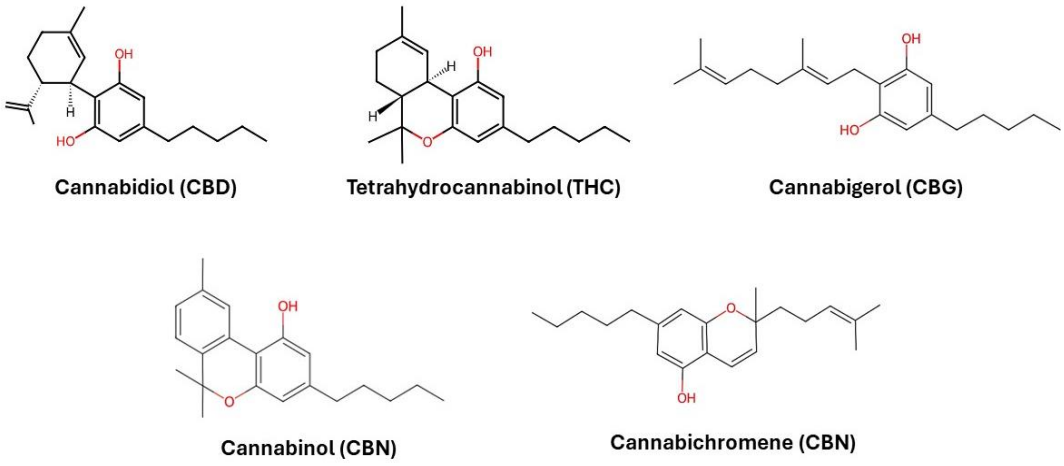

Figure 2. Examples of phytocannabinoids structures.

Phytocannabinoids are the primary metabolites of this plant genus, produced naturally through biochemical synthesis and naturally accumulated in their acidic form. Through non-enzymatic catalytic phenomena, such as drying and heating processes, primary decarboxylation of these structures occurs, leading to their neutral forms, which exhibit bioactive and/or psychoactive properties (ALIFERIS; BERNARD-PERRON, 2020a; BONINI et al., 2018; FORDJOUR et al., 2023).

The main biosynthesis pathway of phytocannabinoids (Figure 3) begins with the precursor molecule, olivetolic acid, which undergoes alkylation with geranyl diphosphate (GPP) through the action of prenyltransferase, resulting in the production of cannabigerolic acid (CBGA). Through the action of specific cannabinoid synthetases, CBGA generates multiple acidic cannabinoids, such as cannabidiolic acid (CBDA), cannabichromenic acid (CBCA), and Δ-9-tetrahydrocannabinolic acid (Δ9-THCA). The primary decarboxylation of these precursors forms neutral cannabinoids, such as cannabidiol (CBD), cannabichromene (CBC), and Δ9-THC. Additionally, another pathway exists through the action of divarinic

acid, which acts as a co-substrate with the same GPP, forming cannabigerovarinic acid (CBGVA). From the CBGVA, other acids such as cannabidivarinic acid (CBDVA), cannabichromovarinic acid (CBCVA), and Δ-9-tetrahydrocannabivarinic acid (Δ9-THCVA) are synthesized. The decarboxylation of these acids forms their respective neutral cannabinoids: cannabidivarine (CBDV), cannabivaricromene (CBCV), and Δ-9-tetrahydrocannabivarine (Δ9-THCV) (BONINI et al., 2018).

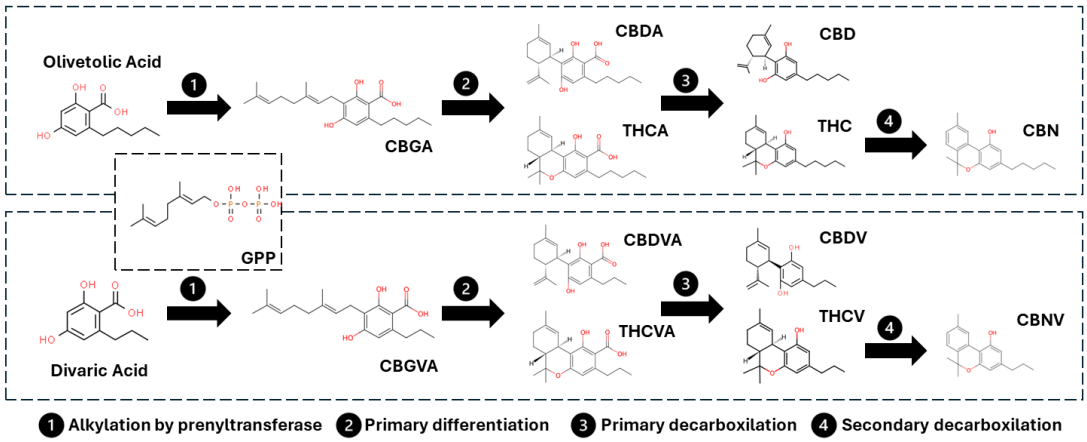

Figure 3. Phytocannabinoids biosynhtetic pathway.

The metabolites Δ9-THC and CBD are the two main cannabinoids found in the plant and the most extensively studied (ALIFERIS; BERNARD-PERRON, 2020a; DE BRITO SIQUEIRA et al., 2023). CBD is widely used for textile purposes, in addition to showing low agonism for endocannabinoid receptors, promoting anticonvulsant, calming, and anxiolytic effects with no psychoactivity (BONINI et al., 2018). On the other hand, Δ9-THC is the substance in *Cannabis* with the most significant psychoactive effects, including euphoria, perceptual alterations, and cognitive deficits. It is classified as a partial agonist of both endocannabinoid receptors and exhibits antiemetic and analgesic properties. The oxidative degradation of Δ9-THC occurs spontaneously but is catalyzed by the presence of light and heat. Following this secondary decarboxylation, cannabidiol (CBN) is generated as a degradation product, showing low affinity for CB1 and CB2 receptors, no psychoactive properties, and no significant therapeutic implications (BONINI et al., 2018; CARVALHO et al.,

2020a).

Medicinal extracts of *Cannabis* used in the treatment of various clinical conditions contain Δ9-THC and CBD as the main active ingredients, with their proportions varying according to the indication. The raw material may contain the degradation product of Δ9-THC (CBN), depending on the storage conditions, such as temperature and light exposure, and the extraction and formulation methods used in the manufacturing process. Monitoring these three cannabinoids is an excellent alternative for monitoring the quality of *Cannabis*-based products (CARVALHO et al., 2020a).

Several advanced analytical techniques can be employed for the determination and quantification of these constituents in *Cannabis* herbal extracts (CHE). Separation methods, such as chromatography, stand out due to the complexity of the matrix involved in the analytical problem. Various detectors in conjunction with both, gas chromatography (GC) and liquid chromatography (LC), can be utilized. However, due to the heat sensitivity of phytocannabinoids, the latter becomes preferable (ALIFERIS; BERNARD-PERRON, 2020a; NIE; HENION; RYONA, 2019).

The most used analytical technique for determining the composition of phytocannabinoids in the plant and derived products is high-performance liquid chromatography with a diode array detector (HPLC-DAD). This is a widely accepted technology due to its ease of use and cost-effectiveness. However, using a mass spectrometer as a detector results in higher selectivity, sensitivity, and superior and critical elucidation power, overcoming limitations such as coelutions and interferences in the analytical problem (NIE; HENION; RYONA, 2019).

Liquid chromatography (LC) is a physicochemical method for separating components of a mixture and is one of the most modern analytical methods. The separation occurs through the differential migration of analytes, resulting from their different interactions with two phases, one stationary and one mobile (COLLINS; BRAGA; BONATO, 2006). HPLC is an interesting analysis alternative, especially because it operates at low temperatures, allowing the separation of thermally unstable compounds,

and has a variety of operational modes, that helps to elucidate various separation mechanisms (COLLINS; BRAGA; BONATO, 2006). The high pressures applied in HPLC results in elevate separation capacity at shorter analysis time. Its remarkable versatility extends the applicability of the technique for different solid or liquid samples (since adequately pre-processed and soluble at the mobile phase), and for distinct analyte groups (molar masses ranging from 32 to 4,000,000 Da) (LEE, 2011).

Reversed-phase chromatography (RPC) is the ideal chromatographic mode for separation of nonpolar or slight polar analytes such as phytocannabinoids. A nonpolar column, typically C8 or C18, is employed with a polar mixture of water and an organic solvent (often methanol and/or acetonitrile) as mobile phase (LEE, 2011). RPC is usually more convenient, robust, and versatile than normal-phase chromatography, and its columns also tend to be more efficient, reproducible, and available in a wider range of dimensions, being the main form of HPLC since the late 1970s. An additional advantage of RPC is generally the faster equilibration of the column after a change in the mobile phase or in between runs, when using gradient elution (LEE, 2011).

The coupling of HPLC to mass spectrometry (MS) combines the advantages of chromatography with the possibility of structural elucidation, by monitoring pseudomolecular masses and/or mass transitions, with increased selectivity. To make this coupling effective, uncontrolled chemical modifications of the analytes and sample loss cannot occur during the transfer in the interface. Therefore, soft ionization modes, such as electrospray (ESI), are used to eliminate mobile phase and introduce the analytes into the high vacuum environment of the MS (CHIARADIA; COLLINS; JARDIM, 2008).

The use of mass spectrometry for analysis of *Cannabis* and its derived products is growing quickly, given the increasingly need for sensitive and selective techniques for quantitative studies. High-Resolution Mass Spectrometry (HRMS), such as that with an Orbitrap analyzer, is among the most recent advances in the molecular analysis area, with vast potential to assess the quality of *Cannabis*-derived products (ALIFERIS;

BERNARD-PERRON, 2020a; NIE; HENION; RYONA, 2019).

For these technology, a sample-solution is introduced into the chromatographic separation inlet and conducted to an electrospray ionization (ESI) source. In ESI, the sample solution is pumped by a capillary highly charged module at atmospheric pressure. Then, by high voltage application and a co-axial gas flow, the solution is distorted into a Taylor cone and generates a fine mist. The droplets emitted from the Taylor cone undergo rapid solvent evaporation until the charge density and the Coulombic repulsion surpass the surface tension at the so-called Rayleigh limit. New even smaller and highly charged droplets are produced via jet fission, this mechanism is constantly repeated until reaching nanometers droplets that are fundamentally gaseous analyte ions (HO et al., 2003; KONERMANN et al., 2013; WILM, 2011).

These ions are accelerated into the mass analyzer. The Orbitrap consists essentially of three electrodes: outer electrodes have the shape of cups facing each other and are electrically isolated, while a spindle-like central electrode holds the trap together and aligns it. When voltage is applied between the outer and the central electrodes, the resulting field is strictly linear along the axis and has a radial component that strongly attracts ions to the central electrode with a tangential velocity. Ions are injected into the volume between the central and outer electrodes, and, with a correct choice of parameters, the ions remain on a nearly circular spiral inside the trap, much like a planet in the solar system. Outer electrodes promote image current detection of these axial oscillations, that are digitized in the time domain and Fourier-transformed into the frequency domain for a mass spectrum conversion (ZUBAREV; MAKAROV, 2013).

For an appropriate LC-Orbitrap analysis, a sample must be properly previously prepared. This step goal is to provide a reproducible and homogeneous sample solution that is suitable for injection into the chromatographic system, ideally free of interferences or matrix effect and that should not provide any damage to the column. It is also desirable to concentrate the analyte, when possible, and modify analytes to the best detection and separation as possible. An ideal sample treatment protocol

should provide quantitative recovery of analytes, involve a minimum number of steps and be easily automated (DEVANSHU et al., 2010; LI; JIAN; FU, 2019).

Liquid extraction is one of the most conventional sample preparation techniques for HPLC analysis and can be properly applied for phytocannabinoids quantification at *Cannabis* products. Based on the differential solubility and partitioning equilibrium of analyte molecules between immiscible phases, it can be also assisted by agitation and/or ultrasonic to facilitate equilibrium portioning between phases. In the end, phases are separated, and the analytes-containing one is traditionally evaporated to dryness and re-suspended with mobile phase or a similar solvent system and then injected onto the chromatographic column (DEVANSHU et al., 2010; LI; JIAN; FU, 2019). Several factors can influence the efficiency of a liquid extraction protocol and therefore need to be optimized, such as solvent volume, solvent polarity, pH, temperature, extraction time, sample/solvent ratio, ultrasonic assistance, and many others (LUQUE DE CASTRO; PRIEGO-CAPOTE, 2007; MAZZOLA et al., 2008).

1.5.2. Metals and metalloids

Cannabis plants have a remarkably capacity of phytoremediation, as they can remove metals and metalloids from the environment and absorb them in their biomass without affecting their heartiness. This phenomenon causes them to concentrate these elements within themselves, which can later be found in *Cannabis*-derived products (NIE; HENION; RYONA, 2019). Therefore, elemental species represents an important monitoring group for quality control and risk assessment approaches.

Metals and metalloids can display different roles in a biotic system, as represented in Figure 4. Some elements are essential to a plant's life, such as Zn, Cu, Fe, and Mn, while others can be potentially toxic and malefic to the final consumer depending on its amount or species, such as Pb, Cd, Hg, and As. Other elements, such as Ca, Fe, Co, Ni and Mn, present

formidable oxidative potential and, thus, major contributions to matrix degradation of *Cannabis*-based products. Beyond environmental sources, the manufacturing process itself can input contaminants to the final product (MILAN; MICHALSKA; JUROWSKI, 2024; NIE; HENION; RYONA, 2019).

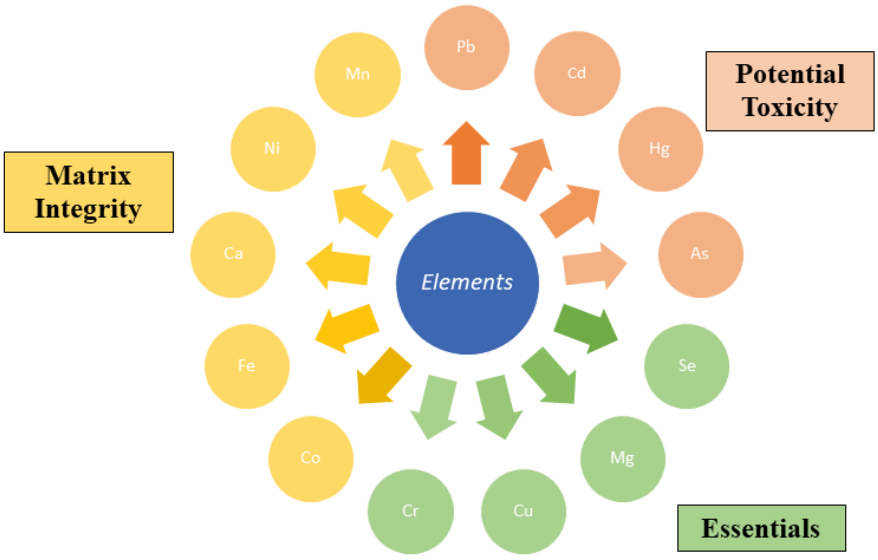

Figure 4. Examples of relevant metals and metalloids.

There is a gap at the toxicological regulatory delimitation for *Cannabis*. While testing laboratories can currently access a growing list of metals and metalloids found in plant material and derived products, jurisdiction strongly differs between each country or local federation. Naturally, the limits are referred to the final product route of administration, i.e., ingestion, topical application, or inhalation, and are typically in accordance with the recommendations provided in the USP <232>/ICH Q3D guidelines for contaminants in herbal medicines (MILAN; MICHALSKA; JUROWSKI, 2024; NIE; HENION; RYONA, 2019).

The metals and metalloids can be quantified by many ways, but atomic absorption spectroscopy (AAS), inductively coupled plasma optical emission spectroscopy (ICP OES), and inductively coupled plasma mass spectrometry (ICP-MS) are the front-runners. Low analytes levels are expected, therefore, sensitivity-enhancing approaches are needed, such as graphite furnace and cold vapor approaches for the AAS technique (GF AAS and CV AAS, respectively) and ultrasonic nebulization (USN) for ICP

OES (USN-ICP OES) or ICP-MS (USN-ICP-MS). Also, due to the monoelemental nature and consequently low analytical frequence provided by AAS is overlooked in this context (NIE; HENION; RYONA, 2019b), the plasma techniques are rather employed in the quality control of *Cannabis* products and were used in this work for the multielement determination of CHE.

Besides the high analytical frequency, ICP-MS takes the lead as the main analytical platform used in modern testing laboratories due to the low sensitivity (close to ng L^{-1} for most elements).

The ICP-MS technique is – briefly – based on an inductively coupled argon-plasma that acts as an ion source, promoting a combination of sequential phenonema of dessolvation, vaporization, atomization, and ionization of the aerosol of a sample solution normally introduced by means of a nebulizer. Monovalent and positive ions are predominantly generated, accelerated, and focalized to a mass analyzer, that separates them by its mass/charge ratio (*m/z*) to be, finally, captured and measured in a detector that translates it as an electrical signal (THOMAS, 2013).

For multielement analysis by ICP-MS, a sample must be properly prepared by digestion, extraction, separation, dilution and/or enrichment approaches. Traditionally, a digestion protocol is applied, in which the matrix is mineralized to remove the organic matter and to alter the original chemical environment into a digest, a solution with the analytes distributed homogeneously in their inorganic chemical forms only (MATUSIEWICZ, 2017).

Sample digestion depends on many factors, such as the nature of the matrix, the analytes to be determined, their concentration levels, and the desired determination confidence. Besides that, it is inherently expensive and time consuming, and responsible for the major source of errors in the various stages of an analytical procedure. A diverse range of methods for organic and inorganic sample materials are available and target protocols must be developed and optimized (MATUSIEWICZ, 2017).

Samples are introduced into the ICP-MS traditionally as solutions, due to its higher simplicity, easier manipulation, and simpler

homogenization. Sample solution is conducted by a peristaltic pump at a steady flow (ideally) to a nebulizer and a spray chamber system. A fine aerosol is formed by mechanical forces of a gas flow (commonly argon) and conducted to a plasma torch for ionization, with only 1-2 % reaching its goal (THOMAS, 2013). While distinct nebulizer designs are available commercially, the concentric one is distinguished as the most used one for conventional applications. In a concentric nebulization, a solution is introduced through a fine-bore capillary tube and meets a rapidly moving flow of gas at a flow of approximately 1 mL min^{-1}, resulting in a Venturi effect that breaks it up into a fine-droplet aerosol, with remarkably stability and sensitivity (THOMAS, 2013).

The function of a spray chamber is to reject the larger aerosol droplets and to smooth out nebulization pulses produced by the peristaltic pump. Double-pass and cyclonic chambers are two of the most popular designs commercially available for ICP-MS, coming in a variety of sizes and materials. Some spray chambers can be cooled for thermal stability of the sample and to reduce solvent loading into the plasma, mainly when introducing organic solvents. It can promote several beneficial effects, such as: (i) reducing plasma energy waste to vaporize solvent and maximizing analytes ionization; (ii) reducing oxide and hydroxide species formation; and (iii) maximizing long-term signal stability. Spray chamber refrigeration is a critical step to enable organic solvent introduction into the plasma for direct diluted sample analysis (THOMAS, 2013).

The plasma torch consists of three concentric tubes, usually made of quartz, passed by a gas flow (usually argon) at a flow rate of 12-17 L min^{-1}, depending on the manufacturer. A second argon flow (auxiliary gas) passes between the middle tube and the sample injector at ~1 L min^{-1}, for protective roles between plasma and torch structure, while a third gas flow (nebulizer gas), also at ~1 L min^{-1}, brings the sample, in the form of a fine-droplet aerosol, from the sample introduction system. The plasma generation occurs due to argon gas interaction with a load coil powered by a high alternate radiofrequency current. With this, starter electrons are made available, accelerated by an electromagnetic field produced by a coil and a

cascade discharge is conducted by consecutives atoms-electrons collisions. While the first plasma gas flow rate is default by the instrumentation supplier, both auxiliary and nebulizer gas flow rates must be optimized for a robust operation (THOMAS, 2013).

Single quadrupole is the most common mass analyzer in ICP-MS instrumentation. A direct current (DC) field and a time-dependent alternating current (AC) of radiofrequency are applied on opposite pairs of four metallics rods. By optimum AC/DC ratio selection, ions of a selected mass are allowed to pass through the rods to the detector, whereas the others are unstable and ejected. Single quadrupole technology provides distinguished resolutions and sensitivities performances, in addition to high-speed analysis, operational simplicity, high stability, and cost, in comparison to others mass analyzers, such as magnetic sector or time-of-flight (THOMAS, 2013).

It is noteworthy to mention that ICP-MS hyphenation with liquid chromatography can also be employed for speciation and quantitation of individual organometallic compounds (NIE; HENION; RYONA, 2019b).

1.6. Methods Optimization

An analytical method must be precise, accurate, reliable, and suitable for the analytical problem. A major step of a method development is its optimization, which is fundamental for achieving the best experimental conditions that produce the best possible analytical performance (FERREIRA et al., 2007a).

The increasing quality requirements of regulatory agencies, the high cost of reagents and feedstocks, the modern urge to green chemical processes and the growing upgrades of advanced analytical instrumentations are some of the reasons that makes optimization steps critical at the modern analytical chemistry scenario (FERREIRA et al., 2007a; WEISSMAN; ANDERSON, 2015).

The univariate optimization strategy of One Factor/Variable At a Time (OFAT/OVAT) is a straight-forward and simple approach that is historically

widely used for methods optimization.

