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REVIEW

Pitfalls in the analysis of phytocannabinoids in cannabis inflorescence

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Abstract

The chemical analysis of cannabis potency involves the qualitative and quantitative determination of the main phytocannabinoids: Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), cannabigerol (CBG), cannabichromene (CBC), etc. Although it might appear as a trivial analysis, it is rather a tricky task. Phytocannabinoids are present mostly as carboxylated species at the aromatic ring of the resorcinyl moiety. Their decarboxylation caused by heat leads to a greater analytical variability due to both reaction kinetics and possible decomposition. Moreover, the instability of cannabinoids and the variability in the sample preparation, extraction, and analysis, as well as the presence of isomeric forms of cannabinoids, complicates the scenario. A critical evaluation of the different analytical methods proposed in the literature points out that each of them has inherent limitations. The present review outlines all the possible pitfalls that can be encountered during the analysis of these compounds and aims to be a valuable help for the analytical chemist.

Keywords Cannabinoid extraction · Cannabinoid analysis · GC-FID · LC-UV · Mass spectrometry · Cannabis flos monograph

Introduction

Cannabis is a chemically complex natural mixture of biologically active compounds spanning flavonoids, amino acids, sugars, phytocannabinoids, and so forth [1–3]. Among these, phytocannabinoids represent the most interesting and most thoroughly studied class of compounds as they are endowed of a wide range of pharmacological activities. In particular, (−)-*trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC) and (−)-*trans*-cannabidiol (CBD) are the best known phytocannabinoids (Fig. 1), with the former characterized by the intoxicant psychotropic activity typical of cannabis [4–6], and the latter void of such effect but known for its anti-inflammatory, anti-oxidant, anti-convulsant, and other numerous properties [7–15]. Although legal restrictions limit the use of cannabis and its psychotropic constituents in research and clinic [16, 17], they have been recognized as valuable treatments for several therapeutic indications, including glaucoma, Tourette syndrome, and neuropathic pain [7, 18–21]. As an example, Sativex and Epidiolex (GW Pharmaceuticals, UK) are standardized cannabis extracts approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) and marketed to treat symptoms of multiple sclerosis and epilepsy respectively. Drug formulation and subsequent production and marketing should follow strict regulations and meet specific quality standards. The analysis of the product plays a key role in all steps of production. In particular, the analysis of phytocannabinoids is extremely important for ensuring a

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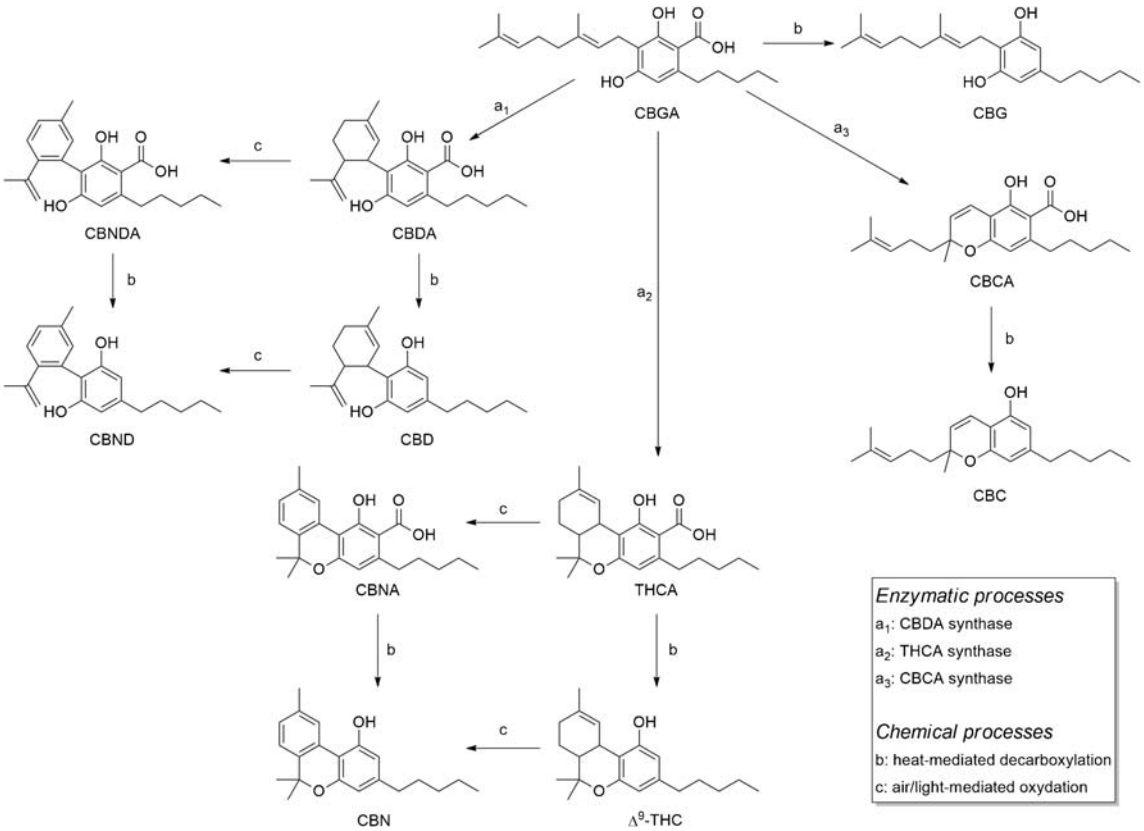


Fig. 1 Schematic representation of the formation of phytocannabinoids. The process is distinguished between enzymatic (**a**), carried out by the plant enzymes, and chemical (**b** and **c**), which takes place independently from the plant metabolism

high-quality product whose chemical profile matches the label exposed.

