Reviews

Development and Early Identification of Cannabis Chemotypes during the Plant Growth: Current Analytical and Chemometric Approaches

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The identification of cannabis chemotypes at an early stage of a plant's growth, which is long before anthesis, has been intensively pursued in order to control the on-target selection of the cultivar type at the beginning of cultivation, so as to avoid economic and legal drawbacks. However, this issue has been systematically addressed by only few and relatively recent studies of analytical chemistry, possibly because result validations require long-term monitoring of the content and ratio of cannabinoids and terpenes in a great number of plant specimens suitably selected and grown. Here, we review the procedures, the chromatographic techniques and the statistics used in topical investigations during the past thirteen years. Through heterogeneous and not easily comparable approaches, they prove the feasibility of chemotypes safe determination within the first month of a plant's life.

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Marco Pallavicini received his PhD in "Chimica del Farmaco" in 1986 at Milan University. In 1990, he became Assistant Professor at the same university and, eight years later, Associate Professor. Since 2005, he has been Full Professor of Medicinal Chemistry at Milan University. From medicinal chemistry, his interest has extended to analytical characterization of biological active compounds and their precursors in unichiral form, in particular by NMR spectroscopy, DSC and chiral HPLC.

Eleonora Casagni obtained her Bachelor's degree in Pharmaceutical Chemistry and Technology at the University of Bari in 2001. She is chemistry lab technician at the Department of Pharmaceutical Sciences of the University of Milan and she is expert in pharmceutical and forensic research. She is involved in scientific projects aiming at developing analytical methods by liquid and gas chromatography techniques for the detection of drugs in different matrices. In recent years her work has focused on the analysis of the main cannabinoids in Cannabis inflorescences and preparations.

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1 Introduction

After a gradual decline in the mid-20th century, principally due to legal restrictions, the medical use of *Cannabis sativa* L. female inflorescence started to increase at the end of the '90s, and it has greatly increased in the last ten years, supported by strong evidence of its effectiveness for treating various disease conditions.[1-7] Moreover, its therapeutic potential seems to be far from being fully exploited, and new important clinical applications are emerging.[8-10] A linear increase in the annual number of scientific publications dealing with *Cannabis*, from a little less than 1000 in 2010 up to about 2000 in 2015, and more than 4000 last year, testifies to the great research interest in this plant and the pressing need to address the many issues concerning its breeding, processing, distribution and use. Each of these activities needs to be continuously supported and monitored by morphological, chemical and biological analysis. The content of active constituents and the presence of contaminants or adulterants[11,12] are the two main aspects that need to be ascertained for a safe, effective, reproducible and regulated medical use of this botanical drug, which is also the most frequently used illicit drug worldwide.[13] Hence, it is no surprise that most of the documented research efforts in this field focus on the development, application and surveillance of fit-for-purpose standard analytical methods.

Cannabis is a heterogeneous matrix containing a complex secondary metabolome with an uneven constituents distribution.[14] Cannabinoids, which are terpenophenolic compounds, are the major constituents and those most widely recognized as responsible for the pharmacological effects of the plant. They are more than one hundred and they are present in all of the aerial parts of the plant, but achieve the maximum concentration in the female flowers, preferentially located in the large-capitate stalked glandular trichomes on female flowers bracts and bracteoles.[15] The most common ones are those with pentyl side chains at the phenol nucleus, while the propyl homologues are generally minor constituents. The pentyl substituted cannabinoids are biosynthesized as prenylated aromatic carboxylic acids from cannabigerolic acid (CBGA), which is then transformed into tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), and cannabichromenic acid (CBCA) by specific synthases (Fig. 1).[16,17] The carboxy group is not very stable, since it is easily lost under the influence of
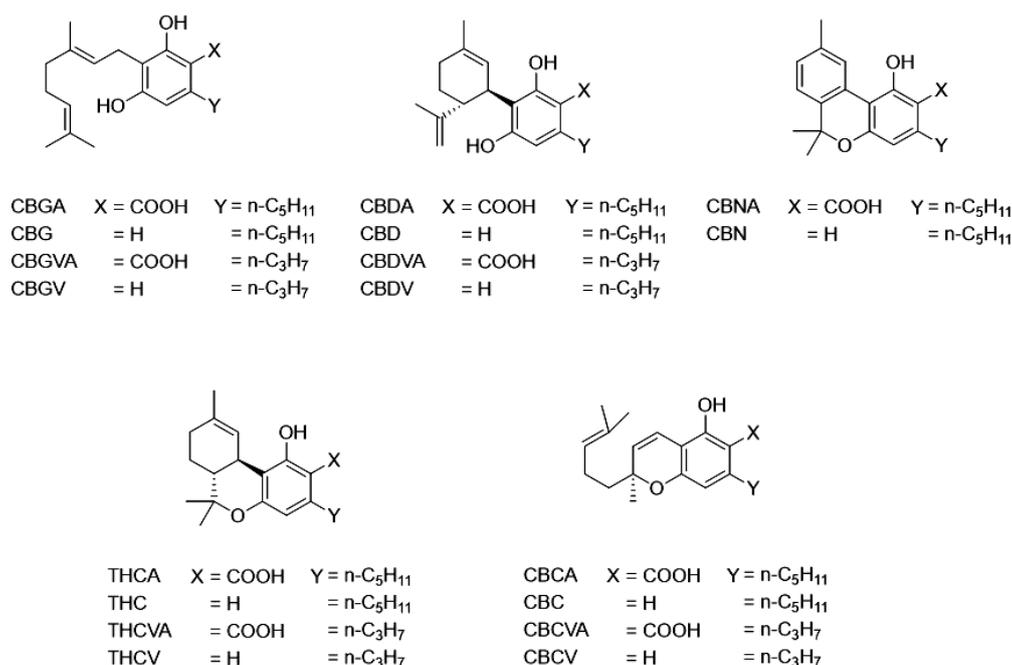

CBGA	X = COOH	Y = n-C_5H_{11}	CBDA	X = COOH	Y = n-C_5H_{11}	CBNA	X = COOH	Y = n-C_5H_{11}
CBG	= H	= n-C_5H_{11}	CBD	= H	= n-C_5H_{11}	CBN	= H	= n-C_5H_{11}
CBGVA	= COOH	= n-C_3H_7	CBDVA	= COOH	= n-C_3H_7		
CBGV	= H	= n-C_3H_7	CBDV	= H	= n-C_3H_7		

THCA	X = COOH	Y = n-C_5H_{11}	CBCA	X = COOH	Y = n-C_5H_{11}
THC	= H	= n-C_5H_{11}	CBC	= H	= n-C_5H_{11}
THCVA	= COOH	= n-C_3H_7	CBCVA	= COOH	= n-C_3H_7
THCV	= H	= n-C_3H_7	CBCV	= H	= n-C_3H_7

Fig. 1   Chemical structures of the major acidic and neutral cannabinoids in *Cannabis*.

