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Current Pharmaceutical Design, 2023, *29*, 394-406

MINI-REVIEW ARTICLE

Entourage Effect and Analytical Chemistry: Chromatography as a Tool in the Analysis of the Secondary Metabolism of *Cannabis sativa* L.

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ARTICLE HISTORY

Received: July 05, 2022
Accepted: September 16, 2022

DOI:
10.2174/1381612829666221103093542

Abstract: *Cannabis sativa* L. has been used as medicine for thousands of years. Since the early identification of tetrahydrocannabinol (THC) in 1960, pharmacological activities were attributed to a group of unique structures named cannabinoids. For decades, research and development were applied to determine different cannabinoids and their medicinal properties. Nowadays there is evidence that the therapeutic benefits of the plant are based on the synergy of cannabinoids and other secondary metabolites such as terpenes and flavonoids. Differences between the medical performance of isolated compounds like cannabidiol (CBD) or THC and full-spectrum plant extracts are notable. Indeed, the superiority of the last one is provoked by the synergy between various different compounds. This improved medicinal effect is called the entourage effect. Chromatography has become the method of choice for the determination of cannabinoids, terpenes, and flavonoids, so it represents an excellent tool for a proper characterization of the plant and plant derived products. The objective of characterization relies not only in analyzing the fingerprint of cannabis, but also to identify different chemotypes for medical purposes. To understand the contributions of each natural product to this "entourage effect", this review presents an in-depth analysis of the utilization of High-performance liquid chromatography (HPLC), Gas chromatography (GC) and other methods for the analysis of phytocomponents of *Cannabis sativa* L. In this sense, a representative number of examples and advances made in the field together with limitations and future needs are provided. It can be concluded that standardized protocols and quality control policies and procedures are necessary for the comprehensive analysis of cannabis extracts and derivatives.

Keywords: Entourage effect, synergy, inflorescences, cannabis, medicinal plant, trichomes, cannabinoids, chemotypes, chromatography.

1. INTRODUCTION

Cannabis sativa L. is a medicinal plant native to Central Asia and belongs to the Cannabaceae family. It is historically well-recognized for its several uses such as textile resource, food, medicinal purposes, or recreational use [1-3].

Regarding its medicinal properties, the female inflorescences are considered the most valuable part of the plant [4]. Glandular trichomes present in *C. sativa* female inflorescences accumulate a greater quantity of exclusive compounds called phytocannabinoids or cannabinoids [5]. These molecules interact in the human body with special receptors belonging to an endocannabinoid system, which function is related to the regulation of many biological processes and homeostasis [6, 7].

The medicinal use of *C. sativa* is recognized in the following pathologies: multiple sclerosis, refractory epilepsy, nausea, vomiting associated with chemotherapy, anorexia in people with HIV, and chronic pain [8-10]. Nevertheless, interest in *C. sativa* medicinal properties keep growing since the identification of tetrahydrocannabinol (THC), the compound responsible for the psychoactive effect [11]. Other cannabinoids were identified, such as cannabidiol (CBD) which is becoming popular for the recent evidence of its use in refractory epilepsy [12, 13]. Potential pharmacological activities and therapeutic implications are being explored in many research scopes. Today there are several cannabis-based drugs, one of them is Sativex, which is an oromucosal delivery system based on an extract with a 1:1 ratio of THC and CBD. Alternatively, Epidiolex, was approved by the FDA in 2018 and is made up of 100 mg/mL of purified CBD for oral administration [14].

At present time research is also being directed at groups of other metabolites present in cannabis [15, 16]. This is due to the hypothesis that the medicinal activity of cannabis is not only influenced by cannabinoids, but also other compounds present in the plant, such as terpenes or flavonoids. The synergy between compounds that improve medical properties is called the entourage effect [17, 18]. Some research on the entourage effect will be cited later, but this concept provides evidence aimed at the characterization of full-spectrum extracts and not the synthesis of isolated or purified compounds. The full characterization of plants derived products would be an emerging requirement to understand the influence of other compounds on therapeutic responses, considering the entourage effect [19, 20].

Several analytical methods for the analysis of the aforementioned molecules are available, but chromatography techniques are the most suitable in terms of qualitative and quantitative determinations [21-24]. This review highlights the relevance of chromatographic techniques as a tool for the characterization of the components responsible for the entourage effect present in *Cannabis sativa* plant extracts.

2. RELEVANT BIOLOGICAL AND MEDICAL ASPECTS

2.1. Cannabinoids

Cannabinoids or phytocannabinoids are the most studied group of compounds in *C. sativa* [15, 16, 25]. These molecules are present only in this species, with a few exceptions. The structure is based on a terpene-phenolic molecule with 21 or 22 carbons [26].

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Current Pharmaceutical Design

Cannabinoids are synthesized in their acid forms in glandular trichomes occurring in female flowers. These molecules share similar biosynthesis pathways with both terpenes and flavonoids [15]. The precursor of all cannabinoids is cannabigerolic acid, and the specific cannabinoid synthase is required for the transformation to the most abundant cannabinoids in *C. sativa*, tetrahydrocannabinol acid (THCA), and cannabidiol acid (CBDA) [13, 15, 16, 26]. Decarboxylation of cannabinoid acids into their corresponding neutral forms (which have a higher pharmacological activity) occurs upon heating. Neutral forms (THC, CBD, *etc*.) are the most cited and reviewed compounds of cannabis. More than 150 different cannabinoids have been reported and they can be classified into 10 subclasses according to their chemical structure [13, 26]. Cannabinoids with only three carbons tail were described, THCv and CBDv. More recently, in 2019, tetrahydrocannabiphorol (THCP) was described. This molecule has more pharmacological activity than its analog THC [27].

Most of the biological properties related to cannabinoids rely on their interactions with the endocannabinoid system mentioned above. The endocannabinoid system includes two G protein-coupled cannabinoid receptors, CB1 and CB2, as well as two endogenous ligands, anandamide, and 2-arachidonylglycerol. Endocannabinoids are synthesized on demand according to the metabolism condition since this system activity is related to the modulation of several physiological processes. The mechanism is the release of excitatory or inhibitory neurotransmitters [6, 7]. Table **1** summarizes the pharmacological activities and medical properties of cannabinoids.

