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Pharmaceutical and biomedical analysis of cannabis extracts: a critical review

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Highlights

- Sample preparation strategies for the extraction of cannabinoids are described for plant and biological matrices
- Techniques for cannabinoids analysis are described with advantages and drawbacks
- Chromatographic methods are compared in terms of selectivity and sensitivity
- Detection methods are presented based on the specific aim of the cannabinoids analysis

Abstract

Cannabis products have recently regained much attention due to the high pharmacological potential of their cannabinoid content. In this review, the most widely used sample preparation strategies for the extraction of cannabinoids are described for the specific application to either plant materials or biological matrices. Several analytical techniques are described pointing out their respective advantages and drawbacks. In particular, chromatographic methods, such as TLC, GC and HPLC, are discussed and compared in terms of selectivity and sensitivity. Various detection methods are also presented based on the specific aim of the cannabinoids analysis. Lastly, critical considerations are mentioned with the aim to deliver useful suggestions for the selection of the optimal and most suitable method of analysis of cannabinoids in either biomedical or cannabis derived samples.

Keywords*: cannabis, cannabinoids, liquid chromatography, gas chromatography.*

Abbreviations: AcOH, acetic acid; ACN, acetonitrile; APCI, atmospheric pressure chemical ionization; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; CBCA, cannabichromenic acid; CBC, cannabichromene; CBDA, cannabidiolic acid; CBD, cannabidiol; CBDVA, cannabidivarinic acid; CBDV, cannabidivarin; CBE, cannabielsoin; CBGA, cannabigerolic acid; CBGAM, CBGA monomethyl ether; CBG, cannabigerol; CBGM, CBG monomethyl ether; CBGV, cannabigerovarin; CBLA, cannabicyclolic acid; CBL, cannabicyclol; CBLV, cannabicyclolvarin; CBNA, cannabinolic acid; CBN, cannabinol; CBT, cannabitriol; CBV, cannabivarin; CFL-A, cannaflavin A; CFL-B, cannaflavin B; $CHCl_3$, chloroform; Chex: cyclohexane; CL, chemiluminescence; CPE, cloud point extraction; DAD, diode array detector; DCM, dichloromethane; EI, electron impact; ESI, electrospray ionization; EtOAc, ethyl acetate; EtOH, ethanol; Et_2O, diethyl ether; FA, formic acid; FID, flame ionization detector; FLD, fluorescence detector; FT-IR, Fourier Transform infrared spectroscopy;

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FUSE, focused ultrasound extraction; GC, gas chromatography; hex, hexane; HFIP, hexafluoroisopropanol; HILIC, hydrophilic interaction LC; HPLC, high performance LC; HPTLC, high performance TLC; HS-SPME, headspace solid phase microextraction; *i*PrOH, isopropanol; IT, ion trap; LC, liquid chromatography; LLE, liquid-liquid extraction; LOD, limit of detection; LOQ, limit of quantification; MeOH, methanol; MEPS, microdialysis-extraction packed sorbent; MS, mass spectrometry; MSTFA, *N*-methyl-(trimethylsilyl) trifluoroacetamide; NCI, negative chemical ionization; NH_4OH, ammonium hydroxide; NMR, nuclear magnetic resonance; ^{1}H NMR, proton NMR; OF, oral fluids; o.n., overnight; PFPA, pentafluoropropionic anhydride; PFPOH, pentafluoropropanol; PLE, pressurized liquid extraction; QqQ, triple quadrupole; Q-ToF, quadrupole-time of flight; RP, reverse phase; r.t., room temperature; SFE, supercritical fluid extraction; SLE, solid-liquid extraction; SPE, solid phase extraction; TFAA, trifluoroacetic anhydride; THCA, tetrehydrocannabinolic acid; THC (or Δ^9-THC), tetrahydrocannabinol; THC-COOH, 11-nor-9-carboxy-THC; THC-COOH-gluc, THC-COOH-glucuronide; THC-gluc, THC-glucuronide; THC-OH, 11-hydroxy-THC; THCVA, tetrahydrocannabidivarinic acid; THCV, tetrahydrocannabidivarin; TLC, thin layer chromatography; TMCS, trimethylchlorosilane; UPLC, ultra-performance LC; UV, ultraviolet.

1. Introduction

Cannabis sativa L. can be considered as the most controversial plant in our society [1]. It is the most widespread drug of abuse due to its intoxicating effects resulting from the psychotropic activity of the best known component (−)-*trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC or simply THC) [2-6]. At the same time, cannabis has been known for centuries all over the world for its undeniable medicinal properties. Nowadays its applications in the clinical world span from multiple sclerosis to epilepsy, neuropathic pain, arthritis, nausea and vomiting due to chemotherapy, appetite stimulation in HIV/AIDS, depression, anxiety disorders, sleep disorders, psychosis, glaucoma, and Tourette syndrome [7-11]. The interest in the chemistry and pharmacology of this annual dioecious plant belonging to the family of Cannabaceae is continuously increasing after the discovery of a unique group of terpenophenolic compounds named phytocannabinoids. At least 90 cannabinoids have been isolated from cannabis and characterized since the early 1940s [2]. An important breakthrough was made in 1964 when Raphael Mechoulam isolated and characterized for the first time the main psychoactive component of cannabis, THC [12,13]. Whether cannabis is intended either as a source of fibers and/or seed production or for therapeutic purposes depends on the ratio between THC and cannabidiol (CBD), which is known to possess several pharmacological properties but not the psychotropic one of THC. In particular, analgesic, antioxidant and antiepileptic activities have been attributed to this compound, which seems also to reduce THC side effects [14-16]. Although CBD and THC have such relevance when talking about cannabis, these molecules are not biosynthesised in the plant, which instead produces cannabidiolic acid (CBDA) and tetrahydrocannabinolic acid (THCA). A chemical reaction triggered by heat leads to the decarboxylation of these compounds to get the corresponding decarboxylated (or neutral) species CBD and THC. The latter are the bioactive components, whereas still very little is known about the activity of the two acid forms.[17-20] CBDA and THCA are the major components of cannabis inflorescence. Other minor cannabinoids are cannabichromenic acid (CBCA), cannabigerolic acid (CBGA), the “stem cell” of the other cannabinoid acids, and cannabinolic acid (CBNA). All these compounds upon decarboxylation lead to the neutral derivatives, respectively cannabichromene (CBC), with anti-inflammatory, antibacterial and antifungal activity, cannabigerol (CBG) with analgesic, antibacterial and antifungal activity, and cannabinol (CBN), which derives from the oxidation of THC as a result of prolonged storage and has potent sedative properties [21]. A schematic representation of the biosynthetic route

of THCA and CBDA, their conversion respectively into THC and CBD and the oxidation of THC to CBN is reported in Figure 1.

Beyond cannabinoids, a substantial part of the about 500 compounds present in this complex matrix is represented by other types of molecules, such as terpenes, flavonoids, stilbenoids, amino acids, fatty acids, alkaloids, hydrocarbons, carbohydrates, and phenols [22]. Terpenes represent the volatile component of the plant and were proved to have a synergic action with cannabinoids [21,23].

As a consequence of the increase in the development of medicinal cannabis preparations, there is an increasing demand of the development of qualitative and quantitative methods for the analysis of the bioactive components of cannabis. As a general rule, the analytical method employed for the determination of cannabinoids needs to match the application required. Analysis of plant material is generally performed for the determination of the type of cannabis (fiber or drug type), for the quality control of the material used for medicinal purposes, or for biosynthetic studies within biotechnological research [24]. Conversely, analysis of biological matrices, such as urine, blood, hair, etc., are mainly necessary to provide evidence of drug abuse or for pharmacokinetics studies [24]. Different aims claim different techniques to be used for both sample preparation and analysis. The literature on the determination of THC and its six main metabolites in human body materials until 2002 has been fully covered in the review by Raharjo and Verpoorte [24], whereas Battista *et al.* evaluated the analytical approaches for the determination of THC and the endocannabinoids anandamide (*N*-arachidonoylethanolamine) and 2-arachidonoylglycerol in several human matrices [25]. This review focuses on the analytical methods employed to analyse both plant materials and biological matrices with respect to both cannabinoid content and other bioactive substances contained in cannabis. The review mainly relates the progress that has been done in the past fifteen years (2002-2016) in the sample preparation and analytical techniques employed.[2]

2. Experimental techniques

2.1 Extraction methodologies and sample preparation

1.1.1 Plant material

Figure 2 shows the molecular structure of the most common cannabinoids and cannabis flavones. In plant material, which commonly corresponds to the plant inflorescence, the most widely employed method of extraction is the solid-liquid extraction (SLE), which involves the use of an appropriate solvent with great affinity for cannabinoids. In order to obtain a selective extraction of either cannabinoid acids or neutral cannabinoids, it is necessary to undertake a different extraction procedure. Acid and neutral cannabinoids can be extracted using common organic solvents or a mixture of more solvents. The most common solvent is ethanol (EtOH) since it has a high extracting efficiency due to its high affinity for cannabinoid molecular structure [2,26,27]. Indeed, a method of extraction with EtOH 96% (v/v) has been recently proposed on a draft of *Cannabis Flos* monograph of the German Pharmacopoeia [28].