The selective analysis of phytocannabinoids represents a hot topic nowadays especially because no standardization has been accomplished notwithstanding all efforts made by private companies and public research institutions [22]. The scenario is rather complex starting from the sampling to the extraction and lastly to the actual analysis and detection. A long series of analytical methods have been developed, validated, and published in the literature, and many others are under development every day around the world [23]. Although more communication and cross-talk between analytical laboratories have been achieved in the last few years, no agreement has been reached among the scientific community on the validation parameters and criteria for the determination of phytocannabinoids in cannabis samples [22].

The monograph of *cannabis flos* of the German Pharmacopoeia [24] and the Union method established by the European Commission for the quantitative determination of the Δ^9-THC content in hemp varieties [25], the latter hereafter referred to as EU method, represent two official methods for cannabis inflorescence analysis, using respectively high-performance liquid chromatography-ultraviolet detection (HPLC-UV) and gas chromatography-flame ionization detection (GC-FID). The two methods have different purpose, sample preparation, extraction procedure, and result expression, therefore not suitable to be compared. In particular, it is possible to refer to the German Pharmacopoeia to carry out purity testing on cannabis drug and analyze cannabinoid quantities indicated in the label with respect to completely dried drug [24], while the EU method was designed to verify the THC content in hemp varieties or in cannabis inflorescence and resin [25]. Although not comparable, they are the only official methods that can be legally accepted and may be taken as a guidance for future development of more accurate and reliable methods.

In order to shed light on this articulate topic, the present review aims to elucidate the pitfalls and tricks in the analysis of phytocannabinoids, hereafter called cannabinoids, in cannabis plant material. Most of the critical aspects discussed herein are generally not clearly addressed in analytical papers but are commonly encountered in the routine practice.

Sampling and sample preparation

The first pitfall in the analysis of cannabinoids occurs in the sampling. Cannabinoids are lipophilic compounds contained in the glandular trichomes on the female flowers and leaves; thus, stems and seeds are void of such compounds unless they are contaminated by the oily resin contained in the trichomes

[26]. When a cannabis sample is collected from the plant, small stems and leaves, and sometimes seeds, are usually included along with the flowers. The part of the plant collected for the analysis is crucial for the result of the final concentration of cannabinoids. Routine analyses have highlighted that different parts of the plant or parts taken from plants located a few meters of distance contain completely different cannabinoid concentrations. Furthermore, if the sample is taken from the basal flowers, which generally reach a lesser degree of maturation, the cannabinoid concentrations will be unavoidably lower than those found in the apical flowers. The most appropriate way to get a representative sample of cannabis inflorescence is to collect and mix flowers and small leaves from the upper third of a reasonable number of plants located at few meters away in the same geographical area. The result will be an average representative of the cannabinoid concentration present in the whole area.

Sample preparation is also very variable among laboratories due to the lack of a standardized procedure. Indeed, this step may include or not a cleaning step where some of these additional parts, such as stems and seeds, are removed as they can dilute the sample and lower the concentration of cannabinoids. Depending on whether and how the sample is cleaned and which parts are included in the sample, the resulting concentration of cannabinoids will be different.

Another adverse effect for cannabinoids comes from light during storage. Already in 1976, Fairbairn et al. demonstrated that significant losses of cannabinoids occur after exposure to either light or air, with the latter also causing the oxidation of THC into its aromatized form cannabinol (CBN) (Fig. 1) [27]. Other authors confirmed the same influence of light and air on cannabinoid contents, thus concluding that herbal sample could be stored for 1 to 2 years in the dark at room temperature and as much as possible in the absence of air [28–30].

Before the analysis, the sample is generally dried to eliminate most of the water. The drying method reported in the German Pharmacopoeia involves the heating of the sample over phosphorous(V) oxide at 40 °C under a pressure between 1.5 and 2.5 kPa for 24 h [24]. However, the German Pharmacopoeia contemplates this step with the aim to verify that the weight loss on drying does not exceed 10% (w/w). Hence, it rather gives directions for a purity test on already dried cannabis material [24].

When harsher conditions are employed (higher temperature) on fresh plant, more water is lost and the concentration of cannabinoids is consequently higher. It is worth noting that the EU method involves the drying of the sample at temperatures as high as 70 °C until a constant weight is reached (no fixed time is indicated) and the moisture content does not exceed the 13% [25]. On the other hand, if water is not properly removed from the plant material, this favors mold growth with subsequent risk for mold spores and/or mycotoxin inhalation and health damage [31].

During sample preparation, the raw material is generally finely ground or micronized in order to break the trichomes and release the oily resin containing cannabinoids. If the sample is not ground, the final recovery of cannabinoids will be lower as a smaller surface will be in contact with the extracting solvent. The EU method involves the grinding of the sample and sifting through a 1-mm mesh sieve until a semi-fine powder is obtained [25]. The German Pharmacopoeia indicates a similar optimal powder size, which is obtained after grinding and sifting through a 710 sieve [24]. One crucial thing to take into account is that, if the ground sample is not extracted within a short time, it is more susceptible to degradation, thus distorting the actual concentrations. It is also important to collect all the solid residues of resin and trichomes as they easily adhere on the grinder walls, thus leading to a significant loss of cannabinoid percentage. This aspect is also highlighted in the Danish Cannabis Monograph, which is based on the monograph "Cannabis inflorescence" (version 7.1, November 2014) and the (non-public) EDQM-working-document (PA/PH/Exp. 13B/T (16) 38, August 2016) published by the Dutch OMC (Dutch Office for Medicinal Cannabis) [32]. The same monograph indicates a 5-mm powder size, underlining that pulverization to a finer size would not improve the situation, but rather lead to the opposite effect [32].

