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Cannabis – review of the issues related to determination of the total content of delta-9-tetrahydrocannabinol (Δ-9-THC) and delta 9-tetrahydrocannabinolic acid (Δ-9-THCA-A).

Summary

Cannabis analyses are amongst the most common types of analyses performed by forensic laboratories, owing to the spread of cannabis-originated drugs on illegal markets. Yet, this subject brings about numerous controversial analytical issues that spark heated debates among specialists in the field.

The present work is a review of cannabis-related and analytical issues pertaining to the determination of the total content of delta-9-tetrahydrocannabinol (Δ-9-THC) and delta 9-tetrahydrocannabinolic acid (Δ-9-THCA-A) in herbal cannabis samples. Such analyses are currently typically performed by means of gas chromatography or to a lesser extent by liquid chromatography. In the case of gas chromatography, the total content is determined as the sum of delta-9-tetrahydrocannabinol (Δ-9-THC) that was originally present in the sample and delta-9-tetrahydrocannabinol (Δ-9-THC) formed as a result of decarboxylation of delta 9-tetrahydrocannabinolic acid (Δ-9-THCA-A). The liquid chromatography method is suitable for assaying both compounds in their natural form. Both methods have a number of advantages and disadvantages that require particular attention while performing analyses. One of the objectives of the present work was to carry out a comparative study of both methods of analysis, including their advantages and disadvantages.

Presented in this article is a review of the literature – particularly, related to the subject addressed herein – published in recent years by police drug experts in "Problemy Kryminalistyki" (eng. Issues of Forensic Science) quarterly. The first part contains the review of issues related to cannabis and their products – cannabinoids, cannabis subspecies, use and effect on the human body, legal issues related to cannabis, instrumental methods of assaying Δ-9-THC and Δ-9-THCA-A. The following part contains a discussion on factors influencing the assays' results such as sampling, stability of cannabinoids in cannabis, plant age, extraction, sample humidity, derivatization, stability of standard solutions, decarboxylation of delta 9-tetrahydrocannabinolic acid (Δ-9-THCA-A), chromatography separation conditions and equipment.

In view of the requirements of the Quality Management System that imposes the need for achieving accreditation of the test procedures, the author hopes that the present work will be helpful for analytical chemists working on developing cannabis assays and it will draw their attention to the factors that need to be considered when drafting budget and assessing total measurement uncertainty.

Keywords cannabis, Δ-9-THC, Δ-9-THCA-A, Δ-8-THC, gas chromatography (GC-FID, GC-MS), liquid chromatography (HPLC).

Introduction

Cannabis sativa L. is the species name for cannabis (hemp) – a plant of the *Cannabaceae* family, order Urticales. This species embraces a number of varieties of cannabis, e.g., cultivated or wild growing in different climate zones. The most popular on the drug market is the Indian variety of cannabis – *Cannabis sativa, varietas indica* [1].

Over the centuries, the applications of cannabis have changed. Initially, they were used in religious practices, then in medicine and textile industry, and over the last few decades they have become the world's most popular and widespread drug drug. The history of cannabis consumption goes back several thousand years when it originated in Asia. The first references to cannabis can be found in Chinese and Indian writings such as Vedian hymns and Indian religious songs, dated back to the first millennium B.C. In these works, the power of "soma" has been praised – a miraculous plant of Gods, capable of sending into the state of ecstasy and giving power. Other historical references

to the use of cannabis for intoxicating purposes can be found in the works of Greek historian Herodotus, who lived in 5th century B.C. Herodotus described the customs of nomadic tribes living in the steppes of Russia, who constructed special tents to be used as saunas and places for smoking cannabis. From China and India the habit of consuming cannabis spread over to Persia, Assyria and the entire Middle East from where it was introduced into Northern Africa by means of the Arabs, who imposed Islam on conquered territories along with cannabis consumption (Islam prohibits alcohol consumption but not so explicitly the use of drugs). From Africa the custom has been transferred to Europe [1, 2].

In Poland, cannabis became a popular hallucinogenic substance at the turn of the 19th and 20th centuries, subsequent to their widespread use for official therapies as efficient, easily accessible medicinal plant. Cannabis was used as a tranquilizer, sleeping medication and a substitute of morphine. Cannabis liqueurs and extracts were available at pharmacies as herbal medicine. Polish medicine book "Farmakopea Polska II" published in 1937 and 1946 still listed Herba Cannabis indiceae species as herbal medicine, although this "medicine" was already absent from "Farmakopea Polska III" edition. It was likely the result of popularizing hallucinogenic properties of cannabis in Europe, alarming reports on the demand for this drug substance and the cases of abusing cannabis-related "medicines" for non-medical purposes. It was believed that withdrawing Herba Cannabis from official health care would prevent risks posed by its hallucinogenic properties. Unfortunately, that was not the case. Nowadays, cannabis-originated drugs are amongst the most popular and most widely used in the world. They are known under different names, most frequently referring to the region of cultivation, predominantly marihuana, dagge, kif, ganja. The illicit cultivations intended for production of drug substances are practically impossible to control [1].

Cannabis are the most widely accessible illegal drug substance in Europe. Their sources are both import and domestic production. In most of the countries cannabis consumption has increased in the 1990s and at the beginning of the 21st century, although the discrepancies between countries are observed. Cannabis can be grown in various environments; in many parts of the world it is a wild-growing plant. The cultivations have been reported in 172 countries and territories (United Nations Office on Drugs and Crime – UNODC, 2009). Such an abundance means that providing estimates of global production is difficult. In 2009, UNODC estimated global marihuana production in the year 2008 for 13 300–66 100 tons. It is conservatively estimated that cannabis has been used at least once by about 75.5 million Europeans, that is over one in five of 15–64 year olds [3].

Cannabis and their products

Cannabis is a herbaceous, annual plant with straight, upright shoots, deep-rooting, growing to the height of 3 m. Its leaves are dark green, palmately compound, the margins serrate, rough on both sides, with separate thread like bracts [1].

Cannabis is dioecious, meaning it comes as plants with separate female (pistillate) and male (staminate) flowers. Only female plants are suitable for drug purposes, due to a higher content of active substances – phenolic compounds, so called cannabinoids, exclusively specific to genus Cannabis [1, 4–5]. Monoecious cannabis varieties with male and female flowers on one plant are also known. Male dioecious cannabis plants called "plaskonie" [no English counterpart – translator's note] are visibly distinct from female plants that form fruits. The apex of the male plant is decorated with a panicle of inconspicuous, greenish-yellow staminate flowers, stem sparingly foliated, leaves lighter and with less intense odor than those of female plants. Male inflorescences and leaves also contain cannabinoids, although in minute quantities, far below those of female inflorescences and leaves [1].

Female plants are larger than their male counterparts, well branched, densely foliated, with light green multifloral inflorescences. They emit characteristic intense and aromatic odor. Cannabis' seeds are its fruits – one-seeded achenes ovoid in shape and slightly flattened. Pericarp is smooth and slightly glossy. The fruits of some of the varieties, practically referred to as the seeds, differ in size, color and surface patterning. Frequently, the seeds supplied for laboratory tests are in different stages of maturity. Unripe fruits (seeds) are small, oval and green or grayish green. Upon ripening, they change color to light brown or brown. However, the fruit (seed) appearance is not a basis for distinguishing between cannabis varieties. Occasionally, this is possible by tracing the entire life cycle and determining the content of active substances. Seeds, stems and fibres do not contain or contain only minute quantities of cannabinoids and thus, those parts are not listed as drug substances in Polish or international legislation.

Top parts of blooming or fruit bearing cannabis secrete the resin rich in substances of psychoactive and hallucinogenic effect. Cannabis products include marihuana, hashish and hash oil [1].

Marihuana

Marihuana is a drug substance obtained from cannabis with almost no processing. It constitutes dried and shredded leaves and inflorescences from upper shoots of different cannabis varieties. Marihuana is available in fragmented or powdered form. In order to decrease the volume of the plant matter, the product is pressed. Marihuana is used for smoking in hand-

rolled and manufactured cigarettes, pipes, frequently mixed with tobacco (joints). Occasionally, it is used to prepare hydroalcoholic extracts [1]. A variety with high content of psychoactive substances is called "sinsemilla" (from Spanish: no seeds), and constitutes of female seedless plants of *Cannabis sativa* [5].

Hashish

Hashish is a preparation of *Cannabis* resin. The resin is produced throughout ripening period – blooming and fruiting. Its function if to protect the flowers from drying out. The resin is abundantly secreted in dry and hot climates, and therefore hashish is mainly produced in two areas of the world, i.e., southern and eastern Mediterranean countries and on the Indian subcontinent. Commercial presentation of hashish is most frequently pressed slabs, blocks, prills or cylinders differing by hardness, shade of color and degree of gloss. Hashish is typically smoked in pipes or added to food [1, 5].

Hash oil

Hash oil is a liquid extract from the plant material or the resin, obtained with the use of organic solvents such as alcohol, petrol or light petroleum. Extraction is a multi-stage process. After evaporation of the solvents, an oily-tar, viscous, water-insoluble liquid is collected, with characteristic odor and blackish-brown color with greenish sheen. Solvent-diluted oil takes on a green or brown color, depending on the degree of ripeness of the plant material and solvent properties. Such a liquid form is more suitable for smuggling, since after placing in tightly sealed containers it is difficult to detect by trained dogs. Hash oil is added to tobacco or marihuana cigarettes. Two to three drops of oil are typically absorbed onto cigarette tobacco and in this way the drug is being "legally" used [1, 5].

As there are no two individuals with identical fingerprints, there are no two identical *Cannabis* products in terms of appearance, properties and composition, due to the fact that they are obtained from different plant material, processed by different methods, enriched and concentrated by different techniques and prepared for smuggling in a different way [5].

Cannabinoids

In Cannabis variety used for drug purposes, 421 substances representing 18 different chemical groups have been identified. Among them are above 50 carbohydrates, including highly carcinogenic benzopyren and benzoanthracene; 103 terpenes, the majority of which have irritant effect on respiratory tracts; 12 fatty acids; 11 steroids; 20 nitrogen containing heterocyclic compounds and above 60 cannabinoids – 21 carbon atom compounds, present exclusively in cannabis.

Two systems of atom numbering are applied to present chemical structure of cannabinoids, first based on dibenzopyran and the second on monoterpenoid structure (Fig. 1 Numbering of atoms in dibenzopyran and monoterpenoid compounds; see Polish version).

The term "cannabinoids" also includes synthetic structural analogs of natural cannabis components and their metabolites produced in living plants.

