

Extraction of naturally occurring cannabinoids: An update

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Abstract

Introduction: Organic molecules that interact with the cannabinoid receptors are called cannabinoids, which can be endogenous, natural or synthetic compounds. They possess similar pharmacological properties as produced by the plant, *Cannabis sativa* L. Before cannabinoids can be analyzed, they need to be extracted from the matrices.

Objective: To review literature on the methods and protocols for the extraction of naturally occurring cannabinoids.

Methodology: An extensive literature search was performed incorporating several databases, notably, Web of Knowledge, PubMed and Google Scholar, and other relevant published materials. The keywords used in the search, in various combinations, with cannabinoids and extraction being present in all combinations, were *Cannabis*, hemp, cannabinoids, *Cannabis sativa*, marijuana, and extraction.

Results: In addition to classical maceration with organic solvents, e.g., ethanol, pressurized solvent extraction, solvent heat reflux, Soxhlet extraction, supercritical fluid extraction, ultrasound-assisted extraction and microwave-assisted extraction, are routinely used nowadays for the extraction of cannabinoids from plant materials and cannabis consumer products. For the extraction of cannabinoids from biological samples, e.g., human blood, and also from food and beverages, and wastewater, solid-phase extraction and its variants, as well as liquid-liquid extraction are commonly used. Parameters for extraction can be optimized by response surface methodology or other mathematical modelling tools. There are at least six US patents on extraction of cannabinoids available to date.

Conclusions: Irrespective of the extraction method, extraction temperature, extraction time and extraction pressure play a vital role in overall yield of extraction. Solvent polarity can also be an important factor in some extraction methods.

Keywords

Cannabis sativa; cannabinoids; extraction; cannabis; marijuana; biological samples

Short abstract

Cannabinoids are organic molecules that interact with the cannabinoid receptors. Before applying any analytical tools to analyse cannabinoids, it is essential to extract cannabinoids from the matrix. This review appraises the literature on the methods and protocols used for the extraction of naturally occurring cannabinoids from various matrices. An extensive literature search was performed incorporating several databases, notably, Web of Knowledge, PubMed and Google Scholar, and other relevant published materials including published books. The keywords used in the search, in various combinations, with cannabinoids and extraction being present in all combinations, were *Cannabis*, hemp, cannabinoids, *Cannabis sativa*, marijuana, and extraction. Several extraction methods, *e.g.*, maceration, refluxing, Soxhlet, ultrasound-assisted, microwave-assisted, supercritical fluid and pressurised liquid extraction as well as various solid-phase extraction methods, for the extraction of different naturally occurring cannabinoids from various matrices are available in the literature, together with a few patents.

1 INTRODUCTION

Organic molecules that interact with the cannabinoid receptors, also known as endocannabinoid system, and possess similar pharmacological properties as offered by the plant, *Cannabis sativa* L. are termed as cannabinoids¹⁻³. Among the cannabinoids, phytocannabinoids (or natural cannabinoids) is the group of terpenophenolic compounds typically found in *C. sativa* and also present in a few other plant species, e.g., *Acmella oleraceae*, *Echinacea angustifolia*, *E. purpurea*, *Helichrysum umbraculigerum* and *Radula marginata*, and include their carboxylic acid analogues and transformation products^{1,3-5}. Figure 1 displays the structures of main naturally occurring cannabinoids. There are well over a hundred naturally occurring cannabinoids, and Δ^9 -tetrahydrocannabinol (**12**, Δ^9 -THC or THC) and cannabidiol (**3**, CBD) are the two principal cannabinoids (Figure 1), biosynthesized by *C. sativa*^{1,2,5}. Δ^9 -THC (**12**) exerts the psychoactive property of *C. sativa*, whereas, cannabidiol (**3**), has antipsychoactive property^{1,5}. Natural cannabinoids are generally accumulated in viscous resins produced in the glandular trichomes of *C. sativa*, and can be structurally classified into eight major classes: cannabichromenes (**1**, CBC), cannabicyclics (**2**, CBCL), cannabidiols (**3**, CBD), cannabigerols (**6**, CBG), cannabinols (**8**, CBN) and tetrahydrocannabinols (**12**, THC), cannabielsoins, iso-tetrahydrocannabinols and cannabicitrans^{1,5}.

Cannabinol (**8**, CBN), a mildly psychoactive cannabinoid, appears to be the first phytocannabinoid discovered in a red oil extract of *Cannabis* by the British Chemist Robert S. Cann, followed by the discovery of cannabidiol (**3**, CBD) and then tetrahydrocannabinol (**12**, THC) and so on^{1,5}. Since the discovery of these major cannabinoids, several analytical tools and methods have been introduced for the detection, identification, quantification and analysis of various naturally occurring cannabinoids, predominantly from the plant *C. sativa*, as well as in various biological matrices, e.g., human blood, urine, hair and nails, often linking to pharmacokinetic studies and/or forensic analysis^{1,2,5}. At the same time, various methods and protocols for the extraction of naturally occurring cannabinoids from various matrices have also been reported. In fact, extraction is one of the key pre-analysis steps for any compounds. As the analysis of *Cannabis* has gained new global importance, mainly for quality control within the legalized recreational and medical *Cannabis* industry, and also for forensic differentiation between drug-type *Cannabis* and legal products such as fibre hemp and CBD (**3**)-rich/THC (**12**)-poor *Cannabis*^{1,5}, the importance of the correct choice of an extraction method for the extraction of cannabinoids from various matrices, e.g., *Cannabis* plant

extracts, hemp food products, biomass, cannabis oils, whole blood, plasma, oral fluids, hair and so on, has become paramount.

The choice of an extraction method relies on the nature of the source material, e.g., dried plant powder, biological materials, soil or water, as well as the target compounds, e.g., cannabinoids⁶. Prior to deciding on a particular extraction method for naturally occurring cannabinoids, one must consider the following: purpose of extraction, quantity of extraction, purification steps to be carried out, purity level of cannabinoids, possible artefact formation, stability of target cannabinoids, physicochemical properties of target cannabinoids, and obviously the cost and environmental impacts. In addition to traditional methods like maceration, distillation or boiling, several other modern extraction methods and techniques can be applied for the extraction of naturally occurring cannabinoids, and those methods include, Soxhlet, accelerated solvent extraction, pressurized liquid extraction, microwave-assisted extraction, ultrasound-assisted extraction, supercritical fluid extraction, solid-phase extraction and solid-phase micro extraction^{4,6}. The choice of solvent for extraction is equally important to have maximum extraction yield. For the extraction of naturally occurring cannabinoids, most often, organic solvents are preferred, because of the lipophilic nature of most of the naturally occurring cannabinoids. Once an extraction method is chosen, the extraction parameters can easily be optimized using modern computational technology and various mathematical models.⁷ In fact, in recent years, there have been remarkable advancements in computational methods and technologies positively impacting on phytochemical methods including methods of extraction. This review article appraises the developments in methods and protocols applied for the extraction of naturally occurring cannabinoids from various matrices.

2 EXTRACTION OF NATURALLY OCCURRING CANNABINOIDS

Since the introduction of CBD-based anticonvulsant drug Epidiolex in 2018, much focus has been given on therapeutic cannabinoids⁸. To date, there are a good number of publications on various extraction techniques applied for the extraction of cannabinoids from natural sources available in the literature^{4,8-14}, highlighting the importance of those techniques as well as remarkable advancements in increasing extraction yields. Maceration with alcohol, albeit an old method for extraction, is still used for the extraction of cannabinoids from plant materials. Alcohol-based extraction is particularly useful for the preparation of *Cannabis* tincture containing cannabinoids. There are various ethanol

extractors commercially available for cannabinoids extraction. One of the disadvantages of this method is relatively poor yield. However, the extraction yield of cannabinoids can be improved by raising the extraction temperature. Most often, better yield can be achieved by using a Soxhlet apparatus, and using alcohol as a solvent for extraction, and at an elevated temperature. Besides alcohol, other extraction solvents like ether, chloroform and hydrocarbons like, butane and propane are also used to extract cannabinoids.

