Chapter

Exploring the Mysteries of *Cannabis* through Gas Chromatography

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Abstract

In the last decades, cannabinoids, the active constituents of *Cannabis sativa* L., have been attracting a strong interest, regarding the health effects associated with the use of *Cannabis* and *Cannabis*-derived products. The progressive legalization of this species in several countries has prompted an increasing concern about the characterization and quantification of cannabinoids in diverse chemotypes of the plant, as well as the obtained final products. Therewith, Process and Product Quality Assurance (PPQA) becomes a mandatory practise to verify the Good Manufacturing Practices (GMP). Gas chromatography is one of the most used techniques in this sense due to its high attainable resolution. However, sample complexity and the thermal lability of cannabinoids hinder the analysis. In this chapter, a fully description of the recent advances in the *Cannabis sativa* L. analysis by gas chromatography will be presented, including different approaches that have come up to solve the obstacles encountered.

Keywords: *Cannabis sativa* L., hemp, complex matrix, cannabinoids, Terpenoids, gas chromatography, quality control assurance

1. Introduction

Cannabis sativa L. is the most thoroughly studied and widely used plant from Central Asia 3000 years before the Christian era. This annual dioecious plant has a complex chemical composition, including cannabinoids, terpenes, flavonoids, stilbenoids, fatty acids, alkaloids, carbohydrates, and polyphenols, among others. Therefore, this plant and its by-products have been widely used in different areas in the production of ropes, cloth, food or oil, being considered one of the most significant agricultural crops over the years. Additionally, since the chemical structure of Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive compound in *Cannabis*, was identified by Mechoulam in the 60s, the studies about the active compounds of *Cannabis* increased dramatically and, owing to its bioactive components, *Cannabis* has been used for recreational, medicinal and scientific purposes [1]. In recent years, as a consequence of multiple scientific evidences, the number of countries around the world where the use of *Cannabis* is being legalized or decriminalized is

continuously growing, like many Latin American countries (Uruguay, Peru, Venezuela, Chile, Colombia, Argentina, and Ecuador), different U.S. states, some countries of Europe (Italy, Czech Republic and Germany) and Canada. In this regulatory scenario, the most common practice is to use the Δ^9-THC content for the discrimination among industrial or medicinal varieties. However, the classification of *Cannabis* plants regarding this parameter varies depending on the legislation of the country of origin. While some countries like Switzerland, Uruguay or Colombia authorize 1.0% of Δ^9-THC for medicinal use, more restrictive legislation in the European Union limits this value to the 0.2% content. It should be noted that obviously only duly registered and certified varieties could be used [2]. When talking specifically about medical *Cannabis*, quality control of cultivars as well as standardization is a requirement for these applications as they are extremely important to ensure the health and safety of medical *Cannabis* users and patients, being necessary the development of reliable methods to quantify bioactive compounds from *Cannabis* [3, 4].

Therefore, several chromatographical techniques have been widely used for the identification and quantification of *Cannabis* constituents, being gas chromatography (GC), especially coupled to mass spectrometry (GC–MS), the most established technique in forensic and clinical toxicology analyses. Considering the similarities of their bioactive compounds in their physicochemical properties, chromatographic separation involves a mandatory step that may be usually considered as time consuming. For this reason, different strategies have been implemented increasing the resolution between isomers and overcoming this problem. In addition, the identification of these compounds is possible thanks to the advanced National Institute of Standards and Technology (NIST) mass-spectral database. In this manner, the analysis of *Cannabis* does not require highly sophisticated equipment being appropriate for laboratories with reduced instrumental availability. Nevertheless, different methods have been reported using high-resolution mass spectrometry allowing the untargeted analysis of *Cannabis* samples [5].

Generally, an internal standard (IS) is a nonendogenous compound, which is naturally similar to the target analyte. In this sense, this methodology is employed to increase the reproducibility of the quantification when there is a source of errors during sample analysis. These inaccuracies may be related to random and systematic errors, due to sample preparation or even the complexity of the sample, among other factors. The IS must be added to each sample in a constant amount, as well as to blank and calibration standards. In this manner, any deviation occasioned during the analysis of the sample will also affect the IS, correcting the result with the relation of both signals. For this reason, the selection of the IS is a critical step to guarantee the precision in the analysis. The stable-isotope labeled IS are the most suitable when a MS detector is used [6].

Furthermore, derivatization processes are generally required, improving the chromatographic resolution as well as peak shape of analytes [7]. For this reason, many derivatization agents have been applied, being the silylation approaches preferred. These derivatization agents are suitable to volatilize and improve mass fragmentation properties of active proton-containing groups. Commonly, N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA), N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), trimethylchlorosilane (TMCS) or N-tert-butyldimethylsilyl-N- methyltrifluoroacetamide (MTBSTFA) are utilized [8]. Although this procedure turns out to be time-consuming, it is worthy since it provides increased sensitivity and reproducibility [6, 9]. Throughout this chapter, the authors will discuss the multiple analytical work modalities and methods that have been used to solve all the inconveniences found in the analysis of *Cannabis* samples using gas chromatography.

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2. Cannabinoids

Cannabinoids are the major constituents of *Cannabis* plant, distributed on aerial surfaces/leaves and female inflorescences of the plants. These compounds are concentrated on resinous secretions produced by glandular trichomes [1, 10]. Their bioactive compounds are acquiring more notoriety because of their physiological effects as well as their medicinal properties, which are applied in the treatment of a wide range of diseases and disorders (e.g. multiple sclerosis, epilepsy, fibromyalgia). They are also employed for alleviating the pain induced by some treatment methods for various diseases, including cancer. Alongside being of great interest for the patient and medical communities, the European medicinal *Cannabis* market is expected to boom in the coming years. Consequently, the development of more efficient qualitative and quantitative methods for the analysis of these compounds is required [11].

Cannabinoids may be classified into three groups based on their source of production, *viz*., endocannabinoids, phytocannabinoids and synthetic cannabinoids [12]. There is an extensive list of these compounds, hence only some of them are depicted in **Figure 1**.

