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Analysis of Cannabinoids in Medicinal Cannabis Products: A Comprehensive Review

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Cannabis plant has been known for its medicinal use for centuries. Recent research into its pharmacology has revealed medicinal properties, promoting the use of cannabis for therapeutic purposes and consequently the growth in the production of medicinal cannabis-based products. The potency of cannabinoids in products for therapeutic purposes is essential to assess their effects on health. Medicinal cannabis products, based on extracts from the cannabis plant, however, lack standardization in both preparation and quality control. In this review, chromatographic methods and alternative strategies are described for qualitative and quantitative analysis of cannabinoids in cannabis-based products. The direct application of portable spectroscopic techniques on cannabis farms for monitoring plant growth is also reported. Finally, a special section has been dedicated to the use of chemometric tools that have been employed in the field of cannabis including methods utilizing chemometric tools such as exploratory analysis, multivariate classification, and calibration.

Keywords: *Cannabis sativa* L., quality control, methods, hemp, chemometrics

1. Introduction

Cannabis, also known as marijuana and hemp, has three different varieties: *Cannabis sativa*, *Cannabis indica*, and *Cannabis ruderalis*. It is a dioecious annual plant belonging to the Cannabaceae family. As dioecious species exhibit inflorescences exclusively male or female on separate plants, the differentiation between male and female plants occurs in the early stages of their vegetative development. Exclusively in female plants, the development of trichomes on the bracts of the flowers is observed. These trichomes consist of a unique set of terpenophenolic compounds found in cannabis, called cannabinoids, also known as phytocannabinoids.[1-3]

C. sativa is one of the plants capable of producing cannabinoids with more than a hundred compounds described. Currently, over 550 compounds have been recognized in cannabis, and 144 distinct cannabinoids have been isolated from the plant. In plant tissues, cannabinoids are synthesized in the form of carboxylic acids. The most common acidic cannabinoids found are Δ^9-tetrahydrocannabinoic acid A (THCA-A), cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA). The last one, CBGA is the direct precursor of THCA, CBDA and cannabichromenic acid (CBCA). CBGA derivatives are formed from different enzymes that cyclize the terpene fraction. The carboxyl group exhibits low stability and, when subjected to heat or light, can readily lost as CO_2. This process leads to the formation of the corresponding neutral cannabinoids: Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabigerol (CBG). In other words, neutral cannabinoids arise from photochemical, oxidation, or isomerization reactions that may take place within the plant, or as a result of external conditions after harvest as, for instance, during heating, drying of harvested plant material, storage or when cannabis is smoked. Cannabinol (CBN) is a common degradation product of

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THC, found in higher quantities in cannabis samples that have been stored for a prolonged period.[1,4-6] The mechanism of cannabinoids is shown in Figure 1.

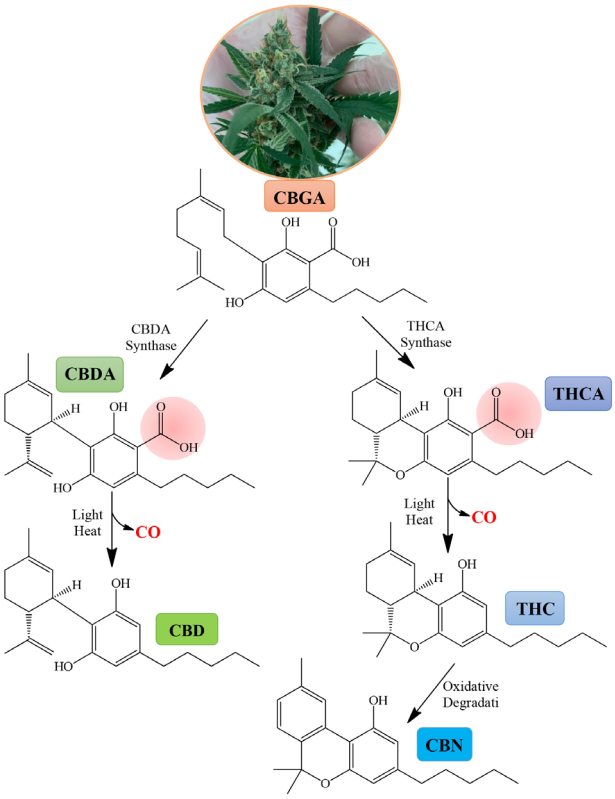

Figure 1. The most common cannabinoids and their biosynthetic pathway from CBGA to THCA and CBDA, conversion of THC and CBD through light and/or heat, and the oxidative degradation process converting THC into CBN.

Interest in the chemistry of cannabis metabolites increased following the discovery of phytocannabinoids, a product of chemical studies carried out in the 1940s and 1960s. Since the early 1940s, researchers[7] have identified and characterized at least 90 cannabinoids from cannabis. A significant discovery occurred in 1964 when Raphael Mechoulam[8] successfully isolated and characterized THC, the primary psychoactive component of cannabis, for the first time. Due to the intoxicating effects resulting from the psychotropic activity of THC, cannabis has become the most widespread drug of abuse, so much so that it is considered illegal in many countries due to its potential for abuse. On the other hand, cannabis has been known for centuries around the world for its medicinal properties. Research into the biological activities of the plants, chemistry and pharmacology has confirmed medicinal properties. For this reason, the study of the plant and its terpenophenolic compounds has sparked increasing interest in the development of medicines and cannabis preparations for various medical applications.[1,2,4,6,9]

1.1. Legal aspects

Following alcohol, cannabis stands as the second most frequently utilized psychotropic substance in the United States.[10] In Europe, its consumption surpasses other substances by approximately fivefold, making it the most commonly used illicit drug.[11] *C. sativa* can grow under different climatic conditions and has excellent genetic adaptability. These characteristics allow the creation of new varieties of genotypes enriched for a specific cannabinoid. As a result, strain varieties have been produced in abundance with limited taxonomic classification, popularly referred to as either medicinal type or marijuana, or industrial type or hemp.[9] To overcome this situation, the United Nations Office on Drugs and Crime[12] has proposed the use of an index based on the amount of the three main cannabinoids to classify cannabis plants. The index is calculated with the relative proportions, that is, the peak area, of THC, CBN and CBD obtained by chromatography, in the expression: {[THC] + [CBN]/[CBD]}. If the index value is greater than 1, THC is predominant and the plant is classified as "drug type" or chemotype I (Δ^9-THC/CBD >> 1.0). When the content of THC and CBD is balanced, a Δ^9-THC/CBD ratio close to 1.0 is for "intermediate type" or chemotype II plants. On the other hand, if the index value is less than 1, CBD is predominant and the plant is classified as "fiber type" or chemotype III (Δ^9-THC / CBD << 1.0). Other chemotypes are also classified as type IV, when cannabigerol (CBG) predominates, and type V when there is an undetectable amount of phytocannabinoids. This approach enables differentiation of the plants based on the proportions of specific cannabinoids and helps classify cannabis varieties.[13-15]

Cannabis with a predominant THC profile is extensively cultivated globally. Approximately 147 million people, constituting around 2.5% of the world population, consume it.[16] Cannabis (fiber type) known as hemp is used for food, textile, and medicinal purposes. The plant is employed for industrial purposes in 50 countries across Europe, Asia, North, and South America. The industrial use of cannabis focuses on the production of more than 2,500 products used in the fields of agriculture, energy, the paper industry, textiles, recycling, personal care products, construction materials, and medical supplements. Regulations by different countries vary the maximum limit of THC in plants allowed. Producing countries require that varieties contain less than 1% THC. In the European Union, the maximum legal limit for cultivation is 0.2% THC, with some exceptions such as the Czech Republic and Austria, with < 0.3%, and Switzerland with 1.0%.[17,18]

In the United States, before 1950, hemp was freely cultivated, mainly to produce fiber for industrial

applications. In 1970, the Federal Controlled Substances Act[19] made the medicinal and recreational use of cannabis, as well as the cultivation of hemp, illegal. This situation, however, changed over time. By February 2022, Medical Cannabis Acts had been enacted in 37 states and the District of Columbia, each with notable variations in specific provisions.[20] As of November 2021, Recreational Marijuana Acts had been passed in 18 states and the District of Columbia.[20] Additionally, in December 2018, the U.S. Congress passed the 2018 Farm Bill,[21] legalizing industrial hemp as an agricultural product. Since then, in the United States, hemp has been defined by law[21] as *C. sativa* that contains no more than 0.3% (m/m) of total THC, calculated as: (Δ-9-THC) + 0.877 × (THCA) based on dry weight. Additionally, and in accordance with Department of Agriculture rules,[22] flower samples from hemp cultivation must be tested 30 days before the expected harvest date. Testing is carried out in registered facilities using traditional chromatography methods.[23-25]

In recent years, *Cannabis* has garnered increased attention in medical research as a therapeutic option, with numerous countries worldwide legalizing its medicinal use. Despite the therapeutic promise of cannabinoids, legislative measures tied to the 1961 United Nations Single Convention on Narcotic Drugs[26] and the 1971 Convention on Psychotropic Substances[27] have significantly impeded research on the pharmacological and therapeutic applications of *Cannabis*. Nevertheless, shifts in national policies concerning medicinal cannabis have taken place in various countries. Between 2012 and 2021, 41 countries (23 in Europe) legalized the use of *C. sativa* and/or cannabis-based products for medical purposes.[18] In 2016, Australia authorized the medicinal use of cannabis, with ongoing investigations into the potential legalization for recreational use.[28] There is confusion regarding the legality and availability of these products, both within and among legal regulatory bodies. Prescription and over-the-counter medicines marketed and approved by regulatory agencies, such as the European Medicines Agency (EMA) and the Food and Drug Administration (FDA), are standardized products and formulated in dosages with proven quality and safety.[29-31] Products from plant-based formulations or artisanal extracts, however, have not been subjected to the quality controls generally associated with legal approval for the marketing of medicines.[5,18,32]

Despite having been legalized in some countries, there are still concerns about how cannabis consumption may affect society. In favor of legalization, there are those who highlight the extinction/minimization of the illegal market, improvement in quality control of inputs and medicines, increase in tax collection, commercial stimulus and reduction in violence linked to gangs and drug trafficking. On the other hand, there are opponents who highlight issues such as chemical dependency, risks to mental health, passive exposure and possible worsening of mental illnesses. The impact of legalization continues to be the subject of debate, with research underway to explore the implications in different jurisdictional areas, although the scientific evidence of the therapeutic potential of cannabis is already well established.[33]

1.2. Medicinal cannabis

For centuries, cannabis has been recognized for its medicinal applications. In recent years, there has been a notable surge in research exploring the medical applications of cannabis, with a number of countries adopting a more flexible stance towards its use as a medicine. Cannabinoids are the main active constituents of the plant and are associated with a broad range of pharmacological activities. Medical cannabis is the term used to describe the therapeutic use of cannabis or cannabinoids, to treat illnesses or alleviate medical symptoms. Medicinal cannabis extracts are obtained from the botanical raw material of the cannabis plant and contain cannabinoids at different levels of purification and refinement.[32,34,35]