Extraction of cannabinoids

Extraction procedure

The monograph of *cannabis flos* indicates dynamic maceration (DM) as the method of choice for the extraction of cannabinoids [24]. Other sources in the literature propose different types of extraction, such as ultrasound-assisted extraction (UAE), as also indicated by the EU method [25], microwave-assisted extraction (MAE), and supercritical fluid extraction (SFE) [33]. DM involves the use of a solvent with several shaking or stirring cycles at room temperature, while UAE and MAE use respectively sound waves and microwaves to accelerate the extraction and increase the yield of cannabinoids. SFE with CO_2 is a promising alternative technique with several advantages including no flammability or toxicity issues, ease of solvent removal, good affinity for lipophilic compounds, and availability at low cost [34]. SFE is not commonly employed as an extraction method for routine analysis but rather for preparative purposes with the aid of a certain percentage of ethanol as co-solvent [34]. Brighenti et al. investigated the four different methods and different solvents in order to suggest the procedure that gives the highest cannabinoid yield [33]. Three cycles of DM with decreasing volumes of ethanol at room temperature resulted the best extraction method for cannabinoids [33]. The sample to solvent ratio is also very important to ensure a high recovery yield. In particular, as

suggested by the same authors and as also indicated by the German Pharmacopoeia, the ideal ratio is 1 g of sample for 100 mL of solvent [24, 33]. A lower amount of solvent, even for a longer time, or just one extraction cycle instead of three can decrease the yield. On the other hand, the EU method requires that the plant material is extracted in one cycle in an ultrasound bath for 20 min with a sample to solvent ratio of 1:50 (g/mL) [25]. Another advantageous extraction technique is the accelerated solvent extraction (ASA) or pressurized liquid extraction (PLE), which does not require a filtration step as the solid raw material is confined in the sample extraction cell [35]. This technique can be used with different solvents and temperatures with the aid of high pressure in order to improve the solubilization of the analytes within a very short time [36, 37]. However, this methodology has not been recognized as an official method for the analysis of cannabinoids and has been confined solely to research purposes, most likely because high pressure has a no negligible effect on cannabinoid stability [35].

Extraction solvent

A number of methods that use different solvents for the extraction of cannabinoids have been developed and reported, but to date, a protocol that encompasses no issues has not been provided. The most widely employed solvent is ethanol, also indicated as the solvent of choice in the monograph of *cannabis flos* [23, 24, 38]. The results obtained by Brighenti et al. suggest that ethanol is the solvent with the highest extractive power compared with methanol, methanol/chloroform 9:1 (v/v), acetone, and hexane due to the polarity of the carboxylated cannabinoids present in fresh plant material [33]. Hexane actually showed the worst performance in terms of cannabinoid yield due to its lower affinity for these species. However, it is noteworthy to mention that using hexane in place of ethanol would result in a cleaner cannabinoid-rich extract as it carries fewer amounts of other contaminants from the plant like flavonoids, alkaloids, and chlorophylls [39]. Moreover, it is worth noting that, being more lipophilic than ethanol, it also extracts the waxy material that covers the trichomes, with the risk of clogging the chromatographic column. It is important to mention that hexane is also used in industry for the crystallization of CBD, suggesting that this solvent can lead to a decrease in the solubility of cannabinoids, which can then precipitate resulting in a lower quantified final concentration. On the other hand, it provides cleaner chromatograms that can be easily interpreted. Indeed, hexane is used for the selective analysis of cannabinoids in the official method established by the European Commission [25]. As a consequence of the lower affinity of hexane for cannabinoids compared with more polar solvents, the analysis would show lower concentrations, thus allowing on the market products with cannabinoid levels outside the permissible limits.

Analysis of phytocannabinoids

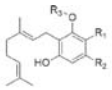
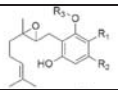
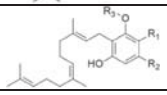
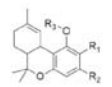
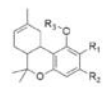
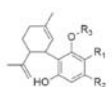
A comprehensive inventory of phytocannabinoids has been recently published by Hanuš et al. [40] with almost 150 molecules and updated with the latest findings on new non-canonical phytocannabinoids like the butyl and heptyl THC and CBD homologs [41–44]. An exhaustive list of the most commonly found phytocannabinoids in hemp material is reported in Table 1. The next paragraphs of the present review outline a series of analytical approaches with pitfalls and strengths for the analysis of these exceptional molecules.