Modern research on cannabinoids was initiated in 1964 when the structure of delta-9-tetrahydrocannabinol (Δ-9-THC) – main psychoactive component of cannabis, was resolved in Mechoulam's laboratory. Delta-9-THC is characterized by stereoselective pharmacological activity – left-handed isomer is, depending on the applied testing method, 6–100 times more active than right-handed isomer. Subsequently, the structures of other cannabis components (referred to as "classical cannabinoids") have been identified, among which, the following deserve particular attention:

- delta-8-tetrahydrocannabinol (Δ-8-THC);
- cannabinol (CBN);
- cannabidiol (CBD);
- tetrahydrocannabinolic acid (Δ-9-THCA-A);
- tetrahydrocannabinolic acid (Δ-9-THCA-B);
- cannabinolic acid (CBNA);
- cannabichromene (CBC) (Fig. 2).

The content of Δ-9-THC in plants depends on their genetic properties, vegetation conditions, method of harvesting, age, soil type and climate. By selecting appropriate conditions, it is possible to increase the content of the psychoactive component. For example, marihuana produced in the years 1979–1982 contained appx. 3% Δ-9-THC, while nowadays it contains 6–10%. Recently, a cannabis variety named Netherweed with Δ-9-THC content of 20% has been bred in the Netherlands [5]. Figure 2 presents chemical formulas of the most significant classical cannabinoids (see Polish version).

Individual plant organs differ by the content of Δ-9-THC. According to the research results, stems do not contain Δ-9-tetrahydrocannabinol, while this compound is present at a concentration of 1% in the calyces, 3% in the inflorescences and 5% in the resin [7]. Cannabis products contain the following concentrations of Δ-9-THC: plant parts (marihuana) – 0.5–5%; resin (hashish) – 2–10%; hash oil – 10–30%. The above are indicative values as the Δ-9-THC content in particular samples of cannabis products may go beyond the defined ranges [5].

Delta-9-tetrahydrocannabinol (Δ-9-THC) is typically present in parallel with cannabinol (CBN), cannabidiol (CBD) and delta-8-tetrahydrocannabinol (Δ-8-THC) [8]. Both CBD and CBN are the compounds with euphoric properties, whereas the effect of Δ-8-THC is similar to that of Δ-9-THC, although weaker [4]. The most important precursor of Δ-9-THC in cannabis is Δ-9-tetrahydrocannabinolic acid (Δ-9-THCA-A) that

decarboxylates to Δ-9-THC at elevated temperature. This process takes place during smoking of cannabis products as well as during their analysis by a gas chromatography method – in the injection port where high temperature is maintained. Other cannabinoids present in cannabis include: cannabinolic acid (CBNA), cannabidiolic acid (CBDA), cannabichromene (CBC), cannabigerol (CBG), cannabigerolic acid (CBGA), cannabivarin (CBV) and tetrahydrocannabivarin (THV) [8].

Cannabis subspecies

According to one of the classifications, cannabis belong to the genus and species Cannabis sativa that can be further divided into three subspecies: wild cannabis, dug cannabis and common cannabis.

Wild cannabis

Wild cannabis are characterized by thick pericarp and the seeds falling off easily. The plants are relatively low-growing. The seeds are small, darkly pigmented and have rich mosaic-like patterning. Fiber content is low, while the Δ-9-THC content reaches 0.5% or even 1%. Wild cannabis grow in Asia (India, Iran, Afghanistan, China). In Europe they can be found in Poland, Czech Republic, Slovakia and the Balkan countries.

Drug cannabis

Dioecious cannabis with drug properties. The stems are highly branched and densely foliated. The leaflets are small, intensely green. Drug cannabis contain little fibre. Drug substances are obtained from female plants. The calyces of the female flower clusters are covered with glandular trichomes that emit intense odor and secrete viscous resin. Drug cannabis belong to the southern type. They can be found in India and all its neighboring countries. In Poland drug cannabis do not mature, although they can be cross-bred with all local types.

Common cannabis

Common cannabis stand out by the richness of forms. They include southern, northern and intermediate types.

Intermediate cannabis – include Polish and Central Russian cannabis. Typically the stems grow up to the height of 125–200 cm, frequently even 250 cm or above. The leaves are larger than in the northern type, composed of 7 leaflets. The seeds are light-colored, without mosaic-like patterning.

Southern cannabis – include French, Spanish, Yugoslavian, Italian and Turkish cannabis. The stems grow up to the height of 250–400 cm. The leaves are large and wide, composed of 9–11 leaflets. The seeds are large, grey or dark grey, without mosaic-like patterning. Typically, southern cannabis do not mature in Poland.

All forms and types of cannabis can be easily cross-bred.

With regard to the delta-9-tetrahydrocannabinol (Δ-9-THC) and cannabidiol (CBD) contents, the cannabis can be divided into three main chemotypes. This classification is facilitated by the fact that a single cannabis plant belongs to a particular chemotype throughout its entire life cycle.

Chemotype I

- Drug type – strong drug effect
- Δ-9-THC > 2%, CBD = 0%

Prevalent in hot climate. Embraces Indian Mexican and South African cannabis.

Chemotype II

- Intermediate type – drug effect
- Δ-9-THC > 0.5% (on average, from 0.3% to 0.9%), CBD > 0.5%

Includes all cannabis cultivated in warm Mediterranean-type climate.

Chemotype III

- Fibre type (hemp)
- Δ-9-THC < 0.3%, CBD > 0.5%

Includes cannabis cultivated in the temperate zone.

Under favorable cultivation conditions, high CBD content can cause Δ-9-THC to exceed the threshold defined for fibre type. This happens in crops grown for several years under artificial conditions (greenhouse, special agrotechnical conditions). The Δ-9-THC content in such crops can exceed 0.2%, which classifies them as drug type despite other plant properties being characteristic for fibre type. Hence, attempts are being made to breed safe cannabis with low Δ-9-THC content. Based on the results of a research work, a distinct chemotype has been defined as follows:

Chemotype IV

- Δ-9-THC appx. 0.001%, CBD appx. 0.2%

The literature gives also another coefficient that can be used for distinguishing the fibre type from the drug type, the so called cannabinoids proportion criterion:

$$PC = \frac{CBN\,\% + ”\ \text{-}\ 9\ \text{-}\ THC\,\%}{CBD\,\%}$$

For PC < 1, cannabis are classified as fibre type. For PC >1, cannabis are classified as drug type.

This criterion has been established based on cannabinoid transformation cycle occurring in plants. As is known, young plants exhibit high CBD concentration, while the Δ-9-THC content remains low. During the maturation period, cannabinoid biosynthesis is activated, which results in the increase in the Δ-9-THC content in plants. Prolonged storage and drying of cannabis causes the conversion of Δ-9-THC to CBN, thereby decreasing its content [7].

Uses of cannabis

Industrial use of cannabis consists of producing fibres suited for spinning, making cordage from hemp straw obtained from the stems and obtaining oil from seeds. Hemp fibres are typically processed in the form of technical fibres in order to obtain ignoble and coarse yarn destined for production of bags, ropes and cordage. The fibres are also utilized by the paper industry. Traditional regions of hemp cultivation in Poland include Lublin, Jarosław and Kielce-Cracow region [1].

It is interesting to note that in Switzerland and Italy herbal cannabis is used as a food additive in such food products as oil, flour, tea, lemonade, beer and liqueur, while essential oils extracted from cannabis by steam distillation are used in the production of cosmetics [9–10].

Drug cannabis are used for – euphemistically speaking – recreational purposes, i.e., causing euphoria, albeit accompanied by toxic effects and the threat of becoming drug-dependent. The effects of drug cannabis have been controversial as early as in antiquity. For some, this plant pointed the way towards Hades, while for others towards paradise [5].

Currently, large, illicit cannabis plantations are localized predominantly in North and South America, the Caribbean, Africa and South-East Asia, in such countries as Afghanistan, Columbia, Iraq, Jamaica, Lebanon, Mexico, Morocco, Pakistan and Turkey. Cannabis plantations can be also found in Europe although, due to the adverse climatic conditions, the plants are low in cannabinoids content. An exception are the special hydroponic cultivations, e.g., in the Netherlands, in which the plants are adequately selected and ensured special growing conditions. Cannabis obtained from such cultivations match or exceed the quality of their counterparts from other regions of the world [2–3].

Effect on the human body

Cannabis preparations not only have intoxicating but also toxic effect. The most frequent symptoms of biological toxicity include anxiety attacks, sleep disturbance, loss of control over bodily functions. A week-long consumption of cannabis can already lead to the symptoms of psychical addiction. Such symptoms are typically developed by people with weak nerves and will. Heavy marihuana and hashish smokers admit that at the first contact with those drugs, they perceived them as being harmless.

The degree of ∆-9-THC absorption depends on the route of administration. In order to achieve euphoria, marihuana cigarettes are smoked, hence the active substance is introduced via respiratory tracts. Less frequently, cannabis preparations are administered orally. Another way of administering ∆-9-THC is the injection, although due to poor water solubility, preparation of injection solution is cumbersome.

During smoking, ∆-9-THC partially decomposes and only appx. 20–70% of the active substance present in marihuana reaches the lungs. The ∆-9-THC content in the smoke depends on the manner of smoking, i.e., increases with less frequent puffing, e.g., one puff per minute, followed by holding the smoke in the lungs for 5s, while it decreases (by appx. 20%) when the smoker draws puffs at shorter time intervals (larger amount of the active substance undergoes thermal destruction).

The presence of ∆-9-THC in an organism induces a state of euphoria and hallucinations, increases sensitivity to musical stimuli and noise, changes spatial and temporal perception of reality, causes motor coordination disorders, impairs short-term memory, slows down learning processes, causes panic or short-term state of anxiety, confusion, or psychosis. For occasional smokers, 2–3 mg ∆-9-THC are sufficient to achieve a pleasant feeling of euphoria. One hand-made cigarette (roll-up) can intoxicate 2–4 rookie smokers. Heavily dependent people smoke 5–8 roll-ups per day [11].

Harmful effects of long-term cannabis consumption are significant. The major hazards include increased risk of chronic bronchitis and upper respiratory tracts carcinomas, risk of becoming addicted, increased risk of giving birth to underweight child (when cannabis has been used during the pregnancy period), incidences of paranoid personality disorders or road accidents.

Social awareness of the hazards related to the use of cannabis is much lower than in the case of alcohol and tobacco due to insufficient studies as well as preventive, and information measures. The main argument against legalization of cannabis products lies in their highly detrimental, toxic effects on respiratory, cardiovascular, reproductive and immunological systems, fetus and brain, caused by uncontrolled, repeated consumption. These adverse effects have a very broad scope – from the molecular level that constitutes the basis of human life, to thinking and personality, being its most perfect signs.