The most common cannabinoids, which have paradoxical effects on the central nervous system, are THC and CBD. THC is psychoactive and euphoric, while CBD is a depressant and has anticonvulsant and anxiolytic properties. Clinical studies involving cannabis, cannabinoids, and synthetic analogues have been reported to be efficient in treating conditions such as chronic neuropathic pain, appetite loss in cancer or AIDS (acquired immunodeficiency syndrome) patients, and multiple sclerosis. For this reason, medicinal cannabis has been used for various therapeutic purposes, seeking to offer relief and treatment for specific medical conditions. Cannabis containing elevated THC levels is employed for managing conditions such as Tourette syndrome, glaucoma, and nausea. A mixture of THC and CBD is utilized to alleviate pain and muscle spasms. CBD mitigates pain, inflammation, and the psychoactive side effects of THC; as well, CBD is used to treat various forms of epilepsy.[2,36,37]

Cannabinoids can be administered in a variety of ways, such as orally, sublingually, topically, or by being smoked, inhaled, added to food, or prepared as a tea. They can be used in the form of natural herbs, extracted directly from the plant, obtained through the isomerization of cannabidiol or produced synthetically.[35] These compounds can activate or modulate the endocannabinoid system (EC), which is extensively integrated into various organ systems of the

brain and body. The EC system plays diverse roles in the homeostatic regulation of humans and certain animals.[38]

Various medicinal cannabis products and magisterial preparations with varying ratios of Δ9-THC and CBD are available on the market. Common formulations include filter bags for cannabis decoction, single-dose inhalation formulations, and cannabis extracts, primarily suspended in olive oil or ethanol.[39] Cannabis oil is the preferred form of preparation, given its ease of adjusting individual administration doses throughout the treatment period and the increased bioavailability of its active constituents.[37] In addition to the medicinal cannabis products mentioned above, several cannabis-based medicines have been developed. Among them, Marinol® (Solvay Pharmaceuticals, Belgium), an oral preparation of Δ^9-THC, Nabilone® (Valeant Pharmaceuticals International, USA), a synthetic analogue of Δ^9-THC, and Sativex® (GW Pharmaceuticals, UK) are the most known, as well as an oral mucosal spray that contains a balanced proportion of Δ^9-THC and CBD.[2]

Cannabinoid concentrations may vary depending on the product. For example, CBD oil products marketed in Japan have CBD concentrations between 29 and 109 mg g^{-1}, with THC detected in trace concentrations of 0.002 mg g^{-1}.[40] E-liquids for electronic cigarettes sold in Switzerland have THC concentrations of less than 0.2%, while CBD varies between 0.182 and 3.346%.[41] In extracts from two strains of *Cannabis sativa*, Bedrocan and Bedrolite, CBD concentrations range from 0.38 to 39.2% and THC concentrations from 18.93 to 86.99%.[42] Cannabis oils for medicinal purposes distributed in Brazil have THC concentrations of around 10 mg mL^{-1} and CBD concentrations of 3 mg mL^{-1}.[36] Techniques used for these analyses include liquid chromatography-tandem mass spectrometry (LC-MS/MS), ultra-performance liquid chromatography with diode array detection (UPLC-DAD), Fourier-transform infrared spectroscopy (FTIR), and high-performance liquid chromatography with diode array detection (HPLC-DAD). To detect low concentrations, the LC-MS/MS technique is more suitable due to its high sensitivity, while HPLC-DAD and UPLC-DAD are more affordable and widely used.

The raw material used in the production of medicinal extracts are the flowers of pistillate specimens rich in THCA and CBDA, which are heated to obtain the active ingredients, THC and CBD. These 4 cannabinoids, together with CBN, are widely used as criteria for the following: to characterize Cannabis plant products; provide useful information about the plant such as age, potency (amount of cannabinoids) and possible geographic origin; and to control the quality of medicinal products.[36,43]

When it comes to the quality of medicinal cannabis products, however, other parameters need to be considered in addition to cannabinoid content. Other classes of analytes found in the plant require analysis: terpenes, other secondary metabolites, heavy metals, residual solvents, microorganisms, pesticides, herbicides, fungicides, rodenticides, and other chemical adulterants that can be harmful to health and the environment. The relevance of their analysis is related to the possible harm that these compounds may inflict if they are ingested directly from the plant or if they are co-extracted with cannabis's primary components and later detected in processed cannabis products.[44,45]

Given these problems, consumers may inadvertently purchase products with undesirable properties. In fact, the Food and Drug Administration (FDA) has been issuing warning letters since 2015[46] regarding unwarranted and illicit medical claims concerning CBD formulations produced or distributed by US companies. Cases have been reported[47] in which some products contained significantly lower cannabinoid content than that indicated on the label, while others contained significantly higher amounts of THC than labeled, placing patients at risk of adverse effects. Research on the quantification of cannabinoids in Brazilian medicinal extracts, carried out by Carvalho *et al.*,[36] in 2020, showed a wide variability in the composition of medicinal extracts used to treat the same disease. In preparing the extracts, different vehicles can be used to dissolve the cannabis resin: olive oil, sunflower oil, coconut oil, soybean oil and even oil mixtures. Differences in phytocannabinoid content have also been reported; in addition to CBD, the extracts were found to have other cannabinoids such as THC, CBN, THCA and CBDA. The presence of CBN indicates the degradation of THC; the presence of acid cannabinoids (THCA and CBDA) indicates that the decarboxylation of the raw material was inadequate.

The lack of established guidelines for use, restrictions on varieties and chemical residues, as well as the absence of proven methods for assessing cannabis products, can expose users to harmful substances or residue levels higher than would be permitted if there were clear regulatory guidelines and regulations.[47]

The objective of this literature review is to record the versatility and evolution of analytical methods used in the quantitative and qualitative analysis of phytocannabinoids in cannabis-based products (especially products with medicinal purposes), and cannabis extracts. The use of chemometric tools in data processing and analysis will also be discussed, as well as the development of cannabinoid prediction models and their application in quality control.

2. Methods for the Review

To find relevant publications for the construction of this review, databases such as Scopus, Google Academic and Web of Science were consulted over a period of 6 months. A combination of keywords such as "medical cannabis", "cannabinoids", "analysis", "control", "quantification" and "methods" were searched. In total, 113 articles published between 1970 and 2023 in English, Portuguese or Spanish were selected. The focus of this review was on the analytical part, the methods used to analyze cannabinoids in the cannabis plant and its extracts, and cannabis-based products (especially with medicinal purposes). Therefore, articles were disregarded which had a different perspective such as clinical studies, classification, and taxonomy, as well as uses and cultivation of cannabis, analysis of cannabinoids in biological matrices. Altogether, 98 articles were read to prepare this work, including 16 articles that were cited only in the introduction part, such as book chapters and review articles.

3. Analysis of Cannabinoids

Due to the growth in production of medicinal cannabis formulations, there is an increasing need for the development of analytical methods. Accurate qualitative and quantitative analyses of phytocannabinoids are indispensable for associating medicinal effects and potential negative health impacts associated with the potency of specific phytocannabinoids and other compounds, such as terpenoids. Chromatographic methods, such as liquid chromatography (LC) and gas chromatography (GC), are the most used mainly due to their ability to separate analytes. THC was isolated for the first time in 1964 by Raphael Mechoulam,[8] using alumina chromatography for analysis. With the advancement of technology and analytical techniques, cannabinoid analysis methods have evolved significantly. Currently, advanced techniques such as high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) are used, coupled with ultraviolet diode array detection (UV-DAD), which enable the accurate identification of cannabinoids by their chromophore groups. At the same time, these detectors reveal the presence of interfering compounds in various sample matrices.[48] Furthermore, the introduction of liquid chromatography coupled with mass spectrometry (LC-MS) has revolutionized the analysis of polar and unstable compounds, increasing sensitivity and selectivity in the detection of analytes in complex matrices. This coupling has made it possible to identify and quantify compounds in complex mixtures by measuring the mass-to-charge ratio of the ions of the separated analytes, thus overcoming the low specificity of conventional detectors.[49,50] When coupled to gas chromatography, mass spectrometry also provides detailed structural information about mass spectrometry analytes, in addition to GC-MS systems having high sensitivity.[51]

One of the primary challenges in analytical assessments for quality control, however, is the proficiency of laboratories to do the analysis. Standardized tests are necessary that meet analytical criteria that would be approved by the competent control authorities. Laboratories also face challenges related to the cost and accessibility of some cannabinoid standards. Another difficulty is related to the complexity of the matrices of medicinal cannabis products. For example, an oily matrix can lead to technical damage to analytical instruments and diminish the operational lifespan of chromatographic columns.[7,18,52,53]

For the development of analytical methods applied to cannabis-based products, it is necessary to extract cannabinoids from plant material, prepare the obtained extract, and then perform detection followed by quantification of the concentration of each cannabinoid found in the plant.[54] The focus of this review is on qualitative and quantitative analytical methods for cannabinoids in plant extracts and products used for medicinal applications.

3.1. Chromatographic techniques

Traditionally, the determination of cannabinoid content has been conducted using HPLC and GC coupled to MS; alternatively, flame ionization detection (FID) has been used for GC or UV-DAD for HPLC, as detectors. The GC method has been widely used in the quantitative analysis of cannabinoids, but it is not capable of distinguishing between cannabinoids and their carboxylic counterparts without prior derivatization, because as the method involves subjecting the sample to heat, causing the acidic forms of cannabinoids to undergo decarboxylation and transform into their neutral counterparts. On the other hand, the HPLC method makes it possible to determine the original composition of cannabinoids in the plant through direct analysis. Unlike GC, no decomposition of cannabinoids occurs during HPLC analysis.[1,17,34,55] The most common analytical technique for determining cannabinoids in plants in most laboratories worldwide is high-performance liquid chromatography coupled with either diode array detection or mass spectrometry. These are preferred for analyzing the phytocannabinoid profile due to their robustness, reproducibility, sensitivity and speed.[18,56] In addition to cannabinoid analysis, liquid chromatography has been used in terpene analysis. Caruso *et al.*[57] reported for the first

time, a two-dimensional liquid chromatography method for the simultaneous separation of terpenes and cannabinoids in cannabis plant material. The proposed method makes it possible to identify 21 terpenes and 10 cannabinoids in cannabis samples, resolving between 40 and 54 peaks in just 65 min. This is due to the distinctive advantage of two-dimensional liquid chromatography, which lies in its superior ability to separate multiple compounds in complex samples.