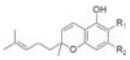
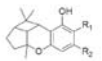
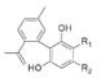
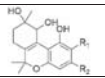
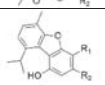
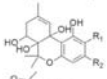
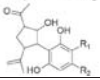
Analysis of decarboxylated cannabinoids

It is now well established that the cannabis plant does not produce the decarboxylated forms of cannabinoids, such as tetrahydrocannabinol (THC), cannabidiol (CBD), cannabichromene (CBC), and cannabigerol (CBG), but rather their carboxylated precursors, tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), cannabichromenic acid (CBCA), cannabigerolic acid (CBGA), etc., via specific enzymatic pathways (Fig. 1) [89]. Only a decarboxylation reaction, generally induced by heat, can convert these acidic forms into the corresponding decarboxylated species. Taking advantage of this conversion under heating, the gas chromatographic technique can trigger this reaction inside the injection chamber, thus revealing only one peak at the retention time of the decarboxylated species corresponding to the sum of the two forms (carboxylated and decarboxylated species). If a derivatization step is carried out prior to the analysis, the decarboxylation of the native forms is prevented, thus obtaining two distinct peaks in the chromatogram. As already reported by Dussy et al., the decarboxylation occurring in a gas chromatograph does not have a fixed rate as it strictly depends on the geometry of the liner and temperature of the injector port [90]. It is estimated that only 70% of the initial THCA injected can be recovered as THC by this kind of analysis [90]. Most likely, some decomposition process with loss of thermally unstable cannabinoids takes place. Besides, it should be taken into account that the decarboxylation rate of THCA does not apply to the other cannabinoids since each one displays a completely different decarboxylation kinetics [91]. A derivatization of these species may overcome the issue, but the method should be validated every time that a new parameter is introduced in the analysis of cannabinoids.

It is noteworthy that gas chromatography coupled to flame ionization detector (GC-FID) without a derivatization step has been chosen by the European Union for the official analysis of cannabinoids run by the authorities [25]. This raises some concern around the reliability of the results. Hence, it might be the case to revise the official method taking all the aforementioned pitfalls in the GC analysis into account.

Table 1 Main cannabinoids commonly found in hemp extracts. For each cannabinoid, the type, structure, common name, and original reference are indicated

Type	Structure	R_1	R_2	R_3	Name	Reference
Cannabigerol (CBG)		COOH	C_5H_{11}	CH_3	Cannabigerolic acid monomethyl ether (CBGMA)	[45]
		COOH	C_5H_{11}	H	Cannabigerolic acid (CBGA)	[46]
		COOH	C_4H_9	H	Cannabigerobutolic acid (CBGBA)	[47]
		COOH	C_3H_7	H	Cannabigerovarinic acid (CBGVA)	[48]
		COOH	CH_3	H	Cannabiorcogerolic acid (CBGOA)	[47]
		H	C_5H_{11}	CH_3	Cannabigerol monomethyl ether (CBGM)	[49]
		H	C_5H_{11}	H	Cannabigerol (CBG)	[50]
		H	C_4H_9	H	Cannabigerobutol (CBGB)	[47]
		H	C_3H_7	H	Cannabigerovarin (CBGV)	[51]
		H	CH_3	H	Cannabiorcogerol (CBGO)	[47]
		COOH	C_5H_{11}	H	6,7-Epoxy-CBGA	[52]
		H	C_5H_{11}	H	6,7-Epoxy-CBG	[52]
		COOH	C_5H_{11}	H	Sesqui-CBGA	[53]
		H	C_5H_{11}	H	Sesqui-CBG	[53]
Δ^9-Tetrahydrocannabinol (Δ^9-THC)		COOH	C_5H_{11}	CH_3	Δ^9-Tetrahydrocannabinolic acid monomethyl ether (Δ^9-THCMA)	[47]
		COOH	C_5H_{11}	H	Δ^9-Tetrahydrocannabinolic acid (Δ^9-THCA)	[54, 55]
		COOH	C_4H_9	H	Δ^9-Tetrahydrocannabutolic acid (Δ^9-THCBA)	[43, 56]
		COOH	C_3H_7	H	Δ^9-Tetrahydrocannabivarinic acid (Δ^9-THCVA)	[57]
		COOH	CH_3	H	Δ^9-Tetrahydrocannabiorcolic acid (Δ^9-THCOA)	[47, 56]
		H	C_5H_{11}	CH_3	Δ^9-Tetrahydrocannabinol monomethyl ether (Δ^9-THCM)	[47]
		H	C_5H_{11}	H	Δ^9-Tetrahydrocannabinol (Δ^9-THC)	[58]
		H	C_4H_9	H	Δ^9-Tetrahydrocannabutol (Δ^9-THCB)	[43, 56, 59]
		H	C_3H_7	H	Δ^9-Tetrahydrocannabivarin (Δ^9-THCV)	[48, 60]
		H	CH_3	H	Δ^9-Tetrahydrocannabiorcol (Δ^9-THCO)	[47, 61]
Δ^8-Tetrahydrocannabinol (Δ^8-THC)		COOH	C_5H_{11}	H	Δ^8-Tetrahydrocannabinolic acid (Δ^8-THCA)	[1]
		COOH	C_3H_7	H	Δ^8-Tetrahydrocannabivarinic acid (Δ^8-THCVA)	[48]
		H	C_5H_{11}	H	Δ^8-Tetrahydrocannabinol (Δ^8-THC)	[1]
		H	C_3H_7	H	Δ^8-Tetrahydrocannabivarin (Δ^8-THCV)	[48]
Cannabidiol (CBD)		COOH	C_5H_{11}	CH_3	Cannabidiolic acid monomethyl ether (CBDMA)	[47]
		COOH	C_5H_{11}	H	Cannabidiolic acid (CBDA)	[62]
		COOH	C_4H_9	H	Cannabidibutolic acid (CBDBA)	[43]
		COOH	C_3H_7	H	Cannabidivarinic acid (CBDVA)	[48]