Attempts are being made to utilize cannabinoids (∆-9-THC, CBD) for treatment of such medical conditions as multiple sclerosis, Parkinson's disease, postoperative conditions, spinal cord injuries, AIDS-associated cachexia, carcinoma, glaucoma, decreased lower esophageal sphincter pressure, peritonitis, intestinal neuropathy [5].

Cannabis in view of the Act on Counteracting Drug Addiction

According to the provisions of the Act on Counteracting Drug Addiction of 29 July 2005 currently in force (Journal of Laws, no. 179, item 1485), cultivation of cannabis is prohibited with the exception

of hemp, whose cultivation is contingent on obtaining proper permissions.

The emergence of genetically modified cannabis with low Δ-9-THC (< 0.20%) and high delta 9-tetrahydrocannabinolic acid (Δ-9-THCA-A, delta-9-THC-2-carboxylic acid) content on the market, necessitated legislative action in order to amend the Act on Counteracting Drug Addiction of 29 July 2005. The amendment came into force on 1 February 2007 and it pertained to the definition of hemp, which was amended to read as follows: "plants of the species Cannabis sativa L., in which the sum of contents of delta 9-tetrahydrocannabinol and tetrahydrocannabinolic acid (delta-9-THC-2-carboxylic acid) in flowering or fruiting tops, from which the resin has not been removed, is below 0.20% by weight calculated on the dry matter". In the previous version of the Act, the only criterion for determining whether or not the herbal cannabis sample was classified as drug was that of the Δ-9-THC content [8].

According to the provisions of the amended Act, competent administrative or criminal proceedings and the ruling on the criminal activity is contingent upon subjecting the collected evidence to proper physicochemical tests. The ruling takes into consideration the classification and distinguishing between the domestic Cannabis sativa L., the so called fibre type (summarized Δ-9-THC and Δ-9-THCA-A content equal to or below 0.2% by weight calculated on the dry matter) and other than fibre type cannabis varieties (summarized Δ-9-THC and Δ-9-THCA-A content exceeding 0.2%). According to Polish legislation, the following substances are considered as drugs: i) herbal cannabis with summarized Δ-9-THC and Δ-9-THCA-A content above 0.2%; ii) resin and other cannabis products, regardless of the Δ-9-THC and Δ-9-THCA-A content [12]. Herbal cannabis, resin, tinctures, tonics and all other extracts have been included in the groups I-N and IV-N of intoxicating substances, while the most active ingredient of cannabis preparations – delta 9-tetrahydrocannabinol (Δ-9-THC) has been classified to the group II-P of psychotropic substances [5].

Instrumental methods for assaying Δ-9-THC and Δ-9-THCA-A content in cannabis samples

The majority of Δ-9-THC assaying methods described in the literature utilize gas chromatography for cannabis products analysis for the purposes of judicial proceedings. Typically, these methods determine the total Δ-9-THC content, which comprises natural Δ-9-THC and additionally, Δ-9-THC formed as a result of decarboxylation of Δ-9-THCA-A under conditions of high temperature inside the GC injector. Delta-9-THC can be analytically distinguished from its precursor by using derivatization of analytes, followed by gas chromatography [8].

Current instrumental methods of analysis of cannabinoids include a wide range of chromatographic techniques coupled with mass spectrometry. The most frequently used technique is gas chromatography – mass spectrometry utilizing a single quadrupole with electron ionization source (GC-EI-MS) or chemical ionization sources (positive or negative) (GD-PCI/NCI-MS). A new application also emerged that consists in two-dimensional gas chromatography coupled with mass spectrometry utilizing a single quadrupole with electron ionization source (GC-GC-EI-MS).

It is increasingly common to analyze cannabinoids by the gas chromatography technique coupled with tandem mass spectrometry performed by various mass analyzers, predominantly triple quadrupole (QQQ) and three-dimensional quadruple ion trap mass spectrometers.

Beside GC-MS, there are increasingly frequent reports on the equally successful applications of liquid chromatography coupled with mass spectrometry (LC-MS) towards analysis of cannabinoids [11].

Factors affecting the analyses

Sampling

The majority of drug analysis methods utilized by forensic laboratories are based on low volume samples. Therefore, it is essential for the sample to be representative of the entire material from which it has been collected. Given the high cost of materials and reagents, and the time efficiency requirement, it is recommended that forensic laboratories apply verified and recognized sampling methods, thereby limiting the number of required quantitative analyses.

Both in the case of cannabis field and indoor cultivations, from each plot that has been visually classified as containing one type of plants, the sample of 30 fruiting or flowering tops (appx. 20 cm in length) is randomly collected, excluding the edges of the crop. The collected sample must be stored in paper packaging. Prior to laboratory analyses, the plant material must be dried. The proper conditions for long-term storage are darkened and cool storerooms.

In the case of sampling of large quantities of herbal cannabis, regard should be given to the present guidelines on representative drug sampling developed by the United Nations Office on Drugs and Crime – UNODC [13]. According to the guidelines, the sampling can be arbitrary or based on statistical formulas. Arbitrary sampling methods are proved in practice, in spite of the fact that they are not based on any statistical calculations. The main disadvantage of arbitrary sampling lies in relatively substantial numbers of samples required for larger amounts of evidence. The United Nations Drug Control Programme – UNDCP recommends a square root arbitrary sampling method, wherein the sample size (n) taken from the

population (N) is calculated, based on the following formula:

for $N < x$ $n = N$
$x \leq N \leq y$ $n = z$
$N > y$ $n = \sqrt{N}$
for $x = 10$, $y = 100$, $z = 10$

The statistical sampling methods include:

- hypergeometric probability distribution method;
- binominal distribution method;
- Bayes' theorem-based method [14].

The hypergeometric probability distribution method assumes that for a total population size (N), at a materiality threshold of α, the probability of at least k100% of objects yielding the same result as randomly collected n objects equals $(1 - \alpha)100\%$. In laboratory practice, the α and k coefficients most frequently assume values of 0.05 and 0.9, respectively [14]. This means that the probability of at least 90% of packages containing the same substance as randomly selected sample of n size collected for the tests equals 95%. If no negative results have been obtained, the analysis stops after n trials. In case of occurrence of one or two negative results, additional sampling from a population is required [15].

The binominal distribution method is simpler than the hypergeometric probability distribution method, although it can only be applied to certain special cases. This method assumes that prior to the analysis of a particular object, the previously analyzed object returns to the population. Although not a standard method of drug sampling, the binominal distribution can be used to describe the hypergeometric probability distribution when dealing with large amount of evidence (at least 50 items) and relatively small sample volumes. However, it should be noted that the binominal distribution method provides merely an estimation and the n value (number of objects tested) will be slightly underestimated. Only in the cases of large amounts of evidence (sometimes in the order of thousands of items) the numbers of objects collected for testing calculated by using hypergeometric and binominal distribution method will be equal.

The method based on Bayes' theorem assumes that the number of tested objects is known and predetermined. Based on this value, the degrees of probability are calculated for unknown amount of evidence, which is the variable in this case. The Bayes' method allows to take into account the sampler's knowledge of the evidence. Although the exact item number is not known, there are some indications as to the type of the evidence. For example, when all plants within the newly established cannabis plantation look alike, they are probably all cannabis. Conversely, a situation is possible, when there is no information available whatsoever on the quantity and the type of drugs which constitute the evidence. On account of such a priori information, various mathematical models are used to estimate the number of objects (n) taken for analyses.

The use of Bayes' theorem is recommended in the case of evidence containing more than 50 items, small in size. The number of objects calculated in this way is overestimated, just like with binominal distribution, however, in this case it is less of a problem. As it is in the case of other statistical methods, the maximum permissible number of negative results has to be assumed and if the assumptions laid down have not been met, the number of analyzed objects needs to be modified. In the most common situation, no negative results are permitted. The Bayes' approach relies on β type probability distribution. This type of distribution requires parameters a and b to be defined a priori.

If no information on the content of the analyzed material (e.g., tablets) is available, it is possible to define both parameters as 1. If there is information available a priori to suggest that either all or none of the tablets contain the drug substance, both parameters can be defined as 0.5. The adoption of values $b = 1$ and $a = 3$ (or above) means that an a priori presumption was made, based on a visual inspection, that all the objects most likely contain the drug substance. An example of the latter may be a plantation of young cannabis plants.

Microsoft Excel macro available on the ENFSI website (www.enfsi.eu) can be used to calculate the number of objects (n) collected for tests for predefined parameters, by applying the hypergeometric probability distribution method, binominal distribution method or Bayes' theorem-based method [14].

In the case of qualitative analysis of the material that has the characteristics of cannabis, the Bayes' theorem-based method of sampling is preferred over the hypergeometric probability distribution method [13]. Nevertheless, there are some problems with practical implementation of this method, arising from the absence of established strict guidelines on the selection of a and b parameters. The key issue in this case is the ability of the sampling person to separate own professional experience from subjective perceptions. For this reason, the methods of hypergeometric probability distribution and binominal distribution seem easier to explain and comprehend. The fact that both methods require more objects to be collected/analyzed when dealing with larger amounts of evidence is compensated by their resilience to potential challenges in the court of law [14].

When preparing an averaged sample, i.e., a mixture of particular samples, it is important that the content of the prepared mixture reflect that of the analyzed material as a whole. By definition, the averaged sample returns a mean value without any data on particular objects. The main problem with this method of sampling lies in insufficient homogeneity of the material [14].

When cannabis samples are collected, it is important to follow the guidelines listed below.

- if a single package containing loose, free flowing herbal cannabis material has been delivered for

analysis, the sampling involves the procedure of collecting a single laboratory sample from the bulk sample;
- if a compressed slab has been delivered, the samples are collected from two different sites of the slab and the slab is being broken to verify the homogeneity of the inner content;
- if more packages have been delivered, a visual inspection is performed to determine whether all the packages contain the same material; if any particular package is distinguished by its appearance from the others, it is subjected to separate analysis;
- if whole plants have been delivered, only the tops are subjected to Δ-9-THC quantitative analysis;
- A bulk sample of the plant tops collected from a single plot is treated as a single package of loose, free flowing herbal cannabis material – the sampling involves the procedure of collecting a single laboratory sample from the bulk sample; one of the methods of reducing the size of the bulk sample must be applied; while collecting the final laboratory sample, before its homogenization, the actions required are similar as in the case of agglomerated material – larger and smaller aggregates of the flowering tops or their fragments are selected for analyses in the same proportions in which they are present in the bulk sample; attention must be paid to the process of segregation (self-sorting);
- the amount of the laboratory sample collected must be sufficient to ensure repeatability of the laboratory tests and, if needed, to allow for performing the referee tests [16].