3.1.1. Gas chromatography (GC)

GC is a chromatographic technique employed for the separation and analysis of compounds from various matrices that can undergo vaporization without decomposition. GC is one of the most frequently used chromatographic techniques for the quantitative analysis of phytocannabinoids in plant material and biological matrices due to its robustness and reproducibility. When coupled with mass spectrometry, it adds high sensitivity and the ability to provide detailed structural information about the analytes.[18,51,58]

The most commonly used solvents for extracting cannabinoids, prior to chromatographic analysis by GC, are methanol,[37,43,59,60] and ethanol.[61-65] Hexane[14] and dichloromethane[66] have also been used. Regarding the columns, non-polar or low-polarity stationary phases, such as dimethylpolysiloxane, have been employed[59] as well as mixtures with phenyl, dimethylpolysiloxane 95%/ phenyl 5%.[60,66,67] Regarding the mobile phase, the carrier gas mostly used is helium; in fact, of the studies mentioned in this article, only one of them used hydrogen.[64] The temperature ramps used mostly range up to 300 °C with analysis times from 12[67] to 39.9 min.[61] Due to the need for high temperatures in the chromatographic column, acidic cannabinoids undergo decarboxylation during analysis, therefore, in GC, the total amount of cannabinoids in a sample is the sum of the acidic and neutral components.[9,58]

The literature gives examples of cannabinoid analysis by GC for various purposes, for example, Broséus *et al.*[14] developed a method for distinguishing between drug-type and fiber-type cannabis based on the relative proportions of the main compounds found in the leaves of cannabis seedlings. With the proposed method, it would not be necessary to wait for the plant to flower to determine the cannabis chemotype. The chemical profile of cannabis was studied using high-resolution mass spectrometry and two-dimensional analysis, making it possible to identify the chemical structures of cannabinoids and other compounds of interest, such as pesticides and degradation products. With the GC-MS system, it is possible to use libraries for structural identification.[59,60,64] Likewise, GC-MS/MS with multiple reaction monitoring (MRM) allowed verification of the legality and screening of cannabinoids in CBD oils.[52] When analyzing galenic preparations of cannabis oil, Cas *et al.*[37] reported that cannabinoid content was significantly linked to cannabis chemotypes and extraction protocols. The authors evaluated samples with and without a previous decarboxylation step. With the help of selected ion monitoring (SIM) by a mass detector, it was possible to evaluate which oils were richer in the neutral active form of cannabinoids (CBD, THC) through a decarboxylation step. Ciolino *et al.*[61] identified cannabinoids in cannabis-based consumer products in the United States. Commercial products analyzed included foods, sweets, beverages, topicals, vapes/e-liquids, oral supplements; hemp seed oils of different origins and generic dronabinol capsules. Most of these products presented CBD as the predominant cannabinoid.

3.1.2. Liquid chromatography (HPLC/UPLC)

HPLC is recognized as a modern, powerful, and versatile chromatographic separation technique. It is the most employed analytical separation method for qualitatively and quantitatively assessing compounds in natural product extracts, fractions, or end products. UPLC, an advanced liquid chromatography technique, requires a short analysis time and uses a minimal quantity of solvent(s) as the mobile phase. UPLC instruments are characterized by smaller particles, less than 2 microns, compared to larger particles, between 2.5 and 10 microns, in conventional HPLC systems. Due to the smaller particle size, UPLC operates at higher pressures (above 6,000 psi) and offers greater separation efficiency of analytes from samples due to the shorter diffusion path between them and the stationary phase. Several types of detection systems can be used in liquid chromatography; for cannabinoid analysis, however, the DAD have emerged as the standard for cannabinoid potency testing, as well as the use of liquid chromatography coupled to mass spectrometry.[24,68] In fact, a bibliometric analysis carried out on publications that contained key words such as analysis and cannabinoids or cannabis, showed a strong connection to liquid chromatography and quality control when the word "cannabinoids" was chosen as the key word on the map, as illustrated in Figure 2. This indicates that liquid chromatography is one of the most recurring topics in publications relating to cannabinoid analysis. There are also other links to cannabis, hemp, THC, synthetic THC (dronabinol), and CBD and its extracts.

In the published literature[1,24,43,44,54,57,59,61-63,66,70-73] on cannabinoid analysis employing chromatographic methods, the majority of studies reported have analyzed extract of the

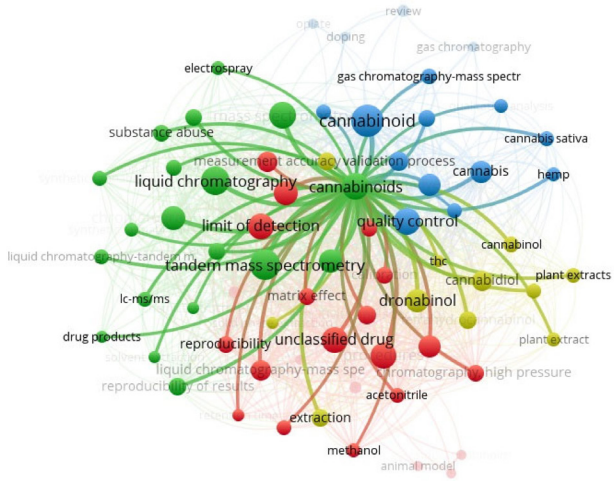

Figure 2. Map based on bibliographical data with co-occurrence analysis of keywords, highlighting the connections with the word cannabinoids. Figure obtained from Vosviewer (Centre for Science and Technology Studies Leiden University).[69]

plant material. Some studies have analyzed cannabinoids from the leaves[14,74] and most of them have analyzed the flowers.[5,13,60,64,65,75-86] Other products have also been analyzed, such as hemp nuts,[87] CBD oil,[40,52] hemp seed oil,[61,62,88] oil preparations, which include diluting the plant extract in oil, or extracting the cannabinoids directly into oil[36,37,39,67,71,89] and generic capsules.[62,70,90]

Prior to a chromatographic analysis, it is necessary to extract the cannabinoids from their respective matrices. The solvent most commonly used for this purpose is methanol, followed by ethanol and lastly mixtures of methanol with various solvents, for example methanol/chloroform;[1,17,34,54,76,89] methanol/acetonitrile;[71,81] methanol/hexane[36,44] and methanol/acetone.[60] The use of hexane as an extraction solvent,[72] as well as the mixture of ethanol and acetonitrile in a 50:50 ratio,[73] have also been reported.

Methods for extracting non-psychoactive cannabinoids such as CBD, CBDA, CBG, CBGA from fiber-type plant material have been evaluated. Brighenti *et al.*[77] tested 4 techniques to optimize the extraction of cannabinoids in pharmaceuticals and hemp. The techniques tested were ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction and dynamic maceration. Ultrasound and microwave assisted extraction are techniques employing sound waves or microwaves to accelerate the extraction process and enhance the yield of metabolites. Supercritical fluid extraction offers the advantage of eliminating flammability or toxicity concerns since the solvent is completely removed. Dynamic maceration involves extracting plant material using a solvent and multiple agitations. The extraction time and temperature were optimized for each technique and were compared under their optimal conditions. Studies report[77] that dynamic maceration with ethanol at room temperature for 45 min was the best technique among those tested. On the other hand, in a different study, Tzimas *et al.*[85] also evaluated cannabinoid extraction techniques: extraction assisted by ultrasound, microwaves and dynamic maceration in fiber-type cannabis inflorescences. The researchers reported more promising results for ultrasound-assisted extraction, given that acoustic cavitation favors the penetration of the solvent into the plant tissue and the diffusion of solutes within the extraction medium. Similarly, Baranauskaite *et al.*[63] pointed out ultrasound-assisted extraction was the best technique for extracting cannabinoids compared to maceration and heat extraction by reflux. Ultrasound-assisted extraction was considered the ideal technique as it required less time, energy and cost. Correia *et al.*[73] also highlight the use of ultrasound in the extraction process. The authors proposed and optimized a methodology for ultrasound-assisted solid-liquid extraction. The extraction process was optimized using experimental design to evaluate different solvents, volumes, steps and sonication time. The mixture of ethanol and acetonitrile (50:50) provided the highest extraction yield, with 1 step of 1 min of sonication and a volume of 10 mL of solvent.

Sample preparation varies depending on the matrix. Among cannabis products for medicinal purposes, the oily matrix is the most common. Preparing cannabis oil for analysis involves accurately measuring a specific amount of oil, typically between 50 and 100 mg, and dissolving it in suitable solvents such as methanol, isopropanol, dichloromethane, acetonitrile, or solvent mixtures such as methanol (9:1 v/v). After adding the solvent, the mixture is vortexed and, in some cases, subjected to ultrasound to ensure complete homogenization. The samples are then centrifuged and filtered using Teflon or nylon filters with a porosity of 0.22 to 0.45 μm. Some protocols include cooling to −20 or −41 °C to facilitate phase separation. This step helps freeze the oil layer, allowing the liquid solvent layer to be pipetted into a new container. Finally, samples can be diluted and mixed with internal standards before being transferred to HPLC vials and stored at 4 °C until analysis.[36,53,75,80,91]

Published literature shows that for cannabinoid analysis with chromatography, the stationary phase most used has been a C18 reversed phase column. Some authors used the following C18 column types with complementary selectivity: C18-AR;[24,64,75] EC-C18, C18 stationary phase with superficially porous particles; [78,88] or the SB-C18 and XB-C18, C18 stationary phase with fully porous particles.[13,39,65,82] The vast majority of mobile phases were used with gradients of water acidified with formic acid (0.1% v/v) and acetonitrile or ammonium formate and

acetonitrile buffers. Some studies have reported the use of acetonitrile acidified with formic acid[5,44,64,81-83,85,87,89] or acetonitrile acidified with acetic acid.[62,86,91] Liquid chromatography has emerged as the gold standard for cannabinoid analysis using DAD as detector, as well as coupled to MS. Several types of mass spectrometry analyzers are used in cannabinoid analysis, each with its specific advantages. The triple quadrupole (tQ) provides high sensitivity and selectivity at a low cost and is commonly used for cannabinoid quantification in routine methods due to its robustness.[5,83,89] Time of flight (ToF) analyzers provide high resolution and rapid analysis and are used for the identification and characterization of cannabinoids due to their ability to provide accurate mass data.[24,92,93] The orbitrap provides better resolution and accuracy of mass/charge ratio data, making it ideal for identifying new cannabinoids and analyzing complex samples.[13]

Additionally, it is possible to observe that recently some works[78,82,83] have reported using sequential mass spectrometry (MS/MS) systems due to the possibilities of obtaining more detailed structural information. This ability to fragment selected ions, enables the acquisition of detailed information about the structure of the cannabinoids and better analytical sensitivity and selectivity. Sequential mass analysis improves detection sensitivity and selectivity, reducing interference and increasing quantification accuracy.

In summary, mass spectrometry is an essential technique for analyzing cannabinoids in medicinal samples, offering high sensitivity, specificity, and the ability to provide detailed information about molecular structure. The use of different types of analyzers and the combination with HPLC systems further improve the accuracy and quality of the data obtained. Table 1 lists works on cannabinoid analysis using liquid and gas chromatography as the main technique.