A specific heat and time ration is crucial for preventing or enhancing inter-conversions of various cannabinoids. Because of the thermolabile nature of some cannabinoids, other modern techniques, for example, CO₂ supercritical liquid extraction (SFE) is often preferred for the extraction of cannabinoids¹⁵. Similarly, ultrasound-assisted (UAE) and microwave-assisted extraction (MAE) of cannabinoids have become popular in recent years. Hydrodynamic extraction technology has been applied for the extraction of cannabinoids from *Cannabis* flowers in industrial scales, and it involves a combination of temperature, pressure, and ultrasonication control parameters to produce quality assured extracts. Extraction of cannabinoids from any other matrices than plant materials, especially from biological samples for forensic analysis, often involve a solid-phase-extraction (SPE) protocol or its variants.

It is noteworthy that the extraction stage, and the correct choice of the extraction method are extremely important for the desired applications of *Cannabis* extracts. For example, extract containing CBD (**3**) for medicinal use is made following a suitable extraction protocol and using ethanol as the solvent of extraction that naturally enriches CBD (**3**) content and makes the extract free from any contaminants. The following sections deal with specific examples of various extraction methods applied to the extraction of naturally occurring cannabinoids from various sources.

2.1 Extraction of cannabinoids in *Cannabis sativa* L. plant samples and *Cannabis* consumer products

Various extraction methods that have been reported for the extraction of cannabinoids from *Cannabis sativa* L. plant samples and *Cannabis* consumer products, e.g., hashish, marijuana and cannabis oils discussed in the following subsections.

Because of low solubility of naturally occurring cannabinoids in water, maceration of ground *C. sativa* L. plant samples with water is not usually a preferred option. However, recently it was demonstrated that selective extraction of cannabinoids from *Cannabis* seeds

could be possible using pressurised hot water extraction⁹. The process ensured removal of polar and semi-polar compounds from the seeds. The benefit of response surface methodology⁷ was cleverly exploited in this extraction method to work out optimum extraction parameters, e.g., extraction time, extraction temperature, and collector vessel temperature, for the extraction of Δ^9 -tetrahydrocannabinol (Δ^9 -THC or THC, **12**), cannabinol (CBN, **8**), cannabidiol (CBD, **3**), cannabigerol (CBG, **6**), and cannabichromene (CBC, **1**). The optimized extraction parameters were: extraction temperature 150°C, vessel temperature 160°C and extraction time 45 min. Pressurized hot water extraction¹⁶ is a form of supercritical fluid extraction (SFE), where instead of CO₂, water is used under its supercritical form as a solvent, which possesses similar solvability capacity to that of methanol and ethanol⁹. It is noteworthy that to keep the water in the supercritical state during the extraction process, it is necessary to use high temperature and pressure. This extraction technique was found to be particularly useful for selectively higher extraction yield of CBD (**3**) than THC (**12**) and CBN (**8**). Pressurized hot water extraction process, when proposed initially about a decade ago, involved sequential steps that occur in the extraction cell filled with sample materials (Figure 2) and a large quantity of sands; the steps are: i. desorption of solutes, ii. diffusion of extraction solvent into the matrix, iii. Partition of solutes from the sample matrix into the extraction solvent, and iv. chromatographic elution out of the extraction cell to collection vial¹⁶ and was comparable to accelerated solvent extraction¹⁷.

The use of high pressure solvents, especially using supercritical CO₂, with or without any co-solvent, has long been a popular option for extraction of naturally occurring cannabinoids from *C. sativa*, especially from hemp, which is normally low in THC (**12**) content, and used routinely for the selective extraction of CBD (**3**)⁸. In fact, SFE is considered as an excellent extraction technique for the extraction of THC (**12**) and other related naturally occurring cannabinoids as this technique utilizes low toxicity solvents, e.g., supercritical CO₂, and produces solvent-free cannabinoids extract. However, this method suffers from the disadvantage of low polarity of supercritical CO₂, which is often adequately addressed by the addition of small amount (>5%) co-solvent like ethanol. In a recent report⁸, several processing parameters, e.g., extraction pressure up to 1300 bar, use of ethanol as a co-solvent, and decarboxylation of feed, in the use of high pressure solvent for the extraction of cannabinoids from the flower buds of hemp, have been compared and contrasted. In addition to the use of supercritical CO₂, near critical propane and dimethyl ether were also tried. However, the extraction yield for cannabinoids was much higher with supercritical CO₂ than with near

critical propane or dimethyl ether. It was observed that the use of 5% ethanol as a co-solvent as well as increased pressure enhanced the extraction efficiency of cannabinoid acids. Earlier, a similar method described the use of pure supercritical CO₂ with ethanol as a co-solvent for the extraction of cannabinoids from *Cannabis* hybrid flowers, comparing with the use of decarboxylation and winterization techniques¹⁰. Winterization is an oil refinement technique that involves dissolving the extract in ethanol, then placing the mixture in a freezer to chill, commonly used in biotechnology. It was reported that decarboxylation increased the extraction yield of cannabinoids. Rovetto et al.¹⁸ reported the extraction protocol employing supercritical CO₂ for *C. sativa* with high concentration of THC (**12**) and Δ⁹-tetrahydrocannabinolic acid (Δ⁹-THCA or THCA, **13**), and evaluated various extraction conditions to maximize the extraction yield of cannabinoids. Extractions were performed both with multi-steps pressure increment at a constant CO₂ flow rate and at constant pressure. It was noticed that the extraction yield was dependant on pressure used, and the starting composition of the plant material. The use of ethanol as the co-solvent was investigated using a constant ethanol flow, and by applying ethanol pulses at different time intervals throughout the extraction process.

THC (**12**) was successfully extracted from *C. sativa* L. plant materials with good yield using SFE at different pressures (15-33 MPa), at different temperatures (40-80°C) and ethanol as a co-solvent (0-5%), combining with solid-phase extraction (SPE) on a octadecyl modified silica gel (5 g, 40-60 μM), eluting with a gradient of aqueous acetonitrile for further purification¹⁹. The experimental design consisted of 19 experimental runs and all the parameters were optimized by using response surface methodology and central composite design. Kitryte et al.¹³ described extractions of cannabinoids from industrial hemp threshing residues using consecutive supercritical CO₂ extraction and enzyme-assisted extraction. Optimized supercritical CO₂ extraction afforded 8.3 g/100 g of lipophilic fraction containing 0.2 and 2.2 of CBD (**3**) and cannabidiolic acid (CBDA, **4**). Enzyme-assisted extraction technique applies specific enzymes to disrupt the cell wall of source material to improve its extraction efficiency and extraction yield. This technique can be combined with various other techniques to enhance the overall recovery of bioactive compounds from source materials. Another SFE protocol has recently been reported for the analysis of cannabinoids in plant biomass and medicinal *Cannabis* resin²⁰.

Nowadays microwave-assisted extraction (MAE), an environmentally friendly technology, has become routinely available in natural products laboratories, and has been

applied for the extraction of various types of natural products²¹. This technique has recently been applied successfully to extract cannabinoids, particularly THC (**12**) and CBD (**3**) from *C. sativa* plant materials¹². During this extraction, different extraction parameters, such as, ethanol concentration (30-70%), extraction time (10-30 min) and solid/liquid ration (5-15 g/mL), were evaluated to maximize the extraction yield, with the help of response surface methodology. The Box-Behnken design⁷ was utilized for the experimental design. Using the optimized protocol, the extraction yields for THC (**12**) and CBD (**3**) were, respectively, approximately 0.03-0.06 mg/mL and 0.22-1.84 mg/mL. For the MAE of cannabinoids, a simple home-made extraction system consisting of a microwave oven connected to appropriate glass apparatus with round bottom flask and condenser was used. Earlier, a MAE method for the extraction of cannabinoids, THC (**12**), CBD (**3**) and cannabinol (CBN, **8**), in hemp nuts and application of response surface methodology for optimization of extraction parameters was reported²². This MAE was compared with extraction efficiency of other methods, such as, heat reflux extraction, Soxhlet extraction, SFE and ultrasound-assisted extraction (UAE), and it was observed that MAE afforded the highest extraction yield of cannabinoids in hemp nuts (6.09 g/g) with the least solvent use and shortest extraction time, whereas the extraction yields for the other techniques, heat reflux extraction, Soxhlet extraction, SFE and UAE, were, respectively, 4.14, 5.81 and 3.73 g/g. In another comparative extraction study for cannabinoids, mainly cannabidiolic acid (CBDA, **4**), CBD (**3**), THC (**12**), THCA (**13**), and CBN (**8**), from *Cannabis*, a MAE method was compared with ultrasound-assisted extraction (UAE)²³.