		COOH	CH_3	H	Cannabidiorcolic acid (CBDOA)	[47]
		COOH	C_5H_{11}	H	Cannabidiolic acid (CBDM)	[63]
		H	C_5H_{11}	H	Cannabidiol (CBD)	[64]
		H	C_4H_9	H	Cannabidibutol (CBDB)	[41-43, 56, 59]
		H	C_3H_7	H	Cannabidivarin (CBDV)	[48]
		H	CH_3	H	Cannabidiorcol (CBDO)	[61]
Cannabichromene (CBC)		COOH	C_5H_{11}	-	Cannabichromenic acid (CBCA)	[65]
		COOH	C_3H_7	-	Cannabivarichromenic acid (CBCVA)	[48]
		COOH	CH_3	-	Cannabiorcichromenic acid (CBCOA)	[65]
		H	C_5H_{11}	-	Cannabichromene (CBC)	[65, 66]
		H	C_3H_7	-	Cannabivarinchromene (CBCV)	[51, 66]
		H	CH_3	-	Cannabiorcichromene (CBCO)	[67]
Cannabicylclol (CBL)		COOH	C_5H_{11}	-	Cannabicyclolic acid (CBLA)	[68]
		COOH	C_3H_7	-	Cannabicyclovarinic acid (CBLVA)	[47]
		COOH	CH_3	-	Cannabiorcicylolic acid (CBLOA)	[69]
		H	C_5H_{11}	-	Cannabicyclol (CBL)	[70]
		H	C_3H_7	-	Cannabicyclovarin (CBLV)	[71, 72]
		H	CH_3	-	Cannabiorcicyclol (CBLO)	[69]
Cannabielsoin (CBE)		COOH	C_5H_{11}	-	Cannabielsoinic acid (CBEA)	[73]
		COOH	C_3H_7	-	Cannabielsovarinic acid (CBEVA)	[74]
		H	C_5H_{11}	-	Cannabielsoin (CBE)	[75]
		H	C_3H_7	-	Cannabielsovarin (CBEV)	[74]
Cannabinol (CBN)		COOH	C_5H_{11}	-	Cannabinolic acid (CBNA)	[46]
		COOH	C_3H_7	H	Cannabivarinic acid (CBNVA)	[48]
		COOH	CH_3	H	Cannabiorcolic acid (CBNOA)	[47]
		H	C_5H_{11}	CH_3	Cannabinol monomethyl ether (CBNM)	[76]
		H	C_5H_{11}	H	Cannabinol (CBN)	[77, 78]
		H	C_3H_7	H	Cannabivarin (CBNV)	[79]
		H	CH_3	H	Cannabiorcol (CBNO)	[71]
Cannabinodiol (CBND)		COOH	C_5H_{11}	-	Cannabinodiolic acid (CBNDA)	[47]
		COOH	C_3H_7	-	Cannabinodivarinic acid (CBNDVA)	[47]
		H	C_5H_{11}	-	Cannabinodiol (CBND)	[80, 81]
		H	C_3H_7	-	Cannabinodivarin (CBNDV)	[80]
Cannabitriol (CBT)		COOH	C_5H_{11}	-	Cannabitriolic acid (CBTA)	[47]
		H	C_5H_{11}	-	Cannabitriol (CBT)	[82, 83]
		H	C_3H_7	-	Cannabitriolvarin (CBTV)	[56]
Miscellaneous		H	C_5H_{11}	-	Cannabifuran (CBF)	[84]
		H	C_5H_{11}	-	Dehydrocannabifuran (DCBF)	[84]
		H	C_5H_{11}	-	Cannabicitran (CBCT)	[85]
		H	C_5H_{11}	-	Cannabiripsol (CBR)	[86]
		H	C_5H_{11}	-	Cannabitetrol (CBTT)	[87]
		H	C_5H_{11}	-	Cannabimovone	[88]

Simultaneous analysis of native and decarboxylated cannabinoids

Our experience, along with a growing body of literature, has taught us that decarboxylated cannabinoids in fresh plant material represent only a minor part, if not just a trace, of the pool of cannabinoids detected [23, 36, 38, 92]. The carboxylated forms are rather the predominant compounds in the chromatographic analysis of fresh cannabis inflorescence or hemp biomass. It is obvious that the ratio of the two species is overturned when a prolonged heating at high temperature (above 60 °C) is conducted for the desiccation of the cannabis sample [29].

Instrumental analysis

In light of the above, it would be correct to analyze both species in a cannabis-derived product. To this end, different methods with some variants can be followed. A recently emerging method has been included, near-infrared (NIR) spectroscopy, which is now being claimed as a promising tool for cannabinoid analysis also for forensic investigations in biological fluids [93]. A summary of all techniques with the related pitfalls is reported in Table 2.

Gas chromatography

Gas chromatography (GC) involves the heating of the sample in the injector port to convert the sample into its vapor form, which is then introduced into a continuous flow of an inert gas, generally helium or hydrogen, which carries the molecules of the sample through a long capillary column.

As already mentioned above, a derivatization can prevent the decarboxylation of the acidic species in the injection chamber to reveal two distinct peaks. Nonetheless, some material is unavoidably lost, thus providing an underestimated quantification [92].

Most GC methods employ FID detection, which is more quantitatively reliable compared with GC coupled to mass spectrometry detectors (GC-MS) that requires instead specific deuterated standards besides pure analytical standards in order to compensate for matrix effects on the ionization of the analytes [92]. At this regard, a deuterated standard is commercially available only for very few plant cannabinoids, specifically THCA (THCA-d_3), CBD (CBD-d_3), Δ^9-THC (Δ^9-THC-d_3), and CBN (CBN-d_3). Otherwise, deuterated standards should be synthesized *in house* for different cannabinoids or, alternatively, some laboratories use the existing ones as internal standards, though making a systematic error throughout the analyses [94].