[2] The literature references discussed and listed herein were found in *scopus* and *web of science* databases by searching for keywords related to: cannabis analysis, cannabinoids analysis, cannabinoids extraction, cannabis and gas chromatography, cannabis and liquid chromatography, LC-MS of cannabinoids, etc. Moreover, the research was limited to the time interval 2002-2016.

Methanol (MeOH), and ethyl acetate (EtOAc) are also widely employed alone or in combination with other solvents (for example MeOH:$CHCl_3$ 9:1 (v/v)) for both chemotype distinction [29,30] and quality control purposes [31,32].

Another solvent which has high lipophilicity that is employed for the extraction of cannabinoids for quality control purposes is hexane [32], or hexane:*i*PrOH 9:1 (v/v) for chemotaxonomic analysis [33]. Hexane has also been used by Mariotti *et al.* in order to classify the plant material on the basis of its THC content, which can be considered a marker of the plant age [6]. Peschel and Politi explored different extraction solvents and combination for chemotype distinction [34]. They proposed a complex procedure involving an extraction with ethyl acetate (EtOAc) and ethanol (EtOH) 40% (v/v) and a parallel defatting with heptane and exhaustive extractions with methanol (MeOH) 70% (v/v). Each extract underwent a fractionation between water and an organic solvent in a liquid-liquid system in several steps, first with dichloromethane (DCM), and secondly with EtOAc. EtOAc extracts were fractionated into a hexane, and an aqueous (8% MeOH (v/v)) fraction [34]. The authors also included in the extracts profiling the characteristic cannabis derived prenylated flavones cannaflavin A (CFL-A) and B (CFL-B).

When only cannabinoid acids are the target of analysis, it is necessary to perform the extraction at room temperature, which ensures no conversion of the actual cannabinoids composition of the plant material. Conversely, in order to make cannabis extract for medicinal use it is important to ensure the presence of active principles represented by neutral cannabinoids. To this end, it is necessary to perform the extraction at high temperature or pass through a preliminary decarboxylation step, which can be carried out in the presence (in water at 100 °C for 2 hours) or absence of a solvent [26,35]. The decarboxylation is a critical step because it does not provide the conversion of cannabinoid acids into equivalent amounts of neutral cannabinoids when it is conducted in an open reactor. The temperature is a key parameter that dramatically affects the conversion process. In order to get the total consumption of cannabinoid acids, it is necessary to heat the sample at a temperature that causes the evaporation and/or decomposition of the neutral cannabinoids [24]. A closed reactor, high temperatures and short time would certainly prevent these side events [24]. A very common hot extraction that can be employed for different purposes like taxonomical species identification, forensic classification and source tracing, consists of the use of a soxhlet apparatus, which however is solvent and time consuming (more than two extraction cycles are generally required) [29,32].

An interesting alternative method for quality control analysis has been proposed by Ameur *et al.*, which consists of a cloud point extraction (CPE) of THC from cannabis resin [36]. This method involves the use of a non-ionic surfactant (Dowfax 20B102) mixed with cannabis resin, a salt (Na_2SO_4) and deionized water. The mixture is shaken and heated at an appropriate temperature (40-90 °C). A separation of two phases, aqueous and surfactant-rich phase, is reached upon heating (45 °C), addition of the salt and centrifugation. In this way, a 60% extraction yield was achieved for THC within one hour (it did not increase over time). The authors suggest that CPE can be a good alternative to other traditional processes and offers many interesting advantages, such as the possibility of extracting and pre-concentrating analytes in a simple single-step procedure, without the use of expensive and potentially toxic organic solvents. Moreover, it does not require the evaporation of the solvent and does not cause any analyte loss, and the extract is compatible with the mobile phase used in reverse phase HPLC.

Supercritical fluid extraction (SFE) is a very efficient way to extract cannabinoids and terpenes from cannabis inflorescence. Supercritical CO_2 is the solvent used to extract the terpene component, while cannabinoids are extracted by means of a co-solvent, usually ethanol (10-20% in CO_2) [5,37]. The

parameters involved in this process are temperature and pressure, which require a fine tune in order to obtain a high extraction efficiency of all compounds. SFE is a useful technique for preserving the stability of thermos-labile and light-sensitive compounds and is scalable up to industrial size [5]. Moreover, Omar *et al.* ensure an extraction yield of cannabinoids up to 90% with EtOH as co-solvent (less than 40% with only CO_2) [37]. SFE is generally employed to separate the aromatic part of cannabis (terpenes), which is cannabinoid free, from the pharmacologically active fraction, which is cannabinoid rich. The latter can be used for cannabis varieties distinction which are to be correlated to the therapeutic effects.

When dealing with medicinal cannabis, common toxic organic solvents are to be avoided. Cannabis tea is indeed a popular preparation to consume medicinal cannabis [38]. However, it is reasonable that the amount of cannabinoids is quite low due their scarce solubility even in hot water [35,39]. Oily preparations are becoming also very popular but there is still the need for a standardized extraction protocol [39,40]. Only recently, a research work has been published by our research group regarding the extraction procedure and the analysis of cannabinoids from medicinal cannabis inflorescence [41]. The extraction procedure proposed involves the heat of the cannabis inflorescence at 110 °C in olive oil and at 78 °C in ethyl alcohol. All the vapours produced are cooled down with a condenser and refluxed into the stirring mix. In this way, it is possible to preserve the terpene component. The results indicated that the procedure is quite efficient due to the reflux and the cannabinoids extraction yield is close to 100% in ethyl alcohol in less than one hour and 70% in olive oil in about two hours [41].

2.1.1 Biological matrices

A recent review regarding the analytical approaches used for the determination of phytocannabinoids and endocannabinoids in human matrices has been published by Battista *et al.* [25]. The most commonly analysed human samples for the detection of cannabinoids are blood (whole blood, serum and plasma), urine and hair. The aim of the analysis in the case of human matrices is generally to prove the precedent or present consumption of illicit drugs. It is important to take into account the time elapsed since the last use, because not all cannabinoids can be detected after a certain time. For example, after 10-12 hours from cannabis consumption THC can no longer be detected [24]. The compounds generally analysed in blood and urine are 11-hydroxy-Δ^9-THC (THC-OH) and 11-nor-9-carboxy-Δ^9-THC (THC-COOH), which are the major metabolites of THC. THC-COOH undergoes glucuronidation and can be found in urine as the most abundant metabolite (in both conjugated, THC-COOH-gluc, and non-conjugated form). CBD and CBN are generally not suitable analytes for cannabis consumption proof in human matrices [25]. The only example of the detection of these two phytocannabinoids was reported by Milman *et al.* in 2012 suggesting that CBD and CBN positive samples indicate unambiguously recent cannabis consumption [42]. The main THC metabolites are reported in Figure 3.

The extraction step of cannabinoids in biological matrices is very critical since it needs to be highly reproducible, highly efficient (in terms of analyte recovery), highly selective and to eliminate as much interference as possible deriving from the matrix.

Detection of phytocannabinoids and their metabolites in human matrices always requires a step of deproteination. It can be achieved by treatment with either formic acid in methanol or ice-cold acetonitrile, or enzymatically [42-53].