Ultrasonic sound-assisted extraction (UAE) is a popular method for the extraction of various phytochemicals and it involves extraction with an appropriate solvent in a glass container having ground plant materials in it, and sonicating the content using a ultrasonic bath (high frequency pulses , 20 KHz), either at room temperature or at an elevated temperature²⁴. In recent years, UAE method has been applied for the extraction of cannabinoids from *C. sativa* L.¹⁴. Using mathematical modelling and computation, e.g., response surface methodology, it is now possible to optimize the extraction parameters like time, input power, temperature, and solvent composition to achieve maximum extraction yields for cannabinoids. In a recent study, it has been demonstrated that UAE of cannabinoids, e.g., THC (**12**) and CBD (**3**), from the inflorescences of fibre-type *C. sativa* L. could significantly improve the extraction yield¹⁴. In that study, a Tesla 150 WS ultrasonic bath fitted with a titanium probe (diameter: 18 mm) was used and the frequency of operation and power output were, respectively, 20 KHz and 150 W.

There are quite a few reports on comparative studies on the use of various commonly used extraction methods for cannabinoids from plant matrices, e.g., maceration, SFE, UAE, MAE, Soxhlet extraction, and reflux heat extraction, and their extraction efficiencies^{11,23,25,26}. The impact of various extraction methods and associated conditions on the extraction yields of CBD (**3**) and CBG (**6**) from the *C. sativa* L. ssp. *santhica*, has recently been reported¹¹. In addition to comparison of methods, e.g., maceration (90% ethanol, for upto 48 h at room temperature) and reflux heat extraction (using different solvent at 95°C under reflux), a simple extraction protocol for CBD (**3**) and CBG (**6**) from hemp was optimized by using UAE method with 96% ethanol, material/solvent ratio of 1:10 and the extraction time of 10 min at room temperature. In another study²³, when comparing the MAE with UAE processes of cannabinoids from *Cannabis*, the best conditions for the UAE in terms of yield of industrial hemp, were achieved with ethanol for 50 min at 60°C. It was noted that with industrial hemp as well as the medicinal one, UAE method was more efficient than MAE yield of both total THC (**12**) and CBD (**3**).

Standardization of *Cannabis* products needs grade categorization for growing the plant, and extracting bioactive cannabinoids accumulated in its inflorescence. Namdar et al.²⁵ evaluated the outcomes from different extraction processes, and their impact on the levels of cannabinoids extracted from inflorescences positioned along the flowering stem of *C. sativa*. It did not come as a surprise that the polarity of the extraction solvent, drying processes and purification methods could influence cannabinoid composition of the extract. However, the new observation was that regardless of extraction protocols and analytical methods, the amounts of cannabinoids and terpenoids in the inflorescences decreased with the position of the collected inflorescence from top to bottom of the flowering stem. This study highlighted the fact that development of optimized growth protocols for *C. sativa* cultivation and appropriate use of optimized extraction methods for the extraction of cannabinoids from the flowers are essential for standardizing *Cannabis*-based products.

In the way of developing a new extraction protocol (optimized dynamic maceration) for nonpsychoactive cannabinoids from fibre-type *C. sativa*, different extraction methods, e.g., dynamic maceration, UAE, MAE and SFE methods were evaluated and compared focusing on extraction yields²⁶. It can be noted here that dynamic maceration is a technique where the extraction takes place by diffusion using organic solvents based on their polarity to extract compounds of interest. It was observed that dynamic maceration for 45 min with ethanol at room temperature was the most suitable technique for the extraction of

cannabinoids in hemp samples. Dynamic maceration was carried out on a certain amount (0.25 g) of a hemp inflorescence sample with ethanol (10 mL) at room temperature for 15 min under magnetic stirring. In this comparative study²⁶, it was found that there was no noticeable difference between ultrasound-assisted extraction and supercritical fluid extraction, which afforded the lowest amounts of CBDA (**4**), CBD (**3**) and CBGA (**7**), whereas the extraction yield for CBDA (**4**) was highest with dynamic maceration. Microwave-assisted extraction (MAE) provided the highest amount of CBD (**3**), whereas there was no significant difference in extraction efficiency of dynamic maceration and MAE for CBGA (**7**). It was concluded that dynamic maceration was the best technique for acidic cannabinoid CBDA (**4**), but MAE was more effective for the extraction of neutral cannabinoid CBD (**3**).

A rapid and simple metabolomics-based protocol using Sorptive Tape-like Extraction coupled with Laser Desorption Ionization Mass Spectrometry (STELDI-MS) was described for the analysis of naturally occurring cannabinoids and additive contents of *C. sativa* L. products²⁷. In that study, *Cannabis* products were obtained from the police. The samples were seized by drugs enforcement agents in Campinas, Brazil, and cannabinoids were analyzed according to their abundance in the samples. The extraction of cannabinoids was carried out using 50% aqueous methanol under vortex for a minute.

Hemp seed oil, also known as *Cannabis* seed oil, is one of the most known, and widely available commercial *Cannabis* consumer products, and in this product, the THC (**12**) and CBD (**3**) contents can vary significantly from sample to sample. Generally, hemp seed oil is obtained by pressing hemp seeds; cold pressed, unrefined hemp seed oil is dark to clear light green in colour and possess a nutty flavour. Zhang et al.²⁸ used an orthogonal test design⁷ to optimize the extraction conditions, namely, time of extraction, and the amount of methanol as the solvent of extraction, for extracting THC (**12**) and CBD (**3**) from hemp seed oil. An experimental design is said to be orthogonal when each factor can be evaluated independently of all the other factors design⁷. In a two-level factorial design, this is achieved by matching each level of each factor with an equal number of each level of the other factors. Addition of 5 mL, two times, of methanol into hemp seed oil for 15 min was found to be the best conditions for extraction of cannabinoids **3** and **12**.

Several decades ago, Fairbairn and Liebmann²⁹ reported a simple and rapid method for the extraction and estimation of cannabinoids from *C. sativa* L. plant and in its products, by using maceration at room temperature as well as at elevated temperature with chloroform and petroleum ether as the solvents for extraction. Whilst the *Cannabis* plant samples were

obtained from an experimental garden in London that used seeds from Nepal to grow *Cannabis* plants for experimental purposes, *Cannabis* consumer product, *Cannabis* resin, samples potentially of Pakistani origin were obtained from a custom seizure in 1969.

In addition to standard routine extraction methods as outlined above for the extraction of cannabinoids from *Cannabis* plants and *Cannabis* consumer products, there are quite a few rather interesting methods appeared in the literature in recent years. One of those methods is the stir bar sorptive extraction method, which has recently been utilized for the extraction of cannabinoids from *Cannabis* inflorescences for preconcentration of metabolites for further analysis³⁰. Stir bar sorptive extraction is a sample preparation technique for chromatographic analysis³¹; it is considered as a valid alternative for several other separation and pre-concentration procedures because of its high recoveries and concentration factors. This method offers a better alternative to classical extraction methods by reducing the consumption of and exposure to the solvent, disposal cost, and extraction time. It was shown that the performance can be enhanced by stir bar surface coating to increase the extraction selectivity and sensitivity. In the extraction protocol as described by Franchina et al.³⁰, stir bars coated with 63 μ L of poly-dimethylsiloxane, 10 mm length x 1.0 mm thickness, were used. The optimum extraction parameters (optimized via experimental design) were found to be: extraction time 60 min, extraction temperature 50°C, extraction solvent water-methanol-acetone (5:4:1) with addition of salt (10% NaCl), for the extraction of CBD (**3**), THC (**12**) and CBN (**8**). Another experiment utilized in-tube solid-phase extraction of THC (**12**) from *Cannabis* leaves as well as from biological samples³², and important parameters affecting extraction efficiency, e.g., extraction and desorption times, pH of the sample solution and flow rates of the sample and eluent solutions, were investigated and optimized.