Among the described sampling methods, no single, optimal method can be pointed out, the reason being the multitude of factors influencing the outcome of the analysis. Such factors include: drug type, amount of confiscated material, aim of the tests, level of experience of the chemist and the court members, and economic factors. In any given case, the sampling method of choice should meet the requirements of courts and prosecutor's offices, which in turn should take into consideration the cost of analysis and operating conditions of the particular laboratory [14].

Stability of cannabinoids in cannabis

Upon harvesting and drying of cannabis, the process of degradation of main cannabinoids is stopped. Nevertheless, Δ-9-THC still remains susceptible to the action of the oxygen in the air, elevated temperature and ultraviolet light (UV) – the factors causing oxidation of Δ-9-THC to CBN. Besides, Δ-9-THC can isomerize to Δ-8-THC, as has been well documented in the works of Mechoulam (1970, 1973) and Razdan (1973). In view of the above, it is recommended that the harvested cannabis plants be stored in dry and darkened storerooms [13, 17–18].

The study carried out at the Institute of Forensic Research (IES) in Cracow revealed that after one-year storage period in paper packages at room temperature, the chemical composition of dried cannabis changed significantly. During storage, the plant material undergoes the process whereby the contents of Δ-9-THC and cannabichromene (CBCh) are reduced, while the content of cannabidiol (CBD) increases. The ratio of CBD to CBN is also changed. The literature confirms the phenomenon of reduction of the Δ-9-THC content in cannabis and hashish samples during storage. Various authors have observed an increase in CBN content and relative stability of CBD content as well as an increase in CBN content and relatively stable CBD/Δ-9-THC ratio throughout the one-year period, followed by slow reduction of CBD content, whereby the complete degradation of Δ-9-THC has taken place within one year after commencement of the storage. The dynamics of changes in the content of cannabinoids can be exploited for the purposes of estimating the freshness of the plant material [16].

Plant age

Article 4 (5) of the Act on Counteracting Drug Addiction of 29 July 2005 (Journal of Laws, no. 179, item 1485) defines fibre type cannabis as: "plants of the species Cannabis sativa L. (commonly known as the hemp) in which the sum of contents of delta 9-tetrahydrocannabinol and tetrahydrocannabinolic acid (delta-9-THC-2-carboxylic acid) in flowering or fruiting tops, from which the resin has not been removed, is below 0.20% by weight calculated on the dry matter". Article 4 (37) of the same Act defines herbal cannabis as "flowering or fruiting tops from which the resin has not been removed or, in plants that have not attained the flowering stage – leaves and stems". On the list of drugs annexed to the current Act (Annex I), HERBAL CANNABIS other than hemp as well as tinctures, tonics and any other extracts from cannabis other than hemp, have been classified to the groups I-N and IV-N of intoxicating substances.

An analysis of the provisions of the Act reveals their inconsistency with respect to the definition of hemp and herbal cannabis. As the definition of herbal cannabis refers to the plants prior to the flowering stage, it seems logical that the same context (stage) should be applied to the definition of hemp.

The above inconsistencies can lead to problems when young or very young cannabis plants are subject to analysis. While analyzing the plants at the vegetative stage, by abiding strictly by the provisions of the Act, one can determine whether the provided material is herbal cannabis. although, without the possibility of classifying it in the particular group (fibre or non-fibre type), as the definition of hemp does not take account of such material.

Such conclusions have been already drawn in the past in the works of Ankus and Sokołowska-Jabłońska, according to whom, the variety of premature cannabis plants with low Δ-9-THC content

cannot be identified with certainty. Therefore, the results of tests on premature plants must be rejected due to noncompliance of the evidence with statutory definition [19].

In the case of young cannabis plants, the problems associated with issuing legal opinions are not only caused by the provisions of legislation of Poland. A similar situation is encountered in other countries. In accordance with an official protocol of the European Union on determination of the total content of Δ-9-THC and Δ-9-THCA-A (converted to Δ-9-THC) in different cannabis varieties using gas chromatography technique, the plants at the flowering stage should be sampled for analyses [20, 21].

In the case, when young plants are delivered for analyses, it would be advisable to continue their cultivation until maturation, as otherwise the determined Δ-9-THC and Δ-9-THCA-A content may not be indicative enough to allow for their classification as fibre or non-fibre type. However, such procedure would be very time-consuming and costly. On the other side, earlier research showed that the cannabis chemotype remains constant and independent of the growth phase and the plant sex, despite changes in the contents of particular cannabinoids. As early as within the first month of age, Δ-9-THC begins to dominate in the drug type, while CBD becomes prevalent in the fibre type. Owing to this, it is possible to identify cannabis chemotype already during this period of growth.

An interesting method of cannabis discrimination has been proposed by Broséus et al. This method is based on determination of the content of the compounds in young leaves by gas chromatography-mass detection technique, followed by processing of analytical data by using Support Vector Machines (SVM). The authors subjected to analyses 11 fibre type varieties approved for cultivation by the Swiss Federal Office for Agriculture and 13 drug type varieties of cannabis. The seedlings have been grown for 28 days and then the leaves were harvested, dried at room temperature and homogenized. For each of the varieties tested, three samples (100 mg each) were extracted with 5 ml of hexane with admixed squalane as the internal standard (35 mg of squalane per 100 ml of hexane). Subsequently, the extracts were sonicated in an ultrasonic bath for 15 min and next, shaken on a rotator for 1h, followed by filtration. The analyses were performed by a Agilent 7890A chromatograph with a Agilent 5975C mass spectrometer equipped with a HP-5 ms column (length: 30 m, diameter: 0.25 mm in diameter, film thickness: 0.25 μm). The initial temperature of 100°C has been increased to 260°C at a speed of 10°C/min and then maintained for 10 min. The following parameters were applied: injection volume: 2 μl; split: 1 : 20, injector temperature: 280°C; transfer line temperature: 250°C; ion source temperature: 230°C; quadrupole temperature: 150°C. Chromatograms were registered in full scan mode (m/z 30–450).

The authors selected 15 chemical compounds commonly found in cannabis seedlings for initial analysis, out of which eight compounds were shortlisted, e.g., guaiacol, γ-eudesmol, bulnesol, α-bisabolol, THV, CBD, THC and CBN that showed the highest discrimination power and thus capability of distinguishing hemp from drug type cannabis. With regard to the total number of plants tested, the rate of false positive indications was below 2%. Furthermore, it has been observed that by using the method of discrimination between drug and fibre type based on quantitative relationship expressed by the formula [THC] + [CBN])/[CBD] recommended by the United Nations Office on Drugs and Crime, 0.6% of fibre type plants and 7.5% of drug type plants were classified incorrectly [20]. The relative ratios between Δ-9-THC, CBN and CBD were also assessed in the study involving mature cannabis plants undertaken in Greece. The false identification ratio observed therein approached 20%, depending on the computation method applied [22].

Extraction

The methods of determination of Δ-9-THC, Δ-9-THCA-A as well as other cannabinoids require the use of highly efficient, selective and reproducible extraction technique. A proper sample preparation is extremely important as it may positively influence the sensitivity and help to reduce interferences caused by the sample matrix. Several extraction systems have been reported in the literature, involving single solvents, e.g., methanol, ethanol, benzene, light petroleum [6], cyclohexane [23], n-hexane [6, 22, 24], acetone, toluene [4], chloroform [25–26], methylene chloride [26], ethyl acetate [27], or the mixtures thereof, e.g., methanol : chloroform (9 : 1) [6, 13, 17, 26, 28], n-hexane : ethyl acetate (9 : 1) [6], methanol:dichloromethane (9 : 1) [9], mixture: hexane : isopropanol (9 : 1) [10] and other.

It is worth to note that by using non-polar solvents such as n-hexane or light petroleum, only neutral forms of cannabinoids can be extracted in a quantitative manner, while other solvents and their mixtures enable quantitative extraction of the acid forms [13].

Herbal cannabis samples destined for extraction which contain only flowers and leaves, should be powdered. It is advisable to carry out this process in a specially designed mill operating at high speed, e.g., 100 rps, followed by passing the sample through a sieve with apertures of 1 mm. Sieving increases sample homogeneity – it may be omitted if the laboratory can prove that the homogeneity of samples prepared by using a different method falls within the allowable margin of tolerance [13].

Alternative methods of sample preparation include shaking, shaking combined with the use of an

ultrasonic bath and extraction in a Soxhlet extraction apparatus [23].

Quantitative analyses are frequently conducted in the presence of an internal standard. To this end, the following compounds are used: 5α-cholestan [6], docosane [6, 27], tetracosane [6, 22], octacosane [25], nonadecane [29], mCPP [8], Δ-8-THC [10], prazepam [17].

Extraction efficiency has a substantial impact on the accuracy of determinations. This factor is particularly important in the case of herbal cannabis samples with Δ-9-THC and THCA-A total content approaching the statutory limit. The study on the effect of extraction efficiency on the results of the determination of Δ-9-THC content was carried out by Ankus and Sokołowska-Jabłońska [4]. The determinations were performed by liquid chromatography, using an apparatus from Waters equipped with a UV detector type 486, a pump type 515 HPLC, an autosampler type 717 plus and a workstation with Millenium software. The samples were subject to isocratic elution on a MN Nucleosil 100–5 column (5 μm, 4,6 x 250 mm), with a mobile phase consisting of acetonitrile : water : concentrated H_3PO_4 (70 : 30 : 0,01), and with the following separation parameters: column temperature: 30°C; eluent flow rate: 1,5 ml/min; detection wavelength:: 230nm; injection volume: 10 μl. The samples were prepared as follows: a test portion of 100 mg of plant material was extracted with 6 ml of methanol : chloroform mixture (9 : 1) and then shaken for 30 min on a shaker or for 15 min in an ultrasonic bath. It has been concluded that both methods were fully interchangeable. In order to evaluate the extraction efficiency under predefined conditions, once a sample had been taken for determination from extraction I, the remaining volume of extract was dumped and subsequently, the same volume of extraction mixture was added, and the extraction process was repeated. In this way, three subsequent extracts of the same sample were prepared. Following the analysis, it has been concluded that for the extraction system used, a single round of extraction was insufficient to determine the total Δ-9-THC content in herbal cannabis sample. After the first extraction, the material still contained 8–12% of its original Δ-9-THC content. Such amount can be significant when herbal cannabis with low, approaching the statutory limit Δ-9-THC content are subject to the tests. Disregarding the Δ-9-THC fraction that remains in the plant material after the first extraction can lead to an underestimation of the actual Δ-9-THC content below the statutory limit [4]. It is very likely that the case with Δ-9-THCA-A extraction is similar.