2.2. Flavonoids

Phenolic compounds constitute a group of highly heterogeneous substances widely distributed in the plant kingdom [28, 29]. They are characterized by the presence of a phenolic functional group attached to an aromatic ring in their structure. More than 10, 000 different structures have been described [28, 29]. Its concentration is variable according to the synthesis mediated by environmental conditions, like humidity or UV exposition [30]. Flavonoids are considered the most numerous polyphenols [28, 31].

More than 150 phenolic compounds and flavonoids have been identified in *C. sativa* [15, 16, 26]. Thirty-four flavonoids were isolated, which could be categorized into seven basic chemical skeletons that can be methylated, glycosylated (C- or O- glycosides), prenylated, or geranylated [26]. The seven chemical structures of the flavonoid aglycones are orientin, vitexin, isovitexin, apigenin, luteolin, kaempferol, and quercetin [15, 26, 32, 33]. Three prenylated/geranylated flavones, cannflavin A, B, and C, were isolated in *C. sativa*, and are described for the first time in this species [34].

As it was mentioned before, flavonoids are considered the most numerous polyphenols in the Plantae Kingdom. They exhibit different applications, among which we can mention anthelmintic, antihepatotoxic, anti-inflammatory, antidiarrheal, antiulcer, antiviral, antiallergic and vasodilator activities [31]. They also inhibit cytotoxic effects and promote tumor growth. They also have a particularly suitable chemical structure to exert an antioxidant action by acting as free radical scavengers, neutralizing dangerous reactive oxygen species, and chelating metal ions [31]. Cannflavins and cannabis flavonoid derivatives have shown medicinal activities for Alzheimer's treatment or for models of pancreatic cancer. Table **1** summarizes the pharmacological activities and medical properties of cannabis flavonoids [33, 35].

2.3. Terpenes

Terpenoids are the most diverse and extensive group of substances from secondary metabolism, with more than 120 molecules identified in cannabis [15, 16, 26, 36, 37]. Indeed, there were described 61 monoterpenes, 52 sesquiterpenoids, 2 triterpenes, one diterpene, and 4 terpenoid derivatives [26]. They are responsible for the aroma and flavor of the different strains [15, 16, 36]. These molecules are classified according to their structure, which can be large or small depending on the number of carbons they have (they are usually different combinations and condensations of a 5-carbon structural package -isoprene-) [26].

The production and distribution of these substances in the plant vary according to various parameters, such as environmental conditions or the maturity of the plant [30]. It has been observed that terpene yield and floral aroma vary with the degree of maturity of female flowers [38]. The site of greatest production are the glandular trichomes.

Monoterpenes dominate generally the volatile terpene profile, including mainly D -limonene, β-myrcene, α- and β-pinene, terpinolene, and linalool [15, 16, 26, 33]. The most abundant sesquiterpene β-caryophyllene has recently regained interest due to its high interaction with the CB2 receptor in the endocannabinoid system.

These molecules play an important role in cannabis taxonomy and have been used as strain markers in this species [39]. Table **1** summarizes the pharmacological activities and medical properties of cannabis terpenoids.

3. ENTOURAGE EFFECT AND CHARACTERIZATION

The entourage effect is defined as the therapeutic superiority of a plant extract compared to isolated compounds, such as medicines based on CBD or THC [17, 18, 36, 40]. This concept is based on the synergy between different secondary metabolites, following the idea that "the sum of the synergia 'coordinated task', and this from gr. (συνεργία) synergy 'cooperation', following the idea of "the whole is more than the sum of the parts" [40]. The entourage effect was first described by Raphael Mechoulam and Shimon Ben-Shabatin, who observed an enhanced affinity of 2AG for the CB2 endocannabinoid receptor-related with the presence of other compounds, palmitoyl glycerol, and linoleyl glycerol [41]. Subsequently, this same effect was observed with the presence of phytocannabinoids [42]. Two classifications related to the type of interaction between compounds emerge from this phenomenon. If the interaction is between metabolites of the same group, it is called intra-entourage, as is the case with the mitigation of secondary effects of THC in the presence of CBD [18, 36]. On the other hand, the interaction between metabolites from different groups is known as inter-entourage, postulating that linalool, due to its anticonvulsant properties, could be combined with the activity of CBD [18, 36].

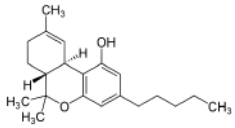
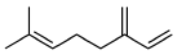
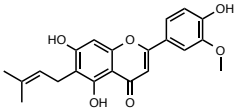
3.1. Basis and Evidence of Synergy

Accumulating evidence suggests that the endocannabinoid system is a promising target for the treatment of a variety of health conditions. Two visions of cannabinoid drug development and therapy have emerged. One approach is focused on developing formulations that are directly derived from the cannabis plant. The other perspective proposes a single-molecule approach with individual phytocannabinoids purified or synthesized for pharmaceutical development [43].

Chronic pain is one of the numerous registries for treatment with cannabis, due to its great analgesic capacity. In an investigation of patients with resistance to opioids, significant improvement in analgesia was demonstrated using an extract with CBD and THC *versus* a THC-predominant variety [44].

The study included three types of treatment, a THC-enriched extract, an endocannabinoid system-modulating analgesic, a whole plant extract, and a placebo. Plant extract treatment was significantly better than placebo and moderately better than THC-enriched extract treatment. Linear correlation has been shown between the dose of a standardized plant extract and the decrease in pain. While in the other treatment, the oral administration of a

Table 1. Pharmacological activities and medical properties of cannabinoids, terpenes, and flavonoids.