Apart from usual organic solvents like butane, hexane, methanol, ethanol, petroleum ether or chloroform, deep eutectic solvents have recently been used for the extraction of CBD (**3**) from industrial hemp leaves³³ as a green extraction medium, and extraction parameters were optimized by response surface methodology providing the highest CBD (**3**) extraction yield of 12.22 mg/g under the optimized conditions. It was concluded that this CBD (**3**) extraction method could offer high extraction yield, simple and environmentally friendly operation, and low cost. It is worth mentioning here that deep eutectic solvents are systems formed from a eutectic mixture of Lewis or Brønsted acids and bases, which can contain a variety of anionic and/or cationic species, and they are considered as good alternatives to conventional organic solvents and ionic liquids for extraction. Morini et al.³⁴ assessed the

extraction efficiency and reproducibility of a commercially available new generation extractor for the extraction of THC (**12**) and CBD (**3**) in *Cannabis*-based products. It was found that the extraction procedure was robust, reproducible and could be effortlessly applied to *Cannabis* preparations. The difference in the results that might exist for the estimation of THC (**12**), THCA (**13**) and CBN (**8**) amount in *C. sativa*, when Soxhlet extraction and pressurized liquid extraction (PLE) are used, was explored³⁵. It was identified that THC (**12**) amounts extracted by *n*-hexane or methanol using the pressurized liquid extraction (PLE) were not much different, while THCA (**13**) extracted by PLE using *n*-hexane was more efficient than the Soxhlet extraction using the same solvent. However, when methanol was used the solvent of extraction, Soxhlet provided better extraction yield than that of the PLE method for this cannabinoid. It was concluded that mutual transformation of THCA (**13**), THC (**12**) and CBN (**8**) could occur not only during Soxhlet extraction, but also during PLE method despite it utilizes much shorter extraction time.

Another rather unconventional extraction method, cloud point extraction, was applied for the extraction of THC (**12**) from *Cannabis* resin³⁶, and the nonionic surfactant Dowfax 20B102 was used. The performance of a cloud point extraction process is usually influenced by factors like the cloud point temperature and concentration of surfactants and the physicochemical properties of solutes themselves. Cloud point extraction, also known as micelle-extraction, micelle-mediated extraction or liquid-concentration technique, is one of those novel green and low-cost techniques for extraction that can be used for the extraction of functional thermally sensitive components from natural matrices³⁷. It utilizes green surfactants as extractants. The key steps involved in cloud point extraction are addition of surfactant to sample → maintenance of suitable temperature for some time → centrifugation → decanting of supernatant → suitable treatment. After addition of surfactant to the sample, generally a salt (e.g., Na₂SO₄) is also added. A cooling phase is necessary between centrifugation and decanting. It can be noted that compared with conventional solid-liquid extraction, this method avoids the use of volatile organic solvents, making this method environmentally friendly. A couple of years ago, a quick, innovative, simple, robust and low-cost extraction protocol was developed for the extraction of THC (**12**), CBD (**3**) and CBN (**8**) from marijuana samples using a hard-cap-espresso extraction method with isopropanol³⁸. The extraction efficiency was determined by comparison of extraction yields for seized samples by the espresso method and a reference methodology based on UAE. In this hard-cap-espresso extraction technique, a Nespresso Essenza Manual XN2003 Krups coffee

machine was utilized. Briefly, 0.2 g of sample was homogenized with 2 g Speed matrix dispersing agent for Applied Separations and placed inside a Nespresso compatible stainless-steel refillable capsule from Mycoffestar. Capsule volume was achieved with further dispersing agent. A borosilicate filter was placed on the top to filter. Filled cap was inserted into the coffee machine and it was extracted with 100 mL of isopropanol in 40 s.

A total of 156 samples of marijuana seized in Brazil were analyzed by ^1H NMR techniques, and prior to analysis, cannabinoids were extracted by classical maceration method using methanol as the solvent of extraction³⁹. Simply, 150 mg of each marijuana sample was weighed into different flasks and extracted with 1 mL of methanol for about 72 h in the refrigerator. The flasks were closed and protected from light. The resultant extract was subjected to filtration and solvent evaporation. The extract was re-dissolved in chloroform-d for NMR analysis. Principal component analysis (PCA)⁷ was used to group samples according to the various criteria.

Cannabis olive oil preparation is a popular *Cannabis* consumer product. In recent years pharmacists had to supply an increasing number of prescribed medicinal products based on *C. sativa* L., and *Cannabis* olive oil preparation is the first choice as a concentrated extract of cannabinoids⁴⁰. A study focusing on understanding the impact of temperature and extraction time on the concentration of cannabinoids in medicinal preparation was published⁴⁰. In addition, the effect of temperature on THCA (**13**) decarboxylation during extraction from starting from *Cannabis* inflorescence was evaluated. A simple methanol-based extraction protocol was used. Recently, in the analysis of medicinal *Cannabis* products, a design of experiments approach was used to optimize CO₂ supercritical fluid extraction⁴¹. Studied key variables included CO₂ flow rate, extraction time and extraction pressure. The highest extraction yield (7.1%) was obtained under high flow rate (150 g/min), with long extraction time (600 min) at high pressure (320 bar), proving the best recoveries of THC (**12**) and CBD (**3**). Comparative study on chemical profiles, mainly CBD (**3**) and THC (**12**) content, of *C. sativa* L. medicinal oil using two different extraction protocols, maceration and Soxhlet extraction, was published by Pegoraro et al.⁴² The Soxhlet extraction of inflorescences exhibited the highest yield of extraction. Four extraction methods, e.g., Soxhlet, UAE, MAE and SFE methods, have recently been evaluated for their extraction efficiency for the extraction of cannabinoids from *Cannabis* medicinal products.⁴³ It was found that the MAE consistently produced completely decarboxylated phytocannabinoid extracts. It was further noted that

temperature and exposure time could play an important role in decarboxylation of cannabinoids.

2.2 Extraction of naturally occurring cannabinoids from biological and forensic samples

Marijuana or hashish, a cocktail of at least 30 different major cannabinoids, generally prepared from crushing the leaves, flowers (inflorescences) and even stems of *C. sativa* L., is one of the oldest recreational and addictive natural products used by humans for centuries^{1,5}. Despite this fact, the nonmedical recreational application of *Cannabis* or marijuana is illegal in many countries, which dictates the necessity for the use of analytical tools, like GC, HPLC and UPLC, to analyse biological and forensic samples like blood, oral liquid, hair and urine, to confirm marijuana usage. After consumption of marijuana, THCA (**13**), present in the crude marijuana, is converted to THC (**12**) by heat during smoking, and excreted in the urine as its glucuronide conjugates¹. Often, cannabinoids and their metabolites or biotransformation products are present in extremely small quantities in biological matrices. Therefore, in order to analyse cannabinoids in biological matrices, e.g., human urine or human blood, an appropriate extraction protocol must be in place to preconcentrate the amounts of metabolites to the detectable limit by any analytical tool. Nowadays, among a few other extraction methods, solid-phase extraction (SPE) and/or micro solid-phase extraction (MSPE), online or offline, have been used routinely for the extraction of cannabinoids and their biotransformation products from various biological matrices⁴⁴. It can be mentioned here that SPE is a widely used technique that uses a sorbent to isolate the target compounds from a given sample. However, when the solid is packed in a cartridge, the efficiency of the interaction sorbent-analyte is limited by the flow-rate selected to percolate the sample^{6,45}. On the other hand, MSPE is SPE in a micro scale, and is more applicable for enrichment of extractants from biological or environmental samples⁴⁶. MSPE utilizes a fibre coated with an extraction phase, which could be a polymeric liquid or a solid adsorbent. Generally, the quantity of analyte extracted by the fibre is proportional to its concentration in the sample⁴⁷.

