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CRITICAL REVIEW ON RECENT TRENDS IN CANNABINOID DETERMINATION ON CANNABIS HERBAL SAMPLES: FROM CHROMATOGRAPHIC TO VIBRATIONAL SPECTROSCOPIC TECHNIQUES

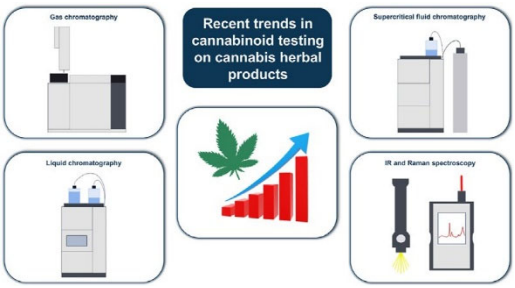
Riccardo Deidda [a, *], Amandine Dispas [a, b], Charlotte De Bleye [a], Philippe Hubert [a], Eric Ziemons [a]

[a] *University of Liege (ULiege), CIRM, Laboratory of Pharmaceutical Analytical Chemistry, B36 Tower 4 Avenue Hippocrate 15, 4000, Liege, Belgium*
[b] *University of Liege (ULiege), CIRM, Laboratory of Medicine Analysis, B36 Tower 4 Avenue Hippocrate 15, 4000, Liege, Belgium*
** Corresponding author. E-mail address: riccardo.deidda@uliege.be (R. Deidda).*

highlights

- Analytical options for cannabinoid analysis in cannabis herbal samples are analyzed.
- Recent trends about this topic are shown and discussed.
- Focus on innovative techniques such as SFC and vibrational spectroscopy.
- Pros & Cons for each technique are deeply discussed.

Graphical abstract



Abstract

Cannabis has been at the center of scientific attention for some years now. Since its pharmacological potential has been highlighted, cannabis has become a hot topic in research laboratories, leading to the publication of many scientific studies. Focusing on analytical chemistry, an enormous number of analytical methods for cannabinoid (CNB) determination have been published, involving various techniques. However, no globally accepted reference method for CNB determination has yet been chosen. This review aims to identify very recent analytical methods developed to analyze phytocannabinoids in cannabis herbal samples. For certain techniques, stagnation in terms of employed operational conditions can be observed. In this context, a reference method of analysis should be proposed and accepted worldwide to standardize CNB determination. In contrast, for other techniques, we are witnessing a scientific ferment, which is resulting in the development of new interesting analytical options. In this regard, particular focus has been given to these niche techniques, which are now emerging in the analytical panorama of cannabis analysis, offering new important perspectives for the future of cannabis testing. Supercritical fluid chromatography

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and infrared spectroscopy showed tangible advantages when applied to CNB determination in herbal samples.

Keywords

Cannabinoids, Cannabinoid potency, Cannabis analysis, Chromatographic techniques, Vibrational spectroscopy

Contents

1. Introduction

1.1 Cannabinoids

The term cannabinoids (CNBs) refers to a class of chemicals that are naturally synthesized, among others, by Cannabis sativa L [1]. More than 120 CNBs have been isolated, in this plant, to date [2-6]. Glandular trichomes, densely expressed on female inflorescences, are the plant structures where CNB synthesis occurs [3,5,7,8]. The latter starts with the reaction between a resorcinol and an isoprenoid group and, involving different enzymes, ends with the formation of various acidic CNBs [8]. The main compounds were cannabigerolic acid (CBGA), cannabidiolic acid (CBDA) and Δ^9-tetrahydrocannabinolic acid (THCA-A). These are carboxylic acid forms, where the loss of the carboxylic group induces the formation of the corresponding neutral forms cannabigerol (CBG), cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC), following the previous example [9,10]. This phenomenon occurs after harvest and under heating or light exposure during sample storage [5,11]. Table 1 shows the chemical structures of the main CNBs found in cannabis plants, together with their molecular weight, CAS number and abbreviation. From a chemical point of view, CNBs are isoprenylated resorcinyl polyketides that can vary on both the isoprenyl residue and the resorcinyl alkyl

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side chain (R_2), giving rise to a high number of different combinations [9]. Moreover, most CNBs are chiral and naturally synthesized in a single enantiomeric form, the *(–)-(R,R)*-form [12].

Among CNBs, THC is broadly known for its psychotropic properties, which have made the cultivation and use of this plant strictly regulated by laws all around the world [13]. In addition to naturally occurring CNBs (also called phytoCNBs), synthetic CNBs have also appeared. Synthetic CNBs are not considered by this review, as they present very different chemical properties from the properties of naturally occurring CNBs and are not to be confused with them [5,14,15].

1.2 Analytical interest

Over the past two decades, there has been a growing interest in cannabis by the scientific world. In fact, in addition to the psychotropic properties exhibited by THC and sought-after for recreational purposes, some evidence about its pharmacological properties has emerged [10,16-18]. This evidence concerns mainly THC; however, CBD and CBG are still largely investigated for various clinical applications [16,18,19]. As a consequence, a strong interest in developing analytical techniques to properly characterize cannabis samples has also grown in parallel [20-22]. Despite the high number of analytical methods developed for this aim, researchers are still looking for better solutions from both methodological and technological points of view.

Two general areas are involved in cannabis sample testing: quality control and forensic laboratories. The former aim to ensure the quality of cannabis samples intended for medical use [3]. The latter are focused mainly on the quantification of total THC to assess whether samples coming from different possible contexts comply with the law [13,23]. In fact, according to the last European drug report of the European Monitoring Centre for Drugs Addiction, cannabis remains the most commonly used illicit drug in Europe [24].

Obviously, medicinal cannabis analysis is broader than forensic analysis since it involves different types of testing needed to comply with the good agricultural and manufacturing practices that strictly regulate these products [25-27]. In addition to CNB potency, other tests are performed on medicinal cannabis: microbiological, pesticide, heavy metal and aflatoxin contamination as well as moisture content determination are mandatory tests [9,25-27]. This review focused only on CNB determination of then herbal material.

1.3 Critical elements in quantitative cannabinoid determination on cannabis herbal samples

Cannabis herbal samples represent a challenging analytical sample asking for many precautions to be taken during the whole analytical process of testing, from the sampling to the final instrumental analysis. Some recent works pointed out the need for worldwide accepted reference methods to harmonize cannabis testing and reduce interlaboratory variations [28-30]. To name one, Jikomes et al. individuated systematic differences in the CNB content reported by different state-certified laboratories in Washington state [29]. In this context, Citti et al. recently outlined the most common pitfalls encountered when analyzing CNBs in cannabis samples [28].

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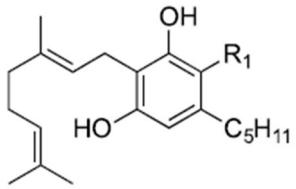
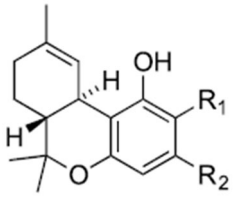
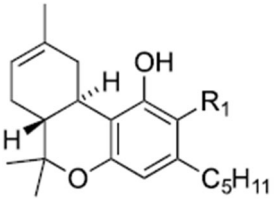
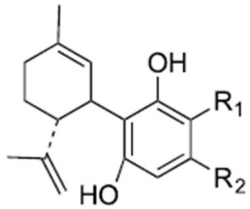
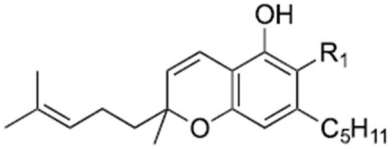
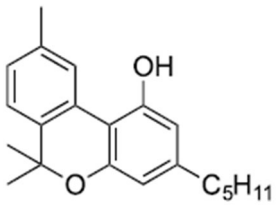
Table 1

Name, chemical structure, molecular weight, CAS number and abbreviation of the main phyto-cannabinoids isolated in cannabis samples.

Name	R_1	R_2	MW (g mol^{-1})	CAS	Abbreviation
Cannabigerolic acid	COOH	NA	360.49	25555-57-1	CBGA
Cannabigerol	H	NA	316.48	25654-31-3	CBG
					
Δ^9-Tetrahydrocannabinolic acid	COOH	C_5H_{11}	358.47	23978-85-0	THCA-A
Δ^9-Tetrahydrocannabinol	H	C_5H_{11}	314.46	1972-08-3	THC
Tetrahydrocannabivarinic acid	COOH	C_3H_7	330.42	39986-26-0	THCVA
Tetrahydrocannabivarin	H	C_3H_7	286.41	24274-48-4	THCV
					
Δ^8-Tetrahydrocannabinolic acid	COOH	C_5H_{11}	358.47	23978-89-4	Δ^8-THCA-A
Δ^8-Tetrahydrocannabinol	H	C_5H_{11}	314.46	5957-75-5	Δ^8-THC
					
Cannabidiolic acid	COOH	C_5H_{11}	358.47	1244-58-2	CBDA
Cannabidiol	H	C_5H_{11}	314.46	13956-29-1	CBD
Cannabidivarinic acid	COOH	C_3H_7	330.42	31932-13-5	CBDVA
Cannabivarin	H	C_3H_7	286.41	24274-48-4	CBDV
					
Cannabichromenic acid	COOH	C_5H_{11}	358.47	185505-15-1	CBCA
Cannabichromene	H	C_5H_{11}	314.46	20675-51-8	CBC
					
Cannabinol	NA	NA	310.43	521-35-7	CBN
					

MW, molecular weight; CAS, chemical abstract service registry number.