The deproteinized samples can then be extracted following two different procedures: liquid-liquid extraction (LLE) or solid phase extraction (SPE). LLE is usually time-consuming and makes the

quantification of cannabinoids very difficult in samples where their level is generally very low, while SPE seems to be a more common technique for biological matrices [3,25]. With SPE the target compounds are absorbed onto a stationary solid phase material, allowing a pre-concentration of the analytes before analysis [51]. Among the advantages of SPE there are high reproducibility, easy automation of the technique and less solvent waste compared to LLE [25,54,55]. The solvents used to elute cannabinoids from SPE are similar to those generally employed in LLE: MeOH, EtOH, hexane and EtOAc, in some case with the addition of acetic acid depending on the nature of the SPE stationary phase. More recently, a μ-SPE clean-up procedure has been proposed by Sergi *et al.*, which involves minimal volumes of organic solvents and only 100 μL of plasma [47]. The difference with the classical SPE consists of a packed sorbent in a pipette tip that does not require vacuum neither for loading or elution. This technique dramatically reduces the time needed for the extraction.
A particular biological matrix that requires a long procedure due to its complexity is hair. The sample preparation involves a wash procedure to eliminate any possible external contamination and a digestion step to liberate the analytes from the matrix [53,56]. Finally, a pre-concentration step generally with SPE has to be included in most cases or, alternatively, a headspace solid phase microextraction (HS-SPME) can be applied in order to extract the analytes of interest [57,58]. The latter consists of dipping a fiber material directly into the digested solution to let the analytes absorb onto it [58].

2.2 Analytical techniques

3.1.1 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) presents some advantages compared to other more sophisticated technologies. In particular, it is largely employed for a preliminary semi-quantitative analysis of the cannabinoid content of plant extracts [26]. The aforementioned draft of the *Cannabis Flos* monograph reports a TLC based method for the qualitative determination of the main cannabinoids in the plant inflorescence (see paragraph 2.1.1) [28]. Hazekamp *et al.* developed and validated a simple rapid high performance TLC (HPTLC) method for the quantification of THC, which was proved to be accurate and reproducible. Moreover, it allowed for the qualitative analysis of other main neutral cannabinoids found in cannabis [26]. The identification of cannabinoids is generally based on the comparison of the retention factor (R_F) value with that of authentic standards, whereas the visual evaluation is obtained by dipping the TLC plate into aqueous fast blue B solution (FBB), which is a selective stain for cannabinoids [26]. In addition, this method can be applied to both polar and non-polar C_{18} silica gel plates, which provide opposite elution orders. Nonetheless, TLC has some limitations in terms of specificity and sensitivity, which result fairly low compared to other analytical platforms and thus the results must be taken with caution.

4.1.1 Gas Chromatography (GC)

Gas chromatography (GC) is one of the most commonly used approaches for the analysis of cannabinoids in both plant materials and biological matrices [33,59,60]. However, this analytical platform does not allow for the direct analysis of the extracted sample because it involves the heating of the sample at high temperature (about 280 °C) prior to the chromatographic separation in order to transform the liquid sample into its gaseous phase. The heating of the sample leads unavoidably to the decarboxylation of the cannabinoids acids to get the corresponding neutral cannabinoids. Therefore, the result is the sum of acid and neutral form. In order to avoid this phenomenon a

preliminary step of derivatization of the cannabinoid acids is necessary and the distinction between acid and neutral form is possible. However, it is important to take into account that a 100% yield for the derivatization of cannabinoid acids by GC is difficult to obtain [61]. Moreover, it has been demonstrated that the thermal conversion of the cannabinoid acids into their neutral derivatives in the GC injection port is only partial yielding an underestimation of the total amount of cannabinoids [62]. In fact, the authors suggest the exact total cannabinoids value should be measured by determining acid and neutral form separately [62]. Nevertheless, GC is the method officially employed by the authorities for the determination of cannabinoids.

GC is generally interfaced to a flame ionization detector (FID) or to a mass spectrometry (MS) detector. The advantage of FID consists of a more accurate quantitative response with respect to MS. In fact, whilst the former bases the quantification of cannabinoids on the use of authentic standards, the latter needs the use of the corresponding deuterated standards, which are expensive and not commercially available for all minor cannabinoids. Anyway, MS allows for a higher specificity compared to FID when similar species co-elute. The sensitivity of GC-FID is also remarkably lower than that of GC-MS. Specifically, the sensitivity of GC-FID is only slightly below 1 μg/mL [2,27,63], whereas it is possible to reach values in the order of or even below 1 ng/mL with GC-MS [33,64,65]. For hair samples, along with LC-ESI-MS, GC-MS with electron impact ionization (GC-EI-MS) is the most employed technique for the analysis of THC and its metabolite THC-COOH. However, the level of the latter are lower than that of its parent drug because of the weak incorporation of the acidic metabolite into the hair matrix. Since it is important to determine this metabolite in cases when discrimination of the external contamination from cannabis consumption is required, the aforementioned technique is not suitable. Given that the cut-off concentration for THC-COOH is 0.2 pg/mg, it is possible to overcome the limitation of GC-MS by using a negative chemical ionization (GC-NCI-MS) with triple quadrupole (QqQ) MS/MS that increases the limit of quantification (LOQ) to 0.05 pg/mg [66,67].

Some authors have suggested that one-dimension GC does not offer enough resolution in the separation of cannabinoids, which actually present very similar chemical structures [5]. Hence, hyphenated techniques, such as two-dimension GC (GC×GC), are much more efficient in the evaluation of the chemical profile of cannabis samples [5,68]. One example was published by Lowe *et al.* in 2007, where a two-dimensional (2D) gas chromatography/electron impact-mass spectrometry (GC/EI-MS) method was developed and validated for simultaneous quantification of THC, THC-OH and THC-COOH in human plasma [69]. The method employs 2D capillary GC (in series) and cryofocusing for enhanced resolution and sensitivity [69]. Limits of quantification (LOQ) reached with this technique were 0.125, 0.250 and 0.125 ng/mL for THC, THC-OH, and THC-COOH, respectively [69].

5.1.1 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography coupled to mass spectrometry (HPLC-MS) has been recently become the method of choice for the qualitative and quantitative determination of cannabinoids in both plant materials and biological fluids [46,70-79]. In contrast to GC, LC based techniques do not encounter decomposition of the sample as they work at room temperature allowing the direct analysis of cannabinoid acids in the extracted sample [61].

The most commonly used columns are based on reverse phase (RP) C_{18} stationary phases, although hydrophilic interaction liquid chromatography (HILIC) stationary phases have also been employed [80]. In our laboratory, we have tested different stationary phases like amino, cyano, and different

C_{18} columns (*unpublished results*). Our results confirmed what reported in the literature on fused core C_{18} columns (e.g. Poroshell C_{18}), which have a high resolution power [27,41,81-83]. This aspect is remarkably important in the case of analytes extracted from cannabis due to the presence of numerous co-eluting cannabinoids. In particular, it is difficult to obtain a baseline resolution for Δ^9-THC and Δ^8-THC, for CBDA and CBGA, and for CBD and CBG [1,27]. Several methods have been recently developed with UPLC columns, which have a sub-2 μm diameter of the particles [27,67,84-95]. The great advantage in the use of such columns lies in fast analyses and high separation efficiency. Ultimately, the stationary phases that have proved to provide an optimal separation of the main cannabinoids in both plant materials and biological fluids are RP C_{18}.

It is noteworthy to point out that phytocannabinoids are optically pure in the plant. Anyway, very few works report the chiral separation of cannabinoids [96]. Hence, it is of utmost important for the scientific community and for the ultimate users of cannabis products to investigate the stereostabilty of cannabinoids not only in the solvents used for the extraction but also in the biological fluids after *in vivo* administration since they are known to undergo a series of metabolic transformations. There are many research works regarding the rapid inversion of configuration of compounds favoured by both solvents [97-104] and biological fluids [105][106]. Indeed, it is known that enantiomers generally possess different pharmacological activities [101,106]. In the particular case of cannabinoids, it has been demonstrated that (+)-CBD, which is the non-natural cannabinoid, has an affinity with CB_1 receptors similar to that of THC. On the other hand, the natural enantiomer, (–)-CBD, has shown no preferential affinity for either CB_1 or CB_2 receptors [107]. An example of chiral column with the ability of offering an optimal separation of the enantiomers of the main cannabinoids is based on the amylose tris(3,5-dimethylphenylcarbamate) stationary phase [96,108].

A considerable improvement in the separation power can be achieved using 2D chromatography [29]. The technique involves the combination of two dimensions of different separation mechanisms in series. The whole eluate (comprehensive 2D chromatography) or selected fractions ("heart-cut" 2D-chromatography) from the first dimension are collected and injected into the second dimension, where they are further separated by an orthogonal separation mechanism [109]. This analytical trick is particularly useful when chromatographic resolution of numerous compounds is desired, especially for cannabinoids, many of which are isomers difficult to separate by only one separation mechanism [29].