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Veniero Gambaro obtained hid degree in Pharmacy in 1972 at the University of Pavia. Since 1977, he has been Associate Professor in Toxicological Chemistry at the University of Milan. His research activity was devoted to the analysis of drugs of abuse on behalf of the judicial authority either on biological and non biological samples.

Michele Dei Cas obtained his PhD in Molecular and Translational Medicine in 2021 at the University of Milan. At the moment, he has a Postdoctoral Fellow in Clinical Chemistry in the Department of Health Sciences. His scientific interests are related to the application of -omics approaches in the fields of laboratory medicine from forensic toxicology to clinical biochemistry.

Gabriella Roda received her PhD in Chemistry in 2000 at the University of Milan; since 2002 she has been Assistant Professor in Medicinal Chemistry at the Faculty of Pharmacy. In 2017, she gained the scientific qualification for Associate Professor in Legal Medicine. She is involved in pharmaceutical and toxicological analysis and much of her research effort is devoted to the drugs of abuse with particular attention to the analysis of cannabinoids in both galenic preparations of medicinal Cannabis and industrial hemp.

heat or light, giving corresponding neutral cannabinoids (CBG, THC, CBD and CBC) (Fig. 1).[18]   Therefore, any chemical classification must take into account of both the content of THC and CBD and the content of THCA and CBDA quantitatively convertible into THC and CBD, so determining the so-called "total THC" and "total CBD".[13] Cannabinol (CBN) is produced by the oxidative degradation of THC and it is commonly found in aged cannabis samples (Fig. 1).[19] Terpenes, mostly mono- and sesquiterpenes, and terpenoids, in all about 140, form the other large category of cannabis secondary metabolites, generally amounting to 2 – 5% of the inflorescence dry weight. They contribute to the organoleptic characteristics of the plant, and may act synergistically with cannabinoids ("entourage effect"). Their content, in terms of quality and quantity, could be a basis for a further phytochemical subcategorization of cannabis.[20]

Indeed, it is the phytochemical profile in cannabinoids, substantially defined by the relative abundance of THC and CBD, that identifies the "chemotype" or "chemical phenotype" of individual plants or of populations of cannabis. It is genetically controlled by the presence at the B locus of two codominant alleles, B_D and B_T, responsible, respectively, for the occurrence of CBDA and THCA in the plant.[21,22] Chemotype I is the THC-dominant one (total THC/total CBD >> 1, total THC > 0.3% of inflorescence dry weight); chemotype II is defined as intermediate THC/CBD (total THC/total CBD close to 1, usually 0.5 – 2); and chemotype III, namely "hemp" or "fibre-type", is the CBD-dominant one (total THC/total CBD << 1, total THC < 0.2 or 0.3% of inflorescence dry weight). Chemotypes IV and V are additional fibre-type plants, the former containing CBGA as the main cannabinoid and the latter containing almost no cannabinoids.[23]

The differential activities of cannabinoids in humans, particularly of THC and CBD, provide a rationale for such chemotype classification based on the THC/CBD ratio, which has become a sound reference to establish regulatory requirements and to develop guidelines for cultivation and processing. In any case, it should be clear that the chemical phenotype is unsuitable as a taxonomic criterion, provided that different cannabis species exist, but it is strictly and usefully related to the "cultivar" concept.[24] A cultivar is made up of plants of the same species that have been selected by man for particular characteristics and that uniformly maintain these characteristics when propagated by appropriate means. This implies that a successful standardization of cannabis materials, necessary for safe medical use and reproducible clinical trials, depends on whether they derive from clonally propagated uniform cultivars that, if grown under defined conditions, can produce consistent chemical profiles. Therefore, analytical methods suitable for recognizing chemotypes and verifying the conditions ensuring their segregation and stability are highly desirable, and they would be even more effective if chemotypes discrimination could be possible in young specimens with high classification performance. In fact, a praecox diagnosis of the bred chemotypes during growth is very important to prove that the plantation is legal at an early stage avoiding illicit cultivation, even if unintentional, and the consequent penalties and economical drawbacks.

Here, we provide an overview of studies that in recent years have addressed the issue of cannabis chemotypes identification through analyses of the cannabinoid and terpene content all along the growth, since the early weeks of the life of the plants with the aim of determining the chemotype as soon as it has definitely stabilized, which is long before plants' flowering.

2 Defining Chemotype and Obtaining Medical Grade Products

In defining the chemotype, qualitative and quantitative aspects must be distinguished. The THC/CBD ratio is the qualitative chemotype, controlled by simple monogenic mechanisms, substantially unaffected by environment and showing a discrete distribution in progenies.   The quantitative aspect of the chemotype is the total cannabinoid content. It is under polygenic control and it has a Gaussian distribution in progenies, and is affected by the environmental conditions.[21,25] The yield in a single target cannabinoid is related to both the qualitative (mutual ratio of cannabinoids) and quantitative (total cannabinoid content, botanical raw material, namely leaf and inflorescence, proportion) aspects. Historically, most effort has been placed on finding and breeding plants with high THC concentrations for recreational use.[26]   Since the late 1990s, breeding programs involving academic institutions and pharmaceutical companies have been committed to selecting other cannabis chemotypes, not only CBD-enhanced and THC-poor. As a result, a collection of chemotypes dominant in each one of the eight cannabinoids (THC, CBD, CBG, CBC and the corresponding propyl homologues) has been created, and thus ensuring quality supplies for medical research and for pharmaceutical product development.[27,28]   The success of these programs was strictly dependent on standardized and controlled growing methods aimed at producing a uniform product and on indoor and outdoor propagation protocols, by cuttings or from all-female seeds, with no detectable variations in morphologic, physiologic, biochemical, and genetic profiles as compared to the mother plants. Within these projects, cannabinoid contents and profiles were monitored by GC-FID after drying, decarboxylating and extracting the harvested biomass under standardized conditions.[28]

Despite the success of such advanced and pioneering production programs, understanding the chemotype development during growth and early differentiating, in a forensic perspective, drug-type plants from nondrug types remain major current issues in the large-scale production of cannabis, which have attracted the investigation interest of more than one research group.