2.1 Endocannabinoids

Endocannabinoids (ECs) are defined as endogenous lipids that are involved in many physiological and pathological conditions, regulating neurological disorders. They are characterized by their cannabimimetic features, activating the cannabinoid receptors (CB1 and CB2) as well as other receptors. For this reason, the development of reliable methodologies to determine ECs levels is mandatory in order to understand the role of these lipid metabolites. Different ECs have been widely analyzed by GC–MS and LC–MS, like arachidonoyl ethanolamide (anandamide, AEA) and 2-arachidonyl glycerol (2-AG) in human biosamples, which are the two most widely studied [13]. Additionally, other ECs like virodhamine and noladin ether has been detected. These compounds present physiological properties comparable to natural and synthetic exogenous cannabinoids. However, the main drawback found in their determination is their low concentration, acute instability of some endogenous compounds as well as the limited sample availability. For this reason, different extraction techniques like liquid–liquid extraction (LLE) or solid phase extraction (SPE) have been used for the quantification of ECs from different samples, to increase the analyte concentration. Moreover, microextraction

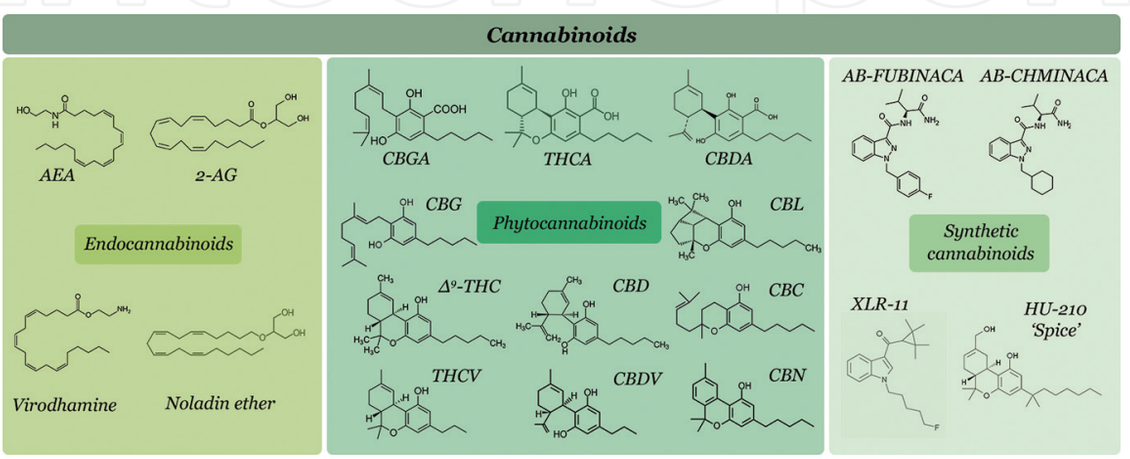

Figure 1.
Classification of cannabinoids based on their source of production.

approaches like solid phase microextraction (SPME) have been employed in the ECs analysis, reducing the required amount of sample [14].

Several strategies using GC–MS have been described in the literature for the determination of ECs from biological samples [15–17]. In this regard, different derivatization reagents like BSTFA or methyl ester of the methoxim dimethyisopropylsilyl (DMiPSi) have been employed, in order to increase the stability of these compounds during their analysis. Additionally, different ionization modes, including electron ionization (EI), positive chemical ionization (PCI) and negative chemical ionization (NCI, also denoted as NICI, ECCI, ECNI and ECNICI) have been utilized. Such is the case of the method reported by Kayacelebi et al., based on the indirect evaluation of 2-AG by the analysis of arachidonic acid (AA) and prostaglandin E_2 (PGE_2), which are hydrolysis products of the EC. This approach employs GC–MS and GC–MS/MS using the NCI mode. This technique provides enhanced selectivity in comparison to EI since only analytes with high electron capture capacity or high electron affinity may be ionized, thereby eliminating potential sample matrix interferences and allowing detections at very low concentration levels (ng/L). This consideration provides an advantage in contrast to LC–MS, since sample matrix is usually one of the major issues in this methodology. In NCI, low energy electrons (around 2 eV) are produced by collision of the reagent gas (generally methane, ammonia or carbon dioxide) with electrons emitted from the filament. The resulting electrons are captured by the analyte producing stable molecular anions. Although only specified analytes fulfill the characteristics to be used by this technique, derivatization processes provide wider applicability to this unusual ionization mode. For instance, the perfluoro group presents high electron affinity, being suitable for NCI. In this manner, an esterification process of AA and PGE_2 was accomplished using pentafluorobenzyl bromide (PFB-Br) as derivatization agent, allowing the controlled fragmentation of both metabolites and accordingly, resulting in a highly sensitive quantitative method [18]. Additionally, PCI has been used for the determination of AEA by GC–MS using TMS as derivatization agent. As with NCI, PCI is based on the generation of protonated molecules by collision of the reagent gas with an electron emitted from the filament. The ionized reagent gas reacts with the analyte, resulting in a positive adduct ion. In both PCI and NCI, information about the molecular weight of the analyte is provide, unlike EI where higher energy electrons collide with analytes creating fragmented positive ions and other species. Even though this methodology may appear simpler, the selectivity reached by NCI outweighs the benefits of PCI, as well as the weaknesses of the technique itself, *viz.,* dissociative reactions by high energy electrons, analyte response suppression by oxygen or water contamination and affected reaction yield and fragmentation behavior due to high ionization chamber temperature [19]. These strategies are summarized in **Table 1**.

Despite the fact that GC–MS has been extensively utilized to determine ECs metabolites from biological samples, in the recent years, LC–MS/MS has become the generally employed instrumentation in the analysis of ECs, being considered in this sense the reference analytical technique. This preference may be attributed to increased sensitivity and selectivity as well as high-throughput capability. In addition, derivatization processes of ECs may be avoided, which are laborious and time-consuming [20].

