Cannabinoids and Synthetic Cannabinoids

Analytical Challenges in the Determination of Cannabinoids

Several preparations of *Cannabis sativa* are estimated to be consumed by 200–300 million people around the world, being the most popular illicit drug of the 21st century [1]. New types of drugs of abuse containing synthetic cannabinoids are becoming increasingly popular mainly in Europe and the USA [2], and they are usually referred to as new psychoactive substances (NPS). NPS are often unregulated drugs, since molecules are modified to circumvent the law, and are designed to produce effects that are similar to those of illegal substances [2].

The analysis of this type of substances is of outmost importance for forensic or clinical toxicology laboratories. It has current application in many scenarios, such as driving under the influence of drugs and occupational accidents [3–5]. Therefore, their detection in human biological specimens helps providing useful information to deal with those situations, and sensitive methods are deemed necessary for that purpose. The main laboratorial challenges involving the extraction and analysis of these compounds in biological samples will be discussed below, and in order to facilitate reading and comprehension the section will be divided in two, whether naturally occurring cannabinoids or synthetic cannabinoids are concerned.

Detecting Naturally Occurring -Cannabinoids in Human Biological Samples

Among the cannabinoids of forensic interest, tetrahydrocannabinol (THC) and its major metabolites, 11-nor-9-carboxy-THC (THCCOOH) and 11-hydroxy-THC (THC−OH) are frequently monitored in blood samples, and are the markers of cannabis consumption.

Determining cannabinoids is a constant analytical challenge, particularly because of their low concentrations in the commonly analysed blood, serum and/or plasma specimens. Modern instrumentation, including gas (GC) [6,7] or liquid chromatographic (LC) systems [8–18] coupled to tandem mass spectrometry (MS/MS), can offer high sensitivity and specificity for identification. Analyses are usually performed using sample volumes from 0.5 to 1.0 mL, but lower amounts (e.g. 100 µL) have been reported [11].
Sample preparation can be done by protein precipitation without any further pretreatment [11,19], or it can be followed by solid phase extraction (SPE) [8,13-15,20]. However, cannabinoids can be strongly bound to endogenous substances, and simple protein precipitation may result in low recovery. As such, some methods have used liquid-liquid extraction (LLE) [7,16,21] or simple SPE [10,12,17,18] procedures instead. Determination of cannabinoids is generally performed in whole blood, but plasma and serum can be used to predict the time of the last exposure to THC [15,20] based on mathematical models that use either the concentration of THC (model I) or the concentration ratio of THCCOOH to THC (model II) [22-24].

Minor cannabinoids have been suggested as markers of recent cannabis use [13,25]. THC-glucuronide, cannabidiol (CBD), and cannabinol (CBN) can exhibit short detection times of less than 4 hours following controlled smoked cannabis administration [25]. CBD cannot be included as a recent use marker because of CBD-rich cannabis, a newly available medicinal product, that is being evaluated. Newmeyer et al. [26] reported that the markers of recent use that exhibited the highest likelihood of detection were cannabigerol (CBG) and CBN, while lower detectability was noted for THC-glucuronide and ∆9-tetrahydrocannabivarin (THCV) after controlled administration studies involving frequent and occasional users.

Urine is a valuable matrix for the detection of cannabis exposure, providing a long detection window. THCCOOH is the major urinary metabolite primarily excreted as its glucuronic-acid conjugate [27-32]. The concentration ranges vary usually between 1 to 600 ng/mL; however, samples with concentration exceeding 1000 ng/mL can be found. THC and THC-OH are also excreted in the form of glucuronides, but at lower concentrations. Cleavage of glucuronic acid is a prerequisite for GC-MS, but it is not strictly required in LC-MS/MS analysis, because this technique allows the direct analysis of such conjugates. Therefore, analytical methods generally include an alkaline or enzymatic hydrolysis step to liberate the free compounds. Basic hydrolysis with NaOH or KOH has lower
cost, time and is the most widely used. Enzymatic cleavage may include the use of β-glucuronidase, and usually requires longer incubation times (3 to 16 hours) [12,33–35].

Some methods do not employ any pretreatment except for a simple dilution [36,37], but a clean-up step is usually recommended, namely LLE [34,35], SPE [33,38–43] or microextraction techniques [44].

In occasional cannabis users, a mathematical model has been developed to determine new cannabis use by comparing creatinine-normalized THCCOOH concentration in two urine sample collections [45,46]. In frequent cannabis users, a mathematical model is available to aid in the differentiation of new cannabis use from residual THCCOOH excretion [47].

From another perspective, free THC, THC-OH, CBD and CBN cannot be considered as markers of recent cannabis intake, since they have not been found in non-hydrolysed urine of frequent and occasional cannabis smokers [48]. In addition, THC and THC-OH can be detected up to 24 days after the last cannabis use in hydrolysed urine samples of frequent cannabis users [33].