Ankus and Sokołowska-Jabłońska investigated the effect of extract storage on the stability of Δ-9-THC concentrations. To this end, the sample was taken from the fresh extract and immediately assayed for Δ-9-THC content, while the remaining extract was stored for 12 days in a sealed container and in a dark place at room temperature. After this period, the extract was retested for Δ-9-THC content. Subsequently, the above procedure was repeated daily until the 16th day following the date of extract preparation. Based on the obtained results, it has been concluded that the Δ-9-THC content in the extract increases, whereas the cannabinolic acids content decreases, over time. It can be inferred that a certain amount of acids convert into Δ-9-THC. The process of conversion is not linear but of a permanent nature [4].

Sample humidity

According to the provisions of the Act on Counteracting Drug Addiction of 29 July 2005 (Journal of Laws, no. 179, item 1485), quantitative determination of the total content of Δ-9-tetrahydrocannabinol (Δ-9-THC) and Δ-9-tetrahydrocannabinolic acid (Δ-9-THCA-A) in herbal cannabis should refer to the dry matter. In view of the possibility of receiving fresh and damp plant material for testing, consideration should be given to the first stage of sample preparation, i.e., the method and duration of drying. It is important to answer the question: "whether and in which cases the quantitative analysis of routinely prepared, air-dried cannabis test portions should take into account the correction for the water content" [30].

The term: "dry matter of the plant material" has not been clarified in the Act. Given the economic importance of hemp as fiber-producing, oil-producing and farming plant, the definition of the above term can be derived from the relevant standards. The following Polish standards concerning chemical-agricultural analyses of the plant material samples collected form field and experimental cultivations lay down the methods for sampling and determination of the dry matter content: PN-88/R04012 „Chemical-agricultural analysis of plants – sampling"; PN-88/R04013 „Chemical-agricultural analysis of plants – determination of air-dried and dry matter content". The standard PN-88/R04013 defines the "dry matter" as "air-dried plant material, fragmented as to pass through a sieve with apertures of 1 mm square, properly mixed and dried in an electric dryer at 105 ± 2°C, for at least 3hrs, until a constant mass is reached". The botany textbooks generally state that: " plant dry matter is obtained by depleting the tissues of any water that can be evaporated at 105°C". In other words, the dry matter represents the difference between the weighed sample portion and the mass of water contained within this sample portion. The air-dried sample is defined by the PN-88/R04012 standard as: "plant material sample, air-dried naturally or artificially at a temperature not exceeding 80°C". Such a sample still contains a certain amount of water [30].

Plant material of various moisture contents, in some cases even including fresh plants, can be submitted for testing. In practice, the analysis is performed on

air-dried plants that have been dried to a dry matter at room conditions. Such plant material is fragmented and subjected to particular procedural stages of high performance liquid chromatography (HPLC) or gas chromatography (GC) [30].

Plant material submitted under practical conditions and then air-dried for 2–3 days at adequate air-circulation and at room temperature, still contains a certain amount of water, which can have an impact on the final result. It should be noted that the total content of delta-9-tetrahydrocannabinol (Δ-9-THC) and Δ-9-tetrahydrocannabinolic acid (Δ-9-THCA-A) in drug type cannabis substantially exceeds the statutory threshold and oscillates between a few and several percent. However, the cannabinoids content in plants from domestic cultivations, which are typically of poorer quality and with lower content than those growing in more favorable climatic zones, can approach the statutory threshold defined by the Act on Counteracting Drug Addiction. Therefore, in case of quantitative analyses, it is important to pay attention to correct determination of the dry matter content in seized cannabis.

Failure to take into account a certain amount of water contained in air-dried samples may, in extreme cases, lead to an understatement of delta-9-tetrahydrocannabinol (Δ-9-THC) and Δ-9-tetrahydrocannabinolic acid (Δ-9-THCA-A) contents, which then fall below the statutory threshold of 0.20% by weight. For a given 5% moisture content of the plant material, by not applying a correction, one can underestimate the result (oscillating around the statutory threshold) by appx. 0.01%. Consequently, for samples containing 0.21% cannabinoids by weight of dry matter and with the moisture content above 5%, not applying the correction will result in a calculated content below the 0.20% threshold. Hence, such non-fibre type cannabis may be incorrectly classified as hemp.

Water content in fresh cannabis plants dried for a few days at room temperature or at 70°C until the leaves attain brittleness, averages 8–13% by weight [13]. After a day-long drying at 35°C, the dry matter content in the sample reaches appx. 95% [30].

Based on a simple relationship between the total content of Δ-9-THC and 9-THCA-A (z) determined for a sample of a moisture content (w), and the total content of Δ-9-THC and 9-THCA-A referred to the dry matter (x)

$$z = \frac{(100 - w)}{100} x$$

it is possible to construct a diagram illustrating the effect of a moisture content on the total contents of Δ-9-THC and 9-THCA-A in samples with the actual Δ-9-THC / 9-THCA-A contents of 0.20; 0.21; 0.22; 0.23; 0.24; and 0.25%, respectively [30].

Figure 3 presents effect of the moisture content on the total contents of Δ-9-THC and 9-THCA-A in samples with the actual Δ-9-THC / 9-THCA-A contents of 0.20; 0.21; 0.22; 0.23; 0.24; and 0.25%, respectively [30] (see Polish version).

Based on the diagram, for a given test sample dried for a day at 35°C, with a dry matter content of 95%, if the obtained result ranges below 0.19%, the actual total content of Δ-9-THC and Δ-9-THCA-A does not exceed the statutory threshold and therefore, the seized plant material is that of hemp, pursuant to the Act on Counteracting Drug Addiction. For the results at or above 0.20%, the actual total content of Δ-9-THC and Δ-9-THCA-A is undoubtedly above the statutory threshold, which eliminates hemp [30].

For the plant material submitted tfor testing that had been dried for at least a few days at room temperature without any auxiliary equipment and assuming a safety margin that sets out the maximum water content in the analyzed samples at 15%, the corresponding threshold below which the sample is classified as hemp equals 0.17%. In this case, the safe value above which the submitted material is classified as "other than hemp" equals 0.24%.

Derivatization

When cannabis extract is introduced into GC injector, decarboxylation occurs in the injection port and in consequence, only the decarboxylated., i.e., neutral forms of cannabinoids can be analyzed by gas chromatography technique. In order to analyze acid forms of cannabinoids such as Δ-9-THCA-A it is necessary to perform derivatization. However, due to the fact that most frequently, the neutral forms are the targets for analysis, a significant number of literature reports concern chromatography methods without derivatization. It should also be noted that achieving the derivatization degree required for quantitative analysis is extremely difficult [6].

The most common derivatization procedures are based on trimethylsiliation which occurs upon applying various reagents introducing trimethylsilyl group such as trimethylsilanes, trimethylsilyl amines, TMS esters and TMS amides, the latter including: N,O-bis(trimethylsilyl) acetamide (BSA); N,O-bis(trimethylsilyl) trifluoro acetamide (BSTFA); N-methyl-N-(trimethylosilyl) trifluoro acetamide (MSTFA) and N-methyl-N (tert-butyldimethylsilyl) trifluoro acetamide (MTBSTFA). Other used compounds include: trimethylchlorosilane (TMCS), trimethylsilylimidazole (TMSI), trimethyliodosilane (TMIS) or potassium acetate. The most frequently used derivatizing reagents are the mixtures of BSTFA with 1% TMCS or BSA in acetonitrile environment. Other methods include alkylation with such reagents as tetrabutylammonium hydroxide [4, 6]. Derivatization of highly polar compounds decreases their polarity, increases volatility and thermal stability. The selection of a proper siliating reagent should be based on

its efficiency in inducing derivatization reaction, by-products formed and the costs of chemicals [24].

Stability of standard solutions

While performing quantitative analysis of herbal cannabis samples, one should take into account the stability of standard solutions used for preparation of the calibration curves. The stability of Δ-9-THC and Δ-9-THCA-A standard solutions has been studied by Zoller et al. [9]. The study found that the basic Δ-9-THC standard solution in methanol is stable for one year when stored at 20°C. A diluted methanolic Δ-9-THC solution used for calibration retains stability for at least one month when stored at +5°C or for at least 5 days when stored at room temperature in the dark (e.g., inside the autosampler unit). Delta-9-tetrahydrocannabinol (Δ-9-THC) is unstable in highly acidic environment. In methanolic solution of Δ-9-THC at a concentration of 4.6 μg/ml, mixed 1:1 with 1.4M aqueous solution of hydrochloric acid, maintained at room temperature (23°C), 25% of Δ-9-THC decomposed within 5 hrs. Delta-9-THC is much more stable in alkaline solutions. After 22hrs, no Δ-9-THC decomposition has been observed in methanolic solution of 3,1 μg/ml Δ-9-THC and 0.62M potassium hydroxide, maintained at 45°C. In methanolic solution of Δ-9-THC at a concentration of 2.6 μg/ml, mixed 1:1 with 1.24M aqueous solution of sodium hydroxide, maintained at 45°C, 9% of Δ-9-THC decomposed within 21 hrs.

A standard solution of delta-9-tetrahydrocannabinol (Δ-9-THC) has lower stability as compared to the standard solution of delta 9-tetrahydrocannabinolic acid (Δ-9-THCA-A). Methanolic Δ-9-THCA-A solution retains stability for at least 3 months when stored at –20°C. The shelf-life of a diluted Δ-9-THCA-A solution stored at +5°C is two weeks, while the same solution stored at room temperature remains stable for appx. 4 days. Decomposition of 5% of Δ-9-THCA-A proceeds within 12 days. In methanolic solution of Δ-9-THCA-A at a concentration of 1,0 μg/ml, mixed 1:1 with 1.4M aqueous solution of hydrochloric acid, maintained at room temperature (23°C), 36% of Δ-9-THCA-A decomposed within 4 hrs. In alkaline environment, THCA-A (0,6 μg/ml) was more stable than in acidic but less stable than in neutral environment. After 24hrs, decomposition of 34% of Δ-9-THCA-A was observed in 0.62M methanolic solution of potassium hydroxide, maintained at 45°C. In methanolic solution of Δ-9-THCA-A mixed 1:1 with 1.24M aqueous potassium hydroxide solution and maintained at 45°C, decomposition of 34% of Δ-9-THCA-A was observed after 17hrs [9].

With regard to the chemical forms of the reference materials, an optimal standard solution should be an easily accessible, inexpensive and highly stable crystalline substance. Unfortunately, in most of the cases, Δ-9-THC and Δ-9-THCA-A standards are accessible as methanolic or ethanolic solutions [25]. However, Δ-9-THCA-A standard is more stable in ethanolic solution than in crystalline form [31].