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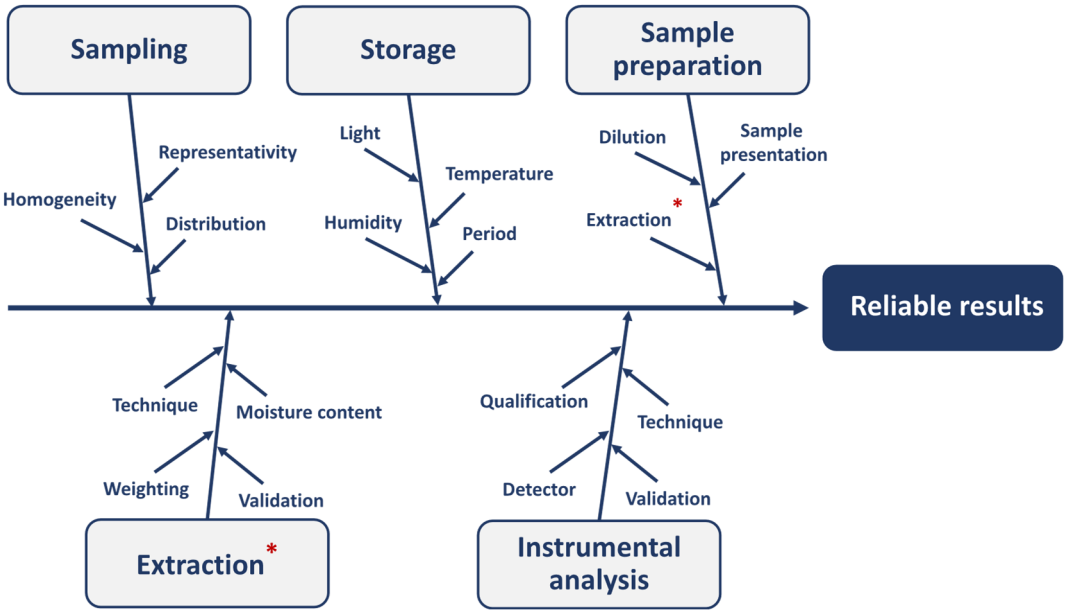

Fig. 1. Fish-bone diagram representing the main potential sources of risk when analyzing cannabis herbal samples. The symbol (*) on "Extraction" indicates that it is an element of "Sample preparation" that has been zoomed in to detail its components.

In summary, when approaching cannabis herbal samples and in particular from a quantitative point of view, one must pay attention to many critical aspects that can easily lead to a lack of accuracy and significant intra- and interlaboratory imprecision. These aspects are summarized in the form of a fish-bone diagram in Fig. 1, where sampling, storage, sample preparation, extraction and instrumental analysis are presented as the main potential sources of risk that can impact the reliability of analytical results. First, the sampling process is a critical step for properly representing the sample under study. In the case of cannabis inflorescence seizures, the totality of the sample (including stalks and leaves when present) should be considered, since quantitative results are commonly expressed in weight-on-weight percentage. The same considerations apply for medicinal products, where a defined CNB concentration is indicated on the label. Homogenization by grinding, for instance, is an essential step before weighing samples prior to extraction. Moisture content should be considered as well. Plant material can contain significant amounts of water, which vary from sample to sample [31]. To measure this parameter, methods based on loss on drying should be avoided, since water is not the only compound that evaporates (other volatile molecules, such as terpenes, are present as well). Near-infrared spectrophotometers calibrated with data coming from methanol-free Karl Fisher titrations could be a relevant and time-saving solution. Sample stability and continuous chemical mutation must be considered during both distribution and storage phases. In fact, taking THCA-A as an example, it can easily decarboxylate into THC, which in return can oxidize into CBN [5]. Consequently, storage, protected from heat and light sources, is essential to avoid (or at least to slow) analyte degradation. This aspect is also crucial for the reference samples commonly used to evaluate extraction yields when developing and validating new extraction procedures. The extraction method may be the most critical step of this analysis since the extraction is intimately correlated to the accuracy of quantitative results. Moreover, cannabis herbal samples represent a complex matrix, since more than five hundred chemical compounds have been identified to date (including terpenes, polyphenolics, alkaloids, waxes, and triglycerides) [4,5]. The extraction yield for each analyte to quantify should be evaluated to verify the reliability of the procedure. However, reference samples are difficult to obtain and manage, and internal standards cannot be representative of the extraction process. One can refer to Refs. [32,33] for an

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exhaustive discussion about the procedures that have been applied to this aim. Unfortunately, even for this aspect of the analysis, there is no harmonization in the approaches.

Obviously, to generate accurate and reproducible results during quantitative CNB determination in cannabis herbal samples, all the abovementioned aspects, directly or indirectly connected with the analysis, should be scrupulously considered by the analytical chemist.

2. Review of the analytical techniques and methods for cannabinoid determination

In this section, analytical techniques and methods recently developed for CNB determination in herbal material are reviewed. Practical details about each reviewed method are provided in the form of tables to facilitate the retrieval of useful information. Both qualitative and quantitative aspects were evaluated and discussed for each technique. Due to the high number of scientific papers about this topic, only recent literature has been considered (from 2015 to 2020). Some application notes provided by equipment manufacturers are included as well.

Fig. 2 shows the evolution of the number of scientific papers published over the past 15 years about the analytical techniques discussed hereafter. An "advanced document search" was conducted using the Scopus® database to search and filter documents by year. Papers were found by searching for the combination of the words "cannabinoid", "cannabis", "analysis" and the analytical technique of interest within titles, keywords and abstracts and limiting the search to the "chemistry", "pharmacology, toxicology and pharmaceutics" and "agricultural and biological sciences" areas. Looking at Fig. 2, the progressively greater application of liquid chromatography (LC) compared to gas chromatography (GC) over the years is evident. In addition, the number of papers on CNB determination has increased over time. The blue and orange colors on the histogram mark the entry of infrared (IR) spectroscopy, Raman spectroscopy and supercritical fluid chromatography (SFC) as new analytical tools in the context of CNB determination. This search aims to provide a snapshot of the recent trends but does not represent an exhaustive analysis. Indeed, it is focused only on academic papers and does not take into account application notes.

As a matter of completeness, other analytical techniques, such as thin-layer chromatography (TLC) and nuclear magnetic resonance (NMR), have also been applied to this scope [10,34-38]. However, these techniques are not discussed hereafter since their implementation is limited by their well-known technical and/or performance limitations in terms of cost and maintenance (for NMR) and lack of accuracy and sensitivity (for TLC) [20,21,28].

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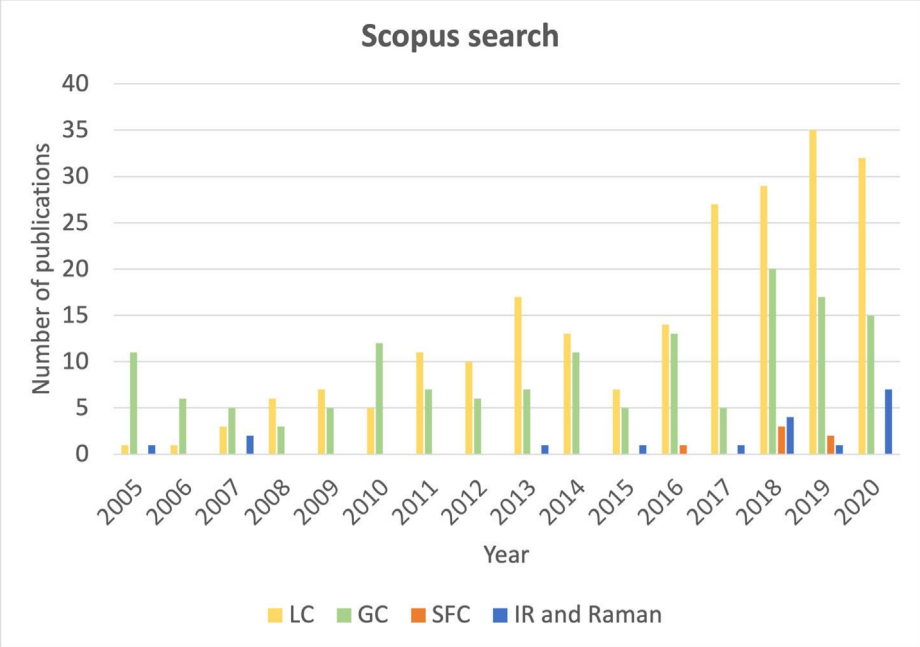

Fig. 2. Histogram representing the results obtained from the following Scopus advanced search (on February 5, 2021): TITLE-ABS-KEY ("cannabinoid" AND "cannabis" AND "analysis" AND "LC" OR "liquid chromatography" OR "SFC" OR "supercritical fluid chromatography" OR "IR" OR "Infrared spectroscopy" OR "GC" OR "gas chromatography" OR "Raman" OR "SERS" OR "Surface enhanced Raman scattering") AND (LIMIT-TO (SUBJAREA, "CHEM") OR LIMIT-TO (SUBJAREA, "PHAR") OR LIMIT-TO (SUBJAREA, "AGRI")).