In contrast, THCCOOH, THC-glucuronide, and THCCOOH-glucuronide were measurable in the urine of frequent and occasional smokers [48]. Cannabis usage within 6 hours can be predicted if there is an absolute difference of fifty percent between two consecutive positive samples for THC-glucuronide with a creatinine normalized concentration ≥ 2 mg/g in the first sample (efficiencies of 93.1% and 76.9% in frequent and occasional smokers, respectively) [48].

With the increase in drug testing in oral fluid it became pertinent to evaluate cut-offs and testing criteria for clinical and forensic drug testing purposes [49]. However, a disadvantage associated to this particular matrix is that many drugs reduce salivary flow, making collection difficult. Indeed, low oral fluid volumes are often observed after smoking or ingesting cannabis [50,51].

Oral fluid samples can be collected by “spitting”, but it is common practice to use collecting devices due to hygienic reasons. In addition, buffers included in the collection systems improve drug stability [52–54]. On the contrary, fluorescent light and exposure to plastic surfaces will result in THC degradation, requiring refrigerated conditions [52,55]. These methods of collection are well documented and are known to affect analytical results, and a great concern exists regarding the adhesion of THC to several surfaces [52].

It is also widely accepted that the parent drug is usually detected in greater concentrations in oral fluid than metabolites [52,56]. There is an immediate contamination of the oral mucosa during cannabis smoking, and as such THC oral fluid concentrations usually exceed 1000 μg/L short time after smoking [50,57,58].

These considerations are important for quantitative analysis and were taken into account when implementing cut-offs. A cut-off of 1 μg/L for THC was proposed for influenced driving situations [49,59], whereas 2 μg/L is used in workplace drug
THC-OH and THCCOOH are also detected, but commonly at very low concentrations. It is important to consider a hydrolysis step to maximize the detection of THCCOOH, since this metabolite has been found to be conjugated in oral fluid [49]. In addition, the THCCOOH detection is considered a key issue regarding the differentiation between passive exposure and actual cannabis use, as this metabolite is not present in cannabis smoke and has not been measurable in oral fluid samples from passively exposed individuals [52,61]. Also, positive oral fluid tests for THC may occur shortly after passive exposure to cannabis smoke, but results are negative within 1 h [57].

A comprehension of cannabinoid oral fluid pharmacokinetics after controlled smoked cannabis might help to determine detection windows, to discover markers of recent smoking, and minimize the risk of passive contamination [62]. Overall, studies have shown that THCCOOH usually has longer detection times than THC [51,62]. Regarding situations of recent cannabis use or exposure, a greater THC concentration is indicative of more recent drug exposure and four criteria have been suggested: (i) THC ≥ 2 ng/mL, and THCCOOH ≥ 20 pg/mL; (ii) THCCOOH (pg): THC (ng) ratio ≤ 4; (iii) THC ≥ 2 ng/mL, and CBD ≥ 0.5 g/mL; and (iv) THC ≥ 2 ng/mL, and CBN ≥ 1 ng/mL [51,52].

The possible correlation with plasma levels has also raised concerns, and recent studies reported that the prediction of plasma THC concentration from oral fluid levels is not feasible due to the large variations observed. Nevertheless, there is enough support to assume that a positive oral fluid THC result is indicative of recent cannabis exposure [63]. Huestis and Cone [57] have also reported a greater variability in oral fluid THC concentrations when compared to plasma, but periodic time intervals evaluations reflected that oral fluid concentrations closely mimic plasma concentrations.

Concerning hair testing, the main challenges involve the ability to determine drugs and metabolites following active consumption, and care should be taken to ensure that the results are not biased by others derived from passive exposure or exogenous application. An issue that may arise is due to cosmetic treatments, which can cause deterioration or even distortion of the findings. In a recent publication, Van Elsué and Yegles [64] conclude that bleaching and perming reduced all cannabinoids concentrations in hair, with THC being more affected than THCCOOH, CBN and CBD. Bleaching caused strong chemical degradation of cannabinoids, while perming exerted more a leaching out effect, and permanent colourings in single applications had only little effects on cannabinoids. When hair analysis is being used to identify drug use, the major limitation is external contamination, which can confuse passive exposure with actual drug use. The issue of external contamination must be addressed through multiple methodologies and cannot be solved through the simple application of any single approach. A simple use of cut-off levels is insufficient because external contamination can occur at any
level. There are several approaches available for decontamination, and the washings should be stored for later analysis, if necessary.