In German laboratories carrying out determinations of Δ-9-THC content in herbal cannabis samples, for many years cannabinol (CBN) has been used for calibration in place of Δ-9-THC. CBN is accessible in the form of a crystalline substance of high stability and purity that is easily verifiable by using e.g., melting temperature tests. In a series of proficiency tests organized in 1997 and participated by over 30 European forensic laboratories, the poorest results have been obtained for determination of the Δ-9-THC content. The standard deviation of Δ-9-THC determination results was 29%, while in the case of cocaine and amphetamine determinations, it amounted to 5 and 8%, respectively. The high error rate observed during the tests can be associated, to a certain degree, with issues related to potential decomposition of Δ-9-THC. CBN and/or CBD can be applied as standard solutions for determination of the Δ-9-THC content in analyses involving the use of gas chromatograph fitted with a flame ionization detector (GC-FID). It is worth emphasizing that the above combination is recommended by the United Nations Office on Drugs and Crime – UNODC [13, 25].

Decarboxylation of delta 9-tetrahydrocannabinolic acid (Δ-9-THCA-A)

Determination of acid forms of cannabinoids, including Δ-9-tetrahydrocannabinolic acid (Δ-9-THCA-A), requires a special attention due to the process of decarboxylation to the corresponding neutral forms, that occurs at elevated temperatures. Moreover, it needs to be considered that the heating of samples at high temperatures, e.g., 200°C for 30 min, results in a loss of neutral forms of cannabinoids through evaporation, even in samples that had been tightly sealed under a nitrogen atmosphere. In a 1990 study, Veress et al. investigated the process of decarboxylation of cannabinoid acids occurring in an open laboratory glassware in the presence of various chemical solvents, including n-hexane, ethylene glycol, diethylene glycol, n-octanol, dioctyl phthalate and dimethylsulphoxide. The study addressed the influence of temperature and heating time applied to the sample as well as the type of surface of laboratory glassware on the efficiency of the decarboxylation process. Included in the study were such types of surfaces as the glass and various types of sorbents. The conducted research led to the following conclusion: optimal conditions for decarboxylation of cannabinoid acids, whether at the presence or absence of organic solvents, always include a proper temperature for evaporation of neutral forms of cannabinoids. As a consequence, it is impossible to convert cannabinoid

acids into corresponding quantities of neutral cannabinoids by simply heating the sample in an open chromatographic reactor. Furthermore, it has been found that the best conditions for decarboxylation are provided by heating the sample at 200°C for 2 min in a closed reactor, [6].

Quality control tests for determination of the total content of Δ-9-THC and Δ-9-THCA-A organized by the European Network of Forensic Science Institutes (ENFSI) and the Swiss Society of Forensic Medicine (SGRM/SSML) revealed that a significant workload is required in order to ensure achieving satisfactory results by different forensic laboratories performing cannabis-related assays. A number of problems related to such tests consisted in the lack of proper Δ-9-THCA-A standard, now available on the market for only a few years. This compelled the forensic laboratories using the high performance liquid chromatography (HPLC) method to perform a conversion of Δ-9-THCA-A to Δ-9-THC by pre-heating the samples under controlled conditions, prior to conducting analyses [27].

A more detailed study of the process of conversion of Δ-9-THCA-A to Δ-9-THC became possible only after a method of obtaining milligram amounts of Δ-9-THCA-A from herbal cannabis has been elaborated by Dussy et al. The purity of the obtained Δ-9-THCA-A determined by H1-NMR method was around 96%. The main contaminant was cannabinolic acid (CBNA) – an oxidized form of Δ-9-THCA-A. The above study, in addition to addressing the process of decarboxylation of Δ-9-THCA-A to Δ-9-THC under different analytical conditions, also focused on decarboxylation that occurs during the simulated process of cannabis smoking. Having sufficient amounts of Δ-9-THCA-A, allowed the determination of parameters influencing decarboxylation process inside GC inlet liner and their comparison with the parameters of decarboxylation occurring in a glass container. In both cases, the degree of decarboxylation was determined by applying High Purity Liquid Chromatography – Diode Array Detector (HPLC-DAD) and Gas Chromatography – Flame Ionization Detector (GC-FID) techniques. After optimization of analytical procedures, the Δ-9-THC and Δ-9-THCA-A contents were determined in a dry and fresh material, consisting of cannabis plants and inflorescences as well as hashish. The combined leaves and flowers were homogenized and subjected to analyses, while the thick stems were disposed of. In addition, the tests covered cigarettes with admixed Δ-9-THC and Δ-9-THCA. The aim of these tests was to determine the Δ-9-THC content that remained after the smoking process [27].

Gas chromatography analyses were carried out with the use of a Carlo Erba GC8000Top chromatograph fitted with a flame ionization detector (FID) with a CTC Combi PAL autosampler. The study involved the use of a DB-5MS capillary column from J&W Scientific (length: 5 m, diameter: 0.25 mm, film thickness: 0.25 μm). The flow rates of helium, hydrogen and air were 0.6 ml/min, 40 ml/min and 300 ml/min, respectively. Additionally, nitrogen was applied as a "make up" gas at a flow rate of 30 ml/min. Split ratio was 50:1. The following temperature program was adopted: initial temperature: 120°C (2 min), temperature ramp 20°C/min, final temperature: 300°C (3 min) [27].

HPLC analysis was performed by a Thermo Finnigan chromatograph equipped with a P4000 pump which allows mixing of four mobile phase components, a UV6000LP photodiode array detector and a AS3000 autosampler. An Allure C-18 column from Restek (length: 150 mm long, diameter: 3.2 mm, particle size: 5 μm) was also used. The detection wavelength was $\lambda = 228$ nm. Linear gradient solution was applied with the following solvents: (A) 0.05 mM acetic acid buffer solution (pH 4.75), (B) acetonitrile, (C) methanol. Initial composition of the mobile phase: (A) 13%, (B) 22% (C) 65% was maintained for 2 min after which period the proportion of solvent (B) was increased to 35%, while solvent (A) was correspondingly reduced [27].

In order to assess the efficiency of the decarboxylation process, a solution containing 500 μg Δ-9-THCA-A was transferred into the container, evaporated under a gentle stream of nitrogen gas and then the container was sealed, placed in the chromatography oven and heated at a constant temperature for 15 min. The reaction products were dissolved in 500 μl of methanol and analyzed by HPLC. A comprehensive diagram included in a work by Dussy et al. [27], showing chromatograms of decarboxylation products obtained at 120°C, 140°C, 160°C and 180°C, revealed the presence of two additional peaks derived from cannabinol and dihydrocannabinol, beginning at 140°C and upwards. The presence of such peaks clearly suggests that the process of decarboxylation does not proceed in a quantitative manner.

After conducting initial tests involving the container heated inside the chromatography oven, Dussy et al. analyzed cannabis samples. Sample preparation involved extraction of a test portion of 50 to 100 mg of cannabis product (dried fresh plants, flowering tops or hashish) with 2 ml of ethyl acetate containing 1 mg/ml docosane ($C_{22}H_{46}$) as an internal standard. Subsequently, the extracts were sonicated in an ultrasonic bath for 15 min. Next, 10 μl of extract was diluted with 390 μl of ethyl acetate and analyzed by GC. In the case of HPLC analysis, 10 μl of extract was spiked with 990 μl of methanol and analyzed using the external calibration method.

The next stage of analysis included optimizing the Δ-9-THC and Δ-9-THCA-A (converted to Δ-9-THC) total content assays, with regard to the type of inlet liner used and inlet temperature. Three liner types have been tested:

- straight liner;
- straight liner with silanized glass wool;
- cup-splitter liner.

With regard to repeatability and the degree of conversion, the best results were obtained for the straight liner with silanized glass wool. The straight liner was characterized by the lack of repeatability, while the cup-splitter liner supported slightly lower degree of conversion. The amount of glass wool had a negligible effect on the degree of conversion. However, it was important that the glass wool be tightly packed, and not loosely inserted. The maximum degree of conversion amounting to 67% was achieved at the temperature of 220°C, at which further Δ-9-THC oxidization produced no detectable cannabinol. This conversion value corresponds to the result obtained by Lehman and Brenneisen.

Subsequently, the study by Dussy et al. [27] addressed the influence by the temperature of Δ-9-THCA-A conversion (performed immediately before HPLC analysis) on the results. However, the authors did not supply information about the method of heating applied, particularly the type of containers used (open vs. closed). An analysis of the obtained results revealed that the conversion curve reaches its maximum corresponding to a 70% conversion degree at a temperature of 150°C. At higher temperatures, Δ-9-THC is oxidized to cannabinol. Interestingly, the sum of Δ-9-THC, Δ-9-THCA-A and cannabinol contents does not add up to 100%, which suggests the possibility of formation of polymers. In the event of a change of the exposure time, it is imperative to repeat the tests in order to determine the maximum value of conversion for the new exposure time. The obtained maximum degree of conversion corresponds to the gas chromatography technique, optimal type of liner and optimal injector temperature.

The optimized analytical procedures were applied by Dussy et al. towards analysis of plants from indoor cultivations, inflorescences and hashish. After extraction with ethyl acetate, the samples were analyzed by GC and HPLC methods, without prior decarboxylation. In line with the expectations, for all samples tested the sum of Δ-9-THC and Δ-9-THCA-A contents determined by HPLC was greater than the total content of Δ-9-THC and Δ-9-THCA-A (converted to Δ-9-THC) measured by GC. The Δ-9-THC content expected for GC technique, based on the assumption of complete decarboxylation, was estimated on the basis of HPLC results with predefined 70% conversion degree, which value was obtained from analyses carried out under optimized conditions, with Δ-9-THCA-A used as a standard. The correlation between estimated and measured Δ-9-THC levels was very strong for the plants, weaker for the inflorescences and unsatisfactory for hashish. It seems that in the case of higher Δ-9-THCA-A contents, decarboxylation is preferred over other reactions and therefore, the measured Δ-9-THC levels surpass the estimated ones. Under applied conditions, the maximum degree of Δ-9-THCA-A to Δ-9-THC conversion reached 70%. The laboratories using HPLC technique for determination of the total Δ-9-THC content considered as a sum of Δ-9-THCA-A and Δ-9-THC levels determined separately, usually report higher values than those applying decarboxylation, followed by GC determination. It is recommended that the laboratories performing determinations of Δ-9-THC and Δ-9-THCA-A total contents define the degree of conversion achieved under their respective testing conditions. When the Δ-9-THC content is determined after decarboxylation, such value represents the minimum, rather than accurate amount of this compound [27].

Following the study by Dussy et al., further assessments have been made by Stanaszek and Zuba [8], who developed and validated a method of determination of the total content of Δ-9-THC and Δ-9-THCA-A in herbal cannabis samples by using High Purity Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS) techniques, and compared the results yielded by both techniques.