2.2 Phytocannabinoids

Phytocannabinoids are naturally occurring cannabinoids found in the *Cannabis* plant. These compounds present a similarity in their chemical structure since they are constituted by a C_{21} structural feature with alterations in the length of their side

CANNABINOIDS	Sample treatment					Instrumental technique				Analytical features					
	Sample	Amount of sample	Metabolite	Derivatization	Extraction	Technique	Ionization source	Column	Analysis Time (min)	LOD	LOQ	Precision (RSD, %)		Recovery (%)	Ref.
												Intra-assay	Inter-assay		
ENDOCANNABINOIDS	Plasma	1.5 mL	AEA and 2-AG	BSTFA/TMCS	LLE	GC–MS	PCI	DB-1 MS	16.2	0.3–0.5 ng/mL	0.35–1 ng/mL	<11	10.6–14.1	42.7–72.2	[13]
	Blood	0.2–5 mL	AEA and 2-AG	DMiPSi	SPE	GC–MS	EI	DB-1	<20	—	—	—	—	—	[15]
	Mouse brain	—	AEA and 2-AG	BSTFA	SPE	GC–MS	PCI	Rtx-5MS	11	0.01 pmol	0.2 pmol	2.5		70.9	[16]
	Mouse brain	30 mg	AEA, 2-AG and PEA	BSTFA	SPE	GC–MS	CI	CP-Sil8 CB	<30	—	—	—	—	—	[17]
	Dog liver	—	2-AG (AA and PGE2)	PFB-Br	—	GC–MS and GC–MS/MS	NCI	OPTIMA-17MS	32	—	10 nM / ≤0.1 μM	2.3–12.9		—	[18]
	Tissue	50–100 mg	NAE (PEA, SEA, AEA)	BSTFA	SPE	GC–MS and GC–MS/MS	EI	Rtx-5MS	—	—	—	—	—	—	[19]
PHYTOCANNABINOIDS			11-OH-THC							0.03 pg./mg	0.1			88	[27]
	Hair			MSTFA	LLE	GC–MS/MS	EI	DB5-MS				9–14	5–9		[27]
			THC, CBD and CBN							0.3–1.4	0.9–4.7 pg./mg			68–97	[27]
	Plant (buds, leaves and stems)	0.2 g	CBD, THC and CBN	—	HCEE	GC-,S	EI	Zebron ZB-5MS	30–60	0.05–0.09 mg/L	0.17–0.30 mg/L	5.6–7.4		97–99	[28]

CANNABINOIDS	Sample treatment					Instrumental technique			Analytical features					
	Sample	Amount of sample	Metabolite	Derivatization	Extraction	Technique	Ionization source	Column	Analysis Time (min)	LOD	LOQ	Precision (RSD, %)		Recovery (%)	Ref.
												Intra-assay	Inter-assay		
	Cannabis seed	5 g	THC, CBD, CBC, CBG and CBN	—	PHWE	GCXGC-QTOF-MS/MS	EI	BPX5	30	—	—	2.87–4.5	1.32–5.60	89–45–92-56	[29]
	Hair	50 mg	THC, CBD, CBN and 11-OH-THC	MSTFA	LLE	GC–MS/MS	EI	DB5-MS	14	0.303–1.4 pg./mg	0.1–4.7 pg./mg	1.84–8.8	2.78–14.05	68–97	[31]
			THC-COOH	PEPA:HFIP			NCI		8						
	Serum and plasma	1 mL	THC, THC-COOH and 11-OH-THC	MSTFA	SPE (AXS)	GC–MS	EI	OPTIMA® 5 MX Accent	<30	0.15–2 ng/mL	0.3–3.3 ng/mL	0.8–4.8	1.9–6.1	76.9–96.5	[32]
	Human breast milk	0.05 mL	THC, CBN and CBD	—	SPME	HS-SPME-GC–MS	EI	HP-5MS	16	10 ng/mL	20 ng/mL	6.5–13.3	2.1–9.2	90.9–118	[33]
	Hair	25 mg	THC-COOH, OH-THC, THC, CBD and CBN	MSTFA	SPE	GC–MS/MS	EI	Zebron ZB-5MSi	68	0.2–2 pg./mg	0.5–5 pg./mg	1.6–5.5	2.3–6.6	19–79	[34]
	Human serum	1 mL	THC, THC-OH, THCA, CBD, CBDA and CBG	MSTFA	LLE	GC–MS	AP	HP-5MS	12	0.05–0.9 ng/mL	0.2–3 ng/mL	0.2–14.6	6.9–14.9	>82	[35]
	Plant (roots, stems, buds and leaves)	100 mg	THCV, CBD, CBC, Δ8-THC, Δ9-THC, CBG, CBN, CBDA, THCAA and CBGA	BSTFA	—	GC-FID	—	DB-1MS	17.5	0.11–0.19 μg/mL	0.34–0.56 μg/mL	0.19–16.79		—	[36]

CANNABINOIDS	Sample treatment					Instrumental technique				Analytical features					
	Sample	Amount of sample	Metabolite	Derivatization	Extraction	Technique	Ionization source	Column	Analysis Time (min)	LOD	LOQ	Precision (RSD, %)		Recovery (%)	Ref.
												Intra-assay	Inter-assay		
	Buccal swabs	5 mg	THC, Δ8-THC, CBN, CBD, CBC, CBG, and CBDV	MSTFA	SPME	HS-SPME-GC–MS	EI	Rxi-35 Sil	12	< 0.04 μg/mg	—	—	—	—	[37]
	Plant (flowers)	—	CBD, THC and CBN	—	SBSE	GCXGC-QTOF-MS	EI	Rxi-5MS	93	0.02–0.15 μg/mL	0.05–0.51 μg/mL	8.8–19.3		—	[5]
	Standard mixtures	—	THCV, CBD, CBC, Δ8-THC, Δ9-THCA, 11-hydroxy-Δ9-THC and 11-nor-9-carboxy-Δ9-THC	BSTFA+1% TMCS	—	GC-UV/VUV	—	Rtx-5	15	5–10 mg/L	—	—	—	—	[11]
	Standard mixtures	—	62 SCs	—	—	GC–MS	PI	DB-5MS	—	—	—	—	—	—	[41]
SYNTHETIC CANNABINOIDS	Seized plant	150 mg	15 SCs	—	—	GC–MS	cokl-EI	DB-5MS	>15	—	8–133 μg/L	2.9–7.3		—	[42]
	Seized plant	—	34 SCs	—	—	GC–MS/MS	EI	DB-5MS	>30	19.9–68.8 ng/mL	—	—	—	—	[43]
							CI			91.1–162.9 ng/mL					