3 Identification of Chemotypes during the Growth

Topical analytical studies on cannabis chemotype development during growth are relatively recent and sporadic over the last thirteen years. Furthermore, those approaching the issue in a systematic way are heterogeneous for sampling plans, considered analytes, samples type, monitored growth period, and analytical techniques. Therefore, a critical comparison would be necessary, but very difficult. Such differences are schematically summarized in Table 1 and detailed in the following paragraphs, each of which has been devoted to one of these investigations.

3·1 Periodical GC-FID quantification of THC, CBD and CBG in leaves from 28 to 103 days after sowing

The first systematic study on chemotype development during the growth of *Cannabis sativa* L was reported by Mandolino *et al.* in 2008.[23] Young expanded leaves periodically picked up from each of 116 monitored plants were the botanical material submitted to analysis after drying at 65°C for 48 h and powdering. Samples of these materials (100 mg, 5% residual humidity) were individually analyzed by GC-FID to quantify THC, CBD and CBG. The powdered materials were extracted

Table 1　Reviewed studies on the chemotype development during cannabis growth

Ref.	Chemo type	Plants no.	Plant material	Sampling plan (DAP: day after plantation; RG: root-growing phase)	Analytes	Analytical technique	Statistics
23 (2008)	I, II, III	116	Leaves	9 samplings over 28 – 103 DAP period	THC, CBD, CBG	GC-FID GC-MS	
			Flowers	1 sampling at 150 DAP			
29 (2010)	I, III	360	Leaves	1 sampling at 28 DAP	THC, CBD, CBN, CBG, CBL, THCV	GC-MS	PCA LDA SVMs
					6 terpenes		
31 (2012)	I	65	Leaves	Weekly samplings until to flowering	THCA, THC, CBDA, CBD, CBGA, CBG, CBN	HPLC-DAD	
			Flowers	Weekly samplings since flowering			
33 (2016)	I, II, III	350	Leaves	23 samplings since RG until to 179 DAP	THCA, THC, CBDA, CBD, CBGA, CBG, CBN, CBC	HPLC-DAD (cannabinoids) GC-MS GC-FID (terpenes)	PCA PLS
			Flowers	9 samplings over 122 – 179 DAP period			
					28 terpenes		
39 (2018)	I, II, III	99	Leaves	1 sampling at vegetative stage 1 sampling at flowering stage	THCA, THC, THCVA, THCV, CBDA, CBD, CBDVA, CBDV, CBGA, CBG, CBN, CBC	HPLC-DAD-MS	R^2 regression analysis
			Flowers	1 sampling at maturation stage			Non-hierarchical k-means cluster analysis

with hexane (5 mL). Squalene and squalane were added as an internal standard and reference standard, respectively. The carrier gas was helium. Compound identities were initially determined by GC-MS, and by comparing the retention times with those of commercial cannabinoids, used as standards. These were also used for the quantitative determinations carrying out calibrations at various concentrations. Response factors were estimated for the three cannabinoids and the total content of the three cannabinoids and that of each of them were expressed as percent of the leaf dry-weight, namely milligrams in 100 mg of dried material extracted and analyzed. The experimental plan consisted of monitoring the content of the three cannabinoids in the leaves of (A) 44 plants of a cannabis variety corresponding to chemotype III, (B) 40 plants obtained by hybridization between a drug- and fibre-variety and corresponding to chemotype II and (C) 32 plants from seized seed corresponding to chemotype I. Monitoring started at 28 days after plantation (28 DAP) and finished at 103 DAP. Nine timetabled leaf sampling were made from each of the 116 plant during this period and an additional inflorescence sampling at 150 DAP and the three cannabinoids contents were determined for each sample (1160 determinations). The results of such analyses can be summarised as follows:

1　The average amount of total cannabinoids in leaves similarly increased for all of the three accessions until about 60 DAP. Starting from 80 – 85 DAP, the amount decreased in chemotypes II and III, while it was relatively constant in chemotype I (Fig. 2). The highest value of total cannabinoid (6.36%) was registered in a sample of chemotype II at 90 DAP.

2　Since the earliest sampling (28 – 40 DAP), CBD was always dominant in chemotypes II and III, and the mean THC was always <0.2% (threshold set to issue subsidies to hemp growers) in chemotype III. THC remained <0.2% also in inflorescences of chemotype III. The mean THC was
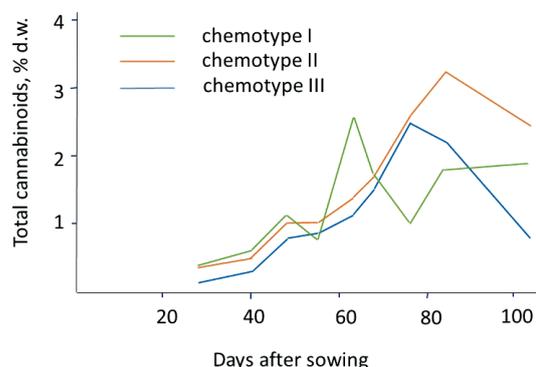

Fig. 2　Time courses of the average total cannabinoid content (percent of the leaf dry-weight) for 1044 leaves of the three chemotypes examined.

always >0.2% in chemotype I (plants from seized seeds) (Fig. 3).

3　The THC/CBD ratios characterizing the three chemotypes (<<1, ≈1, >>1) remained strongly segregated during the 103 days of monitoring. The chemotype was maintained by all plants during the entire life cycle, from the earliest leaves to the anthesis. Among 1044 samples, a switch from the chemotype III to the chemotype II was observed only in four specimens. Furthermore, one of the 44 plants of chemotype III should be assigned to chemotype IV, since its CBG content was systematically higher than both CBD and THC. No CBG prevalent plants were found in the other two accessions.

Based on these results, the authors concluded that it is not necessary to wait for anthesis, and to analyze flowers to get safe and reliable information about the chemotype. As already stably

configured 28 – 40 days after sowing, the chemotype can be determined at an early life stage. This is very important not only for assessing eligibility for fibre hemp subsidies, but also for forensic and legal purposes as well.