Liquid chromatography

Liquid chromatography (LC) operates with completely different conditions from the GC technique [92]. In particular, no heating is applied to the sample; thus, no decarboxylation is encountered either before, during, or after the analysis. This technique detects the naturally occurring species without altering the original chemical composition. Therefore, no derivatization of the carboxylated cannabinoids is needed prior to the analysis. Notwithstanding the lower sensitivity compared with a mass spectrometric detector, UV is the detection method that suits the most for the analysis of cannabinoids [23, 92]. Although carboxylated and decarboxylated species have different absorption wavelengths (λ_{max}), specifically 306 and 270 nm for the former and 225 nm for the latter, which can be detected using a diode array detector (DAD), both types of cannabinoids can be easily detected at 228 nm with good sensitivity using a single wavelength UV detector [23, 92].

On the other hand, the operator must be able to provide a method with good resolution of all the analytes under investigation and use appropriate analytical standards. Indeed, the method proposed in the monograph of *cannabis flos* included in the German Pharmacopoeia is based on the LC-UV technique [24]. The other relevant drawback of LC-UV-based methods is the low dynamic range detected by the instrument. Especially when dealing with hemp biomass, which contains high percentages of CBD and very low percentages of THC, it is difficult to provide an accurate quantification of both species with a single analytical run. Therefore, two different dilutions should be prepared in order to get both CBD and THC within the limits of the linear range.

Given the abovementioned considerations for the LC technique, the use of a different detector like mass spectrometry (MS) may result in an increase of sensitivity, with limits of quantification (LOQ) below the nanogram per milliliter, but carrying some arduous issues [94, 95]. It is reasonable to think that a large number of species can be detected at 228 nm with an LC-UV-based method and that MS detection can overcome this hypothetical loss in specificity. However, it has been demonstrated that both MS and UV detectors lead to overlapping results, thus proving to be both sensitive and specific for cannabinoid detection [23].

Along with pure analytical standards, LC-MS methods require the use of deuterated analytical standards for the same reasons explained for GC-MS [92].

In recent years, the field of high-resolution mass spectrometry detection has come up with new highly sensitive solutions for the determination of even the least common cannabinoids in a cannabis-derived sample [36, 43, 44, 96]. This kind of instrumental platforms is becoming increasingly popular among the scientific research community, but is still very far from being included in the official routine analysis due to the need of specialized personnel required.

Table 2 Characteristics and pitfalls of the analytical methods for cannabinoid quantification

Method	Extraction procedure/ solvent	Detector	Analytical standards		Derivatization	Cannabinoids detected	Advantages	Pitfalls	Ref.
			Authentic	Deuterated					
GC	Any	FID	Yes	No	Yes	Carboxylated/decarboxylated	Ease of use, no need for deuterated standards	Underestimation of actual concentrations[a]	[90, 92]
					No	Decarboxylated	Ease of use, no need for deuterated standards	Underestimation of actual concentrations[a]	[25, 90, 92]
		MS	Yes	Yes	Yes	Carboxylated/decarboxylated	Ease of use, high sensitivity	Underestimation of actual concentrations[a], need for deuterated standards	[90, 92]
					No	Decarboxylated	Ease of use, high sensitivity	Underestimation of actual concentrations[a], need for deuterated standards	[90, 92]
LC	Any	UV	Yes	No	No	Carboxylated/decarboxylated	Ease of use, high accuracy, no need for deuterated standards	Low sensitivity, low dynamic range	[24, 92]
		MS	Yes	Yes	No	Carboxylated/decarboxylated	High sensitivity, high accuracy	High cost, qualified users, need for deuterated standards	[23, 92]
TLC	Any	UV-Vis	Yes	No	No	Carboxylated/decarboxylated	Ease of use, quick results	Low accuracy, low sensitivity	[24, 92, 97]
NMR	Any	–	Yes	No	No	Carboxylated/decarboxylated	Quick results	High cost, qualified users	[100, 102]
NIR	None	–	Yes	No	No	Carboxylated/decarboxylated	Quick results, no need for extraction	Low accuracy	[93, 107]

[a] The underestimation of the actual concentrations is due to the loss of cannabinoid mass in the injection chamber at high temperature

Thin layer chromatography

Thin layer chromatography (TLC), as also already discussed in a previous review [92], is characterized by the great advantage of being faster, easier, and less expensive than the other chromatographic techniques. However, the results are not as accurate as those obtained by GC and LC and the sensitivity is not high enough when very low limits of psychotropic cannabinoids have to be detected. Nonetheless, a reliable quantitative high-performance TLC method has been published by Fischedick et al. [97]. This technique, regardless of the improvements made [98], is still far from being recognized and included among the official methods for cannabinoid analysis.

Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) is another technique employed in the analysis of cannabinoids, though it is not as sensitive as a mass spectrometry-based method. Still, it remains one of the few techniques capable of elucidating the chemical structure and stereochemistry of an unknown compound [99].

Besides its use as a qualitative technique, NMR is an accurate and reliable quantitative platform for the analysis of cannabinoids in a very short analysis time, especially with the latest highly sensitive probes [100–103]. Notwithstanding these great advantages, this technique is not very common due to the high instrumental costs (purchase and maintenance) and the need of highly qualified personnel.