CANNABINOIDS	Sample treatment					Instrumental technique				Analytical features					
	Sample	Amount of sample	Metabolite	Derivatization	Extraction	Technique	Ionization source	Column	Analysis Time (min)	LOD	LOQ	Precision (RSD, %)		Recovery (%)	Ref.
												Intra-assay	Inter-assay		
	Standard mixtures		6 SCs	—	—	GC–MS	EI	Rtx-200	21	—	—	—	—	—	[44]
						GC-IR	—	BPX5							
	Plant	10 mL	11 SCs	—	—	GC–MS	EI PCI NCI	HP-5MS	30	—	—	—	—	—	[45]
	Urine							J&W scientific	21	1–5 μg/L	5 μg/L	6.1–16.2		54–98.2	[6]
		2 mL	29 SCs	BSTFA	UADLLME	GC–MS	EI	5% phenyl-methylsilicone		1–5 μg/L	5 μg/L	0.5–19.7		93.8–105.3	[6]
	Blood														
	Plant	20 mg	44 SCs	—	SPME	HS-SPME-	EI	DB-5 ms	23	—	—	—	—	—	[46]
	Blood	500 μL				GC–MS									
	Saliva	455 μL	5 SCs	—	MEPS	GC–MS	EI	HP-5 MS	26	10–20 μg/L	30–60 μg/L	3.6–8.9		62–124	[47]

2-AG: 2-arachidonyl glycerol; ***AA:*** *arachidonic acid;* ***AEA:*** *anandamide;* ***AXS:*** *anion exchange sorbent;* ***BSTFA:*** *N,O-bis (trimethylsilyl) trifluoroacetamide;* ***CBC:*** *cannabichromene;* ***CBD:*** *cannabidiol;* ***CBDA:*** *cannabidiolic acid;* ***CBDV:*** *cannabidivarin;* ***CBG:*** *cannabigerol;* ***CBGA:*** *cannabigerolic acid;* ***CBN:*** *cannabinol;* ***DMiPSi:*** *methyl ester of the methoxim dimethyisopropylsilyl;* ***EI:*** *electron ionization;* ***FID:*** *flame ionization detector;* ***GC:*** *gas chromatography;* ***HCEE:*** *hard cap espresso extraction;* ***HFIP:*** *hexafluoroisopropanol;* ***IR:*** *infrared;* ***LLE:*** *liquid–liquid extraction;* ***MEPS:*** *micro extraction by packed sorbent;* ***MS:*** *mass spectrometry;* ***MSTFA:*** *N-Methyl-N-(trimethylsilyl) trifluoroacetamide;* ***NAE:*** *N-acylethanolamines;* ***NCI:*** *negative chemical ionization;* ***PCI:*** *positive chemical ionization;* ***PEA:*** *palmitoylethanolamide;* ***PFB-Br:*** *pentafluorobenzyl bromide;* ***PFPA:*** *pentafluoropropionic anhydride;* ***PGE_2:*** *prostaglandin E_2;* ***PHWE:*** *pressurized hot water extraction;* ***PI:*** *photoionization;* ***SBSE:*** *stir bar sorptive extraction;* ***SEA:*** *stearoylethanolamide;* ***SPE:*** *solid phase extraction;* ***SPME:*** *solid phase microextraction;* ***THC:*** *tetrahydrocannabinol;* ***THCAA:*** *tetrahydrocannabinolic acid A;* ***TMCS:*** *trimethylchlorosilane;* ***QTOF:*** *quadrupole time-of-flight;* ***UADLLME:*** *ultrasound-assisted dispersive liquid–liquid microextraction;* ***UV/VUV:*** *ultraviolet/vacuum ultraviolet.*

Table 1.
Analytical platforms for the analysis of cannabinoids in Cannabis *samples.*

chain (C1-C5) attached to the aromatic ring. Cannabigerolic acid (CBGA) is a precursor molecule, which can be converted through a series of enzymatic reactions into Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA), cannabidiolic acid (CBDA), or cannabichromenic acid (CBCA). Acidic phytocannabinoids may be subsequently decarboxylated into their corresponding active neutral form by decarboxylation processes, losing a COOH group. This process may occur over time, under heating or alkaline conditions [10]. Consequently, the previously mentioned cannabinoids will be transformed into cannabigerol (CBG), Δ^9-THC, cannabidiol (CBD) and cannabichromene (CBC). All these cannabinoids are characterized by a composition of five carbon atoms in their side chain. Additional compounds have been detected varying the length of their side chain, thereby varinoids compounds, such as tetrahydrocannabivarinic acid (THCVA), cannabidivarinic acid (CBDVA), cannabidivarin (CBDV) and tetrahydrocannabivarin (THCV) are naturally produced in the plant, with a three-term alkyl chain. Some other compounds, like Δ^8-tetrahydrocannabinol (Δ^8-THC), cannabinol (CBN), cannabicyclol (CBL), cannabinolic acid (CBNA), and cannabicyclolic acid (CBLA) have also been reported. Additionally, the exposition to environmental agents like oxygen may lead to the alteration of these compounds. The oxidative degradation of Δ^9-THC to CBN under oxygen exposition is a representative example of this condition [21].

Currently, almost 150 phytocannabinoids have been detected in *Cannabis* plant, although most of them have never been isolated or characterized. Therefore, the list is still under construction since new cannabinoids are continually being discovered [22]. Recently, new phytocannabinoids have been detected, *viz.,* cannabidibutol (CBDB) and Δ^9-tetrahydrocannabutol (Δ^9-THCB), characterized by four carbon atoms in their side chain, as well as cannabidiphorol (CBDP) and tetrahydrocannabiphorol (Δ^9-THCP), being the seven-term homologs of CBD and Δ^9-THC, respectively [23]. Δ^9-THCP proved to be as active as Δ^9-THC but at lower doses, being necessary further analyses to evaluate the pharmacological effect of this potent phytocannabinoid.

For many years, *Cannabis* has had a negative connotation due to the psychotropic effects associated with Δ^9-THC. However, researchers have been working for a long time against this situation due to the beneficial health components found in the plant. All the bioactive components contribute in different manner to health, being used as treatment against different diseases. Among them, CBD has been the most widely studied phytocannabinoid, since it presents a series of medical properties like antioxidant, anti-inflammatory, antibacterial, anti-proliferative and neuroprotective effects. However, all this flood of information has diverted the attention from some other minor cannabinoids with more remarkable and interesting characteristics. In this sense, CBDV, CBG and Δ^9-THCV have been barely investigated since the low concentration of these cannabinoids in the plant hinder their isolation for further studies. Nevertheless, there is evidence of the anti-inflammatory and anti-proliferative properties of CBG and CBC, together with a significant antibacterial activity [24, 25]. Thus, the genetic selection of *Cannabis* varieties rich in other minor phytocannabinoids would confer the opportunity of extract these compounds.