2.2.1 Human blood samples

Among the biological and forensic samples, the blood is probably one of the most popular samples for cannabinoid analysis; it can be whole blood, plasma or serum^{1,5}. Human blood, plasma and serum samples are widely used in forensic analysis to detect the consumption of cannabinoids. Analysis of cannabinoids in any blood sample requires an extraction step, online or off-line. Asiabi et al.³² described an in-tube SPE method for the

extraction of THC (**12**) in biological samples including human serum and plasma. In this study, a new plate-like nano-sorbent based on copper/cobalt/chromium layered double hydroxide was synthesized, and the synthesized nanoparticles were introduced into a stainless-steel cartridge using a dry packing method. The packed cartridge was utilized as an on-line "packed in-tube" configuration followed by HPLC analysis for the determination of trace amounts of THC (**12**) from biological samples and cannabis leaves.

An online extraction protocol linked with HPLC-MS method was reported for the simultaneous quantification of 11 cannabinoids in human plasma and urine samples⁴⁸. The extraction protocol involved transfer of ten microlitre of 20-fold aliquots of 200 μ L of the calibrator, quality control, or blank sample (urine or plasma) into a low-binding polypropylene vial (1.5 mL). Eight hundred μ L of 0.2 mol/L ZnSO₄ 30% water/70% methanol containing the internal standards (5 ng/mL) were added. Samples were vortexed for 10 min followed by centrifugation (at 27,500 g, 4 °C, 10 min). The supernatant was transferred into an HPLC autosampler vial for LC-MS analysis. The plasma (352) and urine (93) samples that were collected as part of various clinical and observational trials investigating the effects of medical marijuana in the context of various conditions such as inflammatory bowel disease, Parkinson's disease, paediatric epilepsy, paediatric patients with brain tumours, and changes in sleep patterns. Cannabinoids extracted and detected in the plasma as well as urine samples were THC (**12**), 11-hydroxy- Δ^9 -tetrahydrocannabinol (11OH-THC, **9**), 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid (THC-COOH, **10**), 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid glucuronide (THC-C-gluc), CBN (**8**), CBD (**3**), CBG (**6**), cannabidivarin (CBDV, **5**), Δ^9 -tetrahydrocannabivarin (THCV, **14**) and 11-nor-9-carboxy- Δ^9 -tetrahydrocannabivarin (THCV-COOH, **15**). Glucuronides are generally the major biotransformation products of cannabinoids found in human plasma and urine.

A molecularly imprinted polymer MSPE protocol in batch mode was applied for the extraction of cannabinoids, mainly THC (**12**), THCA (**13**) and 11OH-THC (**9**), from human plasma and urine samples collected from marijuana abusers⁴⁹. The extraction method was as follows: plasma and urine samples (0.1-1.0 mL) were placed into 25 mL flasks and were diluted to 5.0 mL with a 0.1 M/0.1 M KH₂PO₄/NaOH buffer solution (pH 6.0). The mixtures were enriched with the internal standard solution. The conditioned molecularly imprinted polymer (MIP)-MSPE device was placed into the buffered sample, and the flasks were transferred to the shaker inside the incubator chamber (40°C) and mechanically stirred at 150 rpm for 12 min. After analyte retention, the MIP-MSPE device was removed and placed into a 25 mL flask

containing 5 mL of 0.1 M/0.1 M $\text{KH}_2\text{PO}_4/\text{NaOH}$ buffer solution at pH 6.0 for rinsing. After discharging the rinsing wastes, 2 mL of methanol/aqueous acetic acid 9:1 was added for elution for 6 min. The MIP-MSPE procedure was the same when performed for method validation, although 5.0 mL of drugfree plasma/urine samples containing the internal standard was spiked at several concentration levels. The eluates were evaporated to dryness under a stream of N_2 at 40°C and redissolved with 100 μL of mobile phase (90% of 0.1% formic acid in acetonitrile and 10 % of 0.1% formic acid in water) for HPLC analysis. Although not for human blood samples, a high-throughput method using automated SPE on Phenomenex Strata-X Drug B SPE cartridges, coupled with ultra-high-pressure liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was developed for the determination of THC (**12**) and its major metabolites from sheep whole blood⁵⁰. This method could be used for the analysis of cannabinoids from human blood samples.

A disposable pipette extraction (DPX) method was applied for the extraction of cannabinoids and their glucuronide conjugates, mainly THC (**12**) and its phase I and phase II metabolites, from whole blood, analysis by LC-MS⁵¹. However, five other minor cannabinoids were also extracted. Extraction recoveries and matrix effects at low- and high-quality control concentrations were 54.0-84.4% and -25.8-30.6%, respectively. Disposable pipette extraction (DPX) is relatively a new SPE method used for rapid extraction of sample. Unlike traditional SPE devices, in DPX, solutions are mixed with the sorbent in a dispersive manner to provide rapid equilibration⁵². A simple SPE method using anion exchange sorbent was employed for the extraction of cannabinoids from plasma and serum, prior to GC-MS analysis⁵³. Earlier, Pelicao et al.⁵⁴ reported a SPE-based extraction method for GC-MS analysis of several illicit drugs including cannabinoids from postmortem blood samples. A combination of protein precipitation and SPE was applied for the extraction of THC (**12**), 11-OH-THC (**9**) and THCA (**13**) from human serum a part of routine forensic toxicology assay⁵⁵. While blank human serum samples were obtained from a transfusion centre and were screened previously for cannabinoids, serum samples of three *Cannabis* consumers were provided by police authorities and analyzed in duplicates or triplicates. Acetonitrile was used as a precipitant. About a decade ago, König et al.⁵⁶ reported an HPLC-MS method for quantitative determination of naturally occurring cannabinoids, mainly THC (**12**) and its two metabolites in human peripheral blood samples. This method was validated for forensic toxicological analysis. In this protocol, protein precipitation, integrated solid-phase extraction and on-line enrichment followed HPLC separation and detection with a triple quadrupole mass spectrometer were employed.

Much earlier, a MSPE method was reported for the extraction of cannabinoids, THC (**12**) and CBD (**3**), from biological samples including, mice blood and brain, and human urine⁵⁷, and a simple SPE method was applied for the extraction of cannabinoids from human whole blood and urine samples⁵⁸. A liquid-liquid extraction method for the extraction of cannabinoids, THC (**12**), CBD (**3**), CBN (**8**), 11-OH-THC (**9**) and THCA (**13**), from post-mortem blood samples, prior to 2D GC-MS analysis, was reported⁵⁹. *n*-Hexane and ethyl acetate (5:1) were used as the organic phase to partition against the aqueous phase.

2.2.2 Human hair samples

Human hair samples frequently used for forensic analysis for drugs of abuse or illegal drugs like *Cannabis*¹. Hair analysis is used to monitor usage of drugs over long periods, and in recent years, solid phase microextraction has become an important extraction method for this analysis. In fact, hair analysis has become a routine procedure in most forensic laboratories since this alternative matrix provides advantages over classical matrices, particularly, wider time window, non-invasive sampling and good stability of the analytes over time¹. However, the major difficulty associated with hair analysis of cannabinoids includes low concentrations of the major metabolite, THC-COOH (**10**). Thus, the use of an effective extraction method is a prerequisite for any LC or GC-based analysis of human hair samples for cannabinoids.

A recent HPLC-MS analysis of human hair samples for cannabinoids utilized a an ultrasonic-assisted methanolic extraction protocol prior to HPLC analysis⁶⁰. Earlier, Montesano *et al.* (2015)⁶¹ published an HPLC-ESI-HRMS/MS method for the analysis of CBD (**3**), CBN (**8**), THC (**12**) and THC-COOH (**10**) in human hair samples. Pressurised liquid extraction (PLE) method was used to extract cannabinoids and their metabolites from experimental hair samples. This method appeared to be fast and accurate the determination of those four cannabinoids in human hair samples, suitable for forensic analysis of hair samples for the presence of cannabinoids.

A micropulverized extraction (MPE) protocol was applied for the extraction of conjugated THC-COOH (**10**) (with glucuronic acid) from a Cannabis user's hair sample⁶², and previously the same researchers utilized the same process for the extraction of non-conjugated THC-COOH (**10**). It was demonstrated that THC-COOH (**10**) could be extracted completely from authentic hair containing THC-COOH (**10**) at the recommended cut-off level using MPE. In addition, MPE with and without hydrolysis afforded the measurement of the percentage of the conjugated form in total THC-COOH (**10**), unlike alkaline dissolution of hair.