Secondary Metabolites					
Structures		**Cannabinoids**	**Terpenes**	**Flavonoids**	**Refs.**
					
Cannabis Sativa L. Studies	Most Cite Pharmacological Activities	❑ Anti-inflammatory ❑ Analgesic ❑ Neuro-antioxidative ❑ Antispasmodic ❑ Anti-anxiety ❑ Anti-emetic ❑ Anti-psychotic ❑ Immunomodulatory ❑ Neuro-protector ❑ Anti-fungal ❑ Anti-bacterial ❑ Sedative ❑ Cytotoxic ❑ Anticonvulsant	❑ Anti-inflammatory ❑ Cytotoxic	❑ Antitumoral ❑ Neuroprotective	Cannabinoids [107] Terpenes [48, 108] Flavonoids [35,109]
Other Medicinal Plants Studies		-	❑ Antitumoral ❑ Anxiolytic ❑ Immunomodulatory ❑ Anti-inflammatory ❑ Analgesic ❑ Anticonvulsant ❑ Anti-bacterial ❑ Anti-fungal	❑ Anthelmintic ❑ Antihepatotoxic ❑ Anti-inflammatory ❑ Antidiarrheal ❑ Anti-ulcerous ❑ Antiviral ❑ Antiallergic ❑ Vasodilators ❑ Antitumor ❑ Cytotoxic ❑ Antioxidant	Terpenes [36, 37, 110] Flavonoids [31, 111]
Found in Cannabis		CBDV, CBD, CBG, THCV, CBN, THC, CBC, CBL, (and respective acid forms)	D -limonene, β-myrcene, α- and β-pinene, terpinolene, and linalool. Sesquiterpene β-caryophyllene	Apigenin, luteolin, kaempferol, quercetin, cannflavin A, B, and C	[13, 15, 16, 26]

purified compound raised a plateau in the correlation of administrated dose and therapeutical effect, representing a limit for clinical studies [45].

In the last years, scientists from the Complutense University of Madrid demonstrated the cytotoxic effects of cannabinoids [46]. Research has been progressing and pharmacological activity has been demonstrated in different types of cancer. This work compared the cytotoxic effects of THC against a whole plant extract (containing low amounts of CBG); the latter formulation was the one that yielded the best results [47]. Another study showed that there are correlations between different groups of terpenes and cannabinoids, showing that certain associations between the two produce a greater cytotoxic response against tumor lines [48].

The benefits that a complete cannabis extract could have in terms of mental health have been postulated in extensive reviews [36], due to the anxiolytic and antidepressant activities of compounds such as terpenes added to the anxiolytic activity of CBD [36]. Terpenes could also be effective in the treatment of chronic pain due to their remarkable anti-inflammatory and analgesia properties [36].

Research has been carried out on the cannabimimetic activity of terpenes, most of the time affinity with the CB1 receptor [49, 50]. However, later works suggested that despite representing viable ligands for the modulation of the affinities of cannabinoids for their receptors, there was no significant modulation, so the mechanism of action of the potential therapeutic effect must be explained in another way [51, 52]. It is relevant to mention that terpenes, by themselves, already exert pharmacological activities that have been cited on numerous other occasions [18, 37, 53].

3.2. Cannabis Chemotypes and Secondary Metabolism Characterization

Historically, *C. sativa* was classified into three varieties according to their morphological characteristics or growth conditions, also according to the concentrations of THC and CBD in each one [3, 17, 54]. Over time these classifications were blurred by the incredible power of hybridization of the plant, however, exhaustive genetic studies have been carried out that generally determined the existence of three chemotypes [54]. Based on their ratio of total THC to total CBD, cannabis cultivars are commonly

classified into high-THC, high-CBD, and hybrid classes [54]. However, the cannabis plant has other cannabinoids and other metabolites with biological activities [15, 16, 26, 55], the terpenoids and flavonoids have not been included in these classification approaches. To better understand the medicinal potential of cannabis, a better classification system is needed based on a range of potentially active components [19, 20, 56] (Fig. **1**). While cultivars in the same class have similar compositions of major cannabinoids, their levels of other cannabinoids and their compositions of terpenes and other compounds may differ substantially [56, 57]. A very useful and essential tool in this type of inquiry and elucidation is the use of chemometric methods, such as multivariate analysis or principal component analysis, which considers more than one variable and groups according to the degree of link between each one [19, 20, 39, 56, 57]. In addition, a criticism of the popular definition of cannabis is the use of the term "strains", this denomination is appropriate for bacteria and viruses. “Chemovar” is the most appropriate denomination since it considers chemical variability, and today the emphasis should be directed to the biochemical and pharmacological distinctions between these chemovars [17, 18, 20].

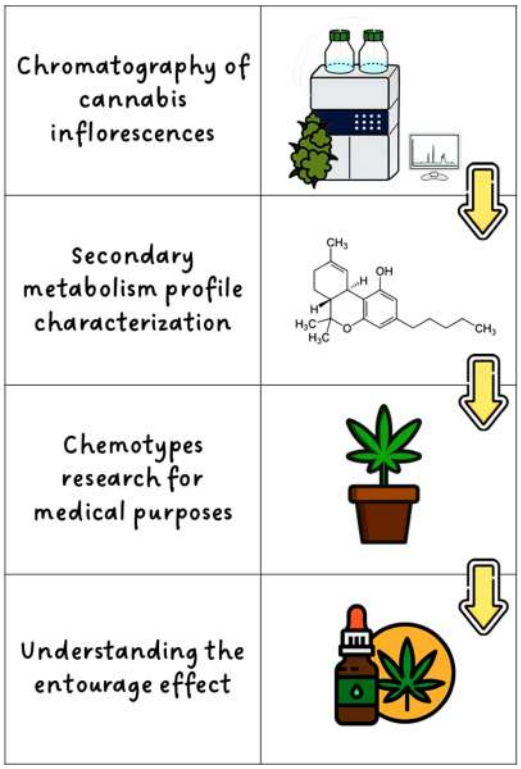


Fig. (1). Flowchart of cannabis chemical characterization relevance for medicinal purposes (understanding entourage effect and defining chemovars). (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

4. ANALYSIS OF MEDICINAL PUZZLE: CHROMATOGRAPHIC ANALYSIS

4.1. High-performance Liquid Chromatography (HPLC)

HPLC has become in recent years the technique of choice for the determination and quantification of cannabinoids in inflorescences and plant material. This technique allows the analysis of the acid forms of cannabinoids (those synthesized by the plant and precursors of the neutral forms) since it operates at room temperature, avoiding the decarboxylation of these molecules. These conditions allow HPLC to characterize the authentic content of cannabinoids present in the sample [21, 23, 24, 58, 59]. Some methods can analyze up to 16 cannabinoids.