The separation of the main cannabinoids by HPLC is not a trivial task, especially with an isocratic elution. In fact, most papers report their separation by gradient elution [1,5,27,31,34,44,47,50,51,60,69-71,73,74,77,78,110-112], and only very few works describe isocratic elution methods maintaining a good resolution for the main cannabinoids [41,63,75,76]. It is interesting to note that the relative elution time of the acidic cannabinoids can be influenced by the pH of the eluent, while the order of elution for the neutral cannabinoids remains the same on RP C_{18} columns [61].

With HPLC different types of detectors can be employed, such as ultraviolet (UV), fluorescence (FLD) and mass spectrometry (MS). UV detection is the most used for the analysis of cannabinoids in plant materials, where the amount of the main cannabinoids is relatively high [1,27,29,34,36,60,63]. Only few scientific works report the use of this detector for the analysis of biological samples as they require more sensitive detectors like MS due to the low level of the analytes of interest [3,113]. UV detection is based on the absorption of the chromophore of the substituted phenolic ring, as this is a common structural element among the tested cannabinoids. The alkyl sidechain does not influence the UV absorbance, as there is no difference between THCA (C5-

sidechain) and THCVA (C3-sidechain). The cyclization of the non-phenolic part of the cannabinoids also has no influence on the absorbance, except when it implies the formation of another aromatic ring (CBN and CBNA) or a conjugated double bond (CBC and CBCA). The draft of the *Cannabis Flos* monograph of the German Pharmacopoeia describes a LC-UV method for the purity test of the main cannabinoids, CBDA, Δ^9-THCA, CBD, Δ^9-THC, Δ^8-THC and CBN [28]. Although UV spectrophotometer is the most widely employed detector, it presents some drawback related to the scarce sensitivity and specificity. This is why it is scarcely used for the qualitative and quantitative determination of cannabinoids in biological fluids. However, a recent study has reported a SPE method for the pre-concentration of the sample to the UV level of sensitivity [3]. The low specificity could be overcome by the use of a photodiode array detector (PDA) since the cannabinoid acids present an absorption spectrum different from that of neutral cannabinoids. Specifically, the wavelength used for neutral cannabinoids is about 220 nm, while cannabinoid acids also show absorption peaks at about 270 and 310 nm [41,61]. Anyway, by setting the UV response at 228 nm it is possible to detect both acid and neutral cannabinoids. The UV detector, however, does not allow to discriminate neutral cannabinoids like CBG and CBD, which result very difficult to separate. In this case, MS provides a higher level of detection since it distinguishes the various cannabinoids depending on the *m/z* of their molecular ion. Given that CBG and CBD have different *m/z*, they could be identified by this method. This is not true for isomers of cannabinoids like Δ^8-THC and Δ^9-THC, which cannot resolved even by MS detection. In fact, the performance of MS depends on the type of mass analyzer used in the analysis. In the case of isomers, a high-resolution fragmentation spectrum could help in the identification on the account of the fragments generated [5]. In this respect, time-of flight analyzers (ToF) are often used in order to obtain the structural information of the target compounds. Coupled to a quadrupole mass filter, Q-ToF mass spectrometers provide accurate mass identification (<5 ppm accepted accuracy threshold for confirmation of elemental composition) for both the precursor and the product ions. This allows differentiating between two different compounds with the same nominal mass but with different elemental composition [5]. One of our ongoing studies has highlighted the presence of five cannabinoids with the same *m/z* 315.2294 (Δppm<5), identical to that of THC and CBD (*unpublished results*) in Bediol® oil and EtOH extracts. Among these cannabinoids, some co-elute, thus making their analysis difficult. The only way to be distinguished is by their high-resolution MS/MS spectrum. Unfortunately, the studies on the high-resolution MS/MS fragmentation of both major and minor cannabinoids are quite poor in the literature [5].

Nowadays, the most widely used analyzers for quantitative determination of cannabinoids and their respective metabolites in biological fluids are QqQ instruments, which have excellent sensitivity and selectivity as they work on multiple reaction monitoring (MRM) transitions. However, since they provide only nominal mass measurements and not a structural identification of non-target compounds, they require the use of deuterated analytical standards (-d_3, -d_6 and -d_9). The use of deuterated standard is essential in order to obtain accurate data, as the major drawback of MS is the matrix effect [114]. Only unreliable values are obtained without these analytical standards, especially for cannabinoid acids, which are generally ionized at about 300 °C in the electrospray ionization (ESI) interface and thus are decomposed to the neutral forms.

MS has proved to be very useful also in the evaluation of the chemical stability of the analytical standards used for the quantitative analysis. In fact, one of our recent works pointed out that the analytical standard purchased in methanol solution (1 mg/mL) of CBD undergo a sort of decomposition with the formation of a new peak of *m/z* 347.0222 in positive ionization mode [41]. The peak was not observed in the standard solution of CBD stored in ethanol [41].

Another MS detector used for the analysis of cannabinoids is the ion trap, which has the advantage of adding other levels of fragmentation (MS^3, MS^4,…, MS^n) of the fragments generated from the fragmentation of the parent molecular ion, thus providing important additional information on the chemical structure of unknown compounds [31].
Very few studies have reported the use of HPLC coupled to FLD since fluorescence spectra of cannabinoids are strongly affected by the pH of the mobile phase [61]. In fact, cannabinoid acids lose completely their fluorescence properties in acidic conditions, CBC does not show any fluorescence signal in a basic environment, and CBN has no fluorescence at all [61].

6.1.1 Other techniques

An alternative method to the conventional HPLC and GC analysis for the determination of cannabinoids is nuclear magnetic resonance (NMR) spectroscopy [34,111,115,116]. In fact, quantitative NMR has been considered as a highly accurate and reproducible technique with relatively short analysis time. In contrast to LC and GC, the major advantage of such technique is the lack of sensitivity toward impurities present in the plant material such as chlorophyll and lipids [35,111,115]. However, this technique is not commonly employed due to the high instrumental costs and to the necessity of highly specialized personnel.
Very few applications to qualitative and quantitative analysis of cannabinoids have been developed and reported with Fourier Transform Infrared (FT-IR) spectroscopy. To the best of our knowledge, the scientific research in this field has been limited to the paper published by Dorado *et al.* in 2001, which describes the analysis of the changes in C/N-modified lignocellulosic substrates from *Cannabis sativa* L. during microbial transformation of hemp [117].
Another technique employed in the determination of cannabinoids is immunoassay (IA). This technique generally provides scarce selectivity due to the difficulty in finding antibodies that are specific for each cannabinoid. It is rather common to have an antibody that recognizes a class of compounds with similar chemical structure. Therefore, an IA is suitable for a preliminary assessment of drug abuse, but a positive IA should always be confirmed with other more sensitive and specific techniques such as either GC-MS or LC-MS [118]. On the other hand, several IA based methods have been recently developed because it offers a rapid screening of synthetic cannabinoids in biological fluids [118-120].

7.1.1 New frontiers in the analysis of cannabis extracts

Cannabis sativa L. is an important medicinal plant of great pharmacological interest. Indeed, it is currently prescribed in form of either oil, tea or tincture for a series of pathologies [7-10]. Nonetheless, the scientific community is still very far from a thorough understanding of its comprehensive chemical composition. So far, about 90 cannabinoids and 500 compounds belonging to different chemical classes have been identified [22]. In order to extend the knowledge on this powerful plant, metabolomics has been used as a new analytical tool for the identification of unknown compounds in both plant materials and biological matrices [121]. In the past few years dramatic developments in high-throughput metabolomics have been achieved, especially due to the aid of bioinformatics technologies [121].
Metabolomics studies can be carried out by using several analytical platforms, such as high-resolution ^{1}H NMR, GC-MS, and LC-MS. In particular, ^{1}H NMR has been widely employed for classification of cannabis cultivars and for structure elucidation exploiting *J*-resolved, ^{1}H-^{1}H COSY, and ^{1}H-^{13}C HMBC spectroscopy [34,115,122-125]. GC-MS is also employed for the discrimination of cannabis

cultivars [2,6,126]. However, a superior level of accuracy and precision for metabolite identification with very low ppm error (<5) is undoubtedly provided by HPLC-MS/MS with detectors like Q-ToF or Orbitrap [127-129]. The great advantage of these detectors is the capability of generating a molecular formula from the molecular ion isotopic pattern. The acquisition of the high-resolution fragmentation spectra and the match with the corresponding authentic standard allows for an unambiguous identification of the compounds under investigation. In spite of this outstanding progress, the chemical composition of cannabis medicinal extracts is dramatically variable due to different temperatures, time and solvents used in the extraction process as reflected in the HPLC-MS chromatogram reported in Figure 4 (Citti *et al.*, *unpublished results*) [130]. In this regard, metabolomics can be considered as a very powerful tool in the scientists' hands for the evaluation of the most significant metabolite changes that affect the pharmacological activity of the extracts. This approach could make a significant breakthrough in the comprehensive chemical characterization of cannabis medicinal extracts and could eventually pave the way towards a standardized extraction procedure.