The percentage of conjugated THC-COOH (**10**) in hair measured using the MPE was approximately 26%. Authentic hair sample was obtained from a *Cannabis* user, washed with an aqueous SDS (1%), water and methanol. The sample was then cut into 2 mm long pieces. The hair (10 mg) was placed in safe-lock tube (2 mL) and spiked with 50% aqueous acetonitrile (10 mL) containing 5 ng/mL THC-COOH-*d*₃, as the internal standard.

2.2.3 Human urine samples

Human urine samples are popular in forensic toxicological analysis for the determination of various illegal drugs, including cannabinoids, and their metabolites¹. One of the earliest analysis of cannabinoids in human urine samples was reported in 1980⁶³, where a simple and rapid method for extraction of urinary cannabinoids by liquid-solid column chromatography was described. Later, in 1987, a similar chromatographic extraction method using a bond-elut-THC extraction column, was reported by Duc⁶⁴. Five years later, King et al.⁶⁵ published a paper on evaluation of a thin-layer chromatographic method for the detection of cannabinoid THC metabolite using microcolumn disk extraction technique. An automated headspace solid phase dynamic extraction method for the determination of cannabinoids in human hair samples was reported⁶⁶, where alkaline hydrolysis and headspace solid-phase dynamic extraction (HS-SPDE) followed by on-coating derivatization and GC-MS analysis were carried out. It applied a hollow needle with an internal coating of polydimethylsiloxane as extraction and pre-concentration medium. The process was quite simple and straight forward, and involved washing of hair samples with deionized water, petroleum ether and finally, with dichloromethane. After the addition of deuterated internal standards, the hair sample was hydrolyzed with NaOH and directly subjected to HS-SPDE. It was observed that this automated HS-SPDE-GC-MS procedure was considerably faster than conventional methods of hair analysis. HS-SPDE is a method for solvent-free extraction of organic compounds from aqueous samples. In a so-called inside needle capillary absorption trap a hollow needle with an internal coating of polydimethylsiloxane is used as the extraction and preconcentration medium⁶⁷.

An online trap and flush extraction LC-MS/MS method for the simultaneous detection of drugs of abuse including cannabinoids in human urine samples was reported⁶⁸. An online extraction protocol linked with HPLC-MS method was reported for the simultaneous quantification of 11 cannabinoids in human urine samples⁴⁸. In that study, APCI-MS/MS in positive ion mode was applied with an HPLC for the detection of CBD (**3**), cannabidivarin (CBDV, **5**) CBN (**8**), CBG (**6**), THC (**12**), CBC (**1**), 11-OH-THC (**9**), THCV (**14**), THC-COOH (**10**),

THCV-COOH (**15**), and THC-C-glucuronide in human urine samples using rather a short column (40 mm). Sanchez-Gonzalez et al. (2017)⁴⁹ utilized a simple HPLC-ESI-MS/MS method for the quantification of THC (**12**) and its major biotransformation products, THC-COOH (**10**) and 11-OH-THC (**9**) in human urine and plasma, where the separation was achieved on a reversed-phase silica C₁₈ analytical column (100 mm x 4.6 mm; particle size: 5 µm) eluting with a Gradient elution with water and ACN, both containing 0.1% HCOOH. The urine samples were extracted using imprinted polymer micro solid-phase extraction. Muller and Opdal (2018)⁶⁹ have recently reported a rapid semi-automated sample preparation with alkaline hydrolysis in a 96-well plate for quantification of THC-COOH (**10**) in human urine samples by UPLC-MS/MS, where an Acquity UHPLC BEH C₁₈ column (100 mm x 2.1 mm; particle size: 1.7 µm) was used with the mobile phase comprising 0.1% HCOOH in ammonium formate (10 mM, pH 3.3) and ACN in a gradient elution mode.

2.2.4 Miscellaneous biological samples from human

Apart from the blood and the urine, or the human hair, there are a few other interesting human samples that can be analyzed for the detection of *Cannabis* use. One of such sample is human fingernails⁷⁰. In fact, drugs, including cannabinoids, remain in nails for long period of time, and nails can provide useful information about an individual's history of exposure to illicit substances. Fingernail clippings, obtained from long-term *Cannabis* users, were assessed as analytical specimens for the detection of cannabinoids⁷⁰. The initial processes involved a decontamination phase and an extraction step. Detergent (0.1% sodium dodecyl sulphate, SDS), water and methanol washes followed by alkaline hydrolysis (1M NaOH) and liquid-liquid extraction were employed for the extraction of cannabinoids from the fingernails.

Oral fluid, a complex matrix, can be used for the detection of *Cannabis* use or abuse. In fact, the evaluation of oral fluids levels is useful in proving drug usage and abuses. A microextraction by packed sorbent (MEPS) coupled to HPLC-MS was reported for the determination of cannabinoids, e.g., THC (**12**), 11-OH-THC (**9**), THC-COOH (**10**), CBD (**3**) and CBN (**8**), in human oral fluid⁷¹. MEPS is one of the newer SPE techniques that operates with small sample volumes, for example, only 125 µL sample was required in this study. Extraction of the analytes was performed using a MEPS syringe (250 µL) packed with C₁₈ Silica sorbent, which was initially conditioned by flushing twice with methanol and another two times with 50% aqueous methanol containing 50 mM formic acid. Oral fluid sample (125 µL) was mixed with 50 mM formic acid in methanol (125 µL) containing internal standards at a concentration

of 0.1 ng/mL for THC-COOH-*d*₃ and 20 for THC-*d*₃. The resulting mixture was sonicated (6 min at room temperature at 12,000 x g for 5 min) to remove protein precipitation. The supernatant was passed through the MEPS dispensing and aspirating five times the same aliquot to improve the extraction. After a washing step analytes elution was achieved using 2 x 25 µL of 50 mM 50 mM NH₄OH in methanol. The eluate was collected in a vial and 10 µL was directly injected in the LC–MS/MS instrument.

Breast milk can also be a good matrix for cannabinoids detection, especially to detect maternal exposure, active or passive, to marijuana during the lactating phase. Wei et al.⁷² reported a sensitive method for the detection of cannabinoids, e.g., THC (**12**), CBD (**3**) and CBN (**8**), in breast milk, where an alkaline saponification-solid-phase extraction was employed to extract cannabinoids for UPLC-MS/MS analysis.

2.3 Extraction of cannabinoids from dietary supplements, food and beverages

Sometimes cannabinoids are present in food and beverages, either as contaminants or as food additives⁷³⁻⁷⁶. Fibre-type *C. sativa* L. (hemp) produces non-psychoactive cannabinoids, and CBD (**3**) is the most important one among them¹. Usually CBD (**3**) and other non-psychoactive cannabinoids are biosynthesized in both female and male inflorescences, and these cannabinoids are used medicinally in various food, beverages and pharmaceutical preparations. The use of cannabinoids in food stuff makes the qualitative and quantitative analysis of cannabinoids in food and food supplements pivotal for quality assurance and the dietary intake control of cannabinoids-containing food items. It is even more important when it comes to widely consumed apiary products, and the bees produce them from different floral sources. For the first time, an HPLC-based method has recently been developed and validated for the analysis of cannabinoids in honey, using both UV and MS detection methods, providing simultaneous detections and quantification of several cannabinoids, CBD (**3**), CBDA (**4**), CBG (**6**), CBGA (**7**), THC (**12**) and THCA (**13**)⁷³. Prior to HPLC analysis, a quick, easy, cheap, effective, robust and safe extraction procedure with an un-buffered method was employed and optimized. Generally, extraction of compounds from honey requires a complex extraction procedure. In this study, two approaches for extraction were tested: an ultrasonication in a water bath, followed by a liquid–liquid purification step, and a SPE. Before being submitted to the extraction procedures, honey samples were heated at 40 °C and manually stirred for homogenization. The SPE method was found to be more effective, and it involved the following process. Samples of honey (2 g) were diluted with 8 mL of water in a centrifuge tube (50 mL) under continuous agitation. Acetonitrile (10 mL) and 50 µL of the