The methods developed for qualitative and quantitative analysis of cannabinoids by liquid chromatography have also been validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, recovery and selectivity.[60,73,75] Validation of the methods was carried out in accordance with International Organization for Standardization (ISO) 17025[94] and SFSTP (French Society of Pharmaceutical Sciences and Techniques);[1] the AOAC (Association of Official Analytical Chemists) and ASTM (American Society for Testing and Materials),[71] as well as the ICH (International Council for Harmonization).[53,54,85,91]

Although, in general, the methodologies for cannabinoid analysis have been the same, that is, by extraction of cannabinoids from the matrix and chromatographic analysis, the purpose or motivations for these studies have been different. For example, for quality control, Carvalho *et al.*[36] quantified THC, CBD, CBN, THCA, and CBDA in artisanal medicinal preparations produced in Brazil by patients' families. The authors found that only 10 of the 68 extracts analyzed had a predominant CBD profile and, even so, the levels were much lower than those determined in American extracts. Furthermore, high levels of THCA, CBDA, and CBN were found in the national samples analyzed. Similarly, de Backer *et al.*[1] analyzed cannabinoids to evaluate the psychoactive potency and quality control of medicinal samples. Li *et al.*[90] developed a method to evaluate the quality and stability of medical cannabis products produced in New York and to confirm the potency described on the labels. Takashina *et al.*[40] tested the quality of CBD oil products sold in Japan, by evaluating the CBD profile and identifying and quantifying THC residues. Some of the samples that revealed traces of THC exhibited concentrations of 0.007 and 0.002 mg g^{-1}. These values are in line with the limits established by the European Union,[95] which has set the maximum THC limit in CBD oils at 0.0075 mg g^{-1}. Citti *et al.*[88] carried out a kinetic study of the decarboxylation of CBDA present in hemp seed oil. The reported results suggest that the best storage condition is 5 °C, in order to preserve the stability of the oil for longer periods. Analysis of THC and CBD and other less abundant cannabinoids has been used to distinguish strains of the cannabis plant;[13,79] for assessment of therapeutic potency;[44] for evaluation of new, faster and more efficient methods to extract cannabinoids and terpenes from plant material;[71] for classification of medicinal cannabis samples based on their cannabinoid and terpene profiles;[65] to study cannabinoids present in different parts of the cannabis plant;[59] to identify hemp retailers based on the chemical content of cannabinoids in hemp samples;[74] to determine the best solvent for extraction;[75,78,81] as well as to determine the best extraction technique.[63,77,85] The time of chromatographic analysis has also been evaluated, with methods lasting 30 min,[1,36] 10 min[74,76] and 8 min *per* sample.[5,70]

3.1.3. Disadvantages of chromatographic techniques

Despite being accurate and standardized for certification purposes, as mentioned above, chromatographic methods (LC and GC) have disadvantages such as sample destruction, high sample preparation costs and run times, involving the extraction of active ingredients with organic solvents, which limit their application in the location where a quick and non-destructive process is preferred. Also

Table 1. Chromatographic methods for analyzing cannabinoids in cannabis extracts and medicinal products

Method	Matrix	Sample preparation	Stationary phase (SP) and mobile phase (MP)	Analyte	Chemometrics tool	Reference
GC×GC-FID	cannabis resin	methanol extraction	SP: 2 columns, dimethylpolysiloxane and polyethylene glycol MP: helium	chemical profile	PCA, HCA	59
GC-FID	hemp plant material	three ethanol extraction techniques: maceration, ultrasound-assisted extraction and reflux heat extraction	SP: capillary column Rxi-5 MS MP: helium	CBD, CBG	N/A	63
GC-FID, GC-MS	cannabis oil preparations	not reported	SP: capillary column DB-5MS UI 5% diphenyl/95% dimethylpolysiloxane MP: helium	THC, THCA, CBD	N/A	67
GC-MS	leaves from different cannabis chemotypes	ultrasound-assisted hexane extraction	SP: capillary column HP-5 ms MP: helium	THV, CBD, THC, CBN	LDA and SVM (support vector machines)	14
GC-MS	plant material	ultrasound-assisted methanol extraction	SP: capillary column J&W HP5 MP: helium	THC, CBD, CBN, CBG	ANOVA and HCA	43
GC-MS	cannabis oil	methanol extraction	SP: capillary column Rxi-5ms MP: helium	CBD, CBDA, THC, THCA, CBN	PCA, HCA, PLS-DA	37
GC-FID	hemp flowers	extraction in hexane	SP: fused silica capillary column (SPB-5) MP: helium	THC, CBD	N/A	72
GC-MS/MS	CBD oil	QuEChERS	SP: fused silica capillary column VF-5 MS MP: helium	CBD, THC, CBN	N/A	52
GC×GC-LR TOF MS; GC×GC-HR TOF MS	*Cannabis* flowers (*Indica*, *sativa* and hybrid types)	extraction in water/ methanol/acetone mixture (5:4:1)	SP: 2 columns, nonpolar Rxi-5MS (5% diphenyl-95% dimethylpolysiloxane phase) and midpolar Rxi-17Sil MS (equivalent to a 50% diphenyl-50% dimethylpolysiloxane phase) MP: helium	THC, CBD, CBN	PCA, HCA, DOE	60
GC-MS HPLC-DAD	THCA-rich plant materials, commercial cannabis consumer products, hemp seed oil, and generic dronabinol capsules	extraction in variations of ethanol (95% aqueous or pure) or acetonitrile (83-91% aqueous or pure)	GC-MS SP: Restek Rxi-35Sil MS MP: helium HPLC-DAD SP: C18-AR. MP: acetonitrile 66:34: 0.5% acetic acid (no pH adjustment, nominal pH 2.9)	D9-THC, D8-THC, THCA, CBD, CBDA, CBN, CBG, CBGA, CBDV, THCV, CBC	N/A	61,62
GC-MS HPLC-DAD	inflorescences of different chemovars of medicinal cannabis	ethanol extraction	GC-MS SP: DB-5 capillary column (5% phenyl, 95% dimethylpolysiloxane) MP: helium HPLC-DAD SP: XB-C18 MP: formic acid and 20mM ammonium formate buffer (pH 2.9) and acetonitrile	CBDA, CBGA, CBG, CBD, THCV, CBN, D8-THC, D9-HC, CBL, CBC, THCA, CBCA	PLS-DA	65

Table 1. Chromatographic methods for analyzing cannabinoids in cannabis extracts and medicinal products (cont.)

Method	Matrix	Sample preparation	Stationary phase (SP) and mobile phase (MP)	Analyte	Chemometrics tool	Reference
GC-FID, HPLC-DAD	cannabis flowers	ethanol extraction	GC-FID SP: column Elite 5MS MP: hydrogen HPLC-DAD SP: C18 MP: 0.1% of formic acid in water and 0.1%	THCA, CBDA, THC, CBG, CBD, CBDVA, CBDV, CBGA, THCV, THCVA, CBC	N/A	64
GC-FID, HPLC-DAD	cannabis plant (aerial parts) (agricultural hemp and smoking product)	GC: dichloromethane extraction	GC-FID SP: DB-5 MS capillary column MP: helium HPLC-DAD SP: CORTECS Shield RP18 MP: 0.1% formic acid in water (v/v) and acetonitrile	THCA, THC, CBDA, CBD, CBN	N/A	66
TLC	dried pistillate inflorescences of cannabis, THC-dominant chemotype	ultrasound-assisted methanol extraction	SP: aluminum sheets pre-coated with silica gel 60 F245. MP: hexane-ethyl acetate-methanol ternary system (70:20:10 v/v/v)	CBDA, THCA, CBGA, CBD, CBG, CBN, D8-THC, D9-THC	DOE	84
HPLC-UV	commercial hemp seed oil	dilution in 2-propanol	SP: EC-C18 MP: 0.1% of formic acid in water and acetonitrile	THCA, THC, CBDA, CBD, CBN, CBG, CBDV	N/A	88
HPLC-UV	cannabis (plant material)	extraction in olive oil	SP: Poroshell 120 EC-C18 MP: 0.1% (v/v) formic acid aqueous phase and 0.05% (v/v) formic acid in methanol	THC, CBD, CBN, THCA, CBDA	ANOVA	71
HPLC-UV	hemp plant material	ethanol extraction	SP: C18. MP: water/acetonitrile in a ratio of 9:31 (v/v), with 0.1% formic acid (v/v) and 10 mM ammonium formate (without pH adjustment)	THCA, D9-THC, D8-THC, THCV, CBDA. CBD, CBDV, CBG, CBN, CBC	N/A	70
HPLC-DAD	drug and fiber cannabis	methanol: chloroform (9:1) extraction	SP: C18 MP: methanol and water containing 50 mM ammonium formate (pH 5.19)	THC, THCA, CBD, CBDA, CBG, CBGA, CBN, D8-THC	DOE	1
HPLC-DAD	dried flowers and cannabis oil	methanol extraction	SP: C18 MP: acetonitrile and 10 mM ammonium formate (pH 3.6)	D9-THC, D8-THC, THCA, CBD, CBDA, CBN, CBG, CBC, THCV	DOE	75
HPLC-DAD	cannabis flowers	methanol: chloroform (9:1) extraction	SP: C18 MP: 25 mM ammonium acetate solution (pH 5.75) and methanol	D9-THC, D8-THC, THCA, CBD, CBDA, CBGA, CBG, CBN	N/A	76
HPLC-DAD	cannabis flowers of different strains	extraction in 80% methanol	SP: C18 MP: 10 mM ammonium formate (pH 3.6) and acetonitrile	THC, CBD, THCA, CBDA, CBN, CBG, CBGA, CBC, CDBVA	PCA, MLR	79
HPLC-DAD	cannabis oil preparations/cannabis olive oil extracts	dilution in THF and methanol	SP: 120 SB-C18 MP: ACN/water mixture containing 5 mM K 2 HPO 4 adjusted to pH 3.45 (range 3.11-3.50) at 75/25 v/v ratio	CBD, THC	AQbD, DOE	39

Table 1. Chromatographic methods for analyzing cannabinoids in cannabis extracts and medicinal products (cont.)