2.1 Gas chromatography

Gas chromatography represents the gold standard technique for the determination of volatile compounds in forensic laboratories due to its rapidity and simplicity of use. High-efficiency separations can be obtained with short analysis times thanks to the use of capillary analytical columns, which nullify the multiple path term in the Van Deemter equation. However, it is not always well suited for CNB determination. In fact, the main drawback of GC is that it does not allow direct determination of the acidic CNB forms due to the high temperatures (typically approximately 280 C) reached by the injection port of the GC systems. In fact, acidic CNBs are thermolabile compounds, and at these temperatures, they turn into their corresponding neutral forms by decarboxylation [20,21]. Moreover, the decarboxylation rate is not fixed, depending on the geometry of the liner and temperature of the injector port [21]. Aspect that can lead to staggered results. For this reason, a derivatization step should be added to an already time-consuming sample preparation process to ensure an accurate estimation of both neutral and acidic CNBs [39]. Derivatization yield should be evaluated as well. If the determination of the acidic CNBs is not necessary for analytical purposes (i.e., when evaluating the total THC content for forensic purposes), as an alternative to derivatization, a heating step of the sample can be implemented prior to extraction and GC analysis [13]. In this way, the decarboxylation of the acidic CNB forms is performed during sample preparation, and GC analysis can be focused only on the neutral CNB forms. Obviously, the decarboxylation rate has to be evaluated to select the optimal operational conditions and assure a maximal decarboxylation rate is obtained before analysis.

Concerning detectors, flame ionization detection (FID) [40-44] and mass spectrometry (MS) [42,43,45-49] are the most employed. FID, contrary to MS detection, allows obtaining more accurate quantitative results without using expensive deuterated compounds as internal standards. MS detection is necessary when a more sensitive response or structural information is desired (i.e., for bioanalytical purposes or for minor

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CNB profiling). When performing quantitative analysis, the use of internal standards, preferentially deuterated standards, is mandatory to minimize signal variability and to correct eventual matrix effects on the ionization of analytes (ion suppression or enhancement). Unfortunately, these standards are commercially available for the main CNBs but not for all [28].

Table 2 shows some practical and operational details in terms of instrumentation, sample matrix, analytes, stationary and mobile phases, chromatographic conditions (temperature of injector, oven, and source), analysis time and derivatization agents described in the analytical methods reviewed for this work [40-49].

Among these operational details, Ibrahim et al. developed an interesting GC-FID method for the determination of both acidic and neutral CNBs in a total run time of 17.5 min. Qualitative and quantitative performances were evaluated for ten CNBs (although the method allows efficiently separating three extra CNBs), providing the highest selectivity in terms of the number of analytes considered among the methods reviewed [44]. Concerning method precision, as an estimate of the quantitative performance, RSD values were below 15% for repeatability and 17% for intermediate precision. Concerning accuracy testing, the relative bias ranged from 2.30 to 1.52. These values are in accordance with the values reported by other papers.

Table 2

GC methods for the analysis of naturally occurring cannabinoids found in recent literature (2015-2020).

Instrumentation	Matrix	Analytes	Column	Carrier gas	Conditions	Analysis time	Derivatization	Reference
GC-FID	Cannabis oil	CBD, THC, CBN	Rtx-1, (10 m l., 0.1 mm i.d., 0.1 μm f.t.)	NS	T_i, 280 °C; T_{FID}, 300 °C; T_o, 180-300 °C	4 min	NA	40
GC-FID	Hemp inflorescences	CGB, CBD	Rxi-5 MS, (30 m l., 0.25 mm i.d., 0.25 μm f.t.)	Helium	T_i, 290 °C; T_{FID}, 330 °C; T_o, 80-310 °C	27.5 min	NA	41
GC-FID	Cannabis inflorescences	THC, THCA-A, CBD, CBN, CBDA	DB-5MS UI, (30ml., 0.25 mmi.d., 0.25 μm f.t.)	Helium	T_i, 280 °C; T_{FID}, 300 °C; T_o, 200-300 °C	12 min	BSTFA and TMCS (99:1 v:v)	42
GC-MS	Cannabis inflorescences	THC, THCA-A, CBD, CBN, CBDA	Rxi-5 MS, (30 m l., 0.25 mm i.d., 0.25 μm f.t.)	Helium	T_i, 280 °C; T_s, 230 °C; T_o, 70-300 °C	21 min	BSTFA and TMCS (99:1 v:v)	42
GC-FID	inflorescences	CBD, CBN, THC, Δ^8-THC	HP-5 (5% phenyl methyl siloxane), (30 m l., 0.32 mm i.d., 0.25 μm f.t.)	Nitrogen	T_i, 250 °C; T_{FID}, NS; T_o, 60-240 °C	10 min	NA	43
GC-MS	inflorescences	CBDA, CBD, CBN, THC, Δ^8-THC, THCA-A	HP-5MS UI (5% phenyl methyl siloxane), (30 m l., 0.25 mm i.d., 0.25 μm f.t.)	Helium	T_i, 260 °C; T_s, 230 °C; T_o, 180-310 °C	10 min	BSTFA and TMCS (99:1 v:v)	43
GC-FID	Cannabis plant material	THCV, CBD, CBC, Δ^8-THC, THC, CBG, CBN, CBDA, THCA-A, CBGA, CBDV, CBL, CBDVA	DB-1MS, (15 m l., 0.25 mm i.d., 0.25 μm f.t.)	Helium	T_i, 275 °C; T_{FID}, 300 °C; T_o, 190-300 °C	17.5 min	BSTFA and TMCS (98:2 v:v)	44
GC-MS	Cannabis inflorescences	THC, CBN, CBD	HP-5MS UI (5% phenyl methyl siloxane), (30 m l., 0.25 mm i.d., 0.25 μm f.t.)	Helium	T_i, 280 °C; T_s, 230 °C; T_o, 50-300 °C	15 min	NA	45
GC-HRMS	Cannabis inflorescences	Untargeted	DB-5MS UI, (30ml., 0.25mmi.d., 0.25 μm f.t.)	Helium	T_i, 250 °C; T_s, 305 °C; T_o, 50-310 °C	120.8 min	BSTFA and TMCS (98:2 v:v)	46
GC-MS	Cannabis plant material	CBD, CBDA, THC, THCA-A, CBN, Δ^8-THC, CBG, CBGA, CBDV, THCV, CBC	Restek Rxi-35Sil MS (35% silphenylene), (30 m l., 0.25 mm i.d., 0.25 μm f.t.)	Helium	T_i, 250 °C; T_s, NS; T_o, 60-300 °C	39.9 min	BSTFA	47
GC-MS	Hemp inflorescences	THCV, CBD, CBC, Δ^8-THC, THC, CBG, CBN, CBDA, THCA-A, CBGA	Restek RTX5 (35% silphenylene), (10 m l., 0.1 mm i.d., 0.1 μm f.t.)	Helium	T_i, 300 °C; T_s, 200 °C; T_o, 180-300 °C	7.5 min	MSTFA and TMCS	48
GC-MS	Cannabis inflorescences	CBD, CBC, THC, CBG, CBN, 11-OH-THC, THCA-A	Agilent HP-5MS, (30 m l., 0.25 mm i.d., 0.25 μm f.t.)	Helium	T_i, 300 °C; T_s, 210 °C; T_o, 100-300 °C	13/20 min	HDMS and TFA in pyridine medium	49

BSTFA, N,O-bis (trimethylsilyl) trifluoroacetamide; DMAP, 4-dimethylaminopyridine; HDMS, hexamethyldisilazane; MSTFA, N-Methyl-N-(trimethylsilyl)trifluoroacetamide; NA, not applicable; T_{FID}, detector temperature for flame ionization detection; T_i, injector temperature; T_o, oven temperature; T_s, source temperature for MS detection; TFA, trifluoroacetic acid; TMCS, trimethylchlorosilane.

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2.2 Liquid chromatography: from high-performance liquid chromatography to ultrahigh-performance liquid chromatography

Liquid chromatography represents a valid alternative to GC for CNB determination, as clearly demonstrated by the trend of LC publications shown in Fig. 2. In fact, reversed-phase LC (RP-LC) is perfectly suited to CNB lipophilic properties. Moreover, LC operational conditions do not involve heating and are compatible with the thermolability of acidic CNBs. Therefore, acidic CNBs can be detected and differentiated from the corresponding neutral forms in their original form. Several works describing both HPLC and UHPLC methods can be found in the literature [22,50]. Table 3 summarizes the operational conditions of some methods published in the last five years, including some application notes from instrument manufacturers [2,6,36,43,45,46,51-73]. Instrumentation, sample matrix, analytes considered, stationary and mobile phases, analysis time and detection technique are reported.

From HPLC to UHPLC, essential technological advances have been made, allowing chromatographic systems to deal with higher pressures and to generate relatively ultrafast separations [74]. The reduction of the total system dead volume allowed gaining efficiency by reducing band broadening. Moreover, the introduction of columns packed with sub2-mm fully porous particles has increased the efficiency, throughput, resolution, and sensitivity of conventional HPLC methods.

Another relevant advance, in terms of column technology, has been achieved with superficially porous particles (also named core shell or fused-core). This type of column chemistry combines the benefits of both fully porous particles and nonporous particles [74]. They can be used with both HPLC and UHPLC systems with advantages in terms of efficiency and analysis time and have been largely applied to CNB determination [2,58,59,64,69,72].