3·2 GC-MS quantification of 8 cannabinoids and 6 terpenes in leaves at 28 day after plantation

Two years later, the differentiation of fibre- and drug-type cannabis plants was the issue addressed by Swiss researchers that started to widen the analysis to the relative proportions of the major compounds found in cannabis seedlings' leaves 28 days after sowing, and to use chemometrics tools to process the obtained data.[29] Fifteen plants of each of 11 legal cannabis varieties (chemotype III) and of 13 illegal ones (chemotype I) were grown under standardized conditions and analyzed by GC-MS according to a protocol similar to the one described above. Areas of targets ions of 14 identified compounds (8 cannabinoids and 6 terpenes) in the chromatograms were determined and normalized to the internal standard squalane. The data were the square-rooted in order to reduce the influence of larger peaks and thus to have the variables on a comparable scale. Finally, data were scaled to zero mean and unit variance. The United Nations Office on Drugs and Crime proposes a value 1 of the index X, which is the $([THC]+[CBN])/[CBD]$ ratio, to discriminate between chemotype I ($X > 1$) and chemotype III ($X < 1$).[30] Assuming that the chemotype classification of the seeds was unquestionable and, consequently, that 15×11 (165) and 15×13 (195) plants should be recognized already 28 DAP as belonging to chemotype III and to chemotype I, respectively, the authors verified that the classification based on the above criterion ($X >$ or < 1) led to misclassify 0.6% of fibre type samples and 7.5% of drug type samples. On the other hand, principal component analysis (PCA) was performed. Eight

initial variables were selected: the content of four among the six analyzed terpenes (guaiol, γ-eudesmol, bulnesol, α-bisabolol) and of four among the eight analyzed cannabinoids (THCV, CBD, THC and CBN). A plot of PC1 against PC2 for each sample clearly showed that some expected fibre type and drug type samples were non-linearly separable leading to and overlapping area. Then, two classification methods, linear discriminant analysis (LDA) and support vector machines (SVMs), were tested. To assess the quality of the model, the analyzed samples were divided into two groups: the training samples, on which the model is built, and the test samples, on which the predictive capability is tested. The separation into training and test sets, randomly created, was repeated 1000 times and then the model was validated by a test performed on an independent set consisting of 44 cannabis seedlings, grown under unknown conditions and belonging to drug-type cannabis. SVMs performed much better than LDA. Over 1000 iterations, it gave, in the test set, 0.3 and 1.3% of what are called by the authors "false positive drug-types" (FPD) and "false positive fibre-types" (FPF), respectively, while the FPD and FPF percentages were 0.3 and 6.0 for LDA (Table 2). Overall, regardless the data processing methods used to minimize the titled "false positives" and the additional analytes selected as diagnostic indicators, the study confirms the feasibility of the chemotype discrimination on four-weeks cannabis seedlings by analysis of leaf constituents.

3·3 HPLC-DAD quantification of neutral and acidic cannabinoids in leaves and flowers all along the growth

Chemotype identification in young specimens of THC-dominant cultivars and evolution of their content in major cannabinoids along the growth were the subject of an analytical study by De Backer et al. in 2012.[31] Classical GC, the most commonly used method for the analysis of cannabis products in those years, as documented by the two above papers, was innovatively replaced by high-performance liquid chromatography with diode array detection (HPLC-DAD), which allows one to detect and to quantify both acidic and neutral cannabinoids. In GC, acidic cannabinoids are decarboxylated and only neutral cannabinoids are detected. However, the partial conversion may cause an underestimation of the total cannabinoid content. Conversely, HPLC is the simplest method for determining the original composition in cannabinoids of plant material. The procedure, developed and validated by De Backer et al. in 2009,[32] was applied to plant material samples (shoots and leaves weekly collected all along the growth) previously dried for 18 h at 35°C under forced ventilation and then powdered. Samples of 200 mg of powder were then extracted with methanol/chloroform (9/1 v/v) and the extracts were evaporated under a gentle stream of nitrogen. The resultant residues were redissolved in 100 μL of a water/methanol mixture (1/1, v/v). Prazepam was used as an internal
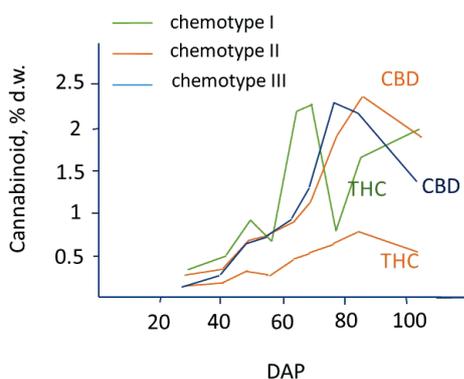

Fig. 3   Time course of the amount of THC and CBD in the leaves of the examined chemotype I and III, respectively, and of both THC and CBD in the examined chemotype II.

Table 2   Results for LDA and SVMs showing mean values of the classification measures over 1000 iterations with the standard deviation in brackets

	Training set			Test set		
	%Performance	%FPD	%FPF	%Performance	%FPD	%FPF
LDA	97.3 (±0.4)	0.3 (±0.2)	5.5 (±0.9)	97.0 (±1.0)	0.3 (±0.5)	6.0 (±2.3)
SVMs	99.7 (±0.1)	0.0	0.7 (±0.3)	99.2 (±0.7)	0.3 (±0.8)	1.3 (±1.2)

FPD: "false positive drug-types"; FPF: "false positive fibre-types". Overall dataset: 870 samples (leaves) from 28 days old plants of chemotype I and III, analyzed for the content of four cannabinoids and four terpenes.

standard. Chromatographic separations were accomplished using a C₁₈ analytical column and a mixture of methanol/water containing 50 mM ammonium formate as a mobile phase according to a pre-specified gradient. The wavelengths monitored were 211 and 220 nm for the neutral and the acidic cannabinoids, respectively. Seven major cannabinoids (THC, THCA, CBDA, CBD, CBGA, CBG, and CBN) were efficiently separated in a single run of 25 min and quantified after calibration with the respective standards. Δ⁸-THC was determined qualitatively. The analyses showed that the mean total THC content, namely THCA plus very small amounts of THC, was already 0.21% (percentage of weight of dry plant material) at the beginning of the vegetative state for the young shoots provided by police with an age estimable of between 1 and 3 weeks (Fig. 4). Then, total THC increased to 1.6 – 2.1% in the subsequent weeks and was stable until the onset of flowering. In the cutting deriving from these mother plants, the total THC was immediately higher than 2%, and remained at around 3% until to flowering. The total THC content increased strongly with the plant age, reaching the highest levels (16 – 22%) five or six weeks after flowering. CBD/CBDA was never detected; if present, the levels were always below the
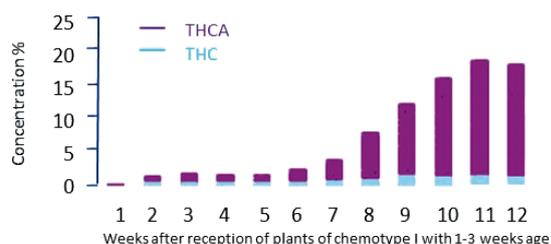

Fig. 4 Evolution of the total THC (THC + THCA) content in 1 – 3 weeks old seedlings, 7 – 15 cm high, of seized chemotype I plants during the successive growth weeks.