It consists of testing one variable at a time, while the others are kept constant. In the beginning of the 19th century, Ronald A. Fisher, conducting studies in the agriculture field, developed the basis of the modern Design of Experiments (DoE), an upward trend and ongoing advance field (DURAKOVIC, 2017). DoE is a methodology for conducting and planning experiments aimed at extracting the maximum amount of information with the least number of analyses. A planned experiment is a critical tool used to collect complex data and to contour resources constraints. Common features of DoE include the planning of tests, the definition of an approach to collect data, simultaneous strategic variation of factors, and a statistical approach of data analysis to understand experimental factors and its responses (VANAJA; RANI, 2007a).

In recent years, more and more chemometric tools have been used for the multivariate optimization of analytical methods. Among its main advantages, the following can be mentioned: (i) a decrease in the number of experiments, (ii) a significant reduction in the consumption of reagents and working time, (iii) a consolidated mapping of the methodology, (iv) an extensive number of analytical insights and (v) an efficient process evaluation (FERREIRA et al., 2007b).

An adequate process optimization must be conducted with as many parameters (called variables) as possible, demonstrated by initial screening designs, which typically include two-level factorial designs, that can be either Full Factorial (Table 1) or Fractional Factorial (Table 2). The initial screening step aims to explore process knowledge and identifies significant variables for further optimization, therefore, experimental data is correlated with variables levels and coded coefficients are calculated in normalized units (Equation 1). A coded coefficient describes the size and direction of the relationship between a variable under study and the system response, thus, its impact while others variables are kept constant. A coefficient relative size and its p-value can be applied to assess the practical significance of the effect a variable has on the response variable.

$$Coefficient = \frac{1}{n_i}\sum_{j=1}^{n_i} y_{i_{(+1)}} - y_{i_{(-1)}} \qquad \textbf{(Equation 1)}$$

Where n_i is the total number of observations, $y_{i_{(+1)}}$ is sum of trial responses when the variable is at its upper-level and $y_{i_{(-1)}}$ the sum of trial responses at its lower-level.

Table 1. Full Factorial Design (coded) for 3 factors at 2 levels (2^3).

Exp	X_1	X_2	X_3
1	-1,00	-1,00	-1,00
2	1,00	-1,00	-1,00
3	-1,00	1,00	-1,00
4	1,00	1,00	-1,00
5	-1,00	-1,00	1,00
6	1,00	-1,00	1,00
7	-1,00	1,00	1,00
8	1,00	1,00	1,00

Table 2. Fractional Factorial Design (coded) for 3 factors at 2 levels (2^{3-1}).

Exp	X_1	X_2	X_3
1	-1,00	-1,00	1,00
2	1,00	-1,00	-1,00
3	-1,00	1,00	-1,00
4	1,00	1,00	1,00

When a Full Factorial is conducted, all the possible combinations of variables are tested while at a Fractional Factorial only a key subset of experiments (a fraction) is tested. The latter design requires fewer trials than the former, being adequate when resources are limited or the number of factors in the experiment is large, however confounding effects are exhibited, obscuring the existence of interactions and compromising full results interpretation. Extra center points can be augmented to check

curvature effects and statistical significances (CAVAZZUTI, 2013; POLITIS et al., 2017)

Once the screening is completed, the elected significant factors are further studied using more comprehensive designs, such as Central Composite Design (CCD) or Box-Behnken Design (BBD), aiming to process optimization that can support quadratic or higher order effects and generate data for Response Surface Methodologies (CAVAZZUTI, 2013; POLITIS et al., 2017).

These designs generates a second-order predictive model, according to the Equation 2, where y is the predicted response, b_n are the regression coefficient and x_n are the coded levels of the independent variables. The models construction, allows a graphic representation of a Response Surface, that allows variables interpretation of a optimized condition with maximum or minimum response, depending on the ultimate goal (CAVAZZUTI, 2013; PEIXOTO; OLIVEIRA; CADORE, 2012; SZPISJÁK-GULYÁS et al., 2023)

$$y = b_o + \sum_{i=1} b_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{j=i+1}^{n} b_{ij} x_i \, x_j, \qquad \textbf{(Equation 2)}$$

A CCD is a 2^k (for k parameters, i.e., variables) full factorial design, to which a central point and the star points are added, generating a design size of 2k+2k+1, as seen at Table 3. CCD is one of the most commonly used optimization designs, originally introduced by Box and Wilson in 1951 as an alternative to the full-level factorial design (SZPISJÁK-GULYÁS et al., 2023). The CCD consists of three parts: factorial points, axial/star points, and center points, therefore, having more samples than those strictly necessary for curvature estimation (CAVAZZUTI, 2013; SZPISJÁK-GULYÁS et al., 2023).

Table 3. Example of Central Composite Design (coded) for 3 factors.

Exp	X_1	X_2	X_3
1	-1,00	-1,00	-1,00
2	-1,00	-1,00	1,00
3	-1,00	1,00	-1,00
4	-1,00	1,00	1,00
5	1,00	-1,00	-1,00
6	1,00	-1,00	1,00
7	1,00	1,00	-1,00
8	1,00	1,00	1,00
9	-1,682	0,00	0,00
10	1,682	0,00	0,00
11	0,00	-1,682	0,00
12	0,00	1,682	0,00
13	0,00	0,00	-1,682
14	0,00	0,00	1,682
15	0,00	0,00	0,00

The Box Behnken Design (BBD) is a rotatable or nearly rotatable second-order design based on three-level incomplete factorial designs (Table 4), built by combining two-level factorial designs with incomplete block designs in a particular manner. BBD main advantages are the limited sample size as the number of parameters grows and the evading capacity of extreme conditions, as it does not contain combinations in which all factors are at the highest or lowest levels at the same time (SZPISJÁK-GULYÁS et al., 2023). In Box-Behnken designs, a block of samples corresponding to a two-level factorial design is repeated over different sets of parameters, while the not-included parameter remains at their mean level throughout the block (CAVAZZUTI, 2013).

Table 4. Example of Box-Behnken Design (coded) for 3 factors.

Exp	X_1	X_2	X_3
1	-1,000	-1,000	0,000
2	1,000	-1,000	0,000
3	-1,000	1,000	0,000
4	1,000	1,000	0,000
5	-1,000	0,000	-1,000
6	1,000	0,000	-1,000
7	-1,000	0,000	1,000
8	1,000	0,000	1,000
9	0,000	-1,000	-1,000
10	0,000	1,000	-1,000
11	0,000	-1,000	1,000
12	0,000	1,000	1,000
13	0,000	0,000	0,000

The design choice to be conducted depends on the analytical problem itself and the analyst know-how. CCD methodology provides a rotatability dimension to the model and gives better information within or beyond the limits of the traditional spinning process. As CCD requires more observations trials than BBD, more relevant data can be provided and novel method's responses can be achieved to the investigation. A BBD provides faster and simpler optimization responses with limited number of experiments, being adequate especially for previously known processes bias and well-known parameters refine (CAVAZZUTI, 2013; POLITIS et al., 2017; SZPISJÁK-GULYÁS et al., 2023).

Multivariate optimization techniques have been applied to the optimization of a diverse number of analytical methods, from pre-concentration strategies and novel extraction approaches to chromatographic methods and even electroanalytical protocols (FERREIRA et al., 2007a). Several reviews and scientific papers have been published on this subject. However, the use of the DoE tool for studies of *Cannabis*

products is relatively recent in a general context, with a small number of reports and plenty of room for greater applications. To this date, only 126 documents were found in a non-exhaustive literature research at Web of Science data for “*Cannabis*” and “Design of Experiments” or “Multivariate Optimization” over a 30-year period, while for Scopus database, the same entry returned 375 results.

When an innovative approach, such as DoE, meets a full of potential study field, such as *Cannabis*, powerful pieces of work can be brought to light.

2. Research Objetives

2.1. General objective

To develop two analytical methods for phytocannabinoids (CBD, THC, and CBN) quantification by UHPLC-MS/MS and multielement quantification by ICP-MS in *Cannabis* herbal extracts.

2.2. Specific objectives

1. To explore Design of Experiments (DoE) as a multivariate optimization approach of the instrumental and sample preparation parameters, identifying significant parameters and achieving maximum analytes responses.
2. To validate methods' analytical and ecological performances by adequate standardized guidelines, such as ANVISA RDC 166/2017 and the 12 principles of Green Chemistry.
3. To conduct a comparative study between methods performances, taking into account analytical reliability, greenness performance and routine viability.
4. To apply the developed optimized methods, as a Proof-of-Concept, to commercial samples, to elucidate analytical application viability and *Cannabis*-based products composition

3. Material and Methods

The presented thesis is based on a article compilation format. Therefore, the Material and Methods section will be presented here and omitted from the respective articles at the Results and Discussion section.

Section 3.1 and Chapter 4 are based on the research article "Phytocannabinoids quantification in *Cannabis* extracts of veterinary applications by UHPLC-HRMS/MS: A novel multivariate optimization approach" (submitted at Journal of Biomedical and Pharmaceutical Analysis) while section 3.2 and chapter 5 are based on the article "Development of a method for multi-elemental analysis of medicinal *Cannabis* oil extracts by inductively coupled plasma mass spectrometry" (submitted at Microchemical Journal).

3.1. Research Article 1 – Phytocannabinoid quantification by UHPLC-HRMS/MS

3.1.1. Chemicals and reagents

For sample preparation, methanol and n-hexane, both GC grade were acquired from Tedia (Fairfield, OH, USA). CBD, THC and CBN standards were provided by AB Científica Ltda (Espírito Santo, Brazil). Carboxy-tetrahydrocannabinol-D_3 (THC-COOH-D3) and carboxy-tetrahydrocannabinol-D_9 (THC-COOH-D9) were purchased from Cerilliant Corporation (Texas, USA). For LC-HRMS/MS analysis, methanol HPLC grade, formic acid 98%-100% and ammonium formate were provided by Tedia (Fairfield, OH, USA), Merck (Darmstadt, Germany) and Spectrum Chemical (Gardena, CA, USA), respectively. Ultrapure water (18.2 MΩ.cm) was obtained from a Millipore Milli-Q purification system (Billerica, MA, USA).

3.1.2. UHPLC-HRMS/MS Instrumentation and analysis

A Dionex Ultimate 3000 ultra-high performance liquid chromatography (UHPLC) system coupled to a QExactive Plus hybrid

quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray ionization (ESI) source was used. Separation was performed in a reversed-phase column (ACE UltraCore 2.5 SuperC18, 50 mm x 2,1 mm; 2,5 µm), at 50 °C, constant flow rate of 400 µL min^{-1} and injection volume of 5 µL. A gradient chromatographic run started at 5% of mobile phase B (methanol with 0.1% formic acid) and 95% of mobile phase A (water with 5 mM ammonium formate and 0.1% formic acid). Mobile phase B was increased to 10% at 0.5 minutes, then to 25% at 1 minute, and then 90% at 6 minutes. After reaching 100% of B at 8 minutes and maintaining this ratio until 9 minutes, the initial chromatographic condition was restored from 9.1 to 11.1 minutes.

The LC effluent was pumped to the mass spectrometer operating in a positive ESI mode, calibrated daily with a manufacture's calibration solution (Thermo Fisher Scientific, Bremen, Germany). ESI parameters were further optimized, and the final setup employed was: spray voltage of 4.00 kV, S-lens voltage of 80 V, capillary temperature of 250 °C, auxiliar gas heater temperature of 350 °C, nitrogen sheath, auxiliary, and sweep gas were set at 30, 10, and 1 arbitrary unit, respectively. Full-scan data were acquired in a range of *m/z* 80 – 800 at a resolution of 70,000 full width at half maximum (FWHM), automatic gain control (AGC) of 1 x 106 and maximum injection time (IT) of 100 ms. Parallel reaction monitoring (PRM) data were acquired at a resolution of 70,000 FWHM with AGC of 1 x 10^5, IT of 50 ms, loop count of 2, and isolation window of *m/z* 1.0. The analytes CBD, THC, and CBN, as well as the internal standard THC-COOH-D9, were selected from an inclusion list for mass fragmentation in a Higher-energy Collisional Dissociation (HCD) collision cell with normalized collision energy (N)CE. Data was acquired and processed using Thermo ScientificTM TraceFinderTM 4.1 software (Thermo Fisher Scientific, Austin, TX, USA), with a ± 5 ppm mass tolerance. Precursor ions of *m/z* 315.23186 (CBD and THC), 311.20056 (CBN), 348.22487 (THC-COOH-D3) and 354.26253 (THC-COOH-D9) were fragmented in a higher energy collisional dissociation (HCD) cell with (N)CE of 40%, 37%, 35% and 35%, respectively. The LC effluent was pumped to the mass spectrometer

operating in a positive ESI mode, calibrated daily with a manufacture's calibration solution (Thermo Fisher Scientific, Bremen, Germany).

3.1.3. Optimization of electrospray ionization source and internal standard evaluation

CBD, THC, and CBN standards were, separately, spiked at a final concentration of 1 µg mL^{-1} to three vials containing 1.00 mL of solvent in a proportion of 60:40 v/v methanol with 0.1% formic acid and water with 0.1% formic acid and 5 mM ammonium formate. To each of them, two internal standards (ISTD) (i.e., THC-COOH-D3 and THC-COOH-D9) were spiked, separately, for fit-for-purpose evaluation.

A Plackett-Burman (PB) screening design was conducted for optimization of the independent variables (X_n), specified in Table 5, for the electrospray ionization (ESI) source. Eight experiments (trials), represented in Table 6, were carried out randomly, and each trial was injected in triplicate (i.e., 8 x 3 = 24) from the same prepared vial. The analyzed response corresponded to the coefficient of variation (CV%) (n=3) obtained for each ratio "Analyte Area/ISTD Area".

Table 5. Variables and levels studied in the Plackett-Burman screening design for the optimization of ESI source parameters.

Code	Variables	Level (-1)	Level (+1)
X_1	Auxiliar gas heater temperature (°C)	250	350
X_2	Capillary temperature (°C)	250	350
X_3	Electrospray voltage (kV)	2.5	4
X_4	Sheath Gas (a.u.)	30	50
X_5	Auxiliary Gas (a.u.)	10	20
X_6	S-lens (V)	50	80
X_7	Sweep gas (a.u.)	0	1

Table 6. Plackett-Burman screening design of trials conducted (trials 1 to 8), with the independent variables (X1 to X7) and their levels.

Trial	Aux. gas heater temp. (°C)	Cap. temp.(°C)	Electrospray voltage (kV)	Sheath Gas (a.u.)	Auxiliary Gas (a.u.)	S-lens (V)	Sweep gas (a.u.)
1	350	350	4.00	30	20	50	0
2	350	350	2.50	50	10	50	1
3	350	250	4.00	30	10	80	1
4	250	350	2.50	30	20	80	1
5	350	250	2.50	50	20	80	0
6	250	250	4.00	50	20	50	1
7	250	350	4.00	50	10	80	0
8	250	250	2.50	30	10	50	0

3.1.4. Optimization of sample preparation

3.1.4.1. Screening design

To select the most important variables and their levels to be used in the sample preparation modelling design, a screening Full Factorial Design (FFD) 2^3 (8 trials) was performed. The sample preparation protocol consisted in a ultrasound-assisted liquid-liquid extraction (UA-LLE) with methanol:hexane 9:1 v/v as an extractor mixture, as previously successfully reported in literature (CARVALHO et al., 2020b).

Twenty-five microliters of a CBD, THC and CBN standard mix at 1.2 ng/µL were added in test tubes and evaporated to dryness in an evaporator at 30°C under N_2 stream. To the dry residue, 100 mg of coconut oils, a sample model for *Cannabis* extracts, were added and mixed in vortex (30 sec) for a complete homogenization. Methanol:hexane 9:1 v/v mixture was added to the samples, which were submitted to the UA-LLE in a shaker at 400 rpm and, then, in a ultrasound. As represented in Table 7, agitation time in shaker (5 or 15 min – coded as X_1); ultrasound time (15 or 45 minutes – coded as X_2); and extractor mixture volume (2.5 or 7.5 mL – coded as X_3) were assessed in two levels, as independent variables, with six extra replicates for a central point (10 min, 30 min, 5 mL, respectively). When submitting the samples to the ultrasound, it is noteworthy to mention that the bath must be open to avoid heating and, consequently, the degradation of phytocannabinoids.

After the UA-LLE, the samples were refrigerated at –30°C for 30 minutes, centrifugated at 4000 rpm for 20 minutes and its organic phases were separated. The final volume was, then, adjusted to 7.5 mL, an aliquot of 975 µL was transferred to a vial, and 25 µL of THC-COOH-D9 10 ug mL^{-1} were added as ISTD.

The samples were subjected to analysis by LC-HRMS/MS, and the ratios “Analyte Area/ISTD Area" were determined for the analytes in each trial. A non-linear regression was used to calculate the coefficients (b_1, b_2 and b_3) as represented in Equation 3 and its relevancy to the experimental

protocol.

$$b_i = \frac{1}{n_i}\sum_{j=1}^{n_i} y_{i_{(+1)}} - y_{i_{(-1)}} \qquad \textbf{(Equation 3)}$$

Where n_i is the number of factors, $y_{i_{(+1)}}$ is the trial response at the upper-level factor and $y_{i_{(-1)}}$ the trial response at the lower-level factor.

Table 7. Screening factorial design matrix (2^3) used for selection of the most important variables in sample preparation. The independent variables (X_1 to X_3), and their levels (not coded) are shown in the matrix. Trial 9 is the central point, which was prepared in 6 replicates.

Trial	X_1 - Agitation Time (min)	X_2 - Ultrasound time (min)	X_3 - Extraction volume (mL)
1	5	15	2.50
2	15	15	2.50
3	5	45	2.50
4	15	45	2.50
5	5	15	7.50
6	15	15	7.50
7	5	45	7.50
8	15	45	7.50
9 (n = 6)	10	30	5.00

3.1.4.2. Modelling design

A Box-Behnken design (BBD) for 3 variables was performed to find the optimum working region for the sample preparation protocol. As described previously, coconut oil spiked with the phytocannabinoids standards CBD, THC and CBN was used as a model for *Cannabis* extracts.

The only difference in sample preparation in relation to the screening design was the final volume adjustment to 10 mL instead of 7.5 mL. To an aliquot of 975 µL of the final solution, 25 µL of THC-COOH-D9 10 µg mL^{-1} was added as ISTD and analyzed by LC-HRMS/MS. Agitation time in shaker (10 or 20 min – coded as X_1), ultrasound time (5 or 25 minutes – coded as X_2), and extractor mixture volume (5 or 10 mL – coded as X_3) were assessed as independent variables, with five extra replicates of a central point (15 min, 15 min, 7.5 mL, respectively). The ratios “Analyte Area/ISTD Area" were determined for the analytes in each trial as dependent responses. Experimental design represented at Table 8.

BBD generalizes a second order predictive model as follows: $y = b_o + \sum_{i=1} b_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{j=i+1}^{n} b_{ij} x_i x_j$, where y is the predicted response, b_n are regression coefficients, and x_n are the coded levels of the independent variables [9]**.** The results of the BBD design were analyzed using regression analysis (Minitab software version 18.1) and the response surfaces were constructed in STATISTICA software version 10.0.0.0. After determining the optimal conditions, the discrepancy between the experimental values and its predicted values were compared to validate the model.

Table 8. Box-Behnken design matrix of experiments (trials) performed to find the robust working region for the sample preparation protocol. The independent variables (from X_1 to X_3, not coded) and their levels are shown in the matrix. (not coded) and its dependent values. Trial 13 is the central point, which was prepared in 5 replicates.

Trial	X_1 - Agitation Time (min)	X_2 - Ultrasound time (min)	X_3 - Extraction volume (mL)
1	10	5	7.50
2	20	5	7.50
3	10	25	7.50
4	20	25	7.50
5	10	15	5.00
6	20	15	5.00
7	10	15	10.00
8	20	15	10.00
9	15	5	5.00
10	15	25	5.00
11	15	5	10.00
12	15	25	10.00
13 (n = 5)	15	15	7.50

3.1.5. Method validation

Following the Brazilian Health Regulatory Agency (ANVISA) resolution 166/2017 guidelines, the method's analytical performance was assessed and validated. Selectivity, defined as the ability to unequivocally identify and/or quantify the analyte in the presence of components that may be present in the sample, was evaluated through the preparation and

analysis of coconut oil, sesame oil, soy oil and olive oil matrices, without fortification of analytes but spiked with the ISTD.

The linearity, demonstrated by the method's ability to respond proportionally to the concentration of an analyte in a sample, was evaluated through the preparation of samples, in triplicate, at five different concentration levels. The determination coefficient (R^2), the data homoscedasticity (Cochran test), and the residual scatter plot were evaluated. The working range was established from the linearity assessment. Calibration curves were prepared following the established working range in the: (i) mixture solvent (methanol:hexane 9:1 v/v) for external standard calibration; (ii) coconut oil matrix for matrix matching calibration approach; and (iii) by standard addition in a real sample. Angular coefficients were compared for adequate matrix effect investigations by F-test for variance equality and t-test for analytical inclination equality.

For precision, defined as the closeness between the results obtained through the analytical method, intra-assay repeatability was estimated through the preparation of triplicates of real samples spiked with CBD, THC, and CBN at three concentration levels (i.e., low, medium, and high), defined from linearity and working range. The results were evaluated through the CV% of the concentrations obtained from the analytical curve of the samples prepared and injected by a single analyst, on a single day and on the same equipment. For inter-assay repeatability, the same sample preparation protocol was conducted by other analyst on a different day and the CV% of the combined dataset was calculated.