A method of sample preparation adopted by Stanaszek and Zuba consisted of collecting 0.5g of dried herbal cannabis and extracting the sample with 5 ml of methanol for 16hrs. For HPLC analysis, a RP-18e Chromolith column (length: 100 mm, particle size: 5 μm) was thermostat-heated to 40°C. The analysis was conducted using the isocratic elution method. The mobile phase consisted of a mixture of water spiked with 100 μl of phosphoric acid per liter (pH=3) and acetonitrile (70:30). The obtained extract was diluted 20 times and subjected to HPLC analysis with the use of a HPLC Elite LaChrom L-2000 System (VWR Merck-Hitachi), featuring a diode array detector. The mobile phase flow rate was 1 ml/min. The injection volume was 20 μl. Quantitative analysis was performed at λ = 210nm for Δ-9-THC and λ = 220nm for Δ-9-THCA-A. Calibration curves for Δ-9-THC and Δ-9-THCA-A were drawn up within the range of 0.05% (2.5 μg/ml) to 2.0% (100 μg/ml). For GC-MS analysis, the extract was spiked with 10 μl of mCPP internal standard (1000 μg/ml) and diluted 20 times with methanol. The analyses were performed with the use of a HP6890 GC System coupled with a mass detector (Agilent 5973 Network Quadrupole MSD), with a split/splitless injector maintained at 250°C. The separation was carried out on a HP-5MS column (length: 30 m, diameter: 0.25 mm, film thickness: 0.25 μm). The volumes of 1 μl were injected in the splitless mode. The carrier gas was helium, at a flow rate of 1 ml/min. The following temperature program was adopted: initial temperature: 75°C (1 min), temperature ramp 25°C/min, final temperature: 275°C (9 min). The detector operated in the range of 40 to 600 m/z. For quantitative analysis, the signal intensities at m/z = 299 (Δ-9-THC) and m/z = 154 (internal standard – mCPP) were used. The calibration curve was drawn up within the range of 0.1% to 0.8%.

In order to compare the High Purity Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS) techniques and evaluate their usefulness in determination of main psychoactive cannabis components, Zuba and Stanaszek analyzed 64 real marihuana samples using previously developed and validated analytical procedures. The results of Δ-9-THC and Δ-9-THCA determinations obtained by HPLC technique were juxtaposed with those obtained by GC-MS in order to investigate the correlation between those variables. Delta-9-THC contents determined by GC-MS technique ranged between 1.6% and 19.6% (average 7.4%) in the dry matter of herbal cannabis. Delta-9-THC contents determined by HPLC technique ranged between 0.1% and 9.6% (average 1.0%) in the dry matter of herbal cannabis. Delta-9-THCA-A contents determined by HPLC technique ranged between 0.7% and 13.7% (average 7.4%) in the dry matter of herbal cannabis. The total Δ-9-THC content (the sum of Δ-9-THC and Δ-9-THCA-A) determined by HPLC technique ranged between 0.8% and 23.0% (average 8.4%) in the dry matter of herbal cannabis.

A comparison of both techniques in terms of the results obtained for the Δ-9-THC content determination in real samples, revealed a good correlation (r = 0.93), which suggests their suitability for determination of the total content of this compound in herbal cannabis and other cannabis products, e.g., hashish. Equation of a straight line passing through the point (0,0) – y = 0,8581x indicates that the contents determined by GC-MS technique amounted on average to appx. 86% of those obtained by using HPLC. Hence, the conclusions concerning decarboxylation process formulated previously by Dussy et al. were further confirmed by Zuba and Stanaszek. Underestimation of the Δ-9-THC content observed for GC-MS technique may be a result of incomplete thermal decarboxylation of Δ-9-THCA-A to Δ-9-THC. It follows that the only means of accurately determining the total Δ-9-THC content is to separately measure the Δ-9-THCA-A and Δ-9-THC contents, and then sum them up. A post-decarboxylation Δ-9-THC level determined by gas chromatography technique reflects merely the minimum and not the total content thereof. Determination of the total Δ-9-THC content in herbal cannabis or cannabis products provides an accurate measure of their psychoactive properties.

The findings by Dussy et al. were reflected in the instruction of the United Nations Office on Drugs and Crime on the methods of analysis of cannabis and cannabis products [13]. The instruction recommends that determination of the total content of Δ-9-THC and Δ-9-THCA-A (converted to Δ-9-THC) should be preceded by decarboxylation achieved by means of heating the extracts in open containers to 150°C. The process of decarboxylation takes place within a few minutes after evaporation of the solvent. However, the laboratories are advised to perform validation of the process under conditions available locally. If decarboxylation is performed in the course of the analyses, it may, depending on the chromatography system, complete in full during the injection or proceed with much lower efficiency, despite identical temperature parameters. Elevated injection temperature may cause Δ-9-THC to decompose inside the injector (inlet) liner. Hence, both the chromatographic system in use and analytical conditions applied must be validated as to ensure complete decarboxylation, without causing decomposition of Δ-9-THC [13].

The results obtained by Dussy et al. suggested that under the adopted analytical conditions, the level of decarboxylation of delta-9-tetrahydrocannabinolic acid (Δ-9-THCA-A) depends on its content in analyzed cannabis samples. Given the identical method of sample preparation, a higher Δ-9-THCA-A content in the sample corresponds to a higher content in the extract, which in turn means that a larger amount of this compound is applied onto the column. Decarboxylation proceeds more efficiently in samples containing higher measurable levels of Δ-9-THCA-A, sourced from the injected extract aliquots. The maximum degree of decarboxylation obtained in the optimization studies launched by the authors amounted to 70%. However, the authors have not revealed the concentration or the content at which the process was optimized, nor whether optimization was performed at one or more concentrations. Given the variability in decarboxylation levels observed by the authors, ranging between 73% and 90% (calculated as the total Δ-9-THC content determined by GC, relative to the total content of Δ-9-THC and Δ-9-THCA-A measured by HPLC), it can be assumed that decarboxylation was optimized at one concentration level. This was likely due to an initial assumption that decarboxylation level does not depend on the Δ-9-THCA-A content. Unfortunately, it was not until the later stage of the study that this assumption proved to be wrong.

In the study by Stanaszek and Zuba, an average degree of conversion calculated on the basis of the test results of 64 real samples amounted to 86%. It might seem that such value is in contrast with a mere 70% conversion degree obtained by Dussy et al. in the tests including a standard solution of Δ-9-THCA-A. In fact, however, the above results appear mutually consistent when one considers different analytical conditions applied by both research teams, particularly as regards the injection. While Dussy et al. applied a split mode of injection with the split ratio of 50:1, Stanaszek and Zuba opted for a splitless mode. Additionally, different adopted methods of sample preparation should be taken into account. As a result, the sample volumes injected by Stanaszek and Zuba most likely contained large amounts of Δ-9-THCA-A, which underwent a high degree of conversion. An average degree of conversion of 86% observed by Stanaszek and Zuba matched the result obtained by Dussy et al. for hashish samples.

In the case of samples containing large quantities of Δ-9-THCA-A, the amount of this compound deposited onto the column is considerable even at the applied split ration of 50:1. It should be stressed that upon validation of self-developed, GC-MS-based analytical method, Zuba and Stanaszek concluded that for the adopted method of sample preparation and analytical conditions, the Δ-9-THC calibration curve remains linear over the range of 0,1–0,8%. Thus, within this range the decarboxylation process is independent of the Δ-9-THCA-A level in the sample.

Based on a detailed analysis of the works by Dussy et al. and Stanaszek and Zuba against the background of other literature sources [9, 17], and on this author's own modest personal experiences, certain concerns could be raised by the absence of any information about the separation of Δ-9-THC from Δ-8-THC achieved by using HPLC technique. The lack of any such information is especially surprising, considering relatively short columns used by both research teams, that hardly support satisfactory separation of these compounds.

The study by Ankus i Sokołowska-Jabłońska on a durability of cannabis extract samples also has indirectly implied the dependence between decarboxylation of delta-9-tetrahydrocannabiniolic acid (Δ-9-THCA-A) and the concentration of this compound. The study revealed an increase in the Δ-9-THC level and a decrease in the cannabinolic acids content in cannabis extract, over time. It can be inferred from this finding that a certain amount of acids convert into Δ-9-THC. The process of conversion is not linear but of a permanent nature [4]. Figure 4 is a reproduction of a diagram from the publication by Ankus and Sokołowska-Jabłońska showing the conversions of Δ-9-THCA-A, including chemical formulas of the particular compounds [4].

Chromatography separation conditions

Chromatography separation conditions play an important role by affecting the accuracy of determination of the total content of Δ-9-THC and Δ-9-THCA-A in herbal cannabis or cannabis products. In the case of gas chromatography coupled with a flame ionization detector (GC-FID) or gas chromatography fitted with a mass detector (GC-MS), Δ-9-THCA-A is not registered on the chromatograms due to its decarboxylation to Δ-9-THC.

Figure 4. presents scheme of conversions of delta 9-tetrahydrocannabinolic acid (Δ-9-THCA-A) [4].

Therefore, it is advisable to verify whether under the adopted separation conditions the peak derived from Δ-9-THC overlaps with the peaks of other cannabinoids. Such verification should be carried out with the use of a control mixture of the corresponding standard solutions. Due to a high degree of structural similarity among particular cannabinoids, their retention times are often very similar. It should also be born in mind that the mass spectra of Δ-9-THC and Δ-8-THC are similar and differ only by relative signal intensities of particular ions, whereby these intensities additionally depend on the type and age of the mass detector in use.

The study on separation of cannabinoids contained in the herbal cannabis samples was conducted by Stambouli et al. [29]. The GC/MS analyses were carried out using a Varian CP-3800 chromatograph coupled with a Saturn 2200 ion trap mass spectrometer. The chromatograph was equipped with a CTC Analytics CombiPAL autosampler and a PTV 1079 injector. Separation was carried out using a HP-5 column (length: 25 m x diameter: 0.2 mm x film thickness: 0.11 μm), with helium as the carrier gas. The following temperature program was adopted: initial temperature: 60°C (2 min), temperature ramp 15°C/min, final temperature 280°C (5 min). The injector operating in the splitless mode was set at 270°C. The analyses were performed across a range of m/z = 35–500. The ion trap temperature was 180°C and the transfer line temperature was 280°C. On the registered chromatograph, Δ-8-THC was eluted immediately before cannabidiol (CBD). Subsequently, the Δ-9-THC (the peaks of both cis and trans geometric isomers were registered) and cannabinol (CBN) peaks were eluted, whereby the Δ-8-THC quantity in the sample was relatively low as compared to Δ-9-THC, CBN and CBD quantities.