In the particular case of cannabinoids, the confirmation of THCCOOH is required to definitively prove the use of cannabinoids. However, the hair incorporation rate of this particular metabolite is very low due to its polar nature. The recommended limits of quantification are of $\leq 0.1$ ng/mg for THC and $\leq 0.2$ pg/mg for THCCOOH [65]; for this reason, high selectivity and sensitivity are deemed necessary, which are provided by tandem mass spectrometry instrumentation.

**Detecting Synthetic Cannabinoids in Human Biological Samples**

Over the last few years, synthetic cannabinoids have been introduced in the illicit market as a form of evading legislation, and the rapid synthesis of such compounds has become a challenge for clinical and forensic laboratories, since method development cannot timely follow up with it. Regarding the complexity of these substances and their metabolites, the scientific community remains focused on developing and validating new methods for these synthetic drugs in several biological samples, since the available immunoassays are unspecific [66,67]. Several limitations can be pointed, from which the most important is the unavailability of adequate reference standards for parent drugs and metabolites [68]. Another disadvantage is the scarcity of suitable libraries to compare mass spectra for identification purposes [67]. Furthermore, the lack of conclusive pharmacokinetic studies makes it difficult to determine which biological specimens are most adequate for analysis. Urine is the most used in the analysis of this class of NPS, as it is easily accessible, abundant and less interferences are present. Nonetheless, metabolic processes of these compounds is extensive and occurs rapidly, thus small amounts of the parent drug are present in opposition to a large number of metabolites, which unfortunately are not characterized in full [2,66,67]. Serum samples have been used in several studies because it allows detecting both parent drugs and metabolites, with prevalence of the former. However, it is not the preferred matrix for screening, considering that large amounts of sample are needed [2]. Similarly, oral fluid presents the same advantage, that is, parent drugs are the main compounds identified. Moreover, sample collection is not invasive and adulteration is difficult. On the other hand, due to the slow diffusion of the substances from the blood stream to oral fluid, it can be stated that their detection in saliva is only possible in cases of recent consumption [69,70]. Blood, despite of being invasively collected, is one of the most used biological specimens in routine laboratorial analysis. This sample allows detecting the active compounds of the parent drug, but it becomes useless in cases of chronic intoxications due to a short detection window. Therefore, if the substances are detected on both blood and urine, a recent exposure can be assumed [67,70]. Lastly, hair has become an emergent sample, not only in cases when other samples are unavailable, but also
because it has larger detection windows; this allows assessing cases of chronic consumption, while its adequacy in cases of recent or sporadic consumption is little. Similarly to what happens in blood, the parent drug is the major compound detected. Notwithstanding, this sample can externally contaminated, which may impair results interpretation [67,68,71].

Overall, the existing studies are inconclusive, and are restricted to a limited number of substances. This is a problem, since new drugs are being synthetized every day and this trend is likely to increase. Therefore, it is necessary to take into consideration aspects such as pharmacokinetics, chemical and toxicological properties of these substances. Thus, it is still a challenge analysing the effects of these synthetic compounds and it is of extremely importance to pursue with further studies on this field, in order to develop new analytical methods and immunoassays to overcome these gaps.

**Conclusions**
The legalization of cannabis for recreational purposes in several countries and the insufficient regulation of its use for medical reasons has allowed the drug to be used for other purposes. On the other hand, the recent appearance of synthetic cannabinoids, on which there are few studies available, means that toxicology laboratories are challenged every day to identify and quantify lower concentrations in order to assist authorities and health professionals. In fact, the consumption of these substances is not only a legal problem in some situations, but a serious public health problem as well, particularly for young people as indicated in the most recent report on drugs from the UN.

**Authors**
Tiago Rosado¹,², Nicolás Fernández³, Mário Barroso⁴, Ângelo Luís¹,², Sofia Soares¹,², João Gonçalves¹,², Ana Y. Simão¹,², Débora Caramelo¹,², Ana P. Duarte¹,², Eugenia Gallardo¹,²

**Affiliations**
¹Centro de Investigação em Ciências da Saúde, Faculdade de Ciências da Saúde da Universidade da Beira Interior (CICS-UBI), Covilhã, Portugal
²Laboratório de Fármaco Toxicologia, UBIMedical, Universidade da Beira Interior (CICS-UBI), Covilhã, Portugal
³Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Toxicología y Química Legal, Laboratorio de Asesoramiento Toxicológico Analítico (CENATOXA, Buenos Aires, Argentina
⁴Servicio de Química e Toxicologia Forenses, Instituto de Medicina Legal e Ciências
Contact

Prof. Eugenia Gallardo
Centro de Investigação em Ciências da Saúde and Laboratório de Fármaco Toxicologia - UBIMedical,
Universidade da Beira Interior, Covilhã, Portugal
egallardo@fcsaude.ubi.pt

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References


Contact

Universidade da Beira Interior, Covilhã, Portugal