Accuracy, defined as the degree of agreement between individual results obtained by the method under study and a value accepted as true, was evaluated through recovery assays of CBD, THC, and CBN in triplicates of real samples at three concentration levels (i.e., low, medium, and high), defined from linearity and working range. The obtained concentration was compared to the theoretical concentration value.

For sensitivity, limits of detection (LOD) and limits of quantification (LOQ) were estimated for the analytes. The former, defined as the lowest concentration of the analyte present in a sample that can be detected but

not necessarily quantified, was calculated by Equation 4, and the latter, defined as the lowest concentration of the analyte in a sample that can be determined with acceptable precision and accuracy, by Equation 5.

$$\text{LOD} = 3.3\ x\ \frac{standard\ deviation\ of\ intercept}{analytical\ curve\ slope} \quad \textbf{(Equation 4)}$$

$$\text{LOQ} = 10\ x\ \frac{standard\ deviation\ of\ intercept}{analytical\ curve\ slope} \quad \textbf{(Equation 5)}$$

For robustness, three critical parameters on sample preparation were altered in approximately 10% and analytes concentrations were monitored for performance comparison. Agitation times of 16 min and 20 min, ultrasound times of 22.5 min and 27.5 min, and solvent volumes of 6.00 mL and 8.00 mL were tested.

3.1.6. Analytical application for veterinary medicine extracts

The developed, optimized, and validated methodology described in this work was applied to real *Cannabis* products. Four CHE commercialized for veterinary medicine applications were purchased in the Rio de Janeiro state (Brazil) and analyzed in triplicate. The label content information and its concentration values obtained by the method were compared.

3.2. Research Article 2 – Multielement analysis by ICP-MS

3.2.1. Chemicals and reagents

Argon 99.996 % and oxygen 99.50 % (both from LINDE, Brazil) were employed in the plasma generation system, the former as principal, auxiliary, and nebulizer gas, and the latter added to the nebulizer gas in the organic solutions introduction to avoid incomplete combustion and carbon deposits. Methane 99.995 % (White Martins, Brazil) was used as the reaction gas in the Dynamic Reaction Cell (DRC). Ultrapure water (18.2 MΩ cm) was obtained from a Millipore Milli-Q purification system (Billerica, MA, USA), and HNO_3 (65 %, VETEC Química Fina, Brazil) was bidistilled in a Duo-PUR sub-boiling distillation system (Milestone, USA), while H_2O_2 was

provided by Supelco (Sigma Aldrich, Germany). The solvents xylene, butanol, and isopropanol (all from VETEC Química Fina, Brazil) were employed for the solutions, standards, and samples preparation in the D&S method. The multielement organic standard S-21 (Conostan, USA) and multi-element aqueous 5 % HNO_3 calibration standards no. 2 and no. 3 (PerkinElmer, USA) were used to prepare the analytical solutions. A setup aqueous solution containing In, U, Mg, Be, and Ce at 1.0 µg kg^{-1} each in 1 % HNO_3 (PerkinElmer, USA) was employed for the plasma optimization. A Rh 1000 mg L^{-1} standard solution (PerkinElmer, USA) was applied as internal standard (IS).

3.2.2. Samples

Six herbal oil extracts were kindly donated by Apoio à Pesquisa e Pacientes de *Cannabis* Medicinal (APEPI), a *Cannabis* research and costumer-supporting association based in Macaé, RJ, Brazil. Sesame oil was acquired at a local market from Rio de Janeiro, RJ, Brazil, and employed as a sample substitute in the blank solutions to mimic the physic-chemical properties of the sample and since it is commonly applied as pharmaceutical vehicle in *Cannabis* herbal extracts.

3.2.3. ICP-MS Instrumentation and conditions

All measurements were performed in a NexIon 300X ICP-MS spectrometer equipped with Universal Cell Technology, produced by PerkinElmer (USA). The plasma gas flow rate was kept at the default value of 17.0 L min^{-1}. Sampler and skimmer cones made of Ni were employed for acid solutions analysis, while Pt cones were utilized for organic solutions analysis. An Al made hyper-skimmer was employed at both sample introduction conditions.

For sample introduction, the standard default system (PerkinElmer, USA) with a Meinhard concentric glass nebulizer, a cyclonic spray chamber, and a 2.0 mm diameter injection tube was employed for the aqueous solutions. For the D&S method, a specific introduction system for organic

solvents was employed, composed of a Meinhard concentric glass nebulizer (Glass Expansion, USA) coupled to a cryogenic desolvation unit kept at −5 °C (IsoMist™, Glass Expansion, USA), and a torch with an injector tube of 0.8 mm diameter (PerkinElmer, USA). Oxygen was introduced between the spray chamber outlet and the injector, and its flow rate was controlled using a 247 D mass flow controller (MKS Instruments, Inc., USA), which was visually adjusted to the minimum flow rate for eliminating the characteristic carbon green emission

Initially, to guarantee the best sensitivity of each introduction condition, acid or organic solutions, with minimum spectral interference, the plasma operational conditions (RF power and nebulizer Ar flow rate) and DRC conditions (CH_4 flow rate) were strategically optimized.

For aqueous sample introduction, the plasma operational conditions were optimized employing a multielement standard containing In, U, Mg, Be, and Ce at 1.0 µg L^{-1} in 1 % HNO_3 and the experimental trials planned based on the Box Behnken Design of 3 parameters, displayed in Table 9. The intensity of In and the Ce^{+2}/Ce^{+} and CeO/Ce^{+} ratios were monitored, in agreement with supplier recommendations for system suitability assessment. The independent variables (from X1 to X3) and their levels (not coded) are shown in the matrix. Trial 13 is the central point, which was prepared in 3 replicates. The best conditions were established as a compromise for the maximum intensities and minimum interferences.

Table 9. Box-Behnken design matrix of experiments (trials) to optimize the plasma conditions for analysis of aqueous solutions.

Trial	X_1 – Nebulizer gas flow rate (mL min^{-1})	X_2 - Auxiliar gas flow rate (mL min^{-1})	X_3 – RF Power (W)
1	0.925	1.0	1000
2	0.925	1.0	1300
3	1.075	1.0	1000
4	1.075	1.0	1300
5	1.0	0.9	1000
6	1.0	0.9	1300
7	1.0	1.1	1000
8	1.0	1.1	1300
9	0.925	0.9	1150
10	1.075	0.9	1150
11	0.925	1.1	1150
12	1.075	1.1	1150
13 (n = 3)	1.0	1.0	1150

For organic solutions introduction, a previous report from our group (VIANA; SAINT'PIERRE, 2019) demonstrated the optimization of the plasma conditions in this same instrument. After a fine tuning with a multielement standard solution in xylene, the best conditions were established as a compromise for the maximum intensities and minimum interferences.

A xylene-based solution containing Ca, Ba, and Ti at 20 µg L^{-1} and xylene as the blank solution were also analyzed by DRC-ICP-MS at different CH_4 flow rates, from 0.50 to 0.75 mL min^{-1} for DRC conditions optimization. The signal-to-blank ratios (SBR) were measured for the following isotopes: ^{44}Ca, ^{138}Ba, and ^{48}Ti. The CH_4 flow rate was optimized for the best analytes SBR and minimum oxides and double-charged ions formation.

3.2.4. Sample preparation methods optimization

3.2.4.1. Open-flask acid decomposition method (oAD)

Based on previous reports for oil samples (ASTOLFI et al., 2021; LEPRI et al., 2011), a diluted acid open-flasks wet digestion (oAD) method was proposed. The most important variables and their levels were initially selected by a screening Full Factorial Design (FFD), and then, optimized by a Box-Behnken Design (BBD) to find the optimum working conditions. Sesame oil fortified with a 12-element organometallic standard (Ag, Ba, Ca, Cd, Co, Cr, Fe, Mg, Mn, Ni, Pb, and Zn) at 10 mg kg^{-1} was employed for the optimizations.

The oAD protocol consisted of a 500 mg sample mass weighed into 15 mL tubes (Sarstedt, Germany), and a volume of a digestion mixture containing 10 % v/v HNO_3 and H_2O_2 was added. After homogenization, the mixtures were heated to 100 ^oC on a hot plate (Fisatom, Brazil), centrifuged, and the fine upper-organic layer was removed. The final volume was adjusted to 13.0 mL with ultrapure water and the sample solutions analyzed by ICP-MS.

For FFD, as presented in Table 10, the HNO_3 volume (2.5 or 7.5 mL – coded as X_1); the H_2O_2 proportion (0 or 50 % v/v – coded as X_2), and the heating time on the hot plate (20 or 60 min – coded as X_3) were assessed in two levels, as independent variables, with six extra replicates at the central point (5.0 mL, 25 %, and 40 min, respectively). “Analyte signal/IS signal" were determined for the analytes in each trial. Non-linear regression was used to calculate each variable’s coefficients (i.e. b_0, b_1, b_2, and b_3), as represented in Equation 6 and its relevancy to the experimental protocol.

$$b_i = \frac{1}{n_i}\sum_{j=1}^{n_i} y_{i_{(+1)}} - y_{i_{(-1)}} \qquad \textbf{(Equation 6)}$$

Where n_i is the number of factors, $y_{i_{(+1)}}$ is the trial response at the upper-level factor and $y_{i_{(-1)}}$ the trial response at the lower-level factor.

Table 10. Screening factorial design matrix (23) for selection of the most important variables in the oAD method. Trial 9 is the central point (n=6).

Trial	X_1 - HNO_3 volume (mL)	X_2 - H_2O_2 proportion (%)	X_3 - Heating time (min)
1	2.5	0	20
2	7.5	0	20
3	2.5	50	20
4	7.5	50	20
5	2.5	0	60
6	7.5	0	60
7	2.5	50	60
8	7.5	50	60
9 (n=6)	5.0	25	40

For BBD, the final volume was adjusted with ultra-pure water to 11.0 mL instead of 13.0 mL for practical reasons. As displayed at Table 11, the HNO_3 volume (1, 3.5 or 6 mL – coded as X_1), the H_2O_2 proportion (25, 50 or 75 % – coded as X_2), and the heating time on the hot plate (45, 60 or 75 min – coded as X_3) were assessed as independent variables, with three extra replicates at the central point (3.5 mL, 50 %, 60 min, respectively). The “Analyte intensity/IS intensity” ratios were determined in each trial as dependent responses.

The BBD generates a second-order predictive model, according to the Equation 7:

$$y = b_o + \sum_{i=1} b_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{j=i+1}^{n} b_{ij} x_i x_j, \qquad \textbf{(Equation 7)}$$

Where y is the predicted response, b_n are the regression coefficient and x_n are the coded levels of the independent variables (PENG et al., 2020)**.** The results of the BBD design were analyzed using regression analysis (Minitab software version 18.1) and the response surfaces were constructed in STATISTICA software version 10.0.0.0. Response surfaces models were constructed based on composite desirability, calculated as an

overall geometric mean of each analyte model response target to maximum analyte/IS signal ratio.

Table 11. Box-Behnken design matrix of experiments (trials) performed to find the optimized working region for the oAD method. Independent variables (from X_1 to X_3, not coded) and their levels (not coded) are shown in the matrix. Trial 13 is the central point, which was prepared in 3 replicates.

Trial	X_1 – HNO_3 volume (mL)	X_2 – H_2O_2 proportion (%)	X_3 – Heating time (min)
1	1.0	25	60
2	6.0	25	60
3	1.0	75	60
4	6.0	75	60
5	1.0	50	45
6	6.0	50	45
7	1.0	50	75
8	6.0	50	75
9	3.5	25	45
10	3.5	75	45
11	3.5	25	75
12	3.5	75	75
13 (n = 3)	3.5	50	60

3.2.4.2. Closed-vessel acid decomposition method (cAD)

A closed-vessel acid decomposition method (cAD) was carried out in a digester block (DAH 904, Berghof, Germany), based on a previous report for oil samples (ASTOLFI et al., 2021). The method consisted of 500 mg sample mass weighed in the digestion Teflon vessels (DAB 3, Berghof, Germany), added HNO_3 and H_2O_2, homogenized, and kept at rest for a pre-heating time. After closing the vessels, samples were heated in the digester block at the temperature program presented in Table 12.

Table 12. Digester block heating program.

Step	Temperature (ºC)	Time (min)	Stage
1	100	10	Ramp
2	100	20	Hold
3	150	10	Ramp
4	150	10	Hold

Analogously to the oAD method, fortified sesame oil was employed and a FFD was conducted to parameters screening. Then, in this case, the more robust Central Composite Design (CCD) was applied to find the optimum working region. For FFD, different volumes of HNO_3 and H_2O_2 were added to a 500 mg weighted sample mass, and different times of pre-heating were applied, as presented in Table 13. The final volume was adjusted to 50.0 mL with ultrapure water, samples were analyzed by ICP-MS and the ratios “Analyte signal/IS signal" were monitored. Non-linear regression was used to calculate each variable’s coefficients (b_1, b_2, and b_3) and its relevancy to the experimental protocol.

Table 13. Screening factorial design matrix (23) used for selection of the most important variables in the cAD method. The independent variables (X1 to X3), and

their levels (not coded) are shown in the matrix. Trial 9 is the central point, which was prepared in 6 replicates.

Trial	X_1 - HNO_3 volume (mL)	X_2 - H_2O_2 proportion (%)	X_3 – Pre-heating time (min)
1	2.5	0	0
2	7.5	0	0
3	2.5	50	0
4	7.5	50	0
5	2.5	0	60
6	7.5	0	60
7	2.5	50	60
8	7.5	50	60
9 (n = 6)	5.0	25	30

For CCD, only 2 variables were assessed, as shown in Table 14. The ratios “Analyte intensity/IS intensity" were determined in each trial as dependent responses. CCD results were analyzed using regression analysis (Minitab software version 18.1), and the response surfaces were constructed in STATISTICA software version 10.0.0.0.

Table 14. Central Composite Design matrix of experiments (trials) performed to find the optimum working region for the cAD method. Independent variables (from X_1 to X_2, not coded) and their levels (not coded) are shown in the matrix. Trial 9 is

the central point, which was prepared in 5 replicates.

Trial	X_1 - HNO_3 volume (mL)	X_2 – Pre-heating time (min)
1	1.5	10
2	4.5	10
3	1.5	60
4	4.5	60
5	0.9	35
6	5.1	35
7	3.0	0
8	3.0	70
9 (n=5)	3.0	35

3.2.4.3. Dilute-and-Shoot method (D&S)

A fixed sample:solvent ratio of 1:9 m/m was defined herein, and three different organic solvents were investigated: xylene, butanol, and isopropanol. Fortification experiments (7.5 ug L^{-1}) were conducted with an multielement organometallic standard added to 400 mg of sesame oil for performance evaluation and solvent selection. Observational studies were conducted to investigate samples-solvent compatibility, solution stability and phase formation comparing spiked and not-spiked samples. Ecological and economical were also taken into account for diluent selection.

3.2.5. Analytical performance evaluation

Following the Brazilian Health Regulatory Agency (ANVISA) resolution 166/2017 guidelines (AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA (ANVISA), 2017), the methods' analytical performances were evaluated. As DoE requires robustness at non-included parameters, the initial optimization step was conducted with an organometallic standard containing only 10 elements, which can guarantee the most efficient fortification and signal acquisition. At the validation stage, with the already optimized conditions, a 28-element standard mix was prepared with butanol

and employed for fortification at all analytical performance assessments. Analytes scope was expanded, strategic elements were added based on previous literature reports for *Cannabis* and Natural Products. The following isotopes were monitored: ^{107}Ag, ^{27}Al, ^{75}As, ^{197}Au, ^{134}Ba, ^{44}Ca, ^{114}Cd, ^{59}Co, ^{53}Cr, ^{133}Cs, ^{63}Cu, ^{57}Fe, ^{202}Hg, ^{39}K, ^{7}Li, ^{26}Mg, ^{55}Mn, ^{98}Mo, ^{58}Ni, ^{31}P, ^{208}Pb, ^{106}Pd, ^{121}Sb, ^{118}Sn, ^{88}Sr, ^{48}Ti, ^{51}V, and ^{66}Zn.

The linearity, demonstrated by the method's ability to respond proportionally to the concentration of an analyte, was evaluated through the preparation of calibration curves, in triplicate, at nine different concentration levels from 0.1 to 100 µg L^{-1} for most analytes. The curves were conducted for Fe from 50 to 300 µg L^{-1}, P from 50 to 250 µg L^{-1}, and Ba from 100 to 350 µg L^{-1} due to their distinct order of magnitude. The determination coefficient (R^2) and the data homoscedasticity (Cochran test) were evaluated.

The sensitivity, instrumental limits of detection (LOD_{inst}) and limits of quantification of the method (LOQ_{met}) were estimated for the analytes in each sample preparation method. The LOD_{inst} is the lowest concentration of the analyte present in a solution that can be detected but not accurately quantified by a method. It was calculated by Equation 8 and reported as µg L^{-1} of the analyte in the sample solution. The LOQ_{met} is the lowest concentration of the analyte in a sample that can be determined with acceptable precision and accuracy. It is reported as µg kg^{-1} of the analyte in the sample, calculated by Equation 9.

$$LOD = 3.3\ x\ \frac{standard\ deviation\ of\ intercept}{analytical\ curve\ slope} \quad \textbf{(Equation 8)}$$

$$LOQ = 10\ x\ \frac{standard\ deviation\ of\ intercept}{analytical\ curve\ slope}\ x\ Dilution\ Ratio \quad \textbf{(Equation 9)}$$

For each sample preparation method, calibration curves were prepared following an established working range (based on linearity and sensitivity

assessments) at three different calibrations approaches: (i) by aqueous acid analytical solutions (HNO_3 10 % v/v) for External Standard (ES) calibration; (ii) by Matrix Matching (MM) calibration approach employing sesame oil to mimic the samples matrix; and (iii) by Standard Addition (SA) employing one *Cannabis* oil sample. Angular coefficients were compared for adequate matrix effect investigations and evaluated by F-test for variance equality and Student's t-test (n=6; $\alpha = 0.05$) for analytical inclination equality.

For precision, defined as the closeness between the results of a sample obtained through the analytical method, intra-assay repeatability was estimated through the preparation of triplicates of the samples spiked with a multielement standard at two concentration levels (i.e., low and high), defined from linearity and working range. The results were evaluated through the relative standard deviation (RSD, %) of the concentrations obtained from 3 replicates of the samples prepared and injected by a single analyst, on a single day and on the same equipment.

Accuracy, defined as the degree of agreement between individual results obtained by the method under study and a value accepted as true, was evaluated through recovery assays of analytes in triplicates of the samples at two added concentration levels (i.e., low and high), defined according to the linearity and working ranges. The obtained concentration was compared to the added concentration and the recovery reported as % of the added concentration.

The developed and optimized methodology will be applied for the analysis of the six *Cannabis* oil extracts, prepared in triplicate. As a baseline report, sesame oil is also analyzed in triplicate. Shapiro-Wilk's test was applied for data normality assessment, and multiple Student's t-tests conducted for the samples and the sesame oil comparison. Spearman correlation coefficients were also calculated.

3.4.6. Ecological performance evaluation

The open software "AGREE: The Analytical Greenness Calculator" was applied to evaluate methods' ecological performance. Based on the 12

principles of green analytical chemistry, an “ecoscale” from 0.0 to 1.0 was assigned and greenness was predicted for the developed methods.

4. Research Article 1 – Phytocannabinoid quantification by UHPLC-HRMS/MS

This section is based on the research article “Phytocannabinoids quantification in *Cannabis* extracts of by UHPLC-HRMS/MS: A multivariate optimization approach for plant-based medicines analysis”, submitted at Journal of Biomedical and Pharmaceutical Analysis (Impact Factor 3.1).

This research article was submitted on July 18, 2024 and is currently under review, undergo by manuscript number JPBA-D-24-01975.

Phytocannabinoids quantification in *Cannabis* extracts of by UHPLC-HRMS/MS: A multivariate optimization approach for plant-based medicines analysis

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Abstract

Cannabis market is in a global steady growth and *Cannabis* herbal extracts (CHE) are one of the most interesting and sought products for therapeutic approaches of a diverse number of clinical conditions. The medical potential of *Cannabis* is mainly attributed to phytocannabinoids biosynthesis, and cannabidiol (CBD), tetrahydrocannabinol (THC) and cannabinol (CBN) are highlighted as major targets of pharmaceutical and

phytotherapy concern. Advanced analytical techniques, such as UPLC-HRMS/MS, portrays powerful alternatives to their monitoring and consequently quality assurance of *Cannabis*-based products. However, further analytical steps need to be critically optimized to keep up with instrumental potential. A CBD, THC and CBN quantification method by UHPLC-MS/MS was developed herein and optimized by Design of Experiments (DoE) by the first time, from the best of our knowledge. Instrumental parameters and sample preparation were explored for maximum performance. Electrospray ionization source was refined to ionization relative variation of 2%, 2% and 5% for CBD, THC and CBN, respectively, while a ultrasound-assisted liquid-liquid extraction with methanol:hexane 9:1 v/v was polished to a compromise condition (6.7 mL extraction mixture volume, 18 min of agitation time and 25 min of sonication time) that maximized these analytes signals to over 80% at the same time. The methods response was validated within regulatory criteria for Brazilian pharmaceuticals and satisfactory parameters of linearity, accuracy, precision, sensitivity and matrix effects were demonstrated, especially when combined to a *Matrix Matching* calibration approach. The mathematical model predictability was cross-validated and credible responses were observed (86% – 120%). A proof-of-concept was conducted by analytical application to a 4-samples batch and its application presented herein arose a significant concern relative to CHE products, as major discrepancy between label description and quantified content (over 100,000-times lower) was observed, not only compromising the therapeutic potential, but also revealing a consumer's blindspot to these crutial natural medicine input. Herein, the method process knowledge was fully detailed with great predictability and its performance was designed to maximum response, with adequate analytical and pharmaceutical requirements. DoE provides a simple, reliable, and effective approach to achieve multivariate optimizations of risk assessment methods for *Cannabis* and many other natural phytotherapy inputs.