Method	Matrix	Sample preparation	Stationary phase (SP) and mobile phase (MP)	Analyte	Chemometrics tool	Reference
HPLC-DAD	capsules, tablets, sublingual oral solutions, tinctures and vaporizer cartridges	extraction in methanol	SP: Poroshell® 120 MP: 0.1% (v/v) formic acid in 25 mM aqueous ammonium formate; and 0.1% (v/v) formic acid in acetonitrile	THCA, CBDA, CBGA, CBDV, THC, CBD, CBN, CBC, CBG, THCV	N/A	90
HPLC-DAD	cannabis and hashish plant material	extraction in methanol: hexane (9:1) assisted by ultrasound	SP: C18 MP: 0.1% formic acid in water and 0.1% formic acid in acetonitrile	THC, THCA, CBD, CBDA, CBN	N/A	44
HPLC-DAD	medicinal extracts in oils	extraction in methanol: hexane (9:1) assisted by ultrasound	MP: ammonium formate buffer as solvent, 50 mM, (pH 5.19) and methanol	THC, CBD, CBN, THCA, CBDA	N/A	36
HPLC-DAD	hemp oil supplements	acetonitrile extraction	SP: C18 MP: 0.5% acetic acid in water and acetonitrile	CBD, CBDA, THC, CBN	N/A	91
HPLC-DAD	dried pistillate flowers of predominant thc chemotype	ultrasound-assisted methanol extraction	SP: C18 MP: 0.05% acetic acid in water adjusted to pH 4.40 ± 0.05 with ammonium hydroxide (30% solution); and acetonitrile	CBDVA, CBGA, CBDA, THCA, CBDV, THCV, CBD, CBG, CBN, D8 -THC, D9 -THC, CBC	DOE, (CCD)	86
HPLC-DAD	dried cannabis inflorescences and cannabis oil	extraction in acetonitrile: methanol (4:1 v/v) assisted by ultrasound	SP: C18 MP: phase A (Milli-Q water buffered with 20 mM ammonium formate and 0.1% formic acid), mobile phase B (acetonitrile) and mobile phase C (methanol buffered with 10 mM ammonium formate and 0.05% formic acid)	D9 -THC, D8 -THC, THCA-A, CBN, CBD, CDBA, CBC, CBDV, CBG, CBGA	N/A	53
HPLC-DAD	cannabis (plant material)	2 extraction methods and 2 solvents ultrasound-assisted extraction and turbo-extraction. methanol: chloroform (9:1) and ethanol	SP: C18 MP: 0.1% formic acid and methanol solution	CBD, CBD, THC, THCA, CBDA	N/A	54
HPLC-DAD	cannabis herbal samples	ultrasound-assisted acetonitrile: ethanol (50:50, v:v) extraction	SP: C18 MP: isocratic separation composed of 75% acetonitrile and 25% ultrapure water with 0.1% formic acid (pH ca. 2.8)	D9-THC, CBD, D8-THC, CBN, D9-THCA, CBDA	DOE	73
HPLC-DAD HPLC-ESI-MS and MS2	female inflorescence of fiber type *C. sativa*	4 extraction techniques evaluated: dynamic maceration, extraction assisted by ultrasound, microwaving and supercritical fluids	SP: C18 MP: water acidified with 0.1% formic acid and acetonitrile	CBDA, CBD, CBGA, CBG	N/A	77
HPLC-DAD, ESI/TOFMS	1 placebo, 8 samples of plant material, cigarette samples, hemp flowers	ultrasound-assisted methanol extraction	SP: column Raptor ARC-18 MP: 0.5 mM ammonium formate plus 0.02% (v/v) formic acid (pH 3.0) and acetonitrile	CBDVA, CBDV, CBDA, CBGA, CBG, CBD, THCV, ACBD, CBCV, THCVA, CBN, CBNA, D9-THC, D8-THC, CBL, CBC, D9-THCA, D8-THCA, CBCA, CBLA, CBT	N/A	24

Table 1. Chromatographic methods for analyzing cannabinoids in cannabis extracts and medicinal products (cont.)

Method	Matrix	Sample preparation	Stationary phase (SP) and mobile phase (MP)	Analyte	Chemometrics tool	Reference
HPLC-MS	powdered hemp nuts	ultrasound-assisted isopropanol extraction	SP: C18 MP: water and acetonitrile containing 0.1% formic acid	THC, CBD, CBN	Design of Experiment, HCA and PCA	87
HPLC-MS	extracts of Bedrocan®, Bediol®, Bedrolite® and mixed preparations	extraction in olive oil dilution in isopropanol	SP: column Acquity® UPLC HSS T3 MP: ACN: water (75: 25 + 0.05% formic acid) and isopropanol:ACN (80: 20 + 0.05%)	THC, CBD, THCA, CBDA, CBN	N/A	80
HPLC-MS/MS	medicinal cannabis extracts, Bediol inflorescences	extraction in ethanol and olive oil dilution in 2-propanol	SP: EC-C18 MP: water acidified with 0.1% formic acid and acetonitrile	CBDA, CBGA, THCA, CBD, THC, CBG, CBN	PCA	78
HPLC-MS/MS	hemp flowers	ultrasound-assisted ethanol extraction	SP: C18-XB MP: water containing 5 mM formic acid and acetonitrile with 5 mM formic acid	THC, CBD, CBC, CBG, CBN, CBDV, THCA, CBGA, CBDA	PCA, PLS-DA	82
HPLC-MS/MS	cannabis and hemp flowers	extraction in methanol: water (80:20)	SP: C18-amide MP: 100:0.1 water: formic acid and 100:0.1 acetonitrile: formic acid	CBDV, THCV, THC, CBD, CBC, CBG, CBN, CBL, CBDVA, THCVA, THCA, CBDA, CBCA, CBGA, CBNA, CBLA	N/A	83
LC-MS/MS	CBD oils products	dilution with isopropanol and water/ methanol	SP: Inertsil ODS-HL column MP: 0.1% formic acid in water and 0.1% formic acid in acetonitrile	CBD, D8-THC, CBN, CBG, CBDA, CBGA, CBD, D9-THC	N/A	40
LC-QQQ-MS	dried cannabis inflorescences	extraction in methanol	SP: C18 MP: water with 0.1% formic acid and acetonitrile with 0.1% formic acid	CBDVA, CBDV, CBDA, CBGA, CBG, CBD, THCV, THCVA, CBN, CBNA, THC, Δ8-THC, CBL, CBC, THCA-A, CBCA	N/A	5
UPLC-DAD	fiber-type cannabis inflorescences of different chemotypes	3 ethanol extraction techniques: dynamic maceration, ultrasound-assisted extraction and microwave-assisted extraction	SP: C18-PFP MP: water and acetonitrile, both containing 0.1% (v/v) formic acid	CBD, CBDA	DOE	85
UPLC-UV-MS	cannabis flowers	ultrasound-assisted extraction in acetonitrile: methanol (80:20)	SP: C18 MP: water containing 0.05% formic acid and acetonitrile with 0.05% formic acid	THC, CBG, CBD, THCA, CBGA, CBDA	N/A	81
UHPSFC/PDA-MS	cannabis extracts, flower, leaf and hashish samples	acetonitrile: methanol (80:20) extraction	SP: column ACQUITY UPC2 BEH 2-EP (2-ethyl pyridine) MP: CO_2 as solvent and isopropanol: acetonitrile (80:20) with 1% water	CBL, CBD, D8-THC, THCV, D9-THC, CBC, CBN, CBG, THCA-A, CBDA, CBGA	PCA and PLS-DA	74
UPLC-HRMS	cannabis flowers	extraction in 96% ethanol	SP: SB-C18 MP: 0.1% (v/v) of aqueous solution of formic acid and acetonitrile	phytocannabinoid profile	PCA, PLS-DA	13

Table 1. Chromatographic methods for analyzing cannabinoids in cannabis extracts and medicinal products (cont.)

Method	Matrix	Sample preparation	Stationary phase (SP) and mobile phase (MP)	Analyte	Chemometrics tool	Reference
UPLC-MS/MS	cannabis oil and tea	methanol: chloroform (9:1) extraction	SP: C18 MP: acetonitrile and acidified water (0.1% formic acid)	THC, THCA, CBD, CBDA, CBN, CBG, CBC	N/A	89

ANOVA: analysis of variance; AQbD: analytical quality by design; CBL: cannabicyclol; CBLA: cannabicyclolic acid; CBD: cannabidiol; CBDA: cannabidiolic acid; CBDV: cannabidivarin; CBDVA: cannabidivarinic acid; CBG: cannabigerol; CBGA: cannabigerolic acid; CBNA: cannabinolic acid; CBC: cannabichromene; CBCA: cannabichromenic acid; CCD: central composite design; DA: discriminant analysis; DAD: diode array detector; DOE: design of experiments; ESI: electrospray ionization; FID: flame ionization detector; HCA: hierarchical cluster analysis; HPLC: high-performance liquid chromatography; LC-QQQ-MS: liquid chromatography triple quadrupole mass spectroscopy; LDA: linear discriminant analysis; MLR: multiple linear regression; MS: mass spectrometry; PCA: principal component analysis; PFP: pentafluorophenyl; PLS: partial least squares; SVM: support vector machine; THC: delta-9-tetrahydrocannabinol; THCA-A: delta-9-tetrahydrocannabinolic acid A; THCVA: tetrahydrocannabivarinic acid; THCV: tetrahydrocannabivarin; THV: tetrahydrocannabivarinic acid; UHPSFC: ultra-high-performance supercritical fluid chromatography; UPLC: ultra-performance liquid chromatography; N/A: not applicable.

required are complex and expensive instruments, well-trained technicians, use of hazardous chemicals, and long chemical analysis times, which represent major challenges for implementing regulatory testing, making them harder to use for quality control within design and manufacturing facilities.[1,23,34,96]

These constraints have prompted the quest for quicker, more user-friendly alternatives to HPLC and GC. At the same time, the development of the hemp and medicinal cannabis industry has presented a demand for fast, robust and cheap methods for large-scale testing, thus encouraging alternative analytical techniques to cover this need.[17,97]

3.2. Alternative methods

The number of publications on cannabinoid analyses has grown considerably since 2004 (Figure 3a). With the advancement of technology, the growing popularity of the use of alternative methods is evident when comparing these with studies that employ classical methods for analysis, as illustrated in Figure 3b. The techniques that make up these alternative methods will be discussed below, as well as their applications.