3. Conclusions

In summary, the methods of choice for the determination of cannabinoids in both plant materials and biological matrices are chromatography-based techniques. Between gas and liquid chromatography, the latter should be preferred as it allows for the determination of the actual cannabinoid composition (acid and neutral species) without the necessity of a derivatization step. If GC is employed without a preliminary derivatization reaction, it unavoidably transforms the cannabinoid acids into their corresponding neutral forms, thus providing a total value of the two species. Furthermore, recent works have suggested that the decarboxylation of the cannabinoid acids is only partial and the results is an underestimation of the actual value. Much attention is to be paid on both the purity of the analytical standards and their storage conditions, as they are easily degradable by light and heat. Their authenticity needs to be assessed each time in order to obtain accurate and reproducible results among different analytical laboratories. Moreover, when a UV spectrophotometer is used as detector, particular attention is to be paid on having a reasonable resolution of cannabinoids that could co-elute. When a co-elution occurs, a mass spectrometer is more suitable as it provides both molecular ions and fragmentation spectra of the cannabinoids under investigation. On the other hand, with a mass spectrometer the use of appropriate deuterated standards is mandatory in order to compensate for the matrix effect especially with an ESI source. Often, these standards are either not commercially available or very expensive. If not all these requisites are satisfied, the analysis is not to be considered as accurate and reliable and would only generate discordant results among different analytical laboratories.

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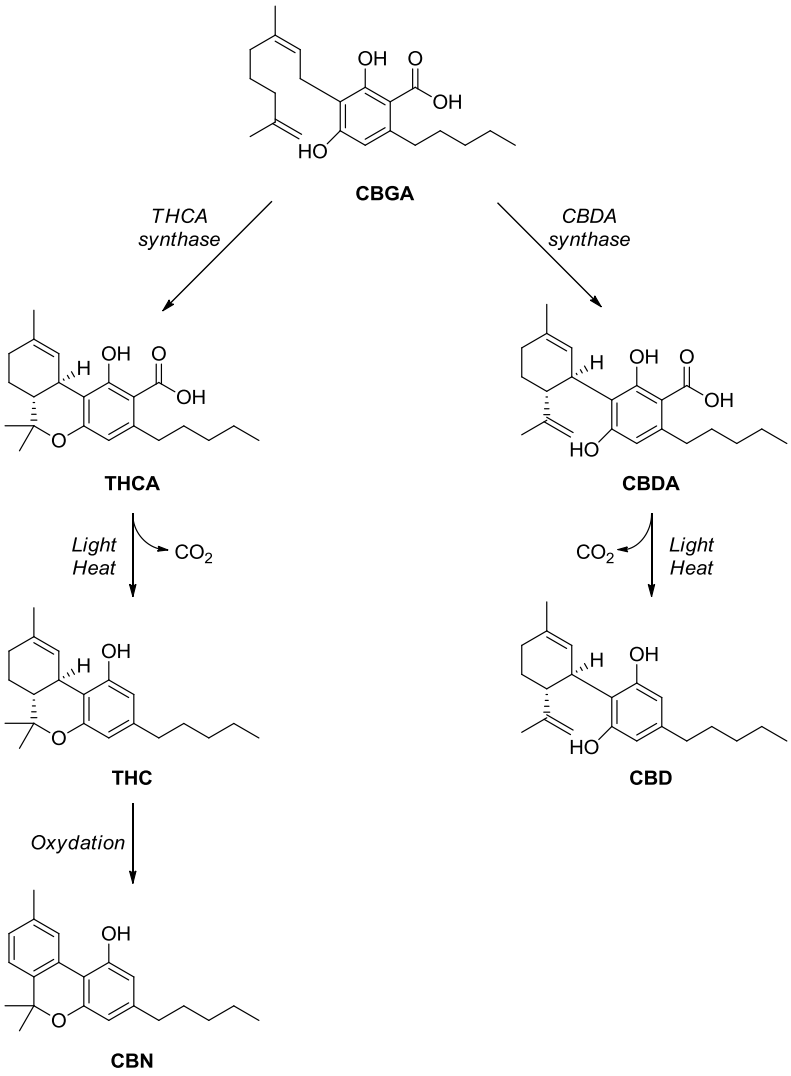

Figure 1. Schematic representation of the biosynthetic route of THCA and CBDA from CBGA, formation of THC and CBD by light and /or heat and oxidation of THC to CBN.

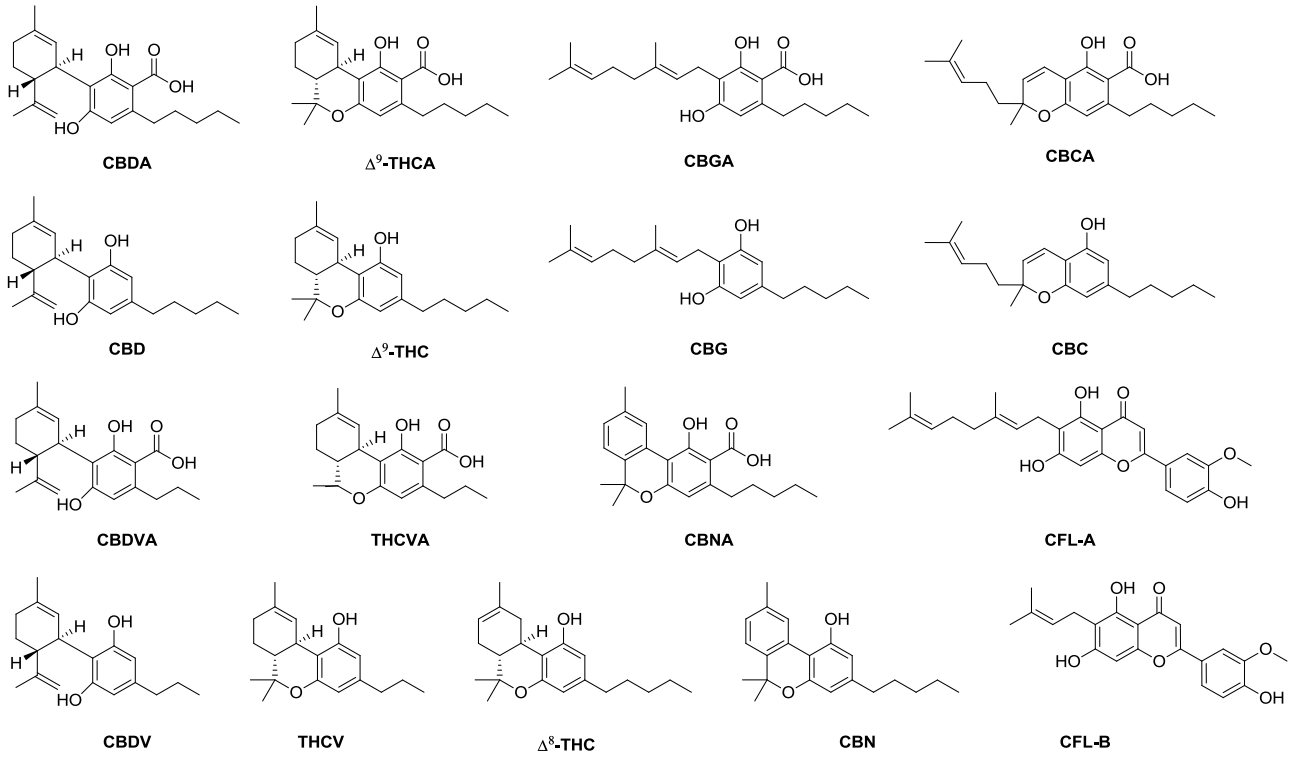

Figure 2. Molecular structure of the most common acid and neutral cannabinoids and flavonoids (cannaflavin A and cannaflavin B).