Keywords

Cannabis; Design of Experiments; Multivariate Optimization; Liquid Chromatography; Mass spectrometry

4.1. Introduction

Cannabis is a highly variable, complex, and polymorphic plant genus, originated from Eurasia, being currently distributed worldwide in variable habitats, climates, and soils conditions (ALIFERIS; BERNARD-PERRON, 2020b). Its commercial potential is historically known for more than 5000 years, including for paper manufacturing, fiber production, and even medicine (HAZZAH et al., 2020). *Cannabis* market is experiencing an impressive growth, with continuous projections of expansion(BEN AMAR, 2006)**.** At the moment, more than 500 different derivative products are commercially available. In therapeutical scenarios, *Cannabis* herbal extracts (CHE) are highlighted as the most interesting and sought products for a variety of applications in both human and animal healthcare (CHICOINE et al., 2020).

Positive feedback and improvement in health conditions are also extensively reported for antiemetic effect(BEN AMAR, 2006; CHICOINE et al., 2020), appetite stimulation (BEN AMAR, 2006; LANDA; SULCOVA; GBELEC, 2016), analgesia (BEN AMAR, 2006; CHICOINE et al., 2020; LANDA; SULCOVA; GBELEC, 2016), multiple sclerosis (BEN AMAR, 2006; CHICOINE et al., 2020), spinal cord injuries (BEN AMAR, 2006)**,** (CHICOINE et al., 2020), Tourette's syndrome (BEN AMAR, 2006), epilepsy (CHICOINE et al., 2020; LANDA; SULCOVA; GBELEC, 2016), glaucoma (BEN AMAR, 2006; CHICOINE et al., 2020), Parkison Disease (BEN AMAR, 2006; CHICOINE et al., 2020), dystonia (BEN AMAR, 2006)**,** osteoarthritis (CHICOINE et al., 2020; DELLA ROCCA; DI SALVO, 2020)**,** cancer (CHICOINE et al., 2020), inflammation(CHICOINE et al., 2020) and Alzheimer's Disease (CHICOINE et al., 2020).

The psychoactive and therapeutical potential of *Cannabis* is mainly

attributed to phytocannabinoids, that are bioactive terpene phenolic substances, and major metabolites produced in female inflorescence's glandular trichomes. Cannabidiol (CBD) and tetrahydrocannabinol (THC), isomers compounds with different affinities to CB1 and CB2 receptors of the endocannabinoid system (ECS), are the two most explored phytocannabinoids for different therapeutical applications. ECS modulates essential regulatory functions for the human body and its biological balance, such as appetite and digestion, energy balance, sleep patterns, immune status, inflammation, and emotional responses (HAZZAH et al., 2020).

CBD is a partial agonist at CB2 and an antagonist at CB1 receptor, with anxiolytic and antidepressant effects, while THC is a partial agonist at CB1 and CB2 receptors with major therapeutical effects of analgesia, muscle relaxation, sleep support and anticonvulsant properties. Both act in synergy for clinical approaches and are ideal targets for *Cannabis* product's potency monitoring. Also, cannabinol (CBN) is an oxidative by-product of THC formed by the exposition to light and/or heat. Despite CBN having a weaker affinity to CB1 and CB2 receptors, its administration together with THC promotes greater therapeutic action by Entourage Effect. As CBN is also found in aged and/or degraded products (HAZZAH et al., 2020), its monitoring is important as a stability indicator.

Pharmaceutical sector and bioanalytical studies are process-based and quality-oriented (POLITIS et al., 2017). Therefore, quality cannot only be tested into the final product, but needs to be taken into account during planning and development stages. In this context, Design of Experiments (DoE) is a well-established statistical approach that enables implementation of these pursued optimum quality in research conditions in a quick, effective, and economical way, promoting operational excellence in the whole analytical process. Thus, this work aims to propose a multivariate optimization, by DoE, for CBD, THC and CBN quantification in CHE by ultra high-performance liquid chromatography coupled to high resolution tandem mass spectrometry (UHPLC-HRMS/MS). The HRMS/MS, with the association of a Full-MS acquisition and a second stage of mass fragmentation, enables the quantification of target substances as well as an

exploratory analysis. The possibility of retrospective search for the assessment of candidates for new formulations is a differential that emphasizes the importance of using state-of-the-art analytical techniques in such context.

4.2. Mass spectrometry identification and chromatographic separation

Collision-induced dissociation (CID) experiments were performed to identify specific fragments for the analytes and to corroborate the data published previously in the literature (BIJLSMA et al., 2011; CITTI et al., 2019; DOS SANTOS et al., 2019; HEHET et al., 2022). The ESI(+)MS/MS spectra are shown in Supplementary Material S1 and its major information are summarize in Table 15.

CBD and THC are constitutional isomers, with similar fragmentation profiles. A neutral loss of 122 Da (*m/z* 315 → 193) is monitored as quantifier ion, that may represent a ether function breaking and a terpene ring cleavage (CITTI et al., 2019; DOS SANTOS et al., 2019), while losses of 56 Da (*m/z* 315 → 259) and 180 Da (*m/z* 315 → 135) are monitored as qualifier ions, standing for pentyl groups cleavage and a terpene moiety commonly found in cannabinoids at positive ionizations mode, respectively (CITTI et al., 2019; DOS SANTOS et al., 2019)**.**

In positive mode, CBN (*m/z* 311) provides a quantifier product ion at *m/z* 223 (80 Da loss), probably given by a CBN-dehydrated without a lateral pentyl group (LELARIO et al., 2021) and a qualifier product ion at *m/z* 241 (70 Da loss) due to aliphatic 5-carbon chain cleavage (FERRER, 2020; LELARIO et al., 2021). Another qualifier product ion of *m/z* 195 was monitored, consequential of a resorcinol moiety and one carbon atom or sequential pentyl lateral chain and two methyl groups losses of a dehydrated CBN (LELARIO et al., 2021).

A 46 Da loss (*m/z* 348 → 302), a 152 Da loss (m/z 348 → 196) and a 18 Da loss transition (*m/z* 348 → 330) were monitored for THC-COOH-D3, while analogues transitions (*m/z* 354 → 308, 196, and 336) were

monitored for THC-COOH-D9. The first one suggests a six-member saturated ring opening (BIJLSMA et al., 2011) while the common *m/z* 196 ion for both THHC-COOH-D3 and THC-COOH-D9 stands for a terminal Cd_3 loss from the latter. A typical 18 Da mass loss suggests a common and non-specifical H_2O loss.

Table 15. Monitored compounds in this work, their molecular formulas, mass transitions, (N)CE applied and in-agreement literature reports.

Compond	Molecular formula [M]	Precursor ion (*m/z*) $[M+H]^+$	Mass error (ppm)	(N)CE (%)	Product ion (*m/z*) $[M+H]^+$	Mass error (ppm)	Reference
CBD	$C_{21}H_{30}O_2$	315.2318	2.85	40	193.1223[a]	2.88	1,2
					259.1691[b]	2.85	1,2
					135.1168[b]	2.87	2
THC	$C_{21}H_{30}O_2$	315.2318	1.40	40	193.1223[a]	1.38	1,2
					259.1692[b]	1.44	1,2
					135.1168[b]	1.40	2
CBN	$C_{21}H_{26}O_2$	311.2006	2.73	37	223.1174[a]	2.74	2
					241.1222[b]	3.00	2
					195.1168[b]	2.22	2
THC-COOH-D3	$C_{21}H_{25}D_3O_4$	348.2249	1.26	35	302.2194[a]	1.40	3
					196.1411[b]	1.05	3
					330.2143[b]	1.50	3
THC-COOH-D9	$C_{21}H_{19}D_9O_4$	354.3225	1.19	35	308.2570[a]	1.52	6
					196.1411[b]	1.99	6
					336.2520[b]	1.18	6

a = quantifier product ion; b = qualifier product ion.

Regarding chromatographic separation, the UHPLC method developed herein with a reversed phase C18 column (ACE UltraCore 2.5 SuperC18, 50 mm x 2,1 mm; 2,5 µm) and a gradient of water (added of 5 mM ammonium formate and 0.1% formic acid) and methanol (with 0.1% formic acid) provided a satisfactory separation of all analytes, mainly considering CBD and THC isomers. Typical extracted-ion chromatograms are presented in Figure 5, obtained in solvent solutions, with major peaks detected at retention times (RT) of 8.15, 8.70, 8.52, 8.04, and 8.03 min for CBD, THC, CBN, THC-COOH-D3 and THC-COOH-D9, respectively. A comparison of chromatograms obtained either in solvent, and in coconut oil showed no variation in retention times. A short chromatographic run time (11.1 min) was achieved with RT reproducibility within 0.1 % RSD.

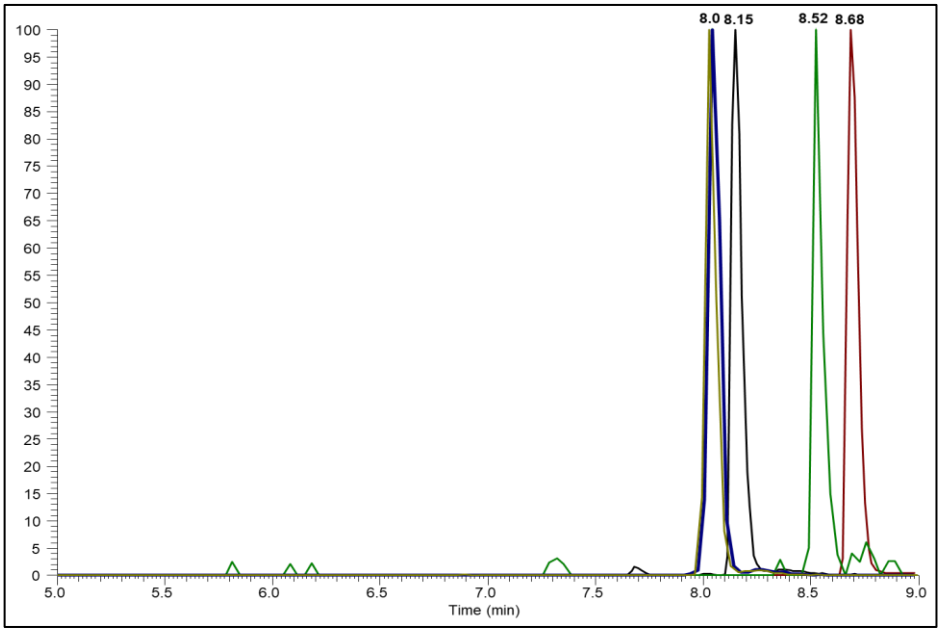

Figure 5. Extracted-ion chromatograms for each analyte monitored in this work and their retention time. Data illustrated, in order, as THC-COOH-D3 (yellow), THC-COOH-D9 (blue), CBD (black), CBN (red) and THC (green).

4.3. ESI optimization and ISTD evaluation

ESI is a well-established atmospheric pressure ionization for substances with medium and high polarity, from low to high molecular mass, including protein analysis with generation of multivalent ions. As ESI

efficiency is directly related to a method's sensitivity and reproducibility, its optimization is a critical step for a method development, which is necessary for optimal detection and/or quantification of the analytes. In this context, multivariate approaches, such as DoE, provides a useful and powerful tool for a faster, simpler, and more economical development, especially for complex matrices, in alternative to the traditional one-factor-at-a-time optimization (GRUENDLING; GUILHAUS; BARNER-KOWOLLIK, 2009)**.** In this work, a Plackett-Burman Design (PBD) for 7 factors were applied for the determination of the optimum ionization conditions.

By the PBD approach, only 8 experiments were needed to evaluate 7 different ionization source parameters, and its impact on ionization phenomena of the analytes. This design screens out unimportant factors, however not taking into knowledge factors interactions, and helps to sort out which factors to concentrate on, reducing the amount of data that needs to be collected for a prime investigation (VANAJA; RANI, 2007b)**.**

As the series of trials were conducted with scrambled combinations of the ion source parameters, the CV% of each ratio "Analyte Area/ISTD Area", with two different ISTD candidates, were evaluated and the results are summarized in Figure 6. In general, THC-COOH-D9 provided greater CV% for all analytes in most of the tested ionization conditions. However, particularly in trial 3, established conditions (350 °C auxiliar gas heater temperature; 250 °C capillary temperature; 4 kV for electrospray voltage; 30 a.u. for sheath gas; 10 a.u. for auxiliary gas; 80 V for S-lens; and 1 a.u. for sweep gas) THC-COOH-D9 granted the greatest instrumental precision observed for all three analytes, with satisfactory CV% of 2%, 2% and 5% for CBD, THC and CBN, respectively. Thus, this was the locally optimized operational condition selected for this work.

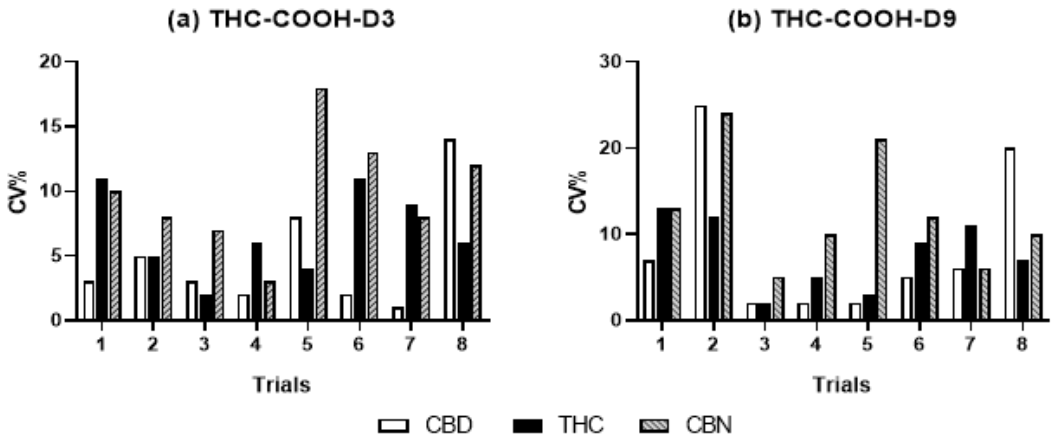

Figure 6.Coefficient of Variation (CV%) of “Analyte area/ISTD area” ratios for each ESI conditions’ trials (1 to 8) and each ISTD candidate (THC-COOH-D3 and THC-COOH-D9).

4.4. Selection of sample preparation parameters

Several factors can influence the efficiency of a liquid-liquid extraction (LLE) protocol as proposed herein, such as solvent volume, solvent polarity, pH, temperature, extraction time, sample/solvent ratio, ultrasonic assistance, and many others (LUQUE DE CASTRO; PRIEGO-CAPOTE, 2007; MAZZOLA et al., 2008). Therefore, a screening of operational parameters was conducted to identify the significant ones in the sample preparation method. A Full Factional Design was conducted (Table 7) and 3 parameters (agitation time in shaker, sonication time and extraction mixture volume) were investigated.

While LLE depends on mass transfer of components between a two immiscible liquid phases system, agitation time and ultrasound (US) time portrays major parameters to be optimized with great impact in LLE efficiency. Greater agitation generally increases LLE efficiency. However, oily extracts are not fully soluble in methanol, and its system agitation promotes stables emulsifications, a non-pursued case for an efficient LLE protocol (PROTTI et al., 2019)**.** Besides that, and excessively high agitation speed can decrease extraction efficiency due to back-mixing. Ultrasonic bath system can effectively stir a system by cavitation without compromising

its chemical properties, while accelerate mass transference and emulsion destruction. Nevertheless, operational refinement is need due to possible emulsion formation (PROTTI et al., 2019).

LLE efficiency is also strongly related to the sample-solvent volume ratio used. High recoveries are typically obtained when large volumes of organic solvent are employed. However, analytical sensitivity is compromised at greater dilution factors (BERMAN et al., 2018; “Chapter 8 Liquid-liquid extraction: High throughput techniques”, 2003; MAZZOLA et al., 2008)**.** This parameter refinement not only represents a major improvement in protocol’s efficiency but also in method’s cost.

From the experimental batch conducted, non-linear regressions were calculated for each analyte dataset and results are reported at Table 16.

Table 16. Analysis of Variance (ANOVA) parameters, calculated coefficients, and their p-value of screening trials for each analyte. Significant parameters are underlined in bold.

	CBD		**THC**		**CBN**	
R^2	0.89835		0.98303		0.98571	
R^2 (Adj.)	0.7357		0.95587		0.96284	
	Coefficient	**p-value**	**Coefficient**	**p-value**	**Coefficient**	**p-value**
Curvature	0.015040	0.000000	0.028643	0,000000	0.043007	0.000000
b_o	**0.011825**	**0.00000318**	**0.019417**	**0.00000123**	**0.028333**	**0.00000116**
b_1	0.000110	0.818684	**-0.003001**	**0.004565**	**-0.004349**	**0.004472**
b_2	-0.000957	0.088661	**-0.004502**	**0.000747**	**-0.007873**	**0.000303**
b_3	0.000397	0.421415	0.000425	0.520152	-0.000223	0.811276
$b_{1,2}$	**0.001423**	**0.025810**	**0.005872**	**0.000214**	**0.009379**	**0.000131**
$b_{1,3}$	-0.000550	0.279443	0.000556	0.407678	0.001043	0.292942
$b_{2,3}$	-0.000055	0.908308	**0.001902**	**0.027101**	0.002009	0.072983
$b_{1,2,3}$	0.001120	0.056671	**0.002298**	**0.013496**	0.002308	0.048208

Coefficients b_1 and b_2, respectively, representative of agitation time and ultrasound time were statistically significant only for THC and CBN, both with negative effects in analytes' normalized signal. Therefore, minimum amounts of both operational parameters were suggested in an optimized protocol to maximize analytical signals. Besides that, their second-order interaction were also significant for all three analytes, reiterating the importance of their inclusion in the next step of sample preparation optimization protocol.

Coded as coefficient b_3, extraction mixture volume was not found statistically significant for any analyte herein. However, a second-order interaction with b_2 and a third-order interaction with b_1 and b_2 was found significant only for THC. However, THC concentrations plays a critical role in *Cannabis* industry. In medicinal and edible products, THC content is often related to the medicine potency and is commonly regulated with established thresholds. In this light, we chose to also incorporate this parameter in a further experimental optimization step, besides its low global contribution.

4.5. Sample preparation multivariate optimization

Agitation time, ultrasound time and extraction mixture volume optimization are not only critical to analytical signal maximization but also are strongly related to the sample preparation cost and time-length. Therefore, operating in their minimum amounts are desirable for defining a low-cost and high-frequency protocol.

As a 3-factor BBD was conducted (Table 8), the model for simultaneous CBD, THC and CBN analytical signals' as a function of agitation time, ultrasound time and extraction mixture volume was assessed. Its results, individual coefficients and model's figure of merit are summarized in Supplementary Material. Besides that, the composed desirability and its two-dimensional (2D) surface responses are also presented in Figure 7.

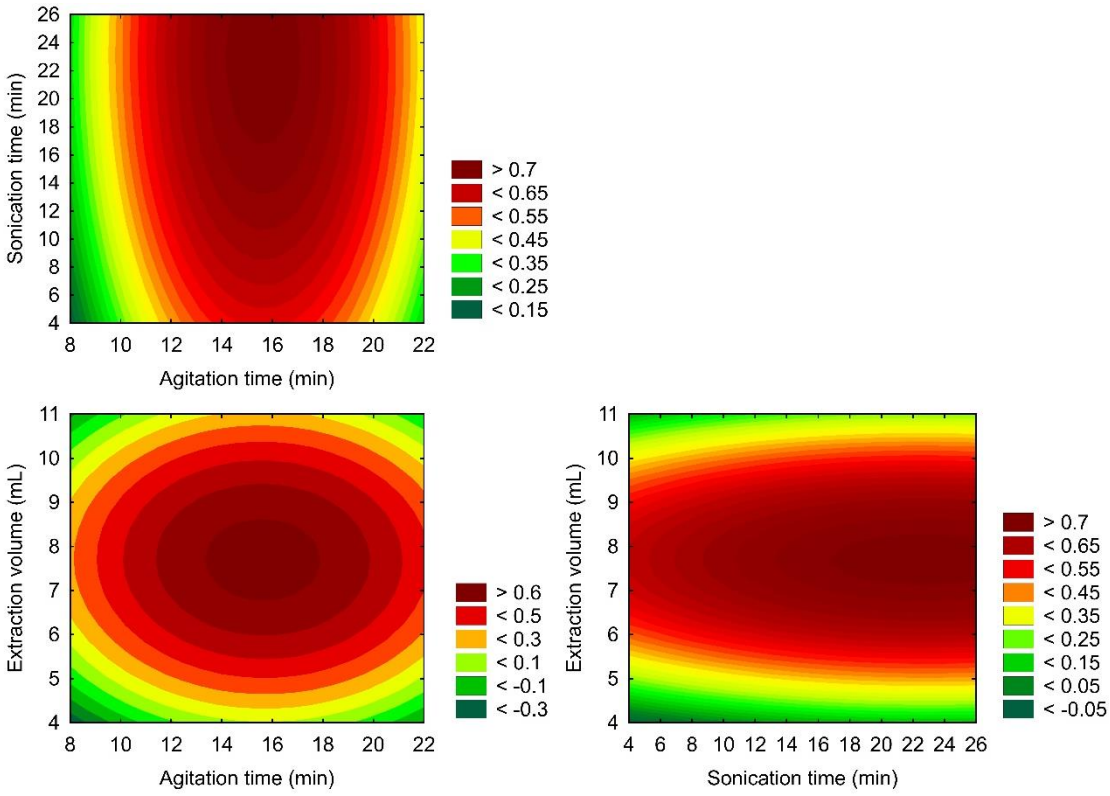

Figure 7. Two-dimensional (2D) surface response of CBD, THC and CBN analytical signal for sample preparation optimization.