The most used columns for this type of analysis are RP C18, due to the lipophilic properties of cannabinoids [60-70]. It is important to mention that one of the great difficulties when analyzing cannabinoids is the co-elution and overlapping of peaks [67]. It is difficult to obtain a correct separation and baseline resolution for some of the cannabinoids with lower retention time, like CBDA and CBG, or CBD and THCV [67]. However, other columns with improved characteristics have been used to increase the efficiency of the system by changing column dimensions like diameter particle or column length (HILIC or UHPLC columns, also core-shell) [69, 71, 72]. For example, our research group validated a method for the determination of 11 cannabinoids using an RP C18 column 250 × 4.6 mm, 5um (unpublished results).

The separation of cannabinoids uses methanol: water or acetonitrile: water as the mobile phase, with proportions of formic acid, phosphoric acid, or TFA to improve the resolution of the acid forms, since the pH of the mobile phase affects its retention time [60-62, 64, 65, 67, 69, 71-74]. In general, a gradient program is used to achieve good peak separation and resolution, however, there is literature that reports isocratic systems [64, 66, 72, 75-77].

The detectors mostly used in cannabinoid determination are the ultraviolet detector (UV), the fluorescence detector (FLD), or mass spectrometry (MS) [21, 23, 24, 58, 59]. UV is one of the most cited for the analysis of plant material where cannabinoid concentrations are high [64, 69, 73, 76, 78], unlike the traces found when biological material is analyzed. This detector is also cheap and simple. Detection is based on chromophore absorption of the phenolic ring, which is a common structural element in all cannabinoids [23]. The presence of the alkyl tails does not influence the UV absorbance, so there would be no difference between the detection of THCA and THCVA. UV detector has certain limitations to discriminate some cannabinoids since the sensitivity and specificity are limited, especially when molecules are not separated during the chromatographic run.

These difficulties can be overcome by using a diode array detector since acidic cannabinoids have a different absorption spectrum than neutral cannabinoids [60-62, 66, 68, 69, 75]. The wavelength used for the detection of neutral cannabinoids is 220 nm, and acid cannabinoids maximum absorptions are at 270 and 310 nm [79]. Detection of both acid and neutral cannabinoids could be determined at 228 nm. Fluorescence detector (FLC) is rarely used because the fluorescence of cannabinoids is affected by the pH of the mobile phase, and there are some cannabinoids such as CBN, that has no fluorescence at all [21, 79].

Despite the virtues that the UV-DAD detector provides for daily routine or characterization analysis, the mass spectrometry (MS) detector is the most specific and sensitive for the analysis of cannabinoids. HPLC-MS detects these molecules based on the charge/mass relation of each. Various modifications and interfaces have been cited in the cannabis analytical literature, but most of these approaches are aimed at detecting cannabinoids in biological samples, not necessarily in plant material [21, 23, 24, 58, 59, 65, 71, 74, 80].

Analytical chemistry approaches to cannabis flavonoids characterization are limited. For the identification of some new flavonoids in cannabis, NMR has been used [62], and for the determination and quantification of flavonoids in plant material, planar chromatography, column chromatography or HPLC, has generally been used [26]. In a recent work, Brighenti *et al*., extracted characteristically cannabis flavonoids (cannflavin A and cannflavin B) with several protocols trying to improve the yield due to the low concentration of these metabolites [70]. Acetone was finally the chosen solvent for the extraction. Analysis was performed using a non-polar column (RP-HPLC) (Table **2**).

Table 2. Representative references and their chromatographic characteristic for HPLC analysis of cannabinoids.