3.2.1. Vibrational spectroscopy

Analytical tools such as infrared (IR) and Raman spectroscopy techniques have become instrumental in monitoring attributes of quality within the pharmaceutical industry. These techniques excel in rapidly screening substantial sample volumes, offering a versatile, non-destructive approach for qualitative and quantitative profiling, as well as identifying growth stages of cannabis plants and extracts.[14] In recent years, there has been a growing use of non-destructive vibrational spectroscopy methods, such as infrared spectroscopy or Raman, for the analysis of marijuana.[98]

Mid-infrared spectroscopy (MIR, 4000-400 cm^{-1}) is a method used to analyze the molecular structure of unknown compounds. By obtaining these patterns, it is possible to obtain information about the molecular structure by comparing spectra to identify specific fingerprints, which is useful in drug quality control. Furthermore, MIR can analyze complex mixtures such as cells and food, identifying small changes in the samples.[99]

Vibrational spectroscopy techniques combined with chemometric tools have become the favored technology for detecting and quantifying materials in the agricultural

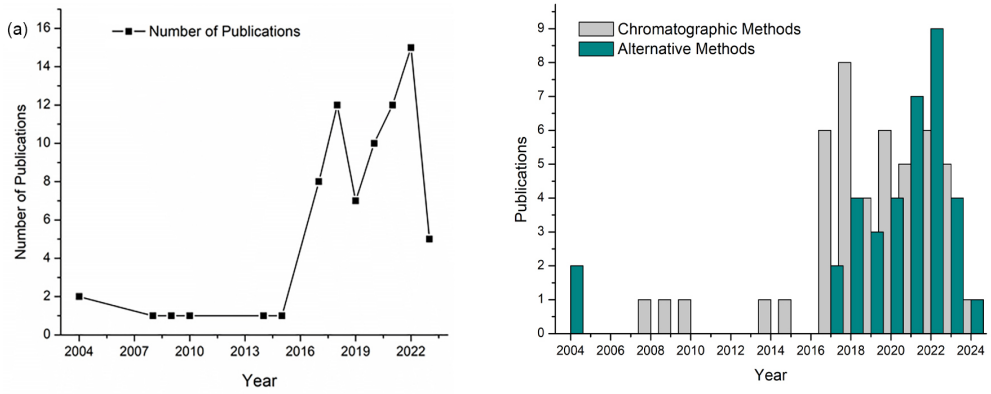

Figure 3. Number of articles published *per* year on analysis of cannabinoids in cannabis extracts and cannabis-based products (a). Distinction of the types of analysis method used (b).

sector.[96] Specifically, near-infrared spectroscopy (NIR), a fast, economical, versatile, robust, and sustainable tool, has been widely used in various fields for qualitative and quantitative analysis of key parameters such as proteins, fats, moisture, ash, starch or sugar, raw materials related to the quality of agricultural products. In contrast to chromatographic methods, NIR offers numerous benefits, including streamlined sample preparation, swift data acquisition, non-destructiveness, cost-effective instrumentation, and reduced consumable usage. These advantages position NIR as a viable alternative for the prompt analysis of hemp oil samples, enabling users to make swift and informed adjustments during product quality control assessments.[17,100]

NIR employs the absorption of electromagnetic radiation within the range of 780 to 2500 nm to create a distinctive spectral fingerprint for each sample. NIR spectra associated with multivariate chemometrics techniques are used to build models to predict composition or for classification purposes.[97] NIR has the capability to measure multiple chemical parameters simultaneously. When coupled with multivariate analysis techniques, it has been applied for both qualitative and quantitative analyses of a wide range of plant materials. This feature makes NIR one of the most appealing non-destructive analytical tools in the fields of agriculture and food industries.[23] In reflectance mode, NIR spectroscopy enables rapid scanning of cannabis to generate NIR spectra. These spectra can be employed for predicting cannabinoid content or distinguishing between cultivars with distinct chemotype profiles.[55]

Studies that used NIR spectroscopy to analyze cannabinoids in industrial hemp samples have shown the ability of the NIR technique to quantify cannabinoids quickly, sensitively, accurately, reliably and non-destructively.[15,17,96,101,102] Additionally, NIR enables the simultaneous prediction of numerous constituents using the same spectral data.[25]

Jarén *et al.*[17] identified the main bands of the NIR spectra of hemp samples. The spectrum region examined exhibited distinct absorption bands at approximately 1210, 1450, 1736, 1762, 1820, 1940, 2060, and 2090 nm, which are indicative of proteins, lipids, water, and various compounds found in hemp, originating from OH, NH, CH, and other bonds. Notably, the band at 1736 nm, corresponding to aromatic hydrocarbons of terpenes, is linked to CBD content, as cannabinoids are classified as terpene-phenolic compounds.

Sample variability has also been studied with NIR and MIR. Gloerfelt *et al.*[97] showed the effectiveness of NIR in quantifying cannabinoids in plant material from different origins from at least 22 countries. The results highlight the feasibility of NIR in conjunction with chemometrics to make accurate prediction of cannabinoids in cannabis plants as raw material. Geskovski *et al.*[42] demonstrated the potential of mid-infrared spectroscopy (MIR) as a process analytical technology (PAT) in the quantification of THC and CBD in extracts and flowers from different origins, considered critical quality parameters in plant production and cannabis extracts. NIR has also been used to distinguish legal and illegal industrial hemp. Su *et al.*[25] developed models for discriminant analysis that achieved an overall accurate classification of 94%, with independent validation, demonstrating an 84% correct classification for legal and illegal industrial hemp.

In addition to plant material, NIR has also been used to quantify cannabinoids in hemp oil,[58,99] commercial products,[41] and pharmaceuticals.[103] Chen *et al.*[100] presented an application of NIR spectroscopy combined with chemometrics to quantify concentrations of CBD and total CBD (CBD and CBDA) in hemp oil samples examined through a glass container. Grafinger *et al.*[41] used Fourier transform infrared (FTIR) to analyze CBD-rich e-liquids sold in Switzerland. All samples complied with legal requirements, with a THC content of less than 1%. On the other hand, they observed a deviation in the quantified CBD content in relation to the labeled CBD content in half of the analyzed samples. The manufacturer's information on the label and packaging varied drastically in relation to the determined CBD content, ranging from 45.9 to 117.9%. Grekopoulos[103] developed and validated a NIR spectroscopic method for the release of pharmaceutical-grade CBD-based liquid formulations. The method enabled direct measurement and rapid quantification of active pharmaceutical ingredients. In this study, NIR spectroscopy emerged as a pivotal choice for the quality control of pharmaceutical products derived from cannabis, attributed to its versatility and efficacy.

One of the advantages of the NIR technique is that measurements in the field can be carried out using portable devices.[96,101,104] Risoluti *et al.*[105,106] proposed a method based on a miniaturized spectrophotometer, the MicroNIR On-Site, for monitoring cannabinoids in hemp seed oil[106] and monitoring the residual content of cannabinoids in hemp flour.[105] Deidda *et al.*[104] evaluated 2 portable spectrophotometers (NIR-S-G1 and MicroNIR) for the quantitative analysis of THC in whole cannabis inflorescences and cannabis resins. Tran *et al.*[55] performed statistical analyses of inflorescences from 734 cannabis plants, using NIR and LC-MS data to create predictive models for 14 cannabinoids. Using different multivariate analysis techniques, they compared two NIR instruments: the Bruker MPA II (bench) and the MicroNIR (handheld). The benchtop instrument showed better results; The

MicroNIR, however, yielded accurate predictions for the primary cannabinoid precursors, such as CBDA and THCA, and was precise in the identification of other cannabinoids, including CBD, THC, CBNA, CBN, and CBCA. Similarly, Duchateau *et al*.[101] created 2 classification methods in accordance with European and Swiss laws[107,108] using benchtop NIR and a portable device on aerial parts of cannabis. These models were accurate by 91 and 95% for the set of tests obtained with bench and portable devices, respectively.

Raman spectroscopy has also been used as a non-invasive and non-destructive means of cannabinoid analysis with the aim of differentiating cannabis plants according to type and gender before flowering. Ramos-Guerrero *et al*.[98] discriminated between different varieties of marijuana. By comparing the Raman spectra of standard cannabinoids with five types of marijuana, they identified common spectral bands useful for plant characterization. The OPLS-DA (orthogonal partial least squares discriminant analysis) model developed provided a classification accuracy of 100%, making it possible to distinguish different varieties of Sativa marijuana based on their Raman spectra. On the other hand, Goff *et al.*[3] explored the feasibility of Raman spectroscopy to differentiate between hermaphrodite, male and female cannabis plants. They collected Raman spectra from these plants, discovering that the differences in biochemical profiles enabled this differentiation. Carotenoid levels are notably higher in female plants, whereas male plants exhibit lower concentrations. Hermaphrodites have lower carotenoid concentrations compared to male and female plants. Using chemometrics, the authors achieved 99.6% accurate differentiation between the chemotypes of cannabis. The portability and sensitivity of Raman spectroscopy makes it possible to use it directly on cannabis farms to monitor and control plant growth. Higgins *et al*.[16] have also been successful in differentiating young male and female hemp plants. The analysis identified, with 90 and 94% accuracy, male and female plants, respectively, before flowering. The non-invasive and fast technique was based on variations in the levels of lutein, an important carotenoid, revealed by Raman spectra. This differentiation can be made non-destructively and accurately in a matter of seconds using a portable spectrometer, paving the way to monitor and control hemp production directly on farms.

3.2.2. NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy presents a viable alternative to traditional chromatographic analysis for assessing cannabinoids. This technique is fast, reliable and does not require special processing or sample preparation. Unlike LC and GC, this method's primary benefit lies in its insensitivity to impurities found in plant material, such as chlorophyll and lipids. Aside from providing shorter analysis times when compared to traditional chromatographic methods, direct NMR analysis circumvents potential alterations in sample composition and loss of analytes during sample preparation. Moreover, NMR enable simultaneous determination of multiple analytes. Despite these potential benefits, the infrequent utilization of NMR methods in cannabinoid determination is attributed to elevated instrumental costs and the need for highly specialized personnel.[9,88,109,110]

Studies in the literature that have used NMR for cannabinoid analysis include various purposes, such as quality control of cannabis cultivars. Choi *et al*.[111] carried out metabolomic analysis of cannabis cultivars using NMR and multivariate analysis. The authors analyzed flowers and leaves of the plants taking 12 min for each procedure. The results showed that the cannabinoid content in the leaves was lower than in the flowers; the main factors that contributed to this differentiation were carbohydrates and amino acids. NMR combined with chemometrics was also used for rapid screening and authentication of cannabis samples based on the chemical profile of plant extracts,[112] discrimination of marijuana seized according to the capture period, using CBN as a marker,[113] screening for cannabinoids in CBD oils[111] and studying the quality of hemp seed oils during storage under different conditions (2-8 and 30 °C) for 30 days.[110]

The performance of cannabinoid analysis by NMR has been verified with the reference method, chromatography. Brighenti *et al*.[56] compared the efficiency of HPLC-DAD and NMR analytical techniques for studying the main cannabinoids in different samples of cannabis inflorescences. In general, agreement was reported between the two methods regarding quantitative cannabinoid data, indicating their reliability in determining active compounds in cannabis extracts. This study underscores the potential of NMR as a promising tool for accurately determining cannabinoids in both plant material and derived products.

3.2.3. Mass spectrometry

Mass spectrometry also stands out as an emerging technique for the direct analysis of complex mixtures. It facilitates the identification of compounds in extracts or medicinal preparations by differentiating analytes by the mass/charge ratio (*m/z*). Modern spectrometers operate by ionizing the molecules of interest and separating the ions based on this ratio (*m/z*), enabling the detection, counting, and characterization of atoms and molecules with different compositions and sizes.[114,115]

Borille *et al*.[116] combined the electrospray ionization (ESI) technique with Fourier transform ion cyclotron resonance

mass spectrometry (FT-ICR-MS) to analyze the chemical profile of cannabis. The results obtained forecast the growth duration of the plants with an approximate prediction error of one week (root mean square error of calibration-RMSEC 0.34 week and root mean square error of prediction-RMSEP 1.01 week). The ESI FT-ICR MS approach was efficient in detecting a wide range of cannabinoids, providing detailed and necessary information about the characteristics and chemical profile of cannabis, without the need for prior information about the samples. The ESI technique offers soft ionization, capturing ions directly from the sample solution into the gaseous environment of the mass spectrometers. ESI was also used to classify cannabis samples according to their origin and composition, employing flow injection analysis (FIA-ESI-MS/MS) and chemometric tools.[6]

Contreras *et al.*[117] introduced an innovative method, thermal desorption ion mobility spectrometry (TD-IMS), to analyze cannabinoids directly from cannabis plant extracts. This method offers fast spectral fingerprints. The TD-IMS technique was combined with chemometric tools to identify cannabis chemotypes by their chemical characteristics, using data pre-processing. With this approach, cannabis samples were successfully discriminated, grouped according to chemotypes, psychoactivity and cannabinoid profiles. This technique allows for quick analysis (less than 2 min) and can be used on-site, making it especially attractive for cannabis growers.