As shown in Figure 7, the optimal region for analytes response is comprised around 18 min of agitation time, 25 min of ultrasound time and 7.00 mL of extraction mixture volume, representing an individual optimization rate of 89%, 80% and 82% for CBD, THC and CBN, respectively. Therefore, a successful mean global optimization rate of 84% was achieved.

Carvalho et al. (2020) proposed a similar UA-LLE sample protocol for *Cannabis* herbal extracts, however employing 10 mL of extraction mixture and 30 min of ultrasound assistance (CARVALHO et al., 2020c). Berman et al. (2018) achieved to establish a metabolomic profile from *Cannabis* inflorescences with 10 mL of ethanol as extraction solvent and 30 min of ultrasound assistance (BERMAN et al., 2018) while Protti et al. (2019) managed to extract cannabinoids by applying 10 mL of pure methanol, but 30 min of ultrasound assistance and another 15 min of

agitation were needed (PROTTI et al., 2019). Our findings represent a faster, cheaper, and simpler protocol in relation to previous reports from literature, with statistically maximized performance. To the best of our knowledge, this is the first study to approach multivariate optimization in sample preparation protocols of *Cannabis* extracts by DoE.

In order to verify the adequacy of the predictive model constructed, experimental values from the Box-Behnken Design were compared to model's prediction ones. Results are summarized in Table 10. Model's accuracy were around 86 – 120 % for CBD, 89 – 110 % for THC and 95 – 104 % for CBN. These findings corroborate with the adequacy of the constructed model for all analytes.

Table 17. Validation of model predictability for quantification of CBD, THC and CBN in *Cannabis* oils by LC-HRMS/MS.

Trial	CBD		THC		CBN	
	Exp.	Predicted (% Accuracy)	Exp.	Predicted (% Accuracy)	Exp.	Predicted (% Accuracy)
1	0.009	0.01 (111)	0.012	0.012 (100)	0.028	0.028 (100)
2	0.007	0.006 (86)	0.012	0.011 (92)	0.031	0.031 (100)
3	0.006	0.007 (117)	0.010	0.011 (110)	0.032	0.032 (100)
4	0.012	0.011 (92)	0.016	0.015 (94)	0.039	0.038 (97)
5	0.007	0.006 (86)	0.009	0.008 (89)	0.024	0.025 (104)
6	0.007	0.008 (114)	0.009	0.010 (111)	0.021	0.023 (110)
7	0.008	0.007 (88)	0.011	0.010 (91)	0.024	0.023 (96)
8	0.005	0.006 (120)	0.011	0.011 (100)	0.034	0.033 (97)
9	0.007	0.007 (100)	0.007	0.007 (100)	0.019	0.018 (95)
10	0.009	0.009 (100)	0.014	0.013 (93)	0.037	0.036 (97)
11	0.009	0.009 (100)	0.014	0.014 (100)	0.034	0.035 (103)
12	0.007	0.008 (114)	0.010	0.010 (100)	0.026	0.027 (104)
13	0.010	0.010 (100)	0.015	0.015 (100)	0.039	0.039 (100)

4.6. Analytical performance validation

4.6.1. Selectivity

By evaluating multiple vegetable oils commonly used in *Cannabis* extracts manufacturing, selectivity was demonstrated. There was no evidence of matrix components interfering with the retention times or mass transitions of monitored analytes herein. No peaks for any mass transitions monitored were detected in blank vegetable oils.

4.6.2. Linearity and Homoscedasticity

6-points analytical curves were constructed by three different calibrations strategies: by external standard (ES), by matrix matching (MM); and by standard addition (SA). All analytes provided $R^2 > 0.99$ in a concentration range between 25 – 150 ng g^{-1}. Cochran's C-test indicates homoscedasticity ($C_{critical} = 0.616$; 95% confidence level) for all analytes in all three calibration approaches.

4.6.3. Matrix effect

Angular coefficients from 6-points analytical curves constructed in triplicates by all three calibration methods were compared for matrix effects investigation. ES angular coefficients (0.0022 ± 0.0001 for CBD; 0.0023 ± 0.0002 for THC; and 0.0044 ± 0.0003 for CBN), showed statistically significant difference from SA angular coefficients (0.0027 ± 0.0008 for CBD; 0.0028 ± 0.0006 for THC; and 0.0047 ± 0.0008 for CBN), while no matrix effect was observed in MM angular coefficients (0.0014 ± 0.0001 for CBD; 0.0014 ± 0.0001 for THC; and 0.0026 ± 0.0002 for CBN) when compared to SA angular coefficients. Therefore, due to its great similarity to real sample's matrix, powerful compensation of matrix effects and adequate analytical reliability, Matrix Matching calibration approach was applied herein.

1.1.1. Precision and Accuracy

Satisfactory precisions and accuracy parameters were obtained for all three analytes in all three concentration levels approached, as seen at

Table 18. Thus, reassuring the method’s pertinence to the analytical question herein.

Table 18. Intra-day precisions (n=3; two analysts' datasets), Inter-day precision (n=6), and recovery for each analyte. Data reported as %RSD for precisions parameters and as % for the accuracy parameter.

	CBD			**THC**			**CBN**		
Level (ng g^{-1}**)**	**50**	**100**	**150**	**50**	**100**	**150**	**50**	**100**	**150**
Intra-assay precision (%)	4	4	7	14	6	7	13	14	6
Inter-assay precision (%)	7	10	15	14	7	14	9	15	13
Recovery (%)	86	84	98	69	77	80	76	86	90

4.6.4. Sensitivity

Limits of Detection (11, 13 and 8 ng g^{-1}) and Limits of Quantification (33, 39 and 23 ng g^{-1}) were estimated for each CBD, THC and CBN, respectively. More than 1,000,000-fold lower than expected concentrations in real samples and, therefore, expressing an adequate quantitative analytical approach.

4.6.5. Carryover

All analytes were not inclined to carryover when spiked at a concentration of 300 ng g^{-1}. Analytical blanks injected after the concentrated sample were absent of CBD, THC or CBN peaks in monitored mass transitions.

4.6.6. Robustness

Novel adjusted conditions promoted less than 8% variation in analytes normalized intensities. As equivalent analytical performances were observed, method's robustness was assured.

4.7. Analytical application for medicine veterinary extracts

4 herbal extracts of *Cannabis* were investigated herein. Label information of these products disclosed the presence of 6 mg g^{-1} of CBD and 0.3 mg g^{-1} of THC. 20:1 CBD (CHICOINE et al., 2020):THC formulations are common applied in clinical applications, designed to provide tranquilizers and sedatives benefits of CBD while minimizing psychoactive effects of THC. Previous studies reported generally well-tolerance to CBD-rich extracts by dogs and cats, with no strong adverse effects observed (CHICOINE et al., 2020; KULPA et al., 2021; VAUGHN; KULPA; PAULIONIS, 2020).

While many studies focus on potential health benefits, pharmacokinetic and safety of *Cannabis* veterinary medicine, the quality control of these treatment products must be emphasized to guarantee its correct therapeutical delivery. Our findings indicates that very low amounts

of CBD (66 ± 7 g g^{-1}) were detected in only half of the samples analyzed, while no THC or CBN was found in any of them. Dosed amounts of CBD were below 0.001% of its label content, virtually not differing from a placebo and exhibiting no therapeutical potential. Besides that, the absence of CBN, a degradation product of THC, even on trace levels denies the hypothesis of THC natural degradation and suggests that none THC was present in any level from the beginning of this formulation.

4.8. Conclusion

Cannabis therapy portrays a versatile and in-steady growth clinical approach in modern medicine. Its efficiency is modulated by phytocannabinoids contents and their entourage effects, therefore CBD, THC and CBN are remarkable target items to be monitored in quality controls methods. Advanced analytical techniques, such as LC-HRMS/MS, portrays powerful alternatives to phytocannabinoids detection and quantification, however, further analytical steps, such as sample preparation and instrumental conditions, needs to be critically optimized to keep up with instrumental performance.

Design of Experiments provided a fast, simple, reliable, and effective approach to optimize the method developed herein. Innovative optimization approaches, such as DoE, are needed in modern study fields, especially in pharmaceutical and bioanalytical applications. In the proposed method, significant operational parameters were identified and globally maximized. Besides that, analytical performance was successfully validated for pharmaceutical requirements and satisfactory analytical parameters were achieved.

The proposed method demonstrated adequate precision, accuracy and sensitivity for the analytical problem addressed, and not only showed viability to veterinary CHE analysis but also for future applications in humans CHE. Its application in real samples unraveled a significant concern in these products' risk assessment. A major discrepancy between label content and quantified content was observed, not only compromising its therapeutic potential but also revealing a consumer's blindspot. Taking into account that, this end-user typically belongs to a sensible clinical trial and

Cannabis medicine represents a unique options of comfort guarantee or even palliative care, CHE’s quality control takes place as a major piece in the compromise to these patient’s life and dignity.

5. Research Article 2 – Multielement analysis by ICP-MS

This section is based on the research article “Development of a method for multi-elemental analysis of medicinal *Cannabis* oil extracts by inductively coupled plasma mass spectrometry”, submitted at Microchemical Journal (Impact Factor 4.9).

This research article was submitted on August 18, 2024 and is currently under review, undergo by manuscript number MICROC-D-24-05454.

Development of a method for multi-elemental analysis of medicinal *Cannabis* oil extracts by inductively coupled plasma mass spectrometry

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Abstract

The *Cannabis* oil extract is an in-expansion medicinal input, indicated for several clinical conditions. Some countries have already regulated quality control parameters for *Cannabis* products. Among them, the presence of metals and metalloids, which are usually in low concentrations in oil samples due to the low solubility, unless when in an organometallic form. Also, an eventual contamination by a toxic element in medicinal *Cannabis* products would be a critical exposure to someone in a health condition. The quantification of elemental contaminants in herbal oily extracts is still a challenging analytical approach due to the matrix

complexity, the presence of different species of the analytes, the potential risks associated to the decomposition of oil samples or working with organic solvents, and the presence of interferences due to the organic matrix, among other problems. In this work, three sample preparation methods (open-flask acid decomposition on a heating plate - oAD, acid decomposition on closed vessels - cAD, and dilute-and-shoot with organic solvent - D&S) were developed for the multielement determination in *Cannabis* oil extracts by inductively coupled plasma mass spectrometry (ICP-MS). The analytical performances of the proposed sample preparation methods were critically evaluated regarding accuracy, precision, sensitivity, and ecological performance. For the first time, to the best of our knowledge, Design of Experiments (DoE) was approached to optimize the sample preparation methods for multielement analysis of *Cannabis* extracts. The method employing the digestion of the samples with diluted HNO_3 in open flasks and a matrix matching calibration approach provided the overall best performance and was applied to analyze 6 *Cannabis* herbal oil extracts and one sesame oil, which was employed to mimic the diluent of the samples, not provided. In general, low metal and metalloid contents were found in the samples and, by comparing *Cannabis* oil extracts and the sesame oil, statistically significant differences were identified for Au, Cu, K, Li, Mg, Mn, Ni, Pb, Ti, and Zn. Lead was found at higher levels in all *Cannabis* extract samples, in a range from 11.7 to 12.4 μg g^{-1}, higher than the limit recommended by the United States Food and Drug Agency (FDA) for potentially toxic elements. Among the *Cannabis* extract samples, Li, Mg, Mn, Ni, Ti, and Zn presented significant differences, suggesting a relevant heterogeneity and non-standardized quality control for these products concerning elemental contaminants.

Keywords

Cannabis; Design of Experiments; Sample Preparation; Inductively Coupled Plasma Mass Spectrometry.

5.1. Introduction

Cannabis is a highly variable, complex, and polymorphic plant genus originating from Eurasia and currently distributed worldwide in variable habitats, climates, and soil conditions (ALIFERIS; BERNARD-PERRON, 2020b; DOS SANTOS; ROMÃO, 2023). Its commercial potential has been historically known for more than 5000 years, and its market is experiencing impressive growth, with continuous projections of expansion (HAZZAH et al., 2020). In therapeutical scenarios, *Cannabis* herbal extracts (CHE) are highlighted among the most interesting and sought *Cannabis*-based products for a variety of applications in both human and animal healthcare.

CHE is a generic term for extracts made from harvested and dried female flowering tops, resinous flowers, and small leaves of the *Cannabis* plant, with high concentrations of cannabinoids, such as cannabinol (CBD) and tetrahydrocannabinol (THC), and a triglyceride-rich vehicle, commonly sesame oil, coconut oil, and olive oil (OFFICE ON DRUGS AND CRIMES, 2023). Several improvements in health conditions by *Cannabis* administration are extensively reported for antiemetic effect (BEN AMAR, 2006; CHICOINE et al., 2020), appetite stimulation (BEN AMAR, 2006; LANDA; SULCOVA; GBELEC, 2016), analgesia (BEN AMAR, 2006; CHICOINE et al., 2020; LANDA; SULCOVA; GBELEC, 2016), multiple sclerosis (BEN AMAR, 2006; LANDA; SULCOVA; GBELEC, 2016), spinal cord injuries (BEN AMAR, 2006; LANDA; SULCOVA; GBELEC, 2016), Tourette’s syndrome (BEN AMAR, 2006), epilepsy (BEN AMAR, 2006; CHICOINE et al., 2020; DELLA ROCCA; DI SALVO, 2020; LANDA; SULCOVA; GBELEC, 2016), glaucoma (BEN AMAR, 2006; LANDA; SULCOVA; GBELEC, 2016), Parkison Disease (BEN AMAR, 2006; LANDA; SULCOVA; GBELEC, 2016), dystonia (BEN AMAR, 2006), osteoarthritis (CHICOINE et al., 2020; DELLA ROCCA; DI SALVO, 2020), cancer (LANDA; SULCOVA; GBELEC, 2016), inflammation (LANDA; SULCOVA; GBELEC, 2016), and Alzheimer’s Disease (LANDA; SULCOVA; GBELEC, 2016).

As judicial and social advances occur regarding the regulation of *Cannabis*, risk assessment methods and an efficient quality control are needed. Metals and metalloids are critical components of these inputs, not only the potentially toxic contaminants capable of bioaccumulation such as

Pb, Cd, Hg, and As, but also essential elements that can be harmful in high concentrations such as Zn, Mg, Fe, and Cu (MILAN; MICHALSKA; JUROWSKI, 2024)**.** *Cannabis* has a remarkable capacity to absorb metals and metalloids not only from its natural surroundings, such as soil, air, and water, but also from contamination sources, i.e., wastewaters, sewage sludge, and organic waste (MILAN; MICHALSKA; JUROWSKI, 2024). Furthermore, the production of its derivatives, obtained from mechanical and chemical operations, like cutting, grinding, and extraction, can often introduce elemental pollutants from machinery and reagents that remain in the final product (MILAN; MICHALSKA; JUROWSKI, 2024)**.**

Despite *Cannabis*-based products benefits, there is a global lack of standardized toxicological regulatory delimitations. Limits regarding potential and essentials elements usually pertain to the final products route of administration, such as ingestion, topical application, or inhalation. Each jurisdiction differs between countries or states, however, many of these limits are in accordance with the recommendations provided in the USP <232>/ICH Q3D guidelines for contaminants in herbal medicines, typically measured in terms of micrograms per gram (μg g^{-1}) (MILAN; MICHALSKA; JUROWSKI, 2024; THE INTERNATIONAL COUNCIL FOR HARMONISATION, 2015; UNITED STATES PHARMACOPEIA, 2017).

The elemental determination, usually carried out by spectrometric techniques, is challenging in oil samples, such as CHE, due to its high organic matter content. It requires a specific sample introduction system, introduces several interferences, causes carbon deposition and instrumental performance loss, and increases maintenance costs (DAMAK et al., 2019). For these reasons, the sample preparation is a critical analytical step in the elemental analysis. Many sample preparation strategies are available, i.e., wet digestion, which is mostly preferred for most samples, (micro) emulsification and (ultrasound-assisted) extraction, rather employed for oil samples, as well as the dilutions in organic solvents, and the less common dry ashing, each one with advantages and limitations (LEPRI et al., 2011; SHAH; SOYLAK, 2022). Therefore, not only the selection of the sample preparation procedure, but also the optimization of the main parameters needs to be quality-oriented and critically considered

in the analytical method development, to provide the best sample preparation protocol. In this context, the Design of Experiments (DoE) is a well-established statistical approach that enables the implementation of the pursued optimum performance in a quickly, effectively, and economically way, thus being an excellent tool in the development of analytical methods(CAVAZZUTI, 2013; POLITIS et al., 2017; SZPISJÁK-GULYÁS et al., 2023).

Focusing on the quality control of *Cannabis* products, the main aim of this work is to compare three sample preparation methods for the multielement determination of *Cannabis* herbal extract samples by inductively coupled plasma mass spectrometry (ICP-MS). Namely an open-vessel acid decomposition in a hot plate (oAD); a closed-vessel acid decomposition in a digester block (cAD); and a dilute-and-shoot with organic solvents (D&S). All methods were optimized, oAD and cAD by using the DoE strategy, and D&S using a univariate-way approach. The three sample preparation methods will be critically and comparatively evaluated regarding feasibility, analytical performance, and green impact. The ultimate goal is to help the industry of *Cannabis* products to recognize the most effective and feasible preparation method for a safe and satisfactory quality control regarding the elemental composition and to help the users to know the quality of the commercialized *Cannabis* products.

5.2. Optimization of the plasma operational conditions

The optimization of the plasma conditions seeking maximum analyte intensity and minimum interference is crucial for ICP-MS analysis. The nebulizer gas flow rate, auxiliar gas flow rate, and RF power are the main parameters that impact the system operation and the quality of the generated data. Therefore, in this work, the plasma conditions were optimized for each sample introduction scenario (aqueous or organic media) following the suppliers' performance guidelines in terms of sensitivity and robustness. Distinct strategies and statistical tools were applied for aqueous and organic media, as detailed below.

5.2.1. Aqueous solutions introduction

Many studies applying multivariate strategies to optimize the plasma parameters can be found in the literature (FROES et al., 2009; NOVAES et al., 2016; PEIXOTO; OLIVEIRA; CADORE, 2012; SANTELLI et al., 2008; TREVIZAN et al., 2005). Box–Behnken design (BBD) is a class of rotatable or nearly rotatable second-order design based on a three-level incomplete factorial design. Thus, it does not contain combinations, in which all factors are at the highest or lowest levels simultaneously, and it is not necessary to perform trials in extreme situations.

A BBD was conducted herein to optimize the plasma conditions. BBD provides faster and simpler optimization responses with a limited number of experiments, especially for previously known processes and responses bias, being, therefore, a strategical approach for refining well-known parameters. A screening step was not conducted due to the already-established significance of these parameters at the dependent signal. Analytes' responses from each trial are reported in Table 19, while the parameters of the model are presented in Table 20 (Supplementary material), and its 2D graphic representations in Figure 8.

Adequate determination coefficients (> 0.8) were achieved for all three dependent variables, suggesting adequate fit between experimental responses and model predictions. A compromise condition for maximum In^{+} signal intensity and minimum CeO^{+}/Ce^{+} and Ce^{+2}/Ce^{+} ratios was aimed and plotted in Figure 8. Better responses were observed at higher levels of auxiliar gas flow rate and RF power, while at medium levels of nebulizer gas flow rate. Therefore, an optimized region was located at a set-up of 1.0 mL min^{-1} for the nebulizer gas flow rate, 1.10 mL min^{-1} for the auxiliary gas flow rate, and 1225 W for the RF power. A global optimization rate of 85 % was successfully achieved and, therefore, this set-up was applied for following experiments introducing aqueous solutions.

Table 19. Responses from Box-Behnken Design (BBD) performed in the optimization of plasma conditions for aqueous solution introduction.