-	Cannabinoids (from Inflorescences)					
	High Performance Liquid Chromatography					
Solvent of Extraction	**Method and Detector**	**Stationary Phase**	**Mobile Phase**		**Cannabinoids Detected**	**Refs.**
$MeOH/CHCl_3$ (9:1)	HPLC-DAD (200-400 nm)	Waters XTerra® MS C18 (250 × 2.1 mm, 5.0 µm)	A	50 mM amonium formate buffer	CBDA, CBGA, CBG, CBD, CBD, CBN, 8THC, 9THC, THCA	[60]
			B	MeOH		
			Gradient			
EtOH	HPLC-DAD (200-400 nm)	Poroshell 120 EC-C18 (150 × 2.1 mm, 2.7 µm)	A	H_2O + 0.1% formic acid	CBDVA, CBDV, CBDA, CBGA, CBG, CBD, THCV, THCVA, CBN, 9THC	[61]
			B	ACN + 0.1% formic acid		
			Gradient			
EtOAc, EtOH, heptane, MeOH	HPLC-DAD-UV (275 nm)	Aces 5® Phenyl (250 × 4.6 mm, 5 µm)	A	H_2O	CBD, CBN, 9THC, THCA	[62]
			B	ACN		
			Gradient			
$MeOH/CHCl_3$ (9/1)	HPLC- DAD (272 nm)	Waters X-Bridge C18 (150 × 4.6 mm, 3.5 µm)	A	50 mM Ammonium formate buffer + 10% ACN	CBDA, CBGA CBG, CBD, THCV, CBN, 9THC, CBC, THCA	[63]
			B	90% ACN		
			Gradient			
02	HPLC -UV (275 nm)	Luna C18 (150 × 3 mm, 3.0 µm)	ACN 80%, 0.1% formic acid 10 mM ammonium formate		CBDV, CBDA, CBGA CBG, CBD, THCV, CBN, 9THC, 8THC, CBC, THC.	[64]
			Isocratic			
EtOH	HPLC-DAD (220 nm, 240 nm, 270 nm and 307 nm)	MacMod ACE 5 C18-AR (250 × 4.6 mm, 5.0 µm)	Acetonitrile/0.5% acetic acid		CBDV, CBDA, CBGA, CBG, CBD, THCV, CBN, 8THC, 9THC, CBG, THCA	[66]
			Isocratic			
$MeOH/CHCl_3$ (9/1)	HPLC-UV (220 nm)	C18 column, Nex-Leaf CBX Potency (150 × 4.6 mm, 2.7 µm)	A	H_2O + 0.1% phosphoric acid	CBDA, CBGA, CBG, CBD, THCV, CBN, D9-THC, D8-THC, CBC, THCA	[67]
			B	ACN + 0.1% phosphoric acid		
			Gradient			
$MeOH/CHCl_3$ (9/1)	HPLC- DAD (210-400 nm,)	Luna C18(2) column (150 × 4.6 mm, 3 µm)	A	H_2O + 0.1% formic acid	CBDA, CBGA, CBG, CBD, THCV, CBN, 8THC, 9THC, CBC, CBL, THCA	[68]
			B	ACN + 0.1% formic acid		
			Gradient			
ACN/H_2O (1:1)	HPLC-UV UPLC- DAD (220 nm)	Kinetex XB-C18 column (150 × 4.6 mm, 2.6 µm) Kinetex XB-C18 column (150 × 2.1 mm, 1.7 µm)	A	H_2O + 0.1% formic acid	CBDV, CBDA, CBGA, CBG, CBD, CBN, THC, THCA	[69]
			B	ACN + 0.1% formic acid		
			Gradient			
(CH3)2CHOH	HPLC-DAD (190-600 nm)	Ascentis Express C18 (150 × 3.0 mm, 2.7 µm)	A	H_2O + 0.1% formic acid	CBDA, CBGA, CBG, CBD	[70]
			B	ACN + 0.1% formic acid		
			Gradient			
Oil, H_2O	UHPLC-QqQ	Acquity UPLC HSS C18 column (150 × 2.1 mm, 1.8 µm)	A	H_2O 0.1% formic acid	CBDA, CBG, CBD, CBN, THC, CBC THCA	[71]
			B	ACN		
			Gradient			

(Table 2) Contd....

Solvent of Extraction	Method and Detector	Stationary Phase	Mobile Phase		Cannabinoids Detected	Refs.
Oil, EtOH	HPLC-DAD (228 nm) HPLC-Q-ToF	Poroshell 120 SB-C18, (100 × 2.1 mm, 2.7 µm)	A	H_2O + 0.1% formic acid	CBDA, CBD, CBN, THC, THCA	[72]
			B	ACN + 0.1% formic acid		
			Isocratic			
EtOH, hexane, hexane/EtOH (7:3)	HPLC- UV/Vis (220-280 nm)	Purospher RP-18 (250 × 4.6 mm, 5.0 µm)	ACN 85%, 0.1% formic acid		CBG, CBD, CBN, 9THC, CBC	[73]
			Isocratic			
Olive oil, EtOH	HPLC-MS/MS (ESI)	Kinetex EVO C18 (100 × 2.1 mm, 5.0 µm)	A	2.0 mM ammonium acetate buffer	CBDA, CBGA, CBG, CBD, CBN, 9THC	[74]
			B	ACN		
			Gradient			
MeOH/hexane (9:1)	HPLC-DAD (210 nm)	LiChrospher® 60 RP (125 × 4 mm, 5.0 µm)	A	TEAP 25mM	CBD, CBN, 9THC, THCA	[75]
			B	64% ACN/35% TEAP 25 mM		
			Isocratic			
Hexane/ethyl acetate 9:1	HPLC-UV (210 nm)	100 RP-18 LiChroCart (125 × 4.0 mm, 5 µm)	66% Acetonitrile/triethylammoniumphosphate 25 mM buffer		THC, THCA	[76]
			Isocratic			
MeOH	HPLC-UV (220 nm)	Lichrocart, C18 (125 × 4.0 mm, 5 µm)	65% ACN—phosphate buffer		CBD, CBN, 9THC	[78]
			Isocratic			
MeOH	LC-QToF MS/MS	Mediterranean (250 × 4.6 mm, 3.0 µm)	A	5% ACN + 0.1% FA	CBDVA, CBDV, CBDA, CBGA CBG, CBD, THCV, CBN, 8THC, 9THC, CBC, CBL, THCA	[80]
			B	5% H_2O + 0.1% FA		
			Gradient			
MeOH	HPLC-MS/MS (ESI)	Waters CORTECS C18 (150 × 2.7 mm, 2.2 µm)	A	H_2O + 0.1% formic acid	CBG, CBD, 9THC	[98]
			B	MeOH + 0.1% formic acid		
			Gradient			
EtOAc	HPLC DAD (228 nm)	Allure C18 (150 × 3.2 mm, 5 µm)	A	13% buffer 0.05 mM acetic acid	THC, THCA	[99]
			B	22% ACN		
			C	66% MeOH		
			Gradient			
MeOH	HPLC-UV (225 nm)	Gemini-NX C18 (150 × 4.6 mm, 3 µm)	A	5% ACN + 0.1% TFA	CBDA, CBGA, CBG, CBD, CBN, CBNA, 8THC, 9THC, CBC, THCA	[100]
			B	95% ACN + 0.1% TFA		
			Gradient			

4.2. Gas Chromatography (GC)

GC has been the most popular technique used for regulatory, toxicological, and forensic purposes due to its specificity and efficiency. However, it is currently considered that it is not a completely adequate technique for the analysis of cannabinoids since it works at high temperatures (280°C), causing the decarboxylation of acidic cannabinoids to their neutral form [21, 23, 24, 58, 59].