From a medical point of view, the administration of these compounds has been normally accomplished by *Cannabis* oil extracts, which are produced from dried *Cannabis sativa* L. inflorescences incorporated in common edible oils (*e.g.,* olive or sunflower), sometimes using these oils as extraction media [26]. Thus, *Cannabis* extraction has been thoroughly studied to obtain highly concentrated content of cannabinoids and other beneficial components. Different organic solvents like ethanol, methanol, acetone, or hexane have been utilized for this purpose, although more natural approaches, like water, have been also evaluated. An exhaustive

control of the potency as well as the standardization of different plant batches may be accomplished to guarantee the correct dosage of phytocannabinoids in the final oil. Accordingly, analyses of the extracts resulted of the maceration in olive oil have been performed by GC coupled to flame ionization detector (FID) and GC–MS, as a way of comparison (see **Table 1**). Results were comparable in terms of precision, accuracy and linearity [27]. Recently, an innovative and rapid method has been reported to the extraction of cannabinoids, using the hard cap espresso extraction (HCEE) methodology coupled to GC–MS [28]. This approach allows the quantitative extraction of THC, CBD and CBN from seized *Cannabis* samples in only 40 s, using 100 mL of isopropanol and GC–MS determination. The obtained results were compared with those acquired by the ultrasound-assisted extraction (UAE) reference method, being both approaches statistically comparable. Similarly, a green extraction method, pressurized hot water extraction (PHWE), has been used to extract cannabinoid compounds from *Cannabis sativa* L. seeds. This technique, based on the supercritical fluid extraction, is an alternative to CO_2 that enables the extraction of polar and semi-polar bioactive compounds from *Cannabis* seeds. In this case, GCxGC-TOF-MS methodology allowed the identification of cannabinoids without the need of using standards [29]. Considering that these concentrated cannabinoids extracts will be used in the pharmaceutical industry, an exhaustive control is mandatory to ensure consumer safety according to the GMP system [30].

These compounds are metabolized in the organism after consumption, being possible the detection of their metabolites. Analysis of these chemical by-products is an excellent solution to distinguish between passive drug exposure and active consumption. In this sense, Δ^9-THC is rapidly adsorbed and metabolized to 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (Δ^9-THC-COOH) and 11-hydroxy-Δ^9-THC (11-THC-OH), using these compounds as *Cannabis* biomarkers of the psychoactive form. However, the low concentration of these metabolites in biological samples implies the use of high sensitive equipment like GC–MS/MS or LC–MS/MS as well as the extraction and preconcentration of the analytes before the analysis, which is time and solvent consuming, especially when liquid–liquid extraction (LLE) is utilized [31]. Alternatives approaches to LLE has been reported, including anion exchange sorbent (AXS) and SPME in combination with head space GC–MS (HS-SPME-GC–MS) [32, 33].

Recently, Beike et al. reported an automated process of sample preparation, which includes extraction, clean up and derivatization of cannabinoids, prior to GC-EI-MS/MS. The determination of THC as well as its metabolites Δ^9-THC-COOH and 11-THC-OH in hair samples was accomplished using a SPE cartridge attached to the module of the MultiPurposeSampler (MPS) autosampler from GERSTEL, allowing the complete automation of the extraction process with relative standard deviation better than 7% [34]. In the same way, distinct GC ionization sources have been applied to achieve enhanced sensitivity, like atmospheric pressure (AP) source. This recently commercialized modality provides better ionization results than electron ionization (EI) or chemical ionization (CI) sources, enhancing selectivity and, consequently, sensitivity. AP source has been used for the quantitative determination of cannabinoids in serum samples at trace levels [35]. This softer ionization technique is based on the ionization of compounds by corona discharge in the presence of nitrogen, operating at atmospheric pressure. However, EI has been employed as the common ionization source for the analysis of *Cannabis*. This modality is characterized by its higher ionization energy, which produces extensive fragmentation of the molecular ion, thus reducing the possibility of using the better known and higher mass precursor ions (typically used in ESI-LC–MS) in selected reaction monitoring mode (SRM) and, on the other hand, the presence of higher mass

precursor ions in CI improves the potential for lower detection limits in CI SRM as compared to EI SRM.

Phytocannabinoids have been widely analyzed by GC techniques, using predominantly FID and MS as detectors. One of the main shortcomings of the analysis of these compounds by GC is the direct analysis of acidic phytocannabinoids, which undergoes decarboxylation in the injector port due to the high temperatures. This drawback can be avoided by derivatization processes, which increase the chemical stability of the acidic compounds [9, 36]. For this reason, generally liquid chromatography (LC) is preferred since derivatization step is avoided. Nevertheless, greater sensitivity is reached with GC methods, still being the instrumental technique of choice. Franklin et al. reported the headspace derivatization of cannabinoids during HS-SPME step. To this end, 5 μL of derivatization reagent, MSTFA, was inserted in a 20 mL sample vial with the aid of a vial insert. The presence of MSTFA effectively derivatized the neutral cannabinoids enhancing the sensitivity of the determination. However, an earlier derivatization of the acidic cannabinoids is necessary since the performance of the headspace derivatization in this case was not so effective [37].

An additional problem associated with the determination of cannabinoids by GC–MS is the difficulty to distinguish between isomers without compromising analysis time. This is the case of Δ^9-THC, which presents four isomers: Δ^8 -THC, CBD, CBC, and CBL. Some strategies have been developed to overcome this problem, like the inclusion of two-dimensional GC (GCxGC) technique, which allows the identification of complex samples, identifying biomarkers and cannabinoid isomers [5]. In this case, stir bar sorptive extraction (SBSE) coated with poly-(dimethylsiloxane) (PDMS) was utilized allowing the preconcentration of a wide range of compounds with different volatilities. The retained metabolites were directly desorbed in a GCxGC–MS system, resulting in a green analytical treatment procedure. Although isomers resolution is solved with this system, analysis time remains a problem since it is necessary to perform an extraction step for at least 60 min in addition to the 93 min per run. Nevertheless, the automatization of the process may reduce human involvement and therefore systematic and random errors could be limited. Conversely, the use of GC with vacuum UV spectroscopy (VUV) could be a potential solution since differentiation among isomers may be solved in shorter analysis times (<15 min). This technology analyses compounds in the UV/VUV spectral range (120–240 nm) and is based on the excitation of chemical bonds [11]. This strategy has been utilized for the determination of different cannabinoids and their metabolites. The deconvolution of co-elution peaks allows the reduction of the chromatographic time and therefore the analysis process. However, this solution constitutes an improvement at the expense of sensitivity.