In the case of HLPC analysis, insufficient separation and partial overlapping of Δ-9-THC and Δ-8-THC peaks can be problematic. Therefore, in order to determine the Δ-9-THC peak area (used for quantitative calculation) it is necessary separate the peaks by using the vertical drop lines which are set manually at the lowest points of the valleys. These issues have been addressed by Zoller et al., who conducted a study on determination of the content of Δ-9-THC and Δ-9-THCA-A present in herbal cannabis and in food containing herbal cannabis as an additive. The analyses were carried out with the use of a LC-10A Shimadzu chromatograph equipped with a LC-10AD pump system, a SIL10 autosampler, a SPD-10AV UV-Vis detector, a RF-10A fluorescence detector, a Gastorr degasser (Omnilab, Mettmenstetten, Switzerland), a CBM-10A communications bus module and a CLASS-LC10 workstation. Separation was carried out on a Nucleosil 120–3 C18 column (length: 125 mm, diameter: 2 mm) (Machery-Nagel, Oensingen, Switzerland), thermostat-heated to 26°C, at a flow rate of 0.2 ml/min. The injection sample volume was 10 μl. The used solvents included acetonitrile (A) and water spiked with 8.6 g of 85% phosphoric acid per liter (B). The following linear gradient was applied: 0 min, 55% A and 45% B; 25 min, 80% A and 20% B; column wash – 26 min 90% A and 10% B; 30 min, 90% A and 10% B; 31 min, 55% A and 45% B, column conditioning

31–40 min. Determination of Δ-9-THC was carried out at the wavelength of λ = 210 nm, while Δ-9-THCA-A at the wavelength of λ = 272 nm. Additional determinations were carried out with the use of fluorescent detector. A sample of 2g of herbal cannabis was homogenized, extracted with 60 ml of methanol-dichloromethane mixture (9:1) and sonicated in an ultrasonic bath for 15 min. For the purposes of analysis, 1 ml of the solution was diluted with methanol to a volume of 10 ml. Within the analytical system employed, Δ-9-THC eluted before Δ-8-THC and the separation conditions allowed to achieve the resolution of both compounds at the level of 1. It has been found that shorter gradient run times and isocratic conditions led to poorer resolution and peak overlap problems. In the case of HPLC analyses, a preliminary optimization of Δ-9-THC and Δ-8-THC separation and elution under gradient conditions is recommended. HPLC-related problems with achieving satisfactory separation can also be experienced in the case of Δ-9-tetrahydrocannabinolic acid (Δ-9-THCA-A) analysis. Yet another problem relates to the identification of compounds with the retention time similar to that of Δ-9-THCA-A. In the instruction of the United Nations Office on Drugs and Crime of 1987, the following method of Δ-9-THC and Δ-9-THCA-A determination in cannabis samples has been recommended: suggested column: C-18 (length: 250 mm, internal diameter: 4.6mm); mobile phase: 0.02N H_2SO_4 solution : methanol (20 : 80); flow rate: 2.0 ml/min; injection volume: 10 μl; detection wavelength: λ = 220 nm or λ = 254 nm; internal standard: di-n-octyl phthalate. According to the instruction, under the above separation conditions and at the retention time close to that of the Δ-9-tetrahydrocannabinolic acid Δ-9-THCA-A), the following order of cannabinoids elution should be observed: cannabichromene (CBC), cannabinolic acid (CBNA), Δ-9-tetrahydrocannabinolic acid (Δ-9-THCA-A). However, the identification of the particular compounds is problematic. This can be further confirmed by the fact that in the study by Ankus and Sokołowska-Jabłońska [4] the peak positioned within the area of chromatogram characteristic for the eluted Δ-9-THCA-A was defined in broad terms as the bandwidth characteristic of cannabinol acids. The following operating conditions were adopted throughout the above study: column: Nucleosil 1005 C-18 (length: 250 mm, internal diameter: 4.6 mm, particle size: 5 μm); mobile phase: acetonitrile : water : concentrated H_3PO_4 (70 : 30 : 0,01); column temperature: 30°C; eluent flow rate: 1,5 ml/min; injection volume: 10 μl; detection wavelength: λ = 230 nm. Interesting results have been reported by Lehman and Brenneisen (1995) [23], who applied liquid chromatography coupled with a diode array detector (HPLC-DAD) for the identification and quantification of neutral and acid forms of cannabinoids. The compounds were eluted by a gradient elution method. The peaks derived from particular compounds were identified with the use of standard solutions and based on cannabinoid-specific ultraviolet (UV) spectra. A C-18 column (length: 200 mm, internal diameter: 2.0 mm) with a corresponding pre-column (length: 20 mm, particle size: 3 μm) was applied. By looking at the published chromatograms of the three basic cannabis chemotypes, i.e., drug, intermediate and fibre type (hemp) [23], it can be easily inferred that each type is characterized by different relative amounts of cannabinoids that elute at the retention time similar to that of Δ-9-THCA-A. The analysis was partially hindered by a relatively high background noise on the chromatograms. Nonetheless, the drug and fibre type samples exhibited an additional, low peak, immediately preceding Δ-9-THCA-A that was not identified by the authors. The same peak was also present in the intermediate type. Presumably, it was derived from cannabinolic acid (CBNA), as shown by the fact that this compound is present as impurity in extracts of Δ-9-tetrahydrocannabinolic acid (Δ-9-THCA-A) obtained from herbal cannabis by preparative chromatography technique [27]. Moreover, in the intermediate chemotype sample, the peak derived form Δ-9-THCA-A partially overlaps with the one of cannabidochromene (CBC), which latter compound is also eluted before Δ-9-THCA-A. Delta-9-tetrahydrocannabinolic acid (Δ-9-THCA-B) was eluted at short retention time. Considering the above described problems with ensuring separation conditions that support obtaining a distinct Δ-9-THCA-A peak that is clearly separable from the peaks derived from other cannabinoids, the lack of information on the obtained results of Δ-9-THCA-A separation in published works [8, 27] seems to be quite puzzling, all the more so as the authors had the diode array detectors (DAD) at their disposal that allowed for simultaneous analysis at multiple wavelengths. Accurate determination of the Δ-9-THC and Δ-9-THCA-A contents requires securing optimal separation conditions, which can be both tedious and time consuming. Once this task has been achieved, however, the original cannabinoids content can be directly determined without the need for Δ-9-THCA-A decarboxylation. Particularly valuable in this context appears to be the study by de Backer et al. [17]. The research part addressed the optimization of conditions for the separation of a mixture of Δ-9-THC, Δ-8-THC, Δ-9-THCA-A, CBD, CBDA, CBG, CBGA and CBN standard solutions. The following method of sample preparation has been adopted: the plant material was dried for 24 hours at 35°C at appropriate ventilation and then homogenized by milling. A sample of 200 mg of the milled material was extracted with 20 ml of methanol : chloroform mixture (9:1) and next shaken for 30 min. using a rotator. The obtained extracts were filtered and diluted. An aliquot of 100 μl of the final extract was evaporated under the stream of

nitrogen and dissolved in 100 μl of water: methanol mixture (5:5). Analyses were carried out in the presence of prazepam (100 mg/l) added as internal standard. The experimental setup included a Hewlett-Packard chromatograph (Agilent Technologies, Boblingen, Germany) equipped with a G1322A four-channel pump, a G1313A degasser of the 1200 series, a G1313A autosampler of the 1100 series and a Waters 2996 photodiode detector with Empower Pro 2.0 software. Analyses were carried out across the wavelength range (λ) 200 to 400 nm. The separation was carried out on a Waters XTerra MS C18 column (length: 250 mm, internal diameter: 2.1 mm, particle size: 5 μm) with a corresponding pre-column (length: 10 mm). The mobile phase consisted of methanol : water mixture spiked with 50 mM ammonium formate (pH 5.19). Initially, the mixture contained 68% of methanol, which amount was gradually increased to 90.5% within 25 min and then to 95% within an additional minute. The above conditions were maintained for 3 min. Subsequently, the mobile phase composition was adjusted to its initial parameters within 1 min, followed by column conditioning for 6 min. The total run time was 36 min, at the flow rate of 0.3 ml/min. The column was thermostat-heated to 30°C. The injection volume was 30 μl. The authors optimized the conditions for determination assays and validated the developed procedure by using the Total Error Approach method. Optimization addressed three factors: percentage of methanol at the beginning of the gradient elution, pH of the aqueous component of the mobile phase and the estimated duration of the gradient elution that is necessary to achieve 95% methanol content in the mobile phase. As a result of the study, it has been concluded that the selectivity of cannabinoids separation can be modified by adjusting the pH of the eluent. This possibility was particularly useful in the case of acid forms of cannabinoids. By optimization of the pH of the eluent and the speed of the gradient elution it was possible to separate all cannabinoid forms subject to the study. The chromatographic peaks overlapped only in instances where CBD and CBG were present at 10%. The developed procedure also allowed for complete separation of Δ-9-THC from Δ-8-THC [17]. The results obtained by de Backer et al. appeared so promising as to induce the purchase of a similar chromatographic column by the Physical Chemistry Department of the Forensic Laboratory of the Rzeszów Province Police Headquarters, with the purpose of conducting tests and implementing the column for the needs of routine casework.

Equipment requirements

Using state-of-the-art equipment for analyses has a great influence on the analytical results obtained. This effect is particularly visible as regards liquid chromatography. For this kind of analysis, it is particularly beneficial to use diode array detector (DAD), which is especially useful in the case of poor separation of chromatographic peaks. The use of DAD enables identification of substances on the basis of their UV-Vis spectra. It is also possible to deduce whether incompletely separated peaks correspond to two different chemical compounds or have appeared as a result of a column overload. A new trend in the development of gas chromatography is a method of ultra performance liquid chromatography (UPLC), which gradually substitutes high performance liquid chromatography (HPLC). The selection of a particle size in the UPLC method is by necessity the result of the compromise. The lower the diameter, the higher the back pressure (resulting from the flow of the mobile phase) that builds up in a given system. Due to relatively low working pressure limits in traditional HPLC chromatographs, it is not possible to load the columns with particles of 2.1 mm or 1.0 mm in diameter which are nowadays commonly used in UPLC. The UPLC system permits up to nine times faster analysis as compared to the traditional system fitted with columns loaded with 5 μm particles and up to three times comparing with columns loaded with 3 μm particles. The back pressure within the UPLC system is nine and three times greater, respectively, than in the traditional system. The UPLC-compatible columns carry smaller sized particles, are shorter and smaller in internal diameter. Increased speed, sensitivity and resolution of analyses are among the advantages of UPLC method. Faster analysis results in reduced consumption of chemicals. Improved resolution of the columns saves time for optimization of assays and validation of newly developed analytical methods [32, 33].

Source:

Figs. 1–4: author

Translation *Rafał Wierzchosławski*