Trial	In^+ intensity (kcps)	Ce^{+2}/Ce^+ ratio (%)	CeO^+/Ce^+ ratio (%)
1	50.3	3	2
2	31.1	1	1
3	292.7	196	7690
4	291.4	15	418
5	228.9	6	146
6	97.2	4	5
7	284.7	8	254
8	121.1	6	12
9	35.1	1	1
10	295.6	34	1071
11	42.6	2	1
12	328.6	66	2052
13	167.5	40	6

Table 20. Determination coefficients (R^2) and calculated regression coefficients (not-coded) of plasma parameters (In^+ intensity, Ce^{+2}/Ce^+ and ratio and CeO^+/Ce^+) by Box-Behken Design (BBD)

	Response variables		
	In^+ Intensity	**Ce^{+2}/Ce^+ ratio**	**CeO^+/Ce^+ ratio**
R^2	0.9393	0.8426	0.8355
b_o	360781	-0.5	-121
b_1	-357	0.0140	0.495
b_2	1195237	- 37.6	- 1377
b_3	- 2329734	20.1	970
$b_{1,2}$	299	- 0.0299	- 1.212
$b_{1,3}$	- 531	- 0.0001	- 0.017
$b_{2,3}$	634068	8.0	245
$b_{1,1}$	0.14	0.000006	0.000291
$b_{2,2,}$	- 430870	33.9	1333
$b_{3,3}$	1228305	- 13.7	- 591

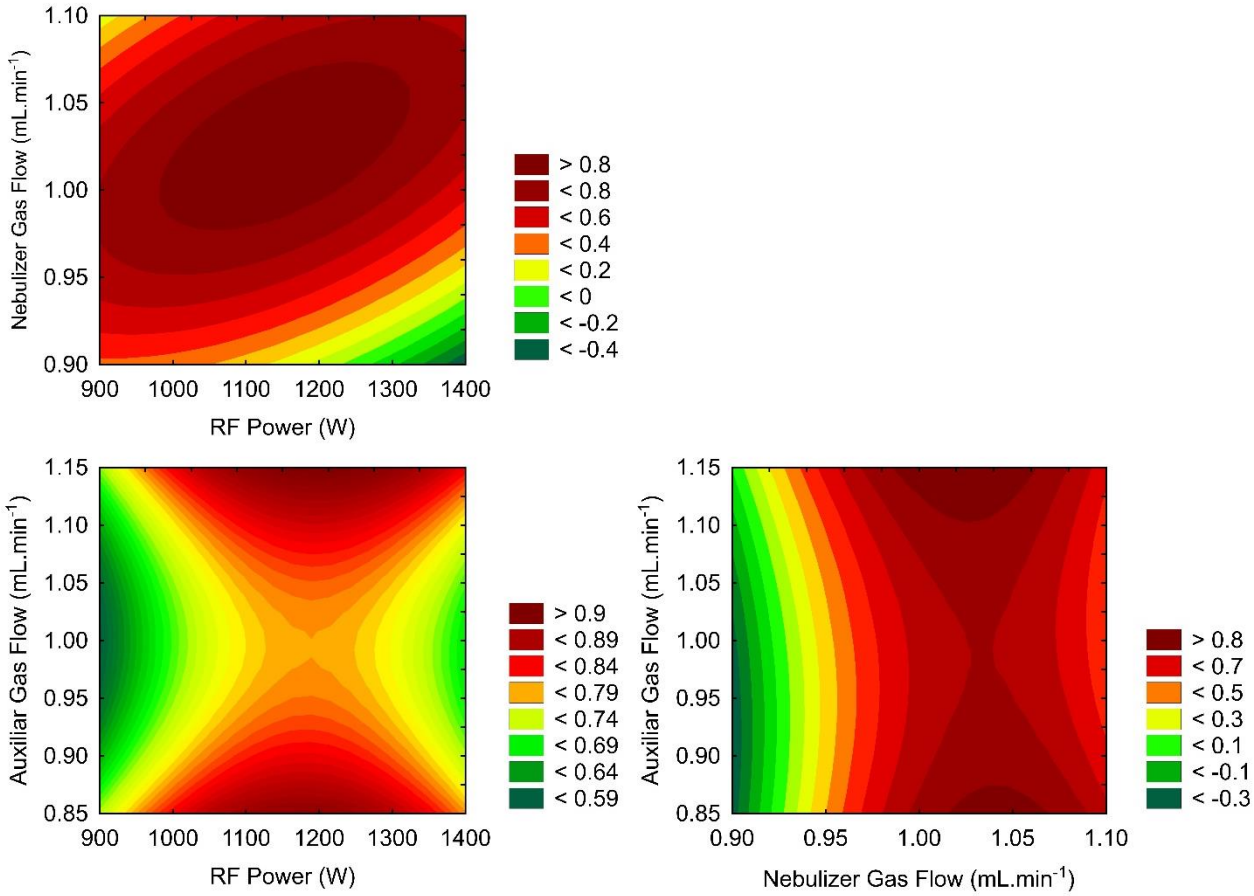

Figure 8. Two-dimensional (2D) surface response plasma parameters optimization.

5.2.2. Organic solutions introduction

Sample dilution with an appropriate organic solvent, i.e., xylene, kerosene, toluene, alcohols, etc., can be applied as a simple, fast, and user-friendly procedure to direct analysis by ICP-MS (LEPRI et al., 2011). However, it requires a specific sample introduction system for the introduction of organic solvents, not always available in a routine analysis lab.

Since the introduction of organic solvents is routinely employed in the laboratory and the plasma parameters have already been optimized in a previous study from our group (VIANA; SAINT'PIERRE, 2019), only a fine adjustment was conducted herein. The pre-set conditions were evaluated for this study, providing adequate and valid responses. The setup of 0.56 mL min^{-1} nebulizer gas flow rate, 1.00 mL min^{-1} auxiliary gas flow rate, and

1300 W RF power was applied for the following experiments. Preliminary experiments demonstrated significant interference on the ^{44}Ca, ^{138}Ba, and ^{48}Ti signals, and then, these analytes were determined employing the dynamic reaction cell (DRC) with methane as reaction gas.

The effect of the CH_4 flow rate was evaluated at the selected analytes by monitoring the signal-to-noise ratio, while the gas flow rate was systematically adjusted. According to previous experiments, the RPq Mathieu parameter was fixed at 0.45 throughout the study. As shown in Figure 9, a 0.55 mL min^{-1} methane flow rate provided the best signal-to-noise ratio for all three analytes and, therefore, was selected for the following experiments.

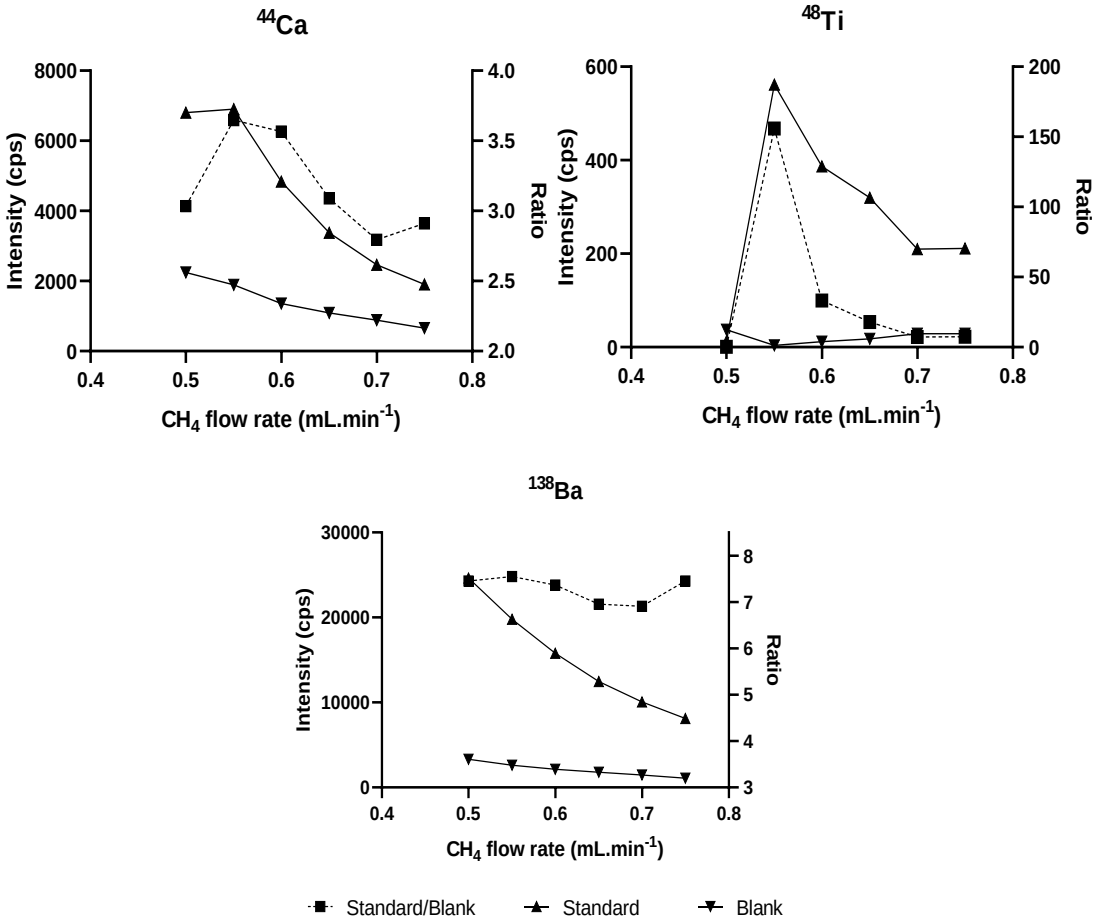

Figure 9. Responses from CH_4 flow rate optimization for the ICP-MS analysis employing the DRC.

5.3. Optimization of the parameters of the sample preparation methods

5.3.1. Open-flask acid decomposition (oAD) method.

Many parameters can influence the open-flask digestion protocol performance, with distinct impacts depending on the analytes' nature. Regarding a multielement analysis, parameters influence needs to be well-studied to select a compromise condition. A screening test was conducted, and the calculated parameters are presented in Table 21.

The regression coefficient b_3 was the most critical operational parameter, with a significant positive influence on all the 12 analytes, indicating that the heating time (variable X3) should be cautiously modeled in the following optimization steps. It is a predicted outcome since the oxidizing power of a digestion reagent shows a marked dependence on the temperature (MATUSIEWICZ, 2003). Besides that, the HNO_3 volume (indicated by the coefficient b_1) significantly impacted most elements (positive for Ba, Cr, Fe, Mg and Mn and negative for Cd and Zn), thus, being also a critical variable for optimization.

Finally, H_2O_2 proportion (indicated by the coefficient b_2) provided significant variations for Mg, Mn, and Zn, with major magnitude. Hydrogen peroxide is a popular oxidizing reagent employed to support digestion agents. However, in some instances of trace metal analysis, its addition is discouraged due to its reactivity and impurities. Then, optimizing the amount of hydrogen peroxide aiming at a minimum could represent an interesting approach.

Table 21. Calculated coefficients from screening trials of oAD method. Significant parameters are in bold.

	b_o	b_1	b_2	b_3	$b_{1,2}$	$b_{1,3}$	$b_{2,3}$	$b_{1,2,3}$
Ag	**0.22113**	-0.00202	-0.00325	**0.012118**	-0.00269	-0.00541	-000626	-0.00252
Ba	**0.050935**	**0.003544**	0.000366	**0.006486**	-2.9E-05	-0.00202	-0.00177	-0.00075
Ca	**2.203089**	0.347217	0.001295	**0.168421**	-0.07338	-0.00473	0.028569	0.01929
Cd	**0.092251**	**-0.00705**	-0.00062	**0.003858**	-0.00139	-0.00168	**-0.00322**	-1.8E-05
Co	**0.606893**	0.006827	-0.008	**0.022711**	-0.00952	-0.01058	**-0.02395**	-0.0075
Cr	**0.049424**	**0.002262**	0.001325	**0.00366**	-0.00135	-0.00073	**-0.00198**	-0.00027
Fe	**0.095031**	**0.008641**	**0.005261**	**0.006175**	0.001153	-0.00033	0.002312	0.001586
Mg	**11.22177**	**1.47783**	**4.7946**	**5.33277**	**0.85778**	**0.47967**	**3.68471**	**0.47295**
Mn	**0.902845**	**0.033495**	**0.049186**	**0.096018**	0.001891	0.001661	0.011863	0.005123
Ni	**0.118116**	-0,00224	0.000675	**0.004866**	-0.00261	-0.00266	**-0.00534**	0.000641
Pb	**0.106461**	0,000671	-0.00226	**0.006192**	-0.0026	-0.0029	-0.00426	-9E-06
Zn	**0.067791**	**-0.00457**	**0.003553**	**0.007075**	-0.00033	-0.00019	0.000072	-0.00033

All three previous parameters were selected as critical parameters for the wet open-flash acid decomposition method optimization. These parameters are vital to the analytical performance and strongly related to the sample preparation cost and the method's time-length. Therefore, operating at minimum values is desirable for defining an environmentally friendly, low-cost, and high-frequency method.

As a 3-factor BBD was conducted, a quadratic model describing the analytes' signals as function of the HNO_3 volume, the H_2O_2 proportion, and the heating time was assessed. Their coefficients and the model's figures of merit are summarized in Table 22 (Supplementary Information), while its trials' responses are reported in Table 23 (Supplementary Information). Besides that, the two-dimensional (2D) surface responses are presented in Figure 10.

High R^2 values (>0.9) and low model p-values (<0.05) were achieved for most analytes, corroborating the very good fit between the constructed model and the trial observations. High lack-of-fit p-values were also obtained, accepting the null hypothesis and assuming a reasonable variable relationship in the model. An optimal region for analytes response is comprised of around 4.3 mL HNO_3, 57 % H_2O_2 (2.5 mL), and 60 min of heating time at 100 ^oC. Therefore, a successful mean global optimization rate (represented by the composite desirability) of 84 % and individual optimization rates ranging from 70 % (Ca) to 98 % (Zn) were achieved.

The developed method agrees with the already reported literature (ASTOLFI et al., 2021). Astolfi et al. (2021) proposed the use of 5 mL of a reagent mixture [10 % (v/v) HNO_3 and 2:1 (v/v) H_2O_2] for olive oil sample decomposition in a water bath (95 ^oC, 40 min), achieving satisfactory results. It is noteworthy to mention that, while their work was based on a univariate optimization with a great number of experiments, ours strategically located a statistical global maximum performance with minimum trials and a significant resolution of parameters, which the first approach could not achieve.

Table 22. Results (analyte intensity/internal standard intensity) from Box-Behnken Design experiments (trials) performed to find the optimized working region for open-flask acid decomposition method. Trial 13 is the central point, which was prepared in 3 replicates.

Trial	Ag	Ba	Ca	Cd	Co	Cr	Fe	Mg	Mn	Ni	Pb	Zn
1	0.080	0.019	0.55	0.07	0.49	0.017	0.050	0.63	0.72	0.094	0.086	0.032
2	0.090	0.031	1.24	0.07	0.48	0.022	0.060	1.14	0.76	0.097	0.097	0.039
3	0.091	0.022	0.59	0.07	0.49	0.020	0.053	0.58	0.75	0.092	0.097	0.033
4	0.109	0.037	1.34	0.07	0.56	0.039	0.096	1.22	0.85	0.119	0.131	0.049
5	0.094	0.026	0.58	0.07	0.54	0.023	0.051	0.57	0.77	0.101	0.108	0.033
6	0.104	0.034	1.29	0.07	0.48	0.027	0.064	1.11	0.75	0.096	0.113	0.042
7	0.086	0.035	0.58	0.07	0.51	0.019	0.049	0.58	0.77	0.099	0.101	0.033
8	0.112	0.039	1.43	0.07	0.53	0.029	0.070	1.28	0.83	0.101	0.120	0.043
9	0.106	0.036	0.96	0.08	0.55	0.023	0.058	0.90	0.83	0.107	0.129	0.038
10	0.120	0.036	0.98	0.08	0.60	0.028	0.061	0.98	0.91	0.1149	0.133	0.042
11	0.099	0.036	0.94	0.07	0.54	0.023	0.061	1.02	0.82	0.102	0.130	0.038
12	0.101	0.037	0.94	0.07	0.51	0.022	0.060	0.99	0.81	0.101	0.128	0.041
13 (n = 3)	0.107 ± 0.004	0.039 ± 0.002	1.05 ± 0.07	0.24 ± .01	0.57 ± 0.03	0.01 ± 0.004	0.082 ± 0.003	1.08 ± 0.09	0.90 ± 0.04	0.130 ± 0.004	0.133 ± 0.009	0.049 ± 0.003

Table 23. Analysis of Variance (ANOVA) parameters, p-values of the predictive model, lack-of-fit and calculated regression coefficients (not-coded) for Box-Behken Design of open-flask acid decomposition method.

	Ag	**Ba**	**Ca**	**Cd**	**Co**	**Cr**	**Fe**	**Mg**	**Mn**	**Ni**	**Pb**	**Zn**
R^2	0.9046	0.8791	0.9853	0.9928	0.7902	0.9837	0.9174	0.9786	0.8442	0.9056	0.8854	0.9682
Model	0.041	0.069	0.000	0.000	0.215	0.001	0.030	0.001	0.119	0.040	0.062	0.003
Lack-of-fit	0.293	0.200	0.650	0.939	0.0510	0.546	0.143	0.987	0.539	0.221	0.500	0.821
b_o	0.1124	0.0213	-0.175	-1.613	0.315	-0.4469	- 0.1947	- 0.762	0.108	-0.1435	0.1056	-0.0677
b_1	0.00781	0.01102	0.1430	0.0951	0.0105	0.02305	0.00532	0.1807	0.0705	0.01452	0.0211	0.00747
b_2	0.001168	0.000655	0.01136	0.01385	0.00541	0.003910	0.001169	0.01002	0.00952	0.001922	0.00101	0.000718
b_3	0.00175	-0.00088	0.0130	0.04487	0.00368	0.01252	0.00750	0.0325	0.0133	0.00660	-0.00150	0.002661
$b_{1,2}$	0.000033	0.000012	0.000292	0.000040	0.000283	0.000057	0.00013	0.000525	0.000215	0.00009	0.000094	0.000040
$b_{1,3}$	0.000105	-0.00002	0.0009	0.000020	0.000580	0.000031	0.00005	0.001030	0.00055	0.00004	0.000094	-0.0000

$b_{2,3}$	-0.000008	0.000001	-0.00001	-0.000008	-0.000048	-0.000004	-0.00001	-0.00007	-0.00006	-0.00001	-0.00001	-0.0000
$b_{1,1}$	0.001799	-0.00118	-0.00878	-0.014122	-0.00832	-0.003700	- 0.0015	-0.02135	-0.01508	-0.00291	-0.00401	- 0.0010
$b_{2,2,}$	-0.000006	-0.00001	-0.0001	-0.000134	-0.000030	-0.000038	- 0.0000	-0.00008	-0.00006	-0.00002	-0.00001	- 0.0000
$b_{3,3}$	0.000013	0.000009	-0.00013	-0.000372	-0.000033	-0.000104	- 0.0001	- 0.0003	- 0.0001	0.00006	0.000011	- 0.0000

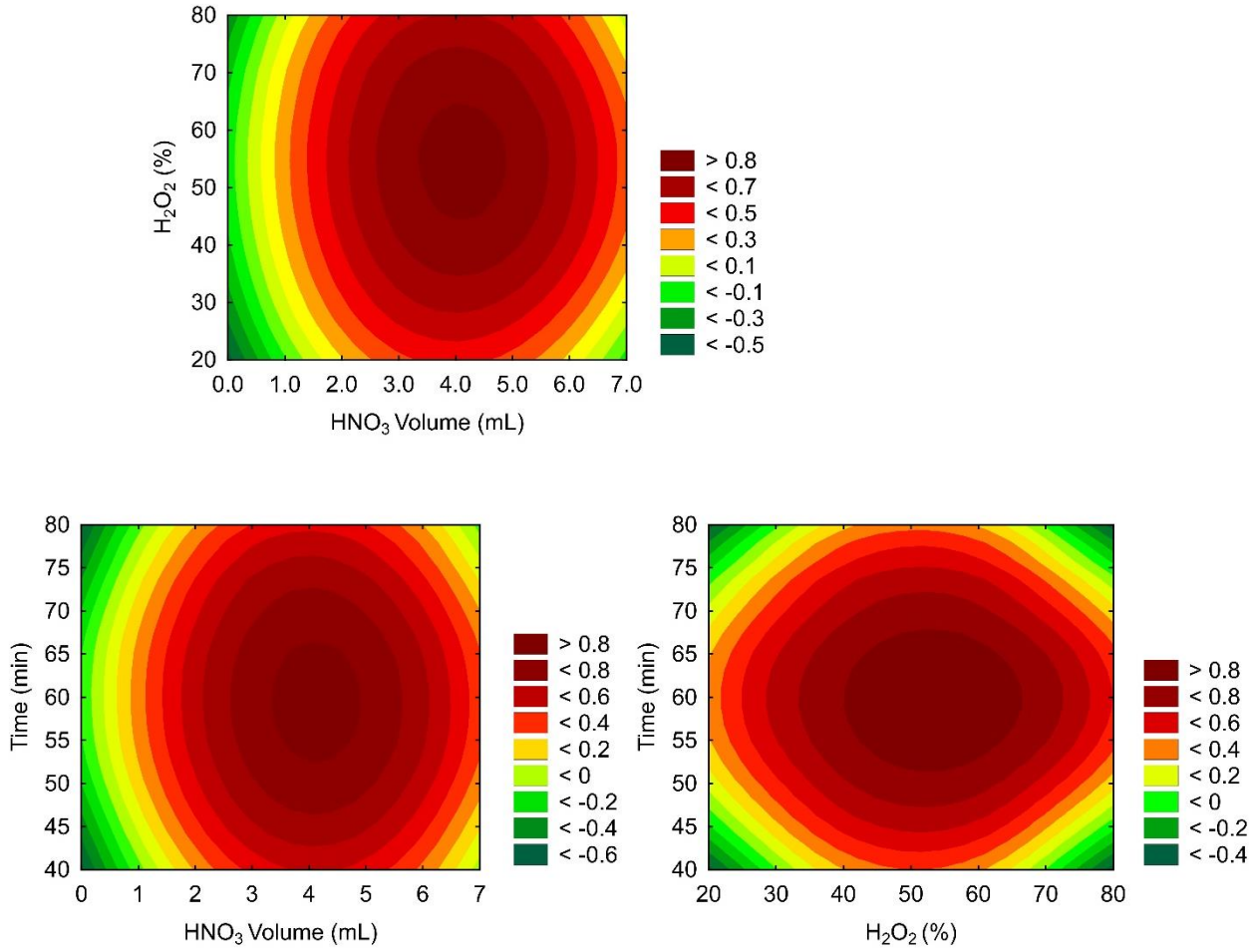

Figure 10. Two-dimensional surface response for open-flask acid decomposition (oAD) method.