2.3 Synthetic cannabinoids

Synthetic cannabinoids (SCs) are a class of designed drugs that simulates the effects of Δ^9-THC towards cannabinoid receptors. Originally, the synthesis of these compounds was intended with therapeutic purposes as a treatment of pain, being later introduced in the recreational drug market [38]. Some of the best-known SCs include AB-FUBINACA (N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-H-indazole-3-carboxamide), AB-CHMINACA (N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluoro-benzyl)-1H-indazole-3-carboxamide), XLR-11 ((1-(5-fluoropentyl)-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl) methanone) and HU-210 (1,1-Dimethylheptyl-11-hydroxy-tetrahydrocannabinol), commercialized in many European and US countries as 'Spice', among others. Some of these cannabinoids are represented in **Figure 1**. There is a real risk consuming

this type of drugs since their chronic abuse often leads to dangerous side effects as well as toxicity and, even in some cases, resulting in death. In addition, new psychoactive substances are constantly emerging, presenting unknown and unexpected effects. Consequently, further research is needed in this field entailing an enormous challenge, since it requires a continual actualization of the analytical techniques with the lack of available chemical reference standards. Chromatographical techniques like GC has become an important tool in this field to determine the prevalence and to assess the risks of SCs [39]. An additional problem is related to the chemical structure of these compounds, which is not well defined and therefore hinder even further their detection. While some of them, denoted as classical SCs, are structurally like natural cannabinoids, others named nonclassical and hybrid SCs present different structural features. For this reason, researchers studied fragmentation pathways of these compounds to facilitate their identification by GC–MS [40]. Although this process appears simple, some compounds result in similar mass spectra, impeding the identification of the SCs. Tandem and high-resolution MS may solve this obstacle, but generally, these equipments are not easily accessible. Therefore, more economically feasible solutions are needed. One cost-effective solution to this issue could be the utilization of different ionization modes, such as photoionization (PI). Akatsu et al. reported the utilization of GC-PI-MS for the identification of 62 SCs. This technique allows the detection of stable neutral compounds with low ionization threshold using a radical cation produced by ultraviolet light radiation which traps an electron from the target molecule. This effect is not possible with EI, which is an adequate technique for relatively high ionization threshold. In this way, with the combination of both ionization modes, it would be possible the identification of SCs [41]. Additionally, alternative ionization sources as cold-EI or CI have been described to overcome the SCs lability towards EI. These techniques increase the abundance of molecular ions allowing the discrimination between structurally-related compounds enhancing at the same time the limit of detection (LOD) and quantification (LOQ) of the procedure (see **Table 1**) [42, 43]. Alternatively, the combination of the GC-EI-MS and GC-IR techniques allowed the separation of six SCs regioisomers with excellent resolution results [44]. Additionally, Umebachi et al. reported the combination of GC–MS using EI, PCI and NCI for the structural elucidation and identification of different SCs in herbal products. The fragmentation of the indole and indazole SC structures was monitored with the different ionization modes, using methane as reagent gas. Although EI gives enough information about the fragmentation pattern of the molecules, PCI and NCI provide the $[M+H]^+$ and $[M-H]^-$ fragment ions, which could be used for the molecular weight estimation [45].

On the other hand, complexity of samples may cause a reduction in the sensitivity of the determination as well as to affect the chromatographic system, particularly the stationary phase of the analytical column. Consequently, routine methods for identification of new psychoactive substances generally require quick, cheap and simple extraction methods. Scientists have been working for many years addressing this deficiency, providing new and upgraded extraction techniques to remove the undesired sample interferences. Mercieca et al. have developed a simple method to determine SCs and their metabolites in urine and blood samples. The procedure included ultrasound-assisted liquid–liquid microextraction (UALLME) and silylation derivatization coupled with GC–MS, being able to effectively identify 36 SCs and related metabolites with acceptable accuracy and precision results [7]. In the same way, HS-SPME-GC/MS has been utilized to determine 40 SCs in herbal mixtures as well as blood samples. The utilization of the SPME methodology allows the reduction of blood sample volume to 500 μL, of great advantage in the forensic analysis [46]. Recently, a new configuration using microextraction by packed

sorbents (MEPS) to isolate five SCs from oral biofluids has been reported. This method allows the quantification of these compounds in the low μg/L level by GC-EI-MS, requiring just 500 μL of sample. MEPs configuration allows the potential automatization of the process for routine analysis, improving the method precision [47].

3. Terpenes

Apart from cannabinoids, *Cannabis sativa* L. is rich in terpene compounds, being the major components of the *Cannabis* essential oils (EO). In the same way to cannabinoids, hemp EO is secreted by glandular trichomes, which are presented in the leaves and inflorescences of the plant. Terpenes represent the volatile fraction, responsible for the characteristic smell of the plant [48]. Moreover, their health benefits and antimicrobial and insecticidal properties have recently increased the interest in the hemp EO. In addition, some studies reveal a synergistic action of terpenes with cannabinoids, known as 'entourage effect', in the treatment of pain, inflammation, depression, anxiety, addiction, epilepsy, cancer, and infections [4]. For this reason, more than 120 terpenes have been identified in *Cannabis* using GC-FID and GC–MS. Furthermore, recent studies have revealed that the terpenoid profile is a useful tool since compositional differences may be used to distinguish between *Cannabis* cultivars that have similar cannabinoid content [49, 50]. Terpenes may be classified according to their number of carbon atoms and the isoprene residues present in their structure as monoterpenes, diterpenes, triterpenes and sesquiterpenes, being the monoterpenes α-pinene, β-myrcene and α-terpinolene, and the sesquiterpenes β-caryophyllene and α-humulene the most abundant compounds in *C. sativa* L (**Figure 2**).