Regarding detectors, UV detection is a straightforward approach because of the presence of chromophores in CNB chemical structures and the linearity of its response [2,6,36,45,53-61,63-65,68-72]. UV detection is, depending on the application, well suited to CNB determination, but only when each analyte can be separated from others prior to detection. Under these conditions, accurate results can be guaranteed with a relatively easy and inexpensive detector. However, this type of detector lacks specificity and is not able to discriminate between similar compounds that may coelute. Structural variants such as nonaromatic cyclization of the isoprenyl residue (CBDA vs. THCA-A for instance) or variations in the length of the resorcinyl alkyl moiety (THCA-A vs. THCVA for instance) are not differentiated by UV spectra [9]. When method selectivity cannot be obtained through the chromatographic part of the analysis or a higher sensitivity is needed, MS detectors can be employed [2,6,43,46,51,52,60,62,63,65-67,73]. In fact, detection based on the mass-to-charge ratio (m/z) by selected ion monitoring could, in some cases, allow discrimination between coeluting analytes. However, with this detection system, one must pay attention to the presence of isomeric compounds (THC vs. Δ^8-tetrahydrocannabinol, THC vs. CBD, for instance) among the analytes (see Table 1) [75]. Only high-resolution mass analyzers such as quadrupole-time-of flight and Orbitrap can possibly discriminate constitutional isomers thanks to different parent and fragment ions that originate from different elemental arrangements in chemical structures [2,46,62,73].

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Table 3

LC methods for the analysis of naturally occurring cannabinoids found in recent literature (2015-2020).

Instrumentation	Matrix	Analytes	Column	Mobile phase composition	Analysis time	Detection	Reference
HPLC-UV	Hemp inflorescences	CBDV, CBDA, CBGA, CBG, CBD, THCV, CBN, THC, Δ^8-THC, CBC, THCA	NexLeaf CBX for Potency (150 × 4.6 mm, 2.7 μm), Shimadzu	ACN, water, and phosphoric acid (gradient)	10 min	UV (λ, 220 nm)	54
HPLC-UV	Cannabis material including flower, leaves, stems and hashish	THCA-A, THC, CBDA, CBD, CBN	Kinetex C8 (100 × 2.1 mm, 2.6 μm), Phenomenex	ACN, water, and formic acid (gradient)	13 min	UV (λ, 210 nm)	57
HPLC-UV	Cannabis plant material including leaves and flowers	THC, CBN, CBD, THCA-A	Nucleodur C18 Gravity (250 × 4.6 mm, 5 μm), Macherey-Nagel	ACN, water and phosphoric acid (isocratic)	5 min	UV (λ, 211 nm and 220 nm for THCA-A)	45
HPLC-UV	Cannabis olive oil	THC, CBD	Poroshell 120 SB-C18 (150 × 2.1 mm, 2.7 μm), Agilent	ACN, water and dipotassium hydrogen phosphate (isocratic)	6 min	UV (λ, 222 nm)	58
HPLC-UV	Plant material	CBDV, CBDA, CBGA, CBG, CBD, THCV, CBN, THC, THCA-A, CBC	Poroshell 120 (150 × 3 mm, 2.7 μm), Agilent	ACN, water, formic acid, and ammonium formate (gradient)	25 min	UV (λ, 227 nm)	59
HPLC-UV	Cannabis inflorescences	THC, CBD, CBN, Δ^8-THC, THCA-A, CBG, CBGA, CBDA	Poroshell 120 SB-C18 (75 × 3 mm, 2.7 μm), Agilent	MeOH, water and ammonium acetate (gradient)	10 min	UV (λ, 235 nm)	64
HPLC-UV	Biomass and extracts	CBDA, CBGA, CBG, CBD, THCV, CBN, THC, Δ^8-THC, CBL, CBC, THCA-A	Luna C18 (150 × 4.6 mm, 3 μm), Phenomenex	ACN, water, and formic acid (gradient)	22.2 min	UV (λ, 220 nm)	68
HPLC-UV	Plant material	THC, CBD, CBG, CBN, THCA-A, CBDA, CBGA	Zorbax RX-C18 (250 × 4.6 mm, 5 μm), Agilent	ACN, water, and TFA (gradient)	55 min	UV (λ, 214 nm)	36
HPLC-UV	Cannabis inflorescences	THCA-A, CBDA, THC, CBD, CBG, CBC, Δ^8-THC, CBN	Poroshell 120 EC-C18 (150 × 2.1 mm, 2.7 μm), Agilent	ACN, water, and formic acid (gradient)	17 min	UV (λ, 214 nm)	69
HPLC-UV	Cannabis inflorescences	THC, THCA-A, CBD, CBDA, CBN, Δ^8-THC, CBG, CBGA, CBC, CBCA, THCV, THCVA, CBDV, CBDVA, CBL, CBLA	Cortecs Shield RP18 (150 × 4.6 mm, 2.7 μm), Waters	ACN, water and TFA (isocratic)	26 min	UV (λ, 228 nm)	70
HPLC-UV	Cannabis inflorescences	CBDV, THCV, THC, CBD, CBG, THCA-A, CBDA, CBN, CBGA, CBC, Δ^8-THC	Poroshell 120 EC-C18 (50 × 3 mm, 2.7 μm), Agilent	MeOH, water, and formic acid (gradient)	11 min	UV (λ, 230 nm)	72
HPLC-UV/MS/HRMS	Cannabis inflorescences	THC, CBD, CBN, THCA-A, CBDA	Poroshell 120 SB-C18 (100 × 2.1 mm, 2.7 μm), Agilent	ACN, water, and formic acid (gradient)	10 min	UV (λ, 228 nm) and ESI-QTOF (positive mode)	2
HPLC-UV/MS/MS^2	Hemp inflorescences, hemp based pharmaceutical products (oil and balm)	CBDA, CBGA, CBG, CBD	Ascentis Express C18 (150 × 3 mm, 2.7 μm), Supelco	ACN, water, and formic acid (gradient)	45 min	UV (λ, 210 nm for neutral and 220 for acidic CNBs) and ESI-Iontrap (positive and negative mode)	65
HPLC- MS/HRMS	Hemp inflorescences	Untargeted	Mediterranean C18 (250 × 4.6 mm, 3 μm), Teknokroma	Water, ACN, and formic acid (gradient)	52 min	ESI-QTOF (positive and negative modes)	46
HPLC-UV/MS/MS^2	Hemp inflorescences	CBGA, CBDA, CBG, CBD	Ascentis Express C18 (150 × 3.0 mm, 2.7 mm), Supelco	ACN, water, and HCOOH (gradient)	37 min	UV (various λ), ESI-IonTrap (positive and negative modes)	60
HPLC-HRMS	Cannabis inflorescences	CBD, THC, CBN, CBG, CBDA, THCA-A, CBGA	Synergi Hydro RP (150 × 2 mm, 4 μm), Phenomenex	ACN, water, and formic acid (gradient)	20 min	HESI-Orbitrap (positive and negative modes)	73
UHPLC-UV	Hemp plant material and resin	CBDV, CBDA, CBGA, CBG, CBD, THCV, CBN, THC, Δ^8-THC, THCA-A, CBC	Luna C18 (2) (150 ×3mm, 3 μm), Phenomenex	ACN, water, HCOOH, and ammonium formate (isocratic)	8.5 min	UV (λ, 275 nm)	53
UHPLC-UV	Plant material	THC, THCA-A, CBDA, CBGA, CBD, CBG, CBN	Acquity UPLC HSS C18 SB (100 × 2.1 mm, 1.8 μm), Waters	MeOH, water and formic acid (gradient)	16 min	UV (λ, 211 nm for neutral and 220 nm for acidic)	55
UHPLC-UV	Cannabis inflorescences	CBDA, CBN, CBC, THCA-A, CBD, THC	Luna Omega C18 (150 × 2.1 mm, 1.6 μm), Phenomenex	ACN, water, and formic acid (gradient)	20 min	UV (λ, 280 nm)	56

(continued on next page)