LOD of the method. The CBGA levels rose up from 0.11 to 1.93% during flowering. Upon receipt, the exact age of the seized seedlings was unknown and only valued at between 1 and 3 weeks. However, at that moment the total THC was already nearby 0.2%, namely the maximum THC content allowed for fibre type, and after 1 week of cultivation it increased sharply. On the basis of all these results, the author concluded that 3 weeks after germination, the chemotype of a high-THC-yielding variety can be safely determined and it is stable throughout the life of the plants.

3·4 GC-MS and HPLC quantification of terpenes and cannabinoids in leaves and flowers all along the growth

GC and HPLC are both used in a more recent and complex analytical investigation that considers all of the first three chemotypes analysing their evolution during the growth not only in cannabinoid, but also in terpene content, thus transcending and completing a forensic perspective exclusively focused on THC/CBD ratio determination and on drug-type and nondrug type differentiation.[33] About fifty clones of each of seven mother plants (three from chemotype I, three from chemotype III and one from chemotype II) were cultivated for six months. Each week, some plants were cut, dried for 1 week at 20°C and 45% humidity, and 100 mg samples were extracted with 1 mL of EtOH/CHCl₃ (9/1, v/v) by sonication. After filtration, they were diluted in EtOH at 1:100 for cannabinoid analysis and at 1:10 for terpene analysis. Phenanthrene and 1-octanol were added as internal standards for cannabinoids and terpenes, respectively. The analysis of cannabinoids was performed using a C₈ analytical column and a binary gradient of MeOH/0.1% aqueous solution of acetic acid at a detection wavelength of 230 nm. Eight major cannabinoids (THC, THCA, CBDA, CBD, CBGA, CBG, CBN and CBC) were separated and quantified with an external calibration performed using the respective standards at concentrations ranging from 0.5 to 400 μg/mL. The analytical fluctuations were corrected with the internal standard phenanthrene. Terpene analysis was performed
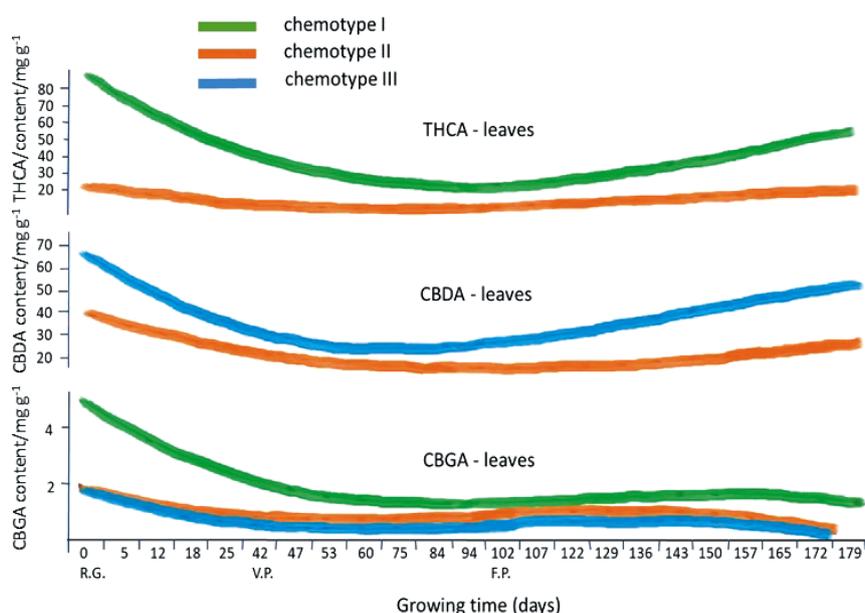

Fig. 5 Evolution of the THCA, CBDA, and CBGA content in the leaves during the growth of plants from chemotype I, II and III. Only the trend lines are shown. The very low THCA content in chemotype III and CBDA content in chemotype I are not shown. R.G.: root-growing phase; V.P.: vegetative phase; F.P.: flowering phase.

via GC-MS using a single quadrupole mass spectrometer. Helium was the carrier gas, and the screened mass range was 50 – 400 amu.   Among the 28 analyzed terpenes, 10 were identified by comparing their retention times and the obtained mass spectra with reference standards, and 18 were identified using the NIST library. The quantitative analysis of terpenes was performed by GC-FID using a DB5 column, as for the GC-MS qualitative analysis and with the same oven gradient and injection and flow conditions, but the carrier was nitrogen. The quantification of all terpenes was made using the average of three sets of standards containing γ-terpinene at concentrations ranging from 0.5 to 1000 µg/mL in EtOH.   Analytical fluctuations were corrected with the internal standard 1-octanol.

As for cannabinoids, the most important conclusion is that the chemotypes of the plants, as defined by THCA/CBDA ratio, were clear from the beginning and stable during the growth. Furthermore, the THCA and CBDA contents in the leaves showed the same time evolution for every chemotype: decreased during the first weeks of the vegetative phase and increased during the last 2 weeks of the vegetative phase (days 84 – 94), then decreased at the first weeks of flowering (from day 102) and then increased again (Fig. 5). As for the flowers, the maximum THCA concentration was observed in chemotype I in the ninth week of the flowering phase (20%, day 165), followed by a decrease, while for chemotypes II and III THCA and CBDA continued to increase until to day 179 (conclusion of the study) (Fig. 6).   This investigation also considered the development of the CBGA levels in leaves during the growth, finding that it was similar to that of THCA and CBDA until to flowering (Fig. 5). After flowering, the CBGA content remained constant in the leaves for chemotype I, while it decreased for chemotypes II and III (Fig. 5). An analogous difference between chemotype I and the other two chemotypes was found in the flowers: CBGA increased in chemotype I flowers until the onset of senescence, whereas it slightly decreased in chemotype II and III flowers (Fig. 6).