Near-infrared spectroscopy

Near-infrared (NIR) spectroscopy has been a less investigated technique and only recently developed as an application to the analysis of cannabinoids. Indeed, it has been mostly employed for qualitative discrimination between drug-type and fiber-type cannabis [104], or between cannabis and other plant species [105], and more recently for the prediction of the growth stage of cannabis plants in indoor cultivations [106]. The literature on the quantitative application of NIR to cannabinoid concentration is rather scarce. Sánchez-Carnerero Callado et al. developed and validated a NIR-based method proving that there is sufficient information in the NIR spectral region for the prediction of cannabinoid concentrations in dried and ground cannabis plant materials [107].

The great advantage of this technique is that the analysis requires very little pre-treatment (e.g., grinding, drying); it is faster, simpler, and does not involve the use of environmental pollutants with respect to the reference analytical methods (LC and GC) [93, 107]. The major drawback of the NIR technique is its lower accuracy compared with the standard analytical techniques like GC and LC as the peaks of cannabinoids can be easily masked by those from other compounds. Nonetheless, with the appropriate improvements and relative calibrations to be validated with the LC technique, NIR spectroscopy may be the best candidate for on-site police testing of cannabis samples and routine cannabinoid analysis.

Analytical standards of cannabinoids

Pure analytical standards, although necessary for a reliable quantification, can easily represent a source of error. First, the carboxylated forms are very unstable and should be stored properly at low temperature. Moreover, cannabinoid standards are generally marketed as solutions in methanol, which compromises their stability. In particular, the neutral species react with methanol forming alkylated derivatives that elute very close to the starting cannabinoids [23, 92]. This can cause an overestimation of the actual cannabinoid concentration. Indeed, if a standard sample has a concentration lower than the amount stated on the label, the subsequent measurements that are based on that false value will be artificially higher. Therefore, the true concentration of cannabinoid standard solutions should be verified by a standardized method on a periodic basis, due to the inherent instability of cannabinoids in solution [108, 109]. Otherwise, such a systematic bias in the analytical method will give, for example, artificially high THC values if the true value of the standard is less than the amount that is claimed on the label with possible legal consequences.

Moreover, it is known that cannabinoids like THC and CBD can easily oxidize in air to cannabinol (CBN) and cannabinodiol (CBND), respectively, which present further aromatization on the terpene ring [40]. Carboxylated cannabinoids can undergo the same reaction; thus, CBDA can oxidize to cannabinodiolic acid (CBNDA) and THCA can oxidize to cannabinolic acid (CBNA) (Fig. 1) [40]. It is therefore important to store the cannabinoid standards at temperatures below − 18 °C for a maximum of 6 months to ensure that no decomposition occurs. More importantly, at the first use of the standard solution, it is recommended to divide it into aliquots in order to prevent decomposition of the whole stock solution each time it is thawed and used.

Last but not least, it is necessary to upgrade analytical methods for the quantitative analysis of cannabinoids by actually verifying standard reference solutions, as also reported by Poortman-van der Meer and Huizer that were the first to raise the issue on the actual content of cannabinoid reference standard solutions [108].

Constitutional isomers and stereoisomers

THC can theoretically exist as seven constitutional isomers with the same molecular formula ($C_{21}H_{30}O_2$) but different

connectivity depending on the position of the double bond on the terpene ring. In particular, the shift of the double bond from position C9–C10 across the terpene ring of Δ^9-THC leads to the formation of $\Delta^{6a,10a}$-THC, $\Delta^{6a,7}$-THC, Δ^7-THC, Δ^8-THC, Δ^{10}-THC, and $\Delta^{9,11}$-THC (Fig. 2) [110]. It is reported that they are not naturally occurring, with the exception of Δ^8-THC, which is commonly found in cannabis extracts due to a thermodynamically favored isomerization of Δ^9-THC [40]. An in-depth analysis of cannabis samples derived from plant material and the use of highly sophisticated and sensitive instrumental platforms, such as ultrahigh-performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS), can reveal the presence of several THC and CBD isomers []. However, the exact chemical structure of each isomer cannot be easily assigned to the corresponding chromatographic peaks even with such instrumentation. In fact, the fragmentation spectra of these peaks obtained at the common collision energies (20, 30, or 40 eV) provide very similar if not identical patterns with respect to the predominant isoforms Δ^9-THC and CBD and could be distinguished only by means of the NMR technique. However, the scarce amount of these isomers in the plant material impedes their physical isolation and characterization. At present, while Δ^9-THC is in Schedule II of the 1971 Convention on Psychotropic Substances, all other THC isomers fall into Schedule I of the same Convention, thus being all under international control [17]. Since no analytical procedure has been developed for the other unnatural isomers, the total THC content in routine cannabis analysis is currently calculated as the sum of the contributions of Δ^9-THC, Δ^8-THC, and THCA.

It is worth noting that Δ^9-THC and CBD are chiral cannabinoids and can exist as four stereoisomers, specifically (−)-*trans*, (+)-*trans*, (−)-*cis*, and (+)-*cis* [110]. The scientific literature teaches that the naturally occurring form for both Δ^9-THC and CBD is (−)-*trans*, which corresponds to the (*R*,*R*)-form, although trace amounts of the "unnatural" form (+)-*trans* of Δ^9-THC have been detected by the Gasparrini group in a medicinal cannabis variety (Bedrocan) [111]. CBC is also a chiral cannabinoid, but it represents a rare case of a natural racemate, although the same authors reported an excess of one enantiomer in the Bedrocan variety [1, 40, 111]. Although these stereoisomers can be only detected by the use of a chiral stationary phase, it would be interesting to analyze the presence of unnatural stereoisomeric forms of Δ^9-THC and CBD in other cannabis/hemp varieties as this field has been only scarcely explored. Indeed, nothing is known about the pharmacological activities of the stereochemical variants of (−)-*trans*-Δ^9-THC and very little about those of CBD [112]. In particular, the unnatural (+)-*trans*-CBD showed a binding affinity for CB_1 receptor comparable with that of Δ^9-THC [112].