working internal standard solution were added. Afterwards, MgSO_4 (4 g) and NaCl (1 g), were added and the tube was shaken immediately for 1 min. The sample was then centrifuged at 4000 rpm for 5 min and the upper organic layer was collected and brought to dryness under vacuum. The residue was dissolved in 2 mM aqueous $\text{CH}_3\text{COONH}_4$ -acetonitrile (50:50, 150 μL) solution for HPLC analysis. Earlier, a rapid HPLC-ESI-MS/MS method for the simultaneous determination of CBD (**3**), CBDA (**4**), CBG (**6**), CBGA (**7**), CBN (**8**), Δ^8 -THC (**11**), THC (**12**), THCA (**13**) and THCV (**14**), in *Cannabis sativa* L. based beverages and food was reported⁷⁵, where an Ascentis Express RP-Amide stainless steel column (50 mm \times 4.6 mm; particle size: 2.7 μm) was eluted with a linear gradient using the mobile phase comprising water-ACN0.1% HCOOH . This method was applied and found to be useful for the analysis of hemp seeds, oil and flour, as well as the food and beverages that contain them. The extraction of cannabinoids from food materials was performed as follows. The food samples were homogenized, a portion (1 g) was weighed in a polypropylene tube (15 mL). 90% methanol in chloroform (10 mL) was added, vortexed for 1 min, and centrifuged at 1700 g and 4°C for 15 min. Further 10 mL of 90% methanol in chloroform was added to the supernatant, and vortexed for a further 15 min using the same conditions. The sample was finally centrifuged before LC-MS analysis. The method for the extraction of cannabinoids from beverages was similar to that of the above with the difference that the liquid sample (1 mL) was mixed with methanol (4 mL) before undergoing the vortex mixing step and centrifugation.

Only a couple of UPLC methods reported for the analysis of cannabinoids in dietary supplements, food and beverages in recent years^{74,76}. A fully validated UPLC-UV-PDA and UPLC-ESI-MS/MS method for the quantification of THC (**12**) in dietary supplements was reported⁷⁴. Forty five samples including those from dietary supplement tablets, capsules, powders, liquids, cookies and candy, collected from Korean markets, were analyzed in this study, and the method was indicated to be useful for adulterant inspection and sample analysis providing targeted screening of cannabinoids in dietary supplement and foods. In that study, the entire contents of the package were crushed to homogenize, and the homogenized sample (1 g) was mixed with methanol. The solution was vortexed briefly and sonicated for 30 min, additional methanol was added to a after cooling, and filtered to make it ready for UPLC analysis. Similarly, a simple UPLC-ESI-MS/MS method has recently been published for the analysis of THC (**12**), THC-COOH (**10**) and 11-OH-THC (**9**) in milk samples⁷⁶. A total of 13 milk samples (whole, semi-skimmed and skimmed), five junior formula milk products were analysed by this method. It was shown that this method was superior to

previously published methods in terms of recoveries percentages obtained, and time needed to determine the analytes (only required 12 min, 24 min with the post-execution). The extraction steps, as above, involved homogenization, addition of solvent (e.g., methanol), vortexing, centrifugation and filtering, and addition of internal standard before it could be analyzed by UPLC.

2.4 Extraction of cannabinoids from wastewater and sewerage

In addition to analyses of cannabinoids in *Cannabis sativa* plant, *Cannabis* products, biological and forensic samples, the detection of cannabinoids present in various environmental samples, e.g., water and wastewater samples, has been under the radar of analytical chemists⁷⁷⁻⁸¹. The analysis of wastewater for the presence of cannabinoids often provides valuable information about community consumption for a defined catchment area. The recovery of cannabinoids from complex wastewater matrices is rather problematic owing to the hydrophobic nature of cannabinoids⁸⁰. In a recent study with wastewater, two sample preparation techniques—liquid-liquid extraction and SPE methods were evaluated, with comparable limits of quantification between 0.001 and 0.5 µg/L in wastewater, prior to LC-MS/MS analysis for the quantification of cannabinoids present in wastewater, particularly cannabis urinary biomarker 11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol (THC-COOH, **10**), cannabidiol (CBD, **3**)⁸⁰. An ultra-high-performance supercritical fluid chromatography (UHPSFC) method was developed for the determination of cannabinoids THC (**12**) and its three major metabolites (monohydroxylated, dehydroxylated and carboxylated) in wastewater⁸¹. A liquid-liquid extraction protocol was employed for sample preparation for the chromatographic analysis.

CBD (**3**), CBN (**8**), 11-OH-THC (**9**) and THC (**12**) were detected and quantified in sewage sludge by a HPLC-ESI-MS/MS method, and provided, for the very first time, the evidence on the occurrence of these cannabinoids in sewage sludge⁷⁷. Extraction of cannabinoids from sewage sludge was carried out by pressurized liquid extraction (PLE) using an automated accelerated solvent extractor ASE 200 (Dionex Corporation). Freeze-dried sewage sludge (0.5 g) was transferred to 11 mL stainless extraction cells partially filled with Al₂O₃. Once in the cell, sewage sludge was spiked with internal standards, in order to correct for potential losses during the analytical procedure, as well as for matrix effects. Then, void spaces in cells were entirely filled up with activated Al₂O₃. The optimized PLE conditions included temperature 50 °C, pressure 1250 psi, preheating time, heating time and static time 5 min each, one static cycle, flush volume 60% and purge time 60 s. Aqueous methanol (90%) was used as extraction

solvent. While Andres-Costa et al. (2016)⁷⁸ developed a method to determine THC (**12**) and THC-COOH (**10**) in water samples using a UHPLC method applying QqTOF-MS/MS in positive ion mode and employing a SPE extraction protocol (Phenomenex Strata-X cartridges; methanol and dichloromethane as solvents), an ESI-MS/MS detection method was used to detect and quantify THC (**12**), THC-COOH (**10**), THC-COOH-glucuronide in wastewater samples utilizing a UPLC separation on a Phenomenex Kinetex C₁₈ column (100 mm x 2.1 mm; particle size: 1.7 µm) and SPE on Strata XC cartridges for initial extraction of cannabinoids⁷⁹.

Waste water analysis is considered to be one of the most useful methods for the determination of various drugs used in in the geographical areas that wastewater treatment plants service, and simply by monitoring human biomarkers in sewage water, the consumption of various drugs, including cannabinoids, can be determined¹. In fact, wastewater-based epidemiology is an innovative approach that uses the analysis of human excretion products in wastewater to obtain information about exposure to drugs in defined population groups⁷⁹.

3. PATENTS

There are at least six patents available covering extraction of cannabinoids from various matrices⁸²⁻⁸⁷ (Table 1). One of the earliest patents on cannabinoid extraction was patented in 2002⁸⁷ and it described a method for the extraction of cannabinoids, cannflavins, and essential oils from hemp, and production of a whole hemp extract without THC (**12**). It was a simple protocol involving extraction of ground industrial hemp with an organic solvent. The extract was then loaded onto a chromatographic column selected to fractionate specific cannabinoids, cannflavins, and essential oils. THC (**12**) was fractionated out of the extract, producing a THC-free hemp extract. Specific cannabinoids and related compounds of interest were also fractionated out, thereby producing purified cannabinoids, cannflavins, and related compounds. The most recent patent on cannabinoid extraction has been published in April 2020⁸², and it presented apparatuses, methods, and systems for extraction, isolation, purification, and conversion of various cannabinoids, and modifications of whole-plant hemp extracts. Earlier, Tegen et al.⁸³ patented processes involved in solvent extraction of cannabinoids, terpenes and flavonoids from biomass using a nonpolar, organic solvent, or a mixture of nonpolar, organic solvent and polar, organic solvent. A method for preparing a purified cannabis extract comprising extraction of a crude extract from cannabis solids, dewatering the crude extract, removing chlorophyll from the crude extract, and distilling the

crude extract thereby creating a purified cannabis extract was described in the patent owned by Towle⁸⁵. The use of lipid for the extraction of cannabinoids was patented in 2017⁸⁶.