Dong *et al.*[118] developed a method involving direct thermal desorption analysis coupled with real-time mass spectrometry (TD-DART-MS) for the classification of diverse cannabis hemp cultivars through multivariate analysis. The method proved to be simple and fast, without the need for chromatography and solvent extraction. The PLS-DA model obtained performed well in the classification rate (> 99%). The findings of this study indicate that the TD-DART-MS method, when combined with chemometrics, was able to classify cannabis cultivars. Chambers *et al.*[93] also reported promising results using direct real-time analysis coupled to high-resolution mass spectrometry (DART-HRMS) and chemometrics to differentiate between hemp and marijuana plant materials. The model achieved an internal accuracy of 98% and 100% accuracy for external validation samples, showing its ability to differentiate accurately *C. sativa* plant materials.

3.2.4. Other analytical methods

Analytical methods such as sensors based on organic semiconductors, hyperspectral imaging, and fluorescence spectroscopy, combined with chemometric tools, have been used for the analysis of cannabinoids in cannabis plants and extracts. The application of each of these methods is summarized below.

Comeau *et al.*[119] created a sensor utilizing organic thin-film transistors (OTFT), comprising two organic semiconductor materials, CuPc and F16-CuPc, coated with an alkaline thin film based on Fast blue BB (4-amino-2,5-diethoxybenzanilide zinc diazotized double salt, or FBBB). This sensor is designed for precisely detecting and characterizing the main cannabinoid components found in the cannabis plant. The profile and chemical compositions (chemotyping) of cannabis extracts was also studied by an ultraviolet microplate reader method.[120]

Lu *et al.*[23] used hyperspectral imaging technology to measure the levels of cannabinoids in industrial hemp floral and leaf materials. Using the wavelength range of 400 to 1000 nm, they identified samples rich/poor in CBD and legal/illegal in THC with 99 and 97% accuracy, respectively, using linear discriminant analysis. Full-spectrum PLS models identified both CBD and THC satisfactorily in floral tissues. Predictions for CBG and its acidic forms, however, were unsatisfactory; these were improved with parsimonious PLS models, employing a wavelength selection procedure to minimize collinearity of variables. This study pointed out the potential of hyperspectral imaging for rapid quantification of cannabinoids in industrial hemp. On the other hand, Nicolas *et al.*[121] used hyperspectral imaging to classify cannabis chemotypes in several cultivars during the cultivation cycle of the plants. Due to the non-invasive and non-destructive nature of the technique, the authors were able to perform *in situ* measurements and classify cannabis plants into their corresponding chemotype based on the quantification of the cannabinoids present in them. This methodology was developed with the aim of ensuring quality control throughout the cultivation process. This non-destructive technique represents a promising alternative for monitoring chemotype in cannabis crops.

Birenboim *et al.*[122] introduced a new method that combined fluorescence spectroscopy with parallel factor analysis modeling (PARAFAC) to identify and quantify key cannabinoids in fresh cannabis inflorescences. The five-component PARAFAC model was successful in predicting four acidic cannabinoids and one neutral cannabinoid. The identity of the cannabinoids was confirmed by comparison with pure standards and by concentrations measured by HPLC. The study showed that there is ample information in the fluorescent spectral region to construct prediction models for cannabinoids in cannabis extracts, demonstrating the viability of this approach as a simple, economical and rapid alternative for cannabinoid analysis. Table 2 compiles the studies that have employed alternative methods for cannabinoid analysis.

Table 2. Alternative methods for analyzing cannabinoids in cannabis extracts and medicinal products

Method	Matrix	Sample preparation	Reference method	Analyte	Chemometrics tools	Reference
FTIR	CBD-rich e-liquids	N/A	UPLC-DAD	CBD, CBDA, THC, THCA, CBN	N/A	41
	cannabis flowers and extracts	N/A	HPLC-DAD	THC, CBD	PLS	42
	drug-type and fiber-type cannabis flowers	N/A	HPLC-MS/MS	THC, THCA, CBD, CBDA, CBG, CBGA, CBN	PLS	102
NIR	hemp samples (plant material)	N/A	UHPLC-MS/MS	THCA, THC, CBDA, CBD	PLS	96
	cannabis leaves and inflorescences	N/A	GC-FID	CBDV, Δ9-THCV, CBD, CBC, Δ8-THC, Δ9- THC, CBG and CBN	PCA, PLS	15
	CBD-based liquid formulations in different doses and flavors	N/A	HPLC-UV-Vis	CBD	PLS	103
	hemp seed oil and hemp flour	N/A	GC-MS	THC, THCA, CBD	PCA, PLS-DA, PLS	105, 106
	aerial parts of cannabis	N/A	GC-FID	THC	PLS-DA, SIMCA	101
	female inflorescences of plants and resins	N/A	UHPLC-UV	THC	PLS	104
	hemp oil	N/A	HPLC	CBD, CBDA	SOSVEN, PLS	100
	Kompolti variety hemp	N/A	HPLC-DAD	THC, CBD	PCA, PLS	17
	plant material	particle size reduction (ground plant material)	HPLC-MS	CBDA, THCA, CBD, THC, CBC, CBGA, CBG, CBDVA, CBDV THCV, THCVA, CBN, CBNA CBCA	PCA, PLS, PLS-DA	55
	plant material from different origins (different countries)	N/A	HPLC-UV	Δ9-THCA, Δ9-THC, CBDA, CBD, CBGA, CBG, THCVA, THCV, CBDVA, CBDV, CBN, CBC	stacked ensemble, gradient boosting machine	97
UV-Vis NIR	dry industrial hemp (plant material)	ethanol extraction	N/A	physical and chemical properties	PCA, artificial neural networks	123
^{1}H NMR NIR	whole and ground hemp seeds	N/A	GC-FID	THC, CBD, CBN, CBG, CBC	PLS	25
^{1}H NMR	plant material, four different cannabis cultivars	extraction in methanol/chloroform (9:1, v/v)	GC-FID	THC, CBD, CBN, THCA, CBDA	N/A	34
	leaves and flowers of 12 cannabis cultivars	extraction in methanol, water and chloroform (25:25:50, v/v)	N/A	THC, CBD, CBN, THCA, CBDA	PCA	111
	cannabis plant buds	extraction in $CDCl_3$	N/A	chemical profile of extracts	LDA, SVM	112
	marijuana samples	methanol extraction	N/A	D8-THC, D9-THC, THCA, CBN, CBV	PCA, PLS-DA, OPLS-DA	113

Table 2. Alternative methods for analyzing cannabinoids in cannabis extracts and medicinal products (cont.)

Method	Matrix	Sample preparation	Reference method	Analyte	Chemometrics tools	Reference
^{1}H NMR	cannabis inflorescences of different chemotypes	dilution in $CDCl_3$	HPLC-DAD	CBDA, CBGA, CBG, CBD, CBN, THC, THCA	N/A	56
	hemp seed CBD oil	dilution in $CDCl_3$	N/A	CBD, THC, CBN, CBG, THCV	N/A	109
	hemp seed oil	dilution in $CDCl_3$	N/A	quality of oils due to differences in spectra	PCA, OPLS-DA	110
Hyperspectral imaging	hemp plant (plant material, leaves and flowers)	N/A	HPLC-UV	THCA, CBDA, CBGA, THC, CBD, CBG	PLS	23
	cannabis plant	images were captured *in situ*	HPLC-DAD	THC, CBD	PLS-DA, HPLS-DA	121
Raman	cannabis (plant material) of 4 sativa genetic varieties	N/A	N/A	THCA, THC, CBDA, CBD, CBN	OPLS-DA	98
	cannabis plants	N/A	N/A	biochemical profile	PLS-DA	3
	fresh hemp plant and collected leaves	N/A	HPLC-PDA	biochemical profile	PLS-DA	16
TD-IMS	cannabis plant of different chemotypes (female inflorescence)	ultrasound-assisted *n*-hexane extraction	N/A	CBD, CBDV, THCV, CBC, D8-THC, D9-THC, CBG, CBN, THCA, CBDA, CBGA	PCA-LDA	117
TD-DART-MS	flowers of hemp chemotypes	N/A	HPLC-MS	THC, THCA, THCV, CBD, CBDA, CBDV	PCA, PLS-DA	118
Ultraviolet microplate reader	cannabis extracts from various species	extraction and dilution in $CDCl_3$	N/A	chemical profile of extracts	PLS-DA, SVM	120
Thin-film organic transistors	cannabis plant	extraction and dilution in acetonitrile	HPLC-DAD	CBD, THC	N/A	119
ESI(±)-FT-ICR MS	plant material	ultrasound-assisted acetonitrile extraction	N/A	chemical profile of extracts	GA-PLS	116
FIA-ESI-MS	cannabis samples (aerial parts and pressed plant material)	extraction in methanol	HPLC-DAD	D8-THC, D9-THC, THCA, CBDA, CBD, CBN, CBGA, CBG	HCA, PCA, PLS-DA	124
Fluorescence spectroscopy	cannabis inflorescences of 16 different chemistries	dilution in ethanol	HPLC-DAD	THCA, CBDA, CBGA, THC, CBD, CBG, CBCA	PARAFAC	122
DART-HRMS	hemp and marijuana plant material	N/A	N/A	biochemical profile	random forest, PCA	93

The sample preparation listed in the table corresponds to the main method and not the reference method. CBD: cannabidiol; CBDA: cannabidiolic acid; CBDV: cannabidivarin; CBDVA: cannabidivarinic acid; CBG: cannabigerol; CBGA: cannabigerolic acid; CBNA: cannabinolic acid; CBC: cannabichromene; CBCA: cannabichromenic acid; DA: discriminant analysis; DAD: diode array detector; DART-HRMS: direct analysis in real-time- high resolution mass spectrometry; ESI: electrospray ionization; FID: flame ionization detector; FT-ICR-MS: Fourier transform ion cyclotron resonance mass spectrometry; FTIR: Fourier transform infrared; GA: genetic algorithm; GC: gas chromatography; HCA: hierarchical cluster analysis; HPLC: high-performance liquid chromatography; IR: infrared; LDA: linear discriminant analysis; MS: mass spectrometry; NIR: near-infrared; NMR: nuclear magnetic resonance; OPLS: orthogonal partial least squares; PARAFAC: parallel factor analysis; PCA: principal component analysis; PLS: partial least squares; SIMCA: soft independent modeling of class analogy; SOSVEN: self-optimizing support vector elastic net; SVM: support vector machine; TD-DART-MS: thermal desorption direct analysis in real time mass spectrometry; TD-IMS: thermal desorption ion mobility spectrometry; THC: delta-9-tetrahydrocannabinol; THCA-A: delta-9-tetrahydrocannabinolic acid A; THCVA: tetrahydrocannabivarinic acid; THCV: tetrahydrocannabivarin; UPLC: ultra-performance liquid chromatography; N/A: not applicable.