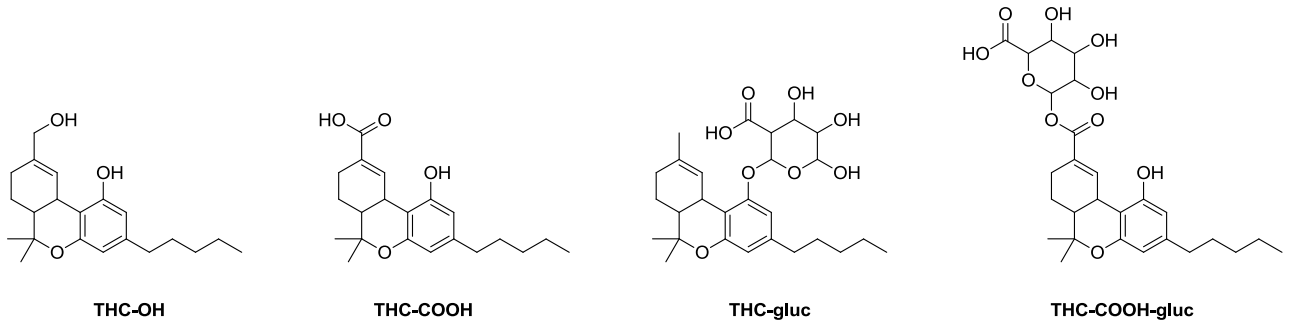

Figure 3. Molecular structure of the most common Δ^9-THC metabolites.

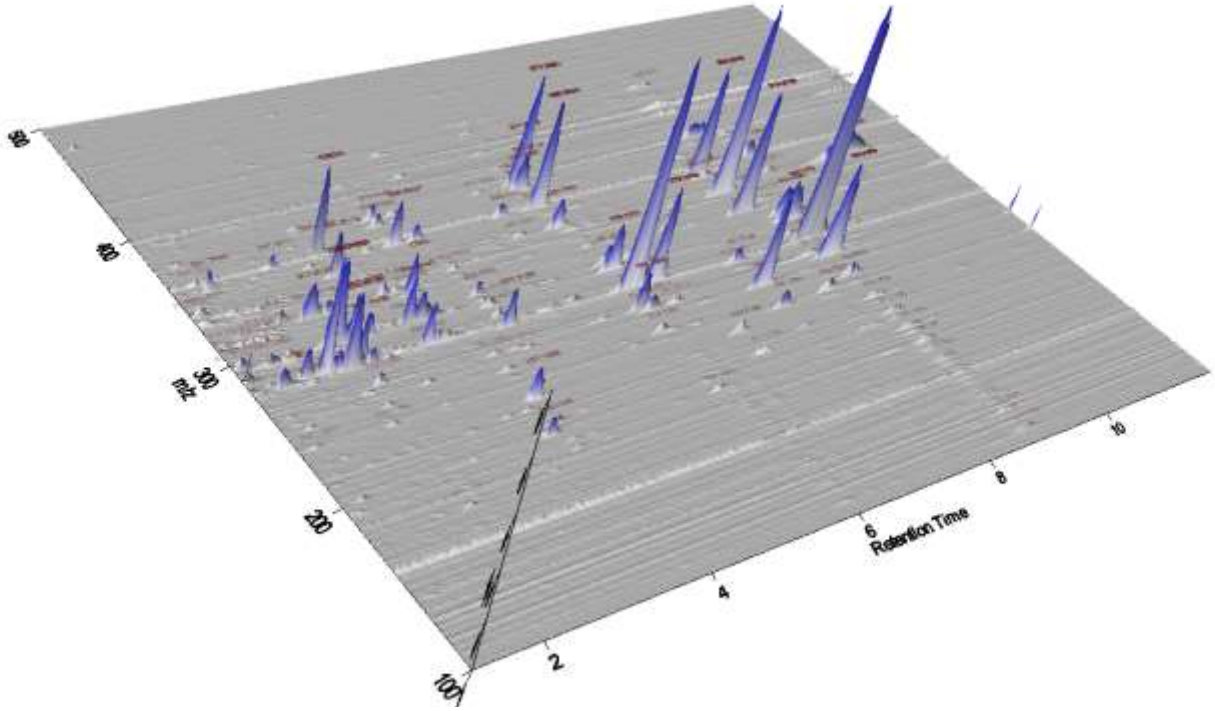

Figure 4. 3D Total Ion Chromatogram (TIC) of a Cannabis sativa L. oil extract in positive ionization (ESI+) mode. The retention time (min) is represented on the x axis, the peak intensity (ion counts) on the y axis and the m/z values on the z axis (Citti et al., unpublished results) [130].

Table 1. Analytical methods for the analysis of cannabis plant material

Analytical technique	*Matrix*	*Identified analytes*	*Extraction methodology*	*Sensitivity*	*Reference*
HPLC-DAD	Seized cannabis and fiber-type plants	Δ^9-THCA, CBDA, CBGA, Δ^9-THC, CBD, CBG, CBN (quantitative), Δ^8-THC (qualitative)	MeOH/$CHCl_3$ 9:1 (v/v)	LOQ 0.125 (THCA, CBGA, CBDA, THC, CBN), 0.188 (CBD), 0.375 µg/mL (CBG)	[1]
GC-FID GC-MS	Medicinal cannabis buds (Bedrocan ®, Bedropuur® and Bediol®)	Terpenes, Δ^9-THC, Δ^8-THC, CBD, CBG, CBC, THCV, CBDV, CBGM	EtOH	0.4-0.5 (terpenes), 0.6 mg/g (cannabinoids)	[2]
HPTLC	Medicinal cannabis cultivars (Bedrocan® and Bediol®)	Δ^9-THC, CBN (quantitative) CBD, Δ^8-THC, THCV, CBG, CBC (qualitative)	Decarboxylation (H_2O, 100 °C, 2 h) and extraction with EtOH	LOD 10 ng, LOQ 50 ng	[26]
HPLC-DAD GC-FID	Flowers	CBDVA, CBDV, THCV, CBGA, THCVA (tentative ID), CBDA, CBG, CBD, CBN, Δ^9-THC, Δ^8-THC, CBC, THCA, terpenes	EtOH	LOQ 5-8 µg/mL	[27]
2D-LC-UV-CL HPLC-ESI-ToF	Industrial-grade hemp	CBDV, CBCV, CBV, CBLV, CBGV, CBN, CBC, CBD, CBL, CBG, CBE, CBT, CBNA, CBCA, CBDA, CBLA, CBGA (tentative ID)	EtOAc 78 °C, 1.5 h with Soxhlet	-	[29]
LC-APCI-IT	Plant	THC, THCA, CBD, CBDA, CBN	MeOH/$CHCl_3$ 9:1 (v/v)	0.1 (CBD), 0.04 (CBDA), 0.03 (CBN), 0.28 (THC), 9.9 g/kg (THCA)	[31]
GC-FID	Seized plants	THC, THCA, CBN	Soxhlet or PLE with MeOH or hex	-	[32]

GC-MS	Inflorescence (from seized seeds)	CBG, CBN, CBD, CBC, THC, terpenes	SLE with hex and ultrasonication	-	[6]
GC-MS	Hemp products (pastilles, seeds, scented grass, beer, liqueur, oil)	THC, CBD, CBN	Hex/*i*PrOH 9:1 (v/v) and derivatization with MSTFA and TMCS	1 (THC, CBN), 2 ng/mL CBD	[33]
^{1}H NMR HPLC-DAD	THC-type, CBD-type, CBG-type, fiber (CBD)-type plants	THC, CBD, CBN, THCA, CFL-A, CFL-B, phenols, flavonoids	Fractionated extraction with EtOAc, EtOH, heptane, MeOH	LOQ 0.5 mg/g (of extract), 10 mg/mL for peak separation	[34]
^{1}H NMR	Bedrocan®, illicit material, CBD-rich and non-cannabinoid type plants	THC, THCA (semi-quantitative)	Hot and cold H_2O extracts and tinctures (EtOH/H_2O)	-	[35]
HPLC-DAD	Resin	THC	Cloud point extraction with Dowfax 20B102, deionized H_2O and Na_2SO_4	LOD 0.04 µg/mL	[36]
HPLC-QTOF HPLC-QqQ	Plants	CBD, THCA, THCV, CBN, THC, CBG (quantitative) Cannabicoumaric acid, CBCA, 10-EtO-9-OH-Δ6a-THC, 4-AcetoxyCBC, CBGA, CBGAM, THCA-C4 (tentative ID)	SFE (CO_2/10%EtOH)	LOD 0.2 (CBD, THCA) 0.05 (THCV, CBN, THC), 0.02 ng/mL (CBG)	[5]
GC-MS	Plants	Terpenes, THC, CBN, CBD	FUSE with *i*PrOH:Chex 1:1 (v/v) or SFE CO_2 (for terpenes) then CO_2/20%EtOH (for cannabinoids)	LOQ 1 µg/mL	[37]
UHPLC-QqQ	Medicinal cannabis inflorescence	THCA, CBDA, THC, CBD, CBC, CBG, CBN	Tea and oil extracts	LOD 0.3, LOQ 1 µg/mL	[39]