This transformation, which occurs within the equipment, can alter the determination of the actual content of cannabinoids present in the sample. In general, determinations for toxicological purposes do not seek to distinguish between acidic and neutral forms, but rather directly the potency of the plant, which is referred to as total THC or total CBD. However, there is evidence indicating that the decarboxylation is not complete during the analysis, it depends a lot on the characteristics of the injector [59, 79, 81].

If the objective is the determination of total cannabinoids, a pretreatment to achieve conversion before analysis is suggested in the literature. To avoid this conversion, there is a pretreatment to derivatize the cannabinoids, thus allowing their discriminatory analysis between acid and neutral forms [82]. This step involves a lot of pretreatment time and must also be standardized to evaluate its performance. Functional groups like COOH or OH can be transformed into more stable and volatile molecules. TMS, BSTFA, MSTFA, and MTBSTFA have been reported as some of the si-

lylating agents for the derivatization of cannabinoids [23, 59, 83-85]. Also, this procedure requires the presence of a catalyst, the most cited being TMCS.

One of the enormous advantages of gas chromatography is that the stationary phase is arranged in a capillary column which greatly improves the efficiency of the system. Regarding the stationary phase, the most used are non-polar, such as DB-5 or DB-1, due to the lipophilic nature of cannabinoids [80, 86-89].

The most used detectors in this technique are the flame ionization detector (FID) and the mass spectrometer (MS), each one has its advantages and disadvantages. The FID detector is much more recommended for accurate quantification, than the MS detector for quantification [23, 81, 89]. Cannabinoid standards can be used (although they are expensive) for quantification, but the use of different internal standards is also recommended. The AHP suggests 4-androstene-3-17-diene and tetracosane and docosane have also been cited. The MS detector is much more specific and sensitive than the FID, being able to characterize the sample despite any interference (matrix effect), however, it requires the use of deuterated standards that are expensive and that there are not for each cannabinoid. These deuterated standards are necessary to minimize signal interference.

For terpenes chromatographic analysis, the extraction from cannabis plant material can be carried out in different ways, with solvents (a mixture of ethanol and hexane, 7:3), and hydrodistillation. Solventless techniques such as SPME, SHS, DSH, SBSE were also cited as showing high advantages over other methods in terms of time, simplicity, and automatization. Qualitative and quantitative analysis of terpenes is done through GC, and the most common stationary phases are non-polar. An FID detector is used for quantification because it is low cost and simple to employ (Table **3**) (Fig. **2**).

5. OTHER TECHNIQUES FOR CANNABINOIDS ANALYSIS

For cannabinoid analysis, some alternative techniques are available to further improve conventional HPLC and GC methods. In 2009, the United Nations suggested that fast and easy qualification of cannabis products could be achieved using a variety of color tests. Fast Corinth V salt test, the Fast Blue salt test, the Rapid Duquenois test, and Beam Test are the most cited procedures to detect different cannabinoids [90, 91].

The Beam test is a centennial technique that determines the presence of cannabidiol (and cannabigerol) [90], this colorimetric reaction is becoming popular in routine domestic analysis for quality control essays (personal observation).

Concerning other chromatographic approaches, thin layer chromatography (TLC) presents some virtues, is a quick technique and the visual detection of cannabinoids can be performed in many samples simultaneously. To improve the characteristics of specificity, sensitivity, and reproducibility, various strategies, and modifications to conventional TLC have been designed. Fischedick *et al.* (2009) developed and validated a simple high-performance rapid TLC (HPTLC) method for THC quantification, which was shown to be accurate and reproducible [92]. Galand *et al*. (2004) developed other planar techniques (AMP, OPLC) that improved the results obtained by conventional TLC [93].