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Table 3 *(continued)*

Instrumentation	Matrix	Analytes	Column	Mobile phase composition	Analysis time	Detection	Reference
UHPLC-UV	Plant material	CBC, CBD, CBDA, CBDV, CBG, CBGA, CBN, THC, THCA-A, THCV, Δ^8-THC	Acquity UPLC BEH Shield C18 (100 × 2.1 mm, 1.7 μm), Waters	ACN, water and ammonium acetate (gradient)	16 min	UV (λ, 220 nm)	61
UHPLC-UV	Cannabis inflorescences	THC, THCA-A, CBD, CBDA, CBN, Δ^8-THC, CBG, CBGA, CBC, CBCA, THCV, THCVA, CBDV, CBDVA, CBL, CBLA	Cortecs UPLC Shield RP18 (100 × 2.1 mm, 1.6 μm), Waters	ACN, water and TFA (isocratic)	11 min	UV (λ, 228 nm)	71
UHPLC-UV/MS/MS	Cannabis extracts	CBDV, THCV, CBDA, CBD, CBGA, CBG, CBN, THC, Δ^8-THC, THCA, CBC	Kinetex C18 core-shell (50 × 2.1 mm, 1.3 μm), Phenomenex	ACN, water, and formic acid (isocratic)	6.5 min	UV (λ, 280 nm) and ESI-Q-trap (positive mode for neutral and negative mode for acidic CNBs)	63
UHPLC-MS	Cannabis decoction and oil	THC, THCA-A, CBD, CBDA	Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7 μm), Waters	MeOH, water, and ammonium formate (gradient)	10 min	ESI-QqQ (positive mode)	51
UHPLC-MS/MS	Hemp seeds, hemp seed oil and hemp protein	CBD, CBN, THC, CBC, CBG, CBDV, THCV, Δ^8-THC, CBDA, THCA-A, CBCA, CBDVA, CBGA, THCVA	Acquity UPLC BEH Shield RP18 (100 × 2.1 mm, 1.7 μm), Waters	ACN, water, and formic acid (gradient)	13 min	ESI-QqQ-IonTrap (positive and negative modes)	52
UHPLC-MS/MS	Cannabis inflorescences	CBDA, CBD, CBN, THC, Δ^8-THC, THCA-A	Luna Omega Polar C18 (100 × 2.1 mm, 1.6 μm), Phenomenex	ACN, water, and formic acid (gradient)	17 min	ESI-QqQ (positive mode for neutral CNBs and negative for acidic CNBs)	43
UHPLC-MS/MS	Inflorescences, leaves and hashish	CBDA, CBGA, CBG, CBD, THCV, CBN, THC, Δ^8-THC, CBL, CBC, THCA-A	Cortecs UPLC C18 (100 × 2.1 mm, 1.6 μm), Waters	ACN, water, and formic acid (gradient)	17.5 min	UV (λ, 220 nm), ESI-SQ (positive mode)	6
UHPLC-MS/HRMS	Cannabis inflorescences	THCA-A, THC, Δ^8-THC, CBN, CBC, CBD, CBG, CBDA, THCV, CBGA, CBDV, CBDVA, CBL	Kinetex C18 core-shell (150 × 2.1 mm, 2.6 μm), Phenomenex	ACN, MeOH, water, and acetic acid (gradient)	20 min	ESI-Orbitrap (negative mode)	62
UHPLC-MS	Cannabis olive oil	CBD, CBN, CBDA, THC, THCA	Acquity UPLC HSS T3 (30 × 2 .1 mm, 1.8 μm), Waters	ACN, water, isopropanol, and formic acid (gradient)	3.5 min	ESI-QqQ (positive mode)	66
UHPLC-MS/MS	Cannabis inflorescences	CBDA, CBG, CBD, CBN, THC, CBC, THCA-A	Acquity UPLC HSS C18 (150 × 2.1 mm, 1.8 μm), Waters	ACN, water, and formic acid (gradient)	10 min	ESI-QqQ (positive mode)	67

Unfortunately, this is not always the case, since sometimes, CNBs share the same fragmentation pattern [76]. For quantitative applications of MS detection, eventual matrix effects associated with the use of liquid phase and electrospray ionization sources must also be considered. In this context, isotopically labelled internal standards are needed to mitigate matrix effects. In conclusion, the choice of the best suited detector relies on the analytical target. In fact, pharmaceutical samples are not supposed to vary in terms of qualitative composition, and a UV detector could be enough to perform the analysis (if selectivity has been demonstrated during the validation step). The same cannot be said for forensic samples, where considerable qualitative variations can be observed over time and depending on their provenance. These variations can potentially result in unexpected peaks that could compromise method selectivity and accuracy during routine analyses.

Many UHPLC methods have been developed and published for the quantitative determination of CNBs [6,43,51-53,55,56,61-63,66,67,71]. Among others, Fekete et al. recently developed a UHPLC-UV method allowing the quantitation of eleven CNBs in a single run of 10 min [61]. This paper is interesting from a methodological point of view since the authors applied a "design of experiments" strategy for screening, optimization, and robustness testing of the operational conditions. Wang et al. validated a UHPLC-UV-MS method selective for 11 CNBs, obtaining interesting quantitative performance. RSD values for repeatability and intermediate precision were below 2.5%, while recovery values between 97 and 105% were obtained for accuracy testing [6]. Several manufacturers proposed application notes for their instruments as well. Among them, Waters Corporation described a HPLC [70] and a UHPLC [71] method for the determination

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of 16 CNBs by employing a mobile phase composed of ACN and water (with 0.1% TFA) in isocratic elutions of 26 and 11 min, respectively. Excellent qualitative performance in terms of peak resolution can be observed in the chromatograms provided. It would be interesting to also evaluate its quantitative performance in terms of accuracy and precision.

2.3 Supercritical fluid chromatography

Since the coming on the market of ultrahigh-performance supercritical fluid chromatographic (UHPSFC) systems, their analytical performance, in terms of efficiency, analysis time and resolving power, has captured the attention of analytical chemists [77-82]. The use of sub2-mm particle analytical columns, as for UHPLC, allows efficient chromatographic separations to be obtained at very low analysis times. The low viscosity and high diffusivity of supercritical carbon dioxide, added to the possibility of using a wide range of solvents as modifiers, make this technique versatile for analyzing a large panel of substances. The use of carbon dioxide (an ecofriendly and cost-effective gas) as the principal mobile phase component allows reproducing normal phase conditions without employing toxic organic solvents. In this context, stationary phases such as diol-, amino- and cyano-bonded silica are available in the form of SFC dedicated columns. By using stationary phases, such as octadecyl- (C18)-, 1-aminoanthracene- (1-AA)-, and 2ethylpyridine- (2-EP)-bonded silica, reversed-phase conditions can also be reproduced [83,84]. In fact, West et al. regrouped stationary phases based on their polarity, showing the large versatility of this technique [82].

Table 4

SFC methods for the analysis of naturally occurring cannabinoids found in recent literature and application notes by equipment manufacturers.

Instrumentation	Matrix	Analytes	Column	Organic modifier in the mobile phase	ABPR	Analysis time	Detection	Make-up solvent for MS detection	Reference
Waters Acquity UPC^2	Inflorescences, leaves and resin	CBL, CBD, Δ^8-THC, THCV, THC, CBC, CBN, CBG, THCA-A, CBDA, CBGA	Acquity UPC^2 BEH 2-EP (150 × 3.0 mm, 1.7 mm), Waters	2-propanol:acetonitrile (80/20, v/v) with 1% of water (gradient, $\%_{max}$ 30% v/v)	1500 psi/103 bar	10 min	UV (λ, 220 nm); ESI-MS (positive and negative modes)	MeOH with 8 mM ammonium formate and 0.5% of formic acid (0.6 mL min^{-1})	86
Waters Acquity UPC2	Inflorescence and resin samples	CBD, Δ^8-THC, THC, CBC, CBN, THCA-A, CBDA, CBG, CBGA	Acquity UPC^2 Torus DIOL (100 × 3.0 mm, 1.7 μm), Waters	2-propanol with formic acid (gradient, $\%_{max}$ 25% v/v)	1738 psi/120 bar	6 min	UV (λ, 214 nm)	NA	87
Waters Acquity UPC2	Hemp samples	CBG, CBD, THC, THCA-A, CBGA, CBDA, and 17 synthetic CNBs	Acquity UPC^2 Torus 1-AA (100 × 3.0 mm, 1.7 μm), Waters	Methanol with 2% of water v/v (gradient, $\%_{max}$ 25% v/v)	1738 psi/120 bar	9.5 min	ESI-MS (positive and negative modes)	MeOH and water 80:20 v/v with 10 mM of ammonium formate	14
Waters Acquity UPC2	CBD oil	CBD, THC and its three isomeric forms	Acquity UPC^2 Trefoil AMY1 (150 × 3.0 mm, 2.5 μm), Waters	200-proof ethanol (gradient, $\%_{max}$ 20% v/v)	2000 psi/138 bar	5 min	UV (λ, 228 nm)	NA	88
Jasco SFC-4000	Standard solutions	CBDV, CBD, CBC, THCV, THC, CBN, CBG, CBDA, THCA-A, CBGA	Lux Cellulose-2 (250 × 4.6 mm, 5 μm), Phenomenex	Ethanol (gradient, $\%_{max}$ 17% v/v)	1738 psi/120 bar		UV (λ, 220 nm)	NA	89
Agilent 1260 Infinity II SFC System	Standard solutions	THC, THCV, 11-OH-THC, CBN, CBG, 11-COOH-THC, CBD	Acquity UPC^2 Torus 1-AA (100 × 3.0 mm, 1.7 μm), Waters	Methanol (gradient, $\%_{max}$ 40% v/v)	1885 psi/130 bar	3 min	AJT-MS^2 (positive mode)	MeOH with 5 mM ammonium formate and 5% of water (0.1 mL min^{-1})	91
Shimadzu UC Nexera	Hemp inflorescences	CBDV, CBD, CBG, CBC, THC, CBN, CBDA, CBGA, THCA-A	Shim-pack GIS C18 (250 × 4.6 mm, 5 μm), Shimadzu	Reagent alcohol (gradient, $\%_{max}$ 12% v/v)	2900 psi/200 bar	6 min	UV (λ, 220 nm)	NA	90
Not specified	Cannabis inflorescences	CBC and the (–)-(R,R) and (+)-(S,S) forms of CBDV, CBD, THC and Δ^8-THC	*(S,S* and *R,R)* Whelk-O 1 (100× 4.6 mm, 1.8 μm), Regis Technologies	Methanol (isocratic, 2% v/v)	1500 psi/103 bar	12 min	UV (λ, 214 nm)	NA	12

SFC, supercritical fluid chromatography; ABPR, active back pressure regulator; 2-EP, 2-ethyl pyridine; 1-AA, 1-amino anthracene; AJT, Agilent Jetstream Technology.

The first application of SFC to CNB determination was described by Bäckström et al. in 1997 [85]. At that time, modern UHPSFC systems were still not available, and instrumental drawbacks overcame benefits in

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the application of this promising but premature technique. Poor sensitivity, repeatability, carryover and lack of robustness were the main weaknesses that affected SFC until the coming of modern UHPSFC in the 2010s [79,82].