HPLC-DAD HPLC-Q-ToF	Medicinal cannabis inflorescence (Bediol®)	THCA, CBDA, THC, CBD, CBN	Oil and ethyl alcohol extracts	LOD 0.05, LOQ 0.1 μg/mL	[41]
GC-MS	Plant	THC, CBD, CBN	Hexane for 10 days then sonication	-	[59]
UPLC-DAD-ESI-MS GC-EI-MS	Herbal products	Synthetic cannabinoids	MeOH and ultrasonication 10 min	LOQ 10 μg/mL	[60]
HPLC-DAD GC-FID	Dried fresh hemp plant, dried hemp flowers and hashish	THCA, THC	EtOAc and sonication 15 min	-	[62]
HRGC-FID HPLC-UV	Hashish	THC, CBD, CBN	MeOH	HRGC-FID: 0.034 (THC), 0.041 (CBD), 0.026 mg/mL (CBN) HPLC-UV: 0.044 (THC), 0.014 (CBD), 0.018 mg/mL (CBN)	[63]
HPLC-QqQ Cryogenic ^{1}H NMR	Laser-microdissected trichomes of medicinal Cannabis (Bediol®)	THCA, CBDA, THC, CBD, CBG, CBN, CBC	MeOH, sonication 10 min and incubation o.n. at r.t. (for LC-MS), $CDCl_3$ (for NMR)	LC-MS: LOQ 3 (CBD, CBG, CBN), 30 ng/mL (CBC)	[111]
^{1}H NMR GC-MS	Medicinal cannabis inflorescence	THCA, CBDA, THC, CBD, CBN	MeOH/$CHCl_3$ 9:1 (v/v) and ultrasonication 2 min 4 °C	NMR: LOQ 0.2 (THCA, CBDA, CBD), 0.1 mg/mL (THC, CBN)	[115]
^{1}H NMR	Medicinal cannabis inflorescence	THCA, CBDA, THC, CBD, CBN, CBG, CFL-A, CFL-B	Fractionated extraction with MeOH/$CHCl_3$ 1:1 (v/v), 90%MeOH, hex, then stepwise gradient of EtOH in acetone	-	[116]

HPLC-UV ^{1}H NMR	Medicinal cannabis inflorescence (Bediol®, Bedrobinol®)	Δ^{9}-THCA, CBDA, CBNA, CBGA, Δ^{9}-THC, Δ^{8}-THC, CBD, CBG, CBN, CBC	MeOH and ultrasonication 10 min	-	[123]
^{1}H NMR	Medicinal cannabis inflorescence	THCA, THC, CBD	50%MeOH:$CHCl_3$ 1:1 (v/v)	-	[124]

Table 2. Analytical methods for the analysis of cannabis in biological samples

Analytical technique	*Matrix*	*Identified analytes*	*Extraction methodology*	*Sensitivity*	*Reference*
2D-GC-EI-MS (THC, THC-OH, CBD, CBN) 2D-GC-NCI-MS (THC-COOH)	OF	THC, THC-OH, THC-COOH, CBD, CBN	Deproteination (ice-cold ACN), SPE: hex/acetone/EtOAc 60:30:20, v/v/v (THC, THC-OH, CBD, CBN), hex/EtOAc/AcOH 75:25:2.5, v/v/v (THC-COOH) and derivatization: BSTFA (THC, THC-OH, CBD, CBN) or HFIP and TFAA (THC-COOH)	LOQ 0.25 (THC, THC-OH, CBD), 1 µg/L (CBN), 5 ng/l (THC-COOH)	[42]
LC-ESI-QqQ	OF	THC, CBD, CBN	DDS buffer, 0.1 M Sørensen's phosphate buffer pH 6, hex/EtOAc 9:1 (v/v) 60 min	LOQ 1 (THC), 2 ng/mL (CBD, CBN)	[43]
LC-APCI-Q-Trap	OF	THC, THC-OH, CBD, CBG, THC-COOH, THCV	Hydrolysis and SPE with CH_2Cl_2:*i*PrOH:NH_4OH 78:20:2 (v/v/v)	LOQ 0.2 µg/L (THC, THC-OH, CBD, CBG, THCV), 15 ng/L (THC-COOH)	[44]
2D-GC-EI-MS (THC, THC-OH, CBD, CBN) 2D-GC-NCI-MS (THC-COOH)	OF^{m}	THC, THC-OH, CBD, CBN, THC-COOH	SPE with hex/acetone/EtOAc 60:30:20 (v/v/v) for THC, THC-OH, CBD and CBN, SPE with hex/EtOAc/AcOH 75:25:2.5 (v/v/v) for THC-COOH, then derivatization with BSTFA for THC, THC-OH, CBD and CBN and with HFIP and TFAA for THC-COOH	LOQ 0.5 (THC, THC-OH, CBD) and 1 ng/mL (CBN), 7.5 pg/mL (THC-COOH)	[45]

μ-flow-LC-Orbitrap	OF^m	THC, THC-COOH, CBD, CBN	Deproteination (ice-cold ACN) and SPE with hexane/EtOAc/AcOH 75:25:1 (v/v/v)	LOQ 0.5 (THC, CBD, CBN) and 0.015 ng/mL (THC-COOH)	[46]
LC-QqQ	OF^m	THC, THC-OH, THC-COOH, CBD, CBN	Deproteination with 50 mM FA in MeOH and elution on MEPS with 50 mM NH_4OH in MeOH	LOQ 0.02 (THC-COOH), 0.25 (THC), 0.30 (CBD, CBN) and 0.40 ng/mL (THC-OH)	[47]
HPLC-ESI-μQqQ	Blood, plasma and serum	THC, THC-OH, THC-COOH, CBD, CBN	Deproteination with ACN-d_3, then on-line or off-line derivatization with dabsyl chloride solution and 0.1 M NaOH	LOQ 0.25 (THC, THC-COOH), 0.30 (THC-OH), 0.40 (CBN) and 0.80 ng/mL (CBD)	[49]
LC-ESI-QqQ	Whole blood	THC, CBN, THC-gluc, THC-COOH-gluc, THC-COOH, THC-OH, CBD	Deproteination (MeOH, ACN) and SPE with 1% AcOH in ACN (v/v)	LOQ 1 (THC, THC-OH, THC-COOH, CBD, CBN), 0.5 (THC-gluc) and 5 μg/mL (THC-COOH-gluc)	[50]
On-line SPE LC-QqQ	Peripheral blood	THC, THC-OH, THC-COOH	Deproteination with ACN and on-line SPE with ACN/H_2O/FA 60:40:0.1 (v/v/v)	0.50 (THC, THC-OH) and 2.5 ng/mL (THC-COOH)	[51]
LC-QqQ	Whole blood, urine, gall bladder fluid, cerebrospinal fluid, gastric contents, cerebrum, liver, lungs, muscle and kidneys	THC, THC-OH, THC-COOH, THC-COOH-gluc, CBD, CBN	Deproteination with ACN and addition of 0.25 M AcOH and EtOAc:hex 1:9 (v/v)	LOQ 0.58 (THC), 0.47 (THC-OH), 5.06 (THC-COOH), 41.1 (THC-COOH-gluc), 0.60 (CBN), 0.44 ng/mL (CBD)	[52]
HPLC-UV	Plasma and urine	THC, THC-COOH	Basic hydrolysis for urine, then SPE with MeOH	LOQ 16 (THC) and 6.4 ng/mL (THC-COOH)	[3]