During the growth, the study systematically also monitored the content of terpenes, 8 monoterpenes and 20 sesquiterpenes, in the leaves and in the flowers of the same plants examined for cannabinoids (Fig. 7).   The expression of specialized terpene synthases can differ among the tissues and the growth stages. During the flowering phase, monoterpene synthases were more abundantly expressed, leading to an increase of the monoterpenes content. Summarizing, the monoterpenes total content showed the same trend as THCA and CBDA in the leaves, while, in flowers, as THCA for chemotype I and as CBDA for chemotypes II and III. On the other hand, in all of the three chemotypes, sesquiterpenes maintained constant levels (2 – 4%) in the leaves and, with major concentrations, also in the flowers. A correlation of higher levels of cannabinoids to higher levels of terpenoids was shown for the first time in 2010 by Fischedick *et al.*,[34] and more recently highlighted by O'Connell *et al.*[35] in an attempt to set a chemotaxonomic discrimination of cannabis based on the determination of monoterpenoids, sesquiterpenoids and cannabinoids in dried flower material from various cannabis strains.

At the end of this study on the levels of cannabinoids and terpenes during growth, the authors had the values of 36 variables (content of 8 cannabinoids and 28 terpenes) for 224 samples (9 sampling of flowers and 23 sampling of leaves for each of seven plants during the growth). PCA analysis of these data was performed. PC1–PC2 projection showed that there were two clearly distinct clusters, the one of chemotype I and the other of chemotypes II and III, and that leaves were separated from the flowers in each cluster.   The loading projection
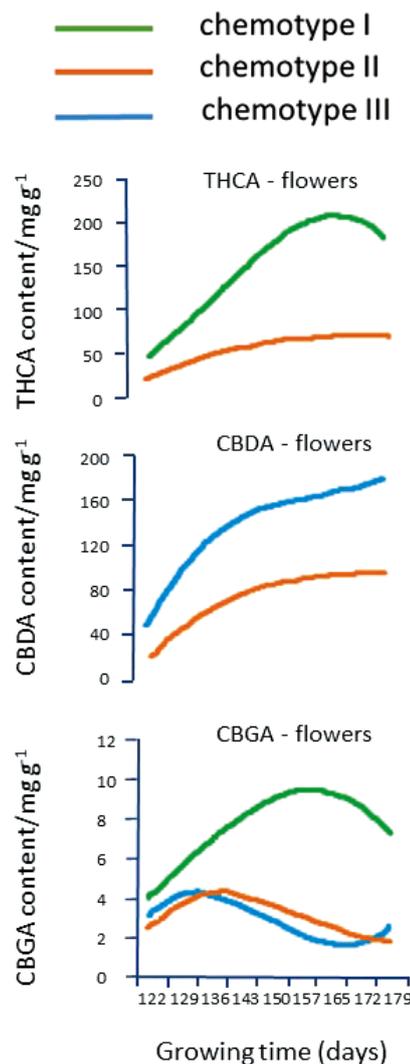

Fig. 6 Evolution of the THCA, CBDA, and CBGA content in flowers during the growth of plants from chemotype I, II, and III. Only the trend lines are shown. The very low THCA content in chemotype III and CBDA content in chemotype I are not shown.

indicated the cannabinoids and the terpenes having a higher content in the THCA-dominant chemotype I. They were CBGA and CBC and the terpenes γ-selinene, β-selinene, α-gurjunene, γ-elemene, selina-3,7(11)diene. In chemotype III, characteristic terpenes were γ-eudesmol, guaiol, α-bisabolene, eucalyptol, β-eudesmol, and α-bisabolol.   A significant enrichment was confirmed for the latter two sesquiterpenes also by a recent investigation on the terpenoids profile in different cannabis chemotypes.[36] In a PLS analysis, the projection of the samples in the PC1–PC2–PC3 space showed the distinction of the three kinds of chemotypes with a slightly clearer definition of the leaves and the flowers. The regression coefficient of each of the 36 variables with the growth time was obtained for chemotypes I and III. The monoterpenes exhibited positive coefficients, while the sesquiterpenes had mostly negative coefficients. Defining these correlations could be useful to select plants producing target blends of cannabinoids and terpenes and to determine the growth time needed to fulfil such requirements.
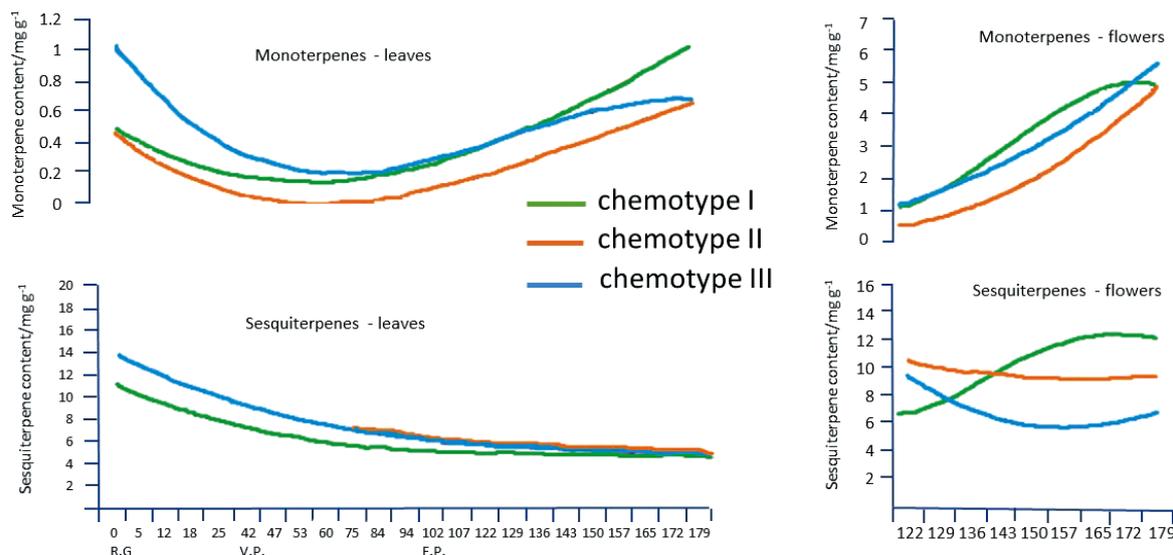
Fig. 7   Evolution of total monoterpene and sesquiterpene content in leaves and flowers during the growth of plants from chemotype I, II, and III. Only the trend lines are shown. R.G.: root-growing phase; V.P.: vegetative phase; F.P.: flowering phase.