Wang et al. followed twenty years after Bäckström's work, with€ the first UHPSFC method aimed to quantify CNBs in cannabis extracts. This work is an example of SFC orthogonality (in terms of analyte elution order) with respect to RP-LC methods when analyzing CNBs [86]. Moreover, the method was validated by estimating both qualitative and quantitative performance according to the ICH guidelines (RSD ranging from 0.83 to 7.55% for repeatability and intermediate precision).

Since then, very few papers describing the development of SFC methods for this purpose have been published. A different application was described by Mazzoccanti et al., who employed the inverted chirality column approach to obtain the chemo- and enantioselective separation of some CNBs [12]. Two chiral stationary phases with the same bound selector but with opposite configurations (R,R- and S,S-Whelk-01 CSP columns) were used to determine the enantiomeric purity of medicinal cannabis extracts. This aspect has also been discussed in a recent review [50]. Jambo et al. developed an SFC-MS method for the quality control of potential counterfeit medicinal cannabis with synthetic CNBs [14]. Synthetic CNBs and some major phytoCNBs were considered during method development, which was performed by applying the analytical quality-by-design strategy. The method was validated, obtaining quite good results (RSD ranging from 2.60 to 5.70% for repeatability and intermediate precision). Recently, Deidda et al. developed a UHPSFC-UV method for CNB determination to compare its quantitative performance to that of a reference UHPLCUV method [87]. A routine application involving ninety-two real cannabis samples was simulated. The quantitative results obtained with both methods were compared by applying the Bland-Altman statistical method, demonstrating their accordance.

Although only a few works can be found in the literature, several application notes have been made available by some instrument manufacturers [88-91]. Table 4 summarizes the operational conditions employed by the methods reviewed in terms of instrumentation, matrix, analytes considered, column, type and ratio of organic modifier in the mobile phase, back pressure, analysis time, detection, and make-up solvent for MS detection [12,14,86-91].

Concerning detectors, the same considerations made for LC could be drawn. Indeed, the success of UV and MS hyphenation to SFC has been largely demonstrated [78,83,84].

Recent literature has demonstrated that the performance of modern SFC is comparable to that of UHPLC [77,79]. In conclusion, SFC is undoubtedly a very competitive alternative to LC analysis for different reasons: orthogonality with respect to RP-LC (different mechanisms of retention) and versatility (wider polarity range of analytes that can be analyzed by modulating the quantity of organic modifier).

2.4 Infrared spectroscopy

Infrared (IR) spectroscopy is divided, according to the spectral range applied, into near-infrared (NIR) (800-2500 nm, respectively 12,500 - 4000 cm^{1}), mid-infrared (MIR), (2500-25,000 nm, respectively 4000 - 400 cm^{1}) and far-infrared (FIR) (25,000-1,000,000 nm, respectively 400 - 10 cm^{1}) spectroscopy [92,93]. The NIR spectral region is associated with combination and overtone bands, which arise from molecular vibrations caused by the interaction between light and molecules. Within these broad bands, both qualitative and quantitative information about sample constituents lies and can be extracted by chemometric tools [94,95]. In the MIR spectral region, fundamental absorption bands give a molecular fingerprint, which is notably

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used for structural investigation and spectral comparison. In fact, the MIR spectral region is richer in bands that are stronger and much sharper than those observed in the NIR region. For this reason, spectral interpretation is easier, bringing more clarity in spectra [96]. The same bands can be used also for quantitative purposes. Concerning the FIR region, it is difficult to access and primarily used for measuring inorganic molecules and is thus not relevant for CNB determination [92,93,97].

IR spectroscopy, in some cases, could undoubtedly be a very interesting alternative to the analytical techniques conventionally used to analyze cannabis. Indeed, it is particularly adapted for all applications demanding rapid analytical responses and directly in the field of interest, such as those encountered in the forensic context, but not only [97-100]. IR analysis is very simple and userfriendly, not requiring highly trained personnel. Indeed, once a chemometric model has been developed and implemented on the device, a few minutes are necessary to acquire a spectrum and obtain the results. The rapidity of the analysis is, without any doubt, a major advantage offered by these techniques. No solvent is necessarily involved, and analyses can be performed directly on the sample without any transformation. Moreover, handheld and/or portable spectrophotometers allow direct analyses in the field, representing a real and unique technological advance [97,99,100]. However, the development of a chemometric model is the critical step of the process since it requires highly qualified personnel to properly manage this task [94]. In fact, in the case of NIR spectroscopy, its spectral signal, raising from combination and overtone bands, is difficult to interpret, and multivariate approaches are necessary to discern and treat the pertinent spectral information. Chemometric tools are commonly used also in MIR spectroscopy for complex matrixes, such as cannabis samples. Various algorithms are available to analysts and can be selected depending on the purpose of the analytical method (e.g., qualitative or quantitative applications). In this context, supervised and unsupervised methods can be distinguished, as described by the general monographs of the European and United States pharmacopoeias (Ph.Eur. 5.21 and USP 1039) and displayed in Fig. 3 [97]. While unsupervised methods exclusively rely on the acquired spectral information, supervised methods require external inputs to relate spectral data to qualitative or quantitative information. For more details about chemometrics, readers are referred to Refs. [101,102]. During model calibration, representative spectra are collected and used to cover all the intrinsic sample variability [92-94]. Then, depending on sample complexity, a high number of samples for calibration could be necessary. In addition to it, also different moisture contents have to be considered for herbal samples, since also water absorbs IR light and can interfere with the analysis. The calibration of quantitative IR models generally relies on data obtained by another analytical technique (GC, LC, SFC, etc.), which is called the reference technique. Another aspect to be considered is the low sensitivity of IR spectroscopy (in general approximately 1% and 0.1% for NIR and MIR spectroscopy, respectively), which can limit the domain of application [92,103]. For all these reasons, sound scientific approaches, as the AQbD strategy, should be implemented for IR method development, as recently discussed by Igne et al. [94].

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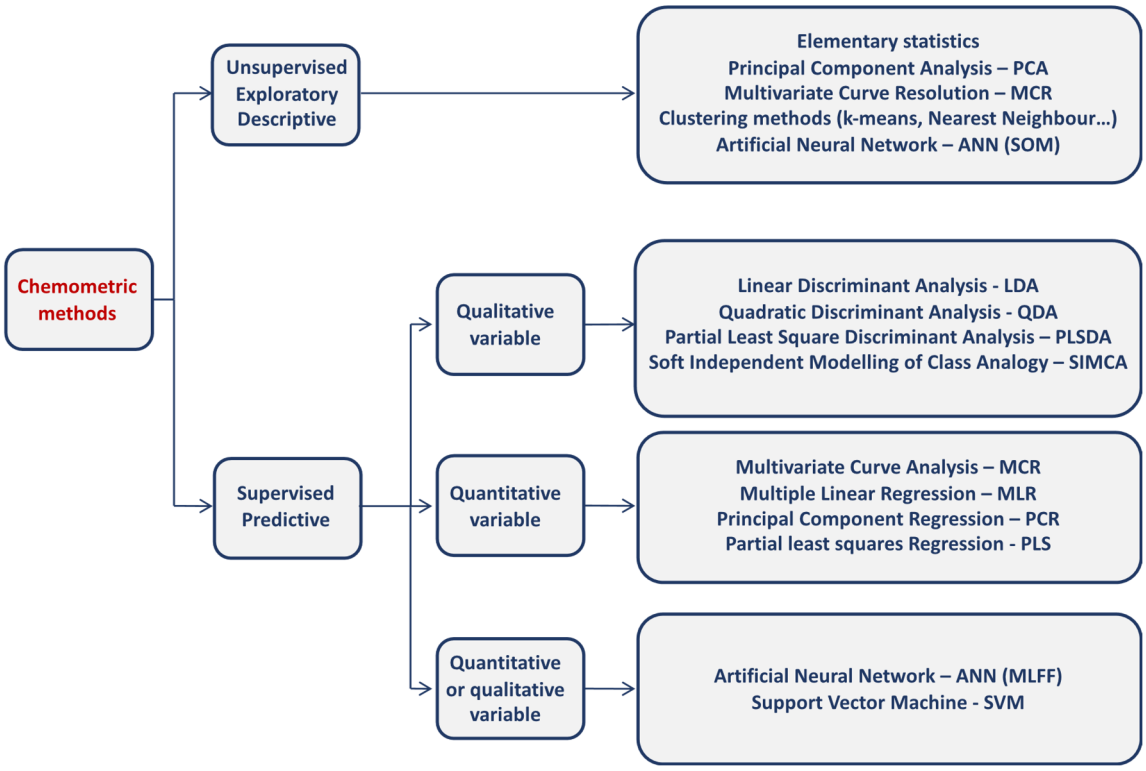

Fig. 3. Chemometric methods from the chapter 5.21. of the European pharmacopoeia 8.7.