4. Chemometric Tools for Cannabinoid Analysis

Throughout section 3, several methods were reported on the use of multivariate statistical analysis for the qualitative and quantitative analysis of cannabinoids. This was particularly evident in sub-section 3.2. “Alternative Methods”, because of the technological advancement of

instrumental methods. This is due to the ability of modern analytical techniques to provide high-dimensional data, where many variables for each sample can be measured at a high speed and by frequency. These alternative methods have increasingly required the use of tools that assist in the processing, evaluation and interpretation of data.[125,126]

It is in this context that chemometrics is important. Chemometrics is often defined as an area of chemistry that employs mathematical and statistical methods for the treatment of multivariate data obtained from chemical systems to extract the maximum amount of information from these systems, enhancing the range of understanding about what the data means. Some of the notable multivariate methods that are planning, supervised and unsupervised pattern recognition, and multivariate calibration, which is one of the most used techniques.[127]

Unsupervised pattern recognition methods are used for exploratory data analysis, to evaluate correlations and similarities/differences between samples. Thus, no pre-existing knowledge about the identity or groupings of these samples is considered. The main unsupervised techniques are: hierarchical cluster analysis (HCA) and principal component analysis (PCA). PCA is one of the most popular techniques employed, generally in the first contact with the data. It recognizes relationships between variables and between samples, identifies patterns and detects and interprets anomalous samples present in the data.[128]

Applications of unsupervised methods for cannabinoid analysis include discrimination, according to cannabinoid content of hemp seed powder products,[87] plant materials,[43,55] hemp seed oil,[106] hemp flours,[105] cannabis cultivars,[111] and even samples of seized marijuana. Leite *et al.*[113] used PCA to differentiate among the samples according to their collection period using CBN as the marker: the concentration of CBN in the marijuana samples exhibited a direct correlation with the seizure time. This study confirmed the use of CBN as a biological marker for estimating the age of the plant or medicinal product. Chambers *et al.*[93] used PCA to differentiate plant materials from hemp and marijuana. The application of PCA to hemp and marijuana data revealed a clear grouping, enabling their distinction.

In supervised pattern recognition techniques, additional information about the identity and measurement of the samples is required to categorize them. A training set with samples from known classes is then used to develop classification models, which are later used to identify unknown samples.[129,130] Some of the main supervised pattern recognition techniques used in analytical chemistry are: independent and flexible modeling by class analogy, such as SIMCA (soft independent modeling of class analogy) and its variations, such as DD-SIMCA;[131] linear discriminant analysis LDA, and PLS-DA. The first is the most popular one-class classification method, which focuses on similarities between samples within the same category, as opposed to variations between categories. LDA and PLS-DA are known as discriminant classification methods, utilized to establish delineations between the assessed classes.[127]

Among the applications of supervised methods for cannabinoid analysis, the most commonly used is PLS-DA with several purposes, including identification of hemp retailers based on the chemical content of cannabinoids in hemp samples, 92% of samples were correctly classified;[85] classification of medical cannabis samples based on their cannabinoid and terpene profiles with a correct classification rate of 96 and 100% for the cross-validation and prediction datasets respectively;[65] classification of various hemp cultivars with classification rate (> 99%);[118] and classification of illegally seized samples.[6]

Cannabis sample classification models using the SIMCA algorithm have also been reported. Duchateau *et al.*[101] formulated PLS-DA and SIMCA models for the classification of cannabis samples based on European and Swiss legislative standards,[107,108] employing both benchtop and portable infrared devices. The SIMCA models demonstrated accuracy of 91 and 93% for the benchtop and portable devices, respectively. The PLS-DA models achieved accuracy of 91% and 95% for the benchtop and portable devices, respectively.

The main objective of multivariate calibration techniques is to establish a mathematical relationship between the instrumental data acquired from a sample, such as a spectra data (**X** matrix) as a reference value represented by a vector **y**. The application of these techniques involves two phases. In the first, called calibration, the data from the **X** matrix, as well as the reference values contained in the **y** vector, are used to build the calibration model. In the second stage, called prediction or validation, this model is used to quantify the property of interest in unknown samples.[129] Among the most popular multivariate calibration methods, the following stand out: multiple linear regression (MLR), principal component regression (PCR), partial least squares regression (PLS) and support vector machine regression (SVMR).[132-134] PLS is the most used in the quantification of cannabinoids.

PLS has been used to quantify cannabinoids using, for the most part, vibrational spectroscopic methods, relating the spectra to the concentration of cannabinoids determined by chromatography. In this way, PLS models have been developed to quantify cannabinoids in hemp samples of the kompolti variety,[13] in liquid formulations

based on pharmaceutical grade CBD,[103] in hemp seed oil,[106] in cannabis extracts,[42] in hemp oils,[100] and in plant material.[23,55,96]

Geskovski *et al.*[42] demonstrated the capacity of mid-infrared spectroscopy (MIR) as a process analytical technology (PAT) for quantifying the primary phytocannabinoids (THC and CBD). The concentrations of CBD and THC in the samples ranged from 0.38 to 39.2% and from 18.93 to 86.99%, respectively. Multivariate models were built to predict THC and CBD content in samples of extracts and flowers originating from various sources. The PLS models accurately predicted THC and CBD concentrations in both cannabis extract samples and decarboxylated cannabis flowers. For cannabis extract samples, the RY (the correlation coefficient of the **Y** matrix) values were 0.95 for THC and 0.99 for CBD, with corresponding RMSEC values of 4.67 and 1.21%, and RMSEP values of 3.79 and 1.44%, respectively. In the models predicting THC and CBD from decarboxylated cannabis flowers, the RY values were 0.99 for both compounds, with RMSEC values of 0.43% for THC and 0.21% for CBD, and RMSEP values of 2.32% for THC and 1.33% for CBD, indicating robust predictive performance. Similarly, Lu *et al.*[23] built quantitative PLS-based models that achieved prediction accuracies of RPD (ratio of prediction to deviation) 2.5 and R^2 0.84 for CBD and THC in hemp flowers.

In the studies that analyzed previously described cannabinoids qualitatively and quantitatively, 63% used multivariate analysis tools, especially in studies employing alternative analytical methods. Of the publications where alternative analytical techniques were employed, 85% used chemometrics. And in the case of classic methods, 43% used chemometric tools (Figure 4a). In the group of studies that used chemometrics, unsupervised pattern recognition was the most common multivariate method, followed by supervised pattern recognition, calibration and lastly, Design of Experiment (Figure 4b). The use of a variety of algorithms has been reported, including PCA, HCA, PLS-DA. PCA-LDA, SIMCA, MLR, GA-PLS, PLS, PARAFAC, SVM, and neural networks. The most frequently algorithms used, however, were PCA, followed by PLS-DA, PLS and HCA (Figure 4c).

5. Conclusions

The growing use of cannabis for therapeutic purposes has encouraged the production of medicinal formulations that range from pharmaceutical products with certified quality to artisanal products without any type of standardization and quality control. This has led to the development of analytical methods to enable quality control, whether in pharmaceutical, artisanal or derived products, and with crops in the growth phase.

Since 2004, the number of publications on cannabinoid analysis has grown considerably. The standard analytical method for quantitative and qualitative analysis of cannabinoids has been and continues to be chromatography, specifically liquid chromatography with DAD detector as well as coupled with MS. This is mainly because liquid chromatography allows direct analysis to determine the original composition of cannabinoids in the plant. Unlike GC, there is no breakdown of cannabinoids during analysis and the process does not require derivatization. LC-MS analysis offers several important advantages: it allows accurate identification of compounds, improves sensitivity for detection and quantitation at low concentrations, and provides high selectivity. These characteristics make chromatography coupled with MS a powerful and efficient tool in the analysis of cannabis products. There are limitations, however, such as the high cost of the instruments and the need for users to master analytical methodologies and the functioning of the equipment. Therefore, these systems are not widely available or

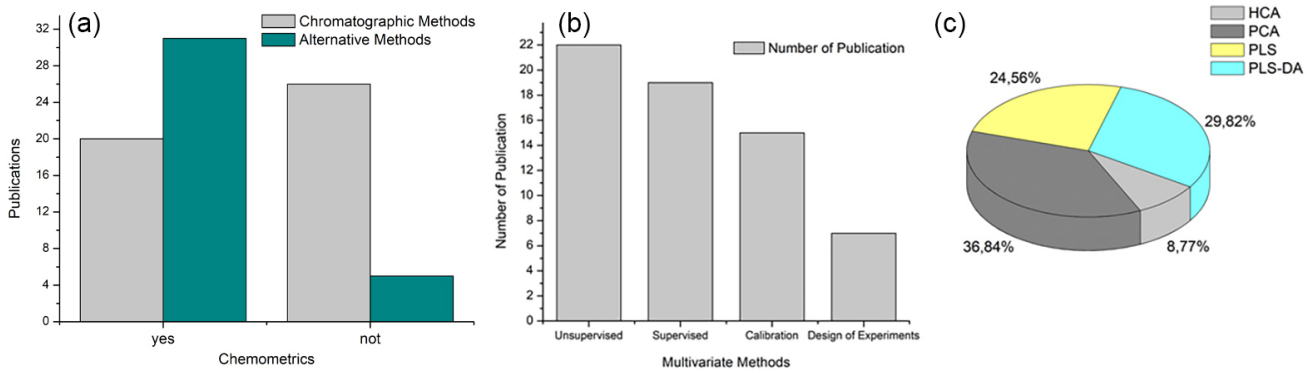

Figure 4. Prevalence of analytical techniques for cannabinoid analysis using chemometrics (a). Comparison of the most used multivariate methods (b). The most frequently used algorithms (c).

routinely used, being mainly intended for scientific research and technological development.

Due to the limitations of chromatographic techniques as a whole, there is an increasing search for alternative methods that are faster and simpler to use. With the technological advancement of instrumental methods and the widespread use of chemometrics techniques, a variety of methodologies have been developed for cannabinoid analysis. These methodologies include the use of vibrational spectroscopy such as NIR and Raman; NMR; and other emerging techniques such as electrospray ionization coupled to mass spectrometry, thermal desorption ion mobility spectrometry, sensors based on organic semiconductors, direct analysis in real time coupled to mass spectrometry, hyperspectral imaging and fluorescence spectroscopy. Most of the proposed methods employ chemometric tools that are essential for the processing, evaluation, and interpretation of the data generated. Chemometric tools are also used in the construction of models that enable classifying and/or predicting the concentration of cannabinoids.

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