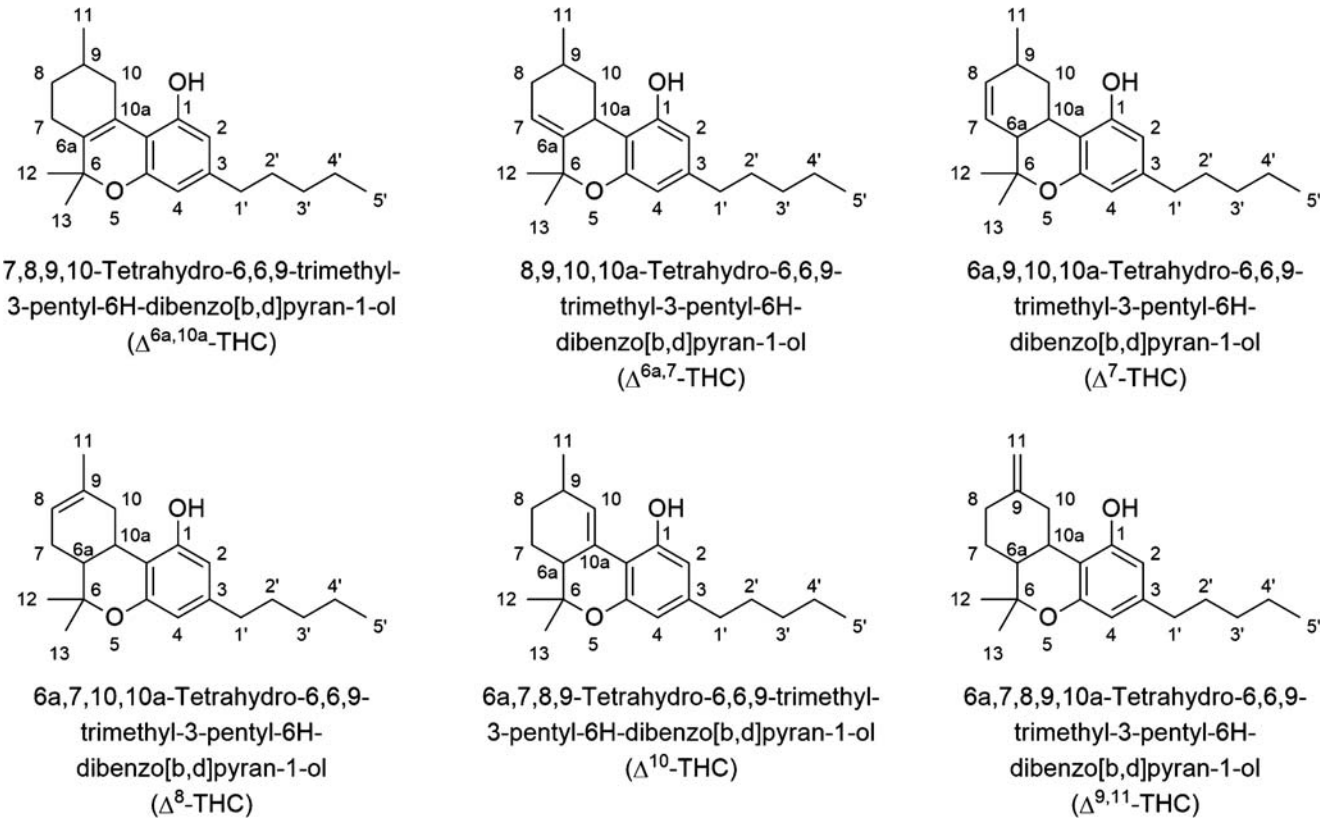


Fig. 2 THC isomers. Chemical structure of the six constitutional isomers of Δ9-THC. For each isomer, the IUPAC name and abbreviation are given

Conclusion

Many are the pitfalls in the analysis of cannabinoids and each step represents a source of variability in the results, thus making inter-laboratory cross validation an arduous task. From the considerations reported in this work, it is clear that the analyst/scientist should pay attention to each variable he/she may encounter during the analysis starting from the sample arrival. Based on the literature results, the method reported by the monograph of *cannabis flos* in the German Pharmacopoeia may be suggested as the procedure of choice for the analysis of cannabinoids.

However, many gaps still need to be filled and many aspects of cannabinoid analysis are still unexplored. For example, all the steps preceding sample arrival to the laboratory, such as harvesting, drying, grinding, sifting, and storage, should not be disregarded and need a more widespread standardization. Several confirmations derived from experimental practice indicate that the concentrations of cannabinoids in fresh inflorescence are significantly different from those in the ground and sifted sample.

It is necessary to carry out relevant inter-laboratory collaborative trials for reproducibility evaluation and eventually achieve the standardization of the analytical procedure, which can contribute to the development of official methods that also meet legal requirements.

The scientific and industrial communities are working very hard to achieve a comprehensive knowledge on this field. The evaluation of the benefits and risks through a reliable analysis of the active principles is highly desirable especially with the growing interest towards cannabis and cannabinoids as a valuable help in modern medicine. We believe that the joint effort of each single scientist will be essential to lay a solid foundation and shed light on a world of confusion.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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