Like SFC, IR spectroscopy applications have also come to light very recently, but both qualitative and quantitative applications are sufficiently documented to provide an overview of the analytical potential of this technique. Table 5 presents some very recent works about the application of both NIR and MIR spectroscopy to CNB determination. Information about instrumentation, spectrometer type, spectral range, sample matrix, analysis type, analytes considered, chemometric model, spectral pretreatment, and quantitative performance are provided [99,104-110]. Focusing first on qualitative applications, Coppey et al. recently developed a platform able to provide analytical results on illicit drug samples in a few seconds using a handheld NIR device. A chemometric model for differentiating between illegal and legal cannabis samples was developed, showing 100% sensitivity and specificity (in terms of true positive and true negative values, respectively) during the validation studies [99]. This type of application is undoubtedly the most interesting since it could allow police forces to implement a user-friendly analytical tool for obtaining analysis results during police checkpoints in a very short time. Moreover, no sample preparation was needed since the analysis was performed directly on entire cannabis inflorescences, which can be translated into an enormous gain in terms of time and consumption in opposition to the conventional tools used for the same purpose (mainly GC but also LC methods, as stated above), which require several hours, including sample preparation, to make analytical results available. Duchateau et al. proposed a similar application but on crushed inflorescences contained in plastic bags as a support to perform the analysis [104]. The NIR potential for quantitative determination of CNBs in cannabis samples was also recently investigated. In this context, Callado et al. applied two benchtop spectrophotometers (a dispersive NIR and a Fourier transform-NIR system) to develop partial least squares (PLS) models to predict the content of eight CNBs in cannabis inflorescences after grinding [105]. Good performances were obtained in terms of coefficient of determination in cross validation (ranging from 0.83 to 0.99) and standard error of prediction (values among 1.5-3 times the standard error of laboratory) for each analyte. Townsend et al. described a similar application but focusing only on total THC and total CBD concentrations [107]. Deidda et al. also

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focused their attention on THC content determination but directly on entire cannabis inflorescences and resins to demonstrate the feasibility of applying two handheld spectrophotometers for in-field-based quantitative analyses without any preparation of the sample [106]. The impact of the spectrophotometer technical features on predictive performance was highlighted, and promising results were obtained. Other NIR applications, but on different analytical targets, are described in Refs. [111-113].

Table 5

IR methods for the analysis of naturally occurring cannabinoids in cannabis herbal samples found in recent literature.

Instrumentation	Spectrometer type	Spectral range	Matrix	Analysis type	Analytes	Chemometric model	Spectra pre-treatment	RMSEP (% w/w)	Reference
NIR handheld device	Dispersive	950-1650 nm (10526-6061 cm^{-1})	Entire inflorescences	Qualitative	CBD and THC	Ensemble regression models	2nd derivative and SNV	NA	99
NIR benchtop system	Fourier transform	1000-2500 nm (10000-4000 cm^{-1})	Ground inflorescence	Qualitative	THC	SIMCA and PLS-DA	SNV and Savitzky-Golay 2nd derivative	NA	104
NIR handheld device	Dispersive	1613-2500 nm (6200-4000 cm^{-1})	Ground inflorescence	Qualitative	THC	SIMCA and PLS-DA	SNV and Savitzky-Golay 2nd derivative	NA	104
NIR benchtop system	Dispersive	400-2498 nm (25000-4003 cm^{-1})	Ground inflorescences	Quantitative	CBDV, THCV, CBD, CBC, Δ^8-THC, THC, CBG, CBN	PLS	SNV, DT, MSC, and Norris-Williams derivative	NS	105
NIR benchtop system	Fourier transform	800-2500 nm 12500-4000 cm^{-1})	Ground inflorescences	Quantitative	CBDV, THCV, CBD, CBC, Δ8-THC, THC, CBG, CBN	PLS	1st and 2nd derivative, 1st derivative with vector normalization, and 1st derivative with MSC	NS	105
NIR handheld device	Dispersive	950-1650 nm (10526-6061 cm^{-1})	Entire, ground and sieved inflorescences and resins	Quantitative	THC	PLS and ensemble regression model	Savitzky-Golay 2nd derivative and SNV	Entire inflorescences, 1.75; resins, 1.46	106
NIR benchtop system	Fourier transform	1000-2500 nm (10000-4000 cm^{-1})	Ground inflorescences	Quantitative	THC and CBD	PCR	NS	NS	107
ATR-MIR benchtop system	Fourier transform	2500-25000 nm (4000-400 cm^{-1})	Decarboxylated inflorescences and extracts	Quantitative	THC and CBD	PLS	Savitzky-Golay smoothing and 2nd derivative	For extracts, 3.79 (THC), and 1.44 (CBD); for inflorescences, 2.32 (THC), 1.33 (CBD)	108
ATR-MIR portable system	Fourier transform	2500-25000 nm (4000-400 cm^{-1})	Ground and entire inflorescences	Quantitative	THCA-A, THC, CBDA, CBD, CBGA, CBG, THCVA	PLS	2nd derivative	NS	110
ATR-MIR benchtop system	Fourier transform	2500-15385 nm (4000-650 cm^{-1})	Extracts	Quantitative	THC and THCA-A	PLS	Mean center and Savitzky-Golay 1st derivative	For distillates, 1 (total THC); for concentrates 5 - 6 (total THC), 6 (THCA-A), 0.8 (THC)	109

ATR, attenuated total reflection; DT, detrending; MSC, multiple scattering correction; NA, not applicable; NS, not specified; PCR, principal component regression; PLS, partial least of squares regression; PLS-DA, partial least of squares regression-discriminant analysis; RMSEP, root mean square error of prediction; SIMCA, soft independent modelling of class analogies; SNV, standard normal variate.

MIR spectroscopy has also been applied to CNB determination [108-110]. Among others, Geskovski et al. recently developed some PLS models based on spectra acquired on cannabis inflorescences (decarboxylated) and extracts, aiming at highlighting the MIR potential as a process analytical technology (PAT) for monitoring the concentration of THC and CBD. These results confirmed its potential for quality attribute monitoring in cannabis products [108]. In addition to it, the concept of using MIR spectroscopy as a PAT for cannabis production processes was reinforced by Smith et al., who published an application note describing the application of MIR spectroscopy to optimize growing conditions (in terms of growing time and lighting) by monitoring CNB concentrations in dried cannabis inflorescences [110]. Seven CNBs were considered as the analytes and a PLS model was built for each analyte. Among these, the models built for CBDA and THCA-A showed good results in terms of absolute errors (±0.80 and ±0.86% w/w, respectively)

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and R^2 (93.2 and 94.6, respectively). Also in this case, real-time quantitative results were obtained, allowing to follow how CNB concentrations change with growing conditions.

2.5 Raman spectroscopy

Like IR spectrophotometers, Raman spectrophotometers can also be miniaturized, allowing in-field-based analyses to be performed with the advantages already discussed [97]. Additionally, in this case, sample preparation is not necessary, nor is the use of organic solvents. Rapidity, greenness, and the possibility of performing in-field analyses are the main advantages of this technique. Through Raman spectroscopy, a specific signature of the main components of a sample can be obtained. Raman spectra are usually easier to interpret than NIR spectra and do not demand complex algorithms to be used to treat chemical information. However, Raman spectroscopy can be highly affected by fluorescence caused by some interfering molecules (such as chlorophyll in the case of cannabis inflorescences), which can cover the weaker Raman signal of the analytes of interest. This drawback makes Raman spectroscopy more challenging to apply to plant material analysis. The signal-to-noise ratio (Raman signal/fluorescence) can be optimized by using higher laser wavelength sources (830 nm for instance) [114,115] or by applying more elaborate pretreatment algorithms. In this way, the Raman scattering signal can be exploited from spectra. Also in Raman spectroscopy, chemometric tools are widely employed. In the literature, some interesting applications can be found. Sanchez et al. applied a handheld Raman spectrophotometer to differentiate between hemp and cannabis. A 100% accuracy was documented [114]. The same research group published another work based on the detection of CBD and CBG in hemp. However, more interestingly, they also provided the spectroscopic signature of the main CNBs (CNG, CBGA, THC, THCA-A, CBD, CBDA) to be used for quantitative purposes [115], representing the cornerstone on which quantitative methods for CNB potency determination can (and should) be developed.

Like NIR spectroscopy, Raman spectroscopy is also affected by low sensitivity. To overcome this drawback, the application of surface-enhanced Raman scattering (SERS) could be envisaged to detect very low quantities of analytes [116]. A progressive application of SERS to illicit drug trace detection and quantification (including CNBs) in different matrices can be observed in the literature [117-122]. However, no SERS application to CNB determination on cannabis samples or on cannabis extracts has been found, despite its potential for this application.

Table 6

Evaluation of the main properties of the analytical techniques used for cannabinoid determination on cannabis herbal sample and reviewed in this work

Analytical technique	Sample preparation	Solvent use	Greenness	Method development complexity	Analysis time	Analysis cost	In-field applicability	Sensitivity
GC-FID	5	2	2	2	5	3	0	4
GC-MS	5	2	2	2	5	5	0	5
LC-UV	3	5	0	3	5	4	0	4
LC-MS	4	5	0	3	5	5	0	5
SFC-UV	3	3	3	3	3	4	0	4
SFC-MS	4	3	3	3	3	5	0	5
IR	0	0	5	5	1	1	5	1
Raman	0	0	5	4	1	2	5	1

The scale of grades ranges from the minimum (0) to the maximum (5) for each aspect.

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3. Discussion

Table 6 aims to grade the main aspects of each analytical technique discussed above to explore the advantages and disadvantages associated with the use of each technique and to guide analytical chemists in their choice. To do this, a scale of grades ranging from the minimum (0) to the maximum (5) has been adopted for the following aspects: solvent use, greenness, method development complexity, analysis time and cost, in-field applicability and sensitivity. Raman and IR spectroscopy, contrary to chromatographic techniques, allow analyses to be performed without sample preparation and do not ask for solvents to perform analysis. This is their first advantage. SFC, contrary to LC, requires small amounts of organic solvent as a modifier of the mobile phase. Furthermore, carbon dioxide can be recycled. For these reasons, SFC was rated as an ecofriendly technique together with Raman and IR spectroscopy. Regarding the complexity of the method development process, GC presents the lowest score since its operational conditions can easily be set. In contrast, IR spectroscopy was highly rated due to the involvement of chemometric tools and reference techniques for this task. The lowest scores for the "analysis cost" and "analysis time" parameters were given to spectroscopic techniques, since they allow analysis results to be obtained in a few seconds without involving expensive material (columns, solvents, materials for extraction, etc.). Moreover, relevant costs related to both equipment acquisition and periodic maintenance must be considered for chromatographic techniques. As extensively discussed in the previous section, handheld and portable spectrophotometers have been developed, allowing Raman and IR analysis to be performed directly in the field of interest.