3·5 LC-MS determination of alkyl cannabinoids across vegetative, flowering and maturation stages

The C_3-alkyl cannabinoids cannabidivarin (CBDV) and Δ^9-tetrahydrocannabivarin (THCV) are emerging as therapeutic entities.[28,37,38]   The characterization of variation in alkyl cannabinoid composition across the vegetative, flowering and maturation stages is the subject of a recent study.[39]   Such chemotypic diversity was profiled on 99 individual cannabis from 20 seed accessions by liquid chromatography–mass spectrometry (LC-MS), an analytical technique which has been usefully applied to natural products and drugs components for its high sensitivity and selectivity, low limits of detection and wide calibration range.[40–42] Fresh leaf tissue was collected from each individual during the vegetative stage and during the flowering stage, and it was then frozen until analysis, when it was ground and extracted with HPLC-grade EtOH.   At maturation, individual plant racemes were collected, dried at 35°C and they too were extracted with HPLC grade EtOH.   LC-MS cannabinoid profiling runs were conducted using a C_{18} column, a binary gradient of 0.005% TFA in water and of 0.005% TFA in acetonitrile. The HPLC instrument was coupled to DAD and a quadrupole mass detector. The run time was 16 min.   Twelve cannabinoids were quantified: 5 acid cannabinoids (THCA, CBDA, CBGA, THCVA and CBDVA) and 7 neutral cannabinoids (THC, THCV, CBD, CBDV, CBN, CBG and CBC). Calibration curves were constructed using commercially available standards; in the case of THCVA and CBDVA, standards were developed after isolation from the plant tissue. The cannabinoid profiles across the three developmental stages were described by four parameters: F_{C3}, namely the fraction of propyl homologues ((THCVA+CBDVA+THCV+CBDV)/total cannabinoids), F_{C5}, namely the fraction of pentyl homologues ((THCA+CBDA+THC+CBD)/total cannabinoids), $F_{dicyclic}$ ((CBDVA+CBDA+CBDV+CBD)/total cannabinoids) and $F_{tricyclic}$ ((THCVA+THCA+THCV+THC/total cannabinoid), namely the fractions of dicyclic and tricyclic cannabinoids. At maturation, as expected, three discrete distributions comprised of low $F_{dicyclic}$:$F_{tricyclic}$, intermediate $F_{dicyclic}$:$F_{tricyclic}$, and high $F_{dicyclic}$:$F_{tricyclic}$ ratios were observed, while the C_3-/C_5-alkyl cannabinoid proportions presented as a continuum, with ratio
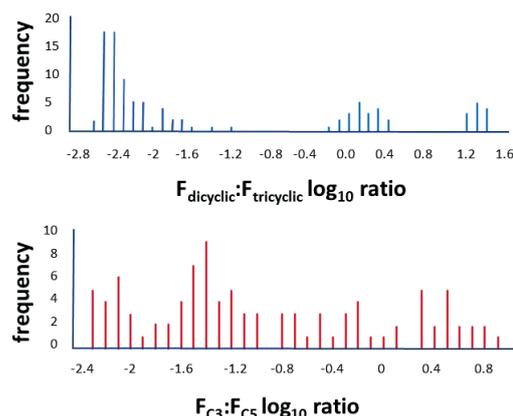

Fig. 8 $F_{dicyclic}$:$F_{tricyclic}$ and F_{C3}:F_{C5} $\log_{10}$ ratios of 99 mature *Cannabis* plants.

values ranging from 0.05 to 8, without any obvious distribution patterns (Fig. 8). At the vegetative stage and at the flowering stage, the $F_{dicyclic}$ and F_{C3} values were predictive of the values at maturation, indicating that cannabinoid fractions show minimal plasticity throughout development maintaining under environmentally uniform conditions, not only the di-/tricyclic, but also the C_3-/C_5-alkyl cannabinoid fraction to be relatively stable. These observations suggest a strong genetic influence on the between-plant variation in the cannabinoid profile, although a greater insight into the genetic mechanisms responsible for alkyl cannabinoid composition is required.

4 Conclusion

In the very crowded panorama of the studies devoted to phytochemicals identification and quantification in the many cannabis varieties and derivatives, those analyzing the composition evolution of the secondary metabolome of this plant (*e.g.*, cannabinoids and terpenes) during the growth in a

rigorous and systematic manner are relatively few and recent. All of this, even though cannabis chemotypes determination, which is mandatory, should be made as early as possible during cultivation for many reasons, mainly economical and legal. All of the studies here reviewed, regardless of the accessions selection and of the sampling design, prove that the chemotypes are clear from the beginning of the plant life, indicatively 3 – 4 weeks after planting, and that they do not change later throughout the entire life cycle. Therefore, an early diagnosis is possible and advisable. As summarized in Table 1, an examination of the analytical methods shows that, after the first systematic investigations utilizing GC-FID and GC-MS in 2008 and in 2010,[23,29] HPLC coupled to DAD and to MS has taken over in the successive studies relegating GC to terpenes determination.[31,33,39,43,44]   Furthermore, over the years, early chemotype classification has improved, not limiting itself to drug- and fibre-type distinction, but succeeding into differentiating the various chemotypes, thanks also to quantification broadening over many terpenes and more cannabinoids than THC and CBD and to the application of multivariate statistics to data analysis.

5 References

1. M. Pratt, A. Stevens, M. Thuku, C. Butler, B. Skidmore, L. S. Wieland, M. Clemons, S. Kanji, and B. Hutton, *Syst. Rev.*, **2019**, *8*, 320.

2. B. Wilsey, T. Marcotte, R. Deutsch, B. Gouaux, S. Sakai, and H. Donaghe, *J. Pain*, **2013**, *4*, 136.

3. J. Corey-Bloom, T. Wolfson, A. Gamst, S. Jin, T. D. Marcotte, H. Bentley, and B. Gouaux, *Can. Med. Assoc. J.*, **2012**, *10*, 1143.

4. L. De Petrocellis, A. Ligresti, A. Schiano Moriello, M. Iappelli, R. Verde, C. G. Stott, L. Cristino, P. Orlando, and V. Di Marzo, *Br. J. Pharmacol.*, **2013**, *168*, 79.

5. B. E. Porter and C. Jacobson, *Epilepsy Behav.*, **2013**, *29*, 574.

6. O. Sagredo, M. R. Pazos, S. Valdeolivas, and J. Fernandez-Ruiz, *CNS Drug Discovery*, **2012**, *7*, 41.

7. E. A. Penner, H. Buettner, and M. A. Mittleman, *Am. J. Med.*, **2013**, *126*, 583.

8. C. M. White, *J. Clin. Pharmacol.*, **2019**, *59*, 923.

9. J. Faim and J. Balteiro, *Eur. J. Public Health*, **2020**, *30*, ckaa040.015.

10. T. Cassano, R. Villani, L. Pace, A. Carbone, V. N. Bukke, S. Orkisz, C. Avolio, and G. Servidio, *Front. Pharmacol.*, **2020**, *11*, 124.