5.3.2. Closed-flask acid decomposition (cAD) method

Closed systems, such as a digester block, provide a wet decomposition procedure with the advantages of isolated atmosphere, less volatile elements loss, and elevated temperature and pressure, thereby improving the decomposition efficiency. Essentially, complete decomposition can be accomplished in shorter times and, in many cases, HNO_3 alone is a sufficiently powerful decomposition agent. However, in this work, preliminary studies demonstrated that a pre-digestion step, although increasing the sample preparation time, was important to ensure a more controlled and milder reaction medium during the cAD method, improving the reproducibility and process safety.

In order to select the most important variables at the cAD method, a screening Full Factorial Design was performed, and the calculated parameters are presented in Table 24. Pre-heating time and HNO_3 volume (indicated by coefficients b_3 and b_1, respectively) were the most important operational parameters, with significant impact on 8 and 7 analytes, respectively, thus, being selected to the following modelling. The H_2O_2 proportion (represented by coefficient b_2) was significant only for 5 of the 12 analytes and all presented negative impacts, therefore, H_2O_2 addition was not used for this sample preparation method.

Table 24. Calculated coefficients from screening trials of closed-flask acid decomposition (cAD) method. Significant values are in bold.

	b_o	b_1	b_2	b_3	$b_{1,2}$	$b_{1,3}$	$b_{2,3}$	$b_{1,2,3}$
Ag	**0.154477**	**-0.007467**	0.003181	0.004023	0.003448	0.003154	-0.001505	**-0.004404**
Ba	**0.041146**	**-0.001523**	0.000142	**0.001136**	-0.000064	**0.000557**	0.000158	**-0.000858**
Ca	**0.604030**	**0.097586**	0.006469	**0.008412**	**0.020226**	-0.000140	-0.004342	**-0.009327**
Cd	**0.072557**	**-0.004859**	**-0.001510**	**0.000647**	**0.001860**	0.000143	**-0.000863**	**-0.001301**
Co	**0.486677**	-0.013455	0.005311	0.009316	0.006002	0.006924	-0.004127	-0.018729
Cr	**0.069236**	**-0.001576**	**-0.003513**	**0.004192**	**0.005441**	**-0.006190**	**-0.004656**	**-0.003399**
Fe	**0.085592**	**0.010246**	**-0.012014**	**-0.007995**	**-0.006899**	**-0,012847**	**0.005465**	**0.006923**
Mg	**0.588873**	**0.020264**	**-0.010350**	**-0.008618**	**0.031094**	**0.026484**	**0.014058**	**-0.037104**
Mn	**0.723885**	-0.003465	**-0.006335**	**0.020602**	**0.013867**	**-0.015724**	-0.002127	**-0.021840**
Ni	**0.137796**	**-0.008424**	0.001729	**0.013292**	0.006244	**-0.023265**	-0.000324	0.006475
Pb	**0.088747**	**-0.005575**	-0.000070	0.001370	0.001584	0.001133	-0.002024	-0.002113
Zn	**0.035931**	-0.001098	-0.000722	-0.000059	0.001219	-0.001058	-0.000062	0.000785

For the sequential optimization step, only HNO_3 volume and pre-heating time were selected as relevant for the CCD approach. Central Composite Design applies a second-order model to correlate analytes response as a function of these previously cited operational parameters. By adding center points and star points to a factorial design, CCD methodology provides a rotatability dimension to the model and gives better information within or beyond the limits of the traditional spinning process. As CCD requires more observations trials than BBD, more relevant data can be provided, and novel method's responses can be achieved to the investigation.

Individual trials responses and ANOVA parameters from CCD predictive model are reported at Tables 25 and 26 (Supplementary Information), respectively. Also, the composed desirability and its two-dimensional (2D) surface responses are presented in Figure 11. An optimal region around 3.5 mL of HNO_3 volume, and 20 min of pre-heating time was identified. It represents a global optimization rate of 86 %, ranging the individual optimization rates from 60 % (Ni) to 96 % (Ba).

Table 25. Central Composite Design experiments (trials) applied to the analyte intensity/internal standard intensity ratios to find the optimized working region for closed-flask acid decomposition (cAD) method. Trial 9 is the central point, which was prepared in 5 replicates.

Trial	Ag	Ba	Ca	Cd	Co	Cr	Fe	Mg	Mn	Ni	Pb	Zn
1	0.0863	0.0205	0.1598	0.0447	0.2675	0.0294	0.0209	0.1575	0.2716	0.0630	0.0879	0.0245
2	0.0847	0.0221	0.2378	0.0364	0.3115	0.0357	0.0296	0.2068	0.3395	0.0789	0.0852	0.0212
3	0.0747	0.0184	0.1825	0.0382	0.2405	0.0281	0.0259	0.1653	0.2743	0.0881	0.0794	0.0272
4	0.0440	0.0120	0.1343	0.0194	0.1658	0.0224	0.0163	0.1199	0.2234	0.0906	0.0471	0.0129
5	0.0693	0.0173	0.1673	0.0421	0.2208	0.0259	0.0291	0.1470	0.2212	0.0501	0.0761	0.0236
6	0.0749	0.0204	0.2554	0.0331	0.2772	0.0332	0.0344	0.2278	0.3029	0.0730	0.0776	0.0224
7	0.0790	0.0194	0.2142	0.0366	0.2613	0.0315	0.0285	0.1802	0.2694	0.1079	0.0790	0.0196
8	0.0885	0.0227	0.2276	0.0413	0.2938	0.0337	0.0256	0.2126	0.3006	0.0609	0.0922	0.0214
9 (n = 5)	0.086 ± 0.002	0.0218 ± 0.0004	0.238 ± 0.005	0.040 ± 0.02	0.29 ± 0.01	0.043 ± 0.001	0.038 ± 0.001	0.51 ± 0.02	0.32 ± 0.01	0.079 ± 0.002	0.089 ± 0.002	0.026 ± 0.001

Table 26. Analysis of Variance (ANOVA) parameters, p-values of the predictive model, lack-of-fit and calculated regression coefficients (not-coded) for Central Composite Design of closed-flask acid decomposition (cAD) method.

	Ag	**Ba**	**Ca**	**Cd**	**Co**	**Cr**	**Fe**	**Mg**	**Mn**	**Ni**	**Pb**	**Zn**
R^2	0.5428	0.4909	0.7042	0.6321	0.5348	0.8984	0.8519	0.5135	0.7398	0.4075	0.5289	0.7759
Model	0.261	0.346	0.074	0.142	0.273	0.002	0.008	0.308	0.050	0.499	0.283	0.031
Lack-of-fit	0.000	0.000	0.001	0.005	0.004	0.019	0.014	0.011	0.0016	0.659	0.001	0.000
b_o	0.0403	0.00851	-0.0043	0.0341	0.1046	-0.00657	-0.00697	0.0352	0.0666	0.0428	0.0442	0.01202
b_1	0.0269	0.00687	0.1030	0.00486	0.0929	0.02314	0.01741	0.0698	0.1166	0.0261	0.0247	0.00504
b_2	0.0007	0.000210	0.00423	0.00029	0.00271	0.000829	0.001098	0.00281	0.00401	-0.00037	0.000824	0.000514
$b_{1,2}$	-0.001	-0.00005	-0.000841	-0.0001	-0.00079	-0.00008	-0.00012	-0.00063	-0.00079	-0.00009	-0.00342	-0.00007

$b_{1,1}$	-0.0037	-0.00084	-0.01011	-0.0009	-0.010119	-0.00324	-0.00210	-0.00631	-0.01297	-0.00312	-0.00001	-0.00068
$b_{2,2}$,	-0.0001	-0.00001	-0.000029	-0.001	-0.00001	-0.00001	-0.00001	-0.00002	-0.00003	0.00001	-0.0002	-0.0001

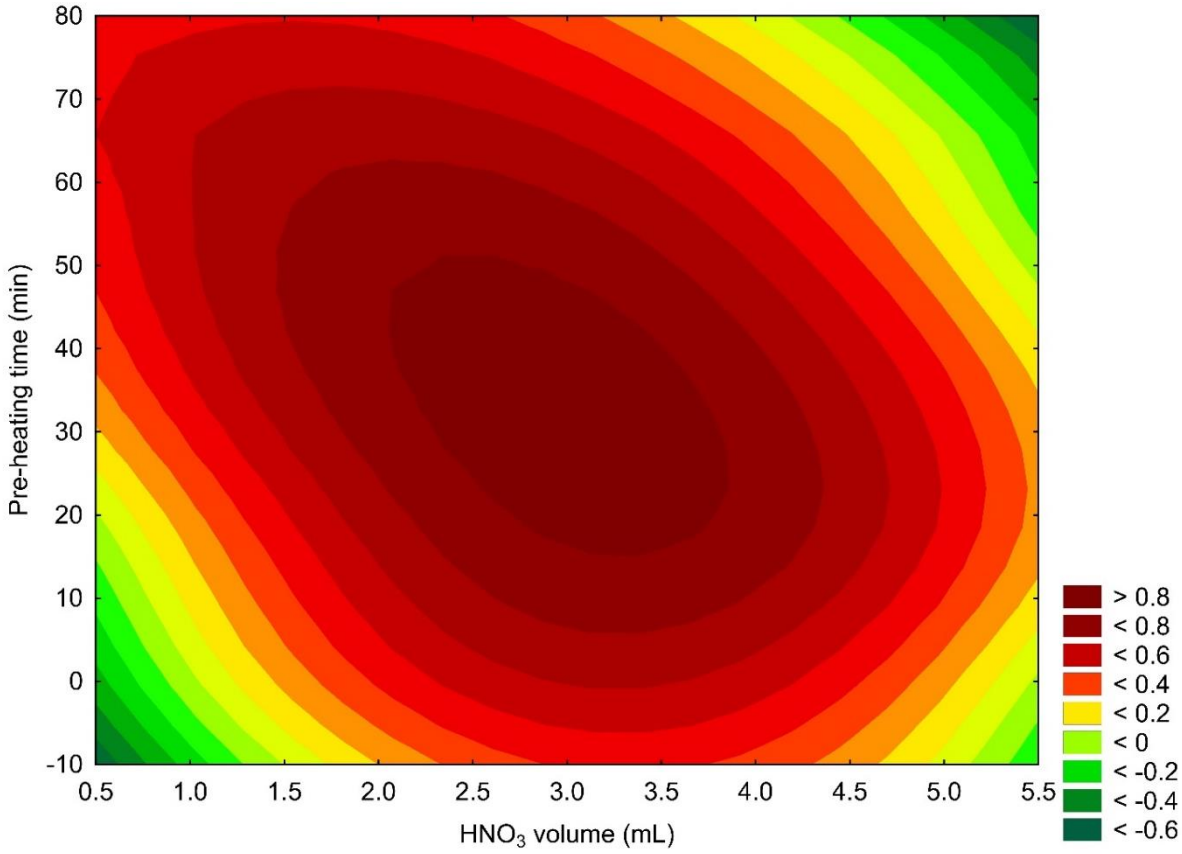

Figure 11. Two-dimensional surface response model for closed-flask acid decomposition (cAD) method.

5.3.3. Dilute-and-shoot method optimization

Sample preparation methods based on oxidizing mineralization, as the previously studied oAD and cAD, are well-known and extensively widespread. Their effectiveness is undoubtable, however, the time-consuming, energy-depending, and waste-generating nature of these methods make fast and on-site analysis impractical and the large batches analysis a non-user-friendly experience. A dilute-and-shoot method, based on simple sample dilution in an organic solvent, is a good approach that can be explored as a sample preparation method for organic liquids and oil samples, as can be fast, straightforward, and easy-to automatize (MARTÍNEZ et al., 2020).

Although dilute-and-shoot methods with organic solvent has been successfully applied to ICP-MS determinations (VIANA; SAINT'PIERRE, 2019), the direct introduction of organic loads into an ICP is not trivial. Lower plasma robustness, high instability, carbon deposits in cones and lenses, and polyatomic interferences are common issues that need to be addressed (MARTÍNEZ et al., 2020; VIANA; SAINT'PIERRE, 2019). Specific sample introduction systems can be used to overcome these limitations. In this work, a sample injector with smaller diameter, a reduced and optimized nebulization rate, and the introduction of O_2 as auxiliary gas were successfully explored to make these determinations viable.

A critical step of this sample treatment approach is the solvent selection. The ideal solvent must adequately dissolve the samples, have low volatility and low toxicity, be compatible with instrumentation, and not induce additional interferences and matrix effects (MARTÍNEZ et al., 2020). However, solvents that are generally employed and commonly reported in the literature are: xylene, toluene, kerosene, and methyl isobutyl ketone (MIBK) (CHAVES et al., 2011; MARTÍNEZ et al., 2020), which do not achieve all these goals and are not compatible with water, thus requiring organic standards for the calibrations.

Regarding its performance similarity to the commonly used solvent (xylene) and its even superior performance in a great number of cases, butanol was selected as a sample diluent in this method in the following experiments. Alcohol-based dilutions are less toxic, volatile, and expensive, while its application can enable the employment of inorganic aqueous standards, with certain limitations, without phase separation in replacement to organometallic standards (CHAVES et al., 2011; VIANA; SAINT'PIERRE, 2019). It is noteworthy to mention that further optimization could be applied to propose solvents mixtures to this method. However, since it is not the purpose of this work to approach three component systems and their interactive mechanisms, it was avoided at this moment.

5.4. Analytical performance

5.4.1. Matrix effect

Analytical curves were constructed with 6 points of concentrations from specifics ranges detailed at 3.3.4., each solution measured in triplicate, for all three calibration strategies (ES, MM and SA). Angular coefficients were compared for matrix effects investigation. The results are presented in Table 27. No matrix effect was observed for oAD and cAD methods, in any scenario, as expected, since the organic matrix was decomposed in the sample preparation. At the Dilute-and-Shoot methodology, 12 analytes (Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Pd, Sr, V, and Zn), from the monitored 28, were affected by matrix effects when external calibration (EC) was applied. Matrix matching calibration (MM) was appropriate for correction of these effects for 10 analytes (except for Co and V), however, additional matrix effects were observed for 3 elements (Cd, Li, and P). Also, Sb and Sn could not be adequately acquired by D&S approach due to polyatomic interferences or any contamination that could not be corrected or described until this moment, therefore, they will not be approached from now on.

Complex sample matrices require complex calibrations strategies. Although the extensive capacity to correct matrix effects, standard addition is a quite laborious and time-consuming approach, while matrix matching and external standard calibration provide reliable analytical results for most elements in many scenarios with minimum bias and considerably less effort (WILSCHEFSKI; BAXTER, 2019). Due to its matrix effect investigation and aiming for best analytical performance, such as satisfactory matrix mimic attempt, adequate analytical reliability and possibility to be prepared in-house and on-line (WILSCHEFSKI; BAXTER, 2019), the SA calibration was

selected herein for oAD and cAD further investigation and application, while MM was selected for D&S.

Table 27. Correlation of matrix effects observed (highlighted in bold underline) between External Standard calibration (ES), Matrix Matching calibration (MM) and Standard Addition calibration (SA) for each analyte by Dilute-and-Shoot (D&S) method.

	D&S	
	ES x SA	**MM x SA**
Ag	No	No
Al	No	No
As	No	No
Au	No	No
Ba	No	No
Ca	No	No
Cd	No	**Yes**
Co	**Yes**	**Yes**
Cr	**Yes**	No
Cs	No	No
Cu	**Yes**	No
Fe	**Yes**	No
Hg	No	No
K	No	No
Li	No	**Yes**
Mg	**Yes**	No
Mn	**Yes**	No
Mo	No	No
Ni	**Yes**	No
P	No	**Yes**
Pb	**Yes**	No
Pd	**Yes**	No
Sr	**Yes**	No
Ti	No	No
V	**Yes**	**Yes**

Zn	**Yes**	No

5.4.2. Linearity

A 9-points analytical curve was constructed for each analyte by Extenal Standard calibration strategy for oAD and cAD and by Matrix Matching for D&S. All analytes provided $R^2 > 0.95$ and Cochran's C-test indicates homoscedasticity ($C_{critical} = 0.616$; 95 % confidence level) for all analytes in these ranges.

5.4.3. Sensitivity

Limits of Quantification (Table 28) were estimated for each analyte and each method. Lowest LOQs were achieved for 15 elements (Ba, Cd, Co, Cr, Cs, Li, Mg, Mn, Mo, Sn, Sr, Ti, V, and Zn) with oAD method and for other 11 analytes (Al, As, Au, Ca, Cu, Fe, Hg, K, Ni, Pb, and Pd) with D&S. The cAD method was the best option only for Ag and Sb. That could be explained by the lower dilution factor intrinsic to those methods (13-times for oAD and 10-times for D&S) in comparison to cAD (100-times).

From a global viewpoint, the LOQ were in the same order of magnitude for most elements. However, it is noteworthy to highlight that some abundant elements, such as Al, Ca, K, Mg, P, and Zn, demonstrated unexpectedly higher LOQ at the cAD method, that can be attributed to methods intrinsic characteristics. Closed-flash acid decompositions performance is strictly related to Teflon-flasks integrity and decontamination efficiency. Teflon, known for its chemical resistance, is not immune to elemental contamination and its decontamination procedures tends to lose efficiency over time.

Table 28. Limits of quantification (ng g^{-1}) for each sample preparation method. Parameters reported as n.d. could not be determined.

Element	oAD	cAD	D&S	Element	oAD	cAD	D&S
Ag	7.6	1.9	3.3	**Li**	0.6	1.0	0.9
Al	37.3	580.5	5.7	**Mg**	31.9	149.4	33.3
As	8.0	22.2	4.2	**Mn**	1.7	4.7	13
Au	7.2	9.9	5.0	**Mo**	1.0	1.8	1.7
Ba	24.6	120.8	262.8	**Ni**	12.5	48.3	9.1
Ca	321.0	2393.6	183.8	**P**	672.7	4257.0	3211.8
Cd	1.1	3.3	3.1	**Pb**	2.0	7.1	1.9
Co	1.1	6.4	2.8	**Pd**	4.8	3.7	0.6
Cr	6.6	32.1	19.6	**Sb**	3.5	1.9	n.d.
Cs	0.3	0.3	2.1	**Sn**	2.0	11.1	n.d.
Cu	4.6	16.6	4.2	**Sr**	0.9	7.0	2.6
Fe	2057.1	1130.9	484.8	**Ti**	7.9	20.8	398.3
Hg	25.5	73.7	7.9	**V**	0.7	1.4	5.3
K	204.2	1485.8	68.8	**Zn**	16.3	222.4	25.9

5.4.4. Working range

Six-points calibration curves were constructed by ES for oAD and cAD methods and by MM for D&S methods for following experiments. Working ranges were established for each analyte and each sample preparation method based on linearity, sensitivity, and expected concentrations, aiming the best analytical application as possible.

For oAD and cAD, curves between 0.1 and 10 µg L^{-1} were defined for Cd, Co, Cs, Li, Mn, Mo, Sr, and V, while 2.5 – 20 µg L^{-1} range was applied for Ag, Al, As, Au, Ba, Cr, Cu, Hg, Mg, Ni, Pb, Pd, Sb, Sn, Ti, and Zn. Besides that, Ca and K were studied between 15 and 40 µg L^{-1} and the duo Fe and P were approached between 50 – 1000 µg L^{-1}.

By D&S, most of elements (Ag, Al, As, Au, Ba, Cd, Co, Cr, Cs, Cu, Hg, Li, Mg, Mn, Mo, Ni, Pb, Pd, Sb, Sn, Sr, Ti, V, and Zn) were approached between 1 – 15 µg L^{-1}, while ranges of 20 – 70 µg L^{-1} were established for Ca, Se, and Ti, and of 100 – 350 µg L^{-1} for Ba. Curves with ranges of 250 – 500 µg L^{-1}, 50 – 300 µg L^{-1} and 5 – 30 µg L^{-1} were constructed for P, Fe, and K, respectively.

5.4.5 Precision

Precision investigation results are presented in Table 29. Repeatability was estimated through relative standard deviation (RSD, %) of spiked samples triplicate at two different concentration levels. Satisfactory repeatability (< 15 %) was obtained for most analytes at both concentration levels by all three methods, assuring methods' pertinence to the analytical question herein.

Due to its similar nature, oAD and cAD methods presented similar precision parameters. Al, Ca, and Sn exhibited unsatisfactory % CV at both concentration levels by both methods (CV > 20 %), while slightly elevated % CV were obtained only for Mg and V at the lower concentrations level (15 > CV > 20 %). Besides that, high deviations were displayed for V at the lower level only by oAD and for K at both levels only by cAD method. It is noteworthy to mention that D&S method provided satisfactory precisions for all analytes at both levels approached.