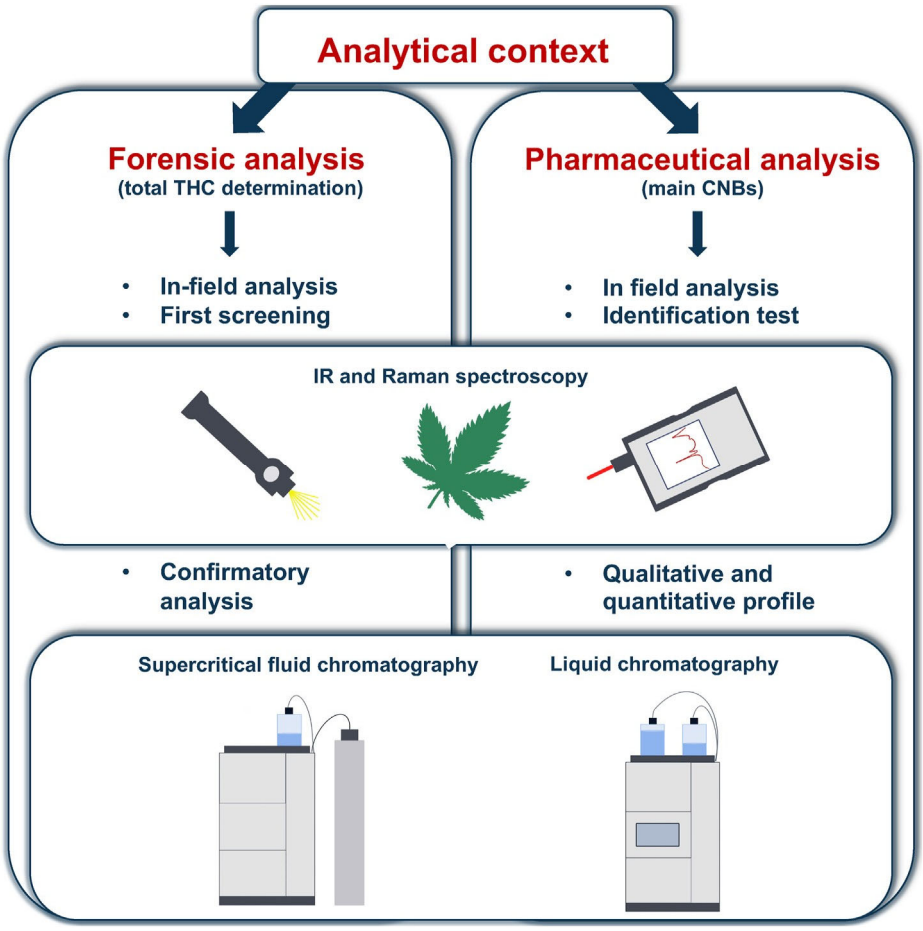

Fig. 4. Scheme showing the possible applications of the discussed analytical techniques for the pharmaceutical and forensic fields.

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This portability is not yet the case for chromatographic techniques. The last parameter evaluated is sensitivity, which varies consistently from one technique to another and obviously regarding detectors. In this context, chromatographic techniques outperform spectroscopic techniques. Quantitative performance data, in terms of precision and accuracy, are not available for the spectroscopic techniques. For this reason, a global evaluation is not yet possible.

Based on these considerations, Fig. 4 proposes a scheme of possible applications of these techniques with respect to the analytical context of reference. In a forensic context, IR and Raman spectroscopy could be very useful as decisional tools for the first screening of potential police seizures (qualitative and semiquantitative applications). Then, confirmatory analyses of illicit samples focused on the accurate determination of total THC content could be performed by LC or SFC (both preferably coupled to MS detectors). GC was excluded because of its constraints in terms of sample preparation for thermolabile compounds. From a pharmaceutical point of view, IR and Raman spectrophotometers could be used for identification tests, as is currently done for drug manufacturing processes. Chromatographic techniques could be used for the determination of acidic and neutral CNBs, with a preference for the greenest SFC.

4. Conclusions

GC and LC have been widely applied to CNB determination in recent decades. The methods reviewed in this work showed that the operational conditions are often repeated, and only small differences are found among the methods (see Tables 2 and 3). This stagnation in terms of inventiveness demonstrates that the main operational parameters for these techniques have now been identified. Both techniques remain undoubtedly reference methods for this analytical purpose. For this reason, they are ready for a definitive worldwide accepted method to be developed and validated on a maximal number of analytes commonly found in cannabis samples. In this context, an analytical quality-by-design approach could be interesting from a methodological point of view, as a support for method development and as a guarantee of robustness [123,124].

Remaining in the context of chromatographic techniques, SFC has now largely demonstrated its analytical value, showing that it can play an important role in quality control and forensic laboratories. This utility has been sufficiently documented in the literature, as it is no longer a young technique, contrary to what some may think. However, its application to CNB determination is very recent, and for this technique, a definitive method for routine analysis should be proposed. Routine data should be documented and analyzed as well to persuade even the most skeptical analyst of its value.

Concerning IR spectroscopy, both qualitative and quantitative approaches have been investigated, and their feasibility for this application was demonstrated. The implementation of handheld spectrophotometers has paved the way for in-field-based analyses, which can be particularly interesting in a forensic context. Now, what is missing is the development of chemometric models based on larger databases, whose predictions could be used to thoroughly analyze and evaluate the performance of this technique in terms of the accuracy of quantitative results.

The same is valid for Raman spectroscopy, which still lacks quantitative applications in the context of CNB determination on cannabis samples.

In conclusion, CNB determination concerns various fields of analytical chemistry, each with its own disparate needs and requirements. For this reason, the various tools in the hands of analysts should be

individually chosen depending on the specific application. Indeed, to date, there is no universal technique that is able to satisfy every need at the same time. As a consequence, this choice should be made by taking into account both advantages and limitations, which are intrinsically linked to each analytical technique.

Author credit statement

Riccardo Deidda: writing (original draft), writing (review and editing), conceptualization, formal analysis, visualization; Amandine Dispas: writing (review and editing); Charlotte De Bleye: writing (review and editing); Philippe Hubert: conceptualization, supervision, funding acquisition, project administration; Eric Ziemons: writing (review and editing), conceptualization, supervision, funding acquisition, project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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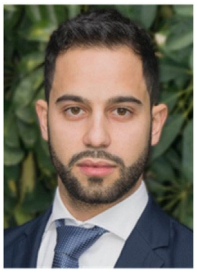
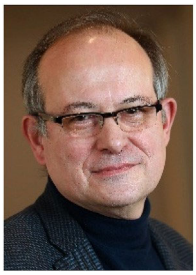


Riccardo Deidda is currently a PhD candidate in analytical chemistry at the University of Liege, Belgium. He earned his master's degree in Pharmacy from the University of Florence, Italy. His research activities are twofold, including the application of both chromatographic and spectroscopic techniques to cannabis analysis and the implementation of risk-based approaches (i.e., analytical quality-by-design) for method development in analytical chemistry.

Amandine Dispas is a postdoctoral researcher in the laboratory of pharmaceutical analytical chemistry and in the laboratory for the analysis of medicines at the University of Liege (Belgium). She received her Ph.D. in 2016 in analytical chemistry. Her research is focused on separation techniques for drug analysis, from conventional LC to SFC and CE.

Charlotte De Bleye was graduated in Pharmaceutical Sciences at the University of Liege in 2011. Thereafter, she had a grant holder form the F.R.S.-FNRS and started a PhD thesis dealing with the investigation of quantitative performances of surface-enhanced Raman scattering (SERS) in the laboratory of Pharmaceutical Analytical Chemistry (LCAP) of the Professor Philippe Hubert. She received her PhD in Biomedical and Pharmaceutical Sciences in 2016. Nowadays, she is still working in the LCAP and her research is focused on SERS and Raman imaging.



Philippe Hubert is full Professor of pharmaceutical analytical chemistry at the department of pharmaceutical sciences, University of Liege (Belgium). He received his Ph.D. in 1994 in analytical chemistry. His research interests are focused on the robust development and validation of analytical methods mainly dedicated to the quality control of drugs and counterfeit medicines using mainly separation techniques and vibrational spectroscopy. He has published more than 330 papers in international journals. He is a member and reviewer of several journals and societies as well as member of the editorial board of Journal of Pharmaceutical and Biomedical Analysis (Elsevier).

Eric ZIEMONS is currently Associate Professor in Pharmaceutical Chemistry at the University of Liege (ULiege) and Director of the ViBra-Sante hub of the Center for Interdisciplinary Research on Medicines (CIRM). He received his PhD in Pharmaceutical Sciences from ULiege in 2006 under the supervision of Professor Philippe HUBERT. They continue to work together on different projects.His research interest span both vibrational spectroscopy and hyperspectral imaging applied to the pharmaceutical and biomedical fields with a particular focus on data analysis and surface-enhanced Raman scattering for trace detection in complex media.He is the author of more than 100 peer reviewed articles.