Some strategies have been developed to elucidate the terpene profile in *Cannabis* samples (see **Table 2**). This is the case of HS, coupled in some cases to SPME [51, 52], and two-dimensional GCxGC [5]. Meiri et al. reported the simultaneous analysis and quantification of 93 terpenoids in *Cannabis* inflorescences by static headspace (SHS) coupled to GC–MS/MS at very low μg/L level [53]. SHS allows the direct analysis of plant materials without sample treatment, which may affect negatively to the terpenoid content. Additionally, direct injection of these complex sample matrices is not recommended because coextracted interferences potentially hinder terpenes determination. On the other hand, low-volatility compounds may

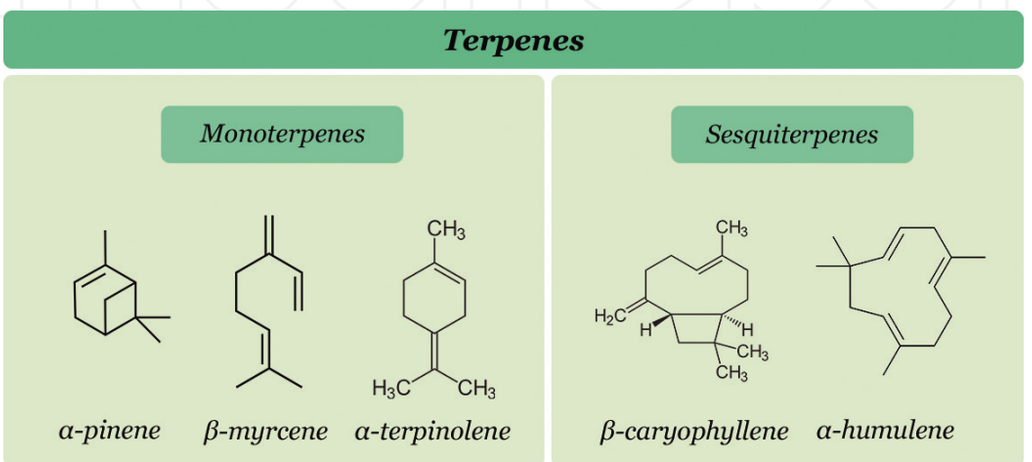

Figure 2.
The most abundant terpenes in Cannabis sativa *L.*

TERPENES	Sample treatment			Instrumental technique				Analytical features				Ref.
Sample	Amount of sample	Derivatization	Extraction	Technique	Ionization source	Column	Analysis time (min)	LOD	LOQ	Precision (RSD, %)		
										Intra-assay	Inter-assay	Recovery (%)	
EO	—	—	SPME	HS-SPME-GC–MS	EI	DB-5	60	—	—	—	—		[51]
Inflorescences and macerated oils	100 mg	—	SPME	HS-SPME-GC–MS	EI	Rtx-Wax	33	—	—	—	—		[52]
Inflorescences	5 mg	—	—	SHS-GC–MS/MS	EI	DB-35MS UI	74	0.001–0.123 μg/mL	0.002–0.374 μg/mL	<11.4	<21.9	81.2–119.6	[53]
Plant (leaves, inflorescence and seeds)	1 g	MSTFA:TMCS, 99:1, v/v	—	fast-GC-FID	EI	Restek RTX-5	<16	0.03–0.27 μg/mL	0.10–0.89 μg/Ml	3.59	7.82	77.52–107.10	[2]
Cannabis oil	400	—	SPME	HS-SPME-GC–MS	EI	HP-5-cross-linked poly-5% diphenyl-95% dimethyl polysiloxane	63	—	—	—	—		[55]

EI: *electron ionization;* ***EO:*** *essential oil;* ***FID:*** *flame ionization detector;* ***GC:*** *gas chromatography;* ***HS:*** *head space;* ***MS:*** *mass spectrometry;* ***MSTFA:*** *N-Methyl-N-(trimethylsilyl)trifluoroacetamide;* ***SHS:*** *static head space;* ***SPME:*** *solid phase microextraction;* ***TMCS:*** *trimethylchlorosilane.*

Table 2.
Analytical platforms for the analysis of terpenes in Cannabis *samples.*

be retained in the injection port requiring more frequent maintenance [54]. Nevertheless, some strategies have been reported by direct injection using fast-GC-FID, analyzing 29 terpenes and CBD from leaf samples in less than 16 min [2]. Despite higher amount of sample is used in the later approach, the sensitivity is affected, being approximately 10-fold lower than the GC–MS/MS procedure, probably due to the utilization of different detection systems. On the other hand, the precision is improved after the derivatization with MSTFA:TMCS.

Sample storage plays an important role in the terpene analysis, having monoterpenes a tendency to evaporate more easily than sesquiterpenes. For this reason, special attention is required when analyzing these compounds. Some strategies have been evaluated such as using frozen samples instead of dried plant material or dynamic maceration at room temperature of plant inflorescences, providing excellent results [9, 55]. Additionally, oxygenated terpenoids, because of the presence of light or oxygen, may reveal some problems during plant storage and processing. Thus, the terpene profile provides interesting information about the EO or the starting plant material, such as the aging of the product, bad storage conditions or quality and breeding conditions. Terpene analysis provides these kind of information to take into consideration [25].

4. Other compounds

As it was described before, *Cannabis sativa* L. is mainly composed by cannabinoids and terpenes. However, some studies were focused on the determination of other minor compounds, like fatty acids and carbohydrates (see **Table 3**). Devi et al. reported the analysis of hemp oil by GC–MS for the determination of fatty acids and other compounds to be exploitable as biofuel [56]. In their study, different solvent extraction techniques were evaluated, such as supercritical fluid extraction (SFE), Soxhlet (SOX) and percolation (PER), among others. Although SOX is the best energy-efficient process, SFE is preferable in terms of purity of the obtained oil. However, alternative strategies might be implemented since large amount of solvents and extended extraction times make this approach economically and environmentally unattractive. On the other hand, an innovative method using hemp steam treatment has been described [57]. Steam and hot water were used to extract different compounds from diverse parts of hemp plants, such as stalk and leaves, using LLE and GC–MS for this purpose. The procedure allows the identification of several compounds like carbohydrates, fatty acids and aldehydes, among others. This procedure represents an improvement in comparison to the Devi et al. approach since analysis time is greatly reduced as well as solvent consumption. Additionally, Delgado-Povedano et al. reported two analytical platforms (GC-TOF/MS) and (LC-QTOF-MS/MS) for the untargeted characterization of *Cannabis* extracts, identifying a wide range of families including lipids, flavonoids, and amino and organic acids, among others, in multiples varieties of medicinal *Cannabis* [50]. The GC platform allowed the identification of 134 compounds in plant extracts in comparison with the 46 compounds identified by LC.