Table 29. Relative standard deviation (%, n=3) observed for each element at two different concentrations and for each sample preparation method. Concentration levels (µg L^{-1}) are reported as “a/b”, being “a” that for open acid decomposition (oAD) and closed acid decomposition (cAD) methods and “b” for dilute-and-shoot. Parameters reported as n.d. could not be determined. Table to be continued.

	Lower level	**oAD**	**cAD**	**D&S**	**Upper level**	**oAD**	**cAD**	**D&S**
Ag	**5/2.5**	2	3	4	**15/10**	2	2	5
Al	**5/2.5**	20	39	5	**15/10**	20	17	3
As	**5/2.5**	3	1	2	**15/10**	2	7	4
Au	**5/2.5**	11	3	1	**15/10**	3	10	2
Ba	**2.5/150**	3	4	1	**10/300**	3	10	4
Ca	**20/30**	33	29	6	**35/60**	31	35	12
Cd	**0.5/2.5**	5	12	4	**5/10**	2	4	1
Co	**0.5/2.5**	6	8	1	**5/10**	1	4	1
Cr	**5/2.5**	6	15	10	**15/10**	6	2	2
Cs	**0.5/2.5**	4	9	4	**5/10**	3	3	4
Cu	**5/2.5**	1	6	4	**15/10**	1	6	2
Fe	**100/100**	3	3	3	**750/250**	3	2	4
Hg	**5/2.5**	1	4	4	**15/10**	2	5	8
K	**20/15**	7	321	1	**35/25**	5	22	3
Li	**0.5/2.5**	8	9	3	**5/10**	4	5	2

Table 29. Relative standard deviation (%, n=3) observed for each element at two different concentrations and for each sample preparation method. Concentration levels (µg L^{-1}) are reported as “a/b”, being “a” that for open acid decomposition (oAD) and closed acid decomposition (cAD) methods and “b” for dilute-and-shoot. Parameters reported as n.d. could not be determined. Table conclusion.

Mg	**5/2.5**	**18**	**16**	**7**	**15/10**	**2**	**12**	**5**
Mn	**0.5/2.5**	3	15	4	**5/10**	2	4	1
Mo	**0.5/2.5**	2	4	4	**5/10**	2	5	5
Ni	**5/2.5**	2	15	2	**15/10**	4	4	1
P	**100/300**	1	2	2	**750/450**	2	5	2
Pb	**5/2.5**	1	2	5	**15/10**	1	2	
Pd	**5/2.5**	2	2	8	**15/10**	2	1	7
Sb	**5/2.5**	1	6	n.d.	**15/10**	4	0.2	n.d.
Sn	**5**	34	87	n.d.	**15**	29	24	n.d.
Sr	**0.5/2.5**	8	7	6	**5/10**	1	5	2
Ti	**5/30**	11	3	1	**15/60**	5	8	2
V	**0.5/2.5**	19	9	1	**5/10**	2	6	2
Zn	**5/2.5**	5	9	5	**15/10**	3	6	1

5.4.6. Recovery

Recovery investigation results are presented in Table 30. The oAD method provided satisfactory recoveries (80 % to 120 %) for most elements at both concentration levels, with exception of Al (67 %) and K (73 %) at the lower level and Ca at both levels (68 % and 71 %).

By cAD method, only Sn (126 %, lower level) and Ca (both levels) demonstrated unsatisfactory recoveries. Flask contamination is suggested due to its recovery discrepancy and abundant nature of these elements. Unfortunately, D&S provided unsatisfactory recoveries for all elements at the upper concentration level.

Table 30. Recovery (%, n=3) for each element at two different concentration levels and for each sample preparation method by External Standard calibration curve. Added concentration levels (µg L^{-1}) are reported as “a/b”, being “a” that for open acid decomposition (oAD) and closed acid decomposition (cAD) methods, while “b” is for dilute-and-shoot method. Parameters reported as n.d. could not be determined. Table to be continued.

	Lower level	oAD	cAD	D&S	Upper level	oAD	cAD	D&S
Ag	**5/2.5**	96	97	96	**15/10**	111	101	127
Al	**5/2.5**	67	88	121	**15/10**	109	99	175
As	**5/2.5**	99	97	104	**15/10**	105	95	151
Au	**5/2.5**	102	100	99	**15/10**	111	107	145
Ba	**2.5/150**	99	100	102	**10/300**	111	107	113
Ca	**20/30**	68	772	113	**35/60**	71	843	107
Cd	**0.5/2.5**	110	107	102	**5/10**	105	113	145
Co	**0.5/2.5**	92	100	98	**5/10**	100	99	145
Cr	**5/2.5**	121	101	95	**15/10**	108	88	140
Cs	**0.5/2.5**	100	104	76	**5/10**	108	109	146
Cu	**5/2.5**	94	90	101	**15/10**	109	97	148
Fe	**100/100**	96	95	133	**750/250**	96	98	64

Table 30. Recovery (%, n=3) for each element at two different concentration levels and for each sample preparation method by External Standard calibration curve. Added concentration levels (µg L^{-1}) are reported as “a/b”, being “a” that for open acid decomposition (oAD) and closed acid decomposition (cAD) methods, while “b” is for dilute-and-shoot method. Parameters reported as n.d. could not be determined. Table conclusion.

Hg	**5/2.5**	**99**	**109**	**95**	**15/10**	**107**	**103**	**141**
K	**20/15**	73	16	231	**35/25**	94	94	106
Li	**0.5/2.5**	96	102	105	**5/10**	100	98	153
Mg	**5/2.5**	104	109	241	**15/10**	114	93	205
Mn	**0.5/2.5**	107	103	101	**5/10**	103	103	148
Mo	**0.5/2.5**	97	115	96	**5/10**	105	98	149
Ni	**5/2.5**	99	95	93	**15/10**	117	98	148
P	**100/300**	100	101	101	**750/450**	96	101	179
Pb	**5/2.5**	101	100	98	**15/10**	112	103	121
Pd	**5/2.5**	97	100	54	**15/10**	114	99	126
Sb	**5/2.5**	102	103	n.d.	**15/10**	118	113	n.d.
Sn	**5**	99	126	n.d.	**15**	117	553	n.d.
Sr	**0.5/2.5**	93	97	94	**5/10**	102	98	122
Ti	**5/30**	104	97	144	**15/60**	112	96	139
V	**0.5/2.5**	85	96	97	**5/10**	111	107	160
Zn	**5/2.5**	99	83	275	**15/10**	105	85	198

5.5. Ecological performance

Environmental friendliness assessment of sample preparation protocols and analytical methods is a critical concern that needs to be addressed in the method development step. At the current point, satisfactory analytical parameters are not a sufficient reward, even to pay for high energy consumption and/or great waste production.

Several approaches can be applied to measure Green Analytical Chemistry (GAC) metrics. The greenness of an analytical procedure is a multivariate and complex parameter that considers environmental, health, and safety issues of the procedure. Therefore, AGREE is a comprehensive, flexible and user-friendly open software that measure method's greenness based on SIGNIFICANCE principles, such as amounts and toxicity of reagents, generated waste, energy requirements, the number of procedural steps, miniaturization degree and automation level (PENA-PEREIRA; WOJNOWSKI; TOBISZEWSKI, 2020).

The AGREE metric system was conducted to compare all 3 sample preparation methods developed herein. The assessment criteria are taken and transformed into a unified 0−1 scale. Data are reported in Table 31.

The D&S method provided the best greenness metric (0.61) in comparison to oAD (0.50) and cAD (0.42) methods. Its major advantages include a few numbers of manual steps, high frequency of samples prepared per batch and lower energy consumption. cAD presented the biggest waste generation and was considered the most dangerous method, while oAD stands as an intermediate candidate, with higher number of samples that can be prepared per hour, with low waste generation and a low volume of hazard materials dependency.

It is noteworthy that all the developed protocols – likewise most of sample preparation methods for multielement analysis – demonstrated great potential to further improvements of greenness. For example, dilution factor reduction and FIA implementation could be applied to minimize residue production.

Table 31. Ecological assessment for each sample preparation method.

No.	Principle	oAD	cAD	D&S
1	Preparation placement	External preparation and batch analysis	External preparation and batch analysis	External preparation and batch analysis
2	Sample economy	0.5	0.5	0.5
3	Measurement position	Off-line	Off-line	Off-line
4	Preparation steps	5 manual steps	4 manual steps	1 manual step
5	Automation	Manual and not miniaturized	Manual and not miniaturized	Manual and not miniaturized
6	Derivatization	Not used	Not used	Not used
7	Waste	7 mL	35 mL	5 mL
8	Sample throughput	30 samples per hour	12 samples per hour	60 samples per hour
9	Most energy consumption step	Hot plate	Assisted extraction	None
10	Sustainability, renewability, and reusability of materials	< 25 % of reagents are sustainable or can be reused	< 25 % of reagents are sustainable or can be reused	< 25 % of reagents are sustainable or can be reused
11	Hazardous materials	Yes (0.4 mL)	Yes (3.5 mL)	No
12	Safety	Oxidazable	Corrosive and Oxidazable	Flammable

Final Score (max. 1.0)	**0.50**	**0.42**	**0.61**

5.6. Analytical application

All methods developed herein presented notorious advantages and disadvantages, at both analytical and ecological points of view. In a commitment condition, the classic oAD method was the one selected to be applied to 6 real samples. Its intermediate greenness performance, satisfactory analytical parameters and user-friendly operational conditions corroborate this one as the best candidate herein. Again, further greenness improvements are identified in this work and encouraged by the authors.

The D&S method represents a fast but limited protocol herein as a significant number of interferences were observed, compromising analytical results and requiring further calibration and corrective mechanisms. The oAD and cAD methods promoted reliable analytical responses, however the latter promoted a lower analytical frequency and a higher contamination susceptibility.

For sum, six samples, in triplicate, were treated with 4.3 mL of HNO_3 10 % v/v, 2.5 mL of H_2O_2 and digested for 60 minutes at hot plate (100 ^oC). 28 elements were quantified by a Matrix Matching calibration curve, constructed with sesame oil. The data are expressed in Table 32. A triplicate sesame oil sample was also prepared and analyzed as the blank for comparison. Results for As, Ba, Ca, Cd, Co, Cr, Cs, Fe, Hg, Mo, Sr, and V were below LOD for all samples, and, therefore, not presented in this table.

Table 32. *Cannabis* oil and sesame oils samples analysis (n = 3). Data reported as µg g^{-1}. Statistically significant differences between *Cannabis* samples and sesame oil are highlighted in bold.

Analyte.	Sesame	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Ag	10.98 ± 0.05	**10.62 ± 0.03**	**10.61 ± 0.03**	**10.74 ± 0.02**	10.92 ± 0.02	**10.60 ± 0.03**	**10.67 ± 0.02**
Al	54 ± 7	**182 ± 30**	**114 ± 36**	**139 ± 11**	**113 ± 5**	**114 ± 12**	74 ± 15
Au	<LOQ	**7.4± 0.1**	<LOQ	**8.0 ± 0.8**	<LOQ	<LOQ	<LOQ
Cu	<LOQ	<LOQ	**4.92 ± 0.07**	**5.4 ± 0.2**	<LOQ	<LOQ	<LOQ
K	<LOQ	**4075 ± 42**	**4195 ± 170**	**670 ± 46**	**630 ± 15**	**460 ± 18**	**336 ± 33**
Li	<LOQ	<LOQ	<LOQ	<LOQ	**2.23 ± 0.05**	**2.1 ± 0.2**	<LOQ
Mg	379 ± 14	**1294 ± 16**	**1312 ± 34**	**450 ± 5**	**445 ± 23**	351 ± 19	**320 ± 14**
Mn	3.5 ± 0.2	**14.8 ± 0.8**	**14.0 ± 0.6**	**7.6 ± 0.5**	**7.1 ± 0.3**	<LOQ	<LOQ
Ni	16 ± 1	<LOQ	<LOQ	**2.7 ± 0.1**	<LOQ	<LOQ	<LOQ
P	<LOQ	**926 ± 75**	**887 ± 71**	<LOQ	<LOQ	<LOQ	<LOQ
Pb	11.1 ± 0.2	**12.0 ± 0.3**	**12.0 ± 0.2**	**12.4 ±0.1**	**12.3 ± 0.1**	**11.69 ± 0.07**	**12.0 ± 0.2**
Pd	5.9 ± 0.3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Sb	8.9 ± 0.1	**7.4 ± 0.1**	**7.4 ± 0.1**	**7.40 ± 0.04**	**7.59 ± 0.09**	**7.41 ± 0.07**	**7.53 ± 0.06**
Sn	10.3 ± 0.1	**4.3 ± 0.3**	**3.92 ± 0.07**	**4.1 ±0.2**	**5.4 ± 0.4**	**5.7 ± 0.2**	**5.5±0.2**
Ti	<LOQ	<LOQ	<LOQ	<LOQ	**11.4 ± 1.1**	**26.0 ± 0.8**	<LOQ
Zn	203.7 ± 2.7	**216 ± 4**	**212 ± 2**	**178 ± 3.4**	**196 ± 5**	178 ± 8	**171 ± 7**

As, Ba, Ca, Cd, Co, Cr, Cs, Fe, Hg, Mo, Sr, and V could not be quantified at any of the *Cannabis* extracts or sesame oils samples. Significantly higher concentrations for the most samples, when compared to the sesame oil, were obtained for Al, K and Mn, while significantly lower concentrations in samples than in sesame oil were observed for Sb and Sn. Although the Student t-test has indicated significant differences among samples and sesame oil for Ag, Pb, and Zn, in general the concentrations were very similar, indicating a probable natural common origin of these elements, which can be from the oil employed in the extraction. Some elements (Au, Cu, Li, Ni, and Ti) presented higher concentration in only few samples, when compared to the sesame oil, which can be from the natural variability of plant samples, although it could also be from different oils employed in the extraction or, more probable, from equipment employed in the extraction of the different samples, since this information was not provided. The same was observed for Mg in two samples, while the others presented concentrations in the same order of the sesame oil.

Higher concentrations of Pb and K and lower concentrations of Sb and Sn were observed for all 6 samples. Mg and Mn were also found at higher concentrations for most samples (5 of 6 and 4 of 6, respectively). Some elements, such as Au, Cu, Li, Ni, P, and Ti were found at higher concentrations for 1 or 2 samples each. Zn was found at higher concentrations for 2 samples while at lower concentrations for 3 samples.

Based on typical consumption of 1 g day^{-1} of the oil extract, USP Chapter 232 and ICH Q3D guidelines dictates a maximum tolerable amount of Pb levels of 5 µg g^{-1} in oral and inhaled *Cannabis* products. Herein, all 6 samples were found at a 2-times higher level. This data is in agreement with previous reports that *Cannabis* user have higher urinary Pb and Cd levels than non-*Cannabis* users (MCGRAW et al., 2023). Chronical lead exposure can lead to several negative health impacts, such as high blood pressure, muscle and abdominal pain, headaches, mood and memory disorders,

reduced sperm count, and many others (KIM et al., 2015).

A significant number of strong correlations were observed. Ag and Pb, potentially toxic metals, demonstrated a strong positive one (ρ = 0.771), suggesting a common source of contamination. That can be attributed to agricultural products and other anthropogenic forms of soil contamination, such as mining, smelting, burning of fuel and industrial and/or vehicular emissions [68,69]. Aluminum provided several strong correlations with essential elements, both positive (K, Mn, and Mg) and negative (Sb and Sn). Previous reports already demonstrated that Al could stimulate micronutrient uptakes in plants (BOJÓRQUEZ-QUINTAL et al., 2017).

Several essential elements to plant growth, e.g., Mn, Mg, K, and Zn, demonstrated strong positives correlations between each other, suggesting common biological mechanisms and sources (K x Mn 0.900; K x Zn 0.771; Mg x Mn 0.900; Mg x K 1.000; Mg x Zn 0.771; Mn x Zn 0.700). It is noteworthy to mention that K, Mg and Mn, essential elements found in remarkably elevated concentrations at the *Cannabis* samples, demonstrated strong negative correlations with the potentially toxic element Sn (ρ = -0.886, ρ = -0.886, and ρ = -0.700, respectively). Ni also demonstrated an absolute negative correlation with Ag and Pb levels (ρ = -1.000) suggesting competitive roles, although this element has been detected in only the sesame oil and one *Cannabis* sample.

5.7. Conclusion

Three sample preparation methods for multielement analysis of *Cannabis* herbal extracts by ICP-MS were developed herein. Design of Experiments was successfully employed to better understand operational parameters influence and achieve maximum performance at 2 of these methods. This is the first time, for the best of our knowledge, that DoE was applied to *Cannabis* treatment for multielement analysis. All three methods demonstrated adequate analytical parameters for most elements when submitted to a partial performance validation based on ANVISA RDC

166/2017 guidelines. The methods' greenness was also assessed and intermediate metrics, with plenty of room for improvements, were obtained. The developed open acid decomposition method was the one selected herein for analytical application to 6 real samples due to its simple conduction, adequate analytical precision, accuracy and sensitivity, and satisfactory ecological performance. A reliable and user-friendly multielement analysis by ICP-MS was achieved and, in comparison to a vegetable oil baseline, statistically significant disparate levels were observed for Au, Cu, K, Li, Mg, Mn, Ni, Pb, Ti and Zn at *Cannabis* herbal extracts for, at least, one analyzed sample.

6. General conclusion

Advanced analytical techniques, such as LC-HRMS/MS and ICP-MS, portrays powerful alternatives to *Cannabis*-based products monitoring, however, further analytical steps, such as sample preparation and instrumental conditions needs to be critically optimized to keep up with instrumental performance. Design of Experiments provided a fast and effective approach to optimize the methods developed herein, ensuring better and more reliable results.

Innovative optimization approaches, such as DoE, are required in modern study fields, especially in pharmaceutical and bioanalytical applications, for quality-oriented designs and for maximum performance.

In this thesis, analytical methods for phytocannabinoids quantification by UHPLC-HRMS/MS and multielement quantification by ICP-MS in *Cannabis* herbal extracts were successfully developed. An ultrasound-assisted liquid-liquid extraction was proposed for the organic analytical approach, while two acid decompositions (open and closed-vessel) and a dilute-and-shoot alternative were explored for the inorganic analytical one.

Distinct optimization strategies were conducted, e.g. Plackett-Burman Design, Full Factorial Design, Central Composite Design and Box-Behnken Design. Its selection took into account analytes abundance, methods nature (i.e. analyte extraction or sample decomposition), literature reports advance, resources availability and, primarily, analytical criteria required.

Optimization was critically designed for its purpose. Process-knowledge was investigated and significant parameters were localized for each protocol, i.e. solvent volume, sonication time and agitation time for UA-LLE method; HNO_3 volume, H_2O_2 proportion and heating time for oAC method; and HNO_3 volume and heating time for cAD method. These parameters levels were delimited for maximum analytes responses.

It is noteworthy to mention that methods optimization for multiple

analytes, such as approached in this work, represents an additional level of difficulty. An absolute optimized condition commonly cannot be found, due to discordant responses for each analyte.

Analytical performance was evaluated by ANVISA RDC 166/2017, the gold-standard validation guidelines for pharmaceutical analytical methods. Methods' parameters regarding linearity, matrix effect, precision, accuracy and sensitivity, were evaluated. Matrix effects correction was studied by distinct calibration strategies such as External Calibration, Matrix Matching and Standard Addition. Acid decomposition methods were efficient enough to reduce matrix contributions and, therefore, EC was a sufficient approach for quantification, while MM was required for UA-LLE and D&S.

Adequate precision, accuracy and sensitivity metrics were achieved for most analytes in all developed methods. The cAD method demonstrated similar precision to oAD for most elements, with lower sensitivity and poor accuracy for abundant elements, that can be attributed to intrinsic contamination issues. The D&S method was the most precise, with satisfactory precisions for all analytes at all levels approached. However, interferences and matrix effects could not be fully corrected, compromising its accuracy for many elements, mainly on high concentrations levels.UA-LLE demonstrated satisfactory accuracy, precision and sensitivity for all analytes.

The D&S method provided the best greenness metric (0.61) in comparison to oAD (0.50) and cAD (0.42) methods. Its major advantages include a few numbers of manual steps, high frequency of samples prepared per batch and lower energy consumption. The cAD method presented the biggest waste generation and was considered the most dangerous method, while oAD stands as an intermediate candidate, with higher number of samples that can be prepared per hour, with low waste generation and a low volume of hazard materials dependency.

Methods application in a *Cannabis* herbal extracts batch

demonstrated significant quality consideration for these products. For phytocannabinoid quantification, major discrepancy between label content and quantified content was observed in these samples. Four formulations containing 6 mg g^{-1} of CBD and 0.3 mg g^{-1} of THC were investigated. No THC or CBN was found in any of the samples. Low amounts of CBD (66 ± 7 ng g^{-1}; 0.001% of its label content) were quantified in only half of the samples. Besides that, the absence of CBN, a degradation product of THC, even on trace levels denies the hypothesis of THC degradation and suggests that THC was not present in any significant level on these products' formulation.

Also, low metal and metalloid contents were determined in the samples. By comparing to a vegetable oil baseline, statistically significant differences were identified only for Au, Cu, K, Li, Mg, Mn, Ni, Pb, Ti, and Zn. Lead was found at higher levels in all Cannabis samples in a range from 11.7 to 12.4 µg g^{-1}, higher than the limit recommended by FDA guidelines for potentially toxic elements. Among the *Cannabis* extract samples, Li, Mg, Mn, Ni, Ti and Zn presented significant differences, suggesting a relevant heterogeneity and non-standardized quality control for these products concerning elemental contaminants.

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