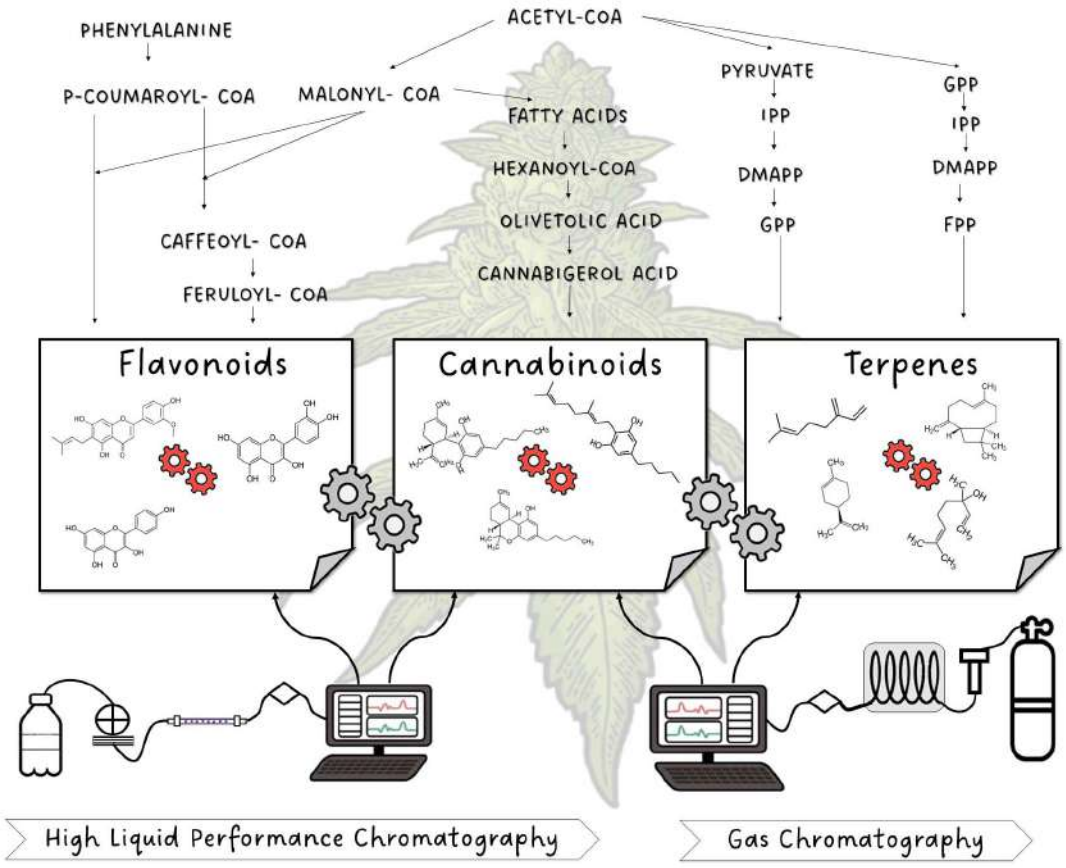


Fig. (2). Biosynthesis of principal secondary metabolites of *C. sativa* and selected methods for their analysis. Red gear symbolizes intra-entourage. Gray gear symbolizes inter-entourage. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Table 3. Representative references and their chromatographic characteristic for GC analysis of cannabinoids.

Cannabinoids (From Inflorescences)					
Gas Chromatography					
Solvent of Extraction	**Method and Detection**	**Stationary Phase**	**Temperature Program**	**Cannabinoids Detected**	**Refs.**
EtOH, hexane	GC-MS	HP-5 MS (30 m × 0.25 mm, 0.25 μm)	50°C increases to 300°C (at 6 C/min) holds for 2 min	CBG, CBD, CBN, THC, CBC	[73]
MeOH	GC-FID	HP5 (1 5 m × 0.32 mm, 0.25 μm)	180 increases to 220°C (at 40°C min), holds for 5 min. Increases to 240°C (at 5°C/min). Finally increases to 280°C (at 25°C/min).	CBD, CBN, 9THC	[78]
Hexane	GC-ToF/MS	DB-5MS-UI (30 m × 0.32 mm, 0.25 μm)	50°C increases to 104°C (2°C/min) holds for 27 min. Increases to 120 °C (at 20°C/min) holds for 0.8 min. Increases to 160°C (at 4°C/min) holds for 10 min. Increases to 232°C (at 25°/min) holds for 2.9 min. Increases to 242°C (at 1.5°C/min) holds for 6.7 min. Increases to 250°C (at 2°C/min) holds for 4 min. Finally increases to 300°C (at 25°C) and holds for 2 min.	CBDV, CBG, CBD, THCV, CBN, 8THC, 9THC, CBC	[80]
ACN :MeOH (8: 2)	GC-FID	DB-1MS (15 m × 0.25 mm, 0.25 μm)	190°C holds for 1 min, before increasing to 230°C (30°C/ min) holds for 2 min. Increases to 250°C (at 5°C/min) holds for 1 min. Increases to 300°C (at 20°C/min), where it was held for 2.75min.	CBDA, CBGA, CBG, CBD, THCV, CBN, 8THC, 9THC, THCA, CBC	[82]
MeOH	GC-FID GC-MS	DB-5MS UI (30 m× 0.25 mm, 0.25 μm) Rxi®-5 ms, (30 m× 0.25 mm, 0.25 μm)	200 increases to 300°C (10°C/min) holds for 2 min 70°C increases to 180°C (at 40°C/min) then increases to 300°C (at 10°C/min) holds for 6.25 min	CBDV, CBD, CBN THC, THCA	[83]
EtOH, ACN	GC-MS	Rxi-35Sil MS (30 m × 0.25 mm, 0.25 μm)	60°C increases to 220°C (25°C/min) holds for 10 min. Increases to 300°C (at 10°C/min) holds for 15 min	CBDV, CBDA, CBGA, CBG, CBD, THCV, CBN, 8THC, 9THC, CBC, THCA	[84]
EtOH	GC-FID GC-MS	DB5 (30 m × 0.25 mm, 0.25 μm)	60°C, increases to 240°C (at 3°C/min) and holds for 5 min	CBDV, CBG, CBD, THCV, CBN, 8THC, 9THC, CBC	[86]
MeOH	GC-FID	DB-1 (30 m × 0.32 mm, 0.25 μm film thickness)	230°C (hold for 7 min), increases to 260°C (at 10°C/min) and holds for 2 min	CBD, CBN, THC	[87]
EtOH	GC-MS	DB-5ms (15 m × 0.25 mm, 0.25 μm)	60°C increase to 240°C (at 8.5°C/min) holds for 2 min	CBDV, CBG, CBD, THCV, CBN, THC, CBC	[88]
Hexane	GC-MS	HP-5MS (30 m × 0.25 mm, 0.25 μm)	100°C (hold for 1 min), increases to 260°C (at 10°C/min) and holds for 10 min	CBG, CBD, CBN, THC, CBC	[101]
Hexane	GC-MS	HP-5ms (30 m × 0.25 mm, 0.25 μm)	100°C, increases to 260°C (at 10°C/min) and holds for 10 min	CBG, CBD, CBN, THC, CBL	[102]
Steam distillation (SD) and hydro-distillation (HD)	GC-MS	HP-5 MS (30 m × 0.25 mm,0.10 μm)	60°C (hold for 5 min) increases to 220°C (at 4°C/min) increases to 280°C (at 11°C/min) holds for 15 min	CBDV, CBD, THC, CBC	[103]
EtOH	GC-FID GC-MS	DB-5 (30 m × 0.25 mm, 0.25 μm)	60°C increase to 240°C at (3°C/min) holds for 5 min	CBG, CBD, THCV, CBN, THC, CBC	[104]
Supercritical fluid extraction (SFE)	GC × GC FID/MS	D: HP-5MS (30 m × 0.25 mm, 0.25 μm) 2 D: DB-17MS (5.0 m × 0.25 mm, 0.25 μm)	60°C increases to 102°C (at 4°C/min), increases to 165°C (at 12°C/min) finally increases to 300°C (at 6°C/min) holds for 5 min	THC	[105]
Hexane/ethyl acetate (60:40)	GC-MS	HP-5 MS (30 m × 0.25 mm, 0.25 μm)	100°C (1 min) to 290°C (10 min) at 20°C/min	CBD, CBN, THC	[106]

Nuclear magnetic resonance (NMR) spectroscopy has been considered a highly accurate and reproducible technique with a relatively short analysis time, and one of its major advantages is the lack of sensitivity toward impurities present in the plant material. However, this technique is not commonly employed due to the high instrumental costs and the necessity of highly specialized personnel [94-96].