In the countries where *Cannabis* has been legalized for medical purposes, the governing agencies have established a series of strict regulations to guarantee the quality, safety, and usefulness of *Cannabis* products. Generally, these guidelines include the necessary quality control of pesticides, residual solvents, mycotoxins, heavy metals, and microbes to agree with the GMP system. The first two contaminants are widely controlled in the pharmaceutical industry using GC methodologies for their analysis. Short- and long-term adverse health effects are associated with the exposure to these contaminants through consumption of *Cannabis* products [58].

OTHER COMPOUNDS	Sample treatment					Instrumental technique				Ref.
	Sample	**Amount of sample**	**Target**	**Derivatization**	**Extraction**	**Technique**	**Ionization source**	**Column**	**Analysis time (min)**	
	Seed oil	—	Fatty acids	—	SFE/SOX/PER/ULT/UTS/STU	GC–MS	EI	DB-5MS	60	[56]
	Plant	2 mg	Carbohydrates	TMCS:HMDS	LLE	GC–MS	EI	HP-5 ms	28	[57]
	Plant (leaves and inflorescences)	100 mg	Untargeted	BSTFA + TMCS	—	GC-TOF	EI	DB-5MS-UI	53	[50]
	—	—	Pesticides	—	—	GC–MS/GC–MS/MS	EI	ZB- multiple residue-1	—	[58]
	Concentrated samples	100 mg	Residual solvents	—	—	HS-GC–MS	EI	SHRXI- 5MS	7	[60]

BSTFA: *N,O-bis (trimethylsilyl) trifluoroacetamide;* ***EI:*** *electron ionization;* ***GC:*** *gas chromatography;* ***HMDS:*** *hexamethyldisilzane;* ***HS:*** *head space;* ***LLE:*** *liquid–liquid extraction;* ***MS:*** *mass spectrometry;* ***PER:*** *percolation;* ***QTOF:*** *quadrupole time-of-flight;* ***SFE:*** *supercritical fluid extraction;* ***SOX****: Soxhlet;* ***STU:*** *Soxhlet treated ultrasonication:* ***TMCS:*** *trimethylchlorosilane;* ***ULT:*** *ultrasonication;* ***UTS:*** *ultrasonication treated Soxhlet.*

Table 3.
Analytical platforms for the analysis of other minor compounds in Cannabis *samples.*

In the cultivation of *Cannabis*, pesticides are often applied to repel or eliminate unwanted pests. Despite the fact that this activity is required from an agricultural point of view, it may also affect human health due to the remain of pesticide residues in *Cannabis* and its derived products. For this reason, much attention has been focused on analyzing these compounds to guarantee the safety and quality of the final pharmaceutical drug. Different analytical methods have been applied to this end, being GC–MS one of the most common encountered techniques [21]. However, one of the main limitations associated with *Cannabis* pesticide analysis is the low-level concentration of these pollutants in the sample in comparison to cannabinoid and terpene content. For this reason, different methods have been reported to remove potential interferences and enable the analysis of residual pesticides, as LLE, SPE, SPME and quick, easy, cheap, effective, rugged, and safe (QuEChERS) [58]. Conversely, in the last years, some researchers have evaluated the potential of *Cannabis* EO as botanical insecticide. The toxicity of these products has been tested with different insect pests like aphids and houseflies, among others [51, 59]. In this way, the exploitation of hemp by-products represents an opportunity of creating a circular economy.

In the analysis of products derived from *Cannabis*, which is generally manufactured from extracts obtained with organic solvents, there are not many publications dealing with residual solvents, despite the high health risk associated with these contaminants. Probably, this is linked to the relatively recent legalization of the medical use of *Cannabis*. As a matter of fact, Raber et al. have reported the use of HS-GC–MS for the qualitative analysis of these impurities in cannabinoid concentrates [60] but it would seem plausible to think that the scarcity of recent scientific publications derives from the existence of official directions for the quantification of such residues, such as national and international alimentary codices and pharmacopeias.

5. Conclusions

Gas chromatography is a well-established tool in the *Cannabis* sector. Owing to the extraordinary health benefits of the bioactive compounds of *Cannabis sativa* L., reliable methods to quantify them are required for quality assurance. Additionally, cannabinoid and terpenoid profiles provide useful information to distinguish between *Cannabis* cultivars. Furthermore, this technique is utilized in the fight against the illegal recreational use of *Cannabis*, detecting Δ^9-THC and their metabolites in different biological samples and identifying new synthetic cannabinoids. For this reason, several analytical platforms have been developed using different modalities, such as (fast)-GC-FID, GC–MS, GC–MS/MS, GC-TOF/MS or GC-IR. Among them, GC–MS is the most utilized technique probably due to their accessibility as well as the quality of the results. Additionally, the features of this modality may be easily modified changing the ionization source, which might enhance the sensitivity and selectivity of the measurements. Thus, several ionization sources like EI, PCI, NCI and AP have been utilized in the analysis of *Cannabis*. On the other hand, although GC-TOF/MS is a more sophisticated technique, which is not as easily accessible as GC–MS, some approaches employing this modality have been already published allowing the untargeted analysis of *Cannabis* metabolites. Regarding the complex chemical composition of *Cannabis* plant, different strategies have been performed avoiding matrix effect and solving simultaneously low concentration, and chromatographic resolution problems in some cases. In addition, different extraction techniques have been utilized to determine *Cannabis* metabolites in different matrixes. Microextraction techniques like SPME, SBSE or MEPS

have been applied, which involve an advantage concerning other extraction modalities since generally solvent and time consuming is minimized. In this sense, there are an extensive range of possibilities to overcome the different problems that may emerge in the analysis of *Cannabis*. Conversely, although sometimes GC approaches are non-preferred in order to avoid time-consuming derivatization steps, this process allows the analysis of instable compounds. This is the case of acidic cannabinoids, which generally are subjected to silylation processes to avoid decarboxylation in the injection port of the GC. Additionally, derivatization procedures improve the chromatographic resolution as well as the peak shape of analytes, enhancing analytical features like sensitivity and reproducibility.

Conflict of interest

The authors declare no conflict of interest.

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