				LOD 6 (THC) and 2.5 ng/mL (THC-COOH)	
LC-QqQ	Living and post-mortem whole blood	THC, CBD, CBN, THC-OH, THC-COOH	Protein precipitation with ACN and on-line SPE	LOD 0.6 (THC), 1.1 (THC-OH), 0.9 (THC-COOH, CBD) and 2.5 ng/mL (CBN) LOQ 1.8 (THC), 3.2 (THC-OH), 2.8 (THC-COOH, CBD) and 7.7 ng/mL (CBN)	[54]
Fast LC-MS/MS	Whole blood	THC, THC-COOH	Deproteination with ACN and SPE with EtOAc/hex 50:50 (v/v) and 2% acetic acid	LOQ 0.25 mg/mL	[55]
LC-ESI-QqQ	Hair	THC, THC-COOH	Washing with MeOH, digestion with NaOH 2.5 M 60 °C and extraction with EtOAc	LOD 1 pg/mg LOQ 3 pg/mg	[56]
GC-IT	Hair	THC, CBD, CBN	Washing with petroleum ether, H_2O, DCM, digestion in 1 M NaOH and HS-SPME	LOD 7 (CBD), 11 (CBN) and 31 pg/mg (THC) LOQ 0.012 (CBD), 0.030 (CBN) and 0.062 pg/mg (THC)	[57]
GC-MS	Hair	THC, CBN, CBD	Washing with H_2O and acetone, digestion with NaOH 1 M 80 °C, HS-SPME with iso-octane and derivatization with BSTFA	LOD 0.012 (THC), 0.013 (CBD) and 0.016 ng/mg (CBN) LOQ 0.037 (THC), 0.038 (CBD) and 0.048 ng/mg (CBN)	[58]
GC-MS	Plasma	THC, THC-OH, THC-COOH, CBD, CBN	SPE and derivatization with BSTFA	LOQ 0.15-0.29 (THC, 11-OH-THC, THC-COOH, CBD) and 1.1 ng/mL (CBN)	[64]
GC-MS	Meconium	THC-OH, THC-COOH	SPE and hydrolysis	LOD 5 ng/g LOQ 10 ng/g	[65]
GC-NCI-QqQ	Hair	THC-COOH	Washing with *i*PrOH, hydrolysis with 1 M NaOH (95 °C, 30 min), acidification,	LOD 0.02 pg/mg LOQ 0.05 pg/mg	[66]

			extraction with hex/EtOAc 9:1 (v/v) and derivatization with PFPOH and PFPA		
Fast GC/NIC-QqQ	Whole blood	THC, THC-OH, THC-COOH	Hex/EtOAc 9:1 (v/v), then derivatization with TFAA and HFIP	LOQ 0.5 (THC, THC-OH) and 2.5 ng/mL (THC-COOH)	[67]
2D-GC-EI-QqQ	Post-mortem blood	THC, CBD, CBN, THC-OH, THC-COOH	1 M phosphate buffer pH 4 and LLE with hex/EtOAc 5:1 (v/v) and derivatization with MSTFA	LOQ 0.25 (THC, CBN, THC-OH) and 0.5 ng/mL (CBD, THC-COOH)	[68]
2D-GC: GC-FID and GC-cryotrap-MS	Plasma	THC, THC-OH, THC-COOH	Enzymatic hydrolysis, deproteination with ice-cold ACN, then SPE with EtOH and derivatization with BSTFA	LOQ 0.125 (THC, THC-COOH) and 0.25 ng/mL (THC-OH)	[69]
LC-QqQ	Breast milk	THC, THC-OH, THC-COOH	Dilution with 100 mM ammonium acetate pH 5.5 and SPE with MeOH	LOD 1 (THC-COOH) and 1.5 ng/mL (THC, THC-OH) LOQ 5 ng/mL	[70]
LC-QqQ (THCA) GC-MS (THC, THC-OH, THC-COOH)	Urine and serum	THC, THCA, THC-OH, THC-COOH	SPE with ACN	LOD 2.5 ng/mL LOQ 5 ng/mL in urine and 7.5 ng/mL in serum	[71]
LC-QqQ	Plasma	THC, THC-OH, THC-COOH	SPE with MeOH and 0.1 M AcOH	LOD 0.2 (THC, THC-OH) and 1.6 ng/mL (THC-COOH) LOQ 0.8 (THC, 11-OH-THC) and4.3 ng/mL (THC-COOH)	[72]
LC-APCI-IT	EDTA-plasma and urine	THC, THC-OH, THC-COOH, CBD, CBN	EDTA-plasma: anion exchange sorbent SPE with ACN:ammonia 98:2 (v/v) Urine: enzymatic hydrolysis and LLE with Et_2O:EtOAc 50% (v/v)	LOD 0.1 (EDTA-plasma) and 0.5-1 ng/mL (urine) LOQ 0.2 (EDTA-plasma) and 1-3 ng/mL (urine)	[73]

LC-ESI-QqQ	Bile	THC, THC-OH, THC-COOH, CBN, CBD, THCA, THC-COOH-gluc, THC-gluc	Dilution with 10 mM ammonium formate buffer pH 6.5 and SPE with MeOH	LOD 0.05-0.5 ng/mL LOQ 0.3-0.8 ng/mL	[74]
LC-ESI-MS	OF, urine and whole blood	THC, THC-OH, THC-COOH	OF and urine: SPE with hex/EtOAc 80:20 (v/v) Blood: deproteination with ACN then SPE	LOD 2 in OF (THC), 0.5 in urine and blood (THC, THC-COOH) and 20 ng/mL in blood and urine (THC-OH) LOQ 5 in OF (THC), 2 in urine and blood (THC, THC-COOH) and 25 ng/mL in blood and urine (THC-OH)	[75]
LC-ESI-QqQ	Whole blood	THC, THC-OH, THC-COOH	Deproteination with MeOH and extraction with hex:EtOAc 90:10 (v/v)	LOD and LOQ 0.5 (THC), 1 (THC-OH) and 2 μg/L (THC-COOH)	[76]
LC-ESI-QqQ	Hair	THCA, CBD, CBN, THC	Washing with H_2O, acetone and petroleum ether and extraction with MeOH	LOD and LLOQ 2.5 (THCA) and 20 pg/mg (THC, CBD, CBN)	[77]
LC-QqQ	Whole blood	THC, THC-OH, THC-COOH	Deproteination with MeOH and SPE with hex/EtOAc/AcOH 88:10:2 (v/v/v)	LOD and LOQ 1 (THC, THC-OH) and 5 ng/mL (THC-COOH)	[79]
UPLC-ESI-QqQ	Urine	THCA (and other drugs of abuse)	Enzymatic hydrolysis and extraction with MeOH/H_2O 60:40 (v/v)	LOD 1 ng/mL LOQ 2 ng/mL	[84]
UPLC-ESI-ToF	Whole blood	THC	Deproteination with MeOH and SPE with 25%NH_4OH/ACN/EtOAc 2:10:88 (v/v)	Not specified	[85]
UPLC-QqQ	Whole blood and urine	Synthetic cannabinoids and their metabolites	SPE with EtOAc	LOD 0.08-0.13 ng/mL LOQ 0.11-0.17 ng/mL	[87]
UPLC-ESI-QqQ	Post-mortem whole blood	Synthetic cannabinoids	Na_2CO_3 buffer pH 10.2 and Et_2O	LOD 0.01 ng/mL LOQ 0.05 ng/mL	[89]

UPLC-ESI-QqQ (OF) GC-MS (serum)	OF and serum	THC, CBD, CBN (OF) THC, THC-OH, THC-COOH (serum)	OF: DrugWipe5S® Serum: SPE with methanol and derivatization with MSTFA	OF: LOD 0.25 ng/mL; LOQ 5 ng/mL (THC, CBD, CBN) Serum: LOD 0.6 (THC), 0.3 (THC-OH) and 0.3 ng/mL (THC-COOH); LOQ 1 (THC), 0.3 (THC-OH) and 3 ng/mL (THC- COOH)	[94]
UPLC-ESI-QqQ	Whole blood	THC, THC-OH, THC-COOH	Deproteination with ACN and SPE with hex:EtOAc 80:20 (v/v)	LOD 0.02 (THC), 0.05 (THC-OH) and 0.1 ng/mL (THC-COOH) LOQ 0.05 (THC), 0.1 (THC-OH) and 0.2 ng/mL (THC-COOH)	[95]
UPLC-ESI-QqQ	Whole blood	THC, THC-OH, THC-COOH	Deproteination with MeOH and SPE with hex/EtOAc/AcOH 88:10:2 (v/v/v)	LOD and LOQ 0.5 µg/L (THC-OH, THC-COOH) LOD 0.2 and LOQ 0.5 µg/L (THC)	[110]