11. E. Valoti, E. Casagni, L. Dell'Acqua, M. Pallavicini, G. Roda, C. Rusconi, V. Straniero, and V. Gambaro, *Forensic Sci. Int.*, **2012**, *223*, e42.

12. V. Gambaro, S. Arnoldi, S. Bellucci, E. Casagni, L. Dell'Acqua, L. Fumagalli, M. Pallavicini, G. Roda, C. Rusconi, and E. Valoti, *J. Chromatogr. B*, **2014**, *957*, 68.

13. N. D. Sarma, A. Waye, M. A. ElSohly, P. N. Brown, S. Elzinga, H. E. Johnson, R. J. Marles, J. E. Melanson, E. Russo, L. Deyton, C. Hudalla, G. A. Vrdoljak, J. H. Wurzer, I. A. Khan, N. C. Kim, and G. I. Giancaspro, *J. Nat. Prod.*, **2020**, *83*, 1334.

14. M. ElSohly and D. Slade, *Life Sci.*, **2005**, *78*, 539.

15. P. G. Mahlberg and E. S. Kim, *J. Ind. Hemp*, **2004**, *9*, 15.

16. O. Aizpurua-Olaizola, J. Omar, P. Navarro, M. Olivares, N. Etxebarria, and A. Usobiaga, *Anal. Bioanal. Chem.*, **2014**, *406*, 7549.

17. S. Sirikantaramas, S. Morimoto, Y. Shoyama, Y. Ishikawa, Y. Wada, Y. Shoyama, and F. Taura, *J. Biol. Chem.*, **2004**,

18. A. Hazekamp, "Cannabis, Extracting the Medicine [thesis]", Leiden, Universiteit Leiden, **2007**.

19. E. B. Russo, *Chem. Biodivers.*, **2007**, *4*, 1614.

20. J. T. Fischedick, A. Hazekamp, T. Erkelens, Y. H. Choi, and R. Verpoorte, *Phytochemistry*, **2010**, *71*, 2058.

21. E. P. M. De Meijer, M. Bagatta, A. Carboni, P. Crucitti, V. Moliterni, P. Ranalli, and G. Mandolino, *Genetics*, **2003**, *163*, 335.

22. G. Mandolino, M. Bagatta, A. Carboni, P. Ranalli, and E. De Meijer, *J. Ind. Hemp*, **2003**, *8*, 51.

23. D. Pacifico, F. Miselli, A. Carboni, A. Moschella, and G. Mandolino, *Euphytica*, **2008**, *160*, 231.

24. E. De Meijer, in "*Handbook of Cannabis*", ed. R. Pertwee, **2014**, Chap. 5, Oxford University Press, 90.

25. K. Hillig, *J. Ind. Hemp*, **2008**, *7*, 5.

26. European Monitoring Centre for Drugs and Drug Addiction, Cannabis Production and Markets in Europe, Luxembourg, Publication Office of the European Union, **2012**, 144, http://dx.doi.org/10.2810/52425.

27. E. De Mejier, in "*Handbook of Cannabis*", ed. R. Pertwee, **2014**, Chap. 5, Oxford University Press, 92.

28. S. Chandra, H. Lata, M. A. ElSohly, L. A. Walker, and D. Potter, *Epilepsy Behav.*, **2017**, *70*, 302.

29. J. Broséus, F. Anglada, and P. Esseiva, *Forensic Sci. Int.*, **2010**, *200*, 87.

30. United Nations Office of Drugs and Crime (UNDOC). Recommended Methods for the Identification and Analysis of Cannabis and Cannabis Products, **2009**, http://www.unodc.org/documents/scientific/ST-NAR-40-Ebook.pdf (retrieved October 2009).

31. B. De Backer, K. Maebe, A. G. Verstraete, and C. Charlier, *J. Forensic Sci.*, **2012**, *57*, 918.

32. B. De Backer, B. Debrus, P. Lebrun, L. Theunis, N. Dubois, L. Decock, A. Verstraete, P. Hubert, and C. Charlier, *J. Chromatogr. B*, **2009**, *877*, 4115.

33. O. Aizpurua-Olaizola, U. Soydaner, E. Öztürk, D. Schibano, Y. Simsir, P. Navarro, N. Etxebarria, and A. Usobiaga, *J. Nat. Prod.*, **2016**, *79*, 324.

34. J. T. Fischedick, A. Hazekamp, T. Erkelens, Y. H. Choi, and R. Verpoorte, *Phytochemistry*, **2010**, *71*, 2058.

35. R. D. Richins, L. Rodriguez-Uribe, K. Lowe, R. Ferral, and M. A. O'Connell, *PLoS ONE*, **2018**, *13*, e0201119.

36. M. Dei Cas, S. Arnoldi, L. Monguzzi, E. Casagni, C. Morano, E. Vieira de Manicor, C. Bolchi, M. Pallavicini, V. Gambaro, and G. Roda, *J. Pharm. Biomed. Anal.*, **2021**, *203*, 114180.

37. V. K. Vemuri and A. Makriyannis, *Clin. Pharmacol. Ther.*, **2015**, *97*, 553.

38. K. A. Jadoon, S. H. Ratcliffe, D. A. Barrett, E. L. Thomas, C. Stott, J. D. Bell, S. E. O'Sullivan, and G. D. Tan, *Diabetes Care*, **2016**, *39*, 1777.

39. M. T. Welling, L. Liu, C. A. Raymond, O. Ansari, and G. J. King, *Front. Plant. Sci.*, **2018**, *9*, 1510.

40. G. Roda, F. Faggiani, C. Bolchi, M. Pallavicini, and M. Dei Cas, *Anal. Sci.*, **2019**, *35*, 479.

41. Q. Chang, Y. Shao, Y. Yang, H. Yu, and R. Wang, *Anal. Sci.*, **2019**, *35*, 201.

42. Z. Huang, Y. J. Bi, Y. F. Sha, W. Y. Xie, D. Wu, and B. Z. Liu, *Anal. Sci.*, **2018**, *34*, 1049.

43. M. Dei Cas, E. Casagni, A. Saccardo, S. Arnoldi, C. Young, S. Scotti, E. Vieira de Manincor, V. Gambaro, and G. Roda, *Forensic Sci. Int.*, **2020**, *307*, 110113.

44. M. Protti, V. Brighenti, M. R. Battaglia, L. Anceschi, F. Pellati, and L. Mercolini, *Med. Chem. Lett.*, **2019**, *10*, 539.

279, 39767.