4. CONCLUSIONS

Extraction of naturally occurring cannabinoids from a matrix is an important step prior to most of the available chromatographic analysis, e.g., GC-MS, HPLC-MS/MS or UPLC-MS/MS. In addition to classical maceration with an organic solvent, e.g., ethanol or methanol, among the other major extraction methods, pressurized solvent extraction, solvent heat reflux, Soxhlet extraction, supercritical fluid extraction, ultrasound-assisted extraction and microwave-assisted extraction, have now been routinely used for the extraction of cannabinoids from plant materials and cannabis consumer products. For the sample preparation or extraction of cannabinoids from biological samples, e.g., human blood and urine, and also from food and beverages, and wastewater, solid-phase extraction (SPE) and its various variants, as well as liquid-liquid extraction are commonly used. There are at least six US patents are available that describe various methods of extraction of cannabinoids, mainly from plant source.

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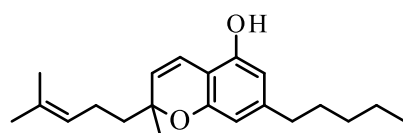
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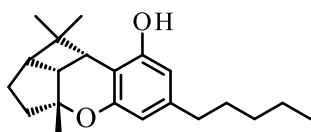
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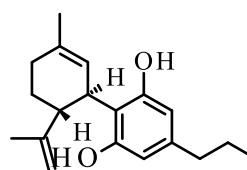
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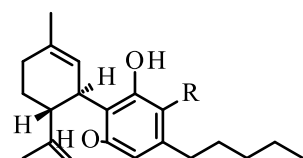
Cannabichromene (**1**, CBC)



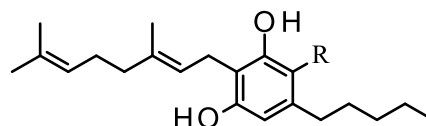
Cannabicyclol (**2**, CBCL)



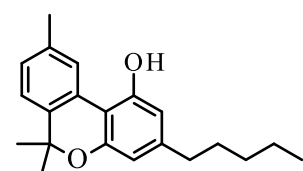
Cannabidivarin (CBDV, **5**)



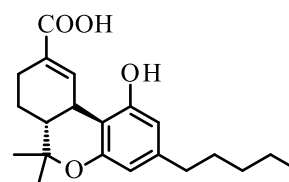
Cannabidiol (**3**, CBD) R = H
Cannabidiolic acid (**4**, CBDA) R = COOH



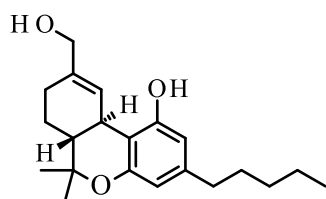
Cannabigerol (**6**, CBG) R = H
Cannabigerolic acid (**7**, CBGA) R = COOH



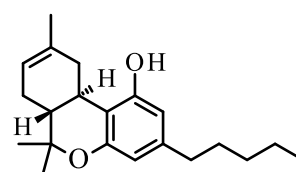
Cannabinol (**8**, CBN)



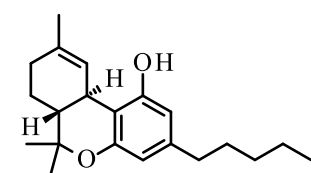
11-Nor-9-carboxy- Δ^9 -tetrahydrocannabinol (11-COOH-THC or THC-COOH, **10**)



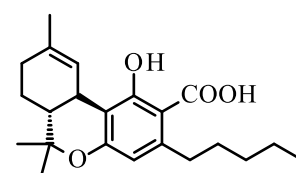
11-Hydroxy-tetrahydrocannabinol (**9**, 11-OH-THC)



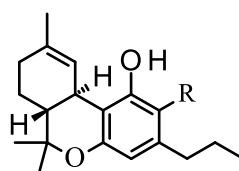
trans-(8)-Tetrahydrocannabinol (**11**, Δ^8 -THC)



Δ^9 -Tetrahydrocannabinol (**12**, Δ^9 -THC or THC)



Δ^9 -Tetrahydrocannabinolic acid (**13**, THCA)



Tetrahydrocannabivarin (**14**, THCV) R = H
Tetrahydrocannabivarinic acid (**15**, THCVA) R = COOH

FIGURE 1 Examples of naturally occurring major cannabinoids

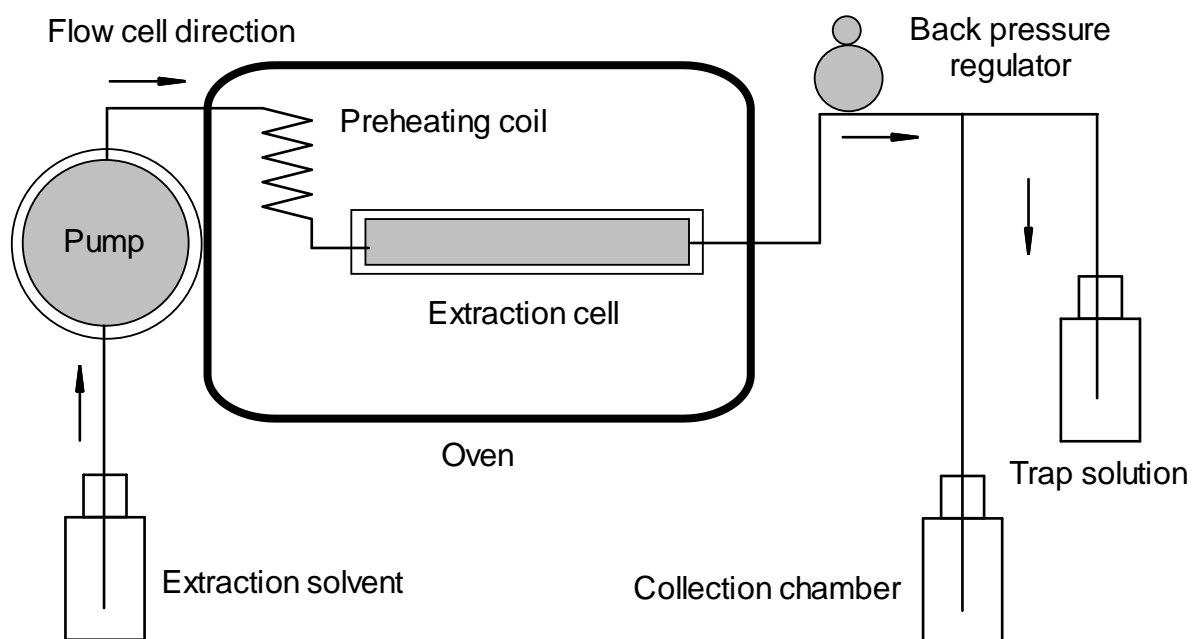


FIGURE 2 Pressurized hot water extraction device for the extraction of naturally occurring cannabinoids (modified from Nuapia et al.¹⁵ and Teo et al.¹⁶)

TABLE 1. Patents on cannabinoids extraction

Patent number	Brief detail	Patent assignee	Inventor(s) and reference	Year
US 10624872	Apparatuses, methods, and systems for extraction, isolation and conversion of various cannabinoids, and modifications of whole-plant hemp extracts therewith	Charlotte's Web Inc	McCorkle et al. ⁸²	2020
US 10413845	Processes for solvent extraction of cannabinoids, terpenes, and flavonoids from biomass	Socati Technologies	Tegen et al. ⁸³	2019
US 10517911	Manufacturing methods, compositions, and medical applications of orally administered cannabis pharmaceuticals using representative/total/complete cannabis extractions (cannabis infused pills)	Harvest Direct Enterprises LLC	Gharib and Gharib ⁸⁴	2019
US 09937218	Systems and methods for cannabinoid and terpene extraction and purification	-	Towle ⁸⁵	2018
US 09808494	Process for the extraction of cannabinoids from cannabis using lipids as an extraction solvent	RM Labs LLC	Barringer ⁸⁶	2017
US 6403126	Cannabinoid extraction methods	Websar Innovations Inc.	Webster et al. ⁸⁷	2002