Very few applications for qualitative and quantitative analysis of cannabinoids have been developed and reported with infrared spectroscopy. MIR (mid-infrared) spectral region is richer in stronger bands, bringing more clarity in spectra. IR spectroscopy is a very interesting alternative to conventional analysis of cannabis constituents since it does not require trained personnel. This is a fast technique and does not require solvents and the analysis is direct, the sample does not need to be transformed. Although this technique has low sensitivity, there are reports of the determination of seven different cannabinoids in dry cannabis inflorescences [23].

Raman spectroscopy is a noninvasive, nondestructive technique and allows the separation of several cannabinoids and its acid forms with 100% accuracy. These results substantially expand the applicability of RS as a tool for hemp cultivation and breeding. Sample preparation is not necessary, and interpretation of data is easier than in IR analysis. One of the disadvantages is the possible interaction with fluorescence compounds like chlorophyll, so direct analysis of plant material is a challenge. However, more interestingly, they also provided the spectroscopic signature of the main CNBs (CNG, CBGA, THC, THCA-A, CBD, CBDA) to be used for quantitative purposes, representing the cornerstone on which quantitative methods for CNB potency determination can (and should) be developed.

Capillary electrophoresis is becoming a sensitive approach for the determination of cannabinoids, although. The first approaches using this technique describe the separation of a few cannabinoids, but in the last year, a fast and cost-efficient method of separation and quantitation of 14 cannabinoids was described. CE is a powerful tool in the analytical chemistry of cannabis and its results are comparable with research employing UHPLC system that resolves 10-16 phytocannabinoids [97-111].

CONCLUDING REMARKS

Interest in *Cannabis sativa* L has been increasing over the last few years. Its medicinal properties or potential applications are being intensively studied for the treatment of a wide spectrum of pathologies [112, 113].

The pharmacological effects of *C. sativa* have been reported and described based on the most abundant metabolites, such as THC or CBD. However, as mentioned in this work, *C. sativa* presents other secondary metabolites, terpenes and flavonoids which also have medicinal properties.

In the last years, research papers have confirmed the synergy of these compounds in the complete extract of the plant, enhancing the therapeutic activity compared to a purified component. These studies demonstrate a superior pharmacological response using complete extracts compared to isolated molecules for different activities such as cytotoxic or anti-inflammatory.

The close relationship that may exist between a certain therapeutic effect with a certain characteristic set of terpenes and cannabinoids has also been mentioned. However, much remains to be understood about the mechanisms of action that underlie this synergistic phenomenon.

The administration of medicinal plants has been based on whole formulations for millennia, and *C. sativa* is not an exception. These conclusions diverge from the reductionist paradigm that establishes the use of a single component for a health condition. Following this line of thought… if pathologies are usually multifactorial, why shouldn't therapy also be the same?

On the other hand, currently, the taxonomic classification of cannabis is based on genetic analysis that considers three chemotypes according to the presence and proportions of CBD and THC in the plant. However, there is evidence that suggests that terpenes have some implication in determining the chemotype since there are packages/sets of terpenes that are synthesized especially in some varieties and not in others.

C. sativa taxonomy should consider and include all compounds directly related to its medicinal potential. For this type of determination, statistical approaches based on multivariate analysis have been very useful in various works.

Considering *C. sativa* as a factory of medicinal compounds that interact with each other synergically represents a challenge from an analytical point of view. The immense variety of compounds and the interactions between them demands the development of methods that answer current questions and allow a better understanding of *C. sativa* medicinal benefits. Quality control and standardized protocols are also necessary for the comprehensive analysis of cannabis extracts and derivatives.

Several methods have been developed to quantify cannabinoids, and although there are no official guidelines on their analysis, most methods are validated and share certain general characteristics. As mentioned earlier in this review, the most suitable technique for the analysis of cannabinoids is HPLC, with the UV detector being the most economical, while the MS detector is the most specific. Both flavonoids and terpenes are analyzed with previously mentioned techniques for this group of metabolites, with some adaptations related to the analysis of new compounds such as cannflavins.

Therefore, the importance of chromatography in chemical characterization relies not only on defining chemovars based on their active compounds, but also on understanding the foundations of the entourage effect.

LIST OF ABBREVIATIONS

ACN	=	Acetonitrile (CH_3CN)
CBC	=	Cannabinochromene
CBD/CBDA	=	Cannabidiol/Cannabidiolic Acid
CBDv	=	Cannabidivarin
CBG/CBGA	=	Cannabigerol/Cannabigerolic Acid
CBN	=	Cannabinol
GC	=	Gas Chromatography
HPLC	=	High Performance Liquid Chromatography
MeOH	=	Methanol (CH_3OH)
9THC/9THCA	=	Tetrahydrocannabinol/Tetrahydrocannabinolic Acid
THCP	=	Tetrahydrocannabiphorol
THCv	=	Tetrahydrocannabivarin
TLC	=	Thin Layer Chromatography

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

F.M.S.S. is grateful for her doctoral fellowship granted by the CONICET and the Universidad de Buenos Aires and grants from the Universidad de Buenos Aires, UBACYT 20020150100056BA and PIDAE 2019 (Martín F. Desimone), which supported this work.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

F.M.S.S. is grateful for her doctoral fellowship granted by the CONICET and the authors would like to acknowledge the Universidad de Buenos Aires and grants from the Universidad de Buenos Aires, UBACYT 20020150100056BA and PIDAE 2019 (Martín F. Desimone), which supported this work.

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